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# DATA ON SOME STORAGE TECHNOLOGICAL PARAMETERS OF FRUITS AND VEGETABLES

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When ripe for storage, the density of apples of the *Jonathan* variety was found to be $0.768 \pm 0.04$ g cm$^{-3}$. This value decreases gradually with the increase of the maximum diameter. The specific volume of these apples is 1.303 cm$^3$ g$^{-1}$, the actual volume of one apple is on the average 150 cm$^3$. The volume of the apple can be calculated accurately and quickly without direct measurement with the help of the relationship described by the authors.

The density of storage-ripe *Hardenpont* pears was established as $0.967 \pm 0.01$ g cm$^{-3}$, their specific volume was 1.033 cm$^3$ g$^{-1}$. No significant deviations in specific volume could be demonstrated by grading according to size. The actual average volume of one pear was 120 cm$^3$. The authors suggest a method for calculating the volume of the fruit taking its shape into consideration.

Cabbage of the *Braunschweig* variety had a density of $0.607 \pm 0.07$ g cm$^{-3}$ when ripe for storage. The specific volume of the cabbage was 1.647 cm$^3$ g$^{-1}$, according to individual measurements the average volume of one cabbage was 3000 cm$^3$. A mathematical correlation is suggested for the quick determination of the volume.

The volume utilization quotient was determined for all three products as a function of the volume of the storage container on the basis of packing tests.

Because of the rapid development of storage and processing technologies of fruits and vegetables it became necessary that, in addition to the biological characteristics, also the physical and chemical parameters of the products should be studied more extensively. These properties of the raw materials are manifested in a rather complex manner and decide in most cases (mainly in industrial practice and in commerce) the value and quality of the goods. Their importance is increasing not only in production and in the design and building of more highly developed production lines and equipment, but also in the field of economic calculations and in the establishment of models.

The physical (physical-mechanical) parameters, such as, for instance, size, average weight, density, volume, specific heat, impact resistance, consistency, seed ratio, etc., or the chemical properties, such as, for instance, solids, starch, sugar, acid or water content and aroma constituents of the various fruit and vegetable varieties are, both separately and collectively, parameters whose usefulness needs no detailed explanation for those skilled in the art of cold storage or preservation.

With the exception of the extensive experiments for the measurement of consistency (texture), the literature about the determination of the physical parameters of fruit and vegetable varieties by means of measurements,

or about the test methods applied, is rather scanty. There have been but few comprehensive and detailed experiments and measurements. The most frequently cited works are those by DANNIES (1950) and NEHRING and KRAUSE (1969), but references to the varieties are practically missing.

An accurate and reliable determination of the physical parameters of the raw product requires a large number of measurements. In this way the information gained experimentally or by calculation will be available for the investigation of some serious problems of both cold storage and processing technology and will provide an opportunity to draw a comparison with chemical and other material parameters.

When determining the characteristic density values of tomato HULSEY and co-workers (1971) compared several methods for measuring the volume. SHAW (1974) found a close relationship, characteristic of the product, between the almost constant decrease of specific gravity during the cold storage of apples of the *Jonathan* variety and the other physical and chemical parameters of the fruit. CASIMIR and co-workers (1967) studied the effect of steam blanching *in vacuo* on the internal gas condition of the apple slice and thereby on the changes in specific gravity.

Among the most important guidelines for cold stores of food, the necessity of knowing some of the physical parameters of the raw materials is mentioned by ALMÁSI and DOBRAY-HORVÁTH (1971), with special reference to calculating the specific cooling requirements. Admitting the demands which might be raised in this field KOMÁNDI-ERDŐDI and TOMCSÁNYI (1971) constructed and applied an instrument to measure the firmness of the product, that is its resistance to injuries. KOMÁNDI-ERDŐDI (1974) carried out mechanical tests on the produce in relation to machine harvesting. The size, mass, density, specific volume and surface of winter apple and apricot of identified varieties prepared for cold storage have been measured by GONDA (1972), similar measurements on winter pears and cabbage, again on known varieties, were made by RICHTER (1974). LÁSZTITY and co-workers (1973) determined the texture and degree of ripeness of peach and pear varieties. It has appeared from the experiments of SASS and HORN (1973) and of GYURÓ and SASS (1974) that the determination of the characteristic dimensions of the varieties and the time of measurement are in no way indifferent, since depending upon the time of harvest and upon the way of cultivation cold stored apple will not only lose weight, but its diameter and specific gravity will also diminish.

The practically important physical parameters of the raw material of the Hungarian food industry were summarized by BALOGH (1972), the physical parameters of liquids processed by the canning industry were described by KÖRMENDY (1972, 1974). We have tried to determine some parameters not tested earlier and needed in engineering design and practice.

In the INSTITUTE OF FOOD TECHNOLOGY AND MICROBIOLOGY OF THE UNIVERSITY OF HORTICULTURE we have been carrying out experiments for several years with respect to the testing of some important physical-mechanical parameters of fruit and vegetable varieties with the aim of improving the technology of cold storage. We have determined, among others, the piece weight of the products, the number making up 1 kg, the density, volume, specific volume and size of the products. If conducted over a period of several years, these experimentally obtained or calculated data might be of value for theoretical calculations, but also in the practice of cold storage for the dimensioning of the stores, of the spacing arrangements, of the necessary cooling performance, *etc.*

We have also started the examination of the utilization of the store room, that is of the boxes and containers. Packaging (filling) tests were carried out with the aim of obtaining a space utilization coefficient which might be used in practice. This is a series of experiments which touches not only upon storage technology but also on the rentability of storage. In this respect the results, obtained by the INSTITUTE OF FRUIT GROWING OF THE UNIVERSITY OF HORTICULTURE in the period between 1965 and 1971 and the analytical work of RÉDAI and KISS (1973) were also taken into consideration.

## 1. Materials and methods

When choosing the *raw material* we assumed that the testing of varieties suitable for long-term winter storage is a topical problem. Thus, we decided to determine the physical parameters of *Jonathan* apples, *Hardenpont* pears and *Braunschweig* cabbage. The apple and pear samples came from the SZIGETCSÉP EXPERIMENTAL PLANT OF THE UNIVERSITY OF HORTICULTURE, the cabbage was a product of a co-operative farm in Székesfehérvár.

With respect to the *time of harvesting* and the pertaining state of ripeness, in the case of apples we relied on the observations of HÁMORI-SZABÓ and SASS (1971). Bearing in mind the changes in the average weight and size of the fruit during ripening, the apples ripe and prepared for storage under controlled atmospheric conditions were picked and tested in both years between 12 and 18 September. The fruit was considered ripe for storage when the starch test gave a value of 2.5 to 3.5 and the firmness of the pulp was around 7.5 kg measured with a manual penetrometer (*Fruit Pressure Tester*, STAZIONE SPERIMENTALE DI ORTIFRUTTICOLTURA, Milano). In the unripe apple the starch represents the greater part of the carbohydrates. During ripening more and more starch is converted into sugar. The measure of this conversion is used for the determination of the stage of ripeness. When the slice of apple is dipped in a solution of iodine–potassium iodide the starch

turns blue. The coloured apple slices are then compared with a numbered scale of printed blue-colour etalons (STANCHINA *et al.*, 1970).

The *Hardenpont* pears were picked and immediately tested in both years between 25 and 29 September when they were ripe for storage (0.4—0.6 mm penetrometer degrees, measured with a *Labor*-type penetrometer). Cabbage samples were harvested in a state of about 85% ripeness (fully developed head, compact and hard) between 3 and 10 November and tested two days later.

Individual pieces of apples and pears were sampled randomly, the size of the samples was 200 kg. In the case of cabbage whole boxes were taken, the sample size was 100 kg.

Determination of the *piece weight* and of the number of fruits making up 1 kg (this of course could not be performed with cabbage) was repeated 10 times. The *length* of the specimens was measured on 100 randomly chosen individual pieces.

The *density* (specific gravity) of the product was determined by means of the *Reimann*-type hydrostatical balance used for the determination of the specific gravity of potatoes, in water. In the case of apples and pears 5 kg, in the case of cabbage, 3 kg samples were tested, the testing of fruits was repeated 10, that of cabbage 20 times. Due to its loose texture cabbage absorbed a fair amount of water during the relatively short time of the measurement which might have distorted the apparent weight loss measured under water, so that the cabbage was re-weighed after each immersion together with the water dripping out of it and the value of the volume was corrected for the value of the relative weight increase compared to the weight in air. The correction factor is valid for a given variety under given conditions for the actual sample only and cannot be applied generally.

*Specific volume* was expressed as the reciprocal of the density.

Grading according to *size* was performed manually on the basis of the maximum diameter with the help of a classifying lath. This method could not be used for cabbage which was classified by measuring each individual and class-intervals were set up according to the frequency distribution.

In addition to the determination of specific gravity and specific volume, the bulk of the samples (partly ungraded and partly graded according to size) was subjected to the *space filling* test. For this the fruit boxes used in cold stores and made of plastic (internal dimensions $56 \times 37 \times 28$ cm, volume 53,872 $cm^3$) were chosen, in addition to the Hungarian *GEV*-type wooden boxes (volume 62.752 $cm^3$, internal dimensions $53 \times 37 \times 32$ cm) used in these experiments for apple in one of the seasons only. The space-filling tests were repeated 10 times with randomly chosen material and without a regular row-type arrangement of the produce.

From the parameters of the *shape*, the volume of the product was assessed mathematically. This was found suitable without individual volume measurements, provided data on the length of the produce are available for quick and comparatively accurate assessment of the characteristic volume of a single piece of the produce. The validity of the mathematical functions was checked by 10 to 15-fold repetition of volume measurements of individual pieces by the overflow method.

The *coefficient of volume utilization of* the product was obtained as the ratio of the theoretically possible packing to the experimentally obtained value in the filling test.

## 2. Results and conclusions

### 2.1. *Piece weight, density and specific volume*

The results obtained by the measurement of mass and of density are summed up in Tables 1 and 2, without showing individual data.

Table 1

*Weight per piece, density and specific volume of* Jonathan *apples*

| Characteristics | Mathematical statistical testing | | | |
|---|---|---|---|---|
| | Arithmetic mean (M) | Standard deviation (s) | Standard error ($s_{\bar{x}}$) | Coefficient of variation (%) |
| Specific number ($kg^{-1}$) | 8.5 | 1.41 | 0.45 | 15.7 |
| Weight per piece (g) | 116.0 | 9.07 | 2.87 | 7.8 |
| Ungraded: | | | | |
| density ($g\ cm^{-3}$) | 0.7676 | 0.04 | 0.01 | 5.2 |
| specific volume ($cm^3g^{-1}$) | 1.3035 | | | |
| Size-graded: | | | | |
| 55—60 mm ⌀ | | | | |
| density ($g\ cm^{-3}$) | 0.7719 | 0.02 | 0.01 | 2.6 |
| specific volume ($cm^3g^{-1}$) | 1.2960 | | | |
| 65 mm ⌀ | | | | |
| density ($g\ cm^{-3}$) | 0.7699 | 0.03 | 0.01 | 3.8 |
| specific volume ($cm^3g^{-1}$) | 1.2995 | | | |
| 70 mm ⌀ | | | | |
| density ($g\ cm^{-3}$) | 0.7568 | 0.02 | 0.01 | 2.6 |
| specific volume ($cm^3g^{-1}$) | 1.3219 | | | |
| 75 mm ⌀ | | | | |
| density ($g\ cm^{-3}$) | 0.7566 | 0.02 | 0.01 | 2.6 |
| specific volume ($cm^3g^{-1}$) | 1.3223 | | | |

Table 2

*Weight per piece, density and specific volume of* Hardenpont *pears and* Braunschweig *white cabbage*

| Characteristics | Mathematical statistical testing | | | |
|---|---|---|---|---|
| | Arithmetic mean (M) | Standard deviation (s) | Standard error ($s_x$) | Coefficient of variation (%) |
| *Hardenpont* pears | | | | |
| Specific number ($kg^{-1}$) | 7.2 | 1.23 | 0.39 | 17.1 |
| Weight per piece (g) | 114.0 | 5.40 | 1.71 | 4.7 |
| Ungraded: | | | | |
| density ($g\ cm^{-3}$) | 0.9673 | 0.01 | 0.00 | 1.0 |
| specific volume ($cm^3g^{-1}$) | 1.0339 | | | |
| Size-graded: | | | | |
| 55–60 mm ⌀ | | | | |
| density ($g\ cm^{-3}$) | 0.9649 | 0.01 | 0.00 | 1.0 |
| specific volume ($cm^3g^{-1}$) | 1.0364 | | | |
| 65 mm ⌀ | | | | |
| density ($g\ cm^{-3}$) | 0.9635 | 0.01 | 0.00 | 1.0 |
| specific volume ($cm^3g^{-1}$) | 1.0385 | | | |
| White cabbage variety *Braunschweig* | | | | |
| Weight per piece (kg) | 1.83 | 0.45 | 0.10 | 24.6 |
| Ungraded: | | | | |
| density ($g\ cm^{-3}$) | 0.6076 | 0.07 | 0.02 | 11.5 |
| specific volume ($cm^3g^{-1}$) | 1.6474 | | | |

Density data appear in a more illustrative manner as a function of size (*i.e.* largest diameter) in Fig. 1 which shows also the standard deviations.

In the case of *Jonathan* apple density is between 0.7500 and 0.7700 $g\ cm^{-3}$, and the density of the graded samples decreases gradually as the diameter increases. The density of the 70- and 75-mm apples differs significantly ($P \geq 95\%$) from that of the ungraded sample and of the 55–60-mm and 65-mm fractions. There is no evidence of a difference between the data obtained in two different years, just as no significant difference was found between the densities of apples grown on wild understock, productive spindle or on Hungarian wattle. The conclusions which can be drawn from the data of specific volume are analogous to those obtained by the determination of density.

The density of the *Hardenpont* winter pear was found to be 0.9673 $g\ cm^{-3}$. The difference between the densities of the ungraded and fractionated

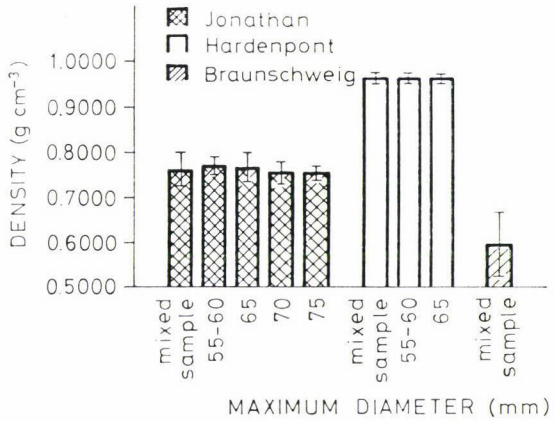


Fig. 1. Density of non-graded and size-graded winter apples, pears and white cabbage and the standard deviations

samples was small, statistically insignificant. On the other hand, it is true that in this case the picture is not complete because fractions with greater diameters have not been examined.

Tested without grading the density of the *Braunschweig* cabbage was 0.6076 g $cm^{-3}$ with rather large deviations. However, the change in the density of the product as a function of size in grading needs further experiments.

### 2.2. *Calculation of the volume*

The shape of the *Jonathan* apple is in good approximation a sphere, hence for a closer determination of its volume the formula known for rotational bodies can be applied. Starting from this observation and placing the specimen into the system of co-ordinates the volume of the *Jonathan* apple can be calculated (Fig. 2) after integration and substitution as follows:

$$V = \frac{\pi}{12}(3b^2a - a^3)$$

where a is the height measured along the middle line in cm,
b is the largest diameter in cm.

Accordingly, the average volume of our *Jonathan* apples, calculated from the measured characteristic dimensions, is 150 $cm^3$. The arithmetical average of the sums of parameters a and b is in close linear relationship with the values obtained for the volume (Fig. 3).

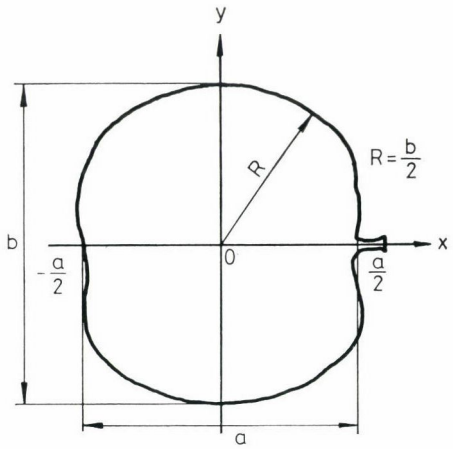


Fig. 2. Characteristics of the shape of *Jonathan* apples

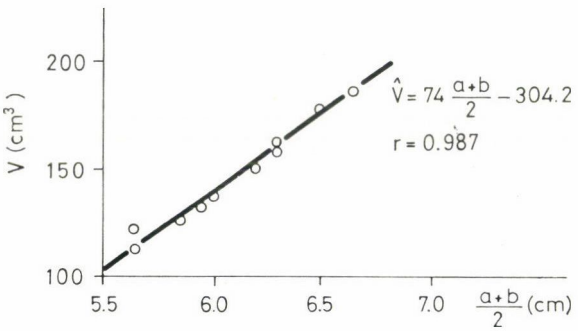


Fig. 3. Arithmetic mean of the maximum diameter and height of *Jonathan* apples related to the values of volume obtained by calculation

The equation of the regression line is

$$V = 74\frac{a+b}{2} - 304.2$$

and the correlation coefficient is

$$r = 0.987$$

The shape of the *Hardenpont* pear can be made up from two truncated cones (Fig. 4).

Thus, the volume of the *Hardenpont* pear can be determined as the sum of two truncated cones, without a significant modification of the data.

Without going into the details of derivation the volume of this fruit is

$$V = \frac{\pi}{12} \cdot b \cdot (a^2 + a \cdot a_3 + a_3^2)$$

where b is the height measured along the middle line in cm.

$$a = \frac{a_1 + a_2}{2}$$

where $a_1$ is the diameter of the fruit near the calyx (on the side of the calyx), in cm,

$a_2$ is the diameter of the fruit on the side of the stem, near the stem, in cm,

$a_3$ is the largest diameter, in cm.

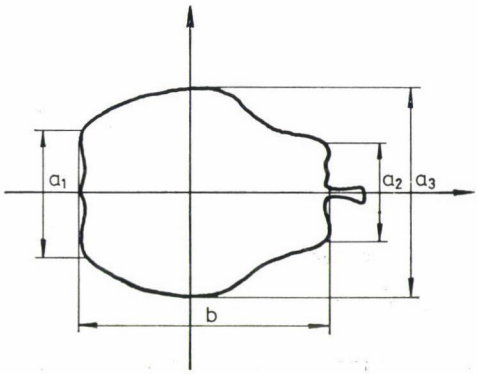


Fig. 4. Characteristics of the shape of *Hardenpont* pears

Both according to calculations and from individual volume determinations the average volume of the *Hardenpont* pear is 20 $cm^3$, and there is no significant difference between the calculated and empirically obtained data. The relationship between the largest diameter and the volume data obtained by calculation is illustrated in Fig. 5.

The relationship is unequivocally close and linear. Regression analysis results in the following equation:

$$V = 52.5\ a_3 - 201.1$$

$$r = 0.982.$$

The relationship between the largest pear-diameter and the individually determined volume data is shown in Fig. 6.

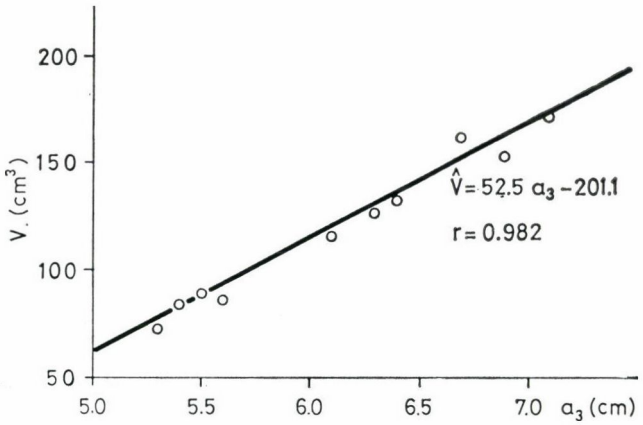


Fig. 5. Maximum diameter of *Hardenpont* pears as related to the calculated volume

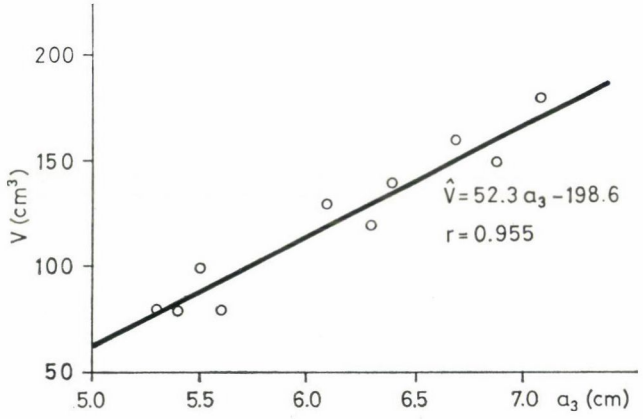


Fig. 6. Maximum diameter of *Hardenpont* pears related to the volume of pears as measured

The correlation is similar to that depicted in Fig. 5, the value of r is somewhat lower: r = 0.955, and the equation of the regression line:

$$V = 52.3\ a_3 - 198.6.$$

From the closely related factors illustrated in Figs. 5 and 6 the value of the volume can be obtained quickly and relatively accurately as a function of the greatest diameter.

The shape of the *Braunschweig* cabbage can be considered as a disk flattened at the edge, or an ellipsoid (discus-shape, Fig. 7).

The volume of individual pieces of the variety can be determined from the following correlation:

$$V = 0.52 \cdot a^2 \cdot b$$

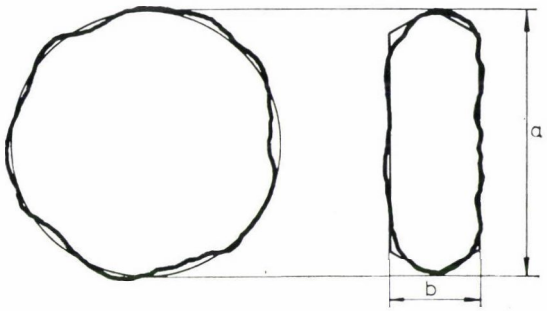


Fig. 7. Characteristics of the shape of white cabbage, variety *Braunschweig*

where a is the diameter of the disk, in cm,
b is the thickness measured in the middle, in cm, and
0.52 is a constant containing the shape parameters.

According to our calculations the average volume of a single *Braunschweig* cabbage is 3000 $cm^3$. There was no significant difference between the arithmetical average of the calculated volume and that determined by individual measurement. No correlation was found between the values of length and volume.

### 2.3. *Space filling*

A non-adequate utilization of the packed volume of stored boxes diminishes the income of the plant, while when this value approaches the optimum it is possible to increase the utilization of a given volume of refrigerated space. Our filling tests were devised to serve this aim.

The results of filling tests with *Jonathan* apples are shown in Table 3.

The standard deviation values of the series of data were compared by means of the *Bartlett* test (WEBER, 1961), and the results are given in Table 4.

Since at the probability level of $P = 99\%$ there was no significant difference in the standard deviation it appears that we have been dealing with a balanced homogeneous series of data.

In the filling tests it was attempted to avoid any surface damage of the fruit and it was found that significantly more of the 65-mm apples and significantly less of the 75-mm fraction than of the mixed sample could be placed into the plastic boxes. Into this particular box definitely the least amount of the 75-mm fraction could be packed. More fruit can be filled into the GEV box than into the plastic one with compartments, but this difference was not significant in our experiment.

Table 3

*Space filling tests with size-graded and ungraded* Jonathan *apples in fruit cases*

| Sample | Average piece per case | Analysis of variance |
|---|---|---|
| Ungraded: | | |
| M | 207 | M *vs.* $M_{65}$ * |
| G | 226 | M *vs.* $M_{70}$ — |
| Size-graded: | | |
| $M_{65}$ | 229 | M *vs.* $M_{75}$ * |
| | | M *vs.* G — |
| | | G *vs.* $G_{70}$ — |
| $M_{70}$ | 220 | |
| $M_{75}$ | 168 | $M_{65}$ *vs.* $M_{70}$ — |
| | | $M_{70}$ *vs.* $M_{75}$ * |
| $G_{70}$ | 239 | |
| | | $M_{65}$ *vs.* $M_{75}$ * |

—: $P < 95\%$

*: $P \geq 95\%$

The experiment consisted of 10 series of parallel measurements
M: Plastic fruit case for use in cold storage
G: Apple case GEV
Subscripts stand for largest fruit diameter (mm)
F: 4.28 Comparison of space filling coefficients
$F_{5\%}$:2.42 relative to size
$LSD_{95\%}$: 21.3

Table 4

*Homogeneity of variance of the space-filling data pertinent to* Jonathan *apples (*Bartlett-*test)*

| Sample | FG | $s^2$ | $\chi^2$ |
|---|---|---|---|
| Ungraded: | | | |
| M | 9 | 189 2 | 13 30 |
| G | 9 | 129.4 | 9.10 |
| Size-graded: | | | |
| $M_{65}$ | 9 | 147.3 | 10.36 |
| $M_{70}$ | 9 | 178.5 | 9.00 |
| $M_{75}$ | 9 | 75.3 | 5.30 |
| $G_{70}$ | 9 | 48.4 | 3.40 |

$\chi^2$: 5.46

From the data of the filling test, of the size of the box and of the true volume of the fruit the utilization of the volume of the plastic box can be calculated. In the case of ungraded apple this has a value of 58 %, in the case of the 65-mm fraction 64 %, of the 70-mm fraction 61 %, of the 75-mm fraction 47 %, in the case of classified fruit the average was 57 %. For the box of the type GEV and ungraded apple this value is 54 %, for the 75-mm fraction 57 %.

The data obtained in the filling tests with *Hardenpont* pear are shown in Table 5.

Table 5

*Space-filling test of* Harderpont *pears in plastic fruit cases*

| Sample | Average piece per case | Result of the general $t$ test |
|---|---|---|
| Ungraded: | 232 | |
| Size-graded: 55–60 mm ∅ | 264 | * |

*: $P \geq 95\%$
n: 10
t: 7.53
$t_{5\%}$: 2.10

In this experiment there was but a single size. It was found that, of the pears of 55 to 60 mm diameter, significantly more could be packed into the box than from the mixed batch, this, however, needs further investigation. The comparatively homogeneous size of the *Hardenpont* pear at the

Table 6

*Space filling test of white cabbage, variety* Braunschweig *in plastic cases*

| Sample | Average piece per case | Analysis of variance | |
|---|---|---|---|
| Ungraded, mixed | 8.7 | Mixed *vs.* size-graded: 29–32 cm ∅ | * |
| Size-graded | | | |
| 29–32 cm ∅ | 11.0 | Mixed *vs.* size-graded: 32–37 cm ∅ | – |
| 32–37 cm ∅ | 6.7 | Size-graded fractions: 29–32 cm ∅ *vs.* 32–37 cm ∅ | – |

–: $P < 95\%$
*: $P \geq 95\%$
n: 10
F: 8.70
$LSD_{95\%}$: 2.16
–: non-significant

time of harvest is indicated by the fact that the difference between packing coefficients of ungraded and graded samples justifies at the utmost the separation and testing of two more fractions.

In Table 6 the results of the filling tests with *Braunschweig* cabbage are summarized.

The results of the *Bartlett* test are given in Table 7.

The fluctuation of the standard deviations at the $P = 99\%$ probability level is not significant in this case either.

Table 7

*Homogeneity* (Bartlett-*test) of variance of space filling data pertinent to white cabbage, variety* Braunschweig

| Sample | FG | $s^2$ | $\chi^2$ |
|---|---|---|---|
| Ungraded, mixed | 9 | 1.79 | 18.09 |
| Size-graded: | | | |
| 29–32 cm ∅ | 9 | 0.44 | 4.49 |
| 32–27 cm ∅ | 9 | 0.45 | 4.61 |

$\chi^2$: 6.11

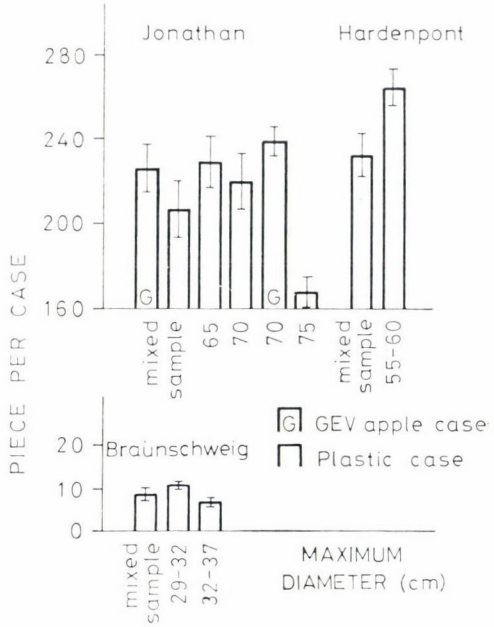


Fig. 8. Space filling test (piece per plastic case and GEV apple case) with ungraded and size-graded winter apples, pears and white cabbage, variety *Braunschweig*

Compared to the mixed sample definitely more of the fraction of relatively uniform size (29 to 32 cm diameter) obtained by grading can be packed into the box. Compared to this class, significantly less of the 32- to 37-cm fraction can be packed into the same box. The volume utilization coefficient of the ungraded sample is 48 %, that of the 29- to 32-cm fraction 61 %, and that of the cabbage with 32-to 37-cm diameter 37 %.

The result of the filling tests for all three investigated products are illustrated in Fig. 8.

We believe that further investigation of the utilized volume of containers might lead beyond the already mentioned targets to a possible revision of the size of boxes used for storing fruit and vegetables.

*

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# PRESERVATION OF OIL SEEDS BY INFILTRATION UNDER VACUUM

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A method of preserving or improving the storage stability of oil-seeds by infiltrating them under vacuum with antioxidant-treated molten fat was developed. The method is suitable for extending the storage stability of walnuts for one year at room temperature. Results were satisfactory in storage experiments with salted groundnuts.

The method is based on the fact that the infiltrating fat blocks the air passages of the plant particle, preventing thereby the oxidation of lipids present in the oil-seeds. At the same time the infiltrated fat prevents the drying out of the seeds.

The technique is easy to carry out under normal factory conditions, by batch or continuous treatment. The plant meterials treated by this method are suitable for all traditional applications.

Oil-seeds are plant particles originating from various taxonomic categories. They generally belong to the reproductive organs of plants (seeds, fruits), and certain fatty substances are accumulated in their vacuoles to serve as reserve nutrients for the young plant.

Oil-seeds are frequently divided into groups on the basis of the characteristic fatty acid in their fatty substances: *e.g.* oleic acid group, linoleic acid group, *etc.*

The fatty substances in the vacuoles are protected from oxidative damage by natural antioxidants. These, having a higher redox potential, are the first to be oxidised. After the exhaustion of the reducing capacity of the natural antioxidants comes the turn of the fatty substances to be oxidised. One type of oxidation damage is rancidity. Evidently, fats of more unsaturated bonds are more prone to oxidation. Thus, under identical conditions, fats belonging to the linoleic acid group have a tendency to become rancid prior to those belonging to the oleic acid group.

Apart from the natural antioxidants, the stability of fats and oils in the vacuoles depends on the histological structure of the oil-seed. GRACZA and co-workers (1969) found that the cotyledon of sunflower seed shows a dense microtexture in the anabiotic state during storage. However, the cell mass becomes gradually loose, the size of the cells increases manifold during germination.

It is evident that the air passages in a looser texture promote a more intense gas exchange than those in a denser texture, therefore the fatty substances in vacuoles of a looser tissue are more exposed to oxidative effects. Since, as shown by HASKÓ (1953), walnuts belong to the linoleic acid group, while hazel-nuts to the oleic acid group, furthermore, since their tissue structures differ considerably, it can easily be explained why walnuts are more prone to rancidity than hazel-nuts.

From this aspect groundnuts are nearer to hazel-nuts, belonging into the oleic acid group and having a denser structure. Thus, they can be stored satisfactorily. In the course of roasting the high temperature causes the proteins to coagulate, the viscosity of the oil is reduced and the oil leaves the vacuoles where it enjoyed certain protection. Roasting has a damaging effect upon the natural antioxidants, too. Thus, the proneness to rancidity in roasted groundnut substantially increases as reported by WOODROFF (1966).

It is a consequence of the facts enumerated above that oil-seeds do not play an important role in staple diet inspite of their high nutritive value. The composition of walnuts and groundnuts is shown in Table 1.

World-wide statistics show clearly that the cultivation and international commercialization of walnuts is fairly low. This may be unambiguously ascribed to their low storage stability. Walnuts are not suitable for longer storage (*e.g.* one year) or only under refrigeration which is costly. It is indicated by the above circumstances that walnuts are treated and consumed all over the world as delicacies. The food industry is for the same reasons averse to the utilization of walnuts and uses them only in the season.

The case of groundnuts is somewhat more favourable. This oil-seed, because of its unpleasant flavour, is not consumed in the raw state, only in the roasted state. However, in the latter state it is a staple food and an important raw material of the food industry (WOODROFF, 1966). In the United States about 50 products, confectionary and bakery goods, are manufactured from groundnuts based on about 100 recipes.

Roasted groundnuts, as mentioned above, are of low storage capacity. To overcome this setback packaging and packaging materials were carefully selected (laminated membrane, vacuum packaging, packaging under gas atmosphere, *etc.*). These procedures are, however, rather expensive. Storage in refrigerated storage space is expensive, too.

There are some other methods for the preservation of oil-seeds, such as the manufacture of canned and jam-like products, but the volume of these is insignificant. The aim of this study was to approach the problem of increasing the storage stability of walnuts and other oil-seeds on an entirely new path and this is infiltration under vacuum.

Table 1

*Oil-seeds. Nutrient and fatty acid composition of walnuts and peanuts, physical and chemical properties of their fat content*

| | Walnuts | Peanuts |
|---|---|---|
| *Nutrient composition*[a] (%) | | |
| moisture content | 4 | 8 |
| fat content | 64 | 47 |
| protein content | 19 | 22 |
| fibre content | 2–3 | 3 |
| mineral salts | 2 | 2 |
| *Fatty acid composition*[b] (%) | | |
| palmitic acid | 4.6 | 7.4 |
| stearic acid | 0.9 | 4.0 |
| arachic acid | — | 6.2 |
| oleic acid | 17.8 | 61.1 |
| linoleic acid | 73.4 | 21.4 |
| linolenic acid | 3.3 | — |
| *Physical and chemical properties of the fat*[b] | | |
| specific gravity at 20 °C | 0.924–0.926 | 0.915 –0.920 |
| refractive index at 25 °C | 1.4740 | 1.4680–1.4720 |
| point of solidification (°C) | –29 | 0 |
| saponification value | 188–194 | 188–194 |
| iodine number | 143–162 | 83–103 |

[a] MACZELKA (1962)
[b] HASKÓ (1953)

*Vacuum infiltration*

According to ERNST (1967), infiltration is a filtration process where the fluid or gas penetrates the discontinuous layer "in contrast to dialysis which is a spontaneous process, by the active intervention of an external force".

The external force is sometimes hidden. For instance the infiltration test applied by *Mollisch* to discover the openness of plant stomata (HARASZTY *et al.*, 1973) or the narcotic infiltration as used in veterinary practice by HEIDRICH and MÜLLING (1958) and others, which appear independent of external forces and are based mainly on physical and chemical forces.

Physico-chemical methods are applied also by NEUMANN and PRINZ (1974) to intensify spontaneous infiltration in beans by the addition of surface active agents.

Infiltration under vacuum, *i.e.* the introduction of a fluid into plant tissues under vacuum as an external force, was developed by plant physiologists. This is due to the fact that the plant organism does not posses such a perfect circulating system as the animal body, thus the introduction of active agents is more complicated.

At the same time in the tissues of plant organs there are many ducts and air passages and gas exchange takes place here. If the plant tissue is immersed in a fluid medium and completely covered by it and the surface of the medium is exposed to vacuum then the air from the intercellular ducts will be expelled and its place taken by the fluid medium. The medium may be pure water (McDonald & Mackeon, 1975), may contain biologically active agents, or even a bacterium suspension. The latter was applied by Groth (1966) to increase bacterial contamination in beans. It is possible to infiltrate under vacuum with a fatty substance and/or a substance of lipophilic character.

Infiltration under vacuum was made use of very little so far in the industry. Occasionally the utilization of this method is reported in the literature. Beelman and McCardle (1975) apply vacuum infiltration to mushrooms directly after picking to increase their water binding and retention capacity. Little was found on the application of the method in the confectionary industry. A procedure is mentioned by Purr (1960) for the promotion of the penetration of fermentation liquor into cocoa beans, using infiltration under vacuum. Taralli and Stefani (1972) used the same method to introduce an alkaline solution into the cocoa beans with the aim of digestion. The infiltration method in itself is very simple, easy to perform. The simplest apparatus is the laboratory-scale equipment assembled as shown in Fig. 1.

The industrial-scale equipment may be suitable for batch or continuous operation. For batch operation any small vacuum equipment, even a vacuum pan, as shown in Fig. 2, may be used.

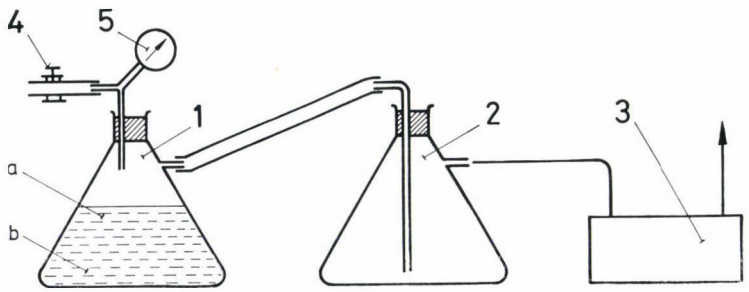


Fig. 1. *Schematic diagram of the laboratory-size apparatus for infiltration.* 1: Vacuum flask; 2: buffer flask; 3: vacuum pump; 4: Hoffmann clamp; 5: manometer; a: substance to be treated; b: liquid medium

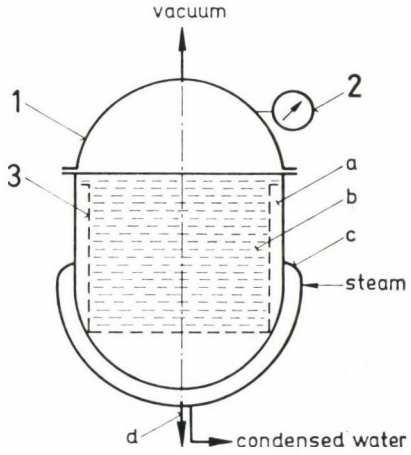


Fig. 2. *Schematic diagram of an industrial vacuum pan.* 1: Vacuum pan; 2: manometer; 3: perforated basket; a: liquid medium; b: substance to be treated; c: steam or hot water jacket; d: outlet

The material to be treated is immersed in the fluid medium in a perforated container or a basket. The equipment is then sealed hermetically and exposed to gradual evacuation. At the end of the predetermined period evacuation is discontinued and, after removing the container, the medium is drained off. Draining may be accelerated by vibration or blowing air on it.

The equipment for continuous operation is somewhat more complicated. Here the space used for infiltration has to be provided with adequate lockage and the rate of in- and output has to be selected. A continuous industrial equipment is shown in the schematic diagram in Fig. 3.

This continuous vacuum infiltration equipment was patented by TARALLI and STEFANI (1972).

If the medium applied is a fatty substance the temperature of infiltration has to be selected so as to keep the fat melted.

High temperature and the unnecessarily high vacuum has to be avoided because these increase the tension of water resulting in the drying out of the material and a waste of energy.

It seems that by the application of infiltration under vacuum the storage stability of oil-seeds prone to rancidity may be increased. The authors presumed that by using for infiltration a fatty substance as medium which is solid at room temperature and contains dissolved antioxidants, the effect will be two-fold, as follows:

- mechanical effect, the fat penetrates the air passages and, after solidification, it blocks them to the penetration of oxygen;
- chemical effect, the antioxidant dissolved in the fat medium increases the reduction capacity of the fats in the cells.

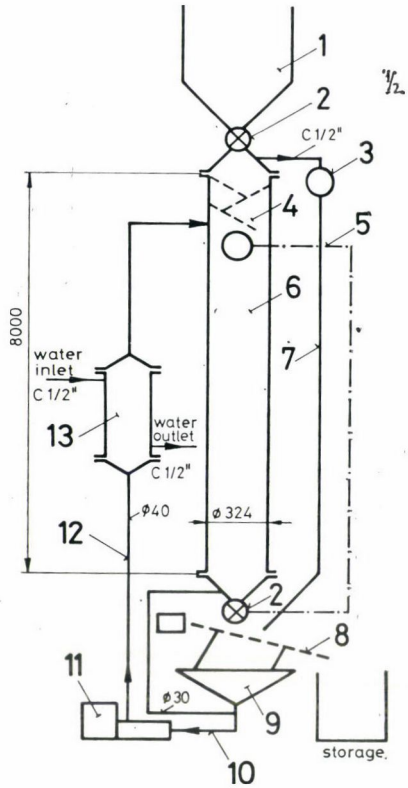


Fig. 3. *Schematic diagram of an industrial vacuum infiltration equipment for continuous operation.* 1: Buffer bin; 2: air closure; 3: vacuum pump; 4: baffles; 5: level control; 6: infiltrating chamber; 7: vacuum pipe; 8: vibro-sieve; 9: fat collector; 10: fat suction pipe; 11: pump; 12: pressure line; 13: calorifer

## 1. Materials and methods

### *1.1. Materials*

*1.1.1. Walnut kernel,* unknown variety, obtained from BUDAPEST CHOCOLATE FACTORY.

*1.1.2. Groundnut kernel,* freshly roasted, obtained from the BUDAPEST CHOCOLATE FACTORY.

*1.1.3. Cocoa butter,* obtained from the cocoa plant of the BUDAPEST CHOCOLATE FACTORY.

*1.1.4. Vegetable fat,* hydrogenated vegetable oil, Swedish product under the fantasy name *Acofect.*

*1.1.5. Butylated hydroxytoluene* (BHT) under the commercial name of *Topanol,* obtained from BUDAPEST MEAT PROCESSING ENTERPRISE.

*1.1.6. Common salt,* edible, fine.

*1.1.7. Packaging material,* 3-layer laminate (polyethylene—paper—aluminium foil), heat-sealed.

*1.1.8. Petroleum ether,* analytical grade.

*1.1.9. Quarz sand,* analytical grade.

*1.1.10. Ethyl alcohol,* 96%, analytical grade.

*1.1.11. Glacial acetic acid,* analytical grade.

*1.1.12. Potassium iodide,* analytical grade.

*1.1.13. Chloroform,* analytical grade.

*1.1.14. Starch,* analytical grade, water-soluble.

*1.1.15. Sodium sulfate,* 0.1 *N*, analytical grade.

*1.1.16. Sodium hydroxide,* 0.1 *N*, analytical grade.

*1.1.17. Ether,* analytical grade.

*1.1.18. Phenolphtalein* indicator in alcohol.

### *1.2. Apparatus*

*1.2.1. Laboratory vacuum infiltration apparatus* as seen in Fig. 1. The volume of the filtering flask is 1 litre.

*1.2.2. Vacuum pan.* Candy boiling pan at the BUDAPEST CHOCOLATE FACTORY of 150 l volume and provided with steam jacket.

*1.2.3. Soxhlet apparatus.*

*1.2.4. Drying oven* adjusted to 103 ± 2 °C.

*1.2.5. Perforated basket with perforated lid* of 25 l volume. Perforation diameter 5 mm.

*1.2.6. Air conditioned storage space.* Average temperature 20.6 ± 2.90 °C, relative humidity 55.8 ± 10.8% (controlled with a thermohygrograph).

*1.2.7. Laboratory candying pan* of 40 cm ∅.

*1.2.8. Laboratory vibro-sieve* with a series of standard sieves.

### *1.3. Methods*

*1.3.1. Infiltration under vacuum in the laboratory.* The apparatus described in para. 1.2.1 was used. About 500 ml melted fat containing 0.1–0.2% antioxidant (45–50 °C) is filled in the vacuum flask, 250 g of the substance to be infiltrated is placed into it. The flask is then sealed by the stopper and gradually placed under vacuum. To apply vacuum gradually is necessary because the content of the flask foames vigorously, due to the departure of steam and air. When the process of filtration is finished as shown by diminishing departure of air, vacuum is cut off (by loosening the *Hoffmann* clamp or turning off the vacuum pump). The treated substance is placed on a sieve (*e.g.* a colander) and the fat is drained. The fat may be reused in the next infiltration and draining may be intensified by the use of a vibro-sieve (para. 1.2.8.).

*1.3.2. Industrial scale infiltration under vacuum.* The vacuum equipment as described in para. 1.2.2 was used. The basket (para. 1.2.5) is filled with the substance to be treated and then placed in the pan containing approx. 100–200 l melted fat with 0.1–0.2% added antioxidant. The fat should cover the basket. The pan is sealed and a vacuum of 100–200 Torr developed. An infiltration period of 10–20 min is applied. At the end the vacuum is cut off, the pan opened and the perforated basket removed and allowed to drain. The treated substance is filled into polyethylene bags and used for storage experiments or, in the case of groundnuts, it is placed on a vibro-sieve to prapare it for salting.

*1.3.3. Salting of groundnuts.* The infiltrated and thoroughly drained roasted groundnut kernels are placed in the laboratory candying pan and salted with 1.5% salt. In order to obtain an evenly salted product it is desirable to blend it for 5 min. The control sample is treated with 1.5% fat and 1.5% salt in the candying pan.

*1.3.4. Determination of the moisture content by drying.* This is carried out at 103 °C.

*1.3.5. Determination of the fat content.* Extraction in the *Soxhlet* apparatus is applied.

*1.3.6. Determination of the acid number.* According to HUNGARIAN STANDARD MSz 3633-67 (1967).

*1.3.7. Determination of the peroxide value.* According to HUNGARIAN STANDARD MSz 19823-73 (1973).

*1.3.8. Sensory evaluation,* according to TAPODÓ and HIRSCHBERG (1975). The panel consisted of seven experienced members. Of each sample 100 pieces were distributed between the panel members. Not more than 6 samples were tested at a time. Water and apples were at the disposal of the panel members. The samples were tested for the following defective characteristics: rancidity, mouldiness, bitterness, by-taste (not foreign to the product, but not harmonizing with it, *e.g.* tartness in walnuts), foreign taste (foreign to the product, *e.g.* petroleum taste). Defective taste was scored in three grades: weak, medium, and strong. Defects were scored as seen on the next page.

The total score for defects is obtained by adding up the points assessed according to the above scale.

The sensory method was tested by the scoring of walnuts of different quality. Results are summarized in Tables 2 and 3.

Although, as it may be seen in the tables, the standard deviation belonging to each degree of spoilage, is different, the method proved to be suitable to follow the oil-seeds from the fresh state to complete spoilage. The 100-kernel method is centred around the product and if the scores for defects do not exceed 12, a product of satisfactory quality may be manufactured from the nuts.

| Nature of defect | | Extent of defect | | |
|---|---|---|---|---|
| Character | Symbol | Grade | Symbol | Score |
| Rancid | Ra | weak | a | 1.0 |
| | | medium | b | 2.0 |
| | | strong | c | 3.0 |
| Mouldy | Mo | weak | a | 0.6 |
| | | medium | b | 1.2 |
| | | strong | c | 2.0 |
| Bitter | Bi | weak | a | 0.6 |
| | | medium | b | 1.2 |
| | | strong | c | 2.0 |
| By-taste | Bt | weak | a | 0.2 |
| | | medium | b | 0.6 |
| | | strong | c | 1.0 |
| Foreign taste | Ft | weak | a | 3.0 |
| | | medium | b | 4.0 |
| | | strong | c | 6.0 |

Table 2

*Sensory evaluation of walnuts stored for 120 days in air-conditioned store-room**

| Serial number of sample | Nature of defect Ra | | Nature of defect Mo, Bi | | Nature of defect Bt | | |
|---|---|---|---|---|---|---|---|
| | Extent of defect | Score of partial defects | Extent of defect | Score of partial defects | Extent of defect | Score of partial defects | Total score for defects |
| 1. | 4a | 4 | 1a | 0.6 | 2a | 0.4 | 5.0 |
| 2. | 3a | 3 | 3a | 1.8 | 3a | 0.6 | 5.4 |
| 3. | 4a, 1b | 6 | 1a | 0.6 | 1b | 0.4 | 7.0 |
| 4. | 2a, 2b | 6 | — | 0.0 | 3a | 0.6 | 6.6 |
| 5. | 5a | 5 | 2a, 2b | 3.6 | 2a, 1b | 1.0 | 9.6 |
| 6. | 1a, 3b | 7 | 2a | 1.2 | — | 0.0 | 8.2 |
| 7. | 2a | 2 | 1a | 1.8 | 2a, 2b | 1.6 | 5.4 |
| Average | | 4.71 | | 1.37 | | 0.66 | 6.74 |
| Standard deviation | | ±1.80 | | ±1.19 | | ±0.51 | ±1.68 |

* Each sample consisted of 100 walnut kernels and was tested by a panel of seven members. Sensory defects under vacuum in cocoa butter with 0.3% Topanol.

