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APPLICATION POSSIBILITIES OF NUCLEAR FILTER-MEMBRANES IN THE FOOD AND BEVERAGE INDUSTRY

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The paper reports on the research of the application's possibilities of nuclear microfilter-membranes in the dairy, distillery, brewery and winery. The membranes with 0.2 and 0.6 μm mean pore diameter and 6.5% porosity were produced in the Institute for Nuclear Research, Dubna, Russia. The investigations were carried out in the Hungarian research institutes of milk, beer, alcohol and wine industries. Mainly the following parameters were studied:

- transmission capacity,
- selectivity,
- effect of filtration on the storability and microbiological status of liquids.

Keywords: beverage, filter-membranes, food industry, irradiation by heavy ions, microbiological measurements, microfiltration, nuclear filter, pore diameter, porosity, selectivity, storability, transmission

It is a well-known fact that the different membrane filtration methods – microfiltration, ultrafiltration, nanofiltration, hyperfiltration (RO) – are of great importance in many fields of the food industry (PORTER, 1975; HAUBS, 1975; SCHNEIDER, 1980).

Figure 1 shows the range of the different filtration processes (OSMONICS, 1990).

The nuclear filters – mainly microfilter membranes produced by nuclear methods – are usually 5–20 μm thick inert layers with 0.1–10 μm pore diameter. The materials of the membranes are artificial substances (e.g. polyethylene-terephtalate, cellulose-acetate) and the production is carried out in accelerators by bombardment with heavy ions (KUZNETSOV, 1981). The average pore diameter depends on the circumstances (alkali concentration, duration, temperature) of the

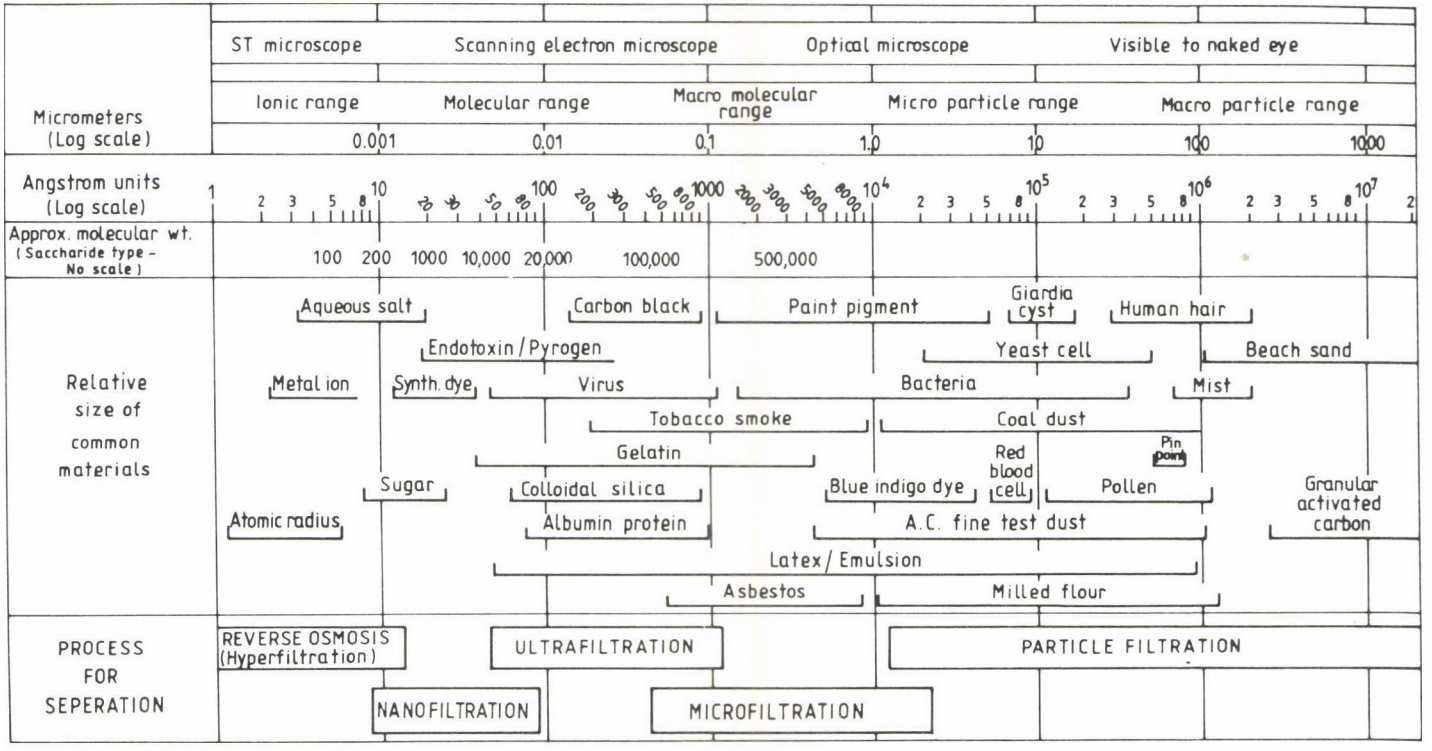

Fig. 1. Range of different filtration processes

chemical treatment of the membrane after the irradiation. The membranes can be applied to ensure microbial safety thereby increasing the shelf-life of liquid foods and drinks.

1. Materials and methods

The investigated nuclear filter-membranes were manufactured by xenone ion radiation treatment of polyethylene-terephtalate foils in the U-300 type cyclotrone in the Laboratory of Nuclear Reactions of the Institute for Nuclear Research, Dubna, Russia. The mean pore diameter was 0.2 and 0.6 μm with 6.5% porosity.

A lot of different measurements were carried out for characterization of the technical parameters of the filter-membranes, in this paper only a few are reported. The experiments were carried out in the Hungarian Dairy Research Institute (Mosonmagyaróvár), Research Station of Brewing Industry (Budapest), Research Institute for Viticulture and Enology (Kecskemét) and Research Institute for Distillery (Budapest) (SZABÓ, 1989a, b, 1990a, b).

The most important investigated parameters and measurements are as follows:

- transmission (filtering) capacity for different fluids (e.g. milk, whey, soft drink) as a function of pressure,
- selectivity for the components of the measured fluids,
- microbiological measurements of the filtered products,
- effect of filtration on the storability,
- applicability of the filters for microbiological investigations.

In the measurements, carried out in Dairy Research Institute a laboratory ultrafiltration equipment (DDS 136–20 UF–HF) was used with a piston pump type Rannie.

2. Results and discussion

Table 1

Selectivity values for milk and whey components

Component	Selectivity (%)	
	Milk	Whey
Dry material	79.1 ± 1.3	79.6 ± 2.0
Total nitrogen	97.6 ± 1.2	98.2 ± 1.6
Protein	99.3 ± 0.7	99.1 ± 0.8
Lactose	33.0 ± 1.5	32.9 ± 1.9
Minerals	74.1 ± 2.1	49.3 ± 3.0

Table 1 shows the selectivity (i.e. the retention %) for milk and whey components of the nuclear filters with 0.2 μm pore size. The selectivity (s) was calculated using the formula:

$$s = [(c_1 - c_2)/c_1]\ 100\%$$

where c_1 and c_2: concentrations in the original and the filtered solutions.

The effectiveness of the filtration process was estimated on the base of the storability (measured organoleptically) of the different filtered and bottled beer (e.g. Kinizsi, Mátyás, Kőbányai világos) samples. Additionally microbiological measurements were performed, too.

Table 2

Filtration of Kinizsi beer without pasteurization and comparison of the storability with pasteurized Kinizsi beer

Applied filter-layer	Storability (day)
Nuclear filter-membrane with 1 refilling	9–11
Nuclear filter-membrane with 2 refillings	9–12
Nuclear filter-membrane with 3 refillings	15–18
Ordinary filtration	7–8
Pasteurized beer	>30

In Table 2 some results of the filtration of "Kinizsi" beer are given. The investigations were carried out using nuclear filters with 0.6 μm average pore diameter.

According to the preliminary measurements the filtering capacity of the nuclear membranes with 0.2 μm pore diameter decreases rapidly as a consequence of fast forming blockages so they are not suitable for industrial wine filtration.

Figure 2 shows the cell count reducing effect of the 0.6 μm pore size nuclear filters in the process of wine filtration in comparison with the W-sterile EK filter-plates (Filtrox). The measurements with the filtered wine samples were carried out in 2 repetitions.

Filtration results of apricot-brandy, liqueur, biological vinegar and sour cherry juice samples are given in Table 3. The measurements were performed applying a mechanical carrier layer (Millipore and Sartorius filter-layers) by a pressure of 0.6 bar.

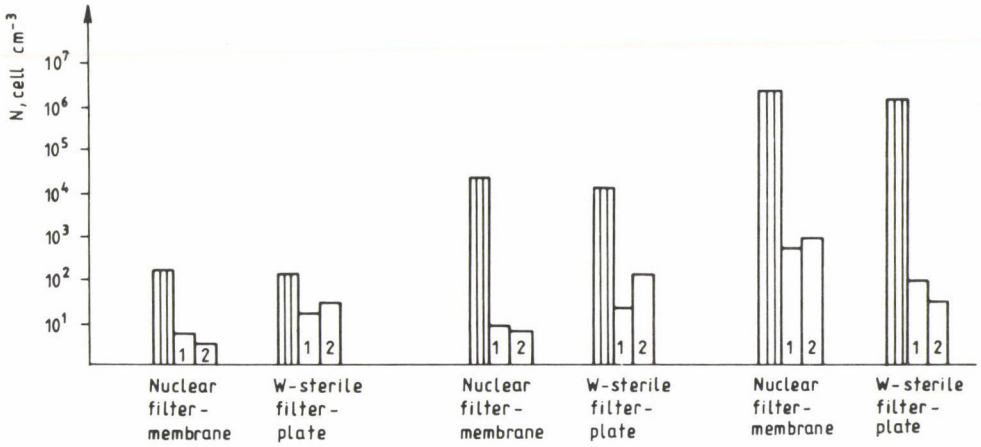

Fig. 2. The effect of filtration on the total cell count of wine. ▥: before filtration, □: after filtration

Table 3

Filtration results of the apricot-brandy, liqueur, biological vinegar and sour cherry juice samples

Pore diameter of the nuclear filter (μm)	Sample	Type of filter layer	Filtration time for 100 cm^3 (s)
0.2	Brandy	Millipore	190
		Sartorius	120
0.2	Vinegar	Millipore	100
	(10%)	Sartorius	60
0.6	Liqueur	Millipore	300
0.6	Sour cherry	Millipore	250
	juice	Sartorius	150

3. Conclusions

The protein retention for milk and whey and the transmission capacity for lactose solution of the 0.2 μm pore size nuclear filters is rather high in comparison with the ultrafilter membranes usually applied in the dairy industry. In contrast the filtering performance for milk and whey was rather low, approx. 50% of the membranes used in industrial scale.

The application of nuclear filter-membranes with 0.6 μm mean pore diameter has a positive effect on the storability of the beer, the storage-period is longer.

Microbiological measurements proved that the nuclear filters perfectly removed the yeasts remaining in the beer after the industrial silica filtration.

The microbiological investigations showed that the cell count reducing effect of the nuclear membranes is similar to that of the W-sterile EK filter-plates because the initial values were reduced by a few orders of magnitude during filtration. It is necessary to mention that the nuclear filter is capable for the filtration of prefiltered wines only, because the high starting permeability values decrease rapidly in consequence of blockages.

According to the results in case of alcoholic and non-alcoholic beverage filtration the nuclear membranes are usable for sterile filtration only as post-filters. As a consequence of blockage the filtration-performance decreased rapidly during process when the dry material content exceeded 10%.

Further disadvantages of the nuclear filters are the rather low mechanical resistance, the easy destruction of the edges and the uneasy mountability into the apparatus.

Finally it has to be mentioned that for industrial scale applications of the nuclear filters also the following criteria should be fulfilled:

- applying the filters with mechanical carrier layer,
- decrease of the electrostatically charged state,
- improvement of the mechanical resistance,
- increase of the filtering capacity.

*

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EFFECT OF IMMOBILIZED YEASTS ON THE QUALITY OF BOTTLE-FERMENTED SPARKLING WINE

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To eliminate the difficult clarification process in bottle fermented sparkling wine production, four variations of Ca-alginate entrapment technique were applied for immobilization of a *Saccharomyces bayanus* starter strain. Biocatalyzer prepared on the usual way of Ca-alginate entrapment technique (A) was compared to that prepared by coating the alginate beads with a cell-free alginate layer (B). Both types of biocatalyzers were investigated in two levels of cell loading (1, 2) and compared to the traditional free-cell fermentation (K) as a control. Chemical analysis of sparkling wines after three months of aging on yeast showed little differences only in few parameters. The organoleptic quality of sparkling wines was not adversely affected by either types of biocatalyzers. The cell retention capacity of biocatalyzers characterized by the limpidity of sparkling wine was not satisfactory using biocatalyzer without coating. A significantly better cell retention could be achieved by the use of cell-free alginate coating, but the adhesion of the coat to the surface of the beads needs further improvement.

Keywords: biocatalyzer, *Saccharomyces bayanus*, sparkling wine production, immobilized yeasts

The champenois method of sparkling wine production has both high labour and winery requirements. It arises primarily from the special clarification process of bottle-fermented sparkling wine. After the second fermentation and aging the yeast sediment is settled on the stopper and "disgorged". The delicate process called "remuage" takes a period of 1–3 months and imposes considerable constraints on the manufacturer.

To simplify the clarification process different methods were studied, including fermentation with immobilized yeasts. In this case the biocatalyzer can rapidly settle because of the gravity. This topic has been studied by several authors and has been recently reviewed by KRÁSNY and co-workers (1992). Calcium alginate was found a convenient carrier for immobilized cells (BIDAN et al., 1978; PARDONOVÁ et al., 1986; DIVIES, 1988; FUMI et al., 1987, 1988; MALIK et al., 1990a, b).

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In some cases some release of cells was reported, because of the effect of wine compounds as well as CO_2 production. To eliminate the cell release, improved methods of coating the biocatalyzer with a compact cell-free layer of alginate have been developed (KLEIN et al., 1986; LALLEMENT, 1990).

The aim of our present study was to compare the alginate entrapment techniques with and without "coating" as well as the traditional technique under the same condition in respect of the clarity, chemical composition and sensory characteristics of sparkling wine.

1. Materials and methods

1.1. Yeast strain

Inoculation was made with Erbslöh company's ChampaFerm dried product of *Saccharomyces bayanus* LW 187–50.

1.2. Base wine

The Chardonnay base wine came from a micro-vinification experiment of the Department of Oenology, University of Horticulture and Food Industry. After blending, the wine was fined with 0.8 g dm^{-3} bentonite (Deriton) and filtrated adding 0.3 g dm^{-3} of perlite (K 195). The wine contained 11.4% (v/v) alcohol, 8.8 g dm^{-3} titratable acid and 20 and 84 mg dm^{-3} free and total SO_2, respectively. It was dosed with 24 g dm^{-3} double refined sugar and filled into sterile bottles of 1.5 dm^3 volume through a membrane filter (0.45 μm).

1.3. Immobilization

Yeast cells were grown in a complete medium containing 5% (w/v) sucrose, 0.1% (w/v) diammonium hydrogen phosphate and 0.1% (w/v) yeast extract (Oxoid) at 25 °C to $1.2 \cdot 10^7$ cell cm^{-3} concentration. Yeasts were centrifuged at 6000 r.p.m. for 10 min and used as starter for both immobilization and free-cell control treatment.

For immobilization of cells 3% (w/v) aqueous solution of Na-alginate (Fluka) was prepared and autoclaved. The biomass appropriately dispersed in sterile water was added to the alginate suspension in two different concentrations (ratio 1:3). Homogeneous suspension was dispensed in a dropwise fashion into 0.2 mol dm^{-3} $CaCl_2$ solution continuously stirred to obtain beads of 3.5–4 mm diameter. After gelation for 2.5 h at room temperature it was washed twice with sterile water. Half of each type of biocatalyzer was covered by Na-alginate free of cells, gelated again in

$CaCl_2$ and washed with sterile water. All biocatalyzers were stored overnight in sterile dry base wine at +5 °C before fermentation, to exchange the free water content of the gel for wine.

1.4. Description of treatments

Five treatments, 40 bottles of each, were prepared, representing 4 different biocatalyzers and control. The immobilized treatments were prepared by calculating the quantity of beads to obtain 10^5 cm^{-3} initial cell content. Suspension of starter was added to the free-cell treatment in the same cell concentration. Bottles were closed with sterile plastic corks and wire caps. Fermentation was carried out at 11 °C to 13 °C. Notations of treatments are summarized in Table 1.

Table 1

Experimental treatments

Code	Type of starter	Starting concentration in biocatalyzer (BC) (cells cm^{-3} BC)
1/A	BC without coating	2.9×10^7
1/B	BC with coating	2.9×10^7
2/A	BC without coating	4.3×10^7
2/B	BC with coating	4.3×10^7
K	control (free cells)	

1.5. Sample analysis

Free cell mass in sparkling wine was expressed in terms of optical density at 540 nm. Alcohol, extract, sugar, titratable acid, tartaric acid, volatile acid, acetaldehyde were determined by the O.I.V. standard methods (ANON, 1990). CO_2 content was calculated from pressure (measured by pressure gauge for bottles) and temperature (JAULMES, 1973). The pH-value was measured with a precision pH-meter (OP–205/1) using a combined glass electrode. Color intensity was characterized spectrophotometrically by absorbances at 420 nm.

The rest of organic acids were measured by high performance liquid chromatography (BÓDY, 1988). Amino acids were determined from filtered samples by an automatic amino acid analyzer (Aminochrom 2), metal and phosphorus contents were measured by inductively coupled plasma atom-emission (ICP) technique.

Sensory evaluation was performed by scoring on a 15-point scale (odor, taste and CO_2, 5 points maximum for each) by 5 experienced judges. Triplicates of treatments were evaluated in three separate series. Each series contained one replicate of every treatment in randomized order. To avoid the recognition of different treatments due to their appearance, the sensory evaluation was conducted in a poorly lighted room using intransparent glasses. The clarity of the wines was visually evaluated after the scoring was finished.

1.6. Statistical analysis

The physical and chemical parameters measured in the individual bottles of bottle fermented sparkling wine are influenced by numerous independent random effects (errors from minor inhomogeneity of cuvée, filling, inoculation, corking, microclimatic conditions, inaccuracy of sampling and measurement, etc.), which allows a presumption of normal distribution. The analytical data of the experiments were evaluated by analysis of variance using the "Statographics 3.0" (Copyright 1985–1988 STSC, Inc. and Statistical Graphics Corporation) computer package. Every time three randomly selected bottles of each treatment were investigated. Depending on the applied analytical method the average of two or three repeated measurements from each bottle was used as an input for analysis of variance. Due to the design, the experimental generalized error was estimated from three different bottles of each treatment. The mean values of five treatments including control were compared by the least significant difference at $P = 0.05$. The mean scores of the sensory evaluation were compared by two-way-analysis of variance for 5 treatments $\times$ 5 judges $\times$ 3 replicates (AMERINE & ROESSLER, 1976).

1.7. Electron microscopic observation

Biocatalyzers were examined by scanning electron microscope (BS 300, Tesla) after fermentation. Gel beads were fixed with 2% glutaric aldehyde in cacodylate buffer at pH 7 and dehydrated in acetone. Samples were dried using the CO_2 critical point drying technique and were coated with gold.

2. Results

During fermentation, weekly analyses of samples were made to observe differences among treatments. The kinetics of fermentation was followed by measuring the alcohol, sugar and CO_2 content. The results showed that the process of fermentation of control samples was significantly faster compared to the samples treated with biocatalyzer. Completion of fermentation in the bottles treated with

immobilized yeasts showed 1–2 weeks delay, which is negligible compared to the total aging period of champenois method. These results were shown elsewhere (BÚSOVÁ et al., 1992). Hereafter we report the results obtained after 3 months aging of completely fermented sparkling wine on yeast in the bottles.

2.1. Clarity

The most important parameter, the clarity of wines characterized by optical density is presented in Fig. 1. Analysis of variance table is shown in Table 2.

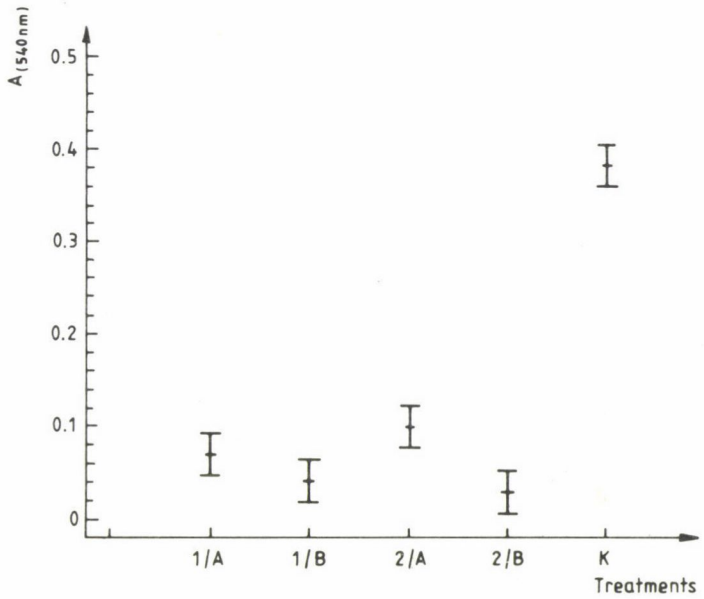

Fig. 1. Optical density of sparkling wines after three months of fermentation. Mean values of absorbance at 540 nm measured from three bottles against the same samples filtered. Vertical bars are LSD at P = 0.05. For codes of treatments see Table 1

The lowest values, corresponding to the highest clarity, were measured in wines treated with coated biocatalyzer (1/B, 2/B). Without coating the biocatalyzer caused a minor increase of optical density but at a level of P = 0.05 the increase was significant only in 2/A. This examination has not displayed exactly the results received by visual evaluation. Examining by naked eyes, treatments 1/B and 2/B were always found significantly clearer then 1/A and 2/A. However, a slight haziness could be observed by scrupulous examination even in samples 1/B and 2/B.

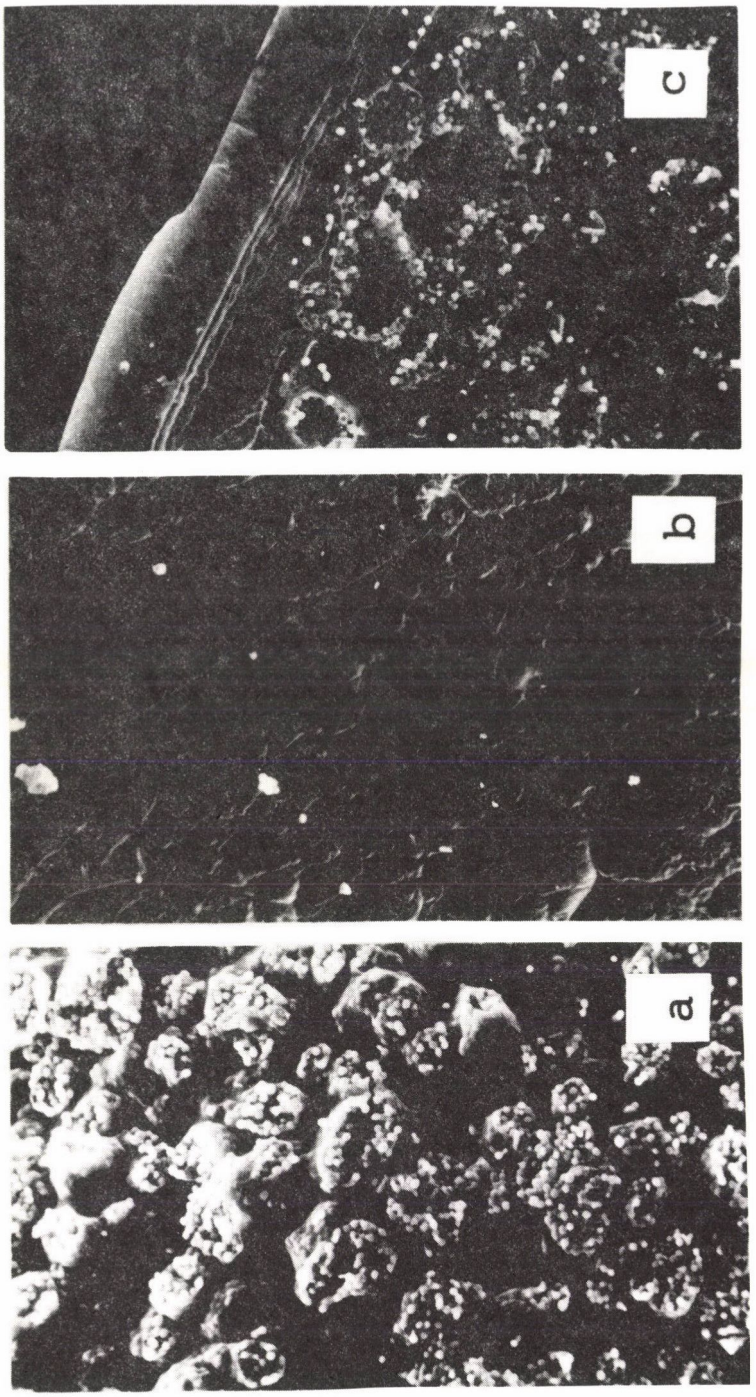

Fig. 2. Scanning electron micrographs of the biocatalyzers after fermentation (250×). Surface of beads without (a) and with (b) coating, section of the bead with coating (c)

Table 2

One-way analysis of variance for the optical density of sparkling wines three months after fermentation

Source of variation	Sum of squares	d. f.	Mean square	F-value	Sig. level
Between treatments	0.2587	4	0.1647	108.66	0.0000
Within treatments (error)	0.0060	10	0.0006		
Total	0.2646	14			

d.f.: degree of freedom

The differences in clarity between the two types of biocatalyzers were consistent with the electron microscopic observation of the biocatalyzers (Fig. 2).

Photos illustrate the surface of beads after fermentation without (Fig. 2a) and with (Fig. 2b) coating. The surface of beads without coating shows lots of breakages which let cells come out of the bead and increase turbidity of sparkling wine. The one with coating shows intact, unchanged surface, so yeasts are unable to get out of the bead. The effect of alginate coating is demonstrated on Fig. 2c.

2.2. Analytical composition

Means and least significant differences of the main analytical compounds of samples are presented in Table 3. There are some statistically significant differences among treatments in the alcohol, sugar, titratable acid, volatile acid, tartaric acid, lactic acid contents and pH-value. Still, these differences are not considerable from professional point of view.

The highest alcohol content of the control sample was consistent with its lowest sugar content.

Sugar content of 1/A, 2/A was smaller than that of 1/B and 2/B. This may be caused by coating, which means an additional barrier for the diffusion of fermentation substrata.

Titratable acid content of control is higher than that of the other treatments, but its tartaric acid content is smaller than that of 1/B and 2/B.

Table 3

Effect of different biocatalyzers on the main analytical components of sparkling wine after three months fermentation

Component	1/A	1/B	2/A	2/B	K	$LSD_{5\%}$
Alcohol (%, v/v)	12.62	12.67	12.33	12.49	12.88	0.18
Sugar ($g\ l^{-1}$)	2.13	3.3	2.37	4.5	1.73	1.15
CO_2 ($g\ l^{-1}$)	16.46	17.21	15.11	15.51	17.01	–
Sugar-free extract ($g\ l^{-1}$)	19.93	20.07	19.83	20.1	20.00	–
pH	3.08	3.08	3.08	3.09	3.05	0.01
Acids ($g\ l^{-1}$):						
titratable	8.0	8.23	8.1	8.17	8.4	0.07
tartaric	3.73	3.93	3.8	3.93	3.77	0.12
malic	2.2	2.3	2.3	2.2	2.4	–
citric	0.163	0.228	0.163	0.173	0.175	–
lactic	0.082	0.066	0.059	0.037	0.059	0.03
shikimic	0.034	0.033	0.032	0.031	0.034	–
volatile	0.31	0.36	0.32	0.32	0.4	0.02
color intensity (A_{420nm})	0.067	0.070	0.068	0.070	0.069	–

Mean values measured in three bottles of each treatment. For codes of treatments see Table 1

LSD: Least significant difference at level of P = 0.05. Not indicated when significance of F-value is lower than 5%

Volatile acidity was significantly lower in every biocatalyzer treatment compared to the control. The quantity of the other acids showed negligible differences.

The pH-value of control was significantly lower than the pH of the other treatments which shows analogy with the titratable acid content.

The amino acid composition, influenced by both the consumption and the autolysis of yeasts, is considered as one of the most important factors affecting organoleptic quality of sparking wines. The amino acid content of experimental treatments is presented in Table 4.

Table 4

Effect of different biocatalyzers of the amino acid composition of sparking wine after three months fermentation

Amino acid	1/A	1/B	2/A	2/B	K	$LSD_{5\%}$
Threonine	4.3	3.1	3.9	3.5	3.5	–
Serine	6.77	8.07	6.77	4.73	6.07	–
Glutamic acid	14.8	9.33	6.63	9.33	3.3	–
Proline	593.3	496.7	506.7	420.0	306.7	114.6
Glycine	5.97	5.97	4.03	3.2	2.97	–
Alanine	7.7	7.17	4.73	6.5	4.77	1.69
Valine	1.4	1.4	2.33	1.87	1.87	–
Methionine	1.5	2.0	3.03	1.5	2.0	–
Isoleucine + Leucine	6.33	3.8	6.67	4.13	6.3	2.14
Tyrosine	4.57	7.23	5.63	5.9	3.27	–
Phenyl-alanine	3.83	4.6	5.37	6.13	6.13	–
Lysine	4.53	5.67	6.8	3.4	2.83	–
Histidine	4.3	2.83	2.1	3.57	3.57	–
Arginine	18.33	17.0	17.33	28.0	30.0	–
γ-aminobutyric acid	53.33	46.0	41.0	30.0	48.0	9.29
NH_4	4.0	2.5	6.5	4.5	5.0	–

Mean values measured in three bottles of each treatment. For codes of treatments see Table 1

LSD: Least significant difference at level of P = 0.05. Not indicated when significance of F-value is lower than 5%

Alanine as well as proline content in the control is significantly lower than in the other treatments except 2/A and 2/B, respectively. Isoleucine and leucine contents in 1/A, 2/A and control are significantly higher, than in 1/B and 2/B. The γ-amminobutyric acid content of control is the same as that of the other treatments except 2/B. There was no significant difference among treatments for the rest of amino acids.

Since the immobilization technique used is based on ionotropic gelation, it is an important question how the biocatalyzer influences the ionic composition of sparkling wine. Table 5 shows the metal and phosphorus content of wines. Considering its particular importance, the Ca-content is separately displayed in Fig. 3 and its analysis of variance is summarized in Table 6.

Table 5

Effect of different biocatalyzers on some metal and phosphorus content of sparkling wine after three months fermentation

Parameter	1/A	1/B	2/A	2/B	K	$LSD_{5\%}$
Ca	114.8	125.2	114.9	125.4	86.4	9.79
K	489.9	484.9	470.1	470.3	474.1	11.17
Na	34.7	31.9	32.9	33.0	36.9	–
Fe	9.90	9.85	9.96	10.13	10.05	0.16
Mg	71.5	69.0	67.6	68.6	69.5	–
Zn	1.94	0.95	1.78	1.09	1.07	0.30
P	70.2	73.5	72.6	74.3	77.4	2.30

Mean values measured in three bottles of each treatment. For codes of treatments see Table 1

LSD: Least significant difference at level of P = 0.05. Not indicated when significance of F-value is lower than 5%

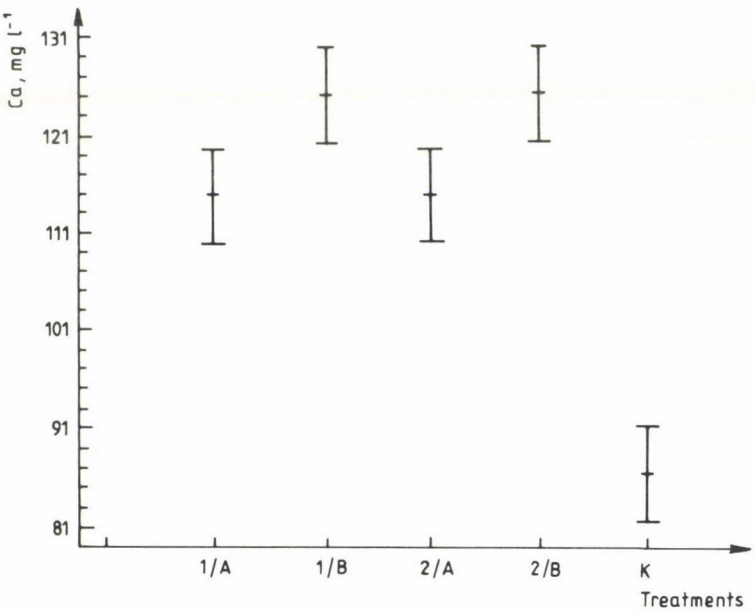

Fig. 3. Effect of the biocatalyzers on the Ca-content of sparkling wines after three months fermentation. Vertical bars are LSD at P = 0.05. For codes of treatments see Table 1

Table 6

One-way analysis of variance for the Ca-content of sparkling wines after three months fermentation

Source of variation	Sum of squares	d.f.	Mean square	F-value	Sig. level
Between treatments	3037.9930	4	759.4982	26.26	0.0000
Within treatments (error)	289.2747	10	28.9275		
Total	3327.2677	14			

d.f.: degree of freedom

As it can be seen in Fig. 3, the Ca-alginate gel caused significant increase in Ca content of sparkling wine. The difference between the control sample and the biocatalyzer without coating (1/A, 2/A) was 29 mg dm^{-3}. The alginate coat resulted in an additional increase of approximately 10 mg dm^{-3} calcium (1/B, 2/B). There were minor differences among treatments in K, Fe, Zn and P content, which may be caused by building these ions into the beads.

2.3. Sensory evaluation

A very important step of examination was the sensory evaluation. Scores for odor, taste and CO_2, when separately analyzed, showed no significant differences among treatments. However, the differences become significant in the overall quality characterized by the total scores (the sum of the scores given for different qualities). As Fig. 4 shows, the control has significantly lower total scores than the treatment 1/B. The analysis of variance table is presented in Table 7.

Table 7

Analysis of variance for the scores of sensory evaluation of sparkling wines after three months fermentation

Source of variation	Sum of squares	d.f.	Mean square	F-value	Sig. level
Main effects	54.4667	8	6.8083	4.85	0.0002
A: Treatments	17.3000	4	4.3250	3.08	0.0241
B: Judges	37.1667	4	9.2917	6.62	0.0002
Interactions A×B	10.5333	16	0.6583	0.47	0.9508
Residual (error)	70.1667	50	1.4033		
Total	135.1667	74			

d.f.: degree of freedom

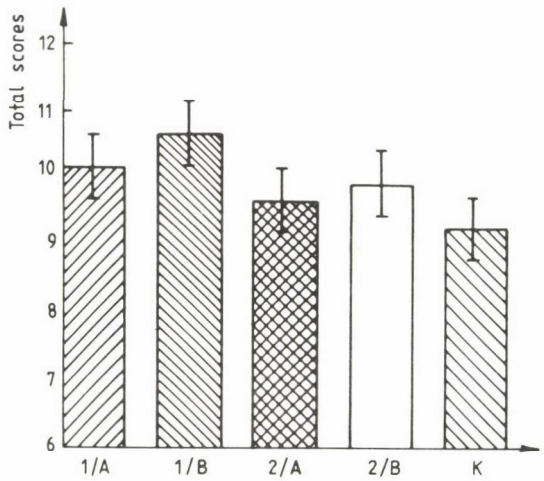

Fig. 4. Total scores obtained by sensory evaluation of treatments after three months fermentation. Each column represents a mean of three parallel samples scored by five judges in three separate series (n = 15). Vertical bars are confidence limits for mean scores at P = 0.05. For codes of treatments see Table 1

3. Conclusions

Results of our experiments, in agreement with other authors (FUMI et al., 1987; FUMI et al., 1988, LALLEMENT, 1990), show that Ca-alginate entrapment technique does not cause considerable changes in the main analytical composition of bottle fermented sparkling wine, compared to the traditional method.

Comparing the two types of biocatalyzers with and without coating we could find significant differences only in two main compounds. The alginate coating as an additional diffusion barrier resulted in a slight increase of residual sugar content, but this sugar concentration still gives a dry sparkling wine according to the standard. The wine fermented with coated biocatalyzers showed a higher tartaric acid content, which cannot be explained by the biological activity of the biocatalyzer. The possible explanation is that the rougher surface of the uncoated biocatalyzer accelerated the tartarate crystallization, removing tartaric acid from the sparkling wine, by increasing the amount of crystal grains. The higher amount of crystals could be visually observed on the surface of the beads without coating.

FUMI and co-workers (1988) found little differences in some amino acids between the traditional and immobilized treatments, which differences varied

depending on the yeast strains, base wines and aging period applied. In general, the differences were too little and variable to draw conclusion as to the pure effect of immobilization.

Our results show no essential differences in amino acid composition of the different treatments, either. It has to be mentioned, that the actual level in sparkling wine on lees is a balance between the amino acids consumed (or produced) by yeasts during fermentation and that released from dead cells due to autolysis. Since the 3 months aging period before analysis is considered too short for an excessive autolysis of cells, the effect of yeast metabolism may predominate in the measured amino acid composition. The higher proline and alanine content in most of the biocatalyzer treatments might be related to the reduced rate of growth and fermentation due to the immobilization. In general, the alginate coating did not influence significantly the amino acid content of sparkling wine, compared to the uncoated biocatalyzer. The effect of immobilization on the autolysis of cells will be evaluated after longer period of aging on yeast.

As it was expected from the previous reports (FUMI et al., 1988; MAGYAR & PANYIK, 1989), the Ca-content of sparkling wine prepared with biocatalyzer, either with or without coating, was significantly higher, compared to the control. MAGYAR and PANYIK (1989) have pointed out that higher content of Ca in biocatalyzer treatments is caused by diffusion of residual free Ca-ions from pores of the biocatalyzer rather, than by the liberation of Ca-ions bounded to the alginate gel. This diffusion is possible to prevent by repeated washing of biocatalyzer before usage. The higher Ca content, which causes higher deposit of tartarate during aging will be removed by disgorging together with biocatalyzer, because tartarate crystals tend to settle quickly by gravity, too.

Results of the sensory evaluation in our experiment confirmed the general opinion, that the Ca-alginate entrapment technique does not have any unfavorable effect on the organoleptic quality of sparkling wine. In fact, the overall quality of sparkling wine fermented by biocatalyzers with lower cell loading was found a bit higher then that of the control. The alginate coat did not affect the flavor and taste.

The main point of our experiment was to compare the cell retention capacity of two different biocatalyzers. Since the immobilized cell system is expected to save the limpidity of wine during the second fermentation, any extent of cell release is unacceptable. Living cells, released from the biocatalyzer before the exhaustion of the sugar content is completed, can grow up to the visible concentration in the bottle. In our experiment, in contrast to some other reports (DZHAOSHVILI et al., 1984; FUMI et al., 1988) and in agreement with BAMBALOV and TSVETANOV (1988), biocatalyzer prepared without coating showed high extent of cell leakages and in this form it cannot be used for elimination of sparkling wine clarification. The cell release was efficiently reduced by the use of biocatalyzer coated with cell-free alginate layer.

However, the release of cells into the wine could not be maintained below the level of visibility, because the coat was lost by a few beads. As it can be seen on Fig. 2b and 2c the intact coating can perfectly retain cells if it is satisfactorily fixed on the surface of beads. Loss of the coat of only one the bead in a bottle may cause turbidity of the sparkling wine after awhile. The aim of our further study is to prevent the loss of the coating of the beads during fermentation and aging by increasing the adhesion of the coating to the surface of the beads.

These results show that wines prepared with immobilized yeasts give differences just in few analytical components, but these differences are not significant from professional point of view. This means that this method will be suitable for preparing sparkling wines after the problem of the adhesion of coating to the surface of biocatalyzers is satisfactorily resolved.

*

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PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL ASPECTS OF FREE RADICALS[a]

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Free radicals are generated in the body both under physiological conditions and in response to exogenous initiators. The author gives a brief account on the possibilities of free radical production and on their role in physiological and pathophysiological processes. The defensive mechanisms against the free radicals are also presented. Finally, the free radical theory of atherosclerosis, cancer and alcohol induced liver diseases is discussed.

Keywords: free radicals, defense systems, diseases

Environmental pollutants, UV light and gamma-radiation are among the many exogenous initiators of free radical reactions, but the most important source of these radical species in vivo are biochemical redox reactions involving oxygen (LUNEC, 1990). These reactions play an important role in many physiological processes, where the highly toxic free radicals are kept under control by several enzyme- and nonenzyme-reactions. However, when these control mechanisms are not effective the free radicals may lead to cellular damage i.e. pathological tissue degeneration.

It is not therefore surprising that the list of diseases in which free radicals have been implicated is growing longer and longer.

The four-electron reduction of molecular dioxygen to water involves the formation of several active intermediates such as the superoxide anion radical, the hydroxyl radical and the hydrogen peroxide. The role of these radicals will be discussed briefly.

Superoxide radical

One-electron reduction of molecular oxygen produces the superoxide anion radical ($O_2 + e^- \rightarrow O_2^{\cdot}$).

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A portion of the $O_2^{\cdot}$ is formed in vivo as a consequence of chemical accident. For example electrons can escape from the electron carriers of the respiratory chain and pass directly onto oxygen resulting in $O_2^{\cdot}$ (FRIDOVICH, 1974).

Sugars, including glucose are oxidized very slowly to produce oxygen radicals, which are efficiently scavenged. In diabetic patients the elevated blood glucose level has a key role in the development of oxidative stress. The mechanisms of this process involve an increased production of free radicals by glycosylated protein autooxidation, a reduced scavenging by superoxide dismutase and a lack of reduced glutathione and ascorbic acid (WOLFF & DEAN, 1987, WOLFF, 1987 NATH et al., 1984, CHARI et al., 1984, JENNINGS et al., 1987).

In many cases the $O_2^{\cdot}$ production in vivo is functional, for example activated phagocytic cells (monocytes, neutrophils, eosinophils, macrophages) generate $O_2^{\cdot}$, which is essential for killing some of the bacterial strains. In chronic granulomatous disease, the $O_2^{\cdot}$ producing membrane-bound NADPH oxidase system in phagocytes is inactive. Due to this fact several engulfed bacterial strains especially *Staphylococcus aureus* are not killed and when phagocytes die they are released in viable form (CURNUTTE & BABIOR, 1987).

The endothelium-derived relaxing factor produced by vascular endothelium is identical to nitric oxide (NO) (IGNARRO et al., 1987, PALMER et al., 1988). This endothelium also seems to produce $O_2^{\cdot}$. These two free radicals can react resulting in a nonradical product. The variations in the productions of NO and $O_2^{\cdot}$ may play role in the regulation of vascular tone (HALLIWELL, 1991).

Extracellular $O_2^{\cdot}$ may effect cell proliferation. According to this hypothesis the small amount of free radical at the physiological generation rate is useful, and it should not be rapidly removed. The role of $O_2^{\cdot}$ in this process may explain the very low activity of the natural antioxidant enzyme, SOD in the extracellular fluids (HALLIWELL, 1991).

Hydroxyl radical

One of the intermediates in superoxide-driven Fenton and Haber-Weiss reactions is a hydroxyl radical (FEHÉR & VERECKEI, 1985). It is a very highly reactive radical and can attack and damage the living system. Its half-life is very short, within a microsecond, it can pluck an electron from almost any organic molecule and initiate further radical or non-radical processes, which are now accepted to be related to a variety of pathophysiological events (FEHÉR, 1988, MÓZSIK et al., 1992). However, in physiological circumstances free radicals exist at a very low steady-state concentrations, $10^{-4} - 10^{-9}$ M/l in pure solutions. In complex biological fluids or tissue, their amount would be much lower (LUNEC, 1992) and they will cause such a damage which is quickly contained and eliminated (DIPLOCK, 1991).

Free radical chain reactions can occur if hydroxyl radicals attack DNA. Chemical alteration of deoxyribose, purine and pyrimidine bases may lead to mutations and DNA strand breakage. Imperfect repair of DNA damage can result in oncogene activation and carcinogenesis. On the basis of the urinary excretion of DNA adducts the oxidative damage to DNA occurs at an estimated rate of about 10^4 hits per day in humans, these lesions are effectively repaired (AUROMA et al., 1989, BREIMER, 1988, KASAI & NISHIMURA, 1986).

Oxidation of polyunsaturated fatty acids by hydroxyl radical has been intensively studied because of its ability to initiate the free-radical chain reaction known as lipid peroxidation (GUTTERIDGE, 1988).

Reactive oxygen species

Superoxide radical and two protons can form hydrogen peroxide, which itself is poorly reactive, but it can be converted into hydroxyl radical in the presence of divalent iron, and probably copper (DIPLOCK, 1991).

Transition metals

Metals such as iron and copper can change their oxidation state in consequence of accepting or donating single electrons. This phenomenon explains that transition metals are good promoters of free radical formation (HALLIWELL & GUTTERIDGE, 1989). Iron and possible copper can be involved in the decomposition of lipid hydroperoxides into alloxyl (fast reaction) or peroxyl (slow reaction) radicals (KAPPUS, 1991).

Recently researchers have suggested a relationship between high iron stores and risk of myocardial infarction, cancer and insulin dependent diabetes mellitus (GORDEUK et al., 1987, ANON, 1989, SALONEN et al., 1992). Moreover elevated serum ferritin are often associated with impaired immunity (DORNER et al., 1983, MORGAN et al., 1983, BASSAN et al., 1985).

Regarding the pathomechanisms of these diseases, peroxidative damage of lipid membranes must also be taken into consideration (GORDEUK et al., 1987). Iron must be liberated from proteins before being involved in free radical reactions. It is believed that iron from ferritin and, or heme proteins can be mobilized by oxidant stress (SALONEN et al., 1992).

Generally, iron overload is a pathological disorder of iron metabolism that results from either excess dietary iron absorption or parenteral iron loading leading to iron accumulation in the form of ferritin (DALLMAN, 1990). In physiological circumstances increased iron fortification of staple foods or medicinal iron ingestion can also fill up the iron stores. However, the production of iron overload in normal individuals by prolonged high iron ingestion has not yet been definitively demonstrated (GORDEUK et al., 1987).

According to our experiments (WACHNIK et al., 1989, WACHNIK et al., 1992, WACHNIK et al., 1993) copper deficiency results in an increased hepatic iron concentration and lipid peroxidation. Moreover the rate of hepatic lipid peroxidation seems to be correlated with the oil content of the diet and the iron concentration of liver. It was proved that in hormonally active females hepatic lipid peroxidation is lower than in males because endogenous estrogens afford protection against lipid peroxidation. In gonadectomized females – contrary the expectation – liver malondialdehyde production still remains low, probably because hepatic iron concentration falls.

In rats with streptozotocin-induced diabetes mellitus, the hormonal lability caused changes in metabolism of copper and iron and subsequently influenced the rate of lipid peroxidation.

Antioxidant defense mechanisms

In living organism a range of mechanisms are developed to prevent or reduce the potentially injurious effects of free radicals (MÓZSIK et al., 1992).

Primary defense, natural antioxidant enzymes. Superoxide dismutase (SOD) removes superoxide radical by catalysing a dismutation reaction. In mitochondria SOD is a manganese containing enzyme, while that in the cytosole, it has a copper-zinc dependent activity. Notwithstanding that free radicals play a role in the aetiogenesis of human cancer, little evidence exists that low dietary intake of Mn, Cu, and Zn is involved in this process (DIPLOCK, 1991), but dietary copper level correlates strongly with Cu, Zn-SOD activity (PROHASKA, 1990).

Gluthathione peroxidase: The selenium dependent form of enzyme degrades hydrogen peroxide and reacts with free fatty acid hydroperoxides. Blood selenium concentration seems to be associated with some kinds of cancer and correlates with gluthathione peroxidase activity (NEVE et al., 1988, BUTLER et al., 1991).

Catalase is a widely distributed enzyme. It is found mainly in peroxisomes and mitochondria which metabolizes hydrogen peroxide resulting in water and oxygen.

Secondary defense, antioxidant vitamins. α-tocopherol is a lipid soluble molecule located in the interior of biological membranes. It protects poly-unsaturated fatty acids of membranes against lipid peroxidation by scavanging peroxyl radicals resulting in tocopherol-O·. Because it prevents further chain propagating steps, α-tocopherol has been described as the only lipid soluble chain breaking antioxidant in human blood plasma and erythrocyte membranes (SIES, 1989). The tocopherol radical can migrate to the membrane surface and be converted back to α-tocopherol by ascorbic acid (MCCAY, 1985, ESTERBAUER et al., 1989a, ESTERBAUER et al., 1989b). α-tocopherol and ascorbic acid protect not only membrane lipids but circulating LDL-cholesterol, too (ESTERBAUER et al., 1989b).

This phenomenon may explain that low plasma concentrations of α-tocopherol and ascorbic acid seem to correlate with an increased incidence of myocardial infarction (GEY et al., 1987).

Ascorbic acid is an aqueous chain-breaking antioxidant, scavenging peroxyl--radicals. The product in this reaction is the ascorbic radical. Thus ascorbic acid is an antioxidant in itself, but it serves as a co-antioxidant by interacting with α-tocopherol (SIES, 1989).

β-carotene scavenges superoxide, decreases the rate of formation of methyl linoleate hydroperoxides and is an efficient quencher of singlet molecular oxygen (KRINSKY, 1989, DI MASCIO et al., 1991).

Free radicals and atherosclerosis

Recent studies have demonstrated that oxidative modification of LDL plays an important role in the development of atherosclerosis. Endothelial cells, smooth muscle cells and macrophages are involved in this process, either directly by generating an oxidizing potential or indirectly by generating oxygen free radical. Oxidative modification of LDL can be inhibited by butylated hydroxy-toluene and vitamin E but can be accelerated by several factors such as cigarette smoking due to increased oxidative stress (STEINBERG et al., 1989, LUC & FRUCHART, 1991, DUTHIE et al., 1989, ANDERSON, 1991).

Native LDL can undergo oxidative modification especially when its concentration is high. The peroxidation of polyunsaturated fatty acids will induce chain reaction of free-radical-mediated lipid peroxidation. Oxidized LDL-s are taken up by the scavenger receptors of macrophages. While the receptor mediated uptake of native LDL is well controlled, the uptake of oxidized LDL is not down regulated. This uncontrolled uptake of cholesterol is thought to be a factor of importance in the pathogenesis of atherosclerosis. Namely when macrophages become overloaded with cholesterol, they are converted to foam cells which accumulate in the arterial intima as a fatty streak (JÜRGENS et al., 1987, STEINBERG & WITZTUM, 1990).

Free radicals and cancer

Human carcinogenesis is a multistep process that extends over several years. Among the mechanisms which are involved in the pathogenesis, free radical formation has to be mentioned. Free radicals can damage DNA, structural proteins, enzymes and membranes. Epidemiological studies indicated that natural antioxidants such as β-carotene, ascorbic acid and tocopherol, have a protective effect against certain types of cancer. These compounds may serve as lipid antioxidants of free radical scavengers (STÄHELIN et al., 1991, BLOCK, 1991, KNEKT et al., 1991).

Free radicals and alcoholic liver disease

Ethanol induced hepatotoxicity is partly due to the production of free radicals. In unfavourable metabolic conditions acetaldehyde, the first metabolite of ethanol, can be converted to acetate, while superoxide radicals are formed. Reactive oxygen species also develop during the function of the microsomal ethanol oxidizing system. These free radicals may play a role in ethanol induced lipid peroxidation. In alcoholic serum, tocopherol concentration is low, so the balance of oxidant and antioxidant systems is also altered (VERECKEI & FEHÉR, 1990).

Conclusion

Evidence indicates that free radical mediated injury plays a role in the etiology of several diseases. Iron overload may promote, but some micronutrients such as α-tocopherol, ascorbic acid, β-carotene and selenium, may reduce the incidence of these diseases. However, a very low concentration of free radicals has a physiological advantage and so their complete elimination would not be beneficial.

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A COMPARATIVE STUDY OF PHYSICOCHEMICAL INDICES (PHENOLIC SUBSTANCES) WHICH CAN AFFECT THE COLOUR OF SPANISH RED WINES[a]

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Physicochemical indices that affect colour of red wines have been studied in seventy-two Spanish red wines. Total phenolic and diverse phenol fractions were determined. Frequently used methods in wineries have been applied in order to distinguish usual data for characterizing red wines. The analytical scheme of anthocyanin equilibria based on simple spectrophotometric measurements has been tried. Results proved a wide variation in physicochemical properties of Spanish red wines, that justifies the application of reliable methods. The effect of differences in varieties, region, cultivars, age and making procedures on anthocyanin and catechin contents of red wines was considered. Differences between recommended methods for anthocyanin determination have been demonstrated. Indices as "Polymeric pigment colour" or features of "Chemical age" were determined, too, as well as their relation with aging status.

Keywords: red wines, colour, polyphenol content, anthocyanin, catechin, flavonoids

The colour is a characteristic and important property of wines (LAY & DRAEGER, 1991). Many factors influence the colour of red wine. Vinification techniques and aging or oxidation processes can produce important variations in the chromatic characteristics of wines (OUGH & AMERINE, 1962; BRIDLE, 1983; KERENYI & KAMPIS, 1984; KAMPIS & ÁSVÁNY, 1985; HEREDIA & GUZMÁN, 1988; SCHNEIDER, 1988). HEREDIA and GUZMÁN (1989) have recently studied the colour characteristics of wines from "Castilla-La Mancha" (Spain).

These variations affect the qualitative and quantitative phenolic composition of wines. The fractionation of phenolic compounds of red wines (OSZMIANSKI et al., 1988) was as follows:

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- Fraction I: phenolic acids
- Fraction II: catechins, procyanidins and anthocyanin monomers
- Fraction III: flavonols
- Fraction IV: anthocyanin polymers

Among flavonoid compounds, the most affected components are anthocyanins, the most directly responsible compound for the colour of red wines (LAY & DRAEGER, 1991).

In this study, physicochemical parameters that influence chromatic characteristics are measured for a group of adequate samples of Spanish red wines, as well as some conclusions concerning the reliability of the proposed methods are given.

1. Materials and methods

1.1. Wines

The study was performed with seventy-two, randomly selected Spanish red wines. They were all bottle samples produced in 31 wineries from diverse Spanish "Origin Appellations" and two experimental wineries. All the wines were elaborated from 1978 to 1988 vintages. Sample selection was as diverse as possible to ensure a representative collection of the grape varieties used to make Spanish red wines.

1.2. Analytical measurements

A Bausch & Lomb "Spectronic 2 000" spectrophotometer and precision glass or quartz cells with 1, 2, 5 or 10 mm pathlengths were used for spectral records.

The traditional "Permanganate Oxidation Number" determination was carried out according to the OIV Methods (CEE, 1990). For total polyphenols, catechins, anthocyanins, flavonoid and non-flavonoid compounds, the recommendations proposed by AMERINE and OUGH (1974) have been considered. The relative standard deviation is offered as a representation for property variability.

To study anthocyanin equilibria, the analytical scheme proposed by SOMERS and EVANS (1974; 1977) and JACKSON and co-workers (1978), based on spectrophotometric measurements, has also been applied. The determination of polyphenols proposed by SOMERS and EVANS (1977) was based on the UV absorption at 280 nm. The technique is only useful for comparative purposes, since results are expressed in terms of absorbance units.

Non-coloured anthocyanins (NA) are evaluated by the absorbance increment due to the increase of colour in an acid medium (HCl treatment), once a weighted

elimination of the absorbance ascribed to polymers has been carried out. This technique defines up to three features of "Chemical Age" (CA), as follows

$$CA(I) = A_{SO_2} / A_{acetaldehyde}$$
$$CA(II) = A_{SO_2} / A_{HCl}$$
$$CA(III) = A_{SO_2} / A_{520}$$

2. Results and conclusions

The values obtained for the "Permanganate Oxidation Number": oscillated between 19 and 83. More than 50% of the samples are located in the zone of "very light" wines, according to the classification of RIBEREAU-GAYON and co-workers (1980). These authors had observed a decrease in this parameter for recent vintages, which has been confirmed with the samples under study.

The total amount of phenolic compounds (mean value = 1 610.8 mg l^{-1}) lies within the marginal values that can be considered as usual for red wines (Table 1), except for a strongly coloured wine from "Garnacha Tintorera", a variety which contains anthocyanic compounds, even in the grape pulp.

Table 1

Results for the polyphenolic components
(n = 71; sample No. 48 was an outlier)

Component	$\bar{x}$ (n = 71)	Range	s
Total polyphenols (mg l^{-1})	1 610.8	2 252.3	398.6
Flavonoids (mg l^{-1})	1 219.7	1 657.5	376.3
Flavonoids (%, w/w)	74.8	41.3	8.6
Non-flavonoids (mg l^{-1})	391.1	1 028.4	141.1
Anthocyanins[a] (mg l^{-1})	65.5	392.9	55.0
Anthocyanins[a] (%, w/w)	3.9	12.0	2.1
Anthocyanins[b] (mg l^{-1})	79.4	404.7	59.9
Catechins (mg l^{-1})	491.4	1 411.2	491.9
Catechins (%, w/w)	29.7	54.5	11.2

[a] By the "pH increasing" method; [b] By the "SO_2 blanking" method; $\bar{x}$: mean value; n: number of measurements; s: standard deviation

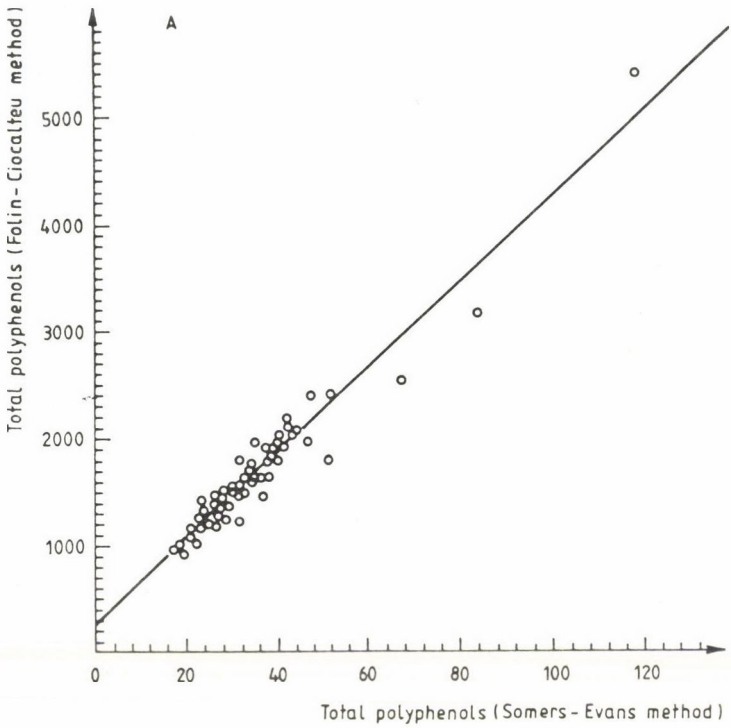

Fig. 1A. Correlation between Folin – Ciocalteu and Somers – Evans methods for total polyphenols

The method for determination of polyphenols presents a good correlation ($r^* = 0.964$, $P < 0.001$) with the Folin – Ciocalteu method (Fig. 1A). This relationship between methods allows to consider a slight modification in the formula used to obtain concentrations of total polyphenols as mg l^{-1}. Regression curve between both determinations is:

$$TP_{(FC)} = 272.5 + 39.9\, TP_{(SE)}$$

where

TP = Total Polyphenols
FC = Folin – Ciocalteu
SE = Somers – Evans.

A similar equation, with respect to A_{280}, is

$$TP_{(FC)} = 112.9 + 39.9\, A_{280}.$$

According to the general content in phenolic compounds, flavonoid concentrations varied from 474.6 mg l^{-1} in wines made of the "Monastrell" variety ("Jumilla" origin appellations), to 4 715.0 mg l^{-1}, that correspond to the "Garnacha Tintorera" sample ("Valencia" origin appellation). The grape variety, as well as wine-growing region, influence decisively, the anthocyanin content (GONZÁLEZ-SAN JOSE et al., 1990; MATTIVI et al., 1991). Nevertheless, flavonoid compounds showed a wide variability with reference to the total phenolics: from less than a half of total (48%) to almost the totality (90%). Variation in the anthocyanin content between cultivars was substantial, and the traditional maceration technique gave the highest yields of anthocyanins (LAY & DRAEGER, 1991; ARFELLI et al., 1992).

An extended range for catechin content is found, too (from 102.3 mg l^{-1} to 1 548.3 mg l^{-1}). As a consequence of the singularity of the method employed, the catechin value of a wine in absolute terms in thus of poor value (SCHNEIDER, 1989) (Table 1). So, with regard to their relative proportion of total phenolics, they oscillate between 8% and 63%. The mean value obtained for catechin (29.7%), is rather similar to that obtained for Italian wines (GIGLIOTTI & BUCELLI, 1992). Both the length of maceration and the presence of large amounts of seeds in contact with the must during the fermentation process led to wines with a higher content of catechins. Nevertheless, there is not any relation between total phenolics and catechin proportion.

Results observed by ALONSO and co-workers (1988) have been confirmed by us. Greater contents of catechins have been found in "Rioja" wines than for "Valdepeñas" wines. This is explained by the proportion of "Garnacha" variety in "Rioja" wines, in addition to "Tempranillo" variety, that is the only one used for elaborating "Valdepeñas" wines.

Anthocyanin determination by the traditional methods ("pH increasing" method and "bisulphite blanking" method) presented some differences, as it was observed by RIBÉREAU-GAYON & STONESTREET (1965). The technique based on decolouration by the SO_2 effect offered the highest values, explained by a partial decolouration in non-antochyanic compounds. Some authors (GLORIES, 1984) offer single factors to calculate the anthocyanin contents by both techniques, but errors up to 7 per cent were found for Spanish wines.

The SOMERS & EVANS (1977) technique depends on the "Anthocyanin Color in Acid" (ACA) (JACKSON et al., 1978), and it offers lower results than the previous methods (mean value = ca. 52 mg l^{-1}) (Table 2). A strong correlation ($r^* = 0.966$, $P < 0.001$; $r^* = 0.929$, $P < 0.001$, respectively) with any of two methods (Fig. 1B) gives the evidence of a systematic error, and it allows us to consider the equation of the curve, taking one of the two methods as reference.

Table 2

Results for the polyphenolic variables obtained by SOMERS *and* EVANS *(1974, 1977).*
(n = 71; sample No. 48 was an outlier)

Component	$\bar{x}$ (n = 71)	Range	s
Free SO_2 (mg l)	0.6	3.3	0.7
Total phenolics (U.A.)	33.7	66.7	10.6
Total anthocyanins (mg l)	51.4	377.9	52.4
Ionized anthocyanins (mg l)	15.8	78.6	12.9
Degree of ionization of anthocyanin (α)	40.8	95.3	12.6
Degree of ionization of anthocyanin (α')	45.3	95.2	21.9
Chemical age (I)	0.7	0.5	0.1
Chemical age (II)	0.3	0.5	0.1
Chemical age (III)	0.7	0.4	0.1
Polymeric pigment colour	1.7	3.6	0.7
Wine colour in acid	5.4	22.1	3.1
Anthocyanin colour	0.8	3.8	0.6
Anthocyanin colour in acid	2.6	18.9	2.6
Non-coloured anthocyanins	1.8	15.5	2.2

$\bar{x}$: mean value; n: number of measurements; s: standard deviation

For the method of "pH increasing", for instance

$$\text{Anthocyanin concentration} = 12.5 + 20.6\ \text{ACA}$$

and through an adequate transformation of the original equation:

$$\text{Anthocyanin concentration} = 20\ \text{ACA}.$$

Most of the wines (80%) under study had a degree of ionization of anthocyanins (α) less than 50%. The obtained values oscillated between α = 5% and α = 100% (Table 2).

"Natural degree of ionization of anthocyanins" (α') is related with SO_2 equilibria in recently made wines. Results proved that high correlation exists between α and α' ($r^* = 0.985$, $P < 0.001$). Any appreciable difference between α and α' was indicative of the SO_2 added to the wine after fermentation. SOMERS & EVANS (1977) found a very wide variation in measures of (α' − α); so, from 0 to 22%. These results have been confirmed in Spanish red wines, since a range from 0 to 16% has been found. Apparently, aging status is not a factor that influences these results; it was observed that young (less than 1 year old) and aged (more than 1 year old) wines showed similar mean values for (α' − α): 4.98% and 4.22%, respectively.

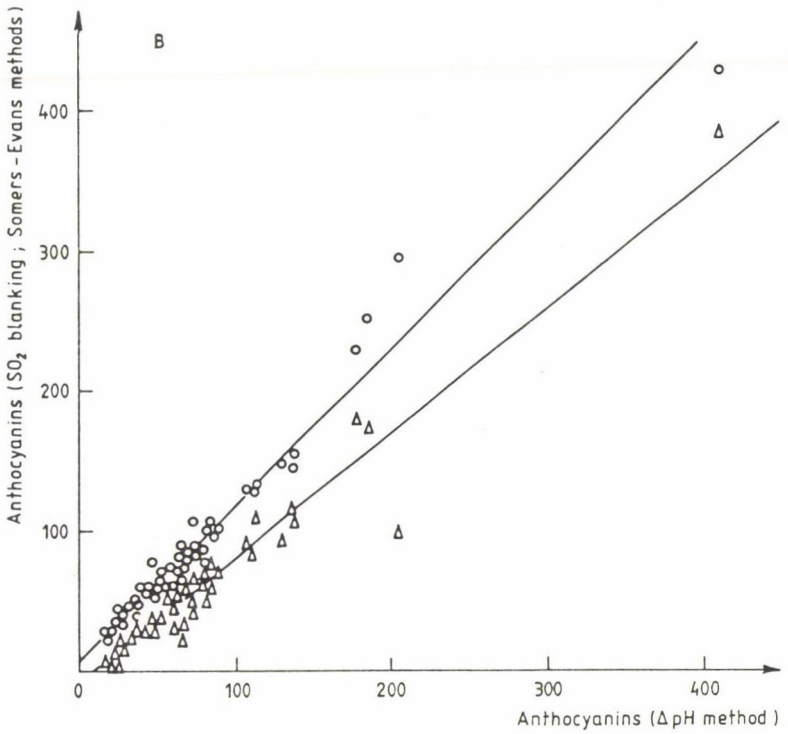

Fig. 1B. Correlation between ΔpH methods and both "bisulphite blanking method" (o) and Somers – Evans method (Δ) for anthocyanin determination

Concentrations of ionized anthocyanins showed a smaller variation than those of total anthocyanins. With the aging, an increase of the ionization degree was observed. Yet it was compensated by a considerable decrease of the total amount of anthocyanins with the time elapsed (DRDAK et al., 1989). After storage of wines total losses of at least 50% of anthocyanin pigments have been shown (ROMMEL et al., 1990). This effect explains the fact that low concentrations of ionized anthocyanins were observed, from 2.8 mg l^{-1} to 81.4 mg l^{-1} (Table 2).

The "Polymeric Pigment Colour" is an index of phenolic compounds non-affected by decolouration effect of SO_2. As red wines are aged, the content of polymeric pigments increased. The variability is in accordance with other results obtained in the wines under study (Table 2).

Mean values of NA is 1.8 units of absorbance (Table 2). Chemical age (II) did not correlate very well with any of the other definitions of chemical age (I and II) ($r^* < 0.8$), and its mean values differed. This means that a differentiative significance

of aging status takes place. Figure 2 shows the correlation lines between the three indexes which attempt to classify the samples by their aging status. The age of wine samples is related to the color (HEREDIA & GUZMÁN, 1988), anthocyanin content (LIAO et al., 1992) and grape variety (ETIEVANT et al., 1988), to a greater extent than methods of vinification (BARILLERE et al., 1988).

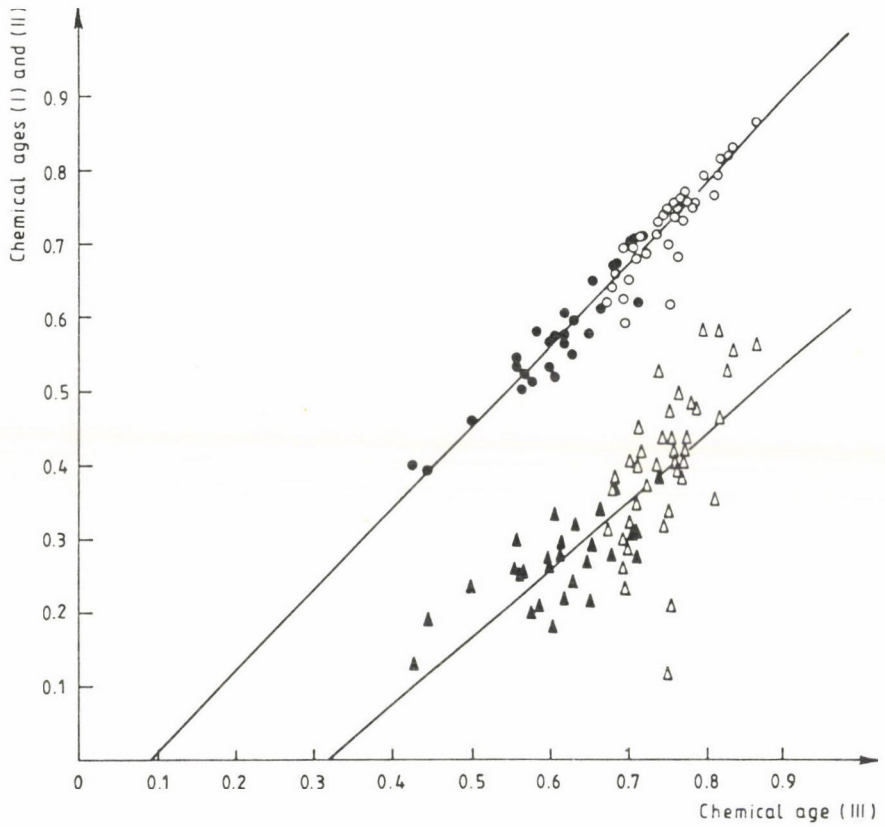

Fig. 2. Correlation of chemical age (III) with both chemical age (I) (• young wines, o aged wines) and chemical age (II) (▲ young wines, Δ aged wines)

Symbols

UV = Ultraviolet
NA = Non-coloured anthocyanins
CA = Chemical age
TP = Total polyphenols
FC = Folin – Ciocalteu method
SE = Somers – Evans methods
A_{280} = Absorbance at 280 nm
ACA = Anthocyanin colour in acid
U.A. = Unity of absorbances

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Acta Alimentaria, Vol. 23 (1), pp. 43–58 (1994)

CONDENSATION OF GLUCOSE BY THE REVERSED HYDROLYSIS REACTION OF GLUCOAMYLASE

I. EFFECT OF BOTH D-GLUCOSE AND STARCH CONCENTRATIONS, TEMPERATURE, AND ENZYME CONCENTRATION

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This investigation has been carried out in order to determine the main factors affecting the condensation of glucose by the reversed hydrolysis reaction of glucoamylase and to establish the optimum conditions which lead to produce refined glucose during the hydrolysis or starch by reducing the predisposition to synthesis of oligosaccharides.

Under the conditions used the synthesis of oligosaccharides was increased by increasing the concentration of dry substance as a starting material, which was either free glucose or starch. The enzyme catalyzed rapid formation of maltose and a slower synthesis of isomaltose from D-glucose as a substrate, but after long reaction times (at equilibrium) the isomaltose concentration was higher than that of maltose. At a higher initial concentration of starch a large amount of oligosaccharides was resynthesised especially isomaltose, and the yield was significantly reduced with prolonged incubation times. By increasing the glucoamylase concentration the process kinetics was increased, but the final product distribution and the equilibrium constants were not affected. High performance liquid chromatography (HPLC) was used to analyze synthetic products.

Keywords: glucoamylase, glucose, starch, synthesis of oligosaccharides

Glucose is produced by hydrolysis of starch from the non-reducing ends of its chain by glucoamylase (amyloglucosidase, α-(1→4)-glucan glucohydrolase, EC 3.2.1.3).

MADGAVKAR and co-workers (1977), KUSUNOKI and co-workers (1982) and TSEKOVA and co-workers (1984) had presented kinetic expressions for the hydrolysis. At low starch concentrations, the enzyme hydrolyzes starch almost completely to yield glucose (KUSUNOKI et al., 1982). At higher initial concentration of dextrin and starch a large amount of isomaltose was synthesized and the glucose yield was

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significantly reduced after very long reaction times (ROELS & TILBURG, 1979; SHIRAISHI et al., 1985).

HEHRE and co-workers (1969) showed that glucoamylase catalyzes the rapid synthesis of maltose and a slower synthesis of isomaltose from β-D-glucose. ROELS and TILBURG (1979) proposed second order rate equations for glucose condensation to maltose and isomaltose. ADACHI and co-workers (1984) have devised the kinetic model for the hydrolysis and synthesis of maltose and isomaltose, and the validity of the model was verified experimentally at 40 °C and pH 5.0. They found that the enzyme catalyzed rapid hydrolysis and synthesis of maltose, but isomaltose was hydrolysed and synthesized more slowly. BESCHKOV and co-workers (1984) studied the kinetic model of maltose and maltotriose hydrolysis, and the kinetic model of glucose condensation at equlibrium at 40 °C and pH 4.6.

The aim of the present study was to establish the effect of both D-glucose and starch concentrations, temperature and enzyme concentration on oligosaccharides synthesis by the reversed hydrolysis reaction of glucoamylase. It is very important for the production of refined glucose during the hydrolysis of starch. By reducing the predisposition to resynthesis or by alteration in conditions the equilibrium could be shifted the reaction sufficiently to give useful proportion of synthetic products, especially isomaltose.

1. Materials and methods

1.1. Materials

Glucoamylase in liquid form was supplied by Miles Kali Chemie, produced from *Aspergillus niger*. Trade name is Optidex L–300 (Hungary).

Soluble starch and D-glucose obtained from Reanal, Fine Chemical Company (Hungary).

Maltose was supplied by Serva Feinbiochemica Heidelberg (Germany).

Isomaltose, panose and isomaltotriose were purchased from Sigma Chemical Co. (USA).

1.2. Methods

1.2.1. Purification of glucoamylase. Industrial enzyme preparations from *A. niger* may contain enzymes with α-amylase, glucoamylase, transglucosidase, and maltase activities (PAZUR & ANDO, 1959). Therefore, Optidex L–300 industrial glucoamylase (of *A. niger* origin) fractionated with alcohol as described by PAZUR and ANDO (1959) was used in the subsequent experiments with an activity to 353.5 GAU cm^{-3}.

1.2.2. Determination of glucoamylase activity. Glucoamylase activity was measured according to the procedure described in MILES LABORATORIES (1973). The unit of glucoamylase activity (GAU) is defined as the amount of enzyme required to liberate one gram of D-glucose per h from 50 cm^3 of 4% (w/v) soluble starch at 60 °C and pH 4.2.

1.2.3. Analytical procedures. Concentration of the carbohydrates were analyzed by High Performance Liquid Chromatography (HPLC) with a Waters Associates Model 21–42. The conditions used were as follows:

Two columns were used: one column of Spherisorb-NH_2 5 μ (250×4.6 mm) was eluted with a solvent system of acetonitrile and water (75:25 by volume) at a flow rate 2 $cm^3\ min^{-1}$, chart speed was 20 $cm^3\ h^{-1}$, at room temperature and products were detected by Waters Differential Refractometer R 401 and Differential Refractometer Electronics unit (at RI 8x) with run parameters of recording integrator were plot baseline position (zero) = 0, attenuate height (AΠ 2↑) = 5, chart speed for plotting (CHT sp) = 0.3, width of peaks at half-heights (PK WD) = 0.16, threshold (THRSH) = 5 and reject peaks with insufficient area (AR REJ) = 5000. The second was column of Aminex HPX–42A (300×7.8 mm) which was heated by water jacket to 85 °C, mobile phase was H_2O at a flow rate 1.5 $cm^3\ min^{-1}$ and at RI 32x.

Products concentrations were calculated from peak areas after calibration with standard solutions.

1.2.4. Operating conditions. The glucoamylase-catalyzed reaction was allowed to proceed under the specified experimental conditions in Erlenmeyer flask immersed in a shaking water bath. The reaction was initiated by addition of glucoamylase to a carbohydrate (D-glucose or starch) solutions. Part of the reaction mixture was taken at different intervals of time from the reaction vessel and kept in a boiling water bath for 10 min for enzyme denaturation, diluted with distilled water, filtered and the concentrations of carbohydrates in the sample solution analyzed by HPLC. When only glucose concentration was measured during the hydrolysis of starch, the Shaffer–Somogyi micro method (A.O.A.C., 1984) was used.

2. Results and discussion

Examination of the enzymatic technological processes proves that the yield of dextrose-production from starch was mostly subject to a reverse synthesis which takes place at a high glucose concentration in the presence of glucoamylase or of hydrochloric acid. In case of high D-glucose levels these reversed products were also responsible for a decrease of the final product's DE value (LEE et al., 1976 and KENNEDY et al., 1985). This result depends on the occurrence of reversion reactions (synthesis), which form disaccharides and other oligosaccharides from hydrolysis

products. Structures of oligosaccharides which could be (re)formed by reversion reactions are as follows.

Maltose:	α-D-Glucopyranosyl-(1→4)-D-Glucopyranoside
Isomaltose:	α-D-Glucopyranosyl-(1→6)-D-Glucopyranoside
Nigerose:	α-D-Glucopyranosyl-(1→3)-D-Glucopyranoside
Panose:	α-D-Glcp-(1→6)-α-D-Glcp-(1→4)-D-Glucopyranoside
Isomaltotriose:	α-D-Glcp-(1→6)-α-D-Glcp-(1→6)-D-Glucopyranoside
Isopanose:	α-D-Glcp-(1→4)-α-D-Glcp-(1→6)-D-Glucopyranoside
Maltotriose:	α-D-Glcp-(1→4)-α-D-Glcp-(1→4)-D-Glucopyranoside

During studying the hydrolysis of starch by glucoamylase, it was found that the reversion products from glucose were formed in parallel with the production of glucose. Maltose was rapidly formed during the reaction, while the isomaltose formation was relatively slower. These events were accompanied by a decrease in glucose yield (SHIRAISHI et al., 1985). Condensation of glucose reactions, the production of maltose, nigerose, panose and isomaltotriose as limited products were obtained, while the main product was obviously the isomaltose (HEHRE et al., 1969 and PAZUR et al., 1977). The reversion products could be ascribed to the origin of glucoamylase (PAZUR & OKADA, 1967 and SHIRAISHI et al., 1985), its activity, pore diffusion effect (MÜLLER, 1987), effect of pH (STOUFFS & VENKER, 1984), immobilization form (KENNEDY et al., 1985) or to the transglucosidase activity, which only contaminates the glucoamylase (BARKER & CARRINGTON, 1953).

For the production of refined glucose during the hydrolysis of starch, it was necessary to investigate the kinetics and mechanism of such an enzymatic reversion. The different factors which affected the condensation of glucose by the reversed hydrolysis reaction of glucoamylase such as glucose concentration, temperature, concentrations of both starch and enzyme were studied.

2.1. Effect of glucose concentration

In order to examine the effects of concentration of glucose and water on the formation of oligosaccharides, the glucose solution of various concentration was incubated with soluble glucoamylase (130 GAU cm^{-3}) at pH 4.2 (0.05 mol l^{-1} acetate buffer) for 24 h at 40, 50, 55 and 60 °C, respectively. The relationship between the yield of oligosaccharides and initial glucose concentration is shown in Fig. 1. The total yield of oligosaccharides was increased almost linearly with the increase of the initial glucose concentration. The concentration of water was calculated from an equation suggested by ADACHI and co-workers (1984):

water conc. (mol l^{-1}) = −6.546 × glucose conc. (mol l^{-1}) + 55.13 and was accompanied with the glucose concentration in Fig. 1. Even a 65% glucose concentration, the water concentration was 31.49 mol l^{-1} in contrast to 3.61 mol l^{-1} of glucose. From Fig. 1, it could be seen that the glucose concentration was one of most important factor to increase or decrease the yield of oligosaccharides.

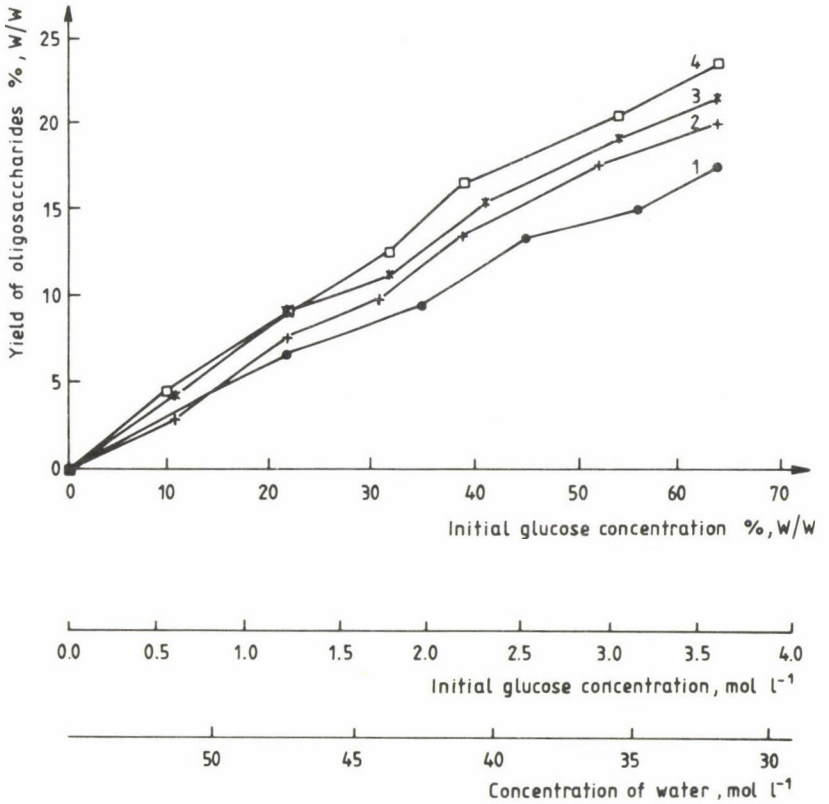

Fig. 1. Effects of concentration of glucose and water on the formation of oligosaccharides. 1: 40 °C; 2: 50 °C; 3: 55 °C; 4: 80 °C

The time course of oligosaccharide formation is presented in Fig. 2. It appears that the yield of oligosaccharides reached its maximum after approximately 3 days at each glucose concentrations used.

The amount of bonded glucose as oligosaccharides was indirectly estimated by quantifying the remaining free glucose in the product mixture by HPLC (Fig. 3).

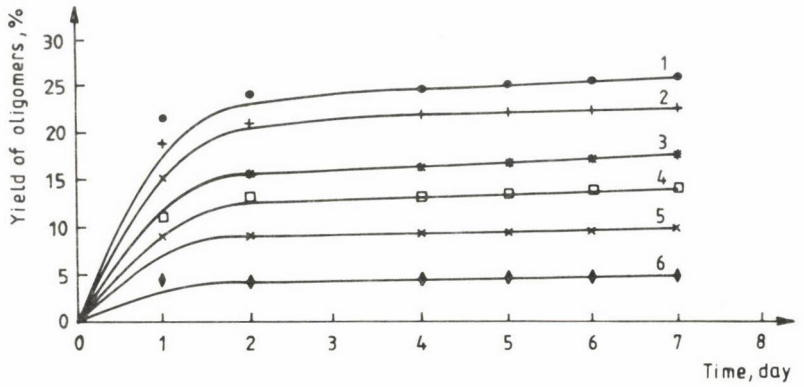

Fig. 2. Effect of initial glucose concentration on the formation of oligosaccharides, at 55 °C pH 4.2, 0.05 mol l^{-1} acetate buffer and 130 GAU cm^{-3}. Initial glucose concentration, 1: 64.85%; 2: 53.15%; 3: 40.45%; 4: 31.27%; 5: 22.00%; 6: 10.65%

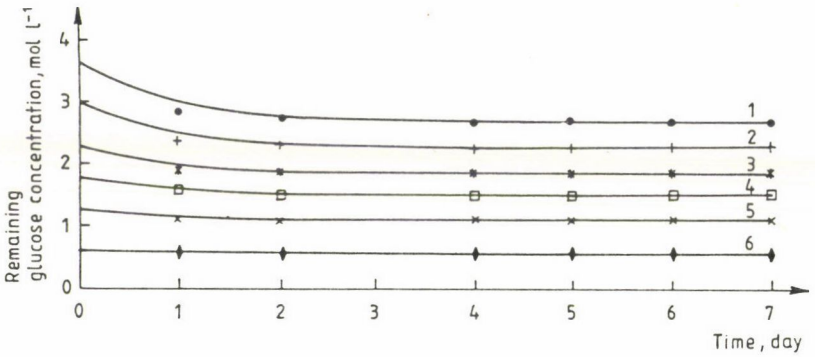

Fig. 3. Effect of initial glucose concentration on the formation of oligosaccharides, at 55 °C, pH 4.2, 0.05 mol l^{-1} acetate buffer and 130 GAU cm^{-3}. Initial glucose concentration (mol l^{-1}), 1: 3.603; 2: 2.853; 3: 2.247; 4: 1.737; 5: 1.222; 6: 0.582

Figure 4 showed that the isomaltose formed the major part of the oligosaccharides in the product mixture after 7 days (at equilibrium). At the highest glucose concentration (65%), isomaltose was increased to 19% (w/w) of the total sugar, while the final maltose and panose concentrations were obtained in comparable amounts (2.5–3.0%, w/w). The molar ratio of isomaltose to maltose and panose were 6.5±0.5 and 9.0±1.0, respectively (after 7 days), in each initial concentration of glucose. A more detailed representation of polymerization products formation from the glucose solutions of various concentration is shown in Figs 5, 6, 7.

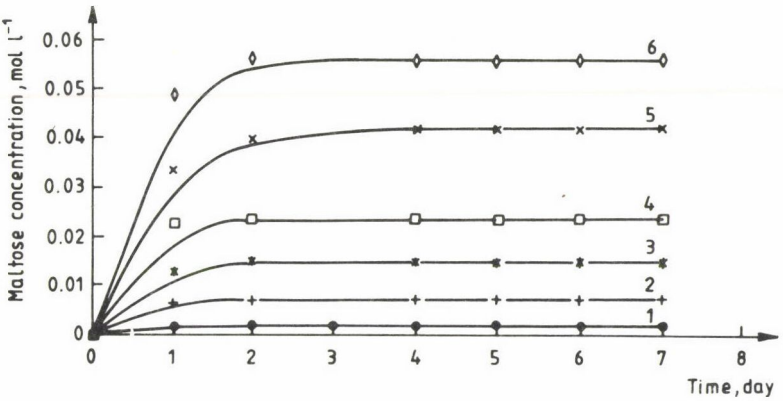

Fig. 4. Effect of initial glucose concentration on the formation of isomaltose. Initial glucose concentration (mol l^{-1}), 1: 0.592; 2: 1.222; 3: 1.737; 4: 2.247; 5: 2.953; 6: 3.603

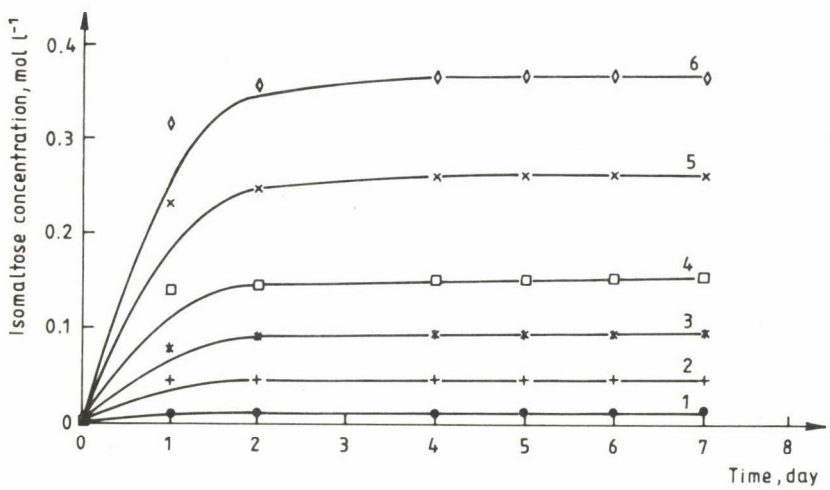

Fig. 5. Effect of initial glucose concentration on the formation of maltose. Initial glucose concentration (mol l^{-1}), 1: 0.592; 2: 1.222; 3: 1.737; 4: 2.247; 5: 2.953; 6: 3.603

The reversion rate of isomaltose, maltose and panose from 54% glucose with glucoamylase (25 GAU cm^{-3}) at 55 °C and pH 4.8 (0.05 mol l^{-1} acetate buffer) is presented in Fig. 8. It was observed that the concentration of maltose rapidly increased, then gradually decreased with slow reversion of glucose mainly into isomaltose. After long reactions times higher isomaltose concentration than maltose was observed. Actually, HEHRE and co-workers (1969) reported that maltose was synthesized rapidly and isomaltose was slowly synthesized by using the condensation activity of glucoamylase.

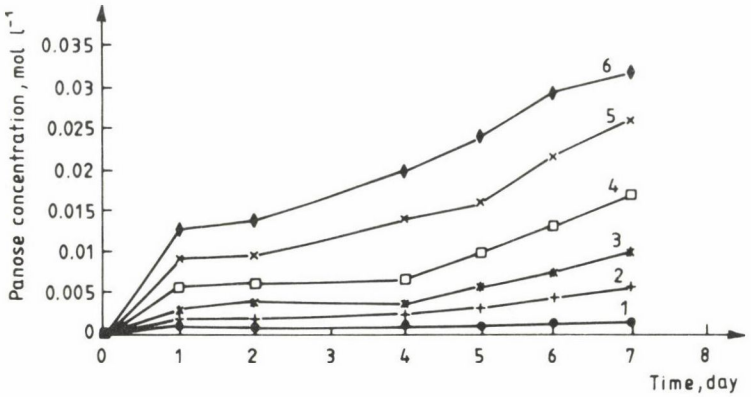

Fig. 6. Effect of initial glucose concentration on the formation of panose. Initial glucose concentration (mol l^{-1}), 1: 0.592; 2: 1.222; 3: 1.737; 4: 2.247; 5: 2.953; 6: 3.603

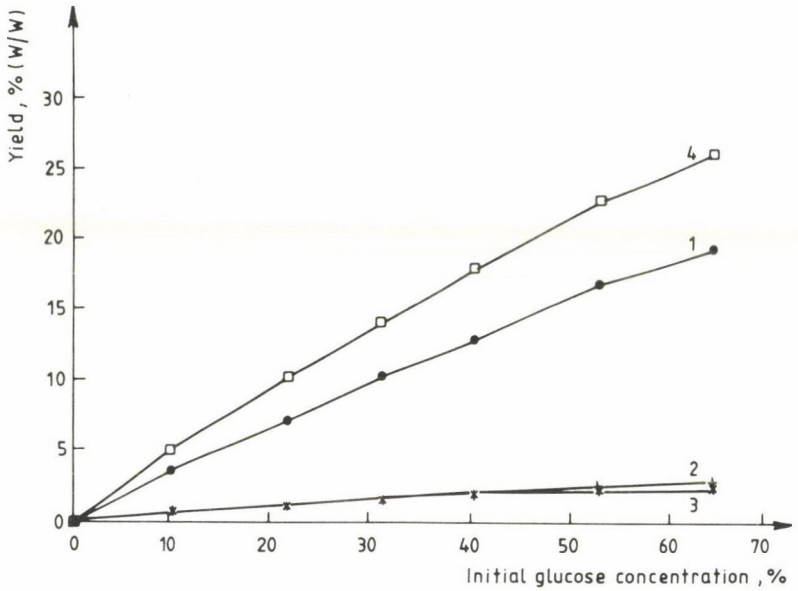

Fig. 7. Effect of initial glucose concentration on yields of isomaltose, maltose, panose and total reversion rate after 7 days. Yields in % after 7 days, 1: isomaltose; 2: maltose; 3: panose; 4: total reversion rate

Generally, in each cases the highest yield of oligosaccharides was attributed to the high glucose concentration used and also to a low water concentration per activity. In a 65% glucose solution, for example, the molar ratio of water to glucose was 8.72, which means that there was a stoichiometric process of glucose hydroxyl groups.

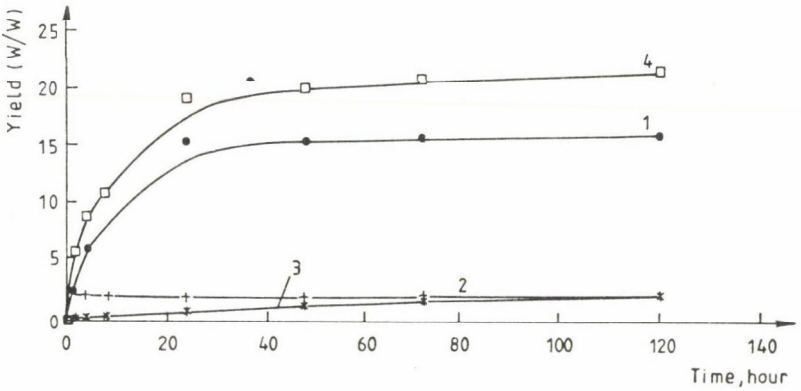

Fig. 8. Reversion rate of isomaltose, maltose and panose by glucoamylase with 54% glucose, pH 4.8, 55 °C and 25 GAU cm^{-3}. 1: Isomaltose, %; 2: maltose, %; 3: panose, %; 4: total reversion rate, %

2.2. Effect of temperature

The effect of temperature on equilibrium constants cannot generally be predicted but must be studied empirically.

From a thermodynamic point of view the system of condensation equilibria in a sugar solution is very complex, since the values for the free energy changes for the hydrolysis of most types of glycosidic bonds in oligosaccharides of different chain lengths are very similar. In this work the temperature effect on the total yield of oligosaccharides was tested by carrying out the reaction at 40, 50, 55 and 60 °C, at glucoamylase concentration of 130 GAU cm^{-3}, pH 4.2 (0.05 mol l^{-1} acetate buffer) and glucose solution of various concentration for 24 h (Fig. 1). It was observed that the yield of oligosaccharides increased by increasing the reaction temperature with each initial concentration of glucose.

The composition of oligosaccharides at 65% glucose after 24 h at 40, 50, 55 and 60 °C is summarized in Table 1 and illustrated in Fig. 9. The obtained results refer to the increase of isomaltose and panose concentrations by approximately 1.3 and 1.8 times, respectively, by raising the reaction temperature from 40 °C to 60 °C, while maltose was obtained in comparable amounts. From Table 1, it is obvious also that the total yield of oligosaccharides increased to about 1.3 times by raising the reaction temperature from 40 °C to 60 °C. But this increase is virtually temperature-independent because the composition of the oligosaccharides formed is independent of temperature. This behaviour was observed also by ROELS and TILBURG (1979).

