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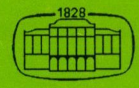

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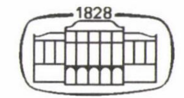

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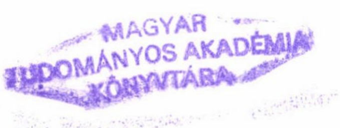

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EFFECTS OF DIETARY SELENIUM AND VITAMIN E ON THE OXIDATIVE STABILITY OF LARD

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The effects of dietary sodium-selenite and Vitamin E on the oxidative stability and fatty acid composition of lard have been investigated. Lard samples were subjected to the accelerated storage at 60 °C in classic oven test. The level of lipid peroxidation was analyzed by measuring the thiobarbituric acid reactive substances and peroxide production, as well as the decrease in a polyunsaturated fatty acid levels. The fatty acid composition of lard was not influenced by dietary factors and prolonged heating. Supplementing the diets with tocopherol-acetate (30 mg kg^{-1} of feed) significantly increased the oxidative stability of lards during heating and the protective effect of vitamin E was comparable with that of butylated hydroxytoluene (BHT). Dietary selenium (0.3 mg kg^{-1} of feed as sodium-selenite) was not effective as an antioxidant in lards. The combination of dietary vitamin E and sodium-selenite showed significant protective effect against peroxidation in lards in comparison to the control group, deficient both in vitamin E and selenium, but this combination was significantly less effective than dietary vitamin E alone.

Keywords: lard, dietary supplementation, vitamin E, sodium-selenite, oxidative stability

The deterioration of fats and oils is mainly due to the oxidation processes. Oxidative deterioration of the polyunsaturated lipids of food leads through formation of hydroperoxides to short-chain aldehydes, ketones, and other oxygenated compounds which are considered to be responsible for the development of rancidity in stored foods. Control of lipid peroxidation in food is of great importance due to the increasing demand of good quality products. The use of antioxidants increases the product's shelf life and heat stability. The most commonly used lipid antioxidants at the present time are butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tertiary butylhydroquinone (TBHQ), and tocopherols. They are added to a wide variety of foods including vegetable oils and animal fats, and their antioxidative effects were established in many reports (PERKHURST et al., 1968; RHEE, 1978; MORRISON et al., 1981; HAWRYSH et al., 1990). The antioxidative properties of many other synthetic or natural compounds were also investigated in order to evaluate their possible efficacy in extending the quality and stability of food products (CORT et al., 1975; CHANG et al., 1977; DAS & PEREIRA, 1990; HOSONO et al., 1991; YEN & DUH, 1993).

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Apart from using either natural or synthetic antioxidative additives there is also a tendency to use dietary compounds to improve the quality of foods of animal origin. The supplemental dietary vitamin E given in large doses is reported to reduce lipid oxidation in pork, broiler meat, beef, milk, and eggs (DUCKLEY et al., 1976; LIN et al., 1989; MONAHAN et al., 1990; MITSUMOTO et al., 1991; CHERIAN et al., 1996). The animal feed is often supplemented with selenium too in order to prevent several pathological disorders related to selenium deficiency and for its protective effect on meat oxidative stability (DE VORE et al., 1983).

This study was conducted to evaluate the effects of physiological doses of vitamin E and selenium added to the animal feed on the oxidative stability of lard, and to compare the possible protective activity of these dietary components with BHT as representative of the currently used synthetic antioxidants.

1. Materials and methods

1.1. Feeding regimen

Twenty-four hogs (Landrace × Duroc × Yorkshire), 8 weeks old, castrated, were equally divided into four groups. The basal diet consisted of corn (58.5%), low-fat soybean meal (33%), sucrose (4%), and was low in natural selenium and vitamin E (0.017 mg of selenium and 10 mg of tocopherols/kg of feed). To the basal diet also was added mixture of necessary minerals and vitamins $Ca_3(PO_4)_2$ (2 g/100 g of feed); $CaCO_3$ (1 g/100 g); NaCl (0.5 g/100 g); $FeSO_4$ (12 mg/100 g); $ZnSO_4$ (9 mg/100 g); $CuSO_4$ (3 mg/100 g); $MnSO_4$ (3 mg/100 g); $CoSO_4$ (3 mg/100 g); KI (1 mg/100 g); vitamin A (500 IJ/100 g); vitamin D_3 (50 IJ/100 g). The first, control group, was fed by basic diet. In the second feeding group the basic diet was supplemented with tocopherol-acetate, 30 mg per kg of feed, in the third group with 0.3 mg of selenium as sodium-selenite per kg of feed, and in the fourth group both with 30 mg tocopherol acetate and 0.3 mg of selenium as sodium-selenite per kg of feed. The food and water were provided *ad libitum* to all the animals during the trial.

Due to certain technical problems, only three pigs (weighing 75±5 kg liveweight) were chosen from each group and slaughtered. To obtain sufficient sample for all analyses six strips of back subcutaneous fat tissue (ca 1.5 kg) were removed from each carcass at 6 h post mortem. The strips were packaged in polyethylene bags and stored at –20 °C prior to analyses. Unfrozen strips of adipose tissue were used to conduct three replications of rendering treatment.

1.2. Sample preparation

Defrosted strips of fat tissue were cut to small pieces and all parts of the skin and residual muscle tissue were removed. The homogenized fat samples were dry-rendered for one hour at 100 °C under vacuum. All solid particles were removed from lards by filtering. The lards obtained from the control group were divided into two batches. To the second batch commercial antioxidant BHT was added (0.01%).

1.3. Accelerated oxidation test

For determining the oxidative stability of lards obtained from differently fed pigs the oven test was used (WOLF, 1968). The lard samples (250 g) were heated at 60 °C in a glass-door oven, under constant outside artificial light (80 W). The light source was equally distanced from all the samples and the surface area/weight ratio was 4.5 $cm^2 g^{-1}$ in all samples.

The peroxide value, thiobarbituric acid number and fatty acid composition of the lard samples were determined in duplicate for each parameter at the beginning of the test, and at the time intervals of 18, 42, 60, and 90 h.

1.4. Chemical analyses

Lard peroxide values were determined according to AOAC standard procedure (AOAC, 1980). The results were expressed as milliequivalents of peroxide per kg of lard.

The lipid oxidation occurring in lard during accelerated oven test was also monitored by measuring the production of malonaldehyde (MAL) and other so-called thiobarbituric acid reactive substances (TBARS) in the 2-thiobarbituric acid (TBA) test. For the extraction of TBARS from lard samples, a distillation procedure of TARLADGIS and co-workers (1964) was used. The colored complex formed in the reaction between lard distillate and 2-TBA was developed in boiling water-bath for 35 min (TARLADGIS et al., 1960). The intensity of pink color was read at 532 nm on PYE UNICAM SP6 500 UV spectrophotometer, and compared with the values on standard absorbance curve. *In situ* formation of MAL by acid hydrolysis of 1,1,3,3-tetraetoxypropane (KE et al., 1984) was used for the standard curve preparation. The oxidation status of lards was reported as TBA number (=mg of MAL per kg of lard).

The analyses of fatty acid composition of lard was accomplished using gas chromatography method on a Varian 1400 Gas Chromatograph equipped with flame ionisation detector on stainless steel column packed with 6% LAC-3-R-728 on 80/100 acid washed Chromosorb G. The preparation of fatty acid methyl esters was done according to the procedure of METCALFE and SCHMITZ (1961).

Copper and calcium contents were determined in lard samples using the flame atomic absorption spectrophotometry after wet ashing of the samples.

The statistical analyses of the experimental data was performed using nonparametric analysis of variance (Kruscal-Wallis one-way analysis by ranks followed by Mann-Whitney test) which gives the possibility to calculate the level of significance between the differences among treatments in the sample of a small size (DANIEL, 1991).

2. Results and discussion

All lard samples obtained by dry-rendering of adipose tissue were of mild, characteristic flavour and white colour. The contents of minerals copper, and calcium as the ones having the highest prooxidant effect (according to TAMATE, 1990) was in lard samples 0.082, and 1.21 mg kg^{-1}, respectively, and they did not significantly vary among the lard batches, so their prooxidant effect was approximately equal in all lard samples.

Table 1

Fatty acid compositions of fresh lards (% of total fatty acids) ($\bar{x}^a$±SE)

Fatty acids	Control group	Vit. E-suppl. group	Se-suppl. group	Vit. E + Se-suppl. group
10:0	tr	0.3 ± 0.07	0.2 ± 0.03	0.2 ± 0.07
12:0	0.1 ±0.12	0.3 ± 0.10	0.2 ± 0.03	0.2 ± 0.03
14:0	1.6 ±0.34	2.0 ± 0.30	1.5 ± 0.31	1.6 ± 0.24
15:0	0.2 ±0.07	0.2 ± 0.03	0.2 ± 0.05	0.2 ± 0.03
16:0	24.8 ±1.16	24.8 ± 1.77	23.2 ± 1.38	23.5 ± 1.18
16:1	2.9 ±0.79	2.0 ± 0.11	2.6 ± 0.46	1.9 ± 0.27
17:0	0.9 ±0.11	0.8 ± 0.43	1.5 ± 0.21	0.6 ± 0.28
17:1	0.7 ±0.07	0.7 ± 0.33	1.3 ± 0.12	0.6 ± 0.07
18:0	13.0 ±0.65	13.9 ± 0.79	14.4 ± 0.53	13.9 ± 1.41
18:1	40.2 ±1.78	41.5 ± 0.25	39.7 ± 0.92	37.9 ± 0.46
18:2	13.3 ±1.19	11.7 ± 1.37	14.4 ± 2.10	16.7 ± 1.94
18:3	0.5 ±0.12	0.4 ± 0.12	0.7 ± 0.07	0.2 ± 0.05
20:0	0.2 ±0.07	0.2 ± 0.00	0.2 ± 0.05	0.6 ± 0.07
20:2	0.8 ±0.19	0.7 ± 0.09	0.2 ± 0.03	0.8 ± 0.09
20:4	0.4 ±0.07	0.4 ± 0.04	0.5 ± 0.21	0.7 ± 0.21
22:0	0.2 ±0.05	0.2 ± 0.03	0.1 ± 0.00	0.3 ± 0.07
Peroxide value	1.82 ±0.528	1.00 ± 0.000	1.61 ± 1.021	0.98 ± 0.043
TBA number	0.97 ±0.723	0.30 ± 0.100	0.67 ± 0.378	0.50 ± 0.100

[a] The results represent mean values of six determinations (2 duplicate analyses × 3 replications)

At the beginning of the accelerated storage test, the oxidative status of all lard samples was low and did not significantly vary among the groups. The peroxide values were below 2 mEq kg^{-1} and TBA numbers below 1 mg MAL kg^{-1} in all the samples. Dietary supplementation of feed with vitamin E and/or selenium had no significant effect on lard fatty acid composition in fresh lard, as could be seen from Table 1. Oleic and palmitic acids were the most abundant fatty acids. Polyunsaturated fatty acids content (PUFA) in all lard samples was in the range of 13.2–18.4% of all fatty acids, and the variations were greater within the groups than among them.

The changes in peroxide and TBA values, as well as changes in polyunsaturated fatty acid content in lards during the oven test are presented in Figs. 1–3. The lard samples from the control group showed the most pronounced peroxidation throughout the whole experiment. The lard samples obtained from the Se-supplemented pigs had

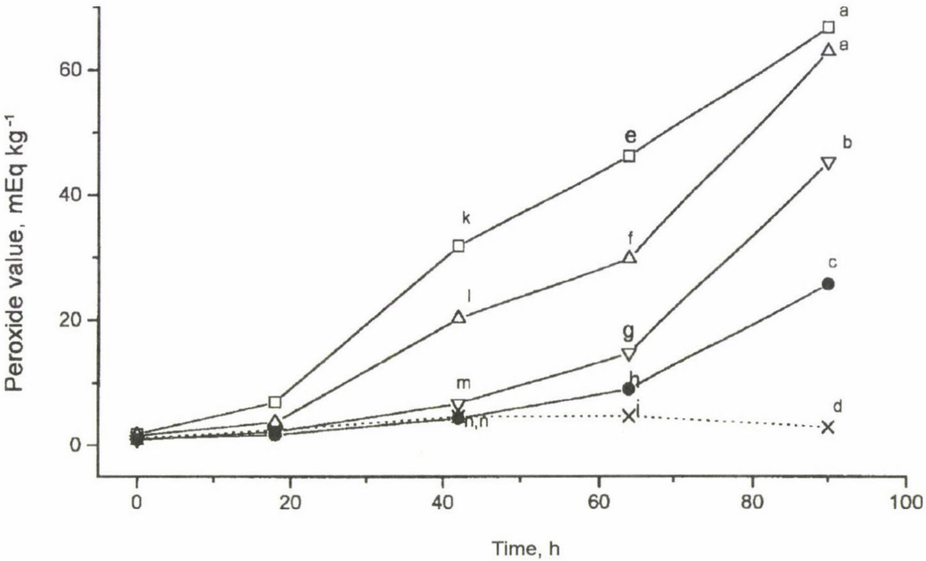

Fig. 1. Changes in lards' peroxide values during oven test. The conditions of accelerated storage of lard samples were: heating at 60 °C in a glass-door oven, under constant outside artificial light (80 W); the surface area to weight ratio was 4.5 $cm^2 g^{-1}$ in all samples. Peroxide value of lard samples (5.00 g) was investigated at the beginning of the oven test and after 18, 42, 60 and 90 h. The method of evaluation was titration with 0.1 mol dm^{-3} sodium-thiosulfate solution after the reaction of peroxides with potassium iodide in acidic medium. All analysis were done in duplicate. a,b,c...: Peroxide values assigned with no common letters were significantly different. □: Control group; •: Vitamin E supplemented group (30 mg kg^{-1} of feed); Δ: selenium supplemented group (0.3 mg kg^{-1} of feed); ∇: Vitamin E + selenium supplemented group (30 mg + 0.3 mg kg^{-1} of feed); x: control group of lards protected with BHT (0.01%).

significantly lower peroxide values than the control group samples during the first 64 h of heating, but after 90 h their values were equalized, so that at the end of the oven test dietary selenium showed no protective effect against peroxide formation in lards. At the same time dietary selenium showed significant protective effect in comparison to the control group against TBARS formation in lards throughout the whole experiment, as shown in Fig. 2.

Dietary vitamin E supplementation to the experimental animals showed an excellent antioxidative protection effect on lards within 42 h of exposure to 60 °C, and during this time peroxide and TBA values were in good correlation with the same values of BHT protected samples. After 42 h the level of oxidation in vitamin E-supplemented samples increased in comparison to BHT samples, but was markedly lower than in lards from all other groups. After 90 h of heating lards, dietary vitamin E had approximately 70% of BHT protective potential, according to both peroxide and TBA values.

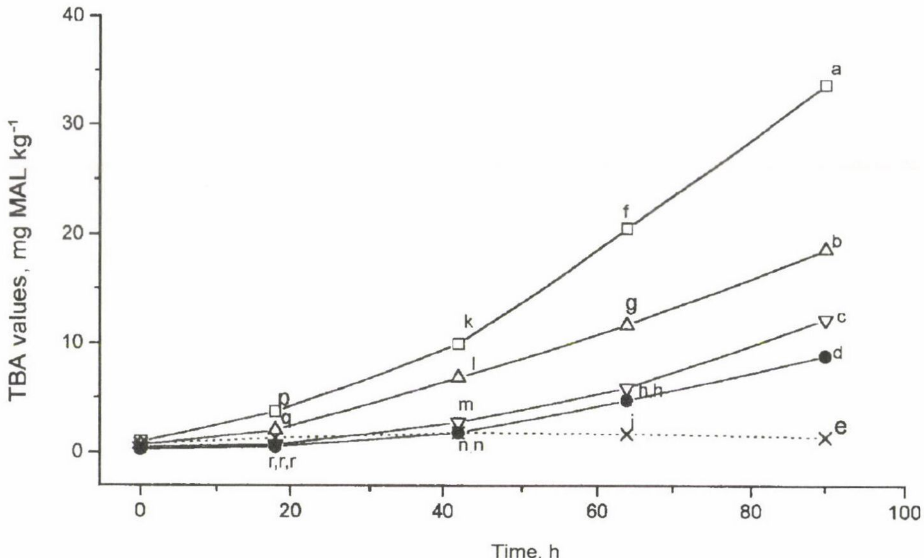

Fig. 2. Changes in lards' TBA values during oven test. After the distillation of TBARS from lard samples (5.00 g) 5 cm^3 of the distillate reacted with 5 cm^3 of 0.02 mol dm^{-3} 2-thiobarbituric acid on the boiling water-bath for 35 min and the light absorption of the formed colored complex was measured at 532 nm against the values from standard curve. The standard curve was prepared by in situ formation of MAL by acid hydrolysis of 1,1,3,3-tetraetoxypropane. a,b,c...: TBA values assigned with no common letters were significantly different. □: Control group; ●: Vitamin E supplemented group (30 mg kg^{-1} of feed); Δ: selenium supplemented group (0.3 mg kg^{-1} of feed); ∇: Vitamin E + selenium supplemented group (30 mg + 0.3 mg kg^{-1} of feed); x: control group of lards protected with BHT (0.01%).

The combination of dietary vitamin E and sodium-selenite had more pronounced antioxidative effect in lards than dietary sodium-selenite alone, but at the same time it was significantly less effective than dietary vitamin E alone. The overall oxidative stability of lard was influenced by dietary vitamin E and selenium supplementation in the following order: no dietary supplementation < sodium-selenite < sodium-selenite + vitamin E < vitamin E.

In contrast to the results of peroxide and TBA tests, the fatty acid composition of all lard samples did not significantly change during the experiment. The content of PUFAs showed slight and insignificant decrease towards the end of the test (2–7%) in all lard samples examined (Fig. 3).

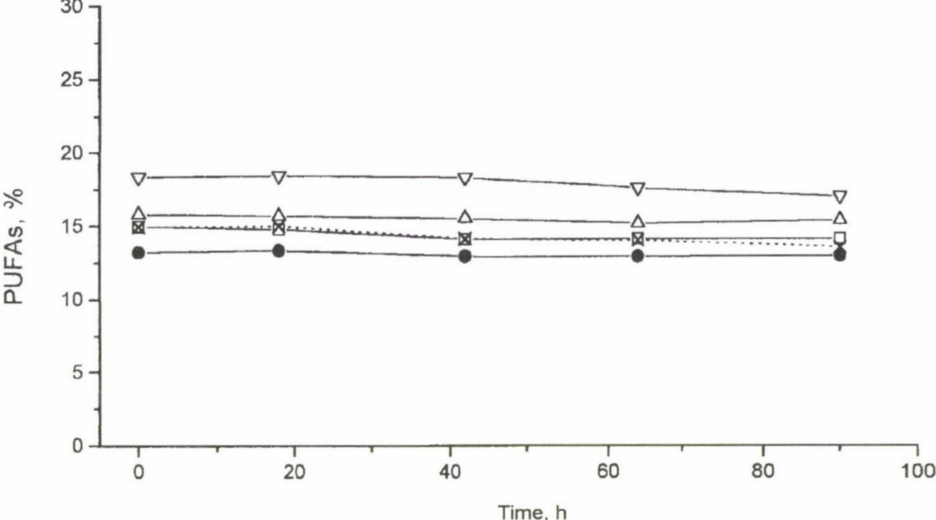

Fig. 3. Changes in PUFAs' content of lards during oven test. After the saponification of lipids fatty acids were extracted and esterified with methanolic boron trifluoride solution. The obtained fatty acid methyl esters in hexane solution were directly injected into the column for gaschromatographic analysis. The conditions of gas-chromatography were:

– 150 cm stainless steel column packed with 6% LAC-3-R-728 on 80/100 acid washed Chromosorb;
– column temperature: 185 °C (isothermally);
– injection port temperature: 200 °C;
– flame ionization detector temperature : 210 °C;
– nitrogen flow: 23 cm^3 min^{-1}.

Fatty acids were identified by comparison of retention times with those of standards. Amounts of each fatty acid were calculated by the peak area method. □: Control group; ●: Vitamin E supplemented group (30 mg kg^{-1} of feed); Δ: selenium supplemented group 0.3 mg kg^{-1} of feed); ∇: Vitamin E + selenium supplemented group (30 mg + 0.3 mg kg^{-1} of feed); x: control group of lards protected with BHT (0.01%).

It has long been recognized that fat-soluble tocopherols can function as antioxidants in different tissues (FARRELL, 1988). The phenolic group of tocopherols reacts as an electron donor, thus interrupting free radical reactions that otherwise can cause PUFA damage and formation of numerous peroxidation products. Directly added natural tocopherols or α-tocopheryl-acetate to the food products showed no consistent results in suppressing oxidation processes (MARKUZE, 1966; ST ANGELO et al., 1988; RAMANATHAN & DAS, 1992; WU et al., 1994). In recent years more interest has been focused on increasing the oxidative stability of foods of animal origin by supplementing the animal feed with antioxidative substances. Dietary tocopherol supplementation results in an elevated level of vitamin E in all cell membranes. This tissue accumulation of vitamin E appears to occur in a dose – and duration – dependent manner (ARNOLD et al., 1993). On the other hand, selenium involved in the enzyme glutathione-peroxidase activity occurs in cytosol and mitochondrial matrix of the cells and tissues of the body. This enzyme functions as a part of a mechanism responsible for the metabolism and detoxification of oxygen (NEVE, 1991). Dietary selenium supplementation results in an elevated level of selenium mainly in muscle tissue and selected organs such as liver, kidneys, heart (EKHOLM et al., 1991). According to ARC recommendation from 1981, and JANSEN and co-workers (1988) vitamin E requirement for growing pigs are 20–50 mg kg^{-1} feed. The selenium requirement for growing pigs has been proposed to be 0.1–0.4 ppm (A.R.C. 1981; MAHAN & MOXON, 1978). The selenium content of locally produced feeds in Serbia is low because the natural concentration of selenium in local soils is low (MAKSIMOVIĆ et.al., 1992). The chosen levels of vitamin E and selenium supplementation in the experiment are generally considered to be adequate to meet pig's requirements for these two nutriments and at the same time not to induce over-exposure of selenium (SHAMBERGER, 1983).

Data presented in several publications show that dietary vitamin E supplementation, above the dietary requirement level, suppresses lipid oxidation in various pork products (BUCKLEY et al., 1989; ASHGAR et al., 1991; MONAHAN et al., 1992). TSAI and co-workers (1978) reported an increase in the stability of adipose tissue with 50–200 ppm long-trial vitamin E supplementation of pigs feed. The results of GRAU and FLEISCHMANN (1966) for lard, made with 100 ppm of dietary vitamin E, were similar. According to the results of our experiment, long-trial supplementation of pig diets with only 30 mg of vitamin E/kg of feed showed excellent antioxidative effects in lards after their exposure to air, light, and increased temperature for 90 h. At the end of the experiment the level of TBARS and peroxides in vitamin E protected lard samples, was 4 and 2.5 times lower, respectively, than in the samples from unsupplemented animals. The antioxidative activity of dietary vitamin E in lard was comparable with the effect of 0.01% BHT in the interval of 60 h of heating.

The protective effect of dietary selenium on muscle tissue oxidative stability was reported by DE VORE and co-workers (1983) and COX and co-workers (1993). The adipose tissue is commonly rich in lipophilic vitamin E, but is generally low in selenium (SHAMBERGER, 1983), so that selenium is of lower importance as an antioxidative cellular agent in adipose tissue. Data obtained in this experiment indicated that dietary selenium at a level of 0.3 $mg\,kg^{-1}$ of feed could not be used as a lard antioxidant, though it had small protective potential, as showed in TBA test, in comparison to the selenium deficient samples.

3. Conclusions

The results of this study indicate that nutrition of pigs with feed deficient in vitamin E and selenium results in lards highly susceptible to oxidative deterioration. Long-term supplementation of feed with 30 $mg\,kg^{-1}$ of vitamin E, which is often used in commercially available diets, is highly effective in increasing oxidative stability of lard in short-term storage. Dietary supplementation with 0.3 $mg\,kg^{-1}$ of selenium as well as the combination of selenium and vitamin E, which is also used in agricultural practice, have significantly lower antioxidative potential, but still enhances lard oxidative stability compared to a nonsupplemented diet.

LIST OF ABBREVIATIONS:

FA: fatty acid
PUFA: polyunsaturated fatty acid
TBARS: thiobarbituric acid reactive substances
MAL: malonaldehyde
2-TBA: 2-thiobarbituric acid
TBA: thiobarbituric acid
BHT: butylated hydroxytoluene

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EPICHLOROHYDRIN ACTIVATED CELLULOSE BEADS FOR THE IMMOBILIZATION OF ALKALINE PROTEASE

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An effective support, cellulose beads were prepared for the immobilization of alkaline protease using the bifunctional agent epichlorohydrin. The specific activity retained by the immobilized alkaline protease was 13.85% when assayed at pH 8.5 using casein as a substrate.The optimum pH shifted towards alkaline side by 0.5 unit as a result of immobilization. The optimum temperature of the cellulose bound alkaline protease increased by 5 °C when compared to the free enzyme.The thermal and pH stability were improved by immobilization.

Keywords: cellulose beads, immobilization, alkaline protease, epichlorohydrin

Natural and synthetic materials are widely used as supports for immobilizing biologically active agents (CHEN & TSAO, 1976; KRAJEWSKA et al., 1990 and CHELLAPANDIAN & SASTRY, 1994). Among the natural supports cellulose, alginate, chitin, chitosan, starch and dextran are used for the immobilization of enzymes with or without modification of physical and chemical properties (CHEN & TSAO, 1977; SUNDBERG & PORATH, 1974; PORATH et al., 1971; PETACH & DRISCOLL, 1994; LENDERS et al., 1985; KRAJEWSKA, 1991 and KAMBOJI et al., 1996). Cellulose can be effectively used as a support because it is cheap, chemically stable, resistant to microbial contamination and physically can easily be modified to the required form. However, the fibrous shape of cellulose lacks the mechanical properties and the poor flow through properties result in high pressure drop and channeling in packed bed reactors.The above difficulties can be overcome by modifying the physical form of cellulose in the form of beads.

In the present investigation cellulose beads were prepared and alkaline protease was immobilized using epichlorohydrin as coupling agent.The effects of various factors such as epichlorohydrin concentration, immobilization pH, enzyme concentration and coupling time were determined on the activity of alkaline protease-cellulose beads.The

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properties like thermal and pH stability, temperature and pH optimum of the free and immobilized alkaline protease were compared.

1. Materials and methods

1.1 Materials

Cellulose acetate, trichloroacetic acid, dimethyl sulfoxide, Folin-phenol reagent from M/s. SD Fine Chemical, India: casein, BSA, tyrosine, epichlorohydrin from M/s. SRL, India; alkaline protease (*Bacillus licheniformis*) from M/s. NOVO Industries, Denmark were used in this investigation.

1.2 Preparation of cellulose beads

Six % (w/v) solution of cellulose acetate was prepared by dissolving cellulose acetate in water miscible organic solvent (a mixture of acetone and dimethyl sulfoxide in the ratio of 6:4). The solution was then dispersed into a water phase, when the dispersed droplets were brought in contact with water, the liquid droplets coagulated and porous particles were formed.The cellulose acetate was then regenerated to cellulose by hydrolyzing with 1*N* sodium hydroxide at 60 °C for one hour.Then the cellulose beads were thoroughly washed with distilled water.

1.3 Immobilization of alkaline protease on cellulose beads

One gram of cellulose beads was mixed with 10 volumes (w/v) of 2% epichlorohydrin in 1*N* sodium hydroxide in the presence of sodium borohydride at 60 °C for 2 h. Then the cellulose-epichlorohydrin conjugate was thoroughly washed with distilled water to remove the excess epichlorohydrin. The epichlorohydrin activated cellulose was treated with 15 volumes (w/v) of borate buffer (pH 8.5) and 5 volumes of alkaline protease solution (50 mg) and was kept for 2 h with occasional shaking at room temperature. The excess and unbound enzyme was removed by washing with 1*N* sodium chloride and distilled water. Finally, the immobilized enzyme was washed with borate buffer (pH 8.5) and stored at 4 °C in borate buffer.

1.4 Estimation of protein and proteolytic activity

The amount of protein coupled with the epichlorohydrin activated cellulose beads was estimated by Folin-phenol method (LOWRY et al., 1951). The activity of the free and immobilized alkaline protease was estimated using casein as a substrate (CHELLAPANDIAN & SASTRY, 1994).

1.5 Properties of free and immobilized alkaline protease

The properties of immobilized alkaline protease were compared with the free enzyme. To find the optimum temperature, the activity of free and immobilized alkaline protease was assayed at increasing temperature in the range of 30 to 70 °C. The thermal stability was determined by exposing the free and immobilized alkaline protease to various temperature (40 to 70 °C) for 30 min. Then the samples were rapidly cooled to 40 °C and then assayed for activity. The optimum pH of the free and immobilized alkaline protease was determined by varying the substrate pH in the range of 6.5 to 11.0 using suitable buffers (0.2M citrate-phosphate buffer, phosphate buffer-mono and dibasic sodium phosphate, borate buffer – boric acid and borax and carbonate – bicarbonate buffer). The pH stability was determined by incubating the free and immobilized alkaline protease in different buffer solutions of varying pH from 5 to 11 for 2 h before assaying for activity.

2. Results and discussion

All experiments were performed in duplicate and the mean values are represented in the figures and the deviation (less than ±2%) are shown in the graphs. The immobilization of alkaline protease involves covalent attachment of the amino group to an available epoxy group present in the epichlorohydrin activated cellulose beads. The maximum activity of the immobilized alkaline protease was obtained at an epichlorohydrin concentration of 3.0 to 3.75%, a pH of 9 to 10, 50 mg of enzyme and enzyme coupling time of 180 min. At the above mentioned conditions, the amount of alkaline protease coupled was found to be 12.06 mg g^{-1} of cellulose beads. The activity of free and immobilized alkaline protease was assayed using casein and haemoglobin as substrate and the immobilized alkaline protease immobilized on anion exchanger retained 13.85% and 18.15% of its original activity with respect to casein and hemoglobin. It was reported that alkaline protease immobilized on anion exchanger retained only 12% (OHMIYA et al., 1978), trypsin on cellulose-g-co-HMA activated by p-benzoquinone retained only 6.25% (GUILBAULD & MASCINI, 1986) and papain on modified polysulfone membrane and hydroxy ethyl coated polysulfone hollow fibers retained only 12% and 25% of its original activity (GANAPATHI et al., 1995). However, cellulase immobilized on Fe_3O_4 retains higher activity of the immobilized enzyme depending on the amount of enzyme coupled, characteristics of the support and the exact conditions employed for coupling of the enzyme.

Normally, the enzyme activity increases with increase in temperature up to a certain point after which due to denaturation of the enzyme protein, a decline in the activity will be observed. The optimum temperature for both free and immobilized

alkaline protease was determined by varying the substrate temperature (30 to 70 °C) using casein as substrate. Results on the effect of temperature on cellulose bound alkaline protease and free alkaline protease are shown in Fig. 1. The optimum temperature of the immobilized alkaline protease was found to be 65 °C and for the free enzymes it was 60 °C. The change in the optimum temperature of the immobilized alkaline protease may be due to resistance to denaturation. Similar results were obtained when invertase was immobilized on acylchitosan (KUSAOKE et al., 1987) and endo-polygalacturonase was coupled onto trimaley chitosan (PIFFERI et al., 1989).

The thermal stability of the free and immobilized alkaline protease was estimated by measuring the residual activity of the alkaline protease.The variation in thermal stability based on relative activity of the free and immobilized alkaline protease as a function of temperature to determine the effect of treatment (immobilization) is shown in Fig. 2. From the figure it was observed that the thermal stability of alkaline protease improved by immobilization. The thermal stability of the free and immobilized alkaline protease was found to be 17.19% and 32%, respectively at 70 °C (Fig. 2). The enhanced thermal stability of the immobilized enzyme is attributed mainly to the prevention of autodigestion and multipoint attachment to a carrier (GOLDSTEIN, 1973).

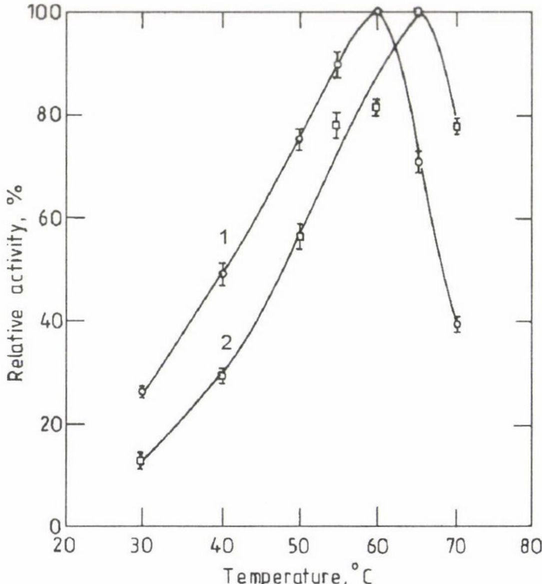

Fig. 1. Optimum temperature of the free and immobilized alkaline protease. 1: Free enzyme; 2: immobilized enzyme

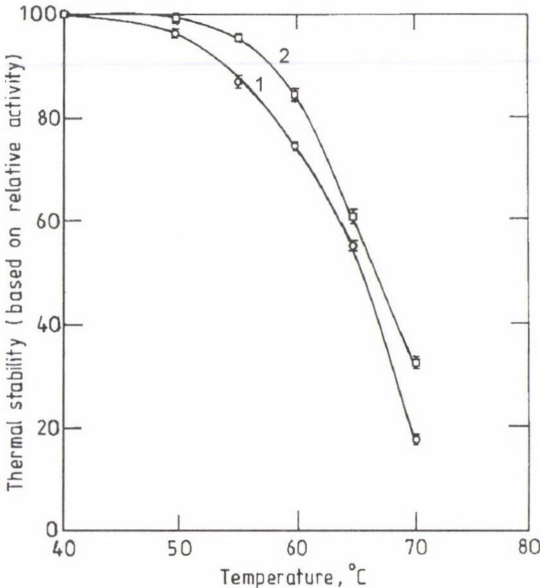

Fig. 2. Thermal stability of the free and immobilized alkaline protease based on relative activity. 1: Free enzyme; 2: immobilized enzyme

Alkaline protease immobilized on nylon (CHELLAPANDIAN & SASTRY, 1994) and β-amylase bound to cellulose (MAEDA et al., 1978) showed increased thermal stability. From the graph it can also be observed that the change in relative activity of free enzyme on account of treatment is significant except at 50 and 65 °C.

In order to determine the optimum pH, the free and immobilized alkaline protease were assayed by varying the substrate pH from 6.5 to 11 by the addition of suitable buffer (0.2M citrate-phosphate, phosphate, borate and carbonate-bicarbonate buffer). The variation of relative activity as a function of pH for the free and immobilized alkaline protease is shown in Fig. 3. It is observed from the figure that the optimum pH of the immobilized alkaline protease shifted towards alkaline side by 0.5 units (8.5–9.0 to 9.5) when compared to the free enzyme. The shift in optimum pH may be due to interaction between the charged groups on the support material and hydrogen ion in the substrate solution (ERARSLAN & GURAY, 1991). Similar results were observed for penicillin acylase immobilized on DEAE-cellulose (WARBURTON et al., 1972; 1973) and for catalase immobilized on alumina (TARHAN & ULSAN, 1990). It was also observed from Fig. 3 that the activity of the free enzyme was better in comparison

with the immobilized enzyme at a lower pH range (6.5 to 9.0). However, the relative activity of the immobilized enzyme was higher in the pH range from 9.5 to 11.

The pH stability of the enzyme is an important criterion for the proper storage of immobilized enzyme. In the present study, the pH stability of the free and immobilized alkaline protease was studied by incubating in suitable buffer (pH 5 to 11–0.2M citrate-phosphate; phosphate, borate and carbonate-bicarbonate buffer) for 2 h at 30 °C and assay was carried out at standard conditions. Figure 4 shows that the free enzyme shows maximum stability at pH 8.0 to 9.0 whereas the maximum stability of the immobilized enzyme was observed at a pH range of 7.50 to 9.0. However, the pH stability of the immobilized alkaline protease was better in the range of 6.5 to 9.0 when compared to the free enzyme.

The working stability and storage stability of the alkaline protease immobilized on cellulose were found to be good. The working stability of the immobilized alkaline protease was studied in a batch reactor hydrolysing casein for 3 h and then washed with borate buffer (pH 8.5). The above process was repeated five times and at the end of fifth run the alkaline protease immobilized on cellulose retained 84% of its original activity. When the immobilized alkaline protease was stored at 44 °C and 25 °C for 60 days, it retained almost 86% and 72% of its original activity, respectively.

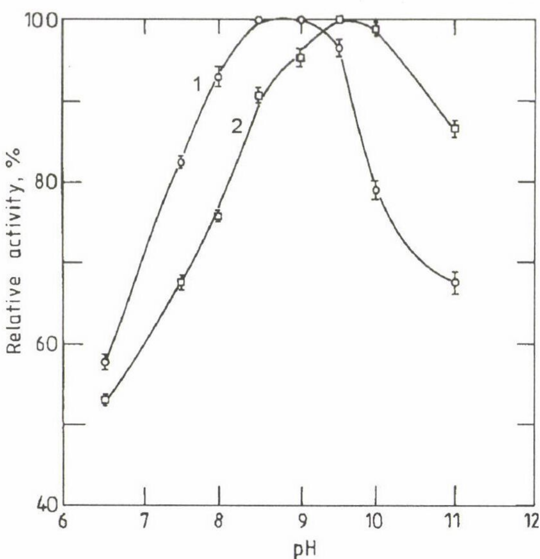

Fig. 3. Optimum pH of the free and immobilized alkaline protease. 1: Free enzyme; 2: immobilized enzyme

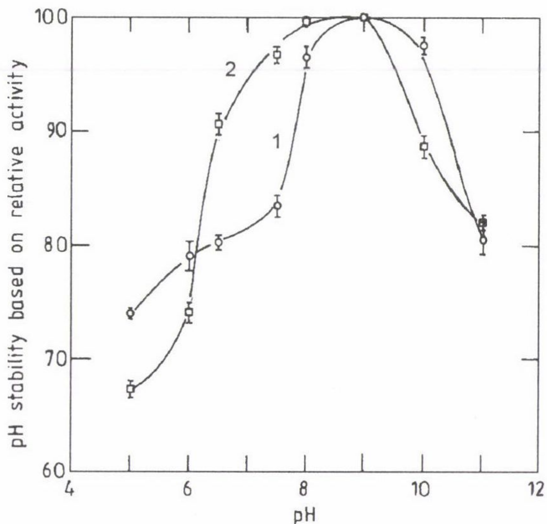

Fig. 4. pH stability of the free and immobilized alkaline protease based on relative activity. 1: Free enzyme; 2: immobilized enzyme

3. Conclusion

Cellulose beads were successfully used for immobilization of alkaline protease in the presence of epichlorohydrin. The immobilized alkaline protease showed higher thermal stability and pH stability when compared to free enzyme. Optimum temperature and pH of the alkaline protease were altered by immobilization.

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PRODUCTION AND USE OF SPROUTING WHEAT SEEDS AS A BIOLOGICALLY VALUABLE FOOD

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Selected samples of wheat seeds (varieties: Balkan, Srbijanka, Rana niska and Evropa) were soaked in distilled water for 24 h then germinated at room temperature for 3 to 5 days. The sprouts were removed and separated into three fractions (root, culm and grain) manually. The yields of root and culm ranged from 4.4 to 8.2% (calculated on dry matter of initial seed).

After drying the gross chemical composition (moisture, ash, raw protein, invert sugar, starch, cellulose, fat), amino acid composition and mineral components (Na, K, Mg, Ca, Fe, Cu, Zn, Mn) were determined.

High protein contents with increased levels of threonine, methionine and lysine (compared to the original seed) and also an increased quantity of magnesium and zinc make the sprouts a valuable source of nutrients and a potential additive for enrichment of some foods (e. g. pies, pasta, sausages, soup etc.).

Keywords: wheat, germination sprouts, chemical composition of sprouts

The overall efforts to improve and make the use of cereals as foods more effective resulted in a growing interest on the possibilities of using cereal germs and sprouts in the human nutrition (OLIVER, 1975; NOCKER, 1983; CHRISTIAN & GREGER, 1985; BUSTORF-HIRSCH, 1987; ŠIBALIĆ, 1988). The production of sprouts includes a process of germination by which components of grain are transformed to components more nutritious for humans (HIATT, 1973; DOZET et al., 1990; ŠARIĆ et al., 1992). These ingredients have potential for the enrichment of human diets which may be deficient in some nutrients (NOCKER, 1983; GRAČAN, 1987; ŠARIĆ et al., 1992).

To produce a high quality sprout, it is necessary to pay attention to the choice of suitable cereal cultivar, to growing conditions, including soil management and weed control and on farm storage (DOZET et al., 1990).

The aim of our research project is to develop adequate technology for sprout production and preservation and for the use of cereal grain sprouts as a valuable food. In this paper we summarize some results about the chemical composition of wheat sprouts.

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1. Materials and methods

1.1. Choice of raw material

Tests were carried out on four Yugoslav wheat varieties, belonging to different technological groups of quality, i.e. Balkan – belonging to the improvers, Srbijanka and Rana niska – belonging to bread wheat varieties and Evropa 90 – a basic variety.

1.2. Germination procedure

Relative humidity of air was maintained at a level higher than 70 %. Soaking of samples was carried out in distilled water. Pouring of water and rinsing during germination were carried out twice a day. Samples were germinated on perforated devices to make rinsing and removal of surplus liquid possible. Germination lasted for 5 days as it was published by ŠARIĆ and co-workers (1992).

The sprouts were removed and dried at 40 °C and then carefully separated into three fractions (root, culm and grain) manually (ŠARIĆ et al., 1992).

1.3. Determination of germination ability and energy

The standard method of investigation of the quality of seed was used (YUGOSLAV REGULATIONS, 1987). Data represent mean values of three replicates. Statistics was done according to HADŽIVUKOVIĆ (1979).

1.4. Determination of chemical composition

All samples and fractions were tested using standard A.O.A.C. (1970) procedures in order to determine the contents of protein, fat, invert sugar, starch and cellulose. Statistics was done according to HADŽIVUKOVIĆ (1979), too. Mineral components were determined according to A.O.A.C. (1970) procedures, and the amino acid composition was determined using an amino acid analyzer after acid hydrolysis.

2. Results and discussion

2.1. Choice of cereal grains

The choice of suitable raw material is a necessary requirement for the development of any food technology. Numerous cereal varieties and hybrids were tested for their energy of germination and the ability to germinate (ŠARIĆ et al., 1995). Visual observation of samples tested and the way how they germinate made it possible to choose the best varieties and hybrids for germination.

2.2. Choice of variety

In order to choose a variety of wheat most suitable for the production of sprouts, the energy of germination and the ability to germinate were determined. Varieties Rana niska and Evropa 90 were slightly superior (Table 1), in comparison to others; the former exhibited better germination energy and germination percentage and the latter had a higher yield of sprouts, but high losses, too, (Table 2). According to the statistical data, energy of germination and germination percentage are independent of the wheat variety (Table 1).

Table 1

The germination energy and germination percentage of some wheat varieties

Wheat variety	Energy of germination (%)	Germination (%)	Length of germ culm (cm) after	
			3 days	5 days
Balkan	89	99	0.2–0.5	4–5
Srbijanka	83	96	1.0–1.5	3–4
Rana niska	93	97	0.5–1.0	3–4
Evropa 90	91	95	1.0–1.5	5–6
Standard deviation	3.84	1.60		
Coefficient of variation	4.31	1.70		

Table 2

Yields and losses during germination

Wheat variety	Yields of culm and root (% d.m. of the initial material)	Losses of culm and root (% d.m. of the initial material)
Balkan	7.2	13.5
Srbijanka	7.1	5.0
Rana niska	8.2	5.8
Evropa 90	4.4	13.0
Standard deviation	1.4	3.9
Coefficient of variation	20.1	42.0

Table 3

The chemical composition of wheat sprouts compared to the initial sample

Indicator	Dry kernel			Dry fraction of sprouts									Statistics: F-distribution	
				Root			Culm			Kernel				Probability 0.05
	$\bar{x}$	±s	v	$\bar{x}$	±s	v	$\bar{x}$	±s	v	$\bar{x}$	±s	v	Calculated	and r_1=3, r_2=17 degree of freedom
Moisture (%)	11.4			10.9			13.4			9.2				
Ash (% d.m.)	1.72	0.10	5.81	4.96	0.51	10.28	4.99	0.61	2.45	1.44	0.13	8.75	24.78	3.20
Raw protein (% d.m.)	14.0	0.45	3.24	28.79	6.10	21.20	34.88	2.12	6.08	12.56	1.10	8.76	8.43	3.20
Invert sugar (% d.m.)	2.3	0.38	16.52	11.85	2.33	19.80	27.39	3.07	11.20	12.81	1.74	13.60	17.77	3.20
Starch (% d.m.)	68.9	1.28	1.86	5.17	4.50	87.04	0.0			67.20	2.48	3.69	153.35	3.20
Cellulose (% d.m.)	1.98	0.27	13.64	15.89	1.19	7.49	5.57	1.80	32.30	1.83	0.84	45.90	24.17	3.20
Fats (% d.m.)	3.32	0.27	8.13	1.57	0.48	30.81	3.31	0.70	21.24	2.69	0.31	11.50	2.07	3.20

$\bar{x}$: Mean value of five replicates;
±s: standard deviation;
v: coefficient of variation, %

2.3. Conditions of germination

The average yields of germ culms and roots for three varieties Balkan, Srbijanka and Rana niska ranged between 7.1 and 8.2. Evropa 90 had the lowest yield because of the greatest sprout length (Table 2). Therefore, varieties that germinated fast exhibit great losses. Coefficient of variation proved that yields and losses of culm and root depend much on the wheat variety (Table 2).

2.4. Chemical and biochemical properties of sprouts

Sprouts, particulary culm and root, are rich in protein (Table 3). During germination an increase of invert sugar is evident contributing to their slightly sweet taste.

Statistical data, especially high values for coefficient of variation (Table 3), indicate that chemical changes in germinated seeds are very intensive, and besides wheat characteristics depend on the length of root and culm (DOZET et al., 1990).

High F-distribution values, (Table 3) prove that there are statistically significant differences between ash, protein invert sugar, starch and cellulose content in dry kernel and dry fractions of sprouts.

Sprouts, especially root and culm are potentially beneficial foods because of increased levels of magnesium, zinc and iron (Table 4).

The amino acid composition of fractions after germination (Table 5) and a high protein content with an increased level of essential amino acids such as: threonine, methionine and lysine make sprouts a valuable source of nutrients. As ŠARIĆ and co-workers (1995) stated, they may be useful, good additives for the enrichment of some foods (i.e pies, pasta, salad dressing, soup, sausages, etc.)

Table 4.

The content of mineral components in dry matter of wheat

Element	Dry grain	Root & culm	Sprout
Macro element (mg/100 g)			
Na	21.98	150.3	20.42
K	495.54	1958.3	428.34
Mg	121.45	173.8	136.95
Ca	0.061	0.272	0.113
Trace element (µg/100 g)			
Fe	43.13	67.34	41.68
Cu	8.02	28.99	8.49
Zn	40.95	126.69	43.21
Mn	44.18	55.09	42.78

Table 5.

Amino acid composition of sprouts (% d.m.)

Amino acid	Dry grain	Fresh sprout	Fresh root & culm
Aspartic acid	1.19	2.89	5.68
Threonine	0.66	1.32	2.19
Serine	0.73	1.39	1.72
Glutamic acid	3.55	6.37	3.73
Proline	1.66	2.92	2.96
Glycine	0.60	1.26	1.89
Alanine	0.62	1.41	3.02
Methionine	0.20	0.56	2.19
Isoleucine	0.71	1.32	2.25
Leucine	1.02	2.31	3.49
Tyrosine	0.81	1.84	3.91
Phenylalanine	0.95	2.09	3.25
Histidine	0.84	2.09	4.38
Lysine	0.70	1.50	2.90

3. Conclusions

Grain sprouts have significant importance as valuable food products, and also as supplements to individual products to improve the biological and nutritive value. Chemical and biochemical analysis and sensory evaluations of sprouts indicate a possibility to apply the sprouts in the human diet successfully.

The addition of fresh sprouts to salad, soup, yoghurt and other meals would improve their nutritive value. Sprouts can be used fresh, but also dried as additives for the production of pies, pasta, sausages, etc.

There is a significant change in chemical composition and nutritive value unambiguously indicating an increase in proteins and invert sugar and decrease in starch in root and culm, as well as the increase of magnesium level which is an essential component of many enzymes. The increase of iron and zinc content is also important because the former is a constituent of hemoglobin and the latter contributes to cellular immunity.

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DEGRADATION OF LOW MOLECULAR WEIGHT FRAGMENTS OF PECTIN AND ALGINATES BY GAMMA-IRRADIATION

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Low molecular weight (LMw) fragments of polygalacturonic acid (PGA) obtained by enzymatic degradation, as well as low molecular weight fragments of pectin (methyl esterified polygalacturonic acid) and alginates obtained by mechanolysis have been used to study the mechanism of radiation-induced degradation.

By means of high performance anion-exchange chromatography (HPAEC), the degradation of specific entities induced by γ-irradiation and the formation of new compounds were monitored. The LMw fragments of PGA, pectin and alginates showed an increasing reduction in the degree of polymerisation upon exposure to increasing irradiation doses. An alginate with a high mannuronic to guluronic acid ratio appeared to be more sensitive towards irradiation than an alginate having a low ratio. Degradation of the PGA fragments by irradiation can be ascribed to hydrolytic cleavage of glycosydic bonds, although HPAEC analysis revealed that also other, unknown products were released during the irradiation process. Storage of various irradiated PGA fractions at –20 °C did not affect significantly their average molecular weight and had no effect on their HPAEC elution pattern.

Keywords: pectin, alginate, oligosaccharides, HPAEC, degradation by γ-irradiation

In a contribution to the International Workshop on Food Irradiation, DELINCÉE (1989) pointed out that radiation chemistry of food components is important for the understanding of possible changes in foodstuffs preserved by ionising radiation. So, understanding of radiation chemical principles will help in the design of processing methods to ensure that high quality foodstuffs be obtained, make it possible to extrapolate the changes resulting from irradiation in one food to another and also help in the development of control methods for identifying whether a particular food has been irradiated or not.

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A nice overview of the radiolytic products derived from carbohydrates after irradiation is given by DELINCÉE (1989). The γ-irradiation of fruits and vegetables results in changes in the physico-chemical characteristics of the tissue (MURRAY, 1990; CLARKE, 1961; YASIA et al., 1987; SJÖBERG, 1987) although the exact mechanisms involved are not clear, and revealing these mechanisms is extremely difficult since it was impossible to distinguish between primary and secondary reactions within the complex plant tissue (SIMIC, 1983; BEYERS et al., 1983). For this reason, the study of model systems of polysaccharides, which are important cell wall components of plant raw materials, is essential in revealing the mechanisms involved. This has been carried out for storage polysaccharides like starches (BACHMAN et al., 1987). SKINNER and KERTÉSZ (1960) already investigated the plant cell wall component pectins, and based on viscosity measurements, they suggested a random hydrolytic fissure of the glycosidic linkages.

This study was aimed at a better understanding of the degradation by irradiation of pectins, important cell wall components of plant raw materials. To be able to also investigate the effect of different constituent uronic acid moieties, two structurally different anionic polysaccharides e.g. pectins and alginates were chosen as model substrates, while advanced chromatographic techniques were used to monitor the reaction mixtures.

1. Materials and methods

Low Mw PGA fragments obtained by enzymatic degradation. Polygalacturonic acid (PGA; ICN Biochemicals Inc., Costa Mesa, California, USA) was degraded by endo-polygalacturonase (PG) purified from the culture liquid of the yeast *Kluyveromyces fragilis* (VERSTEEG, 1979). Enzymatic degradation was performed by incubation of a solution of PGA (0.5% w/v) in 0.05M Na-acetate buffer (pH 5) containing 0.01% (w/v) NaN_3 with 0.4 U of PG during various times at 30 °C. The enzyme was inactivated by heating the solution at 100 °C for 10 min. The digest was analysed by high-performance size-exclusion chromatography (HPSEC).

Sample preparation. PGA was used directly as obtained from the manufacturer. Desalted high-methyl pectin (HMP; Obipectin Ltd., Bischofszell, Switzerland) was prepared by dissolving 1 g of HMP in 200 ml of distilled water under stirring. During additional gentle stirring for 10 min 7.5 g chlorine-free Amberlite IR 45 and 7.5 g Dowex-OH-form 50 W (50–100 mesh) as ion exchanger were added to the solution. The mixture was then poured slowly into a glass column. The ion-exchange column was washed with distilled water and the filtrate was concentrated using a rotating evaporator and precipitated in ethanol/water 80% (v/v). The desalted HMP was subjected to repeated washings with ethanol/water 80% (v/v) until the filtrate was free

from sugars as detected by the phenol-H_2SO_4 assay (DUBOIS et al., 1958). The alcohol insoluble residue of HMP was finally rinsed with 96% ethanol. The insoluble residue was dried by solvent exchange (aceton followed by ether) and dried at room temperature for 3 days. The dried material was pulverised to a fine powder (0.7 mm) using a Cullatti DFH hammer mill prior to its use.

The alginates M/G 2.5 and M/G <1 were transferred to the acid form according to the method described in the FOOD CHEMICAL CODEX (1981). Samples of 5 g were transferred into beakers and stirred for 10 minutes with a mixture of 5 ml concentrated hydrochloric acid and 100 ml of 60% isopropyl alcohol. The mixtures were then centrifuged at 39 900 g for 20 min. The alcohol insoluble residues were washed 6 times with 15 ml portions of 60% isopropyl alcohol until the filtrates were chloride free. The residues were dried at room temperature by solvent exchange, i.e. aceton and ether.

Low Mw fragments of pectins and alginates obtained by mechanolysis. Low Mw pectin fragments were prepared from an apple pectin with a degree of esterification (DE) of 76% (high-methyl pectin, HMP, Obipectin Ltd., Bischofszell, Switzerland) and low Mw alginate fragments were prepared from an alginate with a high mannuronic acid/guluronic acid ratio (M/G 2.5) and from an alginate with a low ratio (M/G <1) obtained from Kelco International Ltd. San Diego, California.

Mechanolysis (ball milling) was carried out according to the method of VAN DEVENTER-SCHRIEMER and PILNIK (1987) by transferring the polysaccharides (18 g) into cylindrical jars filled with porcelain cylinders. The jars were vibrated with a Vibratom SM (Siebtechnic GmBH, Muhlheim, FRG) at a frequency of 1420 min^{-1} and an amplitude of 1.75 mm at room temperature. After every two h of milling the jars were cooled to 4 °C. At certain time intervals within the mechanolysis process, samples were taken and the progress of the degradation was monitored by HPSEC. An average degree of polymerisation (DP) of ca. 20 was obtained after 55 h mechanolysis treatment for HMP and after 84 h for the alginates.

Fractionation of the degraded polysaccharides. The degraded polysaccharides (»350 mg) were each dissolved in H_2O (3 ml) and applied onto a Sephacryl S100 column (100×5 cm; Pharmacia, separation range for dextrans 500–30,000 Da) and eluted with 0.1M Na-acetate buffer pH 4.0. Fractions were analysed by automated methods for uronic acid (AHMED and LABAVITCH, 1977; THIBAULT, 1979) and total neutral sugars (TOLLIER & ROBIN, 1979).

HPSEC was performed on a SP8800 HPLC (Spectra Physics) equipped with three Bio-Gel TSK columns (300×7.5 mm) in series (40XL, 30XL, and 20XL; Bio-Rad Labs) in combination with TSK XL guard column (40×6 mm) and elution at 30 °C with 0.4M acetic acid/Na-acetate (pH 3.0) at a flow rate of 0.8 ml min^{-1} (SCHOLS et al., 1990). The eluate was monitored by a Shodex SE-61 Refractive Index detector.

HPAEC of the degradation products was performed on a Dionex Bio-LC system (Sunnyvale, California, USA) including a quarternary gradient pump, eluent degas (He) module, and pulsed electrochemical detector (PED) in the pulsed amperometric mode (PAD), completed with a Spectra Physics SP8800 autosampler and a Spectra Physics Winner data handling system. A CarboPac PA100 column (4×250 mm) with matching guard column (Dionex) was used at a flow rate of 1.0 ml min^{-1}. The gradient was obtained by mixing solutions of 0.1M NaOH and 1M Na-acetate in 0.1M NaOH. For galacturonic acid oligomers, after an equilibration step of 15 min with 0.2M Na-acetate in 0.1M NaOH, 20 µl of the sample was injected and a linear gradient to 0.7M Na-acetate in 0.1M NaOH within 40 min was started. Finally, the column was washed for 5 min with 1M Na-acetate in 0.1M NaOH and re-equilibrated for the next injection. For alginate oligomers, after an equilibration step of 15 min with 0.25M Na-acetate in 0.1M NaOH, 20 µl of the sample was injected and a linear gradient to 1M Na-acetate in 0.1M NaOH within 45 min was started. Finally the column was washed for 5 min with 1M Na-acetate in 0.1M NaOH and re-equilibrated for the next injection.

Irradiation and storage stability. Sealed tubes (Kimax) containing 2 mg sample and 2 ml of deoxygenated distilled water were irradiated with doses of 5, 10 and 15 kGy at a dose rate of 5.01 kGy h^{-1}. Irradiation using a Co-60 source was performed at the Institute for Atomic Sciences in Agriculture (ITAL) Wageningen, The Netherlands. Samples of control and irradiated PGA stored at –20 °C for 3 and 6 weeks were analysed to study their storage stability.

2. Results and discussion

Our strategy was to use oligosaccharides to study the effect of irradiation and to reveal the mechanism of degradation. Oligomers of appropriate length were isolated since they were not commercially available. The degradation of PGA by PG was monitored by HPSEC (Fig. 1A). Using commercial GPC software, it was calculated that the average DP of PGA digested for 2.5 h at 30 °C was ca. 10, which was in the range of analysis by HPAEC. However, the digest still had a rather broad molecular weight (Mw) distribution. Therefore it was considered that a further chromatographic separation over a preparative Sephacryl S100 column might be useful to obtain more homogeneous populations in sufficient amounts. The separation obtained on Sephacryl S100 was very similar to that obtained by HPSEC, and the fractions were pooled into 3 subfractions having a relatively high, medium or low molecular weight (HMw, MMw and LMw fraction respectively) and analysed again by HPSEC (Fig. 1B).

The characteristics of the pools before and after irradiation with respect to their weight average- and number, average molecular weight (Mw and Mn), the ratio Mw/Mn and the DP values determined from these data are shown in Table 1. It can be

seen that the Mw/Mn ratio of the starting material before irradiation were close to 1, indicating that the fractions are rather homogenous, although, especially for the LMw subfraction, distinct peaks for the individual oligomers can be recognised (Fig. 1B). The calculated DP values of 40, 13 and 4 for HMw, MMw and LMw PGA subfractions respectively will enable monitoring the influence of irradiation by HPAEC.

The elution profiles of the irradiated MMw PGA subfraction as monitored by HPSEC are shown in Fig. 2. It can be seen from the patterns that the molecular weight decreased significantly during irradiation and the shift in Mw appeared to depend on the irradiation dose. For the MMw subfraction as well as for the two other fractions, the effect of three different irradiation doses is summarised in Table 1. It can be concluded that the effect of degradation depends on the size of the oligomers under investigation since 6.7% of all bonds present in the HMw subfraction were hydrolysed (5 kGy dose),

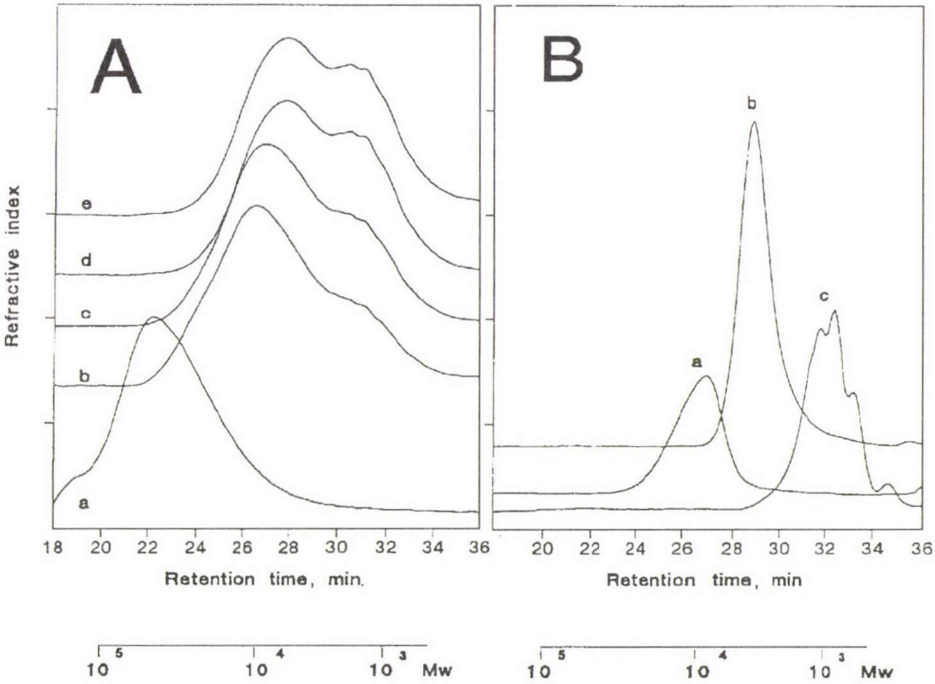

Fig. 1. High-performance size-exclusion chromatography of A) polygalacturonic acid before (a) and after incubation with polygalacturonase for 1 h (b), 1.5 h (c), 2 h (d) and 2.5 h (e). Figure B showed the HPSEC elution patterns of the pooled fractions of the PG digest of polygalacturonic acid obtained after incubation for 2.5 h and chromatography over Sephacryl S100: High Mw fraction (a), Medium Mw fraction (b) and Low Mw fraction (c)

Table 1

Weight average molecular weight (Mw), number average molecular weight (Mn), Mw/Mn ratio, degree of polymerization (DP) and % hydrolysis of unirradiated and irradiated HMw, MMw and LMw subfractions of PGA digest as measured by HPSEC

Subfr.	Radiation dose (kGy)	Mw (Da)	Mn (Da)	Mw/Mn	DP	% Hyd.[a]
HMw	0	9600	8100	1.2	40.4	0
	5	4100	2200	1.9	10.9	6.7
	10	2600	1400	1.9	7.2	11.4
	15	2100	1200	1.8	6.1	14.0
MMw	0	3200	2700	1.2	13.4	0
	5	2970	2150	1.4	10.8	1.8
	10	2600	1900	1.4	9.3	3.3
	15	2400	1600	1.5	8.1	4.9
LMw	0	1030	840	1.2	4.2	0
	5	1000	810	1.2	4.1	0.8
	10	1000	740	1.4	3.7	3.1
	15	980	730	1.3	3.6	3.7

[a] % hydrolysis gives the percentage of glycosidic linkage split during the treatment as calculated from the Mn value determined by HPSEC (SCHOLS et al., 1990)

while the degradation for the MMw and LMw subfractions was only 1.8 and 0.8%, respectively. These values were almost doubled when an irradiation dose of 10 kGy was used, while the extra effect of the 15 kGy dose was less pronounced.

Figure 3 illustrates typical elution profiles of non-treated samples of galacturonic acid oligomers (MMw subfraction) as measured by HPAEC. It should be realised that in contrast to HPSEC with refractive index detection, examination of the HPAEC elution patterns is not an appropriate method for absolute quantification of oligomers formed, because the response factor of the PAD detector for the oligomers might be different (LEE, 1990). For this reason, the contribution of the higher oligomers in the digest is underestimated. As a result of irradiation, the major peaks in the elution pattern (galacturonic acid oligomers of DP 5–11) are degraded, and peaks eluting at lower salt concentrations increased in intensity. Using standards present in our laboratory, it was demonstrated that these oligomers formed were "normal" galacturonic acid oligomers having a lower DP and were the result of a hydrolytic cleavage of the glycoside bonds. The results suggest that using irradiation, galacturonic acid oligomers (DP >8) were preferentially degraded in a random fashion, comparable to the mechanism as described for PG by PILNIK and ROMBOUTS (1981). These findings

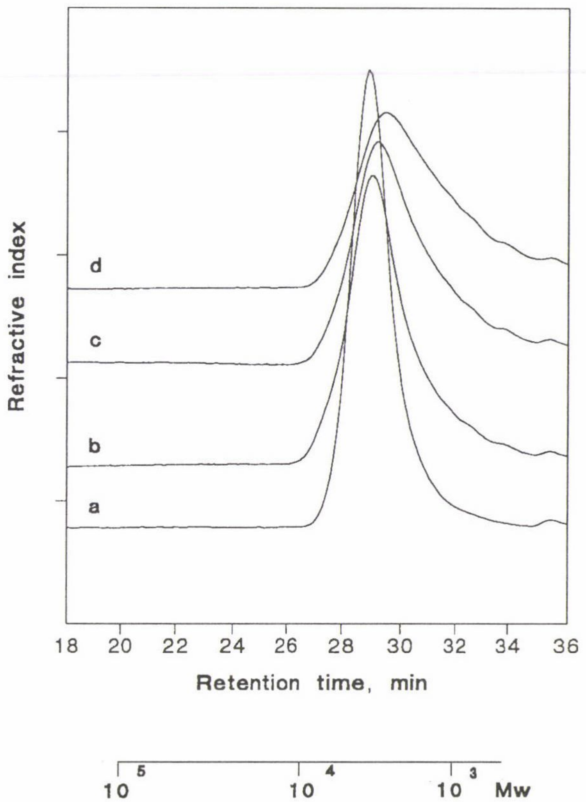

Fig. 2. High-performance size-exclusion chromatography of oligomeric fragments of polygalacturonic acid (subfraction MMw) before (a) and after irradiation using different doses: 5 kGy (b), 10 kGy (c), and 15 kGy (d)

are also in agreement with those of SKINNER and KERTÉSZ (1960), who stated on basis of viscosity measurements and electrophoreses experiments that a hydrolytic fissure of the glycosidic linkages is expected. Like these authors, we also found that the degradation increased by increasing irradiation dose. However, especially irradiation at high irradiation doses also resulted in the formation of some unknown compounds eluting in between the known galacturonic acid oligomers, suggesting a more complex mechanism than hydrolysis of the glycosidic linkages only. Although the amount of these unknown compounds is much lower than the amount of the 'normal' saturated oligogalacturonic acids, these compounds might be crucial intermediates in the elucidation of the degradation pathway.

Since it has been known that irradiation might induce chemical changes in the polymer molecules as result either from the direct-effect or indirect-effect reactions (CHARLESBY, 1960), the irradiated samples were stored at –20 °C for several weeks and analysed again after certain time intervals (Table 2). The data revealed that the irradiated samples did not show any continuation of the degradation process during storage at low temperatures. The small variation in DP values found for the various fractions during storage are probably caused by experimental errors. The storage stability found is not in contrast with findings of NAWAR (1983), who stated that irradiated molecules may continue to produce excited and ionised molecules which immediately begin to react with each other to form some new, stable chemical radiation products. It might also be expected that during storage at temperatures below 0 °C, the formation of radiolytic products and radiolysis after irradiation will be diminished due to protection by ice.

Since the above findings demonstrated that the selected methods enabled a more detailed examination of irradiated oligo-galacturonides, also oligosaccharides derived from other charged polymers were studied using the same approach.

Pectin and alginate samples were mechanically degraded by a mechanolysis method, which resulted in fragments with unchanged degree of esterification as was also found in the study of VAN DEVENTER-SCHRIEMER and PILNIK (1987). The molecular weight of high methyl pectin and alginates monitored by HPSEC decreased significantly after 55 h and 84 h milling, respectively and the average degree of polymerisation obtained was about 20. The degraded polymers were separated on Sephacryl S100 as described above and the fractions obtained were pooled into the subfractions: HMw, MMw and LMw. These three subfractions of HMP and the alginates were then characterised with respect to their molecular weight as measured by HPSEC. DP values of 28; 22 and 10 were calculated for the pectin samples, respectively. The corresponding values for the alginate having a M/G ratio of 2.5 were 38; 20 and 7 and for the alginates with a M/G ratio <1; 25, 22 and 8, respectively.

The results of irradiation-induced degradation on the subfractions derived from the various degraded pectin and alginate samples is shown in Table 3. It is found that for all subfractions, the average DP decreased with increasing irradiation dose. This is in agreement with results obtained by KING (1994), and KUME and TAKEHISA (1983) for alginate polymers, which showed a reduction in viscosity and in gel strength upon molecular degradation induced by radiation of high viscosity Na-alginates. The effect of irradiation towards oligomers of pectin is rather similar to HPSEC patterns (not shown), decrease in average DP (Table 3) and Mw and Mn (not shown) as compared to the effects measured for oligomers of PGA. Unfortunately, the degree of esterification of the pectin oligomers was not measured. However, it has been shown before that radiation did not affect the degree of esterification of pectin (SJÖBERG, 1987).

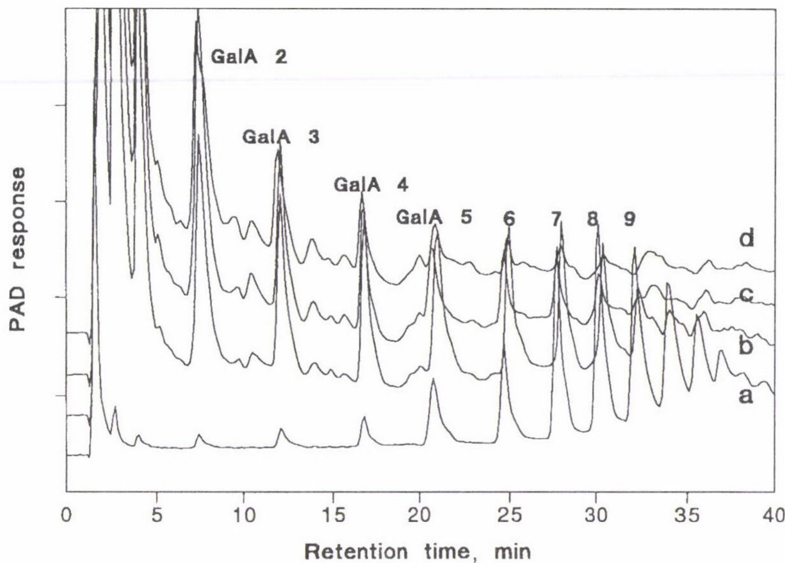

Fig. 3. High-performance anion-exchange chromatography of oligomeric fragments of polygalacturonic acid (subfraction MMw) before (a) and after irradiation using different doses: 5 kGy (b), 10 kGy (c), and 15 kGy (d)

Table 2

Effect of irradiation and storage on degree of polymerization (DP) of HMw, MMw, LMw subfractions of PGA digest as measured by HPSEC

Storage time (week)	Radiation dose (kGy)	Subfraction		
		HMw	MMw	LMw
0	0	40.4	13.4	4.2
	5	10.9	10.8	4.1
	10	7.2	9.3	3.7
	15	6.1	8.1	3.6
3	0	42.6	13.4	4.6
	5	10.5	10.9	4.3
	10	7.3	8.1	4.5
	15	4.5	7.1	3.9
6	0	42.5	13.6	4.5
	5	12.1	10.3	4.2
	10	8.1	7.6	4.0
	15	6.6	6.6	4.0

Table 3

Effect of irradiation on DP of HMw, MMw, LMw subfractions of pectin, alginate M/G 2.5 and alginate M/G< 1 fragments as measured by HPSEC

Sample		Radiation dose (kGy)			
		0	5	10	15
Pectin	HMw	28.6	19.2	14.8	12.8
	MMw	22.0	16.1	12.1	10.3
	LMw	10.0	7.3	6.5	6.3
Alginate	HMw	38.0	6.0	4.2	3.6
M/G 2.5	MMw	20.2	12.1	7.6	6.3
	LMw	6.9	6.1	5.6	5.2
Alginate	HMw	25.3	9.6	6.9	5.6
M/G<1	MMw	21.9	13.0	8.7	7.1
	LMw	7.8	6.7	6.4	5.8

Comparison of results obtained for pectin with those obtained for alginates showed that alginates are more sensitive to irradiation than pectin. It can be seen in the HMw subfractions that an alginate having a M/G ratio of 2.5 seems to be more sensitive than an alginate having relatively more guluronic acid residues (M/G <l; Table 3). Also in studies of heat induced degradation, it was found that alginates with a high M/G ratio were more susceptible to degradation. From this it appears that the uronic acid composition and arrangements of the uronic acid residues (blockwise, alternating) effects the stability of the alginates.

The degradation of pectin and alginate fractions by irradiation was also measured by HPAEC (Figs 4 and 5). In the elution pattern of the irradiated pectin fragments, the most pronounced degradation products could be identified as galacturonic acid oligomers. However, due to the HPAEC elution conditions, methyl esters which still might be present linked to the carboxyl groups of the galacturonic acid residues, will be saponified during the analysis and no information will be obtained on the esterification after irradiation. Comparison of the elution pattern of irradiated pectin oligomers (Fig. 4) with that of irradiated PGA oligomers illustrates that the mechanism of degradation of uronic acid oligomers as provoked by irradiation is not influenced significantly by methyl esterification. And on the other hand, it is assumed that degradation after irradiation is not stimulated by negatively charged carboxyl groups. Nevertheless, it should be stated that small differences between the HPAEC patterns of the methyl esterified and the non-methyl esterified galacturonic acid oligomers can be recognised, especially within the ratio of minor, unknown peaks towards the more dominant galacturonic acid oligomers formed after treatment.

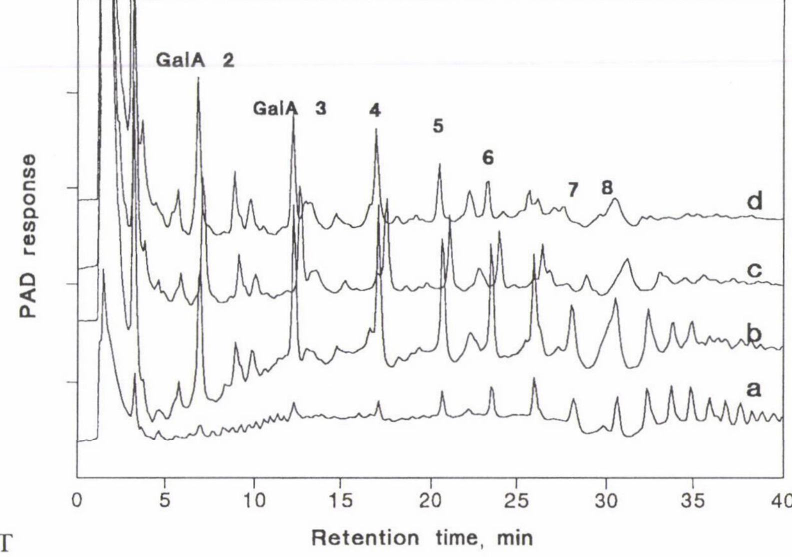

Fig. 4. High-performance anion-exchange chromatography of methyl esterified oligomeric fragments of pectin obtained after mechanolysis (LMw) before (a) and after irradiation using different doses: 5 kGy (b), 10 kGy (c), and 15 kGy (d)

The elution profiles of irradiated alginates are much more complicated, since 'simple' hydrolysis of the glycoside linkages (as was found for galacturonic acid oligomers) may result in numerous oligomers having the same size, but differing in building blocks (guluronic acid versus mannuronic acid) or sequences of these residues. Consequently, PGA digest is only applicable as standard to indicate radiation-induced degradation of pectin. The elution profiles of the two different irradiated alginate oligomers (M/G = 2.5 versus M/G = <1) showed great similarities, although the chromatogram of the alginate having a M/G ratio of 2.5 showed more unknown peaks. The degradation can be ascribed to radiation-induced fracture of the main chain.

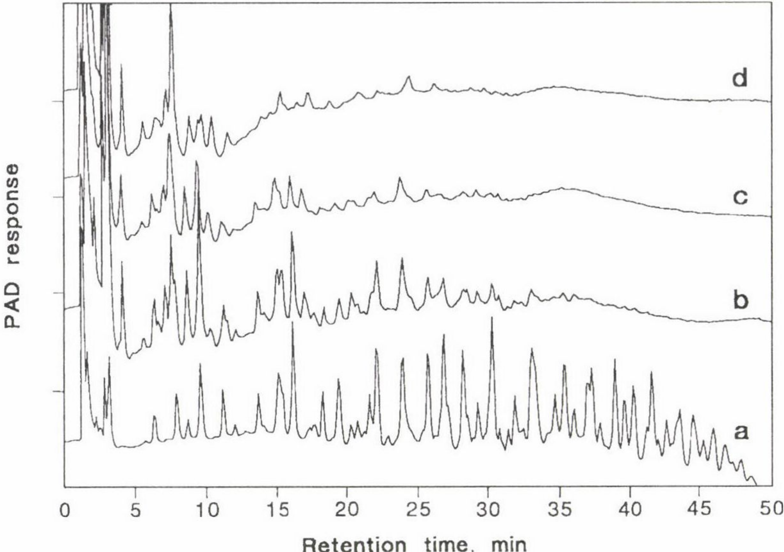

Fig. 5. High-performance anion-exchange chromatography of oligomeric fragments of alginate (M/G ratio of 2.5) obtained after mechanolysis (HMw) before (a) and after irradiation using different doses: 5 kGy (b), 10 kGy (c), and 15 kGy (d)

3. Conclusions

The degradation by irradiation of oligomeric fractions obtained from PGA, high methyl pectin and alginate samples by enzymatic and mechanical degradation was studied. Fragmentation of polysaccharides to oligomers within a molecular weight range, which enables chromatographic characterisation of the fragments provided good model substrates to study their degradability under irradiation conditions and to monitor the products formed by HPSEC and HPAEC methods. The low Mw fragments of PGA, pectin and alginates exposed to irradiation show a reduction in average molecular weight. Degradation by irradiation can be ascribed to hydrolytic cleavage of glycosidic bonds resulting in new fragments with lower degree of polymerisation and formation of other reaction products appearing on chromatograms between known peaks, which could not be identified by methods used in this study.

*

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A COMPARATIVE STUDY OF KARYOTYPING, RAPD–PCR AND nDNA/nDNA HOMOLOGY METHODS FOR IDENTIFICATION OF YEASTS ISOLATED FROM INDIAN BEVERAGES

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A survey of three different methods in yeast strain identification (karyotyping, RAPD–PCR and nDNA/nDNA reassociation) has been carried out in order to differentiate, with industrial purposes, the strains present in red wine, beer and bakers yeast originating from India. All the three methods were able to successfully identify the strains. However, RAPD–PCR was more rapid than the other two.

Keywords: *Saccharomyces sensu stricto,* electrophoretic karyotyping, RAPD–PCR, nDNA/nDNA reassociation

Numerous yeast strains are being used for commercial wine fermentation, beer brewing and bakery products. Two reasons have made necessary the characterization of these new strains: quality control in dry yeast production to ensure that the final product is identical with the original strain; and fermentation process control to ensure that it is really conducted by the inoculated yeast. As most of the industrial yeast strains belong to the group of *Saccharomyces sensu stricto,* the species of which cannot be readily distinguished and identified, especially *Sacch. pastorianus* and *Sacch. bayanus,* by classical biochemical methods, molecular methods have recently been employed. These comprise analysis of protein patterns, DNA sequence polymorphism, mt DNA restriction patterns, etc. (QUEROL et al., 1992; DEÁK, 1995). However, the complexity of some of these methods renders their industrial application difficult. In this regard, a method intended for industrial application must be fast, able to clearly differentiate between strains, reliable and economical (CASEY et al., 1990; LAVELLÉE et al., 1994).

In this study we have carried out identification of two strains isolated from Indian red wine, using three currently available methods to characterize yeast (electrophoretic karyotyping, RAPD–PCR analysis and nDNA/nDNA homology) in order to determine the most rapid and sensitive method to differentiate isolated yeast. Both strains were earlier characterized by traditional identification methods and were tentatively identified as *Sacch. cerevisiae.*

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1. Materials and methods

1.1. Strains investigated

The strains investigated were Sh 1 and Sh 4, isolated from red wine, as well as type strains of *Sacch. cerevisiae* CBS–1171 and *Sacch. bayanus* CBS–380, *Sacch. pastorianus* CBS–1513, *Sacch. ellipsoideus* CBS–1395, *Sacch. uvarum* CBS–395, and *Sacch. monacensis* CBS–1503.

1.2. Electrophoretic karyotyping

Oakley-Gutowsky's method (OAKLEY-GUTOWSKY et al., 1992) was adopted for isolating chromosomal DNA. Chromosome separation was carried out in agarose gel which consisted of 0.9% (w/v) agarose gel (Sigma A–2929), in 0.25×TBE, by Rotating Field Electrophoresis, using Rotaphor R22 (Biometra, Germany). The electrophoresis was run at 180 V (6 V/cm), at 12 °C, for 36 h. Pulsing time interval changed from 120–20 s logarithmically over the electrophoresis duration at an angle of 115–95°. After electrophoresis, the gel was stained with 0.5 μg ml^{-1} ethidium bromide in 0.25× TBE for one hour at room temperature and destained in 0.25×TBE for one hour at room temperature. The gel was observed with short wave UV illumination and photographed.

1.3. RAPD–PCR assay

Isolation of DNA was carried out according to procedure of MESSNER and PRILLINGER (1995).

Ten μl of template DNA was amplified in a final volume of 10 μl of 10×PCR buffer, 50 ng primer, 0.2 mM of each nucleotide triphosphate, 1 unit Taq polymerase (Promega M–186A), and 1.5 mM $MgCl_2$. The primer for amplification was primer 21 (BALEIRAS-COUTO et al., 1994) with a sequence: 5' – GCT CGT CGT T – 3'. Amplification was performed on Quatro TC–40 thermocycler. The initial pre-denaturation occurred at 95 °C for 3 min. This was followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 36 °C for 1 min and elongation of target DNA at 72 °C for 2 min.

For the electrophoretic separation, the PCR products were loaded into 1.2% (w/v) of standard agarose gel (Sigma A–2929) in TAE buffer containing 0.3 μg/ml ethidium bromide. Buffer and gel were pre-cooled to 4 °C, and separation was performed without further cooling at 5 V/cm for 1 h, DNA fragments were visualized in UV illumination and photographed.

Scoring of similarities between individual lanes was done by hand according to the formula of NEI and LI (1979), as follows:

$$\text{Similarity} = 2\times\frac{\text{(bands in common)}}{\text{total bands}}$$

1.4. DNA extraction, purification and nDNA/nDNA reassociation

Strains studied were Sh 1, Sh 4 and type strain of *Sacch. cerevisiae* CBS–1171. Extraction and purification of DNA was performed according to MARTINI and PHAFF (1973) with minor modification. nDNA/nDNA reassociations were determined in 2× SSC using a Response 2 (Gilford, USA) spectrophotometer equipped with a thermoprogrammer.

2. Results and discussion

2.1. Electrophoretic karyotyping

The results of electrophoretic karyotyping are presented in Fig. 1. Strains Sh l, Sh 4 and *Sacch. cerevisiae* type strain show chromosomal bands in all the three size ranges, namely, heavy (>1000 kb), medium (500–1000 kb) and light (<500 kb). This is a feature characteristic of *Saccharomyces sensu stricto* but rarely exhibited by non *Saccharomyces sensu stricto* species. In addition, several authors reported numerous bands at molecular sizes less than 500 kb in *Saccharomyces sensu stricto* group (CARDINALLI & MARTINI, 1994). These properties clearly separate the four taxa of *Saccharomyces sensu stricto* from all of the non *Saccharomyces* yeast species. The karyotypes of strains Sh 1 & Sh 4 contain high numbers of chromosomal bands (16–17), with molecular sizes ranging from approximately 250–2200 kb.

Strains Sh 1 & Sh 4 showed chromosome length polymorphism (CLP) and/or changes in ploidy which are indicated by the bright bands on the ethidium bromide stained gels. BAKALINSKY and SNOW (1990) pointed out the advantage of industrial strains in maintaining a stable but unbalanced set of chromosomes. There is also a known tolerance for aneuploidy among brewing yeast (PARRY & COX, 1970). In addition, meiosis is not a regular part of the life cycle of industrial strains, so the possession of extra or incomplete sets of chromosomes protects the strains' integrity. The domestic strains of *Saccharomyces sensu stricto* are hence expected to show aneuploidy or polyploidy. Hence it is not surprising to find that strains Sh 1 & Sh 4, both of which are of industrial origin, showed co-migration, CLP or variations in ploidy.

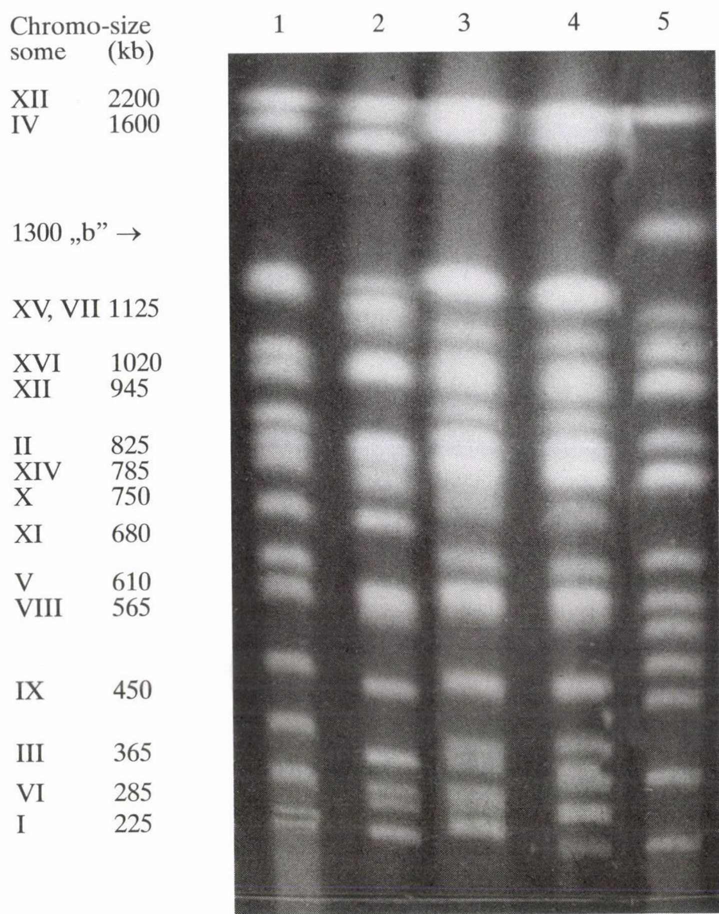

Fig. 1. Analysis of chromosomal DNA from yeasts by Rotating Field Electrophoresis. Lane 1: Biorad Standard *Sacch. cerevisiae* (YNN 295); lane 2: *Sacch. cerevisiae*^{T}; lane 3: Sh 1; lane 4: Sh 4, lane 5: *Sacch. bayanus*^{T}. Electrophoretic conditions: agarose conc. 0.83% (w/v), 180 V (6 V/cm), temperature = 12 °C, running time = 36 h; pulsing time = 120–20 s. logarithmically; angle = 115°–95°

A gross comparison of the number of heavy bands to that of medium and light bands showed, on an average, that the number of medium and light bands exceed the number of heavy bands. This is in line with earlier reports on karyotypes of the genus *Saccharomyces* that there exists an inverse relationship between chromosome number and relative size of individual bands (VAUGHAN-MARTINI et al., 1993). These findings generally fit into the hypothesis that more evolved taxa, which have a greater dependence upon a specific habitat, are often characterized by larger number of chromosomes in their respective phylogenetic lines (MILLER et al., 1989).

2.2. *Elimination of* Sacch. bayanus

Sacch. bayanus strains can be differentiated from *Sacch. cerevisiae* strains in that *Sacch. bayanus* shows a specific chromosomal band (~1300 kb) marked 'b' in Fig. 1. Chromosomal band 'b' lies between chromosomes IV and XV, VII of *Sacch. cerevisiae* YNN 295. *Sacch. cerevisiae* is characterized by the presence of chromosomal bands at about 1600 kb which corresponds to chromosome IV of *Sacch. cerevisiae* YNN 295. *Sacch. bayanus* lacks a chromosomal band at ~1600 kb. Apart from this difference in the chromosomal bands at about ~1600 and ~1300 kb which can differentiate *Sacch. bayanus* from *Sacch. cerevisiae*, there are also other ways to distinguish *Sacch. bayanus* from its siblings since it displays specific chromosome pattern in the medium chromosomes, wherein a set of about 5 chromosomes are seen well separated but close together. This specific pattern is independent of CLP (Fig. 1). In line with the above arguments, since strains Sh 1 & Sh 4 lacked chromosomal bands at ~1300 kb as well as the specific patterns that is common to all *Sacch. bayanus*, it can rationally be concluded that these strains are not *Sacch. bayanus.*

2.3. *Elimination of* Sacch. pastorianus

Sacch. pastorianus strains are characterized by the chromosomal band at ~1300 kb as well as another at ~1600 (TORNAI-LEHOCZKI & DLAUCHY, 1996). Since strains Sh 1 & Sh 4 showed only a chromosomal band at ~1600 kb and none at all at ~1300, it is concluded that these species are not *Sacch. pastorianus.*

2.4. *Confirmation of* Sacch. cerevisiae

In view of the elimination of strains being *Sacch. bayanus* or *Sacch. pastorianus*, the only remaining domesticated species of *Saccharomyces sensu stricto* is *Sacch. cerevisiae.* As pointed out earlier, *Sacch. cerevisiae* strains are characterized by a chromosomal band at ~1600 kb and none at ~1300, Sh 1 & Sh 4 also exhibit a chromosomal band pattern very similar to that of *Sacch. cerevisiae.* Hence, unequivocally, based on electrophoretic karyotyping, it is established that the strains Sh 1 & Sh 4 are *Sacch. cerevisiae.*

2.5. *Random Amplification of Polymorphic DNA analysis*

RAPD-PCR profile of all the strains studied is presented in Fig. 2. Based on the profile similarity values were calculated by using the formula of NEI and LI (1979). A similarity matrix based on the calculated similarity values is presented in Table 1.

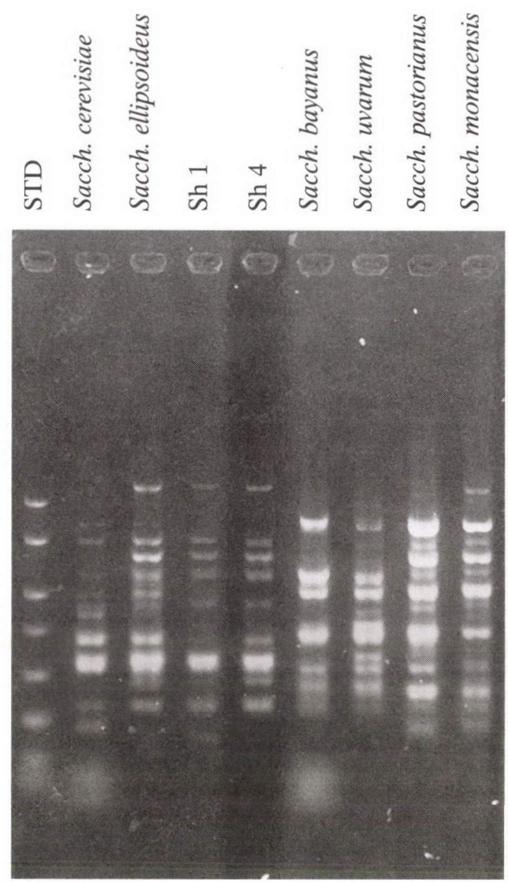

Fig. 2. RAPD–PCR analysis of 8 yeast strains generated with primer 21 (BALEIRAS-COUTO et al., 1994). Lane 1: molecular size marker; lane 2: *Sacch. cerevisiae*; lane 3: *Sacch. ellipsoideus*; lane 4: Sh 1; lane 5: Sh 4; lane 6: *Sacch. bayanus*; lane 7: *Sacch. uvarum*; lane 8: *Sacch. pastorianus*; lane 9: *Sacch. monacensis*

Similarity values between Sh 1 and *Sacch. cerevisiae*, *Sacch. bayanus* and *Sacch. pastorianus* are on an average 92%, 73% and 72%, respectively, while, between Sh 4 and *Sacch. cerevisiae*, *Sacch. bayanus* and *Sacch. pastorianus* are 92, 71 and 70 (all in %), respectively.

Table 1

Similarity matrix for the strains investigated

Strains	Similarity values (%)							
	Sacch. cerevisiae	*Sacch. ellipsoideus*	Sh 1	Sh 4	*Sacch. bayanus*	*Sacch. uvarum*	*Sacch. pastorianus*	*Sacch. monacensis*
Sacch. cerevisiae	100	92	92	92	72	74	71	73
Sacch. ellipsoideus	92	100	92	92	72	74	71	73
Sh 1	92	92	100	92	72	74	71	73
Sh 4	92	92	92	100	70	72	69	71
Sacch. bayanus	72	72	72	70	100	92	80	83
Sacch. uvarum	74	74	74	72	92	100	81	81
Sacch. pastorianus	71	71	71	69	80	81	100	91
Sacch. monacensis	73	73	73	71	83	81	91	100

The numerical value of similarity of RAPD–PCR is not sufficient to identify a strain. MESSNER and co-workers (1994) showed that RAPD–PCR similarity values below 20% did not give information with respect to phylogenetic relatedness of different organisms. However, very high similarity coincides with high nDNA/nDNA homology values.

