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DETERMINATION OF THE CAPSAICIN CONTENT IN RAW GREEN PAPRIKA

E. KOZMA-KOVÁCS, E. KEVEI-PICHLER, E. and I. LENDVAI

(Received May 7, 1975)

Two procedures were developed for the determination of capsaicin in raw green paprika. It is expedient to carry out the two determinations successively from the same stock solution.

By means of the first rapid procedure, a colour reaction developed on filter paper, it may be established whether or not the paprika is pungent. At the same time the approximate capsaicin content may be determined (capsaicin quantities above 0.5 μg can be detected).

If the above reaction is positive a quantitative determination is performed by thin-layer chromatography.

Two adsorbents (ready-made DC SIL G-25 UV 254 layer and Kieselgel G layer, made in the laboratory), two detection reagents (ferric chloride–potassium ferricyanide and *Gibbs* reagent) and a common solvent mixture (chloroform–ethyl alcohol, 99 : 1) were used.

The average standard deviation of the method in the first detection was $\bar{s} = 0.0714$ mg% and in the second $\bar{s} = 0.1085$ mg%.

It was established that the error of the method did not vary significantly with any of the solvents and/or adsorbents.

The time requirement of the capsaicin determination, including the informatory test, is 3 to 3.5 h. However, if only pungency has to be tested, 2 to 3 min suffice to give final information.

The literature on this subject matter is mostly concerned with the capsaicin content of ground red paprika and of oleoresins.

Classical methods are based on some colour reaction of capsaicin which is evaluated by photometry (HIPPENHEIMER, 1949; NORTH, 1949; BENEDEK, 1953, 1959; SCHULTE & KRÜGER, 1955; SPANYÁR *et al.*, 1956, 1959). The common shortcoming of these methods is that the elimination of pigments, fats and of other troublesome substances is difficult and they are not sufficiently sensitive, they are not suitable for the determination of capsaicin below 10 mg in 100 g.

In recent years methods of thin-layer and gas chromatography have been developed.

Column, thin-layer and paper chromatography were first used only for the purification of the capsaicin containing extract, since after chromatography pigments and other substances did not disturb determination by photometry (HOLLÓ *et al.*, 1957; FRIEDRICH & RANGOONWALA, 1965; KARAWYA *et al.*, 1967; GOVINDARAJAN & ANANTHAKRISHNA, 1974). The drawback of these methods is that they are lengthy and the preparations cause losses, therefore

they are not suitable for determination of low capsaicin contents. Extracts containing 50–150 μg capsaicin were applied to the layer and these correspond to a capsaicin content of 500–1000 mg per 100 g sample.

Literature on quantitative thin-layer chromatography of ground paprika is listed in Table 1.

Table 1

Thin-layer chromatographic methods for the quantitative determination of capsaicin in ground paprika

Authors	Adsorbent	Developing agent	Detecting reagent	Sensitivity of the detecting reagent
HEUSSER (1964)	Kieselgel HF_{254}	1. Twice in chloroform (to eliminate lipoids) 2. Chloroform–methanol–acetic acid (95 : 1 : 5)	a) 0.4% 2,6 dibromoquinone chloroimide dissolved in methanol b) Ammonia atmosphere	1 μg
SPANYÁR and BLAZOVICH (1969)	Kieselgel G	Chloroform–methanol (99 : 1)	15% $FeCl_3$; 0.5% $K_4[Fe(CN)_6]$, 1 : 1 aqueous solution	0.1 μg
RIOS and DUDEN (1971)	Kieselgel G	Chloroform–ethyl acetate–isopropanol (33 : 9 : 3)	281 nm *Zeiss-Chromatogram-Spectralphotometer*	2 μg
ANDRÉ and MILE (1972)	Kieselgel G	Diethyl ether	a) 0.2% dichloroquinone chloroimide dissolved in methanol b) 0.05 *N* NaOH, 50% solution in methanol	0.2 μg

The first method applying thin-layer chromatography was developed by HEUSSER (1964). The procedure is lengthy because capsaicin is separated after running twice in chloroform in order to eliminate lipoids. The chromatograms could be evaluated visually and then capsaicin could be eluted to determine its quantity by spectrophotometry. However, this procedure requires an extract containing 100–200 μg capsaicin.

The method of SPANYÁR and BLAZOVICH (1969) is exceptional because it is suitable for the analysis of ground paprika samples containing above or below 10 mg capsaicin in 100 g. The extraction procedure is different for samples of low and high capsaicin content. An extract containing 0.2–0.6 μg capsaicin is needed for determination.

Based on the method of SPANYÁR and BLAZOVICH (1969), RIOS and DUDEN (1971) as well as ANDRÉ and MILE (1972) developed thin-layer chroma-

tographic methods. The former authors used remission spectrophotometry, for evaluating the separated capsaicin. The latter authors used *Gibbs* reagent, specific for capsaicin. However, these methods, because of the long and complicated extraction procedure, are rather tedious.

Except for SPANYÁR and BLAZOVICH, the other authors do not mention the time requirement of their methods. According to SPANYÁR and BLAZOVICH (1969) the analysis of samples containing capsaicin above 10 mg% takes about 2–2.5 h, while that of samples of low capsaicin content 4–4.5 h.

Studies on the structure of capsaicin by thin-layer chromatography are also worth mentioning. These methods are listed in Table 2.

Table 2

Thin-layer chromatographic methods for the study of the structure of capsaicin obtained from ground paprika

Authors	Adsorbent	Developing agent	Detecting reagent
FRIEDRICH and RANGOONWALA (1965)	Polyamide	Water–dioxane (2 : 1)	0.5% Echtblausalz 0.1 *N* aqueous KOH
TYIHÁK and co-workers (1966)	Kieselgel G	Benzene–ethyl acetate (1 : 1)	15% $FeCl_3$ 0.5% $K_4[Fe(CN)_6]$ aqueous solution 1 : 1 mixture
JUHÁSZ and TYIHÁK (1969)	Cellulose MN 300	0.1 *M* NaOH 0.2 *M* Na-acetate 0.3 *M* Na_2CO_3 (2 : 3 : 5)	15% $FeCl_3$ 0.5% $K_4[Fe(CN)_6]$ aqueous solution 1 : 1 mixture
RANGOONWALA (1969)	Polyamide DC + + 10% cellulose DC MN 300	0.1 *N* NaOH—N,N-dimethyl formamide (9 : 1)	0.5% aqueous diazobenzene sulfonic acid 1 *N* NaOH in methanol

It was found by these methods that capsaicin is not a homogeneous substance but consists of two components present in the proportion of 2 : 1. By investigating the structure FRIEDRICH and RANGOONWALA (1965) established that the substance present in greater amount is *trans* capsaicin, while the other component is a dihydro derivative of capsaicin.

TYIHÁK and co-workers (1966) and JUHÁSZ and TYIHÁK (1969) discovered that a phenolic hydroxyl group is responsible for pungency. The substance of higher R_f value is less pungent but reacts in the same way as capsaicin.

The thin-layer chromatographic method of RANGOONWALA (1969) is suitable for the investigation of capsicum extracts mixed with synthetic products. Capsaicin, *cis*-capsaicin, pelargonic acid-vanillyl amide and dihydrocapsaicin may be determined by this method.

The aim of developing gas chromatographic methods was also to throw

more light on the structure of capsaicin and promote quantitative determination.

The capsaicin obtained from ground paprika was hydrolysed by TODD and PERUN (1961) and the fatty acids were transformed into methyl esters and determined by gas chromatography. The method provided valuable information on the fatty acid chains of capsaicin and enabled the differentiation between synthetic compounds.

Paprika oil was studied by HOLLÓ and co-workers (1969).

Other methods were aiming at the elucidation of the structure of the capsaicin molecule and its homologues. This was achieved either by siliconizing the capsaicin containing extract (HARTMAN, 1970), or by using a siliconized column and SE-30 silicone rubber stationary phase (MÜLLER-STOCK *et al.*, 1973).

Most of the papers mentioned were concerned mostly with the capsaicin content of ground paprika and were rather poor in information on green paprika.

This problem was studied already earlier at the CENTRAL FOOD RESEARCH INSTITUTE, Budapest. SPANYÁR and co-workers (1958) carried out the determination by a diazotization reaction on filter paper with the carbon tetrachloride extract of paprika ribs. The threshold value of the method is 100 μg capsaicin. It has the advantage of taking only a few minutes to decide whether the paprika is pungent.

SPANYÁR and BLAZOVICH (1968) tried to determine quantitatively the capsaicin content of green paprika by thin-layer chromatography. The method developed by them has the disadvantage of lengthy preparatory steps. The paprika pericarp is homogenized, then suspended in 57% alcohol. After centrifuging and distilling, the aqueous residue is repeatedly extracted with ether. Finally dehydration is followed by repeated evaporation. The solution in ether, thus obtained, is used for thin-layer chromatography, proven successful in capsaicin determination in ground paprika. The time requirement of the method is not mentioned. The average standard deviation of the method $\bar{s} = 0.18$ mg%.

Since the above described methods did not come into general use this study was aiming at developing a rapid method for the determination of capsaicin in green paprika.

To judge pungency in green paprika has been a problem of long standing since the available sensory method is not sufficiently exact and it is tedious. Because of increased export the regular control of green paprika samples became necessary. By means of sensory evaluation this is not possible, panel members get tired (and erratic) of tasting quickly. It is important therefore to replace sensory evaluation by a more accurate, objective method.

The method developed in the course of this study is intended to replace

sensory evaluation and it may be used for quantitative determination of capsaicin. The analysis is carried out in steps. First pungency is tested by rapid paper chromatography and at the same time an approximate value of the capsaicin content is obtained.

The same stock solution is used to carry out the quantitative determination according to SPANYÁR and BLAZOVICH (1969), complemented by the application of a new adsorbent (ready-made DC SIL G-25 UV_{254} layer) and a detection reagent specific for capsaicin (*Gibbs* reagent).

The preparative operations of the method are simple and rapid. The homogenized paprika pericarp is extracted with carbon tetrachloride, filtered through anhydrous Na_2SO_4. The filtrate is evaporated and dissolved in CCl_4 and used as stock solution.

The simple preparatory operations and the use of the ready made layer permit of carrying out the analysis rapidly.

Determination including the paper chromatography of informatory character takes about 3– 3.5 h. (The average standard deviation of the method, depending on the detection reagent used, is $\bar{s} = 0.0714$ and $\bar{s} = 0.1085$ mg%.)

Since this method is more simple and rapid than those hitherto used it is proposed for practical use.

1. Materials and methods

1.1. Green paprika samples

The following varieties were used:
Bogyiszlói csemege (Dessert paprika, Bogyiszló)
Újmajori csemege (Dessert paprika, Újmajor)
Alma csemege (Apple dessert paprika)
Cecei csemege (Dessert paprika, Cece)

Variety *Bogyiszlói csemege* was bought on the market at various points of time.

The other varieties were obtained from Székkutas by courtesy of the NATIONAL INSTITUTE FOR AGRICULTURAL VARIETY TESTING.

1.2. Preliminary experiments

1.2.1. Adsorbents. — Apart from the adsorbent proposed by SPANYÁR and BLAZOVICH (1969) the following were found suitable for capsaicin determination: *DC SIL G-25 UV_{254} ready-made layer (Macherey-Nagel);* thickness: 0.25 mm; needs not be activated before use; *POLYGRAM SIL G ready-made layer (Macherey-Nagel);* thickness: 0.25 mm, it has to be activated before use at 105 °C for 30 min.

The advantage of both layers is their perfectly even thickness and surface, thus it is easy to apply the solution. At the same time the time requirement is reduced by the omission of layer preparation and occasionally of activation.

The above mentioned adsorbents are of similar type (silicagel) and thus are suitable for capsaicin determination and may be substituted for one another.

1.2.2. Developing agents. — Apart from the developing agents mentioned in the literature: chloroform — ethyl alcohol, 99 : 1 (SPANYÁR & BLAZOVICH, 1969), diethyl ether (ANDRÉ & MILE, 1972), the following reagents were tested:

ether–petroleum ether (40–70 °C) (98 : 2)

chloroform–ether–absolute alcohol (50 : 50 : 1).

By means of any of these developing agents capsaicin may be efficiently separated.

1.2.3. Detecting agents. — Two detecting agents were used and both were found equally suitable.

1. *Gibbs'* reagent:

a. 0.4% 2,6 dichloroquinone chloroimide dissolved in absolute alcohol

b. 0.1 *N* NaOH dissolved in water.

The layer is first sprayed with solution *a* and, after drying, with solution *b*.

Sensitivity of the detecting agent: 0.5 μg capsaicin. This reagent is specific for capsaicin being the most sensitive reagent of the phenolic hydroxyl group.

2. Detecting agent:

a. 15% aqueous solution of ferric chloride

b. 0.5% aqueous potassium ferricyanide.

Detection is carried out with the 1 : 1 mixture of the two reagents directly before use.

Sensitivity: 0.2 μg capsaicin.

The detecting agent is very sensitive, but not specific and therefore other substances may also give the blue reaction. Determination is, however, not disturbed thereby if the accompanying substances had been previously separated by thin-layer chromatography.

On the basis of the preliminary experiments two adsorbents: Kieselgel G and DC SIL G-25 UV_{254} ready-made layers and chloroform–ethyl alcohol (99 : 1) as developing agent were used. Capsaicin was detected by the two reagents mentioned above.

1.3. Preparation of the green paprika sample

Since the moisture content of green paprika is relatively high (70–93%) and its capsaicin content low (0–7 mg%) the extraction operation has to be ex-

tremely reliable, otherwise this operation may become the source of serious errors.

Testing a number of extraction procedures described in the literature showed that carbon tetrachloride as suggested by SPANYÁR and co-workers (1958) is the most suitable. The results obtained by this method were compared with other methods (SPANYÁR & BLAZOVICH, 1969; ANDRÉ & MILE, 1972) and extraction with carbon tetrachloride was found very efficient.

Reagents: carbon tetrachloride, analytical grade; Na_2SO_4 anhydrous, analytical grade; quartz sand.

Extraction: An average sample of 400–500 g was used. The pericarp of the paprika was cut into pieces then homogenized either in a *Turmix* apparatus or with a meat mincer. From the paprika pulp thus obtained 20 g were rubbed with 10 g anhydrous Na_2SO_4 and 2 g quartz sand in a porcelain mortar then washed into a 200 ml *Stift* flask and filled to mark with carbon tetrachloride. After shaking several times during 30 min the solution was filtered through a folded filter. Hundred ml of the filtrate were dehydrated with anhydrous Na_2SO_4 and filtered. The dehydrating agent was washed with CCl_4 and thereby the capsaicin was eluted, the solution was evaporated to 15–20 ml over a water bath under nitrogen stream. After evaporation the residue is cooled again under nitrogen stream and washed into a 25-ml measuring flask and made to mark with CCl_4. This is the stock solution used for the pungency test as well as for the determination.

1.4. Analytical methods

In the knowledge of relevant literature and the results of the preliminary experiments two methods, independent but complementary, were developed. The first method enables one to determine whether a volume of 0.05 ml of the extract contains a detectable amount (at least 0.5 μg) of capsaicin and to assess its approximate quantity; this takes only a few minutes.

The second method is suitable to determine the quantity of capsaicin by means of thin-layer chromatography.

1.4.1. Detection of pungency in green paprika. Informatory test. — Reagents: 20 $\mu g\ ml^{-1}$ capsaicin solution in CCl_4 was used for comparison; quantitative filter paper, MN 640 m *(Macherey–Nagel); Gibbs* reagent: *a.* 0.4% 2,6–dichloroquinone chloroimide dissolved in absolute alcohol; *b.* 0.1 *N* NaOH dissolved in distilled water.

Principle of the method: With a known amount of the carbon tetrachloride paprika extract (1–2 drops) a microreaction is carried out on filter paper. As little as 0.5 μg capsaicin may be detected by the blue compound formed on the addition of *Gibbs* reagent.

Procedure: Using a micropipette (0.1 ml volume of 100 divisions) 0.05 ml is dropped on the pencil-marked center of a MN 640 M filter paper of 7 cm

diameter. The drop should spread in a not too large but round circle. After the evaporation of the solvent (accelerated by hot air from a hair-drier) 0.015–0.02 ml of the *a* component of *Gibbs* reagent, after drying the same amount of the *b* component are applied to the spot.

If the extract contained no capsaicin a grey-brown spot becomes visible. In the presence of capsaicin, as soon as the alkali is applied, a blue ring appears around the spot and on drying it becomes more intense. The dried filter paper retains the blue colour for days.

The sensitivity of this method is good, 0.5 μg capsaicin is enough to give a positive reaction.

To establish the approximate capsaicin content of the stock solution volumes equivalent to 0.5 and 1.0 μg capsaicin of a standard capsaicin solution are applied to a filter paper each (*e. g.* from a stock solution of 20 μg ml^{-1} 0.025 and 0.05 ml, resp.). When the colour reaction is accomplished the two filter papers are compared to that having the green paprika spot. On the basis of colour intensity the capsaicin content may be assessed.

If the results of the informatory test are not satisfactory, for instance the spot is much paler than that of the 0.5 μg capsaicin standard, the stock solution has to be concentrated. If the colour is much more intense than that of the 1.0 μg capsaicin standard, the stock solution has to be diluted.

1.4.2. Determination of the capsaicin content by thin-layer chromatography. — As mentioned above two ready-made thin-layers, one developing agent and two detecting reagents were used. Reagents: 20 μg ml^{-1} capsaicin solution in CCl_4 for comparison; chloroform, analytical grade; 96% ethyl alcohol.

Given the capsaicin concentration in the stock solution as established by the informatory test, the most suitable volume of stock solution for chromatography may be seen in Table 3.

Data in the table may be used irrespective of whether *Gibbs* reagent or Berlin-blue reagent is applied.

Procedure: On the activated Kieselgel G layer or on the DC SIL G-25 UV_{254} layer 6 points, identically spaced, are marked. Depending on the detecting agent used, various amounts of the stock solution are applied to these start points.

In case the *Gibbs* reagent is used 0.05, 0.085 and 0.12 ml, resp., are applied alternately from the 20 μg ml^{-1} standard solution and the stock solution.

In case the ferric chloride–potassium ferricyanide reagent is used the alternately applied volumes are 0.02, 0.04 and 0.06 ml, resp.

The thin-layer is developed till the solvent front reaches the upper edge of the layer with the chloroform–ethyl alcohol (99 : 1) reagent. The chromatogram is dried with a hair-drier. Drying is important, because even a minute amount of solvent may cause trouble. With the second detecting agent traces of the solvent may hinder evaluation.

Table 3

Correlation between the capsaicin content as established in the preliminary test and the volume of stock solution used in thin-layer chromatography

Capsaicin content (mg%)	Volume of stock solution required for chromatography (ml)
1	5
2	10
3	15
4	20
5	25
6	30
7	35

In case the *Gibbs* reagent is used for detection, first the thin-layer is sprayed with component *a*. After drying component *b* is applied. In order to achieve even development of the colour the alternate application of the two components has to be repeated several times. After detection, clean-cut blue spots appear the size and intensity of which is directly related to the capsaicin concentration. The evaluation of the spots may be expediently carried out half an hour after the last spraying.

The second detecting agent is the 1 : 1 mixture of 15% ferric chloride and of 0.5% potassium ferricyanide. It is important to apply the reagent evenly, because the yellowish-green colour of the background may disturb the evaluation of the blue capsaicin spots. Evaluation has to be carried out within 1–2 min after the spraying of the layer, because other spots may appear around the capsaicin spot and the colour of the background may merge with the colour of the spots.

The capsaicin content of the sample was established by way of comparing the size and intensity of the spots of the stock solution with the spots of the standard solution, according to the semi-quantitative method of BLAZOVICH and co-workers (1969). If the spots are not comparable the procedure has to be repeated.

Figs. 1 and 2 show chromatograms suitable for the quantitative evaluation of the capsaicin content of the green paprika extract. The chromatogram in Fig. 1 was made visible with *Gibbs* reagent.

The chromatogram in Fig. 2 was sprayed with the 1 : 1 mixture of ferric chloride and potassium ferricyanide.

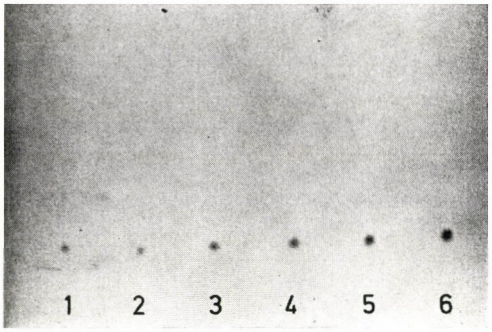

Fig. 1. Determination of the capsaicin content of paprika extract by quantitative thin-layer chromatography, using *Gibbs* reagent. Adsorbent: DC SIL G-25 UV_{254}, ready-made. Developing agent: chloroform–ethanol (99 : 1). Detecting agent: *Gibbs* reagent. 1. Stock solution of paprika, 0.05 ml. 2. Standard capsaicin solution (20 $\mu g\ ml^{-1}$), 0.05 ml. 3. Stock solution of paprika, 0.085 ml. 4. Standard capsaicin solution (20 $\mu g\ ml^{-1}$), 0.085 ml. 5. Stock solution of paprika, 0.12 ml. 6. Standard capsaicin solution (20 $\mu g\ ml^{-1}$), 0.12 ml

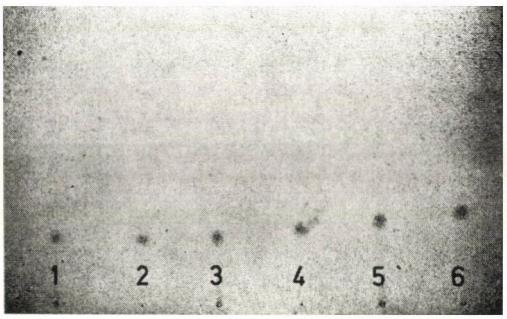

Fig. 2. Quantitative determination of the capsaicin content of paprika extract by thin-layer chromatography, using ferric chloride–potassium ferricyanide reagent. Adsorbent: Kieselgel G (0.3 mm) drawn layer. Developing agent: chloroform–ethanol (99 : 1). Detecting agent: aqueous 0.5% potassium ferricyanide and aqueous 15% ferric chloride solutions mixed directly before use (1 : 1). 1. Stock solution of paprika, 0.02 ml. 2. Standard capsaicin solution (20 $\mu g\ ml^{-1}$), 0.02 ml. 3. Stock solution of paprika, 0.04 ml. 4. Standard capsaicin solution (20 $\mu g\ ml^{-1}$), 0.04 ml. 5. Stock solution of paprika, 0.06 ml. 6. Standard capsaicin solution (20 $\mu g\ ml^{-1}$), 0.06 ml

2. Results and conclusions

The usefulness of the rapid chromatographic method for the determination of the capsaicin content of green paprika has been demonstrated in various ways.

The efficiency of the extraction procedure was studied. A pulp was prepared from the green paprika variety *Cecei*, containing no capsaicin, and various amounts of capsaicin (1–7%) were added. The recovered capsaicin was evaluated by thin-layer chromatography. The chromatograms were made visible with both detecting agents. Results are given in Tables 4 and 5.

Table 4

Determination of capsaicin added (1–7 mg%) to non-pungent green paprika, using Gibbs reagent and ready-made DC SIL G-25 UV_{254} layer

Amount of added capsaicin (mg%)	Recovered capsaicin (mg%)	Average (mg%)	s (mg%)
1.0	1.03 1.01 1.17 1.17 1.02	1.080	0.082
2.0	1.97 1.96 1.93 1.94 2.04	1.968	0.043
3.0	3.04 3.08 2.97 3.02 2.98	3.018	0.045
4.0	4.23 3.91 3.98 4.04 4.14	4.060	0.13
5.0	5.11 5.08 5.22 5.22 5.25	5.176	0.076
6.0	5.96 6.03 6.11 5.94 6.10	6.028	0.075
7.0	7.06 6.94 6.94 7.01 6.97	6.984	0.051

Table 5

Determination of capsaicin added (1–7 mg%) to non-pungent green paprika, using the ferric chloride–potassium ferricyanide reagent and ready-made DC SIL G-25 UV_{254} layer

Amount of added capsaicin (mg%)	Recovered capsaicin (mg%)	Average (mg%)	s (mg%)
1.0	1.15 1.11 1.15 1.04 1.15	1.120	0.023
2.0	2.00 2.11 1.98 2.08 2.01	2.036	0.056
3.0	2.91 3.07 2.94 2.80 3.28	3.00	0.183
4.0	3.95 4.01 4.05 3.96 3.84	3.962	0.079
5.0	5.13 5.00 4.92 4.85 4.91	4.962	0.106
6.0	6.03 5.75 5.90 6.10 5.96	5.948	0.134
7.0	7.21 7.08 7.10 7.08 6.85	7.064	0.131

The *Bartlett* test was used to see whether the deviations belong to the same distribution. The results have shown the deviation to originate at 90% probability level from the same distribution in the range of 1–7 mg% capsaicin content. The average standard deviations obtained are as follows:

using *Gibbs* reagent $\bar{s} = 0.0714$ mg%

using the other reagent $\bar{s} = 0.1085$ mg%.

The values of recovered capsaicin obtained by the procedures, differing only in the detecting agents used, were compared by two-factor variance analysis (Table 6).

Table 6

Variance analysis of the amounts of capsaicin recovered by the use of two different detecting agents (Tables 2, 3)

	Sum of squares	Degree of freedom	s^2	F
Total	55.21	13		
Samples	55.17	6	9.195	
Detecting agents	0.0037	1	0.0037	0.63
Residual	0.0356	6	0.0059	

The average standard deviation on the application of the two different detecting agents $\bar{s} = 0.076$ mg%; $F_{table\ 0.95} = 6.0$

On the basis of the table of variances the conclusion was drawn that the difference between the results obtained by the two detecting agents was non-significant ($F = 0.63 \ll F_{table\ 0.95} = 6$).

The average standard deviation of the detecting agents = 0.077 mg%.

The amount of recovered capsaicin in the range of 1–7 mg% added capsaicin was investigated. In this case the average of the capsaicin values obtained by the use of the two detecting reagents was taken into account. Data were evaluated by linear regression analysis. Equation of the regression line:

$$y = 0.059 + 0.993x$$

where

y = recovered capsaicin, mg%

x = capsaicin added, mg%.

The correlation coefficient: $r = 0.9999$, the standard deviation about the regression line: $s^2_{yx} = 0.0016$.

The difference between the regression line and the $y = x$ line was investigated. As the difference between the y-intersept and 0 and the tangent and 1 was found non-significant, the added capsaicin was recovered to 100%, and the calibration curves obtained with the two detecting agents may be characterized by a common regression line.

Further, from the same green paprika homogenate two portions were weighed in and 5 parallel chromatograms were made with both reagents. Thus conclusions could be drawn as to the error in weighing in and in replicates.

The average of standard deviations belonging to replicates, as computed by single factor variance analysis, $\bar{s} = 0.0624$.

There is no significant difference between weighings ($F = 0.31 \ll F_{table\ 0.95} = 5.1$).

Results are given in Table 7.

Table 7

Capsaicin content of the pericarp of Bogyiszlói csemege *paprika calculated on the basis of two weighings and 5 parallel chromatograms (Kieselgel G layer, both detecting agents)*

Weighing in	Serial number of chromatogram	Capsaicin content, mg%	
		Detecting agent	
		1	2
I	1	4.95	4.87
	2	4.93	4.86
	3	4.90	4.97
	4	4.95	5.02
	5	4.84	4.95
Average capsaicin content, mg%		4.91	4.93
Standard deviation, s mg%		0.046	0.068
II	1	4.90	4.91
	2	4.97	4.82
	3	4.98	4.95
	4	4.93	5.02
	5	4.95	5.04
Average capsaicin content, mg%		4.95	4.95
Standard deviation, s mg%		0.031	0.088

Average standard deviation $\bar{s} = 0.062$ mg%

Variance of error in weighing in and replication

	Sum of squares	Degree of freedom	s^2	F
Total	0.0663	19		
Parallel	0.0037	3	0.0012	0.31
Residual	0.0626	16	0.0039	

$F = 0.31 < F_{table\ 0.95} = 5.1$

Further it was proven by mathematical calculations that for the comminution of green paprika the *Turmix* apparatus and chopping up by hand are equally satisfactory if all other conditions of preparation are strictly observed. Calculations were carried out on the basis of averages of two weighings and 5 parallel chromatograms for both reagents. Results are shown in Table 8.

Table 8

Variance analysis of capsaicin contents of green paprika homogenized by two different methods (using Turmix apparatus or hand-chopped)

Weighing in	Method of comminution	Serial number	Capsaicin content, mg%	
			Detecting agent	
			1	2
I–II	*Turmix*	1	4.93	4.89
		2	4.95	4.84
		3	4.94	4.96
		4	4.94	5.02
		5	4.90	4.99
Average capsaicin content, mg%			4.93	4.94
Standard deviation, s mg%			0.020	0.074
III–IV	hand-chopped	1	4.92	4.89
		2	4.99	4.80
		3	4.91	4.98
		4	4.96	5.03
		5	5.01	4.98
Average capsaicin content, mg%			4.96	4.94
Standard deviation, s mg%			0.043	0.091

Average of standard deviations of turmixed samples $\bar{s}$ mg%	0.07
Average of standard deviations of hand-chopped samples $\bar{s}$ mg%	0.014
Common average standard deviation of the two comminution technologies, $\bar{s}$	0.011

	Sum of squares	Degree of freedom	s^2	F
Total	0.00048	3	0.00016	
Preparation	0.00023	1	0.00023	1.8
Residual	0.00025	2	0.00013	

$F_{table\ 0.95} = 19$

Hereafter four homogenates were made of the paprika variety *Bogyiszlói csemege*, one portion was weighed of each and 3 parallel chromatograms were

prepared, using both detecting reagents, and the capsaicin content was determined.

The average capsaicin contents as obtained from different weighings were very different and the consequent error was significantly greater than other sources of error of the method, 1.087 mg%.

However, parallel chromatography from the same weighing resulted in corresponding capsaicin contents. Thus it follows that parallel weighings have to be prepared from separate homogenates. Results are given in Table 9.

It can be seen from the Table that the average values obtained with the two reagents do not differ, while the results of two different weighings differ significantly.

Table 9

Error and variance analysis of capsaicin contents determined from different homogenates of the same green paprika sample

Weighing in	Capsaicin content, mg%	
	Detecting agents	
	1	2
1	3.93	3.86
	3.96	3.95
	3.88	3.96
Average capsaicin content	3.92*	3.925*
2	3.11	2.91
	3.045	2.975
	2.99	3.04
Average capsaicin content	3.04	2.976
3	2.04	1.98
	2.1	2.03
	2.07	2.03
Average capsaicin content	2.07*	2.01*
4	3.09	3.00
	3.00	3.00
	3.01	3.02
Average capsaicin content	3.03	3.00

Average of the four weighings 2.996 mg%

$\overline{x}_{\text{detecting agent1}} = 3.015$ mg%
$\overline{x}_{\text{detecting agent2}} = 2.978$ mg%

* deviation from the common average

	Sum of squares	Degree of freedom	s^2	F
Total	3.55	7		
Replicates	3.5457	3	1.1819	2.300
Detecting agents	0.00278	1	0.00278	5.45
Residual	0.00154	3	0.0051	

$$\text{L.S.D.} = \text{sm} \cdot t \cdot \sqrt{\frac{1}{3}} = 0.041 \qquad \text{(least significant difference)}$$

Finally the averages of capsaicin contents obtained from different paprika varieties were tabulated. For evaluation both ready-made layers: DC SIL G-25 UV_{254} and Kieselgel G, as well as both detecting reagents were used in three replicates each. As in the case of the detecting reagents, the difference between the adsorbents is non-significant as well. Results are tabulated in Table 10.

The rapid method developed for the determination of capsaicin in green paprika, including the informatory test, takes about 3–3.5 h. Thus 5–6

Table 10

Capsaicin content of different paprika varieties determined on two adsorbents with two detecting agents

Paprika variety	Capsaicin content, mg%			
	Detecting agent			
	1		2	
	DC SIL G-25 UV_{254}	Kieselgel G	DC SIL G-25 UV_{254}	Kieselgel G
Bogyiszlói, pointed, dark green	5.98	5.84	5.96	6.00
Bogyiszlói, white	4.96	4.98	5.00	4.95
Bogyiszlói, white	0.92	0.90	0.92	0.88
Alma	1.15	1.10	1.00	1.15
Újmajori	0.43	0.42	0.43	0.45

Variance analysis of results obtained on the two different adsorbents.

Variance analysis of results obtained on the two different adsorbents

	Sum of squares	Degree of freedom	s^2	F
Total	106.52	19		
Samples	106.49	4	26.62	
Replicates	0.0053	3	0.0018	0.741
Residual	0.0288	12	0.0024	

$F_{table\ 0.95} = 4.9$

samples may be analysed in one day. The method is suitable for the investigation of pungency.

The capsaicin content of green paprika may be determined on both adsorbents by the application of the 99 : 1 mixture of chloroform and ethyl alcohol as the developing agent and either of the two detecting reagents. It is advisable to carry out prior to thin-layer chromatography the microcolour reaction on filter paper. To obtain accurate results it is expedient to start with at least two homogenates of 500 g each.

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STUDY OF THE PHOSPHO- AND GLYCOLIPIDS OF SOYA

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After the determination of the ratio of non-lipid and lipid components by a multistep analytical procedure, the neutral and polar components were fractionated according to lipid groups. Within the non-lipid components of soya-lecithin, water soluble non-lipids (salts, amino acids, carbohydrates) are predominant. In addition to neutral lipids (triglycerides), one phospholipid and two glycolipid fractions were obtained by fractionation of the lipid groups. On the average, the ratio of glycolipids to phospholipids was 15 : 85, which is in good agreement with literature data.

For the fractionation of purified lipid components, a column chromatographic method was developed, using *Sephadex* LH-20 as gel bed. Structure elucidation of the fractionated phospholipid components was carried out by IR spectroscopy and gas chromatography. It has been established that, depending on the nature of the alcohol fraction linked to the phosphate group, a marked difference can be observed in the fatty acid composition.

The practical aim of our investigations was the elaboration of a method for determination of the efficiency of industrial fractionation (degumming).

Knowledge of the qualitative and quantitative composition of lecithin obtained in the procedure may lead to a revised technology and, through this, to the utilization of Hungarian soya-lecithin in the food industry.

Extensive research has recently been carried out to study qualitative and quantitative changes in polar lipids during the ripening period of oilseeds.

In the course of maturation of seeds, the most significant change observed in the lipid composition of soybeans is an increase in the amount of triglycerides and a parallel increase of the synthesis of oleic and linoleic acids. Although the total amount of glyco- and phospholipids shows a much slower increase during ripening, these compounds may be assumed to take an active role in metabolism (Privett *et al.*, 1973).

Singh and Privett (1970) studied immature and mature soybean samples of the *Chippewa* 64 variety 44 and 88 days after flowering. After extraction and evaporation, proteolipids were removed by filtration and the filtrate was washed out with 0.5% NaCl solution to remove non-lipid contaminations. The total lipid extracts were fractionated by chromatography on a silicic acid column. After separation the fractions were studied by thin-layer chromatography in one- and two-dimensional systems. The spots were detected by means of iodine vapour and developed with a specific reagent (ammonium molybdate). The total amount of organic material on the thin-layer was shown by application of a sulphuric acid spray and charring at 180 °C. The results are given in Table 1.

Table 1

Polar lipids of immature and mature soybean according to SINGH *and* PRIVETT *(1970)*

Main components in the fractions	Immature soybean (44 days)	Mature soybean (88 days)
Neutral lipids (TG, ST, SE)	62.2%	92.0%
Sterolglucosides (ESG, SG)	18.2%	—
Glycolipids (CE, SL, DGDG)	11.2%	1.9%
Phospholipids (PA, PI, PE, PC, PS)	7.9%	6.1%

Nomenclature: TG (triglycerides); SE (sterol esters); ESG (esterified sterolglucosides); SG (sterolglucosides); CE (cerebrosides); SL (sulpholipids); DGDG (digalactosyl diglycerides); PA (phosphatic acid); PI (phosphatidyl inositol); PE (phosphatidyl ethanolamine); PC (phosphatidyl choline); PS (phosphatidyl serine); LPC (lysophosphatidyl choline); LPE (lysophosphatidyl ethanolamine); MGDG (monogalactosyl diglyceride); GPL (glycophospholipid); PG (phosphatidyl glycerol); DPG (diphosphatidyl glycerol)

PRIVETT and co-workers (1973) gave a report on changes in the lipid composition of the soybean variety *Anoka G. max.*, observed during different stages of the development process. Extraction and removal of the non-lipid components were carried out under conditions described above. Lipid components were separated by adsorption chromatography using a silicic acid column treated with ammonium hydroxide. After fractionation of the groups, phospholipids and glycolipids were separated by thin-layer chromatography and, applying the above method of charring on the plate, quantitative analysis of the spots was carried out by using densitometric techniques. In agreement with the above findings, it has been stated that in the course of maturation, the amount of neutral lipids increases, while polar lipids decrease. Within this group, glycolipids show a constant decrease, while in the case of phospholipids, after an initial decrease, a certain degree of increase could be observed again (Table 2).

Table 2

Some data cited from the analytical measurements published by PRIVETT *and co-workers (1973) about lipid groups of soybean during ripening*

Main components in the fraction	Immature soybean (55 days)	Mature soybean (97 days)
Neutral lipids	88.6%	88.1%
Glycolipids I–II total	2.1%	1.6%
I (ESG, SG)	(0.2%)	(0.7%)
II (MGDG, CE, DGDG, GPL)	(1.9%)	(0.9%)
Phospholipids (PG, DPG, PE, PC, PI, PA)	8.6%	9.8%

Nomenclature: see Table 1

ERDAHL and co-workers (1973) studied commercial soya-lecithins and established that the composition of products marketed by the firms ASSOCIATED CONCENTRATES and CENTRAL SOYA was very similar. The soya-lecithin, defatted in the course of production, was fractionated on a silicic acid column with application of a solvent gradient. In addition to the small amount (2.5%) of neutral lipids, the ratio of phospholipids and glycolipids was determined (Table 3).

Table 3

Lipid fractions of the Azolectin preparation from data published by ERDAHL *et al., 1973*

Main components	Weight %
Neutral lipids	2.5
Glycolipids I–II total	15.7
I (ESG, SG)	(6.2)
II (CE, DGDG and others)	(8.8)
Phospholipids (PE, PI, PC, PA, and others)	82.5

Nomenclature: see Table 1

On the basis of the above results, the change in the ratio of phospholipids (PL) and glycolipids (GL) was compared according to ripeness and the variety examined. The data and the PL/GL ratio of *Azolectin* to soya-lecithin are summarized in Table 4.

Table 4

Distribution of polar lipids of soya into phospho- and glycolipids according to data in the literature

Weight (%)	*Chippewa* 64+ soybean		*Anoka G. max*++soybean		Azolectin+++ commercial soya-lecithin
	immature (44 days)	mature (88 days)	immature (55 days)	mature (97 days)	
Phospholipid	7.9	6.1	8.6	9.8	82.5
Glycolipid	29.4	1.9	2.1	1.6	15.0
PL/GL ratio	21 : 79	76 : 24	80 : 20	86 : 14	84 : 16

\+ SINGH and PRIVETT (1970)
++ PRIVETT and co-workers (1973)
+++ ERDAHL and co-workers (1973)

As can be seen, qualitative and quantitative distribution of phospho- and glycolipids during the ripening of soybeans is very much dependent on

the variety. In addition, the change in the ratio of glycolipids (GL) and phospholipids (PL) is also considerable.

There is a marked difference in the PL/GL ratio of immature *Chippewa 64* soybeans, especially in comparison to the values obtained with immature *Anoka G. max.* beans.

The fatty acid composition of glycolipid components separated by various methods has been studied by several researchers. The change in the fatty acid composition of digalactosyl diglycerides (DGDG) and esterified sterol glucoside (ESG) components in the course of ripening of soybeans has been determined by SINGH and PRIVETT (1970).

The fatty acid composition of the monogalacto- and digalactolipids of soybeans was studied by KUNDU and co-workers (1974).

The fatty acid composition of esterified sterol glucoside components isolated from soybeans was investigated by LE PAGE (1964) and the same components isolated from soybean oil were studied by KIRIBUCHI and co-workers (1966). The results of these studies are presented in Table 5.

Table 5

Fatty acid composition of soya glycolipids according to literature data

Carbon chain	MGDG	DGDG	DGDG		ESG		ESG soybean (+++)	ESG soya oil (++++)
	soybean (+)		immature	mature	immature	mature		
			beans (++)		beans			
10 : 0	—	—	—	—	—	—	—	—
12 : 0	0.4	0.4	—	—	—	—	—	—
14 : 0	2.0	1.5	—	—	—	—	—	—
16 : 0	30.0	27.0	11.6	11.4	38.2	33.9	33.7	42.7
16 : 1	—	—	—	—	—	—	0.9	—
18 : 0	6.5	7.2	5.3	5.7	17.3	6.2	7.0	19.6
18 : 1	35.8	27.2	6.2	5.3	7.0	9.1	8.8	13.7
18 : 2	19.2	29.2	13.2	31.0	23.4	43.2	47.4	31.2
18 : 3	5.8	7.2	63.7	46.6	14.0	7.6	2.2	2.8

Nomenclature: see Table 1
\+ KUNDU and co-workers (1974)
++ SINGH and PRIVETT (1970)
+++ LE PAGE (1964)
++++ KIRIBUCHI and co-workers (1966)

Of recent reports in the literature, we wish to refer to the results obtained by SEN GUPTA (1974), who studied the components of raw soybean oil and found, in addition to the major components (triglycerides), also 3% of phospholipids, 90–95% of which was composed of glycerophospholipids and 5–10% sphingophospholipids.

During the past few decades, the production and industrial processing of soybeans in our country was not significant, compared to the production of seeds of high oil content (sunflower, rape-seed, linseed). Due to the shortage of protein fodder and soaring prices on the world market, domestic production of high protein content soybeans and the marketing of its oil and defatted soybean flour have become a matter of great importance.

In addition to the problem of processing an increased amount of soybeans and the difficulties involved, separation of the polar lipids of soybean oil and the extraction and marketing of soya-lecithin also presented problems to be coped with. Of the lipid components of mature soybeans, the ratio of polar lipids (phospho- and glycolipids) was relatively high. While the polar lipids of mature sunflower seeds constitute at most 2% of all lipids, in the case of soybeans, this value may be as high as 8% (SINGH & PRIVETT, 1970) and may even reach 11.4% (PRIVETT *et al.*, 1973).

Industrial utilization of the growing amount of lecithin formed in the course of soya processing justified qualitative and quantitative studies on the components and examination of the phosphorus content.

There is no generally accepted method for the analysis of polar lipid components. This is especially true for industrial products, which contain various amounts of neutral and polar lipids, depending on the technology applied. The very few data available in the literature on commercial products and those given in the catalogues of manufacturers show remarkably different values.

1. Materials and methods

A study has been made for the quantitative and qualitative determination of polar lipids, and within this, for the measurement of phospho- and glycolipid components and fatty acid composition of the soya-lecithin produced by the CSEPEL VEGETABLE OIL FACTORY, Budapest.

1.1. Column chromatography

1.1.1. Partition column chromatography. — The non-lipid components of lecithin samples were separated by partition chromatography (ROUSER *et al.*, 1967). A crude fraction of *Molselect* G-25 (REANAL product, Budapest) was used and the gel, swelled in a (1 : 1) mixture of methanol–water, was poured into a 1×30 cm glass column up to a height of 25 cm. In order to prevent floating, a 2 cm layer of glass pearls was applied on top of the gel. For elution of the fractions, the following solvent mixtures were used:

1. chloroform–methanol (19 : 1), a mixture saturated with water;
2. a (19 : 1) mixture of 850 ml chloroform–methanol and 170 ml glacial acetic acid, and water to saturation;

3. a (9 : 1) mixture of 580 ml chloroform–methanol and 170 ml glacial acetic acid, and water to saturation;
4. a (1 : 1) mixture of methanol–water.

The gel was poured into the column and washed twice with solvent mixtures 1 to 4 in the following order: 25 ml, 50 ml, 25 ml and 50 ml.

Then the column was allowed to stand in solvent 4 until just before application of sample, when the column was washed with 50 ml of eluant 1.

A soya–lecithin sample (200–250 mg) was dissolved in eluant 1 and applied to the column. Elution was carried out at a flow rate of about 0.5 ml min^{-1}, using eluants in the order and amount given above. The eluates were distilled *in vacuo* and the ratio of fractions was determined by weight measurements. Separation was checked by thin-layer chromatography.

1.1.2. Adsorption chromatography. — The lipid components purified by partition chromatography were separated into lipid groups on acid treated *Florisil* (60–100 mesh) adsorbent according to CARROLL (1963) and the method of CARROLL and co-workers (1968).

Acid treated and activated *Florisil* (9 g) was suspended in chloroform and poured in a 40×1 cm column up to a height of 35 cm. The samples (50–100 mg) were dissolved in 5 ml chloroform and applied to the column. Elution of the lipid groups was carried out at flow rate: 2 ml min^{-1}, using 60 ml of each eluant: chloroform, chloroform–acetone (1 : 1), acetone, methanol.

The eluates were distilled *in vacuo* and determined by weight measurements. The fractions were identified by thin-layer chromatography.

1.1.3. Column chromatography on Sephadex LH-20 gel. — Beside separation of lipid groups by adsorption chromatography, the purified lipids were simultaneously fractionated on *Sephadex* LH-20 gel, too.

The gel (a PHARMACIA product) was allowed to swell in a (2 : 1) volume mixture of chloroform–methanol, then the purified sample (100–150 mg) in 5% solution was applied to the column. The components were eluted with a 2 : 1 mixture of chloroform–methanol at a flow rate of 0.5 ml min^{-1}. Considering the geometric parameters of the column, the rate of elution corresponds to 9.6 cm h^{-1} linear volume flow.

The fractions were collected in 2.5 ml portions by means of a *Labor MIM* OE 604 type automatic receiver. Refractive indices of the fractions were determined at 20 °C by means of a *Zeiss Abbe* type refractometer. The fractions were combined on the basis of refractive index differences (15–17 peaks). The fractions were then identified by thin-layer chromatography.

1.2. Thin-layer chromatography

Thin-layer chromatographic measurements were made on *Kieselgel* G plates prepared by the method of *Stahl*, with modifications suggested by

ABRAMSON and BLECHER (1964), and ROUSER and co-workers (1964). Before application of the fractions, the air-dry plates were defatted in a (3 : 1) mixture of petroleum ether–acetone. Lipid contaminations on the plate were scraped off in a 1.5 cm stripe. The layers were activated for one hour at 110 °C and applied within 3–4 hours. Layer thickness was approx. 0.2 mm.

Preparative thin-layer chromatography was carried out applying a suspension of 25 g *Kieselgel* G with 50 ml 0.01 *M* NaCl on each plate. Layer thickness was approximately 2 mm.

For all chromatograms analytical grade solvents (REANAL products, Budapest) were used. A 65 : 25 : 4 mixture of chloroform–methanol–water was applied, according to WAGNER and co-workers (1961).

The components were identified with special developing reagents:

— for phosphorus compounds: ammonium–molybdate and perchloric acid (HANES & ISHERWOOD, 1949);
— for the determination of free amino groups: a ninhydrin reagent (DÉVÉNYI, 1970);
— for the identification of choline–phosphatides: the *Dragendorff* reagent (BEISS, 1964);
— for the determination of molecules with sugar content: an orcinol-sulphuric acid reagent (SKIPSKI *et al.*, 1967);
— for compounds containing sterol: an antimony trichloride reagent (STAHL, 1969).

1.3. Infrared spectroscopy

Identification of the major components was carried out by IR spectroscopy using a *Zeiss* UR-20 instrument.

1.4. Gas chromatography

After esterification (CHALVARDJIAN, 1966), the fatty acid composition of the original soya-lecithin samples, of purified lipids and separated fractions was determined by gas chromatographic measurements.

Fatty acids with carbon atom numbers higher than 20 are often observed in polar lipids (phospho- and glycolipids), therefore measurements were carried out under conditions suitable for both short- and long-chain components. For this purpose, we used silicon oil, which is applicable up to 300 °C.

Considering the fact that unsaturated fatty acid methyl esters cannot be separated according to the number of unsaturated bonds on apolar stationary phase, and given the apolar chromatogram, we performed another chromatographic measurement on polar stationary phase, which has a shorter range of measurement, but separates monoene-, diene-, triene-, *etc.* unsaturated

C_{14}–C_{22} fatty acid components. This phase is more selective than the apolar phase, where sterols, hydroxy fatty acids are also eluted.

1.4.1. Gas chromatography on apolar stationary phase. — *May-Baker* silicon oil was used in a 20 m, 0.25 mm ∅ glass capillary [layer thickness: approx. 150–200 Å ($1.5–2.0 \cdot 10^{-9}$ m); splitting 1 : 250]. Conditions: programmed temperature increase 8 °C min^{-1} in the range of 70–270 °C; carrier gas N_2, $p_0 = 1.4$ ata; FID, in a *Pye* 105 apparatus.

1.4.2. Gas chromatographic measurement on polar stationary phase. — Measurements were carried out using 10% EGS-X on *Gas Chrom* W packing (100–120 mesh); isothermic conditions at 185 °C, carrier gas N_2, $p = 2.8$ ata; FID, in a *Chromatron* GCHF 18.3 instrument.

Evaluation of the chromatograms was made by computing percentages of peak areas and calculation of peak heights × half widths. In measurements on apolar stationary phase in a capillary, due to programmed temperature increase, the homologous fatty acid methyl esters appeared as symmetrical peaks, thus, there was good agreement between area and peak height ratios.

2. Results

The non-lipid components of industrial lecithin samples were separated on *Molselect* G-25 gel. Distribution of the lipid and non-lipid components is summarized in Table 6.

Table 6

Composition of lipid and non-lipid substances of our soya-lecithin preparation fractionated on Molselect G-25 gel

Distribution into fractions	Weight (%)	Main components
Lipids (No.1)	81.00	neutral lipids, free fatty acids, phospho- and glycolipids
Non-lipids I (No.2)	1.98	solvent soluble non-lipids, degraded phosphatides, urea, gangliosides
Non-lipids II (No.3)	0.45	aromatic amino acids, non-defined non-lipids
Non-lipids III (No.4)	16.30	water soluble non-lipids: sugars, salts, amino acids

In thin-layer chromatographic measurements fraction No. 2 showed no reaction with the specific reagent used, therefore cannot be considered as lipid-type substance.

Fraction No.3 is negligible, while the amount of water-soluble non-lipids is considerable. Fraction No.4 of the sample showed positive reaction with ninhydrin at a value of 0.1 R_f (Fig. 1). This means that the fraction contains also aminoacids (peptides) in addition to NaCl used in the hydration technology.

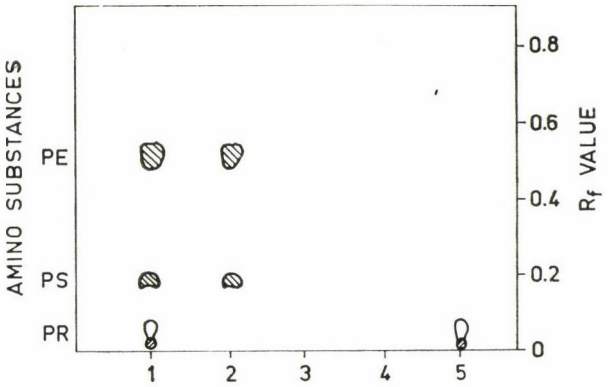

Fig. 1. Purification of soya-lecithin samples from non-lipid contaminants on *Molselect* G-25 gel. Detection on thin-layer chromatogram: *Kieselgel* G plate; elution: chloroform–methanol–water 65 : 25 : 4 mixture; 25 °C; ninhydrin spray development of amino-groups. On the plate: 1. original soya-lecithin; 2. lipid fraction; 3–4. solvent soluble non-lipids; 5. water soluble non-lipids. For abbreviations for lipid groups see Table 1. PR — protein-type contaminations

The lipid components previously purified on a *Molselect* G-25 column were further fractionated on a *Florisil* column pretreated with acid. These studies were carried out with two kinds of gradient elution. Using solvent mixtures of different polarity as suggested by CARROLL (1963), we found the following distribution within polar lipids (Table 7).

Table 7

Polar lipid components of Csepel soya-lecithin fractionated according to CARROL*'s method (1963)*

Eluent	Components	Weight (%)
Chloroform–acetone 1 : 1 v/v	MGDG, CE	13.0
Acetone	DGDG, SG, PA	11.7
Chloroform–methanol 9 : 1 v/v	PE, PS	29.0
Chloroform–methanol 1 : 1 v/v	PC, LPC	30.2
Methanol	PC, LPC	16.1

Nomenclature: see Table 1

According to the separation, the ratio of phospholipid to glycolipid was 75.3 to 24.7. The fact, however, that, in the case of acetone, phosphatidic acid becomes eluted in addition to the glycolipid components, must also be taken into consideration. We obtained more satisfactory results with the gradient elution system of CARROLL and co-workers (1968). The results are given in Table 8.

Table 8

Distribution of lipid groups in soya-lecithin

Main components	Weight (%)
Neutral lipids (NL), sterols (ST), free fatty acids (FFA)	52.2
Glycolipids I (SG, ESG)	4.9
Glycolipids II (CE, DGDG, *etc.*)	2.8
Phospholipids (PE, PC, LPE, LPC, PI, PS)	39.9
Phospholipid-glycolipid ratio	84/16

Nomenclature: see Table 1

Detailed qualitative study of both original and purified samples as well as of the fractions separated by column chromatography was carried out by thin-layer chromatographic measurements. According to the chromatograms, the results of separation are in good agreement with literature data, illustrated in Fig. 2.

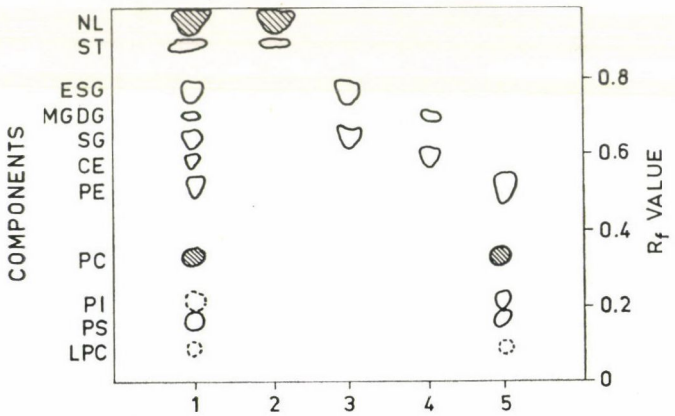

Fig. 2. Fractionation of soya-lecithin on acid-treated *Florisil* column. Detection on thin-layer chromatogram: *Kieselgel* G plate; elution: chloroform–methanol–water 65 : 25 : 4 mixture; 25 °C; development with $K_2Cr_2O_7$–H_2SO_4 at 180 °C. On the plate: 1. purified lipids; 2. neutral lipids; 3. glycolipids I; 4. glycolipids II; 5. phospholipids. For abbreviations see Table 1

From the phospholipid fraction (Fraction 4) of soya-lecithin samples separated on a *Florisil* column, the two major components, phosphatidyl choline (lecithin) and phosphatidyl ethanolamine (cephalin) were separated and their fatty acid methyl esters were studied by gas chromatography (Fig. 3).

Among the fatty acids of phosphatidyl ethanolamine, the ratio of C_{18} unsaturated fatty acid was found to be lower while that of C_{16} (palmitic acid) was higher.

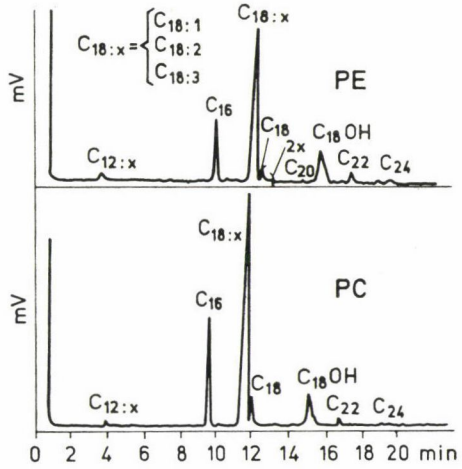

Fig. 3. Gas chromatogram of phosphatidyl ethanolamine and phosphatidyl choline fraction of *Csepel* soya-lecithin fatty acid methyl esters. Experimental conditions: 20 m long, 0.25 mm ⌀ glass capillary, with *May-Baker* silicon oil; programmed temperature increase 8 °C min^{-1} in the range of 70–270 °C; FID, N_2 1.4 ata; on a *Pye* 105 chromatograph

The fatty acids of the two main glycolipid fractions were also investigated. In the gas chromatogram of the fatty acid methyl esters in Fraction 1 of glycolipids, the appearance of both short and long carbon chains could be observed in addition to the C_{16} and C_{18} fatty acids (Fig. 4).

In Fraction 2 of glycolipids, fatty acids with C_{26} and C_{28} carbon atoms were also detectable, although in much lower amounts (Fig. 5).

Comparison with Fraction 1 (Fig. 4) shows that in this chromatogram, the fraction (approx. 5%) eluting according to carbon atom number 17, is presumably the alcohol, derived from the cerebroside compounds, eluted on

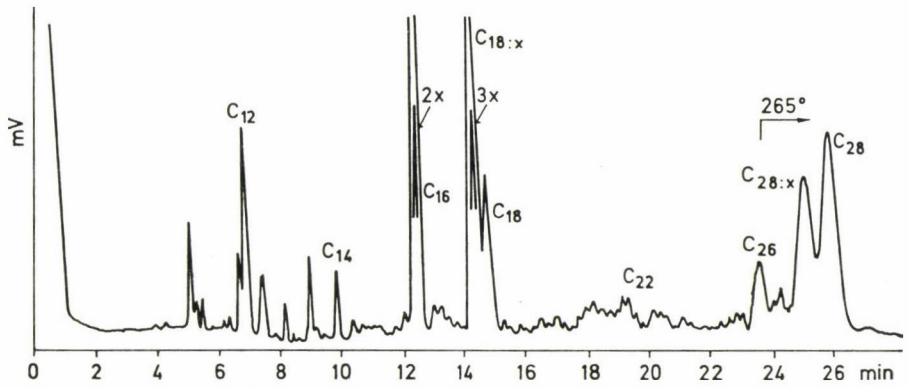

Fig. 4. Gas chromatogram of glycolipid I fraction of *Csepel* soya-lecithin fatty acid methyl esters. Conditions: see Fig. 3. On the chromatogram: 2× attenuation increase

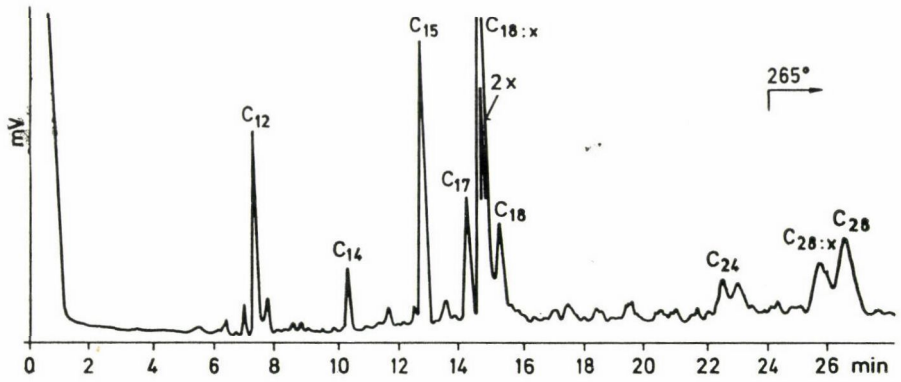

Fig. 5. Gas chromatogram of glycolipid II fraction of *Csepel* soya-lecithin fatty acid methyl esters. Conditions: see Fig. 3

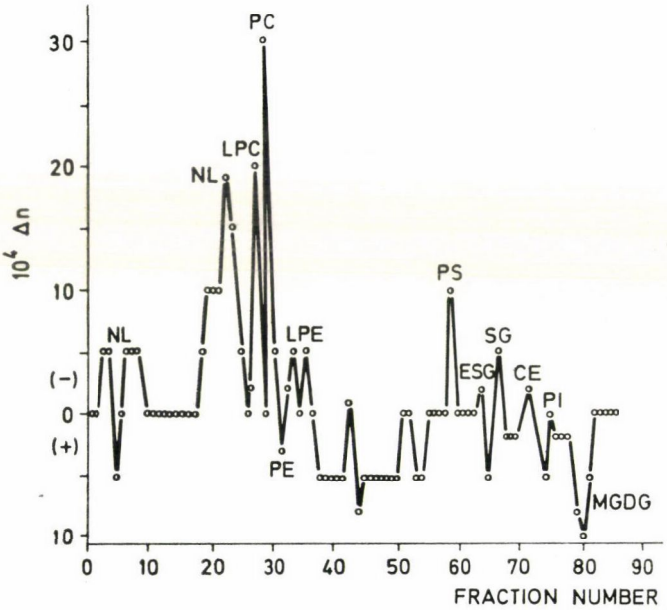

Fig. 6. Gel chromatogram of purified *Csepel* soya-lecithin lipids on *Sephadex* LH-20 (a PHARMACIA product). Column: 105 × 2 cm. Eluent: chloroform–methanol 2 : 1 v/v. Flow rate: 0.5 ml min^{-1}; linear flow rate: 9.6 cm h^{-1}. Fractions collected in 2.5 ml portions by means of a *Labor MIM* OE 604 type automatic fraction collector. Detection by refractive index measurement. For abbreviations see Table 1

silicon phase. This may be identified by a combined method of gas chromatography and mass spectrometry.

The adsorption chromatographic method outlined above was used for the separation of the polar lipids of soya into main groups. In subsequent experiments of this study, the polar lipids of soya have been further fractionated on *Sephadex* LH-20 gel.

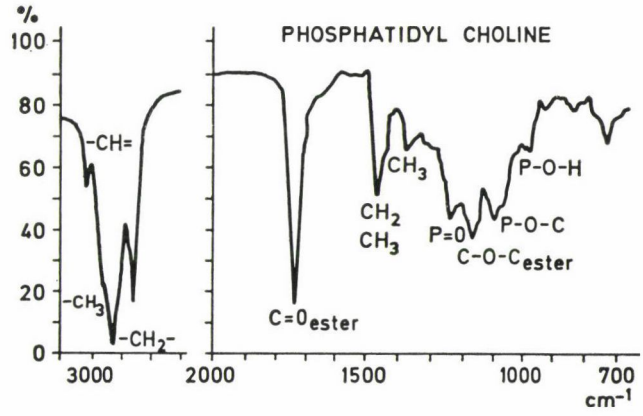

Fig. 7. Infrared spectrum of phosphatidyl choline (PC) fraction. UR-20 spectrophotometer (ZEISS, Jena). Samples taken in KBr disks

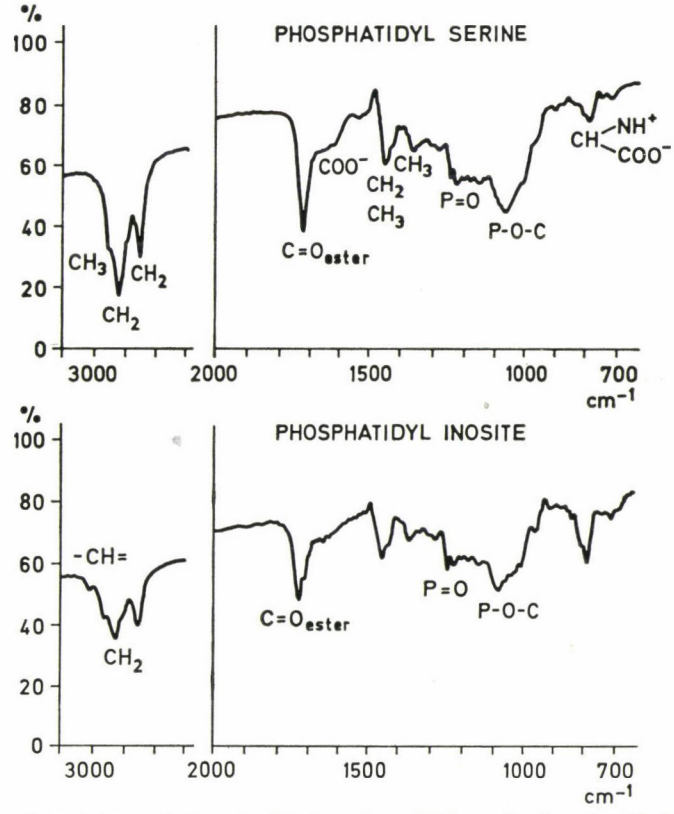

Fig. 8. Infrared spectra of phosphatidyl serine (PS) and phosphatidyl inosite (PI). Conditions see Fig. 7

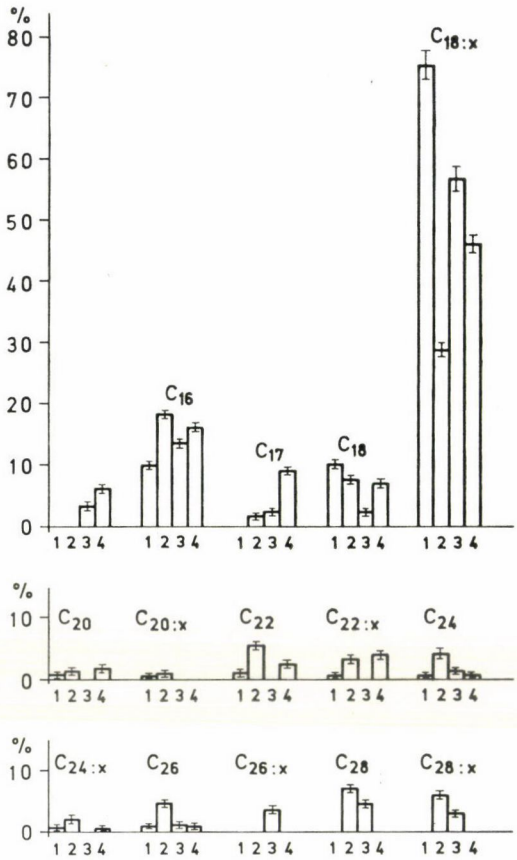

Fig. 9. Distribution of fatty acids in different phospholipid fractions of *Csepel* soya-lecithin. Gas chromatographic measurement data from apolar stationary phase. Abbreviations of the PL fractions on the diagram (columns from left to right): 1. phosphatidyl choline (PC); 2. phosphatidyl ethanolamine ; 3. phosphatidyl serine (PS); 4. phosphatidyl inosite (PI). Columns show the averages of 3 measurements. The vertical bars indicate the value of the standard deviation

In earlier experiments we have observed that the non-lipids of commercial lecithin considerably decreased the efficiency of separation, in our further investigations, therefore, the non-lipid components were previously removed from the samples.

The gel chromatogram detected with the refractive index difference is presented in Fig. 6.

Identification of the fractions was made by thin-layer chromatography as described above. The major, chromatographically pure components were studied by IR spectroscopy. This method allowed mainly the identification of phospholipids which are, in fact, the main components of lecithin. We wish to present here the IR spectra of soya phosphatidyl choline (Fig. 7), soya

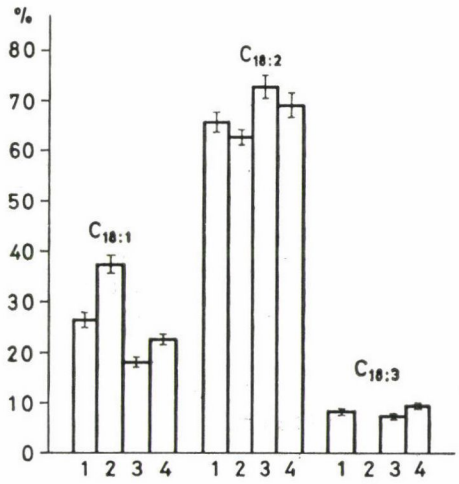

Fig. 10. Distribution of unsaturated C_{18} fatty acids in different phospholipid fractions of *Csepel* soya-lecithin. Gas chromatographic measurement data from polar stationary phase. Abbreviations and signs see in Fig. 9

phosphatidyl serine and soya phosphatidyl inositol (Fig. 8). Owing to the rather low amount of glycolipid fractions, no evaluable spectrum could be obtained.

Gas chromatographic study of the fatty acid composition of phospholipid components identified by the above methods revealed some characteristic features. Significant difference could be observed in the fatty acid distribution of phospholipid components in fatty acids with carbon atom numbers higher than C_{20}, as can be seen in Fig. 9. In phosphatidyl inositol, the amount of fatty acids with carbon atom number higher than 20 is above 8%, and in the case of phosphatidyl ethanolamine and serine, this value proved to be even higher than that.

Chromatograms taken on EGSS-X phase under isothermic conditions provided some information on the ratio of C_{18} unsaturated fatty acids (oleic, linoleic and linolenic acid), as illustrated in Fig. 10. The absence of $C_{18:3}$ linolenic acid from fatty acids of phosphatidyl ethanolamine and at the same time, a relatively high ratio of $C_{18:1}$ oleic acid can be observed.

Measurement showed the ratio of $C_{18:2}$ linoleic acid to be the highest and that of oleic acid to be the lowest among fatty acids of phosphatidyl serine.

3. Conclusions

Comparison of these results with a large number of literature data led to the conclusion that there is no uniform method for the separation of phospho- and glycolipids of vegetable oils, including soya oil. The non-lipid components

of industrial lecithin decrease the efficiency of chromatographic measurements. Removal of these components was carried out on a *Molselect* G-25 gel and, in addition to the lipid components, three further fractions have been obtained.

The most efficient method for the separation of lipid groups was carried out with acid treated *Florisil* and the best results for the separation of components were obtained with a *Sephadex* LH-20 gel. By the latter procedure phosphatides (PC, PE, PS and PI) could be isolated in satisfactory purity and the structure of these compounds was studied by IR spectroscopy. Detailed gas chromatographic investigations revealed a substantial difference in the fatty acid composition of the various phospholipids.

The fatty acids of phosphatidyl choline showed greatest resemblance to the fatty acid composition of triglycerides of the original oil. Molecules bound to phosphatidic acid in the order ethanolamine — serine — and inositol showed an increasing divergence in the fatty acid composition.

In summary, it may be stated that the fatty acids of phospholipid fractions separated by gel chromatography differ depending on the variety of the plant and, within the molecule, on the nature of the alcohol fraction. This observation supports the results of our earlier investigations on sunflower and rapeseed lecithin, according to which hydrophility of the alcohol fraction is compensated by the markedly liophilic character of fatty acid components.

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A STUDY OF PUROTHIONIN ISOLATED FROM THE PETROLEUM ETHER EXTRACT OF WHEAT FLOUR

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Purothionin, the protein component of lipoprotein of flours milled from wheat grown in Hungary was studied in order to obtain data on the structure of the lipid complexes of flour protein and to clarify their composition.

A detailed investigation was made of the steps of purothionin isolation. By repeated, simple petroleum ether extraction of flour, marked BL-112, quantitative data were obtained as regards the isolation of the apolar lipid fraction of wheat flour. Extraction repeated twice proved to be the optimal for the isolation of purothionin.

The acid concentration and reaction time resulting in maximum yield and maximal purity of the product of cleavage by HCl of the petroleum ether extract were found to be 24 mval g^{-1} and 15 min, resp.

A multi-step method was developed for the purification of purothionin. In the course of this study the substance treated as impurity by the methods described in the literature, proved to be a high molecular weight component (M_s = 130 000) of purothionin.

The pure purothionin can be separated by gel filtration into six, by polyacrylamide gel electrophoresis into eight components. The amino acid composition, the N- and C-terminal amino acids and the molecular weights of these fractions were determined. The amino acid composition of all the fractions is characteristic of purothionin and differs from that of other wheat proteins (relatively high basic amino acid and cysteine contents, relatively low glutamic acid and proline contents and the complete absence of tryptophan). Molecular weights of the fractions are: 130 000; 116 000; 50 000; 5200; 5200 and 4300, resp.

A homogeneous protein preparation was obtained by repeated simple gel filtration, a method suitable also for preparative use, from the purothionin fraction known as α-purothionin.

The study of lipid and protein complexes is one of the most rapidly developing fields of biochemistry and food chemistry. This is partly due to the important role played by lipoproteins in many vital processes, partly to the fact that study of a number of technologies in the food industry infers the knowledge of data referring to the structure, properties and stability of the lipoproteins of the given material.

Both aspects are valid for the lipoproteins of wheat flour. An increasing volume of practical experience and research results point to the important role played by the lipoproteins or protein-lipid complexes of wheat flour in the technological properties of pastry and in the quality of products of the bakery industry prepared from wheat flour.

As early as in 1921, data were published on the effect of the phosphatide content on the plasticity of gluten.

McCAIG and McCALLA (1941) were the first to prove experimentally

that during the hydration of wheat proteins in the presence of phosphatides protein-lipid complexes are formed as an effect of interaction. The lipid binding capacity of flour during wetting and dough formation was the subject of a number of studies, *e. g.* that of OLCOTT and MECHAM (1947), or recently of BALDWIN and co-workers (BALDWIN *et al.*, 1963, 1965).

As a result of these experiments it became indisputable that as an interaction between hydrated wheat proteins and polar lipids (phospholipids, glycolipids) lipoproteins or proteolipids are formed. In the last ten years, particularly as a consequence of the work done by POMERANZ and co-workers (POMERANZ & FINNEY, 1969; POMERANZ & WEHRLI, 1969; POMERANZ, 1973) the protein-lipid complexes of glycolipid content came in the forefront of interest.

The above experiments did not touch upon the problems whether the protein-lipid complexes are formed secondarily in the wetted gluten or in the dough, or are present originally in the wheat flour.

Experiments carried out in the last three decades have shown that wheat flour contains protein-lipid complexes, of which the most is known about the substance isolated and named purothionin, by *Balls*. It is prepared by cleavage with alcoholic HCl from the petroleum ether extract of wheat flour (BALLS & HALE, 1940). As it was considered already by BALLS and co-workers (1942) the purothionin is lipoprotein, that is the protein component of lipopurothionin. REDMAN and FISHER (1968) succeeded in isolating the lipopurothionin.

Besides the lipoproteins obtained by petroleum ether extraction, some other wheat lipoproteins are known, which can be isolated by different methods. Among others CARTER and co-workers (1956), COOKSON and co-workers (1957) and HOSENEY and POMERANZ (1970) separated lipoproteins from wheat flour. Several papers were published (LÁSZTITY *et al.*, 1969; VARGA *et al.*, 1975; BÉKÉS & MONORI, 1975; NEDELKOVITS & TELEKY-VÁMOSSY, 1974) on protein-lipid complexes of Hungarian wheat varieties.

The aim of this study was to throw more light on wheat proteins summarized under the name purothionin, which is definitely not a product formed in the dough, but a substance present in wheat flour and under *in vivo* conditions interacting with lipids. The investigation of the structure and properties of purothionin, may lead to the knowledge of the physiological characteristics of the plant. The data obtained by the examination of the properties and structure of purothionin may shed some light on the structure and function of the protein-lipid complexes which are formed during gluten and dough formation and which influence greatly the baking quality of wheat flour.

Another aim of this study was to obtain a homogeneous *pure protein preparation* and thereby lay the foundations of an extensive research project on wheat proteins, carried out at the Department. After the amino acid

sequence test of purothionin fractions, the final aim will be the investigation of the complete structure of lipopurothionin.

An essay was made to define more exactly the concept of purothionin as the name for a group of proteins isolated from wheat flour. BALLS and coworkers (1942) gave this name to this substance on the basis of its high cystine content. Later, however, research was almost completely restricted to the α- and β-purothionin fractions (FISHER *et al.*, 1968; AXFORD *et al.*, 1968; REDMAN & ELTON, 1969; REDMAN & FISHER, 1968, 1969; NIMMO *et al.*, 1968, 1974; *etc.*). Therefore it was considered important to study the fractions hitherto treated by the researchers as impurities.

1. Materials and methods

1.1. Materials

The raw material used in the experiments was flour, marked BL-112, prepared from wheat grown in 1972 and 1973 and marketed for bread baking, with an ash content of 1.12%. The main properties of the flour were as follows: moisture content 13.21%; nitrogen 2.93%; raw fat 0.36%.

1.2. Isolation of purothionin

Purothionin was isolated basically by the method of *Balls* (Fig. 1) (BALLS & HALE, 1940). However, because of the incompleteness of literary data, and due to conclusions drawn from the results of the preliminary experi-

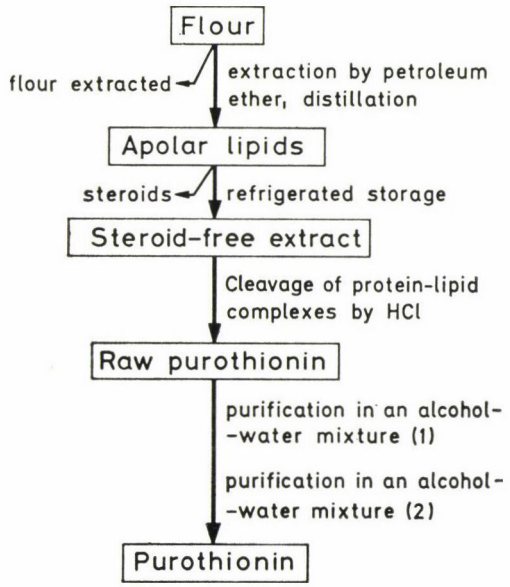

Fig. 1. Schematic diagram of purothionin isolation (BALLS & HALE, 1940)

ments, it seemed reasonable to gain deeper insight into the steps of the original method, to obtain quantitative data in order to elucidate the optimum conditions of each step.

1.2.1. Petroleum ether extraction of wheat flour. — Preliminary experiments have shown that by a 6–8-h extraction with petroleum ether, only about 40% can be extracted of the lipid components soluble in petroleum ether. In order to see whether the extracts obtained in repeated extractions differed from one another, or how many repetitions are needed, in default of data in the literature, a detailed study was made of repeated simple extractions of wheat flour.

Two thousand g of flour were shaken for 2 h in a 5-litre flask with 1 600 ml petroleum ether of b. p. 40–60 °C, in a shaking apparatus. After 15 min first the clear supernatant then the wetted flour were filtered through a *Büchner* funnel. The extraction was repeated five times with a fresh portion of petroleum ether, each. The solutions thus obtained were distilled under vacuum. The residue was made free of petroleum ether by drying in a vacuum oven overnight at 40 °C. The five lipid fractions and the flour samples removed between extractions were tested for the following properties:

- nitrogen and raw fat contents in the flour samples according to LÁSZTITY and TÖRLEY (1971)
- group fractionation of lipid fractions according to MANGOLD (1961) and investigation of the quantitative distribution of fractions by densitometry of the thin-layer chromatograms
- thin-layer chromatography of the phospholipids precipitated with acetone from the lipid fractions (MANGOLD, 1961)
- total fatty acid composition of the lipid fractions by gas chromatography (MONORI & BÉKÉS, 1975).

1.2.2. Splitting of the protein-lipid complexes with hydrochloric acid. — The lipid fractions obtained by extraction in petroleum ether were stored for 10 days at —10 °C. The steroids were separated from the oil-like extract by centrifuging with a table centrifuge cooled to 0 °C, at 6 000 rpm for 20 min.

An assay was made to determine the conditions for obtaining the maximum yield and the relatively purest product by treating the extract with HCl.

Ten g of the steroid-free extract were dissolved in 10 ml ether, to which various quantities of HCl reagent were added and the final volume made up to 100 ml with absolute alcohol. (The reagent was prepared by absorption in absolute alcohol of the hydrochloric acid vapours liberated from concentrated HCl by concentrated sulphuric acid; concentration of reagent: 4.1 *N*.)

The HCl concentrations related to oil were as follows: 3, 6, 12, 18 and 24 mval g^{-1}, resp.

The reaction was carried out at 25 °C for 15, 30 and 60 min, resp. The reaction mixtures were then cooled to —5 °C. The raw purothionin was ob-

tained from the refrigerated mixtures by centrifugation at 6 000 rpm for 15 min. The sediment was washed immediately with absolute alcohol, then in 1 : 1 mixture of diethyl ether and absolute alcohol, finally in diethyl ether. The purothionin thus obtained was air-dried.

To compare the various preparations their nitrogen and phosphorus contents (BARTLETT, 1959) were determined, as well as their thermogravimetric diagrams and infrared spectra (MONORI & BÉKÉS, 1975) and the possibility of fractionation by molecular size on a *Sephadex* G-75 column was tested (as described in para. 1.4.1).

1.3. Purification of the purothionin preparation

1 000 mg raw purothionin (t_0) were dissolved in 150 ml distilled water. After cooling, 135 ml absolute alcohol were added. The opalescent solution was kept overnight at —5 °C, then centrifuged in a *MOM* 120 ultracentrifuge, using a preparative rotor at —7 °C and 35 000 rpm, for 45 min. A pale yellow sediment settled on the wall of the centrifuge tubes (t_1). By evaporating the clear solution at 35 °C in a vacuum oven the once cleaned purothionin was obtained (t_2).

The preparation (t_2) cleaned once, was dissolved in 15 ml distilled water and 100 ml absolute alcohol were added. It was kept overnight at —5 °C, then centrifuged as described above (t_3). The twice-cleaned purothionin (t_4) preparation was obtained by evaporating the solution.

The twice-cleaned preparation was dissolved in 60 ml of the (1 : 1) mixture of chloroform and methanol, then sufficient water was added to yield a two-phase mixture of chloroform, methanol and water (1 : 1 : 0.9). The milk-like emulsion was refrigerated overnight. The next day it separated into two phases, with a milk-like layer in between. The upper phase was sucked down and 5 ml of the above mixture were added in small portions, while shaking. A milk-like emulsion was formed again, which was centrifuged for 1 h at 8 000 rpm. Here again two phases were formed with a boundary layer in between. After sucking down the upper phase and completing the volume, purification was repeated twice more. Finally the united upper phases (t_7) the lower phase (t_6) and the boundary layer (t_5) were each evaporated under vacuum. To control the efficiency of the steps of purification, each substance was tested for N and P content.

For further examination phase t_7, that is purified purothionin was used.

1.4. Analysis of purothionin

1.4.1. Fractionation according to molecular size. — Fractionation according to molecular size was carried out by the gel filtration technique. A dextran

gel, *Sephadex* G-75, manufactured by PHARMACIA, was used. Several eluents were tested (BÉKÉS, 1975) and the following was found the best for sharp separation: an aqueous solution of 0.05 *M* acetic acid plus 0.01 *M* KCl. Preliminary fractionation was carried out on a 3.8×40 cm preparative column (v_0 = 130 ml). Individual fractions were eluted on a 2.2×22 cm (v_0 = 27 ml) column. The fractions were collected and gel filtration was followed up on a LKB *Uvicord* automatic fraction collector. The UV detector was used at 280 nm. The rate of through-flow was 3.5 and 0.8 ml min^{-1}, resp. Fractions were taken every 5 min.

1.4.2. Ultracentrifuge tests. — For these tests a *MOM* 120 type apparatus (MAGYAR OPTIKAI MŰVEK, Budapest) was used. The sedimentation rate was studied by the *Schlieren* technique. The tests were carried out at 20 °C with 20 to 45 000 rpm, depending on the sedimentation velocity of the sample under examination. The sedimentation constants of the purothionin fractions extrapolated to zero concentration (based on a 1/S concentration diagram) were calculated by computer on the basis of the data obtained by examining the fractions at 5 different concentrations (0.5–1.8%) each (MONORI & BÉKÉS, 1975), as well as taking into consideration the dilution effect.

Molecular weights were determined by a high speed equilibrium experiment, by the use of interference optics, at 25 000 rpm. The concentration of the samples was 0.05%. Here, too, a computer was used for evaluation (MONORI & BÉKÉS, 1975).

1.4.3. Determination of the amino acid composition. — Ten mg of the samples were digested at 105 ± 1 °C in 5.6 *N* HCl, applied in 500 fold excess, in ampulles freed from air by nitrogen rinsing. By applying various hydrolysis periods (24, 48 and 72 h, resp.) and testing two additives (thioglycolic acid and phenol) the optimum hydrolysis time was established and the extent of amino acid decomposition during hydrolysis was determined. It was found, that in case the experiment was carried out with care the purothionin fractions can be hydrolized by HCl in 24 h, without any additive.

Subsequent to hydrolysis the samples were evaporated in nitrogen stream and dissolved in citrate buffer of pH = 2.2. The analysis of the amino acids was carried out on an AAA-881 type automatic amino acid analyser (manufactured in Czechoslovakia at the MICROTECHNA). The single column, three-buffer-exchange method developed by DÉVÉNYI (1968) was used.

To determine the tryptophan content of the samples alkaline hydrolysis and *Fixion* (CHINOIN, Budapest) ion-exchange thin-layer chromatography were applied.

1.4.4. Determination of N-terminal amino acids. — N-terminal amino acids were determined by the dinitro-fluorobenzene (DNF) technique modified by VARGA and LÁSZTITY (1970) for wheat proteins.

1.4.5. Determination of C-terminal amino acids. — The determination

of the C-terminals of purothionin fractions was carried out by the mixed enzyme method with carboxy peptidase, according to NEDELKOVITS and WÖLLER (1970). The quantitative determination was carried out as described in para. 1.4.3.

1.4.6. Electrophoretic tests on polyacrylamide gel. — Model-69 apparatus (a product of REANAL, Budapest) was used with an acid system of 10 and 15% low polar gel (pH 4.5; 3.5; 3.1). In some of the experiments the low polar gel contained 6% urea. Protein fractions were identified by *Coomassie*-brillant blue (SERVA) (KERESE, 1975). *Chromoscan*-type densitometer was used for quantitative evaluation.

2. Results

2.1. Studies on the methodology of isolation of purothionin

2.1.1. Extraction of wheat flour by petroleum ether. — The quantity of lipids obtained by repeated simple extraction of flour BL-112 and the composition of the extracted flour are shown in Table 1.

Table 1

Changes in the nitrogen and raw fat content of wheat flour BL-112 and the quantity of lipids obtained upon repeated, simple extraction with petroleum ether

Serial number of extraction steps	Nitrogen	Raw fat	The quantity of lipids from 1 g flour (mg)
	content related to flour solids ($mg\ g^{-1}$)		
0 (original flour)	29.35	9.6	
1	32.20	5.4	4.2
2	31.95	2.8	2.6
3	31.90	1.6	1.2
4	31.75	1.1	0.5
5	31.80	0.8	0.3

In each extraction step 2000 g flour were shaken in 1600 ml petroleum ether for 2 h.

In Table 2 and Fig. 2 the composition of some lipid extracts obtained in different extraction steps is summarized. In Fig. 2 the quantitative changes of fractions of lipid extracts separated by thin-layer chromatography, are illustrated. Table 2 contains data on the compositional changes of gross fatty acid content.

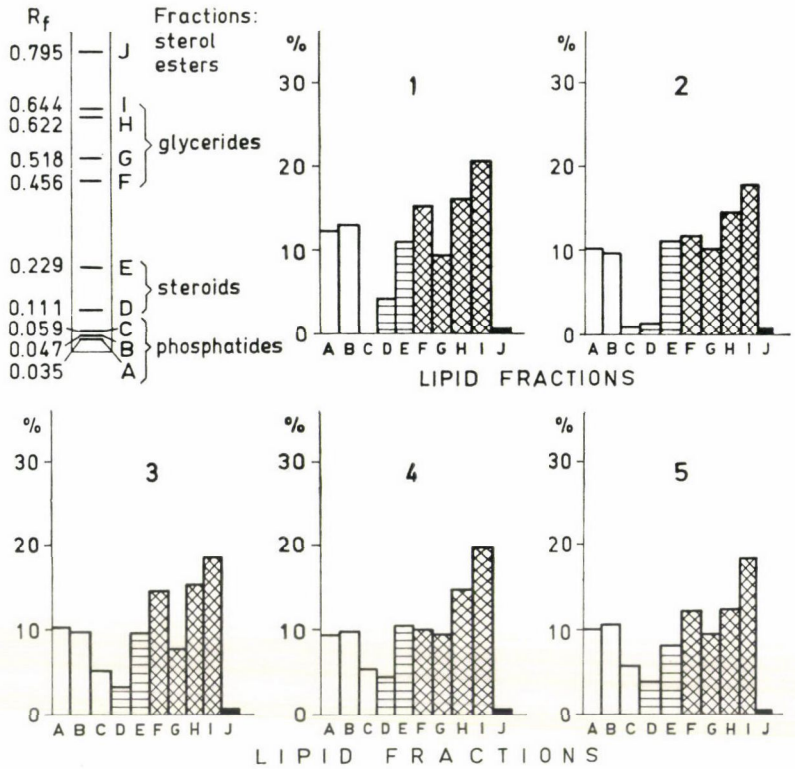

Fig. 2. Distribution of the fractions obtained by thin-layer chromatography from the lipid fractions extracted from wheat flour BL-112 by repeated simple extraction with petroleum ether. The analysis was carried out according to MANGOLD (1961). (Layer: Kieselgel G; developing solvent: petroleum ether–ether–acetic acid, 100 : 100 : 2.) The fractions obtained in the subsequent steps of extraction did not differ significantly

Table 2

Gross fatty acid composition of the lipids obtained from wheat flour BL-112 by repeated, simple extractions with petroleum ether

Fatty acid		Fatty acid content, %				
		Serial number of extractions				
		1	2	3	4	5
Miristic acid	14 : 0	1.3	0.9	1.1	1.1	1.1
Palmitic acid	16 : 0	20.9	19.2	20.2	20.0	20.1
Stearic acid	18 : 0	0.8	0.9	0.8	0.8	0.7
Oleic acid	18 : 1	17.6	18.7	18.3	19.0	18.7
Linolic acid	18 : 2	56.6	57.2	57.4	56.0	56.2
Linolenic acid	18 : 3	1.2	1.3	1.0	1.1	1.3
Arachic acid	20 : 0	1.5	1.7	1.2	1.2	1.3

2.1.2. Effect of the conditions of hydrochloric acid cleavage upon the quantity and properties of raw purothionin. — The quantity and the N%/P% factor calculated from the N and P content of raw purothionin were investigated under different reaction circumstances — *i. e.* at reaction times 15, 30, 60 min, at HCl concentrations 6, 12, 18, 24 mval g^{-1}, and under all possible combinations. The results are shown in Fig. 3 as a function of the parameters.

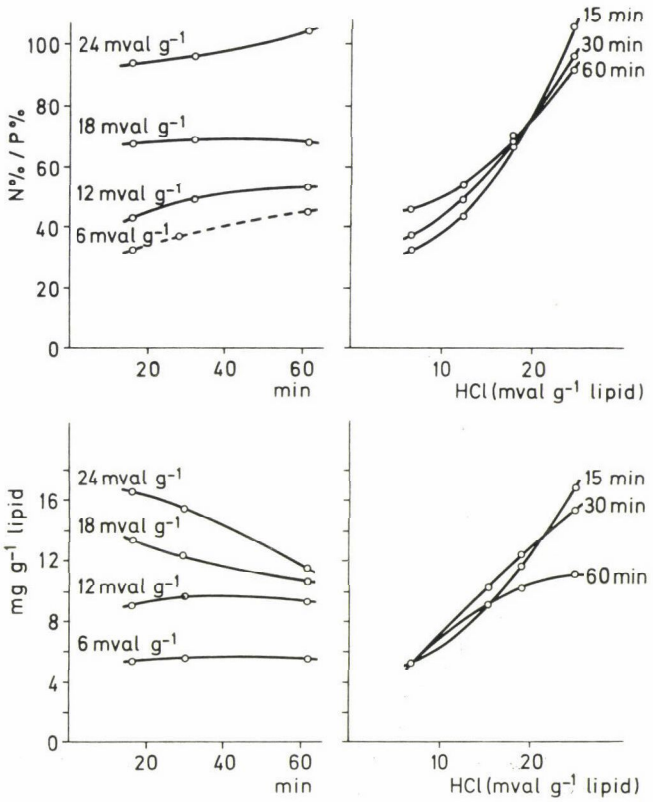

Fig. 3. The effect of the conditions of HCl-cleavage on the quantity (mg g^{-1}) and purity (N %/P %) of raw purothionin. The reaction times are 15, 30 and 60 min, the concentrations of HCl are 6, 12, 18 and 24 mval g^{-1}

The thermo-gravimetric curves of samples obtained under different conditions do not display significant differences. On evaluating the curves the moisture content of the samples was found to be between 4.28 and 5.36% and the ash content between 6.0 and 8.4%.

The infrared spectra differed only in the intensity of the –CO–NH– and P–O–C bands.

In studying the separability according to molecular size it was found that the quantity as well as the number of fractions separated from raw purothionin obtained under different conditions, varied. In Fig. 4 the elution curves for the two extreme examples are shown: one of them is the curve belonging to purothionin obtained at low acid concentration during a long reaction period, while the other at high acid concentration during a short reaction period.

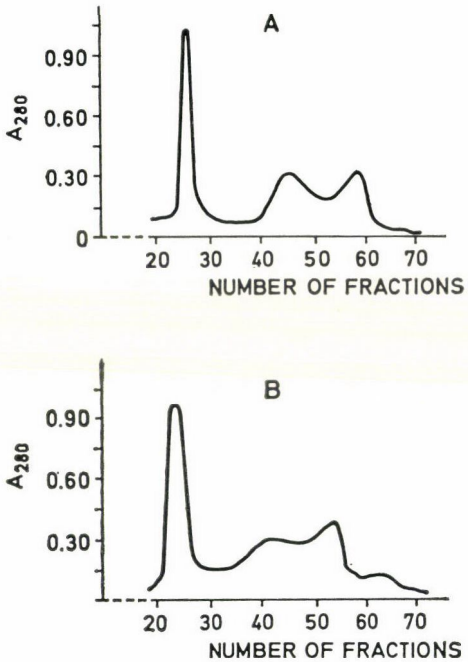

Fig. 4. Distribution of two purothionin samples according to molecular size obtained by extraction under different conditions. Molecular size was determined by gel filtration on *Sephadex* G-75. Marked "A" is the elution curve obtained at lower acid concentration during longer reaction period (6 mval g^{-1}; 60 min). Curve "B" was obtained at higher acid concentration and shorter reaction period (24 mval g^{-1}; 15 min)

2.2. Purification of the raw purothionin

Table 3 gives quantitative data on purothionin purification, the phosphorus content of the pure product as well as of the impurities.

The material balance of the isolation of purothionin is presented in Table 4.

Table 3

Quantity and phosphorus content in purothionins of different degrees of purity and in the separated impurities

Symbol of sample	Sample	Material obtained from 1 000 g raw purothionin (g)		Phosphorus content (%)	
t_0	Raw purothionin	1000.0		0.17	
t_1	Impurities in 1st step of purification		41.5		0.41
t_2	Purothionin purified once	871.0		0.09	
t_3	Impurities in 2nd step of purification		31.9		0.13
t_4	Purothionin purified twice	716.2		0.04	
t_5	Boundary layer of 3rd purification		18.5		0.17
t_6	Lower phase of 3rd purification step		190.7		0.20
t_7	Purified purothionin	504.7		<0.01	

Table 4

Material balance of the isolation of purothionin

	Raw purothionin	Steroid-free extract	Raw extract	Flour
	(mg g^{-1})			
Raw extract				9.60
Extract free of steroids			880.0	8.45
Raw purothionin		16.00	14.1	0.12
Pure purothionin	504.0	8.06	7.1	0.06

Data in the table show the amount (mg) of the substance found in 1 g of the material in the respective columns (*e.g.* from 1 g steroid-free extract 16.0 mg raw purothionin may be obtained).