Table 1

Temperature dependence of the yield of disaccharides and trisaccharides. Glucoamylase (130 GAU cm^{-3}) at 65% (w/v) D-glucose and pH 4.2 for 24 h

Temperature (°C)	Composition of oligosaccharides (%, w/w)			Yield (%, w/w)
	Isomaltose	Maltose	Panose	
40	13.65	2.18	1.51	17.34
50	15.67	2.30	1.92	19.89
55	16.78	2.28	2.25	21.31
60	18.34	2.34	2.68	23.36

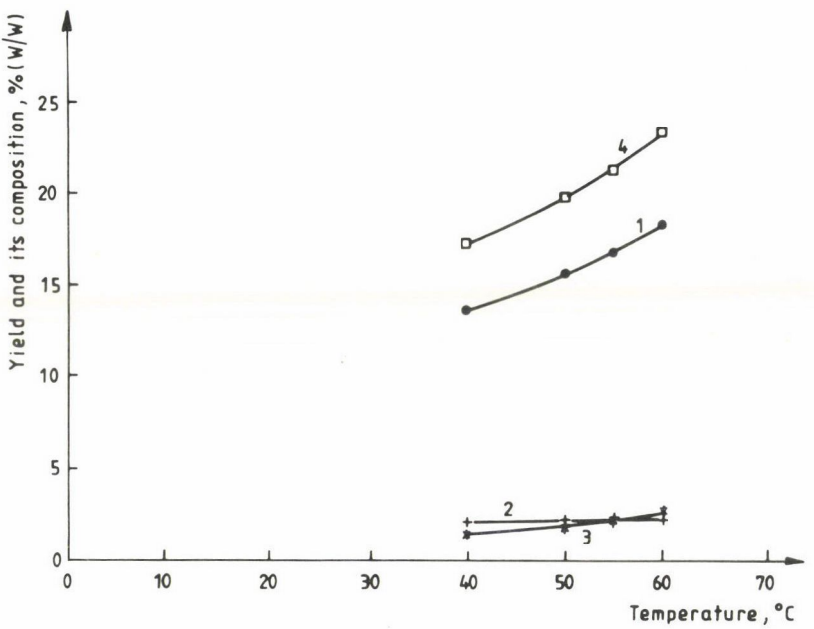

Fig. 9. Effect of temperature on yield and composition of oligosaccharides after 24 h. 1: Isomaltose (%); 2: maltose (5); 3: panose (%); 4: yield (%)

2.3. Effect of starch concentration

The appearance of glucose in hydrolysis of starch was carried out at 55 °C and pH 4.2 (0.05 mol l^{-1} acetate buffer) by enzyme concentration of 707 GAU per 100 cm^3 of starch solution using 10, 20, 30, 40 and 45% starch concentration. The results are illustrated in Fig. 10. It is obvious that the glucose concentration reached its maximum in a few hours after the start of the reaction. These results showed that

the maximum glucose yield from starch hydrolysis were 95.49, 94.95, 94.56, 94.25 and 93.65% attained by 10, 20, 30, 40 and 45% starch solutions with the corresponding unconverted fraction of substrate and resynthesis products 4.51, 5.05, 5.44, 5.75 and 6.35%, respectively. It means that the glucose was the only significant reaction product at the lowest starch level. Glucose concentrations as maximum conversions were reached in early stage and then gradually decreased as shown in Fig. 10. These observations also indicate that the reduction of the glucose concentration was due to the rapid formation of maltose and a slower synthesis of isomaltose as reported by HEHRE and co-workers (1969).

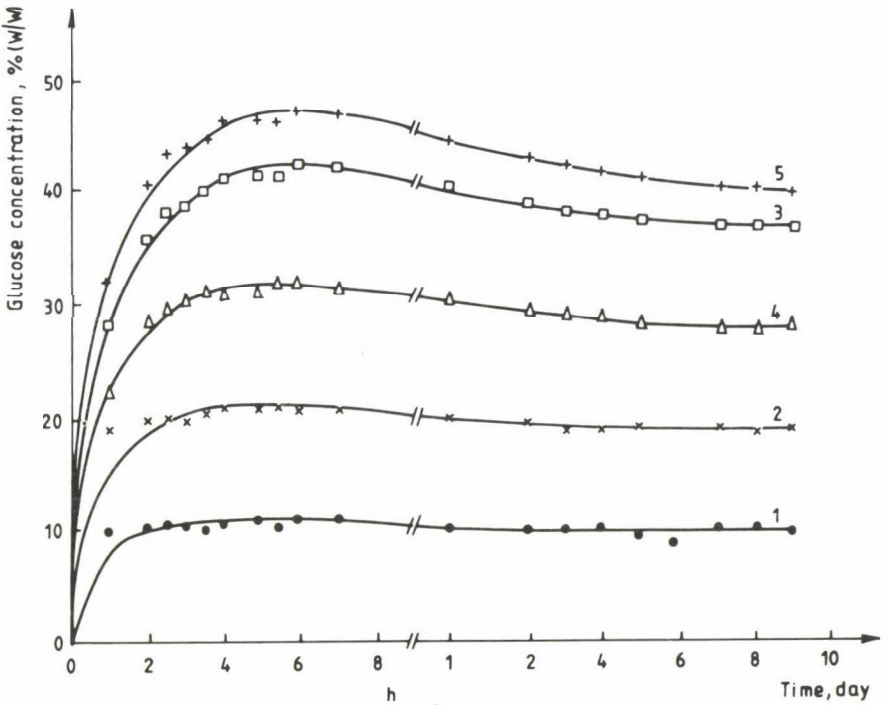

Fig. 10. Appearance of glucose concentration in hydrolysis of starch during 9 days. Initial starch concentrations in %, 1: 10; 2: 20; 3: 30; 4: 40; 5: 45

However, the appearance of oligosaccharides formation in hydrolysis of starch at the same conditions as above is represented in Fig. 11. It was observed that high yield of oligosaccharides was reached by increasing the glucose concentrations as maximum conversions attained from hydrolysis of starch. The yield of oligosaccharides reached maximum after about 9 days, which was 4.88, 7.79, 10.18, 12.16 and 14.73% with 10.61, 21.10, 31.52, 41.89 and 46.78% as maximum yield of glucose, respectively.

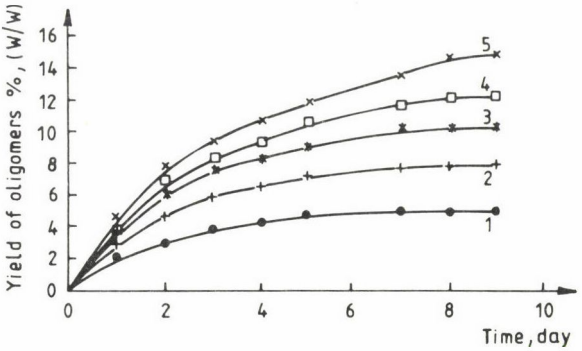

Fig. 11. Effect of starch concentration on the reversion rate of oligosaccharides. Starch concentration (w/v), 1: 10%; 2: 20%; 3: 30%; 4: 40%; 5: 45%

Figure 12 showed the effect of concentration of glucose hydrolyzed by using different starch concentrations on the synthesis of isomaltose as main by-product. It was clear that the concentration of isomaltose increases gradually, whereas becomes significant at the higher starch concentrations, at least after sufficiently long reaction times. Equilibrium or near equilibrium was reached after approximately 9 days, which contained an initial starch concentration of 10, 20, 30, 40 and 45% (w/w), isomaltose levels up to 3.8, 7.8, 9.4, 11.0 and 12% (w/w), respectively.

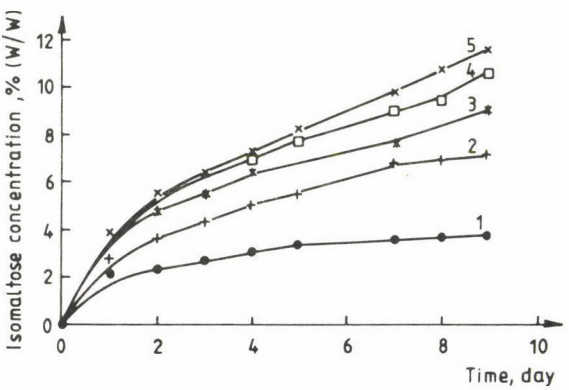

Fig. 12. Effect of starch concentration on the formation rate of isomaltose as main by-product. Starch concentration (w/v), 1: 10%; 2: 20%; 3: 30%; 4: 40%; 5: 45%

On the other hand, Fig. 13 exhibits yields of glucose, oligosaccharides and isomaltose at equilibrium or near equilibrium (after 9 days), maximum in glucose concentration as maximum conversion for hydrolysis of starch, and unconverted

fraction of starch at different initial concentration of starch. The order of the magnitude isomaltose yield was almost identical to the result demonstrated by BESCHKOV and co-workers (1984) and SHIRAISHI and co-workers (1985). It was evident that, when high concentrations of starch were hydrolyzed by glucoamylase, complete conversion to D-glucose was never obtained. Instead, the yield of D-glucose passes through a maximum and then decreases with time, and that was due to the reversion reactions (resynthesis), which reform oligosaccharides. The extent of that reversion became significant with increasing the initial concentration of starch and at prolonged incubation.

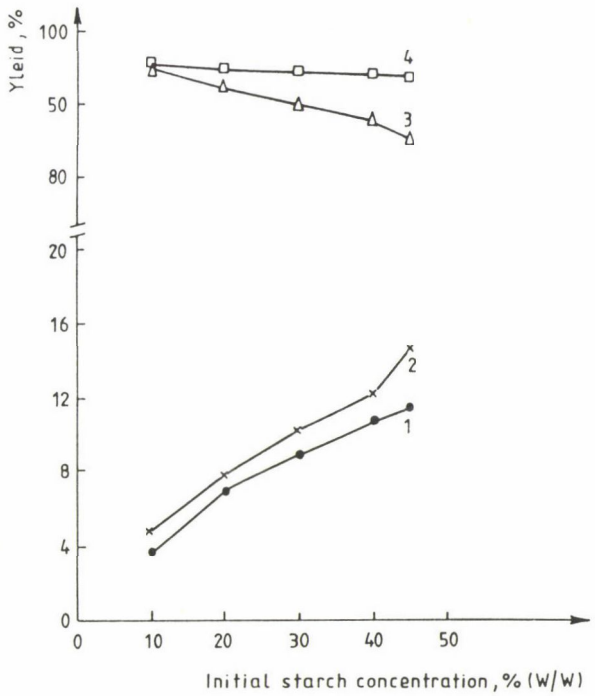

Fig. 13. Effect of initial starch concentration on yield in hydrolysis of starch at equilibrium condition after 9 days. 1: Isomaltose (%); 2: oligosaccharides (%); 3: glucose yield (%) after 9 days; 4: max. glucose concentration (%) after 7 h

2.4. Effect of enzyme concentration

The reaction was carried out at 50 °C, pH 4.4 (0.05 mol l^{-1} acetate buffer), 52% initial glucose concentration with seven enzyme concentrations (10, 20, 30, 40,

80, 160 and 200 GAU cm^{-3}). The results are shown in Fig. 14. The percentage of reversion rate of oligosaccharides after 2 days with 10, 20, 30, 40, 80, 160 and 200 GAU cm^{-3} glucoamylase was 4.94, 7.41, 11.33, 14.52, 18.06, 20.87 and 21.13%, respectively, while after 10 days was 18.98, 20.19, 21.73, 22.33, 23.00, 23.04 and 22.99%, respectively.

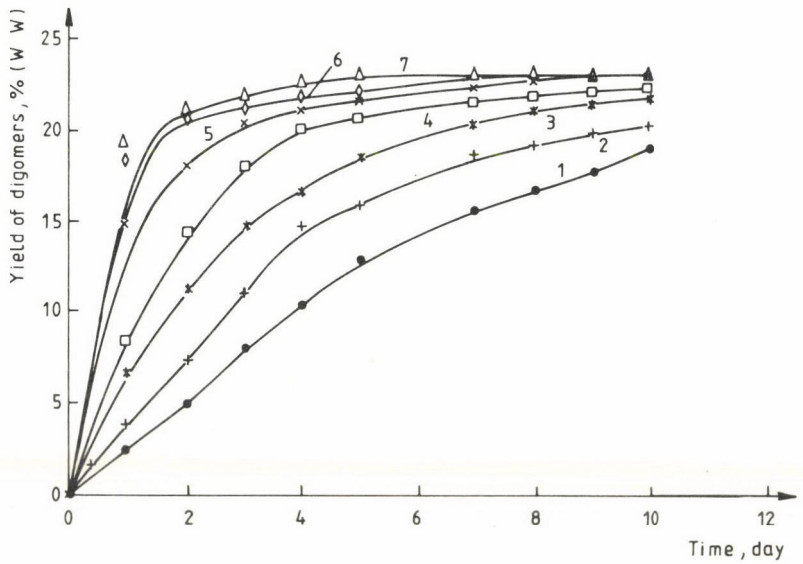

Fig. 14. Effect of enzyme concentration on reversion rate of oligosaccharides. Enzyme concentrations: 1: 10 GAU cm^{-3}; 2: 20 GAU cm^{-3}; 3: 30 GAU cm^{-3}; 4: 40 GAU cm^{-3}; 5: 80 GAU cm^{-3}; 6: 160 GAU cm^{-3}; 7: 200 GAU cm^{-3}

From Fig. 15, it was observed that after 2, 3, 4, 7 and 9 days equilibrium was reached (3.0±0.3) for isomaltose with 200, 160, 80, 40 and 30 GAU cm^{-3} glucoamylase, respectively, while with 10 and 20 GAU cm^{-3} glucoamylase the time required to reach equilibrium would be greater than 10 days. It was clear that the speed of process kinetics increased with increasing glucoamylase concentration. The final product distribution and the equilibrium constant were affected (Fig. 16). According to our knowledge the effect of soluble glucoamylase concentration on the reversion rate of oligosaccharides have not been reported in the literature, although they were of practical and theoretical importance.

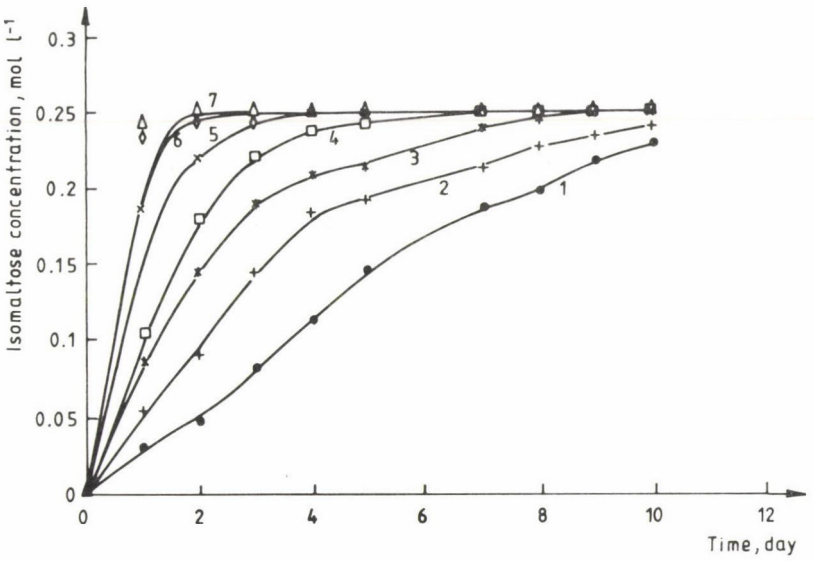

Fig. 15. Effect of enzyme concentration on the formation of isomaltose. Enzyme concentrations: 1: 10 GAU cm^{-3}; 2: 20 GAU cm^{-3}; 3: 30 GAU cm^{-3}; 4: 40 GAU cm^{-3}; 5: 80 GAU cm^{-3}; 6: 160 GAU cm^{-3}; 7: 200 GAU cm^{-3}

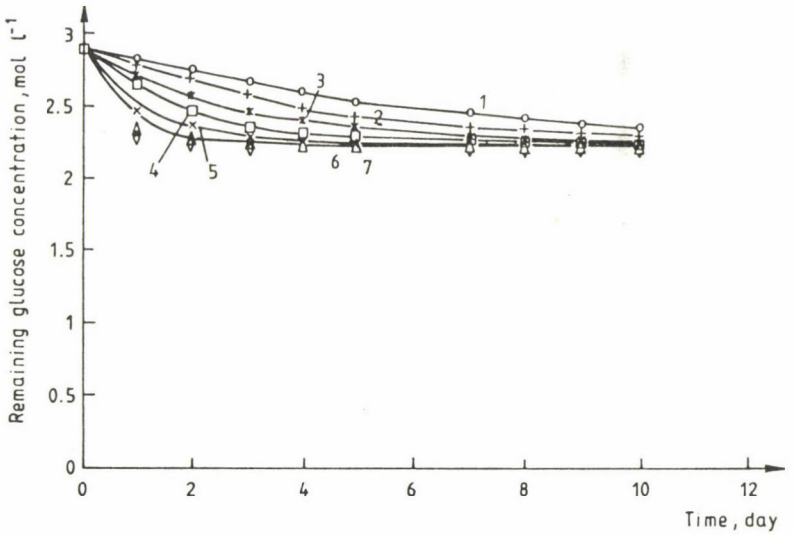

Fig. 16. Effect of enzyme concentration on remaining glucose concentration at pH 4.2, 50 °C and initial glucose concentration (52%, w/w). Amount of enzyme: 1: 10 GAU cm^{-3}; 2: 20 GAU cm^{-3}; 3: 30 GAU cm^{-3}; 4: 40 GAU cm^{-3}; 5: 80 GAU cm^{-3}; 6: 160 GAU cm^{-3}; 7: 200 GAU cm^{-3}

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CONDENSATION OF GLUCOSE BY THE REVERSED HYDROLYSIS REACTION OF GLUCOAMYLASE.

II. EFFECT OF INHIBITION OF GLUCOSE DERIVATIVES AND pH

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Glucose has two anomer types (α- and β-D-glucose). The inhibiting effect of the methyl-α- and methyl-β-D-glucoside, and 6-deoxy-D-glucose on synthesis of isomaltose as main product in the reaction mixture were investigated. It was found that the K_I values for methyl-α-D-glucoside and methyl-β-D-glucoside were 7.5 and 1.0 mol l^{-1}, respectively, which means that the role of the β-configuration of glucose for synthesis was confirmed and supported well. Thus, the K_I value for 6-deoxy-D-glucose was 0.41 mol l^{-1}, which indicates a much stronger inhibition compared to the methyl-α- and methyl-β-D-glucoside.

The effect of pH on the synthesis of isomaltose was also studied at 55 °C over the range of pH 3.0 to 7.0 and 54% (w/v) glucose solution. The optimum pH for the synthesis of isomaltose by the reversed hydrolysis reaction of glucoamylase from *A. niger* was approximately 4.8, which was in accordance with the values reported in the literature for the hydrolysis of starch (pH 4.5), maltose and isomaltose (pH 5.0). It means that both of the function groups of the glucoamylase take part in synthesis and hydrolysis, too.

Keywords: glucoamylase, glucose, glucose derivatives, synthesis

Evaluation of the enzymatic technological processes proves that the yield of dextrose-production from starch was mostly subject to the reverse synthesis which takes place at a high glucose concentration in the presence of glucoamylase or of acid. In the presence of high D-glucose levels these reversion products were also responsible for a decrease of the final product's dextrose equivalent (DE) value (KENNEDY et al., 1985). The reaction kinetics and the mechanism of the acid catalyzed reversion were investigated by HOLLÓ and co-workers (1967a and b).

The condensation of D-glucose to maltose and isomaltose by glucoamylase have been investigated by HEHRE and co-workers (1969) and the evidence obtained suggests that in the reversion reaction the enzyme requires donor substrates of specific anomeric configuration (β-D-glucopyranose) and that the mechanism of the condensation was one of glucosyl transfer in a hemiacetal-carbinol condensation.

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They have shown that the glucoamylase catalyzes the rapid synthesis of maltose and a slower synthesis of isomaltose, and low and limited production of other oligosaccharides. When maltose was the substrate under the same conditions, maltotriose and panose, but not isomaltose, were found in addition to D-glucose.

ONO and co-workers (1964) reported that β-D-glucopyranose was produced by hydrolysis of disaccharides by the glucoamylase from *Rhizopus delemar.*

PAZUR and ANDO (1960) treated the methyl-α-D-glucoside with amyloglucosidase and found that glucose was not produced in the digest even on prolonged incubation times.

HOLLÓ and co-workers (1973) investigated the inhibiting effect of various glucose derivatives on the joining, orientation and affinity of the substrate bonding at the active centre of glucoamylase which were promoted induced by the hydroxyl group on C_3, but as presumed by THOMA and KOSHLAND (1960), the fitting of the enzyme molecule was induced by the hydroxyl group on C_4 of the non-reducing chain end.

However, HOSCHKE (1978) studied the structure of active centre of glucoamylase in different pH regions, and he found that the active centre was in an active state at pH 2.0–5.1, but when there was an increase in pH and the pH value exceeds 5.1, this active structure gets loosened, then opens up and the enzyme losed its activity.

PAZUR and ANDO (1960) hydrolyzed the maltose and isomaltose by glucoamylase and found that the maximal values were obtained at pH 5.0 and have been used for calculating the relative activity (%) of the enzyme at the other pH values.

In the present study, the effect of D-glucose concentration on the formation of isomaltose only (as main product in the reaction) without inhibitor and in the presence of methyl-α-D-glucoside or methyl-β-D-glucoside or 6-deoxy-D-glucose as inhibitors were studied for indication of affinity of the substrate bonding at active centre of the enzyme molecule. Also, the effect of pH on synthesis of isomaltose was investigated to determine the pH-optimum for synthesis.

1. Materials and methods

1.1. Materials

For glucoamylase, D-glucose, isomaltose, maltose, isomaltotriose and panose the same sources as described in Part I were used.

Methyl-α-D-glucoside, methyl-β-D-glucoside and 6-deoxy-D-glucose were purchased from Sigma Chemical Co. (USA).

1.2. Methods

Determination of glucoamylase activity and analytical methods applied were previously described in Part I.

Conditions for reaction were carried out to study the inhibiting effect of glucose derivatives (methyl-α-D-glucoside, methyl-β-D-glucoside and 6-deoxy-D-glucose) on the formation of isomaltose. The glucoamylase-catalyzed reaction was allowed to run at pH 4.8 with 0.05 M acetate buffer at 50 °C in test tube immersed in a shaking water bath. The reaction was initiated by addition of glucoamylase solution (20 GAU cm^{-3}) to a D-glucose solutions (1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mol l^{-1}) without inhibitors and in the presence of inhibitors as methyl-α-D-glucoside, methyl-β-D-glucoside and 6-deoxy-glucose with concentration of 1.4, 0.5 and 0.5 mol l^{-1}, respectively. A part of the reaction mixture was taken to a boiling water bath for 7–10 min for enzyme denaturation, diluted with distilled water, filtered, and the products concentrations (specially isomaltose as main product) in the sample solution were analyzed by HPLC.

The hydrolysis of methyl-α-D-glucoside and methyl-β-D-glucoside by glucoamylase were tested under the same conditions as above, but no glucose indicating any hydrolysis was found after 24 h incubation. Also, the inhibiting effect of the 6-deoxy-D-glucose on the formation of isomaltose was studied under the same conditions by using an inhibitor concentration of 0.5 mol l^{-1} for 10 h, but no yields were obtained.

The examine the effect of pH on the synthesis of isomaltose, 54% D-glucose solution containing glucoamylase (25 GAU cm^{-3}) was incubated at 55 °C at pH ranging from 3.0 to 7.0 with 0.05 mol l^{-1} acetate buffer for 6 h.

2. Results and discussion

2.1. Inhibiting effect of glucose derivatives on the synthesis of isomaltose

HEHRE and co-workers (1969) stated that when starting the reaction with pure β-D-glucopyranose, the reversion rate was found to be much faster than with pure α-D-glucopyranose. ONO and co-workers (1964) reported that β-D-glucopyranose was produced by the hydrolysis of disaccharides by the glucoamylase from *Rh. delemar*. It means that if we want to explain the process of synthesis properly, the rate of mutarotation must taken into account whether we start the reaction with equilibrium glucose or with a pure β-D-glucose.

Concerning the above explanation by using the substrate analogue, which was α- and β-glucosidic hydroxyl blocked, the methyl-β-D-glucoside was expected to have a stronger blocking effect on synthesis than the methyl-α-D-glucoside.

Figure 1 shows the chromatograms for synthesis products by an initial glucose concentration of 3.5 mol l^{-1} at 50 °C, pH 4.8 (0.05 mol l^{-1} acetate buffer) and in the presence of 1.4 mol l^{-1} methyl-α-D-glucoside. The peaks identified with the standards were as follows:

1st methyl-α-D-glucoside	($k'_{rel.}$ = 0.6)
2nd glucose	($k'_{rel.}$ = 1.0)
4th maltose	($k'_{rel.}$ = 1.8)
5th isomaltose	($k'_{rel.}$ = 2.0)
7th panose	($k'_{rel.}$ = 3.4)
8th isomaltotriose	($k'_{rel.}$ = 4.2)

The third and the sixth peaks could probably be identified as methyl-α-isomaltose ($k'_{rel.}$ = 1.3) and methyl-α-panose ($k'_{rel.}$ = 2.7), respectively. However, in the series of chromatograms, it was obvious that the concentration of maltose increases rapidly, then gradually decreases. The methyl-α-dimer and isomaltose peaks were increased when the maltose peak reached its maximum, which indicated the consecutive reaction behaviour.

The hydrolysis of methyl-α- and methyl-β-D-glucosides by glucoamylase was tested, but no yields indicating any breakdown was found. This result is in agreement with the results of the isotopic measurements carried out by PAZUR and ANDO (1960).

Effect of glucose concentration (1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mol l^{-1}) on the formation of isomaltose only (as main product in the reaction) without inhibitor (Fig. 2) and in the presence of 1.4 mol l^{-1} of methyl-α-D-glucoside or 0.5 mol l^{-1} of methyl-β-D-glucoside (Figs 3 and 4) were investigated Based on the conversion curves, the initial velocity rates were determined graphically (Table 1) and plotted them versus the substrate concentrations according to the method of Lineweaver and Burk, the plot of $1/V_0$ against $1/G_0$ (Fig. 5), and according to the method of Hanes, the plot of G_0/V_0 against G_0 (Fig. 6).

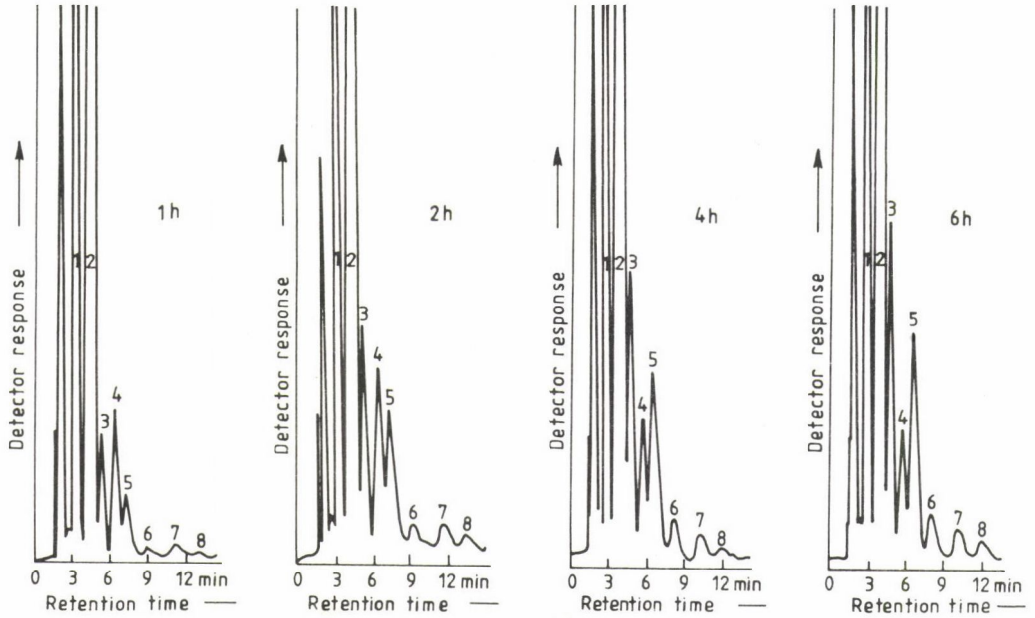

Fig. 1. HPLC chromatograms of oligosaccharides synthesized by glucoamylase from D-glucose in the presence of methyl-α-D-glucoside under these conditions: G_0 = 3.5 mol l^{-1}, methyl-α-D-glucoside = 1.4 mol l^{-1}, E_0 = 25 GAU cm^{-3} at 50 °C and pH 4.8 (Spherisorb-NH_2, 250×4.6 mm, $CH_3CN:H_2O$ = 75:25, 2 cm^3 min^{-1} and RI 8×). Peaks: 1. M-α-D-glucoside, 2. glucose, 3. M-α-isomaltose, 4. maltose, 5. isomaltose, 6. M-α-panose, 7. panose and 8. isomaltotriose

Table 1

Initial velocity for synthesis of isomaltose by glucoamylase with and without inhibitors at 50 °C, 20 GAU cm^{-3} and pH 4.8

Initial glucose conc. (mol l^{-1})	Initial velocity (M/h) × 10^{-3}			
	Without inhibitor	With inhibitor		
		m-α-D-glucoside (1.4 mol l^{-1})	m-β-D-glucoside (0.5 mol l^{-1})	6-deoxy-D-glucose (0.5 mol l^{-1})
1.0	5.24	–	4.10	–
1.5	7.61	6.57	–	3.48
2.0	9.13	7.81	7.52	4.57
2.5	11.57	10.50	–	5.38
3.0	13.18	–	10.43	–
3.5	–	12.68	–	6.59
4.0	15.85	–	12.39	–

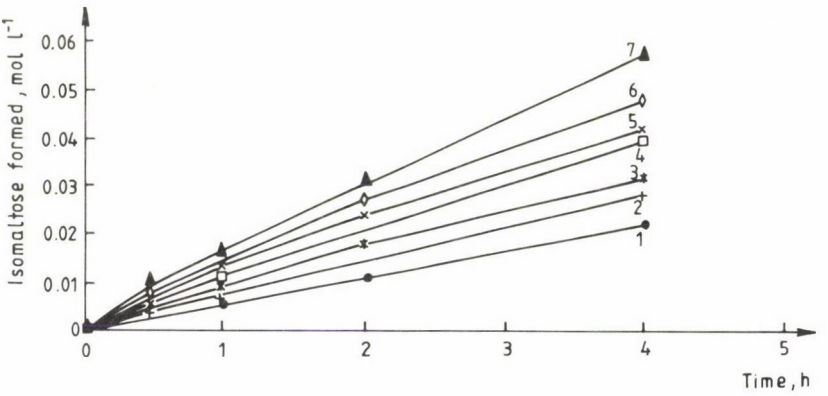

Fig. 2. Formation of isomaltose vs. time with different glucose concentrations. Initial glucose concentration (mol l^{-1}), 1: 1.0; 2: 1.5; 3: 2.0; 4: 2.5; 5: 3.0; 6: 3.5; 7: 4.0

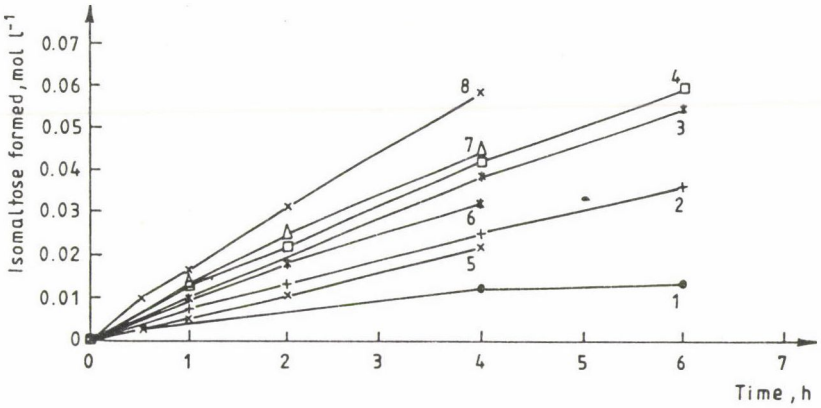

Fig. 3. Formation of isomaltose vs. time with different glucose concentrations in the presence of methyl--α-D-glucoside (1.4 mol l^{-1}). Inhibitor and glucose concentration (mol l^{-1}), 1: I+G 1.5; 2: I+G 2.0; 3: I+G 2.5; 4: I+G 3.5; 5: G 1.5; 6: G 2.0; 7: G 2.5; 8: G 3.5

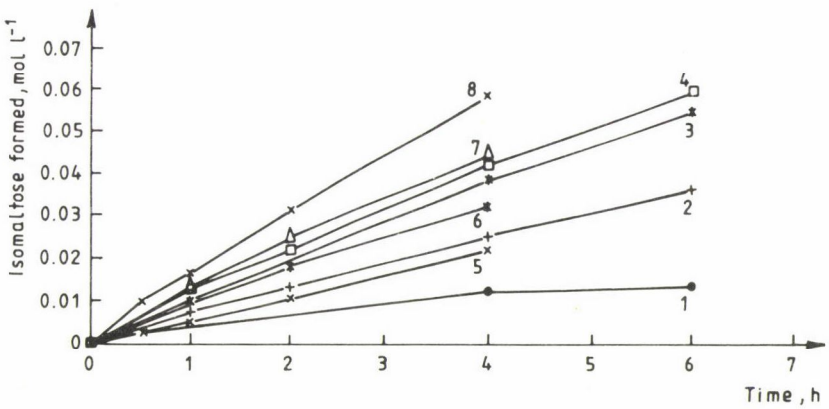

Fig. 4. Formation of isomaltose vs. time with different glucose concentrations in the presence of methyl--β-D-glucoside (0.5 mol l^{-1}). Inhibitor and glucose concentration (mol l^{-1}), 1: I+G 1.0; 2: I+G 2.0; 3: I+G 3.0; 4: I+G 4.0; 5: G 1.0; 6: G 2.0; 7: G 3.0; 8: G 4.0

As it can be seen in the Figs 5 and 6, the inhibition was purely competitive. The abscissa sections of the Lineweaver–Burk plots were $1/Km = -0.12$, $-1/Km(1+[I]/KI) = -0.10$ or $= -0.08$ for the enzymatic reactions determined without inhibitor (only glucose), with methyl-α-D-glucoside or methyl-β-D-glucoside, respectively. The KI values for methyl-α- and methyl-β-D-glucoside were 7.5 and 1.0 mol l^{-1}, respectively. It means that the role of the β-configuration of glucose for synthesis was confirmed and supported well by inhibition kinetics.

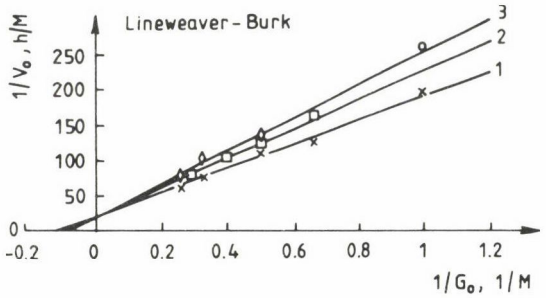

Fig. 5. Velocity of isomaltose formation depending on initial glucose concentration with/without the presence of methyl-α-D-glucoside or methyl-β-D-glucoside. 1: G. without inhibitor, $y = 20.1 + 170.51\ x$, $r^2 = 0.99$; 2: G. + M-α-D-glucoside, $y = 20.2 + 200.94\ x$, $r^2 = 0.97$; 3: G. + M-β-D-glucoside, $y = 21.3 + 228.90\ x$, $r^2 = 0.99$

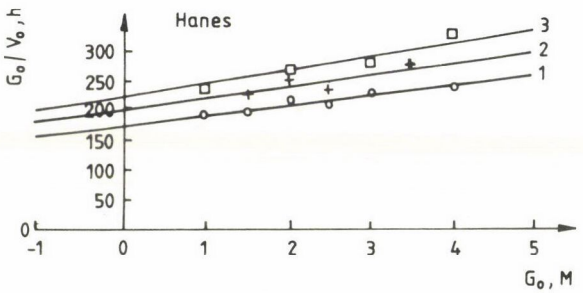

Fig. 6. Velocity of isomaltose formation depending on initial glucose concentration with/without the presence of methyl-α-D-glucoside or methyl-β-D-glucoside. 1: G. without inhibitor, $y = 173.85 + 18.29\ x$, $r^2 = 0.97$; 2: G. + M-α-D-glucoside, $y = 201.32 + 20.34\ x$, $r^2 = 0.83$; 3: G. + M-β-D-glucoside, $y = 223.20 + 23.30\ x$, $r^2 = 0.97$

The inhibiting effect of the 6-deoxy-D-glucose on the formation of isomaltose was also studied under the same condition of the methyl-glocusidic experiments by using an inhibitor concentration of 0.5 mol l^{-1}. The importance of this experiment is twofold. First, the main product in the reaction during the synthesis was α-(1→6)--glycosidic bond (isomaltose). Second, the joining, orientation and affinity of the substrate binding at the active centre were promoted and induced by the hydroxyl groups (THOMA & KOSHLAND, 1960 and HOLLÓ et al., 1973).

The pure 6-deoxy-D-glucose was incubated with glucoamylase, then experiments were carried out at various glucose concentrations. The curves and relationships developed by Linewaver–Burk and Hanes were illustrated in Figs 7, 8 and 9, respectively.

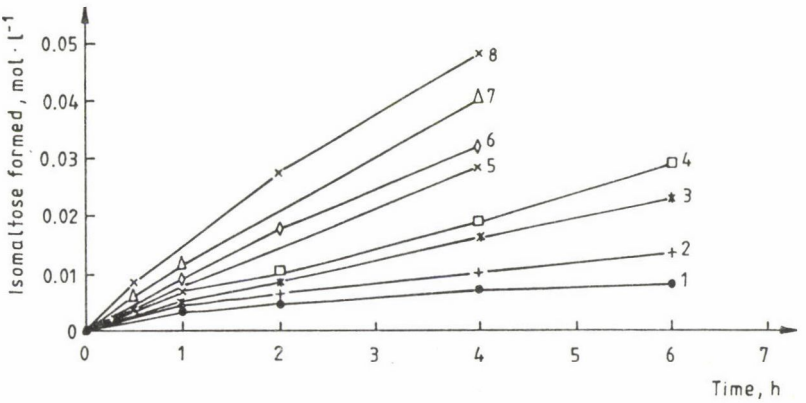

Fig. 7. Formation of isomaltose vs. time with different glucose concentrations in the presence of 6-deoxy-D-glucose (0.5 mol l^{-1}). Inhibitor and glucose concentration (mol l^{-1}), 1: I+G 1.5; 2: I+G 2.0; 3: I+G 2.5; 4: I+G 3.5; 5: G 1.5; 6: G 2.0; 7: G 2.5; 8: G 3.5

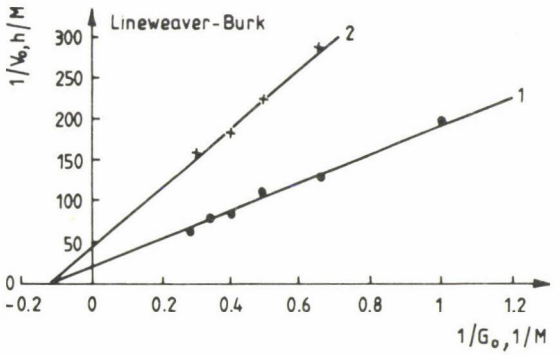

Fig. 8. Velocity of isomaltose formation depending on initial glucose concentrations with/without the presence of 6-deoxy-D-glucose, 1: G. without inhibitor, $y = 20.1 + 170.51\ x$, $r^2 = 0.99$; 2: G. + 6-deoxy-D-glucose, $y = 44.65 + 357.62\ x$, $r^2 = 0.98$

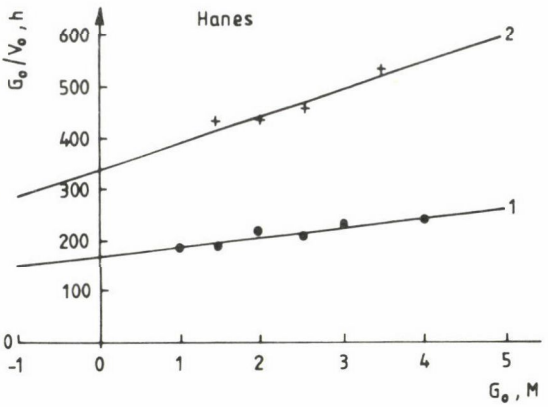

Fig. 9. Velocity of isomaltose formation depending on initial glucose concentrations with/without the presence of 6-deoxy-D-glucose, 1: G. without inhibitor, y = 172.85 + 18.29 x, r^2 = 0.97; 2: G. + 6-deoxy-D-glucose, y = 341.98 + 52.26 x, r^2 = 0.95

When incubating the 6-deoxy-D-glucose only with the enzyme at 50 °C for 6 h, no products were obtained as synthesis of α-6-deoxy-D-glucopyranosyl-(1→4)-6--deoxy-D-glucose or α-6-deoxy-D-glucopuranosyl-(1→3)-6-deoxy-D-glucose, which means that due to the absence of primary alcohol group (hydroxyl group on the C_6), no reactive-complex can be formed.

From Fig. 8, it was observed that the abscissa sections of Lineweaver–Burk plots (−1/Km) was equal to −0.12 for both the uninhibited reaction (only glucose) and in the presence of 6-deoxy-D-glucose, while the intercepts of ordinate were $1/V_{max}$ = 20.10 and $1/V_{max}$ (1+[I]/KI) = 44.65, respectively. The value of inhibition constant was calculated and it is equal to K_i = 0.41 mol l^{-1}, which indicates a much stronger inhibition compared to the methyl-α- and methyl-β-D-glucoside.

2.2. Effect of pH

The pH-dependence of the enzymatic hydrolysis was already known. GHALI and co-workers (1980) studied the pH-dependence of the initial rates of hydrolysis of soluble starch by the soluble glucoamylase from *A. niger* and found that the pH-optimum was 4.5 (starch No. 1), while SZAJÁNI and co-workers (1985) showed that the optimum pH was 4.0 (starch No. 2). However, PAZUR and ANDO (1960) hydrolyzed the maltose and isomaltose by glucoamylase and found that the maximum values were obtained at pH 5.0, and have been used for calculating the relative activity (%) of the enzyme at other pH values. Also we have determined the pH optimum for synthesis, too. We believe that if the measured data of pH optimum for isomaltose synthesis correspond with the data measured by the above authors for

hydrolysis, that would support and prove our hypothesis discussed below (synthesis mechanism).

The effect of pH on the synthesis of isomaltose: 54% (w/v) glucose solution containing glucoamylase (25 GAU cm^{-3}) from *A. niger* was incubated at 55 °C and at pH ranging from 3.0 to 7.0 for 6 h. The results were listed in Table 2 and illustrated in Fig. 10. It could be recognized from Table 2 and Fig. 10 that an optimum pH for the synthesis of isomaltose by reversed hydrolysis reaction of glucoamylase was approximately 4.8, which agreed with the values reported in the literature for the hydrolysis of starch, maltose and isomaltose (PAZUR & ANDO, 1960; GHALI et al., 1980 and SZAJÁNI et al., 1985). The difference between the dissociation constants for the two catalytic groups of the glucoamylase was much wider (WHITAKER, 1972). Since we could be right to assume that both of the function groups of glucoamylase active centre take part in synthesis and hydrolysis, too.

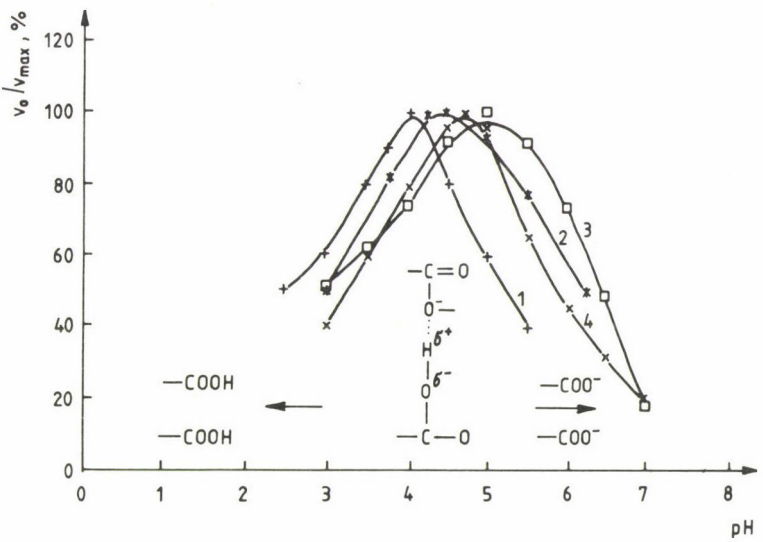

Fig. 10. Effect of pH on the reaction velocity during: hydrolysis of starch and isomaltose, and synthesis of isomaltose. 1: Hydrolysis of starch 1; 2: hydrolysis of starch 2; 3: hydrolysis of isomaltose; 4: synthesis of isomaltose

Table 2

Effect of pH on the formation of isomaltose by glucoamylase. At 55 °C, 54% (w/v) of D-glucose and 25 GAU cm^{-3} for 6 h

pH	Isomaltose formed		Rel. velocity
	(g/100 cm^3)	(mol/l) × 10^{-2}	(V_0/V_{max})
3.0	1.53	4.48	0.40
3.5	2.30	6.72	0.60
4.0	3.03	8.85	0.79
4.5	3.68	10.75	0.96
4.8	3.83	11.20	1.00
5.0	3.63	10.64	0.95
5.5	2.49	7.28	0.65
6.0	1.72	5.03	0.45
6.5	1.22	3.57	0.32
7.5	0.77	2.25	0.20

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CHARACTERISTIC OF *LEUCONOSTOC OENOS* STRAINS OCCURRING IN HUNGARIAN WINES

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Twenty-one strains of *Leuconostoc oenos* were isolated and identified from wines of region Eger. These strains are of great technological importance, because they are able to convert malic acid to lactic acid by malolactic fermentation (MLF). This is a slow but useful process, specially in red wines.

The most frequently occurring strain of *Leuconostoc oenos* was isolated from red wines "Egri bikavér", in 1992. This strain was studied in detail, and its growth characteristics were determined.

Keywords: *Leuconostoc oenos*, malolactic fermentation (MLF), specific growth rate, tomato juice factor (TJF)

Leuconostoc oenos occurs only in wines and related habitats, so it seems to be well adapted to MLF. The species was described by GARVIE (1967).

Since then it has been frequently found in musts and wines of different countries all over the world, and reported on the factors of its MLF activity and carbohydrate fermentation (MEYRATH, 1969, LAFON-LAFOURCADE, 1975, KUNKEE, 1975, WIBOWO et al., 1985).

Malolactic fermentation is an important process in winemaking. Malic acid is converted to lactic acid and CO_2 by various species of *Lactobacillus*, *Pediococcus* and *Leuconostoc*. This activity results in an increasing pH, so it is desirable in wines of high acidity (COSTELLO, 1983).

Earlier, enologists have traditionally relied on the development of the natural bacterial contaminants of grapes to perform MLF, but this process was slow (KUNKEE, 1965).

The new method is to inoculate the wines with selected strains. Since *Leuconostoc oenos* is better adapted to high acidity than lactobacilli or pediococci, this species is most frequently used in France in winemaking (LAFON-LAFOURCADE et al., 1983). However, the SO_2 and ethanol content are limiting factors for the

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bacterial growth (CARR et al., 1976). *Leuconostoc oenos* strains are usually inhibited by 40–50 mg l^{-1} total SO_2 and at 12–15% alcohol content (BRITZ, 1990).

Synthetic media, such as MRS or modified Rogosa broth permit a poor growth or *Leuconostoc oenos* (PILONE et al., 1976). The tomato juice factor, glucopanthenate has an important stimulatory effect on the growth of *Leuconostoc oenos*, because it prefers this glucoderivate form of panthenic acid (AMACHI, 1971). Fructose is a natural substrate in musts and wines besides malic acid, so it is recommended for heterofermentative lactic acid bacteria utilizing fructose (ZUNIGA et al., 1992).

The aim of present work is to study the growth of *Leuconostoc oenos* as a function of pH and selected the best medium for culturing.

1. Materials and methods

1.1. Isolation

The strains were isolated from wines of Eger region (Table 1).

Table 1

Sources of isolates

Wine name	Type	Year	No. of isolates
Egri kékfrankos	red	1989	3
Egri kékfrankos	red	1990	1
Egri bikavér	red	1991	4
Egri bikavér	red	1992	12
Egri leányka	white	1993	1

The characteristic parameters of the wines from which bacteria were isolated are summarized in Table 2.

Table 2

Characteristic parameters of the wines

Parameter	Red wines	White wine
pH	3.14–3.52	3.32
Total SO_2 (mg l^{-1})	34–44	8
Free SO_2 (mg l^{-1})	6–12	2
Malic acid (g l^{-1})	0.68–2.05	2.5
Alcohol (%)	9.5–12.5	10.9

Strains were isolated from young wine samples inoculated in C–104 broth. (Yeasts were inhibited by the addition of 0.01% cycloheximide.)

The tubes were sealed with molten Vaspar and incubated at 27 °C for 7–10 days.

1.2. Identification

Identification was performed according to Bergey's manual (SNEATH et al., 1986). The morphological, physiological and biochemical characteristics were tested regularly, as described by GARVIE (1967, 1980, 1986).

The following tests were made: microscopic morphology, motility, Gram stain, catalase reaction, type of fermentation, hydrolysis of arginin, growth at 5, 15, 25, 30, 40 °C, growth at pH 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, dissimilation of malate, tolerance of 10% ethanol, stimulative effect of TJF.

The fermentation of amygdalin, arabinose, cellobiose, esculin, fructose, galactose, lactose, maltose, mannitol, mannose, melecitose, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, saccharose, trehalose and xylose were determined at 27 °C, after 2 weeks.

1.3. Growth media

Composition of the four different growth media used in comparative studies are summarized in Table 3.

1.3.1. C–104 broth. It was recommended as a medium for isolation of MLF bacteria by the Hungarian Winemaking Committee.

1.3.2. MRS broth. This medium is widely used for cultivation of lactic acid bacteria (MAN et al., 1960). Manganese, acetate and oleic acid esters, especially Tween 80, are essential for most species therefore these compounds are included in MRS broth.

1.3.3. MRS-TJ broth. Supplementation of the MRS broth with tomato juice can give a stimulatory effect, especially for strains, requiring gluconopanthenate.

1.3.4. F–104 broth. Leuconostoc oenos is a wine-adapted bacterium, consequently fructose and malic acid are most commonly used substrates by this species. Hence, the F–104 broth is expected to be a rich medium supporting well the growth.

Table 3

Composition of the isolation and growth media

Components ($g\,l^{-1}$)	Type of media			
	C-104	MRS	MRS-TJ	F-104
Pepton	5	10	10	5
Meat extract	–	10	10	–
Yeast extract	5	5	5	5
Glucose	–	20	20	–
Fructose	–	–	–	20
L-malic acid	3	–	–	3
K_2HPO_4	–	5	5	–
Tween 80	0.1 cm^3	1 cm^3	1 cm^3	0.1 cm^3
$MgSO_4 \cdot 7H_2O$	0.05	0.5	0.5	0.05
$MnSO_4 \cdot 2H_2O$	0.05	0.2	0.2	0.05
$(NH_4)_2$-citrate	–	2	2	–
Na-acetate	–	5	5	–
Tomato juice	250 cm^3	–	250 cm^3	250 cm^3
Cyclohexamide	0.1	–	–	–
pH	4.5	4.5	4.5	4.5

1.4. Preparation of filtered tomato juice

Hundred g tomato paste (Hungarian trade mark "Aranyfácán", product of 25% Brix value) was 10-times diluted in distilled water and after 30 min setting in was centrifuged at 1500x g for 5 min, and the supernatant was filtered through Sartorius membrane filter (pore size: 1,2 μ).

1.5. Growth experiments

From each broth 10–10 cm^3 were dispended into steril tubes. (All media were sterilized by autoclaving for 20 min at 115 °C.)

The inoculum was prepared in the appropriate broth. Fresh 3-day cultures were used for inoculation, in 1% (v/v) ratio. The inoculated tubes were incubated at 27 °C.

Growth was followed by measuring of absorbance at 560 nm (SPECOL spectralcolorimeter), there times every day for a week.

Each experiment was carried out in two replicates.

1.6. Determination and comparison of growth characteristics

Growth curves were estimated by a computer program designed especially for the calculation of growth characters (REICHART, 1984). The specific growth rate (u_{max}, h^{-1}) and the generation time (g, h) were determined.

The maximum of absorbance (A_{max}) is also useful for characterizing the growth of microorganisms, because it reflects the cell biomass produced.

For this reason the A_{max} values were determined after 7 days, when the stationary growth phase was attained in all cases.

The specific growth rates and the maximum of absorbance of *Leuconostoc oenos* 1 were compared at different pH and in different broth.

Data were evaluated by one-way analysis of variance, according to SVÁB (1973).

The F-test of the one-way analysis of variance showed significant differences between the treatments at 99.9% level of probability. So the means of growth characteristics, u_{max} and A_{max} were compared at 99.9, 99 and 95% level of probability, respectively.

2. Results and discussion

2.1. Characterization of Leuconostoc oenos *strains*

Twenty-one strains were isolated from wines of region Eger.

Table 4

General characteristics of isolated Leuconostoc *strains*

Morphology:	coccoid cells in pairs and chains
Motility:	–
Gram strain:	+
Catalase reaction:	–
Growth, aerobic:	+ } aerotolerant – anaerobic
Growth, anaerobic:	+
Fermentation:	Heterolactic (acid and CO_2 from glucose and gluconate)
Growth temperature	5 °C: –
	15 °C: +
	25 °C: +
	30 °C: +
	40 °C: –
Growth at initial	pH = 3.5: +
	pH = 4.5: +
	pH = 5.5: +
	pH = 6.5: +
Stimulatory effect of TJF:	+
Dissimilation of malate:	+
Tolerance to 10% ethanol:	+
NH_3 from arginine:	–

All were similar in 20 fundamental tests. The results of morphological and physiological tests are summarized in Table 4.

Accordingly, these strains belonged to the well-defined Gram-positive, heterolactic group, the *Leuconostoc* genus.

An easy species assignment could be made, because all strains were able to grow at initial pH = 4.5 and 3.5, which is characteristic in this genus only to *Leuconostoc oenos*.

2.2. Biochemical properties of Leuconostoc oenos *strains*

The carbohydrate fermentation patterns of isolates are summarized in Table 5.

Results of 20 carbohydrate fermentations supported the identification of isolated strains with *Leuconostoc oenos*, according to Bergey's criteria determined by methods of GARVIE (1986).

All strains were characterised by strong fermentation of fructose, ribose and a moderate fermentation of trehalose and esculin. Strains were alike in failing to ferment amygdalin, arabinose, lactose, maltose, mannitol, melicitose, raffinose, rhamnose and sorbitol.

As it is shown in Table 5, five types of *Leuconostoc oenos* strains could be distinguished according to wine samples and year of isolation. These were only "ecological" types within the species because delayed fermentation of cellobiose, salicin and xilose are allowed by Bergey's criteria for *Leuconostoc oenos*.

The strains isolated from red wines were able to ferment neither arabinose nor xylose. These characters were remarkable, because in the French literature of winemaking and microbiology numerous references have been made to similar strains as *Leuconostoc "gracile"*. However, this name is not recognized in current classification, and these strains are considered only for a type of *Leuconostoc oenos* (WIBOWO et al., 1985).

The most frequently isolated type, *Leuconostoc oenos* 1, was examined in details.

The growth characteristics of this type of *Leuconostoc oenos*, isolated from wine of Egri bikavér was measured at different pH values and in different broth media.

Table 5

Carbohydrate fermentation patterns of Leuconostoc strains

Carbo-hydrates	*Leuconostoc oenos*	Type of strain isolated from region Eger				
		Red wines				White wine
		1	2	3	4	5
Acid from	Borgey's criteria 1986	Egri bikavér 1992	Egri bikavér 1991	Egri kékfrankos 1989	Egri kékfrankos 1990	Egri leányka 1993
Amygdalin	d	–	–	–	–	–
Arabinosa	d	–	–	–	–	–
Cellobiose	d	–	(d)	(d)	(d)	–
Esculin	+	+	+	+	+	+
Fructose	+	+	+	+	+	+
Galactose	d	(d)	–	–	+	–
Lactose	–	–	–	–	–	–
Maltose	–	–	–	–	–	–
Mannitol	–	–	–	–	–	–
Mannose	d	–	–	–	–	+
Melicitose	ND	–	–	–	–	–
Melibiose	d	–	–	–	–	+
Raffinose	–	–	–	–	–	–
Rhamnose	–	–	–	–	–	–
Ribose	+	+	+	+	+	+
Salicin	d	–	–	+	–	+
Sorbitol	ND	–	–	–	–	–
Saccharose	–	–	–	–	–	–
Trachalose	+	+	+	+	+	+
Xylose	d	–	–	–	–	+
Number of strains		17	4	3	1	1

Symbols: d : 11–89% strains positive
(d): delayed reaction
ND: not determined
+ : 90%–100% strains positive
– : 90%–100% strains negative

2.3. The effect of pH on the growth of Leuconostoc oenos *1*

The effect of the different pH-values was evaluated by one way analysis of variance.

The means of specific growth rates at different pH values are demonstrated in Fig. 1.

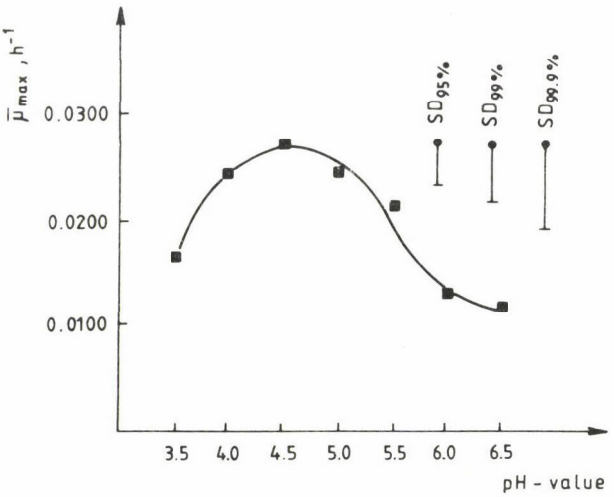

Fig. 1. The pH-optimum curve of *Leuconostoc oenos* 1 in MRS broth at 27 °C

Figure 1 shows the pH optimum curve of *Leuconostoc oenos* 1, with the significant differences at 95, 99, and 99.9% level of probability. The pH-optimum was found at pH = 4.5.

The specific growth rate decreased with increasing pH. At pH = 5.5, the difference was significant at 95% level of probability, compared to the growth at pH = 4.5.

The growth of *Leuconostoc oenos* 1 was very slow at pH = 6.0 and at pH = 6.5. These specific growth rates differed very highly from the optimum, at 99.9% level of probability.

The specific growth rate was also significantly less at pH = 3.5 than at pH = 4.5. The difference between these values was very highly significant.

Nevertheless, the growth rate was significantly larger at pH = 3.5 than at pH = 6.5, the difference was significant at 95% level of probability. It can be concluded that *Leuconostoc oenos* 1 is better adapted to acidity than to the neutral pH range.

2.4. Effect of media composition on the growth of Leuconostoc oenos *1*

Figures 2–5 show the growth curves of *Leuconostoc oenos* 1 at 27 °C, in the basal and supplemented broths.

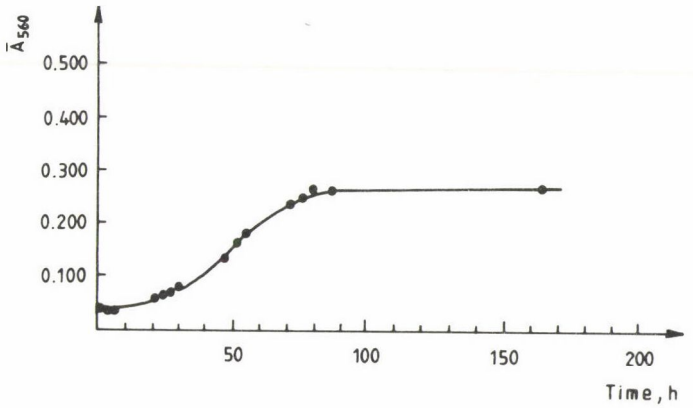

Fig. 2. Growth curve of *Leuconostoc oenos* 1 in MRS broth, at pH = 4.5, at 27 °C

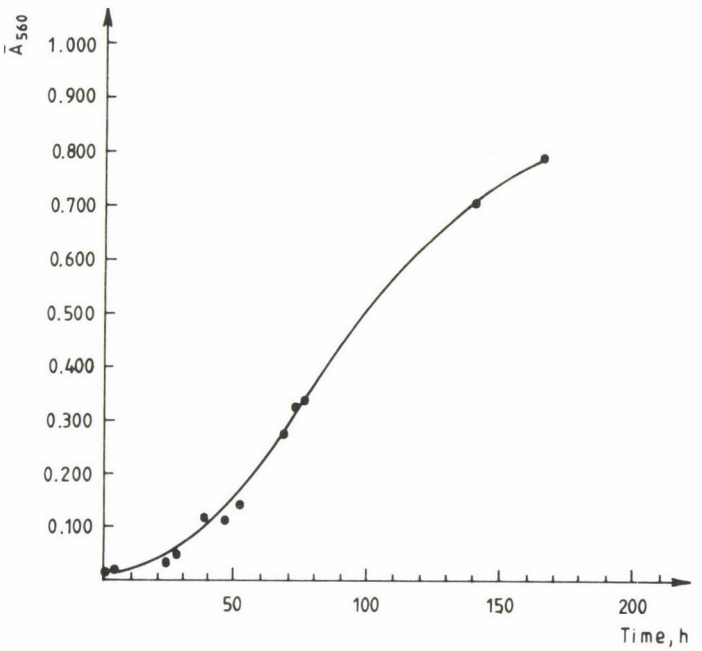

Fig. 3. Growth curve of *Leuconostoc oenos* 1 in MRS–TJ broth, at pH = 4.5, at 27 °C

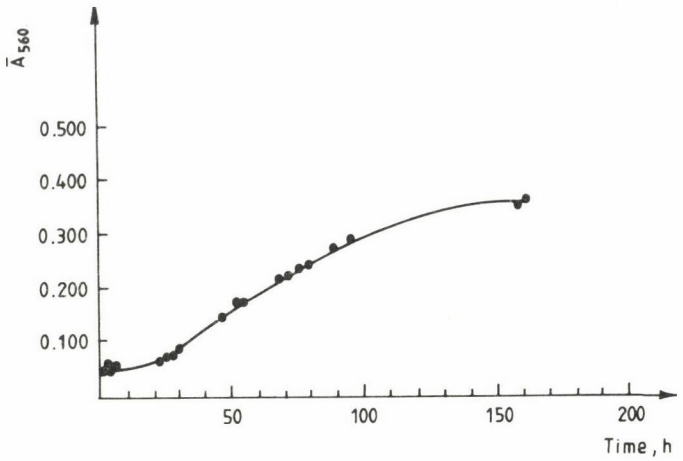

Fig. 4. Growth curve of *Leuconostoc oenos* 1 in C – 104 broth, at pH = 4.5, at 27 °C

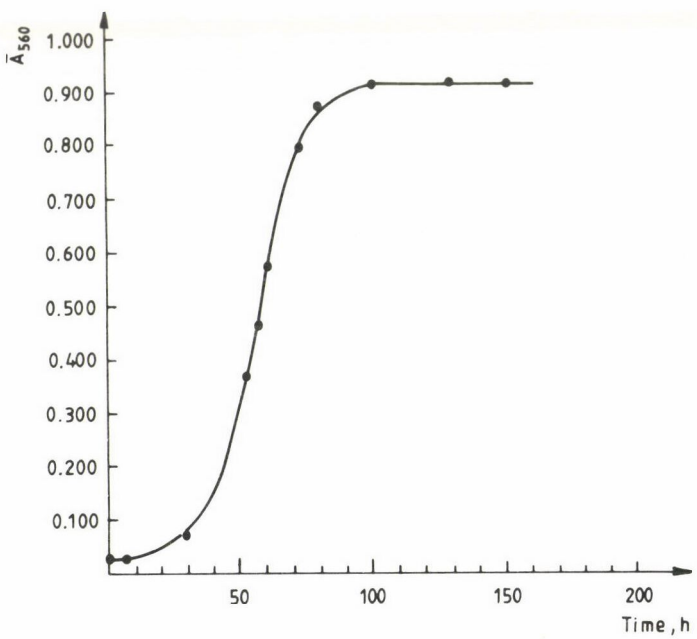

Fig. 5. Growth curve of *Leuconostoc oenos* 1 in F – 104 broth, at pH = 4.5, at 27 °C

As it can be seen from the figures, depending on the composition of the broth, the growth curves differ from each other.

The specific growth rates of *Leuconostoc oenos* 1, in different broths were evaluated by one way analysis of variance.

The significant levels for the comparison of these specific growth rates are demonstrated in Fig. 6.

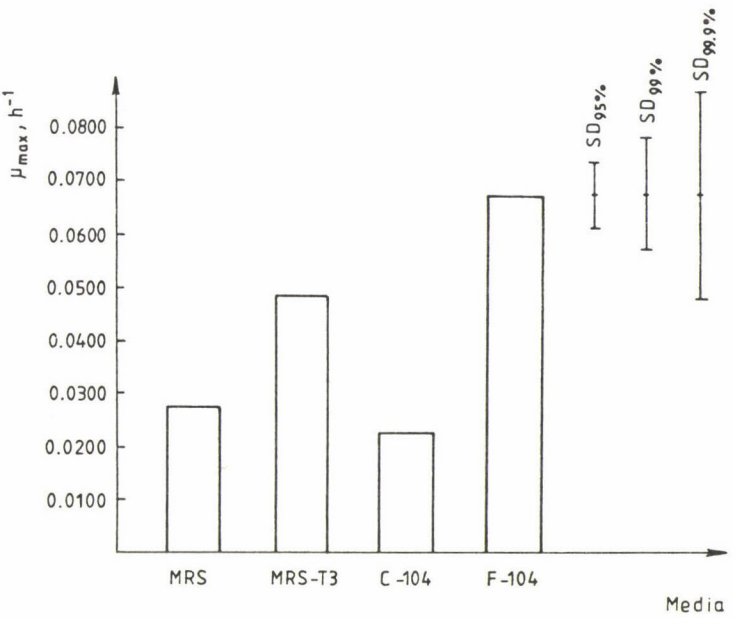

Fig. 6. Effect of growth media on the specific growth rate of *Leuconostoc oenos* 1 at pH = 4.5 and 27 °C

The maximum values of absorbance, as characteristic data of populations in different broths were also evaluated by one way analysis of variance.

The means of maximum absorbances in different broths and their significant differences are presented in Fig. 7.

Mathematical-statistical evaluation showed the F–104 broth the most favourable growth medium. This broth was superior to the C–104 and the MRS broth at 99.9% level of probability, and differed from the MRS–TJ broth, too, at 99% level of probability.

The MRS–TJ broth, compared with the simple MRS broth was significantly better, at 99% level of probability.

The specific growth rates in MRS and C–104 broths did not differ from each-other but the maximum of absorbance, was less at 95% level of probability in simple MRS broth.

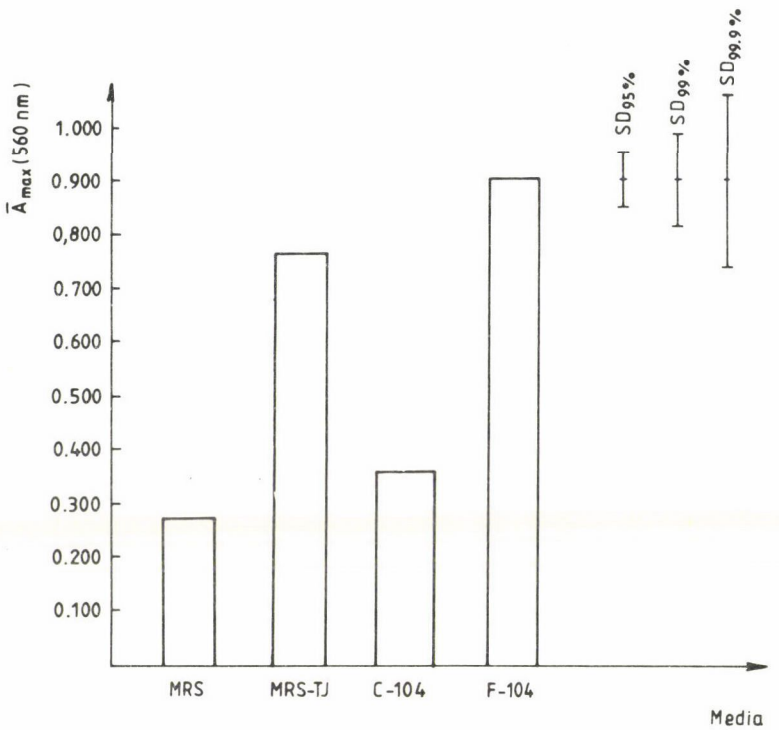

Fig. 7. Effect of growth media on the maximum of absorbance of *Leuconostoc oenos* 1 at pH = 4.5 and 27 °C

The stimulatory effect of TJF was significant for the growth of *Leuconostoc oenos* 1.

The favourable effects of TJF and fructose resulted in greater growth rate and population of F–104 broth.

The generation time, calculated from the mean of u_{max} in this broth was 10.25 h.

It was half as that measured in simple MRS broth. Similarly, in modified Rogosa medium about 22 h generation time was recorded (PILONE et al., 1976).

3. Conclusions

Twenty-one strains of *Leuconostoc oenos* were collected and identified from Hungarian wines, from the region of Eger.

The metabolic activity of these strains to carry out MLF in wines may be useful. For this reason it was important to study the pH optimum and growth characteristics.

Leuconostoc oenos 1, which appeared to be an acid tolerant MLF bacterium had an optimum at pH 4.5. It can be used as a selective starter for induction of malic acid fermentation in wines. This process will be faster than the spontaneous MLF, particularly if an appropriate number of bacterial cells are inoculated. The most vigorous population was obtained in F – 104 broth, it was the most preferable medium.

*

Thanks are due to O. REICHART, Head of the Microbiological Department (University of Horticulture and Food Science) for providing the computer program of growth modelling.

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SEPARATION OF A METHIONINE-ENRICHED FRACTION FROM ENZYMATICALLY MODIFIED CASEIN BY FAST PROTEIN LIQUID CHROMATOGRAPHY

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α-Chymotryptic casein hydrolysate and a methionine-enriched EPM-product from this proteolysate were investigated by ion-exchange chromatography. Two types of anion-exchange resins were used for the separations. MONO Q column was applied to monitor the differences between the two protein mixtures. A methionine-enriched fraction of the α-chymotryptic hydrolysate of casein was separated on Q-Sepharose column.

Keywords: EPM, casein, methionine-enriched fraction, FPLC, ion-exchange chromatography

The aim of our work was to separate the methionine-enriched fractions from the enzymatically modified casein because the role of bound methionine in food proteins is of special importance from the point of view of food safety (HAJÓS et al., 1993).

The applied analytical and preparative methods were carried out according to JUILLERAT and co-workers (1989) for phosphopeptides from whole casein by fast protein liquid chromatography using MONO Q strongly basic anion-exchange resin both for analytical and preparative separations. The tryptic peptides were dissolved in Tris buffer (20 mmol, pH 8.0) and eluted by linear salt gradient.

In case of α-chymotryptic hydrolysate of casein and its methione-enriched product segmented gradient (HAANOOT et al., 1986, BARREFORS et al., 1985) was applied on the MONO Q HR 5/5 column. The preparative separation was performed on Q-Sepharose resin at low pressure and fast flow rate.

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1. Materials and methods

1.1. Samples

Hydrolysate: casein (Hammersten, Reanal) was hydrolyzed with α-chymotrypsin for 2 h at 37 °C (α-chymotrypsin: Sigma, EC 3.4.21.1., 47 U mg^{-1} solid, from bovine pancreas).

EPM-product: enzymatic peptide modification was performed with an α-chymotrypsin hydrolysate of casein. α-chymotrypsin was used as catalyst also in the EPM-reaction. The reaction was carried out at 37 °C, pH: 6.0 for 16 h, the concentration of casein hydrolysate was 20% (w/v) (HAJÓS et al., 1988).

1.2. Chromatographic separation

Pharmacia fast protein liquid chromatography (FPLC) system with UV-M monitor and OH-850 recorder (Radelkis) was used. For analytical purposes a prepacked anion-exchange MONO Q column (HR 5/5/ Pharmacia) was applied. The buffer of low strength contained 0.02 mol Tris, and was adjusted to different pH values (see figures). The buffer of high strength had the same composition with the addition of 1 mol NaCl. The buffers were filtered through a 0.22 μm Millipore filter (GVWP 04700). A sample volume of 300 μg was injected, the eluent flow rate was 1 cm^3 min^{-1} and the absorbance was measured at 280 nm.

The salt gradient profiles are represented in Fig. 1 and Fig. 2. Preparative separation was carried out on a Q-Sepharose HR 10/30 column (Pharmacia), eluent flow rate 4 cm^3 min^{-1}, and the sample load was 200 mg/run. Other conditions were the same as described above.

1.3. Carboxypeptic digestion and thin-layer cation-exchange chromatography

The samples were incubated with a mixture of carboxypeptidase A and carboxypeptidase B (1:1) in 100 μl 0.1 mol NH_4HCO_3 buffer. Samples (10 μl) were investigated after incubating for 0, 15, 30, and 60 min, respectively, at which times each sample was immediately mixed with an equal volume of conc. HCl to stop the enzyme reaction. Free amino acids were separated by thin-layer ion exchange chromatography (HAJÓS et al., 1986).

The chromatography was evaluated by a Biotec-Fischer video-densitometer.

2. Results

Figures 1a, b, and c show the chromatograms of the hydrolysate and the modified products on MONO Q HR 5/5 anion-exchanger at different pH values: pH 8.0, pH 8.5, pH 8.8, respectively. The difference between the chromatograms of the hydrolysate and the EPM-product is better revealed at higher pH value (Fig. 1c).

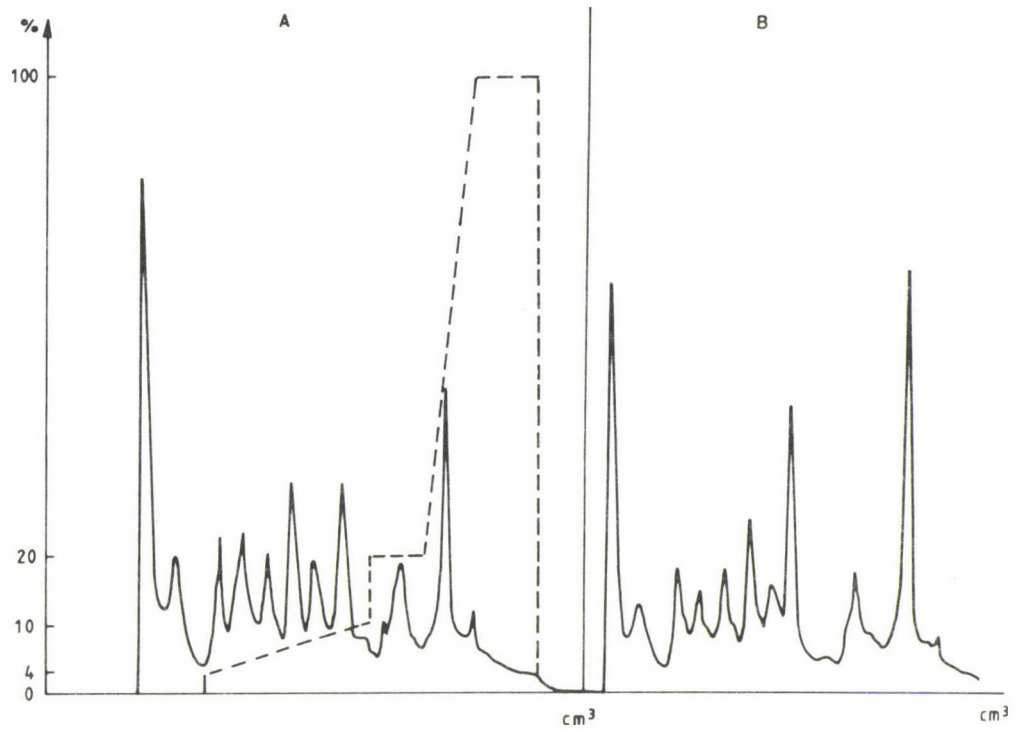

Fig. 1a. Separation of the α-chymotryptic casein hydrolisate (A) and the methionine-enriched EPM-product (B) on MONO Q resin, column HR 5/5, AU 0.01, pH 8.0

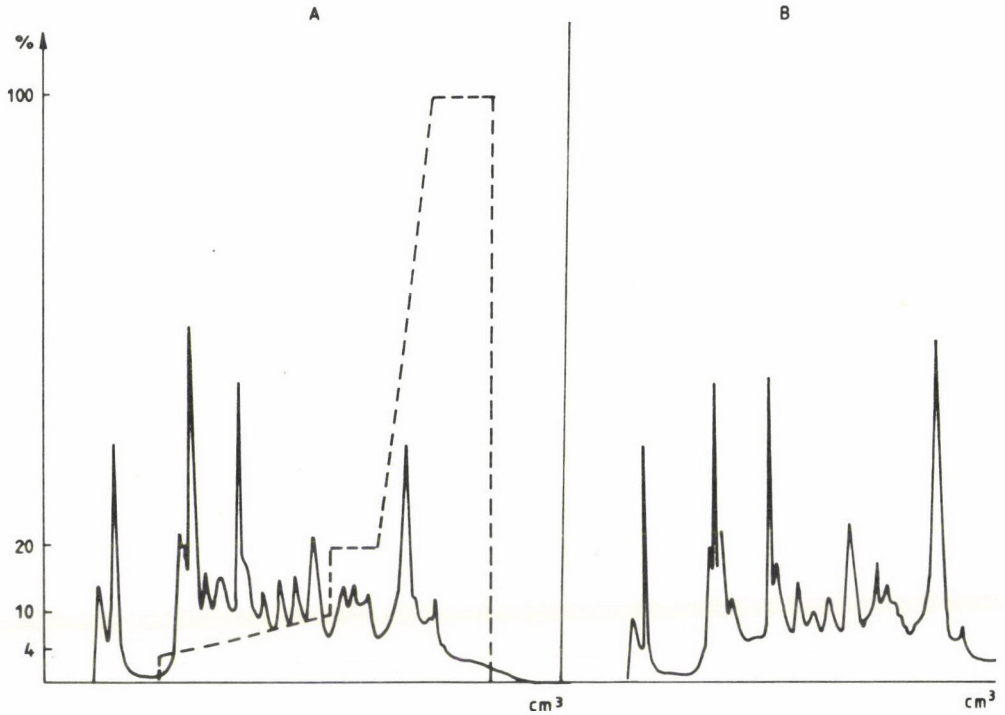

Fig. 1b. Separation of the α-chymotryptic hydrolysate (A) and the methionine-enriched EPM-product (B) on MONO Q resin, column HR 5/5, AU 0.01, pH 8.5

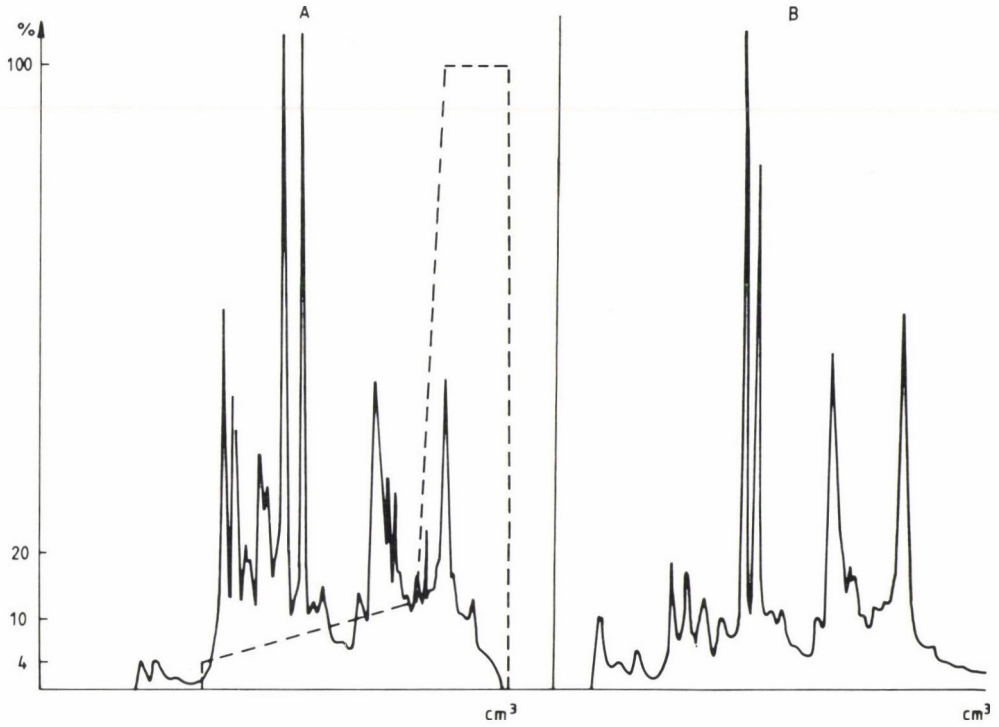

Fig. 1c. Separation of the α-chymotryptic casein hydrolysate (A) and the methionine-enriched EPM-product (B) on MONO Q resin, column HR 5/5, AU 0.01, pH 8.8

On the Q-Sepharose HR 10/30 preparative column the modified product was separated with the same gradient, which was developed on MONO Q. By this way the EPM-product was separated to seven fractions (Fig. 2). The incorporated methionine content of fractions was investigated after the digestion with exopeptidases to compare with that of hydrolysate. The second fraction (Fig. 3a), which was eluted at the start of salt gradient, contains remarkably high percent of methionine bound at the end of the peptide chains.

Table 1

Quantitative evaluation of methionine-content of the samples separated by cation exchange thin-layer chromatography

No.	Position (mm)	Area	Area (%)
1	7.3394	810	4.5
2	20.9174	2292	12.8
3	36.6972	2640	15.8
4	53.5780	2126	11.9
5	69.3578	1569	8.8
6	85.5046	49	0.3
7	100.5505	3612	20.2
8	115.9633	1225	6.8
9	133.5780	735	4.1
10	148.9908	746	4.2
11	159.2661	420	2.3
12	179.0826	99	0.6
13	193.0275	1567	8.8
		17,890	100.0

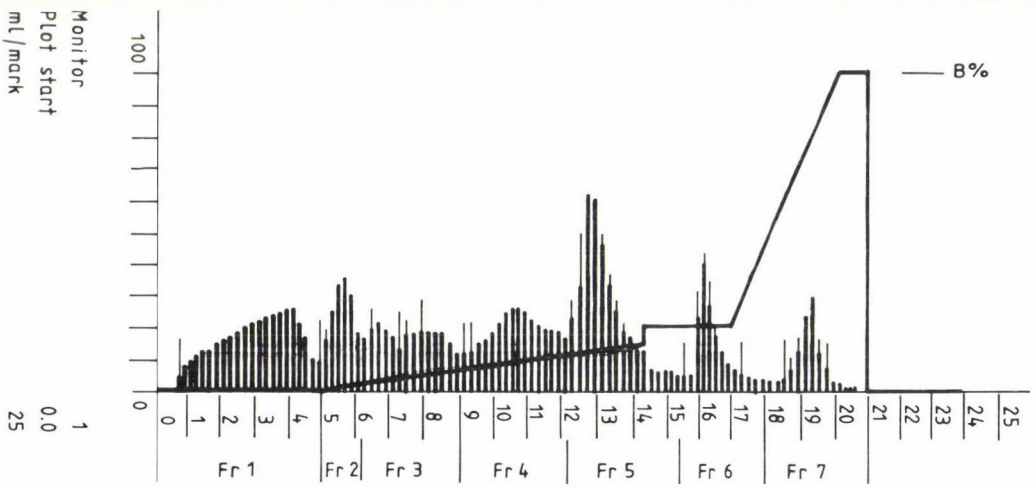

Fig. 2. Separation of the methionine-enriched EPM-product on Q Sepharose resin, column HR 10/30, AU 2.0, pH 8.0

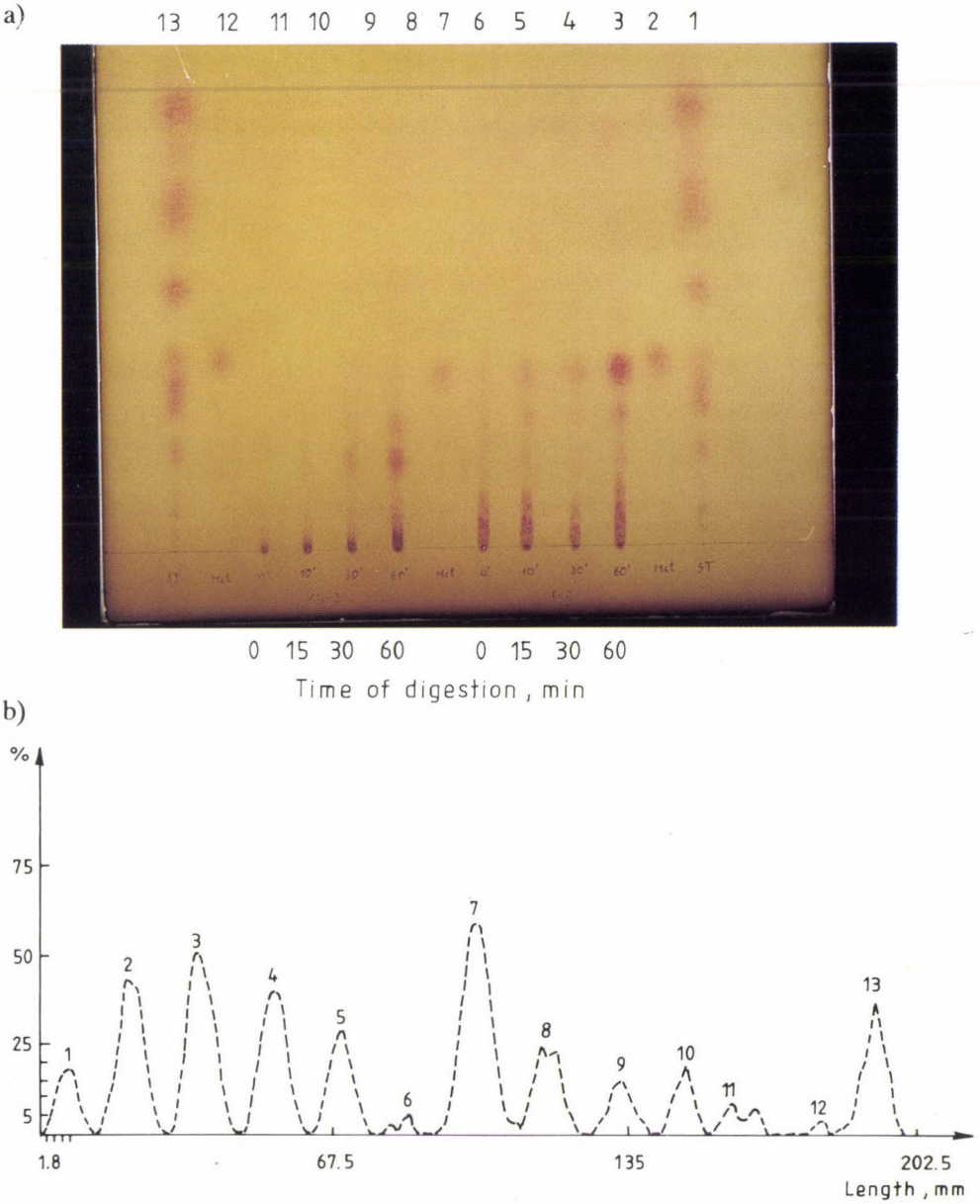

Fig. 3a. Cation-exchange thin-layer chromatography of α-chymotryptic casein hydrolysate and the second fraction of the methionine enriched EPM-product separated on Q-Sepharose anion-exchange resin, after carboxypeptic digestion. 1 and 13: amino acid standard, 2: methionine, 3–6: samples from reaction mixture (2nd fraction), 7: methionine, 8–11: samples from reaction mixture (hydrolysate, 12: methionine; (b) Densitometric evaluation of the methionine spots on the chromatoplate. 1 and 13: amino acid standard, 2: methionine, 3–6: samples from reaction mixture (2nd fraction), 7: methionine, 8–11: samples from reaction mixture (hydrolysate), 12: methionine

The densitometric evaluation shows (Fig. 3b, Table 1) that the methionine content of the EPM-product was three times higher than that of the hydrolysate.

3. Conclusions

The anion-exchange separation was suitable for the analytical separation of α-chymotryptic casein hydrolysate and the methionine-enriched EPM-product.

This fact can be explained, that the covalently incorporated methionine modifies the net charge of certain molecules in the peptide mixture an that modifies their binding properties to the ion-exchange matrix.

The preparative separation of the methionine-enriched EPM-product resulted in a fraction of high percent of covalently bound methionine.

The aim of this study was to find a reliable and sensitive separation technique that could distinguish between peptides differing in one amino acid only, in this case in methionine. The salt concentrations at which the enzymatically modified peptides of casein were eluted showed great reproducibility between runs.

By the use of ion exchange columns Mono Q and Q-Sepharose developed for FPLC it was possible to find a suitable separation method for a proteolysate and a methionine-enriched EPM product of casein.

*

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A POTENTIAL PROTEIN ENGINEERING SITE IN *ASPERGILLUS NIGER* GLUCOAMYLASE: VICINITY OF DISULPHIDE BRIDGE 449–222

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Thermal denaturation and kinetic studies have been performed on glucoamylase and on its truncated forms obtained by papain proteolysis. Substrates of differing length had significant effect neither on stability of the enzyme forms nor on proteolytic susceptibility of the native enzyme. Formation of disulphide-bridged oligomers seems to be on the denaturation pathway but it is not the rate-determining step in irreversible inactivation. Results indicate that stabilization around the accessible disulphide of the protein might retard thermodenaturation but not catalysis.

Keywords: glucoamylase, limited proteolysis, thermoinactivation

Glucoamylase (glucan 1,4-α-glucosidase, E.C. 3.2.1.3.) catalyses the release of glucose from the non-reducing end of starch and related poly- and oligosaccharides. As one of the industrial enzymes used in the largest quantities in the world (SAHA & ZEIKUS, 1989), it is a frequented target of protein engineering.

The kinetics and thermodynamics of catalysis have been thoroughly elucidated (HIROMI et al. 1973; 1983) and the role of active site residues established (CLARKE & SVENSSON, 1984; SIERKS et al., 1989a, 1990). However, despite several results (HOSCHKE, 1978; MUNCH & TRITSCH, 1990), a reverberating success in increasing the stability of the enzyme is still missing. This is not surprising if one considers that catalysis by glucoamylase is supposed to be accompanied by large fluctuations (SIERKS et al., 1989b). Though inventive studies have unveiled important topological features (BELSHAW et al., 1990; EVANS et al., 1990), the lack of detailed knowledge of the molecular events taking place during catalysis and inactivation of the enzyme still prevents the dissection of the catalytically indispensable reversible local unfolding of the protein from that leading to inactivation.

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Location of the specific structural region where irreversible unfolding starts (ULBRICH-HOFMANN et al., 1992) would substantially simplify the task of stabilization. Thermostability studies in which the enzyme incubated at high temperature is cooled down and assayed for residual activity at ambient temperature provide informations on the irreversible denaturation process with few relation to its topology. Treating enzymes under non-denaturing conditions with proteases, on the other hand, is an effective way to locate the most flexible parts of structures (FONTANA, 1992), but these likely origos for reversible unfolding do not necessarily coincide with those for irreversible denaturation. Presence of ligands in saturating concentration selectively protects enzyme parts they bind to against both processes. Here we report interpretation of such studies with the help of the recently determined three-dimensional structure of a glucoamylase (glucoamylase-II(471) from *Aspergillus awamori*, identical to *Aspergillus niger* glucoamylase) by ALESHIN and co-workers (1992), and of its complex with 1-deoxynojirimycin (HARRIS et al., 1993). The results, partly presented earlier by VÁRALLYAI (1993), might allow for outlining a rational stabilization strategy that would not impair catalytic activity of glucoamylase.

1. Materials and methods

Commercial enzyme preparation of *Aspergillus niger* ATCC 22343/65 was produced in the Bioengineering Dept. of Central Food Research Institute (HOSCHKE et al., 1993).

All chemicals and reagents were of analytical grade commercial preparations used without further purification.

1.1. Purification of G1 and G2

The G1 and G2 forms of glucoamylase of *Asp. niger* were purified by the modified method of SVENSSON and co-workers (1982) as described by SASVÁRI (1993). All operations were carried out at 4 °C. To the crude enzyme solution ammonium sulfate was added to 60% saturation. The resulting precipitate was isolated by centrifugation (15 min, 10000 g), redissolved in 25 mmol Tris pH = 7.0 buffer containing 1 mmol EDTA, applied to Sephadex G-25 gel filtration column and eluted with the same buffer. The first, protein containing peak was separated to G1 and G2 by ion exchange chromatography using DEAE-Sephacel and the same buffer with linear gradient of NaCl from 0 to 0.5 mol.

1.2. Proteolytic digestion of glucoamylase

Purified glucoamylase (50 mg) – G1 or G2 – was dialysed against water, liophylised, and redissolved in 0.2 mol phosphate buffer pH = 7.0, containing 5 mmol L-cystein and 2 mmol EDTA, to 10 mg cm^{-3} final protein concentration. 5 mg papain was added to this solution and the reaction mixture was incubated at 30 °C in a shaking bath. The reaction was stopped by diluting the sample to 2 mg cm^{-3} glucoamylase concentration, adjusting the pH to 3.6 with HCl and cooling to 4 °C.

1.3. Purification of G' from the digestion mixture

The digestion mixture was applied to a Q-Sepharose ion exchange column, equilibrated with 20 mmol Tris pH = 7.0 buffer and eluted with linear gradient of NaCl from 0 to 0.5 mol. Resulting peaks were desalted by ultrafiltration (Amicon YM 10 000), liophilized and stored at 4 °C.

1.4. Analyses

HPLC analyses were carried out using RPC18 column eluted with a 30 min linear gradient of 0.1% TFA – water, 0.1% TFA – acetonitril.

For FPLC analysis we used MONO–Q ion exchange column and a linear gradient of 0–0.5 mol NaCl in 20 mmol Tris pH = 7.0 buffer.

SDS-gel electrophoresis was performed in 10% polyacrylamide gels.

N-terminal sequences were determined by Dr. A. Patthy in the Analysis Laboratory in the Institute for Biochemistry and Protein Research.

1.5. Assays

Protein concentrations were determined by using the extinction coefficient $E = 1.37 \times 10^5$ mol^{-1} cm^{-1} for G1 and $E = 1.09 \times 10^5$ mol^{-1} cm^{-1} for G2 and G' (STOFFER et al., 1993).

Activity measurements were performed in 0.05 acetate buffer pH: 4.5 at 45 °C. 3 μg pure enzyme was added to 1.5 cm^3 preincubated buffer containing 10 mmol maltose or 1% raw starch. Aliquots were withdrawn after 0, 5, 10, 20, 40 min of incubation. The released glucose was measured with the glucose-oxidase/peroxidase method using o-dianizidin as chromogen. The PNPG (paranitrophenyl-α-D--glucopyranosid) hydrolyzing activity was calculated from measuring the increase in absorbance at 340 nm in a reaction mixture containing 30 μg enzyme and 0.3 mg substrate in 3 cm^3 buffer.