Both Sh 1 & Sh 4 show a clearly higher similarity to *Sacch. cerevisiae* strains than to *Sacch. bayanus* and *Sacch. pastorianus.* The pattern produced was also more similar to *Sacch. cerevisiae* than *to Sacch. bayanus* or *Sacch. pastorianus.* The number of bands exhibited by *Sacch. pastorianus* was more than either *Sacch. bayanus* or *Sacch._cerevisiae.* A higher similarity was found between *Sacch. bayanus* and *Sacch. pastorianus* than between *Sacch. cerevisiae* and *Sacch. pastorianus* or between *Sacch. bayanus* and *Sacch. cerevisiae.* This higher percentage of similarity is expected as both the species also show a high nDNA/nDNA optical reassociation between them. However, RAPD–PCR analysis does not provide any evidence to support the assumption that *Sacch. pastorianus* is an amphidiploid of either *Sacch. cerevisiae* and *Sacch. bayanus or Sacch. cerevisiae* and *Sacch. paradoxus.*

The genetic variability within the different species of *Saccharomyces sensu stricto* group in previous studies were placed at 0–17%, more specifically *Sacch. cerevisiae* 88–100%, *Sacch. bayanus* 83–100% and *Sacch. pastorianus* 87–100%. The

similarity matrix (Table 1) shows that similarity values between *Sacch. cerevisiae* & *Sacch. ellipsoideus, Sacch. bayanus* & *Sacch. uvarum* and *Sacch. pastorianus* & *Sacch. monacensis* were well within this range.

Therefore, based on RAPD–PCR analysis it can be said that since strain Sh 1 and Sh 4 were more similar to *Sacch. cerevisiae* than to *Sacch. bayanus* or *Sacch. pastorianus,* these two strains are indicated to be *Sacch. cerevisiae.*

2.6. nDNA/nDNA reassociation

The results of nDNA/nDNA reassociation experiments are shown in Table 2. Samples were run in duplicates, thus giving our results by using different permutation combinations. The nDNA–nDNA homology calculated was an average value of these measurements.

Table 2

Results of reassociation studies of strains Sh 1, Sh 4 and Sacch. cerevisiae (CBS 1171)

Strain combination	Estimated reassociation values % for Sh 1	Estimated reassociation values % for Sh 4
A,B,C	89.69	93.21
B,D,E	95.16	95.16
B,E,C	89.71	89.65
A,B,D	95.93	91.42
Average	92.62	92.35

A: *Sacch. cerevisiae*^{T}
B: Sh 1/Sh 4
C: Mixture of *Sacch. cerevisiae*^{T} & Sh 1/Sh 4
D: Mixture of *Sacch. cerevisiae*^{T} & Sh 1/Sh 4
E: *Sacch. cerevisiae*^{T}

The interpretation of nDNA–nDNA hybridization studies is not always straight forward, since the values obtained are not absolute. They depend very much on the method and specific experimental conditions used. It is evident from Table 2 that both Sh 1 and Sh 4 showed very high nDNA/nDNA homology, i.e., 92.62 and 92.35, respectively.

It has been widely accepted that strains exhibiting 80% or higher nDNA/nDNA relatedness can be considered conspecific (KURTZMAN et al., 1980). In this investigation the DNA homology values of about 92% with *Sacch. cerevisiae*^{T} unquestionably establishes that the strains Sh 1 & Sh 4 are *Sacch. cerevisiae.*

Evidently, electrophoretic karyotyping allows us to differentiate both the strains studied. However it is a complex and time consuming technique (taking more than 3 effective working days, including sample preparation and electrophoresis) and does not permit the analysis of large number of samples. RAPD–PCR is sensitive and helps differentiate the strains studied. In addition it is rapid and produces results within one day, and allows several strains to be analysed simultaneously. nDNA/nDNA homology, though is very reliable, takes 5 effective days, including sample preparation and to measure the homology. Moreover, effectively only 2 samples can be measured in a single experiment if spectrophotometric method is used.

In view of the above advantages and disadvantages, RAPD–PCR was found to be the most effective for industrial application compared to the other two methods.

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MODELLING MODIFIED ATMOSPHERE PACKAGING OF PERISHABLE PRODUCE: KEEPING QUALITY AT DYNAMIC CONDITIONS

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To study the dynamics of modified atmosphere packaging (MAP), a complete dynamic and integrated mathematical MAP model was developed. The main objective of MAP is preserving product quality by reducing the rate of the metabolic processes of the packed product. This study describes how keeping quality, in relation to the gas exchange rate of the packed product, is incorporated in the MAP model. Simulations, under several constant and dynamic conditions, show the effect of MAP on keeping quality of the packed product.

Keywords: modified atmosphere packaging, modelling, keeping quality, perishable products, MAP, shelf life

To retain the quality better, perishable products can be packed using plastic films (HENIG, 1978). The principle of Modified Atmosphere Packaging (MAP) is based on reducing the rate of the metabolic processes in the product as a consequence of the changed composition of the atmosphere induced by the product itself (BURTON, 1978). Quality degradation, being in part a normal metabolic process, will also be inhibited. The success of modified atmosphere packaging strongly depends on the interactions between the packed product, the properties of the package and the environmental conditions (KADER et al., 1989; CAMERON et al., 1995). As different products differ in their behaviour and as MA packages will be exposed to a dynamic environment, each MA package has to be tuned to specific demands. The most vital part in a MAP system is the product itself with a limited lifetime to be prolonged as much as possible.

To study packed products at different dynamic conditions, a model, which describes the complete system of MAP is essential. Therefore, an integrated model was developed (HERTOG et al., 1997b) describing all processes active in MAP as outlined in Fig. 1, dynamically in time and temperature. Several sub-models (keeping quality, gas exchange of the product and temperature dependence of the permeability of the film for O_2, CO_2, N_2, H_2O) were validated separately. The integrated MAP model will be validated by the current research in the fields of packaging, respiration and keeping quality.

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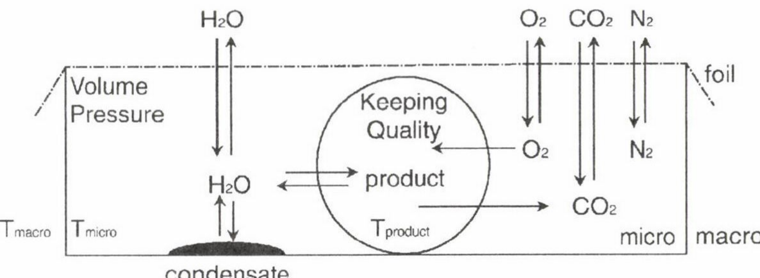

Fig. 1. An outline of the processes and interactions occurring in MAP. Three major components can be distinguished: the packed product, the surrounding macroclimate and the enclosed microclimate with the film being the diffusion barrier between macro- and microclimate. Gas and heat exchange will subsequently take place between the three components.

The most dominant factor affecting all elements of MAP is the environmental temperature. It influences, among other things, the gas exchange rate of the product and the diffusion characteristics of the film. Furthermore, aspects such as keeping quality of the product, relative humidity, the formation of condensate, partial pressures and the volume (in case of a flexible package) are all influenced, directly or indirectly, by the temperature of the environment.

Several MAP models, with different levels of detail, have been developed over the years (ZAGORY & KADER, 1988), but the current model is the first which includes keeping quality of the packed product and is completely dynamic. This study will focus on how keeping quality is modelled and implemented in the overall MAP model. Using simulations, it will be shown how keeping quality of perishable produce is affected by modified atmospheres and dynamically changing temperatures.

1. Keeping quality at constant conditions

Quality of perishable products is becoming increasingly important to the consumer. At the end of a logistic distribution chain the consumer decides whether or not a product is still acceptable. Quality, as assigned by the consumer, is affected by fundamental processes in the product, influencing the various quality attributes relevant for the specific product (SLOOF et al., 1996). Keeping quality is defined as the time a product remains acceptable (TIJSKENS & POLDERDIJK, 1996) and it can be used as a general indication of the overall product quality.

Tijskens (TIJSKENS, 1995; TIJSKENS & POLDERDIJK, 1996) developed a generic model for keeping quality, which basically incorporates all aspects of it, including the

effects of initial quality, limits of acceptance, dynamic temperature conditions and the different biochemical mechanisms for the reactions underlying quality decay. The static part of the model was validated for more than 60 different horticultural crops. For constant environments keeping quality can be described as:

$$KQ = \frac{f(Q_0, Q_l)}{\sum_{i=1}^{n} k_i} \tag{1}$$

with k_i being the rate constants affecting the involved quality attributes. The quality function $f(Q_0, Q_l)$ is an expression in terms of the initial quality Q_0 and the quality limit Q_l. The exact form of this expression depends on the underlying mechanism of quality decay (TIJSKENS, 1995; TIJSKENS & POLDERDIJK, 1996). Each rate constant k_i depends on temperature, presumably according to Arrhenius' law:

$$k_i(T) = k_{i,ref} \cdot e^{\frac{Ea_i}{R_{gas}}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)} \tag{2}$$

where R_{gas} = gas constant (8.314 J mol^{-1}·K^{-1}). The parameter $k_{i,ref}$ stands for the rate constant at the arbitrarily chosen reference temperature T_{ref}(K). The activation energy Ea_i expresses the dependence of the rate constant k_i on temperature T(K).

Keeping quality at reference temperature can be formulated as:

$$KQ_{ref} = \frac{f(Q_0, Q_l)}{\sum_{i=1}^{n} k_{i,ref}} \tag{3}$$

By combining Eq. 1 and 3, keeping quality can be formulated completely independently of the actual mechanism of the underlying processes, assuming the initial quality and the quality limit being constant:

$$KQ = \frac{KQ_{ref} \cdot \sum_{i=1}^{n} k_{i,ref}}{\sum_{i=1}^{n} k_i} \tag{4}$$

Of course, the type of kinetic mechanism is still of major importance for the quality attributes themselves. This formula is an adaptation of the formula by TIJSKENS (TIJSKENS, 1995; TIJSKENS & POLDERDIJK, 1996) to obtain a more handsome formula for computer implementation. Originally KQ_{ref} was defined as the keeping quality at

reference temperature for the first attribute only without considering an effect of the other attributes. When more than one attribute was involved the value of KQ_{ref} became a virtual one. In the current definition KQ_{ref} is the actual keeping quality at reference temperature irrespective of the number of attributes involved. To prevent over parameterisation, Tijskens (TIJSKENS, 1995; TIJSKENS & POLDERDIJK, 1996) set the first reference rate constant to one. In the current approach the summation of $k_{i,ref}$ was set to one, thereby simplifying Eq. 4 to:

$$KQ = \frac{KQ_{ref}}{\sum_{i=1}^{n} k_i} \tag{5}$$

The applied adaptations do not affect the value of KQ_{ref}, k_{ref} and Ea for products with only one limiting attribute as published by Tijskens (TIJSKENS, 1995; TIJSKENS & POLDERDIJK, 1996). The parameter values for products with two limiting attributes, however, are affected. The adjusted values for these products are presented in Table 1.

Table 1

Estimated parameters[a] for products with two limiting attributes. Data from the SPRENGER INSTITUTE (1986)

Product	R^2_{adj} [b]	KQ_{ref}	$k_{1,ref}$	Ea_1	$k_{2,ref}$	Ea_2
Bean, French	80	5.7	0.9480	9705	0.0520	–32659
Bean, runner	92.5	5.7	0.9986	11174	0.0014	–57922
Bean, slicing	85.1	4.9	0.9635	8732	0.0365	–34026
Beetroot	99.1	72.3	0.9548	21129	0.0452	–18978
Bell pepper	98.6	11.1	0.9388	15474	0.0612	–40380
Cucumber	97.6	9.0	0.5964	4333	0.4036	–19239
Kohlrabi-leaf	96.2	41.5	0.9664	13316	0.0336	–12647
Papaya[c]	95.5	24.8	0.6523	6387	0.3477	–21391
Tomato	98.6	6.3	0.2409	9375	0.7591	–50647

[a] KQ_{ref} = keeping quality at reference temperature T_{ref} (= 10 °C); $k_{i,ref}$ = reaction rate constant at reference temperature of attribute *i*; Ea_i = activation energy of reaction rate constant k_i

[b] R^2_{adj} = percentage variance accounted for

[c] data from PAULL (1993)

2. Keeping quality, dynamic in time and temperature

During a product's lifetime temperature changes and different temperature scenarios will result in different remaining quality. This remaining quality determines keeping quality. With dynamically changing temperatures acting on a product, the keeping quality remaining at a certain standard temperature can be predicted, assuming the limits of acceptance being known and constant. Keeping quality of products at a certain standard temperature (index *st*) for which quality depends on multiple limiting quality attributes, can be described as:

$$KQ_{st} = \frac{KQ_{ref} - \int_0^t \sum_{i=1}^n k_{i,dyn} \cdot dt}{\sum_{i=1}^n k_{i,st}} \tag{6}$$

with the integral describing the quality breakdown during the dynamic scenario with rate constants $k_{i,dyn}$ as function of the dynamically changing temperature. This formula is a correction of the earlier published formula (TIJSKENS, 1995; TIJSKENS & POLDERDIJK, 1996).

3. Respiration and keeping quality

The positive effect of MAP on keeping quality is based on reducing the overall rate of the metabolic processes. Respiration can be considered to be a fair measure for the general activity of the product and therefore for the rate of quality decay as well. The lower the total activity, the slower quality decays are (BRASH et al., 1995). Generally, the data on keeping quality are gathered using products stored at normal gas conditions (21% O_2, 0% CO_2). When measuring products at other gas conditions the activity can be expressed relative to the activity at normal gas conditions at the same temperature. Therefore, relative respiration was introduced as the ratio between the actual respiration level and the respiration at 21% O_2 and 0% CO_2 at the same temperature.

$$Rel_{resp} = \frac{Resp(O_2, CO_2, T)}{Resp(21\%\ O_2, 0\%\ CO_2, T)} \tag{7}$$

However, respiration is only a measure for the aerobic metabolic activities. To include fermentative activities as well, relative respiration was calculated using the total gas exchange expressed in terms of CO_2 production. The gas exchange of the product

was modelled and calculated according to HERTOG and co-workers (1997a). Relative respiration is now used in the formula for keeping quality to account for the changed rate of quality decay as a consequence of an overall change in the metabolic rate during MA packaging resulting in:

$$KQ_{st} = \frac{KQ_{ref} - \int_0^t Rel_{resp} \cdot \sum_{i=1}^{n} k_{i,dyn} \cdot dt}{\sum_{i=1}^{n} k_{i,st}} \qquad (8)$$

The same approach was, in fact, applied by POLDERDIJK and co-workers (1995) where the rate constant describing colour development of broccoli buds appeared to depend on the atmospheric conditions (modified atmosphere, controlled atmosphere, normal air). A deviant respiration, because of changed atmospheric conditions, only influences the rate constants of quality decay at the dynamic conditions ($k_{i,dyn}$). The other parameters from Eq. 8, KQ_{ref} and $k_{i,st}$, do not need correction using relative respiration as KQ_{ref} describes an initial condition, which is defined independently of the storage condition and as $k_{i,st}$ is defined for a shelf life at normal air conditions. Eq. 8 is used in the current MAP model.

4. Simulations

To illustrate the effect of modified atmospheres and temperature changes on keeping quality, several simulations were conducted and are discussed. Figure 2 illustrates the effects of temperature and gas conditions on keeping quality of chicory at a standard temperature of 10 °C. Chicory is a product with only one limiting quality attribute. So, the lower the storage temperature, the less the quality breakdown during storage and the greater the remaining keeping quality are. When storing at normal air in 10 °C (the same temperature as the reference temperature chosen) the product will lose one day of keeping quality for each day of storage. Chicory, stored at 5 °C, reaches its quality limit and becomes unacceptable only after 11 days. During storage at 20 °C this is already after 1.5 day. The added effect of applying low oxygen to decrease quality breakdown strongly depends on temperature. Applying low oxygen conditions at 5 °C, keeping quality of chicory can be extended by four days. At 20 °C the benefit of applying low oxygen is only half a day.

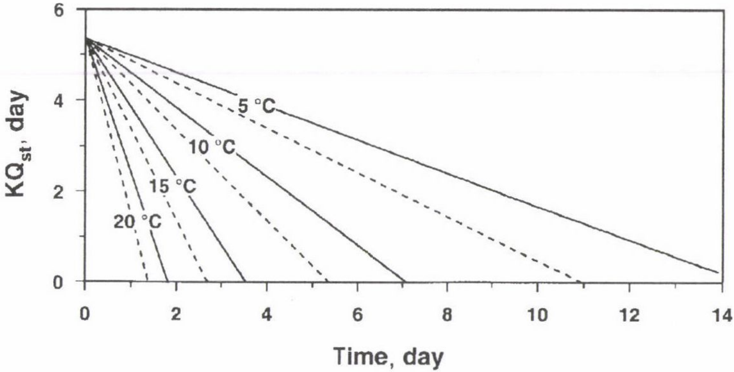

Fig. 2. A simulation of keeping quality (KQ_{st}) of chicory at a standard temperature (T_{st}) of 10 °C after storage at different constant temperatures (5, 10, 15 and 20 °C) applying two different atmospheric conditions, --- normal air (21% O_2 and 0% CO_2) and — low oxygen conditions (5% O_2 and 0% CO_2).

In Fig. 3 a stepwise increase in temperature is imposed on chicory stored in normal air. The keeping quality remaining at 10 °C is calculated. The product temperature needs some time to adapt to the imposed discrete temperature steps in the environment, resulting in a continuously changing product temperature. The rate of quality decay follows the temperature changes of the product resulting in an increasing decline of keeping quality.

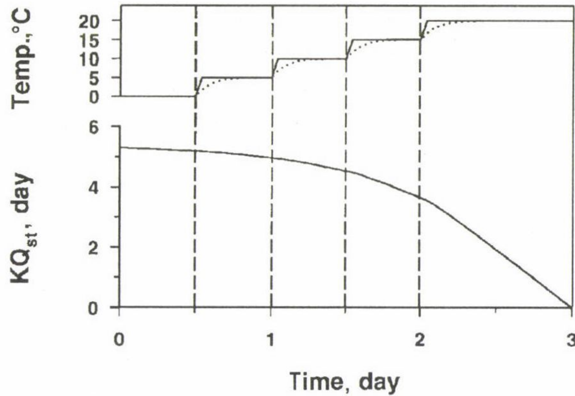

Fig. 3. Simulation of a stepwise increase in temperature imposed on chicory stored in normal air. The simulated remaining keeping quality is calculated at a standard temperature of 10 °C. ·····: Product; —: air

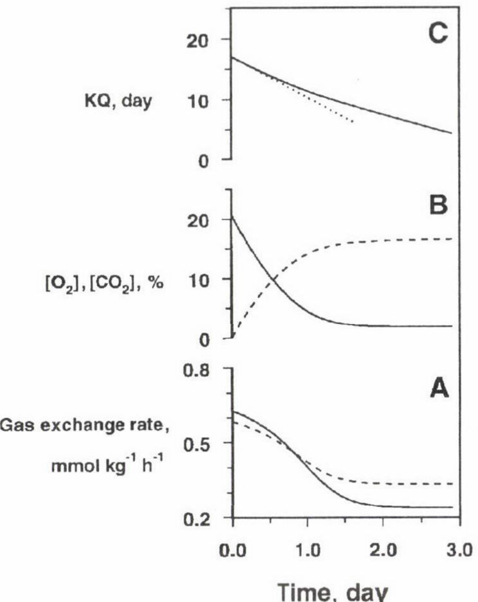

Fig. 4. A simulation of the initial period of modified atmosphere packaging of chicory at a constant temperature. A: gas exchange by the product (O_2 consumption (—) and CO_2 production (----)); B: levels of CO_2 (----) and O_2 (—) in the package; C: keeping quality in function of time. The dotted line is to emphasize the non linearity of keeping quality.

The positive effect of MA packaging on keeping quality can be seen in Fig. 4. Chicory is packed in normal air (21% O_2, 0% CO_2). During the first two days the atmosphere of the package changes by respiration of the product (to about 2% O_2 and 16% CO_2; Fig. 4B), thereby reducing respiration to about half the initial rate (Fig. 4A). This reduction of metabolic rate results in a decrease in the rate of quality decay as can be seen in Fig. 4C.

A complete dynamic situation is outlined in Fig. 5. Apples are packed and kept at 20 °C for two days. This situation is comparable with the situation depicted in Fig. 4. However, at day 2, the MA conditions were disturbed by removing the plastic film for several hours. This might happen when controlling the quality or by damaging the film. When the package is closed again, MA conditions are again generated by the product. The opening of the package resulted in a clear change in the rate of quality decay as can be seen in Fig. 5D. Starting from day 4, a temperature fluctuating around 7 °C is imposed on the packed product (Fig. 5A). This is comparable to temperatures imposed

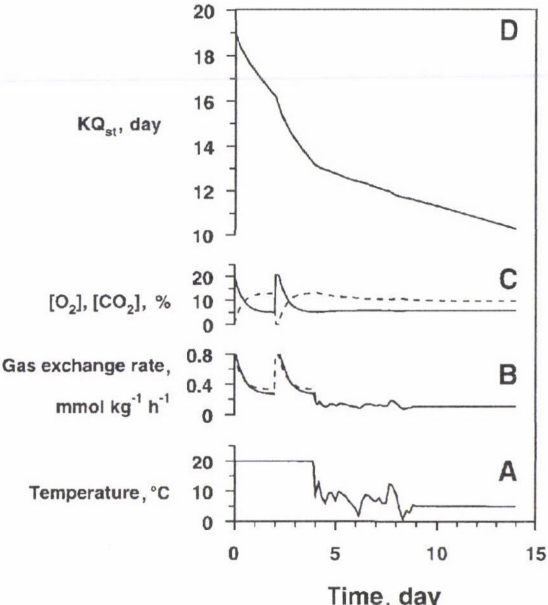

Fig. 5. A simulation of modified atmosphere packaging of apples. A: temperature; B: gas exchange by the product (O_2 consumption (—) and CO_2 production (----)); C: levels of CO_2 (----) and O_2 (—) in the package; D: keeping quality remaining at a T_{st} of 10 °C as a function of time.

on the packed product during transportation. Lowering the temperature from 20 to around 7 °C induces a clear instant decrease in the rate of quality decay (Fig. 5D). The subsequent temperature fluctuations have relatively small effects on keeping quality as can be deduced from the quite straight line for KQ_{st} from day 4 to 9.

The preceding simulations showed that the MAP model enables the user to explore modified atmosphere packaging and the effect on the keeping quality of the packed product. As the model includes the complete dynamics of the concerned processes, a good insight is given into how a certain steady state situation is realized.

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INFLUENCE OF DIFFERENT GENOTYPES ON THE MEAT QUALITY OF CHICKEN KEPT IN INTENSIVE AND EXTENSIVE FARMING MANAGEMENTS

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Three genotypes: 1. Broiler 2. Transylvanian naked-neck and 3. Hungarian speckled chicken kept in "extensive" and "intensive" farming managements were investigated. The legs and breasts of male and female of these chicken genotypes were used for the study. There were the following significant differences between the components in the meats of the Transylvanian and Broiler genotypes kept "intensively".

Transylvanian chicken meat had lower muscle to bone ratio, higher pH-value, higher water-holding capacity (in legs), less fat content, lower conjugated diene level, higher iron, zinc (only in male legs) and copper (only in female legs) concentration and higher riboflavin level (in legs).

For fatty acids, the Transylvanian group had higher linoleic and lower myristic and palmitic acid concentration.

Practically, no differences were observed in protein, cholesterol and thiamin concentrations and also in the thiobarbituric acid reactive substances (TBARS) between the genotypes.

In some cases similar results were found for the muscles of the Hungarian speckled genotype, but the differences were statistically not significant partly due to the small number of samples.

The superoxide dismutase activities in the male breast samples of the Transylvanian naked-neck and Hungarian speckled chickens were significantly lower than in the Broiler kept "extensively".

Keywords: genotypes, farming managements, pH, WHC, fatty acids, conjugated diene, trace elements, vitamins

In the past decade, a marked emphasis has been placed on the nutritional value of food in both raw and processed form. Consumers become more and more aware of nutritional values and many make their food purchases based on those qualitative characteristics. Chicken as an important item in the diet need much more attention than

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are paid to. Chicken is comparable to beef, lamb, and pork in composition and nutritive value.

Genetic improvement of broiler performance has focused primarily on increases in growth rate, more recently however, new pressures have arisen (LIZHEN & MCMILIAN, 1991).

Fatty acid composition of breast and leg meat affected by genotype were investigated by AMAD and co-workers (1992).

Genetic and phenotypic association of body weights, muscle weights, (breast, thigh and leg) and total fat (RANJIT & TREHAM, 1994; and HRDINKA et al., 1995) were examined.

The changes in genetic strains have raised some concerns about the processing quality of the Broiler muscles. Genetic manipulation altered the chemical composition, biochemical characteristics and quality of Broiler muscles. Protein content increased in the meat of Broilers along with the age. Broilers processed at age of the weeks had the highest shear values (EVANS et al., 1976).

Very scarce information is available with respect to genotype and farming system. The present study reports on the effect of genotype on the chemical components of chickens' meat, including the interaction of farming systems.

1. Materials and methods

1.1. Animals and farming systems

Three genotypes of chicken (Broiler, Transylvanian naked-neck and Hungarian speckled chickens) were used in these experiments. Male and female chickens were received from the Institute for Small Animal Research, Gödöllő, Hungary.

The chicken were kept in "extensive" and "intensive" farming managements.

In the "intensive" farming managements the chicken were placed in a closed area of a big building. Density of chickens was 25 kg m^{-2} as a chick unit for growing stock. The air temperature was 32 °C in the first week, then it was decreased by 2 °C weekly to 18 °C. Twenty-four-hour light was performed both naturally and artificially. The chicken were fed with adequate diet for 52 days. The feeding of the animals was performed according to the prescription of the University of Agricultural Science, Gödöllő. Details for the feeding of the animals kept in "intensive" management are given in Table 1.

In the "extensive" farming management, the chicken were placed after hatching in a bigger building with higher ceiling as a fold unit for growing stock and fed in the first week with the same diet as the other group followed by a grower diet of seeds (maize, barley, rye, sunflower seed) and green forage until day 117.

Table 1

Composition of feeds for chicken kept in an intensive way

Feed	Starting feed till 22nd day (%)	Raising feed till 43rd day (%)	Finishing feed till 52nd day (%)
Maize	55.7	59.0	61.6
Wheat	5.0	5.0	5.0
Soy	28.5	26.2	24.0
Fish meal	5.0	4.0	–
Sunflower seed	–	–	3.0
Grease powder[a]	2.5	2.5	3.0
Methionine	0.1	0.1	0.05
Mono-calcium-phosphate	1.0	1.0	1.0
$CaCO_3$	1.5	1.5	1.5
NaCl	0.2	0.2	0.3
Starter Broiler premix 210 C[b]	0.5	0.5	0.5
Total	100.0	100.0	100.0
Energy [MJ kg^{-1}]	12.5	12.6	12.1
Digestible raw protein [%]	19.5	18.2	16.1

[a] 40% lard on the surface of corn flakes

[b] produced by Bábolna Ltd., containing a mixture of vitamins and trace elements

After two weeks the chicken were put in a "free range" situation, so the composition of their diet cannot be given. Temperature and light were influenced by the actual climate conditions during May in Hungary. Density of the animals was 12 kg m^{-2}.

1.2. Analytical methods

The cuts of chicken used in these experiments were breasts and legs. After the removal of the skin from breast and leg of each bird individually, they were deboned by hand and the weight of bone and meat was recorded. The meat was ground twice through a kitchen meat grinder.

Meat samples were analyzed immediately after grinding. Moisture, crude fat and protein were measured according to the analytical manual of the A.O.A.C. (1984, 24.003, 24.005, 24.038).

The pH values were assayed by a Radelkis pH meter (Type, PO-264/I Hungary) and measured according to KONIECKO (1979).

Water holding capacity was determined according to HAMM (1960).

Cholesterol content was determined as follows: after digestion with HCl, the fat was extracted and then saponified. The non-saponified part was cleaned by thin-layer chromatography and the sterols were separated on GLC. The stationary phase was Chromosorb Q with 3% liquid phase of JXR, using nitrogen as carrier gas at a temperature of 270 °C and employing a flame ionization detector. Stigmasterol was applied as internal standard.

Determination of fatty acid composition: fat was extracted with a mixture of chloroform–methanol (2:1, v/v). After evaporation, the residue was dissolved in hexane and then transesterified with 2 mol l^{-1} sodium methylate in absolute methanol. After neutralization with acetic acid, water was removed by $CaCl_2$ and the solution was injected into the gas chromatograph of Carlo Erba Fractovap 2400 with nitrogen as carrier gas. Chromosorb Q as a stationary phase and a liquid phase 5CP and 10C were used. Separation was carried out for both phases using a thermal program between 180–220 °C and a flame ionization detector.

The activity of superoxide dismutase (SOD) was measured photometrically at 480 nm by the inhibition of adrenaline autooxidation as described by SUN and ZIGMAN (1978).

Thiobarbituric-acid-reactive substances (TBARS) were determined by RAMANATHAN and DAS (1992) and the results were given as malondialdehyde (MDA) ng g^{-1} meat.

Conjugated dienes were assayed at 233 nm in extract of 2 g samples in 10 cm^3 isooctane as described in A.O.A.C.(1984, 24.054b).

The determination of iron, zinc and copper were carried out by atomic absorption spectroscopy (AAS) technique after dry-ashing (PERKIN-ELMER MANUAL, 1971).

Thiamine (vitamin B_1) was evaluated by a microbiological assay (GYÖRGY & PEARSON, 1967). Riboflavin (vitamin B_2) was assayed by HPLC technique (BARNA & DWORSCHÁK, 1994).

1.3. Statistical evaluation

For statistical evaluation a two-tailed Student's t-test and analysis of variance were used where appropriate.

2. Results

It was found that the muscle weights obtained from Broiler chicken were much higher than those of the other genotypes kept in both managements which are due to inherent genotype characteristics. These well-known data are not shown in this paper.

Table 2

Effect of three genotypes on meat to bone ratio of male and female chicken kept "intensively" and "extensively", respectively (mean ±SD)

Genotype	Meat to bone ratio							
	Extensive				Intensive			
	Male		Female		Male		Female	
	leg	breast	leg	breast	leg	breast	leg	breast
Broiler	3.1±1.1	2.0±0.9	2.9±0.9	4.1±1.2	3.7±0.5	5.9±0.6	4.0±0.6	5.9±1.6
n	11	11	13	13	10	10	9	9
Transylvanian naked-neck	2.4±0.3	2.9±0.5	2.1±0.6	3.4±0.7	2.5±0.4***	3.4±0.7***	2.6±0.4***	3.8±0.9**
n	10	10	4	4	20	20	20	20
Hungarian speckled	2.3±0.3*	3.1±0.8						
n	14	14						

Significant differences were determined in comparison to the Broiler sample within the columns
***P<0.001; **P<0.01; *P<0.02

The meat to bone ratio was significantly less for Transylvanian naked-neck genotype than for the Broiler kept intensively. However there was no difference between them when kept in "extensive" farming system (Table 2).

The pH values (Table 3) were higher in the meats of Transylvanian chicken than in those of Broiler kept "intensively" and no significant differences were observed between the genotypes kept "extensively" except for breast meat of Hungarian speckled genotype. The higher pH values remained in the normal range (NORTHCUTT et al., 1994). Table 4 shows that there were significant differences in water holding capacity only in the legs of Transylvanian naked-neck genotype kept intensively. These results are coincided with those obtained by NORTHCUTT and co-workers (1994). They reported that water-holding values were significantly influenced by the type of meat (breast and leg). Leg meat had higher held water for fresh and cooked sample than breast meat.

We found no significant differences in the protein content between the genotypes kept under the two different farming managements. These results are not shown here.

As can be seen in Table 5 the crude fat content of Transylvanian naked-neck genotype kept "intensively" was significantly lower as compared to the Broiler chicken. There were no differences between the genotypes kept "extensively".

Table 3

Effect of three genotypes on pH of male and female chicken kept "intensively" and "extensively", respectively (mean ±SD)

Genotype	pH value							
	Extensive				Intensive			
	Male		Female		Male		Female	
	leg	breast	leg	breast	leg	breast	leg	breast
Broiler	5.75±0.23	5.51±0.19	5.73±0.11	5.48±0.22	5.88±0.31	5.31±0.4	5.70±0.26	5.47±0.29
n	11	11	13	13	10	10	9	9
Transylvanian naked-neck	5.90±0.15	5.52±0.09	5.95	5.63	6.25±0.18***	5.78±0.31***	6.29±0.09***	5.83±0.35**
n	5	5	2	2	10	10	10	10
Hungarian speckled	5.73±0.11*	5.21±0.04***						
n	7	7						

Significant differences were determined in comparison to the Broiler sample within the columns
***P<0.001

Table 4

Effect of three genotypes on water-holding capacity (WHC) of male and female chicken kept "intensively" and "extensively", respectively (mean ±SD)

Genotype	Water holding capacity (%)							
	Extensive				Intensive			
	Male		Female		Male		Female	
	leg	breast	leg	breast	leg	breast	leg	breast
Broiler	43.66±3.10	42.98±5.46	44.47±5.92	42.89±6.0	38.49±2.46	40.59±8.31	37.12±2.55	38.28±3.36
n	9	11	12	13	10	10	9	9
Transylvanian naked-neck	44.99±5.19	42.37±3.06	50.26	40.59	42.64±2.59***	40.41±4.24	42.55±5.17**	40.33±3.23
n	5	5	2	2	10	9	10	10
Hungarian speckled	42.79±10.28	35.37±9.64						
n	7	7						

Significant differences were determined in comparison to the Broiler sample within the columns
***P<0.001; **P<0.01

As for fatty acid composition Table 6 shows that the meats of Transylvanian naked-neck had higher level of linoleic acid when kept intensively than in the Broiler muscle. The same results were found for the leg parts of Transylvanian naked-neck and Hungarian speckled kept "extensively". For the linolenic acid there were small,

however significant differences in favour of the Transylvanian genotype. Concerning the myristic and palmitic acid concentration that can be regarded as risk factor in atherogenic processes, it was significantly lower in the muscles of Transylvanian naked-neck genotype kept "intensively" than in the Broiler. No explanation was found for the reason of these findings.

Cholesterol levels did not show any significant differences between the genotypes.

As for the lipid degradation products the amounts of conjugated dienes were found much smaller in the meats of Transylvanian naked-neck than those of Broiler kept intensively. The results unambiguously show the inclination of the Broiler's muscle to lipid peroxidation probably due to their generally higher fat level and looser membrane structure. There were no differences between the genotypes kept in extensive farming management (Table 7).

TBARS determination is also one of the most widely used method for measuring lipid peroxidation in muscle food. We found no significant differences between the genotypes except for the female breasts kept "intensively". "Extensive" Hungarian speckled legs have significantly lower TBARS values than those of Broiler (Table 8).

As for the enzymatic defence system, Table 9 indicates that the meat of Transylvanian naked-neck and also the muscles of Hungarian speckled chickens kept "extensively" had significantly lower SOD activity as compared to the Broiler.

Table 5

Effect of three genotypes on crude fat contents of male and female chicken kept "intensively" and "extensively", respectively (mean ±SD)

Genotype	Crude fat (g/100 g)							
	Extensive				Intensive			
	Male		Female		Male		Female	
	leg	breast	leg	breast	leg	breast	leg	breast
Broiler	3.18±1.38	1.18±0.58	4.20±1.68	1.71±1.08	7.2±0.91	3.5±0.89	7.6±1.5	3.2±1.12
n	5	6	10	10	7	9	8	8
Transylvanian naked-neck	1.8±0.59	1.19±1.4	6.3	3.2	3.9±0.68***	1.58±0.34***	3.8±0.75***	1.38±0.49***
n	3	4			7	9	8	9
Hungarian speckled	2.6±0.9	1.29±0.79						
n	7	7						

Significant differences were determined in comparison to the Broiler sample within the columns
***P<0.001

Table 6

Effect of three genotypes on fatty acid content of male and female chicken kept, "intensively" and "extensively", respectively (mean ±SD)

Genotype	Fatty acid (g/100 g fatty acids)							
	Extensive				Intensive			
	Male		Female		Male		Female	
	leg	breast	leg	breast	leg	breast	leg	breast
				Myristic acid (C14)				
Broiler	0.6±0.1	0.5±0.15	0.6±0.2	0.6±0.2	0.7±0.1	0.8±0.1	0.8±0.1	0.7±0.1
n	10	10	13	13	10	10	9	9
Transylvanian naked-neck	0.5±0.2	0.4±0.2	0.2	0.2	0.6±0.1*	0.6±0.1****	0.7±0.1	0.7±0.1
n	5	5	2	2	10	10	9	10
Hungarian speckled	0.5±0.1	0.4±0.2						
n	7	7						
				Palmitic acid (C16)				
Broiler	22.5±2.1	23.4±2.0	23.0±2.4	24.1±2.3	25.7±1.1	26.6±1.7	25.7±1.4	26.6±1.4
n	10	10	13	13	10	10	9	9
Transylvanian naked-neck	20.4±2.7	22.1±2.3	21.2	22.5	21.1±1.0****	23.6±0.9****	21.2±0.7****	23.0±0.8****
n	5	5	2	2	10	10	10	10
Hungarian speckled	20.2*±1.3[b]	21.9±1.2						
n	7	7						
				Linoleic acid (C18=2)				
Broiler	14.2±2.5	14.9±2.6	13.7±2.2	14.4±3.4	15.3±2.0	14.8±1.5	14.0±1.3	13.6±1.8
n	10	10	13	13	10	10	9	9
Transylvanian naked-neck	18.2±1.4**	16.9±1.8	11.8	12.6	25.6±1.5****	22.8±1.8****	25.8±2.1****	23.2±1.5****
n	5	5	2	2	10	10	10	10
Hungarian speckled	17.3±2.0***	16.4±1.1						
n	7	7						
				Linolenic acid (C18=3)				
Broiler	1.4±0.7	1.2±0.7	1.6±0.7	1.4±0.5	1.1±0.2	0.9±0.3	1.0±1.2	0.9±0.3
n	10	10		13		10	9	9
Transylvanian naked-neck	1.2±0.5	0.9±0.3	1.4	1.1	1.3±1.2*	1.0±0.2	1.2±0.2	0.9±0.3
n	5	5	2	2	2	10	9	10
Hungarian speckled	1.4±0.7	2.1±0.8*						
n	7	7						

Significant differences were determined in comparison to the Broiler sample within the columns

****$P<0.001$; ***$P<0.01$; **$P<0.02$; *$P<0.05$

Table 7

Effect of three genotypes on conjugated dienes in male and female chicken kept "intensively" and "extensively", respectively (mean ±SD)

Genotype	Conjugated dienes A_{233}							
	Extensive				Intensive			
	Male		Female		Male		Female	
	leg	breast	leg	breast	leg	breast	leg	breast
Broiler	0.403±0.492	0.218±0.152	0.620±0.530	0.186±0.097	2.12±0.59	0.572±0.239	3.19±0.88	0.684±0.261
n	11	11	11	13	10	10	9	9
Transylvanian naked-neck	0.184±0.108	0.115±0.052	0.422	0.206	0.417±0.192***	0.167±0.057***	0.400±0.210***	0.158±0.0.60***
n	5	5	2	2	10	10	10	10
Hungarian speckled	0.156±0.084	0.102±0.056						
n	7	7						

Significant differences were determined in comparison to the Broiler sample within the columns
***P<0.001

Table 8

Effect of three genotypes on TBARS of male and female chicken kept "intensively" and "extensively", respectively (mean ± SD)

Genotype	TBARS and MDA ng/100 g meat							
	Extensive				Intensive			
	Male		Female		Male		Female	
	leg	breast	leg	breast	leg	breast	leg	breast
Broiler	114.6±36.6	88.0±41.8	92.2±25.3	71.9±19.1	147.8±26.1	106.7±25.5	150.6±40.0	126.2±26.7
n	11	11	12	11	10	10	9	9
Transylvanian naked-neck	77.5±29.2	59.7±17.2	65.0	57.8	130.0±35.0	92.0±29.4	122.2±34.9	78.5±33.3***
n	5	5	2	2	10	10	10	10
Hungarian speckled	63.0±8.0**	59.2±10.1						
n	7	7						

Significant differences were determined in comparison to the Broiler sample within the columns
***P<0.001; **P<0.01

As can be seen from Table 10 the iron levels of the muscle from Transylvanian naked-neck kept "intensively" were much higher than those of Broiler. The zinc and copper levels showed small differences. These data are comparable to our earlier results (LATIF et al., 1996).

Table 9

Effect of three genotypes on superoxide dismutase (SOD) in male and female chicken kept "intensively" and "extensively", respectively (mean ±SD)

Genotype	SOD (U/mg protein)							
	Extensive				Intensive			
	Male		Female		Male		Female	
	leg	breast	leg	breast	leg	breast	leg	breast
Broiler	705±131	494±91	540±68	321±54	257±81	174±30	202±66	175±40
n	11	11	13	13	10	10	9	9
Transylvanian naked-neck	400±34**	211±25**			233±83	218±93	171±45	194±58
n	5	5			10	10	10	10
Hungarian speckled	640±196	230±52***						
n	7	7						

Significant differences were determined in comparison to the Broiler sample within the columns
***P<0.001; **P<0.01

As Table 11 shows, practically there are no differences in the thiamin content between the meats of the genotypes. However, riboflavin levels are generally higher in the muscles of Transylvanian genotype compared to the Broiler.

3. Discussion and conclusion

The results of the present study clearly demonstrate that the meat of various genotypes of chicken showed significant differences in chemical components and nutritive value of the muscle and these were further modified by the farming managements.

It must be taken into consideration, that the animals from different farming managements were not slaughtered at the same time. But from nutritional and organoleptic viewpoints the different times (52 and 117 days, respectively) should be regarded as the physiologically "grown up" state and an optimal condition for the meat consumption. After all, the time of slaughtering had no significant effect on our results.

Table 10

Effect of three genotypes on zinc, iron and copper levels of male and female chicken kept "intensively" and "extensively", respectively (mean ±SD)

Genotype	Extensive				Intensive			
	Male		Female		Male		Female	
	leg	breast	leg	breast	leg	breast	leg	breast
				Zn (µg/g)				
Broiler	25.96±11.2	14.0±4.3	22.5±3.2	10.88±4.1	13.96±4.30	9.71±4.61	16.45±2.51	9.26±2.79
n	9	10	12	12	10	10	9	9
Transylvanian naked-neck	22.72±8.09	13.66±6.19	23.27	12.22	17.43±3.04**	8.96±2.1	16.77±2.63	11.14±5.1
n	5	5	2	2	10	9	9	9
Hungarian speckled	21.23±4.6	7.84±0.54****						
n	7	7						
				Fe (µg/g)				
Broiler	15.64±7.78	9.65±4.65	12.75±4.64	7.07±3.47	7.24±2.80	5.83±2.96	7.36±1.31	5.52±2.37
n	10	11	12	13	10	10	9	9
Transylvanian naked-neck	16.70±6.36	10.37±5.70	12.16	12.46	10.45±3.78****	6.18±0.9***	13.50±3.92*****	8.61±3.22**
n	4	5	2	2	10	9	10	9
Hungarian speckled	13.63±4.61	5.55±1.75*						
n	7	7						
				Cu (µg/g)				
Broiler	0.75±0.26	0.49±0.14	0.65±0.25	0.41±0.12	0.53±0.11	0.41±0.12	0.50±0.12	0.35±0.18
n	10	10	12	13	10	10	9	9
Transylvanian naked-neck	1.09±0.77	0.36±0.13	0.98	0.51	0.71±0.32	0.53±0.20	0.74±0.16***	0.49±0.17
n	4	5	2	2	10	8	10	9
Hungarian speckled	0.94±0.52	0.41±0.25						
n	7	6						

Significant differences were determined in comparison to the Broiler sample within the columns
*****P<0.001; ****P<0.002; ***P<0.01; **P<0.02; *P<0.05

Table 11

Effect of three genotypes on thiamin and riboflavin content of male and female chicken kept "intensively" and "extensively", respectively (mean ±SD)

Genotype	Extensive				Intensive			
	Male		Female		Male		Female	
	leg	breast	leg	breast	leg	breast	leg	breast
				Thiamin (μg/100 g)				
Broiler	84.7±31.7	66.4±29.3	88.2±18.4	61.5±16.2	57.5±19.8	51.1±18.5	58.5±15.8	51.5±17.6
n	11	11	13	13	10	10	9	9
Transylvanian naked-neck	87.2±8.5	49.7±9.2	132.5±26.2*	80±4.2	48.2±15.0	39.9±12.4	49.8±20.6	37.0±17.7
n	5	5	2	2	10	10	10	10
Hungarian speckled	103±33.7	61.7±22.1						
n	7	7						
				Riboflavin (μg/100 g))				
Broiler	171±33.9	70.0±14.7	165.5±30.0	72.7±19.96	119±44.0	81±17.7	109±25.5	83±27.7
n	10	11	13	13	10	10	9	9
Transylvanian naked-neck	243±20.5	74.6±13.9	190.5±38.9*	86±19.8	198±57.6*	75±26.3	161±32.6***	94±28.4
n	5	5	2	2	10	10	10	10
Hungarian speckled	193.6±27.3	59±5.7						
n	7	7						

Significant differences were determined in comparison to the Broiler sample within the columns
**P<0.002; *P<0.01

Transylvanian naked-neck chicken were generally less influenced by "intensive" farming system, especially for the quality characteristics. The meat of Transylvanian genotype was richer in riboflavine and trace elements (Fe, Zn and Cu) during the "intensive" farming management which is advantageous from nutritional viewpoint. The meats of Transylvanian naked-neck chicken had significantly lower percentages of fat content, myristic and palmitic acids, which are atherogenic factors, compared to those of Broilers kept "intensively".

From economical point of view the Broiler chicken showed the most favourable effect in the increase of muscle weight when kept in "intensive" and "extensive" farming managements, but on the other hand it was coupled with the loss of numerous qualitative characteristics, respectively, partly pre-determined genetically.

Neither genotype nor farming management had any influence on either the protein content or cholesterol level of legs or breast meat of chicken.

From the point of water-holding-capacity Transylvanian naked-neck chicken was superior to Broiler kept "intensively".

Transylvanian naked-neck chicken kept "intensively" produce "healthier" basic foods (less in conjugated diene content and in some cases lower TBARS level) because these degradation products of lipid peroxidation promote some atherogenic and tumour-initiating reactions.

*

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COMPARISON OF MEDIA FOR ENUMERATION OF *CLOSTRIDIUM SPOROGENES* PA3679/S BY CONDUCTANCE MEASUREMENT

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The efficiency of Plate Count Broth (PCB), Yeast Peptone Tryptone Dextrose Medium (YPTD), Modified PA3679 Broth (MPA), Yeast Extract Broth (YEB), Thioglycollate Broth (Thiogl) – without NaCl, Malthus Special Peptone Yeast Extract Medium (Malthus), Differential Reinforced Clostridial Medium (DRCM), Tryptone Soya Broth (TSB) – without NaCl, Modified Tryptone Peptone Glucose Medium (MTPG), Tryptone Peptone Glucose Yeast Broth (TPGY) for measuring conductance changes during the outgrowth of *Clostridium sporogenes* PA3679/S was tested in a RABIT instrument. With PCB, YPTD, MPA and YEB conductance curves could not be obtained. In the case of Thiogl, Malthus, DRCM and TSB the correlation between the detection times and the initial cell counts was not adequate. The best results were received with the MTPG and the TPGY media.

Keywords: conductance technique, RABIT, *Clostridium sporogenes* PA3679, medium

It has been known for years that when microbes are present in a medium the conductance of the medium changes as a result of their metabolic activities. This change can be measured and plotted against time. However, for some microbes, such as the clostridia, it is not easy to find a medium which is suitable both for the instrument and the bacteria themselves. For the cultivation of clostridia the normally used media contain salts which make them unsuitable for a conductance measurement as the presence of NaCl higher than 0.5% makes it impossible for the instrument to work. To eliminate this problem the indirect technique was developed. However, in some cases when growth properties (growth rate, lag-phase) are to be examined the indirect technique is not suitable, since the microbe reaches the stationary phase of its growth before the detection begins, thus the characteristics of the plotted curve can not be interpreted as growth properties.

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The aim of our work was to examine the applicability of some media suggested for cultivation of clostridia in the literature for conductance measurements with the RABIT instrument.

Materials and methods

1.1. Test organism

The measurements were carried out with *Clostridium sporogenes* PA3679/S anaerobic mesophilic spore forming bacteria. Spores were obtained by using a biphasic cultivation technique according to SCOTT and BERNARD (1982) with the difference, that in the solid phase the component ground defatted beef heart (500 g) was replaced with meat extract (16.7 g). Harvested spores were washed with distilled water by centrifugation and stored in distilled water at 4 °C.

1.2. Culture media

Plate Count Broth (PCB), Differential Reinforced Clostridial Medium (DRCM) and Malthus Special Peptone Yeast Extract Medium (Malthus) were commercially available mixtures from Merck and Malthus, they were prepared as recommended on the label. Thioglycollate Broth (Thiogl) and Tryptone Soya Broth (TSB) was prepared without the addition of NaCl, Yeast Peptone Tryptone Dextrose Medium (YPTD) was prepared according to the formula of MALLIDIS and SCHOLEFIELD (1986), Modified PA3679 Broth (MPA) was prepared as described by GRISCHY and co-workers (1983), Yeast Extract Broth (YEB) was made by the formula of PFLUG and co-workers (1979) studied by POLVINO and BERNARD (1982), GRISCHY and co-workers (1983), Modified Tryptone Peptone Glucose Medium (MTPG) and Tryptone Peptone Glucose Yeast Broth (TPGY) were prepared as described by SCOTT and TAYLOR (1981).

The composition of the media is shown in Table 1.

1.3. Conductance measurement

For the conductance measurement a RABIT instrument was used. Prior to examination the spore suspension was heat activated at 80 °C for 10 min. Decimal dilutions from 10^8 to 10^1 cm^{-3} were made and from each dilution 0.5 cm^{-3} was transferred to 9.5 cm^{-3} medium in two parallels in the case of each medium. Each measurement was carried out at 37 °C for up to 48 h. Detection times were defined as the time at which a 7 μS increase was detected above the baseline. Detection times were then plotted against the *Clostridium sporogenes* count in RCM agar obtained by traditional plating and incubation at 37 °C for 5 days in anaerobic jar.

Table 1

Composition of media (g l^{-1}) examined in the RABIT instrument for cultivation of Clostridium sporogenes PA3679/S

Name	Composition of media (g in 1000 cm³)											Reference
	Tryptone	Yeast extract	Glucose	L-Cysteine	Na-thioglycollate	Soya Peptone	Prot. Peptone	Lab-Lemco	K_2HPO_4	Starch	Na-acetate	
PCB	5	2.5	1									Merck
DRCM	5	1	1	0.5			5	8		1	5	Merck
TSB	17		2.5			3			2.5			Merck
YPDT	3	1	1				5	2.5		1		Mallidis & Scholefield (1986)
Malthus		x					x					Malthus
TPGY	50	20	4		1		5					Scott & Taylor (1981)
Thiogl	15	5	5.5	0.5	0.5							Merck
MTPG	50		4	0.5			5					Scott & Taylor (1981)
MPA	10	2			x				2	2		Pflug et al. (1979)
YEB		10	x		x				2	1		Pflug et al. (1979)

x – exact amount is not known

2. Results

Using PCB, YPTD, MPA and YEB media no curves were obtained with the RABIT conductance measurement. That means that either the growth of the bacteria or the quality of their metabolic activities was not enough to induce a change in the conductivity of the media sufficient to reach the minimum detection level of the instrument.

In case of Thiogl medium a good correlation (R^2=0.9813) between the cell count and the detection time was found but only when the initial count was above 10^6 cm^{-3}. In the tubes inoculated with fewer bacteria the changes in the conductivity due to the metabolic activity of the bacteria were not enough to reach the minimum detection level of the instrument (Fig. 1).

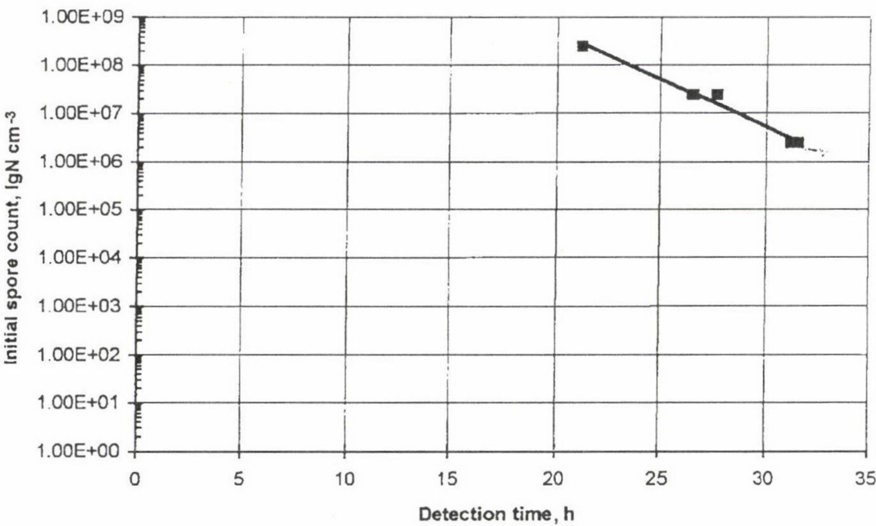

Fig. 1. Calibration curve for *Clostridium sporogenes* PA3679/S in Thiogl medium for RABIT conductance measurement (n=2); $y=4E+12e^{-0.4526x}$; $R^2=0.9813$

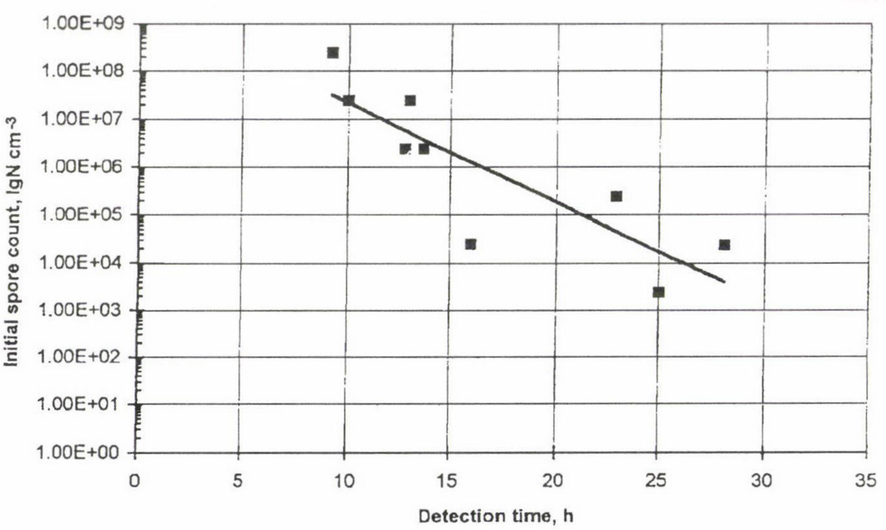

Fig. 2. Calibration curve for *Clostridium sporogenes* PA3679/S in Malthus medium for RABIT conductance measurement (n=2); $y=3E+09e^{-0.4756x}$; $R^2=0.7215$

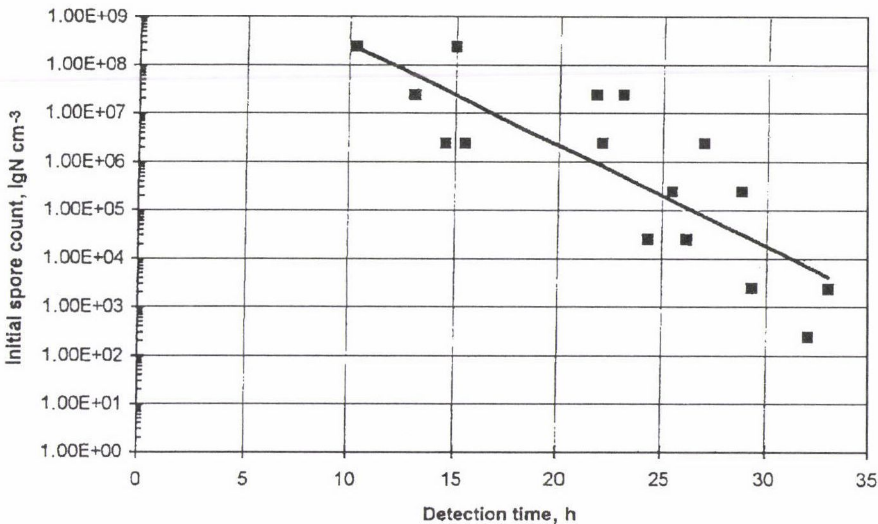

Fig. 3. Calibration curve for *Clostridium sporogenes* PA3679/S in DRCM medium for RABIT conductance measurement (n=2); $y=3E+10e^{-0.4806x}$; $R^2=0.6907$

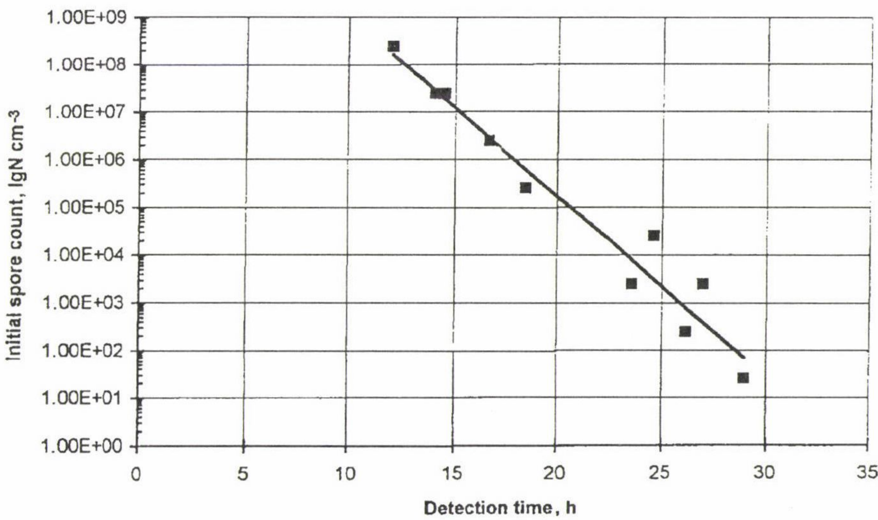

Fig. 4. Calibration curve for *Clostridium sporogenes* PA3679/S in TSB medium for RABIT conductance measurement (n=2); $y=5E+12e^{-0.8619x}$; $R^2=0.9546$

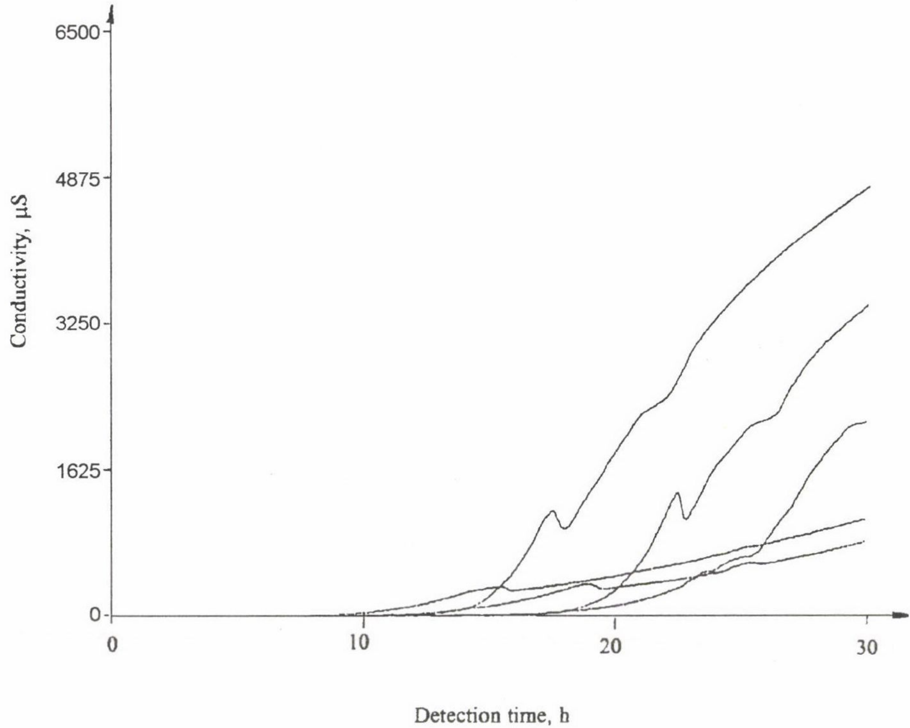

Fig. 5. Curves obtained with *Clostridium sporogenes* PA3679/S in TSB medium with the RABIT instrument

With the media Malthus (Fig. 2) and DRCM (Fig. 3) curves could be obtained with initial spore counts as low as 10^2–10^3 cm^{-3}. The quality of the curves were acceptable too, but the correlations between the initial cell counts and the detection times are not reliable (R^2=0.7215 and 0.6907).

The calibration with TSB showed a very good correlation (R^2=0.9546) between the initial cell counts and the detection times (Fig. 4), but the quality of the growth curves were very poor (Fig. 5). With the addition of supplements highly metabolised by *Clostridium sporogenes* we have tried to improve the quality. Cysteine (0.05%), tryptophane (0.2%) and Na-carbonate (0.14%) were added to the medium, but they proved to be inefficient.

MTPG (Fig. 6) and TPGY (Fig. 7) proved to be the best media of the ones studied. The correlation between the initial cell counts and the detection times was very good (R^2=0.9711 and 0.9765). Curves were obtained even with initial cell counts of 10^1 cm^{-3}. The obtained growth curves were smooth (Fig. 8).

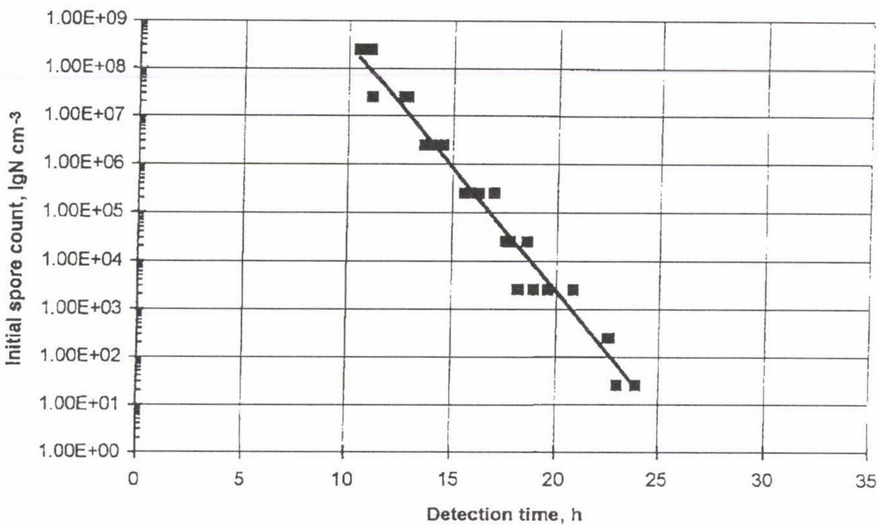

Fig. 6. Calibration curve for *Clostridium sporogenes* PA3679/S in MTPG medium for RABIT conductance measurement (n=6); $y=4E+13e^{-1.1798x}$; $R^2=0.9711$

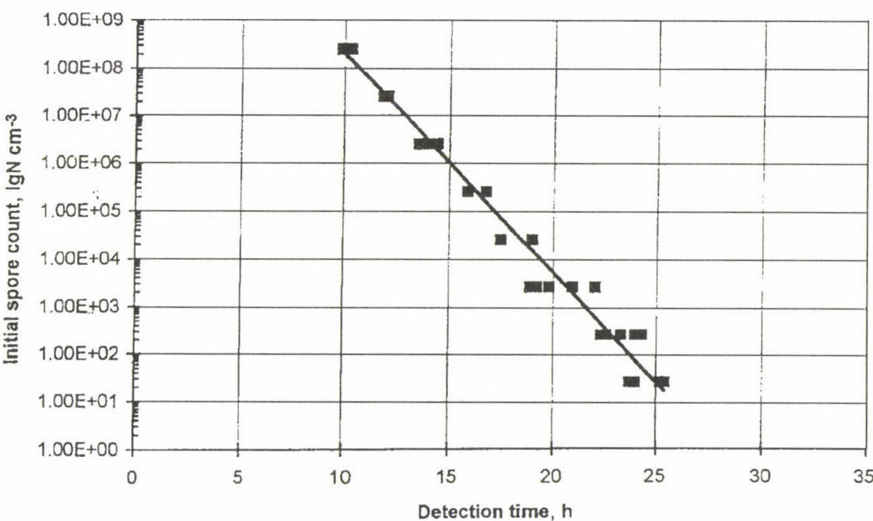

Fig. 7. Calibration curve for *Clostridium sporogenes* PA3679/S in TPGY medium for RABIT conductance measurement (n=6); $y=8E+12e^{-1.0584x}$; $R^2=0.9765$

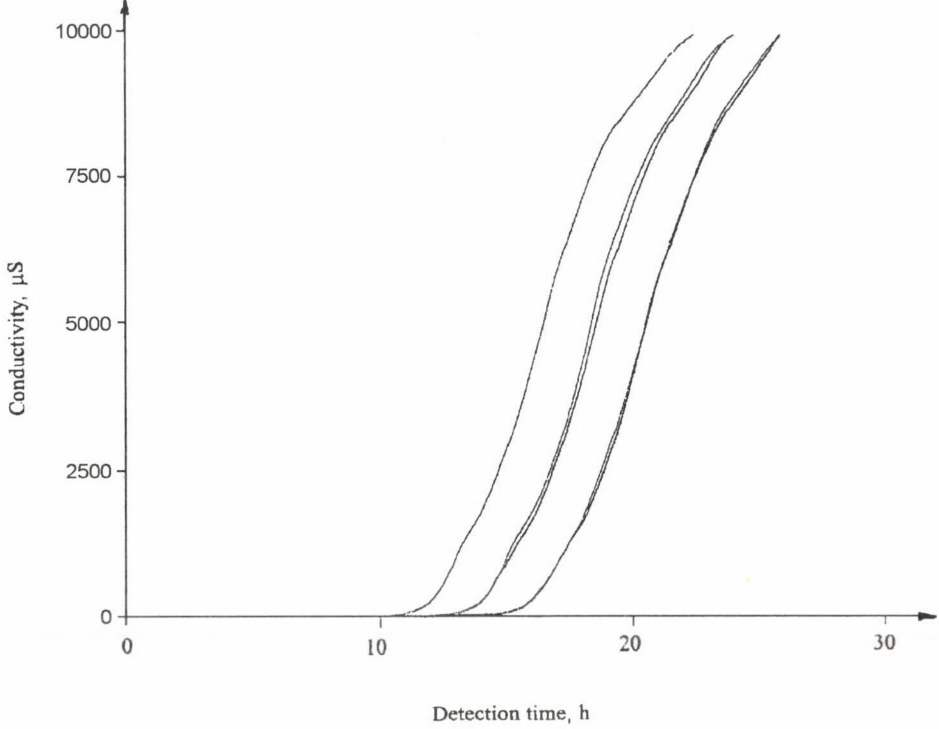

Fig. 8. Curves obtained with *Clostridium sporogenes* PA3679/S in TPGY medium with the RABIT instrument

3. Conclusions

Our main goal was to find a medium that meets the requirements of the direct conductance measurements in the RABIT instrument and provides a good cultivation medium for *Clostridium sporogenes* PA3679/S as well.

From the obtained results it can be concluded that PCB, YPTD, MPA and YEB can not be used, Thiogl, Malthus, DRCM and TSB only with modifications and further investigations are necessary.

MTPG and TPGY media satisfy the demands raised by conductance measurement. The correlation between the initial spore counts and the detection times are highly significant (R^2=0.9711 and 0.9765, respectively) and the quality of the curves is good.

*

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DETECTION OF WHEAT BY ADAPTED POLYMERASE CHAIN REACTION (PCR) METHODOLOGY

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The wheat-PCR methodology, elaborated by ALLMANN and co-workers (1993), was adapted to detect wheat in different foodstuffs. Protocols of the cited paper were used except for extraction of DNA, the preparation of which was performed by three phase partitioning as well as DNA binding. Amplicons (109 bp wheat-DNA segments) were obtained for flours and heat-treated foodstuffs. This methodology provides for quick investigation of DNA in different food products.

Keywords: wheat-PCR, three-phase partitioning of DNA, heat treated foodstuffs

Since 1985, polymerase chain reaction (PCR) has been one of the most powerful method in molecular biology. In a programmable thermocycler, the target-DNA flanked by two oligonucleotide primers can be amplified by thermostable Taq DNA-polymerase on condition that length, temperature and number of denaturation, annealing, extension as well as $MgCl_2$ concentration are optimized. Detailed information on polymerase chain reaction is given in the book of INNIS and co-workers (1990) and that of MCPHERSON and co-workers (1991).

The species-specific DNA fragment, produced in easily detectable quantity by polymerase chain reaction, is a uniquely useful signal e.g. for identification of pathogen microorganisms. Work done in this field of food research is very intense (CANDRIAN & LÜTHY, 1991; CANDRIAN, 1994), though beyond the scope of the work presented. Detection of a food component in foodstuffs by analysis of specific nucleic acids based on hybridization, including polymerase chain reaction, is a promising new area of food investigation. Intentional or accidental adulterations have brought about speedy development in food analysis. Presence or absence of wheat in special products is of primary importance to people suffering from wheat intolerance. After successful amplification, amplicons can be separated and visualized traditionally by electrophoresis, often characterized additionally by restriction endonucleases (ALLMANN et al., 1992a and b (wheat), MEYER et al., 1993 (soya), ALLMANN et al., 1993 (wheat), MEYER et al., 1994 (pork)). To detect presence of wheat in the food

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matrix, genomic DNA must be isolated. Efficient purification of DNA was achieved by a novel application of three phase partitioning, a method used successfully for the purification of horseradish peroxidase (SZAMOS & HOSCHKE, 1992).

PCR, applied for food component investigation, is a technique of quick detection and identification, however, its quantification can only be solved by additional experiments.

The aim of our work was to initiate a carefully elaborated wheat PCR methodology to acquire experience on DNA-based analysis.

1. Materials and methods

1.1. Materials

1.1.1. Samples. Wheat flour (BL 55), breadcrumbs, biscuit (Sire Sire), and bouillon cube (Maggi) were of commercial origin, other cereals were made available for us by the National Institute of Food Hygiene and Nutrition, Budapest.

1.1.2. Chemicals. Absolute ethanol, acrylamide, ammonium persulfate, ammonium sulfate, boric acid, bromophenol blue, ethylenediamine tetraacetic acid (EDTA), HCl, LiCl, NaCl, N,N'-methylene bisacrylamide, N,N,N',N'-tetramethyl-ethylenediamine, sodium lauryl sulfate, tert butanol and Tris base were of analytical grade (Reanal). Proteinase K was from Merck, Ampli Size DNA Standard, Ampli Size Agarose and ethidium bromide solution were from BioRad. Gene Amp Lambda Control Set (Perkin Elmer) was used for instrument control. For each amplification Master Mix (Boehringer) was used as reaction mixture. For final purification of DNA, Wizard DNA Clean Up System (Promega) was used.

1.1.3. Primers. For priming two 25-mers for wheat PCR (ALLMANN et al., 1993), synthesized by Pharmacia, were used.

1.1.4. Reagents for ELISA. Gliadin was from Fluka, all other chemicals were of analytical grade. Antigliadin-rabbit IgG and antigliadin-rabbit IgG-HRP conjugate were prepared at the Central Food Research Institute. Horseradish peroxidase conjugated goat anti-human IgG (Human), HRP-conjugated goat anti-rabbit IgG (Human) and 3,3',5,5'-tetramethyl benzidine (TMB) substrate were used. Human sera of children containing anti-gliadin IgG antibody were from Madarász Children Hospital Gastroenterological Centre.

1.2. Methods

1.2.1. Gliadin extraction. Ground samples (100 mg) were mixed with 1 cm^3- of 70% (v/v) aqueous ethanol in capped centrifuge tube and extracted with vigorous shaking on a flash shaker (IKA-Schüttler MTS 4, 700 rpm for 30 min at 4 °C).

Following extraction, the samples were centrifuged (T 24 D, 5000 rpm for 20 min at room temperature), the supernatant was separated and used for ELISA.

1.2.2. Desoxyribonucleic acid extraction. Half a cm^3 or 1.0 cm^3 extraction solution (0.1 mol Tris-HCl (pH 7.5), 0.1 mol NaCl, 0.01 mol EDTA, 2% (w/v) sodium dodecylsulphate, 0.5 mg cm^{-3} proteinase K) was added, resp., to 50 mg flour or 100 mg of powdered food sample weighed in 1.5 cm^3 Eppendorf tubes. Samples were digested on a Stat Fax incubator, set to 575 rpm for 4 h at 37 °C, by shaking the tubes positioned horizontally in a case made of foam plastic. After digestion, the samples were centrifuged at 13 000 g for 15 min and supernatants were processed by the reference method (ALLMANN et al., 1993) or by three phase partitioning and Wizard DNA Clean Up procedure, respectively.

In the reference method, DNA-isolation was attained by addition of saturated NaCl solution and subsequent ethanol precipitation. In the partitioning procedure used for DNA isolation, at first 0.5 cm^3 of the supernatant was added to 300 mg powdered, sifted ammonium sulfate in a 1.5 cm^3 Eppendorf tube and vortexed. After dissolving the salt, 215 μl tert. butanol was added to the solution containing abundant precipitate, well shaken and kept at 4 °C for at least 1 h. (If the initial supernatant contained large amount of proteins, a more efficient removal of them was achievable by a two stage three phase partitioning as follows: 0.5 cm^3 supernatant was added to 88 mg of ammonium sulfate (30% relative saturation) and mixed. After addition of 215 μl tert. butanol, the system was well shaken then let to stand for 1 h. Phase separation was completed by low speed centrifugation (800–1000 rpm for 5 min), the result of which was a protein midlayer between the organic and aqueous liquid phases. The aqueous phase was transferred to another 1.5 cm^3 Eppendorf tube containing 200 mg ammonium sulfate. After dissolution of the salt, 215 μl tert. butanol was pipetted onto the solution, mixed thoroughly by shaking, thereafter left at 4 °C for at least one h. The midlayer formed by low speed centrifugation was separated from the liquid phases and dissolved in 0.2–0.5 cm^3 of 0.01 mol Tris-HCl –0.001 mol EDTA-buffer (pH 7.5).

A_{260}/A_{280} ratios of extracted DNA ranged from 1.0 to 1.5 (one-stage three phase partitioning), or from 1.4 to 1.7 (two-stage TPP), respectively, depending on composition and solubility relationships of the sample matrix. DNA prepared by the partitioning step was further purified by Wizard DNA Clean up System (Promega) according to the instruction of the manufacturer.

1.2.3. PCR conditions and DNA analysis. Wheat DNA amplifications were carried out in a final volume of 50 μl in 0.5 cm^3 thermocycler tubes (Marsh). DNA solutions were diluted to 20–40 ng μl^{-1}, and 5 μl of the dilution was added to each tube, containing primers pipetted previously. The solution was supplemented with sterile water to 25 μl (tubes for reaction control contained all the components, but in place of template DNA 5 μl of sterile water was added). The tubes were placed in the

thermocycler (PDR-91, BLS) and DNA was denatured at 95 °C for 2 min; in the pause, 25 μl of Master Mix preheated to 50 °C was added and the DNA-program (see Table 1) started. Thermocycler control was performed by amplification of Gene Amp Lambda Control DNA.

Table 1

Wheat-PCR thermocycling parameters

Operation	Setting of thermocycler
Hot Start	94 °C/2 min
Denaturation	95 °C[a]/10 sec
Annealing	68 °C[a]/30 sec
Extension	72 °C[a]/10 sec[a]
Cycle number	35[+]

[a] data of reference publication

PCR reaction mixtures were analyzed on 8% polyacrylamide gel in Midget Electrophoresis Unit (LKB). Ten cm^3 of gel solution was composed of 2.65 cm^3 acryl stock (29:1), 1 cm^3 of tenfold concentrated Tris-boric acid-EDTA buffer, 6.36 cm^3 distilled water, 8 μl TEMED and 80 μl of 10% ammonium persulfate. 1.1 μl of tenfold concentrated loading buffer (20% Ficoll 400, 100 mmol Na_2 EDTA, 1% SDS, 0.25% bromophenol blue, pH 8.0) was added to 10 μl of PCR reaction mix and 10 μl of this dilution was loaded onto the well of the slab gel (AUSUBEL et al., 1989). The gel was run at 5 V cm^{-1} for 3 h, stained in 0.5 μg cm^{-3} ethidium bromide solution for 15 min and washed with distilled water for 15 min. The transilluminated gel was photographed by means of a yellow filter. The ethidium bromide waste was destructed by the sodium nitrite/hypophosphorous acid method (LUNN & SANSONE, 1990).

1.2.4. Allergenic test. Indirect competitive ELISA was used to determine allergenicity of food samples. Five μg cm^{-3} gliadin, fifty μl sample (200–20 000 μg cm^{-3}) and gliadin standard (0.2–2 000 μg cm^{-3}) diluted in 0.1 mol PBS (pH 7.4), was added to each well of ELISA plates coated with 5 μg cm^{-3} gliadin, respectively. Human sera of children containing antigliadin IgG antibody (1:100), were added. Determinations were performed in triplicate for each data point. After incubation at 37 °C for 1 h, the wells were washed with PBST (pH 7.4) and 0.1 cm^3 of horseradish peroxidase-conjugated goat anti-human IgG diluted in 0.1 mol PBS (pH 7.4, 1:1500) was added to each well. Reagents were then incubated at 37 °C for 1 h and the excess of reagents was removed by washing with PBST (3×0.3 cm^3/well). 0.1 cm^3 of OPD-H_2O_2 (0.4 mg cm^{-3} o-phenylene diamine in 0.05 mol phosphate/citrate buffer,

containing 0.01% (v/v) H_2O_2, pH 5.0) was added to each well. After 5 min the reaction was stopped by adding 0.05 cm^3 4 mol sulfuric acid and optical densities were measured at λ=490 nm using a Dynatech (UK) ELISA reader. Results were calculated in mg g^{-1} sample on the basis of calibration curve. Sensitivity of indirect competitive ELISA was 200 μg cm^{-3}. Allergenic activities were expressed in percentage of the reference serum sample.

1.2.5. Sandwich ELISA for gliadin. The immune-serum for sandwich ELISA was raised in rabbits by injecting whole gliadin. Wells of ELISA plates were incubated overnight at 4 °C with anti-gliadin rabbit IgG antibody diluted (1:200) from stock solution in 0.05 mol Na-carbonate-bicarbonate buffer (pH 9.6). Following washing, the wells were incubated with samples diluted in 0.1 mol PBS (1:50, 1:100, 1:300, pH 7.4) and gliadin standard diluted in 0.1 mol PBS (3 000 ng, 1 000 ng, 300 ng, 100 ng, 50 ng, 25 ng, 12.5 ng, 6.25 ng, pH 7.4) for 1 h at 37 °C. After 3 cycles of washing anti-gliadin IgG-HRP-conjugate was added, then the plate was washed and dried. Solution of 42 mmol TMB, containing 0.01% (v/v) H_2O_2, was added to each well. After 15 min the reaction was stopped by adding 0.07 cm^3 of 2 mol sulfuric acid and optical densities in the wells were measured at λ=450 nm. Sensitivity of sandwich ELISA for gliadin was 50 ng cm^{-3}. Results were calculated in mg g^{-1} samples on the basis of a calibration curve.

2. Results

The application of an optimized reaction mixture and Hot Start technique in the adapted PCR-methodology resulted in strong signals from wheat samples, when the reaction mixture composition and thermocycling parameters were selected as presented in Table 1. Effect of primer concentration on wheat-PCR signal (109 bp) and amplification of λ-DNA are illustrated in Fig. 1. Strong signals were obtained at 0.5 μmol primer concentration for wheat flour (BL 55) and bio-wheat flour. At 0.9 μmol primer concentration the 109 bp amplicon is accompanied by a greater amount of smaller products than in the amplification with 0.7 μmol primer. In further experiments primers were used in 0.5 μmol concentration. Performance of PDR-91 thermocycler was verified by amplification of λ-DNA.

Amplification experiments done without Hot start, in most cases, resulted in several strong (109 bp included) and a few minor products (data not shown). In Fig. 2, amplicons from different flour samples are presented. Signals of Öthalom wheat flour and MV-19 wheat flour were obtained for DNA prepared by the reference procedure (it is to be noted that the ethidium bromide fluorescence on the gel was much stronger for these bands than shown by the photo). The 109 bp product was not detected in rice meal and potato starch.

Presence of wheat was detected in mixtures of meals and in heat treated foodstuffs as it is presented in Fig. 3. For 1% wheat in rice meal strong signal was obtained and the advantage of amplification of small DNA-segment for identification of components in heat treated food products, emphasized by the authors of the wheat-PCR-methodology, is demonstrated. In Table 2, results of gliadin assays and wheat PCR are presented. Gliadin content of different wheats is influenced by numerous factors, thus the measured differences can not be regarded as significant. As it can be seen, at low level of gliadin, wheat PCR proved to be the method of confirmation.

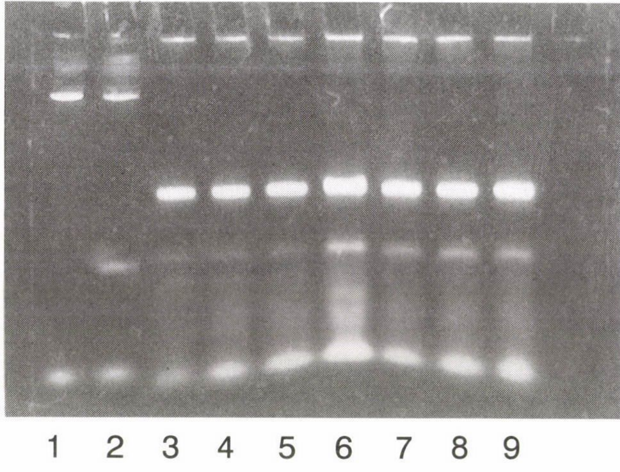

Fig. 1. Amplification of λ-DNA and wheat DNA. 10 μl PCR-mixture/well was loaded onto the gel and run at 34 V for 3 h, except for lanes 5 and 9, where 15–15 μl was loaded, resp. 1: 500 bp segment of λ-DNA, 0.4 ng template, 40 μl reaction volume; 2: as 1., but 0.5 ng/50 μl; 3–6: 109 bp segment of wheat DNA from biowheat sample at 0.5 μmol (3), 0.7 μmol (4 and 5) and 0.9 μmol (6) primer concentration, resp.; 7–9: 109 bp segment of wheat from wheat flour (BL 55) at 0.5 μmol (7) and 0.7 μmol (8 and 9) primer concentration, resp.

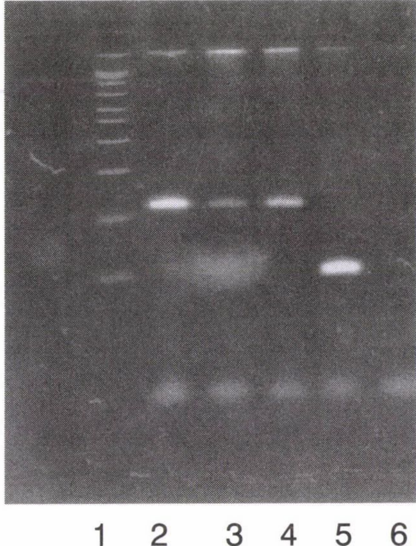

Fig. 2. Wheat PCR of different flours. Electrophoretic conditions were the same as described in Fig. 1. 1: 5 μl of Ampli Size DNA marker; 2: biowheat; 3: Öthalom wheat; 4: MV-19 wheat; 5: rice; 6: potato

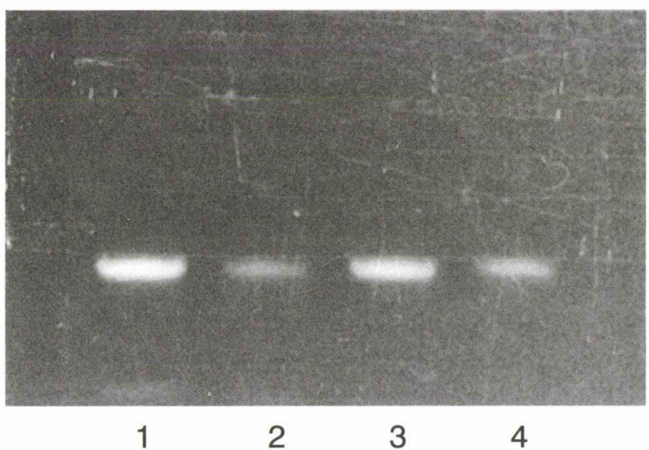

Fig. 3. Wheat in mixtures and heat treated foodstuffs. Electophoretic conditions were the same as described in Fig. 1. 1: 5% wheat in rice meal; 2: 1% wheat in rice meal; 3: biscuite; 4: bouillon cube

Table 2

Results of gliadin, allergenicity and genomic wheat DNA analysis obtained for different food samples

Samples	Gliadin mg/g[a]	Gliadin mg/g[b]	Allergenic activity (%)	Wheat-PCR
5% wheat in rice meal	–	0.40(0.0)	25.6(1.4)	++
1% wheat in rice meal	–	0.04(0.0)	9.8(3.6)	++
biscuit	–	5.34(0.9)	42.8(1.1)	++
bouillon cube	2.10(0.2)	28.15(4.2)	53.8(1.1)	++
potato flake	0.01(0.0)	0.01(0.0)	12.4(1.1)	–
bio-wheat flour	1.62(0.6)	21.59(0.6)	52.2(0.2)	+++
durum wheat meal (Multidur)	3.71(0.3)	5.09(0.7)	44.5(0.0)	+++
aestivum wheat meal (Tiszatáj)	6.03(0.5)	25.06(0.0)	51.5(1.5)	+++
rice flour	0.01(0.0)	0.02(0.0)	4.7(1.5)	–

[a] anti/gliadin rabbit IgG
[b] human sera

3. Conclusion

Success of amplification experiments requires stringent control of numerous conditions, the number of which may be comparable to those of conventional immunoassays. The initiated wheat-PCR methodology, modified to a slight extent, worked reliably as low as 1% wheat in rice meal. Result of DNA extraction basically influences the chance of success of PCR (PCR is inhibited by around 50 different substances, ROSSEN et al., 1992), and the simplification of DNA isolation essentially improved feasibility of the whole methodology. One stage phase partitioning was the method for samples containing highly degraded DNA, while two stage TPP yielded high quality DNA from flours. Risk of contamination was greatly reduced by the usage of optimized reaction mix, however, PCR operation demands the highest carefulness and purity in manipulations. Finally, it can be concluded that PCRs amplifying short species specific segments will gain importance in the investigation of heat treated foodstuffs.

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Short communication

DETECTION OF MOULDS IN PAPRIKA POWDER BY ENZYME-LINKED IMMUNOSORBENT ASSAY

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A commercial ELISA kit was used for detection of *Penicillium* and *Aspergillus* species in paprika samples. The cells of a plate are coated with antibodies raised against the heat stable, water soluble polysaccharide antigens specific to these fungi. Viable mould count was determined using three different media (oxytetracycline-glucose yeast extract agar, rose-bengal chloramphenicol and dichloran rose-bengal chloramphenicol agar), which showed almost the same results. The kit is suitable for detection of both viable and non-viable moulds. There was a good correlation between mould colony count and titer of ELISA reaction for untreated samples. In (supposedly) decontaminated samples there was no correlation between mould colony count and ELISA titer. The results revealed that viable mould counts does not reflect the actual microbiological quality of the products. Results indicated that ELISA-mould tests could be used as a rapid, reliable method for screening paprika powders and possibly other spices, for mould contamination.

Keywords: moulds, ELISA, paprika powder

Detection of mould content of food is important because of economic losses due to fungal spoilage and the public health hazard (many mould species are able to produce mycotoxins). Testing for moulds in processed foods provides an index of quality, indicating whether food products have been prepared from uncontaminated, high-quality materials or not. Methods able to recognise moulds in foods under all circumstances would be useful for evaluating the possible risks related to fungal contamination.

Different methods are available for detecting moulds in food. They include cultural methods, electrical measurements using conductance and other changes in

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electrical properties of culture media, detection of heat-stable mould components like chitin, microscopic methods for detecting mycelium, etc. These methods have been summarized by JARVIS and co-workers, (1984) and by HARRIS and KELL (1985). Of these methods the mould colony count and Howard Mould Count (HMC) are used in many countries as a standard method for quality control purposes. Most of the current methods have disadvantages.

With traditional culture methods heat-damaged or inactivated moulds escape detection and they are time consuming (time requirement is 5–8 days). Microscopical detection of mycelium lacks precision, using for example the ATP assay, has not widely been applied due to the problems of separating mycelium from food materials. Chemical methods based on detection of fungal chitin (COUSIN et al., 1984) are not totally acceptable because the chitin content varies among species of mould.

Alternative techniques have been developed to replace HMC. One such method is the quantification of ergosterol as an indicator of fungal contamination (SEITZ et al., 1977). PETTIPHER and co-workers (1985) applied the direct epifluorescent filter technique to detect moulds in tomato concentrates. Recently some immunological methods were described for detection moulds in foods (NOTERMANS et al., 1986; LIN et al., 1986; LIN and COUSIN, 1987; TSAI and COUSIN, 1990). They reported serological methods using enzyme-linked immunosorbent assay (ELISA) for detection of extra-cellular polysaccharides (EPS) produced by various moulds. A sensitive technique of latex agglutination was described by KAMPHUIS and co-workers (1989), and NOTERMANS and KAMPHUIS (1990).

The aim of this work was to study the ability of the enzyme-linked immunosorbent assay (ELISA) for detection moulds in paprika powders. Two methods have been compared in this work for detection moulds in paprika powder: plating technique as a general standard method and enzyme-linked immunosorbent assay (ELISA) as a commercially available immuno-assay.

1. Material and methods

1.1 Samples

Two types of the samples were prepared for the experiments. The first set of the paprika powders were collected randomly from different commercial stores in The Netherlands. In the second set mould-free paprika powder and artificially contaminated samples (with *Aspergillus niger)* were blended in various proportions.

1.2 Plating technique

Viable mould counts from sample homogenates and their dilution series were determined on agar plates containing 15 ml of three different media. One ml-aliquots of the samples were pipetted into petri-dishes and overpoured with molten OGYEA (Oxytetracycline-Glucose Yeast Extract Agar, CM 545), RBCA (Rose-Bengal Chloramphenicol Agar, OXOID CM 549) or DRBC (Dichloran Rose-Bengal Chloramphenicol Agar, CM 727) agar in duplicate. Plates were incubated at 24 °C for 4 to 5 days. DRBC was chosen because KING and co-workers (1979) showed that this medium restricted the growth of *Mucor* and *Rhizopus* group, thus avoiding overgrowth by spreading of these moulds.

1.3 Estimation of genus distribution

Moulds were isolated from the agar plates after counting the colonies and were investigated by microscope for morphological parameters. The ratio of occurrence of *Aspergillus* and *Penicillium* species was obtained comparing the number of isolates belonging to these genera to the total mould counts.

1.4 ELISA for detecting moulds

ELISA procedure for detecting moulds of the *Penicillium/Aspergillus* group was used according to NOTERMANS and HEUVELMAN (1985). The ELISA is based on detection of the immunologically active extracellular, heat-stable polysaccharides (EPS) released by moulds. The EPS are an essential part of the mycelium cell wall, and are not found to be present in non-moulded food.

One g of the paprika powder was diluted with 10 ml of phosphate-buffered saline containing Tween (PBS-T: 2.86 g $Na_2HPO_4 \times 2H_2O$; 0.55 g $NaH_2PO_4 \times H_2O$; 8.7 g NaCl; 1 ml Tween 20 in 1 l distilled water, pH 7.2–7.4) and homogenized with a stomacher mixer for 1 minute. The clear liquid after the settle of the deposit was used directly in the immunoassays.

RIDASCREEN Moulds A/P commercial ELISA kits (obtained from R-Biopharm GmbH, Darmstadt, Germany) were used according to the manufacturers instructions. The wells in the microtiter strip are coated with specific antibodies to *Aspergillus* and *Penicillium* EPS. Hundred µl of the positive control, the negative control or 100 µl of a sample in duplicate were added to the wells and incubated for 30 min at room temperature. By adding the sample solution or the positive control to the wells, EPS present bind to the specific capture antibodies. The wells were washed with 250 µl of washing buffer (repeated three times) to remove sample components not bound by the antibodies. The bound antigen is detected by a specific antibody conjugated to peroxidase (enzyme conjugate). Hundred µl of diluted enzyme conjugate was pipetted

to all wells and incubated for 30 min at room temperature. The wells were washed again with 250 μl of the washing buffer (repeated three times) to remove any unbound enzyme conjugate. Fifty μl of substrate (urea peroxide) and 50 μl of chromogen (tetramethylbenzidine) were added to each well and incubated for 30 min at room temperature in the dark. Bound enzyme conjugate converts the colourless chromogen into a blue product. Hundred μl of the stop reagent were pipetted to each well leading to a colour change from blue to yellow. The absorbance was measured at 450 nm against an air blank.