2.3. The study of purothionin

Subsequent to the elucidation of the conditions of isolation, sufficient pure purothionin was prepared for further experiments, by processing 200 kg of flour.

2.3.1. Fractionation of purothionin by molecular size. — In Fig. 5 the elution curves obtained upon the fractionation of purothionin by molecular size, are shown.

2.3.2. Investigations by gel electrophoresis. — The results of these experiments show that fractions by molecular size may be separated into sub-fractions in an electric field. At the same time it was observed that the fractions obtained by gel filtration contaminate each other, although to a small extent. Figs. 6 and 7 show the results of these experiments.

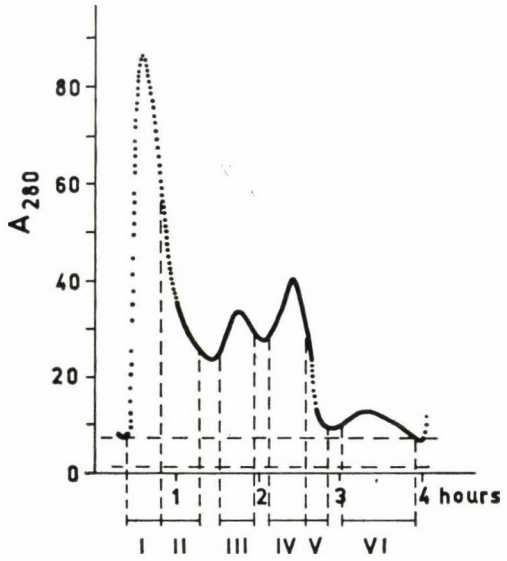

Fig. 5. Elution curve of purified purothionin fractionated on *Sephadex* G-75 column. In the diagram obtained on the LKB *Uvicord* apparatus the horizontal lines show the substances eluted in one fraction and the intervals belonging to the collected fractions are also given (I–VI)

2.3.3. The gross amino acid composition of purothionin fractions. — The gross amino acid composition of the fractions, considered uniform on the basis of their molecular size, as well as that of the contaminating protein isolated in the course of purification, marked t_6, are shown in Table 5.

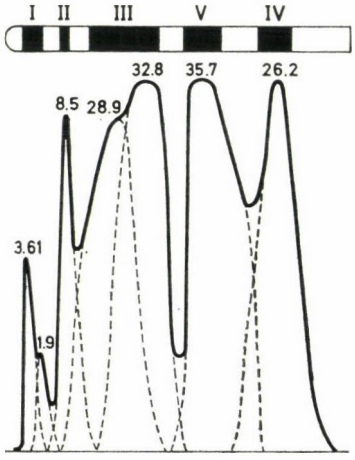

Fig. 6. Schematic diagram of the polyacrylamide disc and the densitogram of a purified purothionin (15% small-pore gel containing 6% urea; pH = 3.1 β-alanine–acetic acid buffer). 300 μg of the substance was applied. Current: 2 mA per tube for 30 min, then 4 mA per tube for 80 min. Proteins were developed with *Coomassie* Brillant Blue. Roman numerals (I–V) mark the fractions obtained on *Sephadex* G-75 (Fig. 5). Arabic numerals stand for the relative quantities of the fractions established from the densitogram

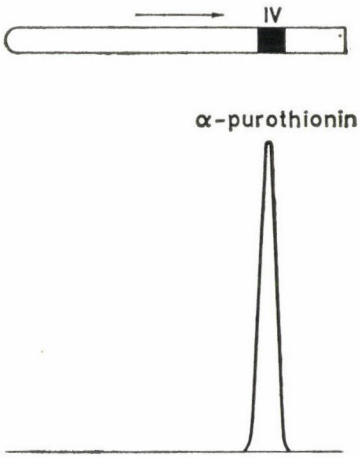

Fig. 7. Schematic diagram of the polyacrylamide disc and the densitogram of fraction IV obtained in fractionation according to molecular size. The substance proved to be homogeneous for molecular size and electrophoretic mobility. On the basis of its "rapid" mobility and other properties it may be considered α-purothionin

Table 5

Gross amino acid composition of fractions obtained on Sephadex G-75 from purified purothionin (I to VI) and of the impurity (t_6) separated on purification

	Mark of fractions						
	I	II	III	IV	V	VI	t_6
ASP	9.13	7.70	8.21	6.69	6.78	4.31	8.34
THR	7.68	9.54	6.00	4.50	4.65	8.88	1.23
SER	3.56	6.41	7.09	7.33	7.31	8.30	4.54
GLU	10.09	9.13	11.60	3.03	3.07	5.80	0.10
PRO	1.87	3.10	3.80	4.45	4.65	5.86	2.59
GLY	4.98	5.00	5.20	6.53	3.70	5.54	4.93
ALA	4.13	4.54	5.50	5.08	3.15	5.00	4.50
VAL	6.87	6.30	5.70	1.41	1.83	2.98	6.27
CYS	6.60	5.05	6.00	14.50	14.40	9.20	7.00
MET	1.50	0.74	2.90	traces	traces	traces	0.83
ILE	3.08	2.71	3.44	1.45	0.55	0.80	2.80
LEU	9.14	7.60	8.60	9.28	11.91	11.90	8.60
TYR	0.50	0.10	1.90	1.73	3.20	traces	0.40
PHE	2.38	4.90	5.40	2.05	3.04	4.61	3.01
LYS	8.94	14.10	8.02	13.69	14.02	13.10	7.90
HIS	1.87	0.19	2.12	0.19	0.60	traces	0.90
ARG	10.00	8.64	7.35	15.52	15.03	14.20	9.25
TRP	0	0	0	0	0	0	0
NH_3	2.87	1.89	1.70	2.70	2.90	2.50	2.30

Data of the table represent 1 g amino acid in 100 g protein. By $Ba(OH)_2$ test for TRP content neither fraction contained TRP.

2.3.4. Studies on terminal amino acids and of molecular weights. — Table 6 contains the N- and C-terminal amino acids and the results of ultracentrifuge tests of the fractions considered homogeneous by molecular size. The same table contains the molecular weights calculated on the basis of gel electrophoretic tests and of the quantitative evaluation of the terminals, as well as the molecular weights calculated from the elution volumes of gel filtration.

Table 6

Terminal amino acids, sedimentation constants and molecular weights of purothionin fractions separated on Sephadex G-75. The molecular weights were determined by different methods: equilibrium method using a centrifuge, gel filtration and calculations on the basis of quantitative data obtained by gel electrophoresis on polyacrylamide and by terminal amino acid determinations

	Fractions							
	I		II	III		IV	V	VI
	I/1	I/2		III/1	III/2			
Sedimentation constant (S_w) (1 Svedberg = 10^{-13}S)	7.50		6.31	1.43		0.76	0.53	—
Molecular weights ($\times 10^{-3}$)								
MW_{VC}	134		120	57.3		8.8	8.8	—
$MW_{gel\ filtration}$	132		125	32.0		11.2	8.9	4.2
$MW_{N\text{-terminal}}$	145	133	132	51.5	49.0	5.9	4.6	5.0
$MW_{C\text{-terminal}}$	133	129	116	43.6	57.0	5.2	5.2	4.3
N-terminal amino acids	ARG	ASP	LYS	GLU	ARG	LYS	SER	ALA
C-terminal amino acids	ASP	ALA	LEU LEU	ASP	VAL	LYS	ARG	GLY

3. Conclusions

Since BALLS and HALE (1940) laid the foundations, about 15 papers have been published on purothionin, its isolation, properties, biological and biochemical functions.

This relatively high interest is partly due to the fact that purothionin is the protein portion of a lipoprotein (thus being the first of the complex proteins of flour to be isolated) and partly to the fact that purothionin is different from

the general composition of wheat proteins, its function and its role in baking technology is not yet clarified.

Though every author followed the path of purothionin isolation elaborated by BALLS and HALE (1940) their results are not suitable for comparison. Few data are available on the amount of purothionin isolated, the composition of the extracted flour was not studied, the contaminants separated upon purification were not analysed, and so on. The most sensitive step in purothionin preparation, the hydrochloric acid cleavage of the protein lipid complexes has not been studied yet.

Therefore in the first part of this study, the questions left open so far have been examined.

The repeated simple extraction with petroleum ether was quantitatively followed and the composition of lipid extracts was investigated. A more thorough study of the composition of extracts was not aimed at. It was intended to see whether the extracts obtained in the individual steps of extraction differed, or whether the extracts obtained in repeated extractions could be combined.

From repeated simple extractions the following conclusions may be drawn:

The quantity of lipid fractions obtained in subsequent extractions decreased, while their composition hardly changed (Table 2, Fig. 2).

Thus, to achieve a higher yield, extraction may be repeated and the fractions obtained in each step may be combined. To decide on how many repetitions are expedient the relation between the quantity of material obtained and the time requirement have to be taken into account. Fig. 8 shows

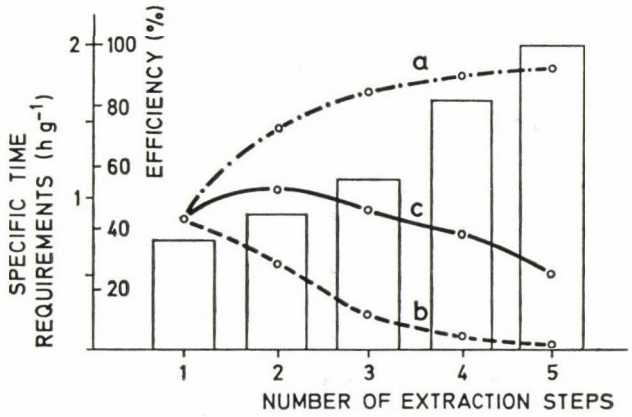

Fig. 8. Efficiency of extraction in the repeated simple petroleum ether extraction of wheat flour BL-112. a — total efficiency related to the total amount of lipids extracted by petroleum ether; b — step-by-step efficiency related to the total amount of lipids extracted by petroleum ether; c — actual efficiency related to the residual lipid content in wheat flour. The column diagram illustrates the time requirement necessary to extract 1 g of lipid

the time necessary to obtain 1 g lipid extract, and the different yield percentages calculated on the basis of the extraction experiments.

The results may be evaluated by means of the material balance equations of drug extraction (BÉKÉS, 1975). It seems expedient to make two extractions. This finding is supported by the percentage yields related to residual lipid concentration in flour (Fig. 8, curve C).

The results of the cleavage experiments show that the conditions of cleavage (reaction period, quantity of HCl applied) have a decisive effect upon the quantity of the product obtained, its composition, the phosphorus and nitrogen contents of the precipitate, and the distribution according to molecular weight.

If a maximum output of high protein but low phosphorus content is aimed at, a relatively high acid concentration and a short reaction period are profitable.

The results of the cleavage reactions permit the setting up of a hypothetic lipopurothionin structure in which some phospholipid components are bound to a single protein molecule, while others being in interrelation with two proteins form a "bridge" (BÉKÉS, 1975).

For the purification of purothionin the methods used by other authors were combined and an efficient three-step method was developed. Changes in the quantity of the material were followed up and so was the phosphorus content (Table 3). The impurity obtained in the third purification step proved to be protein by *Biuret* reaction. Making a detailed analysis of this protein (t_6) it was found to be the mixture of purothionin fractions (marked I and II) of high molecular weight. The molecular weight, determined by gel filtration, was found to be 130 000. The amino acid composition is shown in the last column of Table 5.

Evaluation of the study of purothionin. From the analysis of purothionin obtained by fractionation on *Sephadex* G-75 column by repeated chromatography the following conclusions may be drawn:

— Based on gel electrophoresis terminal amino acid analysis, purothionin proved to be a mixture of at least eight protein fractions.

By gel chromatography 4 fractions, and by repeated running, 6 sharply separated fractions could be distinguished (Fig. 5). These main fractions could be separated into subfractions on the basis of their mobility in electric fields (Figs. 6 and 7).

— The purothionin fractions marked α and β which were thoroughly examined by other authors, were identified as fractions marked IV and V in this study. Their identity was proved beyond doubt by the mobility of the fractions in the electric field, their molecular size and first of all by their amino acid composition (Table 5).

— As it may be seen from data of amino acid composition, the α and β

purothionin enjoyed a particular place not because of their special composition, but because components of higher molecular weight have not been studied so far.

— On the basis of gel filtration and amino acid composition the substances isolated in the third purification step were found to be identical with the high molecular weight fractions I and II of purothionin, therefore they cannot be considered impurities. Earlier authors have removed these and fraction III at the outset from the material, under examination. But, as it may be seen in Table 5, the amino acid composition of the first three fractions differs from the usual wheat protein composition and resembles much more that of fractions IV and V (α and β purothionin). Analysis has shown only two essential differences: relatively higher glutamic acid and lower cystine content. On the other hand similarity is proved by the total absence of tryptophan, low methionine, histidine and tyrosine contents and the exceptionally high arginine and lysine contents (Table 5).

The N- and C-terminal amino acids of all of the subfractions separated by electrophoresis were determined from each main fraction. However some uncertainty was observed in the determination of the C-terminals of fraction III, because the doublet obtained by electrophoresis had three C-terminals of identical order of magnitude.

— The molecular weights of the isolated proteins were determined by four different methods: gel filtration, UC equilibrium test, evaluation of the quantities of N- and C-terminals (Table 6).

— It was fraction IV (α-purothionin) which could be prepared free of any other protein component by gel filtration. This fact was supported by the results of gel electrophoresis (Fig. 9) and by each N- and C-terminal result obtained in the terminal amino acid tests. Thus it has become possible to investigate the primary structure of α-purothionin by more fundamental studies.

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STUDY INTO THE MAILLARD REACTION OCCURRING BETWEEN METHIONINE AND TRYPTOPHAN ON THE ONE HAND AND GLUCOSE ON THE OTHER

PART I — STUDIES IN AQUEOUS SOLUTIONS

E. DWORSCHÁK and F. ÖRSI

(Received November 15, 1975)

The *Maillard* reaction of methionine and tryptophan, forming the limiting amino acids in proteins of animal and plant origin and hitherto not sufficiently studied, was investigated in model solutions. The aqueous solutions buffered in the range of pH 3 to 13 contained 0.02–0.2 *M* methionine, 0.01–0.07 *M* tryptophan and 0.01–1 *M* glucose, resp. The experiments were carried out in the temperature range of 110–140 °C, within the time interval of 10 to 180 min.

The quantity of brown substances formed in the course of the reaction and of the amino acids was determined. Amino acids were determined by the ninhydrin reaction, characteristic of the alpha-amino group and by reactions specific for amino acids. The changes of the amino acids with time were evaluated by calculating the kinetic parameters (reaction rate constant, order of reaction).

For both amino acids a maximum in the reaction rate was observed at the amino acid–glucose ratio corresponding to the formation of mono- and difructosyl derivatives. At higher sugar ratios the kinetic parameters suffered a substantial change. Kinetic analysis of the reaction has shown the amino acids to react also with the degradation products of glucose (chiefly with trioses). In the presence of excess sugar the degradation products of sugar were found to increase.

In the case of methionine the *Maillard* reaction takes the course of a mono-amino–monocarbonic acid reaction.

The dissimilar behaviour of tryptophan is due to the reactivity of the -NH group of the indole ring. Thus the bifunctional tryptophan is more active in this reaction than methionine and its kinetic parameters are different, too.

The dependence of the reaction rate constants on pH points to a strong basic catalytic effect with a maximum at pH 11. Above this pH the competing acid formation, chiefly that of lactic acid, comes into prominence as against the *Maillard* reaction.

Methionine is the limiting amino acid in most proteins of animal origin. In most of the plant proteins used to make up for protein deficiency, for instance in soy and yeast proteins, methionine is contained at a low level. It is expedient to investigate the damage done to food proteins by the *Maillard* reaction from the aspect of the limiting amino acids, since their degradation exerts a decisive effect upon the nutritive value of these proteins. *Maillard* reaction as occurring between methionine and sugar was studied earlier mainly from the aspect of the formation of aromatic substances (BARYLKO-PIKIELNA *et al.*, 1974; LINDSAY & LAU, 1972; WAINRIGHT *et al.*, 1972).

Maillard reaction of tryptophan was studied by relatively few authors, probably because its determination in proteins is not completely resolved (FRIEDMAN & FINLAY, 1971). Of its two basic amino groups the pk_B-value of the aliphatic one is 4.6, while that of the secondary amino group in the indole ring is lower by three orders of magnitude (BRUCKNER, 1974). Thus, its reaction

with sugar is probably more intricate than the reaction of monoamino–monocarbonic acids. According to recent data (BRÄUTIGAM & SEVERIN, 1974) tryptophan reacts with xylose by the formation of carboline-skeleton compounds.

The investigation of tryptophan from this aspect is indicated by the fact that it turns into the limiting amino acid when the *Maillard* reaction is advanced in powdered milk. Its moisture dependent degradation differs also from that of lysine (DWORSCHÁK & HEGEDÜS, 1974). In stored baby foods tryptophan proved to be one of the most easily degraded amino acids (DWORSCHÁK & CZUCZY, 1975).

Based on the above considerations model systems consisting of glucose and methionine as well as of glucose and tryptophan were studied for browning and chemical changes accompanying browning. In order to be able to evaluate the changes occurring in the course of the reaction the kinetic parameters of the reactions were established. From the order of reaction it was possible to draw conclusions as to the mechanism of the reaction.

The experiments were carried out in aqueous solutions as well as in melts. In the present paper the results obtained in aqueous solutions are given.

1. Materials and methods

1.1. Materials

Analytical grade DL-methionine, DL-tryptophan and D-glucose (REANAL, Budapest) were used to prepare the model solutions.

To study inhibition of the reaction DL-N-acetyl-methionine and alpha-DL-N-acetyl-tryptophan were prepared as described in the manual of GREENSTEIN and WINITZ (1961).

1.2. Preparation of the glucose-amino acid solutions

A 0.20 *M* phosphate buffer (a mixture of Na_2HPO_4 and 0.1 *M* citric acid or 0.1 *M* NaOH) was used in the range of pH 3 to 13. The concentration of methionine varied between 0.02 and 0.2 *M*, while that of tryptophan, because of its lower solubility, between 0.01 and 0.07 *M*. The amino acids and the glucose were dissolved separately in the cold buffer and the solutions were mixed only prior to heating. The glucose concentration was varied between 0.01 and 1.00 *M*.

1.3. Heating of the samples

About 8 ml of the solution prepared according to para. 1.2. were heated in a closed ampoule in the *Kutesz* Type 615 aluminium block test tube thermostat. Heating times varied between 10 and 180 min.

1.4. Quantitative determination of the brown colour formed

The colour substance formed in the aqueous solution was diluted according to need and characterized by absorbance at 465 nm as suggested by CHOI (1949).

1.5. Determination of the alpha-amino nitrogen

The quantitative determination of both methionine and tryptophan was carried out according to COCKING and YEMM (1954) by means of ninhydrin dissolved in methylcellosolve.

1.6. Specific methionine determination

Sullivan's photometric procedure as modified by *Korpáczy* and specific for the $-S-CH_3$ group of methionine was used (KORPÁCZY, 1954) with absorbance measured at 520 nm. The N-acetyl-methionine was also evaluated by the same method.

1.7. Specific determination for tryptophan

The *Spies–Chambers* method specific for the indole ring, was used for tryptophan determination, with absorbance measured at 590 nm. Alpha-N-acetyl-tryptophan was evaluated by the same method (SPIES & CHAMBERS, 1948).

In measurements according to paras. 1.4, 1.5, 1.6 and 1.7 *Spectromom* Type 203 (MOM, Budapest) photometer and 1 cm cuvette were used.

1.8. pH measurements

The pH of the aqueous solutions was measured with *Radelkis* Type OP-meter at 25 °C, prior to heating. Since the reactions were carried out at temperatures above 100 °C the pH of buffer-systems was determined above 100 °C, too. The difference in pH values was non-significant, therefore corrections were not effected.

1.9. Calculation of kinetic parameters

The overall kinetic orders of the components of the *Maillard* reaction between amino acids and glucose were determined by the method of initial velocity, as described in the manual of SCHWETLICK (1971). The initial rate of amino acid degradation was determined by a graphic method. The partial order was estimated from the slope of the line determined by the method of

least squares from the experimental values of the logarithm of the initial concentration of the component in question and of the logarithm of initial decomposition velocity. The results were tested at the confidence level of 95%.

2. Results

The absorbance values determined according to para. 1.4 and characteristic of browning *vs.* glucose and amino acid concentrations, are shown in Figs. 1 and 2. In the case of methionine only one maximum is seen in the presence of 0.08 *M* amino acid and 0.12 *M* glucose corresponding to the mole ratio of 2 : 3. As regards tryptophan maximum browning is seen at 0.022 *M* amino acid and 0.028 *M* glucose and at 0.013 *M* amino acid and 0.037 *M* glucose, corresponding to 3 : 4 and 1 : 3 mole ratios, resp. (Figs. 1 and 2).

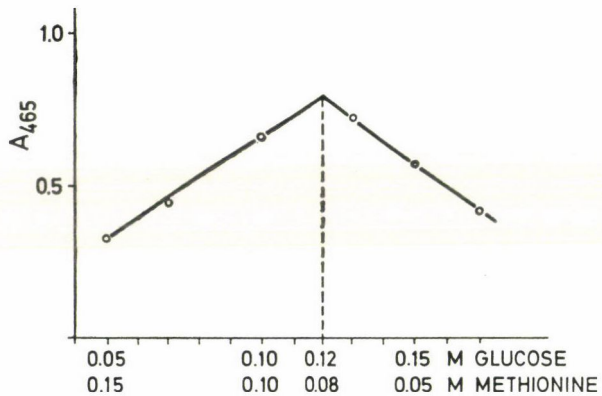

Fig. 1. Effect of changes in glucose and methionine concentration on the formation of brown colouring substances (A_{465}) (heat treated at 130 °C, for 2 h, pH = 7.0)

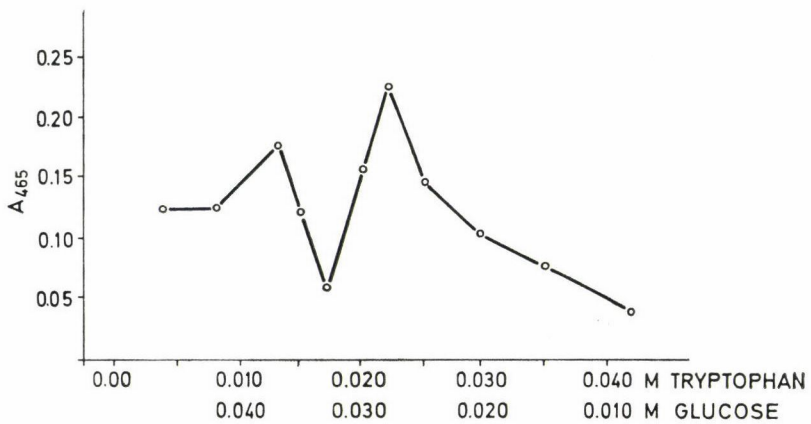

Fig. 2. Effect of changes in glucose and tryptophan concentration on the formation of brown colouring substances (A_{465}) (heat treated at 130 °C, for 1 h, pH = 10.0)

In order to establish the partial order of glucose in methionine decomposition, measurements were carried out at concentrations corresponding to maximal browning (0.08 *M* methionine) (Fig. 3).

The partial order established for glucose was found to be 1/2 below a glucose concentration of 0.12 *M*, while above this concentration it was practically 0.16. By means of the method of least squares the deviation of the latter from 0 is non-significant.

In the range studied the reaction is of first order related to methionine (Fig. 4). No difference was found between the results obtained by the two different methods used (paras. 1.5 and 1.6).

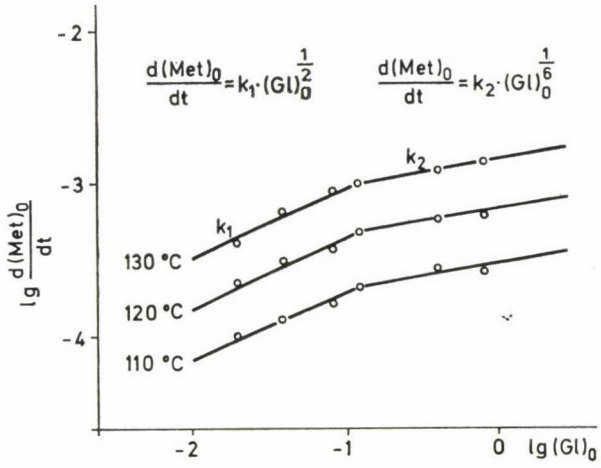

Fig. 3. Logarithm of the initial decomposition rate of methionine as a function of the logarithm of glucose concentration (pH = 10.0). Equations of the regression lines and the correlation constants:

below 0.12 *M* glucose concentration

110 °C: $\lg W_0 = 0.445 \lg (Gl)_0 - 3.27$ $r = 0.982$
120 °C: $\lg W_0 = 0.473 \lg (Gl)_0 - 2.99$ $r = 0.975$
130 °C: $\lg W_0 = 0.463 \lg (Gl)_0 - 2.57$ $r = 0.981$

above 0.12 *M* glucose concentration

110 °C: $\lg W_0 = 0.163 \lg (Gl)_0 - 3.53$ $r = 0.921$
120 °C: $\lg W_0 = 0.174 \lg (Gl)_0 - 3.16$ $r = 0.939$
130 °C: $\lg W_0 = 0.174 \lg (Gl)_0 - 2.84$ $r = 0.956$

The decomposition of methionine in the range tested is described by the following reaction kinetic equations:

glucose concentration below 0.12 *M*: $$-\frac{d[Met]}{dt} = k[Met][Gl]^{1/2} \quad (1)$$

glucose concentration above 0.12 *M*: $$-\frac{d[Met]}{dt} = k[Met]. \quad (2)$$

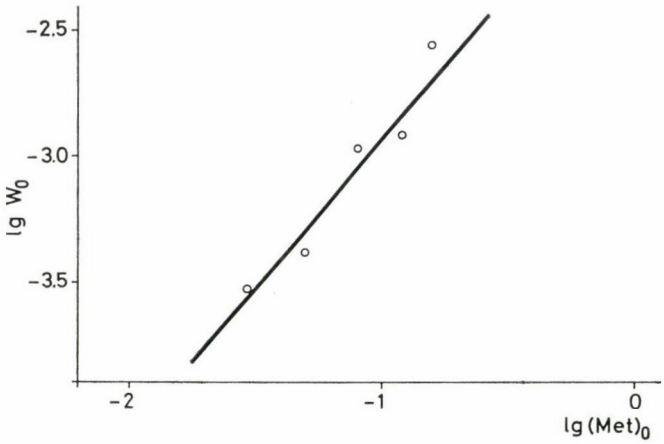

Fig. 4. Logarithm of the initial decomposition rate of methionine (W_0) as a function of the logarithm of the initial methionine concentration (pH = 10.0, 130 °C)
$\lg W_0 = 1.06 \lg (Met)_0 - 1.89$, $r = 0.887$

The partial order for glucose at a tryptophan concentration of 0.05 *M*, determined by alpha-amino nitrogen, was found to be 1.40 ± 2.23 below a glucose concentration of 0.1 *M*, and 0.49 ± 0.04 above this concentration (Fig. 5).

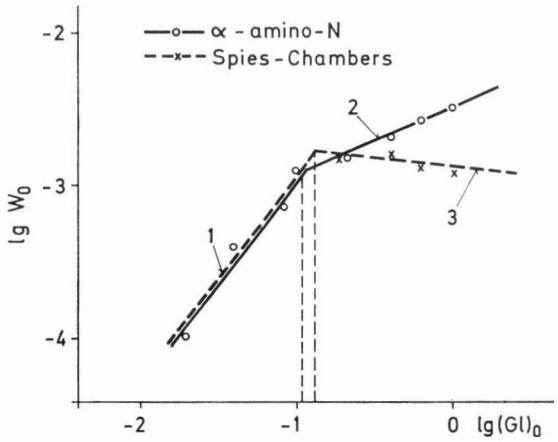

Fig. 5. Logarithm of the initial decomposition rate of tryptophan as a function of the logarithm of the initial glucose concentration (pH = 10.0, 130 °C) below 0.1 *M* glucose concentration:

1. $\lg W_0 = 1.40 \lg (Gl)_0 - 1.54$ $r = 0.813$

above 0.1 *M* glucose concentration

2. $\lg W_0 = 0.49 \lg (Gl)_0 - 2.48$ $r = 0.971$
3. $\lg W_0 = -0.10 \lg (Gl)_0 - 2.86$ $r = 0.856$

The partial order for tryptophan, similarly to that for methionine was independent of the amino acid concentration and was found to be 0.38 ± 0.22 (Fig. 6).

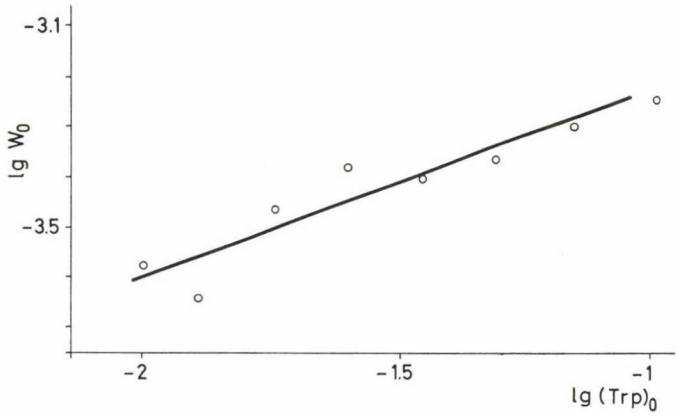

Fig. 6. Logarithm of the initial decomposition rate of tryptophan (W_0) as a function of the logarithm of the initial tryptophan concentration (pH = 10.0, 130 °C)
$\lg W_0 = 0.38 \lg (Trp)_0 - 2.84$, r = 0.924

If the decomposition of tryptophan was followed by alpha-amino nitrogen determination, the following equations were obtained:

glucose concentration below 0.1 *M*: $$-\frac{d[Trp]}{dt} = k[Trp]^{0.38}\,[Gl]^{1.4} \qquad (3)$$

glucose concentration above 0.1 *M*: $$-\frac{d[Trp]}{dt} = k[Trp]^{0.38}\,[Gl]^{0.5}. \qquad (4)$$

When tryptophan decomposition was studied by the method of SPIES and CHAMBERS (1948) the reaction kinetic equation did not differ from (3) and (4). The difference was observed in the partial order of glucose above a glucose concentration of 0.1 *M*. The difference between the slope (0.106 ± 0.69) of the equation related to partial order (Fig. 5) and 0 was non-significant. The difference however, was significant ($P < 5\%$) between slopes 0.106 and 1.4, characteristic of the range of glucose concentrations below 0.1 *M*. The intercept of the ordinate was nearly identical for both methods.

A detailed discussion of differences is given in para. 3.2.

In comparing the reactivity of amino acids with that of the N-acetylated derivatives the decomposition velocity of alpha-N-acetyl-tryptophan was found to be reduced to one ninth of that of tryptophan (pH 10; 130 °C). Under identical conditions the decomposition speed of N-acetyl-methionine was reduced to one twentieth of that of methionine.

The velocity constants of methionine and tryptophan decomposition were studied as a function of pH. The logarithm of the decomposition velocity constants of the reaction stages occurring in the presence in amino acids in excess is shown in Fig. 7. It can be seen that the experimental points may be

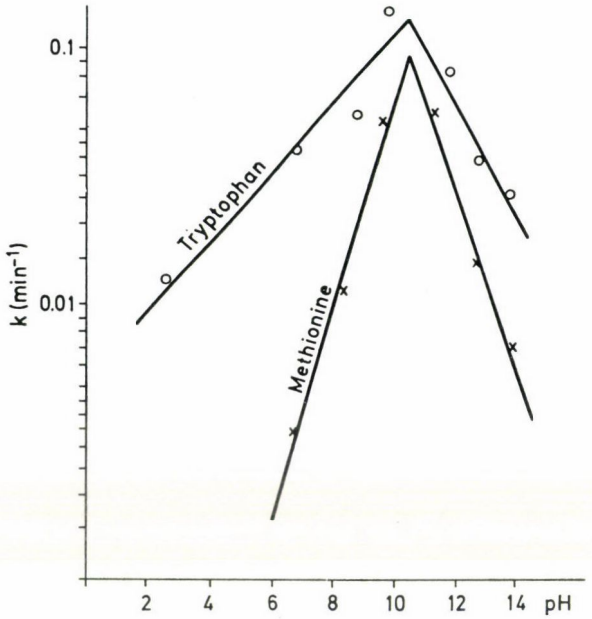

Fig. 7. Changes in the rate constants of the degradation of tryptophan and methionine in the presence of glucose as a function of pH (130 °C)

Regression lines:

Methionine		Tryptophan	
$\lg k_1 = 0.38\,pH - 5.18$	$r = 0.999$	$\lg k_1 = 0.128\,pH - 2.30$	$r = 0.957$
$\lg k_2 = -0.37\,pH - 0.135$	$r = 0.937$	$\lg k_2 = -0.194\,pH + 1.09$	$r = 0.959$

approximated by a line. The decomposition rate constants of both amino acids increase with increasing pH value to a maximum (pH 10.75 for methionine and 10.55 for tryptophan). At pH-s above these values the constants decrease. A significant difference ($P < 0.1\%$) was found in the slopes of the lines from the maxima to lower pH ranges of methionine and tryptophan, resp.

3. Conclusions

3.1. Results of the study into model systems containing methionine

In the methionine containing solution the maximum of brown colouring substances was reached at an amino acid–glucose mole ratio of 2 : 3 (Fig. 1). The order of reaction related to glucose changed at the same mole ratio (Fig. 3). From this observation it may be concluded that in the course of a *Maillard* reaction between methionine and glucose primarily two *Amadori* products:

mono-fructose-methionine (mole ratio 1 : 1) and di-fructose-methionine (mole ratio 1 : 2) are formed at nearly identical proportions (ANET, 1959). As seen from the investigations into the reaction order (Fig. 3) the irreversible bonding of methionine is due to carbonyl compounds of 3 carbon atoms, the trioses. (According to VELISEK and co-workers, 1972, *Schiff*-base is formed during the reaction.) The reaction order of 0.5 is explained by the splitting of glucose in two and by the participation of derivatives in the step determinant for

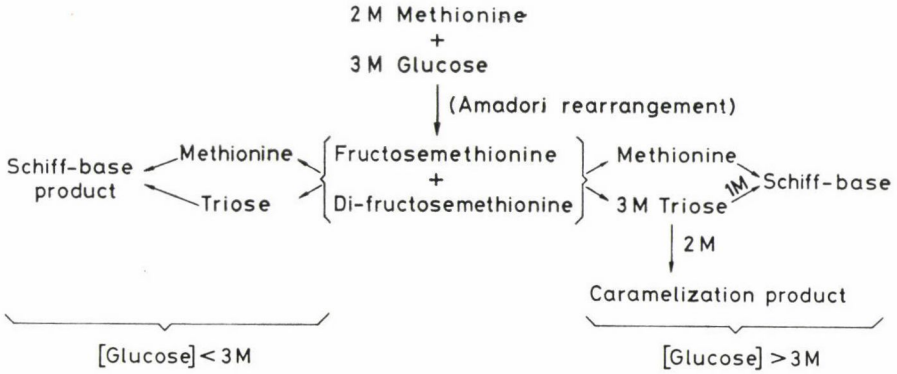

Fig. 8. Suggested pathways for the main reactions between methionine and glucose in aqueous solution

speed. ÖRSI (1969) found the role of trioses and methylglyoxal decisive in glucose caramelization. These processes are modified when the glucose is in excess of the 2 : 3 mole ratio. In this case the sugar in excess provides such an amount of derivatives that the step decisive for velocity is transferred to another place in the chain of reactions and the reaction becomes nearly independent of glucose concentration. The derivatives of excess sugar are probably polymerized whilst caramel substances are formed. The reaction paths, suggested on the basis of kinetic measurements, are shown in Fig. 8.

The *Maillard* reaction with methionine was intensified by the increase of pH (the same is valid for tryptophan) and this is a proof of the reaction being catalyzed by a base (SPARK, 1969). However the velocity constants decreased above pH 10.75. These findings show correlation with the data of VUKOV (1964) who found that in sugar solutions above pH 11 lactic acid and glycolic acid formation increases at the expense of brown colouring substances. The acids thus formed do not participate either in the *Maillard* reaction or in caramelization.

3.2. Results of the study into model systems containing tryptophan

The reaction between tryptophan and glucose is more intricate than that of methionine. In aqueous solution the first maximum of browning occurs

at nearly the same mole ratio as with methionine. The second maximum shows that in the presence of higher sugar concentrations more than two sugar molecules may react with tryptophan (Fig. 2).

The results of these experiments permit of the conclusion that in spite of its mild basic character the –NH group in the indole ring reacts with glucose. The following facts and some data found in the literature seem to support this conclusion.

a. The partial order as found for tryptophan in equations (3) and (4), 0.38 is close to 0.5 (the difference between them is non-significant) and this may be explained by two active centres within the molecule.

b. As seen in Fig. 7 the decomposition rate in the presence of tryptophan is higher ($k = 0.115$) than in the presence of methionine having only one active centre ($k = 0.0865$). At the same time the pH gradient of the rate constant of tryptophan is less steep than that of methionine. This is a consequence of the aromatic character of the indole ring, or of the shaded location in space of the –NH group in the ring, thus less polarizable by a basic catalyzer.

c. The reactivity of alpha-N-acetyl-tryptophan is higher than that of the acetyl derivative of methionine.

d. Earlier findings related to heated powdered milk and egg powder (DWORSCHÁK & HEGEDÜS, 1974) show tryptophan degradation, depending on moisture content in an irregular fashion, to be due to the specific *Maillard* reaction of the nitrogen in the indole ring.

e. According to data in the literature (SUNDBERG, 1970) 3-methyl indole is capable of additioning carbonyl compounds on the nitrogen.

The order of the reaction pertinent to glucose changes at a glucose concentration of 0.1 *M* in the presence of 0.05 *M* tryptophan as shown in Fig. 5. In comparison to methionine in this case, before glucose is formed in excess, probably only difructose-tryptophan is present as intermediary.

On the basis of equations (3) and (4) the hypothetical pathways of irreversible tryptophan degradation are shown in Fig. 9.

As long as, in relation to difructose-tryptophan, intermediary tryptophan is present in excess the partial order of glucose is 1.5. Thus it is probable that in the development of the intermediary determining speed both glucose and triose, formed from glucose, take part. Probably the less active hexose reacts with the alpha-amino group, while the more active triose with the –NH group of the indole ring and the compound thus formed is the intermediary of the reaction. Similarly to the case of methionine when glucose is present in excess, the order of the reaction drops, but only as low as 0.5, thus in this case the reaction of amino acids with trioses determines velocity.

Tryptophan degradation, as characterized by the *Spies–Chambers* colour reaction, is essentially independent of glucose concentration if glucose is in

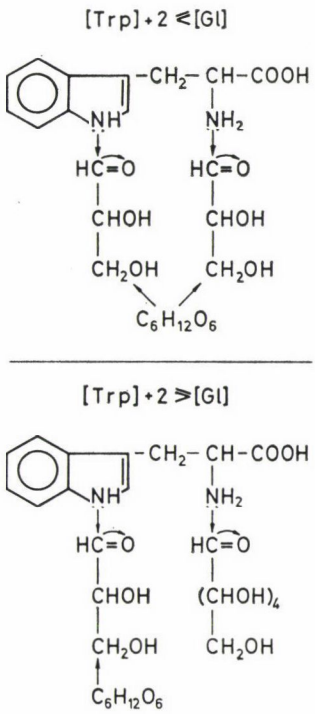

Fig. 9. Suggested pathways for the main reactions between tryptophan and glucose in aqueous solution

excess of difructose-tryptophan. In this range a difference is apparent in the kinetic equations based on alpha-amino nitrogen and on the *Spies–Chambers* colour reaction, specific for the indole ring, resp. Since substitution in position 2 of the indole ring is not possible (FRIEDMAN & FINLEY, 1971) when *Spies–Chambers* colour reaction occurs, substitution is probable at a relative excess of glucose. This finding is in accordance with the results obtained by BRÄUTIGAM and SEVERIN (1971). These authors found that during the *Maillard* reaction with tryptophan on the second carbon atom of the indole ring substituted compounds were formed with carboline structure.

3.3. Main conclusions drawn from the results

a. The pathway of the *Maillard* reaction with methionine follows the pathway of the *Maillard* reaction of monoamino monocarbonic acids and the initial step is the formation of mono- and difructose methionine, by way of the *Amadori* rearrangement.

b. The kinetic analysis of the above reaction has shown the derivatives of glucose (primarily the trioses) to react with the amino acid, too.

c. The different behaviour of tryptophan is due to the participation of the –NH group of the indole ring in the *Maillard* reaction.

d. It was observed for both amino acids that in the presence of glucose in excess the kinetic character of the reaction changed and this may be traced to the increase in the concentration of sugar derivatives. The formation of sugar derivatives is no more the determinative factor of reaction speed. The speed determining step is transferred to another location in the reaction chain.

e. The dependence of the reaction rate constant on pH points to a strong basic catalysis with a maximum at pH 11. Above this pH concurrent acid formation reactions, primarily that of lactic acid comes to the foreground against the *Maillard* reaction.

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COMPARISON OF SENSORY AND INSTRUMENTAL METHODS IN THE STUDY OF THE COLOUR OF CELERY

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The colour of three celery varieties: *Hegykői*, *Frigga* and *Imperator* was studied as a function of radiation dose (0, 15, 150 and 250 krad) and storage time (0 and 5 weeks), resp. The celery was stored at 9–11 °C and 70–85% relative humidity (RH).

The method used is based on tristimulus reflectance measurement of surfaces of cut pieces of celery and calculations to obtain colour difference values (ΔE) which are suitable for statistical evaluation. The results were found to show good correlation with sensory evaluation.

The colour difference between the three celery varieties was not significant statistically.

The tendency to blacken increased with increasing radiation dose. Blackening tendency is enhanced immediately upon radiation treatment. This tendency was not observed in cooked celery.

Blackening during storage remained visible after cooking.

The basic task of the measurement of colour is to determine x, y and Y colour coordinates by which the colour of the sample is established in a standard way. With foods, a further task is the exact determination of the changes occurring as a consequence of some treatment. In this case the colour prior to treatment is the basis for comparison. However it is not sufficient to determine the x, y and Y coordinates of the colour prior and subsequent to treatment and to calculate Δx, Δy and ΔY characterizing the change, because these data do not furnish direct information on the change as observed sensorically.

A relation is established between the change in colour coordinates and the sensory capacity to observe the colour difference by calculating ΔE colour difference. $\Delta E = 1$ is considered the threshold value of the sensory capacity to observe colour difference. Differences $\Delta E < 1$ are not yet observed, differences $\Delta E > 1$ may be observed.

Several methods are known to calculate ΔE colour difference. A simple method, suitable for use in the industry was published by MCLAREN in 1970. The method is based on the following 42nd formula of *Adams-Nickerson* (AN42) and V_x V_y V_z values are described in the XYZ conversion tables:

$$\Delta E_{AN42} = 42\{[0.23V_y]^2 + [\Delta(V_x - V_y)^2] + [0.4\Delta(V_y - V_z)^2]\}^{1/2}.$$

The aim of colour measurement in vegetables may vary. In certain cases the colour of the surface is of interest, while in other cases the inside colour, *i.e.* the colour of the cut surface is the goal. There are cases in which the determination of the combined colour of the surface and inside is the task.

The aim of colour measurement is determined by the field of application where colour is a parameter of quality. Since celery is always peeled before use, the inside colour is of interest.

To measure the colour of the inside of vegetables different methods are available.

SPIESS investigated the colour of carrots in 1964. The samples were peeled, then chopped and finally homogenized. The homogenized sample was filled immediately into the sample holder and measured. This method is suitable to measure the colour of the purée prepared from the produce, thus for measurement of the colour of baby foods.

If the celery is judged by the colour of cut surface, the colour measurement must be carried out on this surface. NEMITZ (1963) published the results of measurements on carrots, FRANCIS (1969) on sweet potatoes. The same method was used in the CENTRAL FOOD RESEARCH INSTITUTE, Budapest, for the study of browning in peaches, apricots, apple and pears (VÁMOS & GAJZÁGÓ, 1974).

The aim of this study was to determine the colour in celery by instrumental and sensory methods and to compare the results of the two methods.

1. Materials and methods

1.1. Raw materials and their storage

Celery varieties *Hegykői*, *Frigga* and *Imperator* were investigated. The samples were obtained from the RESEARCH STATION, Fertőd, of the INSTITUTE FOR HORTICULTURAL RESEARCH.

The samples were stored in cases in a single layer at 9–11 °C and 70–85% RH.

1.2. Radiation treatment

The samples were irradiated with 0, 15, 150 and 250 krad [0, 0.15, 1.50 and 2.50 kGy (kilogray)], resp. Irradiation was carried out in the IRRADIATION PLANT of the CENTRAL FOOD RESEARCH INSTITUTE, Budapest with a ^{60}Co radiation source of 50 kCi [1.185 TBq (terabecquerel)] activity, at a dose rate of 200 krad h^{-1} (2 kGy h^{-1} or 0.56 Gy s^{-1}).

1.3. Instrumental colour measurement

Measurements were carried out with a *Momcolor* tristimulus instrument for colour measurement (MAGYAR OPTIKAI MŰVEK, Budapest).

The instrument may be used for the photoelectric measurement of the colour of surfaces, powders, transparent materials and fluids in the internationally accepted CIE colour system. Colour components X, Y and Z are determined.

To make measurements more exact the white member No. 69-003-00 of the No. 003 etalon series of the HUNGARIAN OFFICE FOR MEASUREMENT was used.

Preparation of the sample. After peeling the raw celery it was cut into prisms of 25 mm length and 12 mm width. Prior to colour measurement a part of the prisms were cooked, the rest was measured raw. Cooking was carried out as described for sensory evaluation. The pieces were cooled in the brine.

One prism was cut of each celery taking care not to include hollow parts of the root. The colour was measured on the prism surface nearest to the skin on an area of 10 mm diameter. The piece was placed on the measuring surface of the instrument and covered with a hood of optically black inner surface.

Results were evaluated by means of a computer.

1.4. Sensory tests

The samples were tested in the raw and in the cooked state. For the raw test the celery was washed, dried and cut in half.

For cooking the celery was peeled, cut up and cooked in a boiling NaCl solution of 1.5% (celery: cooking brine, 1 : 2) for 20 min. After cooking the samples were kept in the cooking brine, because otherwise they blackened rapidly.

The samples were scored on a 5-pont scale, 5 scores corresponding to the best, 1 to the poorest sample.

The averages of the results were ranked according to KRAMER (1960) and evaluated.

2. Results

2.1. Instrumental colour measurement

The colour of the samples treated with 0, 15, 150 and 250 krad, resp. and stored for 0 and 5 weeks was measured in the raw and in the cooked state (Tables 1 and 2).

Table 1

The colour as measured in raw celery samples related to radiation

Variety	Dose (krad)	Storage time (week)	N	x		y		X	
				mean	standard deviation	mean	standard deviation	mean	standard deviation
Hegykői	0	0	3	0.3143	0.0138	0.3530	0.0081	67.63	6.9834
	15		3	0.3653	0.0371	0.2995	0.0651	61.73	0.7875
	150		3	0.3229	0.0059	0.3522	0.0200	68.18	0.1811
	250		3	0.3874	0.0624	0.3886	0.0634	62.86	5.6090
	0	5	3	0.3482	0.0199	0.3560	0.0153	61.68	8.1981
	15		3	0.3461	0.0074	0.3583	0.0019	54.82	4.4473
	150		3	0.3574	0.0056	0.3589	0.0133	47.03	6.7596
	250		3	0.3864	0.0096	0.3807	0.0057	35.64	0.5664
Frigga	0	0	3	0.3305	0.0056	0.3381	0.0049	58.59	4.7227
	15		3	0.3483	0.0067	0.4683	0.0021	65.16	6.7370
	150		3	0.3278	0.0117	0.3457	0.0042	65.85	6.9741
	250		3	0.3196	0.0037	0.3394	0.0051	62.68	6.2444
	0	5	3	0.3423	0.0103	0.3639	0.0138	63.30	4.9117
	15		3	0.3317	0.0050	0.3609	0.0301	68.15	10.3359
	150		3	0.3557	0.0184	0.3556	0.0096	55.66	16.6604
	250		3	0.3829	0.0088	0.3678	0.0103	33.26	7.1921
Imperator	0	0	3	0.3470	0.0156	0.3512	0.0155	65.77	7.3735
	15		3	0.3473	0.0068	0.3665	0.0202	63.23	3.9554
	150		3	0.3362	0.0048	0.3511	0.0090	65.61	5.8694
	250		3	0.3865	0.0286	0.3847	0.0374	57.23	10.7406
	0	5	3	0.3517	0.0040	0.3587	0.0064	65.18	4.9323
	15		3	0.3375	0.0102	0.3498	0.0147	59.58	6.9149
	150		3	0.3660	0.0266	0.3546	0.0169	52.09	12.6034
	250		3	0.3889	0.0115	0.3789	0.0045	37.56	5.7423

dose and storage time after radiation treatment (at 9–11 °C)

Y		Z		V_x	V_y	V_z	ΔE_{AN42} Related to 0 krad
mean	standard deviation	mean	standard deviation				
75.70	3.7677	73.02	5.9814	8.528	8.861	8.151	
51.97	15.5240	57.30	11.2760	8.212	7.582	7.363	43.09
74.41	4.6431	68.58	4.3757	8.559	8.798	7.942	4.69
63.25	7.5557	59.85	10.3674	8.273	8.232	7.503	16.85
63.27	9.2627	54.12	16.2850	8.212	8.232	7.186	
56.67	3.3716	46.72	2.4073	7.816	7.863	6.750	3.91
47.46	8.3599	37.03	3.7408	7.326	7.298	6.109	9.55
35.12	0.3379	21.47	1.5741	6.508	6.414	4.822	20.38
70.17	5.0900	68.99	7.7845	8.580	8.591	7.962	
65.97	7.2286	56.18	7.7500	8.402	8.376	7.303	7.90
69.29	5.3119	65.18	2.5815	8.439	8.546	7.775	4.71
66.42	8.2815	66.27	7.9888	8.267	8.397	7.830	5.44
67.18	3.9177	54.80	8.9625	8.300	8.438	7.226	
73.28	6.6656	64.46	16.8800	8.559	8.745	7.740	5.00
56.35	18.4369	47.29	20.1274	7.869	7.845	6.787	9.26
32.19	7.9733	21.55	4.5870	6.325	6.239	4.832	23.46
66.72	7.6735	58.68	14.6567	8.434	8.412	7.439	
66.59	3.1579	52.34	7.1586	8.294	8.407	7.084	8.17
68.41	5.2240	61.31	9.0265	8.423	8.500	7.576	4.32
57.19	12.4954	33.14	9.7962	7.957	7.892	5.822	19.19
66.46	5.1031	53.82	6.2083	8.402	8.402	7.169	
61.60	5.6533	55.30	7.7036	8.095	8.139	7.253	6.62
51.53	15.8811	41.97	18.1634	7.652	7.551	6.451	9.52
36.75	6.4110	22.75	5.5348	6.662	6.546	4.948	19.56

Dose (krad)	Comparison of data on 0 day and after 5 weeks (ΔE_{AN42})		
	Hegykői	*Frigga*	*Imperator*
0	15.54	11.25	4.47
15	32.27	9.66	8.31
150	19.16	9.97	12.19
250	22.88	26.76	15.38

Table 3

The colour as measured in cooked celery samples related to

Variety	Dose (krad)	Storage time (week)	N	x		y		X	
				mean	standard deviation	mean	standard deviation	mean	standard deviation
Hegykői	0	0	3	0.3225	0.0213	0.3374	0.0177	20.13	4.1972
	15		3	0.3258	0.0136	0.3410	0.0226	18.36	4.8597
	150		3	0.3217	0.0163	0.3591	0.0026	16.97	0.4065
	250		3	0.3263	0.0236	0.3355	0.0137	16.86	2.8980
	0	5	3	0.3283	0.0436	0.3700	0.0056	20.50	4.4270
	15		3	0.3738	0.0119	0.3803	0.0122	15.87	3.4648
	150		3	0.4000	0.0262	0.3696	0.0161	15.22	7.6364
	250		3	0.3946	0.0411	0.3674	0.0079	19.87	9.6788
Frigga	0	0	3	0.3108	0.0248	0.3297	0.0258	18.86	3.4286
	15		3	0.3336	0.0085	0.3498	0.0161	20.51	1.8931
	150		3	0.3320	0.0247	0.3327	0.0214	18.30	7.6226
	250		3	0.3273	0.0301	0.3337	0.0067	16.12	2.6449
	0	5	3	0.3184	0.0171	0.3159	0.0400	20.98	2.3134
	15		3	0.3444	0.0298	0.3287	0.0295	13.18	2.9271
	150		3	0.3495	0.0359	0.3467	0.0484	15.83	2.1102
	250		3	0.3875	0.0089	0.3990	0.0106	13.26	2.6073
Imperator	0	0	3	0.3451	0.0171	0.3421	0.0318	18.62	1.5824
	15		3	0.3359	0.0106	0.3512	0.0093	21.27	0.8694
	150		3	0.3187	0.0167	0.3417	0.0161	17.07	3.2761
	250		3	0.3794	0.0457	0.3429	0.0303	18.36	0.8563
	0	5	3	0.3254	0.0232	0.3445	0.0245	21.06	3.0485
	15		3	0.3850	0.0216	0.3536	0.0326	31.40	5.8596
	150		3	0.3811	0.0456	0.3958	0.0320	35.51	10.6002
	250		3	0.4102	0.0035	0.3742	0.0079	12.27	0.3496

radiation dose and post-irradiation storage time (at 9–11 °C)

Y		Z		V_x	V_y	V_z	ΔE_{AN42} Related to 0 krad
mean	standard deviation	mean	standard deviation				
21.02	4.0944	20.70	0.2901	5.081	5.135	4.742	
18.91	4.2321	18.59	4.3112	4.887	4.902	4.522	2.79
18.97	0.6698	16.87	1.2280	4.719	4.914	4.332	7.05
17.34	2.6999	17.46	3.0696	4.707	4.715	4.400	4.68
22.98	2.9615	18.53	2.6068	5.125	5.343	4.511	
16.04	2.8922	10.34	1.9097	4.581	4.554	3.454	13 57
14.12	7.2219	8.14	2.7159	4.490	4.303	3.076	20.84
18.00	8.3007	10.67	3.9865	5.059	4.798	3.506	22.18
19.99	3.5286	20.69	2.6954	4.945	5.026	4.742	
21.61	2.9988	19.38	1.1952	5.125	5.198	4.607	5.43
18.00	6.4814	17.54	4.4289	4.876	4.798	4.400	7.29
16.73	4.0664	17.07	4.6464	4.607	4.641	4.355	4.21
20.84	3.3790	24.07	4.0913	5.179	5.113	5.070	
12.64	3.0021	12.22	2.3171	4.215	4.090	3.736	11.45
15.55	1.1722	14.69	5.9595	4.568	4.490	4.067	8.79
13.66	2.7497	7.16	0.6211	4.229	4.248	2.8843	23.98
18.46	2.2108	17.07	3.6800	4.911	4.856	4.355	
22.25	1.1241	19.70	2.7092	5.212	5.271	4.639	6.62
18.28	3.3778	17.80	0.9647	4.732	4.833	4.434	6.78
16.80	2.3396	13.76	3.3189	4.887	4.654	3.952	8.43
22.15	1.8041	20.80	3.3254	5.190	5.261	4.752	
29.24	8.0485	21.47	4.7152	6.167	5.926	4.822	17.69
36.88	11.1560	22.46	12.2737	6.501	6.553	4.920	22.65
11.15	0.1224	6.42	0.2811	4.077	3.867	2.727	20.81

Dose (krad)	Comparison of data on 0 day and after 5 weeks (ΔE_{AN42})		
	Hegykői	*Frigga*	*Imperator*
0	10.29	7.43	6.58
15	12.68	14.13	16.81
150	20.24	3.00	26.65
250	22.18	18.51	10.62

Table 3

Tendency to blacken in various celery

Variety	Dose (krad)	Time after cooking (min)	N	x		y		X	
				mean	standard deviation	mean	standard deviation	mean	standard deviation
Hegykői	0	10	3	0.3225	0.0213	0.3374	0.0177	20.13	4.1972
	15		3	0.3258	0.0136	0.3410	0.0226	18.36	4.8597
	150		3	0.3217	0.0163	0.3591	0.0026	16.97	0.4065
	250		3	0.3263	0.0236	0.3355	0.0137	16.86	2.8980
	0	120	3	0.3174	0.0153	0.3425	0.0319	19.17	3.6213
	15		3	0.3223	0.0106	0.3365	0.0340	17.53	3.5915
	150		3	0.3330	0.0048	0.3561	0.0084	17.51	0.4631
	250		3	0.3330	0.0177	0.3459	0.0167	18.70	1.5240
Frigga	0	10	3	0.3108	0.0248	0.3297	0.0258	18.86	3.4286
	15		3	0.3336	0.0085	0.3498	0.0161	20.51	1.8931
	150		3	0.3320	0.0247	0.3327	0.0214	18.30	7.6226
	250		3	0.3273	0.0301	0.3337	0.0067	16.12	2.6449
	0	120	3	0.3079	0.0136	0.3302	0.0139	18.63	2.9962
	15		3	0.3274	0.0077	0.3428	0.0089	19.49	1.8209
	150		3	0.3219	0.0299	0.3013	0.0062	17.26	5.1644
	250		3	0.3225	0.0137	0.3185	0.0290	18.14	2.8108
Imperator	0	10	3	0.3451	0.0171	0.3421	0.0318	18.62	1.5824
	15		3	0.3359	0.0106	0.3512	0.0093	21.27	0.8694
	150		3	0.3187	0.0167	0.3417	0.0161	17.07	3.2761
	250		3	0.3794	0.0457	0.3429	0.0303	18.36	0.8563
	0	120	3	0.3210	0.0165	0.3353	0.0242	18.14	2.0789
	15		3	0.3212	0.0053	0.3413	0.0012	21.73	1.3026
	150		3	0.3132	0.0115	0.3243	0.0267	18.44	2.0529
	250		3	0.3367	0.0290	0.3599	0.0011	17.10	1.1125

varieties as a function of time after cooking

Y		Z		V_x	V_y	V_z	ΔE_{AN42} realated to 0 krad
mean	standard deviation	mean	standard deviation				
21.02	4.0944	20.70	0.2901	5.081	5.135	4.742	
18.91	4.2321	18.59	4.3112	4.887	4.902	4.522	2.79
18.97	0.6698	16.87	1.2280	4.719	4.914	4.332	7.05
17.34	2.6999	17.46	3.0696	4.707	4.715	4.400	4.68
20.62	4.6136	20.03	0.3087	4.980	5.092	4.670	
17.96	2.3452	18.74	4.6684	4.780	4.798	4.533	5.53
18.72	0.1713	16.35	0.9835	4.780	4.879	4.274	3.75
19.45	1.4357	18.06	2.7249	4.922	4.970	4.467	3.24
19.99	3.5286	20.69	2.6954	4.945	5.026	4.742	
21.61	2.9988	19.38	1.1952	5.125	5.198	4.607	5.43
18.00	6.4814	17.54	4.4289	4.876	4.798	4.400	7.29
16.73	4.0664	17.07	4.6464	4.607	4.641	4.355	4.21
20.00	3.2528	21.60	1.4292	4.911	5.026	4.832	
20.42	1.9926	19.54	0.6894	5.014	5.070	4.618	5.01
15.92	3.4658	19.64	3.0800	4.756	4.541	4.628	15.37
18.10	4.1618	19.89	0.9694	4.852	4.810	4.660	6.96
18.46	2.2108	17.07	3.6800	4.911	4.856	4.355	
22.25	1.1241	19.70	2.7092	5.212	5.271	4.639	6.62
18.28	3.3778	17.80	0.9647	4.732	4.833	4.434	6.78
16.80	2.3396	13.76	3.3189	4.887	4.654	3.952	11.75
19.53	2.4754	19.14	4.3323	4.852	4.970	4.576	
23.11	1.5860	22.83	1.5995	5.254	5.353	4.948	3.79
19.17	3.0670	21.13	1.1887	4.887	4.937	4.782	4.94
18.52	2.8508	15.61	4.1960	4.732	4.856	4.178	4.90

Dose (krad)	Comparison of data obtained after 10 and 120 min (ΔE_{AN42})		
	Hegykői	*Frigga*	*Imperator*
0	2.52	2.08	7.57
15	2.18	2.69	4.24
150	4.06	4.28	4.73
250	4.34	4.25	5.13

The colour of the cooked samples was measured within 20–30 min after cooking. Ten min were required for cooling, because the samples could not be measured hot. As can be seen in Table 3, there is no difference between samples as a function of time elapsing between cooking and measurement.

In Fig. 1 the ΔE values were plotted as a function of radiation dose. The ΔE value was obtained by comparing the irradiated sample to the untreated one. Therefore the figure shows the difference in the colour of samples irradiated at different dose levels and of the untreated sample.

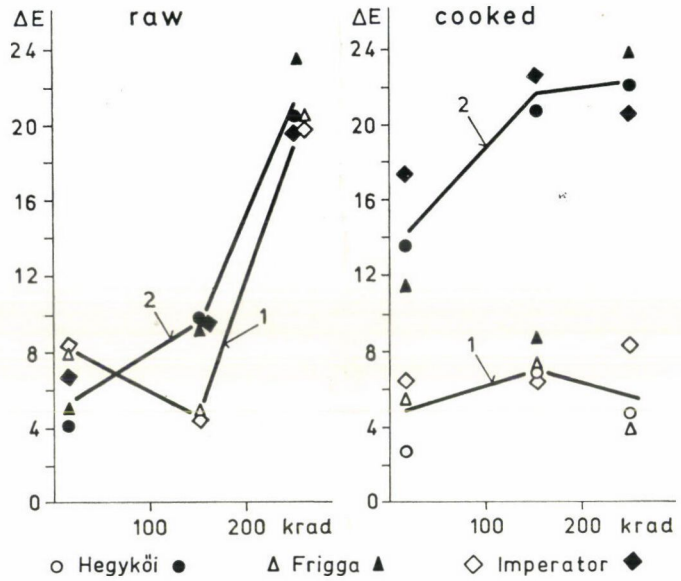

Fig. 1. Comparison of the colour of various celery varieties as measured in the raw and in the cooked vegetable, resp. Irradiated samples were compared with untreated samples. The ΔE values thus obtained were plotted as a function of radiation dose. 1: samples on 0 day (open circle); 2: samples stored at 9–11 °C for 5 weeks after irradiation (solid circle); A: *Hegyköi;* B: *Frigga;* C: *Imperator*

The difference between the colour of raw celery on 0 day and after 5 weeks storage was non-significant. There was no change in the colour of the cooked samples with radiation dose, when measured on 0 day. After 5 weeks the cooked samples were darker than initially.

When the colour of the two other varieties was compared to that of *Frigga* (having the whitest flesh) they did not differ on the basis of their ΔE value.

When the three celery varieties were compared by t test at each dose level the raw sample irradiated with 250 krad was found to blacken but this colour difference could be eliminated by cooking.

After 5 weeks of storage of intact celeries the raw samples treated with 150 and 250 krad showed blackening. Upon cooking the samples treated with 250 krad remained significantly blacker than the other samples (Table 5).

Table 4

Comparison of the colour of various celery varieties based on the ΔE value[+]

Variety	Raw		Cooked	
	0 day	5 weeks	0 day	5 weeks
	experimental t values			
Hegykői	1.40	1.61	1.06	1.51
Imperator	0.90	2.35	1.30	1.30

tabulated t values = $t_{95\%} = 2.57$ $t_{99\%} = 4.03$ $t_{99.9\%} = 6.86$

[+] Varieties *Hegykői* and *Imperator* were compared to variety *Frigga*, since the colour of this was the lightest.

2.2. *Sensory colour evaluation*

Fig. 2 shows the data obtained by ranking according to KRAMER (1960).

On 0 day the difference between raw and cooked samples was non-significant. The raw sample treated with 250 krad and a *Frigga* sample cooked and treated with 150 krad formed exception, they were darker than the other samples. The difference was significant at the probability level of 99%.

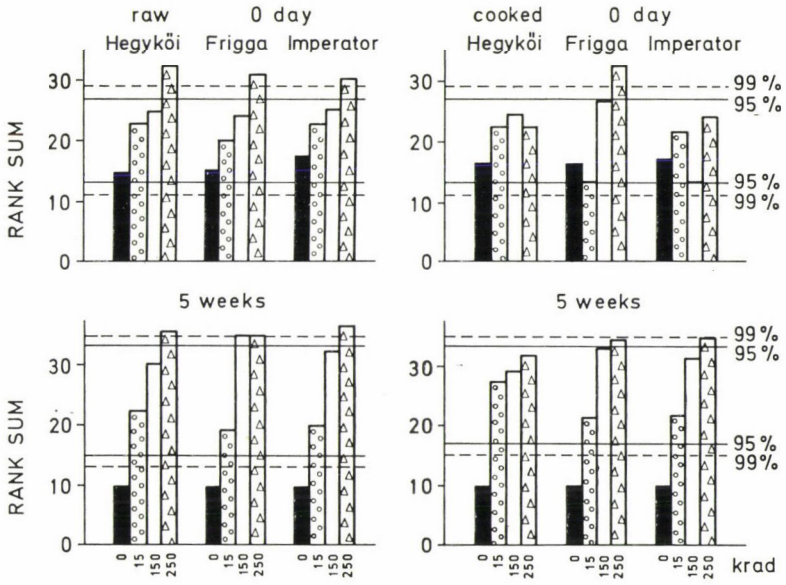

Fig. 2. Colour of various celery varieties as established by scoring *vs.* radiation dose and post-irradiation storage time at 9–11 °C. The lower the rank sum the lighter the colour of the samples

Table 5

Comparison of the colour of various celery varieties on the basis of their ΔE values (The ΔE values in the table are related to 0 krad)

	Dose (krad)	Variety	Raw			Cooked		
			ΔE	ΔE	s	ΔE	ΔE	s
0 day	15	*Hegykői*	not evaluable			2.79		
		Frigga	7.90			5.43	4.95	1.96 (v%=39.6)
		Imperator	8.17			6.62		
	150	*Hegykői*	4.69			7.05		
		Frigga	4.71	4.57	0.22 (v%=4.8)	7.29	7.04	0.26 (v%=3.7)
		Imperator	4.32			6.78		
	250	*Hegykői*	16.85			4.68		
		Frigga	20.38	18.93	1.85 (v%=9.8)	4.21	5.77	2.31 (v%=40.0)
		Imperator	19.56			8.43		
5 weeks	15	*Hegykői*	3.91			13.57		
		Frigga	5.00	5.18	1.36 (v%=26.3)	11.45	14.24	3.17 (v%=22.3)
		Imperator	6.62			17.69		
	150	*Hegykői*	9.55			20.84		
		Frigga	9.26	9.44	0.16 (v%=1.7)	22.65	17.43	7.53 (v%=43.2)
		Imperator	9.52			8.80		
	250	*Hegykői*	20.38			22.18		
		Frigga	23.46	21.13	2.06 (v%=9.7)	23.98	22.32	1.59 (v%=7.1)
		Imperator	19.56			20.81		

s = standard deviation v = coefficient of variation

t-test

	krad \ krad	Raw		Cooked	
		150	250	150	250
0 day	15			2.25	0.58
	150		16.37***		1.16
5 weeks	15	6.59**	13.74***	0.89	4.83***
	150		12.02***		1.35

$t_{95\%}$ = 2.57: * $t_{99\%}$ = 4.03: ** $t_{99.9\%}$ = 6.86: ***

After a storage period of 5 weeks the untreated raw and cooked samples are significantly ($P > 99\%$) better than the rest. Significantly worse than the rest of cooked samples is variety *Hegykői* (treated with 250 krad). All the raw samples treated with 150 or 200 krad were significantly darker than the rest.

2.3. Comparison of the results obtained by instrumental measurement and sensory evaluation

The ΔE values were plotted as a function of the average score. Since it had been established that the tendency of blackening was independent of variety, it was possible to plot all the results on the same graph (Fig. 3).

The closest correlation was found between the cooked samples after 5 weeks storage ($r = 0.965$). Correlation was virtually non-existant between sensory scores and instrumental measurements with cooked samples on 0 day. Further study seems necessary to clarify the phenomenon.

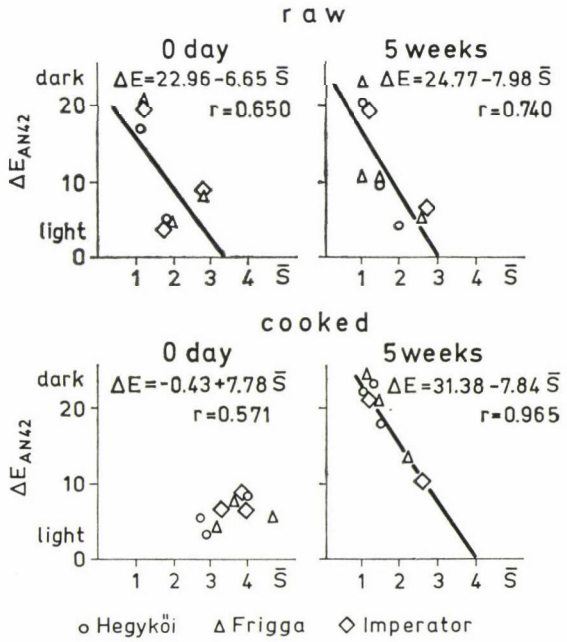

Fig. 3. ΔE values plotted as a function of the average score ($\bar{s}$). The ΔE values were obtained by relating the samples treated at different dose levels to the untreated sample. r = correlation coefficient

3. Conclusions

The colour of celery varieties *Hegykői*, *Frigga* and *Imperator* was studied as a function of irradiation with 0, 15, 150 and 250 krad and of 0 and 5 weeks storage time.

Both the instrumental measurements and the sensory evaluation permitted of the same conclusions.

No statistically significant difference between the colour of various celery varieties could be detected.

The tendency of turning black increased with increasing radiation doses. The tendency increased immediately upon treatment. However, in cooked celery this tendency was not observed.

Blackening occurring during storage could not be eliminated by cooking.

The main purpose of this study was to compare instumental colour measurement with sensory evaluation.

The objective measurement and the calculations applied led to a value (ΔE) by which the statistical evaluation of the results became possible.

The ΔE values were plotted as a function of the average score.

The higher the ΔE value, the blacker was the sample.

The rather high values of the correlation coefficient seem to justify the comparison between the two methods (instrumental and sensory).

The ΔE value is of higher informative value if it is supported by sensory data. It has the advantage of enabling statistical evaluation and comparison. But being a relative figure it can only show differences.

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DETERMINATION OF CALCIUM IN FEEDS AND FEED COMPONENTS BY ATOMIC ABSORPTION

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The aim of this study was to develop a method suitable for the determination of calcium in a wide concentration range without the necessity of weighing-in very small portions or diluting the stock solution in case of samples of high calcium or phosphorus content. With such an objective, calcium determination does not fall within the traditional scope of the atomic absorption technique of measurement. This branch of instrumental analysis was developed specifically for the determination of metal ions in extremely low concentrations. However, in the present case the use of this technique was indicated not by its extreme sensitivity, but by its rapidity and methodological simplicity. This aspect prompted the author to test also the flame emission technique, however, the higher stability of the 0 point and the higher optical selectivity of the absorption method led him to decide in favour of the latter.

The main feature of this process is that the ash is dissolved in diluted nitric acid solution and solutions of $(NH_4)_2HPO_4$, Na_2EDTA and triethanolamine are added. The added solutions eliminate the possibility of various chemical errors, stabilize the degree of calcium ionization in the flame and reduce the sensitivity of atomic absorption measurement to the required level. The three additives fulfil various functions simultaneously. In the presence of the fairly large amount of added $(NH_4)_2HPO_4$ the effect of the inherent PO_4^{3-} ions becomes negligible ("infinite disturbance") while the sensitivity of calcium determination decreases, which in this case is important.

The disturbing effect of some other ions (small amounts of aluminium and sulfate) is eliminated by Na_2EDTA and the sodium thus introduced behaves in the flame as a buffer for ionization. The triethanolamine ensures the desirable pH level for the stability of the EDTA complex, at the same time contributes to the reduction of sensitivity by slowing down atomization.

Measurements were carried out with a *Pye Unicam* SP 1900 double beam apparatus and thus results were obtained in percentage without graphic interpretation (linearization of the curve and expansion of scale).

The method has no inherent error, the relative standard deviation of individual results is ± 2.24 rel.% for samples of $\sim 3\%$ calcium content. The method is recommended mainly for the determination of calcium concentrations above 1%.

As it is well known, in the presence of phosphate the amount of free Ca atoms available for absorption in acetylene—air flame is reduced. In the case of feed materials and mixed feeds of high phosphate content this disturbing effect is increased. A serious error may be caused by the presence of phosphate in feeds where the absolute phosphate concentration is low, but the P : Ca ratio is high. In the selection of the experimental conditions it is expedient to take into account other chemical sources of error beside the disturbing effect of phosphate. In some cases the amount of aluminium and sulfate present may not be neglected, either.

Mixed feeds and their components may contain substantial amounts of sodium (common salt), and these, according to the well known mechanism (WELZ, 1975b), reduce the degree of calcium ionization in flame thereby increasing the absorption index. In plant materials the effect of inherent potassium on the degree of Ca ionization may not be neglected either. Since in some of the materials tested the calcium concentration is high, the high sensitivity of the atomic absorption technique is not an absolute advantage. In view of accurate results the weighing of too small test portions or extensive dilution is not advantageous. In developing the method the authors aimed at making it suitable for use within wide limits of measurement. A further aim was to avoid expensive reagents. (The lanthanum salts suggested for the elimination of disturbing chemical effects are for instance fairly expensive.)

1. Materials and methods

1.1. The role of additives

First it was tried to eliminate disturbing chemical effects with Na_2EDTA (WELZ, 1975a), however, in relation to phosphate under the given conditions this did not prove sufficiently effective (Fig. 1).

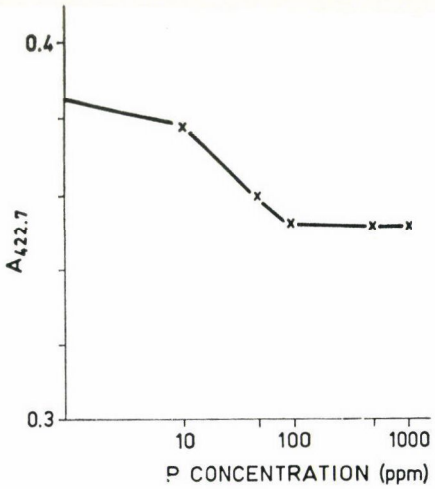

Fig. 1. Change in the absorption (A) of calcium in acetylene–air flame as affected by P concentration [added in the form of $(NH_4)_2HPO_4$)]. The solution contained 0.05 *M* Na_2EDTA and 10% triethanolamine. Parameters of measurement: $\lambda = 422.7$ nm; 0.8 l · min^{-1} acetylene; 4.8 l · min^{-1} air; burner turned by 10°

However, results were satisfactory when a large amount of $(NH_4)_2HPO_4$ was introduced in addition to the complex forming agent to the sample solution as well as to the standard solutions. Thus, in practice the absorption

index became independent of the phosphate content of the sample and other sources of error (aluminium, sulfate) while the sensitivity of the method was reduced. (In this method the use of Na_2EDTA is indicated not so much by the presence of phosphate, but by that of other ions of disturbing effect.) With Na_2EDTA a fair amount of sodium is introduced and this behaves like ionization buffer in the flame reactions. The pH needed to stabilize the CaEDTA complex is provided by triethanolamine. The triethanolamine containing solutions used had a pH above 9 and this was satisfactory, since at pH = 8 the complexometric titration of Ca is possible (SAJÓ, 1973). The reason for choosing this basic substance was that it reduces the rate of atomization and thereby the sensitivity of the reaction (Fig. 2).

1.2. Materials

96% ethyl alcohol

5 *N* HNO_3

Solution **Z** to eliminate disturbing effects: 102 g $Na_2EDTA \cdot 2H_2O$, 13 g $(NH_4)_2HPO_4$ and 550 ml triethanolamine are dissolved in about 3 l distilled water and made to the mark in a volumetric flask of 5 l. This solution is stored in a polyethylene balloon (the solution contains ~0.055 $M \cdot l^{-1}$ Na_2EDTA and ~550 ppm P).

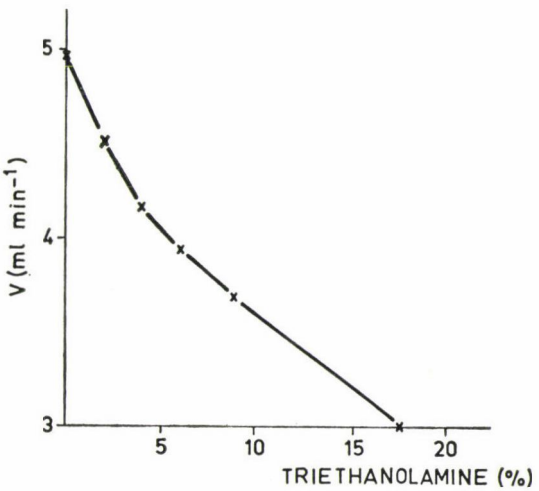

Fig. 2. Dependence of atomization rate (V) on the triethanolamine content of the solution. Flow rate of air: 4.8 l · min^{-1}

Ca^{2+} solution of 2 000 ppm: 4.9944 g of $CaCO_3$, dried at 105 °C, are weighed and dissolved without loss in 5 *N* HNO_3, evaporated to dryness over a water bath. The residue is dissolved in distilled water and washed into a volumetric flask of 1 000 ml and made to the mark.

Standard solutions containing 0, 50 and 100 ppm Ca^{2+}, resp.: 0, 25.00 and 50.00 ml, resp. of the 2 000 ppm Ca^{2+} solution are transferred into volumetric flasks of 1 l, 5 ml of 5 *N* HNO_3 and 900 ml of solution Z are added to each. The three volumetric flasks are filled to the mark. The solutions are stored in polyethylene flasks. (In the case of the available instrument these three standard solutions are sufficient. During the development of the method, however, more standard solutions were used within concentration limits 0 to 150 ppm. Of the reagents used, triethanolamine was of the "purissimum" grade, the others of analytical grade.)

1.3. Mineralization and preparation of solutions

From samples having a Ca content above 0.5% 1.00 g of the ground material was weighed-in, damped with alcohol, charred and incinerated at 500 °C. Of samples of lower Ca content 5.00 or 10.00 g were weighed-in. The ash was dissolved in 5 *N* HNO_3 and washed with hot distilled water through a filter, into a 100 ml volumetric flask. When cool, the flask was made to the mark. Directly prior to measurement 1.00 ml portions are transferred to test tubes and 9.00 ml solution Z added to each.

1.4. Atomic absorption measurement

Instrument: PYE UNICAM LTD., Type SP 1900, double beam atomic absorption spectrophotometer of *Ebert* optical arrangement. Measuring parameters: flame: acetylene 0.8 l min^{-1}, air = 4.8 l min^{-1}; rate of atomization: 3.6 l min^{-1}; burner turned by 10°; wave-length 422.7 nm; slit width: 0.1 mm. Calibration: After setting the 0 point the absorbance of standard solutions of 50 and 100 ppm Ca^{2+} are measured. Using the operation instructions "Conc" the absorbance *vs.* Ca (ppm) function is linearized. Thereafter the scale is expanded to show the figure 5.00 at the digital display when the Ca^{2+} solution of 50 ppm is atomized. By setting the instrument in this way the percentage Ca content of samples may be read directly if the weight of the sample was 1.00 g. (No graphic evaluation is necessary.)

2. Results and conclusions

Curve A in Fig. 3 shows absorbance as a function of the calcium content in case of the use of additives, described above, while curve C is another graphic illustration of absorption as a function of concentration. The latter is essentially the illustration of certain transformation of the absorbance function, effected in the given instrument in two steps, under electronic interventions

in accordance with para. 1.4 (linearization of the curve and scale expansion). The advantage of the function obtained by transformation (curve C) is that its direction tangent (= sensitivity) is constant in a wide concentration range and that the value of the function corresponds numerically to the percentage Ca in the sample if operations are carried out in accordance with paras. 1.3, and 1.4 and a test portion of 1.00 g is weighed in (Fig. 3).

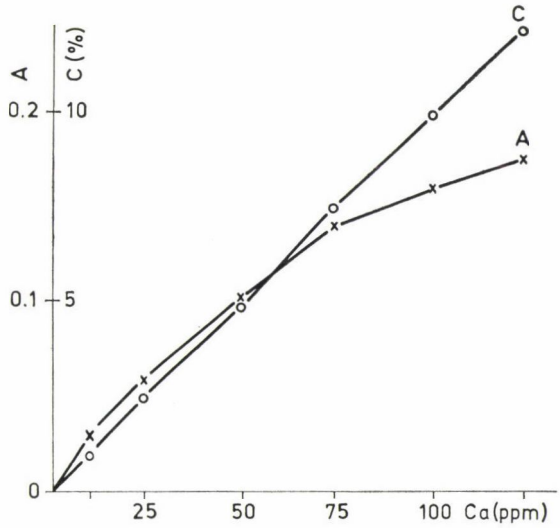

Fig. 3. Dependence of the absorption signal on the calcium content. The solutions contained ~ 0.025 *M* HNO_3, ~ 0.05 *M* Na_2EDTA, 10% triethanolamine and $(NH_4)_2HPO_4$ corresponding to $\sim$ 500 ppm P; pH = 9.1. Parameters of measurement the same as in Fig. 1. A: curve of absorbance; C: curve obtained by linearization and scale expansion

To characterize the accuracy of the method parallel determinations were performed with a feed concentrate. Accuracy was tested by the so called addition method and the permanganate method generally used in Hungary. As can be seen in Tables 1, 2 and 3, results satisfy practical requirements if the calcium content is above 1%. However with concentrations below 1% the relative differences were high (Table 3). This is probably due to the unfavourable effect, in this range, of reduction of sensitivity upon the accuracy of results. Presumably the comparative method is not sufficiently accurate if calcium concentration is some tenth of a percent or even below. Since differences found in results at very low calcium concentrations were not investigated the reliability of the method developed is considered proven only for samples with calcium contents above 1%.

The advantage of the method lies in its applicability at relatively high calcium concentrations without the need for weighing-in very small test portions or for excessive dilutions. Another advantage of the method is that

Table 1

Accuracy of the method of calcium determination by atomic absorption

Experimental material: "Calf starter" concentrate

	Ca (%)	rel. %
Mean value	2.99	—
Standard deviation	±0.067	±2.24
Deviation from the mean	±0.021	±0.70

Number of parallel tests: 10

Table 2

Accuracy of the atomic absorption method of calcium determination as checked with the addition method

Added calcium 1.50%, related to the sample

Serial number	Sample	Calcium measured (%)		Deviation from the calculated value	
		in the original sample	after addition of Ca	%	rel. %
1.	wheat bran	0.31	1.75	−0.06	−3.3
2.	feed	1.23	2.80	0.07	2.6
3.	feed	1.50	3.00	0.00	0.0
4.	feed	3.20	4.79	0.09	1.9
5.	feed concentrate	2.99	4.38	−0.11	−2.5
6.	feed concentrate	3.36	4.85	−0.01	−0.2
7.	feed concentrate	3.50	4.95	−0.05	−1.0
8.	feed super-concentrate	7.80	9.46	0.16	1.7
9.	mineral additives	8.00	10.42	−0.08	−0.8

Table 3

Comparison of results obtained by the atomic absorption technique and permanganate titration, resp.

Serial number	Sample	P	Na	K	Ca (%)		Deviation	
		%			atomic absorption*	permanganate	%	rel. %
1.	corn (moisture 11%)	0.28	—	0.29	0.024	0.029	−0.005	−17.2
2.	wheat bran	0.79	—	0.15	0.31	0.35	−0.04	−11.4
2.	fermented alfalfa	0.36	—	1.61	2.17	2.09	0.08	3.8
4.	feed	0.61	0.68	0.31	1.23	1.26	−0.03	−2.4
5.	feed	0.75	0.55	0.28	1.50	1.52	−0.02	−1.3
6.	feed concentrate	1.64	1.66	0.66	2.99	2.88	0.11	3.8
7.	feed super-concentrate	2.79	1.09	1.62	7.80	7.62	0.18	2.4
8.	mineral additives	8.13	10.44	0.0	8.00	8.19	−0.19	−2.3

* Mean value of three measurements

the reagents used for eliminating the disturbing compounds are not expensive.

It seems worth mentioning that the diluted nitric acid solution used in calcium determination is suitable for the determination not only of other metals but also of phosphate and chloride.

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INVESTIGATIONS INTO THE ENZYMIC BROWNING OF APRICOT CULTIVARS

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(Received 31 May, 1976)

Five apricot cultivars from the same orchard were investigated for enzymic browning as well as for polyphenol oxidase (PPO) and o-dioxy phenol (ODP) concentrations. According to enzyme and substrate concentrations the cultivars could be divided into two groups each, while the values of initial browning rate (BA) suggested division into three groups (Table 1). The ratios of the highest to the lowest values determined were 10 for ODP, 2 for PPO and 3 for BA. A significant correlation ($r = 0.54$) was established between BA and ODP concentration (Fig. 1). The relative effect of substrate content on browning as calculated from standardized partial regression coefficients was about twice as great as that of enzyme concentration. These findings are in disagreement with earlier results of the authors obtained with apple cultivars, the initial browning rates of which were found to depend primarily on enzyme concentration. The contradiction was assumed to be due to different ratios of PPO/ODP in the two kinds of fruits: the concentration of enzyme as related to that of substrate was 5 times higher in apricots than in apples. Thus, while in the latter case inactivation of the enzyme by reaction products seemed to be the limiting factor of browning, in the former case it was substrate depletion.

A study carried out earlier in this laboratory on the enzymic browning of apples (VÁMOS-VIGYÁZÓ *et al.*, 1976) resulted in the establishment of considerable differences in the browning tendencies of different cultivars, whereby the rate of discolouration was found to depend primarily on polyphenol oxidase (PPO) concentration in the fruits. Since findings published in the literature on the participation, in the phenomenon of enzymic browning, of PPO and its substrates as present in horticultural products are rather divergent, it was thought interesting to extend the work to other kinds of fruits, too. The present paper deals with investigations into the rate of enzymic browning, polyphenol oxidase concentration and o-dioxy phenol content of apricot cultivars.

1. Materials and methods

1.1. Apricot cultivars

Five apricot cultivars were supplied by the CEGLÉD RESEARCH STATION OF THE HORTICULTURAL RESEARCH INSTITUTE, Budapest. The signs and names of the cultivars were as follows: C 235 *Magyar* (Hungarian), C 244 *Bíbor* (Purple), C 333 *Rakovszky*, C 568 *Mandula* (Almond) and C 732 *Keverék* (Mixed lot). The fruits were stored in a cold room at +5 °C until utilization.

1.2. Determination of browning activity, polyphenol oxidase content and o-dioxy phenol concentration

The methods of determining the initial rate of browning of the fruit flesh as well as the respective concentrations of enzyme and substrates have been described in detail elsewhere (VÁMOS-VIGYÁZÓ *et al.*, 1976; MIHÁLYI & VÁMOS-VIGYÁZÓ, 1976; VÁMOS & GAJZÁGÓ, 1974; VÁMOS-VIGYÁZÓ *et al.*, 1973), thus only the principles of the methods and the dimensions and units used will be mentioned here.

Initial browning rate (hereafter referred to as browning activity, BA) of peeled apricot slices was determined by reflectance measurement in the spectrocolorimeter *Spekol* (CARL ZEISS, Jena) at 580 nm and 100-fold sensitivity. Changes in reflectance were plotted against time and the initial linear section of the resulting curve was used to calculate browning activity by linear regression. Activity was expressed in terms of changes in reflectance per min ($\Delta R\ min^{-1}$) as indicated by the instrument and a change corresponding to 1 scale division per min was adopted as the unit.

Polyphenol oxidase (PPO) activity of appropriately diluted homogenates of peeled fruits was determined at pH 5.4 in the presence of chlorogenic acid substrate added in excess. Reaction mixtures were kept at 30 °C for various intervals up to 20 min and the changes in optical density (OD) as caused by the coloured substances formed from the substrate upon enzyme action were read in a spectrophotometer *MOM 203* (MAGYAR OPTIKAI MŰVEK, Budapest), at 420 nm. Enzyme activity was indicated by the slope of the linear section of the OD *vs.* reaction time plot and was calculated from the latter by linear regression. The enzyme was considered to be of unit activity if it brought about a change in OD of $10^{-4}\ min^{-1}$. Activity was related to 1 g of fruit flesh (wet-weight basis) and will be referred to as enzyme concentration.

A modified version of the method of ALMÁSI and MOLNÁR (1961) based on the *Hoepfner* reaction (ARNOW, 1937) was applied to determine the o-dioxy phenol (ODP) content in the methanol extracts of peeled and comminuted apricots. Apricot content ranged from 0.1 to 0.25 g ml^{-1} in the extracts which were reacted in turn with HCl, $NaNO_2$ and NaOH solutions. OD-s of the resulting pink solutions were determined at 520 nm with the spectrophotometer *MOM 203*. ODP content was expressed as chlorogenic acid on the basis of a calibration curve.

1.3. Calculation of correlations between browning activity, polyphenol oxidase concentration and o-dioxy phenol content

Linear regression analysis with two variables as well as with two independent variables was applied to establish relationships between the characteristics studied (SVÁB, 1973).

2. Results

2.1. o-Dioxy phenol and polyphenol oxidase contents as well as initial browning rates of apricot cultivars

The results obtained with the five cultivars studied are summarized in Table 1, the statistical evaluation of the data obtained is shown in Table 2.

Table 1

o-Dioxy phenol content, polyphenol oxidase concentration and browning activity of apricot cultivars

Sign and name of apricot cultivar	o-Dioxy phenol content^{+} mg %		Polyphenol oxidase concentration, Ug^{-1}		Browning activity, U	
	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s
C 235, *Magyar*	68	17	2690	62	1.7	0.1
C 244, *Bíbor*	6.7^{++}	3.2	4380	109	0.6	0.1
C 333, *Rakovszky*	9.7	3.7	4030	135	1.1	0.1
C 568, *Mandula*	42	15	2070	131	0.9	0.1
C 732, *Keverék*	6.5	1.7	3720	78	1.0	0.2

$\bar{x}$ = mean of 6 parallel determinations
s = standard deviation
$^{+}$ = expressed as chlorogenic acid
$^{++}$ = mean of 4 parallel determinations

Table 2

Significance levels of the differences in o-dioxy phenol content, polyphenol oxidase concentration and browning activity of apricot cultivars

Sign	ODP				PPO				BA			
	C 244	C 333	C 568	C 732	C 244	C 333	C 568	C 732	C 244	C 333	C 568	C 732
C 235	***	***	*	***	***	***	***	***	***	***	***	***
C 244		0	**	0		**	***	***		***	**	*
C 333			***	0			***	0			0	0
C 568				***				***				0

Significance levels refer to differences in the data of Table 1 as calculated by *Student*'s *t*-test.

ODP = o-dioxy phenol content; PPO = polyphenol oxidase concentration; BA = browning activity.

Significance levels of differences, %: *** = 99.9; ** = 99; * = 95; 0 = difference not significant.

According to ODP content the five cultivars may be divided into two groups: the ODP contents of the cultivars *Bíbor* and *Rakovszky* as well as *Keverék* are below 10 mg% and do not differ significantly from each other,

while those of the *Magyar* and the *Mandula* apricots are very much higher (68 mg% and 42 mg%, resp.). The ratio of the highest and lowest value was more than 10.

The cultivars studied proved — with the exception of *Keverék* and of *Rakovszky* apricots — different at significance levels of 99 or 99.9%, with respect to PPO concentration. Nevertheless, the values of this characteristic, too, suggest dividing the 5 cultivars into two groups: *Magyar* and *Mandula* apricots had enzyme contents below 3 000 U g^{-1}, while in the other three cultivars these values were around 4 000 U g^{-1}. It may be noted that enzyme concentrations were lower in the cultivars of higher substrate contents. The ratio of the highest to the lowest PPO content was around 2.

BA was highest (1.7 U) in *Magyar* apricot of highest ODP concentration and lowest (0.6 U) in *Bíbor* which belongs to the low substrate content group. BA-s of the rest of the cultivars lay between these values and did not differ significantly from each other. Thus, with respect to BA, the five cultivars studied could be divided into 3 groups. The ratio of the highest to the lowest activity value was nearly 3.

With respect to the characteristics studied the 5 apricot cultivars showed the most considerable differences in o-dioxy phenol content.

2.2. Correlations between initial browning rate, o-dioxy phenol content and polyphenol oxidase concentration

Results obtained with the 5 apricot cultivars studied suggest some relationship between BA and ODP content, while the concentrations of ODP and PPO seem inversely related to each other. The data were subjected to mathematical analysis in order to express these relationships in an exact way.

As a result of linear regression analysis with two independent variables the correlation between initial browning rate, ODP content and PPO concentration may be described by the following equation:

$$Y = 1.4148 + 0.0189X_1 - 0.000265X_2$$

where Y = browning activity; U
X_1 = polyphenol oxidase concentration; U g^{-1}
X_2 = o-dioxy phenol concentration; mg%
n = 59 (= number of pairs of data)
R^2 = 0.35 (= squared correlation coefficient).

Analysis of variance according to Table 3 showed the multiple correlation coefficient to be significant at a probability level of 99.9%.

Table 3

Analysis of variance of the linear correlation between the dependent variable browning activity and the independent variables o-dioxy phenol content and polyphenol oxidase concentration as determined in apricots

Source of variance	SSQ	DF	MS	F
Total	16.5500	58		
Regression	5.8287	2	2.9143	15.22
Error	10.7213	56	0.1914	

The critical value, at the level of P = 0.1%, of F for DF 2–60 is 7.76.
SSQ = sum of squares
DF = degree of freedom
MS = mean square
F = value of F-test

Standardized partial regression coefficients were calculated to establish the relative effects of ODP and PPO concentrations on BA (SVÁB, 1973). The respective values of 0.88 for ODP and —0.43 for PPO showed the effect of substrate content on initial browning rate to be about double that of enzyme concentration.

The correlation was further analyzed by testing the direct and indirect effects of the independent variables (the concentrations of ODP and PPO) on the dependent variable BA by path analysis. Expansion of the squared multiple correlation coefficient to direct and common (indirect) effects led to the results summarized in Table 4.

Table 4

Participation of o-dioxy phenol content and polyphenol oxidase enzyme concentration in the variance of the initial rate of enzymic browning of apricots as calculated by expansion of the squared multiple correlation coefficient R^2

	Components of effect	Relative participation of direct and joint effects, %
Direct	ODP	77.8
	PPO	18.4
Joint	ODP · PPO	— 61.0
	Total, R^2	35.2
	p^2E	64.8
	Total	100.0

ODP = o-dioxy phenol concentration
PPO = polyphenol oxidase enzyme concentration
$p^2E = 1-R^2$, square of the path-coefficient of deviation

As can be seen, the variance of browning is mainly due to the substrate content of apricots, *i.e.* 77.8% of the variance are caused by the direct effect of ODP concentration. The direct effect of enzyme concentration is but slight (18.4%), while the common effect of the two independent variables is negative (—61.0%). Thus, only 35.2% of the variance of browning may be accounted for by the direct and joint effects of ODP and PPO contents, whereas the majority, 64.8% is due to factors not included in this study.