1.6. Thermal inactivation

The enzyme was incubated at 70 °C in 3.0 and 0.3 mg cm^{-3} concentration in the buffer used in activity measurements. Samples withdrawn at appropriate time intervals were placed into ice-water bath, and residual activity was assayed with PNPG and/or with maltose. For determination of the effect by ligands the same system was used but the buffer contained 10 mmol gluconolacton, 20 mmol maltose or 1% raw-starch, respectively.

2. Results and discussion

2.1. Limited proteolysis

If proteins without having been denaturated are treated with proteolytic enzymes, cleavage occurs at the most flexible parts of the structure. Subtilisin digestion of glucoamylase apparently dissected the latter into functional starch--binding and catalytic domains (STOFFER et al., 1993). Our treatment of G1 (the large isoenzyme) with papain under non-denaturing conditions yielded two forms: one that appears identical with natural isoenzyme G2, and G', a protein significantly smaller than G2 (see Fig. 1). In contrast to the subtilisin digestion, we were not able to separate the intact starch-binding domain, probably because it is susceptible to further fragmentation by papain but not by subtilisin. G' seems to be pure as it eluted in one peak on RP-HPLC, on MONO-Q ion-exchange chromatography-FPLC and appeared as a single band on SDS-PAGE (not shown).

The gelelectrophoretic pattern is the same if starting material is G2, i.e., practically only G' is formed (not demonstrated). As G' has the same N-terminal sequence as G1 and G2, the proteolytic cleavage certainly occurs at the C terminal part of G1 and G2, most likely somewhere in the outskirts of Ser/Thr rich region between residues 440-450.

The formation of G' from G2 as well as from G1 shows that the site of cleavage is the same in G1 and G2. Accordingly, loss of the so-called "raw starch binding domain" does not influence the location of the most sensitive part towards proteolysis by papain: the most flexible area remains the same. This agrees with the finding by WILLIAMSON and co-workers (1992), that the tertiary structures of the catalytic and the (raw starch) binding domain do not decisively influence each other.

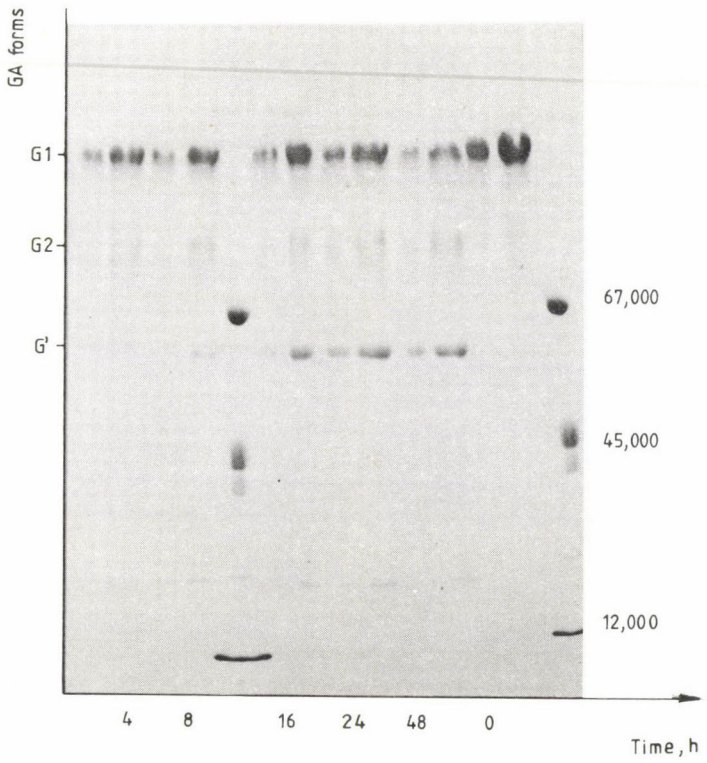

Fig. 1. SDS-PAGE of limited proteolysis of G1 with papain. Enzyme in 10 mg cm^{-3} concentration was incubated at 30 °C in 50 mmol phosphate, pH 7.0 in the presence of 1 mg cm^{-3} papain for the time indicated

The activities of the different enzyme forms are shown in Table 1. The very low raw starch degrading ability of G2 and G' is obviously due to the lack of the starch binding domain. However, all three forms display similar catalytic activities towards the substrates maltose and PNPG. Furthermore, there is no difference between the two smaller enzymes concerning their activities towards raw starch. This indicates that while the loss of the starch binding domain practically abolishes activity towards raw starch as expected, a further loss of approximately 60 amino acid residues from the smaller isoenzyme G2 has negligible effect on catalysis. A similar conclusion was reached earlier by STOFFER and co-workers (1993) in their subtilisin-nicking experiments.

Table 1

Activity of different forms of glucoamylase

Enzyme forms	Activity [U[a]/μmol enzyme]		
	Raw-starch	Maltose	PNPG
G1	41±2	51±2	4.0±0.6
G2	2.6±2	52±2	5.3±0.6
G'	3.6±2	58±2	2.8±0.6

[a] One U of enzyme activity catalyses the hydrolysis of 1 μmol maltose min^{-1} at 45 °C and pH = 4.5

Activities were measured as described in M&M (in 0.05 mol acetate buffer pH: 4.5 at 45 °C; 3 μg pure enzyme was added to 1.5 cm^3 preincubated buffer containing 10 mmol maltose or 1% raw starch. 100 μl aliquots were withdrawn and the released glucose was measured with the glucose-oxidase/peroxidase method using o-dianizidin as chromogen. The PNPG (paranitrophenyl-α-D--glucopyranoside) hydrolizing activity was calculated from measuring the increase in absorbance at 340 nm in a reaction mixture containing 30 μg enzyme and 0.3 mg substrate in 3 cm^3 buffer)

Another conclusion of the proteolytic cleavage experiment is apparent from Fig. 2. G', the "nicked" enzyme form, appears to be rather stable as it is practically not digested further by papain in 5 days. Accordingly, it displays a rather compact structure with no area with flexibility comparable to that of the two main flexible points in G1, the one around residue 512, where G1 is also in vivo cleaved to G2, and the other, most likely around residue 450. If in vivo G2 is indeed resulting from a postsynthetic proteolysis of G1, the question why G' is then not formed remains open.

The gelelectrophoretic pattern of proteolysis by papain is not altered in the presence of saturating concentration of effectors (Fig. 2). This holds for the smallest ligand, the transition-state analogue gluconolactone (LÁSZLÓ et al., 1978) as well as for the largest one, raw starch. This clearly indicates that neither location of, nor susceptibility at, the most flexible point is altered when it is the enzyme-effector complex rather than the free enzyme that is cleaved. In stereochemical terms this means that the weak point for reversible denaturation should be without the substrate binding area(s).

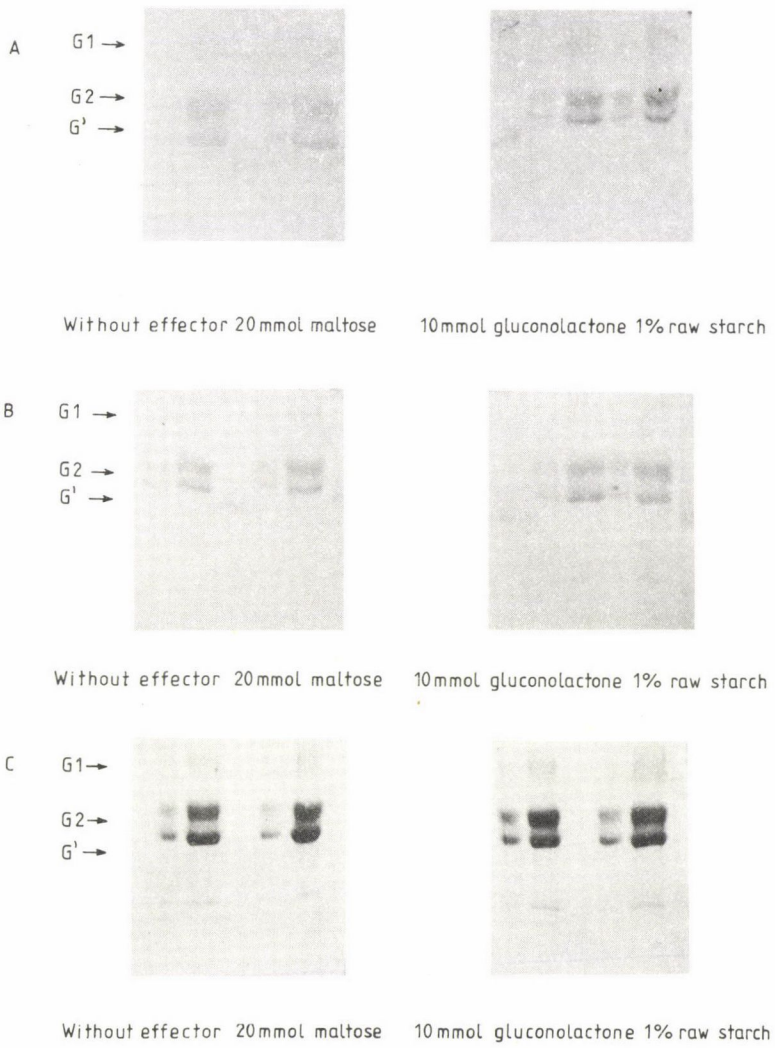

Fig. 2. Time course of an effector influence on limited proteolysis of GA2 by papain. A: 24 h incubation time, B: 48 h incubation time, C: 5 days incubation time. GA2, pure but containing both G1 and G2, was incubated in 10 mg cm^{-3} concentration at 30 °C in 50 mmol phosphate, pH 7.0 in the presence of 1 mg cm^{-3} papain and of effectors (10 mmol gluconolactone, 20 mmol maltose, 4% soluble starch) for 24, 48 and 120 h as indicated

2.2. *Thermal denaturation*

The time course of irreversible loss of activity of the different enzyme forms was determined to obtain information on the mechanism of this process as well as on the stability of the enzyme (Table 2). The results show that the difference in between the rate of irreversible activity loss of the three different forms of glucoamylase is within the experimental error. Neither can any significant protecting effect of ligands be seen. Consequently, the decisive point of irreversible inactivation is outside the active site area just as is the most flexible point (cf. above). From these results it might be concluded that the weak point is certainly not close to the active site.

Table 2

Half-life time of different forms of glucoamylase with and without effectors

Enzyme forms	Effectors			
	–	10 mmol gluconolactone	20 mmol maltose	1% raw starch
	$t_{50\%}$ (min)			
G1	6.5±1	6.8±1	6.7±1	5.5±1
G2	6.6±1	5.2±1	7.5±1	8.2±1
G'	6.8±1	7.7±1	7.1±1	7.4±1

Enzyme (3 mg cm^{-3} was incubated in the presence and absence of effectors at 70 °C and samples withdrawn at appropriate time intervals were assayed against both maltose and PNPG as substrates at 45 °C. $t_{50\%}$ is the time when activity decreased to half of the original value

The time course of irreversible loss of activity has also been determined with 0.3 mg cm^{-3} enzyme concentration (not shown). In that case $t_{50\%}$ values increased not more than by approx. 10%. Since thus an order of magnitude change in the protein concentration only slightly influenced the rate of activity loss, the latter seems to be a monomolecular process.

2.3. *Role of disulphide bonds in denaturation*

The general mechanism of irreversible thermoinactivation of proteins involves aggregation and changes in primary structure (MOZHAEV, 1993), with cysteines playing important roles. In their study MUNCH and TRITSCH (1990) on a commercial *Aspergillus niger* glucoamylase containing the isoforms observed high-molecular weight aggregates of glucoamylase of presumably disulphide-bridging origin. In the present work, SDS-PAGE run without any reducing agent (for G2 see Fig. 3) also

indicated the formation of higher molecular weight aggregates, probably dimers. No such high molecular weight forms were detected with mercaptoethanol present in the sample buffer, neither could any smaller fragments be seen, so the aggregates are likely to be disulphide-linked species.

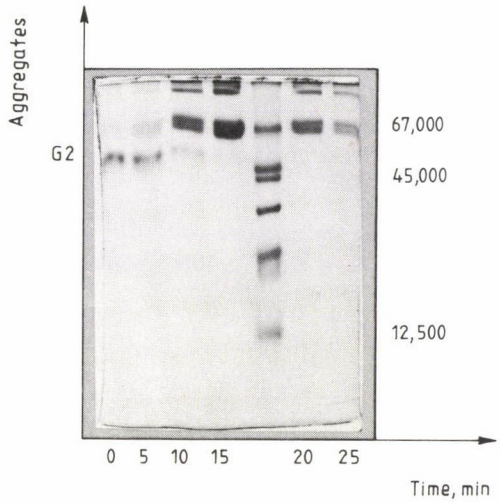

Fig. 3. SDS-PAGE of G2 incubated at 70 °C. Enzyme (3 mg cm^{-3}) was incubated at 70 °C. Aliquots at indicated time were withdrawn into an ice-water bath and stored at 4 °C till assaying for activity

From the sequence data it is apparent that G1 contains nine, and G2 eight cysteine residues. G' definitely shorter by at least about 40 amino acids than G2, should have lost Cys-507 and be left with seven cysteines.

An inspection of the X-ray structure of glucoamylase-471 (ALESHIN et al., 1992), probably a good representation of G', reveals that disulphide bridges are opposite the active site region, with Cys-210-Cys-213 bridge completely and 262-270 partly buried, whereas 449-222 appears to be accessible. The only unpaired cysteine, Cys-320 seems buried in the core region. This is consistent with our finding that no thiol groups could be titrated in neither forms of glucoamylase and with similar results obtained earlier (FREEDBERG et al., 1975). Being buried, direct participation of Cys-320 in the aggregation is rather unlikely (though it cannot be excluded yet). A more plausible assumption is that a disruption of the surface 449-222 cysteine bridge occurs with the formation of intermolecular disulphide bonds. Further experiments to clarify this problem are in progress.

When the time course of decrease of catalytic activity was compared to that of aggregate formation, it appeared that enzyme samples with higher than 50% aggregate concentrations as estimated from gels still retained significantly more than 50% activity. This together with the fact that $t_{50\%}$ did not substantially change with the protein concentration indicates that aggregation, though likely to lie on the pathway, is not the rate-determining step in irreversible thermal denaturation.

3. Conclusions

Since the absence of the raw-starch binding domain decreases neither the catalytic activity towards soluble substrates nor the stability of the catalytic domain, fermentation for industrial purposes might well aim at producing glucoamylase with higher G2:G1 ratios.

As ligands have a significant effect neither on $t_{50\%}$ of inactivation nor on proteolytic nicking of the enzyme, the "weak point" of the protein is not in the active site area. The accessible disulphide bridge between cysteines 449 and 222, suggested to be a weak point on the basis of the results on aggregation, is remote from the active site indeed. Accordingly, scaffolding there might effectively stabilize the enzyme. Another prospective site for stability engineering is Cys-320. This residue, not essential for catalysis, might play a role in intermolecular disulphide-bridge formation either directly or indirectly by triggering a disulphide-exchange process. Elimination of this possibility might prevent aggregate formation and thus increase the stability of glucoamylase.

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EFFECT OF IRRADIATION ON FOLATE LEVELS AND ON BIOAVAILABILITY OF FOLATES IN DEHYDRATED FOODSTUFFS

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A method for the assessment of the bioavailability of folates in foods is described. It involves measuring the response of selected tissue stores (plasma and liver) to test diets in the folate-depleted rat.

Electron-irradiation of dried spinach (10 kGy) and dried asparagus (5 kGy), and γ-irradiation of egg yolk powder (2 kGy) caused no significant loss of folates, neither in analytically determined folate levels nor in bioavailability of folates.

Keywords: folate analysis, folate bioavailability, irradiation effects, dehydrated foodstuffs, HPLC-analysis

The incidence of salmonellosis and other foodborne diseases is increasing in many countries, and irradiation of foods is considered to be one of the more promising methods to fight this trend. Officials of the World Health Organisation have voiced the opinion that food irradiation may be one of the most significant contributions to public health to be made by food science and technology since the introduction of pasteurization (KÄFERSTEIN & MOY, 1993). The FAO/IAEA/WHO Joint Expert Committee on Food Irradiation, having reviewed the results of numerous chemical, toxicological and microbiological studies, concluded that the irradiation of any food commodity up to an overall average dose of 10 kGy presents no toxicological hazard and introduces no special nutritional or microbiological problems. However, the Committee found that there was little recent information on the effect of radiation on folic acid, and recommended additional studies on representative folate-containing foods (JECFI, 1981).

In countries where irradiation of certain foodstuffs is permitted, radiation processing of dried food products, such as spices, dehydrated vegetables, egg powder and gum arabic for the purpose of assuring their hygienic quality, is one of the commercially interesting applications of this technology (DIEHL, 1990). From this

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group of products we have selected three items, dried spinach, dried asparagus and egg yolk powder, to study the effect of irradiation on folate levels and on the bioavailability of folates. Little information exists on the influence of food processing on folate bioavailability (ARAMOUNI, 1986). Concerning the irradiation of foods, no studies on effects of this treatment on the bioavailability of food folates have been reported previously.

1. Materials and methods

1.1. Tested foods and diets

Deep-frozen spinach was purchased locally, finely chopped, freeze-dried (Suwelak, Billerbeck) and finely powdered. Fresh asparagus was also purchased locally, peeled and blanched. After blanching it was cut in 1×1 cm cubes, deep-frozen, ground, freeze-dried (Dereko, Ludwigsburg) and finely powdered. Dried egg yolk was obtained from Eto (Ettlingen). All dried foodstuffs were vacuum-packed: one half was kept at −20 °C, the other half was irradiated at room temperature and kept at −20 °C. Electron irradiation was applied to dried spinach and asparagus at dose levels of 10 and 5 kGy, respectively. Dried egg yolk was γ-irradiated with a dose of 2 kGy.

Table 1

Composition of the folate-deficient basal diet

Constituent	g kg^{-1} diet
Casein, vitamin-free (Merck)	210.0
Sucrose	203.0
Wheat starch (Roth)	377.0
Maize oil (Mazola)	97.7
Cellulose (Roth)	53.5
Mineral mixture[1)]	44.0
Vitamin mixture[2)]	0.3
DL-Methionine (Merck)	5.0
Cholinchloride (Merck)	2.0
Succinylsulphathiazole (Sigma)	10.0

[1)] Mineral mixture provided (per kg of diet): $CaCO_3$ (Merck) 9.2 g, $Ca_3(PO_4)_2$ (Merck) 6.6 g, KH_2PO_4 (Merck) 13.6 g, NaCl (Merck) 4.6 g, KCl (Merck) 5.3 g, $MgSO_4 \cdot 7H_2O$ (Merck) 8.1 g, $MnSO_4 \cdot H_2O$ (Merck) 0.48 g, $FeSO_4$ (Merck) 0.65 g, $ZnCO_3$ (Merck) 0.11 g, $CuSO_4$ (Merck) 17.2 mg, NaF (Merck) 25.1 mg, KI (Merck) 2.2 mg, $KAl(SO_4)_2$ (Merck) 4.0 mg.

[2)] Vitamin mixture provided (per kg of diet): thiamine-HCl (Sigma) 20 mg, riboflavin (Sigma) 20 mg, pyridoxol-HCl (Merck) 15 mg, nicotinic acid (Sigma) 50 mg, calcium D-pantothenic acid (Sigma) 40 mg, D-biotin (Sigma) 1 mg, cyanocobalamin (Merck) 20 μg, DL-α-tocopheryl acetate (Merck) 100 mg, all trans retinol acetate (Sigma) 5 mg, menadione (Sigma) 5 mg, cholecalciferol (Sigma) 25 μg

The composition of the folate-deficient basal diet fed to all animals during the folate depletion period is shown in Table 1. Standard diets fed in the folate repletion period were identical, except for the addition of crystalline folic acid (FA) from Sigma (Deisenhofen). Test diets containing a natural source of food folate, which were fed in the folate repletion period, were adjusted to be isocaloric and isonitrogenous in comparison to the basal diet. Table 2 shows the levels of folate sources in the standard and test diets of the repletion period.

Table 2

Levels of folate sources in the standard and test diets of the repletion period

Experiment No.	Repletion diets
1. dried spinach[2)]	B + 0, 0.5, 1 or 2 mg FA/kg diet [4)]
	B[1)] + 10% or 20% dried spinach[5)]
	B[1)] + 10% or 20% dried spinach *[5)]
2. dried asparagus[3)]	B + 0, 0.5, 1 or 2 mg FA/kg diet[4)]
	B[1)] + 7.5% or 15% dried asparagus[5)]
	B[1)] + 7.5% or 15% dried asparagus *[5)]
3. dried egg yolk[2)]	B + 0, 0.25, 0.5, 1 or 2 mg FA/kg diet[4)]
	B[1)] + 0, 0.25, 0.5 or 1 mg FA/kg diet + 17% dried egg yolk[5)]
	B[1)] + 0, 0.25, 0.5 or 1 mg FA/kg diet + 17% dried egg yolk *[5)]

B = basal diet; * = irradiated; [1)] all test diets were adjusted to be isocaloric and isonitrogenous to basal diet; [2)] ad libitum feeding during total assay time, blood collection from non-fasted animals; [3)] ad libitum feeding during depletion period, 18 g diet/day during repletion period, blood collection from overnight fasted animals; [4)] standard diets; [5)] test diets

1.2. Protocols of rat bioassays

Male weanling Sprague-Dawley rats (60–80 g) were purchased from Charles River Wiga GmbH (Sulzfeld). The lightest and heaviest animal and one animal of median weight were sacrificed on the first day of the assay to determine initial plasma and liver folate concentration. During 21 days of depletion the rats were fed a folate-depleted basal diet that contained 20 $\mu g\ kg^{-1}$ residual folate from "vitamin-free" casein. Positive controls were kept for 21 days on a folate-enriched basal diet (2 mg FA kg^{-1} diet). After 21 days some of the depleted rats and the positive controls were sacrificed to determine plasma and liver folate concentration. The remaining depleted animals were randomized according to body weight and allocated to folate-enriched standard and test diets as shown in Table 2 for a repletion period of 14 days. In experiment 1 positive (2 mg FA kg^{-1} diet) and negative controls (folate-deficient basal diet) were kept during the whole assay-period of 5 weeks,

whereas in experiment 3 only negative controls were kept throughout this assay-period. In experiment 2 neither positive nor negative controls were kept.

After the 14-d repletion period the animals were anesthetized. Blood was collected from Vena cava into heparinized tubes. Plasma was separated by centrifugation, transferred to glass tubes and stored at −20 °C until analysed for folate content with radio-proteinbinding-assay. Livers were excised, flushed with cold physiological NaCl-solution and immediately analysed for folate content with HPLC (fluorimetric detection). Body weights were recorded twice a week, food intakes every two days.

Due to the high content of fat in dried egg yolk a maximum of 17% could be added to the test diets so that they were still isocaloric and isonitrogenous in comparison to the basal diet. As a consequence this diet contained only about 400 μg folate kg^{-1} diet; test diets were therefore prepared with added folic acid (see Table 2).

1.3. Analytical methods for assessing folate content in biological materials and diets

1.3.1. Radio-proteinbinding-assay for plasma folate. Folate levels in rat plasma were determined in duplicate using a commercial radio-proteinbinding-assay from Becton Dickinson (Heidelberg). This kit is based on the principle of competitive protein binding between radiolabeled folic acid and folates deriving from the tested sample. The amount of bound [^{125}I]-folic acid is inverse-proportional to the amount of free folates in the sample.

1.3.2. HPLC-analysis for liver folates. In experiment 1 and 2 livers of each diet group were pooled and immediately analysed in duplicate. Extraction, preparative chromatography and HPLC-analysis of folates were carried out with a modified method of GREGORY and co-workers (1984). To weighed liver sample (ca. 2 g) four volumes of 0.05 M sodium acetate buffer (pH 7.5, pH-optimum of proteinase-K) containing 1% (w/v) ascorbic acid were added. The mixture was homogenised, an aliquot of 1 g of liver was placed in a 50 cm^3 screw-cap polypropylene centrifuge tube and 1 cm^3 proteinase-K-solution (containing 1 mg of this enzyme) was added. After flushing the sample with nitrogen, the tube was sealed tightly and incubated in a water bath for 1 h at 37 °C, then placed in a boiling water bath for 10 min. After cooling the sample on ice, the tube was centrifuged (10.000 × g, 15 min, 2 °C), the supernatant removed and the pH adjusted to 7.0. The extract was cleaned up by anion-exchange-chromatography with DEAE-Sephadex A−25 and an aliquot of the eluate was analysed by HPLC. Separation of folate monoglutamates (THF, 5−CH_3−THF and 5−CHO−THF) was performed isocratically with a flow of 0.8 cm^3 min^{-1} on a 5 μm-ODS-stationary phase with a mobile phase of 33 mM potassium dihydrogenphosphate, 7% acetonitrile at pH 2.3. The native fluorescence

of the reduced folates was monitored at 295 nm excitation and 356 nm emmission wavelengths.

In experiment 3 the livers of each diet group were not pooled, but separately analysed in duplicate. The extraction-method varied from the description mentioned before in applying heat-treatment (5 min in a boiling water bath) to non-minced liver samples prior to homogenisation. The inactivation of the endogenous deconjugase allowed determination of monoglutamates in one part of the liver extract. The other part of the extract was incubated with exogenous hog kidney deconjugase (pH 4.9, 1 cm^3 hog kidney preparation/aliquot of 1 g of liver, 37 °C, 4 h). It thus contained the sum of monoglutamates endogenously present and those liberated from polyglutamates.

1.3.3. HPLC-analysis for assessing folate content in foodstuffs and diets. Extraction, preparative chromatography and HPLC-analysis of folates in dried spinach, asparagus and egg yolk were performed in the way described for experiment 3 (4 determination for each sample). Folate content (in μg) was expressed in terms of folic acid equivalents. Dried egg yolk was treated with proteinase-K. In spinach and asparagus the incubation with proteinase-K was omitted because of their low protein content. Incubation with hog kidney deconjugase was not carried out on egg yolk extracts, because earlier tests showed they contained no polyglutamates.

Diets were analysed for actual folate content before use (4 determinations for each sample) to account for variations in weighing and mixing that may occur during preparation. For diets containing dried spinach, asparagus or egg yolk the described HPLC-analysis with fluorimetric detection was used.

In experiment 3 determination of folic acid in diets containing added folic acid was based on HPLC-analysis with post-column-derivatisation and fluorimetric detection. Although folic acid has no native fluorescence, it can be cleaved oxidatively to fluorescing pterine derivatives. A 1% (w/v) triplesalt-solution ($2KHSO_5KHSO_4K_2SO_4$) is pumped to the mobile phase with a flow of 0.5 ml min^1. Breakdown of folic acid occurs in a 10 m reaction-loop at 70 °C. At the wavelength combination of 365/450 nm pterine derivatives show fluorescence (MÜLLER, 1993).

1.3.4. Microbiological assay for folic acid in standard diets. In experiment 1 and 2 the analysing method for diets containing folic acid was the microbiological assay with *L. casei*. This growth assay was based on the A.O.A.C. method (1980) for folate determination. A commercial medium (Vitamin folic acid assay broth base for microbiology, Merck, Darmstadt) was prepared as described by the manufacturer. Aliquots of sample extracts were diluted with the extraction buffer (0.1 M KH_2PO_4, 1% ascorbic acid, pH 7.0) to appropriate concentration. The assay tubes contained 10 cm^3 of the commercial medium, 1 to 5 cm^3 of the diluted sample extract plus sufficient bidistilled water to yield a final volume of 20 cm^3. Folic acid standards were run over the range of 0–0.5 ng per 20 cm^3 assay tube. The tubes were autoclaved

10 min at 115 °C, inoculated with a folate-depleted suspension of *L. casei* (ATCC 7469), then incubated for 40 h at 37 °C. A spectrophotometer was used to determine turbidity by monitoring absorption at 580 nm.

1.4. Statistical analysis

Regression equations were determined for the values of plasma or liver folate concentration (y-variable) and folate intake from the standard diets (diets containing FA) in the repletion period (x-variable). The values for plasma or liver folate concentration after repletion with test diets were substituted into these regression equations to estimate biological activity of folates from tested foods in relation to folic acid standard. The obtained x-values were divided by the measured folate intake of the respective test diet and multiplied by 100 to yield a relative bioavailability (%). The slope-ratio-assay is an additional possibility to determine bioavailability. Regression equations were not only calculated for repletion with standard, but also with test diets. The ratio of the slopes of regression equations for test to standard diets is the relative bioavailability. The requirements for applying the slope-ratio--assay are linearity of regression equations and common y-intercepts.

2. Results and discussion

2.1. HPLC-analysis to determine folate levels in biological materials and foodstuffs

The method of GREGORY and co-workers (1984) for extraction of folate was modified in several ways. An additional proteinase-K-treatment of samples with a high protein content, like rat liver or dried egg yolk, improved the folate extraction by about 25% (PFEIFFER 1993).

Gregory proposed a 60-min heating-time for folate extraction, to achieve complete conversion of 10- to 5-CHO-THF and to carry out a sum-determination of these two derivatives, which cannot be separated chromatographically. But even after heat-conversion of 10- to 5-CHO-THF a high probability of obtaining a mixture of folate derivatives at pH 2.3 of the mobile phase exists, because of the high degree of interconversions between 5-CHO-THF, 10-CHO-THF and 5,10-CH=THF at acidic pH. A short heating time of 10 min was found to be sufficient and it provided a higher yield of the heat-labile folates (PFEIFFER, 1993).

Attention must be paid to the endogenous deconjugase, an enzyme with high activity in rat liver, spinach and asparagus. When this enzyme is active at the moment of homogenisation, folate polyglutamates are rapidly hydrolysed to monoglutamates and no differentiation between mono- and polyglutamates is possible. Therefore in experiment 3 liver samples were heated prior to homogenisation. As a result of the

inactivation of endogenous deconjugase, monoglutamates and polyglutamates (hydrolysed to monoglutamates by exogenous hog kidney deconjugase) could be determinated. This provided not only information on the native pattern of folate derivatives but also on the ratio of mono- to polyglutamates.

2.2. Effect of irradiation on folate levels

Folate concentrations in unirradiated/irradiated dried spinach were $8.3 \pm 0.4/8.5 \pm 0.3$ μg g^{-1}, in dried asparagus $8.2 \pm 0.3/7.9 \pm 0.3$ μg g^{-1} and in egg yolk powder $2.6 \pm 0.3/2.4 \pm 0.2$ μg g^{-1}, respectively. Differences between folate levels in unirradiated or irradiated samples of the same product are not significant. This agrees with earlier studies showing high radiation resistance of folates in meat (ALEXANDER et al., 1956; THAYER, 1990), and in a chicken diet (RICHARDSON et al., 1958).

2.3. Rat bioassays for determining folate bioavailability

Even though chemical analysis shows no effect of irradiation on folate levels, it could be argued that irradiation might affect the food matrix so as to impair the bioavailability of folates. A variety of experimental approaches have been used to determine the bioavailability of food folates. They include measurement of changes in hematological values and urinary folate excretion in humans or of changes in growth and tissue folate levels in chicks and rats in response to known intakes of folic acid of folate containing foods. The variety of protocols has contributed to the variability of current estimates of food folate bioavailability. Relatively low estimates of availability were obtained in human studies using folate loading and urinary excretion (TAMURA & STOKSTAD, 1973). Other studies have reported high availability of food folate in the rat assay (HOPPNER & LAMPI, 1986; ABAD & GREGORY, 1987).

2.3.1. Folate concentrations in plasma and liver. Weight gain and food consumption of rats fed diets with tested foods were not different from those of rats fed the standard diets.

During the depletion period plasma and liver folate concentrations fell to 1/5th until 1/10th of the starting level. Table 3 shows the increase of the analysed parameters during the repletion with standard or test diets. The changes in plasma and liver folate concentration during depletion and repletion period were similar in all experiments. No significant differences occurred between plasma or liver folate concentrations after feeding test diets containing unirradiated or irradiated dried foodstuff.

Plasma folate concentrations of overnight fasted animals in experiment 2 were higher than plasma folate concentrations of non-fasted animals in experiment 1 and 3. As PIETRZIK (1993) has reported an elevation of plasma folate levels in human volunteers after a fasting period of 8 h to 2 days, an additional experiment was carried out, to examine whether fasting led to an elevation of folate levels in rat plasma. This experiment showed that overnight-fasting (14 h) of rats led to a significant increase in plasma, urin and fecal folate concentration. Liver folate concentration was not affected by fasting (PFEIFFER, 1993).

In experiment 3 the native folate pattern of rat liver was determined. In all groups the proportion of monoglutamates to total folate was between 10 and 20%. The monoglutamate pattern of folate derivatives consisted of THF (10–30%) and 5–CH_3–THF (70–90%), the total folate pattern consisted of the three main reduced folates THF (5–25%), 5–CH_3–THF (45–65%) and 5–CHO–THF (15–35%).

2.3.2. Folate content of repletion diets. Table 4 shows the measured folate content of applied repletion diets and the calculated folate intake during the repletion period (measured folate content of the diet × food consumption during repletion period). In experiment 3 repletion test diets contained not only folate from the tested foodstuff (dried egg yolk) but also from folic acid.

The expected values for folate content of repletion test diets – calculated from measured folate levels in the foodstuffs (2.2.) – were:

- 10%/20% dried spinach (as fed to test group E/F): 832/1664 μg kg^{-1} diet
- 10%/20% dried, irradiated spinach (as fed to test group I/J): 847/1694 μg kg^{-1} diet
- 7.5%/15% dried asparagus (as fed to test group E/F): 615/1229 μg kg^{-1} diet
- 7.5%/15% dried, irradiated asparagus (as fed to test group I/J) 595/1189 μg kg^{-1} diet
- 17% dried egg yolk (as fed to test group E): 447 μg kg^{-1} diet
- 17% dried, irradiated egg yolk (as fed to test group I): 413 μg kg^{-1} diet.

The comparison of these values with the data in Table 4 shows a good agreement between expected and measured values for folate content in the test diets with asparagus and egg yolk. In test diets containing dried, unirradiated spinach the measured values corresponded to the expected values, whereas the measured folate values in test diets with dried, irradiated spinach were inexplicably 20–30% lower than expected.

Expected values for folic acid concentration in standard diets were 250, 500, 1000 or 2000 μg kg^{-1} diet. Measured folic acid contents in standard diets of experiment 1 and 2 were in good agreement with the expected values. In experiment 3 the measured folic acid concentration was too low, not only in the standard diets but also in the test diets. The deviation from the expected values may be attributed to the small quantity of added folic acid.

Table 3

Plasma and liver folate concentrations

	Experiment 1 (spinach)			Experiment 2 (asparagus)			Experiment 3 (egg yolk)		
	n	Plasma (nmol l^{-1})	Liver (nmol g^{-1})	n	Plasma (nmol l^{-1})	Liver (nmol g^{-1})	n	Plasma (nmol l^{-1})	Liver (nmol g^{-1})
Initial	3	118.6±11.2	22.5±0.8	3	155.2±20.8	16.2±1.0	3	172.2±20.7	25.5±1.9
Depletion (3 wks)	3	12.4±1.9	3.8±1.0	3	22.1±3.9	4.1±0.4	3	11.6±5.2	2.6±0.5
Pos. control (3 wks)	3	69.8±11.8	13.1±0.3	2	106.5±22.4	9.1±0.3	2	94.6±16.8	10.9±0.6
Repletion:									
St. Gr. A	–	–	–	–	–	–	3	10.3±2.7	4.3±0.6
St. Gr. B	3	22.3±7.3	4.6±0.6	4	35.1±4.6	7.7±0.5	3	20.0±3.8	8.4±1.5
St. Gr. C	3	37.4±4.8	9.4±0.1	4	41.3±5.7	8.4±0.9	3	28.0±4.7	9.5±1.9
St. Gr. D	3	64.9±6.4	12.1±1.2	4	62.9±9.3	14.4±1.1	3	65.3±22.0	12.5±1.5
T. Gr. E	3	41.2_a±2.9	11.0_c±1.0	4	39.2_e±8.1	7.3_g±0.6	3	9.5_j±4.1	3.5_n±0.6
T. Gr. F	3	89.9_b±5.7	14.8_d±0.1	4	146.0_f±37.3	11.1_h±1.0	3	24.5_k±5.6	7.5_o±0.6
T. Gr. G	–	–	–	–	–	–	3	31.9_l±5.8	8.3_p±2.1
T. Gr. H	–	–	–	–	–	–	3	66.1_m±5.6	11.5_s±1.5
T. Gr. *I	3	48.0_a±4.5	11.6_c±0.1	4	36.1_e±4.8	6.0_g±0.5	3	8.6_i±2.7	2.8_n±0.4
T. Gr. *J	3	86.1_b±8.2	18.3_d±1.8	4	118.4_f±24.0	10.5_h±1.2	3	21.1_k±1.2	4.6_o±0.2
T. Gr. *K	–	–	–	–	–	–	3	31.0_l±2.9	6.9_r±0.2
T. Gr. *L	–	–	–	–	–	–	3	57.4_m±2.8	9.0_s±1.3
Neg. control (5 wks)	3	12.2±3.0	1.3±0.1	–	–	–	2	2.5±0.3	1.2±0.2
Pos. control (5 wks)	3	86.9±11.4	6.7±0.1	–	–	–	–	–	–

St. Gr. = standard groups:
A: 0.25 mg FA kg^{-1} diet
B: 0.50 mg FA kg^{-1} diet
C: 1.00 mg FA kg^{-1} diet
D: 2.00 mg FA kg^{-1} diet

T. Gr.(*) = test groups with unirradiated (irradiated) foodstuffs:
E(I): 10% dried spinach (*) or 7.5% dried asparagus (*) or 17% dried egg yolk (*)
F(J): 20% dried spinach (*) or 15% dried asparagus (*) or 17% dried egg yolk (*) + 0.25 mg FA kg^{-1} diet
F(K): 17% dried egg yolk (*) + 0.50 mg FA kg^{-1} diet
H(L): 17% dried egg yolk (*) + 1.00 mg FA kg^{-1} diet

n = number of rats in a diet group; Values for plasma folate are means±standard deviation from n rats in a diet group; Values for liver folate in exp. 3 are means±standard deviation from n rats in a diet group; in exp. 1 and 2 values for liver folate were means±standard deviation from 2 determinations of pooled livers; Within a given column, results sharing the same subscript showed no significant difference ($P > 0.05$)

Table 4

Measured folate content of repletion diets and calculated food intake during repletion period

	Experiment 1 (spinach)		Experiment 2 (asparagus)		Experiment 3 (egg yolk)			
	folate content ($\mu g\ kg^{-1}$ diet)[1]	food intake (μg)	folate content ($\mu g\ kg^{-1}$ diet)[1]	food intake (μg)	from FA	folate content ($\mu g\ kg^{-1}$(diet)[1] from egg yolk	sum	food intake (μg)
St. Gr. A	-	-	-	-	286 ± 83	-	286 (83)	94 (30)
St. Gr. B	487 ±172	176 (72.2)	675 ±106	170 (27.4)	395±123	-	395 (123)	134 (56)
St. Gr. C	805 ± 98	287 (35.4)	1116 ±136	279 (37.9)	589±129	-	589 (129)	189 (72)
St. Gr. D	2030 ±257	699 (144.8)	2140 ±198	539 (51.2)	1494±308	-	1494 (308)	507 (134)
T. Gr. E	823_a ± 39	298 (64.3)	715_e ± 10	177 (4.8)	-	441_h±49	441 (49)	137 (22)
T. Gr. F	1659_c ±218	536 (100.3)	1428_f ± 64	352 (22.4)	350±151	462_h±35	812 (186)	269 (72)
T. Gr. G	-	-	-	-	435±127	456_h±37	890 (164)	311 (66)
T. Gr. H	-	-	-	-	655±164	451_h±34	1106 (198)	378 (74)
T. Gr. *I	665_b ± 53	236 (36.8)	731_c ± 47	184 (12.6)	-	439_h±43	439 (43)	140 (24)
T. Gr. *J	1215_d ± 99	419 (60.9)	1337_f ± 71	322 (22.6)	188 ± 64	447_h±20	635 (84)	199 (54)
T. Gr. *K	-	-	-	-	253 ± 85	451_h±20	704 (105)	216 (55)
T. Gr. *L	-	-	-	-	363 ± 98	451_h±27	814 (125)	277 (85)

Description of Table 4 is identical to description of Table 3

[1] Values are means ± standard deviation from 4 determinations; Values in parentheses are the maximum possible error calculated from error--reproduction-rule; Within a given column, results not sharing the same subscript differed significantly ($P < 0.05$)

2.3.3. Determination of folate bioavailability. Bioavailability was estimated on the basis of regression equations determined for plasma and liver folate concentration and of folate intake from the standard diets in the repletion period. Calculated regression equations showed linearity in experiment 1 and 2 for both parameters plasma and liver: $y = 11.100+0.078x$, $r = 0.950$ (exp. 1, plasma), $y = 1.199+0.005x$, $r = 0.984$ (exp. 1, liver), $y = 21.394+0.076x$, $r = 0.870$ (exp. 2, plasma) and $y = 3.971+0.0189x$, $r = 0.983$ (exp. 2, liver). In experiment 3 a linear regression equation could be determined only for plasma: $y = 1.096+0.129x$, $r = 0.947$. For the second parameter liver a saturation curve was fitted: $y = C+A(1-e^{-Bx})$ with $A = 12.754$, $B = 0.006$ and $C = 0.380$.

The values for plasma or liver folate concentration after repletion with test diets were substituted into these regression equations. The obtained folate uptake was divided by the calculated folate intake and multiplied by 100 to yield relative bioavailability. Using either plasma or liver folate concentration as a response variable, bioavailability estimates are shown in Table 5.

Table 5

Relative bioavailabilities (in %) of folate in dried spinach, asparagus and egg yolk

	Regression analysis		Slope-ratio-assay	
	Plasma	Liver	Plasma	Liver
Unirradiated, dried spinach	$160_a \pm 34$	170	170	169
Irradiated, dried spinach	$215_b \pm 21$	252	252	272
Unirradiated, dried asparagus	$132_c \pm 59$	104	–	108
Irradiated, dried asparagus	$105_c \pm 34$	83	–	106
Unirradiated, dried egg yolk	$47_d \pm 23$	$34_e \pm 6$	121[1)]	–
Irradiated, dried egg yolk	$41_d \pm 12$	$25_e \pm 3$	147[1)]	–

Within a given column, results not sharing the same subscript differed significantly ($P < 0.05$); No standard deviation indicated for liver in experiments 1 and 2, because livers of each diet group were pooled for analysis; [1)] Bioavailability for the system folic acid + folate from unirradiated or irradiated dried egg yolk

Bioavailabilities can also be determined with the slope-ratio-assay, if two or more concentrations of tested food were incorporated in the repletion diets. Further requirements for employing this test are linearity of the calculated regression equations and common y-intercepts. The ratio of the slopes of regression equations for test to standard diets reflects the relative bioavailability. Table 5 shows bioavailabilities calculated by slope-ratio-assay in comparison to regression analysis.

In all experiments bioavailability estimates from plasma are in good correspondence with those from liver, so that these two parameters are equally useful indicators of bioavailability of food folates in short-term bioassays. Where slope-

-ratio-assay could be applied (spinach, both parameters; asparagus, parameter plasma) the calculated bioavailabilities with this test agree with the bioavailabilities calculated by regression analysis.

The irradiation of dried spinach with a dose of 10 kGy gave rise to a significantly higher folate bioavailability, whereas the irradiation of dried asparagus (5 kGy) and dried egg yolk (2 kGy) had no significant effect. As indicated above (2.3.2.), folate concentration in the test diet with irradiated dried spinach was 20 to 30% lower than expected on the basis of folate concentration in the irradiated dried spinach added to these diets. Bioavailability being calculated as the ratio of folate uptake in the body to folate intake via diet, an erroneously low intake figure would result in an erroneously high bioavailability. In view of the lack of an effect of irradiation on folate concentration in plasma and liver (Table 3) we conclude that the calculated increase in bioavailability in the case of dried spinach was more apparent than real. It may have resulted from an erroneously low result of folate analysis of the diet.

Estimated folate bioavailabilities from dried spinach and asparagus were higher than 100% (Table 5). In dried spinach folate availability was 160% calculated from plasma and 170% from liver. The bioavailability of folates in dried asparagus was 132% from plasma and 104% from liver. For folates from asparagus no value of bioavailability was found in the literature. Using folate excretion in urine as parameter BABU and SRIKANTIA (1976) measured a folate bioavailability in spinach of 63%. Using liver or serum folate concentration in rat-bioassay as a response parameter the availability of folates in dried spinach were 97–131% (HOPPNER & LAMPI, 1986), 84% (BABU & LAKSMAIAH, 1987) and 75% (CLIFFORD et al., 1991).

Many reasons can lead to bioavailabilities higher than 100%. An incomplete in vitro extraction of folates from the food matrix would underestimate folate content of test diets and so overestimate folate bioavailability. Since an additional enzyme--treatment with cellulase and α-amyloglucosidase provided no higher folate content in spinach and asparagus, incomplete extraction of folates seems unlikely.

Another reason for a bioavailability above 100% can be the presence of a folate--protecting factor in the food matrix. Ascorbic acid is known to protect labile, reduced folates from oxidation. FARRAR and BLAIR (1989) suggested that the major site of catabolism of folates is the gastrointestinal tract, particularly the stomach. Plasma kinetics indicated a 2–3-fold rise in plasma $5-CH_3-THF$ when dosed with ascorbate, compared to without ascorbate. The antioxidant ascorbate apparently promotes the entry of folates into the body by preventing oxidative breakdown.

Since spinach and asparagus are high in vitamin C (52 mg/100 g fresh spinach and 21 mg/100 g fresh asparagus; SOUCI and co-workers 1989/90) and a considerable amount of these dried materials (10 or 20% of dried spinach; 7.5 or

15% of dried asparagus) was mixed into the test diets, a high vitamin C intake occurred for the animals fed these test diets (ca. 5–20 mg per day and rat). In contrast, animals fed the standard diets had no vitamin C intake. In the two above-mentioned rat-bioassays (HOPPNER & LAMPI, 1986; CLIFFORD et al., 1991) only 0.8–3.2% dried spinach was mixed to the test diets. This may be the reason for estimated bioavailabilities in these experiments being somewhat lower than in our study.

Calculated bioavailabilities of folate in dried egg yolk (47% in plasma and 34% in liver) are in agreement with literature values. Using folate excretion in urine as parameter BAEU and SRIKANTIA (1976) reported a folate bioavailability of 72.4% (35-137%) for chicken whole egg. TAMURA and STOKSTAD (1973) found the availability of folate in hard boiled egg yolk to be 39% (0-129%). The much lower bioavailability for folates in egg yolk (no vitamin C content) agrees with the hypothesis that foodstuffs high in vitamin C content have also a higher folate bioavailability.

3. Conclusion

A depletion period of 21 days is sufficient to minimize plasma and liver folate concentrations, so that in a following 14-days repletion period a clear and concentration-dependent rise of folate levels in plasma and liver occurs.

To obtain a linear regression line between the folate intake in the repletion period and the plasma or liver folate concentration, the content of folic acid in the standard diet should not exceed 2 mg kg^{-1} diet.

As changes in plasma and liver folate concentration during depletion and repletion period were similar, these two parameters are equally useful indicators of bioavailability of food folates in short-term bioassays. This is also shown by the good agreement in bioavailability calculated from plasma and liver data.

For the two vegetables spinach and asparagus – high in folate and ascorbic acid content – calculated bioavailabilities were higher than 100%. Bioavailability of folates in egg yolk, which is lacking ascorbic acid, was about 40%. We propose that a high vitamin C level in the diet leads to high folate bioavailability.

Analysis of folates in irradiated dried spinach (10 kGy), asparagus (5 kGy) and egg yolk (2 kGy) revealed no significant difference as compared to the unirradiated products. Bioavailability of folates, as determined in folate-depleted rats, was unaffected by irradiation in dried asparagus and egg yolk. In dried spinach irradiation caused a questionable increase in bioavailability of folates.

Abbreviations

FA	folic acid	*	irradiated
THF	tetrahydrofolic acid	ODS	octadecylsilyl-phase
5-CH_3-THF	5-methyltetrahydrofolic acid	DEAE	diethyl-aminoethyl
5-CHO-THF	5-formyltetrahydrofolic acid	HPLC	high-performance liquid chromatography
5,10-CH=THF	5,10-methenyltetrahydrofolic acid		

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Acta Alimentaria, Vol. 23 (1), pp. 119 (1993)

BOOK REVIEW

Radioecology and environmental protection

Ellis Horwood Series in Environmental Management, Science and Technology

A. S. SZABÓ

Ellis Horwood Ltd., Chichester and Akadémiai Kiadó, Budapest, 1993, 258 pages

Environmental protection is a very important activity of the world population. Factors of natural and/or artificial origin influence our environment. The radio-environmental science or radio-environmental biology call it radioecology and it involves the radiation, radioactive isotopes in environment, and their interactions with living materials, plants, animals and humans. Our duty is to know as much as possible about this field. The author did his best to collect the newest information about radioecology. We should be in aware of the fact that radiation and radioactive materials are part of our life, we should live together with them, we have to use them with responsibility.

The author discuss related problems in 4 chapters, on 258 pages, with 100 tables and a great number of references. The main topic is the radioactivity in the biosphere and the relationship with the atmosphere, soil, plants, animals and man. The author gives a good survey of the properties of radioactive elements and their role in food chain, and some possibilities of decontamination during food processing. The application of radiation in agriculture and food industry is also discussed.

An interesting statement of the book is, that the background radioactivity is gradually increasing as a consequence of the nuclear weapon tests, emission from the nuclear power plants and different human activity with radioactive isotopes (medical therapy, experimental work, etc.). However, we should accept that they belong to our life. The question is, what is the acceptable level of radioactivity? How can the radioactive contamination in the biosphere, in our environment be prevented and/or reduced?

The radioecological research and organizations in Hungary are also outlined. This chapter informs us about the activity and results of the control/monitoring system of food and food chain.

I. F. KISS

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ACTA ALIMENTARIA

VOLUME 23 NO. 1 – 1994

CONTENTS

Application possibilities of nuclear filter-membranes in the food and beverage industry
SZABÓ, A. S. 3
Effect of immobilized yeasts on the quality of bottle-fermented sparkling wine
BÚSOVÁ, K., MAGYAR, I. & JANKY, F. 9
Physiological and pathophysiological aspects of free radicals
ANTAL, M. 25
A comparative study of physicochemical indices (phenolic substances) which can affect the colour of Spanish red wines
HEREDIA, F. J. & GUZMÁN-CHOZAS, M. 33
Condensation of glucose by the reversed hydrolysis reaction of glucoamylase. – Part I. Effect of both D-glucose and starch concentrations, temperature, and enzyme concentration
EL-SAYED, H. & LÁSZLÓ, E. 43
Condensation of glucose by the reversed hydrolysis reaction of glucoamylase. – Part II. Effect of inhibition of glucose derivatives and pH
EL-SAYED, H. & LÁSZLÓ, E. 59
Characteristic of *Leuconostoc oenos* strains occurring in Hungarian wines
JUHÁSZ-ROMÁN, M. 71
Separation of a methionine-enriched fraction from enzymatically modified casein by fast protein liquid chromatography
DEMECZKY, ZS., HUSSEIN, S. & HAJÓS, GY. 85
A potential protein engineering site in *Aspergillus niger* glucoamylase: vicinity of disulphide bridge 449 – 222
VÁRALLYAY, É., SASVÁRI, ZS., HOSCHKE, Á. & ASBÓTH, B. 93
Effect of irradiation on folate levels and on bioavailability of folates in dehydrated foodstuffs
PFEIFFER, C., DIEHL, J. F. & SCHWACK, W. 105
Book review 119

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THE COMPETITIVENESS OF HUNGARIAN FOOD INDUSTRY

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(Received: 4 October 1993; accepted: 21 April, 1994)

The increase of the competitiveness of Hungarian food industry is of vital importance, not only for the branch, but also for the whole Hungarian economy. Using PORTER's four-dimension model of competitiveness, the article analyses the competitiveness of the food industry as a function of factor and demand conditions, industrial strategies- and structure and condition of supporting and related industries. The authors underline the importance of the optimal use of the agroecological potential. The article analyses the structural changes in the Hungarian food industry and proves that the concentration of the Hungarian food industry is comparatively low. The lack of effective market competition is breaking the development of food-industrial competitiveness on foreign market, too. Privatisation gives a good change for the innovation in food industry, but the first aim of buyers is to buy new market chances. On the base of the system-analysis of competitiveness some strategical suggestions are given for further development.

Keywords: food economy, food industry, competitiveness, food policy, privatisation

The Hungarian food industry plays a determinative role in satisfying the home-demands and is a very important net foreign-exchange importer (Table 1).

Table 1

The role of food industry in the Hungarian national economy

	Share in gross output of national economy	Share in GDP (%)	Share in export (%)	Share in import (%)
Agriculture	14.8	13.9	7.3	2.0
Food industry	8.9	3.0	18.0	3.8
Sum	23.7	16.9	25.3	5.8

Source: ANON (1992)

0139-3006/94/$ 4.00

The effectiveness of food-product export is better than in most fields of Hungarian national economy, that is why the food-industrial export is of vital importance from the viewpoint of the external balance of the national economy. The majority of agricultural products is processed in the food-industrial sphere, so the competitiveness of the food industry is a determinative factor for the rural development and employment, too. The rush and dynamic changes in foreign markets of Hungarian food-industry (collapse of the COMECON, the emergence of the European Single Market) and the increasing Hungarian food import (as a consequence of the Hungarian approach to EC) increase the need and the importance of an up-to-date analysis of the competitiveness of the Hungarian food industry.

The results of this analysis can be used in the long-range planning of national economy, in the strategical management of the Hungarian agribusiness and in the formation of the strategy and the tactics of privatisation.

1. Methods of determination of competitiveness – a theoretical approach

There is a long history of efforts to explain international success in industries in the form of international trade (HARROD, 1957, DIXIT & NORMAN, 1980), but the rush changes in character and form of international competition in the post-World War II period (technological development, coexistence of globalisation and protectionism in international trade relations) increase the need for a new approach of competition.

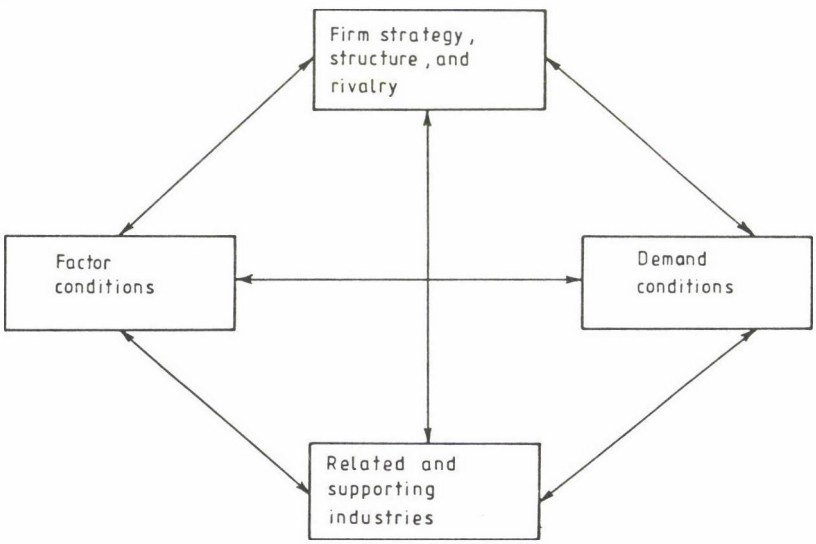

Fig. 1. The determinants of national advantage (PORTER, 1990)

PORTER (1990) underlines the importance of a complex and dynamic approach of competitiveness. His model (Fig. 1) explains the competitiveness of a given nation's industry as a function of four main determinants. This model can be used as an effective tool to determine the competitive position of Hungarian food industry and to outline the most important directions of development.

2. Results

2.1. The factor-conditions of Hungarian food industry

In the cost-structure of food-industrial production the costs of raw materials play an essential part (40–90% of the whole production-cost depending on the grade of processing), so the agroecological potential and the level of agricultural production is a main factor of food-industrial competitiveness. The level of Hungarian agroecological potential is rather high (CSETE, 1980), but the increasing environmental pollution (taking into consideration the comparatively low technical level of Hungarian economy) and the intensive pressure of the Western-European governments on their firms with environmentally risky activity to relocate their plants into less developed (e.g. Central- and Eastern-European) countries. Figure 2 shows that there is a considerable decrease in agriculturally usable land.

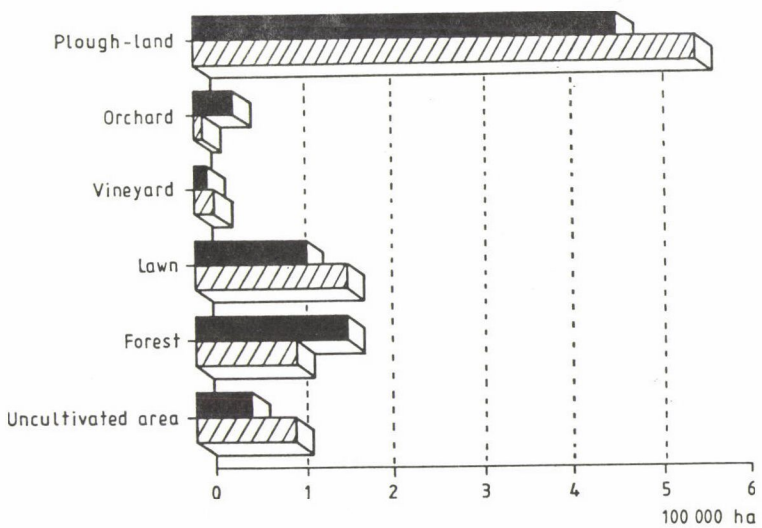

Fig. 2. Land-area by branches of cultivation (1925/30–1985/90). ■ : 1925–1930; ▨ : 1985–1990

The efficient use of natural resources is determined by the structure of economic activities. From the viewpoint of competitiveness in the last four decades the following errors and mistakes have been made:

- Inadequate geographical distribution of various agricultural activities, not taking into consideration the agroecological conditions (e.g. locate fruit- and wine plantations in regions with a high level of belated frost-hazard).
- Use of inadequate (corn cultivars with high productivity, but low level of product quality), one-sided and/or obsolete species and cultivar-structure (the apple production has been turned into the most important part of Hungarian fruit-growing, and in the apple production the obsolete Jonathan-production plays a determining part).
- Low utilization of special agroecological possibilities (e.g. there is no up-to-date system of origin-certification in Hungarian wine-making).

The high-quality human resources and the long traditions of agricultural production are important primary factors of the competition, too.

The development of agricultural production may be an effective tool in the decreasing of unemployment. From this viewpoint the labour-intensive horticultural production plays a key role.

The most important fields of the qualitative development of human resources on every level are as follows:

- Increasing business and farm-management knowledge and skills;
- Widespreading the ability to use modern information-resources and data-processing techniques;
- Increasing the knowledge of environmentally protective methods of agricultural and food-industrial production;
- Better use of foreign language on middle and top-management level.

2.2. Demand condition for products of food industry

The intensity and sophistication of home demand plays a determining role in the international competitiveness of a given industry.

During the last four decades there have been considerable changes in Hungarian food consumption.

The most important characteristic features of the change of food consumption are:

- The increasing consumption of more valuable food products (meat, fruits),
- Decreasing consumption of less valuable products (cereals),
- Mass consumption of products which formerly were specialities and delicacies (coffee).

The Hungarian food consumption is too quantity-oriented, the energy-intake is comparatively high (ANON, 1992).

The most important problems of the Hungarian home-food market are:

– After the long decades of equalization of personal revenues the diversification of purchasing power increases. There is an increasing pauperisation and an increasing primitive capital accumulation parallel, and a need for a middle-class but this class has not formed yet.

– Increasing smuggle and illegal turnover of the goods with a governmental inland revenue.

The too high level of governmental taxation creates a considerable difference between home and foreign prices and this is incentive for smuggling and illegal trading. The result is the decrease of aggregate revenue at product groups of coffee, spirits, tobaccos, etc. both on enterprise and national level.

– Low level of consumption culture at a wide variety of products.

For example there is a considerable over-production of wines, and one of the most important channel of problem-elimination could be the increase of home consumption but the culture of wine consumption is on a comparatively low niveau (lack of wine-stubes, refined bottles and bottle-labels, high qualified, foreign-language speaking salespersonnel), so there are no practical chances of short-run solution of this problem.

2.3. Firm strategy, structure and rivalry

CSIZMADIA and SZÉKELY (1986) provide a detailed account of the development of the Hungarian food industry and its relationship with agriculture before the recent beginning of wholesale economic reform. Here, attention is focussed on some of its distinguishing characteristics.

After World War II the reorganisation of food industry took place on the base of centrally-planned economy. One of the most important aims of reorganisation was the formation of homogeneous companies which could be directed centrally. After the partial economic reforms of 1968, the structure of Hungarian food industry was progressively changed. The most important aspects of change were as follows:

– The decentralisation of food industry by liquidation of trusts between 1979 and 1991;

– The construction of numerous small- and medium-scale factories by co-operatives and municipalities;

– The introduction of freedom of choice of activity's field of food-industrial firms.

As a result of this processes the concentration of Hungarian food industry considerably decreased. Using various indicators of concentration (Figs 3, 4) it can be

proved, that the generally-accepted theory about the "too high" rate of concentration in Hungarian food economy is not acceptable on the base of up-to-date facts.

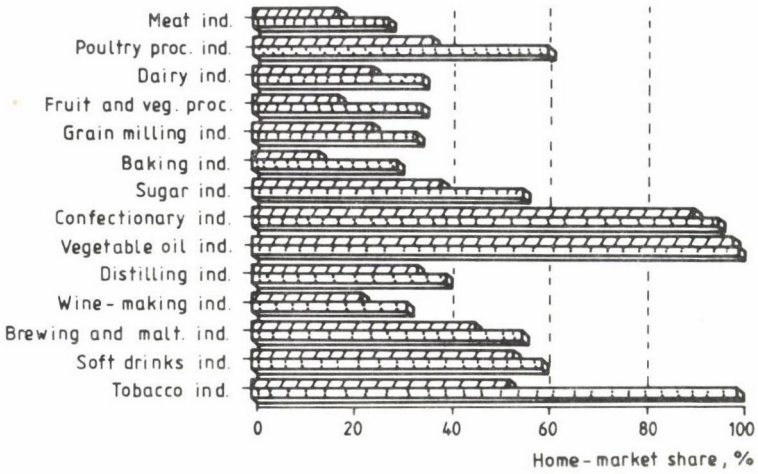

Fig. 3. The share of the largest, and the four largest companies in Hungarian food market. ▨ : Branch-largest co.; ⊡ : four largest co.

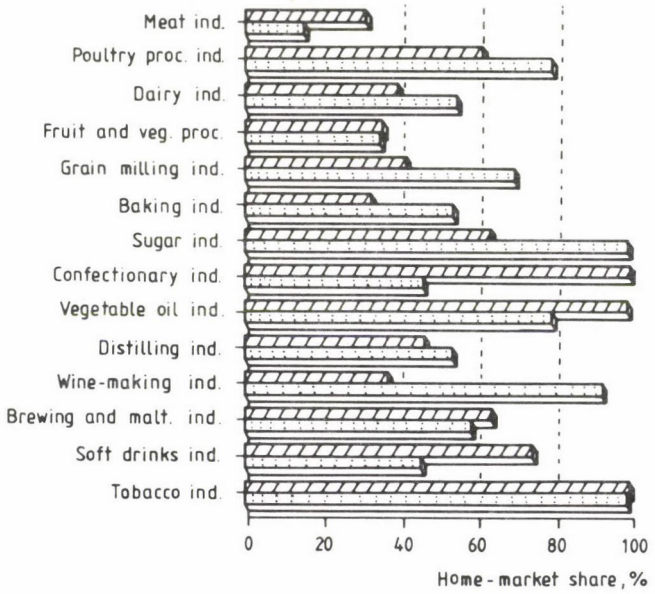

Fig. 4. Market share of the five largest food companies in Hungary and Great Britain. ▨ : Hungary; ⊡ : Great Britain

The comparatively low level of concentration is only a necessity but not a sufficient precondition of economic competition. Still, in the Hungarian food market real competition has not been formed, at a considerable high number of firms, because

- For the majority, of Hungarian firms (mainly in canning, wine-making, poultry-processing, grain and milling industrial firms) the most important field of realisation was the low demanding COMECON market.
- In most branches the increasing of (highly dotated) convertible export played an important role from the viewpoint of governmental supports and personal interest system of managers;
- The firm import-regulation system braced the import of food-industrial products, so there was no import competition on the home-market of various products;
- Even in the '80s there was a seasonal and/or geographical shortage of some product groups in Hungary (e.g. brewery products, products of vegetable oil industry).

It can be seen, that the economic environment did not give enough incentive for a real competition on home-market, and the former COMECON states needed low-quality products because of lack of purchasing power.

In this situation the competitiveness of Hungarian food industry considerably decreased.

The privatisation of food-industrial firms means a new era in the development of food industry and plays role in the competitiveness.

Table 2

The share of privatised companies in Hungarian food industry by branches as a per cent of capacities (Dec. 1993)

	%
Meat and meat processing industry	5
Poultry processing industry	3
Grain milling industry	6
Dairy industry	4
Sugar industry	42
Vegetable oil industry	100
Fruit and vegetable processing industry	44
Confectionary industry	100
Distilling industry	29
Brewing and malting industry	60
Wine-making industry	10
Tobacco industry	100

The privatisation (Table 2) of Hungarian food industry is mainly based on foreign capital, because the home-based purchasing power is considerably small. The rate of privatisation is slower than it was anticipated at the beginning of the process five years ago. The most important causes of this situation are:

- General recession on the world market;
- Too large supply of production capacities in former COMECON-member states;
- Uncertain political situation in Central Europe (e.g. civil war in Yugoslavia);
- Lack and insufficiency of legal environment.

The most important lessons of privatisation up to this date are:

- One of the most important driving force of buying from the viewpoint of investors is the safe home-market. The branches with mono- (e.g. vegetable oil industry) or oligopolistic (e.g. sugar industry) position are highly preferred. It is often the case, that firms which formerly were enthusiastic advocates of free trade and competition after the privatisation fight for a more protectionalist governmental policy.
- The branches with a more simple, standard technology (e.g. tobacco industry, brewery industry) have a more preferred position;
- The branches, producing mainly for the former COMECON market have a backward position in the process of privatisation;
- It is often believed that privatisation and competition go hand in hand, but the real events do not prove this theory, because the former independent firms are often bought by the same companies;
- All over the world there is no experience concerning such large-scale privatisation. So there are no patterns of privatisation before the Hungarian government. This, and the strong effect of political forces in the course of privatisation is one of the main issue of the lack of governmental strategy of privatisation in food industry. This lack of strategy has caused numerous mistakes, and often weakened the competitiveness of food industry.

The privatised firms try to increase their own competitiveness. The most important general behaviour patterns are the following:

- General rationalisation and automatization of various firm's activities;
- Restructuring of product-sortiments, pruning the number of various products;
- More emphasis is laid on marketing activities;
- Intensive orientation to neighbouring countries;
- Improvement of financial management and managerial accounting system;
- Improvement of raw-material quality using incentive for paying systems.

For the further development of food industry the increasing profitability of production is a natural precondition. It can be seen from Fig. 5 that in food industry original income practically is not produced. The high rate of income centralisation by the state makes the situation even worse from year to year (Fig. 6).

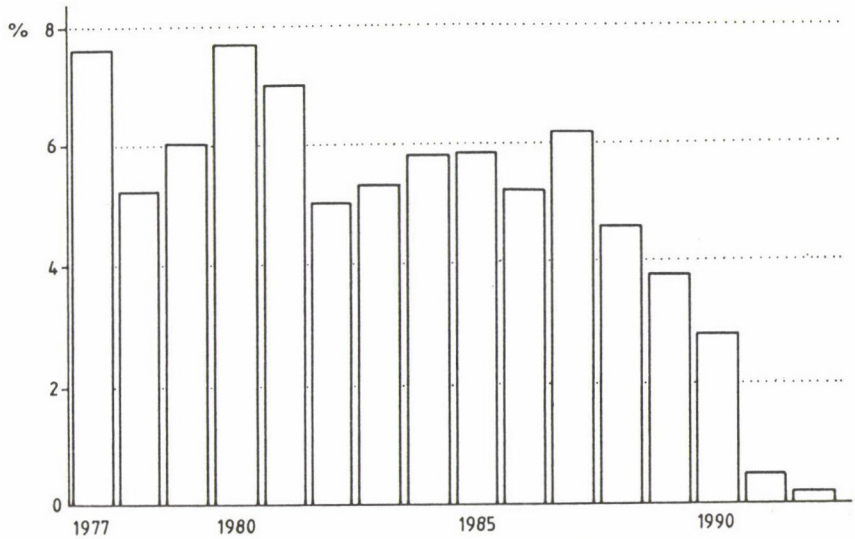

Fig. 5. The decrease of rate of capital return in the Hungarian food industry

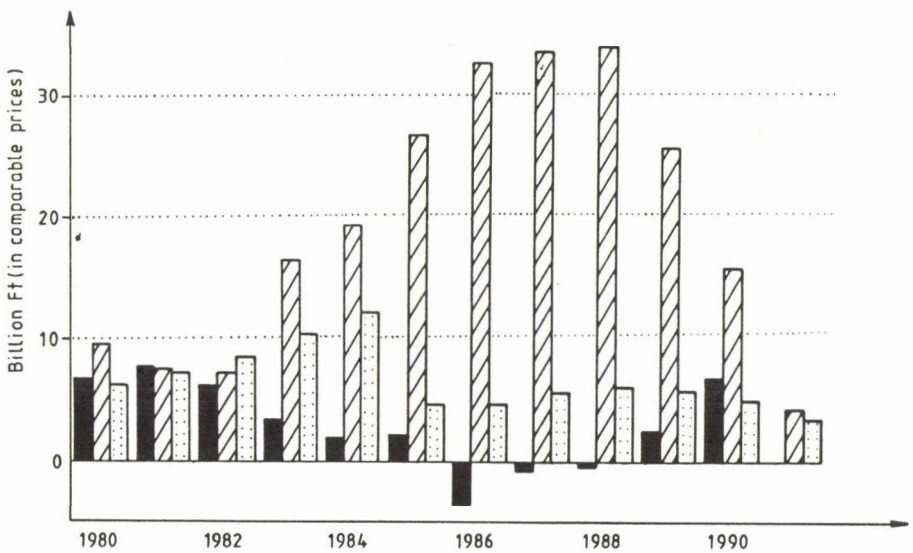

Fig. 6. The change of original income, financial support and drain of income in Hungarian food industry (1981–1991). ■ : Original income; ▨ : dotation; ⊡ : skimmed sum

2.4. The level of related and supporting industries

The increasing disproportion between the princes of industrial inputs and agricultural outputs is an effective barrier of increasing the quality of agricultural products for industrial use. The considerable decreasing of revenue of agricultural producers gives no possibility of safe, predictable, convenient use agricultural raw-material production for industrial purposes. For example the irrigable land-area is decreasing from year to year, so the agricultural production shows a considerable variance. The large state-farms and co-operatives reacted upon the market changes much more slowly, than the numerous small-farms, so by increasing the number of small agricultural producers more rapid changes can be predicted.

The above examples underline, that the uncertainty of agricultural production is an effective brake of food-industrial competition.

The supporting industries have developed comparatively slowly, and one-sidedly in years of centrally-planned economy and distribution of work within COMECON states. The industrial-input producers often had monopolistic position (e.g. chemical or paper industry) or are rather obsolescent and fragmented (e.g. in Hungary 11 producers produce tins for canning industry in a yearly amount of 350 million tins. In a developed state only one plant is enough to produce such quantity).

The privatisation gives new perspectives in some branches for technical development by foreign working capital (e.g. in paper industry), so from the viewpoint of international competitiveness Hungarian food industry will have better background than in the past. The development of small-industry sector is an effective tool for the satisfaction of small-scale producers who produce products for the niche market on base of specific orders (e.g. small-capacity printing houses for label-printing).

The logistical system of the society is of vital importance for the food distribution sector. In the centrally-planned economy the infrastructure of commerce was comparatively less developed, than other spheres of society, so there was little or no possibility to use up-to-date commercial techniques. The privatisation gives a good possibility of increasing the technical level of food-retailing trade because here there is no need for a large-scale capital investment, and the personal capital of Hungarian inhabitant can be effectively used.

The technical development of wider infrastructure (communication, transportation, banking-services, etc) needs a longer period, and an inflow of foreign capital.

3. Conclusions

Analysing the competitiveness of Hungarian food industry using Porter's general model of competitiveness, the following conclusion could be fored:

– In the reconstruction of Hungarian food economy a special emphasis should be laid on the optimal use of agroecological potential taking the viewpoints of environmental protection into consideration;

– The low price of human resources is only a short-run competitive advantage of Hungarian food industry. In the future more emphasis should be laid on qualitative development of human resources;

– The food-industrial firms must learn, how to adapt to a differentiated consumer's demand structure;

– An adequate strategy must be worked out for the Hungarian food-import policy. This strategy should deal with three problems:

  – the role of export in increasing the competition on home-market;

  – the stopping of smuggling and private import;

  – the defense of food-producers from short-run dumping measures of foreign producers.

– In privatisation the viewpoint of assurance of long-run competitiveness of Hungarian food-industrial sphere must be taken into account with more emphasis.

– Lowering the rate of centralised income is a necessary precondition for the increase of competitiveness, because in this situation there are practically no resources of any development.

– The stabilisation of agricultural production adapting various methods of modern market regulations is of vital importance for food industry.

– The development of the infrastructure is a necessary precondition of the further increase of competitiveness of Hungarian food industry.

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COMPARATIVE STUDY ON SOME FERMENTATION PROPERTIES OF *STREPTOCOCCUS THERMOPHILUS* AND *LACTOBACILLUS ACIDOPHILUS* IN MILK AND MODIFIED MILK MEDIA

PART II. EFFECT OF THE INCREASED SOLID NOT FAT ON THE FERMENTATION PROPERTIES OF THE MIXED CULTURE

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Growth and acid production of the mixed culture of *Streptococcus thermophilus* and *Lactobacillus acidophilus* were investigated in milk and modified milk media as a function of the solid not fat content. The microorganisms were grown on sterilized cow's milk, cow's milk + 1% glucose and cow's milk + 6% tomato juice media, containing 8.5, 10.34 and 12.18% solid not fat (controlled by addition of skim milk powder). Fermentations were run at 45 °C temperature with 3% starters. Results showed that addition of 1% glucose or 6% tomato juice to the cow's milk had a slight stimulatory effect on the growth and acid production of the mixed culture. Increase of the solid not fat up to 12.18% in milk media does not affect the cell counts and acid production significantly but decreases the whey syneresis in the fermented yoghurt-like products. The sensory quality of the fermented products proved to be the best in the case of the fermentation of cow's milk with addition of 1% glucose, containing 10.34% solid not fat.

Keywords: *Lactobacillus acidophilus*, *Streptococcus thermophilus*, fermentation, acid production

Consumption of products containing *Lactobacillus acidophilus* is considered beneficial for maintaining good health and combating intestinal infections (HOOD & ZOTTOLA, 1988). Fermented milk products containing *L. acidophilus* organisms include the following (MARSHALL et al., 1982): Bioghurt, made with *L. acidophilus* and *Streptococcus thermophilus*, although *L. bulgaricus* may be added to enhance acidification (RASIC & KURMANN, 1978); Biogarde, containing *Bifidobacterium bifidum* in addition to *L. acidophilus* and *Str. thermophilus*, ACO yoghurt, made by adding lyophilized culture of *L. acidophilus* to traditional stirred yoghurt after completion of the incubation.

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The basic problem of the *L. acidophilus* fermentations is that this microorganism grows slowly in milk and the sensory quality of this fermented product is not sufficient for the consumers. To overcome this problem some additives have been applied in the fermentation processes as it is partly reviewed in the first part of this paper (BADRAN & REICHART, 1993).

The effect of the nutrient additives (1% glucose and 6% tomato juice), temperature of the fermentation and the inoculum size on the microbial activity was dealt with in the previous part. The conclusions were as follows:

The highest acidity and cell counts were produced by the mixed culture of *Streptococcus thermophilus* and *Lactobacillus acidophilus*. The addition of tomato juice to the milk media exerted a stimulatory effect on the growth of the strains tested and the growth at 45 °C resulted in higher acidity and cell counts than the incubation at 40 °C.

Present work deals with the effect of the increasing solid not fat on the fermentation properties and sensory quality of the yoghurt-like fermented milk product.

The use of reconstituted dried milk in the yoghurt manufacture is an obvious solution when a shortage of milk occurs although the price of the dried milk is gradually increased (EL-SHIBINY et al., 1977). TODORIC and BAJIC (1979) studied the effect of the dried milk addition on the quality of yoghurt. Yoghurt was made from milk alone and with addition of 0.5, 1, 2 and 3% dried skim milk, incubated at 42 °C with 2% starter. Dried skim milk addition improved the viscosity of the product. Over 1% the addition markedly accelerated the acid production and thus reduced the shelf life.

LEDER and THOMASOW (1973) studied the use of gelatin in yoghurt manufacture. Gelatin was used in 0.1–1% concentration (at 10 different levels) in the manufacture of plain and fruit yoghurt from skim milk, milk with 0.5–3.5% fat and whole milk. For improving the consistency and reducing whey separation in yoghurt, 0.3–0.6% high bloom gelatin was added and gave good results without the necessity of increasing the milk solids content.

DAVIS and co-workers (1977) studied the use of milk concentrated by reverse osmosis for the manufacture of yoghurt. The preparation of yoghurt from milk concentrated by reverse osmosis to 12.5 and 15% total solids was compared to that from milk adjusted to comparable concentrations by addition of dried skim milk. Culture growth, acid production, acetaldehyde production, viscosity and flavor scores of yoghurt prepared from the reverse osmosis concentrates were at least equal to those of yoghurt made from conventionally concentrated milk, suggesting that the former constitutes a suitable yoghurt base.

Fortification of milk is an important step in yoghurt making as an increase in solids markedly improves consistency. Commercial yoghurt in the UK is usually

made from milk containing 16–17% total solids. This fortification can be achieved either by addition of skim milk powder, or by evaporation, or by ultrafiltration (ABRAHAMSEN & HOLMEN, 1980).

The effect of the increased solid not fat content on the behavior of the yoghurt microflora and the suitability of these milk media for yoghurt manufacture were studied by MOUSA and co-workers (1985). Results showed that the increase of solid not fat in milk media improved the resultant yoghurt quality and decreased the whey syneresis not only at the end of the fermentation but after 24 h storage in refrigator as well. Also, the increase of solid not fat increased the acidity. The total and lactic bacterial counts of milk media containing 14% solids not fat were higher than those of milk media containing 12 or 14% solids not fat.

BATAWY and co-workers (1987) reported on a cultured butter milk (yoghurt type) product made from cow's butter milk after fortification with 3, 6, 9, 12 and 15% (w/v) dried skim milk. Results showed increase in total solids, total N, acidity, acetaldehyde, lactose and curd tension with increasing concentration of dried skim milk. Acidity and curd tension increased gradually during the storage, while acetaldehyde and lactose decreased. Product obtained by adding 6% (w/v) dried skim milk had the highest sensory score, both when fresh and after storage.

GUIRGUIS and co-workers (1987) studied the whey syneresis of yoghurt made from concentrated skim milk. They found that the control yoghurt prepared from fresh skim milk (8.7% total solids) fortified to 14% total solids with dried skim milk had a syneresis value of 14.5 $cm^3/100$ g. In case of yoghurt prepared from reconstituted skim milk powder alone the syneresis value was 20.2 $cm^3/100$ g.

The aim of present work is to study the effect of the increased solids not fat in milk media on the growth of the mixed culture of *L. acidophilus* and *Str. thermophilus* and on the quality of the fermented yoghurt-like product.

1. Materials and methods

Materials and the chemical and microbiological methods are partly summarized in our previous paper (BADRAN & REICHART, 1993). New methods applied in present work are as follows.

1.1. Increase of the solids not fat in milk media

Solid not fat in milk media was increased by addition of commercial skim milk powder. The average composition of skim milk powder: protein 35.9%, lactose 52.3%, fat 0.8%, minerals 8.0% and moisture 3.0%.

1.2. Determination of whey syneresis

Whey syneresis was determined after centrifugal separation according to HARWALKAR and KALB (1983).

1.3. Sensory evaluation of the products

Products were judged by a panel of 5 members on a 100 points arbitrary scale (flavour 45, body and texture 35, appearance and colour 10, acidity 10).

1.4. Fermentation experiments

The fermentation experiments were run at 45 °C in Erlenmeyer flasks containing 200 cm^3 sterilized milk or modified milk media using 3% inoculum of mixed culture. Temperature was controlled by water bath with accuracy of 0.1 °C. Samples were taken from the flasks at zero time and in every hours until coagulation (3 h). From the samples total acidity, pH and the number of colony forming units (cfu) were determined.

1.5. Preparation of yoghurt-like products

Yoghurt-like fermented milk products were made according to HAMDY and co-workers (1972). The fermentations were run in 200 cm^3 plastic containers (used in the industry for fermented milk products) at 45 °C temperature started with 3% inoculum of mixed culture. After reaching the complete coagulation the products were stored in refrigerator at 6 °C for 24 h and judged sensorically. The whey syneresis was determined immediately after the coagulation and after 24 h refrigerator storage.

1.6. Mathematical statistical evaluation

For the mathematical statistical evaluation of the experimental results the STATGRAPHICS 5.1. program package was used.

2. Results and discussion

2.1. Acid production and growth

In the fermentation experiments the combined effects of the nutrient additives and the increasing solids not fat (SNF) on the fermentation properties were investigated.

Levels of the nutrient additives:

Skim milk
Skim milk + 1% glucose
Skim milk + 6% tomato juice

Levels of SNF: 8.5, 10.34 and 12.18%.

Recovery media for cell counting:

Tryptone Glucose Yeast Extract (TGE) agar
Elliker medium
Modified Elliker (M. Ell.) medium

The experimental results are recorded in Tables 1–3. The values of lg cfu represent algebrical means of 3 parallels.

Table 1

Growth and acid production of the mixed culture
(T = 45 °C, inoculum = 3%)
Skim milk

SNF (%)	Time (h)	T.A. (%)	pH	Means of lg cfu cm^{-3}		
				TGE	Elliker	M. Ell.
8.5	0	0.20	6.46	7.13	7.07	7.09
	1	0.25	6.16	7.54	7.49	7.51
	2	0.47[a]	5.46	8.07	8.01	8.02
	3	0.68[a]	4.89	8.15	8.08	8.10
10.34	0	0.21	6.41	7.20	7.09	7.13
	1	0.27	6.07	7.66	7.53	7.55
	2	0.49[a]	5.39	8.09	8.06	8.07
	3	0.71[a]	4.79	8.19	8.13	8.15
12.18	0	0.22	6.38	7.10	6.99	7.03
	1	0.30	5.99	7.73	7.56	7.59
	2	0.51[a]	5.34	8.14	8.07	8.10
	3	0.75[a]	4.73	8.28	8.21	8.23

T.A.: Total acidity in lactic acid (%)
[a] Coagulated
95% confidence interval for lg cfu means = 0.068

Table 2

Growth and acid production of the mixed culture
(T = 45 °C, inoculum = 3%)
Skim milk + 1% glucose

SNF (%)	Time (h)	T.A. (%)	pH	Means of lg cfu cm^{-3}		
				TGE	Elliker	M. Ell.
8.5	0	0.20	6.45	7.16	7.02	7.07
	1	0.25	6.15	7.68	7.56	7.59
	2	0.48[a]	5.42	8.15	8.10	8.11
	3	0.69[a]	4.86	8.19	8.14	8.16
10.34	0	0.21	6.40	7.18	7.10	7.14
	1	0.27	6.06	7.70	7.57	7.60
	2	0.49[a]	5.38	8.17	8.11	8.13
	3	0.72[a]	4.77	8.25	8.20	8.22
12.18	0	0.22	6.38	7.05	7.00	7.01
	1	0.30	5.98	7.74	7.64	7.69
	2	0.51[a]	5.32	8.17	8.12	8.13
	3	0.76[a]	4.72	8.30	8.25	8.26

T.A.: Total acidity in lactic acid (%)
[a] Coagulated
95% confidence interval for lg cfu means = 0.068

Table 3

Growth and acid production of the mixed culture
(T = 45 °C, inoculum = 3%)
Skim milk + 6% tomato juice

SNF (%)	Time (h)	T.A. (%)	pH	Means of lg cfu cm^{-3}		
				TGE	Elliker	M. Ell.
8.5	0	0.23	6.39	7.18	7.06	7.13
	1	0.28	6.05	7.81	7.61	7.64
	2	0.49[a]	5.38	8.17	8.11	8.12
	3	0.74[a]	4.76	8.28	8.18	8.20
10.34	0	0.24	6.37	7.19	7.11	7.17
	1	0.30	5.98	7.84	7.67	7.73
	2	0.51[a]	5.35	8.21	8.14	8.16
	3	0.76[a]	4.71	8.31	8.21	8.25
12.18	0	0.25	6.35	7.14	7.04	7.08
	1	0.32	5.96	7.88	7.71	7.76
	2	0.54[a]	5.28	8.22	8.17	8.19
	3	0.80[a]	4.65	8.34	8.28	8.29

T.A.: Total acidity in lactic acid (%)
[a] Coagulated
95% confidence interval for lg cfu means = 0.068

Comparing the total acidity (T.A.) values belonging to the same time, it can be established that the addition of 1% glucose does not increase the acid production of the mixed culture significantly. At the end of the fermentations the differences do not exceed the 0.01% value. The addition of 6% tomato juice to the milk media resulted in higher acidity. The difference is caused partially by the acidity of the tomato juice (0.03% in lactic acid at the beginning) but at the end of the fermentations they reach the 0.05–0.06% values.

The effect of the SNF on the acid production is similar in every cases; the increased SNF improves the acid production (Fig. 1).

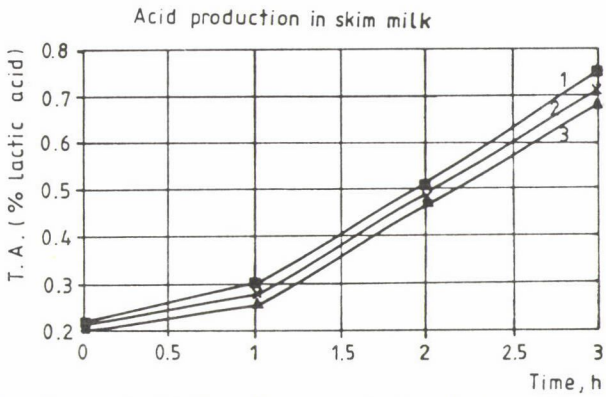

Fig. 1. Effect of the solids not fat (SNF) on the acid production of mixed culture of *L. acidophilus* and *Str. thermophilus* in milk media. (T = 45 °C, inoculum = 3%). 1: SNF = 12.16%; 2: SNF = 10.34%; 3: SNF = 8.5%

Table 4

Analysis of variance for lg cfu

Source of variation	Sum of squares	d.f.	Mean square	F ratio
Main effects				
Time	63.773653	3	21.257884	6043
Nutrient additives	0.497588	2	0.248794	70.73
SNF	0.158714	2	0.079357	22.56
Recovery media	0.445337	2	0.222668	63.31
Residual	1.104460	314	0.0035174	
Total	65.979752	323		

As it can be seen from the horizontal rows of Tables 1–3, the recovery media have affected the cfu values. This effect was evaluated by the variance analysis of the

measured cell numbers (including all of the lg cfu data). The results are summarized in Table 4.

The F values and significance levels in Table 4 demonstrate that all the factors (time, nutrient additives, SNF and recovery media) have significant effect on the cell number. These effects are demonstrated by Figs 2–5.

Effect of the time:

During fermentation the cell number is increasing significantly. The average growth curve of the mixed culture is demonstrated by Fig. 2.

Effect of the nutrient additives:

Both nutrient additives significantly improve the growth. The highest cell number can be achieved by using 6% tomato juice (Fig. 3).

Effect of the increased SNF:

Increasing the SNF from 8.5% to 10.34% the cell numbers have increased significantly. Further increase in SNF to 12.18% resulted in positive effect on the cell counts, but the difference proved to be not significant (Fig. 4).

Effect of the recovery medium:

The TGE medium gave the highest cell numbers, followed by the modified Elliker and Elliker media (Fig. 5).

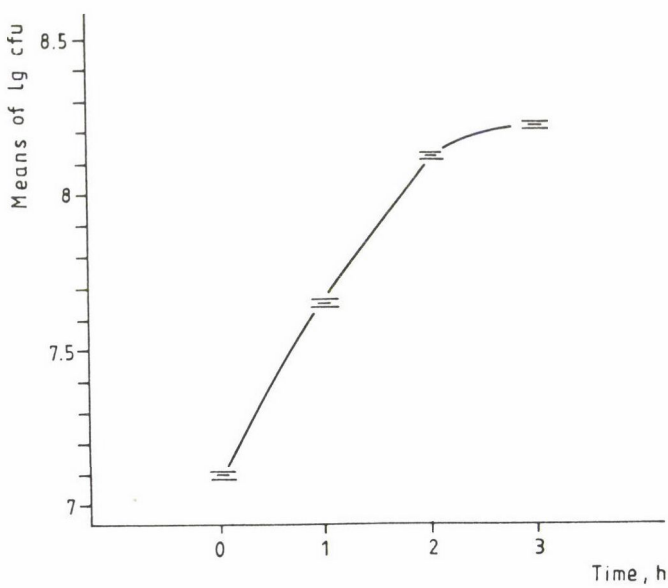

Fig. 2. Average growth curve of the mixed culture of *L. acidophilus* and *Str. thermophilus* in milk media. (T = 45 °C, inoculum = 3%)

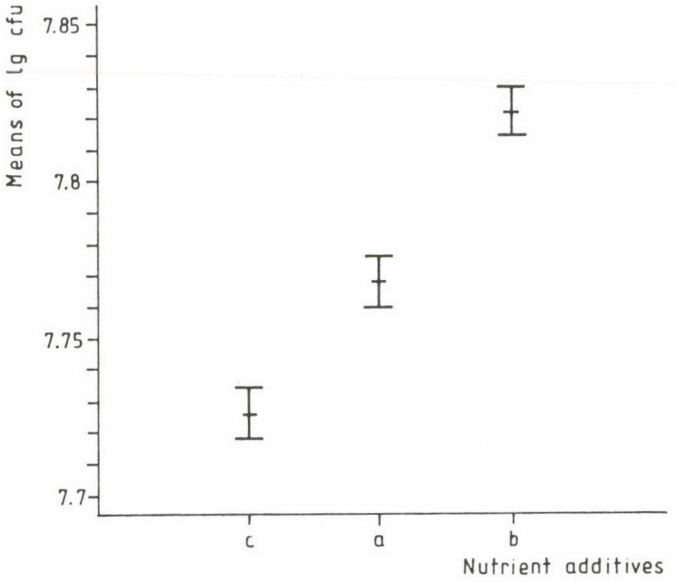

Fig. 3. Effect of the nutrient additives on the colony forming units (cfu). Mixed culture of *L. acidophilus* and *Str. thermophilus* at 45 °C. Abbreviations: c: skim milk, a: skim milk + 1% glucose, b: skim milk + 6% tomato juice

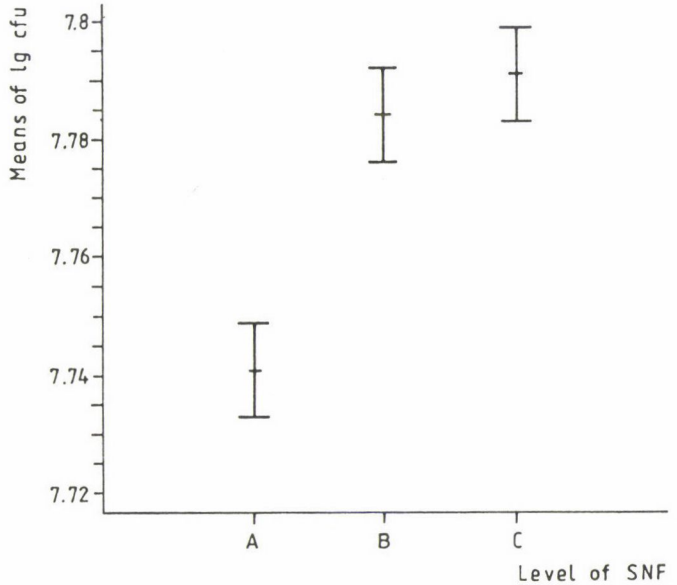

Fig. 4. Effect of solids not fat (SNF) on the colony forming units (cfu). Mixed culture of *L. acidophilus* and *Str. thermophilus* at 45 °C. Abbreviations: A: skim milk, SNF = 8.5%, B: SNF = 10.34%, C: SNF = 12.18%

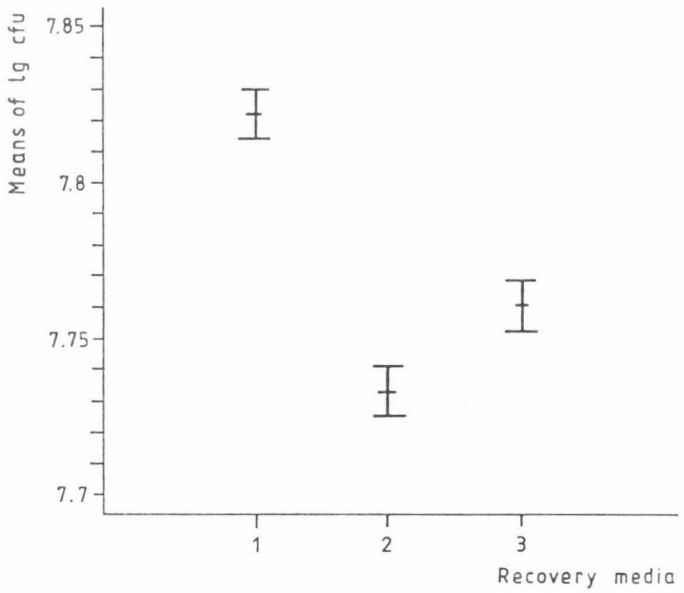

Fig. 5. Effect of recovery media on the colony forming units (cfu). Mixed culture of *L. acidophilus* and *Str. thermophilus* at 45 °C. Abbreviations: 1: TGE agar, 2: Elliker medium, 3: Modified Elliker medium

Table 5

Whey syneresis of yoghurt-like milk products at the complete coagulation and after 24 h storage in refrigerator
(Whey syneresis in % of the initial volume)

SNF (%)	Milk media	At coagulation (%)	After storage (%)
8.5	Cow's milk (control)	46.7	32.2
	Cow's milk + 1% glucose	46.1	31.5
	Cow's milk + 6% tomato juice	53.5	42.0
10.34	Cow's milk (control)	33.4	28.4
	Cow's milk + 1% glucose	31.0	24.1
	Cow's milk + 6% tomato juice	47.0	35.0
12.18	Cow's milk (control)	17.5	12.8
	Cow's milk + 1% glucose	16.5	11.9
	Cow's milk + 6% tomato juice	21.9	15.6

2.2. Whey syneresis

Table 5 shows the measured whey syneresis of the fermented milk products at the complete coagulation and after 24 h storage in refrigerator.

From data summarized in Table 5 the following conclusions can be drawn.