Table 3

*Sensory evaluation of walnut kernels stored for 160 days in conditioned store**

| Serial number of sample | Nature of defect Ra | | Nature of defect Mo, Bi | | Nature of defect Bt | | Total score for defects |
|---|---|---|---|---|---|---|---|
| | Extent of defect | Score of partial defects | Extent of defect | Score of partial defects | Extent of defect | Score of partial defects | |
| 1. | 8,a 6b, 2c | 26 | 3a, 1b, — | 4.0 | 9a, 2b, 2c | 5.0 | 35.0 |
| 2. | 12a, 3b, 4c | 30 | 2a, 4b, 1b | 8.0 | 13a, 6b, — | 6.2 | 44.2 |
| 3. | 13a, 6b, 1c | 28 | 2a, 2b, — | 3.6 | 15a, 9b, — | 8.4 | 40.0 |
| 4. | 6a, 6b, 4c | 30 | 1a, 2b, 2c | 7.0 | 10a, 4b, 3c | 7.4 | 44.4 |
| 5. | 7a, 7b, 5c | 36 | — 2b, 1c | 4.4 | 8a, 7b, 1c | 6.8 | 47.2 |
| 6. | 10a, 8b, — | 26 | 2a, 1b, 1c | 4.4 | 10a, 5b, 3c | 8.0 | 38.4 |
| 7. | 9a, 5b, 4c | 31 | 1a, 3b, 1c | 6.2 | 12a, 6b, 2c | 8.0 | 45.2 |
| Average: | | 29.6 | | 5.4 | | 7.1 | 42.1 |
| Standard deviation: | | ± 3.46 | | ±1.69 | | ±1.20 | ±4.36 |

* Each sample consisted of 100 walnut kernels and was tested by a panel of seven members. Sensory defects were summarized for each sample separately. For symbols see para. 1.3.8.

## 2. Results

### *2.1. Infiltration of walnuts*

*2.1.1. Experiments carried out in the laboratory.* Several experiments were carried out. One of the experiments is described in the following.

Cocoa butter containing 0.3% Topanol was used. About 1 kg walnut-kernel was infiltrated under vacuum in the laboratory on February 5, 1975. A 20-min infiltration period was applied. Analysis of the drained product gave the results below:

| | Control | Infiltrated |
|---|---|---|
| Moisture content | 6.20% | 4.29% |
| Fat content | 54.62% | 56.04% |
| Acid number | 0.63% | 0.68% |
| Peroxide value | 2.44% | 3.33% |

Both the control and the infiltrated samples were placed each in an aluminium dish of 2 l volume and covered with paper. The samples were stored for 10 months and from time to time exposed to sensory control.

Results are given in Table 4.

Table 4

*Sensory evaluation of infiltrated and control walnut kernels stored in conditioned air and the laboratory*

| Number of days | In air-conditioned store | | | | | | | | | | In the laboratory without air-conditioning | | | | | | | | | | Note |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Infiltrated | | | | | Control | | | | | Infiltrated | | | | | Control | | | | | |
| | Ra | Mo Bi | Bt | Ft | Score for defects | Ra | Mo Bi | Bt | Ft | Score for defects | Ra | Mo Bi | Bt | Ft | Score for defects | Ra | Mo Bi | Bt | Ft | Score for defects | |
| 0 | | | | | 2a | | | | | 2 | | | | | 2a | | | | | 2 | Prior to infiltration |
| 112 | 5a | 3a | | | 6.8 | 2a | 1a | 3a | | 3.5 | 3a | 5a | 5a | | 7.5 | 12a | | | | 12 | |
| 150 | 2a<br>1b | 1a | | | 4.7 | 23a<br>4c | 4a<br>1b | 4a | | 39.8 | 2a<br>1b<br>1c | 2a | | | 10.2 | 37a<br>3b<br>8c | 13a<br>1b<br>2c | 4a | | 81.2 | |
| 205 | | | | | | 11a<br>14b<br>6c | 5a<br>1b<br>1c | 1c | | 64.2 | | | | | | 37a<br>5b<br>8c | 4b | | | 75.8 | |
| 302 | 1a<br>1b<br>1c | 1b | | | 11.4 | 10a<br>9b<br>6c | 20b<br>1c | | | 72.0 | 2a<br>2b | 1a<br>1c | | | 8.6 | 12b<br>65c | 4<br>2c | | | 227 | |

For symbols see para. 1.3.8.
Infiltration under vacuum with cocoa butter containing 0.3% Topanol.

Table 5

*Sensory evaluation of walnut kernels infiltrated with cocoa butter in the factory*

| Number of days | Stored in air-conditioned space | | | | | | | | | | Stored in a room without air-conditioning | | | | | | | | | | Note |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Infiltrated | | | | | Control | | | | | Infiltrated | | | | | Control | | | | | |
| | Ra | Mo Bi | Bt | Ft | Score for defects | Ra | Mo Bi | Bt | Ft | Score for defects | Ra | Mo Bi | Bt | Ft | Score for defects | Ra | Mo Bi | Bt | Ft | Score for defects | |
| 0 | 1a | 5a | 3a | | 10.5 | 1a | 3a<br>2b | | | 4.0 | 1a<br>1b | 5a<br>1b | | 3a<br>4b | 10.5 | 1a | 3a<br>2b | | | 4.0 | Day of treatment |
| 180 | 3a<br>1b | 5a<br>2b | 7a | | 12.5 | 18a<br>12b<br>9c | 6a<br>5b<br>2c | 6a | 2a | 92.8 | 1a | 10a<br>2b | | 3a | 13.3 | Not evaluable, completely rancid! | | | | | |

For symbols see para. 1.3.8.

The conclusions drawn from the results are:

- a slight decrease of moisture content accompanies infiltration,
- the fat content increases by about 10% as was observed when weighing the samples, too,
- the control sample, whether stored in an air-conditioned space or not, was spoiled in the 7th month,
- the samples treated, whether or not stored in air-conditioned space, were of good hedonic quality during the whole storage period.

*2.1.2. Infiltration in the factory.* The experiment was carried out on March 20, 1975. The vacuum pan, as described in para. 1.2.2 was used and

Table 6

*Sensory evaluation of walnuts infiltrated with shortening in the factory and stored in conditioned room*

| Infiltrated | | | | | | Control | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Number of days | Ra | Mo Bi | Bt | Ft | Score for defects | Ra | Mo Bi | Bt | Ft | Score for defects | Note |
| 0 | | | | | | 3a | | | | | Day of the treat- |
| | | | | | | 1b | 1b | — | — | 6.2 | ment |
| 124 | 2a | 5b | 7a | | | | | | | | |
| | 1b | 2b | | | 12.5 | 11a | 9a | 7a | 1a | | |
| | | | | | | 14b | 4b | | | 88.0 | |
| | | | | | | 8c | 5c | | | | |

cocoa butter containing 0.3% Topanol. Two hundred kg walnuts were treated, during a 20 min infiltration period at about 50 °C. At the end of the treatment the drained walnut-kernel was filled warm as it was, in polyethylene bags. A part of the sample was stored in conditioned space, the rest without air-conditioning, for 6 months. The results of sensory tests carried out at the end of the storage period are shown in Table 5.

The results given in the table prove the efficiency of infiltration under vacuum, even under non-conditioned circumstances of storage.

*2.1.3. Infiltration in the factory using shortening.* Essentially the same experiment as above, only using shortening instead of cocoa butter. The results of the sensory evaluation are shown in Table 6.

The results prove that infiltration with shortening is also suitable to extend the storage life of walnuts at 20 °C till the next harvest.

*Note:* In every experiment the walnut-kernels obtained a greyish fat coating on cooling. This coating was not disadvantageous if the nuts were used to make confectionary products, *e.g.* fillings, enriched chocolate, etc. When rinsed in warm alcohol the nuts became suitable for decorating purposes. The treated nuts retain their scrunchy consistency during storage.

### *2.2. Peanuts*

*2.2.1. Infiltration of peanuts with cocoa butter.* Peanuts were treated as described in para. 2.1.1. Draining was made more efficient by using vibrosieve. The peanuts were then salted and the product was found almost without defect. The nuts were stored in heat sealed bags and tested for sensory value after 1 year storage. Results are shown in Table 7.

Table 7

*Sensory evaluation of peanuts infiltrated with cocoa butter in the laboratory after one year of storage in air-conditioned space*

| Infiltrated | | | | | Control | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Ra | Mo Bi | Bt | Ft | Score for defects | Ra | Mo Bi | Bt | Ft | Score for defects |
| | | 4a | 1a | | 2a | 2a | 3a | | |
| | | | | 6.2 | 6b | 1b | 4b | — | 15.7 |
| | 1c | | | | 2c | | | | |

The control was made by salting peanuts with cocoa butter, without infiltration. As may be seen in the table, cocoa butter in itself prevents rancidity or the development of bitter taste, however, infiltration under vacuum offers even better protection.

*2.2.2. Infiltration of peanuts with shortening.* This experiment was carried out according to para. 2.1.3. The peanuts were kept under vacuum for 10 or 20 min. A part of the product was used to make salted peanuts. After 8 months storage the samples were exposed to sensory evaluation and chemical analysis. Results are given in Table 8.

As it may be seen infiltration with shortening is efficient, too. The time of treatment seems to be important as well. The sample treated for 20 min scored high, while the defects which have been observed were due mainly to the development of bitter taste rather than rancidity.

The scrunchy consistency was maintained during storage.

Table 8

*Sensory evaluation of peanuts infiltrated with shortening and chemical analysis after 8 months storage*

(Sample consisting of 200 kernels, results converted to 100 kernels)

| Control | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Analysis | | | |
| Ra | Mo Bi | Bt | Ft | Score of defects | moisture content % | fat content % | acid number | peroxide value |
| 9.5a | | | | | 3.01 | 50.47 | 1.39 | 6.98 |
| 13.5b | | | | 139.9 | | | | |
| 28c | | | | | | | | |

| Infiltrated during 10 min | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Analysis | | | |
| Ra | Mo Bi | Bt | Ft | Score of defects | moisture content % | fat content % | acid number | peroxide value |
| 2a | 1.5a | 4a | 10a | | 2.33 | 50.85 | 1.01 | 1.74 |
| 3b | 25b | 4b | | 66.1 | | | | |
| 2.5c | 1.5c | 2c | | | | | | |

| Infiltrated during 20 min | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Analysis | | | |
| Ra | Mo Bi | Bt | Ft | Score of defects | moisture content % | fat content % | acid number | peroxide value |
| 5a | 5.5a | 2a | | | 2.33 | 51.38 | 0.99 | 4.16 |
| 1b | 0.5b | | | 19.9 | | | | |
| 0.5c | | | | | | | | |

For symbols see para. 1.3.8,

## 3. Conclusions

The results of the experiments described show that the application of infiltration under vacuum is an extremely promising way of increasing the storage stability of oil-seeds. The fact that walnuts after treatment retained their sensory quality for a year when stored at room temperature may lead to their utilization in a wider range. Since originally walnut is a plant indigenous under warmer climate, it may be cultivated on a larger scale in countries, where cold storage is expensive and limited.

Further advantages of infiltration under vacuum are the following:

- complicated, specific equipment is not required,
- the process is of low energy requirement, therefore cheap,
- the surplus fat, introduced in the produce may be accounted for in confectionary recipes,
- no change is caused in the sensory quality of the produce,
- infiltration may be developed into a continuous operation, thus it is productive,
- expensive packaging materials and methods are rendered unnecessary.

The advantages of the infiltration technique may be utilized for other produce, too, to extend their storage stability. The method is successful if the technology, the kind of fat or acetyl glycerides, lately propagated, are used and the antioxidants are appropriately selected.

*

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# CHANGES IN COMPONENT FATTY ACIDS IN PULP LIPID OF RIPENING BANANA

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The fatty acid composition of pulp lipid from two banana varieties *e.g. Giant Cavendish* and *French Plantain* during ambient temperature ripening was studied. Although similar types of fatty acids were present in both the varieties, considerable differences in their quantitative distribution were observed at each stage of ripening. Irrespective of the variety, both palmitic acid and linolenic acid of pulp lipid increased with an appreciable decrease in linoleic acid content during the ripening of the fruit.

The contribution of lipids especially fatty acids to the development of numerous aroma compounds in fruits during ripening has been a subject of many investigations in recent years (TANG & JENNINGS, 1968; FORSS, 1969; TRESSL & JENNINGS, 1972; GHOLAP & BANDYOPADHYAY, 1975). However, reports on the influence of fatty acid constituents of banana pulp lipid and their compositional changes during ripening on the subtle differences in the aroma characteristics of different varieties are scanty in the literature. Studies on pulp lipid of ripening banana (variety *Valery*) indicated a marked change in the fatty acid composition of pulp lipid with ripening (GOLDSTEIN & WICK, 1969). TRESSL and DRAWERT (1973) investigated the enzymatic conversion of linoleic and linolenic acids into volatile carbonyl compounds in banana pulp homogenate. It has been observed that palmitic, linoleic and linolenic acid increased in contentration before climacteric rise but decreased in the maximum formation of aroma substances in *Gros Michel* banana variety (TRESSL *et al.*, 1970). The present paper reports on a comparative study on the fatty acid composition of pulp lipid from two varieties of banana during ripening at ambient temperature.

## 1. Materials and methods

### *1.1. Materials*

Two varieties of mature unripe banana *viz., Giant Cavendish* (local name: *Harichal*) and *French Plantain* (local name: *Rajeli*) were procured from a local market within 48 h after harvest. The respective variety in two batches was kept for ripening at ambient temperature (28–32 °C) in

perforated polyethylene bags. The samples were selected by a panel of five expert judges according to the state of ripeness determined organoleptically as follows:

- Raw: the peel was green and the pulp was hard and astringent without any ripe aroma.
- Half ripe: the peel was yellowish green and the pulp was slightly soft and astringent with perceptible characteristic ripe aroma.
- Fully ripe: the peel was uniformly yellow in case of *Rajeli* and yellowish green in case of the other variety. The pulp in both the cases was devoid of astringency and each had its own characteristic ripe flavour and aroma. The solvents used were of *Analar* (BDH) quality. Silica gel was from *E. Merck*, Federal Republic of Germany.

### *1.2. Analytical procedures*

*1.2.1. Pulp lipid.* Each sample was peeled, cut into small cubes and the pulp lipid was extracted with isopropanol followed by chloroform-isopropanol mixture (2 : 1, v/v) according to the method described by GOLDSTEIN and WICK (1969).

*1.2.2. Thin-layer chromatography (TLC).* TLC separation of lipid components of each sample was carried out on a silica gel plate (20×20 cm) using petroleum ether (B. P. 40–60 °C) : diethyl ether : acetic acid (90 : 10 : 1, v/v) as solvent system according to the previously described method (BANDYOPADHYAY & GHOLAP, 1973). The major components of pulp lipid were identified by comparing the $R_f$ value of authentic tripalmitin (HORMEL INSTITUTE, Minnesota), oleic acid (SIGMA), cholesterol (SIGMA), distearin (SIGMA) and lecithin (SIGMA).

*1.2.3. Glyceride and phospholipid content.* Glyceride and phospholipid content of each lipid extract was estimated colourimetrically according to the method of VAN HANDEL (1961) and FISKE and SUBBAROW (1925), resp.

*1.2.4. Fatty acid methyl esters.* Each lipid extract was saponified with alcoholic 2*N* KOH (A.O.C.S., 1958). Non-saponifiables were removed by repeated extraction of diluted soap solution with a mixture of petroleum ether : diethylether (1 : 1, v/v) and the fatty acids were recovered by acidification of the soap solution by 1*N* $H_2SO_4$ followed by extraction with diethyl ether and finally methylated by refluxing for 2 hrs with anhydrous methanol and 2 drops of *conc.* $H_2SO_4$.

*1.2.5. Gas liquid chromatography (GLC).* A *BARC* model gas chromatography equipment with a flame ionisation detector and with a stainless steel column (183 × 0.635 cm O · D), packed with 20% ethylene glycol succinate on acid-washed 60/80 mesh *Chromosorb* P (APPLIED SCIENCE LABORATORY, U.S.A.) was used for the determination of fatty acid composition of pulp

lipid of each sample. The oven and detector temperature was maintained at 190 °C, while that of injection port was at 235 °C. Nitrogen at a flow rate of 40 ml $min^{-1}$ was used as a carrier gas. The fatty acids were identified by comparison of their retention times with those of reference standards (SIGMA CHEMICALS, USA). Gas chromatograph peak areas were measured by multiplying peak height by peak width at half height.

## 2. Results

### *2.1. Pulp lipid*

Fig. 1 shows the TLC separation of pulp lipid of *Harichal* and *Rajeli* varieties at different ripening stages. It appeared from Fig. 1 that the pulp lipid constituents of both the varieties seemed to be identical and that there was no appreciable qualitative change in major lipid constituents of the respective variety during ripening of the fruit.

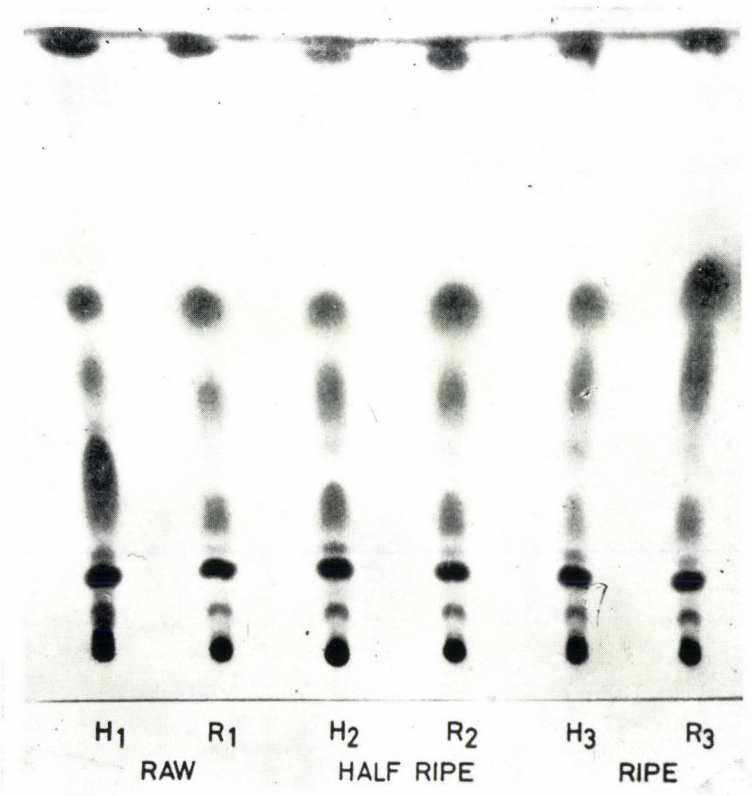


Fig. 1. TLC separation of pulp lipid from two varieties of ripening banana on silica gel plate. See experimental section for TLC details.
H: *Harichal* R: *Rajeli*

The major spots in both cases were identified as triglycerides ($R_f$: 0.55), free fatty acids ($R_f$: 0.42), sterols ($R_f$: 0.13), diglycerides ($R_f$: 0.07) and phospholipids ($R_f$: 0). It reveals from Fig. 1 that in both the banana varieties the free fatty acid content ($R_f$: 0.42) appeared to increase while that of unidentified constituent ($R_f$: 0.24) seemed to decrease during the ripening of the fruit.

Table 1 presents the pulp lipid, glyceride and phospholipid content of two varieties of banana during ripening. At ambient temperature storage, the *Harichal* variety took about 11 days to reach fully ripe state, whereas the other variety attained the same state after 15 days. The fully ripe banana pulp of *Harichal* was softer in texture and had stronger aroma and flavour as compared to that of *Rajeli*. It appeared that lipid, phospholipid and glyceride content of banana pulp remained practically constant within the same variety during ripening. However, phospholipid and glyceride content of pulp lipid of *Rajeli* was higher than that of *Harichal* at respective ripening states indicating that the varietal differences possibly influenced the pulp lipid composition.

Table 1

*Pulp lipid, glyceride and phospholipid content of banana pulp (variety* Harichal *and* Rajeli)[a] *at different ripening stages of banana stored at 28–32 °C*

| Ripening stage | Harichal | | | Rajeli | | |
|---|---|---|---|---|---|---|
| | Pulp lipid (% wt. of wet pulp) | Glyceride (% wt. of pulp lipid) | Phospholipid (% wt. of pulp lipid) | Pulp lipid (% wt.) | Glyceride (% wt. of pulp lipid) | Phospholipid (% wt. of pulp lipid) |
| Raw | 0.21±0.05 | 44.5±4.50 | 6.4±0.60 | 0.24±0.04 | 50.0±1.50 | 11.1 ±0.64 |
| Half ripe | 0.18±0.02 | 44.5±4.20 | 6.6±0.30 | 0.22±0.05 | 48.5±3.0 | 10.75±1.03 |
| Full ripe | 0.19±0.02 | 44.0±3.50 | 6.8±1.20 | 0.20±0.01 | 54.5±1.50 | 11.40±0.60 |

[a] Mean values ± standard deviation

## 2.2. *Distribution of fatty acids in pulp lipid*

The fatty acid composition of the pulp lipids of the two banana varieties at different ripening states is given in Table 2. Similar types of fatty acids were present in both the varieties, except for a difference in the presence of a minor amount of lower fatty acids in *Rajeli*. The most predominant fatty acids in pulp lipids of both varieties of banana were found to be palmitic and linoleic acids. Linolenic acid content of *Harichal* was observed to be much higher than in the other variety, where this fatty acid was virtually absent at raw state. On the other hand, *Rajeli* had higher linoleic acid content than *Harichal*. Irrespective of variety, both palmitic acid and linolenic acid

content increased with an appreciable decrease in linoleic acid content during the ripening of the fruit. Thus, the amount of unsaturated fatty acids decreased, though the linolenic acid showed some increase during ripening.

Table 2

*Fatty acid composition (% wt) of pulp lipid*[a] *of ripening* Harichal *and* Rajeli *banana during storage at 28–32 °C*

| Fatty acids | Harichal | | | Rajeli | | |
|---|---|---|---|---|---|---|
| | Raw | Half ripe | Fully ripe | Raw | Half ripe | Fully ripe |
| $C_{8:0}$ | — | — | — | — | 0.10±0.01 | 0.11±0.01 |
| $C_{10:0}$ | 0.18±0.04 | 0.30±0.01 | 0.52±0.05 | — | 0.20±0.02 | 0.40±0.01 |
| $C_{12:0}$ | 0.42±0.01 | 0.68±0.02 | 0.91±0.05 | 0.14±0.02 | 0.18±0.07 | 0.11±0.01 |
| $C_{14:0}$ | 0.54±0.07 | 0.61±0.09 | 1.48±0.38 | 0.82±0.03 | 1.39±0.15 | 0.69±0.01 |
| $C_{14:1(?)}$ | — | — | — | 0.41±0.02 | 0.50±0.01 | 0.50±0.03 |
| $C_{15:0(?)}$ | 0.27±0.02 | 0.29±0.05 | trace | 0.40±0.02 | 0.39±0.08 | 0.23±0.04 |
| $C_{16:0}$ | 37.17±3.10 | 38.30±0.20 | 44.10±4.25 | 33.20±2.50 | 34.70±4.40 | 38.40±1.14 |
| $C_{16:1}$ | 10.37±2.05 | 7.74±0.01 | 6.84±0.60 | 2.40±0.41 | 2.37±0.55 | 1.78±0.08 |
| $C_{16:2(?)}$ | 1.46±0.15 | 2.51±0.01 | 1.21±0.33 | trace | 0.88±0.07 | 0.73±0.08 |
| $C_{18:0}$ | 1.58±0.16 | 1.59±0.01 | 1.55±0.41 | 1.36±0.21 | 1.67±0.01 | 1.70±0.09 |
| $C_{18:1}$ | 10.98±0.20 | 10.80±0.40 | 10.84±0.46 | 5.43±0.33 | 5.10±0.16 | 4.80±0.40 |
| $C_{18:2}$ | 21.10±0.10 | 20.00±0.56 | 10.11±1.01 | 39.10±1.75 | 34.40±2.20 | 27.60±0.78 |
| $C_{20:0}$ | 9.76±0.42 | 8.58±0.13 | 9.38±0.95 | 17.20±0.50 | 15.60±1.20 | 20.42±0.85 |
| $C_{18:?}$ | 5.65±0.51 | 8.29±0.36 | 14.40±1.50 | trace | 2.54±0.17 | 3.30±0.90 |

[a] Mean value ± standard deviation.

## 3. Conclusions

The preponderance of various acyl esters of fatty acids and carbonyl compounds in banana aroma concentrate (TRESSL *et al.*, 1970) could presumably be explained due to the participation of certain fatty acids in the aroma biogenesis. Oleic, linoleic and linolenic acids (HULTIN & PROCTOR, 1962; TRESSL & DRAWERT, 1973) have been reported to be the precursors of various aromatic principles of banana. In the present study, though no definite correlation between fatty acid composition and aroma and flavour characteristics of banana could be established, it is likely that the differences observed in the quantitative distribution and changes in the fatty acids of pulp lipids during ripening might contribute to the subtle differences in aroma characteristics of different varieties of banana.

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# STUDY INTO THE MAILLARD REACTION OCCURRING BETWEEN METHIONINE AND TRYPTOPHAN ON THE ONE HAND AND GLUCOSE ON THE OTHER. PART II.— STUDIES IN MELTS

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The *Maillard* reaction, as occurring between methionine and tryptophan on the one hand and glucose on the other, in melts of temperatures above 100 °C was studied derivatographically.

The changes observed in the derivatogram were evaluated on the basis of earlier studies, by chemical analysis and by comparison with the derivatograms of pure substances of high molecular weight obtained by gel chromatography. The molar ratio of the sugar and the amino acid participating in the reaction was established by studies on those sections of the derivatogram which correspond to the *Maillard* reaction. It was found that 1 mole glucose cannot react with more than 0.67 mole methionine or 0.43 mole tryptophan. In accordance with the observations in aqueous solutions, the study of the reaction between tryptophan and glucose has shown both functional groups of tryptophan participating in the reaction.

Our knowledge of the *Maillard* reaction is based mostly on model experiments carried out in aqueous and organic solvents (Dworschák & Örsi, 1977). However, in the case of foodstuffs this reaction occurs frequently between solid phases or their melts (roasting, storage of dried vegetables, bread-crust formation, *etc.*). The study of these systems involves many difficulties. One of these is, for instance that the reaction is very fast at these high temperatures. In such a case the heating and cooling periods must not be neglected and isothermic studies may be accompanied by substantial error.

In earlier studies (Örsi, 1969; Örsi, 1972; Telegdy-Kováts & Örsi, 1973) in the course of the evaluation of the thermogravimetric curves of glucose and fructose, much useful information was gained on the mechanism of caramelization. From changes in the derivatograms occurring upon the addition of amino acids in these experiments, conclusions were drawn as to the mechanism of the reaction.

The same two amino acids, methionine and tryptophan, used in the experiments in aqueous solutions (Dworschák & Örsi, 1977) were chosen for the experiments presented here.

## 1. Materials and methods

### *1.1. Materials*

D-glucose, DL-methionine and DL-tryptophan, products of REANAL, Budapest, were used.

### *1.2. Methods*

*1.2.1. Production of colour substance fractions of high molecular weight.* In a beaker of 300 ml 600 mg D-glucose and 400 mg of the amino acid (DL-tryptophan or DL-methionine) were heated in a drying oven at 160 °C for 30 min. The mixture was then dissolved in 50 ml distilled water and the insoluble residue was removed by filtration. The colour substances were separated on a *Sephadex* G-25 gel column (Product of PHARMACIA, Uppsala, Sweden). A 10-ml portion of the reaction mixture was applied to the water-soaked gel column of 50 cm length and 40 mm diameter. It was eluted with distilled water at a rate of 50 ml $h^{-1}$. The eluate was collected in 10 ml fractions and the optical density of each fraction was measured against distilled water in a *Spectromom* 401 (MOM, Budapest) photometer at 400 nm. The elution curves are shown in Fig. 1. Fractions 16 to 30 contained the colour substances of high molecular weight. These were then combined and evaporated over a water bath. The colouring substance was dissolved in distilled water and eluted again to remove the impurities of low molecular weight.

*1.2.2. Derivatograph.* A *Paulik-Paulik-Erdey* derivatograph (MOM, Budapest) was used in the study. Either 100 mg of the D-glucose-amino acid mixture or 100 mg of the colour substance was weighed in the largest platinum dish. The temperature of the samples was raised from room temperature to

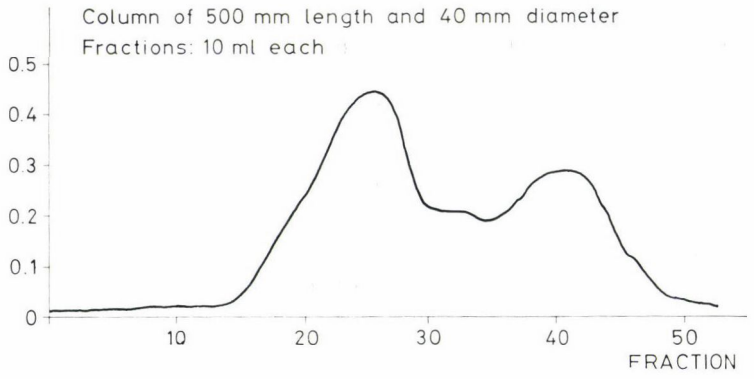


Fig. 1. Elution curve of the products of the *Maillard* reaction. *Sephadex* G-25 column 50×4.0 cm. Eluted in distilled water. Sample was prepared by thermal treatment of glucose and tryptophan as described in the text

700 °C at a rate of 6 °C $min^{-1}$, the process taking about 100 min altogether. During this time the temperature, change in weight, rate of weight change (differential thermogravimetry, DTG) and the differential thermoanalysis (DTA) curves were recorded.

From the DTG curve the beginning and end points of each reaction phase accompanied by weight change can be established while from the DTA curve the endothermic or exothermic character and the extent of the reaction can be determined.

*1.2.3. Recovering the D-glucose and the amino acids by thin-layer chromatography.* The amount of amino acids and of glucose in samples taken at various phases of the heat treatment and cooled was determined by thin-layer chromatography. Ten $\mu$l of the solution to be tested was applied to a *Kieselgel* G layer and developed in the 5 : 4 : 1 mixture of aceton, butanol and water. The solvent was removed by drying the layer in an oven of 100 °C. To detect the amino acids the layer was sprayed with 0.5% ninhydrin solution in ethyl alcohol, then heat treated at 100 °C. The layer was then sprayed with a 5 : 5 : 1 mixture of 4% solution of aniline in ethanol, 4% of diphenylamine in ethanol and 85% solution of phosphoric acid and was heat treated at 100 °C for 15 min to detect the sugars. The sugars appeared as brown spots on a light yellow background. The semi-quantitative determination was carried out by comparing the spots, obtained by applying solutions of known concentration of amino acid or sugar with those obtained from solutions of unknown composition. The $R_f$ values obtained were as follows: glucose 0.3, tryptophan 0.35, methionine 0.45.

## 2. Results

In order to clarify changes occurring in the course of the reactions the derivatograms of the pure amino acids and glucose and their mixtures of different composition were recorded. The derivatograms thus obtained were compared also with the derivatograms obtained with the colour substance of high molecular weight and separated by gel chromatography. At various phases of the reaction the derivatograph was opened, the substance was dissolved in water, then studied by thin-layer chromatography.

### *2.1. Results obtained by derivatography*

The derivatograms of the two amino acids are shown in Figs. 2 and 3.

The thermogravimetric (TG) and differential thermogravimetric (DTG) curves show that at temperatures below 220 °C the weight of amino acids does not change. The behaviour of methionine is particularly interesting

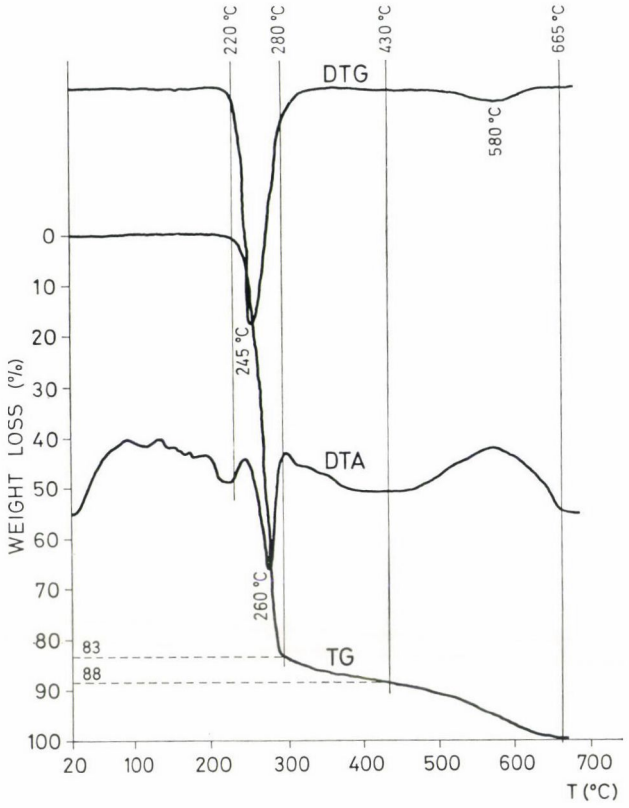


Fig. 2. Derivatogram of methionine. Tested: 100 mg. DTA and DTG sensitivity 1/5.

inasmuch as it suffers 80 % weig ht lossin the temperature range of 220–260 °C. Tests carried out in test-tubes have shown methionine to sublime in this temperature range, leaving only a small residue to become brownish char and to burn between 260 and 600 °C. Degradation of tryptophan occurs stepwise, sublimation was not observed.

Fig. 4 shows the derivatogram of glucose.

Changes start with the melting of glucose at 155 °C. As shown by the TG curve, up to and during melting no loss of weight occurs. If the sample, taken at this stage, is cooled a glassy amorphous product appears showing the signs of decomposition only by its light yellow colour. Decomposition is more significant in the range of 185–190 °C and the first break-down phase ends at 250 °C. On the basis of earlier experiments caramelization of glucose corresponds to this phase. Here, simultaneously with the formation of a substantial amount (*ca.* 20 %) of volatile substances, coloured intermediaries of low molecular weight are formed which later transform into coloured

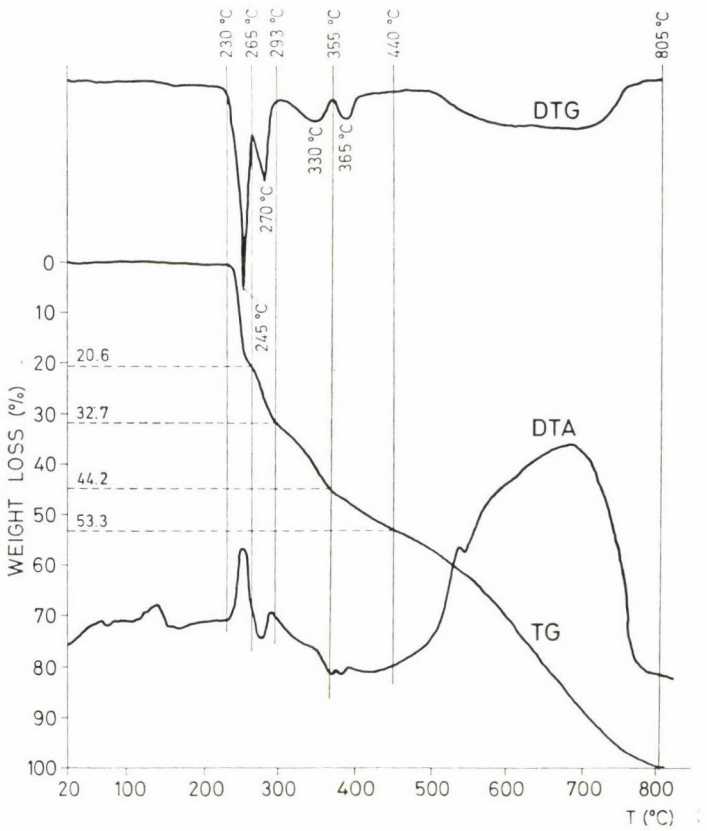


Fig. 3. Derivatogram of tryptophan. Tested: 100 mg. DTA and DTG sensitivity 1/5.

polymers of high molecular weight (10 to 20 thousand Dalton). A substantial amount of reversible oligo- and polysaccharide is also formed. In the 250 to 300 °C range further volatile substances appear while the polymers turn insoluble. In this range the oligosaccharides formed by thermopolymerization degrade.

In the range of 300–370 °C further weight loss may be observed due to the pyrolysis of the colour substances. The pyrolysis products form a very intricate mixture consisting of a great number of components. Thermopolymers of higher degree of polymerization, formed upon heat treatment from glucose, break down, too.

In the 370–600 °C range the residues of pyrolysis burn with the infiltrating oxygen to form carbon dioxide and water.

After elucidating the behaviour of the pure substances mixtures of the following ratio were prepared from amino acids and glucose: 5 : 95; 10 : 90; 15 : 85; 20 : 80; 30 : 70; 40 : 60; 50 : 50; 60 : 40; 70 : 30; 80 : 20, and 90 : 10.

Of these mixtures 100 mg each were taken and studied in the derivatograph under the above conditions.

Fig. 5 shows the derivatogram of the mixture of 60 mg glucose and 40 mg methionine.

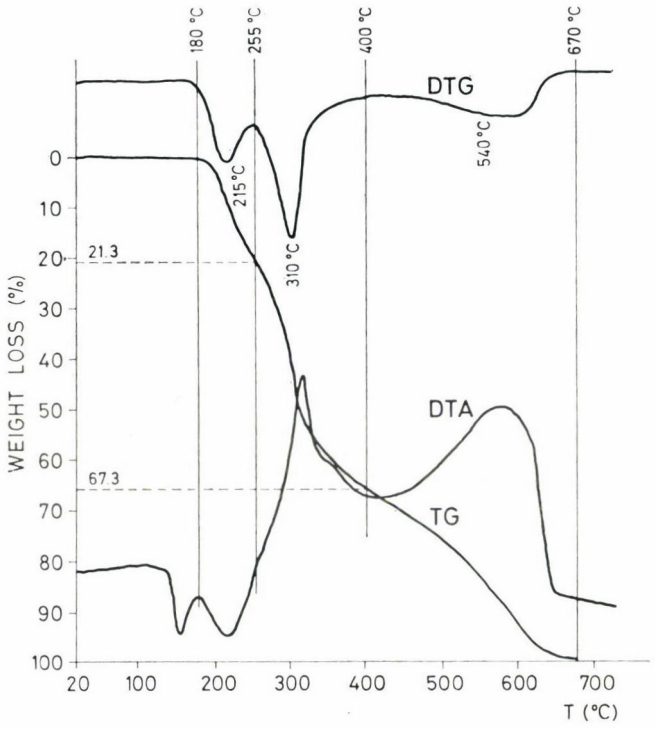


Fig. 4. Derivatogram of glucose. Tested: 100 mg. DTA and DTG sensitivity 1/5.

As it may be seen, decomposition begins already at 140 °C. In the range of 140–200 °C a process accompanied by substantial weight loss occurs, not seen in either of the derivatograms of glucose or methionine. The phase characterized by insolubilization can be found between 200 and 240 °C. The phase in the 240–370 °C range, accompanied by significant weight loss, and characteristic of the pyrolysis of the colour substance and the polysaccharides, is missing, or the weight loss is substantially lower. Above 400 °C the residue is oxidized in the infiltrating air.

Fig. 6 shows the derivatogram of the mixture of 30 mg glucose and 70 mg tryptophan.

The derivatogram in some aspects resembles, in others differs from, the previous derivatogram. Decomposition sets in at 150 °C and the phase corresponding to the *Maillard* reaction comes to an end at 220 °C. Decomposition occurs in two stages as distinct from that of methionine. The end of the first stage is at 170 °C as is apparent in the DTG curve.

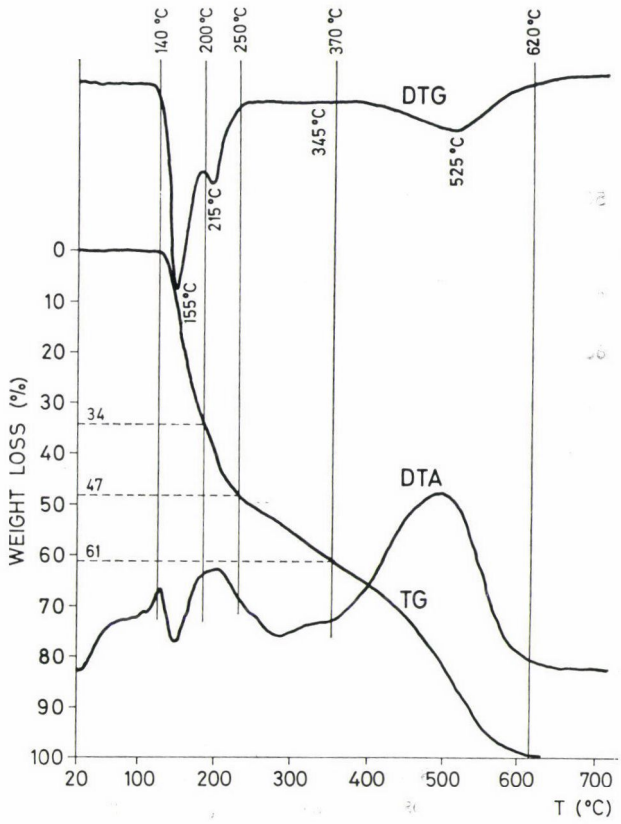


Fig. 5. Derivatogram of the mixture of 60 mg glucose and 40 mg methionine. DTA and DTG sensitivity 1/5.

The exothermic reaction observable in the 220–250 °C range is pertinent to the decomposition of DL-tryptophan and is found also in the derivatogram of pure DL-tryptophan along with the weight loss in the 255–290 °C range. In comparison to pure glucose the weight loss in the 300–600 °C range is lower. The residue of this phase is substantial and burns in the 400–600 °C range in an exothermic reaction.

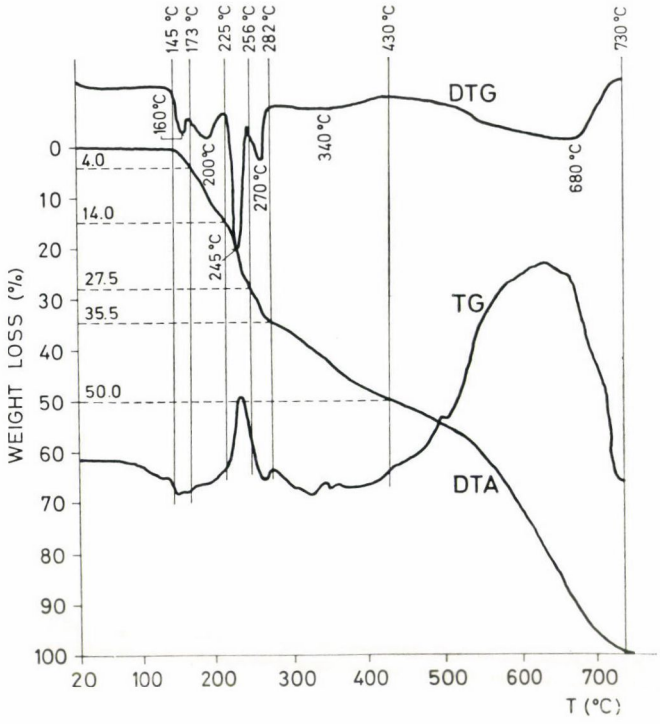


Fig. 6. Derivatogram of the mixture of 30 mg glucose and 70 mg tryptophan. DTA and DTG sensitivity 1/5.

## 2.2. *Construction of weight loss isotherms*

To characterize the glucose–amino acid mixtures of various composition, taking into account processes belonging to identical temperature ranges or of the same character, composition–weight loss diagrams were constructed from the derivatograms, where the isothermic points corresponding to the start and end points of the processes were connected by straight lines.

In Fig. 7 the composition–weight loss isotherms of the thermal break down of methionine–glucose mixtures are shown.

Open circles mark the start and end points of stages accompanied by weight loss as seen in the derivatograms. The points corresponding to identical stages of mixtures of various composition (start or end of a step), mostly of identical temperature, were connected by lines. The individual fields in the figure correspond to the sections of the thermal break-down processes.

From the aspect of this study the most important range was 150 to 190 °C where the *Maillard* reaction takes place. If methionine is in surplus (above 50% w/w) this stage is accompanied by the 220–270 °C range of

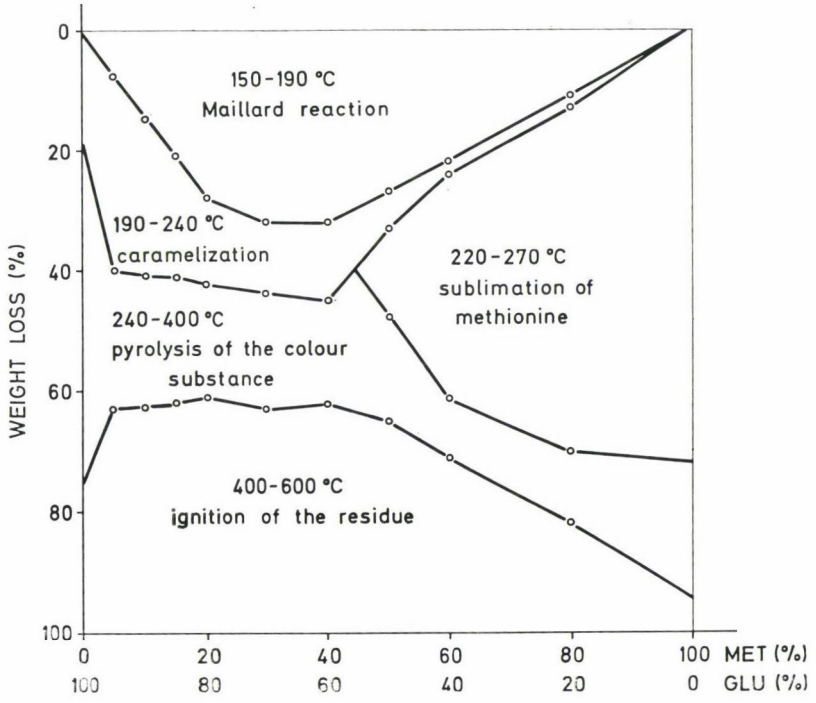


Fig. 7. Weight loss isotherms of methionine–glucose mixtures constructed from the derivatograms

methionine sublimation. However, at lower methionine concentrations this is not observed, although the aqueous extract of the sample contains methionine even at 250 °C. This is formed probably during dissolution in water. The break-down of glucose is rapid, at 190 °C only traces are found. In spite of this, with high glucose concentrations, in the 190–240 °C range a phase characteristic of caramelization may be observed. In the presence of methionine in excess the character of the process changes and shows weight loss accompanied by the colour substance becoming insoluble.

With increasing methionine content the weight loss occurring in the range of 240–400 °C and that connected to oxidizing of the residue in the 400–600 °C range, decrease. This is due to the fact that the surplus methionine evaporates and enhances the residue only to a small extent. At the same time the diminishing glucose produces less colour substance, too. At lower methionine concentrations it is the colour substance that first undergoes pyrolysis, while at higher concentrations the thermopolymerization products of methionine pyrolyse. This concept is supported by the increase in weight loss between 80 and 100% methionine content.

Fig. 8 shows the weight loss isotherms of tryptophan–glucose mixtures.

It is evident at first sight that decomposition processes become complicated by steps in the decomposition of amino acids. The section corresponding to the *Maillard* reaction separates into two well distinguishable sections as shown by the DTG curve. The first section falls in the range between 150 and 170 °C and the second between 170 and 200 °C. The second reaction is probably related to the tryptophan point of lower activity, since, as it may be seen in Fig. 8, it appears only at higher tryptophan concentrations (above 15%).

The steps of tryptophan decomposition in the ranges of 230–250 and 250–270 °C appear only at higher tryptophan concentrations, in the 250–270 °C range only above 50% tryptophan. The difference in the appearance of decomposition steps may be due to two reactive groups of the tryptophan molecule of differing activity. At 30% tryptophan concentration a part of the molecule is still bound, while the second part of the molecule is free and takes part in the decomposition reaction.

In the case of mixtures of high glucose concentration, residual glucose, not participating in the *Maillard* reaction, undergoes caramelization.