1.5 Aerobic plate counts and DEFT counts

Total aerobic plate counts (APC) and microscopic total count estimated by the direct epifluorescent filter technique (DEFT) have been performed as described elsewhere (KISKÓ et al., 1997).

2. Results and discussion

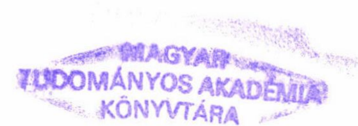
Results from the first sample set tested with plate count and ELISA test are summarised in Table 1.

Table 1

Viable mould count, proportion of Aspergillus and Penicillium isolates (genus distribution), the logarithm of ELISA- and DEFT-APC values of different paprika powder

	Viable mould count (log CFU/g)			Genus distribution	log ELISA-titer[a]	DEFT-APC	Possibly decontaminated
No.	OGYEA	RBCA	DRBC	(% Pen, Asp)	Pen/Asp		
1.	6.3	6.2	6.2	59	5.0	0.9	–
2.	6.0	6.0	6.0	65	4.0	0.8	–
3.	NT	NT	3.8	48	3.0	2.1	–
4.	4.3	3.9	4.3	42	3.0	NT	–
5.	3.4	3.1	3.3	16	2.0	1.6	–
6.	NT	NT	<0.1	NT	4.0	3.2	X
7.	NT	NT	1.0	100	3.5	3.1	X
8.	NT	NT	2.6	26	3.0	2.4	X
9.	<0.1	1.0	1.0	<10	3.0	3.0	X
10.	NT	NT	<1.0	NT	3.0	NT	10 kGy irrad

[a] ELISA titer is reciprocal of the highest sample dilution giving a positive result
NT: not tested


MAGYAR TUDOMÁNYOS AKADÉMIA KÖNYVTÁRA

The three different media provided about the same results for the viable mould count. Counting of plates was rendered more difficult by overgrowing by *Mucor/Rhizopus* group especially on OGYEA agar. Table 1 demonstrates that highly contaminated samples showed relatively higher ELISA titers. The maximal measurable ELISA-titer was 7 at a pure mould biomass (*Penicillium aurantiogriseum* F 1296/3C) (not shown in Table 1). As for log ELISA-titer for *Penicillium* and *Aspergillus* strains the estimated values ranged between 2 and 5. This agree with the value reported by NOTERMANS and co-workers (1988) for paprika powder. The samples showed in some cases very low mould count, although, these samples showed a fairly high ELISA titer. This confirmed our supposition that they were treated (e.g. irradiated or fumigated) on the basis of results given by logarithmic DEFT-APC values (KISKÓ et al., 1997). This can be clearly observed from the results comparing samples 4 and 10. Sample 10 is the same as sample 4, but irradiated by us with 10 kGy radiation dose. Consequently, the viable mould count is not a good reflection of the real state of mouldiness of a food product. If products are treated with an antifungal treatment, moulds are no longer detectable by viable cell counting.

There was a strong correlation between viable mould count (DRBC) and the titer of the ELISA reaction in the samples without decontamination in the first sample set (R=0.94) (curve not shown). In treated products, there was no correlation between colony count and ELISA titer (R=–0.46) (curve not shown) and relatively high ELISA titers were detected as can be seen in Table 1.

Mould antigens are stable under various conditions of heating or low and high pH (NOTERMANS et al., 1987) and it can be seen from this work it is also stable after irradiation as determined by ELISA. Therefore, the ELISA reaction represents the real mould content of the samples.

Results of the mixture samples tested by ELISA reaction are given in Fig. 1. There was a slight increase in the ELISA titer values with increasing percentage proportion of mouldy paprika in the samples.

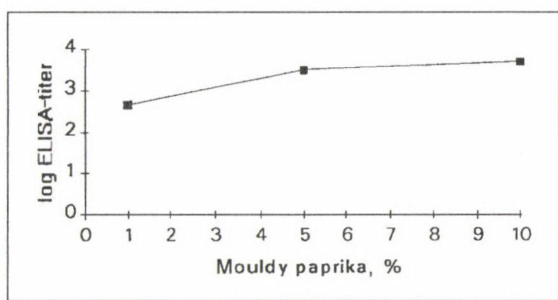


Fig. 1. Log-ELISA titer as a function of the percentage proportion of mouldy paprika in the samples

According to the U.S. FDA's Regulatory Action Levels for Mould Defects in Foods defect action level for *Capsicum* is the average of 3% or more insect infested and/or mouldy pods by weight. In our studies this contamination level corresponded to an ELISA-titer of 2.8. The ELISA kit is able to detect mould contamination below and above this value therefore it can be used for screening mould contamination level of paprika powder.

Penicillium/Aspergillus ELISA gave promising results for screening mycological quality of paprika powders, moreover it was possible to determine moulds after irradiation or in samples treated in unknown way. The ELISA results might be improved by using also other (e.g. *Mucor/Rhizopus*) ELISA tests. However, application of these mould ELISA kits in practice is hindered yet by high price of the kits.

*

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Acta Alimentaria, Vol. 27 (1), pp. 105–108 (1998)

Book reviews

Chemical and functional properties of food components

Z.E. SIKORSKI (Ed.)

Technomic Publishing Co., Basel, 1997, 293 pages

The book is a good result of international co-operation and activity on the field of chemical science with special regard to the properties of food components. It contains twelve chapters which are dealing with the significant food components as important and determining factors of quality.

The first chapter is the Food components and their role in food quality. It is the introduction part of the book and it deals with the main food components, the quality of foods, the functional properties of food components, the role of chemistry and processing factors. The second part is dealing with the importance of water from the aspect of food quality. It emphasizes the importance of water molecule structure and the effect of that on the properties of food. The title of the third part is Mineral components. The contents and role of minerals in food have a stochastic relationship with the nutritive value of diets but it also determines the toxicity of fresh and processed foods. The fourth chapter is the part of saccharides, their structure, reactivity and functional properties. It mentions, very briefly, their roles in biodegradable plastic production. The next is the part of lipids, their chemical and functional properties and in addition the effect of refining and hardening the composition of fats. The largest part is the chapter of proteins. The basic idea is the correlation between the chemical structure and properties, furthermore the functional properties of proteins. After that it deals with changes due processing, the enzymatic changes and the chemical modification. It is unfortunate that the part of edible coating is the shortest one. Rheological properties of food systems is the title of the seventh chapter of the book. It summarizes the rheological properties of foods from the aspect of food processing. The last part discusses the importance of rheological properties for process design and control. The subject of the next chapter is the food colorants. They are ranked in two categories: natural pigments and synthetic organic colours. The ninth chapter deals with flavour compounds. Important part of the chapter is the flavour changing during food storage and processing. The next chapter surveys the role of main functional additives from the aspect of food quality. Food safety is the subject of the next, i.e. eleventh

chapter. Interesting part of this is the nutritional evaluation of food processing. The last chapter is dealing with the mutagenic and carcinogenic components in food. The author summarizes the latest results of the field in this chapter. Interesting part is the foodborne mutagenes and carcinogens connected with the food safety, and it also shows the effects of anticarcinogenic food components to give a complete picture of the problem.

At the end of the chapters list of references is given to help the researchers to study the field in more detail.

Summing up, the outstanding book could be recommended for those who are working in the food industry, teaching in high school and university, furthermore who are engaged in research and development in the field of food science and technology.

I. VARSÁNYI

Distribution packaging for logistical systems

D. TWEDE and B. PARSOS

Pira International, Surrey, UK, 1997, 40+A110 pages

The publication is a literature review as it is announced by the authors. This review is intended to provide a functional model for logistical packaging analysis. The text indicates the performance required of packaging in various logistical activities, including transport, warehousing and customer service.

The publication has six parts. The Introduction deals with the value of packaging in logistical systems. The next part embraces Logistical packaging functions and value in 17 pages and it deals with the protection function, utility function, communication function and value analysis. The Packaging/logistic system integration is the next part and it contains the following aspects: transportation issues, warehousing issues, returnable packaging, and packaging postponement, the extension of which is 14 pages. The part of Conclusions is short and concised in one page. And then is the part of Abstracts, which includes 103 abstracts each of which providing an informative summary of the original document. It has to be mentioned that in most cases, a copy of the original document can be obtained from the Pira Information Centre. Fifty-three additional references between 1950 and 1995 are at the end of Abstracts. The book is a comprehensive and new packaging review dealing with the value of packaging in logistical systems.

I. VARSÁNYI

Engineers' guide to cleaner productions technologies

P. M. RANDALL (Ed.)

Technomic Publishing Co., Lancaster, Basel, 1996, 240 pages

The book is intended as an aid and a guide to the practicing engineer, who has the responsibility for process improvement and design to reduce or eliminate wastes into the environment. At the same time the work proposes and realises a review of the engineering problems which appear in the technologies of waste reduction.

The authors define cleaner production technologies as industrial processes that emit little or no hazardous waste materials. Cleaner production technologies incorporate changes in process design or processing parameters, different or purer starting materials, and/or substitution of nontoxic solvents or reactants for toxic or hazardous materials.

Also, it is essential that the final product quality be reliably controlled to meet acceptability standards and that the cost of applying the new technology relative to the cost of similar technologies should be considered.

It is important to note: the concept of cleaner production is not new. Many similar terms exist today, such as clean technology, waste reduction and waste minimization.

The cleaner technologies described in this book are divided into five sections:

- Section 1.0 discusses process improvement alternatives in cleaning and degreasing operations. The main focus of the technologies covered is the elimination or reduction of the·use of hazardous solvents applied in cleaning processes.
- Section 2.0 discusses alternatives to chlorinated solvents in cleaning and degreasing operations.
- Section 3.0 describes cleaner technologies that can be used to reduce or eliminate waste in coating removal operations.
- Section 4.0 describes cleaner technologies that can be used to reduce emissions and wastes from paints and coatings application.
- Section 5.0 discusses alternatives to metal finishing.

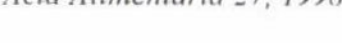
Each section divides the technologies into commercially available and emerging types.

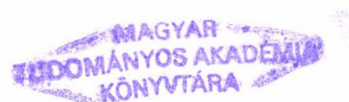
Also, this book discusses pollution prevention srategies. It deals with the various technical, economic, and regulatory factors that influence the selection and use of a cleaner technology.

Detailed information is presented to select one or more candidate technologies for further analysis and in-plant testing. The book does not recommend any technology over any other.

On the whole, the book makes a successful analysis of the processes which occur in the cleaner production technologies. The material addresses specialists interested in this field.

I. HORVÁTH




MAGYAR TUDOMÁNYOS AKADÉMIA KÖNYVTÁRA

Announcement

IFU – Fruit Juice Symposium '98
"The world of tropical fuits"
celebration of 50th anniversary of IFU
Fortaleza (Ceará), Brazil
November 3–6, 1998

organized by IFU in co-operation with the Brazilian Fruit Juice Association

Main topics:

1. juices and juce products manufactured from tropical fuits and vegatables;
2. fruit juice technology / fruit juice industry in tropical (and subtripocal) countries;
3. fruit production in tropical (and subtropical) countries;
4. general developments in fruit juice technology;
5. commercial aspects of tropical fruit juices;
6. logistical aspects of tropical fuits / fruit processing / juice marketing;
7. new developments in fruit juice technology (tropical and non tropical) incl. processing, storage, handling, logistics;
8. quality-assurance, analytical aspects of tropical fruits / tropical juices.

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- language: English;
- manuscript must be in English and will be published in an IFU-symposium-report;
- please submit an abstract of one page maximum in English, including name and exact addresses of the author(s) and the speaker.

Call for posters:

results of original research work directly connected with the field of the main topics of this workshop could be presented as posters;
posters have to be presented in English language;
for the selection we need an English summary of max. 1 page A-4 including the names and addresses of the author(s).

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BATTISTUTTA, F., D'ANDREA, E., DA PORTO, C. & FLOCEA, V.

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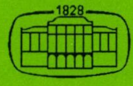

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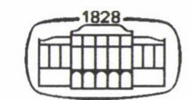

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MICROBIOLOGICAL QUALITY OF GOUDA AND EDAM TYPE CHEESES

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A microbiological study was undertaken of the indicators of health and hygiene of Gouda and Edam type cheeses consumed in Santa Cruz de Tenerife. The methods used were those of the Standard Methods for Examination of Dairy Products and those put forward by the National Centre of Nutrition and by Mossel for the microbiological analysis of foodstuffs. The presence of coliforms, *Enterobacteriaceae*, *Enterococcus*, yeasts and moulds was examined.

The presence of these microorganisms is related to contamination during production and distribution. The non observance of the maturing period might also justify the counts shown.

The presence of the pathogens *Salmonella spp.*, *Shigella spp.*, enterotoxigenic *Staphylococcus aureus*, and *Escherichia coli* was not detected in any of the samples.

Keywords: microbiological quality, Gouda and Edam type cheeses, food safety

Spanish governmental regulations require cheeses made from raw milk to be ripened for at least 60 days before sale or consumption to eliminate pathogenic microorganisms. However, some reports about the survival of different pathogens during manufacturing and ripening of different types of cheeses suggest that, in some cases, 60 days ripening may not be enough to inhibit some bacterial pathogens (SANCHEZ et al., 1993, RODRÍGUEZ et al., 1995). For this reason it is important to analyse the presence of pathogens in every single kind of cheese at this age.

According to the Quality Standards (MINISTERIO DE RELACIONES CON LAS CORTES Y SECRETARÍA DEL GOBIERNO, 1975), Edam and Gouda type cheeses are of a semi-hard consistency, spherical in shape with a slight depression on the top and bottom as regards the Edam type, and cylindrical or a prismatic block, covered with paraffin, wax, plastic or by a film of vegetable oil as regards the Gouda type.

Firm texture and straw-like colour, it may have regularly or irregularly distributed spaces inside. Consumption is not recommended until it has matured for at least five weeks, so that it attains all of its features.

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The most important part of its composition is pasteurised cow milk to which dairy ferments and rennet or other coagulating enzymes are added.

Aside from the shape, size and colour of the wax covering, Gouda and Edam are very similar; however, the former is slightly richer in fat and it is a little softer in texture.

These cheese types may suffer the same defects which afflict hard cheeses such as the growth of mould (mainly *Penicillum* and *Aspergillus)*, gas formation and the appearance of abnornal smells and bitter taste (CHAPMAN & SHARPE, 1987).

The purpose of our work was to analyze the microbiological quality and the occurrence of some pathogens such as *Staphylococcus aureus*, *Salmonella* in Gouda and Edam type cheeses.

1. Materials and methods

A total of 180 cheese samples, 90 corresponding to the Gouda type and 90 to the Edam type, were analysed. At the same time, they were divided into three brands (30 samples each) considered to be indicative of the greatest consumption on the Canarian market.

The samples were obtained aseptically from whole wheels of cheeses. Ten grams of each sample, taken according to the APHA methods after discarding the rind of the cheeses, were homogenized with 90 ml of 2% sterile sodium citrate solution for 1 min in a Stomacher 400 Lab Blender (Seward Medical, London), thus making a 1/10 dilution. Decimal dilutions were prepared by mixing 1 ml with 9 ml of 0.1% sterile peptone water.

The microorganisms studied were:

- Coliform total count (Most probable number: MPN)
- *E. coli* count and identification
- *Enterobacteriaceae* total count
- Isolation and identification of *Salmonella spp.* and *Shigella spp.* in 25 g
- Count and identification of enterotoxigenic *Staphylococcus aureus*
- Count of *Enterococcus*
- Count of *Clostridium perfringens*
- Count of yeast and mould

The methods used were those of the Standard Methods for Examination of Dairy Products (RICHARDSON, 1985) and those recommended by the National Centre of Nutrition (PASCUAL-ANDERSON, 1989, 1992) and by MOSSEL & MORENO (1995) for the microbiological analysis of foodstuffs.

2. Results and discussion

Pathogens *Salmonella spp.*, *Shigella spp.*, enterotoxigenic *Staphylococcus aureus* and *Escherichia coli* have not been detected in any samples studied. This was not the case in the rest of the hygiene-health markers analysed.

The study of the average count obtained in the microbiological parameters analysed is shown in Table 1. For total coliforms, the average count (cfu g^{-1}) was 5.3×10^2, 2.2×10^5 cfu g^{-1} for total *Enterobacteriaceae*, 1.4×10^2 cfu g^{-1} for the *Enterococcus*, 0.3 cfu g^{-1} for *Clostridium perfringens*, 5.1×10 and 1.1×10 cfu g^{-1} for yeast and moulds, respectively.

In order to establish if there were significant differences between brands, type and brand/type interaction for the microorganisms studied, a variance analysis was carried out into the logarithm plus one of the average counts. The average counts of the logarithm could not be carried out as in many cases the variable is zero and the logarithm of zero is not defined. Once the differences are detected through analysis of variance the Duncan Test was applied to affirm where these differences existed.

The analysis of variance of the total coliform count (log total coliforms +1) detected significant differences between brands, type of cheese and in the brand/type interaction ($\alpha<0.01$) (Table 2).

Duncan's Multiple Comparation study determined that for the total coliform count the average of brand C is greater than B and at the same time is similar to brand A. It also showed that Edam type presented higher counts than those of Gouda and in the interaction the average of C, for both Gouda and Edam types, is greater than the rest with the average count of brand B Gouda type being lower.

Table 1

Average values of the microbiological parameters (cfu/g) of Edam and Gouda type cheeses

		Brand			Type	
Microorganisms	Total	A	B	C	Edam	Gouda
Total coliforms	5.3×10^2	4.7×10^2	1.9×10^2	9.4×10^2	8.0×10^2	2.7×10^2
Total *Enterobacteriaceae*	2.2×10^5	6.7×10^5	3.2×10^2	1.3×10^3	4.4×10^5	1.8×10^2
Enterococcus	1.4×10^2	6.9×10^1	2.3×10^1	4.3×10^2	9.6×10^1	1.9×10^2
Clostridium perfringens	3.0×10^{-1}	2.0×10^{-1}	5.0×10^{-1}	3.0×10^{-1}	3.0×10^{-1}	4.0×10^{-1}
Yeasts	5.1×10^1	1.1×10^2	–	4.0×10^1	1.1×10^2	1.5×10^1
Moulds	1.1×10^1	1.9×10^1	2.2×10^1	1.1×10^1	1.6×10^1	5.8×10^1

Table 2

The analysis of variance of log total coliforms + 1

Source	Sum of square	Degrees of freedom	Mean square	Observed *f* ratio	Significance level
Mark	416.93501	2	208.4675	39.216	0.000
Type	83.70244	1	83.70244	15.746	0.001
Interaction	81.323216	2	40.661608	7.649	0.007
Error	924.96851	174	5.315911		
Total	1506.9292	179			

Table 3

The analysis of variance of log Enterobacteriaceae *+ 1*

Source	Sum of square	Degrees of freedom	Mean square	Observed *f* ratio	Significance level
Mark	311.54339	2	155.77169	25.401	0.000
Type	184.26078	1	184.26078	30.046	0.001
Interaction	66.697458	2	33.348729	5.438	0.051
Error	1067.072	174	6.1325978		
Total	1629.5736	179			

The analysis of the variance of the *Enterobacteriaceae* count (log *Enterobacteriaceae* +1) detected significant differences between brands and types of cheese ($\alpha<0.01$) and in the brand/type interaction ($p<0.05$) (Table 3).

Table 4

The analysis of variance of log Enterococcus *+ 1*

Source	Sum of square	Degrees of freedom	Mean square	Observed *f* ratio	Significance level
Mark	761.99216	2	380.99608	157.956	0.0000
Type	2.04087	1	2.04087	0.846	0.3687
Interaction	1.1316246	2	0.5658123	0.235	0.7912
Error	419.69567	174	2.4120441		
Total	1184.8603	179			

Table 5

The analysis of variance of log Clostridium + *1*

Source	Sum of square	Degrees of freedom	Mean square	Observed *f* ratio	Significance level
Mark	0.6977566	2	0.3488783	1.905	0.1519
Type	0.0793167	1	0.0793167	0.433	0.5183
Interaction	0.504948	2	0.252474	1.379	0.2546
Error	31.859456	174	0.1831003		
Total	33.141477	179			

Duncan's Multiple Comparation study indicated (α<0.01) that brand A presents a greater *Enterobacteriaceae* count than brands B and C with no notable differences between these two. Furthermore, Edam cheese presents a greater count for this group of bacteria than Gouda (α<0.01). Regarding brand/type interaction, Edam-type brand A presented the highest count and brand B Edam-type was the lowest. No significant differences were found in the rest of the interactions.

The analysis of variance of the count of *Enterococcus* (log *Enterococcus* +1) only detected significant differences between brands (α<0.01) (Table 4).

The study of Duncan's Multiple Comparation determined that brand C presented the greatest average count with no significant differences between brands A and B.

The analysis of variance of the count of *Clostridium perfringens* (log *Clostridium* +1) did not detect notable differences between brands, types and interaction (Table 5).

The analysis of variance of the yeast counts (log yeasts +1) showed significant differences between brands, type of cheeses and in the brand/type interaction (α<0.01) (Table 6).

Table 6

The analysis of variance of log yeast + 1

Source	Sum of square	Degrees of freedom	Mean square	Observed *f* ratio	Significance level
Mark	119.00772	2	59.50386	23.737	0.000
Type	143.41586	1	143.41586	57.211	0.000
Interaction	78.124382	2	39.062191	15.566	0.000
Error	436.17879	174	2.5067746		
Total	776.72674	179			

Table 7

The analysis of variance of log mould + 1

Source	Sum of square	Degrees of freedom	Mean square	Observed *f* ratio	Significance level
Mark	22.038287	2	11.019144	6.712	0.0016
Type	18.74201	1	18.74201	11.416	0.009
Interaction	28.965913	2	14.482956	8.821	0.002
Error	285.67265	174	1.6417968		
Total	355.41886	179			

The study of Duncan's Multiple Comparation determined that, for the yeast count, brand B presented a lower value than brands A and C without significant differences. Edam type presented greater counts than Gouda. In the interaction the average of brand C, both Gouda and Edam type, is greater than the rest.

The analysis of variance of the mould counts (log mould totals +1) detected significant differences between brands, type of cheeses and in the brand/type interaction ($\alpha<0.01$) (Table 7).

The study of Duncan's Multiple Comparation determined that, for the mould count, brand B presented a lower value than brands A and C though, there were not significant differences. Edam-type cheese presented greater counts than Gouda and in the interaction, the averages of brand C, both in Gouda and Edam and brand A Edam type, are greater than the rest.

3. Conclusions

In assessing microbiological quality, the frequency of samples exceeding the reference values permitted for consideration as suitable or unsuitable for consumption was used studying the differences between the frequencies found by the X^2 method of Pearson (MILTON & TSOKOS, 1987). The microbiological tolerances put forward by the National Centre of Foodstuffs and Nutrition (PASCUAL-ANDERSON, 1989) and by MOSSEL and MORENO (1995) were applied.

In Table 8, the percentages of the samples of Gouda and Edam cheese types, which satisfied or otherwise, the microbiological values of applied reference, are shown for each of the microbiological parameters studied.

A high percentage of samples, which have exceeded the reference values, both for the coliform count (71.7% of non suitable samples) and for the *Enterobacteriaceae* totals (67.2% of unsuitable samples), was obtained. There were significant differences between the brands ($\alpha<0.01$) and the Gouda and Edam types, the greatest percentage of

unsuitable samples corresponding to brand C, Edam type. The presence of these microorganisms was connected to post-pasteurisation contamination, and to a lesser extent to the use of raw milk with an excessively high bacterial content (GARCÍA et al., 1980).

The count of *Enterococcus* was positive in 39.4% of all the samples; 76.7% of the total samples were considered suitable or not exceeding the reference value (1×10^2 cfu g^{-1}). Significant differences were not observed between the Gouda and Edam types, but rather between the brands ($\alpha<0.01$). The highest percentage of the samples considered unsuitable (23.3%), corresponded to brand C (22.8%), compared to 0.5% of brand A. These microorganisms possess a series of characteristics which enable them to withstand extreme conditions, tolerate wide ranges of temperature, saline and acidic concentrations, which results in, unsurprisingly, their presence in most cheeses. Contamination usually occurs during production (HERNÁNDEZ et al., 1989, MARCOS, 1986, ORDÓNEZ, 1978, RAMOS, 1982, SUÁREZ et al., 1983). If they are detected in large quantities, this might point to inadequate hygienic conditions at the moment of handling, although there is no concrete proof that their presence supposes a health risk. These microorganisms, especially the enterococcus, cannot replace *E. coli* as indicators of recent fecal contamination, neither can they be related to the simultaneous presence of pathogenic microorganisms of enteric origin (BANWART, 1989).

Clostridium perfringens was detected in 11.1% of all the samples analysed, but at no time did it exceed the microbiological reference value (10 cfu g^{-1}). The detection of clostridia is of great interest, owing to the fact that they may be the cause of irregularities in the cheeses, if the water activity enables them to grow, especially in the cases of *C. pasteurianum, C. tyrobutyricum, C. butyricum* and *C. sporogenes*. The thermal treatment employed in the production process of this type of cheese is enough to destroy the vegetative cells, but the bacterial spores may survive and produce gas, which causes cracking. For this reason, the addition of nisine, an antibiotic which blocks the germination of spores, is authorised (SOMERS & TAYLOR, 1987, WARBURTON et al., 1986).

87.2% of all the samples were considered suitable, because they did not exceed the reference values applied, either for the mould count or for the yeast count.

As far as the yeast count is concerned, significant differences were obtained ($\alpha<0.01$) between the brands, Gouda and Edam types. The samples from the Edam type revealed considerably higher counts, with 12.8% of the samples being unsuitable; whereas in all the samples of Gouda type cheese counts lower than 1×10^2 cfu g^{-1} were obtained. According to brand distribution A and C showed 6.7% and 6.1%, respectively of unsuitable samples, and B none.

Table 8

Acceptability of cheese samples according to the microbiological reference limits (in percentage)

Microorganisms	Microbiological reference values (cfu/g^{-1})	Total	Brands			Sig. (α)	Type		Sig (α)
			A	B	C		Edam	Gouda	
Total coliforms	Suitable: Absence	28.3	12.8	15.5	0	<0.01	11.1	17.2	<0.01
	Not suitable: Presence	71.7	20.6	17.8	33.3		38.9	32.8	
Total *Enterobacteriaceae*	Suitable $\leq 1\times10^1$	32.8	12.2	19.4	1.1	<0.01	12.2	20.5	<0.05
	Not suitable $> 1\times10^1$	67.2	21.1	13.9	32.2		37.8	29.4	
Enterococcus	Suitable $\leq 1\times10^2$	76.7	32.8	33.3	10.5	<0.01	38.3	38.3	N.S.
	Not suitable $> 1\times10^2$	23.3	0.5	0	22.8		11.7	11.7	
Clostridium perfringens	Suitable $\leq 1\times10^1$	100	33.3	33.3	33.3	–	50	50	–
	Not suitable $> 1\times10^1$	0	0	0	0		0	0	
Yeasts	Suitable $\leq 1\times10^2$	87.2	26.7	33.3	27.2	<0.01	37.2	50	<0.01
	Not suitable $> 1\times10^2$	12.8	6.6	0	6.12		12.8	0	
Moulds	Suitable $\leq 1\times10^1$	87.2	27.8	31.7	27.8	<0.05	40.5	46.7	<0.01
	Not suitable $> 1\times10^1$	12.8	5.5	1.7	5.5		9.4	3.3	

Significance: – There are not significance facts
N.S. There are not significant differences

In the mould count once again it was the Edam type which gave the higher percentage of unsuitable samples (9.4%), compared with the Gouda type (3.3%). For brand distribution, the same as for the yeast count, brand B gave lower counts with only 1.7% of unsuitable samples, compared to 5.5% of brands A and C. For the distribution according to brands, as with the yeast count, brand B was the one which showed lower counts with only 1.7% of unsuitable samples compared with 5.5% for brands A and C.

As far as the health factor was concerned, the action of yeasts was merely infective, whereas the greatest problem caused by moulds was concerned with their capacity to produce mycotoxins (*Aspergillus spp.*, *Fusarium spp.* etc). Several authors have shown that the very mature cheeses may contain aflatoxins, although their incidence does not seem to be great in commercially produced cheeses (ALVAREZ et al., 1990, GARCÍA et al., 1980).

In studies undertaken by ALVAREZ and co-workers (1990), it was established that the stability of the aflatoxins during the maturing process and storage of cheeses varied greatly according to the type, length of the maturing process or storage, presence of a covering, salt content, etc.

Cheese types Gouda and Edam usually have a covering of paraffin, wax or a film of vegetable oil, which creates a barrier against contamination.

In conclusion, evaluating our results, and applying the aforementioned reference limits, it can be stated that the hygiene-health quality, from the microbiological point of view, of the Gouda and Edam cheese types analysed is unsatisfactory. We believe that the presence of coliforms and *Enterobacteriaceae* totals in a high percentage of the samples is basically due to contamination during production and distribution. The fact that the maturing process is not respected might also justify the counts observed.

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INVESTIGATION OF PROLAMIN CONTENT OF CEREALS AND DIFFERENT PLANT SEEDS

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Some percent of the population can not tolerate several cereals like wheat, rye, triticale, barley, oat, which is due to the coeliac disease or gluten sensitive entheropathy. The only therapy of the disease is the strict gluten-free diet, therefore, we sought new sources for gluten-free foodstuffs.

We measured the gliadin content of wheat varieties, wheat products, and different plant seeds by sandwich ELISA method. The ethanol soluble protein fractions (prolamins) were studied with SDS-PAGE as well.

The prolamin concentration of amaranth seed (red and white), buckwheat and millet was under the permitted limit in gluten-free foodstuffs. In our examinations the prolamin content of sorghum reached the permitted limit in gluten-free foodstuffs. The toxic prolamin bands of different plant seeds like amaranth (red and white), millet were diffuse on the gel slab and could be found on the low molecular mass area. The oilseeds (sesame, flax) did not contain any prolamin band and its concentration was not detected by ELISA method.

Our examinations revealed that the plant seeds: amaranth (red and white), buckwheat, millet, sesame, flax can be sources of gluten-free foodstuffs. Their prolamin content was low enough that they are sufficient for feeding patients with an injured gut membrane, if their antinutritive effect can be neglected. Analytical results suggest that the mentioned plant grains could be used in coeliac diet after clinical trial and validation.

Keywords: gluten-free diet, plant seeds, SDS-PAGE, sandwich ELISA

The cereals are important and major components of the human diet, providing complex carbohydrate, fibre and protein. However, smaller number of the population can not tolerate several cereals like wheat, rye, triticale, barley, oat. Gluten is the storage protein of wheat consisting of gliadin and glutenin.

The gliadins are monomeric proteins with unusually high glutamine and proline content and low concentration of charged groups. It is known that all sub-groups of the gliadins are toxic to coeliac mucosa in vitro. The only therapy of the illnes is the strict gluten-free diet. In the international recommendation the limit of gliadin content in gluten-free foodstuffs is 10 mg gliadin/100 g dry material.

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Determination of toxic proteins need a sensitive, specific and reliable method. The sandwich ELISA method (WINDEMANN et al., 1982, TRONCONE et al., 1986, MILLS et al., 1989, SKERRITT & HILL, 1990) is accepted to determine plant flour gliadin content nowadays. The toxic prolamin bands can be studied by SDS-PAGE method as well. In this paper we looked for a new source for gluten-free foodstuffs. For this reason we examined the gliadin content and harmful prolamin bands of cereals, other plant flours and seeds by sandwich ELISA and SDS-PAGE.

1. Materials and methods

Wheat cultivars "bio" wheat (organic cultivation), Bonadur, Multidur, Gk Öthalom, Gk Tiszatáj, MV-19 (flour and grain), wheat products, rye and different type of plant seeds, which are used in the human nutrition (sesame, amaranth, sorghum, buckwheat, millet, flax) were examined.

The gliadin antigen 30 kD was purchased from SERVA (Germany). The Low Molecular Weight kit was obtained from Pharmacia (Sweden), the electrophoresis chemicals were bought at Janssen and SERVA. All other chemicals were of analytical grade or better and were purchased from Reanal Co. (Hungary).

Prolamin samples were extracted with 70% (v/v) ethanol and the supernatant was separated by centifuge or filtration.

For the SDS-PAGE analysis of extracted samples the modified discontinuous buffer system (LAEMLI, 1970), stacking gel 5% PAA (polyacrylamide) (T 5%, C 2.7%), separating gel 10% PAA (T 10%, C 2.7%) were used. The vertical SDS-PAGE was carried out with the BIO-RAD Mini Protean II Electrophoresis Cell. The slabs were stained at room temperature in a solution of 0.2% Coomassie brillant blue R-250 dissolved in 25% (v/v) methanol l–10% (v/v) acetic acid. Destaining was performed by the same solvent mixture. The gels were evaluated by BIOTEC-Fisher laser video densitometer, equipped with D analyse software. A-gliadin immune serum was developed in rabbits bought at Breading and Feeding Research Institute for Small Animals, Gödöllő. The titer of hyperimmune serum was established by indirect ELISA. The conjugatum was made according to NAKANE and KAWASI (1970) in our laboratory. ELISA microtiter plates were purchased from the the Sülysápi Műszeripari and Műanyag Feldolgozó GMK, Hungary. The sandwich ELISA method was used according to MILLS and co-workers (1989), and our work was carried out according to SKERRIT and HILL (1990).

A-gliadin immune serum with the coating buffer l:400 was adsorbed onto the microplates and incubated overnight. After incubation the plates were washed three times with PBST (0.1 M phosphate buffered saline containing 0.2% Tween 20, pH 7.4). The free binding sites were covered with PBSG (0.1 M phosphate buffered saline

containing 1% gelatine, pH 7.4.) The gliadin standards in quantity of 5.0 μg, 2.5 μg, 1.25 μg, 0.62 μg, 0.31 μg, 0.16 μg, 0.08 μg, 0.04 μg and samples of wheat varieties, wheat products diluted in 1:50, 1:500 and plant seeds diluted in 1:2, 1:4 were filled into wells. After 1 h incubation at 37 °C and 3 cycles of washing with the Titertek Microplate Washer (Labsystem, Finland), the conjugatum was added to the wells. The plates were incubated for 1 h at 37 °C, washed 3 times and dried. Then to each well 0.1 cm^{-3} TMB (3,3',5,5'-tetramethyl benzidine) substrate was added and the plates were incubated for 15 min. The reaction was stopped with 4 mol l^{-1} sulfuric acid, 70 μl/well. The absorbance of the wells was determined by Dynatech MR 7000 plate reader.

2. Results and discussion

As shown in Fig. 1 the SDS-PAGE of different wheat cultivars are different. On the basis of molecular weight distribution of proteins, the wheat gliadins can be identified. The molecular mass of the main characteristic subunits of wheat gliadin are as follows: 103 kD, 98 kD, 88 kD, 74 kD, 59 kD, 43–31 kD, 25 kD, 16–14 kD. There are no bigger differences between the bands of gliadin of wheat flour than there are between the wheat cultivars. The flours have more high molecular mass subunits (103–88 kD) than the ground whole seeds. The reason of it could be the 18% ratio of husk in the wheat seed. The gliadin content of flours and seeds of wheat varieties measured by ELISA were different, parallel with the results of gel electrophoresis (Table 1). The gliadin content in the flours was higher than in the whole seeds which was coincident with the results of SDS-PAGE (Fig. 1, Table 1). The wheat bran and germ (Fig. 2) have plenty bands of prolamins with the same molecular mass, as has wheat gliadin. On the basis of seed SDS-PAGE of these products the wheat origin and the presence of wheat gliadin could be identified. The wheat starch (protein content 0.5%) has detectable protein only at 16 kD, but not at 67–30 kD, which are characteristic of the wheat gliadin (Fig. 2). The wheat flake and puffed wheat are heat-processed foods. The heat treatment decreased the detectiability of gliadin. In case of wheat flake, the gliadin could be detected in a perfect way (the flake gets a wet, 15–20 min long heat treatment below 100 °C). The puffed wheat gets a wet, short (30 s) heat treatment but at high tempetature (270–290 °C) during its production, which strongly disturbs the detectiability of gliadin. Definite prolamin band around 67 kD can be detected, but there is a weak appearance of prolamins with 67–30 kD.

Table 1

Gliadin content of flours and seeds of wheat varieties measured by ELISA

Samples No	Wheat samples	Concentration mg g^{-1} ±s
1	"Bio" wheat seed	1.616±0.62
2	Bonadur seed	4.408±0.37
3	Multidur flour	3.712±0.29
4	GK Öthalom flour	4.408±0.51
5	GK Öthalom seed	2.83±0.38
6	GK Tiszatáj flour	6.030±0.49
7	GK Tiszatáj seed	4.880±0.47
8	MV-19 flour	5.00±0.52
9	MV-19 seed	4.610±0.34

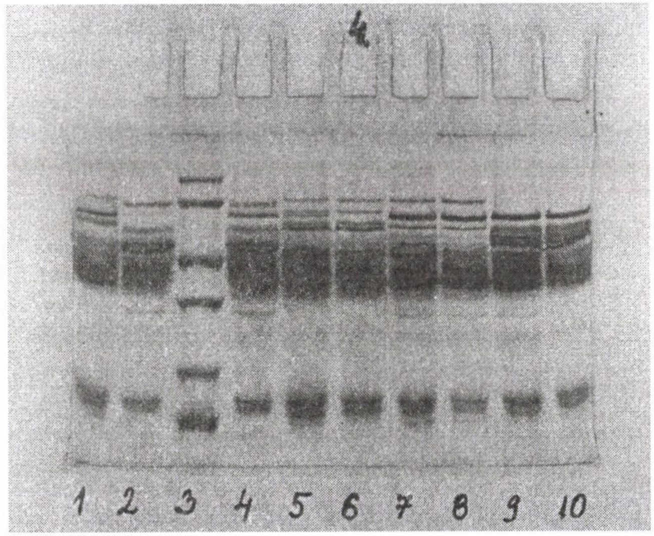

Fig. 1. SDS-PAGE of prolamins of different wheat cultivars (flour and seed) Lane 1: "Bio" wheat seed; Lane 2: Bonadur wheat seed; Lane 3: MW standard ; Lane 4: Multidur wheat flour; Lane 5: GK Öthalom wheat flour; Lane 6: GK Öthalom wheat seed, Lane 7: GK Tiszatáj wheat flour, Lane 8: GK Tiszatáj wheat seed; Lane 9: MV 19 wheat flour; Lane 10: MV-19 wheat seed

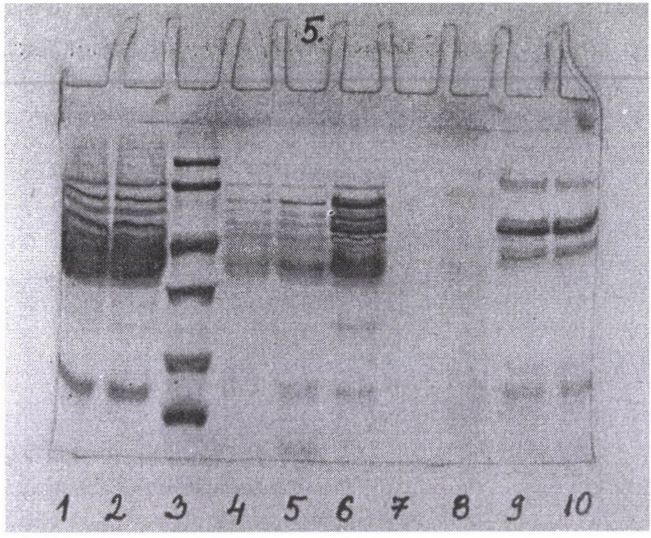

Fig. 2. SDS-PAGE of prolamins of different wheat products. Lane 1: Gluten; Lane 2: Gluten; Lane 3: LMW standard; Lane 4: Wheat germ; Lane 5: Wheat bran; Lane 6: Wheat flake; Lane 7: Wheat starch; Lane 8: Puffed wheat; Lane 9: Rye, Lane 10: Rye

Sample preparation of wheat products (flakes, bran, extruded products) for ELISA analysis was not satisfactory. We could not add ethanol to the product in a simple way because the solvent adsorbed onto the surface of grinded samples, therefore, sample preparation of heat treated cereal products needs improvement. The gliadin content of heat treated cereal products was not measurable with polyclonal immune serum (Table 2), it is not suitable to detect heat stable gliadins. The a-gliadin rabbit serum indicates only the reactive groups (epitops) of the flakes and puffed wheat. The measured absorbance was low and could not be fitted to the standard curve and therefore, the quantity of gliadin could not be estimated (Table 2). The 70% ethanol extract of the other plant seeds used in the human nutrition has characteristic and species specific molecular mass distribution on SDS-PAGE slab (Fig. 3). On the bases of these results the following seeds could be identified and distinguished from each other and from the wheat gliadin. The molecular mass distribution of different plant seeds is as follows: in rye 75, 48, 35, 19 and 16 kD, in amaranth 9 kD, in buckwheat 12 kD, in millet 13 and 25 kD, in sorghum 20 and 28 kD. The sesame and flax have no detectable prolamin bands (Fig. 3). The prolamin concentration (Table 3) measured by ELISA in case of amaranth seed (red, white), buckwheat, millet was low enough, it was under the permitted limit for gluten free foods. In our examinations the prolamin

content of sorghum approximated the permitted limit. The toxic prolamin bands of different plant seeds in Fig. 3 (amaranth red, amaranth white, millet, buckwheat) were diffuse on the gel slab and could be found in the low molecular mass area. The sorghum has a lot of toxic bands. The oilseeds did not contain any prolamin bands on the basis of our results. The results were similar to SDS-PAGE and ELISA methods (Table 3).

Table 2

Gliadin concentration of different wheat products and rye detected by ELISA

Samples No	Wheat products	Concentration mg g^{-1} ±s
1	Wheat germ	0.03 or nd
2	Wheat bran	nd
3	Wheat flake	nd
4	Wheat starch	0.01
5	Puffed wheat	0.07 or nd
6	Rye	1.80±0.20

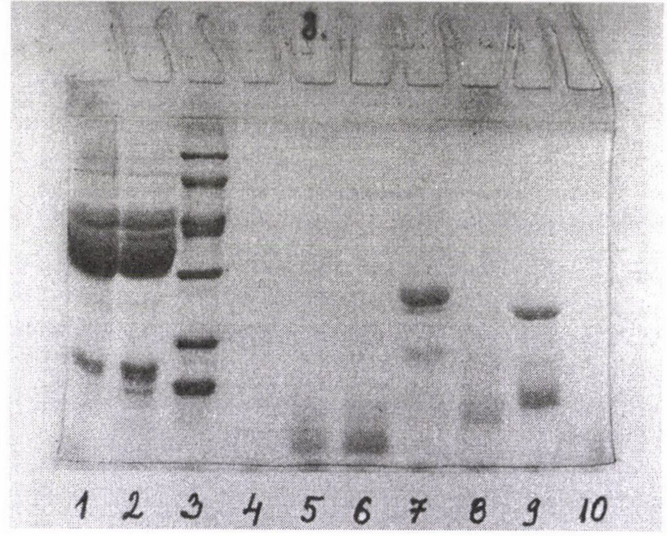

Fig. 3. SDS-PAGE of prolamins of different plant seeds. Lane l: Gluten; Lane 2: Wheat flour; Lane 3: LMW standard; Lane 4: Sesame; Lane 5: White amaranth; Lane 6: Red amaranth; Lane 7: White sorghum; Lane 8: Buckwheat, Lane 9: Millet; Lane 10: Flax

Table 3

Prolamin content of different plant seeds

Samples No	Plant seeds	Concentration mg g^{-1} ±s
1	Sesame	–
2	Amaranth white	0.029±0.016
3	Amaranth red	0.059±0.026
4	Sorghum	0.098±0.044
5	Millet	0.054±0.014
6	Flax	nd
7	Buckwheat	0.034±0.010

3. Conclusion

Our examination revealed that the plant seeds: amaranth (red and white), buckwheat, millet can be a source of gluten-free foodstuffs. Their prolamin content was probably low enough for feeding patients with an injured gut membrane, if their antinutritive effect can be neglected. It is of the utmost importance to make a clinical trial before feeding coeliac patients with plant grains tested.

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MICROBIAL CHANGES IN NATURAL AND ALGAL YOGHURTS DURING STORAGE

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Storage experiments were carried out so as to study the changes in the microbial flora of stirred yoghurt samples enriched with a microalgal biomass and in that of control samples for 35 days at 4 °C and 15 days at 15 °C. Along with the microbiological investigations, the pH of the samples was also measured. A characteristic viable cell count of over 10^8 cfu g^{-1} was detected both in the control and in the algal yoghurt samples, regardless of storage temperature. However, the viable cell counts were significantly higher in the case of the algal yoghurt at 4 °C. The count of moulds and yeasts amounted to a level of 10^1 cfu g^{-1} by the sixth day and to a level of 10^5 cfu g^{-1} by the fifteenth day of the storage period at 15 °C, while the algal yoghurt, if stored at 4 °C, is guaranteed to have a shelf-life of one month even if manufactured under non-aseptic conditions.

Keywords: natural yoghurt, algal yoghurt, storage, viable starter bacteria, microbial contaminants

Due to post-acidification, degradation of protein or fat resulting in smell and taste defects and due to spoilage by microbial contaminants, the traditional yoghurt containing viable cells has a limited shelf-life (ROHM & LECHNER, 1989; MAYER, 1990). Even if stored at a temperature below 10 °C (at 4–6 °C) yoghurts have a shelf-life of 2–3 weeks (up to 4–6 weeks in the case of aseptic packaging).

Some European countries have established standards regarding the content of viable starter bacteria in yoghurt. The values range between 10^6 and 10^8 cfu g^{-1} (RAŠIĆ & KURMANN, 1978; GLÄSER, 1992). The heat treatment of fermented dairy products is contrary to the regulations in Austria and both natural and flavoured yoghurts with a characteristic viable population of less than 10^6 cfu g^{-1} are considered to be imitations (BUNDESKANZLERAMT, 1990). The draft of CODEX ALIMENTARIUS HUNGARICUS (1996) suggests that yoghurts should contain at least 10^7 g^{-1} viable bacteria of starter culture origin. This is in conformity with the draft of an International Dairy Federation (IDF) Standard suggesting that fermented dairy products should contain at least 10^7 g^{-1} characteristic viable starter bacteria at the time of sale. ROHM and LECHNER (1988 and

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1989) are of the same opinion. According to GLÄSER (1992), yoghurts of high quality can be characterized by 10^8 g^{-1} lactic acid bacteria at the time of consumption. He suggests that a *Streptococcus thermophilus/Lactobacillus bulgaricus* ratio of 1:1 to 3:1 and a defined level of lactase activity should also be required at the time of sale, in the European Union.

Storage experiments were conducted to study the changes in the microflora of stirred yoghurt samples enriched with a microalgal biomass at a concentration of 3 g l^{-1} and also in the microflora of control samples for a period of 35 days at 4 °C and 15 days at 15 °C in order to follow the quantitative changes in the characteristic and undesirable microbial flora under non-sterile manufacturing conditions. Along with the microbiological investigations, the pH of the samples was also measured so as to obtain data concerning the degree of post-acidification.

1. Materials and methods

The algal stirred yoghurt samples and the control ones needed for the storage experiments were manufactured in the pilot plant of the Hungarian Dairy Research Institute Inc. at Mosonmagyaróvár.

The raw material used was bulk milk with a fat content of 3% (w/w) being free of substances which might impede the development of the yoghurt culture. It was homogenized at a temperature of 65 °C and at a pressure of 15 MPa (150 bar) in a high-pressure homogenizer. The heat treatment was five minutes at 90 °C. FYE-43 yoghurt starter culture (kindly supplied by the Hungarian Dairy Research Institute Inc. at Mosonmagyaróvár) was employed at a concentration of 3% (v/v). The incubation took 2.5 h at 42 °C.

In the case of algal yoghurt the dried biomass of *Spirulina platensis* was added to the product when pH dropped to 5.2, at a temperature of 25 °C. Both the algal and the control (natural) samples were filled into 40 retail containers apiece which were then sealed with aluminium foil. Half of the samples was put into a cooling and heating thermostat at 15 °C while the rest of them was stored in a refrigerator at 4 °C after a day's pre-cooling. Three retail containers (n = 3) were opened after 0, 3, 6, 9, 12, and 15 days of storage apiece in the case of algal and control yoghurt samples stored at 15 °C and after 0, 7, 14, 21, 28, and 35 days of storage in the case of algal and control samples stored at 4 °C. The following microbial groups were determined using the FIL/IDF standard methods: total microbial count, counts of *Streptococcus salivarius* subsp. *thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, yeasts and moulds, total enterococci and coliform organisms (INTERNATIONAL DAIRY FEDERATION, 1971, 1985 and 1988). The parameters tested are shown in Table 1.

Table 1

Major parameters of the determination of microbial counts in the storage experiment

No.	Microorganism (group)	Method	Culture medium[a]	Incubation	
				time (h)	temperature (°C)
1	*Lact. bulgaricus*	Pour-plate	MRS Agar	72[b]	37
2	*Strep. thermophilus*	Pour-plate	M17 Agar	48	37
3	Total microbial count	No.1+No.2.[c]	–	–	–
4	Yeasts and moulds	Pour-plate	YGC Agar	96	25
5	Coliform organisms	MPN	BRILA Broth	24–48	37
6	Enterococci	Pour-plate	KEA Agar[d]	72	37

[a]: all culture media were purchased from Merck KGaA, [b]: incubated anaerobically, [c]: *Lact. bulgaricus* count + *Strep. thermophilus* count (calculated values), [d]: Kanamycin Esculin Azide Agar

2. Results and discussion

Figures 1–3 illustrate the survival of the characteristic microbial flora of yoghurt samples stored at 15 °C.

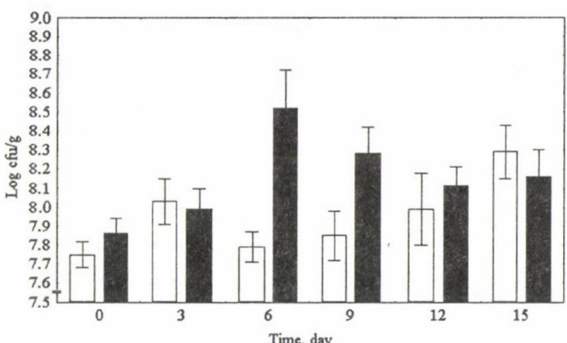

Fig. 1. Changes in the *Lact. bulgaricus* count during storage at 15 °C. White bars (□): natural yoghurt, black bars (■): algal yoghurt, whiskers (I): standard deviation (±s), n = 3

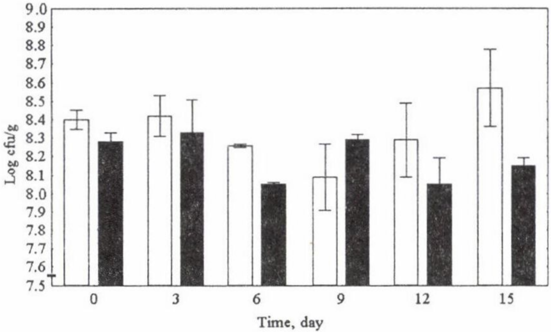

Fig. 2. Changes in the *Strep. thermophilus* count during storage at 15 °C. White bars (□): natural yoghurt, black bars (■): algal yoghurt, whiskers (I): standard deviation (±s), n = 3

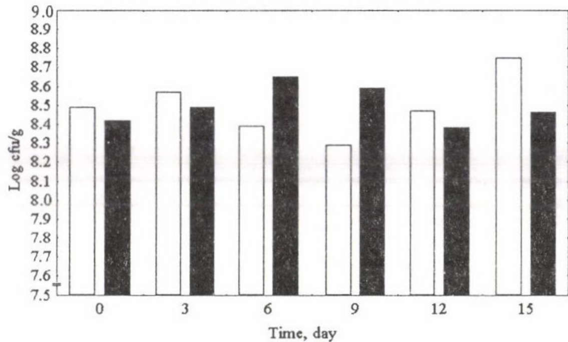

Fig. 3. Quantity changes in the characteristic viable cell count during storage at 15 °C. White bars (□): natural yoghurt, black bars (■): algal yoghurt, calculated values (*Lact. bulgaricus* count + *Strep. thermophilus* count), n = 3

As shown in Figs 1 and 2, the viable cell counts of both starter culture bacteria constituting the characteristic microflora of yoghurt exceeded the value of 10^8 cfu g^{-1} in the samples enriched with algal biomass. The stimulatory effect of the algal biomass on *Lact. bulgaricus* was noticeable throughout the storage period, with the most pronounced stimulation observed on day 6. This phenomenon can be attributed to the presence of peptone, adenine and hypoxanthine in the algal biomass because these nitrogenous substances are capable of significantly ($P = 0.05$) stimulating the growth and acid production of *Lact. bulgaricus* (VARGA et al., 1998). Since the data plotted in Fig. 3 are calculated values, these were solely influenced by the results illustrated in Figs 1 and 2. As for the total viable cell counts, both the control and the algal yoghurt samples

met even the most stringent requirements detailed in the introduction of this paper (Fig. 3).

Owing to the non-sterile manufacturing conditions and the high storage temperature, the count of yeasts and moulds amounted to a level of 10^1 cfu g^{-1} by the sixth day and to a level of 10^5 cfu g^{-1} by the fifteenth day of the storage period. No significant difference was observed between the control and the algal samples in this regard.

Enterococci and coliform organisms were detected neither in the control nor in the algal yoghurt samples during the entire storage period.

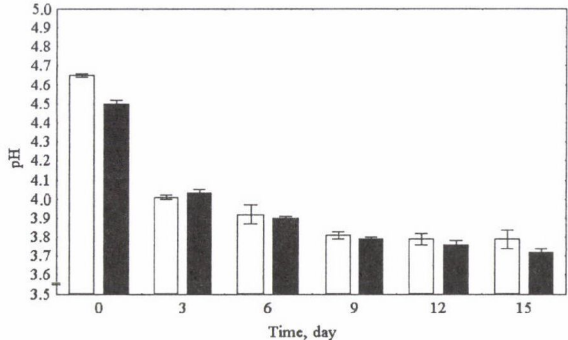

Fig. 4. Post-acidification of yoghurt samples stored at 15 °C. White bars (□): natural yoghurt, black bars (■): algal yoghurt, whiskers (I): standard deviation (±s), n = 3

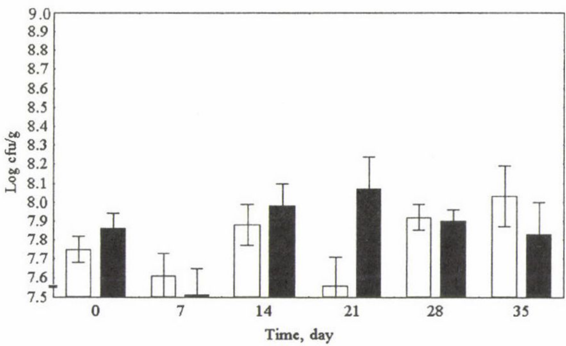

Fig. 5. Changes in the *Lact. bulgaricus* count during storage at 4 °C. White bars (□): natural yoghurt, black bars (■): algal yoghurt, whiskers (I): standard deviation (±s), n = 3

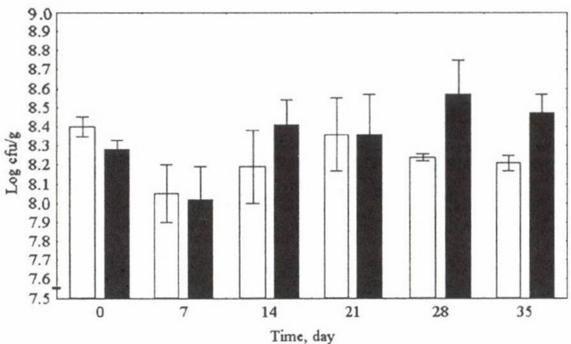

Fig. 6. Changes in the *Strep. thermophilus* count during storage at 4 °C. White bars (□): natural yoghurt, black bars (■): algal yoghurt, whiskers (I): standard deviation (±s), n = 3

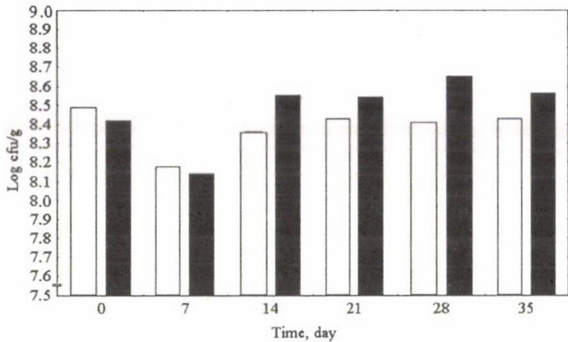

Fig. 7. Quantity changes in the characteristic viable cell count during storage at 4 °C. White bars (□): natural yoghurt, black bars (■): algal yoghurt, calculated values (*Lact. bulgaricus* count + *Strep. thermophilus* count), n = 3

As the pH values show there was a pronounced post-acidification, obviously due to the high storage temperature (Fig. 4). There was no significant difference between the control and the algal yoghurt samples regarding post-acidification.

Figures 5–7 illustrate the quantity changes in the characteristic microbial flora of yoghurt samples stored at 4 °C.

Figure 5 shows that – both in the case of the control yoghurt samples and in that of the algal ones – the viable cell counts of *Lact. bulgaricus* did not change basically during the storage period at 4 °C. Although the data show some fluctuations, these are within the range of tolerance characteristic of microbiological determinations, i.e.

approximately ±0.1–0.2 log cycles. The same tendency was observed in the case of *Strep. thermophilus* (Fig. 6).

As for the characteristic viable cell counts, both the control yoghurt samples and the algal ones met even the most stringent requirements of the European Union throughout the storage period (GLÄSER, 1992). Furthermore, the viable cell counts were found to be significantly higher in the algal yoghurt at a storage temperature of 4 °C (Fig. 7).

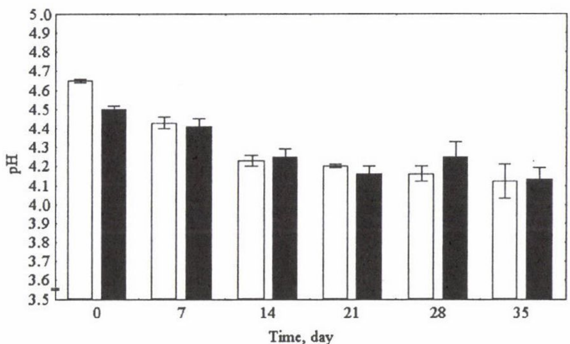

Fig. 8. Post-acidification of yoghurt samples stored at 4 °C. White bars (□): natural yoghurt, black bars (■): algal yoghurt, whiskers (I): standard deviation (±s), n = 3

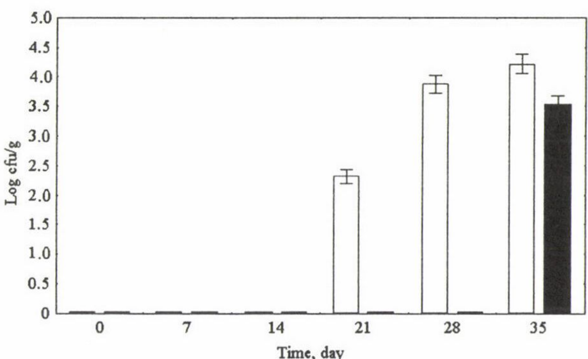

Fig. 9. Changes in the counts of moulds and yeasts during storage at 4 °C. White bars (□): natural yoghurt, black bars (■): algal yoghurt, whiskers (I): standard deviation (±s), n = 3

As shown in Fig. 8, no excessive post-acidification was observed. Neither the pH of the control yoghurt samples nor that of the algal ones dropped below a value being still characteristic of such products during a storage period of 35 days. Regarding post-acidification, there was no significant difference between the control and the algal yoghurt samples.

Figure 9 shows how intensively the algal biomass inhibited the growth of moulds and yeasts in the product during storage at 4 °C.

On the twenty-first day of the storage period the counts of yeasts and moulds found in the control yoghurt samples slightly exceeded the value of 10^2 cfu g^{-1}, while the algal yoghurt is guaranteed to have a shelf-life of one month even if manufactured non-aseptically.

None of the samples contained a detectable count of enterococci and coliform organisms during the storage period.

The literature provides explanation of these findings. DE CANO and co-workers (1990) tested phenolic compounds extracted from *Nostoc muscorum* cyanobacteria for antifungal properties. The algal phenolic compounds were found to inhibit the growth of *Candida albicans* significantly (89.1%). Extracellular products were extracted from a wide range of cyanobacteria by the same workers (DE CAIRE et al., 1993). One third of the 36 strains tested proved to produce compounds responsible for growth inhibition of *C. albicans*. In another experiment by MIURA and co-workers (1993), three out of ten *Chlorella* strains produced an inhibitory effect on the growth of *Saccharomyces cerevisiae* under dark conditions. A light-activated compound responsible for the antimicrobial effect was isolated. Two polyhalogenated aromatic compounds extracted from the blue-green alga *Fischerella ambigua* were found to exhibit antibacterial activity against *Escherichia coli* and antifungal activity against *Penicillium oxalicum* (FALCH et al., 1992). FALCH and co-workers (1995) tested 54 cyanobacterial extracts for antibacterial and antifungal activities, 46 of which were active against *Bacillus subtilis*, *E. coli* and/or *Micrococcus luteus* and 13 against *P. oxalicum*.

On the whole, the findings of the experiments are in line with the ones gained by ROHM and co-workers (1990) having examined the storage-induced changes in the populations of viable yoghurt starter organisms and microbial contaminants in randomly selected Austrian natural-set yoghurts. Typically, numbers of *Strep. thermophilus* and *Lact. bulgaricus* remained above 10^8 cfu g^{-1} in yoghurt stored at 10 °C until the sell-by-date (15–20 days after manufacture). Both elevated storage temperature and storage periods markedly reduced the survival of yoghurt starter bacteria. Depending on the sample origin, the yeast populations increased from less than 10 g^{-1} to above 10^6 g^{-1} when yoghurts were stored at 10 °C until the sell-by-date.

The algal stirred yoghurt does have a shelf-life of one month regarding each parameter, even if manufactured under non-aseptic conditions.

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CHEMICAL AND PHYSICAL CHARACTERISATION OF SUGAR BEET FIBER

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Sugar beet fiber used in this study was analyzed for protein, ash, nitrogen, sucrose, acid and neutral detergent fiber, hemicellulose, cellulose, lignin, direct and bulk densities, cation exchange and water holding capacities. Moisture sorption isotherms of fine and coarse sugar beet fibers were determined at 25 °C by the static gravimetric method. BET, Freundlich, Oswin and GAB equations were applied to fit the experimental moisture sorption data for the sugar beet fibers. The parameters of each equation were determined and presented. Goodness of fit (from regression coefficient) indicated that GAB and Oswin equations were the best fitting ones for the sorption data over the a_w range of 0.07 and 0.78.

Keywords: sugar beet fiber, dietary fiber, moisture sorption isotherm, GAB equation

The benefits of dietary fiber were generally accepted and reported by many researchers (TROWELL, 1976; WISKER et al., 1985; ANDERSON et al., 1984). It was found that soluble fibers such as pectins, beta glucans and vegetable gums have been associated with enhanced glucose tolerance and serum cholesterol-reducing properties of certain dietary fiber sources (ANDERSON, 1986; ANDERSON et al., 1984). The consumption of insoluble fibers such as cellulose, some hemicelluloses and pectins, have been associated with reduced risks of diverticulosis, appendicitis, irritable bowel syndrome, and possibly colon cancer (ANON, 1979). Besides wheat bran, the pulp from sugar beet appears as an attractive source of fiber since it is available in large amounts from the sugar refining industry and contains high levels of low-energetic dietary fiber (20% of soluble fiber), devoid of starch and phytate (BERTIN et al., 1988). The sugar beet fiber is mainly composed of pectins (25%), hemicelluloses (25%) and cellulose (20%) (SCHWEIZER & WURSCH, 1979; MICHEL et al., 1988). Pectin is primarily composed of α-D-galactopyranosyluronic acid units, partially esterified with methanol. They contain in a lesser extent L-rhamnose residues and other neutral units such as galactose, arabinose and xylose (ANON, 1993). Hemicellulose is a heterogeneous group

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containing a number of sugars in its backbone and side chains. Cellulose is a linear polymer of glucose with beta 1–4 links (SCHNEEMAN, 1986).

One of the objectives of this study was to determine chemical and physical properties of sugar beet fiber such as protein, ash, nitrogen, sucrose, acid and neutral detergent fiber, hemicellulose, cellulose and lignin contents, direct and bulk densities, cation exchange and water holding capacities.

Sorption isotherms are important tools used extensively to study the water binding properties of food materials. The typically sigmoid shaped sorption isotherm consists of three regions and adsorbed water can be partitioned into its 'monolayer', 'multilayer' and 'condensed' forms. Water in the initial 'monolayer region' is held by strong hydrophilic bonds on polar sites in the food solid. A high water uptake in this region usually reflects high levels of hydrophilic proteins and polysaccharides. In the second region (multilayer), the water is more loosely held by hydrogen bonds within the pores or matrix structure of the fiber. The least firmly bound water occurs when the water activity level rises above 0.5. In this region, 'condensed water' is mechanically entrapped within the void spaces and has many of the characteristics of liquid water. However, in terms of physical characteristics of food fibers, the absolute amount of water held may be less important than the manner by which that water is held (EASTWOOD et al., 1983). For instance, strongly bound water has been found to have no effect on stool weight, whereas loosely associated water readily increases stool weight (ROBERTSON & EASTWOOD, 1981). In sugar beet fiber, the carboxyl groups in pectin and in the side chains of hemicellulose, and the hydroxyl groups in pectin, cellulose and hemicellulose are expected to participate in water binding. Sorption data for several food fibers have been reported by CHEN and co-workers (1984) and WALLINGFORD and LABUZA (1983). Mathematical models such as the Guggenheim-Anderson-DeBoer (GAB) equation (BIZOT, 1983) have been developed which enable the sorption behaviour of water in foods to be better understood. This approach allows fiber types to be selected that increase water binding in the gastrointestinal tract and still satisfy the requirements of the food industry (CADDEN, 1988). Taking this point into account, the sorption isotherm of sugar beet fibers was determined and the applicability of the experimental data to some isotherm equations was tested.

1. Materials and methods

1.1. Materials

Sugar beet pulp was obtained from Ankara Sugar Factory, Ankara, Turkey. It was collected from the sucrose extraction process at the stage where the pulp had been dewatered by pressing. The fresh sugar beet pulp was stored in the deep-freezer before usage.

1.2. Preparation of sugar beet fiber (SBF)

After thawing the pulp was first cleaned manually from the dark coloured beet pieces and homogenized in ethyl alcohol (96%) by using Waring blender for one minute. The ground pulp was then kept in ethyl alcohol for several times until the filtrate became colourless. Following this procedure, alcohol was separated by filtration. The pulp obtained in this way was air-dried at 60 °C overnight and then at 35 °C for 24 h. The dried pulp was milled by using a Hammer mill and sifted through a series of sieves having 160, 315, 500 and 720 μm apertures. Our previous studies have shown that the effect of particle size of sugar beet fiber on the quality of bread, cookies and extrusion products was found to be different (KÖKSEL et al., 1995; ÖZBOY et al., 1995; 1996). Therefore the sugar beet fibers with particle sizes of 160–315 μm and 500–720 μm which generally gave the good results for all of these products were used in the sorption experiments of SBF. Throughout this paper, the fine granulated sugar beet fiber (160–315 μm) and coarse granulated sugar beet fiber (500–720 μm) will be referred as FG-SBF and CG-SBF, respectively. The samples smaller than 160 μm were not used because of the probable existence of soil and sand particles. After sieving, all fractions were brought together to have the composite SBF and analyzed for the physical and chemical composition.

1.3. Physical and chemical composition

The fibers used in sorption isotherm determinations were analyzed for moisture (A.O.A.C., 1990), neutral detergent fiber (NDF) (VAN SOEST & WINE, 1967), acid detergent fiber (ADF) (VAN SOEST, 1963), hemicelluloses, cellulose, lignin, ash, density (direct and bulk), cation exchange capacity (MICHEL et al., 1988), water holding capacity (MONGEAU & BRASARD, 1982), sucrose, and nitrogen (ANON, 1990). Hemicelluloses and cellulose were obtained by the difference between NDF and ADF and between ADF and lignin, respectively.

1.4. Sorption experiments

The experimental procedure includes three steps: preparation of the sample for sorption experiments, exposing the sample to different relative humidities in small cabinets at constant temperature until the equilibrium is attained and determination of the samples and relative humidities of the cabinets. For adsorption studies, FG-SBF and CG-SBF samples were dried at ambient temperature in a desiccator containing P_2O_5. At the end of drying, the water activities (a_ws) of FG-SBF and CG-SBF were determined to be 0.023 and 0.032, respectively and their dry weights were found to be less than 1% by weight. The samples for desorption were kept in closed jars containing distilled water, and at the start of the experiments the a_w of FG-SBF was measured as 0.924 and

that of CG-SBF as 0.908. AquoLab Model CX2 water activity meter (reading sensitivity = 0.003) was used to determine a_w values.

Gravimetric method was used in equilibrium studies. It consisted of sorption containers, supports for weighing bottles and the weighing bottles in which the sample was exposed to humid atmosphere in the containers. Sulfuric acid (Merck) solutions of different concentrations were used to provide the desired atmospheres (TROLLER & CHRISTIAN, 1978). Nine different sulfuric acid solutions plus distilled water were employed to provide a_ws in the range of 0.07 and 1.0. Each solution was transferred into a 1 l hygrostat (glass jar) and a support was placed in each jar. The weighing bottles containing the samples (app. 1 g) were placed on the supports in the hygrostats which were then tightly closed. Thus, a set of ten jars were kept in an oven (Memmert-ULM 100: temperature sensitivity ±1 °C), maintained at 25 °C for equilibration of the samples. The samples were weighed after 3 weeks and the equilibrium was judged to have been attained when the difference between the two consecutive sample weighings was less than 1 mg g^{-1} solids, as stated by LABUZA and co-workers (1985). The analytical balance used was Shimadzu Libror AEG-220, with the sensitivity of ±0.0001 grams. The a_ws of sulfuric acid solutions were measured both at the start and at the end of the experiment and the values determined at the end have been taken into consideration. The dry weights of the samples were determined according to Association of Official Analytical Chemists (A.O.A.C., 1990). Equilibrium moisture contents (EMC) of the samples were expressed as grams of moisture per 100 grams of dry weight. Although ten samples were taken into experiment at the start, samples with a_ws greater than 0.78 were not taken into consideration because of the mould growth within the first week.

Oswin, BET (IGLESIAS & CHIRIFE, 1982), GAB (CADDEN, 1988), Freundlich (BOKI & OHNO, 1991) equations were tested to fit the experimental moisture sorption data. In the case of BET, the equation was linearized and the constants were found by linear regression. GAB equation was transformed to a second degree polynomial and evaluated by nonlinear regression analysis. SPSS for Windows Release 5.0.1 was used for both the linear and nonlinear regression analysis.

2. Results and discussion

2.1. Physical and chemical composition of sugar beet fiber

Various physical and chemical properties of sugar beet fiber used in this study are given in Table 1.

Table 1

Properties of sugar beet fiber

Moisture (%)	10.05
Ash (%)	3.24
Nitrogen (%)	1.39
Neutral detergent fiber (%)	56.90
Acid detergent fiber (%)	29.10
Cellulose (%)	27.20
Hemicellulose (%)	22.10
Lignin (%)	1.85
Cation exchange capacity meq OH g^{-1}/(as H+) (%)	0.64
Water holding capacity (g/g)	16.60
Direct density (mg cm^{-3})	250.00
Bulk density (mg cm^{-3})	305.00

The chemical composition of sugar beet fiber agreed with previously published data (MICHEL et al., 1988). However, the amount of ash (3.24%) was particularly low and the protein content was found to be slightly high (8.69%). The cation exchange capacity is mainly related to the free carboxyl group content in pectin and to a lesser extent to those in hemicelluloses. The cation exchange capacity values published by MICHEL and co-workers (1988) ranged from 0.59 meq g^{-1} to 0.64 meq g^{-1}. Sugar beet pulp and fiber behave as weak monofunctional cation exchangers, as is the case with most vegetable fibres, whereas cereal fibers act as polyfunctional cation exchange resins (MICHEL et al., 1988).

The water holding capacity (WHC) values are difficult to compare with the data in the literature because they are greatly affected by the measurement techniques and by the experimental conditions. In this study, WHC of sugar beet fiber was found to be 16.60 g g^{-1}. This is lower than the values previously reported (SCHALLER, 1978;

SCHWEIZER & WURSCH, 1979) for sugar beet fibers (26–29 g g^{-1}). However, it was reported that commercial sugar beet fiber preparation (FIBREX) has an even lower value (7 g g^{-1}) for WHC. Cereal fibers usually exhibit poor water holding capacity values as compared to SBF (MC CONNELL et al., 1974: 2.4 and 7.3 g g^{-1}). Direct and bulk densities of sugar beet fiber were much higher than those values determined by MICHEL and co-workers (1988). Sugar beet fiber is especially rich in dietary fiber. In this study, NDF and ADF values of SBF were found to be 56.9% and 29.1%, respectively. While ADF values of SBF was slightly higher than the one determined by MICHEL and co-workers (1988): 25%, NDF values of SBF was found to be almost equal (56.0%).

2.2. Sorption isotherm and models

Adsorption and desorption isotherms of FG-SBF and CG-SBF at 25 °C are shown in Figs 1 and 2 where the equilibrium moisture content is plotted as a function of a_w. Hysteresis was observed in both isotherms at a_w values greater than 0.1 and no difference has been detected between the equilibrium moisture contents of FG-SBF and CG-SBF samples. The shape of the isotherm for a food product represents the integrated hygroscopic properties of its constituents (LABUZA, 1968). Even though the moisture contents corresponding to a_ws greater than 0.78 were not obtained, the isotherms drawn by joining the experimental data points showed the usual Type II sigmoid shape common to most food products. Sorption isotherms for the two different sized SBF were found to be similar. In the related literature similar sigmoidal shaped sorption isotherms were reported for food fibers (oat bran, wheat bran, carboxymethylcellulose and guar gum) (MAZZA, 1980; VAN DEN BERG & BRUIN, 1981; CADDEN, 1988).

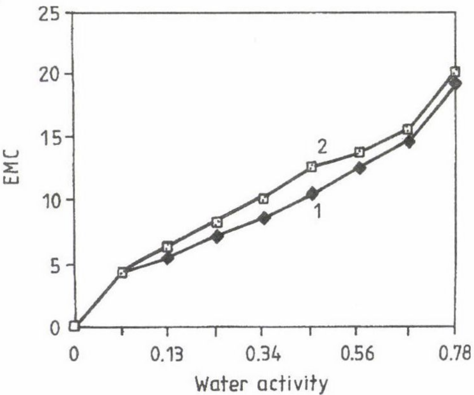

Fig. 1. Sorption isotherm of fine granulated sugar beet fiber at 25 °C.
1: Adsorption; 2: desorption

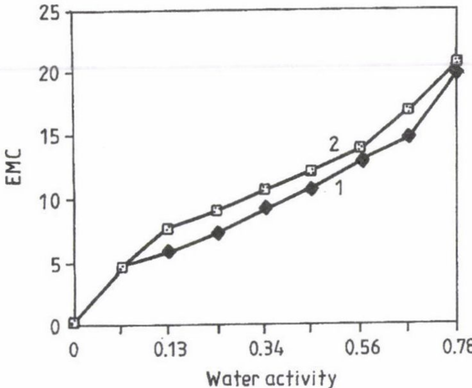

Fig. 2. Sorption isotherm of coarse granulated sugar beet fiber at 25 °C.
1: Adsorption; 2: desorption

There are many equations in the literature describing the sorption characteristics of food and other agricultural products. In this paper, BET, Freundlich, Oswin and GAB equations were used. The constants of the equations were calculated by nonlinear regression analysis except for the BET equation.

The isotherm model in food systems most generally used is that of BRUNAUER and co-workers (1938), which is expressed by Eq. 1.

$$\frac{X}{X_m} = \frac{Ca_w}{(1-a_w)(1-a_w+Ca_w)} \tag{1}$$

where X is the equilibrium moisture content, X_m is the monolayer value, a_w is the water activity and C is a factor related to heat of sorption. Linear forms of BET equation were evaluated by linear regression and the regression coefficients (r^2) were found to be 0.93–0.98. The monolayer coverage value was calculated as 4.80–5.25 g/100 g dry solid. When the applicability of BET equation over the region of 0.07–0.78 a_w was studied, in all cases it was noticed that the y-intercepts were found to be a negative value. This means that the C values are also negative. Physically this is not a possible case and may be the result of assumptions in the derivation of BET equation. Since the assumptions are not entirely true for most materials, the BET isotherm usually holds only for the water activities less than 0.5 (LABUZA, 1968). Therefore, it was concluded that the BET equation did not fit experimental data over the a_w range of 0.07–0.78.

Freundlich isotherm was proposed as an improvement over the Langmuir model. The isotherm is based on the assumption that it is composed of a series of monolayers adsorbing on a surface composed of heterogeneous sites and for this reason it has been successively applied to various foodstuffs such as starches, sugars, egg powders, etc. (RIGANAKOS et al., 1989; DEMERTZIS et al., 1989). The mathematical expression of this model is given by Eq. 2.

$$X = ka_w^{1/n} \quad (2)$$

where n and k are constants related to the sorptive efficiency of the sorbent. Constant k is known as the sorptive capacity of the material and analogous to the monolayer value in the BET equation. Sorption data of sugar beet fiber were analyzed by nonlinear regression analysis and the constants are given in Table 2. Experimental data gave an acceptable fitting to the Freundlich equation ($r^2 = 0.94–0.97$). Values of k and n for granulated SBF were found to be 21.08–22.18 and 1.34–1.59, respectively. Similar k values were obtained for coarse and fine fibers in the case of both adsorption and desorption. The application of the sorption data for SBF or a similar product was not available in the related literature. Therefore, our constants were not compared to those of similar materials. On the other hand, k values for water sorption by wheat flour was reported as 46.4 g/100 g solids (25 °C) by RIGANAKOS and co-workers (1989). For the raffinose, this value was obtained as 3 g/100 g solids (30 °C) by DEMERTZIS and co-workers (1989). DEMERTZIS and KONTOMINAS (1988) have found a k value of 35.2 g/100 g solids (40 °C) for the whole egg powder. The k values obtained in this study were not found to be very distinct from the ones mentioned above.

Table 2

The parameter constants and regression coefficients of Freundlich equation

	k	n	r^2
FG-SBF (ads)	21.08	1.34	0.95
FG-SBF (des)	21.96	1.48	0.97
CG-SBF (ads)	21.63	1.36	0.94
CG-SBF (des)	22.18	1.59	0.95

Table 3

The parameter constants and regression coefficients of Oswin equation

	A	B	r^2
FG-SBF (ads)	11.54	0.40	1.00
FG-SBF (des)	12.70	0.37	0.99
CG-SBF (ads)	11.93	0.40	1.00
CG-SBF (des)	13.29	0.35	0.99

The Oswin equation has been found as a good-fit model for the sorption of various food products up to an a_w of 1.0 (MITTAL & USBORNE, 1985; MOK & HETTIARACHCHY, 1990). The mathematical expression of this model is given by Eq. 3.

$$X = A\left[\frac{a_w}{1-a_w}\right]^B \tag{3}$$

where A and B are constants. Experimental sorption data for the fiber were evaluated by non-linear regression analysis and A and B values are presented in Table 3. Regression coefficients ($r^2 = 0.99$–1.00) suggested that the Oswin equation might be a good model for sugar beet fiber.

The GAB equation was recently favoured by several European and American research laboratories as a standard method for comparison as well as the mathematical modelling of isotherms. GAB equation is the modified form of the BET equation and also includes the physical adsorption in multilayers. Therefore, the model provides a good fit over a range of water activity values up to an a_w of 0.9 (WEISSER, 1986; RIGANAKOS et al., 1989). The GAB equation can be written as Eq. 4.

$$\frac{X}{X_m} = \frac{Cka_w}{(1-ka_w)(1-ka_w+Cka_w)} \tag{4}$$

where X_m is the monolayer moisture content, C is the Guggenheim constant related to the heat of sorption of the first layer and k is a correction factor related to the total heat of sorption of multilayer. Equation 4 was transformed to a second degree polynomial and evaluated by nonlinear regression analysis. The parameter constants were given in Table 4. Regression coefficients ($r^2 = 0.99$–1.00) show that the data fit the GAB model very well. The GAB model can be considered as an extension of the well known and often used BET model of multilayer localized homogenous adsorption, taking into

Table 4

The parameter constants and regression coefficients of GAB equation

	Xm	k	C	r^2
FG-SBF (ads)	7.23	0.82	18.88	1.00
FG-SBF (des)	9.10	0.73	15.68	1.00
CG-SBF (ads)	7.30	0.83	21.15	1.00
CG-SBF (des)	8.92	0.75	21.87	0.99

account the modified properties of sorbed molecules in the multilayer region. The three GAB constants depend on the product characteristics and to some extent on temperature. The GAB monolayer values obtained for fine and coarse granulated SBF ranged between 7.23–9.10 g/100 g solids. The monolayer values calculated from the GAB equation are higher than those calculated from the BET equation. VAN DER BERG (1985) observed the same trend for starch. Also LABUZA and co-workers (1985) reported greater magnitude of the GAB monolayer value than the BET monolayer value for fish flour and corn meal.

In this study, the GAB constants obtained for fine and coarse granulated SBF were found to be similar. Particle size seems to have no effect on X_m and k values and there is a slight increase in C value as the particle size gets coarser.

The reported X_m, C and k values of GAB equation for microcrystalline cellulose (MCC) and guar gum are in the same range as the ones found in the present study (CADDEN, 1988). The similarities in the GAB constants might be attributed to the existence of hydroxyl groups in SBF, MCC and guar gum as the main water binding groups. For cellulose, the crystallinity can decrease the hydration behavior since the water sorption process is limited to the amorphous parts. Minor differences in the GAB constants may be due to the presence of other chemical species such as carboxyl and carboxymethyl groups in pectins of SBF.

3. Conclusions

The ADF and NDF contents and WHC of the SBF were high while cation exchange capacity was found to be moderate. These results proved that SBF is a good source of dietary fiber. The sorption isotherms of SBF were not affected by the particle size of the SBF. Among the models studied, the GAB and the Oswin equations were the best ones in fitting the sorption data for the granulated SBF. The sorption data for the SBF indicates that it has physiologically suitable dietary fiber properties. However, the findings presented here are not meant to be directly applied to predict the physiological

efficiency of SBF. The interrelation between the sorption data and physiological properties of SBF needs to be studied in more detail.

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SEPARATION OF PAPRIKA COMPONENTS USING DENSE CO_2

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The separation of aromatic and colouring components of paprika with supercritical CO_2 as solvent was examined.

The literature solubility data of some paprika components were considered and the operating parameters for a semicontinuous extraction of ground paprika were determined. It was observed, that the selectivity of CO_2 is higher at lower temperature, i.e. at 40 °C. The optimal operating pressure for the extraction of aromatic components from paprika is 15 MPa and for the separation of colouring components the pressure is 40 MPa. Under these conditions the obtained extract was of high colour units (CU) value (180 000), the yield was 1.9%. The mass of CO_2 needed for extraction of aromatic components per kg of raw material was determined and is approximately 51.4 kg CO_2 per kg of paprika.

In the case of continuous countercurrent concentration of commercially available paprika oleoresins, the yield of extraction was 40% at 40 °C and 15 MPa and the CU value of obtained oleoresin, which was free of organic solvents, was 130 000.

Keywords: dense carbon dioxide, extraction, paprika, process parameters

Paprika (*Capsicum annuum*) is one of the oldest and most important source of natural colourants used in the food and cosmetic industries. Red colour of paprika is caused by lipophylic carotenoids, which are partially esterified with fatty acids. The main carotenoid is capsanthin. Hot components of paprika present various amides with common name capsaicinoids. Among them the most important component is alkaloid capsaicin. Besides hot components characteristic taste of paprika is given by etheric oils and glucose and saccharose. Fresh paprika contains 4 to 6 times more vitamin C than lemon juice, but dried and ground fruit contains greater amount of ascorbic acid no more. Paprika contains also flavonglycosides, fatty acids and esters (linoleic, palmitic, stearic, oleic, myristic) and vitamin E as natural antioxidant.

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Liquid extract of paprika is called paprika oleoresin. It is intensively red coloured viscous oil. It is highly desirable that oleoresin should not taste and smell on paprika. To obtain oleoresin with such characteristic the extraction process with dense CO_2 as solvent was examined.

The extraction process of paprika with organic solvents has shown some disadvantages (solvents can not be completely removed, high temperatures of destillation can cause extract denaturation, extracts contain by-products such as fatty acids, capsaicin, etc.) which were minimised using supercritical fluids (SCF) as an extraction solvent.

A semicontinuous process for the production of paprika oleoresin from ground paprika with SC CO_2 is relatively well described in literature (COENEN et al., 1982; COENEN & KRIEGEL, 1983; COENEN & HAGEN, 1983; KNEZ et al., 1991; KNEZ & ŠKERGET, 1994). Extraction of ground paprika is usually performed in two steps:

1st step – extraction of aromatic components at lower operating conditions due to higher solubility in SC CO_2;

2nd step – extraction of colouring components at higher pressure.

The extraction of paprika can be carried out also at maximal pressure, where all components are extracted. The obtained product is then fractionated at different operating parameters in two separation steps into aromatic and colouring components.

The obtained colouring extract is free of organic solvents and of high colour units value (CU), which could be varied dependently of operating parameters.

Described processes have some disadvantages, which are:

- a ratio between mass of CO_2 used for extraction and mass of paprika extracted is very high,
- mass of obtained colouring extract per kg of paprika is low,
- operating costs of the process are very high due to low yield,
- CU value of the obtained extract depends on the quality of raw material. Under the same operating conditions obtained extracts can have different CU values, depending on quality of raw material, therefore, the operating conditions must be optimised for every raw material separately.

In order to avoid above mentioned disadvantages, a new continuous countercurrent concentration process of paprika oleoresin with lower CU value, obtained by conventional solvent extraction with organic solvents was examined.

For the design of SCF extraction process for a substance, knowledge about the equilibrium solubility data of contained components are highly important. The solubility data for some paprika components in CO_2 can be found in literature and are summarised in Table 1. In the present work the solubility data of paprika components were studied and operating parameters for separation of aromatic and colouring paprika components were determined.

Table 1

Solubility data of paprika components in CO_2

System	Conditions	Density of CO_2 (kg m^{-3})	Literature
β-Carotene – CO_2	20–50 MPa, 313–343 K	660.3–992.3	CYGNAROWICZ et al. (1990)
	9.8–29.8 MPa, 308–323 K	363.6–929.5	SAKAKI (1992)
	5–50 MPa, 288–328 K	825.0–1063.0	JAY and STEYTLER (1992)
	10–30 MPa, 298–313 K	633.1–967.7	ŠKERGET et al. (1995)
	20–35 MPa, 313–353 K	594.2–935.4	JOHANNSEN and BRUNNER (1997)
Capsaicin –CO_2	7–40 MPa, 298–333 K	155.6–1005.1	KNEZ and STEINER (1992)
β-Carotene–capsaicin–CO_2	10–31.2 MPa, 298–313 K	633.1–972.8	ŠKERGET and KNEZ (1997)
Oleic acid – CO_2	10.1–25.3 MPa, 313–333 K	296.8–883.1	CHRASTIL (1982)
	7.1–28.8 MPa, 313–333 K	159.0–904.7	ZOU et al. (1990)
	13.8–27.6 MPa, 313–333 K	554.1–897.7	MAHESHWARI et al. (1992)
	10.2–30 MPa, 313–353 K	259.6–910.5	BHARATH et al. (1992)
Stearic acid – CO_2	10.1–25.3 MPa, 313–333 K	296.8–883.1	CHRASTIL (1982)
	13.8–41.2 MPa, 308–328 K	612.5–977.8	MAHESHWARI et al. (1992)
Linoleic acid – CO_2	6.3–27.1 MPa, 313–333 K	133.7–894.7	ZOU et al. (1990)
	13.8–27.6 MPa, 313–333 K	554.1–897.7	MAHESHWARI et al. (1992)
Palmitic acid – CO_2	13.8–41.1 MPa, 308–328 K	612.5–978.5	MAHESHWARI et al. (1992)
Myristic acid – CO_2	13.9–41.9 MPa, 308–333 K	559.1–980.3	MAHESHWARI et al. (1992)
Lauric acid – CO_2	13.9–26.9 MPa, 308–318 K	720.0–914.0	MAHESHWARI et al. (1992)

1. Materials and methods

1.1. Materials

β-Carotene with a minimum purity of 95% was obtained from Sigma Chemical (St.Louis, Mo. USA), Cat. No. C-9750. Capsaicin with a minimum purity of 98% was obtained from Sigma Chemical (St. Louis, Mo. USA), Cat. No. M-2028. Chloroform was 99% pure and was obtained from Kemika (Zagreb, CRO). CO_2 was supplied by Linde (Celje, SLO) and was 99.97% pure.

Paprika oleoresin with CU value 80 000 was obtained from Campania Industrial del Lukus, S. A. Larache, Marocco. Ground paprika was obtained from Droga (Portorož, SLO).

1.2. Methods

1.2.1. Extraction. Semi batch experiments were performed on an apparatus produced by the company UHDE-GmbH-Hagen BRD. The technical data of the plant are:

– the volume of the semi batch operated extractors V_{C1}=4 l and V_{C2}=0.5 l
– the maximal working pressure and temperature are 50 MPa and 120 °C.

To this equipment two continuously operated countercurrent columns were built. Column KO1 of active height of 3 m has a diameter of 52 mm, the maximal working pressure and temperature are 30 MPa and 100 °C. Column KO2 is 2.5 m high and has a diameter of 25 mm. The maximal working pressure and temperature are 50 MPa and 100 °C. The flow rate of CO_2 can be varied between 5 and 33 kg h^{-1}, that of the liquid phase between 0.05 and 20 kg h^{-1}.

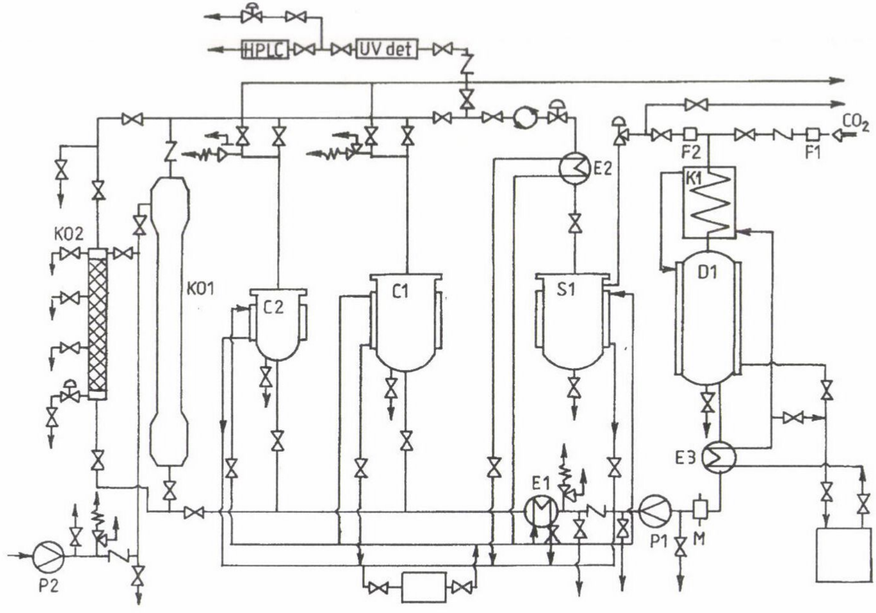

Fig. 1. Apparatus for supercritical extraction. C: autoclave; D: reservoir; E: heat exchanger; F: filter; K: condenser; KO: column; M: flow meter; P: pump; S: separator

The apparatus for extraction is presented in Fig. 1. CO_2 flows through the filter Fl and condenser K1 where it is cooled and condensed into the reservoir D1. From reservoir it flows through the heat exchanger E3, where it is additionally cooled to 0 °C, to the high pressure membrane pump P1. Pump P1 brings CO_2 to operating pressure and pumps the fluid through the heat exchanger where it is heated to the operating temperature. Then it flows to the extractor C1 or C2 or to one of the columns KO1 or KO2.

In case of semicontinuous extraction one of the extractors C1 or C2, which are equipped with the heating jackets, is preliminarly loaded with an amount of raw material. The solute is solubilized in solvent (CO_2). The separation is carried out in the separator S1, where with help of the expansion valve and with heating in the heat exchanger E2 the two phase area is attained. The solute can be taken with help of the valve at the bottom of separator. The remained CO_2 flows through the filter F2 to the condenser and reservoir from where it is recycled to the process. This procedure is repeated until the desired degree of extraction is reached.

The continuous extraction is carried out in one of the columns. The liquid sample flows with the help of the piston pump to the top of the column where it comes in contact with CO_2 in countercurrent flow. Both columns are packed with Sulzer packing. The column KO1 has the sampling valve at the bottom, the column KO2 has valves placed on different heights of the column.