After 24 h storage in refrigerator the syneresis decreases in all cases.

Addition of 1% glucose to the skim milk has slightly decreased the syneresis, while the tomato juice as nutrient additive increases the whey syneresis in all treatments.

Increased SNF decreases the whey syneresis.

2.3. Sensory scores

Table 6 shows the average sensory scores of the yoghurt-like fermented products. The effects of SNF and nutrient additives on the organoleptic properties were evaluated by variance analysis of the total scores (including all data). The results of the variance analysis are summarized in Table 7.

Table 6

Sensory scoring of fermented milk products
Means of the scores of the five-members panel

SNF (%)	Milk media	Flav. 45	Text. 35	Acid. 10	App. 10	Total 100
8.5	Cow's milk	40.0	30.0	7.0	9.5	86.5
	Cow's milk + 1% glucose	40.0	30.5	7.5	9.5	87.5
	Cow's milk + 6% tomato juice	24.0	25.0	6.0	8.5	64.0
10.3	Cow's milk	41.0	31.5	6.0	9.0	87.0
	Cow's milk + 1% glucose	43.0	32.5	6.0	9.0	86.5
	Cow's milk + 6% tomato juice	27.0	28.0	6.5	8.5	69.5
12.2	Cow's milk	38.0	32.0	6.5	9.0	85.5
	Cow's milk + 1% glucose	38.0	33.0	6.5	9.0	86.5
	Cow's milk + 6% tomato juice	27.5	31.5	7.0	8.5	74.5

Abbreviations:
Flav.: Flavour, Text.: Body and texture, Acid.: Acidity, App.: Appearance

Table 7

Analysis of variance for sensory scores

Source of variation	Sum of squares	d.f.	Mean square	F ratio	Sign. level
Main effects					
SNF	85.2778	2	42.6389	2.58	0.0879
Nutrient additives	3235.2778	2	1617.6389	98.08	0.0000
Residual	659.7222	40	16.493		
Total	3980.2778	44			

As the sensory scores summarized in Table 6 show, the nutrient additives have significant effect on the organoleptic properties of the fermentation products. Adding 6% tomato juice to the skim milk, the scores of flavour, texture and appearance decrease and the score of acidity (except for the case of 8.5% SNF) increases. The total scores of the products made with tomato juice additive are significantly less than those of the other fermented milk products. The differences are demonstrated in Fig. 6.

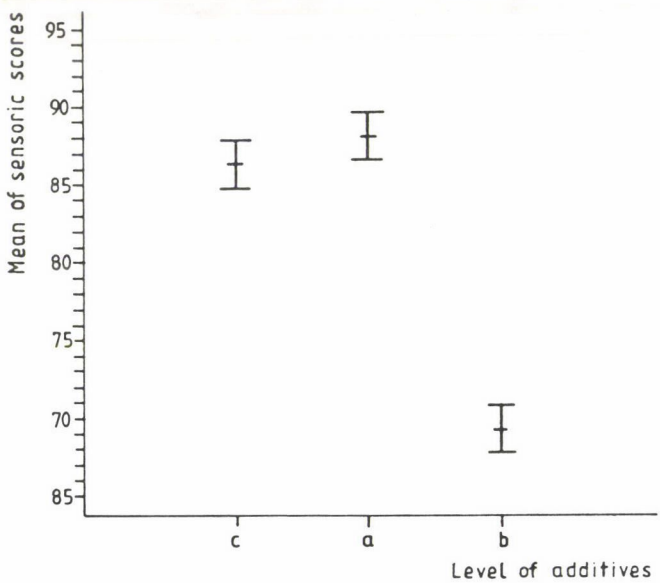

Fig. 6. Effect of the nutrient additives on the sensoric scores of the fermented yoghurt-like products. Abbreviations: c: skim milk, a: skim milk + 1% glucose, b: skim milk + 6% tomato juice

The increased level of SNF has positive effect on the texture and negative consequence on the acidity and appearance. The effect on the flavour is depending on the level of SNF. The flavour proved to be the best in the case of 10.34% SNF and the worst at 12.18%. The total scores increased significantly by increasing the SNF, but the significance level was less than that in case of nutrient additives (Fig. 7).

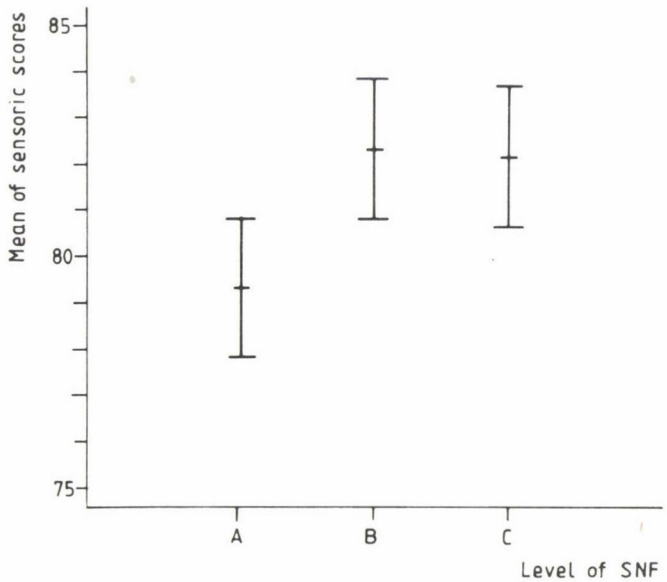

Fig. 7. Effect of the solid not fat (SNF) on the sensoric scores of the fermented yoghurt-like products. Abbreviations: A: skim milk, SNF = 8.5%, B: SNF = 10.34%, C: SNF = 12.18%

As a summary of the effects of the nutrient additives and the SNF on the sensory properties of the yoghurt-like fermented milk products the following conclusions can be drawn.

Addition of tomato juice to the skim milk resulted in an unfavorable quality for consumption.

Addition of 1% glucose to skim milk has slight effect comparing to the sensory quality of the fermented skim milk.

The increased SNF significantly reduces the negative effect of the tomato juice, but does not improve the quality of the other fermented products.

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EFFECT OF CALCIUM ADDITION, ACIDIFICATION AND FERMENTATION ON THE QUALITY CHARACTERISTICS OF CANNED CARROTS (*DAUCUS CAROTA* L.)

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A study was conducted on calcium chloride treatments of canned carrots acidified with one of five organic acids or by lactic fermentation. The quality of the processed carrots was determined by physical, chemical, microbiological and sensory analyses after a two months storage period at room temperature.

The results indicated the possibility of processing high quality canned carrots by small canneries, with low cost equipment and low energy requirements. The acidification procedures also ensure minimal risk of botulism.

Keywords: carrots, thermal processing, acidification, fermentation, calcium

Most vegetables are considered as low acid foods (FOOD & DRUG ADMINISTRATION, 1976) and should therefore be processed at high temperatures to avoid development of *Clostridium botulinum* (RIVERO, 1973; ITO & CHEN, 1978). In the United States the incidence of botulism is frequently associated with the ingestion of home made preserves having pH values above 4.6 (FRAZIER, 1967; AYRES, 1975). In Brasil, GRANER and co-workers (1978) called attention to the risks represented by the canning of home preserves using recipes that do not take into consideration the final pH of such products.

Vegetables have been generally processed by the high energy-using methods of freezing or conventional canning. Considering the high costs of energy and equipment, alternative methods must be sought to reduce cost and to maintain high safety standards from the microbiological point of view, high nutritional quality and good palatability (KOSUP & SISTRUNK, 1982).

The use of acidification or lactic fermentation to lower the pH value of vegetables below 4.6 seems to be an alternative technique to save energy during processing and to provide a product of acceptable quality (KOTZEKIDOU & ROUKAS, 1987; MEURER & GIERSCHNER, 1992).

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Many vegetables cannot be canned because of their poor texture after heat sterilization. Calcium has long been regarded as an important agent to maintain the texture of fruit and vegetables, apparently through its interaction with cell wall pectic materials (VAN BUREN, 1979). For this reason, calcium has been used by several researchers on a variety of commodities to effect firming or maintain firmness (VAN BUREN, 1968; FENJIN HE et al., 1989; MEURER & GIERSCHNER, 1992).

The purpose of this work was to evaluate the effect of calcium, acidification and fermentation and provide information, currently lacking, on the quality of canned carrots processed in these ways, at atmospheric pressure.

1. Material and methods

1.1. Raw Material

Samples of carrots used in this study were commercially grown in Piedade county, State of S. Paulo, Brasil.

1.2. Analyses of raw and/or processed material

1.2.1. Titrable acidity, drained weight and pH were performed by standard methods (A.O.A.C., 1980).

1.2.2. Total soluble solids were determined by direct reading with a refractometer (Atago model N–1).

1.2.3. Texture. Objective measurements were obtained on 100 grams of drained processed carrots using a Food Technology Corporation shearpress model TP–2, with registrar TR–1 fitted with a TG–1A–3000 electronic texture gauge and a CS standard cell. Ram descending rate was 20 cm min^{-1}. The same procedure was used for the unprocessed carrots.

1.2.4. Sensory evaluations. Color, texture and flavor were evaluated by eight trained panelists on a 1 to 9 points hedonic scale, according to LARMOND (1977). The panels were conducted in a room equipped with individual booths, water and indoor red and fluorescent lighting. The red lighting was utilized for evaluation of texture and flavor. Each panelist was presented with an individual sample (50 g) portioned into white cups coded with random numbers.

Objective texture and sensory attributes were evaluated in comparison with a non-acidified control with no calcium added, prepared at the same time as the samples, following the procedures described before for processing the acidified product.

The overall quality attribute was obtained by taking the average of the sum of color, texture and flavor given to each sample.

1.2.5. Microbiological analyses were performed using two cans of each treatment and tested for commercial sterility according to the APAH (CORLETT & DENNY, 1984). Results were reported in terms of presence (+) or absence (−) of biological activity.

1.2.6. Vacuum was determined, as inches Hg, by piercing the cans with a Marshalltown vacuum gauge.

1.2.7. Statistical analyses. The experiment was designed as a factorial with six treatments (five organic acids and fermentation), two levels of firmness (with and without calcium) and two replications. All analyses were conducted in duplicate and data analyzed by the analysis of variance using a randomized complete block design (PIMENTEL GOMES, 1990).

1.3. Processing

The carrots were washed, peeled, cut into slices (1.5 cm thick), blanched in boiling water for 3 min and immediately cooled in tap water at room temperature. After cooling the carrots were divided into two lots.

The first lot was acidified by adding the right amount of either acetic, citric, lactic, malic, or tartaric acid to the canning brine to reach a 4.3 equilibrium pH in the final products, as determined by acidification curves, previously obtained for each acid (ZAPATA & QUAST, 1975).

Before filling, half of this lot was treated by immersing into a 0.5% calcium chloride solution at 50 °C for 10 min. Carrots were filled to a weight of 400 g of slices into 401 × 411 lacquered cans and 420 ml of 1.5% NaCl solution were added.

The other lot was fermented at 35 °C by adding 1 g of a commercial starter (*Lactobacillus bulgaricus* and *Lactococcus lactis*, 1:1) to 10 l of a 1.5% NaCl solution, until products reached a 4.3 equilibrium pH.

The carrots were filled into cans as described before, using the fermented brine solution. Half of this lot was also treated with calcium by adding 0.2% (w/v) of calcium chloride to the fermented canning solution.

All the filled cans were exhausted at 85 °C (can center temperature), sealed and processed for 30 min in boiling water. After two months storage at room temperature of 25 to 31 °C, the final products were completely evaluated.

2. Results and discussion

Chemical and physical characterization of the unprocessed carrots presented the following data: total soluble solids 6.5±0.2%; pH 6.2±0.2 and texture 9.7±0.3 Lbf g^{-1}, as an average of three samples.

The acidification curves used to determine the amounts of acid needed to acidify carrots, from a pH 6.2 to a pH 4.3, indicated that this varied according to the acid used, following the decreasing order: acetic, citric, malic, lactic and tartaric acid (Fig. 1). Such variation was probably due to the buffering capacity of the carrots and the acid dissociation constant (BERNHARDT, 1989).

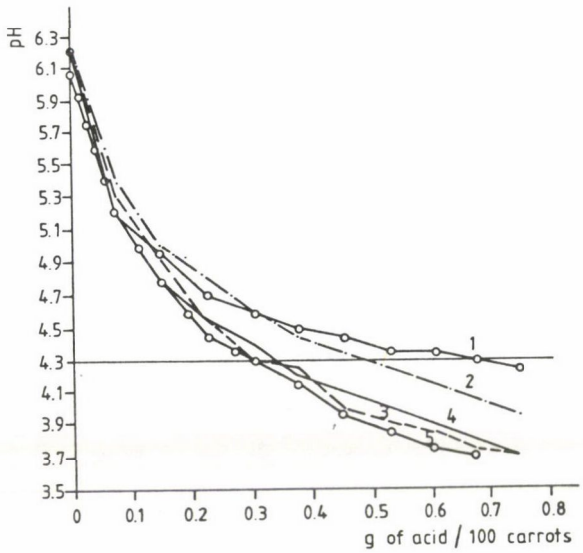

Fig. 1. Acidification curves of carrots with five organic acids to a pH 4.3. 1: Acetic acid = 0.675 g; 2: citric acid = 0.480 g; 3: lactic acid = 0.300 g; 4: malic acid = 0.325 g; 5: tartaric acid = 0.300 g

Acidification with lactic acid, without calcium addition, was the best treatment for color. However, when calcium was present panelists preferred citric and malic acid. These data indicated that the color of canned carrots was affected by calcium (Table 1).

Texture was greatly improved either by direct acidification or by fermentation, especially for calcium treated carrots for which the sensory evaluation showed a better acceptability for citric and lactic acid (Table 1). However, according to shearforce values (Figs 2 and 3) carrots treated with calcium and acidified by fermentation or acetic acid presented firmer textures. The most efficient acid-calcium interaction for texture was found to be that with malic acid, presenting a 70% increase of shearforce values (Fig. 2).

Table 1

Effect of acidification and addition of calcium on the sensory characteristics of canned carrots

Treatments		Average of evaluations (16 replications)			
		Color	Texture	Flavor quality	Overall
Control		7.44^{ab}	5.37^{d}	6.38^{ab}	6.40^{a}
Acetic acid	I	7.37^{ab}	6.56^{abcd}	6.63^{a}	6.85^{a}
	II	6.50^{bc}	6.87^{ab}	7.06^{a}	6.81^{a}
Citric acid	I	7.31^{ab}	6.56^{abcd}	6.06^{ab}	6.65^{a}
	II	7.37^{ab}	7.13^{a}	5.69^{abc}	6.73^{a}
Lactic acid	I	7.62^{a}	6.63^{abc}	6.56^{a}	6.94^{a}
	II	6.06^{c}	6.88^{ab}	6.56^{a}	6.50^{a}
Malic acid	I	6.87^{abc}	6.00^{abcd}	5.81^{ab}	6.23^{a}
	II	7.31^{ab}	6.63^{abc}	5.88^{ab}	6.60^{a}
Tartaric acid	I	7.00^{abc}	6.37^{abcd}	5.88^{ab}	6.42^{a}
	II	6.87^{abc}	6.69^{abc}	6.19^{ab}	6.58^{a}
Fermentation	I	3.69^{d}	5.56^{cd}	4.31^{c}	4.52^{b}
	II	3.56^{d}	5.88^{bcd}	5.00^{bc}	4.81^{b}

I = Without calcium; II = with calcium. Numbers followed by the same letters are not statistically significant by different at the 5% level.

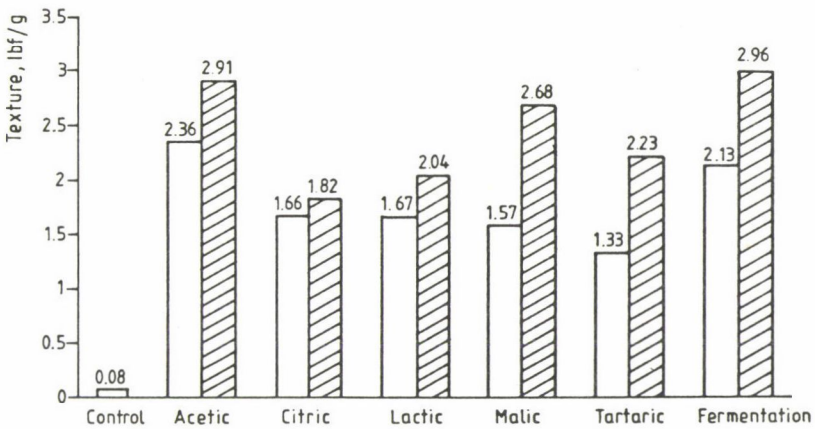

Fig. 2. Effect of acidification and addition of calcium on the texture of canned carrots. □: Without calcium; ▨: with calcium

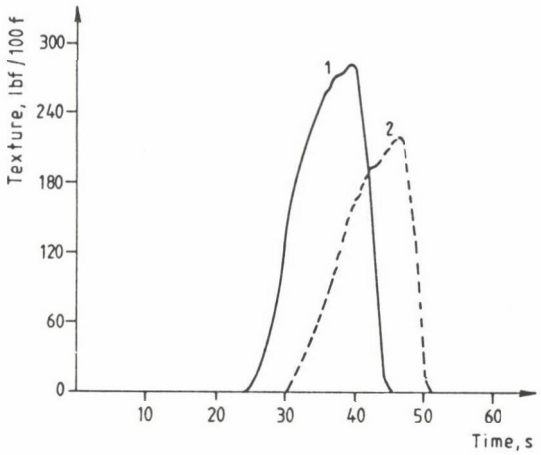

Fig. 3. Typical shearpress curves of acidified (acetic acid) canned carrots. 1: With calcium; 2: without calcium

Although the calcium-citric acid interaction was first in the preference of panelists (Table 1) its objective texture was the lowest observed (Fig. 2). This can be explained by the chelation hypothesis, proposed by FRENCH and co-workers (1989), that softening occurs when a chelator, such as citric acid, removes structural calcium from the cell wall, once cell membranes are affected by heat.

Differences in texture values (Table 1), when treatments with and without calcium were compared, can be attributed to complexes of pectic substances and calcium (VAN BUREN, 1968). When comparisons were done with the control and acidification treatments, such values were probably due to the bonding effect between acid and mucilaginous material during storage (KOTZEKIDOU & ROUKAS, 1987).

Acidification with acetic or lactic acids improved the flavor of canned carrots for both with and without calcium treatments. The other acids did not affect this quality attribute at all. However, fermentation adversely influenced flavor (Table 1), due to the development of off-flavors, probably as a result of microbial cell decomposition during heating and storage. Replacement of the fermented brine by a fresh one could avoid this problem, and also the brine turbidity of the final product.

The sensory panel found fermented carrots the least acceptable in terms of overall quality. Minor differences statistically not significant, were observed among the acidified and control treatments (Table 1 and Fig. 4). However, the results indicated that acidification with acetic or lactic acid improved all sensory attributes of the canned carrots, mainly flavor which is the most important factor for consumers' final decision in selecting foods.

Table 2

Chemical and physical analyses of canned carrots

Analyses	Treatments											
	Acetic		Citric		Lactic		Malic		Tartaric		Fermentation	
	I	II	I	II	I	II	I	II	I	II	I	II
Net weight (g)	806.5	808.0	800.5	799.5	799.0	803.0	803.5	802.5	798.0	799.0	795.0	795.0
Drained weight (g)	409.5	412.5	412.5	411.0	407.5	411.0	408.5	412.5	410.0	412.5	412.5	407.5
Vacuum (pol.Hg)	18.5	18.0	16.5	18.5	18.0	17.2	17.0	18.0	18.5	18.0	17.0	17.2
Soluble solids	3.7	4.0	4.0	4.5	3.7	4.2	3.7	4.0	4.0	4.5	3.2	3.7
Equilibrium pH	4.15	4.15	4.25	4.20	4.25	4.25	4.25	4.25	4.05	3.95	4.45	4.40
Total acidity	0.414	0.411	0.297	0.297	0.247	0.247	0.221	0.214	0.206	0.225	0.175	0.175

I = Without calcium; II = With calcium. All data not exceeding a ± 5% deviation according to the technique used

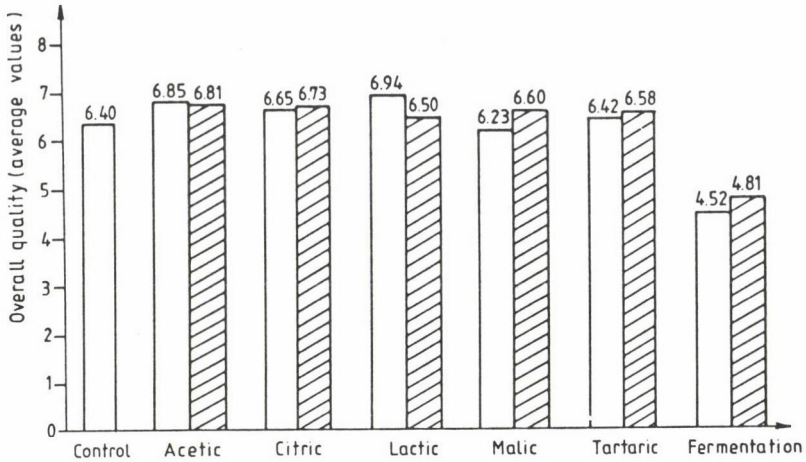

Fig. 4. Effect of acidification and addition of calcium on the overall quality of canned carrots. Sensory evaluation. □: Without calcium; ▨: with calcium

In general, the chemical and physical analyses did not reveal any great influence of the treatments (Table 2). Drained weight slightly increased in relation to that of product filled into cans. Soluble solids decreased in the processed product. Total acidity was higher for carrots acidified with acetic acid, followed by those with citric, lactic, malic, tartaric and fermented. Vacuum values were considered as adequate and no paneling of cans was observed.

Microbial analyses showed no biological activity in any of the final products after the two months storage period.

In conclusion, acidification yielded desirable organoleptic qualities besides requiring less energy input and lower cost, compared with the conventional retorting procedure for low-acid foods. The procedures resulted in a non-favorable environment for the occurrence of botulism.

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THERMAL DENATURATION OF BACTERIAL CELLS EXAMINED BY DIFFERENTIAL SCANNING CALORIMETRY

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Differential scanning calorimetry (DSC) with a microcalorimeter was used to detect thermal transitions in cells of *Listeria monocytogenes*, *Escherichia coli* and *Lactobacillus plantarum*, suspended in pH 6.8 physiological phosphate buffer. DSC-scanning at 0.5 °C min^{-1} showed multi-peaked thermograms. Thermograms taken from 10 to 99 °C temperature range showed an exothermic heat flow below the maximum temperature of growth and a series of endothermic transitions began to appear between 50 to 60 °C, in the temperature range, where the heat destruction of vegetative cells occurred. Our observations strengthen the premise that the process responsible for thermal death is the irreversible thermal denaturation of a crucial proteinaceous component of the cells and it is strongly influenced by the pH of the suspending citric/phosphate buffer. Heat denaturation/melting of DNA required a higher temperature than cell killing, and did not correlate with thermal stability of the bacteria. Heat denaturation of the DNA in whole cells was more or less reversible when heated at near-neutral pH. However, it became irreversible when they were heated in acidic buffer.

Keywords: DSC, thermal denaturation, bacteria

Differential scanning calorimetry (DSC) is a technique increasingly used for studying various heat-related phenomena in materials by monitoring associated changes in enthalpy. Previous studies on the DSC of vegetative bacterial cells or bacterial spores (VERRIPS & KWAST, 1977; MILES et al., 1986; MACKEY et al., 1988, 1991; ANDERSON et al., 1991; STEPHENS & JONES 1993) revealed that the DSC technique may help to obtain better understanding of heat inactivation of microorganisms, which is of eminent importance to many food processing technologies. The thermal death of cells is usually considered being related to heat denaturation of some crucially important biopolymers by pseudo-first-order kinetics and having parameters that correlate with those of proteins (LEPOCK et al., 1988). The unfolding of proteins in solvents is usually accompanied by an endothermal heat effect due to the rupture of inter- and intramolecular bonds which may be observed by DSC as a function of time and temperature.

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It is also well documented that the pH of the heating menstrum affects heat resistance of miroorganisms (INGRAM, 1971). Generally, bacteria show maximum heat resistance over the range of pH 6.0–8.0, with a sharp decrease in survival on either side of the optimum range (WHITE, 1963; STRANGE & SHON, 1964).

The aim of our experiments was to study comparatively the thermal stability of vegetative cells of some food-borne bacteria both by counting viable fractions and by determining DSC thermograms of their suspensions. Heat destruction of these bacteria and their DSC thermograms as affected by the pH of the suspending media were also investigated. For the sake of high sensitivity of thermal analysis a DSC-microcalorimeter was used, enabling us to detect very small heat flows.

1. Materials and methods

1.1. Test organisms

Lactobacillus plantarum NCAIM B 00509 from the culture collection of the University of Horticulture and Food Industry, Budapest, *Listeria monocytogenes* 4ab received from the National Meat Research Institute, Budapest, and *Escherichia coli* No. 9270 obtained from Ms. Louise Fielding, University of Reading, U.K., were studied as test organisms.

1.2. Preparation of bacterial suspensions for heat treatments

The organisms were inoculated into suitable media (BHI-broth, Oxoid CM225, for *L. monocytogenes*, MRS-broth, Merck 10661, for *Lactobacillus plantarum*, and nutrient broth, Oxoid CM1, for *E. coli*). They were batch-cultured for 24 h under slow shaking at 30 °C for *Listeria* and at 37 °C for *E. coli*, and incubated in unshaken static culture for *Lactobacillus plantarum* at 30 °C. The cells from these stationary-phase cultures were harvested by centrifugation for 20 min at 6400 r.p.m. in a Beckman GA–10 type refrigerated centrifuge at 5 °C, washed with ice-cold, sterile physiological phosphate buffer of pH 6.85 and resuspended in the same buffer to approx. 10^{11} CFU cm^{-3} and stored in a refrigerator until use on the same day. The 'physiological' phosphate buffer was composed of KH_2PO_4 3.5 g, $Na_2HPO_4 \cdot 7H_2O$ 10.9 g and peptone 1 g in 1000 cm^3 distilled water and sterilized by autoclaving for 15 min at 121 °C.

1.3. DSC measurements

Portions of 0.9 cm^3 of the buffered suspensions of bacteria were dispensed into tightly closed sample holders of a SETARAM Micro-DSC-II calorimeter interfaced

to a computer for data acquisition and data processing, and heated up from 5 °C to 99 °C at 0.5 °C min^{-1} scanning rate with the same volume of cell-free buffer as reference sample. (It is known from previous literature referred to in the Introduction that additional transitions may occur at temperatures higher than 100 °C, which are beyond the temperature range of our DSC microcalorimeter. However, such processes have no relevance to the subject of these studies.) After heating, the sample holders were cooled down to their initial temperature and re-heated at the same scanning rate as in the first run to investigate the reversibility of the thermograms.

1.4. Loss of viability as a function of heat treatments

Thermal death of bacterial cells in buffered suspensions of approx. 10^8 CFU cm^{-3} initial viable cell count was estimated by heating the suspensions with a programmed heat treatment with a similar low heating rate as in the MicroDSC calorimeter. During heating samples were removed at predetermined temperatures, cooled on ice and survivors were counted following serial dilution, plating into BHI-agar, for *L. monocytogenes*, MRS-agar for *Lactobacillus plantarum*, and nutrient agar for *E. coli*, and incubation at 30 °C and 37 °C, resp., for 72 h.

1.5. Buffers for DSC studies at reduced pH levels

For DSC studies at reduced pH levels, centrifuged and washed pellets of *Lactobacillus plantarum* cells prepared as described under 1.2 were re-suspended in citric acid (0.1 mol l^{-1}) -sodium-hydrogen-orthophosphate (0.2 mol l^{-1}) buffer mixes of pH 5.0, 4.6, 3.5, and 2.7.

2. Results and discussion

2.1. Characteristics of DSC thermograms of suspensions of whole vegetative cells

An exothermic heat flux could be observed from approx. 25 °C onward on the DSC thermograms of vegetative bacterial cells suspended in pH 6.85 phosphate buffer. This exothermic flux reached a maximum at around 45 °C in case of *Listeria monocytogenes* and at approx. 49 °C with *Escherichia coli*. In case of *Lactobacillus plantarum*, this exotherm appeared to be less pronounced than with the other two test organisms. It is assumed that this exothermic heat flow is related to oxidative metabolism of the whole cells (LEPOCK et al., 1988) increasing as the temperature is increased until about the maximum temperature of growth.

At further increasing temperature, a series of endothermic transitions began to appear within the 50 to 60 °C range, where the heat destruction of cells occurred during wet heat treatment. Unfortunately, the onset temperature of denaturation could not be determined because no realistic base line estimation was possible, due to the preceeding exotherm process. The highest temperature endothermic peak within the investigated temperature range occurred at 89 to 94 °C, depending on the test organism.

The lowest temperature endothermic transitions appearing under 60 °C seemed to be overlapped by a more excessive endothermic process with a peak temperature of 63 to 66 °C, again depending on the type of bacteria. Concerning the large number of protein domains in the cells, the number of potential endothermic transitions must be many more than the number of endotherms detectable by the DSC. Therefore, each endothermic signal must represent a combination of numerous transitions, a transition in a component present in a high concentration, or a set of complex transitions. Unfolding of DNA and RNA, alterations of complex cellular structures and denaturation-induced aggregation of macromolecules contribute also to the thermogram. However, the latter event being exothermic, its enthalphic contribution should be small (LEPOCK et al., 1988).

When the cell suspensions heat treated by DSC microcalorimetry were cooled down and a second run was performed, the thermograms obtained by the second heating showed that the exothermic and endothermic processes of the first heating were irreversible transitions, except the highest-temperature endotherm, which appeared more-or-less pronounced during the second run, too. According to previous literature (VERRIPS & KWAST, 1977; MILES et al., 1986), this reversible, or partially reversible transition was the 'melting' process of the intracellular DNA, which is probably combined with denaturation of a component of the cell wall (MACKEY et al., 1991). It is worthwhile to remark that this DNA melting appeared to occur at a few degree lower in the suspension of *L. monocytogenes* than with the other two bacteria, although they were less heat resistant than *L. monocytogenes*. However, the first endothermic transition during programmed heating occurred at somewhat lower temperature range in case of the more heat sensitive test-organisms than with the *Listeria*. Observations summarized above are illustrated by Figs 1 to 3 and Table 1. The reproducibility of transition temperatures was very good. The standard deviations determined for three independent scans were less than ±0.3 °C for the two major endothermic peaks of the *L. monocytogenes* thermogram.

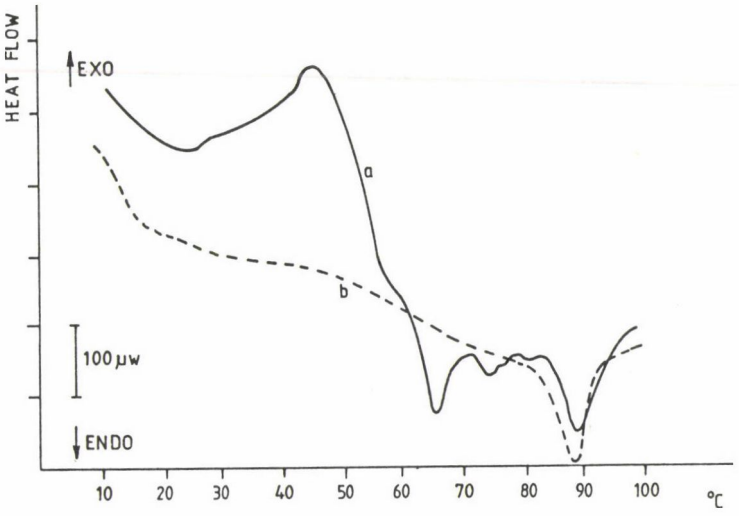

Fig. 1. DSC thermograms of *Listeria monocytogenes* cells suspended in pH 6.8 phosphate buffer. a: First heating; b: second heating. Scanning rate: 0.5 °C min^{-1}, Ampli range: 250 µV

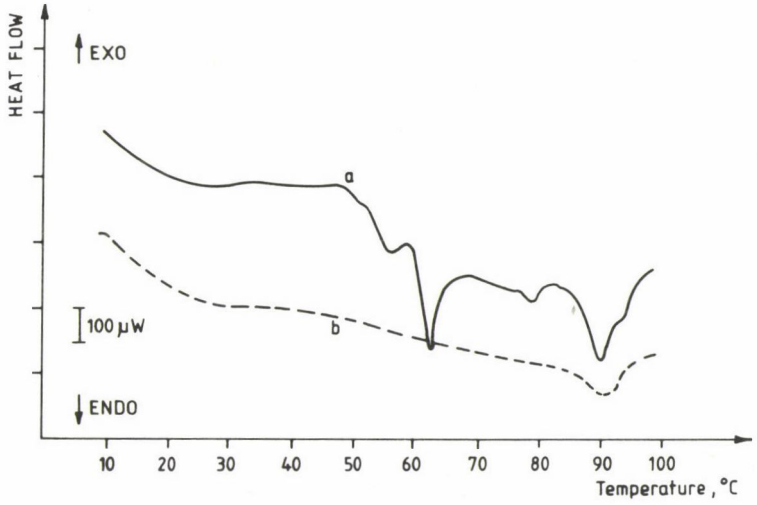

Fig. 2. DSC thermograms of *Lactobacillus plantarum* cells suspended in pH 6.8 phosphate buffer. a: First heating; b: second heating. Scanning rate: 0.5 °C min^{-1}, Ampli range: 250 µV

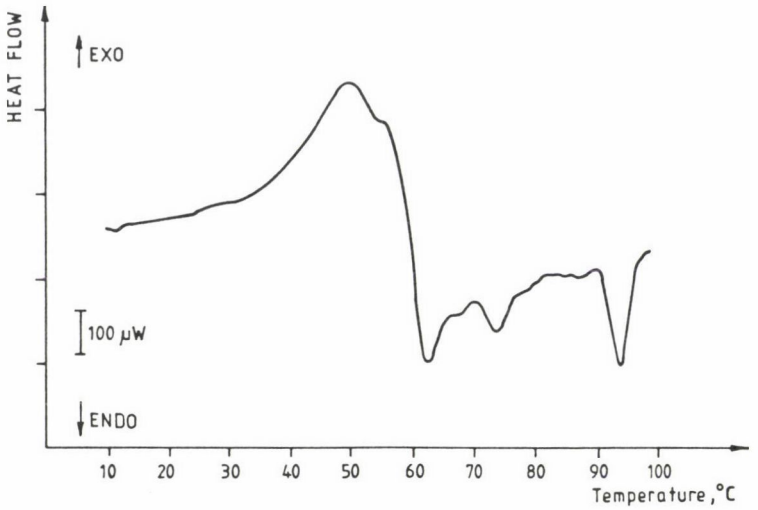

Fig. 3. Difference thermogram of initial heating run minus re-run of *Escherichia coli* cells suspended in pH 6.8 phosphate buffer

Table 1

Some characteristic temperatures of DSC thermograms of pH 6.8 buffered suspensions of vegetative bacteria

	Maximum of the exothermic heat flux (°C)	First irreversible endotherm (°C)	Reversible endothermic peak (°C)
Listeria monocytogenes	45	59 (shoulder)	88 – 89
Lactobacillus plantarum	wide and less pronounced max. below 50	56 (peak)	90 – 91
Escherichia coli	49	55 (shoulder)	94

The differences between the positions of the DSC thermograms obtained during the first run and those determined on re-running the DSC are related to the heat capacity changes caused by irreversible processes taking place during the first heating (MILES et al., 1986).

Unlike Figs 1 and 2, Fig. 3 is a difference thermogram plotted when the computer of the DSC apparatus substracted the data obtained at the second run from the data collected during the first run.

DSC thermograms were published for an other *Escherichia coli* strain by MILES and co-workers (1986) and MACKEY and co-workers (1991), and for another *Listeria monocytogenes* strain by ANDERSON and co-workers (1991) using regular DSC instruments of the Perkin Elmer Company. Considering the temperature range comparable to our studies, the exothermic processes were more pronounced in our investigations and heat denaturation endotherms were observed at temperatures lower by a few degree than in thermograms found in the literature showing, however, the features are similar as far as the major transition peaks are concerned. The aforementioned differences might be related to the difference in heating rates (0.5 °C min^{-1} in our case, and 10 °C min^{-1} in the others). It is well documented that low heating rates reduce the peak temperature (Td) of denaturation for a number of pure proteins and food proteins (ARNTFIELD et al., 1990) which is not due to thermal lag associated with the instrumentation, but rather, is related to the kinetics of denaturation. At the slow heating rate, sufficient time is available so that protein denaturation is complete at lower temperatures. Therefore, a Td value determined at a heating rate of 10 °C min^{-1} may be overestimated and comparison of Td values to denaturation temperatures obtained by other heating conditions of thermal analysis or other techniques of investigation often involves extrapolation of the Td values to a 'zero heating rate' (ARNTFIELD et al., 1990).

2.2. Comparison of the lowest temperature range of heat inactivation of vegetative bacteria with their DSC transitions

Figure 4 shows that during heating the loss of viability of the overwhelming majority of bacteria occurred in a narrow temperature range, which coincided with the lowest-temperature minor endothermic transition during DSC heating. The temperatures which have been reached when 50% of populations have been inactivated were 55 °C for *Escherichia coli*, 52 °C for *Lactobacillus plantarum*, and 58–59 °C for *Listeria monocytogenes*. These temperature values agreed well with the positions of the first irreversible endotherms in the DSC thermograms given in Table 1.

Similar small endotherms preceeding a major peak corresponded with the loss of viability of *Citrobacter freundii* cells in the pioneering studies of VERRIPS and KWAST (1977). According to MACKEY and co-workers (1991) the first irreversible denaturation events are associated with melting of the 30S ribosomal subunit and denaturation of some of the soluble cytoplasmic proteins.

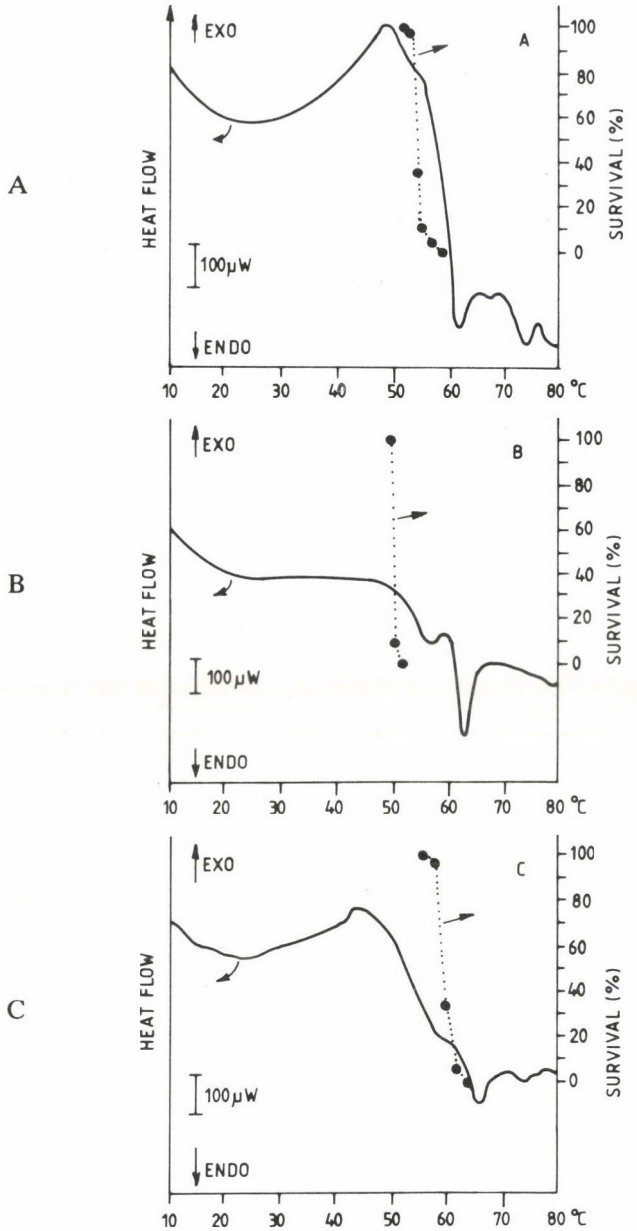

Fig. 4. Comparison of the lowest temperature range of heat inactivation of vegetative bacteria with their DSC transitions at pH 6.8 of the suspending medium. A: *Escherichia coli*; B: *Lactobacillus plantarum*; C: *Listeria monocytogenes* 4ab

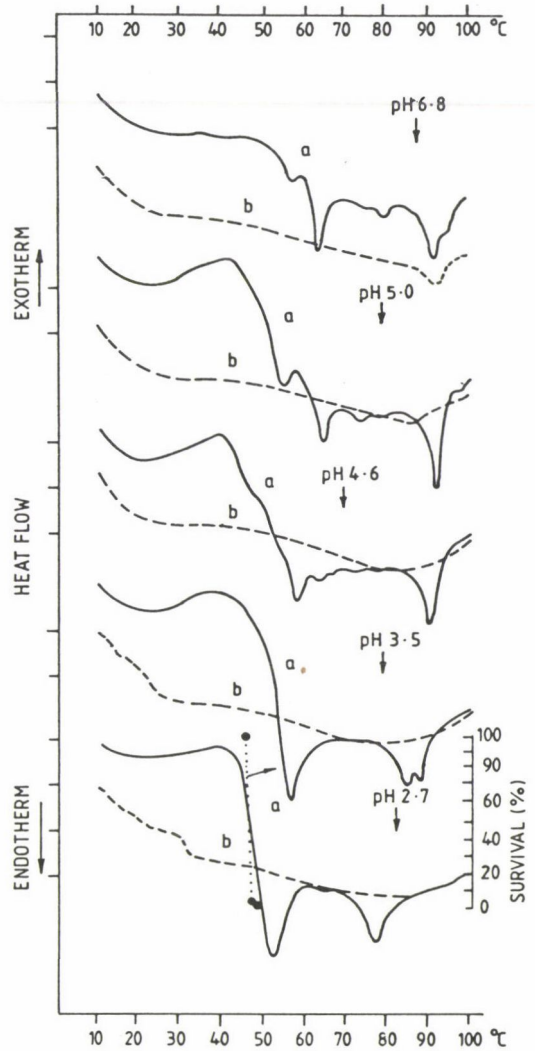

Fig. 5. Effect of pH of suspending medium on the heat resistance and DSC thermograms of whole cells of *Lactobacillus plantarum*. a: First heating; b: second heating

2.3. Effect of pH of suspending medium on the heat resistance and DSC thermograms of whole cells of Lactobacillus plantarum

Unfortunately, the DSC signal of the endothermic transition in bacterial cells which coincides with the lowest temperature range of their loss of viability cannot be estimated accurately due to its overlapping with the previous exothermic process and

its subsequent endothermic peak. This makes it impossible to estimate a base line for the DSC thermogram. Nevertheless, at DSC studies performed the same way as described above but with reduced pH levels the thermograms showed step-wise shifts of transitions towards the lower temperatures, and they became less-and-less abundant in endothermic peaks. This can be seen in Fig. 5.

Figure 5 shows that at the first reduction of pH of the suspending medium the exothermic heat flux observed on the DSC thermogram increased somewhat and was shifted a few degrees lower in the range of the upper part of the growth temperatures. Regarding the endothermic transitions in the lethal temperature range, the basic profile of the DSC thermogram changed little when the pH was reduced from 6.8 to 5.0. However, a shift of endothermic signals towards lower temperatures were already noticeable at pH 4.6. At pH 3.5 and even more at pH 2.7, a drastic reduction of distinguishable endothermic transitions was noted. At the second heat treatment in the DSC apparatus the reversible peak of DNA melting was absent in thermograms taken at pH 4.6 or lower, and instead an uncharacteristic, very broad and flat minimum appeared on the thermograms. This might be caused by the remelting of ribosomal RNA (MACKEY et al., 1991), the melting of which could be superimposed by other denaturation events during the first run. RNA melting must contribute to the DSC profile, but on the basis of total cellular content, protein denaturation must be the dominant endothermic process occurring over the melting temperature of lipids. Under the conditions prevailing in the heated suspension in low-pH citrate/phosphate buffer the renaturation of DNA was hindered. Regarding the DNA signal during the first heating, which occurred at 90–91 °C at pH 6.8, at low pH, such as 3.5, a double endothermic peak appeared only, while at pH 2.7 the highest-temperature transition process took place with a maximal rate already at 77 °C.

It is well documented that extremes of pH in either the acid or alkaline region resulted in lower Td values of proteins (ARNTFIELD et al., 1990).

Diminishing complexity of the multi-peaked DSC thermograms of whole cells at low pH values seemed to be related to a conformational transition in the biopolymers before the thermal scanning.

3. Conclusions

Our observations strengthen the premise that the process responsible for thermal death of microorganisms is the irreversible thermal denaturation of a crucial proteinaceous component of the cells and it is strongly influenced by the pH of the suspending medium. The critical target comprises a small fraction of total denaturable material and the heat killing process appears to be a part of the first endothermic transition detectable by DSC under our experimental conditions. The

critical transition involves protein denaturation of thermolabile proteins. There may be membrane proteins (LEPOCK et al., 1988). Heat denaturation/melting of DNA requires much higher temperatures than cell killing and does not correlate with thermal stability of the bacteria. Heat denaturation of the DNA in the whole cells is more or less reversible at near-neutral pH values, however, it becomes irreversible when the cells are heated in acidic media. Since citrate/phosphate buffers were used for studying effect of reduced pH, which is Mg^{2+}-chelating (STEPHENS & JONES, 1993), it remains to be seen whether the apparent pH-effects were due or not to destabilization of ribosomes via Mg^{2+}-loss.

Much further work similar to those published by MACKEY and co-workers (1991) is required to identify the particular cell components associated with thermogram peaks, and to define the sequence of denaturation events that occurs during thermal destruction of bacteria.

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FAT CONTENT, FATTY ACID COMPOSITION AND VITAMIN CONTENT OF MARE'S MILK

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Changes in the fat content and fatty acid composition as well as the vitamin content of mare's colostrum and milk during the first 45 days of lactation were studied. Milk samples (300–800 cm^3) from 29 lactating mares, were collected daily at the beginning of the lactation, and weekly from 5 to 45 days postpartum. Colostrum and early milk samples were obtained by hand, without oxytocin administration, while the foal nursed. Later milk samples were taken from mixed milk of the totally-milked udder. Each sample was analysed for total solids, fat content and fatty acid composition by conventional methods and Packard gas chromatograph.

The total solids and the fat content of the colostrum and milk were 24.25 to 26.28% and 2.85 to 2.93% on the first day of lactation, 12.15 to 12.78% and 2.05 to 2.17% on the 2nd to 5th days and 10.37 to 10.61% and 1.04 to 1.32%, respectively, on the 8th to 45th days of lactation. The concentrations of octanoic, decanoic, dodecanoic, miristic and palmitoleic acids increased with time while stearic, oleic, linolic and linolenic acids decreased. The fatty acid composition of mare's milk fat was very different from that of cow's milk fat. Mare's milk fat contained octanoic, decanoic, dodecanoic, linolic, linolenic, stearic, miristic and palmitic acids, in ratios of approximately 9.6, 3.1, 2.1, 4.4, 224, 0.2, 0.6 and 0.5 times those of concentrations in cow's milk. On the basis of the differences in fatty acid composition, a new method for determining the amount of cow's milk mixed with mare's milk was developed.

Content of vitamins A, D_3, K_3 and C of colostrum (0.88, 0.0054, 0.043, 23.8 mg/kg) was found one and half time – twice higher than that of normal milk (0.34, 0.0032, 0.029, 17.2 mg/kg). There was no significant difference in vitamin E content (1.342 and 1.128 mg/kg). Vitamin content of mare's milk was very similar to that of cow's milk.

Keywords: mare's colostrum and milk, fat content, fatty acid composition of milkfat, determination of cow milk in mare's milk, vitamin content

The relative importance of the horse industry in Hungary has changed greatly in recent years due to mechanisation in agriculture. In 1991, there were 74 000 horses in Hungary, with most of the industry being associated with sport horses and slaughter horses. Currently, there is considerable interest in the use of mare's milk for human consumption in Western Europe. There have been some promising

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experiences in the use of mare's milk for curing metabolic and allergic diseases and, consequently, the price paid for mare's milk has increased greatly. This suggests a new possibility for producing income from the horse industry. It also suggests that research is needed to evaluate the value of mare's milk as a human food. Most of the earlier research on composition of mare's milk was directed toward evaluating the value of milk as related to nutrition of the foal.

Duration of lactation has been reported to be 5 to 8 months (FEIST & MCCULLOUGH, 1976; TYLER, 1972) and estimated milk production of mares was 2000–3000 kg (NEUHAUS, 1959). During a single milking, composition of the milk changes, so the mixed milk of the totally milked udder must be sampled (LINTON, 1937, CSAPÓ, 1984). Butterfat content undergoes the largest change (DOREAU et al., 1986; NEUHAUS, 1959), and can be 10 to 20 times more at the end than that at the beginning of milking (DITTRICH, 1938; DOREAU et al., 1986; DYUSEMBIN, 1972). Sampling method and milking interval (GIBBS et al., 1982; MARTIN-ROSSET et al., 1978) also influence the composition of milk. Most authors suggest that foals should be present at sampling (ASCHRAFT & TYZNIK, 1976; BOULOT, 1987; BOUWMAN & VAN DER SCHEE, 1978; GIBBS et al., 1982; JOHNSTON et al., 1970; LINZELL, 1972; MIRAGLIA, et al., 1986; NESENI et al., 1958; OFTEDAL et al., 1983; ZIMMERMAN, 1985). Some others advise an injection of oxytocin (LINZELL, 1972; OFTEDAL et al., 1983).

The composition of mares' colostrum was analysed by several authors (JOHNSTON et al., 1970; MCGUIRE et al., 1972; ROUSE & INGRAM, 1970). Colostral period of mares was found to be much shorter than that of cows, and the colostrum showed significant differences from normal milk only on the first day after foaling (JOHNSTON et al., 1970; NESENI et al., 1958; ULLREY et al., 1966). The dry matter content of mare's milk decreased drastically from colostrum to normal milk, primarily due to a decrease in protein content (INTRIERI & MINIERI, 1970; NESENI et al., 1958); the fat content and fatty acid composition of milk fat showed much smaller changes over time (BASE & ZADRAZIL, 1982; INTRIERI & MINIERI, 1970).

The fat content of mare's milk is very low (DOREAU & BOULOT, 1989; JENNESS, 1974). However, it can be influenced by environment and ranged from 0 to 7.9% (NESENI et al., 1958; NEUHAUS, 1959). Analysis of the fatty acid composition of butterfat of mare's milk showed (BASE & ZADRAZIL, 1982; INTRIERI & MINIERI, 1970; JAWORSKI et al., 1982; KULISA, 1986) that it contains very small quantities of stearic and palmitoleic acids, and high quantities of linolenic and linolic acids. This could be explained by the fact that unsaturated fatty acids are not hydrogenated in the digestive system and horses consume a very large amount of forage, which is rich in unsaturated fatty acids.

Among the factors influencing milk composition, stage of lactation is the most important, but the stage of lactation may (INTRIERI & MINIERI, 1970) or may not

(JAWORSKI et al., 1982) influence the fatty acid composition of milkfat. Most authors (KULISA, 1977; NESENI et al., 1958) have not reported breed to affect milk composition, but BOULOT (1987) reported a significant effect of breed. Increasing the fat content of feeds did not increase the quantity of milk and caused no change in milk composition (ZIMMERMAN, 1985), which differed from effects on cow's milk (DOREAU et al., 1988). Others found higher milkfat content to be associated with higher fat input (DAVISON et al., 1987).

Based on the fact that data on the fatty acid composition of mare's milk are limited and that there are virtually no data comparing the fatty acid composition of mare's milk with cow's milk in the same trial, a study was initiated at the PANNON Agricultural University to evaluate the composition of mare's milk and cow's milk simultaneously. Additional objectives of the experiment were to evaluate time changes in milk composition from foaling to 45 days after foaling and to obtain more detailed information of the quantities of polyunsaturated essential fatty acids present in mare's milk.

HOLMES and co-workers (1946) and KULISA (1986) published data on water soluble vitamin content of mare's milk. Data on fat soluble vitamin content of mare's milk could not be found.

1. Materials and methods

In the spring of 1991, the experiments were initiated and milk samples of 29 mares (16 Hungarian Draughts, 4 Haflingers, 6 Bretons and 3 Boulonnais) were collected and analysed. Mares were on pasture of relatively good quality (1 ha per mare-foal/pair), from spring to autumn; supplemental feeding, when 3 kg oats per day was needed. Winter feeding was 3 kg hay, 2 kg concentrate and ad lib. straw daily.

Mares were milked on the following schedule: they were driven in the stable at 06:00 a.m. Each mare and foal were tied. First milking was started at 09:00 and finished at 11:00 a.m. During milking time, foals were released, but retied following milking. Second milking time was between 11:15 and 12:30 a.m. The foals were not retied after the second milking because mares and foals were returned to the pasture until 06:00 a.m. the following day. Milking of mares was accomplished with a Westfalia milking machine (model RPSZ 400).

Colostrum and milk samples (80–100 cm^3) were taken directly after foaling and on the second and third days of lactation by hand milking. On the 5th, 10th, 30th and 45th days of lactation, the mixed milk of the totally milked udder was sampled. Colostrum and milk samples were frozen and stored at −25 °C. At the time of analysis frozen material was thawed and mixed. Dry matter of colostrum and milk samples was determined by HUNGARIAN STANDARD (1981) by drying to constant

weight at 105 °C. Fat content was determined by the Gerber method according to HUNGARIAN STANDARD (1982), and determination of fatty acid content of milkfat was accomplished using a Packard M 419 type gas chromatograph, as described by CSAPÓ and co-workers (1986).

To determine vitamin A-, D- and E-content 5 cm^3 of milk samples was saponified by alcoholic pirogallol solution and 2.5 cm^3 80% potassium hydroxide, then extracted in an alcohol-*n*-hexane system. Extractum was distilled and the rest was diluted in 200 μl methanol, 20 μl of it was injected on the 250×5 mm column with 10 μm granulation Partisil ODS charged of a Pye UNICAM LC-XP HPLC. Elution was carried out by solution of methanol:water of 85:15 ratio at 1.4 cm^3 min^{-1} drift speed. To the quantitative evaluation vitamin standards made by MERCK were used.

For vitamin K determination the milk was extracted with chloroform and the alkalescent substrate was detected at 251 nm. Vitamin C content of milk samples was determined by RADEFF (1938) method.

2. Results and discussion

Dry matter content and changes associated with stage of lactation (colostrum to 45 days) for mares of the four breeds are shown in Table 1. Changes in the fat content of colostra and milks for the same period are presented in Table 2. Table 3 shows fatty acid contents of milkfat and time changes, expressed as relative percentages of fatty acid methyl esters. Investigations related to comparison of fatty acid composition of mare's milk, cow's milk and blends are summarised in Table 4.

Table 1

Dry matter contents of the colostrum and milk of mares
(g per 100 g milk)

Breed	No. of mares		Days postpartum		
			0–0.5	2–5	8–45
Haflinger	x	4	24.25	12.87	10.61
	S.D.		4.34	1.49	2.12
Breton	x	6	24.65	11.93	10.39
	S.D.		6.38	2.05	1.24
Boulonnais	x	3	25.42	12.15	10.37
	S.D.		4.12	2.22	1.73
Hungarian Draught	x	16	26.28	12.78	10.40
	S.D.		3.16	1.64	1.57
Mean		29	25.57	12.55	10.42
	S.D.		4.10	1.32	1.54

Table 2

Fat contents of the colostrum and milk of mares
(g per 100 g milk)

Breed	No. of mares		Days postpartum		
			0–0.5	2–5	8–45
Haflinger	x	4	2.87	2.05	1.04
	S.D.		0.462	0.183	0.610
Breton	x	6	2.91	2.10	1.32
	S.D.		0.381	0.214	0.483
Boulonnais	x	3	2.85	2.17	1.29
	S.D.		0.294	0.331	0.390
Hungarian Draught	x	16	2.93	2.16	1.26
	S.D.		0.455	0.163	0.540
Mean		29	2.91	2.13	1.25
	S.D.		0.431	0.189	0.499

Table 3

Mean and standard deviations for the fatty acid composition of the lipids in colostrum and milk of mares
(Relative percentages of the fatty acid methyl esters)

Fatty acids	Days postpartum							
	Mare						Cow[a]	
	0–0.5		2–5		8–45		5–270	
	$\bar{x}$	S.D.	$\bar{x}$	S.D.	$\bar{x}$	S.D.	$\bar{x}$	S.D.
Octanoic	1.39	0.18	2.56	0.94	2.79	0.91	0.29	0.022
Decanoic	5.41	0.47	8.59	2.89	8.05	2.25	2.61	0.219
Dodecanoic	7.90	1.57	9.89	3.19	8.97	2.10	4.35	0.362
Miristic	6.30	0.26	9.67	1.89	8.72	1.97	14.00	0.998
Palmitic	21.32	1.58	25.63	2.99	23.28	3.58	44.06	2.10
Palmitoleic	2.80	1.97	5.07	1.14	3.96	1.52	2.08	1.009
Stearic	2.36	0.53	1.63	0.51	1.55	0.79	7.94	1.001
Oleic	17.12	0.21	13.77	5.38	13.72	2.58	17.25	1.533
Oleic (ω6)	0.78	0.29	0.74	0.21	0.69	0.24	[b]	
Linoleic	9.78	0.83	6.40	0.90	7.53	1.47	1.72	0.198
γ-Linolenic (ω6)	0.75	0.13	0.51	0.03	0.61	0.19	[b]	
Linolenic	24.11	2.57	15.53	1.99	20.12	4.12	0.09	0.02

[a] from our earlier investigations
[b] not determined

Table 4

Fatty acid composition of mare's milk blended with different proportions of cow's milk
(calculated values)

Fatty acid	Percentage of cow's milk in the blend (Relative percentages of fatty acid methyl esters)						
	Mare milk	1	5	10	25	50	Cow[a] milk
Octanoic	2.79	2.73	2.45	2.23	1.62	0.98	0.29
Decanoic	8.05	7.92	7.28	6.82	5.42	4.07	2.61
Dodecanoic	8.97	8.88	8.29	7.93	6.39	5.62	4.35
Miristic	8.72	8.87	9.24	9.94	11.21	12.56	14.00
Stearic	1.55	1.70	2.29	3.00	4.55	6.20	7.94
Linoleic	7.53	7.38	6.71	6.21	4.80	3.31	1.72
Linolenic	20.12	19.61	17.40	15.55	10.71	5.56	0.09
f-values							
mean	2257	1840	816	390	57	5.3	0.005
S.D.	112	62.4	16.2	12.8	4.45	0.44	0.0007
99% confidence limits for f [Mean ± 3.25 (S.D.)]:							
Upper limit	2621	2043	869	432	72	6.7	0.007
Lower limit	1893	1778	763	348	43	3.9	0.003

[a] from our earlier investigations; $f = \frac{\text{octanoic} \times \text{decanoic} \times \text{dodecanoic} \times \text{linoleic} \times \text{linolenic}}{\text{miristic} \times \text{stearic}}$

The dry matter content of colostrum immediately after foaling ranged from 14.65 to 29.35%. The mean and standard deviation were 25.57% and 4.10. Due to the large variation, breed differences were not significant ($P > 0.25$). Dry matter content decreased quickly following foaling, and values found on day 2 differed only slightly from those obtained 5 days after foaling. The dry matter content of transition milk, on days 2 to 5 of lactation, averaged 12.55%. The dry matter content of normal milk obtained on days 8 to 45 averaged 10.42% with S.D. = 1.54. There were no significant differences among breeds ($P > 0.25$) in the dry matter content of their colostrum or milk samples.

The fat content of colostrum immediately after foaling averaged 2.91% while that of transition milk and normal milk, averaged 2.13 and 1.25%, respectively. There were no significant differences ($P > 0.25$) among breeds in the fat content of their colostrum, transition milk or normal milk. These changes in the fat content of colostrum and milk over time after foaling confirm results of other authors (LINTON, 1931; JOHNSTON et al., 1970). Those authors who reported an increase in the fat

content of colostrum to 48 h (ULLREY et al., 1966) or to day 7 of lactation (FORSYTH et al., 1975) probably made a sampling mistake due to the fact that the udder cannot be easily milked totally immediately after foaling and the fat content increases dramatically during milking. No relationship was found between milk quantity and fat content.

Comparison of the fatty acid composition of butterfat of colostrum and milk showed that the fat of colostrum contained less octanoic, decanoic, dodecanoic, miristic, palmitic and palmitoleic acids than that of normal milk. On the other hand, the fat of normal milk contained less stearic, linoleic and linolenic acids than that of colostrum. There were no significant differences among breeds regarding fatty acid content, and data shown in Table 3 represent the arithmetic average of 29 mares, ignoring breed.

Results of these analyses were in agreement with the data in the literature. Exceptions were linoleic acid, which was significantly less and linolenic acid, which was significantly higher than data in the literature. These differences can be explained by the composition of the diet of mares. The fatty acid composition of feedstuffs have a greater influence on the fatty acid composition of milkfat in case of horses than of ruminants.

When comparing the fatty acid composition of milkfat of mares at day 45 of the lactation and cows, it can be stated that the fat of mare's milk contains 2.1 times as much dodecanoic acid, 3.1 times as much decanoic acid, 4.9 times as much linoleic acid, 9.6 times as much octanoic acid and 224 times as much linolenic acid as cow's milkfat. On the other hand, the fat of mare's milk contains only 0.62 as much miristic acid, 0.53 as much palmitic acid and 0.2 as much stearic acid as cow's milkfat. This huge difference between the fatty acid contents of the milkfat of the two species suggested that we could use the fatty acid composition to detect the presence of cow's milk in a blend of milks from the two species.

The fatty acid contents of cow's milk and mare's milk are shown in the first and last columns of Table 4. A ratio of fatty acid contents, designated as the f-factor, was calculated as the product of fatty acids higher in mare's milk divided by the product of fatty acids higher in cow's milk.

$$f = \frac{\text{octanoic} \times \text{decanoic} \times \text{dodecanoic} \times \text{linoleic} \times \text{linolenic}}{\text{miristic} \times \text{stearic}}$$

Based on the averages, the f-value for mare's milk was 2257 and that of cow's milk was 0.005. Using day 45 milk of 10 mares and milk of 10 individual cows, f-values were calculated for each individual and the standard deviations were calculated as 112 and 0.0007, respectively, for mares and cows.

Assuming the fat contents of mare's and cow's milks to be 1.5 and 4.0%, respectively, the fatty acid contents of various blends were calculated and entered in Table 4. The f-values for 1, 5, 10, 25 and 50% cow's milk were 1840, 816, 390, 57 and 5.3, respectively. Mare-cow pairs were formed randomly at each blend and 10 f-values were calculated for each blend. These values were used to calculate the standard deviations for each blend, which are shown in Table 4. The t-value for 9 degrees of freedom and probability of 0.01 (t = 3.25) was used to calculate the 99% confidence band for each blend. Confidence limits = Means ± 3.25 (S.D.). The confidence limits are shown at the bottom of Table 4 and it can be seen that we could not be 99% certain of detecting adulteration with cow's milk at a level of 1%, but we could be 99% certain of detecting cow's milk at the level of 5%.

All of the above results were based on calculations. The method was tested by creating five blended samples of individual mares and cows at the level of 5% cow's milk. The f-values ranged from 763 to 824 and, when compared with the calibration curve developed from the calculated values, predicted 4.95% cow's milk. Based on the results, the f-value can be used as a means of detecting small quantities of cow's milk blended with mare's milk and the rate of dilution can be quite accurately predicted.

Analysing the vitamin content of colostrum and milk (Table 5) it was found that colostrum contains 2.6 times more vitamin A, 1.7 times more vitamin D_3, 1.4–1.5 times more vitamin C and K_3 and only little more vitamin E, than mare's milk milked between 8th and 45th day of lactation. Mare's milk contains practically the same amount of vitamins A, D_3, E and K_3 than cow's milk, vitamin C content of it is slightly higher.

Table 5

Vitamin content of mare's colostrum and milk

Vitamin (mg/kg)		Days postpartum		
		Mare		Cow[a]
		0–0.05	8–45	5–270
A	x	0.88	0.34	0.352
D_3	x	0.0054	0.0032	0.0029
E	x	1.342	1.128	1.135
K_3	x	0.043	0.029	0.032
C	x	23.8	17.2	15.32

[a] From our earlier investigations

In consequence of the above-mentioned data, that since the fat content of cow's milk is 2 and a half, 3 times higher than that of mare's milk, butterfat or mare's milk contains 2 and a half, 3 times more liposoluble vitamins than that of cow. There are no comparable data for vitamins A, D_3, E and K_3 in the literature. For vitamin C content of mare's milk we have got by 2.5 mg/kg more than HOLMES and co-workers (1946).

*

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PROTEIN CONTENT, AMINO ACID COMPOSITION, BIOLOGICAL VALUE AND MICRO- AND MACROELEMENT CONTENT OF MARE'S MILK

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Changes in the ash, macro- and microelement, protein content, protein fractions and amino acid composition of mare's colostrum and milk, and biological value of milk protein during the first 45 days of lactation were studied. Milk samples (averaging 300 – 800 cm^3) from 29 lactating mares were collected daily at the beginning of the lactation and weekly from the 5th to 45th days postpartum. Colostrum samples were obtained by hand milking without oxytocin administration, while the foals nursed and milk samples were obtained from the mixed total daily production. Each sample was analysed for total protein, true protein, whey protein, true whey protein, casein and non-protein nitrogen (NPN) content using a Kjel-Foss nitrogen analyser and for amino acid composition by LKB amino acid analyser. The biological value of the milk protein was calculated on the basis of amino acid composition by the method of Morup and Olesen (1976).

The total protein, whey protein, casein and NPN contents, respectively, were 16.41, 13.46, 2.95 and 0.052% for colostrum immediately after parturition; 4.13, 2.11, 2.02 and 0.043% for milk between the 2nd and 5th days and 2.31, 1.11, 1.20 and 0.031% for milk in the 8th to 45th days of lactation. The ratios of true protein and whey protein to total protein decreased, while the comparable ratios of casein and NPN increased from foaling to 45 days. The amino acid contents of colostrum and milk decreased during the first 45 days of lactation. Most of the essential amino acids (threonine, valine, cystine, tyrosine, lysine) decreased, while glutamic acid and proline increased in the milk protein after parturition. Therefore, the biological value of the milk protein is highest (132.3) immediately after parturition due to very high levels of threonine and lysine. This value decreases in the course of 5 days to 119.7 and to 107.9 on the 45th day of lactation. The essential amino acid composition and biological value of mare's milk protein was much higher than that of bovine milk proteins.

Ash content of colostrum was significantly higher (0.5923%) than that of normal milk (0.4051%). Among the macro elements, calcium content is lowest right after foaling (747.7 mg/kg), reaches its maximum at about the 5th day (953.7 mg/kg) and later on decreases during the lactation, just right the other macro elements. Among micro elements zinc and copper content decreases after its maximum at the 5th day, manganese content increases till the 5th day of lactation and later on stays on a constant level in the analysed section of the

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lactation. For the macro- and micro element content of colostrum (first data) milked right after foaling and that of normal milk (second data) – in average of genotypes – the following data were obtained: potassium: 928.6 and 517.2 mg/kg; sodium: 320.0 and 166.6 mg/kg; calcium: 747.7 and 822.9 mg/kg; phosphorus: 741.7 and 498.8 mg/kg; magnesium: 139.7 and 65.87 mg/kg; zinc: 2.95 and 1.99 mg/kg; iron: 0.996 and 1.209 mg/kg; copper: 0.606 and 0.249 mg/kg; manganese: 0.0447 and 0.0544 mg/kg.

Keywords: Mare's colostrum and milk, protein content, protein fractions, amino acid composition, biological value, micro- and macroelements

The first report in this series (CSAPÓ et al., 1994) reviewed the reasons for initiating studies on the composition of mare's milk and reported dry matter, fat and vitamin contents of mare's colostrum and milk and the fatty acid composition of milkfat. This report concerns contents of micro- and macroelement, protein and protein fractions of colostrum and milk, the amino acid composition of milk and milk protein, and the biological value of mare's milk protein.

Mare's colostrum was reported to contain more than 10% protein and almost 80% of the protein content was immunoglobulins (DESKUR et al., 1978; INTRIERI & MINIERI, 1970; NESENI et al., 1958). Those concentrations decreased rapidly after foaling. Most foals are born with agammaglobulinaemia or hypogammaglobulinaemia, and colostrum with a high immunoglubulin content is needed as soon as possible to endow passive immunity (CONTI et al., 1984; MCGUIRE et al., 1972; MCGUIRE et al., 1975).

Following the colostral period, the total protein content of mare's milk (N% × 6.38, NESENI et al., 1958) was 1.7 to 3% (BOUWMAN & VAN DER SCHEE, 1978; DUREAU et al., 1990; GIBBS et al., 1982; KULISA, 1977, 1986; MINIERI & INTRIERI, 1970; MIRAGLIA et al., 1986; NESENI et al., 1958; SMOCZYNSKY & TOMCZYNSKI, 1982; ULLREY et al., 1966). Casein, as a proportion of total protein, was less than 50% in most cases, which means that whey protein was more than 50%. In mare's milk, the percentage of non-protein nitrogen (NPN) was surprisingly great compared with other farm animal species, and it may represent 10% of total nitrogen content. The NPN fraction contains much free amino acids (DOREAU et al., 1988). The proteose-peptone fraction of mare's milk was reported to be 0.16–0.19% (STORCH, 1985; URBINISOV et al., 1981). After the colostral period, the whey protein fraction of mare's milk contains 11–21% immunoglobulin, 2–15% serum albumin, 26–50% α-lactalbumin and 28–60% β-lactoglobulin (MINIERI & INTRIERI, 1970; SENFT & MEYER, 1980; URBINISOV et al., 1981; USTINOVA et al., 1983).

There are few reliable data on the amino acid composition of colostrum and milk of mares. The amino acid composition of protein showed little change during the colostral period, and was similar to that of ruminants with the exception of arginine and threonine, and was very similar to that of sow's milk (SARKAR et al.,

1953). Others (DUISEMBAEV, 1973; KULISA, 1986; PELTONEN et al., 1980) reported that the amino acid composition of mare's milk differed significantly from that of other farm animals due to higher cystine and glycine contents. Mare's milk contains much free serine and glutamic acid but is low in methionine.

Among factors that influence protein composition of mare's milk, stage of lactation is the most important. The protein content of mare's milk decreases rapidly to the second week of lactation and continues to decrease slowly to the end of second month (BOUWMAN & VAN DER SCHEE, 1978; GIBBS et al., 1982; LUKAS et al., 1972). There are no literature data available describing later stages of mare's lactation. NPN content did not change during the first two months of lactation (OFTEDAL et al., 1983). After the colostral period, stage of lactation had no significant effect on either the ratio of milk protein fractions (DESKUR et al., 1978; KULISA, 1977; MINIERI & INTRIERI, 1970; MIRAGLIA et al., 1986), or the total amino acid composition (DOREAU et al., 1990).

Most authors (DESKUR et al., 1978; DUISEMBAEV & AKIMBEKOV, 1982; KULISA, 1977; NESENI et al., 1958) reported no breed effect on milk composition, but BOULOT (1987) reported a significant effect. Though there were considerable individual variations (BALBIERZ et al., 1975; BOULOT, 1987; JOHNSTON et al., 1970; OFTEDAL et al., 1983), there was a low correlation between milk yield and milk composition.

The protein content of mare's milk decreased when the energy content of feed increased (DOREAU et al., 1990), which differs from the results experienced with cattle (RÉMOND, 1985). Several authors (GIBBS et al., 1982; PELTONEN et al., 1980) have not found a relationship between the nitrogen content of feed and the total protein content of mare's milk, while others reported a dramatic decrease in the levels of protein and NPN in milk if nitrogen content of feed was reduced (DOREAU et al., 1988).

Most authors determined only the main components of the protein of mare's milk and very few of them determined the total and free amino acid composition of milk. There is very little information on how protein content and amino acid composition of milk protein change after parturition and during the lactation. There are very few data concerning the free amino acid content of mare's milk and the amino acid composition and biological value of mare's milk protein in comparison with cows' milk and bovine milk protein determined at the same time and by the same method.

PEAKER and co-workers (1979) analysing mammary glandular secretion of halfblooded mares during 3 weeks before foaling state that sodium and chloride concentration decrease, potassium, calcium, magnesium and phosphorus concentration increase during this period. Change in calcium content of mammary glandular secretion is used for estimation of expected foaling data.

LINTON (1931) stated, that increasing body weight increases ash content of milk, so at Shetland pony it was 0.38% and at Shire breed 0.54%. Furthermore he calls attention that during that heat period – among others – ash content of milk increases. According to his judgement colostrum is much richer in minerals (0.53–0.77%) than milk. ULLREY and co-workers (1966) analysed colostrum and milk composition of 3 Arabic and 2 Quarter horse mares till the end of the fourth months of lactation. It was found that ash-, magnesium, sodium- and potassium contents of colostrum decrease suddenly till the 12th h after foaling and continuously later on. Phosphorus content of colostrum increases continuously till 24–48 h after foaling than it decrease. Calcium content of colostrum decreases during the first 12 h reaches its normal value on the 8th day and decreases during the lactation, just like the phosphorus. JOHNSTON and co-workers (1970) has an opposite opinion. They have not found any change in tendency of ash content of mare's milk during the first 14 days after foaling.

Mare's milk – compared to that of other farm animals – is very poor in minerals. Most of the authors have measured ash content of mare's milk between 0.3–0.5%, but extremities were 0.2 and 0.7% (Table 1). Low mineral content may be related to low protein content of mare's milk, due to which calcium and phosphorus content of mare's milk are also low. Among minerals 61% of calcium, 31% of phosphorus and 16% of magnesium is in colloid form in mare's milk (DAVIES et al., 1983). Analysis of macro elements was achieved by SCHRYVER and co-workers (1986) in mare's milk. Calcium content was found 500–1500 mg/kg, phosphorus content 200–1200 mg/kg, magnesium content 40–110 mg/kg, sodium content 70–200 mg/kg, potassium content 300–800 mg/kg. Chloride content of mare's milk was found 300–600 mg/kg by NESENI and co-workers (1958) and DESKUR and co-workers (1978).

There are not many reliable data available on trace element content of mare's milk. Data of Table 2 – according to improvement of analytical methods – are reliable, but great differences can be observed in concentration of some trace elements. Differences can be explained partly by methodical problems, major cause is probably the different feeding of examined mares, which affects the trace element content of milk.

What does influence mineral content of mare's milk? BOUWMAN and VAN DER SCHEE (1978) analysing milk composition of Dutch warm-blooded saddle horse mares between 24 h after foaling till 28 day of lactation realised, that ash-, calcium- and phosphorus content of milk decrease in examined period after increase during the 2–3rd day. DOREAU and co-workers (1990) states that calcium content of milk decreases between 7th and 56th days of lactation. According to LINTON (1937) and SCHRYVER and co-workers (1986) ash content of milk decreases during lactation, and decrease true for majority of macro elements and a part of micro elements.

Besides the above-mentioned ones statements of SHUTTON and co-workers (1977) are worth to site, who have not found relationship between energy content and mineral content of milk. LINTON (1937) measured lower phosphorus content and higher chloride ions due to mastitis.

Table 1

Macro element content of mare's colostrum and milk

Author	Time after foaling (day)	Ash (%)	Potassium (mg/kg)	Sodium (mg/kg)	Calcium (mg/kg)	Phosphorus (mg/kg)	Magnesium (mg/kg)
DOREAU et al.	7	–	–	–	1350	460	–
(1990)	28	–	–	–	1180	420	–
	56	–	–	–	970	360	–
HOLMES et al.	28	–	790	–	1060	710	112
(1947)	112	–	640	–	1020	630	90
KULISA (1986)	–	0.295	–	–	–	394	29
LINTON (1937)	28	0.35	–	–	1265	1205	–
	112	0.26	–	–	945	865	–
OFTEDAL et al. (1983)	–	0.42	–	–	–	–	–
NESENI et al.	28	0.45	624	112	847	580	–
(1958)	112	0.30	303	75	485	467	–
SCHRYVER et al.	7	0.61	664	237	1345	943	118
(1986)	28	0.45	469	161	1070	659	86
	105 – 119	0.32	341	115	700	540	43
SUTTON et al.	1	–	–	–	1000	900	–
(1977)	30	–	–	–	1000 – 1200	500 – 600	–
ULLREY et al.	28	0.46	580	186	1186	358	65
(1966)	112	0.27	370	161	614	216	43

Table 2

Micro element content of mare's colostrum and milk

Author	Time after foaling (day)	Zinc (mg/kg)	Iron (mg/kg)	Copper (mg/kg)	Manganese (mg/kg)	Lead (mg/kg)	Iodine (mg/kg)
AMOUNT & TRESSOL (1986)	–	–	–	–	–	–	0.004 – 0.016
KAZHMURATOVA (1976)	–	–	–	–	–	–	0.013 – 0.025
KULISA (1986)	–	0.89	1.46	0.25	–	–	–
LONNERDAL et al (1981)	–	1 – 2	0.3 – 1.0	0.2 – 0.4	–	–	–
SCHRYVER et al. (1986)	7	3.1	–	0.85	–	–	–
	28	2.2	–	0.55	–	–	–
ULLREY et al. (1974)	0	6.4	1.31	0.99	–	–	–
	0.5	2.8	0.95	0.83	–	–	–
	1	3.6	1.05	0.73	–	–	–
	8	3.3	0.88	0.44	–	–	–
	35	2.2	0.71	0.25	–	–	–
	120	2.4	0.49	0.20	–	–	–
UNDERWOOD (1981)	–	–	–	0.20 – 0.36	0.05	–	–
WILLOUGHBY & BROWN (1971)	–	–	–	–	–	0.05	–

1. Materials and methods

Protein and protein fraction contents, amino acid composition and biological value of milk and milk protein of colostrum and milk produced up to the 45th day of lactation by 16 Hungarian Draught, 4 Haflinger, 6 Breton and 3 Boulonnais mares were determined. The feeding, milking and sampling techniques were described previously (CSAPÓ et al., 1994).

Milk samples, frozen to −25 °C, were thawed in water of +35 °C and blended. Total protein content and protein fractions of colostrum and milk were measured by the Kjel-Foss nitrogen analyser (protein content = N% × 6.38). Separation of protein fractions was done as described by CSAPÓ (1984). The amino acid composition of

milk protein was measured by automatic amino acid analyser (type: LKB 4101). Protein was hydrolysed by 6 mol HCl, as reported by CSAPÓ and co-workers (1986). The biological value of milk protein was calculated by the method of MORUP and OLESEN (1976) on the basis of amino acid composition.

Ash contents of samples were determined by the relevant HUNGARIAN STANDARD (1978). At determination of macro- and micro element content of milk samples the gained metal oxides were converted to chlorides by hydrochlorid acid, after this the metals taken into solution were determined by UNICAM SP – 191 type atomic absorption spectrophotometer. Determination of phosphorus content was achieved by Spekol photometer through measurement of blue colour created by ammonium molibdenate.

2. Results and discussion

The concentration of protein and protein fractions, their changes in mare's colostrum and milk to the 45th day of lactation and distribution of protein fractions are shown in Table 3. Table 4 shows the free amino acid content of mare's colostrum and milk while the total amino acid concentrations in mare's colostrum and milk, expressed as g/100 g fluid and g/100 g protein, are shown in Table 5.

Table 3

Means and standard deviations of protein contents and protein fractions of mare's colostrum and milk (g/100 g milk), *and distribution of mare's milk protein fractions as percentages of total protein*

Protein fraction	Days postpartum					
	0–0.5		2–5		8–45	
	$\bar{x}$	S.D.	$\bar{x}$	S.D.	$\bar{x}$	S.D.
Total protein	16.41	3.21	4.13	0.77	2.31	0.50
True protein	16.08	2.98	3.86	0.72	2.11	0.48
Whey protein	13.46	2.63	2.11	0.61	1.11	0.32
True whey protein	13.13	2.41	1.84	0.50	0.91	0.25
Casein	2.95	0.34	2.02	0.26	1.20	0.14
NPN × 6.38	0.34	0.041	0.27	0.015	0.20	0.052

Protein fractions	Days postpartum		
	0–0.5	2–5	8–45
Total protein	100	100	100
True protein	97.96	93.44	91.38
Whey protein	82.02	51.09	48.05
True whey protein	79.98	44.53	39.43
Casein	17.98	48.91	51.95
NPN × 6.38	2.04	6.56	8.62

Table 4

Free amino acid content of mare's colostrum and milk

Amino acid	Free amino acid content			
	mg free amino acid/100 g milk		g free amino acid/100 g free AA	
	colostrum	milk	colostrum	milk
Aspartic acid	2.90	0.60	4.6	1.9
Threonine	2.90	3.57	4.6	11.5
Serine	5.59	8.97	8.8	28.8
Glutamic acid	9.21	9.92	14.5	31.8
Proline	2.50	1.61	3.9	5.2
Glycine	4.01	1.01	6.3	3.2
Alanine	3.68	0.66	5.8	2.1
Cystine	0.53	0.06	0.8	0.2
Valine	9.21	1.67	14.5	5.4
Methionine	0.39	0.03	0.6	0.1
Isoleucine	1.84	0.16	2.9	0.5
Leucine	3.75	0.35	5.9	1.1
Tyrosine	1.38	0.28	2.2	0.9
Phenylalanine	1.51	0.57	2.4	1.8
Lysine	6.32	0.88	9.9	2.8
Histidine	6.38	0.66	10.0	2.1
Arginine	1.58	0.19	2.5	0.6
Totals	63.68	31.19	100.2	100.0

There were no significant differences among the four breeds with regard to the protein content of colostrum, transition milk or milk. Breed was found not to influence distribution of protein fractions, amino acid composition or biological value of colostral or milk protein. The results reported are for 29 individual draught mares.

The total protein content of colostrum immediately after foaling ranged from 13.2 and 22.0% and averaged 16.41%. This value decreased to 4.13% in transitional milk (2nd to 5th days) and to 2.13% in milk (8 to 45 days after foaling). Due to the fact that the true protein content was calculated by subtraction of NPN from total protein, the changes in true protein coincided with those of total protein. There were similar large changes in whey protein and true whey protein contents over time. These two components, respectively, decreased from 13.5 to 13.1%, measured immediately after foaling, to 2.1 and 1.8% days 2 to 5 and to 1.1 and 0.9% days 8 to 45.

Corresponding changes in casein and NPN contents were much smaller. The casein content of colostrum was 2.95% immediately after foaling, 2.02% days 2 to 5 and to 1.20% days 8 to 45. The NPN content of colostrum was about 20% higher than that of transition milk and 40% higher than that of normal milk.

Table 5

Amino acid composition of mare's colostrum and milk, amino acid composition of colostrum and milk proteins and amino acid composition of cow's milk and cow's milk proteins

Amino acid	Days postpartum						Cow's[a] milk	Cow's[a] milk protein
	0–0.5	2–5	8–45	0–0.5	2–5	8–45		
	g AA/100 g milk or colostrum			g AA/100 g protein			g AA/100 g milk	g AA/100 g protein
Aspartic acid	1.543	0.404	0.246	9.3	9.7	10.4	0.26	7.8
Threonine	1.132	0.235	0.101	6.9	5.7	4.3	0.15	4.5
Serine	1.444	0.306	0.147	8.7	7.4	6.2	0.16	4.8
Glutamic acid	2.281	0.702	0.474	13.8	16.9	20.1	0.77	23.2
Proline	1.346	0.339	0.197	8.1	8.2	8.4	0.32	9.6
Glycine	0.558	0.124	0.045	3.4	3.0	1.9	0.06	1.8
Alanine	0.673	0.157	0.076	4.1	3.8	3.2	0.10	3.0
Cystine	0.164	0.033	0.014	1.0	0.8	0.6	0.02	0.6
Valine	0.853	0.198	0.097	5.2	4.8	4.1	0.16	4.8
Methionine	0.213	0.054	0.035	1.3	1.3	1.5	0.06	1.8
Isoleucine	0.492	0.132	0.090	3.0	3.2	3.8	0.14	4.2
Leucine	1.444	0.388	0.229	8.7	9.3	9.7	0.29	8.7
Tyrosine	0.771	0.182	0.101	4.7	4.4	4.3	0.15	4.5
Phenylalanine	0.738	0.200	0.111	4.5	4.8	4.7	0.16	4.8
Lysine	1.444	0.351	0.189	8.7	8.4	8.0	0.27	8.1
Histidine	0.492	0.116	0.056	3.0	2.8	2.4	0.10	3.0
Arginine	0.706	0.186	0.123	4.3	4.5	5.2	0.11	3.3
Tryptophan	0.229	0.054	0.028	1.4	1.3	1.2	0.05	1.5
Totals	16.523	4.161	2.359	100.1	100.3	100.0	3.33	100.0

[a] from our earlier investigations

The distribution of protein fractions, expressed as percentages of total protein (Table 3), changed over time. The true protein content decreased from 97–98% for colostrum to 91% for milk. Contents of whey protein and true whey protein, respectively, decreased from 80 and 82% to 39 and 48%, while casein and NPN contents, respectively, increased from 18 to 52% and from 2.0 to 8.6% during 45 days after foaling. Most of the observed changes occurred during the first 24 h after foaling, and the remainder of the changes from colostrum to milk occurred gradually over the first 5 days.

The composition of milk secreted after the 5th day of lactation was almost identical to that of milk on the 45th day of lactation. The authors cited earlier published very few data on colostrum, and these data had much greater standard deviations than we observed. The range of 4.8–25.0% for the protein content of

colostrum reported by LINTON (1937) and the range of 10.6–25.0% reported by ROUSE & INGRAM (1970) indicated great differences among individual mares. The smaller range in present study (13.2–22.0%) was probably due to use of consistent sampling methods. We considered first milked colostrum to be only that sample which was taken immediately after foaling before the foal could suckle. If the foal suckles prior to sampling, the sample will be diluted due to initiated milk secretion and the composition is altered significantly. Precise timing of the first sample is much more important for mares than for cows because the quantity of colostrum is much less than that in the mammary gland of cows; therefore, the dilution after sucking is much greater. The value of 16.41% for the total protein content of first milked colostrum measured by us was 2.5% lower than that reported by ULLREY and co-workers (1966) and 5–8% higher than that measured by SHUTTON and co-workers (1977).

There are no previous reports concerning the protein fractions of colostrum. The results reported here for the protein fractions of milk are difficult to compare with data of authors cited earlier because they reported casein contents ranging from 41 to 65% of the total protein while we obtained a value of 49 to 52%. There are no reports on changes in protein fractions from colostral period to 45th day of lactation. Values observed for the NPN content of mare's milk were slightly higher than those discussed earlier.

The free amino acid content (Table 4) of colostrum, with the exception of threonine, serine and glutamic acid, were about twice as high as those of normal milk. When the composition of free amino acids is expressed in percentages, it is clear that colostrum contained approximately five times as much basic (histidine, lysine, arginine) amino acids, and only about 1/3 to 1/2 as much acidic amino acids as normal milk. The concentration of free amino acids in colostrum was 63.68 mg/100 g, which is 19.01% of NPN. These values in case of milk were 31.19 mg/100 g and 15.67%, respectively. Therefore, approximately 16–20% of NPN of colostrum and milk of mares is in the form of free amino acids. It has been reported (KULISA, 1986) that mare's milk contains more free serine and glutamic acid than free methionine. The values in Table 4 would indicate that this statement could be expanded to say that the proportion of all other investigated free amino acids were greater than that for methionine. The sulphur-containing amino acids (methionine and cystine) represented much lower proportions of the free amino acids than any of the other amino acids investigated.

The amino acid compositions of colostrum and milk (Table 5) show that changes in amino acid content parallel those of total protein content relative to time after foaling. It means that each amino acids decreases, without exception, from colostrum to milk (8 – 45 days). For example, threonine content declined from 1.13 g/100 g colostrum to 0.10 g/100 g milk and the corresponding change for

glutamic acid was 2.28 to 0.47. When the amino acid composition was expressed as g AA/100 g protein, the changes were much less apparent. Threonine decreased from 6.9 to 4.3 g/100 g protein while glutamic acid increased from 13.8 to 20.1 g/100 g protein. The sum of five essential amino acids (threonine, valine, cystine, tyrosine and lysine) decreased from 26.4 to 21.3 g/100 g protein, while the total of two nonessential amino acids (glutamic acid and proline) increased from 21.9 to 28.5 g/100 g protein.

These results seem to contradict data reported by DOREAU and co-workers (1990) who reported no change in the amino acid composition of mare's milk protein between the 7th and 56th day of lactation. However, they did not investigate the critical period of the first five days after foaling when the largest changes occurred in our investigation. We did not find a publication which reported data on the amino acid composition of colostrum milked immediately after foaling.

Data for the amino acid composition of milk protein, with the exception of the two containing-sulphur amino acids, agree with results of DOREAU and co-workers (1990) and PELTONEN and co-workers (1980). In the case of some amino acids, our results differed from results reported by KULISA (1986) and SARKAR and co-workers (1953).

The biological value of milk protein was calculated by the method of MORUP and OLESEN (1976) based on amino acid composition. The biological value of colostrum milked immediately after foaling (132.3) almost reached the maximum of the method (140), which was due to the very high threonine and lysine contents. During days 2–5, this value decreased to 119.7 due to the reduced quantities of essential amino acids. From the 8th to the 45th day, the biological value of milk protein was 107.9. This is a very high biological value compared to that of cow's milk which was 80.2 based on data in Table 5. These differences can be explained by the higher proportion of whey protein and higher quantities of essential amino acids, especially threonine, in mare's milk. There are no comparable data in the literature.

Ash content of mare's colostrum was measured as 0.5923% in average of samples taken in the first 48 hours of lactation, where extremes were 0.8040% and 0.5150% (Table 6). These values have decreased to 0.5126% (extremes: 0.5420% and 0.4970%) between days 3 and 5, to 0.4051% (extremes: 0.4790% and 0.3010%) between days 8 and 45. Ash content determined in colostrum is not comparable to results in the literature since in Table 1 the nearest value to colostrum for ash content were for the 7th day. In this Table the value of 0.61% (SCHRYVER et al., 1986) coincides well with 0.5923% measured by us. Similarly to colostrum our data on transition milk could not be compared to literature due to lack of data. Our value of 0.4051% for mare's milk coincides well with published ones, though the value of 0.27% (ULLREY et al., 1966), 0.295% (KULISA, 1986) and 0.30% (NESENI et al., 1958) seems too low for us.

Table 6

Macro element content of mare's milk

Macro elements (mg/kg)	Days postpartum							
	Mare						Cow[a]	
	0-2		3-5		8-45		5-270	
	Means	S.D.	Means	S.D.	Means	S.D.	Means	S.D.
Ash (%)	0.5923	0.0905	0.5261	0.0185	0.4051	0.0630	0.7534	0.0312
Potassium	928	75	709	138	517	65	1204	68
Sodium	320	86	177	44	167	72	504	34
Calcium	748	190	953	86	823	125	1287	143
Phosphorus	742	109	638	121	499	83	996	111
Magnesium	140	81	86	15	66	16	139	12

[a] from our earlier investigations

Evaluating the changes of macro elements during the first one and half month it was found that each macro elements except for the calcium decrease in colostral period and in the beginning of lactation. Decrease is most evident in case of magnesium, but significant decrease was experienced in cases of potassium and sodium. At the beginning of the lactation decrease is significantly lower in phosphorus, so this element shows definite change only after the 5th day of lactation. Studying the quantity of calcium absolutely other change was experienced to the previous ones. Calcium content of mare's colostrum is the lowest right after foaling (747.7 mg/kg), it reaches a maximum of 953.7 mg/kg at about 5th day of lactation and later on – similarly to the other macro elements – decreases during the lactation. The maximum curve of calcium content well coincides with observations of ULLREY and co-workers (1966), though they have got maximum calcium quantity on the 8th day after foaling. They have also described the changes of phosphorus content in colostrum by maximum curve. This slightly explains our data which show the lowest decrease in phosphorus content during the colostral period. Our survey data cannot be compared to that of literature due the lack of data.

Evaluating the macro element content of mare's milk it was established that 517.2 mg/kg for potassium and 166.6 mg/kg for sodium coincides well with data in the literature. Values of 822.9 mg/kg for calcium and 498.8 mg/kg for phosphorus content coincide well with most of the results in the literature but in some cases there are slightly lower ones than those. The value of 65.87 mg/kg measured for the magnesium content of milk practically agrees with other's results.

Analysing the change in micro element content of mare's colostrum and milk (Table 7) in consequence of time passed after foaling it was found that zinc and copper decrease continuously, iron decreases after the maximum reached the 5th

day, manganese increases till the 5th day of lactation, and after it stays constant. Only ULLREY and co-workers (1974) published reliable data on the micro element content of mare's colostrum. Comparing their data to our results it was found that they have measured higher zinc-, iron- and copper content of colostrum right after foaling, but differences became negligible after this period. In the period of transition milk the two series of measuring practically coincide.

Table 7

Micro element content of mare's milk

Micro elements (mg/kg)	Days postpartum							
	Mare						Cow[a]	
	0–2		3–5		8–45		5–270	
	Means	S.D.	Means	S.D.	Means	S.D.	Means	S.D.
Zinc	2.95	1.36	2.08	0.50	1.99	0.28	5.63	0.19
Iron	0.996	0.542	1.581	0.901	1.209	0.631	1.07	0.32
Copper	0.606	0.298	0.249	0.122	0.228	0.091	0.302	0.055
Manganese	0.0447	0.0254	0.0534	0.0223	0.0544	0.0293	0.093	0.013

[a] from our earlier investigations

Comparing the micro element content of mare's milk to the literature it can be stated that the 1.99 mg/kg for zinc content coincides well with about half of the published data, but slightly lower than the others. Iron content (1.209 mg/kg) shows a good agreement, too, but a part of the data is slightly lower than those we obtained. The value for copper content of mare's milk (0.228 mg/kg) coincides well one third of published data and significantly lower than the others. The value of 0.0544 mg/kg measured by us for manganese content of milk is practically the same as published by UNDERWOOD (1981).

Comparing the macro- and micro element content of mare and cow milk, it can be stated that cow's milk contains almost twice as much ash, potassium, phosphorus, magnesium and manganese, one and half time more calcium, iron and copper and almost three times more sodium and zinc than mare's milk. Among listed ones the low sodium content of mare's milk deserves special attention, since this can be consumed by such people suffering from cardiovascular troubles and high blood pressure. For these people the approx. 500 mg/kg sodium content of cow's milk is too high.

*

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ASSESSMENT OF WHOLESOMENESS OF IRRADIATED FOODS
(A REVIEW)

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Although the World Health Organization recommends food irradiation as one of the most promising methods to fight the increasing trend in the incidence of foodborne diseases, industrial application of this technology is still very limited. Some consumer initiatives are vigorously opposed to the marketing of irradiated foods, and as a consequence, the food industry in most countries is hesitant to make use of existing legal permissions to irradiate certain foods. A recurring theme in the argumentation of those opposed to food irradiation is the claim or suspicion that consumption of irradiated foods may entail negative chronic health effects. This review considers the arguments of opponents and summarizes the scientific evidence demonstrating radiological, microbiological and toxicological safety as well as nutritional adequacy of irradiated foods.