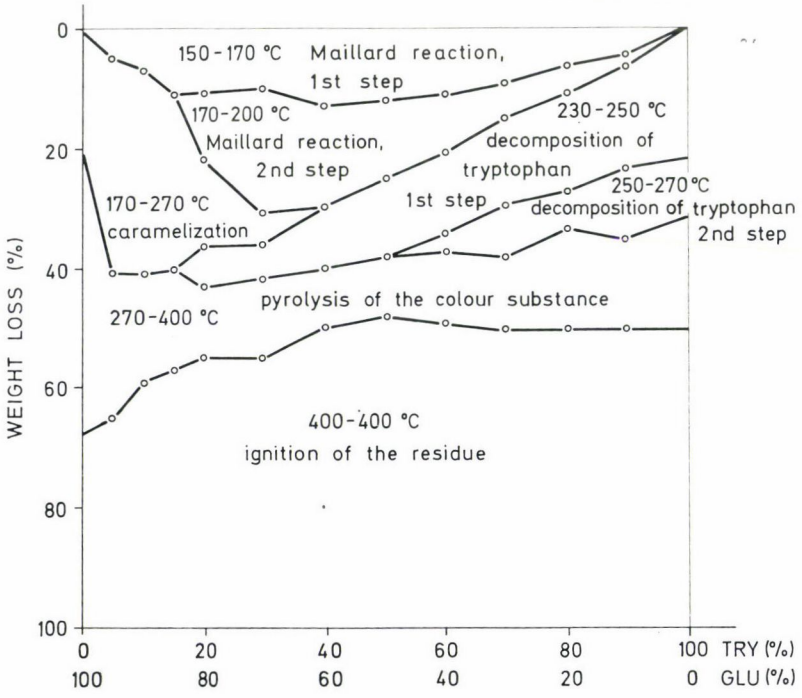


Fig. 8. Weight loss isotherms of tryptophan–glucose mixtures constructed from the derivatograms

Pyrolysis of the colour substance and the oxidization of the residue hardly changes with tryptophan concentration. At low tryptophan concentrations the weight loss upon pyrolysis is substantially reduced while the amount of residue increases. However, at high tryptophan concentrations there is no substantial change. This becomes clear taking into account that tryptophan does not sublime but is converted to char during degradation.

### *2.3. Derivatography of colour substances of high molecular weight*

In order to clarify processes observable in the derivatograms the derivatograms of the colour fractions of high molecular weight obtained by the technique described in para. 1.2, were recorded. These are shown in Figs. 9 and 10.

As it may be seen degradation becomes substantial only above 200 °C subsequent to the evaporation of moisture in the 20–170 °C temperature range. This was the range in which the colour substance became insoluble and pyrolysed. Keeping the sample in a desiccator above sulfuric acid no weight loss occurs in the range of 20 to 170 °C.

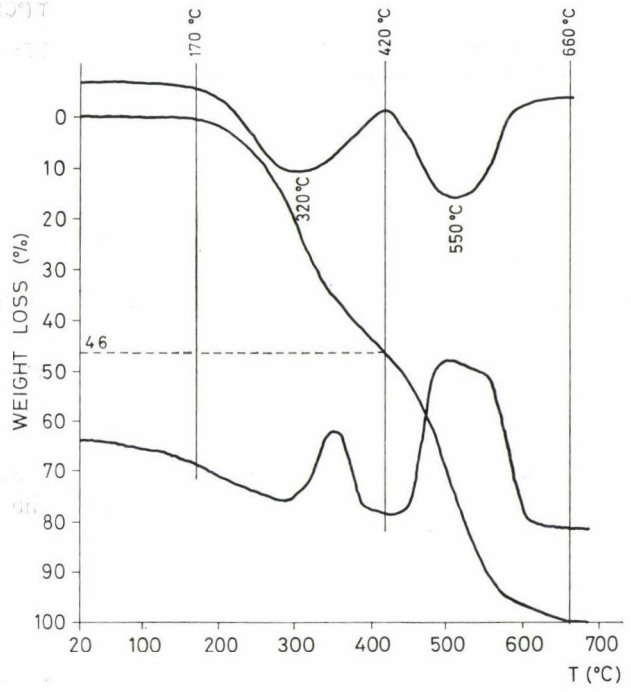


Fig. 9. Derivatogram of 100 mg colour substance isolated from a methionine–glucose mixture dried above sulfuric acid. DTA and DTG sensitivity: 1/5.

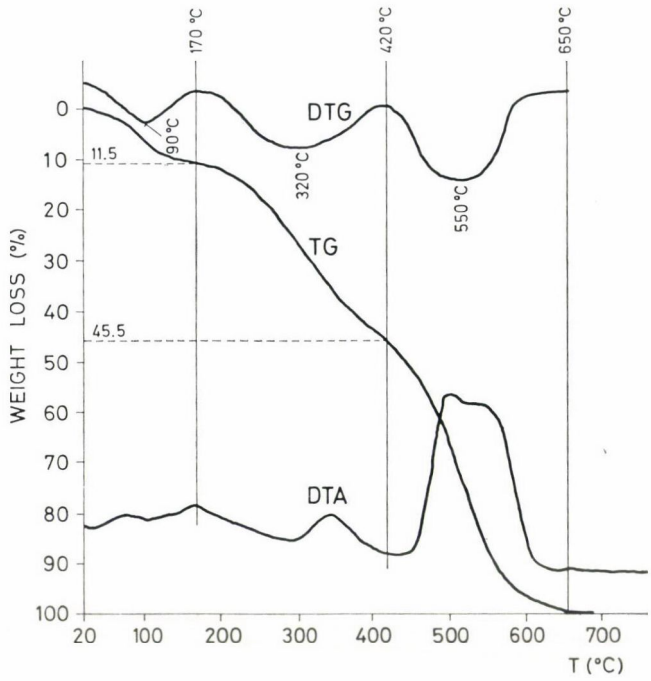


Fig. 10. Derivatogram of 100 mg air dried colour substance isolated from a tryptophan--glucose mixture. DTA and DTG sensitivity: 1/5.

## 3. Conclusions

In order to perform a more detailed analysis of the initial phase, below 200 °C, the range most important as regards the *Maillard* reaction, the relative value of weight loss, pertinent to each component was investigated as a function of composition.

The weight loss of methionine–glucose mixtures as observed in the range of 140–200 °C, expressed as percentage of glucose content, was plotted as a function of methionine content in Fig. 11.

As it may be seen in the Figure the weight loss of the mixture shows a linear increase with increasing methionine content in the range of 0 to 24 % methionine. Above this range it becomes independent of the amount of methionine. This proves methionine to be present above this range in excess and thus it does not participate in the *Maillard* reaction.

This assumption is supported by the set of isotherms shown in Fig. 7. The sublimation range of methionine between 220 and 260 °C appears only above 35 % methionine concentration. This proves that methionine in excess does not react with D-glucose and does not participate in the *Maillard* reaction.

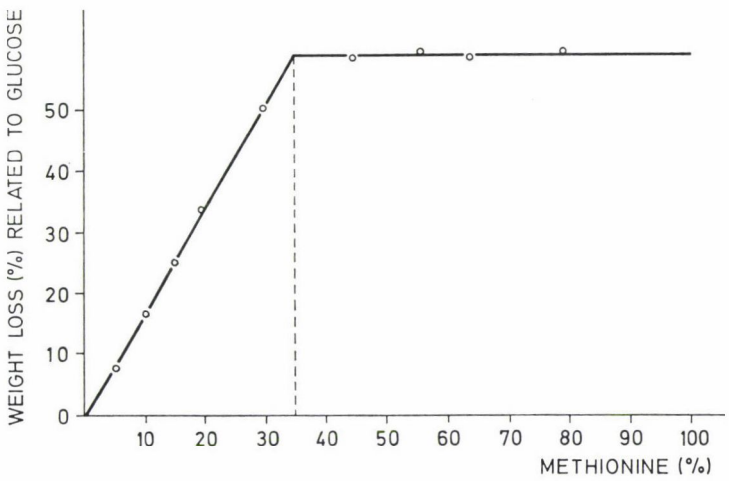


Fig. 11. Weight loss of glucose in the course of the *Maillard* reaction between methionine and glucose as a function of the methionine concentration

Below 35% methionine glucose is in excess, since the weight loss as occurring in the course of the *Maillard* reaction is determined by the amount of methionine present.

A methionine concentration of 35% corresponds in the mixture to 3 : 2 glucose–methionine ratio and corresponds to the ratio of concentrations where a breaking point is observable on the reaction rate *vs.* concentration curves. Above this ratio of concentrations the decomposition of methionine was found to proceed as a reaction of 0 order. The molecular ratio of 3 : 2 shows that in the initial series of reactions the formation of a unique, well defined *Schiff* base cannot be taken into account, as assumed by several authors. It seems that a part of the *Schiff* base rapidly passing through the *Amadori* transformation turns into monofructose-methionine which, reacting with a further molecule of glucose, turns into difructose-methionine.

Given the observed molecular ratio of 3 : 2, 50% of the methionine present reacts with two glucose molecules while the rest only with one.

In the case of tryptophan–glucose mixtures the *Maillard* reaction occurs in two distinct steps. Thus, the studies included both steps and the process in its entirety. The three curves are shown in Fig. 12.

The weight loss *vs.* tryptophan concentration curve shows two breaking points in the range of 150–170 °C. The first breaking point appears at the tryptophan concentration of 15%, corresponding to about 6 : 1 glucose tryptophan ratio. The second appears at 30–35%, in the same place where the curve of the second step as well as the curve of the summary reaction comes to a limit. The latter tryptophan concentration of 33% corresponds

to a 2.3 : 1 glucose–tryptophan molecular ratio. Above this ratio the first step shows slow increase, while the second one a slow decrease, and the net result of the two reaches a constant value as observed with methionine. The two partial steps did not give a constant value probably because their separation even by means of the DTG curve was not complete.

The molecular ratio of 2.3 : 1 is very near to the amino acid–sugar ratio in aqueous solution where the ratio of amino acid decomposition becomes independent of the sugar concentration upon increasing the latter, because then sugar is present in excess.

The assumption based on observations of the reaction in aqueous solution, namely that in the case of tryptophan beyond the participation

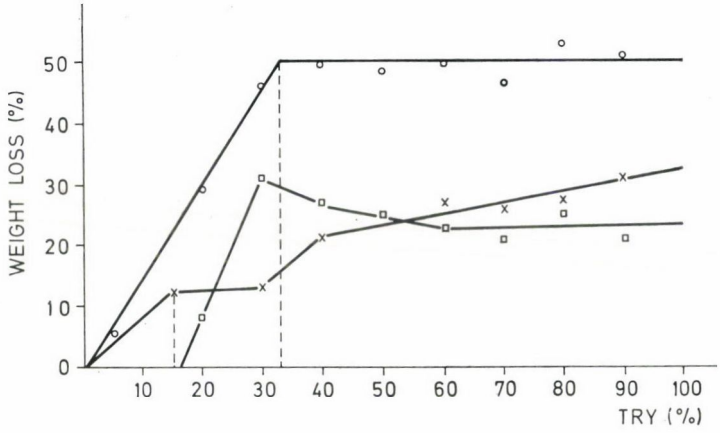


Fig. 12. Weight loss of glucose in the course of the *Maillard* reaction between tryptophan and glucose as a function of the composition of the mixture.
— o — marks the weight loss of the complete reaction, — x — that of the first phase, — □ — that of the second phase

of free amino groups the less basic imino groups of the indol ring are also taking part in the *Maillard* reaction, is supported by the occurrence of the *Maillard* reaction in two steps at a molecular ratio of 2.3 : 1 and the binding of more glucose at higher glucose levels. Although this reaction occurs only at high glucose surplus or at higher temperatures, it cannot be neglected.

Sublimation as observed with methionine does not occur with tryptophan, however, weight loss accompanying decomposition becomes apparent. Decomposition occurs in two distinct phases. However, the two phases differ. The first phase appears at a tryptophan concentration above 15% and the second becomes observable above 40%. Assuming the first step to be related to the splitting of the sensitive indole ring and the second one to the branched chain of amino acids, this may be considered a further proof of the different

participation in the *Maillard* reaction of the two reaction centres. However, to prove this more detailed study of the decomposition of tryptophan upon heating is required.

Summing up the conclusions it may be said that on the basis of the derivatograms of amino acid–glucose mixtures of different compositions, the phases accompanied by weight loss could be correlated with some actual and/or presumed phases of the *Maillard* reaction and the subsequent heat decomposition.

The weight loss as observed in the course of the *Maillard* reaction permitted of the establishment of the molar quantity of amino acid reacting with glucose.

Half of the methionine was found to react with two molecules of glucose and the other half with one molecule only.

In the case of tryptophan it now appears that in addition to the primary amino group the secondary amino group of the indole ring takes part in the reaction, too.

Thus, tryptophan is capable of reacting with more than two molecules of glucose. However, in the presence of amino acid in excess a phenomenon characteristic of the heat degradation of free tryptophan is also observable.

It seems expedient to continue the experiments with other amino acids. The investigation of later phases of the degradation processes may lead to useful findings. The reaction kinetic evaluation of the initial phase corresponding to the *Maillard* reaction is also in progress.

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# THE ACTIVITIES OF POLYPHENOL OXIDASE AND PEROXIDASE IN FRUITS AND VEGETABLES AS RELATED TO pH AND TEMPERATURE

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The pH and temperature dependences of the activities of polyphenol oxidase and peroxidase were studied in homogenates of various fruits and vegetables. The relationships between the enzyme activities and these two factors were found to vary with the product and cultivar. In the case of polyphenol oxidase the relationships were also dependent on the substrate applied.

Between 5 and 60 °C, the rates of pyrogallol and of hydrogen peroxide oxidation as catalyzed by polyphenol oxidase and by peroxidase from the homogenates of all fruits and vegetables studied gave a straight line in the *Arrhenius* plot. In homogenates of the apple cultivars *Jonathan* and *Starking*, oxidation of chlorogenic acid by polyphenol oxidase reached its maximum rate at 30 °C and 25 °C, resp. In the temperature range investigated, *i. e.* between 10 °C and 50 °C, the reaction rate of enzymatic chlorogenic acid oxidation gave a broken line in the *Arrhenius* plot.

The relationship between polyphenol oxidase activity and pH could be described, for different substrates, products and cultivars, by different curves. On chlorogenic acid substrate, enzyme activity in potatoes or apples was maximum at pH values of 6.1 to 6.45, whereas on pyrogallol it was found to increase up to pH 6.7 and 7.3, resp. In the pH range between 3.5 and 6.6 polyphenol oxidase activities of different cultivars of the same kind of fruit (*e. g.* the apples *Jonathan* and *Starking* or the peaches *Ford* and *Elberta*) varied in a more or less similar way. There were, however, striking differences in pH dependence between the two kinds of fruits. In the pH range between 3.4 and 8.1 peroxidase activity varied according to a maximum curve. For potatoes the maximum was observed at pH 5.0 and for kohlrabi in a broad range, between pH 5.1 and 6.6.

Of the enzymes producing undesirable changes during processing of horticultural products, polyphenol oxidase and peroxidase have been studied in the authors' laboratory for several years. From the very beginning of the investigations, when optimum pH and temperature had been selected for activity measurements, the high sensitivity of both enzymes towards these variables became obvious. In the course of these studies it could be established that not only the ranges of pH and temperature in which enzyme activities reached their maxima were different for the various products investigated, but that the relationships between reaction rates and above variables yielded curves of different characters for the two enzymes as present in different products. Findings published in the literature with respect to these relationships are divergent, too. Moreover, most of the communications deal with purified enzyme preparations. Therefore it seemed interesting to study more

thoroughly the pH and temperature dependences of these enzyme activities in the homogenates of some vegetables and fruits.

Since pH and temperature are factors which might play an important role in preventing enzymic browning, a more detailed study of the problem seemed indicated also from the point of view of industrial processing of fruits and vegetables.

## 1. Materials and methods

### *1.1. Materials*

All fruits and vegetables were harvested at the stage of full ripeness and kept at 4 °C until use.

*1.1.1. Materials used in the study of polyphenol oxidase (PPO).* Temperature dependence of PPO was studied in homogenates of apples (cultivars *Jonathan* and *Starking*), apricots (cultivar *Magyar*) and potatoes (cultivar *Gül baba*). pH dependence of the enzyme was determined in homogenates of the two apple cultivars, of potatoes *(Gül baba)* and of the peach cultivars *Ford* and *Elberta*.

Two substrates were used alternately: chlorogenic acid and pyrogallol. Temperature and pH dependences of PPO in *Jonathan* apples as well as the latter relationship for potatoes were established on both substrates. The rest of measurements were carried out on chlorogenic acid substrate, with the exception of the activity – temperature relationship for PPO in potatoes which was determined on pyrogallol.

*1.1.2. Materials used in the study of peroxidase.* Temperature and pH-dependences of peroxidase were studied in homogenates of potatoes and kohlrabi, (cultivar *Kék szalonna* = Blue Lard) the former relationship was established for the enzyme in apricots as well.

### *1.2. Methods*

*1.2.1. Assay of polyphenol oxidase (PPO) activity.* The methods used are based upon spectrophotometric measurement of the optical densities of the coloured compounds formed from the substrate by enzyme action.

In activity measurements on pyrogallol (VÁMOS-VIGYÁZÓ *et al.*, 1973) substrate concentration was 0.25 $M$, on chlorogenic acid (MIHÁLYI & VÁMOS-VIGYÁZÓ, 1976a) it was $3.4 \cdot 10^{-2}$ $M$. These values were necessary to ensure substrate saturation.

Enzyme activity was characterized by initial reaction rate. The enzyme producing a change in optical density of $1 \cdot 10^{-4}$ $min^{-1}$ was considered to be of unit activity (U).

*1.2.2. Assay of peroxidase activity.* Peroxidase activity was determined in reaction mixtures containing $7.4 \cdot 10^{-3}$ *M* $H_2O_2$ and *o*-phenylene diamine as chromogenic oxygen acceptor (MIHÁLYI & VÁMOS-VIGYÁZÓ, 1975).

Activity as characterized by initial reaction rate was considered to be of unit value (U), if the enzyme brought about a change in optical density of $1 \cdot 10^{-3}$ $min^{-1}$.

The activities of both polyphenol oxidase and peroxidase were related to 1 g of product, *i.e.* expressed in the dimension of enzyme concentration (U $g^{-1}$).

*1.2.3. Temperature and pH ranges of measurements.* Temperatures of activity measurements were varied between 5 °C and 60 °C and pH values between 2.45 and 8.1, by methods published elsewhere (VÁMOS-VIGYÁZÓ *et al.*, 1973; MIHÁLYI & VÁMOS-VIGYÁZÓ, 1976a; VÁMOS-VIGYÁZÓ *et al.*, 1977a). In most cases activities were below the limit of detectability well above the lower limit of the pH range selected. The upper limit of the pH range was set by rapid auto-oxidation of the substrates in the alkaline region. Incipient starch degradation, especially in potatoes, and the resulting unfiltratable turbidity of the reaction mixtures made OD readings at temperatures above the range indicated impossible. The lower limit of the temperature range was selected for technical reasons, *i.e.* the equipment used was not suitable to thermostating around 0 °C.

## 2. Results

### *2.1. Dependence of enzyme activities on temperature*

*2.1.1. Polyphenol oxidase.* Polyphenol oxidase activities of apples, apricots and potatoes as plotted against temperature are shown in Fig. 1.

PPO activity as determined on pyrogallol increased in both potato and *Jonathan* apple homogenates in the entire temperature range investigated, *i.e.* from 5 °C to 35 °C. Between 15 °C and 35 °C the increase was nearly linear.

On chlorogenic acid activity maxima were reached with both apple cultivars and with apricots in the range of 25 to 30 °C. The activity of the cultivar *Starking* steadily increased from 10 °C up to 25 °C where it reached its maximum to decrease somewhat steeper, but more or less at a steady rate up to 40° C, the limit of the range investigated. The activity – temperature curve obtained for the cultivar *Jonathan* is more complex: between 15 °C and 25 °C activity remains practically constant, then abruptly increases to reach a maximum at 30 °C, whereafter a similarly steep decrease takes place up to 35 °C. From this value onward, up to 50 °C, decrease in activity

with increasing temperature is less marked. The corresponding curve obtained for apricot PPO bears some resemblance to that obtained for *Starking* apples: maximum was reached, after a steady increase, at 25 °C and followed by a slow decrease.

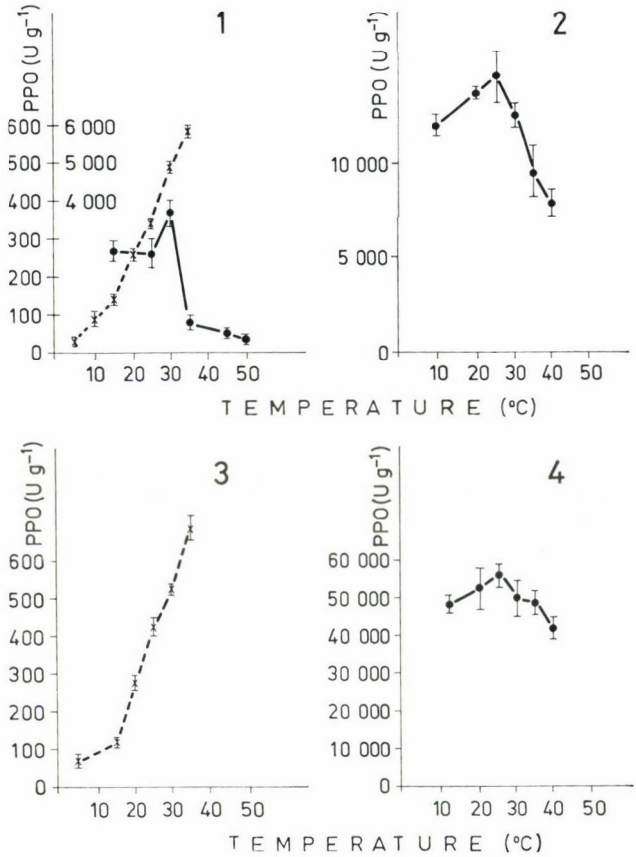


Fig. 1. Temperature dependence of polyphenol oxidase (PPO) activity in homogenates of *Jonathan (1)* and *Starking (2)* apples, *Gül baba* potatoes *(3)* and *Magyar* apricots *(4)*. Substrates: chlorogenic acid (full line); pyrogallol (broken line). Individual points of measurement were determined from the following numbers of OD readings. *1*: 9–21; *2* : 21–24; *3* : 21; *4* : 9–18. OD = optical density. The vertical bars represent standard deviations

*2.1.2. Peroxidase.* Temperature dependences of peroxidase in potato, kohlrabi and apricot homogenates are shown in Fig. 2.

The activity *vs.* temperature relationships obtained for the enzyme as present in various products are fairly different. In the temperature range investigated the curve of potato peroxidase did not reach a maximum. The

increase in activity was less marked between 7 °C and 35 °C, while considerable from 35 °C to 45 °C.

Activity of the kohlrabi enzyme was found to increase with temperature from 15 °C to 35 °C. Between 35 °C and 45 °C variations were not significant, whereas a further increase in temperature up to 50 °C led to a marked decrease in activity.

The activity of peroxidase in apricot homogenates increased in the temperature range from 15 °C to 50 °C and started to diminish above 50 °C. The decrease in activity was, however, not significant up to 55 °C.

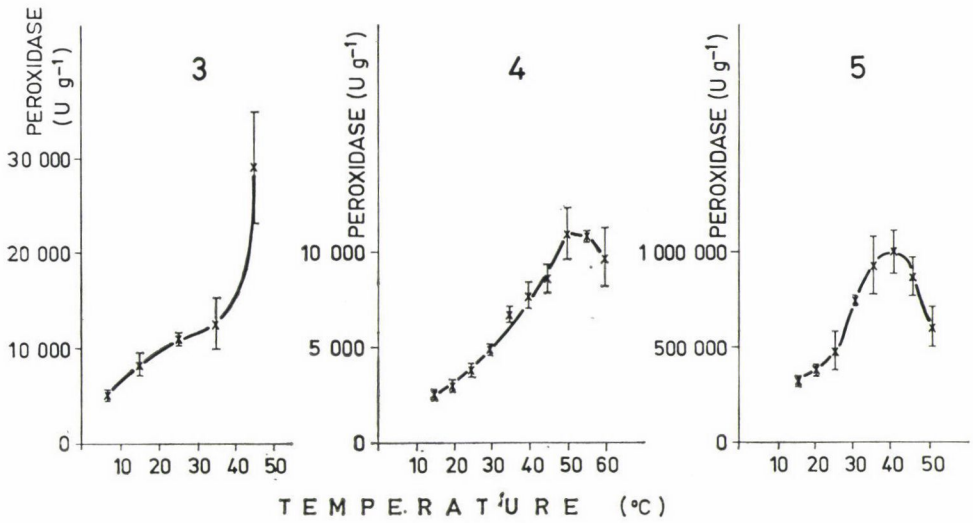


Fig. 2. Temperature dependence of peroxidase activity in homogenates of *Gül baba* potatoes *(3)*, *Magyar* apricots *(4)* and *Kék szalonna* kohlrabi *(5)*.
Substrate: $H_2O_2$ + *o*-phenylene diamine. Individual points of measurement were determined from the following numbers of OD readings. *1* : 36; *2* : 12–15; *3* : 9–24. The vertical bars represent standard deviations

*2.1.3. Activation energies of the enzyme reactions studied.* With the exception of chlorogenic acid oxidation as catalyzed by polyphenol oxidase, reaction rates were found to vary with temperature according to the *Arrhenius* rule. Apparent activation energies of these reactions were calculated (VÁMOS-VIGYÁZÓ *et al.*, 1976a; 1977a) and are summarized in Table 1.

Apparent activation energies of pyrogallol oxidation as catalyzed by polyphenol oxidase of potatoes and apples *(Jonathan)* were 13 000 and 16 000 cal $mol^{-1}$, resp. For the reactions catalyzed by the peroxidase of the products investigated the corresponding values were lower (between 5 290 and 9 690 cal $mol^{-1}$).

Table 1

*Apparent activation energies of the reactions catalyzed by polyphenol oxidase (PPO) and peroxidase in fruit and vegetable homogenates*

| Enzyme | Source of enzyme | Substrate | Apparent activation energy (cal $mol^{-1}$) | |
|---|---|---|---|---|
| | | | mean | standard deviation |
| PPO | Potato *(Gül baba)* | Pyrogallol | 13 000 | 1 340 |
| PPO | Apple *(Jonathan)* | Pyrogallol | 16 000 | 1 590 |
| Peroxidase | Potato *(Gül baba)* | $H_2O_2$ | 5 290 | 1 000 |
| Peroxidase | Kohlrabi *(Kék szalonna)* | $H_2O_2$ | 9 690 | 1 140 |
| Peroxidase | Apricot *(Magyar)* | $H_2O_2$ | 8 030 | 480 |

Apparent activation energies were established graphically from the relationships of reaction rate ($\Delta$ OD $min^{-1}$) *vs.* reciprocal absolute temperature ($K^{-1}$). The relationships were obtained from 4 to 7 points of measurement (reaction rate values) whereby individual points were determined from 9 to 36 OD-readings.

PPO and peroxidase activities were determined at pH 6.2 and 5.0, resp.

## *2.2. Dependence of enzyme activities on pH*

*2.2.1. Polyphenol oxidase.* The activity *vs.* pH relationships of PPO in homogenates of potatoes and *Jonathan* apples as assessed on pyrogallol and chlorogenic acid as well as the corresponding curves obtained on the latter substrate for the enzyme in two lots of *Starking* apples and in the peach cultivars *Ford* and *Elberta* are illustrated in Fig. 3.

The different behaviour, on a given substrate, of the enzyme as present in various products as well as of PPO of a given product on different substrates are clearly visible from the Figure.

On chlorogenic acid, PPO activity of apples was higher in the whole range investigated (from pH 4.6 to 6.6) than in potatoes. On the substrate pyrogallol the opposite was true for pH values above 5.7.

On chlorogenic acid PPO activity in apple and potato homogenates increased in the pH ranges of 4.6 to 6.2 and 5.0 to 6.2, resp. The slight decrease observed between pH 6.2 and 6.5 did not prove significant in either case, and the curves obtained for both products had a similar, somewhat sigmoidal shape. On pyrogallol the increase in PPO activity with increasing pH could be described by an exponential equation (VÁMOS-VIGYÁZÓ & KISS-KUTZ, 1974) for potatoes, while in the case of apples it followed an irregular curve up to pH 6.7, the upper limit of the range investigated. At pH values around 5, PPO activity in potatoes was, on both substrates, below the limit of detectability.

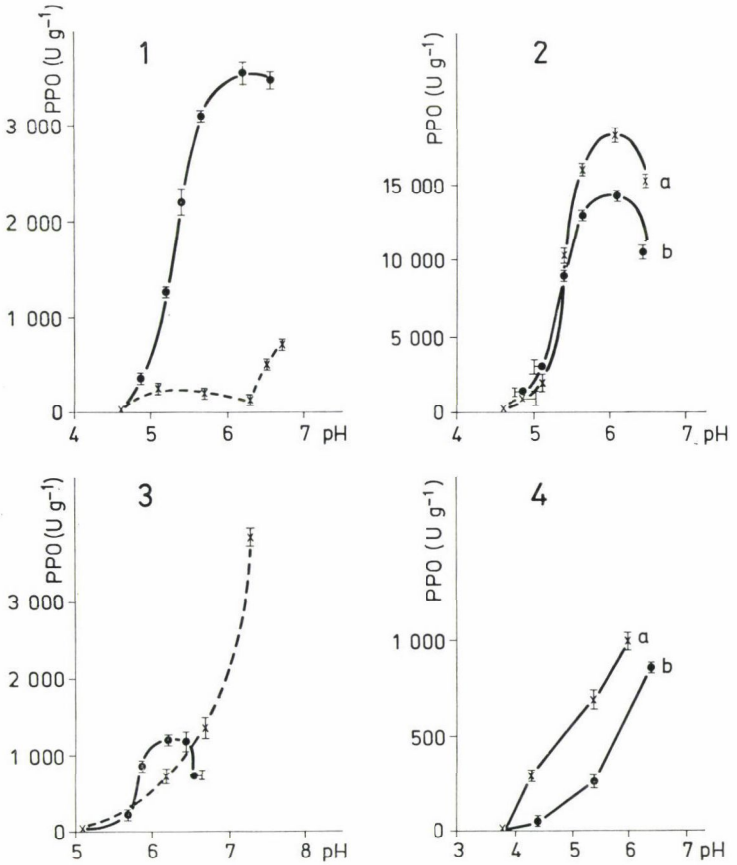


Fig. 3. pH dependence of polyphenol oxidase (PPO) activity in homogenates of *Jonathan (1)* and *Starking (2 a* and *b)* apples, *Gül baba* potatoes *(3)* as well as of *Ford (4 a)* and *Elberta (4 b)* peaches. *2 a* and *b* represent two lots of the same cultivar. Substrates: chlorogenic acid (full line); pyrogallol (broken line). Individual points of measurement were determined from the following numbers of OD readings. *1* : 12–25; *2* : 9–21; *3* : 12; *4* : 9–30. The vertical bars represent standard deviations

Variations, with pH, of PPO activity in peaches as observed on chlorogenic acid substrate were strikingly different from the behaviour of the two other products investigated. In apples, PPO activity was below the limit of measurability at pH 4.6, in potatoes at pH 5.1, while at the former value it is considerable in peaches of the cultivar *Ford* and slight, but measurable in those of the cultivar *Elberta*. At pH 5.1 both peach cultivars have fairly measurable PPO activities. *Starking* apples exhibited a well-defined activity maximum of PPO at pH 6.1, *Jonathan* apples at pH 6.2 and potatoes between 6 and 6.5, while in both peach cultivars enzyme activity steeply increased up to pH 6 and 6.4, resp., *i.e.* the upper limits of the range investigated.

In the whole pH range covered activities, where measurable, were lower in the cultivars *Jonathan* and *Elberta* as compared to *Starking* and *Ford*, resp.

The increase in activity with pH was found to be different even for different lots of the same apple cultivar *(Starking)*, especially in the range of the highest values (from pH 5.6 to 6.45). The maximum was, however, observed at the same pH for both lots.

*2.2.2. Peroxidase.* The variations of peroxidase activity of potatoes and kohlrabi with pH are shown in Fig. 4.

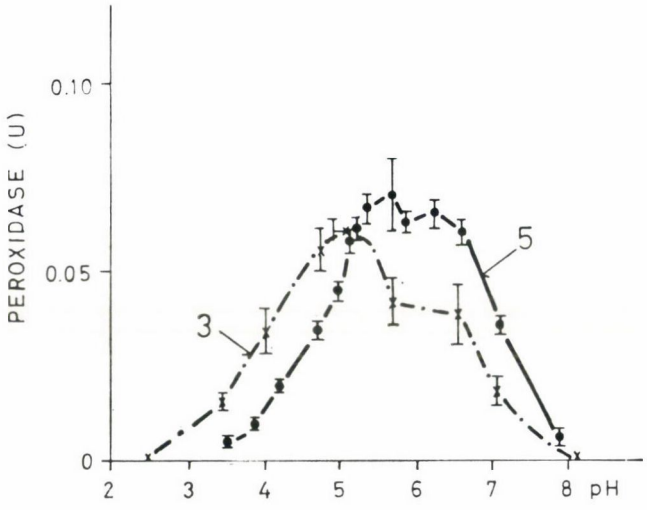


Fig. 4. pH dependence of peroxidase activity in homogenates of *Gül baba* potatoes *(3)* and *Kék szalonna* kohlrabi *(5)*. Substrate: $H_2O_2$ + *o*-phenylene diamine. Individual points of measurement were determined from 12 to 40 OD readings. The vertical bars represent standard deviations

In the pH range between 3.1 and 8.1 peroxidase values of both products were found to vary according to maximum curves. For potatoes peroxidase activity was maximum at pH 5 and the curve had a shoulder between pH 5.7 and 6.5. For the kohlrabi enzyme the range of maximum activity was broader, between 5.1 and 6.6. The slight decrease at pH 5.9 was not significant, neither were the two local maxima at pH 5.7 and 6.3. Instead of activity values as related to unit weight of product (U $g^{-1}$) reaction rates ($\Delta$ OD $min^{-1}$) were compared in this case, because differences in the enzyme contents of the two kinds of vegetables were of several orders of magnitude (MIHÁLYI & VÁMOS-VIGYÁZÓ, 1976b).

## 3. Conclusions

The results presented revealed considerable differences in the pH and temperature dependences of the two enzymes investigated in fruit and vegetable homogenates. The relationships between the parameters studied were found to vary with the kind of horticultural product, even with the cultivar and with the substrate applied. Hence, the validity of results obtained with either of these enzymes is restricted to a given cultivar grown in well-defined environmental conditions (VÁMOS-VIGYÁZÓ *et al.*, 1977a, b) as well as to identical experimental procedures (substrate, pH, *etc.*). This creates a somewhat embarrassing situation when comparing data from the literature with each other or with one's own experimental results. One of the objectives of the present study was to demonstrate, on well-defined fruits and vegetables and by means of well-established methods, the inherent character of these differences.

Some examples to illustrate the differences in the pH dependence of the activity of polyphenol oxidases from different sources shall be cited here. Activity optimum of apricot PPO was established between pH 5 and 6 (SOLER MARTINEZ *et al.*, 1965), that of the egg-plant enzyme at pH 7 (RHOADES & CHEN, 1968) while for a preparation from apple peel chloroplasts optimum pH was found to be 5.1 (HAREL *et al.*, 1964). It is easily conceivable that the action of pH on a soluble purified enzyme preparation is different from that on a mainly cell-bound enzyme as it occurs in tissue homogenates. Purified polyphenol oxidase preparations obtained from different sources, *e.g.* potatoes, apples or peaches were reported to have more than one pH optimum of activity (ALBERGHINA, 1964; HAREL *et al.*, 1964; PATIL & ZUCKER, 1965; WONG *et al.*, 1971). This is attributed, by some authors, to the presence of isoenzymes as formed during ripening (JEN & KAHLER, 1974).

The action of pH on the activity of polyphenol oxidase of a given product was found to be highly substrate dependent. Therefore, substrate specificity measurements have to be carried out in a broad range of pH to avoid erroneous conclusions. *E.g.* the values of PPO activity on potatoes as assessed on pyrogallol and chlorogenic acid are very similar in the pH range of 5 to 5.7, while at pH 6.2 the latter substrate yields considerably higher values. SCHWERDTFEGER (1970) reported on the similarity of the results of activity measurements as carried out with potato homogenates on above two substrates without specifying the pH. According to the results presented in Fig. 3, pH must have been below or around 5.7. It might be of interest that substrate specificity of enzymatic browning of apple slices was found to be pH dependent, too (VÁMOS-VIGYÁZÓ & GAJZÁGÓ, 1978).

The action of temperature on the activities of polyphenol oxidase and peroxidase was also found to depend on the substrate applied. The rate of

oxidation of chlorogenic acid, a natural substrate of PPO, did not vary with temperature according to the *Arrhenius* equation. Apparent activation energies as calculated from the *Arrhenius* plot for the reaction catalyzed by peroxidase in different products varied according to the source of enzyme and, in some cases, differed also from the value of 10 000 cal $mol^{-1}$ generally accepted for enzyme reactions (GUTFREUND, 1965). The same applies to PPO-catalyzed pyrogallol oxidation.

From the industrial aspect, the results presented might throw some light on the complexity of preventing enzymic browning, by demonstrating the differences in the behaviour of the enzymes of different products. Although enzyme activities are negligible at the natural pH values of the products, they are sufficient to cause browning. As to the temperature dependence of enzyme activities, it ought to be stressed that the slight percentage not destroyed by heat treatment and generally considered as negligible, may reflect the survival of heat resistant isozymes. These may, on the one hand, give rise to ulterior browning in the processed goods (SOLER MARTINEZ *et al.*, 1965) and, on the other, promote, in some products, reactivation of the enzymes (VÁMOS-VIGYÁZÓ, *et al.*, 1977a).

From the practical aspect measurements of enzyme activies in tissue homogenates are very important, because – as could be seen from the comparison of the results obtained to findings published in the literature – investigations carried out with enzyme preparations do not permit of any conclusions as to the behaviour of enzymes mainly bound to cell particles. At the same time, for reasons already mentioned, the knowledge of the isoenzyme system is of primary importance and, from the point of preventing enzymatic browning, indispensable. Work in this direction has been considered.

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# DETERMINATION OF PARTICLE SIZE OF VEGETABLE TISSUES BY A SEDIMENTATION TECHNIQUE AFTER ENZYMATIC DISINTEGRATION

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Tissues of seven kinds of vegetables (potato, carrot, squash, green pepper, parsley, celery and kohlrabi) were disintegrated by endo-polygalacturonase. After enzyme treatment dispersoid analytical investigations were performed on the tissue particles (cells and cell agglomerates smaller than 250 $\mu$m) of the filtrate of the disintegrated vegetable tissues by a sedimentation technique. The purpose of this work was to compare the granulometric data and the degree of dispersion of the various disintegrated vegetables and to determine the characteristic size of cells and particles of the individual vegetables following enzyme treatment.

According to the results of the sedimentation method extreme end values of the dominant particle sizes were found in case of carrot and potato. The smallest particles have been found in greater quantity in the filtrate of the disintegrated carrot tissues, while in the filtrate of potato tissues the largest particles were prevalent.

The characteristic particle sizes have been found in the finer size range (carrot and parsley: 10 $\mu$m; kohlrabi: 20 $\mu$m; squash and celery: 30 $\mu$m). Several vegetables can be characterized by two dominant particle sizes (green pepper and kohlrabi with 20 and 60 $\mu$m; parsley with 10 and 60 $\mu$m and celery with 30 and 60 $\mu$m) where, with the exception of green pepper, the frequency of the smaller particles was higher.

Vegetable tissues were treated with endo-polygalacturonase (endo-PG) enzyme in order to obtain individual cells or very small cell agglomerates. Vegetable suspensions produced by this enzymatic disintegration are suitable for the production of baby foods, dehydrated soups, sauces, as well as for vegetable juices and cocktails.

The stability of the juices and cocktails is influenced by the size of vegetable particles. The size of the disintegrated tissues is also not indifferent when the enzyme treatment is performed to produce basic materials for baby foods. The determination of the size of tissue particles can give useful information about the efficiency of enzyme treatment as well.

The sedimentation technique seemed to be the most suitable for the determination of the size of vegetable particles after enzymatic disintegration. This method has already been successfully used for studying the disintegrating effect of ultrasonics in the case of fruit juices (URBÁNYI, 1968) and the performance of an equipment (continuous bead mill) used for the mechanical disintegration of fungal mycelia (ZETELAKI-HORVÁTH *et al.*, 1974).

Sedimentation test was performed in the case of seven vegetables in this work, in order to determine the size distributions of the tissue particles after endo-PG treatment under optimal conditions (optimal pH, temperature and time of incubation).

## 1. Materials and methods

### *1.1. Vegetables used for the tests*

Carrot, parsley, potato, kohlrabi, celery, green pepper and squash tissues were disintegrated by endo-PG. The variety of the vegetables was not identified.

### *1.2. Enzyme treatment*

50 g of vegetables, grated into pieces of 10–30 mm were placed into 100-ml portions of a *McIlvain* buffer at various pH values (optimal for the enzymatic disintegration of the individual vegetables: *pH 3.0*: potato, celery, parsley: *pH 3.5*: carrot, squash, green pepper; *pH 4.0*: kohlrabi; ZETELAKI-HORVÁTH & GÁTAI, 1977) containing 25 mg of endo-PG and were incubated for 3 h at 50 °C on a shaker at 330 rpm. The activity of endo-PG given into the reaction mixture ($SPA_{75}^{Na\text{-}P}$) was 1 000 l $h^{-1}$ (determined by a viscosimetric method; ZETELAKI-HORVÁTH & VAS, 1972).

After incubation the reaction mixture was filtered through a set of screens (0.25 and 1.00 mm).

The filtrates of the disintegrated vegetables were used for the sedimentation test.

### *1.3. Preparation of samples for sedimentation*

Cells were separated from the filtrates by centrifugation at 2 800 rpm for 25 min. The centrifugal sediment was adjusted to the original volume with distilled water and centrifuged again. This procedure was repeated three times. The clean, washed cells and cell agglomerates were suspended again in distilled water and the dry matter content of the suspensions was determined. The individual samples were adjusted to approximately identical dry matter content (about 0.15%).

### *1.4. Description of the sedimentation technique*

Prior to examinations it was established at which particle diameters the measurements should be made. The largest diameter had to be determined in such a way that no particle greater than that diameter be present

in the system. The following diameter range was selected: 230, 150, 110, 80, 60, 40 and 20 $\mu$m. The settling times related to the individual diameters were calculated by means of *Stokes'* law as described in previous publications (URBÁNYI, 1968; ZETELAKI-HORVÁTH *et al.*, 1974).

Data of the results calculated, were plotted to obtain granulometric curves. Particle size distribution values were calculated from the granulometric data – at sites between points of measurement by interpolation – and plotted to obtain curves for the distribution of the degree of dispersion. The distribution was calculated for particle width intervals of 5 $\mu$m. In the figures the equivalent particle diameters have been designated with $d$.

### *1.5. Mathematical statistical calculations*

The standard deviation of the results of the 5 parallel sedimentation columns was calculated for each fraction and each vegetable. Granulometric data of the individual vegetables at the sizes of 16, 42 and 80 $\mu$m were compared to those of potatoes and carrots by means of the $t$ test and their significance levels were determined.

## 2. Results

### *2.1. Granulometric data*

Sedimentation test was performed from the suspensions of cells and cell agglomerates of various vegetable tissues (potato, carrots, parsley, kohlrabi, celery and squash) after enzymatic (endo-PG) disintegration. Samples of each vegetable were sedimented in five replicates.

The way of calculation of the granulometric data is shown by the results of one sedimenting column of kohlrabi (Table 1).

Mathematical means of the replicates were plotted out for granulometric curves (Fig. 1). The ordinate showing the percentage distribution and the abscissa the equivalent particle diameters *(d)* in $\mu$m. Granulometric data of all the tested vegetables are summarized in Table 2. (Data of Fig. 1 and Table 2 represent the mathematical means of the five sedimentation columns, calculated according to Table 1).

Data of granulometric curves indicate the total percentile quantity of particles the diameters of which are equivalent to those given on the abscissa or larger than these.

As it is shown in Fig. 1, data of the individual vegetables are rather different. In the case of potato, 94% of the sample belonged to particle diameters larger than 16 $\mu$m, while in the case of green pepper, squash,

Table 1

*Granulometric data of kohlrabi particles disintegrated by endo-polygalacturonase* (Enzyme concentration: 0.5%; temperature; 50 °C; incubation period: 3 h)

| Fraction | d ($\mu$m) | t (min) | $\Delta$t (min) | $\Delta$G (mg) | Pt (mg) | $\frac{\Delta G}{\Delta t}$ | $P_{II}$ $\left(t\frac{\Delta G}{\Delta t}\right)$ | $Pt-P_{II}$ | % |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 230 | 1.05 | 1.05 | 0.001 3 | 0.00193 | 0.00141 | 0.00153 | 0.00000 | 0.00 |
| 2 | 150 | 2.14 | 1.09 | 0.00138 | 0.00291 | 0.00120 | 0.00267 | 0.00024 | 2.09 |
| 3 | 110 | 3.32 | 1.18 | 0.00151 | 0.00442 | 0.00116 | 0.00409 | 0.00033 | 2.87 |
| 4 | 80 | 5.53 | 2.21 | 0.00151 | 0.00593 | 0.00054 | 0.00269 | 0.00185 | 16.11 |
| 5 | 60 | 7.55 | 2.02 | 0.00160 | 0.00753 | 0.00064 | 0.00530 | 0.00223 | 19.42 |
| 6 | 42 | 12.08 | 4.53 | 0.00165 | 0.00918 | 0.00059 | 0.00476 | 0.00442 | 38.50 |
| 7 | 28 | 18.17 | 6.09 | 0.00134 | 0.01052 | 0.00021 | 0.00385 | 0.00669 | 58.28 |
| 8 | 16 | 28.22 | 10.05 | 0.00096 | 0.01148 | 0.00009 | 0.00255 | 0.00893 | 77.99 |

Table 2

*Granulometric data of the particles of various vegetable tissues, disintegrated by endo-polygalacturonase enzyme* (Shown are averages of 5 parallel sedimentation tests; experimental conditions see: Table 1)

| Vegetables tested | Diameters of particles ($\mu$m) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 230 | 150 | 110 | 80 | 60 | 42 | 28 | 16 |
| | Quantity of particles (%) | | | | | | | |
| Potato | 0.00 | 2.15 | 20.45 | 57.88 | 69.12 | 84.13 | 89.51 | 93.72 |
| Green pepper | 0.00 | 0.77 | 2.13 | 14.88 | 19.38 | 51.81 | 63.02 | 79.25 |
| Squash | 0.00 | 6.09 | 10.04 | 11.81 | 18.56 | 26.04 | 42 70 | 76.82 |
| Parsley | 0.00 | 2.01 | 3.49 | 11.56 | 28.11 | 49.13 | 60.37 | 76.23 |
| Celery | 0.00 | 2.17 | 3.33 | 19.25 | 24.97 | 39.31 | 53.96 | 76.05 |
| Kohlrabi | 0.00 | 1.21 | 6.83 | 13.30 | 19.17 | 40.60 | 53.39 | 74.10 |
| Carrot | 0.00 | 1.74 | 10.12 | 18.30 | 23.07 | 36.54 | 49.67 | 68.20 |

parsley, kohlrabi and carrots, these quantities proved to be only 79.5, 77, 76, 74 and 68%, resp.

The difference among the vegetables is even higher in the fraction containing particles the diameter of which is equal to or larger than 60 $\mu$m. The quantity of potato particles belonging to this size range was 68% while, in the case of parsley, celery, carrots, green pepper, kohlrabi and squash, quantities as low as 28, 24.5, 23, 19.5, 19 and 18.5%, resp., have been found.

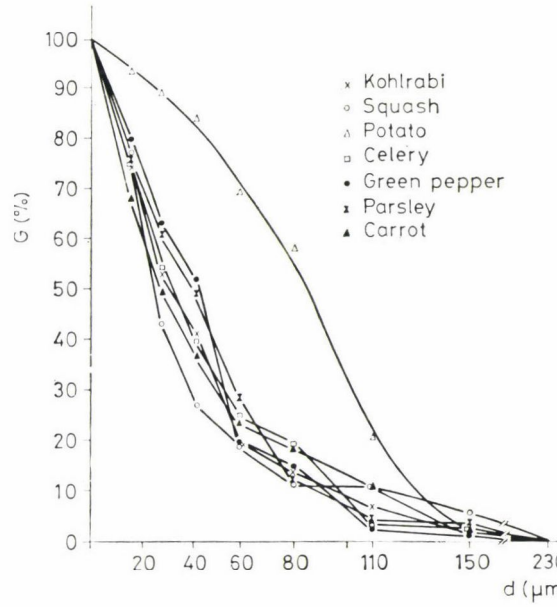


Fig. 1. Granulometric curves of vegetable suspensions prepared by enzymatic disintegration using endo-polygalacturonase. (Parameters of enzyme treatment as in para. 1.2; $d = 2r$, equivalent diameter of the particles; $G$ = weight of the mycelial particles in %)

Data of Fig. 1 and Table 2 clearly show that carrot particles have shifted towards finer distribution while those of the potatoes towards coarser distribution.

The standard deviations of the weights of the individual fractions collected from the 5 sedimentation columns are given in Table 3.