1.2.2. Analyses of samples

1.2.2.1. Gravimetrical analysis – Sample taken from the bottom of separator was weighed (±0.01 g) and yield of extraction was calculated:

$$\text{yield} = \frac{\text{mass of extract} \cdot 100}{\text{mass of paprika}}$$

1.2.2.2. CU value determination – Method MSD10 was used: 1 g of oleoresin was weighed and solubilized in acetone in 100 ml volumetric flask. One ml of the solution was transferred into 100 ml volumetric flask and diluted to the mark with acetone. Absorbance of this solution was measured with spectrophotometer at 462 nm and CU value was calculated as follows:

$$\text{CU} = \frac{A(462\,\text{nm}) \cdot 65000}{\text{mass of oleoresin (g)}}$$

2. Results and discussion

Experiments of semicontinuous extraction of paprika at different operating conditions are presented in Table 2, Figs 2 and 3. At the first step of extraction of aromatic components (Fig. 2) it can be observed, that at 9 MPa and constant flow of CO_2 the change in temperature has no big influence on the amount of extract. At 40 °C the amount of extract is increasing with increasing pressure, the highest amount of extract was obtained under the conditions 15 MPa and 40 °C. In the second step (extraction of colouring components) the remaining paprika is extracted at 40 °C and 40 MPa. The results are presented in Fig. 3. In that case, when the aromatic components were previously (first step) extracted at 15 MPa and 40 °C, the yield in the second step is much lower, only 1.5% compared to the extraction when aromatic components were extracted at 9 MPa and the same temperature. The yield in second step was in that case 6%. On the other hand, the CU value for the obtained colouring extract is, in the case of higher pressure in the first step of extraction, much higher (190 000 CU) compared to that obtained at lower pressure (40 000 CU). Further, such extract is also much more viscous.

Table 2

Operating parameters and results of semicontinuous extraction of paprika

Extraction run		1	2	3	4	5
	t (h)	6	6	6	6	6
1st step	P_e (MPa)	15	15	9	9	9
aromatic	T_e (°C)	40	42	60	40	20
components	P_s (MPa)	5.5–6.5	5.5–6.5	5.5–6.5	5.5–6.5	5.5–6.5
	T_s (°C)	40	40	40	40	40
	t (h)	6	6	6	6	6
2nd step	P_e (MPa)	40	40	40	40	40
colouring	T_e (°C)	40	40	40	40	40
components	P_s (MPa)	5.5–6.5	5.5–6.5	5.5–6.5	5.5–6.5	5.5–6.5
	T_s (°C)	40	40	40	40	40
Yield (%)	aromatic components	11.5	12.1	6.1	5.1	4.7
	colouring components	1.7	1.9	6.2	6.6	7.0
CU	oleoresin	190 000	180 000	43 000	40 000	41 000

For all extraction runs: Φ=28.25 kg CO_2 h^{-1}, mass of paprika extracted = 1.65 kg
Index: e: extraction, s: separation

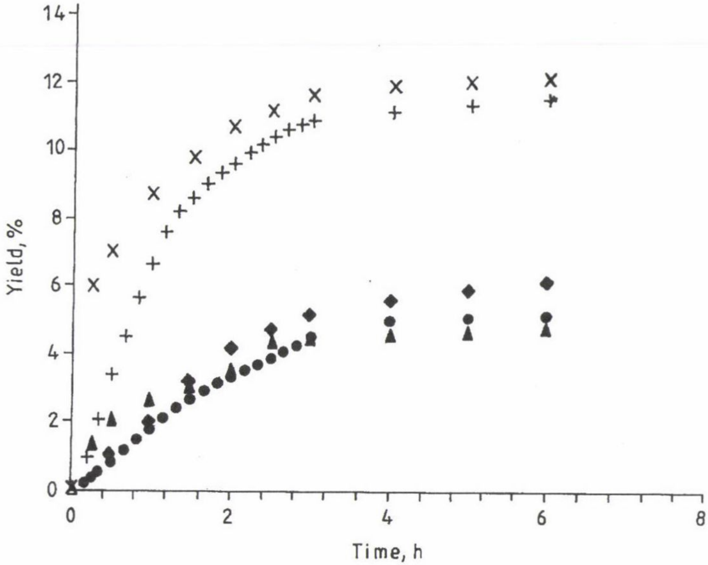

Fig. 2. Kinetics of semicontinuous extraction of paprika: 1st step extraction of aromatic components (yield =100 mass of extract/mass of paprika). ◆: 60 °C, 9 MPa; ●: 40 °C, 9 MPa; ▲: 20 °C, 9 MPa; x: 42 °C, 15 MPa; +: 40 °C, 15 MPa

The results of extraction can be explained with the solubility data of paprika components from the literature (Table 1).

From the solubility data of β-carotene and capsaicin in the ternary system β-carotene – capsaicin – CO_2 (ŠKERGET & KNEZ, 1997) the extraction ratio K is defined as:

$$K = \frac{[y_{C2} / y_{C1}]_{\text{SCF phase}}}{[y_{C2} / y_{C1}]_{\text{solid phase}}}$$

K was calculated and is presented in Table 3 and Fig. 4. It can be observed that the extraction ratio decreases with increasing the pressure at 40 °C independently of mass ratio of both components in the mixture.

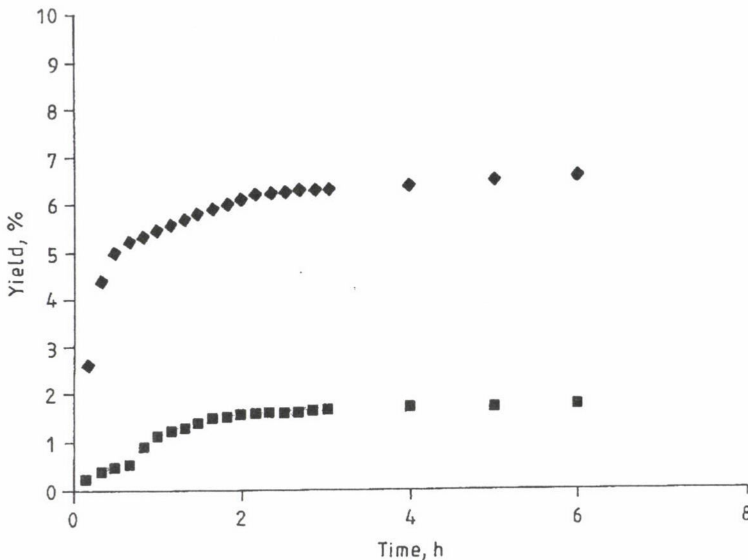

Fig. 3. Kinetics of semicontinuous extraction of paprika: 2nd step extraction of colouring components (yield =100 mass of extract/mass of paprika). ◆: 1st step at 40 °C and 9 MPa; ■: 1st step at 40 °C and 15 MPa

Further, the solubility of oleic and linoleic acid in SC CO_2 increases with pressure and decreases with temperature (ZOU et al., 1990; BHARATH et al., 1992; MAHESHWARI et al., 1992). The solubility of palmitic, stearic and myristic acids increases with pressure and temperature (MAHESHWARI et al., 1992). It can be concluded, that with higher pressure in the first step of extraction, higher amounts of fatty acids and esters are extracted, i.e. separated from colouring components. Therefore, colouring extract obtained in the second step is much more viscous and is of higher CU value.

From the extraction experiments of paprika, the mass of CO_2, needed for extraction of aromatic components per kg of raw material was calculated. From kinetic plot of extraction (Fig. 2) it can be observed, that after 3 h of extraction the yield doesn't change significantly and the mass of CO_2 needed for extraction of aromatic components is then 51.4 kg per kg of paprika. This value is close to the theoretically calculated value from the solubility measurements of capsaicin in binary solid mixture with carotene in CO_2. Solubility of capsaicin at 40 °C and 15 MPa (Table 3) is approximately $3.2 \cdot 10^{-5}$ mole fraction. Assuming that the content of all capsaicinoids in paprika is 0.9 weight % and that capsaicin represents all capsaicinoids in paprika, the

theoretical mass of CO_2 needed for extraction of hot components from paprika can be calculated and is 40.5 kg per kg of paprika.

Operating parameters and results of continuous extraction (concentration) of paprika oleoresin obtained by organic solvents extraction are summarised in Table 4. The fractionation-concentration of paprika oleoresin with CU value of 80 000 was carried out in the column KO2. The concentration was performed at 40 °C and pressures of 9 and 15 MPa. In both experiments the yield of extraction was much higher (55% and 40%, respectively) than in the case of semicontinuous extraction of paprika. The obtained extracts have higher CU value than inlet liquid sample (110 000 and 130 000, respectively) and were free of organic solvents.

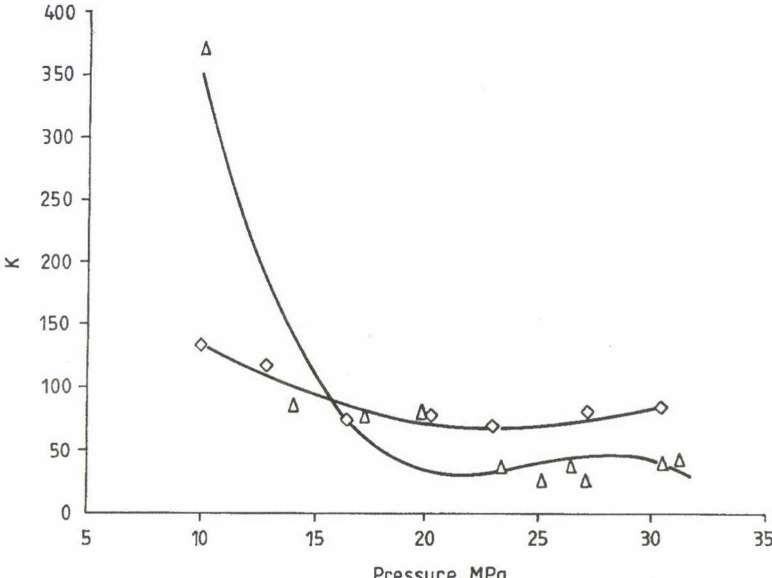

Fig. 4. Extraction ratios K of β-carotene and capsaicin at 40 °C as function of pressure. Δ: 40 °C, $w_{C1}:w_{C2}=1:1$; ◊: 40 °C, $w_{C1}:w_{C2}=2:1$

Table 3

The equilibrium solubility data for ternary system β-carotene (C1) – capsaicin (C2) – CO_2 at different mass ratios (w) of solid components

$w_{C1}:w_{C2}$ = 1:1							
25 °C				40 °C			
P (MPa)	$y_{C1}\times10^7$	$y_{C2}\times10^5$	K^a	P (MPa)	$y_{C1}\times10^7$	$y_{C2}\times10^5$	K^a
10.7	3.028	1.958	36.79	10.0	0.658	4.240	366.32
14.2	3.048	1.967	36.72	14.1	4.120	6.133	84.69
16.7	3.130	1.860	33.82	17.2	4.127	5.692	78.46
18.5	2.949	2.284	44.05	19.9	5.590	7.735	78.72
21.9	2.446	3.543	82.41	23.3	4.043	2.586	36.39
26.0	2.736	3.311	68.85	25.2	5.801	2.610	25.60
				26.5	4.312	2.893	38.17
				27.1	5.493	2.576	26.68
				30.4	4.415	3.164	40.76
				31.2	4.774	3.540	42.18
$w_{C1}:w_{C2}$ = 1:1							
25 °C				40 °C			
P (MPa)	$y_{C1}\times10^7$	$y_{C2}\times10^5$	K^a	P (MPa)	$y_{C1}\times10^7$	$y_{C2}\times10^5$	K^a
10.1	1.898	2.630	157.65	10.0	1.941	2.252	131.98
13.0	2.855	2.756	109.83	12.9	2.914	2.946	115.01
15.9	1.892	2.771	166.66	16.4	5.139	3.267	72.32
20.2	2.948	2.853	110.09	20.3	5.950	4.013	76.74
23.5	2.690	2.860	120.97	23.0	5.134	3.016	66.84
27.6	2.405	2.646	125.18	27.2	4.563	3.178	79.24
				30.3	5.769	4.206	82.94

[a] $K=[y_{C2}/y_{C1}]_{\text{SCF phase}}/[y_{C2}/y_{C1}]_{\text{solid phase}}$

3. Conclusions

From the obtained solubility data of β-carotene and capsaicin in the ternary system β-carotene – capsaicin – CO_2, it can be concluded that at lower pressures a higher separation of both components can be achieved. The dependency of extraction ratio on the pressure confirms the results of paprika extraction experiments.

Table 4

Operating parameters and results of continuous extraction of paprika oleoresin with CU value 80 000

Extraction run		1	2
P_e (MPa)		15	9
T_e (°C)		40	40
P_s (MPa)		1	1
T_s (°C)		40	40
Φ (kg CO_2 h^{-1})		14.12	14.12
Φ (g extract h^{-1})		80	80
Yield (%)	aromatic components	60	45
	colouring components	40	55
CU	oleoresin	130 000	110 000

Index: e: extraction, s: separation

It was found that the temperature has no big influence on the amount of extract and with higher pressure the amount of extractable substances in the first step was increased due to higher solubility of fatty acids and esters in CO_2. The paprika oleoresin, obtained in the second step of extraction, is in that case more viscous and has higher CU value, but the yield of extraction is much lower compared to extraction where aromatic components are previously removed at lower pressure.

The optimal operating pressure for the extraction of aromatic components at temperature of 40 °C is 15 MPa and for the separation of colouring components the pressure is 40 MPa. Under these conditions the obtained extract was of high CU value (180 000), the yield was 1.9%. The yield can be increased with decreasing the pressure for extraction of aromatic components, but at the same time the CU value of oleoresin is decreased.

In the case of continuous concentration of paprika oleoresin, obtained by extraction with organic solvents, the yield was much higher compared to semicontinuous extraction of paprika. At 40 °C and 15 MPa the yield was 40% and CU value of the obtained oleoresin was 130 000. Oleoresin obtained after treatment with CO_2 doesn't contain organic solvents, because they were removed by CO_2. Advantages of continuous countercurrent extraction of paprika oleoresin over semicontinuous extraction of paprika are:

- yield of extraction is higher, time needed for extraction is lower,
- operating parameters of the process are lower,

- CU value of extract can be varied with pressure, temperature and flow of CO_2 or extract and the CU value of the inlet extract,
- obtained extracts are free of organic solvents, which are removed with extraction.

List of symbols

CU: color units
K: extraction ratio
y: mole fraction
w: mass ratio
Φ: mass flow (kg h^{-1})

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DETERMINATION OF MONOTERPENOLS IN WINE USING HRGC WITH ON-COLUMN INJECTION

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Different ratios of hexane–chloroform and hexane–acetone mixtures in different volumes were examinated in order to obtain the best extraction of free and bound terpenols in wine by a sorbent cartridge C_{18} (100 mg). The best recovery was obtained using 3 ml of 1:2 hexane–chloroform mixture. The boiling temperature of this solvent mixture permits the on-column injection of the sample.

Using the on-column injection, the usual concentration phase of the sample required by other methods is eliminated and consequently small quantities of sample and solvent are needed. This method is rapid and inexpensive.

Keywords: HRGC, on-colum injection, C_{18} cartridge, terpenols, wine

Aromatic grapes can be distinguished from grapes with simple flavour because of the presence of monoterpenoid compounds in the fruit. These compounds are present partly as free, volatile forms and partly as glycosidically-bound non volatile precursors (BOIDRON et al., 1989; CORDONNIER et al., 1986; DI STEFANO, 1982; GUNATA et al, 1985a,b, 1986; USSEGLIO-TOMASSET, 1987; WILLIAMS et al., 1980a,b). These glycosidic, non-volatile precursors can give rise to flavoring aglycones when acidic and/or enzymic hydrolytic reactions take place (GUNATA et al., 1990, 1992; WILLIAMS et al., 1981, 1982).

Many methods have been developed for extraction and analysis of terpenols. RAPP and co-workers (1976) suggested liquid–liquid extraction, but this technique is both time consuming and requires large volumes of solvents. Solid phase extraction (GUNATA, 1984; DIRNINGER et al., 1993) has also been used, but it is expensive and a long time is required for resin-conditioning and elution. The method suggested by DI STEFANO (1991) is very interesting but it has some disadvantages: it requires a large quantity of wine sample and consequently of solvent for cartridge elution; the concentration phase, using a Vigreux column, limits the reproducibility of the method

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and the HRGC analysis by split injection should be better using a small volume of sample with a high concentration of compounds.

The method suggested in this paper provides the use of small quantities of sample and solvent for the extraction of terpenols. HRGC analysis provides on-column injection and consequently the concentration phase of the sample is eliminated and high volume of sample with low concentration can be injected.

1. Materials and methods

1.1. Reagents

The solvents and the reagents used in this study were RS grade (Carlo Erba, Milano, Italy). Monoterpenol standards: linalool, geraniol, nerol, β-citronellol and α-terpineol (97% purity) were obtained from Aldrich Chemie (Steinheim, Germany). The extraction was carried out using C_{18} cartridge (EC, 100 mg) (IST. Mid Glamorgan, U.K.). Commercial glycosidases produced by *Aspergillus niger* (Pectasin FL) from Genecor International (Verona, Italy) was used to release aglycones from the glycosidically bound forms.

1.2. Extraction method

A sample of 2 ml of wine was added to 6 ml of citrate/Na-phosphate buffer, pH 7.0 ($C_6H_8O_7$ 0.1 M + $Na_2HPO_4 \cdot 2H_2O$ 0.2 M; volume ratio 0.21). 1-nonanol (100 µl of 0.14 mM solution in ethanol) was added as internal standard. The wine sample was applied to a C_{18} cartridge, which had been previously washed with 1 ml of methanol and then with 2 ml of citrate/Na-phosphate buffer, pH 7.0. Water-soluble compounds were eluted with 2 ml of citrate/Na-phosphate buffer, pH 7.0 and then the cartridge was dried. The free and bound fractions of terpenols were retained by the cartridge.

The free fraction was eluted with 3 ml of a 1:2 chloroform:hexane mixture, after each 1.5 ml the cartridge was dried. The extract was dried with Na_2SO_4 and after the addition of 1-dodecanol (100 µl of 0.11 mM solution in ethanol), the solvent was partially removed under a stream of nitrogen.

The bound fraction was eluted with 5 ml of methanol, after each 2.5 ml the cartridge was dried. Methanol was eliminated using a vacuum centrifuge (Univapo 100 H, UNIEQUIP, Martinsried, Germany). The residue added with 3 ml of citrate/Na-phosphate buffer, pH 5.0 ($C_6H_8O_7$ 0.1 M + $Na_2HPO_4 \cdot 2H_2O$ 0.2 M; volume ratio 0.94) and 200 µl of Pectasin FL was placed in a water-bath for 15 h at 38 °C. After addition of 1-nonanol (100 µl of 0.14 mM solution in ethanol) as internal standard, the free terpenols were liquid–liquid extracted twice with 1 ml of a 1:2 chloroform:hexane

mixture. The organic phase was dried with Na_2SO_4 and the sample, added with 1-dodecanol (100 µl of 0.11 mM solution in ethanol), was concentrated using a stream of nitrogen.

1.3. Equipment and chromatographic conditions

HRGC determination of monoterpenols were performed with a Carlo Erba GC 8560 Mega 2 (Milan, Italy) equipped with on-column injector and FID using a linear speed of 35 cm sec^{-1} (flow rate: 1.7 ml min^{-1}). The column used was a Supelcowax (60 m×0.32 mm i.d., film tickness: 0.25 µm). The oven temperature was programmed as follows: initial temperature 65 °C (3 min isothermal), then 20 °C min^{-1} to 80 °C, from 80 °C to 165 °C with rate 3 °C min^{-1}, and to 230 °C with rate 10 °C min^{-1} (10 min isothermal). The detector temperature was 240 °C.

2. Results and discussion

A model solution containing ethanol 10% vol., 5 g l^{-1} of tartaric acid and 1 mg of linalol, geraniol, nerol, β-citronellol, α-terpineol and 1-nonanol (as internal standard) adjusted to pH 3.2 underwent a recovery study. Different ratios of hexane–chloroform mixture (1:2 v/v and 1:3 v/v) and hexane–acetone mixture (1:3 v/v and 1:4 v/v) in different volumes (l, 2 and 3 ml) were used as eluents in the extraction phase. Tables 1 and 2 show the results of the recovery study. The best recovery was obtained using 3 ml of 1:2 hexane–chloroform mixture. The boiling temperature of this solvent mixture permits the on-column injection of the sample without any problem for the equipment cooling.

The repeatability of the extraction method and HRGC analysis was tested using a Muscat wine sample prepared and analysed six times (Fig. 1). The results reported in Table 3 show that the relative standard deviations (RSD%) are good and lower than 8%. It was verified that free and bound terpenols are retained entirely on the cartridge during wine sample application and bound fractions are not eluted during the fractionation of the free fraction.

Table 1

Results of recovery study of terpenols in model solution passed through C_{18} cartridge and eluted with different ratios of hexane:chloroform mixture

Compound	2 ml 1:3		3 ml 1:3		1 ml 1:2		2 ml 1:2		3 ml 1:2	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Linalool	51.4	5.0	57.0	6.2	80.7	3.5	84.9	4.2	90.0	3.5
α-Terpineol	88.1	8.6	99.9	4.3	103.5	6.0	98.6	5.2	107.5	4.6
β-Citronellol	59.2	9.2	62.0	8.4	81.1	6.2	89.2	5.1	87.4	3.2
Nerol	43.6	8.2	45.1	6.1	83.5	7.5	88.2	6.0	91.0	4.3
Geraniol	60.5	9.3	64.0	9.0	97.3	5.3	100.5	5.1	105.9	5.5
Nonanol (IS)	60.6	7.0	61.3	5.2	75.9	5.1	80.8	4.1	83.3	4.0

Each value represents the mean of three replications
RSD: relative standard deviation

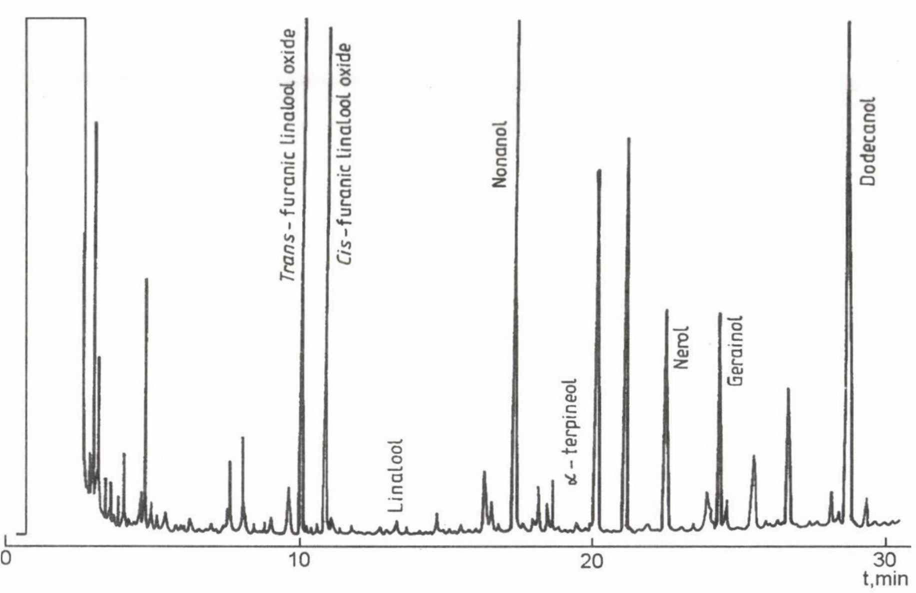

Fig. 1. Chromatogram of bound monoterpenols in commercial aromatic wine

Table 2

Results of recovery study of terpenols in model solution passed through C_{18} cartridge and eluted with different ratios of hexane:acetone mixture

Compound	2 ml 1:4		3 ml 1:4		1 ml 1:3		2 ml 1:3		3 ml 1:3	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Linalool	24.7	16.5	56.9	8.1	81.1	7.3	80.1	4.1	82.4	6.2
α-Terpineol	55.1	9.0	89.4	7.2	95.4	8.0	96.7	8.5	106.1	7.1
β-Citronellol	34.1	6.1	53.2	7.3	83.7	5.0	83.6	6.2	89.3	5.4
Nerol	25.8	12.4	38.9	12.2	83.3	11.3	82.8	4.7	84.8	3.1
Geraniol	37.7	9.5	55.1	8.3	97.7	5.4	96.4	5.1	104.1	7.0
Nonanol (IS)	32.6	11.2	57.0	13.1	79.9	9.1	75.6	10.2	76.0	9.5

Each value represents the mean of three replications
RSD: relative standard deviation

Table 3

Replicate analysis (n= 6) of Muscat wine

Compound	Free terpenols		Bound terpenols	
	Mean[a]	RSD (%)	Mean[a]	RSD (%)
Linalool	284	5.4	420	6.2
α-Terpineol	905	6.0	53	4.6
Nerol	n.d.	–	178	7.1
Geraniol	267	6.1	474	6.1
Trans-furanic linalool ox	90	2.3	420	3.2
Cis-furanic linalool ox	60	2.4	315	4.1

[a] expressed as ppb of 1-dodecanol
n.d.: not detectable
RSD: relative standard deviation

3. Conclusions

The suggested method is easy to use and has the advantage of using small quantities of sample and solvent. In addition, the on-column injection permits to eliminate the concentration phase of the sample. About 90 min are needed for sample preparation and 30 min for a chromatographic run. This method is rapid and inexpensive and can be used as routine analysis.

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THE EFFECTS OF SELENIUM SUPPLEMENTATION IN FEEDING OF LAMBS

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Selenium is an essential trace element for both man and animals whose dietary intake is not sufficient in many parts of the world. Some countries with an originally low dietary selenium intake have instituted fertilisation programmes in order to raise cereal selenium concentrations and thus boost dietary intakes.

An alternative approach to improve the daily dietary selenium intake would be to increase the selenium concentration of carcass meat by supplementation of meat animals for a limited period prior to slaughter. Thus, animal diseases related to selenium deficiency can also be prevented.

Sixteen Scottish Blackface lambs were stratified according to liveweight and then randomly allocated to one of four groups: unsupplemented, 3.5, 7.0 or 10.5 mg sodium selenite/head/week. After 14 weeks the lambs were slaughtered and samples of shoulder and thigh muscle, liver and kidney were obtained for analysis. All three treatments caused an increase in whole blood glutathion peroxidase and plasma selenium concentrations over controls. Shoulder, thigh and liver selenium concentration exhibited a dose response relationship to treatment. Kidney selenium concentrations were unaffected by treatment.

As it is well documented that selenium and vitamin E play a complementary role as antioxidants, plasma vitamin E concentrations were also determined at the beginning, in the middle and at the end of the experiment. They showed a continuous decline probably due to the low dietary vitamin E intake of the lambs during the trial. Tissue vitamin E concentrations were largely unaffected by the dose of selenium supplementation.

It can be concluded that supplementation of meat animals with selenium is an effective means to increase human dietary intakes of the element.

Keywords: selenium, lambs, human dietary intake, glutathione peroxidase, selenium supplementation

Selenium (Se) has been recognized as an essential trace element since it had been demonstrated (SCHWARTZ & FOLTZ, 1957) that it prevents dietary liver degeneration in rats fed a vitamin E deficient diet. For humans, epidemiological studies, especially from

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Finland, have linked a lower Se status to an increased risk of cancer (SALONEN et al., 1985). Associations were established between cardiovascular death, myocardial infarction and serum Se by several authors (KORPELA et al., 1989). Selenium exerts its effects, by our present knowledge, mainly through the antioxidant properties of the selenoenzyme glutathione peroxidase (GSHPx) (EC 1.11.1.9). Although, several other selenoenzymes have been identified in recent years, their exact way of mechanism is still unclear.

Vitamin E is another essential antioxidant which acts principally in the intracellular compartment. It plays – among others – an important part in the metabolism of arachidonic acid, and it is the precursor of a number of compounds including prostaglandins, leukotrienes, thromboxane which play a crucial role in the organism.

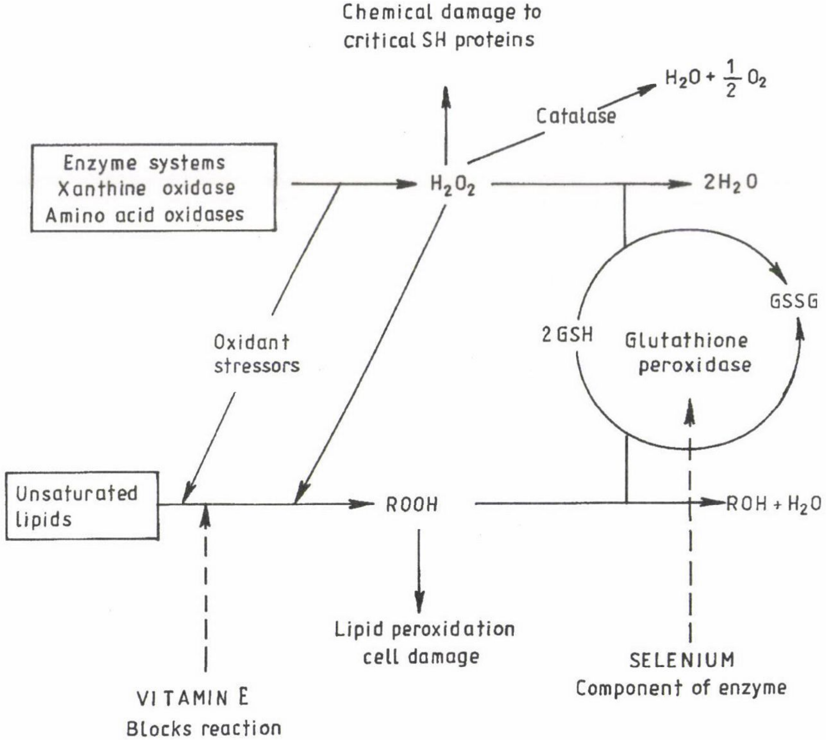

Fig. 1. Intracellular antioxidant activities of vitamin E and selenium

The biochemical action of Se and its relationships with vitamin E were not clarified until it was shown (ROTRUCK et al., 1973) that Se was an integral part of the enzyme GSHPx. HOEKSTRA (1975) then proposed a theory linking vitamin E and GSHPx in the prevention of lipid peroxidation within cell membranes (Fig. 1).

The aim of this study was to determine whether supplementation of lambs with selenium would raise the selenium content in the food chain and how the lambs themselves would respond to such treatment. The glutathione peroxidase activity of the lambs was recorded weekly throughout the experiment. Furthermore, plasma selenium and vitamin E concentrations were measured on several occasions to determine whether tissue concentrations could be predicted through this measurement.

l. Materials and methods

1.1. Animals and diets

Sixteen 4-month-old Scottish Blackface lambs weighing an average of 20.7 kg were stratified according to their live weight and randomly allocated to four different treatment groups. These were nil (treatment 1), 3.5 (treatment 2), 7.0 (treatment 3) and l0.5 mg (treatment 4) Se/head/week per os, respectively. All lambs were fed the same basal diet containing hay, milk powder, maize and barley with a basal Se concentration displayed in Table l. Blood samples were collected by jugular venepuncture weekly into heparinized vaccutainers and used for the determination of glutathione peroxidase activity. The rest of the blood was centrifuged for 15 min. at a speed of 3000 r.p.m. and then the plasma was removed and frozen.

The weight of the lambs was recorded each week.

After fourteen weeks the lambs were slaughtered and samples of shoulder, thigh, liver and kidney were retained for further analysis.

1.2. Determination of Se concentration

Sample preparation for Se determination was carried out using the nitric acid-perchloric acid-sulphuric acid digestion procedure (HERSHEY et al., 1988). Se in the digest solution was then determined by atomic absorption spectrometry via hydride generation used by the same authors.

The plasma samples were analysed as received, while the tissue samples were dried with the final result calculated in all cases on a 100% dry matter basis. The analytical method was verified by analysis of a human reference serum (Seronorm Trace Elements Serum, batch No. 010017). The results (95 $\mu g\ l^{-1}$) agreed with the certified Se level of 94.5±2 $\mu g\ l^{-1}$.

1.3. Determination of GSHPx activity

GSHPx was measured in whole blood by the method of PAGLIA & VALENTINE (1967) employing the RANSEL kit (RANDOX, Belfast). The reaction was carried out at 30 °C. Internal quality control was maintained by use of a standardized control supplied by RANDOX. External quality control was assured by comparison of results obtained by analysing identical samples at the Scottish Animal Diseases Research Institute in Edinburgh.

1.4. Determination of vitamin E concentration

Vitamin E concentration was measured by the HPLC-method reported by the Association of Official Analytical Chemists (A.O.A.C., 1990) and modified by THACKER (1993). The sample was first saponified with pirogallol-ethanol and potassium hydroxide, then extracted with petroleum ether.

For HPLC analysis a polar column was used with reverse phase. The setting for the vitamin E was 295 nm for absorption and 330 nm for emission. For verification a human whole blood standard reference sample was determined for its vitamin E concentration.

Table 1

Se concentration (μg kg^{-1}) of feed components and the Se intake of lambs

Feed component	Selenium concentration		Se intake of lambs (μg)	
	Dried feed	Fresh feed	Lambs up to 20 kg	Lambs more than 20 kg
Hay (d.m.=0.822 g kg^{-1})	47.6	39.1	6.6	6.6
Milk powder (d.m.=1.000 g kg^{-1})	169.7	169.7	15.3	15.3
Maize (d.m.=0.903 g kg^{-1})	47.0	42.4	2.5	3.8
Barley (d.m.=0.899 g kg^{-1})	13.1	11.8	2.0	2.5
Total	–	–	26.4	28.2

d.m.: dry matter

1.5. Statistical analysis

The differences between means of groups were tested by the two-tailed two sample Students's *t* test. Correlation coefficients were obtained and the significance between them tested. All statistical analysis was performed by the Minitab 7 and Excel 4.0 packages.

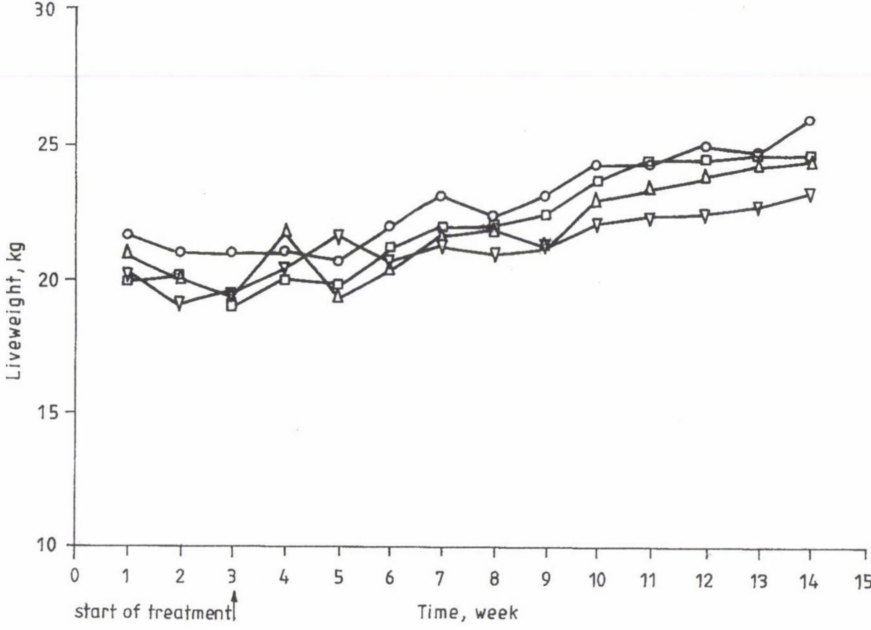

Fig. 2. Change in lamb liveweight of the different treatment groups with time. O: Treatment 1; Δ: treatment 2; ∇: treatment 3; □: treatment 4

2. Results

2.1. Lamb liveweight

The results of the weekly weighing of the lambs are displayed in Fig. 2. No significant differences in lamb liveweight among the different treatment groups were recorded at any stage of the experiment.

2.2. Glutathione peroxidase activity in lamb whole blood

The weekly glutathione peroxidase (GSHPx) activity values can be seen in Fig. 3, which shows the differences according to treatment.

At the beginning of the experiment there were no significant differences among the GSHPx activities of the four treatment groups (p=0.654). After supplementing for twelve weeks the glutathione peroxidase activity of all three treated groups was significantly greater than that of the control group (p=0.0001). However, no significant

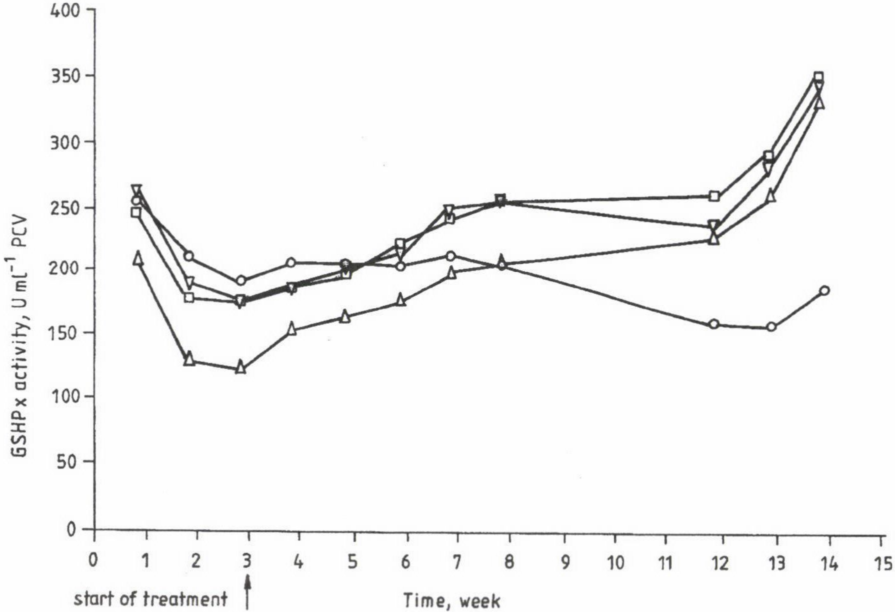

Fig. 3. Changes in lamb whole blood glutathione peroxidase activity with treatment. O: Treatment 1; Δ: treatment 2; ∇: treatment 3; □: treatment 4

differences could be found among the treated groups (p=0.772). There was a significant decline in GSHPx activity of the control group from 255 U ml^{-1} cells in the 1st week to 188 U ml^{-1} cells in the 14th week. All three treatment groups showed a significant increase in GSHPx activity from week 3, when supplementation started, to week 14 when it finished.

2.3. Se concentration of lamb plasma

The results are presented according to treatment in Fig. 4.

There was no significant difference (p=0.747) among the plasma selenium concentrations of the four groups of lambs at the beginning of the experiment. A highly significant difference in selenium concentration between the three treated groups and the control group was found after the 8th week (p=0.0001) and was maintained after 14 weeks (p=0.001). However, there were no significant differences among the three treated groups 2, 3 and 4 in the 8th week (p=0.739) nor between groups 2 and 3 in the 14th week (p=0.665). The selenium concentration in the lambs receiving treatment 4

had increased further. The selenium concentration of the untreated group (treatment 1) did not differ significantly after 8 and 14 weeks from its initial concentration (p=0.74 and 0.64, respectively). On the contrary, the treated lambs exhibited a significant increase in selenium concentration in plasma by the 8th week and this was maintained until the end of the experiment (p=0.0002, 0.0089 and 0.017 in the 8th week and p=0.0007, 0.0001 and 0.015 in the 14th week for treatments 2, 3 and 4, respectively).

2.4. Se concentration of different lamb tissues after fourteen weeks of supplementation in the control group and treated groups

The selenium concentrations found in shoulder, thigh, liver and kidney of the control and treated groups are detailed in Figs 5a and 5b.

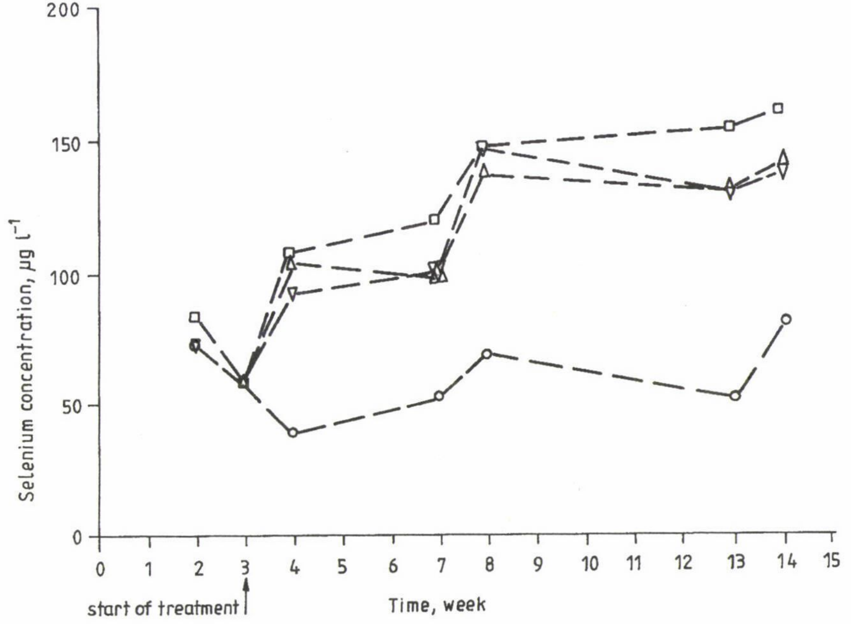

Fig. 4. Changes in lamb plasma selenium concentration with treatment. O: Treatment 1; Δ: treatment 2; ∇: treatment 3; □: treatment 4

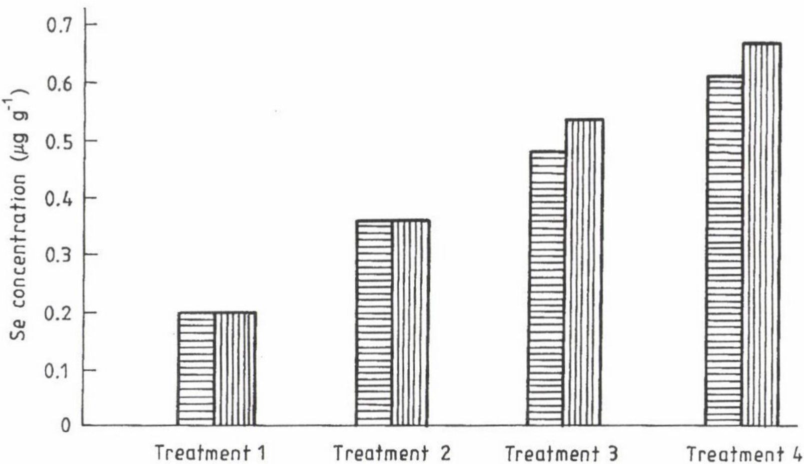

Fig. 5a. Comparison of shoulder and thigh Se concentrations in the four treatment groups. :Shoulder; : thigh

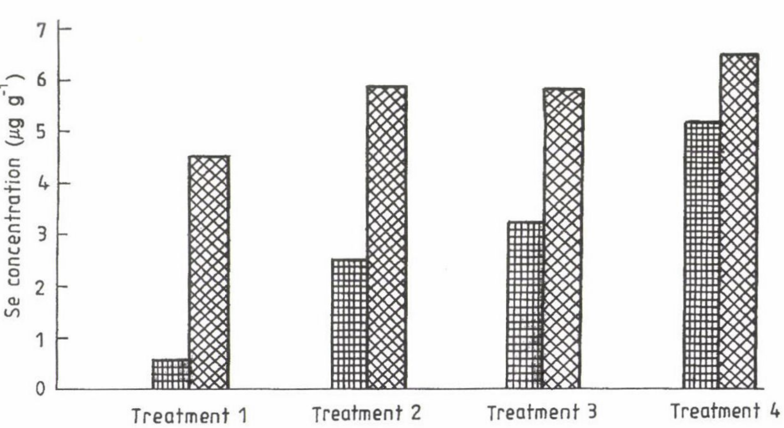

Fig. 5b. Comparison of liver and kidney Se concentrations in the four treatment groups. : Liver; : kidney

2.6. Statistical analysis

The results for the correlation between the plasma selenium and vitamin E level, as well as the glutathione peroxidase activity in whole blood of all the lambs can be seen in Table 2. No significant positive correlation could be found for week 2, 3, 4, 7, and 8. In the 13th and 14th week of the experiment the correlation increased from 0.143 to 0.752 and 0.747.

Table 2

Correlation between plasma selenium concentration, whole blood glutathione peroxidase activity and plasma vitamin E concentration over the time of the experiment

Time period	Parameters	n	r	Significance
Week 2	1. Se/GSHPx	16	0.143	–
	2. Se/vitamin E	16	–0.169	–
	3. GSHPx/vitamin E	16	–0.513	**
Week 3	Se/GSHPx	16	0.237	–
Week 4	Se/GSHPx	15	–0.092	–
Week 7	Se/GSHPx	16	0.130	–
Week 8	1. Se/GSHPx	16	0.377	–
	2. Se/vitamin E	16	0.054	–
	3. GSHPx/vitamin E	16	–0.150	–
Week 13	1. Se/GSHPx	16	0.752	***
	2. Se/vitamin E	16	–0.542	**
	3. GSHPx/vitamin E	16	–0.692	***
Week 14	Se/GSHPx	16	0.747	***
Whole period	1. Se/GSHPx	111	0.574	***
	2. Se/vitamin E	48	–0.224	-
	3. GSHPx/vitamin E	48	–0.490	***

***: P<0.01
**: P<0.05
*: P<0.1
–: no significance

The results for the correlation between lamb plasma and tissue selenium concentrations can be seen in Table 3. The correlation was statistically significant (P<0.01) in all 4 cases. It can be concluded that the plasma selenium concentration is reflected in the tissue selenium concentration.

Table 3

Correlation between plasma and tissue selenium concentrations

Parameters	n	r	Significance
Lamb plasma/Shoulder	16	0.745	***
Lamb plasma/Thigh	16	0.785	***
Lamb plasma/Liver	16	0.760	***
Lamb plasma/Kidney	16	0.707	***

*** P<0.01

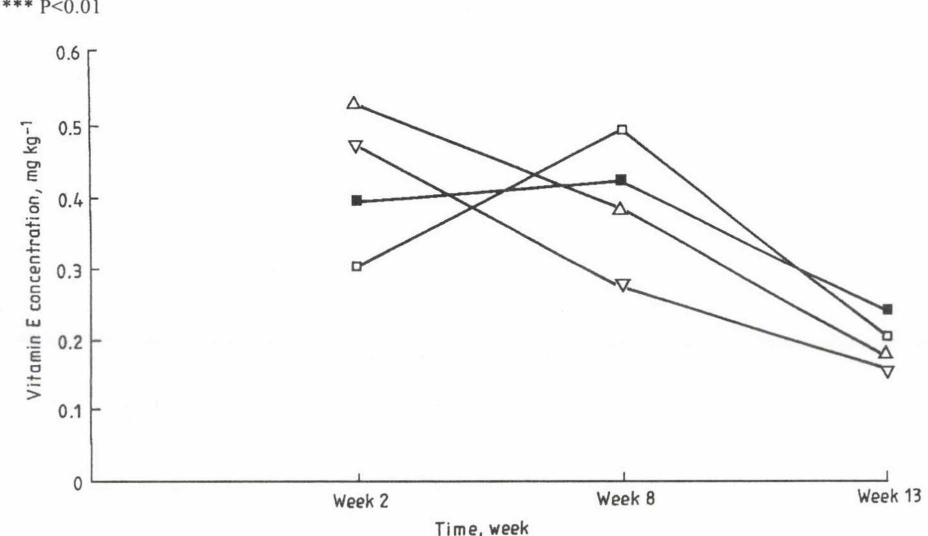

Fig. 6. Vitamin E concentration of lamb plasma according to treatment. ■: Treatment 1; Δ: treatment 2; ∇: treatment 3; □: treatment 4

2.7. Effect of Se supplementation on vitamin E concentration in lamb plasma and tissues

The vitamin E concentrations of lamb plasma of week 2, 8, and 13 can be seen in Fig. 6. These results show the continuous and marked decline of the vitamin E concentration ($P<0.05$) of lamb plasma over the time of the trial.

The vitamin E concentration of lamb tissues is displayed in Fig. 7. The vitamin E concentration of the kidney was the highest among all tissues in all treatment groups and it declined according to treatment. The differences in vitamin E concentration of

the other tissues did not show a particular pattern according to treatment and there was no significant difference between the vitamin E concentration of muscle meat and liver.

3. Discussion

There were no significant differences in growth efficiency between sheep on different dietary Se levels. These results are in accord with certain data reported in literature (McCLURE & MAHAN, 1988) when growing lambs were supplemented with 0.25 to 2.0 ppm Se. ZACHARA and co-workers (1993) also reported similar findings when supplementing lambs with 0.25, 0.41 and 0.58 ppm Se. Significant differences in growth rates between Se supplemented and control animals were only observed when pasture Se levels were below 0.03 mg kg^{-1} d.m. (GRACE, 1984).

According to the data shown in Fig. 3 there was no significant difference between the glutathione peroxidase activity of the four treatment groups at the beginning of the experiment. After 12 weeks of supplementation the glutathione peroxidase activity increased markedly, while the GSHPx activity in the untreated group dropped from a mean value of 255 to 188 U l^{-1} in whole blood. However, there was no significant difference between the GSHPx activity values for the three treated groups, thus indicating that the lowest level of supplementation (group 2) provided an adequate supply of the element for full expression of the enzyme.

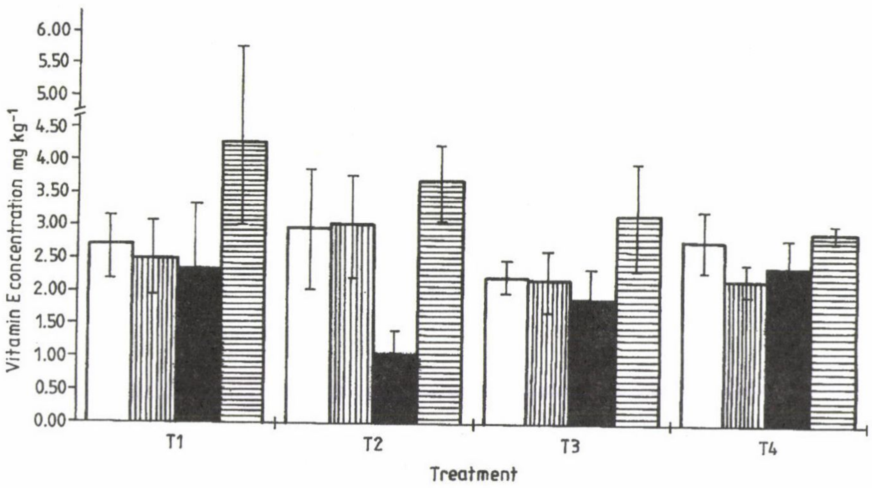

Fig. 7. Vitamin E concentrations of lamb tissues according to treatment. □: Shoulder; ▥ : thigh; ■: liver; ▤: kidney

The results shown in Fig. 4 indicate that lamb plasma selenium concentrations increased following supplementation with Se, but were unaffected by the level of supplementation. The fact that the selenium concentration in the supplemented lambs did not increase further after the 8th week leads to the conclusion that a plateau in Se concentration was quickly reached and that the plasma had already become saturated after 5 weeks of supplementation for all treatment groups. The selenium concentration in the unsupplemented lambs did not decline thus indicating that the basal diet provided sufficient selenium to maintain baseline values.

It can be seen in Fig. 5b that the kidney contained significantly higher amounts of Se than liver, and that the level of dietary supplementation did not substantially influence its concentration. These findings are in close agreement with the data reported by ZACHARA and co-workers (1993).

Shoulder, thigh and liver, however, responded linearly to treatment with a tendency for thigh muscle to take up slightly more selenium than shoulder at the higher levels of supplementation. It can be concluded that skeletal muscle of Se supplemented lambs could be a major source of selenium in the food chain.

The vitamin E concentration of lamb plasma declined markedly ($P<0.005$) over the time of the experiment. One explanation for this rather unexpected result could be an antagonistic effect of Se on vitamin E. On the contrary, theoretically a sparing effect of Se on vitamin E concentration could have been expected in the treated, Se supplemented groups. However, as the vitamin E intake of the lambs amounted only to about 50% of the recommended intake for lambs of that weight and age and was thus insufficient, this seems to be the more likely reason for the significant decrease of vitamin E concentration in lamb plasma.

The vitamin E concentrations of shoulder, thigh and liver varied from lamb to lamb within treatment, a particular pattern could not be observed. Only the vitamin E concentrations of the kidney showed a decreasing tendency as the selenium supplementation increased. This finding was not significant due to the high variation within the group. There was no interaction between the Se content and the vitamin E concentration of the tissues which confirms findings previously reported by WALSH and co-workers (1993).

The results for the correlation between plasma selenium level and glutathione peroxidase activity in whole blood seemed to be rather weak apart from the 13th and 14th week when it was 0.752 and 0.747, respectively. This may be explained by the different source of material i.e. the glutathione peroxidase activity was measured in whole blood, while the selenium concentration was determined in plasma. However, a correlation between the plasma glutathione peroxidase and plasma selenium concentration cannot always be observed. This may be owing to differences in the initial selenium status of the animals.

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CHANGES OF α-CHYMOTRYPSIN DURING ENZYMATIC PEPTIDE MODIFICATION

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We have recently elaborated a special proteinase catalysed reaction, the "enzymatic peptide modification" (EPM) for protein-tailoring and for covalent incorporation of methionine into peptide chains (HAJÓS, 1986; HAJÓS et al., 1990). Methionine incorporation during α-chymotrypsin catalysed EPM shows an optimum curve in function of the content of the added methionine ethylester in the reaction mixtures. In our present study, the maximal enrichment of Met was at the ratio of 48.7 g of methionine ethyl ester/100 g of proteolysate of soy protein isolate and thereafter the amount of the methionine incorporated decreased. A possible interpretation of this fact could be the changes in the reaction environment, which modifies the conformation of the enzyme, namely, the structure of chymotrypsin. CD (circular dichroism) data showed that conformation of chymotrypsin was modified at 37 °C with addition of methionine ethyl ester (as one of the substrates in the EPM reaction). The hydrolytic activity of α-chymotrypsin, which was measured with artificial substrate, N-benzoyltyrosine ethyl ester, was reduced with increasing amount of methionine ethyl ester.

Keywords: conformation of α-chymotrypsin, EPM (enzymatic peptide modification), soy protein isolate, methionine-enrichment of soy protein isolate

Generally, supplementing and fortifying foods with complementary proteins increase the overall nutritional and physicochemical properties of the available protein (WHITAKER & PUIGSERVER, 1982; KIM et al., 1988). Nowadays, in addition to these values of food as described above, new foods or food ingredients, which have the potent physiological functions, are in highlight as the third function of foods. Many efforts are being put to screen and develop novel foods which may have hypoallergenic and anticarcinogen effect (SCHMIDL, 1992). Originally, incorporating the essential amino acids such as methionine and lysine have been studied to improve the nutritional value of proteolytic hydrolysates of food proteins (HAJÓS & HALÁSZ, 1982; HAJÓS et al., 1988), and to improve the nutritive value or reduce the antinutritive character of proteins (CORDLE, 1994, GELENCSÉR et al., 1994, HAJÓS et al., 1996).

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Among the several useful methods to incorporate essential amino acids covalently into peptide chains, enzyme-catalysed modification has been used most effectively due to the high specificity of the appropriate protease (ASO et al., 1977; 1985; SUKAN & ANDREW, 1982; HAJÓS et al., 1990). According to previous results (HAJÓS et al., 1988; 1990), the amount of the covalently incorporated methionine during the actual EPM reaction showed an optimum curve as a function of the methionine ethyl ester content in the reaction mixture. The methionine enrichment was gradually increased with increasing the methionine ethyl ester up to a certain point of the substrate concentration. However, smaller amount of methionine was incorporated when an excessive amount of methionine ethyl ester (over 48.7 g of methionine ethyl ester per 100 g of hydrolysate) was added to the reaction mixtures. It is not unusual that the amount of an enzyme-catalysed product is decreased with the increase of the substrate concentration (LEHNINGER, 1975). In the present study, we have investigated the reasons why the enzyme catalysis was modified during the reaction, assuming that activity of chymotrypsin can be changed by a given amount of methionine ethyl ester in the reaction mixture. It was supposed that probably one of the substrates (the methionine ethyl ester) binds to the site different from the active one of the enzyme and results in altering the enzyme activity by changing the conformation. We also considered the major substrate and product such as soy protein hydrolysate and methionine enriched soy protein products, which could change the structure of α-chymotrypsin and affect the reaction activity like substrate or product inhibition. However, there is no report on substrate and product inhibition for a serine protease like chymotrypsin or trypsin, so we concentrated on the effects of the methionine ethyl ester on the structural change and incorporation rate of methionine during the EPM. In fact, it is not easy to investigate the effects of the main substrate and product because in this case the major substrate and the product were composed of a great number of peptides (HAJÓS et al., 1989). Anyhow, the same concentration of peptide hydrolysate was applied to reduce this factor in this study.

In our work, supposing that α-chymotrypsin catalysis in the peptide mixture could be suppressed by an excess of methionine ethyl ester, we attempted to elucidate the potent effect of substrate or product on the α-chymotrypsin catalysis for covalent methionine enrichment into the peptide hydrolysates using the same amount of soy protein hydrolysate.

1. Materials and methods

1.1. Materials

Soy protein isolate (type: Borostyán, supplied by Boly Agr. Ltd. Hungary) was prepared according to the method of WISSER and THOMAS (1987). For the preparation of soy protein isolate, defatted soybean meal (50 g) was extracted with 500 ml of distilled water for 1 h at room temperature under magnetic stirring. The pH of the mixture was kept constant at 8.0 with the addition of 1 M NaOH solution. After centrifugation at 5,000×g for 20 min, the pH of the supernatant was adjusted to 4.5 by adding 1 M HCl. The resulting precipitate was collected by centrifugation (parameters mentioned above) and freeze dried.

The α-chymotrypsin, N-benzoyl-L-tyrosine ethyl ester (artificial substrate) and methionine ethyl ester were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Phenylisothiocyanate (PITC) was purchased from Pierce Co. (Rockford, IL, U.S.A.).

Other chemicals were of guaranteed analytical grade.

Soy protein hydrolysate (peptic hydrolysate) was used as the substrate of the EPM reactions (HAJÓS et al., 1988). Soy protein isolates were hydrolysed with pepsin at pH 2.0. The incubation was carried out at 37 °C for 2 h. The enzymatic reaction was stopped by freezing. The reaction mixture was stirred during hydrolysis and then freeze-dried.

1.2. Methods

1.2.1. Enzymatic peptide modification. Peptic hydrolysate of soy protein (25%, w/w) was used to produce EPM products in the presence of α-chymotrypsin as catalyst with the various ratios of methionine ethyl ester to peptic hydrolysate as described previously (HAJÓS et al., 1988). EPM 0, 1, 2, 3, 4 is equal to 0.0, 0.21, 0.49, 0.70, 0.90 g methionine ethyl ester/g hydrolysate, respectively . The incubation was carried out at pH 6.0 and at 37 °C for 16 h. The ratio of peptide to enzyme used in the reaction was 100:1 (w/w). The free methionine ethyl ester was removed by dialysis through cellophane membrane (MW cut off: 1000 Da) against distilled water.

1.2.2. SDS-PAGE. SDS-polyacrylamide gel electrophoresis was done with a conventional method (LAEMMLI, 1970; DELINCÉE & HAJÓS, 1984) to analyse the molecular mass distribution of the protein fraction using 15% acrylamide gel. The protein zones were stained with Coomassie Brillant Blue.

1.2.3. Amino acid determination. The amino acids were determined by using Waters Pico-Tag Analysis System (Millipore Co., Milford, MA, U.S.A.; Pico Tag Manual, 1989). In the pre-column steps, protein and peptide samples were first hydrolysed with 6 M HCl, then derivatized with phenylisothiocyanate (PITC) to

produce phenylthiocarbamyl amino acids. These amino acid derivatives may then be analysed by HPLC in amounts as low as 1 picomole. A fixed wavelength detector operating at 254 nm was used.

1.2.4. Enzyme activity determination. Instead of protein hydrolysate an artificial chymotrypsin substrate, N-benzoyltyrosine ethyl ester was used to investigate the simple effect of methionine ethyl ester on α-chymotrypsin activity. Assay for proteolytic activities of chymotrypsin to synthetic substrates was essentially carried out by the methods of WALSH and WILCOX (1970). Briefly, 0.05 M of N-benzoyltyrosine ethylester and 0.15 mg ml^{-1} of α-chymotrypsin was incubated in 80 mM Tris-HCl buffer with 0.1 M $CaCl_2$ at 37 °C in the absence or presence of various concentrations of methionine ethyl ester. Time-dependent increase in absorption at 256 nm for determining the produced N-benzoyltyrosine was recorded for determining and activities were calculated from the linear slope region.

1.2.5. CD measurement. Conformational changes of α-chymotrypsin during the reaction were determined by observing the mean residue ellipticities (CD, circular dichroism spectra) of α-chymotrypsin in the absence and presence of methionine ethyl ester (WOODY, 1985). All experiments were carried out with a temperature-controlled Jasco 710 spectropolarimeter (Jasco Co., Hachioji, Japan). Mean residue ellipticity, [Θ] (degree cm^2 $dmol^{-1}$) was calculated by Θ/c l, where Θ is observed ellipticity, c is the average molar concentration and l is light path length (ADLER et al., 1973). All the samples were degassed in vacuum conditions just before CD determinations. Protein concentration was 0.15 mg ml^{-1}, light path 1 mm, unless otherwise specified, and protein was dissolved in 10 mM sodium phosphate, pH 6.0 adjusted with 0.1 M NaOH. Temperature was controlled by Peltier type programmer (Jasco PTC-343) at 37 °C. The folding amount of secondary structure was estimated with CD spectrum using Yang's equation (YANG et al., 1986) supplied by Jasco.

2. Results

2.1. EPM and methionine incorporation

Figure 1 shows the changes in the molecular weights to the transpeptidation processes during EPM with methionine enrichment. The protein patterns, before and after enzymatic modifications, were investigated by SDS-PAGE separation. Figure 2 shows the amount of enzymatically incorporated methionine into soy protein hydrolysate with α-chymotrypsin determined by amino acid analyser. The amount of methionine bound to the peptide chains gradually increased with increasing amount of methionine ethyl ester up to 48.7 g of methionine ethyl ester/100 g of protein hydrolysate, which was consistent with our previous data (HAJÓS et al., 1990).

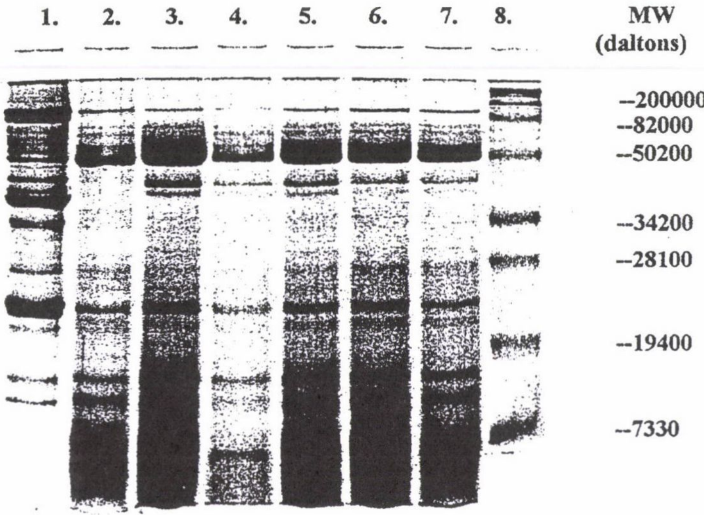

Fig. 1. Electrophoretic patterns of SPI and enzymatically modified products by SDS-PAGE. Lane 1: Soy protein isolate (SPI); lane 2: peptic hydrolysate of SPI; lane 3: EPM product of peptic hydrolysate of SPI (produced in the presence of α-chymotrypsin) without Met-enrichment; lane 4, 5, 6, 7: EPM products of peptic hydrolysate of SPI (produced in the presence of α-chymotrypsin) with different Met-enrichment (lane 4, 0.21 g; lane 5, 0.49 g; lane 6, 0.70 g; lane 7, 0.90 g added methionine ethyl ester/g hydrolysate); lane 8: LMW (low molecular weight controls)

2.2. Effect of methionine ethyl ester on chymotrypsin conformation in a model system

To elucidate the reason of altered α-chymotrypsins' catalytic effect on peptide modification, we gave attention to the possibility of conformation alteration in the presence of methionine ethyl ester, one of substrates in EPM. The used methionine ethyl ester concentration was 13, 26, 39, 52, 65 and 78 mM, which were the methionine ethyl ester concentration used for previous EPM (HAJÓS et al., 1988; 1992). As shown in Fig. 3, conformation of chymotrypsin was altered by adding only 13 mM methionine ethyl ester at 37 °C. That is to say, the intact chymotrypsin has two minimum ellipticity, firstly at 206 nm and secondly around 230 nm (instead of 220 nm characteristic of the typical α-helix structure, (JOHNSON, 1988). The α-chymotrypsin has five β-bulge structures (RICHARDSON et al., 1978). However, adding 13 mM methionine ethyl ester to chymotrypsin results in having one minimum at 218 nm (called γ_{min} at which

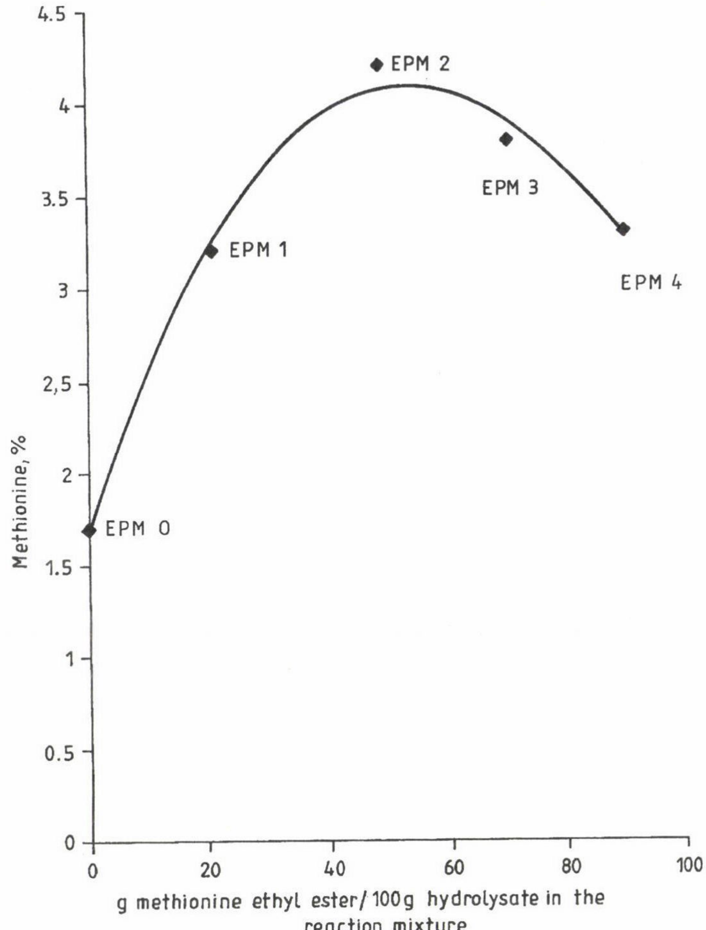

Fig. 2. Methionine incorporation during the α-chymotrypsin catalysed EPM in function of methionine added to the reaction mixtures. Methionine content measured by amino acid analyse (Pico-Tag Amino Acid Analysis System)

ellipticity CD spectrum shows minimum). The CD spectra of α-chymotrypsin in the presence of methionine ethyl ester showed γ_{min} shifted to longer wavelength from 206 nm. Addition of methionine ethyl ester over 26 mM led to the change of the whole profile of CD spectrum of chymotrypsin, but the intensity of molar ellipticity was also decreased with increasing the concentration of methionine ethyl ester. This means that

folding stability of α-chymotrypsin is gradually lost with the increase of methionine ethyl ester due to its instabilization of α-chymotrypsin structure (KWON, 1994a; 1994b).

2.3. Effect of methionine ethyl ester on hydrolytic activity of α-chymotrypsin

The effect of methionine ethyl ester, one of the substrates of EPM, on the hydrolytic activity of α-chymotrypsin was investigated with the artificial substrate,

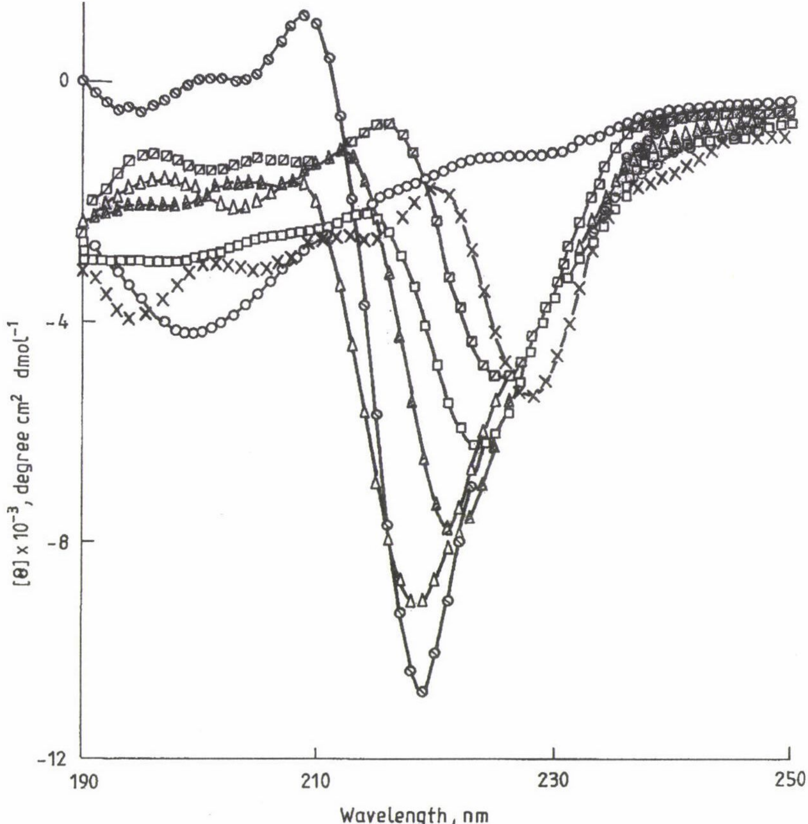

Fig. 3. Methionine ethyl ester-induced conformational change of α-chymotrypsin. Far-UV CD spectra were measured with different concentrations of methionine ethyl ester at 37 °C. Symbols: O: intact chymotrypsin; ●: 13 mM; Δ: 26 mM; ▲: 39 mM; □: 52 mM; ■: 65 mM; +: 78 mM methionine ethyl ester added to 0.15 mg ml^{-1} chymotrypsin in 10 mM sodium phosphate at pH 6.0

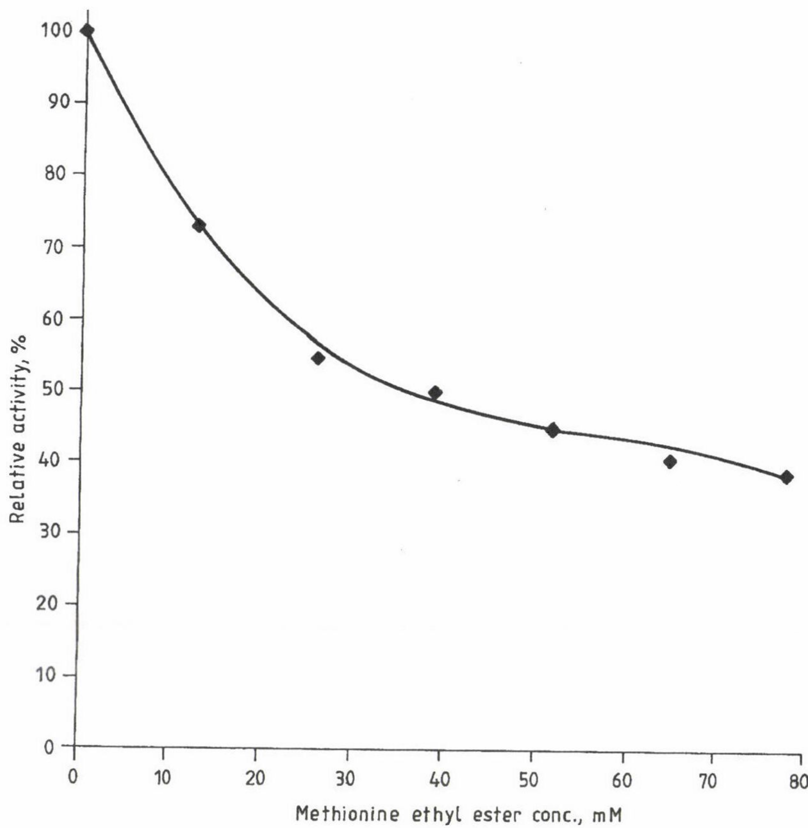

Fig. 4. Relative enzyme activity of α-chymotrypsin in the presence of methionine ethyl ester for the artificial substrate of N-benzoyltyrosine ethylester. 0.0 mM methionine ethyl ester: EPMO; 17.8 mM methionine ethyl ester: EPM1; 41.5 mM methionine ethyl ester: EPM2; 59.3 mM methionine ethyl ester: EPM3; 76.2 mM methionine ethyl ester: EPM4

N-benzoyltyrosine ethyl ester, assuming that great amount of methionine ethyl ester inhibits the enzymatic catalysis or destabilises the enzyme structure. The same concentrations of methionine ethyl ester, as used in CD measurement, were added to the chymotrypsin-artificial substrate mixture, and the hydrolytic activity was measured spectrophotometrically. Enzyme activity was 100% when we did not use methionine ethyl ester. Figure 4 shows that the addition of 13 mM methionine ethyl ester drastically decreased the hydrolytic activity of chymotrypsin to 73%, 26 mM

methionine ethyl ester below 50%. Further increasing the methionine ethyl ester concentrations causes only a slight decrease in the activity of enzyme.

On the basis of the results, our hypothesis is that the methionine ethyl ester modifies the conformation of α-chymotrypsin and modifies the enzyme activity during the enzyme-catalysed reaction.

3. Discussion

Our results support that enzyme-catalysed methionine incorporation into soy protein hydrolysate during EPM shows an optimum curve in function of the content of the added methionine ethyl ester in the reaction mixture. That is, by addition of more than 48.7 g of methionine ethyl ester to 100 g soy protein hydrolysate the amount of incorporated methionine decreased. To monitor this surprising reaction, the conformational alteration of α-chymotrypsin was studied in a model system. We were tempted to elucidate the relationship between a structural characteristic and hydrolytic enzyme activity under these conditions. The hydrolytic activity of α-chymotrypsin to artificial substrate (N-benzoyltyrosine ethyl ester) significantly decreased to 76% and 45% in the presence of 13 mM and 26 mM methionine ethyl ester, respectively, and the hydrolytic enzyme activity slightly decreased in the concentration interval of methionine ethyl ester, which was used during EPM reaction (Fig. 4). We have also considered the impact of conformational alteration on the enzymatic activity with various concentrations of methionine ethyl ester. According to CD profiles of α-chymotrypsin (Fig. 3), the secondary structure was changed with 13 mM methionine ethyl ester and the structure was further modified with increasing concentration of methionine ethyl ester. These results are strongly related to the potential ability of chymotrypsin to catalyse peptide modification. The catalyst-chymotrypsin might be altered by more factors, for instance by the substrate (methionine ethyl ester) before substantial methionine-incorporation, because chymotrypsin might maintain this high initial velocity only for a few seconds due to the deactivation of substrate. At a low concentration of methionine ethyl ester, incorporation is retarded by the low initial velocity (v_0) despite the stable conformation of chymotrypsin. At higher concentrations of methionine ethyl ester, the initial velocity would be high enough, but the enzyme activity considerably decreased. These effects of substrate on the enzyme activity made that methionine incorporation during chymotrypsin catalysed EPM shows an optimum curve as a function of methionine ethyl ester concentration.

Major apparent substrate and product such as soy protein hydrolysate and methionine-incorporated product can probably bind to the active site or other site of the enzyme and can change the structure of chymotrypsin and modify its reaction activity. Usually, in case of substrate binding to the active site of enzyme, this enzyme-substrate

complex can be much more stable than the free enzyme (CREIGHTON, 1984), however, enzyme-substrate complex can be hardly captured and its structure cannot be analysed because bound substrate is easily catalysed. Further information of this kind of substrate effect can be obtained by conducting research with using a model ligand (substrate inhibitor) binding system.

We have concluded that the conformation of the enzyme was modified and thereafter enzymatic catalysis for enzymatic peptide modification was altered by addition of methionine ethyl ester to the reaction mixture. That is in line with our earlier studies that the addition of a great amount of amino acid derivatives significantly modifies the reaction and gives a new direction to the transpeptidation.

This finding might be useful in optimisation of EPM reactions or in the industrial practice of the enzymatic modification processes of proteins.

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Acta Alimentaria, Vol. 27 (2), pp. 193–202 (1998)

Short communication

EFFECT OF MICROWAVE ENERGY ON LIPASE INACTIVATION AND STORAGE STABILITY OF BROWN RICE

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Microwave energy was used as a thermal treatment for the inactivation of lipase and consequent improvement in the long-term storage stability of brown rice. The improvement of brown rice stability during storage would lead to an increase in its consumption. A greater utilization of brown rice could increase its economic value and nutritional benefits.

The three parameters considered most important were studied: 1) microwave energy level (maximum and medium); 2) initial moisture content (13.4, 14.6 and 17.3%) and 3) time of treatment (20 to 140 s).

Microwave energy treatment was shown to be effective in the control of the lipase activity of brown rice with the maximum inactivation rate at the maximum power of microwave energy, a treatment time of 80 s and initial moisture content of 14.6%. Moisture content of brown rice treated by microwaves presented a reduction. During storage of the samples, a decrease in the production of free fatty acids was observed.

Keywords: brown rice, lipase activity, microwave energy, storage stability

Brown rice has a higher nutritional value than polished rice because it has more protein and B-complex vitamins, especially thiamin, riboflavin and niacin. Moreover, the process for its production results in a greater total yield with fewer broken grains (JULIANO, 1985). In spite of the fact that brown rice has several advantages over polished rice, it is not consumed on a large scale.

One of the main reasons for this is the instability of brown rice during storage mainly due to the rapid decomposition of bran lipids. The low stability of brown rice has been attributed to the action of lipase enzyme contained in the rice bran on the lipids (TAKANO, 1993). Brown rice lipids are readily hydrolyzed by lipase releasing free fatty acids (FFAs). FFAs are precursors of off-flavours and off-odours associated

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with the lipid degradation products generated in subsequent oxidation reactions (CHAMPAGNE & HRON, 1992).

In addition, degradation of the lipid by hydrolysis and oxidation will lead to rancification, which is the main limiting factor for storing high fat content products (EKSTRAND et al., 1992).

The location of lipases in rice grain and its characteristics have been reviewed. The utilization of heat treatments, both conventional dry and moist, use of chemicals (DESIKACHAR, 1977), gamma irradiation, and storage under low temperature or inert atmospheres for enzyme inactivation have been suggested. However, they have not been accepted as reliable, practical procedures. Also, several methods exist for stabilizing bran rice separated during the milling of raw milled rice (SAYRE et al., 1982). The evaluated processes included extrusion cooking (RANDALL et al., 1985) and hydrothermal treatment (LU & CHEN, 1996). Other processes for stabilizing brown rice kernels have been proposed such as lipolytic hydrolysis by liquid ethanol extraction (CHAMPAGNE et al., 1990; CHAMPAGNE & HRON, 1991), ethanol vapor treatment (CHAMPAGNE & HRON, 1992) and ethanol containing chelators/acidulants (CHAMPAGNE & HRON, 1994). The feasibility of microwave energy for the inactivation of α-amylase in wheat and wheat flour has been successfully probed by EDWARDS (1964) and AREF and co-workers (1969). They showed that the levels of enzyme activity decreased without damaging the flour with respect to its capacity to make dough, maintaining its viscoelastic properties. Also, microwave energy has been effective in rice bran stabilization. VETRIMANI and co-workers (1992) used microwave energy to inactivate lipase and lipoxygenase in rice bran, germ and soybean. This treatment led to considerable inactivation of the lipase and complete inactivation of the lipoxygenase present in these materials. JIAXUN-TAO and co-workers (1993) reported stabilization of rice bran by microwave heating at 2450 MHz for 3 min, for up to 4 weeks in storage. Due to the several advantages that microwave energy presents over conventional heat treatment and its efficiency in bran stabilization, the objective of this work was to improve the stability of brown rice during storage by inactivation of lipase enzyme by the use of microwave radiation.

1. Materials and methods

1.1. Preparation of samples

A Brazilian variety of paddy rice (IAC-120) was used to obtain brown rice and polished rice. The milling (1 kg lots of paddy) was carried out in a D'Andrea mill in two operating steps.

The first step of the milling process was the removal of the external rice hull thus obtaining the brown rice sample. To obtain the polished rice sample, the brown rice was transferred to the second step of the process where the aleurone fraction was removed by an abrasive type machine employing a carborundum stone.

1.2. Microwave energy treatment

Each sample of 80 g of brown rice was placed in a petri dish (9.4 cm diameter; 1.4 cm high and 0.2 cm thick) and placed at the center point of the microwave oven (Kenmore, 750 W and 2.450 MHz). The samples inside the petri plates with respective initial moisture contents of 13.4, 14.6 and 17.3% were exposed to microwave energy for 20 to 100 s at maximum (highest) power and 40 to 120 s at medium power.

At the levels applied, the energy absorbed by the product using the medium power was 82.76% of that absorbed using the maximum power. This value was determined according to KINGSTON and JASSIE (1988). After half of the treatment time, the position of the petri dish was inverted to improve energy distribution to the brown rice sample.

1.3. Extraction of crude extract of lipase enzyme

The extract of lipase enzyme was obtained from defatted brown rice, according to the method used by DRAETTA and co-workes (1978). A 40-g sample of rice was ground using the break and reduction Brabender Senior Mill passing through an 80-mesh screen, then washed five times successively with petroleum ether using a shaker apparatus (47 cycles min^{-1}, 30° of oscillating angle) for each washing time. The sample of defatted rice was dried for three hours at room temperature.

A solution of calcium chloride (0.01 mol l^{-1}) was added to the sample in a ratio of 5:1 (p/v). The suspension was adjusted to pH 6.0 with NaOH (0.1 mol l^{-1}) and shaken for three h at 5 °C in a shaker (psychrotherm), and then centrifuged at 12.000×g at 5 °C for 20 min. The float fraction was reserved as the crude extract and used to determine lipase activity.

1.4. Enzyme assay

Lipase activity in the sample was measured with tributirin (99% degree of purity) as substrate. The hydrolysis reaction was followed by titration with 0.01 (mol l^{-1}) NaOH in a pH-STAT (Radiometer Type). The extent of hydrolysis at pH 7.5 and 35 °C was estimated from the amount of 0.01 mol l^{-1} NaOH added during the zero order enzyme reaction. The activity is expressed as mol l^{-1} of fatty acids formed during 5 min reaction time. The reaction mixture consisted of 0.2 ml tributirin, 0.1 ml of potassium chloride (0.05 mol l^{-1}), 1.0 ml calcium chloride (0.005 mol l^{-1}), 1.0 ml of enzyme

solution and sufficient deionized water to give a total volume of 10 ml (AIZONO et al., 1973; FUNASTSU et al., 1971).

1.5. Storage studies

Samples treated by microwave radiation and untreated brown rice were stored at room temperature during 4 months after packing as 500 g samples in glass bottles. To measure the extent of lipolytic hydrolysis in the brown rice sample during storage, the FFAs contents of both treated and control samples were determined at periodic intervals using the A.A.C.C. (1969) 02–04 method.

2. Results and discussion

2.1. Effect of microwave energy treatment and moisture content on the lipase activity of brown rice

The effect on lipase activity of exposing brown rice with different initial moisture contents (13.4, 14.6, and 17.4%) to different power levels of microwave energy (maximum and medium) is shown in Figs 1, 2, and 3. Brown rice with 13.4% initial moisture content exposed to both power levels of microwave radiation showed a slight decrease in the lipase activity as indicated by lipid hydrolysis. The reduction of free fatty acids in brown rice after 80 s of exposure time was from 0.30 to 0.23 and 0.21 mol l^{-1} min^{-1} for medium and maximum power, respectively. However, a greater effect was observed on the sample with 14.6% initial moisture content exposed to maximum power energy. Brown rice conditioned at 14.6% moisture and treated under maximum power of microwave energy, presented a greater inactivation rate of lipase than any other treatment (13.4 and 17.3% moisture) or control sample (brown rice not exposed to microwave energy). However, the lowest production of FFAs was observed in the brown rice with 17.3% of initial moisture content treated with the maximum power of microwave energy during 100 s of exposure time. The brown rice treated under these conditions showed a 65.2% decrease in FFAs production compared with untreated brown rice. The longer the exposure, the greater the decrease in enzyme activity.

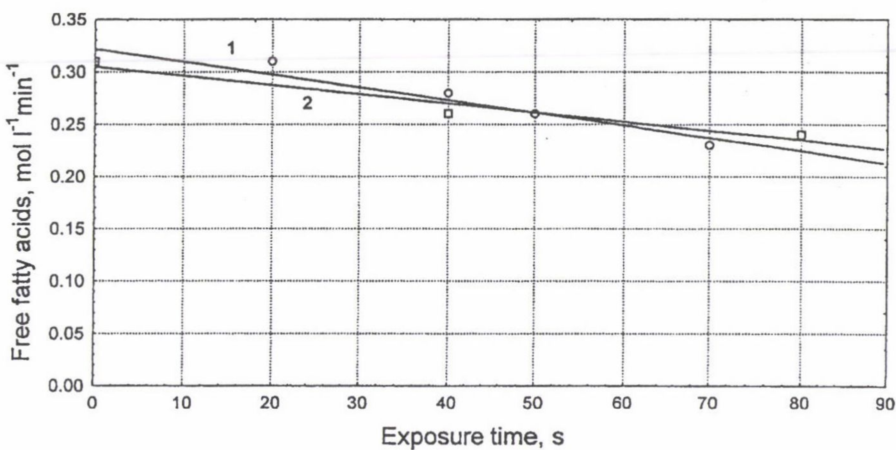

Fig. 1. Effect of microwave energy on the hydrolysis of lipid by brown rice lipase (initial moisture content of rice: 13.4%). Y_1 (P max.) = –0.001 x+0.32, r=0.9576, SD=0.3059; Y_2 (P med.)= –0.001 x+0.305, r=0.9707, SD=0.2944; (P max. and P med.: maximum and medium power, resp.); r: correlation coefficient; SD: standard deviation

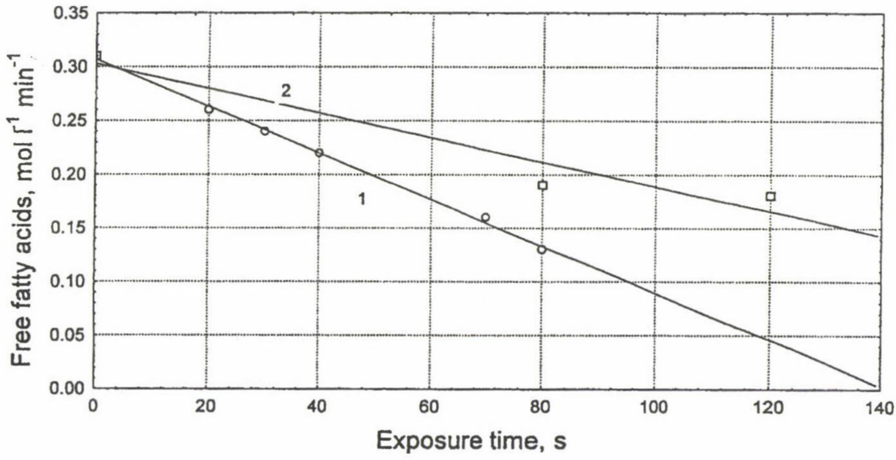

Fig. 2. Effect of microwave energy on the hydrolysis of lipid by brown rice lipase (initial moisture content of rice 14.6%). Y_1 (P max.)= –0.002 x+0.307, r=0.9986, SD=0.06603; Y_2 (P med.)= –0.001 x+0.303, r=0.9652, SD=0.7234; (P max. and P med. : maximum and medium power, resp.)

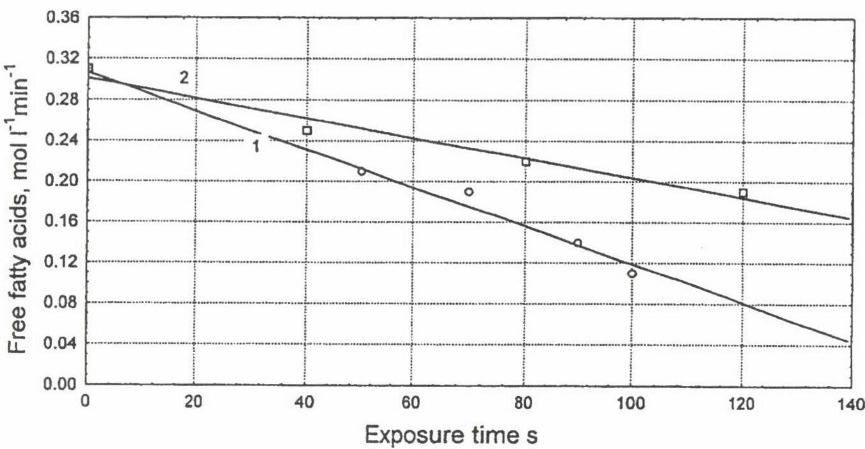

Fig. 3. Effect of microwave energy on the hydrolysis of brown rice lipase (initial moisture content of rice 17.3%). Y_1 (P max.)= –0.002 x+0.306, r=0.9916, SD=0.06532; Y_2 (P med.)= –0.001 x+0.301, r=0.9818, SD=0.03742; (P max. and P med.: maximum and medium power, resp.)

The sample exposed to medium power energy required more exposure time to attain similar results to those obtained with the maximum power energy level.

BARBER and co-workers (1977) reported that the heat resistance of rice-bran enzymes depends on temperature and time of treatment, as well as on moisture content. The higher the moisture content, the lower the heat resistance. VETRAMINI and co-workers (1992) found that 30% lipase activity persisted even after 4 min microwave treatment of rice bran. Storage studies of the microwave treated rice bran showed that the bran is stabilized; there was a much smaller increase in its free fatty acid content after 1 month compared to that in untreated samples. CHAMPAGNE and HRON (1992) showed that during 6 months of storage at 36 °C, free fatty acids increased very little in brown rice kernels treated with EtOH vapours for 3–10 min. Flours produced from treated kernels had low residual lipase activity. Treated kernels and flours prepared from them were more susceptible to oxidase deterioration than untreated kernels and flours, as indicated by increases in the conjugated diene hydroperoxide content during storage.

2.2. *Effect of the microwave energy treatment on moisture content of brown rice*

Figure 4 shows the behaviour of the moisture content of brown rice after exposure time to microwave energy. Brown rice exposed to both levels of microwave energy showed decreased moisture contents. However, the great effect was observed in the sample exposed to the maximum power for the longest exposure time.

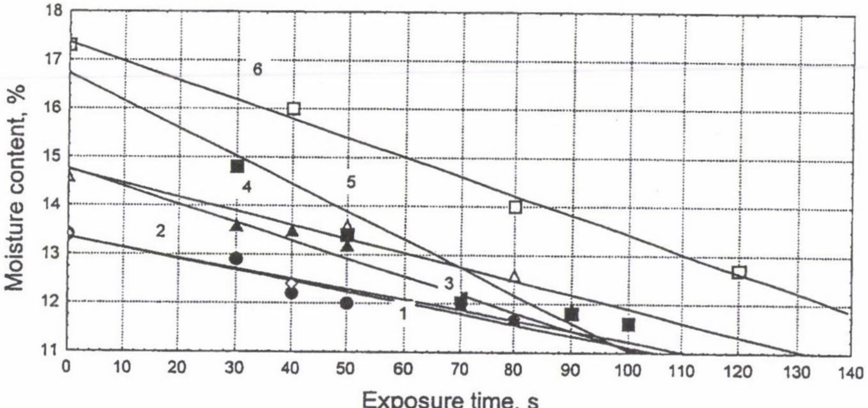

Fig. 4. Effect of microwave energy exposure time on the moisture content variation of brown rice. Moisture content of rice 13.4%: Y_1 (P max.)=–0.022 x+13.351 (r=0.9279, SD=0.62450) Y_2 (P med.)= –0.021 x+13.35 (r=0.9948, SD=0.8544). Moisture content of rice 14.6%: Y_3 (P max.)= –0.037 x+14.759 (r=0.9827, SD=1.0807), Y_4 (P med.)= –0.028 x+14.741 (r=0.9761, SD=1.1786). Moisture content of rice 17.3%: Y_5 (P max.)= –0.057 x+16.733 (r=0.9715, SD=2.2181); Y_6 (P med.)= –0.039 x+17.37 (r=0.9960, SD=2.0477), (P max. and P med.: maximum and medium power, resp.)

Moreover, in this study the initial moisture content was considered to be one of the main limiting factors for long exposure times of microwave treatment.

For the conditioning of initially low moisture content brown rice the microwave exposure time could not be increased, since this resulted in fissuring and possible burning of the product. Therefore, if it is necessary to increase the exposure time for microwave treatment, the initial moisture content of the sample must be increased. It is generally recognized that moist heat is more effective than dry heat. The method of steaming bran for 3 min at 100 °C, followed by drying to the initial moisture content and cooling can yield satisfactory results. Cooking and extrusion under high pressure are other alternatives (LUH et al., 1991).