Keywords: food safety, foodborne disease, irradiation, nutritional quality, public health

1. Introduction

In the last two decades foodborne diseases of microbial aetiology have become an increasing public health problem all over the world. The increasing incidence must be described as dramatic in some countries. In the Federal Republic of Germany, for instance, the morbidity of infectious enteritis has risen from about 10 000 reported cases in 1970 to about 50 000 in 1980, and to over 100 000 in 1990 (DIEHL, 1993). Infants and young children, pregnant women, the immunocompromised, the hospitalized and the elderly are particularly vulnerable to foodborne diseases; many of the estimated 7 000 people that die each year in the United States from such diseases belong to one of these groups (ROBERTS, 1989). Some of the world's leading hygienists have advocated irradiation of feed materials and of certain foods as one of the most promising measures to fight this serious threat to public health (MOSSEL,

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1977, 1987; FARKAS, 1987a; KAMPELMACHER, 1985, 1989), and the World Health Organization (WHO) has endorsed this view (WHO, 1988). KÄFERSTEIN and MOY (1993), WHO Food Safety Unit, have described food irradiation as possibly the most significant contribution to public health to be made by food science and technology since the introduction of pasteurization of milk. After previous clearance by the United States Food and Drug Administration (FDA, 1990), the United States Department of Agriculture has published the final rule permitting irradiation of poultry "to reduce the potential for foodborne illness" (USDA, 1992).

Under these circumstances one should expect rapid growth in the practical use of this new technology. In fact, however, growth has been slow. France, Belgium and the People's Republic of China are the only countries where significant progress in the industrial application of food irradiation has occurred in recent years, and even there the annual tonnage of food irradiated is quite small. In the Netherlands, formerly a leader in this field, a new food act became effective in 1992, which severely limited the list of clearances and introduced very strict labelling requirements for irradiated foods, resulting in a considerable reduction in the amount of radiation-processed food in that country in 1993 (NEIJSSEN, 1993). Efforts of the European Commission to harmonize the legal control of food irradiation in the European Community have repeatedly failed, due to the resistance of some of the member country governments and of the European Parliament. Faced with threats of boycott and picketing by consumer activists (PSZCZOLA, 1990; SATIN, 1993) the food industry is making little use of the existing options to market irradiated foods. WHO has expressed its concern that the unwarranted rejection of this process may endanger public health and deprive consumers of the choice of food processed for safety (KÄFERSTEIN & MOY, 1993).

Doubts about the safety of consumption of irradiated foods are a recurring theme in the argumentation of those opposed to the use of this technology. The repeated rejection by the European Parliament of the European Commission's draft directive on food and food ingredients treated with ionizing radiation was primarily based on a report submitted to the Parliament which claimed that irradiated foods are no more than an empty shell with no vitamins or nutritional value (DIEHL et al., 1991). In the United States of America, some states (Maine, New Jersey, New York) have enacted legislation to ban irradiated foods temporarily or permanently, also based on claims that irradiated foods are unwholesome, potentially toxic or health-threatening; New Jersey's temporary ban expired in 1992.

It therefore appears desirable to reconsider such claims and to review and summarize the scientific background of decisions taken by many governments and international organizations to permit the marketing of certain foods irradiated under specified and controlled conditions.

2. Radiological safety

Elements present in foods can be converted to radioactive isotopes if the food is irradiated with electrons or photons having a very high energy. Below a certain threshold energy, which depends on the type of radiation and on the element, such nuclear reactions do not occur. Based on experimental studies and on theoretical estimates, an FAO/IAEA/WHO Joint Expert Committee on Irradiated Foods (WHO, 1981) has recommended to restrict the radiation sources for irradiation of food to the following:

- Gamma rays from the radionuclides ^{60}Co (maximum energy 1.33 MeV) or ^{137}Cs (maximum energy 0.66 MeV); [MeV = million electron volt]
- X-rays generated from machine sources operated at or below 5 MeV;
- Electrons generated from machine sources operated at or below 10 MeV.

No measurable induced radioactivity is produced when food is treated with radiation emitted by these sources. These restrictions have been incorporated into the Codex General Standard for Irradiated Foods (CODEX ALIMENTARIUS, 1984) and into national regulations in all 38 countries where irradiation of certain foods is permitted. In its regulations on food irradiation FDA (1986) firmly stated: "Because no evidence has been submitted to contradict FDA's finding that the irradiation of food does not cause the food to become radioactive, no further discussion of this issue is necessary."

The different types of radiation described above are sufficiently energetic to cause ejection of electrons in the medium through which they pass. This process is called ionization, and these types of radiation are called ionizing radiations. The ions and free radicals primarily formed when ionizing radiation passes through a food are mostly unstable. They can react with each other and with constituents of the food. "Radiolysis" is a summary term describing the chemical reactions resulting from radiation treatment.

3. Microbiological safety

The effect of ionizing radiation on microorganisms depends primarily on the radiation dose although other factors, such as oxygen partial pressure, water activity and temperature prevailing during irradiation are also of great influence.

With high-dose irradiation aimed at achieving commercial sterility of the food (Table 1) no public health problems can be foreseen (ANELLIS et al., 1979). At lower doses the microorganisms that survive irradiation may become a health risk. However, virtually the same problems arise secondary to other preservation methods that do not lead to complete sterility, such as heat pasteurization. Precautions must be taken, such as refrigerated storage or increasing intrinsic colonization resistance

by ensuring low pH or low water activity, in order to prevent growth of pathogens having survived the preservation treatment.

Table 1

Purposes of food irradiation

Low-dose applications (less than 1 kGy[a])
⇒ Inhibition of sprouting of potatoes, onions, garlic, yam, thus allowing long-term storage without use of chemical sprout inhibitors
⇒ Killing or sexual sterilization of insects, thus preventing losses caused by insects in stored cereal grain, flour, dried fruits, nuts, and pulses without use of chemical fumigants. Prevention of the spreading of insect pests in food trade; use as a quarantine treatment instead of chemical fumigants
⇒ Destruction of parasites in food, e.g. trichinae, tapeworms, *Entamoeba histolytica*, *Toxoplasma gondii*
Medium-dose applications (1–10 kGy)
⇒ Reduction of populations of bacteria, molds and yeasts present on the surface or in the interior of foods, thus improving keeping properties and preventing food poisoning due to *Salmonella*, *Shigella*, *Campylobacter*, *Vibrio*, *Yersinia*, *Listeria* and other non-spore-forming pathogens
High-dose applications (10–45 kGy)
⇒ Destruction of populations of spoilage organisms and pathogens, including spore formers such as *Clostridium botulinum*

[a] Gy (gray) is the unit of radiation dose. It is equal to one joule of energy absorbed per kg of matter being irradiated. A commonly used multiple is the kGy (kilogray) = 1000 Gy. The previously used unit was called rad; 1 Gy = 100 rad.

Fortunately, the most common and – from a public health standpoint – most troublesome bacteria, such as *Salmonella*, *Shigella*, *Yersinia*, *Campylobacter*, and *Listeria spp.*, are most sensitive to radiation and can be reliably eliminated by doses far below 10 kGy (MAXCY, 1982; MOSSEL, 1987; DIEHL, 1990). The more resistant organisms, like spores of *Clostridium botulinum* or *Bacillus cereus*, will at least be reduced in numbers, and the surviving flora is generally less resistant to heat, pH-changes, salt concentration and some other environmental factors (FARKAS & ANDRÁSSY, 1985).

Concerns that radiation-induced mutations in bacteria might cause enhanced pathogenicity or increased radiation resistance have not been substantiated. On the contrary, loss of virulence and infectivity as a result of radiation treatment has been identified early (INGRAM & FARKAS, 1977), and has been confirmed by more recent

studies (FARKAS, 1988). It is indeed possible, under highly specific laboratory conditions, to obtain strains of bacteria that have higher radiation resistance than the parent strain. However, such pure cultures can only survive under conditions where they do not have to compete with the large variety of wild forms occurring in all foods in a natural environment.

In mould increased production of aflatoxin was observed when spores of *A. flavus* (JEMMALI & GUILBOT, 1969; APPLEGATE & CHIPLEY, 1973) or *A. parasiticus* (BULLERMAN & HARTUNG, 1974; SCHINDLER et al., 1980) or cultures derived from such spores were irradiated. Similar observations were made concerning production of ochratoxin by *Aspergillus ochraceus* (now designated *A. alutaceus*) (PASTER et al., 1985). However, results were not clear cut; increased or decreased production of toxin being observed in different experiments.

The size of the inoculum exerts a decisive influence on toxin formation, and this was not taken into account in some of these studies. As demonstrated with *A. parasiticus* (SHARMA et al., 1980) and with *A. flavus* (ODAMTTEN et al., 1987), a medium inoculated with a high number of spores will develop less aflatoxin than one inoculated with a lower number of spores. A reduction in the number of spores by about 4 log cycles, either by simple dilution or by irradiation, caused a twofold increase in toxin production by *A. parasiticus*, and an up to 12-fold increase in toxin production by *A. flavus*. Toxin production is apparently suppressed when the number of inoculated spores per unit volume of substrate exceeds a certain level (KARUNARATNE & BULLERMAN, 1990).

A different experimental design was used by PRIYADARSHINI and TULPULE (1976) who irradiated various foods, which were subsequently heat-sterilized, inoculated with spores of *A. parasiticus*, and incubated. Aflatoxin formation was higher than in unirradiated control samples. However, no practical situation is conceivable in which a food would be first irradiated, then heat-sterilized and then stored under conditions permitting infection and moulding. BORSA and co-workers (1992) have demonstrated that fumigation (methyl bromide, phosphine) as well as irradiation increases the formation of ochratoxin in barley inoculated with *A. alutaceus*.

Experiments in which irradiation stimulated toxin formation were usually done with a massive inoculum of spores. In a 'real life' situation such conditions will hardly occur. Experiments carried out under conditions simulating practical application have either found unchanged or – more frequently – decreased production of mycotoxins in irradiated foods (OGBADU, 1980; CHANG & MARKAKIS, 1982; CHIOU et al., 1990). When an Indian group of authors (BEHERE et al., 1978) studied spontaneous formation of aflatoxin in wheat (i.e. without challenging the wheat with an inoculum of spores) storing the wheat at 90% relative humidity at 28 °C – conditions not unusual in tropical countries – they found either the same or lower

levels of aflatoxin in irradiated samples when compared with unirradiated controls, as demonstrated in Fig. 1.

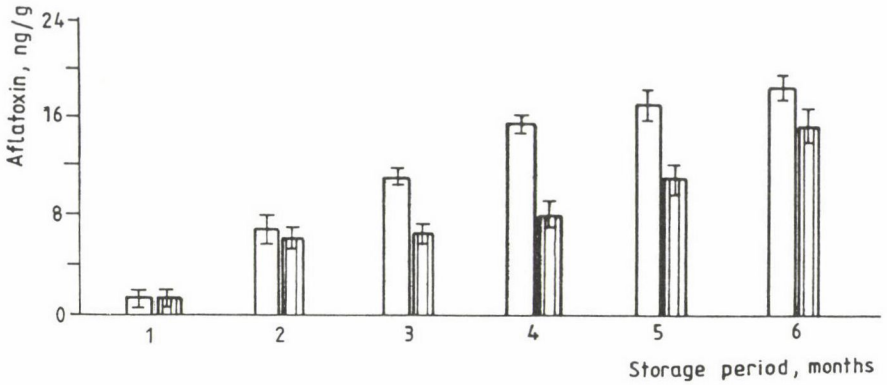

Fig. 1. Aflatoxin levels in control and gamma-irradiated (0.2 kGy) wheat during storage at 90% R.H. and 28 °C. From BEHERE et al. (1978). □: Unirradiated; ▥: irradiated

A study of various strains of *A. flavus* subjected to many repeated cycles of sub-lethal irradiation doses and growth showed that such treatment more frequently led to a complete loss or to a decrease of aflatoxin production that to an increase (FRANK et al., 1971). In summary, there are no indications that any food irradiated and stored under conditions prevailing in practice would be at risk of increased formation of mycotoxins.

4. Nutritional adequacy

Studies of effects of ionizing radiation on nutrients have been reported in hundreds of publications and the results have been summarized in many reviews (e.g. JOSEPHSON et al., 1978; KRAYBILL, 1982; MURRAY, 1983; DIEHL et al., 1991; THAYER et al., 1991). There is general agreement that the metabolizable energy of macronutrients (carbohydrates, lipids, proteins) is unaffected by radiation doses up to 10 kGy and even considerably beyond. Among micronutrients, the essential amino acids, essential fatty acids, minerals, trace elements and most vitamins suffer no significant losses in foods irradiated under conditions of actual or potential commercial application.

A few vitamins, especially vitamin B_1 among the water soluble vitamins, and vitamin E in the fat soluble group, are partially lost (Table 2). The extent of loss depends on the radiation dose applied, the oxygen partial pressure and the composition of the food. The comparative sensitivity of vitamins indicated in Table 2 should therefore be interpreted with caution. If appropriate conditions are chosen,

even vitamins E and B_1 are quite stable (DIEHL et al., 1991). The most effective precaution to be taken to minimize losses of Vitamins E and B_1 is the exclusion of oxygen by packaging under nitrogen or vacuum; irradiation at low temperature can also improve the retention of the less radiation-resistant vitamins (JOSEPHSON et al., 1978; DIEHL, 1979).

Table 2

Relative radiation sensitivity of vitamins

Most sensitive ⟶ least sensitive
Fat soluble vitamins
Vitamin D → carotene → vitamin A → vitamin D → vitamin K
Water soluble vitamins
Vitamin B_1 → vitamin C → vitamin B_6 → vitamin B_2 → folate, niacin, vitamin B_{12}

In order to avoid undesirable changes in taste, odor and texture, irradiation of foods at dose levels above the presently approved limit of 10 kGy would generally require exclusion of oxygen as well as low temperature processing. Under these conditions vitamin losses can be avoided completely or be kept to a minimum, as demonstrated in Table 3 for chicken meat irradiated at 58 kGy (THAYER, 1990). The only significant loss was that of thiamin in gamma-irradiated meat – and even that did not exceed the loss found in the heat-sterilized product. Significant increases, as here observed with riboflavin and folic acid in electron-irradiated and vitamin B_{12} in gamma-irradiated meat have repeatedly been reported by other authors. It is not clear whether this is the result of radiation-induced conversion of a precursor to the vitamin or whether irradiation only improved the availability of the vitamin for analysis in these instances.

High losses of vitamin C were observed when aqueous solutions of ascorbic acid or fruits were irradiated with doses above 10 kGy. These findings are without practical significance. Most fruits will not be processed at doses >2 kGy because they would develop radiation damage, such as pitting of the skin or softening. Many studies have shown that below this dose radiation induced losses of vitamin C are insignificant (DIEHL et al., 1991).

After reviewing the research reports on irradiation of poultry FDA concluded that "irradiation at the doses used does not have a deleterious effect on the levels or the bioavailability of the nutrients in chicken" and "irradiation of poultry at doses of up to 3 kGy will not have an adverse impact on the nutritional value of a person's diet" (FDA, 1990).

Table 3

Vitamin contents of frozen, thermally processed, gamma-irradiated, and electron-irradiated enzyme-inactivated chicken meat.
Adapted from THAYER (1990)

Vitamin	Process			
	Frozen control	Heat-sterilized	Gamma-irradiated 58 kGy at −25 °C	Electron-irradiated 58 kGy at −25 °C
Thiamin-HCl, ppm	2.31	1.53[a]	1.57[a]	1.98
Riboflavin, ppm	4.32	4.60	4.46	4.90[b]
Pyridoxine, ppm	7.26	7.62	5.32	6.70
Niacin, ppm	212.9	213.9	197.9	208.2
Pantothenic acid, ppm	24.0	21.8	23.5	24.9
Biotin, ppm	0.093	0.097	0.098	0.103
Folic acid, ppm	0.83	1.22	1.26	1.47[b]
Vitamin A, IU/kg	2716	2340	2270	2270
Vitamin D, IU/kg	375.1	342.8	354.0	466.1
Vitamin K, ppm	1.29	1.01	0.81	0.85
Vitamin B_{12}, ppm	0.008	0.016[b]	0.014[b]	0.009

Vitamin concentration on dry weight basis. [a] Significantly lower than frozen control. [b] Significantly higher than frozen control.

5. Toxicological safety

5.1. Studies not showing adverse effects

Extensive animal feeding studies designed to detect any toxic factors possibly present in various irradiated foods were carried out in the 1950s and 60s, mostly in the United States and in the United Kingdom. Based on these studies the U.S. Army Surgeon General concluded in 1965 that "Foods irradiated up to an absorbed dose of 5.6 Mrad (56 kGy) with a cobalt-60 source of gamma radiation or with electrons with energies up to 10 million electron volts have been found to be wholesome; i.e. safe, and nutritionally adequate" (CONGRESS OF THE UNITED STATES, 1965).

At about that time, however, the Food and Drug Administration began to demand more stringent evidence of safety. In 1968 it withdrew the clearance for irradiated bacon that it had granted at the request of the U.S. Army in 1963. Evidence from animal feeding studies found acceptable in 1963 was now considered to be insufficient. In response, the Army embarked on a massive programme of testing the safety of radiation sterilized beef and, a few years later, of radiation sterilized chicken meat. Bacon was not retested in view of anticipated confounding

effects arising from nitrosamine formation in nitrate cured meat. Health authorities in other countries also demanded additional studies.

Animal feeding studies of this kind are very costly and it seemed unnecessary to duplicate such projects in different parts of the world. In order to coordinate and rationalize the various efforts, the International Project in the Field of Food Irradiation was created in 1970. Under the sponsorship of FAO, the International Atomic Energy Agency (IAEA) and the Organization for Economic Cooperation and Development (OECD) 24 countries pooled their resources. WHO became associated in an advisory capacity. Feeding studies contracted by the International Project were carried out with irradiated wheat flour, potatoes, rice, iced ocean fish, mangoes, spices, dried dates and cocoa powder. This selection of foodstuffs was based on a consideration of the interest likely to be accorded to the product as a staple food entering international trade, its usefulness to developing countries, and its technological and economic suitability for radiation preservation by doses in the range below 10 kGy. The research programs also included screening tests for mutagenicity and the evaluation of radiolytic effects determined by chemical analysis. None of the studies have given any indication of the presence of radiation-induced carcinogens or other toxic factors. The Project was terminated in 1982 when the member countries found that it had fulfilled its purpose of clearly answering the question of wholesomeness of foods irradiated in the dose range not exceeding 10 kGy.

While the International Project proceeded, numerous toxicological studies were carried out within national research programs. A review of over 1200 studies on the wholesomeness of some 280 different irradiated foods and feeds published in the period from 1925 to 1978 was presented by BARNA (1979). A working group convened by WHO to re-evaluate these older as well as newer studies designed to test the wholesomeness of irradiated foods has recently completed its task (WHO, 1994).

An experimental study differing from the usual routine procedure of feeding irradiated foods to test animals was performed to test the safety of irradiated starch. A mixture of 9 compounds that had been identified in irradiated starch (i.e. formic acid, hydrogen peroxide, methylalcohol, acetaldehyde, formaldehyde, glycolaldehyde, glyceraldehyde, malonaldehyde and glyoxal) was fed to rats at a level of 0.3 g per kg of body weight daily to rats. This corresponded to ingestion of radiolytic products 800 times exceeding that of a baby consuming 30 g of irradiated starch per day. No toxic effect was found even at this highly exaggerated dose level (TRUHAUT & SAINT-LEBE, 1978).

The fact that irradiation gives rise to highly reactive free radicals has often been considered as a reason for special concern about the wholesomeness of foods so treated. A long-term rat feeding study especially designed to assess possible health

effects of a diet containing a high concentration of free radicals was carried out in Germany. No harmful effects were found when this diet, irradiated with a dose of 45 kGy was fed to the animals during their whole life, neither with regard to tumor formation (RENNER & REICHELT, 1973) nor with regard to mutagenicity (RENNER et al., 1973).

For physiological reasons the irradiated food to be tested should not constitute more than a certain percentage of the test animal's total diet. For commodities such as potatoes or rice the level chosen is usually 30%, but this may be far too high when materials such as onions or spices are to be tested. Another approach to the wholesomeness testing of irradiated foods is the feeding of irradiated complete diets. Irradiation-produced toxic substances should be more readily detected when 100% of the diet was irradiated, rather than only 30%. Many generations of animals have been raised on such completely irradiated diets and again no carcinogenic or other toxic effects were found (IAEA, 1979).

Because of the extent of the investigation and the high dose applied, a feeding study with radiation-sterilized chicken meat, carried out in the United States, is most pertinent. The design included four groups of animals receiving chicken meat (a) electron-irradiated at an average dose of 58 kGy, (b) gamma-irradiated at an average dose of 58 kGy, (c) heat-sterilized (F_0 = 6), and (d) enzyme-inactivated (blanched) and stored frozen. The average dose of 58 kGy involved a dose range of 47 to 71 kGy in electron-irradiated as well as in gamma-irradiated meat (A. BRYNJOLFSSON, personal communication). Long-term feeding studies were carried out with dogs, rats and mice. Tests for mutagenicity also utilized the fruit fly, *Drosophila melanogaster*. Teratology studies on several species of animals were also included. More than 230.000 eviscerated broilers were used to produce the 134 metric tons of chicken meat needed for these studies. The whole enterprise took 7 years and cost some US $ 8 million. It was undoubtedly the most extensive toxicological evaluation of any food or food process ever conducted anywhere. No adverse effects attributable to the ingestion of irradiated chicken meat were observed (FDA, 1986; THAYER et al., 1987).

5.2. Studies that caused concern

Whereas the vast majority of all animal feeding studies has demonstrated that irradiated foods exert no harmful effects, some results have necessitated careful re-evaluations. Subsequent analysis or repeat studies have, as a rule, shown that the apparent detrimental effects were due to biological variability, faulty experimental design or biased evaluation.

For example, one author reported that some mice fed irradiated foods had acquired a severe dyspnea caused by dilatation of the left auricle of the heart

(MONSEN, 1960). The study was repeated on almost 5000 mice of the same strain and not a single case of such a heart lesion was found (THOMPSON et al., 1965). Another reason for presumed toxic effects may be an insufficient vitamin supply in the irradiated diet. Thus, rats receiving a diet containing 35% of radiation sterilized beef develop internal bleeding after long-term feeding, and this obviously caused considerable concern at the time (JOHNSON et al., 1960). It was later shown that the level of vitamin K in this diet was very low to begin with, i.e. before irradiation, while the high dose of irradiation applied destroyed the vitamin to the extent of causing symptoms of deficiency. Addition of vitamin K to the diet completely prevented the bleeding (MATSCHINER & DOISY, 1966).

Another study which is frequently cited as evidence that irradiated foods may cause adverse effects, is a report of increased polyploidy (i.e. occurrence of cells containing twice or more the normal number of chromosomes) in lymphocytes of malnourished Indian children who had consumed freshly irradiated wheat (0.75 kGy) for 4 to 6 weeks (BHASKARAM & SADASIVAN, 1975). This effect was not seen when wheat stored for 12 weeks after irradiation was used. The incidence of polyploid cells varies to a certain extent between individuals, and even in one individual from day to day. Normally there will be about 0.1 to 1% polyploid cells in the bone marrow and among the lymphocytes in the circulating blood. The biological significance of an increased incidence of polyploid cells is not known.

In the study with malnourished children 0% polyploid cells was found in the reference group of 5 children fed non-irradiated wheat, 0.8% in the group of 5 children receiving irradiated wheat for 4 weeks, and 1.8% in the group of 5 children ingesting irradiated wheat for 6 weeks. The authors interpreted this as an indication of a dose-related cytotoxic effect of freshly irradiated wheat.

In the study, only 100 cells were counted for each child, which is far too little to recognize an event that is seen in only about one out of 100 cells. The normal (0.8%) or perhaps slightly elevated (1.8%) incidence of polyploidy in the groups of children receiving irradiated wheat may not be surprising, but the below normal (0%) incidence in the reference group is. Remarkably, no differences among the three groups of children with regard to the generally recognized indicators of genetic damage such as chromosomal breaks, gaps, and deletions were observed. Because of the world-wide attention this study received and the debates it induced, the Indian Ministry of Health appointed an Expert Committee to review this work and some other studies which had registered polyploidy in different species of animals fed freshly irradiated wheat. When the original investigators were requested to rescore their own slides, the values obtained were very different from the figures reported earlier on the same slides. The Expert Committee in its review of the data, including review of the slides that had been scored previously and were rescored, did not find any evidence for an increased incidence of polyploid cells as result of consumption of

freshly irradiated wheat. The Committee consequently concluded that the experiments alleged to show increased incidence of polyploidy were not well designed and the results imprecise, and that the data failed to demonstrate any mutagenic potential of irradiated wheat (KESAVAN, 1978).

When the FAO/IAEA/WHO Joint Expert Committee on Irradiated Foods examined this issue in 1976, it arrived at the conclusion that the significance of observations on polyploidy was not clear since the range of incidence of polyploidy normally varies considerably, and the toxicological implications of an increased incidence of polyploidy are not understood. The Committee approved irradiation of wheat and ground wheat products unconditionally, up to a radiation dose of 1 kGy (WHO, 1977). National health agencies or committees of experts who have examined the safety of irradiated foods since then have also come to the conclusion that the Indian studies did not demonstrate an adverse effect of irradiation. Studies carried out in the People's Republic of China on adult human volunteers consuming various irradiated foods for periods of 7 – 15 weeks have given no indication of increased polyploidy (JIN & YUAN, 1988; HAN et al., 1988).

A study recently carried out in Switzerland has once more contradicted the claim that consumption of freshly irradiated wheat causes polyploidy. Groups of rats were fed for 4 or 90 days either with 70% wheat freshly irradiated at 0.25, 0.75 or 2.25 kGy and 30% complementary feed or with an unirradiated control diet. No accumulation of polyploid cells was found in animals receiving the irradiated diets (MAIER et al., 1993). The authors concluded, on the basis of their own study and after re-evaluating related studies of others, that the consumption of irradiated wheat does not pose any health risk to humans.

6. Chemical studies

During the early years of food irradiation research, considerations concerning the wholesomeness of radiation processed foods were based almost exclusively on animal feeding studies. More recently, studies relying on radiation chemistry have supplemented earlier animal feeding experiments. It was suggested that evaluations of the safety of irradiated foods should take estimates of radiolytic changes in foods into account (DIEHL & SCHERZ, 1975; TAUB et al., 1976). The FAO/IAEA/WHO Joint Expert Committee on Irradiated Foods accepted this strategy when it stated: "The general principle of radiation chemical reactions, as revealed by analytical studies, will reduce considerably the extent to which toxicological testing is needed and will simplify the testing procedures" (WHO, 1977).

Quantities of various radiolytic products formed from a variety of food constituents, and the factors which affect their formation, such as temperature, humidity and the presence or absence of oxygen have been studied. The most

important modifying factor is radiation dose. At low doses, such as required for insect disinfestation in grain (below 1 kGy), it is difficult to detect any chemical changes in the irradiated food. At high doses, such as required for sterilization (above 10 kGy), considerable changes may occur but these are still much less intensive than those caused by cooking (SCHUBERT, 1978).

Although experiments on pure amino acids, vitamins, sugars, and other constituents of food often indicate extensive destruction, even secondary to relatively low doses of radiation, the same compounds are invariably more resistant when irradiated in the complex food matrix (DIEHL, 1982). Sensitive analytical methods have permitted the identification of numerous compounds formed as a result of radiolysis of food constituents, among them carbon dioxide, ammonia, hydrogen and many alkanes, alkenes, and aldehydes (NAWAR, 1983; SIMIC, 1983).

Interestingly, recent studies on the effects of ultrasound treatment (sonication), used in food processing, particularly to improve emulsification, causes chemical changes in components of food that are largely identical with the changes provoked by ionizing radiation. Sonication of aqueous solutions of glucose or lactose, for instance, lead to the same reaction products as irradiation, and by the same reaction mechanisms (HEUSINGER, 1990, 1991. See also MURALI KRISHNA et al., 1988; PORTENLÄNGER & HEUSINGER, 1992).

Because of the low yields and the unspecific nature of the radiation-induced compounds, attempts to find reliable methods for the identification of irradiated foods have had little success in the past. With the highly sophisticated analytical methods now available, much progress in this field has been made recently, and it is now possible to reliably identify most irradiated foods (DELINCÉE, 1993; SCHREIBER et al., 1993). The most successful methods are electron spin resonance spectroscopy on foods containing bones, shells or other hard components (GRAY & STEVENSON, 1989; HELLE et al., 1992; DESROSIERS et al., 1993), thermoluminescence measurements on foods containing traces of mineral particles (DANGL et al., 1993; PINNIOJA et al., 1993; SCHREIBER et al., 1994), and gas chromatography on lipid-soluble extracts of fat-containing foods (BIEDERMANN et al., 1992; LESGARDS et al., 1993; MOREHOUSE et al., 1993). Collaborative interlaboratory tests sponsored by IAEA and by the Community Bureau of Reference of the European Community have demonstrated the reliability of these methods when applied to centrally supplied coded samples (DELINCÉE, 1993; RAFFI et al., 1993). In some countries these methods are being used for routine control of irradiated foods (BELLIARDO, 1993). These developments should go a long way towards reassuring consumers that food irradiation can be effectively controlled by governmental authorities.

In 1979, FDA established the Bureau of Foods Irradiated Food Committee (BFIFC) to review the existing agency policy and make recommendations regarding the assessment of the safety of irradiated foods. In full agreement with the radiation

chemistry oriented approach taken by the Joint Expert Committee at that time, BFIFC recommended that safety assessments of irradiated food should be based on (1) projected level of human exposure to the food, (2) estimates of the identity, amount, and potential toxicity of new chemical constituents generated in the food by the irradiation process, and (3) state-of-the-art, sensitive toxicological tests.

BFIFC completed its review and submitted its final report in July 1980 (PAULI & TAKEGUCHI, 1986). In considering a possible worst case scenario in its evaluation, the BFIFC hypothesized the formation of compounds that uniquely occurred in irradiated foods, although such substances had not been identified when the report was written. Although the search for unique radiolytic products (URPs) went on in many laboratories, and some exotic compounds were indeed discovered when pure food constituents were irradiated and analyzed, URPs in irradiated foods have remained elusive. At one time it was thought that o-tyrosine was a URP in irradiated meat, but more recent work has shown that it can also be found in unirradiated meat (CHUAQUI-OFFERMANNS, 1993). Gas chromatographic studies on irradiated fat-containing foods have demonstrated the presence of cyclobutanones that have not (or not yet) been identified in unirradiated foods (CRONE et al., 1992, 1993). The observation that sonication initiates largely the same reaction mechanisms as ionizing radiation suggests that all radiolytic compounds detected in irradiated foods will eventually be also detected as sonolytic compounds in food treated with ultrasound.

The chemical changes mentioned before are the direct or indirect result of radiolytic effects. They can be detected immediately after irradiation. In the dose range of interest for food processing radiation-induced product formation generally increases linearly with radiation dose. A very different type of chemical change occurs when fresh plant tissues are irradiated. A physiological response, similar to the responses caused by heat, cold or mechanical damage, develops within hours or days after irradiation, and may lead to measurable changes in enzyme activities, CO_2 production, O_2 consumption, conversion of starch to glucose, and other metabolic parameters. The dose response relationship is not linear in this case, and the analytical results obtained depend very much on the time elapsing between irradiation and analysis (DIEHL, 1990).

7. Evaluation of the wholesomeness of irradiated foods by international agencies

At its meeting in 1980 the FAO/IAEA/WHO Joint Expert Committee on Food Irradiation concluded on the basis of reports submitted by the International Project and of numerous other studies carried out within national research programs "that the irradiation of any food commodity up to an overall average dose of 10 kGy presents no toxicological hazard; hence, toxicological testing of foods so treated is no longer required". It also concluded that irradiation at this dose level "introduces no

special nutritional or microbiological problems". The Committee did not consider approval of dose levels above 10 kGy because the available data were insufficient for this purpose (WHO, 1981). Since then the results of the feeding study with chicken meat irradiated with an average dose of 58 kGy have been published (THAYER et al., 1987). Thus, there is now substantial evidence that the 10 kGy average dose limit set by the Joint Expert Committee assures a large margin of safety.

With the aim of providing countries with the necessary means to develop national legislation on the irradiation processing of food and its control, the Codex Alimentarius Commission adopted a Recommended International General Standard for Irradiated Foods and a Recommended International Code of Practice for the Operation of Radiation Facilities Used for the Treatment of Foods. The Joint Expert Committee's conclusions concerning the safety of any food irradiated with an overall average dose of up to 10 kGy have been fully adopted by the Codex Alimentarius Commission. The prominent role played by the eminent Hungarian scientist Professor Károly Vas in the preparation of these Codex documents (and in many scientific endeavours), until his premature death in 1981, has been acknowledged by several authors (FARKAS, 1987b; HOLLÓ, 1987; LOAHARANU, 1987).

In the European Community, the Commission of the EC has asked its Scientific Committee on Food (SCF) for advice on the wholesomeness of foods irradiated by suitable procedures. In its report the committee has verified the conclusions of the Joint FAO/IAEA/WHO Expert Committee (WHO, 1981) and has confirmed that no further animal feeding studies need be carried out in order to assess the safety of a food irradiated up to a dose of 10 kGy (SCF, 1989).

At the request of the Australian Ministry for Community Services and Health, the World Health Organization has commissioned an updated, comprehensive analysis of the safety and nutritional adequacy of irradiated food. The report of this evaluation has been published as a WHO monograph (WHO, 1994). The panel of reviewers came to the following overall conclusions:

- Irradiated food produced under established GMP (Good Manufacturing Practice) is considered safe and nutritionally adequate because the process of irradiation will not introduce changes in the composition of the food which, from a toxicological point-of-view, would impose an adverse effect on human health;
- The process of irradiation will not introduce changes in the microflora of the food which would increase the microbiological risk to the consumer;
- The process of irradiation will not introduce changes in the composition of the food(s) which, from a nutritional point-of-view, would cause nutrient losses to a degree that would impose an adverse effect on the nutritional status of individuals or populations.

This most recent review of the available evidence has confirmed earlier evaluations and strengthened the positive attitude towards food irradiation held by WHO for many years. The Agency's position was summarized by its Director General at an international conference in the following terms: "WHO is satisfied regarding the safety of irradiating any food commodity up to an overall average dose of 10 kGy. This, of course, does not imply that food having been exposed to higher doses of irradiation would automatically be rendered unsafe... Since the availability and safety of food are important components of the primary health care approach, WHO is concerned that the unwarranted rejection of this process, often based on lack of understanding of what food irradiation entails, may hamper its use in those countries which may benefit most" (NAKAJIMA, 1989).

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USE OF IMMOBILIZED LIPASE IN TRANSESTERIFICATION OF TRIGLICERIDES

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The lipase of Rhizopus origin has been used in immobilized enzyme form for hydrolysis and transesterification of triglycerides.

The hydrolytic and transesterification activities of the enzyme fixed on Doulite ES 568, celite, cyclodextrin polymer, diatomaceous silica carriers were determined. The fatty acid composition of bioconversion products has been analyzed with Chrom 5 gas chromatograph and with Beckman HPLC apparatus.

The multiple use of the immobilized enzyme is highly economical. Keeping the optimal temperature, pH value and reaction time, the modification of triglycerides becomes a controllable biotechnological procedure.

Keywords: immobilized lipase, Rhizopus lipase, transesterification, hydrolysis of triglicerides.

The vegetable oils and animal fats serving as basic materials of our fatcontaining foods are well known in the circle of consumers. Fats more valuable from biological points of view (fish oil, olive oil) arrive mostly as imported goods into the Hungarian trade. The transformation of raw materials to modified products consumes much energy and leads to the generation of intermediate products harmful to human health. One of the main processes of enzymatic transformation of vegetable, and animal oils and fats is the enzymatic transesterification, resulting in the change of fatty acid composition and of their position inside the molecular structure of triglycerides, in a desired degree and direction.

Valuable materials can be produced from raw materials by bioconversion (BÁNKY, 1990).

1. Materials and methods

For our trials, lipase of Rhizopus origin, product of the company SERVA, was used. Among the conditions influencing enzymatic hydrolysis, the substrate specificity

0139-3006/94/$ 4.00

of the enzyme is essential. This depends on the origin of the enzyme and on the purity of the preparation (IWAI & TSUJISAKA, 1984). In our trials, the fatty acid specificity of lipase of Rhizopus origin was found to be advantageous considering the chain length and insaturation and its high sensitive hydrolyzing- and transesterificating-activity.

For immobilization of the enzyme (MACRAE, 1983), cation exchange resin (Doulite ES 568), diatomaceous silica and CDP-25 cyclodextrin pearlpolymer carriers were used.

The enzyme was fixed on the carrier by adsorption binding method in aqueous solution at pH 8.0, with adding the icy organic solvent agitating the mixture in refrigerator (GODERIS et al., 1987; ANON, 1986). Then, drained dry, and dried in the lyophilizer, it was suitable for further use or for storage. Hydrolytic activity in our trials meant the number of micromols of free fatty acids liberated on the influence of lipase (fixed lipase) from olive oil or triolein substrate within a hour. Transesterification activity was the number of the micromols of fatty acids attachment from a triglyceride in one hour. This was a smaller value by order than the former one. Activity was measured by titrimetric method (COLOWICK & KAPLAN, 1955).

2. Results and discussion

Results are summarized in Table 1. Several methods are used according to references for the measurement of transesterification activity. According to the method by Graille, the 20:1 mixture of triolein and palmitic acid has to get into the petroleum ether solution with immobilized enzyme, at 40 °C (GRAILLE & MUDERHWA, 1987). In other experiments, the incorporation of myristic acid into the triglycerids of the medium fraction of palmitic oil was measured (WISDOM et al., 1984).

Table 1

Activity of immobilized lipase of Rhizopus origin

Carrier sample	Hydrolytic activity ($U\ mg^{-1}$)	Transesterification activity 2 h reaction time ($U\ g^{-1}$)
Rh. lipase (not fixed)	64.0	22.0
Duolite ES 568	4.0	6.7
Celite	4.6	1.4
CDP-25	4.2	1.9
Diatomaceous silica	3.0	1.1

Transesterification with immobilized lipase was studied by help of model substances. Triolein was deacidified, the values of concentration being determined in pre-trials, the ratio was ten mole palmitic acid per one mole trioleine, palmitic acid concentration was 112 mmol l^{-1}.

Studying the transesterification between triglycerides, a 1:1 weight ratio of trioleine and tricapryline was used. The amount of immobilized lipase, together with the carrier, was 200–250 mg 10 cm^{-3} substrate. The transesterification was carried out in petroleum ether, at 40 °C shaking for a given time period. After incubation the fixed lipase was separated from the reaction mixture with filtration. Reaction mixture was deacidified with Varion AT 600 resin. The amount of dry resin and the time needed for the ion exchange for the deacidification of the reaction mixture were determined. The removal of free fatty acid content was controlled by titration with 0.01 mol l^{-1} alcoholic potassium hydroxide. Free fatty acids could be removed with ion exchange resin (5 cm^3) within 2 h. After deacidification, the product was hydrolyzed and methylester was prepared for gas chromatography.

2.1. Analytical tests for qualification of product

Basic condition of the evaluation of results is the use of suitably precise and simple analytical method for the qualification of product. Out of the analytical methods used in the lipid chemistry, thin layer chromatographic techniques were applied first. The thin layer chromatography did not give an acceptable help for detection of slight changes. Gas chromatographic and HPLC results could be quantitatively analysed. Gas chromatographic measurements were performed on Chrom 5 apparatus. Column: 120 cm × 3 mm stainless steel. Chromosorb 80–100 mesh, support, 8–10% SP–255, phenyl-silicone wetting, detection: FID detector, temperatures: column 190–270 °C, temperature program, injector 250 °C, detector 280 °C.

HPLC measurements were made on Beckman apparatus, with RI detector, column: Octadecyl C_{18} 10 μm filling, at 40 bar pressure, temperature 30 °C, mobile phase: acetonitril-acetone 93:7 (v/v) and 37:63 (v/v), the latter resulted in a better separation.

GC chromatogram of the transesterification made with lipase immobilized on celite carrier has been presented in Fig. 1. As a control, the initial mixture was used. Palmitic acid concentration (number 1) changed from 5% to 15% in the percentage of the total fatty acid concentration.

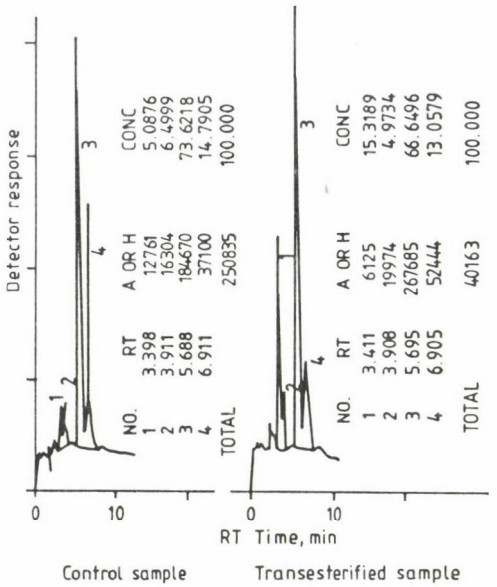

Fig. 1. GC chromatogram of triolein transesterification with palmitic acid and immobilized lipase of Rhizopus on Celite carrier. Peaks: 1. palmitic, 2. palmitoleic, 3. oleic, 4. elaidic. For gas chromatographic conditions see the text

With immobilized lipase on diatomaceous silica carrier, the transesterification was carried out with triolein and tricaprylin mixture according to the following scheme:

Reaction scheme of transesterification:

$$2\begin{bmatrix}R_1\\R_1\\R_1\end{bmatrix} + 2\begin{bmatrix}R_2\\R_2\\R_2\end{bmatrix} \xrightarrow[\text{Rh. lipase}]{\text{immobilized}} \begin{bmatrix}R_2\\R_1\\R_2\end{bmatrix} + \begin{bmatrix}R_1\\R_2\\R_1\end{bmatrix} + \begin{bmatrix}R_2\\R_1\\R_1\end{bmatrix} + \begin{bmatrix}R_2\\R_2\\R_1\end{bmatrix}$$

R_1 is for oleic acid, R_2 for caprilic acid on the glyceride, Rh. = Rhizopus.

The changes in the product concentration ratios after reaction times of 1 h and 4 h are presented in Fig. 2.

The more sensitive HPLC detected more components. The identification of the product needs further examinations, thus approximative qualitative conclusions could be drawn from the chromatograms only. After 1 h reaction time, the main component peaks decreased significantly and new products appeared due the transesterification. The peak areas of 0.2 mg tricaprylin and 2 mg triolein chromatograms were quantitatively determined (Table 2). Knowing the percentual change compared to the initial mixture, with identification of the products of

transesterification of the components, bioconversion gets manageable with the determination of reaction time temperature and pH value.

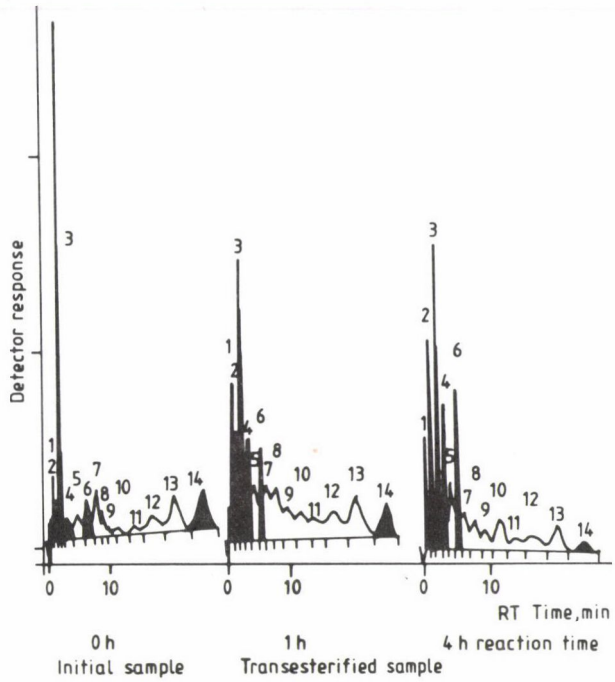

Fig. 2. HPLC chromatogram of the trioleate and tricaprylate transesterification products. For conditions of HPLC separation, see the text and Table 2

Table 2

Amount of transesterification products after 1 and 4 h reaction time
(relative values on the base of peak areas in Fig. 2)

Reaction time	Change in the peak area (% of the original value)				
	Peak numbers of reaction products				
h	2	3	4	6	14
0	100	100	100	100	100
1	130	64	380	280	90
4	216	71	620	650	30

In Table 3, the composition of transesterification product of triolein with palmitic acid has been given as a function of carriers of lipase enzyme. The results of gas chromatography served as base for calculations.

Table 3

Effect of carrier for immobilization of lipase enzyme on the composition of transesterified product
(Reaction temperature: 40 °C. Substrate: composition of the reaction mixture before enzyme addition)

Carrier	Immobilized lipase (mg)	Reaction time (h)	In the product: palmitic acid (%)	In the product: oleic acid (%)
Substrate	–	0	5	76
Doulite ES 568	300	1.5	53	28
Celite	300	1.5	15	66
Celite	300	2.5	46	35
CDP-25	300	1.5	7	74
CDP-25	500	1.5	18	63
CDP-25	500	2.5	15	66
Diatomaceous silica	200	0.5	5	76

Greatest transesterification activity was experienced with the lipase adsorbed on celite. With this preparation, the theoretical palmitic acid incorporation approached to 70% within 1.5 h. The transesterification can be carried out with the use of immobilized lipase enzyme product both with triglyceride-triglyceride and with triglyceride-fatty acid reaction mixtures. This gives the possibility to a good exploitation of the optimal conditions and to a multiple use of immobilized enzyme. Based on research results, the possibility is given to achieve biotechnological processes catalyzed by lipase and to transform the triglycerides forming the main mass of vegetable and animal fats and oils.

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MICROBIAL SAFETY ASSURANCE OF MINIMALLY PROCESSED VEGETABLES BY IMPLEMENTATION OF THE HAZARD ANALYSIS CRITICAL CONTROL POINT (HACCP) SYSTEM

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Pathogens associated with minimally processed (MAP) vegetables include *Listeria monocytogenes*, *Yersinia enterocolytica* and *Aeromonas hydrophila*. Since there is no kill step or application of inhibitory additives to reduce or destroy microorganisms, any organism, pathogenic or not, which are present on the product initially or through contamination during processing or handling will be present on the product. The documented presence of these pathogens, coupled with poor temperature control in the chill chain, indicates the need for a better safety control of minimally processed vegetables.

Microbial safety is assured by implementing the Hazard Analysis Critical Control Point (HACCP) system. HACCP is a systems which identifies hazards for a specific product and process from harvest to consumption and specifies measures for their control. In this article, the seven basic principles of HACCP are outlined and applied to assure microbial safety in the production of minimally processed endive.

Keywords: HACCP, minimally processed vegetables, Modified Atmosphere Packaging, safety assurance.

Microbial quality of our food is of major concern to food processors and public health authorities. Various foods are known to serve as vehicles of food-borne pathogens and toxins (BRYAN, 1988a). Furthermore, faulty practices, procedures and processes (e.g. inadequate refrigeration or heating) contribute to the occurrence of outbreaks of these diseases (BRYAN, 1988b). Driven by the never-ending changing consumer needs, the food industry is aggressively seeking preservation technologies today which deliver convenience products which are 'fresh-like', 'chef-like' and have the image of invisible manufacturing. The emphasis on 'natural' foods has led to the use of fewer preservatives, less salt and sugar, and as a consequence to a loss of intrinsic preservation and to a potential loss of protection from processing through

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less severely processing or heating (GOULD, 1992). Thus, the need for control of microbial safety and quality of these new type 'natural' foods is increasing.

One of these 'new' technologies consists of refrigeration in combination with Modified Atmosphere Packaging (MAP) of minimally processed vegetables. Minimally processed vegetables or 'grade 4' products refer to ready to eat vegetables and include fresh, washed, cut or peeled vegetables ready for use and packed in a sealed polymeric film or tray. This fourth form of trading – preceded by fresh, canned and frozen – originated in the 1980s as an answer to an emerging consumer demand for convenience products which are fresh-like, less severely processed, preservative free and of high quality. Over the last years, the market of minimally processed vegetables is growing both in sales volume and assortment of vegetables e.g. vegetable salads of endive, lettuce, carrots, peppers, potatoes, onions, cabbages, soy beans, both as single vegetable and in mixtures, completed with sauces and dressings, as well as vegetable mixtures for soup or stew (ANON, 1988, SCANDELLA & LETEINTURIER, 1989, SARACINO et al., 1991).

The extension of the shelf-life is achieved by a combination of correct refrigerated storage throughout the entire chill chain, Modified Atmosphere Packaging (MAP) and good manufacturing and handling practices, starting with high quality raw materials. MAP is a food preservation technology whereby the composition of the atmosphere surrounding the product is different from the composition of air. In these minimally processed vegetables the gas composition in the package is modified through respiration of the vegetative tissue (passive modification). Good hygiene and temperature control are very important factors in the extension of the shelf-life, but the addition of a suitable modified atmosphere can give an even greater extension (CARLIN et al., 1990, O'BEIRNE, 1990).

The principal spoilage mechanisms affecting the minimally processed vegetables are microbial growth, oxidation (enzymatic browning) and moisture loss. MAP is effective at inhibiting these spoilage mechanisms, as well as reducing respiration, delaying ripening and decreasing ethylene production, however, MAP will not eliminate the need for refrigeration (WILLOCX et al., 1993). The actual shelf-life of the refrigerated minimally processed vegetables depends on the temperature conditions throughout the entire chill chain since the integrated effect of time and temperature allow the proliferation of pathogenic and spoilage microorganisms as well as the organoleptic and nutritional quality to deteriorate. As a consequence the responsibility for safety and quality of these minimally processed vegetables lies with the producer and distributor as well as the consumer. However, retail display cabinets and domestic refrigerators are known to be critical points in the chill chain of minimally processed vegetables (and other refrigerated products) and temperatures well above 10 °C are frequently observed (WILLOCX et al., 1994).

Pathogens associated with minimally processed vegetables include *Listeria monocytogenes*, *Yersinia enterocolytica* and *Aeromonas hydrophila* (ICMSF, 1988, BERRANG et al., 1989, BEUCHAT & BRACKETT, 1990, CARLIN et al., 1990, ROSSET, 1990, FARBER, 1991, SCHOFIELD, 1992). These infectious pathogens are psychrotrophic and are able to grow at refrigeration temperatures. The crop grows close to the ground were splashing from rain and irrigating sprays soil the leaves and where it is easily reached by insects, snails, slugs and other small animals (ICMSF, 1988). ABDUL-RAOUF and co-workers (1993) also reported the possible contamination (contaminated irrigation water), survival and growth of the enterovirulent *Escherichia coli* O157:H7 on MAP shredded lettuce and sliced cucumber. *Clostridium botulinum* is frequently present in soil and organic fertilizers and due to the close contact of fruits and vegetables with these, they in turn become contaminated (RHODEHAMEL et al., 1992, NOTERMANS, 1993). Furthermore the possible anaerobic conditions created in temperature abused MAP vegetables, can create ideal conditions for growth and toxin production of psychrotrophic non-proteolytic strains of *C. botulinum* when the competitive flora is inhibited (PALUMBO, 1986, BRACKETT, 1987, HOTCHKISS, 1988, HOTCHKISS & BANCO, 1992, RHODEHAMEL et al., 1992). At chill temperatures the growth rate of the non-proteolytic types is slow and so requires control in products where the designed shelf-life exceeds about 10–14 days (BROWN & GOULD, 1992). Other groups of food-borne pathogens which have received little attention today are parasites (*Ascaris sp.*, *Entamoeba histolytica*) and viruses (Hepatitis A and Norwalk virus). These organisms normally gain access to fruit and vegetables before harvest as a result of using contaminated irrigation water or sewage (BRACKETT, 1987).

Since there is no kill step or application of inhibitory additives to reduce or destroy micoorganisms, any organism, pathogenic or not, which are present on the product initially or through contamination during processing or handling will be present on the product at the moment of consumption. The documented presence of infectious pathogens like *Listeria monocytogenes*, *Yersinia enterolytica* and *Aeromonas hydrophila* on minimally processed leafy vegetables, coupled with poor temperature control in the chill chain, indicates the need for a better safety control.

1. The HACCP approach: seven basic principles

One of the best ways to assure food safety is the application and implementation of the Hazard Analysis Critical Control Point (HACCP) system. HACCP involves the systematic assessment of all steps involved in food manufacturing operations from farm to consumption and the identification of those steps which are critical with respect to food safety. The HACCP approach was

originally developed in 1959 by a team at the Pillsbury Company as a means of assuring the safety of foods produced for the US space program (BAUMAN, 1990).

HACCP is a system which identifies specific hazard(s) and specifies measures for their control. By definition, a hazard can be any biological, chemical or physical property that may cause food to be unsafe for consumption (NACMCF, 1992). Biological hazards include bacterial (*Clostridium botulinum*, *Salmonella spp.*, *Staphylococcus aureus*,...), viral (Hepatitis A, Norwalk virus, ...) and parasitic hazards (protozoa and worms). The second hazard category, chemical, can be further divided into naturally occurring (aflatoxins, scrombotoxin, ...) and added chemicals (pesticides, antibiotics, food additives, toxic elements, ...). A number of these chemical hazards are not allowed in food and other have established allowable limits. Proper raw material specifications and vendor certifications will help to prevent the occurrence of added chemical hazards. Physical hazards are foreign bodies like pieces of glass, wood, stone, metal, plastic, or bones. Raw material specifications and vendor certifications next to maintenance and cleaning programs for machinery and equipment are preventive measures for physical hazards (RHODEHAMEL, 1992). In order to assure food safety properly, the HACCP system must also consider these chemical and physical hazards in addition to biological ones. However, in the context of this article, control of chemical and physical hazards of minimally processed vegetables are not considered.

In Table 1 the basic seven principles (NACMCF, 1992; FLAIR, 1994) of the HACCP system are outlined. During the hazard analysis (principle 1), the potential significance of each hazard should be assessed by considering its *risk* and *severity* (NACMCF, 1992). Risk is an estimate of the likely occurrence of a hazard (probability), whereas the severity is its seriousness. Hazards which are of a low risk and not likely to occur (for the product and process under consideration) would not require further consideration. The HACCP team has to decide which hazards are significant and must be controlled in the HACCP plan. The term 'control' in this context means 'to have under control' and should not be confused with testing, checking or verification.

Table 1

Summary chart of the seven basic HACCP principles
(FLAIR, 1994)

1	Identify the potential hazard(s), associated with food production at all stages from growth, processing, manufacture and distribution until the point of consumption. Assess the likelihood of occurrence of the hazard(s) and identify the preventive measures for their control: Hazard Analysis.
2	Determine the points/procedures/operational steps to be controlled to eliminate the hazard or minimize its likelihood of occurrence: identification of Critical Control Points(s) (CCP).
3	Establish critical limits which must be met to ensure each CCP is under control.
4	Establish a monitoring system to ensure control of the CCP by scheduled testing or observation.
5	Establish the corrective action to be taken when monitoring indicates that a particular CCP is not under control.
6	Establish procedures for verification to confirm (by supplementary procedures and tests) that the HACCP system is working effectively.
7	Establish a documentation system including all procedures and records appropriate to all the principles and their application.

2. Application of HACCP to minimally processed vegetables

Since its development, the HACCP concept has continued to evolve so that today several variations have been proposed. A number of international organisations established guide-lines for application and implementation of the HACCP principles into all sectors of food and drink manufacturing, distribution, retailing and catering (ICMSF, 1988; NACMCF, 1992; CODEX ALIMENTARIUS COMMISSION, 1993; FLAIR, 1994). In our application a sequence of 14 stages was used to assure microbial safety in the production and distribution of packaged cut endive, as recommended in the FLAIR HACCP User Guide (FLAIR, 1994).

2.1. Stage 1: Scope of the HACCP study

The objective of the HACCP study is to assure microbial (bacterial) safety in the production and distribution of packaged cut endive. The following HACCP plan will be directed primarily at control of pathogens but will also enhance the total microbial quality of minimally processed endive (vegetables).

2.2. Stage 2: Selection of the HACCP team

HACCP's successful application requires the full commitment and involvement of management and the workforce. It also requires a team approach. The HACCP system must be developed by a multidisciplinary team of individuals with the

necessary expertise, knowledge and experience, relating to the product and process being studied. In practice the team should comprise a small group (maximum 6 persons). Remark that the successful application and implementation of a HACCP system need to be accompanied by both education and training of all members of the company.

2.3. Stage 3: Assembling product data

A complete description of packaged minimally processed endive is given in Table 2. The description includes all applicable information on the product which will help in the hazard analysis and establishing CCPs. For minimally processed vegetables sold in the Belgian supermarkets the indicated shelf-life varies between 2 to 4 days, day of production not included, whereas in France shelf-life is extended up to 7 days if the products are kept at maximum 4 °C (CARLIN et al., 1990). In Belgium, there is no specific temperature control legislation for minimally processed vegetables and they fall into the category of refrigerated products, where air and product temperature during transport, display and storage must be 7 °C at maximum with a tolerance up to 10 °C in the warmest spot.

Table 2

General information and physico-chemical characteristics of packaged minimally processed endive. The worksheet should be dated and signed

Cut endive	Date Authorised by
1. General characteristics	
Composition: curled endive	
Volume: 250 g	
Package material: PVDC (polyvinylidene chloride)	
storage conditions on site: maximum 48 h at 4 °C	
2. Physico-chemical characteristics	
pH: 5.8 – 6.2	
water activity: 0.96 – 0.98	
initial microbial flora:	
Mesophilic aerobic count:	$10^5 - 10^6$ cfu/g
Psychrotrophic Gram – negative count:	$10^5 - 10^6$ cfu/g
Lactic acid bacteria:	$10 - 10^2$ cfu/g
3. Details on package	
shelf-life: 4 days	
instructions for storage: keep refrigerated	
instructions for use: wash before consumption	

2.4. Stage 4: Intended use of the product

The packages of minimally processed endive are distributed through supermarkets to the consumer and the vegetables are consumed raw.

2.5. Stage 5: Product flow diagram

The process from harvest of raw materials until consumption of the finished product is described in Table 3. The purpose of the flow diagram is to provide a clear, simple description of all the steps involved in the process, while the format is a matter of choice. The product life of minimally processed endive (vegetables) is divided into four segments: (1) pre-harvest and harvest practices; (2) production and processing; (3) distribution and retailing operations; (4) and consumer practice and use (ANON, 1988, SCANDELLA & LETEINTURIER, 1989). It is necessary to consider the complete product life for the HACCP plan to be successful. For many refrigerated products, factors before and after processing will influence the microbial safety and control at these steps need to be evaluated into the HACCP study. However, control will not always be possible. For example it is not possible to control improper handling of products during distribution or in the homes, but the effect of temperature abuse must be considered.

Table 3

Flow diagram for minimally processed endive from harvest to consumption

1. Pre-harvest practices and harvest (field)
Irrigation and fertilization – harvest – rinsing cut surface – packing – transport
2. Production and processing
Rapid chilling on receipt – storage – preparation and selection – cutting – washing – rinsing – centrifugation – packaging – labelling – secondary packaging – refrigerated storage
3. Distribution and retailing
Refrigerated transport – storage in supermarket – display cabinets
4. Consumer practice and use
Transport – storage – consumption

2.6. Stage 6: Confirmation of the flow diagram

All members of the HACCP team should be involved in the on-site verification of the accuracy and completeness of the flow diagram. The flow diagram is validated throughout all operational periods (e.g. day, night or weekend shift).

2.7. Stage 7: Hazards analysis and preventive measures (principle 1)

From the hazard analysis conducted above, it was concluded that raw vegetables can be an important vehicle in the transmission of psychrotrophic, infectious pathogens like *Listeria monocytogenes*, *Yersinia enterolytica* and *Aeromonas hydrophila*. From the flow diagram (Table 3) it can be seen that there is no microbial inhibition or reduction step in the processing of minimally processed endive. Pathogens, present on the raw vegetables or which contaminate the product during processing, will remain on the finished product. Contamination by personnel, material and equipment, as well as growth of pathogens are considered as hazards.

Upon completion of the hazard analysis, the significant hazards associated with each step in the flow diagram should be listed along with any preventive measures to control them (NACMCF, 1992). Preventive measures are those actions and activities which are required to eliminate hazards or reduce their occurrence to acceptable levels. No attempt is made at this stage to identify critical control points, but the tabulated form provides a basis. In Table 4, preventive measures are listed for the identified hazard in the pre-harvest and harvest steps. Contamination of the raw materials by organic fertilizers or sewage can be reduced if the food processor works with contractors which effectively apply these preventive measures. If possible only synthetic fertilizers should be used.

Table 4

Hazards and preventive measures during the pre-harvest and harvest step

	Process step	Hazard	Preventive measure(s)
1	Irrigation and fertilization	contamination with pathogens	no sewage and organic fertilizers two weeks before harvest determined in contract with supplier
2	Harvest	contamination	cleaning and disinfection of tools education of personnel in proper harvesting techniques
3	Rinsing cut surfaces	contamination	use of drinking-water, no reuse of rinsing water

2.8. Stage 8: Determination of Critical Control Points (principle 2)

A Critical Control Points (CCP) is defined as a point, step or procedure at which control can be applied and a food safety hazard can be prevented, eliminated or reduced to acceptable levels. CCPs in the production of minimally processed endives were identified using a decision tree presented in Fig. 1. All identified hazards (stage 7) must be addressed. Application of the decision tree will determine

whether or not the process step is a CCP for each specific identified hazard. The number of identified CCPs is dependent on the complexity and nature of the product and process under consideration and the scope of the study. There is no limit on the number of CCPs that may be identified in a study. In Table 5 preventive measures and identified CCPs in the production of minimally processed endive for the hazard of contamination and growth of pathogens are given.

Table 5

Identified hazard, preventive measures and determined CCPs in each step of the production of minimally processed endive

	Process step	Hazard	Preventive measures	CCP
1	Chilling	growth	vacuum cooling, moisturing	CCP1
2	Storage	growth	temperature control, stock rotation	CCP2
		contamination	cleaning and disinfection of storage room	CCP3
3	Preparation	growth	time/temperature control	CCP4
		contamination	regular waste removal	CCP5
			hygiene preparation area	
			good personal hygiene and training	
4	Cutting	contamination	cleaning in-place and disinfection	CCP6
5	Washing	growth	temperature control of wash water	CCP7
			chlorination of wash water, no reuse	
6	Rinsing	growth	drinking water, no reuse	CCP8
7	Centrifugation	contamination	cleaning in-place and disinfection	CCP9
			control of centrifugation speed and time	
8	Packaging	contamination	hygiene of packaging machine	CCP10
			integrity test	
9	Labelling			
10	Packaging			
11	Storage	growth	temperature control, stock rotation	CCP11

When the raw vegetables are purchased from local suppliers, the harvested vegetables are transported unrefrigerated to the production plant. Rapid chilling (CCP1) to remove the initial or 'field heat' from freshly harvested vegetables is essential to inhibit growth of pathogenic and spoilage microorganisms, to restrict enzymatic and respiratory activity, to inhibit water loss and to reduce ethylene production by the product. The precooling operation is the first step in good temperature management (HARDENBURG et al., 1990). Vacuum cooling is a standard commercial method of chilling leafy vegetables. Moisture loss during vacuum chilling (ranges from 1.5 to 5 per cent) is reduced by prewetting or misting the vegetables.

Refrigerated storage of both raw materials (CCP2) and finished products (CCP11) are identified as critical control points for growth of pathogens. A single

process step of refrigerated storage may not allow increase of the hazard to unacceptable levels, but over a number of process steps the total increase may reach unacceptable levels. Therefore temperature should also be reduced in the processing area to limit the potential for growth (CCP4). Refrigeration throughout the complete chill chain of the minimally processed vegetables is necessary to prevent the outgrowth of psychrotrophic pathogens (ANON, 1988, SCANDELLA & LETEINTURIER, 1989).

For the preparation of the raw materials (CCP5), removal of the outer leafs is an essential step in reducing the overall contamination (GARG et al., 1990). Removing wilted, slimy or damaged leaves will improve the overall quality, but microorganisms reach even the new leaves at the inner growing tip through surface moisture films on surrounding leaves. Contamination from food handlers can be reduced by good personal hygiene practices and training, including change of protective clothing (e.g. hair covers, gloves) when entering or leaving the area, hand washing, reporting of sickness (ICMSF, 1988, SCANDELLA & LETEINTURIER, 1989). Processing areas should be designed and operated to prevent growth and contamination of pathogens by temperature control and hygienic equipment and constructions design. Separation of storage facilities for raw materials and finished products eliminates contamination. Working surfaces must be both hygienic and technically efficient. Cut or scratched surfaces are impossible to keep in an hygienic condition and are a potent source of contamination. Effective cleaning should remove all food debris so that microorganisms cannot grow and subsequent production is not contaminated. Disinfection procedures should destroy any micoorganisms left on cleaned surfaces (CCP3, CCP6 and CCP9). The efficient removal and disposal of waste from the manufacturing area is essential to maintaining the hygiene level (BROWN & GOULD, 1992). Suitable storage facilities and containers should be provided and the design and operation of waste disposal systems should prevent product contamination.

Cutters and slicers can be potent sources of contamination (CCP6) because they are usually mechanically complex, providing many inaccessible and uncleanable sites which can harbour bacteria (GARG et al., 1990). Moreover, if heat from motors or gearboxes is not conducted away into the machine, localized hot spots are created, providing ideal growth temperatures (BROWN & GOULD, 1992).

Washing (CCP7) only partially removes the microorganisms that are intrinsic to the vegetables (GARG et al., 1990). The efficacy is often improved by the inclusion of anti-microbials in the wash water, including citric acid, sorbate, lactic acid, antibiotics, but the most widely used is a hypochlorite solution containing 50 to 100 ppm available chlorine (ADAMS et al., 1989). The effectiveness of chlorine depends on concentration and contact time, temperature, pH and organic content of the washing water (MAZOLLIER, 1988). Despite the fact that using chlorine in direct

contact with food is not allowed in Belgium, it is nevertheless common practice. Residual chlorine concentrations are reduced by the following rinsing operation.

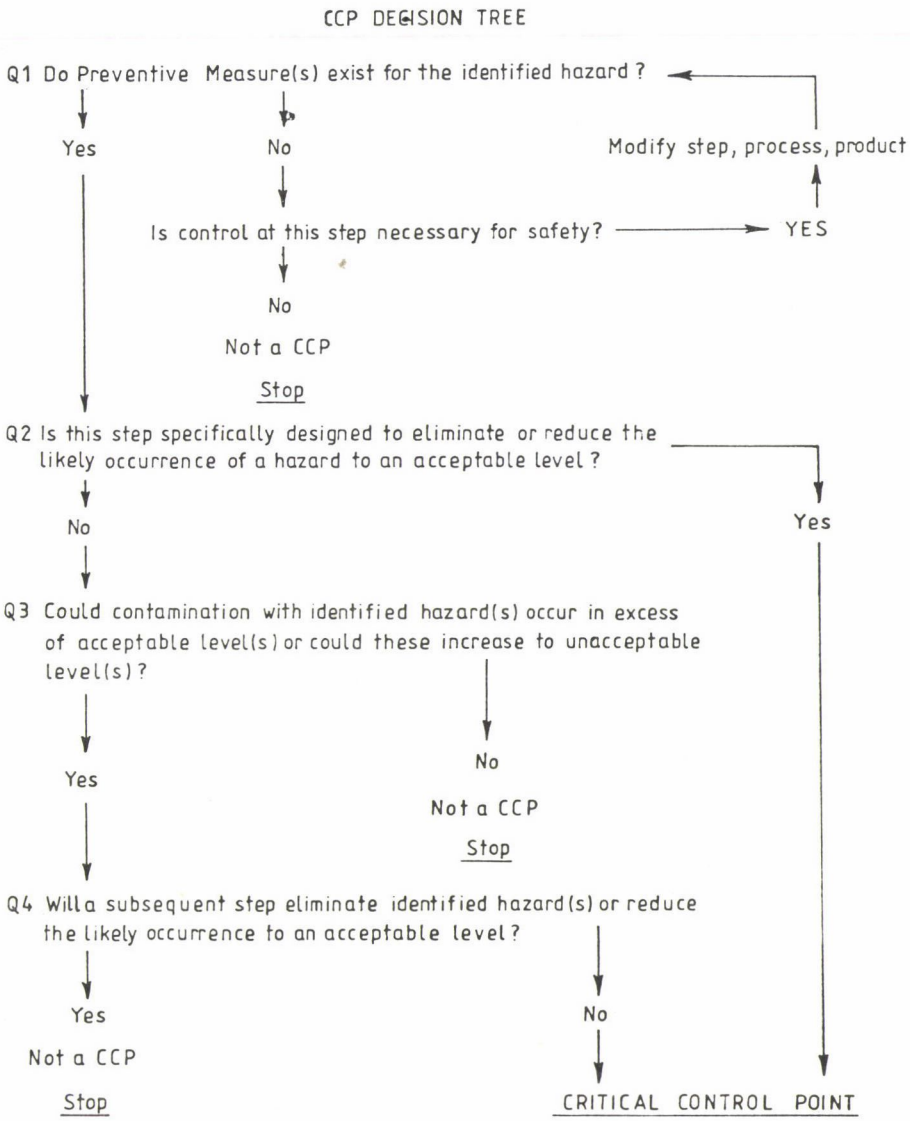

Fig. 1. Decision tree for the determination of the Critical Control Point (CCP) (FLAIR, 1994). Answer each question (Q) in sequence at each step of the process with each identified hazard

Rinsing and drying by centrifugation (CCP8 and CCP9) of the cut vegetables is an important step in extending its storage life. Cellular fluids, released during cutting

are removed by rinsing with water, followed by centrifugation to remove added surface moisture (BOLIN & HUXSOLL, 1991). Only drinking water should be used for washing and rinsing.

Correct permeability characteristics of the packaging film (CCP10) must be chosen to realize full benefits of the modified atmosphere. If an impermeable film is chosen, O_2-concentrations will fall to very low concentrations where anaerobic respiration will be initiated. Anaerobiosis is usually associated with undesirable odours and flavours. In addition, there is a risk of the growth of anaerobic pathogens, such as *Clostridium botulinum* (DAY, 1992). Through gas analysis or visual inspection, seal defects will be detected. Heat sealing remains a delicate operation and a badly sealed pack must be reject (SCANDELLA & LETEINTURIER, 1989).

For the process steps identified as CCP, critical limits must be defined for proper control (*stage 9*). These CCPs must be monitored at a frequency sufficient to ensure process control (*stage 10*) and corrective actions – to be taken when monitoring indicates that a CCP is not under control – must be established in the HACCP plan (*stage 11*).

2.9. Stage 9: Critical limit(s) for each CCP (Principle 3)

A critical limit is defined as a value which separates acceptability from unacceptability. Each CCP will have one or more parameters that must be properly controlled to assure prevention, elimination or reduction of hazards to acceptable limits. Examples of some common measured parameters include temperature, residence time, flow rate, moisture level or water activity, pH and weight. Those critical limits of the parameters serve as boundaries of safety of the CCP. Prior to determining critical limits, all parameters or factors associated with the CCPs must be identified. The second question that needs to be answered is at what point or level would each of these parameters become a safety hazard? To answer this question, information on critical limits can be derived from sources such as regulatory standards and guide-lines, literature surveys, experimental studies and experts.

In Table 6 critical limits for the critical parameters of the CCPs for growth of pathogens are described. For chilled storage of minimally processed vegetables, the product centre temperature is taken as reference for temperature control due to the heat production by respiration of the produce. Although in Belgium, the legal maximum temperature during storage is 7 °C with a tolerance up to 10 °C in the warmest spot, 4 °C was taken as critical limit (ANON, 1988). Remark also that the critical limits for product temperature and residence time of CCP11 are identical to CCP2. Critical limits based on subjective data such as visual inspection must be strengthened by clear specifications of what is acceptable and what is not acceptable. Training will be necessary.

Table 6

Critical limits, monitoring system and corrective action, established for the CCPs for the hazard of growth of pathogens in the production of minimally processed endive

CCP	Critical limits	Monitoring system	Corrective action plan
CCP1	product centre temperature < 4 °C	temperature data logging each batch, two samples	reprocess until product centre temperature reach limit
	cooling time < 30 minutes	automatic registration of fill weight, pressure, and temperature of vacuum chamber	adjust cooling rate of vacuum chamber
CCP2 (CCP11)	product centre temperature < 4 °C	air temperature chart recording check product temperature of each shift	adjust air cooling rate
	storage raw materials maximum 48 h	labelling (bar code) of raw materials continuous stock monitoring	adjust production scheme place product on hold, investigate, and take appropriate action
CCP4	air temperature of preparation room < 12 °C	temperature chart recording visual inspection every 2 h	adjust air cooling rate
	residence time of vegetables < 10 min	supervision	adjust line speed
CCP7	temperature of washing water < 4 °C	continuous temperature monitoring	adjust cooling rate of wash water
	water flow rate > 5 l/kg product	supervision at start of each batch	adjust flow rate
	available chlorine > 100 ppm	laboratory check on chlorine level	adjust chlorine dosage
CCP8	temperature of rinsing water < 4 °C	continuous temperature monitoring	adjust cooling rate of water

2.10. Stage 10: Monitoring system for CCPs (Principle 4)

The monitoring system describes the methods by which management is able to confirm that all CCPs are operating within specification and it also produces an accurate record of performance for future use in verification. Monitoring procedures must be able to detect loss of control at the CCP. Ideally, monitoring should provide this information in time for corrective action to be taken to regain control of the process before there is a need to segregate or reject products. As a consequence, microbiological testing is seldom effective for monitoring CCPs due to their time-consuming nature. In order to establish and effectively conduct monitoring procedures, a plan must be drawn what is to be monitored, how, where and when, and who is responsible.

In Table 6 monitoring systems for the CCPs for the hazard of growth are given. Where possible, continuous, control-based, on-line measurements are used to adjust process conditions in response to deviations of measured parameters from the critical limits. Such measurements are generally possible for temperature, pressure, flow rate and fill rate, although even for these physical parameters there are process environments and food types for which standard instruments cannot be used satisfactorily. In the case of monitoring air temperature of walk-in chill stores for example, it is necessary to use a number of sensors, depending on its size and the number of cooling units, to check the temperature distribution. WILLOCX and co-workers (1994) showed that for display cabinets, temperature differences higher than 5 °C were measured and differences between the actual and read-out temperature up to 10 °C were observed. Positioning of the sensors should give an indication of the warmest air temperature and hence the warmest food in the store or display cabinet.

Monitoring hygiene of personnel and hygienic handling of foods is a difficult task but nevertheless essential for food safety. Educating, training, motivating and observing workers to wash hands after using toilet, after coughing or sneezing in the hand, after handling raw materials, and after hands are otherwise contaminated or soiled will minimize the hazard of contamination (ICMSF, 1988).

2.11. Stage 11: Corrective action plan (Principle 5)

When monitoring indicates that there is a deviation from a critical limit, a specific corrective action plan must be in place to ensure that the CCP has been brought under control. The procedure must also include authorisation and disposition actions for food products that has been produced during the time period that the CCP was 'out of control'. Both corrective action and disposition action should be documented in the HACCP record keeping. In Table 6, (limited) corrective action plans for each CCP are tabulated.

2.12. Stage 12: Establish record keeping and documentation (Principle 7)

Efficient and accurate record keeping is essential to the successful application of HACCP to a food process. This system must include all documentation relating to all CCPs identified: the procedures, modes of operation instructions that are recorded, worked out for the control, piloting, monitoring of the process. Documentation of HACCP procedures at all steps should be assembled and included in a manual and/or integrated into a controlled Quality Management System. Therefore the records must be signed and dated, indexed, suitable for modification and updating and stored for a defined period.

2.13. Stage 13: Verification of the HACCP plan (Principle 6)

The aim of verification is to determine if the HACCP system is in compliance with the plan (quality control) and if the current HACCP plan is appropriate and effective for the product and process under study (quality assurance).

2.14. Stage 14: Review of the HACCP plan (Principle 6)

In addition to the verification system, it is necessary to have a system in place that will automatically lead to a review of the HACCP plan prior to any changes to raw material, product, process, consumer use, etc.

3. Conclusion

Hazard Analysis Critical Control Point is a documented and verifiable approach aimed at food safety assurance. Using the HACCP approach to assure product safety will help to enhance product quality. The emphasis is moved from retrospective quality control to preventive quality assurance. There will however, always be a need for some end product testing particularly for verification purposes.

European Community (EC) directives and Unites States legislation are increasingly including a requirement for HACCP procedures to be adopted by food and drink manufactures. In the new EC Council Directive on the hygiene of foodstuffs [COM(91)525], approved on June 14th 1993, all stages of the lives of foodstuffs are covered and the application of HACCP by manufactures has been specifically referred to give confidence in the safety of products. In the context of product liability, the effective and successful implementation of the HACCP plan can help the food producer to guarantee the safety of food products and to comply with various regulations (local and European), and can also help to defend him when

problems occur. HACCP is a common approach to food safety and will facilitate trade opportunities within and beyond EC.

The successful application of the HACCP principles requires a well defined and consistent methodology. For the microbial safety assurance of minimally processed vegetables (endive), a logical sequence of 14 stages was used. Identified CCPs, critical limits, established monitoring systems and corrective action plans are specific to the product and process under consideration. Furthermore the HACCP plan has to be implemented by the workers and management of the company. As a consequence, there is no unique solution to assure safety of the food product. It must be stressed that this elaborated HACCP plan only serves as an example.

*

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BOOK REVIEW

First Hungarian representative nutrition survey (1985–1988). Results

Vol. 2

GY. BÍRÓ (Ed.)

Medical Officer Head Office, Institute of Health Promotion and National Institute of Food Hygiene and Nutrition, Budapest, 1993, 202 pages

Published by György Bíró, the second volume of the First Hungarian representative nutrition survey appeared at the end of 1993. Already the first volume of the survey's results brought about considerable international response.

The 202 page work – coordinated by the National Institute of Food Hygiene and Nutrition – contains further results of the comprehensive nutrition-epidemiological examination.

Highly valuable and much professional information containing part of the study is the chapter dealing with anthropometric data. The consistent evaluation of the comprehensive anthropologic examination of nearly 17000 adults – representing on a 0.02% level the country's population – performed according similar principles and methods, provides essential data both for nutritionists and clinicians (body mass, body height, Broca index, modified Broca index, optimal body mass, body mass index: BMI, body fat%, Ruffer index, ponderal 1, 2, 3 indexes, Möhr index, arm muscle area etc.). The data are presented according to sex, age groups and domicile, in means and in per cent distributions. The results suggest that a considerable part of the population is overweight.

The chapter of the work examining the frequency of food use gives a detailed picture about the food preferences of the population. It is thought-provoking and might be a major cause of the deficient calcium supply that 20% of women and 25% of men never drink milk or only 1-3 times per month and the distribution of those never or very seldom consuming vegetables is similar.

Results of the comprehensive laboratory examinations are also discussed (hemoglobin, hematocrit, BUN, total protein, albumin/globulin, total cholesterol, HDL-cholesterol, LDL-cholesterol, triglyceride, iron, tibc, copper, zinc, calcium, potassium, magnesium, sodium, ETK, folic acid, wbc ascorbic acid, uric acid). E.g. serum total cholesterol values were in the normal range in 53% of the population, HDL-cholesterol levels were lower than 0.99 and 1.16, respectively, in 15% of the population. Under-the-normal-value folic acid levels were observed in 22% of the examined persons and wbc ascorbic acid levels in 36%.

The survey comprised dietary habits and life styles, too. Forty five percent of the interviewed persons cooked with oil, 43% with fat; 55% (in the country 60%) prepared vegetables with thickening. Thirty one percent of men (in the country 41.7%) drank alcohol daily. One third of the interviewed persons wished to lose weight and 6% wanted to gain weight. Nearly 96% of the women and 55.3% of the men cook regularly, 75-81% of them learned about cooking in the family. These data are also relevant with the very slow changing of dietary habits.

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In the course of clinical examinations, a high percent of the participants had heart complaints (chest pain in rest, chest pain on exercise, dyspnoe). Seventeen percent of men and 37% of women complained about flatulance. Every 10th men and more than one quarter of women presented obstipation.

The discussion of all valuable information well arranged and elaborated according to different aspects, exceeds the possibilities of a book review. The book is an indispensable work of reference for physicians dealing with nutritional research, working in the fields of clinical medicine, prevention or health education and at the same time also for professionals of the food-economy.

M. BARNA

(The book is available in the National Institute of Food Hygiene and Nutrition, 1097, Budapest, Gyáli út 3/a)

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CONTENTS

The competitiveness of Hungarian food industry
LAKNER, Z. & KÓBOR, K. 121
Comparative study on some fermentation properties of *Streptococcus thermophilus* and *Lactobacillus acidophilus* in milk and modified milk media. Part II. Effect of the increased solid not fat on the fermentation properties of the mixed culture
BADRAN, I. I. & REICHART, O. 133
Effect of calcium addition, acidification and fermentation on the quality characteristics of canned carrots (*Daucus carota* L.)
NOGUEIRA, J. N., CANTARELLI, P. R., GALLO, C. R. & MATSUURA, F. C. A. U. 147
Thermal denaturation of bacterial cells examined by differential scanning calorimetry
MOHÁCSI-FARKAS, CS., FARKAS, J. & SIMON, A. 157
Fat content, fatty acid composition and vitamin content of mare's milk
CSAPÓ, J., STEFLER, J., MARTIN, T. G., MAKRAY, S. & CSAPÓ-KISS, ZS. 169
Protein content, amino acid composition, biological value and micro- and macroelement content of mare's milk
CSAPÓ-KISS, ZS., STEFLER, J., MARTIN, T. G., MAKRAY, S. & CSAPÓ, J. 179
Assessment of wholesomeness of irradiated foods (A review)
DIEHL, J. F. & JOSEPHSON, E. S. 195
Use of immobilized lipase in transesterification of triglicerides
TEMESVÁRI, J., BÁNKY, E. & BIACS, P. A. 215
Microbial safety assurance of minimally processed vegetables by implementation of the Hazard Analysis Critical Control Point (HACCP) system
WILLOCX, F., TOBBACK, P. & HENDRICKX, M. 221
Book review 239

ACTA ALIMENTARIA

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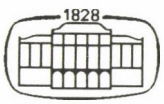

AKADÉMIAI KIADÓ
BUDAPEST

Acta Alimentaria, Vol. 23 (3), pp. 241 – 244 (1994)

In Appreciation to Professor János Holló (Member of the Hungarian Academy of Sciences) on the occasion of his 75th birthday

It is our pleasant and most rewarding duty to extend respectful and warmest congratulations to professor János Holló on the occasion of the 75th anniversary of his birthday (20 August). Generations have received education in food industry under his guidance and, as a true friend, he followed with great interest the activities of his students in the course of their further career, as well. We all feel grateful that – in the best of health – he is still actively participating in activities in food science and greatly contributing with unsparing efforts, to the international acknowledgement of our scientific results.

We owe grateful thanks to him for the foundation of MITE (legal predecessor of our Scientific Society) and his valuable contribution in starting publication of the "Agriculture and Industry", predecessor of our current scientific journal, the Élelmezési Ipar.

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It may be stated, without exaggeration, that our Society has made some contribution to the development of the Hungarian food industry through the foundation and coordination of work as well as development of a sense of vocation among experts in the field. This allowed the food industry to further develop throughout decades and – in spite of unworthy intrigues – to attain world recognition in various branches of the industry.

As founder and first General Secretary, then President, and currently Honorary President of the Society, Professor Holló has worked with unfailing energy to raise the level of scientific achievement as he was well aware that this can be attained only through the joint efforts of well qualified experts. With this in view, as professor of the Budapest Technical University, he actively took part for many decades in the training of young experts, orienting their interests towards the food industry.

Various activities conducted on the level of a handicraft have developed into a manufacturing industry of international standards.

We still have contemporaries, who have devoted – often at the expense of their health – the best of their knowledge to the development of the industry. In addition to science, organization of work was often the driving force integrating central decisions on industrial development and scientific social activities. It may also be of interest to mention that at the time of rapidly developing industrialization, when Professor Holló was offered the post of Deputy Minister of Food and Agriculture, he has chosen to further pursue his true vocation: professor of university, offering him lower material assets but a highly rewarding opportunity to exert an enduring effect of further development in the field.

As many of his associates and former students are still active, I wish to take this opportunity to introduce Professor János Holló to members of the new generation.

After obtaining his degree in chemical engineering from the József Nádor University of Technical and Economic Sciences, Budapest, in 1941 and the Dr.-ing. degree in 1947, he worked through 1941-46 as production and research engineer for various factories of the chemical industry. From 1946-48 he was plant supervisor and head of laboratory, and from 1948-52 technical director of the Kőbánya Brewery. These years in the brewing industry had been greatly stimulating to him and exerted a lasting effect on his further career as well. Even today, he considers himself – with pride – to be a brewery engineer.

He was appointed professor and held the post of Head of the Department of Agricultural Chemical Technology of the Budapest Technical University from 1952-91. He was elected Dean of the Faculty of Chemical Engineering for the academic terms of 1955-57 and 1963-72. In 1972 he was offered the post of scientific director of

the Central Research Institute for Chemistry of the Hungarian Academy of Sciences and is participating in activities there as research professor up to this day.

In 1967 he was elected corresponding member and in 1976 full member of the Hungarian Academy of Sciences.

His scientific activities covered various fields. Just to mention a few, his doctoral thesis: "Comparison of the liquefying and saccharifying diastatic effect of barley germinated at various temperatures"; biological and food industrial technologies; physical and chemical properties of raw materials; industrial enzymes; biological and physico-chemical waste water treatment, etc. The high level work of his own trainees has greatly contributed to the excellent results attained.

In the course of his scientific career, numerous patents have been filled. In 1964, 1965, 1969: the Vepex procedure; in 1964: the enzymatic and in 1981 the high spec. gravity brewing technology; in 1973: production of iso-syrup, etc. In his capacity as university professor, he developed and introduced in 1952 pilot plant training and production planning techniques in the education of chemical engineers.

His activities have raised worldwide interest in international scientific circles, as well. He was elected honorary member of the Polish Scientific Association as well as the Finnish Academy of Technical Sciences and the Academy of Sciences of the GDR, nominated honorary doctor of the Universities of Vienna, Berlin-Charlottenburg, the Budapest Technical University and the University of Horticulture and Food Industry. He served as Chairman, then Vice-Chairman of the International Committee of Agricultural and Food Industries, was elected President and is now Honorary President. For two cycles he acted as President of the International Society of Fat Science, for 30 years of the Committee of Agriculture and Food Products and of the Sub-Committee for Cereals and Legumes of the ISO. He serves on the Board of the Biotechnological and Fat Sciences Committee of IUPAC, is founding member of the European Federation of Biotechnology, President of the Complex Committee of Food Science of the Hungarian Academy of Sciences.

In 1975 he was awarded the Hungarian State Prize (1st degree) and is recipient of numerous foreign distinctions and prizes as well, e.g. Chevalier de l'Ordre des Palmes Académiques, etc.

His scientific results have been recorded in various national and international journals: his number of publications is over 600, he is author and co-author of over 20 books, among them several university text-books.

He is Editor-in-Chief of the Acta Alimentaria, serves as Chairman on the Editorial Board of "Élelmiszervizsgálati Közlemények" and member of the Editorial Board of various other journals: Kémiai Közlemények, the Journal of Applied Microbiology and Biotechnology, the Food/Die Nahrung, the Starch/Die Stärke, the Industries Agricoles Alimentaires, the Biotechnological Equipment, etc.

It would be difficult to enumerate all distinctions and honors awarded to him in appreciation of his activities. Continuous reference has been made to his publications, for instance, his book entitled "Applications of Molecular Distillation", published in English by Akadémiai Kiadó in 1971 was recently referred to by Dean Legall, Dep. President of Pope Scientific Inc. (Wisconsin, USA), with the following comment: "I haven't ever since seen a study comprising all necessary up-to-date information, as compiled in this monograph."

In preparing for this article, I have asked Professor Holló what he would offer to the younger scientific generation as useful advice for successful results in their activities. His answer was: "Never feel bored, never spend time on useless activities, seek for new challenges, never shrink from difficulties and preserve – under all circumstances – human dignity and honesty."

I. TÓTH-ZSIGA

Acta Alimentaria, Vol. 23 (3), pp. 245–255 (1994)

PHASE EQUILIBRIA AND THERMODYNAMIC INCOMPATIBILITY IN PROTEIN CONTAINING SYSTEM

PART I. INVESTIGATION OF CASEIN – POLYSACCHARIDE – WATER SYSTEMS[a]

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Phase equilibria and thermodynamic incompatibility of protein–polysaccharide–water model systems were investigated. Casein, pectin and dextran were the biopolymers used in experiments. Under experimental conditions (temperature: 10–25 °C, concentration of biopolymers 0–10%, pH 6.0–7.8) in every ternary system phase separation – indicating incompatibility – was observed. Temperature and ion concentration were the factors most affecting the incompatibility.

It is suggested that in interaction the role of hydrophilic–hydrophobic equilibria is much greater than ionic (electrostatic) interactions.

Keywords: biopolymers, protein systems, phase equilibrium, thermodynamic incompatibility, protein–polysaccharide interaction

From point of view of colloid chemistry the majority of foods is a multicomplex colloidal system, where in the continuous phase of water different biopolymers are dissolved (dispersed). The most typical biopolymers occurring in the foods are proteins and polysaccharides.

Under given conditions the system is in a dynamic equilibrium as a result of interactions of the components. Any change in the conditions may cause changes in the system leading to a new equilibrium. The direction and the rate of changes depend on the summarized effect of interactions and on the energetical conditions.

In some cases a phase-separation of the originally homogeneous system may occur. The solution mixtures of biopolymers undergo liquid-phase separation, with the macromolecular components concentrating in different phases. This phenomenon is known and in the scientific literature is named thermodynamic incompatibility (TOLSTOGUZOV, 1988, 1993; SEMENOVA et al., 1990). Although the

[a] The research project is financed by OTKA (Hungarian Scientific Research Found)

research on problems of thermodynamic compatibility started about one hundred years ago, the investigation of food systems from this point of view is relatively new. Nevertheless the first results suggest that a better knowledge of food systems from this point of view will be useful for the processing technology and may prevent undesired physical, physicochemical changes (TOLSTOGUZOV, 1988).

As in many fields basic research is starting with less complicated model systems. Although the obtained results are of limited value, nevertheless they should give some information about general tendencies. If a water solution of polymer X (concentration X_k) and a solution of polymer Y (concentration Y_k) is mixed in a given ratio, the parameters of the mixture may be characterized by coordinates of point K (Fig. 1).

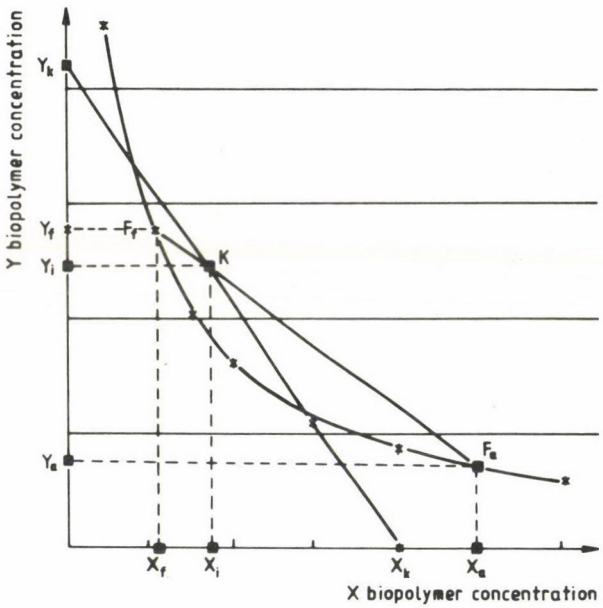

Fig. 1. The phase diagram of the ternary system biopolymer X – biopolymer Y – water. (X_k) X biopolymer solution. (Y_k) Y biopolymer solution. (K) mixture of solutions X and Y. (F_a) lower and (F_f) upper phases. *: The points of binodal

If the two polymers (X and Y) are thermodynamically incompatible after a given time a phase separation will occur and a lower (F_a) and an upper (F_f) phase will be formed (POLYAKOV et al., 1985). Generally one of the phases contain higher concentration of X and the other higher amount of polymer Y.

In our case the concentration of X is higher (higher than X_i corresponding to the homogeneous mixture) and is also higher than X_k. In the upper phase polymer Y is concentrated.

The phenomenon mentioned above gives a possibility to concentrate a biopolymer without any drastic treatment purely with an appropriate selection of two polymers and their concentration (e.g. separation of enzymes from fermentation liquid).

Such process may be named membrane less osmosis (TOLSTOGUZOV, 1988, TOLSTOGUZOV et al., 1985), while the water transport from one phase to the other one is continuous until the thermodynamic equilibrium reached.

The series of points representing F_a and F_f phases determine the phase-equilibrium curve characterizing the incompatibility and this curve is generally hyperbolic.

The area under the curve represents concentrations at which – under given thermodynamic parameters – the biopolymers form homogeneous mixture.

If the concentrations correspond to a value in the upper area the separation of the mixture to two (or more) phases will occur. The measure of the incompatibility may be characterized with the parameters of the phase-equilibrium curve with the area under the curve.

In the experiments presented in this paper protein–polysaccharide–water model systems were studied under varying conditions (temperature, ionic concentration, pH).

1. Materials and methods

Casein was used as protein and four different polysaccharides (apple pectin with 35% methylation, apple pectin with 70% methylation, dextran 500 KD, dextran 2000 KD). Main characteristics of materials are summarized in Tables 1 and 2.

A Britton-Robinson buffer solution was used for adjusting the desired pH-values. The ionic concentration was changed by addition of sodium-chloride solution.

The casein was dissolved in the buffer in concentration range from 3 to 10% (w/v) and the polysaccharides in concentration range from 0.25 to 5.0%.

The standard temperature was maintained by water bath thermostat (±0.2 °C). The separation of phases was followed visually until further changes were not observed. In some cases the phase separation was helped by centrifugation (15 min, 1400 g).

After phase separation a sample was taken by micropipette from both phases. After dilution (1:1000) the protein concentration of solutions was measured by Lowry method and those of polysaccharides by phenol–sulfuric acid method (DUBOIS et al., 1956).

Table 1

Characteristics of experimental materials

Name	Sign	Average molweight	Producer
Casein		35 000	Reanal
Apple pectin with 35% methylation	p (35)	150 000	Herbstreith & Fox
Apple pectin with 70% methylation	p (70)	150 000	Herbstreith & Fox
Dextran	d (500)	500 000	Sigma Chem. Co.
Dextran	d (2M)	2000 000	Sigma Chem. Co.

Table 2

Chemical composition of casein and pectin samples

Component (%)	Casein	Pectin Methylation degree	
		35%	70%
Nitrogen content	15.6	0.18	0.19
Moisture	6.0	9.8	10.2
Ash	2.1	1.8	1.7
Fat	0.2	–	–
Free acid	0.2	–	–
Lactose	0.5	–	–

2. Results and discussion

The effect of temperature on the phase-equilibria of casein – apple pectin with 35% methylation-water is shown in Fig. 2.