Table 3

*Standard deviation of the fractions of 5 replicates during the sedimentation test of the various vegetables disintegrated by endo-PG enzyme*

| Vegetables tested | Diameters of vegetable particles (μm) | | | | | | | Average deviation (%) |
|---|---|---|---|---|---|---|---|---|
| | 150 | 110 | 80 | 60 | 42 | 28 | 16 | |
| | Standard deviation of the quantity of vegetable particles (%) | | | | | | | |
| Potato | 0.722 | 7.260 | 5.834 | 5.712 | 4.226 | 3.145 | 2.192 | 4.155 |
| Green pepper | 0.537 | 0.070 | 3.191 | 4.804 | 6.911 | 4.937 | 2.525 | 3.282 |
| Squash | 2.534 | 2.234 | 2.004 | 2.604 | 3.349 | 3.411 | 6.511 | 3.378 |
| Parsley | 0.644 | 2.267 | 5.753 | 5.207 | 3.963 | 5.594 | 5.605 | 4.111 |
| Celery | 1.061 | 1.541 | 4.878 | 3.213 | 5.814 | 5.140 | 2.955 | 3.514 |
| Kohlrabi | 0.361 | 3.622 | 3.671 | 1.154 | 1.323 | 1.628 | 4.216 | 2.282 |
| Carrot | 1.756 | 3.764 | 2.037 | 4.512 | 4.238 | 5.518 | 2.895 | 3.531 |

Seven fractions were collected from each column and one kind of vegetable was sedimented in 5 columns, resulting in a total of 35 fractions. As shown in Table 3 the values of the average standard deviation proved to be between 2.3–4.2% in the case of the seven tested vegetables.

Granulometric data of the tested vegetables were compared to those of potato and carrot tissues, representing the two extreme dominant particle sizes, in the range of 16, 42 and 80 $\mu$m, by the $t$ test (Table 4).

Table 4

*Comparison of the size of vegetable particles, disintegrated by endo-PG enzyme, using the* t *test* (DF = 8)

| | $t$ values | |
|---|---|---|
| diameter: 16 $\mu$m | | |
| Vegetables tested | Potato | Carrot |
| Squash | 1.94* | 0.91 — |
| Parsley | 3.02** | 1.23 — |
| Celery | 3.43*** | 1.34 — |
| Kohlrabi | 3.06** | 0.83 — |
| Green pepper | 3.07** | 2.03* |
| Carrot | 5.02*** | — |
| diameter: 42 $\mu$m | | |
| Squash | 7.66*** | 1.81 — |
| Parsley | 4.27*** | 1.54 — |
| Celery | 4.46*** | 0.28 — |
| Kohlrabi | 7.84*** | 0.73 — |
| Green pepper | 2.90** | 1.37 — |
| Carrot | 5.62*** | — |
| diameter: 80 $\mu$m | | |
| Squash | 5.88*** | 1.61 — |
| Parsley | 4.00*** | 0.87 — |
| Celery | 3.60*** | 0.14 — |
| Kohlrabi | 4.69*** | 0.88 — |
| Green pepper | 4.77*** | 0.65 — |
| Carrot | 5.03*** | — |

* significant
** highly significant
*** very highly significant

The quantity of particles with a diameter larger than 16 $\mu$m attained 93.7 % in the case of potato. The quantities of carrot and celery particles were very highly ($P \geq 99.9\,\%$) significantly lower in this size range, while the amount of particles of parsley, kohlrabi, pepper and squash differed highly significantly ($P \geq 99\,\%$) and significantly ($P \geq 95\,\%$), resp., from those of the potato.

The quantity of potato particles, the diameter of which was larger than 42 $\mu$m was 84.1 %, was highly significantly higher than the value for green pepper and significantly higher than the values for all the other vegetables tested.

57.9 % of the potatoes consisted of particles larger than 80 $\mu$m, while among the particles of the other tested vegetables this size was found in a very highly significantly lower quantity (11–18 %).

When carrot was compared to the other vegetables a significant difference was found in the case of green pepper only, the suspension of which consisted of particles larger than 16 $\mu$m in a significantly higher proportion than that of carrots. According to the comparison between the size of potato and carrot particles in the above three fractions, the quantity of carrot particles larger than 80 $\mu$m proved to be highly significantly lower, while the quantity of those larger than 42 and 16 $\mu$m were present in a very highly significantly greater proportion in carrot than in potato digest.

Evaluation of size ranges was performed and cumulative distributions were presented for each of the given size categories. Figs. 2 and 3 show the granulometric data of the samples as assessed for each range.

Largest quantities (20 and 39 %) of carrot particles were found in the size categories of 0–10 and 0–20 $\mu$m in diameter, while in the size ranges of 0–30, 0–40 and 0–50 $\mu$m squash particles were found in the largest and carrot particles, in the smallest percentage. In the size category of 0–40 $\mu$m, the quantity of celery particles was also large.

Ninety per cent or more of the vegetables could be found in the size category of 0–100 $\mu$m with the exception of potatoes the particles of which occurred in the above range only to the extent of 70 %.

### *2.2. Particle size distribution*

Distribution constants were calculated from the granulometric data (at sites between points of the curve by means of interpolation) which were plotted to obtain the distribution of size. Distributions of the degree of dispersion for each vegetable related to intervals of 10 $\mu$m were calculated. The frequency distribution of particle sizes of the various vegetables was plotted against particle diameters *(d)* in Fig. 4.

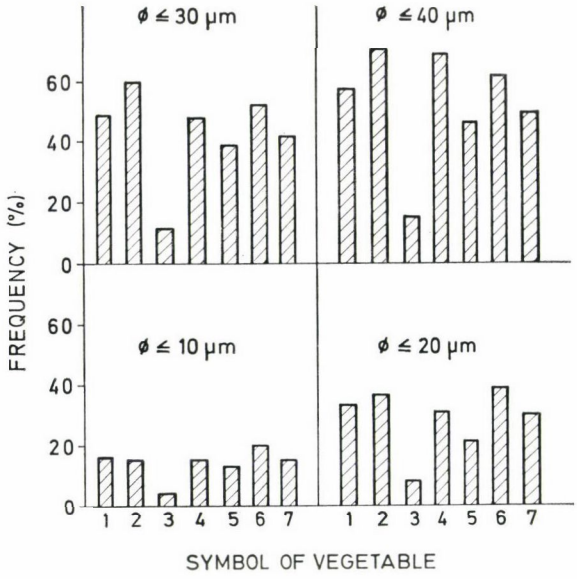


Fig. 2. Cumulative frequency in the 0–40 $\mu$m range of particle diameters in suspensions of vegetable tissues disintegrated by endo-polygalacturonase. 1: Kohlrabi, 2: Squash, 3: Potato, 4: Celery, 5: Green pepper, 6: Carrot, 7: Parsley

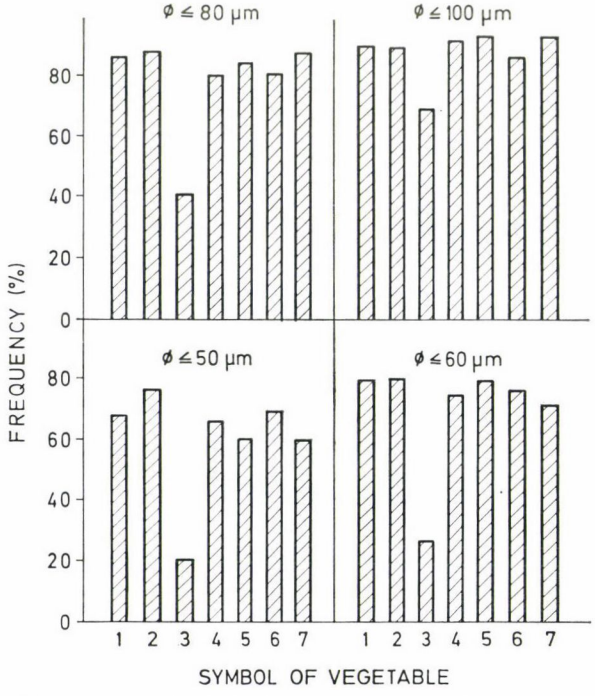


Fig. 3. Cumulative frequency in the 5–100 $\mu$m range of particle diameters in suspensions of vegetable tissues disintegrated by endo-polygalacturonase. 1. Kohlrabi, 2: Squash, 3: Potato, 4: Celery, 5: Green pepper, 6: Carrot, 7: Parsley

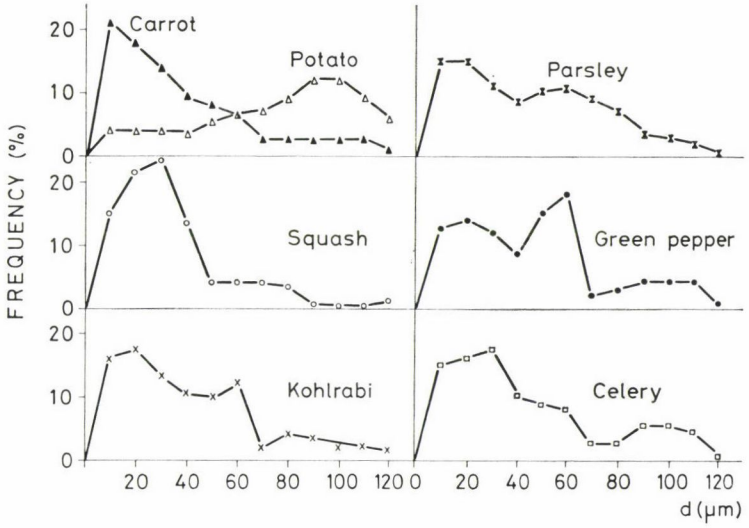


Fig. 4. Particle size distribution of the various vegetable tissues disintegrated by endo-polygalacturonase. 1: Kohlrabi, 2: Squash, 3: Potato, 4: Celery, 5: Green pepper, 6: Carrot, 7: Parsley

According to the Fig. 4 10 $\mu$m proved to be the most frequent particle size in the case of carrot and parsley, 20 $\mu$m in the case of kohlrabi while 30 $\mu$m with squash and celery tissues. 60 $\mu$m was a characteristic size of the cells and cell agglomerates of the enzymatically disintegrated green pepper tissues while 90 and 100 $\mu$m of those of the potato.

In the case of several vegetables two dominant particle sizes have been found, in suspensions of green pepper and kohlrabi 20 and 60 $\mu$m, of parsley 10 and 60 $\mu$m, and of the celery tissues 30 and 60 $\mu$m. With the exception of green pepper the frequency of the smaller sizes proved to be the higher.

## 3. Conclusions

Vegetable tissues were disintegrated by endo-PG enzyme, resulting in vegetable suspensions consisting mainly of single cells and small cell agglomerates. This enzyme is suitable for the production of vegetable juices and cocktails, the stability of which is dependent on the particle size, too. This is why the determination of the distribution of the degree of dispersion of the vegetables is important.

The sedimentation technique applied in this work is suitable for the determination of the distribution of the various particle sizes in the tested vegetables in spite of the fact that the size and shape of the cells differ according to their function and are far from being sphere-shaped.

The frequency distribution of the tested seven vegetables showed similar tendency with the exception of green pepper and potato. The highest peak of frequency among particle sizes were found in the finer size range 10, 20 and 30 $\mu$m. In the case of vegetables having two characteristic particle sizes, the second peaks were obtained at 60 $\mu$m. Green pepper proved to be the only exception where the first peak (at 20 $\mu$m) was the least prevalent.

Potato was shown to be entirely different from the other vegetables tested, having dominant particle sizes around 90 and 100 $\mu$m. In the regions of finer particle sizes, the occurrence of potato cells and particles is rather infrequent.

As the size of the particles of the disintegrated tissues is a result of enzyme action, the effects of enzyme concentration and the time of incubation on the particle size distribution of vegetables, after endo-PG treatment, will next be studied.

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# SUBSTRATE SPECIFICITY OF THE ENZYMIC BROWNING OF APPLES

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Earlier findings of the authors lead to the conclusion that the enzyme polyphenol oxidase as present in apple cultivars of different browning tendency might differ not only in concentration, but also in qualitative characteristics. In order to support this assumption substrate specificity of the browning reaction was investigated with two cultivars of highly different browning tendency as the enzyme source. Eight kinds of substrates buffered to pH 3.6. and 5.4, resp., were applied in concentrations of 1.6, 3.2 and 6.4 $M \cdot 10^{-3}$ each. Browning rates (BA) were determined by reflectance measurements on apple slices dipped in the subtrate solutions for 2 min each.

Browning rates for both cultivars were highest on 4-methyl catechol at pH 5.4, whereby the value obtained for *Starking* was about 1.6 times that for *Jonathan*. Some minor, but distinct differences could be established between the substrate specificities of the two cultivars. Catechol was the second best substrate for both kinds of apples, followed by (+)-catechin hydrate for Starking and chlorogenic acid for *Jonathan*. Some of the differences were pH dependent.

Substrate inhibition as observed in some cases was found to be both pH and cultivar dependent.

For substrates yielding linear *Lineweaver-Burk* relationships apparent $V_{max}$ and $K_m$ were calculated. Several significant differences were found between the values of both characteristics as obtained for the two cultivars and, within the same cultivar, pH dependence of kinetic data could be established as well.

Some of the results were found to be consistent with literature data and seem to support the assumption outlined above.

Enzymic browning of fruits and vegetables is the result of catalytic oxidation of endogenous o-dioxy phenols, followed by polymerization or condensation, complexing with amino acids or proteins and secondary oxidative reactions of the quinones formed (MATHEW & PARPIA, 1971). The enzyme involved in the catalytic process, polyphenol oxidase (PPO, E. C. 1.10.3.1.), as obtained from various sources of vegetable origin, was found to be more or less different with respect to substrate specificity, temperature and pH optimum or sensitivity towards inhibitors (VÁMOS-VIGYÁZÓ & BALÁZS-SPRINCZ, 1970). In recent work carried out in the authors' laboratory differences were established between temperature and pH optima of the enzyme as present in homogenates of different cultivars of the same kind of apples (MIHÁLYI & VÁMOS-VIGYÁZÓ, 1978). These differences were interpreted as the consequence of different PPO isoenzyme compositions in the cultivars tested and it was assumed that this might affect substrate specificity, too.

Since enzymic browning of apples was found to be proportional to their PPO contents (VÁMOS-VIGYÁZÓ *et al.*, 1977a), substrates were compared by direct measurement of discolouration rates of fruit flesh.

## 1. Materials and methods

### *1.1. Apples*

*Jonathan* and *Starking* apples (of slight and pronounced browning tendencies, resp.) were purchased from the RESEARCH STATION OF THE UNIVERSITY OF HORTICULTURE, Ujfehértó (North-Eastern Hungary) in mid-September 1975 and kept at 5 °C in normal atmosphere until use.

### *2.2. Substrates*

Eight substrates, some of which are known to occur in apples, were used in 3 concentrations (0.0016, 0.0032 and 0.0064 *M*) and at two values of pH (3.6 and 5.4) each. The compounds used as substrates were chlorogenic acid, 4-methyl catechol, (+)-catechin hydrate, (+)-catechin, (−)-epi-catechin (all supplied by FLUKA AG, Buchs SG), catechol and pyrogallol (from REANAL, Budapest) as well as DL-3,4-dihydroxy phenylalanine (DOPA), (from CALBIOCHEM, San Diego, Calif.).

Substrate solutions were buffered to the pH desired with 0.1 *M* phosphate-citrate buffers.

### *1.3. Measurement of enzymic browning*

Browning rate of the fruit flesh was determined by a reflectance method developed by the authors and described in detail elsewhere (VÁMOS & GAJZÁGÓ, 1974). The instrument used was the spectrocolorimeter *Spekol* 32-G 315 (CARL ZEISS, Jena) equipped with a reflectance attachment (type 45/0) to which a cuvette and a black cuvette holder were designed in this laboratory.

Slices of the fruit were immersed for 2 min in the buffered substrate solution immediately after cutting. After removal, the surface was blotted with filter paper and the slice placed in the cuvette holder of the instrument. The progress of the reaction was indicated by the needle of the instrument previously adjusted to 100. The appropriate wave-length and sensitivity for measuring enzymatic browning of apple slices were 540 nm and 20–fold, resp. Initial browning rates were calculated from the linear sections of the curves obtained when plotting changes in reflectance ($\Delta$R) *vs.* reaction time. Initial browning rate or browning activity (BA) was expressed in $\Delta$R $min^{-1}$ (1 U = 1 scale division $min^{-1}$). Measurements were carried out in 4 to 7 replicates.

*1.4. Evaluation of the results*

Browning activities were plotted against substrate concentrations. For substrates which did not inhibit the reaction under the conditions applied, *Lineweaver-Burk* relationships were established and kinetic characteristics (apparent $K_m$ and $V_{max}$) calculated.

Results obtained for the two cultivars under identical conditions or for the same cultivar at different pH values were compared by *Student*'s *t*-test.

## 2. Results

*2.1. Browning activities*

Browning activities of *Jonathan* and *Starking* apples as determined on 8 substrates in 3 concentrations each at pH 3.6 and pH 5.4, resp., are shown in Tables 1 and 2.

Browning rates varied in a wide range with the substrate used. Activity was highest for both cultivars on $6.4 \cdot 10^{-3}$ *M* 4-methyl catechol at pH 5.4, whereby the value obtained for *Starking* was about 1.6 times that for *Jonathan*. At pH 3.6 no significant difference was observed under the same conditions between the browning rates of the two cultivars as can be seen in Table 3.

As expected, browning rates at a given pH were, with most substrates, higher for *Starking* than for *Jonathan*. There are, however, some exceptions. For example, at pH 3.6 there was no significant difference between the browning rates of the two cultivars with the two higher chlorogenic acid concentrations, whereas at pH 5.4 the same applied to the lowest concentration of this substrate.

pH affected browning rates as measured on different substrates to different extents. In the majority of cases browning rates were higher at the higher pH value. However, on catechin hydrate and on pyrolgallol of identical concentrations BAs of *Jonathan* apples were found to be identical at the two pH values investigated. In the case of *Starking* this was only partly true. On the whole, catechins seemed to be, in most cases, more readily oxidized at pH 3.6 by the enzyme of either cultivar.

Some of the substrates, when applied in higher concentrations, partially inhibited the reaction. For example, $6.4 \cdot 10^{-3}$ *M* chlorogenic acid decreased the browning rate of *Jonathan* at pH 5.4, whereas no such phenomenon could be observed at pH 3.6 with this cultivar, nor with the other one at either pH. Substrate inhibition was found to occur at pH 5.4 with $6.4 \cdot 10^{-3}$ *M* of catechin, epi-catechin and DOPA, resp., in the case of *Jonathan* and with the above concentration of the former two compounds in the case of *Starking*.

Table 1

*Browning activities (BA) of* Jonathan *apples on different substrates*

| Substrate | | pH | | | | | |
|---|---|---|---|---|---|---|---|
| | | 3.6 | | | 5.4 | | |
| Name | Concentration ($M \cdot 10^{-3}$) | BA (U) | | n | BA (U) | | n |
| | | $\bar{x}$ | s | | $\bar{x}$ | s | |
| 4-Methyl | 1.6 | 4.0 | 0.32 | 6 | 5.8*** | 0.53 | 6 |
| catechol | 3.2 | 6.1 | 0.39 | 7 | 7.3*** | 0.43 | 6 |
| | 6.4 | 8.4 | 0.63 | 6 | 10.2** | 0.82 | 5 |
| Chlorogenic | 1.6 | 2.0 | 0.13 | 6 | 3.7*** | 0.29 | 6 |
| acid | 3.2 | 3.2 | 0.18 | 5 | 6.5*** | 0.34 | 6 |
| | 6.4 | 4.0 | 0.15 | 6 | 5.0** | 0.52 | 6 |
| Catechol | 1.6 | 2.1 | 0.31 | 6 | 2.7** | 0.32 | 6 |
| | 3.2 | 3.4 | 0.32 | 6 | 6.9*** | 0.51 | 6 |
| | 6.4 | 4.5 | 0.33 | 5 | 7.3*** | 0.53 | 6 |
| Catechin | 1.6 | 2.1 | 0.19 | 5 | 2.1ø | 0.29 | 6 |
| hydrate | 3.2 | 2.4 | 0.50 | 6 | 1.5** | 0.20 | 6 |
| | 6.4 | 3.7 | 0.50 | 6 | 2.9* | 0.54 | 6 |
| (+)-Catechin | 1.6 | 1.7 | 0.32 | 6 | 1.3ø | 0.28 | 5 |
| | 3.2 | 1.6 | 0.17 | 6 | 2.0** | 0.19 | 5 |
| | 6.4 | 2.0 | 0.33 | 6 | 1.4* | 0.35 | 4 |
| (−)-Epi-catechin | 1.6 | 1.8 | 0.42 | 6 | 1.7ø | 0.12 | 6 |
| | 3.2 | 1.5 | 0.13 | 6 | 1.1*** | 0.11 | 5 |
| | 6.4 | 1.9 | 0.17 | 5 | 1.2** | 0.31 | 5 |
| Pyrogallol | 1.6 | 0.6 | 0.14 | 4 | 0.6ø | 0.09 | 6 |
| | 3.2 | 0.6 | 0.10 | 5 | 0.6ø | 0.09 | 6 |
| | 6.4 | 0.8 | 0.14 | 5 | 0.8ø | 0.12 | 4 |
| DOPA | 1.6 | 0.1 | 0.03 | 4 | 0.5*** | 0.04 | 4 |
| | 3.2 | 0.4 | 0.07 | 5 | 1.2*** | 0.12 | 6 |
| | 6.4 | 0.9 | 0.10 | 4 | 1.0ø | 0.17 | 5 |

$\bar{x}$ = mean of parallel measurements;
n = number of parallel measurements;
1 BA U = 1 scale division $min^{-1}$ as determined from the linear sections of reflectance *vs.* reaction time plots obtained from measurements carried out at 540 nm on slices of apples in the spectrocolorimeter *Spekol*. Significance levels of differences between BAs obtained at different pH with identical concentrations of a given substrate:
* $P \geq 95\%$;
** $P \geq 99\%$;
*** $P \geq 99.9\%$; ø not significant, $P < 95\%$ (by *t*-test).

Table 2

*Browning activities (BA) of* Starking *apples on different substrates*

| Substrate | | pH | | | | | |
|---|---|---|---|---|---|---|---|
| | | 3.6 | | | 5.4 | | |
| Name | Concentration ($M \cdot 10^{-3}$) | BA/U | | n | BA (U) | | n |
| | | $\bar{x}$ | s | | $\bar{x}$ | s | |
| 4-Methyl | 1.6 | 5.4 | 0.13 | 7 | 8.1*** | 0.33 | 6 |
| catechol | 3.2 | 8.2 | 0.64 | 6 | 9.3** | 0.47 | 6 |
| | 6.4 | 8.9 | 0.70 | 6 | 16.2*** | 0.50 | 6 |
| Chlorogenic | 1.6 | 2.6 | 0.30 | 5 | 3.9*** | 0.36 | 6 |
| acid | 3.2 | 3.3 | 0.26 | 5 | 5.0*** | 0.42 | 6 |
| | 6.4 | 4.2 | 0.34 | 5 | 5.8*** | 0.47 | 6 |
| Catechol | 1.6 | 5.8 | 0.38 | 6 | 7.5*** | 0.67 | 6 |
| | 3.2 | 9.9 | 0.72 | 6 | 12.9*** | 0.65 | 6 |
| | 6.4 | 13.3 | 0.70 | 6 | 13.0Ø | 0.55 | 5 |
| Catechin | 1.6 | 5.8 | 0.79 | 6 | 5.1Ø | 0.58 | 6 |
| hydrate | 3.2 | 5.4 | 0.41 | 6 | 5.4Ø | 0.68 | 6 |
| | 6.4 | 4.4 | 0.32 | 6 | 4.0Ø | 0.42 | 6 |
| (+)-Catechin | 1.6 | 3.3 | 0.50 | 6 | 2.7* | 0.20 | 5 |
| | 3.2 | 4.6 | 0.38 | 6 | 4.0* | 0.36 | 6 |
| | 6.4 | 4.7 | 0.36 | 6 | 2.0*** | 0.23 | 5 |
| (–)-Epi-catechin | 1.6 | 3.3 | 0.35 | 6 | 1.8*** | 0.27 | 6 |
| | 3.2 | 2.4 | 0.55 | 6 | 2.0Ø | 0.23 | 5 |
| | 6.4 | 3.4 | 0.25 | 6 | 1.6*** | 0.25 | 5 |
| Pyrogallol | 1.6 | 2.1 | 0.38 | 5 | 1.2*** | 0.19 | 6 |
| | 3.2 | 1.5 | 0.24 | 5 | 1.1* | 0.23 | 5 |
| | 6.4 | 1.5 | 0.27 | 5 | 1.8Ø | 0.39 | 6 |
| DOPA | 1.6 | 1.3 | 0.31 | 5 | 1.6Ø | 0.17 | 5 |
| | 3.2 | 0.6 | 0.10 | 4 | 3.0*** | 0.12 | 5 |
| | 6.4 | 1.3 | 0.05 | 5 | 4.2*** | 0.45 | 6 |

Symbols as in *Table 1*

To obtain a clearer picture of the differences in substrate specificity of the enzymic browning reaction of the two cultivars, BA values were expressed as percentages of the highest reaction rates (observed at pH 5.4 on $6.4 \cdot 10^{-3}$ *M* 4-methyl catechol). Relative BAs are shown in Fig. 1.

Table 3

*Significance levels of differences between browning activities of* Jonathan *and* Starking *apples as measured at identical pH values with identical concentrations of a given substrate* (Established from the data of Tables 1 and 2)

| Substrate | | pH | |
|---|---|---|---|
| Name | Concentration ($M \cdot 10^{-3}$) | 3.6 | 5.4 |
| 4-Methyl catechol | 1.6 | *** | *** |
| | 3.2 | *** | *** |
| | 6.4 | Ø | *** |
| Chlorogenic acid | 1.6 | ** | Ø |
| | 3.2 | Ø | *** |
| | 6.4 | Ø | * |
| Catechol | 1.6 | *** | *** |
| | 3.2 | *** | *** |
| | 6.4 | *** | *** |
| Catechin hydrate | 1.6 | *** | *** |
| | 3.2 | *** | *** |
| | 6.4 | * | ** |
| (+)-Catechin | 1.6 | *** | *** |
| | 3.2 | *** | *** |
| | 6.4 | *** | * |
| (−)-Epicatechin | 1.6 | *** | Ø |
| | 3.2 | ** | *** |
| | 6.4 | *** | Ø |
| Pyrogallol | 1.6 | *** | *** |
| | 3.2 | *** | *** |
| | 6.4 | *** | ** |
| DOPA | 1.6 | *** | *** |
| | 3.2 | ** | *** |
| | 6.4 | *** | *** |

At pH 5.4 the "best" substrate for the enzyme of both cultivars was 4-methyl catechol, followed by catechol for *Starking* and catechol and chlorogenic acid for *Jonathan*. The order of the less suitable substrates was catechin hydrate, (+)-catechin, (−)-epi-catechin, DOPA and pyrogallol for *Jonathan*, while with *Starking*, DOPA preceded (+)-catechin.

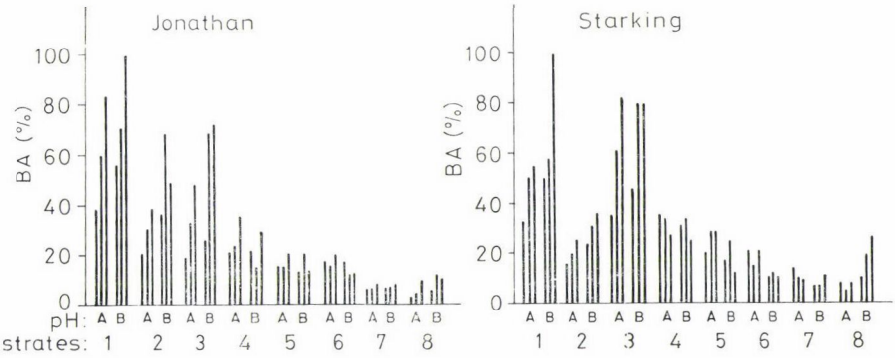


Fig. 1. Relative rates of enzymic browning of apples on various substrates. A = pH 3.6, B = pH 5.4. Substrates: 1. 4-methyl catechol, 2. chlorogenic acid, 3. catechol, 4. catechin hydrate, 5. (+)-catechin, 6. (−)-epi-catechin, 7. pyrogallol, 8. DOPA. Concentrations of the substrates from left to right: 1.6, 3.2 and 6.4 $M \cdot 10^{-3}$

At pH 3.6 the order remained practically unchanged for *Jonathan*, whereas catechol proved a definitely better substrate for *Starking* apples than chlorogenic acid, while pyrogallol preceded DOPA.

### *2.1. Kinetic parameters*

For the substrates which did not inhibit the browning reaction in the concentration range investigated *Lineweaver-Burk* relationships are shown in Fig. 2.

Enzymic browning as catalyzed by *Jonathan* PPO was found to follow *Michaelis-Menten* kinetics with 4-methyl catechol at both pH values tested, with chlorogenic acid and with catechol at pH 3.6. In the case of the enzyme in *Starking* apples the same was established with 4-methyl catechol, catechol and (+)-catechin at pH 3.6, with chlorogenic acid at both pH values and with DOPA at pH 5.4. For the rest of substrates and conditions, resp., the browning reaction was not found to follow *Michaelis* kinetics: *Lineweaver-Burk* relationships did not prove to be significantly linear. (In the case of *Jonathan*, *Eadie-Hofstee* plots were linear only on 4-methyl catechol, chlorogenic acid and catechol at pH 3.6 and, in the case of *Starking*, on chlorogenic acid at both pH values tested.) These findings, too, point to the differences in the nature of PPO as present in the two cultivars.

Apparent $V_{max}$ and $K_m$ values were calculated from the equations in Fig. 2a (*Jonathan*), 2b (*Starking*) and are listed in Table 4.

According to the values of $V_{max}$ substrates could roughly be divided into 3 groups: the highest value was found, in the case of *Starking*, on catechol at pH 3.6. On 4-methyl catechol and on DOPA $V_{max}$ values were medium. In the rest of the cases low values were obtained.

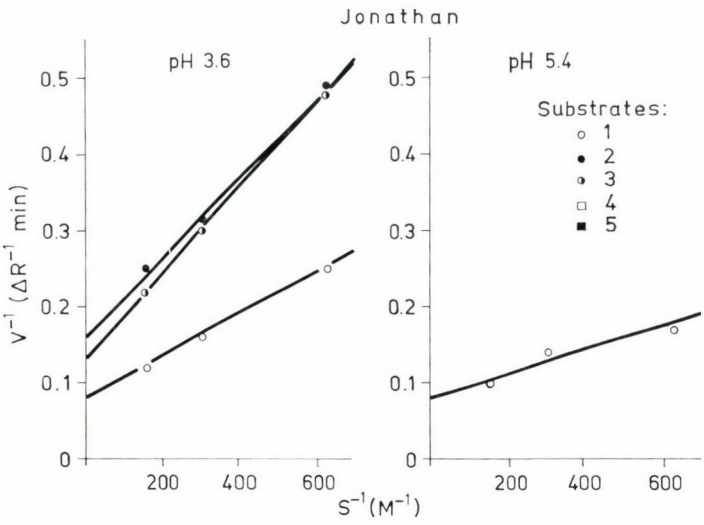


Fig. 2a. *Lineweaver-Burk* plots of the enzymatic browning reaction of *Jonathan* apples on different substrates. 1. 4-methyl catechol, 2. chlorogenic acid, 3. catechol, 4. (+)-catechin, 5. DOPA. Regression equations of the curves and coefficients of determination ($r^2$) are on the next page in Table, under Fig. 2b.

Table 4

*Kinetic parameters of the enzymatic browning reaction of apples on various substrates at two pH values*

| Substrate | pH | Cultivar | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Jonathan | | | | Starking | | | |
| | | $V_{max}$($\Delta$R min$^{-1}$) | | $K_m$(M · $10^{-3}$) | | $V_{max}$($\Delta$R min$^{-1}$) | | $K_m$(M · $10^{-3}$) | |
| | | $\bar{y}$ | s | $\bar{x}$ | s | $\bar{y}$ | s | $\bar{x}$ | s |
| 4-Methyl catechol | 3.6 | 12.5 | 2.66 | 3.38 | 0.72 | 12.5 | 0.00 | 2.13 | 0.00 |
| | 5.4 | 12.5 | 1.36 | 2.00 | 0.22 | — | — | — | — |
| Chlorogenic acid | 3.6 | 6.25 | 0.94 | 3.25 | 0.49 | 5.00 | 0.01 | 1.55 | 0.003 |
| | 5.4 | — | — | — | — | 6.67 | 0.62 | 1.13 | 0.19 |
| Catechol | 3.6 | 7.69 | 0.72 | 4.30 | 0.40 | 29.0 | 6.25 | 5.25 | 1.13 |
| (+)-Catechin | 3.6 | — | — | — | — | 5.88 | 0.93 | 1.24 | 0.20 |
| DOPA | 5.4 | — | — | — | — | 11.1 | 3.7 | 9.3 | 3.1 |

$\bar{x}$ and $\bar{y}$ = reciprocal absolute values of the intercepts on the x and y axis, resp., of the linear regression curves in Fig. 2

s = standard deviations of $\bar{x}$ and $\bar{y}$

— = the reaction did not follow *Michaelis* kinetics

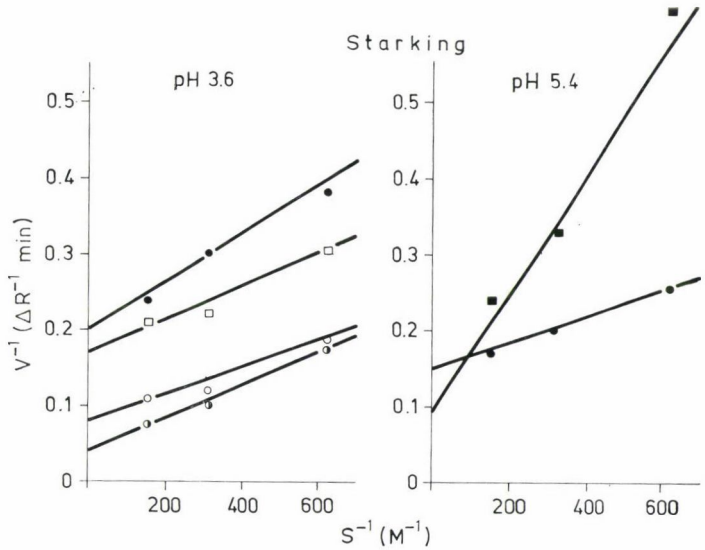


Fig. 2b. *Lineweaver-Burk* plots of the enzymatic browning reaction of *Starking* apples on different substrates. 1. 4-methyl catechol, 2. chlorogenic acid, 3. catechol, 4. (+)-catechin, 5. DOPA. (For symbols of substrates see Fig. 2a.) Regression equations of the curves and coefficients of determination ($r^2$):

| Substrate | pH | Cultivar | Equation | $r^2$ |
|---|---|---|---|---|
| 1 | 3.6 | J | $V^{-1} = 0.08 + 0.00027\ s^{-1}$ | 0.999*** |
| 1 | 5.4 | J | $V^{-1} = 0.08 + 0.00016\ s^{-1}$ | 0.952* |
| 1 | 3.6 | S | $V^{-1} = 0.08 + 0.00017\ s^{-1}$ | 0.957* |
| 2 | 3.6 | J | $V^{-1} = 0.16 + 0.00052\ s^{-1}$ | 0.995** |
| 2 | 3.6 | S | $V^{-1} = 0.20 + 0.00031\ s^{-1}$ | 0.985** |
| 2 | 5.4 | S | $V^{-1} = 0.15 + 0.00017\ s^{-1}$ | 0.999*** |
| 3 | 3.6 | J | $V^{-1} = 0.13 + 0.00056\ s^{-1}$ | 0.998*** |
| 3 | 3.6 | S | $V^{-1} = 0.04 + 0.00021\ s^{-1}$ | 0.994** |
| 4 | 3.6 | S | $V^{-1} = 0.17 + 0.00021\ s^{-1}$ | 0.916* |
| 5 | 5.4 | S | $V^{-1} = 0{,}09 + 0.00084\ s^{-1}$ | 0.990** |

J = *Jonathan;* S = *Starking;* V = reaction rate ($\Delta$ R $min^{-1}$); s = substrate concentration (*M*).

*, ** and *** = correlations significant at the probability levels of 95, 99 and 99.9%, resp.

Each point of measurement represents the mean of 4 to 7 parallel determinations of V. Standard deviations of values are shown in Tables 1 and 2

The affinity of the enzyme in *Starking* apples was highest towards chlorogenic acid and (+)-catechin at pH 5.4. Then followed, in the order of decreasing affinity, chlorogenic acid and 4-methyl catechol at pH 3.6, catechol and DOPA. For the enzyme in *Jonathan* apples the order was 4-methyl

catechol at pH 5.4, the same substrate and chlorogenic acid at pH 3.6 and, finally, catechol.

The reaction was found to follow *Michaelis* kinetics with the enzymes of both cultivars on three substrates only. With two out of these, *i.e.* 4-methyl catechol and chlorogenic acid, $K_m$ values were higher for the enzyme in *Jonathan*. With catechol, $K_m$ values did not differ significantly for the enzymes in the two cultivars.

## 3. Conclusions

The results outlined above confirm the assumption that substrate specificity of the enzymic browning reaction as induced by PPO might be different for different cultivars of the same kind of fruit. The dependence of reaction rate on molar concentration of the substrate as well as on pH was found to be different for the enzymes as present in *Jonathan* and *Starking* apples. Substrate inhibition and other kinetic anomalies also occurred differently in the browning reaction of the two cultivars. The difference between the behaviour of (+)-catechin hydrate and (+)-catechin was unexpected and, so far, could not be interpreted.

Substrate inhibition of the browning of *Jonathan* apples was observed with $6.4 \cdot 10^{-3}$ *M* chlorogenic acid at pH 5.4, while in the case of the cultivar *Starking*, the reaction proceeded, in the concentration range studied, according to *Michaelis* kinetics. In a method developed earlier in this laboratory for determining PPO activity in potato homogenates on chlorogenic acid at pH 6.2 and 30 °C, inhibition was found to occur at substrate concentrations higher than $3.4 \cdot 10^{-2}$ *M* (MIHÁLYI & VÁMOS-VIGYÁZÓ 1976). MACHEIX (1970) did not observe substrate inhibition with chlorogenic acid concentrations up to $5 \cdot 10^{-2}$ *M* when measuring PPO activity in apple homogenates. This discrepancy led the authors of the paper cited (MIHÁLYI & VÁMOS-VIGYÁZÓ, 1976) to the assumption that the concentration threshold of substrate inhibition depended not only on the substrate, but also on the source of enzyme. This has found further corroboration by the results reported here. The difference in the behaviour, towards chlorogenic acid, of PPO as present in *Jonathan* and *Starking* apples, cannot be attributed to different enzyme contents, since at pH 5.4 and a chlorogenic acid concentration of $1.6 \cdot 10^{-2}$ *M* reaction rates were identical with the two cultivars.

In evaluating the results endogenous substrate content of the apples and browning as caused by these substrates have to be taken into account. These were rather different for the two cultivars. BA values were 0.73 and 2.6 U, *o*-dioxy phenol contents 1.4 and 2.2 m*M* (expressed as chlorogenic acid), for *Jonathan* and *Starking*, resp. As these are of the same order as the concentrations of the substrates added and the browning rates measured

in the presence of these substrates, their interference with the results obtained seems probable. With some of the less suitable substrates browning rates were lower than on endogenous polyphenols alone. This might be due to substrate inhibition caused by increased polyphenol concentrations. With chlorogenic acid this is, however, obviously not the case. If inhibition were the result of increased polyphenol concentration due to the presence of endogenous substrates, it ought to have occurred with *Starking* and not with *Jonathan*.

The reactivity of the substrates with PPO as judged from apparent $K_m$, $V_{max}$ or browning rates at the substrate concentrations investigated was found to differ not only with the cultivar and pH, but in the case of a given cultivar, also according to the parameter considered. At pH 3.6, for example, the enzyme in *Starking* apples exhibited maximum affinity towards chlorogenic acid, while apparent $V_{max}$ and browning rate in the concentration range investigated were maximum on catechol and 4-methyl catechol, resp. It would seem obvious to express substrate specificity in terms of apparent $K_m$ but for the fact that, with a considerable part of the substrates tested, the reaction did not proceed according to *Michaelis* kinetics. Deviations were caused, in the majority of cases, by substrate inhibition which might have been avoided by the use of different concentration ranges for the different substrates. It had been shown earlier in this laboratory for PPO in potato homogenates that substrate saturation could be attained with rather different molar concentrations when different substrates were applied (MIHÁLYI & VÁMOS-VIGYÁZÓ, 1976; VÁMOS-VIGYÁZÓ *et al.*, 1973).

Papers published in the literature do not entirely agree on the substrate specificity of apple PPO. This may be due partly to differences in experimental conditions, but partly also to the different nature of the enzyme in the cultivars tested. Of the substrates applied in this study 4-methyl catechol was found by HAREL and co-workers (1964) to give highest activity with particulate PPO of *Grand Alexander* apples. The same authors found a nearly identical activity on chlorogenic acid, values about half as high on l-epicatechin and on catechol, while activity on pyrogallol reached only 1/10 of that obtained on 4-methyl catechol. 3,4-Dihydroxy phenyl alanine proved a specific substrate of chloroplast PPO. Others found initial reaction rates of PPO from *Sturmer Pippin* apples to be higher on chlorogenic acid than on catechol; D-catechin proved less suitable and DOPA gave the lowest results (WALKER, 1964). The latter author found $K_m$ values as obtained from measurements of initial reaction rates in the presence of excess ascorbic acid to be $1.66 \cdot 10^{-3}\,M$ for chlorogenic acid and $1.48 \cdot 10^{-3}\,M$ for (+)-catechin. Taking into account the differences in experimental conditions, these findings may be considered to be in remarkably good agreement with the results obtained with *Starking* apples in this study.

On the whole it can be said that, in spite of some deficiences inherent in the method (such as different endogenous substrate contents of the two cultivars), results seem convincing as to the existence of qualitative differences of PPO in the two cultivars tested and encourage further research in this direction.

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# STUDIES ON RHEOLOGICAL AND BAKING PROPERTIES OF IRRADIATED INDIAN WHEAT

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Effects of gamma radiation on wheat have been studied with special reference to rheological and baking properties. Maximum gelatinization viscosity of starch, water absorption capacity as well as elasticity of gluten are significantly influenced by radiation treatment of wheat at 0.2 to 10 kGy (20 to 1000 krad) dose levels. The baking quality of wheat, irradiated at lower doses is improved, resulting in increased loaf-volume. No significant changes due to radiation treatment [up to 2 kGy (200 krad)] in external and internal sensory attributes of bread are observed, when evaluated by taste panel. However, the breads prepared with wheat irradiated at 10 kGy (1 Mrad), are not acceptable.

Control of insect infestation in stored wheat by irradiation has been extensively investigated and its feasibility is demonstrated. Wheat, disinfested by irradiation, has been shown to be wholesome for use as human food. At Trombay, multi-generation feeding trials with rats have established the safety for use of wheat, gamma irradiated at insect sterilization [200 Gy (20 krad)] or even at ten times higher dose level [2 kGy (200 krad)] (Aravindakshan *et al.*, 1970). Other studies on dominant lethals in mice using wheat-based diet have also shown that irradiation does not cause cytotoxicity in wheat (Vakil *et al.*, 1973). Subtle compositional changes in macro-nutrients like starch or protein may nevertheless take place either by direct or indirect (Oreshko *et al.*, 1962) action of radiation, increasing their sensitivity to their respective hydrolases (Ananthaswamy *et al.*, 1970a; 1970b; Srinivas *et al.*, 1972). This is because of the fragmentation of starch and proteins to low molecular weight entities, easily susceptible to amylolytic and proteolytic actions. These changes, though not of significance in influencing nutritional quality, may affect the visco-elastic properties and in turn the inherent baking quality of wheat. The present studies relate to the effects of ionizing radiation [0.2–10 kGy (20–1000 krad)] on (*i*) rheological properties of wheat, measured by different *Brabender* instruments, *(ii)* baking quality, and *(iii)* evaluation of organoleptic acceptance of bread and chapati by taste panels. A dose of 10 kGy (1 Mrad) was included because the adverse effects on quality, if any, at 2 kGy (200 krad) dose can be expected to be more pronounced at this level.

## Materials and methods

### *1.1. Irradiation*

Samples of *Vijay* variety of wheat, procured from NIPHAD AGRICULTURAL RESEARCH STATION, Nasik (Maharashtra State) were made into 1 kg packets, heat-sealed in polythene bags (0.0075 cm) and irradiated at 25 °C in $^{60}$Co Gamma Cell 220 (ATOMIC ENERGY OF CANADA LTD.) having a flux of 250 Gy min$^{-1}$ (25 krad min$^{-1}$), at 0.2 to 10 kGy (0.02 to 1 Mrad) dose levels. Absorption of ionizing radiation was checked with ferrous sulfate and ceric sulfate dosimetry (WEISS, 1952). The overdose ratio was about 30%. Unirradiated wheat samples were treated as control. Samples were stored for 2 to 3 weeks and ground in *Buhler*'s experimental mill (Model MLU-202) with 3 break systems and 3 reduction rolls. Wheat was conditioned to 15% moisture level prior to milling. The extraction of flour was about 70%.

### *1.2. Rheological properties of wheat*

Rheological properties of wheat flour doughs were measured using *Brabender* instruments, essentially by approved methods described by AACC (1962). Gelatinization viscosity of wheat starch was measured in *Amylograph* and expressed in Amylograph Units (A.U.). Wheat flour (80 g) was mixed with distilled water (450 ml) to form a lump-free suspension and heated in a revolving container (75 rpm) with a steady rise of temperature (1.5 °C min$^{-1}$), until complete gelatinization occurred. Water absorption capacity and dough development time to obtain 500 Brabender Units (B. U.) were measured in a *Farinograph*. Mechanical Tolerance Index (M.T.I.) was calculated from the drop in consistency after mixing for 12 min. Plain dough of flour–salt–water system was prepared in *Farinograph* and kept at 30 °C for 135 min. This was then stretched to the tearing point and individual factors, chatacterizing the gluten properties, were determined from the extensogram.

### *1.3. Baking test*

Breads were prepared by straight-dough lean formula as described earlier (RAO *et al.*, 1976). Ingredients were, 100 g wheat flour, 2.5 g dry yeast suspended in warm water, 2.5 g sugar, 4.0 g each of skimmed milk powder and shortening (Dalda), 1.5 g salt and 10 ppm potassium bromate. Water was added as required for obtaining optimum dough consistency (500 B. U.) and mixed in a dough mixer at a speed of 125 rpm. The dough was fermented for 2.5 h at 30 °C punching at 45 and 100 min. It was then moulded, put into slightly greased baking pans and proofed for 60 min. Baking was done

at 210 °C for 20 to 25 min. The loaf was cooled and the volume was measured by mustard seed displacement method.

Bread crust (1 g) was extracted in 25 ml of 70% ethanol and colour intensity measured in terms of % transmittance at 550 nm in a *Bausch and Lomb Spectronic* 20.

General appearance of bread was adjudged by subjective analysis and expressed in three broad terms, namely, satisfactory (S), not satisfactory (NS) and questionably satisfactory (QS).

For making *chapati* (unleavened Indian bread), the dough was prepared by mixing 100 g whole wheat flour with 1.5 g salt, 10 g refined groundnut oil and about 65% water. This was kneaded thoroughly and kept for 30 min for gluten development. The dough was divided into small balls (25 g each) and rolled into a *chapati*, having a diameter of 15.24 cm. These were roasted on a hot iron plate till well done.

### *1.4. Sensory evaluation*

Overall preference for *bread* and *chapati* was evaluated by 9 point hedonic scale, ranging from "like extremely" to "dislike extremely". Intensity scale (1 to 10 points) was used for scoring the off-characteristics such as discoloration, off-odour, irradiation flavour, etc., in breads. Other desirable characteristics of bread such as loaf volume, crumb colour, texture, cell structure, flavour, taste, chewability, *etc.*, were evaluated by using "technical evaluation sheet".

Freshly prepared bread (centre slice) or *chapati* (quarter piece) samples were served to the panel members. At least three taste panels of 12 members each, were involved for each product, and the average scores calculated.

Statistical analysis of individual mean scores was carried out using *Student*'s *t*-test (ALDER & ROESSLER, 1972).

## 2. Results

### *2.1. Gelatinization viscosity of wheat*

Wheat flour prepared from wheat irradiated at 2 kGy (200 krad) (550 A. U. at 84 °C) and 10 kGy (1000 krad) (230 A. U. at 82 °C) exhibited lower maximum gelatinization viscosity compared to control (980 A. U. at 89 °C, Table 1). Evaluation of amylograms points out that gelatinization of wheat flour started at 62 °C in all the samples. Whereas, the time taken to reach the amylogram peak, was significantly decreased with the increase in radiation dose.