2.3. Effect of the microwave energy treatment on the development of free fatty acids during storage

The development of FFAs during storage in brown rice with different moisture contents when exposed to the maximum microwave power is shown in Fig. 5. Brown rice treated with microwave energy produced less FFAs during storage than brown rice without treatment. However, brown rice with initial moisture contents of 14.6% and 17.3% exposed to microwave energy for 80 and 100 s, respectively, resulted in lower

FFA development than in brown rice with 13.4% of initial moisture content exposed to 70 s of treatment. Although, the samples treated with microwave energy showed a reduction in FFA production during storage, there was still residual lipase present in the brown rice. LUH and co-workers (1991) cited that the use of dry heat to stabilize rice bran has been investigated extensively. Recommended treatment conditions differ dramatically and results are very often discordant. In general, dry heat does not inactivate lipases totally. According to YOKOCHI (1977), the recommended moisture level is in the range of 3–6% to prevent an FFA rise. During dry heat the exposure time is an important variable for stabilizing rice bran. SRIMANI and co-workers (1977) heated the bran at 120 °C for 15 min in a pan with stirring, but it did not arrest lipase activity; 20 min at 110 °C was needed for inactivation.

Rice bran, stabilized by microwave heating at 2450 MHz for 3 min, was stable for up to 4 weeks of storage. Free fatty acid (FFA) content of microwave stabilized bran increased from 4.0% to 4.9% in long grain rice bran and from 4.6% to 6.25% in medium grain rice bran, even under the unfavourable storage conditions used (33 ±2 °C, 75±5% RH). In contrast, increases in untreated bran FFA ranged from 4.0% to 68.3% and 4.6 to 56.8% in long and medium grain bran respectively (JIAXUN-TAO et al., 1993).

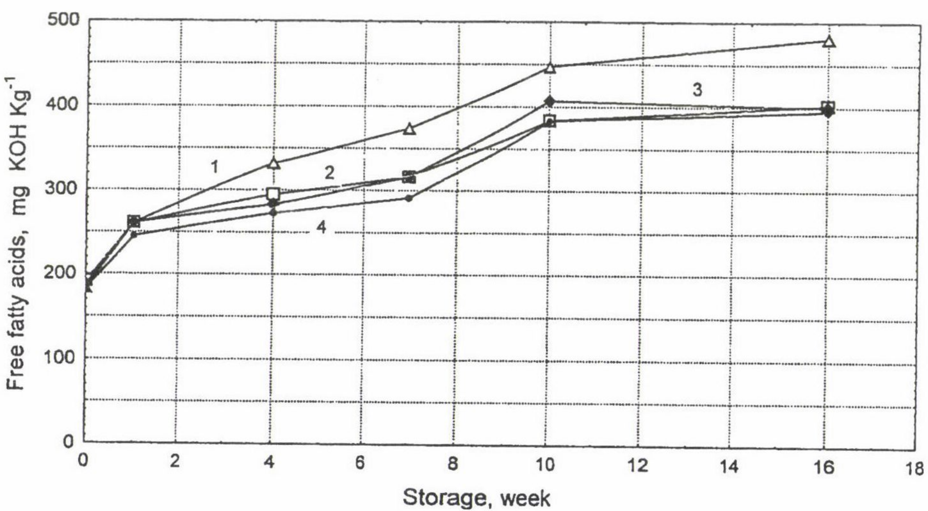

Fig. 5. Effect of maximum power of microwave energy on free fatty acid development in brown rice during storage. 1: 0 s and 13.4%; 2: 70 s and 13.4%; 3: 80 s and 14.6%; 4: 100 s and 17.3%; exposure time and moisture content, resp.

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Therefore, microwave energy treatment under the conditions employed in this study was not sufficient to complete inactivating the lipase of brown rice.

To attain more efficient enzyme inactivation we must increase the microwave exposure time combined with an increased initial moisture content of the brown rice. These two parameters are considered the most important to reach the target of inactivation of the lipase enzyme. However, care must be taken not to change the starch properties such as gelatinizing and to avoid physical changes due to burning and cracking of the brown rice during severe treatment.

3. Conclusions

Results from this work demonstrated the feasibility of the application of microwave energy as a short time heat treatment for lipase enzyme inactivation.

The best conditions for inactivation of lipase enzyme in brown rice were 14.6% of initial moisture content and exposure to maximum (highest) power of microwave energy for 80 s.

However, by increasing both the range of initial moisture content and the exposure time of the microwave energy, it may be possible to eliminate the lipase activity more efficiently without damaging the quality of the product.

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Acta Alimentaria, Vol. 27 (2), pp. 203–205 (1998)

Book reviews

Food safety 1996

Food Research Institute, Department of Food Microbiology and Toxicology, University of Wisconsin, Madison (USA)

C. E. STEINHART, M. E. DOYLE & B. A. COCHRANE (Eds)

Marcel Dekker, Inc., New York–Basel–Hong Kong, 1996, 618 pages

The "Food Safety 1996", the latest volume of this series, gives a very good overview of results and conclusions of this field, covering the literature published during the second half of 1994 and the first half of 1995. The book refers to 4220 published papers. The Editors divided the book into three equal parts, and altogether into 13 chapters. Part 1: Diet and health, Part 2: Safety of food components, Part 3: Foodborne microbial illness. Each part and chapter contain the main conclusions of papers together with data, very briefly.

In Part 1 (Diet and health) the relations between diet and health and the effects of different compounds of diets on metabolic processes are very clearly demonstrated: the influence on the health of fats, fibre, vitamins, proteins, alcohol, the nutritionally balanced diet, the antimutagens and carcinogens as well as that of the different risk factors. The effects of diet and conditions on cardiovascular diseases, cancer, inflammation and immune related diseases, food allergy and food intolerance, endocrine system, nervous system, behaviour, other diseases and ageing are also discussed.

In Part 2 (Safety of food components) the editors collected a lot of very important knowledge about "Assessment of food safety", the problem of risk and risk assessment. "Toxic and carcinogenic activities of chemicals do not depend simply on external concentrations of the substances in the diet or the environment." Different other factors have also influence on the diet and environment, and the interaction between these factors should be considered. The chapter of intentional (direct) additives (preservatives, nitrate, nitrite, antioxidants, sweeteners, colours, vitamins, etc.) has very useful information on one hand on the effect, and on the other hand on the analysis of compounds. It is not clear, why the editors put food irradiation in this chapter. The irradiation is not an additive, it is a technological process. "The indirect additives,

residues and contaminants" chapter is very rich in information (antibiotics, drugs, pesticides, PCBs, PAHs, packaging materials, heavy metals, radionuclides). The "Naturally occurring toxicants and food constituents of toxicological interest" chapter contains many information about food components naturally existing or produced during processing (treatment or storage) of food. Food allergy and food intolerance is an interesting point. This chapter has the largest number of references (1595).

Part 3 (Foodborne microbial illness) can be considered one of the most important parts, because "world-wide, each year, food poisoning kills more than one million people", and from year to year the number of foodborne illnesses are increasing. The food-related risks are living problems at all levels from the harvest to the consumption. The food safety education is of great importance. The mycotoxins, bacterial intoxications and infections are every-day problems world-wide. It is a very large field, where different factors interact, this type of risk is the greatest and causes the highest number of foodborne illnesses and death. The appearance of pathogenic bacteria in different food products and processing lines is frequent and results in serious illnesses and not rarely death. This chapter recounts a very rich list of cases of pathogens in foods, evaluates the different conditions, effects and some control possibilities (irradiation, chemicals, sanitation, HACCP), which are all very edifying. This chapter informs about new detection and identification methods of micro-organisms. At the end of the book very short reviews on "Foodborne parasitic infections" and "Food- and water-associated viruses" are found.

I. F. KISS

New developments in refrigeration for food safety and quality

Proceedings of the Meeting of Commission C2, with Commissions B2 and D2–3, Lexington, Kentucky, US, Oct. 2–4, 1996. Issued for the International Institute of Refrigeration

American Society of Agricultural Engineers, 1996, viii + 318 pages ISBN 0-929355-80-6

Food safety and quality are critical factors of food supply internationally. The conference documents recorded in the book cover various aspects of these subjects in relation to refrigeration science and technology. Following the banquet keynote address by P. J. CLERKIN entitled "From Farm to Table: Challenges and Opportunities for Meat and Poultry Safety", the 44 papers from 12 countries (in a few cases only the abstracts

were available) are grouped according to the following main headings (the number of presentations in each group are given in brackets):

I. Refrigerated transport (3)
II. Optimal temperature and humidity control (2)
III. Ammonia refrigeration applications (4)
IV. Biochemistry of freezing (3)
V. Food property sensor development (3)
VI. Useful shelf life of refrigerated and frozen foods (4)
VII. Improvements in display cases and refrigeration systems (3)
VIII. Safe processing and bacterial growth in refrigerated and frozen foods (4)
IX. Use of packaging and modified atmospheres to preserve shelf life (4)
X. International shipment and inspection issues (1)
XI. Refrigeration of fruits and vegetables I (4)
XII. Refrigeration economics (2)
XIII. Refrigeration of fruits and vegetables II (4)
XV. Cryogenic and flash freezing processes (2).

Two-third of the papers were originated from the United States. The volume is completed with an author index.

The book is recommended to all who wish to gain a comprehensive and practical view of the above important interdisciplinary R & D area.

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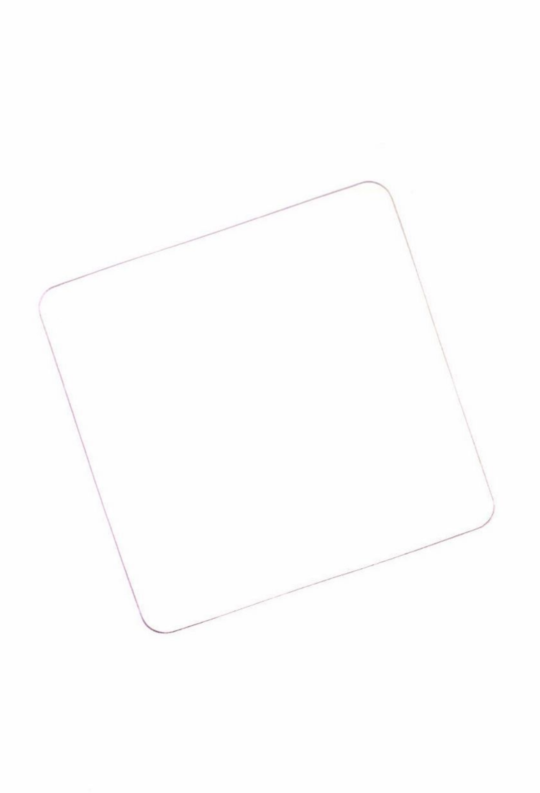
Erratum

Acta Alimentaria Vol. 27 No 1, pp. 53–62 (1998); in the paper Modelling modified atmosphere packaging of perishable produce: keeping quality at dynamic conditions,
written by Hertog, M. L. A. T. M. and Tijskens, L. M. M.
in the header row of Table 1 should be:

Ea_1/R_{gas} and Ea_2/R_{gas} instead of Ea_1 and Ea_2.



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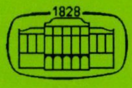

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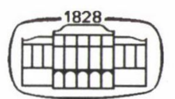

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POSTHARVEST PERMEABILITY CHANGES OF THE CELL SURFACE OF APPLE TISSUE

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The investigation and the mapping of the changes of fruits during storage have fundamental importance in the development of suitable storage technologies. The condition of plant cells, the permeability of the cell wall-membrane complex can be well characterized by the measurement of ion leakage from the plant tissue into an isotonic solution. The parameters of the function describing the changes of the ion leakage during storage refers to the change of the mechanism of ion leakage. The curve of ion leakage may be well approached by a quadratic equation. The initial rate of ion leakage changes according to a maximum curve during storage, the maximum was found after 5 months storage. A hypothesis was established to explain these observations. Before storage and at the beginning of the storage the condition of the membrane determines the ion leakage, later the cell wall takes over the regulation. During senescence the structure of the membrane loosens, its permeability increases however the structure of the cell wall is degraded, which decreases the permeability. As a result of these processes the rate of ion leakage has a maximum during storage.

Keywords: apple, ion leakage, conductivity, storage

The increasing permeability of the membrane during storage has already been observed for a long time (EILAM, 1965). In the opinion of the researchers the degradation of the cell membrane characterises the fruit senescence. The change of ion leakage from fruit tissue is based on the modification of the membrane structure. The membranes become more permeable during senescence, so the diffusion of the dissolved components increases. The membranes are sensitive to both heating and cooling, which causes permeability changes. The changes of membrane structure have been the subject of research for a long time. The cooling decreases the ion leakage from the fruit tissue. Comparing the cooled and control fruits the speed of the ion leakage at 21 °C is higher than at 1 °C (FURMANSKI & BUESCHER, 1979). The extent of the ion leakage follows the senescence of the fruit samples. Fruits were stored between 0.5 °C

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and 3.5 °C until 3.5 for 7.5 months in controlled atmosphere (CA) (3% O_2 and 5% CO_2) and at low pressure (6.7–8 kPa) respectively. The firmer apples stored at low pressure had smaller ion leakage than apples stored in CA (BERNARD & LOUGHEED, 1982). SALTVEIT (1989) described the kinetics of ion leakage by an exponential curve. According to his supposition the ions come from two compartments of the cells into the solution. One of these is the cell wall which looses ions rapidly and contains a small amount of ions, the other is the vacuole which looses ions slowly and contain a large amount of ions. The two compartments are separated by the tonoplast, the citoplasm and the cell membrane. KOVÁCS and co-workers (1988) established that combined postharvest treatment (1 kGy gamma irradiation and calcium chloride) resulted in a good preservation of the cell compartments (with a lot of starch in the plastids, remaining essentially unchanged cytoplasm), but it could not prevent the breakdown of the middle lamella.

Our newer experiments are directed towards finding a connection between the ion leakage rate and the cell surface structure. In our experiments the condition of fruit cells was followed by measuring ion leakage through the cell surface during storage. The aims of the present paper were to apply a conductometrical method to follow the change of the permeability of the cell surface and to study the effect of the storage on the ion leakage and to compare a few cultivars that have or should have greater importance in the future in Hungary. The kinetics of the ion leakage was described and the factors influencing the parameters of the function fitted to the ion leakage were studied. This paper reports some methodological establishments for the further work.

1. Materials and methods

In 3 one-year experiments six cultivars (Jonnee, Jonagold, Jonathan M41, Golden spur, Gloster and Mutsu) were investigated at three different stages of ripeness (7 day intervals). The picking date was optimized for the storage based on the growers' experiences. Samples were picked in September, the optimum time for the longterm storage, as well as one week before and after that date. Apples were stored at 2–3 °C, 92–94% RH in normal atmosphere. Ten pieces of apples were examined from each cultivar, and from each of the three picking dates. The measurements were carried out at the harvest date and after 5, 6 and 7 months of storage. In the second year we included a measurement after 2 months of storage as well.

A 100 cell conductometer (LABVIG OE-420) connected to a computer was used for the measurement of ion leakage, in which 100 parallel samples can be measured at the same time. The ion leakage is expressed as the current (I) measured in μA-s.

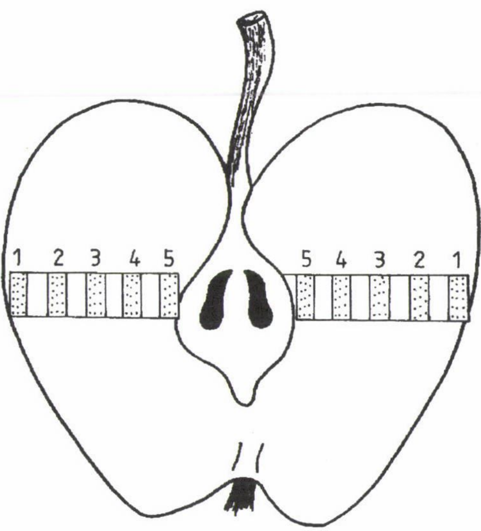


Fig. 1. Sampling. (Figures from 1 to 5 represent the sampling loci from the peel to the core)

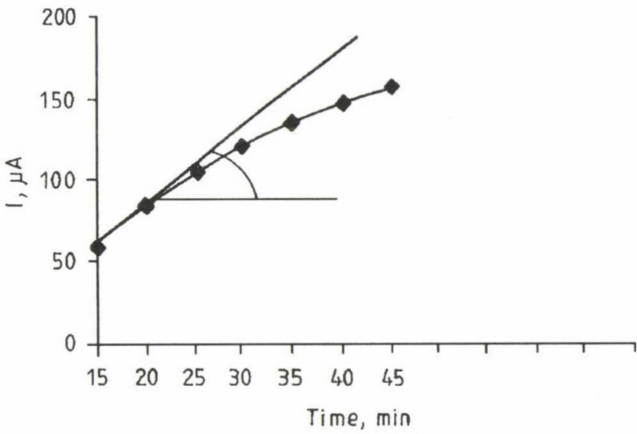


Fig. 2. The determination of the initial rate of ion leakage

Five cylindrical samples (10 mm diameter, 2 mm height) were cut out from both sides of each apple (Fig. 1). At first the apple slices were soaked in 3 cm^3 of mannitol

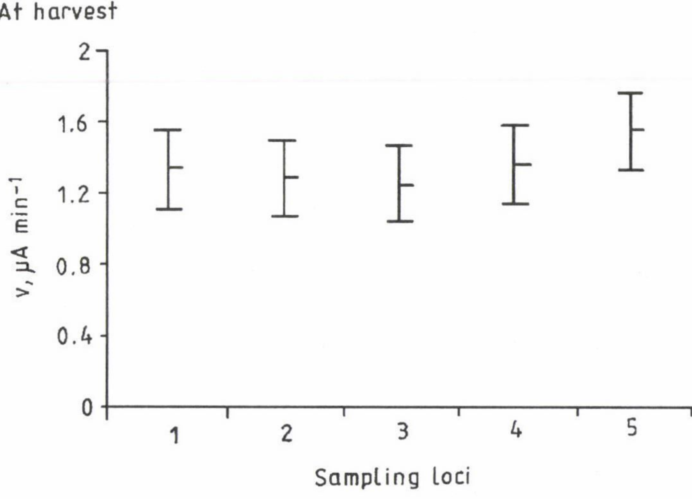
solution containing 0.5 cm^3 2-mercapto-ethanol per dm^3 for the inhibition of browning for 15 min. After moving them into an ion-free mannitol solution the current, that is proportional to conductivity was measured for half an hour. The reason for the soaking is to allow the majority of ions from the cut cells and from the intercellular spaces to migrate into the isotonic solution. For the characterization of the ion leakage curve the initial rate (velocity, v [μA min^{-}]) of ion leakage was determined, which is the slope of the line fitted on the first five measured plots (Fig. 2). Its value corresponds to the kinetic constant of transport processes and characterizes the permeability of the cell wall-membrane complex.

2. Results

The relative standard error of the measurement of the conductivity is approximately 15–20%. There are several reasons for this relatively high rate. The ion distribution in apples is not homogenous, the physiological condition of each apple is also different, for example significant differences in colour can be found. There are always 1 or 2 out of 10 apples that have a significantly higher or lower ion leakage than the average. Therefore numerous repetition are needed to achieve the necessary level of accuracy.

Figure 3 shows the distribution of the initial rate of ion leakage depending on sampling at harvest and after 5 months storage. It can be seen along the maximum cross-section from the peel to the core in Jonagold apple. This profile can be noticed in all cultivars, however there are differences in the degree of deviation. There is no significant deviation among samples taken from different places, which refer to a homogeneous condition of the apple tissue at harvest. However after storage the distribution of the initial rate of ion leakage depending on sampling became stronger. The kinetic constant is an average value in the cells under the peel, then it increases through a minimum towards the core. After five months storage the difference in the rate of ion leakage of the peel and the core is very high and significant. These deviances indicate that the metabolic processes are going on in the apple with different intensity in different parts of the tissue. At the core the permeability increases much more than in the outer part.

Eliminating the samples taken from the nearest tissue to the peel and to the core, the standard deviation does not decrease. This means that the deviation among apples in different physiological conditions has a more important role in the variance of the measurement, than the place of sampling from the same apple.



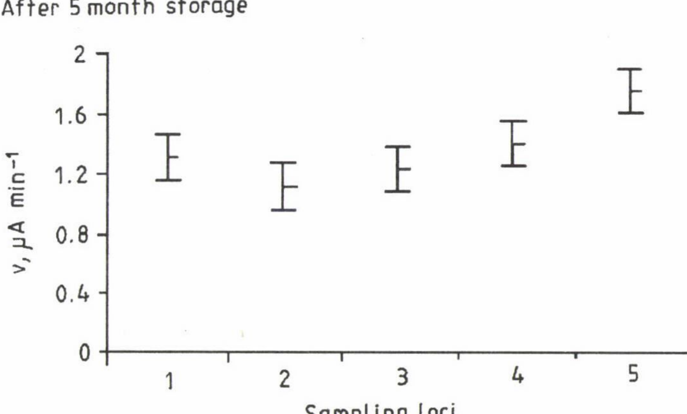


Fig. 3. The changes of the initial rate of ion leakage from the peel (1) to the core (5) in Jonagold in September and February

The cells contain potassium ions in large amount. The majority of this ion can be found in the vacuole, the largest part of the fruit cell. The potassium ion is free in the cell and has a small hydrate shell. The leakage of the potassium ion is of the largest rate among the cations. This is proved by observing the ion composition of the measured solution. The concentration of potassium ion is one order higher in the solution than that of the other cations (Mg^{2+}, Ca^{2+}, Na^{+}).

Our aim was to obtain information about the permeability of apple cells from the curve of ion leakage. There are two theoretical ways to solve this problem:

1. If the leakage mechanism is known, a mathematical model can be set up by making an appropriate mathematical description of the partial processes. The measured data are then used to determine the parameters of the model.

2. If the leakage mechanism is not known, we consider it as a black box. The data have to be analysed and a model that fits the data the best can be found.

Probably different transport processes take part simultaneously in the ion leakage, so the measured data are the results of these processes. The mechanism of the potassium ion leakage can be separated into the following partial processes (Fig. 4):

– Ions are transported through the tonoplast
– Diffusion in the thin cytoplast
– Transport across the membrane
– Diffusion through the cell wall
– Getting out of the tissue across the intercellular space
– Diffusion in the measuring solution

A leakage model can be established from the individual equations of the partial processes. In our cases the values of numerous parameters in the analytical descriptions are not known (for example cell surface, volume of intercellular space). The process,

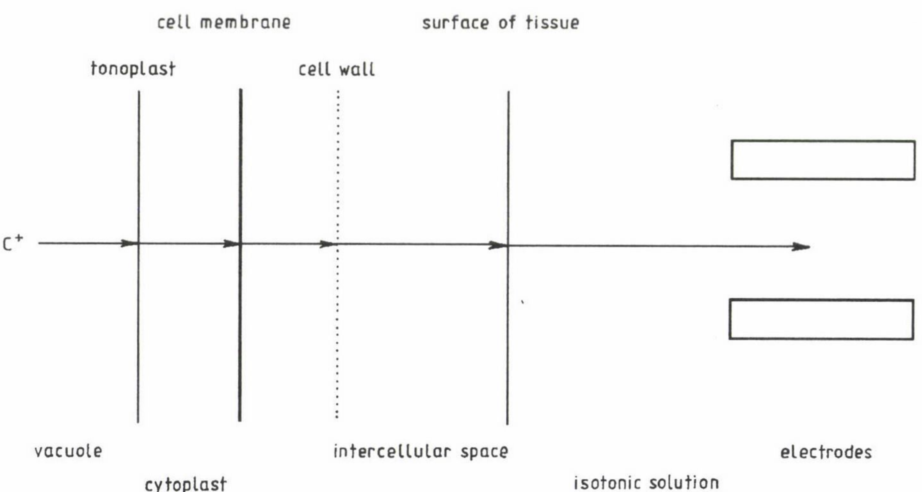


Fig. 4. The cation (c^+) leakage from the tissue

that determines the transport speed, is undefined too. So the mathematical equation describing the kinetics can not be solved. That is why the second way was chosen.

Different types of functions were applied to the measured data. The accuracy was evaluated by statistical parameters. The fitted functions were:

linear:	$I = at+b$
multiplicative:	$I = at^b$
exponential:	$I = \exp(a+bt)$
reciprocal:	$I = a/t+b$
saturational:	$I = t/(a+bt)$,

where I: the measured current [νA]; t: the measuring time [min]; a,b: the constants of the functions.

The correlation coefficient of the linear function was the highest (>0.99) among fitted functions, the multiplicative and the exponential (>0.97) were the next, finally the saturational curve followed (between 0.93 and 0.98) them. The explanation for this change is the pre-soaking step, since the abrupt initial part of the leakage curve was eliminated (Fig. 5).

In the first year during storage the correlation of the reciprocal function decreases, but those of the multiplicative and exponential increase (Fig. 6). The change is not significant in the case of the saturational curve. These results show that the mechanism of ion leakage has changed and the ratio of the speeds of the different processes has shifted during storage. In consequence of the increasing membrane permeability during storage, the importance of the processes described by an exponential function increases and the other physiological processes described by saturational and reciprocal equations fall into the background.

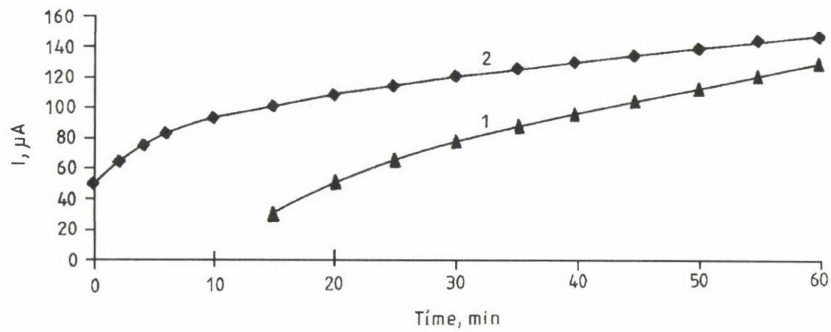


Fig. 5. The ion leakage curve with (1) and without (2) solution changing

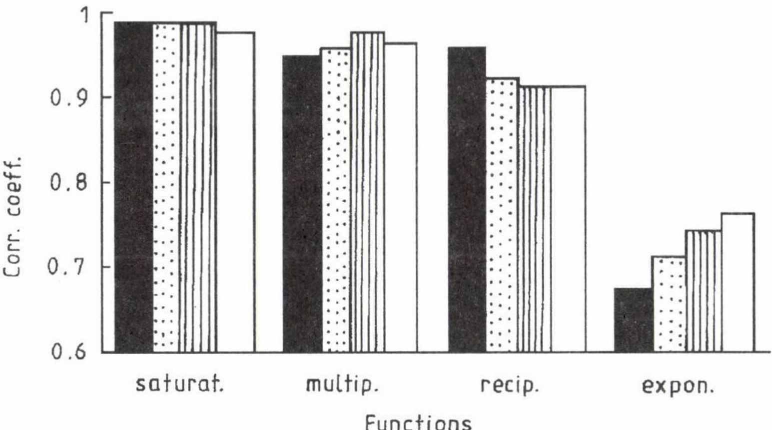


Fig. 6. The changes of correlation coefficients of functions fitted on the ion leakage curve during storage in the first year. ±s = saturational: 0.021; multiplicative: 0.043; reciprocal: 0.034; exponential: 0.062.

■ = 0 month; ▒ = 5 months; ▥ = 6 months; □ = 7 months

In support of the assumption mentioned above, the changes of the curve parameters were investigated, besides the correlation coefficients. The storage time has an effect on the parameters of the functions. This fact also confirms the change of the mechanism of ion leakage during storage (Fig. 7 and Fig. 8).

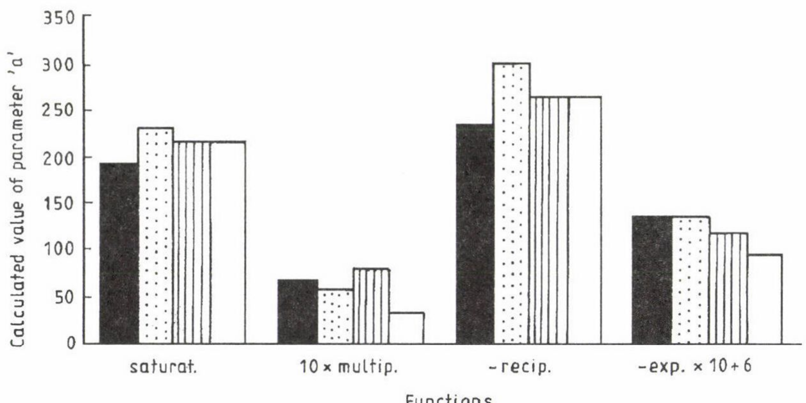


Fig. 7. The change of the 'a' parameter versus storage time in the first year. ±s = saturational: 4.24; multiplicative: 1.69; reciprocal: 5.11; exponential: 4.72 10^{-6}. ■ = 0 month; ▒ = 5 months; ▥ = 6 months; □ = 7 months

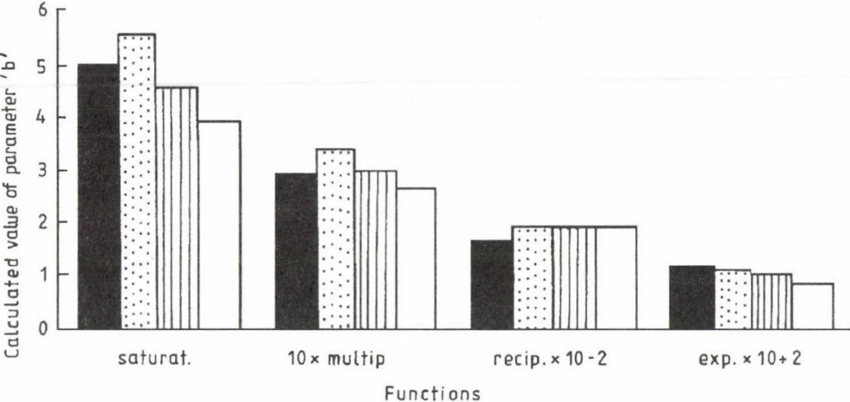


Fig. 8. The change of the 'b' parameter versus storage time in the first year. ±s = saturational: 0.30; multiplicative: 0.006; reciprocal: 2.61; exponential: 2.62 10^{-4}. ■ = 0 month; ▒ = 5 months; ▥ = 6 months; □ = 7 months

In the second and third years in spite of the fact that on the basis of the correlation coefficient the fitting of the linear function suits the measured data at every storage time, it can not be said that the ion leakage from the tissue is linear versus time. This is proved by the fact that, the distribution of the residuals between the measured points and the linear function is not random, but described by a curve which has a maximum (Fig. 9).

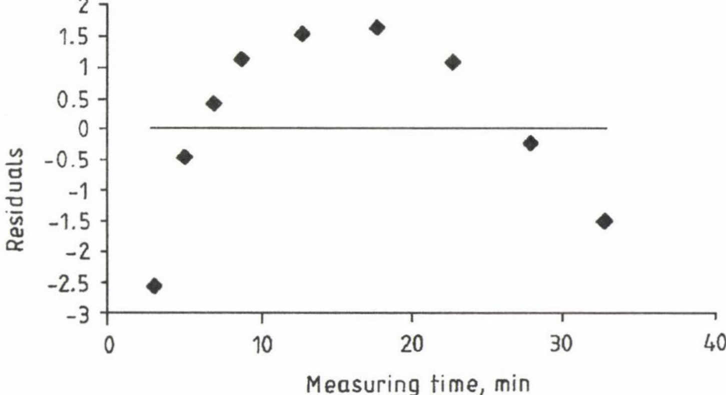


Fig. 9. Residuals between measured data and data calculated on the basis of a linear equation fitted to the leakage data

Because of the parabolic tendency of residuals the linear function was completed by a quadratic element and the fitting of this new equation was investigated:

$$Y = a + bt - ct^2.$$

The correlation coefficient is above 0.995 and the distribution of the residuals is normal. It follows that the quadratic polinom describes the ion leakage curve better than the linear one. The storage time and the cultivar have an effect on the parameters 'a' and 'b' of the quadratic polinom, but no factors have any effect on the parameter 'c' and the correlation coefficient.

The initial rate of ion leakage was also determined. There are significant differences among the initial rates of ion leakage of the 6 investigated cultivars (Fig. 10). The initial rate of ion leakage of Golden is the lowest, that of Gloster is the highest and the rate of Jonathan is significantly higher than the average. The differences might be caused by the differences of the cell structures of cultivars.

The change of the kinetic constant during storage yields the most information on the modification of cell wall and membrane. Averaging the results for all cultivars, the constant changes according to a curve, which has a maximum after 5 months of storage (Fig. 11).

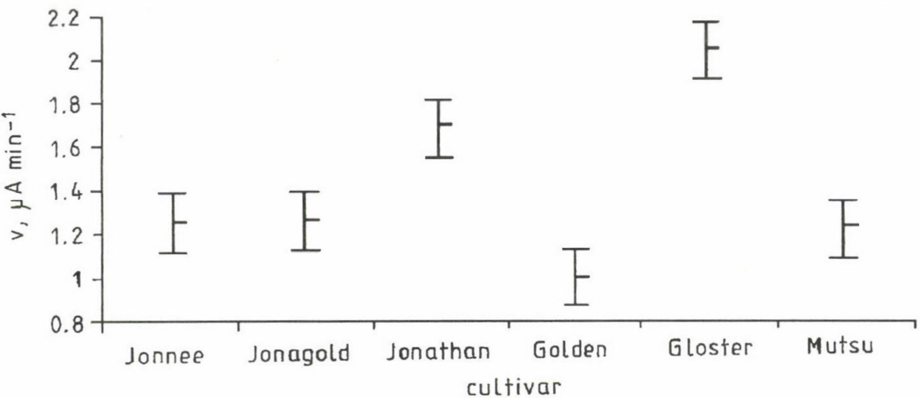


Fig. 10. The effect of the cultivars on the initial rate of ion leakage

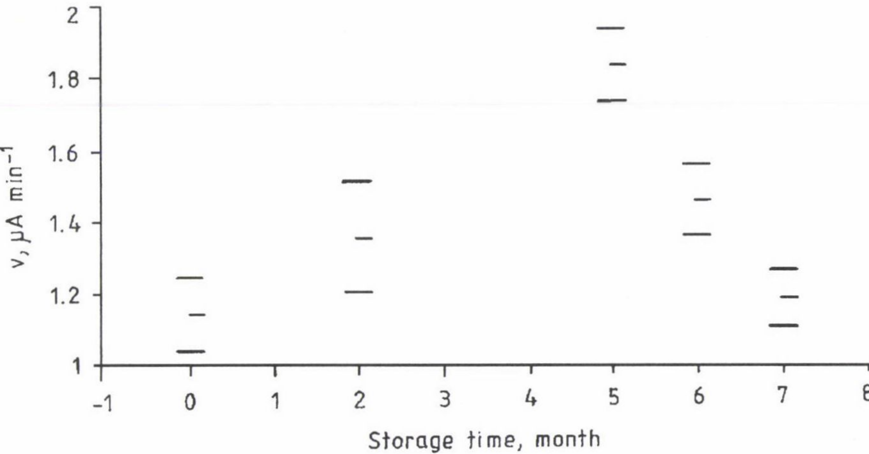


Fig. 11. The change of the initial rate of ion leakage during storage

The different levels of the changes in the initial rate of ion leakage during storage in case of each cultivar refer to significant differences among the cultivars (Fig. 12). Consequently the speed and the character of the senescence, metabolic processes are different. The measurement confirms that these cultivars have different storability, in some cultivars the metabolic reactions run faster than in others under the same conditions.

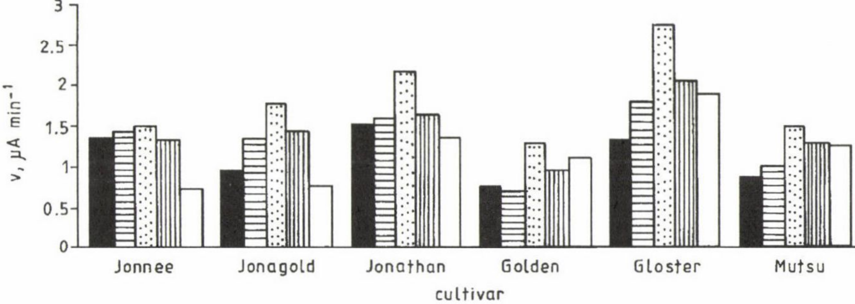


Fig. 12. The change of the initial rate of ion leakage during storage by cultivars. Mean ±s = 0.08. ■ = 0 month; ▤ = 2 months; ▒ = 5 months; ▥ = 6 months; □ = 7 months

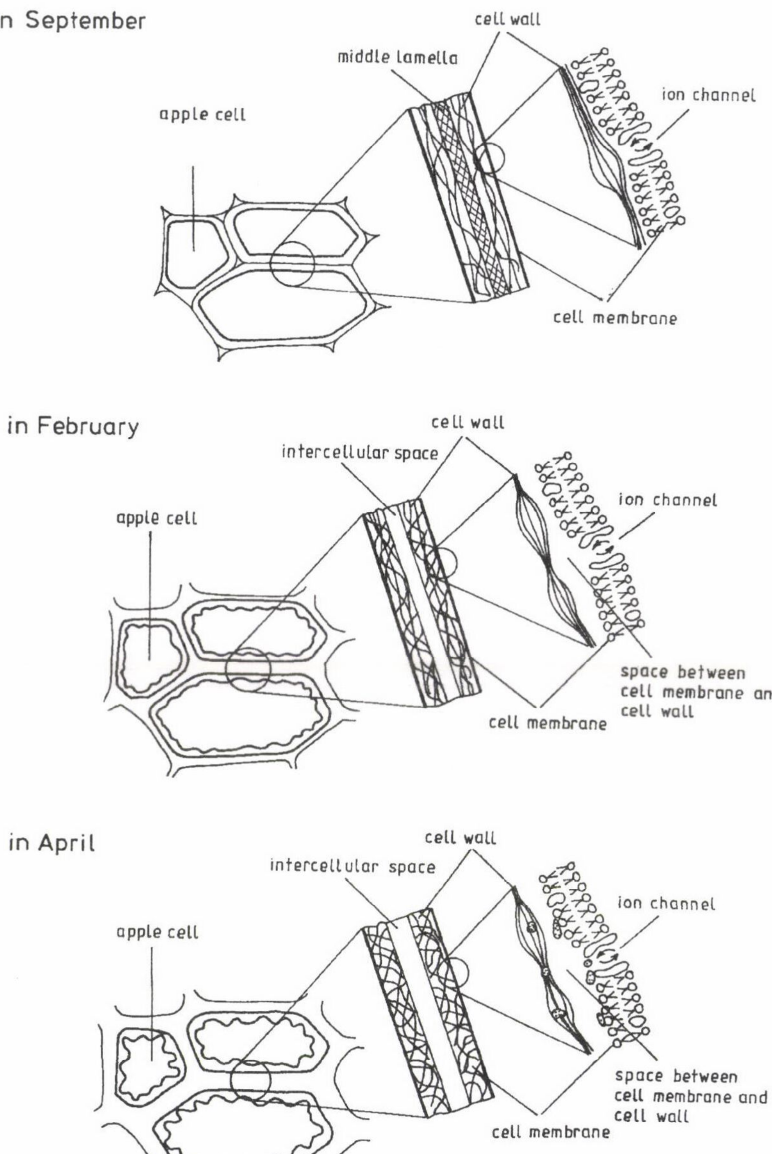


Fig. 13. The changes of the structure of the cell wall-membrane complex during storage

3. Conclusions

The measurement of the ion leakage from the tissue into an isotonic solution characterizes well the condition of plant cells, the permeability of the cell wall-membrane complex. The change of the fitting and the parameters of the mathematical functions describing the ion leakage curve relates to the change of the mechanism of ion leakage process.

The ion leakage curve measured after solution changing can be described best by a quadratic polinom:

$$Y = a + bt - ct^2.$$

The change of the initial rate of ion leakage is characterized by a curve with a maximum after 5 months of storage. Some researchers have obtained similar tendency in changes of the ion leakage.

A hypothesis was set up to explain this phenomenon: at the harvest and at the beginning of the storage the condition of the membrane determines the ion leakage process. The middle lamella between the cells contains mainly pectin. During storage this lamella breaks down, the membrane and the wall separate, the structure of both membrane and cell wall becomes loose. As a result the permeability increases. Later oligomers and other metabolic products of macromolecules bind to the membrane surface and into the wall. This process hinders the path of the ions, so the permeability decreases. In comparison to the highest value after 5 months of storage the initial rate of ion leakage decreases significantly after 7 months of storage (Fig. 13). The hypothesis is supported by present measurements, however, its verification requires further experiments (for example electron microscopy).

*

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BIOPROTECTION ON FRANKFURTER SAUSAGES

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The possibility of prolonging the shelf-life of refrigerated sausages by the utilization of *Lactobacillus alimentarius* was studied. Frankfurter sausages were inoculated with a suspension of *Lactobacillus alimentarius* (flora meat L-2 by Chr. Hansen), by sprinkling, resulting in approximately 10^7 CFU cm^{-2} on its surface. The sausages were then vacuum-packed and stored at +5 °C (±0.1 °C) and +10 °C (±0.1 °C) for 8 weeks. Each week, the water activity of the sausages was recorded. Furthermore, measurements of pH and bacterial counts for psychrotrophic microorganisms, aerobic mesophyles, Gram-negative and lactic acid bacteria were done. Simultaneously, a sensorial analysis was conducted for taste determination, texture, colour, aroma and overall acceptability. The samples inoculated with *Lactobacillus alimentarius* and kept at 10 °C showed a more rapid decrease in pH on day 7, whereas the rest of the samples showed a progressive pH decrease during storage. In control samples stored at 5 °C the pH decrease was observed only after 5 weeks of storage. When stored at 10 °C the same pattern of pH decrease was observed in the third week. The utilization of *Lactobacillus alimentarius* was successful for microbiological control, being effective against Gram-negative microorganisms. Indeed, inoculated samples stored at 5 °C presented counts which were 2 logarithmic cycles lower than control samples kept at the same temperature during storage up to the 6th week. Samples treated with *Lactobacillus alimentarius* and kept at 10 °C, presented a fall of one logarithmic cycle when compared to the control samples for the same storage period. In the treated samples, the counts for psychrotrophic microorganisms and aerobic mesophyles were high at the beginning (approximately 10^6 CFU cm^{-2}), due to the growth of the inoculated lactic acid bacteria. Whereas in the control samples the counts started with a lower number (10^2 CFU cm^{-2}), they increased during storage, exceeding the microbial counts of treated samples. The control samples, when compared to the treated ones, were considered unacceptable for consumption.

Keywords: sausage, bioprotection, *Lactobacillus alimentarius*

Meat can be highly perishable when improperly refrigerated due to its high water activity, its pH close to neutrality and the great quantity of nutrients (FRAZIER & WESTHOFF, 1993). Meat industrialization lengthen meat shelf-life, as in sausage, which

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can be preserved for a long time under refrigeration. In the industrialization process, there is a potential risk of incorporating bacteria to the sausage surface, during packing, which can cause alterations capable of shortening the shelf-life of the product (SARANTOPOULOS et al., 1992).

Recently, the antimicrobial effect of lactic acid bacteria for meat products biopreservation has been emphasized. These antimicrobial effects are probably due to a synergistic effect of the various antimicrobial properties of the lactic acid bacteria such as the production of organic acids, peroxide, carbon dioxide, the lowering of the oxidation-reduction potential, nutrient competition and bacteriocin production (ANDERSEN, 1995; JACK et al., 1995; PIARD & DESMAZEUD, 1992; KLAENHAMMER, 1988).

The major mechanism of action of *Lactobacillus alimentarius* is through nutrient competition with bacteria that causes product alterations as spoilage. Therefore, the addition of high levels of *Lactobacillus alimentarius* in the inoculum (10^7 CFU g^{-1}) is recommended to ensure a high level of initial activity (ARMSTRONG, 1996).

The fermentation of glucose and sucrose by *Lactobacillus alimentarius* produces very low acidity which has many advantages as there is no effect on flavour and taste of treated samples, due to the limited lipolytic and proteolytic activity of these bacteria (ARMSTRONG, 1996).

Lactobacillus alimentarius was tested in various meat products such as: sliced ham, ground beef, Frankfurter sausage, vacuum packed fresh meat and bacon (ARMSTRONG, 1996; LAULUND, 1996).

ANDERSEN (1995) demonstrated that *Lactobacillus alimentarius* was capable of inhibiting *Listeria monocytogenes* in Frankfurter sausage and bacon cubes without altering either the pH or the sensorial characteristics of the samples. Product quality was maintained during storage because *Lactobacillus alimentarius* was capable of inhibiting microorganisms that produce slime or bad smell.

The objective of this work was to evaluate *Lactobacillus alimentarius* performance on Frankfurter sausage. The samples were stored at 5 °C and 10 °C based on the fact that during sausage transport and storage, there are possibilities of variations on recommended refrigeration temperatures.

1. Material and methods

1.1. Samples

In this experiment Frankfurter sausage was used with the following ingredients: beef, poultry meat (mechanically separated), curing salts, polyphosphates, starch, spices and salt.

1.2. Lactobacillus alimentarius *inoculation*

A suspension of *Lactobacillus alimentarius,* consisting of 100 ml of destilled water and 50 g of FLORA CARN L-2 (Christhen Hansen Valinhus S.A.), was used to inoculate sausage samples. The inoculum suspension was sprayed on the sausage surface resulting in approximately 10^7 CFU cm^{-2} of *Lactobacillus alimentarius.*

1.3. Sample packaging and storing

After spray application of the *Lactobacillus alimentarius* suspension, the sausages were vacuum packed in packages weighing approximately 250 g and stored for 8 weeks at 5 °C(±0.1 °C) and 10 °C (±0.1 °C), respectively.

1.4. Microbiological analyses

The sausages were analysed right after treatment and every week. Swab tests were done on sausage surface at three sampling points resulting in an area of evaluation of 12 cm^2. The swab was mixed with 12 ml of peptone water, and this dilution was considered the zero dilution, which was used to do the other dilutions. The dilutions were plated for all microbiological analyses. Total count for aerobic mesophilic and psychrophilic microorganisms were determined using standard agar (MERCK), incubated at 32 °C for 48 h and at 7 °C for 8 days, respectively. Lactic acid bacteria counts were done using MRS agar with double layer and were incubated at 32 °C for 5 days.

Also, evaluation of Gram-negative microorganisms were done utilizing standard agar for counting (MERCK) and by adding 1 ppm of crystal violet and 50 ppm of 2-3-5-triphenyl tetrazolium chloride and incubating at ±32 °C for 48 h (GILLILAND & SPECK, 1975).

1.5. Water activity

The water activity determination was done after spray application following the method of TERRA and BRUM (1988).

1.6. pH

pH determination was carried out using a DIGIMED potentiometer equipped with a glass electrode, at the same time as the microbiological analysis (TERRA & BRUM, 1988).

1.7. Sensorial analysis

Simultaneously to the microbiological analysis, the sausages were evaluated by a taste panel that graded the product in a scale from 0 to 10 for product colour, flavour, taste, texture and appearance. Grades ranging from 9 to 10 were considered very good, from 7 to 8.9 good, from 5 to 6.9 regular, from 3 to 4.9 bad and from 0 to 2.9 unacceptable. Sausages were warmed up for 5 minutes before sensorial analysis started.

2. Results

The positive effects of *Lactobacillus alimentarius* utilization on sausage biopreservation was observed in the control of Gram-negative microorganisms, on samples treated with *Lactobacillus alimentarius* and maintained at 5 °C (Fig. 1). Reductions of two logarithmic cycles for Gram-negative microorganisms, were obtained when compared with controls, by the 6th week of storage. At the same time, when samples treated with *Lactobacillus alimentarius* were stored at 10 °C, a reduction of one logarithmic cycle was observed (Fig. 1).

Samples treated with *Lactobacillus alimentarius* showed increased aerobic mesophilic (Fig. 2) and psychrotrophic microorganisms (Fig. 3) counts at the beginning (10^6 CFU cm^{-2}) due to the growth of the inoculated lactic acid bacteria. In contrast, the control samples presented very low initial counts (approximately 10^2 CFU cm^{-2}), with increasing number of microorganisms during storage, outnumbering the counts of treated samples.

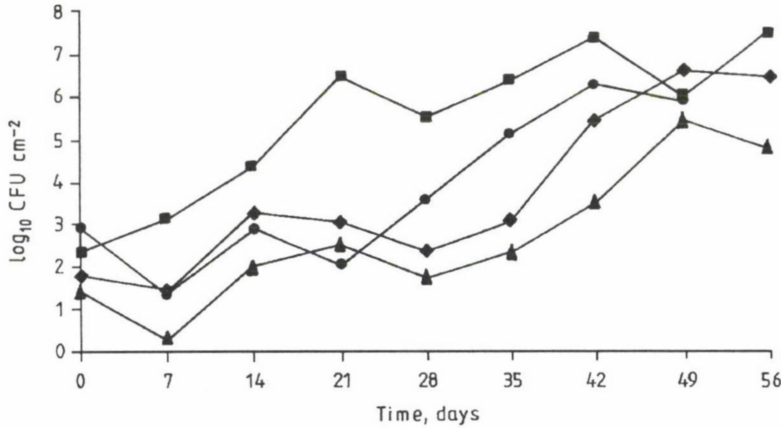

Fig. 1. Development of Gram-negative bacteria (log of CFU cm^{-2}) on sausages during storage at 5 °C (±0.1 °C) and at 10 °C (±0.1 °C). C (◆) = Control sausages stored at 5 °C; Cl (■) = control sausages stored at 10 °C; La (▲) = sausages inoculated with *Lactobacillus alimentarius* stored at 5 °C; Lal (●) = Sausages inoculated with *Lactobacillus alimentarius* stored at 10 °C

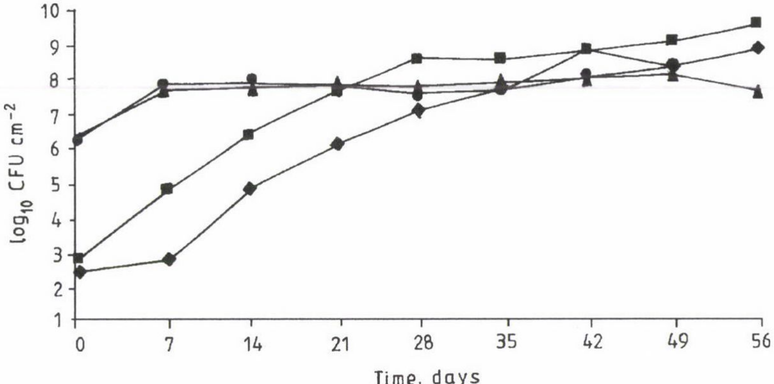

Fig. 2. Development of aerobic mesophilic microorganisms (log of CFU cm^{-2}) in sausages during the storage period at 5 °C (±0.1 °C) and at 10 °C (±0.1 °C). C (◆) = Control sausages stored at 5 °C; Cl (■) = control sausages stored at 10 °C; La (▲) = sausages inoculated with *Lactobacillus alimentarius* stored at 5 °C; Lal (●) = sausages inoculated with *Lactobacillus alimentarius* stored at 10 °C

Samples inoculated with *Lactobacillus alimentarius* presented higher levels of lactic acid bacteria right after inoculation (approximately 10^7 CFU cm^{-2}) compared with the controls (approximately 10^1 CFU cm^{-2}). However, lactic acid bacteria counts in control samples increased during storage reaching the same number as inoculated ones (Fig. 4).

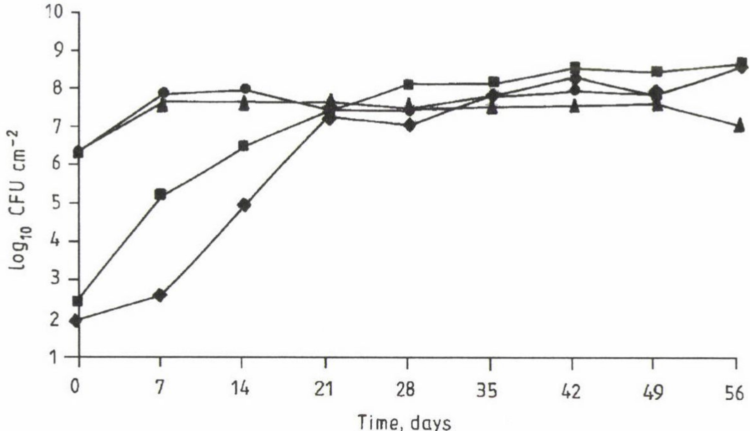

Fig. 3. Development of psychrotrophic microorganisms (log of CFU cm^{-2}) in sausages during the storage period at 5 °C (±0.1 °C) and at 10 °C (±0.1 °C). C (◆) = Control sausages stored at 5 °C; Cl (■) = control sausages stored at 10 °C; La (▲) = sausages inoculated with *Lactobacillus alimentarius* stored at 5 °C; Lal (●) = sausages inoculated with *Lactobacillus alimentarius* stored at 10 °C

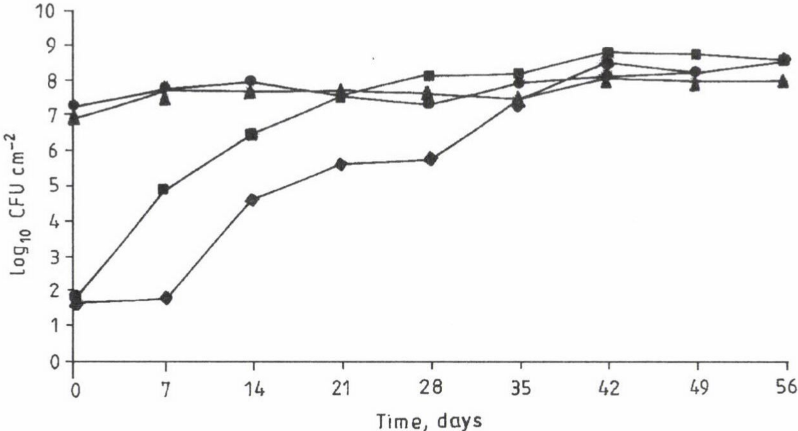

Fig. 4. Development of lactic acid bacteria (log of CFU cm^{-2}) in sausages during the storage period at 5 °C (±0.1 °C) and at 10 °C (±0.1 °C). C (◆) = Control sausages stored at 5 °C; Cl (■) = control sausages stored at 10 °C; La (▲) = sausages inoculated with *Lactobacillus alimentarius* stored at 5 °C; Lal (●) = sausages inoculated with *Lactobacillus alimentarius* stored at 10 °C

On average, the scores attributed to the sensorial properties of sausage samples treated with *Lactobacillus alimentarius* were slightly lower than that of the controls at the beginning of the experiment. However, after some time, the mean score increased when compared to the controls, indicating that *Lactobacillus alimentarius* contributed to the maintenance of the general quality of the samples during the storage period.

In our study, during storage, the development of a white liquid on the surface of control samples as well as gas production within the packages was observed, which contributed to the loss of vacuum. These alterations were observed in control samples stored at 10 °C after the 28th day, and for samples stored at 5 °C after 35th day of storage. The sausages inoculated with *Lactobacillus alimentarius* and stored at 10 °C also showed these alterations, at the same time period, but with lower intensity than the control samples. On the other hand, samples inoculated with *Lactobacillus alimentarius* and stored at 5 °C, did not show these alterations and had a very good appearance.

Samples treated with *Lactobacillus alimentarius* and kept at 10 °C presented a rapid drop in pH by the 7th day, while the controls gradually lowered the pH during storage (Fig. 5).

Sausage inoculation with *Lactobacillus alimentarius* did not affect its water activity, since all samples presented a value of 0.97 for water activity after the treatment.

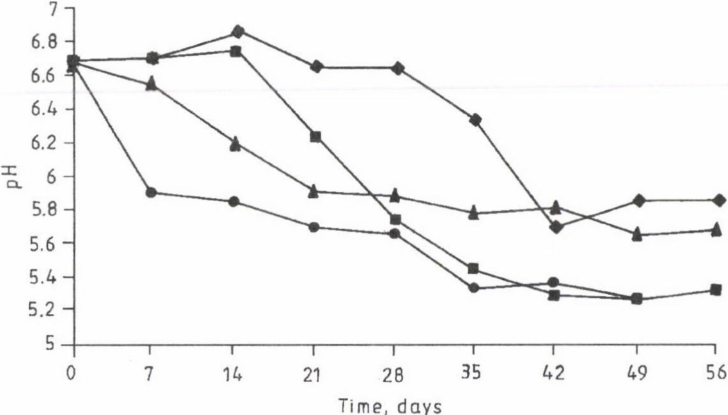

Fig. 5. Sausage pH during the storage period at 5 °C (±0.1 °C) and at 10 °C (±0.1 °C). C (◆) = Control sausages stored at 5 °C; Cl (■) = control sausages stored at 10 °C; La (▲) = sausages inoculated with *Lactobacillus alimentarius* stored at 5 °C; Lal (●) = sausages inoculated with *Lactobacillus alimentarius* stored at 10 °C

3. Conclusions

LAULUND (1996) suggested that total meat quality and meat products quality is influenced not only by total cell counts, but also by bacterial flora composition. In our study high count of mesophilic and psychrotrophic microorganisms in treated sausage samples was observed, initially, due to the growth of *Lactobacillus alimentarius* added to the samples. A high level of bacteria in the inoculum is very important, since it increases the initial activity, ensuring the inhibitory mechanism of nutrient competition (ARMSTRONG, 1996).

In this context, the microbiological examinations confirm that *Lactobacillus alimentarius* is able to suppress the Gram-negative bacteria by two logarithmic cycles. In this manner, the inhibition of undesirable microrganisms such as spoilage Gram-negative bacteria occurs when the microflora is dominated by harmless bacteria. These results agree with that of ANDERSEN (1995) who found a suppression of indigenous flora and the growth of *Listeria monocytogenes* when used a *Lactobacillus alimentarius* on Frankfurter-type sausages and bacon cubes.

On the other hand, SARANTOPOULOS and co-workers (1992) demonstrated that inside vacuum packages, "indigenous" heterofermentative lactic acid bacteria, that produce gases can develop. In our work, the loss of the vacuum was also observed in control samples, although the *Lactobacillus alimentarius* treated samples stored at 5 °C, did not show any of these alterations. The results of this experiment agree with that of

ARMSTRONG (1996), who concluded that starter cultures are capable of suppressing these alterations.

ROSSET (1994) has drawn attention on water activity variation on meat surface. The water activity variation has a great impact on superficial microbial growth, as the higher the water activity of the medium the more intense is the microbial development. With regard of the water activity measured in our experiment, the inoculation with *Lactobacillus alimentarius* on sausage surface did not affect its water activity (0.97), maintaining the same conditions as control samples and therefore, reinforcing the extreme importance of this inoculation on inhibiting growth of undesirable microorganisms.

Based on the results, we can conclude that the culture of *Lactobacillus alimentarius* was effective in controlling undesirable microorganisms in vacuum packages, being more effective when kept at 5 °C. When abusing temperature (10 °C), the culture assured a better general product quality, but was not as effective as at 5 °C. Furthermore, the overall quality of the sausages is improved during the storage because *Lactobacillus alimentarius* inoculation was able to inhibit the bacteria responsible for the production of white liquid on the surface of Frankfurter sausages as well as off-flavour and off-odour.

Therefore, the application of a bioprotective culture on sausage surfaces, by spray, should be considered as an additional safety factor in combination with careful food manufacturing practice and cold storage of the food. The appearance and nature of white liquid on the control samples is currently under investigation on our laboratory.

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EFFECT OF STORAGE TEMPERATURE, TIME, DISSOLVED OXYGEN AND PACKAGING MATERIALS ON THE QUALITY OF ASEPTICALLY FILLED ORANGE JUICE

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The interaction effects of beverage packaging materials (laminated cartons, high density polyethylene packs (HDPE), polyethylene-terephthalate (PET), and glass) on the quality and shelf life of commercially prepared orange juice were evaluated at two temperatures (4 and 24 °C) during 60 days of storage. Browning, loss of ascorbic acid, dissolved oxygen and absorbed d-limonene into packs or retained in juice were used as indicators for juice quality. The extent of browning and loss of ascorbic acid were found to be higher in HDPE packs than in cartons and lowest in PET and glass. The ascorbic acid content and rate of browning were significantly affected by the level of dissolved oxygen in the juice. The content of d-limonene in juice stored in HDPE packs and cartons was reduced by 33.6% and 21%, respectively, compared with 11% and 9.1% for PET and glass after 15 days of storage at 24 °C. The reduction of d-limonene content in juice was related to the absorption rate of each type of package. Absorption of d-limonene was greater in HDPE packs (5.3 mg dm^{-2}) and cartons (4.2 mg dm^{-2}) than in PET (0.26 mg dm^{-2}). The conditions which lead to the change in the quality of juice are discussed.

Keywords: orange juice, packaging materials, d-limonene absorption, ascorbic acid, dissolved oxygen

Many recent developments in food packaging are related to the barrier properties of plastics in extending the shelf life of foods (ARORA et al, 1991). These include the development of different types of polymers, copolymers, laminates and other packaging materials. Considerable improvement in design effectiveness has been achieved with the introduction of new materials and processes, making this area one of the most effective in food product development. Hence, this growth has been paralleled by an increasing attention to the interaction between plastic packaging materials and foods (KIM-KANG, 1990) and the effects that these might have on food quality.

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The quality of orange juice is related mainly to its ascorbic acid content, colour, and flavour ingredients. Temperature, level of oxygen, storage time and pack types are the most critical factors influencing the quality. Various studies have been conducted to evaluate the effect of polymeric packaging materials on orange juice quality and its flavour ingredients. It is reported that dissolved oxygen, storage time and temperature affected the ascorbic acid degradation and non-enzymatic browning. Flavour was lost from the fresh orange stored in polyethylene containers after three months of storage at room temperature (DURR et al., 1981). Significant absorption of citrus flavour into polyethylene within a few days at 24 and 35 °C is reported (HIROSE et al., 1988). An acceleration of ascorbic acid degradation and browning was observed due to contact of orange juice with polyethylene film (MANNHEIM et al., 1987). The effect of storage temperature of citrus juice on the degradation rate of ascorbic acid (NAGY, 1980; GRAUMLICH et al., 1986; KAANANE et al., 1988; and KENNEDY et al., 1992) and non-enzymatic browning have been studied (KANNER et al., 1982). The adverse effect of dissolved oxygen on the quality of citrus juices has been reported. Higher dissolved oxygen causes an increase in ascorbic acid degradation (BISSET & BERRY, 1975; KACEM, 1987; KACEM et al., 1987; KENNEDY et al., 1992; SIZER et al., 1988; SOLOMON et al., 1995; TRAMMELL et al., 1986) and browning (KACEM et al., 1987; ROBERTSON & SAMANIEGO, 1986; SOLOMON et al., 1995; TRAMMELL et al., 1986).

However, in rare of the previous studies the combined impact of the variables or quality indicators have been studied or quantified. Moreover, no studies have been published regarding the stability of orange juice in PET bottles. Hence, the aim of this study was designed to compare the effects of the most known citrus juice pack types: glass, laminated cartons, high density polyethylene (HDPE), and polyethylene-terephthalate (PET) on the quality and shelf life of orange juice at two temperatures (24 and 4 °C), taking ascorbic acid degradation, d-limonene content in package and juice, and rate of browning as indicators of the overall quality of juice.

1. Materials and methods

1.1. Materials

1.1.1. Packs. Transparent glass bottles with screw caps were used. Laminated carton packages consisting of four layers (polyethylene/ paper board/ aluminium/ polyethylene) and packed with orange juice were obtained from the factory immediately after packaging. New high density polyethylene (HDPE) and polyethylene-terephthalate (PET) bottles were obtained from SOLVAY Brussels (ELTEX B 4020). The bottles were sterilized by hydrogen peroxide (H_2O_2) 10% (FAO/WHO, 1982), followed by rinsing three times with sterile distilled water.

1.1.2. Juice. Juice samples in laminated carton packages consisted of ready-to-drink commercial orange juice. These were prepared from orange juice concentrates (60–65 °Brix) that were reconstituted with sterile water to give single-strength orange juice at 12 °Brix. They were obtained from the manufacturer immediately after production and packaging. The packages contained 1 l of juice.

1.2. Methods

1.2.1. Preparation. Reconstituted orange juice from laminated carton packs was immediately transferred to sterilized glass jars and plastic packages (HDPE and PET) (1 l) in an anaerobic cabinet (Forma Scientific model 1024: 84% N_2, 8% CO_2, 8% H_2 atmosphere). The glass, carton and plastic packages were stored in the dark at 4 °C and 24 °C for 60 days. The experiments were run in triplicate. Analyses were done in duplicate.

1.2.2. Quality indicators. Ascorbic acid was determined by HPLC consisting of a Gilson pump (Model 805) at a flow rate of 2.5 ml min^{-1}, a 250×4.6 mm Li Chr NH_2 column, (Gilson) U.V. detector (118 UV/VIS) set at 254 nm, and Shimadzu recorder (C-R1B Chromatobac). The mobile phase was 75:25 (v/v) acetonitrile/0.05 mol l^{-1} KH_2PO_4, pH 5.95 (TUAN & WYATT, 1987). Orange juice (1 ml) was transferred to a 10 ml volumetric flask and made to volume with distilled water. The diluted orange juice was then filtered through a 0.45 μm filter and kept in ice for a few minutes before being injected into the HPLC (SOLOMON et al., 1995). D-limonene in juice and in packaging material was determined by the bromide-bromate titration method (SCOTT & VELDHUIS, 1966). In order to extract the d-limonene from carton and plastic materials, the whole carton or plastic packages was cut up (1×0.5 cm pieces) and rinsed with distilled water. The package pieces were then placed into a distillation flask. 150 ml of isopropanol was added so that the sample was completely immersed. The sample/isopropanol mixture was allowed to stand for approximately 24 h. This extraction step was repeated three times to ensure the recoveries of all d-limonene from carton and plastic (HIROSE et al., 1988). 100 ml of water was then added to the sample and distilled to determine the amount of d-limonene in packs by the bromide-bromate titration method.

Optical density was measured as an indicator of browning. The juice was centrifuged at 900 g for 20 min before adding ethanol (1:1) to the filtrate and then filtered. The optical density of clear juice was read at 420 nm in a Bausch & Lomb Spectronic 1001 spectrophotometer (MEYDAV et al., 1977). This test was carried out in addition to visual inspection of colour changes.

Dissolved oxygen was measured immediately after opening the pack using a dissolved O_2 electrode Monitor (KELMA-BVBA, Belgium). The samples were

continuously shaken at a reproducible rate to ensure a constant oxygen supply to the sensor. The reading was taken when a steady value was reached (5 min).

1.2.3. Statistical analysis. Every treatment was performed three times. Two analyses were taken from the test samples at each specific time interval. Mean values and standard deviations were calculated at each time interval and analyzed by SPSS version 6 (SPSS Inc. 444 N. Michigan Avenue. Chicago, Illinois, 60611) for analysis of variance (one way, two way, and three way), F-ratio ($P \leq 0.05$) and Multiple regression.

2. Results and discussion

2.1. Oxygen content and juice quality

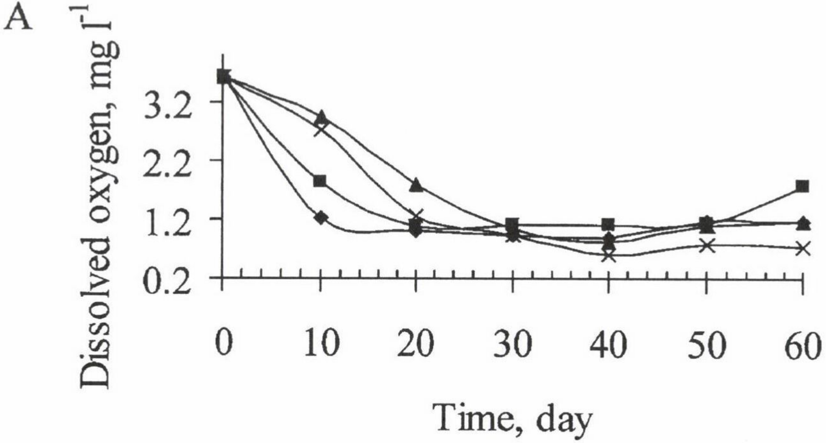
On the packaging day, the concentration of dissolved oxygen in the orange juice in different types of containers was 3.63 mg l^{-1}. The difference in the initial dissolved oxygen content between the packs was negligible since the filling was performed under anaerobic conditions. As seen in Fig. 1 A, B, the oxygen content tended to decrease during the storage in all types of packs at both storage temperatures (24 and 4 °C). In glass and PET packs, gradual and consistent loss of oxygen in orange was noticed up to 60 days of storage. In the laminated carton and HDPE oxygen content decreased within 20 days. Thereafter, further increases occurred during the rest of storage. It is reported that the PET film has low permeability characteristics compared to high permeable HDPE film (GEORGIN, 1986) and poor oxygen barrier laminated cartons (MARSHALL et al., 1986). It is reported that the oxygen content in orange juice stored at 8 °C in glass container with carton lid increased from 1 mg l^{-1} to 8.3 mg l^{-1} during 52 days of storage. Similar findings have been reported in other studies about the effect of oxygen on the quality of orange juice (KENNEDY et al., 1992; SOLOMON et al., 1995).

In the present study, in HDPE and laminated carton packs, it is observed that in some cases the dissolved oxygen was higher at 4 °C than at 24 °C. This could be due to a high consumption rate of oxygen through different types of reactions at a higher storage temperature (GRAUMLICH et al., 1986).

2.2. Ascorbic acid

The change in ascorbic acid in the orange juices in different packaging materials during 60 days of storage at 24 and 4 °C is presented in Fig. 2 A, B. The contact of orange juice with laminated cartons, HDPE and PET packs accelerated ascorbic acid loss compared to similar juices stored in glass jars at the same temperatures. The 3-way analysis of variance revealed that the interaction between the three variables (temperature, time, and type of packages) had a significant effect ($P \leq 0.05$) on ascorbic

acid content. The ascorbic acid degradation in HDPE packs (22.9%) was more pronounced than in laminated cartons (5.9%), PET bottles (5.1%) and glass (4.6%), after 10 days storage at 24 °C. Further, after 20 days of storage the loss increased to 23% and 11% in HDPE packs and laminated cartons, respectively, while it was 10.3% in PET and 9% in glass bottles. Generally, there is a rapid degradation of vitamin C in orange juice in the early stage of storage followed by a gradual loss. Meanwhile, these rates were lower at 4 °C. The regression analysis showed a good correlation between storage time and degradation of ascorbic acid and followed a first order reaction. Other workers (KENNEDY et al., 1992) reported similar results in the degradation of ascorbic acid stored at 4, 20, and 37 °C in TetraBrik carton packs. Other reports (KANNER et al., 1982) showed that the loss of ascorbic acid in 58 °Brix orange concentrates followed first order reaction kinetics at temperatures of 25 °C and below. The rate of oxidation of ascorbic acid, however, is temperature dependent, it is affected by the dissolved oxygen level (SOLOMON et al., 1995; ROBERTSON & SAMANIEGO, 1986). This reflected in increasing the rate of ascorbic acid loss in laminated carton and HDPE packs compared to PET bottles. These results are in accordance with the results reported in other previous studies (KENNEDY et al., 1992; SOLOMON et al. 1995; NAGY & SMOOT, 1977). Hence, during the time of storage the permeability of each type of pack affects the ascorbic acid content. In the study of the effect of pack types (carton and glass) on the quality of fruit juices (GHERARDI, 1982) high oxygen and low carbon dioxide content was found in the head space of carton packs versus low oxygen and high carbon dioxide content in glass bottles. It is concluded that the main cause for the difference in keeping quality between products in carton packs and glass jars was due to higher oxygen transfer rates into the former package (GHERARDI, 1982). Furthermore, in aseptic orange juice, it is reported that although, ascorbic acid destruction could continue in the absence of dissolved oxygen, it is present in the product or in the container headspace or permeating through the container accelerates these processes and reduces shelf-life (GRAUMLICH et al., 1986). Moreover, the storage temperature had also a significant effect ($P \leq 0.05$) on the ascorbic acid loss. After 60 days of storage at 24 °C and 4 °C the loss of ascorbic acid were 51.65 and 46.7% in HDPE packs, 28.45 and 13% in laminated cartons and 25.1% and 13.9% in glass jars, respectively. The ascorbic acid retention was found to be better at low temperature in glass containers, LDPE and HDPE containers which contained model solutions and were stored for 60 days at 4, 23 and 50 °C (ADSULE & ANAND, 1977). Moreover, previous studies concluded that the most important factor in determining the shelf-life of aseptic orange juice and its affect on the degradation of ascorbic acid is storage temperature (GRAUMLICH et al., 1986; KAANANE et al., 1988; KENNEDY et al., 1992 and KANNER et al., 1982).



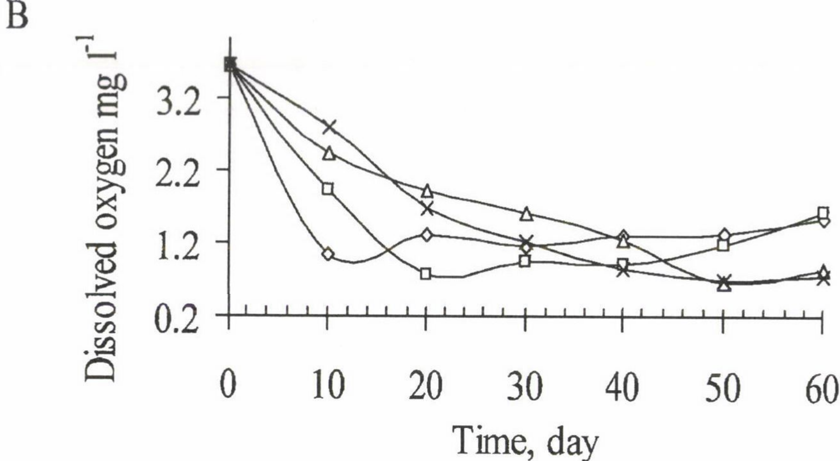
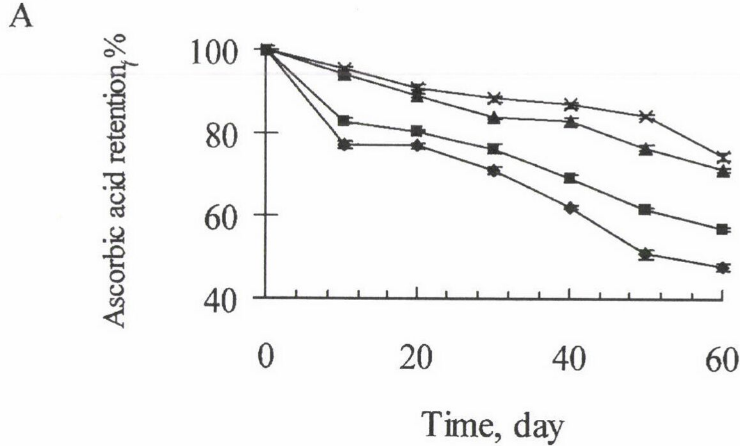


Fig. 1. Dissolved oxygen in orange juice packed into glass bottles ×, laminated carton packs ■, □, HDPE packs ◆, ◇, and PET packs ▲, Δ during 60 days, stored at 24 (A) and 4 °C (B) respectively



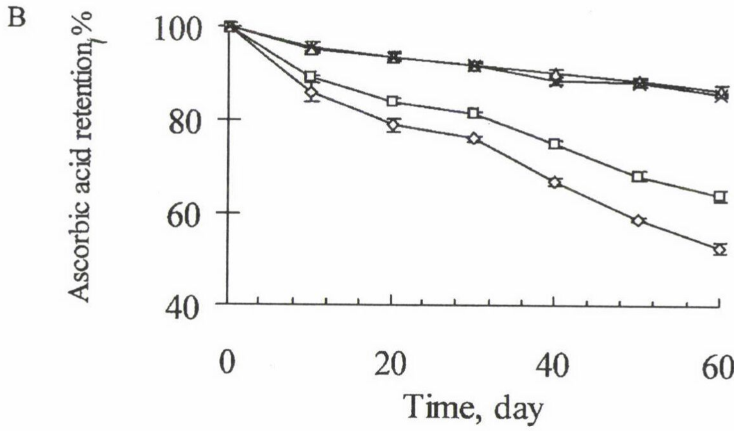


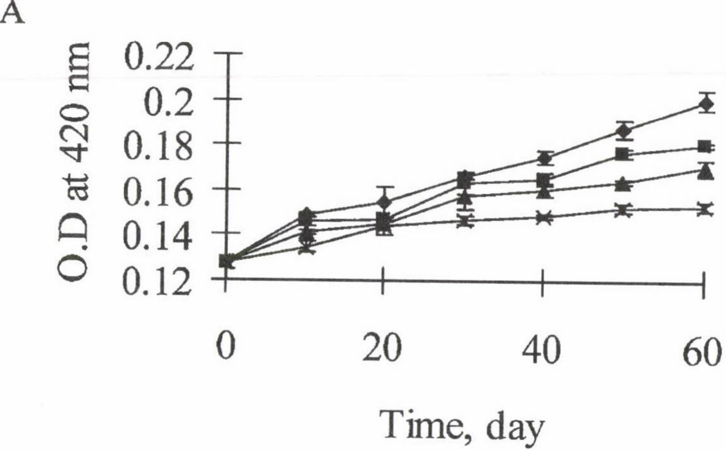
Fig. 2. Ascorbic acid retention in orange juices aseptically packed into glass bottles ×, laminated carton packs ■, □, HDPE packs ◆, ◇, and PET packs ▲, Δ during 60 days, stored at 24 (A) and 4 °C (B) respectively

2.3. Browning

The rate of browning in different packaging materials during 60 days of storage at 24 and 4 °C is presented in Fig. 3 A, B. The rate of browning was affected in a similar manner as ascorbic acid. The 3-way analysis of variance revealed that the interaction between the three variables (temperature, time, and package type) had a significant effect ($P \leq 0.05$) on the browning rate. The results showed that the package type plays a significant role on the rate of browning. After 60 days of storage at 24 °C the rate of browning had increased more in HDPE packs (57% than in laminated carton (41%), and to a lower extent in PET (33.6%) and in glass jars (20.3%). As it was postulated before for ascorbic acid degradation, the linear regression analysis showed a good correlation between storage time and the rate of browning, which also followed a first order reaction. In the present study there was also a significant effect ($P \leq 0.05$) of storage temperature on the colour of orange juice. The rate of browning was significantly less at 4 °C than at 24 °C for the three package types (Fig. 3). It is noticed also that there is a relation between browning and ascorbic acid loss during storage. It is showed that increased levels of dissolved oxygen result in increasing oxidation of ascorbic acid, and thus, increasing the development of brown pigment in the juice (SOLOMON et al., 1995).

2.4. D-limonene content in orange juice

The reduction of d-limonene in orange juice stored at 24 and 4 °C for 60 days in the different type of packs is presented in Fig. 4 A, B. A rapid reduction in d-limonene content in orange juice in HDPE packs (33.6%) and laminated cartons (20.9%) was noticed in the first 10 days at 24 °C and to a lesser extent in PET (11.0%) and in glass jars (9.1%). The rate of reduction was more pronounced in HDPE packs and laminated cartons after 30 days of storage at 24 °C. In the same manner, at 4 °C the d-limonene content decreased significantly in HDPE and laminated cartons after 10 days. Moreover, temperature significantly affected ($P \leq 0.05$) the d-limonene content in orange juice. Overall, the main tested parameters (package type, temperature and time) had a significant effect on the limonene content. The 2-way analysis of variance showed a significant interaction effect between the package types and time.



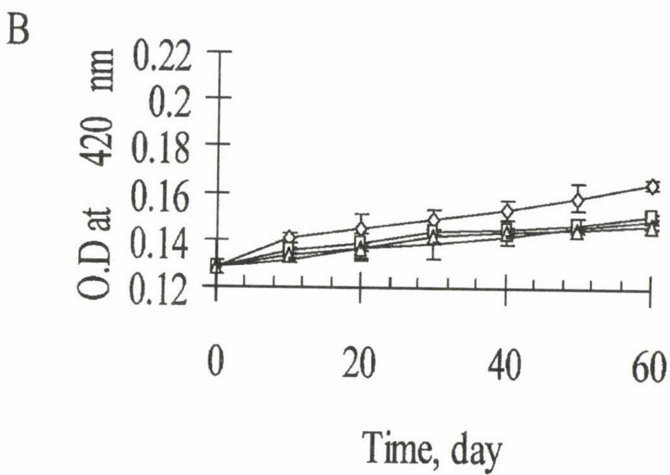
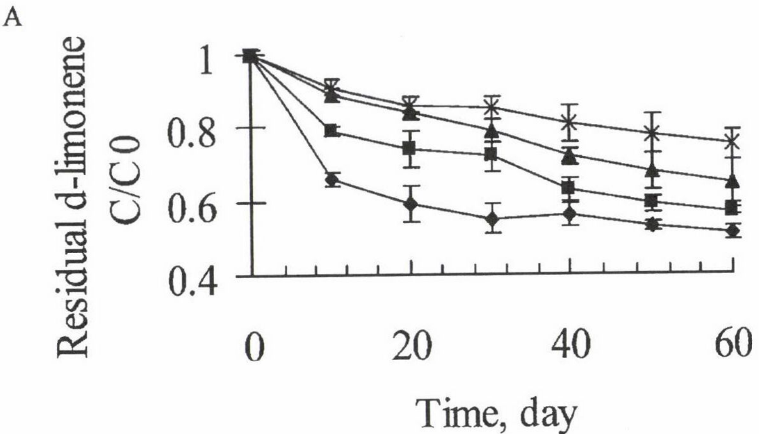


Fig. 3. Browning of aseptically filled orange juices in glass bottles ×, laminated carton packs ■, □, HDPE packs ◆, ◇ and PET packs ▲, Δ during 60 days, stored at 24 (A) and 4 °C (B) respectively



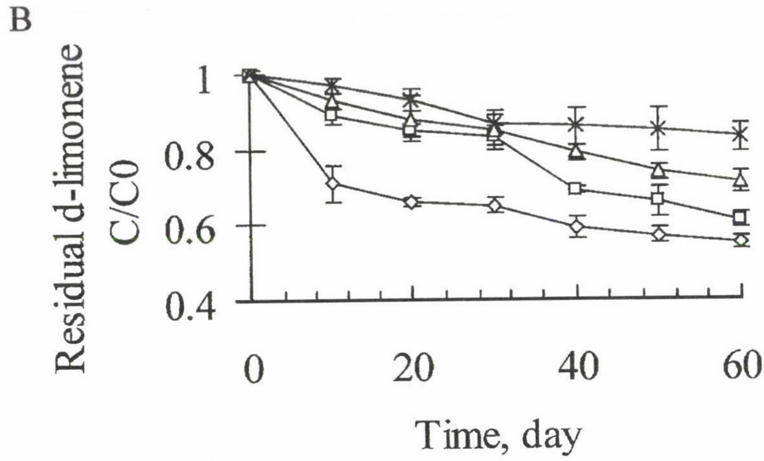


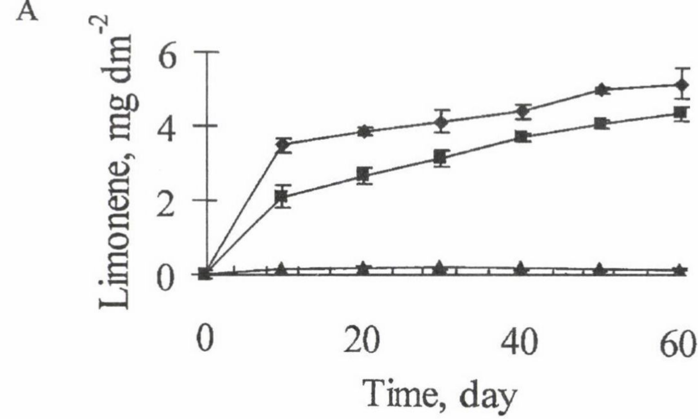
Fig. 4. d-limonene residual in orange juices aseptically packed into glass bottles ×, laminated carton packs ■, □, HDPE packs ◆, ◇ and PET packs ▲, Δ during 60 days, stored at 24 (A) and 4 °C (B) respectively

In the present study, at the end of storage at 24 °C the loss of d-limonene was 47.5%, 40.9%, 32.0% and 20.9% in HDPE packs, laminated cartons, PET and glass, respectively. It is reported (MANNHEIM et al., 1987) that the loss of d-limonene was from 80 to 60 mg l^{-1} (25%) in carton pack juice during the first five days of storage at 25 °C. An other report (HIROSE et al., 1988) observed the same rate of d-limonene loss in the orange juice stored in carton packs, from 25 mg to 19.1 mg (20.4%), at 24 °C within three days of storage. This was apparently due to absorption of limonene by the polyethylene contacting surface. It is also reported (HIROSE et al., 1988) that rapid absorption rate of d-limonene into polyethylene occurred in the beginning of storage and decreased after 12 days of storage when the saturation state was reached. The loss of d-limonene for the samples stored in glass could be due to a consumption rate of oxygen through storage time.

2.5 Limonene in packs

The absorption of d-limonene into HDPE packs, laminated cartons and PET bottles was determined at 24 and 4 °C (Fig. 5 A, B). A higher absorption rate observed in HDPE packs followed by laminated cartons (LDPE layer) and PET, respectively. There was a significant effect of the main tested parameters (packages, temperature and time) on the limonene content of the packs. In the 2-way analysis of variance there were significant effects of package types and time on the absorption rate of limonene. Further, there was a significant difference evaluated by three way analysis of variance which confirmed the role of the three variables on limonene absorption.

It is shown that the absorption of citrus oil constituents was high with LDPE, because of its large amorphous area and low crystallinity, while absorption was less in the case of HDPE and PP (CHARARA et al., 1992). The same trend for LDPE was reported (SADLER & BRADDOCK, 1991). In the present study the absorption of limonene was higher in HDPE compared to LDPE (laminated carton). This could be explained by the difference of thickness between the two types of polyethylene employed. HDPE had a thickness of 5×10^{-1} mm, ten times more than that of LDPE (5×10^{-2} mm). The present results showed that the limonene absorption to laminated cartons was 3.2 mg dm^{-2} after 30 days at 24 °C which was in good agreement with the previous results (HIROSE et al., 1988). Our results showed that the dissolved oxygen is an additional factor that can be affected by the d-limonene content. Limonene absorption produced an increase in oxygen permeability which appeared proportional to limonene concentration (SADLER & BRADDOCK, 1990).



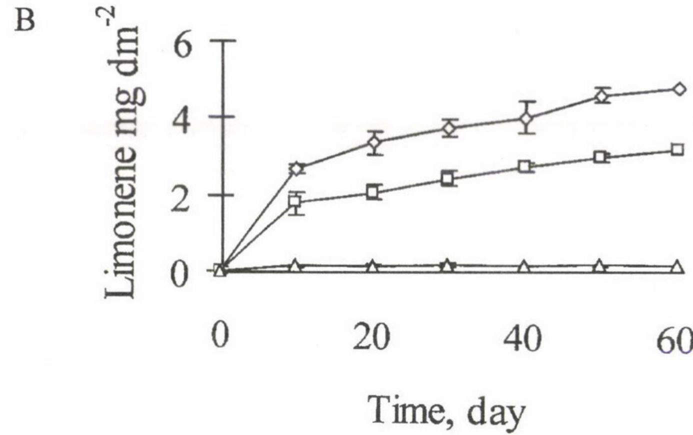


Fig. 5. Absorption of d-limonene (mg dm^{-2}) into laminated carton packs ■, □, HDPE packs ◆, ◇, and PET packs ▲, Δ during 60 days, stored at 24 (A) and 4 °C (B) respectively

3. Conclusion

Our study clearly indicated the effect of package types on the quality of aseptically filled orange juice. The best keeping quality was observed with glass bottles, PET bottles, then laminated cartons and the lowest were with HDPE packs. Reactions, such as ascorbic acid degradation and browning were accelerated in laminated cartons

and HDPE packs as compared to PET bottles and glass jars. The level of dissolved oxygen is an important factor in the degradation of ascorbic acid and browning. Rate of ascorbic acid degradation and browning followed first order reaction kinetics. D-limonene concentration was reduced rapidly in HDPE packs and laminated cartons. This loss is related to the increase in absorption rate of d-limonene into plastics which was observed to be higher in HDPE than in laminated cartons and less in PET bottles. This result confirmed that the amount of d-limonene absorbed into different polymer materials depends on the nature of the polymer. Storage temperature and time greatly influenced the quality of aseptically packaged orange juice.

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OSMOTIC DEHYDRATION IN APPLE: INFLUENCE OF VARIETY, LOCATION AND TREATMENT ON MASS TRANSFER AND QUALITY OF DRIED RINGS

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Golden Delicious and Red Gold varieties grown at three different agroclimatic locations, with or without pretreatments were evaluated for mass transfer behaviour upto 4.5 h of osmotic dip and the effect of the dip on the quality of osmo-vac dried rings was compared with conventionally dried rings. Data for mass reduction (MR), water loss (WL) and solid gain (SG) during osmotic dip and for chemical constituents and overall acceptability of dried rings were recorded, which differed significantly. Apple rings made out of wet temperate and high hills (L_2) location exhibited higher MR and WL. In general, Golden Delicious variety, irrespective of any location, yielded good quality osmo-vac dried rings. However, the rings from high hills and cold desert (L_3) location were the most acceptable, owing to crunchy texture, good taste/flavour with higher ascorbic acid retention and less browning. Treatment of prepared rings with potassium metabisulphite/ascorbic acid proved essential in checking oxidation during handling and drying, whereas blanching resulted in undesirable penetration of sugars into the rings.

Keywords: apple, osmotic dip, dehydration, mass reduction, water loss, solid gain, chemical parameters, overall acceptability

Apple (Malus x domestica Borkh.) is undoubtedly the most important temperate fruit of the world. Out of 48.890 million tonnes of the world apple production, India produces 1.238 million tonnes (F.A.O., 1994). Himachal Pradesh, Jammu and Kashmir and hill districts of Uttar Pradesh are the main states producing apple in India. Increased production in recent years has resulted in more amounts of processing grade fruit and industry seeks new methods of processing.

Apple is processed into a variety of products (WAY & MCLELLAN, 1989), amongst which juice, concentrate, canned sauce, canned slices, dried slices and frozen slices are the most important. Conventional dehydration of apple slices leads to a product of dark colour, leathery texture and poor flavour with a loss of nutritive value.

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Innovation of novel techniques is taking place constantly and the ultimate aim is to keep the initial wholesomeness in the final product. Osmotic dehydration is one of these techniques.

Many authors have studied different aspects of osmotic dehydration in apple. The quantity and rate of water removal and solid gain are affected by several variables and processing parameters. In general, it has been shown that the weight loss and solid gain in osmosed fruit depends upon the solute concentration in the osmotic solution (PONTING et al., 1966; FARKAS & LAZAR, 1969; BOLIN et al., 1983), temperature (PONTING et al., 1966; LE MAGUER, 1988), sample to solution ratio (BONGIRWAR & SREENIVASAN, 1977; CONWAY et al., 1983), sample size and shape (ISLAM & FLINK, 1982; LERICI et al., 1985) and pretreatments (LERICI et al., 1985; SAUREL et al., 1994; LAZARIDES et al., 1995). However, there is lack of information on variety and agroclimatic location effect on mass transfer behaviour during osmotic dip and quality of osmotically treated dried rings. Therefore, in this study, two varieties from three different agroclimatic locations of Himachal Pradesh, with or without any pretreatment were tested in order to evaluate their effect on mass reduction (MR), water loss (WL), and solid gain (SG) for different periods of osmotic dip and the quality of osmo-vacuum and conventionally dried rings were evaluated.

1. Materials and methods

1.1. Raw material

Medium size fruits of Golden Delicious and Red Gold varieties from three different agroclimatic locations of Himachal Pradesh viz., (i) sub temperate sub humid mid hills (L_1), (ii) wet temperate high hills (L_2) and (iii) dry temperate high hills and cold desert (L_3) were harvested at optimum maturity during the year 1995. The fruits were manually peeled and cut into uniform rings (13 mm thickness) with slicer.

1.2. Treatments

To study the effect of different treatments on mass transfer behaviour and quality of dried rings, the following pretreatments were given:

T_0 = steam blanching (3 min) + potassium metabisulfite dip (0.5% for 30 min)
T_1 = osmotic dip
T_2 = potassium metabisulfite dip (0.5% for 30 min) + osmotic dip
T_3 = steam blanching (3 min) + osmotic dip
T_4 = ascorbic acid dip (0.5% for 30 min) + osmotic dip

1.3. Osmotic solution and process

A 70 °Brix sucrose solution, temperature maintained at 50 °C was used as an osmotic solution for dip. The prepared rings, according to treatments (T_1–T_4), were immersed in the solution in 1:3 ratio for varying periods (0.5, 1.5, 3.0 and 4.5 h) without agitation. Two kg rings were used for each treatment and the experiment was replicated thrice.