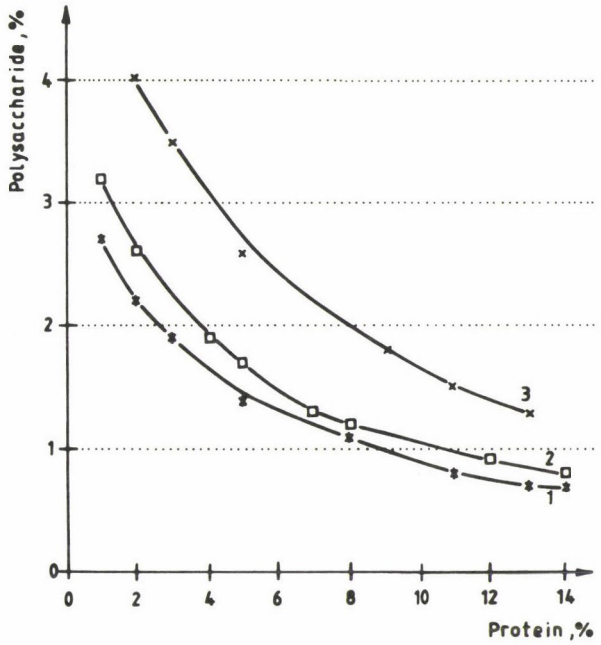

Fig. 2. Temperature dependence of incompatibility of casein – apple pectin – water system. (pH 7.8, 0.085 mol l^{-1} NaCl). 1 = 10 °C, 2 = 18 °C, 3 = 25 °C

As it is seen the increase of temperature caused a significant decrease in the incompatibility of the system. To explain such changes first at all the structure of casein in the solution should be studied. According to the general view – summarized recently in the excellent review of HOLT (1992) – in solution casein micelles are present. The micelles of casein are composed of different subunits and have more or less spherical shape.

The surface of the micelles is not smooth but has a "hairy" outer layer.

The majority of experimental data confirms the view that the surface contains κ-casein subunits having a macroglycopeptide part with hydrophylic properties.

The outer hydrophylic layer results in sterically stabilized particles in aqueous media. When two sterically stabilized particles approach one another, entropic and enthalpic interactions between the lyophylic particles lead to a repulsion that is sufficiently strong to overcome the dispersion forces of attraction. Although the

casein micelles have a high stability in aqueous solutions, changes of temperature may cause partial dissociation of κ-casein subunits.

Due to the presence of acidic carbohydrate components, phosphoric acid and dissociable amino acid side chains, changes of pH and ionic concentration may affect the surface charge and surface properties. The polysaccharide components of the model system are typical hydrophylic molecules. In addition, pectin – depending on the degree of methylation – may have weak ionic character and through methyl groups some hydrophobicity.

Thermodynamic incompatibility of two polymers in common solvent occurs at least at one of the two conditions: (1) the polymer interaction parameter I_{24} is substancially positive; (2) the difference between the interaction parameters of each polymers in the solvent (I_{12} and I_{14}) is not too small. Thus the change of incompatibility may be connected with changes of I_{24} or the difference between I_{12} and I_{14}. The necessary condition of the full stability of the ternary system may be expressed by the following equation:

$$\frac{(A_{12}{}^{*}A_{14})}{A_{24}{}^{2}} \geq 1$$

where A_{12} and A_{14} are the components of the second virial coefficient of the solution which characterize polymer-solvent interactions, and A_{24} is the component of the second virial coefficient which characterizes polymer-polymer interactions (CVETKOV et al., 1964).

The observed lower incompatibility is probably a result of more factors. One of them may be the excluded volume effect depending on the ratio of entropic and enthalpic effects and also on the temperature.

In addition, weakening of hydrogen bonds – playing a role in casein–polysaccharide interaction – with the increase of temperature and the more intensive Brownian-motion with hindrance of phase separation may also play a significant role.

Experiments made with other casein–polysaccharide mixtures showed the same tendencies. The effect of sodiumchloride was opposite than that of the temperature (Fig. 3).

It was observed that even addition of small quantities of salt results in an increase of incompatibility. The polysaccharide content of the lower phase is decreasing and asymptotically reaches a constant low level.

It seems that over a given ionic concentration the polysaccharide content of the protein-rich phase remains constant. Investigating the behavior of other polysaccharides in such systems we observed the same phenomenon. Over given ionic concentration the polysaccharide content of protein-rich phase is constant and does not depend on ionic concentration.

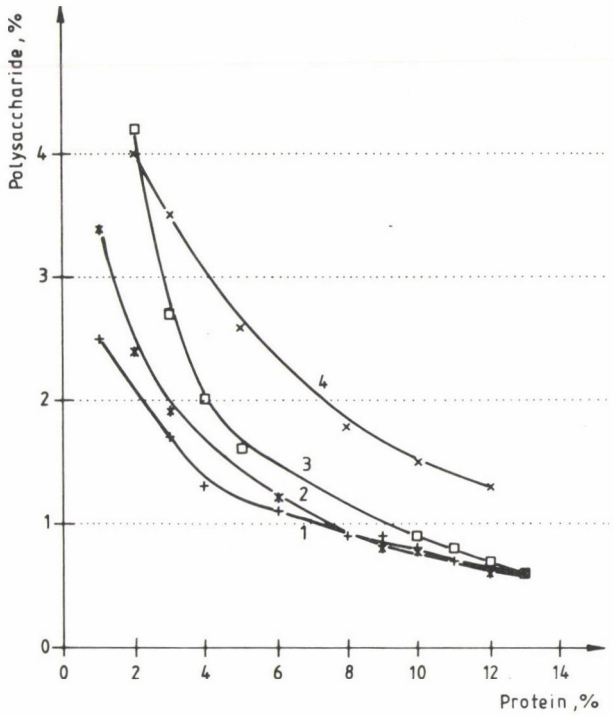

Fig. 3. Dependence of incompatibility of casein – apple pectin – water system (pH 7.8, 25 °C) on ion concentration. 1 = 0.340 mol l^{-1}, 2 = 0.255 mol l^{-1}, 3 = 0.170 mol l^{-1}, 4 = 0.085 mol l^{-1}

These results may be explained first of all with the changes of the structure of casein micelles. As it is known the acid precipitated casein has a much lower mineral (ion) content than the rennet casein. Addition of sodium chloride causes an interaction of ions with casein components leading to partial dissociation of micelles and changes of their surface.

As a result the interaction parameters of casein with water are changing and the difference between the interaction parameters of two polymers with solvent will increase, supposing that pectin – water interaction will not be significantly influenced by the presence of sodium chloride. The constant polysaccharide concentration of the protein-rich phase over a given ion concentration may be explained with a "saturation" of casein with sodium ions.

Out of the data on the effects of pH changes on the incompatibility as example results for the casein – pectin – water ternary system are shown in Fig. 4.

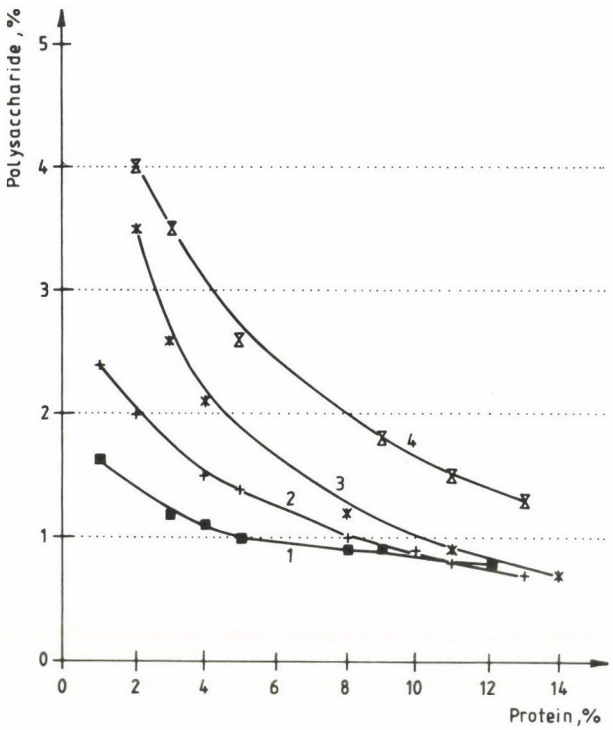

Fig. 4. Dependence of incompatibility of casein – apple pectin – water system (25 °C) on pH. 1 = pH 5, 2 = pH 6, 3 = pH 7, 4 = pH 7.8

As it can be seen in Fig. 4 shifting of the pH to acidic region caused an increase of incompatibility. On the contrary higher pH-values are connected with lower incompatibility. These observations may be explained with the changes of charges of polysaccharide and protein. Both polymers have ionizable groups. Being in a pH region over the isoelectric point of casein and taking the carboxylic groups of pectin in mind, the increase of pH causes growing electrostatic repulsion and an increase of incompatibility.

To investigate the phase-equilibrium in conditions when the polysaccharide has no dissociating groups, a casein – dextran – water system was studied and compared with casein – pectin – water system under the same conditions. The results are summarized in Fig. 5.

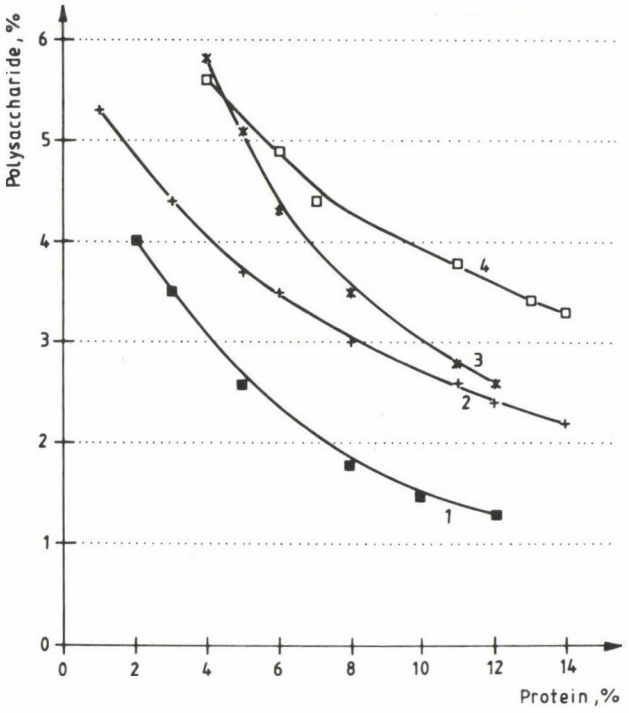

Fig. 5. Incompatibility of the casein – polysaccharide – water ternary systems at constant (0.085 mol l^{-1}) ion concentration. (pH 7.8, 25 °C). (Designations in figure are defined in Table 1.) 1 = p (35), 2 = p (70), 3 = d (500), 4 = 2 (2M)

The results suggest that not only the surface charge of the molecules (differences between two pectins being methylated to an extent of 35% and 70%), but also the molecular size of polymers may have an effect.

It is interesting that at another pH-value the differences between two pectins and two dextrans practically diminished. This may be explained that at this pH-value the role of charges is not significant or that the higher possibility of hydrophobic interactions due to the higher methylation are compensated with charge changes occurring during shifting the pH of solution from 7.8 to 6.0 (Fig. 6).

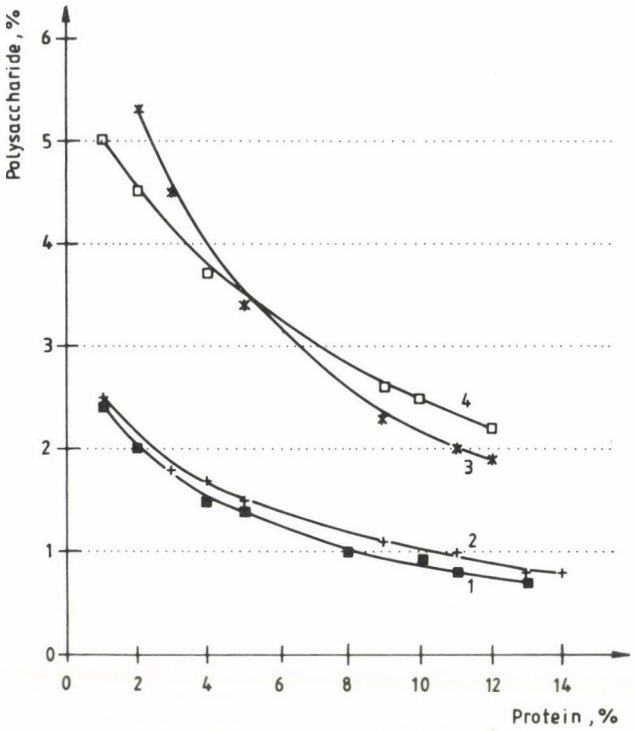

Fig. 6. Incompatibility of the casein – polysaccharide – water ternary systems at constant (pH 6) pH value. (Ion concentration 0.170 mol l^{-1}, 25 °C.) (Designations in figure are defined in Table 1.) 1 = p (35), 2 = p (70), 3 = d (500), 4 = d (2M)

In every case it is clear that the changes in compatibility could not be explained solely with surface charge, but these are of complex nature where the non-Coulombic interactions play a significant role.

This suggestion is supported by the experiments of BRAUDO and ANTONOV (1993), too. The influence of the shape and size of molecules particularly that of proteins is also important as it was shown in experiments with systems containing bovin serum albumin (BSA) and different polysaccharides (HARDING et al., 1993).

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DETERMINATION OF TRYPTOPHAN AND METHIONINE BY MERCAPTOETHANESULFONIC ACID HYDROLYSIS AT ELEVATED TEMPERATURE

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Three mol l^{-1} mercaptoethanesulfonic acid (MES–OH) was used for the hydrolysis of different samples (pure proteins, free tryptophan and milk powder with high milk sugar content). Different temperatures (160, 170 and 180 °C) and time periods (15–90 min) were compared under standard conditions to minimise side reactions during 3 mol l^{-1} mercaptoethanesulfonic acid hydrolysis for the best recovery of the amino acids (especially tryptophan and methionine). The following materials were used for testing the hydrolysis methods: Bovine ribonuclease, lysozyme, citochrom C, free tryptophan and mare's milk powder. The hydrolysis at high temperature was successfully applied for the amino acid analysis of milk powder with high contents of carbohydrate and pure proteins. It is stated that the higher temperature and the shorter time give results which are very similar to the original method (125 °C/24 h). In some cases, such as tryptophan and methionine determination at 160–170 °C for 15–30 min, the results were better than those obtained by the original method. A disadvantage of MES–OH hydrolysis method is that it reduces cystine to cysteine, which coelutes with proline from the ion exchange column and it may interfere with the determination of proline in high-cystine proteins.

Keywords: protein hydrolysis, mercaptoethanesulfonic acid, high temperature, methionine and tryptophan determination

The problems associated with rapid and accurate determination of the tryptophan content in polypeptides, food and feed proteins have not yet been perfectly resolved. Tryptophan can be determined indirectly in intact proteins by spectrophotometric methods if the quantity of tyrosine is known. The tryptophan content can also be determined directly after basic hydrolysis of proteins, (OELSHLEGEL et al., 1970), but this method is time consuming. The hydrolysate is not suitable for total amino acid analysis because some amino acids (especially

0139-3006/94/$ 4.00

arginine) are damaged under the conditions of hydrolysis. During acid hydrolysis of proteins, tryptophan is partly or, in the presence of carbohydrates, totally decomposed.

In the last twenty years, attempts have been made to eliminate the destruction of tryptophan residues. MATSUBARA and SASAKI (1969) performed the hydrolysis of proteins with 6 mol l^{-1} HCl containing 0.5–5% thioglicolic acid, LIU and CHANG (1971) used 3 mol l^{-1} p-toluenesulfonic acid containing 0.2% tryptamine, while PENKE and co-workers (1974) and CSAPÓ and co-workers (1986b) used 3 mol l^{-1} mercaptoethanesulfonic acid for mild acid hydrolysis of peptides and proteins in order to increase precision of determination of tryptophan.

Recently, some hydrolysis methods were developed at elevated temperature (CHIOU & WANG, 1988) and by means of microwave technology (CHEN et al., 1987, 1989; PICKERING & NEWTON, 1992). By the help of microwave irradiation the temperature in the hydrolysis vessels is higher than 150 °C, and this offers the advantage of shortening the hydrolysis time from 24–27 h to 15–45 min. In previous reports, the authors reported use of a mixed acid solvent of hydrochloric acid and trifluoracetic acid or hydrochloric acid and propionic acid (CHIOU & WANG, 1988) at temperatures higher than 150 °C. In this report, 3 mol l^{-1} mercaptoethanesulfonic acid at 160, 170 or 180 °C was used for amino acid analysis of pure proteins, free tryptophan and milk powder with high milk sugar content. Both different temperatures and different hydrolysis times were compared under standard conditions. The aims were to minimise side reactions during 3 mol l^{-1} mercaptoethanesulfonic acid hydrolysis for efficient recovery of the single amino acids (especially tryptophan and methionine), and to find a rational hydrolysis method for amino acid analysis of food and feed samples. The final objective was to identify a new hydrolysis method that enables accurate and very rapid hydrolysis of proteins.

1. Materials and methods

1.1. Hydrolysis and processing of the hydrolysate

Pyrex reusable hydrolysis tubes having 8 mm i.d. (Pierce Chemical Company, Rockford, IL, USA) were used for hydrolysis of proteins or for treating free tryptophan. The tubes can contain up to 8 cm^3 of hydrolysing agent without contact with the PTFE (polytetrafluoroethylene) sealing cup. In each case, 1 cm^3 of 3 mol l^{-1} mercaptoethanesulfonic acid (MES–OH) was added to the tubes for the preparation of protein and peptide hydrolysate. Each tube had two PTFE sealing caps to get complete leak-free operation during heating at 160, 170 or 180 °C.

One mg peptide, protein or free tryptophan or 20 mg fat-free milk powder was weighed into Pyrex tubes previously washed with hydrochloric acid and deionised water. One ml 3 mol l^{-1} MES-OH was added to each sample (MES-OH was obtained from Pierce Chemical Company, Rockford IL, USA) and nitrogen was bubbled for five minutes through the hydrolysing agent by glass capillary. After bubbling with nitrogen, the Pyrex tubes were immediately closed, and put into the heating oven at 160 °C for 15, 30, 45, 60 or 90 min, at 170 °C for 30, 45, 60 or 90 min or at 180 °C for 15, 30, 45, 60 or 90 min. One sample of each examined material was hydrolysed at 110 °C for 24 h, according to the method of MOORE and STEIN (1951), with 6 mol l^{-1} HCl, and one sample by 3 mol l^{-1} MES-OH at 125 °C for 24 h as suggested by LIU and CHANG (1971). After hydrolysis, the tubes were cooled at room temperature and pH of the samples was set to 2.2 with 4 mol l^{-1} NaOH. During neutralisation, the temperature was held below 30 °C with the help of a sodium chloride-ice mixture. After dilution with citrate buffer (pH = 2.2), the hydrolysates were filtered and applied to the automatic amino acid analyser.

1.2. Materials tested

The following materials were used for testing the hydrolysis methods: Bovine ribonuclease, lysozime and citochrom C, free tryptophan and mare's milk powder. The protein content of the milk powder (22.7%) was determined using a Kjel-Foss 16200 (Foss Electric, Denmark) rapid nitrogen analyser. The protein content was calculated from the nitrogen % using a conversion factor of 6.38.

1.3. Amino acid analysis

The peptides and proteins were hydrolysed at different temperatures for different times, and the amino acid contents of the hydrolysates were determined by an LKB 4101 type (LKB Biochrom, England) automatic amino acid analyser using Merck (Merck, Germany) amino acid calibration standard. Otherwise the analyses were carried out as described by CSAPÓ and co-workers (1986a).

2. Results and discussion

2.1. Amino acid composition of bovine ribonuclease

Bovine ribonuclease was hydrolysed by 6 mol l^{-1} HCl at 110 °C for 24 h, 3 mol l^{-1} MES-OH at 125 °C for 24 h and 3 mol l^{-1} MES-OH at 160, 170 and 180 °C for different (15-90 min) times. The amino acid compositions of ribonuclease after the two 24 h hydrolyses and elevated temperatures for shorter times are in

Tables 1 and 2. The results in Table 1 show that the conventionally used 6 mol l^{-1} HCl at 110 °C and the MES – OH hydrolysis method at 125 °C gave very similar results, except in the case of the two very sensitive amino acids (threonine and methionine). For these amino acids, the 3 mol l^{-1} MES – OH method gave higher results than the 6 mol l^{-1} HCl method. Using the mercaptoethanesulfonic acid hydrolysis method, it was found that the cystine content of the sample was reduced to cysteine or the free SH-group of cysteine which can react with that of mercaptoethanesulfonic acid, and resulted in the formation of a small amount of 2-amino-3-(2-sulfoethyl-thio)propionic acid. A further finding was that, using mercaptoethanesulfonic acid hydrolysis, the cysteine (formed from the cystine) appears in the chromatogram in the place of proline and, particularly in the case of proteins of high cystine content, this results defective determinations.

Table 1

Amino acid composition of bovine ribonuclease hydrolysed by MES – OH at 160 °C for different times

Amino acids	Theoretical value	110 °C/24 h 6 mol l^{-1} HCl	125 °C/24 h 3 mol l^{-1} MES – OH	160 °C for 15 min	30 min	45 min	60 min	90 min
Asp	12.54	12.40	13.03	15.16	14.32	13.35	13.46	12.83
Thr	7.48	7.19	7.63	8.15	8.11	7.65	6.64	7.46
Ser	9.90	9.73	10.13	9.76	9.81	9.99	10.16	9.91
Glu	11.11	12.18	12.30	12.73	11.84	11.38	11.32	11.10
Pro	2.89	2.96	5.00	4.43	4.53	4.70	5.03	5.22
Gly	1.41	1.64	1.49	2.37	2.22	2.06	1.70	1.70
Ala	6.71	6.92	6.92	7.81	7.84	7.27	7.29	7.08
Cys	6.08	5.15	—	4.25	4.02	3.89	2.22	1.42
Val	6.62	6.66	6.82	4.10	4.32	5.24	6.02	6.78
Met	3.75	3.26	3.86	3.87	3.85	3.92	3.95	3.86
Ile	2.47	2.52	2.54	0.84	1.71	2.15	2.42	2.56
Leu	1.65	1.75	1.68	1.37	1.54	1.66	1.70	1.68
Tyr	6.83	6.78	7.02	5.84	6.32	6.51	6.69	6.98
Phe	3.11	3.36	3.42	2.69	2.93	3.05	3.15	3.34
Lys	9.18	9.18	9.54	9.67	9.72	9.81	9.60	9.45
His	3.90	3.83	4.05	2.87	2.95	3.17	3.23	4.13
Arg	4.37	4.49	4.57	4.09	4.06	4.19	4.42	4.50

Data are expressed as gram amino acid per 100 gram protein. The values in the Table 1 represent the means of triplicate determinations. Hydrolysis conditions were 3 mol l^{-1} mercaptoethanesulfonic acid 125 °C/24 h and 160 °C for different times using Pyrex No. 9826 tubes. The theoretical values of different amino acids were calculated from the protein sequence.

Table 2

Amino acid composition of bovine ribonuclease hydrolysed by MES – OH at 170 and 180 °C for different times

Amino acids	170 °C for				180 °C for			
	30	45	60	90	15	30	45	60
	min				min			
Asp	13.44	13.40	12.68	13.04	13.73	13.28	12.93	12.96
Thr	7.69	7.70	7.55	7.68	7.77	7.50	7.52	7.53
Ser	10.03	10.04	9.84	7.94	10.16	9.94	9.94	9.81
Glu	11.27	11.21	10.88	11.32	11.70	11.46	11.61	11.81
Pro	4.73	5.99	6.14	6.55	5.93	5.94	6.08	6.35
Gly	2.06	1.70	1.71	1.76	2.11	1.70	1.64	1.67
Ala	7.27	7.13	6.99	7.08	7.42	6.99	6.88	6.93
Cys	3.87	2.20	1.44	0.91	2.40	1.99	0.84	0.15
Val	5.22	5.89	6.63	6.89	4.51	5.72	6.64	6.78
Met	3.93	3.92	3.80	3.66	4.04	3.70	3.73	3.61
Ile	2.16	2.41	2.52	2.62	1.35	2.21	2.51	2.58
Leu	1.64	1.66	1.64	1.71	1.64	1.62	1.67	1.68
Tyr	6.47	6.70	6.93	6.98	6.63	6.86	6.87	6.85
Phe	3.05	3.12	3.32	3.46	3.11	3.09	3.16	3.24
Lys	9.80	9.37	9.45	9.72	9.99	9.47	9.28	9.31
His	3.17	3.23	4.03	4.11	3.22	4.13	4.13	4.17
Arg	4.20	4.33	4.45	4.58	4.29	4.40	4.57	4.57

As can be seen from the results of Table 1, 45 min was not sufficient time to hydrolyse all of the peptide bonds, and shorter hydrolysis times yielded incomplete recoveries of several amino acids (valine, leucine, isoleucine), and 60 min at 160 °C was not sufficient time for total cleavage of the peptide bonds adjacent to valine. The results of hydrolysis at 160 °C for 60 min was very similar (except valine) to those of conventionally used hydrolysis methods. Recoveries of valine were comparable to 100% only at 160 °C for 90 min. At 170 °C and 180 °C, respectively, 60 and 45 min were needed for total hydrolysis of peptide bonds. The higher temperatures did not yield decreased amounts of threonine and methionine.

In summary it can be stated that the optimum hydrolysis times, for all amino acids, were 90, 60 and 45 min, respectively, at 160, 170 and 180 °C. At shorter times, the recovery of valine and isoleucine was much lower, and the recovery of threonine and methionine did not change.

2.2. Recovery of free tryptophan

In Table 3, recoveries of free tryptophan as percentages, after treatment by 3 mol l^{-1} MES – OH at various temperatures for various times are shown. The

tryptophan totally decomposes during acidic hydrolysis, but 93.2% of tryptophan remained unchanged after 125 °C/24 h hydrolysis. This is very similar to the results which were obtained by the 160 °C/90 min, 170 °C/60 min and 180 °C/45 min hydrolysis methods. With increasing temperature and hydrolysis time, decomposition of free tryptophan increased. However, at the highest temperature and longest hydrolysis time (180 °C/60 min) 91.2% of free tryptophan remain unchanged. Accordingly, decomposition rate of tryptophan at lower temperature and longer time is very similar to that at higher temperature and shorter time.

Table 3

Percentage recovery of free tryptophan

Hydrolysis	110 °C/24 h 6 mol l^{-1} HCl	125 °C/24 h 3 mol l^{-1} MES–OH	160 °C for 15 min	30 min	45 min	60 min	90 min
Recovery of tryptophan (3 runs)	–	93.2	99.8	98.3	96.4	95.2	93.1

Hydrolysis	170 °C for 30 min	45 min	60 min	90 min	180 °C for 15 min	30 min	45 min	60 min
Recovery of tryptophan (3 runs)	96.2	95.3	94.8	92.1	94.3	94.1	93.1	91.2

2.3. Analysis of tryptophan containing pure proteins

The lysozyme and citochrom C were hydrolysed with 3 mol l^{-1} MES–OH at various temperatures and for various times. The results of these amino acid analyses are given in Tables 4 and 5. In the higher tryptophan containing lysozyme, recovery of tryptophan was higher than that found for citochrom C with lower tryptophan content. Similar to the results on recovery of free tryptophan, it can be stated that, with increasing temperature and hydrolysis time, the decomposition of tryptophan will be higher. It seems that the optimum hydrolysis method for tryptophan determination would be a shorter time and lower temperature such as 160 °C/45 min or 170 °C/30 min. Recovery of tryptophan from citochrom C at 180 °C/15 min was only 89.0%. When choosing optimum hydrolysis time and temperature, one must consider that the lower temperature and the shorter hydrolysis time is not sufficient to cleave the peptide bonds between hydrophobic amino acids.

Table 4

Tryptophan contents of lysozyme after various hydrolysis methods

Hydrolysis	160 °C for			170 °C for			180 °C for		
	45	60	90	30	45	60	15	30	45
		min			min			min	
Tryptophan content (3 runs)	7.18	7.03	6.94	7.09	6.94	6.82	7.08	6.91	6.74
Recovery of tryptophan (3 runs)	96.9	94.9	93.7	95.7	93.7	92.0	95.5	93.3	90.9

Theoretical value: 7.41 (g tryptophan/100 g protein)

Table 5

Tryptophan contents of cytochrom C after various hydrolysis methods

Hydrolysis	160 °C for			170 °C for			180 °C for		
	45	60	90	30	45	60	15	30	45
		min			min			min	
Tryptophan content (3 runs)	1.49	1.44	1.40	1.42	1.39	1.37	1.38	1.36	1.29
Recovery of tryptophan (3 runs)	96.1	92.9	90.3	91.6	89.7	88.4	89.0	87.7	83.2

Theoretical value: 1.55 (g tryptophan/100 g protein)

2.4. Analysis of milk powder

The tryptophan contents of milk powder following various methods of hydrolysis are presented in Table 6. The 6 mol l^{-1} HCl hydrolysis method (110 °C/24 h) and the 3 mol l^{-1} MES – OH hydrolysis method (125 °C/24 h) gave very similar data for amino acid composition of milk powder except for methionine and cystine. The methionine content of milk powder is higher after 3 mol l^{-1} MES – OH hydrolysis due to the reducing effect of MES – OH. The cystine content of milk powder cannot be determined by the MES – OH hydrolysis method, because the cystine content of the milk protein will be reduced to cysteine and this amino acid

appears in the chromatogram in the place of proline. Fortunately, in the case of milk powder, the very low cystine content does not seriously disturb the determination of proline. The reduction of cystine to cysteine is influenced by the time and temperature of hydrolysis. At 160 °C/30 min and 45 min and 170 °C/45 min, part of the cystine can be determined after 3 mol l^{-1} MES–OH hydrolysis. After longer times and/or higher temperatures of hydrolysis, most of the cystine will convert to cysteine.

Table 6

Amino acid composition of milk powder hydrolysed by MES–OH at different temperatures for different times

Amino acids	110 °C 24 h 6 mol l^{-1} HCl	125 °C 24 h 3 mol l^{-1} MES–OH	160 °C for 30 min	160 °C for 45 min	160 °C for 60 min	170 °C for 45 min	170 °C for 60 min	180 °C for 30 min	180 °C for 45 min
Asp	10.4	9.2	9.5	9.4	9.2	9.3	9.7	9.6	9.7
Thr	4.3	4.3	4.6	4.4	4.3	4.4	4.3	4.3	4.2
Ser	6.2	6.4	6.6	6.4	6.4	6.4	6.2	6.3	6.2
Glu	21.2	20.1	21.0	20.6	20.5	21.1	20.8	20.5	20.8
Pro	7.7	8.1	8.0	8.0	8.0	8.2	8.1	8.1	8.2
Gly	1.9	1.7	1.8	1.7	1.7	1.8	1.8	1.9	1.8
Ala	3.2	3.3	3.3	3.2	3.3	3.2	3.2	3.3	3.3
Cys	0.7	—	0.4	0.1	—	0.2	—	—	—
Val	4.1	4.5	3.7	4.3	4.5	4.3	4.4	4.4	4.5
Met	1.8	2.2	2.3	2.3	2.2	2.2	2.1	2.2	2.1
Ile	3.8	4.6	3.7	4.2	4.5	4.3	4.5	4.4	4.6
Leu	9.7	9.7	9.3	9.5	9.6	9.5	9.6	9.6	9.4
Tyr	4.3	4.8	4.9	4.7	4.7	4.7	4.8	4.8	4.6
Phe	4.7	4.6	4.8	4.8	4.7	4.7	4.7	4.7	4.7
Lys	8.4	8.2	8.2	8.2	8.1	8.0	8.0	8.1	8.0
His	2.4	2.6	2.3	2.6	2.6	2.3	2.5	2.4	2.4
Arg	5.2	4.4	4.2	4.3	4.5	4.1	4.2	4.3	4.5
Trp	–	1.30	1.34	1.29	1.27	1.29	1.14	1.09	1.01
Recovery of									
Trp[a]	0	100	103.1	99.2	97.7	99.2	87.7	83.9	77.7
Met[a]	81.0	100	104.5	104.5	100	100	95.4	100	95.4

[a] 125 °C/24 h, 3 mol l^{-1} MES–OH = 100%

As it can be seen from the results of Table 6, 30 or 45 min is not sufficient time to hydrolyse the peptide bonds adjacent to valine and isoleucine. The results of hydrolysis at 160 °C/60 min is very similar to the results of 6 mol l^{-1} HCl 110°C/24 h and 3 mol l^{-1} MES–OH 125 °C/24 h hydrolysis methods. Recoveries of aliphatic

amino acids were also very good. The methionine and threonine contents of milk protein were decreased with increasing hydrolysis time and/or temperature. This decrease was quite small and methionine content of milk protein was higher at the highest temperature and longest hydrolysis time than the values obtained by 6 mol l^{-1} HCl hydrolysis method. The recovery of methionine by the elevated temperature and short time hydrolysis method was higher than that obtained by the 125 °C/24 h method.

The recovery of tryptophan, when compared with the 125 °C/24 h method, was reduced by longer hydrolysis time and/or higher temperature. It seems that 180 °C is too high for tryptophan determination. The optimum hydrolysis conditions for tryptophan determination are: 160 °C/30, 45 or 60 min or 170 °C/45 min.

In conclusion, high temperature hydrolysis with 3 mol l^{-1} mercaptoethanesulfonic acid was successfully applied to the amino acid analysis of pure proteins and milk powder with high carbohydrate content. The aim of these investigations was to introduce a rapid method which is suitable for complete hydrolysis of protein and the hydrolysate is suitable for complete amino acid determination including tryptophan. In the original method proposed by PENKE and co-workers (1974), the protein was hydrolysed by 3 mol l^{-1} MES-OH at 110 °C for 24 and 72 h similarly by to the conventionally used 6 mol l^{-1} HCl hydrolysis. In this report, we state that the higher temperature and the shorter time yielded results which were very similar to the original methods. In some cases (such as tryptophan and methionine determination at 160 or 170 °C for 15–30 min) the results were superior to those obtained by the original method. The great disadvantage of this method is, that MES-OH reduces cystine into cysteine, which appears in the chromatogram in the place of proline and this, particularly in the case of proteins with high cystine content, may falsify the results of proline determination.

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DEFORMATION VERSUS LOAD RELATIONS OF TINPLATE CANS. INTERPOLATION BETWEEN EXTREME LOADING CYCLES

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Extreme loading cycles were applied to tinplate cans Type 7/2–153 (V_0 = 3232 cm^3) for measuring volume change vs. pressure difference relations ($\Delta V = f(\Delta p)$). The term deformation is understood as volume change in the publication. Interpolation formulas of empirical nature have been created for the calculation of $\Delta V = f(\Delta p)$ relations inside the domain of the extreme loading cycles. Measured and calculated results have been compared, earlier results on a can of smaller size (V_0 = 860 cm^3) also have been included. The ratio of the mean difference (d) between measured and calculated volume changes to the mean deviation of parallels (s) has been found between 0.91 to 4.81.

Keywords: deformation of tinplate cans, sterilization of food

Rather significant saving might be expected in a canning factory by reducing the wall thickness of tinplate cans (FERENCZY & KÖRMENDY, 1984). However, a mere empirical approach regarding sterilization and subsequent storage is probably too expensive and lengthy. It seems to be more efficient to start with modelling calculations and treat experimentally only the recommended versions from previous modelling.

Modelling itself means here calculations, that handle measured results on deformation versus load relations of the actual tinplate can, the conditions at filling and closing operations, the variation of retort and can temperatures, retort pressure during sterilization process. Modelling concepts and experimental results have been recently published by PALMIERI and co-workers (1992). Neither permanent outbulging of the end plates, nor collapse of the can from transgressing critical pressure differences may occur under sterilization conditions or subsequently (KÖRMENDY & FERENCZY, 1989; KÖRMENDY, 1989). As a general rule, large volume cans are more susceptible to damages in the sterilization process.

The authors of this work applied two types of extreme loading cycles to a selected type of can and prepared so-called interpolation formulas to calculate

0139-3006/94/$ 4.00

deformation vs. load inside the domain of the extreme loading cycles. They performed experiments with reduced loads and compared the results with values calculated by the interpolation formulas. The principle of the measuring apparatus (DELI, 1988) and other fundamentals have been based on the early work of RIGHI (1955).

The interpolation formulas, as of empirical nature, were created on the previous experimental concepts and results of FERENCZY (1986), KÖRMENDY and FERENCZY (1989). Deformation means volume change in this publication. The fact, that an actual volume difference at a load (pressure difference) depends on the previous loading history of the can, has been regarded to some extent inside the structure of the interpolation formulas.

According to sterilization practice loads and deformations surpass the limits of reversible elasticity. The limit values of elasticity are approximately $|\Delta p| = 0.05$ bar and $|\Delta V| = 6.3$ cm^3 for the cans under investigation in this publication (FELFÖLDI et al., 1994).

1. Materials and measurement methods

1.1. Type of the can used for experiments

Cans used for experiments were manufactured from lacquered tinplate of 0.28 mm thickness in the Debreceni Tartósítóipari Kombinát (Integrated Food Preservation Factory of Debrecen, Hungary). Hungarian denomination of the can is type 7/2 – 153, being the Anglo-American equivalent about #603 × 700. Main geometric data are presented in Fig. 1, an undulated part is found on the cylindrical mantle.

Can volume was measured by filling the cubic content using water. Water density vs. temperature was taken from the data of RAZNJEVIC (1964). Hardness (temper) of tinplate was determined according to the Rockwell 30T method (INTERNATIONAL STANDARD, 1978).

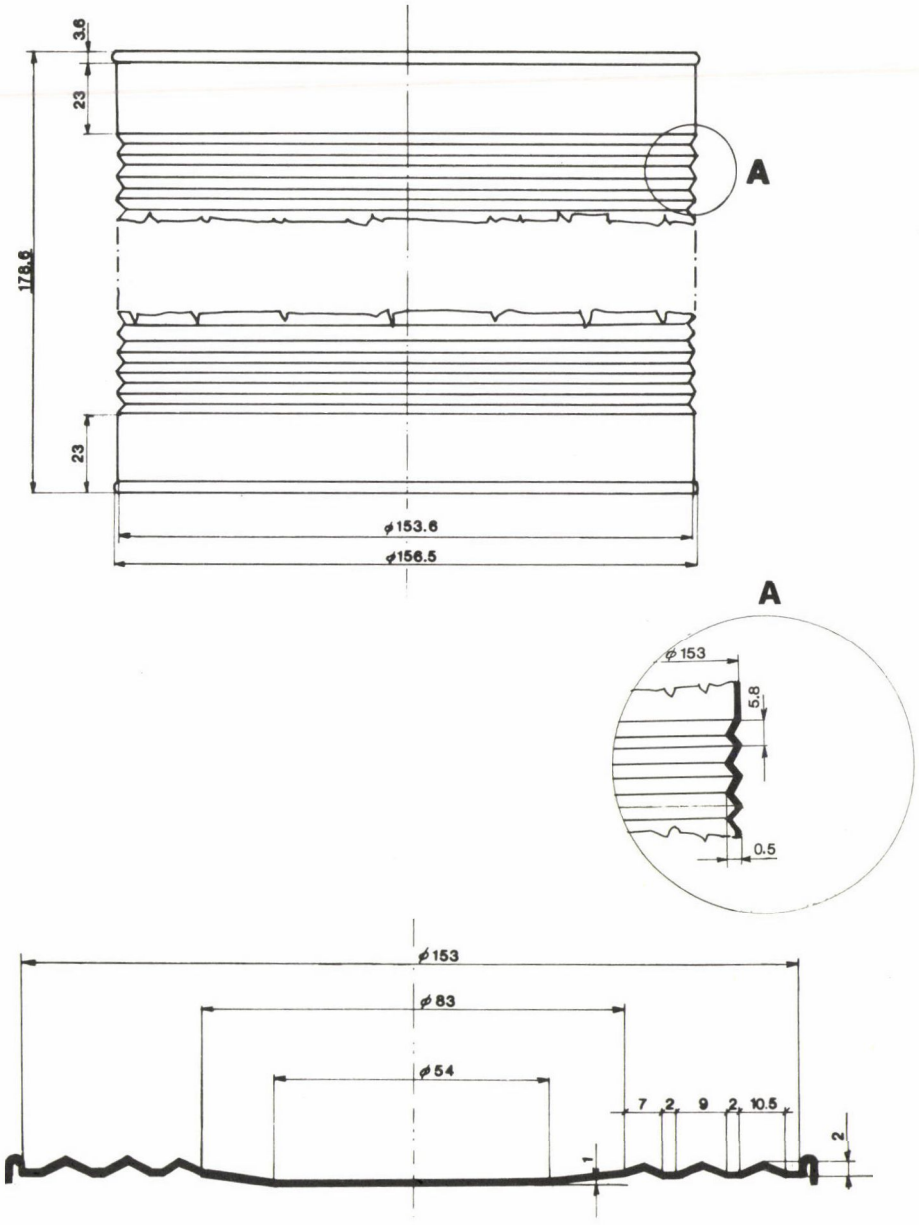

Fig. 1. Major geometric data of tinplate cans

1.2. Apparatus for measuring deformation vs. load relations

The working principle of the apparatus can be found in previous publications (RIGHI, 1955; KÖRMENDY & FERENCZY, 1989; PALMIERI et al., 1992). The apparatus used by the authors is a unique design of DELI (1988) and is apt for handling cans up to the outside diameter: 157 mm and height: 248 mm (Anglo-American equivalent size: #603 × 913).

Instrument for measuring pressure difference (Δp): coiled tube pressure gauge (manovacuum gauge) with 1 − 0 − 1.5 bar range. Grade of accuracy: 1% of the (greater) end value. Scale division 0.05 bar.

Device for measuring the volume change (ΔV) of the can: Scaled glass tube of 15 mm inside diameter. Standard deviation from repeated reading of the scale: 0.036 cm^3.

Location of the stub (inside diameter: 4 mm) connecting the inside space of can with the pressure transmission tube: 12 mm from the end of the can.

1.3. Determination of critical pressure differences

Determinations were carried out in the open apparatus of para. 1.2., without filling it up with water. Inside overpressure was regarded as measured critical one (Δp_{ci}), when the first radial line appeared at the edge of the outbulged end plate of the can. Measured critical pressure difference from outside overpressure (Δp_{co}) caused the abrupt collapse of the can at the mantle.

Actual extreme differences (Δp_{m1}, Δp_{m2}) for model calculations have been obtained by reducing Δp_{ci} or increasing $\Delta p_{co} < 0$ by at least three times of the standard deviation from renewed experiments (with new cans).

1.4. Determination of the extreme loading cycle Type A

The volume change (or difference, ΔV, see Eqn (2) has been measured with the apparatus in para. 1.2., by changing the pressure difference (Δp, see Eqn (1)). Variation of Δp was created by stepping forward and backward with the fixed step $|\delta(\Delta p)_m| = 0.1$ bar in the following sequence: $0,...,\Delta p_{m1},...,0,...,\Delta p_{m2},...0$ (increase from zero up to Δp_{m1}, return to zero, decrease to Δp_{m2}, increase up to zero). Lines 3A, 4A, 5A, 6A illustrate the cycle in Figs 2 and 5.

1.5. Determination of the extreme loading cycle Type B

The same procedure has been applied here as for the cycle Type A, but the sequence of pressure differences proceeded as follows: $0,...,\Delta p_{m2},...,0,...,\Delta p_{m1},...,0,...,\Delta p_{m2},...,0$. Lines 1B, 2B, 3B, 4B, 5B, 6B illustrate such a cycle in Figs 2 and 6.

1.6. Determination of volume change vs. pressure difference relations inside the domain of extreme loading cycles

Similar procedure was applied here as for the extreme loading cycles, but instead of Δp_{m1} and Δp_{m2} step $\delta(\Delta p)_m$ changed direction already at $\Delta p_{d1} < \Delta p_{m1}$ and Δp_{d2} (or $x_{dH2} = \Delta p_{dH2}$) $> p_{m2}$. More strictly: At least one of the absolute values of Δp_d is less than the absolute value of its respective extreme load Δp_m. Such cycles are illustrated in Figs 2, 7 to 10. Respective lines are marked by 1B, 2H, 3H, 4H', 5H, 6H'.

2. Equations for the calculation of ΔV vs. Δp relations

2.1. General principles, symbols and indices

All equations used for calculating ΔV vs. Δp relations include the results of the extreme loading cycle experiments and consequently are named interpolation equations.

Previous experimental concepts and results of FERENCZY (1986), KÖRMENDY and FERENCZY (1989) have been incorporated into the interpolation formulas, regarding to some extent the previous loading history before the actual load. Accordingly the equations are of empirical nature and further improvement may be expected in the future.

The domain of Δp has been divided into the zone of "Vacuum" ($\Delta p < 0$) and of "Overpressure" ($\Delta p > 0$).

The following rules were derived from experiments:

Increasing *absolute value of* Δp is always followed by increasing *absolute value of* ΔV. The greatest part of the volume change is due to the formation of convex or concave domes at the end plates (FERENCZY & KÖRMENDY, 1984). Decreasing absolute value of Δp effects the reduction of the absolute value of ΔV. Volume change is retarded now by the propping effect of the domes, either convex or concave.

Not so self evident as before, but rather interesting was the finding that in a section, where propping effect is present (see e.g. section 2B, 4H', 6H' in Fig. 2), the

repeated change of the direction of loading does not effect considerably the relation between ΔV and Δp. Accordingly, calculation could be simplified.

Pressure difference is understood as

$$\Delta p = p - p_R, \tag{1}$$

p is the inside and p_R is the external pressure of the can.

Volume change or difference is understood as

$$\Delta V = V - V_0, \tag{2}$$

where V is the volume of the can at Δp, V_0 is the original volume of the unloaded can ($\Delta p = 0$). V and V_0 belong to the same actual temperature of the measuring apparatus.

The general symbol for relations ΔV vs. Δp is $\Delta V = f(\Delta p)$. Typographic simplification demanded the introduction of the symbols

$$x = \Delta p, \; y = \Delta V.$$

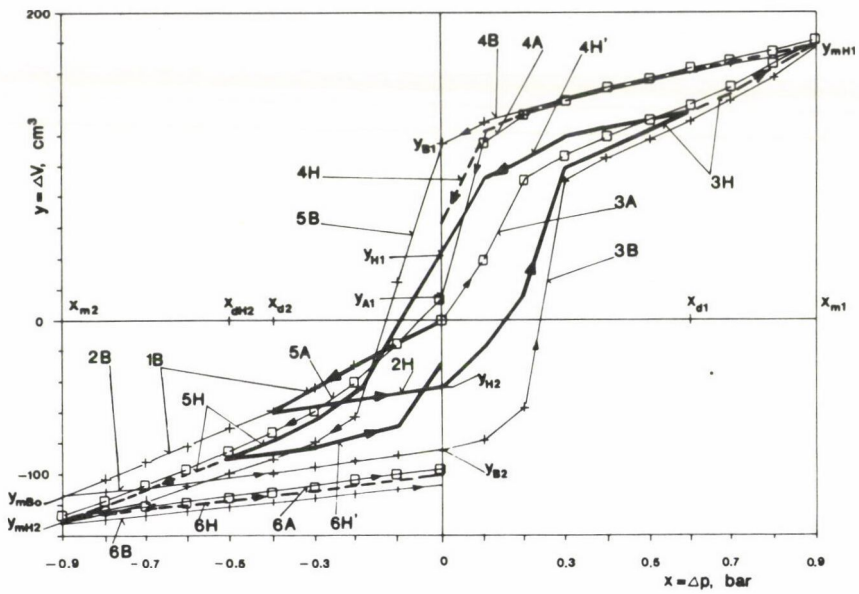

Fig. 2. Sections and corresponding lines illustrating the extreme loading cycles (Types A and B) and a loading cycle inside their domain (Type H). Markings relate to sections in para. 2.2. Symbols are listed in Table 1

Two basic formulas have been evolved, each in original and modified version. One refers to dome formation and creates an average between relations Type A and

Type B (see Eqn (3) and Eqn (3.1)). The other takes into account the propping effect (see Eqn (4) and Eqn (4.1)).

Symbols and indices used in the interpolation equations are listed in Table 1.

Table 1

Symbols used in the equations of para. 2.2.
Graphic representation: Fig. 2 to Fig. 10

Symbol	Interpretation
δ	Elementary step for the variable following the symbol
$f_{3A}(x),...,f_{6A}(x)$	Sections of the extreme loading cycle Type A
$f_{1B}(x),...,f_{6B}(x)$	Sections of the extreme loading cycle Type B
$f_{2H}(x)$	Section created by stepping backwards from $x = x_{d2}$
$f_{3H}(x)$	Section created after entering into the zone of "Overpressure" at $x = 0$, $y = y_{H2}$
$f_{4H}(x)$	Section created by stepping backwards from $x = x_{m1}$
$f'_{4H}(x)$	Section created by stepping backwards from $x = x_{d1} < x_{m1}$
$f_{5H}(x)$	Section created after entering into the zone of "Vacuum" at $x = 0$, $y = y_{H1}$
$f_{6H}(x)$	Section created by stepping backwards from $x = x_{m2}$
$f'_{6H}(x)$	Section created by stepping backwards from $x = x_{dH2} > x_{m2}$
x	$= \Delta p$, simplified symbol of the pressure difference
(x_{d1}, y_{d1})	Coordinates belonging $\begin{cases} f_{3H}(x), f_{3A}(x) \end{cases}$
(x_{d2}, y_{d2})	to step direction $\begin{cases} f_{1B}(x) \end{cases}$
(x_{dH2}, y_{dH2})	change in sections: $\begin{cases} f_{5H}(x) \end{cases}$
x_{m1}	Maximum of Δp in the zone "Overpressure"
x_{m2}	Minimum of Δp in the zone "Vacuum"
y	$= \Delta V$, simplified symbol of the volume change
y_{A1}	$= f_{4A}(0) = f_{5A}(0)$
y_{B1}	$= f_{4B}(0) = f_{5B}(0)$
y_{H1}	$= f_{4H}(0)$ or $= f'_{4H}(0)$
y_{B2}	$= f_{2B}(0) = f_{3B}(0)$
y_{H2}	$= f_{2H}(0)$
y_{mBO}	$= f_{1B}(x_{m2}) = f_{2B}(x_{m2})$
y_{mH1}	$= f_{3H}(x_{m1}) = f_{4H}(x_{m1})$
y_{mH2}	$= f_{5H}(x_{m2}) = f_{6H}(x_{m2})$

δx marks a step in pressure difference, δx_d marks a step in shifting the potential point of reversal along the x axis. $\delta x = \delta x_d$ means simultaneous shift of x and x_d.

2.2. Equations

2.2.1. Original equations are based on the following formulas:

$$y = b + [f_A(x) - a][1 - (a-b)/(a-c)] + [f_B(x) - c](a-b)/(a-c) \quad (3)$$

$$y = f_1(x) - [f_1(x) - f_2(x)](f_1(x) - d)/(f_1(x) - e) \quad (4)$$

Modified formulas, replacing the original ones are as follows:

$$y = b + [f_A(x) - a](1 - (|a-b|)/(|a-b| + |b-c|) + \\ + [f_B(x) - c](|a-b|)/(|a-b| + |b-c|) \quad (3.1)$$

$$y = [1 - (f_1(x) - d)/(f_1(x) - e)] \cdot [(f_1(x) + y_0 - d - e)/(y_0 - e)] \cdot f_1(x) + \\ + (y_0 - d)/(y_0 - e) \cdot [(f_1(x) - d)/(f_1(x) - e)] \cdot f_2(x) \quad (4.1)$$

Experiments and calculations start always from the point x = 0, y = 0.

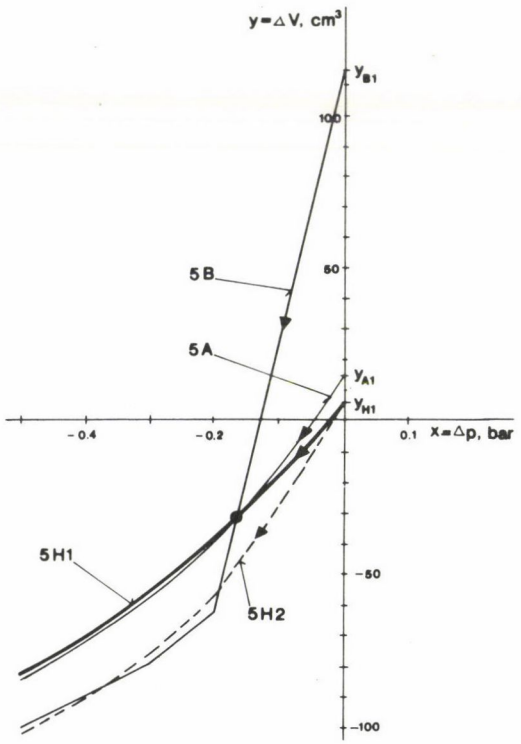

Fig. 3. Difference between interpolated results using Eqn (3) and Eqn (3.1). a = y_{A1}, b = y_{H1}, c = y_{B1} are to be substituted. y_{H1} lies outside the domain $y_{B1} \leq y \leq y_{A1}$ and lines 5A and 5B cross each other. Markings relate to sections in para. 2.2. Symbols are listed in Table 1. 5H1, 5H2: lines of section 5H, calculated by Eqn (3) and Eqn (3.1), respectively

The details in paras 2.2.2., 2.2.3., 2.2.4. refer to the modified formulas, however, the same substitutions are valid for the original ones (except for y_0, which appears only in the modified equations).

The previous formulas are of empirical nature without special theoretical background. The application of the formulas can be followed in para. 2.2.2. and Fig. 2. Sections of the extreme loading cycles are illustrated in Figs 5 and 6. Figures 3 and 4 are demonstrating the previous equations as well.

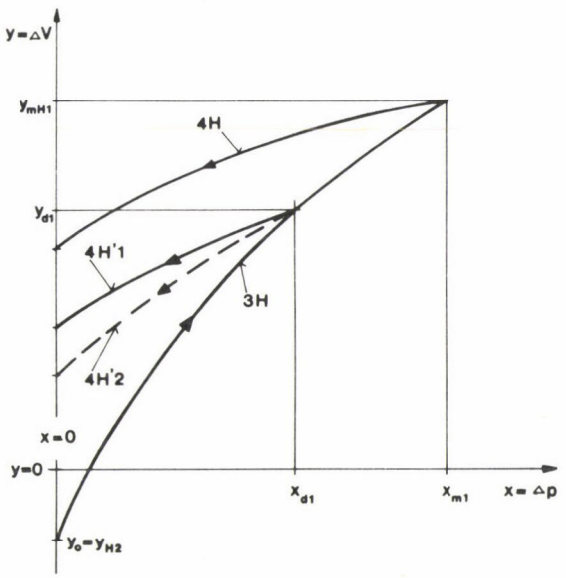

Fig. 4. Difference between interpolated results using Eqn (4) and Eqn (4.1). d = y_{d1}, e = y_{mH1}, y_0 = y_{H2} are to be substituted. Markings relate to sections in para. 2.2. Symbols are listed in Table 1

Equations (3) and (3.1) serve to describe dome formation and create weighted averages from measured relations $f_A(x)$ and $f_B(x)$. The sum of the weighting factors is 1. The factors are determined through the values, which $f_A(x)$, $f_B(x)$ and y uptake at $x = \Delta p = 0$ (at the end of the previous section), as

a is the volume change $f_A(0)$ at $x = \Delta p = 0$ from Type A measurement,

b is the interpolated volume change y(0) at $x = \Delta p = 0$ as the result of previous calculations.

c is the volume change $f_B(0)$ at $x = \Delta p = 0$ from Type B measurement.

The formation of averages is illustrated in Fig. 3, where a = y_{A1}, b = y_{H1}, c = y_{B1}. Further information is provided in para. 2.2.5.

The example in Fig. 4 serves for explanation of Eqn (4) and Eqn (4.1). Line 3H represents dome formation at increasing inside overpressure. Line 4H illustrates how the $\Delta V = f(\Delta p)$ relation ($y = f(x)$) is modified, when reverting the step direction at

$x_{m1} = \Delta p_{m1}$. Both lines are calculated averages using Eqn (3) or Eqn (3.1) (see para. 2.2.2., Section 3H and 4H). Line 4H'1 and line 4H'2 were calculated by Eqn (4) and Eqn (4.1) respectively. Both lines start at $x_{d1} = \Delta p_{d1}$, i.e. the pressure difference of step direction change and demonstrate how the propping effect works. The proper substitutions into Eqn (4) and Eqn (4.1) are as follows (see para. 2.2.2., Section 4H'):

$f_1(x) = f_{3H}(x)$, $f_2(x) = f_{4H}(x)$;
$d = y_{d1}$, the volume difference, where step direction reverts;
$e = y_{mH1}$ is the same but belongs to x_{m1};
$y_0 = y_{H2} = f_{3H}(0)$, the volume change at $x = \Delta p = 0$ in the section of dome formation. Further details are provided in para. 2.2.5.

2.2.2. First sections in the zone "Vacuum", $x \leq 0$.

Section 1B, starting at $x = 0$, $y = 0$.
Conditions: $\delta x = \delta x_{d2} < 0$, $x = x_{d2} \geq x_{m2}$
Result: $y = f_{1B}(x)$
Section 2H
Conditions: $x_{d2} < x \leq 0$, or $x = x_{d2}$ and $\delta x > 0$
Substitutions into Eqn (4.1): $d = y_{d2}$, $e = y_{mBo}$, $y_0 = 0$, $f_1(x) = f_{1B}(x)$, $f_2(x) = f_{2B}(x)$; if $f_{1B}(x) = d = e$, then $[f_{1B}(x) - d]/[f_{1B}(x) - e] = 1$
Result: $y = f_{2H}(x)$

2.2.3. Sections in the zone "Overpressure", $x \geq 0$.

Section 3A, starting at $x = 0$, $y = 0$
Conditions: $\delta x = \delta x_{d1} > 0$, $x = x_{d1} \leq x_{m1}$
Result: $y = f_{3A}(x)$
Section 3H
Conditions: as before for section 3A
Substitutions into Eqn (3.1): $a = 0$, $b = y_{H2}$, $c = y_{B2}$, $f_A = f_{3A}(x)$, $f_B(x) = f_{3B}(x)$
Result: $y = f_{3H}(x)$
Section 4H
Conditions: $\delta x < 0$, $x_{d1} = x_{m1}$
Substitutions into Eqn (3.1): $a = 0$, $b = y_{H2}$, $c = y_{B2}$, $f_A(x) = f_{4A}(x)$, $f_B(x) = f_{4B}(x)$
Result: $y = f_{4H}(x)$
Section 4H'
Conditions: $x_{d1} > x \geq 0$, or $x = x_{d1}$ and $\delta x < 0$
Substitutions into Eqn (4.1): $d = y_{d1}$, $e = y_{mH1}$, $y_0 = y_{H2}$, $f_1(x) = f_{3H}(x)$, $f_2(x) = f_{4H}(x)$; if $f_{3H}(x) = d = e$, then $[f_{3H}(x) - d]/[f_{3H}(x) - e] = 1$
Result: $y = f'_{4H}(x)$

2.2.4. Additional sections in the zone "Vacuum", $x \leq 0$.

Section 5H
Conditions: $\delta x = \delta x_{dH2} < 0$, $x = x_{dH2} \geq x_{m2}$
Substitutions into Eqn (3.1): $a = y_{A1}$, $b = y_{H1}$, $c = y_{B1}$, $f_A(x) = f_{5A}(x)$, $f_B(x) = f_{5B}(x)$
Result: $y = f_{5H}(x)$
Section 6H
Conditions: $\delta x > 0$, $x_{dH2} = x_{m2}$
Substitutions into Eqn (3.1): $a = y_{A1}$, $b = y_{H1}$, $c = y_{B1}$, $f_A(x) = f_{6A}(x)$, $f_B(x) = f_{6B}(x)$
Result: $y = f_{6H}(x)$
Section 6H'
Conditions: $x_{dH2} < x \leq 0$, or $x = x_{dH2}$ and $\delta x > 0$
Substitutions into Eqn (4.1): $d = y_{dH2}$, $e = y_{mH2}$, $y_0 = y_{H1}$, $f_1(x) = f_{5H}(x)$, $f_2(x) = f_{6H}(x)$; if $f_{5H}(x) = d = e$, then $[f_{5H}(x) - d]/[f_{5H}(x) - e] = 1$
Result: $y = f'_{6H}(x)$

2.2.5. Differences between Eqn (3), Eqn (3.1) and Eqn (4), Eqn (4.1).

There is no difference between Eqn (3) and Eqn (3.1), when $a < b < c$ or *vice versa*, i.e. b is located between a and c. Otherwise results differ, because the weighting factors for calculating the mean of $f_A(x)$ and $f_B(x)$ are reduced to $(a-c)$ in Eqn (3), instead of $|a-b| + |b-c|$ as in Eqn (3.1). Figure 3 illustrates results in this respect.

Equation (4) has the property of shifting $f_2(x)$ in the direction of the axis of y up or down to the point (x_d, y_d), if the condition $[e - f_1(x)]/[e - f_2(x)] = \text{constant}$ is met. Equation (4.1) corrects this shifted line in a way that the nearer x_d is located to $x = 0$, the more it bends towards $f_1(x)$ from the shifted $f_2(x)$, as illustrated in Fig. 4.

2.2.6. Examples

Figures 5 and 6 illustrate measured lines of the two extreme loading cycles, while Fig. 2 illustrates an example for interpolational calculations. Thin lines with empty squares and crosses represent cycle Type A and Type B, respectively. Thick full lines illustrate calculated final results, broken lines demonstrate intermediary results.

Calculation started at $x = \Delta p = 0$, $y = \Delta V = 0$, then proceeded along line 1B with decreasing pressure differences (calculated and measured values coincide here). Step direction changed at $x_{d2} = -0.4$ bar and pressure difference started now to increase first up to $x = 0$ (line 2H), then up to $x_{d1} = 0.6$ bar (line 3H), where step direction reverted again. Next followed the calculation of lines 4H' and 5H and at $x_{dH2} = -0.5$ bar step direction reverted again to calculate line 6H', which ended at $x = 0$.

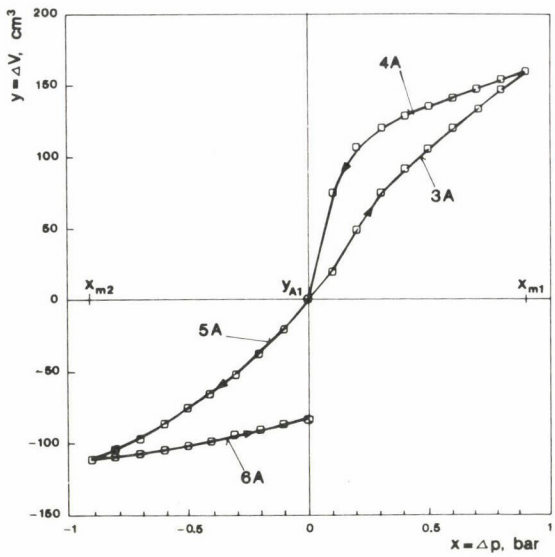

Fig. 5. Measured values of the extreme loading cycle Type A. Markings relate to sections in para. 2.2., symbols are listed in Table 1

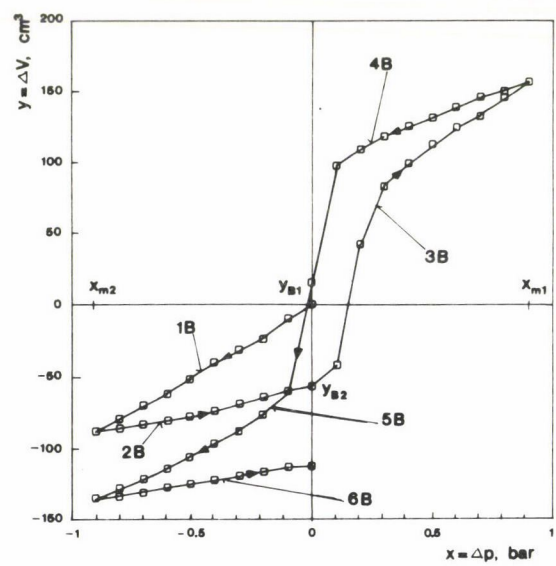

Fig. 6. Measured values of the extreme loading cycle Type B. Markings relate to sections in para. 2.2., symbols are listed in Table 1

Line 3H and 5H was calculated by Eqn (3), while Eqn (4) served for the calculation of line 2H, 4H' and 6H'. Broken lines 4H and 6H were calculated using

Eqn (3) and results were used for the subsequent calculation of line 4H' and 6H', respectively.

2.3. Mean difference of measured and calculated volume changes

Mean difference ($\bar{d}$) has been calculated by approximating the following relation:

$$\bar{d} = [(1/X \int_0^X (y_m - y_c)^2 d|x|]^{0.5} \quad (5)$$

where $X = N|\delta x|$

Index m and c mark measured and calculated values, N is the number of steps $|\delta x|$. Approximation means assumed linear change of y within one step.

3. Results

3.1. Can volume and tinplate temper (hardness, see para. 1.1.)

Eight different closed cans' volume has been measured, each twice. The mean volume was $V_0 = 3232.0$ cm^3 at $T_0 = 18$ °C, standard deviation of 16 determinations was $s = 13.2$ cm^3. Only slight difference between individual can volumes could be detected on 5 percent probability level, and no difference at 1 percent level, according to an analysis of variance.

Three tinplate cans were taken for temper (hardness) measurements. Samples for testing were cut from four different parts of each can (two from end plates, two samples from the mantle). Each determination has been repeated three times. Altogether 36 determinations were carried out. Analysis of variance of hierarchical type resulted in statements as follows: No difference has been found between individual cans. No significant difference could be defected between the average of the two end plates and the mantle average (probability level 5 percent).

Mean temper was 61.61 R30T degrees, standard deviation from 36 measured values: 0.54 R30T degrees, standard deviation from three can averages: 0.32 R30T degrees.

3.2. Critical pressure differences (see para. 1.3.)

Six cans for critical inside overpressure (Δp_{ci}) and six cans for critical outside overpressure (Δp_{co}) have been examined.

The average of Δp_{ci} was 1.05 bar, with a standard deviation $s = 0.04$ bar. The relating extreme pressure difference for model calculations has been taken for

$$\Delta p_{m1} = 0.9 \text{ bar} < 1.05 - 3(0.04) = 0.93 \text{ bar}$$

As no can collapsed at the available greatest negative pressure difference: −0.955 bar, $\Delta p_{m2} = -0.9$ bar has deliberately been choosen for model calculations. No collapse has ever occurred in later experiments at this value.

3.3. Experimental results regarding the extreme loading cycle Type A (see para. 1.4.)

Experiments have been repeated 4 times with new cans (parallels). Mean values are presented in Fig. 5. Sum of square of deviations (SSQ) has been calculated from the differences between means of ΔV and their respective 4 parallels. Degrees of freedom is

D.F. = K(P−1) = 36(4−1) = 108, where K is the number of fixed pressure differences, P is the number of parallels. Mean deviation (s) is the square root of the mean square (SSQ/D.F.), s = 1.95 cm^3 has been obtained.

3.4. Experimental results regarding the extreme loading cycle Type B (see para. 1.5.)

Experiments have been repeated 4 times with new cans. Mean values are presented in Fig. 6. Statistical evaluation is understood as in para. 3.3.: P = 4, K = 54, D.F. = 162, s = 2.09 cm^3.

3.5. Experimental results inside the domain of the extreme loading cycles (loading cycles Type H)

Experiments were carried out in two versions. In Version 1 the pressure difference has been varied as follows: Δp = 0, (−0.1),..., (−0.4), (−0.3),..., 0, 0.1,..., 0.6, 0.5,..., 0, (−0.1),..., (−0.5), (−0.4),..., 0. Number of parallels was P = 2. Results are illustrated in Figs 7 and 8.

The pressure difference in Version 2 has been varied according to Δp = 0, (−0.1),..., (−0.4), (−0.3),..., 0, 0.1,..., 0.6, 0.5,..., 0, (−0.1),..., (−0.5), (−0.6),..., (−0.9), (−0.8),..., 0. Number of parallels was P = 3. Results are illustrated in Figs 9 and 10.

The first and greater part of the two versions have been contracted, i.e. $P_1 = 5$ parallels have been considered up to the first 25 steps. Therefore, in Figs 7 to 10 the lines numbered 1 are identical. Statistical evaluation treated the two versions jointly, considering SSQ and D.F. For example

D.F. = 25(5−1) + 5(2−1) + 13(3−1) = 131 for altogether K = N = 43 steps in pressure differences. Results are presented in Table 2.

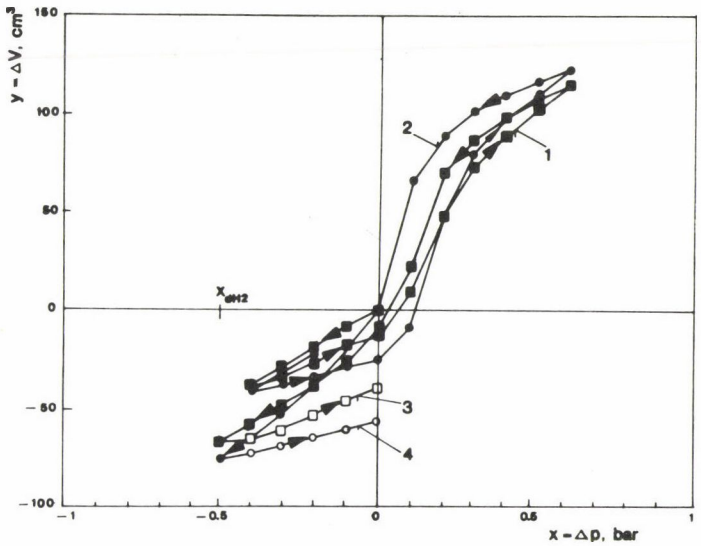

Fig. 7. Difference between measured and calculated results, regarding Version 1 in paras 3.5. and 3.6. and applying Eqns (3) and (4). 1: measured values, up to $x_{dH2} = -0.5$ bar; 2: calculated values up to $x_{dH2} = -0.5$ bar; 3: measured values in section 6H' (see para. 2.2., Fig. 2); 4: calculated values of section 6H'

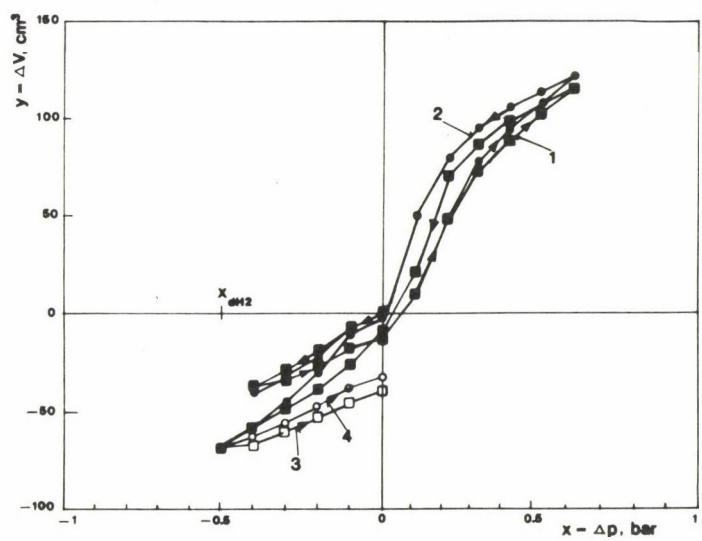

Fig. 8. Difference between measured and calculated results, regarding Version 1 in paras 3.5. and 3.6. and applying Eqns (3) and (4.1). 1: measured values up to $x_{dH2} = -0.5$ bar; 2: calculated values up to $x_{dH2} = -0.5$ bar; 3: measured values in section 6H' (see para. 2.2., Fig. 2); 4: calculated values of section 6H'

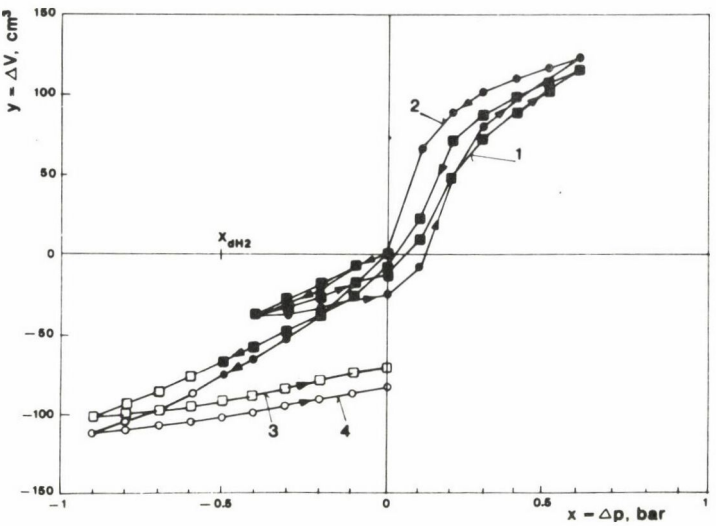

Fig. 9. Difference between measured and calculated results, regarding Version 2 in paras 3.5. and 3.6. and applying Eqns (3) and (4). 1: measured values up to $x_{dH2} = -0.5$ bar; 2: calculated values up to -0.5 bar; 3: measured values in section 6H (see para. 2.2., Fig. 2); 4: calculated values of section 6H

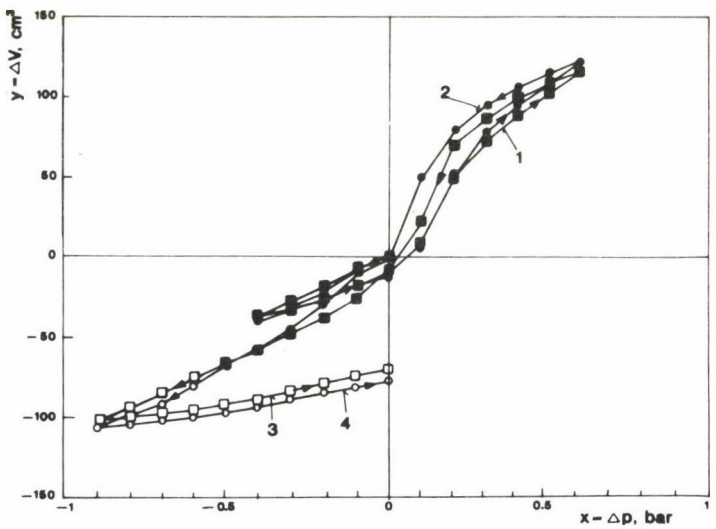

Fig. 10. Difference between measured and calculated results, regarding Version 2 in paras 3.5. and 3.6. and applying Eqns (3) and (4.1). 1: measured values up to $x_{dH2} = -0.5$ bar; 2: calculated values up to -0.5 bar; 3: measured values in section 6H (see para. 2.2., Fig. 2); 4: calculated values of section 6H

Table 2

Comparison of measured and calculated results, using interpolation equations
Symbols are explained in paras 2.3., 3.3. to 3.6.

Denomination	Interpolation formulas	$\bar{d}$ (cm^3)	N	s (cm^3)	D.F.	K	P	$\bar{d}/s$
Earlier results	Eqn (3),	2.36	170	2.59	1027	$K_1=481$	$P_1=3$	0.91
(para. 3.6.)	Eqn (4)					$K_2=65$	$P_2=2$	
Version 1 +	Eqn (3),	11.33				$K_1=25$	$P_1=5$	4.08
Version 2	Eqn (4)					$K_2=5$	$P_2=2$	
(paras 3.5.and	Eqn (3),	6.42	43	2.78	131	$K_3=13$	$P_3=3$	2.31
3.6.)	Eqn (4.1)							
	Eqn (3.1)	13.38						4.81
	Eqn (4.1)							

3.6. Calculated results inside the domain of the extreme loading cycles (calculated cycles Type H)

Calculated results have been compared with experimental results by using the mean difference ($\bar{d}$) of para. 2.3. the results of both experimental versions in para. 3.5. have been compared first by calculating with Eqn (3) and Eqn (4), then by using Eqn (3) and Eqn (4.1), at last by the use of Eqn (3.1) and Eqn (4.1). Results are partly illustrated in Fig. 7 to Fig. 10. The mean difference has been reduced to the mean deviation ($\bar{d}/s$). Results of the two experimental versions and the due calculated results have been united for the evaluation of $\bar{d}$ and s (see para. 3.3. and 3.5.). Results are demonstrated in Table 2.

A part of earlier results of FERENCZY (1986), KÖRMENDY and FERENCZY (1989) also has been treated in the same way, but using only Eqn (3) and Eqn (4). Measured results, as having had been presented under serial number 7 (Table 1) and in Fig. 6 (para. 2.2.) in the publication of KÖRMENDY and FERENCZY (1989), were investigated here. Results of these investigations are included in Table 2. Can volume at room temperature was V_0 = 860 cm^3.

4. Conclusions

Interpolation equations (Eqn (3), Eqn (4)) have been created on earlier results of FERENCZY (1986), KÖRMENDY and FERENCZY (1989). These are of pure empirical nature and include measured data of extreme loading cycles of tinplate cans. Mean difference of measured and calculated volume changes reduced to the mean deviation of measured data ($\bar{d}/s$, see Table 2) is considered acceptable ($\bar{d}/s = 0.91$).

The same (original) equations have been applied to a can of larger volume and with undulated mantle and $\bar{d}/s = 4.08$ could be obtained as a less satisfactory result. By using the original Eqn (3) and the modified Eqn (4.1) instead of Eqn (4) $\bar{d}/s = 2.31$ could be obtained. Figures 7 to 10 illustrate how calculation approximates measured results in the previous cases.

The use of Eqn (3.1) and Eqn (4.1) worsened the fitting as resulting in $\bar{d}/s = 4.81$. Further improvement seems to be reasonable regarding the interpolation equations by relating them on a sound (not necessarily complicated) theoretical basis.

Though theoretically not well founded, the authors checked if the mean difference d is less or greater than the significant difference (SD) belonging to the mean deviation s (regarding the t-value at 5 percent probability and the number of parallels).

It was found that $\bar{d}$ was always greater than SD for the present experimental work, and was found less than SD for earlier results (see para. 3.6.).

*

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EFFECT OF GERMINATION ON A RANGE OF NUTRIENTS OF SELECTED GRAINS AND LEGUMES

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Common available seeds, wheat, barley, lentil, soya were soaked in distilled water and germinated for 48–96 h at ambient temperature and normal day–night light. The germinated seeds were analyzed for macronutrients – fats, protein, fibre, saccharides and some micronutrients – amino acids, minerals and vitamines. It was found that germination considerably increases the nutritive value of seeds.

Keywords: germination, grains, legumes, nutritive value

Germination of seeds is one of the possibilities how to increase the nutritive value of foods by natural way. This method is known for a very long time. In our country, the people in countryside used to prepare special meals from germinated rye or pea (in spring months). Recently there is a renewed interest in germination of seeds.

It is evident from the literature too, that both the research and the practical application of germinated seeds in nutrition is the most widespread in Japan and USA. In Slovakia germination is popular among vegetarians only.

Different seeds can be germinated: all legumes (bean, pea, lentil, soya) (IDOURAINE, 1989, TAJIRI, 1985; BEDNARSKI, 1985; ABDULLAH & BALDWIN, 1984; BERRY, 1988; MABESA, 1984), grains (rye, wheat, barley, oats) (SWANSTON & TAYLOR, 1990; REDDY, 1984; WAGINGER & HABERDA, 1985;), rice, rape seeds, corn, amarant seeds, millet, sorghum (MIRIC, 1988; DOHMEN, 1987; THOMPSON & SERRAINO, 1985; COLMENARES DE RUIZ & BRESSANI, 1990; GLENNIE, 1985; BALDINI, 1982). Period of germination is usually about 24–72 h. The germinated seeds are used either as whole (fresh, dried and milled to flour, etc.) or as 7–10 cm long sprouts (TATSUO, 1977; YOSHIHIDE HAGIWARA, 1986).

The first phase in the process of germination is soaking of the seeds. In seeds after uptake of water activation of different enzymes begins – i.e. amylolytical, proteolytical, lipolytical enzymes, carboxypeptidase, chitonase, proteinase and the others (BERRY, 1988; KRIKUNOVA, 1983; AHOKAS & NASKALI, 1990; EVANS & TAYLOR, 1990). Enzymatic activity brings about many other biochemical changes.

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The content and composition of fats are changed (MOSTAFA, 1987; GHAZALI & CHENG, 1991). The fibre content, digestibility of protein and content of some vitamines essentially rise (BEDNARSKY, 1985; WATZL & LEITZMANN, 1984; ABDEL-HAMID & ABDEL-RAHMAN, 1984; ABDEL-KADER, 1984). According to MABESA (1984) the relative nutritive value increases nearly seven times. Published changes of the nutritive components during germination are often rather different, depending on many conditions (mode of germination, temperature, moisture, quality of raw material, etc.).

In our work we dealt with the changes of nutritive components in two grains – wheat and barley and two legumes – soya and lentil.

1. Material and methods

1.1. Material

The seeds selected for germination were as follows: wheat cv. Viginta, spring barley cv. Rubín, lentil cv. Lenka and soya cv. Evans. All seeds were purchased from agricultural and seed-dealing societies Lehnice, Sládkovicovo, Jablonec.

All seeds before germination were thoroughly cleaned with tap water and finally distilled water. Soya, lentil and wheat seeds were then soaked for 12 h in 5-fold volume of distilled water at nearly 20–23 °C. After soaking seeds were spread in layer of 0.5–1 cm on plastic plates and covered with moist flax matter (maintained). The germination was carried out at ambient laboratory temperature (ca. 22–23 °C) in day–night light. Soya, lentil and wheat seeds were germinated for 96, 48 and 52 h, respectively. Samples were washed with distilled water in every 8 h. Barley was soaked for 14 h in 5-fold volume of distilled water, than spread on plastic plates in 0.5 cm layer, covered with moist matter and germinated at ambient temperature for 10 h. In further system we changed 2×8 h soaking and 16 h germination. All germination time for barley was 58 h.

1.2. Preparation of samples for analysis

The dry seeds were grounded to powder in vibration stainless mill VM 4 (CSFR). The homogenized samples were stored in tightly closed glass vessels at 4–6 °C, before analysis. Germinated seeds were crushed wet to fine consistence with plunge mixer, than sealed in plastic bags (BOPP foil), freezed and stored at –18 °C before analysis (max. three days). For determination of vitamin C samples were homogenized separately with the use of stabilizer.

1.3. Methods

Moisture was determined gravimetrically by drying to constant weight at 105 °C. Crude fat was determined gravimetrically after 6 h extraction in Twisselman apparatus with petroleum ether and followed by evaporation of organic phase. Samples were ashed at 550–600 °C and ash dissolved in ca. 10% HCl. From ash the following mineral components were determined: Ca by complexometric titration, Fe by photometric method. Samples were mineralized with H_2SO_4 and H_2O_2 and the total N was determined by micro Kjeldahl method. Crude protein was calculated from the total N content by multiplying this value with the coefficient for protein of grain (% N × 5.7). Automatic amino acid analyzer AAA T 339 (ČSFR) was used for the amino acid assay after the acid hydrolysis of sample with 6 mol HCl for 24 h at 105 °C in sealed tube. The column cartridge was Ostion LG ANB in Na^+ cycle. Ascorbic acid was determined by titrimetric method with 2,6-dichlorphenolindophenol with 2% monohydrogenphosphoric acid as stabilizer. Thiamin was oxidized to thiochrom and the fluorescence of thiochrom was measured. The measurement of fluorescence at 370 nm was used for the determination of riboflavin after exposure of sample to 40 W 40 min^{-1} and carrying out riboflavin to lumiflavin. Fibre was determined by Scharrer-Kürschner method. Carbohydrates content was calculated as a percent of dry matter. All the determinations were carried out in 2 or 3 parallels.

Statistical analysis: An estimate of relative deviation ($s_R = k_n \cdot R$) was made from differences of limiting values (R) and tabulated coefficient $k_n = 0.8862$ (if $n = 2$) or $k_n = 0.5908$ (if $n = 3$) (ECKSCHLAGER et al., 1980).

2. Results and discussion

2.1. Dry matters, ash, fibre

Seeds absorb a great amount of water during soaking, legumes 60%, grains 50% of their weight. Decreased content of ash was observed in germinated barley (–10%), lentil (–12%) and soya (–23%), on the contrary, 7% increase in case of wheat. It corresponds well with literature data. It could be explained by leaching of minerals into the soaking water during relatively long soaking of seeds. Substantial increasing of fibre content was detected, in all four germinated seeds. The highest increase was observed in soya, 74%, smaller in wheat, 58%, barley 48% and lentil 44%. Our diet is generally short dietary fibre, therefore this increasing might be of importance.

Table 1

Content of some macronutrients in dry and germinated seeds

Sample	Dry material (%)		Ash (%)		Fibre (%)		Total N (%)		Crude fat (g kg^{-1} d.m.)		Sacchar. sol. (g kg^{-1} d.m.)	
	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s
Wheat D	90.64	0.42	16.25	0.97	25.28	0.92	22.42	1.16	15.68	0.42	814.99	0.76
Wheat G_1	42.34	0.11	17.32	0.71	40.39	1.08	22.64	0.11	6.23	0.30	807.01	1.24
Wheat G_2	43.12	0.32	17.54	0.64	39.99	1.01	22.51	0.69	5.98	0.12	808.18	0.86
Barley D	91.19	0.16	24.21	0.88	51.76	0.48	15.72	0.46	20.78	0.18	813.65	0.88
Barley G_1	43.13	0.35	21.70	0.30	76.15	0.67	15.93	1.24	7.73	0.41	803.62	0.69
Barley G_2	43.56	0.11	21.93	0.64	76.73	0.74	15.85	0.64	7.35	0.27	805.36	1.59
Lentil D	89.36	0.21	37.38	0.92	43.29	1.08	40.38	0.92	15.62	0.39	673.54	1.08
Lentil G_1	33.90	0.04	32.91	1.11	62.13	0.76	40.72	1.00	7.37	0.41	665.49	1.11
Lentil G_2	32.74	0.37	33.12	1.01	62.87	0.49	40.59	1.08	7.22	0.28	665.43	0.92
Soya D	91.88	0.21	54.36	1.09	64.04	1.22	46.30	0.97	172.21	0.83	445.48	0.92
Soya G_1	35.49	0.20	41.49	0.86	111.88	0.81	46.36	0.81	143.07	0.71	439.32	0.59
Soya G_2	35.05	0.26	41.74	0.79	112.11	0.58	46.54	0.52	143.65	0.42	437.22	0.70

d.m.: dry matter; D: dry; G: germinated; $\bar{x}$: mean value; s: standard deviation

2.2. Protein, fats, carbohydrates

The protein content did not change in any of the samples. In some cases 1–2% increase was observed, but it was not conclusive. Essential changes were observed in fats and not significant ones in saccharides. Both components are the main energy sources in the biochemical processes of germination. The fat content decreased in wheat, barley, lentil and soya by about 60, 60, 53, 17%, respectively. The results of analysis in dry and germinated seeds are summarized in Table 1.

2.3. Amino acids, minerals, vitamines

In consequence of rapid increasing of enzymatic activity comes to fragmentation of protein into amino acids during germination of seeds. Protein turned more digestible. This fact is in agreement with literature data, too (SWANSTON & TAYLOR, 1990; AHOKAS & NASKALI, 1990). Of the total amino acids (AA) especially the essential AA-s were investigated, as presented in Table 2. During germination, as it can be seen, rather important increasing of essential AA occurs in grains, by 14 to 23%, less in legumes, up to 5%. The content of AA methionine increased by more than 100% in legumes and about 50% in case of phenylalanine in grains.

Table 2

Content of essential and total amino acids in dry and germinated seeds
(g kg^{-1} d.m.)

Amino acids	Wheat		Barley		Lentil		Soya	
	D	G	D	G	D	G	D	G
Essential								
Leu	6.63	7.65	5.39	5.67	15.47	15.87	18.21	19.99
Ileu	2.85	3.40	2.49	2.83	7.76	7.68	10.24	10.40
Lys	3.42	4.06	3.95	4.55	17.43	17.60	17.19	18.10
Val	4.96	5.47	5.24	5.71	11.65	11.13	11.86	11.75
Phe	4.61	6.96	3.64	4.94	11.64	11.82	12.60	12.60
Thre	3.02	3.59	3.26	3.40	9.28	9.43	10.62	10.67
Met	1.15	1.70	0.79	1.16	0.25	0.88	0.73	1.65
SEAA	26.68	32.84	24.74	28.10	73.48	74.40	81.46	85.65
SOAA	72.65	82.20	55.06	59.87	141.88	148.27	173.81	165.19
STAA	99.33	115.04	79.80	87.97	215.36	222.67	255.27	250.84

D: dry
G: germinated

SEAA: sum of essential AA
SOAA: sum of other AA
STAA: sum of total AA

Table 3

Content of minerals and vitamines in dry and germinated seeds

Sample	Ca (g kg^{-1} d.m.)		P (g kg^{-1} d.m.)		Fe (g kg^{-1} d.m.)		L-ascorbic acid (mg kg^{-1} d.m.)		Thiamine (mg kg^{-1} d.m.)		Riboflavin (mg kg^{-1} d.m.)	
	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s
Wheat D	1.700	0.21	1.440	0.21	37.935	0.08	0		5.64	0.25	3.75	0.42
Wheat G_1	1.491	0.16	1.570	0.74	13.630	0.45	393.62	2.09	5.10	0.49	7.36	0.58
Wheat G_2	1.571	0.20	1.671	0.42	14.136	0.35	398.86	1.80	3.05	0.51	7.35	0.50
Barley D	0.633	0.32	1.495	0.63	28.303	0.29	0		3.94	0.60	2.08	0.59
Barley G_1	0.550	0.21	0.510	0.28	10.431	0.32	440.67	0.90	3.67	0.43	5.79	0.39
Barley G_2	0.482	0.30	1.488	0.48	11.654	0.24	444.21	1.20	3.98	0.52	6.23	0.61
Lentil D	0.786	0.34	2.393	0.28	37.250	0.35	0		8.58	0.50	2.46	0.41
Lentil G_1	0.738	0.42	2.390	0.43	23.914	0.42	386.66	0.88	7.30	0.61	4.23	0.35
Lentil G_2	0.724	0.36	2.374	0.40	23.689	0.40	380.12	1.00	6.90	0.58	4.83	0.42
Soya D	2.503	0.36	3.261	0.29	46.475	0.16	0		13.77	0.60	2.62	0.50
Soya G_1	1.861	0.12	2.420	0.32	20.599	0.52	422.96	0.93	7.98	0.12	5.53	0.43
Soya G_2	1.943	0.38	2.385	0.40	20.845	0.13	423.73	0.78	8.25	0.36	5.68	0.38

d.m.: dry matter; D: dry; G: germinated; $\bar{x}$: mean value; s: standard deviation

The ash was analysed for Ca, P and Fe. All mentioned minerals decreased in all samples. We suppose that it was caused by leaching of minerals into soaking water. The content of Fe decreased by about 30 to 60%, Ca 7 to 24%. The phosphorus content ranged from slight decreasing to small increasing. It is known that P in grain is bound in the form of phytate or phytic acid. Decreased content of P during soaking is probably balanced with P liberated from the bound form.

Biosynthesis of ascorbic acid is one of the most interesting and important factors in the germination process. Ascorbic acid, which is not present in dry seed at all, reaches measurable values in germinated samples. The content was nearly 20 mg per 100 g of fresh germinated sample (38–44 mg per 100 g d.m.) which means an important contribution of vitamin C in human diet if an acceptable food product could be prepared. In all seeds during germination the decrease of thiamin was registered. This 9 to 45% decreasing could be explained by the fact, that thiamin is a cofactor of many enzymatic systems. The rapid increase of enzymatic activity in the process of germination is known. In a sample of barley, where germination runs not so rapidly, decreasing of thiamin content was not detected. On the other hand, the content of riboflavin in germinated seeds appreciably increased by 90 to 180%. The results of analyses of minerals and vitamines are summarized in Table 3.

3. Conclusions

Germination as the first step in the process of growth of plants is accompanied with great changes in the chemical composition of the seeds. These changes are different and they are dependent mainly on the duration of germination. It may be a short time germination, which was used in our experiments (48–96 h) or long time (7–10 days).

During soaking seeds absorb water and many biochemical reaction starts (RAHMA, 1987; REDDY, 1984). A great part of the storage matter is degraded, new compounds are synthetized or the content of originally present compounds increases. In our experiments soya and lentil seeds absorbed more water (57% of their weight) while wheat and barley about 10% less.

The ash content of the germinated seeds was about 10–20% less than that of the dried seeds. This decreasing of total mineral content can be explained by leaching minerals into soaking water (especially when distilled water was used). Essentially smaller permeability of aleurone layer of wheat and barley seed caused slower water absorption and less losses of minerals by the soaking. Regarding the microelements, Fe content decreased (30–60%) more than that of the Ca (7–24%). It is known, that part of Ca in corn may be bound in form of phytate (content of phytic acid in wheat and barley is about 0.5–1.0%). Phytic acid and phytate (Ca, Mg) are in a great part degraded during germination and Ca, P, Mg become biologically available. It is

confirmed in works of GHAZALI and CHENG (1991); LARSSON and SANDBERG (1993) and BORADE (1984). During germination not only degradation of phytic acid occurs but that of the other antinutritive factors, too (tripsin inhibitor, tannin glucosinolate) (SHARMA & SEGHAL, 1992; MARERO et al., 1991).

During short time germination of barley, wheat, soya and lentil no significant changes in the content of total protein was determined. The increase of the essential AA content (5–23%) is important. The increased content of total amino acids in germinated seeds (at some AA up to 50–100%) testifies, that in the process of germination the degradation of protein occurs, they become easier digestable. Similar results are found in works of SAVELKOUL (1992) and GHAZALI and CHENG (1991).

The crude fat content during germination of wheat, barley, soya and lentil greatly decreased, by about 17 to 60%. Similar decreasing of fat content was noticed by GHAZALI and CHENG (1991) in black gram and BOG LEE and KI WOON (1993) in soya beans at short time germination (1 to 3 days). However, last authors also observed that total fat and tocopherols increased when seeds were germinated until the length of the sprouts was nearly 10 cm.

The content of carbohydrates decreased slightly in our experiments in agreement with the results of FURUTA and co-workers (1991) (lentil) and VIDAL--VALVERDI and FRIAS (1992) (soya).

The greatest change was noticed in the fibre content during germination; 44–47% increase was measured in all kind of seeds. The content of soluble carbohydrates decreased during the germination by only about 1%. It is, therefore, possible to suggest, that the components of fibre (lignin, cellulose) were built from polysaccharides (mainly starch). VIDAL-VALVERDI and FRIAS (1992) dealt with this problem in details in case of two var. of lentil and they have achieved similar results as we in our experiments.

The initiation of the biosynthese of L-ascorbic acid during germination is remarkable. Within 2–4 days the content of this vitamin has reached nearly 400 mg kg^{-1} of fresh weight level. This value, in our geographic position, can be considered as middle high in comparison with the content of L-ascorbic acid in fresh fruits and vegetable. The content of L-ascorbic acid in germinating barley, wheat, lentil and soya is even higher than it is in tomatoes, vegetables and green peas. Among fruits only strawberries, black currant and hips have higher L-ascorbic acid content than germinated seeds. Similar results were obtained by WATZL and LEITZMANN (1984); GHAZALI and GOH (1991); GHAZALI and CHENG (1991); VIDAL-VALVERDI and FRIAS (1992). The same intense increasing of carotenoid content was found by GHAZALI and GOH (1991) in germinating beans.

In germinating seeds (in all four samples) a remarkable increasing of riboflavin content (90–180%) was observed. GHAZALI and CHENG (1991) documented thiamin

content in germinated black gram; while in our experiments the content of this vitamin decreased. The results obtained for the nutritive components in germinated seeds are thus rather different. The comparison of the results is not quite reliable because of the different conditions during germination.