Table 1

*Evaluation of amylograms of irradiated wheat*

Wheat flour (80 g) — water slurry was heated in revolving container with a steady rise in temperature of 1.5 °C $min^{-1}$ until complete gelatinization occurred. Temperature and time at which maximum gelatinization took place, were noted. Results are averages of three experiments ± S.E.

| Parameter | Radiation dose (kGy) | | | |
|---|---|---|---|---|
| | 0 | 0.2 | 2 | 10 |
| Maximum gelatinization viscosity (A. U.) | 980±5.0 | 830±7.0 | 550±5.0 | 230±8.0 |
| Temperature (°C) | 89 | 85 | 84 | 82 |
| Time (min) | 42±1.0 | 39±0.6 | 38±0.04* | 37±0.4** |

* $P \leq 0.05$
** $P \leq 0.01$

## 2.2. *Evaluation of farinograms*

Farinograms obtained with control and irradiated wheat flour samples, were evaluated and results are summarized in Table 2. To obtain a normal titration curve, which determines the dough consistency at 500 B. U., unirradiated samples required 62% water, whereas, those irradiated at 0.2, 2 and 10 kGy (20, 200 and 1000 krad) dose levels, absorbed 65, 69 and 73%

Table 2

*Evaluation of farinograms of irradiated wheat*

Wheat flour (300g) was taken in *Farinograph* mixing chamber equilibrated at 30 °C, distilled water was added from the burette until a dough of 500 B.U. consistency was obtained. The behaviour of the relaxed dough towards continuous mechanical action was recorded after the resting period of 1 h. The water absorption capacity is corrected on 14% moisture basis. Results are averages of three experiments ± S.E.

| Parameter | Radiation dose (kGy) | | | |
|---|---|---|---|---|
| | 0 | 0.2 | 2 | 10 |
| Water absorption (%) | 62±1.5 | 65±1.0* | 69±1.7* | 73±1.2** |
| Dough development time (min) | 3.5±0.05 | 3.5±0.05 | 3.0±0.04* | 2.5±0.04* |
| Dough stability (min) | 2.0±0.04 | 2.5±0.05* | 2.5±0.05* | 3.0±0.03* |
| Mechanical tolerance index M.T.I. (B.U.) | 80±2.0 | 50±1.5 | 40±1.6 | 40±1.5 |
| Drop in dough consistency after 60 min rest (B.U.) | 20±1.2 | 30±1.5 | 30±1.5 | 50±1.7 |

Probability levels of significance of difference between radiation treatment and control:
* $P \leq 0.05$
** $P \leq 0.01$

water, resp. Similarly, dough development time (2.5 to 3.5 min) and dough stability (2–3.0 min) were significantly ($P \leq 0.05$) affected by irradiation. The mechanical tolerance index was considerably improved in irradiated samples. Further drop in dough consistency on fermentation, was about 20 units in control compared to 30 to 50 units in irradiated samples.

### *2.3. Evaluation of extensograms*

Evaluation of extensogram data, obtained with dough after 135 min of rest period at 30 °C, is shown in Table 3. Total energy used for stretching the dough (A) was increased considerably in irradiated (10 kGy=1000 krad) samples (108 $cm^2$) compared to control (45 $cm^2$). Measurement of the extensogram height after 50 mm stretching (B), showed that irradiated samples offered better resistance [520, 620 and 780 B. U. in 0.2, 2, 10 kGy (20, 200 and 1000 krad), resp.], compared to unirradiated one (310 B. U.). The "ratio figure" (D) which indicates the behaviour of the dough strength and potentiality of baking volume was larger in irradiated samples [4.7, 5.1 and 6.0 for 0.2, 2, 10 kGy (20, 200, and 1000 krad), resp.] compared to control (2.7).

Table 3

*Evaluation of extensograms of irradiated wheat*
The salt-water dough was prepared in *Farinograph* and stretched to its tearing point after 135 min rest period in *Brabender Extensograph*. Average value of three experiments ± S.E.

| Parameters | Radiation dose (kGy) | | | |
|---|---|---|---|---|
| | 0 | 0.2 | 2 | 10 |
| A. Energy ($cm^2$) | 45±4.0 | 78±5.6* | 92±5.2** | 108±5.5** |
| B. Resistance of stretching (B. U.) | 310 | 520 | 620 | 780 |
| C. Extensibility (mm) | 115 | 110 | 120 | 130 |
| D. Ratio B/C | 2.7±0.04 | 4.7±0.05* | 5.1±0.04* | 6.0±0.05** |

Probability levels of significance of difference between radiation treatment and control:
* $P \leq 0.05$
** $P \leq 0.01$

### *2.4. Baking quality*

Data on the external characteristics of yeast-leavened bread such as specific loaf volume and crust colour, prepared from control and irradiated wheat flour are given in Table 4. Photographs of representative bread loaves are depicted in Figure 1. Specific loaf volume, which directly measures the lightness or fluffiness, was significantly more for the breads prepared from

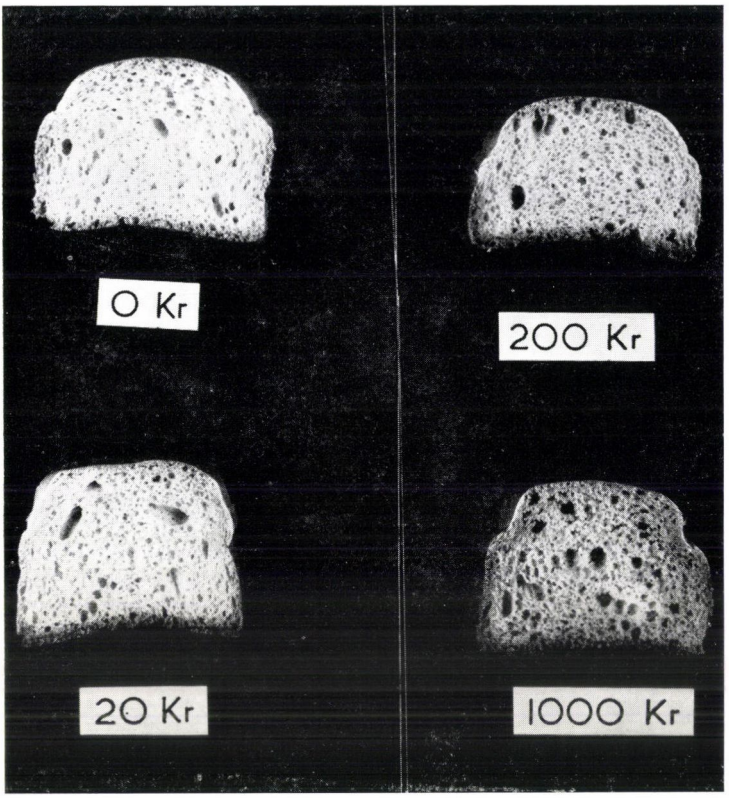


Fig. 1. Breads were prepared from unirradiated and irradiated wheat using straight dough lean formula. Quantity of water added was determined by *Farinograph*. The dough was fermented (2.5 h), proofed (60 min), moulded and baked at 210 °C (20 min)

Table 4

*Effect of irradiation on the baking quality of wheat*
The breads were prepared by straight dough lean formula as described in the text. Results are averages of three experiments, ± S.E.

| Parameter | Radiation dose (kGy) | | | |
|---|---|---|---|---|
| | 0 | 0.2 | 2 | 10 |
| Specific loaf volume ($cm^3\ g^{-1}$) | 3.5±0.05 | 3.9±0.05* | 4.1±0.09* | 2.7±0.08** |
| Crust colour (% trasmittance) | 97 | 92 | 90 | 76 |
| General appearance | Satisfactory | Satisfactory | Satisfactory | Not satisfactory |
| Mean score for breads | 6.0±0.4 | 6.4±0.3 | 5.3±0.4 | 4.6±0.3 |
| Mean score for *chapati* | 7.7±0.2 | 7.8±0.2 | 7.5±0.2 | 6.7±0.3 |

* $P \leq 0.01$
** $P \leq 0.001$

wheat irradiated at 0.2 and 2 kGy (20 and 200 krad). However, the intensity of crust colour was higher in samples irradiated at 2 and 10 kGy (200 and 1000 krad) level indicating high dose of radiation to cause more browning. The general appearance was satisfactory for the breads prepared only up to 2 kGy (200 krad). The specific loaf volume was very highly significantly ($P \leq 0.001$) lower in breads prepared with wheat irradiated with 10 kGy (1000 krad).

*2.5. Sensory evaluation of breads*

Results on the organoleptic evaluation of bread and *chapati* are given in Tables 4, 5 and 6. Bread prepared with 2 kGy (200 krad) wheat, although scoring lower than the control, scored sufficiently high to be considered as acceptable by majority of consumers (Table 4). However, scoring was significantly low ($P \leq 0.1$) at 10 kGy (1 Mrad). The mean score for *chapati* did not exhibit any significant difference in rating, which could be attributed to irradiation treatment. Each individual undesirable characteristic, if any, in bread prepared from irradiated wheat, was evaluated on intensity scale of 1–10 points by trained panel members. Examination of the values (Table 5) revealed that intensity of discoloration, off-odour, irradiated flavour and unfavourable textural changes, seem to be functions of dosage used. The initial intensity of off-characteristics shifted from lower to higher ranges in irradiated samples. However, even at 2 kGy (200 krad), only slight discoloration (2.4) and traces (2.0) of other undesirable characteristics were identified. These were more pronounced at 10 kGy (1000 krad).

Further, each desirable (+) and undesirable (−) – external and internal – characteristic in breads, was evaluated and described in appropriate descriptive terms. Averages of the frequency distributions of opinions of the three panels each consisting of 12 members are shown in Table 6. When 50% or more of the total membership of the panel approved the description of the positive characteristics, the sample was considered as acceptable. The majority of them (11 out of 12), preferred the bread prepared from 0.2 kGy (20 krad) the most, because of its large and even volume, light crust colour and spongy uniform cell structure. Most of the members disapproved the dark crust colour of the bread, prepared from wheat irradiated at higher doses [2 and 10 kGy (200 and 1000 krad)]. Irradiated flavour and taste were detected by many members in 2 kGy (200 krad) samples, but these were not evident in 0.2 kGy (20 krad) bread. However, the rating for chewability of control bread was highest because of its softness and lowest for 10 kGy (1000 krad) samples because of its rubbery texture as noticed by several panel members. These ratings were reflected in overall acceptability; this was highest (91%) for 200 Gy (20 krad) bread and lowest (25%) for 10 kGy (1 Mrad) samples.

## 3. Conclusions

The compositional changes in irradiated wheat starch (ANANTHASWAMY *et al.*, 1970a, b) and proteins (SRINIVAS *et al.*, 1972) were directly reflected in the physical properties, measured in present studies. At a constantly increasing temperature, the gelatinization property of starch, which is a process similar to the first stage of baking and governed by modifications brought about by amylases during the heating period, is markedly affected by radiation treatment. The decrease in amylogram peak height (Table 1) in the irradiated samples, seems to be a function of dose and may be attributed to increased susceptibility of starch degradation products to amylolysis (LEE, 1960). This may result in stimulation of gas production up to certain limit in dough (LEE, 1959) which gives higher loaf-volume (LAI *et al.*, 1957; TIPPLES, 1969). The beneficial effect of irradiation on bread volume is evident only when baking formula contains less sugar (2.5% in the present studies) but not when rich formula (more than 6% sugar) is employed (RAO *et al.*, 1975). Physically damaged smaller particle-sized starch may also help in increasing the available surface area of hydration (FINNEY *et al.*, 1960; MEREDITH, 1966) as confirmed by increased water absorption capacity of irradiated samples (Table 2). GREER and STEWART (1959) have shown that dextrin chain length in starch governs the hydration rate of wheat flour. The differences in dough consistency at the start and 12 min later in control and irradiated samples (Table 2), also suggest that mixing tolerance of

Table 5

*Technical evaluation of off-characteristics of breads prepared with control and irradiated wheat*

| Off-characteristics | Average score on control | [a]Intensity scale for breads Irradiated (kGy) | | |
|---|---|---|---|---|
| | | 0.2 | 2 | 10 |
| Discoloration | 1.2±0.21 | 1.8±0.25 | 2.4±0.28 | 4.9±0.30 |
| Off-odour | 1.0±0.22 | 1.3±0.22 | 1.9±0.20 | 3.7±0.37 |
| Irradiated flavour | 1.0±0.20 | 1.3±0.25 | 1.9±0.22 | 4.9±0.32 |
| Off-flavour (other than irradiation) | 1.1±0.28 | 1.8±0.28 | 2.0±0.30 | 4.0±0.35 |
| Texture | 1.1±0.25 | 1.2±0.22 | 2.0±0.28 | 5.5±0.32 |

[a] Intensity scale

| | |
|---|---|
| 1 None | 6 Above moderate |
| 2 Trace | 7 Strong |
| 3 Slight | 8 Very strong |
| 4 Below moderate | 9 Extreme |
| 5 Moderate | 10 Very extreme |

Results are averages of three panel tests ± S.E.

Table 6

*Frequency distribution of preference test for bread characteristics*
Results are the averages of the opinion of the three taste panels, each one consisting of 12 members. Frequency distribution expresses the number of members, describing either positive or negative characteristics

| Characteristics | Description | | Frequency distribution of number of panel members | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Irradiated (kGy) | | | | | | | |
| | Positive + | Negative — | 0 | | 0.2 | | 2 | | 10 | |
| | | | + | — | + | — | + | — | + | — |
| Bread volume | Large | Small | | | | | | | | |
| | even regular | uneven irregular | 7 | 5 | 11 | 1 | 6 | 6 | 4 | 8 |
| Crust colour | Light brown | Dull brown | | | | | | | | |
| | | dark with spots | 9 | 3 | 10 | 2 | 4 | 8 | 3 | 9 |
| Crumb texture | Soft | Hard brittle | 9 | 3 | 9 | 3 | 8 | 4 | 4 | 8 |
| Cell structure | Spongy | Not spongy | | | | | | | | |
| | uniform | irregular | 9 | 3 | 11 | 1 | 8 | 4 | 4 | 8 |
| Flavour | Pleasant | Flat irradiated | 9 | 3 | 10 | 2 | 5 | 7 | 3 | 9 |
| Taste | Acceptable | Bitter burnt | | | | | | | | |
| | | irradiated | 8 | 4 | 9 | 3 | 6 | 6 | 3 | 9 |
| Chewability | Soft | Tough gummy | | | | | | | | |
| | | rubbery | 11 | 1 | 8 | 4 | 7 | 5 | 4 | 8 |
| Overall | Acceptable | Non-acceptable | 10 | 2 | 11 | 1 | 9 | 3 | 3 | 9 |

irradiated wheat flour was increased, resulting in better dough strength and blending value. The dough development by mixing, a critical step in bread making may be affected by hydration rate of proteins. Thus, a lower mixing requirement of dough from irradiated wheat is both desirable and economical when mechanical mixing is done on a commercial basis. Slight increase in dough stability supports heavier mechanical handling and stronger fermentation of dough prepared from irradiated wheat. Evaluation of extensogram (Table 3) indicates some alteration in the gluten quality, which affects the resistance and extensibility of the dough (MILNER, 1957; PAPE, 1973) as a result of irradiation. Larger volume of the loaves from irradiated flour (Table 4) can be expected from the observed extensogram height and ratio figure of hydrated gluten. Thus, fariongraph and extensograph tests support the view that the overall bread-making characteristics of wheat flour are improved up to 2 kGy (200 krad) dose level. However, at higher doses, 10 kGy (1 Mrad), these are adversely affected (MILLER *et al.*, 1964). Overall external and internal characteristics of bread prepared from wheat irradiated upto 2 kGy (200 krad) were not affected by radiation treatment. However, low loaf volume, dark colour of the crust and off flavour, which

are not desired by consumers, contributed in lowering the hedonic scale rating of the samples, irradiated at higher doses (Tables 5 and 6). The dark colour can be attributed to *Maillard* type reaction (POMERANZ *et al.*, 1962), since the liberation of reducing sugars and tyrosine from starch and protein, respectively, is significantly increased in irradiated wheat (ANANTHASWAMY *et al.*, 1971). The increased tyrosine level may also stimulate tyrosinase activity and subsequent polymerization of quinones to melanin, responsible for the browning of various biological materials and darkening of wheat dough (ABROL, 1971; ABROL & UPRETI, 1970).

The changes in rheological properties were reflected more in baking characteristics of leavened bread prepared from irradiated [2 and 10 kGy (200 and 1000 krad)] wheat, than in *chapati*. The hedonic scale rating was comparable for all the samples. Similar observations were also made by AHMAD, *et al.*, (1973) for Pakistani unleavened bread *(roti)*, prepared from wheat irradiated upto 1 kGy (100 krad).

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# SENSORY AND INSTRUMENTAL MEASUREMENT OF COLOUR CHANGES OCCURRING DURING STORAGE IN SOME CARROT VARIETIES

E. KOVÁCS, L. HORVÁTH and J. BENCZE-BŐCS

(Received November 1, 1976; accepted February 22, 1977)

The colour of different carrot varieties was studied as a function of storage time (at 9–11 °C and 70–85% RH). Some of the varieties were suitable for direct consumption *(Amsterdamer Treib, Gonsenheimer, Nantoise)*, the others for industrial processing and storage *(Chantenay, Flakker, Fertődi Vörös)*.

The colour of the samples was established instrumentally and by sensory evaluation.

Close correlation was found between the $\Delta$ E values and saturation values ($r = 0.915$). The correlation coefficient for saturation and sensory scores was $r = 0.834$. Panel members were found to consider mostly saturation of the colour characteristics. The correlation coefficient between $\Delta$ E values and average scores was found to be $r = 0.770$.

Since panel members valued mostly the saturation value while the $\Delta$ E value stands for the joint change of saturation and hue, the lower correlation coefficient is explicable.

Carrot varieties were investigated with a view to finding varieties suitable for storage and industrial processing.

In home use or in industrial processing of horticultural produce their colour may be of decisive importance, sometimes only on account of the attractive appearance, on other occasions, as with carrots, on account of the valuable carotene content.

The selection of the appropriate instrument for the measurement of the colour of various products and that of evaluation methods suitable for comparison are problems of importance.

## 1. Materials and methods

### *1.1. Raw materials and storage*

The raw material was obtained from the FERTŐD STATION OF THE INSTITUTE FOR HORTICULTURAL RESEARCH. The varieties tested were:

early varieties *Amsterdamer Treib*
*Gonsenheimer*
*Nantoise*

varieties suitable for processing and storage

*Chantenay*

*Fertődi Vörös*

*Flakker*

Characterization of the varieties is given in an earlier paper of KOVÁCS and VÖRÖS (1976).

The samples were stored in cases at 9–11 °C and 70–85 % relative humidity.

*1.2. Instrumental colour measurement*

The *Momcolor* tristimulus colorimeter (manufactured by MAGYAR OPTIKAI MŰVEK, Budapest) was used for the measurements.

The instrument is suitable to measure objectively the colour of surfaces, powders, transparent materials and liquids in the internationally accepted CIE colour system. Tristimulus values X, Y and Z are determined with the instrument.

To calibrate the instrument the No. 69-003-00 etalon of series 003, prepared in the NATIONAL OFFICE FOR MEASURING in 1969, was used.

Data were evaluated by the following principle: $\Delta E$ expresses the colour difference between the chromaticity co-ordinates and the colour difference sensing capacity of man. $\Delta E = 1$ is the threshold value of the colour difference sensing capacity of man. A difference of $\Delta E < 1$ falls below the sensing capacity, while differences $\Delta E > 1$ are sensed.

Several methods are available to calculate the $\Delta E$ value. The method described by MCLAREN (1970) is easy to calculate and widely used in the industry. The *Adams-Nickerson* colour difference formula, where $V_x$, $V_y$ and $V_z$ are the transformed values found in X, Y and Z tables, is given below (MCLAREN, 1970).

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

The values obtained by instrumental measurement were calculated by the above formula as in our earlier publication (KOVÁCS *et al.*, 1977).

*1.3. Preparation of the sample*

The colour of the outer surface of carrots was measured. Prisms 12 mm wide and 25 mm long were cut. Three parallel measurements were made of each variety.

*1.4. Sensory colour evaluation*

The colour of the washed carrots was scored by a panel of constant composition. Samples were characterized with scores ranging from 1 to 5, the latter corresponding to the best sample. The average of sensory scores was calculated.

## 2. Results

Figure 1 illustrates the yellowish-red region of the CIE triangle. It may be seen in the Figure that the dominant wave-length of the samples changed in the region of 582–593 nm. The saturation percentages have been marked, they were found to fall between 40–60%. The points in the Figure correspond to the average colour values of the varieties on the basis of the x and y colour coordinates after storage periods of 0.5 and 11 weeks, resp.

One way of demonstrating colour differences consists in showing the distance between the two points by the axis of the *MacAdam* ellipse of the desired direction (x, y) or in other words, in presenting the factor by which the threshold value of the sensitivity of the human eye ($\Delta$ E) has to be multiplied to equal the colour difference.

McLaren's formula (1970) was used to calculate the $\Delta$ E values.

The correlation between the $\Delta$ E values and the scores given in sensory testing or the percentage of saturation, as well as between saturation percentage and sensory scores, was assessed.

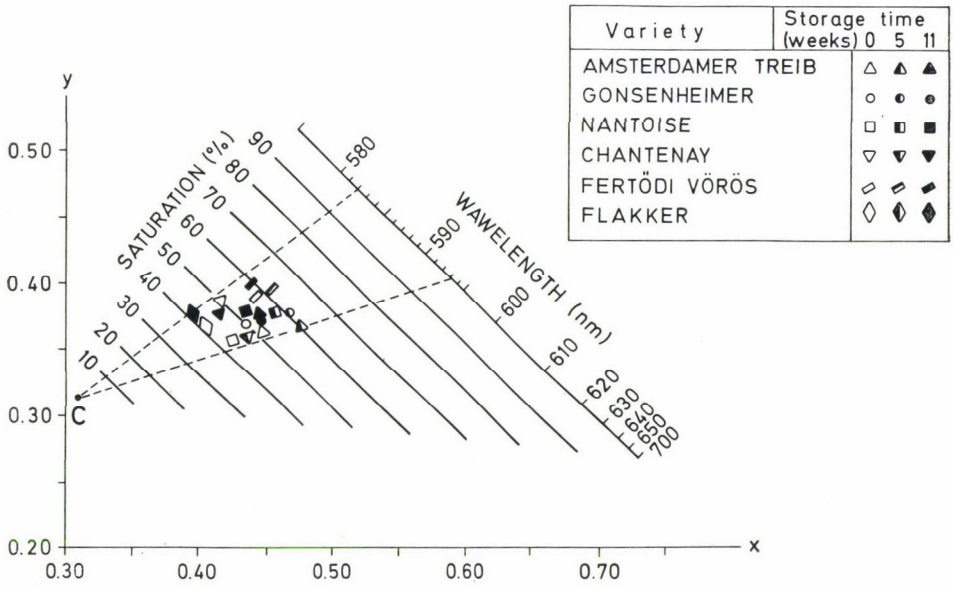


Fig. 1. Colour of the different carrot varieties on the basis of chromaticity coordinates x, y at 0 day and after storage for 5 and 11 weeks, resp. *C* is the CIE light-source corresponding to day light.
Measurements were carried out with a *Momcolor* tristimulus industrial laboratory colorimeter (Magyar Optikai Müvek, Budapest) To calibrate the apparatus the No. 69–003–00 etalon of series 003, prepared in the National Office for Measuring in 1969, was used

Results are shown in Figs. 2, 3 and 4.

The correlation between saturation and the $\Delta$ E value is close (r = 0.925): the more vivid the colour of the sample the lower is the $\Delta$ E value. Dull colour is characterized by a high $\Delta$ E value (Fig. 2).

The correlation between saturation and sensory scores is also close (r = 0.83). This reflects a satisfactory correlation between the results of instrumental measurement and sensory evaluation (Fig. 3). In comparing the $\Delta$ E values to the sensory scores a correlation coefficient of r = 0.77 was obtained.

Table 1

*Colour changes in different carrot*

The samples were stored at 9–11 °C and 70–85% RH. Measurements were carried out with

| | Storage period (week) | n | x | | y | |
|---|---|---|---|---|---|---|
| | | | Average value | Standard deviation | Average value | Standard deviation |
| Early varieties | | | | | | |
| *Amsterdamer Treib* | 0 | 3 | 0.4540 | 0.0184 | 0.3673 | 0.0073 |
| | 5 | 3 | 0.4797 | 0.0076 | 0.3741 | 0.0086 |
| | 11 | 3 | 0.4461 | 0.0096 | 0.3775 | 0.0072 |
| *Gonsenheimer* | 0 | 3 | 0.4410 | 0.0078 | 0.3735 | 0.0173 |
| | 5 | 3 | 0.4739 | 0.0077 | 0.3883 | 0.0051 |
| | 11 | 3 | 0.4511 | 0.0113 | 0.3765 | 0.0110 |
| *Nantoise* | 0 | 3 | 0.4280 | 0.0153 | 0.3637 | 0.0082 |
| | 5 | 3 | 0.4632 | 0.0058 | 0.3797 | 0.0044 |
| | 11 | 3 | 0.4351 | 0.0142 | 0.3823 | 0.0021 |
| Varieties for industrial use and storage | | | | | | |
| *Chantenay* | 0 | 3 | 0.4189 | 0.0250 | 0.3898 | 0.0107 |
| | 5 | 3 | 0.4412 | 0.0477 | 0.3561 | 0.0110 |
| | 11 | 3 | 0.4214 | 0.0106 | 0.3780 | 0.0050 |
| *Fertődi vörös* | 0 | 3 | 0.4477 | 0.0042 | 0.3713 | 0.0083 |
| | 5 | 3 | 0.4757 | 0.0152 | 0.3708 | 0.0191 |
| | 11 | 3 | 0.4547 | 0.0166 | 0.3910 | 0.0133 |
| *Flakker* | 0 | 3 | 0.4146 | 0.0075 | 0.3721 | 0.0074 |
| | 5 | 3 | 0.4396 | 0.0308 | 0.3679 | 0.0067 |
| | 11 | 3 | 0.4031 | 0.0096 | 0.3836 | 0.0092 |

Panel members were strongly influenced by the vividness (saturation) of colour and the $\Delta E$ value stands for the joint change of saturation and hue which seems to explain the lower correlation coefficient, $r = 0.77$.

A deficiency of the $\Delta E$ value is that it is not quite realistic when a change in saturation is accompanied by a change in colour. It would be of interest to develop a more specific statistical evaluation method from the $\Delta E$ values and the regions of the *MacAdams* ellipse concerned which would reflect the changes in colour more accurately than the present procedure.

Results pertinent to varieties are given in Table 1.

*varieties as a function of time*

*Momcolor* tristimulus industrial laboratory colorimeter (MAGYAR OPTIKAI MÜVEK, Budapest)

| X | Y | Z | | | | $\Delta E_{AN42}$ | |
|---|---|---|---|---|---|---|---|
| Average value | Average value | Average value | $V_x$ | $V_y$ | $V_z$ | Between 0 and 5 Between 0 and 11 weeks | Change (%) between the 5th and 11th weeks* |
| 27.80 | 22.49 | 10.93 | 5.851 | 5.292 | 3.546 | | |
| 35.52 | 27.55 | 10.83 | 6.501 | 5.783 | 3.531 | 11.81 | |
| 34.18 | 28.92 | 13.32 | 6.397 | 5.900 | 3.885 | 7.85 | + 33.5 |
| 38.74 | 32.78 | 16.32 | 6.745 | 6.230 | 4.262 | | |
| 40.19 | 32.41 | 12.03 | 6.855 | 6.198 | 3.705 | 0.65 | |
| 37.95 | 31.71 | 14.36 | 6.693 | 6.140 | 3.899 | 4.93 | —658.5 |
| 27.74 | 23.56 | 13.49 | 5.842 | 5.403 | 3.912 | | |
| 31.22 | 25.61 | 10.61 | 6.150 | 5.598 | 3.496 | 11.46 | |
| 29.77 | 26.18 | 12.53 | 6.029 | 5.654 | 3.775 | 7.46 | +34.9 |
| 22.63 | 21.00 | 10.19 | 5.348 | 5.135 | 3.430 | | |
| 26.26 | 21.07 | 11.75 | 5.712 | 5.145 | 3.668 | 15.35 | |
| 34.44 | 30.92 | 16.58 | 6.413 | 6.072 | 4.297 | 10.59 | +31.0 |
| 32.88 | 27.31 | 13.27 | 6.292 | 5.756 | 3.885 | | |
| 32.03 | 25.03 | 10.35 | 6.217 | 5.540 | 3.455 | 7.24 | |
| 32.61 | 28.11 | 11.08 | 6.267 | 5.828 | 3.569 | 7.72 | —6.6 |
| 17.68 | 15.85 | 9.10 | 4.804 | 4.541 | 3.248 | | |
| 26.94 | 22.85 | 12.56 | 5.769 | 5.333 | 3.789 | 11.37 | |
| 25.06 | 23.99 | 13.35 | 5.598 | 5.443 | 3.899 | 10.69 | +6.4 |

* — paling
\+ darkening

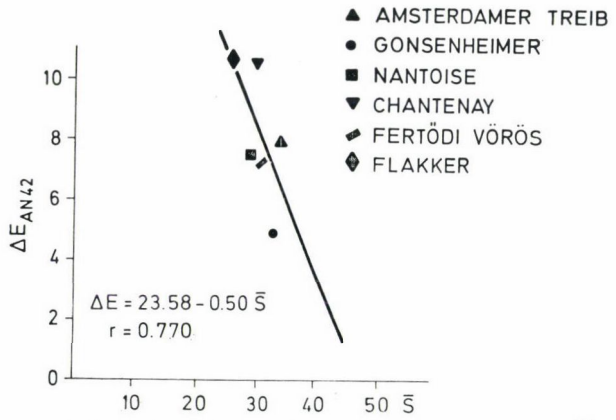


Fig. 2. Comparison of the $\Delta$ E values and average sensory scores ($\bar{S}$) of carrot samples stored for 11 weeks.
The $\Delta$ E values are the averages of 3 parallel measurements. Scores are the averages of those of 10 panel members

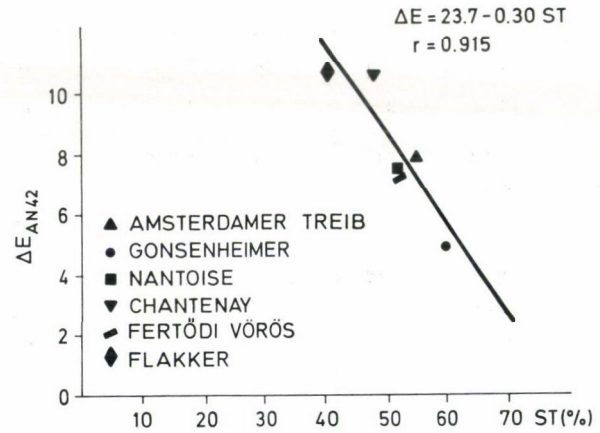


Fig. 3. Comparison of the $\Delta$ E values and saturation values (ST) of different carrot varieties after a storage period of 11 weeks. The $\Delta$ E values are the averages of 3 parallel measurements

*Comparison of samples stored for 0 and 5 weeks*

The colour of variety *Gonsenheimer*, not sensitive to harvesting and storage, changed but slightly, while that of the other varieties, including *Fertődi Vörös*, altered. The change consisted in reduction of saturation, the samples became paler or the yellow component increased at the expense of the orange-red component.

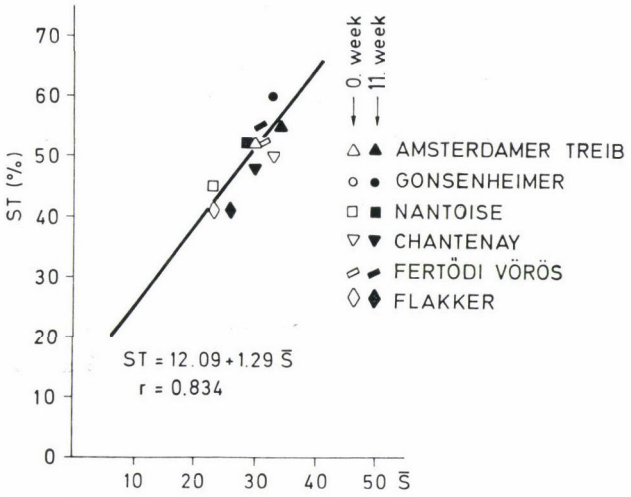


Fig. 4. Comparison of saturation values (ST) and average scores by regression analysis. Samples on 0 day and after 11 weeks storage were used for comparison. The $\Delta$ E values are averages of 3 parallel measurements

*Comparison of the 11-week sample with the original*

The change was slightest in variety *Flakker* (6.4%) and in *Fertődi Vörös* (–5.8%). The colour of varieties *Nantoise*, *Amsterdamer Treib*, *Chantenay* turned more vivid (*cca.* 30%). Percentages show the changes observed between the 5th and 11th weeks. The colour of the *Gonsenheimer* variety exhibited a rapid change, visible to the naked eye, it became more and more pale (Table 1).

## 3. Conclusions

The colour of different carrot varieties was studied in order to find out whether the results obtained by the objective instrumental method applied were comparable with those of the sensory test.

Close correlation was established between the two methods. According to the results the following varieties are suitable for industrial processing: *Fertődi Vörös* and *Flakker* are most suitable because their colour exhibited the slightest change during the storage period of 11 weeks. *Chantenay*, *Nantoise* and *Amsterdamer Treib* are also suitable for storage. Variety *Gonsenheimer* seemed least suitable for industrial processing.

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# FEED UTILIZATION EFFICIENCY OF ENZYME-CONTAINING FEEDS IN LIVE-STOCK RAISING

## PART I. – ALFA-AMYLASE-CONTAINING FEEDS IN THE RAISING OF BROILER CHICKENS

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Sexed chicklings (one-day-old female and male chicks) were bred during 56 days on enzyme-free and two kinds of alfa-amylase-containing rations. The starting ration contained 5 SKB U $g^{-1}$ (0.2% enzyme preparation), the breeding ration 10 SKB U $g^{-1}$ (0.4% enzyme preparation) for 4 groups of each variant, consisting of 120 chicks each. Four groups of each variant, consisting of 120 chicks each were started and fed on rations containing 10 SKB U $g^{-1}$ (0.4 % alfa-amylase preparation).

At the end of the experiment, the female chicks started on lower amylase content feed produced a weight increase which was 34 g higher than that of the enzyme-free control group, while those fed 0.4% alfa-amylase preparation gained 56 g more. For male chicks the additional increase was 55 g and 75 g, resp. The excess increase in weight of female and male chicks fed 0.2% enzyme preparation was significant at a probability level of 95%, while those fed 0.4% showed an extra increase which was significant at the 99% level of probability.

With female chicks fed on rations containing enzyme, 70–80 g feedstuff was saved per 1 kg live-weight as against the control group, the difference being significant at the 90% probability level. With male chicks the saving was 80 g and 160 g, resp., proven to be significant at 95% and 99% probability.

The results obtained with the two types of enzyme ration were also compared with each other at the end of the breeding period. The average body weight of female chicks given 0.4% enzyme preparation during the whole period was 1431 g, 22 g more than the body weight of female chicks (1409 g) started on rations containing 0.2% enzyme preparation and receiving 0.4% amylase preparation in their feed after the 29th day. The average body weight of male chicks fed rations complemented with 0.4% enzyme preparation was 1714 g on the 56th day, *i. e.* 20 g heavier than that of male chicks given 0.2% enzyme preparation for 28 days and 0.4% from the 29th day (1694 g). The difference between the average body weights of chicks fed on the two kinds of ration (20–22 g) was not found to be significant at the probability level of 90%.

The specific feed consumption of female chicks bred on the two kinds of enzyme- supplemented diets was almost identical (2.39 kg and 2.38 kg). The male chicks utilized the ration of higher enzyme content better in that they needed 80 g less feed for the production of 1 kg live-weight (2.36 and 2.28 kg). The higher feed utilization is not significant at the 90% probability level.

On slaughter the weight of broilers given enzyme exceeded the weight of the control group. The eviscerated chicks were heavier by 88 g when fed on 0.2 and 0.4% enzyme preparation and by 93 g when fed on 0.4% enzyme preparation throughout. The weight of legs was higher by 34 g and 29 g, resp., the breast by 25 g and 28 g, resp. The higher gain was proven at the 99% probability level.

The experiments have shown that in broiler breeding it is sufficient to start with the addition of 5 SKB U $g^{-1}$ enzyme while it is expedient to complement the feed by 10 SKB U $g^{-1}$ in a later stage of breeding.

Little is known of the enzymic degradation of mixed feed in the live animal. Beside the hydrolytic enzyme present in the pancreatic juice, the

mixed feed is degraded by the microflora and by the tissue enzymes present in the feed.

The degree of degradation depends on the kind of enzyme and the kind of starch (REED, 1966). Experiments carried out with male chicks permitted of the same conclusion. The starch of corn was utilized better than potato starch. The difference in the digestion of the two types of starch was ascribed to the amylase specificity of broilers (NITSAN & BARTOV, 1972).

The degradation of starch in the feed may be improved by the addition of enzyme preparations produced by microorganisms.

The use of microbial enzyme preparation in the food industry is fairly general. Their use as "food additive" was permitted in a number of countries (ILANY, 1975).

Several hundreds of enzyme preparations are manufactured and used in Japan. The volume of production in the U.S.A. amounted to 34 million dollars in 1971 and a 66 % increase is envisaged for 1980 (VÁMOS-VIGYÁZÓ 1974).

The Swiss firm UFA manufactures enzyme preparations specifically for animal feeding purposes (ANON, 1970; BICKEL, 1971). According to the information leaflet of the Swiss firm BÜHLER modern premix factories utilize not only hormones and medicaments but also enzymes (ANON, 1975).

Diets complemented with alfa-amylase were mainly used in the breeding of broilers. The aim of this study was to compare the parameters of chickens fed on diets free of enzyme and complemented with alfa-amylase, resp.

## 1. Materials and methods

### *1.1. Materials*

*1.1.1. Animals.* The experiments were carried out with broiler hybrids obtained from the RESEARCH INSTITUTE FOR SMALL ANIMALS, Gödöllő. The birds are identified under the name *Mezőturi*.

*1.1.2. Diets.* Commercial starter and breeding diet were used in the experiment. Their composition is shown in Table 1.

The starter feed was given till the 28th day. From then on till the end of the experiment (56th day) breeding feed was administered.

*1.1.3. Enzyme preparation.* Bakterie Amylase Novo 240 (Denmark) prepared by the fermentation of *Bacillus subtilis* was used in this study. The concentration of the preparation was 3 000 SKB U $g^{-1}$.

### *1.2. Methods*

*1.2.1. Circumstances of rearing.* The chicklings were sexed and then vaccinated. Each chick was weighed and provided with a wing-mark. The distribution of weights between groups is shown in Table 2.

Table 1

*Composition of diets*

| Serial number | Ingredient | Starter diet | Breeding diet |
|---|---|---|---|
| 1. | Corn meal | 62.0 | 68.0 |
| 2. | Wheat germ | 2.0 | — |
| 3. | Soya bean meal (50% protein) | 21.5 | 19.6 |
| 4. | Fish meal (70% protein) | 3.0 | 3.0 |
| 5. | Meat meal (58% protein) | 2.7 | 1.8 |
| 6. | Animal feeding yeast | 1.2 | — |
| 7. | Fat | 3.0 | 3.0 |
| 8. | ÁP 17 (Ca : P) | 1.2 | 1.3 |
| 9. | Feeding limestone | 1.9 | 1.7 |
| 10. | Feeding salt | 0.2 | 0.3 |
| 11. | Premix *Mabro* | 0.5 | — |
| 12. | Premix *Mabro* | — | 0.5 |
| 13. | Methionine-containing premix | 0.8 | 0.8 |
| | Total | 100.0 | 100.0 |

Table 2

*Weight distribution of chicklings*

| Body weight (g) | Female chicks (%) | Male chicks (%) |
|---|---|---|
| 38 | 28 | 27 |
| 40 | 36 | 34 |
| 42 | 24 | 25 |
| 44 | 12 | 14 |
| Total | 100 | 100 |

The experiments were carried out with 12 groups of 120 female chicks of almost identical weight (40.4 g) each and with 12 groups of 120 male chicks each. Of the 12 groups

— 4 were fed on enzyme-free diet;

— 4 groups obtained starter ration complemented with 5 SKB $Ug^{-1}$ and breeding ration complemented with 10 SKB U $g^{-1}$ of alfa-amylase (~0.2 and 0.4% of the commercial preparation): hereinafter enzyme concentrations will be given in terms of concentration (%) of enzyme preparation in the diet: this will be called the "0.2→0.4% diet" or "0.2→→0.4% ration";

— 4 groups were raised on feed containing 0.4% alfa-amylase preparation thoroughout (hereinafter: "0.4% feed" or "0.4% ration").

*1.2.2. Processing of data.* Each bird was weighed every 14 days, taking notice of the wing-marks. Feed comsumption of each group was established simultaneously. The average body weight of each group, the average daily gain, the average daily feed consumption and the amount of feed required for the production of 1 kg live-weight were calculated.

*1.2.3. Measurement of the alfa-amylase concentration.* The alfa-amylase concentration in the enzyme preparation and in the feed was measured by the method of *Wohlgemuth* as modified by SANDSTEDT, KNEEN and BLISH (SANDSTEDT *et al.*, 1939) and was expressed in SKB units (U).

*1.2.4. Slauthering.* When the experiment was finished the broilers were slaughtered in an experimental slaughterhouse. The eviscerated birds, the legs and the breasts were weighed.

*1.2.5. Biometrical treatment of data.* The average body weights of the chicks fed on different rations, the feed utilization and the data obtained on slaughtering were compared by *Student*'s *t* test.

The normal distribution was calculated from the empirical frequency distribution of body weights. The groups were compared on the basis of average body weights calculated from the normal distribution.

The correlation between the daily average gain and the average daily feed consumption was expressed by a polynomial of the second order. The maximum of the curve was determined by partial regression coefficients (SVÁB, 1973).

## 2. Results

### *2.1. Gain of weight*

The average body weights, on every 14th day, of the female and male chicks bred on enzyme-free and two kinds of enzyme-containing rations are presented in Table 3.

The weight of the female chicks raised on enzyme-free ration averaged 1375 g. After 56 days, the average body weight of female chicks raised on 0.2→0.4% ration was significantly higher than the control by 34 g (1409), while the weight of those on a 0.4% ration was highly significantly more by 56 g (1431 g). The 22 g difference between the average body weights of female chicks raised on two different types of enzyme-containing ration was not significant at the 90% probability level.

On comparing the average body weights of male chicks again the 0.4% feed resulted in the highest average body weight, 1714 g. The average body weight of the male chicks given the feed containing less enzyme was 1694 g.

Table 3

*Average body weights of broilers*

| Age (days) | Alfa-amylase concentration (%) | | | | | |
|---|---|---|---|---|---|---|
| | Female chicks | | | Male chiks | | |
| | Free of enzyme | 0.2→0.4 % diet | 0.4 % feed | Free of enzyme | 0.2→0.4 % diet | 0.4 % feed |
| | $\bar{x}\pm s$ | $\bar{x}\pm s$ | $\bar{x}\pm s$ | $\bar{x}\pm s$ | $\bar{x}\pm s$ | $\bar{x}\pm s$ |
| 1–14 | $183\pm3$ | $182\pm2^{\varnothing}$ | $179\pm2^{\varnothing}$ | $165\pm7$ | $164\pm4^{\varnothing}$ | $166\pm1^{\varnothing}$ |
| 1–28 | $516\pm14$ | $521\pm11^{\varnothing}$ | $526\pm4^{\varnothing}$ | $555\pm19$ | $564\pm22^{\varnothing}$ | $560\pm7^{\varnothing}$ |
| 1–42 | $924\pm18$ | $931\pm16^{\varnothing}$ | $964\pm37^{*}$ | $1052\pm25$ | $1015\pm39^{\varnothing}$ | $1061\pm45^{\varnothing}$ |
| 1–56 | $1375\pm6$ | $1409\pm27^{*}$ | $1431\pm29^{**}$ | $1639\pm19$ | $1694\pm26^{*}$ | $1714\pm25^{**}$ |

Average body weight of chicklings: $40.4\pm2.0$ g
$\bar{x}$ = average body weight of four groups (g)
$\pm s$ = standard deviation (g)
Difference in comparison to the enzyme-free control:
$\varnothing$ = not significant at the 90% probability level
* = significant at the 95% probability level
** = highly significant at the 99% probability level

In comparison to the 1639 g average body weight of the control group, the weight increase of 75 g was highly significant, while the 55 g increase was significant. The 20 g difference in the average body weights of the groups fed on the two kinds of enzyme-containing feeds was not significant at the 90% probability level.

*2.2. Distribution of body weights*

The individual body weights of female and male chicks raised for 56 days on identical ration was summarized and calculated. It was found that the empirical frequency distribution of the individual weights of chicks raised on three kinds of ration did not differ significantly from the normal distribution.

Calculated on the basis of the normal distribution, the average body weight of female chicks raised on the control ration amounted to 1377 g, that of male chicks to 1653 g. These average weights were considered limit values and the body weight distribution of female and male chicks raised on the two kinds of enzyme-containing feed was compared to these (Table 4).

When the starter feed contained 0.2% and the breeding ration 0.4% alfa-amylase preparation, 60% of the 445 female chicks and 61% of the 434 male chicks possessed body weights higher than the average body weights of the corresponding control group.

Table 4

*Body weight distribution calculated on the basis of the normal distribution*

| Ration | Female chicks | | | | | | Male chicks | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Calculated average, body weight ± standard deviation (g) | Number of birds below average body weight of control | | above average body weight of control | | Total number of birds (no.) | Calculated average body weight and ± standard deviation (g) | Number of birds below average body weight of control | | above average body weight of control | | Total number of birds (no.) |
| | | (%) | (no.) | (%) | (no.) | | | (%) | (no.) | (%) | (no.) | |
| 1. Free of enzyme (control) | 1377 ±138 | 50 | 227 | 50 | 227 | 454 | 1653 ±139 | 50 | 225 | 50 | 224 | 449 |
| 2. 0.2→0.4% diet | 1043 ±108 | 40 | 178 | 60 | 267 | 445 | 1694 ±152 | 39 | 169 | 61 | 265 | 434 |
| 3. 0.4% feed | 1428 ±104 | 30 | 136 | 70 | 318 | 454 | 1709 ±146 | 35 | 157 | 65 | 291 | 448 |

On administering 0.4% feed, 70% of the 454 female chicks and 65% of the 448 male chicks were of body weights higher than the average of the control group.

*2.3. Correlation between the average daily feed consumption and the average daily weight gain*

The correlation between the average daily feed consumption and the average daily weight gain of broilers raised on enzyme-free and two different enzyme-containing rations, was investigated. The correlation of the breeding parameters was described by an equation of the second order.

The second order polynomials belonging to the female and male chicks are illustrated separately (Figs. 1 and 2).

The quadratic equations, the total correlation coefficients ($R$), the complete determinant coefficients ($R^2$) and the number of linear and quadratic members calculated, as well as the maxima of the curves are shown in the figures.

The quadratic correlation of the six groups investigated is close and significant according to the $F$ test. The linear and quadratic effects as calculated from the total regression were also studied by the $F$ test. The variation in the average daily weight gain is explained in 96–97% by linear and in about 2% by quadratic terms.

The average daily weight gain of both female and male chicks raised on rations containing enzyme was higher than that of the control group.