1.4. Observations for mass transfer

The rings, after different intervals (0.5, 1.5, 3.0 and 4.5 h), were taken out from the osmotic bath, drained, washed in warm water to remove the adhering syrup and observations for MR, WL, and SG were recorded:

$$\mathrm{MR}\ (\%) = \frac{\mathrm{IM} - \mathrm{MT} \times 100}{\mathrm{IM}}$$

where IM = initial mass of the sample

MT = mass of the sample at time T (T=0.5, 1.5, 3.0, and 4.5 h)

$$\mathrm{WL}\ (\%) = \frac{\mathrm{IW} - \mathrm{WL(T)} \times 100}{\mathrm{IM}}$$

where IW = initial water content of the sample

WL (T) = water loss at time T (T = 0.5, 1.5, 3.0, and 4.5 h)

IM = initial mass of the sample

$$\mathrm{SG}\ (\%) = \%\mathrm{WL} - \%\mathrm{MR}$$

1.5. Dehydration

Dehydration of rings of T_0 treatment was carried out in a cabinet drier maintained at a temperature of 60±2 °C to a constant weight (11–13% moisture). However, osmotically treated rings (T_1–T_4) as per treatments mentioned above were dehydrated after 4.5 h dip in a vacuum drier maintained at 82±2 °C having a vacuum of 640 mm Hg to a moisture content varying from 5 to 8 per cent.

1.6. Physico-chemical determination

Fruit firmness (1b/sq") was measured by using Magness pressure tester. Specific gravity was determined by water displacement method as described by GUSTAFSON (1926). Moisture (%), total soluble solids (°Brix), titratable acidity (% malic acid), pH, reducing sugars (%), total sugars (%), ascorbic acid (mg/100 g), pectin (% calcium pectate), total ash (%), and non-enzymatic browning (optical density at 440 nm) were determined by following standard procedures outlined by RANGANNA (1986).

1.7. Sensory evaluation

Dried rings were evaluated for overall acceptability based upon the observation for colour, taste/flavour, and texture by a panel of 7 judges on 9 point Hedonic scale (AMERINE et al., 1965). To analyse the results, numerical values are assigned to each point on the scale, viz., like extremely = 9, like very much = 8, like moderately = 7, like slightly = 6, neither like nor dislike = 5, dislike slightly = 4, dislike moderately = 3, dislike very much = 2 and dislike extremely = 1.

1.8. Statistical analysis

The data were analysed by using completely randomised design (CRD) three factorial as described by COCHRAN and COX (1963).

2. Results and discussion

The physico-chemical composition of Golden Delicious and Red Gold varieties from three locations is presented in Table 1.

In general, Golden Delicious variety recorded higher values for different parameters except total ash. Golden Delicious variety from L_3 location was superior in quality as compared to Red Gold from all the three locations and the same variety from L_1 and L_2 locations.

2.1. Effect on mass reduction (MR), water loss (WL), and solid gain (SG)

MR, WL, and SG in the apple rings from Golden Delicious and Red Gold varieties grown at three different locations, with or without pretreatments at various contact times are shown in Tables 2–4.

These data illustrate the difference in mass transfer (MR, WL, and SG) behaviour of the two varieties grown at three different locations. Golden Delicious variety recorded higher MR, WL and SG as compared to Red Gold at different contact time intervals (0.5, 1.5, 3.0 and 4.5 h) of osmotic dip. Rapid mass transfer took place in the initial 1.5 h of osmotic dip and thereafter slowed down. After 4.5 h dip, the rings from L_2 had the highest MR (42.29%) and WL (56.01%), whereas the solid gain was maximum (13.78%) in L_1. The difference in data for mass transfer amongst varieties and locations seems to be due to the different compactness of the tissues and variation in the physico-chemical composition of the fruit (Table 1.) The higher WL and MR in Golden Delicious variety from L_2 are probably due to higher polysaccharides content of the fruit as indicated by high firmness of fresh fruits (15.60 lbs/sq"). Starch, pectin, cellulose and hemi-cellulose are the main polysaccharides present in apple. The amount

of these polysaccharides is definitely higher in firmer fruits than in that of less firm ones as the degradation of these compounds results in increased total soluble solids and fruit softening. CONTRERAS and SMYRL (1981) and BOLIN and co-workers (1983) have reported that higher polysaccharides content results in higher MR and WL during osmotic dip. Increase in MR (44.71%) and WL (56.45%) was more pronounced in rings submerged in osmotic solution without any pretreatment (T_1) after 4.5 h dip, whereas the SG was lowest (11.73%) when compared with other treatments. Blanching of rings (T_3) showed the most adverse effect on the MR (37.52%) and WL (52.37%) and resulted in higher SG (14.84%). The reduction in MR and WL and increase in SG in T_3 might be due to the loss of semi-permeability of cell wall by heat treatment. In fruits, the cell wall membranes are living biological units which can stretch and expand under the turgor pressure generated inside the cells. The membrane freely allows the solvent molecules to pass through but to a lesser extent the passage of solute molecules. Therefore, when the heat treatment (blanching) of rings is done, the semi-permeability of the membrane is disturbed, thus resulting in a higher intake of solute from the osmotic solution with less water loss.

2.2. Effect on chemical constituents

Significant differences were seen in various chemical parameters among varieties, locations, and treatments (Table 5).

Golden Delicious variety recorded higher acidity (1.01%) as compared to Red Gold (0.99%). Location L_1 had comparatively lower acidity (0.99%) compared to L_2 and L_3, which were non significant. The variation in acidity among varieties and locations may be attributed to the initial acid content of the fruit. On the other hand, the treatment effect on the acidity was more pronounced and it was noticed that the T_0 treatment (conventional drying) dried rings had maximum acidity of 1.27%, whereas in osmotically treated followed by vacuum dried rings, the acidity levels were low indicating that during osmotic process the leaching of acid might have taken place. But, this contributed to pleasing taste in the dried apple rings as opposed to the objectional taste/sugar-acid blend of conventionally dried rings of T_0 treatment. DIXON and JEN (1977) and NANJUNDASWAMY and co-workers (1978) have also reported changes in acid content of osmotically treated and dried apple slices. A proportional increase in reducing and total sugars was observed in both varieties from three locations. Golden Delicious variety recorded comparatively higher reducing (38.73%) and total sugars (80.21%). Among locations, maximum reducing (37.64%) and total sugars (80.92%) were recorded for L_3 and minimum for L_1. Conventionally dried rings (T_0) recorded higher reducing sugars (51.59%) and lower total sugars (72.66%), whereas reverse in treatments T_1–T_4. This may be due to osmotic dip given in treatment T_1–T_4, which has resulted in penetration of sucrose into the rings.

Like in fresh fruit, maximum ascorbic acid content was observed in Golden Delicious variety and L_3 dried rings. After drying retention of more ascorbic acid was recorded in rings treated with sulfur dioxide and given osmotic dip (T_2) followed by vacuum drying. Althogh, T_0 rings were also treated with sulfur dioxide before drying but, the ascorbic acid in this treatment was lost by oxidation in air dehydration. Optical density (OD) which represents the index of browning of dried rings also varied for different varieties, locations and treatments. Golden Delicious variety as a whole and L_3 location rings in particular showed minimum browning of 0.011 and 0.009, respectively. There appears to be more browning in T_1 treatment as no blanching/sulfur dioxide/ascorbic acid dip was given, indicating that these pretreatments are important to check the oxidation during handling and dehydration.

Table 1.

Physico-chemical composition of apple fruit from different locations

Parameter	Golden Delicious			Mean	Red Gold			Mean
	L_1	L_2	L_3		L_1	L_2	L_3	
Fruit firmness (lbs/sq")	15.96	15.60	13.74	14.80	14.66	14.65	14.08	14.46
Specific gravity	0.99	0.97	1.03	1.00	0.98	0.95	1.00	0.98
Total soluble solids (°Brix)	12.12	11.20	13.82	12.38	12.40	10.58	12.46	11.81
Titratable acidity (% malic acid)	0.34	0.36	0.34	0.35	0.34	0.32	0.37	0.34
pH	3.73	3.67	3.60	3.66	3.68	3.64	3.70	3.67
Reducing sugars (%)	6.72	6.90	8.00	7.21	7.06	6.52	7.65	7.06
Total sugars (%)	8.60	8.42	9.85	8.96	8.80	7.85	8.53	8.39
Ascorbic acid (mg/100g)	8.90	9.38	11.96	10.08	7.35	8.54	6.23	7.39
Pectin (% calcium pectate on dwb)	1.36	2.06	2.16	1.86	1.46	1.95	2.20	1.82
Total ash (%)	1.48	1.80	2.04	1.77	1.60	1.94	2.48	2.01

L_1: Mid hills, L_2: high hills, L_3: dry temperate, dwb: dry weight basis

Table 2

Effect of variety, location and treatment on % mass reduction (MR) of apple rings during osmotic dip at different intervals

Interval (h)	Variety	Location				Treatment					CD (0.05)					
		L_1	L_2	L_3	Mean	T_1	T_2	T_3	T_4	Mean	V	L	T	V×L	V×T	L×T
0.5	Golden Delicious	14.39	14.16	14.32	14.29	15.47	14.50	12.26	14.93	14.29						
	Red Gold	15.89	12.68	11.98	13.52	14.11	14.40	11.56	13.99	13.52						
	Mean	15.14	13.42	13.15		14.79	14.45	11.91	14.46		0.28	0.34	0.40	0.47	0.56	0.69
1.5	Golden Delicious	28.73	25.69	28.52	27.65	29.76	27.53	23.58	29.73	27.65						
	Red Gold	25.82	24.21	25.39	25.14	26.41	24.72	23.22	26.22	25.14						
	Mean	27.27	24.95	26.95		28.08	26.12	24.40	27.97		0.06	0.07	0.08	0.10	0.12	0.14
3.0	Golden Delicious	39.81	38.00	38.35	38.72	41.31	39.44	35.21	38.94	38.72						
	Red Gold	32.86	33.27	33.88	33.34	34.65	33.05	31.93	33.71	33.34						
	Mean	36.33	35.63	36.11		37.98	36.24	33.57	36.32		0.18	0.22	0.26	0.32	0.37	0.45
4.5	Golden Delicious	42.53	44.32	42.52	43.13	47.25	42.67	39.43	43.15	43.13						
	Red Gold	38.18	40.27	39.43	39.29	42.18	40.00	35.62	39.38	39.29						
	Mean	40.35	42.29	40.97		44.71	41.33	37.52	41.26		0.08	0.10	0.11	0.14	0.16	0.20

L_1: Mid hills, L_2: high hills, L_3: dry temperate, CD: critical difference, V: variety, L: location, T: treatment

Table 3

Effect of variety, location and treatment on % water loss (WL) of apple rings during osmotic dip at different intervals

Interval (h)	Variety	Location				Treatment					CD (0.05)					
		L_1	L_2	L_3	Mean	T_1	T_2	T_3	T_4	Mean	V	L	T	V×L	V×T	L×T
0.5	Golden Delicious	25.15	22.06	22.70	23.30	23.47	22.89	22.93	23.94	23.30						
	Red Gold	23.74	22.57	20.58	22.29	21.48	22.85	21.87	22.98	22.29						
	Mean	24.44	22.31	21.64		22.47	22.87	22.40	23.46		0.21	0.33	0.37	0.50	0.54	0.86
1.5	Golden Delicious	41.26	37.26	38.71	39.07	40.76	38.97	35.60	40.99	39.07						
	Red Gold	37.11	36.60	35.33	36.35	36.82	34.99	36.15	37.44	36.35						
	Mean	39.18	36.93	37.02		38.79	36.98	35.87	39.21		0.30	0.36	0.43	0.51	0.59	0.74
3.0	Golden Delicious	52.78	50.13	49.68	50.86	52.74	51.75	48.08	50.88	50.86						
	Red Gold	44.84	46.51	44.37	45.24	45.34	44.36	45.76	45.49	45.24						
	Mean	48.81	48.32	47.02		49.04	48.05	46.92	48.18		0.65	0.87	1.72	1.11	2.39	2.98
4.5	Golden Delicious	56.31	57.19	54.86	56.13	59.06	55.83	54.00	55.60	56.13						
	Red Gold	50.97	54.84	50.52	52.10	53.85	52.13	50.74	51.72	52.10						
	Mean	53.64	56.01	52.69		56.45	53.98	52.37	53.66		0.12	0.15	0.31	0.19	0.43	0.54

L_1: Mid hills, L_2: high hills, L_3: dry temperate, CD: critical difference, V: variety, L: location, T: treatment

Table 4

Effect of variety, location and treatment on % solid gain (SG) of apple rings during osmotic dip at different intervals

Interval (h)	Variety	Location				Treatment					CD (0.05)					
		L_1	L_2	L_3	Mean	T_1	T_2	T_3	T_4	Mean	V	L	T	V×L	V×T	L×T
0.5	Golden Delicious	10.23	8.21	8.16	8.86	7.71	8.25	10.50	9.01	8.86						
	Red Gold	7.85	9.89	8.59	8.78	7.37	8.45	10.30	8.99	8.78						
	Mean	9.04	9.05	8.37		7.54	8.35	10.40	9.00		0.05	0.07	0.02	0.03	0.07	0.08
1.5	Golden Delicious	12.53	11.57	10.19	11.42	10.99	11.44	12.02	11.27	11.42						
	Red Gold	11.29	12.39	9.94	11.21	10.42	10.25	12.93	11.22	11.21						
	Mean	11.91	11.98	10.06		10.70	10.84	12.47	11.24		0.12	0.15	0.29	0.19	0.41	0.52
3.0	Golden Delicious	12.97	12.13	11.32	12.14	11.44	12.32	12.87	11.95	12.14						
	Red Gold	11.99	13.24	10.49	11.91	10.69	11.32	13.84	11.78	11.91						
	Mean	12.48	12.68	10.90		11.06	11.82	13.35	11.86		0.04	0.05	0.12	0.07	0.16	0.20
4.5	Golden Delicious	13.78	12.87	12.34	13.00	11.81	13.16	14.57	12.45	13.00						
	Red Gold	12.79	14.56	11.09	12.81	11.66	12.14	15.11	12.34	12.81						
	Mean	13.78	13.71	11.71		11.73	12.65	14.84	12.39		0.09	0.12	0.26	0.17	0.39	0.49

L_1: Mid hills, L_2: high hills, L_3: dry temperate, CD: critical difference, V: variety, L: location, T: treatment

Table 5

Effect of variety, location and treatment on chemical characteristics and quality of osmo-vac and conventionally dried apple rings

Parameter	Variety	Location				Treatment						CD (0.05)					
		L_1	L_2	L_3	Mean	T_0	T_1	T_2	T_3	T_4	Mean	V	L	T	V×L	V×T	L×T
Acidity (% malic acid)	Golden Delicious	0.99	1.03	1.01	1.01	1.29	0.93	0.95	0.94	0.93	1.01						
	Red Gold	1.00	0.97	1.00	0.99	1.25	0.89	0.95	0.91	0.94	0.99						
	Mean	0.99	1.00	1.00		1.27	0.91	0.95	0.92	0.93		0.002	0.002	0.005	0.003	0.008	0.01
Reducing sugars (%)	Golden Delicious	36.44	42.02	37.73	38.73	51.66	36.69	39.55	31.31	34.43	38.73						
	Red Gold	33.35	32.10	37.56	34.33	51.53	33.36	28.67	27.23	30.89	34.33						
	Mean	34.89	37.06	37.64		51.59	35.02	34.11	29.27	32.66		0.03	0.03	0.07	0.04	0.09	0.11
Total sugars (%)	Golden Delicious	79.30	79.75	81.59	80.21	73.52	80.77	82.24	81.53	83.04	80.21						
	Red Gold	79.59	79.84	80.26	79.90	71.81	82.29	81.82	81.93	81.64	79.90						
	Mean	79.44	79.79	80.92		72.66	81.53	82.02	81.73	82.34		0.03	0.04	0.09	0.06	0.13	0.16
Ascorbic acid (mg/100 g)	Golden Delicious	23.75	20.51	31.71	25.33	20.76	23.29	29.91	23.64	29.03	25.33						
	Red Gold	20.07	19.11	17.60	18.93	16.30	17.26	21.83	17.67	19.94	18.93						
	Mean	21.91	19.81	24.65		18.53	20.27	25.87	20.65	24.48		0.64	0.88	1.76	1.16	2.50	3.06
NEB (OD at 440 nm)	Golden Delicious	0.015	0.012	0.007	0.011	0.010	0.015	0.009	0.012	0.009	0.011						
	Red Gold	0.013	0.014	0.011	0.013	0.012	0.017	0.011	0.012	0.011	0.013						
	Mean	0.014	0.013	0.009		0.011	0.016	0.010	0.012	0.010		0.0003	0.0003	0.0007	0.0001	0.001	0.001
OAA	Golden Delicious	6.94	6.47	7.12	6.84	5.62	6.91	7.57	6.96	7.17	6.84						
	Red Gold	6.60	6.28	6.43	6.44	5.37	6.60	7.09	6.61	6.53	6.44						
	Mean	6.77	6.37	6.77		5.49	6.75	7.33	6.78	6.85		0.11	0.13	0.30	0.19	NS	NS

L_1: Mid hills, L_2: high hills, L_3: dry temperate, CD: critical difference, V: variety, L: location, T: treatment, NS: not significant, NEB: non-enzymatic browning, OAA: overall acceptability on 9 point Hedonic scale, OD: optical density

2.3. Effect on overall acceptability

The dried rings of Golden Delicious from L_3 location were adjudged best due to highest score (7.13) obtained for texture, appearance and taste. Pretreatments with sulfur dioxide and ascorbic acid combined with osmotic dip and vacuum drying improved the quality of the product to a greater extent. Conventionally dried rings (T_0) got minimum score (5.49) mainly due to the leathery texture and poor taste of the product. On the other hand, the rings with osmotic dip followed by vacuum dehydration resulted in a crunchy and high flavoured product.

3. Conclusions

– Golden Delicious variety suited best for production of osmo-vac dried rings.

– Apple grown in wet temperate and high hills climatic location (L_2) resulted in higher mass reduction (MR) and water loss (WL) during osmotic dip.

– Potassium metabisulphite dip prior to immersion of rings in osmotic (sucrose) solution proved to be mandatory in production of good quality vacuum dried rings.

– Blanching of rings before osmotic dip should be avoided as it interferes in water removal during osmotic dehydration, resulting in undesirable solid penetration into the rings.

– Vacuum drying of osmotically treated rings yielded a dried product having crunchy texture, good taste/flavour with more retention of ascorbic acid and less browning.

– Variety, location and treatment play a significant role in mass reduction (MR), water removal (WL) and solid gain (SG) during osmotic dehydration as well as in the quality of finished product.

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THE CORRELATION OF REDOX POTENTIAL AND SOME CHEMICAL PARAMETERS IN SPINACH PURÉE DURING PROCESSING AND FROZEN STORAGE

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The aim of this work was to study the changes of the principal compounds in spinach during processing into purée and frozen storage and to correlate these with the changes of redox potential (rH) depending on technological parameters and storage conditions (time and temperature). For that purpose two hybride varieties of spinach, Martina and Taurus RZ, were used.

A good correlation was found among the changes of rH and the levels of nitrates, oxalates and total chlorophylls as well as peroxidase activity in spinach during processing into purée. The rH significantly diminished during blanching process and reached the lowest value during purée preparation.

The rH was also found to be a good indicator of variation in the content of total chlorophylls in spinach purée during frozen storage.

Keywords: spinach purée, total chlorophylls, oxalic acid, nitrate, peroxidase, redox potential, processing, frozen storage

Spinach is an important article of commerce, and is highly valued in nutrition. However, apart from desirable components (vitamins and valuable mineral food elements) it contains components that may be harmful in cases of improper storage, processing and use (BENGSTON, 1969; WITTE, 1970).

The major indicator of the overall acceptability and value of this vegetable are nitrate, oxalate, flavour, texture and its characteristic colour, originated from green chlorophyll pigments. Chlorophyll degradation can occur during processing and storage and depends on temperature, pH, time, enzyme action, oxygen and light. Most of the chemical pathways involved have been established but a method for complete stabilization has not yet been achieved (HENDRY et al., 1987; YAMAUCHY & WATADA, 1991).

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Redox potential (rH) is believed to be an important selective factor in all environments. In spite of its obvious significance relatively little work on the rH of foods has been published.

The objective of our research was to study the correlation between rH and total chlorophylls, nitrate, oxalic acid levels and peroxidase activity in spinach during processing into purée and the correlation between rH and total chlorophylls in spinach purée during frozen storage. The aim was to explore the relationships among these parameters. The correlation coefficients were used as a linear regression measure.

1. Materials and methods

Spinach was grown locally on the complex of the Agriculture Faculty and harvested in the summer 1996 at the optimum maturity for processing. For the investigation two hybrid varieties of spinach, Taurus RZ and Martina RZ were used. These were new selections of spinach with smooth and thick leaves, green to dark green colour and firm stems. Taurus had shorter stems than Martina.

The spinach leaves were sampled from a factory line for preservation by freezing, after they had been harvested, transported, stored before processing, washed, blanched 3 min at 90 °C, cooled and chopped. After each processing stage the samples were packed in polyethylene bags of about 2 kg capacity, frozen at –35 °C in a stream of cold air and all of them were stored for 8 days at –18 °C before the analysis started. If changes occurred they were of the same intensity in all samples. The bags with frozen spinach purée of Martina variety were placed in a freezer at two different temperatures (–18 and –30 °C) and analysed after 15, 45 and 105 days of storage. All the samples for analysis were homogenized while still frozen in laboratory mixer. Subsamples were examined to determine analytical variability.

The dry matter, pH and oxalic acid analysis were performed according to the Official A.O.A.C. Methods of Analysis (A.O.A.C., 1990). The nitrate level in the samples was determined by the colourimetric method of SEN and DONALDSON (1978). Determination of total chlorophyll pigments was carried out by the spectrophotometric assay of VERNON (1960). Peroxidase activity was determined by the spectrophotometric assay of HEMEDA and KLEIN (1990) using quaiacol and hydrogen peroxide as a substrate. Enzyme activity was expressed in units of peroxidase per gram of dry weight of vegetable tissue at 20 °C. One unit of activity was defined as the amount of enzyme that caused an increase of 0.01 in absorance at 470 nm per min.

Redox potential was measured by a platinum redox electrode model 96-78 on an Expandable Ion-Analyser (Model Orion EA 940) (DIKANOVIĆ-LUČAN & PALIĆ, 1992).

Results are statistically interpreted using t-test, F-test, ANOVA and linear regression analysis (DAVIES, 1961).

2. Results

The effect of technological parameters on the changes of physical and chemical parameters in spinach of two selected varieties during purée preparation is shown in Table 1.

After harvest there were no differences between varieties with respect to dry matter and peroxidase activity. The oxalate and nitrate contents were higher in the Martina than in the Taurus variety. The results for the total chlorophyll pigment contents showed higher pigment concentrations in the Martina than in the Taurus cultivar (17.36 to 16.65 mg g^{-1} dry matter, respectively). The mean pH values for these cultivars ranged from 5.75 to 5.87 and the rH from 24.77 to 23.97.

The results for redox potential were in agreement with literature data cited by NOJEIM and co-workers (1981) (Eh=341 mV) and MONTVILLE and CONWAY (1982) (Eh=318 mV) for canned spinach, the only values found in the literature for spinach.

Table 1

The results of physico-chemical analysis of spinach during processing into purée[a]
(mean of 6 determinations ±SD)

Analysis	Cultivar	Processing stages					
		Harvest	Transport	Storage	Washing	Blanching	Chopping
Dry matter [%]	Martina	10.78±1.05	10.91±0.34	10.06±0.50	6.65±0.50	6.51±0.38	5.82±0.36
	Taurus	11.11±0.32	11.57±0.26	11.41±0.27	7.05±0.24	6.89±0.44	5.92±0.35
Total chlorophylls [mg g^{-1} dry matter]	Martina	17.3±0.34	17.40±0.36	16.63±0.72	16.80±0.28	16.60±0.45	15.10±0.67
	Taurus	16.65±0.73	16.73±0.48	16.89±0.63	16.05±0.53	15.72±0.54	13.91±0.69
Peroxidase activity [units g^{-1} dry matter]	Martina	10.07±0.52	13.05±0.51	14.00±0.58	10.69±0.33	0.00	0.00
	Taurus	10.95±0.40	14.07±0.94	14.50±0.58	11.00±0.56	0.00	0.00
Nitrate [mg g^{-1} dry matter]	Martina	8.44±0.25	8.60±0.17	8.30±0.32	9.70±0.38	7.60±0.16	7.70±0.14
	Taurus	2.88±0.31	2.85±0.11	2.40±0.42	5.00±0.14	3.28±0.20	1.02±0.25
Oxalic acid [mg g^{-1} dry matter]	Martina	93.80±0.56	102.50±6.47	112.90±4.4	90.01±0.99	65.91±0.64	65.93±0.75
	Taurus	84.40±0.69	86.01±0.43	93.30±0.42	79.96±0.56	65.87±0.53	60.12±0.82
rH value	Martina	24.77±0.31	25.26±0.16	25.51±0.43	25.59±0.03	20.49±0.22	21.03±0.08
	Taurus	23.97±0.09	24.40±0.26	24.76±0.57	25.18±0.31	20.39±0.08	20.59±0.25
pH value	Martina	5.75	5.81	5.82	5.84	6.90	6.88
	Taurus	5.87	5.84	5.88	5.90	6.74	6.89

[a] Each value represents the mean of three replicates and two batches

During transport to the processing plant and 13 h of standing there at a temperature of 5 °C (before processing) the proportion of total chlorophyll, oxalate and nitrate contents as well as peroxidase activity increased in both cultivars probably because of the tissue dehydration. An accumulation of oxalate in the tissues can be related to the shift in equilibrium in favour of biosynthesis rather than degradation (HITOMI et al., 1992). After 13 h of storage in the plant the peroxidase activity increased and reached the highest value during spinach processing into purée. During these two stages of examination (the transport and storage) the rH values slightly increased.

The washing led to a decline of oxalate content and peroxidase activity. The fraction of the chlorophyll pigments remained the same as before washing. The nitrate content after washing increased that could have been due to the presence of nitrate in the water used (60 mg l^{-1} NO_3).

At the same time a trend towards increased rH value was observed. One reason for such a behaviour might be a presence of high water content with dissolved oxygen on the spinach after washing. The other reason might be an increase of nitrate level and its faster conversion into nitrite in damp spinach (AWORTH et al., 1980).

The blanching and cooling of spinach involved a loss of total chlorophylls, oxalic acids and nitrate contents and complete inactivation of the enzyme peroxidase, suggesting overblanching. Spinach treated by heating for 3 min at 90 °C accumulated reducing substances and had a lesser amount of dissolved oxygen than untreated spinach. These conditions lowered the rH from 24.77 to 20.49 in Martina and from 23.97 to 20.39 in Taurus cultivar. Decreasing the rH can provide useful information for evaluating the blanching procedure.

After chopping, the quantities of almost all the spinach components investigated decreased additionally, due to the removal of insoluble (cellulose and hemicellulose) leaf parts and the addition of water for the purpose of obtaining a uniform amount of final dry matter in the purée (5.92% in the Taurus and 5.82% in the Martina cultivar). In the final product an addition of water, likely because of dissolved oxygen, increased the rH value in both cultivars.

To establish the significance of differences among cultivars and processing stages, with respect to quality parameters, an analysis of variance was calculated (Table 2).

Table 2

Variance analysis of data in Table 1

Chemical parameters	Source of variation	SS	df	MS	F	F crit.
Dry matter	Cultivar	0.864	1	0.864	9.06	6.61
	Process.stages	62.122	5	12.424	130.32	5.05
	Error	0.476	5	0.095		
	Total	63.463	11			
Total chlorophylls	Cultivar	1.347	1	1.347	10.44	6.61
	Process. stages	9.492	5	1.898	14.72	5.05
	Error	0.645	5	0.129		
	Total	11.484	11			
Peroxidase activity	Cultivar	1.061	1	1.061	13.31	10.13
	Process. stages	20.753	3	6.918	86.72	9.27
	Error	0.239	3	0.080		
	Total	22.054	7			
Nitrate	Cultivar	88.303	1	88.303	254.56	6.61
	Process. stages	8.691	5	1.738	5.01	5.05
	Error	1.734	5	0.347		
	Total	98.728	11			
Oxalic acid	Cultivar	323.918	1	323.918	12.53	6.61
	Process. stages	2521.445	5	504.289	19.51	5.05
	Error	129.189	5	25.837		
	Total	2974.553	11			
Redox potential (rH)	Cultivar	0.842	1	0.842	20.48	6.61
	Process. stages	50.657	5	10.131	246.61	5.05
	Error	0.205	5	0.041		
	Total	51.704	11			

Table 3

Correlation between redox potential and the chemical variables during processing for spinach cultivars Martina and Taurus

Chemical variables	Martina		Taurus	
	r	Regression equation	r	Regression equation
Total chlorophylls	0.68	y=11.11+0.23x	0.68	y=8.22+0.33x
Peroxidase activity	0.98	y=–54.48+2.63	0.95	y=–60.90+2.99x
Nitrate	0.79	y=2.35+0.25x	0.51	y=4.27+0.31x
Oxalic acid	0.92	y=–88.61+7.45x	0.90	y=–45.39+5.33x
pH	–0.98	y=11.73–0.23x	–0.97	y=11.31–0.22x

There were no significant differences in dry matter, total chlorophyll and total oxalic acid contents and peroxidase activity with cultivars and technological stages. There were significant differences in nitrate content and rH with cultivar. Technological parameters had an important influence on the rH but not on the nitrate level in spinach.

The results obtained demonstrated that changes in the rH value occurred whenever there were changes in almost all of the parameters investigated in spinach during purée preparation. The correlation coefficients (r) between the rH and the chemical parameters in the two cultivars ranged from 0.68 to 0.98 for the Martina and from 0.51 to 0.97 for the Taurus cultivar. The lowest correlation coefficients were found between the rH and chlorophyll pigments and nitrate contents (Table 3).

In order to elucidate how the rH changes of spinach purée correlate with the total chlorophylls during frozen storage, the Martina cultivar and two storage temperatures were used (-18 and –30 °C). The total chlorophyll content in spinach purée after freezing at –35 °C was 16.82 mg g^{-1} dry matter.

The results showed that immediately after freezing the changes of rH and the total chlorophylls in spinach purée were not significant. The values of those parameters were slightly lower than before freezing (Fig. 1).

During frozen storage the chlorophyll pigments and rH decreased until 45 days and after this period they increased considerably at both temperatures. These changes were more pronounced in spinach purée stored at –18 than at –30 °C.

The spontaneous regreening process of spinach has been attributed to the formation of green metallocomplexes of chlorophyll derivatives with small amounts of copper, zinc or aluminum. The formation of these complexes during food processing for preservation and during storage after processing are well known and were reported a long time ago (JONES et al., 1962).

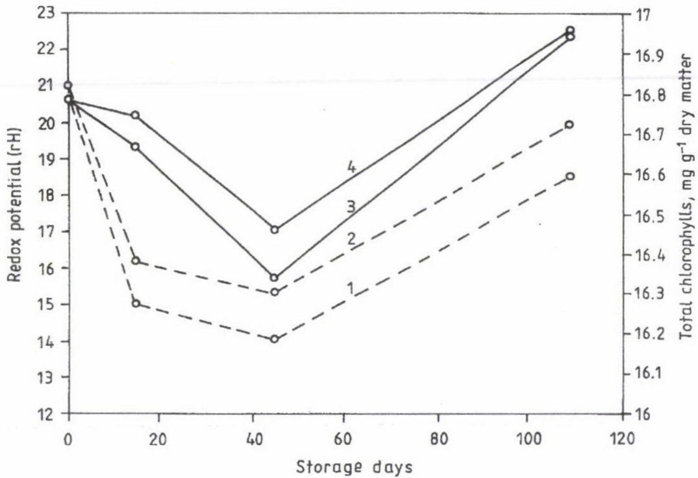

Fig. 1. Changes of redox potential and total chlorophylls in frozen spinach purée Martina cultivar during storage at –18 and –30 °C. 1: chlorophylls, –18 °C; 2: chlorophylls, –30 °C; 3: rH, –18 °C; 4: rH, –30 °C

Analysis of variance showed that the change of total chlorophylls and rH depends significantly ($P<0.05$) on the storage time but not on the storage temperature (Table 4).

The electrochemical measurements in spinach purée during freezing and frozen storage revealed that the rH changes followed the changes in the total chlorophyll pigments. The correlation coefficient (r) between those parameters was 0.75 at both temperatures and it was higher than in spinach during purée preparation (r=0.66 and 0.68).

Table 4

Variance analysis of data in Fig. 1

Chemical parameters	Source of variation	SS	df	MS	f	F crit.
Chlorophylls	Temperature	0.016	1	0.016	8.83	10.13
	Storage days	0.448	3	0.149	81.54	9.27
	Error	0.005	3	0.002		
	Total	0.470	7			
Redox potential (rH)	Temperature	0.690	1	0.691	3.66	10.13
	Storage days	38.968	3	12.989	68.95	9.27
	Error	0.565	3	0.188		
	Total	40.223	7			

3. Conclusion

The obtained results have shown that there was a good correlation between changes of the rH and the chemical parameters in spinach purée during preparation and frozen storage. The rH might be the potential relevant quality factor in industrial practice, especially in evaluation of blanching process.

Further investigation is needed on the method development for measuring of the rH in processed vegetables.

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COMPARISON OF SYNTHESIS FOR DETERGENT GLUCOSIDES IN ORGANIC SOLVENT–WATER MIXTURES USING NATIVE GLUCOSIDASES

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The behaviour of native α-glucosidase and β-glucosidase was studied in reverse hydrolytic processes to compare their activity in binding various alcohols to glucose with the aim to synthesize detergent O-alkyl or aralkyl glucosides. In the presence of glucose as substrate glucosidases retained after 48 h incubation at room temperature about 50% of their original acitivity depending on water content and type of alcohol.

It was found that native α- and β-glucosidases have significant O-glycosylation acitivity with glucose as substrate and with different alcohols serving both as reaction partners and solvents. The yields were moderate (about 20%) because inactivation of both glucosidases was rather fast even in the presence of glucose substrate. The best results were found at room temperature with alcohols containing 10–15% water and at reaction times not longer than 72 h.

Keywors: α- and β-glucosidase, O-glycosylation, organic solvents, reverse hydrolysis

In aqueous solutions enzymatic hydrolytic reactions catalyzed by hydrolases are practically irreversible, but hydrolysis can be converted to synthesis by reducing the water content of the medium. Hydrolases can be used for reverse hydrolysis in organic solvents (JAKUBKE & KUHL, 1982) but in most cases some water is required to maintain the native conformation of the enzyme (ADLERCREUTZ, 1993). In most cases solubility, stability and activity of enzymes are reduced in the presence of organic solvents (CANTARELLA et al., 1991 and GUPTA, 1992).

Proteases, esterases and glycosidases are used in biotranformations to catalyse the synthesis of biologically active peptides, esters and of glycosides first of all in transpeptidation, transesterification and transglycosidation reactions using activated substrates (CHAIKEN et al., 1992; CHEN & SIH, 1989; CROUT et al., 1992 and TRICONE et al., 1995). Stereoselectivity and regioselectivity of these reactions are far higher than in chemical syntheses (FLOWERS, 1987 and PAULSEN, 1982). It was found that, with the exception of glycosidases, enzymes of carbohydrate metabolism give too tight enzyme-

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product complexes, therefore, they do not work in organic solvents (KIEBOOM, 1990). There are only a few published data for reverse hydrolytic reactions of glucose by glycosidases e.g. glucoamylase and β-glucosidase (EL-SAYED & LÁSZLÓ 1994, and LAROUTE & WILLEMOT, 1992).

At present we examined the behaviour of α-glucosidase and β-glucosidase using glucose as substrate in reverse hydrolysis, in order to compare their activity in binding various alcohols to glucose. This process may be useful in the synthesis of detergent O-alkyl or aralkyl glucosides (DE GRIP, 1979). A few of such alkyl glucosides were synthesized by chemical syntheses (REEVES & MAZZENO, 1954) and/or transglycosidation of phenyl glucoside as a substrate (MITSUO et al., 1984). The activity of native β-glucosidase in reactions of a number of alcohols with glucose was tested earlier (LAROUTE & WILLEMOT, 1992 and VULFSON et al., 1990) but the behaviour of α-glucosidase in similar reactions has not been examined.

1. Materials and methods

1.1. Materials

α-Glucosidase from baker's yeast (EC 3.2.1.20), β-glucosidase from almonds (EC 3.2.1.21) and the alcohols (n-butanol, n-pentanol, n-hexanol, 2-phenylethylethanol, 2-methoxyethanol and cyclohexanol) were products of Sigma Chemical Co. (USA).

1.2 Methods

1.2.1. Measurement of acitivity of glucosidases. Activity of α-glucosidase and β-glucosidase in the reaction mixtures was measured according to the literature (LARNER, 1960) with substrate maltose (0.12 mol l^{-1}) and cellobiose (0.25 mol l^{-1}), respectively, in different alcohols of 0–30% water content.

1.2.2. Reverse hydrolytic reactions. Reaction mixtures (0.5 ml) containing α-glucosidase or β-glucosidase (2 mg ml^{-1}) and glucose (0.10 mol l^{-1}) in different alcohols of 0–30% water content were shaken on IKA-VIBRAX-VXR shaker at a speed of 200 min^{-1} at 0–30 °C for 24–96 h. Two-phase reaction mixtures were diluted with distillated water (2 ml), then their glucose content was analysed immediately. Samples for glucose analysis were taken from diluted, well stirred, two-phase reaction mixtures.

1.2.3. Determination of glucose content of reaction mixtures. Concentration of unreacted glucose was measured directly in reaction mixtures both by enzymatic analysis using hexokinase, glucose-6-phosphate dehydrogenase and NADP (BEUTLER et al., 1974) and by determining glucose concentration as a reducing sugar by 3,5-dinitrosalicylic acid (HOSTETTLER et al., 1951).

1.2.4. Determination of alkyl glucoside content of reaction mixtures. After measuring the glucose concentration of diluted, two-phase reaction mixtures, solvents were evaporated in vacuo. The alkyl glucoside content of residues was analysed by Chrompress LABOR-MIM (Hungary) HPTLC (KLAUS et al., 1989) and Ultrascan XL LKB laser densitometer. Yields are given on the basis of HPTLC method.

Quantity of alkyl glucosides was controlled by an indirect method, as well. Separated alkyl glucosides were dissolved in sodium acetate buffer (0.1 mol l^{-1}, pH 5.1, 1 ml) then hydrolysed by α- or β-glucosidase (2 mg ml^{-1}), finally glucose concentration of mixtures was measured by the methods described above. The results of these experiments were within ±5% of the values measured by HPTLC method.

All data are mean values obtained from three parallel experiments.

2. Results and discussion

2.1. Effect of the nature of alcohol on the activity of α- and β-glucosidases

Inactivation of both gucosidases was fast, it was practically completed within 5 h at 0–30 °C temperature in the alcohols studied (n-butanol, n-pentanol, n-hexanol, 2-phenyl-ethylethanol, 2-methoxyethanol and cyclohexanol) containing 0–50% water. Incubation time was 24–72 h. It is known that there are many factors that enhance stability of enzymes, e.g. reversible interactions between enzyme and substrate molecules (MOZHAEV & MARTINEK, 1984) therefore, inactivation of both glucosidases in the presence of glucose was also studied.

We found that in the presence of glucose substrate (0.10 mol l^{-1}) as stabilizing agent glucosidases retained 30–50% of their original activity depending on water content and type of alcohol even after 48 h incubation at 25 °C. The effect of temperature was less pronounced than that of water content and incubation time. Since between 10 °C and 25 °C (room temperature), deactivation curves of glucosidases were practically the same, reverse hydrolitic processes were carried out at room temperature.

Deactivation of both glucosidases in some other alcohols containing 10% water at room temperature for 1, 5 and 36 h incubation time are presented in Tables 1 and 2. These data demonstrate that the process of deactivation was similar in all the alcohols studied but deactivation of α-glucosidase was slightly faster than of β-glucosidase.

A characteristic example for the behaviour of glucosidases in alcohols is presented in Fig. 1. Incubation of β-glucosidase in n-butanol containing 1% water for 1 h at 10 °C reduced the activity of enzyme by 70% and after 5 h it was completely deactivated. In the presence of glucose (0.10 mol l^{-1}), but otherwise under the same conditions, activity of β-glucosidase was reduced only by 40% after 1 h, by 60% after 5 h and by 70% after 36 h of incubation at the same parameters.

Table 1

Deactivation of α-glucosidase on incubation in different alcohols of 10% water content at room temperature

Reaction mixture	Residual activity after 1 h (%)	Residual activity after 5 h (%)	Residual activity after 36 h (%)
1–1	18	0	0
1–2	50	38	28
2–1	20	2	0
2–2	60	47	38
3–1	21	2	0
3–2	53	40	30
4–1	20	3	0
4–2	59	43	35

(1–1): n-butanol, (1–2): n-butanol+0.10 mol l^{-1} glucose; (2–1): n-hexanol, (2–2): n-hexanol+0.10 mol l^{-1} glucose; (3–1): cyclohexanol, (3–2): cyclohexanol+0.10 mol l^{-1} glucose; (4–1): 2-methoxyethanol, (4–2): 2-metoxyethanol+0.10 mol l^{-1} glucose

Table 2

Deactivation of β-glucosidase on incubation in different alcohols of 10% water content at room temperature

Reaction mixture	Residual activity after 1 h (%)	Residual activity after 5 h (%)	Residual activity after 36 h (%)
1–1	28	3	0
1–2	60	40	32
2–1	25	4	0
2–2	65	53	42
3–1	25	3	0
3–2	58	45	34
4–1	23	4	0
4–2	60	45	37

(1–1): n-butanol, (1–2): n-butanol+0.10 mol l^{-1} glucose; (2–1): n-hexanol, (2–2): n-hexanol+0.10 mol l^{-1} glucose; (3–1): cyclohexanol, (3–2): cyclohexanol+0.10 mol l^{-1} glucose; (4–1): 2-methoxyethanol, (4–2): 2-methoxyethanol+0.10 mol l^{-1} glucose

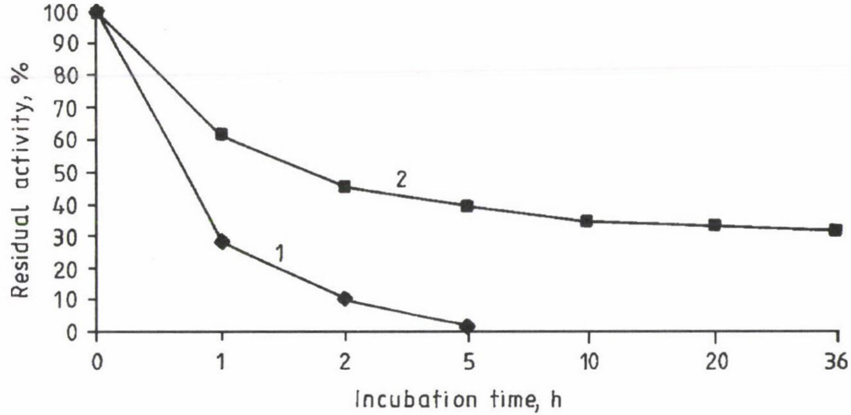

Fig. 1. The effect of glucose on the activity of β-glucosidase incubated in n-butanol containing 1% water at 10 °C (1: without glucose, 2: in the presence of glucose 0.10 mol l^{-1})

Deactivation of enzymes in organic solvents can be caused by many factors. Often the electrostatic interactions in enzymes are altered as a result of the presence of organic solvents (WEETAL & VANN, 1976), or enzymes undergo conformational changes in organic solvents (KLYOSOV et al., 1975). We found no correlation between deactivation data of glucosidases, solubility of the alcohols in water, solubility of water in alcohols and dielectric constants of the alcohols.

2.2. Reverse hydrolytic reactions

The reverse hydrolytic reactions were carried out under similar conditions as described for the deactivation processes. Glucosidases (2 mg ml^{-1}) were shaken in different alcohols of 0–20% water content containing glucose (0.10 mol l^{-1}) in the first series of experiments at 0–30 °C for 24–96 h and later at room temperature for 72 h.

It was found that α- and β-glucosidases had significant O-glycosylation activity with glucose as substrate and with different alcohols as both reaction partners and solvents. The dependence of the yields on the water content of alcohols and reaction time was rather similar with different alcohols.

Highest yields (~18%) were detected for all alcohols studied at 10–15% water content at room temperature and 72 h reaction time. It was found that, because of the deactivation of the enzyme, prolongation of reaction time practically did not increase the yields of reverse hydrolytic reactions. As an example time dependence of the reaction in cyclohexanol is presented in Figs. 2 and 3 for α- and β-glucosidase, respectively.

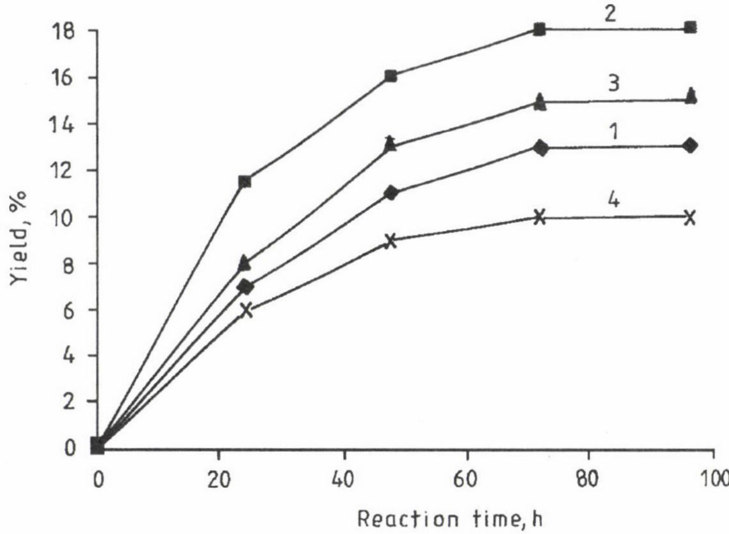

Fig. 2. Yield of cyclohexyl α-D-glucopyranoside in its α-glucosidase catalysed synthesis as a function of water content (1: 5%, 2: 10%, 3: 15%, 4: 20%) cyclohexanol and reaction time at room temperature

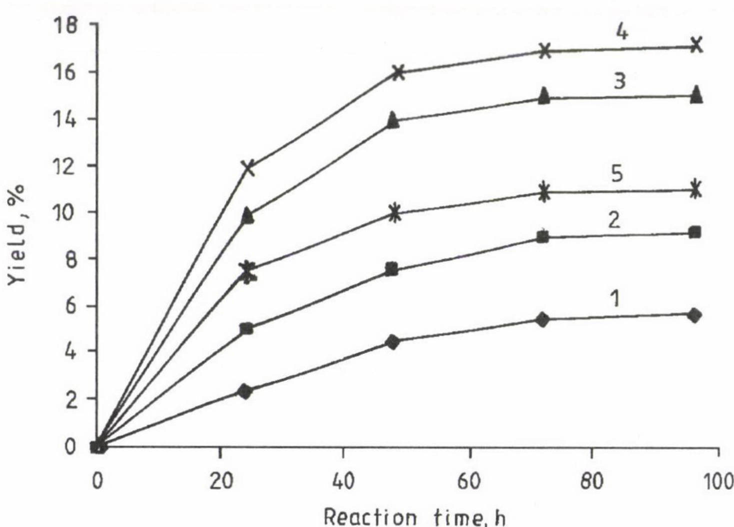

Fig. 3. Yield of cyclohexyl β-D-glucopyranoside in its β-glucosidase catalysed synthesis as a function of water content (1: 1%, 2: 5%, 3: 10%, 4: 15%, 5: 20%) cyclohexanol and reaction time at room temperature

We compared the effect of water content on the activity of α- and β-glucosidase in binding various alcohols to glucose in reverse hydrolysis at optimum parameters (room temperature and 72 h reaction time). The results are presented in Figs 4–7.

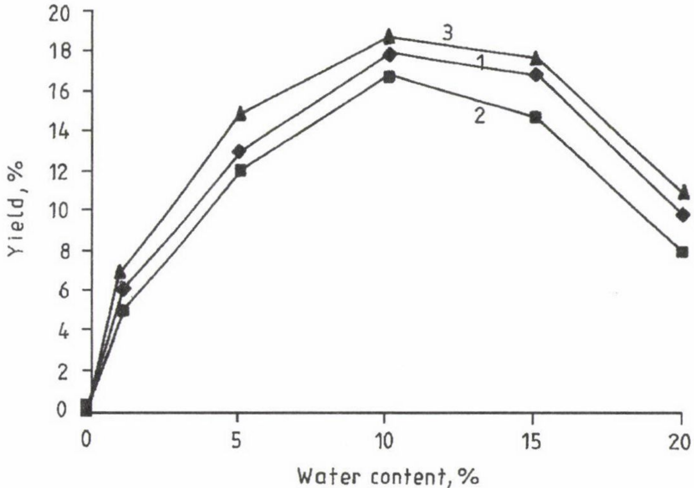

Fig. 4. Effect of water content of n-butanol (1), n-pentanol (2) and n-hexanol (3) on the yield of reverse hydrolytic reactions catalysed by native α-glucosidase (2 mg ml^{-1}) in a reaction mixture containing 0.10 mol l^{-1} glucose at room temperature for 72 h

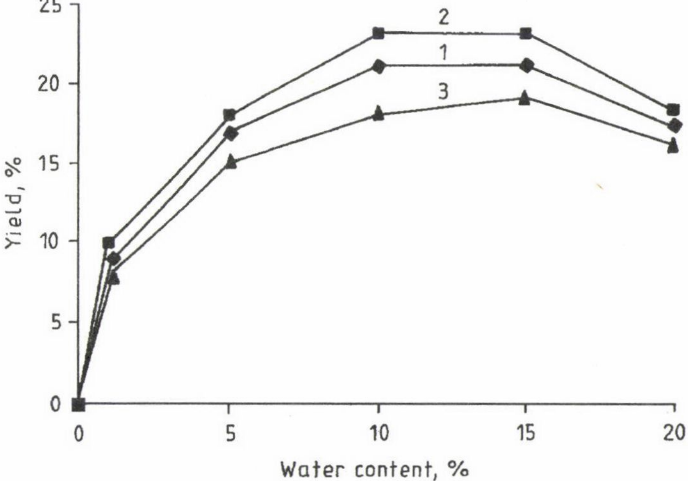

Fig. 5. Effect of water content of n-butanol (1), n-pentanol (2) and n-hexanol (3) on the yield of reverse hydrolytic reactions catalysed by native β-glucosidase (2 mg ml^{-1}) in a reaction mixture containing 0.10 mol l^{-1} glucose at room temperature for 72 h

Yields were slightly better using β-glucosidase (Fig. 5 and Fig. 7) than α-glucosidase (Fig. 4 and Fig. 6) but the difference was insignificant. It seems that n-butanol, n-pentanol and n-hexanol are almost equally suitable for reverse hydrolytic synthesis with both glucosidases (16–18% for α-glucosidase and 18–21% for β-glucosidase) at 10–15% water content.

In cyclohexanol the yields were almost the same for both glucosidases (18% in cyclohexanol of 10% water content). In water-miscible 2-methoxyethanol of 10% water content in the reaction catalysed by α-glucosidase the yield was almost twofold (23%) than by β-glucosidase (14%). On the contrary, in 2-phenoxyethanol of 10% and 15% water content in the reaction catalysed by α-glucosidase the yields were only 9% and 8%, respectively, while with β-glucosidase 17% and 18%, respectively.

We suppose that enzyme stability is a decisive factor in reverse hydrolytic reactions between glucose and different alcohols catalysed by glucosidases. We found no correlation between yields, solubility of the alcohols in water, solubility of water in alcohols and dielectric constants of the alcohols.

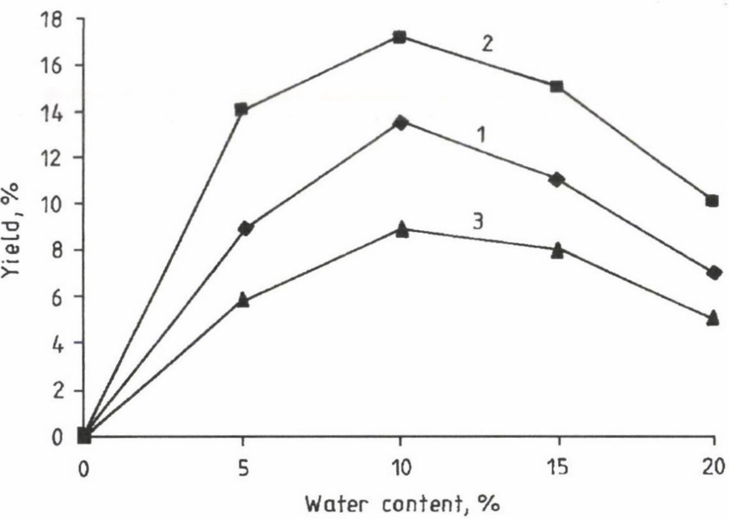

Fig. 6. Effect of water content of 2-methoxyethanol (1), cyclohexanol (2), and 2-phenoxyethanol (3) on the yield of reverse hydrolytic reactions catalysed by native α-gucosidase (2 mg ml^{-1}) in a reaction mixture containing 0.10 mol l^{-1} glucose at room temperature for 72 h

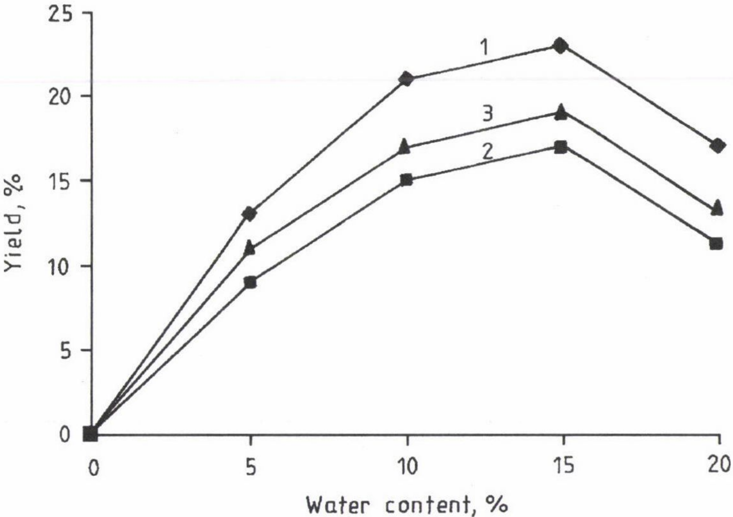

Fig. 7. Effect of water content of 2-methoxyethanol (1), cyclohexanol (2), and 2-phenoxyethanol (3) on the yield of reverse hydrolytic reactions catalysed by native β-glucosidase (2 mg ml^{-1}) in a reaction mixture containing 0.10 mol l^{-1} glucose at room temperature for 72 h

Complex heterogeneity of reaction mixtures of reverse hydrolytic processes is well known (KUHL et al., 1990 and LOPEZ-FANDINO et al., 1994) and it can be attributed to several factors. In our case on the one hand except 2-methoxyethanol, the alcohols studied are immiscible with water and on the other hand both enzymes and substrate glucose are only sparingly soluble in these alcohol-water mixtures.

Due to this heterogeneity when the best reactions were repeated on a larger scale (in tenfold quantity) and the alkyl gucosides were isolated by evaporation in vacuo and chromatography of residue, we found reduced yields (5–8%) consequently. The chemical structure and detergent properties of the synthesized O-glucosides have not been studied in detail yet. Modification of glucose-enzyme ratio in reaction mixtures was attempted, as well. Neither more enzyme nor more glucose enhanced the yield of the reactions.

*

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Short communications

IDENTIFICATION OF LACTIC ACID BACTERIA ISOLATED FROM TARHANA DURING FERMENTATION

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In this study three different types of tarhana were produced and 107 lactic acid bacteria were isolated during the fermentation of tarhanas. For isolation, MRS agar and M17 agar were used. Only 86 out of 107 isolates could be subjected to the further cultural and biochemical identification tests. Of these, 35 isolates could be well identified as *Lactobacillus plantarum* (14 isolates), *Lact. casei* subsp. *pseudoplantarum* (7 isolates), *Lact. delbrueckii* subsp. *bulgaricus* (1 isolate), *Lact. helveticus* (2 isolates), *Lact. fermentum* (2 isolates), *Lact. brevis* (6 isolates) and *Leuconostoc mesenteroides* subsp. *mesenteroides* (3 isolates). Forty two isolates could also be identified but they differed from typical strains of related lactic acid bacteria on the basis of their one or more cultural and biochemical characteristics. The remaining 9 isolates could not be correctly characterized into species by the identification tests used in the present study.

Keywords: tarhana fermentation, lactic flora, isolation, characterization

Tarhana, a traditional fermented food, has been known for centuries by Turkish people. It is also known with different names in various Middle East and European countries today, such as "kishk" in Syria, Palestine, Jordan, Lebanon and Egypt; as "kushik" or "kushuk" in Iraq and as "trahanas" in Greece. However, the processing procedure is quite similar in each country, the amount and the kinds of the ingredients used may differ depending on customs and availability of the raw material. The Hungarian "tarhonya" and Finnish "talkuna" are the non-fermented tarhana-like products. Tarhonya is a granulated dried pastry made of flour and eggs without lactic acid fermentation. In talkuna production, cereal flour is heat treated and it is mixed with yogurt before consumption (SIYAMOĞLU, 1961; HAMAD & FIELDS, 1982; EL-GENDY, 1983; STEINKRAUS, 1983; ABO-DONIA, 1984; TUOMINEN et al., 1988; JANDAL, 1989; TAMIME & O'CONNOR, 1995).

Generally tarhana is produced as dry powder form and used in soup preparation. It is often produced by fermenting the mixture of yogurt, wheat flour, various

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vegetables, herbs and spices. Mixture is fermented for 1–5 days, dried, powdered and stored (ANON., 1981; TEMİZ & PIRKUL, 1990; 1991). Tarhana production is principally based on lactic acid fermentation. For the lactic acid fermentation, yogurt or naturally fermented skimmed milk are commonly used as inoculum. In some regions of Turkey, concentrated yogurt, a Turkish traditional product which is prepared by filtration of yogurt through a cloth bag, or buttermilk, a traditional by-product of the butter production from yogurt, may be used as well. In all cases, yogurt or other naturally fermented milk products undergo a typical lactic acid fermentation in tarhana. Baker's yeast may also be added to tarhana mixture before fermentation (SIYAMOĞLU, 1961). In this way, lactic acid fermentations by lactic acid bacteria and ethyl alcohol fermentation by yeasts are performed together in the mixture.

The studies about the identification of principal microorganisms in fermented yogurt-wheat mixture products are very limited (EL-GENDY, 1983). ÖZBILGIN (1983) and TEMİZ and PIRKUL (1990) examined the microbiological and chemical changes on tarhana during fermentation. The latter researchers also determined the counts of lactic streptococci and lactobacilli in the tarhana mixture during 5 days of fermentation. However, we are not aware of any study related to the identification of principal lactic acid bacteria of tarhana fermentation.

The objective of this study was to investigate and identify the lactic acid bacteria carrying out the lactic acid fermentation in tarhana.

1. Materials and methods

1.1. Tarhana preparation

Three types of tarhana were produced. Plain yogurt (PY), manufactured at the Dairy Plant of Hacettepe University Food Engineering Department by using the Chr. Hansen's yogurt starter (CH-1) was used as inoculum in the first type of tarhana. In the second type of tarhana concentrated yogurt (CY), purchased from a local market in Ankara, was used as inoculum. The PY together with the compressed fresh baker's yeast (Pak Maya Co., Izmit, Turkey) (PBY) was used as inoculum in the third type of tarhana. For preparation of tarhana ingredients, onions were peeled and then peeled onions, tomatoes, fresh green and red peppers were washed. They were chopped to smaller pieces, stem and seeds of peppers were removed. These ingredients were mixed together (360 g of onion, 750 g of tomato, 300 g of fresh green pepper and 200 g of fresh red pepper) as indicated in the study of TEMİZ and PIRKUL (1990) and salt (60 g) was added. The mixture was heated in a stainless steel container to the boiling point and boiled for 10 min over medium heat with continuous stirring. After being allowed to cool to 30 °C or below, the mixture was distributed into three stainless steel containers

equally. The wheat flour (570 g) and yogurt (500 g) were added to each mixture. In this step, PY, CY and PBY were used as inoculum for the first, second and third type of tarhana, respectively. The PBY contained 7 g of compressed fresh baker's yeast. The mixtures were kneaded for 10 min and then allowed to ferment for 4 days at 30 °C in an incubator (Dedeoğlu Inc., Turkey).

1.2. Isolation of lactic acid bacteria

For isolation of lactic acid bacteria, MRS agar (Merck, 10660) and M17 agar (Oxoid, CM 785) were used according to the manufacturer's directions. Actidione (cycloheximide) (Serva, 10700), an antifungal antibiotic, was added into the MRS agar medium with the procedure described by HARRIGAN and MCCANCE (1976) for inhibition of probable yeast and mould growth in the medium. The dilutions of tarhana samples were pour plated with the agar media immediately after inoculation and then at each successive fermentation day. The plates were incubated at 37 °C for 3 days (ANDERSSON, 1988; ANON., 1990). The colony groups with different morphologies grown in or on the agar media were determined during the incubation and a certain number of colonies in each group were randomly selected and isolated according to their relative numbers. Isolates were subcultured in M17 broth (Oxoid, CM 817) or MRS broth (Merck, 10661) for 2–3 times. Purity of subcultures were controlled by restreaking on the agar media. The stock cultures in 12.0% reconstituted skim milk were also obtained at 37 °C and they were stored at 4 °C.

1.3. Differentiation of lactic acid bacteria isolates into genera and species

The differentiation scheme for lactic acid bacteria by SCHILLINGER and LUCKE (1987) was principally used for identification of the isolates into genera. In the first step, Gram reaction, microscopic morphology and catalase activity of the isolates were examined. Homo- and heterofermentative organisms were distinguished by measuring gas production from glucose. Then the isolates were subjected to some other preliminary identification tests such as growth at different temperatures, growth in presence of 6.5% NaCl and ammonia production from arginine. After that the isolates were further categorized on the basis of some distinguishable biochemical and physiological characteristics. Taxonomic descriptions from the literature were used for identification of the isolates.

1.4. Identification tests

Vigorously growing cultures of the isolates were used for the identification tests. Unless otherwise stated the temperature of incubation was 37 °C.

Presence of catalase and pseudocatalase activity was tested on MRS agar or M17 agar (HARRIGAN & MCCANCE, 1976) and on the Basal medium described by WHITTENBURY (1964), respectively. Gas (CO_2) production from glucose was observed in modified MRS broth described by SCHILLINGER and LUCKE (1987) after 3 days of incubation. Ammonia production from arginine was tested in modified MRS broth described by SCHILLINGER and LUCKE (1987). Ammonia was detected using Nessler's reagent at 2nd and 7th days of incubation (HARRIGAN & MCCANCE, 1976; JAYNE-WILLIAMS, 1976). Growth at 15 °C (in a precision cooled incubator, Memmert ICE 400), 37 °C and 45 °C was investigated in MRS broth or M17 broth. Inoculated tubes were examined for growth daily for 2–14 days (GARVIE, 1967a;b; JAYNE-WILLIAMS, 1976; ANON., 1990). Growth in different salt concentration was tested in MRS broth containing 6.5% and 10.0% NaCl or in M17 broth containing 4.0% and 6.5% NaCl. The results were obtained after 7 days of incubation (HARRIGAN & MCCANCE, 1976). For carbohydrate fermentation tests, the test sugars (obtained from Sigma) were added to the MRS basal medium (SCHILLINGER & LUCKE, 1987) in test tubes. Inoculated tubes were examined after 2–7 days of incubation. Aesculin hydrolysis was tested in modified MRS broth described by GUNTHER and WHITE (1961) and JAYNE-WILLIAMS (1976) or in M17 broth without lactose (JAYNE-WILLIAMS, 1976). The black precipitate formation was accepted as positive result after 2–7 days of incubation. Growth at different pH was tested in MRS broth adjusted to pH 4.2 and 4.4 or in M17 broth adjusted to pH 9.2 and 9.6 (HARRIGAN & MCCANCE, 1976). Growth in litmus milk was tested in Litmus milk (Oxoid, CM 45) after 10 days of incubation (SMITH & SHATTOCK, 1962; HARRIGAN & MCCANCE, 1976; JAYNE-WILLIAMS, 1976). Acidity test was only applied to homofermentative lactobacilli according to ROGOSA and co-workers (1953) and HARRIGAN and MCCANCE (1976). The production of dextran from sucrose was tested on MRS agar in which glucose had been replaced by 5% (w/v) sucrose (HARRIGAN & MCCANCE, 1976; SCHILLINGER & LUCKE, 1987). Diacetyl formation from glucose was examined according to modified Barritt's method after 2–7 days of incubation (HARRIGAN & MCCANCE, 1976). Methylene blue reduction test was applied to the *Streptococcus* strain according to JAYNE-WILLIAMS (1976). The results were controlled at 1st, 2nd and 7th days of incubation for color change in indicator. The configuration of the lactic acid isomers: D- and L(+) lactic acid was determined enzymatically by using D- and L(+) lactic acid dehydrogenase (Boehringer Mannheim GmbH, Mannheim) (ANON., 1992). Total lactic acid required to determine the configuration of lactic acid isomers was detected according to STEINSHOLT and CALBERT (1969).

2. Results

2.1. Identification of lactic acid bacteria as genera

During the tarhana fermentation 107 isolates of lactic acid bacteria were isolated from the MRS and M17 media. All isolates were Gram-positive. In our experiments, it was observed that some isolates formed weak bubbles during the application of catalase tests. The further identification tests of these isolates were continued considering the statements of several researchers about catalase and pseudocatalase activity of lactic acid bacteria (WHITTENBURY, 1964; SHARPE et al., 1966; COLLINS & TAYLOR, 1967; PREMI & BOTTAZZI, 1972; SHARPE, 1979). However, two isolates in tetrad morphology formed vigorous bubbles with the catalase tests and so they were eliminated. Besides these, some isolates were lost during the steps of their purification and identification. Therefore only 86 lactic acid bacteria isolates could be subjected to the further identification tests. Of these, 69 were differentiated into *Lactobacillus*. Ten of them were identified as *Leuconostoc* and 6 of them were *Pediococcus*. The bacterium isolated from M17 agar was identified as *Streptococcus*. On the basis of production of gas from glucose, 51 lactobacillus isolates were homofermentative.

2.2. Differentation of lactobacilli isolates into species

The biochemical and physiological characteristics of 69 lactobacillus isolates are shown in Table 1. Sixty out of 69 isolates were differentiated into nine different species by comparing with the taxonomic descriptions reported in the literature (ROGOSA et al., 1953; ROGOSA et al., 1961; SHARPE, 1962; 1979; SHARPE et al., 1966; 1972; GASSER, 1970; REUTER, 1970; BUCHANAN & GIBBONS, 1974; HARRIGAN & MCCANCE, 1976; JAYNE-WILLIAMS, 1976; KRIEG & HOLT, 1984; SCHILLINGER & LUCKE, 1987; TEMİZ, 1989). The characteristics of 32 isolates out of 60 agreed well with those of *Lactobacillus plantarum* (14 isolates), *Lact. casei* subsp. *pseudoplantarum* (7 isolates) *Lact. delbrueckii* subsp. *bulgaricus* (1 isolate), *Lact. helveticus* (2 isolates), *Lact. fermentum* (2 isolates) and *Lact. brevis* (6 isolates). The characteristics of 28 of 60 isolates were very similar to those of *Lact. plantarum* (8 isolates), *Lact. casei* subsp. *casei* (3 isolates), *Lact. curvatus* (9 isolates), *Lact. delbrueckii* subsp. *bulgaricus* (6 isolates) and *Lact. fermentum* biotype *cellobiosus* (2 isolates). However, they differed from typical strains on the basis of their one or more biochemical and physiological characteristics. The remaining 9 isolates could not be identified at the species level by using the identification tests in the present study because of important deviations in their characteristics. They were named as non-identified isolates. Among these, only the strain LP16 was homofermentative.

Table 1

Biochemical and physiological characteristics of the lactobacilli investigated

Typical species (a)	Isolate codes (b)	Fermentation of																			
		Glucose	Lactose	Sucrose	Maltose	Xylose	Arabinose	Melibiose	Cellobiose	Sorbitol	Galactose	Fructose	Raffinose	Salicin	Mannitol	Ribose	Aesculin	Growth at 45 °C	Growth at 15 °C	Lactic acid config.	Acidity (%)
Lact. plantarum		+(c)	+	+	+	V	V	+	+	+	+	+	+	+	+	+	+	V	+	DL-	0.30-1.20
	LC1,LC6,LC7-1,LC7-2,LC8-1, LC8-2,LC11,LC12,LC17-1, LC17-2,LC20,LP8-1(WI)	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	+	DL-	0.24-0.82
	LC19,LPB15(WI)	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	V	+	ND	0.30-0.62
	LC9-1,LC9-2,LP3-1,LP3-2,LP22	+	+	V	+	-	-	+	+	V	+	+	V	+	+	+	+	-	+	DL-	0.39-0.70
	LC15-1,LC15-2	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	ND	0.65,0.69
	LP21	+	+	+	+	-	-	+	+	-	+	+	+	+	+	w	+	w	+	DL-	0.41
Lact. casei subsp. *pseudoplantarum*		+	+	+	+	-	-	-	+	+	+	+	-	+	+	+	+	?	+	DL-	?
	LC2-1,LC2-2,LC5 LC18-11,LC18-12, LC18-21,LC18-22(WI)	+	+	+	+	-	-	-	+	+	+	+	-	+	+	+	+	V	+	DL-	0.33-0.39
Lact. casei subsp. *casei*		+	+	+	V	-	-	-	+	+	+	+	-	+	+	+	+	V	+	L(+)-	1.20-1.50
	LC16-1	+	+	+	+	-	-	-	+	-	+	+	-	+	+	+	+	-	+	ND	1.08
	LC16-21,LC16-22	+	+	+	+	-	-	-	+	-	+	+	-	+	+	w	+	-	+	DL-	0.92,ND
Lact. curvatus		+	V	-	+	-	-	-	+	-	+	+	-	+	-	+	+	-	+	DL-	?
	LP3,LP14-1,LP14-2 LP15,LP17 LP23,LP24,LP25,LP26	+	V	+	+	-	-	-	+	-	V	+	-	+	-	V	+	-	+	ND	0.12-0.37
Lact. delbrueckii subsp. *bulgaricus*		+	+	-	-	-	-	-	-	-	+	+	-	-	-	-	-	+	-	D-	1,70
	LP7,LP8-2,LP18,LP19	+	+	-	-	-	-	-	-	-	-	V	-	-	-	-	V	+	-	D-	1.04-1.20
	LP10(WI)	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	ND	1,13
	LP20-1,LP20-2	+	+	+	-	-	-	-	-	-	-	w	-	-	w	w	-	+	-	D-	1.03,1.16

Table 1 (cont.)

Typical species (a)	Isolate codes (b)	Fermentation of																			
		Glucose	Lactose	Sucrose	Maltose	Xylose	Arabinose	Melibiose	Cellobiose	Sorbitol	Galactose	Fructose	Raffinose	Salicin	Mannitol	Ribose	Aesculin	Growth at 45 °C	Growth at 15 °C	Lactic acid config.	Acidity (%)
Lact. helveticus		+	+	-	+	-	-	-	-	-	+	w	-	-	-	-	-	+	-	DL-	2,70
	LC3-1,LC3-2(WI)	+	+	-	+	-	-	-	-	-	+	+	-	-	-	-	w	+	-	ND	1.67,1.96
Lact. fermentum		+	+	+	+	V	V	+	-	-	+	+	+	-	-	+	-	+	-	DL-	?
	LC4-1,LC4-2(WI)	+	+	+	+	+	-	+	-	-	+	+	+	-	-	+	-	+	-	DL-	ND
Lact. fermentum biotype *cellobiosus*		+	w	+	+	V	-	+	+	-	+	+	+	w	-	+	+	V	V	DL-	?
	LC23,LC25	+	+	+	+	+	-	+	+	V	+	+	+	+	+	+	+	+	V	DL-	ND
Lact. brevis		+	#	V	+	V	+	+	-	-	#	+	-	-	#	+	V	-	+	DL-	?
	LPB8,LPB11,LPB12,LPB13(WI)	+	V	V	+	+	+	+	-	-	+	+/w	-	-	-	+	+/w	-	+	ND	ND
	LPB10,LPB14(WI)	+	+	V	+	+	+	+	-	-	+	+/w	-	-	V	+	-	-	+	DL-	ND
Non identified isolates																					
	LC10,LC13-2,LC14	+	+	+	+	-	+	+	-	-	+	+	+	-	-	-	-	+	+	DL-	ND
	LC13-1,LC21,LC22,LC24-1,LC24-2	+	+	+	+	V	V	+	-	-	+	+	+	-	-	+	-	+	+	ND	ND
	LP16	+	+	+	+	-	-	+	-	-	+	-	-	+	+	-	+	-	+	ND	ND

(a) Taxonomic descriptions from the literature

(b) Code number symbols of the isolates: [P] isolated from the tarhanas produced by using PY; [C] isolated from the tarhanas produced by using CY; [PB] isolated from the tarhanas produced by using PBY; [WI] well-identified isolates

(c) Symbols: [+] positive reaction; [–] no reaction or no growth; [V] variable in reaction; [w] weak; [#] weak or late or negative in reaction; [D-], [DL-], [L-] lactic acid isomers formed by strain; D- or L(+)-: the isomer recorded makes up 90% or more of total lactic acid, DL-: 25–75% of total lactic acid are of L(+)- configuration. [ND] not determined; (?) no knowledge in the literature used.

Table 2

Biochemical and physiological characteristics of the leuconoctocs investigated

Typical species (a)	Isolate codes (b)	Growth at 37 °C	Dextran from sucrose	Growth in litmus milk	Aesculin	Growth at pH 4.2	Fermentation of														
							Glucose	Lactose	Sucrose	Maltose	Xylose	Arabinose	Melibiose	Cellobiose	Galactose	Fructose	Raffinose	Salicin	Mannitol	Ribose	Lactic acid config.
Leuc. mesenteroides subsp. *mesenteroides*		V(c)	+	-	V	-	+	V	+	+	V	+	V	?	+	+	V	V	V	+	D-
	SP15,SP16,SPB8-1(WI)	+	+	-	+	-	+	+	+	+	+/w	+	+	V	+	+	+	+	-	+	ND
	SPB1-1	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	ND	ND	ND	ND
	SPB8-2	+	+	-	+	-	+	+	+	+	w	+	+	-	+	+	+	+	-	-	D-
	SPB2-1	+	+	-	+	-	+	+	+	+	w	+	+	-	+	+	+	+	w	-	ND
	SP4-1,SPB9,SPB14-2,SC11	+	+	-	+	-	+	+	+	+	+/w	+	+	V	+	+	+	V	-	-	ND

(a) Taxonomic descriptions from the literature

(b) Code number symbols of the isolates: [P] isolated from the tarhanas produced by using PY; [C] isolated from the tarhanas produced by using CY; [PB] isolated from the tarhanas produced by using PBY; [WI] well-identified isolates

(c) Symbols: [+] positive reaction; [–] no reaction or no growth; [V] variable in reaction; [w] weak; [?] no knowledge in the literature used; [D-] lactic acid isomer formed by strain; the isomer recorded makes up 90% or more of total lactic acid; [ND] not determined.

Table 3

Biochemical and physiological characteristics of the pediococci investigated

									Fermentation of															
Typical species (a)	Isolate codes (b)	Growth at 37 °C	Growth at 45 °C	Growth at pH 4.4	Growth in 6.5% NaCl	Growth in 10% NaCl	Diacetyl from glucose (VP)	Glucose	Lactose	Sucrose	Maltose	Xylose	Arabinose	Melibiose	Cellobiose	Galactose	Fructose	Raffinose	Salicin	Mannitol	Ribose	Sorbitol	Aesculin	Lactic acid config.
Ped. pentosaceus		+(c)	+	+	+	w	+	+	+	-	+	-	+	+	+	+	+	-	+	-	+	-	+	DL-
	SPB3-1,SC10-1	+	+	+	+	w	+	+	+	-	+	-	-	+	+	+	+	-	+	-	+	-	+	L(+)-
	SPB1-2, SPB3-21,SPB3-22 SC10-2	+	+	+	+	w	+	+	+	-	+	-	-	+	+	+	+	-	+	-	+	-	+	ND

(a) Taxonomic descriptions from the literature

(b) Code number symbols of the isolates: [C] isolated from the tarhanas produced by using CY, [PB] isolated from the tarhanas produced by using PBY.

(c) Symbols: [+] positive reaction; [-] no reaction or no growth; [w] weak;
[ND] not determined; [DL-], [L-]lactic acid isomers formed by strain.
DL-: 25–75% of total lactic acid are of L(+)- configuration,
L(+)-: the isomer recorded makes up 90% or more of total lactic acid.

2.3. Differentiation of Leuconostoc *isolates into species*

The biochemical and physiological characteristics of 10 leuconostocs are presented in Table 2. All isolates were characterized as *Leuconostoc mesenteroides* subsp. *mesenteroides* according to the taxonomic descriptions from the literature (SMITH & SHATTOCK, 1962; SHARPE et al., 1966; GARVIE, 1967 a; b; BUCHANAN & GIBBONS, 1974; HARRIGAN & MCCANCE, 1976; JAYNE-WILLIAMS, 1976; SHARPE, 1979; KRIEG & HOLT, 1984; ANON., 1990). However, the characteristics of 3 isolates only agreed well those of *Leuc. mesenteroides* subsp. *mesenteroides*.

2.4. Differentiation of Pediococcus *isolates into species*

The characteristics of 6 pediococcus isolates are shown in Table 3. All of them were typed as *Pediococcus pentosaceus* by comparing with the taxonomic descriptions from the literature (GUNTHER & WHITE, 1961; SHARPE et al., 1966; BUCHANAN & GIBBONS, 1974; HARRIGAN & MCCANCE, 1976; JAYNE-WILLIAMS, 1976; SHARPE, 1979; KRIEG & HOLT, 1984). However, these isolates differed from *Ped. pentosaceus* by not using arabinose. In addition, the isolates tested in terms of lactic acid configuration formed L(+) lactic acid whereas typical strains of *Ped. pentosaceus* produce DL-form.

2.5. Identification of the Streptococcus *isolate into species*

The strain of SP6-1 isolated from M17 agar was identified as *Streptococcus thermophilus* according to the taxonomic descriptions from the literature (SHARPE et al., 1966; HARRIGAN & MCCANCE, 1976; SHARPE, 1979). However, this isolate formed DL-lactic acid whereas typical strains of *Strep. thermophilus* form L(+) lactic acid.

3. Conclusions

Eighty six lactic acid bacteria isolated from the tarhanas during fermentation were subjected to some preliminary and distinguishable identification tests. Thirty five out of 86 isolated strains could be well identified according to the results of the biochemical and physiological tests examined in the present study. Of the well identified isolates, 32 were lactobacilli (Table 1) and remainders were *Leuconostoc mesenteroides* subsp. *mesenteroides* (Table 2). Fourteen of the lactobacilli isolates were identified as *Lactobacillus plantarum* (Table 1). But two of them (LC19 and LPB15) differed from the others by fermenting arabinose. The strains of *Lact. plantarum* fermenting arabinose were typed as *Lact. arabinosus* by BUCHANAN & GIBBONS (1979). One lactobacilli isolate (LP10) had similar characteristics to the typical strains

of *Lact. delbrueckii* subsp. *bulgaricus*. But this isolate failed to ferment fructose. ROGOSA and co-workers (1953) stated that some strains of *Lact. delbrueckii* subsp. *bulgaricus* were unable to ferment fructose or they fermented it lately. Two isolates of lactobacilli were characterized as *Lact. helveticus* since they developed high acidity in milk. Six heterofermentative lactobacilli isolates were identified as *Lact. brevis*. Three of them (LPB11, LPB12, LPB13) differed from other *Lact. brevis* isolates by not fermenting lactose and one of them (LPB10) by fermenting mannitol. However, the fermentation of lactose and mannitol by typical strains of *Lact. brevis* are weak or late or negative (Table 1). The biochemical and physiological characteristics of the other lactobacilli agreed well with those of *Lact. casei* subsp. *pseudoplantarum* (7 isolates) and *Lact. fermentum* (2 isolates). As a result, it is evident that *Lact. plantarum*, *Lact. casei* subsp. *pseudoplantarum* and *Lact. brevis* formed a major group of the well identified lactic acid bacteria isolated from the tarhana samples during fermentation. It is mentioned that *Lact. plantarum*, *Lact. casei* and *Lact. brevis* are prominent in kish fermentation (EL-GENDY, 1983).

In this study, the biochemical and physiological properties of 42 isolates out of 86 were very similar to those of *Lact. plantarum* (8 isolates), *Lact. casei* subsp. *casei* (3 isolates), *Lact. curvatus* (9 isolates), *Lact. delbrueckii* subsp. *bulgaricus* (6 isolates), *Lact. fermentum* biotype *cellobiosus* (2 isolates), *Pediococcus pentosaceus* (6 isolates), *Leuc. mesenteroides* subsp. *mesenteroides* (7 isolates) (Table 1–3) and *Streptococcus thermophilus* (1 isolate). However, they differed from typical strains of the related lactic acid bacteria on the basis of their one or more biochemical and physiological characteristics. For example, three lactobacillus isolates were typed as *Lact. casei* subsp. *casei* because of developing higher amount of acidity in milk than those of *Lact. casei* subsp. *pseudoplantarum* strains (Table 1). However, these were unable to ferment sorbitol. According to ROGOSA and co-workers (1953), some strains of *Lact. casei* subsp. *casei* were unable to ferment sorbitol. But two isolates (LC16-21, LC16-22) tested in terms of lactic acid configuration produced DL-lactic acid instead of L(+) form. HITCHENER and co-workers (1982) pointed out that, in particular, atypical betabacteria strains isolated from vacuum-packaged beef in their study appeared to form only L(+) isomer of lactic acid whereas typical betabacteria form both isomers. Two heterofermentative isolates whose properties were similar to *Lact. fermentum* were identified as *Lact. fermentum* biotype *cellobiosus* since they differed from the strains of *Lact. fermentum* by fermenting cellobiose and salicin, and hydrolyzing aesculin. However these two isolates differed from the typical *Lact. fermentum* biotype *cellobiosus* by fermenting mannitol, and one of them also by fermenting sorbitol (Table 1). The isolates of *Ped. pentosaceus* formed L(+)- lactic acid and the isolate of *Strep. termophilus* formed DL-lactic acid. However, the production of DL-lactic acid by *Ped. pentosaceus* and L(+)-lactic acid by *Strep. thermophilus* is considered to be one of the

most typical and characteristic properties of these bacteria. As mentioned by SHARPE (1979), owing to the size of analytical error difficulty arises when one of the isomers constitutes less than 10% of the total lactate formed. On the other hand, in most DL-lactic acid formers the ratio of the two isomers depends on the growth phase (KANDLER, 1983). KRIEG and HOLT (1984) mentioned that some DL- lactic acid formers produce predominantly L(+)- or, in a few cases, D(–)-lactic acid during the early growth phase, and therefore, care must be taken to analyze cultures after they have reached the stationary growth phase. It is indicated that the ratio of D/L lactate formed by *Ped. pentosaceus* may vary with growth conditions (BACK, 1978). Electrophoretic mobility of the lactate dehydrogenases migth be useful to differentiate the species in these problematical cases. BACK (1978) found that *Pediococcus pentosaceus* forms DL-lactate and L(+) and D(–)-lactic acid dehydrogenases. Moreover, *Streptococcus thermophilus* has two lactic acid dehydrogenases which form L(+)-lactate (GARVIE, 1978).

The properties of 8 non-identified heterofermentative strains closely resemble to those of biotypes of *Lact. fermentum* and they may be considered to be *Lact. fermentum* biotype *cellobiosus* in their patterns of growth at both 15 °C and 45 °C (BOTTAZZI, 1988). However, they could not ferment cellobiose. On the other hand, 4 of them presented typical characteristics of *Lact. fermentum*, except growth at 15 °C. The non-identified homofermentative strain (LP16) was typed as an atypical streptobacterium. The isolates which differed from typical strains of lactic acid bacteria, were generally considered to be atypical. For example, HITCHENER and co-workers (1982) and SCHILLINGER and LUCKE (1987) mentioned the difficulties of identification of atypical streptobacteria and heterofermentative bacteria in their studies. It is also pointed out that the relationships between various groups of organisms designated as atypical streptobacteria are not clear, since different criteria were used in each study (HITCHENER et al., 1982).

Producing three different kinds of tarhana enhanced the chance to isolate different strains of lactic acid bacteria (Table 1–3). In fact, we are mainly interested in whether the tarhana fermentation at all influenced by the kind of inoculum and microbial interaction among the lactic strains, also between the lactic strains and baker's yeast. The main goal of the study was to determine lactic flora of tarhana and the tarhanas from which the isolates were isolated. In this view, for example most of the isolates of *Lact. plantarum*, all isolates of *Lact. casei* subsp. *plantarum*, *Lact. helveticus* and *Lact. fermentum* were isolated from the tarhana produced by using CY whereas all isolates of *Lact. delbrueckii* subsp. *bulgaricus* were isolated from the tarhana produced by using PY, and *Lact. brevis* from the tarhana produced by using PBY as inoculum (Table 1). The isolates of *Ped. pentosaceus* could not be isolated from the tarhana produced by using PY (Table 3) whereas the isolates of *Leuc. mesenteroides* subsp.

mesenteroides were isolated from all tarhana samples (Table 2). The results indicate that the inoculum and microbial interaction may influence the lactic flora in tarhana fermentation.

The biochemical and physiological criteria used in the present study were not sufficient to differentiate some isolates into species. In fact, the presence of meso-diaminopimelic acid (mDpm) in cell wall and the configuration of lactic acid formed by bacterium is very helpful for the differentiation of the isolates due to the greater taxonomic value of these stable characters. But, the carbohydrate fermentation pattern of heterofermentative lactobacilli is similar, and they do not differ in the configuration of lactic acid produced (DL) nor in murein type (Lys-D-Asp) of the cell wall (SCHILINGER & LUCKE, 1987). However, most recent advances of classifying and identifying the lactic acid bacteria have come from molecular systematics based on 16s rRNA sequence data analysis and DNA–DNA hybridization (KLIJN et al., 1991; DRAKE et al., 1996). They promise a greater taxonomic value for lactic acid bacteria. However, the utilization of these recent approaches should be considered in coordination with the more classical identification tests.

M17 agar is recommended as an improved medium for the growth and the enumeration of lactococci by the manufacturers and it has been recommended by IDF (International Dairy Federation) for selective enumeration of *Streptococcus thermophilus* from yogurt (ANON., 1990). In the present study, although a number of streptococci have been isolated from M17 agar, only one of them isolated from the tarhana produced by using PY could be maintained and identified as *Strep. thermophilus* by using M17 media. It is expected that the strains of the genus *Lactococcus* are involved in tarhana fermentation. But the temperature used with M17 was obviously too high to isolate the lactococci. We have already carried out another study with a similar experimental design in order to compare two plating temperatures of 30 °C and 37 °C by using the media of M17 agar and Neutral red chalk lactose agar (NRCLA). NRCLA is another recommended medium for the detection of lactococci in milk and milk products (HARRIGAN & MCCANCE, 1976).

In conclusion, the present report on the identification of lactic flora of tarhana fermentation will be able to help in the selection of appropriate cultures for controlled, pure culture fermentations. However, more work is needed for further determination of lactic flora involved in tarhana fermentation. Most recent advances of molecular systematics should also be included in order to differentiate the isolates into well identified species.

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QUALITY REQUIREMENTS OF SPECIALITY FOODSTUFFS IN THE EU AND THEIR APPLICATION IN HUNGARY

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Entering the final phase of the preparation for negotiations on EU accession, the main area of work of our department is the co-ordination of the adaptation of European legislation in the Hungarian agricultural and foodstuffs sectors. Our specific field of work is the assessment of the EU's horizontal and vertical foodstuffs quality provisions, and the elaboration of their possible application in Hungary. This paper focuses particularly on the scientific analysis of the adoption of EC provisions regarding speciality products that are of high importance from the point of view of the exportability of Hungarian foodstuffs.

Keywords: speciality foodstuffs, EU food quality legislation, scientific guidelines for the harmonization process, exportability of Hungarian foodstuffs

The new Hungarian food legislation has been designed to be in full harmony with EU food legislation by the end of 1998. In the framework of the Hungarian legal harmonization process this paper concentrates particularly on the scientific analysis of the adoption of EC regulations pertaining to speciality products that are of particular importance for Hungarian export.

These regulations have a particular significance in that, by taking into account the speciality agricultural products and foodstuffs, they allow the upgrading of these traditional products of individual countries on the market instead of making the products of the single market uniform. They contribute to the acceptance by consumers of indications of speciality, by providing an adequate quality guarantee with the products. It is of vital importance to Hungary that these rules make joining the system possible for third countries as well, if they meet the requirements set by law. In order to ensure a similar protection for Hungarian products on EU markets, it is essential to adopt the relevant EC legislation (KISÉRDI-PALLÓ, 1997a).

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1. Materials and approaches

In the course of research the in-depth analysis of those particularly important quality provisions of the EU was carried out which are feasible prior to the accession of Hungary. These are the following:

– Council Regulation (EEC) No 2092/91 on organic production of agricultural products and indications referring thereto on agricultural products and foodstuffs (EEC, 1991)

– Council Regulation (EEC) No 2081/92 on the protection of geographical indications and designation of origin for agricultural products and foodstuffs (EEC, 1992a)

– Council Regulation (EEC) No 2082/92 on certificates of specific character for agricultural products and foodstuffs (EEC, 1992b)

After analysing the complex text of these EC regulations, using the special literature available, their application was studied in practice in various EU member states. During the comparative analysis of the experience gathered from the different practices, particular attention was paid to scientific interrelations, to the difficulties of implementation and to possible critical notes. Subsequently, impact analyses were conducted with regard to the possible Hungarian application of EC quality provisions, and, after studying the Hungarian legislative and institutional background, proposals were outlined as to their upgrading.

2. Results and discussions

2.1. The analysis of foreign legislation concerning organic production and certification system

Inspection of the end product cannot suffice to establish conclusively whether a product has been obtained organically. Effective inspection depends on the inspection body being able to check every stage of production. Given the particular task of effectively monitoring compliance with the rules on organic production, a regular inspection system is needed.

Step 1: Regulation No 2092/91 (EEC) on organic production and indications of agricultural products and foodstuffs (EEC, 1991), the USA Organic Food Production Act (USA ACT, 1990) and the Hungarian situation were compared from the point of view of the quality control and certification system (Table 1).

Table 1

State of the quality control and certification system of organic production in USA, EC and Hungary

	USA	EC	HUN
Regulation	Organic Foods Production Act of 1990	Regulation 2091/92 (EEC)	Draft ministerial decree (1997)
Scope	agricultural products whether raw or processed including any product derived from livestock	unprocessed agricultural crop products and foods composed of plant origin	unprocessed agricultural crop products and foods composed of plant origin; organic livestock farming
Control system	certifying agent	designated inspection authorities and/or approved private bodies	private inspection body ("Biokultúra" Association)
Certification system	State-Organic Certification Program	Organic Farming-EEC Control System	Organic Production-Hungarian Control System

Step 2: Analysing the state of the organic production and of the quality control system in USA, EC and Hungary, my conclusion was that on the basis of EC provisions it is necessary to draw up national legislation in Hungary that takes into account Hungarian specificities and possibilities. This is justified by a growing demand on the part of consumers for organic products, by the need to ensure a fair market competition and to provide consumers with appropriate information.

Step 3: An impact study was prepared to assess the possibility of the elaboration of Hungarian legislation concerning organic production (KOMÁROMY et al., 1992).

2.2. The elaboration of their application in Hungary

2.2.1. Governmental side. The primary task set in the impact study, that is the elaboration of national legislation concerning organic production, is underway at the Ministry of Agriculture in accordance with the Hungarian Act No XC/1995 on foodstuffs (HUNGARIAN ACT, 1995). The regulation on organic production is based on EC rules, and its publication is expected by 1998.

2.2.2. Inspection side. At present, pursuant to Regulation No. 314/97 (EC), the bodies recognised by the Commission of the EU as the inspection and certification

body of unprocessed crop products and foodstuffs in Hungary are the "Biokultúra" Association and SKAL (EC, 1997). The Hungarian association for organic production (Biokultúra) is full member of IFOAM since 1987 and its accreditation according to European Standard EN 45011 is underway (EN, 1990).

2.2.3. Consumer side. A promotional programme to make consumers aware of the importance of the indications of organic production by Hungarian Collective Agricultural Marketing Centre (AMC) was launched last year.

2.3. The analysis of the European system of protection of origin and the certification of specific characters

Regulation No 2081/92 provides essential definitions in the relevant field (EEC, 1992a). It distinguishes two categories of protected names: geographical indications (PGI) and designation of origin (PDO). The distinction between the two categories depends on how closely the products are linked to the specific geographical area whose name they bear. The Regulation also defines generic names, which cannot be protected (e.g. camembert, emmental, brie, mozzarella, edam, cheddar).

Only groups of producers working with the same product may apply for the registration. The application must be accompanied by precise product specification. The Regulation therefore sets up an appropriate inspection structure. The inspections may be carried out by designated inspection authorities, or by private bodies.

Under Regulation No 2082/92, traditional character is an essential additional element for the purposes of the certification of specific character (EEC, 1992b). The procedure for registering a certificate of specific character is very similar to that of geographical indications and designations of origin. There is also a clear parallel between the two Regulations in the rules on inspection: they are virtually identical.