It can be therefore said that the total nutritive value of germinated seeds is much higher than that of their dry originals. Germination decreases the content of fat and that of the antinutrition factors. Proteins of germinated seeds are more digestible and the essential amino acid content is increased. During germination the L-ascorbic acid, riboflavin and fibre content essentially arises. Figure 1 shows the changes in the content of nutritive components of germinated samples (wheat, barley, soya and lentil) in the percent of the original counterpart.

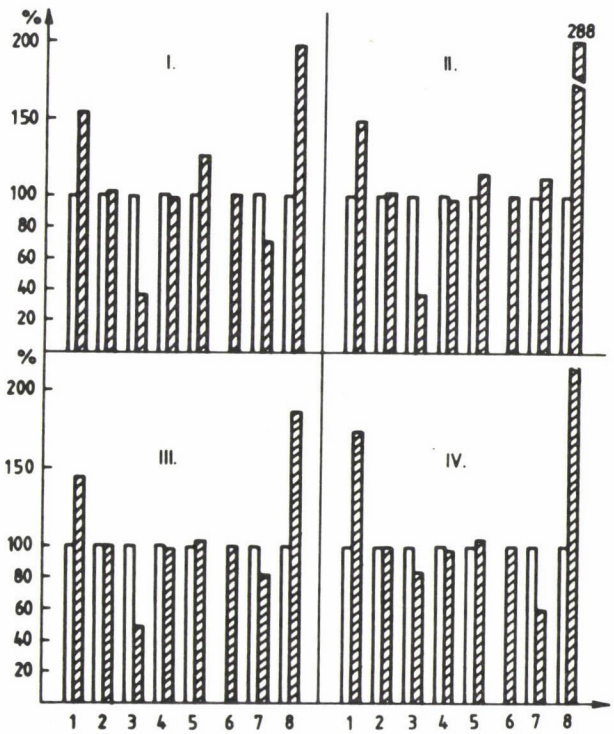

Fig. 1. Comparison of percentual content of some nutrients in dry □ and ▨ germinated: I: wheat, II: barley, III: lentil, IV: soya. 1: fibre, 2: total N, 3: crude fats, 4: saccharides, 5: essential AA, 6: ascorbic acid, 7: thiamine, 8: riboflavin

Wheat, barley, soya and lentil seeds with 1 cm long sprouts are shown in Fig. 2 after short time (2–4 days) germination. They can be consumed as whole corn and sprouts. Germinated wheat is consumable even without heat treatment. Legumes, because of the antinutritive factors content (germination do not degrade them totally) must be heat treated before eating. Germinated corns, legumes and other seeds could significantly enrich the human diet, mainly in winter season.

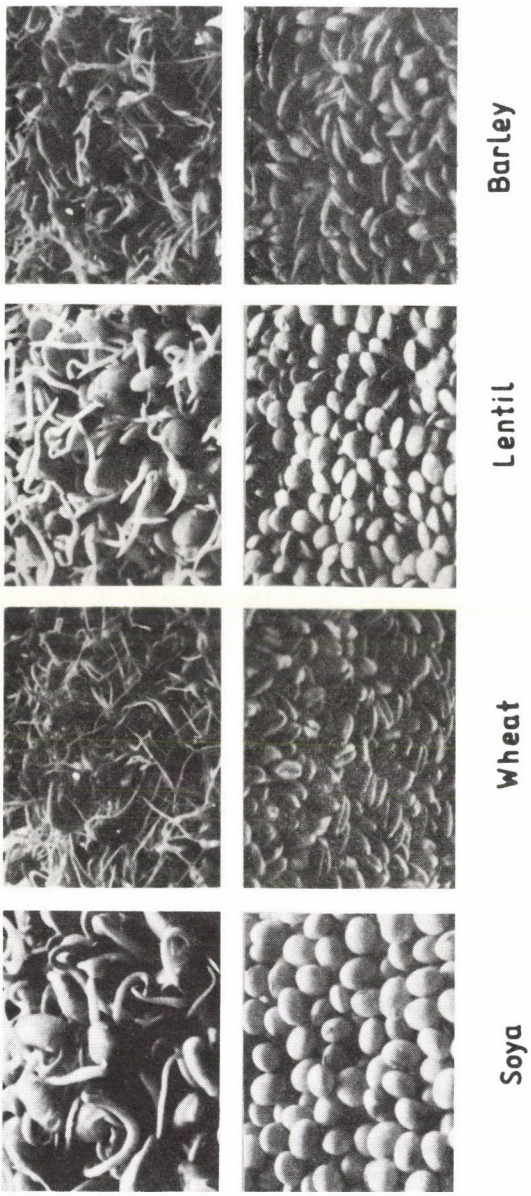

Fig. 2. Appearance of dry and germinated seeds

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ANALYSIS AND QUANTITATION OF SUGARS IN HONEY OF DIFFERENT BOTANICAL ORIGIN USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Sugars of different unifloral honeys from acacia, limetree, sweet chestnut, sunflower, eucalyptus, sage, mixed floral, cockshead, common milkweed and honeydew were determined as potential floral origin characterising parameters. The HPLC separation system was composed of an amino-bound silica gel column (Supelcosil-NH_2 250×4.6 mm, 5 μm particle size) and 83–17% (v/v) acetonitril–water as eluent. The sugars were detected by a Refractive Index Detector.

Ten sugars could have been identified: fructose, glucose, sucrose, turanose, maltose, isomaltose, erlose, melecitose, maltotriose and raffinose. Most of the chromatograms contained 2–3 more peaks, probably disaccharides.

Keywords: honey, sugar composition, HPLC

Organoleptic properties and pollen analysis have been used for long to distinguish between honeys of different botanical origin. Certain color ranges, aromas, flavors and pollens are usually characteristic of a given floral type. The sensory assessment of these organoleptic properties is generally highly subjective and the pollen content and spectrum are influenced by different factors.

The chemical composition of honey is complex and the contents of individual constituents vary considerably (ZANDER & KOCH, 1975; WHITE 1978). Surveys of floral honey composition have established that the three major components are fructose, glucose and water, averaging 38.2%, 31.3% and 17.2% respectively (DONER, 1977). Besides them di- and trisaccharides and some higher sugars, especially in honeydew honey, have been identified.

GLC analysis of trimethylsilyl (TMS) derivatives of sugars and sugar oximes have been reported by several authors (HADORN & ZÜRCHER, 1974; IVERSON & BUENO, 1981; BROBST & SCOBELL, 1982; LOW & SPORNS, 1988). These methods are accurate and sensitive but sample derivatization is time-consuming and often not quantitative.

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The separation, identification and quantitation of carbohydrates by high performance liquid chromatography (HPLC) have recently received an increasing attention. The HPLC separations require specific columns e.g. alkylamine bonded phases (BINDER, 1980; BOGDANOV & BAUMANN, 1988; MAUCH et al., 1987; MÜLLER & SIEPE, 1980; NIKOLOV et al., 1984), cation and anion exchange resins (BROBST & SCOBELL, 1982; POURTALLIER et al., 1990; SWALLOW and LOW, 1990) or α- and β-cyclodextrin bonded phases (ARMSTRONG & JIM, 1989). The detectors were refractive index or pulsed amperometric ones. The amino-bonded silica columns use acetonitril–water mobile phase and are probably the most commonly used packings for the separation of carbohydrates today.

The aim of the works in the papers cited above was the employment of new methodologies, mainly.

The object of the recent study was to investigate the sugar differences of composition in honeys of known botanical origin.

1. Materials and methods

1.1. Apparatus

Moisture determination was performed by first measurement of the refractive index value (Abbe refractometer at 20 °C). Average refractive index values were converted to honey moisture contents using the table developed by Wedmore (AOAC, 1990).

The HPLC column used was Supelcosil NH_2, 250 × 4.6 mm i.d., packed with 5 μm average particle size stationary phase, the mobile phase was acetonitril-water (83–17) as eluent. The pump (Labor MIM Liquopump 312/1) was run at a flow rate of 1.8 $cm^3\ min^{-1}$. Samples were injected using a 20 μl loop and detected by a Hewlett Packard Refractive Index 1047A detector at 35 °C. Peak areas were integrated by a Hewlett Packard 3396A computing integrator.

1.2. Sample preparation

The honey samples were provided and the sensory assessment and pollen analysis were carried out by the Lukács és társa Laboratórium BT (Budapest, Hungary). Altogether 35 samples, 7 acacia (*Robinia pseudoacacia*), 8 limetree (*Tilia cordata*), 5 sweet chestnut (*Castania sativa*), 3 common milkweed (*Asclepias syriaca*), 5 mixed floral, 2 sunflower (*Helianthus annuus*), 1–1 sage (*Salvia officinalis*), bear's onion (*Allium ursinum*), eucalyptus (*Eucalyptus sp.*), cockshead (*Onobrychis viciifolia*) and honeydew honey were investigated. Sample treatment for HPLC analysis: 4.6–4.8 g sample was dissolved in 25 cm^3 distilled deionised water and

diluted with acetonitril to 50 cm^3. Each standard was injected separately to determine the retention time. Mixed sugar standards were prepared in the same way as honey solutions. To determine the concentration of the carbohydrates multilevel calibration method was used. Before injection, all solutions were filtered through a Samplex-NH_2 (0.45 μm) cartridge (Bio-Separation Technology, Hungary). All samples were analysed in three parallels.

2. Results and discussion

The results of the analysis are summarised in Tables 1–4. Table 1 shows the total sugar content of different unifloral honeys, the amount of individual sugars, the fructose–glucose ratio and the range of these values. In case of saga, eucalyptus, cockshead, bear's onion and honeydew honeys the mean values of the subsequent analysis of the individual samples could have been presented only.

The total sugar content of floral honeys were 76.59–80.20% and that of the honeydew honey was 71.70%. These values are similar to the findings of WHITE (1978) and BOGDANOV and BAUMANN (1988). Since the moisture content of the samples varied from 14.8% to 19.9% for comparison the individual sugars were expressed in dry matter. Among the 34 floral honeys the fructose content of acacia honeys was the highest and the glucose content was the lowest with 49.59% and 30.44%, respectively. On the basis of the fructose content honeys could be classified into 5 groups as follows:

- acacia honey (~49%),
- common milkweed, sage, sweet chestnut and mixed floral honeys (45–46%),
- limetree, sunflower and eucalyptus honeys (42–43%),
- cockshead honey (~36%),
- honeydew honey (~32%).

The percentages of sucrose were low except for cockshead honey. Considering the sucrose content cockshead honey was supposed to be adulterated. During 10 months storage DEIFEL and co-workers (1985) found a considerable decrease in sucrose, less decrease in erlose and an increase in maltose content. As the HPLC analysis was carried out after a 6–7 months waiting period of the samples the low sucrose content might be the consequence of the storage time.

The average erlose content was about 3% in acacia, common milkweed and sage honeys, 1% in mixed floral, sunflower, sweet chestnut, limetree and honeydew honeys and 0.18–0.62% in eucalyptus, bear's onion and cockshead honeys.

No, or only trace amounts of melecitose and raffinose have been found in floral honeys except for one sweat chestnut honey, but in honeydew honey 6.17% and 2.00% of these sugars were found, respectively. This property distinguishes mostly the floral honeys from the honeydew honey (DONER, 1977).

Table 1

Sugar contents of honeys of different botanical origin

Parameters	Acacia n=7			Limetree n=8			Sweet chestnut n=5			Common milkweed n=3		
	$\bar{x}$	min	max	$\bar{x}$	min	max	$\bar{x}$	min	max	$\bar{x}$	min	max
Total sugar %	79.93	77.25	83.07	78.67	74.35	82.07	78.89	77.3	83.60	80.2	78.62	81.53
Fructose %[a]	49.59	47.65	51.17	42.96	39.96	49.95	45.51	44.11	49.55	46.10	44.71	46.81
Glucose %[a]	30.44	28.70	32.85	37.04	34.06	37.61	33.1	29.31	37.61	32.06	29.47	33.64
Sucrose %[a]	1.13	0.13	3.78	0.44	0	0.67	0.23	0	0.54	1.02	0.77	1.49
Turanose %[a]	3.06	2.70	3.62	2.28	1.83	3.18	2.71	2.13	3.11	2.69	2.49	2.98
Maltose %[a]	4.22	3.66	4.72	3.51	2.19	5.25	4.55	4.47	4.90	4.44	3.79	5.09
Isomaltose %[a]	0.55	0.42	0.78	1.61	0.53	3.31	0.91	0.49	0.87	0.43	0.38	0.47
Erlose %[a]	2.94	1.23	3.62	1.00	0.15	2.57	1.12	0.57	1.61	2.91	2.34	3.35
Melecitose %[a]	0.00	0.00	0.00	0.04	0	0.08	1.04	0	5.08	0.00	0.00	0.00
Maltotriose %[a]	0.15	0.05	0.30	0.17	0.05	0.22	0.20	0.03	0.32	0.23	0.16	0.29
Raffinose %[a]	0.00	0.00	0.00	0.01	0	0.08	0.01	0	0.03	0.04	0.01	0.06
Fructose - Glucose ratio	1.63	1.56	1.68	1.16	1.09	1.44	1.37	1.24	1.63	1.44	1.32	1.58

continued on p. 301

Table 1 (continued)

Parameters	Mixed flower n=5			Sunflower n=2			Sage n=1	Eucalyptus n=1	Bears onion n=1	Cockshead n=1	Honeydew n=1
	$\bar{x}$	min	max	x	min	max	$\bar{x}$	$\bar{x}$	$\bar{x}$	$\bar{x}$	$\bar{x}$
Total sugar %	78.84	76.78	80.67	77.35	75.99	78.93	78.95	77.11	76.85	76.59	71.70
Fructose %[a]	45.09	42.48	47.39	42.9	41.62	44.25	46.23	42.54	44.09	36.42	32.20
Glucose %[a]	37.07	32.63	40.1	39.51	37.34	41.74	31.20	37.95	39.46	34.27	29.50
Sucrose %[a]	0.48	0.12	0.64	0.53	0.42	0.64	2.87	0.53	0.28	17.62	0.00
Turanose %[a]	1.90	1.08	2.71	1.19	0.91	1.50	2.80	2.21	1.74	0.00	2.24
Maltose %[a]	3.81	2.98	5.72	2.40	2.21	2.47	4.57	4.86	2.20	0.86	4.80
Isomaltose %[a]	0.39	0.2	0.53	0.11	0.03	0.18	0.47	0.68	0.71	0.08	2.37
Erlose %[a]	1.33	0.54	2.62	1.05	0.91	1.16	2.83	0.62	0.18	0.21	1.14
Melecitose %[a]	0.05	0.02	0.15	0.00	0.00	0.00	0.00	0.16	0.19	0.00	6.17
Maltotriose %[a]	0.14	0.01	0.26	0.09	0.08	0.10	0.06	0.21	0.00	0.06	0.24
Raffinose %[a]	0.03	0	0.05	0.03	0	0.04	0.00	0.02	0.00	0.06	2.00
Fructose - Glucose ratio	1.22	1.09	1.44	1.09			1.48	1.12	1.12	1.06	1.09

n: number of samples;
$\bar{x}$: mean value;
min–max: range of measured values;
[a]: expressed in dry matter

Table 2

Composition of acacia honeys

Components	Sample No.								
	1	2	3	4	5	6	7	$\bar{x}$	s
Robinia pollen %	41	36	43	44	41	31	40	39.43	4.50
Cruciferae pollen %	8	7	10	3	6	4	1	5.57	3.10
Moisture %	15.5	18.3	18.2	18.5	19.6	16.2	15.7	17.43	1.60
Total sugar %	82.66	78.08	79.96	77.32	78.59	82.28	80.6	79.93	2.06
Fructose %	43.43	42.54	44.33	43.36	42.83	43.05	43.18	43.25	0.57
Glucose %	26.34	26.99	27.26	25.82	27.47	25.79	26.05	26.53	0.70
Sucrose %	2.26	0.28	0.12	0.26	0.2	3.29	0.57	1.00	1.26
Turanose %	2.9	2.31	2.54	2.6	2.56	2.62	3.18	2.67	0.28
Maltose %	4	3.11	3.44	3.41	3.5	4.08	4.25	3.68	0.42
Isomaltose %	0.48	0.39	0.55	0.6	0.46	0.39	0.47	0.48	0.08
Erlose %	3.1	2.36	1.6	1.18	1.43	3	2.67	2.19	0.78
Maltotriose %	0.15	0.1	0.12	0.09	0.14	0.06	0.23	0.13	0.05
Fructose - Glucose ratio	1.65	1.58	1.63	1.68	1.56	1.67	1.66	1.63	0.05

$\bar{x}$: mean value of the samples;
s: standard deviation

Table 3

Composition of limetree honeys

Components	Sample No.									
	1	2	3	4	5	6	7	8	$\bar{x}$	s
Tilia pollen %	13	18	86	14	43	19	83	14	36.25	31.32
Moisture %	15.3	19.6	18.4	17.6	18.2	18.9	17.1	16.5	17.70	1.38
Total sugar %	78.51	80.49	80.09	74.95	76.42	79.99	78.89	80.06	78.68	2.00
Fructose %	38.02	39.43	42.51	36.68	36.23	37.62	37.34	35.81	37.96	2.16
Glucose %	34.08	32.85	29.5	29.97	32.57	34.03	32.94	36.29	32.78	2.21
Sucrose %	0.25	0.14	1.17	0	0.54	0.21	0.25	0.52	0.40	0.39
Turanose %	1.79	2.41	2.36	2.63	1.62	1.83	1.95	1.49	2.01	0.41
Maltose %	3	3.75	1.91	4.44	3.86	2.39	3.18	2.3	3.10	0.88
Isomaltose %	0.68	0.76	0.49	0.95	0.68	2.86	2.34	2.63	1.42	1.00
Erlose %	0.56	0.98	2.03	0.15	0.67	0.73	0.62	0.73	0.81	0.55
Melecitose %	0	0	0	0.04	0.06	0.05	0.07	0.07	0.04	0.03
Maltotriose %	0.12	0.17	0.13	0.05	0.12	0.27	0.19	0.19	0.16	0.07
Raffinose %	0	0	0	0.03	0.06	0	0	0	0.01	0.02
Fructose - Glucose ratio	1.12	1.2	1.44	1.22	1.11	1.1	1.13	0.99	1.16	0.13

$\bar{x}$: mean value of the samples;
s: standard deviation

Table 4

Composition of sweet chestnut honeys

Components	Sample No.						
	1	2	3	4	5	$\bar{x}$	s
Castania pollen %	76	96	91	56	94	82.60	16.82
Moisture %	15.2	16.8	17.8	17.2	15.3	16.46	1.16
Total sugar %	80.13	78.12	77.41	82.14	77.58	79.08	2.03
Fructose %	39.66	43.21	42.29	41.37	34.78	40.26	3.33
Glucose %	30.76	26.95	26.09	32.89	29.78	29.29	2.79
Sucrose %	0.35	0.11	0	0.32	0.25	0.21	0.15
Turanose %	2.71	2.5	2.69	2.09	2.02	2.40	0.33
Maltose %	4.33	4.01	4.03	4.22	3.57	4.03	0.29
Isomaltose %	0.7	0.76	0.7	0.45	1.42	0.81	0.36
Erlose %	1.39	0.52	1.43	0.67	0.78	0.96	0.42
Melecitose %	0	0	0.07	0.06	4.53	0.93	2.01
Maltotriose %	0.23	0.06	0.09	0.06	0.43	0.17	0.16
Raffinose %	0	0	0.03	0.02	0	0.01	0.01
Fructose - Glucose ratio	1.29	1.6	1.62	1.25	1.17	1.39	0.21

$\bar{x}$: mean value of the samples;
s: standard deviation

According to the Hungarian standard honeys of different botanical origin are distinguished according to their sensory assessment and pollen contents. Tables 2–4 show the composition of the individual acacia, limetree and sweet chestnut honeys. These tables contain the main pollens, moisture and sugar contents, the average and standard deviation data. From these tables the variations of the individual unifloral honey samples can be seen.

Table 2 shows the composition of the individual acacia honey samples. Acacia honeys have to contain 30% of Robinia pollens. All the samples contained more than that. There were no great differences in the amounts of the sugars except for sucrose and erlose. Every sample showed a high fructose–glucose ratio, that is typical for acacia honeys.

Table 3 shows the composition of the individual limetree honeys. Though the limetree pollen content should be 20%, 5 of the samples contained less. According to their sensory assessment they were limetree honeys. In sample 3 Tilia pollen was very high, the sugar composition was similar to those of acacia honeys. No, or negligible amounts of melecitose and raffinose were detected. Limetree and acacia honeys could be distinguished by their fructose–glucose ratio.

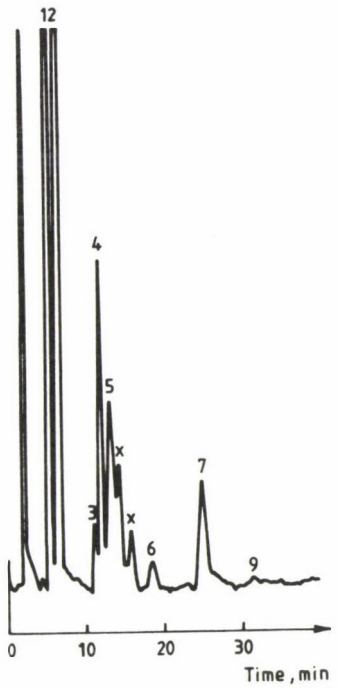

Fig. 1. Chromatogram of an acacia honey. Column: Supelcosil NH_2 (250×4.6 mm i.d.); mobile phase: acetonitril/water (83/17); flow rate: 1.8 cm^3 min^{-1}; column temperature: 35 °C; refractive index detector. Peaks, 1: fructose, 2: glucose, 3: sucrose, 4: turanose, 5: maltose, 6: isomaltose, 7: erlose, 9: maltotriose, x: unidentified disaccharides

Table 4 shows the composition of sweet chestnut honeys. Although very high amount of Castania pollen could be detected in each sample, samples 2 and 3 were similar to acacia honeys on the basis of sugar composition and fructose–glucose ratio. According to fructose, glucose and melecitose contents sample 5 was similar to honeydew honey.

Figures 1–3 show the chromatograms of acacia, limetree and sweet chestnut honeys, respectively. As it can be seen on the chromatograms the dominance of the mono- and disaccharides and the presence of erlose were typical of floral honeys.

Figure 4 shows the chromatogram of the honeydew honey. Besides of fructose and glucose, melecitose and raffinose were dominant.

Some peaks could not be identified in lack of analytical standards. On the basis of retention times they were most probably the disaccharides kojibiose, nigerose and maltulose.

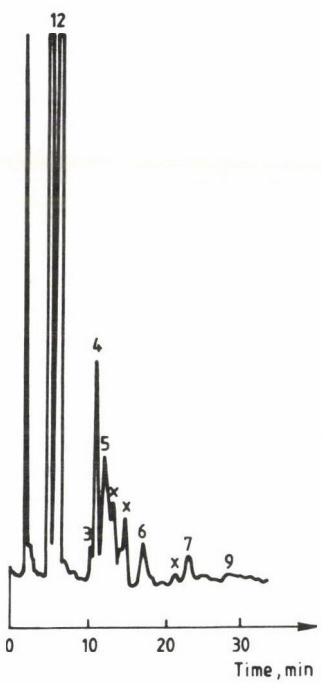

Fig. 2. Chromatogram of a limetree honey. Column: Supelcosil NH_2 (250×4.6 mm i.d.); mobile phase: acetonitril/water (83/17); flow rate: 1.8 cm^3 min^{-1}; column temperature: 35 °C; refractive index detector. Peaks, 1: fructose, 2: glucose, 3: sucrose, 4: turanose, 5: maltose, 6: isomaltose, 7: erlose, 9: maltotriose, x: unidentified disaccharides

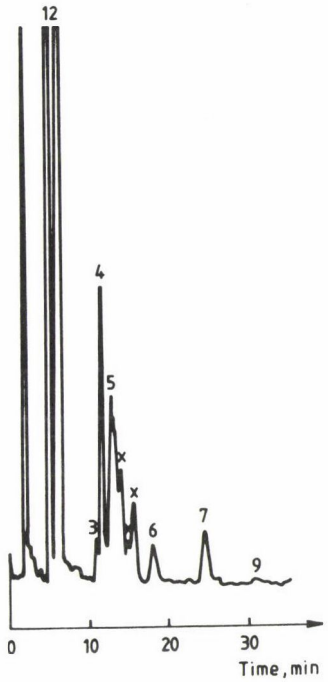

Fig. 3. Chromatogram of a chestnut honey. Column: Supelcosil NH_2 (250×4.6 mm i.d.); mobile phase: acetonitril/water (83/17); flow rate: 1.8 cm^3 min^{-1}; column temperature: 35 °C; refractive index detector. Peaks, 1: fructose, 2: glucose, 3: sucrose, 4: turanose, 5: maltose, 6: isomaltose, 7: erlose, 9: maltotriose, x: unidentified disaccharides

3. Conclusion

Thirteen different sugars were separated by HPLC technique in the investigated honey samples. Ten of them were identified and the others are probably disaccharides.

The sugar composition alone was not enough to identify the botanical origin of the honeys, but according to the monosaccharide and trisaccharide composition and quantity, floral honeys could be distinguished from honeydew honey. These findings were similar to those of BOGDANOV and BAUMANN (1988), DONER (1977) and WHITE (1978).

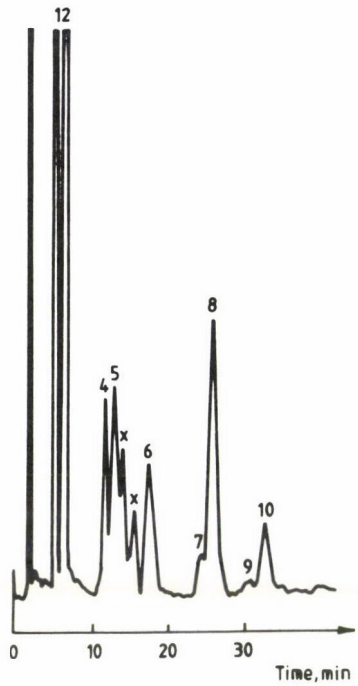

Fig. 4. Chromatogram of a honeydew honey. Column: Supelcosil NH_2 (250×4.6 mm i.d.); mobile phase: acetonitril/water (83/17); flow rate: 1.8 cm^3 min^{-1}; column temperature: 35 °C; refractive index detector. Peaks, 1: fructose, 2: glucose, 4: turanose, 5: maltose, 6: isomaltose, 7: erlose, 8: melecitose, 9: maltotriose, 10: raffinose, x: unidentified disaccharides

All acacia honeys stand out regarding their high fructose content and fructose–glucose ratio. This was in agreement with the findings of KRAUZE and ZALEWSKI (1991).

On the basis of the fructose content honeys could be classified into 5 groups. More physical and chemical parameters should be investigated (BONVEHI & COLL, 1993; KRAUZE & ZALEWSKI, 1991) to find characteristic differences among floral honeys.

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CHARACTERIZATION OF THE LIPOLYTIC SYSTEM OF GERMINATED SUNFLOWER SEEDS

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Current interest in bioengineering has been extended recently to the exploration of lipase-catalyzed reactions for the commercial processing of oils and fats. Sunflower seedlings, but not dormant seeds, contain a very active lipolytic system associated with oil bodies located in cotyledon cells, showing two peaks of maximum activity (pH 3.2 and 8.6) and being notably stimulated by Triton X–100 and higher temperatures (75–85 °C). GLC of fatty acids evidenced that the lipolytic system of sunflower shows higher affinity towards saturated fatty acids, and HPLC analysis of the residual triacylglycerols (TAG) revealed lesser affinity for TAG with higher oleic acid content. Some properties of the lipolytic system of sunflower could be potentially useful in bioengineering processes.

Keywords: sunflower, lipase, *Helianthus annuus*, oil bodies

Applications of lipase-catalyzed reactions, such as hydrolysis of fats and oils for the production of fatty acids and esterification or interesterification of fats and other lipids for the preparation of diverse products in food and no-food industries, are increasing. At present, the application of lipases in biotechnological processes seems to be economically feasible and appropriate mainly for the preparation of specific products of high commercial value, which cannot be prepared conveniently by chemical synthesis (MUKHERJEE, 1990).

Most of the lipase preparations are of microbial origin (MACRAE, 1983), but oilseeds have a good potential as a source of lipases, as seedlings are easily to grow and harvest and crude lipase preparations can be obtained by extraction with buffer solutions or as acetone powder (HASSANIEN & MUKHERJEE, 1986). Nevertheless, only a few number of seeds have been studied for lipase content (ARRIBÉRE, 1993) and solely three of them have been purified to homogeneity (HAMMER & MURPHY, 1993).

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In the present paper we report the biochemical properties of the lipolytic system located in cotyledon cells of sunflower seedlings and point out its valuable characteristics for application in biotechnological processes.

1. Materials and methods

1.1. Plant material

Sunflower seeds (*Helianthus anuus*, L. cv. ACA 871) were provided by Asociación de Cooperativas Argentinas C. L. (Pergamino, Argentina). The seeds were soaked in running tap water for 24 h at room temperature (20 °C) and then allowed to germinate on moist paper towels at 30 °C in darkness (ARRIBÉRE, 1993).

1.2. Enzyme preparations

Cotyledons from 2-day-old seedlings were carefully removed from the rest of the plant material, washed with distilled water and homogenized for 1 min (Omnimixer, Sorval) with 0.1 M Tris-HCl buffer (pH 7.5) at 4 °C (2 cm^3 of buffer per g of cotyledons). The homogenate was filtered through a piece of nylon cloth (pore size: 0.20 mm^2) and the filtrate ("cotyledon extract") was centrifuged at 11,000 g for 30 min at 4 °C. The fatty layer floated to the top and was carefully removed with a spatula, resuspended in the same buffer (100 mg cm^{-3}), and centrifuged again as above: the resulting fatty layer containing the washed, isolated oil bodies (HUANG, 1992) was resuspended in distilled water (100 mg cm^{-3}). The supernatants were collected and the pellets separated and resuspended in 0.1 M phosphate buffer (pH 7.0).

1.3. Determination of lipolytic activity

Lipase activity was assayed by a colorimetric method based on the estimation of the absorbance at 715 nm of the isooctane soluble copper complexes formed by the fatty acids released during lipolysis (KWON & RHEE, 1986).

The assay mixture contained 2 cm^3 buffer solution, 5 cm^3 isooctane, and 2 cm^3 resuspended fatty layer (containing both enzyme and endogenous substrate), added in that sequence. In the case of the supernatant and pellet 0.5 cm^3 of sunflower oil (native substrate) was dissolved in 4.5 cm^3 of isooctane prior to be added to the assay mixture.

The mixture was stirred for 2 min at 37 °C, then the reaction was stopped by adding 2 cm^3 6 N HCl. Three cm^3 isooctane was withdrawn, mixed with 0.6 cm^3 5% (w/v) copper acetate solution (pH 6.1) and stirred for 90 s in a vortex; the

absorbance of the solution was measured at 715 nm and the results expressed as nmol fatty acids equivalent to oleic acid per min per pair of cotyledons. Blank assays (free fatty acids eventually present in the sample) were made by pouring the sample (2 cm^3) upon 2 cm^3 6 N HCl and stirring the mixture for 1 min before the addition of buffer and isooctane. The reaction was carried out in a specially designed equipment to perform multiple thermostatized reactions in a two-phase system (CAFFINI et al., 1990).

1.4. Partial characterization of the lipolytic system

Lipolytic activity was assayed in the fatty layer as indicated above within the range of pH 2.6 – 10.6 at 37 °C. The following buffer systems were used (STOLL & BLANCHARD, 1990): citrate – phosphate (pH 2.6 – 7.0), boric acid – sodium borate (pH 7.6 – 9.2), and glycine – sodium hydroxide (pH 8.6 – 10.6).

The effect of temperature on lipolytic activity was followed by incubating samples of resuspended fatty layer at pH 3.5 and 8.5 for 2 min at 37 °C, 45 °C, 55 °C, 65 °C, 75 °C, 85 °C, and 90 °C.

The action of different chemicals was examined by incubating 2 cm^3 of resuspended fatty layer for 10 min at 37 °C with 0.2 cm^3 different solutions: 1 mM phenylmethylsulphonyl fluoride (PMSF), 0.1 mM $HgCl_2$, 1 mM and 10 mM $CaCl_2$, $Cu(CH_3COO)_2$, EDTA, KCl, $MgCl_2$, and NaCl, 1% (w/v) SDS, and 1% (v/v) Triton X – 100. Then, residual activity was measured as indicated above.

1.5. Enzyme specificity assays

1.5.1. Lipid extraction. Total lipids were extracted from cotyledons with chloroform-methanol (2:1) according to RADIN (1969). The extract was gently mixed with 0.2 vol. of 0.9% (w/v) NaCl solution and the organic lower phase was drained off and evaporated under nitrogen current. The residual oil was further analyzed by chromatographic procedures described later.

1.5.2. Gas-liquid chromatography (GLC) of fatty acids (FA). Methyl esters of FA were prepared by transesterification of the residual oil (0.5 g) dissolved in 30 cm^3 methanol – benzene (3:1) containing 0.5 cm^3 concentrated sulfuric acid (HERRERA et al., 1991). After cooling, the mixture was extracted for 1 min with 50 cm^3 hexane. The hexane extract was washed up to neutrality with 10% (w/v) sodium chloride and dehydrated with anhydrous sodium sulfate. The hexane was evaporated to dryness and the sample redissolved in hexane (1:10 dilution). One mm^3 of this dilution was injected into the cromatograph.

A Hewlett Packard chromatograph (model 5890 – A, integrator 3392) and a SP 2330 column were used for the FA analysis. Best resolution was achieved with a

nitrogen flow rate of 20 cm^3 min^{-1}, an injector temperature of 250 °C and a column temperature of 188 °C. The standards used consisted of an equimolecular mixture (10 mg cm^{-3}) of myristic (14:0), palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1), linoleic (18:2), arachidic (20:0) and behenic (22:0) acids (Sigma Chemical Co.).

1.6. High performance liquid chromatography (HPLC) of triacylglycerols (TAG)

HPLC of TAG was carried out in a Konik chromatograph using a Bio-Sil C-18 HL 90-5 S column (250 × 4.6 mm, particle size 5 μm, Bio-Rad). A small quantity of residual oil was redissolved in chloroform (ca. 5 mg cm^{-3}) and injected into the chromatograph. The samples were eluted with ethanol-acetonitrile (7:3) with a flow rate of 1.3 cm^3 min^{-1}. TAG detection was carried out at 210 nm with a UVIS 204 UV-detector. The chromatograms were recorded and integrated with a SP 4600 integrator. Tentative identification of TAG was based on comparing retention times of standards (Sigma Chemical Co.) and elution orders of sunflower oil TAG obtained from the literature (DONG & DI CESARE, 1983, BARRON & SANTA-MARIA, 1991).

2. Results and discussion

When sunflower cotyledon extracts were separated into fatty layer, supernatant and particulate fraction, the lipolytic activity was detected in the fatty layer of seedlings only, but activity was null in ungerminated seeds. No lipolytic activity on sunflower oil (native substrate) could be detected in supernatant or pellet fractions. These results prove that the lipolytic system of sunflower is associated with the oil bodies of cotyledon cells. Negative results previously reported (HUANG, 1984) were probably due to the assay conditions, as for in vitro lipolysis could be detected in organic solvent (like isooctane) and continuous stirring are indispensable.

Highest lipase activity was reached at days 2-3 of germination (data not shown). The effect of pH on lipolytic activity in lipid bodies isolated from sunflower seedlings is rather different from those noted in case of most oil seed lipases as yet studied, owing to the unusual presence of two optimum pH values (Fig. 1), one of them was located in the acid zone (pH 3.2) and another in the basic one (pH 8.6). With the exception of *Ricinus communis* (ORY, 1969), *Brassica campestris* (VILLALOBOS et al., 1987), and *Lupinus alba* (SANZ & OLIAS, 1990), containing an "acid" lipase in ungerminated seeds, most of known lipases show maximum activity at alkaline pH values (ARRIBÉRE, 1993).

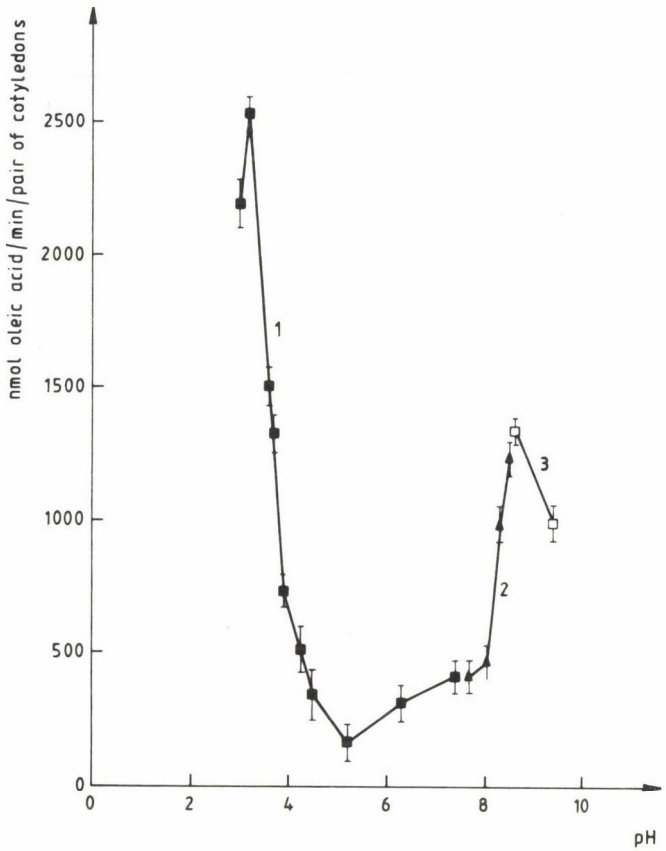

Fig. 1. Effect of pH on lipolytic activity in oil bodies (fatty layer) isolated from 2-day-old sunflower seedlings. Buffers were citrate – phosphate (pH 2.6 – 7.0), boric acid – sodium borate (7.6 – 9.2) and glycine – sodium hydroxide (pH 8.6 – 10.6). Data points represent the mean value of five determinations and each experiment was repeated three times. 1: citrate – phosphate, 2: boric acid – borax, 3: glycine – NaOH

Lipolytic activity was notably stimulated by higher temperatures at both optimum pH values (Fig. 2): maximum activity is reached at 85 °C (pH 3.2) and 65 – 75 °C (pH 8.6). Though no literature information is available for experiments on endogenous substrate, lipases isolated from other oil seeds show much lower optimum temperature (DAOOD & AL-ANI, 1988).

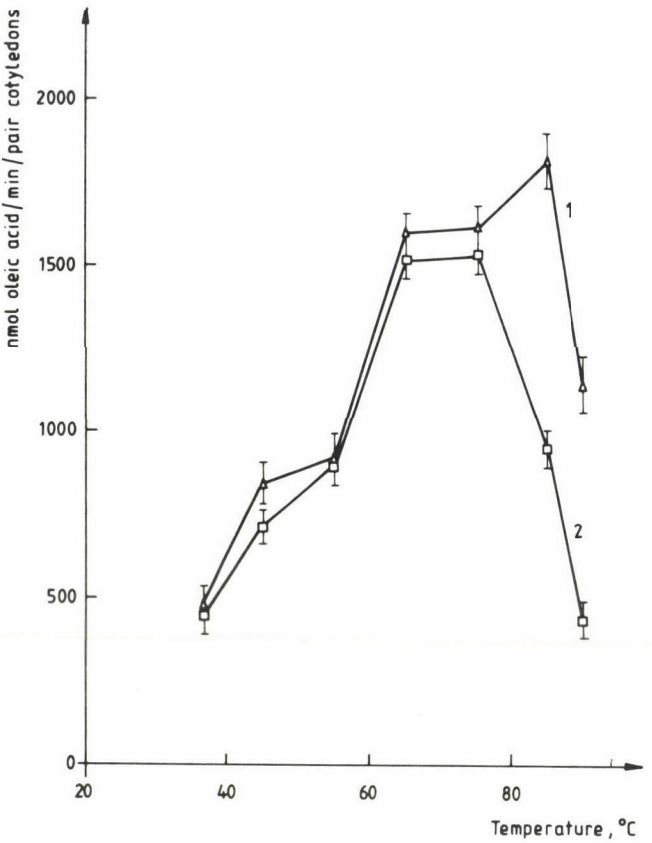

Fig. 2. Effect of temperature on lipolytic activity in oil bodies (fatty layer) isolated from 2-day-old sunflower seedlings. Data points represent the mean value of five determinations and each experiment was repeated three times. 1: pH 3.5, 2: pH 8.6

The action of different chemicals at both optimum pH values is shown in Table 1. BENZONANA and DESNUELLE (1968) had postulated that the fatty acids liberated during lipolysis would make the lipase difficult to approache to the TAG molecules located at the oil–water interphase, and that bivalent cations (as fatty acid acceptors) could prevent this type of inhibition, but this effect has not been noted in the lipolytic system of sunflower oil bodies, as in the assay conditions most bivalent ions depressed lipolytic activity. On the other hand, SANZ and OLIAS (1990) proposed that monovalent cations could be able to supress enzyme inhibition by interphase charge effect; we have tested the action of sodium and potassium chloride, and lipolytic activity was depressed or not affected in the concentrations assayed.

Table 1

Effect of ions, enzyme inhibitors and detergents on lipolytic activity of oil bodies isolated from 2-day-old sunflower seedlings at the optimum pH values.
The consigned data are the mean values of at least five assays and each experiment was repeated twice

Chemical	Relative lipolytic activity	
	pH 3.2	pH 8.6
None	100	100
1 mM potassium chloride	34	73
10 mM potassium chloride	18	84
1 mM sodium chloride	91	53
10 mM sodium chloride	104	54
1 mM calcium chloride	93	83
10 mM calcium chloride	40	55
1 mM cupric acetate	32	68
10 mM cupric acetate	94	40
1 mM magnesium chloride	74	63
10 mM magnesium chloride	59	48
0.1 mM mercuric chloride	58	65
1 mM mercuric chloride	44	59
1 mM EDTA	53	27
10 mM EDTA	27	27
1 mM PMSF	37	62
1% Triton X-100	155	140
1% SDS	55	19

The effect of detergents was not uniform: SDS depresses lipolytic activity, but when Triton X-100 was added to the assay mixture, both at acidic and alkaline optimum pH, activity notably increases. This behaviour is opposed to that showed by corn (LIN et al., 1983a), rape (LIN & HUANG, 1983b), and glycoxysomal castor bean lipases (MAESHIMA & BEEVERS, 1985).

BRADY and co-workers (1990) have reported that the catalytic centre of *Mucor miehei* lipase is closely similar to the catalytic triad of serine proteases. The results obtained after the addition of protease inhibitors to the sunflower oil bodies suspension provides little information on the hydrolytic mechanism of the enzyme system responsible for the mobilization of internal TAG, as the activity is depressed either by inhibitors of cystein (Hg^{2+}), serine (PMSF), or metalo-proteases (EDTA).

The existence of two optimum pH values in sunflower oil bodies could suggest the presence of two lipolytic enzymes. The only report on the existence of two lipases associated with oil bodies with different optimum pH values relates to castor bean (HILLS & BEEVERS, 1987), where an acidic lipase is present in dry seeds and a neutral lipase appears at day 3 of germination. On the contrary, the effect of different

chemicals on lipolytic activity (Table 1) and thermal behaviour of the enzyme system at the two optimum pH values (Fig. 2), as well as the similar patterns of lipolytic activity vs. days of germination and TAG specificities at those pH values (ARRIBÉRE, 1993) do not provide unequivocal evidence for the existence of two different lipases within the lipolytic system of germinated sunflower seeds.

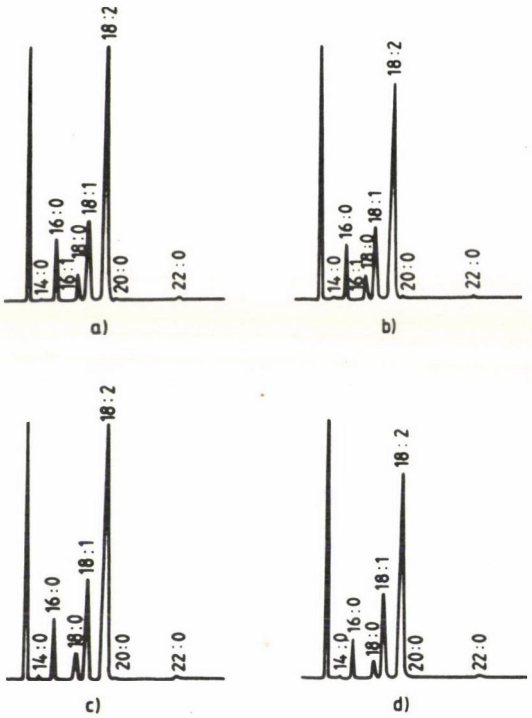

Fig. 3. Fatty acid composition of sunflower oil by GLC. a: ungerminated seeds, b: day zero (end of the imbibition period, 24 h), c: 2 day-old seedlings, and d: 3 day-old seedlings. For conditions, see the text

Figure 3 shows the chromatograms corresponding to the fatty acid composition of the oil present in dry sunflower seeds, in day zero seeds (end of the imbibition period), and in 2 and 3 day-old sunflower seedlings. Changes in TAG composition of the same oil samples were analyzed by HPLC and the chromatograms are shown in Fig. 4.

Sunflower oil obtained from dry seeds contains in 85.3% unsaturated fatty acids, mainly linoleic and oleic acids. After 3 days of germination the GLC analysis of

fatty acids shows that the relative composition of linoleic acid decreases only by 4% and that of oleic acid notably increases (Table 2). Results obtained by HPLC of TAG confirm GLC information, as while trilinoleoyl-glycerol (LLL) decreases after three days of germination (6%), dilinoleoyl-oleoyl-glycerol (LLO) and dioleoyl-linoleoyl-glycerol (LOO) increase in the same period (13% and 73%, respectively), revealing lesser affinity of the enzyme system for TAG with higher oleic acid content (Table 3). On the other hand, the relative composition of saturated fatty acids, though representing the minor fraction, notably decreases in the same period (Table 2), suggesting that the lipolytic system of sunflower shows higher affinity towards saturated fatty acids, as occur in other plant lipases (DAOOD & AL-ANI, 1986).

On the basis of the described results, some properties of the lipolytic system of sunflower could be potentially useful in bioengineering processes: a) the presence of two peaks of maximum activity at acid and alkaline zones and its lower but actual activity at neutral pH makes it a versatile reagent in lipolytic processes where the pH of the reaction is a restrictive condition; b) its higher thermic resistance turns it into an attractive natural lipasic bioreactor, specially for hydrolysis of triacylglycerols with high content of saturated fatty acids, which needs high temperatures for melting; c) owing to its specificity it could be a valuable tool in the interesterification processes of fats and oils.

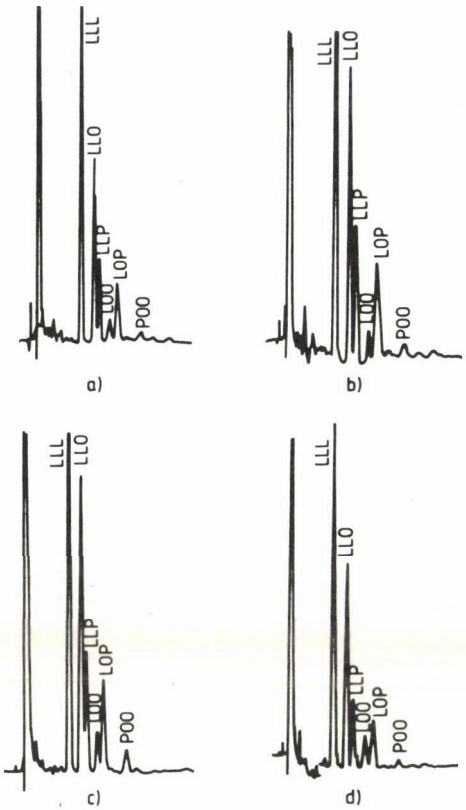

Fig. 4. Triacylglycerol composition of sunflower oil by HPLC. a: ungerminated seeds; b: day zero (end of the imbibition period, 24 h), c: 2 day-old seedlings and d: 3 day-old seedlings. For conditions, see the text

Table 2

Fatty acid composition (%) of oils from ungerminated and germinated sunflower seeds (day zero means the end of the imbibition period, 24 h).
Results correspond to GLC relative areas of methyl esters of the next fatty acids: miristic acid (14:0), palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), arachidic acid (20:0), and behenic acid (22:0). Values are the average of three determinations and the standard deviation is less than 1%

Fatty acids	dry seeds	day zero	2 day-old	3 day-old	% variation after 3 days
14:0	0.357	0.135	0.175	0.390	+9
16:0	7.832	7.582	6.531	6.204	−21
16:1	0.190	0.108	0.102	0.266	+44
18:0	4.817	4.761	4.804	4.038	−16
18:1	17.513	17.680	19.543	21.821	+24
18:2	67.603	68.168	66.463	65.009	−4
20:0	0.605	0.389	0.402	0.369	−39
22:2	1.081	0.970	1.034	0.985	−9

Table 3

Changes in triacylglycerol composition during germination of sunflower seeds (day zero means the end of the imbibition period, 24 h).
Values consigned represent relative areas of TAG separated by HPLC: trilinoleoyl-glycerol (LLL), dilinoleoyl-oleoyl-glycerol (LLO), dilinoleoyl-palmitoyl-glycerol (LLP), dioleoyl-lineleoyl-glycerol (LOO), and linoleoyl-oleoyl-palmitoyl-glycerol (LOP). Values are the average of three determinations and the standard deviation is less than 1%

TAG	dry seeds	day zero	2 day-old	3 day-old	% variation after 3 days
LLL	46.1	46.1	44.7	43.3	−6
LLO	26.8	26.2	28.2	30.3	+13
LLP	13.2	13.1	12.0	11.0	−20
LOO	3.3	3.7	4.2	5.7	+73
LOP	10.6	10.9	10.6	9.7	−8

*

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PHOTOACOUSTIC SPECTRA OF SKIM MILK POWDER AND OF MILK PROTEIN CONCENTRATE

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In this study a possible application of photoacoustic spectroscopy (PAS) on skim milk powder and UF milk protein concentrates is presented. Measurements were made in the 200–400 nm wavelength range. Photoacoustic spectra of milk protein concentrates are compared with the photoacoustic spectra of amino acids.

Keywords: amino acids, milk proteins, photoacoustic spectroscopy, ultrafiltration

The photoacoustic effect was discovered by BELL in 1880. Application of photoacoustic effect to solid samples began to blossom only from the 1970s, with the development of measuring technique (PARKER, 1973; ROSENCWAIG & GERSHO, 1976; TAM, 1986).

If a light absorbing sample is placed in a sealed chamber equipped with a microphone, and a modulated (chopped) light beam with frequency f is directed onto the sample through transparent (glass or quartz) window, sound waves of frequency f – equal to that of the chopping – can be detected by the microphone. The sound waves are created by that part of absorbed light-energy which is converted to heat. The heat-waves originate from the spot of absorption and part of them reaches the surface of the sample and heats up the gas layer in the immediate vicinity. This heating up, because of the modulated light, is periodical. It causes therefore periodical pressure changes of identical frequency in the sealed chamber. They are detected by the microphone and called PA (photoacoustic) signal.

Photoacoustic spectrum can be obtained by determining the PA signal of the sample as a function of the wavelength of the incident light. In formation of the signal, optical and thermal parameters of the sample play decisive role (TAM, 1986).

Advantages of the method are as follows:

- it is non-destructive;
- it does not require sample preparation;
- powders, opaque and gelatinous samples can be investigated;

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- it is relatively fast (1–2 min);
- no reagents are needed.

These advantages make the method suitable for testing biological (BALASUBRAMANIAN & MOHAN RAO, 1986), medical (ALTER, 1983; BINDRA et al., 1992), agricultural and food (KOCSÁNYI et al., 1988; BELTON & TANNER, 1983) samples.

The number of photoacoustic measurements performed on milk, milkpowder and dried milk products is relatively small.

MARTEL and co-workers (1987) carried out photoacoustic analyses of milk products in visible and ultraviolet (250–600 nm) regions. During heat-sterilization an absorption-peak emerged at 280 nm which is attributed to the Maillard reaction.

N'SOUKPOÉ-KOSSI and co-workers (1988) tested the kinetics of Maillard-browning in milk-powder tablets. Degree of reaction was given as the quotient of photoacoustic signals measured at two different wavelengths. The possibility for applying this method in milk industry was discussed.

MARTEL and co-workers (1990) determined the water content of the milk by measuring the amplitude and phase of photoacoustic signal in the near infrared region. Results agree rather well with the water content. However, this method is not recommended as a fast technique for water determination because of inherent phase fluctuations in the samples analysed.

DÓKA and co-workers (1991a) compared the photoacoustic and photoinduced X-ray fluorescence (PIXE) measurement of milk protein concentrate mixed with Fe-gluconate to determine the Fe content. In the visible spectral region, the possibility of photoacoustic measurement of Fe content and the nonlinearity of measured photoacoustic signals were discussed.

Determination of protein content can be performed by applying chemical analysis (by wet chemistry) or by spectrophotometric methods (NAKAI & LE, 1970; BIGGS, 1972). A condition for the applicability of both the ultraviolet and the infrared spectrophotometric methods is whether the proteins can be brought into solution. The basis of spectrophotometric methods in ultraviolet region is, on the one hand, the characteristic absorption of aromatic amino acids in the 250–300 nm wavelength region, on the other hand, the absorption of peptide-bonds in the range of 180–220 nm.

The photoacoustic method presented previously does not require such condition and offers directly a relatively fast way for protein analysis.

1. Materials and methods

The photoacoustic spectrophotometer (DÓKA et al., 1991b) was equipped with a 300 W xenon lamp (ILC Technology, Cermax LX300UV) and a monochromator

(Joben-Yvon, Modell H – 10) equipped with a stepping motor. The monochromatic light was modulated by a light chopper of variable r.p.m. The modulation frequency was set to 63 Hz and the intensity-modulated light was directed on the sample in the photoacoustic chamber. The electronic signal of the coupled microphone was transmitted on phase sensitive (lock-in) amplifier (Stanford SR – 530). The filtered and in phase amplified signal of the microphone was processed by a computer as a function of the wavelength. Since the intensity of the light beam as a function of wavelength is not constant, the received photoacoustic signal has to be normed for the emission spectrum of the lamp. For emission spectrum determination, carbon powder is generally used as a standard, because it absorbs similarly at each wavelength and in this way it represents well the light energy radiated by the lamp.

Components and their concentrations (g/100 g) of the tested skim milk powder and milk protein concentrates are as follows: protein content for skim milk powder in average 36.6; protein content for concentrates 75, 80, 85; water (max.) 3, 5, 5, 5; fat (max.) 0.7, 1, 1, 1; lactose 51, 10 – 11, 5 – 6, 1 – 1.5; ash 8.2, 7.5 – 7.8, 7.5 – 7.7, 7.4 – 7.7. Aromatic amino acid content of 100 g protein of protein concentrates – in accordance with tests carried out by amino acid analyser – was as follows: tryptophane 1.7 g, tyrosine 4.6 g, phenylalanine 4.7 g; data referring to skim milk powder were: 1.4, 5.3, 5.4 g.

The products were made from cow's milk by application of the following technological procedures: skimming, heat treatment, membrane separation (ultrafiltration), vacuum-evaporation and spray-drying.

The separation (UF) and concentration (EV, SD) of the protein was performed only by physical methods, therefore, the structure and the state of protein was not changed during the manufacturing process.

The diameters of the particles were between 10 – 40 μm.

2. Results and discussion

The photoacoustic spectra of skim milk powder and milk protein concentrates are presented in Fig. 1. In the investigated interval characteristic absorption can be seen in the 250 – 300 nm wavelength range. The maximum absorption is at 275 nm, which corresponds to the results in the literature (MARTEL èt al., 1987). Moreover, significant difference of photoacoustic spectra of skim milk powder and concentrates can be seen in Fig. 1. By increasing protein content the photoacoustic signal also increases.

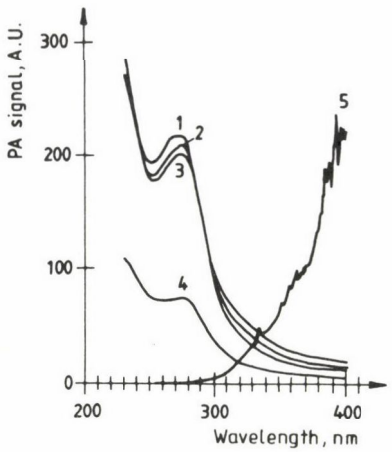

Fig. 1. Photoacoustic spectra of milk protein concentrates containing protein at 1: 85%; 2: 80%; 3: 75% and skim milk powder (4) containing protein 36.6%. Curve 5 was determined by dividing curve a by spectra of amino acids in Fig. 2

If we compare the photoacoustic spectra of concentrates with photoacoustic spectra of aromatic amino acids (Fig. 2) – taking into account that nonaromatic acids do not absorb in the range of 250–300 nm – we can see that photoacoustic peaks of milk protein concentrates are derived exclusively from tyrosine, tryptophan and phenylalanine amino acids. To verify this, we divided the photoacoustic spectrum of concentrate with 85% protein by the spectra of amino acids. It is distinctly visible that photoacoustic peak disappears (curve 5 in Fig. 1).

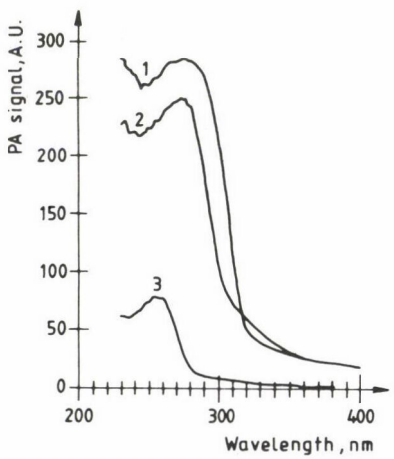

Fig. 2. Photoacoustic spectra of tryptophan (1), tyrosine (2), phenylalanine (3)

The dependence of photoacoustic signal on concentration of aromatic amino acids is determined in Fig. 3. The photoacoustic signal was proportional to absorption and the latter was proportional to the concentration. The g/100 g units might be converted to mol/100 g units. For the three amino acids (mentioned previously) the following mol/100 g concentration were used: 0.02565 in skimmed milk powder, 0.04544 in concentrate containing 75% protein, 0.04848 in 80%, 0.05152 in 85% concentrates.

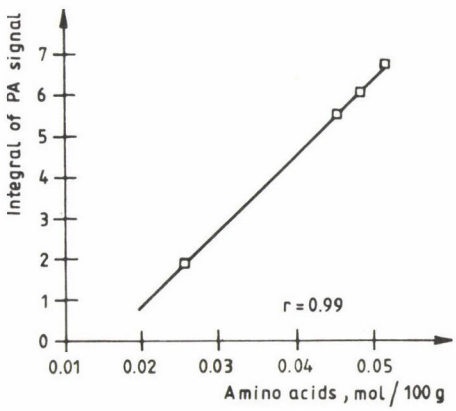

Fig. 3. Integral of photoacoustic signal (see in text) as a function of the amino acids content

When the relationship between the photoacoustic signal and aromatic amino acid is examined, this correlation is found to be nonlinear at the maximum of the signal.

If the 250–300 nm region is taken as basis, the linear relationship shown in the figure was received.

Values of ordinate were defined as follows: Values of ordinates were defined as the area between the measured spectral curves and an exponential curve which was fitted to the spectrum without the peak in the 250–300 nm wavelengths range.

The values obtained were presented as a function of concentration (Fig. 3). Correlation coefficient of the received relation by repeated measurements was $r = 0.99$.

On the basis of the obtained results a possibility was found for using the photoacoustic technique as a fast and non-destructive analytical method in the investigation of the dried milk products.

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PHOSPHORUS CONTENT OF EDIBLE WILD MUSHROOMS OF HUNGARY

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The phosphorus content of some common edible mushroom species from Hungarian habitats were determined. The average concentration was relatively high (8.21 g/kg d.m.) and unrelated to taxonomical position. The wood-destroying fungi (*Aphyllophorales* species) and the *Russulales* species had lower average concentrations (5.97 and 4.57 g/kg d.m., respectively), whilst the *Boletales* species had higher (6.69 g/kg d.m.) and the *Agaricales* species the highest (10.73 g/kg d.m.) concentrations. The highest phosphorus concentration was measured in the fruit bodies of *Lepista nuda* (19.38 g/kg d.m.). The analysed edible macromycetes can be important sources of phosphorus in the human diet.

Keywords: phosphorus, edible wild mushrooms

Phosphorus is an essential component of the biological macromolecules of living organisms (nucleic acids, phosphoproteins, phospholipids). Foods of plant and animal origin have been objects of chemical investigations for long, however, there are only a few data on the edible mushroom species. Most of the available data concerns different varieties and species of cultivated edible mushrooms (SOLOMKO et al., 1986; GERGELY et al., 1986; LELLEY, 1991; VETTER, 1989, 1990, 1994a). There is, however, only a small amount of information on edible, but not cultivated wild mushrooms (OHTONEN, 1982; VASS & TÖLGYESI, 1979; TÖLGYESI & VASS, 1984; TYLER, 1980; VETTER, 1993). The aim of this work was therefore to determine and compare the phosphorus contents of some edible, wild mushrooms of Hungary.

1. Materials and methods

The mushroom samples were gathered from different sites (mainly from the mountains) in Hungary. After cleaning and drying, the mushrooms were milled. The samples (200 mg mushroom powder) were digested in closed teflon bombs [+2 cm^3 HNO_3 (65%) +2 cm^3 H_2O_2 (30%)], at 1.56×10^5 Pa pressure. The material was filtered and diluted, and the phosphorus content was determined by plasma

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generated spectroscopy (ICP) (in four replications). The phosphorus contents of samples were characterized with arithmetical means (g/kg d.m.) and standard deviations (s).

2. Results and discussion

The analytical data are summarized in Table 1 according to the systematical position of the fungi. The average phosphorus content was 8.21 g/kg (s: 4.12), thus the variability in the phosphorus content of the fungi was not great (essentially greater values were published by TÖLGYESI & VASS, 1984; VETTER, 1989). Considering the phosphorus status of different fungal groups, the wood-destroying *Aphyllophorales* and *Polyporales* species had relatively low concentrations (arithmetical mean: 5.97 g/kg d.m.) but the species *Laetiporus sulphureus* had only 1.93 g/kg d.m.; *Pleurotus pulmonarius* contained 12.84 g/kg d.m.

The average content of the group *Boletales* was 6.59 g/kg d.m. and this concentration was relatively stable (s: 1.54). The *Agaricales* species were heterogenous, not only morphologically, but chemically, too. The highest phosphorus concentrations were measured in the fruiting bodies of *Lepista nuda* (17.13 – 19.38 g/kg d.m.), those of *Agaricus* species were between 7 and 15 g/kg d.m., confirming our previously published data on non-cultivated *Agaricus* species (VETTER, 1990). Other genera and species from this family have lower phosphorus concentrations. The *Russulales* species (from the genera *Russula* and *Lactarius*) have an average concentration of 4.57 g/kg d.m., and the standard deviation is small. Among the greater taxonomical groups (orders or families) or fungi there were not significant differences. It seems that the phosphorus level and the taxonomical status of analyzed fungi are more or less independent (this situation is the same with the potassium concentrations of wild mushrooms: VETTER, 1994b).

In conclusion, wild edible mushrooms species in Hungary are of general importance as good sources of phosphorus, i.e. the majority of these fungi would provide important supplements for human nutrition.

Table 1

Phosphorus content of analyzed wild edible mushrooms from Hungary (g/kg d.m.)

Species	Habitat	Phosphorus content ($\bar{x}$)	Standard deviation (s)
Taxonomical position			
Aphyllophorales			
Laetiporus sulphureus (Bull.: Fr.) Murrill	(M. Budai: Normafa)	4.48	0.29
Hirneola auricula-judae (Bull.: St. Anm.) Berk.	(M. Budai: Normafa)	1.93	0.22
Polyporales			
Pleurotus pulmonarius (Fr.) Quél.	(M. Bükk: "Őserdő")	12.84	0.31
Pleurotus pulmonarius (Fr.) Quél.	(M. Budai: Normafa)	6.84	0.20
Pleurotus ostreatus (Jacq. et Fr.)	(Gemenc)	3.79	0.12
Boletales			
Suillus granulatus (L.) Kuntze	(M. Pilisi: Pilisszentkereszt)	4.96	0.09
Suillus granulatus (L.) Kuntze	(SBK)	6.77	0.87
Suillus granulatus (L.) Kuntze	(M. Karancs: Zagyvapátfalva)	6.37	0.28
Boletus edulis Bull.	(M. Budai: Csillebérc)	6.27	0.08
Boletus edulis Bull.	(M. Budai: (Normafa)	4.50	0.85
Boletus edulis Bull.	(Budakeszi)	7.63	0.66
Boletus luridus Schff.	(M. Budai: Csillebérc)	6.87	0.28
Xerocomus crhysentheron (Bull.) Quél.	(M. Pilis: Pilisszentkereszt)	10.12	0.68
Leccinum aurantiacum (Bull.: St. Am) S. F. Gray	(M. Karancs: Salgóvár)	5.84	0.34
Gomphidius glutinosus (Schff.) Fr.	(M. Pilis: Pilisszentkereszt)	6.60	0.21
Agaricales			
Flammulina velutipes (Curt. Fr.) Sing.	(Érd)	8.95	0.27
Clitocybe odora (Bull.: Fr.) Kummer	(M. Pilis: Pilisszentkereszt)	13.80	1.49
Lepista nebularis (Fr.) Harmaja	(Őriszentpéter)	10.69	0.47
Lepista nebularis (Fr.) Harmaja	(M. Bükk: "Őserdő")	5.04	0.10

$\bar{x}$: mean value of 4 measurements;

s: standard deviation

Species	Habitat	Phosphorus content ($\bar{x}$)	Standard deviation (s)
Lepista nebularis (Fr.) Harmaja	(M. Bükk: "Őserdő")	10.90	0.88
Lepista nuda (Bull.: Fr.) Cke.	(Őriszentpéter)	19.38	1.40
Lepista nuda (Bull.: Fr.) Cke.	(Hűvösvölgy)	17.13	0.73
Tricholoma albobrunneum (Pers.: Fr.) Kummer	(SBK)	4.10	0.11
Tricholoma terreum (Schff.: Fr.) Kummer	(M. Pilis: Pilisszentkereszt)	5.82	0.20
Armillariella mellea (Vahl.: Fr.) Karst.	(M. Mátra: Parádsasvár)	8.00	0.54
Leucopaxillus giganteus (Fr.) Sing.	(M. Pilis: Pilisszentkereszt)	15.21	0.47
Leucopaxillus giganteus (Fr.) Sing.	(M. Karancs: Zagyvapátfalva)	12.58	0.10
Clitopilus prunulus (Scop.: Fr.) Kummer	(M. Pilis: Pilisszentkereszt)	12.73	0.10
Pluteus atricapillus (Secr.) Sing.	(Herend)	11.84	0.82
Pluteus atricapillus (Secr.) Sing.	(M. Budai: Normafa)	13.48	0.73
Agaricus abruptibulbus Peck	(M. Bükk: "Őserdő")	14.05	0.12
Agaricus purpurellus (Moell.) Moell.	(M. Bükk: "Őserdő")	13.43	0.58
Agaricus silvaticus Schff.: Fr.	(M. Börzsöny: Királyrét)	13.09	0.26
Agaricus silvaticus Schff.: Fr.	(M. Budai: Csillebérc)	15.24	0.53
Agaricus augustus Fr.	(M. Budai: Normafa)	7.71	0.18
Agaricus arvensis Schff.: Fr.	(M. Bükk: "Őserdő")	13.95	0.12
Macrolepiota procera (Scop.: Fr.) Sing.	(Cap)(M. Karancs: Zagyvapátfalva)	11.53	0.11
Macrolepiota procera (Scop.: Fr.) Sing.	(Stipe)(M. Karancs: Zagyvapátfalva)	7.36	0.45
Macrolepiota procera (Scop.: Fr.) Sing.	(Cap)(M. Karancs: Zagyvapátfalva)	9.72	0.46
Macrolepiota procera (Scop.: Fr.) Sing.	(Stipe)(M. Karancs: Zagyvapátfalva)	8.38	1.00
Coprinus micaceus (Bull.: Fr.) Fr.	(M. Budai: Normafa)	8.36	0.28
Psathyrella hydrophyla (Bull.) R. Mre. et Werner	(M. Budai: Csillebérc)	8.83	0.15
Hypholoma capnoides (Fr.: Fr.) Kummer	(M. Bükk: "Őserdő")	3.70	0.37

Species	Habitat	Phosphorus content ($\bar{x}$)	Standard deviation (s)
Cortinarius nemorensis (Fr.) Lge.	(M. Karancs: Zagyvapátfalva)	6.22	0.37
Russulales			
Russala cyanoxantha Schff. Fr.	(M. Budai: Normafa)	3.87	0.22
R. heterophylla (Fr.) Fr.	(M. Karancs: Zagyvapálfalva)	4.08	0.12
R. heterophylla (Fr.) Fr.	(M. Budai: Normafa)	4.82	0.16
R. heterophylla (Fr.) Fr.	(M. Börzsöny: Királyrét)	4.84	0.10
R. rosacea Pers.: S. F. Gray	(M. Karancs: Zagyvapátfalva)	3.35	0.18
R. rosacea Pers.: S. F. Gray	(M. Budai: Normafa)	0.35	0.07
R. vesca Fr.	(M. Pilis: Pilisszentkereszt)	4.89	0.10
R. xerampelina (Schff.: Secr.) Fr.	(M. Pilis: Pilisszentkereszt)	5.13	0.71
Lactarius azonites Bull.: Fr.	(M. Pilis: Pilisszentkereszt)	4.74	0.20
L. piperatus (L.: Fr.) S. F. Gray	(M. Pilis: Pilisszentkereszt)	4.37	0.91
L. piperatus (L.: Fr.) S. F. Gray	(M. Börzsöny: Királyrét)	3.24	0.08
L. quetius Fr.	(Budapest)	5.87	0.16
L. subdulcis (Pers.: Fr.) Fr.	(M. Bükk: "Őserdő")	5.30	0.13
L. subdulcis (Pers.: Fr.) Fr.	(M. Budai: Normafa)	6.22	0.24
Gasteromycetes			
Langermannia gigantea	(M. Bükk: "Őserdő")	13.68	0.50

(Abbreviations: M: Mountain; SBK: Botanical Garden of Soroksár)

*

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CONTENTS

In appreciation to Professor János Holló on the occasion of his 75th birthday
TÓTH-ZSIGA, I. 241
Phase equilibria and thermodynamic incompatibility in protein containing system. Part I. Investigation of casein – polysaccharide – water systems
NAGY, J., LÁSZTITY, R. & PERNECZKY, A. 245
Determination of tryptophan and methionine by mercaptoethanesulfonic acid hydrolysis at elevated temperature
CSAPÓ, J., CSAPÓ-KISS, ZS., CSORDÁS, E., FOLESTAD, S., TIVESTEN, A. & NÉMETHY, S. 257
Deformation versus load relations of tinplate cans. Interpolation between extreme loading cycles
KÖRMENDY, I., KONCZ, L. & SÁRKÖZI, I. 267
Effect of germination on a range of nutrients of selected grains and legumes
DANIŠOVÁ, C., HOLOTNÁKOVÁ, E., HOZOVÁ, B. & BUCHTOVÁ, V. 287
Analysis and quantitation of sugars in honey of different botanical origin using high performance liquid chromatography
FÖLDHÁZI, G. 299
Characterization of the lipolytic system of germinated sunflower seeds
ARRIBÉRE, M. C., PRIOLO, N. S. & CAFFINI, N. O. 313
Photoacoustic spectra of skim milk powder and of milk protein concentrate
DÓKA, O., SZÖLLŐSY, L. & NOVÁK, Á. 325
Phosphorus content of edible wild mushrooms of Hungary
VETTER, J. 331

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EFFECT OF THE DEPRIVATION OF MINERAL AND VITAMIN SUPPLEMENT ON LIPID PEROXIDATION IN PIGS

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Kahyb piglets, 30 kg body weight, were divided into three groups. The control group received 0.54 g 100 g^{-1} ISV–647 mineral and vitamin supplement (premix) in the feed. Group 2 was deprived of the supplement when the animals reached a body weight of 75 kg. No supplement (premix) was given to animals in group 3.

From economic point of view body weight gain appeared favourable in partial supplement deprivation group. Fat content was highest in the spare ribs of control pigs. Malondialdehyde levels were higher in the pork tissues having high fat content. Supplement deprivation had no effect on malondialdehyde levels in pork and liver, probably due to a reduction in dietary levels of prooxidants and antioxidants, too. Conjugated dienes were significantly elevated in the group of total supplement deprivation. The intensity of lipid peroxidation processes was higher in certain pork tissues originally having a high fat content.

Keywords: mineral and vitamin supplement, pig, lipid peroxidation, malondialdehyde, conjugated dienes, fat content, superoxide dismutase

In recent years detrimental effects have been observed in the physico-chemical characteristics and composition of meat and meat products mainly due to improper feeding and animal keeping practices (PROHÁSZKA et al., 1987). During the feeding of meat animals the requirement of vitamins, essential macro- and micro-elements is met by the addition of an adequate mixture of vitamins and mineral salts ("premix") to the feed. The "premix" requirement depends on the period of growth, but in practice the amount of the added mixture does not change during the lifetime of the animals (DWORSCHÁK & PROHÁSZKA, 1986). A supplement containing micro- and macro-elements has a catalyzing activity on formation of free radicals (DILLARD et al., 1982).

The presence of free radicals in the animal organism is essential to maintain the normal physiological conditions. The functional balance is kept by chemical and enzyme defence mechanisms (ZINDENBERG-CHERR et al., 1983). The deterioration

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or deficiency of protecting agents result in the excess of the free radicals. Their reactions and their intermediates may damage the structure of polyunsaturated fatty acids. The lipid peroxides and some of their degradation products formed during the free radical reactions cause extensive damage to the membrane structure, resulting in a decrease of the electrical resistance and membrane integrity (CAPEL & THORNLEY, 1982; ISLAM, 1982). Changes in the permeability of tissue might cause degenerative processes and later some detrimental changes in the quality of food (BUCKLEY et al., 1989; LINGNERT et al., 1989). These reactions can play a role in the etiology of various diseases in human organism (e.g. tumorigenic and atherogenic processes) (SCHAMBERGER, 1980; MCCORD & FRIDOVICH, 1982; COMPORTI, 1985).

Free radical reactions may induce unfavourable changes in flavour and colour during improper feeding of meat animals or after slaughtering during storage and meat processing (DWORSCHÁK et al., 1990; SPANIER et al., 1990; DWORSCHÁK et al., 1991). The presence of degradation products of lipid peroxidation in meat must also be considered from a food hygienic point of view (CAPEL & THORNLEY, 1982). According to statistical data from 37 countries, the occurrence of breast and colon cancer showed a strong correlation with fat intake (SCHAMBERGER, 1980).

Our aim was to investigate the effect of supplement containing micro- and macro-elements and vitamins on some characteristics of lipid peroxidation in pigs.

1. Materials and methods

Piglets of genotype Kahyb received the same piglet feed containing 0.6 g 100 g^{-1} ISV 647 supplement until they attained to the body weight of 30 kg. The nutrient composition of the feeds and the quantity given by ISV 647 supplement are shown in Table 1. The feed contained maize, wheat, soya meal (46 g 100 g^{-1} protein), Ca-phosphate, sodium chloride.

Then the pigs intended to fatten were divided into three groups, each containing about 40 animals. The animals in each group received the traditional Hungarian porker feed (Table 1), containing the same components as the piglet feed but in different distribution and also containing meat flour (58 g 100 g^{-1} protein).

The control group received the porker feed containing 0.54 g 100 g^{-1} ISV 647 supplement during their whole lifetime.

The animals in group 2 were deprived of the supplement ("premix") when they reached a body weight of 75 kg.

Only porker feed without supplementation was given to animals in group 3.

The fattening period lasted 131 days. The average weight gain was 16.2 kg per month. As average 4.01 kg feed produced 1 kg weight gain in the animals. At the end of the period, after attaining 100 – 120 kg body weight, the pigs were slaughtered.

Table 1

Nutrient composition of the feeds and the nutrient surplus by the added vitamin and mineral supplement

			Piglet feed	Supplement ISV 647 0.6 g 100 g^{-1} in the feed	Porker feed	Supplement ISV 647 0.54 g 100 g^{-1} in the feed
Protein	g	100 g^{-1}	18.4	–	15.1	–
Carbohydrate		"	48.4	–	49	–
Fat		"	3.2	–	6.0	–
Crude fibre		"	1.6	–	2.6	–
Ca	mg	100 g^{-1}	1001	117	641	105
P		"	585	79	552	70
Fe	mg	1000 g^{-1}	33.6	47	31.9	42
Cu		"	2.1	36	3.8	30
Zn		"	22.7	47	43.1	42
Mn		"	16.2	23	18.4	21
I	μg	100 g^{-1}	1.6	70	1.7	63
Se		"	25	7.2	28.8	65
Vit A		"	34.2	179	32.9	162
Vit D_3		"	–	1.5	0.02	1.35
Vit E	mg	100 g^{-1}	1.67	0.75	1.8	0.67
Vit B_1	μg	100 g^{-1}	468	37	500	34
Vit B_2		"	198	131	237	118
Vit B_6		"	585	95	760	85
Niacin		"	2320	360	4458	323
Vit B_{12}		"	–	1.1	0.3	1.0
Butylhydroxytoluene (BHT)	mg	1000 g^{-1}	–	48	–	43

The chosen parameters were determined from spare ribs, chops, hams and livers of six pigs from each group.

Two hundred and fifty g of meat and liver samples were minced twice in a mincer, then mixed with tenfold volumes of 0.154 mol l^{-1} KCl buffer solution. Homogenization took 5 min using a homogenizer (Janke und Kungel KG, Staufen i. Breisgan).

Malondialdehyde (MDA) was assayed photometrically with the thiobarbituric acid reagent in meat and liver homogenates after an ascorbic acid induction (OHKAWA et al., 1979).

Superoxide dismutase (SOD) activity was determined by the inhibition of adrenaline autooxidation in meat and liver homogenates after centrifuging 600 r.p.m. (SUN & ZIGMAN, 1978).

Conjugated dienes extracted from 1 g sample were assayed by measurement of the absorbance at 233 nm in 100 cm^3 iso-octane (A.O.A.C., 1984).

The fat content of the samples was measured by extraction with CCl_4 after digestion with HCl.

Protein was estimated as described by LOWRY and co-workers (1951) using bovine serum albumin as standard.

Selenium content of the samples was determined after wet digestion by atomic absorption based on hydride generation technique (MHS – 10 PE, Perkin Elmer 403 AAS, λ = 196 nm).

Numerical data represent mean ±SD of six measurements. For the statistical evaluation two-tailed Student's t-test and an analysis of variance were used where appropriate.

2. Results

The effect of supplement deprivation on body weight in every pigs intended to fatten is shown in Table 2A. Less than half of the animals reached the body weight above 95 kg in the group of total supplement deprivation.

The growing rates of the pigs in the other two groups were higher, more than eighty percent of animals had more than 95 kg of body weight. Body weight of the experimental animals (n = 6) can be seen in Table 2B. The average, the smallest and the biggest body weights were the highest in the group of partial supplement deprivation.

According to the data given in Tables 2A and 2B, from economical point of view the partial supplement deprivation in the feed appeared the most favourable feeding condition of pigs selected to fatten.

Table 2A

Effect of supplement deprivation on growing rate of pigs

	Number of animals		
	Control	Partial supplement deprivation	Total
Total	39	38	45
Body weight above 95 kg	32 (82%)[a]	33 (87%)[a]	20 (44%)[a]
Body weight under 95 kg	7 (18%)[a]	5 (13%)[a]	25 (46%)[a]

[a]: in the percent of all animals in the same group

Table 2B

Effect of supplement deprivation on body weight of experimental animals
(n = 6)

	Body weight (kg)		
	Control	Partial supplement deprivation	Total
Average	99.2	116.0	89.0
Minimum	75.0	91.0	75.0
Maximum	123.0	133.0	112.0

Average fat content of the samples is shown in Table 3. In consequence of supplement deprivation fat content decreased considerably in spare ribs with high fat content. There were no changes in meat containing less fat. The fat content of the liver was significantly higher in the supplement deprived pigs. It was presumed that in the control group the lipid metabolism was enhanced and the fat was stored in the muscle tissues.

Table 3

Effect of supplement deprivation on fat content of different pork meat products and pig liver

Tissue	Fat content (g 100 g^{-1})		
	Control	Partial supplement deprivation	Total
Spare rib	29.3±6.8	23.2±3.6	15.7±5.1
Chop	8.1±2.3	8.3±1.7	9.0±1.5
Ham	7.9±2.5	5.7±2.1	5.0±1.7
Liver	3.3±0.3	4.4±0.8*	4.6±1.0*

n = 6; mean ± SD; * significant difference to control $P < 0.01$

The levels of conjugated dienes are shown in Table 4. The level of conjugated dienes was lower in liver than in the other tissues examined. It was found that in spare ribs with high fat content the lipid peroxidation is significantly more intensive than in chops. Total supplement deprivation induced a significantly less intensive lipid peroxidation in spare ribs and chops as compared to the controls.

Table 4

Effect of supplement deprivation on conjugated dienes in different pork meat products and pig liver

Tissue	Conjugated dienes (absorbance at 233 nm × 100)		
	Control	Partial supplement deprivation	Total
Spare rib	25.5 ± 8.9[a]	20.3 ± 13.6	12.7 ± 7.2[a']
Chop	9.2 ± 3.2[b]	6.1 ± 2.0	4.0 ± 2.2[b']
Ham	4.3 ± 2.1	6.6 ± 3.6	4.6 ± 2.6
Liver	1.5 ± 0.2	1.6 ± 0.2	1.6 ± 0.2

n = 6; mean ± SD; a – a' and b – b' significant difference in the same part of body using analysis of variance

Concentrations of MDA, one of the degradation products formed during the lipid peroxidation can be seen in Table 5. Supplement deprivation had no effect on MDA levels. In pork with high fat content the MDA level was higher than in those containing less fat. Higher level of MDA were observed in the liver than in the muscles.

Table 5

Effect of supplement deprivation on malondialdehyde level in different pork meat products and pig liver

Tissue	MDA (nmol 100 g^{-1})		
	Control	Partial supplement deprivation	Total
Spare rib	34.1 ± 19.9	31.8 ± 13.7	34.3 ± 19.4
Chop	18.3 ± 6.9	17.5 ± 6.3	18.4 ± 9.4
Ham	18.8 ± 9.5	17.1 ± 7.4	16.3 ± 5.4
Liver	72.5 ± 38.2	84.0 ± 35.6	66.8 ± 26.8

n = 6; mean ± SD

SOD eliminates the superoxide radicals formed in the free radical reactions, and inhibits the enhancement of the lipid peroxidation. The enzyme activities are shown in Table 6. The enzyme activity is high in spare ribs probably in connection with the high fat content and the high level of conjugated dienes. On the contrary, in liver the activity of SOD was lower. Enzyme activity decreased moderately due to the effect of supplement deprivation. These changes were not significant.

Table 6

Effect of supplement deprivation on superoxide dismutase activity in different pork meat products and pig liver

Tissue	SOD (U mg^{-1} protein)		
	Control	Partial supplement deprivation	Total
Spare rib	211 ± 98	180 ± 67	193 ± 65
Chop	157 ± 88	118 ± 22	111 ± 31
Ham	145 ± 62	129 ± 20	112 ± 34
Liver	85 ± 17	111 ± 51	108 ± 34

n = 6; mean ± SD

Selenium content of pork and liver shown in Table 7. There was no significant difference between the groups, but the supplement given to the animals probably resulted in a higher selenium content in the muscles.

Table 7

Effect of supplement deprivation on selenium level in different pork meat products and pig liver

Tissue	Se ($\mu g\, g^{-1}$)		
	Control	Partial supplement deprivation	Total
Spare rib	0.152 ± 0.055	0.112 ± 0.074	0.121 ± 0.058
Chop	0.130 ± 0.058	0.120 ± 0.061	0.103 ± 0.038
Ham	0.166 ± 0.058	0.104 ± 0.045	0.094 ± 0.021
Liver	0.190 ± 0.079	0.204 ± 0.125	0.182 ± 0.066

n = 6; mean ± SD

Supplement deprivation did not cause any change in protein content and the level of micro- and macro-elements (numerical data are not introduced).

3. Discussion

Our results show that from economical point of view the partial supplement deprivation has the most favourable effect on the increase of body weight.

Lipid metabolism in pigs gets moderately changed by feeding with the supplement containing trace elements and vitamins. It is probable, that the lipid storage was diminished in spare ribs rich in fat due to supplement deprivation, and the fat content decreased in the liver having different fatty acid composition.

Based on the results of conjugated dienes, lipid peroxidation processes significantly increased in the supplement added group in certain pork tissues having originally high fat content. This increased reaction may be attributed to the elevated lipid content of muscles tissues (LINGNERT et al., 1989). The high surplus of Fe, Cu, Zn and Mn supplied by the supplement probably exerted a more intensive catalytic effect in the lipid peroxidation than the amounts originally present in the feed (OHKAWA et al., 1979; DILLARD et al., 1982).

The malondialdehyde occurs later in the lipid peroxidation than the conjugated dienes, therefore the supplement deprivation had no effect on MDA levels.

SOD, glutation peroxidase, catalase and glutathione-S-transferase belong to the enzymes protecting the living organism from free radicals and lipid peroxidation. SOD activity is higher in pork tissues of the animals fed with the supplement showing an increased requirement to compete with free radicals. The increased activity may be due to the much higher intake of Mn produced by the supplement, too (ZINDENBERG-CHERR et al., 1983).

Summing up, partial or total premix deprivation has a favourable effect on production, fat content and conjugated dienes in pigs intended to fatten. It seems that antioxidants (vitamin A, BHT) present in the supplement cannot protect perfectly the organism from lipid peroxidation induced by the surplus trace elements found in the supplement, too. In our experiment the supplement (premix) deprivation meant trace element, vitamin and antioxidant reduction, so that the prooxidant and antioxidant effects were nearly balanced in supplement deprived and added groups, too. The treatment had no strong effect on lipid peroxidation processes but the results point to the possibility how to eliminate the deleterious products of those reactions in meat and meat products.

These arguments underline the necessity of avoiding improper feeding practice of animals for meat production. Further investigations are necessary to optimize the supplement rate to every period of animal life corresponding to the requirements attaining favourable body weight and fat content as well as avoiding the enhancement of free radical reactions.

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STUDY ON THE PHYTIC ACID CONTENT OF FOOD PRODUCTS OF CEREAL BASE

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Phytic acid content is determined in an indirect way by our method. From the excess iron quantity not used for iron-phytate precipitate production from the iron(III) containing solution, the original phytic acid content of the sample is deduced on the base of calibration curve prepared from Na-phytate standard solutions.

The test should be preceded by a thorough desintegration, homogenation, extraction for one hour at least.

The sample size, the quantity of the extraction agent used and the amount of the aliquot extract for precipitation have to be in strict harmony with each other based on the principles given above. The precipitation is carried out in a water-bath at 100 °C temperature for 45 min. Photometric measurement is made 30 min after the onset of the colour reaction.

Following the principles mentioned above, our test method is appropriate to determine phytic acid content of food products with a phytic acid concentration higher than 1.0 g per 100 g, with an accuracy of ±5%.

Keywords: phytic acid, composition of cereals, cereals products

Phytic acid is not ubiquitous in the plant kingdom. The presence and concentration of phytates in food depend largely on the part of the plant consumed and its stage of maturity at harvest (O'DELL et al., 1972). The phytate concentration in whole grain cereals and oilseeds has been reported to be approximately 1% (CAMIRE & CLYDESDALE, 1982).

Beside the favourable characteristics of foods of vegetable origin, it should be well recognized that natural foods also contain undesirable components. Numerous foods of plant origin contain antinutritive substances inhibiting the utilization of important nutritives (KOMANDI, 1987; PETRES & CZUKOR, 1986; PETRES et al., 1988) (Table 1).

Since phytic acid does not have a characteristic absorption spectrum and because there are no specific reagents that identify phytate, phytic acid determination has been an analytical problem for 30 years. Precipitation methods have been

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subdivided into (a) direct methods in which the ferric phytate is removed and determined as phosphorus or inositol (McCANCE & WIDDOWSON, 1935) and (b) indirect methods in which an excess of ferric chloride is added to precipitate the phytate and the iron in the ferric phytate precipitate is determined while the phytate concentration is calculated.

Table 1

Antinutritive substances in foods of vegetable origin

Heat-sensitive substances	Heat-resistant substances
Protease inhibitors	Phytic acid
Trypsin inhibitor	Tannic substances
Chemotrypsin inhibitor	Phavism factors
Hemagglutinins	Oestrogen isoflavonol derivates
Goitrous factor	Flatulence factors
Antivitamins	Saponins

1. Materials and methods

1.1. Materials

The quantity of phytic acid, being a characteristic representative of heat-resistant antinutritive substances, was studied with our method in various foods of plant origin.

With cereal flakes, bran, sezam seeds, soy granulate, being all components of müzli mixtures, and with bakery products made of whole-meals, great quantities of phytic acid get into the diet of humans. This, combined with an unbalanced nutrition, can lead to deficiency illnesses, mainly in patients being in their phase of development.

1.2. Methods

A fundamentally new analytical method has been developed based on the quantitative conversion of determination of phytic acid in foods.

Principle

Phytic acid content is determined in an indirect way, the original phytic acid content of the sample is deduced on the base of calibration prepared from standard Na-phytate used for iron phytate percipitate.

Reagents

Use only reagents of recognized analytical grade, unless otherwise specified, and distilled or demineralized water of equivalent purity.

Na-phytate, for analysis

Na-phytate solution: 1.1754 g Na-phytate $\times 9\ H_2O$ was dissolved in 100 cm^3 3% (v/v) solution of trichloroacetic acid

Trichloroacetic acid, for analysis (TCA)

TCA solution: 3% (v/v) in distilled water

Iron-ammonium-sulfate, $Fe/NH_4/_2/SO_4/_2 \times 12\ H_2O$ for analysis

Iron-ammonium-sulfate solution: 8.637 g iron-ammonium sulfate was dissolved in 500 cm^3 distilled water and 50 cm^3 conc. hydrochloric acid, and 1000 cm^3 quantitatively taken to volume with distilled water in 1000 cm^3 volumetric flask.

Hydrochloric acid, conc./d = 1.639 g/1000 cm^3/

Ammonium-acetate, for analysis

Ammonium-acetate solution: 20% (v/v) in distilled water

Hydrochloric acid solution, c/HCl/ = 10% (v/v)

Oxammonium.HCl, for analysis

Oxammonium.HCl solution: 10% (v/v) in distilled water

α, α'-dipyridile, for analysis

α, α'-dipyridile solution: 1.0% (v/v) in distilled water

Apparatus

Usual laboratory equipments and, in particular, the following ones:

Bulb pipette, 5, 50 cm^3

Graduated pipette, 1, 10 cm^3

Automatic pipette, 5 cm^3

Erlenmeyer flask, 500 cm^3

Stift flask, 100 cm^3

Volumetric flask, 25 cm^3

Centrifuge tube, 50 cm^3

Analytical balance, accurate to within 0.0001 g

Hammer-mill

Centrifuge, n = min 15.000 min^{-1}

Spectrometer, single-beam or double-beam, suitable for measurement of absorption at 520 nm, and fitted with cells of 1 cm path.

Sampling

A recommended sampling method is given in INTERNATIONAL STANDARD (1980).

Preparation of the sample

In accordance with INTERNATIONAL STANDARD (1981) until the required particle size is obtained, then homogenize.

Selection of-weighing-in parameters according to the measuring range of the method in Fig. 3.

Procedure

In accordance with Fig. 3 and Fig. 1.

Calculation

In accordance with Fig. 6 and Fig. 4.

The difference between the results of the measurements obtained in accordance with Fig. 7.

Test report

The test report shall specify

- the sampling method
- the method used,
- the test result(s) obtained, and
- if the reproducibility has been checked, the final quoted result is obtained.

2. Results and discussion

2.1. As a new step the iron not built-in into the precipitate has been separated from the iron(III) containing solution where it was used in excess in a known quantity; and the free iron was determined instead of the iron bound into the iron-phytate precipitate. α, α'-Dipyridile colour reaction was used, the mixture was measured photometrically against its own control at 520 nm. The results were evaluated with the help of a calibration line based on Na-phytate standard solutions (THOMPSON & ERDMAN, 1982; LÁSZTITY et al., 1987; LÁSZTITY & TÖRLEY, 1987) (Fig. 1).

The steps of phytic acid determination from the sample are as follows:

- sample desintegration – homogenisation,
- extraction of phytic acid, phytate from the food sample by 3% TCA (trichloro acetic acid),
- production of an iron-phytate precipitate on a water-bath at 100 °C temperature,
- separation of the iron-phytate precipitate by centrifugal sedimentation,

– iron determination from supernatant (actually, it means α, α'-dipyridile colour reaction and photometrical measuring and finally evaluation of the results from the calibration curve). (See the annex for the detailed description of the method.)

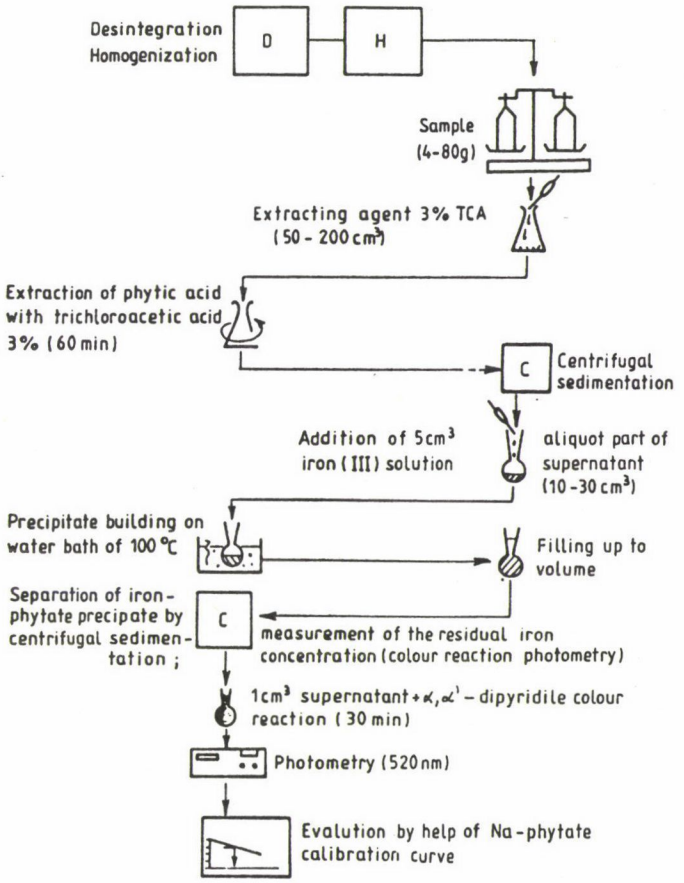

Fig. 1. Flow-chart of the phytic acid testing method

In our first series of trials, decrease of the phytic acid concentration was measured in various phases of bread preparation.

Tests were made with müzli components and flours of various ash content, where an appropriate homogenization was of highest importance as the samples were of different stabilities.

The components have different phytic acid content and thus, it was necessary to determine the optimal sample size as a function of the prospective phytic acid content. Thus, 4–5 g was found to be the optimal size for analysis.