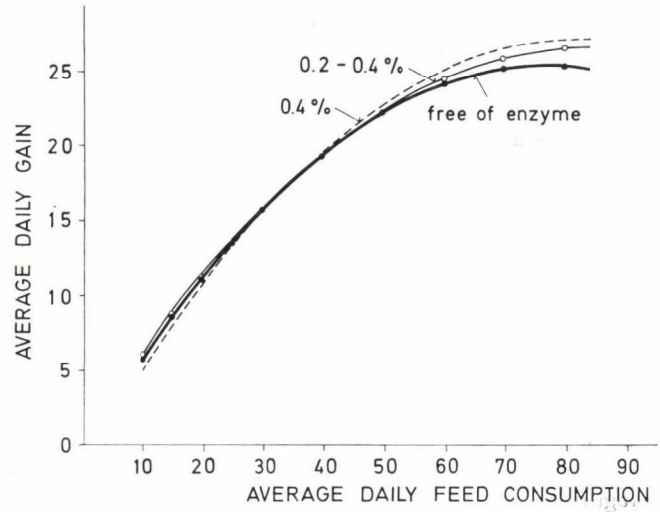


Fig. 1. Correlation between the breeding parameters for female chicks

| | | R | $R^2$(%) | $r^2_{linear}$(%) | $r^2_{quadratic}$(%) | $x_{maxima}$ |
|---|---|---|---|---|---|---|
| Free of enzyme: | $Y' = -0.440 + 0.666x - 0.0043x^2$ | 0.9965 | 99.30 | 96.80 | 2.50 | 77.44 |
| 0.2 → 0.4 % diet: | $Y' = -0.142 + 0.639x - 0.0038x^2$ | 0.9941 | 98.83 | 96.96 | 1.87 | 83.42 |
| 0.4 % feed: | $Y' = -1.695 + 0.704x - 0.0043x^2$ | 0.9985 | 99.71 | 87.30 | 2.41 | 81.75 |

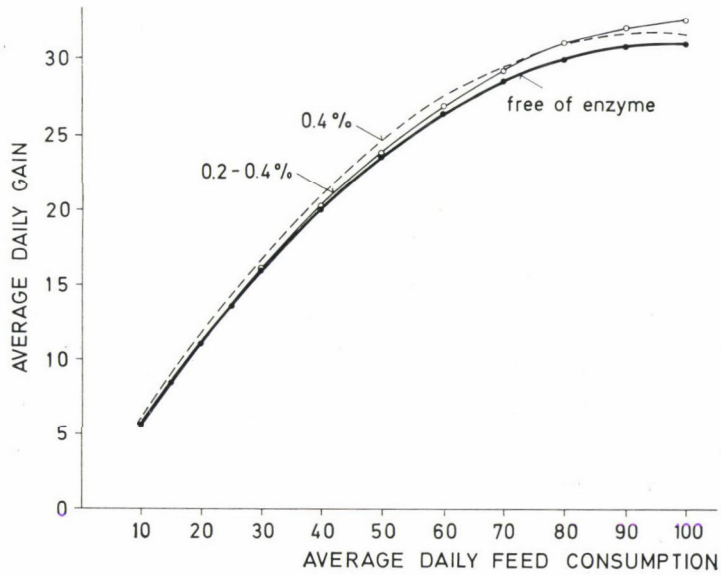


Fig. 2. Correlation between the breeding parameters for male chicks

| | | R | $R^2$(%) | $r^2_{linear}$(%) | $r^2_{quadratic}$(%) | $x_{maxima}$ |
|---|---|---|---|---|---|---|
| Free of enzyme: | $Y' = -0.905 + 0.651x - 0.0033x^2$ | 0.9972 | 99.45 | 97.05 | 2.40 | 97.3 |
| 0.2 → 0.4 % diet: | $Y' = -0.585 + 0.647x - 0.0031x^2$ | 0.9923 | 98.47 | 96.49 | 1.98 | 103.1 |
| 0.4 % feed: | $Y' = -1.163 + 0.706x - 0.0037x^2$ | 0.9969 | 99.39 | 96.92 | 2.47 | 93.7 |

Table 5

*Specific feed consumption of broilers*

| Age (days) | Alfa-amylase concentration | | | | | |
|---|---|---|---|---|---|---|
| | Female chicks | | | Male chicks | | |
| | Free of enzyme | 0.2→0.4 % diet | 0.4 % feed | Free of enzyme | 0.2→0.4 % diet | 0.4 % feed |
| | $\bar{x}\pm s$ | $\bar{x}\pm s$ | $\bar{x}\pm s$ | $\bar{x}\pm s$ | $\bar{x}\pm s$ | $\bar{x}\pm s$ |
| 1—14 | $1.78\pm0.03$ | $1.80\pm0.03^{\varnothing}$ | $1.82\pm0.04^{\varnothing}$ | $1.82\pm0.10$ | $1.83\pm0.07^{\varnothing}$ | $1.79\pm0.02^{\varnothing}$ |
| 1—28 | $1.91\pm0.03$ | $1.93\pm0.04^{\varnothing}$ | $1.96\pm0.04^{\varnothing}$ | $1.99\pm0.13$ | $1.88\pm0.12^{\varnothing}$ | $1.82\pm0.05^{*}$ |
| 1—42 | $2.24\pm0.03$ | $2.18\pm0.02^{*}$ | $2.13\pm0.09^{*}$ | $2.16\pm0.05$ | $2.13\pm0.10^{\varnothing}$ | $2.08\pm0.03^{*}$ |
| 1—56 | $2.46\pm0.04$ | $2.39\pm0.04^{x}$ | $2.38\pm0.07^{x}$ | $2.44\pm0.02$ | $2.36\pm0.05^{*}$ | $2.28\pm0.07^{**}$ |

$\bar{x}$ = average specific feed consumption of 4 groups ($kg \cdot kg^{-1}$)
$\pm s$ = standard deviation (kg)
Difference relative to the enzyme-free control:
$\varnothing$ = not significant at the 90% probability level
x = significant at the 90% probability level
* = significant at the 95% probability level
** = highly significant at the 99% probability level

### *2.4. Specific feed consumption*

The specific feed consumption of female and male chicks raised on three different types of ration is shown in Table 5.

At the end of the experiment the specific feed consumption of female chicks given the control ration was 2.46 kg, while that of those raised on feed complemented with alfa-amylase was 2.39 kg and 2.38 kg, resp. In comparison to the control group the lower specific feed consumption (by 70 and 80 g, resp.) is significant at the 90% probability level. Feed utilization of female chicks raised on the two kinds of enzyme-containing ration was nearly identical.

The specific feed consumption of male chicks raised on enzyme-free ration amounted to 2.44 kg on the 56th day. On ration 0.2→0.4% it was 80 g less (2.36 kg), with the 0.4% feed 160 g less was required and the difference was significant or highly significant, resp. The 80-g difference between the feed requirement of the groups raised on the two types of enzyme-containing rations was not significant.

### *2.5. Slaughter weight*

The experiment was finished by slaughtering the chicks in an experimental scale slaughterhouse. The eviscerated birds, the legs and the breasts of the 64 broilers were weighed. Weights are summarized in Table 6.

It may be seen from the data that the slaughter weight of the eviscerated birds, the legs and breasts of the broilers raised on enzyme containing rations

Table 6

*Slaughter weights on the 56th day*
(g)

| Parts of the body | Ration | | | | | | Difference between rations | |
|---|---|---|---|---|---|---|---|---|
| | 1 | | 2 | | 3 | | | |
| | Control (22 birds) | | 0.2→0.4% diet (20 birds) | | 0.4% feed (22 birds) | | | |
| | $\bar{x}$ | ±s | $\bar{x}$ | ±s | $\bar{x}$ | ±s | 2–1 | 3–1 |
| Eviscerated body | 1123 | 134 | 1211 | 138 | 1216 | 102 | 88* | 93* |
| Leg | 297 | 35 | 331 | 37 | 326 | 24 | 34** | 29** |
| Breast | 229 | 28 | 254 | 35 | 257 | 30 | 25* | 28** |

$\bar{x} \pm s$ = average ± standard deviation
* = significant at the 95% probability level
** = highly significant at the 99% probability level

were significantly or highly significantly higher than those of the control group. The weights of birds raised on the two different enzyme-containing rations were almost identical.

## 3. Conclusions

The average body weights of broilers raised on the two types of enzyme-containing feed were higher than those of the control group. The weight increase was significant at the 95 and 99% probability levels, resp. To produce unit live-weight less enzyme-containing feed was required than enzyme-free feed. Savings on the feed could be demonstrated for female chicks at 90%, for male chicks at the 95 and 99% levels of probability.

The difference between the average body weights of female chicks as well as for male chicks raised on feeds of different enzyme content amounted to 20–22 g (not significant at the probability level of 90%). The same can be shown by the comparison of the body weight distribution for female and male chicks (Table 4). The reduction of the enzyme content of the starter ration from 0.4 to 0.2% affected the weight distribution on the 56th day very little. Only 10% of the female chicks and 4% of the male chicks had lower body weights at the reduced enzyme content.

Specific feed consumption of female chicks on two different enzyme concentrations was nearly the same (Table 5). Male chicks, however, utilized the feed of higher enzyme content better by 3.4%. The increase in the enzyme content of rations from 0.2% to 0.4% in the second half of the breeding period

resulted in better feed utilization. This is shown by the polynomials of second order (Fig. 2).

Of the quadratic curves representing male chicks raised on two kinds of enzyme-containing ration the one for the higher enzyme content shows a slighter slope at an average daily consumption of 50 g feed ($0.2544 < 0.2705$). Thus, the maximum is reached at the consumption of 93.7 g as against 103.1 g. The direction of the curves and the maxima suggest that the difference in the curves is due to the change in the enzyme concentration in the feed.

Upon slaughter, broilers raised on feed of two different enzyme concentrations were found to have higher eviscerated body, leg and breast weights than the birds raised on feed free of enzyme and the difference was significant at the 95% and 99% probability levels, resp.

On the basis of the results it may be concluded that if the starch content of the starter ration is hydrolysable by alfa-amylase the concentration of the enzyme preparation used may be reduced from 0.4% to 0.2%. The breeding feed is expediently complemented by 0.4% of the alfa-amylase preparation.

*

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# COMPARATIVE ANALYSIS OF THE PROTEOLYTIC ACTIVITY IN ENZYME PREPARATIONS

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The proteolytic activities of Pronáz, prepared by the fermentation from *Bacillus subtilis* and *Streptomyces fradiae, rimosus* and *griseus* and of other neutral and alkaline protease preparations were compared. Casein and serum albumin served as the substrate. The quantity of amino acids formed during degradation was determined with an amino acid analyzer. It was established that the proteins used in the experiments were degraded to free amino acids at a high degree of efficiency by protease preparations [Pronáz from *Streptomyces rimosus* (CHINOIN) 48.8%, Pronase from *Streptomyces griseus* (CALBIOCHEM): 62.0%, conditions of degradation: 0.5% human serum albumin, 5.4 PUK enzyme per mg albumin, pH: 8.5, at 40 °C, 24 h]. Protease preparations of bacterial origin produced a lower amount of amino acids (alkaline protease of *B. subtilis:* 8.9%). On the application of Hungarian Pronáz preparations beside the free amino acids non-hydrolyzed proteins (2–7% of total N), and peptides (48–88%) were present [Pronase (CALBIOCHEM): 3.5 and 34.5%, resp.]. As a result of proteolysis by Pronáz preparations a wide range of amino acids was found in the hydrolysate. On the basis of their properties Pronáz preparations seem to be useful for pretreat to animal feedstuffs. The specific proteolytic capacity of the Pronáz preparations proved of advantage in the determination of *Scheffner*'s Protein Digest Residue index, used for *in vitro* determination of biological value.

The demand for enzyme preparations of proteolytic activity is steadily increasing in the textile, paper and food industries. Another useful application seems to be enzyme pretreat to feedstuffs. In accordance with the various fields of application enzyme preparations of animal, plant and bacterial origin and of different degrees of purity are marketed. Recently the preparations *Pronase* (CALBIOCHEM) and *Pronáz* (CHINOIN), containing an enzyme complex of a wide activity range and manufactured with *Streptomyces* strains, proved to be effective.

In recent years the protease production of *Streptomyces, Aspergillus* and *Actinomyces* strains was studied by many researchers (*Streptomyces griseus:* CHALOUSKA, 1955; NOMOTO & NARAHASHI, 1956a, b, c; SHIRATO & NAGATSU, 1965; SHISHKINA, 1967; WÄHLBY, 1968; *Streptomyces fradiae:* FENIKSOVA *et al.*, 1965; *Aspergillus flavus:* SIPEROVICH & LISENKOV, 1967; *Actinomyces levendulae, Actinomyces violaceus:* BEKHTEREVA *et al.*, 1958). These studies were aimed at the isolation of pronase producing strains, at developing fermentation technology suitable for pronase production and at the isolation of enzymes from the fermentation broths and at developing methods for their purification.

In these experiments we wished to compare the proteolytic activity of Hungarian Pronáz to that of *Streptomyces griseus* Pronase, manufactured by CALBIOCHEM U.S.A., available on the market. These preparations were compared also with preparations of bacterial origin.

A method was worked out to assay the protein degrading capacity of the enzymes investigated. Results obtained in these experiments promote further investigations into the enzymic preparation of animal feeds.

## 1. Materials and methods

### *1.1. Enzyme preparations*

| Preparation | Manufacturer | Specific activity (PUK $g^{-1}$) |
|---|---|---|
| *Streptomyces fradiae* Pronáz | CHINOIN | 29 000 |
| *Streptomyces rimosus* Pronáz | CHINOIN | 23 000 |
| *Streptomyces griseus* Pronase | CALBIOCHEM | 45 000 |
| *Bac. subtilis* neutral protease | CHINOIN | 14 000 |
| *Bac. subtilis* alkaline protease | CHINOIN | 8 000 |

*Protein used in the proteolysis experiments* Hammarsten casein (commercial product); crystalline human serum albumin (Prepared by HUMAN INSTITUTE FOR SEROBACTERIOLOGICAL PRODUCTION AND RESEARCH, Budapest).

### *1.2. Preparation of samples for amino acid analysis*

The protein solutions were hydrolyzed with 6 *N* hydrochloric acid under nitrogen gas pressure for 20 h at 105 °C. On completion of hydrolysis the hydrochloric acid was removed by evaporation on a *Rotadest* evaporator under mild vacuum. 0.5- to 0.1-ml samples were applied to the column. After enzymatic hydrolysis 5 ml of the hydrolysates were acidified with 0.05 ml conc. acetic acid. 0.5- to 1.0-ml samples were applied to the column.

### *1.3. Determination of amino acids*

Determination was carried out on a *Biocal* type automatic amino acid analyzer by the two-column method on *Aminex* A 5 and *Aminex* A 6 resin (BIOCAL, 1969). The columns were eluted with buffer solutions of pH 3.25–4.25 and 5.25, resp. Analysis took 220 or 88 min, resp. To correct for degradation during hydrochloric acid hydrolysis and evaporation the following corrections were applied: threonine 8%, serine 6%, cysteine 4%, tyrosine 10%. Two parallel determinations were carried out with each sample, if the difference between the parallels was not higher than 5%.

*1.4. Ultrafiltration*

To isolate peptides formed during the degradation of proteins the digest was exposed to ultrafiltration. A membrane filter of 5 nm pore diameter of the *Sartorius* system, type SM 121336, was used. According to specifications molecules greater than 10 000 are retained by this filter.

*1.5. Determination of the proteolytic activity of the enzyme preparations*

Determination was carried out by the method of the KAKEN LABORATORY in Japan (CALBIOCHEM, 1970). The PUK unit (Pronase Unit of Kaken Laboratory) is understood to mean the quantity of enzyme that liberates from a 0.2% casein solution in a phosphate buffer of pH 7.38 during 1 h at 40 °C an amount of tyrosine which produces an optical density of 1.00 with *Folin* reagent at 660 nm in a photometer.

*1.6. Non-degraded proteins*

Residual protein, not degraded in the course of proteolysis was determined by the method of LOWRY and co-workers (1951) after precipitation with trichloroacetic acid. Four parallel measurements were made from each sample.

## 2. Results

*2.1. Casein degradation with* Streptomyces fradiae *Pronáz*

In the first stage of the experiments the optimum enzyme: substrate ratio and the effect of temperature upon proteolysis was studied with *Streptomyces fradiae* Pronáz preparation.

Casein (5 mg $ml^{-1}$) was used as a substrate in an aqueous solution, on the basis of preliminary experiments. The pH of the solution was set at 8.5 with a 0.5 *M* sodium hydroxide solution. Of the solution thus prepared 9-ml portions were distributed into test tubes and 1-ml portions of the enzyme solution were added to each. The enzyme: substrate ratio was varied by altering the amount of casein between 1.35 and 6.75 mg $ml^{-1}$. Proteolysis was carried out at 28 or 40 °C in an ultrathermostat, for 24 h. During digestion the initial pH of 8.5 was reduced to 6.9. After 24 h the samples were treated as described in para. 1.2 to 1.6. Results are shown in Table 1.

As it may be seen in the table by changing the enzyme: substrate ratio the degree of degradation, and the free amino acid content of the digest varied. The efficiency of degradation was highest at an enzyme: substrate ratio of 5.4 PUK $mg^{-1}$ casein. In this experiment 26.74% of the casein was

Table 1

*Protein degradation with* Streptomyces fradiae *Pronáz*

| Sample | Enzyme concentration (PUK $ml^{-1}$) | Enzyme: substrate ratio (PUK $mg^{-1}$) | Digestion temperature (°C) | Digestion period (h) | Total amount of amino acids as % casein weighed in |
|---|---|---|---|---|---|
| 1. | 6.75 | 1.35 | 40 | 24 | 17.39 |
| 2. | 13.00 | 2.60 | 40 | 24 | 20.40 |
| 3. | 20.25 | 4.05 | 40 | 24 | 22.56 |
| 4. | 27.00 | 5.40 | 40 | 24 | 26.47 |
| 5. | 33.75 | 6.75 | 40 | 24 | 24.57 |
| 6. | 27.00 | 5.40 | 28 | 24 | 24.48 |
| Casein upon acidic hydrolysis | — | — | — | — | 95.60 |

degraded to amino acids. When the optimum enzyme : substrate ratio was applied the free amino acid content of the digest was higher at 40 °C temperature than at 28 °C.

An aqueous casein solution (5 mg $ml^{-1}$), set at pH 8.5 and hydrolyzed with hydrochloric acid (para. 1.2) was used as control. The total amino acid content of the sample was determined. 95.6 % of the casein were recovered as free amino acid.

Beside free amino acids and intact protein, the digests contained peptides, too. The intact protein was determined in the digested samples according to the method of LOWRY and co-workers (1951). The presence of peptides was shown by the non-identifiable peaks in the chromatograms: in the acidic-neutral runs in the 31st, 53rd, 57th, 66th, 104th and in the 109th min, while in alkaline runs before arginine. These peaks were not observed in the diagrams recorded after the hydrochloric acid hydrolysis of the digests. The ultrafiltration of digests, as described in para. 1.4, proves that the molecular weight of the peptides was below 10 000. The result of amino acid analysis in samples prior to and after ultrafiltration was identical. The non-identifiable peaks as observed in the chromatograms were considered as low-member peptides by HAMILTON (1963) and other authors, as well.

The study of the proteolytic activity of *Streptomyces fradiae* Pronáz aroused interest in various ideas. On studying the amino acid composition in the digest (Table 1) and comparing the essential amino acids with the result of proteolysis carried out with the enzymes of the animal organism (using data of SCHEFFNER *et al.*, 1956) it was found that proteolysis with *Streptomyces fradiae* Pronáz resulted in an amino acid ratio utterly different from that obtained

Table 2

*Percentage of the free essential amino acids in the hydrolysate obtained by acidic and enzymatic degradation of casein*
(Using data by SCHEFFNER, 1967)

| Hydrolyzing agent | Quantity of the free amino acids (percentage of the sum of 9 amino acids) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Tre | Val | Met | Ile | Leu | Phe | Try | Lys | His |
| 6 *N* hydrochloric acid[a] | 8.6 | 15.1 | 6.3 | 12.1 | 20.5 | 11.5 | 2.4 | 16.8 | 6.6 |
| pepsin[a] | 18.6 | 1.6 | 0.2 | 8.8 | 57.0 | 7.2 | 6.0 | 0.5 | 0.1 |
| pepsin-trypsin[a] | 13.6 | 8.2 | 2.9 | 11.2 | 28.4 | 9.1 | 3.1 | 21.1 | 2.3 |
| pepsin-trypsin-erepsin[a] | 12.8 | 11.5 | 3.0 | 10.1 | 25.2 | 10.8 | 3.2 | 20.9 | 2.5 |
| *Streptomyces fradiae*—Pronáz (CHINOIN) | | | | | | | | | |
| average (n = 6) | 7.94 | 7.99 | 9.46 | 11.02 | 20.75 | 27.54 | 2.09[a] | 9.35 | 3.80 |
| standard deviation | 1.05 | 1.15 | 0.29 | 2.50 | 3.20 | 0.75 | — | 1.20 | 0.65 |

[a] SCHEFFNER's data

with pepsin, trypsin or peptidase. Of the essential amino acids methionine and phenylalanine are present in the digest in substantially higher ratio than in samples hydrolyzed with the other enzymes. The results of calculations are presented in Table 2.

*2.2. Comparison of the protein degrading capacity of proteolytic enzyme preparations*

In the second stage of the experiments the proteolytic activity of *Streptomyces fradiae* Pronáz was compared with that of *Streptomyces rimosus* Pronáz and *Streptomyces griseus* Pronase as well as of the neutral and alkaline proteases of *Bacillus subtilis*. The enzyme preparations are specified in para. 1.1. The substrate used was human serum albumin in a 5 mg $ml^{-1}$ aqueous solution. To improve digestibility, the solution was kept for 1 h at 85 °C. Then the pH was set at 8.5 with a 0.5 *M* sodium hydroxide solution. Nine-ml portions of the pretreated albumin solution were distributed into test tubes, to each of which 1 ml of the enzyme solution was added. The enzyme concentration was adjusted to an enzyme: substrate ratio of 5.4 PUK $mg^{-1}$ uniformly. The test tubes were kept for 24 h in the thermostat at 40 °C during which the pH was controlled. Amino acid analysis was carried out as described in para. 1.3. Results are presented in Table 3.

When proteolysis was finished in an aliquot of the samples the non-degraded protein was precipitated with trichloro-acetic acid and determined

Table 3

*Comparative analysis of protein degradation by different proteolytic enzyme preparations*

| Enzyme preparation | Enzyme concentration in the digest (PUK $ml^{-1}$) | Enzyme: substrate ratio (PUK $mg^{-1}$) | Distribution of N fractions | | |
|---|---|---|---|---|---|
| | | | Free amino acids (%) | Peptides (%) | Protein forming precipitate with trichloro-acetic acid (%) |
| *Streptomyces fradiae* Pronáz | 27.00 | 5.40 | 32.0 | 66.0 | 2.0 |
| *Streptomyces rimosus* Pronáz | 27.00 | 5.40 | 48.8 | 48.2 | 3.0 |
| *Streptomyces griseus* Pronase | 27.00 | 5.40 | 62.0 | 34.5 | 3.5 |
| *B. subtilis* neutral protease | 27.00 | 5.40 | 7.1 | 85.9 | 7.0 |
| *B. subtilis* basic protease | 27.00 | 5.40 | 8.9 | 87.7 | 3.4 |

by LOWRY's method. On deducting the quantity of non-degraded protein and the total amount of amino acids, as obtained by amino acid analysis, from the quantity of protein weighed in, the amount of peptides was obtained. The presence is suggested by the non-identifiable peaks on the chromatogram of acid-neutral run at proteolysis induced by *Streptomyces* Pronázes, appearing in the 58th, 83rd, 105th, 119th and 143th min. In alkaline separation these peaks appear in the 23rd, 27th and 54th min. Further non-identifiable peaks appeared in the proteolytic digest with neutral *Bacillus subtilis* protease in the 123rd and 128th min of the acid-neutral run. In the digest of *Bacillus subtilis* alkalase a non-identifiable peak also appeared in the 25th min of the basic run.

It may be seen in the table that the total amount of non-degraded protein is low (2–7 %) in the case of the various protease preparations of bacterial origin and of the different pronase preparations.

The decisive difference in the activity of the pronase and protease preparations studied appeared in the free amino acid content of the digests. Thus when we speak of the high proteolytic capacity of pronase preparations this means that in comparison to other proteolytic enzyme preparations they degrade a large portion of the proteins to free amino acids.

Data in Table 3 show that the degree of degradation efficacy of *Streptomyces griseus* (CALBIOCHEM) Pronase is satisfactory; the liberated amino acid in the digests amounted to 32.0 and 48.8 %, resp. The free amino acid content of digests obtained with bacterial enzyme preparations was substantially lower, only about 7.1 and 8.9 %, resp. of the proteins were degraded to amino acids.

## 3. Conclusions

Beside the satisfactory degrading capacity of the enzyme preparations manufactured by CHINOIN their broad substrate specificity deserves interest. When compared with proteolytic enzymes of animal origin the essential amino acid composition of the hydrolysate obtained with *Streptomyces fradiae* Pronáz reminded of the composition obtained with pepsin. However, it differed from the latter by the presence of alkaline amino acids, the cleavage of methionine bonds and the absence of glutamic acid.

*Scheffner*'s Protein Digest Residue index is determined with pepsin. Because of the characteristic degradation specificity of pepsin AKESON and STAHMANN (1964) tried to find an enzyme the proteolytic activity of which would produce end products more closely resembling the end products of proteolysis in the living organism. It would be of interest to investigate the possibility of using *Streptomyces fradiae* Pronáz for the determination of the PDR index. To investigate this problem seems the more desirable, since during heat treatment the quantity of methionine is reduced (SCHEFFNER, 1967). In the shortened PDR index determination, where the quantity of only three amino acids: lysine, methionine and tryptophan is taken into account, the advantages of using a pronase preparation would be even higher.

In comparison to the products of hydrolysis with hydrochloric acid the low ratio of acidic amino acids, the high concentration of aromatic and branched-chain amino acids and of methionine is striking in the product of enzymatic hydrolysis. The absence of proline is explained by its known resistance to the proteolytic effect of enzymes. Proline appears in the urine in peptide bond, too (ALBANESE, 1967).

Ultrafiltration has shown that the molecular weight of the peptides is below 10 000. In the degradation by pepsin the peptides consist of 7 amino acids on the average (BELOFF & ANFINSEN, 1958). In the case of Pronáz, further breaks of bonds point to peptides of even lower numbers of units. Investigations by HORVÁTH (1971) have shown the peptides to consist of four amino acid residues on the average.

The degree of degradation efficiency of bacterial proteases was substantially lower than that of the *Streptomyces* proteases. Of the two bacterial proteases that of *Bacillus subtilis* neutral protease was better than the alkaline protease and this is in accordance with data in the literature (TSURU *et al.*, 1966). The specificity of bacterial proteases is lower as shown by the free amino acid composition in the digest and the appearence of more peptide peaks in the chromatogram. In the case of neutral protease from *Bacillus subtilis* peaks appeared in the 123rd and 128th min, while with *Bacillus subtilis* alkalase a further peak appeared in the 25th min on the basic column. This is in accordance with the general opinion that protein degradation by microbial proteases

is effective in a wide range (HAGIHARA, 1958) although some researchers observed narrow specificity with *Bacillus subtilis* proteases (MCCONN *et al.*, 1964; FEDER, 1967).

Bacterial proteases are known to disrupt generally *Ala*, *Val*, *Leu*, *Phe*, *Tyr* bonds (GRIFFIN & PRESCOTT, 1970). The *Bacillus subtilis* neutral protease studied by the authors disrupted, in addition, *Glu* and *Met* bonds as well as those of basic amino acids.

With the spreading of industrial animal husbandry the problem of the proper preparation of animal feedstuffs arose particularly in relation to feeding suckling animals. To achieve better digestion the application of enzymatic predigestion seems to be expedient. The degradation efficiency and specificity of the enzymes studied allow for their use in the preparation of feedstuffs.

The Pronáz produced by *Streptomyces fradiae* found industrial application (PHYLAXIA COMPANY FOR VETERINARY BIOLOGICALS AND FEEDSTUFFS, Budapest). Its significance lies in its capacity to improve the utilization of soya by degrading the trypsin inhibitor present in soya.

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# COUNTER-CURRENT HYDROLYSIS OF WASTE FEATHER WITH ACID AND ENZYME

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Economical digestion of feather by chemicals is impeded by its low volume weight. In order to eliminate this drawback a multi-stage acid–enzyme–acid hydrolysis process was evolved. It was established that the degradation of feather may be accomplished with a mineral acid concentration of 10% instead of the concentrated acid or base hitherto used. Thereby the quantity of salt formed when neutralizing the acid, is reduced. It was shown further that alkaline protease does not satisfactorily liquefy feather but forms a gel from the feather meal set at pH 9. By introducing multi-stage digestion, 50 g feather of 50 g $dm^{-3}$ volume weight could be pulped with 0.25 $dm^3$ of 10% mineral acid. The catalyzing effect of hydrochloric acid was found stronger.

The waste feather of 50 g $dm^{-3}$ volume weight, used in the experiments, yielded a 4–5% hydrolysate when digested with 10% mineral acid at 100 °C during 24 h, whereas the proposed multi-stage process yielded a hydrolysate of 16—18% measured refractometrically. This is in agreement with earlier results obtained in the course of wheat gluten hydrolysis. The solid content of feather used was recovered at 95% under laboratory conditions.

The protein deficiency of the world could be mitigated by a more efficient utilization of keratinous waste materials. Although the amounts available are not too large, the complete chemical degradation of these wastes would be very useful. For instance feather waste is at present added to animal feeding stuffs in the form of feather meal. The protein content of this, however, is not fully utilized by the animals. According to MÉNESI *et al.*, (1964) and BAJNÓGEL (1969) the utilization of feather meal falls between 27 and 63%. The result obtained in feeding experiments by MORAN and SUMMERS (1968) was similar. They fed a diet consisting of feather and soya meal to young broiler cocks. Increasing the ratio of feather meal in the diet, weight gain was slowed down in comparison to cocks fed on pure soya meal. The flesh of the birds was of lower quality, too. BARBER (1965) came to similar conclusions in piglet feeding experiments. He added 5% feather meal to the diet.

Apart from using keratinous waste materials for animal feed assays were made to utilize the processed material for human consumption. SHORLAND and BENTLEY (1969) made an attempt to transform wool for this purpose. The protein was dissolved in sodium sulphide, then made insoluble by acid treatment and separated from the solution. The digestibility of the product was about 77%. Papain was also used to prepare digestible protein from wool. The products of chemical and enzymic digestion were mixed to the raw material

of different foods in a proportion of 40–60 %. Consumers could not tell the foodstuff containing the substitute from the control.

One of the drawbacks hindering the utilization of feather as feed or supplementary food lies in its low volume weight. The present experiments were aimed at eliminating this difficulty.

## 1. Materials and methods

### *1.1. Materials*

*1.1.1. Feather.* A product known as "hygienic down for bedding" was used in the experiments. Since this product was washed and disinfected it could not have suffered degradation. To obtain a uniform raw material 15 kg feather was bought from the same batch of 50 g $dm^{-3}$ volume weight. The volume weight of moist feather, the by-product of the poultry industry, was also investigated. The wet weight of the feather after the plucking machine was found to be 608.0 ($\pm$61.7) g $dm^{-3}$ with a solids content of 31.51 ($\pm$3.06) %. Thus the solids content of 191.0 g $dm^{-3}$ was available for being turned into feed or food material.

*1.1.2. Concentrated sulphuric acid*, analytical grade

*1.1.3. Concentrated hydrochloric acid*, analytical grade

*1.1.4. Sodium hydroxide*, analytical grade

*1.1.5. 35 % formalin*, analytical grade

*1.1.6. Calcium carbonate*, analytical grade

*1.1.7. Commercial calcinated lime*

*1.1.8. Activated carbon*, type Decolor A

*1.1.9. Alkaline protease.* Activity 1.50 Anson U $g^{-1}$, optimum conditions: pH = 9, 60 °C

*1.1.10. Filter.* Macherey-Nagel Nr. 640 w.

*1.1.11. pH paper.* Macherey-Nagel 516, universal. Feinchemie K–H. Kallies KG, range: pH 6–7.5

### *1.2. Methods*

*1.2.1. Formol titration.* One of the simplest methods for the determination of the quantity of amino acids and peptides, used by SØRENSEN already in 1907 (BRUCKNER, 1961). In the experiments to 20 ml sample neutralized to pH 7, 10 ml 35 % formalin were added, it was then titrated with 0.5 *N* NaOH to re-adjust pH to 7. Because of the dark brown colour of the samples, the end point was determined with an indicator paper with a scale of 0.3-pH intervals. The extent of degradation was characterized by the amount (ml) of titrant used.

*1.2.2. Pulping with mineral acid and hydrolysis.* The sample was treated with mineral acid at 100 °C, using an apparatus consisting of an electric heater

and a 2-l round bottom flask, fitted with thermometer, sampling outlet and reflux condenser.

The time periods of hydrolysis are considered to mean the period during which the temperature of the digest is kept at 100 °C.

*1.2.3. Enzymatic treatment.* Subsequent to pulping the pH of the pulp was set at 9 with NaOH, then 0.5% alkaline protease related to the original weight, was added. The protease used was the experimental product of the CHINOIN FACTORY, Budapest, and had a concentration of 1.5 Anson U $g^{-1}$. The thermostat used for liquefaction was adjusted to 60 °C.

*1.2.4. Determination of the time needed for pulping.* The process of pulping could be visually followed in the glass flask. Changes in the material were recorded by photography. Pulping was considered finished when the original form of the feather became unrecognisable and an about 1.5 cm thick layer appeared over the liquid. The time requirement of the process was measured with a timer.

## 2. Results

### *2.1. Mineral acid and time requirements for pulping*

It is important from the aspect of both economy and quality to use the minimal amount of catalyser for the degradation of the material. Thus it was important to use as little catalyst as possible in the processing of feather. This is further justified in the use of hydrochloric acid the removal of which from the digest is rather complicated. Treatment with enzyme was applied in order to carry out digestion mildly and without loss. Earlier protein-containing raw materials were degraded with concentrated alkali (MALGARA, 1965) or with concentrated mineral acid (HERSICZKY & SIMONYI-SIKLÓS, 1966).

*2.1.1. Pulping with sulphuric acid.* The degrading effect of sulphuric acid was studied as a function of acid concentration. At each concentration four parallel measurements were carried out. The time of interruption of the degradation process was determined visually, the extent of chemical degradation was checked by formol titration and related to the initial composition. The process of the experiment is illustrated in Fig. 1.

*2.1.2. Pulping with hydrochloric acid.* The pulping experiments were carried out as with sulphuric acid. Results are shown in Fig. 2.

### *2.2. Enzymic liquefaction*

The extent of enzymic liquefaction *vs* increasing degradation period was studied at identical acid concentrations. It was thought that the influence of mineral acid beyond liquefaction facilitates protease enzyme action. In

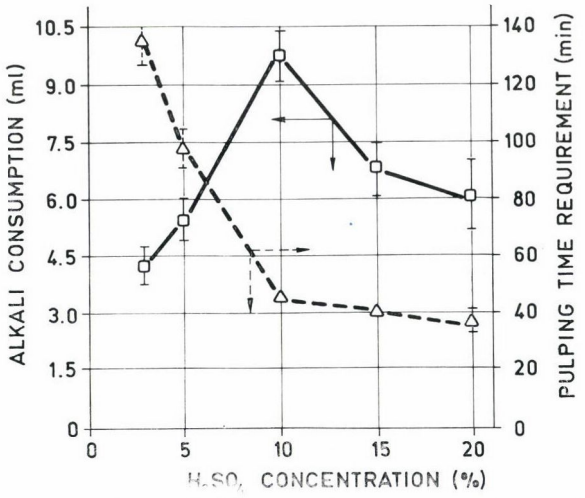


Fig. 1. Effect of concentration of $H_2SO_4$ used for pulping on the alkali consumption of feather (ml 0.5 *N* NaOH per 20g) of formaldehyde and on the time requirement for pulping. Vertical bars stand for the standard deviation when N = 4. Concentration: 50 g feather in 450 ml acid

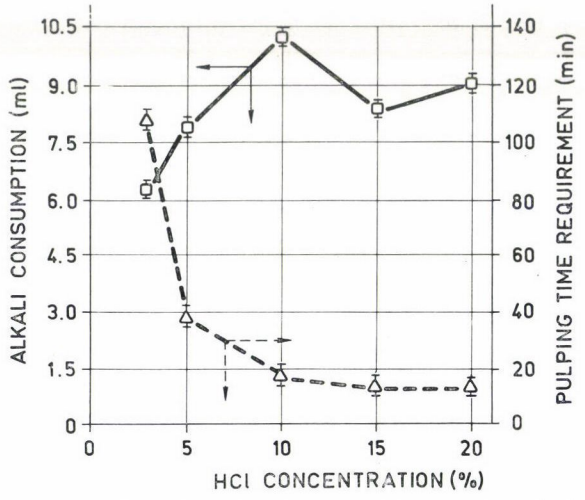


Fig. 2. Effect of concentration of HCl used for pulping on the alkali consumption of feather (ml 0.5 *N* NaOH per 20 g) in the presence of formaldehyde and on the time requirement for pulping. Vertical bars represent standard deviation when N = 4. Concentration: 50 g feather in 450 ml acid

these experiments 50 g feather and 450 ml acid were used. In order to avoid excessive increase of volume the pH optimal for enzyme action was set with 40% NaOH. The same experimental series was used to establish the optimum period of enzymic treatment. Results are summarized in Tables 1 and 2.

Table 1

*Effect of 40–45-min digestion with mineral acids on the subsequent enzymatic degradation of feather*

Enzyme used: alkaline protease at a concentration of 0.5% related to the solids of feather. Number of parallel measurements: 4

| Period of enzyme treatment (h) | Formol-alkali consumption (ml) | | | | | |
|---|---|---|---|---|---|---|
| | 5% HCl | | 10% HCl | | 10% $H_2SO_4$ | |
| | mean | standard deviation | mean | standard deviation | mean | standard deviation |
| 0 | 5.2 | 0.56 | 5.6 | 0.12 | 3.25 | 0.12 |
| 1 | 6.05 | 0.31 | 5.95 | 0.40 | 3.45 | 0.12 |
| 3 | 6.20 | 0.14 | 6.45 | 0.12 | 3.35 | 0.12 |
| 5 | 6.15 | 0.12 | 6.37 | 0.31 | 3.48 | 0.02 |
| 8 | 6.20 | 0.16 | 6.60 | 0.56 | 3.40 | 0.12 |
| 24 | 6.60 | 0.16 | 6.65 | 0.40 | 3.90 | 0.02 |
| 30 | 6.35 | 0.12 | 6.55 | 0.46 | 3.57 | 0.16 |
| 48 | 6.40 | 0.16 | 6.65 | 0.12 | 4.05 | 0.15 |

Table 2

*Effect of 105-min digestion with mineral acids on the subsequent enzymatic degradation of feather*

Enzyme used: alkaline protease at a concentration of 0.5% related to the solids of feather. Number of parallel measurements: 4

| Period of tratment with enzyme (h) | Formol-alkali consumption (ml) | | | | | |
|---|---|---|---|---|---|---|
| | 5% $H_2SO_4$ | | 10% $H_2SO_4$ | | 5% HCl | |
| | mean | standard deviation | mean | standard deviation | mean | standard deviation |
| 0 | 2.8 | 0.15 | 4.05 | 0.12 | 5.6 | 0.16 |
| 1 | 3.57 | 0.07 | 3.82 | 0.17 | 5.8 | 0.50 |
| 3 | 4.32 | 0.60 | 3.75 | 0.12 | 6.25 | 0.12 |
| 5 | 3.90 | 0.10 | 4.12 | 0.33 | 6.60 | 0.16 |
| 8 | 3.82 | 0.20 | 4.05 | 0.10 | 6.35 | 0.36 |
| 24 | 4.92 | 0.80 | 4.07 | 0.18 | 6.45 | 0.15 |
| 30 | 4.60 | 0.10 | 4.20 | 0.21 | 6.30 | 0.16 |
| 48 | 5.27 | 0.20 | 5.15 | 0.08 | 6.20 | 0.70 |

It may be seen in the tables that the formol-alkali consumption is hardly affected by the period of enzymic treatment, thus the incubation time was fixed at 17 h for reasons of work organization. However, a definite difference was observed in the consistency of the control and the sample treated with

enzyme. As an effect of enzymic treatment the feather pulp turned into a gel. Using a rotation viscosimeter (EPPRECHT *Rheomat*-15), increase in viscosity was measured during gel formation.

### *2.3. The multi-stage "acid–enzyme–acid" feather processing technique*

One aim of this study was to eliminate the drawbacks due to the low volume weight of feather. In these experiments 100 g feather could be filled in the 2 litre flask (at 50 g $dm^{-3}$ volume weight).

To fill fresh feather in the reaction chamber, while the previous portion is being degraded is not without danger even under laboratory conditions. In the case of industrial autoclaves under pressure technical difficulties may arise.

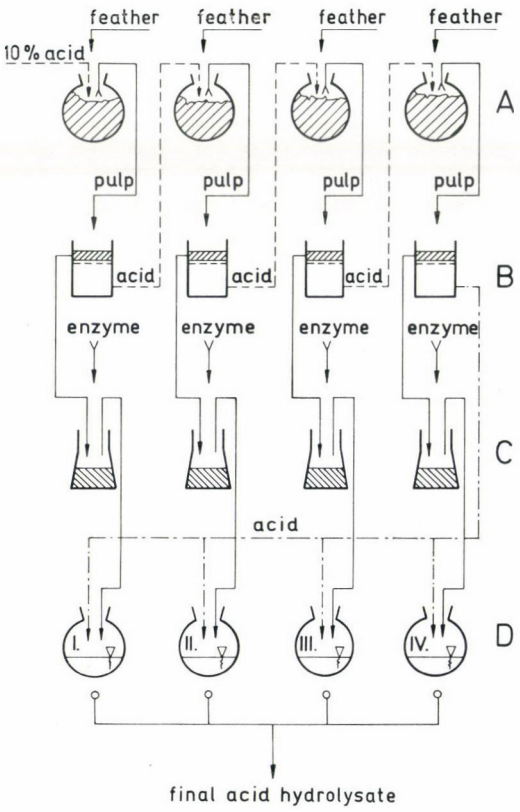


Fig. 3. Schematic diagram of the multi-stage hydrolysis of feather. *A*: four-stage pulping with mineral acid; *B*: filtration of the degraded feather and washing with water; *C*: neutralization of the pulp and treatment with enzyme; *D*: division of the acid residue into four portions

Table 3

*Numerical data of the multi-stage pulping with sulphuric acid and liquefaction by enzyme*

| Number of uses of the same $H_2SO_4$ | Quantity of feather weighed in (g) | Volume of $H_2SO_4$ (ml) and refractometer value (%) | | | | Suspended pulp set at pH = 9 | | |
|---|---|---|---|---|---|---|---|---|
| | | prior to use | | after use | | | Formol-alkali consumption | |
| | | (ml) | (%) | (ml) | (%) | Volume (ml) | prior to enzyme (ml) | after treatment (ml) |
| | | Operation A | | Operation B | | Operation C | | |
| | | *First series* | | | | | | |
| I. | 50 | 1000 | 9 | 900 | 12 | 250 | 1.5 | 2.8 |
| II. | 50 | 900 | 12 | 820 | 12.5 | 250 | 1.6 | 3.0 |
| III. | 50 | 820 | 12.5 | 720 | 13.5 | 260 | 1.8 | 3.7 |
| IV. | 50 | 720 | 13.5 | 650 | 15 | 263 | 2.0 | 3.9 |
| | | *Second series* | | | | | | |
| I. | 50 | 1000 | 9 | 930 | 9 | 260 | 1.8 | 3.3 |
| II. | 50 | 930 | 9 | 850 | 11 | 300 | 2.0 | 3.2 |
| III. | 50 | 850 | 11 | 780 | 12 | 300 | 1.9 | 2.9 |
| IV. | 50 | 780 | 12 | 690 | 14 | 300 | 2.0 | 3.1 |
| | | *Third series* | | | | | | |
| I. | 50 | 1000 | 9 | 920 | 10 | 285 | 1.5 | 2.7 |
| II. | 50 | 920 | 10 | 850 | 12 | 255 | 1.7 | 2.8 |
| III. | 50 | 850 | 12 | 790 | 14.5 | 260 | 1.8 | 2.6 |
| IV. | 50 | 790 | 14.5 | 720 | 16 | 290 | 1.7 | 2.9 |

Taking the above into account a multi-stage process seemed to be suitable to eliminate the drawbacks of low volume weight. On the basis of preliminary measurements and calculations it was found that under the given conditions after a four-stage process the residual acid is still sufficient to catalyse final degradation.

A schematic diagram of the complex process is given in Fig. 3.

The results of operations *A*, *B* and *C*, carried out as shown in the diagram, are summarized in Tables 3 and 4.

In Fig. 4 the volume ratios of the above operations are shown in a random example.

In an industrial process the recycling of the acid washing water may be accomplished in order to render the process more economical.

It was found that the residual acid solution divided in four, is sufficient to continue hydrolysis. The final hydrolysis was continued for 24 h under a reflux condenser.

Table 4

*Numerical data of multi-stage pulping with hydrochloric acid and subsequent liquefaction by enzyme*

| Number of uses of the same $H_2SO_4$ | Quantity of feather weighed in (g) | Volume of $H_2SO_4$ (ml) and refractometer value (%) | | | | Suspended pulp set at pH = 9 | | |
|---|---|---|---|---|---|---|---|---|
| | | prior to use | | after use | | Volume (ml) | Formol-alkali consumption | |
| | | (ml) | (%) | (ml) | (%) | | prior to enzyme (ml) | after treatment (ml) |
| | | Operation A | | Operation B | | Operation C | | |
| | | *First series* | | | | | | |
| I. | 50 | 1000 | 17 | 990 | 21 | 163 | 1.6 | 2.3 |
| II. | 50 | 990 | 21 | 950 | 24 | 245 | 1.7 | 2.6 |
| III. | 50 | 950 | 24 | 910 | 26 | 250 | 2.1 | 3.0 |
| IV. | 50 | 910 | 26 | 850 | 28 | 290 | 2.4 | 3.2 |
| | | *Second series* | | | | | | |
| I. | 50 | 1000 | 17 | 970 | 20 | 205 | 1.6 | 2.5 |
| II. | 50 | 970 | 20 | 940 | 22 | 215 | 1.7 | 2.6 |
| III. | 50 | 940 | 22 | 910 | 25 | 235 | 1.8 | 3.1 |
| IV. | 50 | 910 | 25 | 850 | 27 | 275 | 2.0 | 2.9 |
| | | *Third series* | | | | | | |
| I. | 50 | 1000 | 17 | 990 | 21.5 | 220 | 1.4 | 2.1 |
| II. | 50 | 990 | 21.5 | 930 | 23.5 | 295 | 1.5 | 2.0 |
| III. | 50 | 930 | 23.5 | 880 | 25.5 | 290 | 1.7 | 2.8 |
| IV. | 50 | 880 | 25.5 | 820 | 27.5 | 290 | 1.8 | 2.5 |

*2.4. Technical data on the hydrolysates of the proposed feather processing technique*

Process-technical investigations were carried out with 45-min degradation period in the case of sulphuric acid and 20-min periods in the case of hydrochloric acid. The final hydrolysates were combined, the pH was set first with slaked lime at 4.8, then with $CaCO_3$ at 6. This technique was chosen to avoid over-alkalization in industrial practice. However, the material balance could be established only in the sulphuric acid hydrolysate. The $CaCl_2$ present in the hydrochloric acid hydrolysate hindered the establishment of the proper refractometer value.

The refractometer value of the filtrate obtained after the neutralization of the sulphuric acid was 18%. On washing the precipitate with water 95% of the solids content of weighed-in feather was regained.

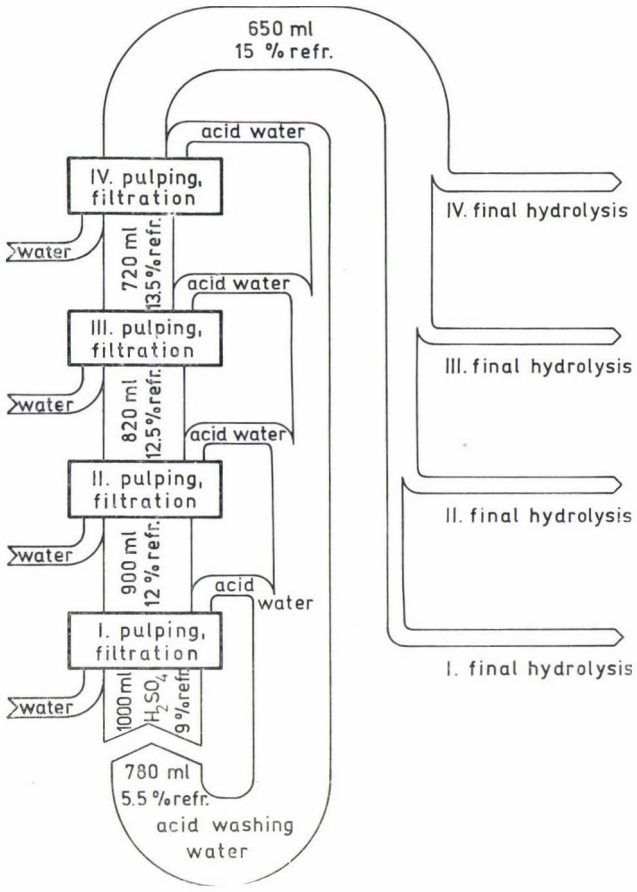


Fig. 4. Multi-stage pulping with $H_2SO_4$ and filtration (Table 3, First series). Volume proportions and de-acidification of the cakes

## 3. Conclusions

The experiments for both sulphuric and hydrochloric acid have shown the 10% solution to be the most efficient in feather degradation (Figs. 1–2).

Enzyme treatment subsequent to acid treatment increased the NaOH consumption in comparison with the control, however this was not sufficiently extensive and was not in direct relation to time. Treatment with enzyme caused the acid digest to turn into gel. The mineral acid used in the final stage was expected to react with the colloidal substance more rapidly and thereby reduce the time requirement of the process.

A significant result of the multi-stage degradation process was the substantial increase in the volume weight of the feather filtrate from 50 g $dm^{-3}$

to 700 g $dm^{-3}$. In the light of this result it is conceivable to prepare pulp from the feather at the different poultry processing plants and collect the pulp for a central factory. In this case washing with water may be omitted because the acid preserves the digest.

Using the two acids in identical concentration the hydrolytic effect of hydrochloric acid is more extensive. In the hydrolysis of starch similar results were obtained by SZEJTLI (1964). It seems desirable to investigate the possibility of a further reduction of acid concentration.