Results were similar when the effects of substrate and enzyme concentrations were tested separately, by regression analysis with two variables (one dependent and one independent). In this case a linear correlation ($r = 0.53$) was obtained between ODP content and BA as represented in Fig. 1.

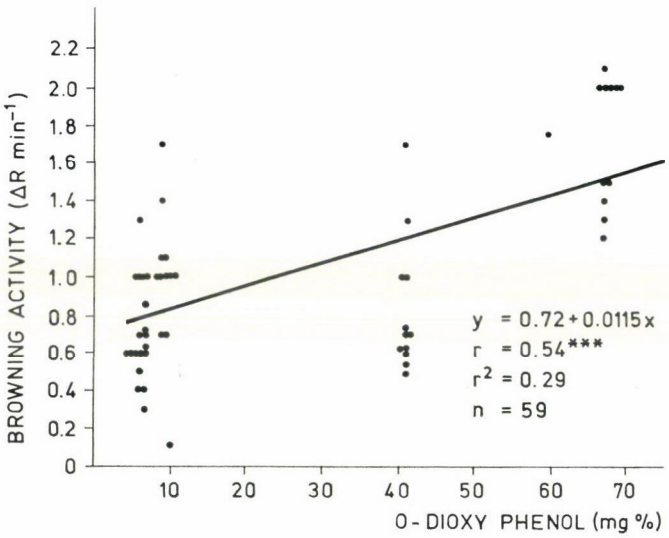

Fig. 1. Correlation between initial browning rate (browning activity) and o-dioxy phenol concentration of apricots. ΔR = change in reflectance; r = correlation coefficient; n = number of data; *** = the correlation is significant at the probability level of 99.9%

The correlation is significant at the probability level of 99.9%. The squared correlation coefficient shows initial browning rates of apricot cultivars to depend to an extent of 29% on substrate concentration. No correlation was found between BA and PPO.

3. Conclusions

3.1. Difference between apricot cultivars with respect to the characteristics studied

In the study presented regional and seasonal variations were excluded. Fruits of a given lot were picked from a maximum of 3 trees grown in the same

orchard. Thus, the differences established might be regarded as due to the cultivars.

The ratio of the highest to the lowest value (68 and 6.5 mg%, resp.) of ODP content was found to be more than 10 (Table 1). The latter value was, as a matter of fact, the lowest ever measured in this laboratory in various kinds of fruits (pears, apples, peaches). In earlier experiments carried out with apricots of unknown cultivars as purchased from small producers, considerably higher ODP contents (150 and 100 mg%, resp.) were found in two different lots (VÁMOS-VIGYÁZÓ *et al.*, 1973).

Standard deviations of ODP concentrations were much higher for apricots than, *e.g.* for apples (s% = 35 and 7, resp., on the average). This is probably connected with the fibrous structure of apricot flesh.

The differences between the five cultivars tested were less marked with respect to PPO concentration: the highest value (4 380 U g^{-1}) was slightly more than double that of the lowest (2 070 U g^{-1}) (Table 1). The enzyme concentrations of two apricot lots of unknown origin were, as determined earlier (MIHÁLYI & VÁMOS-VIGYÁZÓ, 1976; VÁMOS-VIGYÁZÓ *et al.*, 1973), 2 160 and 2 650 U g^{-1}. These values are within the limits established in the present study.

Browning activities of the five cultivars from Cegléd were between 0.58 and 1.71 U, *i.e.* the ratio of the highest to the lowest value was about 3 (Table 1). The cultivar *Bíbor* apricot, of the slightest tendency to browning may be best recommended, at least from this point of view, for processing. In earlier experiments (VÁMOS-VIGYÁZÓ *et al.*, 1973) BA of two lots of apricots of unknown cultivars were found considerably higher, 4.4 and 4.5 U, resp. These lots were identical with those of higher substrate contents (150 and 100 mg%, resp.) mentioned above.

3.2. Relationships between initial browning rate and the concentrations of polyphenol oxidase and o-dioxy phenols

Linear regression analysis with two variables showed browning activity of apricots to depend to about 30% on o-dioxy phenol concentration (Fig. 1), while no correlation could be established between browning and polyphenol oxidase. Linear regression analysis with two independent variables allowed a deeper insight into the relationship between enzymic browning and the enzyme and the substrates involved in the process: in agreement with the results of regression analysis with two variables the relative effect of o-dioxy phenols was found to prevail in the browning phenomenon over that of polyphenol oxidase as expressed by the ratio of the standardized partial regression coefficients.

This finding is in disagreement with earlier results obtained in similar experiments with apple cultivars (VÁMOS-VIGYÁZÓ *et al.*, 1976). In the study

cited initial browning rate was found to depend to an extent of 56% on polyphenol oxidase concentration, while the role of o-dioxy phenols proved negligible (the squared correlation coefficient was about 0.1).

For the sake of comparison the results of the regression analysis with two independent variables as carried out with data obtained earlier for the six apple cultivars from North-Eastern Hungary (VÁMOS-VIGYÁZÓ *et al.*, 1976) shall be tabulated here (Table 5).

Table 5

Participation of o-dioxy phenol content and polyphenol oxidase enzyme concentration in the variance of the initial rate of enzymic browning of 6 apple cultivars from North-Eastern Hungary[+] *as calculated by expansion of the squared multiple correlation coefficient* R^2

	Components of effect	Relative participation of direct and joint effects, %
Direct	ODP	0.7
	PPO	66.5
Joint	ODP · PPO	5.4
	Total, R^2	72.6
	p^2E	27.4
	Total	100.0

[+] Experimental data relative to the apple cultivars have been published earlier VÁMOS-VIGYÁZÓ *et al.*, 1976).

For symbols see Table 4.

As can be seen, the variance of the initial rate of browning is due to the extent of 66.5% to polyphenol oxidase concentration. The effect of substrate concentration is negligible (0.7%) and the joint (positive) effect of the two independent variables is slight (5.4%). 27.4% of the variance of browning activity cannot be ascribed to either of the two independent variables.

As calculated from the standardized partial regression coefficients, the relative importance of PPO concentration was found to be about 12 times that of ODP content in apples.

The results obtained with apples and apricots as to the participation of polyphenol oxidase and its substrates in the phenomenon of enzymic browning are in striking contrast as can be seen also from Fig. 2. This seems to justify the numerous contradictions found in the literature on the subject, as discussed earlier (VÁMOS-VIGYÁZÓ *et al.*, 1976).

A possible explanation of the apparent contradiction of our findings obtained with apricots and with apples, resp., may be given by comparing the ratios of PPO and ODP concentrations in the two kinds of fruits. Con-

sidering the means of all the cultivars included in the study the value of this ratio is 127 for apricots and 26 for apples. This means that in apricots the enzyme content as related to substrate concentration is, on the average, nearly 5 times higher than in apples. The determining role of polyphenol oxidase concentration in the browning of apples was interpreted as a consequence of the inactivation of the enzyme by its reaction products while substrate was still present (WHITAKER, 1972). In the case of apricots it seems possible

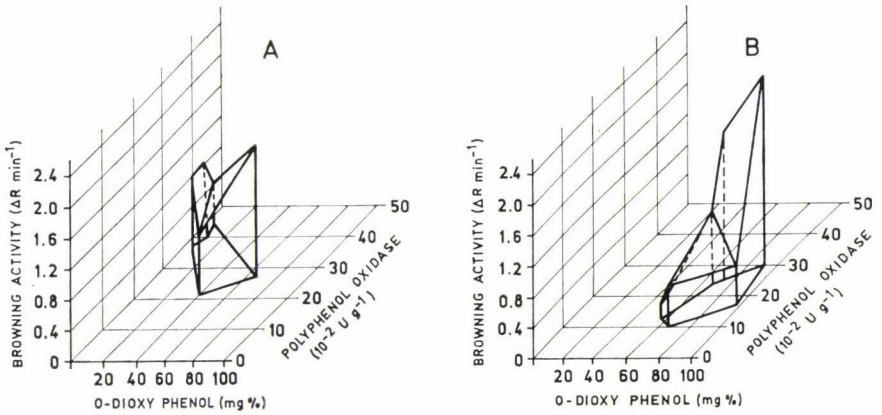

Fig. 2. Comparison of initial browning rates of apricots (A) and apples (B) as function of the concentrations of o-dioxy phenol and polyphenol oxidase. ΔR = change in reflectance

that the oxidation of the total amount of substrate available is insufficient to form the amount of reaction product necessary to inactivate the enzyme, consequently, the limiting factor of the reaction is the depletion of the substrate. Thus, in the case of a given product, the *ratio* of enzyme and substrate contents seems to determine which of the two characteristics plays the decisive role in browning.

Another point shall be stressed here. In the case of apples the variance of browning was caused to an extent of 72.6% by the direct and common effects of PPO and ODP and only 27.4% were due to other factors. In the case of apricots only 35.2% of the variance of browning could be accounted for by the characteristics studied, while the greater part, 64.8%, was due to factors not investigated (Tables 4 and 5). It is assumed that in apricots other enzymes, *e.g.* peroxidase might be present in higher concentrations and contribute to browning. Peroxidase concentration of apples is, as recently established (MIHÁLYI, 1976) among the lowest, therefore enzymic browning of this fruit is considered to be caused almost entirely by PPO. This is also in agreement with the results presented here.

*

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RHEOLOGICAL CHARACTERISTICS OF FRESH AND FROZEN CHICKEN MUSCLES AND THEIR RELATION TO TENDERNESS MEASURED BY SENSORY METHODS*

A PRELIMINARY STUDY

J. Dobrzycki, E. Pietrzak and A. Hoser

(Received February 20, 1976)

The rheological characteristics (measured as shear force on an *Instron* apparatus modell UTM 1112) of some chicken muscles after various chilling and freezing processes were studied.

In parallel tests sensory evaluation of tenderness of the same muscles was carried out by two methods and the results were compared with those obtained using the instrument.

The results show that the method of chilling and freezing is reflected in the shear force values. Therefore this measurement can be helpful in process control and in the development of new processes.

Upon various technological treatments, similar texture changes could be detected whether the two sensory methods, or the instrumental measurements were applied.

Thus, it may be concluded that shear force measurement of chicken meat reflects to some extent the sensory perception of tenderness justifying the use of the instrumental method.

The progress in animal husbandry and the development of new breeds and hybrids brings about the necessity of objective quality assessment of animal raw materials, using objective and comparable quality characteristics whenever possible. A demand for objective quality assessment concerns also the development of new preservation and storage methods as well as new technological processes.

For frozen poultry meat rheological properties of the muscles seem to be one of the most important factors of over-all quality from the technological and consumer points of view.

Rheological characteristics of muscles measured by instrumental methods do, of course, not reflect in all complexity the sensory perception of tenderness, however, simplicity, reproducibility of results and objectiveness are unquestionable advantages.

Rheological characteristics of the product can be expressed in many ways, among which the shear force is often used (Kramer & Szczesniak, 1973).

The objective of this study was to measure the rheological characteristics (measured as shear force on an *Instron* apparatus) of some chicken muscles after various chilling and freezing processes.

* Paper presented at the 3rd Methodological Symposium on Food Analysis, 9–11 October 1975, Szentendre, Hungary.

The question was how great was the influence of the above mentioned processes on the rheological properties of the muscles and how exactly could these be followed by shear force measurements.

1. Materials and methods

1.1. Materials

Eight-week chicken broilers of *Sussex-Cornish* breed of an average weight of 1450 g, served as the experimental material.

Slaughtering was made on the line in the processing plant. The group of 180 chickens was divided into three lots: one lot was chilled in iced water (as it is done on the processing line), the second chilled in nitrogen vapour at —50 °C. The third lot remained unchilled until frozen. Two methods of freezing were applied: conventional method (air-blast freezing tunnel, air temperature —35 °C, freezing time, approx. 20 h) and liquid nitrogen spray (LN_2) in a tunnel (down to a core temperature of the carcasses of —30 °C; freezing time, approx. 20 min). The chicken carcasses not frozen, but quickly chilled in N_2 vapour to inner temperature 0 — + 2 °C. Prior of analysis, chicken carcasses wrapped in vacuum-closed polyethylene bags and they were uniformly thawed in the running water of 30 °C. The hichen carcasses were uniformly cooked in 1% NaCl solution (ratio of carcass to NaCl solution 1:2). The thermal treatment was finished when the inner temperature of the thickest chicken muscle reached +80 °C. Then, the carcasses were uniformly cooled to +20 °C in laboratory glass-ware to avoid excessive moisture losses. For instrumental as well as for sensory evaluations *musculus pectoralis superficialis* was used throughout.

1.2. Instrumental determination of rheological characteristics

The investigations were carried out on an *Instron* universal testing machine, table model TM 1112. Basically, this instrument is used by mounting the sample on the table connected to the strain gauge. Changes in force applied to the sample by horizontal crosshead and shearing blunt edge (*Warner–Bratzler* type) were observed with a strip-chart recorder which plots a force *vs.* time curve for every test.

Operating parameters: crosshead speed: 5 cm min^{-1}; chart speed: 5 cm min^{-1}, tension-compression cell, 2, 4 and 10 kg full deflection. The maximum force for shearing the sample was determined and also the work of shearing (*i.e.* the area under the curve shear *vs.* time was measured by planimetry).

1.3. Sensory evaluation

Sensory evaluation was made by an experienced 10-member panel. Every technological treatment (combination of chilling and freezing) was evaluated in three replicates.

Two methods of sensory analysis were applied:

First method: scoring of texture on a 5-point scale, according to TILGNER (1957); second method: comparative-profile method according to DAGET (1974), modified at the SENSORY ANALYSIS LABORATORY of the INSTITUTE OF FOOD AND NUTRITION in Warsaw. In this method four texture characteristics were evaluated: fibrousness, hardness, tenderness and juiciness, for their deviations from the same characteristics in the reference sample.

2. Results and discussion

The results of the investigations are shown in Table 1. The instrumental characteristics are expressed as shear force and shearing work in absolute values, obtained with the apparatus (G shear g^{-1}, and G cm g^{-1}, resp.) and also in relative values (as % of values for the reference sample taken as 100%). For the sake of comparison the results of sensory evaluation of the experimental material (average scores) have also been presented in the table.

Table 1

Instrumental and sensory characteristics of chicken chilled and frozen by different methods

Chilling method		Material								
		non-frozen			frozen					
					traditionally			in liquid nitrogen		
		Instron data	%	SC	*Instron* data	%	SC	*Instron* data	%	SC
Water	SF	—			284	93	3.4	298	99	3.7
	WS				591	124		518	109	
N_2	SF	301	100	3.9	330	109	3.8	268	90	4.1
	WS	474	100		628	132		448	94	
No chilling	SF	—			312	104	3.4	297	99	3.7
	WS				519	109		525	110	

SF — shear force value, G g^{-1} of sample; WS — work of shear, G cm g^{-1} of sample; SC — sensory score, 5-point scale.

One can see that both chilling and freezing methods have influence on the texture characteristics of chicken muscles and also, that these effects are interrelated.

The best effect was observed with rapid chilling in nitrogen vapour.

The texture of the samples thus prepared scored higher than those chilled in water or not chilled. This tendency was observed independently of the method of freezing.

The sensory profiles of chicken frozen traditionally and frozen in liquid nitrogen are about the same. However, by freezing in LN_2 the texture was significantly better. With the latter method of freezing, results of shear force measurement fully confirm those, obtained in sensory evaluation. However, with traditional freezing, this direct relationship between sensory and instrumental data was not obtained.

To clear up this controversy, the results obtained with the same material by the profile method (Fig. 1) had to be studied.

In this method the texture was evaluated as four separate factors: fibrousness, softness, tenderness, juiciness. It can be seen that the method of chilling and freezing mainly influences tenderness and juiciness, while fibrosity and softness are affected to a lesser degree.

One can presume, that the relationship between instrumental data and sensory evaluation of these two separate factors (tenderness and juiciness) would be closer and more clearly expressed.

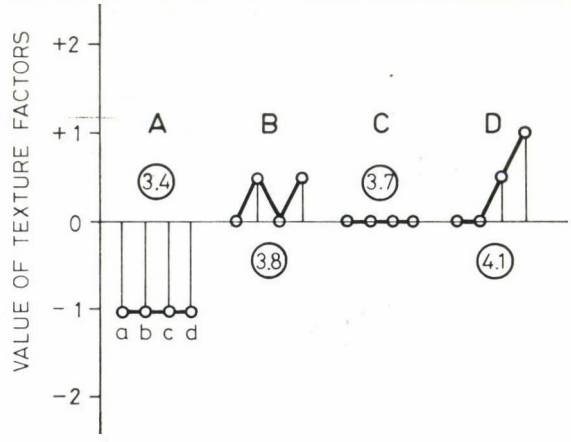

Fig. 1. The results of sensory evaluation of chicken chilled and frozen by different methods. Mean scores, obtained by 5-point scale method are given in the circles. The results, obtained by the comparative-profile method, are given in the plot. Evaluated texture factors: a — fibrosity; b — softness; c — tenderness; d — juiciness

	Method of chilling	freezing
A	in water	traditional
B	in N_2	traditional
C	in water	in LN_2
D	in N_2	in LN_2

Above presented preliminary data show the complexity of the problem of instrumental and sensory evaluation.

Since the study is being continued, it can be expected, that more complete experimental material will shed some more light on the whole problem.

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A WHEAT ENDOSPERM MICROHARDNESS INDEX*

H. GASIOROWSKI and S. POLISZKO

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This paper presents ways of determining a generalized index of wheat endosperm microhardness allowing for structural heterogeneity and thus representing the real hardness of kernel. Four varieties of Polish common wheat and one *Canadian Amber Durum* were used in this investigation. All samples of wheat were stored at constant relative humidity for obtaining hygroscopic equilibrium. Wheat kernels were cut longitudinally with a microtome and microhardness of the disclosed endosperm was determined with the microhardness tester PMT-3. The specific gravity of grain was determined stereometrically in the *Breuil* mercury volumeter. The *Brabender* hardness tester was used also, to measure kernel hardness. Some scores of kernels completely vitreous and completely floury were distinguished and the average microhardness was measured. Then a generalized index of endosperm microhardness was calculated.

The experiments show interdependence between specific gravity of the kernels and microhardness of their endosperm. The densimetric method of determining the generalized index of wheat endosperm microhardness is very suitable for the control of grain quality.

Comparison between the index of kernel hardness determined on the *Brabender* tester and generalized wheat endosperm hardness index does not show close correlation.

The results of the experiment point to the possibility of classification of wheat varieties on the basis of wheat endosperm microhardness.

Kernel hardness is defined as resistance of the grain to deformation by an outside force. There are two types of methods of determining this property. One of them is to subject a given quantity of wheat to the abrasive action of a grinding stone for a specified period of time and calculating the percentage grain weight loss (ANDERSON *et al.*, 1966; SYMES, 1969; TAYLOR *et al.*, 1939). The second type of the methods for determining kernel hardness (microhardness) is to test individual kernels or even structural fragments of a single kernel of grain (CHUSID, 1958; GROSH & MILNER, 1959).

Indices of kernel hardness in a given quantity of wheat are valuable in determining tempering and milling parameters, but they are less important for forecasting the quality of mill product, which is due to the fact that the indices of hardness depend not only on the mechanical properties of kernel endosperm but also on the same properties which are characteristic of their bran. NAUMOV and co-workers (1970) observed that the removal of kernel bran as a result of scouring off 10 per cent from the total quantity of grain causes more than 30 per cent decrease in kernel hardness.

* Paper presented at the 3rd Methodological Symposium on Food Analysis, 9–11 October 1975, Szentendre, Hungary.

Measurements of endosperm microhardness in relation to the quantity of grain to be milled has been so far a weak point of the results of such measurements. It is heterogeneity connected with a defined degree of vitreousness of grain that is responsible for the low representativity of the results of measurements usually carried out on some scores of kernels.

This paper presents ways of determining generalized indices of wheat endosperm microhardness allowing for structural heterogeneity and thus representing the real hardness of grain.

1. Material and methods

1.1. Materials

Four varieties of Polish common wheat and one *Canadian Amber Durum* were chosen for experimental purposes. Their characteristics can be found in Table 1.

Table 1

Characteristics of grain

Wheat	Weight		Protein content (%)	Vitreousness (%)
	1000 kernels (g)	hectoliter (g l^{-1})		
Canadian Amber Durum	46.80	828	12.77	98
Kaukaz	42.80	808	8.30	32
Mironowska 808	40.12	766	10.28	58
Grana	40.22	764	9.05	40
Opolska	35.92	742	9.65	10

All samples of wheat were stored at constant relative humidity to obtain hygroscopic equilibrium.

1.2. Measuring of wheat endosperm hardness

Aiming at measuring wheat endosperm hardness the kernels were fixed at one third of their height in epoxy resin and the upper part was cut off with a microtome at two thirds of their height. Microhardness of the disclosed endosperm was determined using the microhardness tester PMT-3 with a *Vickers* diamond indenter. Calculations were carried out on the basis of a well known equation:

$$H = \frac{1.854\ P}{d^2} \tag{1}$$

where H means microhardness (kg mm^{-2}), P = load of indenter (P = 0.1 kg), d = length of diagonal indentation (mm).

Then the *Brabender* hardness tester was used to measure kernel hardness (in a given quantity). In this tester maximum torque was measured in *Brabender* units. Afterwards the specific gravity of grain was determined with the *Breuil* mercury volumeter.

2. Results and discussion

Hardness can be defined as the medium energy necessary to press the volume unit of a material (GRIGOROVICH, 1960). As the resulting figures directly depend on the geometrical shape of the indenter which is selected at random by the producer, it cannot be treated as a physical constant as, for instance, modulus of elasticity or specific gravity. However, when the conditions of experiments are strictly adhered to, the hardness can be treated as a conventional index of density of energy of interatomic bonding in solids.

If we accept the above interpretation of hardness as true also in relation to organic solids and to wheat endosperm, the following generalized equation for all kernel endosperm hardness will result from the energy balance:

$$H_e = H_v \cdot V + H_f \cdot (1 - V) \tag{2}$$

where H_v and H_f mean hardness of vitreous or floury structural constituent, resp., and V = volume share of the vitreous structural constituent in kernel endosperm. In order to determine generalized indices of endosperm microhardness of the investigated samples, 30 kernels completely vitreous and as many completely floury were selected. Then the average microhardness of their disclosed endosperm was measured. Generalized indices of endosperm microhardness of the samples were calculated from equation (2), assuming that the volume share of vitreous structure constituents in the endosperm equals the relative share of vitreous structure constituents in the kernel investigated. The results of the measurements (H_v and H_f) and of the calculations (H_e) are shown in Table 2.

The possibility of applying a wheat endosperm microhardness index calculated from equation (2) for the control of grain quality is impeded by the time-consuming preparation of samples and measurements

It is assumed that the densimetric method of determining the generalized index of wheat endosperm microhardness may be more suitable here. This method is based on the correlation between specific gravity of kernels and microhardness of their endosperm.

Figure 1 demonstrates changes in endosperm microhardness of vitreous and floury structure constituents.

Table 2

The results of measurements of wheat endosperm microhardness
Means (m) and standard deviations (s) of 30 parallel measurements

Wheat	Relative vitreousness (V)	Specific gravity (D) g cm^{-3}	Microhardness in kg mm^{-2}				Generalized endosperm microhardness index		WHI (*Brabender* units)
			vitreous phase (H_e)		floury phase (H_f)				
			m	s	m	s	H_e	H_d	
Canadian Amber Durum	0.98	1.353	15.03	0.68	8.53	0.49	14.90	14.6	114.0
Kaukaz	0.32	1.305	10.65	0.51	5.45	0.30	7.12	6.6	63.7
Mironowska 808	0.58	1.284	6.40	0.25	4.40	0.15	5.57	5.0	63.0
Grana	0.40	1.281	7.07	0.37	3.61	0.22	5.00	4.8	27.7
Opolska	0.10	1.242	4.36	0.24	2.60	0.16	2.80	3.2	41.5

The shape of the curve in Fig. 1 indicates that the interrelation is characterized by the same function common both for vitreous and floury structure constituents of wheat.

Generalized indices of wheat endosperm microhardness may be determined indirectly on the basis of estimations of medium specific gravity of the wheat kernels. Respective values of indexes (in *Vickers* units) may be obtained

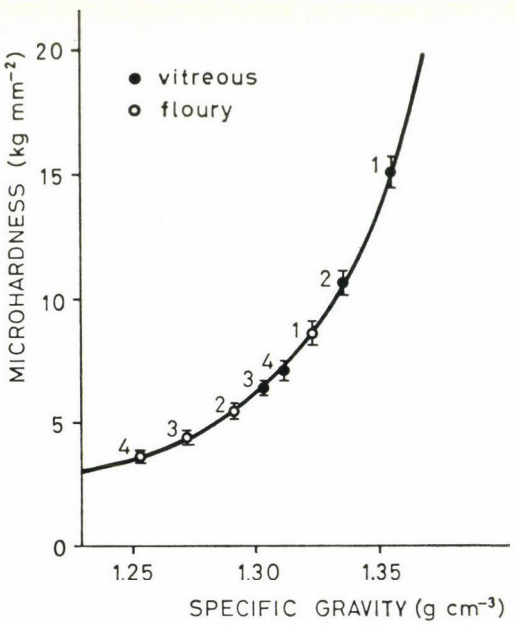

Fig. 1. Correlation between specific gravity of vitreous and floury wheat kernels and endosperm microhardness. 1 — *Canadian Amber Durum;* Polish varieties: 2 — *Kaukaz,* 3 — *Mironowska* 808, 4 — *Grana.* Vertical bars show standard deviation

by the method of interpolation from the curve in Fig. 1 or by calculations with the experimental equation:

$$H_d = 0.00189\ D^{29} + 2.31 \tag{3}$$

where H_d measured in kg mm^{-2} is the generalized endosperm microhardness index of wheat characterized by kernel specific gravity D (in g cm^{-3}).

The differences between the values determined in the two above mentioned ways were found to be within 2 to 14 per cent and are probably resulting from a relatively great error made during the visual determination of grain vitreousness.

Comparison between the index of kernel hardness in a given quantity determined with the *Brabender* hardness tester (WHI in Table 2) and the generalized wheat endosperm microhardness index does not exhibit close correlation especially when the vitreous fraction is low and the bran thick. Although variety *Opolska* has the lowest endosperm microhardness index of all the investigated varieties (H_d = 3.2 kg mm^{-2}), its kernel hardness index in a given quantity is 50 per cent higher than that of *Grana* wheat (H_d = 4.8 kg mm^{-2}). The results of the experiment point at the possibility of errors at classification of wheat into definite groups of hardness on the basis of the indices which have been in use so far.

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ASSESSMENT OF MEAT TENDERNESS ON THE BASIS OF PENETRATION MEASURED IN RAW TISSUE*

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A penetrometer AP 4/1 (VEB FEINMESS, DDR) provided with a 10-needle probe was used to measure a value of penetration in fresh meat. A correlation between the depth of probe penetration into meat and the tenderness score of roasted beef was found to be high and significant. In case of the probe movement perpendicular to muscle fibers the coefficient of the correlation was 0.82, significant at $P < 0.01$. The high value of the correlation coefficient shows that the method can be used as a practical physical test to predict from measurement on fresh meat the tenderness of the cooked meat.

In the paper a detailed description of a measurement procedure is given. Moreover, some experimental results are presented to show an influence of meat pH on the depth of penetration and also some observations to illustrate a phenomenon of cold contraction known to reduce tenderness of quickly chilled meat.

For the average consumer tenderness and colour are the most important attributes of meat quality. The colour can be relatively easily characterized by measurable physical parameters. The tenderness of meat, however, is of a more complicated nature and efforts to correlate sensory judgement with results of physical tests have not yet brought a satisfactory solution. The ideal solution should relate properties of raw meat to tenderness of meat after cooking. Such a test could serve as a means to select beef of guaranteed tenderness at meat factory level. According to SZCZESNIAK and TORGESON (1965) attempts to relate *Warner-Bratzler* shear values on raw meat to those obtained after cooking have resulted in low or in non-significant correlation coefficients. Promising results have been reported by HANSEN (1971) who used the *Armour* tenderometer with the ten-needle probe. He measured the resistance of meat to the penetrating probe and found the correlation coefficients with panel score to be near to 0.8.

1. Experimental

1.1. Measuring penetration

In our experiments the ten-needle probe devised by HANSEN (1971) was used in connection with an automatic penetrometer AP 4/1. The depth of the probe penetration during 5 seconds, under constant weight of 900 p was

* Paper presented at the 3rd Methodological Symposium on Food Analysis, 9–11 October 1975, Szentendre, Hungary.

measured. The meat sample was cut to fit into a holder ($4.5 \times 4.5 \times 12$ cm) in order to prevent deformation of sample.

The holder with meat was placed on the penetrometer table and lifted so that the needles just touched the surface of the meat. At this position the probe was released and after 5 seconds stopped automatically. The depth of needle penetration can be read with an accuracy of ± 0.1 mm.

In the performed experiments samples of beef were cut in two different ways:

— With meat fibers running parallel to the direction of probe movement;

— With fibers running perpendicularly to the direction of probe movement.

For every sample four measurements were made.

1.2. Sensory investigation of tenderness

Sensory tests were performed by a trained panel of 5 to 6 persons. Meat for the sensory test was cut from the same muscles as taken for penetration measurements and roasted at 160 °C till the internal temperature reached 80 °C. Meat was tested cold according to the following scale: 1 — very tough, 2 — tough, 3 — slightly tough, 4 — tender, 5 — very tender.

2. Results

In case of penetration measured in the direction parallel to meat fibers the correlation was found to be low and non-significant. When results of sensory tests were compared with measurements in perpendicular direction a distinct correlation was found with a high coefficient, $r = 0.82$ ($P \leq 0.01$) (Fig. 1).

The regression equation is as follows

$$Y = 0.73 + 0.30\,X$$

where Y stands for mean sensory score (points) and X stands for the depth of penetration (mm). According to this equation, for very tender meat samples, the penetration value should reach 12 mm, for tender meat from 9 to 12 mm and for very tough meat less than 7 mm. Analysis of error shows that the standard error of the mean penetration value is 0.5 mm and the standard error of the mean sensory score is 0.2 points. A confidence interval for tenderness expressed in points of sensory scale but calculated from regression approaches ± 0.7 points. Such precision is sufficient for practical meat selection. It can be, however, upset by two factors — high pH of meat and the phenomenon of cold contraction. As it is shown in Fig. 2 when the pH of the meat exceeds 6.0 the penetration value is decreased and does not follow the calcu-

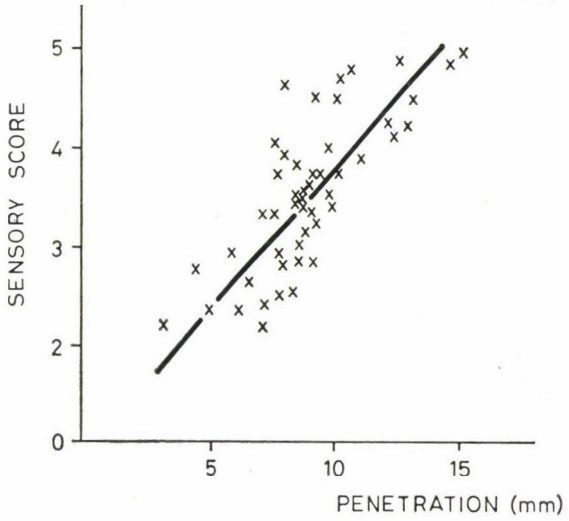

Fig. 1. The correlation between sensory score of tenderness and penetration depth of the ten-needle probe

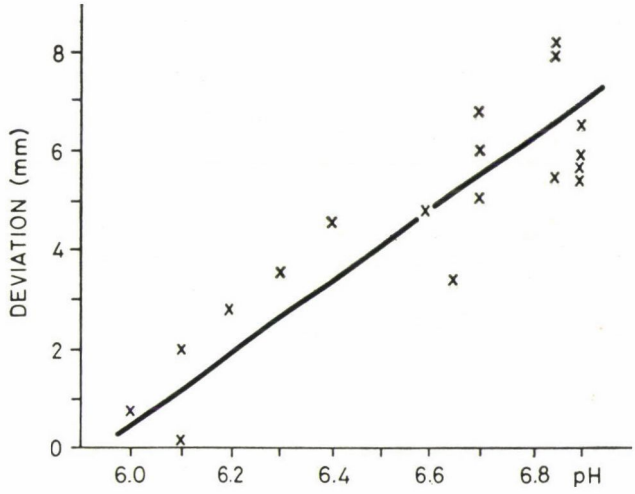

Fig. 2. Deviations of penetration values for meat with pH over 6.0

lated equation of regression. It can be seen (Fig. 2) that a difference between an expected and a measured value is strictly related to the pH of the meat. Hence, an appropriate correction can be calculated by the equation

$$D = 7.2 \ (\mathrm{pH} - 6.0).$$

For instance, in one of our experimental groups the mean pH of meat was extremely high, 6.67. The tenderness of meat with high pH is usually

good and in this case it was scored by a panel at 4.7 points. The penetration value would be expected in the range of 12 to 14 mm, but the observed value reached barely 8.3 mm. The correction

$$D = 7.2\ (6.67 - 6.0) = 4.4\ \text{mm}$$

when added to 8.3 mm, gives the expected value of about 13 mm, which is in good agreement with 4.7 points of sensory score. Meat with pH over 6.0 does not occur often and is undesirable mainly for its low keeping properties. Its higher resistance to the penetrating probe results probably from characteristic sticky consistency of this kind of meat.

Cold contraction is a phenomenon known to occur when meat is quickly chilled (BENDALL, 1973). This additional contraction of muscle fibers induced by low temperature in refrigerators gives rise to undesirable changes of meat tenderness. In beef it develops mainly in the surface layers which are overflown by cooling air. When samples are cut for penetration measurements the results obtained on the side more intensely cooled are always about 30% lower than those obtained for internal parts of the meat. In the course of sensory testing the whole cross-section of muscle is tasted and the panel score represents its average tenderness. If one does not measure penetration always on the same side, it will introduce an additional source of error and lower the precision of tenderness assessment. In order to reduce this kind of error it is advisable to measure penetration on both sides of the meat sample and to correlate its mean value with the sensory score. This sensitivity of penetration to cold contraction offers otherwise a valuable tool for controlling conditions of meat chilling because if the rate of cooling during the first few hours after slaughter is chosen properly the cold contraction and its adverse effect on meat tenderness can be eliminated.

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MICROBIOLOGY OF FOODS PASTEURISED BY IONISING RADIATION*

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The general effects of irradiation pasteurisation (whether for radicidation or radurisation) are, first, to reduce greatly the numbers of all micro-organisms in foods.

Because pathogenic species of bacteria or fungi are not among the more resistant vegetative forms, they tend to be readily and preferentially eliminated; this is the basis of well known proposals for radicidation treatments.

On the whole, irradiation has not been found to increase toxin production by toxigenic organisms. A conceivable small increase in individual cases would be offset by a much larger diminution in the occurrence of the organism.

Noteworthy among the surviving flora are enterococci, yeasts, and spores of *Bacillus* and *Clostridium*. No radiation resistant pathogens have come into prominence. Occasionally there are novel, highly resistant bacteria, which have not been found pathogenic, and are not concerned in spoilage.

The foreseeable risks to public health arise from survival of spores of pathogenic species of *Bacillus* and *Clostridium*, but only in foods in which these spores can develop and so become harmful. The risk seems significant with *Cl. botulinum* type E associated with fish and sea-foods, where storage below 3 °C has consequently been recommended. With other foods, the associated species are common contaminants of normal foods. They are mesophilic, and only become dangerous under warm conditions of storage; hence no restrictions might be thought necessary. The problems here are exactly similar to those with the corresponding cooked foods, where administrative restrictions have been found desirable on packaged fish, but not on other foods.

Except with relatively low doses, irradiation pasteurisation causes foods to spoil in an unusual manner. This is not regarded as serious. The same has long been familiar with cured foods and is now becoming familiar with packaged foods. Unlike traditional putrefaction, the spoilage changes which occur have no known significance for health.

With respect to mutation, so far there is no evidence that, as a result of irradiation, non-pathogenic species acquire pathogenicity; hence, attention can be confined to possible changes in known pathogens.

Similarly, there is still no evidence of significant increases in pathogenicity of pathogens connected with food; irradiation more usually causes decrease. If increases appear, the first sensible step would be to undertake surveillance to discover their significance (as with transferable drug resistance). Concern would be limited to foods which support good growth of the species in question.

If difficulty were to arise as the result of re-cycling, this could readily be prevented by operating the irradiation plant at a temperature low enough to restrict multiplication of the relevant organisms.

Alternatively, control could be effected by storing (after irradiation) at temperatures sufficient to prevent growth.

* This review is based on the Technical Report IFIP-R33 of the International Project in the Field of Food Irradiation and published with the kind permission of the Project Director.

The relevant species tend to be different in different foods. Accordingly, necessary limiting temperatures are likely to be different for different foods, and in any case substantially above 0 °C. There are indications indeed that irradiation may raise the minimum growth temperature.

Repeated irradiation can cause changes sufficient to confuse strain differentiation; so far, they have not sufficed to prevent recognition of general identity *e.g.* in *Salmonella* serotypes.

Possible mutational changes as above would have to be offset against the accompanying large reductions in frequency of occurrence of the organisms caused by irradiation. An increase in radiation resistance, conceivably but not probably arising through re-cycling, might make this reduction smaller than now appears.

Irradiation pasteurisation produces definite and large improvements in the microbiological status of foods and feeds, by eliminating most pathogens and extending storage life. The associated risks seem relatively small and are still not demonstrated, except in the case of fish and sea-foods where storage below 3 °C is recommended. The balance of advantage to public health seems heavily in favour of such a process, as in heat pasteurisation or cooking of food.

CONTENTS

The wide variety of foods consumed by man might be contaminated at some time by almost any kind of micro-organism, and the corresponding diversity of micro-environments in food provides, at some time, environmental conditions that could permit growth of many species. However, the properties

of the food and its habitual treatment favour particular species, which accordingly multiply to form the associations characteristic of spoilage of particular foods under particular conditions of use; these general relations were reviewed by MOSSEL and INGRAM (1955). All methods of preservation depend on inhibition of the characteristic species.

Many traditional methods nevertheless leave a large — though different — surviving microflora: *e.g.* curing (SYMPOSIUM, 1957), smoking (HANDFORD & GIBBS, 1964), or even simply packaging (CAVETT, 1968). Particularly interesting in the present context are non-sterilising heat treatments, cooking or "pasteurisation", which may be used either simply to extend storage life (*e.g.* pasteurised cheese, canned ham and other "semiconserves") or specifically to eliminate relatively heat-sensitive pathogens (*e.g.* tubercle bacilli from milk, or salmonellas from egg pulp or feedstuffs).

The application of pasteurising doses of ionising radiation, in radurisation or radicidation respectively, is directly analogous to these non-sterilising heat treatments. On a broad view, irradiation at substerilising dose levels is simply one of many microbiologically selective methods of food processing, which does not present specially novel or unfamiliar problems.

The important questions are those related to public health (THATCHER, 1963). For example, whether selective changes in the microflora might make known pathogens more likely to occur or bring into prominence unfamiliar pathogens; whether "mutational" (including adaptive) changes might make known pathogens more virulent or more difficult to recognise, and whether new pathogens might arise in this way. (The term "pathogen" here implies, of course, organisms significant in foods, particularly those involved in food poisoning). Finally, there is the possibility that development of resistant strains might render an irradiation process ineffective.

Since the lower micro-organisms are more resistant to radiation than higher forms of life, the latter would be eliminated by radiation treatment at levels relevant to the former; hence problems related to protozoa, nematodes, insects etc. are not considered here. This review does not attempt to harmonise doses quoted as roentgens, r, r.e.p., or rads; for present purposes the differences are insignificant.

1. Changes in the microbial flora of foods caused by non-sterilising ("pasteurising") radiation treatment

The selective action of irradiation obviously depends on the relative resistance of the different species involved, and special interest is attached to those species which are involved in food poisoning. It is therefore advantageous to begin with a brief review of this topic before proceeding to observations on the mixed flora of foods or feeds.

1.1. The relative radiation resistance of micro-organisms important in irradiated foods

Information about the radiation resistance of various micro-organisms was published some time ago by several authors (*e.g.* DUNN *et al.*, 1948; THORNLEY, 1963a). Table 1 attempts to review some of the observations. It can only be a rough guide, because different observers worked with different conditions (*e.g.* types of radiation: not important; or suspending media: important; or degree of anaerobiosis: important but seldom specified). Nevertheless, it serves to indicate the relative resistance of the principal groups of interest.

1.1.1. Radiation resistance of bacteria.

Despite considerable overlapping, *Gram*-negative bacteria, including the common spoilage organisms of many foods *(e.g. Pseudomonas)* and particularly enteric species including pathogens *(e.g. Salmonella, Shigella)*, are generally more sensitive than vegetative *Gram*-positive bacteria, some of which (notably *Streptococcus faecium* — cf. also CHRISTENSEN & KJEMS, 1965) are rather resistant. The spores of *Gram*-positive genera *Bacillus* and *Clostridium* are more resistant still. Rarely, even more highly resistant forms may be encountered, typified by *Micrococcus radiodurans* (ANDERSON *et al.*, 1956).

With regard to food poisoning, most of the *Gram*-negative pathogens, together with *Escherichia coli* and the associated members of the family *Enterobacteriaceae*, have low resistance to radiation; though there are a few strains in the intermediate range.

The same applies to staphylococci (Table 1), where there is a particularly wide scatter in the resistance recorded for *Staph. aureus*. The uniquely high level recorded by QUINN and co-workers (1966) is suspect; it is taken from their Table 1, which quotes values apparently higher than the original data in Fig. 19, and the experiments were carried out under conditions where there were large "tails" on survival/dose curves.

There is also a rather wide spread of resistance among *Salmonella* serotypes, from *S. typhi* to *S. enteritidis*, reaching at the upper end well into the intermediate range of resistance for vegetative bacteria. Unlike the situation in water, or in heating wet foods, the usual enteric indicator organisms are generally less resistant to irradiation than the associated salmonellas; also in dry foods, however, it is known that salmonellas may persist longer than enteric indicators (*e.g.* BROWN & GIBBONS, 1950).

There is also a wide spread of resistance among *Streptococcus* species. The enterococci are unusually resistant, especially the species *Strep. faecium*, which is among the most resistant of vegetative forms. In the frozen state (MATSUYAMA *et al.*, 1964) or the dry state, (CHRISTENSEN, 1964) it is as resistant as the less resistant spore formers.

The latter, except for *Cl. botulinum* type E, belong to species not involved in food poisoning, whether of *Bacillus* or *Clostridium*. On the other hand, some strains of *B. cereus* are among the most resistant *Bacillus* strains. Similarly, *Cl. botulinum* type A includes strains long established as having about the most resistant of all spores, though some food-poisoning strains of *Cl. perfringens* almost equal them (ROBERTS, 1968).

The group of bacterial species responsible for the spoilage of meat, milk and poultry at low temperatures belong to the so-called *Pseudomonas-Achromobacter* association (MOSSEL & INGRAM, 1955); the group *Achromobacter* has however now been renamed *Acinetobacter* or *Moraxella* (see INGRAM & SHEWAN, 1966; THORNLEY, 1967). Whereas the *Pseudomonas* species of this association (*e.g. Ps. geniculata*, Table 1) have very low resistance to radiation, there is a wide spread of resistance in the *Acinetobacter-Moraxella* group (THORNLEY, 1963a; TIWARI & MAXCY, 1972c), so that strains belonging to this group are apt to survive and form a major element in the flora of perishable foods after exposure to moderate radiation doses (INGRAM & THORNLEY, 1959; TIWARI & MAXCY, 1972c). The latter workers (*loc. cit.* 1972c) recorded that whereas most *Acinetobacter* isolates had low resistance, a few *Moraxella* strains were as resistant as enterococci (see Table 1). Among numerous isolates from beef irradiated up to 270 kr, TIWARI & MAXCY (1972c) record D values of the order of 100 kr for the most resistant which were identified as *Moraxella*.

(i) Effect of freezing. To prevent damage to flavour, it is sometimes recommended to irradiate in the frozen state, which increases radiation resistance by a factor of about two (MATSUYAMA *et al.*, 1964; ANELLIS *et al.*, 1973). However, this happens more or less equally with all vegetative bacteria, as Table 2 indicates. Hence the general order of comparative resistance is not changed by freezing before irradiation, though the scale of dosage is perhaps doubled. The same probably applies to drying.

1.1.2. Radiation resistance of moulds and yeasts.

The sensitivity of fungi to ionising radiation has been established by many authors (*e.g.* DUNN *et al.*, 1948; BRIDGES *et al.*, 1956; MALLA *et al.*, 1967; CHOU *et al.*, 1970). In general the radiation resistance of moulds is of the same order as that of vegetative bacteria except for the more sensitive ones. For example, SOMMER (1973) quotes the following doses for 0.01% survival: *Penicillium expansum*, 200; *Mucor*, 250; *Botrytis cinerea*, 300; *Rhizopus*, 350; *Alternaria citri*, 400; *Cladosporium herbarum*, 450; *Alternaria tenuis*, 550 kr. These figures indicate a respective range from 300 to 840 kr for the doses corresponding to the million-fold inactivation which Table 1 represents.

Such figures are only approximate, because dose levels may be modified by relevant circumstances. For example, BUCKLEY and co-workers (1969)

Table 1

Approximate radiation doses (in krad) needed to reduce viable numbers a million-fold for various micro-organisms; (potential) pathogens are italicised

The values are taken from the various references indicated below, sometimes directly, sometimes by calculation *via* D-values, on the basis of the likely populations in the irradiated cultures if no precise information was given. Where two entries appear for the same species and author, they represent the range among several strains examined

1. Bridges and co-workers (1956)
2. Erdman and co-workers (1961a, b)
3. Koh and co-workers (1956)
4. Niven (1958)
5. Pepper and co-workers (1956)
6. Lewis and co-workers (1972)
7. Maxcy and Tiwari (1973)
8. Matsuyama (1972)
9. Roberts (1968)
10. Quinn and co-workers (1966)
11. Tiwari and Maxcy (1972c)

< 50 krad		100—150 krad		200—300 krad		500—750 krad		1000—2000 krad	
Ps. aeruginosa	3	Aer. aerogenes	2	*Bruc. abortus*	7	*Moraxella*	11	*Staph. aureus*	10
geniculata	4	Alc. ammoniogenes	3	*E. coli*	10	*Salm. paratyphi* B	10	Strep. faecium	4
V. parahaemolyticus	8	*Prot. vulgaris*	10	coli II	2	*Strep. faecalis*	2, 10	Bac. coagulans	5
Aer. aerogenes	3	*Coryneb. acne*	3	*Shig. dysenteriae*	10	Lactobacillus (homof.)	4	pumilus	5
Serr. marcescens	3	*Mycob. tuberculosum*	2	*Salm. pullorum*	10	Bac. coagulans	5	subtilis	5
Salm. typhi	3			Lactobacillus (heterof.)	4	Debaryom. kloeckeri	4	stearothermophilus	4
Prot. vulgaris	3			Leuconostoc	4	Sacch. ellipsoideus	1	Clostridium spp	4, 5
E. coli	3			Microb. conglomeratus	3			*Cl. botulinum* E	2
Bacteroid. ovatus	3			thermosphac-				Cand. krusei	1
vulgatus	3			tum	4			parakrusei	1
Staph. albus	3			Bacillus (vegetative)	3				
aureus	4			*Staph. aureus*	2				
				Asp. fischeri	1				
				flavus	1				
				terreus	1				
				versicolor	1				
				Tor. candida	4				

50—100 krad		150—200 krad		300—500 krad		750—1000 krad		2000—3000 krad	
Ps. fluorescens	7	*Ps. aeruginosa*	3	*Salm. cholerae-suis*	10	*Cand. albicans*	1	*Bac. cereus*	6
V. parahaemolyticus	8	Aer. cloacae	2	*enteritidis*	7, 10	*Cryptoc. neoformans*	1	pumilus	5
Acinetobacter sp	11	*E. coli* I	2	*gallinarum*	2	Sacch. cerevisiae	1	*Cl. perfringens*	9
Alc. viscosus	3	*Shig. paradysenteriae*	10	*paratyphi* A	10	fragilis	1	*botulinum* A	2, 4
Leuc. dextranicum	3	*sonnei*	10	*typhi-murium*	7	*Tor. histolytica*	1		
mesenteroides	3	Asp. fumigatus	1	*typhosa*	10				
Staph. aureus	3	oryzae	1	*wichita*	10				
Penic. camembertii	1	Penic. notatum	1	Sarc. lutea	3				
				Micrococcus spp	4				
				Staph. aureus	2, 7				
				Strep. faecalis	4				
				Asp. niger	1			> 3000 krad	
				Candida spp	4				
				Schizos. octosporus	1			Micr. radiodurans	4
				Tor. cremoris	1			roseus	4
				rosei	1			*viruses*	4

observed, with spores of *Rhizopus stolonifer*, that radiation sensitivity is greatest at early stages of germination; while SOMMER and co-workers (1964) reported that doses for 1% survival were in air only about 60% as great as the doses required under anaerobic conditions.

Table 2

Approximate radiation doses (in kr) needed to reduce viable numbers a million-fold, for various bacteria irradiated in frozen media

(data of ANELLIS *et al.*, 1973; potential pathogens are italicised)

<300+	300—600	600—900	1200—1500
Ps. aeruginosa		Alc. faecalis	Strep. faecium 2/4
			Strep. faecalis QM
Aerob. aerogenes 2/3++	Aerob. aerogenes 1/3	*Strep. faecalis* 1539	
E. coli 5/6	*E. coli* 1/6		
Prot. vulgaris			
Salmonella 4/6	*Salmonella* 2/6		
Serr. marcescens			
Staph. aureus 6/6			
		900—1200	>3000
Lactob. arabinosus	Strep. lactis		
Lactob. casei		Strep. faecium 2/4	Micr. radiodurans

+ The lowest dose tested.

++ Number of strains/total number tested.

The yeasts are distinctly more resistant than the moulds, as resistant as the more resistant bacteria, and with especially resistant species such as *Candida krusei* are as resistant as the more sensitive bacterial spores. Because of their resistance also to anti-bacterial antibiotics, such species have especially predominated after combined antibiotic/irradiation treatments (THORNLEY *et al.*, 1960; INGRAM, unpublished observations).

1.1.3. Highly resistant species.

The use of radiation has revealed bacterial species which are unusually resistant; it is noteworthy that, though these must have been present before, they were so inconspicuous as to be unknown. *Micrococcus radiodurans* was the first to be isolated (ANDERSON *et al.*, 1956), from meat; but similar species are apparently widely distributed in the environment. MATSUYAMA (1972) recorded about 10 highly resistant isolates from various natural radioactive sources; MATSUYAMA mentioned however that, on the basis of the ecological findings, there may be no direct correlation between back-

ground radioactivity and the occurrence of these resistant strains in nature. More recently, KRISTENSEN (1974) has examined the flora of three reactor pools, and from each an isolate was obtained which was about as resistant as *M. radiodurans*. It was assumed that these bacteria (*Gram*-positive non-sporing rods) were mutants characterised by their high radiation resistance. But 15 clones isolated from three locations were apparently identical, an extremely improbable occurrence if all were mutants. More probably, the radiation field was simply selecting resistant organisms normally present in water at very low levels.

A number of observations suggest that the remarkable resistance of the above-mentioned vegetative bacteria arises from their capacity to effectively repair DNA damage, including double-strand scissions (MOSELEY & LASER, 1965; KITAYAMA & MATSUYAMA, 1968; MOSELEY, 1968; MATSUYAMA, 1972). Accordingly, the lethal effect of radiation should be enhanced by inhibiting the DNA repair processes; hence sub-lethal heating before or after irradiation, and in certain cases, salting should be effective in reducing the number of survivors of radioresistant vegetative bacteria in foods. All the exceptionally radiation resistant vegetative bacteria are heat sensitive (GOLDBLITH, 1971).

There seem to be no records yet of outstandingly resistant moulds or yeasts corresponding to the highly radiation resistant bacteria.

1.2. Observations on foods

The microbiological safety of irradiated foods has been investigated by many workers, particularly with regard to foods of animal origin. The Report of the Joint FAO/IAEA/WHO Expert Comittee (REPORT, 1964), dealing with the technical basis for legislation on irradiated food, warned that "the nature of the surviving organisms and the likelihood of their subsequent activity need consideration, especially for those species likely to be of concern in public health" (p. 32). On wet foods of near-neutral reaction (*e.g.* eggs, fish, meat, milk), bacteria usually develop. On foods which are acid (*e.g.* fruits) or partly dry (*e.g.* cereal products), moulds tend to predominate. The two have usually been considered separately.

1.2.1. Foods spoiled by bacteria.

These foods include in particular the valuable and perishable foods such as meats and sea-foods, which are liable to present problems of public health. Work on meats has been reviewed by NIVEN (1958); and on fish and sea-foods by COLEBY and SHEWAN (1965) and by SHEWAN (1971).

(i) Spoilage. Where these foods are kept in air under cool conditions below about 15 °C, the important spoilage bacteria are psychrotrophic species of *Pseudomonas, Aeromonas, Vibrio, Achromobacter (i.e. Acinetobacter* or *Moraxella)*, some cold-tolerant *Enterobacteriaceae* and *Micrococcus*. The *Gram*-negative

species are greatly reduced in number even by doses of the order of 100 kr but are not usually completely eliminated. Accordingly, in air-permeable packages, spoilage follows a fairly normal course with a delay corresponding to the diminution in number. For example, the storage life of iced fish can be more than doubled if a radiation dose of about 100 kr is applied soon after catch, while the microbial count is still low, but fish so irradiated produces essentially the same spoilage odour as unirradiated fish, although delayed (MEYER, 1971; DIEHL, 1973).

Greater dosage eliminates the normal spoilage flora, with a greater delay in spoilage which then however takes atypical, and usually less obnoxious forms where unusual bacteria predominate (COLEBY & SHEWAN, 1965; INGRAM & ROBERTS, 1966). Since the more resistant bacteria are commonly less active metabolically than the typical spoilage species (ALUR *et al.*, 1971; TIWARI & MAXCY, 1972b), the abnormal spoilage may be produced only when the numbers of bacteria reach unusually high levels. This however has no particular significance when there are no associated pathogens.

The surviving bacterial flora is predominantly *Moraxella-Acinetobacter*, *Micrococcus*, or lactic acid bacteria including enterococci (as might be expected from observations on pure cultures, such as those presented in Tables 1 and 2). The same range of types is found in large measure in irradiated seafoods, poultry and red meat. The importance of the *Moraxella-Acinetobacter* group has been demonstrated, for example, in irradiated poultry (INGRAM & THORNLEY, 1959; IDZIAK & INCZE, 1968), red meat (TIWARI & MAXCY, 1972c), fish (LEWIS *et al.*, 1972), and oysters (LIUZZO *et al.*, 1967; these workers used out-of-date terminology — see THORNLEY, 1967).

When fish was vacuum-packed, however, *Lactobacillus* sp. came to predominate after post-irradiation storage (PELROY & EKLUND, 1966). Other workers have observed micrococci, or coryneforms (MASUROVSKY *et al.*, 1963; MAVINKURVE *et al.*, 1967). Similar favouring of the *Gram*-positive elements by packaging might be expected with meats: WOLIN and co-workers (1957) noted a predominance of *Microbacterium thermosphactum* in packaged irradiated beef (ground?); and DRAKE and co-workers (1960) recorded a flora of *Lactobacillus plantarum* in canned hams irradiated at 210 kr. In these cases, spoilage took the form of souring. Because enterococci are the most frequent cause of spoilage in pasteurised canned ham and because they are relatively resistant to radiation, DRAKE and co-workers (1960) concluded that no tolerable radiation treatment was likely to improve the stability of that foodstuff.

A Japanese study on the changes in the microflora of Vienna sausage after gamma irradiation revealed that the main species in aged smoked sausage were *Micrococcus*, *Acinetobacter*, *Lactobacillus*, *Serratia* and yeasts, while *Acinetobacter* was predominantly found after 200 kr (MATSUYAMA,

1972). The appropriate dosage for radurisation appears to lie between 300 and 500 kr for Vienna sausage.

Yeasts are on the whole more resistant than non-sporing bacteria, so that their relative importance is increased by irradiation (especially with intermediate doses). Hence, they may be a major element in the postirradiation flora of foods on which they are initially fairly numerous (*e.g.* frankfurters — DRAKE *et al.*, 1958; poultry — THORNLEY *et al.*, 1960).

There are several reports of highly resistant bacteria having been isolated from irradiated foods. The first was *Micrococcus radiodurans* (ANDERSON *et al.*, 1956). COLEBY and co-workers (1961) also detected resistant organisms in meat and noted the resistance of enterococci; CHRISTENSEN & KJEMS (1965) found that some of the survivors from irradiated *Strep. faecium* cells were as resistant as *M. radiodurans*. *Micrococcus sp.* RBD (LEWIS, 1971) were isolated from meat and fish. Japanese workers reported that from the microflora of rice grain irradiated at up to 1.36 Mr, strains of a resistant "red *Pseudomonas*" were isolated (IIZUKA & ITO, 1968; ITO *et al.*, 1969); after further study they were named *Pseudomonas radiora* (ITO *et al.*, 1971, 1972). These species, typified by *Micrococcus radiodurans*, are non-pathogenic, biochemically modest, slow growing and do not take part in spoilage (DAVIS *et al.*, 1963; KRABBENHOFT *et al.*, 1965; LEWIS, 1971). The radiation-resistant micrococci all grow poorly or not at all at temperatures as low as 10 °C (LEWIS *et al.*, 1972). MATSUYAMA (1972) has pointed out that the presence of perhaps as little as 2% NaCl or mild heating, would entirely prevent the occurrence of such bacteria. Consequently, though they may be expected to occur in irradiated foods, their numbers should remain small and they do not seem likely to be important.

(ii) Pathogens. Most food poisoning pathogens are sensitive to radiation. Taking a relatively resistant *Salmonella* (*e.g. S. enteritidis* — Table 1) as about the most resistant of vegetative pathogens (except enterococci whose involvement in food poisoning is uncertain), a dose of 200 kr would reduce their numbers by about 100-fold or more, depending on circumstances. The elimination of *Salmonella* by this kind of treatment (radicidation) is now well-known in principle (PANEL PROCEEDINGS, 1963). Such a treatment would be expected to reduce to correspondingly greater degrees more sensitive pathogens such as *Staph. aureus*, other *Salmonella* serotypes, *Shigella*, *E. coli*, *Brucella* and *Vibrio* species, and with the more sensitive species the expected degrees of inactivation are very high, even with such a fairly low dose.

There are some experiments which appear to demonstrate a better growth of pathogens in irradiated foods because the normally competing saprophytic flora has been suppressed. (This phenomenon explains why staphylococcal food poisoning is much more frequent from ham sold cooked than sold raw.) EDDY and INGRAM (1962) demonstrated inhibition of *Staph.*

aureus by the indigenous flora of vacuum-packed bacon (micrococci and lactobacilli), whereas in packs where the indigenous flora had been killed by irradiation, a *Staph. aureus* inoculum multiplied rapidly. Similar observations were made by MATCHES and LISTON (1968), when they inoculated cells of *Salmonella* into control and irradiated sea-foods. However, such experiments are unrealistic in adding many unirradiated cells of the pathogen to an already irradiated food. They ignore the fact that, in practice, the cells of the pathogen would be few initially, would be irradiated in the food, and suffer diminution in number and loss of competing power like the saprophytic flora. SLABYJ and co-workers (1965) took the argument a step further, by showing that *Staph. aureus* ceased to be inhibited by the spoilage flora of crabmeat after irradiation at 100 or 200 kr, even when the staphylococci were added before irradiation. However, they added very large numbers of staphylococci so as to produce quite unrealistic ratios of staphylococci to saprophytes before and after irradiation, and their data show an increasing inhibition when this ratio was brought down to less high (but still unrealistic) levels. As these authors point out, a dose of 200 kr would in practice be expected to eliminate staphylococci entirely from crabmeat.

So far as the writers are aware, none of the recognised non-sporing food-poisoning bacteria has ever been isolated from a normal food or feed irradiated at this sort of dose level, which plainly produces an enormous general improvement in public health status (cf. MAXCY & TIWARI, 1973). The problems are those of the survival of more resistant species.

The classical strains of *Moraxella* were isolated from human sources, and some have been associated with pathogenic conditions other than food-poisoning. Hence, the suggestion arose that "enrichment" of *Moraxella*s by irradiation increased the risk to human health; a suggestion considered and rejected by IDZIAK and INCZE (1968). Detailed study of this group by THORNLEY (1967) showed that the isolates from humans (all in her Phenon 2) are taxonomically different from many isolates from poultry (in Phenons 3 and 4). The precise taxonomic position and significance of the especially resistant *Moraxella* strains identified by TIWARI and MAXCY (1972b) have not yet been established.

The enterococci are probable survivors, and there are suggestions that they may have been implicated in food poisoning. *Strep. faecium* is more resistant than *Strep. faecalis*, the latter being the species to which the suspect strains belong. However, enterococci occur in rather large numbers in various foods without known ill-effect (*e.g.* cheese; packaged bacon). In particular, they survive thermal treatments and hence used to cause extensive spoilage of pasteurised canned hams without causing any illness (cf. SYMPOSIUM, 1955). Hence it is not customary in the West to regard enterococci as of public health significance, save in particular circumstances where they may be

"indicators" of faecal pollution or of unsanitary practice (*e.g.* THATCHER & CLARK, 1968). In Eastern Europe the enterococci appear to be regarded more strictly, and there might be a greater tendency to require storage under cool conditions for the relevant irradiated foods (as required for the corresponding heat-pasteurised canned ham, but to control spoilage). However, because of the ability of enterococci to grow at 0°, even such precautions would be effective only for a limited period, short of freezing.

Since yeasts play a relatively important part in the flora of irradiated poultry (THORNLEY *et al.* 1960), and since *Candida albicans* is among the more resistant yeasts (see Table 1), it was feared that low-dose irradiation might favour the appearance of this pathogen on poultry carcasses, as treatment with antibiotics favours *Candida parapsilosis* (NJOKU-OBI *et al.*, 1957). Observations (unpublished) made by the first writer (M.I.) failed, however, to detect *C. albicans* on poultry carcasses, treated with radiation alone or radiation plus chlortetracycline, *C. lipolytica* being the predominant yeast species in each case.

The significant problems arise from the likely survival of spores of pathogenic bacteria in foods where they can grow. These spores, too, are reduced in number by irradiation, *e.g.* a dose of 200 kr would reduce the relatively sensitive spores of *Cl. botulinum* type E by 10 to 100-fold or of the resistant type A by 3 to 10-fold. Nevertheless, given the natural frequency of occurrence of these spores, such reductions would still leave sufficient survivors to cause risk. The problem is most serious with *Cl. botulinum* type E, because it grows relatively quickly under cool conditions. As type E is virtually confined to fish and sea-foods, these are the foods of particular concern. The general nature of the problem was aptly illustrated by outbreaks of botulism in the U.S.A. in 1963 caused by hot smoked fish, which became toxic without undergoing a recognised form of spoilage. With ordinary fish at normal temperatures, though the eventual development of *Cl. botulinum* type E is possible or even likely, danger is avoided because the fish spoils first and is therefore not eaten. Packaging alone raises similar problems: when ABRAHAMSSON and co-workers (1965) studied toxin production by *Cl. botulinum* type E in fresh herring stored at 20 °C for 48 hr, they found that toxin developed more rapidly in vacuum-packed than in air-packed or open-packed fish; KAUTTER (1964) showed that vacuum packaging delayed normal surface spoilage of white fish at 10 °C to such an extent that *Cl. botulinum* type E cells multiplied and developed toxin without the product appearing spoiled.

Similarly the effect of irradiation, which has much more effect on the spoilage organisms than on spores (cf. Table 1) is, by delaying spoilage, to reduce the time interval and margin of safety between spoilage and toxicity. With a radiation dose high enough to destroy the normal flora and prevent

the usual signs of spoilage, the margin of safety disappears, as in the case of smoked or packaged fish. Numerous and extensive experiments with irradiated fish (PANEL PROCEEDINGS, 1970) lead to the following conclusions:

(i) It is necessary to avoid doses in the approximate range of 300 to 2000 kr, which suffice to suppress normal spoilage and greatly extend organoleptic life without certainly eliminating *Cl. botulinum* type E (EKLUND & POYSKY, 1967).

(ii) Toxin formation occurs the more readily as the temperature is raised above 5 °C (*e.g.* HOBBS, 1966).

(iii) The margin between spoilage and toxin formation is less, the greater the number of spores inoculated. Usually these numbers have been abnormally high, giving an exaggerated impression of the risk.

(iv) The danger is avoided, of course, if the irradiated food is held at temperatures at which the surviving spores cannot develop and give rise to toxin. Growth of *Cl. botulinum* type E has been recorded down to 3.3 °C by SCHMIDT and co-workers (1961), but other workers (including SCHMIDT *et al.* themselves, — pers. comm. C. F. SCHMIDT) have not obtained growth again at so low a temperature, and it is rare to observe toxin production below 5 °C. Hence storage at 3 °C is regarded as sufficient to provide an adequate margin of safety.

With radiation-pasteurised meat and poultry, the corresponding problem would be the survival of the spores of *Cl. perfringens*, or (rarely) *Cl. botulinum* types A or B. These organisms of course occur at present in the unirradiated foods, and the question is whether irradiation would make their development more likely. It seems unlikely that problems would arise at temperatures below 20–25 °C because these species grow relatively slowly at such temperatures. Spoilage before danger is therefore to be expected unless the doses used were so high as to eliminate most other species, for example enterococci. No experimental evidence of risk has been published. There is a corresponding problem with cooked foods, but only when the foods are kept warm.

So far, little attention has been paid to the survival of *Bacillus* spores, which are usually more numerous than those of clostridia. The spores of *Bacillus cereus* are relatively resistant and some strains of this species cause food poisoning though not severe. It is not known whether the pathogenic strains are equally radiation resistant. Similar questions may arise with *B. licheniformis*. These organisms resemble the mesophilic clostridia in requiring warm conditions for extensive multiplication before they become hazardous.

If irradiation is combined with heating, only the most resistant mesophilic spores are likely to remain, the radiation-resistant vegetative types being heat sensitive, as already noted. The combination of blanching (80 °C,

4 min) and irradiation (250 kr), with tropical shrimps resulted in the predominance of *Bacillus* (84%), as against *Lactobacillus* (24%), *Aeromonas* (13%) and *Achromobacter* (14%) in shrimps subjected to blanching treatment only (KAMAT & KUMTA, 1974). Moreover, there are synergistic actions between heat and radiation, which result in degrees of inactivation much larger than the sum of the two treatments individually *(e.g.* GRECZ *et al.*, 1966; HANSEN, 1969).

1.2.2. Foods spoiled by moulds.

Corresponding to the sensitivity to radiation of most fungi, even relatively low doses of the order of 100 kr suffice to produce significant reductions of the flora and delays in spoilage where moulds are the cause.

(i) Spoilage. Thus, radiation doses of 50 to 200 kr reduce mould spoilage of various fruits, extending their shelf-life by several days (DENNISON & AHMED, 1966; DHARKAR & SREENIVASAN, 1966; SOMMER & FORTLAGE, 1966; SOMMER & MAXIE, 1966; ECKERT & SOMMER, 1967; BARKAI-GOLAN *et al.*, 1968; FARKAS *et al.*, 1972).

The same applies to mould spoilage of bread (STEHLIK, 1967) or cereals (WATANABE *et al.*, 1971). With rye bread irradiated at doses from 50 to 500 kr, a retardation of moulding was observed by STEHLIK (1967). By applying 50 kr to flour and subsequently to bread, HARTUNG and co-workers (1973) reduced the visible and total mould which developed up to 20 weeks (this treatment was applied to extend the storage life of fresh bread used on manned psace flights). MILNER (1957) reported that it was possible to control growth of fungi in stored wheat which had been exposed to gamma ray doses above 250 kr. WEBB and co-workers (1959) have reported that the dose of irradiation required to inhibit or delay mould growth on ground corn increased as the moisture content of corn increased.

For several fungi (as for bacteria), mild heating has been found to sensitise them to radiation with a high degree of synergism (SOMMER *et al.*, 1967; PADWAL-DESAI, 1974). This was observed despite the fact that, at the stage of maximum resistance to radiation, the fungi were most sensitive to heat (50 °C for 5 min) (PADWAL-DESAI, 1974). Hence it is now recognised that a promising means of increasing the effectiveness of low-dose irradiation (25–150 kr) for controlling fungal spoilage of fruits, without affecting normal quality attributes, is to combine irradiation with mild heating *e.g.* 50 °C for 5 min (MAXIE & ABDEL-KADER, 1966; SOMMER *et al.*, 1967; 1968; AHMED *et al.*, 1969; BARKAI-GOLAN *et al.*, 1969; BEN-ARIE & BARKAI-GOLAN, 1969; PADWAL-DESAI *et al.*, 1973; PADWAL-DESAI, 1974). Similarly, chapaties and bread slices, packed in polycell pouches, and subjected to 50 kr followed by 35 min at 65°, were free from mould and shelf-stable up to 100 weeks at 28° to 32 °C (PADWAL-DESAI *et al.*, 1973; PADWAL-DESAI, 1974).

(ii) Toxin formation. Special interest is attached to certain moulds, in genera often associated with foods, which produce toxic metabolites (mycotoxins) when they grow on foods, or feeds (MARTH, 1972). Most important are the carcinogenic aflatoxins, produced by certain strains of *Aspergillus flavus* and *A. parasiticus* (generally more potent than *A. flavus*).

It was reported by BRIDGES and co-workers (1956) that spores of *Aspergillus flavus* were inactivated by an ionising radiation dose of 300 kr; this agrees with the findings of ZIRKLE and co-workers (1952) who used γ- and X-rays to inhibit the growth of air-dried *Aspergillus* spores. WEBB and co-workers (1959) observed that *A. flavus* strains were destroyed by a dose of 350 kr in ground corn samples containing 12.5–23% moisture, but JEMMALI & GUILBOT (1969b) noted that mycelial development of *A. flavus* and subsequent aflatoxin production could occur following irradiation with 500 kr (contrast Table 1). MÜNZNER and DIEHL (1969) and FRANK and co-workers (1971) compared the susceptibility of toxigenic and apparently non-toxigenic strains of *A. flavus* to repeated sublethal doses of irradiation. No difference in radiation resistance between toxigenic and the non-toxigenic strains was detected.

The relation between physiological state and radiation responses was observed by PADWAL-DESAI (1974) with *Aspergillus flavus* (toxigenic) and *A. flavus oryzae* (non-toxigenic). Conidia were more sensitive than mycelia. Conidia of a toxigenic strain isolated from groundnuts and later confirmed as *A. flavus* ATCC 15517, showed exponential survival curves both in saline ($D_{10} \sim 30$ kr) and potato-dextrose broth ($D_{10} \sim 40$ kr) when irradiated at 25 °C with γ-rays. Germinating conidia were most sensitive. There is evidence that wet spores of *Aspergillus terreus* are more sensitive to γ-rays than dry spores (JEMMALI & GUILBOT, 1969a). SCOTT and co-workers (1971) demonstrated a germination inhibitor of spores of *Aspergillus nidulans;* dilution or removal of this substance may cause "accelerated" germination in culture, giving irradiated cells less time to repair damage so that fewer cells survive.

With *Aspergillus flavus*, too, the doses of radiation required for inactivation can be much diminished by combination with heating. 30 min at 65° plus only 50 kr was found to inactivate toxigenic *A. flavus;* preinoculated packs of groundnuts (approximately 10^5 conidia into each 150 g pack) showed no proliferation of fungi after that combination treatment (PADWAL-DESAI, 1974).

The selective effect of radiation treatment might indeed cause a favourable change in composition of the microflora of food in some cases. HARTUNG and co-workers (1973) reported that irradiation (50 kr) of flour reduced the total number of viable moulds by approximately half. These moulds were grouped as *Aspergillus*, *Penicillium* and unclassified in the non-irradiated flour, the distribution percentages were 85% *Aspergillus* plus *Penicillium* and

15% unclassified, and in the irradiated flour, 45% and 55% resp. Similar changes were observed in the irradiation treatment of white bread, and of raisin bread. The authors considered these observations noteworthy, since the mould genera *Aspergillus* and *Penicillium* are specially significant for the production of toxic and carcinogenic metabolites.

The above observations suggest that *Aspergillus flavus* is among the more sensitive moulds. It is therefore the less likely to be among the survivors of a radiation treatment aimed at controlling moulds, unless the folly were committed of irradiating material already heavily contaminated with that species. This would be doubly dangerous because aflatoxin might already have been formed and inactivation of preformed aflatoxin seems practically impossible by irradiation (KILLEBREW *et al.*, 1968). MIYAKI and co-workers (1967) reported that aflatoxins were resistant to radiation; doses as high as 30 000 kr, and 18 000 kr degrade only about 10% of aflatoxin in the dry state (AIBARA & MIYAKI, 1969). Moreover, attempts to reduce or eliminate the toxicity of aflatoxin-containing foods and feeds by heating were also reported to be largely ineffective (BLOUNT, 1961). Proposals for radiation treatments exclude the use of material which is already mouldy.

Aspergillus flavus does not grow at temperatures below 10°, and does not produce much toxin below 15 °C, even when not irradiated; neither does it grow on grain which is drier than about 83% relative humidity, the limit depending on temperature (DAVIS & DIENER, 1968). Obviously, therefore, no aflatoxin problem will arise if irradiated grain is stored under cool, dry conditions. The question whether the limiting conditions are less strict for the irradiated fungus does not seem to have been investigated. Similar information is needed about other toxigenic species.

1.3. Discussion of selective effects

The application of a given dose of radiation produces the same microbiological effect whatever the intention. Consequently "radicidation", besides destroying pathogens will also extend storage life; while "radurisation" to extend storage life likewise simultaneously diminishes risk from pathogens. It is therefore convenient to treat the two together under the general term "pasteurisation".

The problems of the surviving micro-flora are clearly different according to the nature of the food and its associated species. Foods too dry to permit growth of micro-organisms, or frozen foods, would present no problems; they would simply carry fewer micro-organisms, of all kinds, after irradiation than before.

Similarly, partially dry or acid foods which support moulds but not bacteria, will not give rise to any problems related to bacteria, whether of food poisoning or spoilage. If they are foods which do not normally support

hazardous species of fungi, there is no reason to expect that these will appear after irradiation, for these fungi are not specially resistant to irradiation, and, as far as is known, the food is not rendered more likely to support them by an irradiation treatment. Thus, no public health problems can be foreseen with the majority of fruits, for example.

The semi-dry grains or pulses, which are normally liable to support toxigenic fungi if mishandled, may present the same problem after irradiation but the indications are that irradiation would diminish it. All fungi are greatly reduced in number. The non-toxigenic types appear to be relatively favoured by irradiation. Toxin-production by the toxigenic species is generally diminished, despite a few reports to the contrary. The possibility of growth and toxin production is avoided if the drystuff is kept under storage conditions which prevent mould growth, as is at least equally necessary for the unirradiated drystuff if risk is to be avoided.

The wet, perishable, high-protein foods, which normally support bacteria, are the ones which raise bacteriological problems when irradiated. Because the vegetative food poisoning bacteria are relatively sensitive, irradiation causes large or very large reductions in their numbers, so that they are not found in irradiated normal foods which, in this respect, are much improved by irradiation. Among the more radiation-resistant vegetative organisms, none have yet been found associated with a pathogenic risk in irradiated foods.

The significant bacteriological problems arise from the resistant spores of a few food poisoning species, notably *Cl. botulinum*, *Cl. perfringens*, *Bacillus cereus* (and perhaps *B. licheniformis*). *Cl. botulinum* type E is especially troublesome, despite its relatively sensitive spores, because it grows well at normal temperatures; but it is fortunately restricted to fish and seafoods. The corresponding problems with vacuum packaged and smoked fish have been signalled by outbreaks of food poisoning. which led to restrictions on those foodstuffs; and it seems clear that restrictions would similarly be desirable with irradiated fish and seafoods. The simplest would be a requirement to hold below 3 °C, which prevents growth of *Cl. botulinum* type E. An IAEA panel (PANEL PROCEEDINGS, 1970) concluded: "Radiation of fresh fishery products is a feasible process if the final products are stored below 3 °C, achieving a two or three times increase in shelf-life with no known health hazard."

Problems of spore survival, exactly analogous in principle, arise with pasteurised foods other than fish whether packaged (INGRAM, 1964) or irradiated; but they are less serious where spores of the mesophilic species are concerned because these require relatively high temperatures to multiply freely. Thus, cooked meat is handled without temperature restrictions in many countries, despite the well-known tendency if it is kept warm to cause outbreaks of

food poisoning from *Cl. perfringens*, with the implication of risk from *Cl. botulinum* also. The risk from irradiated meat would be similar, although conceivably less, because spores of *Cl. perfringens* tend to require heat activation before they will germinate (ROBERTS, 1968). Similar considerations probably apply to the mesophilic *Bacillus cereus*. The purist might argue that, to avoid any risk, all irradiated meats, for example, should be stored at temperatures at which *Cl. perfringens* or *Cl. botulinum* cannot grow: about 10 °C according to MICHENER and ELLIOTT (1964). But higher temperatures clearly suffice to reduce the risk to proportions which are generally tolerable, with the exactly similar hazard from cooked meats, hence it may be suggested that no special temperature restrictions might be necessary for irradiated meats, at least in temperate climates. The Food Protection Comittee in the U.S.A. remarked that the spores of *Cl. perfringens* and *Cl. botulinum* are so common in soil and dust that they may be expected to occur in normal foods (REPORT, 1964, p. 34).

These effects of irradiation-pasteurisation of food on the associated microbiological risks to public health may be summarised as follows. It produces obvious and large improvements as regards the various vegetative species significant in foods. It raises familiar problems through the survival of spores: in the case of fish and seafoods sufficient to justify limitations in processing and storage temperature; in the case of meats apparently not so, at least in temperate climates. There is a balance of advantage to be evaluated here; in many applications (*e.g.* radicidation of meats) the balance seems to be in favour of using ionising radiation to combat non-sporing species.

Clearly, the foregoing generalisations might be invalidated if a food contained unusually large numbers of some pathogen initially, thereby making its survival in greater numbers more likely. Irradiation processes are based on assumptions of normal hygienic quality in the foods treated. In this respect they are no different from other food processes: it is well understood by manufacturers, for example, that packaging will not restore spoiled food, or that safe canning procedures are impossible with unusually contaminated raw materials.

The likelihood that spoilage will take unusual forms has been understood for some time. If, for example, irradiation causes lactic acid bacteria to replace a normally *Gram*-negative flora, spoilage will take the form of souring rather than "putrefaction"; and, since souring is less objectionable than putrefaction, a larger chemical change with more bacteria may have to develop before spoilage is recognised. Exactly similar situations arise with cured or packaged foods, which often carry very large numbers of bacteria without being regarded as spoiled (cf. INGRAM, 1962; CAVETT, 1968). With irradiated foods, despite extensive scrutiny of the important cases, there is still no indication that the surviving flora is of significant public health

interest, nor that it constitutes a spoilage problem of unusual economic significance. While organoleptic quality may be a criterion of acceptability from a commercial standpoint, it is not necessarily so from the public health point of view; and there is no reason to expect that new criteria of organoleptic quality would be unacceptable to consumers, especially if it were understood that they are associated with a diminished risk to public health.

In these effects of irradiation on the bacterial flora of foods, there is a noteworthy parallelism with the effects of heating, despite occasional differences in detail. For example, as was pointed out some time ago (INGRAM & ROBERTS, 1966), lower doses may be used for acid or cured foods in appertisation by radiation as in appertisation by heat, and for similar reasons. The same parallelism exists for lower dose pasteurising treatments, because the ranking of the important organisms (fungi as well as bacteria) in order of resistance to radiation is similar to that for resistance to heat, although the range of resistance is much less for radiation (about 100-fold) than for heat (many million-fold) (ECKERT & SOMMER, 1967; INGRAM, 1971; PADWAL-DESAI, 1974). Micro-organisms may be listed in order of increasing resistance to either heat or irradiation as follows: the *Gram*-negative bacteria are on the whole most sensitive, especially the psychrotrophs important in spoilage; salmonellas and staphylococci are intermediate in resistance, together with some yeasts and mould; a few *Gram*-positive species, notably enterococci, are more resistant; finally there are the spores, with those of *Cl. botulinum* type E being about the least resistant and those of type A about the most resistant. The survivors of pasteurising radiation treatments, whether aimed at radicidation or radurisation, are therefore likely to be similar to the survivors of heat pasteurisation, or of cooking, and to present generally similar problems.

With regard to pathogens, an important case where irradiation differs from heating is for viruses. The viruses are among the most radiation resistant pathogens, so that radiation is not likely to be useful to "clean up" a virus-infected food. On the other hand, irradiation produces some inactivation of viruses while controlling bacteria, and has not been found to make a virus any more likely to grow on the food after irradiation than before (*e.g.* BALDELLI, 1966). In this respect irradiation is similar to refrigeration.

The problem of the changes in composition of the microflora of radiation-pasteurised perishable foods, although each individual case should be taken into thorough consideration, is not a unique problem, but rather one common to all food treatments not leading to a sterile product. Therefore the problem is not held by food microbiologists and technologists to be so dangerous and unsolvable as to justify rejection of the merits of radiation-pasteurisation. Recent developments have strengthened the attitude that there is no "absolute safety". A margin of risk remains even when the most sophisticated experi-

ments have not given evidence of danger. While the absolute safety of food irradiation, just as that of any food preservation process, cannot be established, the probability of risk, from the microbiological viewpoint, seems low enough compared to the envisaged benefits.

2. The significance of "mutations"

Ionising radiations are well known as mutagenic agents (TATUM, 1946). This property could conceivably be significant in food irradiation: mutations increasing radiation resistance (RR) would make micro-organisms more difficult to destroy; pathogens might be increased in virulence or toxin production; their characters might be changed so that they become difficult to recognise.

The changes brought about by a single irradiation treatment are trivial, as a rule, especially at modest doses which are likely to leave appreciable numbers of surviving organisms. Where changes are produced, they are commonly restored in the first few cell divisions following irradiation, so that the eventual population is normal. An exception appears in the production of toxins, where a single irradiation sometimes does produce significant change.

Usually, noteworthy changes are brought about only by repeated irradiation and, when coupled with intermediate or concomitant periods of growth, develop as "adaptations" to the treatment, the most obvious being an increased resistance to irradiation. Increases in pathogenicity, or changes making cells difficult to identify, are not likely to become important unless a few mutant cells became greatly enriched by coupling with increased radiation resistance, or superior growth rate, or by both.

For these reasons, this report considers first the effects of single irradiation, mainly on toxin formation, and then the effect of repeated cycles of irradiation plus growth on radiation resistance, diagnostic characters, growth and pathogenicity.

2.1. One-irradiation treatments

The literature on single irradiation treatments is very extensive and cannot be summarised adequately. It is only possible to pick out a few items of particular interest.

With rare exceptions, the changes produced by a single sub-lethal irradiation are temporary, and are restored in the first few cell divisions after irradiation. Hence the eventual population is virtually normal, possessing the characters of the parent culture. However, a large proportion of the irradiated cells may be "damaged" and not survive the first few cell-divisions if ecological conditions are unfavourable. Heated cells behave similarly.

2.1.1. Virulence of food-poisoning pathogens.

No reports have been found to indicate increased virulence as a result of irradiation. On the other hand, there are reports from the medical field of loss of virulence (*e.g.* LESCHKOVICH, 1958). Similarly, TIWARI and MAXCY (1972a) noted a diminished infectivity and lethality to chick embryos of *Salmonella typhimurium* irradiated only once with 68 kr of ^{60}Co γ-rays.

2.1.2. Reduced vitality of organisms surviving irradiation.

Because of its ability to produce deleterious genetic and/or non-genetic changes in the exposed organisms, ionising radiation in general results in populations with reduced fitness. Many investigations have shown that microbial populations surviving irradiation became more exacting in their environmental requirements, and more sensitive towards anti-microbial agents (KRABBENHOFT *et al.*, 1964; FARKAS, 1970; ROBERTS, 1970). ROBERTS and co-workers (1965) found that γ-radiation rendered the spores of *Cl. sporogenes* and *Cl. oedematiens* type C more sensitive to post-irradiation inhibition by sodium chloride. Similarly, FARKAS and co-workers (1967) found that, after doses of 400 kr, irradiated spores of *Bacillus cereus*, *B. pumilus* and *B. coagulans* were recovered much less well, by factors up to ten-fold, if the pH of the recovery medium was removed up to 1 unit from the optimum.

2.1.3. Diagnostic media.

Such "damaged" cells cannot be exposed to unfavourable media, such as selective media often are, without prior resuscitation in favourable media, after which they may be expected to behave normally. This point is well known, and also in relation to dried or heated foods (TECHNICAL REPORT, 1970). With irradiated cells, the relations are apparently similar (*e.g.* CORRY *et al.*, 1969; LICCIARDELLO *et al.*, 1970; TIWARI & MAXCY, 1972a). This is doubtless the reason, in general terms, why the highest counts are obtained on rich culture media when making viable counts on irradiated foods (FREEMAN & BRIDGES, 1960).

2.1.4. Antibiotic resistance.

Resistance to antibiotics may sometimes be increased by a single irradiation. With 4 *Salmonella* serotypes, PREVITE and co-workers (1971) found that the relative frequency of resistance to a particular level of tetracycline or streptomycin was increased up to 77-fold by irradiation. The effect was diminished with tetracycline and was about the same with streptomycin for increasing doses from 50 to 500 kr. However, as the authors noted, because of the greatly increased lethality, doses in the higher end of this range produce a large decrease in the absolute numbers of resistant cells: for example a population of 10^9 cells of *S. thompson*, containing 81 000 streptomycin-resistant individuals before irradiation, would be reduced to 10^3 cells containing only 6 resistant cells after irradiation with 500 kr.

2.1.5. Clostridium botulinum toxin.

Much has been published on the control of *Cl. botulinum* by irradiation and, occasionally, the reports contain information about toxin production. From such reports, it appears that the effect of sub-lethal irradiation may be either to increase or to diminish toxin production even in circumstances where full growth has taken place.

For example, earlier and greater toxin production by type E in fish was recorded by EKLUND and POYSKY (1967); and greater toxin production by type F in culture media was noted by WILLIAMS-WALLS (1969), despite inhibition of cell lysis. On the other hand, delayed toxin production at 30 °C, and inhibition at 20° with a dose of 300 kr, was observed by ABRAHAMSSON and co-workers (1966) under circumstances resembling those in the experiments of EKLUND and POYSKY (1967). The reasons for such differences are not clear.

Such questions with *Cl. botulinum* toxin are, however, more or less academic, as the aim is always to maintain circumstances where no growth — and hence no toxin formation — can occur. Sub-lethal irradiation makes growth less likely under sub-optimal conditions such as reduced water activity, as noted above.

2.1.6. Mycotoxin formation.

The possibility that moulds surviving radiation treatment might have altered patterns of toxin production has recently raised concern.

JEMMALI and GUILBOT (1969a, 1970a, b) observed that radiation in the dose range 10–500 kr influenced the ability of *A. flavus* V-3734-10 to produce aflatoxin B_1, increased or decreased production being observed in different experiments. Several apparently non-toxigenic strains of *A. flavus* were induced by irradiation to produce measurable levels of aflatoxin (JEMMALI & GUILBOT, 1969a). These effects were clearly due to some influence of irradiation of the fungus itself, for they occurred on the irradiation of spores alone, which excludes any possible complicating influence of irradiation on the substrate. Also, the irradiation of aflatoxin, dry or in the presence of organic matter, has virtually no direct effect at the dose levels (order $\ll$ 1000 kr) under consideration here (cf. FRANK & GRÜNEWALD, 1970).

In the last few years, several closely related papers were published by APPLEGATE and CHIPLEY (1973a, b, 1974a, b), attempting to demonstrate increased aflatoxin production by *Aspergillus flavus* after γ-irradiation. Because they are repetitive, these papers need not all be discussed in detail.

From the earlier papers (APPLEGATE & CHIPLEY, 1973a, b), the observations most worthy of mention are that exposure of the non-toxigenic *A. flavus* NRRL-A-12268 conidial spores to 25–600 kr of 60-Co γ-radiation did *not* induce the production of aflatoxins on either wheat or synthetic medium; these results contradict those of JEMMALI and GUILBOT (1969b).

Studies on the toxigenic *A. flavus* NRRL-3145 were described in more detail in the recent papers. APPLEGATE and CHIPLEY (1974a) irradiated wet pellets of *A. flavus* spores with single doses of γ-rays up to 600 kr, and then transferred about 10^6 irradiated spores into replicate flasks of either a liquid synthetic culture medium or moist wheat grains (50 g H_2O/100 g wheat), kept 1–10 days at 28 °C. Flasks were withdrawn daily and the content of aflatoxins estimated. Doses of 400 kr or more prevented spore germination entirely, hence no aflatoxin was produced.

At lower doses, results regarding aflatoxin B_1 were quite clear in relation to the synthetic medium. The flasks containing irradiated spores had more aflatoxin than zero-dose controls (which contained almost none!), by an amount which increased from days 1–5, and then fell again until day 10, by which time the irradiated spore cultures were virtually the same as controls. With increasing dose, the rise was delayed, and became greater and more transient; with 200 kr, irradiated spore cultures produced at first less toxin than controls (possibly an effect of delayed growth) so that, despite a pronounced peak on days 7–9, the product of "concentration × time" was roughly the same as for controls. In the wheat cultures, results were confused by great variability; nevertheless, a similar picture was indicated. There was little effect up to doses of 150 kr; at 200 kr less aflatoxin was produced up to day 4, from days 6–8 there was perhaps twice as much aflatoxin in the irradiated samples as in the controls, by day 10 levels in the 200 kr cultures had declined till they were nearly the same as controls. At 300 kr aflatoxin production was similar but more transient, and a dose of 400 kr killed all the spores.

For aflatoxin B_2, the contents in samples incubated between 4 and 10 days are given in Table 3.

Table 3

Aflatoxin B_2 contents (μg) of substrates inoculated γ-irradiated conidia of A. flavus NRRL-3145, after 4 to 10 days irradiation at 28 °C

(data of APPLEGATE & CHIPLEY, 1974a)

Radiation dose (kr)	Per 25 g wheat medium	Per 25 ml synthetic medium
0	0.032–0.062	0.018–0.029
25	0.037–0.051	0.000–0.060
50	0.025–0.046	0.054–0.102
100	0.032–0.056	0.035–0.170
150	0.010–0.070	0.000–0.046
200	0.030–0.069	0.005–0.057
300	0.010–0.038	0.005–0.012

The authors concluded that in wheat medium "generally, the amounts of this toxin produced by irradiated cultures were equal or less than those produced by the controls", and in the synthetic medium "both the 50 and 100 krad exposed cultures demonstrated a significant increase in their toxin

Table 4

The effect of 60-Co irradiation on aflatoxin B_1 and B_2 production by Aspergillus flavus NRRL-3145[+]

(from APPLEGATE & CHIPLEY, 1974b)

B_1				B_2			
Dose (krad)	Wheat[++]	Dose (krad)	Synthetic medium[+++]	Dose (krad)	Wheat[++]	Dose (krad)	Synthetic medium[+++]
300	0.2288^{a}	300	0.0089^{a}	300	0.0194^{a}	300	0.0057^{a}
150	0.2351^{a}	0	0.0278^{b}	150	0.0273^{ab}	150	0.0181^{b}
50	0.2561^{ab}	150	0.0307^{b}	50	0.0280^{ab}	0	0.0194^{b}
0	0.2575^{ab}	25	0.0489^{bc}	100	0.0338^{bc}	200	0.0198^{b}
25	0.2857^{ab}	200	0.0626^{bc}	25	0.0382^{c}	25	0.0403^{c}
100	0.3200^{b}	100	0.0909^{c}	200	0.0396^{c}	100	0.0444^{c}
200	0.3547^{b}	50	0.0953^{c}	0	0.0399^{c}	50	0.0562^{c}

[+] Total aflatoxin yields from 25 g of wheat or 25 ml of synthetic medium from day 1 through day 10 of incubation.

[++] Means (μg) of three replications in the same column having different superscripts are significantly different ($P < 0.05$).

[+++] Means (μg) of three replications having different superscripts are significantly different ($P < 0.01$).

producing capacities over those of the control", and "significant increases in B_2 production were observed also when spores were exposed to 200 krad". Results for aflatoxins G_1 and G_2 resembled those for B_2.

A slightly modified account of the same work was published more recently (APPLEGATE & CHIPLEY, 1974b). In this, the mean contents and the results of statistical analysis are given for aflatoxins B_1 and B_2 in Table 4.

The conclusion was: "In general, the findings of this study indicate that, irrespective of substrate (wheat or synthetic), the exposure of *A. flavus* spores to radiation levels of from 100 to 200 krad should result in statistically significant increases in aflatoxin production over the amounts produced by non-irradiated spores". On the basis of Table 4, this conclusion appears

incorrect, the authors having misinterpreted their own statistical analysis. Only the means followed by different single superscripts differ significantly from each other at the given probability of error, in which case the only conclusion to be drawn is that samples inoculated with 300 kr irradiated spores contained, in general, less aflatoxins than the other samples.

These results and conclusions of APPLEGATE and CHIPLEY have to be treated with reserve, for several reasons:

(i) It is doubtful whether they are valid statistically; and, even if they were, they could hardly be considered as technologically or toxicologically meaningful.

(ii) There was a marked lack of dependence on dose, as Table 4 strikingly illustrates.

(iii) Responses with time were similarly erratic; the stimulation was transient. Where there was evident stimulation, it appeared to have almost disappeared again by day 10 after irradiation. Unfortunately, observations were not continued over longer times likely to be of practical interest.

(iv) The highest aflatoxin concentrations reported were still quite low. Calculated on the basis of their 25-g wheat samples, a content of 25 μg is 1 ppm; their maximum transient values in 200-krad irradiated samples were around 0.50 μg, *i.e.* 20 ppb, which is still well below the 30 ppb limit proposed by FAO, or the 25 ppb limit considered as the highest acceptable aflatoxin concentration of "good lots" in the U. S. sampling plans, used to estimate aflatoxin concentration in shelled peanuts (WHITAKER *et al.*, 1974). The actual value in the moist wheat would have been still lower, about 15 ppb.

(v) The experimental procedure of adding irradiated spores to unirradiated cracked, moistened and autoclaved wheat of water activity near 100%, was wholly unrealistic for grain storage.

(vi) The doses emphasised (100 kr upwards) are distinctly higher than the range 20–50 kr authorised (and needed) for insect disinfestation of wheat and flour (TILTON & BROWER, 1973).

BULLERMAN and co-workers (1973) studied the effect of γ-irradiation on the ability of two toxigenic strains of *A. parasiticus* to survive and produce aflatoxin in fresh bread. White bread slices were inoculated with approximately 10^2 and 10^6 conidial spores/slice of *A. parasiticus* strains NRRL 2999 or NRRL 3000. The inoculated slices were individually packaged in polyethylene pouches, irradiated at 0, 100 and 200 kr at ca. 25°, stored for 10 days at 25 °C, and then analysed for aflatoxins and total mould content. Storage studies were extended, the bread slices being examined also at 1, 2, 4 and 6 weeks after irradiation for aflatoxin content, total moulds, yeasts and bacteria. After storage at 25 °C for 10 days, visible mould was evident to varying degrees on all control samples; the authors observed that "the control cultures

of both strains produced more apparent growth at the 10^2 spores inoculum than the 10^6 spore level", and stated, "This may represent some type of competitive effect or auto-inhibition that is related to spore concentrations and limited growth conditions". There was no appreciable mould on the slices treated at 100 kr; and it was completely lacking on slices given 200 kr with strain NRRL 2999. Reduction in mould growth did not result in an increase in bacterial numbers (possibly because the bread was too dry). As the authors stated, "The 200 krad dose essentially eliminated aflatoxin production in either strain at both levels of spore inoculation during 10 days of storage. The 100 krad treatment level also reduced the amount of aflatoxins detected in inoculated bread at the end of the 10 day storage period". After storage for up to 6 weeks, the amount of aflatoxin produced is given in Table 5.