Under the Community Regulations, negotiations can be undertaken with interested non-member countries, with a view to establishing a protection and inspection system equivalent to the Community system. Such registration is in the interest of Hungarian producers who want the names of their products to be protected Europe-wide. The geographical names thus become a useful advertising tool of Hungarian products (KISÉRDI-PALLÓ, 1992).

2.4. Preparation of national legislation of speciality foodstuffs

At the starting point of the implementation of EC Regulations it was established that such a type of registration and certification system had never been operated in Hungary apart from the wine sector. We did not have a catalogue of geographical indications which had protection in Hungary. We had no specifications which determined the region of origin or the methods of production. We had no experience in

operating such a system, and the interested sectors of the public were not used to such an approach.

Step 1: Studying the EU Commission's registration procedure practice and the list of products approved by the EU's Commission. The list consists of 415 products as of June 20, 1997 (KISÉRDI-PALLÓ, 1997b).

Step 2: Presentation of a proposal concerning the elaboration of the Hungarian registration procedure (Fig. 1).

Applications shall be submitted to the Hungarian Food Codex Commission together with the specification. The Hungarian Food Codex Commission members are representatives of the academia, the economy, the governmental food inspection authority, the consumer organisation and ministries.

Step 3: Creation of a list of Hungarian products which are candidates for protection of designation of origin or geographical indication (Table 2).

Step 4: Creation of a list of Hungarian products which are candidates for certification of specific character (Table 3).

Step 5: Preparation of a proposal concerning the inspection system.

According to the Hungarian Act No XC/1995 on foodstuffs, the Veterinary and Food Control Stations (Stations) are competent for the supervision and control in their area of agricultural products and foodstuffs (HUNGARIAN ACT, 1995.). These Stations will be the competent authorities to establish that the production of registered foods meets the criteria laid down in their product specification, as these bodies fulfil the requirements laid down in EN 45011 (EN, 1990). The costs of the inspection – according to the official price list of testing – shall be paid by the producer.

Table 2

Some candidates of Hungarian products for protection of designation of origin or geographical indication

1.	"Makói" vöröshagyma	onion
2.	"Makói" fokhagyma	garlic
3.	"Szegedi" paprika	Capsicum annuum
4.	"Kalocsai" paprika	Capsicum annuum
5.	"Magyar" szalámi	Hungarian salami
6.	"Csabai" kolbász	sausage
7.	"Gyulai" kolbász	sausage
8.	"Alföldi" búzakenyér	wheat bread
9.	"Kékkúti" ásványvíz	mineral water
10.	"Margitszigeti" kristályvíz	mineral water

Application by a producer group
(accompanied by specification)
⇓

Hungarian Food Codex Committee
(competent authority)
⇓

Formal investigation — Rejection ⇒ — No publication

⇓ Acceptance

First publication
(in Official Journal of Ministry of Agriculture)
⇓

Objection procedure — Rejection ⇒ — No registration

⇓ Acceptance

REGISTRATION
(of main points of the application in Hungarian Food Codex)
⇓

FINAL PUBLICATION
(of name in Official Journal of Ministry of Agriculture)

Fig. 1. Proposal for national registration procedure in Hungary

Table 3

Some candidates of Hungarian foods for certification of specific character

1.	Nagymama lekvárja	Grandmother's jam
2.	Házi szilvalekvár	Traditional plum jam
3.	Háztáji csirke	Farm chicken
4.	Háztáji pulyka	Farm turkey
5.	Csusza tészta	Traditional pasta torn to pieces by hand
6.	Tarhonya	Pasta of specific shape, garnishing of meat dishes
7.	Rétes	Hungarian strudel filled with apple/poppy seed/walnuts/sour cherries/cabbage
8.	Szilvás derelye	Pastry of specific shape filled with plum jam
9.	Gulyás	Goulash – traditional recipe
10.	Halászlé	Fisherman's soup – traditional recipe

2.4. Tasks in the future

2.5.1. Governmental side. Pursuant to the Hungarian Act XI/1997 on the protection of trademarks and geographical indications, a governmental decree has to be released to regulate in detail the protection of geographical indications and designation of origin for agricultural products and foodstuffs in Hungary (HUNGARIAN ACT, 1997). The Hungarian Patent Office and the Ministry of Agriculture are jointly responsible for the elaboration of the decree. The entry into force of the decree is expected by the first half of 1998.

Pursuant to the Hungarian Act No XC/1995 on foodstuffs (HUNGARIAN ACT, 1995), the national legislation concerning the special characteristics of agricultural products and foodstuffs was elaborated by Ministry of Agriculture. The regulation based on EC rules was published in January of 1998 (MINISTRY OF AGRICULTURE, 1998).

After establishing the national legislation and institutional background the most important task will be to enter negotiations on the accession to the EU system.

2.5.2. Inspection side. Hungary had no tradition of inspection services geared to specific foods or a centralised system of certification so it is an essential task to set up the methods of inspection services at a national level based on a harmonized approach.

2.5.3. Producer side. To encourage manufacturers to have their product registered the elaboration of product specifications – complying with EU requirements – will have to start. As a part of this work the elaboration of the product specification of "Hungarian salami" is underway. This will serve as a model.

2.5.4. Consumer side. Launching a promotional programme to make consumers aware of the importance of the indications of speciality by Hungarian Collective Agricultural Marketing Centre (AMC).

2.5.5. Researchers' side. The development of a scientific and technical basis for speciality products (e.g. analysis of consumer expectations, development of methods of investigation, identification of the criteria for product quality).

3. Conclusions

To sum up, the reform of Common Agricultural Policy (CAP) assigns a major role to European quality policy in the interest of both consumers and producers. Regulations (EEC) No 2092/91 or organic production, No 2081/92 on the protection of geographical indications and designation of origin and No 2092/92 on certificates of specific character for agricultural products and foodstuffs form important instruments of European quality policy.

This package of European legislation provides for a new structure for the monitoring of the speciality foods. All products registered under these Regulations will be subject to inspection to ensure that the requirements of the registered specification are met. In Hungary applicants must nominate an inspection body which shall meet the provisions laid down in European Standard EN 45011 (EN, 1990) by 31 December 1998 at the latest.

Hungary's preparation for the accession to the quality control system of the organic production, the protection system of designation of origin and certification system of specific character of the EU enhances the appropriate recognition and protection on the single market of special Hungarian products manufactured with traditional methods. This contributes to the improvement of the competitiveness of Hungarian goods.

*

The author wishes to express her gratitude to Professor P. MOLNÁR President of HNC of EOQ for his invaluable assistance. I am also extremely grateful to Professor P. BIACS, Member of the Editorial Board, for his encouragement.

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Acta Alimentaria, Vol. 27 (3), pp. 303–306 (1998)

Book reviews

Processing vegetables
Science and technology

D. S. SMITH, J. N. CASH, W.-K NIP and Y. H. HUI (Eds)

Technomic Publishing Company, Inc., Lancaster and Basel,
1997, ISBN 1-56676-507-2, 415 pp. + index

Vegetables are more than just possible food products. Due to their sensorial and nutritional value, vegetables are becoming more and more popular in every part of the world, and the increasing consumption of vegetables transforms and makes healthier the daily food intake of people. Vegetables can be consumed in several forms, their role in meals is not limited any longer to soups and side-dishes but also serve as foods providing culinary assortment as well.

Nowadays a higher percentage of vegetables is being processed commercially with mass production than ever before. For consumers with increased and different requirements these vegetable products preserved with gentle processing from good quality raw materials, and having long shelf-life and stable quality provide in the households a range of foods that can be prepared at any time quickly and easily.

This book consisting of 2 parts and 14 chapters have been written by 17 authors who are all specialist and recognised authority in their own field. The authors have given special attention to harvesting, process procedures and equipment and the handling of problems encountered during mass production in relation to quality assurance. Of the modern technologies available today in vegetable processing the authors dealt mainly with the canning process and its application and paid less attention to quick freezing which is also deservedly popular in this field. The food processing technology options fail to include the use of ionising radiation and the biological type preservation used in many parts in Europe in industrial-scale vegetable processing. Interesting and detailed information is provided to the readers on advanced automation methods and packaging systems. In some chapters processing is rightly connected with cultivars and the necessary storage. The book excellently details the processing technology of some major vegetables, it is unfortunate that some important raw material such as cauliflower, broccoli, leaf-vegetables, etc. are not included. There are many handy tables, figures and photos in the book. At the end of each chapter there is a list of references which, especially in Chapters 5 to 14 contain useful information. The 18-page long Index provides excellent orientation in the book.

The first part of the book deals with basic theoretical knowledge of vegetable processing (Fundamentals in Processing Vegetables). Separate chapters are devoted to canning (Chapter 2) and to drying and freeze-drying (Chapter 3). One of the best chapters of the book is the uniform and concise summary of the microbiology of vegetable processing (Chapter 4). 147 references can be found at the end of the chapter.

The second and larger part of the book (Chapters 5 to 14) includes the description of industrial procedures, technologies used for producing vegetable products (Processing Vegetable Commodities). Separate chapters are devoted to the processing of carrots, cucumbers, edible mushrooms, onions, pepper,

potatoes, snap beans, sweet corn and taro (*Colocasia esculenta* [L.]) and tomato. The structure of content and the complexity of these chapters vary greatly. The authors unfortunately very rarely use the SI units. The chapters on the processing of edible mushrooms (Chapter 7), potatoes (Chapter 10), sweet corn (Chapter 12) and tomato (Chapter 14) are well written and of high standard. The chapter on cucumber processing (Chapter 6) has a highly different approach than that of the other chapters.

The book provides valuable information for vegetable processors and for those engaged in growing or supplying vegetables for processing. The publisher issued this book as part of its high-standard series, following the publication of the successful Processing Fruits (Vol. 1–2).

T. SÁRAY

Math concepts for food engineering

R. W. HARTEL, T. A. HOWELL, Jr. and D. B. HYSLOP

Technomic Publishing Co., Inc., Lancaster, Basel, 1997,
ISBN: 1-56676-564-1, 177 pages

Food engineering laboratory manual

G. V. BARBOSA-CANOVAS, LI MA and B. BARLETTA

Technomic Publishing Co., Inc., Lancaster, Basel, 1997,
ISBN: 1-56676-541-2, 141 pages

Two new books have been published in 1997 by the Technomic Company on the field of food engineering for educational purposes in universities and academies. Both books have paperback covers.

The first book, with title: Math Concepts for Food Engineering, covers 177 pages and is divided into ten chapters:

Algebra; Interpolation of data in tables; Graphs and curve fitting; Calculus; Problem solving; Gases and Vapors; Mass Balances; Energy Balances; Fluid Mechanics; Heat Transfer. The first 4 chapters can be regarded as a repetition of the most useful mathematical basics, with examples from food engineering practice. Altogether 40 to 45 examples enable the students to connect abstract knowledge with practice. The following 6 chapters contain 58 examples on different areas of food physics, referring to already known results of food engineering, food physics. The authors of this book are professor, graduate research assistant and senior lecturer of the University of Wisconsin (Department of Food Science and Department of Biological Systems Engineering). They created the book on experiences of their tutorial activity.

The second book, with title: Food Engineering Laboratory Manual, contains 141 pages and 12 chapters: Planning experiments; Friction losses determination in a pipe; Convective heat transfer coefficient determination; Thermal processing of foods: Part I, heat penetration; Thermal processing of foods: Part II, lethality determination; Freezing of foods; Drying of foods: Part I, tray drying; Drying of foods: Part II, spray drying; Drying of foods: Part III, freeze drying; Extrusion of foods; Evaporation; Physical separation.

The book covers the most important preservation fields and a few food processing operations as indicated by the chapter titles. Altogether 17 examples are included, each chapter is provided with a number of questions for controlling one's progress. A few important references also have been cited at chapters' ends. The book is of great help for carrying out laboratory experiments, both for students and teachers. Authors are members of the Washington State University and possess Ph. D. and M. S. graduations.

In essence, both books belong to the pre-computer era, which fact has special advantages and also some drawbacks. It can be considered as advantage that students can concentrate on the problem to be solved without spell-bound by the mirage of PC technics. It seems to be simple to give tutorial amendment if more sophisticated computer technics is involved. However, the complete lack of numerical methods, such as of finite differences and finite elements, is disadvantageous, especially in the fields of thermal processing, freezing and drying. Microwave technics is also lacking.

I. KÖRMENDY

Microorganisms in foods 6: microbial ecology of food commodities

Editorial Committee: ROBERTS, T. A., PITT, J. I., FARKAS, J., GRAU, F. H.

ICMSF, Blackie Academic & Professional, London, 1998,
ISBN 0 75140430 6, 615 pages

The book is an updated version of Microbial Ecology of Foods, Volume 2, published by the Academic Press in 1980. The revised edition is written by 19 scientists from 11 countries, plus 12 consultants and 12 chapter contributors, selected by the International Commission on Microbiological Specifications for Foods (ICMSF). The book considers the developments in food processing and packaging, new ranges of products, and foodborne pathogens that have emerged since 1980.

It is emphasised that the traditional control of food safety by inspection, hygiene regulations and end-product testing is not adequate, and the information collected and edited in this book, together with those in Microorganisms in Foods 5: Characteristics of Microbial Pathogens (1996, Blackie Academic & Professional) serve as basis in the effective implementation of the HACCP system outlined in the 4th volume of Microorganisms in Foods: Application of the Hazard Analysis Critical Control Point (HACCP) System to Ensure Microbiological Safety and Quality (ICMSF, Blackwell Scientific Publications, 1988).

The book principally retains the structure of the first edition, however, it also includes new points. For each commodity the followings are discussed: (i) the important properties of the food commodity that affect its microbial content; (ii) the initial microflora at slaughter or harvest; (iii) the effect of harvesting, transportation, processing and storage on the microbial content; (iv) the means of controlling processes and the microbial content.

The chapters, i.e. food commodities are as follows: 1. Meat and meat products., 2. Poultry and poultry products, 3. Fish and fish products, 4. Feeds and pet foods, 5. Vegetables and vegetable products, 6. Fruits and fruit products. 7. Spices, dry soups and oriental flavourings, 8. Cereals and cereal products, 9. Nuts, oilseeds and dried legumes, 10. Cocoa, chocolate and confectionery, 11. Oil- and fat-based foods, 12. Sugar, syrups and honey, 13. Soft drinks, fruit juices, concentrates and fruit preserves, 14. Water, 15. Eggs and egg products, 16. Milk and dairy products, 17. Preventing abuse of foods after processing, and in the Appendices (I–IV) useful information can be found on ICMSF.

Chapter 1 (Meat and meat products) is detailed to represent the structure: the (I) Introduction (Definitions, Important properties, Methods of processing and preservation, Types of meat product) is followed by the (II) Initial microflora (Ruminants, Pigs). In section (III) Primary processing the (A) Effects of processing on microorganisms (Ruminants, Pigs, Chilling, Carcass storage and transport), (B) Spoilage, (C) Pathogens and (D) Control are discussed. Section (IV) deals with the Carcass cutting and packaging, and the subsequent 10 sections (V–XIV) detail the different meat products (frozen meat, raw comminuted meats, raw cured shelf-stable meats, dried meats, cooked perishable uncured meats, fully retorted shelf-stable uncured meats, cooked perishable cured meats, shelf-stable cooked meats, snails, froglegs). Each section discusses the effects of processing on the microorganisms, the specific spoilage and pathogenic microorganisms of concern, and factors to be considered for process control are listed.

The structure of chapters on foods of plant origin is similar, there next to the raw material, the frozen, dried, canned, fermented and acidified products, sprouts and other speciality foods are separately dealt with. Emphasis has been given to foods in international trade, too.

References (in alphabetical order) at the end of each chapter are further source of information (the majority of the cited papers were published after 1980, the latest in 1996).

The book is a logical development of previous ICMSF publications, and is of the usual high standard in content and printing.

This volume of the series of Microorganisms in Foods is intended and highly recommended for those interested primarily in applied aspects of food microbiology such as food processors, food microbiologists, food technologists, veterinarians, public health workers and regulatory officials. It cannot be missing from your bookshelf.

J. BECZNER

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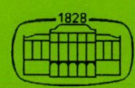

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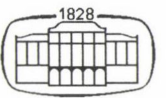

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Acta Alimentaria, Vol. 27 (4), pp. 307–318 (1998)

EFFECT OF WOOD TREATMENT ON CHEMICAL AND SENSORY QUALITY OF PEACH WINE DURING AGEING

V. K. JOSHI and P. K. SHAH

Department of Postharvest Technology, Dr YS Parmar University of Horticulture and Forestry, Nauni-Solan (H. P.) 173230. India

(Received: 10 July 1997; revision received: 27 May 1998, accepted: 3 June 1998)

Chemical composition and sensory quality of peach wines of eight cultivars aged with three different wood chips viz., *Quercus*, *Bombax* and *Albizia* species were determined and compared with that aged without wood chips. Significant changes in biochemical characteristics of the wines traceable to the treatment of wines with wood chips were observed. Wines aged with *Quercus* wood had higher total phenols, aldehyde and more total esters contents while the wines with *Albizia* had higher alcohols content. Wood treated wines showed slight increment in volatile and titratable acidity over the control. Sensory evaluation indicated that wines aged with wood chips were rated better than control and that treated with *Quercus* was the best wine. The wines from Flavorcrest, Redhaven, Sunhaven and Richahaven behaved better than others for higher tannin extraction, formation of more esters and less changes in higher alcohol and aldehyde contents during maturation of one year. However, changes were more pronounced during the first six months of maturation than in the later period. The wine of all the cultivars were acceptable, though that of Richahaven, Sunhaven, J. H. Hale, Flavorcrest, and Kateroo were rated better than others.

Keywords: peach wine, ageing, wood chips

There are numerous reports on the use of *Quercus* barrels in aging of table wine (AIKEN & NOBLE, 1985; SINGLETON, 1974; QUINN & SINGLETON, 1985) but only scanty information on the use of wood chips in wines is available (WILKER & GALLANDER, 1988; JOSHI et al., 1994). The extraction of wood components and increased but limited oxygen exposure are desirable factors during wine storage in wooden barrels. However, glass and stainless steel containers have many advantages such as lower term cost, greater protection against oxygen over exposure and smaller evaporation losses over wooden barrels. The addition of wood chips to the wines for producing barrel-aged characteristics is one of the reasons while using glass containers in maturation of wines (SINGLETON, 1974).

Astringency and bitterness are good characteristics of wine which increase with storage in *Quercus* wood treated wines due to hydrolysis of catechin from *Quercus*

wood chips (FISCHER & NOBLE, 1994). WILKER and GALLANDER (1988) studied the effect of *Quercus* wood chips in wine and found higher level of gallic acid during storage. New aromatic aldehydes and total phenol extracted from wood chips during maturation of wines made a wine with desirable flavour and better aroma (AMERINE et al., 1980; VIGUERA et al., 1994). Treatment of wines from apricot, plum and mixed fruits with wood chips have been reported (JOSHI et al., 1994). However, there is no information on the treatment of peach wine with wood chips. Effect of treating the peach wine with wood chips of *Bombax*, *Quercus* and *Albizia* was studied and the results are reported here.

1. Materials and methods

1.1. Peach varieties

The eight different varieties of peach viz. Richahaven, Sunhaven, Redhaven, Flavorcrest, Stark Early Giant, J. H. Hale, Kateroo and July Elberta used in this experiment were obtained from the orchard of Dr. YS Parmar University of Horticulture & Forestry, (Nauni) Solan, H. P.

1.2. Yeast culture

The culture of *Saccharomyces cerevisiae* var. *ellipsoideus* strain UCD 595 used in the study was obtained from the department of Enology and Viticulture, California, Davis.

1.3. Wood chips

Wood chips of different trees viz., *Quercus* sp. (W_1), *Bombax* sp. (W_2) and *Albizia* sp. (W_3) were obtained from the local forest. They were cut into sticks of 3.5 cm long and 0.2 mm thickness. Before adding to the wines, the chips were oven dried followed by slight roasting. A set without wood chips was used as a control (W_0).

1.4. Preparation of wine

For the preparation of must, fruits of different cultivars were washed and cooked separately in a pressure cooker till pressure was developed. The cooked material was passed through a tomato pulper to make the pulp. The pulps were diluted in 1:1 ratio, raising the initial TSS to 24 °B with sugar syrup (70 °B), adding pectinol and diammonium hydrogen phosphate (DAHP) at the rate of 0.5 and 0.1 per cent, respectively. To each must, SO_2 in the form of potassium metabisulphite was added at the rate of 100 ppm. Musts were kept over night, before inoculation with active yeast

culture. The fermentations were carried out in 10 litre glass bottles fitted with air locks, which were initiated by addition of active yeast culture of *Saccharomyces cerevisiae* var. *ellipsoideus* at the rate of 5 per cent at a temperature of 22±1 °C. After completion of fermentation siphoning/racking and filtration were carried out. Initially after every 15 days and thereafter, one month, racking/siphoning was done. The wines were further clarified prior to and after maturation with wood chips. The prepared peach wine was filled in clean beer bottles, adding 100 ppm potassium metabisulphite and were sealed with wooden corks.

1.5. Treatment with wood chips

The wines of each cultivar were matured with three different types of wood chips as detailed earlier, along with control for a year (JOSHI et al., 1994). The wood chips were roasted slightly and 3 g per beer size bottle of chips were added. The quality of wood chips was kept at the same level to see the effect of wood chips on the physico-chemical and sensory qualities of wine. This was a screening type of experiment.

1.6. Analyses

Analysis of different wines was carried out before start of maturation process (initial values) and after an interval of 0 (M_0), 3 (M_1), 6 (M_2) and 12 (M_3) months, while the sensory evaluation were performed only after 6 and 12 months intervals. All analyses were performed in triplicates.

Titratable acidity (% malic acid) and the volatile acidity (g/100 ml acetic acid) of wines were measured by standard methods (AMERINE et al., 1980). The total phenols (tannin contents) in wines were analysed by Folin-Ciocalteu procedure given by SINGLETON and ROSSI (1965), while total esters were estimated by the method of LIBERATY (1961). Ethanol content in the wines was estimated by colorimeteric method (CAPUTY et al., 1968). Aldehyde and higher alcohols contents were determined in wines as per the method of AMERINE and OUGH (1979) and GUYMON and NAKAGIRI (1952), respectively. Different wines were analysed for sugars (total and reducing) as per the method described by RANGANNA (1986). The sensory analysis of different wines was conducted by a panel of 10 judges. Each sample was evaluated for different quality attributes such as colour and appearance, astringency, aroma, bouquet, sweetness etc. as per the proforma of AMERINE and co-workers (1980). Sum of scores for all the attributes was made for a specific treatment out of a total score of 20.

1.7. Statistical analysis

The data of quantitative estimation and sensory analysis were analysed by completely randomized factorial design (CRD) and randomized block design (RBD),

respectively (O'MAHONY, 1986). The data of chemical analysis of the entire experiment were analysed taking into account 3 factors i.e. cultivar, wood chips or intervals (maturation periods). To see the effect of different wood chips or the maturation periods, the data of wines with or without different wood chips obtained for each cultivar at different intervals were averaged. Similarly, the means for maturation periods are irrespective of wood chips. The data of sensory evaluation were analysed only for two periods (6 and 12 months). The results are presented, either as the effect of different wood chips or intervals, on the behaviour of wines of different cultivars.

2. Results and discussion

2.1. Effect of wood chips addition

The results revealed that total phenols, total esters and aldehyde content of wines of different cultivars differed significantly (Table 1). The maturation of wines with wood chips increased the total phenolics over the control. The amount of phenols however, was found higher than that reported for wood treated plum and apricot wines (JOSHI et al., 1994) which might be the contribution of the peach fruits. Further, higher quantity of phenols in the wood treated wines than in the control ones indicates the extraction of phenolic compounds (flavonoides and non-flavonoides) from wood during maturation (QUINN & SINGLETON, 1985; WILKER & GALLANDER, 1988; VENKATARAMU et al., 1982; DEVES, 1994). All the wood treated wines recorded higher total esters than the untreated wines. Oxidation of ethanol into acetic acid and their interaction and the consequent increase in the ester content of wine during maturation might have contributed to the increase (CASTINO et al., 1993). Total esters impart fruity aroma to the wines during maturation in *Quercus* wood barrels (AMERINE et al., 1980; QUINN & SINGLETON, 1985; GZOCHUKWU et al., 1994). However, the increase in ester content is proportional to their initial values in the respective cultivars.

The amount of aldehyde in wines treated with different wood chips was significantly different (Table 1). It ranged between 45–69 mg l^{-1}. Low aldehyde content of the wines is desirable, though the range of aldehyde obtained in this study does not seem to have sensory significance as observed earlier (AMERINE et al., 1980). Similar increase in aldehyde content during maturation of grape wine with wood chips has been reported (WILDENRADT & SINGLETON, 1974). The aromatic aldehydes are known to impart the flavour and colour to the wines, and the increase is attributed to their extraction from wood chips (VIGUERA et al., 1994).

Table 1

Effect of addition of different wood chips on total phenols, total esters, aldehyde and higher alcohols of peach wine of different cultivars

Cultivar	Total phenols (mg l^{-1})				Total esters (mg l^{-1})				Aldehyde (mg l^{-1})				Higher alcohol (mg l^{-1})			
	W_0	W_1	W_2	W_3	W_0	W_1	W_2	W_3	W_0	W_1	W_2	W_3	W_0	W_1	W_2	W_3
Richahaven	253.8	264.6	256.0	251.3	115.1	122.2	121.7	120.6	53.26	52.78	51.54	49.11	125.4	123.1	129.9	127.5
J. H. Hale	270.5	279.4	270.2	271.0	109.3	118.1	117.0	114.9	48.81	47.96	51.43	51.41	148.6	147.2	149.0	140.8
Redhaven	315.9	320.4	315.9	313.5	115.2	122.1	119.5	119.3	55.10	56.56	48.91	46.99	144.6	139.1	150.8	164.1
Flavorcrest	317.8	337.5	322.0	321.2	114.9	120.8	119.2	118.2	60.17	56.70	58.87	51.41	126.9	134.3	133.3	129.0
Sunhaven	290.5	307.0	295.0	291.2	120.3	127.3	124.2	125.1	56.91	60.81	55.38	57.91	138.1	130.1	142.5	156.8
Stark Early Giant	223.2	241.2	227.2	226.2	116.7	121.9	157.4	122.1	68.83	66.80	68.58	68.40	154.6	158.2	161.1	167.4
Kateroo	288.2	309.3	298.9	293.4	109.0	114.3	116.6	113.8	54.04	53.66	50.30	50.56	159.8	197.9	185.6	185.0
July Elberta	214.4	230.6	221.0	218.8	108.0	113.3	111.7	111.8	73.31	73.54	74.31	75.64	215.6	250.8	245.7	256.5
$C.D._{(P \geq 0.05)}$ Cultivar =	1.1				8.2				0.92				0.5			
Wood =	0.7				5.8				0.65				0.4			

W_0 = Control (No chips), W_1 = *Quercus* chips, W_2 = *Albizia* chips, W_3 = *Bombax* chips.
The means are irrespective of maturation periods

The addition of wood chips significantly enhanced the amount of higher alcohols in the wine (Table 1). However, different peach cultivars maintained their initial trend even after maturation with wood chips. The amount of higher alcohols in the table wines (grapes) varies between 140 to 420 mg l^{-1} and quantities higher than this are considered to be detrimental to the quality of wine (AMERINE et al., 1980; BALDWIN, 1993). The quantities of higher alcohols obtained in our study were within the range. Changes in concentration of higher alcohol during maturation were observed earlier (LITCHEV, 1989).

Though statistically significant, the wood treated wines showed only a slight increase in the acidity over the control (Table 2) attributable to the release of fixed acid from the *Quercus* wood as observed for grape wine (WILKER & GALLANDER, 1988). The results are in confirmation with the earlier work (JOSHI et al., 1994). Furthermore, the marginal increase in acidity of the wood treated wines is not expected to upset the acid/sugar balance and consequently, the acceptability of the treated wines.

In general, the wood treated wines had significantly higher volatile acidity than the control but the values were within the prescribed limit (0.040%) (Table 2). The *Quercus* wood treated wines differed significantly from those treated with wood of *Albizia* and *Bombax*. These results are in further confirmation with those reported earlier for grape, plum and apricot wine (WILKER & GALLANDER, 1988; JOSHI et al., 1994).

Wines of different cultivars and treated with wood chips were significantly different for total and reducing sugar contents (Table 2). The increase in reducing and total sugars in wood treated wines could be due to breakdown and/or hydrolysis of wood components like hemicellulose, lignin, cellulose etc. due to long contact period of maturation (REAZIN, 1981; QUINN & SINGLETON, 1985; WILKER & GALLANDER, 1988).

2.2. Changes in contents of chemical constituents during maturation

Total esters of different wines increased significantly differently during maturation but the increase during the first 6 months was more pronounced than in the later period (Table 3). Increase in total esters during maturation is attributed to the phenomenon of ageing (AMERINE et al., 1980). The same trend was observed for volatile acidity during the first 6 months (Table 4) which might be due to the extraction of volatile acids from wood (WILKER & GALLANDER, 1988). After a year of maturation, the volatile acidity varied between 0.027 to 0.040 per cent (still within the range), indicating the soundness of wine during maturation (AMERINE et al., 1980). Titratable acidity of wines also increased significantly during maturation (Table 4), probably due to extraction of fixed acid from the wood chips (WILKER & GALLANDER, 1988) and/or evaporation of water from wine, thus concentrating the acid content of wine (AMERINE et al., 1980).

Table 2

Effect of addition of different wood chips on content of volatile and titratable acidity, total and reducing sugars of peach wine of different cultivars

Cultivar	Volatile acidity (% a.a.)				Titratable acidity (% M.A.)				Total sugar (%)				Reducing sugar (mg l^{-1})			
	W_0	W_1	W_2	W_3	W_0	W_1	W_2	W_3	W_0	W_1	W_2	W_3	W_0	W_1	W_2	W_3
Richahaven	0.024	0.026	0.026	0.025	0.802	0.841	0.837	0.836	1.26	1.26	1.20	1.29	372.3	371.3	375.6	379.4
	(0.724)	(0.725)	(0.725)	(0.725)	(1.142)	(1.158)	(1.157)	(1.156)	(1.12)	(1.12)	(1.09)	(1.13)				
J. H. Hale	0.029	0.032	0.031	0.030	0.782	0.813	0.817	0.820	1.17	1.14	1.17	1.20	354.8	351.8	354.4	364.3
	(0.727)	(0.729)	(0.729)	(0.728)	1.132)	1.146)	1.148)	1.148)	(1.08)	(1.07)	(1.08)	(1.09)				
Redhaven	0.024	0.029	0.029	0.028	0.748	0.782	0.782	0.801	1.09	1.10	1.09	1.12	336.7	336.9	338.1	346.3
	(0.724)	(0.727)	(0.727)	(0.727)	(1.117)	(1.132)	(1.132)	(1.140)	(1.04)	(1.05)	(1.04)	(1.06)				
Flavorcrest	0.032	0.038	0.034	0.036	0.842	0.889	0.885	0.889	1.14	1.15	1.14	1.18	328.3	334.4	331.6	338.2
	(0.729)	(0.733)	(0.730)	(0.732)	(1.159)	(1.179)	(1.177)	(1.177)	(1.06)	(1.07)	(1.06)	(1.09)				
Sunhaven	0.033	0.037	0.037	0.036	0.668	0.717	0.721	0.728	1.18	1.19	1.18	1.22	332.3	329.3	316.1	341.2
	(0.730)	(0.733)	(0.733)	(0.732)	(1.081)	(1.103)	(1.105)	(1.108)	(1.08)	(1.09)	(1.08)	(1.10)				
Stark Early	0.021	0.025	0.023	0.025	0.714	0.757	0.773	0.781	1.17	1.22	1.24	1.27	344.1	344.8	342.6	357.3
Giant	(0.722)	(0.725)	(0.723)	(0.724)	(1.102)	(1.121)	(1.123)	(1.131)	(1.08)	(1.10)	(1.11)	(1.12)				
Kateroo	0.028	0.033	0.033	0.033	0.651	0.681	0.676	0.690	1.15	1.22	1.40	1.20	368.5	370.2	367.1	379.1
	(0.726)	(0.736)	(0.730)	(0.730)	(1.073)	(1.087)	(1.085)	(1.091)	(1.07)	(1.06)	(1.06)	(1.09)				
July Elberta	0.038	0.039	0.039	0.037	0.620	0.668	0.670	0.668	1.21	1.22	1.22	1.26	380.4	381.2	385.5	389.5
	(0.733)	(0.734)	(0.734)	(0.733)	(1.058)	(1.081)	(1.081)	(1.081)	(1.10)	(1.10)	(1.10)	(1.12)				
C.D. $_{(P \geq 0.05)}$ Cultivar =	0.004				0.001				0.005				0.33			
Wood =	0.0003				0.001				0.003				0.24			

W_0 = Control, W_1 = *Quercus* chips, W_2 = *Albizia* chips, W_3 = *Bombax* chips, M. A. = malic acid, a.a. = acetic acid
Values in parentheses are the transformed values

Table 3

Changes in total phenols, total esters, aldehyde and high alcohol of peach wine during maturation

Cultivar	Total phenols (mg l^{-1})				Total esters (mg l^{-1})				Higher alcohols (mg l^{-1})				Aldehyde (mg l^{-1})			
	M_0	M_1	M_2	M_3	M_0	M_1	M_2	M_3	M_0	M_1	M_2	M_3	M_0	M_1	M_2	M_3
Richahaven	241.8	244.2	256.0	269.1	98.23	100.0	125.6	134.1	113.6	133.2	124.4	121.8	48.23	47.69	51.89	55.43
J. H. Hale	259.0	259.0	273.3	286.1	90.27	92.2	120.1	132.1	126.9	137.6	155.3	146.2	43.30	42.33	50.32	57.02
Redhaven	301.7	302.7	319.2	333.4	98.40	99.9	121.8	135.3	121.4	157.4	140.1	143.4	45.53	45.62	52.75	57.30
Flavorcrest	306.1	306.6	324.9	342.4	94.30	95.6	123.6	135.6	154.4	127.9	138.4	126.3	49.32	48.44	57.82	64.09
Sunhaven	278.6	278.6	299.9	309.9	101.50	100.9	131.8	140.0	131.6	145.3	146.1	141.0	53.97	51.51	56.94	64.83
Stark Early Giant	206.0	209.0	230.6	247.9	97.80	98.6	130.5	139.4	144.2	164.5	161.8	154.6	65.73	64.77	67.80	71.88
Kateroo	230.4	275.8	297.4	319.1	97.37	93.2	116.2	130.3	154.3	199.1	186.1	176.1	56.83	50.47	50.78	55.16
July Elberta	203.5	203.0	223.5	237.1	92.50	96.3	112.9	124.3	215.6	233.1	252.2	233.6	63.10	72.82	74.51	75.27
C.D. $_{(P \geq 0.05)}$ Cultivar =	1.1				9.2				0.5				0.092			
Wood =	0.6				5.0				0.3				0.056			

M_0 = Without maturation, M_1 = 3 months, M_2 = 6 months, M_3 = 12 months

Table 4

Changes in titratable and volatile acidity, total and reducing sugar of peach wines during maturation

Cultivar	Titratable acidity (% M.A.)				Volatile acidity (% a.a.)				Total sugar (%)				Reducing sugar (mg l^{-1})			
	M_0	M_1	M_2	M_3	M_0	M_1	M_2	M_3	M_0	M_1	M_2	M_3	M_0	M_1	M_2	M_3
Richahaven	0.66	0.820	0.804	0.863	0.023	0.022	0.026	0.027	1.33	1.19	1.25	1.31	344	358.6	377.5	387.7
	(0.81)	(1.149)	(1.142)	(1.168)	(0.723)	(0.723)	(0.725)	(0.726)	(1.15)	(1.09)	(1.12)	(1.15)				
J. H. Hale	0.71	0.720	0.810	0.894	0.029	0.025	0.033	0.032	1.24	1.13	1.16	1.22	321	341.3	360.4	367.0
	(0.84)	(1.105)	(1.45)	(1.81)	(0.727)	(0.725)	(0.730)	(0.72)	(1.12)	(1.06)	(1.08)	(1.10)				
Redhaven	0.72	0.701	0.784	0.850	0.023	0.022	0.031	0.030	1.26	1.04	1.10	1.16	369	325.6	339.5	353.4
	(0.85)	(1.096)	(1.133)	(1.162)	(0.723)	(0.722)	(0.728)	(0.72)	(1.12)	(1.02)	(1.05)	(1.08)				
Flavorcrest	0.80	0.839	0.890	0.896	0.029	0.029	0.039	0.037	1.19	1.08	1.16	1.23	295	321.5	337.6	340.1
	(0.89)	(1.147)	(1.179)	(1.182)	(0.727)	(0.727)	(0.734)	(0.73)	(1.09)	(1.04)	(1.08)	(1.10)				
Sunhaven	0.63	0.665	0.706	0.755	0.022	0.031	0.039	0.038	1.22	1.02	1.23	1.34	318	268.7	339.5	343.5
	(0.79)	(1.080)	(1.098)	(1.120)	(0.725)	(0.728)	(0.734)	(0.73)	(1.05)	(1.01)	(1.10)	(1.16)				
Stark Early Giant	0.74	0.670	0.735	0.856	0.026	0.021	0.025	0.025	1.32	1.03	1.19	1.47	363	322.5	348.8	360.3
	(0.86)	(1.082)	(1.111)	(1.164)	(0.725)	(0.721)	(0.725)	(0.72)	(1.15)	(1.01)	(1.09)	(1.21)				
Kateroo	0.61	0.623	0.682	0.718	0.029	0.028	0.034	0.033	1.22	1.25	1.15	1.28	333	354.5	376.1	384.5
	(0.73)	(1.060)	(1.087)	(1.104)	(0.727)	(0.726)	(0.731)	(0.73)	(1.10)	(1.06)	(1.07)	(1.10)				
July Elberta	0.70	0.598	0.669	0.704	0.020	0.034	0.040	0.040	1.36	1.15	1.23	1.30	320	373.1	390.1	389.2
	(0.82)	(1.048)	(1.081)	(1.097)	(0.721)	(0.731)	(0.735)	(0.73)	(1.17)	(1.07)	(1.11)	(1.14)				
C.D. $_{(P\geq 0.05)}$ Cultivar =	0.0013				0.004				0.005				0.33			
Wood =	0.0010				0.003				0.003				0.21			

M_0 = 0 month, M_1 = 3 months, M_2 = 6 months, M_3 = 12 months, M. A. = malic acid, a.a. = acetic acid

During maturation, both total and reducing sugars in different wines increased significantly (Table 4). The increasing trend of reducing sugars is apparently the result of hydrolysis of total sugar during maturation (AMERINE et al., 1980). During ageing, significant changes in the higher alcohols content of wines of various cultivars took place. During first half of maturation, the higher alcohols content of all the wines were significantly enhanced followed by a decrease (Table 3).

The aldehyde and total phenols significantly increased during maturation and it varied between 55 to 75 mg l^{-1} (Table 3). During ageing, due to oxidation of ethanol, the amount of aldehyde in wines is generally increased (AMERINE et al., 1980) but comparatively low amount of aldehyde in all the wines shows their microbiological stability in maturation. During ageing with wood chips development of flavour of aged wines reportedly occurs due to the increase in aromatic aldehyde content (AMERINE et al., 1980). Increase in total phenols in wines treated with *Quercus* wood during maturation is established earlier, which gave astringency and bitter taste to wine mainly due to gallic acid (SINGLETON et al., 1971; QUINN & SINGLETON, 1985; FISCHER & NOBLE, 1994; JOSHI et al., 1994).

Based on the results discussed so far, it is clear that during maturation, the wines from Redhaven, Sunhaven, Flavorcrest and Richahaven were better than others due to higher increase in total phenols and ester contents and comparatively less changes in volatile acidity, titratable acidity, higher alcohols and aldehyde contents.

2.3. Sensory evaluation

Wines from all the cultivars treated with different wood chips along with control and their interactions with maturation period differed significantly (Table 5). The treatment of all the wines with *Quercus* wood chips has shown clear improvement in sensory quality over the control wine. *Quercus* treated wines from all the cultivars were found to be the best (Table 5). However, the wines from Richahaven and J. H. Hale cv. treated with *Quercus* wood chips were superior to the wines from all other cvs. Better quality of *Quercus* wood chips treated wine could be correlated with the process of extraction of phenolic substances which are known to improve the astringency and colour quality of wines (SNELTEN & SCHAAFSMA, 1992). During maturation, the wine of Redhaven, Sunhaven, Flavorcrest and Richahaven became more pleasant with respect to desirable astringency and improved in fruity flavour, probably due to more tannins and esters content than others. The total score of the wines after maturation with wood chips indicate that all the eight cultivars gave wine of commercial acceptability. However, Richahaven, Sunhaven, J. H. Hale, Redhaven Flavorcrest and Kateroo were rated superior to others in overall sensory quality impression.

Table 5

Sensory evaluation of peach wine of different cultivars treated with different wood chips

Cultivar	Overall score (6 months)				Overall score (12 months)			
	W_0	W_1	W_2	W_3	W_0	W_1	W_2	W_3
Richahaven	13.42	14.54	12.82	13.50	14.58	16.61	14.73	14.90
J. H. Hale	12.94	13.66	12.10	12.66	13.92	16.00	14.14	15.08
Redhaven	13.46	14.82	13.44	13.20	14.47	15.57	14.13	14.44
Flavorcrest	12.60	14.46	11.76	13.50	13.57	15.53	14.28	13.96
Sunhaven	12.92	14.86	13.48	13.24	14.07	16.09	14.76	15.32
Stark early Giant	11.96	13.60	11.70	12.34	12.92	14.56	13.27	13.57
Kateroo	12.48	14.66	13.32	14.00	13.22	15.22	13.49	14.26
July Elberta	12.86	14.26	11.80	12.56	12.64	14.00	11.89	12.93
$C.D._{(P \geq 0.05)}$ Cultivar =		1.61				1.29		
Wood =		1.61				1.29		

W_0 = Control (no wood addition), W_1 = *Quercus* chips, W_2 = *Albizia* chips, W_3 = *Bombax* chips

3. Conclusions

– The *Quercus* wood treated peach wine had more desirable physico-chemical characteristics than those treated with wood chips from *Albizia* and *Bombax*.

– The changes in various physico-chemical characteristics were broadly similar to those occurring in grape wines.

– *Quercus* wood chips gave wine of superior sensory quality than *Albizia* and *Bombax*.

– The peach wine continuously improved during one year of maturation, though changes were more pronounced during the first six months than the later period.

The results suggest that use of oak wood chips for maturing peach wine would be advantageous like the grape, plum or apricot wine. However, to have more pronounced desirable changes, the quantities of chips could be optimized further.

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INFLUENCE OF SOME PROCESS CONDITIONS ON THE REHYDRATION OF APPLE CUBES

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Dried fruit and vegetables are widely used as ingredients in many food formulations such as pastries, confectionery products, ice-creams, frozen desserts and yoghurts. The different water content of the dried fruit in respect to that of other ingredients may enhance the mass transfer (water or solutes) with problems of phase separation or loss of textural properties. Nevertheless it is not easy to obtain, only by a drying treatment, fruit pieces with "adjusted" a_w values; hence, it could be profitable to have a standard dried fruit rehydrated in water or sugar solutions under appropriate conditions in order to obtain an optimum a_w value.

In this work, different rehydrating trials have been carried out on air dried apple cubes by varying the rehydration medium concentration (from 0 to 30 °Bx), temperature (from 20 to 80 °C) and agitation rate (from 0 to 250 r.p.m.). During rehydration, water content, water uptake and solid loss or gain of samples were measured.

Results have shown that the rehydration time was the main important parameter for the water uptake and for the solid loss, while the medium concentration influenced only the solid gain of apple cubes.

Keywords: rehydration, reconstitution, apple cubes, sugar solutions

Dried cubed, sliced or powdered fruits and vegetables are widely used as ingredients in the bakery, dairy and ice cream industries; the compatibility of a generic ingredient with other components of a formulated food basically depends on the equilibrium between the respective water activities (MASTROCOLA et al., 1995).

The most important quality of dried products is their rehydration capacity when put in contact with some rehydration media such as water, water-alcohol solutions, fruit juices, milk, or sugar syrups. The reconstitution capacity of dried fruit and vegetables can be evaluated as rapid and complete rehydration, in terms of high quantity water uptake and/or low total solids loss; other important parameters considered during the rehydration process are the skin tenderness, the texture firmness, the size and shape

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similar to the fresh state (MALTINI et al., 1993). The method of rehydration, besides the drying technique, undoubtedly influences the amount of water absorbed by dried foods; the rate of rehydration of dried celery increased with increasing the rate of agitation and temperature (NEUBERT et al., 1968), even if at high temperatures cooking phenomena occurred. Other authors (ZRNIC & SAVIC, 1975) showed that during rehydration of dried prunes, the water uptake decreased with the increase of ethanol concentration in water. NEUMANN (1972) considered the effects of predrying treatments on the reconstitution of sliced celery, while CURRY and co-workers (1976) investigated the effect of sodium chloride concentration of the rehydration kinetics of freeze-dried carrots.

In this paper, the results of a research on the influence of different rehydration conditions on the reconstitution kinetics of dried apple cubes are reported. On this product, several rehydrations were carried out by varying (i) the rehydration medium concentration (from 0 to 30 °Bx), (ii) temperature (from 20 to 80 °C) and (iii) agitation rate (from 0 to 250 r.p.m.). During the rehydration process of the dried apple cubes, water content, water uptake and solid loss or gain were measured.

1. Materials and methods

Apples (*Malus domestica*, cv. Golden Delicious) were hand peeled and cut into cubes of 1×1×1 cm. Prior to drying, the fresh apple cubes were treated with 0.5% $Na_2S_2O_5$ solution for 3 min at 20 °C in order to avoid enzymatic browning.

The sulfited apple cubes were air dried in a laboratory pilot plant at 100 °C for 20 min then 80 °C until 4% of water content on dry basis was reached. The drying equipment was a suction air-flow drier (Sandvik Process Systems, Milan, Italy), as described in previous papers (BARBANTI et al., 1994, 1995, 1996). The product load on the tray was of 4 kg m^{-2}. The starting air conditions were: absolute humidity: 0.012 kg H_2O/kg dry air at 20 °C; velocity: 1.5 m sec^{-1}.

The rehydration of dried apple cubes was performed with a fixed liquid/solid ratio of 20:1 in 500 ml glass beaker and the product was submerged by a plastic screen; sugar solutions were obtained by dissolving in water sucrose for analysis (Carlo Erba Reagents, Milan, Italy) at 12, 15 and 30% w/w concentrations (=°Bx); different temperatures (20, 40, 60 and 80 °C) and agitation rates (0, 60, 100 and 250 r.p.m.) were obtained with a thermostated stirrer.

At fixed time intervals, the rehydrated product was withdrawn from the beaker, drained for 3 min on a plastic tray and weighed.

1.1. Analitical determinations

On each sample of rehydrated apple cubes the water content (w.c.%) was measured after drying in vacuum oven at 75 °C for 12 h; (A.O.A.C., 1995). The solution concentrations were controlled by a laboratory refractometer Unirefrax (Bertuzzi, Milan, Italy) under diffused daylight.

The weight increment on 100 g of product (X) due to rehydration was calculated as: X = (rehydrated weight × 100)/initial weight; the amount of water uptake Wu, (g H_2O/100 g) was obtained as: Wu=X×w.c.%; the amount of total solids (Ts, g/100 g) was calculated as: Ts=X×(100–w.c.%).

In this way, the mass balance as water absorption, solid gain (s.g.) or solid loss (s.l.) was evaluated (MASTROCOLA et al., 1988; 1989).

In order to evaluate differences among replicates, a rehydration trial (water, 20 °C, 100 r.p.m.), was repeated three times. A low coefficient of variation was found (lower than 5% in all trials) and then it was decided to run single trials.

1.2. Data analysis

The effects of rehydration parameters such as time, concentration, temperature and agitation rate have been analysed by least square fit of response surfaces (Response Surface Methodology, RSM), according to VUATAZ (1981) and GACULA and SINGH (1984). The quadratic polynomial model used had the general formula:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{12} A \times B,$$

where Y = estimated value; β_0 = the intercept; β_1 = linear effect of the first independent variable (time); β_2 = linear effect of the second independent variable (temperature, concentration, agitation rate); β_{11} = the quadratic effect of the first independent variable (time); β_{22} = the quadratic effect of the second independent variable (temperature, concentration, agitation rate); β_{12} = the interaction effect of the first and the second independent variable (time-temperature, time-concentration, time-agitation rate); β_{ij} = parameters to be estimated.

2. Results

During the drying process, an isotropic volume reduction (shrinkage) of apple cubes of about 66% occurred; during the first period of rehydration (about 2–3 min) an almost complete volume recovery of apple cubes was measured (about 90% of the fresh).

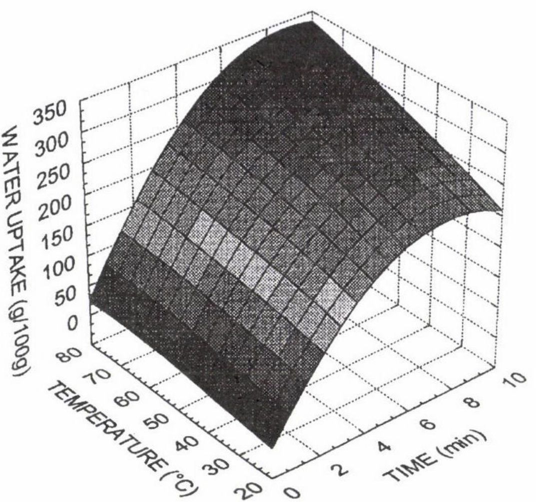

Fig. 1. Response surface of apple water uptake (g/100 g) as a function of the rehydration time and temperature. Rehydrating medium: water; agitation speed: 100 r.p.m.

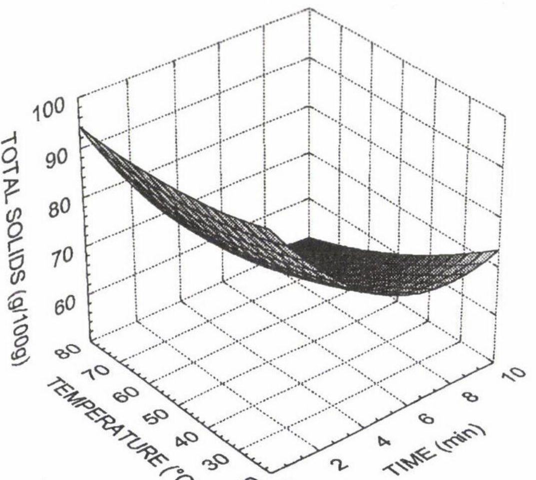

Fig. 2. Response surface of apple total solids (g/100 g) as a function of the rehydration time and temperature. Rehydrating medium: water; agitation speed : 100 r.p.m.

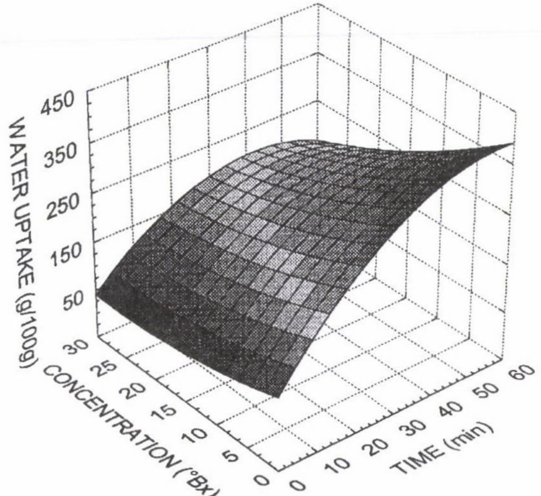

Fig. 3. Response surface of apple water uptake (g/100 g) as a function of the rehydration time and medium concentration. Medium temperature: 20 °C; agitation speed: 100 r.p.m.

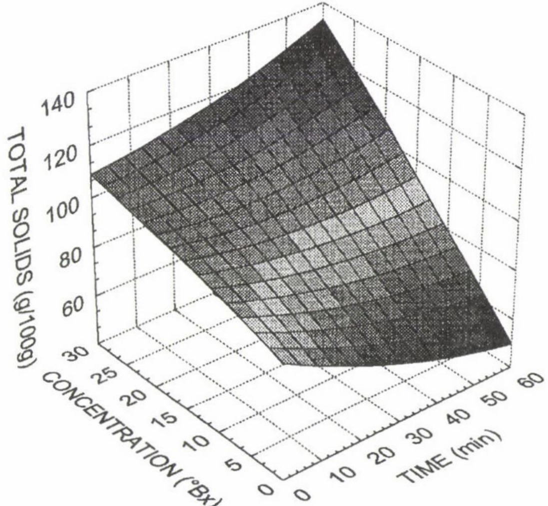

Fig. 4. Response surface of apple total solids (g/100 g) as a function of the rehydration time and medium concentration. Medium temperature 20 °C; agitation speed: 100 r.p.m.

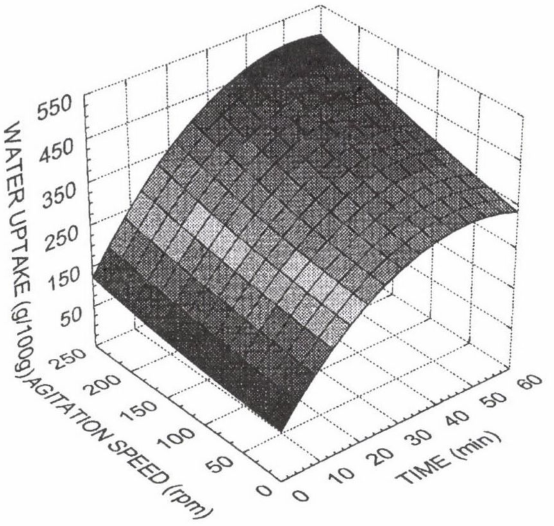

Fig. 5. Response surface of apple water uptake (g/100 g) as a function of the rehydration time and agitation speed. Rehydrating medium: water; temperature: 20 °C

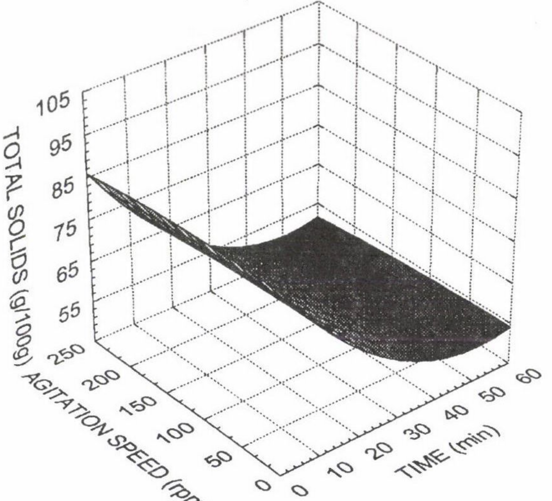

Fig. 6. Response surface of apple total solids (g/100 g) as a function of the rehydration time and agitation speed. Rehydrating medium: water; temperature: 20 °C

The rehydration kinetics of dried apple cubes from 0 min to the end of each experiment have been represented as response surfaces; in each graph, the rehydration time is assumed as the first independent variable, while temperature, concentration and agitation speed are sequentially considered as the second independent variable.

The water uptake and the solid loss or gain are dependent variables (z axis).

In Fig. 1 the response surface of water uptake as a function of time and temperature at 100 r.p.m. of agitation speed is reported. The increase of water uptake is particularly evident as a function of the rehydration time rather than of the temperature: during the first 4–6 min the maximum quantity of water uptake inside the product is reached and then this mass transfer tends to reach equilibrium. On the other hand, the rehydration temperature did not influence the water uptake; moreover, some problems arose with high temperatures (T>50 °C); under these conditions, the rehydration time was limited (max. about 8–10 min) in order to avoid cooking phenomena, breaking of cell walls and high solid loss.

For the same set of variables, the response surface of the apples total solids is shown in Fig. 2. Also in this case, the influence on the solids loss was mainly due to the rehydration time rather than to the temperature.

In Figs 3 and 4 are represented respectively, the response surfaces of the changes of water uptake and total solids as a function of the rehydration time and the medium concentration at 100 r.p.m. of agitation rate.

Time significantly influenced the water uptake (Fig. 3); the total solids have been statistically affected both by the medium concentration and by the rehydration time; in particular, the solid balance is positive (solids gain) for rehydration medium concentration over 10–12 °Bx; conversely, for lower medium Brix values, the apple cubes lost almost the 40% of their original solids content.

The water uptake (Fig. 5) and the total solids changes (Fig. 6) are independent of the agitation speed; moreover, the total solids balance is always negative (solids loss), also in the case of no agitation.

The statistical model fitting the results of the response surfaces (Figs from 1 to 6) have been summarized in Table 1. For each variable, the respective t value and its significance level has been reported. Some values have not been reported when a α was >0.5.

Table 1

Summary of the statistical model fitting, results of the response surfaces

(Figs from 1 to 6). α = significance level; --- = α>0.5, not significant.

Figure No.	Variable	t value	prob α
1 (water uptake)	time	6.961	<0.001
	temperature	0.683	<0.5
	time^2	6.188	<0.001
	temperature^2	0.497	–
	time × temperature	1.934	<0.1
2 (total solids)	time	6.319	<0.001
	temperature	1.406	>0.1
	time^2	5.628	<0.001
	temperature^2	1.174	>0.2
	time × temperature	2.997	>0.005
3 (water uptake)	time	6.312	<0.001
	concentration	0.481	–
	time^2	3.687	<0.005
	concentration^2	0.156	–
	time × concentration	2.669	<0.05
4 (total solids)	time	4.071	<0.001
	concentration	2.734	<0.05
	time^2	1.384	<0.2
	concentration^2	0.892	>0.2
	time × concentration	7.047	<0.001
5 (water uptake)	time	6.855	<0.001
	agitation speed	0.136	–
	time^2	4.826	<0.001
	agitation speed^2	0.373	–
	time × agitation speed	1.186	>0.2
6 (total solids)	time	7.306	<0.001
	agitation speed	0.02	–
	time^2	4.663	<0.001
	agitation speed^2	0.279	–
	time × agitation speed	0.066	–

3. Conclusions

From the results obtained in these experiments, brief but interesting conclusions are outlined.

Under our experimental conditions, it has been verified that the water uptake and the apple solid loss are mainly influenced by the rehydration time and the solid loss by the medium concentration only.

The effects of agitation speed and temperature on water uptake and solid loss were not statistically significant, even if some little variations of water uptake were measured.

However, it should be possible to optimize the levels of the water uptake and of the solids of rehydrated apple cubes by modifying the rehydration time and the medium concentration.

Hence, from the same fruit we could obtain different products characterized by different water and solid content, only by using an appropriate rehydrating low-cost step.

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EFFECT OF DIFFERENT PACKAGING CONDITIONS ON STORAGE OF ROASTED AND SALTED CASHEW NUT

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Roasted and salted cashew nuts (*Anacardium occidentale*) were stored for 360 days at 30 °C and 80% relative humidity in 200 g pouches. Three flexible packaging materials were used, polypropylene/polyethylene (PP/PE); metallized polyethylene terephthalate/polyethylene (PETmet/PE); polyethylene terephthalate/aluminum foil/low density polyethylene (PET/Al/LDPE). Control samples, packaged in PET/Al/LDPE with N_2, were maintained at –18 °C. The stability of nuts was periodically evaluated by texture tests, moisture content, hexanal content and sensory acceptability. Tocopherol content, acid and peroxide values were measured in the lipid fraction. Acid value, tocopherol and hexanal contents were nearly constant. Moisture content increased for all materials, except for nuts packaged in PET/Al/LDPE. Differences were observed in texture tests, mainly in nuts with higher moisture content. Peroxide value did not have a good response. Shelf life based on significant decrease in sensory acceptability for nuts, was 210 days for PP/PE packaging and 310 days for PETmet/PE packaging. Significant decrease was not observed in sensory acceptability of nuts packaged in PET/Al/LDPE in 360 days of storage.

Keywords: cashew nut, packaging, shelf life, storage

The marketable world production of cashew nuts is concentrated in only 5 countries: India, Brazil, Mozambique, Tanzania and Kenya, which are responsible for 98% of the production. The consumption of nuts, for its turn, happens at the high income countries, among which USA is the greatest consumer (EDIBLE NUT MARKET REPORT, 1995). The exploitation of cashew in Brazilian Northeast has great social and economical importance by the employment and foreign exchange credits generation for Brazil (LEITE, 1994). High lipid content (45–47%) and low moisture (2–6%) of cashew nuts can lead to oxidation and texture changes (SAMSON, 1986). Good packaging conditions can reduce deterioration and insure the availability of high quality nuts. The

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effect of packaging conditions in almond nuts was studied by GUADAGNI and co-workers (1978), SENESI and co-workers (1991) and SATTAR and co-workers (1991); in Brazil nuts by RIBEIRO and co-workers (1993); in walnuts by JAN and co-workers (1988); in pecan nuts by HOLADAY and co-workers (1979) and DULL and KAYS (1988); and in macadamia nuts by CAVALETTO and YAMAMOTO (1971). However, there is no data about the effect of different packaging conditions on cashew nut stability. This study was aimed to evaluate the sensory and physico-chemical behavior of roasted and salted cashew nuts stored in different flexible packaging materials for 360 days.

1. Material and methods

Shelled, roasted and salted cashew nuts were obtained from a local company in Fortaleza city, Brazil. For the experiment the nuts were packaged in bags of 3 different flexible materials, PP/PE, PETmet/PE and PET/Al/LDPE. Their characteristics are shown in Table 1. Each package, containing 200 g nuts, was closed with normal air and kept at 30 °C, 80% RH (storage space outside the packages), in the dark. Control samples, packaged in PET/Al/LDPE, were evacuated, filled with nitrogen gas (<1% O_2) and stored at –18 °C. All samples were stored for 360 days.

Packages were taken at random from each treatment every 40–50 days, and whole nuts were analyzed for texture and sensory acceptability. Ground nuts were used for hexanal determination and moisture content. Lipid fraction, obtained by cold extraction according to BLIGH and DYER (1959), was used for tocopherol content, acid and peroxide values determinations. Fatty acid composition of the lipid fraction was obtained.

Table 1

Characteristics of flexible packaging materials

Material	Water vapor permeability rate g H_2O/m^2 day (38 °C/90% RH)	O_2 permeability rate cm^3 O_2/m^2 day (25 °C/1 atm)
PP/PE	5.0	1135
PETmet/PE	2.6	6.63
PET/Al/LDPE	0.036	0.32

PP/PE-polypropylene/polyethylene; PETmet/PE-metallized polyethylene terephthalate/polyethylene; PET/Al/LDPE-polyethylene terephthalate/aluminum foil/low density polyethylene

Fatty acid composition was measured through gas chromatography of the fatty acids methyl esters, obtained according to HARTMAN and LAGO (1973). The analysis was done in a Perkin Elmer Sigma 3B gas chromatograph with flame ionization detector. The column was a 10% SILAR 10C with 4m×1/8". The temperatures were 165 °C in the column, and 215 °C in the detector and injector.

Moisture content (Ca 2c-25), acid value (Ca 5a-40) and peroxide value (Cd 8-53) were measured according to A.O.C.S. (1988).

Hexanal content was obtained by gas chromatography, according to FRITSCH and GALE (1977). The equipment was a Perkin Elmer Sigma 3B gas chromatograph with flame ionization detector. The column was a 3% OV 17, with 2m×1/8". The temperatures were 50 °C in the column, and 110 °C in the injector and detector. The internal standard used was 4-heptanone.

Instrumental texture evaluations (puncture test) were carried out using a texturometer TAXT2 (Texture Technologies Corp.) with a needlelike probe TA 9. The speed was 2 mm s^{-1} and the distance 8 mm. The energy absorbed during the test was determined as the area below the curves obtained, and expressed as gs.

Tocopherol content was measured by high performance liquid chromatography (HPLC) in a Perkin Elmer LC 250 liquid chromatograph with a UV/Vis LC 290 detector. The column was a HP C18 SIL X - 10, with 4.6 mm ID X 25 cm. The wavelength used was 295 nm and the mobile phase was methanol/water (97:3). Nut oil diluted in hexane was injected in the column without treatment.

Sensory acceptability was measured with thirty consumers at each period of analysis. The nuts were placed in coded plastic jars and presented to the judges. The judges were asked to rate each sample on a structured hedonic scale (9=like extremely, 5=neither like nor dislike, 1=dislike extremely). Red ambient light was used in order to mask the products appearance so, the acceptability depends only on nut's aroma and flavor. The end point was considered as the time when significant decrease (Tukey test at 5% level of significance) was observed in sensory acceptability.

Statistical analysis was performed by analysis of variance and average values were compared through Tukey test at 5% level of significance.

2. Results and discussion

Fatty acid composition of lipid fraction of cashew nuts is shown in Table 2. In spite of the high unsaturated fatty acid content (82.1%), the linolenic acid content is low (0.7%), leading to a good oxidative stability.

Table 2

Fatty acid composition of lipid fraction of cashew nuts

Fatty acid	%
C16:0 (palmitic acid)	9.1
C16:1 (palmitoleic acid)	0.4
C18:0 (stearic acid)	8.3
C18:1 (oleic acid)	60.0
C18:2 (linoleic acid)	21.0
C18:3 (linolenic acid)	0.7
C20:0 (arachidic acid)	0.5
Unsaturated fatty acids	82.1
Saturated fatty acids	17.9

The average acid values for the 4 samples of cashew nuts are shown in Fig. 1. For all samples, acid values at the end of storage period were lower than at the beginning of the experiment. Those changes in acid values were not statistically significant ($P \leq 0.05$).

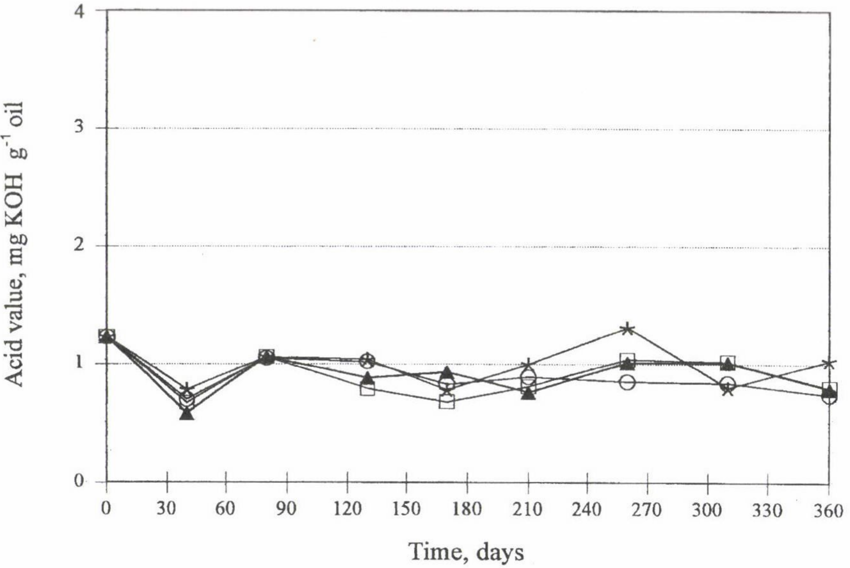


Fig. 1. Acid value of cashew nut oil as a function of storage time. *: PP/PE; □: PETmet/PE; ▲: PET/Al/LDPE; O: control

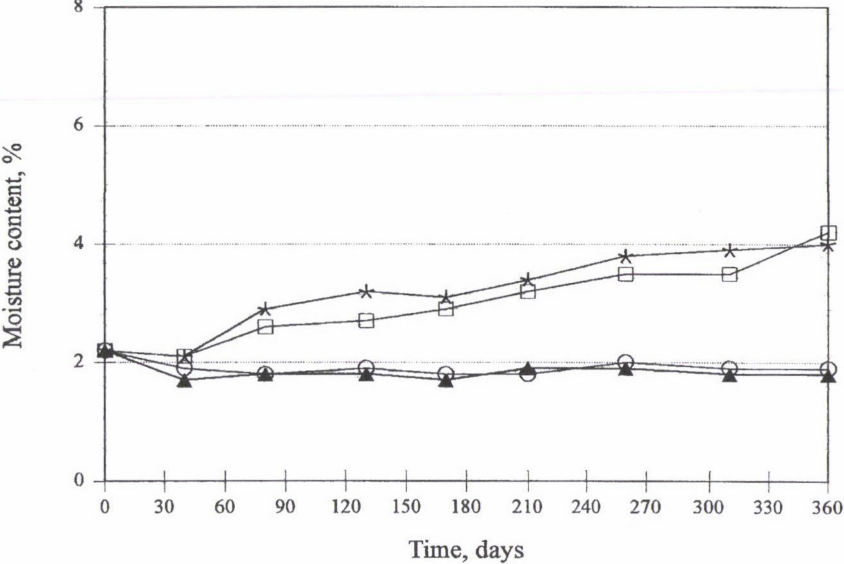


Fig. 2. Moisture content of cashew nut as a function of storage time. *: PP/PE; □: PETmet/PE; ▲: PET/Al/LDPE; O: control

Figure 2 shows moisture content behavior of nuts during storage. Initial moisture content was 2.2% for all treatments. Average moisture content for nuts in PET/Al/LDPE after 360 days of storage was 1.8% for samples at 30 °C and 1.9% for control sample. No increase in moisture content was observed for nuts in these packaging materials due to the lower water vapour permeability rate of PET/Al/LDPE film. For nuts packaged in PETmet/PE the average moisture content at the end of storage period was 4.2% and for nuts in PP/PE was 4.0%. For both materials there were significant increases in moisture content, when statistically analyzed (Tukey test, 5% level of significance). QUAST and TEIXEIRA NETO (1976) reported that moisture content of cashew nuts must be between 4 and 5% in order to avoid breakage and microbial growth.

Energy absorbed during the texture tests increased significantly ($P \leq 0.05$) as a function of storage time for all treatments. In Fig. 3, it can be seen that for nuts packaged in PP/PE and PETmet/PE energy increased at a higher rate than for nuts packaged in PET/Al/LDPE due to the higher moisture content of those samples. The results agreed with OLOSO and CLARKE (1993), who carried out compression tests on roasted cashew nuts and also observed that absorbed energy increased when moisture content was higher. Energy slowly increased also for nuts in PET/Al/LDPE, although the moisture contents were nearly constant, probably due to the aging of nut cells.

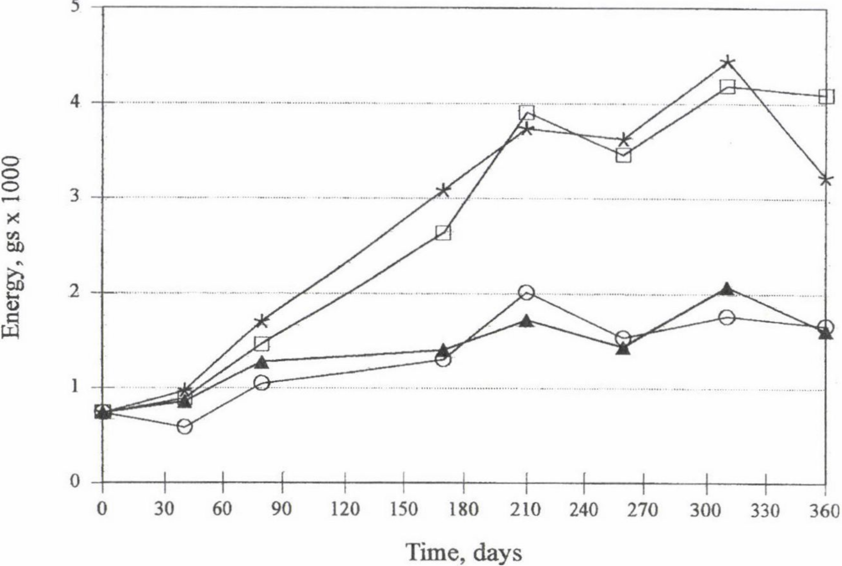


Fig. 3. Texture (energy) of cashew nut as a function of storage time. *: PP/PE; □: PETmet/PE; ▲: PET/Al/LDPE; O: control

The average peroxide values of the 4 samples of cashew nut oil are shown in Fig. 4. The results indicated that peroxide values were significantly ($P \leq 0.05$) higher at the end of the experiment than the initial values, although there was not a defined response. Peroxide values increased in some periods and decreased in others, but they were always below 3.2 meq/1000 g oil for all treatments. According to LABUZA (1971) peroxides are instable compounds and can be easily decomposed in the lipid extraction phase, especially when they are in small concentrations. This can lead to modification of peroxide values during the storage time.

Hexanal traces were observed from 40 and 80 days to the end of storage period in nuts packaged in PP/PE and PETmet/PE, respectively. For nuts in PET/Al/LDPE at 30 °C, hexanal values ranging from 1.8 to 1.9 ppm were detected after 80 days of storage, and only for this treatment it was high enough to be quantified. Hexanal was not detected in control samples. Low hexanal contents were due to reduced oxidative reactions, which agreed with the low peroxide values obtained.

Results of tocopherol determination are shown in Figs 5 and 6. There were oscillating changes in tocopherol values during storage time. Similar behavior was observed by ERICKSON and co-workers (1994) for raw and roasted pecans. At the end of storage

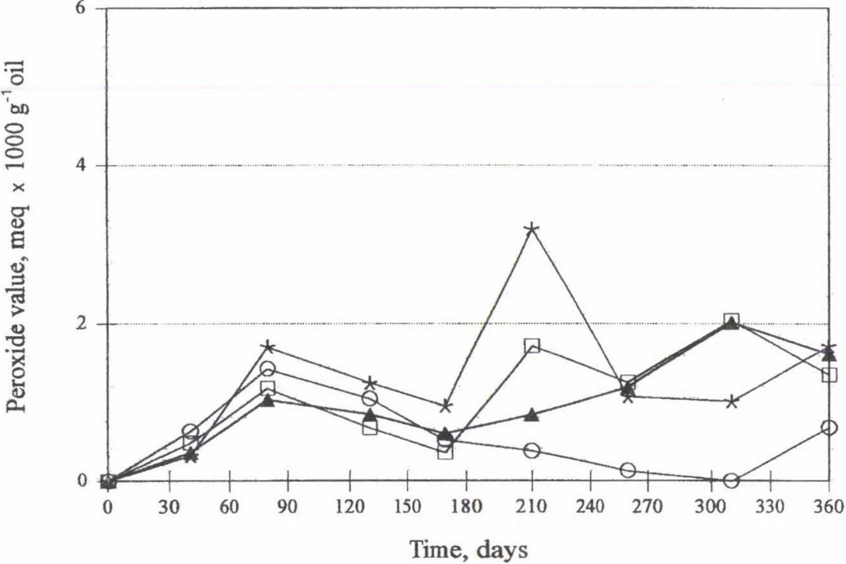
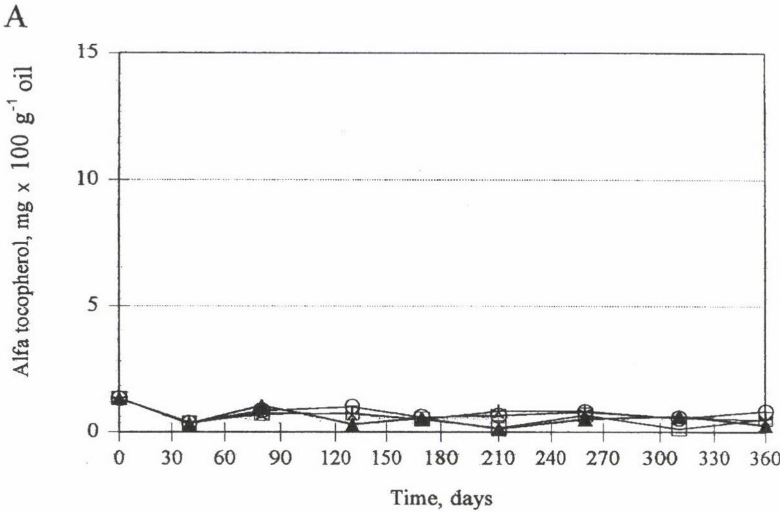


Fig. 4. Peroxide value of cashew nut oil as a function of storage time. *: PP/PE; □: PETmet/PE; ▲: PET/Al/LDPE; O: control

time, tocopherol contents were considered high when compared to the initial content. There were no significant differences ($P \leq 0.05$) among the different treatments and storage time. Tocopherol contents at initial time were 1.33, 9.44 and 3.48 mg/100 g oil, respectively, for α, $\beta+\gamma$ and δ tocopherols. Total tocopherol content for cashew nut oil (14.25 mg$\times$100 g^{-1} oil) is low when compared to other vegetable oils like corn and soybean oil, which have 56 to 114 mg$\times$100 g^{-1} oil and 107 to 144 mg$\times$100 g^{-1} oil, respectively (GUNSTONE et al., 1994).

Sensory acceptability values decreased from 7.36 to 3.87, from 7.36 to 5.83 and from 7.36 to 6.70 for PP/PE, PETmet/PE and PET/Al/LDPE packaging, respectively, during 360 days of storage. For control samples the sensory acceptability changed from 7.36 to 7.56 at the end of storage period. Figure 7 shows sensory acceptability behavior as a function of storage time. Shelf life, based on significant decrease (Tukey test, 5% significance level) in sensory acceptability of the nuts, was 210 days for PP/PE packaging and 310 days for PETmet/PE packaging. Shelf life of cashew nuts packaged in PET/Al/LDPE was higher than 360 days, because no significant decrease ($P \leq 0.05$) was observed in sensory acceptability for these samples during the storage period. Control samples did not show decrease in sensory acceptability.



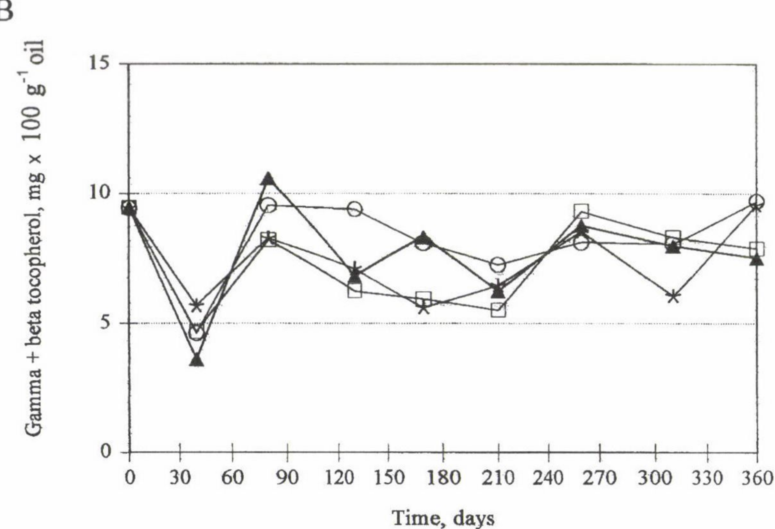
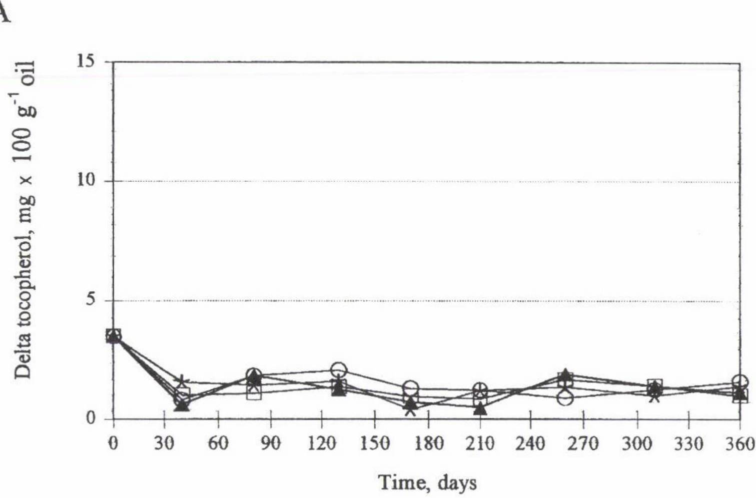


Fig. 5. Tocopherol content of cashew nut oil as a function of storage time (A: α tocopherol, B: β+γ tocopherol). *: PP/PE; □: PETmet/PE, ▲: PET/Al/LDPE; O: control



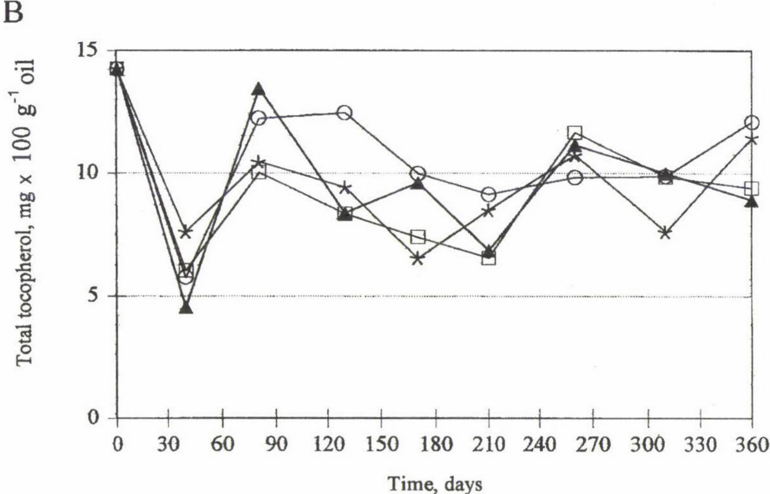


Fig. 6. Tocopherol content of cashew nut oil as a function of storage time (A: δ tocopherol, B: total tocopherol). *: PP/PE; □: PETmet/PE, ▲: PET/Al/LDPE; O: control

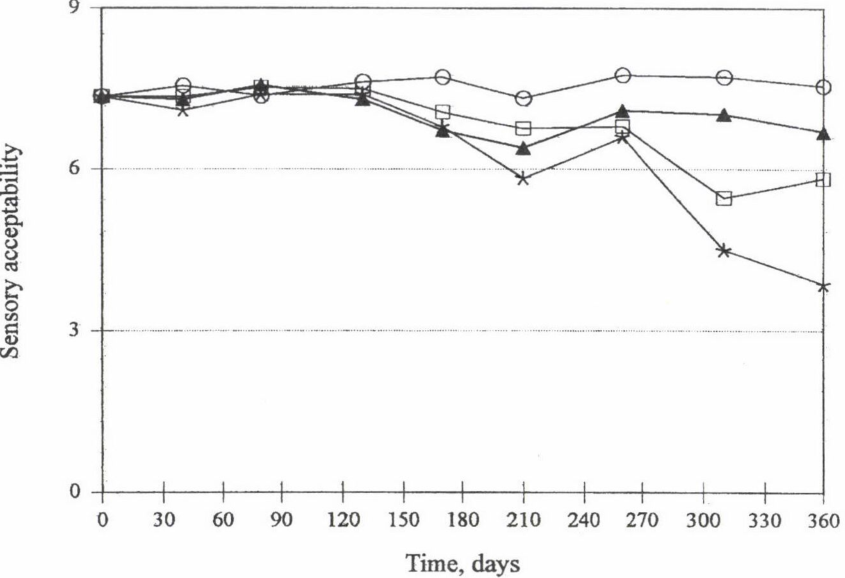


Fig. 7. Sensory acceptability of cashew nut oil as a function of storage time. *: PP/PE; □: PETmet/PE; ▲: PET/Al/LDPE; O: control

3. Conclusions

Flexible packaging materials with lower water vapor permeability rate showed greater nut protection, retarding texture changes and moisture absorption and giving higher shelf life. Oxidative reactions were low during the storage period studied. Results indicated that cashew nuts could be held for 7 months stored at 30 °C and 80% RH without a significant loss of sensory quality in all packaging materials studied.

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EFFECT OF CARBONIC MACERATION ON PHENOLIC COMPOSITION OF RED WINES

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The formation of phenolic constituents is significantly affected by the nature and the maturity grade (year) of the grapes as well as by the vinification method adopted. In case of wines made by carbonic maceration, free-run wines show higher total phenolic level than press wines and the control skin-fermented one.

In MC-wines (made by carbonic maceration), the longer carbonic maceration time (i.e. 14–20 days) made the amount of phenolic constituents (anthocyanin, catechin, leucoanthocyanin) rather much increased.

Wines prepared by cold carbonic maceration (16–18 °C) contained less phenolics than the control products and the warm (30–32 °C) MC wines, respectively.

The effect of an additional skin-fermentation (2 days) performed subsequently to the carbonic treatment, proved advantageous, when it was aimed to prepare red wine.

Keywords: carbonic maceration, anaerobic metabolism, phenolic constituents, red wine

Detailed presentation of carbonic maceration method in vinification as well as the examination of its effect had been discussed in our previous papers (LŐRINCZ et al., 1995, 1996, 1997). The work presented here is aimed at expounding the effects of carbonic maceration on phenolic composition of red wines.

Vinification in carbon dioxid atmosphere significantly alters the phenolic structure of the red wine. CARNACINI and co-workers (1991) observed that the wine produced in anaerobic way had higher total phenol and procyanidine content and lower anthocyanin level. Consequently, these wines displayed moderately proportioned colour compared to the pigmentation of the skin-fermented (control) wines. Similarly, TIMBERLAKE and BRIDLE (1976) determined more total phenolics and catechin-type phenols in wines that had been produced by carbonic maceration.

Anaerobic treatment reduces the tannin extraction (FÜLEKI, 1974). Also the examinations by BEELMAN and MCARDLE (1974) proved that wines of carbonic maceration were significantly poorer in tannin. Experiments of NAVARRO and co-

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workers (1988) showed higher phenolic content in free-run juice than in press must at the conclusion of the first phase in the vinification.

Significantly more polymeric colouring substances could be found in wines made by carbonic maceration than in the skin-fermented products (DUCRUET et al., 1983; NAVARRO et al., 1988). SITTERS and co-workers (1986) estimated that, when a brief (in general 36 h) skin-fermentation process was effected after the anaerobic treatment, a beneficial result on colour was induced.

Ethanol developed in the metabolic course makes the enzymes in berry tissue denaturalize and, thereafter, also destroys the berries. At this point, the berry pulp is invaded by substances emerged from the skin, as phenolics, containing nitrogen and aromatic compounds. Driving of phenolics (and anthocyanins among them) is the bases of the pigmentation of the pulp and juice, during carbonic maceration treatment. The extraction of phenolics towards the pulp shows an increase by temperature rise. The diffusion of the red pigments precedes the tannin migration (FLANZY et al., 1987).

Diffusion conditions of the three phases in course of carbonic maceration are shown in Fig. 1.

In the inner part of solid phase, the anaerobic metabolism as well as the diffusion of the gas and liquid phases are running synchronously. In the course of the anaerobic metabolism, the power of resistance in the berries decreases because of the degradation

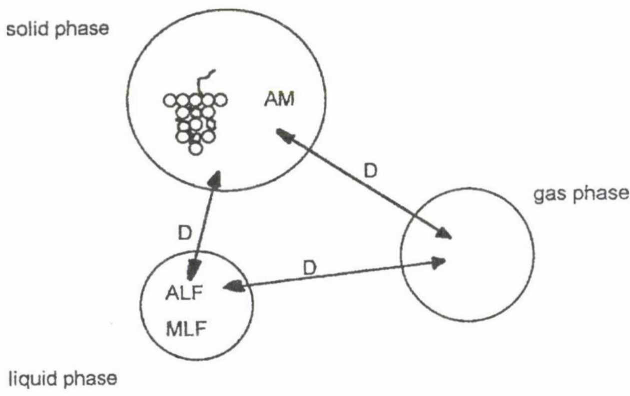

Fig. 1. Alternating changes in three phases of CO_2 atmospheric vinification (FLANZY et al., 1987). AM = anaerobic metabolism; ALF = alcoholic fermentation; MLF = malolactic fermentation; D = exchange by diffusion, solid phase = grape berries, liquid phase = free-run juice, gas phase = CO_2 + volatile substances

of the cell walls. By this, the bursting of the berries will take place, thus juice content of the berries will run into the liquid phase. Consequently, the composition and volume of the latter will continuously change. A tendency to achieve the equilibrium state in relation to the grapes in the gas phase of the container and the gas volume can be observed. Through the fermentation of the must gathered in the bottom of the container, the alcohol in the gas room increases and, consequently by diffusion of this, the alcohol level in the interior of the berries will be raised as well.

1. Materials and methods

A four-year experimentation was performed aiming at the adoption of carbonic maceration. The methods of the experiments were basically identical. Investigations were performed on carbonic maceration in cold and warm, respectively, as well as on the effects of certain technological combinations (e.g. anaerobic treatment followed by skin-fermentation) on wine quality.

1.1. Raw materials

Wines were made from four different grape varieties (Kékoportó, Kékfrankos, Zweigelt and Merlot) harvested in Eger wine-district beetwen 1993 and 1996. In case of Zweigelt and Kékfrankos there were two harvest-days (1 = first harvest, 2 = two weeks later) in 1993.

1.2. Treatments

Grapes were emptied into a container presaturated with CO_2, then the material was stored at warm (30–32 °C) and ambient temperature (16–18 °C), respectively, for 7–12 days, in general. The CO_2 gas absorbed by the berries was regularly recovered in the first 24 hours. Following these treatments, the free-run juice was drawn off, then the grapes were crushed and destemmed. This was followed by pressing. The juices (free-run juice and press juice) were separately fermented. In certain experiments, following the treatment, an additional, two-day skin-fermentation was applied.

Control treatments were performed by applying skin-fermentations of 7–8 days and 19 days, respectively. The treatments were marked as summarized in Table 1. The experiments consisted of three replications.

Table 1

Treatments and their marks

Mark	Treatment
C	Control, skin-fermentation
MC	Carbonic maceration
MCC	Cold carbonic maceration
LMC	Long carbonic maceration
LMCC	Long cold carbonic maceration
+S	+ Skin-fermentation
f	Free-run wine
p	Press wine

1.3. Chemical analysis

Total phenolics, catechin, anthocyanin and leucoanthocyanin contents of the experimental wines were put to analysis. The examinations were performed according to determination methods defined by O.I.V. (1978, 1990) and the standard methods, respectively. The percentage values of polymers (URBÁN, 1985) were examined, as well.

Analysis of variance was done using the STATGRAPH software (95% significance level).

1.3.1. Determination of polymer ratio. Potassium meta-bisulphite solution (0.2 ml, 20%) was added to 5 ml of wine. After 15 min, light absorption values at 420 nm and 520 nm were taken, compared to distilled water and using cuvets of 0.2 cm width. The control sample for this determination was prepared by adding 0.2 ml dist. water to 5 ml of control wine.

$$\text{Polymer ratio } (\%) = \frac{(A_{420} + A_{520})_{\text{K-pyrosulphite}}}{(A_{420} + A_{520})_{\text{control}}} \times 100$$

2. Results and discussion

As for wines of 1993 vintage year, total phenol content in free-run wines (representing the smaller amount) proved higher than the same substance in press wines in every case.

The wines prepared through cold maceration method (marked by MCC) proved to have half content of total phenolics compared to the values in the other samples. Though, this amount could be significantly increased by applying additional skin-fermentation (marked by MCC+S).

Figure 2 shows the development of total phenolics in dependence of the vinification method.

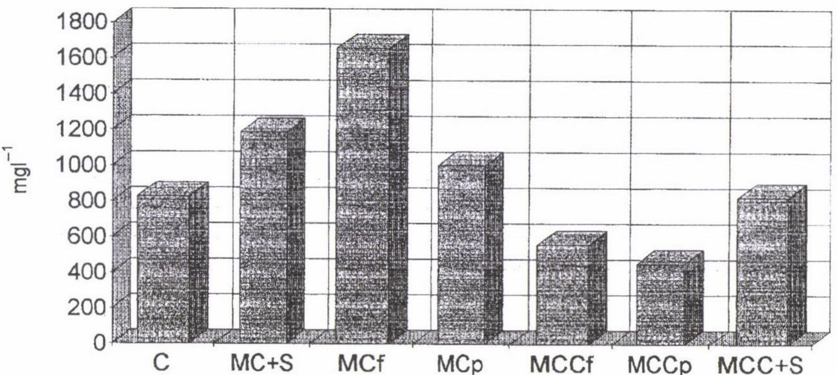

Fig. 2. Total phenol contents of Zweigelt 2 wines (1993). C = Control, skin-fermented; MC = carbonic macerated (30 °C, 7 days); f = free-run wine; p = press wine; MC+S = carbonic macerated (30 °C, 7 days) followed by skin-fermentation (2 days); MCC = carbonic macerated cold (16 °C, 7 days); MCC+S = carbonic macerated cold (16 °C, 7 days) followed by skin-fermentation (2 days)

Table 2

Anthocyanin level (mg l^{-1}) of experimental wines (1993)

Treatment	Kékoportó	Zweigelt 1	Zweigelt 2	Kékfrankos 1	Kékfrankos 2
Control	345	337	250	311	303
MCf	319	410	440	350	287
MCp	279	208	310	225	266
MC+S	343		358		234
MCCf			99		
MCCp		43	89	78	85
MCC+S	311	237	306	295	204

Control = skin-fermented (7 days); MC= carbonic macerated (30 °C, 7 days); f = free-run wine; p = press wine; MC+S = carbonic macerated (30 °C, 7 days) plus subsequently skin-fermented (2 days); MCC = carbonic macerated cold (16 °C, 7 days); MCC+S = carbonic macerated cold (16 °C, 7 days) plus subsequently skin-fermented (2 days)

The anthocyanin level (Table 2) was generally lower in the press wines produced by traditional carbonic maceration than in the skin-fermented products (except for Zweigelt 2). The same is verified by literature, according to which, vinification in CO_2 atmosphere does not really favour the development of intense colour, because the alcoholic fermentation takes place but to a limited extent, in course of the treatment. On the other hand, the anthocyanin content in the free-run wines was found nearly in the same quantity or higher than in the control.