Aiming at the extraction of phytic acid from the food sample, extraction tests were carried out. Changing the added amounts of extracting agent and extraction times, the optimal parameters were determined considering constant food sample size. The effectiveness of the extraction was measured by phytic acid addition:

– case a, the measurement of yield: a given quantity of Na-phytate stock solution was added to the original food sample, then extraction and phytic acid determination were carried out;

– case b, measurement of the recovery %: a given amount of phytic acid stock solution was added to the prepared sample stock solution and then, the processes written above were carried out. With these methods, we wanted to measure the matrix effect.

In our trials, extraction for one hour resulted in a yield of 98% for phytic acid (Fig. 2).

The extraction was followed by centrifugal sedimentation, the determination being carried out from an aliquot of the supernatant fluid.

According to our experience, the aliquot should contain 14.5–29 mg phytic acid to correspond to the measuring range. This requirement can be fulfilled by the appropriate sample size, amount of extraction agent and quantity of the aliquot extract. The experimental parameters mentioned above can be determined by the equation (Fig. 3).

After precipitation, the iron-phytate precipitate has to be separated by centrifugal sedimentation and the iron concentration is determined from the supernatant fluid. Thus, the phytic acid content is measured indirectly by α, α'-dipyridile colour reaction. The colour reaction is measured after 30 min.

For interpretation of absorbance data from the procedure, a phytic acid calibration curve should be prepared (Fig. 4). For the calibration curve, Na-phytate stock solution (10 mg cm^{-3} density), and an iron-ammonium-sulfate standard iron solution (1 mg cm^{-3} density) is prepared (Fig. 5). The iron solution is stable for 5 days, in the presence of nitric acid, stored in dark bottle, in refrigerator.

The calibration curve shows a negative slope, as the concentration of iron not built-in into the iron-phytate precipitate decreases with the increase of the quantity of added phytic acid, and thus, the absorbance of the colour reaction mixture also decreases.

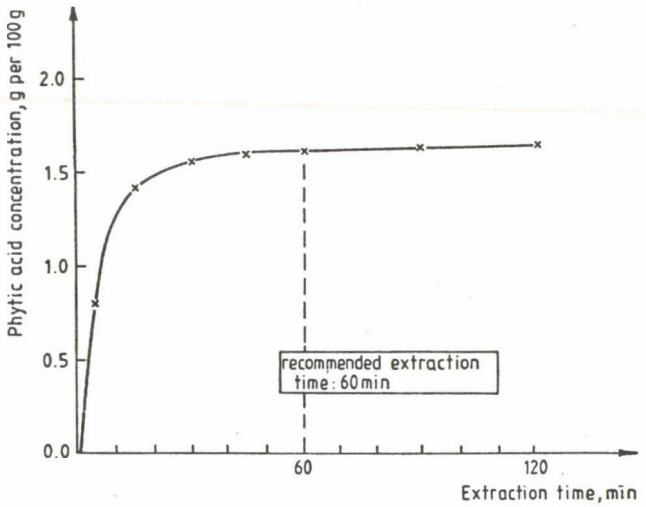

Fig. 2. Phytic acid content as a function of extraction time

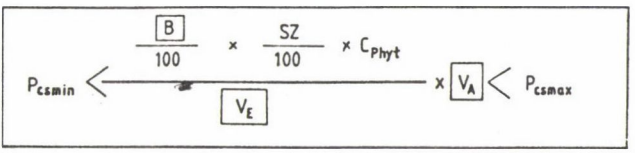

Fig. 3. Selection of sample size according to the measuring range of the method. B: sample size, g; SZ: solid substance content, %; C_{phyt}: expected phytic acid content, g per 100 g; V_E: amount of extracting agent, cm^3; V_A: amount of the aliquot part for precipitation, cm^3; P_{csmin}: phytic acid quantity minimally needed for precipitation, g; P_{csmax}: phytic acid quantity maximally needed for precipitation, g.

Conditions:

B/V_E = max 50% v

$V_A = f_v/B,V_E/ \rightarrow$ $V_E \uparrow \rightarrow V_A \uparrow, B$ = const.

$V_A \downarrow \rightarrow B \uparrow, V_E$ = const.

$B \uparrow \rightarrow V_E \uparrow, V_A$ = const.

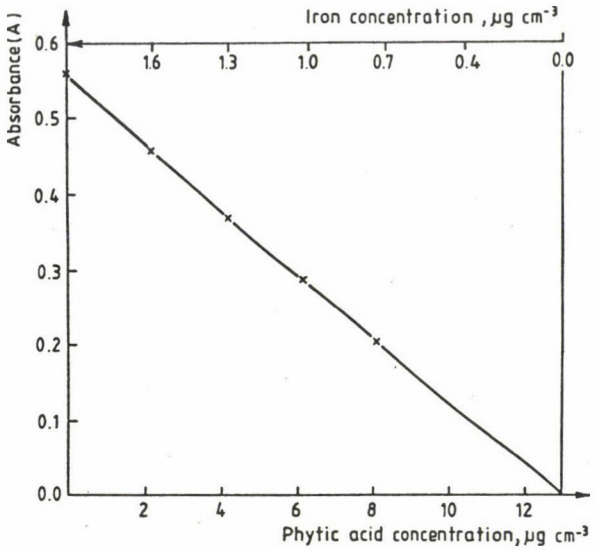

Fig. 4. Phytic acid calibration curve. $A = -0.0437 \times C_{phyt} + 0.545$

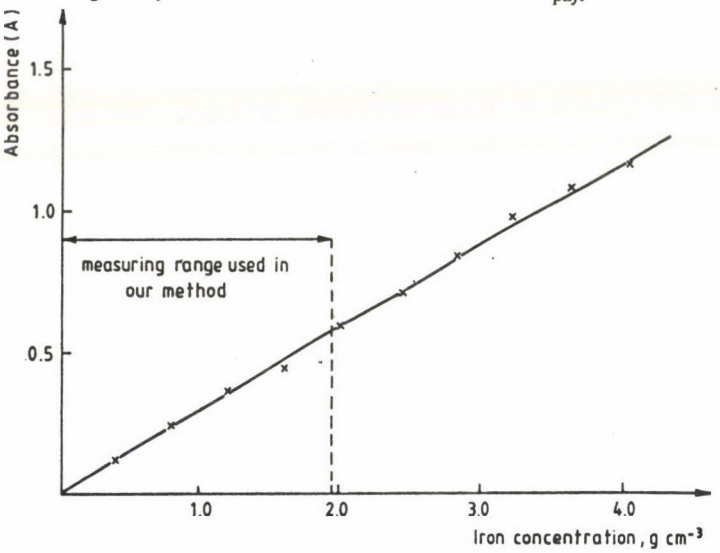

Fig. 5. Iron calibration curve. Results of computer evaluation: standard deviation error square. $A = 0.285 \times C_{Fe}$

Based on our measuring parameters, our method falls into the linear region of α, α'-dipyridile method. Taking into consideration the calibration curve, the dilution, the sample size and the solid substance content, the following equation can be applied for the calculation of phytic acid content (Fig. 6):

$$C_{phyt}\,(g/100g) = \frac{C_{calc} \times H \times 10^4 \times 25}{B \times SZ \times 10^6}$$

$$C_{calc} = \frac{A - A_0}{m}$$

where: f factors for dilution and measuring unit conversion

Fig. 6. Equation for the calculation of phytic acid concentration. C_{phyt}: phytic acid content of the sample, g per 100 g sample; C_{calc}: phytic acid concentration to be calculated from the calibration equation, $\mu g\ cm^{-3}$; H: dilution; B: sample size, g; SZ: solid substance content of sample, %; A: absorbance of the sample; A_0: absorbance (for 0 $\mu g\ cm^{-3}$ phytic acid concentration) of the blank (no phytic acid); m: slope of calibration curve

2.2. The phytic acid content of 9 food samples was measured (Fig. 7). Phytic acid was found in the 0.25 – 2.7 g per 100 g concentration range. For each column, the following information is given: exact phytic acid content, mean deviation in %, standard deviation, phytic acid content of the measured sample extract for precipitation, number of parallel measurements. From the data, the improvement of standard deviation can be observed due to the correctly selected measuring parameters. The tested müzli sample was of commercial origin, containing raisins, dried banana slices, cereal flakes and hulled sunflower seeds. The sample was desintegrated and homogenized without separation of components.

C	0.251	0.312	0.842	1.299	0.251	1.022	1.603	2.605	2.691
Δ%	4.58	1.66	0.91	3.5	48.0	2.13	2.1	3.5	0.66
σ	0.012	0.005	0.01	0.06	0.12	0.031	0.041	0.072	0.028
P(g)	0.018	0.024	0.027	0.009	0.001	0.014	0.013	0.018	0.010
n	6	6	6	6	8	6	6	6	6

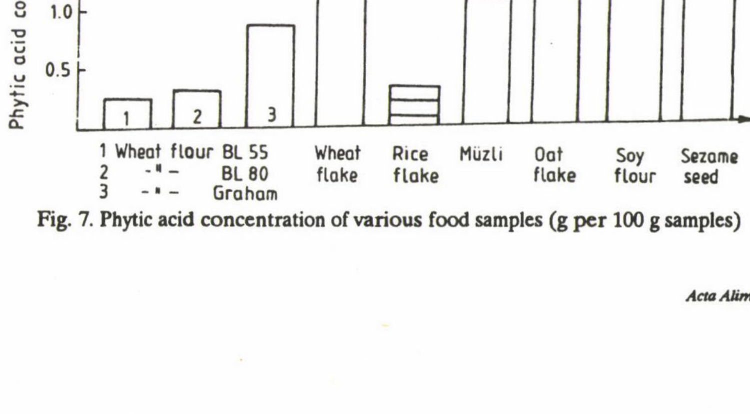

Fig. 7. Phytic acid concentration of various food samples (g per 100 g samples)

Table 2

Phytic acid content of selected food items
(g per 100 g dry weight)

Sample	Phytic acid content (g per 100 g dry weight)			
	SOUCI - FACHMANN - KRAUT		BÍRÓ - LINDNER	Our results
	range	mean		
Rice	0.14 – 0.32	0.24	0.160	0.215 flake
Wheat	0.62 – 1.35	0.99	–	1.299 flake
Oat	0.72 – 1.35	0.90	–	1.603 flake
Soy	1.00 – 1.5	1.25	1.47	2.605 flour
Sesam seed	–		–	2.691
Müzli mix	–		–	1.092
Wheat flour	–		–	0.251 BL 55
				0.312 BL 80
				0.842 Graham-flour

In Table 2 the phytic acid content of various foods is presented, and data from the literature are given for comparison. It is interesting to mention that the BÍRÓ and LINDNER (1988) table of nutritive substances gives wheat flour without any qualification though it is quite obvious that the phytic acid content is highly dependent on the degree of extraction by milling. According to some authors, the phytic acid content of a given plant i.e. leguminous grains is highly influenced by the phosphorus concentration of the soil, the phosphorus in the fertilizer and by the ripeness of the grain.

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CONDENSATION OF GLUCOSE BY THE REVERSED HYDROLYSIS REACTION OF GLUCOAMYLASE

III. EFFECT OF ORGANIC SOLVENTS AND IMMOBILIZATION FORMS

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Enzymatic synthesis of oligosaccharides by glucoamylase in the presence of organic solvents and type of support material used for immobilization were investigated. Using an organic solvent caused an increase in the yield of oligosaccharides by reducing the water content and it appears that the yield of oligosaccharides in water-miscible solvent is smaller than in water-immiscible solvent.

The data obtained clearly demonstrated that the type of carrier used for enzyme immobilization has influence mainly on isomaltose synthesis. The higher yield of oligosaccharides (especially isomaltose) was attained when using immobilized glucoamylase preparation resulted from pore diffusion and tortuosity effects.

Keywords: glucoamylase, organic solvents, immobilization, resynthesis of oligosaccharides

In the last few years, an increasing interest is being devoted to enzyme catalysis in organic solvents as media for enzymatic reactions (for an up-to-date review, see KHMELNISKY et al., 1988). Therefore, the application of enzymes to organic synthesis is attracting attention from academic and industrial viewpoints. Many hydrolytic enzymes have successfully been used for the synthesis of peptide bonds (OOSHIMA et al., 1985; DORDICK, 1989) and ester bonds (MARLOT et al., 1985; RIZZI et al., 1992) in the organic solvents. But for the synthesis of glucosidic bonds, few studies have been conducted. Synthesis of oligosaccharides can also be done from reverse hydrolytic activity of glycosidases (BESCHKOV et al., 1984; AJISAKA et al., 1987). The use of organic cosolvent has some advantage in addition to the enhancement of solubility of nonpolar substrate (NAGAMOTO et al., 1986; DORDICK, 1989): 1) reverse synthesis is available for hydrolases, 2) enhanced thermal stability of enzymes and 3) contamination by microbes can be avoided.

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NILSSON (1987) found that the yield of transglycosylation by α-galactosidase with various concentrations of dimethylformamide decreased with increasing solvent concentration. While, LAROUTE and WILLEMOT (1989; 1992) synthesized α- or β-glucosides and also oligosaccharides by β-glucosidase or glucoamylase, and found that the maximum yields were obtained with 10% (v/v) of water in primary alcohol systems and 15% in diol systems.

VULFSON and co-workers (1990) achieved the alkyl-β-glucoside synthesis from D-glucose by β-glucosidase in the presence of 90–95% immiscible alcohols.

However, most published data for the hydrolysis of starch indicate that in soluble enzyme technology higher glucose content is obtained than in immobilized glucoamylase technique (WEETALL et al., 1975; LEE et al., 1980; KENNEDY et al., 1985; MÜLLER, 1987).

TANAKA and OI (1985) found that cellobiose and gentiobiose synthesis as products from D-glucose by immobilized β-glucosidase covalently bound to polyacrylamide beads were higher than by entrapped enzyme, but when calculation per enzyme activity unit was used, the entrapped enzyme synthesized more cellobiose and gentiobiose than the covalently bound enzyme.

KENNEDY and co-workers (1985) showed that the final product's DE (dextrose equivalent) value of the D-glucose syrup for saccharification by gelatin-glutaraldehyde surface-bound glucoamylase (98.1 DE) is higher than that by gelatin-entrapped glucoamylase (94.2 DE), where the gelatin-entrapped glucoamylase produces relatively high levels of isomaltose (9.8% of total carbohydrate) and a small quantity of isomaltotriose (0.6%), while with the gelatin-glutaraldehyde surface-bound glucoamylase there is no indication whatsoever of the presence of any (1–6) linked reversion products.

Therefore, the present study was carried out to investigate the effect of organic solvents and support materials used for immobilization of glucoamylase on the reversion products.

1. Materials and methods

1.1. Materials

Glucoamylase, D-glucose, isomaltose, maltose, isomaltotriose and panose from, the same sources as described in Part I were used (EL-SAYED & LÁSZLÓ, 1994).

Dextran T 10 (MW = 10,000) was obtained from Pharmacia Fine Chemicals AB Uppsala (Sweden).

Ethanol was purchased from Reanal, Fine Chemical Co. (Hungary). Propanol and butanol were supplied by Prolabo. Hexanol was obtained from Sigma Chemical Co. (USA). Ethanol was of 95% purity and all other solvents had a 99% purity.

Amberlite IR-45 (OH^-) resin, 20-50 mesh, anion exchange, weakly basic was obtained from British Drug House LTD Duolite S 761 resin, weak anion exchange resin (phenol-formaldehyde) was purchased from Rohm and Haas Co. (Merck). Lewatit MP 62 ST (OH^-), anion exchange resin was obtained from Bayer AG (Leverkusen). Bio-Gel TE-2, 50-200 mesh (wet), porous anion exchange gel and Dowex 1×2 (Cl^-), 50-100 mesh, were purchased from Serva Feinbiochemica, Heidelberg (Germany). Molselect DEAE-25 (diethyl aminoethyl cross-linked dextran bead polymer), particle size (dry gel) 40-120 μm, capacity 3-4 meq g^{-1} was obtained from Reanal, Fine Chemical Co. (Hungary). Active carbon was obtained from El-Nasr Pharmaceutical Chemical Co. (Egypt).

1.2. Methods

The same determination of glucoamylase activity and analytical methods previously described in Part I were applied (EL-SAYED & LÁSZLÓ, 1994).

1.2.1. Immobilization of glucoamylase.

1.2.1.1. Immobilization of glucoamylase on Amberlite IR-45 resin and Lewatit MP 62 ST – The resin was activated according to the method described by PARK and LIMA (1973).

The binding of glucoamylase to resin was determined according to the method described by GHALI and co-workers (1980).

1.2.1.2. Immobilization of glucoamylase on Molselect DEAE-25, Dowex 1×2 and Bio-Gel TE-2 – The carriers were prepared by the same method described above for Amberlite but by cycling between 0.5 *N* HCl and 0.5 *N* NaOH and finally equilibrated with acetate buffer, then the binding of glucoamylase to treated resins was similar to that described by GHALI and co-workers (1980).

1.2.1.3. Immobilization of glucoamylase on Duolite S761 resin – The resin was activated according to the method described in CENTRAL FOOD RESEARCH INSTITUTE (1987) bulletin, as follows:

1) One hundred cm^3 of 2 *N* NaOH solution was filled to 20 g of resin (the colour of resin changed from bright brown to dark brown). After stirring (mixing) for some min (5-10 min), the alkaline solution (supernatant) was decanted. This process was repeated until the filtrate was free of protein as judged by lack of light absorption at 280 nm (for less than 0.2).
2) Forty cm^3 of 0.5 *N* HCl solution was poured to previous resin (colour of resin beginning lighten) with stirring, then decanted and 80 cm^3 of 0.5 *N* HCl

solution was poured onto it. The colour of resin changed with stirring several times (10 min) until standstill, then decanted. After this mixing the resin with 80 cm^3 of 0.5 *N* NaOH until the colour constancy (10 min), then decanted.

3) After washing the resin with distilled water until neutral pH, it was equilibrated with acetate buffer (0.05 mol l^{-1}, pH 4.2) by suspending it several times in this buffer until reaching pH 4.2, then the resin was finally suspended in the same buffer and stored at 4 °C for long time (one month).

Immobilization technique: The activated resin was filtered and then the same procedure in the binding of glucoamylase to Amberlite IR – 45 was applied.

1.2.1.4. Immobilization of glucoamylase on activated carbon – The carbon was washed with hydrochloric acid according to the method described by CHO and BAILEY (1978).

Immobilization technique was carried out according to the procedure described by DANIELS and FARMER (1981).

1.2.2. Determination of the porosity (k) of the particles. The values of the void volume between particles (ε) and the porosity of the particles (k) were determined by the pulse response experiment as described by SIROTTI and EMERY (1983).

1.2.3. Measurement of the diffusion coefficient in carriers. The effective diffusion coefficient was measured from the concentration change of the solutes in the well-stirred solution suspending the beads according to the equilibrium-state method (VAN HEUVEN et al., 1984).

The concentration of glucose was determined in liquid phase colorimetrically according to the method described by JOHNSON and co-workers (1964).

1.2.4. Determination of particle size. Particles were confirmed to be spherical by observation with a microscope. The diameter was measured for at least 20 particles, and the arithmetic mean was calculated.

1.2.5. Examination of organic solvent effect's. In order to test the effect of organic solvent on synthesis of oligosaccharides from D-glucose, soluble glucoamylase (10 GAU) or glucoamylase immobilized on Amberlite IR – 45 (0.21 GAU g^{-1}, moisture content = 18%) was incubated in a reaction mixture (5 cm^3) containing 0.5 g D-glucose in case of soluble enzyme or 0.2 g in case of immobilized enzyme, various organic solvents and additional water were used. The reactions were carried out in a shaking water bath at 40 °C for 24 h, then stopped by heating at 100 °C for 10 min just after a dilution with distilled water to reduce percentage of solvent in the medium and then to increase thermal denaturation of enzyme.

2. Results and discussion

2.1. Effect of organic solvent

The products of glucose condensation were analyzed by HPLC (Fig. 1).

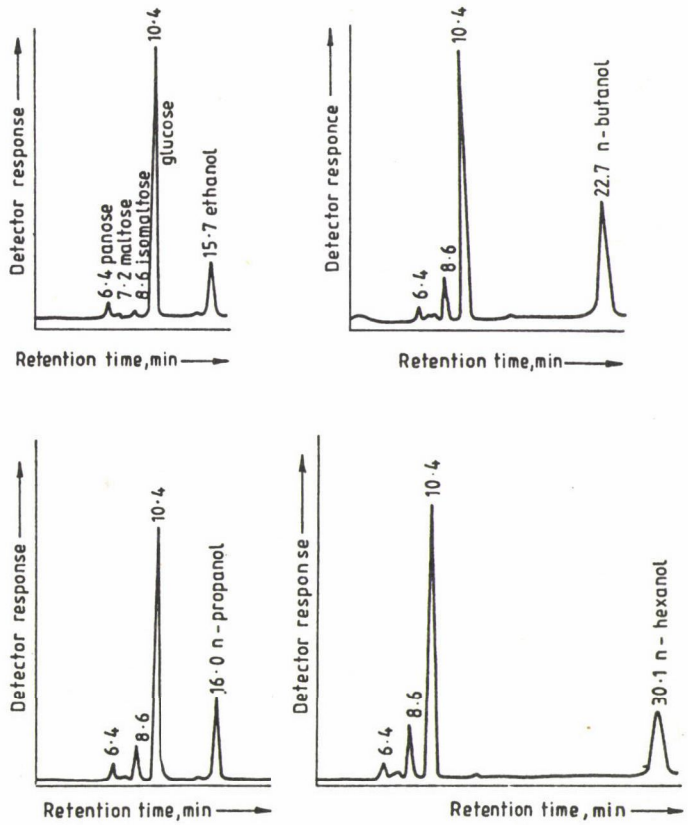

Fig. 1. HPLC chromatograms of oligosaccharides synthesis from D-glucose (10%) by soluble glucoamylase (10 GAU) in the presence of different organic solvents (40%) in 5 cm^{-3} reaction mixture at 40 °C for 24 h (aminex HPX-42A, 300 × 7.8 mm, mobile phase was water, 1.5 cm^3 min^{-1} and RI 32X)

Enzymes exhibit their catalytic properties to the full extent only when they have a strictly defined (native) conformation. In turn, the conformation of the enzyme molecule in solution was determined by a complicated network of both hydrogen bonds and electrostatic and hydrophobic interactions (KHMELNITSKY et al., 1988). In order to combine these interactions to ensure exactly the native conformation, the enzyme molecule should have a definite hydration shell (KUNTZ & KAUZMANN,

1974; RUPLEY et al., 1983). The loss of the shell, or strong distortion of the shell resulting from the introduction of organic solvent into the enzyme solution, entailed the loss of the catalytic properties, too.

Therefore, the effect of water content on synthesis of oligosaccharides by soluble glucoamylase or immobilized enzyme, at high concentration of organic solvents was evaluated. The results were illustrated in Figs 2 and 3. It was observed that the yield of oligosaccharides in organic solvents was increased by reducing the water content because of the water activity coefficient and the dielectric constant decrease by adding organic solvent (HOLLÓ et al., 1973; NAGAMOTO et al., 1986; KASCHE, 1986). Maximum yield was obtained in all organic solvents (in case of immobilized enzyme) with 10% (v/v) of water at 40 °C for 24 h (Fig. 3), while in case of soluble enzyme the maximum yield of oligosaccharides was obtained with 30 to 40% (v/v) water content in water-miscible solvents and 15 % (v/v) water content in water-immiscible solvents (Fig. 2). LAROUTE and WILLEMOT (1989; 1992) determined the necessary water content with glucoamylase and found that it was 10% (v/v) for synthesis of oligosaccharides in monoalcohol systems, while 15% (v/v) for diols. Also, they found that without water, the synthesis was not observed and with high water content, the hydrolytic activity was dominant. Thus, Figs 2 and 3 indicate the effect of solvent type on reversion rate of oligosaccharides. It was cleared that the yield of oligosaccharides in water-miscible solvent is smaller than in water-immiscible solvent. This was attributed to the maximal contact between enzyme and water-miscible solvent (homogeneous system) which results in unstability of the enzyme. While in water-immiscible solvents (two-phase system), in the aqueous phase both the enzyme and highly concentrated glucose are present with a small amount of immiscible solvent which could be dissolved. This soluble fraction of solvent could be responsible for the greater yields of oligosaccharides obtained in this system.

With other words, Figs 2 and 3 illustrate the effect of solvent on enzyme stability (soluble and immobilized enzyme forms). The yields of oligosaccharides were almost similar, although the immobilized enzymes were often more stable than the soluble enzymes in organic solvents (KHMELNITSKY et al., 1988). This may be due to a protection of a part of the soluble enzyme activity from denaturation or irreversible inactivation by aggregation and precipitation (where the enzyme concentration in soluble form was higher than in immobilized form), which was not possible for the immobilized enzyme form.

Yields and relative proportions of products obtained by immobilized glucoamylase (onto Amberlite IR-45) and by using various organic solvents in reaction mixtures containing 10% (v/v) water with an initial glucose concentration of 4% (w/v) at 40 °C for 24 h were reported in Table 1. The results showed that the condensation of glucose in 90% (v/v) ethanol, *n*-butanol and *n*-hexanol were 4.71, 14.08, 20.07 and 26.75% (w/v), respectively. Isomaltose was the major product in the

reaction mixture in each medium. While in the presence of ethanol, the isomaltose concentration was almost equal to maltose. The molar ratio of isomaltose to maltose in ethanol, *n*-propanol, *n*-butanol and *n*-hexanol after 24 h was 0.9, 2.5, 6.2 and 3.6, respectively. It appears that the maltose synthesis was faster or it rehydrolysis was lower in ethanol solution than in propanol solution. Ethanol has a higher dehydration effect and smaller dielectric constant.

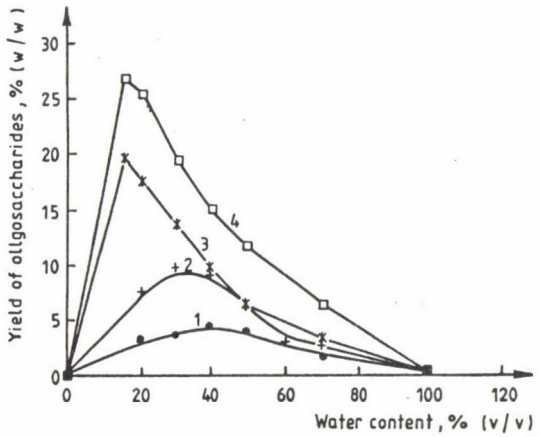

Fig. 2. Effect of water content and type of organic solvent on the yield of oligosaccharides by soluble glucoamylase in reaction mixtures (5 cm^3) containing 10 GAU and 0.5 g D-glucose at 40 °C for 24 h. 1: Ethanol; 2: *n*-propanol; 3: *n*-butanol; 4: *n*-hexanol

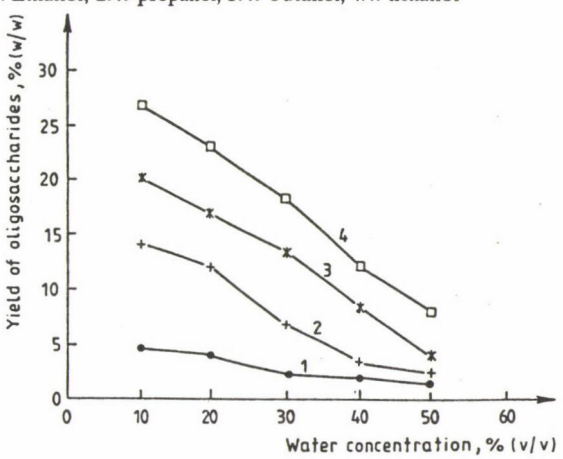

Fig. 3. Effect of water content and type of organic solvent on the yield of oligosaccharides by immobilized enzyme (onto Amberlite IR–45) in reaction mixture (5 cm^3) containing 0.2 g D-glucose and 0.21 GAU g^{-1} at the concentrations of various organic solvents and additional water, at 40 °C for 24 h. 1: Ethanol; 2: *n*-propanol; 3: *n*-butanol; 4: *n*-hexanol

Table 1

Yields and relative proportions of products obtained by immobilized glucoamylase (onto Amberlite IR-45) in various organic solvents. The reaction mixtures containing 10% water and initial glucose concentration was 4% at 40 °C for 24 h

Type of organic solvent	Composition of oligomers (%, w/w) panose	maltose	isomaltose	Yield (%, w/w)
Ethanol	trace	2.55	2.16	4.71
n-Propanol	2.72	3.22	8.14	14.08
n-Butanol	6.04	2.36	14.67	20.07
n-Hexanol	4.58	4.84	17.33	26.75

2.2. *Effect of pore diffusion*

2.2.1. Determination of the porosity (k) of the particles. The values of the void volume between particles (ε) and the porosity (k) of the particles were determined from the first statistical moment of the elution curve for Dextran T-10 (MW 1×10^4) and for glucose. The first-order normalized statistical moment (μ_1), i.e., the average residence time was related to ε and k by equation (SIROTTI & EMERY, 1983).

$$\mu_1 = \frac{V}{Q} [\varepsilon + (1-\varepsilon)k]$$

where V = the volume of the empty reactor (m^3) and Q = the flow rate ($m^3 sec^{-1}$). The data obtained experimentally (for instance, for Amberlite IR-45) were shown in Fig. 4, where μ_1 was plotted against V/Q.

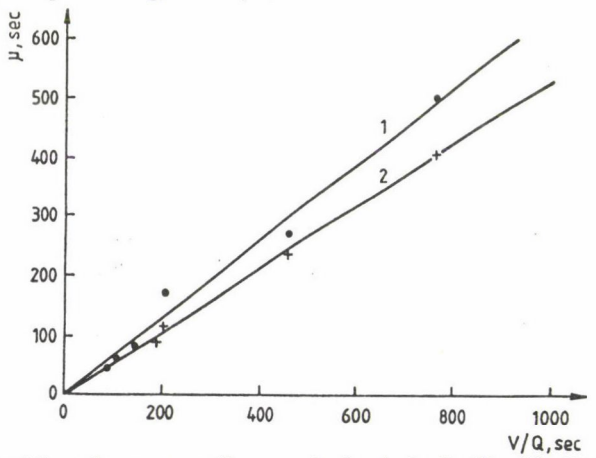

Fig. 4. First moment of the pulse response (for example, for Amberlite IR-45). Slope of the line is the volume accessible to the solute: 1: glucose ($y = 0.717x - 11.50$, $r^2 = 0.98$) and 2: dextran ($y = 0.550x - 2.38$, $r^2 = 0.99$)

The slope of the plot for glucose = $\varepsilon+(1-\varepsilon)k$ as the total void fraction of the column. While for solute (Dextran) excluded from the pores, the internal void volume is effectively zero ($k = 0$) and therefore the slope of the plot is ε, which means the external void fraction. The values of ε and k for various carriers were summarized in Table 2.

Table 2

Determined values of the parameters for a column of various carriers

Carrier type	Particle diameter, dp (mm)	External void fraction, ε (-)	Internal porosity, *k* (-)
Amberlite IR-45	0.22-0.65	0.50	0.17
Amberlite IR-45 and enzyme		0.48	0.15
Bio-Gel TE-2	0.06-0.27	0.54	0.50
Bio-Gel TE-2 and enzyme		0.51	0.49
Duolite S 761	0.20-0.97	0.53	0.19
Duolite S 761 and enzyme		0.50	0.16
Lewatit MP 62 ST	0.35-0.71	0.50	0.16
Lewatit and enzyme		0.49	0.14
Dowex 1×2	0.15-0.47	0.47	0.34
Dowex and enzyme		0.46	0.30
Molselect DEAE-25	0.07-0.19	0.44	0.52
Molselect DEAE-25 and enzyme		0.41	0.49
Active carbon	0.89-1.20	0.48	0.46
Active carbon and enzyme		0.42	0.41

The results showed that the internal porosity of Molselect and Biogel was the largest and that of the Lewatit was the smallest. The internal porosities of carriers change only in slight degree after the enzyme immobilization.

2.2.2. Measurement of the diffusion coefficient in carrier. The effective diffusion coefficient is an important parameter in characterizing heterogeneously catalyzed reactions. It is possible that limited rates of diffusion of substrate and product within the catalyst particles influence the overall reaction rate (PETERSEN, 1965). This influence could be expressed by an effectiveness factor μ defined as the ratio between the actual overall reaction rate and the reaction rate that would be obtained if no internal mass-transfer limitation occurred. The value of μ depends on the intrinsic kinetics and the rate of diffusion in the particle. The latter process is difficult to describe exactly, because of the complicated inner structure of the catalyst.

Therefore an approximation was used, in which it was assumed that the diffusion could be characterized by a single effective diffusion coefficient, D_e, which was correlated to the molecular diffusion coefficients and the internal structure of the catalyst.

To be able to determine this unknown quantity, which is a measure of the rate of transfer of the solute (glucose) in a catalyst sphere a method similar to that by VAN HEUVEN and co-workers (1984) was used.

Spheres of the carrier material were mixed with a liquid phase in which the solute has been dissolved. The solute penetrates into the spheres, resulting in a decrease in concentration in the liquid phase. Using the change of concentration with time for the solute in solution it is possible to determine the effective diffusion coefficient of the solute in the carrier material.

The concentration inside the spheres is both place and time dependent: in the liquid phase concentration was assumed to be the same everywhere. At the same time the external mass transfer resistance was neglected (vigorous agitating). Diffusion inside the spheres is radial and the diffusion coefficient supposed to be constant.

The change in concentration of the solute was described by VAN HEUVEN and co-workers (1984):

$$\text{inside the spheres: } \frac{\partial C_b}{\partial t} = D_e\left[\frac{1}{r^2}\frac{\partial}{\partial_r}\left(r^2\frac{\partial C_b}{\partial_r}\right)\right]$$

with the starting condition: $t = 0 \rightarrow C_b = 0$

and the boundary conditions: $r = R \rightarrow C_b = \frac{C_f}{m}$,

$$r = 0 \rightarrow C_b = \text{finite}$$

$$\text{For the liquid phase: } -4\pi R^2 n D_e \left.\frac{\partial C_b}{\partial r}\right|_{r=R} = V_f \frac{\partial C_f}{\partial t}$$

$$\text{with n = number of spheres} = \frac{V_b}{\frac{4}{3}\pi R^3}$$

and the starting condition: $t = 0 \rightarrow C_f = C_{f,o}$,

where C_b = concentration in spheres, C_f = concentration in liquid, $C_{f,o}$ = concentration at t = 0, m = distribution coefficient ($m = C_f/C_b$ at equilibrium-state), r = sphere coordinate, t = time, D_e = effective diffusion coefficient, R = radius of sphere, V_b = volume of liquid.

Making these equations dimensionless gives:

Spheres	Liquid phase
$\frac{\partial A}{\partial W}=\frac{1}{U^2}\frac{\partial}{\partial U}\left(U^2\frac{\partial A}{\partial U}\right)$	$\frac{\partial B}{\partial W}=-\frac{3}{H}\frac{\partial A}{\partial U}\Big\vert_{U=1}$
$W=0\rightarrow A=0$	
$U=1\rightarrow A=B$	$W=0\rightarrow B=1$
$U=0\rightarrow A=$ finite	

with: $A=\frac{mC_b}{C_{f,o}}$, $B=\frac{C_f}{C_{f,o}}$, $U=\frac{r}{R}$, $W=\frac{D_{et}}{R^2}$ and $H=\frac{mV_f}{V_b}$.

This set of equations could be solved by the way of a Laplace transformation, and the result was presented in Fig. 5. The values of D_e of some carriers used for immobilization of enzyme and the effect of enzyme on it were reported in Table 3.

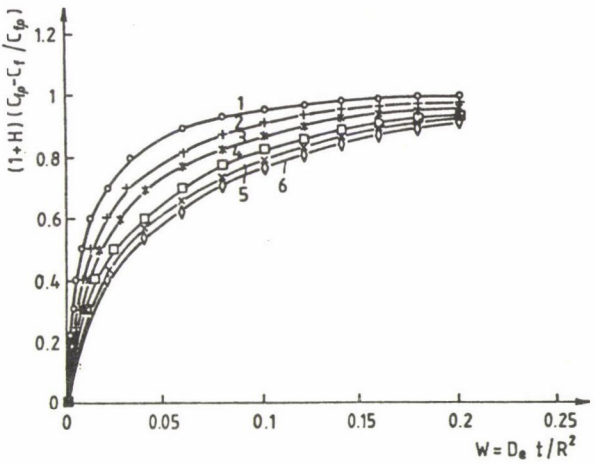

Fig. 5. Determination of D_e through concentration measurement in the liquid phase. Values of H: 1: 0.5; 2: 1; 3: 2; 4: 4; 5: 10; 6: infinite

The obtained data clearly demonstrated that the effective internal diffusivity was the largest in case of active carbon carrier and the regularity of pores was the best in this case, too.

The effective internal diffusion coefficient was empirically related (SATTERFIELD, 1970) to the free solution diffusivity D by

$$D_e = \frac{D.k}{\tau}$$

where the tortuosity τ is a function of the size and shape of the pore, and the diffusing molecule. As mentioned before the molecular diffusion coefficient of glucose in solution at 25 °C was 6.8×10^{-10} m^2 sec^{-1} (GLADDEN & DOLE, 1953), hence difference between D_e and D was showed in Table 3. The ratio D_e/D was expressed as a function of a particle porosity k and tortuosity factor τ which accounts for both tortuosity and varying pore cross-section, i.e., $D_e/D = k/\tau$.

Table 3

The characterization of carriers used and the effect of immobilized glucoamylase on these parameters

Type of support	$D_e \cdot 10^{11}$ (m^2 sec^{-1})	m	D_e/D	τ
Amberlite IR-45	2.15	1.85	0.031	5.5
Amberlite IR-45 and enzyme	1.85	2.03	0.027	5.6
Bio-Gel TE-2	7.34	1.44	0.106	4.7
Bio-Gel TE-2 and enzyme	7.10	1.50	0.103	4.8
Duolite S 761	2.90	2.13	0.042	4.5
Duolite S 761 and enzyme	2.45	2.22	0.035	4.6
Lewatit MP 62 ST	1.50	2.33	0.022	7.3
Lewatit MP 62 ST and enzyme	1.30	2.45	0.019	7.4
Dowex 1×2	3.36	1.70	0.049	6.9
Dowex 1×2 and enzyme	3.00	1.78	0.043	7.0
Molselect DEAE-25	7.26	1.46	0.107	5.0
Molselect DEAE-25 and enzyme	7.00	1.52	0.103	4.8
Active carbon	8.48	1.63	0.123	3.7
Active carbon and enzyme	7.51	1.56	0.109	3.8

2.2.3. Effect of type of carrier used for enzyme immobilization on glucose reversion. Various supports could be used for glucoamylase immobilization. A comparison of some of them in efficiency of oligosaccharides synthesis, especially isomaltose synthesis, was examined by incubating the immobilized glucoamylase preparations with D-glucose (30%, w/v) in 0.05 mol l^{-1} acetate buffer at pH 4.2 and 50 °C for several days. The synthesis of oligosaccharides determines the quality and the final dextrose equivalent (DE) value of the product which can be produced from the enzymatic hydrolysis of starch, and in the commercial processes the resynthesis must be minimized (KENNEDY et al., 1985). The total yield of oligosaccharides and the isomaltose formed (%, w/w) were summarized in Tables 4 and 5, respectively.

The obtained data clearly demonstrated that the type of support had influence mainly on isomaltose synthesis.

Apparently, the yield of oligosaccharides increases by increasing the isomaltose synthesis. From Tables 4 and 5 it was observed that immobilized enzyme on Active carbon, Lewatit MP 62 ST and Doulite S 761 produced relatively high levels of isomaltose as 5.13, 4.99 and 4.28% (w/w), respectively and oligosaccharides as 7.47, 7.40 and 6.26 (w/w), respectively, and determined as total yield produced per all the amount of enzyme activity used in the reaction mixture after 7 days. While with immobilized enzyme on Molselect DEAE–25, Bio-Gel, TE–2, Dowex 1×2 and Amberlite IR–45 there were synthesized low levels of isomaltose equal to 3.10, 2.88, 2.12 and 3.10% (w/w), respectively and oligosaccharides as 5.14, 4.30, 3.17 and 4.60% (w/w), respectively. Although the reversion rate and isomaltose formation (as total yield produced per all the amount of enzyme activity used in the reaction mixture) were obtained in the highest values when the reaction proceeded with glucoamylase immobilized on active carbon, with the use of glucoamylase immobilized on Lewatit MP 62 ST the highest values were also obtained in the reversion rate and isomaltose synthesis as expressed as the total yield obtained per unit of enzyme activity.

Table 4

Effect of type of carrier used for enzyme immobilization on the synthesis of oligosaccharides. At 50 °C, pH 4.2 (0.05 mol l^{-1} acetate buffer) and 30% initial glucose concentration. The reversion ratio was determined as total yield produced per all the amount of enzyme activity used in the reaction mixture

Carrier and activity	Reversion ratio (%)[c] Time (days)											
	1		2		4		5		7		8	
	a	b	a	b	a	b	a	b	a	b	a	b
Amberlite (1.69)	2.90	1.72	3.12	1.85	3.89	2.30	4.18	2.47	4.60	2.72	4.58	2.71
Bio-Gel (2.77)	2.52	0.91	2.98	1.08	3.65	1.32	4.07	1.47	4.30	1.55	4.54	1.65
Duolite (16.44)	3.76	0.23	5.39	0.33	5.64	0.34	6.08	0.37	6.26	0.38	6.23	0.38
Lewatit (1.66)	3.02	1.82	5.61	3.38	6.17	3.72	7.42	4.47	7.40	4.46	7.55	4.55
Dowex (0.96)	1.84	1.92	2.03	2.11	2.71	2.82	2.99	3.11	3.17	3.30	3.56	3.71
Molselect (2.48)	2.46	0.99	2.87	1.16	3.79	1.53	4.42	1.78	5.14	2.07	5.67	2.29
A. carbon (73.54)	3.20	0.06	5.86	0.08	6.41	0.09	–	–	7.47	0.10	–	–

[c] R. ratio (%) = (initial glucose conc. – glucose conc. at time t/initial glucose conc.) × 100

a: Isomaltose formed as total yield produced per all the amount of enzyme activity used in the reaction mixture

b: Isomaltose formed as expressed as the total yield obtained per unit enzyme activity

The higher yield of oligosaccharides (especially isomaltose) was attained when using immobilized glucoamylase preparations resulted from pore diffusion and tortuosity effects. In particular, it was observed that glucose inside the smaller pores or in tortuous pores of an immobilized glucoamylase preparation (Lewatit MP 62 ST) tends to have a longer residence time in a glucoamylase environment than glucose inside the larger pores of the support (Active carbon). It could be explained that glucose diffusion in the smaller pores was slower than in the larger pores of the support. Since prolonged contact of glucose with glucoamylase in the smaller pores leads to increase reversion product, especially the formation of (1→6) linked oligosaccharides because the (1→4) linked oligosaccharides could be rehydrolyzed almost as fast as it is synthesized while (1→6) linked oligosaccharide (slow hydrolysis) accumulated.

Table 5

Efficiency of isomaltose synthesis by immobilized glucoamylase preparations

Type of carrier	Isomaltose formed (%) Time (days)											
	1		2		4		5		7		8	
	a	b	a	b	a	b	a	b	a	b	a	b
Amberlite IR-45	2.07	1.22	2.30	1.36	2.92	1.73	3.03	1.79	3.10	1.83	3.20	1.89
Bio-Gel TE-2	1.90	0.69	2.00	0.72	2.44	0.88	2.71	0.98	2.88	1.04	3.03	1.09
Duolite S 761	2.81	0.17	3.61	0.22	3.89	0.24	4.06	0.25	4.28	0.26	4.50	0.27
Lewatit MP 62 ST	2.27	1.37	3.75	2.26	4.64	2.79	4.96	2.99	4.99	3.01	5.07	3.05
Dowex 1×2	1.35	1.41	1.54	1.60	1.82	1.89	2.00	2.08	2.12	2.21	2.14	2.23
Molselect DEAE-25	1.85	0.74	1.92	0.77	2.53	1.02	2.90	1.17	3.10	1.25	3.42	1.38
Active carbon	3.14	0.04	4.32	0.06	4.81	0.07	–	–	5.13	0.08	–	–

a: Isomaltose formed as total yield produced per all the amount of enzyme activity used in the reaction mixture

b: Isomaltose formed as expressed as the total yield obtained per unit enzyme activity

3. Conclusions

The effect of water content on synthesis of oligosaccharides by soluble glucoamylase or immobilized enzyme, at high concentration of organic solvents was evaluated. It was observed that the yield of oligosaccharides in organic solvents increased by reducing the water content because of the water activity coefficient and the dielectric constant decrease by adding organic solvent. Maximum yield was obtained in all organic solvents (in case of immobilized enzyme) with 10% (v/v) of water at 40 °C, while in case of soluble enzyme the maximum yield of oligosaccharides was obtained with 30 to 40% (v/v) water content in water-miscible solvents and 15% (v/v) water content in case of water-immiscible solvents.

Isomaltose was the major product in the reaction mixture in each medium. However, in the presence of ethanol, the isomaltose concentration was almost equal to maltose. The molar ratio of isomaltose to maltose in ethanol, *n*-propanol, *n*-butanol and *n*-hexanol after 24 h was 0.9, 2.5, 6.2 and 3.6, respectively. It appears that the maltose synthesis is faster or its rehydrolysis is lower in ethanol solution than in propanol solution.

The porosity (k) of the particles were determined from the first statistical moment of the elution curve for Dextran T−10 and D-glucose. The results showed that the internal porosity of Molselect and Biogel were the largest and that of the Lewatit was the smallest. The internal porosities of carriers change only to a slight degree after the enzyme immobilization.

The diffusion could be characterized by a single effective diffusion coefficient, D_e, which was correlated to the molecular diffusion coefficients and the internal structure of the catalyst. The effective internal diffusivity was the largest in case of active carbon carrier and the regularity of pores was the best in this case, too.

The comparison of supports in the efficiency of oligosaccharide synthesis, especially isomaltose synthesis, was examined. The obtained data clearly demonstrated that the type of support influenced mainly the isomaltose synthesis. Apparently, the yield of oligosaccharides increases by increasing the isomaltose synthesis.

Higher yield of oligosaccharides (especially isomaltose) was attained when using immobilized glucoamylase preparations, due to the pore diffusion and tortuosity effects. In particular, it was observed that glucose which was inside the smaller pores or in tortuous pores of an immobilized glucoamylase preparation (Lewatit MP) tends to have a longer residence time in a glucoamylase environment than glucose which was inside the larger pores of the support (active carbon).

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NEW METHOD FOR DETECTION OF PICOGRAM QUANTITIES OF STAPHYLOCOCCAL THERMONUCLEASE

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The development of an enzyme immunoassay for the detection of *Staphylococcus aureus*-TNase is described. To microplate well in which commercially available DNA was immobilized, TNase enzyme was applied. The treatment of immobilized DNA with TNase reduced the quantity of immobilized DNA in comparison with untreated DNA. The detection of residual quantity of DNA was performed by enzyme immunoassay method.

The entire procedure can be carried out in one day and allowed a detection of TNase in solution as low as 2 pg cm^{-3}. The test enabled the detection of TNase from contaminated milk and from cheese extract.

Keywords: *Staphylococcus aureus*, staphylococcal thermonuclease, ELISA method

Staphylococcal thermonuclease (TNase) is an extracellular protein secreted by *Staphylococcus aureus* (*S. aureus*). This enzyme shows a remarkable resistance to heat, low pH, prolonged storage and microbial activity in foods and broth (ABRAMSON, 1971; TATINI, 1981). TNase presence in food is used as an indicator of current or previous staphylococcal contamination.

There are several different procedures for detecting TNase activity: viscosimetry (LASKOWSKI & SEIDEL, 1945; CUNNINGHAM et al., 1956) spectrofotometry (KUNITZ, 1950) turbidimetry (HOUCK, 1959) agar diffusion systems (LACHICA et al., 1971; 1972; TATINI et al., 1976; KOUPAL & DEIBEL, 1978; MAJOR & EZEPCHUK, 1982) and recently "sandwich" ELISA method (POHL & BECKER, 1992). Among the agar diffusion systems the most often used are those based on the metachromatic property of toluidine blue. All five procedures could quantify 1–10 ng cm^{-3} of TNase in solution.

The detection of *S. aureus* in food with traditional methods requires 1–2 days and together with the identification of the strains, 2–4 days. The purpose of this study was to evaluate, based on the new TNase detection method, a procedure for the detection of *S. aureus*, more sensitive than the known methods and, at the same time, highly reliable.

1. Materials and methods

1.1. Materials

DNA sodium salt, high polymer from chicken erythrocytes and Tween 20 were purchased from Reanal (Budapest, Hungary). Bovine albumin and methylated bovine albumin (MBSA) were obtained from Serva (Heidelberg, Germany). Complete Freund adjuvant and Brain heart infusion (BHI) broth from Difco Laboratories (Detroit, Mich., USA) were used. Horseradish peroxidase labelled goat anti-mouse IgG (H+L) was from Pierce (Rockford, IL, USA). Micrococcal nuclease from *Staphylococcus aureus* Foggi strain (activity: 107 units mg^{-1} protein) was purchased from Sigma (Deisenhofen, Germany). *o*-Phenylendiamine was from Calbiochem (La Jolla, CA, USA). Polystyrene microplates (Microstrip) was from Labsystem (Helsinki, Finland).

Light absorbance measuring were made with a Microelisa Minireader MR 250 (Dynatech Laboratories Inc., Denkendorf, Germany).

S. aureus strain FRI-262 a producer of SEB was used in this investigation. A 24 h culture of this organism in 3.7% BHI broth at 37 °C was obtained. *S. aureus* cells were harvested by centrifugation, washed using sterile 0.9% NaCl solution, and diluted appropriately to be used as inoculum.

1.2. Methods

1.2.1. Incubation conditions. The inoculated BHI and skimmed milk were incubated at 37 °C with shaking. Samples were withdrawn after different incubation periods (2, 3, 4, 5 and 6 h) for staphylococcal count and TNase determinations. For the absorption of staphylococcal protein A, 2.5% of normal rabbit serum was added to each samples.

1.2.2. Microbial counts. S. aureus count was determined by surface plating of appropriate sample dilutions on blood agar plates and incubating for 24 h at 37 °C.

1.2.3. Experimental animals. Balb/C mice were bread from stock. Animals were housed in plastic cages with open stainless steel grids and were allowed to consume food and water ad libitum. Female animals of all ages were used.

1.2.4. Preparation of immunizing antigen. High polymer DNA was dissolved in 0.15 mol l^{-1} NaCl to a concentration of 0.5 mg cm^{-3}. The DNA was denaturated by boiling the solution at 100 °C for 10 min and chilling it rapidly in ice-water, after which a 1% solution of MBSA in water was added with mixing until the final weight ratio of MBSA to DNA was 1. A volume of the complex was then emulsified with an equal volume of complete Freund adjuvant (PLESCIA et al., 1964).

1.2.5. Preparation of antisera. Balb/C female mice were given at weekly intervals 4 injections of freshly prepared antigen containing 0.125 mg cm^{-3} DNA, administered subcutaneously (0.1 cm^3). The mice were bled by use of an axillary "pocket" method (WILLIAMS & CHASE, 1967) 7–10 days following the last injection.

1.2.6. Immobilization of DNA in microtiter wells. DNA stock solution was prepared at a concentration of 0.125 mg cm^{-3} in 10 mmol l^{-1} Tris, 1 mmol l^{-1} EDTA and 150 mmol l^{-1} NaCl pH 8.0 (TSE). Working solution of DNA test antigen were prepared immediately before each assay by adding reagents in the following volumes: 0.1 cm^3 10 mmol l^{-1} NaOH, 0.1 cm^3 stock DNA and 9.8 cm^3 of 1.2 mol l^{-1} NaCl in 10 mmol l^{-1} Tris, 1 mmol l^{-1} EDTA pH 8.0 (TE). Samples (0.1 cm^3) of DNA (0.00125 mg cm^{-3}) were applied to polystyrene microplates. Plates were incubated for 30 min at room temperature (LACY & VOSS, 1989). Microplates were then washed three times with phosphate-buffered saline pH 7.2 (PBS) containing 0.05% Tween 20 (PBS–TW).

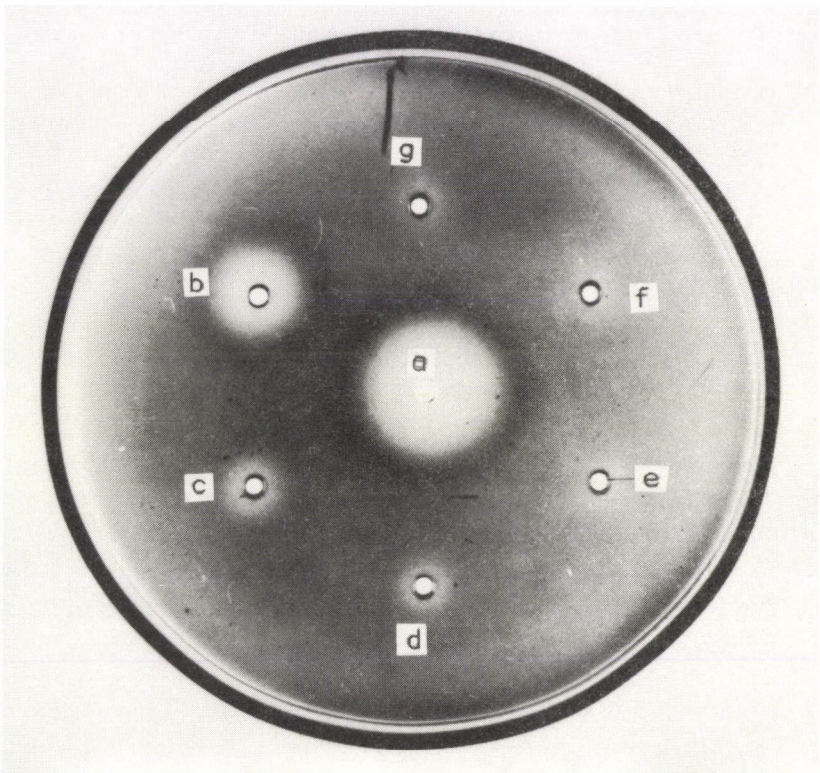

Fig. 1. Toluidine-blue DNA agar diffusion test. A constant volume of 0.01 cm^{-3} of TNase was added to each well and varying zones of DNA hydrolysis were obtained with different concentration of TNase. a = 5 μg cm^{-3}; b = 0.5 μg cm^{-3}; c = 0.05 μg cm^{-3}; d = 0.005 μg cm^{-3}; e = 0.0005 μg cm^{-3}; f = 0.00005 μg cm^{-3}; g = 0.000005 μg cm^{-3}

1.2.7. Assay of TNase activity. A/. TB–DNA agar-diffusion method: the toluidine-blue DNA (TB–DNA) agar was prepared as described by LACHICA and co-workers (1971). After 4 h incubation at 37 °C the TB–DNA plates were observed for a pink halo around each well which was measured (Fig. 1).

B/. ELISA method: to microplate wells in which DNA was immobilized 0.1 cm^3 of micrococcal nuclease diluted in 50 mmol l^{-1} Tris-HCl/10 mmol l^{-1} $CaCl_2$ pH 9.0 buffer was applied to give concentration ranging from 0 to 1500 pg cm^{-3} (0 to 0.00015 U cm^{-3}) of nuclease. Each sample was tested in triplicate. Statistical significance (0.05 level) was determined by analyzing results for variance (SNEDECOR & COCHRAN, 1980).

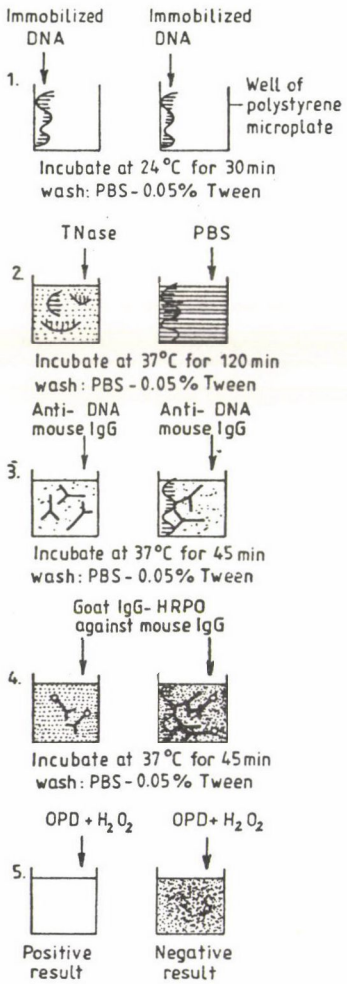

Fig. 2. Diagram of the principle of enzyme immunoassay for detection of nuclease. Each procedure was optimized and the conditions for every step in the assay are shown

Microplates were incubated at 37 °C for 60 or 120 min, washed with PBS–TW as before and 0.1 cm^3 of a 1/300 dilution of antisera (mouse anti-DNA antibody) in PBS–TW was added. Microplates were incubated at 37 °C for 45 min, washed with PBS–TW as before and 0.1 cm^3 of a 1/4000 dilution of Horseradish Peroxidase labelled Goat Anti-Mouse IgG conjugate in PBS–TW was applied. Microplates were incubated at 37 °C for 45 min washed three times with PBS–TW and 0.1 cm^3 of substrate solution containing 0.4 mg cm^{-3} of *o*-Phenylendiamine and 0.006% of H_2O_2 in 150 mmol l^{-1} citrate-phosphate pH 5.0 was added to each well. Colour development was stopped after 30 min by adding 0.025 cm^3 4*N* H_2SO_4 and the absorbance was measured at 490 nm on a Dynatech MR 250 microplate reader (Fig. 2).

1.2.8. Recovery of nuclease added to BHI, skimmed milk and cheese samples. To microplate wells in which DNA was immobilized 0.1 cm^3 of micrococcal nuclease diluted in 3.7% BHI broth or skimmed milk was applied to give concentrations ranging from 0 to 1500 pg cm^{-3} (0 to 0.00015 U cm^{-3}) of nuclease.

To evaluate the efficacy of recovery of TNase from cheese, volumes of a stock solution of micrococcal nuclease were added, in duplicate, to samples of 20 g of hard (40% fat content) sheep milk cheese to obtain concentrations ranging from 0 to 1500 pg cm^{-3} (0 to 0.00015 U cm^{-3}). All samples were obtained from a single complete cheese. The inoculated samples were extracted by the procedure of TATINI and co-workers (1981).

The amount of enzyme present in BHI broth, skimmed milk and the extracts of cheese samples was determined according to the method described above.

2. Results and discussion

The principle of the new method is based on our earlier observation that the treatment of DNA-immobilized plastic wells with TNase, resulted in a reduction in the quantity of immobilized DNA (iDNA) in comparison with untreated DNA-immobilized plastic wells.

In subsequent experiments, optimum conditions for DNA concentration, for mouse anti-DNA antibody and HRPO labelled goat anti-mouse IgG conjugate dilutions, for incubation temperature and time were standardised.

Results presented in Fig. 3 show that treatment of iDNA with TNase for 1 h at 37 °C reduced the quantity of iDNA in comparison with untreated iDNA and the detection limit in this experiment was 6 pg cm^{-3}. The treatment of iDNA with TNase for 2 h at 37 °C decreased the detection limit to 2 pg cm^{-3}.

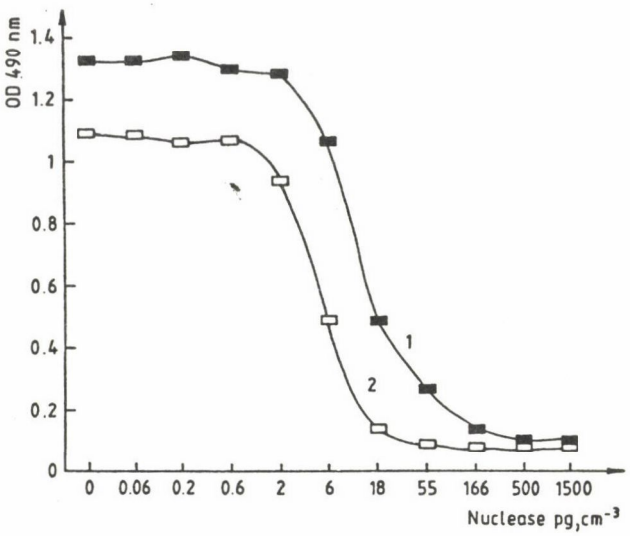

Fig. 3. Degradation of DNA (1.25 μg cm^{-3}) due to different concentration of TNase (0 – 1500 pg cm^{-3}). Assay conditions: pH 9; Incubation time 60 min and 120 min at 37 °C. 1: 60 min, 2: 120 min

In the case when micrococcal nuclease was added to skimmed milk and hard cheese, respectively, the detection limit of TNase was 2 pg cm^{-3} from milk (directly) and 20 pg cm^{-3} from cheese (after extraction).

Tables 1 and 2 show the results of comparative examination in BHI and skimmed milk, respectively, after incubation with *S. aureus*.

Table 1 shows that when 10^2 cm^{-3} *S. aureus* count was given into BHI, TNase activity could not be measured with TB-DNA agar diffusion method during the examination. With ELISA method positive result was achieved first after 6 h of incubation when CFU reached the 10^5 cm^{-3} level. In milk (Table 2), with 10^2 cm^{-3} CFU, TNase activity could not be measured with either methods. In the case of initial count of 10^3 cm^{-3} after 4 h of incubation, at 4.8×10^4 cm^{-3} and 4.6×10^4 cm^{-3} *S. aureus* counts, TNase activity could be detected both in BHI and in milk using ELISA method. With initial cell count of 10^3 cm^{-3} *S. aureus* the presence of TNase could not be detected by TB-DNA agar method. According to our examination, the time of detection decreased furthermore, when the initial count of *S. aureus* was 1×10^4 cm^{-3}. In this case, with ELISA method, TNase activity could be detected in BHI after 2 h and in milk after 3 h incubation; with TB-DNA agar method, TNase activity was observed at first after 6 h incubation in BHI (CFU being 1.1×10^7 cm^{-3}). At 1.0×10^5 cm^{-3} *S. aureus* count, using the ELISA method, TNase activity was measured in all cases in BHI and milk; with TB-DNA agar method, TNase activity was observed at first after 5 h incubation (*S. aureus* count being 1.0×10^7 cm^{-3}).

Finally, with an initial count of 10^6 cm^{-3} *S. aureus*, TNase activity could be detected practically in all cases (with the exception of milk, after 2 h incubation), using either methods. Results with ELISA method are presented in Figs 4 and 5.

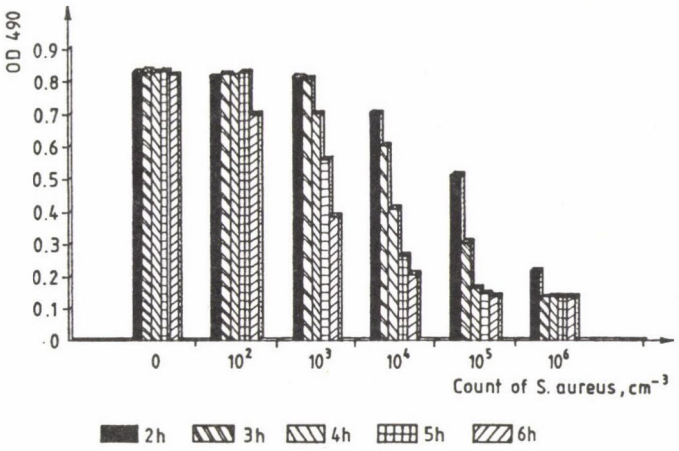

Fig. 4. Relationship between count of *S. aureus*, incubation time and OD (490 nm) of TNase activity in BHI

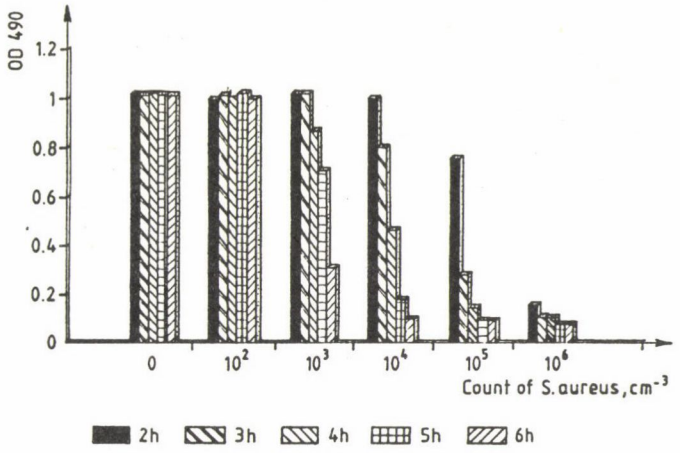

Fig. 5. Relationship between count of *S. aureus*, incubation time and OD (490 nm) of TNase activity in skimmed milk

Table 1

Comparison of detected of TNase activity by ELISA and TB-DNA methods in BHI

Initial count of *S. aureus* CFU cm^{-3}	Incubation time of *S. aureus* in BHI														
	2 h			3 h			4 h			5 h			6 h		
	Activity by: ELISA method	TB-DNA agar	Count of *S. aureus* CFU cm^{-3}	Activity by: ELISA method	TB-DNA agar	Count of *S. aureus* CFU cm^{-3}	Activity by: ELISA method	TB-DNA agar	Count of *S. aureus* CFU cm^{-3}	Activity by: ELISA method	TB-DNA agar	Count of *S. aureus* CFU cm^{-3}	Activity by: ELISA method	TB-DNA agar	Count of *S. aureus* CFU cm^{-3}
10^2	–	–	8.0×10^2	–	–	1.0×10^3	–	–	2.0×10^3	–	–	2.0×10^4	+	–	1.0×10^5
10^3	–	–	4.0×10^3	–	–	1.3×10^4	+	–	4.8×10^4	+	–	1.5×10^5	+	–	1.5×10^6
10^4	+	–	4.0×10^4	+	–	1.5×10^5	+	–	1.0×10^6	+	–	3.0×10^6	+	+	1.1×10^7
10^5	+	–	7.0×10^5	+	–	9.0×10^5	+	+	4.0×10^7	+	+	$>1.0\times10^8$	+	+	$>1.0\times10^8$
10^6	+	+	1.2×10^7	+	+	3.0×10^7	+	+	1.0×10^8	+	+	$>1.0\times10^8$	+	+	$>1.0\times10^8$

(–) negative result of TNase activity
(+) positive result of TNase activity

Table 2

Comparison of detection of TNase activity by ELISA and TB-DNA agar methods in skimmed milk

Initial count of *S. aureus* CFU cm^{-3}	Incubation time of *S. aureus* in skimmed milk														
	2 h			3 h			4 h			5 h			6 h		
	Activity by: ELISA method	TB-DNA agar	Count of *S. aureus* CFU cm^{-3}	Activity by: ELISA method	TB-DNA agar	Count of *S. aureus* CFU cm^{-3}	Activity by: ELISA method	TB-DNA agar	Count of *S. aureus* CFU cm^{-3}	Activity by: ELISA method	TB-DNA agar	Count of *S. aureus* CFU cm^{-3}	Activity by: ELISA method	TB-DNA agar	Count of *S. aureus* CFU cm^{-3}
10^{2}	–	–	1.0×10^{3}	–	–	1.0×10^{3}	–	–	4.0×10^{2}	–	–	1.0×10^{4}	–	–	2.0×10^{4}
10^{3}	–	–	4.0×10^{3}	–	–	9.0×10^{3}	+	–	4.6×10^{4}	+	–	8.0×10^{4}	+	–	6.0×10^{5}
10^{4}	–	–	2.0×10^{4}	+	–	1.1×10^{5}	+	–	5.0×10^{5}	+	–	1.6×10^{6}	+	–	5.0×10^{6}
10^{5}	+	–	7.0×10^{5}	+	–	1.0×10^{6}	+	–	4.0×10^{6}	+	+	1.0×10^{7}	+	+	3.5×10^{7}
10^{6}	+	–	4.0×10^{6}	+	+	2.0×10^{7}	+	+	5.0×10^{7}	+	+	9.0×10^{7}	+	+	1.2×10^{8}

(–) negative result of TNase activity
(+) positive result of TNase activity

The figures show that as compared to control samples (*S. aureus* count in BHI and in milk = 0), due to the TNase activity of *S. aureus*, the quantity of iDNA, detectable by ELISA method, changes with the incubation time and initial *S. aureus* count. Thus, when at 490 nm, significantly lower extinction was measured, as compared to control, the presence of TNase enzyme was presumable.

Our suggested new ELISA method proved to be significantly more sensitive than the widespread TB-DNA method (LACHICA et al., 1971) and recently described "sandwich" ELISA method (POHL & BECKER, 1992). According to literature data, the minimum detectable level of nuclease by TB-DNA method is 5 ng cm^{-3} and by ELISA method of POHL & BECKER is 1 ng cm^{-3}, while by our method it is 2 pg cm^{-3}.

The duration of examination is in case of TB-DNA and new ELISA methods 4–4.5 h. In case of "sandwich" ELISA method the assay can be completed in 3.5–4 h, provided that antibody coated microtiter plates are available.

According to our experiments, the new method is suitable for the indirect detection of *S. aureus* in culture, in milk and in cheese. The presence of TNase in the food suggests the presence or former presence of staphylococci.

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CORN GERM OIL EXTRACTION BY A NEW ENZYMATIC PROCESS

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Aqueous enzymatic extraction of corn germ oil was investigated. Several commercial enzyme preparations (Gamanase 1.5 L, Pextinex Ultra SP-L, Celluclast 1.5 and SP-348) were used individually or in mixtures. The results have shown that the following operations should be applied as to increase the efficiency of enzyme activity: hydrothermal pretreatment of corn germ, grinding, enzymatic treatment and centrifugal separation of oil. A quite new procedure for oil extraction was developed as a technological phase in the course of integral corn processing in the starch technology. The economy of the process was preliminarily analyzed from the aspect of energy consumption.

Keywords: aqueous-enzymatic extraction, biotechnology, corn germ, corn germ oil, wet corn milling

The high content of essential fatty acids and tocopherols includes the corn germ oil in the group of oils with highest biological value. The presence of γ-tocopherol makes the corn oil more stable to oxidation than the sunflowerseed and many other edible oils. Usually, the corn oil is produced from corn germ after wet milling in the course of starch technology. Wet-milled corn germ contains more than 50% moisture and about 20% oil. KARLOVIĆ and co-workers (1988) report that the corn germ has to be dried to 2–7% moisture for processing by conventional procedure (pressing or combination of pressing and extraction). The dry corn germ contains 40 to 50% oil, depending on the corn type and the process used for corn germ separation.

The production of corn germ oil involves several technological problems. Long-term studies of corn-germ oil on our market have shown that the quality varies widely. The main cases are the deterioration processes which start during storage and steeping, as the result of lipoxidase activity and other enzymes present in the germ. These processes are difficult to be slowered down or to be prevented. Drying and conditioning are performed under adequate circumstances. A number of factors influence the quality of corn germ.

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Corn germ is characterized by the content of oxidation and some coloured products that are transferred to the oil during pressing and extraction making the oil refining difficult (VOLOTOVSKAJA et al., 1975; FAUR, 1981; KARLOVIĆ et al., 1985; KARLOVIĆ et al., 1988).

The conventional process is tedious, and in addition, the energy consumption is high, and the recovery of raw material is relatively low.

Screw presses are mainly used for pressing. Full-press expellers are used in small-scale plants. Probably due to the specific morphological structure of corn germ it is very difficult to maintain 5% residual oil content in the cake. LEIBOVITZ and RUCKENSTEIN (1983) report 6% residual oil in the cake after full-pressing. KARLOVIĆ and co-workers (1988) found up to 10% and even higher residual oil content in the cake. The residual oil content of the cake depending on the method of obtaining and processing corn germ is presented in Table 1 (REINERS, 1978).

Table 1

Oil content of corn germ cake and meal depending on obtaining and processing

Separation of		Residual oil content (%)
corn germ	oil	
Wet or dry milling	Full pressing	7–10
Wet milling	Pre-pressing + solvent extraction	1–3
Dry milling	Solvent extraction	1–2

Considering the specific energy consumption of cold pressing in case of different oil seeds, the corn germ proved to be the least economical as shown in Table 2 (STEIN, 1984).

It is evident from the presented data that the yield during corn germ processing is lower than that of other typical oil-bearing materials.

The separation of oil by pressing and extraction is difficult due to the specific morphologic structure and chemical composition of corn germ (KARLOVIĆ et al., 1991).

The solution of the above-mentioned problem is probably the improvement and optimization of the present processing procedure or the development of a new technology as to eliminate the shortages of the conventional method.

Table 2

Specific energy consumption during cold pressing of different raw materials

Raw material	Specific energy consumption kW_h/t
Rapeseed	30 – 35
Sunflowerseed	35 – 40
Safflowerseed	about 35
Linseed	35 – 40
Corn germ	about 50 – 55
Copra	40

An alternate way of oil production – the aqueous enzymatic extraction – has recently attracted interest. Due to the mechanical and enzymatic degradation of the cell walls, lipid bodies i.e. oil, as well as protein and carbohydrates are liberated from the cells (CHRISTENSEN, 1991).

Enzymatic oil extraction was applied to olives (CHRISTENSEN, 1991; DI GIOVACCHINO, 1991), rapeseed (KOFOD, 1988), coconut (MC GLONE & LOPEZ-MUNGUIA CANALES, 1986), linseed (OLSEN, 1988), palm mesocarp (CHEAH et al., 1990), avocado fruit (BUENROSTRO & LOPEZ-MUNGUIA CANALES, 1986), and plum kernels (PIČURIĆ et al., 1991). HITZE and co-workers (1975) were the first to describe the application of this process for corn germ. According to CHRISTENSEN (1991) the data on enzymes of corn germ cell structure degradation are insufficient, so this area is still a challenge for the scientists.

Our previous investigations (BOCEVSKA et al., 1991; BOCEVSKA et al., 1992; BOCEVSKA et al., 1993; KARLOVIĆ et al., 1993), showed that the efficiency of aqueous enzymatic oil extraction is influenced by several factors, as presented in Fig. 1.

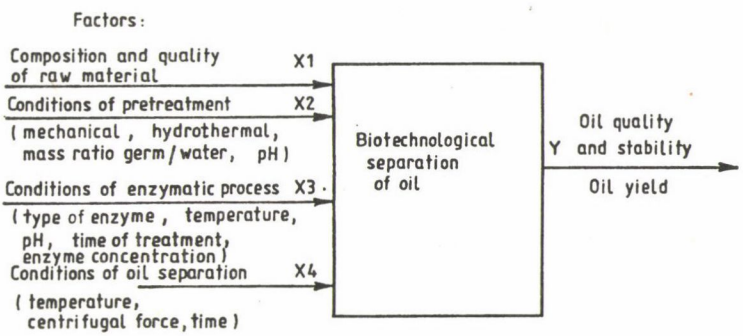

Fig. 1. Factors influencing the yield and quality of oil obtained by biotechnological separation from corn germ

The chemical composition of cell wall (which has to be ruptured) is of special importance. The available data have shown that the chemical composition of cell wall of different oil-bearing plants is rather different, particularly considering the polysaccharides (Table 3).

Table 3

Approximate composition of cell wall polysaccharides of some of the oil-containing crops

Polysaccharide components	Corn germ (%)	Rapeseed (%)	Coconut (%)
Pectic substances	<1	39	–
Mannan	–	–	61
Galactomannan	–	–	26
Arabinogalactans	–	8	some
Cellulose	39	22	13
Hemicellulose:			
xyloglucan	–	29	–
arabioxylan	50	–	–
Other	10	2	–

According to CHRISTENSEN (1991), hemicellulose and cellulose are equally represented in corn germ cell walls. However, it was observed earlier (OLSEN, 1988) that the content of hemicellulose components is higher. The rupture of corn germ cell walls by enzymatic action is, therefore, a very serious problem. Namely, HOLLÓ (1988), considers the enzymatic degradation of lignine (hemicellulose) to be one of the biggest challenges of food and feed technology. A well-known fact is, also, that technological problems arise during enzymatic degradation of cellulose, due to the strong and stable structure of this substrate. The pathway of hydrolysis of cellulose to glucose is still not known completely. It seems that joint sequential action of three main enzymes present in the cellulose-complex is necessary for the hydrolysis of crystalline cellulose (HOLLÓ, 1988).

The complete hydrolysis of cellulose to glucose is not necessary for the aqueous enzymatic oil extraction. Namely, due to the enzymatic treatment the cell wall gets fragmented, the cell content is transferred into the polar water phase. As oil is nonpolar, it can be separated by centrifugation or some other separation procedure.

The economic effect of aqueous enzymatic extraction of rapeseed oil is positive (KOFOD, 1988). The optimization of aqueous enzymatic procedure during coconut processing could also yield economically available results (BARRIOS et al., 1990).

The aim of the present work was to investigate the possibility of application of some commercial enzymes to obtain oil from corn germ by aqueous enzymatic extraction.

1. Materials and methods

1.1. Material

Corn germ used in the experiments was obtained by wet milling in a modern starch plant by hydrocyclone separation. Samples were taken after dewatering by pressing. The germ thus obtained was preserved in a frozen state at −18 °C to the day preceeding the experiment, when it was defrosted in the refrigerator at +4 °C. Basic chemical composition of the wet and dry corn germ is presented in Table 4.

Table 4

Chemical composition of corn germ

Composition	%
a Wet corn germ:	
moisture	53.4
oil	19.1
b Absolutely dry matter	
oil	41.0
proteins	14.9
starch	6.2
pectin	0.7
cellulose[a]	11.2

a: calculated on fat-free material

The characteristics of the applied commercial enzymes and samples obtained from the producers are shown in Table 5.

Table 5

Enzymes used in the experiments

Enzyme	Microorganism	Main activity	Declared activity
Pectinex Ultra SP-L[a]	*Aspergillus niger*	- Polygalacturonase - Pectinesterase - Pectintranseliminase - Hemicellulase	26.000 PG (pH = 3.5 T = 20 °C)
Gamanase[b]	*Aspergillus niger*	- Hemicellulase (endoenzyme) Galactomanase	1.500,000 VHCU g^{-1} (Viscosity Hemicellulase Ac.)
SP-348[a]	*Hemicola insolens*	- Cellulase (egzoenzyme)	-
Celluclast 1.5 L[b]	*Trichoderma reseei*	- Cellulase (endoenzyme)	1500 NCU g^{-1}

[a]: Novo Ferment AG, CH-4243 Dittingen, Switzerland
[b]: Novo Industri A/S, Copenhagen, Denmark

The amounts of the enzymes used in the experiments are presented in Table 6.

Table 6

Dosage of enzymatic preparations in the experiments

No.	Name	Calculated on germ mass %
1	Celluclast 1,5 L	2
2	Celluclast 1,5 L + SP 348	1+1
3	Gamanase 1,5 L	2
4	SP-348	2
5	Gamanase 1,5 L + Pectinex Ultra SP-L	1+1

1.2. Procedure

To obtain oil from corn germ, the following operations were carried out (Fig. 2):

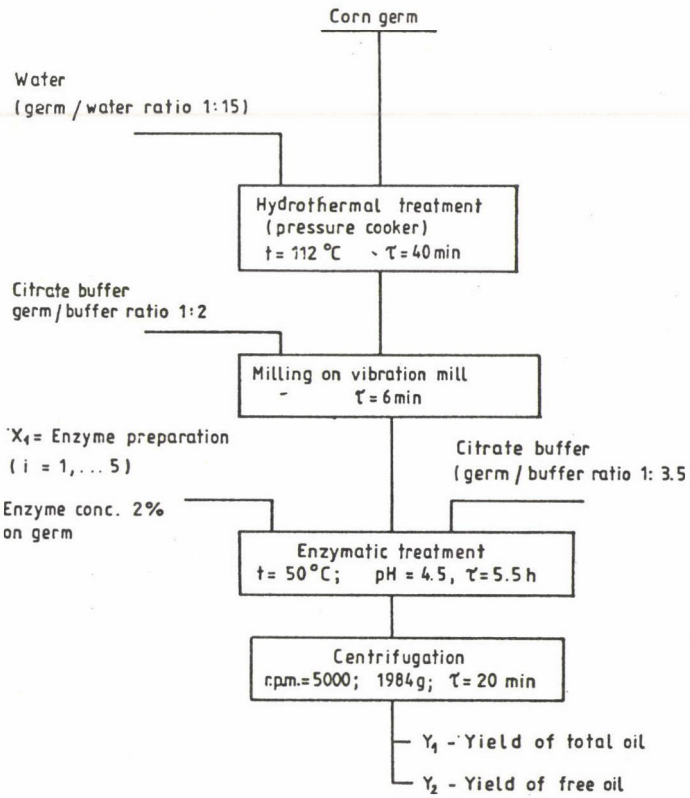

Fig. 2. Plan of experiments $Y_i = f(X_i)$

1.3. Analytical methods

The content of total and free oil was determined using the method developed by BOCEVSKA (1993).

2. Results and discussion

2.1. Analysis of the possible aqueous enzymatic extraction in the integral processing of corn germ in corn wet milling

The flow sheet of the conventional oil obtaining method presented in this work is given in Fig. 3.

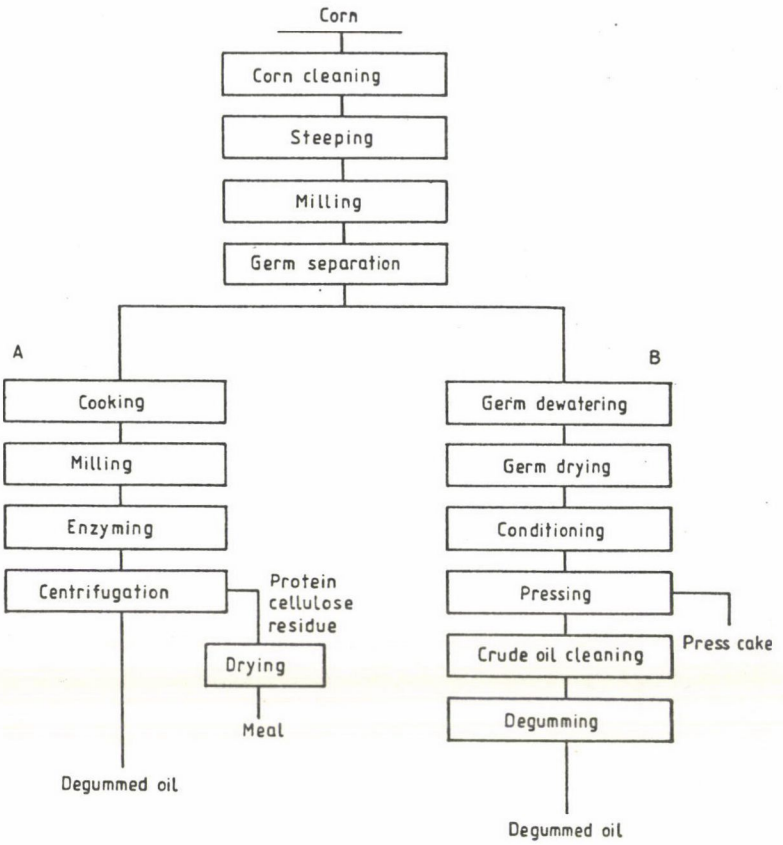

Fig. 3. Alternative ways of oil obtaining from corn germ in the course of integral starch processing of corn. A: new corn germ processing; B: conventional corn germ processing

The investigation of the effect of enzymes on ground corn germ clearly indicates that high oil yield can be obtained following the sequence of operations shown in Fig. 3 and applying the enzyme Celluclast (Fig. 4).

Our investigations show that the yield of oil obtained by the new procedure in even higher than that of obtained by the conventional method. However, the economy of this technological procedure can be estimated after the economic analysis of the process.

The analysis was performed assuming that the oil yield is the same in both processes. In that case, the economy is affected by the cost of energy and enzyme.

The data for the analysis, i.e. the specific consumption of steam and energy for the operations of the conventional procedure were supplied by a modern starch factory, whereas the data for the new biotechnological process were found in literature (HOFMANN, 1989; HAGENMAIER et al., 1975; STEIN, 1984).

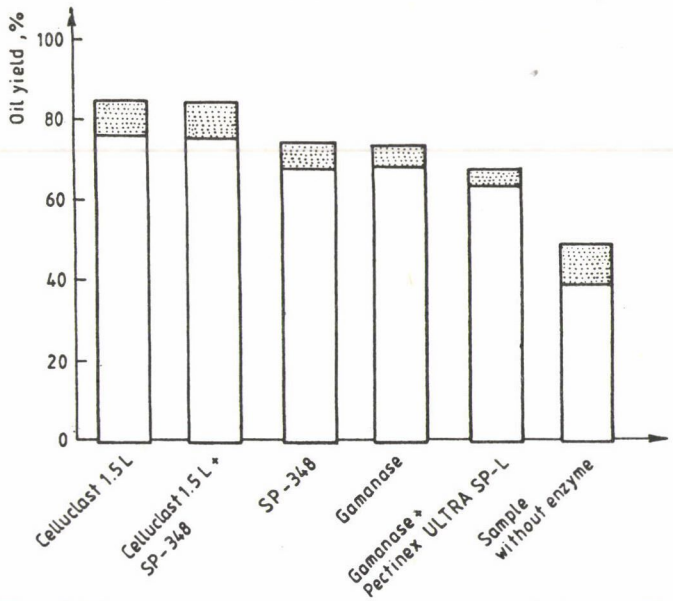

Fig. 4. Effect of different enzymatic preparations on corn oil yield, □: Free oil; ⊠: emulsion

The results presented in Table 7 point out that the cost of enzymes is the key--factor which completely annuls the effects achieved by energy saving during the biotechnological procedure. For that reason, the optimization of enzyme consumption will be the aim of our further investigations. Probably the enzymatic reaction should be performed with smaller amounts of enzymes. However, such experiments were not performed till now, as we were engaged with the optimization of other parameters of enzymatic reaction.

The economic analysis containing a complete material balance and the price of the finished product is still not possible. However, there are several alternative ways for the improvement of economy of the biotechnological procedure. BARRIOS and co-workers (1990) report on the possibility of repeated use of enzymes with water recirculation. It is possible to solve the processing of by-products (protein flour, carbohydrate solution) more economically and adequately. The drying of these products is not the only way of their valorization. BOCEVSKA (1993) considers that the processing of by-products obtained in the first cycle of enzymatic process should be treated in a different way. It may be possible to join the obtained carbohydrate fraction, containing the enzyme after the recirculation, and the starch hydrolyzate or some other substrate, in the process of fermentation.

Table 7

Analysis of specific energy consumption of corn oil extraction by conventional and aqueous enzymatic extraction procedure

Operation	Conventional[a]		Biotechnological	
	Steam (ton/ton)	Electricity (kWh/t)	Steam (ton/ton)	Electricity (kWh/t)
Corn germ dewatering by pressing	–	16.8	–	–
Corn germ drying	1.6	24.0	–	–
Cooking	–	–	0.2	5.0
Germ milling	–	–	–	20.0
Conditioning	0.8	9.6	–	–
Full pressing	–	80.0	–	–
Enzymatic treatment	–	–	0.2	5.0
Crude oil "cleaning" (settling, filtration)	1.6	19.0	–	–
Oil centrifugation	–	–	–	4.5
Drying or protein-cellulose residue	–	–	1.0	9.0
Oil degumming	0.1	4.5		
Total Energy:	4.1	153.9	1.4	43.5
Price in DM/t	225.0	23.1	70.0	6.6
Consumption and price of enzymes	–	–	20 kg × 27.5 = 550 DM	
Total (DM/t)	248.1		626.6	

[a] Plant Data: Starch Factory "IPOK" Zrenjanin, Yugoslavia
[b] Novo Information, Personal communication, 1994
Electric energy 0.15 DM/KWh;
steam 50.0 DM/t;
enzymes (Celuclast 1.5L)[b] 27.5 DM/kg

The characteristics of the obtained oil are more favourable compared to the conventionally obtained oil. Namely, the quality of the oil obtained by the new procedure is equal to the quality of degummed oil intended for physical refining. Practically, it means one operation less in the processing of the oil. Further, it has been proved that due to the work in aqueous media, simultaneously to the liquefaction of cell structure, the phospholipids are separated from the oil. BOCEVSKA (1993) reports that such oil contains about 8 ppm of phosphorous.

The starch processors have a significant experience with the application of enzymes, in contrast to oil industry. The introduction of aqueous enzymatic treatment of corn germ into the integral cereal-oil processing would be beneficial.

*

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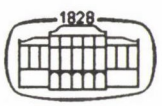

AKADÉMIAI KIADÓ
BUDAPEST

CONTENTS

VOLUME 23
1994

Physiological and pathophysiological aspects of free radicals
ANTAL, M. 25

Characterization of the lipolytic system of germinated sunflower seeds
ARRIBÉRE, M. C., PRIOLO, N. S. & CAFFINI, N. O. 311

Comparative study on some fermentation properties of *Streptococcus thermophilus* and *Lactobacillus acidophilus* in milk and modified milk media. – Part II. Effect of the increased solid not fat on the fermentation properties of the mixed culture
BADRAN, I. I. & REICHART, O. 131

Book reviews 119, 237

Effect of immobilized yeasts on the quality of bottle-fermented sparkling wine
BÚSOVÁ, K., MAGYAR, I. & JANKY, F. 9

Determination of tryptophan and methionine by mercaptoethanesulfonic acid hydrolysis at elevated temperature
CSAPÓ, J., CSAPÓ-KISS, ZS., CSORDÁS, E., FOLESTAD, S., TIVESTEN, A. & NÉMETHY, S. 255

Protein content, amino acid composition, biological value and micro- and macroelement content of mare's milk
CSAPÓ-KISS, ZS., STEFLER, J., MARTIN, T. G., MAKRAY, S. & CSAPÓ, J. 177

Fat content, fatty acid composition and vitamin content of mare's milk
CSAPÓ, J., STEFLER, J., MARTIN, T. G., MAKRAY, S. & CSAPÓ-KISS, ZS. 167

Effect of germination on a range of nutrients of selected grains and legumes
DANIŠOVÁ, C., HOLOTNÁKOVÁ, E., HOZOVÁ, B. & BUCHTOVÁ, V. 285

Separation of a methionine-enriched fraction from enzymatically modified casein by fast protein liquid chromatography
DEMECZKY, ZS., HUSSEIN, S. & HAJÓS, GY. 85

Assessment of wholesomeness of irradiated foods (A review)
DIEHL, J. F. & JOSEPHSON, E. S. 193

Photoacoustic spectra of skim milk powder and of milk protein concentrate
DÓKA, O., SZÖLLŐSY, L. & NOVÁK, Á. 323

Condensation of glucose by the reversed hydrolysis reaction of glucoamylase. – Part I. Effect of both D-glucose and starch concentrations, temperature, and enzyme concentration
EL-SAYED, H. & LÁSZLÓ, E. 43

Condensation of glucose by the reversed hydrolysis reaction of glucoamylase. – Part II. Effect of inhibition of glucose derivatives and pH
EL-SAYED, H. & LÁSZLÓ, E. 59

Condensation of glucose by the reversed hydrolysis reaction of glucoamylase. – Part III. Effect of organic solvents and immobilization forms
EL-SAYED, H. & LÁSZLÓ, E. 359

Analysis and quantitation of sugars in honey of different botanical origin using high performance liquid chromatography
FÖLDHÁZI, G. 297

A comparative study of physicochemical indices (phenolic substances) which can affect the colour of Spanish red wines
HEREDIA, F. J. & GUZMÁN-CHOZAS, M. 33

Characteristic of *Leuconostoc oenos* strains occurring in Hungarian wines
JUHÁSZ-ROMÁN, M. ... 71
Corn germ oil extraction by a new enzymatic process
KARLOVIĆ, DJ., BOCEVSKA, M., JAKOVLEVIĆ, J. & TURKULOV, J. ... 389
Deformation versus load relations of tinplate cans. Interpolation between extreme loading cycles
KÖRMENDY, I., KONCZ, L. & SÁRKÖZI, I. ... 265
The competitiveness of Hungarian food industry
LAKNER, Z. & KÓBOR, K. ... 119
Effect of the deprivation of mineral and vitamin supplement on lipid peroxidation in pigs
LUGASI, A., DWORSCHÁK, E., ENYINGI, T., GERGELY, A., ZSINKA, Á. & BÍRÓ, GY. ... 337
New method for detection of picogram quantities of staphylococcal thermonuclease
MAJOR, P. ... 377
Thermal denaturation of bacterial cells examined by differential scanning calorimetry
MOHÁCSI-FARKAS, CS., FARKAS, J. & SIMON, A. ... 155
Phase equilibria and thermodynamic incompatibility in protein containing system. – Part I. Investigation of casein – polysaccharide – water systems
NAGY, J., LÁSZTITY, R. & PERNECZKY, A. ... 243
Effect of calcium addition, acidification and fermentation on the quality characteristics of canned carrots (*Daucus carota* L.)
NOGUEIRA, J. N., CANTARELLI, P. R., GALLO, C. R. & MATSUURA, F. C. A. U. ... 145
Effect of irradiation on folate levels and on bioavailability of folates in dehydrated foodstuffs
PFEIFFER, C., DIEHL, J. F. & SCHWACK, W. ... 105
Application possibilities of nuclear filter-membranes in the food and beverage industry
SZABÓ, A. S. ... 3
Use of immobilized lipase in transesterification of triglycerides
TEMESVÁRI, J., BÁNKY, E. & BIACS, P. A. ... 213
Study on the phytic acid content of food products of cereal base
TOLNAY, P., HARKAY-VINKLER, M. & PETRES, J. ... 347
A potential protein engineering site in *Aspergillus niger* glucoamylase: vicinity of disulphide bridge 449 – 222
VÁRALLYAY, É., SASVÁRI, ZS., HOSCHKE, Á. & ASBÓTH, B. ... 93
Phosphorus content of edible wild mushrooms in Hungary
VETTER, J. ... 329
Microbial safety assurance of minimally processed vegetables by implementation of the Hazard Analysis Critical Control Point (HACCP) system
WILLOCX, F., TOBBACK, P. & HENDRICKX, M. ... 219

RECENTLY ACCEPTED PAPERS

Measured and calculated pressures and pressure differences of tinplate cans under sterilization conditions
KÖRMENDY, I., KONCZ, L. & SÁRKÖZI, I.

Metabolism of octacosanol in liver and muscle of rat
KABIR, Y. & KIMURA, S.

Relationship between high molecular weight subunits of glutenin and breadmaking quality of Hungarian grown wheats
KÁRPÁTI, M., BRIEBER, K., KURÓCZI, L. GY., CZIRÁK, L. & LÁSZTITY, R.

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ÖZCAN, M. & AKGÜL, A.

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VOLUME 23 NO. 4 – 1994

CONTENTS

Effect of the deprivation of mineral and vitamin supplement on lipid peroxidation in pigs
LUGASI, A., DWORSCHÁK, E., ENYINGI, T., GERGELY, A., ZSINKA, Á. & BÍRÓ, GY. 337
Study on the phytic acid content of food products of cereal base
TOLNAY, P., HARKAY-VINKLER, M. & PETRES, J. 347
Condensation of glucose by the reversed hydrolysis reaction of glucoamylase. – Part III. Effect of organic solvents and immobilization forms
EL-SAYED, H. & LÁSZLÓ, E. 359
New method for detection of picogram quantities of staphylococcal thermonuclease
MAJOR, P. 377
Corn germ oil extraction by a new enzymatic process
KARLOVIĆ, DJ., BOCEVSKA, M., JAKOVLEVIĆ, J. & TURKULOV, J. 389