Table 5

Aflatoxin production in sliced bread inoculated with spores of Aspergillus parasiticus, after γ-irradiation

(data of BULLERMAN *et al.*, 1973)

Storage time (week)	μg Aflatoxins ($B_1 + G_1$)/g bread											
	strain 2999						strain 3000					
	10^2 spores/slice			10^6 spores/slice			10^2 spores/slice			10^6 spores/slice		
	Control	100 krad	200 krad	Control	100 krad	200 krad	Control	100 krad	200 krad	Control	100 krad	200 krad
1	182	N.D.	N.D.	16	111	N.D.	105	N.D.	N.D.	15	6	N.D.
2	46	0.03	N.D.	540	640	0.05	370	N.D.	N.D.	24	122	0.02
4	70	N.D.	N.D.	113	41	13	16	N.D.	N.D.	5	81	N.D.
6	4	N.D.	N.D.	132	*1061*	N.D.	34	N.D.	N.D.	264	227	N.D.

N.D. = not detected.

Surprisingly, the authors concluded from these results: "stimulation of aflatoxin production may have occurred at the 100 krad treatment level in samples inoculated with 10^6 spores after 1, 2 and 6 weeks of storage." No attempt was made to evaluate the data statistically. Apart from one anomalous observation (italicised) the data for both spore concentrations and dose levels show clearly the efficacy of radiation treatment in preventing aflatoxin formation.

It should be realised that it is difficult to interpret quantitative determinations of mycotoxin content in food samples because there are large errors of estimation due to the heterogeneous composition of food, and the inhomogeneous distribution of moulds and their metabolites, as well as

uncertainties inherent in the analytical methods (WHITAKER & DICKENS, 1974). Collaborative studies with aflatoxins have shown variation coefficients to be 25 to 110% (SHOTWELL & STUBBLEFIELD, 1972; COON *et al.*, 1973; FRANK, 1974) depending on the type of food and toxin, as well as the method of evaluation.

In a recent study (BULLERMAN & HARTUNG, 1974) spores or growing vegetative mycelia of *Aspergillus parasiticus* strains NRRL 2999 and NRRL 3000 were irradiated at 100 and 200 krad, and the effects on growth and aflatoxin production in still cultures in yeast-extract sucrose broth were measured. After 7 days at 25 °C, the cultures were heated, the mould mats separated, and the aflatoxin concentration of the broth was determined. Mould growth was expressed as milligrams of mycelial dry weight. Results can be summarised in Table 6.

Table 6

Aflatoxin production by irradiated cultures of Aspergillus parasiticus

(BULLERMAN & HARTUNG, 1974)

Treatment (krad)	Aflatoxin B_1 (μg/mg dry mycelium)				Aflatoxin G_1 (μg/mg dry mycelium)			
	NRRL 2999		NRRL 3000		NRRL 2999		NRRL 3000	
	Spores	Mycelium	Spores	Mycelium	Spores	Mycelium	Spores	Mycelium
0 (control)	2.9	2.6	1.3	0.6	14.5	12.1	19.6	6.1
100	3.6	0.7	1.1	0.3	17.4	3.2	17.5	1.9
200	3.1	0.3	0.7	<0.1	9.4	1.3	0.5	<0.1

The authors mention "the variation of these observations as determined by the standard deviation was such that it could be concluded that no actual differences existed between treatment means". Despite this, they interpret their results as a stimulation of aflatoxin B_1 and G_1 production with spores of strain NRRL 2999 after 100 krad irradiation.

These ill reported investigations of APPLEGATE and of BULLERMAN provide various convincing illustrations of the power of irradiation to diminish aflatoxin production, without (in the writers' view) any convincing illustrations of an increase. Nevertheless, an occasional increase seems conceivable. As pointed out by FRANK *et al.* (1971), *Aspergillus* is heterocaryotic, and results should be interpreted accordingly. Accumulation of nuclei with or without the genetic basis for aflatoxin synthesis appears possible, in the course of many transfers to fresh medium. This may lead to loss, recovery, or gain of the ability to produce aflatoxins. Nuclei with different genetic

patterns may not react uniformly to irradiation. Destruction of the capacity for aflatoxin biosynthesis, or the return of aflatoxin production in some cases, can be understood as a recombination of a pattern of nuclei. The occasional occurrence of cultures having higher aflatoxin-producing ability after irradiation (as compared to the parent strain) would not be surprising under these circumstances. Concerning the appearance of aflatoxin-forming subcultures of an originally non-toxigenic strain, it is supposed that some nuclei of the large number in the heterocaryon have the genetic code for aflatoxin synthesis. Normally, these nuclei remain a minority. However, after irradiation some of them might get a selection advantage and dominate the other types.

After their elaborate investigation of the response of toxigenic and non-toxigenic strains of *A. flavus* to irradiation, FRANK and his co-workers (1971) concluded "we do not believe that there is an increased risk from a possible industrial application of food irradiation. Such treatment has more frequently led to a complete loss or to a decrease of aflatoxin production than to a gain or increase". Looking through the literature, the reviewers agree with this conclusion.

2.2. Repeated irradiation cycles

Experiments related to food irradiation have so far concentrated primarily on the obvious effect of re-cycling in increasing radiation resistance (RR), and have been based on procedures simulating (if remotely) the conceivable cycle of events in industrial practice. Observations on important factors like growth rate, diagnostic characters or virulence have been secondary, on the reasonable basis that these characters are of lesser importance unless coupled with increased RR. Consequently, this review begins with observations on development of RR.

Acquisition of resistance by repeated exposure is a phenomenon not peculiar to irradiation. It occurs also with food preservatives (*e.g.* benzoate, sulphur dioxide) and even with sanitising agents (SOPREY & MAXCY, 1968; MAXCY *et al.*, 1971).

2.2.1. Development of increased radiation resistance.

That repeated sublethal treatments with radiation can lead to permanently increased resistance has been known in principle since WITKIN (1946) developed the B/r strain from *E. coli* strain B using ultra-violet light. GADEN and HENLEY (1953) observed a corresponding difference between the strains B and B/r in resistance to γ-rays. They produced a roughly threefold increase in RR of *E. coli* B by 17 cyclic treatments each consisting of γ-irradiation with 240 kr followed by incubation for 17 hours at 37 °C. The more resistant strain B/r behaved similarly, but the maximum level of RR attained was about the same for

both strains. However, *E. coli* ATCC 9723 became more sensitive (RS) after 2 cycles, with no further change up to 5 cycles.

ERDMAN and co-workers (1961b) examined the effect of exposing several species important in food hygiene to repeated doses of 200 kr of γ-radiation interspersed with periods of growth up to stationary phase (22 hours at 35 °C). They showed that, with different numbers of cycles up to 12, resistance was permanently increased in *E. coli*, *Strep. faecalis*, *Staph. aureus* and *Cl. botulinum*, by factors up to 2-fold. They failed, however, to induce any increase of RR in *Salmonella gallinarum*.

The increased RR acquired by re-cycling vegetative cells may apparently be passed on (perhaps in minor degree) to the spores, and transmitted through the spores to subsequent vegetative cells. Observations of ERDMAN and co-workers (1961b) showed this with *Cl. botulinum* type A.

Twelve cycles of 250 krad with six different *Staph. aureus* cultures produced only small increases of RR, up to 25% (IDZIAK & INCZE, 1968).

More recent work has concentrated on the problem with *Salmonella*. All the earlier re-cycling experiments were made in highly artificial media. IDZIAK and INCZE (1968) used modified meat: they increased the RR of *S. anatum*, *enteritidis*, and *give* by 100%, and that of *S. typhimurium* by 50%, with 12 cycles of 250 kr plus 24–48 hours at 37 °C in poultry meat base; storage for several months on laboratory media (under conditions unfortunately not described) increased this resistance still further, by up to 50%. IDZIAK and INCZE (1968) found it impossible to cycle *Salmonella* strains at 5°, there being no survivors after the first cycle; attenuation might have been expected, because salmonellas will not grow at 5°. It seems possible, in addition, that there may be some lethal effect on RR cells surviving irradiation.

LICCIARDELLO and co-workers (1969a), also, were able by 20-cycle irradiation in culture media to induce several-fold increased RR in *Salmonella heidelberg*, *newport*, *thompson* and *typhimurium*. They confirmed that the increased resistance was retained after many sub-cultures, an observation repeated by subsequent workers. It is interesting that strains of *S. newport* and *S. typhimurium* which developed increased resistance on re-cycling failed to do so when exposed for 5 hours at 37° to a dose rate of 60–160 r/min such that growth rate was approximately equal to death rate (LICCIARDELLO *et al.*, 1969a); it is a pity that this experiment was not continued longer. The increased resistance developed by re-cycling stationary phase cultures (and, obviously, manifest at that phase) is manifest at all phases of growth (LICCIARDELLO *et al.*, 1968). The response of RR cells to temperature during irradiation is similar to that for parent (P) cells.

CORRY and ROBERTS (1970) re-cycled 3 strains of *Salmonella (S. typhimurium* phage type 1/1; *S. typhimurium* phage type 2c/14; and *S. dublin)* irradiating to 0.1% survival. D values were estimated from survival curves

over 6 decimal reductions. After 13 cycles only one strain (*S. typhimurium* phage type 2c/14) showed a significant increase in RR, the D values about doubling. PREVITE *et al.* (1971) failed to obtain any increase of RR after 10-fold re-cycling of 3 strains of *S. typhimurium* at a dose level of 500 kr.

EPPS and IDZIAK (1970) reported increased RR as a result of recycling several *Salmonella* serotypes. With doses of 200 kr one of two *S. montevideo* and *pullorum* cultures was eliminated after 3 cycles (and *Paracolobactrum arizona* after 4), though all three survived 12 cycles at 180 kr. Similarly one of two *S. blockley* and *enteritidis* failed after 7 cycles of 200 kr. Intermediate growth was for 24 hours at 37°. *S. anatum, give, senftenberg, typhimurium* and *worthington* all survived 12 cycles. Some of these cultures were further developed in RR, by a further 12 cycles with 250 kr followed by 12 at 300 kr, leading to D values of 50–100 kr compared with 20–50 for the P strains.

DAVIES and SINSKEY (1973) increased the resistance of *S. typhimurium* strain LT2 up to ten-fold by 84 irradiations to 0.1% survival each followed by growth to stationary phase, the D value rising steadily (not "step-wise" as they state) from 30–40 to 300–330 kr. The resistance increased throughout most of the cell population; their data are, of course, unreliable as indicators of possible minority populations, because they were based on 100 single-cell isolates.

The increased RR consists of two elements, a large increase in "shoulder" on the log inactivation/dose curve, with a decrease in slope (*i.e.* increase in D value) of the linear portion. The two are apparently distinct. In the *E. coli* and *Strep. faecalis* described by ERDMAN and co-workers (1961b), there was little change in "shoulder" and the change in RR almost entirely reflected changes in D value. On the other hand, with the *S. newport* of LICCIARDELLO and co-workers (1969a) the increased RR almost entirely represented increased "shoulder", the D values being almost the same for normal and resistant strains. With 10-cycle *Cl. botulinum*, vegetative cells exhibited increased D value and spores increased "shoulder" (ERDMAN *et al.*, 1961b). These differences confuse comparisons of radiation resistance. For example, LICCIARDELLO and co-workers (1969a) used percentage survival after a dose of 100 kr as their index of resistance. On this basis they observed a roughly 500-fold increase of "resistance" (0.001 to 0.5%) when the D value had simply tripled. Also, their measure of "resistance" appeared to increase in a markedly stepwise manner, though the increase was regular in terms of D value.

PARISI and ANTOINE (1974) recently reported a 4- to 5-fold increase in RR of vegetative cells of *Bacillus pumilus* E 601, one of the internationally recognised biological standards for irradiation sterilisation of medical supplies, as a result of re-cycling under γ-radiation. CHRISTENSEN (1974) reports doubling of RR by 30 cycles with spores of *B. sphaericus* EMBORG and ERIKSEN (1974b) suggest the deliberate development by re-cycling of strains of *B. sphaericus*

and *B. cereus* of graded resistance, in order to use the spores as biological "go/no go" indicators of dosage in irradiation processes. In the investigations with *B. pumilus*, even the radiation resistance of the most RR strain (23 cycles) did not approach that of the non-spore former *M. radiodurans*, or of RR *S. typhimurium* (DAVIES & SINSKEY, 1973). Radiation resistance was not transferred from vegetative cells to spores in the experiments of PARISI and ANTOINE (1974). This is in contrast to the earlier studies of ERDMAN and co-workers (1961b) revealing a transfer of radiation resistance from vegetative *Cl. botulinum* type A cells to their spores. Re-cycling may, however, apparently increase the RR even of already highly resistant organisms. LEWIS (1973) did not obtain any increase in RR of the resistant *Micrococcus* species of LEWIS (1971), re-cycled 10 times through doses of 1.0 Mr. On the other hand, EMBORG and ERIKSEN (1974a) did produce a considerable increase in RR of *M. radiodurans*, by 25 cycles of irradiation to a survival level of 10^{-4}, the corresponding required dosage increasing from 4.5 to 12 Mrad; between irradiations, survivors were grown to colonies on plates. The increased resistance was mainly manifest as further extension of the already large "shoulder" on the inactivation/dose curve.

The RR strains have been found to be stable, preserving their resistance (ERDMAN *et al.*, 1961b) and other characteristics (IDZIAK & THATCHER, 1964) even over years of maintenance on standard media. LICCIARDELLO and co-workers (1969b), CORRY and ROBERTS (1970) and EPPS and IDZIAK (1970) made similar observations. The latter also observed that 5 repeated chick passages of an RR strain of *S. typhimurium* did not result in any reversion to the P form, and maintained morphological peculiarities, and responses to biochemical tests and to polyvalent and group antisera.

Increased RR is usually paralleled by increased resistance to UV (*e.g.* GADEN & HENLEY, 1953; DAVIES & SINSKEY, 1973). It is not accompanied by increased heat resistance (LICCIARDELLO *et al.*, 1969a). The effect of temperature of irradiation on survivor/dose response seems to be the same for RR as for P strains (ERDMAN *et al.*, 1961b).

A feature of re-cycling is the apparent variability of response. GADEN and HENLEY (1953) found that *E. coli* 15 became more radiation sensitive after repeated γ-irradiation treatments, whereas *E. coli* B and B/r increased in resistance. WINKLE (1966), however, could not select out and gradually built up a different strain of *E. coli;* the population that grew from their most resistant organisms had exactly the same distribution of sensitivity as the original culture. Similar differences were found with salmonellas. Working with *S. typhimurium* under apparently similar conditions, LEY and co-workers (1970) actually noted reduced resistance after re-cycling; CORRY and ROBERTS (1970) noted a modest increase; DAVIES and SINSKEY (1973) a large increase still progressing after 80 cycles. Similar differences with *M.*

radiodurans were just noted above. The reason for these discrepancies is not known.

It is clear from the above discussion that different micro-organisms respond differently to re-cycling treatments. This is true of closely related strains, *e.g.* different serotypes of *Salmonella* or even different strains of the same serotype. It is hard to believe that such closely related strains could differ in any fundamental way. The different responses probably result from minor quantitative differences in radiation resistance or mutation frequency, for strains which cannot be re-cycled at one dose level can sometimes be re-cycled at a slightly lower dose level, as noted already.

The amount of re-cycling needed to produce RR, and the limiting number of effective cycles, are also both very variable. ERDMAN and co-workers (1961b) observed no increase beyond 6 up to 21 cycles (200 krad) with *Strep. faecalis*, and no increase beyond 12 up to 18 cycles with their *E. coli* strain (self-isolated). On the other hand, DAVIES and SINSKEY (1973) were still obtaining an increase of RR in *S. typhimurium* LT2 after 84 cycles, with no sign of falling off, rather the reverse.

(i) Diminished resistance. While attention has concentrated on the increase of resistance following re-cycling, it should be remembered that irradiation — even repeated — can result in decreased resistance. This was observed at the outset, for example by HUBER (1952) with vegetative bacteria, by WITKIN (1952) with the B_s strain of *E. coli* B, and by GADEN and HENLEY (1953) with *E. coli* ATCC 9723; and more recently by LEY and co-workers (1970) with *S. typhimurium*. Increased sensitivity at low survival ratios used to be regarded as a general phenomenon, ascribed to the accumulation of "non-lethal hits" gradually rendering organisms more sensitive (*e.g.* ROEPKE & MERCER, 1947). MÜNZNER and DIEHL (1969) subjected cultures of 7 strains of *Penicillium viridicatum* and 6 of *A. flavus* to 16 cycles of electron irradiation at different dose levels below 180 krad which prevented growth. All the strains of *P. viridicatum* and 3 of *A. flavus* became less resistant at all dose levels. In no case was any generally increased resistance observed.

(ii) Mechanism. The mechanisms whereby irradiation-cycling increases RR might be simply a selection of RR cells already existing in normal populations, or the induction and selection of RR mutations by the repeated irradiation. Analysis of the data of WITKIN (1946) suggested that U. V. resistant cells occurred spontaneously in a normal population of *E. coli* strain B, with an average frequency near 10^{-5} per organism per generation; this suggests 2×2^{10} or about 2000 RR cells in a population of 10^8. However, when strain B was exposed to 3800 ergs/mm^2 of U. V. energy (roughly equivalent to 150 kr of X-rays on the basis of survival curves) the mutation frequency among the survivors averaged 4% (WITKIN, 1947). From the outset, therefore, it appeared that the important element was the "direction" by persistent selective irradia-

tion of otherwise random mutations in the direction of increased resistance. This view was confirmed for ionising radiations by GADEN and HENLEY (1953), who calculated the induced mutation frequencies as 4×10^{-7} and 5×10^{-6} per roentgen per cell, for *E. coli* strains B and B/r, respectively.

WRIGHT and HILL (1968) cycled *E. coli* at a survival level of 10^{-3}, and increased the D value about 40% after 7 cycles in organic media, but only about 20% in inorganic media. They speculated that an organic medium might be more mutagenic than an inorganic one, following reports (HAAS & DOUDNEY, 1957) that mutational response of *E. coli* to U. V. light was increased by cultivation in the presence of yeast extract.

The mutation hypothesis was, however, questioned by LICCIARDELLO and co-workers (1969a). They estimated the frequency of RR cells as $1/9\times10^{6}$ cells for *S. newport* and $1/19\times10^{6}$ for *S. typhimurium*. (Such figures must, obviously, depend on the dose selected as criterion of "resistance", in this case 60 kr); and in a culture of *S. newport* grown after irradiation with 75 kr to a survival of about 10^{-7}, the corresponding frequency was $1/16\times10^{6}$ cells, no greater than in the parent culture. Hence they concluded that there is no induction of resistant mutants; although the experiment does not appear convincing.

The proportion of resistant cells and their resistance relative to the rest, influence the radiation dose to be used in re-cycling because it must suffice to remove most of the sensitive cells while leaving some of the resistant ones. The question is complicated by the fact that as re-cycling proceeds, and the population as a whole increases in resistance, the proportion of survivors will become greater unless the dose is increased. Different investigators have operated differently. IDZIAK and INCZE (1968) and LICCIARDELLO and co-workers (1969a) worked with a constant radiation dose. However, the more usual plan has been to increase radiation dose at intervals, so as to maintain the proportion of survivors roughly constant; but the actual level chosen may be very different. In the work of ERDMAN and colleagues, radiation dosage was increased at each cycle to maintain survival at a level of only 10^{-6}, under which conditions a 1.9-fold increase of RR was attained after 12 cycles (ERDMAN *et al.*, 1961b). No further increase was possible, 12-cycle cultures failing to tolerate any further increase in the applied dose which had then been raised to 2080 kr (PONTEFRACT & THATCHER, 1965). EPPS and IDZIAK (1970) also increased the dose applied, but only every 12 cycles; from their data, the survival levels must have varied from about 10^{-8} to 10^{-3} among the different strains they used. DAVIES and SINSKEY (1973) operated similarly, but only every 14 cycles, and to maintain a relatively high survival level of about 10^{-3}. Because of these differences, it is impossible to judge the effect of "survival level" by comparing results of different workers. The results of LICCIARDELLO and co-workers (1969a, their Figs. 1 and 2) might be

Table 7

Nucleic acid contents of parent and 12-cycle RR strain of E. coli

(IDZIAK & THATCHER, 1964)

Strain	DNA (μg)		RNA (μg)	
	per mg dry weight	per cell $\times 10^{10}$	per mg dry weight	per cell $\times 10^{10}$
Parent P	24.3	98.2	66.5	279
12-cycle RR	16.0	94.9	38.2	229

taken to indicate that cycling at high doses leads to a slower increase of RR, but continuing over a greater number of cycles before arriving at a plateau; unfortunately, these figures express resistance in an ambiguous way.

PREVITE and co-workers (1971) suggested that mutants are obtained more readily from *Salmonella* cultures re-cycled at 250 kr or less than from cultures re-cycled at 500 kr. But it seems to the reviewers that this suggestion is not valid. It was based on comparison of a very mixed culture with a pure culture and, as already noted, the changes observed in the former are plausibly explained as being due to selection among the 10 serotypes included in the mixture. This, in practice, very important question calls for more careful investigation.

(iii) Basis of the increased resistance. ERDMAN and co-workers (1961b) observed that their re-cycled RR strains of *E. coli* possessed long cells. It was shown by PONTEFRACT and THATCHER (1965) that these cells possessed unusually large numbers of nuclear bodies (as judged by Feulgen stain), and that they were devoid of cross walls. If each filament were really multinucleate, this would account for the appearance of a "shoulder" on the dose/inactivation curve for RR cultures, of a size which ought to increase with the number of nuclei — this point does not seem to have been investigated.

It appears, however, that these "nuclei" cannot be normal, for the content of nucleic acids per *cell* does not increase as does the number of nuclei. The observations of IDZIAK and THATCHER (1964) suggested only small differences between parent and re-cycled cells in nucleic acid content (Table 7) per cell; though the RR cells had of course distinctly lower contents on a dry weight basis corresponding to the larger size.

DAVIES and SINSKEY (1973) have recently made corresponding observations with RR and P *Salmonella* cells. However, both HILL and SIMON (1961) and WOODSIDE (1965) observed no differences in DNA, RNA and protein content on a dry weight basis between normal, sensitive (B_s) or resistant (B/r) cells of *E. coli* strain B.

Comparing post-irradiation DNA synthesis in B/r and B_s strains of *E. coli*, MCGRATH and WILLIAMS (1966) found that 20 kr of X-rays reduced the

average M.W. of the DNA to half in either strain, but the resistant strains exhibited rapid restoration of the M.W. in post-irradiation growth, leading to the hypothesis that the basis of radiation resistance is an efficient DNA-repair mechanism (cf. GINOZA, 1967). Subsequently, higher rates of DNA-synthesis after irradiation (STAVRIC *et al.*, 1968a) and lower rates of DNA-degradation (STAVRIC *et al.*, 1968b) were observed in the RR as compared with their P strains of *E. coli*.

Similar results have been obtained by DAVIES and co-workers (1973), with the highly RR strain of *S. typhimurium* developed by DAVIES and SINSKEY (1973). Irradiation produced similar leakage of DNA in both RR and P strains, but the RR strain had a lower rate of post-irradiation DNA-degradation and its DNA synthesis was not inhibited by 200 kr as was that of the parent strain. DAVIES and SINSKEY (1973) observed greater activities of DNA polymerase I and polynucleotide ligase in RR compared with P cells of *S. typhimurium* LT2, hence they conclude that the basis of RR in re-cycled strains is the same: namely an unusual ability to repair, and therefore tolerate, radiation-induced strand lesions in DNA.

ALLWOOD and JORDAN (1970) however concluded that this was not the reason for radiation-resistance in strains of *S. thompson*. It contrasts with the effect of irradiation on normal bacteria where, though DNA degradation is reduced, so are rates of DNA synthesis (*e.g.* POLLARD & ACHEY, 1966).

(iv) Development of heat resistant microbial mutants by re-cycling. It is noteworthy that such development of resistance is not unique to radiation treatment. NIVEN and co-workers (1954) doubled the thermal tolerance of a lactobacillus by repeatedly growing the survivors from heat treatments; also heat-resistant strains of *Staph. aureus* (GRUN & YO, 1964) have been obtained by repeated sublethal heating, in a way similar to that used for the development of radiation-resistant mutants.

Using *S. typhimurium* phage type 1(1), CORRY and ROBERTS (1970) found that, after 13 cycles, the strain had a $D_{55°}$ value about double that of the original; after 26 cycles it was almost 3 times as heat resistant as the original; and after 39 cycles it attained the same order of resistance as *S. senftenberg* 775 W, the most heat resistant *Salmonella* known at a_w values close to 1 (GOEPPERT & BIGGIE, 1968; RIEMANN, 1968; NG *et al.*, 1969). The augmented heat resistance of the 26-cycle strain was not lost even after 14 subcultures without heating. No apparent increase in pathogenicity or significant change in common biochemical characters was observed. Re-cycled strains contained, however, extremely low proportions of the smooth colonies required for serological and phage typing. The heat resistance of strains *S. typhimurium* phage type 2c(14) and *S. dublin* was not changed significantly after 13 cycles.

2.2.2. Changes in diagnostic characters.

The desire to be able to identify re-cycled cells correctly, especially important with pathogens, requires that they should retain the crucial diagnostic characters of the parent strains. In fact, changes may occur.

(i) Morphological changes. The temporary development of long filamentous cells, after single exposure to sub-lethal doses of radiation, has been observed in several *Enterobacteriaceae:* with *E. coli* and U.V. light (GATES, 1933); with *E. coli* and X-rays (LEA *et al.*, 1937); and with *Proteus vulgaris* and *S. typhi* and γ-rays by SPENCER (1935). In the γ-radiation re-cycled *E. coli* mutants of ERDMAN and co-workers (1961b), this character was stabilised and maintained through years of sub-culture. IDZIAK and THATCHER (1964) note that the filamentous forms reported by the early workers in response to one irradiation represent a passing phase, heralding the death of the organism unless the cause is removed, whereupon the survivors recover the normal shape after several generations. On the other hand, their filamentous RR form (that of ERDMAN *et al.*, 1961b) was stable. Correspondingly, they note, earlier workers had found the filamentous forms less resistant, not more resistant as in the case of the ERDMAN strain.

On the other hand, it was observed by WOODSIDE (1965) that whereas the RS mutant B_s resembled the parent *E. coli* strain B in having long cells in young cultures and giving characteristic rough (R) colonies, the RR strain B/r had short cells and gave typical smooth (S-type) colonies which were stable. Similarly, LICCIARDELLO and co-workers (1969a) found that RR cells of *Salmonella* strains obtained by 20-cycle treatment were predominantly little longer, but fatter and therefore larger and more coccoid than the rod-shaped P strains.

However EPPS and IDZIAK (1970), using similar *Salmonella* serotypes but more severely re-cycled, observed that some produced filamentous cells *(S. anatum, enteritidis, pullorum, senftenberg* and *worthington),* some coccoid cells *(S. blockley),* and some produced both *(S. typhimurium).* There were corresponding abnormalities in cell division. These characters were stable.

Motility was impaired, but not destroyed, in a 20-cycled strain of *S. newport* (LICCIARDELLO *et al.*, 1969a). However, sufficient re-cycling entirely prevented motility of vegetative cells of *B. pumilus* (PARISI & ANTOINE, 1974). Similarly, the ability of *B. pumilus* to form spores was lost after 15 cycles (PARISI & ANTOINE, 1974).

It appears, therefore, that morphological response to re-cycling may be diverse, and sufficient to make recognition on this basis impossible. A weaker *Gram*-stain was observed in re-cycled cells of *B. pumilus* (PARISI & ANTOINE, 1974).

(ii) Biochemical tests. LUCKIESH and KNOWLES (1948) observed that resistant *E. coli* obtained after 6-cycle U.V. treatment had less metallic sheen

Table 8

Phage patterns of parent and irradiated (0.2 Mrad, 0.02% survival) strains of Staph. aureus

(from ERDMAN *et al.*, 1961b)

Strain	Treatment	Resist.	Colour	Phage pattern
CS 68	Parent	Normal	Yellow	(7, 54, 75±)/81
	14-cycle	Normal	Yellow	81
ML 15	Parent	Normal	Yellow	6/7/47/53/54/75/81
	14-cycle	Normal	Yellow	ditto
			White	ditto
584600	Parent	Normal	Yellow	80/81
	14-cycle	1.25 N	Yellow	52/52A/80/81
			White	52/52A/80/81

on EMB agar and failed to ferment dulcitol, but 8 other biochemical reactions were unchanged.

Similarly ERDMAN and co-workers (1961b) observed, with their 12-cycle γ-irradiated *E. coli*, that most colonies on EMB agar produced no sheen, though the character tended to be restored on sub-culture; this strain of *E. coli* also took longer to develop on *Endo* agar (IDZIAK & THATCHER, 1964). However, with re-cycled *S. heidelberg*, *newport*, *thompson* and *typhimurium*, LICCIARDELLO and co-workers (1969b) noted no differences between RR and P strains in colony appearance or reaction on *brillantgreen*, bismuth/sulfite, *Salmonella-Shigella*, or *McConkey* agars. DAVIES and SINSKEY (1973), too, noted no difference in "qualitative reactions on selective media" with RR *S. typhimurium*.

The general experience with ionising radiation is, as with U.V. light, that re-cycling does not change the majority of characters, except perhaps to delay responses somewhat. For example, ERDMAN and co-workers (1961b) found that re-cycled RR *Strep. faecalis* showed no differences from the parent in colonial appearance or in the Sherman criteria; similar examples with *Salmonella* will be detailed shortly below. Some re-cycled cells of *Staph. aureus*, however, produced colonies lacking the typical yellow colour, which may or may not correspond with other changes (*e.g.* in phage pattern — cf. Table 8).

The 12-cycle *E. coli* cultures of ERDMAN and co-workers (1961b) did display differences from the parent. They had become indol-negative, though other IMVIC reactions were unchanged. On EMB agar, most colonies produced no sheen; some of these failed to ferment lactose at 44.5 °C and others at 44.5° and 37 °C.

IDZIAK and THATCHER (1964) then observed that the above strain of *E. coli* was the same as the parent in its responses to various amino-acids and growth factors. On the other hand, the RR strain failed to produce acid and gas from 7 sugars which were thus attacked by the parent. However, one regards all these observations with suspicion because the parent strain grew on a basal medium (A) ostensibly lacking any C-source, while both parent and RR strains grew on the basal medium (B) ostensibly without N-source (their Table II). Lactose was one of the sugars tested by IDZIAK and THATCHER (1964), and manometric experiments demonstrated a decline of ability to respire that sugar from parent through 1-, 6- and 12-cycle strains. The failure of some RR strains to attack lactose was related to their inability, in contrast to the parent, to produce mucoid colonies on Endo agar at 25 °C.

Increasingly RR strains commonly exhibit decreasing ability to utilise a variety of substrates and increasing requirements for particular substrates and growth factors. For example, in subsequent work (ROBERN & THATCHER, 1968a): the 1-cycle strain did not require amino-acids, purines or pyrimidines for growth; the 6-cycle required leucine, methionine and proline; the 12-cycle required eight amino-acids, and uracil or cytosine. Compounds stimulatory to strains of intermediate resistance became absolute requirements for strains of higher resistance, and consequently the same authors suggested (1968b) that intermediate mutants have lower intracellular pools of these substances though not so low as to exhibit a total requirement for them. With the *Bacillus pumilus* studied by PARISI and ANTOINE (1974), also, increase of RR by re-cycling was accompanied by an increased nutritional requirement for specific amino-acids. The irradiation treatment apparently created sufficient genetic damage so that enzymes critical in the synthesis of certain amino-acids were either altered to such an extent that they were non-functional or lost. DAVIES and SINSKEY (1973) similarly observed that, after about 50 cycles, increasingly re-cycled and RR strains of *S. typhimurium* began more frequently to be incapable of utilising various C-sources.

In the important case of the salmonellas, ERDMAN and co-workers (1961) observed at the outset that re-cycling made *S. gallinarum* citrate and H_2S negative, though the RR was not changed.

The four 20-cycled *Salmonella* serotypes of LICCIARDELLO and co-workers (1969b) all gave typical reactions on Triple-sugar-iron, lysine-iron and phenylalanine agar, and on malonate, urea, tryptone and KCN broths. 11/50 RR single-cell cultures of *S. newport*, and 5/50 of *S. thompson*, failed to grow on Simmons citrate agar; and of the latter five, two failed to produce H_2S. IDZIAK and INCZE (1968) also found that re-cycled *Salmonella* strains could be identified following standard methods.

Like CORRY and ROBERTS (1970), EPPS and IDZIAK (1970) observed that 12-cycled RR *Salmonella* cultures gave results in biochemical tests similar to

those of the P cultures, save for some delayed reactions. More detailed study of single cell isolates from 36-cycled cultures quite frequently showed a significant proportion of cells lacking a particular reaction; but, out of 120 tests among 10 strains, there were only 5 where the proportion of deficient cells exceeded half, and only 1 (*S. enteriditis* on deoxycholate-citrate agar) where almost all the population was defective (their Table 2). The authors conclude that such changes are not likely to prevent proper identification. DAVIES and SINSKEY (1973) observed that RR *S. typhimurium* produced less H_2S than P cultures on Triple-sugar-iron agar.

The notable changes here seem to be the failure of some RR strains to utilise citrate and to produce H_2S. There are serotypes which are normally negative in both these reactions, so the diagnosis would be changed only if these characters were used instead of serotyping, which they are not. Diagnosis as a *Salmonella* would not be affected.

Perhaps the most troublesome changes, so far, are those recorded at the outset by ERDMAN and co-workers (1961b) with *E. coli*. Though the vital changes were few (indol, temperature tolerance for lactose fermentation), they sufficed to change the diagnosis from *E. coli* type I to type II or irregular II: a small change, but significant in this highly sub-divided taxon.

IDZIAK and INCZE (1968) suggested that changes in biochemical characters might be produced only in circumstances where the applied dose is increased with progress of the cycles, so as to hold the number of survivors at a low level throughout. This was based on comparison of their own observations of no biochemical change at constant dosage with those of ERDMAN and co-workers (1961b) who observed biochemical changes after cycling at progressively increased doses. From the more extensive subsequent observations of LICCIARDELLO and co-workers (1969b), also made with constant dosage, it is clear that changes do occur, the outcome more likely depending on the severity of the treatment. The ultimate dose of ERDMAN and co-workers (1961b) was 2000 kr; several times that used by IDZIAK and INCZE (1968).

(iii) Serological, phage and antibiotic reactions. Repeated irradiation may weaken serological reactions. LICCIARDELLO and co-workers (1969b) compared their re-cycled strains of *S. heidelberg*, *newport*, *thompson* and *typhimurium* with corresponding P strains, using polyvalent H antisera. One hundred RR colonies of *S. typhimurium* and 100 P colonies all gave positive agglutination with poly-O serum, but many RR strains were weak; 25 colonies of each reacted positively with appropriate O and H group antisera. With *S. heidelberg* and *thompson*, RR strains did not react as strongly as P strains with poly-O or H antisera. With *S. newport*, 70 RR cultures were stated to react weakly with poly-O antiserum, and weakly or not at all with group-O or group-H antisera. Grouping was still possible, however, and a culture of *S. newport*, giving most difficulty, was

serotyped correctly by a reference laboratory (though, according to the authors' statement, it would have had no group reactions !). Weaker immunological responses of RR cells were also recorded in more detail by EPPS and IDZIAK (1970) with *S. typhimurium*. Antisera from adult chickens, if prepared by injection of P cells, strongly agglutinated both P and RR cells, but did not if prepared by injecting RR cells (their Table 4). Further, whereas prior absorption of a P antiserum by P cells entirely prevented subsequent agglutination by P cells, similar absorption by RR cells had almost no effect.

As regards phage pattern, there may be diversity of response to radiation cycling. Table 8 records observations of ERDMAN and co-workers (1961b) on 3 strains of *Staph. aureus*, showing that re-cycled cells may lose or gain phage responses, or may remain unchanged in this respect.

EPPS and IDZIAK (1970) observed that RR cells of several different *Salmonella* strains were altered in phage response. All P cultures gave numerous plaques with 0–1 phage; RR cultures gave none or few. Observation of equal and effective lysis (by von *Seefried*) was, however, noted by EPPS and IDZIAK (loc. cit.). Phage typing of RR cultures was not possible because of their "rough" character. It appears that, in groups where phage pattern is important in diagnosis, re-cycling might well hinder strain identification, but more information is desirable.

There is a suggestion in the work of DAVIES and SINSKEY (1973, their Fig. 4) that re-cycled RR cells reactivate irradiated phage particles much better than P cells, which DAVIES and co-workers (1973) regard as consistent with the hypothesis that RR cells repair DNA more efficiently.

Although quantitative changes in antibiotic sensitivity are sometimes observed as a result of irradiation, re-cycling does not appear to lead to any systematic change. LICCIARDELLO and co-workers (1969a) observed little difference between RR and P strains of 4 *Salmonella* serotypes in resistance to penicillin, and (1969b) none in responses to chloramphenicol, tetracycline, and three different concentrations of ampicillin. Antibiotic sensitivities of 12-cycled RR strains of *Salmonella* were also examined by EPPS and IDZIAK (1970). They, too, observed no differences from P strains when making 84 tests (12 with each of 7 serotypes); with the exception of three — *S. blockley* became less resistant against streptomycin, and *S. typhimurium* against streptomycin or nitrofurazone. The cycled RR strains of CORRY and ROBERTS (1970) did not differ from their P strains in resistance or sensitivity to 25 antibiotics. When PREVITE and co-workers (1971b) exposed two strains of *S. typhimurium* to 10-fold re-cycling with a dose of 500 kr, they observed a decrease in resistance to ampicillin, chloromycetin, streptomycin and tetracycline; the alterations were, however, smaller than those occurring spontaneously during similar subculturing without irradiation. Repeating the experiment with a mixed culture of 10 *Salmonella* serotypes, increases in frequency

of antibiotic resistance were observed. Increases in radiation resistance ($\times 1.5$) were also observed, plausibly attributable to selection of the more resistant serotypes. It seems probable that the reported increases in antibiotic resistance might have arisen similarly, because later work (PREVITE *et al.*, 1971a) with several normal serotypes showed that those most radiation resistant had the highest frequency of antibiotic resistant cells.

2.2.3. Growth of re-cycled cells.

Except on occasions where a resistant cell is the sole survivor of the whole irradiated population (which seem likely to be rare), it will have to compete with other surviving cells during any period of subsequent growth. If resistant cells are so much slower during growth that the proportion of resistant cells falls to such a degree that subsequent irradiation fails to restore it, re-cycling will eliminate the resistant and favour the sensitive cells. Their relative rates of growth are therefore important.

In fact, it is common experience that RR cells grow more slowly than normal cells (CHRISTENSEN, 1967), and much more slowly on sub-optimal media. For example, ROBERN and THATCHER (1968a) compared the growth of parent, 1-, 6- and 12-cycle strains in a basal mineral salts + glucose medium, a chemically defined medium, and in a peptone medium; the parent and 1-cycle strains grew in all three, but the 1-cycle had a long lag in the basal medium; the 6-cycle and 12-cycle strains did not grow at all in the basal medium, but grew in the other two after a lag of several hours. IDZIAK and THATCHER (1964), making similar comparisons, had observed longer lag phases, lower exponential growth rates, and lower maximum populations, with increased re-cycling. However, the growth rates of RR strains of *Salmonella* were virtually the same as for their P strains, over a range of conditions, according to the observations of LICCIARDELLO and co-workers (1969b).

The maximum population (M-concentration) tends to be slightly less with RR than with the corresponding P strains. This was first observed by WITKIN (1946) in comparisons of *E. coli* B with B/r. It was confirmed by ERDMAN and co-workers (1961b) with *Strep. faecalis*, but with their *E. coli* strain, the 12-cycle RR strain had perhaps 10-fold the greater M-concentration. IDZIAK and THATCHER (1964), using the same or daughter cultures of *E. coli*, observed lower M-concentrations from RR cells. The data of LICCIARDELLO and co-workers (1969b), covering several *Salmonella* serotypes grown at widely different temperatures, aerobically or anaerobically, indicate a greater M-concentration in the RR strain by about 2-fold for *S. newport*, about 1.5-fold with *S. heidelberg* and *S. typhimurium*, and no difference with *S. thompson*.

There appeared to be a noticeable tendency for the RR strains of LICCIARDELLO and co-workers (1969b) to grow more slowly than the P strains after longer lag periods, at temperatures of 20 °C and below. Unfortunately

this temperature zone, the one of most practical interest, was not thoroughly investigated. The observation of ANDO and co-workers (cited by MATSUYAMA, 1972), that apparent D values for spores of *Cl. botulinum* type E are diminished with lower temperatures of incubation of the recovery medium after one irradiation, indicates that a high proportion of spores surviving irradiation have elevated minimum temperatures. The same may apply after repeated irradiation, perhaps more so.

2.2.4. Changes in pathogenicity.

Food poisoning may arise in two different ways, either as a result of an infection by bacterial cells, or through intoxication by a toxin produced by them. The power to infect is termed "virulence", and must be tested by infecting experimental animals; toxin production can sometimes be tested in other ways.

(i) Virulence. Reduced virulence as a result of repeated irradiation of microorganisms is known in a medical context. For example, LESCHKOVICH (1958) noted a marked reduction in the virulence of *Pasteurella pestis* after subjecting a culture to only three X-ray treatments. There have, however, been comparatively few tests of the effect of re-cycling on virulence, with species important in food microbiology.

LICCIARDELLO and co-workers (1969b) compared the virulence of parent *Salmonella* serotypes: *S. newport* (ATCC 6962) and *S. typhimurium* (7823), with corresponding 20-cycle RR derivates; and *S. heidelberg* (ATTC 8326) and *S. thompson* (8391) with 15-cycle RR derivates. The tests were made in two ways: (i) by inoculation into chick embryos, where a few cells sufficed to kill and the process was clearly infective; or (ii) by intra-peritoneal injection into mice, where the LD_{50} was of the order of 10^7–10^8 cells and death might have been due to intoxication. The RR strains were not significantly different in virulence from the normal parent, with one exception. With *S. typhimurium* the RR strain was less virulent, the LD_{50} of the mouse being 10 times greater. Another criterion of virulence is the speed with which test animals die: the authors state that there was no difference between RR and P strains in this respect. A test was then made to see whether animal passage might differently affect the virulence of an RR and its P strain. Cells from cultures of *S. thompson*, with a mouse LD_{50} of 350 and 150 million cells for the RR and P strains respectively, were used to kill chick embryos; then cultures derived from the latter were tested similarly in mice, yielding an LD_{50} of 118 and 35 million, resp. Both P and RR strains behaved similarly, the virulence in both being increased to a similar degree, by this single passage. The experiment was unfortunately not pursued further. Without giving details, DAVIES and SINSKEY (1973) also recorded no change in virulence to mice, as a result of re-cycling *S. typhimurium*.

PREVITE and co-workers (1971) noted that 10-fold re-cycling of several

Table 9

Mortality in 50 chicks inoculated with P and RR (36-cycle) cells of Salmonella serotypes

(simplified from EPPS & IDZIAK, 1970)

Salmonella serotype	Cell type	Total 10-day mortality
anatum	P	9
	RR	2
enteritidis	P	20
	RR	2
infantis	P	24
	RR	8
typhimurium	P	45
	RR	0

Salmonella strains at the 500 kr dose level, produced moderate decline in virulence on injection into mice; in this case, however, the treatment did not change resistance to radiation. However, a much reduced virulence in RR cells was indicated from the results of EPPS and IDZIAK (1970) with four extensively re-cycled *Salmonella* serotypes (Table 9). Their data show further that, whereas 36-cycle cells retained some virulence (killing 8–14/50 chicks), where some virulence remained, it was possessed by only a small fraction (order 10^{-4}) of the total cell population. From this it appears that re-cycling gradually eliminated the virulent cells. Several chick passages of the weakened 24-cycle culture of *S. typhimurium* did not increase its virulence. Heat-killed P cultures had no effect; embryos inoculated with cell extracts showed the same mortality from P as from RR cells. Hence it was concluded that deaths were due to infection, not intoxication by the relatively large numbers of RR cells injected.

Pathogenic bacteria usually yield "smooth" (S) colonies, "rough" (R) colonies which arise by mutation usually consisting of non-virulent cells. In salmonellas, the (S) → (R) change is associated not only with loss of virulence, but also with loss of somatic O-antigens, all three being supposed to be related (cf. ROANTREE, 1967). These relations may apparently be disturbed by radiation re-cycling. For example, LICCIARDELLO and co-workers (1969b) observed weakened response of RR *Salmonella* cells to O-antisera (especially for the relevant group), in most cases without any difference in virulence to mice or chick embryos. Again, EPPS and IDZIAK (1970) observed that with increasing re-cycling, *Salmonella* cultures tended to give (R)-type colonies and non-uniform turbidity in broth cultures with agglutination in

0.002% aqueous acriflavine; and, as just noted above, they concomitantly diminished in virulence. Nevertheless, all resistant cultures were agglutinated by parental O-antisera, and there is no indication that the reactions were weaker. CORRY and ROBERTS (1970), however, found that their 13-cycled strain of doubled RR had become very rough and, while retaining its H-antigens, had lost O-antigens 4 and 5, so that it could still be identified as a *Salmonella* but not as *S. typhimurium*. Neither could it be phage-typed. These changes were similar to those with their heat-cycled strains mentioned above.

Irradiation of an R-type culture might apparently be a likely way of inducing virulence. LICCIARDELLO and co-workers (1969b) irradiated a "rough" (R) culture of *S. newport* on an agar plate (for a different purpose), but did not report the conversion of (R) to (S) colonies; unfortunately no details are given, but there was evidently no trial of the effect of re-cycling.

(ii) Toxin production. ERDMANand co-workers (1961b) tested the effect of re-cycling on toxin production by *Cl. botulinum* type A, comparing the parent strain with a 10-cycled RR strain of about 1.3 times the parental D value. The P culture produced about $4 \cdot 10^6$ mouse MLD/ml, and the RR showed "no decrease". However, a duplicate culture "handled in a manner identical with that of the irradiated one but receiving no irradiation" produced only $5 \cdot 10^5$. These results might be taken to indicate, either that the irradiation cycling produced no change, or that it favoured toxin formation. Further investigation is necessary.

FRANK and co-workers (1971) subjected conidia-bearing cultures of *Aspergillus flavus*, 3 aflatoxin-producing and 3 non-toxigenic strains, to cyclic irradiation with up to 16-fold dosage of up to 240 kr. The intervals between irradiations were relatively long (several months) and not constant and the physiological state of cultures at irradiation was apparently variable also, it being stated that at the first few irradiations the cultures were still growing. The immediate result of this treatment was always to diminish aflatoxin production, under optimal conditions. With 3 different toxigenic strains only 3 out of 10, 0 out of 8 and 5 out of 10 sub-cultures could still produce aflatoxins on rice shortly after the irradiation programme, *i.e.* 8 out of 28. However, when the irradiated cultures were sub-cultured on artificial medium without irradiation (at relatively long intervals, over several years), the responses varied, even between sister sub-cultures from a single parent culture after irradiation: in some the ability to produce aflatoxin gradually disappeared; in others it was gradually restored; in a few it eventually attained levels 10 times greater than in the parent. By this time, 13 of the original 28 strains were producing toxin. A striking observation was the appearance of aflatoxin production in 6 out of 14 sub-cultures of the 3 strains which were non-toxigenic before irradiation, following a year or more of sub-cultivation after the cyclic irradiation. As noted earlier, this kind of erratic behaviour

probably follows from the heterocaryotic nature of the organism. Despite their observations of increased aflatoxin production, the authors concluded that, because single or cyclic irradiation much more frequently leads to a decrease or complete loss of aflatoxin production, such treatment does not increase health risk.

2.2.5. The probability of re-cycling.

In practice, the likelihood appears small that such repeated cycling would occur with foodstuffs under industrial conditions, for reasons first indicated by THORNLEY (1963b). These are essentially because, in any pasteurisation treatment, precautions (for example, cleaning of conveyors, and packaging) are necessary to prevent contamination of the treated product from the initial material. Such precautions would be equally effective in reverse, in protecting the initial product from contamination by the treated product, which must happen with some regularity if re-cycling is to occur. On the other hand, not all foods are packaged: for example, packaging might not normally be thought necessary for iced fish or for fresh poultry irradiated to destroy salmonellas. In such circumstances, the risks of recontamination and hence re-cycling might be increased. With animal feedstuffs, cyclic recontamination might be more likely because they are less carefully handled and packaged. The finding of *Enterobacteriaceae* in heat-treated feedstuffs is often regarded as evidence of such recontamination; but, as a rule, the possibility that the contamination might have been caused by external rodents or birds was not excluded. LICCIARDELLO and co-workers (1969a) also thought it improbable that events in industry would follow "the exact sequence" used in re-cycling, though there are no data to show that an exact sequence is necessary.

The problem could only exist in foods which favour microbial growth. One which does not permit vigorous growth of micro-organisms is not likely to permit their re-cycling: for example, re-cycling of bacteria is impossible on a dry food or feedstuff, as is that of fungi if the material is sufficiently dry.

IDZIAK and INCZE (1968) tried to evaluate systematically the likelihood of re-cycling salmonellas on poultry carcasses. They argued that:

(i) with the dose (250 kr) they used for re-cycling and with an upper D value of 50 kr, a carcass must carry 10^5 salmonellas if any is to survive; and

(ii) the carcass must then be stored at a sufficiently high temperature for long enough to permit the survivor to multiply to near 10^5, otherwise there would be no survivor at the second irradiation.

They justly remark that carcasses with so many salmonellas would probably be spoiled and rejected. Hence they conclude that the probability of re-cycling is negligible.

The above arguments, however, seem too simple. In the case of (i), they ignore the numbers of poultry carcasses involved, which are enormous:

100,000 carcasses with 1 salmonella each (entirely realistic figures) might apparently lead to a survivor. In the case of (ii), resistance is assumed to be the same at the second irradiation as at the first, ignoring the possibility of mutation; but it is true that a rather large change would be needed to weaken this argument substantially — even a 20% increase in RR would still require the population to reach at least 10^4 for any to survive the second irradiation, and there is seldom so large an increase in resistance at the first irradiation.

It may be added, further, that (ii) implies the improbable assumption that *all* the multiplied survivors find their way into the second irradiation, which would only occur on repeated irradiation of the same packet — a contingency to be guarded against. If only a small proportion of the multiplied survivors get into the second irradiation, they would need to have multiplied to correspondingly greater numbers. Argument (ii) therefore seems valid: it is unlikely that sufficient growth could regularly occur between irradiations to make re-cycling possible. Moreover, with regard to their first point, in justice to IDZIAK and INCZE *(loc. cit.)*, it should be recorded that they recommended a dose of 500 kr for practical use, which makes the probability of survivors lower by a factor about 10^{-5}, and requires an almost impossible degree of multiplication ($\times 10^{10}$) before the second irradiation.

Others, however, have recommended lower doses. With a dose as low as 150 kr, following the above arguments, every thousand salmonellas would suffice to leave one survivor and multiplication by only $\times 10^3$ (or less with successive irradiations) would suffice to permit re-cycling. This begins to look more like realistic possibility, hence it may be suggested that with foods like poultry where salmonella contamination is known to be regular and appreciable, radiation treatments should not use dose levels below 200–250 kr. Similar considerations might hold for staphylococci.

The argument looks different again in other circumstances. For example with a spore-bearer, even with a relatively sensitive one such as *Cl. botulinum* type E, 250 kr would scarcely reduce surviving spores by 10^{-2}, so that less than 100-fold multiplication into *spores* would suffice to make re-cycling potentially possible; and this is a species capable of multiplication at cool temperatures. On the other hand, the little evidence so far available suggest that re-cycling may not be important with *Cl. botulinum*, though further information is necessary here. To reduce the probability of re-cycling to the same order as with salmonellas would seem here to require doses of order 1000 kr. Again, the probability of significant re-cycling looks appreciable with *Aspergillus flavus*, unless doses approaching 400 kr are used; and here, apparently, re-cycling need not be rapid, frequent, or particularly regular.

The temperature at which re-cycling is effected would be important, however. A temperature too low for growth will certainly prevent re-cycling and merely to retard growth might suffice. *A. flavus* grows at temperatures

down to 10 °C, but produces appreciable amounts of aflatoxin only at temperatures above 15 °C. Possibly, therefore, re-cycling at 15° might eliminate toxin formation without preventing growth. This seems worth investigating as a general principle. All re-cycling experiments have so far been carried out with cultures maintained at near-optimal temperatures (30 °C), which is unrealistic.

The effect on radiation resistance of presumed irregular re-cycling in actual practice was examined some time ago by ANELLIS (1961). A psychrotrophic *Pseudomonas* grew on organic material accidentally submerged in the water pool used to shield a cobalt source. Here, convection currents appeared likely to effect some re-cycling though the circumstances were not established. The organism proved, however, to be sub-normal in radiation resistance.

2.3. Discussion of effects of "mutation"

There is some risk of regarding ionising radiation as if it were unique in the possibility of inducing mutations. On the contrary, there are other known mutagenic agents which act on the micro-organisms in our foods: either as "natural" environmental factors (*e.g.* cosmic rays); as natural components or additives (epoxides, nitrite, *etc.* — LOVELESS, 1951; BRUCH, 1961; VOGEL & RÖHRBORN, 1970; FISHBEIN *et al.*, 1970; GOODMAN & GILMAN, 1970; HOLLAENDER, 1971; STOLTZ *et al.*, 1974); or as widely used treatments (*e.g.* U.V. irradiation — MCDONALD & WYSS, 1959; ZAMENHOF & REDDY, 1967; RAO & JOSEPH, 1972; FILIPPOV & LEKEVICHUS, 1973). For example, it has long been known that U.V. light is mutagenic; that, like ionising radiation, it can induce resistance; and that resistance can be increased by re-cycling (*e.g.* GADEN & HENLEY, 1953). Resistance to U.V. light and to ionising radiation commonly but not always go together; a large increase in resistance caused by re-cycling under ionising radiation has been accompanied by similarly increased resistance to U.V. light (DAVIES & SINSKEY, 1973). Nevertheless, U.V. light finds widespread application in the treatment of foods, in the sterilisation of beverages and water and in the disinfection of air in food plants. Heat treatment, too, has been proved to be mutagenic, by several authors. EIGNER and co-workers (1961) demonstrated that mild heating can cause DNA strand breakage and could perhaps cause mutations or death like ionising radiation; by exposure of *B. subtilis* strain Marburg to 80–100 °C, NORTHROP and SLEPECKY (1967) produced mutants as a result of de-purination. AHMED and KAPLAN (1964) demonstrated that heating induced nutritional requirements in *E. coli* and *Serratia marcescens*. BRIDGES and co-workers (1969) discovered a correlation between sensitivities to γ-radiation and to incubation at 52 °C, among various strains of *E. coli*. ZAMENHOF (1960) reported the induction of mutants by heating dry spores *in vacuo* at

temperatures from 130 to 155 °C. Subsequently, CHIASSON and ZAMENHOF (1966) showed that, in the dry state, temperatures above 105 °C were needed for strong mutagenic action on spores of *B. subtilis* strain 168 ind$^-$; requirements for 5 amino acids were produced. Genetic changes can be brought about even by various conditions of drying; and these have been reported as a problem in the maintenance of stock cultures (WEBB, 1967, 1969; WEBB & WALKER, 1968; SERVIN-MASSIEU & CRUZ-CAMARILLO 1969).

The general mutational effect of irradiation is evidently to cause damage in genetic and biochemical mechanism, creating impairments of function and biological demands where none existed before. While this is likely to make organisms more difficult to grow and recognise, it does not seem likely to make them more pathogenic, rather the reverse.

2.3.1. Review of changes in properties.

In fact, no reports have been found relating to the acquisition of pathogenicity by a non-pathogen in this way, in particular by the saprophytic organisms which are common in foods. Moreover, irradiation of foods has not led to the appearance in foods of alien pathogens. Hence it seems reasonable, for the present, to concentrate on the effects of radiation on changes in the usual food-borne pathogens, which are relatively few.

Of the infective pathogens in foods, the salmonellas have now been examined several times after many repeated irradiations with doses which (due to acquisition of resistance) were relatively high. The evidence is unambiguous: if virulence was changed, it was reduced. No other infective pathogen has been tested similarly.

With regard to toxin production, the evidence is ambiguous. Irradiation sometimes increases toxin production and sometimes diminishes it. With *Cl. botulinum*, it appears that increases might be more frequent than decreases; with *Asp. flavus*, the opposite has predominantly occurred. There are no clear indications as to why the one or the other should happen on a particular occasion; in general terms, such behaviour might be expected on the basis of random mutations.

Irradiation-induced changes in diagnostic characters seem less serious than might be feared. The greatest changes are in shape, and often make species unrecognisable morphologically; but these changes are temporary and are rapidly reversed upon resuscitation, except in extensively re-cycled cells. The changes in biochemical pattern, or in growth response on selective media, are small and also can usually be restored by a short period of resuscitation.

With re-cycled cells, resuscitation may take longer or become ineffective. But even very extensively re-cycled salmonellas have responded normally to selective media (though numerical response has not been carefully checked). Such changes as have occurred did not suffice to obscure general identity, though they occasionally made precise identification more difficult. Never-

theless, in groups like *E. coli* where small differences in biochemical reaction are significant, difficulties might arise; and there is a hint that phage typing might be confused. These difficulties, which deserve further investigation, have been indicated only with highly re-cycled cells.

Medical authorities might well enquire whether irradiation has any effect on transferable drug resistance. The topic has not yet been investigated directly, although this would be desirable. Nevertheless, the investigations with re-cycled salmonellas, which failed to reveal any notable diminutions in sensitivity among several antibiotics and drugs, suggest that this phenomenon is not important.

There is now no doubt that increases in radiation resistance, by factors of the order of ten-fold, and ample to render radicidation or radurisation processes ineffective, could conceivably occur as a result of extensive re-cycling. But the significance of this possibility is hard to assess, for several reasons:

(i) different investigators, working under apparently similar conditions, may get very different results;

(ii) there may be big differences between closely related strains;

(iii) the effect of dose level (presumably in relation to the resistance of the organism) is not clear; and

(iv) the effect of the age of the culture at re-cycling is also not known.

It is noteworthy that all re-cycling experiments with bacteria have used growth periods of many hours at temperatures near 30° or 35 °C, which are likely to take cultures well into the stationary phase of growth, even starting from small numbers of resistant cells. This has two significant consequences; the general levels of radiation resistance are 2 or 3 times higher, and the selection pressure for growth rate among irradiated cells much lower, than if cultures had been cycled only to the logarithmic phase of growth.

Delayed or slower growth in RR mutants is understandable in the light of parallel observations on their biochemical and other properties. Further, because of the increased nutritional demands of RR cells, it might be expected that they would be at a greater disadvantage in ecologically unfavourable situations where nutritional demands are apt to be increased: for example, in poor media or at marginal temperatures. It would be useful to obtain more information about this, and about the influence of other significant ecological factors like pH and a_w. If it is correct that growth of RR strains is relatively slower than of P strains under cool conditions, re-cycling in the logarithmic growth phase — prolonged under cool conditions — would put RR cells at a disadvantage. No experiments under these conditions have yet been made.

What is clear is that, in a process using an irradiation dose large enough to produce a high degree of inactivation, a correspondingly large degree of

multiplication between doses is necessary if re-cycling is to continue. This is impossible save in favourable circumstances, and it could be prevented by appropriate hygienic measures or by simple reduction of temperature in the irradiation plant.

In these respects, the application of ionising radiation to food is no different in principle from its application in any other context where micro-organisms can grow. The fact that the widespread medical use of radiation (unlike antibiotics) has caused no trouble from altered or more virulent forms of micro-organisms is the best possible indication that analogous difficulties are not probable with food. The doses of radiation normally used in medicine may be relatively small, but the total amount of radiation hitherto used must be very large.

It should always be remembered that to confine attention to mutational increase in pathogenicity etc. neglects the effect of irradiation on the probability of its occurrence at all. For example, a dose of 200 kr which occasionally might perhaps double the likely production of aflatoxin, will reduce the number of surviving *Aspergillus flavus* spores about a thousand-fold, more than correspondingly decreasing the number producing any toxin whatever. Larger doses, moreover, have diminished aflatoxin production while reducing the proportion of surviving spores still more.

2.3.2. Possible restrictions.

Supposing it were concluded that there is some risk from the development of more virulent and resistant mutant microorganisms in irradiated food, one should consider whether restrictions on conditions of storage should necessarily follow.

In similar situations, corresponding action is sometimes not thought to be immediately necessary. For example, by a change similar to mutation, exposure of *Enterobacteriaceae* to an antibiotic can produce enhanced resistance, not only to the same antibiotic but also to other antibiotics and drugs. Such multiple resistance is equivalent to an increased virulence in the sense that the resulting diseases are much more difficult to cure; and this property can be transmitted from one species to another. Yet the phenomenon, occurring with well-recognised pathogens, has not been thought in many countries even to warrant prohibition of the use of antibiotics in feedstuffs for animals. Instead, the situation is monitored on a "wait and see" basis, to look out for the appearance of the altered strains and assess their significance. A similar system of surveillance would apparently suffice in the case of irradiation, until the size of the problem became evident in practice. Routine monitoring of isolates would present no special difficulty in the case of toxin-forming strains or in respect of associated radiation resistance. With infective pathogens like *Salmonella* the routine assessment of virulence would be more troublesome, but quite possible. The concept of the Hazard Analysis Critical Control Point

(HACCP), now established in the U.S.A. (BAUMANN, 1974; PETERSON & GUNNERSON, 1974), could be used to establish the source of any risk which might develop.

Alternatively, if the difficulty arose as the result of re-cycling as seems most likely, it would suffice to operate the irradiation plant near or below the minimum growth temperature for the pathogen, so as to prevent sufficient re-growth of survivors which is essential for re-cycling. This precaution would be relatively simple; and seems entirely practicable, as similar requirements operate in most meat-handling plants.

Even if it were agreed that precaution was necessary to prevent multiplication of mutants after irradiation, a general requirement to store at temperatures below 0 °C should not be necessary. As regards the organisms which usually cause food poisoning, they are well known to be controlled adequately by temperatures in the range 5–15 °C. Different foods might require different temparature limits, according to the hazards presented. With fish, where *Cl. botulinum* type E is a known hazard, slightly lower temperatures might be advisable, *e.g.* 3 °C.

A general need to store below 0° would arise only in either of two eventualities:

(i) if, in existing pathogens, increased virulence or radiation resistance were to become associated with a distinct lowering of minimum growth temperature permitting multiplication near 0°;

(ii) if existing psychrotrophic organisms, already able to multiply at 0° and at present non-pathogenic, were to acquire unusual pathogenic properties.

In the case of (i), various attempts have been made to produce psychrotrophic mutants from (non-pathogenic) mesophiles for experimental purposes, using a variety of mutagens including ionising radiation. These attempts rarely succeed. AZUMA and co-workers (1962) obtained psychrophilic mutants by ultra-violet irradiation of mesophilic pseudomonads. However, the usual effect of mutagens is to narrow temperature limits (HAGEN, 1971). Consequently, possibility (i) seems improbable. Actually the opposite seems likely, that radiation will *raise* minimum temperature, though this needs confirmation.

In the case of (ii) we have already noted the absence of reports that non-pathogenic species can acquire alien pathogenic properties *de novo*, as a result of ionising irradiation, or otherwise. Further, even if this were to happen in some particular species, it need not create a general problem because the psychrotrophic spoilage flora is apt to be characteristic of a particular type of food, and hence the problem would be limited to those foods on which the species in question occurred. A comparable example is the very different treatments accepted for "neutral" foods which present hazards from *Cl. botulinum* and for "acid" (pH < 4.5) foods which do not.

Finally, it is worth recalling that heating can produce mutations, like radiation, though they have been studied to a comparatively small extent. Because heat resistance can develop in a similar way to radiation resistance, yet heating remains an effective commercial process, the development of radiation resistance seems unlikely to become a problem in radiation processing. Moreover, the latter process is expected to be performed under much more strictly controlled conditions than the widespread and variable use of heat processing.

*

The present review has been prepared with the invaluable assistance of reports on different aspects by Prof. R. B. Maxcy (university of nebraska, Lincoln, Nebraska 68508, U.S.A.) and Dr. D. B. Rowley. (Head, food microbiology group, food sciences laboratory, united states army development center, Natick, Massachusetts 01760, U.S.A.

Since the present review was prepared, our attention has been drawn to an important paper by Idziak (1973), in a now journal. It too considers conceivable hazards from increases in radiation resistance, pathogenicity, toxicity and antibiotic resistance, and from alterations in biochemical characteristics and ecological responses. It concludes that there is no indication of special bacteriological hazards associated with radiation processing.

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THE EFFECT OF IRRADIATED DATES ON THE DEVELOPMENT OF *ORYZAEPHILUS SURINAMENSIS* (L.), *COLEOPTERA CUCUJIDAE*

M. S. H. Ahmed, S. B. Lamooza, N. A. Ouda and J. Farkas

(Received June 5, 1975)

Date fruits were treated with different high doses of gamma radiation and used as a full diet for *Oryzaephilus surinamensis*. Eggs of this beetle were seeded on treated and untreated dates. Four doses were applied and parallel controls (unirradiated dates) on the experimental series were also run. After 30 days of incubation at 25 °C and 60—70% RH, the dates infested with eggs were thoroughly examined and all available developmental stages were recorded.

Statistical analysis of the data indicated that the dates irradiated with doses of 1250, 2500 and 5000 krad resp., resulted in a significant difference in developmental delay and in killing effects. While the lower dose of 625 krad did not show any significant difference in comparison with the controls. Therefore, disinfestation of dates by low economical doses might not have any adverse effect on date quality.

Studies on radiation preservation of date fruits have been started some time ago (Mayahi & Ahmed, 1970; Ahmed, 1970; Auda & Al-Rikabi, 1971). These studies include mainly: disinfestation of stored dates, extension of shelf life of fresh dates, and chemical changes induced by radiation. The main objective of the date irradiation programme is to develop a means of reducing date spoilage caused by insects.

Studies so far have shown that disinfestation can be achieved by rather moderate doses of gamma radiation from ^{60}Co. However, wholesomeness tests must be carried out before any international clearance could be granted to date disinfestation by radiation since it is generally accepted that no known hazard to health should be caused in the utilization of irradiated foods (IAEA, 1970). Experiments on wholesomeness have been intensified since the thalidomyde episode. More accurate toxicological tests on differentiated and undifferentiated cells are being carried out to assess the effect of any substance of potential utility which, of course, include preservation of foodstuffs by radiation. Thus, different techniques for the assessments of the effect of irradiated foods to experimental organisms were used, such as: short- and long-term feeding, injection, treated media, etc. Experiments on the wholesomeness and safety of irradiated foods migth be carried out by feeding animals at a level of 35% of the total diet on a dry-weight basis or in some cases at a 15% wet weight of the total diet (Fowler, 1971). A large number of data on

the possible induction of radiomimetic effects by feeding upon irradiated food has been accumulated (SCHUBERT, 1969; MOUTSCHEN-DAHMEN *et al.*, 1970). The biological effects of irradiated food might be brought about by possible indirect effect of ionizing radiation (DE *et al.*, 1969).

It is thought that the period of feeding and the ratio of irradiated to unirradiated components of the foods in the media play an important role in this respect to reveal the actual effect. On the other hand, short- and long-term feeding may not lead to any effects, hence giving rise to inaccurate conclusions.

It is interesting to note that stored dates constitute an adequate diet for all developmental stages of the saw-toothed grain beetle, *Oryzaephilus surinamensis*. Therefore, it was thought advisable to carry out exploratory studies on the effect of irradiated dates, used as a full diet, on the development of *O. surinamensis*.

1. Materials and methods

Eggs of *Oryzaephilus surinamensis* were obtained from laboratory cultures (AHMED *et al.*, 1971). The eggs, 1—48 hours old, were collected and divided into 10 batches of 200 eggs each.

Irradiation of dates (Zahdi variety) was carried out with different doses of gamma radiation from a ^{60}Co source of the type Gammacell 220 at a dose rate of approximately 528 krad h^{-1}. The doses used were: 0 (control), 625, 1250, 2500, and 5000 krad. Each treatment comprised 2 replicates (two 600-ml beakers). In each beaker 35 date fruits were placed. Then for *wholesomeness* tests, 200 eggs were put in each beaker. All the irradiated and unirradiated samples were held at a temperature of 25 $\pm$ 1 °C and 60—70 % RH. After a period of 30 days the dates in each beaker were carefully examined. This incubation period is quite enough for the length of the life cycle of *Oryzaephilus surinamensis* (KURTZ & HARRIS, 1962). The numbers of larvae, pupae and adults were separately recorded.

2. Results

The results of the examination of irradiated and unirradiated dates infested with eggs of *O. surinamensis* are shown in Table 1. The numbers of larvae, pupae and adults of the saw-toothed grain beetle are separately depicted for each replicate. In Table 2 the average numbers and their standard deviations of all stages taken together and that of the adults alone are shown.

Table 1

Development results of 200 Oryzaephilus surinamensis *eggs reared on irradiated and unirradiated dates* (Zahdi *variety*)

Development stage	Control (unirradiat.)		625 krad		1250 krad		2500 krad		5000 krad	
	Replicate 1	Rep. 2	Rep. 1	Rep. 2	Rep. 1	Rep. 2	Rep. 1	Rep. 2	Rep. 1	Rep. 2
Larvae	0	0	2	1	0	0	0	2	0	0
Pupae	6	4	7	8	8	27	43	58	27	28
Adults	104	106	98	88	65	50	8	26	19	12

It is worth mentioning that some increasing doses of gamma radiation caused an obvious softening and in some cases rupturing of many irradiated date fruits, especially when high doses were applied. This phenomenon is, generally, in agreement with the results obtained after irradiating fruits (SREENIVASAN *et al.*, 1971).

Table 2

Average numbers with standard deviations of insects developed from 200 eggs of Oryzaephilus surinamensis *reared on irradiated and unirradiated (control) dates*

Radiation dose, krad	No. of adults	No. of larvae + pupae + adults
0	105.0 ± 1.4	110.0 ± 0.0
625	93.0 ± 7.1	102.0 ± 7.1
1250	57.5 ± 10.6	75.0 ± 2.8
2500	17.5 ± 12.7	68.0 ± 24.7
5000	15.5 ± 4.9	43.0 ± 4.2

From Tables 1 and 2 it can be conspicuously noticed that the number of insects decreased as the radiation dose of the dates increased. The decrease in number was consistent in all cases in which insects were raised on gamma-irradiated dates compared with those which developed on unirradiated (control) dates. All the data were subjected to statistical analyses. The variance ratio tests (F) showed that the effect of irradiated dates on the number of adult insects developed from 200 eggs during 30 days of incubation is highly signifi-

cant since $F = 49.3$ (Table 3) and is significant at less than 0.1% level. At the same time from Table 4 it is noticed that the irradiation has a significant effect on the numbers of larvae, pupae and adults taken together. However, F is significant at the 5% but insignificant at 1% level.

Table 3

Analysis of variance for the effect of irradiated dates on the number of adult insects developed from 200 eggs during 30 days of incubation

Source of variance	D.f.	S.S. (Sum of squares)	M.S. (Mean squares)
Treatments	4	13841.4	3460.35
Residual	5	351.0	70.2

Table 4

Analysis of variance for the effect of irradiated dates on the number of insects in all stages of life cycle developed from 200 eggs during 30 days of incubation

Source of variance	D.f.	S.S.	M.S.
Treatments	4	5819.6	1454.9
Residual	5	688.5	137.7

Table 5 shows the *Duncan* test for the average number of individuals developed from a batch of 200 eggs as affected by the irradiated dates with different doses of gamma radiation. This Table obviously shows a significant delay in development at radiation doses higher than 625 krad (see the first row of the Table). Doses of 1250, 2500 and 5000 krad also caused a significant increase in the killing effect as it is evident in the second row of Table 5.

Table 5

The average number of individuals from 200 eggs as affected by the irradiation of dates used for rearing Orizaephilus surinamensis *(Within the same row the average values not marked with the same letter significantly differ at the 5% level. Otherwise the difference is insignificant)*

No. of individuals	0 krad	625 krad	1250 krad	2500 krad	5000 krad
Number of adults	105 a	93 a	57.5 b	17 c	15.5 c
Number of larvae + pupae + adults	110 a	102 a b	75 b c	68.5 c d	43.0 d

3. Conclusions

From the data presented it is evident that dates treated with a dose of 525 krad of gamma radiation did not cause either a killing effect or a significant difference in the developmental period of *Oryzaephilus surinamensis* as compared with the unirradiated (control) date fruits. This dose is about 10 times the dose required for delayed killing of the most radioresistant stage of this insect (AHMED, 1970). However, even prompt killing requires doses in the range of 5×10^5 rad, but much smaller doses may suffice for delayed effects (O'BRIEN & WOLFE, 1964). Furthermore, delayed killing at economical low doses might not have any adverse effect on dates.

On the other hand, the delay in development and the killing effect of dates treated with high doses (1250, 2500 and 5000 krad) might be ascribed to toxicity or/and softness of the dates or to other factors induced by gamma radiation. At this stage of the work it is difficult to conclude which factor (or factors) has resulted in the above-mentioned effects. However, developmental delay is not always considered as a guide to mutagenicity, and genetical damage could be negatively correlated with developmental delay (AUERBACH, 1966). Therefore, the present experiments did not necessarily demonstrate the mutagenic effect of irradiated dates.

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THE STORAGE OF WHEAT AND CORN OF HIGH MOISTURE CONTENT AS AFFECTED BY IONIZING RADIATION

I. KISS and J. FARKAS

(Received July 29, 1976)

Preliminary experiments were carried out with varieties *Fertődi* and *Bánkúti* at 23% moisture content. These samples were stored in hermetically sealed containers at room temperature. Mould growth on the wheat samples of 23% moisture content was successfully inhibited by treatment with 0.4 and 0.8 Mrad radiation doses. Mildew in the untreated samples and those given a treatment below 0.2 Mrad appeared after a storage period of 1.5 months.

Varieties *Fertődi 1201* and *Bánkúti*, with their moisture contents adjusted to 18.5%, were kept in a space of equilibrium relative humidity (ERH) at room temperature and aerated. Under these conditions their shelf life (the time till the appearence of mildew) was doubled (increased from 12 to 26—48 days) by treatment with 0.4 and 0.8 Mrad. Total aerobic viable cell count and mould count in the samples thus treated were lower by about two orders of magnitude, even after 57 days of storage, than those of the untreated samples. The increase during storage of alpha-amylase activity in wheat of high moisture content was completely inhibited by treatment with 0.8 Mrad.

Under conditions ensuring drying, in paper bags at 18—25 °C, untreated batches of the varieties *Kiszombori* and *Bezostaya* of 18.2 and 15.5% initial moisture content, resp., were free of mould during a storage period of 2.5 months. The differences in cell counts between samples irradiated and untreated were apparent during the whole storage period. The microbial contamination of samples given 0.15—0.16 Mrad treatment was lower by 1—2 orders of magnitude than that of the untreated samples throughout the storage period. At the beginning, alpha-amylase activity in the variety *Bezostaya* was about four-fold as compared to the other wheat varieties and was found to remain constant in the first two months of storage and to increase slowly only afterwards. With the variety *Kiszombori* the alpha-amylase activity of the radiation treated and the untreated samples remained almost identical, while with the variety *Bezostaya* lower activity of the treated samples was observable during storage, while the difference between the activities of the treated and untreated samples increased. After a storage period of three months the wheat samples were milled. Differences in the rate of extraction, in total protein, ash content, gluten content and colour of the flour to be traced back to radiation treatment, were not observed. Baking tests have shown an increasing tendency in the volume and form quotient of the bread baked from the flour of irradiated wheat in comparison to the bread made from untreated flour. However, differences were of no statistical significance.

Spoilage in shelled corn of 31.2% moisture content and stored in jute sacks at about 5 °C could be retarded for a relatively short time when treated with radiation doses up to 0.6 Mrad. The untreated samples were spoiled in two weeks. At the same time the samples irradiated with 0.15—0.6 Mrad were found acceptable when tested for musty smell. However, after 4 weeks these were spoiled, too.

Corn on the cob of 22.6% moisture content did not resist spoilage during the two months storage in jute sacks at 2—5 °C. Without dehydration the moisture content was not reduced to 15% at which value the corn would have been safely storable. However, a radiation dose of 0.075 Mrad was sufficient to keep cell count at a low level throughout the two-month storage period.

In mechanical harvesting cereals are collected at a relatively high moisture content. This fact increases the danger of spoilage and thereby the difficulties during storage.

The danger of microbial spoilage may be reduced by the decrease of the moisture content by drying or by the application of chemical agents (propylene oxide, propionic acid, hydrogen peroxide).

Since dehydration is a lengthy and fuel requiring process and output requirements are high and spasmodic at the time of harvest, great difficulties arise. The use of chemicals involves the danger of residues. Thus in this study the applicability of ionizing radiation for the extension of the storage capacity of high-moisture wheat and corn, was investigated.

1. Materials and methods

1.1. Materials

1.1.1. Wheat varieties. The wheat varieties used in the experiments were as follows: *Bánkúti*, *Fertődi 1201*, *Bezostaya* and *Kiszombori*. In the preliminary experiments with wheat of high moisture content (23.0%) the varieties *Bánkúti* and *Fertődi* were used. In further experiments wheats of lower moisture contents were used: *Bánkúti* and *Fertődi* with 18.5%, *Kiszombori* with 18.2% (the moisture content of these samples had to be increased by spraying with water because their initial moisture content was about 16%, only) and the variety *Bezostaya* with 15.5%.

1.1.2. Corn. Experiments were carried out with shelled corn and corn on the cob. Samples were obtained from the MARTONVÁSÁR ESTATE OF THE AGRICULTURAL RESEARCH INSTITUTE OF THE HUNGARIAN ACADEMY OF SCIENCES. Moisture contents of the corn on the cob and of the shelled corn were 22.6% and 31.2%, resp.

1.2. Irradiation

In the preliminary experiments with wheat of high moisture content samples were irradiated with the X-ray apparatus "*Stabil 250*" (15 mA, 200 kV) at a dose rate of 0.2 Mrad (2 kGy) h^{-1}. For this treatment the samples of high moisture content were sealed in polyethylene film in order to prevent the decrease of the moisture content.

In storage experiments of larger volume both wheat and corn were treated in a panoramic radiation source of 50 kCi (1850 TBq) nominal activity. Wheat samples were treated with 0.05, 0.075, 0.10, 0.15, 0.20, 0.30, 0.40, 0.60 and 0.80 Mrad, resp., corn with 0.075, 0.15, 0.3 and 0.6 Mrad, resp. The

temperature of the chamber was 15 °C at 75% RH. The dose rate of the source was 0.2 Mrad h^{-1}. A solution of ferrous sulfate was used for dosimetry (WEISS *et al.*, 1955).

1.3. Storage

1.3.1. Wheat. The effect of radiation treatment was studied in the preliminary experiments with wheat samples of 23.0 and 18.5% moisture content. (According to the sorption isotherm constructed on the basis of data in the literature these moisture contents correspond to 93 and 87% ERH, resp.)

After treatment the samples of 23.0% moisture content were poured aseptically into wide-mouth bottles with ground stopper. To ensure hermetic sealing the stopper was poured round with paraffin. These samples were stored in a thermostat of 25 °C. Under these conditions no loss of moisture could occur. However, after a longer storage period in the closed space the accumulation of respiratory carbon dioxide was expected, which in its turn could have affected the growth of highly aerobic fungi. For this reason in the preliminary experiments the wheat of 18.5% moisture content was kept not in bottles but in muslin bags of loose texture and the bags were placed in large glass containers. Through the container air of RH equivalent to the equilibrium relative humidity of wheat was pumped and thus CO_2 accumulation was prevented. To adjust the relative humidity of air to the level required, gas washing bottles, filled with sulfuric acid of the appropriate concentration were used.

This method of storage approximated the storage conditions of wheat kept in aerated space better than thermostating in sealed bottles. The glass containers were kept at room temperature. In the common air space the possibility of mutual contamination of the wheat samples kept in loose muslin bags arose, thus the resistance of the radiation-treated samples to contamination could be judged.

The varieties *Kiszombori* and *Bezostaya*, of 18.2% and 15.5% moisture content, resp., were stored in paper bags at 18–20 °C and 60% RH for 4 months, hereafter at 5 °C in a storage space of 75% RH. Each bag contained 10 kg wheat, two bags to each treatment level.

1.3.2. Corn. Both corn on the cob and shelled corn were kept in jute bags at 0–5 °C and 70–90% RH.

1.4. Determination of the mesophilic total and fungal cell count

For the microbiological test the *wheat* samples were homogenized, 20 g were weighed to cg accuracy. Eighty ml sterile water were added and the test portion was suspended in an Ato-mix blender (MSE, 14 000 rpm) by 2×1-

min-treatment. A dilution series was prepared and of this 1 ml each were inoculated into nutrient broth containing 0.5 % tryptone, 0.25 % yeast extract and 0.15 % glucose (pH 7.0) determining mesophilic total viable cell count and on slant malt nutrient (5 % refr. index) solidified with agar, three parallel samples at each dilution level, resp., determining fungal cell count. The inoculated samples were incubated at 30 °C for 7 days. The viable cell count was determined with the aid of *Hoskins*' tables on the principle of the most probable cell count. Results were related to 1 g of wheat.

Of the *shelled corn* 100 g were weighed in a sterile flask and shaken for 5 min with 400 ml sterile water containing 0.2 ‰ Tween 80. A dilutions series was prepared from the suspension. *Corn on the cob* was shaken for 5 min with 1300 ml Tween 80 solution. Cell counts were determined as above.

1.5. Determination of moisture content

The samples were milled in a cereal mill. Ten g portions were dried to constant weight in an "*Ultra-X*" (System *Gronert*) infrared apparatus for moisture determination. The moisture content in % was read directly from the apparatus (DEMEL-GYÖNÖS, 1958).

1.6. Determination of equilibrium relative humidity

The ERH of the samples was determined by the crystal liquefaction method (VAS & CSONTOS, 1956).

1.7. Determination of protein content

The raw protein content (total nitrogen) was determined by the micro-*Kjeldahl* method according to *Wagner-Parnas* (HUNGARIAN STANDARD, 1966).

1.8. Determination of ash content

The flour was ignited at 550 °C to constant weight.

1.9. Determination of the gluten content and spreading of gluten

Twenty four g of flour were mixed with 12 ml tap water. The dough was allowed to stand for 30 min under a glass bell. Then the gluten was washed over a sieve of 200 micron mesh with a water jet of 20 °C. From the gluten thus obtained 5 g were weighed, formed into a ball and allowed to stand under a beaker in humid space for an hour during which the spreading of the ball was measured.

1.10. Determination of the Zeleny number

To 3.2 g flour bromophenol blue solution was added and the mixture was shaken. Then isopropanol solution containing lactic acid was added and the mixture was again shaken. The volume of the swollen flour grains was read in the measuring cylinder. The value obtained was used to calculate the sedimentation value. The flour was classified on the basis of this value.

1.11. Determination of the colour of flour

The colour of the flour was determined by the *Pekár* test (HUNGARIAN STANDARD, 1953).

1.12. Determination of alpha-amylase activity

The method of SANDSTEDT and co-workers (1939) was used to determine alpha-amylase activity. Five g of flour were shaken for 1 h in a glass bottle with ground stopper in 40 ml distilled water. The suspension was filtered through a folded filter paper into a 50-ml graduated flask. The precipitate on the filter was washed with distilled water and the flask made up to the mark. Ten ml of this solution were mixed with 20 ml buffered amylodextrin solution and placed in a water bath of 30 °C. Every minute 0.6 ml of the mixture was added to an iodine solution till the colour became equal to that of the comparative solution. The activity as calculated from the degradation time was given in SKB units (SKBU).

1.13. Baking test

The baking test was carried out according to the HUNGARIAN STANDARD (1953). Three hundred g of wheat flour were used to make the dough and 400 g dough were made into bread. The volume and the form quotient of the bread were determined.

2. Results

2.1. Experiments with wheat

2.1.1. Storage life

2.1.1.1. The storage life in closed space of wheat of 23% moisture content as affected by radiation treatment. — The microbial spoilage of wheat of this moisture content may be retarded throughout a long period by treatment with

0.4 Mrad. On samples treated with 0.4 and 0.8 Mrad resp., mildew was not observed after a 5-month storage period. On samples untreated or treated at lower dose levels mould growth started after about 1.5 months of storage.

2.1.1.2. The effect of radiation treatment on wheat of 18.5% moisture content in aerated space of equilibrium relative humidity. — In this experiment *Fertődi 1201* and freshly harvested *Bánkúti* wheat varieties were used. Since the moisture content of the samples was as low as 16.1% at the start, the samples were sprayed with a calculated amount of water and kept for 24 h in a closed space to allow for the equalization of the moisture content. The moisture content of the samples rose to 19.3%. Hereafter the wheat was divided into bags. The bags were placed in large glass containers of 88% RH, equivalent to 18.5% moisture content. (The containers were kept at the required moisture level by an air flow of controlled moisture content, see para. 1.3.1.) As seen from the results mould growth on the wheat was successfully retarded by treatment with 0.4 and 0.8 Mrad. The shelf life of the treated samples was increased to 2–4-fold of the control, even under conditions when contamination of the treated samples was not excluded. The time elapsing till the beginning of mould growth is shown in Fig. 1.

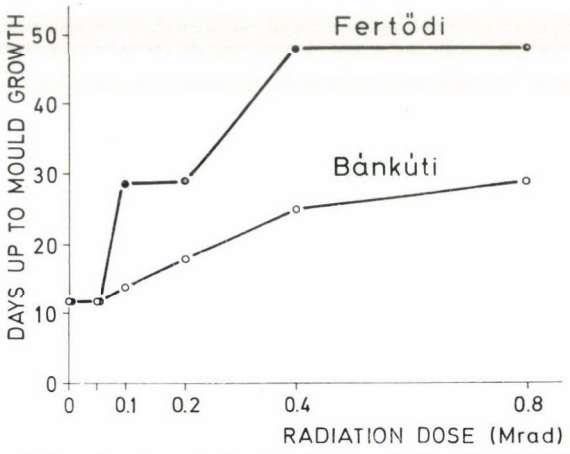

Fig. 1. Moulding of the wheat varieties *Bánkúti* and *Fertődi* of 18.5% initial moisture content as a function of radiation dose (at 22 °C and 88% RH)

The difference in the tendency to spoil of the two wheat varieties may be due to their fungal flora of different resistance or to the difference in their initial microbial contamination.

The efficiency of the radiation treatment is illustrated by the fact that after a storage period of nearly two months (57 days) the difference in the total aerobic and fungal cell counts between the treated and untreated samples was still significant (Fig. 2).

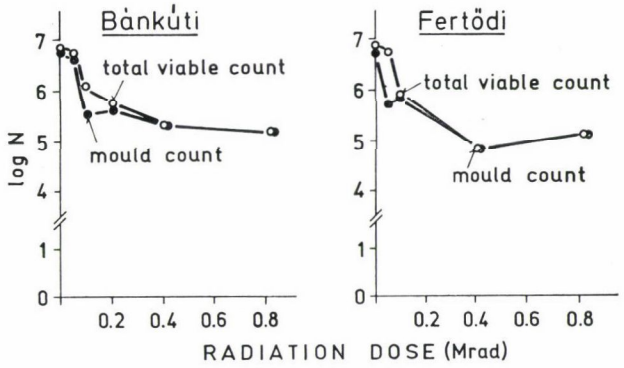

Fig. 2. Mesophilic total viable cell count and mould count in wheat of 18.5% moisture content after a 57-day storage period as a function of radiation dose (at 22 °C and 88% RH)

2.1.1.3. The effect of radiation treatment upon the microbial count of the varieties Kiszombori and Bezostaya stored in an environment ensuring drying.— The varieties *Kiszombori* and *Bezostaya* of 18.2 and 15.5% moisture content, resp., were not stored in a space of ERH but under conditions generally found in practice, loosing moisture to the environment. Difficulties were not encoun-

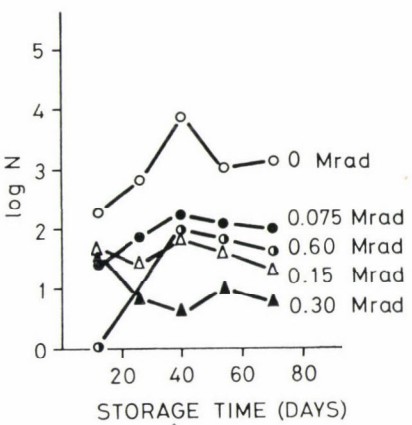

Fig. 3. Mould count in the variety *Kiszombori* of 18.3% initial moisture content as a function of radiation dose and storage period (at 18–20 °C and 60% RH)

tered in their storage. Thus, it was possible to study the effect of irradiation upon their baking quality. The mould count was lower by five orders of magnitude than that of the varieties *Bánkúti* and *Fertődi*. However, at given dose levels the microbial count was reduced to an identical extent. In samples treated with 0.15 Mrad or at higher dose levels, the mould count remained at the initial low level while that of the untreated samples increased during the first month of storage (Figs. 3 and 4).

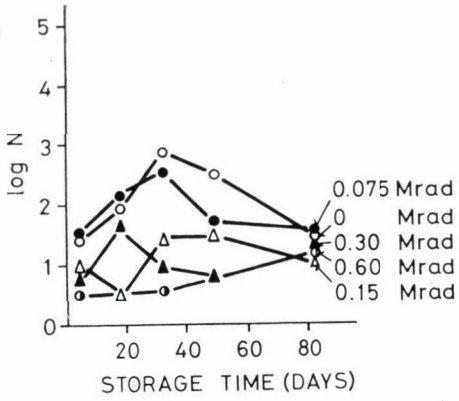

Fig. 4. Mould count in the variety *Bezostaya* of 15.5% initial moisture content as a function of radiation dose and storage period (at 18–20 °C and 50% RH)

In the experiments with the variety *Kiszombori* it was found that fungi surviving treatment with 0.3 Mrad were in the majority Fusaria. From wheat samples treated with 0.6 Mrad yeasts were isolated.

2.1.2. Change in alpha-amylase activity during storage

During the storage of wheat varieties of high moisture content (18.5%, para. 2.1.1.2) it was established that radiation doses ensuring microbial stability affect the biochemical processes preceding germination, too. The changes induced by germination processes as initiated during storage in atmospheres of high moisture content are unfavourable in relation to the baking quality. Since of the enzymes in the cereal grain alpha-amylase becomes activated, among others, only when germination starts, it seemed reasonable to observe amylase activity in order to detect the early stages of germination. Alpha-amylase activity was determined by the method of SANDSTEDT and co-workers (1939). Activity per g sample was calculated on the basis of degradation times.

The results of activity measurements show that if the wheat sample displayed alpha-amylase activity at the beginning *(Fertődi)* this activity was reduced directly by radiation treatment to an extent increasing with increasing dose levels. Alpha-amylase activity rapidly increased during storage in wheat samples of 18.5% moisture content, not treated or given 0.05 Mrad treatment. After fifty days the activity was by two orders of magnitude higher than initially. The activity of wheat samples treated with 0.1 Mrad just doubled during the same period, while that of samples treated at higher dose levels did not change at all. However, after a storage period of four months, the activity in samples treated with 0.2 Mrad increased, while in those treated with 0.4

and 0.8 Mrad, resp., it remained at its original level. It may, further, be concluded from the results that the rate of increase in alpha-amylase activity of the varieties *Bánkúti* and *Fertődi* is different, the rate being higher in the former than in the latter (Figs. 5 and 6).

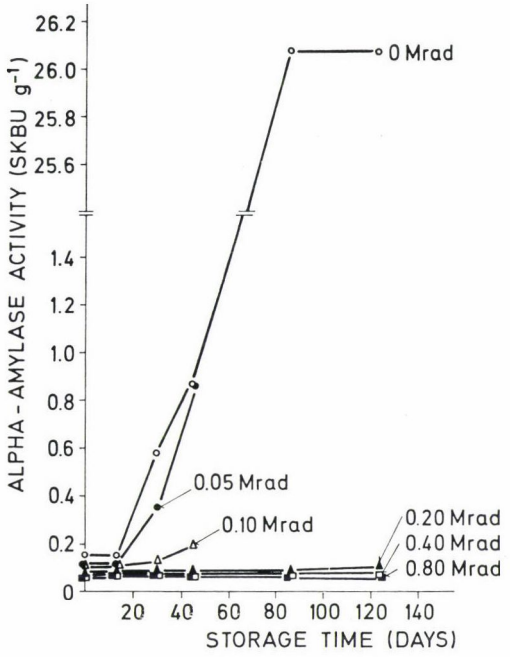

Fig. 5. Alpha-amylase activity, in SKBU g^{-1}, of the variety *Bánkúti*, of 18.5% initial moisture content, as a function of radiation dose and storage period. The moisture content of the wheat was maintained at the initial level (22 °C and 88% RH)

It was found that the alpha-amylase activity of the variety *Kiszombori* with an initial moisture content of 18.3% reduced to the value of 12.8% during storage, was independent of the radiation dose and was identical with that of the untreated sample. In contrast, the activity of the variety *Bezostaya*, of 15.5% moisture content when not treated, increased to three-fold as compared to the irradiated samples. However, the activities of the irradiated samples were independent of the dose level and identical (Figs. 7 and 8).

2.1.3. Extraction rate, Pekár test, Zeleny number

Fractions of the milled product were weighed and it was established, by analysis of variance, that the respective proportions of bran, semolina and flour were not affected by the different doses of irradiation (Table 1).