In general, the anthocyanin level of the wines made by the MC-treatment and additional skin-fermentation (marked by +S) did not overpass the amount found in the wines made by control vinification (the exception was Zweigelt 2).

By using cold (16 °C) carbonic maceration, very little anthocyanin substance could be released from the pigment-pouches of the skin-cells. At this temperature, a seven-day maceration could not effect significantly the destruction of the skin tissue. Though, in this case, the additional skin-fermentation induced significant rise of the anthocyanin level.

Figure 3 demonstrates the quantity of characteristic phenolics in dependence of the vinification method applied. (Grape variety was Kékfrankos.)

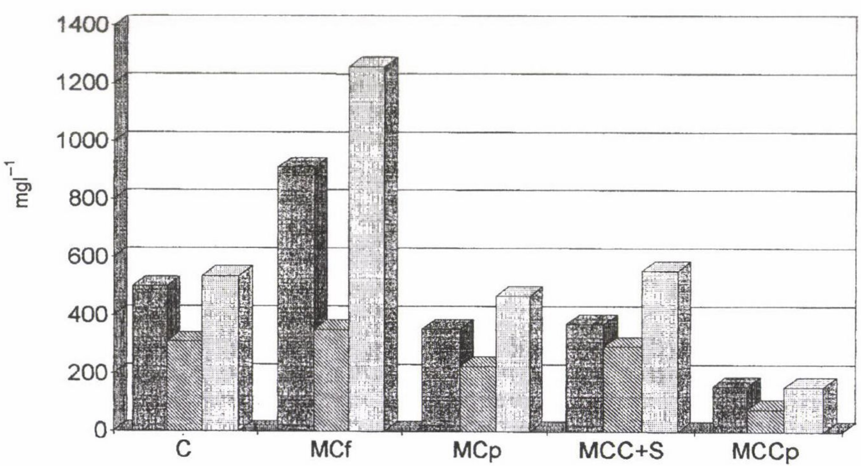

Fig. 3. Characteristic phenol-components of Kékfrankos 1 wines (1993). C = Control, skin-fermented; MC = carbonic macerated (30 °C, 7 days); f = free-run wine; p = press wine; MCC = carbonic macerated cold (16 °C, 7 days); MCC+S = carbonic macerated cold (16 °C, 7 days) followed by skin-fermentation (2 days). ▩: leucoanthocyanin; ▧: anthocyanin; ▦: catechin

Table 3

Polymer ratio (percentage) in experimental wines (1993)

Treatment	Kékoportó	Zweigelt 1	Zweigelt 2	Kékfrankos 1	Kékfrankos 2
Control	36	28	30	21	19
MCf	44	31	29	30	19
MCp	34	38	36	34	33
MC+S	46		34		37
MCCf			56		
MCCp		41	55	65	54
MCC+S	35	29	32	24	33

Control = skin-fermented (7 days); MC = carbonic macerated (30 °C, 7 days); f = free-run wine; p = press wine; MC+S = carbonic macerated (30 °C, 7 days) plus subsequently skin-fermented (2 days); MCC = carbonic macerated (16 °C, 7 days); MCC+S = carbonic macerated (16 °C, 7 days) and subsequently skin-fermented (2 days)

The forming of the polymer ratio is illustrated in Table 3. In control wines, the polymer ratio was generally less than 30%. The polymerisation showed an increase by applying carbonic maceration. This was particularly revealed in considerable degree, when cold CO_2 maceration (MCC) took place. By these samples, the additional skin-fermentation seemed to promote the formation of lower polymerisation values (MCC+S).

In 1994 vintage year, total phenolics, anthocyanin and catechin levels of MC-press wines proved generally lower than the same values of control wines and the values of MC-free-run wines, respectively. The exception was Kékfrankos grape variety, which showed higher values in press wine than in control.

Table 4

Contents (mg l^{-1}) of total phenolics (TP), catechin (C) and anthocyanin (A) in experimental wines (1994)

	Merlot			Kékfrankos			Zweigelt		
	TP	C	A	TP	C	A	TP	C	A
Control	859	304	354	703	207	223	1056	346	219
MCf	860	138	262	1088	274	234	1370	465	221
MCp	432	117	141	986	234	245	910	265	148
LMCf				2290	336	273	2140	865	258
LMCp				1394	212	329	1470	554	171

Control = skin-fermented (8 days); MC = carbonic macerated (30 °C, 7–8 days); f = free-run wine; p = press wine; LMC = long carbonic macerated (30 °C, 15 days)

The result are shown in Table 4. In regard to total phenol content, it was the free-run wines which showed the highest level. This was also supported by higher catechin level in cases of Zweigelt and Kékfrankos varieties.

Anthocyanin content of free-run wines are nearer to the levels in control wines than to those of press wines. The increasing alcohol content in free-run juices is supposed to intensify the extraction power to the skin-cells in the crushed berries.

Longer maceration time (15 days) made the anthocyanin, total polyphenol and catechin content of the (LMC) wines higher, in comparison to wines prepared by shorter (7–8 days) first phase; even the similar values of the control wines were overpassed in most cases.

In vintage year 1995, the phenolic constituents showed the same trend, in nearly all cases. This can be clearly observed in Fig. 4. The free-run wines made by carbonic maceration contained higher amount of total phenolics, anthocyanin, leucoanthocyanin and catechin than the press wines.

By comparing the data of the control wine to those of other wines, it appears that, nearly every phenolic component of the treated wines show higher levels (may be with the exception of catechin). Similar enrichment can be observed in the mentioned components of the wines made by long carbonic maceration in vintage year 1994.

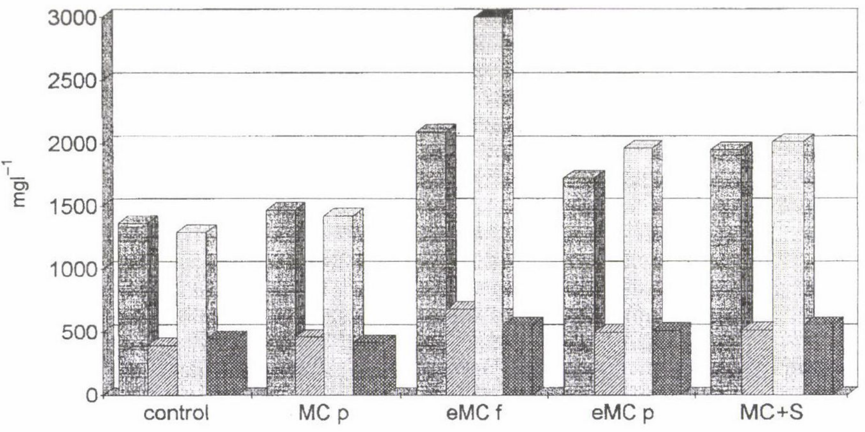

Fig. 4. Phenolic constituents in experimental wines of Kékfrankos (1995). C = Control, skin-fermented; MC = carbonic macerated (26 °C, 19 days); f = free-run wine; p = press wine; e = treated with enzyme preparate; MC+S = carbonic macerated (26 °C, 19 days) followed by skin-fermentation (6 days). ▩: Total phenol; ▧: leucoanthocyanin; □: anthocyanin; ■: catechin

Investigation were performed on the formation of phenolics by CO_2 atmospheric treatment (first phase), in wines of 1996 vintage year. The results are illustrated in Figs 5–8.

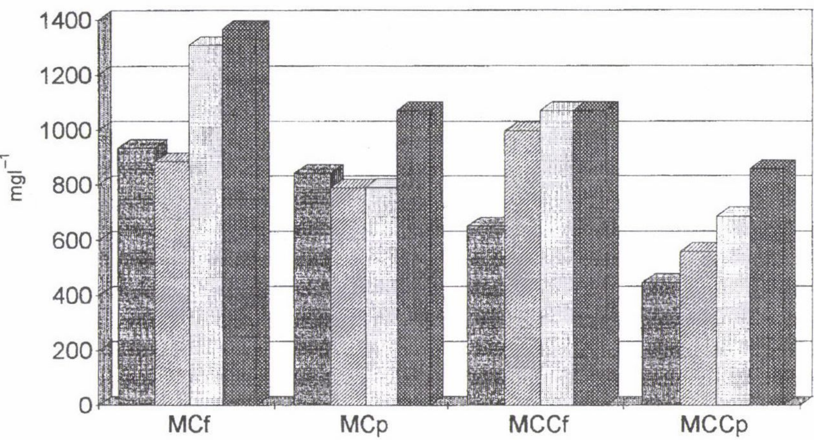

Fig. 5. Formation of total phenol content between 4th and 14th days of several CO_2 treatments (1996). MC = carbonic macerated (30–32 °C); f = free-run wine; p = press wine; MCC = carbonic macerated cold (16–18 °C). ▦: 4 days; ▧: 8 days; ▢: 11 days; ■: 14 days of treatments

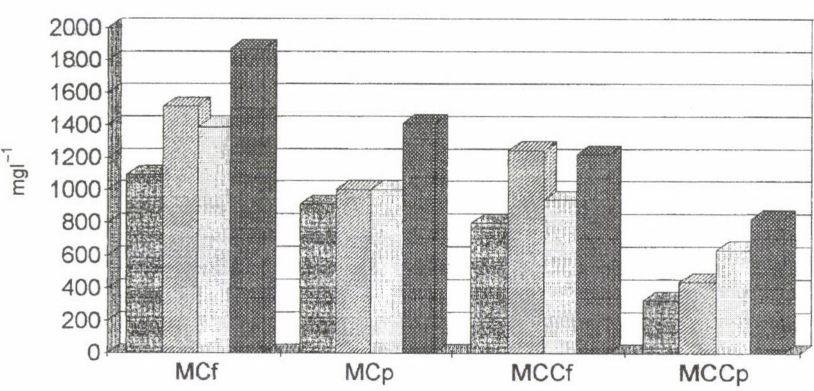

Fig. 6. Formation of leucoanthocyanin content between 4th and 14th days of several CO_2 treatments (1996). MC = carbonic macerated (30–32 °C); f = free-run wine; p = press wine; MCC = carbonic macerated cold (16–18 °C). ▦: 4 days; ▧: 8 days; ▢: 11 days; ■: 14 days of treatments

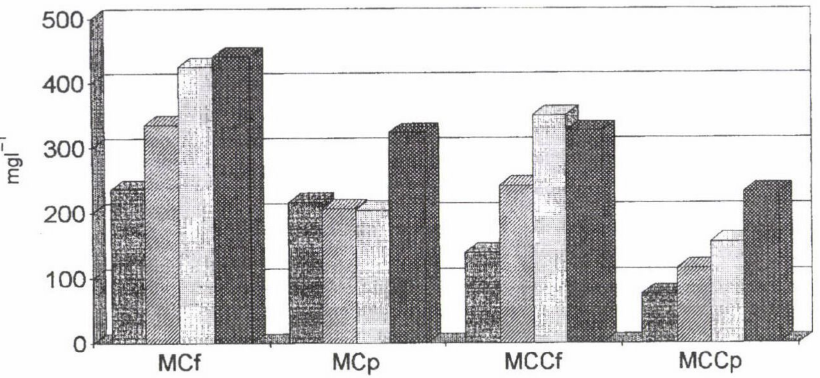

Fig. 7. Formation of catechin content between 4th and 14th days of several CO_2 treatments (1996). MC = carbonic macerated (30–32 °C); f = free-run wine; p = press wine; MCC = carbonic macerated cold (16–18 °C). ▩: 4 days; ▨: 8 days; ▢: 11 days; ■: 14 days of treatments

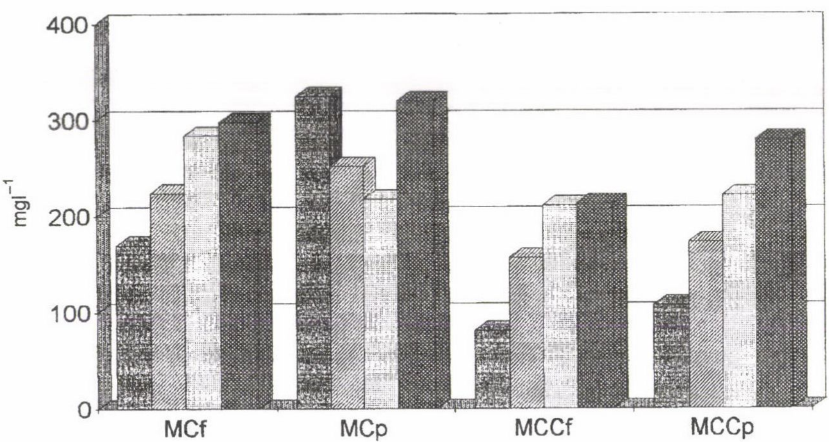

Fig. 8. Formation of anthocyanin content between 4th and 14th days of several CO_2 treatments (1996). MC = carbonic macerated (30–32 °C); f = free-run wine; p = press wine; MCC = carbonic macerated cold (16–18 °C). ▩: 4 days; ▨: 8 days; ▢: 11 days; ■: 14 days of treatments

In free-run juices, the growth of total phenolics, leucoanthocyanin and catechin contents proved larger than in press products. The components mentioned diffused from the skin into the press juice. This migration was affected by the temperature applied in course of the treatment.

Steady enrichment in phenolics could be observed in the press juices, while making cold carbonic maceration. The amount of this was lower compared to that of in the press juice of warm carbonic maceration. As to the quantity of phenolics, fluctuation and decrease (striking transitory character) in the 8th and the 11th days could be observed. Presumably, by this time, the skin tissue of the berries was properly disintegrated and, in the components, a momentary distribution was holding. On one part, already, by this time, the berries continuously let the juice run, on the other, a diffusion from skin towards the berries was functioning, as well.

The provisional decrease could be accounted for a possible rise of tannin phenolics, i.e., condensated pigments by this time. (Higher temperature advances condensation and polymerisation processes.)

As regards the free-run juices, continuous decrease could be observed in total phenol, leucoanthocyanin, catechin and anthocyanin levels.

Anthocyanin level proved higher in press juice than in free-run musts. Even the value taken in the control sample could be achieved by the warm-macerated wines (MCp) after an 8-day treatment.

Through the application of carbonic maceration (for 8 and 14 days), red wines could be prepared, which were richer in the anthocyanin and total phenolics than the wines of the skin-fermentation process (8 days). This is to be seen in Fig. 9.

Carbonic maceration made the quantity of phenolic constituents significantly increase. In case of warm (traditional) treatment, the anthocyanin content presented itself more increased than by cold treatments. By the technology, the different phenolic compounds were extracted selectively. This was backed up by the fact that, in cold (16 °C) carbonic macerated free-run wines anthocyanin level proved lower, though total phenol content was similar.

In every case, total phenol level in free-run wines proved higher than those in press wines. However, anthocyanin level was higher in press wines.

Catechin and leucoanthocyanin levels were higher in free-run wines than in press wines (Fig. 10).

Fig. 9. Formation of total phenol and anthocyanin levels in experimental wines of 1996 year. Control = skin-fermentation (8 days), MC = carbonic macerated (30 °C); MCC = carbonic macerated (16 °C); f = free-run wine; p = press wine; 1 = 8-day maceration, 2 = 14-day maceration. ▩: Total phenol, ▥: anthocyanin

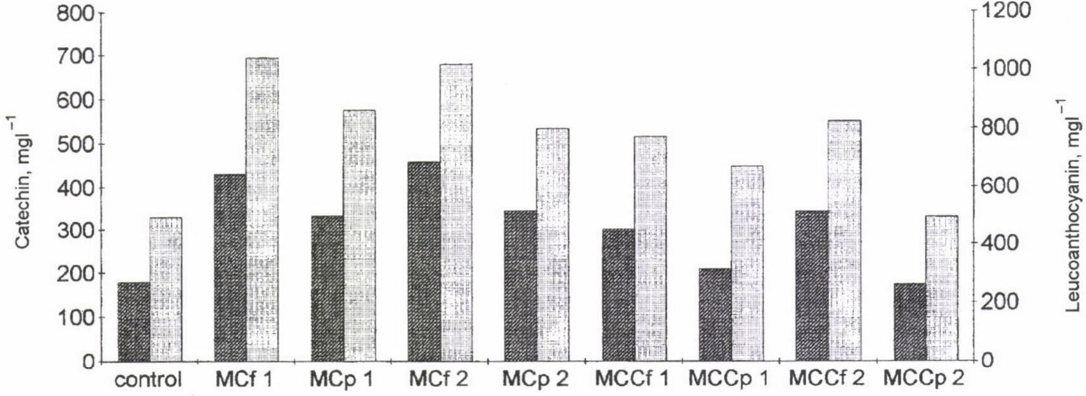

Fig. 10. Formation of catechin and leucoanthocyanin levels in experimental wines of 1996 year. Control = skin-fermentation (8 days), MC = carbonic macerated (30 °C); MCC = carbonic macerated (16 °C); f = free-run wine; p = press wine; 1 = 8-day maceration, 2 = 14-day maceration. ▩: Catechin, ▥: leucoanthocyanin

3. Conclusions

The formation of phenolic constituents is significantly affected by the nature and the maturity grade (year) of the grapes as well as by the vinification method adopted. Relying upon the examinations, we observed the effects of carbonic maceration on phenolic components as follows:

Carbonic gas atmosphere effects difference in phenolic composition of wines made by carbonic maceration and those produced by skin-fermentation (control wines).

Many years' experience suggests that, in case of wines made by carbonic maceration of normal period (i.e. 7–8 days), free-run wines (having the smaller ratio) show higher total phenolic level than press wines and/or the control one.

Anthocyanin content in free-run wines shows similar or higher values compared to skin-fermented and/or press wines.

Also the leucoanthocyanin amount taking part in the browing processes as well as the catechin content proved much higher in free-run wines than in the control samples mostly.

In MC-wines, the longer carbonic maceration time (i.e., 14–20 days) made the amount of phenolic constituents (anthocyanin, catechin, leucoanthocyanin) much increased. In press wines, anthocyanin level generally proved higher, while in free-run wines more leucoanthocyanin and catechin were found. Vinification of longer CO_2 maceration time proved suitable to prepare very mellow red wines.

Wines prepared by cold carbonic maceration contained less phenolics than the control products and the warm MC wines, respectively. In these cases, lesser metabolism degree and skin tissue degradation, respectively, took place, and consequently the extraction of the substances notably influencing the colour turned poorer. Even by using cold carbonic maceration, the free-run juices (produced in limited quantities) proved richer in total phenolics, leucoanthocyanin and catechin substances compared to press juices.

An additional skin-fermentation process which followed the warm carbondioxid-atmosphere treatment (i.e. fermenting the free-run juice and crushed berries together) resulted in somewhat higher amount of phenolic substances (leucoanthocyanin, catechin, anthocyanin) compared to the press wines. The finished wines prepared by this method contained leucoanthocyanin, catechin and anthocyanin substances in quantities similar to the control, or even more.

The effect of skin-fermentation performed subsequently to the cold carbonic treatment proved advantageous, when it was aimed at preparing red wine. The crush of the skin made the phenolic components (and also anthocyanin among them) more easily extractable. Through the adoption of additional skin-fermentation, the polymer ratio was significantly lower in MCC+S wines compared to MCCp ones.

*

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EMULSIFYING PROPERTIES, SURFACE HYDROPHOBICITY AND THERMAL DENATURATION OF PEA PROTEIN FRACTIONS

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The emulsifying activity, emulsion stability, protein surface hydrophobicity (S_0) of albumin, total globulin, vicilin (7S) and legumin (11S) fractions and thermal denaturation of albumin and total globulin fractions of five pea varieties were studied. The emulsifying properties and the S_0 of the protein fractions of a pea variety differed from each other in the case of all varieties. In general, the total globulin and 7S fractions had better emulsifying properties and higher S_0 than the albumin and the 11S fractions. The emulsifying properties and S_0 of the studied pea varieties were different from each other; as well. The thermal (DSC) studies showed that the albumin fractions had one exothermic peak, the total globulin fractions one endothermic peak. The peak temperatures and enthalpies of albumin and total globulin fractions significantly differed from each other. The origin of the protein fractions did not influence the thermal properties.

Keywords: pea protein, emulsifying properties, protein surface hydrophobicity, thermal denaturation

Legumes such as pea are among the most important plant protein sources for human food and animal feed. The use of this protein source as functional agents in food products increases year by year. Increased utilization has led to increased research on their physicochemical properties.

The proteins in pea seed are classified into two categories, albumins and globulins. Albumins are defined as proteins soluble/extractable in water, globulins are proteins soluble/extractable in soline solutions at neutral pH but not in water (OSBORNE, 1924). Major interest has centred on globulin fractions, which have become synonymous with the seed storage protein families vicilin (7S) and legumin (11S). The albumin, legumin and vicilin contents and ratios in peas are genotype specific, but also environment dependent (SCHROEDER, 1982; GUEGUEN & BARBOT, 1988; PEREZ et al., 1993).

The physicochemical properties of pea proteins, which are important properties in food manufacture can also vary significantly between cultivars and fractions. BOROWSKA and co-workers (1996) and SADOWSKA and co-workers (1996) have

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studied the basic composition and physical properties such as firmness and cookability of pea varieties. They observed that the physical properties of seeds depended on the pea variety and crop year. Emulsifying and other functional properties of legumin, vicilin and mixed globulin fractions extracted from peas were investigated by DAGORN-SCAVINER and co-workers (1987) and KOYORO and POWERS (1987). The vicilin fractions, which were shown to be more surface active at interfaces, had better emulsifying properties than legumin fractions. In the case of vicilin-legumin mixtures, better emulsifying results were obtained when vicilin was the major protein (DAGORN-SCAVINER et al., 1987). In the work of KOYORO and POWERS (1987) the vicilin preparation produced more stable foams and emulsions, whereas the legumin gave greater foam expansion, emulsion capacities and surface hydrophobicity.

The aim of the present study was to get further information about some physicochemical properties of albumin, total globulin, vicilin and legumin fractions of five Hungarian cultivated pea varieties.

1. Materials and methods

1.1. Pea samples

Five pea varieties – Hunor, Junak, Türkiz, UM-1073, UM-1095 – were obtained from the Research Station of Vegetable Crops Research Institute, Újfehértó, Hungary.

1.2. Preparation of albumin, globulin, 7S and 11S fractions

Pea protein was extracted with phosphate buffer (0.1 mol l^{-1}, pH 7.0) containing K_2SO_4 (5 g/100 ml) at 20 °C. Albumin and globulin fractions were separated on the basis of their water solubility after dialysis against distilled water according to the method of GUEGUEN and BARBOT (1988). Vicilin and legumin fractions were prepared from globulin fractions after solubilization in NaCl solution (10 g/100 ml). The solubilized globulin was dialyzed against Na-acetate buffer (0.1 mol l^{-1}, pH 4.7) containing 0.2 mol l^{-1} NaCl for 72 h at 4 °C. The precipitated legumin was separated by centrifugation (12000 r.p.m., 20 min., 15 °C) and washed by distilled water, the solubilized vicilin fraction was dialyzed against distilled water for 72 h at 4 °C. All pea protein fractions were freeze dried.

1.3. Determination of physicochemical properties

1.3.1. Emulsifying properties. The emulsifying activity index (EAI) and the emulsion stability index (ESI) were estimated according to the turbidimetric technique of PEARCE and KINSELLA (1978) with slight modification. Two milliliters of sunflower

oil were dispersed into 6 ml protein solution (5 mg ml^{-1}) with a Ultra Turrax stirrer operated at 15,000 r.p.m. for 60 sec. Aliquots (100 µl) of the emulsions were diluted immediately and after 10 min 100 fold with a solution of phosphate buffer (0.01 mol l^{-1} pH 7.4) containing 0.05% sodium dodecyl sulfate (SDS). The turbidity of dilutions was measured in a 1 cm path length glass cuvette at 500 nm. The emulsifying activity index was calculated from the following equation: $EAI(m^2\ g^{-1})=2T/\phi c$, where c is the weight of protein per unit volume of aqueous phase before the emulsion is formed, T and ϕ are turbidity and volume fraction of dispersed phase, respectively. The emulsion stability index (ESI) was calculated from the following equation:

$$ESI(\%) = EAI_{10}/EAI_0 \cdot 100$$

where EAI_{10} is the emulsifying activity index of a dilution that was diluted after 10 minutes of emulsion preparation, EAI_0 is the emulsifying activity index of immediately diluted dilution.

1.3.2. Protein surface hydrophobicity (S_0). The ANS (1-anilinonaphthalene-8-sulfonate) fluorescent probe method of KATO and NAKAI (1980) with modifications was used to measure surface hydrophobicity of protein fractions. The protein samples were serially diluted with 0.01 mol l^{-1} phosphate buffer, pH 7.4, containing 0.002% SDS to obtain protein concentrations ranging from 0.01% to 0.05%. Then, 25 µl methanol solution of ANS (8 mmol l^{-1}) was added to a 5 ml aliquot of each solution. An MPF-2A Hitachi spectrofluorometer was used to measure the relative fluorescence intensities of ANS-protein conjugates. The measurements were made in a 1 cm path length quartz cuvette at an excitation wavelength of 370 nm and an emission wavelength of 480 nm. The instrument was standardized so that the mixture of buffer and 25 µl ANS and methyl acohol and 25 µl ANS had a fluorescence intensity of 0% and 100%, respectively. Surface hydrophobicity was measured as the initial slope of the curve of % fluorescence intensity vs % protein plot.

1.3.3. Thermal denaturation. Differential scanning calorimetry (DSC) was used to determine the thermal denaturation of protein fractions. Accurately weighed samples (900 mg) of protein solutions (10 mg ml^{-1} in distilled water) were sealed in DSC cells. An equal weight of distilled water was sealed in a matching cell and used as a reference. Samples were analyzed in a Setaram micro DSC using a programmed heating rate of 1 °C min^{-1} over a temperature range of 20–100 °C. After heating all the samples were cooled to 20 °C and rescanned to confirm that all transitions were irreversible.

The tests for emulsifying properties and surface hydrophobicity were repeated three times. The results of EAI, ESI and S_0 were presented as the average value of three measurements. To compare the results Student's *t*-test was used.

Table 1

Emulsifying properties and surface hydrophobicity of pea protein fractions

Varieties	Fractions	EAI (m^2g^{-1})		ESI (%)		S_0	
		$\bar{x}$	±s	$\bar{x}$	±s	$\bar{x}$	±s
Hunor	Albumin	29.50	1.12	67.09	0.72	108.88	6.94
	Globulin	21.81	1.03	33.83	1.79	226.11	2.54
	7S	33.03	2.41	75.13	3.27	212.78	9.47
	11S	19.69	0.72	64.16	3.61	162.77	12.28
Junak	Albumin	27.23	0.29	46.56	2.29	90.36	5.25
	Globulin	43.11	2.75	79.25	0.78	141.66	4.41
	7S	37.52	2.05	74.86	2.52	214.44	1.92
	11S	26.47	1.54	55.60	2.41	172.11	3.65
Türkiz	Albumin	32.49	0.75	58.48	1.42	129.99	2.94
	Globulin	26.23	2.19	47.98	2.21	205.55	9.62
	7S	42.26	2.12	81.14	4.29	192.78	8.55
	11S	28.19	1.12	65.85	1.02	176.66	6.66
UM-1073	Albumin	30.98	1.28	48.88	2.42	101.11	1.92
	Globulin	42.81	2.45	76.86	1.58	208.33	8.82
	7S	29.18	1.13	74.63	3.56	176.66	6.66
	11S	24.96	1.07	71.12	2.96	182.22	12.51
UM-1095	Albumin	34.95	1.06	46.74	2.83	89.63	8.98
	Globulin	38.22	1.68	83.26	2.15	141.11	5.91
	7S	39.76	1.73	74.06	1.64	204.44	7.69
	11S	33.56	3.02	64.92	0.71	162.22	2.54

$\bar{x}$: mean
±s: standard deviation

2. Results and discussion

The emulsifying properties and surface hydrophobicity of albumin, total globulin, vicilin and legumin fractions are shown in Table 1.

UM-1095 albumin had the highest, Junak albumin had the smallest EAI values which significantly differed from the values of other albumin fractions. The EAI values of Türkiz and UM-1073 albumin fractions and Hunor and UM-1073 albumin fractions were similar, but the EAI values of Türkiz and Hunor albumin fractions significantly differed from each other. The EAI values of Junak and UM-1073 globulin were similar and significantly higher than the values of other varieties. The EAI value of Hunor globulin was the smallest which significantly differed from the values of other globulin fractions. The ESI value of Hunor albumin was the highest, which significantly differed from the values of other albumin fractions. The ESI values of Junak, UM-1073 and

UM-1095 albumin were similar and significantly smaller than the value of Türkiz albumin. Among the globulin fractions UM-1095 had the highest and Hunor had the smallest ESI value, which significantly differed from the values of other globulin fractions. The ESI values of Junak and UM-1073 globulin fractions were similar and significantly higher than the values of Hunor and Türkiz. In the case of 7S and 11S fractions the 7S fractions had higher EAI and ESI values. These results agree with those obtained by DAGORN-SCAVINER and co-workers (1987) and by KOYORO and POWERS (1987). In their works it was also observed that the vicilin fractions extracted from peas had better emulsifying properties than legumin fractions. The greater emulsifying properties of vicilin compared to legumin may be explained by the fact that vicilin contains fewer cysteine residues and therefore fewer potential disulfide bridges, allowing for a better diffusion rate of proteins to the interface and a film formation behavior of proteins at the interface.

The conclusion can be drawn that while in the case of albumin and total globulin fractions the variety of peas can determine the emulsifying properties, in the case of 7S and 11S fractions the 7S fractions have better emulsifying properties than 11S fractions, independently of varieties. The results confirmed those observation whereas the emulsifying properties of albumin and globulin fractions of pea seeds were variety-dependent and 7S and 11S globulins had significantly different emulsifying characteristics, which are important properties in food manufacture (WRIGHT & BUMSTEAD, 1984; DAGORN-SCAVINER et al., 1987; WRIGHT, 1987).

The protein surface hydrophobicity of the protein fractions showed that among the albumin fractions Türkiz albumin had the highest S_0. This value significantly differed from the values of the other albumin fractions. The S_0 of Hunor and UM-1073 albumin fractions were similar and significantly differed from that of Junak and UM-1095 albumin fractions. Comparing to the other globulin fractions Hunor globulin had a significantly higher S_0. The S_0 values of Junak and UM-1095 globulin fractions were similar and significantly differed from the values of Türkiz and UM-1073 globulin fractions. In contradistinction to the results of KOYORO and POWERS (1987) the S_0 values of 7S fractions were generally higher than the values of 11S fractions, which may be explained by unfolding of the protein molecules exposed to buried hydrophobic regions.

Similarly to the emulsifying properties the S_0 of albumin and total globulin fractions changes depending on varieties. The 7S fractions had generally higher S_0 than 11S fractions independently of varieties. However, the surface hydrophobicity of 7S fractions was higher than that of 11S fractions similarly to the emulsifying properties, S_0 was not necessarily a good predictor of emulsifying properties. For example, although the S_0 values of Hunor and Türkiz 7S fractions were similar their emulsifying properties significantly differed from each other.

Table 2

Thermal denaturation of pea protein fractions

Varieties	Fractions	T_{max}(°C)	Enthalpy (mJ mg^{-1})	Character of peak
Hunor	Albumin	60.1	0.074	exotherm
	Globulin	81.7	0.156	endotherm
Junak	Albumin	60.7	0.080	exotherm
	Globulin	80.6	0.151	endotherm
Türkiz	Albumin	59.6	0.062	exotherm
	Globulin	81.6	0.214	endotherm
UM-1073	Albumin	57.5	0.075	exotherm
	Globulin	82.5	0.176	endotherm
UM-1095	Albumin	52.8	0.059	exotherm
	Globulin	81.7	0.175	endotherm

The thermal denaturation parameters of pea protein fractions are shown in Table 2. The albumin fractions had one major exothermic peak. The peak temperatures were between 52.8 and 60.7 °C and the enthalpies between 0.059 and 0.080 mJ mg^{-1} respectively. In the case of total globulin fractions one major endothermic peak was observed as a result of overlapping transition of two major globulin fractions. The denaturation temperatures were between 80.7 and 82.5 °C, the enthalpies between 0.151 and 0.214 mJ mg^{-1}. WRIGHT and BOULTER (1980) observed at least two overlapping thermal processes during the investigation of cowpea globulins. PEREZ and co-workers (1993) reported that the T_{max} of vicilin and legumin fractions extracted from peas was very near, it was at 365 K in the case of vicilin and at 375 K in the case of legumin. Our results have shown that the denaturation temperature and enthalpy values of albumin and globulin fractions significantly differed from each other. However, the origin of protein fractions did not influence the thermal properties.

3. Conclusions

In the case of all varieties the albumin, total globulin, 7S and 11S protein fractions had different emulsifying properties and protein surface hydrophobicity. In general the total globulin and 7S protein fractions had better emulsifying properties and higher surface hydrophobicity than the albumin and 11S fractions. The measured physicochemical properties of pea varieties were different from each other.

The denaturation temperatures and enthalpy values of albumin and total globulin fractions significantly differed from each other. The albumin fractions had one major exothermic, the globulin fractions one endothermic peak. The origin of the protein fractions did not influence the thermal properties.

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ANTIOXIDANT CONTENT AND COLOUR LEVEL IN DIFFERENT VARIETIES OF RED PEPPER (*CAPSICUM ANNUUM* L.) AFFECTED BY PLANT-LEAF Ti^{4+} SPRAY AND PROCESSING

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Seasoning paprika is, economically, one of the most important vegetable spices and some of the factors that most likely determine its quality may be red colour and antioxidation capacity. This study was carried out to evaluate the varietal influence on the colour levels and on the most effective biological bioantioxidants: ascorbic acid and α-tocopherol. The variations induced during the drying and grinding operations were also determined and the effect of a biological activity inductor (Ti^{4+}-ascorbate) on the composition of these quality parameters in both the fresh and the dried fruit pericarp was analysed. An important decrease of all the parameters studied was observed during processing, especially on the ascorbic acid content (about 75%), red colour (14–58%), and on the level of α-tocopherol (25–30%). Red colour retention in paprika was strongly related to the total bioantioxidant diminution. All the compounds studied were increased when two leaf Ti^{4+} treatments were applied to plants during the crop cycle, but there was some important varietal differences in the intensity of the response.

Keywords: ascorbic acid, α-tocopherol, red pigments, titanium, paprika

Fresh or well-processed foods, mainly fruits, vegetables and cereals, are the best source of antioxidant vitamins. The stability of these compounds has been attributed to a number of factors, including cultivar, plant nutrition status, moisture content, stage of ripeness at harvest, and endogenous antioxidant content (KANNER et al., 1979; LIU & LUH, 1979; DAVIES & HOBSON, 1983; HARALAMPU & KAREL, 1983; KETSA & WONGVEERAKBAN, 1987; BIACS et al., 1989; 1992; BIACS & DAOOD, 1994; DAOOD et al.,1989; 1994; 1996a;b). Peppers are a good source of these natural compounds, mainly α-tocopherol and ascorbic acid, but little is known about the content in paprika which is a very common ingredient of human diet. These vitamins act as protectors of pigments preserving them from chemical and biochemical oxidation, since they act by counteracting the oxidizing effects on lipids by scavenging highly reactive oxygen free

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radicals (HARALAMPU & KAREL, 1983; BORENSTEIN, 1987; DAOOD et al., 1989, 1996b). However, during paprika manufacture, there are some steps which produce a decrease of the bioantioxidants and pigments, which implies an important quality reduction (DAOOD et al., 1996b; CARVAJAL et al., 1997).

On the other hand, titanium is regarded as a beneficial element for the physiological activity of some plants as it usually increases some important metabolic processes (ALCARAZ et al., 1994; CARVAJAL et al., 1994a;b; FRUTOS et al., 1996; LÓPEZ-MORENO et al., 1996; PASTOR et al., 1996, PAIS, 1983). For example it has been shown that Ti increased the stability of chlorophyll and lutein against photo-oxidative damage in tomato (DAOOD et al., 1989) and the development of the colour in pepper fruits (MARTINEZ-SÁNCHEZ et al., 1993; CARVAJAL et al., 1995).

The objective of this work was to study the content of α-tocopherol, ascorbic acid and red colour on six varieties of paprika pepper: 2 Spanish, 2 Hungarian and 2 Bulgarian (main European paprika-producing countries), the effect of processing them and Ti^{4+}-ascorbate application to plant leaves.

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 purchased by 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 crop conditions

The crop was grown in a greenhouse equipped with automatic system to control the 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 two plants were grown. Six experimental blocks (ten containers/block) were randomly disposed in the greenhouse for each cultivar. 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 after flowering (15 ml/plant), and the second one (35 ml/plant), 30 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 control. 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. At harvest (over-ripe red fruit), one fruit sample/block (10 fruits/container, 50 fruits each individual sample) was taken. In the laboratory, peduncles and seeds were removed and samples of the fruit pericarp were washed with 10 g l^{-1} BRIJ 35 solution (non-ionic detergent) and then rinsed three times with deionised 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 50 °C for 2 days. The dried pericarps were ground simulating the paprika manufacture and stored at –20 °C in darkness until analysis.

1.3. Tocopherols determination

Lipid fractions including carotenoids and tocopherols from fresh and dried-ground pericarp were extracted according to a method previously described (BIACS et al., 1989, 1992; DAOOD et al., 1996a) using methanol to remove the water after disintegration of fresh pericarp. Tocopherols were then extracted by a mixture of 1:1 CCl_4-chloroform containing methanol up to 20%. A mixture of 2:1:1 chloroform-acetone-isopropanol was applied to the lipid fractions extracted from ground pericarp samples. The lipid fraction was saponified by reflux with a 4 ml of 30% methanolic KOH for 40 min at the boiling point of methanol in the presence of 0.5 g ascorbic acid. After cooling of the flask and adding 20 ml of salted water, the tocopherol homologues were extracted twice by shaking with 40 ml of petroleum ether in a separating funnel. The ether fractions were collected, washed 3 times with 40 ml of distilled water and dried over anhydrous Na_2SO_4. The solvent was evaporated under vacuum and the residues were re-dissolved in 5–10 ml of HPLC-grade hexane.

Tocopherols were analysed by HPLC using the method described by SPEEK and co-workers (1985). The extract was injected into Licosorb C_{18} 10 mm, 250×4.6 mm and the compound was detected with a fluorescence detector at 320 nm emission and 290 nm excitation. The solvent used was n-hexane:ethanol, 99.5:0.5 with a flow rate of 1.5 ml min^{-1}. For peak identification, the retention times and maximum absorption spectra of tocopherol were compared with those of standard materials (Sigma, USA) which were used also for quantification.

1.4. Ascorbic acid

An aqueous extraction using 4% metaphosphoric acid solution was carried out followed by centrifugation and filtration of the extracts. In the case of fresh pericarp, disintegration of the samples with quartz sand in a mortar before extraction was necessary (DAOOD et al., 1994, 1996a; CARVAJAL et al., 1997).

Ascorbic acid was analysed by HPLC using the method described by DAOOD and co-workers (1994, 1996b). The extract was injected into a Licosorb C_{18} 5 mm, 250×4.6 mm and the compound was detected at 225 nm. The solvent used was 0.01 mol l^{-1} KH_2PO_4-MeOH, 97:3 with a flow rate of 1 ml min^{-1}. For peak identification, the retention times and maximum absorption spectra of ascorbic acid were compared with those of standard materials (Sigma, USA) which were used also for quantification.

1.5. Colour (ASTA, 1968)

Fresh pericarp cut into small pieces and dried-ground pericarp (0.5 g) were extracted with 100 ml of acetone for 24 h in the dark. The supernatant (5 ml) was diluted to 50 ml with acetone and the absorbance was read at 460 nm against an acetone blank. The colour was expressed as ASTA units, according to the equation: [Colour]= 165 A I_f w^{-1}; in which: A = sample absorbance; I_f = deviation factor of the spectrophotometer, which was calculated using a standard 2030 NBS filter that indicates the relationship between the theoretical (A_t) and real (A_R) absorbances at 460 nm; w = sample weight; 165 is the molar extinction coefficient of 1% capsanthin solution in acetone.

1.6. Red/yellow pigments ratio

Pericarp was extracted as for the ASTA determination. The absorbances were measured at 470 nm for red pigments and at 455 nm for the yellow ones (NAVARRO & COSTA, 1993).

1.7. Data analysis

All the measurements were repeated at least three times and for comparing the results from fresh and dry-ground samples, the humidity was taken into account. The data were analysed using the unpaired *t*-test.

2. Results and discussion

2.1. Varietal influence

Results obtained from fresh pericarp are shown in Table 1. Fruit pericarp of control plants from the Spanish varieties (Albar and Negral) contained the highest ascorbic acid levels, whereas the Bulgarian cultivars presented the lowest ones. However, only the Negral variety showed levels significantly different from the rest. In the same way, the two Spanish cultivars presented greater levels of α-tocopherol and a

higher red/yellow pigments ratio than the other fruits studied. Nevertheless, only Albar showed a higher colour level than the rest, followed by the two Bulgarian varieties (Buketen-50 and Gorogled-6).

In a previous work (CARVAJAL et al., 1994a) it was demonstrated that the enhancement of both the biomass production and the biological activity in Ti^{4+}-ascorbate sprayed plants was induced by the cation (Ti^{4+}), whereas the anion (ascorbate) did not have any effect. Furthermore, MOZAFAR and OERTLY (1993) established that the external application of ascorbic acid to roots and leaves of soybean did not increase the ascorbate concentration of the other plant organs. Nevertheless, in this experiment, the application of ascorbic acid to control plants in similar doses than those used in Ti^{4+}-ascorbate treatments, was used to dissipate any possible doubt in this regard, especially because the ascorbic acid is one of the antioxidants the level of which was studied.

Table 1

Bioantioxidants and red colour in fresh pericarp from several Spanish, Hungarian and Bulgarian red pepper (paprika, Capsicum annuum *L.) cultivars*

	Ascorbic acid mg g^{-1} (dm)	α-tocopherol, mg g^{-1} (dm)	Total antioxidants, mg g^{-1} (dm)	Colour, as ASTA units	Red/Yellow ratio
			Control plants		
Albar	9.11 ± 0.31de	2.67 ± 0.05h	11.79 ± 0.36d	806 ± 4h	1.0076 ± 0.0010ef
Negral	10.65 ± 0.70e	2.03 ± 0.05g	12.68 ± 0.75de	401 ± 2a	1.0108 ± 0.0018f
KM-622	8.50 ± 0.31cd	1.35 ± 0.03e	9.85 ± 0.34cd	390 ± 2a	0.9879 ± 0.0018ab
Mihályteleki	8.41 ± 0.19cd	0.66 ± 0.02a	9.07 ± 0.21c	463 ± 7cd	0.9988 ± 0.0017cd
Buketen-50	7.46 ± 19bc	0.87 ± 0.02bc	8.33 ± 0.21bc	478 ± 2de	0.9971 ± 0.0010cd
Gorogled-6	4.17 ± 0.15a	1.02 ± 0.02cd	5.19 ± 0.17a	512 ± 12ef	0.9891 ± 0.0026abd
			Leaf-Ti sprayed plants		
Albar	10.18 ± 0.42de	2.80 ± 0.04h	12.98 ± 0.80de	877 ± 11i	1.0014 ± 0.0014de
Negral	14.90 ± 0.58f	2.56 ± 0.04h	17.39 ± 0.63f	417 ± 2b	1.0094 ± 0.0006f
KM-622	13.92 ± 0.20f	1.54 ± 0.03f	15.46 ± 0.23ef	436 ± 5bc	0.9948 ± 0.0010bcd
Mihályteleki	11.13 ± 0.43e	0.86 ± 0.02b	11.99 ± 0.45d	567 ± 4g	0.9922 ± 0008abc
Buketen-50	10.19 ± 0.28e	1.05 ± 0.04cd	11.24 ± 0.32d	487 ± 2e	0.9963 ± 0.0007cd
Gorogled-6	6.73 ± 0.11b	1.06 ± 0.02d	7.79 ± 0.13b	555 ± 9fg	0.9829 ± 0.0022a

Data are the average value from three individual samples and are expressed as the mean ±SE (standard error). Means followed by the same letter are not significantly different at p:0.05 level within the column (unpaired *t*-test).

All cultivars increased their fruit pericarp antioxidant concentrations when the plants were leaf-sprayed with Ti^{4+} during the crop cycle (Table 1). This effect could be due to the previously demonstrated biological activation induced by the trace element in these plants (MARTÍNEZ-SÁNCHEZ et al., 1993; CARVAJAL et al., 1994a; b; 1995; CARVAJAL & ALCARAZ, 1995; ALCARAZ et al., 1994). It is interesting to point out that the ascorbic acid reached a higher level than α-tocopherol, being the average increase 40% for ascorbic acid and 15% for α-tocopherol. This higher effect of Ti^{4+} on the synthesis of ascorbic acid than on α-tocopherol was probably due to their different distribution in the fruit. So, ascorbic acid mainly accumulates in the pericarp and α-tocopherol is generally located in seeds. Bearing in mind that seeds were removed from the pericarp, the high effect of titanium on the concentration of the ascorbic acid seems to be a logical result. In addition, it is evident that there is an important varietal influence on the increase of both bioantioxidants. So, the Hungarian KM-622 and the Bulgarian Gorogled-6 were the varieties with the highest ascorbic acid enhancements and Negral and Buketen-50 showed the highest α-tocopherol increases.

In the same way for the antioxidant concentrations, the fruits from Ti^{4+} leaf-sprayed plants presented an important enhancement of colour. The most positive effect was shown by the Mihálytelеki variety (18%) and in all the other cases the effect of titanium on pericarp colour could be quantified between 2% (Buketen-50) and 10.5% (KM-622).

2.2. Effect of drying

CARBONELL and co-workers (1986) reported that drying conditions can substantially influence the quality attributes of paprika. However, no attention has been paid to the antioxidant vitamin content as affected by the drying operation. This aspect has been considered recently by DAOOD and co-workers (1996a) and by CARVAJAL and co-workers (1997), though the data presented are not always coincident, probably due to assay with different paprika varieties.

It is well known that spice paprika is usually ground to a powder of about 0.5 mm particle. This process increases the surface area and thus allows higher lipid oxidation. The detrimental effect of lipid oxidation on the whole biological system can be eliminated or controlled if the natural antioxidants are available to perform their role (BIACS et al., 1992; DAOOD et al., 1996a). The level of the antioxidants on ground pericarp after both drying and grinding operations are shown in Table 2. The concentrations of ascorbic acid and α-tocopherol were higher in the two Spanish varieties. Equivalent results were observed in the red/yellow rate. However, Mihálytelеki showed the highest colour levels. A similar effect than that observed in fresh pericarp was appreciated in Ti-treated samples when compared to control. Higher levels of antioxidants and colour were obtained for all the varieties.

Table 2

Bioantioxidants and red colour in dry pericarp from several Spanish, Hungarian and Bulgarian red pepper (paprika, Capsicum annuum *L.) cultivars*

	Ascorbic acid mg g^{-1} (dm)	α-tocopherol, mg g^{-1} (dm)	Total antioxidants, mg g^{-1} (dm)	Colour, as ASTA units	Red/Yellow ratio
			Control plants		
Albar	2.57 ± 0.05b	1.91 ± 0.03fg	4.48 ± 0.08f	335 ± 6bcd	1.0194 ± 0.0004d
Negral	2.48 ± 0.06b	1.45 ± 0.02e	3.93 ± 0.08de	318 ± 4abc	1.0240 ± 0.0005e
KM-622	1.99 ± 0.04a	0.96 ± 0.04d	2.95 ± 0.08ab	307 ± 3ab	1.0007 ± 0.0004bc
Mihályteleki	2.00 ± 0.07a	0.47 ± 0.01a	2.47 ± 0.08a	398 ± 4f	1.0039 ± 0.0007c
Buketen-50	2.06 ± 0.05a	0.62 ± 0.01b	2.68 ± 0.06a	297 ± 4a	1.0015 ± 0.0010bc
Gorogled-6	1.74 ± 0.08a	0.72 ± 0.02c	2.46 ± 0.10a	302 ± 5ab	0.9965 ± 0.0009ab
			Leaf-Ti sprayed plants		
Albar	2.83 ± 0.06b	2.00 ± 0.03g	4.83 ± 0.10f	368 ± 3e	1.170 ± 0.0008d
Negral	3.78 ± 0.06d	1.82 ± 0.03f	5.61 ± 0.09g	336 ± 4cd	1.0193 ± 0.0004d
KM-622	3.33 ± 0.08c	1.10 ± 0.02d	4.43 ± 0.10ef	355 ± 2de	0.9991 ± 0.0011abc
Mihályteleki	2.59 ± 0.08b	0.61 ± 0.01b	3.20 ± 0.09bc	500 ± 4g	1.0033 ± 0.0013bc
Buketen-50	2.76 ± 0.06b	0.76 ± 0.01c	3.52 ± 0.08cd	317 ± 4abc	1.0011 ± 0.0012bc
Gorogled-6	1.93 ± 0.07a	0.76 ± 0.02c	2.69 ± 0.08ab	340 ± 5cd	0.9953 ± 0.0008a

Data are the average value from three individual samples and are expressed as the mean ±SE (standard error). Means followed by the same letter are not significantly different at p:0.05 level within the column (unpaired t-test).

A dramatic decrease was observed in the concentration of ascorbic acid in paprika when it was compared with that found in fresh pericarp (more than 70%) as well as in the levels of α-tocopherol (about 30%). According to some data offered by DAOOD and co-workers (1996a) and CARVAJAL and co-workers (1997), the highest loss shown by the ascorbic acid concentration could be caused either by the sensibility of vitamin C against heat (55 °C during 48 h under our experimental conditions, plus the heating produced by grinding), or by the capacity of vitamin C to act as an antioxidant. If α-tocopherol is considered less thermolabile than ascorbic acid, a similar loss percentage attributed to the antioxidant (about 25–30% in both cases) could be expected. The rest of the loss shown by ascorbic acid could be due to heat.

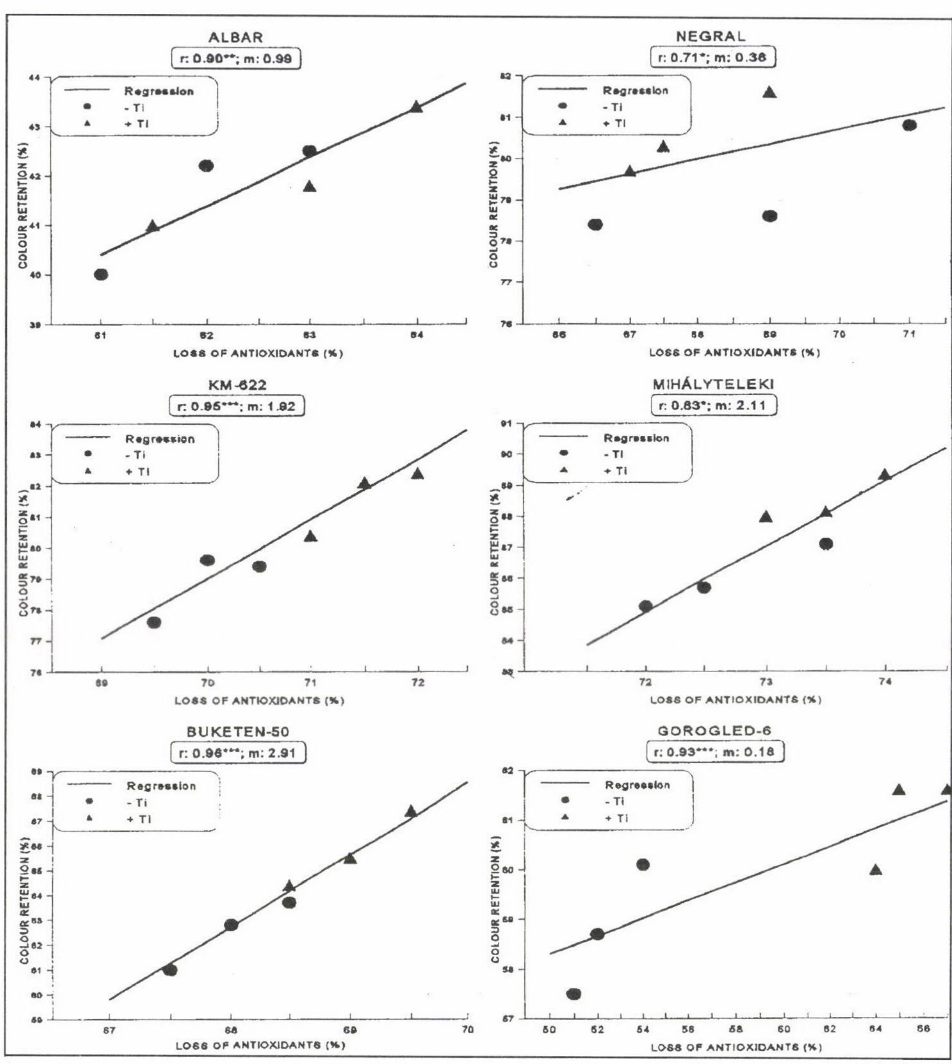

Fig. 1. Correlations between the percentages of loss of antioxidants (X) and colour retention (Y) in paprika from several Bulgarian, Hungarian and Spanish varieties

On the other hand, Fig. 1 shows the relationship between colour retention of dried pericarp and the reduction intensity of all the antioxidants during the drying and grinding process. It is interesting to remark that a significant level was not obtained when colour retention was related to each particular antioxidant. However, the decrease of all the antioxidants was always significantly related to colour retention. Therefore, the bioantioxidants could be considered as the first barrier against the colour decomposition, as was previously suggested by DAOOD and co-workers (1996a). There is a varietal incidence on the slope of the regression lines, but this effect seems to be more dependent on the cultivars than on the content of both parameters in the fresh pericarp studied.

3. Conclusion

Commercial paprika is usually packed and stored at room temperature and, in some cases, not well protected against light effects. Under these conditions the loss of antioxidants can reach values higher than 80%. Therefore, it is interesting to highlight that paprika presents high values both of antioxidants and red colour before packaging. Bearing in mind these considerations, paprika with the highest colour intensity would be from Mihályteleki and Albar varieties, and the most stable from Albar and Negral. Furthermore, when plants were treated with a biological activator titanium Mihályteleki and Albar showed also the highest colour intensity and Negral, Albar and Km-622 were the samples with the highest antioxidants level.

*

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Short communications

PRETREATMENT EFFECT ON THE QUALITY OF WHITE AND RED WINES USING CROSS-FLOW CERAMIC MEMBRANE FILTRATION

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The aim of our study was to investigate and compare the influence of different pretreatment methods and membrane separation methods on the filtration rate and the quality of Hungarian wines.

Four Hungarian wines, two red and two white ones were investigated. The applied pretreatment methods were as follows: a.) once racked, b.) racked + clarified, c.) racked + clarified + filtered by diatomaceous earth.

ITI-70 (SCT, France) ceramic micro- and ultrafiltration membranes were used in a laboratory scale cross-flow membrane equipment. The influence of recycle flow rate on the permeate flux and the quality of wines was measured under constant pressure and temperature. The applicability of ceramic membranes in the filtration of Hungarian wines was studied.

On the bases of measurements it was established, that the ITI-70 ceramic membranes can be used for cross-flow filtration of white and red wines, and that the micro- and ultrafiltration can replace the pretreatment with chemicals.

Keywords: ceramic membrane, pretreatment effect, wine filtration

Sterilizing by microfiltration and ultrafiltration has become an accepted practice within the wine making industry. Several wineries are using crossflow membrane filtration to clarify, stabilize and enhance their wines. The micro- and ultrafiltration are being used to remove thermally unstable proteins, tannins, colour components, yeast, carbohydrate gums and oxidized phenolics (SCHMITT et al., 1987; WUCHERPHENNIG & DIETRICH, 1989; MILTENBERGER et al., 1990; DIETRICH et al., 1992; RIPPERGER, 1993).

It is a well known fact, that the quality and price of wines are influenced by the pretreatment, as well (SZÖVÉNYI & KÁLLAY, 1991; DIETRICH et al., 1992).

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The ceramic membranes are nowadays widely used in fermentation technologies for separation of the product (NAKANO et al., 1993 and 1996; ISHIZAKI et al., 1993), for membrane bio-reactor (MORGANDO et al., 1996), milk separation (SINGH et al., 1995; SANCHEZ-DIAZ-LOZANO et al., 1995; POULIOT et al., 1996; SAVELLO et al., 1997) and in the fruit juice industry (PADILLA-ZAKOUR et al., 1993; DAY & SHEU, 1996; JIRARATANANON et al., 1997), while in the wine making industry the ceramic membrane filters are not yet widespread (MILTENBERGER et al., 1990; DIETRICH et al., 1992).

Different Hungarian wines of the same vintage (Table 1) were investigated in a laboratory scale cross-flow membrane equipment using ceramic micro- and ultrafiltration membranes.

The temperature and pressure were kept constant: t=20 °C, p=3 bar,

The varied parameters (Table 2) influence the permeate flux, the composition and the quality of wine. The permeate flux and the composition of the filtered wine (concentration of colloids, sugar, etc.) were measured and analysed. The quality of the filtered product were checked by sensory analysis, as well.

Table 1

Investigated Hungarian wines

Name	Colour	Region of origin	Vintage	Type
Királyleányka	white	Villány	1995	quality
Kékoportó	red	Villány	1995	quality
Veltelini	white	Jászberény	1995	light
Kékfrankos	red	Egerszalók	1995	light

Table 2

Varied parameters

Pretreatment	a) once racked (R) b) racked + clarified (RC) c) racked + clarified + filtered by diatomaceous earth (RCF)
Membrane pore size (SCT-70)	a) 50 nm/0.05 μm/(ultrafiltration) b) 500 nm/0.5 μm/(microfiltration)
Recycle flowrate	a) 100 l h^{-1} b) 400 l h^{-1}

1. Materials and methods

Experiments were carried out with single tube ceramic modules, type ITI–70, (SCT, France) 250 mm long with diameter of 7/10 mm and filtration area of 55 cm^2. Two different modules were used: the ultrafiltration membrane was made of zirconium oxide with mean pore size of 50 nm (0.05 μm), the microfiltration membrane was made of aluminum oxide with mean pore size of 500 nm (0.5 μm). The two membrane modules were used alternately.

The experimental apparatus is shown in Fig. 1. The feed solution (white or red wine) was set up into the 3 l feed tank. The recycle flow rate was circulated with a rotary-vane pump using a by-pass valve, the adjusted values were 100 l h^{-1} and 400 l h^{-1}. At low recycle rate the hydrodinamics in the membrane module was transient (Re=5040), while at high recycle rate the flow was turbulent (Re=20160). The transmembrane pressure i.e. the feed pressure was kept constant (300 kPa) with a regulation valve. The permeate flowrate was measured by measuring cylinders and stop-watch. The experiments were carried out at a room temperature of 20 °C, which was adjusted by the cooling of wine in the feed tank. The experimental runs were finished when the collected permeate volume reached 500 ml. The membrane module was cleaned after each run and the cleaning was repeated until the standard waterflux was reached.

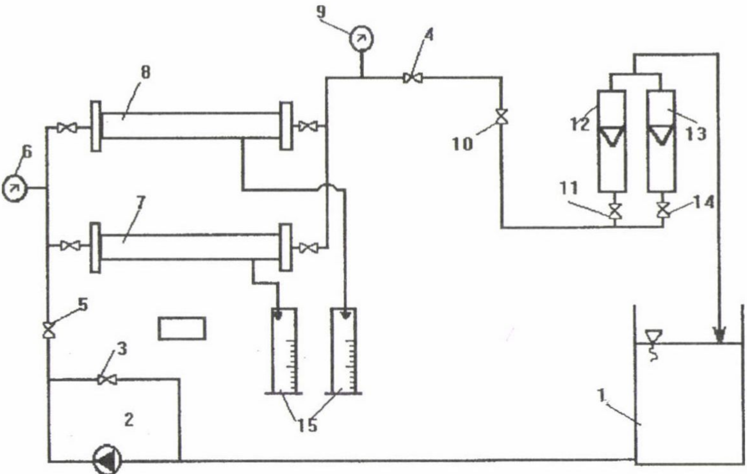

Fig. 1. The experimental apparatus. 1: feed tank; 2: rotary-vane pump; 3: by-pass valve; 4: pressure regulation valve; 5, 10: discharge valves; 6, 9: manometers; 7, 8: membrane modules; 11, 14: ball valves; 12, 13: flow meters; 15: permeate measuring cylinders

Each type of wine was pretreated (R, RC, RCF see Table 2), then filtered by micro- or ultrafilter using 2 recycle flowrates. It means that 48 filtration curves were measured. To control the reproducibility 6 measurement curves were repeated and a good agreement was established.

The permeate samples and the control wines (before membrane filtration) were analysed according to the HUNGARIAN STANDARD (1985). The following parameters were determined: concentrations of alcohol, sugar, sugar-free extract, total acid, tartaric acid, polyphenols, proteins, colloids, pH and colour intensity of the wine.

The characteristic parameters were measured by SPEKOL 11 (Karl Zeiss, Jena) type spectro-photometer. In the case of tartaric acid, ammonium-meta-vanadate was used as a reagent. The polyphenols were expressed in gallus acid using folin-ciocaltens phenol. The soluble proteins were determined by the Breadford method. The colour intensity was measured using 420 nm wavelength in the case of white wine and 420 nm and 520 nm in the case of red wine. The colloid content was precipitated after the well known alcoholic reaction.

2. Results and discussion

The colloid size changes in a large interval (0.02–0.8 μm), so the origin and type of wine influence the colloid concentration very much. The measured colloid concentration of permeate can represent the stabilization effect of membrane filtration.

In Fig. 2 the results of permeate analysis of Kékoportó (red, quality wine) are shown as an example. The more complex the pretreatment, the less colloid content is achieved after membrane filtration. Comparing the colloid concentration with that of the controls, the RCF has the lowest value. The pore size and recycle rate have less influence on the colloid content than expected, although, using the smaller pore size membrane less colloid content was measured.

To compare the influence of pretreatment on polyphenol content of permeate, the concentration in the Kékfrankos (red, light wine) is shown in Fig. 3. The polyphenol concentration decreased with clarification and filtration (by diatomaceous earth), as well. The membrane filtration continued to decrease the polyphenol content to a small extent.

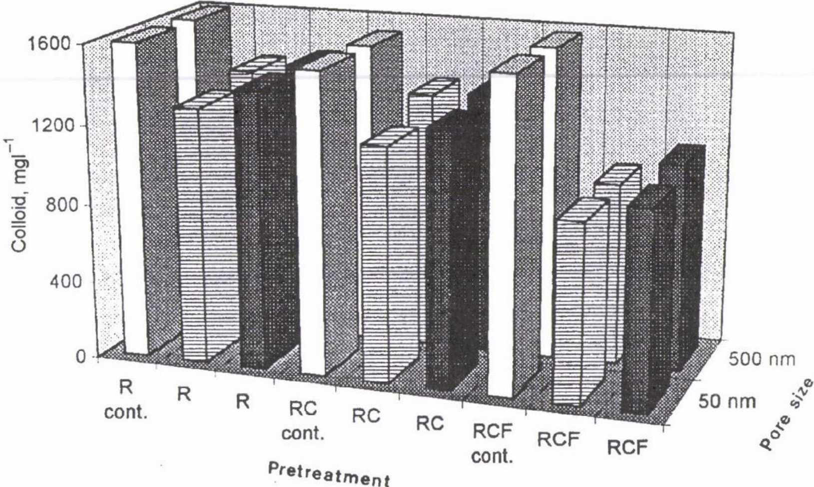

Fig. 2. Effect of pretreatment and membrane pore size on colloid concentration. Kékoportó, red quality wine. R: racked; RC: racked + clarified; RCF: racked + clarified + filtered by diatomaceous earth. □: Control; ▤: recycle rate 100 l h^{-1}; ■: recycle rate 400 l h^{-1}

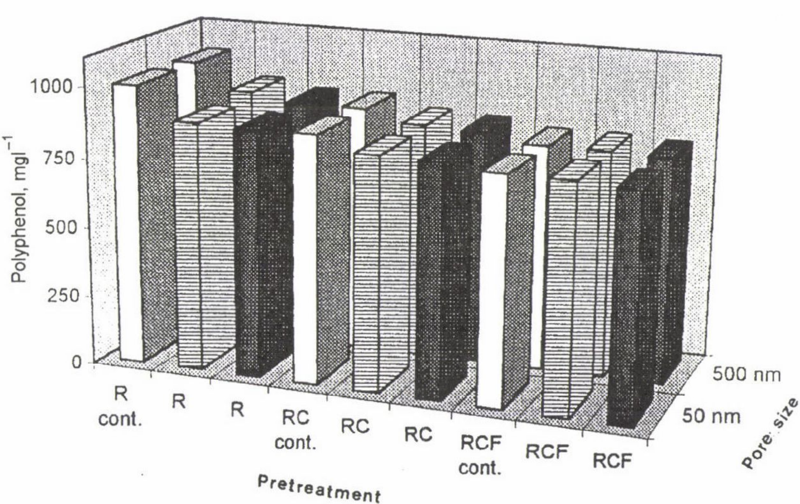

Fig. 3. Effect of pretreatment and membrane pore size on polyphenol concentration. Kékfrankos, red light wine. R: racked; RC: racked + clarified; RCF: racked + clarified + filtered by diatomaceous earth. □: Control; ▤: recycle rate 100 l h^{-1}; ■: recycle rate 400 l h^{-1}

The protein content is presented in Fig. 4 on the example of Veltelini (white, light wine). The clarification and filtration (by diatomaceous earth) decreased the protein content of the wine by some percent, while the membrane filtration removed the 30–70% of the protein. One can observe a significant effect of pore size, the smaller membrane pore size decreased, in some cases halved the protein content.

Typical permeate flux curves are shown in function of the permeate volume in Figs 5 and 6. As the gel layer begins to be formed on the membrane, the permeate flux decreases and reaches its steady state value. Similar curves were measured by GUPTA and ENFERT (1996), the permeate flux strongly decreased in 450 nm pore size ceramic membrane tube. The more pretreated the wine, the higher is the permeate flux. In Fig. 5 pretreated wines are filtered by microfiltration, while in Fig. 6 ultrafiltration is used. As can be seen the flux change is higher at smaller pore size.

In the industrial practice the flux reduction is eliminated by periodical back flash. GUPTA and ENFERT (1996) reduced the gel layer by using turbulence promotor in the tube. In our measurements the increase of the Re number strongly decreased the gel resistance, but higher Re number in not advisable to take care of the wine quality.

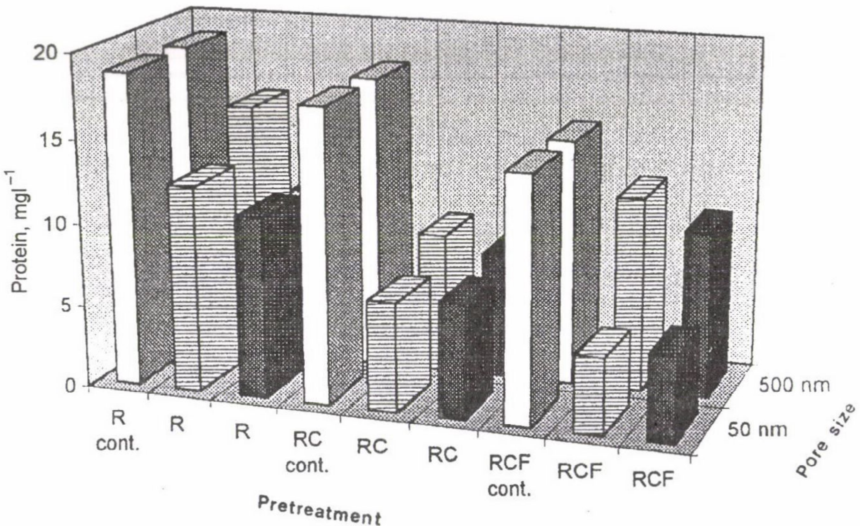

Fig. 4. Effect of pretreatment and membrane pore size on protein concentration. Veltelini, white light wine. R: racked; RC: racked + clarified; RCF: racked + clarified + filtered by diatomaceous earth. ☐: Control; ▤: recycle rate 100 l h^{-1}; ■: recycle rate 400 l h^{-1}

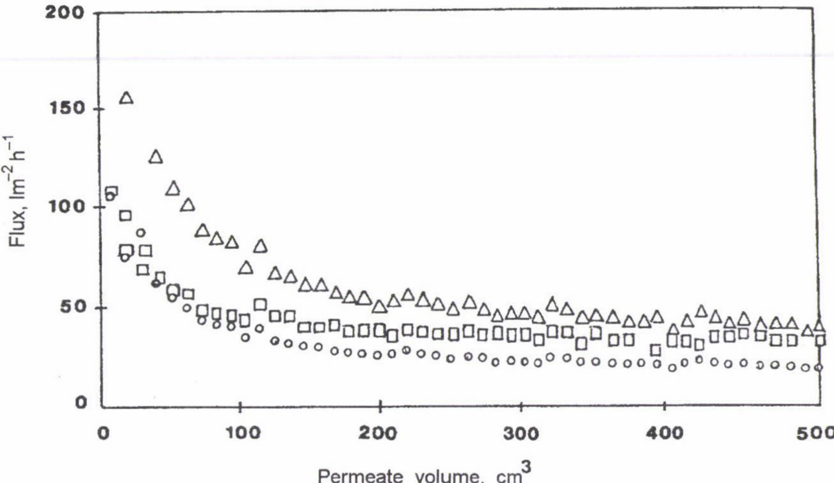

Fig. 5. Influence of pretreatment on permeate flux. Veltelini, white light wine. Microfiltration, pore size 500 nm. Recycle rate 400 $l h^{-1}$. O: Racked; □: racked + clarified; Δ: racked + clarified + filtered by diatomaceus earth

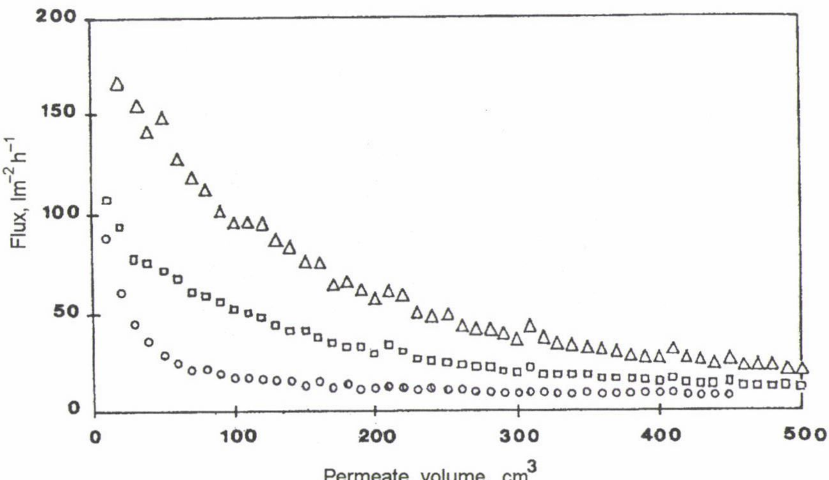

Fig. 6. Influence of pretreatment on permeate flux. Veltelini, white light wine. Ultrafiltration, pore size 50 nm. Recycle rate 100 $l h^{-1}$. O: Racked; □: racked + clarified; Δ: racked + clarified + filtered by diatomaceous earth

As an example, the average flux of Kékoportó (red quality wine) is plotted against pretreatment and pore size of membrane (Fig. 7). The average permeate flux was calculated as an arithmetic mean of the flux values of a flux curve. After the pretreatment steps the permeate flux gradually increased. In case of higher recycle rate in the membrane tube, the gel layer is thinner, the flow resistance is lower, and this fact makes a higher permeate flux possible.

The influence of the pore size was less than expected. Comparing Figs 2–7 it can be concluded, that the pore size in the range of 50 nm (ultrafiltration) and 500 nm (microfiltration) slightly influenced the quality of wines (except for the protein content, Fig. 4).

The pretreatment – mostly the clarification – decreases the colour of the red wine (Fig. 8).

All the 70 wine samples (48 membrane filtered + 6 repetitions + 16 control wines) were checked by sensory analysis with the help of wine experts. The qualification of the samples were done on the basis of colour, taste and smell of wine. It was established that the membrane filtration, practically, did not reduce the organoleptic values of the samples, while the filtration stabilized the wines.

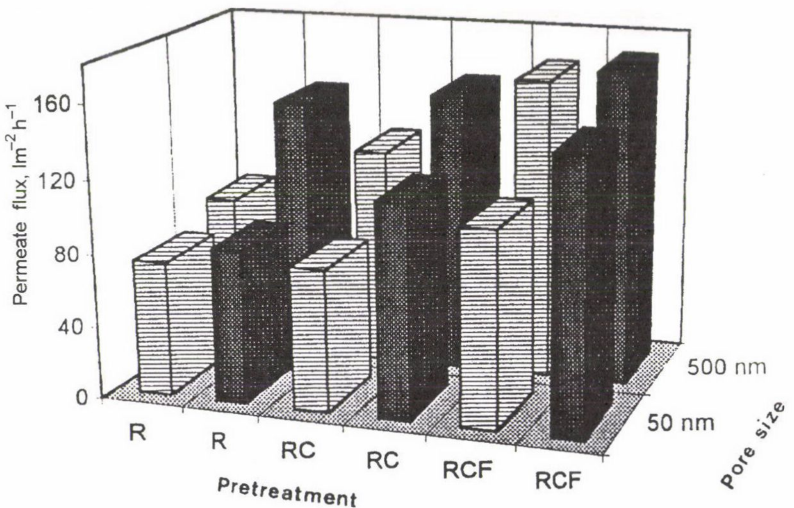

Fig. 7. Effect of pretreatment and membrane pore size on avarage flux. Kékoportó, red quality wine; R: racked; RC: racked + clarified; RCF: racked + clarified + filtered by diatomaceous earth. ▤: Recycle rate 100 l h^{-1}; ▩: recycle rate 400 l h^{-1}

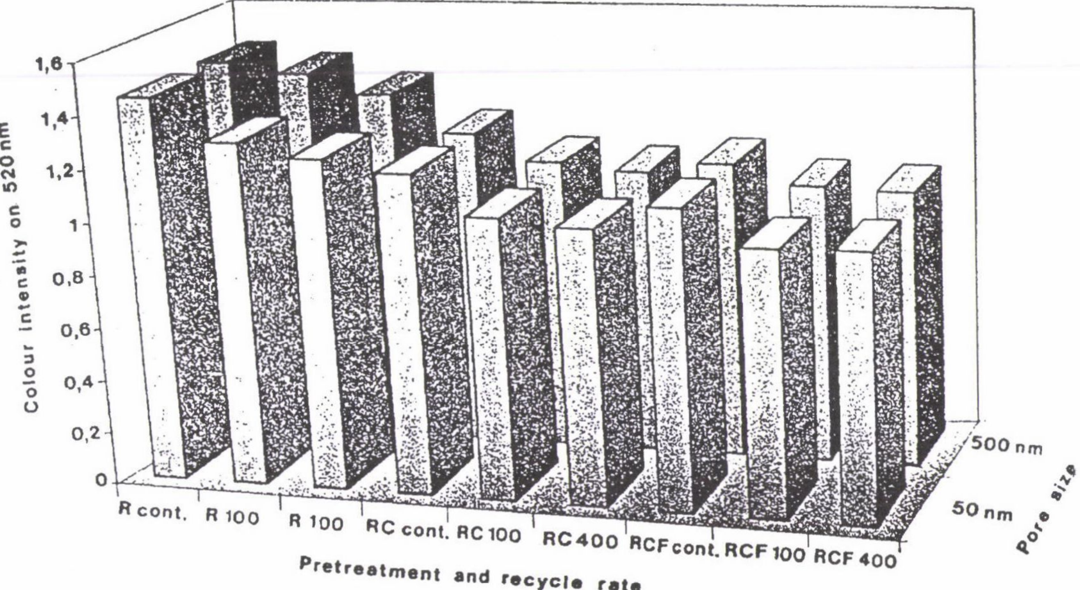

Fig. 8. Effect of pretreatment, recycle rate and membrane pore size on colour intensity of red wine. Kékoportó, red quality wine. R: racked; RC: racked + clarified: RCF: racked + clarified + filtered by diatomaceous earth, control wine (not filtered by membrane), 100: recycle rate 100 l h^{-1}; 400: recycle rate 400 l h^{-1}

3. Conclusions

As determined from experimental results, the pretreatments have positive effect on the performance of the filtration.

The ceramic membranes are suitable for cross-flow filtration of wine. Using 50–500 nm pore size membranes the filtration, practically, did not cause negative organoleptic changes in wines.

The measured colloid concentration, as an index number, can represent the stabilization effect of the membrane filtration.

On the basis of the obtained experimental data it can be concluded, that the crossflow micro- and ultrafiltration, in some cases, can replace the pretreatment with chemicals (clarifiers, diatomaceous earth, etc.), decrease waste disposal and save energy in the wineries.

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EQUIVALENCY LAW IN THE METAL REQUIREMENT OF THE LIVING ORGANISMS?

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From information referring to metal requirements of the human organism as well as metal contents of human and cow's milk and cereal grains it was concluded that an Equivalency Law exists in the metal balance of the living organisms. According to this law the alkali metal requirement (mainly potassium and sodium) is chemically equivalent to that of polyvalent metals (namely calcium, magnesium, zinc, iron, etc.). Theoretical considerations are given for proving the existence of the Equivalency Law.

Keywords: equivalency law, living organisms, metal requirements

With the development of science, more and more knowledge has been accumulated on nutrient requirements ensuring optimal function of human, animal (and plant) organisms. In addition to protein, carbohydrate, vitamin, etc., the requirements apply to mineral substances, macro-, meso- and microelements, as well. The values recommended on the basis of the most recent information concerning nutrient requirements of the human organism, are collected in the Recommended Dietary Allowances (RDA, 1989) by the Food and Nutrition Board of the National Research Council, the National Academy of Sciences in the US, and is republished from time to time. Surveying RDA data, the following regularity can be observed: the daily alkali metal requirement (ΣM^{+}, mainly potassium and sodium) of the human organism is chemically equivalent to the sum of the polyvalent metal requirement (ΣM^{z+}, namely calcium, magnesium, zinc, iron, etc.). Taking into account metal contents of samples of biological origin (milk and cereal grains) as well as simple theoretical considerations, it can be concluded that this regularity exists in the metal balance of all the living organisms on the Earth.

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1. Discussion

The regularity mentioned may be termed as Equivalency Law, which can be described by the following formula: $\Sigma M^{+}=\Sigma M^{z+}$. Respective RDA data referring to adult men aged 19–24 years may represent the relevancy of the Equivalency Law (see Table 1).

The data in Table 1 show there is hardly any difference between alkali metal requirement, amounting to 92.085 milliequivalent/day (meq day^{-1}), and polyvalent metal requirement, which is 89.853 meq day^{-1}. According to RDA data, the difference is greater, for example, in the case of children aged 7–9 years (Ca: 800 mg, Mg: 170 mg, Fe: 10 mg, Zn: 10 mg, Mn: 2–3 mg, Cu: 1–2 mg, Na: 400 mg, K: 1600 mg); on the basis of which alkali metal requirement is 58.322 meq day^{-1} and the sum of polyvalent metal requirement is 54.849 meq day^{-1}. This difference, however, can not question the existence of the Equivalency Law, since RDA tables give data estimated as optimal values.

It is advisable to express the deviation from the Equivalency Law by calculating relative deviations, in percent, for example, by considering half the equivalents of the total amount of metals as standard. Thus, the relative deviation is 3.07% (A+) for children. ("A+" denotes alkali metal excess.) [E.g. (58.322+54.849)/2=56.586, the deviation from this is 58.322–56.586=1.736, which amounts to 1.736·100/56.586=3.07%]. Relative deviation is 1.23% (A+) for adult men.

Table 1

Metal requirement of adult men aged 19–24 years

Metal	mg day^{-1}	milliequivalent day^{-1}
Ca	1200	59.883
Mg	350	28.801
*Fe	10	0.448
Zn	15	0.459
*Mn	3.5 (2–5)	0.191
Cu	2.25 (1.5–3)	0.071
Na	500	21.749
K	2750 (2000–3500)	70.336
Alkali metals	ΣM^{+}	92.085
Polyvalent metals	ΣM^{z+}	89.853

* The equivalents are calculated for iron with 2.5+ charge, and for manganese with 3+ charge

The Equivalency Law can also be expressed by the formula: $\Sigma M^{+}/\Sigma M^{z+}=1$. In this way the deviation from the law ("quotient of equivalency") can be calculated by: $EQ=\Sigma M^{+}/\Sigma M^{z+}$. The value of EQ is greater than one for alkali metal surplus and is lower for the opposite case.

There are data referring to the Equivalency Law in the vegetable kingdom, as well. Results of my measurements concerning metal content of natural cereal flakes and cereal flake flours produced in 1994 in Hungary are given in Table 2. (After digestion with nitric acid and hydrogen peroxide, the metal contents were measured by a plasma emission spectrometer, Thermo Jarrell Ash Corp., AtomScan 25 type ICP.)

Table 2

Metal content of cereal flakes and flake flours [mg kg^{-1}]

Metal	Barley		Wheat		Rye		Oat	
	flake	f. flour	flake	f. flour	flake	f. flour	flake	f. flour
Ca	256.7	244.8	297.2	325.3	329.4	420.0	663.1	444.9
Cu	4.179	3.848	3.243	3.697	2.472	3.462	4.236	3.431
Fe	30.03	28.93	35.52	40.42	18.22	28.09	36.71	29.90
K	2890	3257	3429	3525	3942	5603	3632	2524
Mg	860.6	875.1	1088	1040	1027	1478	1357	921.0
Mn	10.13	10.06	34.20	35.93	18.62	27.96	46.26	39.42
Na	52.85	72.35	13.23	20.40	13.69	12.55	20.01	15.94
Zn	22.69	21.47	19.14	22.49	17.35	26.66	24.19	22.36

Table 3

Cereal flakes and flake flours and the Equivalency Law

	ΣM^{+} [meq kg^{-1}]	ΣM^{z+} [meq kg^{-1}]	$(\Sigma+\Sigma)/2$ [meq kg^{-1}]	Δ [%]	EQ
Barley flakes	76.216	86.349	81.283	6.23 (A–)	0.8827
Barley flake flour	86.451	86.848	86.650	0.229 (A–)	0.9954
Wheat flakes	88.278	108.50	98.389	10.28 (A–)	0.8136
Wheat flake flour	91.045	105.95	98.499	7.57 (A–)	0.8593
Rye flakes	101.42	103.39	102.41	0.96 (A–)	0.9810
Rye flake flour	143.85	146.29	145.07	0.840 (A–)	0.9833
Oat flakes	93.763	149.80	121.78	23.01 (A–)	0.6259
Oat flake flour	65.249	102.27	83.760	22.10 (A–)	0.6380

On the basis of data in Table 2, Table 3 shows alkali metal contents (ΣM^+), polyvalent metal contents (ΣM^{z+}), half of the total metal contents ((Σ+Σ)/2) and relative deviations from the Equivalency Law (Δ[%] and EQ respectively). Iron and manganese contents are calculated as above, "(A–)" denotes the lack of alkali metal.

Table 4

Metal content of human milk during the first 4 months of lactation [meq kg^{-1}]

Metal	Lactation (months)				Overall
	1	2	3	4	
Ca	14.8211	15.0207	14.5716	14.2223	14.6714
Mg	2.2218	2.4686	2.6332	2.7981	2.4686
Fe	0.0108	0.0091	0.0082	0.0072	0.0088
Zn	0.0704	0.0459	0.0336	0.0306	0.0459
Cu	0.0114	0.0100	0.0088	0.0084	0.0097
Na	5.8721	4.6107	4.6542	4.3497	4.8717
K	11.9188	11.5351	11.1770	10.6399	11.3305
ΣM^+	17.7909	16.1458	15.8312	14.9897	16.2022
ΣM^{z+}	17.1355	17.5543	17.2554	17.0666	17.2044
(Σ+Σ)/2	17.4632	16.8501	16.5433	16.0281	16.7033
Δ [%]	1.877 (A+)	4.180 (A–)	4.304 (A–)	6.479 (A–)	3.00 (A–)
EQ	1.0383	0.9198	0.9175	0.8783	0.9418

Table 5

Metal content of human and cow's milk [meq l^{-1}]

Metal	Human milk	Cow's milk
Ca	17.4659	59.8832
Mg	2.4686	9.8745
Fe	0.0313	0.0224
Zn	0.0092	0.0107
Cu	0.0126	0.0063
Na	6.5246	21.7486
K	15.3461	38.3651
ΣM^+	21.8706	60.1137
ΣM^{z+}	19.9877	69.7971
(Σ+Σ)/2	20.9292	64.9554
Δ [%]	4.498 (A+)	7.454 (A–)
EQ	1.0942	0.8613

The data for barley, wheat and rye seem to "conform" to the Equivalency Law surprisingly well, the highest deviation (for oat) is "only" 23%. At the same time, e.g. the ratios of sodium-potassium and calcium-magnesium in each sample considerably deviate from those considered to be optimal, according to RDA. Quantitative ratios of the various metals do not necessarily correspond to human requirements however, since the nutrients are stored in the seeds of cereals in order to satisfy the needs of their "descendants", the seedlings in their first phase of life.

The composition of milk also points to a genetically programmed supply of food requirements for descendants i.e. new-born children. BUTTE and co-workers (1987) gave data on the composition of human milk (and intake of human milk on 45 healthy mother-infant pairs) and to its changes during the first four months of lactation. Table 4 shows these data recalculated in accordance with the above.

PORTER (1978) published data of the composition of human and cow's milk. Table 5 gives values obtained by recalculating these data.

The data mentioned before unanimously verify the existence of the Equivalency Law, but are not sufficient to answer all questions. It is not evident from the data of BUTTE and co-workers (1987), whether there is any significant change in the metal composition of human milk from the range of alkali excess to the steadily increasing deficiency. This question could be answered either by re-elaboration of the original, individual data, taking into account the Equivalency Law or by new measurements. An answer to this question may be of particular significance for producing baby food of optimal composition. In 1989 a dairy product for babies (namely Drikkeklar Allomin, Beauvais Industri A/S, Tåstrup, Sweden) contained the following metals [mg l^{-1}]: Na: 160, K: 585, Ca: 470, Mg: 50, Fe: 7, Zn: 4, Cu: 0.4. Relative deviation from the Equivalency Law is calculated 12.2% (A–). Investigations related to the Equivalency Law may lead to significant practical results e.g. in food science, stock-raising, plant cultivation and in biotechnology as well. It would be feasible to carry out research related to the Equivalency Law for determining concentration of further metals (e.g. Li, Co, Cr, etc., and Al, Cd, Pb, etc.), micro or toxic metals, present in low concentration. The metals mentioned could influence, however, the sum of equivalents only at a small extent. The equivalents of metals with varying valences, e.g. Fe, Mn, etc., should be calculated by taking into consideration a uniform degree of oxidation; e.g. the average of the most common degrees of oxidation in biological systems. Thus, iron and cobalt could be calculated with 2.5+ and manganese with 3+ charge.

2. Theoretical conclusions

A long list of literature data could be enumerated here to verify the existence of the Equivalency Law, but it can be proved theoretically as well. As it is well known, in the aqueous electrolytes of living organisms metals change ligands as positive ions. As strict chemical stoichiometrical rule of these cation exchange processes is that they proceed with the exchange of an equivalent amount of positively charged counter-ions. The various polyvalent metals can hardly act as counter-ions of each other, since they are present as free ions in the electrolytes of organisms only in low concentration and form far more stable compounds (complexes) with various proteins and simple acids. Apart from a few exceptions, the different polyvalent metals cannot act as counter-ions of each other because they cannot substitute each other at the specific bonding sites of the individual protein molecules (enzymes, transport proteins, etc.). Ammonium ions cannot act as counter-ions of polyvalent metals either, since they are present in the electrolytes of living organisms only in rather low concentration. Hydrogen ions (or more precisely, H_3O^+ ions) can also be counter-ions of polyvalent metals only in a very low ratio, as the pH value in living organisms is nearly neutral, thus, the concentration of hydrogen ions is very low; it is in the range of 10^{-7} mol l^{-1}. Contrarily, the total concentration of alkali metals (mainly potassium and sodium ions) is of 0.1 mol l^{-1} order of magnitude. Thus, in ligand exchange processes of polyvalent metal ions, primarily alkali metal ions can act as counter-ions. Life on Earth is uniformly based on proteins. Chemical and physical properties of proteins and other macromolecules allow vital processes in the electrolytes of organisms only at a very low concentration range. Economical utilization of energy and food is a common characteristic of living creatures. It does not allow any surplus of metal ions. Finally, it is commonplace that every living organisms are related genetically. Consequently, it may be supposed that the Equivalency Law, referring to the alkali and polyvalent metals, must exist in the metal metabolism processes of various living creatures on Earth.

It should be mentioned, that the metal content of oceanic and sea-water is significantly shifted from the Equivalency Law towards alkali metal excess ($\Delta \approx 57\%$). During the evolution, after the appearance of living creatures with calcareous skeleton, limestone layers constituting today the enormous calcareous mountain ranges of continents have accumulated in the depths of oceans and seas. Thus, it may be assumed that many million years ago the water of primordial oceans and seas contained more calcium and more magnesium, iron, zinc, etc. than recently. This greatly depended on the carbon dioxide content of the primordial atmosphere. In the course of time, the amount of calcium, magnesium, etc. decreased in water. At the same time, due to the higher solubility of alkali metal compounds, the amount of these did not change substantially. Consequently, millions of years ago the metal content of the water of

primordial oceans and seas must have been closer to the EQ=1 value, as compared to present-day metal content, which may have played an important role in the course of biological evolution.

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Book review

Bioavailability and analysis of vitamins in foods

G. F. M. BALL

Chapman Hall, London, etc., 1998, ISBN 0 412 78090 9, 569 + IX pages

A truly comprehensive book got into the reader's hand. The problems of vitamins are treated from physiological, chemical, analytical aspects, consequently in a real complexity. For this reason both chemists and medical professionals, nutritionists can use it in their everyday work or for research purposes. The main target is to highlight the fundamental factors influencing the bioavailability of vitamins. The chemically detectable amount of vitamins in foods is by no means related to their quantity utilized in human organisms.

The process of utilization is influenced by several exogenic and endogenic constituents. The digestion, absorption play important role because the gastrointestinal tract is highly selective in allowing nutrients to enter the body. There are many exogenic factors, e.g. ingredients of diet (nutrients and non-nutritive compounds), alcohol consumption, drugs, food processing and cooking methods (not only the vitamin losses) that have an effect on bioavailability. Age and state of health seem to be modifying factors, too.

The first two chapters contain a general introduction regarding the physiological aspects of vitamin bioavailability (the role of vitamins, vitamin enhancement of foods, digestion, absorption and transport of vitamins, the concept of bioavailability) and the laboratory procedures in general (assessment of vitamin activity in foods, laboratory facilities, sampling, extraction, clean-up procedures, chromatographical methods, capillary electrophoresis, continuous-flow analysis, microbiological methods, etc.).

In the following thirteen chapters the different vitamins are shown one by one. The structure of each chapter is the same. After a short general introduction the chemical features and nomenclature are treated, then the physiological and biochemical functions, deficiency syndromes, physicochemical properties, analytical methods, bioavailability, dietary intake and, finally, a concise summary (synopsis) and a list of references.

The statement on bioavailability start in each chapter with a review of physiological aspects, including metabolic considerations. It will be continued by the explanation of dietary sources and their bioavailability, effects of confounding dietary factors, effects of food processing and cooking, dietary intake, and of high intake and assessment of vitamin supply, nutritional status.

The analytical part first of all summarizes the scope of analytical techniques in foods. Then it deals with extraction techniques, and lists, highlights the suitable methods.

The reader finds data on the following vitamins: vitamin A (and the provitamin A carotenoids), vitamin D, vitamin E, thiamin, riboflavin (and other flavins), niacin (and tryptophan, an essential amino acid as precursor of niacin), vitamin B_6, pantothenic acid, biotin, folate, vitamin B_{12}, vitamin C. A well compiled register is included.

The author gives information: on up-to-date data, modern methods, newest opinions. The text is very clear, easy to read. The description of methods seems suitable for the reproduction or the application in analytical work respectively. The author has brought about a new and valuable book devoted to very important problems of vitamins. Both the introductory and detailed parts are well-founded and argumented. A thorough reading of the book gives the right to state that it consists of an outstanding scientific work demonstrating the crucial problems of modern vitaminology and giving a practical handbook for the analytical practice in this scope.

G. BÍRÓ

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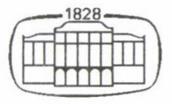

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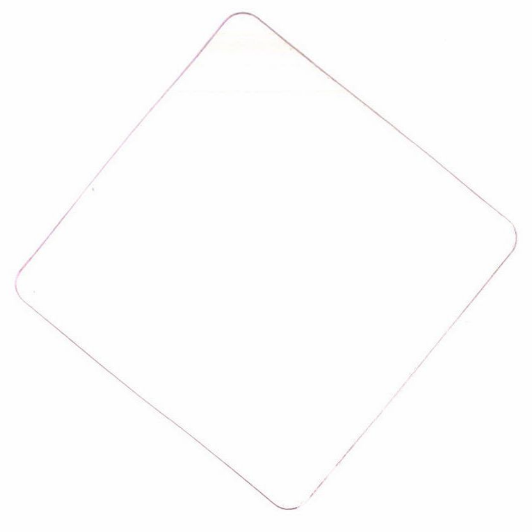

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