The result of the experiments may be summarized in the success of obtaining a concentrated hydrolysate from the feather of low volume weight. The hydrolysate resembles that obtained in the hydrolysis of wheat gluten (HERSICZKY & SIMONYI-SIKLÓS, 1966). The 95% yield deserves also interest and it is due to the mild degradation process.

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# ASSESSMENT OF QUALITY OF SINGLE-CELL PROTEINS WITH *TETRAHYMENA PYRIFORMIS* W

M. HEGEDÜS and GY. ZACHARIEV

(Received February 14, 1977; accepted May 21, 1977)

The quality of the proteins of some yeasts and moulds was assessed by a method using the protozoon *Tetrahymena pyriformis* W as the test organism.

The protein content and protein quality in mycelia produced by five mould strains, and in the yeast strains *Candida guilliermondii*, grown on *n*-paraffin and *Candida utilis*, grown on distillers' solubles, were determined.

In the case of yeasts thiamin, riboflavin, vitamin B-6, niacin and pantothenic acid were also determined.

The averages of the relative nutritive values of yeasts cultured on *n*-paraffin and distillers' solubles (54.10 and 53.71, resp.) did not differ significantly. The relative nutritive value of yeasts grown on *n*-paraffin agreed with the biological indices as published in the literature (BV, NPU), determined in rat feeding experiments. The vitamin content of the yeasts was within the range reported in the literature.

The relative nutritive value of yeasts as determined with Tetrahymena (Tetrahymena-RNV) and the vitamin content did not show close correlation, while a significant negative correlation ($P \geq 99.9\%$) was found between Tetrahymena-RNV and the protein content of the yeasts ($r = -0.83$).

The spread of the Tetrahymena-RN values, protein and vitamin contents of yeasts substantially exceeded the error inherent in the methods used for their determination. The unexpectedly high error may be traced back probably to the novelty of the applied technology and to the technical difficulties accompanying it. The quality of the protein of the five mould strains placed them between the Tetrahymena-RNV of wheat and of yeasts.

*Tetrahymena pyriformis* W was found to be a suitable test organism for the assessment of single-cell protein.

It is a requirement of long standing to have a rapid, inexpensive and reliable method for the determination of the quality of food and feed proteins.

Chemical indices, calculated from the amino acid composition of proteins are frequently adequate, however, in many cases they are not correlated to the results of biological methods. This is particularly true as regards the availability of amino acids changed in the course of some processing operations. *Biological* methods, making use of some higher animals, are well known for their high time and resource requirements. Thus, increasing attention is being paid to *microbiological* methods.

Microbiological methods are applied for various purposes: for the quantitative determination of amino acids in protein hydrolysates (BARTON-WRIGHT, 1952), for the determination of the relative nutritive value of proteins of various origin and for the determination of the availability of essential amino acids. In these experiments the relative nutritive value of proteins was studied with *Tetrahymena pyriformis* W as the test organism.

Among the microorganisms suitable for protein determination a special place is allotted to the protozoon *Tetrahymena pyriformis* W. It produces extracellular proteases, thus it is capable of proliferating in media containing non-hydrolysed proteins (DICKIE & LIENER, 1962). Its amino acid requirement resembles that of rats in growth: lysine, tryptophan, histidine, leucine, isoleucine, phenylalanine, methionine, valine, threonine and arginine (HOLZ, 1973).

ROCKLAND and DUNN (1949) were the first to suggest the use of *Tetrahymena pyriformis* for the determination of protein quality. They carried out experiments with strain *H* and measured the quantity of the acid formed during an incubation period of 41 days. ANDERSON and WILLIAMS (1951) used strain W. They determined the extent of growth by colorimetry after a 4-day incubation period. ROSEN and FERNELL (1956) succeeded in adjusting the composition of the medium so as to direct the growth of the test organism primarily by the utilization of protein or amino acids. STOTT and co-workers (1963) simplified the technique and made it suitable for serial tests.

TEUNISSON (1961), BAUM and HAENEL (1965), KAMATH and AMBEGAOKAR (1968) succeeded in using *Tetrahymena pyriformis* W to rank proteins of various origin according to quality.

Investigations of some authors pointed to difficulties in the analysis of samples of a certain type. Thus, according to BAUM and HAENEL (1965) a high starch content in the sample hinders the proliferation of the protozoa, therefore, Tetrahymena determinations in such substances give lower values. The requirement of Tetrahymena in sulphur-containing amino acids is lower than that of rats (RØLLE & EGGUM, 1971) and its lysine, valine and tryptohan requirement is lower than both of man and rats (RØLLE, 1975). It would follow that by Tetrahymena higher values are obtained for the proteins poorer in amino acids than by higher organisms.

HAENEL and KHARATYAN (1973), as well as HEGEDÜS (1973, 1974) came to the conclusion that the relative nutritive values obtained by Tetrahymena are applicable to higher animals only on the basis of the corresponding regression correlations and the method is suitable rather for the detection of relative changes in protein quality.

DWORSCHÁK and HEGEDÜS (1974) used this method successfully to follow the decrease in the nutritive value of milk protein due to heat treatment. In the mixtures containing appropriate proportions of powdered egg and wheat flour or potato protein (HEGEDÜS & WÖLLER, 1974) and in cakes baked from wheat flour enriched with soya protein (KÁRPÁTI & ZACHARIEV, 1974), the nutritive-value-increasing effect of protein complementation, could be demonstrated.

The aim of this study was to utilize the *Tetrahymena pyriformis* W method in a new field, in the evaluation of single-cell protein.

## 1. Materials and methods

### *1.1. Yeast samples*

The *Candida guilliermondii* strain was used for yeast propagation on hydrocarbon medium. Propagation was carried out in the DISTILLING PLANT, Szabadegyháza with *n*-paraffin as carbon source (BRITISH PETROLEUM, Dinslaken, FRG). A 30-m³ *Vogelbusch* fermentor was used in the experimental cultivation according to the technique developed at the CENTRAL FOOD RESEARCH INSTITUTE, Budapest (SIMEK *et al.*, 1967; SIMEK & SZÉCHÉNYI-MÁRTON, 1969a, b).

The separated yeast cells of 8–10 % solids content were evaporated in a vacuum evaporator at 35–48 °C to a solids content of 20–25 %. Final drying was carried out on a drum drier. Four samples were exempted from this treatment. The concentrated yeast-milk was extremely viscous, therefore it was heated to 70–80 °C so that it might be pumped to the drier.

The *Candida utilis* samples were prepared in the DISTILLING PLANT, Óbuda, on distillers' solubles. The final drying was carried out on a drum drier.

Two years elapsed between the manufacture of the samples and this study. The samples were stored in the meantime in polyethylene bags at 10–15 °C in a cellar.

### *1.2. Mould samples*

The mycelia of *Actinomucor repens*, *Rhizopus cohnii*, *Rhizopus arrhizus* as well as of the two Mucor strains were prepared at the FERMENTATION DEPARTMENT of the CENTRAL FOOD RESEARCH INSTITUTE, Budapest. Bread waste was used as the carbon source (ZETELAKI-HORVÁTH & VAS, 1975; ZETELAKI-HORVÁTH *et al.*, 1976; ZETELAKI-HORVÁTH & VAS, 1976). The samples were dehydrated by washing in aceton and dried in the air. They were stored in paper bags at room temperature (20–25 °C) for 1–2 months and were then analysed.

### *1.3. Reagents used in protein quality determination*

Vitamins and nucleotides used in the test-medium were manufactured by FLUKA (BUCHS SG., Switzerland), while the mineral salts and other reagents were products of REANAL, Budapest.

### *1.4. Method for the determination of protein quality*

*1.4.1. Maintenance of the strain.* The *Tetrahymena pyriformis* W strain, also known under the names *Glaucoma pyriformis* and *Tetrahymena geleii*, was cultured on liquid medium in test tubes, under sterile conditions at 25 °C. Light is not required for its cultivation. The composition of the maintenance

medium: 20 g *Bacto* pepton (DIFCO), 1 g yeast extract (DIFCO), 5 g glucose and 1 g NaCl, dissolved in 1000 ml distilled water and adjusted to pH 7.1. The medium was then sterilized at 121 °C for 15 min in an autoclave. The strain was inoculated with a *Pasteur* pipette under sterile conditions every week.

*1.4.2. Preparation of the nutrient medium.* The medium consists of the chemically defined assay medium and the protein to be tested. The composition of the medium is as follows:

2 ml nucleotide solution
2 ml mineral salt solution
4 ml protein suspension (equivalent to 3 mg nitrogen)
1 ml vitamin solution
1 ml glucose solution.

The protein suspension is prepared by measuring to the nearest tenth of a mg from the sample prepared as described in para. 1.4.3, an amount equivalent to 3 mg nitrogen, and suspending it in 4 ml distilled water.

The nucleotide and mineral salt solutions and the protein suspension are mixed and sterilized together. The vitamin and glucose solutions are sterilized separately at 121 °C for 10 min.

*1.4.2.1. Composition of the nucleotide solution.* – Seventy-five mg guanylic acid (3′) + 2′ (-GMP-$Na_2$), 62.5 mg cytidylic acid (3′) + 2′ (-CMP), 50.0 mg adenylic acid (3′) + 2′ (-AMP) and 25.0 mg uracil are dissolved in distilled water and made up to 100 ml.

*1.4.2.2. Mineral salt solution.* – Dissolve 2.8 g $MgSO_4 \cdot 7H_2O$, 1.25 g $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 0.025 g $MnCl_2 \cdot 4H_2O$ and 0.0025 g $ZnCl_2 \cdot 2H_2O$ in 200 ml distilled water (solution *a*). Dissolve 600 mg $CaCl_2 \cdot 2H_2O$, 60 mg $CuCl_2 \cdot 2H_2O$ and 15 mg $FeCl_3 \cdot 6H_2O$ in 200 ml distilled water (solution *b*). Dissolve 3.5 g $KH_2PO_4$ and 3.5 g $K_2HPO_4$ in 200 ml distilled water (solution *c*). Unite 5 ml each of solutions *a*, *b*, *c* and make it up to 100 ml with distilled water.

*1.4.2.3. Vitamin solution.* – Prepare a dry mixture of the following vitamins: 25 mg Ca pantothenate, 25 mg nicotinamide, 250 mg pyridoxine hydrochloride, 25 mg pyridoxal hydrochloride, 25 mg pyridoxamine dihydrochloride, 25 mg riboflavin, 250 mg thiamin hydrochloride, 25 mg myo-inositol and 25 mg *p*-amino benzoic acid and homogenize the mixture (mixture *a*). Dissolve 625.0 mg choline chloride in 100 ml distilled water (solution *d*). Dissolve 31.3 mg biotin in 500 ml distilled water (solution *e*). Dissolve 31.3 mg folic acid in 500 ml 0.1*N* NaOH (solution *f*). Dissolve 10 mg DL-α-lipoic acid in a few drops of ethyl alcohol and make it to 500 ml with distilled water (solution *g*). To prepare the vitamin solution take 33.8 mg from mixture *a* and add 2 ml each of solutions *d*, *e*, *f* and *g* and make it up to 200 ml with distilled water.

*1.4.2.4. Glucose solution.* – Dissolve 15 g glucose in 100 ml distilled water.

*1.4.3. Preparation of the sample.* The air-dry samples are comminuted and extracted with diethyl ether twice for 8 h each. After further grinding they are put through a sieve of 200 $\mu$m mesh. The nitrogen content is determined by the *Kjeldahl* method.

*1.4.4. Inoculation, incubation and evaluation. Erlenmeyer* flasks of 100 ml volume were used. The final volume of the experimental material was 10 ml. The nutrient medium was inoculated with 0.2 ml three-day Tetrahymena culture of $10^3$ ml$^{-1}$ average initial cell count. Three parallels were weighed from each sample. After inoculation the test tubes were incubated at 25 °C for four days, then mixed. The cultures thus obtained were used to prepare dilution series (2 ×, 5 ×, 10 ×, 20 ×) in a 0.8 % NaCl solution containing 1 % formaldehyde. The cells developed were then photographed in a *Fuchs-Rosenthal*-type haemocytometer through the microscope. The cell counts were plotted as a function of dilution on log–log scale paper and extrapolated to zero dilution. Whole egg powder was used as a standard (product of the National Enterprise for the Poultry Industry, Hungary). The cell counts of the individual samples were expressed as percentage of the cell count obtained with the standard. The index thus obtained is the Relative Nutritive Value (RNV). The error inherent in the method is below $\pm 15$ %.

### *1.5. Microbiological methods for the assessment of members of the vitamin B complex*

Determinations were carried out by the methods recommended by the Vitamin Work Group of the Hungarian Scientific Association for the Food Industries (1971) and the Difco Laboratories (1969). The test organisms used were as follows: thiamin – *Lactobacillus fermenti* ATCC 9338; riboflavin – *Lactobacillus casei* ATCC 7469; Vitamin B-6 – *Saccharomyces carlsbergensis* ATCC 9080; niacin – *Lactobacillus plantarum* ATCC 8014; pantothenic acid – *Lactobacillus plantarum* ATCC 8014.

### *1.6. Mathematical statistical evaluation of the results*

In order to be able to compare spread of the data as measured and the error of the methods used for their determination the coefficients of variation were calculated (CV). The relation between the relative nutritive value and the quantities of chemical components of the yeasts studied were checked by correlation analysis. The significance of differences between averages was established by *Student*'s $t$ test.

## 2. Results

### *2.1. Studies on yeasts*

The nitrogen content and the Tetrahymena-RNV, *i.e.* the relative nutritive values for *Tetrahymena pyriformis* W, of 21 *Candida guilliermondii* samples grown on *n*-paraffin and 7 *Candida utilis* samples grown on distillers' solubles, were determined.

Since in animal feeding the crude protein content is taken into account, the nitrogen values multiplied by 6.25 are also presented. The results obtained with the two yeast strains are listed in Table 1. The results are related to the defatted, air-dry samples.

The average values of the properties studied, the related standard deviations and the results of *Student*'s *t* test used to compare the relative nutritive values of the two yeast strains, are contained in the Table. The average Tetrahymena-RNVs ($54.1 \pm 16.3$) of the yeast strain grown on *n*-paraffin did not differ significantly at $P = 95\%$ from the average Tetrahymena-RNV of the yeast strain fermented in distillers' solubles ($53.7 \pm 11.4$). The standard deviation of the RNVs substantially exceeded the standard error of $\pm 15\%$ inherent in the method. On examining the cause of spread the fluctuation in protein, the most important of the macrocomponents, was studied. It may be seen in the Table that the protein content of yeasts grown on *n*-paraffin varied in a wide range between 41.9 and 61.9%. The extent of spread ($s = \pm 5.8$; $CV = 11.3\%$) substantially exceeded the error of the protein determination method.

In order to find out whether the Tetrahymena-RNV of the yeasts was dependent on their protein content correlation calculations were carried out. The following regression equation was obtained: $Y = 170.17 - 2.27X$, where X is the protein content and Y the Tetrahymena-RNV. The correlation coefficient, $r = -0.83$, was significant at the 99.9% probability level. The regression coefficient was found to be highly significant, too ($p < 0.001$). Thus between the protein content and the RNV of the *C. guilliermondii* a close negative correlation was established. The regression line is shown in Fig. 1.

The protein content of *C. utilis* samples, grown on distillers' solubles, yielded lower spread ($s = \pm 3.3$; $CV = 7.4\%$) and here the Tetrahymena-RN values did not correlate well with the protein values ($r = -0.36$).

The relative nutritive values as determined in this study with Tetrahymena were compared with biological values (BV), net protein utilization (NPU) and the protein efficiency ratio (PER), taken from the literature and determined in rat experiments (Table 2).

It may be seen that the average Tetrahymena-RNV of yeast samples grown on *n*-paraffin showed a satisfactory correspondence with indices de-

Table 1

*Relative nutritive values of Candida yeasts grown on* n-*paraffin and distillers' solubles*

| Serial numbers of samples | *C. guilliermondii* grown on *n*-paraffin | | | *C.utilis* grown on distillers' solubles | | |
|---|---|---|---|---|---|---|
| | Nitrogen[a] % | Protein[a] (N×6.25) % | Tetrahymena RNV | Nitrogen[a] % | Protein[a] (N×6.25) % | Tetrahymena RNV |
| 1 | 8.5 | 53.1 | 43 | 6.8 | 42.5 | 63 |
| 3 | 7.1 | 44.4 | 69 | 7.9 | 49.5 | 65 |
| 3 | 7.4 | 46.3 | 75 | 7.6 | 47.5 | 43 |
| 4 | 8.6 | 53.8 | 41 | 6.8 | 42.5 | 63 |
| 5 | 7.9 | 49.4 | 45 | 7.0 | 43.8 | 37 |
| 6 | 7.8 | 48.8 | 59 | 6.6 | 41.3 | 63 |
| 7 | 7.9 | 49.4 | 56 | 8.0 | 50.0 | 42 |
| 8 | 7.3 | 45.6 | 55 | | | |
| 9 | 9.2 | 57.5 | 31 | | | |
| 10 | 9.9 | 61.9 | 30 | | | |
| 11 | 6.7 | 41.9 | 87 | | | |
| 12 | 6.7 | 41.9 | 78 | | | |
| 13 | 9.6 | 60.0 | 49 | | | |
| 14 | 7.7 | 48.1 | 60 | | | |
| 15 | 9.4 | 58.8 | 40 | | | |
| 16 | 7.2 | 45.0 | 72 | | | |
| 17 | 8.5 | 53.1 | 61 | | | |
| 18 | 8.2 | 51.3 | 66 | | | |
| 19 | 8.2 | 51.3 | 33 | | | |
| 20 | 9.3 | 59.1 | 39 | | | |
| 21 | 8.9 | 55.6 | 48 | | | |
| Mean | 8.2 | 51.2 | 54.1 | 7.2 | 42.3 | 53.7 |
| Standard deviation | 0.9 | 5.8 | 16.3 | 0.5 | 3.3 | 11.5 |
| Coefficient of variation (CV) | 11.4% | 11.3% | 30.2% | 7.5% | 7.4% | 21.3% |

$t$ test of difference between the mean RNVs : 0.06

the critical value of $t$ at $P = 5\%$, DF = 26 is: 2.06

[a] refers to defatted air-dry samples

termined with rats. The average Tetrahymena-RNV of *C. utilis*, grown on distillers' solubles was higher than the pertinent biological indices.

Since the high nutritional value of yeasts depends, beside the protein content, on their significant vitamin content and the members of the vitamin

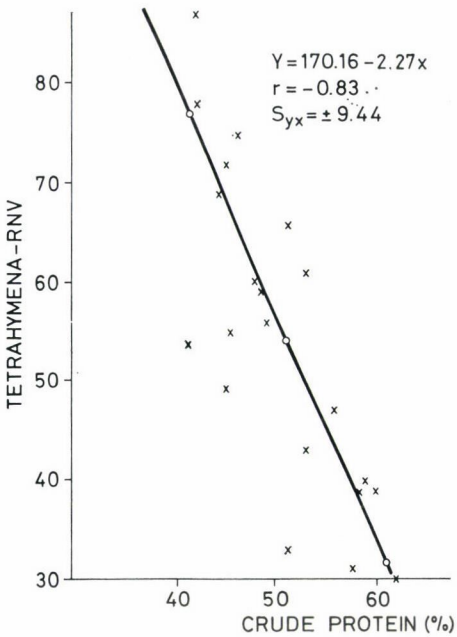


Fig. 1. Correlation between the protein content (x) and Tetrahymena-RNV (y) of *Candida guilliermondii* grown on *n*-paraffin based on the data obtained from 21 yeast samples (n = 21). Pairs of data are presented in Table 1. Data refer to defatted, air-dry samples

Table 2

*Comparison of the Tetrahymena-RNVs and biological indices of yeasts*

| Type of carbon source | Tetra-hymena-RNV | BV | NPU | PER* |
|---|---|---|---|---|
| *n*-paraffin | 54[a] | 59[b] | 50[b] | |
| distillers' solubles | 54[a] | 47[c] | 41[c] | 54[c] |
| | | 32–48[d] | | 23–36[d] |
| unspecified | | 54–62[e] | 44–56 | – |

[a] Present study
[b] SHACKLADY, C. A., 1969
[c] FAO, 1970
[d] POKROVSKIY, A. A., 1972
[e] PALMER, R. & SMITH, R. H., 1971
* as percentage of the PER value (= 3.92) of whole egg

B complex are essential for the Tetrahymena as well, the quantity of thiamin, riboflavin, vitamin B-6, niacin and pantothenic acid in the yeast samples, was also determined. The individual vitamin contents of the samples grown on *n*-paraffin and the average vitamin contents as obtained on distillers' solubles are presented in Table 3.

Table 3

*Vitamin contents of yeast samples*
(mg per 100 g)[a]

| Serial numbers of samples[b] | Thiamin | Riboflavin | Pyridoxine | Nicotinic acid | Pantothenic acid |
|---|---|---|---|---|---|
| 1 | 1.4 | 13.5 | 2.1 | 42.5 | 24.8 |
| 2 | 1.2 | 14.1 | 1.6 | 37.2 | 33.7 |
| 3 | 0.8 | 18.3 | 1.7 | 33.9 | 18.7 |
| 4 | 0.8 | 1.4 | 1.8 | 27.4 | 22.0 |
| 5 | 0.7 | 11.9 | 1.7 | 26.8 | 23.1 |
| 6 | 1.2 | 13.4 | 1.9 | 31.2 | 22.1 |
| 7 | 0.9 | 11.2 | 1.1 | 31.7 | 21.9 |
| 8 | 1.0 | 11.7 | 1.1 | 35.2 | 24.6 |
| 9 | 0.8 | 9.7 | 1.3 | 38.3 | 26.9 |
| 10 | 0.8 | 7.9 | 1.1 | 34.3 | 19.4 |
| 11 | 1.6 | 5.2 | 1.3 | 20.1 | 12.4 |
| 12 | 1.5 | 6.2 | 1.4 | 28.9 | 10.0 |
| 13 | 1.5 | 6.7 | 2.1 | 24.4 | 7.3 |
| 14 | 0.8 | 14.3 | 2.0 | 40.5 | 18.8 |
| 15 | 1.1 | 11.9 | 1.8 | 39.5 | 15.9 |
| 16 | 2.4 | 8.6 | 1.7 | 26.0 | 12.7 |
| 17 | 1.0 | 10.8 | 1.0 | 36.2 | 19.3 |
| 18 | 3.8 | 6.1 | 2.0 | 24.8 | 4.8 |
| 19 | 1.4 | 15.1 | 1.3 | 32.0 | 24.4 |
| 20 | 1.1 | 15.2 | 2.2 | 29.4 | 30.3 |
| 21 | 0.8 | 11.2 | 2.7 | 39.4 | 18.5 |
| Mean | 1.3 | 11.3 | 1.7 | 32.4 | 19.6 |
| Standard deviation | 0.7 | 3.5 | 0.4 | 6.1 | 7.2 |
| *Candida utillis* grown on distillers' solubles (average) | 1.4 | 3.1 | 1.5 | 31.8 | 2.0 |

[a] vitamin contents refer to air-dry defatted samples
[b] *Candida guilliermondii* grown on *n*-paraffin

The average values related to the air-dry *C. guilliermondii* samples, grown on *n*-paraffin were as follows: thiamin: 1.3 mg%; riboflavin: 11.3 mg%; vitamin B-6: 1.7 mg%; niacin: 32.4 mg%; pantothenic acid: 19.6 mg%. For the sake of comparison the average values of *C. utilis* samples, grown on distillers' solubles, were presented also at the bottom of the Table. While

Table 4

*Comparison of the mean vitamin contents of Candida yeasts grown on* n*-paraffin and distillers' solubles with the literature values*
(mg per 100 g)

| | Candida grown on *n*-paraffin | | Candida grown on distillers' solubles | |
|---|---|---|---|---|
| | Present study | SHACKLADY & GATUMEL (1972) | Present study | POKROVSKY (1972) |
| Thiamin | 1.3 | 0.4 | 1.4 | 0.5 |
| Riboflavin | 11.3 | 18.0 | 3.1 | 4.5 |
| Pyridoxine | 1.7 | 2.5 | 1.5 | 3.3 |
| Nicotinic acid | 32.4 | 43.0 | 31.8 | 41.7 |
| Pantothenic acid | 19.6 | 12.5 | 2.0 | 3.7 |

no substantial difference was found in the thiamin, vitamin B-6 and niacin contents of the two yeast strains grown on different media, the average riboflavin content of the yeast, grown on *n*-paraffin, was about three-fold and the pantothenic acid content higher by nearly an order of magnitude than that of yeasts, grown on distillers' solubles. The thiamin, riboflavin, pyridoxine, niacin and pantothenic acid contents as determined in this study fell in the range as reported in the literature (Table 4).

As regards the vitamin contents the scatter is even higher than with the RNV. Variation coefficients are as follows: niacin 18.9%, vitamin B-6 26.7%, riboflavin 31.2%, pantothenic acid 36.9% and thiamin 56.4%.

Correlation calculations were carried out in order to detect whether the thiamin, riboflavin, vitamin B-6, niacin and pantothenic acid content of yeasts grown on *n*-paraffin could have affected the RNVs. The results of calculations are presented in Table 5.

It may be seen that significant correlation does not exist between the vitamin B content and the Tetrahymena-RNV of the yeasts. The significant limit value is approximated only in the case of pantothenic acid – RNV relation ($r = -0.43$). The correlation coefficient was positive for the thiamin – RNV relation, only.

### *2.2. Study of moulds*

In contrast to yeasts data in the literature on moulds are rare. Therefore the method was tested on the mycelia of five different mould strains, prepared at the CENTRAL FOOD RESEARCH INSTITUTE, Budapest. The protein content and Tetrahymena-RNV of the mould samples are presented in Table 6.

Table 5

*Correlation between the relative nutritive values and the vitamin B contents of* Candida guilliermondii *samples*

| Regression equations | *t* test of regression coefficient | correlation coefficient (*r*) |
|---|---|---|
| $y = 43.28 + 8.56\ x_1$ | 1.75 | 0.37 |
| $y = 67.48 - 1.19\ x_2$ | 1.15 | −0.26 |
| $y = 59.27 - 3.14\ x_3$ | 0.36 | −0.09 |
| $y = 86.80 - 1.01\ x_4$ | 1.80 | −0.38 |
| $y = 72.52 - 0.94\ x_5$ | 2.0 | −0.42 |
| Critical values (DF = 19) | | $t = 3.88$ $r = 0.43$ $t_{5\%} = 2.09$ (at P = 5% probability level) |

y = Tetrahymena RNV
$x_1$ = thiamin
$x_2$ = riboflavin
$x_3$ = B-6 vitamin
$x_4$ = niacin
$x_5$ = pantothenic acid

Table 6

*Quality of some mycelial protein assayed with* Tetrahymena pyriformis *W*

| Origin of mycelia | Nitrogen[a] % | Protein (N × 5.25)[a] % | Tetra-hymena-RNV |
|---|---|---|---|
| *Rhizopus cohnii* (222) | 7.28 | 45.5 | 21 |
| *Actinomucor repens* (207) | 5.09 | 31.8 | 33 |
| *Actinomucor repens* (217) | 4.85 | 30.3 | 38 |
| *Rhizopus arrhizus* (225) | 5.30 | 33.1 | 40 |
| *Mucor* sp. (219) | 6.34 | 39.6 | 44 |
| *Mucor mucedo* (224) | 5.54 | 34.6 | 50 |
| Some other protein sources:[b] | | | |
| Wheat flour | 1.96 | 12.3 | 23 |
| Sunflower-seed meal | 7.56 | 47.3 | 38 |
| Rape-seed meal | 6.56 | 41.0 | 38 |
| Soya-bean meal | 8.15 | 50.9 | 62 |
| Skim-milk powder | 5.86 | 36.6 | 77 |
| Whole egg | 11.20 | 70.0 | 100 |

[a] Refers to defatted, air-dry sample
[b] HEGEDÜS (1974)

It may be seen in the Table that the protein of *Rhizopus cohnii* was accorded the lowest value by the protozoon (RNV = 21). The protein of the Mucor strains achieved the highest value (RNV = 44 or 50). For the sake of comparison the Tetrahymena-RNVs of some other protein sources, as determined by HEGEDÜS (1974), are also presented.

Comparative data have shown that Tetrahymena ranks the protein in *Rhizopus cohnii* equal to wheat protein and the protein in the two *Actinomucor* strains and in *Rhizopus arrhizus* equal to the protein of oil-seeds. The protein quality in the two Mucor strains, particularly that of *Mucor mucedo* approximates the protein quality of yeasts (Table 1).

## 3. Conclusions

The protein determination method utilizing *Tetrahymena pyriformis* W was found suitable for use in ranking single-cell proteins with the main protein sources.

The numerical correspondence of the Tetrahymena-RNVs of yeasts grown on two different carbon sources is considered only accidental. However, the difference would not be significant even at higher deviations between averages ($SD_{5\%} = 13.78$).

Spread higher than expected in the relative nutritive values and in the components examined, is due probably to the novelty of the applied technology and materials and to technical difficulties inherent in them.

The reason for the negative correlation between protein content and protein quality is not known. This phenomenon occurs in higher plants, however, the authors are well aware of the significant differences in the metabolism of plants and microorganisms (SCHULZ, 1973). The correlation observed shows that by increasing the protein yield in yeasts, grown on *n*-paraffin, its biological value may decrease. This observation may be of interest to fermentation experts.

The analysis of samples of fungal origin shows that valuable protein sources may be found among moulds. Naturally they have to pass a thorough toxicological examination in order to find out whether they produce mycotoxins. It is known that the mould strain *Paecilomyces varioti* is successfully used in Finland to utilize spent sulfite liquor in fermenting feed-protein of high value (TAMPELLA AB., Tampere, Finland).

Literature on the biological value of proteins in moulds is not available. According to JOHNSON essential amino acids in *Aspergilus niger* amount to 9.2%, in *Rhizopus nigricans* to 9.6%, in *Penicillium notatum* to 13% of the solids content, while in the case of yeast strains: *Saccharomyces* and *Torula* they are between 17 and 30%. This is in concord with the results obtained

in this study, inasmuch moulds proved to be poorer protein sources than yeasts in determinations with Tetrahymena.

As is known, the indices of biological value (BV, NPU, PER) in food and feed proteins are higher than the RNVs as determined by Tetrahymena. The latter may be used in relation to higher organisms only in the knowledge of the appropriate regression correlations (HEGEDÜS, 1974). In this study the Tetrahymena-RNV corresponded practically to the results obtained in animal tests.

To sum up the afore-said, Tetrahymena seems to be suitable for the evaluation of single-cell proteins. It appears particularly useful at the laboratory level of developing fermentation technologies, where sufficient material for animal tests is not yet available and conditions are safe for microbiological work.

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# OPTIMIZATION OF THE COMPOSITION OF THE MEDIUM FOR THE PRODUCTION OF MILK-CLOTTING ENZYME OF MICROBIAL ORIGIN IN SUBMERGED CULTURE

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Experiments were carried out to increase the milk-clotting activity of the fermentation broth of *Endothia parasitica* grown in shaken culture in the laboratory.

The aim of the experiments was to find the optimum ratio of the components in the medium under fermentation conditions developed earlier. Other conditions of cultivation, such as preparation of the inoculum, aeration, qualitative composition of the medium, were not altered.

The medium contained the following components: extracted soybean meal, glucose, $CaCO_3$ or $Ca(NO_3)_2$. Of the mathematical models frequently applied in recent years for the optimization of their ratio the multi-factor design was used. The possible variants of the component composition were designed by increasing or reducing the ratio of individual components in comparison to the original composition. The effect of each change was recorded and characterized by the milk-clotting activity of the variant.

The nutrient composition producing the best result formed the basis of a new optimization experiment. This was carried out by the so called steep approximation of the optimum, that is the intervals between component concentrations were narrowed down.

The milk-clotting effect of the fermentation broth of optimum composition exceeded that of the fermentation broth of initial composition by 119–130%.

In earlier experiments a method for submerged cultivation of *Endothia parasitica* was developed for the production of a milk-clotting enzyme preparation. In order to increase the enzyme yield it was considered desirable to optimize the ratio of components of the media, leaving unaltered other, earlier developed conditions of cultivation (KISS, 1974).

In the first part of the optimization procedure the $2^3$ factorial experiment was applied as a variant of the multi-factor designs described by FISHER (1935). In the course of developing the principles of analysis of variance the possibility of creating mathematical models for planning and evaluation arose (FISHER, 1935). The methods of factorial experiment and its variants are frequently used for the optimization of fermentation processes (BOX & HUNTER, 1957; HOERL, 1959; GOLOZUBOVA *et al.*, 1970; GRACHEV *et al.*, 1970; YEGOROV *et al.*, 1971; MAKSIMOV, 1971; GRACHEVA *et al.*, 1973; JANZSÓ, 1973, 1974; JANZSÓ *et al.*, 1975).

The $2^3$ factorial experiments were followed by the steep approximation of the optimum (BOX & WILSON, 1951; BOX & DRAPER, 1963) setting thereby more accurate limits to the optimum conditions of cultivation.

## 1. Materials and methods

### *1.1. Cultivation of the microorganism*

The *Endothia parasitica* (CBS 25051, Baarn) strain was maintained on malt agar.

The medium for the inoculum was seeded with the aqueous spore suspension of a 1–2-month surface culture. Inoculation was carried out in two steps (KISS, 1974). In the first step 100 ml of the medium were inoculated with $6.5 \cdot 10^8$ spores and aerated by shaking for 24 hours at 30 °C. In the second step 100 ml of the medium were inoculated with 5 ml of the first inoculum and cultured for 18 h under the same conditions as mentioned in the first step.

The fermentation medium was inoculated with 5% of the second-step culture. The composition of the medium was as follows:

| | |
|---|---|
| extracted soybean meal | 1.75% |
| glucose | 3.00% |
| $CaCO_3$ or $Ca(NO_3)_2$ | 0.50% |
| antifoam agent | 0.20% |

Cultures were aerated by shaking at 30 °C and evaluated after 48 h.

### *1.2. Determination of the milk-clotting enzyme activity*

The 48 mold cultures were filtered from mycelia. The milk-clotting activity of the filtrate was determined by SOXHLET's method (1877) and expressed in Soxhlet units (SU $ml^{-1}$).

### *1.3. Optimization of the medium*

To determine the optimum ratio of the components (factors) of the medium first *Fisher*'s $2^3$ factorial experiment was used: the composition of the medium was varied in comparison to the original composition ($x_{0_i}$) given in para. 1.1 by increasing or reducing the concentration of the factors ($x_i$) by $\lambda_i$ intervals. Factors and their numerical values are shown in Table 1.

If the effect of the increase or reduction of individual components is considered simultaneously the number of nutrient variants — variation levels, $N = 2^i$ (where $i$ is the number of factors).

The complete design of factors is shown in Table 2.

Column $N$ contains the number of variation levels (the number of factors in the present case was 3, thus the number of variation levels $N = 2^3 = 8$); The signs of colums $x_i$ ($x_0$, $x_1$, $x_2$, $x_3$, $x_1x_2$, $x_1x_3$, $x_2x_3$, $x_1x_2x_3$) are needed to calculate the free member and coefficients of the regression equation characterizing the changes. The concentrations of factors belonging to each variation

Table 1

*Initial levels and intervals in the multi-factori design*

| Factor ($x_i$) | Initial level ($O_{0i}$, K) | Interval ($\lambda_i$) | Altered concentrations | |
|---|---|---|---|---|
| | | | + | − |
| Extracted soybean meal ($x_1$) | 1.75% | 0.4% | 2.15% | 1.35% |
| Glucose ($x_2$) | 3.00% | 0.5% | 3.50% | 2.50% |
| Ca salt ($x_3$) ($CaCO_3$ or $Ca(NO_3)_2$) | 0.50% | 0.1% | 0.60% | 0.40% |

level ($x_1$, $x_2$, $x_3$) are shown. Data in the columns under results will be discussed in para. 2.1.

Experiments were carried out in two series with 3 parallels at each variation level. In one of the series the nutrient contained $CaCO_3$, while in the other $Ca(NO_3)_2$.

The correlation between the changing quantities of the factors and changes in the activity was expressed by a three-variable regression equation: $\hat{y} = f(x_1, x_2, x_3)$. By omitting the members of high order an eight-member regression equation was obtained:

$$\hat{y} = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{1,2}x_1x_2 + b_{1,3}x_1x_3 + b_{2,3}x_2x_3 + b_{1,2,3}x_1x_2x_3$$

The free member ($b_0$) and regression coefficients ($b_1$; $b_2$; $b_3$; $b_{1,2}$; $b_{1,3}$; $b_{2,3}$; $b_{1,2,3}$) were obtained by reducing the numerical values in column $\bar{y}_N$ of Table 2 according to the signs of $x_0$ and $x_i$ and deviding it by the number of variations ($N$), in this case by 8:

$$b_0 = \frac{\sum^{N} (\bar{y}_N x_0^N)}{N} \quad \text{and} \tag{1}$$

$$b_i = \frac{\sum^{N} (\bar{y}_N x_i^N)}{N} \tag{2}$$

where

$\bar{y}_N$ = the average of the enzyme concentration values of $k$ parallels with $N$ variants; $x_i^N$ is the sign of the factor with $N$ variants as given in Table 2.

After the calculation of the regression coefficients their significance was tested by applying the following inequality:

$$|b_i| > S[b_i] \cdot t_p(f) \tag{3}$$

Table 2

*Factorial design and experimental results*

| Factorial design | | | | | | | | | | | | Results (SU ml$^{-1}$) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | design | | | concentrations (%) | | | interactions | | | | $x_3 = CaCO_3$ | | | | $x_3 = Ca(NO_3)_2$ | | | |
| N | $x_0$ | $x_1$ | $x_2$ | $x_3$ | $x_1$ | $x_2$ | $x_3$ | $x_1x_2$ | $x_1x_3$ | $x_2x_3$ | $x_1x_2x_3$ | $y_N^I$ | $y_N^{II}$ | $y_N^{III}$ | $\bar{y}_N$ | $y_N^I$ | $y_N^{II}$ | $y_N^{III}$ | $\bar{y}_N$ |
| 1 | + | − | − | − | 1.35 | 2.50 | 0.40 | + | + | + | − | 223 | 267 | 246 | 245 | 291 | 331 | 281 | 301 |
| | | | | | | | | | | | | 342 | 399 | 416 | 386 | 505 | 565 | 480 | 517 |
| 2 | + | + | − | − | 2.15 | 2.50 | 0.40 | − | − | + | + | 102 | 38 | 50 | 63 | 55 | 0 | 0 | 18 |
| | | | | | | | | | | | | 63 | 33 | 29 | 42 | 88 | 36 | 54 | 59 |
| 3 | + | − | + | − | 1.35 | 3.50 | 0.40 | − | + | − | + | 533 | 533 | 417 | 494 | 246 | 253 | 253 | 250 |
| | | | | | | | | | | | | 473 | 512 | 528 | 504 | 667 | 667 | 696 | 677 |
| 4 | + | + | + | − | 2.15 | 3.50 | 0.40 | + | − | − | − | 67 | 71 | 71 | 70 | 40 | 60 | 50 | 50 |
| | | | | | | | | | | | | 87 | 51 | 32 | 56 | 89 | 31 | 32 | 51 |
| 5 | + | − | − | + | 1.35 | 2.50 | 0.60 | + | − | − | + | 505 | 640 | 565 | 570 | 565 | 600 | 436 | 533 |
| | | | | | | | | | | | | 636 | 592 | 571 | 599 | 941 | 667 | 640 | 749 |
| 6 | + | + | − | + | 2.15 | 2.50 | 0.60 | − | + | − | − | 274 | 331 | 310 | 304 | 38 | 42 | 38 | 39 |
| | | | | | | | | | | | | 172 | 165 | 121 | 153 | 102 | 123 | 70 | 98 |
| 7 | + | − | + | + | 1.35 | 3.50 | 0.60 | − | − | + | − | 480 | 686 | 686 | 617 | 348 | 686 | 640 | 558 |
| | | | | | | | | | | | | 635 | 688 | 690 | 971 | 762 | 842 | 842 | 815 |
| 8 | + | + | + | + | 2.15 | 3.50 | 0.60 | + | + | + | + | 457 | 505 | 480 | 480 | 30 | 40 | 25 | 32 |
| | | | | | | | | | | | | 220 | 251 | 218 | 230 | 126 | 110 | 133 | 123 |
| K | | | | | 1.75 | 3.00 | 0.50 | | | | | 275 | 386 | 311 | 324 | 204 | 253 | 274 | 244 |
| | | | | | | | | | | | | 334 | 353 | 310 | 332 | 436 | 355 | 267 | 350 |

N = number of variation levels;
K = initial composition of the medium ($x_{0_i}$);
$x_0$ = signs needed to calculate the free member of the regression equation; $x_1$, $x_2$, $x_3$ (design) and $x_1x_2$; $x_1x_3$; $x_2x_3$; $x_1x_2x_3$ (interactions) = signs required to calculate the regression coefficients;
Concentrations (%) = concentration of the nutrient components at each variation level;
$y_N^I$, $y_N^{II}$, $y_N^{III}$ = results of parallel experiments (SU ml$^{-1}$);
$\bar{y}_N$ = average of parallels

where $|b_i|$ is the numerical value of the regression coefficients, $S[b_i]$ is the variance of the coefficients, $t_p(f)$ the coefficient of *Student*. Degree of freedom $f = N(k-1)$.

If inequality exists the given regression coefficient significantly deviates from zero; if there is no inequality it may be ignored.

The variance of the coefficients is calculated as follows:

*A)* Calculation of variance at the different variation levels

$$S^2[y_N^k] = \frac{\Sigma(\bar{y}_N - y_N^k)^2}{k-1} \tag{4}$$

*B)* Average variance of the variation levels

$$S^2[y] = \frac{\Sigma S^2[y_N^k]}{N} \tag{5}$$

*C)* Variance of the mean value

$$S^2[\bar{y}] = \frac{S^2[y]}{k} \tag{6}$$

*D)* Variance of the regression coefficients

$$S^2[b_i] = \frac{S^2[\bar{y}]}{N} \tag{7}$$

Standard deviation of the regression coefficients

$$S[b_i] = \sqrt{S^2[b_i]} \tag{8}$$

Of the members of the regression equation only those were taken into account, for the coefficients of which inequality (3) was valid.

The equations were analysed and the possibility of dropping members of high order or of interaction, was examined.

To examine members of high order the following inequality was used:

$$|\bar{y}_0 - b_0| > \sqrt{\bar{\bar{S}}^2} \cdot \sqrt{\frac{N+k}{Nk}} \cdot t_p(f), \tag{9}$$

where $f = N + k - 2$

$$\bar{\bar{S}}^2 = \frac{(N-1)\cdot S^2[b_i] + (k-1)\,S^2[\bar{y}_0]}{N+k-2} \tag{10}$$

$$S^2[\bar{y}_0] = \frac{\Sigma(\bar{y}_0 - y_0^k)^2}{k(k-1)} \tag{11}$$

If inequality is non-existent the members of high order are negligible.

To test the possibility of dropping the members of interaction *Fisher*'s test was applied. Taking into account the linear section of the regression equations the values of $y$ belonging to each variation level were calculated by reducing the coefficients at each variation level pertinent to the factor of the same sign and the deviation was calculated from the enzyme concentration values as measured ($\bar{y}_N$). The difference in the sums of squares as measured and calculated is characteristic:

$$S^2 = \frac{\Sigma(\bar{y}_N - \hat{y}_N)^2}{N - i - 1} \tag{12}$$

The calculated values:

$$F_{cal} = \frac{S^2}{S^2[y]} \tag{13}$$

Value in the *Fisher* test table:

$$F_{table} = F_p(f_1; f_2) \ , \tag{14}$$

where $f_1 = N\text{–}i\text{–}1$
$f_2 = k\text{–}1$

If $F_{cal} < F_{table}$, the members of the interaction may be dropped.

Information may be derived from the regression coefficients as to the effect of individual factors [when inequality (3) exists] or to their inefficiency (when the inequality is non-existent) and to the direction of effect (increasing or reducing) according to the signs of the coefficients. They show predominantly the direction of change in factor concentrations.

Using the method of steep approximation of the optimum according to Box and Wilson (1951), the optimum nutrient composition may be found in the preconceived concentration series based on the results of the multi-factorial design. The concentration series was selected so as to encompass the nutrient composition found optimal in the multi-factorial design.

## 2. Results

### *2.1. Results obtained by the method of complete factorial design*

In the first part of the experiments the $2^3$ factorial design was applied as shown in Table 1.

In Table 2 the milk-clotting enzyme concentrations as measured at the different variation levels of the multi-factor design, are illustrated (SU $ml^{-1}$). Under Results, columns $y_N^{I}$, $y_N^{II}$, $y_N^{III}$ indicate the fermentation values

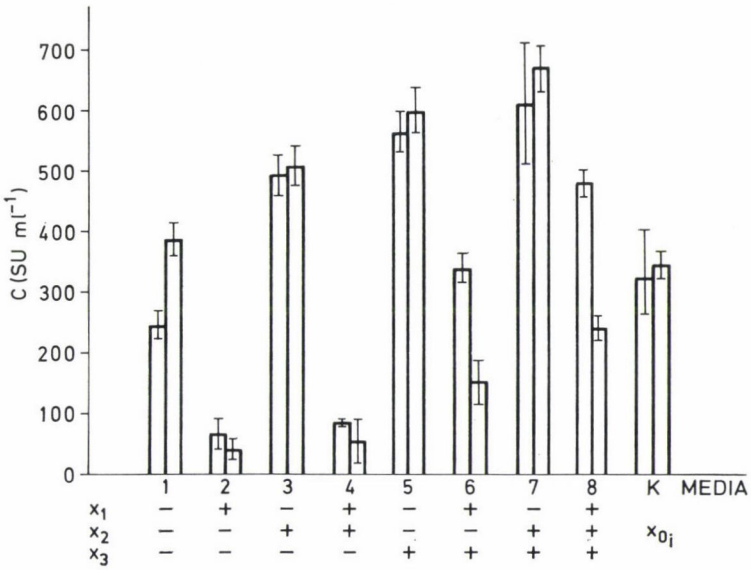


Fig. 1. Milk-clotting activity (means and standard deviations: columns and bars) in the fermentation broth ($C$) as a function of the ratio of nutrient components in a medium containing $CaCO_3$

Legend:
signs below the abscissa mark the increase (+) or decrease (−) of the concentration of the given component (factor) relative to the initial level ($x_{0i}$); K = milk-clotting activity in the medium of initial composition; numbers on the abscissa (from 1 to 8) indicate variation levels listed in column N of Table 2; $x_1$, $x_2$, $x_3$ = components of the medium (factors); number of experiments = 2; number of parallels (n) = 3

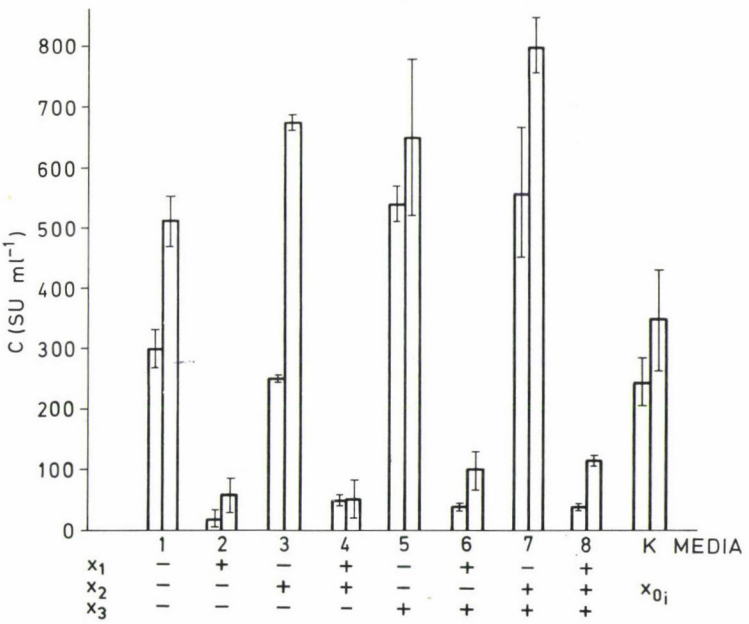


Fig. 2. Milk-clotting activity (means and standard deviations: columns and bars) in the fermentation broth ($C$) as a function of the ratio of nutrient components in a medium containing $Ca(NO_3)_2$

Legend as in Fig. 1

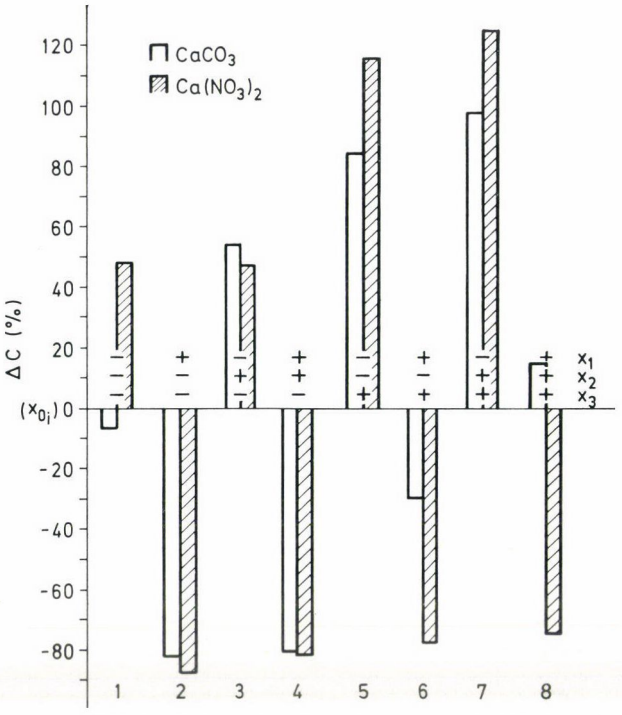


Fig. 3. Changes in the milk-clotting activity of the fermentation broths ($\Delta$ C) at different variation levels as percentage of the initial activity

Legend:
signs on the abscissa mark the increase (+) or decrease (−) in the concentration of the medium components (factors: $x_1$, $x_2$, $x_3$) related to the initial value ($x_{0i}$); numbers 1 to 8 = variation levels as listed in column N of Table 2

obtained in parallel fermentations, while $\bar{y}_N$ represents their average. The results of fermentations at different variation levels differed from one another and from the initial level ($K$). This is illustrated in Figs. 1 and 2.