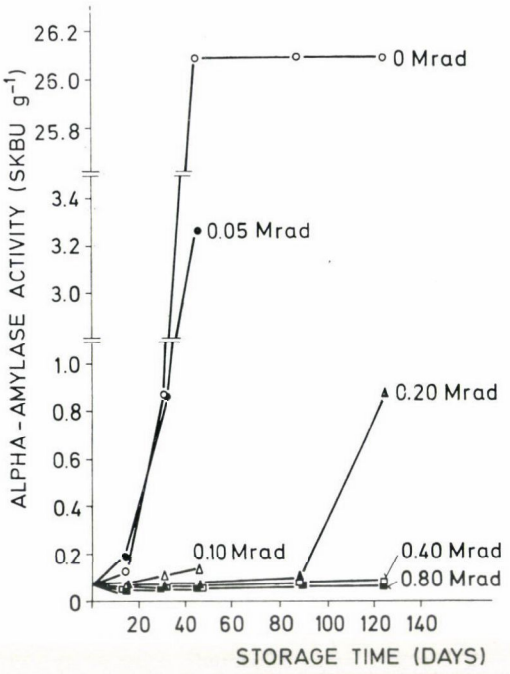

Fig. 6. Alpha-amylase activity, in SKBU g^{-1}, of the variety *Fertődi*, of 18.5% initial moisture content, as a function of radiation dose and storage period. During storage the moisture content was constant (22 °C and 88% RH)

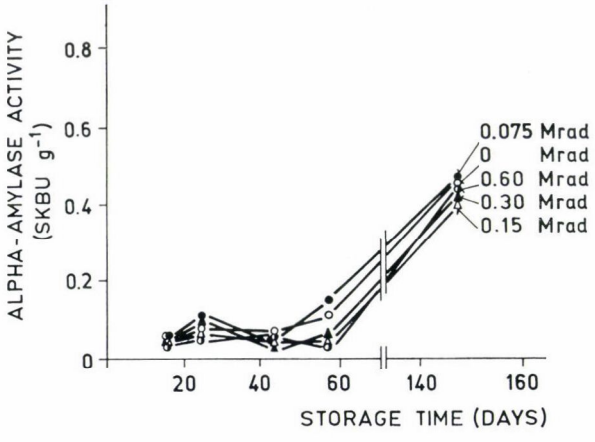

Fig. 7. Alpha-amylase activity, in SKBU g^{-1}, in the variety *Kiszombori* of 18.3% moisture content, as a function of storage period and radiation dose. During 70 days of storage the moisture content decreased to 15.5% (stored at 18–20 °C and 60% RH)

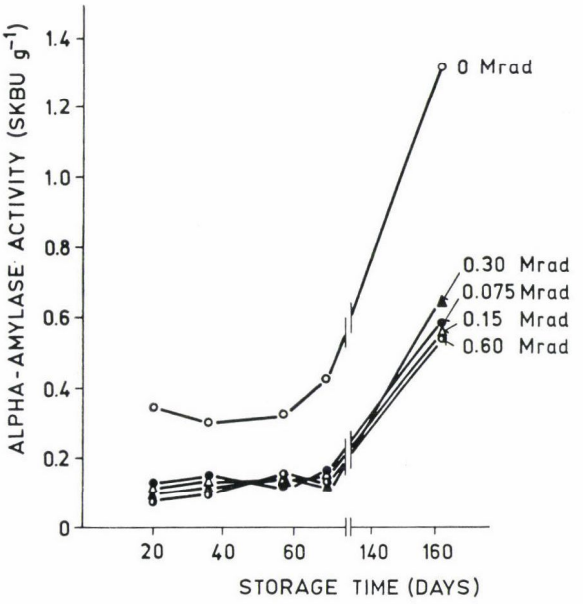

Fig. 8. Alpha-amylase activity, in SKBU g^{-1} in the variety *Bezostaya*, of 15.5% moisture content, as a function of storage period and radiation dose. During 70 days of storage the moisture content decreased to 14.7% (stored at 18–20 °C and 60% RH)

In the *Pekár* test the colour of the flour from the variety *Kiszombori* was slightly darker than the colour sample, the colour of *Bezostaya* was nearly identical. Radiation treatment did not cause changes in colour. In the sedimentation test (*Zeleny* number) no clear-cut tendency could be detected

Table 1

Percentage distribution of the milling fractions of wheat as a function of radiation dose

Bezostaya

Milling fraction		Radiation dose (Mrad)					Variance ratio (F)
		0	0.075	0.15	0.30	0.60	
Bran	$\bar{x}$	10.77	10.78	10.54	10.56	10.24	4.34
	s	0.15	0.04	0.06	0.13	0.26	
Semolina	$\bar{x}$	37.29	38.16	38.36	36.91	35.95	1.63
	s	0.46	0.75	0.37	0.37	2.20	
Flour	$\bar{x}$	51.95	51.06	51.06	52.62	53.82	1.93
	s	0.32	0.78	0.37	0.37	2.45	

Kiszombori

Milling fraction		Radiation dose (Mrad)					Variance ratio (F)
		0	0.075	0.15	0.30	0.60	
Bran	$\bar{x}$	11.26	11.64	11.32	11.66	11.87	0.99
	s	0.37	0.0	0.62	0.28	0.24	
Semolina	$\bar{x}$	37.43	37.23	38.42	38.86	38.44	1.70
	s	0.37	0.28	0.63	0.45	0.42	
Flour	$\bar{x}$	50.66	51.13	50.27	49.49	49.69	3.54
	s	0.93	0.28	0.01	0.73	0.67	

	mesh
Bran	2 000 μm silk sieve
Semolina	400 μm silk sieve
Flour	150 – 200 μm wire sieve

$\bar{x}$ = mean value (%)
s = standard deviation
Critical F value for degrees of freedom $V_1 = 4$, $V_2 = 5$ at 95% probability level: $F = 5.19$

upon treatment with increasing radiation doses, only a fluctuation was observed. The mean *Zeleny* number for the variety *Kiszombori* was found to be 34.1 ± 1.1 and for the variety *Bezostaya* 48.3 ± 4.0.

2.1.4. Protein and ash content

The total nitrogen related to solids content of *Bezostaya* was 2.65%, that of *Kiszombori* 2.47% in the untreated sample and in the one treated with 0.6 Mrad. The ash content of *Bezostaya* was 0.75 ± 0.12%, that of *Kiszombori* 0.98 ± 0.06%.

2.1.5. Baking value of the flour obtained from irradiated wheat samples

Baking tests were performed to find out how the bread was affected by irradiation. In earlier experiments (FARKAS & KISS, 1964) no difference was found between breads made of flour from untreated wheat and of that from 0.8-Mrad-treated wheat. Similar experiments were carried out in the RESEARCH INSTITUTE OF THE HUNGARIAN CEREAL TRUST (1968) and the limit value for sensory changes was found to be 0.2 Mrad.

The volume of the bread showed an increasing tendency with increasing radiation doses with both the varieties *Kiszombori* and *Bezostaya*. This improvement was apparent with *Kiszombori* wheat up to the dose level of 0.15 Mrad. The form quotient displayed also an increasing tendency as a function of dose.

No differences were, however, found in gluten content as a function of dose level. The spreading of gluten was in an inverse correlation with the dose level with the variety *Kiszombori*, while in direct correlation with *Bezostaya*. Results are shown in Figs. 9 and 10.

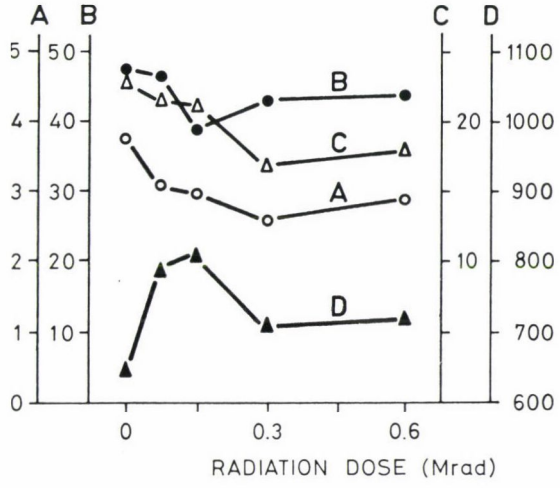

Fig. 9. Comparison, in the flour of *Kiszombori* wheat, of gluten content B (%), spreading of gluten C (mm), form quotient of the bread A and volume of bread D (ml) as a function of radiation dose

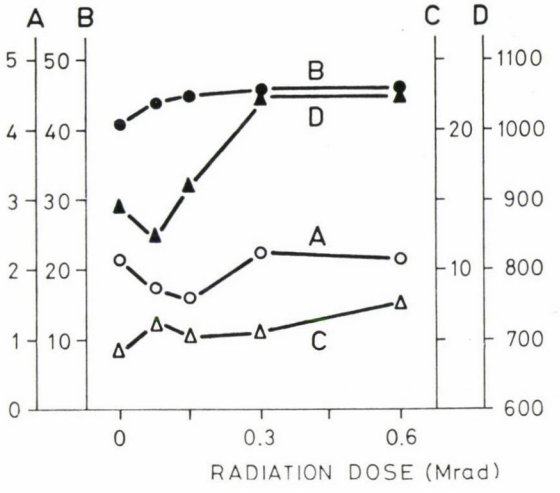

Fig. 10. Comparison in the flour of *Bezostaya* wheat of gluten content B (%), spreading of gluten C (mm), form quotient of the bread A and volume of bread D (ml), as a function of radiation dose

The volumes of breads made from flour of treated and untreated wheat were compared, irrespective of dose levels, by the t test. At the probability level of 95% the differences were non-significant. The structure of bread seemed looser at higher doses (Table 2).

Table 2

The volume of bread as affected by irradiation

Wheat variety *Kiszombori* of 18.2% and *Bezostaya* of 15.5% moisture content were radiation treated at various dose levels (0.075–0.6 Mrad) and the volume of breads prepared from the flours of the above wheat varieties was compared to that of breads baked from untreated flour by means of the t test

	Kiszombori		Bezostaya	
	untreated	treated	untreated	treated
Mean ($\bar{x}$)	670.00	778.75	890.00	926.25
Standard deviation (s)	90.18	155.70	71.65	188.83
Number of measurements (n)	4	8	4	8
t	1.275		0.364	
P%	95%		95%	

The wheat samples were milled 3 months after irradiation. The flour was used 3 weeks subsequent to milling

2.2. *Experiments with corn*

The storage quality of the corn of relatively high moisture content (22.6–31.2%) was reduced by microbial spoilage, mainly caused by moulds. The problems were similar to those found with wheat, but the moisture content had to be reduced below 15% on drying. The initial mesophilic count was 10^4–10^5 g^{-1}, while the mould count for both corn on the cob and shelled corn was 10^3 g^{-1}.

The initial moisture content of 31.2% of the shelled corn samples was reduced during two weeks of storage to about 25% (Table 3). Because of the reduced microbial count, due to radiation treatment the spoilage in irradiated samples was slower than in untreated samples. In the first few weeks of storage this became apparent not only in the difference in microbial counts, but in the musty smell of the untreated samples as well. Table 4 contains the average scores for colour and odour of the samples as found by a panel of 9 members on the 14th day of storage.

The storage life of the shelled corn of high moisture content could not be extended even by a treatment with 0.6 Mrad. After three weeks the cell count increased in the irradiated samples, too. Since on the 26th day of storage

Table 3

Change in the percentage moisture content of shelled corn and corn on the cob (including treated and untreated samples) Stored at 0–5 °C and 70–90% RH

Storage period (day)	Shelled		On the cob	
	$\bar{x}$	s	$\bar{x}$	s
0	31.2	0.7	22.6	0.8
7	26.9	0.8	18.6	0.9
16	25.2	1.2	17.1	0.9
29	24.4	0.8	17.0	0.5
42	—	—	20.3	0.3
63	—	—	18.7	1.1

$\bar{x}$ = mean of the moisture contents of 5 samples (%)

s = standard deviation

Table 4

Sensory evaluation by scoring of shelled corn (25.2% moisture content) on the 14th day o storage. The lowest quality was marked by 5 scores

Radiation dose (Mrad)	Colour		Odour	
	$\bar{x}$	s	$\bar{x}$	s
0	4.4	1.3	4.2	1.3
0.075	3.3	1.0	3.6	0.8
0.15	2.3	1.4	2.8	1.2
0.30	2.8	0.9	2.6	1.2
0.60	2.2	1.4	1.8	1.4

$\bar{x}$ = mean score (of nine panel members)

s= standard deviation

the mould count reached 10^5–10^6 g^{-1} and the mesophilic count 10^8–10^9 g^{-1}, the storage experiment was discontinued.

The corn on the cob of 22.6% moisture content at the time of radiation treatment lost only 4–5% moisture during storage and later, probably due to the relative humidity of the environment, fluctuated between 17 and 20% (Table 3). The reduction in cell count of corn on the cob of 22.6% moisture content achieved by irradiation and the cell counts during storage are illustrated in Figs. 11 and 12.

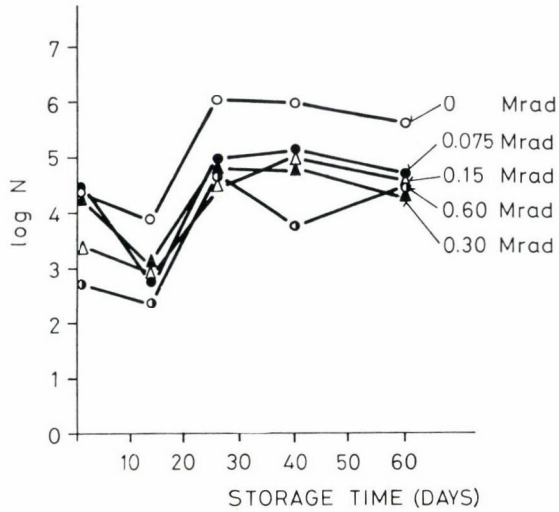

Fig. 11. Mesophilic total cell count in corn on the cob as a function of radiation dose and storage period. (Initial moisture content 22.6% storage temperature 0–5 °C at 70–90% RH)

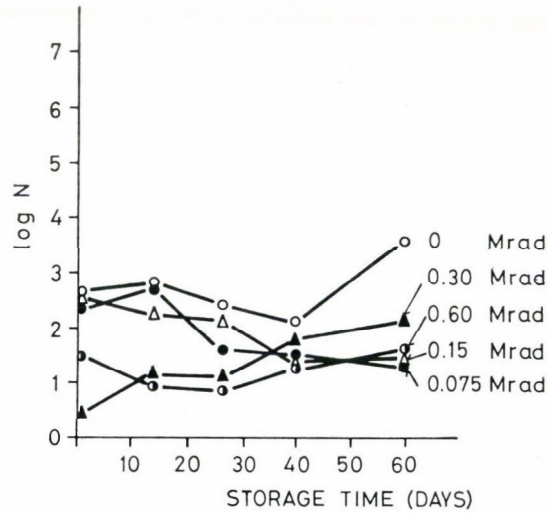

Fig. 12. Mould count in corn on the cob as a function of radiation dose and storage period. (Initial moisture content 22.6%, storage temperature 0–5 °C at 70–90% RH)

3. Conclusions

3.1. Experiments with wheat

It was found in the experiments that the microbial contamination of wheat may be as high as 10^5–10^7 g^{-1} (Fig. 2). Microbes adhere to the surface of the grain and the high moisture content (above 20%) ensures favourable conditions for growth. At a moisture content of 23% spoilage could be inhibited by a treatment with 0.4 Mrad.

Samples were stored in a closed space, therefore the effect of the radiation treatment could have been influenced by respiratory CO_2 accumulation and the decrease in O_2 content.

In wheat samples stored in a ventilated space, which, however, was kept in equilibrium with the 18.7% moisture content of the sample, mould growth could be retarded by treatment with 0.4–0.8 Mrad, thereby increasing their storage life 2–4-fold (Figs. 1 and 2).

In the experiments, where the conditions of storage permitted of drying of the wheat during storage (variety *Kiszombori* of 18.2% and *Bezostaya* of 15.5% moisture content) and the microbial infection was also at a low level (10^2 g^{-1}) mould growth was not observed on the untreated samples either and the cell count did not increase substantially either in the untreated, or in the irradiated samples (Figs. 3 and 4).

Reduction of the mould count may be of importance even if the moisture content is not sufficient to enable rapid mould growth. Namely, several authors succeded in isolating aflatoxin producing aspergilli from bread. These strains originated from the flour of which the bread was baked (SPICHER, 1970; PITT, 1973). In earlier experiments the authors of this paper isolated from bread an *Aspergillus flavus* strain (KISS *et al.*, 1970).

Data in the literature show *Aspergillus*, *Rhizopus* and *Penicillium* species relatively radiation sensitive, while *Alternaria* and *Fusarium* species were more resistant (WEBB *et al.*, 1959; IIZUKA & ITO, 1968; ITO *et al.*, 1969; MOHYUDDIN & SKOROPAD, 1970).

The observations related to the alpha-amylase activity (Figs. 5–8) and the baking quality (Figs. 9–10, Tables 1 and 2) of wheat as affected by irradiation are supported by many data found in the literature.

The quality of bread depends on the stage of maturity, or, in other words, on the time of harvesting of the wheat. Flour obtained from wheat harvested at the beginning or during the stage of waxen ripeness was of the best quality (POLLHAMER, 1970). High moisture content and temperature enhance the activities of enzymes in the grain. Therefore it seems desirable to maintain the high moisture content for some time (intensive alpha-amylase synthesis) and reduce it only later (OLERED & JÖNSSON, 1970). However, the activities

of other enzymes appear at the same time, germination starts and this affects flour quality. A treatment with 0.05–0.10 Mrad is sufficient to reduce respiration intensity (WOODSTOCK & JUSTICE, 1967) and, at a high moisture content, germination is inhibited by radiation with 0.1 Mrad (YEN *et al.*, 1956). Low radiation doses suffice to reduce germination in malting barley, without affecting enzyme activity (FARKAS *et al.*, 1963; VON SZILVINYI, 1967).

According to certain data radiation treatment brings about changes in the chemical composition of wheat which in their turn may cause changes in the biochemical and colloid chemical properties. The water-soluble pentosane fraction increases as an effect of 0.1 Mrad, a change is caused in the gas forming capacity and as an effect of 0.5 Mrad the gas retention capacity is reduced (SEIBEL, 1960; FINNEY *et al.*, 1960; MILNER, 1963). The reducing sugar content increases and the *Maillard* reaction comes to the foreground. High doses activate the amylolytic enzymes as well, thereby increasing attackability of the substrate (MILNER & YEN, 1956).

In these experiments the alpha-amylase activity was shown to be reduced in the varieties *Fertődi* and *Bánkúti* by treatments with 0.1 Mrad, in the variety *Bezostaya* with 0.075 Mrad. The alpha-amylase activity in the variety *Kiszombori* did not differ from that of the untreated sample when given a treatment of 0.6 Mrad. The structure of bread seemed looser, the volume larger with increasing radiation doses. The reduction of the spreading of gluten with the variety *Kiszombori* is probably due to the formation of disulfide bridges.

Data are available in the literature on the improvement of the baking quality of poor wheat upon radiation treatment (SOSEDOV & VAKAR, 1963). The sensory quality of bread does not change on treatment below 1 Mrad, the bread is palatable, its digestibility satisfactory (MILNER & YEN, 1956; SOSEDOV *et al.*, 1957; METTA & JOHNSON, 1959).

In experiments carried out a few years earlier the authors of this paper did not observe differences in the quality of bread baked from untreated flour and flour gained from wheat irradiated with 0.8 Mrad.

The radiation doses required for improving storability are more than sufficient for the disinfestation of the cereals, as well (FARKAS, 1965; DESCHREIDER, 1969).

3.2. Experiments with corn

The spoilage of *shelled corn* of 31.2% moisture content was retarded only for a short period by radiation treatment. The untreated samples deteriorated within two weeks. At this time the test for mustiness showed the samples irradiated with 0.15–0.6 Mrad yet suitable for human consumption, after four weeks these, too, were spoiled (Table 4).

The initial moisture content of 22.6 % of *corn on the cob* was not reduced below 15 %, the level of safety, during two months of storage without drying (Table 4), however, treatment with 0.075 Mrad was sufficient to retard microbial growth (Fig. 11) and the samples were still of satisfactory microbial quality after two months of storage.

Thus, the temporary preservation of corn in the first few months, most critical from the aspect of storage quality, may be achieved by radiation treatment. This conclusion may be of use in the better utilization of drying capacities.

The reduction of mould count or inhibition of mould growth is of great significance from the sanitary aspect of animal feeding because aflatoxin producing *Aspergillus flavus* may be present in mouldy corn. The moisture content of 18.5 % and 25–35 °C temperature are very favourable for growth and toxin production of this species (TRENK & HARTMAN, 1970).

The conclusion that irradiation may reduce the danger of deterioration of corn of high moisture content is supported by the findings of some other authors, particularly in the case when irradiation and drying are applied in combination (WEBB *et al.*, 1959; SAINT-LEBE *et al.*, 1966; MEDVED'SKAYA & KUDRYASHEVA, 1967).

*

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DETECTION OF SOYA AND MILK PROTEINS IN THE PRESENCE OF MEAT PROTEINS

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Investigations were carried out with raw and heat treated beef and pork samples using polyacrylamide-gel disc electrophoresis. The protein fractions of raw and deep-frozen meats and electroferograms of samples from different body parts of the same species were checked. Detection of soya and milk proteins beside meat was worked out. The meat, non-meat protein mixtures were heat treated at 74 °C for 150 minutes to weaken the intensity of meat protein fractions; meanwhile the fractions of soya or milk proteins remained unchanged or became stronger. The sample treated according to the method described was dissolved in water or in 8 M urea. Urea solutions were used to detect soya protein, while for the identification of milk protein aqueous solutions proved suitable. These solutions were separated into protein fractions by using 7.5 per cent acrylamide concentration and a basic buffer system. The described method is suitable to detect proteins from mixtures of beef and pork as well. The electrophoretic pattern may be used to detect soya and milk proteins from both raw and heat treated samples.

The use of milk and soya proteins in the meat industry has become widespread abroad for several years now and is coming into general use in Hungary, too (JUDGE *et al.*, 1974; PFAFF, 1974; ZIVKOVIC & ROSEG, 1974; KÁRPÁTI & ZACHARIEV, 1974; KÖRMENDY & MIHÁLYI, 1974; VIRÁGH, 1974). These facts necessitate to deal with the possibilities of the use of these proteins and with their detection.

By their emulsifying effect, milk and soya proteins employed as additives in small amounts (below 2 per cent) make meats of poor water holding capacity but satisfactory protein content suitable for processing into different meat products. They are also useful when added in greater amounts as complementary protein to meat products with low protein content (e.g. sausages). Employed as complementaries they are suitable to manufacture products with high (25–30 per cent) protein content. These products may be used in special fields of nutrition: dietetics, nutrition of sportsmen, etc.

The proteins mostly employed in Hungary: sodium caseinate, precipitated milk protein, soya proteins (among them primarily Promine D) are innocuous but as their net protein utilization (NPU) differs from that of the meat proteins their employment and ratio to meat protein are not indifferent from the point of view of nutrition. Taking NPU of the whole egg protein as 100,

other types of protein are of the following NPU rate: beef 80, pork 77, casein 72, soya flour 56 (XIVth SESSION OF THE COMECON COMMITTEE. . . 1969).

The usual method for the determination of the nutritive value of proteins, the amino acid analysis, is not sufficiently informative in this case because soya protein contains only half as much of the limiting amino acid, methionine, than does meat protein. Thus, the addition of 20 per cent soya protein during processing results only in a 10 per cent difference in the limiting amino acid content. This difference is not enough to perform reliable, reproducible experiments.

Hitherto examinations of meat protein fractions were carried out by numerous scientists. Some of them already employed the method of polyacrylamide-gel disc electrophoresis the same method that was used by us (HÖYEM & THORSON, 1970; FISCHER & BELITZ, 1971; COHEN, 1966; MAIER & FISCHER, 1966; EHERMANN & BARNA, 1972; CODURI & RAND, 1972; HOFMANN & PENNY, 1971; HOFMANN & PENNY, 1973). However their objectives differed from ours.

There are several data in the literature dealing with the detection of non-meat proteins in meat (KRAACK, 1973; OLSMAN, 1969; SPELL, 1972; FREIMUTH & KRAUSE, 1969) but the methods cited are difficult to perform and are not convenient to make parallel examinations. None of the methods carried out by polyacrylamide-gel disc electrophoresis could be used widely because of the complicated preparatory methods, the use of special equipment and other difficulties in realization (GUY *et al.*, 1973; FROUIN *et al.*, 1973; LOTZ *et al.*, 1973; FREIMUTH & KRAUSE, 1969).

1. Materials and methods

1.1. Materials

1.1.1. Meat samples. The beef and pork samples used in the experiments were fresh meats commercially bought or purchased from the slaughterhouse. To get homogeneous samples available during a longer time period thoroughly minced meat was frozen in a freezer of −18 °C (in about two hours) and stored there until use. This type of meat sample was used in every experiment except for the cases where fresh meat is mentioned.

1.1.2. Soya protein. The employed Promine D was the product of the CENTRAL SOYA COMPANY (USA). Its protein content in the dry substance was at least 90 %. Seventy-five % of the protein was water soluble. Promine D is a spray-dried product of 20–110 μ grain size.

1.1.3. Sodium caseinate. The employed sodium caseinate EM–HV was a product of the ZUID-NEDERLANDSCHE MELKINDUSTRIE N. V. (The Netherlands.) Its protein content was more than 90% of the whole dry substance. At least 90% of the whole protein content was water soluble.

1.1.4. The chemicals used for preparing gel rods, buffer solutions and reagents for staining were purchased from REANAL (Hungary). These chemicals are the following: acrylamide, N,N-methylenebisacrylamide (BIS), N,N,N',N'-tetramethylethylenediamine (TEMED), 2-amino-2-(hydroxymethyl)-1,3 propanediol (TRIS), ammonium persulfate, glycine. Amidoblack 10 B, urea.

Hydrochloric acid was a MERCK product. Acetic acid was produced by ERDŐKÉMIA (Hungary).

1.1.5. Apparatus. To perform electrophoresis, REANAL's electrophoretic apparatus (Model 1969) was used. It contains carbon electrodes and 12 parallel runs may be performed at the same time. During the electrophoretic procedure the apparatus was cooled in a refrigerator at 2–3 °C. The power supply used to get direct current was *Statron* type 301 E, produced in the GDR.

1.2. Methods

1.2.1. Treating and dissolving of samples

1.2.1.1. — Sodium caseinate was dissolved in warm water (about 50 °C), at a concentration of 2 mg ml^{-1} (used as the control medium).

1.2.1.2. — Promine D was dissolved in 8 *M* urea because when using only aqueous solution no characteristic electrophoretic pattern was achieved. The concentration of Promine D was 6.6 mg ml^{-1} (used as the control medium).

1.2.1.3. — Meat samples were weighed in — 30 g each — and homogenized in a mixer with 30 ml water or 30 ml 8 *M* urea solution according to the non-meat protein to be detected. When the mixture was made from different meats (beef, pork) the solvent volume proportionally increased.

1.2.1.4. Heat treatment. — Meat samples or meat–soya, meat–casein mixtures were heat treated for 150 min at 74 °C applying dry heat. Solutions were prepared after treatment. In the experiments both raw and heat treated samples were used.

1.2.2. Gel and buffer. Polyacrylamide gels and buffers were prepared according to the Ornstein–Davis system (ORNSTEIN, 1964; DAVIS, 1964).

Table 1

Protein fractions in the electrophoretic patterns of meat and milk proteins in aqueous solution

Sample	Protein fractions (symbols and R_m values)															
	A 0.12	**B** 0.14	$\mathbf{C}_{O(B)}$ 0.18	**C** 0.27	$\mathbf{D}_{(B)}$ 0.31	$\mathbf{D}_{(P)}$ 0.32	**E** 0.36	$\mathbf{E}_2$—**X** 0.40	$\mathbf{F}_{(P)}$ 0.46	$\mathbf{F}_{(B)}$ 0.51	$\mathbf{G}_{(P)}$ 0.54	$\mathbf{G}_{(B)}$ 0.59	$\mathbf{H}_{(P)}$ 0.60	$\mathbf{I}_{(P)}$ 0.67	$\mathbf{H}_{(B)}$ 0.69	**Y** 0.70
Sodium caseinate, raw								++								++
heat treated								++								++
Milk powder, raw								++								++
heat treated								++								++
Pork, raw	++	++		++		++	+	+	+		++		+	++		
heat treated	+	+				+					+			+		
Beef, raw	++		+	++	++		+	+		++		+			++	
heat treated	+									+					+	
Pork mixed with sodium caseinate	++	++		++		++		++			++			++		++
mixed with sodium caseinate and heat treated	+	+						++						++		++

mixed with milk powder	++	++				++	++	++			++			++		++		
mixed with milk powder and heat treated	++	++				++		++			+			+		++		
Beef mixed with sodium caseinate		++			++	++		+	++		++		+				++	
mixed with sodium caseinate and heat treated		+							++		+						++	
Beef, pork, sodium caseinate mixture, raw	++	++			++		++			++			+			++		++
heat treated	+	+							++							++		++

Fractions marked with index (B) are appearing only in beef, those marked with index (P) are the same for pork. X, Y are casein fractions, all the other fractions are common for beef and pork;

$G_{(P)}$ is the myoglobin fraction characteristic of pork, $F_{(B)}$ is the same of beef;

\+ fraction of weak intensity, ++ fraction of strong intensity;

\+ or ++ placed between two columns means that the two fractions are blended

Table 2

Protein fractions in the electrophoretic patterns

Sample	Protein fractions (symbols and R_m values)								
	A 0.10	**J** 0.17	**B** 0.19	**$C_{(P)}$** 0.24	**$C_{(B)}$—K_1** 0.25	**K_2** 0.30	**$D_{(B)}$** 0.30	**$D_{(P)}$** 0.33	**L** 0.35
Soya protein, raw		++			++	++			++
heat treated		++			++	++			++
Pork, raw	+		+	+				+	
heat treated								+	
mixed with soya protein[a]	+		+	+		++		++	
mixed with soya protein and heat treated						++			
Beef, raw	+		+		+		++		
heat treated							++		
mixed with soya protein[a]	+		+		++	+			
mixed with soya protein and heat treated		++				++			
Beef, pork mixture, heat treated									
mixed with soya protein and heat treated			++			++			

The gels and buffer solutions were made to contain 8 *M* urea or no urea at all according to the method of dissolving the samples. Neither sample nor spacer gels were used, only separation gel was employed. The samples containing 400 μg of protein were mixed with glycerol, buffer and Bromophenol Blue and were layered on the gel surface. The acrylamide concentration chosen was 7.0% and BIS 0.184%. Using separation gel of larger pore, the mobility of the protein fractions is greater but the outlines of the bands are diffuse, the background cannot be sufficiently destained. In case of smaller-pore separa-

of meat and soya proteins in urea solution

and R_m values)

$E_{(B)}$ 0.38	M 0.40	$F_{(B)}$ $E_{(P)}$ 0.41	$F_{(P)}$ 0.46	$G_{(B)}$ 0.48	$G_{(P)}$ 0.55	$H_{(B)}$ 0.59	N 0.60	O 0.68	$H_{(P)}$ 0.78	$I_{(B)}$ 0.80	P 0.99
	++						+	+			++
	++						+	+			++
		+	+		+				+		
			++								
		+	++								
			++								
++		+		++		++				+	
				++		++					
				++		++					
				++		++					
			++			++					
			++			++					

Fractions marked with index (B) are appearing only in beef, those marked with index (P) are the same for pork. J, K_1, K_2, L, M, N, O, P are soya fractions, all the others are common for beef and pork. $F_{(P)}$ is the myoglobin fraction characteristic of pork, $H_{(B)}$ is the same of beef;
+ fraction of weak intensity, ++ fraction of strong intensity;
[a] the electrophoretic pattern of the sample contains other non-indicated fractions, too

tion gel the bands are sharper than those obtained at 7 % acrylamide concentration but, since the distance between them is very small, the identification and measurement of the possible non-meat protein fractions is difficult. Acrylamide concentrations of 3, 5, 7, 9, 11 and 13 % were examined.

1.2.3. Electrophoresis. The upper reservoir was connected to the power supply as cathode (origin) the lower reservoir as anode (termination). The electrophoresis was carried out with a current of 1.5–2.0 mA per tube. It was

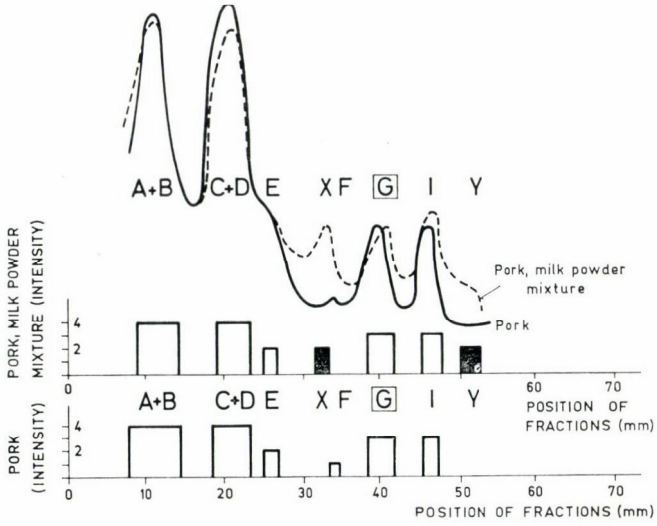

Fig. 1. Polyacrylamide gel protein patterns and densitograms of pork; pork, milk powder mixture. Dark fractions are characteristic casein fractions. □ marks myoglobin fraction. The intensities marked in the figure correspond to densitometer readings, but are not calculated from them. "1" means weak, "2" medium, "3" strong, "4" very strong intensity. Position and width of columns are the same as on the gel rods. The examination was carried out in aqueous solution

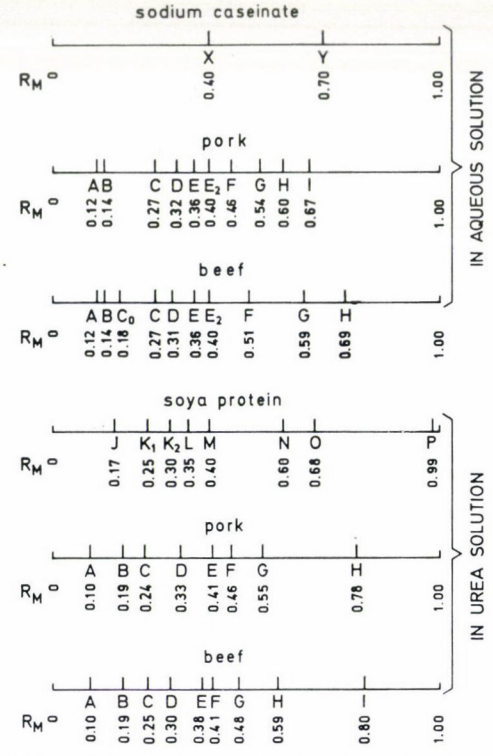

Fig. 2. Relative mobilities (R_m) of the protein fractions of sodium caseinate, pork, beef and soya protein samples

continued until the front of the Bromophenol Blue reached a line about 5 mm from the lower end of the separation gel. The time for electrophoresis was about 3–3.5 hours.

1.2.4. Staining. The removed gel rods were immersed in a 7% aqueous acetic acid solution containing 0.1% Amidoblack dye. This solution served both for staining and fixation of proteins. Destaining was carried out by washing the gel rods in 7% aqueous acetic acid for about 48 hours.

1.2.5. Evaluation of the experiments. The length of the gel rods and the distance of Bromophenol Blue's front from the origin were measured before staining. The migration distance of the protein bands and the length of the gel rod were measured after destaining. These data served for estimating the relative mobilities (R_m) of the fractions. Identification of protein fractions was based on their relative mobilities. The patterns were measured by Chromoscan MK II. (JOYCE LOEBL, England) densitometer. The densitograms were used only to obtain an objective measurement for the qualitative estimation of the electroferograms.

Neither literary data, nor the author's findings made it possible to use densitograms for quantitative determination of proteins. The position of the fractions of meat protein and that of soya protein and sodium caseinate are shown in Figs. 1 and 2.

The distribution of protein fractions of raw, heat treated and mixed samples is demonstrated in Tables 1 and 2.

2. Results and discussion

2.1. Effect of deep-frozen storage on meat proteins

As the meat samples were kept according to para. 1.1.1, it was necessary to investigate the effect of deep-frozen storage on the meat proteins. Beef and pork had been stored deep-frozen for 35 days and their protein patterns were examined several times during this period. None of the samples showed essential differences from the electrophoretic pattern of the fresh meat, there were only slight dissimilarities in the intensity of the fractions. No new fractions appeared and those available in fresh meat did not disappear.

The characteristic fractions of the meat species in aqueous solution are the myoglobin fractions: the **F** fraction for beef ($R_m = 0.51$) and the **G** fraction for pork ($R_m = 0.54$). (Table 1.)

2.2. Electrophoretic patterns of the different cuts of beef and pork

It was necessary to know whether there is any difference in the electrophoretic patterns using different parts of the same species. Twenty-two types

of beef samples taken from different parts of the animal were examined, among them buttock, entrecote, leg, head meat and tail. Some difference was found in the intensity of the fractions. Only two fractions of weak intensity, $\mathbf{C}_0$ and $\mathbf{E}_2$, appeared or disappeared in the samples, all the others were stable.

In the case of pork samples, 10 extremely different parts of the animal were chosen, among them tail, thin flank, shank, spare rib and chop. Although there were differences in the intensities, the number of fractions was identical in all samples (only **A** and **B** fractions of the tail were not separable).

Summarizing the findings of paras. 2.1 and 2.2 it was ascertained that, when using any cut of beef or pork, fresh or deep-frozen, neither in the distribution nor in the intensity of the fractions did any difference occur to disturb the appearance and identification of the possible non-meat protein fractions.

2.3. Identification of soya protein

To detect soya protein, meat+Promine D mixtures were dissolved in 8 *M* urea (according to para. 1.2.1.3). Meat and Promine D samples were used as controls. Using raw meat samples, both the beef–Promine D and the pork–Promine D mixture resulted in too many fractions that made the evaluation of the electroferograms almost impossible (Table 2). To decrease the number of the fractions the known fact was used that meat proteins are rather sensitive to thermal denaturation. They are more sensitive than soya protein or casein. When the same samples were heat treated according to para. 1.2.1.4 the number of fractions decreased so much that the protein fractions of Promine D became well observable (Table 2). The most characteristic fraction of Promine D when mixed with meat is $\mathbf{K}_2$ ($R_m = 0.30$). Using greater Promine D concentration, fractions **J** ($R_m = 0.17$), $\mathbf{K}_1$ ($R_m = 0.25$), **L** ($R_m = 0.35$), **N** ($R_m = 0.60$), **P** ($R_m = 0.99$) may appear in the electroferogram. The myoglobin fractions in the urea solutions are **H** ($R_m = 0.59$) for beef and **F** ($R_m = 0.46$) for pork. The method is suitable for the detection of Promine D in the presence of beef, pork and their mixtures. The lowest Promine D concentration detected is 1.0%.

2.4. Detection of milk proteins

Meat samples containing milk proteins (casein or milk powder) were dissolved according to para. 1.2.1.3. Performing electrophoresis according to para. 1.2.3, the characteristic casein fractions — marked **X** ($R_m = 0.40$) and **Y** ($R_m = 0.70$) — were well detectable in aqueous systems. The **X** fraction appeared as a separate band in every case, the **Y** fraction coincided with **H** protein fraction found in beef samples but with none of those found in pork samples (Table 1). Although the casein fractions were detectable in

raw samples already, the electrophoretic patterns of heat treated samples (heat treating according to para. 1.2.1.4) were much easier to survey and evaluate (Table 1). The minimal detectable concentration of casein was 1.0%. The identification of milk powder was performed also by the detection of its casein content (Table 1).

The reported method is suitable for serial examinations, there is no need for special equipment and it may be performed easily and reproducibly.

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XIVth SESSION OF THE COMECON COMMITTEE FOR COORDINATION SCIENTIFIC AND TECHNICAL RESEARCH WORK. (Budapest, Sept. 1969.) Main aspects of research work in the production of good quality foodstuffs relying upon the scientific accomplishments of agriculture, biology, chemistry and biochemistry.

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DISINTEGRATION OF VEGETABLE TISSUES BY ENDO-POLYGALACTURONASE

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pH optima of the enzymatic disintegration of several vegetable tissues by endo-polygalacturonase were tested in the present work within a pH-range of 3.0–7.0. In the majority of the vegetables tested, the pH optimum was found to be between 3.0 and 3.5. In the case of kohlrabi, squash and carrot, the enzyme treatment proved to be successful in a wider pH range (3.5–4.5 and 3.0–4.5, resp.). Disintegration of the above vegetables into single cells was studied by filtration through a set of screens and was almost complete as the filtrates contained about 96–99% of the original tissues and only negligible parts were retained on the fine mesh screens. The disintegration was rather incomplete in the case of cauliflower, when only 82% of the original tissue was disintegrated into single cells and small cell agglomerates.

The colour, odour and flavour of the enzymatically disintegrated fractions were equal to those of the original tissues.

The production of endo-polygalacturonase enzyme from moulds grown in batch or continuous culture has been the subject of research for several years in the CENTRAL FOOD RESEARCH INSTITUTE of Budapest. Along with the production of the first enzyme preparations, experiments on possible applications were started immediately.

For enzyme treatment, vegetables of high dry matter content were chosen which could be considered as raw material for valuable aromatic vegetable juices and cocktails without any loss to their original vitamin content. The vitamins inside the cells do not suffer any loss during the enzyme treatment as the enzyme attacks only the space between the cells, and an injury to the cell membrane is rare.

Suspensions of cells and tissue agglomerates of the enzymatically disintegrated vegetable tissues can also be utilized as ingredients of baby foods or as basic materials in the manufacture of sauces and dehydrated cream soups.

Enzymatically disintegrated vegetable tissues have already been applied as raw materials for baby foods and dietary food preparations in Yugoslavia (SULC & VUJICIC, 1973). Hundreds of tons of enzymatically decomposed carrots have been used as baby foods in Italy (CHARLEY, 1969).

The enzymatically disintegrated vegetable tissues could be used as valuable basic materials for those vegetable juices and cocktails which are

very popular in England, the Federal Republic of Germany and Bulgaria (USCHEWA & AROLSKI, 1970).

This paper gives an account of the initial experiments where the effect of pH was tested on the enzymatic disintegration of the tissues of various vegetables (potato, squash, carrot, parsley, cauliflower, and kohlrabi).

1. Materials and methods

1.1. Enzyme preparation

Endo-polygalacturonase of *Asp. awamori* was used in the present work having an activity of 4000 $l\ h^{-1}\ g^{-1}$, determined viscosimetrically in a sodium polypectate solution at 50 °C, for 60 minutes.

1.2. Preparation of the vegetables and enzyme treatment

Vegetables were grated into pieces of 5×15 mm. Ten g of the grated vegetables were placed into 250 ml Erlenmeyer flasks, 25 ml McIlvaine buffer, containing 0.5 g enzyme, were added to each and the reaction mixtures were incubated on a shaker (130 stroke min^{-1}) at 50 °C for 3 h.

The effect of pH was tested within a pH range of 3.0–7.0 at intervals of pH 0.5.

After the enzyme treatment the reaction mixtures were poured through fine screens (mesh: first 1.0 and then 0.25 mm resp.). Particles remaining on the screens were removed and dried at 105 °C to constant weight. Dry matter content was determined together with the supernatant and the original tissue. The percentage of degradation into predominantly single cells and tissue particles smaller than 1.0 mm and 0.25 mm, resp. was calculated from the dry weights.

pH values were considered optimal when their use resulted in the highest percentage of the original tissues passing into the filtrate.

2. Results

The degree of enzymatic disintegration of the potato and squash tissues differed according to the pH of the treatment (Fig. 1).

pH 3.0 and 3.5 proved to be optimal for the degradation of potato and squash tissues.

In the case of both vegetables a 97–98 % recovery was obtained, which means that in a reaction mixture of optimal pH, the tissues were decomposed into particles smaller than 0.25 mm. With the increase of pH the quantity

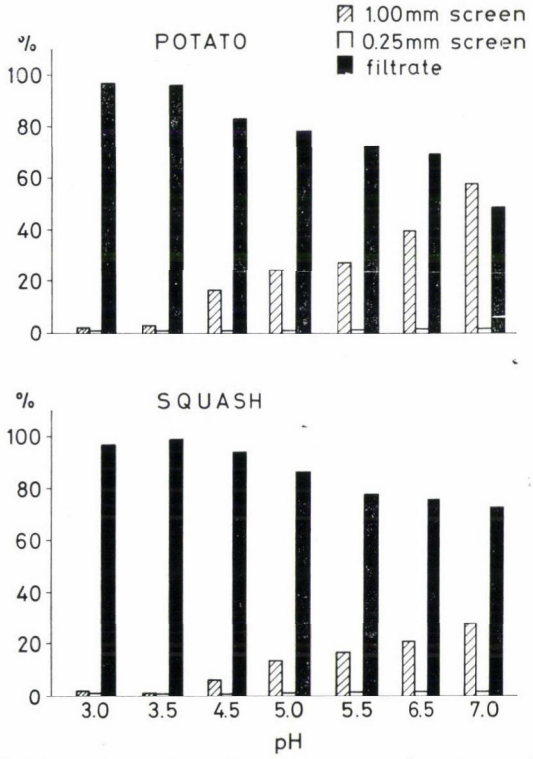


Fig. 1. Enzymatic disintegration of potato and squash tissues as a function of pH

of particles exceeding 1.0 mm increased. In the case of potato and squash tissues the use of pH 7 resulted in 57 and 27%, resp., of tissue agglomerates larger than 1.0 mm.

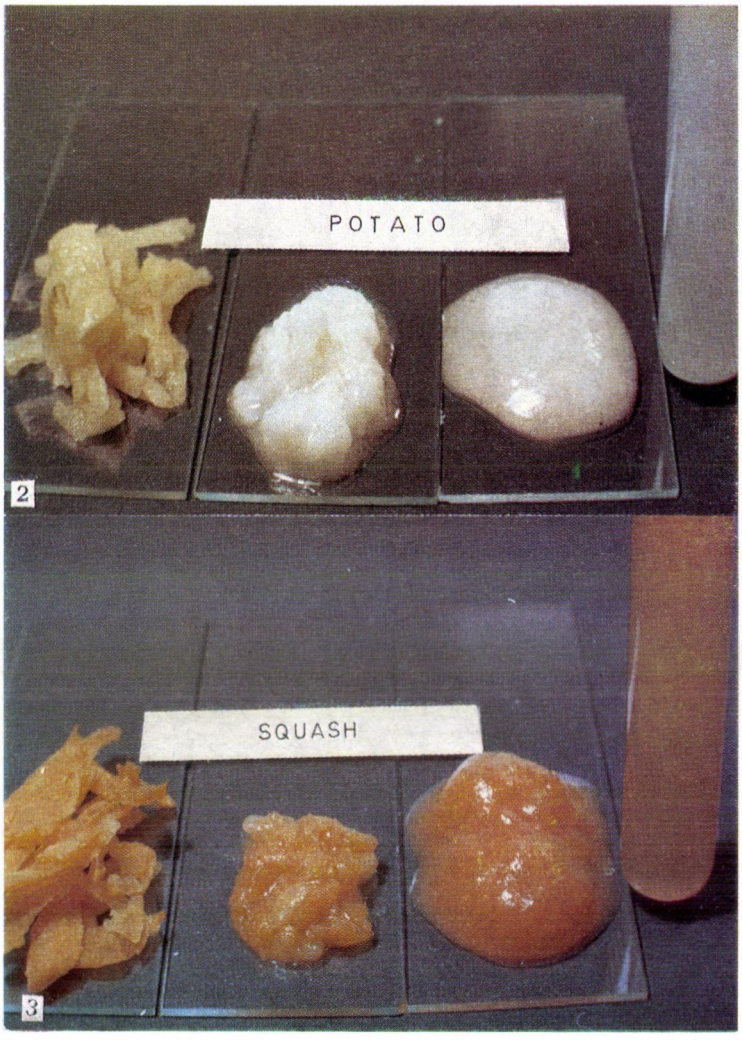
Photographs of the different fractions of the enzymatically disintegrated potato and squash tissues are shown in Figs. 2 and 3.

As can be seen from the Figures, the enzymatic maceration caused no alteration in the colour of the samples and filtrates have the same colour as the original tissues. Oxidation of the potato tissue could be eliminated when placing the slices into enzyme solution immediately after grating.

The consistency of the three fractions was rather different. Residues on the first screen (mesh 1.0 mm) contained several coarser pieces but were already purée-like, while residues remaining on the finer screen (mesh 0.25 mm) were perfectly smooth. The filtrate contained also a few cell agglomerates, but seemed to be a completely homogeneous suspension.

The consistency of the residue of squash tissues on the first screen was smoother than that of the potato tissue.

Photomicrographs of the particles and cells of potato and carrot tissues (found on the 1 mm screen and in the filtrate, resp.) are given in Figs. 4 and 5.



Figs. 2 and *3*. Fractions of enzymatically disintegrated potato and squash tissues. First slides (from the left): untreated tissues prepared for enzymatic maceration, 2nd and 3rd slides: tissue residues after maceration retained on the 1.0-mm and 0.25-mm screens; test tube: filtrate containing single cells and small cell agglomerates

As the Figures show it, tissue residues of the above two vegetables remaining on the 1.0 mm screen consisted of loosely coherent cells, which means that the enzymatic maceration of the squash and potato tissues was very efficient.

The degree of the enzymatic decomposition of parsley and carrot tissues as a function of the pH can be seen in Fig. 6.

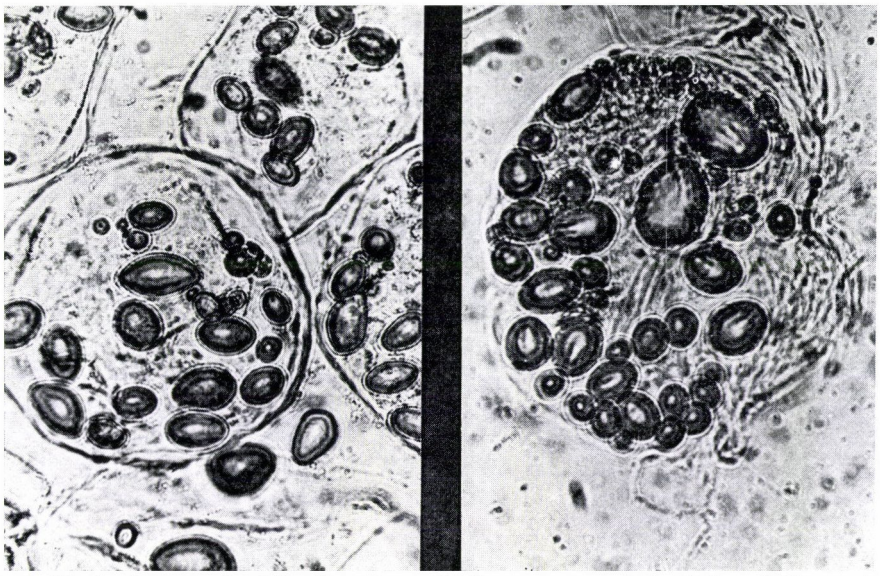


Fig. 4. Photomicrograph of the fractions of 1.0-mm screen (left side) and filtrate (right side) of potato tissues after endo-polygalacturonase treatment (magnification: ×95)

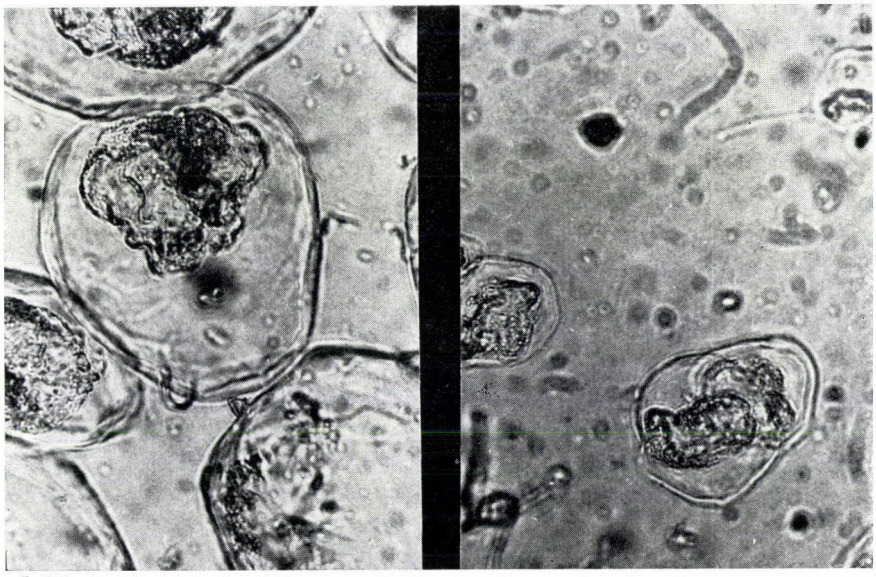


Fig. 5. Photomicrograph of the squash tissues after endo-polygalacturonase treatment (magnification: ×190)

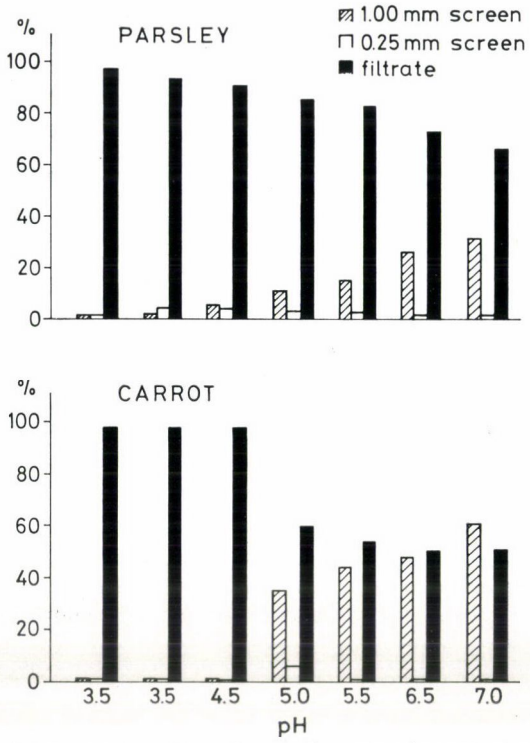


Fig. 6. Enzymatic disintegration of parsley and carrot tissues as a function of the pH

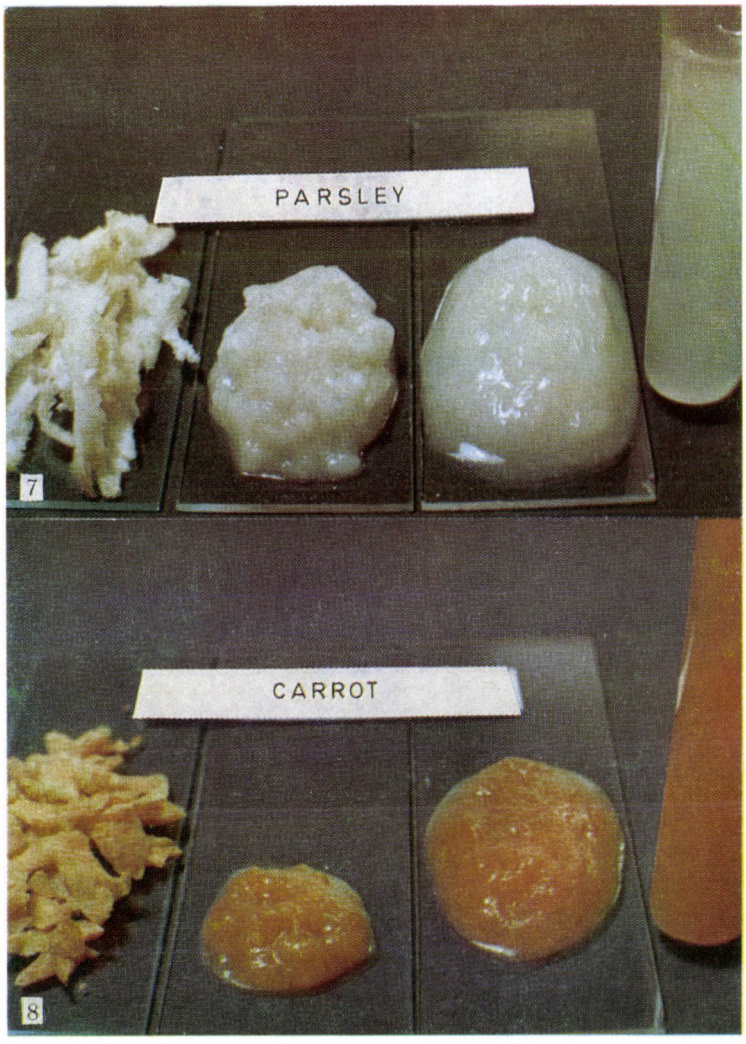
A pH value of 3.0 was optimal for the degradation of parsley tissues while in the case of carrot the degree of decomposition was almost complete in a wider pH range (pH 3.0–4.0) as well. When tissues were macerated at optimal pH, a recovery of 98% was obtained in the case of both the above vegetables. With the increase in the pH the recovery decreased and this was more significant in the case of carrot (only 40% of the original tissue was found in the filtrate at pH 7.0) than in the case of parsley.

Photos of the fractions of parsley and carrot tissues separated on fine screens are given in Figs. 7 and 8.

The colour of the vegetable solutions and purées were identical with that of the original tissues. The creamy consistency of the second fractions of both vegetables can clearly be seen in the Figures, but the first fraction (residue on the 1.0-mm screen) of the carrot tissue was finer than that of parsley.

Structures of the tissue residues and cells of parsley and carrot tissues on the 1.0-mm screen and in the filtrates, resp. are shown, in Figs. 9 and 10.

Photomicrographs illustrate the dimensional and morphological differences among the cells of the individual tissues as well as among the cells of the different vegetables.



Figs. 7 and *8*. Fractions of enzymatically disintegrated carrot and parsley tissues. (Slides with the different fractions are in the same sequence as those in Fig. 2)

The effect of the pH on the disintegration of cauliflower and kohlrabi tissues can be seen in Fig. 11.

The macerating action of endo-polygalacturonase was highest in the pH range of 3.5–4.5 when the recovery of cauliflower tissue was 98%.

The disintegration of kohlrabi tissues by endo-polygalacturonase was more difficult than that of the cauliflower tissues. The pH optimum of the enzyme action on kohlrabi substrate was pH 3.5, when 83% recovery was

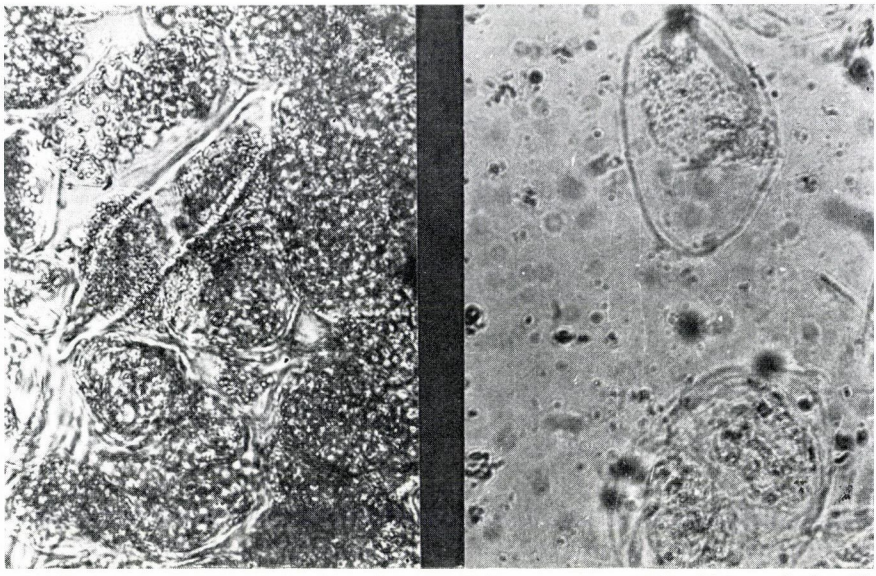


Fig. 9. Photomicrograph of the fractions of 1.0 mm screen (left side) and the filtrate (right side) of parsley tissues after maceration with endo-polygalacturonase (magnification: ×190)

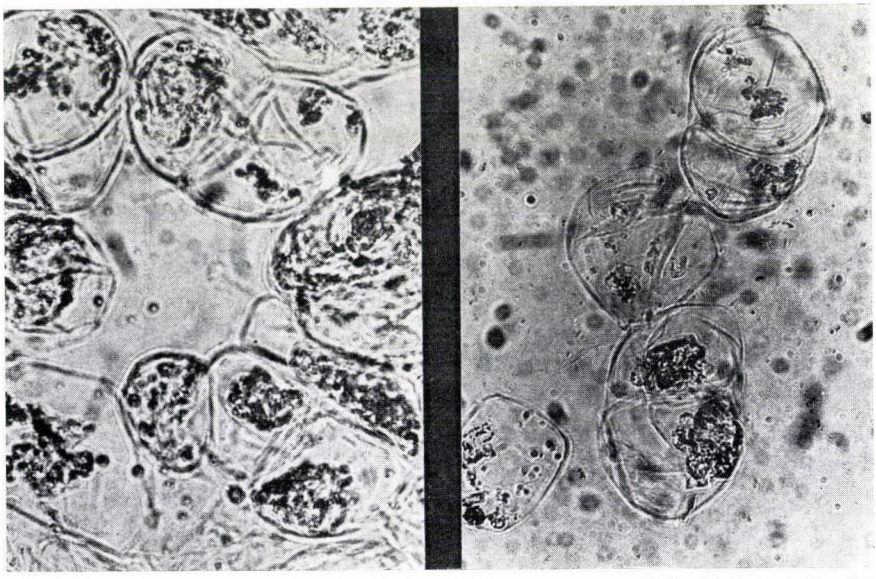


Fig. 10. Photomicrograph of the fractions of 1.0 mm screen, (left side) and the filtrate (right side) of carrot tissues after maceration with endo-polygalacturonase (magnification: ×190)

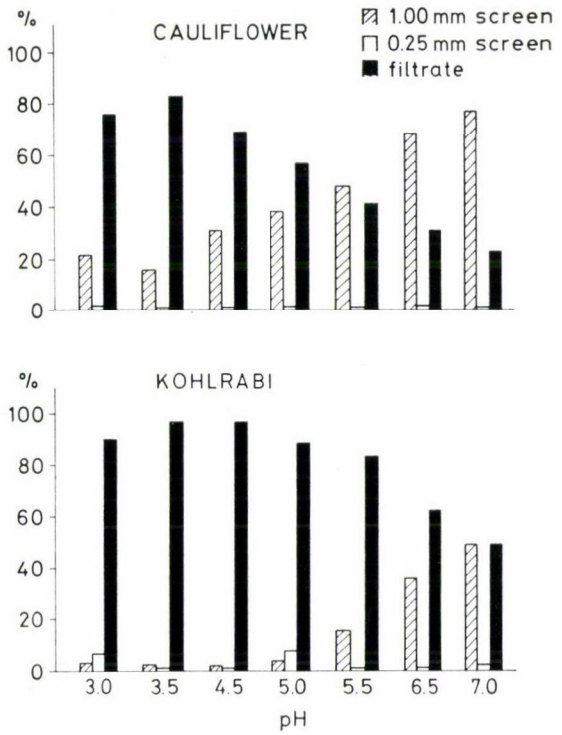


Fig. 11. Enzymatic disintegration of cauliflower and kohlrabi tissues as a function of pH

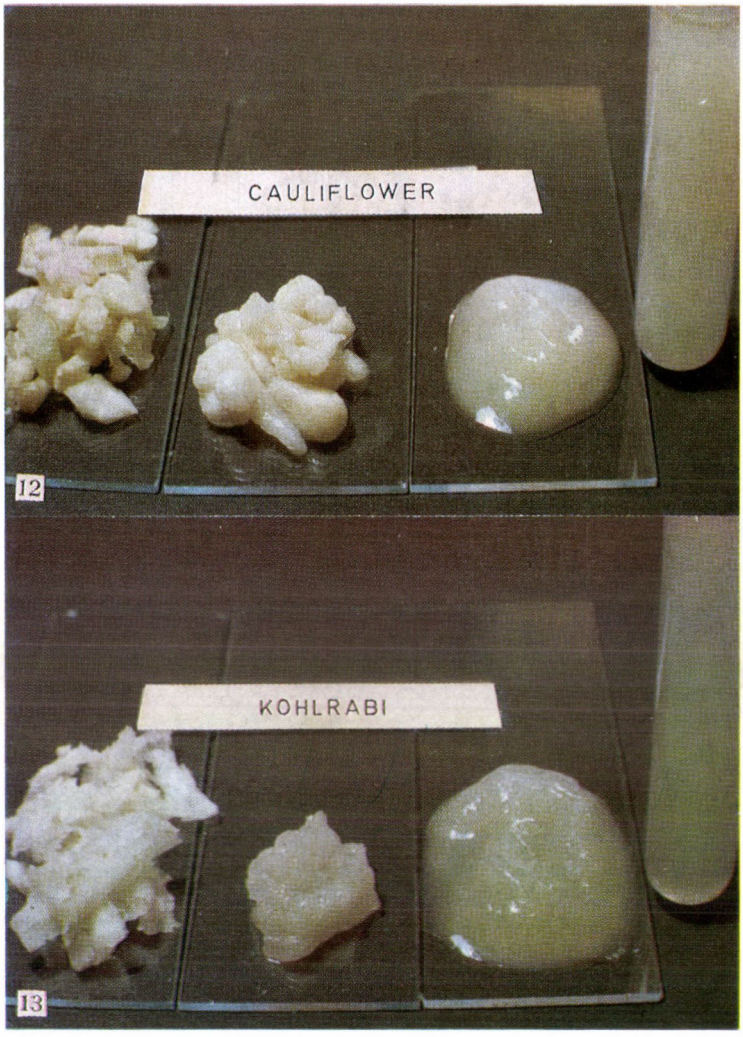
obtained. At pH 7, only 23% of the original tissue were found in the supernatant.

Photos of the fractions of the enzymatically macerated cauliflower and kohlrabi tissues are shown in Figs. 12 and 13.

Enzymatic maceration of cauliflower was less efficient than that of kohlrabi. Fractions of the cauliflower tissue retained on the 1.0-mm screen consisted of rather coarse particles.

Photomicrographs of the residues of cauliflower and kohlrabi tissues are illustrated in Figs. 14 and 15.

The fact that the enzymatic maceration of cauliflower tissues was more difficult than that of kohlrabi, can be explained by the differences in structure of the above tissues. Fig. 14 shows that among tissue residues of cauliflower recovered on the 1.0-mm screen intact tissues can also be found.



Figs. 12 and *13.* Fractions of the enzymatically disintegrated cauliflower and kohlrabi tissues. (Slides with the different fractions are in the same sequence as those in Fig.2)

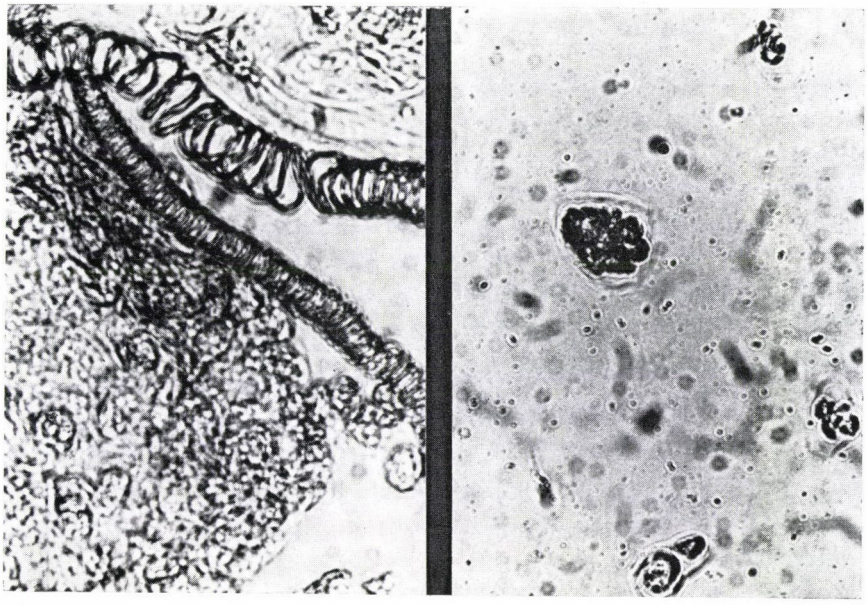


Fig. 14. Photomicrograph of the fraction of 1.0 mm screen, (left side) and the filtrate (right side) of cauliflower tissues after maceration with endo-polygalacturonase (magnification: ×190)

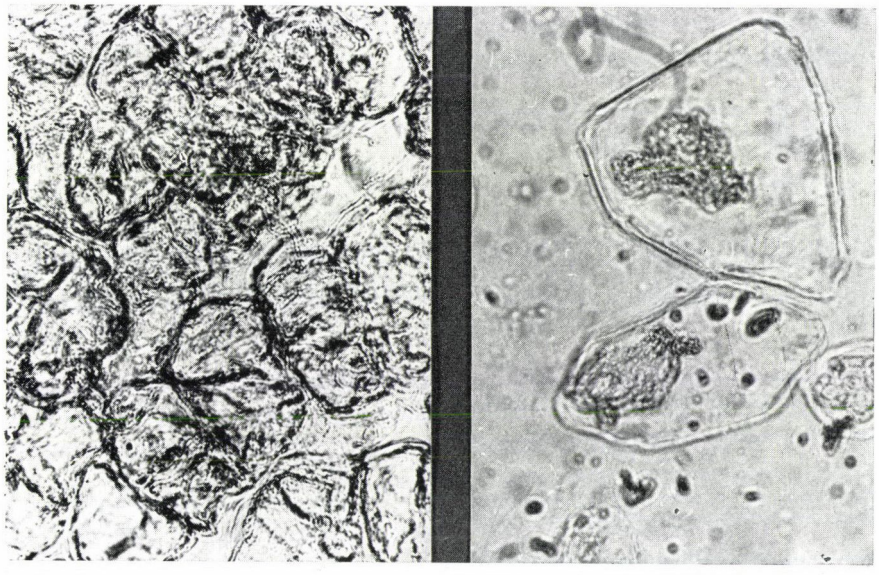


Fig. 15. Photomicrograph of the fraction of 1.0 mm screen, (left side) and the filtrate (right side) of kohlrabi tissues after maceration with endo-polygalacturonase (magnification: ×190)

3. Conclusions

Maceration of plant tissues by pectolytic enzymes has been reported by many authors (BROWN, 1965; MUSSEL & MORRE, 1969; WOOD, 1960; KIM & LEE, 1971; BOCK *et al.*, 1972; RZEDOWSKI, 1972). Hydrolytic type of endo-polygalacturonase (*Rohament* P, ROHM & HAAS, Darmstadt) acting by degradation of low-ester insoluble pectins was succesfully used for the maceration of vegetable tissues. Carrot and potato tissues were degraded by the above enzyme in order to obtain single cells (GRAMPP, 1969). CHARLEY (1969) also used a *Rohament* P preparation for the disintegration of carrot tissues obtaining a recovery of 94% after treatment.

Results of the experiments given in this paper were in agreement with the above results when another endo-polygalacturonase preparation (No. PG-225; pilot product of the CENTRAL FOOD RESEARCH INSTITUTE, Budapest) was used. Macerating activity of this preparation proved to be satisfactory as the use of half of the enzyme concentration given by the above authors resulted in a high (98%) recovery.

Results of our experiments prove that the pH value of the reaction mixture plays an important role in the maceration of vegetable tissues during enzyme treatment. It is clear that pH optima for the endo-polygalacturonase treatment are in the acidic pH range in the case of all the vegetables treated.

The increase of pH resulted in a lower degree of enzymatic disintegration with lower recovery. When potato tissues were macerated by endo-polygalacturonase, highest recovery was obtained at a pH value of 3.0 and at pH 4.5 the recovery was significantly lower. In contrast to our results GRAMPP and co-workers used a pH of 4.5 for the maceration of potato tissues by a hydrolytic type of enzyme (*Rohament* P) while macerating activity of a lyase type pectolytic enzyme proved to be the best at pH 5.0 (ISHII & YOKOTSUKA, 1975).

According to this work the endo-polygalacturonase treatment of squash, cauliflower and parsley tissues showed maximum efficiency at pH values of 3.5 and 3.0, while the maceration of carrot and kohlrabi tissues was successful in a wider pH range (3–4.5 and 3.5–4.5 resp.).

The enzyme treatment will increase the biological value of these juices saving those valuable components and vitamins of the native vegetables, which are normally lost in the press cake, in the course of the usual pressing operations.

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KINETIC ANALYSIS OF PROTEIN SYNTHESIS IN FUNGI

PART IV. THE EFFECT OF pH CONTROL ON GROWTH AND PROTEIN FORMATION OF *RHIZOPUS COHNII*

K. ZETELAKI-HORVÁTH and K. VAS

(Received November 1, 1976)

In our previous work we studied the growth and protein synthesis as well as the kinetic behaviour of several fungal strains (*Mucor*, *Actinomucor* and *Rhizopus* sp.) in synthetic, semi-synthetic and natural media.

In connection with the above work this paper tries to detect the cause of diauxic growth without aiming at completeness, and to investigate the effect of pH as one of the suspected causes of diauxie.

The aim of our work was to establish whether pH control has any influence on the type of growth of *Rhizopus cohnii* as well as on the kinetic constants of its growth and protein synthesis in semi-synthetic (with glucose and sucrose carbon source) and in natural (waste bread) media.

The effect of pH control was investigated from the point of view of protein and carbohydrate content of the mycelia and of the mycelial and protein yield as well.

According to the results of this work pH control had no effect on the type of growth of the culture whichever of the above media was used. Stabilisation of the pH, on the other hand, increased the production of protein in the culture.

In the course of our experiments, the aim of which was to produce protein for human nutrition, the biomass and protein production of twenty filamentous fungi, belonging to the *Actinomucor*, *Mucor* and *Rhizopus* sp., were tested under various cultivation conditions.

With typical representatives of the above species kinetic analysis of growth and product formation was performed (ZETELAKI-HORVÁTH *et al.*, 1975; ZETELAKI-HORVÁTH *et al.*, 1976; ZETELAKI-HORVÁTH & VAS, 1976).

In our previous experiments, the growth and product formation of the kinetically analysed *Rhizopus cohnii* strain followed the pattern of a regular four-phase growth curve when synthetic glucose (**No. 1**) and natural bread medium (**No. 5**) were used. In synthetic sucrose (**No. 3**), semi-synthetic glucose (**No. 2**) and sucrose media (**No. 4**) (the latter two were supplemented with corn-steep liquor) the growth of *Rhizopus cohnii* was always diauxic.

The cause of diauxic growth was attempted to be explained in the course of the kinetic analysis of various polygalacturonase producing *Aspergillus* strains. According to this work, diauxie can be explained by the fact that the assimilation of various sugars proceeds one after another (following enzymatic decomposition of sucrose into glucose and fructose). When the medium con-

tained only a single carbon source, the primary utilization of the amino acids of the corn-steep liquor seemed to be the cause of diauxic growth (ZETELAKI-HORVÁTH, 1972).

The present paper gives information about the work in which the kinetic behaviour of *Rhizopus cohnii* was tested, using media (**Nos. 2**, **4** and **6**) in which the growth of this strain proved to be diauxic. The aim of this work was to establish how pH control can influence the type of growth of the micro-organism, whether diauxic growth can be eliminated by pH control. Our second aim was to establish whether the protein synthesis of the tested strain can be influenced by pH control.

1. Materials and methods

1.1. Microorganism

Rhizopus cohnii (No. 222) was used as a test organism. Stock cultures were maintained on malt agar slants.

1.2. Media of cultivation

The following composition was used for *inoculation medium:* yeast 5.0 g (in the form of extract); soluble starch 15 g; KH_2PO_4 1.0 g and $MgSO_4 \cdot 7H_2O$ 0.5 g adjusted to 1000 ml with tap water and to pH 5.5.

For *cultivation*, semi-synthetic (**Nos. 2** and **4**) and natural (**No. 6**) media were used the composition of which is indicated (in g l^{-1}) in the following table:

Components	Serial number of medium					
	1	2	3	4	5	6
Glucose	80	80	—	—	—	—
Sucrose	—	—	80	80	—	—
Ground-bread	—	—	—	—	40	40
Corn-steep liquor	—	20	—	20	—	20
$(NH_4)_2SO_4$			7.00			
$ZnSO_4$			0.25			
$MnSO_4$			0.10			
KH_2PO_4			1.00			

The pH was adjusted to 4.0. (The method of bread hydrolysis was given in a previous paper: ZETELAKI-HORVÁTH *et al.*, 1975.)

1.3. Method of cultivation

100 ml of the inoculum medium was inoculated with 10^6 spores and incubated for 24 h on a rotary shaker (rpm: 330; stroke: 20 mm; O_2-transfer rate in the flasks 17–19 mmole O_2 l^{-1} h^{-1}) at a temperature of 28 °C.

Fermentations were carried on in 10-liter glass fermenters. Six liters of the medium were inoculated in the fermenter with 600 ml of the above 25-h vegetative culture. The speed of agitation was 460 rpm and the volume of air bubbled through the cultures was 1.0 $l\,l^{-1}$ min^{-1}. The oxygen transfer rate in the fermenter was 50.8 mmole O_2 l^{-1} h^{-1} determined by the method of COOPER and co-workers (1944).

Experiments using the above media with and without pH control were carried on at the same time in two parallel fermenters using identical inocula.

The pH was adjusted every four hours to maintain it at approximately the original level of ca. 4.0. Without this correction the pH soon dropped and reached a stable value of about 2.0 after 24–36 h.

1.4. Preparation of the mycelia

Mycelia were obtained from the fermentation broth by filtration through a nylon cloth. The residues of the medium were removed by thorough washing in water, then the mycelia were dried at 105 °C to constant weight.

1.5. Protein determination

The protein content of the mycelia was determined by the biuret method, modified for protein determination in whole cells by HERBERT and co-workers (1971a).

1.6. Carbohydrate determination

Reducing sugars were measured by the method of SOMOGYI (1952) while the total carbohydrate content of the fermentation broths was determined by the phenol method (HERBERT *et al.*, 1971b).

1.7. Kinetic analysis

In the case of regular growth curves kinetic analysis of growth and protein formation were performed according to the equations of KONO (1968) and KONO and ASAI (1968). Kinetic analysis of diauxic growth was carried out according to KONO and ASAI (1971), while that of product formation in the diauxic cycle according to our previous work (ZETELAKI-HORVÁTH & BÉKÁSSY-MOLNÁR, 1973).

2. Results

2.1. Effect of pH control on mycelia and protein yields

Growth and protein synthesis of *Rhizopus cohnii* in media **Nos. 2**, **4** and **6** with and without pH control as a function of the cultivation period can be seen in Fig. 1.

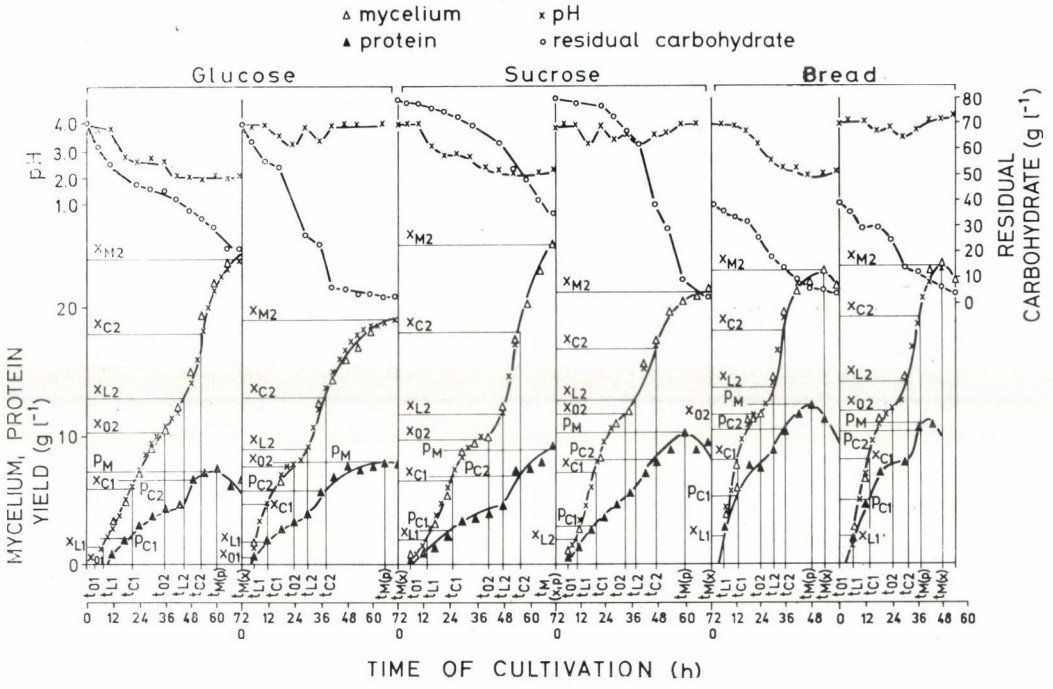

Fig. 1. The growth and protein formation of *Rhizopus cohnii* as a function of the time of cultivation in semi-synthetic glucose (**No. 2**), sucrose (**No. 4**) and in natural bread medium (**No. 6**) with and without pH control. (For other cultivation conditions: see Chapter 1)

The behaviour of *Rhizopus cohnii* in *glucose-* and *sucrose*-containing media was similar. pH control accelerated the assimilation of the sugars, the sugar consumption of the culture increased. In the case of pH control mycelial yield increased in the first part of diauxie and decreased in the second part, while protein was formed contrarily, it decreased in the first and increased in the second phase.

In the case of *bread* media, in contrast to the above, pH control resulted in an increase in the mycelial yield and a decrease in the protein yield in the second part of the diauxic cycle.

2.2. Effect of pH control on the rate of mycelium and protein formation

In accordance with the above results, pH control altered the rate of growth and product formation (Fig. 1). In the first part of the diauxie in *glucose*-containing medium, the effect of pH control resulted in an increase in the specific rate of growth (k) from 0.083 to 0.114 h^{-1} as well as in the specific rate of protein formation (k_{p1}) from 0.038 to 0.041 h^{-1} (Fig. 2 and Table 1), while the first part of the diauxie shortened by 9 hours at the expense of the second part (Fig. 1 and Table 1).

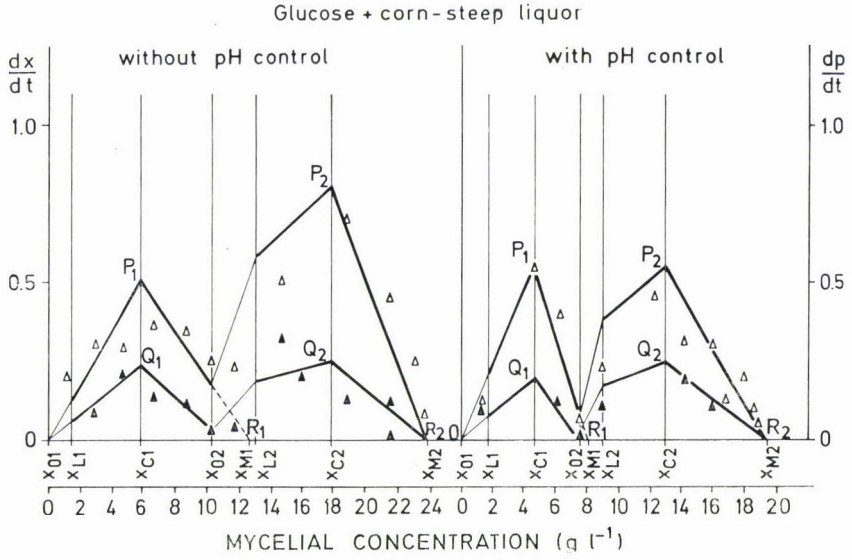

Fig. 2. Rate of growth and protein formation of *Rhizopus cohnii* as a function of mycelial concentration in semi-synthetic glucose medium (**No. 2**) with and without pH control

In *sucrose*-containing medium when pH was controlled, the specific rate of growth (k) also increased (from 0.083 to 0.100 h^{-1}) together with the specific rate of product formation (k_{p1}; from 0.040 to 0.049 h^{-1}) in the first part of the diauxic cycle (Fig. 3 and Table 1). As in the glucose medium, here, too, pH control resulted in the shortening of the first part of the diauxie (Fig. 1 and column "t_0" in Table 1).

The *Rhizopus* culture was less responsive to the effect of pH control in *bread* medium, although the change of pH in uncontrolled media was similar to that in the above ones. In the case of glucose and sucrose media, the specific rate of growth and product formation showed similar alterations. The effect of pH control resulted in an increase in the specific rate of growth (k) from 0.106 to 0.118 as well as in the specific rate of product formation (k_{p1}) from 0.075 to 0.088 h^{-1} (Fig. 4 and Table 1).

Table 1

Kinetic constants of the growth of Rhizopus cohnii (No. 222) *when cultivated in glucose,*

Carbon source	pH control	Part of diauxie	t_O	t_L	t_C	$t_{M/x}$
Glucose	–	1	6	7	21	–
		2	36	44.5	54	60
	+	1	3	6.0	12	–
		2	24	30.0	38.5	72
Sucrose	–	1	3	14.5	25.5	–
		2	42	48.0	55.5	72
	+	1	3	11.5	19.5	–
		2	33.5	39.0	48.0	72
Bread	–	1	3	5.0	13.0	–
		2	24	30.0	35.0	54
	+	1	3	6.0	13.5	–
		2	24	31.0	37.0	48

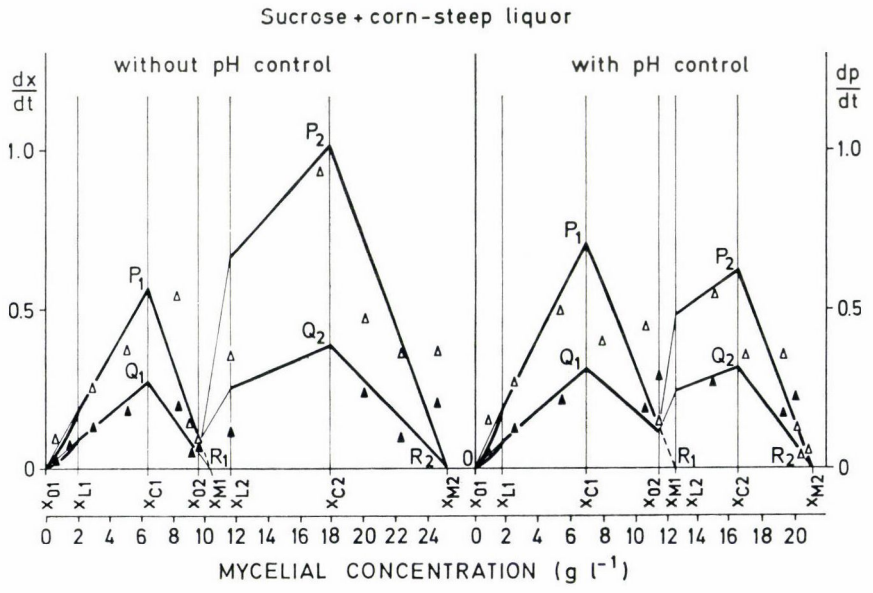

Fig. 3. Rate of growth and protein formation of *Rhizopus cohnii* as a function of mycelial concentration in semi-synthetic sucrose medium **(No. 4)** with and without pH control

sucrose and hydrolysed bread media with (+) and without (−) pH control

x_O	x_L	x_C	x_M	p_M	k	k_{p1}	$t_{M/p}$
0	1.5	6.0	12.8	4.60	0.083	0.038	
10.4	13.0	18.0	23.7	7.20	0.044	0.014	60
0	1.4	4.6	8.1	3.50	0.114	0.041	
7.6	9.0	13.0	19.0	7.80	0.042	0.019	60
0	1.8	6.4	10.4	4.30	0.083	0.040	
9.6	11.6	18.0	24.8	9.00	0.055	0.021	72
0	1.8	7.0	12.5	5.70	0.100	0.049	
11.6	12.6	16.6	20.9	10.20	0.037	0.019	60
0	2.0	8.0	12.0	8.00	0.106	0.075	
11.4	13.6	18.0	22.6	12.20	0.053	0.018	48
0	2.0	8.0	12.0	7.60	0.118	0.088	
11.6	14.0	19.0	23.0	10.58	0.040	0.020	48

The duration of the first part was not influenced by pH control (Fig. 1 and Table 1).

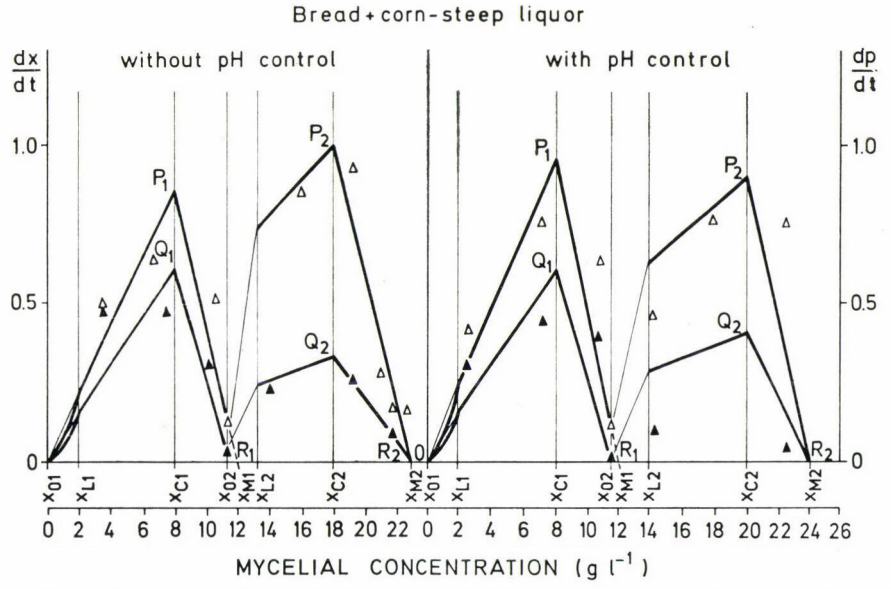

Fig. 4. Rate of growth and protein formation of *Rhizopus cohnii* as a function of mycelial concentration. The fungus was grown in natural bread medium (**No. 6**) with and without pH control

2.3. Effect of pH control on the composition of mycelia

When investigating the composition of mycelia of the *Rhizopus* culture grown on different carbon sources with and without pH control, it was found that pH control can eliminate the decrease in the protein content of the mycelia at the end of the fermentation (Fig. 5).

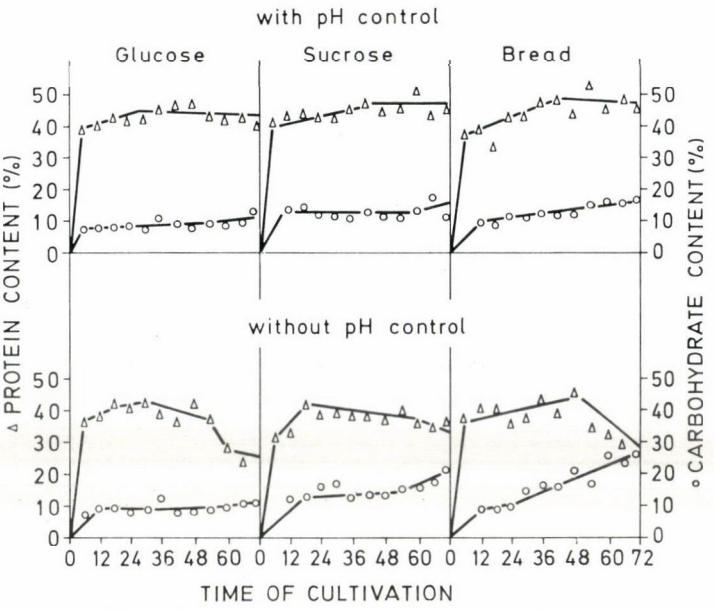

Fig. 5. Protein and carbohydrate content of the mycelia of *Rhizopus cohnii* as a function of the time of cultivation in semi-synthetic glucose, sucrose and natural bread media with and without pH control

The effect of pH control can also be seen in the carbohydrate content of the mycelia. In pH-controlled media the increase in the carbohydrate content of the mycelia as a function of the cultivation period was less significant than in those without pH control.

In *glucose*-containing medium, without pH control the values of the protein content of the 9- and 48-h *Rhizopus* cultures were within the limits of 39 and 42%. After 48 h the protein content of the mycelia gradually decreased to 24% at the end of cultivation, while in media of controlled pH its value varied between 40 and 45% during the whole process of fermentation. The carbohydrate content of the mycelia showed only a slight change during cultivation in glucose-containing media either with or without pH control.

When *sucrose* was used as a carbon source without pH control, the protein content of *Rhizopus* mycelia was the highest in the 18-h culture (42%), later decreasing gradually, while in the case of pH control its values were more constant and higher.

The carbohydrate content of the mycelium revealed an increasing tendency in both cases, showing maximum values of 16 and 22%, resp. with and without pH control.

In the case of *bread* medium, when pH was uncontrolled, the protein content of the mycelia of the 48-h *Rhizopus* culture attained a maximum of 45%, then, with a rapid decrease it dropped below 30% till the end of cultivation. In the pH-controlled medium a 48-h maximum in the protein content was similarly observed, exceeding 50%, this was followed by a decreasing tendency, but its minimum value was equal to the maximum of that without pH control. The carbohydrate content of the mycelia increased with ageing of the culture in both cases, but its maximum was 10% lower in the case of pH control than in the one without pH control.

3. Conclusions

In agreement with our previous results (ZETELAKI-HORVÁTH & VAS, 1976; Fig. 6) the growth of *Rhizopus cohnii* was diauxic when grown in glucose and sucrose media containing corn-steep liquor.

According to the results of the present work, the regular monoauxic growth of the *Rhizopus* culture in bread medium (Fig. 6) could also be altered to a diauxic one when the medium was supplemented with corn-steep liquor (Fig. 4).

When *Rhizopus cohnii* is cultivated without pH control as in our previous experiments, the lack of pH control might be supposed to be the cause of the diauxie. That is why the main purpose of this work was to establish whether the type of growth of the culture can be influenced by pH control. As it is clearly shown in Figures 1, 2, 3 and 4, the occurrence of diauxic growth was independent of pH control in the case of all culture media used.

Mycelial and protein yields of the *Rhizopus* culture have varied according to the media used in the case of pH control.

In glucose- and sucrose-containing media, the mycelial and protein yields of the culture as well as the duration of the two parts of the diauxic cycle have changed similarly. When the above media were used the first part of the diauxie shortened as a result of the pH control, while the maxima of mycelial yield became lower, and that of the protein yield higher.

In bread media, pH control resulted in higher mycelial yield and a lower protein yield, in contrast to the above media.

As a result of pH control the protein content of the mycelia increased, and their carbohydrate content decreased.

The change of the specific rate of growth of *Rhizopus cohnii*, cultivated with pH control, showed an increasing tendency in the case of all the media used.

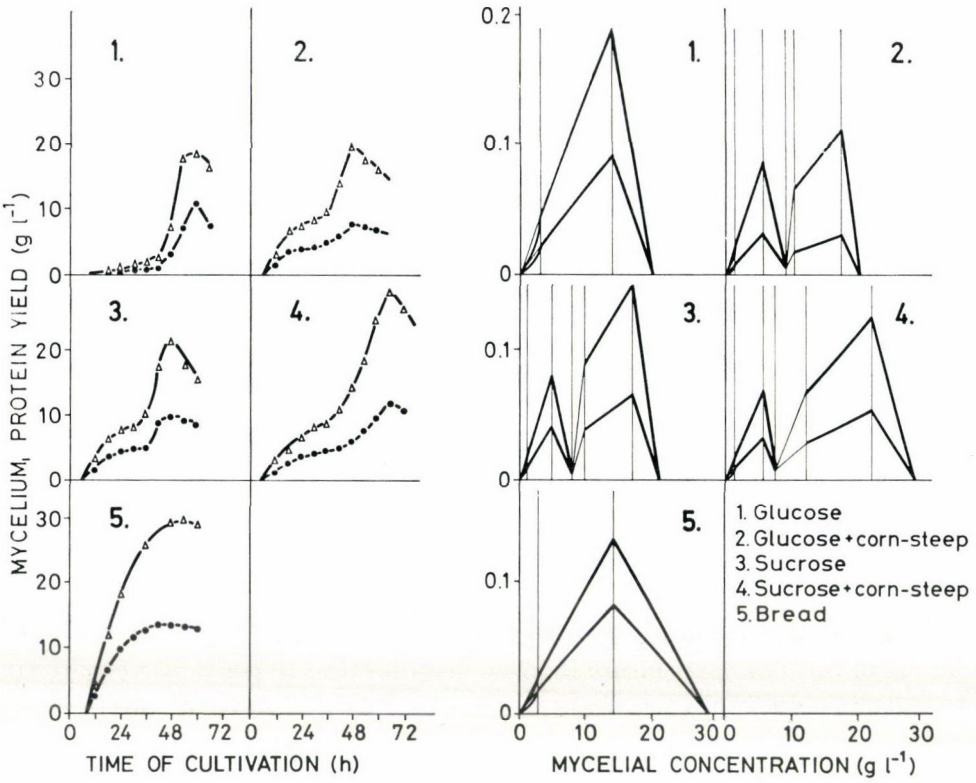

Fig. 6. Growth curves and protein production curves as functions of the time of cultivation, as well as the rate of growth and protein formation of *Rhizopus cohnii* as a function of mycelial concentration. The fungus was grown in synthetic (**Nos. 1** and **3**) semi-synthetic (**Nos. 2** and **4**) and natural media (**No. 5**), respectively

Symbols

k	growth rate constant, h^{-1}
k_{p1}	production rate constant of the growing cells
k_{p2}	production rate constant of the resting cells
t	time, h
x	cell concentration, g l^{-1}
p	protein concentration, g l^{-1}

Subscripts

O	refers to the boundary of an induction phase and a transient phase
L	refers to the boundary of a transient phase and an exponential growth phase
C	refers to the critical point that is the boundary of an exponential growth phase and a declining growth phase
M	refers to theoretical maximum value of cell and product concentration, respectively. The slopes of $\overline{OP}$, $\overline{OQ}$, and $\overline{OR}$ represent the values of k, k_{p1} and k_{p2} respectively.
$t_{M/x}$	time of maximum cell concentration
$t_{M/p}$	time of maximum product concentration

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HEALTH PROTECTION ASPECTS OF FOOD IRRADIATION AT THE PASTEURISATION LEVEL

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Food irradiation at pasteurisation level [radurisation or radicidation (GORESLINE *et al.*, 1974)] should not be looked upon solely as a potential menace to the health of consumers, but rather also, or perhaps primarily, as a source of benefit – just as milk pasteurisation. This is particularly so since absence of chronic oral toxicity of such radiation processed foods seems to have been well established, while the tools for the identification and an assessment of their long-term toxicity of particular radiolytic reaction products are available.

The following aspects of microbiological nature are discussed in this paper.

1. Mutations in micro-organisms secondary to irradiation are not at all unique to radiation processing and, generally, do not render these organisms more robust.

Occasionally observed increased toxin production after irradiation is compensated greatly by a reduction in the numbers of the toxin producing cells. Suitable methodology for monitoring will control any possible slight changes in diagnostic characters. Increasing resistance, resulting from extensive recycling, is accounted for by modern modes of assessment of dose requirements.

2. Potential problems related to micro-organisms surviving irradiation and causing less usual or unusual spoilage patterns can easily be controlled. Application of customarily used microbial ecological methodology allows assessment of the nature of the organisms and their metabolic activities and also enables designing methods for arresting their proliferation.

3. Food virology being a young branch of medical food microbiology and new enteric viruses being isolated regularly, research enabling assessment of health hazards inherent to food-transmitted viruses should be greatly encouraged.

Food irradiation is not aggravating any problems in this area, rather reducing the counts of infective units, albeit not to the extent of heat processing, which it is in most instances not intended to replace, however.

4. Mycotoxicoses are serious though rare diseases; their nature, aetiology and prevention are well known, in contrast to the situation prevailing in the area of food virology.

Mould-prone commodities should be protected against attack by fungi. Even if irradiation of moulds present in such products would lead to increases in toxin formation – a possibility so far not yet demonstrated beyond doubt – such unfavourable possibilities have to be carefully weighed against the reduction in numbers of mould propagules, as a result of irradiation.

General aspects of consumer protection

It seems useful to emphasize a few fundamental aspects of food irradiation that, although in part expressed previously (EUROPEAN MEETING, 1961; TECHNICAL BASIS, 1965; CONSULTANTS' MEETING, 1974; MOSSEL, 1976a), are still quite pressing.

The first point is, that, in principle, the irradiation of food should always be carried out with the purpose of serving the interest of the consumer in a world-wide sense. The benefits to the consumer of the elimination of enteric pathogens (MOSSEL, 1976a) and other harmful micro-organisms (MOSSEL *et al.*, 1968) from food by low dose irradiation are obvious. However, the use of ionizing radiation for food preservation is, indirectly, also serving a general public health interest, since it decreases losses of foods, which at present may amount to no less than 10 per cent of the world food supply (FAO, 1969). The logical inference from this principle is that public health legislators cannot very well neglect this contribution of irradiation to both qualitative and quantitative factors in human nutrition. It would be sad indeed if the lesson emerging from the deplorable battle for the pasteurization of milk fought four decades ago (WILSON, 1933) would not have been learned meanwhile.

Clearly, this does not detract from public health officials having to exert great care in authorizing the use of radiation for the processing of food. The main point here is not a microbiological one; yet it should be mentioned in passing. It seems to have been well established recently that acute or chronic toxicological side effects from consumption of food irradiated at the pasteurising level need not be feared (WHO, 1970; VAN LOGTEN *et al.*, 1972; THIEULIN *et al.*, 1972; HICKMAN, 1973; ELIAS, 1976). However, it has been questioned whether, secondary to the irradiation of food, components might be formed in traces by radiolytic reactions that, in the long run, could harm the consumer. At present relevant gas chromatographic and/or mass spectrometric data are available (MERRITT, 1972; ANGELINI, 1975; DIEHL & SCHERZ, 1975; TAUB *et al.*, 1975) to allow the assessment of such potential risks.

One of the main points of concern of microbiological nature has always been the justified fear that permitting the use of ionizing radiation in food processing could, or rather would, lead to neglect of sanitary measures or effective use of the cold chain that are essential to Good Manufacturing and Distribution Practices ('GMP'). First of all, neglect of these matters threatens, in principle, all food processing techniques: heat treatment, fermentation and chemical preservation alike (MOSSEL, 1975). Ethical food manufacturing industry has come, long ago, to the empirical conclusion that neglecting measures leading to low initial counts of foods to be processed terminally, is utterly unwise. Firstly, this will make more intensive processing compulsory and hence lead to loss of organoleptic quality (MOSSEL, 1975) — the last thing the food industry is after. In addition there are so-called 'remanent' effects of the building-up of relatively elevated microbial populations in materials to be processed subsequently, *viz.* (i) the formation of thermostable toxins; (ii) secretion of high levels of enzymes partly resisting the ultimate treatment; and (iii) initial attacks on the structural integrity of essential food components that will eventually lead to quality loss in the processed commod-

ity upon storage (MOSSEL, 1975). Besides, there can hardly be a less non-conspicuous type of factory than a food irradiation plant, generally requiring authorization by environmental protection authorities. Hence, in granting this permission and during the monitoring of the plant, full control of any deficient microbiological practices can be exerted.

Food irradiation thus being not likely to be applied without supporting advanced technology, is hence unique in its availability for inspection and control. It should be added, in fairness, that it is also unique in some of its beneficial effects. Human health is threatened all over the world (MOSSEL, 1976b) by outbreaks of enteric febrile diseases ultimately caused by contaminated fresh meats and poultry. Presently no other mode of controlling these diseases than by low dose gamma irradiation of the chilled or frozen commodities is available or even known (MAXCY & TIWARI, 1973; MOSSEL, 1976a). It seems to be almost an obligation for public health authorities to keep this possibility in mind.

Specific microbiological aspects

Concerns over the possibility of negative microbiological consequences of food irradiation can be categorized in four groups. The first pertains to the problem of mutation due to irradiation, changing resistance or biochemical attributes of microorganisms. The second group of concerns deals with the consequences of ecological nature, principally with how to control microorganisms surviving radiation treatment. The third type of observations stresses the problem of viruses in foods which, as is well known, show a much greater radiation resistance than bacteria. Finally, data on probably increased toxigenicity in mycotoxinogenic moulds have aroused some concern that needs the attention of professional microbiologists.

These aspects will be dealt with in this order in the following sections.

1. The significance of mutations

It seems to be useful to reiterate first of all two fundamental principles of microbial genetics, pertaining to this area.

First, deliberately used ionizing radiation is not at all unique in inducing mutations in microorganisms. Many conventional environmental factors have mutagenic effects; among these are heat, low a_w; cosmic rays, ultraviolet radiation; nitrites and epoxy compounds.

Next, the general effect of mutagenic agents is impairment of function, expressed in (i) increased requirements for growth; and (ii) loss of pathogenicity, rather than the reverse, *i.e.* acquisition of disease producing properties *e.g.* in the saprophytic microbial association of foods.

With regard to other changes of medical significance the following may be concluded from the literature. Increases in toxin production have been observed in *Cl. botulinum* and in mycotoxinogenic moulds, such as *Aspergillus flavus* (INGRAM, 1975). It has not yet been analysed whether this is a 'real' effect or just results from random mutations provoked by environmental influences *(vide supra)*, because the effects are far from consistent in the sense that toxin production sometimes increases and in other experiments diminishes. The best approach to study the actual significance of such effects in terms of health protection is a careful evaluation of the *net* effect of food irradiation, *i.e.* the integrated result of radiation-induced reduction in numbers of cells and possibly increased toxin formation per surviving colony forming unit (cfu).

Modifications of diagnostic characters of enteropathogenic organisms induced by irradiation have indeed been observed (INGRAM, 1975). However, they have mostly been restricted to minor traits, and no case of *e.g.* a saltation into a different taxon has yet been reported. As has been suggested many years ago (MOSSEL, 1958) these potential difficulties in the assessment of surviving cells can be eliminated, for all practical purposes, by relying on an enumeration of all *Enterobacteriaceae*, in addition to the specific assessment of surviving cfu of *Salmonella*, *Shigella* or *E. coli* (DRION & MOSSEL, 1977).

Finally, the evidence of increased radiation resistance due to 'recycling' and other mechanisms is unambiguous. However, such increases virtually never exceed differences in radiation resistance observed in 'wild' populations of *e.g.* different sero- or biovars of the same genus. The former as well as the latter fluctuations are fully accounted for, however, by the modern mode of assessment of 'adequate' doses for radicidation. This is based on the use of the integrated parameter $MPED_n$ (= most probable effective dose for attaining n overall decimal reductions; MOSSEL *et al.*, 1965, 1968; MOSSEL, 1976a).

2. Ecological aspects

Repeatedly, concern has been expressed about the activities of the microorganisms surviving irradiation at pasteurisation level. Once again, there is nothing new nor unique in the effects of the organisms that survive processing by radiation (MOSSEL & INGRAM, 1955).

It has been established very many years ago by microbial ecologists that, according to the nature of a food (pH, a_w, mode of processing, usual conditions of storage, *etc.*) every specific item will develop its own, typical microflora, customarily indicated as microbial association (WESTERDIJK, 1949). Thus, fresh proteinaceous foods will become slimy in the course of chilled storage, cured meats tend to microbial souring, fruit juices are fermented, semi-dry staples turn mouldy and heat-pasteurized foods such as milk spoil

due to the development of heat-resistant bacterial species; particularly spores of *Bacillaceae* and, less commonly, Lancefield group D streptococci (MOSSEL, 1971).

The changes in the composition of the associations of irradiated foods and the resulting customary, less usual, or sometimes quite unusual forms of microbial spoilage are therefore common to all processed foods. This does not detract from the need to accord each individual commodity thorough experimental consideration. Well-established methodology is available for this purpose. It consists, in essence, of following the course of microbial events by appropriate quantitative culturing methods, as well as chemical methods for the determination of metabolites, characteristic of spoilage, such as low and/or high molecular-weight fatty acids, keto bodies, ammonia and amines, H_2S and mercaptans *etc.*; *cf.* Fig. 1. Following approximate taxonomic grouping of the predominating microorganisms (MOSSEL, *et al.*, 1977) and choosing appropriate selective methods for the assessment of their numbers of cfu/g (MOSSEL, 1975), the monitoring of the events henceforth becomes a matter of simple routine.

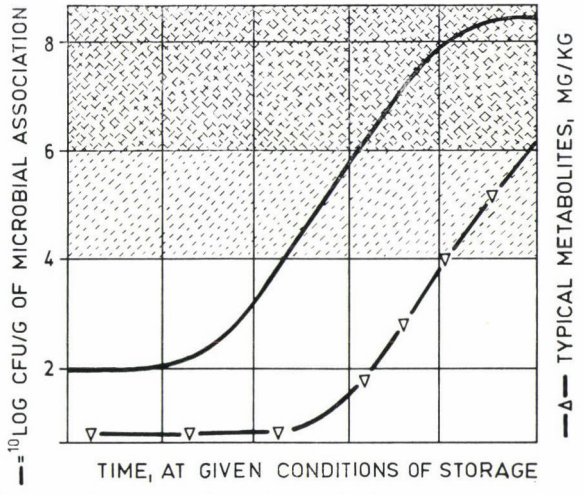

Fig. 1. Number of colony-forming units and metabolite concentration, resp., as a function of the storage time

Once this method of monitoring is available, adequate control of spoilage becomes an easy task. Generally, storage temperatures — attained in the foods themselves — of ≤ 5 °C will control surviving pathogens, with the exception of *Cl. botulinum* type E, being inhibited only by temperatures of ≤ 3 °C. Bacteria of the genera *Listeria* and *Yersinia* may require still lower temperatures for complete inhibition (MOSSEL, 1975), but their occurrence in fresh foods is rather sporadic and, given their low radiation resistance, their presence in radiation-treated foods therefore highly unlikely. In addition they

will not develop to hazardous numbers before truly psychrotrophic, more radiation resistant bacteria will have spoiled the food. Long-term inhibition of psychrotrophic organisms can only be achieved by freezing as currently practised.

3. Viruses transmitted by foods

As resistance of organisms to ionizing radiation increases with decreasing size, it was no surprise to observe that viruses were much more radiation resistant than bacteria. As a first approximation D-values of viruses are of the order 0.6 Mrad (JORDAN & KEMPE, 1956; JOHNSON, 1965; SULLIVAN *et al.*, 1971, 1973; DI GIROLAMO *et al.*, 1972), whereas most non-sporing rod-shaped bacteria show D-values only occasionally exceeding 0.06 Mrad (INGRAM, 1975).

The transmission of some viruses by foods has been demonstrated (CLIVER, 1971; GERBA *et al.*, 1975) and also their 'complete' elimination by customary heat processing (CLIVER, 1971). However, irradiation will reduce the numbers of infective units (i.u.) to some extent, perhaps even sufficiently, given their natural occurrence in low numbers of i.u./g only. This has, obviously to be investigated in more detail by competent virologists. It should be kept in mind, however, that this is much more than can be said for refrigeration of fresh foods of animal origin (CLIVER, 1971), the only mode of processing applied to the greater part of the production of this potentially dangerous staple food nowadays.

At any rate, the area of food virology is indeed showing the most 'unknowns' in the entire field of modern medical food microbiology. It is therefore strongly recommended to continue or initiate the virological research required to assess the health hazards of the most prevalent, food-transmitted viruses. This should be done irrespective of food irradiation which as we have just indicated might only mitigate the risks inherent to the consumption of fresh foods of animal origin.

4. Increased risk of mycotoxicoses

Diseases of a toxic nature caused by the long time consumption of foods severely contaminated with particular mould species (mycotoxicoses) are not at all examples of recently discovered pathology. Ergotism, a classical example of this type of diseases was known, although barely recognized as such, since the Middle Ages. From about 1800 it had been observed that mouldy feeds could harm farm animals. Since the turn of the last century evidence was obtained in Russia and Japan that mould-infested cereals and rice were probably aetiologically related to serious disease syndromes in man. Final proof of the role of mouldy cereals in causing diseases in human consumers came from

the most unfortunate outbreak of alimentary toxic aleukia in the U.S.S.R. in 1943. At the present time some 250 mycotoxinogenic mould types have been identified and approximately 30 mycotoxins isolated and characterized (MOREAU, 1974).

All mycotoxins are relatively low molecular-weight compounds of high thermal resistance. Hence, once formed in foods, they will be carried over, irrespective of most modes of processing customarily applied in food technology. The diseases provoked by intake over long duration of mycotoxins are serious: the organs attacked are predominantly the haematopoietic system, liver, kidneys, endocrine system and parts of the nervous system. In the latter sense mycotoxins are unique in that some of them are tremorgenic, an effect virtually never observed in naturally occurring substances (MOREAU, 1974).

Fortunately the formation of mycotoxins is easy to control. Referring again to Fig. 1 all that is required is inhibiting the germination of mould spores and the proliferation of mycelia. This can be achieved by reducing the water content of the commodities prone to become mouldy to values corresponding to $a_w \leq 0.60$ (MOSSEL, 1975). Where this cannot yet be consistently achieved due to failing technology, the addition of 0.1–0.2% of the completely innocuous preservative propionic acid will control the development of moulds and hence subsequent formation of mycotoxins (MOSSEL, 1975).

In this context the concern about risks of mycotoxicosis in relation to food irradiation seems to be barely justified. It was triggered by the observation of 10–50-fold increase in the formation of mycotoxins after irradiation of mould spores (APPLEGATE & CHIPLEY, 1973a, b, 1974; BULLERMAN & HARTUNG, 1975). Even if one refrains from quite justified reserve with regard to the conclusion of these investigations (INGRAM, 1975) and accepts the authors' own inferences, there is little need for concern. Such changes in toxin formation will have to be offset against the reductions in counts of mould propagules effected by the irradiation process; *cf.* 1 above.

In addition an essential aspect of food irradiation should be reiterated. It has been postulated that this mode of processing should never be dissociated from GMP. The latter involves, in the case of storage of cereals and other mould-prone products that either the final a_w should be below the value allowing germination of mould spores or the commodity should be adequately preserved. Hence, when GMP are respected there is no chance of germination of mould spores and subsequent formation of mycotoxins in irradiated foods.

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Abstracts

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ENZYME PREPARATIONS IN THE FOOD INDUSTRY

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Enzymes, as catalysts of the chemical processes taking place in biological materials, are natural components of most food raw materials. In order to improve and to control the effect of enzymes producing favourable changes in the quality of food, enzyme preparations of reproducible activity have been added to food since the beginning of the 20th century. The rapidly developing fermentation industries promote the economical production of these preparations.

The enzyme preparations used in the food industry might be classified according to the reaction catalyzed, the effect desired, the applying industries, the source and physico-chemical state of the preparations.

A survey of the situation in enzyme production all over the world is presented with special emphasis on the problems encountered in Hungary.

Recently developed enzyme preparations applied or applicable in the food industry (glucose isomerase, lactase, iso-amylase, lipase, nonspecific proteases, flavour enzymes) are discussed. Of the new production technologies immobilization on solid carriers is stressed and carrier-bound enzyme preparations used in the food industry as reported in the literature are listed (amino acid acylase, glucoamylase, invertase, proteases, lactase, glucose isomerase, catalase, glucose oxidase, etc.).

Of the new fields of application the use of enzyme preparations in the food and allied industries, in animal feeds and in environmental protection are, along with some other special fields with emphasis on certain urgent problems, awaiting solution.

Finally, the importance of enzyme preparations in the analysis of foodstuffs is briefly surveyed.

"ENZYME ENGINEERING" IN THE FOOD INDUSTRY

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The term "enzyme engineering" is defined and its objectives outlined. These are: preparation (by means of fermentation in case of enzymes of microbiological origin) and application of enzymes; development of processes on scientific foundation to replace empirical methods, for instance, the study of enzyme formation during fermentation or the interpretation of enzyme production and application based on the knowledge of molecular mechanism.

The following problems pertaining to this field have been investigated.

1. Microbiological enzyme production: optimization of the fermentation of *Bac. macerans* amylase from the aspect of cell growth and enzyme production. Since the quantity and life-time of mRNA and tRNA can already be measured, it remains to optimize and to influence these genetically.

2. Enzyme production: so far non-specific methods have been applied, the goal is to replace these by the industrial application of so-called biospecific methods like affinity chromatography. The possibilities of the binding, by affinity chromatography and of the recovery of amylases of various types are being studied.

3. Use of dissolved enzymes: substitution of empirical methods by means of elucidation of the molecular mechanism. Study of the subsite theory and its application to the enzymes alpha-amylase and glucose isomerase.

4. Immobilized enzymes: several methods for immobilizing enzymes are known, but various problems of application technology still need elucidation (modification of the surface, immobilization of the coenzyme, etc.).

USE OF PROTEOLYTIC ENZYME PREPARATIONS FOR THE PARTIAL HYDROLYSIS OF MEAT PROTEIN RICH IN CONNECTIVE TISSUES

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Experiments were carried out in collaboration with several canning factories to extract the protein content of minced beef, giblets and crushed bony poultry residues by means of proteolytic enzyme preparations.

The enzyme preparations used in the experiments were: a neutral protease manufactured by Chinoin, Budapest and "Bakterienproteinase N" and "Corolase" of the firm Röhm.

A laboratory device was constructed for the enhancement of the effect of enzyme treatment by simultaneous application of a mechanical effect.

The experiments have proved that the protein content of beef can be dissolved to the desired degree by the use of a protease preparation.

By means of the combined treatment protein corresponding to 300–500 g of meat can be obtained from 1000 g of giblet or crushed bony poultry residue.

EFFECT OF ENZYME-SUPPLEMENTED FEED ON THE BREEDING PARAMETERS OF CERTAIN TYPES OF BROILERS

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The breeding data of sexed hybrid broilers of the Mezőtúr and Hubbard strains reared on enzyme-free and alpha-amylase containing feed, respectively, were compared. The composition of the diets and the conditions of rearing were the same for both strains.

The difference in the average final weights of the Mezőtúr and Hubbard females reared on the control feed was, on the 56th day, 115 g (1375 and 1490 g, resp.) and 121 g (1639 and 1760 g, resp.) in the case of males. A comparison of the sexes with respect to specific feed consumption showed a difference of only 20 g in both groups.

The average weight of females of both types reared on feed containing 0.4 % of alpha-amylase was higher by 4 % than that of the controls. Independently of the strains feed utilization of the enzyme-fed stock was higher by 3 %

per kg of live weight, as compared to the control group. The excess weight of the males fed with enzyme-containing feed was 4.0 and 4.6% higher than that of the control. The saving in feed calculated for unit live weight was 6.6% in the case of the Mezőtúr strain, and 5.3% in the case of the Hubbard strain.

The superiority of the breeding parameters of the poultry fed enzyme-containing feed was confirmed by biometric calculations.

The results suggest that the effectiveness of alpha-amylase depends presumably not on the strain of broilers but primarily upon the degree to which the starch in the feed is hydrolyzed by the enzyme.

OPTIMIZATION OF THE COMPOSITION OF THE CULTURE MEDIUM TO OBTAIN, BY SUBMERGED FERMENTATION, A MILK-CLOTTING ENZYME PREPARATION OF MICROBIAL ORIGIN

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Experiments were carried out to increase the milk-clotting activity of the fermentation broth obtained by submerged cultivation of *Endothia parasitica* under laboratory conditions in shaken cultures.

The aim was to determine the optimum ratio between the components of the culture medium of the enzyme-producing fermentation under the fermentation conditions developed earlier. Other conditions of fermentation, like the preparation of the inoculum, aeration, the qualitative composition of the culture medium, were left unchanged.

Mathematical models as widely used lately were applied to the optimization of the culture medium. The possible variations of the components of the culture medium were established by increasing and reducing, resp., the quantities of the various components as compared to their amounts in the initial culture medium. The effects of these changes as characterized by the clotting activity of the filtrates obtained from the varied culture media were recorded.

The components of the culture medium were: extracted soybean, glucose, $CaCO_3$ or $Ca(NO_3)_2$.

Taking into consideration the increase in enzyme activity obtained by applying the apparently optimal culture medium composition the variation range of the concentrations of the components in the culture media was further narrowed. The results were compared to the milk-clotting activity in the initial culture medium.

The results showed that a higher soybean concentration greatly reduced and a higher $CaCO_3$ or $Ca(NO_3)_2$ concentration markedly enhanced the milk-clotting activity of the fermentation broth, while the concentration of glucose had hardly any effect.

Compared to the milk-clotting activity obtained in the initial culture medium that of the optimum culture medium was higher by nearly 100%.

EXPERIMENTS TO OBTAIN AN IMMOBILIZED MILK-CLOTTING ENZYME PREPARATION

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The use of carrier-bound enzymes is spreading in the food industry. From their potential field of utilization in the dairy industry immobilization of milk-clotting enzymes might be particularly important, since it might promote solving the problem of continuous cheese production by providing for the continuous clotting of milk.

The Department of Enzymology of the Central Food Research Institute developed a milk-clotting enzyme preparation by submerged fermentation of the mould strain *Endothia parasitica* and immobilized the enzyme by means of ionic bonds on DEAE-cellulose. The preliminary experiments were performed with technical grade and gel chromatographically purified enzyme preparations, resp.

Protein binding was studied in relation to protein content, ionic strength and pH of the enzyme solution as well as to temperature. The enzyme bound to the DEAE-cellulose carrier was characterized by its clotting capacity, that is by the quantity of milk coagulated within 40 min by 1 g of wet DEAE-cellulose containing the enzyme.

According to the results DEAE-cellulose incubated with a 1% technical grade or with a purified enzyme solution containing about 0.6 mg ml^{-1} of protein had the highest clotting capacity.

INVESTIGATIONS INTO THE PROTEOLITYTIC CAPACITY OF *LACTOBACILLUS CASEI* STRAINS USED IN CHEESE PRODUCTION

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The taste of some types of Hungarian cheese is not sufficiently characteristic. This is particularly true for the "trappist" cheese ripened in plastic foil. Beside the conditions of manufacturing, salting and ripening, the enzymes of the applied clotting agent and bacterial cultures (starters) will primarily contribute to the development of the taste of cheese. Since in the development of cheese flavour the proteolytic enzymes are of decisive importance and so far the cultures used in the Hungarian cheese industry have not been studied from the aspect of their production of proteolytic enzymes, the strains applied have now been subjected to an investigation of this type.

In cheese manufacture the strains are used together with butter culture and milk-clotting enzyme preparations, hence the effects of these must also be taken into consideration. A synergistic or antagonistic effect might exist between the enzymes of the strains in the butter and cheese cultures as well as the milk-clotting enzyme preparation applied and these effects may have a decisive influence on proteolysis in cheese.

The proteolytic capacities of the strains in butter and cheese cultures and of the clotting enzyme were tested separately and concomitantly under laboratory conditions. The degree of proteolysis was recorded as the change of the formol titer of the samples.

According to the results the *Lb. casei* strains used in Hungary can be classified, from the aspect of proteolytic action, into three groups: into those of poorly, fairly and intensely proteolytic strains.

When the strains were grown together with the butter starter culture, no influence on their proteolytic capacity was observed, but the presence of the clotting enzyme increased the activity of the strains. The simultaneous effect of the butter starter culture and of the clotting enzyme caused a decrease in the proteolytic capacity of the strains.

Experiments of cheese production on the large scale are needed to decide which of the strains in the proteolytic group is the most advantageous from the aspect of obtaining a satisfactory cheese flavour.

THE USE OF ENDO-POLYGALACTURONASE FOR FRUITS AND VEGETABLES

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By destroying the pectin-containing binder between the cells of vegetable tissues the enzyme endo-polygalacturonase (endo-PG) forms a suspension consisting of individual cells or smaller cell agglomerates.

In the case of vegetables the pH optimum of decomposition was studied by the addition of 0.5% of the endo-polygalacturonase (pilot product of Central Food Research Institute). When disintegration was performed at optimum pH values the filtrates with particles smaller than 0.25 mm contained about 90% or more of the solids of the original tissue. Disintegration around 80% was observed in the case of green paprika, celery, onion, kohlrabi, cauliflower and savoy, while a 60% degradation was achieved in French beans and beetroot and less than 40% in the case of green peas, spinach and garden sorrel.

The endo-PG treatment of fruits resulted in a 10 to 40% increase in juice yield. The specific viscosity of the juices was 20 to 40% of the control value, with the exception of plums, sour cherry, quince and black-berry. The specific viscosity of plum juice was significantly lower, less than 10% of the control value, while the specific viscosities of sour cherry and blackberry were almost the same (90%), as that of the control. The optimum enzyme concentration for sour cherry and blackberry was 0.01%, for quince 0.005% and for the other fruits investigated 0.05%.

In the case of tomatoes the effect of temperature, enzyme concentration and the duration of the incubation period on juice yield, dry matter yield, on specific viscosity and on the duration of the evaporation was tested for the variety K3 (containing 60% red, 30% straw-coloured and 10% green fruits) and the variety Peto Mac developed for mechanical harvesting.

Depending upon the variety and temperature used, the addition of 0.005% or 0.01% endo-PG enzyme increased the dry matter yield of the samples by 12 to 20%. After an incubation period of 6 h the enzyme-treated samples required a 20 to 30% shorter time for complete evaporation.

Treatment of rose-hips with 0.05% of endo-polygalacturonase resulted in an 8 to 10% higher utilization of the dry matter content together with an 11 to 30% higher yield of vitamin C and a 50 to 70% decrease in specific viscosity.

Treatment with endo-polygalacturonase improved the stability of the vegetable juices. Tomato juice could be stored without sedimentation more than 5 months.

The storage stability of the various vegetable juices (green paprika, celery, carrot, turnip, etc.) could be extended to more than 4 months by simultaneous endo-PG and ultrasonic treatment.

TISSUE ENZYMES OF FOODS AND OF THE RAW MATERIALS OF THE FOOD INDUSTRY

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In vegetable and animal organisms every material is subject to a permanent process of synthesis and degradation. The changes due to this dynamic state of living organisms affect the properties and quality of the nutrients. As long as the vegetable or animal organism lives under natural conditions its vital processes will be in order, governed by a central control mechanism. In the harvested or picked plants and in the isolated parts of the living organisms this organization of the vital processes no longer prevails and dissimilation processes will dominate. Under certain conditions this state might lead to the deterioration of foodstuffs.

Of the changes brought about by tissue enzymes in foods of animal origin the maturing and autolytic processes of meat are the most important.

Several types of processes are known to take place in picked vegetable parts. In the course of the respiratory processes first of all the quantity of carbohydrates tends to decrease; in unripe vegetable parts mainly glycolysis occurs, while in the ripe ones the pentose cycle plays an important part. The sugar-starch conversion processes might proceed parallel to respiration.

Ripening, too, is accompanied by complicated enzymatic reactions, such as the formation of hormones which influence ripening, and parallel to it the degradation of colouring matter: hydrolysis and Mg-loss of chlorophyll, as well as the oxidation of the carotenoids to epoxides. A change in tissue structure is manifest at the time of ripening which can be best characterized by the enzymatic degradation of protopectin. The enzymatic mechanism of the formation of flavour substances in the course of ripening is a little investigated and elucidated field.

The tissue enzymes play an important role also in processing. The predominance and control of hydrolytic processes are of great importance in many branches of the industry; for instance the effect of amylases and proteases in baking technologies or in the manufacture of wort and in general in all processes involving the use of malt or flour.

The action and the possibility of inhibition of polyphenoloxidases playing a negative role in the technological processes have been studied by several

authors. Of the hypotheses concerning the mechanism of action of the copper-containing enzyme the most probable appears to be the one which suggests that the copper atom of the activated phenolase containing Cu(I) forms a link with one of the hydroxyls of *o*-diphenol, then oxidizes the latter to *o*-quinone with the help of oxygen bound by a co-ordinative bond. The bivalent copper is reduced by the hydroxylation of a monophenol into the monovalent ion and thus the cycle continues. The mechanism of action of phenolases containing bivalent copper is unknown.

ON THE ENZYMATIC BROWNING OF FRUITS

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The enzymatic browning of fruits is attributed primarily to the action of the enzyme polyphenol oxidase. This enzyme which occurs in many forms in nature and its large number of substrates have been the subject of research for a long time. In the accumulated material opinions differ whether the enzyme or the substrate is the decisive factor in browning.

From the simultaneous measurement of the initial rate of actual browning, of enzyme activity and of the substrate concentration in apple and apricot homogenates the conclusion was drawn that, independently of the fruit, the ratio of enzyme to substrate concentration will decide which of the two factors will determine the rate of browning in the product. If the numerical value of this ratio (in the units used by the authors) does not exceed 20 the initial rate of browning will be in good approximation a linear function of enzyme activity ($r^2 = 0.81$). This ratio was found in apple cultivars which had been tested for several years and in two apricot cultivars. A ratio higher than 35 was found in 7 batches of apricots of different cultivars tested for two seasons. In this case a very close correlation was found between the rate of browning and the concentration of the substrate ($r^2 = 0.96$) assuming a saturation relationship. For the explanation of this phenomenon it is suggested that in the first case substrate in the fruit is sufficient for the formation of reaction products in a concentration inactivating the enzyme, hence the enzyme is the limiting factor in the reaction. In the second case even total depletion of the substrate is not sufficient to produce a concentration of the reaction product which would inhibit the enzyme, thus substrate content will limit the reaction.

In order to prove the general validity of this assumption experiments will be extended to other kinds of fruit.

pH AND TEMPERATURE DEPENDENCE OF THE POLYPHENOLOXIDASE AND PEROXIDASE ACTIVITIES OF FRUITS AND VEGETABLES

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The authors have studied, for several years, polyphenol oxidase and peroxidase as enzymes which are detrimental from the aspect of processing horticultural products. In relation to the inhibition of the action of these two enzymes, their activities, temperature and pH dependences were investigated in the homogenates of various vegetables and fruits.

Polyphenol oxidase activity in potatoes increased between 25 and 35 °C, whereas it reached a maximum at 30 °C in apples and at 25 °C in apricots. Peroxidase activity in potatoes increased between 7 and 55 °C, reached a maximum in kohlrabi at 40 °C and in apricots at 50 °C. The apparent activation energies of the enzymes occurring in the various products were calculated from the activity–temperature relationships.

Polyphenol oxidase activity increased in the pH range between 4.5 and 6.5 in apples, apricots and peaches, while in potatoes the enzyme was practically inactive at a pH value lower than 6. The activity—pH relationship of a given product was influenced by the cultivar and substrate, too.

Peroxidase activity in potatoes was highest in the pH range between 5 and 5.4, in kohlrabi — depending upon the variety — between pH 5.1 and 5.9–6.0. At pH 6.0, the peroxidase activity in apricots was more than seven times higher than at the pH value of the fruit (pH 3.5).

Comparison of these results with published data shows considerable differences between the relationships from one product to the other and suggests that from experiments with purified enzyme preparations no conclusions can be drawn as to the behaviour of enzymes bound to cell particles.

THE EFFECT OF WHEAT AMYLASES ON WHEAT STARCH

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The deterioration of the quality of bread made of germinated wheat flour is caused mainly by an unfavourable change in the amylolytic state due to increased activity of the amylases and the enhanced accessibility of starch.

The accessibility of starch of various wheat varieties was measured as a function of germination time. The effect of the amylolytic wheat enzymes of

different activities on starch was studied on its dependence upon variety and the state of germination.

Ten different varieties of wheat were allowed to germinate for 4 days and the activities of the amylase extracts and the amylolytic state were measured. The accessibility of starch was determined in the control sample and in the amylase extract of flour obtained from wheat germinated for 4 days.

It was found that the degree of amylolysis was considerably increased by enzyme extracts of high activity, while the accessibility of starch extracted from the flour of wheat in different states of germination was not significantly raised. The effect of the variety of wheat on the accessibility of starch is stronger if degradation is induced by an extract of high amylase activity than if the extract of the control flour is used for degradation.

STUDY OF WHEAT PROTEASES

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The enzymatic state occupies an important position among the parameters which determine the technological value of wheat. The amylases and proteases play here a particularly important role. While the interest of investigators is focussed on the amylases and there are many publications dealing with their properties and their role, only some very scanty information is available on the proteases of wheat.

The authors studied the activity of proteases in various varieties of wheat both at rest and in the germinated state. They prepared, by means of extraction and gel chromatographic methods, several protease concentrates and investigated the characteristic properties (molecular weight, pH optimum, character of the active centre, etc.) of the protein fractions showing protease activity. The results obtained so far seem to indicate that the views which consider wheat proteases uniform, papain-like SH-enzymes, ought to be revised.

BIOCHEMISTRY OF FOOD LIPIDS

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The processes (biochemical changes) taking place in foods are extremely manifold and this is also true for the lipid components. The quantity and quality of fats and fat-like substances (lipids and lipoids) change dynamically in these biological materials which encumbers the comprehensive treatment of the biochemical aspects. During the storage of food and of the raw materials of the food industry, as for instance during the ripening of fruits, the enzymatic conversion of cheese or the fermentation of tobacco, endogenous lipid metabolism responsible for the degradation of lipids might gain predominance as the activities of certain groups of enzymes come into the foreground while those of others are repressed. This is closely linked with the changes caused by processing, such as the change in the degree of aggregation of enzymes in the course of comminution or the induction and activation of processes taking place as a result of the abolishment of steric separation of substrate from enzyme bound to the tissues. Hence the bulk of the raw materials and products of the food industry is a permanently changing biological material subject to metabolism and only a small fraction of it can be considered to be in a steady state (refined oils), from the biochemical aspect. In the latter the causes of changes are mainly of a chemical nature (auto-oxidative processes).

It is therefore advisable to treat separately the biological state of the raw materials and the products of the consequent lipid metabolism, further the enzymatic changes (lipoxygenase and lipolytic enzymatic reactions) taking place during storage and finally the changes and activations caused by processing. In the case of lipids the situation is extraordinarily complicated by the outstanding reactivity of some of the components, since even the presence of a single double bond might drastically increase the rate of chemical changes (auto-oxidation) and alter the stability of the bond between enzymes and substrates. Determination of the products of intermediary metabolism (e.g. of fatty acids) is considered important, since they might be the primary indicators of the lipid-chemical state of the raw material to be processed and thus might determine the parameters of the technology to be applied.

SOME BIOCHEMICAL ASPECTS OF THE ACCUMULATION BY WEIGHT OF VEGETABLE OILS

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In plant cultivation photosynthesis produces a new value. The seed output of some oleoferous plants (sunflower, rape, oil-flax, poppy) might be less than that of other plants, but their use value is greater.

In agriculture the output in weight is considered as production and sales are also mostly according to weight.

Previously, the author has worked out certain parameters to evaluate outputs as products of photosynthesis where in addition to the quantity the composition of the products has also been taken into consideration.

Changes in the ratio of the weights of elements in the various organic compounds are accounted for as primary carbohydrates and expressed in elementary equivalents. The minimum possible weight change — from primary carbohydrates — obtainable by means of biological processes is expressed as the minimum equivalent. Biological changes are unavoidably accompanied, with respect to the formation of compounds, by changes in weight, mostly by loss of weight.

The elementary equivalents of the saturated fatty acids which are important components of plants indicate a weight loss of about 50% which corresponds to about 2 elementary equivalents. This value is somewhat higher for oleic, linoleic and linolenic acids. The values related to the triglycerides of fatty acids are only slightly higher than those related to fatty acids.

It follows from the minimum equivalents of the corresponding fatty acids that a loss in weight of about 65% is incurred on the minimum biological route. This value is only slightly exceeded by oleic, linoleic and linolenic acids.

The minimum equivalents of vegetable oils are: 2.90 for oil seeds in general, 2.95 for cereals and leguminous seeds, and 3.00 for roughage, bulbs and roots.

In some animal species fat accumulation is expressed by the *Kellner* starch value. If, in addition to the vitally important fodder, the possibility of maximum fat accumulation is also taken into account (pig, duck, horse) then, on the basis of biogeneses (glycolysis, acetate-path, etc.), a relation from the aspect of quantities is obtained between the accumulation of animal fat and vegetable oil.

Due to their minimum equivalents vegetable oils can be produced, because of stoichiometric and biochemical reasons, in practically reduced quantities as compared to other vegetable materials (sugar, starch, fibre, protein),

though their high productivity is indicated also by their high heat of combustion.

If in the evaluation of the production of photosynthesis a minimum equivalent of around 3 is assigned to the vegetable oils, then the seed output of oleoferous plants might represent a higher production than that of other plants.

GLYCOLIPIDS OCCURRING IN WHEAT PROTEIN COMPLEXES

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The proteins of wheat flour — the proteins of gluten — are complex proteins containing lipids and carbohydrates. The lipids bound to the protein are polar phospho-, sulpho- and glycolipids. The quality of the proteins of gluten and the lipid composition of the complex determines the technological value of flour.

The lipid–protein–carbohydrate complex was extracted from wheat flour BL 55 after removal of the fat with petroleum ether with a mixture of organic solvents (chloroform: methanol 2:1 v/v). The lipids were fractionated by means of thin-layer chromatography. The components were identified by using standard substances chromatographed under identical conditions, by means of special colour reactions and infrared spectroscopy. The fatty acid components of the glycolipids were determined as methyl esters by way of gas chromatography. The sugar components were studied by thin-layer chromatography on a layer impregnated with boric acid.

Of the polar lipids bound to the protein complex the following ten fractions were identified:

1. Choline phosphatide; 2. Inosite phosphatide; 3. Ethanolamine phosphatide; 4. Glycerol phosphatide; 5. Sulphoglycolipid (SL); 6. Digalactosyl diglyceride (DGDG); 7. Polyglycerine phosphatide; 8. Phosphorus- and hexose-containing fraction; 9. Monogalactosyl diglyceride (MGDG); 10. Steroyl glycoside (SG).

By means of gas chromatographic investigation into the fatty acid composition of the fractions SL, DGDG, MGDG and SG, beside palmitic, stearic, oleic and linoleic acids which occur very frequently in natural fats and oils, also some rarer fatty acids could be identified, notably C_{17} acid in MGDG, C_{21} acid in SL and MGDG, lignoceric acid in SL and SG and unsatu-

rated C_{24} acids in MGDG and DGDG. Investigation of the sugar components showed that the complex contains mainly galacto- and a lesser quantity of arabino-lipids. IR spectroscopy revealed that in the SL fraction sulphur is bound to the galactose not in the form of sulphate but by a sulphonic acid link.

MUSCLE LIPIDS: LIPIDS OF MYOFIBRILLAR PREPARATIONS, THEIR ROLE AND THEIR PEROXIDES

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Muscles as nutrients provide the organism not only with amino acids and vitamins but also with small quantities of lipids. These lipids contain essential fatty acids and phosphorus which are of the highest importance for the organism. From the striated skeletal fresh rabbit muscle free of connective tissues 1.8–3.0% (white muscle) and 2.5–4.5% (red muscle) of lipid can be isolated. Not so very long ago the lipids in the muscle were believed to be situated mainly in the membranes, the sarcoplasmatic reticulum, the mitochondria and in other particles. With the currently available methods, however, pure myofibrillar preparations can be gained from the muscle and it has been found that 20% of the solids content of the myofibrils consists of lipids. The myofibrillar lipids are mainly mixtures of phospholipids and more than 40% of their fatty acid components is made up of unsaturated fatty acids.

Within the myofibrillar substances the proteins responsible for contractibility also contain lipids; notably actin contains about 2.5–4.0 mole/mole, myosin — depending upon the origin of the muscle — 2.5–12.0 mole/mole or more phospholipids, some free fatty acids and other lipids.

Purification by means of chromatography removes hardly any phospholipid from the myosin of the white muscle. Thus, after chromatography from the myosins of the red and smooth muscle are obtained much the same phospholipids than from the white longissimus muscle of the back, 2.0–2.5 mole/mole of protein. Comparison of the myofibrillar lipid contents of different muscles suggests considerable differences between them.

The experiments have shown that the lipids, primarily the phospholipids are normal components of the myofibrillar proteins and are necessary to maintain the structure and function of the protein molecule. An interaction takes place when the freshly obtained lipid fraction is brought into contact with lipid-free denatured and freshly isolated myosin. In addition, they form complexes with metal ions and morphologically different anisotropic chelate crystals are obtained (Figs. 1, 2).

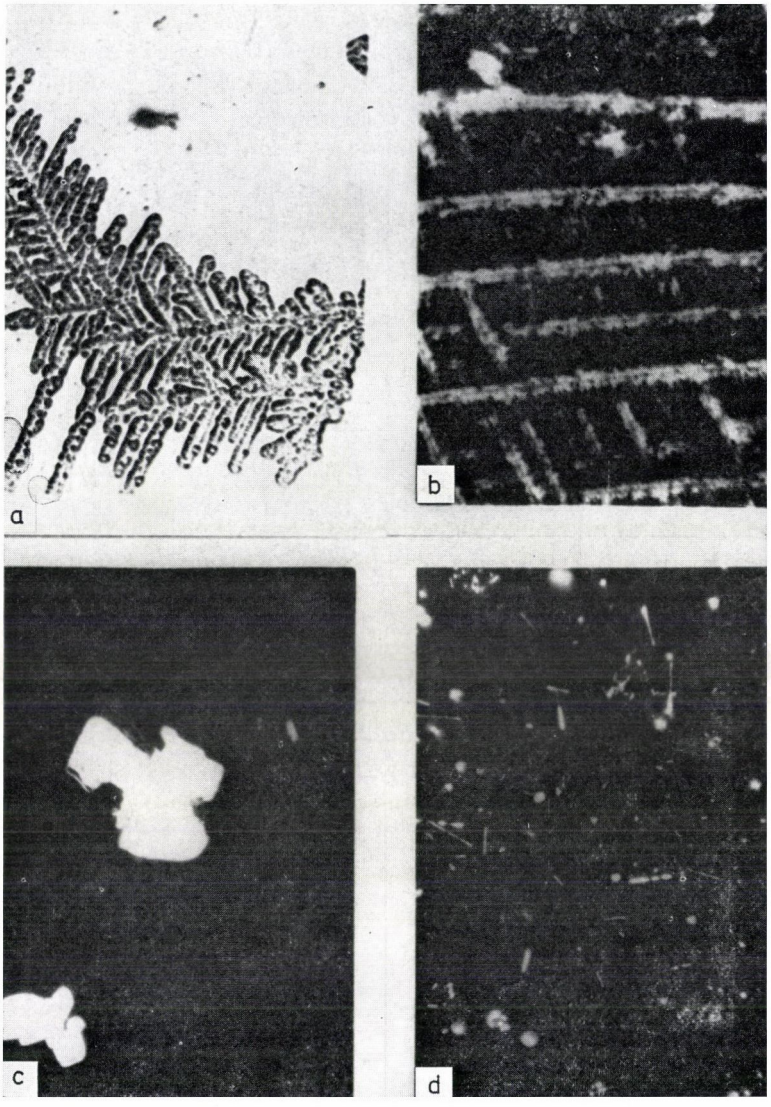

Fig. 1. Amorphous pseudo-figures of acetone-extracted lipid fraction forming from small lipid drops. . .a, double pseudothreadlike lattice-work of ATP–lipid interaction complex. . .b, forming from transveral short pins. Strong anisotropic Mg–lipid crystal-aggregates. . .e, and heterogeneous Ca–lipid microcrystals. . .d.

Treatment with a lipid solvent abolishes the swelling capacity of myofibrils, and of actomyosin gels, as well as their biological functions and the typical properties of actin and myosin. This is most markedly manifest in their lack of solubilization in dilute buffer and aqueous KCl solutions.

From the aspect of the products of the meat industry the water absorp-

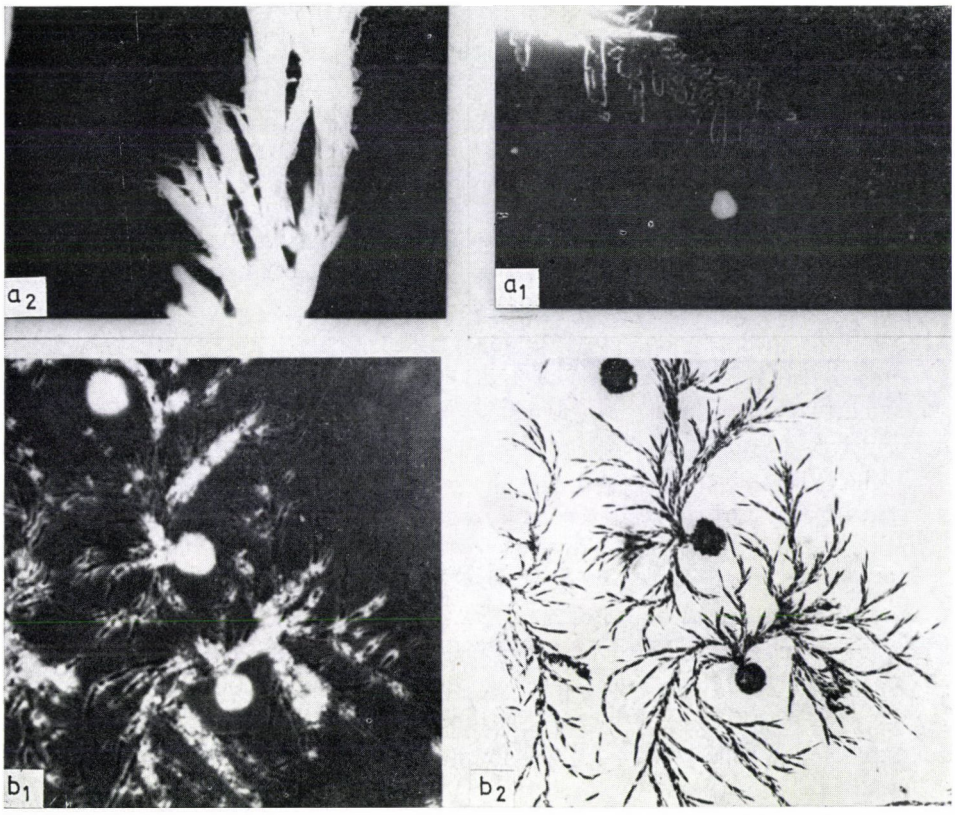

Fig. 2. Development of Arg–lipid interaction pin-aggregates . . . a_1, bunches of developed crystals . . . a_2. Moss-like formation of Lys–lipid aggregates. Polarization . . . b_1, and native. . .b_2 photographic recording

tion capacity of minced meat is not indifferent. This depends upon the water binding ability of the myofibrils and within it upon the properties of the myofibrillar proteins.

The lipid fractions extracted from myofibrillar preparations undergo a rapid auto-oxidation and are converted into yellow or brown, non-specifically polymerized heterogeneous wax-like products. Presumably the initial auto-oxidation of the lipid components and the altered mobility of the metal ions (mainly of inter-molecular Ca^{++}) play an important role in the loss of homogeneity of actin and myosin during storage, just as in the deterioration of the biological functions and of the swelling capacity of freshly minced meat, since the above factors cause an extensive change in the original structure of the myofibrils and of the individual proteins.

INVESTIGATION INTO THE ANTHOCYANINS OF SOUR CHERRIES

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The anthocyanin compounds of the sour cherry cultivars *Pándi* and *Cigány* (Gypsy) were studied. The colouring matter was extracted with 1% methanolic HCl solution and separated on filter paper Whatman No. 3 using *n*-butanol–acetic acid–water (4:1:5) as solvent mixture. By means of this method three colouring components were separated from each cultivar.

Since anthocyanins possess a glycoside structure, they were further identified in three steps: the aglycone part, the sugar moiety and finally the glycoside structure was determined.

The aglycone part was identified after acid hydrolysis of the anthocyanin components by means of a colour reaction with aluminium chloride and borate buffer as well as by means of paper chromatography. The results suggest that the aglycone component of all the three colouring components in both cultivars of sour cherries might be peonidine.

The glycoside structure was determined by means of paper chromatography, fluorescence tests and partial acidic hydrolysis. All anthocyanin components were found to have 3-monoglycoside structures. Of the sugars bound to the aglycone part the presence of galactose could be detected.

RECENT RESULTS AND TRENDS IN THE BIOCHEMISTRY OF FOOD PROTEINS

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Spreading of continuous technologies, intensification and automated control of the technological processes are characteristics of the development of technologies in the food industry. All these objectives require a thorough knowledge of the composition of the raw materials of animal or vegetable origin and of the biochemical processes taking place in them. A detailed analysis of the processes occurring as a result of the action of tissue enzymes, microorganisms and different technological factors (thermal effect, mechanical stress, effect of additives, etc.) is an indispensable condition of the creation of a modern food industry.

Biochemical research related to food proteins is conducted basically in four main directions:

– elucidation of the composition and structure of proteins occurring in foods;

– study of the changes occurring in proteins during technological processes and of the mechanism of these changes;

– protein nutrition, new sources of protein;

– bioregulation problems related to food proteins.

Research concerning the composition and structure of food proteins has brought many new results mainly in the field of animal proteins (establishment of the complete structures of haemoglobins, myoglobin, of some of the egg-proteins, albumins in egg and milk and of certain casein fractions, resp.).

The structure of the first plant protein was elucidated only some years ago. Recently the complete structure has been disclosed. An acceleration of development is expected particularly in the study of the proteins of meat, egg, milk and food enzymes.

Of the results achieved in the study of the changes taking place in the course of the technological processes of the food industry elucidation of the mechanism of post mortem changes in meat and fish, the renneting action on casein and the changes occurring in wheat proteins during the production of dough ought to be mentioned.

Research concerning the genetic problems related to the biosynthesis of food proteins as well as the isolation and identification of the genetic variants of certain food proteins (casein, wheat proteins) deserve special attention. The results might open a rational perspective before the efforts directed to ensure protein raw materials optimum from the aspect of nutrition. Similar long-term hopes are attached to the research concerning natural enzyme inhibitors, weed killers and their relation to the biosynthesis of proteins.

It is expected that a full acquaintance with and control of the processes on the molecular level will be gradually attained.

BIOLOGICAL EVALUATION OF NUTRIENT PROTEINS

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The protein biosynthesis of the organism lays certain claims on the quality of the proteins introduced with the food. Of the various *in vivo* methods used for the evaluation of food proteins human long-term nitrogen equilibrium experiments as carried out by Kofrányi were the only ones to provide accurate numerical results on the biological value of food proteins: the biological value of a protein is the higher the less of it is required for the maintenance of the

nitrogen balance. Biological value can be expressed numerically as the reciprocal of the minimum quantity of protein required for the maintenance of the nitrogen balance of the organism, taking whole egg protein as 100.

The long-term human nitrogen balance experiments have disproved the validity of all indices based on chemical analysis alone, and by the addition of various pairs of proteins, the thesis of limiting amino acids was replaced by the rules of the optimum ratio of essential amino acids. From KOFRÁNYI's results of human nitrogen balance experiments MØRUP and OLESEN developed a mathematical formula for the prediction of the biological value of proteins.

The biological value of a protein cannot be determined from *in vitro* experiments which might provide information only on the preliminary condition of utilization, namely enzymatic decomposition.

CHARACTERIZATION OF SOME BASIC PROTEINS ISOLATED FROM CALF LIVER

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In recent years the N-methylated derivatives of basic amino acids have been detected in a great variety of proteins (e.g. in myosin, actin, cytochrome C, histones, etc.).

These amino acid derivatives (N^E-methylated lysines, N^G-methylated arginines and imidazole-N-methylated histidines) might occasionally fundamentally modify the biological function of a given protein and in the free state possess considerable biological activity.

It follows from the above that in protein-containing foods these amino acid derivatives might occur both in the free and in the bound state. When this is taken into consideration a new aspect is introduced into the biochemical and trophophysiological characterization of certain foods.

For the sake of demonstration some basic proteins and free amino acids of calf liver were chosen since the latter is not only one of the fundamental objects of biochemical research, but also an important raw material of the food industry.

Calf liver was made into a pulp with 0.01 *N* hydrochloric acid and the precipitate obtained from the supernatant with Reinecke's salt was tested for amino acids and proteins.

For the characterization of the proteins electrophoresis in polyacrylamide gel (15% acrylamide, 130 V, pH 2.7), analytical and preparative iso-

electric focussing (polyacrylamide gel and sucrose density gradient), amino acid analysis (automatic amino acid analyzer, thin-layer chromatography on organic and inorganic layers) were used.

USE OF COMPLEX BIOCHEMICAL ANALYSIS IN SELECTING WHEAT FOR PROTEIN AND LYSINE CONTENT

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From a nutritional point of view wheat cannot be considered as an ideal basic foodstuff. Its average protein content is not more than 13% and due to its low lysine content the biological value of wheat protein is also inferior. For this reason, breeding programmes have been initiated all over the world to increase both protein and lysine content of bread wheat. Since in wheat there is a negative correlation between the protein and lysine levels, the main objective of these programmes is to find and utilize the so-called "correlation breaking" varieties. This work requires an efficient and reliable screening method. The methods used so far for determination of protein and lysine contents are more or less unsatisfactory, especially in large-scale investigations. The authors suggest a two-step procedure in which the varieties will be separated first into groups of high and low protein content, respectively, using the Neotec Grain Quality Analyzer (GQA). This instrument operates on the principle of infrared reflexion and is provided with a built-in analogue digital computer unit. In the next step, lysine content will be determined after an acid hydrolysis and chromatography on a Fixion thin-layer with the Chinoin "Telechrom" videodensitometer. For the determination of protein by means of GQA, about 5 g of meal is required, and since this is a non-destructive method, the same sample can be used for the lysine estimations. Using the suggested analytical system, 200 to 300 analyses can be performed in a day provided there is no limitation by grinding and other manipulations. Improvement of the analytical efficiency and computer processing of the obtained data is possible.

The authors have analyzed 1436 wheat selections by means of this complex procedure and found three varieties with a protein content over 16 per cent and lysine content higher than 3 per cent (on a protein basis). The analytical method described above can be used not only for selecting wheat or other cereals for improved protein, but also in selecting oil seeds for oil content and quality (for instance, breeding rapeseed of zero erucic acid).

CHARACTERIZATION OF THE ORIGIN OF MUSCLE TISSUES AND MODE OF PROCESSING BY MEANS OF POLYACRYLAMIDE GEL ELECTROPHORESIS

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Proteins were studied by means of the method of polyacrylamide gel electrophoresis in order to find answers to the following questions:

— whether the gel electrophoretic pattern is suitable 1. for the characterization of various muscle groups of different animal species;

— and 2. for the detection of the degree of protein denaturation occurring in the course of the various cooking procedures in which different heat treatments are used.

The basic instrument and reagents marketed by REANAL were used in the experiments. The protein extracts were prepared by making use of the natural buffer system of muscle meat pulped with water in the proportion of 1:3.

Summing up the results it was found that:

1. muscles from the same parts of animals of the same species and of the same age are very similar with respect to their soluble protein fraction;

2. evaluation of the densitograms of raw beef loin and round meat and of raw pork fillet and loin has shown a similarity from more than one aspect between the soluble protein fractions of muscle tissues in different anatomical positions of the two animal species and a considerable difference between the myoglobin fractions of pork and beef;

3. there is a considerable difference between the ratios of the soluble protein fractions in the musculature of chicken leg and breast, as well as between the individual fractions;

4. when beef tender loin or sirloin was underdone in aluminium foil some of the protein fractions underwent denaturation. Treated in a similar manner in a teflon-coated tin protein denaturation was less but in the case of roasting with high frequency it was more marked and uniform. The protein fractions of underdone beef tender loin were almost the same as those of the raw meat. Similar experience was gained with beef sirloin;

5. when pork fillets were underdone the same trends were observed using the various roasting technologies mentioned;

6. investigation of the mixture of different proteins of animal origin showed that on the electrophoretogram of beef + egg white the bands are not additive but can nevertheless be used in a certain sense for characterization.

STUDIES ON THE AMINO ACIDS, PEPTIDES AND PROTEINS IN TOMATO FRUIT

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Amino acids, peptides and proteins play an important role partly as primary taste carriers and partly as flavour precursors in the development of the taste and odour of fruits and vegetables.

The presence of these compounds in ripe and raw tomato was investigated from this aspect.

By means of parallel application of several thin-layer chromatographic methods it was found that in tomato serum glutamine, glutamic acid, gamma-aminobutyric acid and alanine are present in the largest quantities. Lysine, histidine, arginine, α-leucine and aspartic acid were detected in smaller quantities, and methionine, serine and phenyl alanine were found only in traces. The presence of gamma-aminobutyric acid in the juice was confirmed also electrophoretically.

The water soluble proteins were separated from the tomato serum by means of a Sartorius ultra-filter and it was found that their total quantity amounted to not more than 0.02%. Protein content calculated from the nitrogen content was at the same time 1.1% which has lead to the conclusion that the bulk of the N-compounds consists of free amino acids and peptides.

The presence of peptides in the deproteinated serum could be proved in two ways. After dinitro-phenylation followed by hydrolysis free amino acids were detected in the solution beside DNP-derivatives while, on the other hand, after hydrolysis of the deproteinated serum the amino acid analyzer revealed fairly large quantities of amino acids which were present only in traces as free amino acids (aspartic acid, threonine, phenyl alanine and lysine).

CHANGES IN CERTAIN ESSENTIAL AMINO ACIDS AND VITAMINS OF THE B GROUP DURING THE MANUFACTURE AND STORAGE OF INFANT FORMULAE

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Samples were taken from the following seven steps in the manufacture of the infant formulae *Robébi* A, *Robébi* B and *Linolac*, all based on milk: raw milk, pasteurized milk, fresh concentrated milk, stored concentrated milk, fresh homogenate, stored homogenate and the final product.

There was no significant change in the quantity of the methionine present in the proteins during manufacture, but the lysine content dropped by about 30% in the intermediaries and in the final product.

In the infant formula Linolac the degradation of tryptophan was brought into correlation with the thiobarbituric acid number which indicates the degree of oxidation of fats and which increased exponentially with the duration of storage. It was confirmed by model experiments that a causal relation exists between the oxidation of fats and the degradation of tryptophan.

The levels of the vitamin B complex (vitamins B_1, B_2, and B_6, calcium pantothenate, nicotinic acid and biotin) were determined in the intermediaries and in the fresh and stored final product by means of a microbiological method. According to the results the declared quality of the final product is determined by the degradation of vitamins which are particularly sensitive to the influences of technology (vitamins B_1, B_2, calcium pantothenate), as well as by the dose of the vitamins added.

MICROMORPHOLOGICAL AND RHEOLOGICAL CHANGES PRODUCED BY CHEMICAL EFFECTS IN WHEAT PROTEINS

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Various chemical influences are known to produce important changes in doughs and gluten prepared from wheat flour. The micromorphological effects occurring at the contacting surface of starch granules and gluten matrix during the rheological changes which can be measured macroscopically by use of the recording kneading machine Valorigraph, were studied by means of the scanning electron microscope. The role of lipids in the development of the membranes surrounding the starch particles was also investigated.

It was found that the membranes which develop during kneading are not identical with the amyloplast membranes surrounding the starch granules in the wheat grain. The presence of proteins in these membranes could be demonstrated, too. Similar membranes are formed when the flour particles are brought into contact with water and rapid hydratation produces fibres and networks between the starch granules.

It could be proved by means of protein staining and wet chamber electron microscopy that these fibres are the results of the breakdown of the membranes mentioned above.

Since great importance is attributed to the structure of membranes in the development of the gas-retaining capacity and rheological properties of

dough, the relationships between the micromorphological and rheological effects of the braking of the disulphide bonds, of the blocking of the sulphydryl groups, of the solvents of the gluten components, of the braking of hydrogen bonds, of the masking of ionic bonds and of the pH were studied.

EFFECT OF ZINC FOLIAR SPRAYS ON THE PROTEIN RELATIONS AND BAKING QUALITY OF WHEAT

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In growing highly productive varieties of wheat, the problem of micro-elements is becoming an important topic of plant management.

In an experiment with micro-element fertilization, zinc was added in the form of foliar spray to the plants. The treated varieties were *GK*-3, *Avrora* and *Kavkaz*. Zinc was applied in three doses, i.e. 65, 97 and 130 g of zinc per hectare, respectively, at the end of boot-stage in the form of $ZnSO_4$ and Zn-Na-EDTA.

The effect of the micro-element on the crude protein, true protein, and urea-soluble protein content of the flour prepared from the seeds as well as on its gluten content, on the consistency, protease activity and amylolytic activity of the gluten, furthermore on the Valorigraph value, volume of the experimental bread and on the Zn-content of the grain was studied.

It was found that the different varieties of wheat respond in a different manner to the zinc foliar spray. An unambiguously negative result was obtained in the case of *Kavkaz* variety as far as baking quality is concerned. The most marked changes were observed in the protein content of the flour, in its amylase activity, rheological properties and in the bread volume. Zinc was, as a rule, more effective when applied in the form of sulphate than in the chelate form.

Since no accumulation of zinc was found in the harvested grain of treated plants, the above effects of the micro-element should be considered as indirect ones.

STUDY OF GRAPE AND WINE PROTEINS BY MEANS OF GEL CHROMATOGRAPHY, POLYACRYLAMIDE GEL ELECTROPHORESIS AND ISO-ELECTRIC FOCUSSING

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The soluble proteins present in the grapes and wines originating from the most important Hungarian wine districts (varieties *Hárslevelű*, *Furmint*, *Olaszrizling*, *Tramini*, *Sauvignon* and *Zöldszilváni*) were studied. The molecular weights of the grape and wine proteins were determined by means of column chromatography on a *Sephadex* G 75 gel. The molecular weight of the grape and wine proteins was, independent of the variety, 18.500 ± 740 dalton.

The proteins were separated, dependent on the grape variety, into 6 to 15 components by means of polyacrylamide gel electrophoresis. The grapes and wines of a given variety contained proteins of similar properties and characteristic of the variety.

Separation of the proteins according to their iso-electric points was performed by means of iso-electric focussing. The proteins were separated into 15 to 32 components by electrofocussing performed in a sucrose density gradient column, and in polyacrylamide gel.

Distribution of the proteins according to their iso-electric points is characteristic of the variety of grape, but a considerable number of components is common in the proteins of the different varieties. The iso-electric points of most of the grape proteins is in the pH range between 3 and 5 and in the wines the proteins are positively charged.

BIOCHEMICAL AND PHYSIOLOGICAL ROLE OF MICRO-ELEMENTS

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Classical analytical methods were adequate for the accurate determination of elements present in larger quantities in samples originating from living organisms. In addition, about 30 elements could be detected in trace amounts. These trace elements are called, by some authors, micro-elements. Modern chemical analytical methods (various spectroscopic procedures, atomic absorption spectrophotometry, mass spectrography, activation analysis as well as the separation of certain metal complexes by means of gas chromatography) have permitted the quantitative determination of elements detected

earlier only in traces. According to the recommendation of one of the IUPAC Committees those elements whose concentration does not exceed 100 ppm (or 100 mg l^{-1}) should be called trace elements.

Part of the microelements detected in the living organisms is absolutely indispensable for the health of the individual. According to our present knowledge the following elements belong into the category of essential micro-elements: F, Si, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Se, Mo, Sn, I.

In addition to the above boron is needed by plants.

A deficiency in the essential elements might initiate various pathological processes. Under natural conditions an insufficient supply might occur only in areas where there is a great shortage of one or the other micro-element in the soil and thus the latter does not reach the food chain through the plants.

Modern methods of food industry (grinding of cereals, purification of sugar) remove a considerable part of the micro-element content of crops, thus in industrially developed countries, beside otherwise satisfactory nutrition conditions, diseases due to micro-element deficiency might still occur.

There is a world-wide trend directed to the recognition of symptoms indicating a deficiency in micro-element supply and to the securing of adequate micro-element uptake.

Several of the micro-elements are known as components or activators of certain enzymes. Others are needed for the development of the native steric structure of proteins. Cobalt has a special role being one of the components of vitamin B_{12}.

BIOSYNTHESIS OF AROMA SUBSTANCES IN *ACHILLEA MILLEFOLIUM* USING RADIOACTIVE ISOTOPES

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Achillea millefolium is a common weed which is known for its characteristic aromatic volatile (essential) oil content. The main component of its essential oil is the sesquiterpene kamazulene which has a significant anti-inflammatory activity. The precursors of it in the plant are some proazulenes.

The formation of essential oils in the plant was studied by the incorporation of $2^{14}C$ Na-acetate (0.5 mCi). The path of radioactivity was followed for 1 to 3 days by means of thin-layer and gas chromatography, autoradiography and scintillation measurements.

It was found that the precursor was already incorporated into certain components of the essential oil within a relatively short time (4 hours). The

first radioactive compound was a bicyclic monoterpene, β-pinene and caryophyllene which is of the sesquiterpene type. After 24 hours the proazulenes will be radioactive. Cineol and borneol became radioactive after 72 hours which indicates that in the formation of the essential oil oxidation processes begin to dominate. This investigation convincingly demonstrated the biosynthetic pathway of aromatic terpenoids.

STUDIES ON THE FLAVOUR SUBSTANCES OF TOMATO FRUIT

I. Szárföldi-Szalma and M. Petró-Turza

Central Food Research Institute, Budapest

Experiments aiming at the separation and identification of compounds responsible for the characteristic flavour of ripe raw tomato fruit forms an important part of the work concerning the aroma substances in tomato.

In the experiments gas chromatography was supplemented by sensory evaluations.

Investigation of the odour of the components eluted from the gas chromatographic column indicated that the group of compounds responsible for the flavour can be found in the most volatile fraction.

The group of compounds to be investigated was removed from the aroma extract after appropriate concentration by bubbling nitrogen gas stream through the liquid and collecting the volatile components in cold traps.

The most volatile fraction collected in the trap cooled with liquid nitrogen to about -180 °C was separated by means of gas chromatography. For the identification of the substances in the peak of tomato odour some microreactions were carried out.

The results indicated that the material corresponding to this peak is not made up of a homogeneous substance. Experiments aiming at its separation and identification are now in progress.

INVESTIGATION OF VOLATILE AMINE BASES IN FOODSTUFFS

E. Madarassy-Mersich and N. Gábor-Heckenast

Central Food Research Institute, Budapest

The results of the gas chromatographic investigation of volatile amine bases is reported as part of the work dealing with the flavour substances in raw tomatoes, in concentrated tomato pulp and in powdered tomato.

The volatile amines were extracted after the sample had been made alkaline, by way of steam distillation and collected in dilute hydrochloric acid. The solution was evaporated to dryness and from the amine hydrochloride salt-mixture residue the liberation of the amine compounds and their dissolution in *n*-hexadecane was performed in sealed glass capillaries.

The amine bases were separated in parallel runs in packed columns of two types, namely 20% *Carbowax* 20M coated with 2% KOH on *Chromosorb*-P and *Chromosorb* 103, applying an appropriately chosen temperature programme.

The results of the gas chromatographic tests suggested that raw tomatoes as well as their heat treated products contained a number of amines. From the components separated 9 amine bases could be identified in all three samples. The identification of the three unknown amine compounds present in larger quantities in the samples will be performed in the future.

It was further found that raw tomato contains the largest quantity of amine compounds which decreases gradually in the course of processing.

CHANGES IN APPLE VOLATILES DURING STORAGE

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and E. Hegedüs-Völgyesi

Central Food Research Institute, Budapest

The Department of Enzymology of the Central Food Research Institute has been engaged for several years in the study of the changes occurring in apple volatiles during storage and of the enzymatic formation of flavour. This work involved, among others, storage experiments with the cultivar *Jonathan* from the region Nyírség and the gas chromatographic study of the changes in flavour substances isolated by means of steam distillation from the pulp or the peel.

During a 159-day storage of *Jonathan* apples the total amount of volatiles isolated from the peel by steam distillation first increased, in agreement with data published, and, on the 68th day of storage was considerably higher than the initial value. In the later stages of storage the volatile content dropped to the initial value and remained more or less at the same level. The volatile content of the pulp as isolated by steam distillation changed in a similar manner but to a lesser degree. During the entire period of storage volatile content obtained from unit weight of peel was, in agreement with the findings of others, higher than that obtained from the pulp.

Several changes took place in the number and ratio of the volatiles of the pulp and peel of *Jonathan* apples during storage. The number of components

found on the 1st and 16th day of storage was larger in the pulp, whereas from the 40th day onward it was larger in the peel. After a temporary decrease the number of flavour components was, both in the peel and the pulp, highest at the end of storage.

In forthcoming work storage experiments will be extended to apples picked at at least two different times and stored at two different temperatures in order to gain a more thorough insight into the changes of flavour composition and into the factors influencing it. This should be the first step in the direction of preventing undesirable changes or of promoting desirable processes of flavour formation.

THE MYCOTOXIN PROBLEM

K. Incze

Hungarian Meat Research Institute, Budapest

After a brief historical survey the most generally known mycotoxins and the most important fungi producing them are described together with the resulting diseases. Even today new mycotoxins are quite often isolated, but aflatoxin is still one of the most important toxins which may produce alimentary mycotoxicosis through food consumption. The greatest danger of a number of mycotoxins is their carcinogenic effect, but their non-mutagenic action should not be ignored either. Since hygienic demands foods have to meet are usually higher than those made on animal feed, mycotoxin contamination occurs more often in the latter, thus animal diseases are of higher incidence than human ones.

This is one of the reasons of the fact that prior to the discovery of aflatoxin mycotoxin research was primarily related to fodder tests, but today in some countries extensive surveys and control tests are carried out and have furnished remarkable results both in the human food and feed industries.

From the aspect of the origin of mycotoxins, thus of mycotoxin danger, the culture medium, that is — the type of foodstuff — plays a decisive role. Strains which produce toxin on an artificial culture medium often fail to produce the same in foodstuffs.

The isolation and identification of mycotoxins still involves many difficulties and there are only few countries in which systematic research is pursued and as for setting and accepting the upper limits (tolerances), we are still at the very beginning of the road.

In order to ensure that foodstuffs are free of mycotoxins, prevention should be attempted and products detoxicated by various procedures should be used mainly as animal feed.

INVESTIGATIONS INTO THE INACTIVATION OF PATULIN OCCURRING IN FOODSTUFFS

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The stability and thermal resistance of the aqueous solution of patulin and the possibilities of its inactivation were investigated.

Ultraviolet spectrophotometry and thin-layer chromatography were used. Preliminary experiments indicated that the best solvent for chromatography is a 60:30:1 mixture of toluene : ethyl acetate : formic acid and the best colour reagent is the mixture of a 0.2% solution of *o*-phenylene-diamine in *n*-propanol with 20% sulphuric acid in the proportion of 1:1.

Thin-layer chromatography revealed no marked change in the concentration of the aqueous solution kept at room temperature for 3 weeks. The compound is heat resistant. No decomposition was observed in aqueous solutions of 1 mg ml^{-1} concentration adjusted to pH values of 5.2 and 3.2, resp., after a 30-min heat treatment at 95 °C.

Further experiments were aimed at inactivating this highly heat resistant toxin by means of an approved food additive. Sulphur dioxide was led into the aqueous solution of patulin, while in another experiment a $KHSO_3$ solution was added. Spectrophotometric tests showed no change in the position of the absorption maximum after mixing with $KHSO_3$; the maximum remained at 277 nm the wave length characteristic for patulin, but optical density decreased gradually during the five hours of the test. Thin-layer chromatographic tests showed no change in the patulin concentration of the solution treated with sulphur dioxide, nor could the formation of a new compound be observed.

Another possibility of inactivation is the reaction of a compound containing a sulphhydryl group (cysteine) with patulin. In the thin-layer chromatographic experiments the molar ratio of cysteine to patulin was 1:1, 2:1 and 3:1, resp. Two and a half hours after the beginning of the reaction the spot of patulin treated with the higher quantity of cysteine was considerably fainter than of those treated with less cysteine and after 27 hours toxin in a quantity of 1–2 micrograms could be detected only in the mixture of 1:1 mole proportion. Spectrophotometry showed a complete change in the character of the absorption curve after 18 hours.

VALUATION OF THE RESIDUES OF PLANT PROTECTING AGENTS OCCURRING IN CERTAIN FOODSTUFFS ON THE BASIS OF BIOCHEMICAL TESTS

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In the quality evaluation of foodstuffs, beyond the determination of various properties, the detection of foreign matter is gaining in importance. The group of plant protecting agents form an important part of the foreign substances and their quantitative determination is often decisive from the aspect of quality evaluation.

The degree of contamination of the foodstuffs with plant protecting agents differs greatly in the various regions of our planet because of the different conditions of cultivation. Bearing in mind that international trade relations are expanding, it has become necessary to introduce an evaluation according to limit values permissible from the aspect of public health. Consequently, biochemical and biological test methods must be introduced which are suitable for the determination of the possible biological effectiveness of the decomposition products or metabolites beside the biologically active molecules present in their original form.

The applicability of laboratory — mainly *in vitro* methods can be judged from the results of a large number of parallel tests *in vivo*.

The rapid test methods internationally applied and used also by the authors are, at present, more suitable for the comparison of the biological activity of different substances than for the determination of the absolute value of efficiency.

The results obtained so far indicate that the test systems used produce clearly observable differences only if the level of contamination exceeds the permissible limit value. However, because of the possibility of accumulation these data are worth of notice.

SURVEY OF THE LITERATURE ON THE TESTING OF ORGANOPHOSPHORUS PESTICIDES WHICH MIGHT OCCUR IN FOODSTUFFS

E. KOZMA-KOVÁCS and N. GÁBOR-HECKENAST

Central Food Research Institute, Budapest

The communications dealing with pesticides were grouped according to mechanism of action, chemical structure and the methods used for their determination.

According to data published organophosphorus pesticides can still be considered the most efficient and most widely used plant protecting agents.

A survey was prepared of the research and control stations in Hungary which deal with the testing of the residues of pesticides and of their main fields of research.

It was found that in Hungary several hundreds of well trained scientists work under adequate conditions in well equipped institutions on problems related to pesticides. The tests include the control of the active agent content of pesticides marketed in Hungary, the determination of residues in agricultural products and in the products of the canning industry, the study of metabolites, the elaboration of new analytical methods as well as various investigations related to environmental protection, such as e.g. the control of the pollution of the soil and waters.

For the more efficient utilization of the results of research it would be desirable to improve the organization of the flow of information and the co-operation between the research stations.

DISTRIBUTION OF THE RESIDUES OF SOME PLANT PROTECTING AGENTS AND GROWTH REGULATORS DURING THE PRODUCTION OF FOODSTUFFS

S. KOUDELA and V. CIELESZKY

Institute of Nutrition, Budapest

During food production and culinary technology the pesticide residues in raw materials may undergo various changes. In some cases these operations might reduce the quantity of the residues resulting in harmless decomposition products, while in other cases in certain phases of processing an increase in residues might occur.

The authors report on some of their own investigations. From the chloromequate active ingredient used against lodging of wheat the ears contained

about 15 mg kg^{-1}, 0.5 mg kg^{-1} was in the grain, in the flour even less (the husks contained 25, the bran 1.4 mg kg^{-1}).

The residue of diquate used as desiccant was around 1.7 mg kg^{-1} in the grain and 0.1–0.4 mg kg^{-1} in the flour obtained from the latter (bran contained 2–3 mg kg^{-1}). In husked rice obtained from grain containing 1.3–4.6 mg kg^{-1} less than 0.1 mg kg^{-1} of the agent was found (in bran 5.10 mg kg^{-1}).

In the course of steam distillation of mint in model experiments increasingly more prometrine — a compound with triazine structure — distils over as the pH value of the distillation mixture is raised: in mixtures with pH 5 in the distillate 90% of the active ingredient can be detected.

In other model experiments in the course of the vacuum evaporation of tomato juice a 2–12% decrease in toluidine derivative (dinitramine, trifluraline, benephine) levels was obtained in contrast to a 25–40% decrease achieved by means of steam distillation.

With respect to the action of various technological processes on the active ingredients under investigation it was found that surface treatment, the removal of parts not meant for consumption (by washing, peeling, grinding) results mainly in the removal of the contact and localized residues. In the case of heat treatment the temperature as a rule does not reach the melting points of the active ingredients, thus their residues are not significantly affected by this operation. Similarly during partition (between aqueous and oily phase, steam distillation, etc.) the physical properties of the active ingredients will prevail.

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DETERMINATION OF VITAMIN D_3 IN PREMIXES AND MIXED FEEDS

F. Békés, É. Berndorfer-Kraszner, R. Lásztity, F. Örsi and I. Dobos

(Received February 24, 1976; revision received April 12, 1977; accepted April 13, 1977)

A simple and rapid two-dimensional thin-layer chromatographic method for the qualitative and quantitative determination of vitamin D_3 in premixes and mixed feeds, was developed. The sensitivity of the method is 5 IU, or 0.125 μg vitamin D_3. The method was tested with 3 premixes and 5 mixed feed samples and a comparatively low variation coefficient (between 10 and 30%) was established. As regards their vitamin D_3 content feeds and premixes may be considered very inhomogeneous. The possibilities of further developing the method are outlined.

The determination of vitamin D_3 in natural substances is an intricate analytical task. The difficulties are partly due to problems of separation, and partly to the problems of qualitative and quantitative detection of low concentrations of the vitamin.

The provitamins of vitamin D (ergosterol, 7-dehydrocholesterol) or the intermediaries formed during vitamin metabolism (*i.e.* lumisterol, tachysterol) differ but slightly from substances of vitamin activity, therefore the determination of vitamins is possible only after they had been eliminated. The same applies to the substances formed from vitamin D upon irradiation.

Another difficulty arises from the fact that in some natural substances as well as in the synthetic premixes and feeds, examined in our study, the amount of vitamin A and its precursors present was higher by several orders of magnitude than that of the vitamins D. Since vitamin A and its precursors react with the traditional vitamin D reagents, their separation prior to quantitative determination is essential.

During vitamin D determination the high sensitivity of this vitamin to oxygen, light and temperature has to be taken into account. Every operation has to be carried out at low temperature under the exclusion of light and possibly of air (in nitrogen atmosphere). The difficulties discussed above indicate the necessity for the use of biological methods in the determination of vitamin D (Kodiček & Lawson, 1967).

In what follows, chemical methods for the determination of vitamin D_3 will be discussed.

As it is shown in the literature, apart from some pure vitamin preparations (*e.g.* pharmaceutical products), vitamin D determination has to be pre-

ceded always by operations aimed at separation. First the lipid content of the sample has to be extracted, then it has to be saponified. A substance rich in vitamin D may be obtained by finer separation of the unsaponifiable part. Figure 1 shows the general scheme of vitamin D_3 determination, including the variants of operations of separation and quantitative determination.

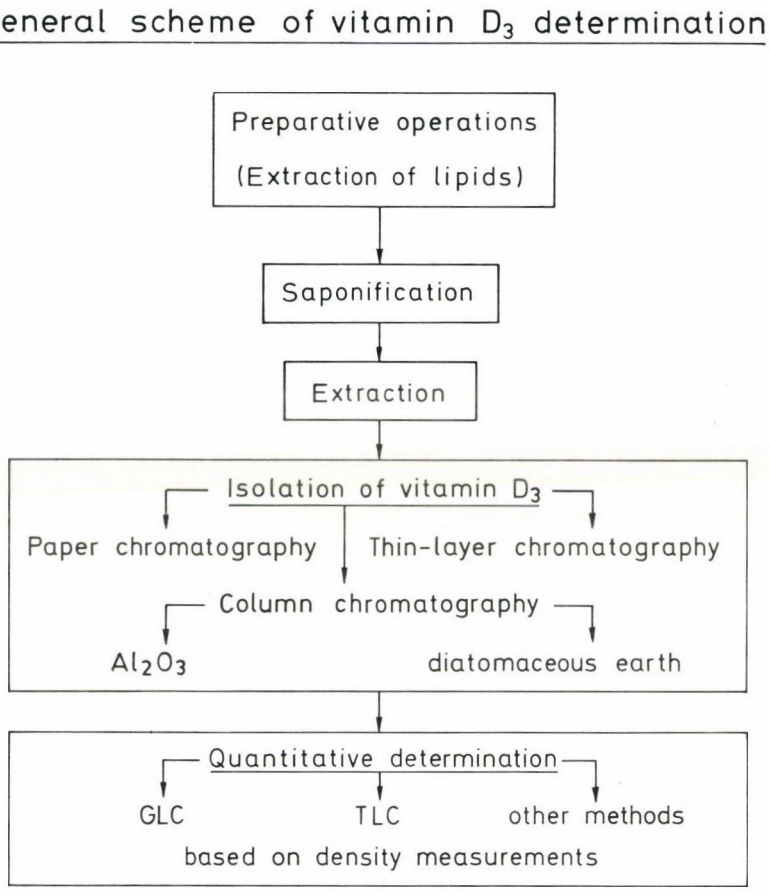

Fig. 1. General scheme of vitamin D_3 determination

By extracting the lipids prior to saponification, contamination of the sample with non-lipid substances, degraded in the course of treatment with alkali, may be prevented.

The vitamin D content of the sample, is found in the non-saponified phase after saponification with alcoholic KOH and may be extracted with ether. Saponification is one of the most delicate operations in vita-

min D determination. Losses may be substantial on account of the oxygen sensitivity of the vitamin. Therefore it is advisable to carry out saponification under nitrogen stream. The use of antioxidants is discussed in several papers (FRITZ & ROBERTS, 1968; MURRAY & *al.*, 1968; ERDŐDY & MURRAY, 1970).

The separation of vitamin D from the ether extract is generally achieved by chromatography.

Column chromatography of high time and reagent requirement is mainly used for the preliminary purification of vitamin D. The columns are packed with aluminium oxide (BROCKMAN, 1936; EVING & *al.*, 1954; BARAU & RAO, 1964; BARAU & RAO, 1965) or with various kinds of diatomaceous earth, (*e.g.* bentonite, frankonite, floridin, *etc.*, NORMAN & DELUCA, 1963; UEDA *et al.*, 1971b). Using aluminium oxide, only the vitamin A esters can be separated, while the major part of vitamin A in the sample is converted into the alcoholic form during saponification. The use of diatomaceous earth involves difficulties due to the undefined binding capacity of the column. If the binding activity is low, impurities remain in the sample, if the activity is too high some of the vitamin D gets absorbed and cannot be eluted.

Paper chromatography, considered an obsolete method already, serves only for qualitative detection (PEERBOOM *et al.*, 1961).

Methods of thin-layer chromatography have been developed to isolate and determine vitamin D in many different substances. In these methods aluminium oxide (DAVIDEK & BLATTNA, 1962; NORMAN & DELUCA, 1963) or silica gel layers were used (CHEN, 1965; LUDWIG & FREIMUTH, 1964; HEYSMAN & SAWYER, 1965; SPANYÁR *et al.*, 1967a, 1967b, 1968). Vitamin D is detected and quantitatively determined by various modifications of the *Brockman–Chen* photometric method, using antimony chloride as reagent. The spot on the thin-layer is either evaluated directly by size or else scratched off and assessed photometrically. To evaluate the purity of the vitamin D spot spectrophotometry in the UV range is more suitable than the $SbCl_3$ reagent (STANLEY *et al.*, 1957; BROWN & BENJAMIN, 1964; CERNY, 1971). However, the latter method is less sensitive and impurities originating from the adsorbents, used as carriers, as well as the losses occurring upon the contact of air and light with the dry adsorbent when the solvent had evaporated, have to be taken into account. Vitamin D detection by UV spectrophotometry is thoroughly reviewed by CERNY (1971) who made a detailed study into the causes and extent of the losses occurring in the course of determination.

Methods of *gas chromatography* have gained popularity in recent years (FRITZ & ROBERTS, 1968; MURRAY *et al.*, 1968; ERDŐDY & MURRAY, 1970; UEDA *et al.*, 1971a; TOUW *et al.*, 1972; KOBAYASHI & YASUMURA, 1972). With these methods, too, the preparation of the sample causes difficulties, since the elimination of impurities and quantitative determination cannot be accomplish-

ed in a single step. However, for the quantitative determination of vitamin D_3 gas-liquid chromatography (GLC) is the most accurate method at present. A difficulty in evaluating results lies in the fact that at the relatively high temperatures of above 220 °C pyro-derivatives are formed. The majority of the authors recommends the determination of silylized derivatives by chromatography.

This study forms part of the research work on the biochemistry and biology of feeds and premixes, carried out at the Department of Biochemistry and Food Technology, Technical University of Budapest. The aim of this study was to develop a simple and reliable method for the determination of vitamin D_3 in premixes and feeds.

1. Materials and methods

After thorough preliminary work, taking into account the problems discussed above, a method of two-dimensional thin-layer chromatography was developed. The scheme of this method is shown in Fig. 2.

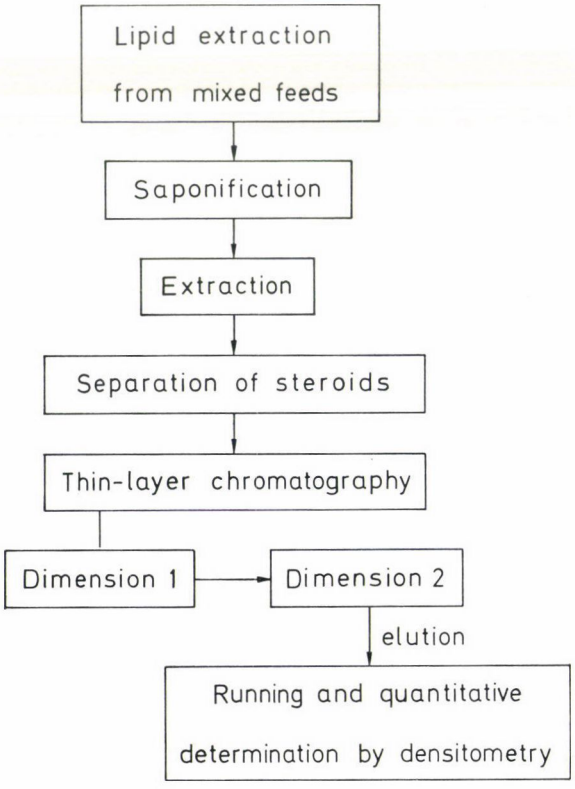

Fig. 2. Scheme of vitamin D_3 determination

The list of laboratory equipment and reagents used is given in the following.

Laboratory equipment:

a) Soxhlet and Besson apparatuses for extraction
b) electrically heated water-bath
c) densitometer *(Vitatron)*
d) vacuum drying oven

Reagents:

a) Standard vitamin D_3 solution: 25 mg vitamin D_3 (Merck) are dissolved in acetone and made up to 100 ml. This solution contains 250 μg, or 10 000 IU in 1 ml.
b) absolute alcohol
c) ether, free of peroxide
d) methanol
e) petroleum ether (Bp. 40–60 °C)
f) digitonine (FLUKA)
g) Na_2SO_4 (anhydrous)
h) chloroform
i) Kieselgel GF_{254} (Merck)
j) acetone
k) 2% ethanolic solution of phosphomolybdate

1.1. *Determination of vitamin D_3 in premixes*

1.1.1. *Saponification.* Twenty g of solid NaOH are put in a 500 ml Erlenmeyer flask, 10 ml of distilled water and 140 ml of absolute alcohol are added, the mixture is heated and shaken till the NaOH is completely dissolved. Five g of the sample previously weighed are added and saponified on a boiling water-bath, under reflux, in 15 min. Subsequently 25 ml distilled water are added, the content of the flask is rapidly cooled and transferred into a 500 ml separating funnel.

1.1.2. *Extraction.* Sixty ml of a 1:1 mixture of ether and petroleum ether are added to the substance in the separating funnel. The mixture is shaken cautiously for about two minutes then allowed to stand till the phases separate (about 10 min.) The lower, aqueous phase is let down into a beaker, the organic phases are collected in another 500 ml separating funnel, in which 200 ml of distilled water had been filled previously.

Extraction is repeated four times with 50 ml ether–petroleum ether mixture each and the organic phases are collected. After the last extraction the aqueous phase is discarded. The collected organic phases are washed to neutral with distilled water under cautious shaking (there is a tendency to

form emulsion). The solution thus obtained is dried above anhydrous Na_2SO_4, the solvent is evaporated to 5–10 ml final volume. This residue is dried in a crystallizing dish in the vacuum drying oven in the dark.

1.1.3. *Separation of steroids.* The dried substance is dissolved in 2 ml methanol, then 0.4 g digitonine dissolved in 10 ml of the 9:1 mixture of methanol and water are added. The sample is covered with aluminium foil and kept in the refrigerator overnight. During this time the greater part of steroids is precipitated and may be separated by filtration. It is advisable to carry out filtration with utensils cooled prior to use, because the precipitate easily re-dissolves at room temperature. The solution is yellowish in colour and absolutely clear if the filtration was carried out carefully. It has to be evaporated to 1 ml under the greatest possible care (cold ventilation).

1.1.4. *Separation by thin-layer chromatography.* The vitamin D_3 content of the premixes is separated from the numerous components by two-dimensional thin-layer chromatography.

On the Kieselgel GF_{254} (Merck) layer the starting point is marked at the lower left-side corner at 20 mm distance from both edges. The end line of the solvent front is marked also in both directions at 160 mm distance from the start. Two developing tanks, possibly of identical size, are prepared. In order to saturate the air space a filter-paper strip is placed in the tank. The developing agents are of the following composition:

First direction: ether–petroleum ether (30:40)
Second direction: chloroform–acetone (90:10)

It is expedient to mix the developing agents 24 hours prior to use and to fill them into the tanks.

One hundred μl from the sample, prepared as described above, are applied to the start point, by a Hamilton syringe.

The glass plate coated with the thin-layer is placed in the tank containing developing agent No. I. and developed till the front reaches the predetermined line. Running the chromatogram takes about 1 h. Then the plate is lifted out from the tank and the developing agent is removed, by cold ventilation, to the last trace. Since vitamin D_3 decomposes easily, it is necessary to place the plate immediately into the second tank, perpendicularly to its direction in the first tank, and develop it again. Development takes about 45 min.

On removing the plate from the tank it is placed under UV light to identify the spot of vitamin D_3. Under the conditions described, vitamin D_3 appears as a brown spot. R_{f_I}: 0.58, $R_{f_{II}}$: 0.33 (Fig. 3).

A line is drawn around the spot with a pin. The spot is then carefully scraped off and vitamin D_3 is dissolved on a glass filter with 2 ml methanol under repeated shaking. Then the filter is washed with 1 ml methanol and the solution is concentrated to 1 ml under cold ventilation, in the dark.

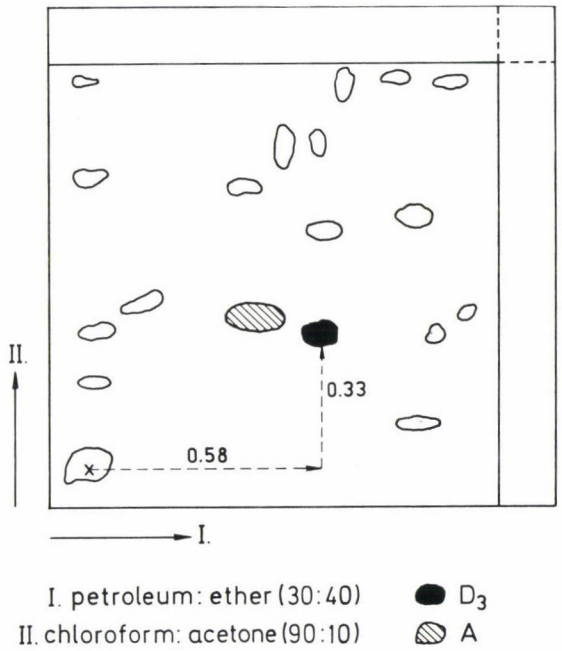

Fig. 3. Vitamin D_3 determination in mixed feeds by two-dimensional thin-layer chromatography

1.1.5. *Re-chromatography in thin-layer and quantitative determination by densitometry.* 0.5 ml of the concentrate is re-run on a Kieselgel GF_{254} layer (Merck) by No. I. eluting system along with the standard. A 2% molybdenum acid solution in ethanol is used for visualisation. After heat treatment for about 15 min the vitamin D_3 appears as a bluish-violet spot on a yellowish background. The intensity of the spots is measured with a *Vitatron* type planimeter and evaluated by means of the calibration curve (Fig. 4).

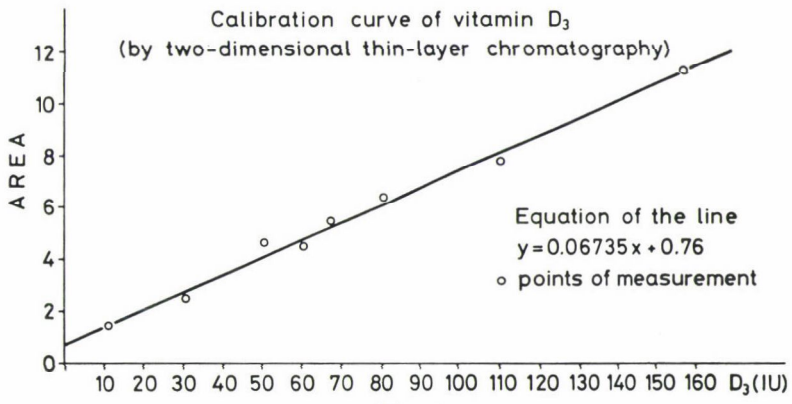

Fig. 4

The vitamin D_3 content, in IU, of 1 kg premix is the value read on the calibration curve multiplied by twenty. The calibration curve is obtained by using a 10-fold dilution of the standard solution (1000 IU = 25 μg D_3 per ml).

1.2. *Determination of the vitamin D_3 content of feeds*

Only those steps are described which differ from the ones applied in vitamin D_3 determination in premixes. Saponification is preceded by the extraction of lipids. This is carried out in a Soxhlet apparatus by dividing 100 g of the feed in 3–4 extraction tubes and extracting it with ether. When the ether refluxed in the extraction chamber is colourless (in about 3–4 hours) the tubes are removed and heating is continued till the total amount of the extract is evaporated to 20 ml. (If the extract is over-concentrated, on saponification, a greenish mucilage may separate.)

Saponification, the precipitation of steroids with digitonine and the separation on the thin-layer is performed in the same way as in the case of premixes. However, the spot, scraped off the layer, is dissolved in less methanol and evaporated to 200 μl, instead of 1 ml and the whole concentrate is applied to the layer for quantitative determination.

To calculate the vitamin D_3 content of feeds the following equation is used:

$$D_3 = 100 \cdot K/0.85$$

where D_3 = the vitamin content of the sample (IU kg^{-1})

K = the value read from the calibration curve.

0.85 is the calibration index obtained by the chromatography and elution of standard vitamin samples of known quantity.

2. Results

To illustrate the above-described method, the results of some vitamin D_3 determinations are given below.

In the case of premixes five parallel weighings were carried out. From each of the five samples 100 μl were used in three parallel two-dimensional TLC-experiments ($5 \times 3 = 15$ parallel experiments).

In the case of feeds three parallel weighings were carried out, and tested as mentioned above. So the number of the parallel data was nine.

The averages of the data obtained by analysis of premixes and feeds are shown in Tables 1 and 2.

To check the accuracy and reproducibility of the method the control tests were carried out by three members of the Department not taking part in

the development of the method. These determinations were made from the same test material used in the above determinations. Thus the averages and standard deviations obtained in the control tests, compared to those in Table 1 and Table 2, respectively, show directly the reproducibility of the method.

Table 1

Vitamin D_3 content of some premixes and the reproducibility of the determinations

(Number of parallel measurements: 15)

Sample	Vitamin D_3 content		
	Average (IUg^{-1})	Standard deviation (IUg^{-1})	Coefficient of variation (%)
Vitamin premix No. 1.	319	76	23.9
Vitamin premix No. 8.	349	15	4.4
Vitamin premix No. 15.	492	43	8.7

Table 2

Vitamin D_3 content of some mixed feeds and the reproducibility of the determinations

(Number of parallel measurements: 9)

Sample	Vitamin D_3 content		
	Average (IUg^{-1})	Standard deviation (IUg^{-1})	Coefficient of variation (%)
Poultry diet	1.760	0.466	26.5
Turkey diet	1.280	0.463	36.2
Sow feed	3.120	0.308	9.9
Piglet diet	2.940	0.588	20.9
Finishing turkey diet	1.545	0.522	33.8

In Table 3 one part of the values obtained in the course of checking by Observer No. 2, are shown. In the first 5 columns the three parallel data obtained from the same extract and in the last column the averages of all 15 or 9 data, respectively, are given, together with the standard deviations.

The results of all control tests are given in Table 4. The reproducibility of results was indicated by the confidence limits at the probability level of 95%. The last column shows the differences in the averages as obtained by the individual observers. Each observer is marked by a serial number.

Table 3

Determinations of vitamin D_3 content in premixes and feeds

Results obtained by Observer No. 2.

Sample	Vitamin D_3 content (IUg^{-1}) Serial numbers of parallel weighings					Average and standard deviation (IUg^{-1})
	1	2	3	4	5	
Vitamin premix No. 1.	350	353	348	350	366	
	340	350	352	340	360	351 ± 7.1
	345	358	350	345	355	
Vitamin premix No. 8.	380	382	380	393	387	
	385	380	375	380	391	385 ± 6.2
	395	380	385	382	395	
Vitamin premix No. 15.	560	570	570	580	568	
	572	568	570	572	570	570 ± 4.4
	568	575	572	570	565	
Sow feed	3.268	2.918	3.502	—	—	
	3.230	3.105	2.915	—	—	3.185 ± 0.229
	3.502	3.268	2.958	—	—	
Piglet diet	2.077	2.255	2.967	—	—	
	2.789	2.611	2.967	—	—	2.651 ± 0.318
	2.967	2.611	2.611	—	—	

Table 4

Results of vitamin D_3 control tests

Sample	Observer No. 1.	Observer No. 2.	Observer No. 3.	Difference obtained between observers	
	Vitamin D_3 content and confidence limits at a probability level of 95% (IUg^{-1})			Ser. Nos. of observers	Difference (IUg^{-1})
Vitamin premix No. 1.	301 ± 110	351 ± 26	—	1 – 2	50
Vitamin premix No. 8.	349 ± 26	385 ± 28	334 ± 30	1 – 2	36*
				1 – 3	15
				2 – 3	51*
Vitamin premix No. 15.	492 ± 78	570 ± 78	483 ± 78	1 – 2	78*
				1 – 3	9
				2 – 3	87*
Sow feed	3.172 ± 0.536	3.185 ± 0.540	3.153 ± 0.538	1 – 3	0.013
				1 – 3	0.019
				2 – 3	0.032
Piglet diet	2.962 ± 0.906	2.651 ± 0.688	—	1 – 2	0.311

* Significant at a probability level of 95%.

3. Conclusions

3.1. *Statistical evaluation of the results*

The random error of the method was examined by analysis of variance on the basis of the experimental plan.

- The results of the control determinations, carried out by different persons, were compared.
- The error originating from the unequal distribution of the components to be determined as well as that originating from the methods of isolation and extraction were separated from the error inherent in the method.
- The spread was calculated in terms of standard deviation values.

The results of control tests summarised in Table 4 show no significant difference ($P \geq 95\%$) between feeds. In the case of premixes a significant ($P \geq 95\%$) but no highly significant ($P \geq 99\%$) difference was observed.

Standard deviations of the results of vitamin D_3 determinations are summarized in Table 5.

The results of control determinations were also drawn into the calculations.

Table 5

The effect of the preliminary operations and of the determination itself on the coefficient of variation in vitamin D_3 determination

Sample	Degrees of freedom	Coefficient of variation of the determination itself %	Coefficient of variation of the preliminary operations (weighing and extraction) %	Overall coefficient of variation %
Vitamin premix No. 1.	20	28.2	0	28.2
Vitamin premix No. 8.	24	4.6	1.8	5.1
Vitamin premix No. 15.	24	12.7	0	12.7
Poultry diet	18	28.3	8.4	29.6
Turkey diet	15	17.4	15.7	23.7
Sow feed	21	9.1	6.4	11.2
Piglet diet	15	12.9	16.0	20.6
Turkey finishing diet	9	9.7	17.4	20.0

As may be seen in Table 5, standard deviations, in the case of premixes, range from 5 to 28%, while those of feeds between 11 and 30% of the means. Compared to methods described in the literature these may be considered satisfactory. As regards the inhomogeneity of the samples this is substantial in the case of feeds.

3.2. *Notes on the applicability and further development of the method*

In comparison with those described in the literature, the method developed by the authors proved of adequate accuracy and of sufficient sensitivity in the case of premixes and feeds. Sensitivity equals 5 IU, or 0.125 μg vitamin D_3. The advantage of the method lies in its rapidity and simplicity. The fact, however, that in the case of premixes substantial differences were found between results obtained by different persons points to the necessity of thorough training in the performance of the method.

In the following some remarks on the advantages, applicability and possibility of further development of the method, are given.

1. Lipid extraction preceding the vitamin D_3 determination has several advantages:

- The disturbing effect of a number of impurities is eliminated by contacting the lipids only with alkali in the course of saponification.
- Though lipid extraction is a time-consuming operation it is counterbalanced by the fact that filtering after saponification becomes unnecessary.
- Aliquots of the ether extract may be used for other determinations, *e.g.* for the determination of essential fatty acids.

As established in experiments carried out with the standard solution of vitamin D_3, the insertion of lipid extraction leads to a more satisfactory recovery and a lower standard deviation between parallel determinations.

2. Since two-dimensional thin-layer chromatography is a very sensitive method and the R_f values are dependent on various parameters, it is desirable to check the R_f value with a standard when a determination is set or whenever a fresh developing agent is used.

It should be noted that the R_f value of vitamin D_3 in the sample may deviate by 5–8% from that measurable with the pure standard, because the migration of the other substances present may influence that of vitamin D_3. Therefore, in similar cases, it is advisable to add a known amount of vitamin D_3 standard to the sample.

3. Following the two-dimensional chromatographic separation of the vitamin, the vitamin D_3 determination method developed by the authors may be modified, or further developed in accordance with the available laboratory equipment.

Where a sensitive gas chromatograph is available the quantitative determination may be carried out with high accuracy with this apparatus.

Quantitative evaluations on thin-layer may be carried out also by using other adsorbent and solvent systems. In laboratories where reflectance detecting densitometer is available this may be used efficiently for inverted phase thin-layer chromatography. Its advantage lies in the fact that from the point

of view of separation it may be considered as chromatography in a third dimension and is useful in separating impurities accidentally present in the sample.

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SENSORY EVALUATION OF A NEW DIHYDROCHALCONE SWEETENER

PART I. — STUDIES IN AQUEOUS SOLUTIONS

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The sweetening capacity of the new artificial sweetener CH-401 decreases significantly with increasing concentration. Compared at sweetness levels corresponding to 9, 18 and 90 ppm solutions of CH-401, the new compound was found to be sweeter than saccharose by factors of 1467, 1022 and 278 respectively.

Compared with saccharin Na, the sweetening value of which was considered to be 440-fold, the sweetening value of CH-401 was between 650 and 700 at the sweetening level of 2 per cent of saccharose.

Sweetening is one of the most important factors in food flavouring. It is used mostly in itself, but the complementary effect of the sweet taste plays an important part also in the modification of other basic flavours. For instance, sweet taste is an important component of the required flavour of salads, various sauces and frequently also of meat products. The consumption of sweeteners found in nature (honey) or produced industrially (cane sugar, beet sugar, dextrose, etc.) has considerably increased the proportion of so-called empty calories in the nourishment of mankind. Instead of the physiologically acceptable average consumption of 15–20 kg per year per person beet sugar, the population of Hungary consumed the double amount: 36 kg yearly (KÖZPONTI STATISZTIKAI HIVATAL, 1974). As a consequence at least 30 per cent of the adult population may be considered as obese (SOÓS *et al.*, 1970, 1971, 1973) and there are many diseases devastating civilized mankind, the suppression of which absolutely requires a considerable reduction of the consumption of calorific sweeteners. Diseases of this kind are diabetes, various secondary cardiopathies and circulatory diseases developing with, resp. because of obesity, as well as caries tormenting even the child with milk teeth.

For this reason in the developed countries considerable attention is paid to the problem to find a sweetener free of calorie, which ensures, like sugar, the required sweetening effect and at the same time is less harmful than natural sweeteners, sugars, and other carbohydrates without sweetening effect. Against the generally used saccharin speaks its taste and thermolability and against the widely popular cyclamates the suspected carcinogenic effect. Requirements on an ideal calorie-free sweetener are manyfold: the taste shall be comparable to that of saccharose, it shall be a readily soluble, stable, thermoresistant

compound at a price acceptable to the consumer. It shall not be carcinogenic, nor objectionable from the aspect of toxicology or teratology in acute or chronic consumption (LINDNER, 1974).

The sweetener meeting the above requirements will of course not replace the customary sweeteners like saccharose, glucose and fructose or even sugar alcohol, sorbite, xylose etc. consumed by diabetics. These, being important sources of energy, are indispensable in the manufacture of full-bodied beverages and other food products. However, about one third of the sugar consumed at present may be replaced by calorie-free sweeteners. Their use is feasible in soft drinks with natural and synthetic aroma, in coffee and tea, canned fruit, jams and confectionary. These products fall into three groups according to the amount of sugar used in their production: coffee and tea require 1–5%, soft drinks 10–16%, canned fruit and jams 35–60% beet sugar.

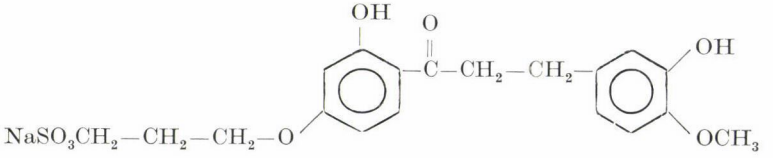
Consequently the relation between concentration and sweetening capacity is a problem of importance.

1. Materials and methods

1.1. *The chemistry of CH-401*

In the course of preparation of several hundreds of flavones, resp. dihydrochalcones FARKAS and co-workers (1973) found a number of compounds with sweet flavour. One of them (marked CH-401) turned out to have such intense sweet flavour that it seemed worth to study its sweetening capacity in more detail.

Structural formula:



Empirical formula: $C_{19}H_{21}O_8SNa$

Molecular weight: 432. Pale cream-coloured crystalline substance.

Melting point: between 351 and 355 °C.

Solubility at room temperature is 1.3 per cent in distilled water. It resists heat treatment at 120 °C for 3 hours in the range of pH 2–7 without change.

In the course of the study of subacute toxicity (feeding rats for 90 days) the general behaviour, change of body weight, hematologic parameters (differential and quantitative blood count, coagulation time) were examined, further

serologic and urine analysis, and after the conclusion of treatment pathologic, resp. histopathologic examinations were carried out. Signs indicative of pathologic alterations were not observed and thus, CH-401 may be considered as non-toxic. More detailed toxicological studies are in progress.

1.2. *Sensory analysis*

With respect to its sweetening property, the artificial sweetener CH-401 was subjected to sensory analysis in aqueous solutions of various concentration by a homogeneous organoleptic panel.

1.2.1. *Selection of panel members.* On the basis of basic taste and threshold value tests 40 out of 80 university students were selected for sensory analysis (ISO, 1973). Their distinguishing ability functioned soundly at the threshold of various flavour concentrations and thus, they were able to give an objective judgement. Their age was between 20 and 24 years, the distribution according to sex was fifty-fifty.

The suitability for sensory analysis was tested with salty, sweet, sour and bitter solutions.

Corresponding to the special task of detecting the sweetness of the CH-401 at the organoleptic limit level with tasters highly sensitive to sweetness, the threshold concentrations were as follows:

sodium chloride:	0.15%
saccharose:	0.4%
citric acid:	0.5%
quinine sulfate:	0.0005%

The tap water used for dilution served as comparison, *i.e.* the objective was the proper identification of the five solutions in four repetitions.

The sensory tests were carried out by tasters who were able to identify the sweet taste at threshold level.

1.2.2. *Method of the sensory analysis of CH-401.* In foods — with the exception of products preserved with sugar and confectionary of high sugar content — saccharose is used in two different concentrations: corresponding to a concentration of 1 to 5 and 10 to 16%, resp. Therefore the examinations were carried out with CH-401 concentrations corresponding to the sweetening effect of 1, 2, 10 and 16% of saccharose, resp., as well as with a saccharin concentration of sweetness equal to 2% saccharose.

In the course of the test a CH-401 solution of constant concentration had to be compared with saccharose solutions of various concentration. The tasters were asked to determine whether the saccharose solution just tasted was more, equally or less sweet than the CH-401 solution.

The samples (one sweetened with CH-401 and 5 containing only saccharose) were presented to the members of the panel in uniform colourless drinking-glasses on individual trays marked with code numbers drawn. Repeated retasting was allowed. Between two tastings, panel members consumed slightly salted biscuit and seltzer water.

The saccharose-containing samples, in comparison to the sample marked M, containing the synthetic sweetener, had to be graded in one of the above categories, and marked by a cross under the proper code number in the evaluation form. The responses collected after each evaluation (representing 200 taste determinations) served for the mathematical evaluation.

The determination of the sweetening value of CH-401 was first carried out according to the mathematical-statistical method of *Pauli* (GUTSCHMIDT & ORDYNSKY, 1961). However, the numerical data of the evaluation were not in accordance with sensory observations. This mathematical-statistical procedure is probably suitable only for the evaluation of certain natural sugars and sugar alcohols (it has been applied recently to xylose and polyalcohols). A special method had to be elaborated for the statistical evaluation of sensory judgement, which permitted of comparing deviating flavour effects with saccharose (sense of taste appearing mainly on the edges and pharyngeal convexity of the tongue).

Since it is a well-known fact that in sensory analysis judgement becomes uncertain at concordant taste levels, an identical number of "less sweet" and "sweeter" judgements were incorporated in the category "identical".

2. Results and conclusions

2.1. *Studies at the sweetness level corresponding to a 9 ppm solution of CH-401*

2.1.1. *Comparison of sweetness.* The concentrations of the sugar solutions were: 0.6, 0.8, 1.0, 1.2 and 1.4%, resp.

Concentration of the CH-401 solution: 9 mg l^{-1}.

Table 1 illustrates the distribution of sensory responses of the 40 tasters and the statistical evaluation of their judgements.

The values in Table 1 were used for the independence test, based on the χ^2 test, in order to determine whether there was a correlation between the number of sensory differentiations and concentration or whether they are independent of each other.

$$\chi^2 = n\left[\sum_{i=1}^{3}\sum_{j=1}^{5}\frac{V_{ij}^2 \cdot V}{V_i \cdot V_j} = 1\right]$$

$$\chi^2 = 200\,[0.3230] = 64.6$$

$$P(\chi^2 > 26.125) = 0.001$$

where χ^2 = the probability distribution of chi-square

n = total number of observations ($n = 200$)

V_{ij} = frequency of the joint occurrence of various sensory observations and concentrations.

$P(\chi^2\ 26.125)$ = probability value determined from the table of χ^2 distribution.

Table 1

Comparison of the sweetness of saccharose solutions to that of a CH-401 solution of 9 mg l^{-1}

Sensory response+	No. of judgements					Total (V_i)
	Saccharose concentration (%)					
	0.6	0.8	1	1.2	1.4	
Less sweet	38	36	32	22	11	139
Identical	2	3	5	8	10	28
Sweeter	0	1	3	10	19	33
Total (V_j)	40	40	40	40	40	200

+ = The saccharose solution is as indicated in the column

V_i = frequency of various sensory observations ($i = 1, 2, 3$)

V_j = frequency of observations belonging to different concentrations ($j = 1, 2, 3, 4, 5$)

Processing the data by computer the following result was obtained (Table 2).

Thus on the basis of the final result it may be excluded with considerable significance that responses and concentrations are independent of one another.

Table 2

Independence test

Sensory response+	No. of judgements					Total (V_i)
	Saccharose concentration (%)					
	0.6	0.8	1	1.2	1.4	
Less sweet	0.2597	0.2331	0.1842	0.0871	0.0218	0.7859
Identical	0.0036	0.0080	0.0223	0.0571	0.0893	0.1803
Sweeter	0	0.0008	0.0068	0.0758	0.2734	0.3568
Total (V_j)	0.2633	0.2419	0.2133	0.2200	0.3845	1.3230

For symbols used see Table 1.

Therefore it is possible to compare the sweetening capacity of the compound to that of saccharose by further mathematical evaluation.

The modified table calculated with the identically sweet values increased by identical number of (±) judgements is shown in Table 3.

Table 3

Corrected table for (±) judgement

Sensory response+	No. of judgements					Total (V_i)
	Saccharose concentration (%)					
	0.6	0.8	1	1.2	1.4	
Less sweet	38	35	29	12	0	114
Identical	2	5	11	28	32	78
Sweeter	0	0	0	0	8	8
Total (V_j)	40	40	40	40	40	200

⨯ inflection point of the less/more judgements.

For other symbols used see Table 1.

The point of inflexion between the saccharose concentrations of 1.2 and 1.4% is clearly visible in Table 1 at the maximum of responses registering "identical" sweetness. This is even more apparent in Table 3 with values "reduced to 0"; the results of basic judgement are illustrated in Fig. 1.

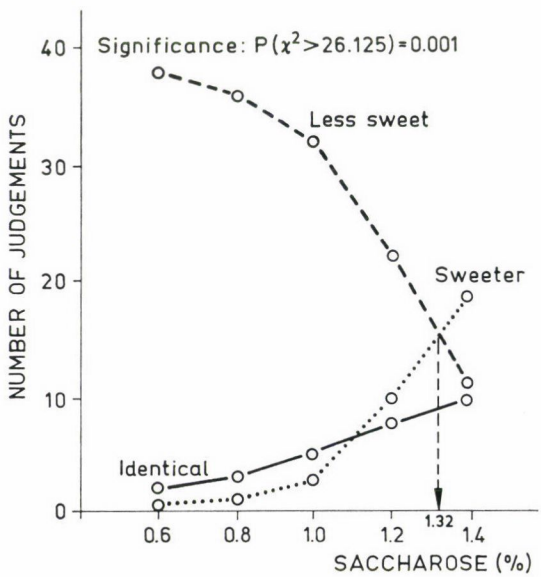


Fig. 1. Determination of the identical "sweetness" point using the judgement curves, using a CH-401 concentration of 9 mg l^{-1}

2.1.2. *Checking the statistical evaluation on the basis of percentage distribution.* This is based on Table 3. Sensory response was quantified as follows:

less sweet than −1
identical with 0
sweeter than 1 (code number).

The relative frequency of the three different judgements was calculated for each concentration and thus, M, the average of quantified judgements was determined by weighting. Table 4 illustrates the results.

Table 4

Code-quantity judgement

Sensory response+	No. of judgements					Code number
	Saccharose concentration (%)					
	0.6	0.8	1	1.2	1.4	
Less sweet	0.95	0.875	0.725	0.3	0	−1
Identical	0.05	0.125	0.275	0.7	0.8	0
Sweeter	0	0	0	0	0	1
Total	1	1	1	1	1	
M	−0.95	−0.875	−0.725	−0.3	0.2	

M = mean-value of judgements


inflection point of the less/more judgements

+ = the saccharose solution is as indicated in the column.

Thus, here too, the concentration considered by most tasters to be identical with the sample containing the synthetic sweetener falls between saccharose concentrations of 1.2 and 1.4%. By linear interpolation this concentration was found to be 1.32%.

It may be concluded from the evaluation that the majority of consumers would find the artificial sweetener CH-401 in aqueous solution at this concentration level 1467 (about 1500) times more sweet than saccharose.

2.2. *Studies at the sweetness level corresponding to an 18 ppm solution of CH-401*

The solutions used for comparison differed by 0.2% from the basic (mean) concentration (2%). The concentration of the CH-401 containing sample was 18 mg l^{-1}.

The following Tables 5 and 6 and calculations comprise the results of the sensory evaluation of 40 tasters.

Table 5

Distribution of judgements

Sensory response+	No. of judgements					Total (V_i)
	Saccharose concentration (%)					
	1.6	1.8	2	2.2	2.4	
Less sweet	12	15	9	5	4	45
Identical	9	6	5	3	4	27
Sweeter	19	19	26	32	32	128
Total (V_j)	40	40	40	40	40	200

For symbols used see Table 1.

It was possible to establish by the independence test of the values in Table 5 a correlation between the sensory differences and the concentrations at a high probability level. In spite of this the sharp inflexion as observed in Table 3 could not be observed here even after the reduction of values to 0 (Table 6).

Table 6

Corrected table for the (±) judgement

Sensory response+	No. of judgements					Total (V_i)
	Saccharose concentration (%)					
	1.6	1.8	2	2.2	2.4	
Less sweet	0	0	0	0	0	0
Identical	33	36	23	13	12	117
Sweeter	7	4	12	27	28	83
Total (V_j)	40	40	40	40	40	200

For symbols used see Table 1.

The sweetening value of CH-401 was found to be 1022 (about 1000) times that of saccharose in a 2% solution, calculated on the basis of the identity value 1.84% obtained from Fig. 2.

2.3. *Studies at the sweetness level corresponding to a 90 ppm solution of CH-401*

It was shown in preliminary experiments that it is expedient to use a 5-member series of saccharose solutions of the concentration of 2.1, 2.3, 2.5, 2.7 and 2.9%, resp. The concentration of the artificial sweetener CH-401: 90 mg l^{-1}.

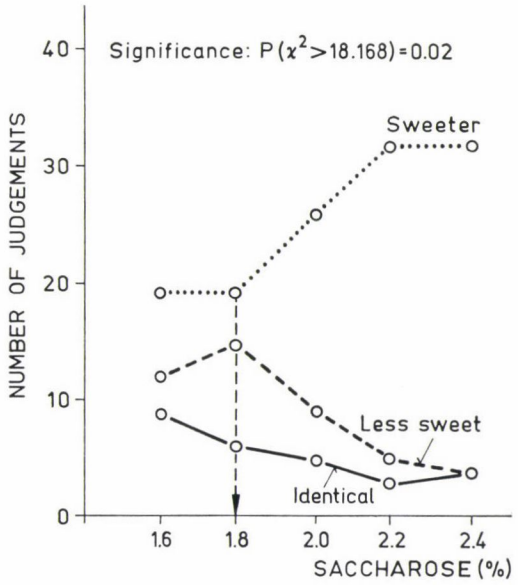


Fig. 2. Determination of the identical "sweetness" point using the judgement curves, using a CH-401 concentration of 18 mg l^{-1}

The results are shown in Table 7.

Table 7

Distribution of judgements

Sensory response+	No. of judgements					Total (V_i)
	Saccharose concentration (%)					
	2i1	2.3	2.5	2.7	2.9	
Less sweet	8	8	7	6	5	34
Identical	2	1	5	4	4	16
Sweeter	10	11	8	10	11	50
Total (V_j)	20	20	20	20	20	100

For symbols used see Table 1.

At the first glance it may be noted that the comparison of the saccharose solutions with the artificial sweetener at the given concentration levels becomes erratic.

The independence test proves likewise that the results are hardly dependent on the sugar concentration. The sweetness of a 90 mg l^{-1} CH-401 solution may be considered approximately equal to that of a saccharose solution of 2.5 or 2.6 %.

Therefore, at this concentration level, CH-401 is not more than 278 times sweeter than saccharose.

Under the given experimental conditions, with increasing CH-401 concentration its sweetening power decreases. Namely, if the CH-401 concentration equivalent to 16% saccharose solution is calculated on the basis of the CH-401 concentration needed to equal a 1% saccharose solution the actual sweetening capacity of the solution is only about one tenth of the value expected.

Figure 3 shows the sweetening capacity of the artificial sweetener of various concentrations as compared to saccharose, and it may be noted that in the lower concentration range the sweetening value decreases considerably with increasing concentration. This decrease may have various explanations. The fatigue of gustatory peripheroceptors may be excluded, because on re-tasting solutions of lower concentration the original intense taste is perceived.

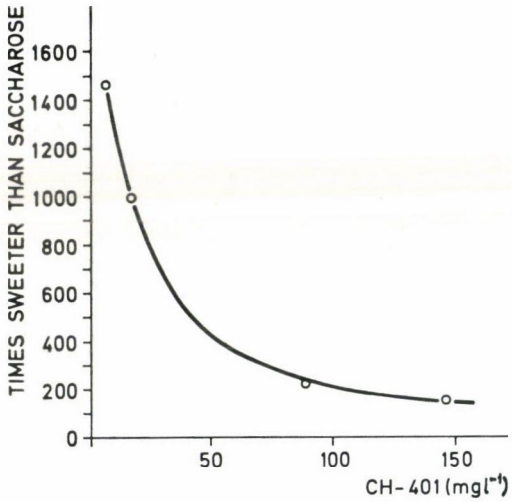


Fig. 3. Correlation between relative sweetness curve and CH-401 concentration

Most likely the dissociation–association of the dihydrochalcone molecules is the determining factor.

2.4. *Comparison with saccharin-Na at the sweetness level of an 18 ppm solution of CH-401*

The concentration of CH-401: 18 mg l^{-1}. The saccharin-Na is considered 440 times sweeter than saccharose. The difference in the concentration of the two successive solutions corresponds to 0.2% saccharose. The results of 40 sensory evaluations are shown in Table 8.

Table 8

Comparison of the sweetness of saccharin solutions to that of a CH-401 solution of 18 mg l^{-1}

Sensory response+	No. of judgements					Total (V_i)
	Saccharin-Na concentration (mg l^{-1})					
	20.0	22.5	25.0	27.5	30.0	
	Corresponding to saccharose concentration (%)					
	1.6	1.8	2.0	2.2	2.4	
Less sweet	23	18	17	12	12	82
Identical	13	9	11	13	13	59
Sweeter	4	13	12	15	15	59
Total (V_j)	40	40	40	40	40	200

For symbols used see Table 1.

As it may be seen in Table 8, differences in taste could not be established with certainty. The independence test resulted in a similar conclusion: concentration values and sensory responses were not independent of one another at the probability level P = 80%.

The statistical evaluation was checked on the basis of percentage distribution applying the method of "reduction to 0". The result is shown in Table 9.

Table 9

Code-quantify judgement of the saccharin-Na/CH-401

Sensory response+	No. of judgements					Code number
	Saccharin-Na concentration (mg l^{-1})					
	20.0	22.5	25.0	27.5	30.0	
Less sweet	0.475	0.125	0.125	0	0	−1
Identical	0.525	0.879	0.875	0.925	0.925	0
Sweeter	0	0	0	0.075	0.075	1
Total	1	1	1	1	1	
M	−0.475	−0.125	−0.125	+0.075	+0.075	

For symbols used see Table 4.

The concentration where the two samples are judged to be of identical sweetness is marked by an arrow in Table 9. By linear interpolation the result is a saccharin concentration of 26.7 mg l^{-1}.

Thus, calculating with a 440-fold sweetness of saccharin Na: 440 × 26.7 = = 11.748 mg saccharose which, taking into consideration the CH-401 con-

centration of 18 mg l^{-1}, means a taste effect which is about the sesquialter of the sweetening capacity of saccharin and at the sweetening level of cca. 2% saccharose represents a sweetening value of 650 to 700.

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SENSORY EVALUATION OF A NEW DIHYDROCHALCONE SWEETENER

PART II. — STUDIES IN FOODS

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The new artificial sweetener CH-401 gives a good overall effect when it is used in solutions having salty or sour flavour. In the presence of bitter taste components as well as in confectionary products containing substantial amounts of egg-yolk and fat, the sweetening capacity diminishes and a metallic by-taste is experienced. With tea and lemonade a sweetening effect almost comparable to saccharose may be attained if the sweetener is combined, to one third of the total sweetening effect, with saccharose. In the case of certain foods (tomato sauce, compotes, etc.) a flavour-equalizing, aroma-enhancing effect may be noted in the presence of CH-401 in addition to a satisfactory sweetening effect.

The sweetening capacity of a new artificial sweetener (CH-401) and saccharose in aqueous solution was compared in a previous paper (LINDNER *et al.*, 1977). In case of lower concentrations these examinations showed CH-401 to be about 1100–1500 times sweeter than saccharose.

In addition to the sweet taste, sour, salty and bitter are considered as basic flavours. It was essential to determine whether the sensory properties of the artificial sweetener CH-401 gain or loose when tasted together with various modifying basic flavours.

1. Materials and methods

The beverages and foods examined are enumerated in the chapter dealing with results.

The examinations were performed by paired comparison (ISO/TC/HGT 2, 1973) with 18 mg l^{-1} CH-401 dissolved in drinking water with and without the addition of

5 g l^{-1} NaCl
5 g l^{-1} citric acid and
50 mg l^{-1} coffeine.

The results are presented in Table 1.

Thus, of the modifying basic tastes, it is the slightly salty taste, which renders the taste sensation somewhat more favourable by the sense of richness.

CH-401 influences favourably the toleration of sour taste, but its sweetening effect falls entirely into the background in the presence of bitter flavour.

Table 1

The sensory properties of the artificial sweetener CH-401 when tasted together with modifying basic flavours

Solutions in 1000 ml drinking water	Modifying basic taste	Evaluation of modified taste of CH-401	Remark
18 mg CH-401 + + 0.5 g NaCl	salty	identical sweet taste	richer
18 mg CH-401 + + 0.5 g citric acid	sour	sweet taste fell slightly into the background	sour taste damped down
18 mg CH-401 + + 50 mg coffeine	bitter	sweet taste fell entirely into the background	acrid, bitter taste develops

2. Results

Sweeteners are used to give a special sweet flavour to beverages, like coffee, tea, soft drinks, as well as to various sweets and confectionary products, cakes filled with cream, ice cream, etc.

For this reason the taste of CH-401 was studied in relation to the complex flavour of the above food items. The new synthetic sweetener was also tasted with the familiar natural sweetener, saccharose.

2.1. Evaluation in beverages

The objective of the experiments was not primarily the determination of the sweetening capacity, but rather the comparison with sugar.

Notwithstanding that the majority of beverages contains more than 1 or 2% saccharose, on account of the reduced intensity of taste mentioned in the sensory analysis at elevated CH-401 concentrations, most of the beverages were tasted at a level corresponding to not more than 1 or 2% saccharose.

The recipe of the beverages was as follows.

2.1.1. Tea

a) Stock solution: 18.4 g Lipton tea infusion in 1580 ml tap water; from this stock solution were prepared the following solutions.

b) Tea sweetened with saccharose: 800 ml stock solution + 200 ml of a 10% saccharose solution corresponding to the sweetening value of 2.0% saccharose.

c) Tea sweetened with CH-401: 480 ml stock solution + 120 ml of the solution of 90 mg CH-401 in 1000 ml water corresponding to the sweetening value of 18 mg l^{-1} CH-401.

d) Tea sweetened with sugar plus CH-401: 469 ml stock solution + 51 ml of a 10% saccharose solution to which 80 ml of the solution of 90 mg l^{-1} CH-401 was added corresponding to the combined sweetening value of 8.5 g l^{-1} saccharose +12.0 mg l^{-1} CH-401.

2.1.2. Coffee

a) Stock solution: 1750 ml black coffee prepared from 144 g ground "Gold Mocca Coffee".

b) Coffee sweetened with saccharose: 960 ml of the above stock solution + 240 ml of a 10% saccharose solution; this mixture corresponds to the sweetening value of 2% saccharose.

c) Coffee sweetened with CH-401: 360 ml of stock solution + 240 ml of a 90 mg l^{-1} CH-401 solution corresponding to the sweetening capacity of 36 mg l^{-1} CH-401.

d) Coffee sweetened with sugar plus CH-401: to 404 ml of the stock solution 36 ml of a 10% saccharose solution and 160 ml of a 90 mg l^{-1} CH-401 solution were added. This mixture corresponds to the combined sweetening value of 6 g l^{-1} saccharose + 24 mg l^{-1} of CH-401 solution.

2.1.3. Lemonade

a) Stock solution: 120 ml freshly squeezed lemon juice was added to 2400 ml seltzer water.

b) Lemonade sweetened with sugar: 960 ml of the stock solution + 240 ml of a 10% saccharose solution corresponding to the sweetening effect of a 2% saccharose solution.

c) Lemonade sweetened with CH-401: 480 ml of the stock solution + + 120 ml of a 90 mg l^{-1} CH-401 artificial sweetener solution; this corresponds to the sweetening value of 18 mg l^{-1} artificial sweetener.

d) Lemonade sweetened with sugar and CH-401: to 480 ml of the stock solution 36 ml of a 10% saccharose solution and 80 ml of 90 mg l^{-1} CH-401 solution were added. This corresponds to the sweetening effect of 6 g l^{-1} saccharose + 12 mg l^{-1} CH-401.

The solutions, poured into glasses marked with a three-digit code number, were tested by a panel of 20 tasters with the possibility of re-tasting. For the neutralization of taste Swiss Gruyere cheese and seltzer water was made available. Between two tests there was an interval of half an hour.

The examinations were carried out in accordance with the INTERNATIONAL STANDARD RECOMMENDATION ISO/TC 34/WG 2 N 80 by triangular test, and the significance of evaluations was determined on the basis of biometrical statistical tables. Table 2 summarizes the results of examinations and the evaluation.

Table 2

Result of judgements of beverages

(Triangular test performed by a panel of 20 members)

Composition of beverage containing artificial sweetener (in comparison to a beverage of 2% saccharose)	Evaluation of the sample containing CH-401	Significance of judgements P(%)	Sweetening value in comparison to saccharose
TEA (1) 18 mg l^{-1} CH-401	tastes better: 4, worse: 16 (correct judgements: 13)	<99	$\ll 1467$
TEA (2) 12 mg l^{-1} CH-401+8.5 g l^{-1} saccharose	tastes better: 2, worse: 14 (correct judgements: 14)	<99.9	
COFFEE (1) 36 mg l^{-1} CH-401	tastes better: 4, worse: 12 (correct judgements: 15)	<99.9	$\ll 550$
COFFEE (2) 24 mg l^{-1} CH-401+6 g l^{-1} saccharose	tastes better: 9, worse: 12 identical: 7 (correct judgements: 14)	<95	
LEMONADE (1) 18 mg l^{-1} CH-401	tastes better: 4, worse: 9, identical: 7 (correct judgements: 11)	<95	$\ll 1022$
LEMONADE (2) 12 mg l^{-1} CH-401+6 g l^{-1} saccharose	tastes better: 3, worse: 9, identical: 8 (correct judgements:11)	<95	

Comments of consumers concerning the taste of individual beverages:

Tea with artificial sweetener: unanimous opinion was that the tea flavoured with sugar was better than that flavoured with CH-401.

Tea with artificial sweetener + one third sugar: tea was in many cases definitely identical, resp., better as compared to tea sweetened only with sugar, and tasted much better as tea sweetened only with CH-401.

Coffee with artificial sweetener: unpleasant after-taste, coffee flavoured with artificial sweetener lacks of sweet taste.

Coffee with artificial sweetener + one third sugar: approximately as the above, *i.e.* taste is not improved by adding sugar.

Lemonade with artificial sweetener: although more sour than the lemonade flavoured with sugar, the total impression of taste is not bad.

Lemonade with artificial sweetener and one third sugar: statistically it is comparable with sugar, but the total impression of taste is improved by adding sugar.

2.2. Evaluation in foods

The recipe of food and basic preparations was as follows (see also Table 3).

2.2.1. Salad dressing

As customary in the catering industry, the sweeteners were added to the dressing consisting of water, vinegar, salt (concentration: 8.8% saccharose, 60 mg l^{-1} CH-401).

2.2.2. Tomato sauce

The tomato puree was diluted with water then seasoned with salt, celery, onion, boiled and thickened with roux and finally sweetened.

2.2.3. Strawberry compote

The washed strawberries were flavoured with cinnamon, lemon slices and cooked for a short time in water (until soft), then sweetened with artificial, resp., natural sweetener.

2.2.4. Parfait

Prepared warm: the egg yolk, milk and solution of sweetener were boiled, then cooled. Finally the mixture was frozen with whipped cream flavoured with the sweetener. Prepared cold: the same, without boiling.

2.2.5. Yellow cream

Milk and the sweetener were mixed and boiled. In the meantime the egg yolk and flour were whisked to a smooth paste and added to the hot milk. Under careful mixing it was cooked till thick. When ready, the whipped egg-white was carefully added.

2.2.6. Butter cream

a) Cooked: the sugar, resp., sweetener solution was boiled down and whisked with butter.

b) Cold: the sweet (sugar or CH-401) solution was whisked with butter without boiling down.

The sensory tests were likewise evaluated in accordance with INTERNATIONAL STANDARD RECOMMENDATION ISO/TC 34/WG 2 N 80.

Table 3 shows the course and results of the sensory tests.

Table 3

Triangular judging with foods and basic preparations

Specification	Sample used for comparison Sugar %	Sample with CH-401 content mg 100 g^{-1}	Result of evaluation Triangular test of CH-401 sample performed by 20 persons	Significance of judgement P(%)
SALAD DRESSING 400 ml water, 5 g salt, 10 ml vinegar (of 10%)	8.8	5.94	Sample slightly worse, rather sickening after-taste. Correct judgements: 19	<99
TOMATO SAUCE 250 g tomato puree 70 g fat, 80 g flour, 16 g salt	9.4	6.62	Identical, richer, slight after-taste. Correct judgements: 20	<99
STRAWBERRY compote 250 ml water, 120 g strawberries	3.1	3.63	Sample less sweet, but with richer after-taste. Correct judgements: 18	<99
PARFAIT (vanilla) 100 ml milk, 4 egg-yolks, 500 ml sweet cream	11.4	12.20	Sample worse because of metallic after-taste. Correct judgements: 20	<99
PARFAIT (chocolate) 30 g cocoa-powder	9.0	12.0	Sample is worse, has metallic after-taste. Correct judgements: 19	<99
YELLOW CREAM 500 ml milk, 35 g flour 15 g cream-powder, 3 eggs	7.5	4.5	Sample is worse, has metallic after-taste. Correct judgements: 19	<99
BUTTERCREAM (cooked) 250 g butter	47.4	102.60	Sample worse, has a strong metallic taste. Correct judgements: 20	<99
BUTTERCREAM (Cold) 250 g butter	47.4	102.60	Sample worse, has a strong metallic taste. Correct judgements: 19	<99

The opinion of the organoleptic panel concerning the individual types of food and basic preparations made with the artificial sweetener CH-401 may be summarized as follows:

The synthetic sweetener is suitable for the preparation of *tomato sauce* and *salad dressing*.

Parfait: prepared cold, has no characteristic taste and prepared warm it has in addition a metallic after-taste.

Strawberry compote: It is sweetened slightly and has a persistent rather sickening after-taste.

Yellow cream: has no characteristic taste and in addition a persistent metallic after-taste.

Butter cream: both the cold and warm preparations have an unpleasant sickening, metallic, slightly bitter after-, resp., by-taste.

3. Conclusions

In combination with other basic tastes, the sweetness of CH-401 creates a good general effect especially in solutions of salty, but also of sour flavour. On the other hand, because of strong by-tastes and reduction of the sweeten-

ing capacity caused by bitter components, it is far less pleasant than in combination with the other two basic tastes.

As for the various beverages and foods, a reduction of the sweetening value of CH-401 may be noted in comparison with pure aqueous CH-401 solutions, partly as a consequence of the increased sweetener concentration, partly because of the combination of substances and tastes.

In the case of beverages, with the exception of those comprising a bitter component, like coffee, the taste is satisfactory to those accustomed to it, however the flavour of sugar to which the customers are used is not achieved, not even in the case of tea and lemonade.

According to the experiments carried out with tea and lemonade, a sweetening effect almost comparable to saccharose (according to some judgements a richer, more balanced flavour) may be attained, if the sweetener is combined with saccharose so that one third of the total sweetening effect derives from sucrose.

In the case of certain foods — like tomato sauce, compotes — flavour equalizing, aroma enhancing effect may be noted in addition to equivalent sweetening effect.

In the case of confectionary products containing much egg-yolk and fats (like cream, parfaits), the sweetening capacity is slight, the metallic by-taste is more distinct.

On the basis of the above it may be stated that the artificial sweetener CH-401 exerts its effect in a way different from natural sugars, or sugar alcohols.

As may be seen in Fig. 1, the sensory experience develops after a few seconds bilaterally on the laryngeal part of the tongue. This means that tastes, especially the accompanying basic tastes appearing in the usual manner with respect to sweet taste, are manifesting themselves earlier and are dominant as primary taste effects. Thus, the first impression of salad dressing, the juice of compote is an intense sour flavour.

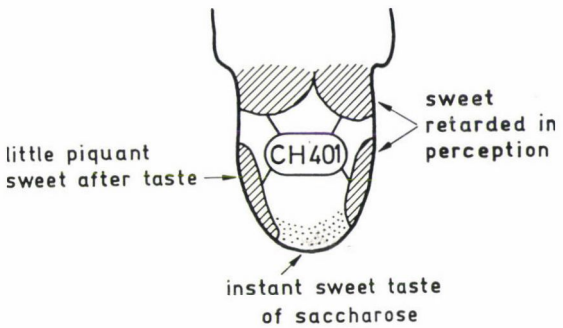

Fig. 1. Distribution of the sensory sensations on the tongue, testing the artificial sweetener CH-401

Another persistent sensation has to be mentioned, namely the slightly sweet, somewhat piquant taste on the oral and lingual mucosa, at first more intensely on both sides of the tip of the tongue.

On the basis of sensory analysis, CH-401 seems to be appropiate for sweetening of selected foods and beverages.

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APPLICATION OF BIAMPEROMETRIC TITRATION WITH POTASSIUM HYPOBROMITE IN THE DETERMINATION OF KJELDAHL-NITROGEN IN ANIMAL FEEDSTUFFS

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In the nitrogen determination by *Kjeldahl*'s method digestion was promoted initially with hydrogen peroxide. After the decomposition of the oxidizing agent the final stage of the process was furthered by the addition of H_2SeO_3 as a catalyst. The NH_4^+ ions formed were determined by titration with KOBr solution without separation by distillation, using biamperometric end-point indication. Since H_2SeO_3 itself takes up some KOBr, the amount of catalyst present in the system had to be known exactly in order to be able to correct the amount of titrant added by a blank value. Therefore, it seemed expedient to dissolve selenium previously in nitric acid and use this solution to introduce a defined amount of catalyst. The effective concentration of the titrant was determined by a standard $(NH_4)_2SO_4$ solution. The reliability of the method developed was checked by the *Kjeldahl-Winkler* method.

The mathematical statistical evaluation of the experimental data has shown that, as regards reproducibility, the biamperometric method is superior to the *Kjeldahl-Winkler* procedure while their accuracy is equal.

The determination of nitrogen by *Kjeldahl*'s method may be substantially accelerated by titrating the ammonium ions formed in the diluted sulfuric acid solution directly instead of after previous separation by distillation. On the basis of the reaction given below various methods were developed, such as bromato-iodometric (WILLARD & CAKE, 1920), amperometric (KOLTHOFF *et al.*, 1953; TSAP & LEONCHIK, 1968; FÜLEKY, 1970), coulometric (ARCAND & SWIFT, 1956), spectrophotometric (HALÁSZ *et al.*, 1973a) and thermometric (HALÁSZ *et al.*, 1973b) methods:

$$2\,NH_4^+ + 3\,OBr^- + 2\,OH^- = N_2 + 3\,Br^- + 5\,H_2O$$

It is known that in addition to elementary nitrogen, nitrogen oxides (KOLTHOFF & BELCHER, 1957) and bromate ions (GROVER & MEHROTRA, 1958) are also formed. However, these by-processes do not exclude the analytical utilization of the main reaction. If experimental conditions are properly selected, hypobromite consumption is well reproducible and is, in practice, in linear correlation with the NH_4^+ content of the solution analyzed. In order to eliminate the error due to the by-reactions it is most expedient to determine the effective concentration of the KOBr reagent with a standard NH_4^+ solution. In the methods mentioned above, generally a pH of 8.2 was found optimal, because at this pH the formation of nitrogen oxides is not too substantial

and the loss of ammonia is practically zero (KOLTHOFF *et al.*, 1953; ARCAND & SWIFT, 1956).

The procedure of TSAP and LEONCHIK (1968) attracted special attention of agricultural laboratories. The principle of this method is that after digestion of the sample with conc. sulfuric acid and perchloric acid the NH_4^+ content of the solution is determined with NaOBr standard solution applying biamperometric indication. This method was modified by FÜLEKY (1970). For digestion, he used hydrogen peroxide instead of perchloric acid. In view of the requirement for the analysis of a great number of samples it seemed expedient to apply further changes.

It is known that digestion by sulfuric acid may be promoted by the addition of hydrogen peroxide only, if it is added in very small portions, while heating is interrupted at intervals (SARUDI, Sr. 1941). This, however, is very cumbersome in the case of large series of samples. This problem was resolved in the present method by adding, beside the oxidizing agent, selenium as a catalyst. Hydrogen peroxide was added to the material prior to commencement of heating. Thus the reaction proceeded to some degree under strong heat development. Heating was applied only after the greater part of the oxidizing agent had decomposed. The role of the catalyst became important in this phase, because it shortened the end phase of the reaction. However, the presence of selenium had to be taken into account in the oxidimetric determination of NH_4^+ ions, because the catalyst itself reacts with hypohalogenites. Thus it was necessary to use a well-defined amount of selenium. Elementary selenium was dissolved in nitric acid and out of the solution containing H_2SeO_3 a predetermined volume was added to the reaction mixture prior to digestion (REMY, 1955). Hereafter, the consumption of the KOBr titrant was corrected by an appropiate blank value.

1. Materials and methods

1.1. Materials

- conc. H_2SO_4
- 30% H_2O_2
- H_2SeO_3 solution: 3.00 g of selenium were dissolved under a hood in 20 ml conc. nitric acid. After evaporating to dryness, it was washed into a volumetric flask of 1000 ml and made to mark.
- 0.05 *N* KOBr solution: 1.39 g of potassium bromate were weighed into a volumetric flask of 1000 ml, 7.5 g of potassium bromide were added and both dissolved in about 300 ml of water. The solution was acidified by the addition of 200 ml *N* hydrochloric acid, then stoppered and allowed to stand for 30 min. Subsequently, 200 ml of 2 *N* sodium hydroxide

were added and the solution made up to the mark with water. The solution was stored in a dark flask and checked for efficiency every day.

- Standard $(NH_4)_2SO_4$ solution: 0.9438 g ammonium sulfate was dried for 2 h at 105 °C, washed into a 1000 m lvolumetric flask. This solution contained 0.2 mg nitrogen per ml.
- Solution for the blank test: 75 ml conc. H_2SO_4, 25 ml H_2SeO_3 solution, and 125 ml 30% H_2O_2 solution were measured into a Kjeldahl flask of 740 ml. The solution was heated until sulfuric acid fumes began to form. After cooling it was transferred into a 500-ml volumetric flask and made to the mark.
- $KHCO_3$ solution: saturated at room temperature.
- Catalyzing and boiling point raising agent: 1:4 mixture of $CuSO_4 \cdot 5H_2O$ and K_2SO_4
- NaOH solution of 33%
- H_3BO_3 solution of 4%
- 0.1 *N* HCl solution
- Indicator solution: 0.1 g methyl orange and 0.15 g indigo carmine dissolved in 100 ml water. The hydrogen peroxide and potassium hydrogen carbonate used were of the purissimum, the other reagents of the analytical grade.

1.2. Equipment used for biamperometric titration

- OH 101/1 type polarograph, manufactured by RADELKIS, Hungary
- Dead-stop titration flask of 120 ml with two built-in platinum electrodes
- OP-912 type magnetic stirrer, manufactured by RADELKIS, Budapest
- 10-ml automatic burette with 0.05-ml graduation.

1.3. Determination of nitrogen by the new method

1.3.1. Digestion. 1.00 g ground sample was weighed in a Kjeldahl flask of 250 ml, 15 ml conc. H_2SO_4, 5.00 ml H_2SeO_3 solution, and 25 ml of a 30% H_2O_2 solution, in several portions, were added. After standing for about 10 min it was heated until it became perfectly clear. When it had reached room temperature, the solution was transferred to a Kohlrausch flask and filled to 100 ml with water. This was then called the "sample solution".

1.3.2. Determination of NH_4^+ nitrogen with KOBr solution and biamperometric end-point indication. Subsequent to digestion, 5.00 ml of the sample solution was measured into the titration flask, 25 ml of the $KHCO_3$ solution were slowly added, then the inner wall of the flask was washed down with 25 ml water. During titration with the KOBr solution intensive stirring was

applied. The polarizing potential was 400 mV, the sensitivity of the measurement of current intensity was $6 \cdot 10^{-8}$ A mm^{-1}. The end point was indicated by the sudden lasting increase of intensity of current: graphical evaluation was not necessary. The $(NH_2)_4SO_4$ standard solution was titrated and the blank determined as described above.

The nitrogen content of the sample:

$$N(\%) = 2\,\frac{a - v}{b}$$

where a = amount (ml) of KOBr solution used for 5.00 ml of the sample, b = = amount of KOBr solution (ml) equivalent to 5.00 ml standard $(NH_4)_2SO_4$, v = blank value ml of KOBr solution used for 5.00 ml of "blank" solution.

1.4. Determination of nitrogen content by the Kjeldahl-Winkler method

1.4.1. Digestion. 1.00 g of the ground sample was digested in 25 ml conc. H_2SO_4, containing $CuSO_4$ and K_2SO_4.

1.4.2. Determination of NH_4^+-nitrogen after separation by distillation. The distilled NH_3 was absorbed in H_3BO_3 solution as recommended by WINKLER (1913). A slight deviation from the original procedure manifested itself in the use of methyl orange-indigo carmine indicator.

If the nitrogen content to be expected exceeded 3% the sulfuric acid-containing solution obtained by digestion was transferred to a Kohlrausch flask and made up to 100 ml with water. Depending on the nitrogen content to be expected, distillation was carried out, from 50.00- or 25.00-ml aliquots. (In the experience of the author limitation of the amount of NH_3 to be distilled is expedient, because thus the loss upon distillation may be neglected.)

2. Results and conclusions

2.1. Study of the blank value in the biamperometric method

In order to be able to decide whether the blank value is dependent on the digestion period after the decomposition of hydrogen peroxide the heating of the sulfuric acid solution ("digestion") containing the catalyst was continued during different periods (Table 1).

As shown in the Table, the blank value is independent of the period of heating in the range studied.

The question of the dependence of the blank value (v [ml]) on the quantity of catalyst was also investigated. In the related five experiments various amounts of the catalyst solution (k [ml]) were added in the range of 5 to 25 ml.

Table 1

Test of the stability of the blank value in biamperometry

For the procedure see para. 1.3.

Heating period of H_2SO_4 containing H_2SeO_3 min	~0.05 *N* KOBr solution ml
20	0.50
40	0.47
60	0.49
180	0.50

On the basis of results the following regression equation was formulated:

$$v = 0.0997\ k + 0.0085$$

The correlation coefficient was found to be 0.9997. Since the additive constant in the equation is very low, it was proven that the value of the blank depends largely upon the quantity of catalyst. (The regression equation is not of general validity, because the value of the additive constant included is a function of the impurities in the reagents.)

2.2. Reliability of the biamperometric method for the determination of NH_4^+-nitrogen in the solution

The NH_4^+-nitrogen content of 100 animal feeding stuff samples was studied in parallel experiments by the biamperometric (B) and the distillation (D) methods in the solution prepared according to para. 1.3.1. One determination was made of each solution. Of the samples studied 45 were mixed feeds, 19 alfalfa, 15 grain, 9 soy flour and 7 of different composition. The nitrogen content of the samples after digestion with sulfuric acid was found to be in the range of 1.2–11.2 %. The arithmetic mean of the results obtained by method B was $\bar{y} = 3.9431\,\%$ and of those by method D $\bar{x} = 3.9400\,\%\ N$.

The null hypothesis H_0: $M(z) = 0$

where the probability variable $z = y - x$. The mean value of y_i–x_i differences: $\bar{z} = 0.0031$

$$\text{The variance: } s^2 = \frac{\sum_{i=1}^{100} (z_i - \bar{z})^2}{99} = 0.002835$$

To test H_0 the single-sample t test was used.

$$t = \frac{\bar{z}}{s} \cdot \sqrt{100} = \frac{0.0031}{0.05325} \cdot 10 = 0.58$$

The parameter of t, $n-1 = 99$. To the latter at 50 % probability a tabular value of 0.677 belongs (FISCHER & YATES, 1963), thus the null hypothesis may not be rejected even on the basis of a statistical test much more severe than the customary (5 % probability level).

Thereafter parallel determinations were carried out by both methods (B, D) with three solutions of different NH_4^+ content and the deviations as well as the mean values were compared. In the former case the F test, in the latter the t test was applied. Where deviations proved to be significantly different the so-called t_f statistics of *Welch* was applied (VINCZE, 1975).

From the parallel samples each 5 g were digested in 750-ml Kjeldahl flasks, the solutions were then transferred into volumetric flasks with water and made up to 500 ml. In this experiment the reagent used was five times that used in the process according to para. 1.3.1 (Table 2).

Table 2

Determination of the nitrogen content of animal feeding stuffs after digestion with sulfuric acid

For all the experiments the solution prepared after modified digestion (para. 1.3.1) was used

	Corn grain		Alfalfa flour		Fish flour	
	Method of analysis					
	B	D	B	D	B	D
n	5	5	5	5	7	7
s	0.011	0.019	0.012	0.032	0.016	0.0695
v(%)	0.80	1.39	0.35	0.93	0.14	0.62
F	$2.98 < F_{0.10}$		$6.83 > F_{0.05}$		$19.35 > F_{0.005}$	
$\bar{y}$ or $\bar{x}$	1.378	1.370	3.430	3.430	11.140	11.200
$\bar{y} - \bar{x}$	0.008		0.000		−0.060	
$\frac{\bar{y} - \bar{x}}{\bar{x}} \cdot 100$	0.58		0.00		−0.54	
t	$0.82 < t_{0.40}$		0.00		$\lvert -2.23 \rvert < t_{0.05}$	

B = biamperometric titration with KOBr solution
D = distillation method with boric acid solution for absorption
n = number of determinations
s = standard deviation (N %)
v = coefficient of variation
$\bar{y}$ = arithmetic mean of results (N %) obtained with method B
$\bar{x}$ = arithmetic mean of results (N %) obtained with method D
' = result of t-test

Conclusions drawn from data summarized in Table 2:

a) The scatter of the results obtained by biamperometric titration was in every case lower than that of the results obtained by the distillation technique. Though on the basis of the F test the difference in the standard deviations with corn samples of relatively low nitrogen content was not found significant even at the 10 % probability level, with alfalfa flour it was significant already at the 5 % probability level. With fish flour of high nitrogen content the difference is significant at the 0.5 % probability level.

b) The standard deviation increased with increasing nitrogen content in the case of both methods, however, the extent of increase is more moderate with the biamperometric method.

c) For both methods, the coefficient of variation is inversely related to the nitrogen content.

d) The results of the biamperometric method show that the coefficient of variation does not reach 1 % even in the least advantageous case.

e) In every case, the absolute deviation between corresponding mean values remained below 0.1 % and the relative difference below 1 %. Deviation is not significant in either case.

On the basis of 100 pairs of data regression test was also performed. The result in equations is as follows:

$$y = 0.9992\,x + 0.0062$$
$$x = 0.9708\,y + 0.1120$$

The correlation between the variables may be characterized by a correlation coefficient of

$$r = 0.9849.$$

2.3. *Reliability of the complete process of nitrogen determination by the method developed*

The results as described above related only to the determination of the NH_4^+-nitrogen content of the solution arrived at by the modified digestion technique (para. 1.3.1). However, it seemed desirable to check the complete procedure (B_c; para. 1.3) including digestion, since the possibility may not be excluded that the combined application of H_2SeO_3 and H_2O_2 may cause the loss of nitrogen or be the source of other error. Control tests were carried out by the Kjeldahl-Winkler technique (D_c; para. 1.4).

Results summarized in Table 3 permit in several respects conclusions similar to those in Table 2. The most important of these are:

a) With the method proposed the standard deviations as well as coefficients of variation were lower than with the method used for comparison. (How-

Table 3

Reliability of the complete procedure as proposed for the determination of nitrogen in animal feeds

	Wheat grain		Alfalfa flour		Soy flour	
	Method of analysis					
	B_c	D_c	B_c	D_c	B_c	D_c
n	5	5	5	5	4	4
s	0.009	0.018	0.017	0.030	0.018	0.045
$v(\%)$	0.445	0.89	0.52	0.90	0.24	0.59
F	$4.00 < F_{0.10}$		$3.11 < F_{0.10}$		$6.25 < F_{0.05}$	
$\bar{y}_c$ or $\bar{x}_c$	2.022	2.014	3.288	3.320	7.650	7.6225
$\bar{y}_c - \bar{x}_c$	0.008		−0.032		0.0275	
$\frac{\bar{y}_c - \bar{x}_c}{\bar{x}_c} \cdot 100$	0.40		−0.96		0.36	
t	$0.88 < t_{0.40}$		$2.08 < t_{0.05}$		$1.15 < t_{0.2}$	

B_c = the "complete" method proposed (para. 1.3)
D_c = "complete" *Kjeldahl-Winkler* method (para. 1.4)
n = number of determinations
s = standard deviation (N%)
v = coefficient of variation
$\bar{y}_c$ = arithmetic mean of the results obtained with method B_c (N%)
$\bar{x}_c$ = arithmetic mean of the results obtained with method D_c (N%)
t = result of t-test

ever, the difference in the corresponding deviations could not be considered significant on the basis of the F test.)

b) The difference between corresponding mean values is not significant at 5% probability in either case.

It is of interest to note that the deviations seen in Table 3 are very near to those in Table 2, though in procedures B_c and D_c the number of sources of error is higher than in procedures B and D (weighing in, digestion, preparation of the solution). One of the reasons for this may be found probably in the fact that the complete procedure was checked by persons more experienced than those performing the earlier experiments (Table 2).

Final conclusion: the biamperometric method is quicker and more precise than the *Kjeldahl-Winkler* method while its accuracy is practically equal to that of the latter.

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CORROSION OF STEEL AND ALUMINIUM CONSTRUCTION MATERIALS IN DIFFERENT FOOD MEDIA

PART II. — ALCOHOLIC BEVERAGES

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It was found that, like in non-alcoholic foods, the extent and rate of corrosion in alcoholic beverages depended very much on the period of exposure. During the first 30 days changes were extensive and rapid, while later the process slowed down.

Beside the changes in the metals, the changes occurring in the alcoholic beverages, used as the aggressive medium, were studied. The iron and aluminium content of the beverages was measured and compared to the theoretical values obtained by calculation on the basis of weight loss of the specimens. The iron content was determined by three different methods and the correlation coefficients of the weight loss of specimens, on the one hand, and the iron content as established by the different methods on the other, was calculated. Apart from one instance the correlation between the weight loss of the carbon steel specimens and the iron content as established by different methods, was very close.

The correlation between the weight loss of aluminium specimens and the aluminium content of wines serving as aggressive media, was very close. In contrast to this, in beverages of higher alcohol content (cherry-brandy and white butter-pear liqueur) the correlation was not so close.

Thus it was established that for the evaluation of the corrosion of carbon steel and aluminium, the knowledge of the duration of exposure is needed in addition to the rate of corrosion because the latter is, among other things, a function of the time of exposure.

In the first part of this study (BÖRÖCZ-SZABÓ, 1976) the corrosion of steel and aluminium construction materials in non-alcoholic foods was discussed. In the present study, an account is given of the same materials in alcoholic beverages.

As it was stressed before, the knowledge of the corrosion of construction materials is of much greater importance in the food industry than in other industrial branches. Metal ions liberated in the course of corrosion contaminate products of the food industry intended for human consumption and may, at the same time reduce their sensory value. Iron and aluminium are not poisonous metals. Iron is an important constituent of the human organism. As regards aluminium, BÁCSKAI (1949) proved in a detailed study that it is not harmful for the human organism. However, more recent investigations have shown that the accumulation of aluminium in the body of haematotherma may lead to the disorderly functioning of certain organs (EVENSTEIN, 1971).

Aluminium is a construction material widely used in the food industries. In prolonged use, wine (KONLECHNER *et al.*, 1958) and fermenting beer (ELZE & MICKSCH, 1970), for instance, may cause severe corrosion in aluminium. ESCHNAUER (1964) studied the amount of aluminium dissolving in wine during

treatment and found that, in contrast to iron, which was easy to remove, it could be removed only by a complicated and expensive procedure. Therefore, it is important to prevent aluminium from contaminating the wine during treatment.

EVENSTEIN (1971) studied the amount of aluminium dissolved in beer of different make in the course of processing. He found that various metals contaminating the aluminium accelerate the rate of corrosion, therefore he suggests the use of high-grade aluminium in manufacturing equipment for beer processing.

The aim of this study was to follow the changes with time in the given construction material as caused by corrosion in the medium used. The correlation between the metal content of the beverages applied as aggressive media and the weight loss of specimens was also investigated.

1. Materials and methods

1.1. Materials

1.1.1. Test specimens. The test specimens were prepared of unalloyed carbon steel plate S3F (HUNGARIAN STANDARD, 1971) and of Al 99.5 foundry aluminium. The nominal thickness of the plates was 2 mm and the size of the specimens 50×100 mm.

1.1.2. Liquid food products used as aggressive media. The following liquid food materials and products were used:

Must: the juice, pressed in the laboratory from grape varieties *Sárfehér* and *Othello* was fermented.

Wine: A white wine *Izsáki Sárfehér* and a red wine *Egri bikavér* were used.

Beer: A light beer purchased on the market (*Kőbányai világos sör*, HUNGARIAN STANDARD, 1969) was used.

Brandy: Commercial cherry-brandy from the stores of the NATIONAL ENTERPRISE OF THE DISTILLING INDUSTRY (ORSZÁGOS SZESZIPARI VÁLLALAT), Budapest.

Liqueur: Commercial white butter-pear liqueur from the stores of the above enterprise.

96% ethyl alcohol (HUNGARIAN STANDARD, 1970).

1.2. Methods

1.2.1. Preparation of the test specimens. The surface of the carbon steel specimens was polished. The surface of the aluminium specimens was lightly roughened and defatted with a 3% solution of 60 °C of Alupon T. The plates were then weighed to the nearest tenth of a mg.

1.2.2. Exposure to corrosion. Five test specimens made of the same material were placed in a glass container, taking care to prevent contact between

specimens. The container was then filled with the selected liquid food product, covered (but not hermetically sealed) and stored at 0 to 5 °C. The number of containers with identical test specimens was equal to the number of inspections to be carried out. Thus, after the passing of a predetermined period of exposure the five specimens contained in the same container were inspected and weighed. Simultaneously the quantity of iron or aluminium dissolved in the aggressive medium was determined. Corrosion rate was expressed in g per m^2 per day (d).

1.2.3. Determination of the iron or aluminium content in the corrosive medium. The iron content of the medium stored with the carbon steel test specimens was determined after wet ashing with nitric acid and perchloric acid (SPANYÁR & KEVEI, 1961a) as a complex formed with α,α'dipiridyl (SPANYÁR & KEVEI, 1961b) or as a complex formed with o-phenantroline (HUNGARIAN STANDARD, 1973) spectrophotometrically. The iron content was further determined in the beverage by atomic absorption spectrometry by means of a *Perkin-Elmer* 290/B type apparatus.

The aluminium concentration in the beverage stored with the test specimens was determined after wet ashing with nitric acid and perchloric acid, as above, by complex formation with aurinetricarbonic acid (SPANYÁR & KEVEI, 1962).

1.2.4. Mathematical statistical evaluation of results. The means, the corrected standard deviations and the coefficients of variation of parallel results were calculated. The correlation between the weight loss of specimens and the iron or aluminium concentration in the respective media was expressed by the correlation coefficient and by calculating the regression line.

2. Results

2.1. Results obtained in the study of carbon steel and aluminium test specimens in wine and grape must

Figure 1 shows the weight loss of carbon steel and aluminium test specimens in white and red wine as a function of time. The surface–volume ratio applied in every case was 0.706 m^2m^{-3}.

Weight loss of the carbon steel and aluminium specimens in white and red grape must is also demonstrated in the figure. After 12 days the weight loss in must was substantially higher than in wine.

The corrosion rate of carbon steel and aluminium in white and red wine was plotted on the basis of the above data in Fig. 2.

Correlation between the weight loss of test specimens and the iron or aluminium concentration in white wine is illustrated in Fig. 3, while data in red wine are shown in Fig. 4.

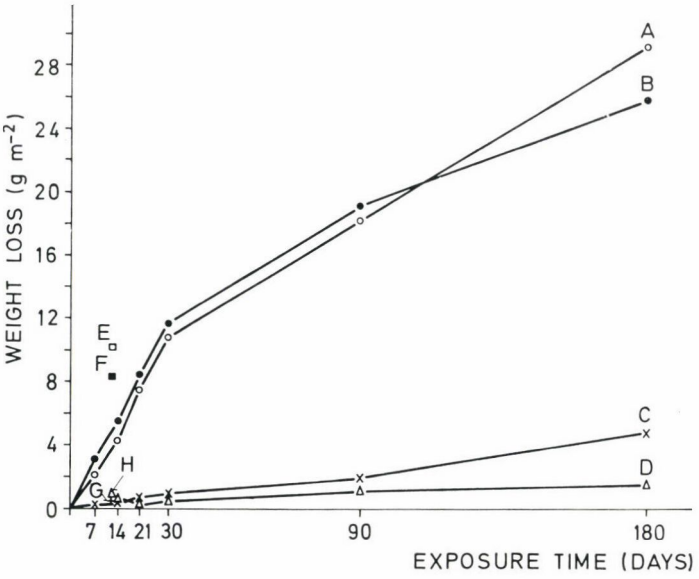

Fig. 1. Weight loss of carbon steel and aluminium test specimens in products of the wine industry as a function of exposure time. A: carbon steel in white wine; B: carbon steel in red wine; C: aluminium in white wine; D: aluminium in red wine; E: carbon steel in white must; F: carbon steel in red must; G: aluminium in white must; H: aluminium in red must

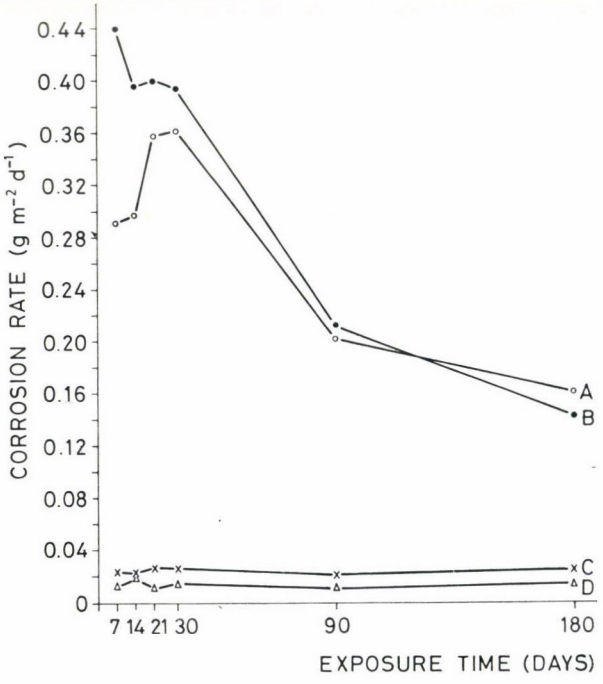

Fig. 2. Corrosion rate of carbon steel and aluminium specimens in products of the wine industry as a function of exposure time. A: carbon steel in white wine; B: carbon steel in red wine; C: aluminium in white wine; D: aluminium in red wine

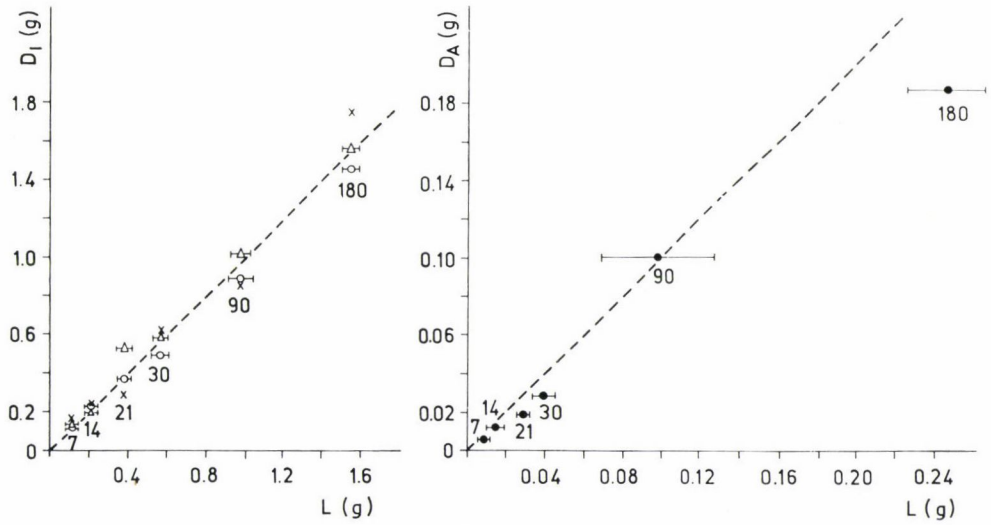

Fig. 3. Correlation between the total weight loss of carbon steel and aluminium specimens (L) and the total amount of iron or aluminium dissolved in white wine (D_I and D_A, resp.) as a function of the period of exposure. The dashed line represents the correlation to be expected on the basis of weight loss, the numbers indicate the exposure period in days, horizontal bars stand for the standard deviation. ⊢○⊣ iron content determined by the α,α'dipiridyl method ($D_I = 0.0002 + 0.9255L$; $r = 0.9993$) ⊢△⊣ iron content determined by the o-phenantroline method ($D_I = 0.0331 + 0.9963L$; $r = 0.9949$); —×— iron content determined by atomic absorption spectrometry ($D_I = 0.0542 + 1.0923L$; $r = 0.9851$); ⊢•⊣ the same data for aluminium ($D_A = 0.0024 + 0.7803$L; $r = 0.9915$)

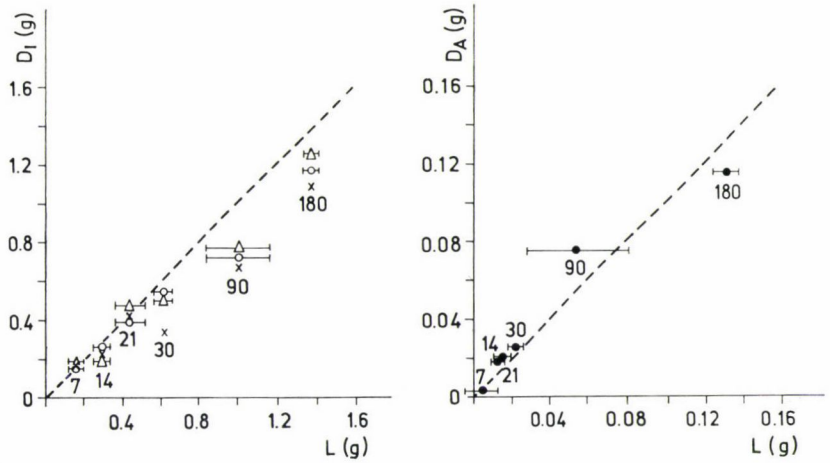

Fig. 4. Correlation between the total weight loss of carbon steel and aluminium specimens (L) and total amount of iron or aluminium (D_I and D_A, resp.) dissolved in red wine as a function of exposure period. The dashed line represents the correlation to be expected on the basis of weight loss, the numbers the exposure time in days and the horizontal bars the standard deviation. ⊢○⊣ iron content determined by the α, α'dipiridyl method ($D_I = 0.0229 - 0.7956L$; $r = 0.9878$); ⊢△⊣ iron content determined by the o-phenantroline method ($D_I = 0.0002 + 0.8612L$; $r = 0.9833$); —×— iron content determined by atomic absorption spectrometry ($D_I = 0.0222 + 0.7144L$; $r = 0.9724$); ⊢•⊣ the same data for aluminium ($D_A = 0.0089 + 0.8436L$; $r = 0.9494$)

A close correlation was found between the weight loss of carbon steel and aluminium test specimens on the one hand and the iron or aluminium concentration of the medium, on the other.

Due to corrosion of the carbon steel test specimens, the iron content of *Sárfehér* must increased from the initial value of 14.22 mg l^{-1} to 712.82 mg l^{-1} in the course of the 12 day fermentation period. The aluminium concentration increased under identical conditions from the initial 2.22 mg l^{-1} to 13.44 mg l^{-1}.

Corresponding values for *Othello* must were as follows: the initial iron content of 17.00 mg l^{-1} increased to 538.07 mg l^{-1}; the initial aluminium content of 3.27 mg l^{-1} increased to 12.03 mg l^{-1} by the end of the experiment.

2.2. *Results obtained in corrosion tests of carbon steel and aluminium specimens in beer*

Because of the limited storage stability of beer, corrosion of carbon steel and aluminium specimens in beer was studied in 10 cycles. Each exposure period lasted 7 days, the specimens were then washed in running water and dried in a hot air stream and finally weighed. The applied ratio of surface to volume was 0.757 m^2m^{-3}. Weight loss of the carbon steel specimens is illustrated as a function of the number of exposure cycles in Fig. 5, and the corrosion rate calculated on the basis of these data in Fig. 6.

The weight loss of the aluminium specimens under the experimental conditions and within the given exposure periods was too slight to be suitable for evaluation.

2.3. *Results of corrosion tests of carbon steel and aluminium specimens in products of the distilling industry*

Carbon steel and aluminium specimens were exposed to cherry-brandy, white butter-pear liqueur and 96 % alcohol. A surface–volume ratio of 0.706 m^2m^{-3} was applied.

Weight loss of the carbon steel specimens is shown as a function of time in Fig. 7. Similarly, the rate of corrosion is plotted as a function of time in Fig. 8.

Weight loss of the aluminium specimens versus time is illustrated in Fig. 9 and the corrosion rate *vs.* time in Fig. 10.

The weight loss of aluminium test specimens in 96 % alcohol was too low to be evaluated.

At the end of the test period and the evaluation of the specimens the iron and aluminium concentration in the medium was determined. The weight loss of the test specimens related to the iron or aluminium concentration in the medium in cherry-brandy is illustrated in Fig. 11 and in white butter-pear liqueur in Fig. 12.

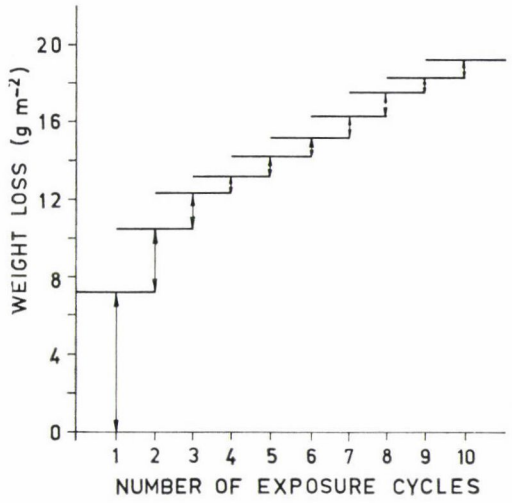

Fig. 5. Weight loss of carbon steel specimens in beer during 10 cycles as a function of time

A close correlation was found between the weight loss of carbon steel specimens and the iron concentration in cherry-brandy.

As it may be seen in Fig. 11, the iron content as determined by atomic absorption spectrometry rather differed from that calculated from the theoretical line based on weight loss data, however, the correlation coefficient indicates close correlation. Indeed, the determined values are located around a straight line. Nevertheless, this line deviates considerably from the line calculated on the basis of weight loss.

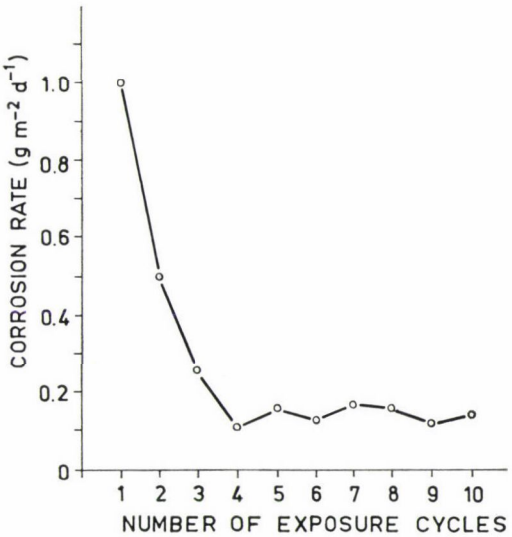

Fig. 6. Corrosion rate of carbon steel specimens in beer as a function of exposure time

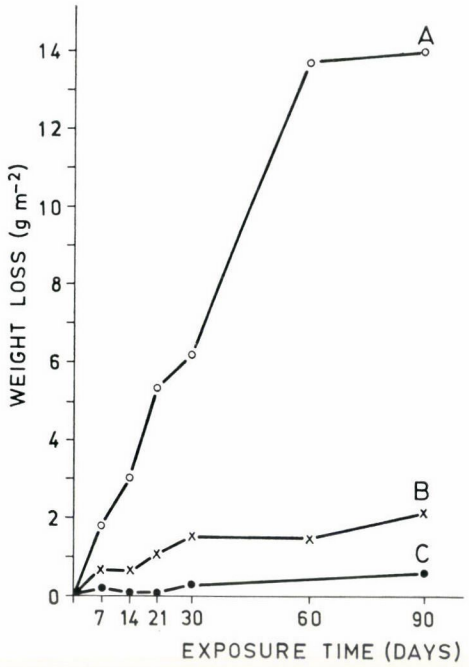

Fig. 7. Weight loss of carbon steel specimens in cherry-brandy, pear liqueur and 96% ethyl alcohol as a function of time. A: in cherry-brandy; B: in pear liqueur; C: in 96% alcohol

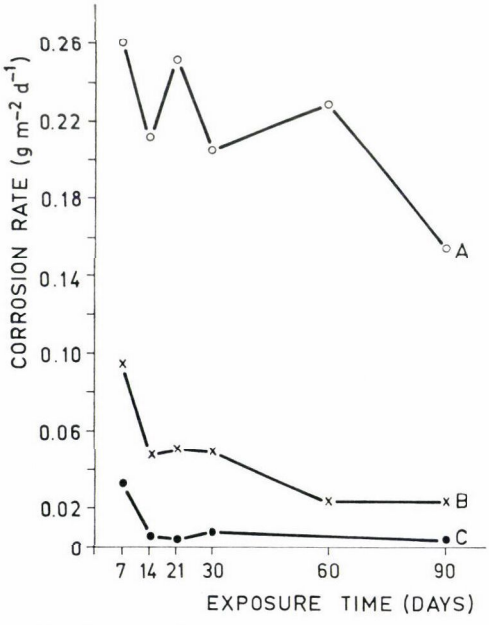

Fig. 8. Corrosion rate of carbon steel specimens in cherry-brandy, pear liqueur and 96% ethyl alcohol as a function of time. A: in cherry-brandy; B: in pear liqueur; C: in 96% alcohol

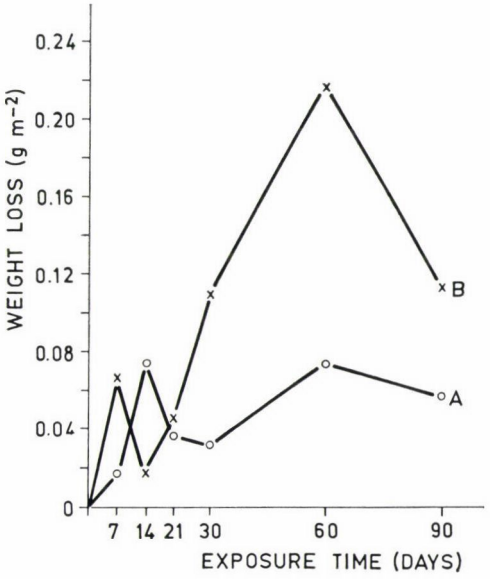

Fig. 9. Weight loss of aluminium specimens in cherry-brandy and pear liqueur as a function of time. A: in cherry-brandy; B: in pear liqueur

The correlation coefficient characterizing the relation between the weight loss of aluminium test specimens and the aluminium content in the corrosive medium was of a very low value. This is shown also by the considerable deviations between values as seen in Fig. 11.

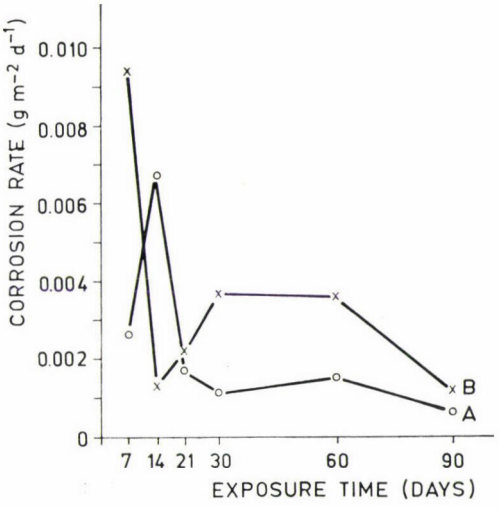

Fig. 10. Corrosion rate of aluminium specimens in cherry-brandy and pear liqueur. A: in cherry-brandy; B: in pear liqueur

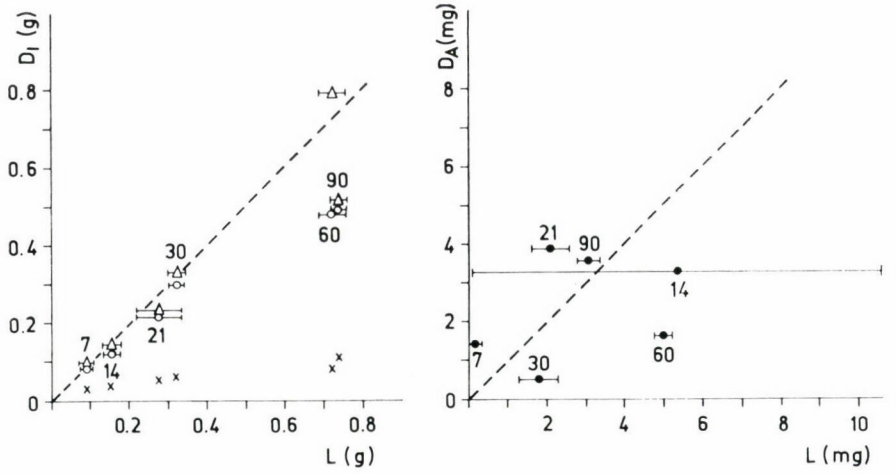

Fig. 11. Correlation between the total weight loss of carbon steel and aluminium specimens (L) and the total amount of iron or aluminium dissolved in the brandy (D_I and D_A, resp.) as a function of time. The dashed line represents the correlation to be expected on the basis of weight loss, the numbers the time of exposure (days) and the horizontal bars the standard deviation. ⊢○⊣ iron content determined by the α, α'dipiridyl method ($D_I = 0.0502 + 0.5962L$; $r = 0.9818$); ⊢△⊣ iron content determined by the o-phenantroline method ($D_I = 0.0094 + 0.8836L$; $r = 0.9330$); — × — iron content determined by atomic absorption spectrometry ($D_I = 0.0195 + 0.1088L$; $r = 0.9858$); ⊢•⊣ the same data for aluminium ($D_A = 0.0017 + 0.2205L$; $r = 0.3152$)

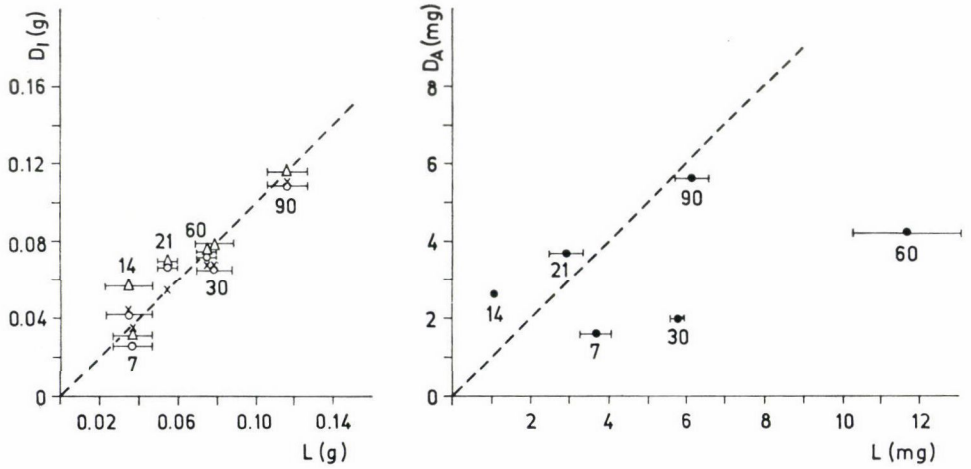

Fig. 12. Correlation between the total weight loss of carbon steel and aluminium specimens (L) and the total amount of iron or aluminium dissolved in white butter pear liqueur (D_I and D_A, resp.) as a function of time. The dashed line represents the correlation to be expected on the basis of weight loss, the numbers the time of exposure (days) and the horizontal bars the standard deviation. ⊢○⊣ iron content determined by the α,α'dipiridyl method ($D_I = 0.0069 + 0.8598L$; $r = 0.8598$); ⊢△⊣ iron content determined by the o-phenantroline method ($D_I = 0.0138 + 0.8598L$; $r = 0.9280$); — × — iron content determined by atomic absorption spectrometry ($D_I = 0.0051 + 0.8542L$; $r = 9822$); ⊢•⊣ the same data for aluminium ($D_A = 0.0024 + 0.178L$; $r = 0.4244$)

The correlation between the weight loss of carbon steel specimens and the iron concentration in white butter-pear liqueur was found to be close.

No correlation was found between the weight loss of aluminium specimens and the aluminium concentration in white butter-pear liqueur, as can be seen in Fig. 12.

3. Conclusions

The corrosion of carbon steel and aluminium specimens was studied in alcoholic beverages. Under the experimental conditions applied (stationary medium, storage temperature) results obtained are characteristic only of storage containers.

The weight loss of carbon steel in both white and red wine gave a very steep line in the first 30 days of storage. After this period the curve became less steep but weight loss still increased substantially. Thus, although the rate of corrosion decreased after 30 days, it did not approach a limiting value even after 180 days of exposure.

Earlier studies indicated the desirability of determining the iron concentration in the aggressive medium by several methods. A good agreement was found between the iron concentrations as determined by the methods given in para. 1.2.3. According to statistical calculations the correlation coefficients point to a close correlation between the weight loss of the specimens and the iron content in the corrosive medium.

The weight loss of aluminium test specimens in white and red wine is about one order of magnitude lower than that of carbon steel specimens. During the 180-day experimental period, the weight loss increases linearly, thus the corrosion rate after 30 days may be considered constant.

Statistical calculations showed that the correlation between weight loss and the aluminium content of the medium was close.

During identical periods the extent of corrosion of both structural materials was higher in grape juice and fermenting must than in wine. Corrosion was intensified by the fermentation process and by CO_2 formation.

In beer the corrosion of carbon steel and aluminium test specimens could be studied only by cycle tests. As can be seen in Fig. 5, during the first three cycles the corrosion of the carbon steel specimens was more intense, while during the subsequent cycles the average weight loss was nearly identical. This may be seen in the corrosion rate curve plotted in Fig. 6.

Weight loss of the aluminium specimens under the prevailing conditions was too low to be evaluated. To obtain weight losses suitable for evaluation aluminium specimens of larger surface and longer periods of exposure should be applied.

Weight loss of carbon steel specimens in commercial cherry-brandy rose rather steeply during the first 60 days, after this, with a sudden change in slope, a very slight loss is observed. The shape of the corrosion rate curve indicates a decreasing tendency, yet this cannot be established unambiguously. A longer experimental period would have served with a more complete picture of the process. This was, however, not projected, because exposure periods longer than 90 days do not occur in practice.

The weight of the carbon steel specimens in pear liqueur was much lower than in brandy. This is due probably to the high sugar concentration. Corrosion follows a decreasing curve approaching a limit value.

As a function of time the weight loss of carbon steel specimens in 96% ethyl alcohol is similar to the curve in liqueur, only of an even lower numerical value. This is probably a consequence of the low moisture content.

The weight loss of aluminium test specimens in cherry-brandy and pear liqueur followed a curve similar to that obtained in non-alcoholic liquid media (sour cherry juice, brines). However, it was more rapid in the liqueur of lower alcohol concentration and higher sugar content than in brandy. The same was true for the corrosion rate. This phenomenon may be traced back probably to the corrosion mechanism of aluminium.

The correlation between the weight loss of carbon steel specimens and the iron dissolved in media of high alcohol concentration may be considered satisfactory as shown by the correlation coefficients in the figures. An interesting phenomenon was observed when the iron concentration was determined by atomic absorption spectrometry in cherry-brandy. The values obtained followed a straight line but this was not identical with the line obtained by calculation on the basis of the weight loss of the specimens. Further investigations on this subject seem necessary.

No correlation was found between the weight loss of aluminium specimens and the aluminium concentration in the media either by plotting (Figs. 11 and 12), or by calculation. Similar observations were made earlier, therefore, it seems desirable to examine this point further.

*

Thanks are due to Ms. G. Szabó, Research Institute for Viticulture and Enology, Head of the laboratory and Mr. G. Sárosi, research assistant for the performance of the atomic absorption measurements and to Ms. K. Liktor-Kudron and Ms. É. Zsarnay for their devoted technical assistance.

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APPLICATION OF ENDO-POLYGALACTURONASE TO VEGETABLES AND FRUITS

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Possibilities of application of an endo-polygalacturonase preparation to 18 vegetables, 11 fruits, 2 varieties of tomatoes were tested, with special regard to tomato varieties developed for mechanical harvesting.

The effect of enzymatic disintegration differed from vegetable to vegetable. Highest recovery of cells and tissue particles was obtained in the case of potato, carrot, parsley, cucumber, squash and red tomato-shaped paprika while the enzymatic disintegration was least effective in the case of garden sorrel and spinach.

After enzyme treatment the larger part of the vegetables consisted of single cells, suitable for further processing into vegetable juices, cocktails, baby foods, dehydrated soups and sauces.

The condensability of the juice of tomato varieties bred for mechanical harvesting could be improved by enzyme treatment, making it possible to produce tomato concentrates of 38–45% dry matter content. In the course of enzyme treatment the loss upon straining decreased by 20% while the juice yield increased to the same extent. The dry matter content of the enzyme-treated samples also increased by 7% as compared to the control.

Endo-polygalacturonase treatment was similarly succesful in the case of fruits. The increase in the juice yield was about 20–50% higher with the majority of fruits tested than that of the control. A simultaneous significant decrease in viscosity and increase in light transmittance of the samples was observed. The colour of the fruit juices became more intensive as a consequence of the enzyme treatment.

The FERMENTATION DEPARTMENT of the CENTRAL FOOD RESEARCH INSTITUTE, has been engaged in the production of endo-polygalacturonase by submerged fermentation.

As the enzyme preparation produced did not contain pectin methyl esterase, or pectin transeliminase and consisted mainly of endo-polygalacturonase, the application experiments aroused special interest.

The first enzyme treatments were carried out with crushed apples from which conclusions as to further possibilities of application could be drawn. The enzyme treatment resulted in a very smooth cream with good consistency (Fig. 1) which, according to microscopic examinations, consisted mainly of single cells (Fig. 2).

This result determined the trend of our further application experiments, leading to the assumption that disintegration of vegetable tissues with endo-polygalacturonase would make it possible to produce vegetable creams and juices consisting mostly of single cells.

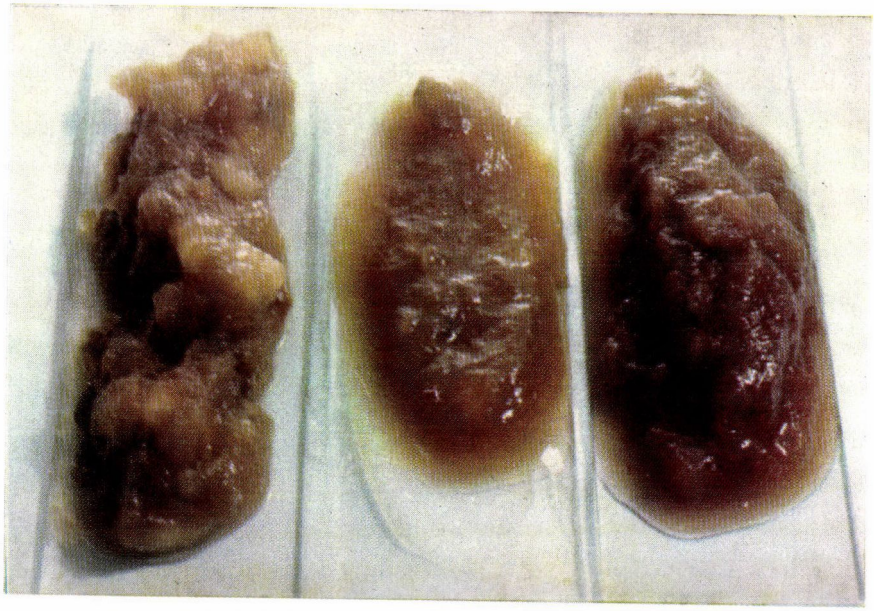


Fig. 1. Crushed apples of the *Jonathan* variety (left side) were treated with 0.5% endo-PG (right side) and 0.5% apple-juice clarifying-PG (*Phylazyme*, in the middle). Incubation: 3 h at 40 °C the pH (3.8 of the apples) was not adjusted

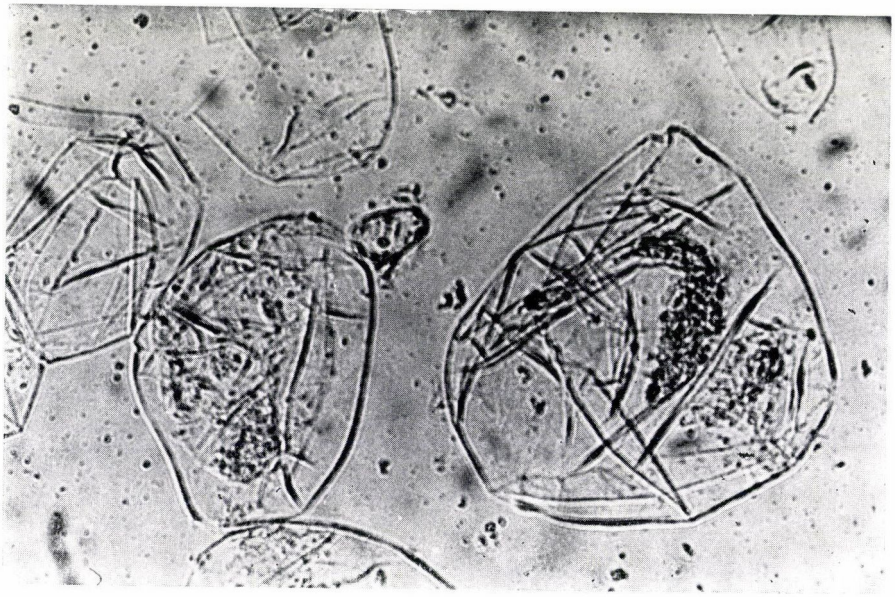


Fig. 2. Photomicrograph of the endo-PG-treated apple cream shown in Fig. 1. (Magnification: × 95)

1. Materials and methods

1.1. Enzyme preparation

Endo-polygalacturonase (endo-PG) (pilot plant product of the CENTRAL FOOD RESEARCH INSTITUTE, Budapest) of *Asp. awamori* was used in the present work, having an activity of 4000 l h^{-1} g^{-1}, determined viscosimetrically in a substrate of sodium polypectate incubated at 50 °C, for 60 min (ZETELAKI & VAS, 1972).

1.2. Variety of fruits and vegetables

The majority of the vegetables and fruits has been bought on the market where it was very seldom possible to identify the varieties.

In the case of tomatoes, varieties of *K 3* (*Kecskemét 3;* a heterosis variety which is not suitable for mechanical harvesting) and *Peto Mec* (bred for mechanical harvesting) were used.

1.3. Preparation of the fruits and vegetables for enzyme treatment and evaluation of the effects

1.3.1. Preparation and enzyme treatment of the vegetables. Vegetables were grated or cut into pieces of 5 × 15 mm. Ten-g quantities of the grated vegetables were placed into 250-ml Erlenmeyer flasks, 25 ml *McIlvaine* buffer, containing 0.5 g enzyme, were added to each and the reaction mixtures were incubated on a shaker (130 strokes min^{-1}) at 50 °C for 3 h.

The effect of pH was tested within a pH range of 3.0–7.0 at intervals of pH 0.5.

After the enzyme treatment, reaction mixtures were poured through a set of screens (mesh: first 1.0 and then 0.25 mm, resp.). Particles remaining on the screens were removed and dried at 105 °C to constant weight. Dry matter was determined together with the filtrate and the original tissue. The percentage of degradation into predominantly single cells and tissue particles (filtrate) and screen fractions larger than 0.25 and 1.0 mm, resp., was calculated from the dry weights.

pH values were considered optimal when their use resulted in the highest portion of the original tissues passing into the filtrate.

1.3.2. Preparation and enzyme treatment of tomatoes. In the case of the variety *K 3*, tomatoes were ground in a mincer and incubated at 15, 20 and 25 °C for 6 h. During incubation, samples were agitated at 100 rpm. Samples were taken every hour of the incubation period. After inactivating the enzyme at 80 °C for 15 min, samples were strained twice. The juice yield, loss during

straining, viscosity and dry matter content of the samples were measured in each case. Following that, samples were concentrated in a *Rotadest* evaporator (KUTESZ, Hungary) for 15 min, at 60 °C and a vacuum of 600 Torr.

After a 15 minute evaporation period, samples were evaluated according to their volume, dry matter content, dry matter yield and viscosity.

At the end of the incubation period, samples were evaporated to the highest technically possible concentration (final evaporation).

In the case of endo-PG treatment of tomatoes bred for mechanical harvesting (variety: *Peto Mec*), a temperature of 15 °C and an incubation period of 4 h were used. After the 4 h incubation period and enzyme inactivation, the strained samples were evaporated for 100 min.

From both tomato concentrates (highly concentrated and concentrated for 100 min), weight, dry matter content, dry matter yield, consistency, fibre content and colour were measured.

1.3.3. Evaluation of enzyme treatment of tomatoes

1.3.3.1. Dry matter yield. – Dry matter yield was obtained by multiplying the mass of the concentrate by its dry matter content (%, w/w) and dividing this by the product of the mass and of the dry matter content of the tomato juice after enzyme treatment. Samples were taken after 15 and 100 min of concentration as well as after final evaporation.

1.3.3.2. Relative viscosity. – Flow times of the samples were determined in an *Ostwald* type viscosimeter and related to that of water. Viscosity was measured three times from each sample at 20 °C.

1.3.3.3. Examination of consistency. – The consistency of the tomato concentrates was tested by spreading of a certain amount of sample in an *Adams* type consistometer (VAS & FÁBRI, 1958). In the course of the experiments, when the optimal parameters for enzyme treatment were determined, the method had to be modified as follows: 1. The dimensions of the hollow truncated cone used for the spreading test were so chosen as to take a sample quantity of exactly 50 g. 2. Before the spreading test, samples were diluted to the same dry matter content (38 %) with distilled water. Spreading tests were repeated six times with each sample.

1.3.3.4. Colour determination. – The colour of the samples was determined in the RESEARCH INSTITUTE OF THE CANNING and PAPRIKA PROCESSING INDUSTRY (Budapest) by measuring their dominant wavelengths (λ_D) with the *Momcolor* apparatus (MOM, Budapest).

1.3.3.5. Fibre content. – The wet fibre content of the samples was determined after centrifuging at 9000 rpm for 15 min. The fibre content was related to the wet weight of the whole sample.

1.3.4. Preparation and enzyme treatment of the fruit. 100 g of fruits, cut into pieces of 10–20 mm were placed into 500 ml Erlenmeyer flasks. Various concentrations of endo-PG dissolved in 10 ml of distilled water were added to each flask, while to samples serving as the control only 10 ml distilled water was given.

Samples were incubated at 40 °C on a rotary shaker (330 rpm) for 1.5 and 3.0 hours. At the end of the incubation period, the enzyme was inactivated at 80 °C for 15 min, and the samples were centrifuged at 2800 rpm.

To study the effect of enzyme treatment, the juice yield, the specific viscosity and optical transmittance of the juices were determined.

1.3.5. Evaluation of enzyme treatment of fruits

1.3.5.1. Juice yield. – The 500-ml tubes of a centrifuge were separated into two compartments by a perforated stainless steel sheet. The enzyme-treated fruit mash was poured into small rugs (made of nylon cloth) and placed on the top of the perforated steel sheet. The clear juice separated into the lower part of the tubes could be easily removed and measured. The juice yield was determined after 20 min centrifuging at 2800 rpm.

1.3.5.2. Specific viscosity. – Specific viscosity was determined in an Ostwald type viscosimeter. The calculation was carried out according to the following equation:

$$\eta_{spec} = \frac{t_{sample}}{t_{water}} - 1$$

where:

t_{sample} = flow time (s) of the fruit samples

t_{water} = flow time (s) of the water

1.3.5.3. Optical transmittance. – Optical transmittance of the samples was measured in *Specord UV–VIS* spectrophotometer (Carl Zeiss, Jena) at 660 nm.

1.3.6. General methods of evaluation

1.3.6.1. Degree of disintegration. – The degree of disintegration was calculated from the dry matter content of the filtrate, as percentage of the dry matter content of the vegetable tissues treated with endo-PG. Dry matter of the various fractions was determined after drying to constant weight in an oven at 105 °C.

1.3.6.2. Tasting test. – In the course of the tasting test the maximum of score sums was 30, 10 scores each for colour, odour and taste of the samples. The number of panelists carrying out the above test was 20.

2. Results

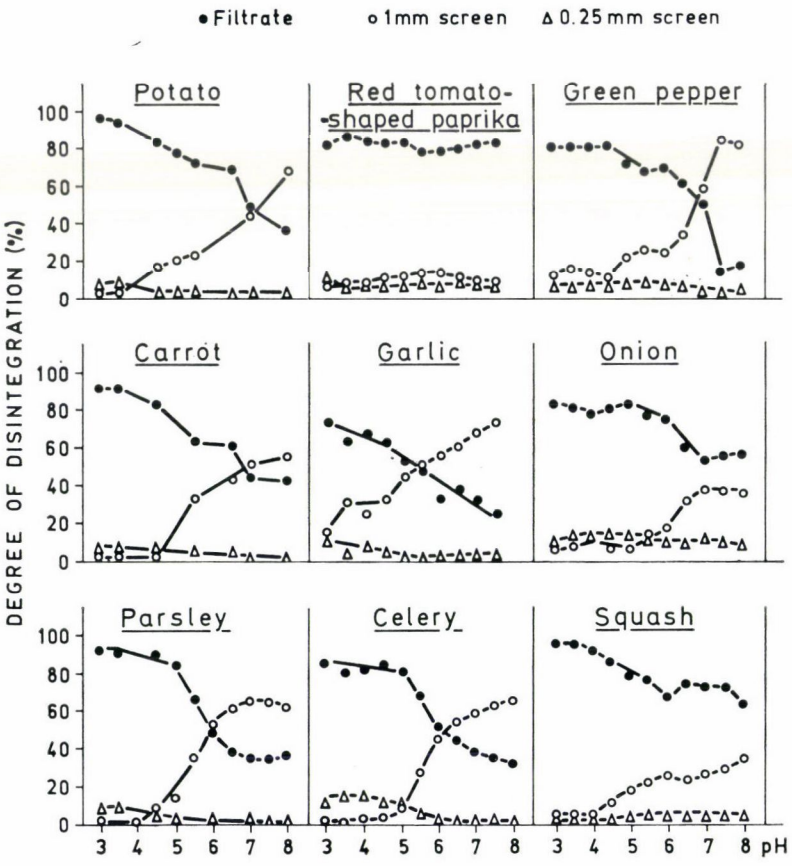
2.1. Disintegration of vegetables by endo-polygalacturonase

pH optima of the disintegrating effect of endo-polygalacturonase were determined with 18 kinds of vegetables (root, bulb, leguminous and leafy vegetables). The optima for enzyme concentration, the time and temperature of the incubation were determined in a previous study.

This paper gives an account of the experiments the purpose of which was to determine the optimal pH for the enzyme treatment.

The fractions (filtrate, 1 mm screen, 0.25 mm screen) of the tissues of potatoes, red tomato-shaped pa*pri*ka, green pepper, carrots, garlic, onions, parsley, celery and squash, are given in Fig. 3 as a function of pH.

The pH optimum of the enzyme treatment proved to be pH 3.0–3.5 for the majority of the vegetables shown in Fig. 3. Some of the vegetables (green



Figs. 3 and 4. Distribution of the fractions after enzymatic disintegration of several vegetables as a function of the pH. (Endo-PG: 0.5%; incubation: 3 h at 50 °C)

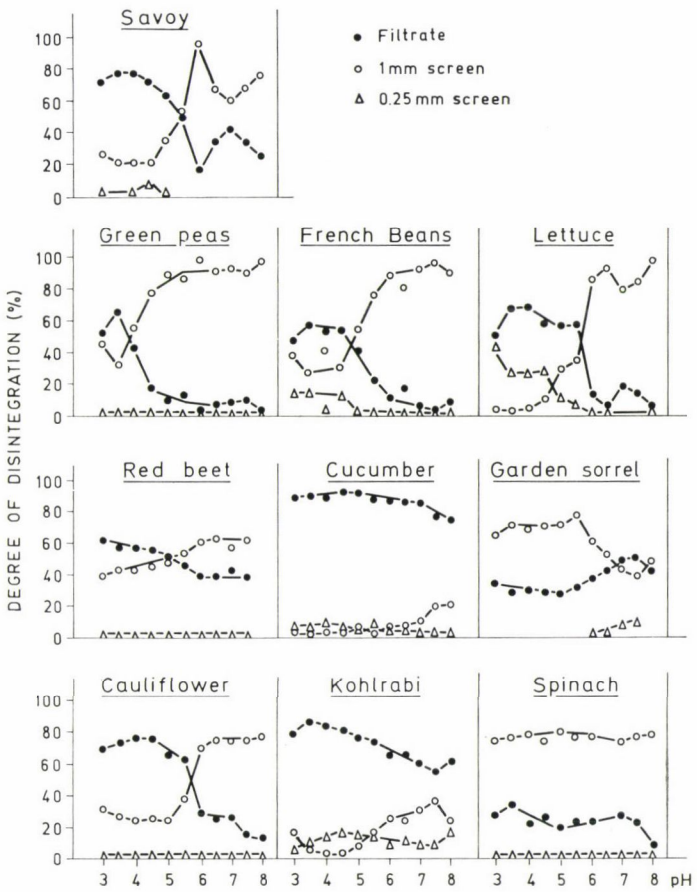
pepper, onions and celery) could be disintegrated succesfully in a wider pH range.

With the exception of red tomato-shaped paprika the increase in pH resulted in an increase of particles larger than 1.0 mm in the case of all the vegetables shown in Fig. 3. The weight of the 0.25 mm fractions was found to be the highest at the pH optimum and near to its pH value.

The enzymatic decomposition of the above vegetables was found to be between 80 and 98 % with the exception of garlic which could be disintegrated only to 72 %.

Fractions obtained after the enzymatic (endo-PG) disintegration of savoy, green peas, French beans, lettuce, beetroot, cucumber, garden sorrel, cauliflower, kohlrabi and spinach tissues, are given in Fig. 4.

The disintegration by endo-polygalacturonase was the least pH dependent when cucumber tissues were used as the substrate. Optimal efficiency was obtained at pH 3.0–3.5 with a recovery higher than 90 per cent.



The pH optimum for the enzymatic disintegration of green peas, lettuce, kohlrabi and spinach proved to be pH 3.5, and 3.0 for beetroot, but in the case of savoy and cauliflower a pH optimum of 4.0 was found. In contrast to the other vegetables, two pH optima were found (pH 4.5 and 7.0) when French beans were treated with endo-polygalacturonase. The enzymatic decomposition of garden sorrel was most efficient at pH 6.5.

The recovery of garden sorrel and spinach was very low (the dry matter content of the filtrate was about 50%) after the endo-PG treatment.

Photographs of the fractions of various vegetables, before and after enzyme treatment, are given in Figs. 5 and 6.

2.1.1. Vegetable juices and cocktails. Vegetables, the enzymatic disintegration of which resulted in a recovery higher than 80–90%, and which possess good aroma, valuable nutritive components and vitamins, were tried for their use as components of vegetable cocktails. A number of various mixtures were prepared, some of which consisted of vegetables only, with or without spices, while others were mixed with fruits.

Some of the most popular mixtures of many tasting tests are shown in Fig. 7. The composition of the mixtures is represented in the upper part of the Figure, while the number of total scores given by the panelists (evaluating colour, odour and taste of the cocktails) can be seen in the lower part of the Figure.

According to the results of these tests, the taste of the vegetable juices prepared after enzyme treatment either did not differ from that of the untreated ones, or proved to be better in several cases.

2.1.2. Stability of vegetable juices. Vegetable juices were stored in a refrigerator at a temperature of 4–5 °C for several days. It was observed that cells and cell-agglomerates sedimented in the juice soon (1–2 days) after treatment. Several tests were made to prevent sedimentation.

Very good stability was obtained when endo-polygalacturonase treatment was combined with ultrasonication. When vegetable juices, prepared by enzymatic disintegration were treated with ultrasonics, opalescent suspensions were obtained, keeping their homogeneity for more than 24 weeks.

The homogeneity of the celery, pepper and carrot juices after endo-polygalacturonase treatment with and without ultrasonication is shown in Fig. 8, while the homogeneity of enzymatically prepared parsley juices with or without ultrasonic treatment can be seen in Fig. 9.

From the photographs it appears that vegetable juices treated with ultrasonics after enzymatic disintegration were stable for more than 4 months, while in those, disintegrated only with endo-polygalacturonase, the cells and tissue particles sedimented.

The stabilizing effect of the endo-polygalacturonase treatment was perfect only in the case of tomato juice. The untreated control samples spon-

Figs. 5 and 6. Fractions of enzymatically disintegrated vegetable tissues. First slides (from the left): untreated tissues prepared for enzymatic maceration, 2nd and 3rd slides: tissue residues after maceration retained on the 1.0 mm and 0.25 mm screens; test tube: filtrate, containing single cells and cell agglomerates. (Endo-PG: 0.5%; incubation: 3 h at 50 °C)

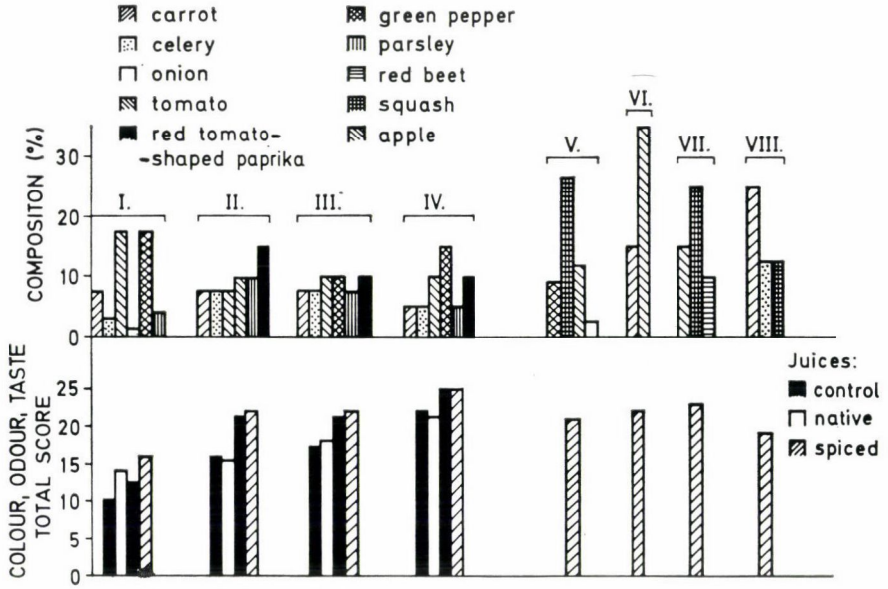


Fig. 7. Composition of the various vegetable cocktails and the scores assigned to the colour, odour and taste of the samples by the panelists. (Columns represent the average total scores of 20 panelists)

taneously separated into serum and fibrous parts, while both treated samples (endo-PG only and endo-PG + ultrasonics) were equally stable for more than 18 weeks (Fig. 10).

2.2. Endo-polygalacturonase treatment of tomatoes

2.2.1. Experiments with tomato variety K 3. Attaining the dry matter content prescribed in tomato paste standards is frequently difficult when juice is used from tomato varieties bred specifically for mechanical harvesting. This was the reason why experiments were performed to improve the condensability, the quality and consistency of the tomato concentrate by endo-PG treatment.

Before harvest, preliminary experiments were carried out with tomatoes of the *K 3* variety. The samples were adjusted to a degree of ripeness which is less favourable than that normally prevailing at mechanical harvesting. The samples contained 60% red, 30% straw-coloured and 10% green fruits. In the course of the enzyme treatment the effect of the temperature, the time of incubation (25, 20 and 15 °C; 1–6 hours) and the effect of enzyme concentration (0.001, 0.005 and 0.01%) was investigated on the juice yield, on the dry

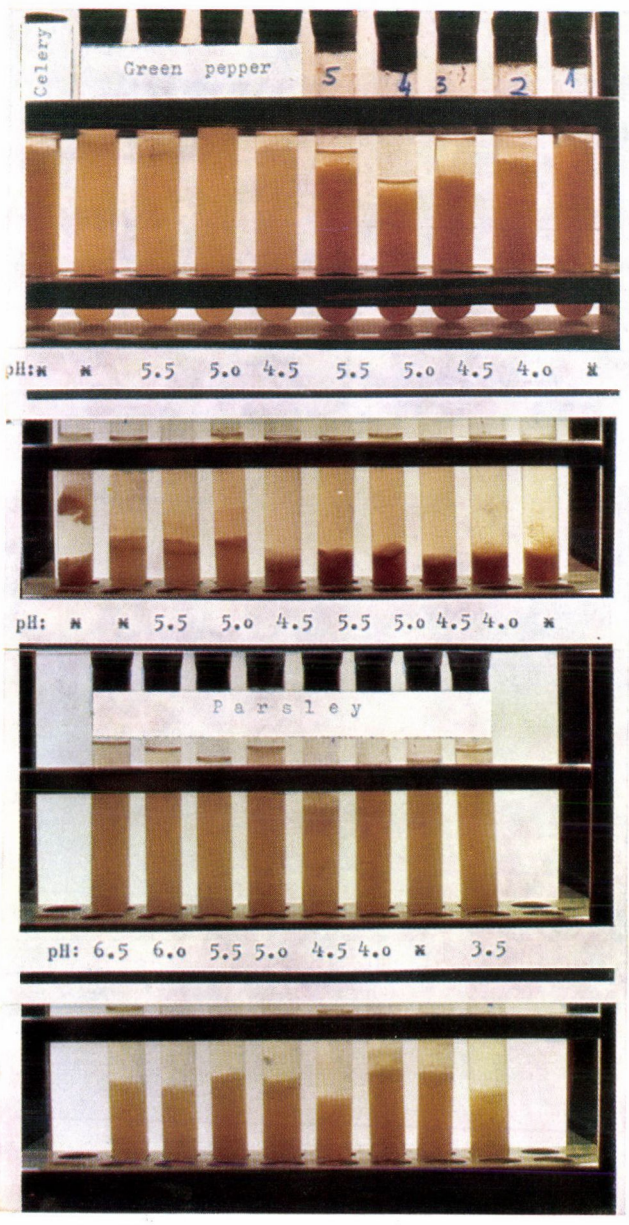


Fig. 8. Stability of the celery, paprika and carrot juices prepared with endo-PG treatment (lower row) and those prepared with the combined treatment of endo-PG + ultrasonics (upper row) after 18 weeks storage. (Endo-PG: 0.5%, incubation: 3 h at 50 °C)

Fig. 9. Stability of parsley juices prepared with endo-PG treatment (lower row), and prepared with the combination of endo-PG + ultrasonics (upper row) after storage of 18 weeks. (Endo-PG: 0.5%, incubation: 3 h at 50 °C)

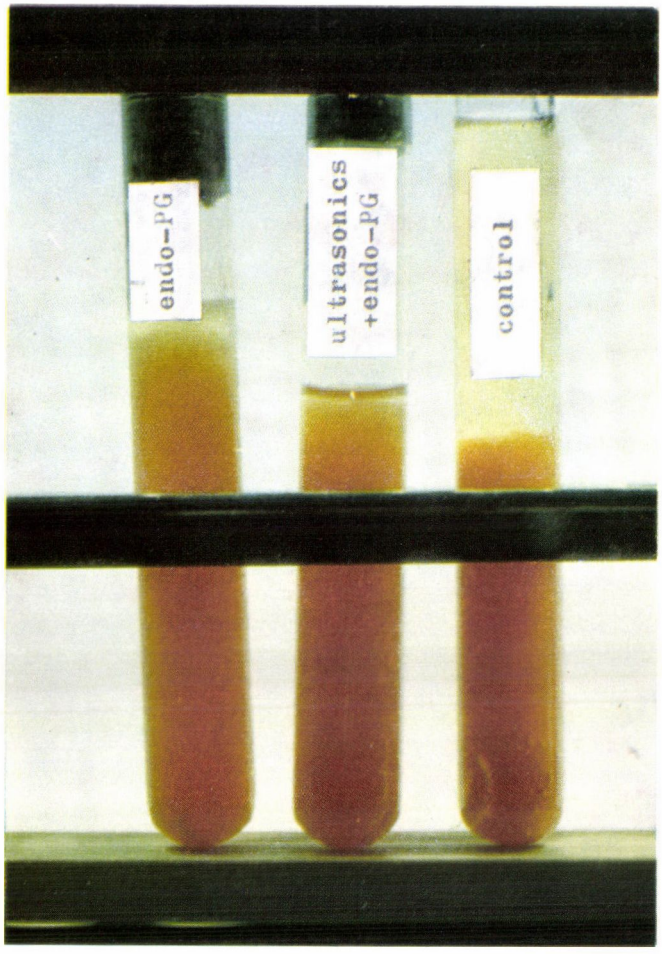


Fig. 10. Stability of tomato juice after 18 weeks of storage. (Endo-PG: 0.1%; incubation: 4 h and 15 °C)

matter content, on the loss at straining and on the dry matter yield of the strained tomato juice. After a 6 hour incubation period the effect of the concentration of endo-PG was examined on the dry matter content, consistency, dry matter yield, the colour and wet fibre content of the concentrates.

2.2.1.1. Evaluation of enzyme treatment after 15 min of evaporation. – Samples, taken every hour during the incubation period, were strained (after enzyme inactivation at 80 °C for 15 min) then were concentrated for 15 minutes in a ROTADEST evaporator at 60 °C and at 600 Torr. After evaporation the volume, dry-matter content and dry-matter yield were determined as a function of the time of incubation and the concentration of enzyme, and were compared to the untreated (control) samples.

The juice yield after enzyme treatment, the dry matter content and the dry-matter yield (after evaporation for 15 minutes) as a function of the time of incubation, are shown in Fig. 11.

The results of the enzyme-treated samples were expressed as per cent of those of the control.

Figure 11 shows that juice yield of the enzyme-treated tomatoes was higher than that of the control at each temperature tested. The maximum of the juice yield was obtained after 1–2 h of incubation at higher temperatures,

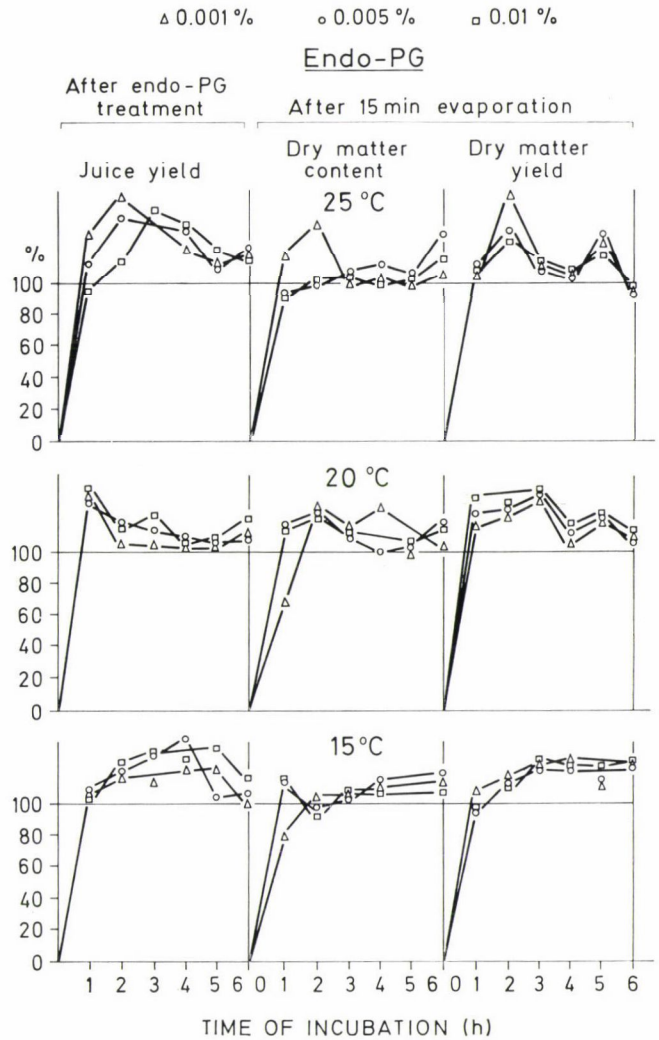


Fig. 11. The effect of endo-PG treatment of tomatoes (variety *K 3*) on the juice yield, dry matter content and dry matter yield at 15, 20 and 25 °C as function of the time of incubation

while at 15 °C a longer (4–5 h) incubation was necessary for the maximum yield.

The dry-matter content of the endo-PG-treated samples was higher at all the three investigated temperatures than that of the control after a 15 min evaporation.

The advantage of the endo-PG treatment can be better seen by comparing the dry matter yield of the samples with that of the control. The use of endo-PG resulted in an increase in the dry-matter yield, the maximum of which exceeded 20 % at each temperature tested. At 25 and 20 °C the maxima of the dry-matter yield were obtained after 2 and 3 h of incubation. These were followed by a certain decrease, but at 15 °C the increase in yield was consistently measurable during the whole incubation period.

Summarizing the results, the excess juice yields, measured from the samples taken every hour during the six hour incubation period, show that the average juice yield increased with increasing enzyme concentration. After 15 min concentration the relative viscosity of the enzyme-treated samples decreased, while their dry-matter content and dry-matter yield increased as compared to the control (Fig. 12). The control samples were taken as 100 %. The columns in the Figure represent the mathematical averages of 18 data.

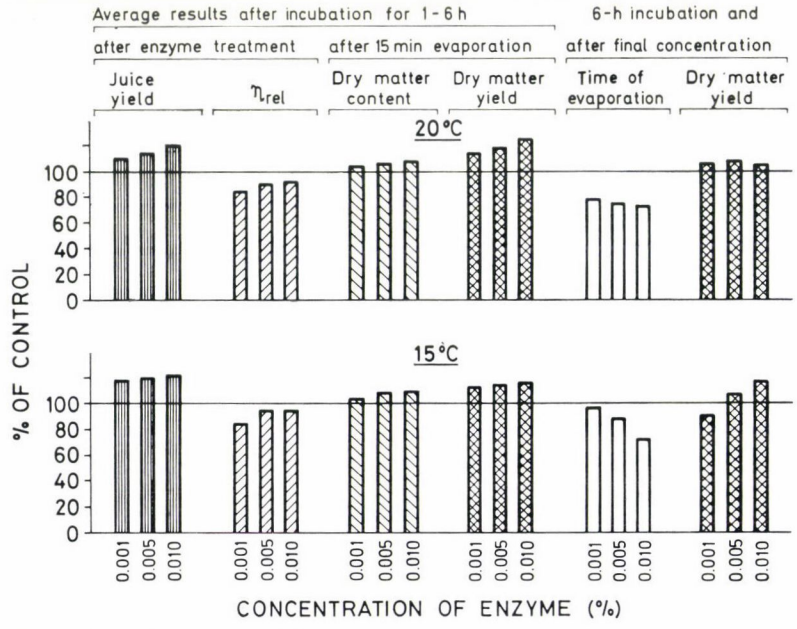


Fig. 12. The effect of the endo-PG treatment of tomatoes (variety *K 3*) on the juice yield (after enzyme treatment for 1 to 6 h) relative viscosity, dry-matter content and dry-matter yield (after 15 min evaporation) and on time of concentration and dry matter yield (after 6 h of incubation) as a function of the enzyme concentration

The difference between the dry-matter yield of the endo-PG-treated samples and that of the control, proves the advantage of application of the enzyme. The highest dry-matter yield was obtained at 20 °C when an enzyme concentration of 0.005 % was used, while at 15 °C the use of 0.01 % endo-PG resulted in the highest yield.

The main advantage of the enzyme treatment is the decrease in the time necessary for the concentration of the samples. This results in a decrease in energy requirement of about 20–40 %.

2.2.1.2. Evaluation of enzyme treatment after final evaporation. – When evaporation was carried on to the technically possible final state, the dry-matter contents of the concentrated enzyme-treated samples and of the control were similar. Considering the dry matter yields of the above samples, the advantage of the enzyme treatment was apparent (Fig. 12). The only exception was the result of the samples treated at a low (0.001 %) enzyme concentration at 15 °C.

The concentration of endo-PG necessary for tomato treatment was determined by mathematical statistical calculations. According to the regression analysis, the increase of the concentration of enzyme from 0.001 to 0.01 % can be recommended only at a temperature of 15 °C, while at 20 and 25 °C the application of 0.005 % of the preparation can give satisfactory results (Fig. 13).

2.2.2. Experiments with tomato variety Peto Mec. After the above mentioned experiments with variety *K 3*, our work was continued with the variety *Peto Mec* developed for mechanical harvesting. The total quantity necessary for a 3-day experiment was bought on one occasion. Fruit samples were stored at 5–7 °C for 3 days, the effect of the time of storage before processing was also taken into account.

Experimental conditions were the same during the three-day periods. The temperature and time of incubation were chosen according to the expected temperature (15 °C) in the tank cars and to the length of time of the transport (4 h) from the crushing plant to the canning factory, in the course of the autumn season. Evaporation was carried on for 100 min in the case of each sample.

The juice yield, the loss at straining after enzyme treatment, the dry matter content, dry matter yield and consistency of the concentrates were given as per cent of the control samples in Fig. 14. (Data in the Figure represent the mathematical average of three experiments.)

As shown in the Figure the enzyme treatment resulted in more than 20 % decrease in the loss at straining and in the same increase in juice yield when compared to the control.

After evaporation for 100 min, the dry matter content of the endo-PG-treated samples was 14 % higher and its consistency 7 % higher than that of the control.

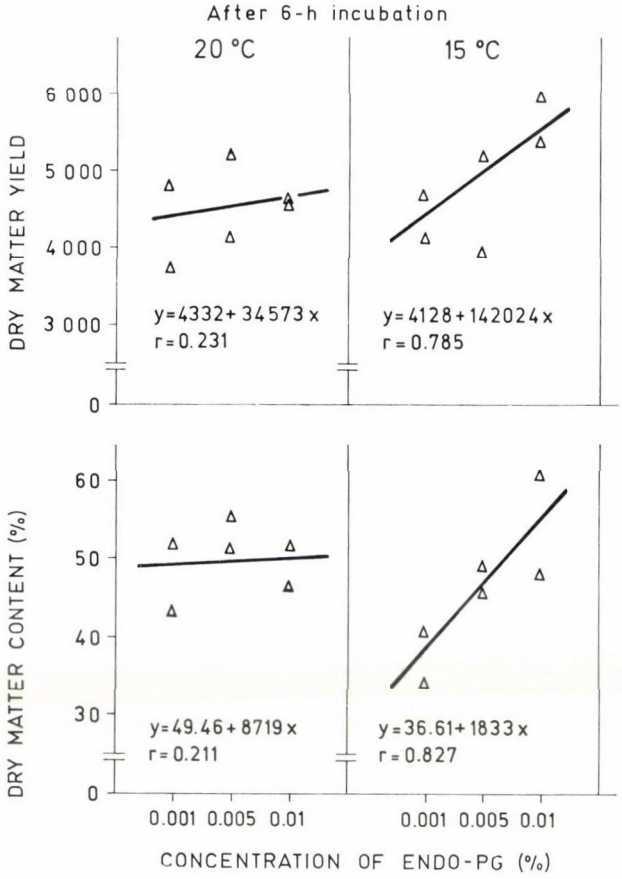


Fig. 13. The effect of the enzyme concentration on the dry matter content and dry matter yield of the tomato concentrates after a 6 h incubation at 15 and 20 °C

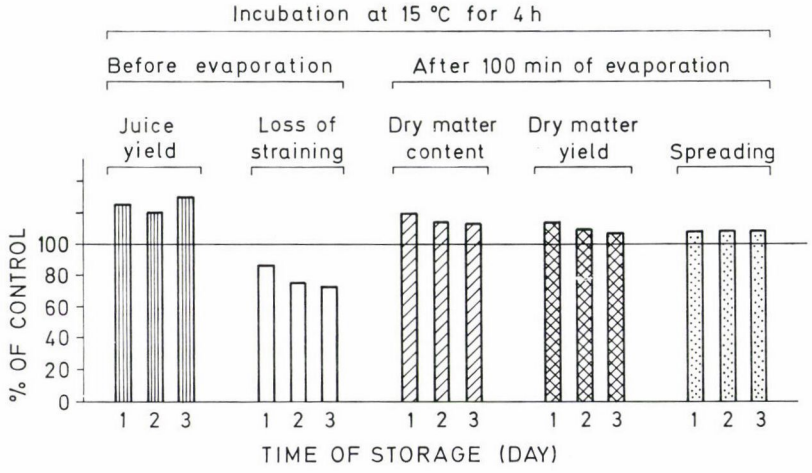


Fig. 14. The effect of the endo-PG treatment on the increase of juice yield and on the straining loss before evaporation and on the dry matter content, dry matter yield and spreading of the samples after concentration for 100 min, as a function of the of storage time of the fruits at 6–8 °C

The colour of the samples proved to be of first class quality according to the dominant wavelength (λ_D).

A fibre content of less than 15 % of the wet weight was found in the case of both enzyme-treated samples and the control, resp.

2.3. Endo-polygalacturonase treatment of fruits

Several berry-fruits and other fruits having high fibre content were treated with endo-polygalacturonase in order to increase the juice yield and decrease the viscosity of the samples. The effects of enzyme concentrations of 0.005, 0.01, 0.05 and 0.1 %, and times of incubation of 1.5 and 3 hours were investigated.

Juice yield, transmittance and viscosity of the juices after enzyme treatment were expressed as per cent of those of the untreated, control samples. Results of the endo-polygalacturonase treatment of raspberry, black-berry, currant, sour cherry, pear and quince are shown in Fig. 15.

It can be seen in the Figure that, in the case of *raspberry*, maximum increase in juice yield (21 %) was obtained at an enzyme concentration of 0.05 % and an incubation period of 1.5 h.

Further increases in the concentration of endo-PG and the duration of incubation resulted in a negative effect on the juice yield. Light transmittance of the samples treated with the optimum enzyme concentration was three times that of the control while its specific viscosity was half of that of the latter.

In the case of juice yield of *currants* an endo-PG concentration of 0.05 % and an incubation period of 1.5 h proved to be optimal. The increase in juice yield was 27 % and its transmission 80 % higher than those of the control, while its viscosity was 65 % lower.

The incubation of *sour cherry* with 0.005 and 0.01 % of endo-PG for 3.0 h, resulted in an excess juice yield of *ca.* 20 %. Under the above conditions the transmittance of the juice increased to four-times of that of the control and its specific viscosity decreased by 30 %.

Results of the endo-PG treatment of *black-berry* proved to be less favourable than in the case of raspberry, but the increase in enzyme concentration to 0.1 % resulted in a juice yield of 16–18 % higher than that measured in the control.

The transmittance of the juices increased as a function of the increase in enzyme concentration when a 1.5-h incubation period was used, but in the case of 3.0 h incubation, there was a strong decrease in transmittance. The specific viscosity of the juices decreased only slightly as a result of increasing the enzyme concentration when a 1.5-h incubation period was used. The lengthening of the time of incubation (at higher endo-PG concentrations of

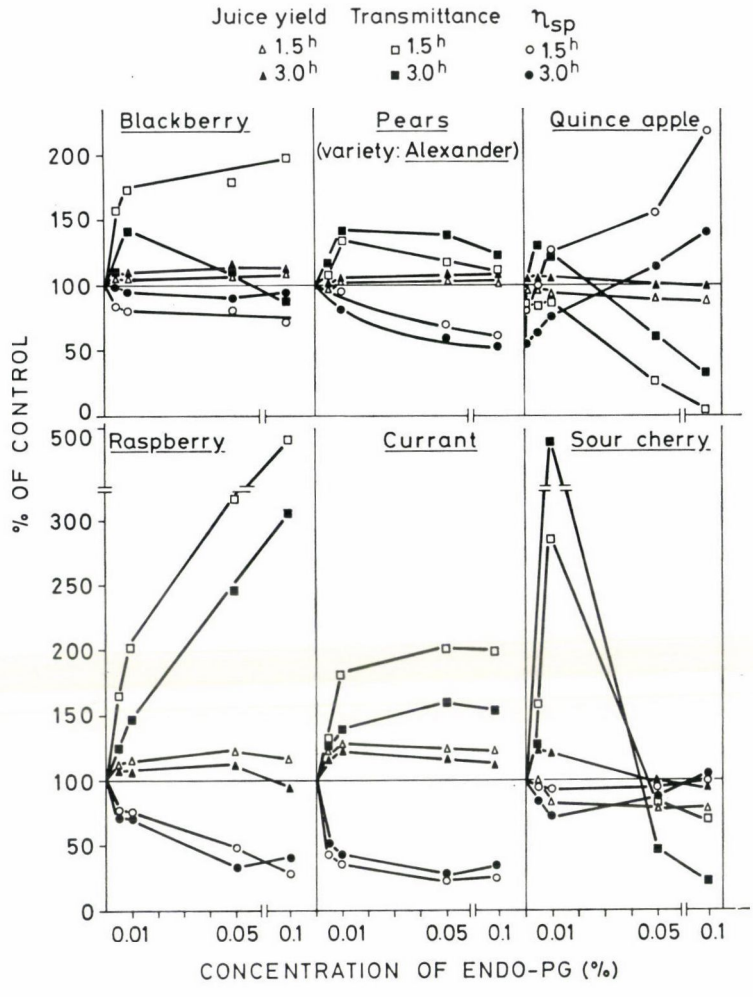


Fig. 15. The effect of the endo-PG treatment on the juice yield, specific viscosity and the transmittance of several fruit juices as a function of the enzyme concentration

0.05 and 0.01 %) resulted in viscosity values equal to or higher than that of the control.

Enzyme treatment of *pears* resulted in positive changes of the juice yield and specific viscosity with the increase of the enzyme concentration and the duration of incubation. Maximum transmittance of light was obtained when 0.01 % endo-PG was added, but further increases in enzyme concentration reduced the transmittance of the samples.

The treatment of pears with 0.1 and 0.05 % endo-PG resulted in a 10 and 8–9 % increase in the juice yield, resp.

In contrast to other fruits, the optimum endo-PG concentration for *quince apples* proved to be 0.005 % resulting in a 7 % excess of juice yield relative to the control.

The colour of the enzyme-treated red fruits was deeper and darker than those of the control treated with clarifying (*Phylazyme*) enzyme. The differ-

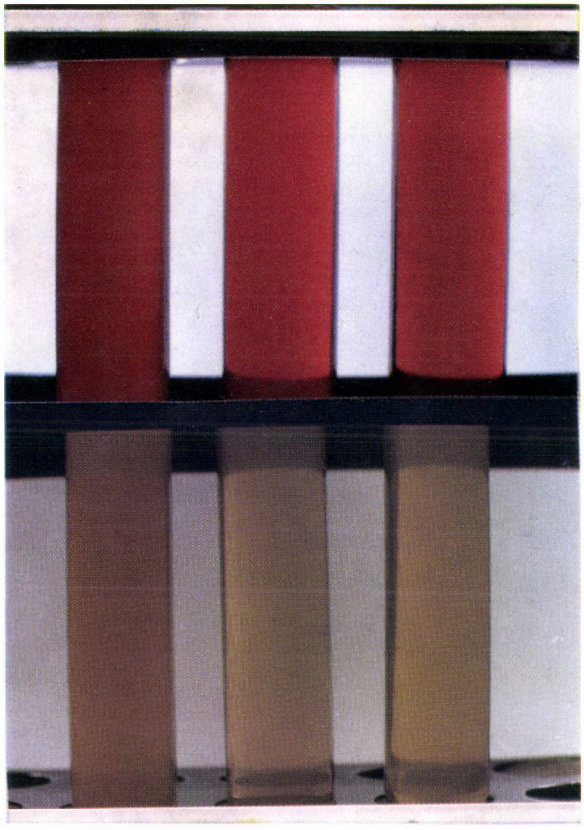


Fig. 16. The effect of the endo-PG treatment on the colour intensity of the raspberry juice. (From the left: tube 1: endo-PG treated samples, tubes 2 and 3: treated with 1 and 2% liquid clarifying enzyme, *Phylazyme*, PHYLAXIA, Budapest; endo-PG: 0.05%; incubation: 1.5 h at 40 °C)

Fig. 18. The effect of the endo-PG treatment on the colour intensity of the peach juice. (From the left: tube 1: endo-PG treated, tubes 2 and 3: treated with 1 and 2% liquid clarifying enzyme *Phylazyme*, PHYLAXIA, Budapest; endo-PG: 0.05%; incubation: 3.0 h at 40 °C)

ence in colour intensity was more remarkable with the increase of the enzyme concentration. This effect of the endo-PG treatment is illustrated by a photo of the raspberry juices (Fig. 16).

Results of the endo-PG treatment of apricots, peaches, red plums, Italian plums and plums of the variety *Besztercei* are shown in Fig. 17.

In the case of *apricot* an endo-PG concentration of 0.05% was necessary to obtain maximum increase in juice yield, which attained 17 and 23% after 1.5 and 3.0 h of incubation, respectively.

The longer incubation period proved to be more advantageous from the points of view of transmittance (175%) and of specific viscosity (25%). Increasing the enzyme concentration beyond 0.05% can not be recommended.

Maximum juice yield was obtained after 3.0 h of incubation and at an endo-PG concentration of 0.05%, in the case of *peaches*, too. The transmittance of the above sample was 2.8-times higher and the specific viscosity 65% lower than that of the control. A further increase in the enzyme concentration does not seem to be advantageous.

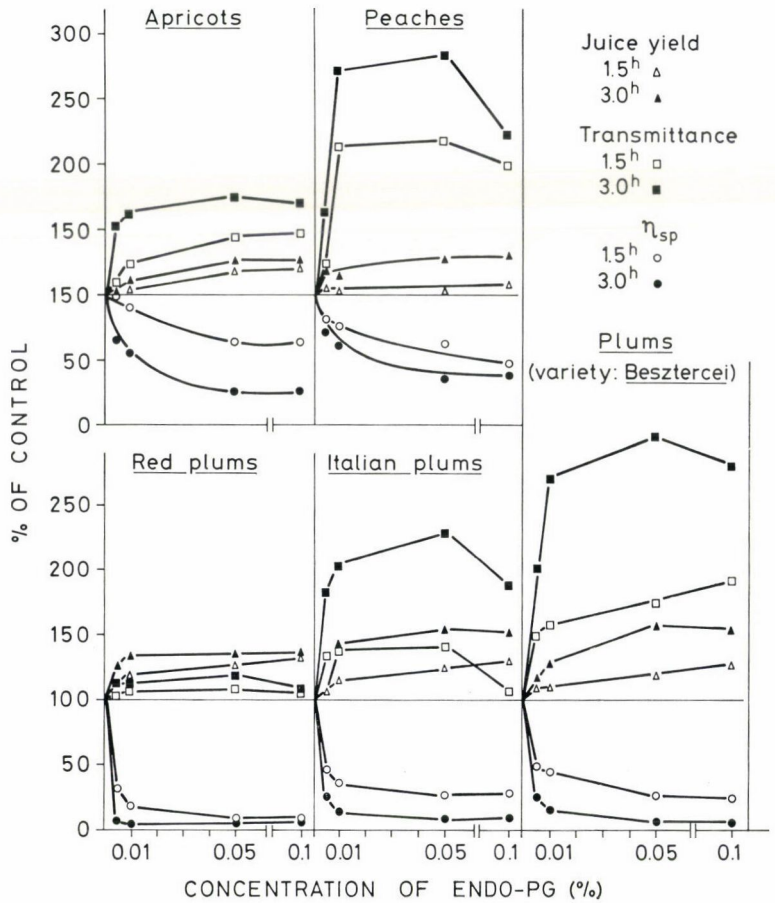


Fig. 17. The juice yield, specific viscosity and transmission of the juices of several endo-PG-treated fruits as function of the enzyme concentration

A significant increase in juice yield was obtained also when *red plums* were treated with endo-PG. Maximum juice yield was attained at an endo-PG concentration of 0.01% and during an incubation period of 3 h and this yield was 23% higher than that of the control. The specific viscosity of the treated samples significantly decreased, being only 5–6% of that of the control.

With *Italian plums* it was found that by increasing the endo-PG concentration to 0.05%, the juice yield and the transmittance of the samples increased with the simultaneous decrease in the specific viscosity. The increases of juice yield relating to the control were 24 and 52%, resp., during 1.5 and 3.0 h of incubation. The transmittance of the samples could also be increased with the increase of the enzyme concentration.

The treatment of other *plums* (variety *Besztercei*) with 0.05 and 0.1% endo-PG, resulted in an increase in juice yield of 20 and 27%, resp., during a 1.5-h incubation period, while its specific viscosity was 70% lower than that of the control. After a 3-h incubation period the specific viscosity of the sample treated with 0.05% endo-PG decreased by 93% as compared to the control.

The colour of the juices was darker, due to its full-bodied character, than those treated with clarifying enzyme *(Phylazyme)* as it is shown in the photo of the peach juices (Fig. 18).

3. Conclusions

It is well-known that pectolytic enzymes can significantly increase the juice yield when added to crushed fruits (HOTTENROTH, 1951; WUCHERPFENNIG *et al.*, 1970) and decrease the viscosity as well (SULC & CIRIC, 1968; GIERSCHNER & REINERT, 1970). Some of the pectolytic enzymes of microbial origin exert macerating activity, too, by being able to disintegrate vegetable tissues into single cells (BOCK *et al.*, 1970; RZEDOWSKI, 1972).

Macerating enzymes are already widely used on an industrial scale. GRAMPP (1969a) applied a hydrolytic type of macerating enzyme (*Rohament P*, ROHM & HAAS, Darmstadt) for the enzymatic disintegration of onion, potato, carrot and cucumber tissues. As it was stated in our previous paper (ZETELAKI-HORVÁTH & GÁTAI, 1977) dealing with the pH dependance of enzymatic disintegration of several vegetables, the efficiency of the macerating activity of the endo-PG preparation used (*PG-225*, pilot plant product of the CENTRAL FOOD RESEARCH INSTITUTE, Budapest) was higher than that of *Rohament P*. In the case of many vegetables (carrot, red tomato-shaped paprika, parsley, potato, cucumber, squash, etc.) a 96–98% disintegration was attained with half of the concentration of *Rohament P* recommended by CHARLEY (1969).

In agreement with our previous observations pH optimum for the endo-PG treatment proved to be in the acid pH range (3.0–4.5) with the only exception of garden sorrel with a pH optimum of 6.5.

In the present work the possibilities of industrial application of endo-PG enzyme were examined. Vegetable tissues were disintegrated for producing soft drinks (vegetable juices and cocktails), baby foods, dehydrated vegetable powders and ice creams. In addition to the above purposes, endo-PG was used to improve the condensability of tomato juice. Results of the latter experiment (decrease in time necessary to attain a prescribed degree of concentration, increase in dry matter content, decrease in straining loss and a significant increase in juice yield, stabilizing effect of the tomato juice for more than 18 weeks) might be the consequence of the enzymatic disintegration of non-soluble pectins of high molecular weight into smaller parts, soluble in water. The above effect of the enzyme treatment can help in preventing the sedimentation of fibrous particles in the juice (PILNIK, 1969; GRAMPP, 1969b).

Endo-PG treatment of several fruits (of high fibre content and of those not easy to press) was also carried out. Enzyme concentrations of 0.05 and 0.01% (at 40 °C for 1.5 and 3 hours) resulted in 8–52% higher juice yields and a 25–95% decrease in viscosity. In the case of raspberry, sour cherry and plums the increase in juice yield was more than 20 and 50% which was much higher than that (10%) obtained by ZACKEL and co-workers (1970) using two types of pectolytic enzymes (produced by surface and submerged culture) in concentrations of 0.1–0.6%.

The taste and aroma of the fruits did not change after enzyme treatment. The colour of the juices became more intensive as a consequence of the enzyme treatment and this colour intensity increased parallel to enzyme concentration.

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THE ROLE OF ENZYME–SUBSTRATE RATIO IN THE ENZYMATIC BROWNING OF FRUIT TISSUE HOMOGENATES

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and V. Nádudvari-Márkus

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The enzymatic browning of fruit is mainly attributed to the action of polyphenol oxidase. The enzyme, as present in multiple forms in nature, as well as its numerous substrates have been, for a considerable period, subjects of extensive scientific research. Knowledge accumulated on the topic is, however, divergent as to which of the two factors, *i.e.* the enzyme or the substrate, plays the decisive role in the overall phenomenon of enzymatic browning.

Simultaneous measurements of the initial rates of actual browning as well as of the concentrations of enzyme and substrate in apples and apricots lead to the conclusion that – independently of the kind of fruit – *the ratio* of the latter two values indicates which of these factors determines the browning rate of the product. If the numerical value of the ratio as expressed in the units adopted in this study does not exceed 20, the initial browning rate is, with good approximation ($r^2 = 0.81$), in linear relationship with *enzyme* concentration. Such ratios were found in different apple cultivars investigated during several years as well as in two apricot cultivars. Ratios > 35 were found in seven lots of apricots of various cultivars investigated during two years. In this case a very close correlation ($r^2 = 0.96$), best described by a saturation equation, could be established between the rate of browning and *substrate* concentration. It was assumed that in the first case the concentration of the reaction products formed from part of the substrate was sufficient to cause enzyme inhibition during the reaction. Thus, necessarily, enzyme concentration was the limiting factor in the reaction. In the second case even total substrate depletion was insufficient for the formation of reaction products in a concentration high enough to inhibit the enzyme. Consequently, in the latter case, the reaction was limited by substrate content.

Some of the research work carried out in this laboratory has been focused, for several years, on enzymatic browning as occurs in the processing of horticultural products. The discolouration, in general unfavourable also with respect to the taste and biological value of the product, is mainly attributed to the action of polyphenol oxidase. This enzyme oxidizes the natural o-diphenols of fruits and vegetables to quinones which in turn are transformed, in secondary non-enzymatic reactions to macromolecular and partly insoluble dark compounds. The polyphenol oxidases of some products catalyze, in addition, the hydroxylation of monophenols (*e.g.* tyrosine) to o-diphenols. The enzyme having no access to its substrates in the intact cell, enzymic browning may develop only in the presence of air (O_2), in vegetable cells damaged by peeling, comminution or bruise.

The tendency to browning of a horticultural product thus depends on its polyphenol oxidase and o-diphenol (plus, in some cases, monophenol)

content. Consequently, the concentration of either of these characteristics ought to lend itself, in principle, as an index of discolouration.

Data published in the literature are, however, divergent as to which of the two factors, *i.e.* the enzyme or the substrate plays the decisive role in the overall phenomenon of enzymatic browning. *E.g.* with apples, some authors did not find any relationship between polyphenol oxidase activity and browning, while the catechins were indicated as main source of discolouration (TÄUFEL & VOIGT, 1962); others stressed the role of chlorogenic acid (HULME, 1958; WALKER, 1962; MONTIES, 1966; SCHEEL MAHN, 1968) while, according to a third group of researchers, enzyme concentration was responsible for browning (WEURMAN & SWAIN, 1955; MACHEIX, 1970). Similar contradictions may be found also in the literature dealing with pears and peaches (SCOTT *et al.*, 1960; LUH *et al.*, 1963; NAKABAYASHI & UKAI, 1963; PECH & FALLOT, 1972; WANG & MELLENTHIN, 1973). In view of these discrepancies it was thought interesting to study this problem more closely.

1. Materials and methods

Initial rates of browning were determined by a method based on reflectance measurement as developed in this laboratory (VÁMOS & GAJZÁGÓ, 1974; GAJZÁGÓ & VÁMOS-VIGYÁZÓ, 1975). Reflectance of apple and apricot slices was measured in the spectrocolorimeter *Spekol* (CARL ZEISS, Jena) at 540 and 580 nm, resp. Polyphenol oxidase concentrations of fruit homogenates and o-dioxy phenol contents of methanol extracts of fruit flesh were assessed by spectrophotometric methods, partly developed in this laboratory (MIHÁLYI & VÁMOS-VIGYÁZÓ, 1976) and partly modified from procedures described in the literature (VÁMOS-VIGYÁZÓ *et al.*, 1976a). Measurements were carried out in the spectrophotometer *Spectromom* 203 (MAGYAR OPTIKAI MŰVEK, Budapest). PPO concentration was read at 420 nm and ODP content — after performing the *Hoepfner*-reaction — at 520 nm.

The dimensions of the characteristics and the abbreviations used are given below:

ODP = o-dioxy phenol content (mg% chlorogenic acid)

PPO = polyphenol oxidase enzyme concentration (kUg^{-1}); $1\ U = 10^{-4}\ \Delta\ OD\ min^{-1}$

BA = initial rate of browning or browning activity ($\Delta\ R\ min^{-1}$); $1\ U = 1\ \Delta\ SD\ min^{-1}$

OD = optical density (1 cm optical path)

R = reflectance

SD = scale division

A total of 21 lots of apples belonging to 6 cultivars and harvested, partly at different stages of maturity, in two regions of Hungary, as well as 9 lots of apricots belonging to 7 cultivars and equally two regions, were investigated with the above methods for 3 and 2 consecutive years, resp. The lots of apricots were partly identical with those dealt with earlier (GAJZÁGÓ *et al.*, 1977). Attempts were made to establish relationships between the characteristics studied.

2. Results

2.1. Browning tendency, polyphenol oxidase and o-dioxy phenol contents of apple cultivars

Initial browning rates, polyphenol oxidase enzyme concentrations and o-dioxy phenol contents as well as the ratios of the latter two characteristics of the 21 lots of apples investigated are shown in Fig. 1.

Considerable differences in browning rate may be observed between the apples according to cultivar and, within the same cultivar, to region and year as well as degree of maturity. The ratio of the highest and lowest values of BA (*Starking*, 1976 and *Jonadel*, 1974) is 35. The differences between the browning rates of the lots are — as proven by analysis of variance — primarily due to the cultivars: the mean browning activities of the lots of *Jonathan*, *Golden delicious* and *Starking* are 0.88, 1.9 and 4.5 U, resp. The differences between these values are significant at the probability level of 95%. The comparison of the mean browning rates of the apples of various cultivars harvested in the same year yielded a significantly higher value for 1976 (3.4 U) than for the two preceding years (1.04 and 1.2 U, resp.), the values of which did not differ significantly. Although considerable (1.01 U), the difference between the mean browning rates of the apples obtained from two regions did not prove significant (VÁMOS *et al.*, 1976c).

A definite parallelism can be observed between the browning rates and polyphenol oxidase concentrations of the apple lots. Browning rates and ODP contents vary in parallel within some groups of apples only (*e.g.* in the case of *Jonadel*, *Jonared* and *Staymared*), while for other groups (*e.g.* the lots of *Golden delicious*) an inverse relationship seems to exist. No apparent relationship can be detected between browning and the ratio of PPO and ODP. The absolute numerical value of the latter as expressed in the units adopted, $|(\mathrm{PPO}/\mathrm{ODP}) \cdot 10^2|$, was, for the population considered, between 1 and 20.

2.2. Correlations between browning rate and polyphenol oxidase as well as o-dioxy phenol content of apples

Taking into account the results obtained attempts were made to establish, in the first place, a relationship between the browning rates and the respective

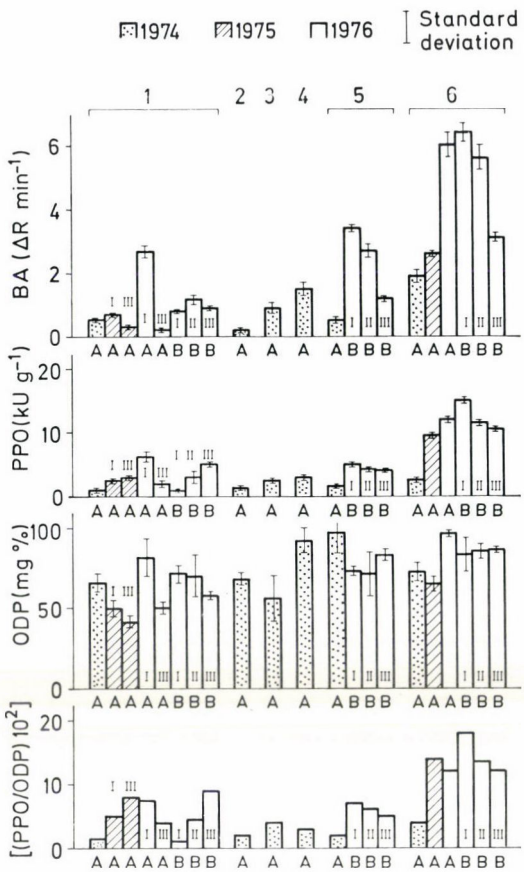

Fig. 1. Initial browning rates (BA), polyphenol oxidase (PPO) enzyme concentrations, o-dioxy phenol (ODP) contents and the ratios PPO/ODP of apple cultivars. *Cultivars:* 1. Jonathan, 2. Jonadel, 3. Jonared, 4. Staymared, 5. Golden delicious, 6. Starking. *Regions:* A = Northern Hungary, B = Central Hungary. *Maturity*: I < II < III. – $1 < |(PPO/ODP) \cdot 10^2| < 20$. In the cases of BA and PPO concentration — where reaction rates are concerned — the vertical bars represent standard deviations of regression coefficients. Regression equations were calculated from the points of measurement of 12 and 6 parallel rate determinations, resp. Each rate curve was obtained from 4–6 points of measurement, thus each value of BA and PPO concentration was calculated from 24 to 72 data

polyphenol oxidase contents of apples. In plotting the means of browning rates *vs.* the means of PPO contents, standard deviations of the latter values were disregarded. As can be seen in Fig. 2, a close linear correlation, significant at the probability level of 99.9 %, was found between these two characteristics for the 21 lots of apples investigated.

As a matter of fact, a positive linear correlation — significant at the probability level of 99 % — could be established also between browning rates and ODP contents. This latter proved to be, however, much less close ($r^2 = 0.34$, $r = 0.58$). The equation describing the correlation is as follows:

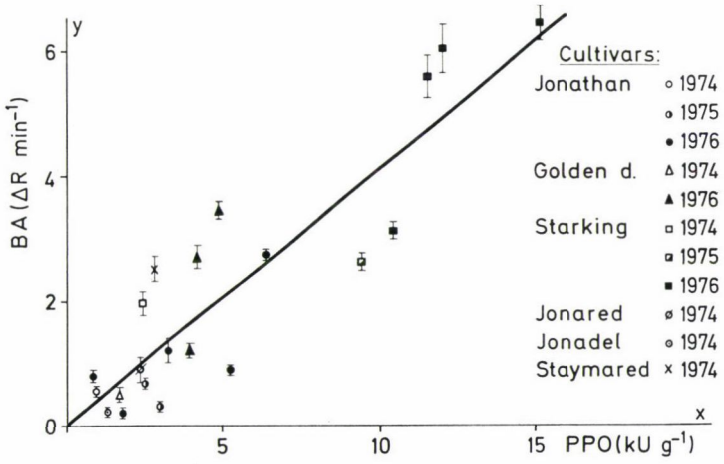

Fig. 2. Correlation between initial browning rates (BA) and polyphenol oxidase (PPO) contents of apple cultivars [$1 < |(PPO/ODP) \cdot 10^2| < 20$]. Equation of the regression curve: BA = 0.02 + 0.41 PPO; $r^2 = 0.81$, $r = 0.90$; number of data pairs, $n = 21$. The correlation coefficient (r) is significant at the probability level of 99.9%. For the meaning of standard deviations see Fig. 1

$$BA = 3.70 + 0.08\ ODP$$

According to these findings, the browning rate of apples is primarily determined by polyphenol oxidase enzyme concentration in the fruits.

2.3. *Browning tendency, polyphenol oxidase and o-dioxy phenol contents of apricot cultivars*

Browning rates, polyphenol oxidase enzyme concentrations and o-dioxy phenol contents as well as the ratios of the latter two characteristics as determined in the total of 9 lots of apricots are illustrated in Fig. 3.

As with apples, region and year as well as cultivar all seem to affect the browning tendency of apricots. Comparing the two cultivars investigated from 2 regions and during 2 years, the samples of *Magyar kajszi* from both years and both regions had higher browning activities than the respective lots of *Rakovszky*. The browning tendency of the population from Western Hungary investigated in 1976 was, on the average, higher than that of the apricots harvested in 1975 in Central Hungary. The ratio of the highest and lowest browning activities as measured in the lots *Szegedi mammut*, 1976 and *Bibor*, 1975, was higher than 6.

In the case of apricots no apparent parallelism seems to exist between browning rate and polyphenol oxidase enzyme concentration. On the other hand, when disregarding the extreme values obtained for the lots *Magyar*, 1975 and *Mandula*, 1975, substrate contents follow the values of browning

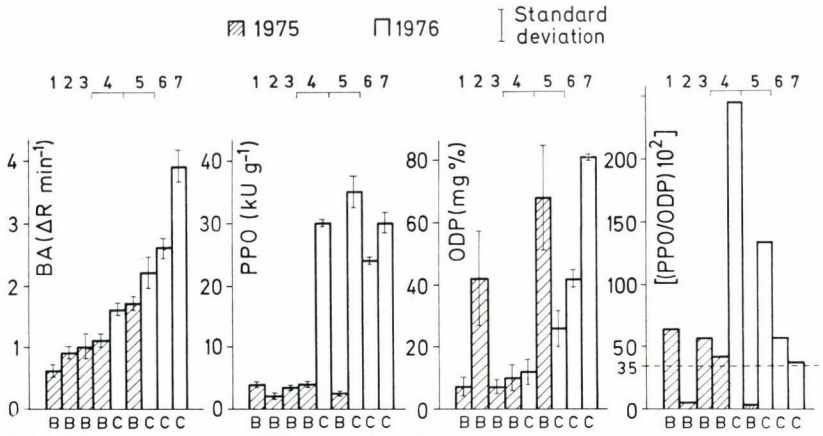

Fig. 3. Initial browning rates (BA), polyphenol oxidase (PPO) enzyme concentrations, o-dioxy phenol (ODP) contents and the ratios PPO/ODP of apricot cultivars. *Cultivars:* 1. Bibor (Purple), 2. Mandula (Almond), 3. Keverék (Mixed Lot), 4. Rakovszky, 5. Magyar (Hungarian), 6. Borsi rózsa (Rose Borsi), 7. Szegedi mammut (Mammoth of Szeged). *Regions:* B = Central Hungary, C = Western Hungary. — $1 < |(\mathrm{PPO/ODP}) \cdot 10^2| < 250$. In the case of BA and PPO concentration — where reaction rates are concerned — the vertical bars represent standard deviations of regression coefficients. Regression equations were calculated from the points of measurement of 12 and 6 parallel rate determinations, resp. Each rate curve was obtained from 4–6 points of measurement, thus each value of BA and PPO concentration was calculated from 24 to 72 data

rates. The ratios of enzyme concentration and substrate content vary, in the apricot population investigated, within wide limits: $4 < |(\mathrm{PPO/ODP}) \cdot 10^2| < 245$; however, their values are — with the exception of the two lots mentioned above — in all cases higher than 35. It is interesting to note how different this ratio may be for lots of the same cultivar (*Rakovszky* and *Magyar*, resp.) obtained in different years and from different regions.

In the apricots *Mandula* and *Magyar* harvested in 1975 the ratios of polyphenol oxidase concentration and o-dioxy phenol content as defined above are ≤ 5 and thus in the range of the respective values obtained for apple cultivars (VÁMOS-VIGYÁZÓ *et al.*, 1976b). If — as already said — these two lots are not taken into account, a highly significant close correlation can be established between the initial browning rates and o-dioxy phenol contents of apricots. The correlation may be equally well described by a saturation equation, a quadratic or a linear relationship. Fig. 4 shows the results obtained by fitting the data to a saturation curve.

The regression equation of the parabolic curve is $\mathrm{BA} = 0.49 + 0.07\ \mathrm{ODP} - 0.00034\ (\mathrm{ODP})^2$; $r^2 = 0.97$, $r = 0.98$, $n = 21$; that of the linear relationship was found to be $\mathrm{BA} = 0.79 + 0.040\mathrm{ODP}$; $r^2 = 0.92$, $r = 0.96$, $n = 7$. (For symbols see Fig. 4.) The latter two correlation coefficients are significant at the probability level of 99.9% and the difference between their values is not significant.

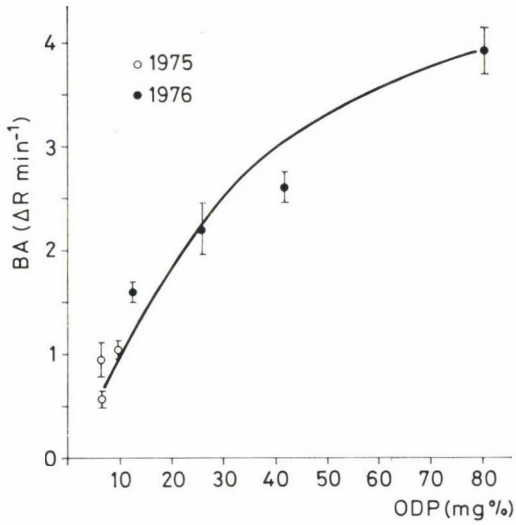

Fig. 4. Correlation between initial browning rates (BA) and o-dioxy phenol (ODP) contents of apricot cultivars [$35 < |(PPO/ODP) \cdot 10^2| < 250$. Equation of the regression curve: $BA = 4.2(1-0.76 \cdot 0.97^{ODP})$; $r^2 = 0.97$, $r = 0.98$; number of data pairs, $n = 7$; the correlation coefficient (r) is significant at the probability level of 99.9%. For the meaning of standard deviations see Fig. 3

Considering the numerical values of the enzyme: substrate ratio on the 1975 samples of the apricots *Magyar* and *Mandula* it was assumed that in the case of these cultivars — as with apples — the rate of browning could be correlated to the polyphenol oxidase content. As can be seen in Fig. 5, neither the

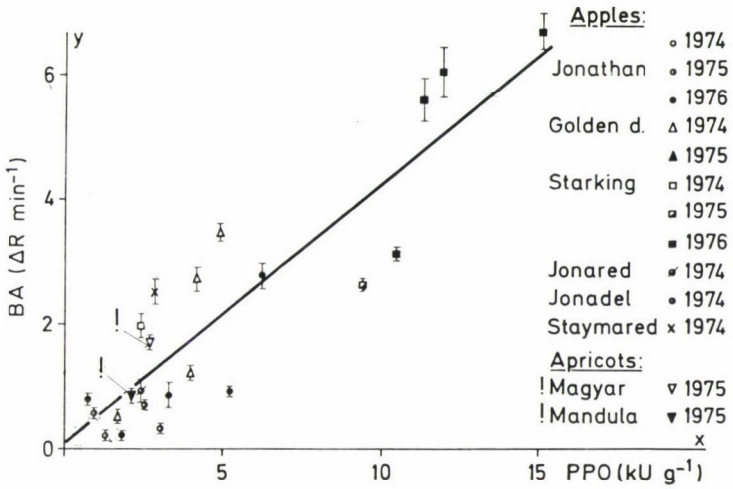

Fig. 5. Correlation between initial browning rates (BA) and polyphenol oxidase (PPO) contents of the apple *and* two apricot cultivars [$1 < |(PPO/ODP) \cdot 10^2| < 20$]. Equation of the regression curve: $BA = 0.06 + 0.41PPO$; $r^2 = 0.81$, $r = 0.90$, number of data pairs, $n = 23$; r is significant at the probability level of 99.9%. For the meaning of standard deviations see Fig. 1

correlation nor the regression coefficient of the linear relationship between browning rate and polyphenol oxidase content of *apples* is altered by including the data of the above two apricot cultivars, only the value of the constant in the equation is slightly modified. This, however, does practically not have any influence on the course of the curve.

The insertion of the same two cultivars in the close (linear) correlation found between browning rates and ODP contents of the rest of the apricots (Fig. 4) lowered the value of the squared correlation coefficient to $r^2 = 0.49$.

In the case of the 7 lots of apricots with an enzyme: substrate ratio > 35, a linear correlation of medium strength, significant at the probability level of 95 %, was found between browning rate and polyphenol oxidase concentration ($r^2 = 0.55$, $r = 0.74$). The correlation may be described by the equation $BA = 0.70 + 0.06PPO$ (symbols as in Fig. 2).

Thus, with respect to the point in question, the lots of apricots investigated did not prove to be uniform. In the majority of cultivars the rate of browning was primarily dependent on substrate concentration.

3. Conclusions

The results obtained showed enzymic browning of horticultural products to depend, in some cases, mainly on polyphenol oxidase, in others on substrate content. The *ratio* of these two characteristics was found to determine which one of them is of primary importance. This ratio may vary considerably, within the same kind of fruit, with the cultivar (*e.g.* in the case of the lots of apricots investigated).

If the numerical value of the ratio — as expressed in the units adopted — was below 20, browning rate was, with good approximation ($r = 0.90$) a linear function of enzyme concentration. Such ratios were found in a number of apple cultivars studied for several years as well as in two apricot cultivars. Ratios exceeding the numerical value of 35 were found in 7 apricot cultivars from two consecutive harvests. In this case a close correlation ($r = 0.98$) between browning rate and substrate concentration was established which could be described satisfactorily by a saturation function.

An interpretation of the phenomenon may be given by assuming that in the first case the amount of substrate present in the fruit is sufficient for the formation of reaction products in a concentration high enough to inactivate the enzyme during the reaction before the substrate is depleted (WHITAKER, 1972). In this case enzyme inactivation is the limiting factor of the reaction. In the second case substrate content in the fruits as related to enzyme concentration was, on the average, lower by one order of magnitude (VÁMOS *et al.*, 1976b; GAJZÁGÓ *et al.*, 1977). Thus, even complete transformation of the

substrate might not yield reaction products in a concentration sufficient to inactivate the enzyme. Consequently, substrate depletion must be the limiting factor of the reaction. This latter assumption is borne out by the fact that the relationship between browning rate and substrate concentration of the population of relatively low substrate content fits a saturation function: the higher the substrate content, the less it influences browning activity.

The hypothesis presented gives a possible interpretation of the contradictions in the literature as outlined in the introduction. Its generalization needs, however, further experiments. The work carried out so far, permits, at any rate, of a conclusion of practical importance: namely, that estimating the browning tendency of a horticultural product from its polyphenol oxidase or o-dioxy phenol content is not possible without the knowledge of the ratio of these two characteristics.

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CHANGES IN ENZYMIC BROWNING, POLYPHENOL OXIDASE AND POLYPHENOL CONTENTS OF JONATHAN APPLES DURING STORAGE

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Changes occurring in enzymic browning rate, polyphenol oxidase, o-dioxy phenol and chlorogenic acid contents of *Jonathan* apples harvested at the same orchard in two consecutive years were studied during prolonged storage in normal atmosphere. The fruits picked in 1974 were kept at 8 °C, those of the following year at 5 °C and 15 °C, resp. Samples were analyzed monthly. Of the characteristics studied, chlorogenic acid was found to vary with storage time according to a maximum curve in all the batches investigated. The maximum value was about 40% higher than the initial one. During the first 4 months of observation browning rate did not significantly exceed the initial value in any of the batches. During the last two months browning tendency decreased in the 1974 batch and doubled in the fruits of the following year. Little or no similarity was observed in the changes of o-dioxy phenol and polyphenol oxidase contents of the apples harvested in different years. On the other hand, all the characteristics studied were found to vary, irrespective of storage temperature, more or less in parallel in the fruits picked at the same time.

Results suggest that during storage browning tendency is more strongly affected by the physiological state of the fruit when harvested than by storage conditions. It seems probable that factors beside those investigated might intervene in the changes observed.

Apples are the main winter fruit grown in Hungary. Part of the fruits harvested in autumn are processed, during nearly the entire storage period, to apple juice concentrate. Since colour is an important factor of juice quality it was considered expedient to investigate the changes which might occur, during storage, in the enzymic browning tendency of apples as well as in the concentrations of the enzyme and substrates participating in the reaction. Investigations were carried out in two consecutive years, during a six-month storage period each, with fruits of the cultivar *Jonathan* which represents the bulk of domestic apple production.

1. Materials and methods

1.1. Apples and storage conditions

Jonathan apples harvested in late September 1974 and in mid-October 1975, resp., were supplied by the RESEARCH STATION OF THE UNIVERSITY OF HORTICULTURE, Újfehértó (North-Eastern Hungary). The fruits picked in 1974

were stored in a cellar of 8 °C average temperature with natural ventilation, those harvested in 1975 were kept partly in a cold-room at 5 °C and partly in an aerated chamber of 15 °C average temperature. Samples for analysis were taken monthly.

1.2. Determination of the rate of browning, polyphenol oxidase concentration, o-dioxy phenol and chlorogenic acid contents

The analytical methods applied had been described earlier, partly in this and partly in other journals. The initial rate of browning, *i.e.* browning activity (BA) was determined by a reflectance method using the spectrocolorimeter *Spekol* (CARL ZEISS, Jena). Measurements were performed at room temperature (22 to 24 °C) on slices of the fruit, immediately after peeling and cutting. Browning activity was expressed in terms of discolouration per min as indicated by the instrument (1 U = 1 scale division min^{-1}) (VÁMOS & GAJZÁGÓ, 1974).

Polyphenol oxidase (PPO) activity was established by spectrophotometric measurement of the initial rate of changes in optical density at 420 nm as induced by the fruit homogenate at 30 °C and pH 5.4, in the presence of added chlorogenic acid substrate (MIHÁLYI & VÁMOS-VIGYÁZÓ, 1976).

o-dioxy phenol (ODP) content was assessed in the methanol extracts of the fruits by a spectrophotometric method based on the *Hoepfner*-reaction (ALMÁSI & MOLNÁR, 1961) as modified in the authors' laboratory (VÁMOS *et al.*, 1976). Optical densities of the reacted extracts were read at 520 nm and expressed as chlorogenic acid on the basis of a calibration curve.

Chlorogenic acid (CA) content of the methanol extracts of the fruits was measured spectrophotometrically at 328 nm (COME, 1971) and evaluated by means of a calibration curve.

1.3. Mathematical statistical treatment of the results

Results were compared by analysis of variance and by Student's *t*-test (SVÁB, 1973). Differences significant at probability levels of 99.9, 99 and 95 % were marked ***, ** and *, resp. Differences not significant were marked Ø.

2. Results

2.1. Characteristics of the apples as determined right after harvest

Browning activities, polyphenol oxidase concentrations, o-dioxy phenol and chlorogenic acid contents of the apples of two consecutive years as determined at the beginning of storage (at "0" time) are shown in Table 1.

Table 1

o-dioxy phenol (ODP), chlorogenic acid (CA), polyphenol oxidase (PPO) contents and initial browning rates (BA) as determined, after different times of storage at different temperatures, in Jonathan apples harvested in 1974 and 1975 in North-Eastern Hungary

Batch →	A							
Storage time (months)	ODP (mg%)		CA (mg%)		PPO (kUg^{-1})		BA (U)	
	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s
0	66.2	5.7	8.0	0.6	0.94	0.21	0.55	0.08
1	—	—	—	—	—	—	0.29	0.06
2	62.2	5.1	7.8	1.6	—	—	0.29	0.03
3	60.0	7.1	11.1	1.1	1.00	0.12	0.52	0.04
4	56.8	0.8	9.5	0.6	0.85	0.12	0.30	0.06
5	51.0	4.3	8.1	0.8	0.75	0.12	0.12	0.03
6	53.7	1.6	8.2	0.0	0.72	0.04	0.19	0.03
Batch →	B							
Storage time (months)	ODP (mg%)		CA (mg%)		PPO (kUg^{-1})		BA (U)	
	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s
0	43.8	4.1	5.6	0.6	2.99	0.07	0.32	0.10
1	46.5	10.9	6.8	2.0	3.37	0.06	0.37	0.21
2	55.8	1.9	6.8	0.3	1.62	0.63	0.30	0.10
3	49.5	3.9	8.2	2.4	1.48	0.13	0.36	0.16
4	47.4	3.0	6.2	1.0	3.04	0.25	0.06	0.05
5	39.1	12.2	6.1	0.1	2.45	0.10	0.56	0.19
6	40.6	7.0	3.7	0.4	1.29	0.19	0.75	0.28
Batch →	C							
Storage time (months)	ODP (mg%)		CA (mg%)		PPO (kUg^{-1})		BA (U)	
	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s
0	43.8	4.1	5.6	0.6	2.99	0.07	0.32	0.10
1	45.5	6.9	8.0	1.6	3.47	0.11	0.37	0.15
2	57.8	1.4	7.8	0.4	1.37	0.15	0.11	0.10
3	50.2	2.6	7.8	1.4	1.52	0.63	0.23	0.16
4	52.0	2.1	6.9	0.8	3.76	0.17	0.20	0.09
5	40.5	4.8	5.1	1.5	2.42	0.46	0.60	0.00
6	—	—	—	—	1.03	0.31	0.65	0.28

A: apples harvested in late September 1974 and stored at an average temperature of 8 °C.

B and C: apples harvested in mid-October 1975 and stored at 5 °C and an average temperature of 15 °C, resp.

O time: beginning of the storage period.

$\bar{x}$ = mean of 3 to 9 parallel measurements.

s = standard deviation.

– = no measurement taken.

The two lots of apples harvested in the same orchard in two consecutive years were rather different with respect to the characteristics investigated. All values, except those of PPO concentration, were higher in the apples picked in 1974. PPO content was about treble in the fruits of the second year as compared to those of the preceding harvest.

2.2. Changes occurring during storage

The values of the characteristics studied as determined monthly are summarized in Table 1. To facilitate evaluation of the changes occurring during storage the values of the characteristics as related to the initial ones were plotted *vs.* storage time and the resulting curves are shown in Fig. 1.

The characteristics investigated were found to vary individually, according to different irregular curves.

2.2.1. Changes in o-dioxy phenol content. In all the lots investigated variations in ODP content were relatively slight. This is also borne out by the analysis of variance the results of which are summarized in Table 2.

Table 2

Significance of the relative variations in o-dioxy phenol content of Jonathan apples on storage in normal atmosphere

Batch →	A						B						C				
Storage time → (months) ↓	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5
0	–	Ø	*	*	*	*	Ø	Ø	Ø	Ø	Ø	Ø	Ø	*	*	*	Ø
1		–	–	–	–	–		Ø	Ø	Ø	Ø	Ø		*	*	*	*
2			Ø	*	*	*			Ø	Ø	Ø	Ø			*	*	*
3				*	*	*				Ø	Ø	Ø				Ø	*
4					*	Ø					Ø	Ø					*
5						Ø						Ø					

Results of the analysis of variance:

A: $F = 4.33^{*}$; $df_1 = 5$; $df_2 = 12$; $SD_{P_{5\%}} = 6.6$.
B: $F = 2.10^{0}$; $df_1 = 6$; $df_2 = 17$.
C: $F = 7.29^{**}$; $df_1 = 5$; $df_2 = 15$; $SD_{P_{5\%}} = 9.4$.

F = *F*-test; Ø = not significant; * and ** = significant at the probability levels of 95% and 99%, resp.
$SD_{P_{5\%}}$ = significant difference (at the probability level of 95%); df = degree of freedom.
– = no measurement taken.
For the rest of symbols see Table 1.

o-dioxy phenol content of the fruits picked in 1974 decreased more or less steadily throughout storage. The changes were not significant during the

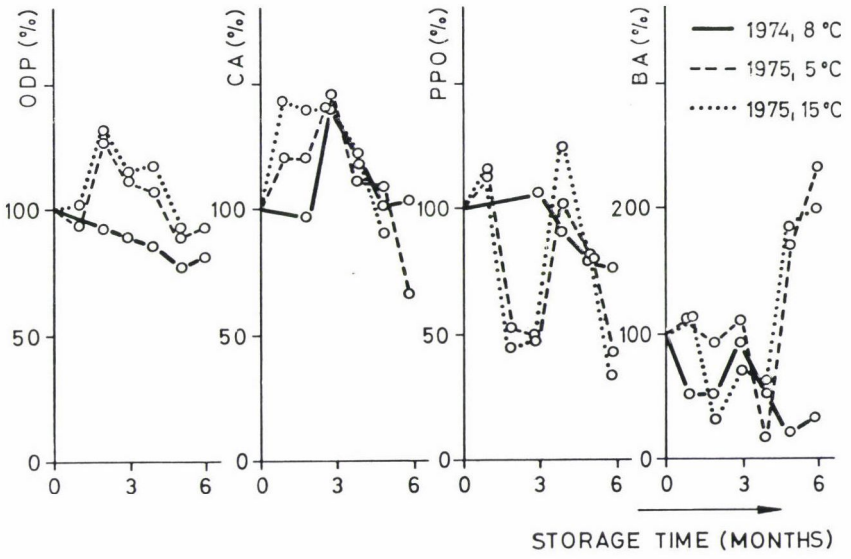

Fig. 1. Relative values of the changes occurring during storage in o-dioxy phenol (ODP)' chlorogenic acid (CA) and polyphenol oxidase (PPO) contents as well as in initial browning rates (BA) of *Jonathan* apples. The apples were harvested in two consecutive years at the same orchard in North-Eastern Hungary and stored in normal (uncontrolled) atmosphere at the temperatures indicated in the Figure

first two months, neither was the slight increase at the end of the period. The variations of the above characteristic showed an entirely different pattern in the apples of 1975: o-dioxy phenol content had a sharp maximum after 2 months of storage, whereafter it decreased with a slight break to reach, by the end of the period of observation, a value near the initial one. These changes, although very similar in trend, proved significant only in the case of the fruits kept at 15 °C, where they were somewhat more pronounced. The similarity of the behaviour of the 1975 apples stored at different temperatures can be clearly seen from Table 3 which contains the comparison, by Student's *t*-test, of the values obtained after identical periods for the lots kept at 5 °C and 15 °C.

No significant differences could be observed in the ODP contents of apples from the same lot, after identical storage periods at different temperatures.

2.2.2. Changes in chlorogenic acid content. As can be seen from Fig. 1 and Table 4, chlorogenic acid content varied, during storage, in a fairly similar way in all three apple batches investigated.

In the lots kept at 5 °C and 8 °C a sharp maximum was observed after 3 months of storage. In the lot stored at 15 °C maximum was reached after 1 and maintained for the two subsequent months. The relative value of the maximum was practically identical for the 3 batches: it ranged from 139 to

Table 3

Comparison of the value of o-dioxy phenol and chlorogenic acid contents as well as of polyphenol oxidase concentrations and browning activities of apples from the same lot, stored for identical periods at different temperatures

Storage time (months)	Significance levels of differences according to Student's t test			
	I	II	III	IV
1	Ø	Ø	Ø	Ø
2	Ø	*	Ø	*
3	Ø	Ø	Ø	Ø
4	Ø	Ø	***	***
5	Ø	Ø	Ø	Ø
6	–	–	Ø	Ø

I = o-dioxy phenol content (mg%).

II = Chlorogenic acid content (mg%).

III = Polyphenol oxidase enzyme concentration (kUg^{-1}).

IV = Browning activity (U).

Ø = difference not significant.

* and *** = differences significant at the probability levels of 95 and 99.9%, resp.

– = not compared.

Apples were picked in mid-October 1975 and stored at 5 °C and 15 °C, resp.

Table 4

Significance of the relative variations in chlorogenic acid content of Jonathan apples on storage in normal atmosphere

Batch →	A						B						C				
Storage time → (months) ↓	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5
0	–	Ø	*	*	Ø	Ø	*	*	*	Ø	Ø	*	*	*	*	*	Ø
1		–	–	–	–	–		Ø	*	Ø	Ø	*		Ø	Ø	*	*
2			*	*	Ø	Ø			*	Ø	Ø	*			Ø	Ø	*
3				*	*	*				*	*	*				Ø	*
4					*	*					Ø	*					*
5						Ø						*					

Results of the analyses of variance:

A: $F = 6.06^{**}$; $df_1 = 5$; $df_2 = 12$; $SD_{P_{5\%}} = 12.0$.

B: $F = 3.83^{*}$; $df_1 = 6$; $df_2 = 17$; $SD_{P_{5\%}} = 16.6$.

C: $F = 4.66^{**}$; $df_1 = 5$; $df_2 = 15$; $SD_{P_{5\%}} = 16.4$.

For the rest of symbols see Tables 1 and 2.

146 % of the initial one. The decrease in relative chlorogenic acid content as observed between the 3rd and 5th month of storage occurred at a similar rate in all the batches. This could be supported by biometrical calculation: no significant differences were found, by analysis of variance, between the chlorogenic acid contents as related to the initial values after 3, 4 and 5 months, in the three apple batches stored at different temperatures. (The respective values of F were $0.07^{\emptyset}$, $0.35^{\emptyset}$ and $0.93^{\emptyset}$ for degrees of freedom of 2 and 6.) After 5 months the chlorogenic acid content practically returned to the initial value. In the apples harvested in 1974 and stored at 8 °C this value was maintained till the end of the storage period, while in those picked one year later and stored at 5 °C it dropped by nearly 40 %. In the lot kept at 15 °C the values of ODP and CA were not assessed at the end of storage.

2.2.3. Changes in polyphenol oxidase concentration. The changes in polyphenol oxidase concentration observed during storage were extremely different in the fruits from different years (Fig. 1, Tables 3 and 5).

In the apples harvested in 1974 PPO concentration remained practically constant for the first 3 months of storage, then slowly decreased to reach, by the end of the 6th month, a value about 25 % below the initial one.

In the apples of the year 1975 PPO concentration increased in the first month — irrespective of storage temperature — by slightly more than 10 %, then dropped abruptly to about half of the initial value to rise again after 4 months to a local maximum followed by a second steep decrease. This lasted till the end of the observation period. Apart from the maximum obtained after 4 months the values determined at identical phases of storage did not differ significantly in the apples kept at 5 and at 15 °C. By the end of the storage period PPO concentration dropped to about 40 % of the initial value.

2.2.4. Changes in initial browning rate. In all cases initial browning rates were found to vary with storage time according to rather irregular curves (Fig. 1). In spite of the differences of these curves as obtained for the batches kept at different temperatures some similarities could be observed during the first 4 months of storage (Table 6): in none of the cases did browning activity significantly exceed the initial value; after 3 months there was a local maximum in all the curves, preceded by a minimum and followed by a descending section. During the first 4 months of storage no significant differences were found, after identical periods, between the browning activities of the batches kept at the two lower temperatures. After the fourth month, however, changes in browning activities of the fruits from the two years took an opposite course: in the apples of the year 1974 the descending section of the curve continued practically till the end of the storage period (the slight increase in the 6th month was not significant). In the fruits picked in 1975 browning activity showed a sudden rise after 5 months which lasted till the end of storage. The final value was very similar for the batches kept at 5° and at 15 °C.

Table 5

Significance of the relative variations in polyphenol oxidase enzyme concentration of Jonathan apples on storage in normal atmosphere

Batch →	A						B						C					
Storage time → (months) ↓	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
0	—	—	Ø	*	*	*	*	*	*	Ø	*	*	*	*	*	*	*	*
1		—	—	—	—	—		*	*	Ø	*	*		*	*	Ø	*	*
2			—	—	—	—			Ø	*	*	Ø			Ø	*	*	Ø
3				*	*	*				*	*	Ø				*	*	*
4					*	*					*	*					*	*
5						Ø						*						*

Results of the analysis of variance:

A: $F = 4.33^{**}$; $df_1 = 4$; $df_2 = 28$; $SD_{P5\%} = 8.8$.
B: $F = 58.33^{***}$; $df_1 = 6$; $df_2 = 35$; $SD_{P5\%} = 11.7$.
C: $F = 3.16^{*}$; $df_1 = 6$; $df_2 = 35$; $SD_{P5\%} = 15.1$.
*** = significant at the probability level of 99.9%.
For the rest of symbols see Tables 1 and 2.

Table 6

Significance of the relative variations in the initial browning rate of Jonathan apples on storage in normal atmosphere

Batch →	A						B						C					
Storage time → (months) ↓	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
0	*	*	Ø	*	*	*	Ø	Ø	Ø	*	*	*	Ø	*	Ø	Ø	*	*
1		Ø	*	Ø	*	*		Ø	Ø	*	*	*		*	Ø	Ø	*	*
2			*	Ø	*	*			Ø	*	*	*			Ø	Ø	*	*
3				*	*	*				*	*	*				Ø	*	*
4					*	*					*	*					*	*
5						Ø						*						*

Results of the analysis of variance:

A: $F = 5.84^{***}$; $df_1 = 6$; $df_2 = 33$; $SD_{P_{5\%}} = 18.2$.
B: $F = 7.09^{***}$; $df_1 = 6$; $df_2 = 29$; $SD_{P_{5\%}} = 37.5$.
C: $F = 5.36^{***}$; $df_1 = 6$; $df_2 = 28$; $SD_{P_{5\%}} = 56.3$.
For symbols see Tables 1, 2 and 5.

3. Conclusions

From the practical aspect the most important result of the work presented is the fact that up to 4 months of storage in normal atmosphere no increase in browning tendency of *Jonathan* apples was observed. This was found to hold for fruits from different years and different picking times and appears to be highly independent of storage conditions. The direction of the changes in browning tendency taking place during storage prolonged over 4 months seems to depend on the physiological state (maturity) of the fruits at harvest rather than on storage conditions: apples of the same batch, but stored at different temperatures (5 °C and 15 °C resp.) were found to behave identically in this respect.

The similarity of the results obtained with the apples of the same batch, but stored at different temperatures proves that the irregularity of the curves describing the variations of the characteristics studied is not the result of the inhomogeneity of the fruit.

The increase in browning activity as observed during the last two months of storage in the apples harvested in 1975 is in agreement with the simultaneous decrease in chlorogenic acid and o-dioxy phenol contents and indicates the transformation of the substrate into coloured products. It is, however, difficult to interpret the drop in enzyme concentration occurring at the same time. It must be assumed that factors other than the ratios of enzyme and substrate might play a role in the browning of apples on prolonged storage. This assumption seems the more justified as none of the parallelisms observed by the authors (VÁMOS-VIGYÁZÓ *et al.*, 1977) and by others (MACHEIX, 1970) between browning and the concentrations of polyphenol oxidase or its substrates in freshly harvested apples were found to exist during storage.

To our knowledge no data on changes in browning tendency of apples during storage are available in the literature. Hence, it was found necessary to extend investigations to other characteristics (*e.g.* respiration) of the fruit, in order to get a clearer picture on the processes involved. Experiments to this effect are in progress.

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