The increase in the quantity of extracted soybean meal ($x_1$), in the presence of both Ca salts, markedly reduced the enzyme concentration in the filtered fermentation broth as related to the initial concentration ($x_0$).

Activity as related to the different variation levels is illustrated in Fig. 3.

Differences in percentage as related to the initial concentration are recorded on the ordinate. The abscissa shows the initial concentration, as well as the composition of the individual variation levels. In the medium of the optimal composition (containing 1.35% extracted soybean meal, 0.60% Ca salt and 3.5% glucose) the increase in the presence of $CaCO_3$ was 104% while with $Ca(NO_3)_2$ it amounted to 125%.

An assay was made to characterize the changes as observed by a three-variable regression equation. By dropping members of high order the following

equations were obtained:
with $CaCO_3$

$$\hat{y} = \mathbf{355} - \mathbf{126}x_1 + \mathbf{60}x_2 + \mathbf{137}x_3 - \mathbf{14}x_1x_2 + \mathbf{25}x_1x_3 + \mathbf{5}x_2x_3 + \mathbf{46}x_1x_2x_3; \quad (15)$$

$$\hat{y} = \mathbf{323} - \mathbf{210}x_1 + \mathbf{39}x_2 + \mathbf{83}x_3 - \mathbf{11}x_1x_2 - \mathbf{12}x_1x_3 + \mathbf{7}x_2x_3 + \mathbf{14}x_1x_2x_3; \quad (16)$$

with $Ca(NO_3)_2$

$$\hat{y} = \mathbf{400} - \mathbf{302}x_1 + \mathbf{32}x_2 + \mathbf{60}x_3 - \mathbf{25}x_1x_2 - \mathbf{31}x_1x_3 - \mathbf{7}x_2x_3 + \mathbf{16}x_1x_2x_3; \quad (17)$$

$$\hat{y} = \mathbf{223} - \mathbf{188}x_1 + \mathbf{0}x_2 + \mathbf{68}x_3 - \mathbf{31}x_1x_2 - \mathbf{67}x_1x_3 + \mathbf{5}x_2x_3 - \mathbf{14}x_1x_2x_3; \quad (18)$$

Of the coefficients only the members in boldface type proved significant.

On analysing the regression equations the members of interaction proved unambigously negligible. The members of high order were found to be on the border of negligibility, thus they were not tested.

The equations point to the necessity of reducing the concentration of extracted soybean meal, increasing that of the Ca salts in order to enhance milk-clotting activity. The effect of glucose proved insignificant.

The extent of increase in activity at the optimum variation level was calculated from the regression equations as related to the initial level. Distribution between factors of the activity-increasing effect was investigated. Results of the calculations are summed up in Table 3.

Table 3

*Activity-increasing effect of individual factors in the nutrient composition found optimal (No. 7)*

| Factor $x_i$ | Interval $\lambda_i$ | Concentration of factors | Activity-increasing effect | | | |
|---|---|---|---|---|---|---|
| | | | $x_3 = CaCO_3$ | | $x_3 = Ca(NO_3)_2$ | |
| | | | SU ml$^{-1}$ | % | SU ml$^{-1}$ | % |
| Extracted soybean meal ($x_1$) | −0.4% ($\lambda_1$) | 1.35% | 165 | 49° | 245 | 79° |
| Glucose ($x_2$) | +0.5% ($\lambda_2$) | 3.50% | 49 | 14 | — | — |
| Ca salt ($x_3$) | +0.1% ($\lambda_3$) | 0.60% | 107 | 32 | 64 | 21 |
| Joint activity-increasing effect as calculated from the regression coefficients | | | | 95 | | 100 |
| Increase in activity at the optimum composition level as calculated from data in Table 2 | | | | 96°° | | 125°° |

° = relative to $b_0$
°° = % deviation from the initial level
Note: in the medium containing $Ca(NO_3)_2$ the effect of glucose was negligible

In the presence of $CaCO_3$ an increase of 96%, in that of $Ca(NO_3)_2$ an increase of 100% was achieved. This effect was mostly due to the reduction of the quantity of soybean.

### *2.2. Results obtained by steep approximation of the optimum*

In the second part of the experiments a concentration series was created from the factors where the intervals were narrower than in the multi-factor design. The initial composition was the same as in the first part. The factor concentrations in the different medium compositions and the pertinent milk-clotting activities are given in Table 4. The effect of factors on the activity is illustrated in Fig. 4.

As may be seen, the medium containing 1.35% extracted soybean meal, 3.5% glucose and 0.6% Ca salt was found to be optimal.

The milk-clotting activity as related to the initial level was increased, in the case of $CaCO_3$ by 119%, while with $Ca(NO_3)_2$ the increase was 130%.

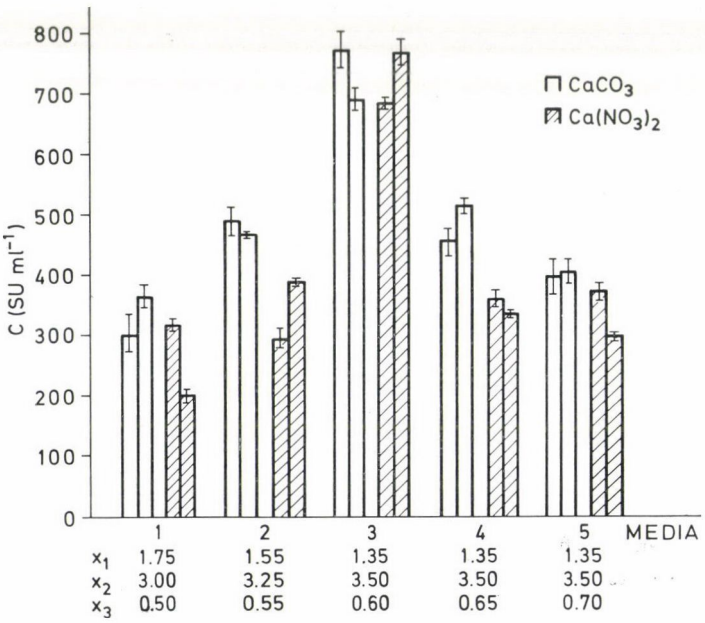


Fig. 4. Results of the steep approximation of the optimum. Milk-clotting activity (C; means and standard deviations: columns and bars) as a function of composition of the medium

Legend:
numbers on the abscissa (1 to 5) indicate serial number of variant of media as listed in Table 4; $x_1$, $x_2$, $x_3$ = components of the media (factors); number of parallels (n): 3; number of experiments: 2

Table 4

*Steep approximation of the optimum*

| Variant of medium | Concentration of factors (%) | | | Results (SU ml$^{-1}$) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | $x_3 = CaCO_3$ | | | | $x_3 = Ca(NO_3)_2$ | | | |
| | $x_1$ | $x_2$ | $x_3$ | $y_N^I$ | $y_N^{II}$ | $y_N^{III}$ | $\bar{y}_N$ | $y_N^I$ | $y_N^{II}$ | $y_N^{III}$ | $\bar{y}_N$ |
| 1 | 1.75 | 3.00 | 0.50 | 325 | 305 | 262 | 297 | 334 | 292 | 329 | 318 |
| | | | | 417 | 369 | 320 | 369 | 216 | 188 | 196 | 200 |
| 2 | 1.55 | 3.25 | 0.55 | 512 | 490 | 452 | 485 | 282 | 291 | 310 | 294 |
| | | | | 470 | 470 | 470 | 470 | 398 | 405 | 360 | 387 |
| 3 | 1.45 | 3.50 | 0.60 | 738 | 812 | 773 | 774 | 640 | 770 | 650 | 687 |
| | | | | 640 | 686 | 738 | 688 | 758 | 751 | 730 | 746 |
| 4 | 1.15 | 3.75 | 0.65 | 472 | 498 | 426 | 455 | 343 | 369 | 380 | 364 |
| | | | | 505 | 565 | 490 | 520 | 361 | 341 | 331 | 344 |
| 5 | 1.15 | 3.75 | 0.70 | 426 | 372 | 398 | 400 | 353 | 390 | 384 | 376 |
| | | | | 457 | 370 | 400 | 409 | 315 | 310 | 282 | 302 |

$x_1$ = extracted soybean meal
$x_2$ = glucose
$x_3$ = $CaCO_3$ or $Ca(NO_3)_2$
$y_N^I$, $y_N^{II}$, $y_N^{III}$ = parallels
$\bar{y}_N$ = average of parallel results

## 3. Conclusions

In experiments aimed at the optimization of the nutrient medium *Fisher*'s multi-factor design was successfully applied. Evaluation of the results has shown in what direction the concentration of nutrients has to be changed to enhance milk-clotting activity. Subsequently, using the method of steep approximation (Box & Wilson, 1951) the optimum ratio of media components was found in a single step.

The ratio of the nutrient components was found to strongly influence the milk-clotting activity of the fermentation broth of *Endothia parasitica*. In order to obtain higher enzyme concentration the quantity of extracted soybean meal had to be reduced while that of the Ca salts had to be increased relative to the initial level. The effect of the change of glucose concentration proved insignificant in the given concentration range.

In the nutrient medium of optimum composition the milk-clotting activity increased by about 119 to 130% as compared to the initial activity.

On the basis of these results it seems to be expedient to extend optimization to other conditions of fermentation not examined in the present study, with a view to increasing milk-clotting activity.

*

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# EFFECT OF AGITATION AND AERATION ON MYCELIAL AND PROTEIN YIELDS OF FUNGI

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Mycelial and protein yields of *Rhizopus*, *Mucor* and *Actinomucor* strains have been examined at three speeds of agitation and three rates of aeration.

The majority of the strains attained the highest mycelial and protein yields at the lowest speed of agitation tested (460 rpm) at an aeration rate of 1.0 l $l^{-1}$ $min^{-1}$ (corresponding to an oxygen transfer rate of 49 mmole $O_2$ $l^{-1}$ $h^{-1}$). Agitation with 700 rpm at an aeration rate of 1.0 l $l^{-1}$ $min^{-1}$ ($O_2$ transfer rate: 100 mmole $O_2$ $l^{-1}$ $h^{-1}$) proved to be optimal only in the case of one strain of *Rhizopus microsporus*. The agitation speed of 940 rpm (with 1.0 l $l^{-1}$ $min^{-1}$ aeration rate, oxygen transfer rate: 126 mmole $l^{-1}$ $h^{-1}$) proved to be optimal also in only one case, in the culture of an *Actinomucor repens* strain.

No noticeable maximum yield was obtained with aeration rates (at 460 rpm) of 0.5 and 1.5 l $l^{-1}$ $min^{-1}$ but the lower aeration rate (0.5 l $l^{-1}$ $min^{-1}$) proved to be optimal to the *Mucor* and *Actinomucor* strains for attaining a rather good yield in the shortest time of cultivation.

Aeration and agitation are basic factors of aerobic fermentation processes. Aeration of a growing microbial culture is primarily undertaken to satisfy its oxygen requirement and at the same time to remove the waste products. The oxygen necessary for the growth and production of fungal culture can be ensured by agitation and aeration of the culture. The efficiency of aeration can be improved by agitation, resulting in an increased interface between gas and liquid. The dependence of yield on each of oxygen supply and mixing of the batch as well, have been reported by many authors (FINN, 1954; MAXON, 1959; STEEL & MAXON 1962). The yield of fermentation products usually declines when the aerating ability of the equipment is inadequate (SHU, 1953; KAROW *et al.*, 1953).

FORTUNE and co-workers (1950) came to the conclusion that the aeration rate is governed by the agitation rate, and at a low agitation speed more air can be used if the foaming properties of the media do not interfere.

Excessive agitation and over-aeration can be harmful by reducing the yield of fermentation product (FORTUNE *et al.*, 1950; HASTINGS, 1952) and by causing alterations in the morphology of mycelia. Thus proper agitation and aeration are essential for optimal yields of fungal mycelium and vary with the organism used (LITCHFIELD *et al.*, 1963). Intracellular nucleotides were found to leak from the mycelium into the water in direct proportion to agitation time in the cultures of *Mucor javanicus* and *Rhizopus javanicus* (TANAKA

*et al.*, 1975a, b). It was also found by the same author that in the aerated and agitated cultures of *Mucor javanicus*, the average length of mycelia was less then 300 $\mu$m and a low agitation speed was necessary to obtain normal growth of the mould.

In the present work the effect of agitation and aeration on the mycelial and protein yields of five different fungus strains *(Rhizopus, Mucor* and *Actinomucor)* was examined. The maximal yields attained in the shortest time of cultivation were also determined in the case of both mycelia and protein.

### *1.1. Microorganisms*

The *Rhizopus*, *Mucor* and *Actinomucor* strains used in this experiment were obtained from the culture collection of the NATIONAL RESEARCH INSTITUTE OF VITICULTURE AND OENOLOGY, Budapest. The names and signs of the investigated microorganisms are listed in Table 1.

Table 1

Rhizopus, Mucor *and* Actinomucor *strains used in the experiments*

| Strain numbers | Fungal species |
|---|---|
| 221 | *Rhizopus cohnii* |
| 223 | *Rhizopus microsporus* |
| 206 | *Mucor racemesus* |
| 219 | *Mucor* sp. |
| 217 | *Actinomucor repens* |

### *1.2. Nutrient media*

Malt agar was used for maintaining the strains.

Nutrient *medium for the inoculum:* yeast extract 5 g, soluble starch 15 g, $KH_2PO_0$ 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, water 1 000 ml, pH 6.5.

*Fermentation medium.* The composition of 1 000 ml of the ground bread medium was as follows: ground bread 40 g; corn-steep liquor 20 g (400 g of dry ground bread were suspended in 940 ml of tap-water. This was heated to 80 °C, then 16 ml of cc. $H_2SO_4$ were added. The hydrolysis was carried out at 120 °C for 60 min. After hydrolysis the pH was adjusted to 4.0 with NaOH. Mineral salts were added to the above medium as follows: $(NH_4)_2SO_4$ 0.25 g; $MnSO_4$ 0.10 g; $KH_2PO_4$ 1.0 g; volume was adjusted to 1 000 ml with tap-water, pH to 4.0).

*1.3. Method of cultivation*

100 ml of the inoculation medium were inoculated with 1 ml of a spore suspension and were incubated for 24 h on a rotary shaker (rpm: 330; stroke: 20 mm; oxygen transfer rate in the flasks: 17–19 mmole $l^{-1}$ $h^{-1}$ at a temperature of 28 °C). The fermentations were conducted in 10-l glass fermentors. The 6 000 ml of medium of a fermentor were inoculated with 600 ml of the above 24-h vegetative culture. The speeds of agitation were 460, 700 and 940 rpm, and the volumes of air bubbled through the culture media were 0.5, 1.0 and 1.5 l $l^{-1}$ $min^{-1}$, resp. Oxygen transfer rate was determined by the method of COOPER and co-workers (1944). In the case of 460 rpm and 0.5, 1.0 and 1.5 l $l^{-1}$ $min^{-1}$ its values were 26, 49 and 60 mmole $l^{-1}$ $h^{-1}$, resp., while at 700 and 940 rpm, at an aeration rate of 1.0 l $l^{-1}$ $h^{-1}$, the oxygen transfer rate proved to be 100 and 126 mmole $l^{-1}$ $h^{-1}$, resp.

*1.4. Preparation of the mycelium*

Samples were taken every 12 h. Mycelia were filtered from the fermentation broth through a nylon cloth (mesh: 60 $\mu$m). The remainder of the medium was washed from the mycelium with water. The mycelium was dried under ventilation at 30 °C for 36–48 h.

*1.5. Protein determination*

Mycelial protein was determined by the *biuret* method (HERBERT *et al.*, 1971).

The effect of agitation speed and aeration rate was examined on the growth and protein formation of several *Rhizopus-*, *Mucor-* and *Actinomucor-strains* in 10-l fermenters. In the first part of the experiment the agitation speed (460, 700 and 940 rpm) was tested (one strain in a week and one agitation speed in three parallel fermentors).

The agitation experiment was followed by the examination of the effect of aeration (aeration rate: 0.5, 1.0 and 1.5 l $l^{-1}$ $min^{-1}$; one strain was tested in a week and each aeration rate in three parallels).

The results [the mycelial and protein yields of the tested strains at various agitation speeds (with a fixed aeration rate: 1.0 l $l^{-1}$ $min^{-1}$) and at different aeration rates (applying the same rpm: 460) as a function of the time of incubation] are shown in Figs. 1–5 and summarized in Table 2.

As the experiments with all the strains could not be carried out during the same period, moreover the effect of agitation and aeration in the case of one and the same strain also had to be tested at different times, not exactly under the same circumstances, the results of the agitation and aeration experiments meant to be performed under "identical" conditions, were not expected to be identical.

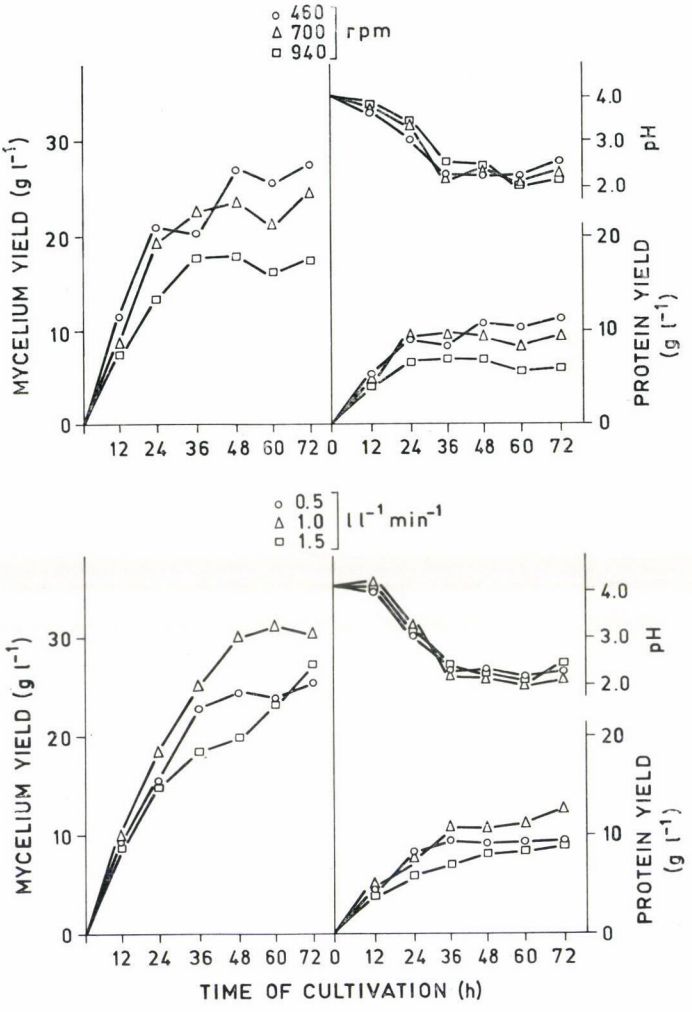


Fig. 1. Effect of agitation speed (at the aeration rate of 1.0 l $l^{-1}$ $min^{-1}$) and aeration rate (at 460 rpm) on the pH and on the mycelial and protein yields of *Rhizopus colnii* (No. 221)

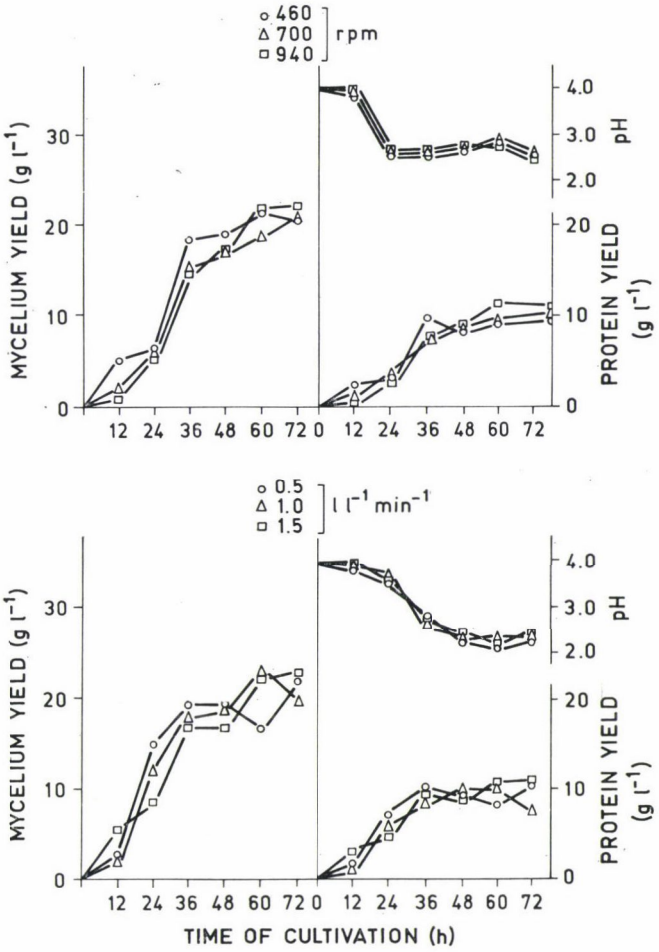


Fig. 2. Effect of agitation speed (at the aeration rate of 1.0 $l\,l^{-1}\,min^{-1}$) and the aeration rate (at 460 ropm) on the pH and on the mycelial and protein yields of *Rhizopus microsporus* (No. 223)

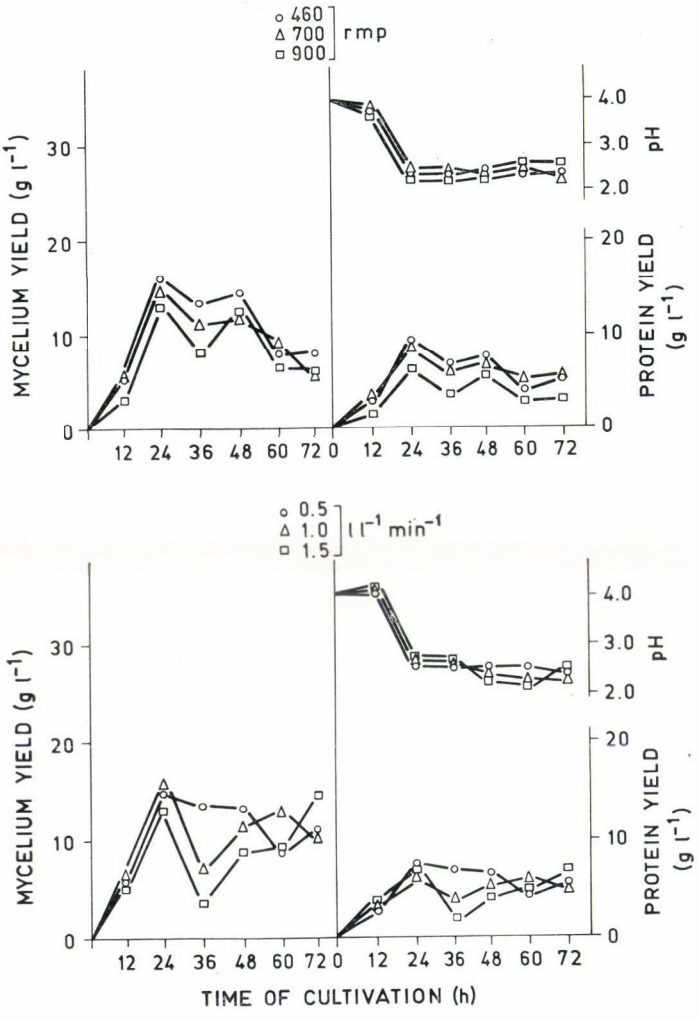


Fig. 3. Effect of agitation speed (at the aeration rate of 1.0 $l\,l^{-1}\,min^{-1}$) and the aeration rate (at 460 rpm) on the pH and on the mycelial and protein yields of *Mucor racemosus* (No. 206)

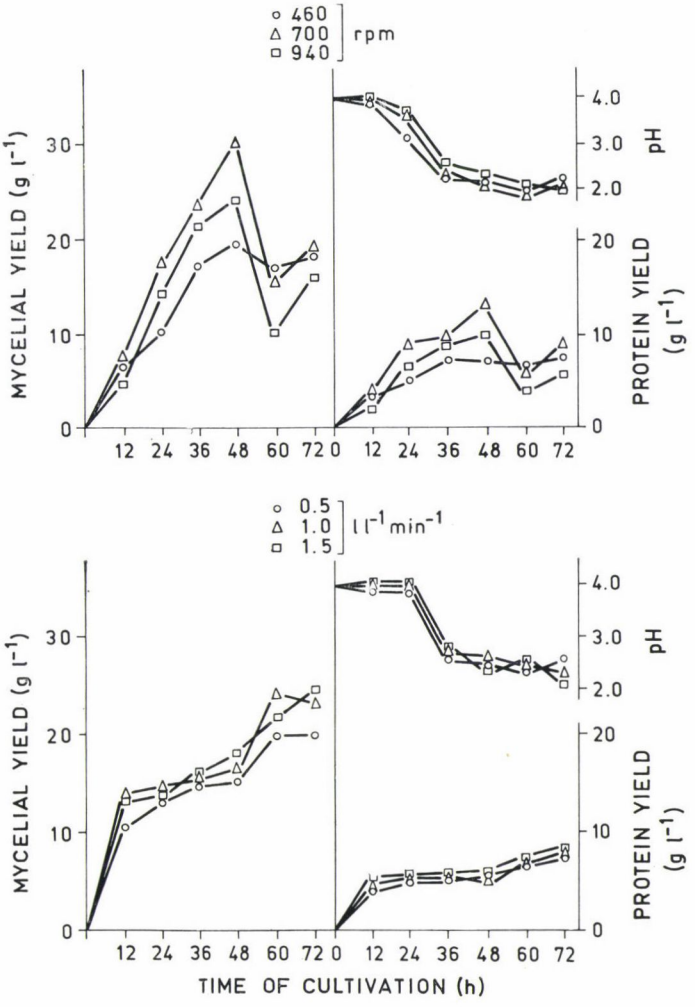


Fig. 4. Effect of agitation speed (at the aeration rate of 1.0 $l\,l^{-1}\,min^{-1}$) and the aeration rate (at 460 rpm) on the pH and on the mycelial and protein yields of *Mucor sp.* (No. 219)

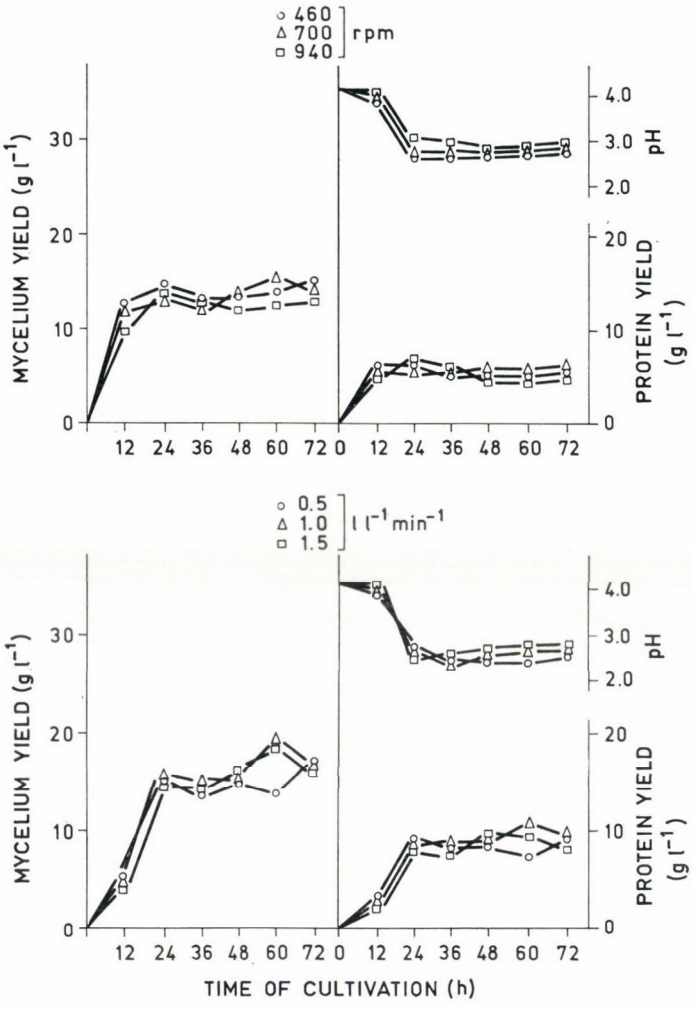


Fig. 5. Effect of agitation speed (at the aeration rate of 1.0 $l\,l^{-1}\,min^{-1}$) and the aeration rate (at 460 rpm) on the pH and on the mycelial and protein yields of *Actinomucor repens* (No. 217)

Table 2

*Maxima of the mycelial and protein yields of the various* Rhizopus, Mucor *and* Actinomucor *strains* [attained at cultivation times (h) indicated in parentheses] *when different speeds of agitation and rates of aeration were applied*

| The strains tested | Maximum yield ($g\ l^{-1}$) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | agitation speed (rpm) at aeration rate 1.0 $l\ l^{-1}\ min^{-1}$ | | | | | | aeration rate ($l\ l^{-1}\ min^{-1}$) at 460 rpm | | | | | |
| | 450 | | 700 | | 940 | | 0.5 | | 1.0 | | 1.5 | |
| | *Mycelium yield* | | | | | | | | | | | |
| *Rhizopus cohnii* | [27.5] | (48) | 24.2 | (48) | 17.5 | (36) | 24.8<br>26.2 | (48)<br>(72) | [29.5]<br>[30.8] | (48)<br>(60) | 27 | (72) |
| *Rhizopus microsporus* | 19.8 | (48) | [30.6] | (48) | 24.2 | (48) | 19 | (60) | [23.8] | (60) | 24 | (72) |
| *Mucor racemosus* | [16.0] | (24) | 14.2 | (24) | 12.8 | (24) | 15.5 | (24) | [16.0] | (24) | 13.5 | (24) |
| *Mucor* sp. | 18.2 | (36) | 20.3 | (72) | [22.0] | (60) | [19.9]+<br>21.8 | (36)<br>(72) | 22.0 | (60) | [22.5] | (72) |
| *Actinomyces repens* | [14.6]*<br>[21.0] | (24)<br>(60) | 13.0<br>15.2 | (24)<br>(50) | 13.8 | (24) | 15.8<br>18.2 | (24)<br>(72) | [16.0]<br>[20.2] | (24)<br>(60) | 15.0<br>19.2 | (24)<br>(60) |
| | *Protein yield* | | | | | | | | | | | |
| *Rhizopus cohnii* | [10.8] | (48) | 9.6 | (36) | 7.0 | (36) | 9.8 | (36) | [11.0]+<br>[13.0] | (36)<br>(72) | 8.5<br>9.0 | (48)<br>(72) |
| *Rhizopus microsporus* | 7.0 | (48) | [12.8]+ | (48) | 9.8 | (48) | 7.5 | (72) | 8.4 | (72) | [9.2] | (72) |
| *Mucor racemosus* | [8.8] | (24) | 8.2 | (24) | 6.3 | (24) | [7.8]+ | (24) | 6.0 | (24) | 6.8 | (24) |
| *Mucor* sp. | 9.8 | (36) | 10.2 | (72) | [11.0] | (60) | [9.8]+<br>10.2 | (36)<br>(72) | 9.5<br>10.2 | (48)<br>(60) | 9.5<br>[11.0] | (36)<br>(72) |
| *Actinomyces repens* | 6.5 | (24) | 5.5 | (12) | [6.8] | (24) | 10.2<br>9.5+ | (72)<br>(24) | [10.2]<br>[10.6] | (60)<br>(60) | 9.5<br>9.5 | (36)<br>(48) |

+ maximum attained during the shortest period of cultivation

[ ] maximum yield in the same row, under one set of conditions (*i.e.* at constant aeration rate and constant agitation speed, respectively)

Maxima of the mycelial and protein yields of the tested strains were not always attained at the same age of the culture. Frequently, it appeared difficult to identify the real maximum yield in cases the maximum, although somewhat lower, was attained earlier. The maximum yields of a fermentation with the corresponding cultivation time at different speeds of agitation and rates of aeration are summarized in Table 2. The maximum of yields, as a function of agitation speed and aeration rate, are indicated by squares, while the highest yields attained at the shortest time of cultivation are marked by crosses.

## 3. Conclusions

LITCHFIELD (1968) states that proper agitation and aeration conditions are essential for optimal mycelial yields of fungi and the optimal conditions vary according to the type of microorganism. Some authors report a dependence of yield on oxygen supply (YOSHINO *et al.*, 1961).

RIPPON (1968) observed that filamentous moulds such as *Penicillium lilacianum* developed budding yeast-like cells when grown in submerged culture under conditions of low redox potential. CARTER and BULL (1971) explained this differentiation by assuming that the fungus is under some physiological stress. FINN (1967) concluded that the control of aeration and agitation in such fermentations may therefore be as important as the control of pH and temperature.

In the experiments reported in this paper it was found (Table 2), that when the effect of the agitation speed was varied, highest mycelial and protein yields (30.6 and 12.8 g $l^{-1}$) were obtained in the culture of *Rhizopus microsporus*. The mycelial and protein yields of *Rhizopus cohnii* (27.5 and 10.8 g $l^{-1}$) and of *Mucor racemosus* (16.0 and 8.8 g $l^{-1}$) gave the next best results in the series.

According to the results of the experiments with varying aeration rates, *Rhizopus cohnii* (No. 221) attained the highest mycelial and protein yields (30.8 and 13 g $l^{-1}$). When considering only the mycelial yield, the second and third best results were obtained in the culture of *Rhizopus microsporus* and a *Mucor sp.*, but when protein is considered to be the final product, *Actinomucor repens* and *Rhizopus microsporus* gave better results.

When the data are evaluated from a different point of view, namely according to the highest yields attained under the shortest period of cultivation, the above sequence changes. In the agitation experiment the highest mycelial yield (27.5 g $l^{-1}$) was obtained in a 48-h culture of *Rhizopus cohnii* but under the same cultivation period *Rhizopus microsporus* attained the best protein yield (12 g $l^{-1}$). Maxima of mycelial and protein yields (16 and

8.8 g $l^{-1}$; 24 h) were obtained in the culture of *Mucor racemosus* in the shortest period of cultivation (24 h).

The protein yield of *Rhizopus cohnii* proved to be the highest (11 g $l^{-1}$) in the shortest cultivation period (36 h) in the aeration experiment. The mycelial and protein yield of *Mucor racemosus* was the best (16.0 and 7.8 g $l^{-1}$) in a 24-h cultivation period.

The majority of strains attained maximum yields at an agitation speed of 460 rpm and an aeration rate of 1.0 l $l^{-1}$ $min^{-1}$. *Rhizopus microsporus* was the only exception, attaining the highest mycelium and protein yields at 700 rpm and an aeration rate of 1.0 l $l^{-1}$ $min^{-1}$. The use of the highest (940 rpm agitation speed (with an aeration rate of 1.0 l $l^{-1}$ $min^{-1}$) or the highest aeration rate (1.5 l $l^{-1}$ $min^{-1}$, with an rpm of 460) resulted in no outstanding optimum yields.

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# BOOK REVIEWS

## Inhibition and inactivation of vegetative microbes

F. A. Skinner & W. B. Hugo (Eds.), Academic Press, London—New York—San Francisco, 1976. xiii + 378 pp.

The book contains the proceedings of a symposium held jointly by the Society for Applied Bacteriology and the North West European Microbiological Group in the summer of 1975, under the same title. Sixteen papers are included mostly from authors in the United Kingdom. The topics of the papers are heterogeneous and due to the character of the book, they are presented in different formats. A wide range of physical and chemical factors affecting bactericides are discussed in the individual papers. The phenomenon of injury, which is of such an importance for the microbiological stability of foods exposed to non-sterilizing stress effects and for the improvement of methods for the detection of microorganisms, was investigated from many aspects. In some of the papers the mechanism of the effect is discussed *e.g.* in "The Inactivation of Vegetative Bacteria by Chemicals" (W. B. HUGO), in others inactivation is viewed as a consequence of various effects upon individual groups of microorganisms, *e.g.* in "Inactivation of Yeast" (I. W. DAWES), in again others the treatment of a certain group of products, *e.g.* in "The Survival of Bacteria in Toiletries" (S. A. MALCOLM), or the influence exerted by a specific technology, *e.g.* in "Inactivation of Bacteria by Freeze-drying" (I. J. BOUS-FIELD & A. R. MCKENZIE). Among papers pertinent to food science and research some relate to a certain branch of food industry, *e.g.* that on "The inactivation of Vegetative Microorganisms by Chemicals in the Dairying Industry" (C. M. COUSINS), others to the resuscitation of damaged bacteria in foods (M. VAN SCHOTHORST). A paper treating water activity mainly in relation to meat deserves attention because of its practical applications (L. LEISTNER & W. RÖDEL), so does the paper on chemical preservatives (B. JARVIS & C. S. BURKE). Extremely valuable and informative are the papers on the antimicrobial activity of $SO_2$ (S. M. HAMMOND & J. G. CARR), on the effect of low temperatures (M. INGRAM & B. M. MACKEY), on thermal injury and inactivation of vegetative bacteria (R. I. TOMLINS & Z. J. ORDAL). On the whole, the book is a collection of very useful information for microbiologists of food, hygienists, technologists and for researchers engaged in food microbiology. It is a useful complement to a book on similar subject, published by the same firm earlier under the title: "Inhibition and Destruction of Microbial Cells" (Ed.: W. B. HUGO, 1971) and to The Bacterial Spore (1969), edited by G. W. GOULD and A. HURST.

J. FARKAS

## Towards an ideal refrigerated food chain

Meeting of Commissions C2, D1, D2, D3 and E1 of the International Institute of Refrigeration Melbourne, September 6—10, 1976.

Experts of 18 countries participated in the meetings and 81 papers were presented in the following topics: General (4 papers); Food Science and Technology (10 papers); Frozen Foods (10 papers); Heat and Mass Transfer in Foodstuffs (7 papers); Construction and Operation of Cold Stores (8 papers); Design and Utilization of Containers (9 papers); Air Transport (3 papers); Specialised Refrigerating Equipment for Processing Fish on Board and on Land (6 papers); Air Conditioning (14 papers).

The results presented at the meetings give a general survey of the research work carried out all over the world in view of the development of food preservation by cooling and freezing.

Of the results reviewed, the following deserve special interest:

The storage life of apples and pears when stored at 3–4 °C or −1.5 −1 °C in $CO_2$ ($CO_2$ concentration = 5–10%, $O_2$ = 5–13%) may be extended by 1–3 months as compared with traditional refrigerated storage conditions.

Bacteria causing the spoilage of fresh milk proliferate rapidly in the temperature range of 10 to 37 °C. Below 15 °C the growth rate is gradually decreased. Different methods of cooling do not degrade the quality of milk if it is cooled to 7 °C within 4 hours. The reaction rate of amino acid aldehyde and myosin aldehyde, affecting the quality of foods and biological substances decreases gradually in the temperature range between 45 and 0 °C, and so does the freezing point of the product. Below 0 °C to −24 °C the reaction rate increases to decrease again to −40 °C. In the phase of freezing the reaction rate is determined decisively by the temperature and not by the concentration of liquid, already frozen (cryoconcentration effegt). The surface of processed poultry is usually contaminated by bacteria. Of the bacteria salmonellae are most sensitive at the frozen storage temperature of −23.3 °C, while *Staphylococcus aureus* is the least sensitive. The vegetative cells of Clostridium perfringens are apparently not sensitive to frozen storage. Even after 27 months of storage at −23.3 °C, bacteria could be isolated from poultry. The fact that bacteria survive long storage at low temperature proves that the microbiological quality of poultry cannot be improved by freezing and frozen storage, thus decontamination must be ensured in the processing phases prior to freezing. The theoretical energy requirement of the different food preservation technologies in units of kWh $t^{-1}$ is as follows: cooling 15, freezing 100, pasteurisation 130, sterilization 225, drying 660. Since freezing seems the most favourable, presumably this method of low energy requirement will be used in the future for the preservation of most foodstuffs.

On comparing the freezing methods of cold air blast, liquid nitrogen and liquid carbon dioxide the advantage of the cryogenic technique was found in its lower weight loss upon freezing, better quality and the simplicity of equipment. In the case of freezing in liquid nitrogen or in carbon dioxide to achieve the temperature of −18 °C, depending on the thickness and wrapping of the product, 4 to 6 min, on freezing by cryogenic freezing technique hardly affects the growth rate of bacteria. At temperatures near the freezing point, the generation times of bacteria amount to several hours. At higher temperatures, however, in the case of cryogenic and cold air, blast freezing techniques the cooling rate is rather high and thus the growth of bacteria is not significant. Thus, neither liquid nitrogen, nor liquid carbon dioxide exert direct bactericidal effect. By electrical carcass stimulation cold shortening thoughness may be prevented in beef. Treatment had the following characteristics: voltage 3600 V, pulse frequency 15 Hz. As an effect of electro stimulation, the rate of glycolysis substantially increases. As a result of stimulation, rapid freezing, ensuring inhibition of loss of quality, is not necessary and slow chilling does not cause toughness in the meat. In stimulated meat the setting in of rigor mortis is deferred by a few hours, thus traditional boning methods may be successfully applied.

Using freon–12 immersion, liquid nitrogen and cold air blast freezing technologies to reduce the temperature of a fish filet of 110 mm thickness from 0 °C to −5 °C 39, 21 and 148 min, resp., are required. The microstructure of the fish does not suffer changes even at the highest freezing rate.

L. Pap

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# A GEL-CHROMATOGRAPHIC METHOD FOR THE FRACTIONATION OF SUNFLOWER AND RAPESEED LECITHIN AND THE GAS-CHROMATOGRAPHIC ANALYSIS OF FATTY ACIDS

P. Biacs, A. Erdélyi, É. Kurucz-Lusztig and J. Holló

(Received December 28, 1975; revision received June 30, 1977; accepted August 14, 1977)

Industrial samples of sunflower and rapeseed lecithin were deoiled, chromathographed on *Sephadex* LH-20 gel (Pharmacia) and eluted with a 2 : 1 ratio of chloroform–methanol solvent mixture. Fractionation was monitored by differential refraction. Our experiments showed that the fractionation of complex polar lipids can be satisfactorily performed on a lipophilic gel and that the order of elution may be attributed to the hydrophilic fraction bound to the phosphate group. The fractionation of industrial lecithin samples afforded components in the following order: phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine and phospatidyl inosite, furthermore lysophosphatides, sterol-glycosides and cerebrosides, in addition to other, mainly protein-like components. Best results were obtained on a 100-cm column (diameter 1 cm) where 12–14 components could be detected by differential refraction.

The fatty acid composition of the different phospholipid components, clearly fractionated by gel chromatography, was determined by a gas chromatographic method. The fatty acids of phosphatidyl choline do not differ much from those of the original sunflower or rapeseed oil, while there is a marked difference in the fatty acid composition of phosphatidyl ethanolamine and phosphatidyl serine, which was found to be much richer in $C_{20}$ polyene fatty acids.

Polar lipids (phospholipids, glycolipids) are present in plants in relatively small amounts, 0.5–2.5% in ripe plant seeds: the phosphatide content of sunflower seeds is about 1.8%.

The primary aim of the industrial processing of oil seeds is the production and raffination of neutral lipids (triglycerides) for nutrition purposes. The oil obtained by pressing and extraction contains polar lipids as contamination, *e.g.*, raw soybean oil contains, on an average, 3% phospholipids in addition to 94% glycerides. The polar lipid content of vegetable oils depends on the species and ripeness of the seeds, and the amount of lipids present in raw vegetable oil increases with the efficiency of the procedure.

The various methods applied in the vegetable oil industry all agree in the following two steps: polar lipids are hydrated with a given amount of water, and the swollen slimes are separated from the oil phase. After being dried, the product is commercialized under the name: *lecithin*.

In estimating the phospholipid content for the quality determination of lecithin, a standardized method of phosphorus determination is applied in the industry. A similar method is used for measuring the phospholipid content of refined and further raffinated edible oils, by which the efficiency

of the procedure can be determined. For selecting the appropriate technology, knowledge of the distribution and ratio of polar lipids, and within this, of the specific phospholipids is also important. Since removability of the components is dependent on the balance of hydrophilic/lipophilic ratios within the individual components, in addition to quantitative determinations, knowledge of the quality distribution is also required.

Various chromatographic methods have been used for the fractionation of complex polar lipid components (KAUFMANN & WESSELS, 1964; MARINETTI, 1967). Comparison of the various literary data shows that the most feasible method is probably the chromatography of a purified polar lipid sample on a silica gel column with a series of eluting solvents with growing polarity, followed by the separation of phospholipids eluted by methanol. This method was used by MCKILLICAN and SIMS (1963) for the fractionation of linseed and safflower phosphatides with hexane–diethyl ether and for the separation of soybean phosphatides by SINGH and PRIVETT (1970). APPELQVIST (1972) obtained glycolipids and phospholipid fractions from industrial rapeseed lecithin by the use of chloroform–acetone.

Phospholipid components were fractionated from safflower seeds on thin-layer by BURKHARDT (1971) and commercial soybean lecithin was produced by EDER (1972) by analitical and preparative thin-layer chromatographic methods. In addition to phosphorus content determination of the fractions scraped off the layer, attempts have been made recently at the determination of the fatty acid composition and distribution as well. Such separations and measurements were reported by KUNDU *et al.* (1974), in their analysis of the fatty acids of mono- and digalactolipids of bean, green pea and soybean seeds.

Lipophilic dextrane gels have been extensively used for the separation of simple lipids (BOROSS & KREMMER, 1974). Available references in this literature, however, have rather scanty data on the analysis of polar lipids by gel-chromatographic methods. The phospholipids of cowmilk were chromatographed by DOWNEY and MURPHY (1970), who applied a solvent gradient, with however, not too satisfactory results, probably due to unsuitable column size. HELMSING (1967) studied gel-chromatographic fractionation of the polar lipids of spinach, although with the aim of separating galactosyl glycerides from phospholipids. Our gel-chromatographic investigations were, thus, carried out with practically no preliminaries in the literature.

## 1. Materials and methods

In our experiments we used industrial samples of sunflower and rapeseed lecithin supplied by different factories of the HUNGARIAN VEGETABLE OIL AND DETERGENT INDUSTRY. The samples were obtained by degumming of

both pressed and extracted oils. The phospholipid content of the oils was 0.5–0.6 % for sunflower and, on an average, 0.7 % for rapeseed, which decreased to 0.3 % after hydration in both cases. Thus, the majority of complex lipids with phosphorus content and other contaminations were found to be present in industrial lecithin.

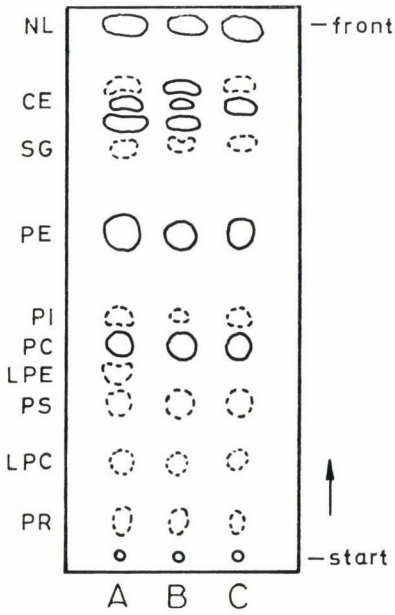


Fig. 1. Thin-layer chromatogram of industrial lecithin samples. Developing reagent: ammonium molybdate–perchloric acid spray, elution: $CHCl_3$—MetOH—$H_2O$ 25 : 25 : 4 v/v. *A* sunflower lecithin (Rákospalota Factory); *B* rapeseed lecithin (Rákospalota Factory); *C* sunflower lecithin (Győr Factory). Symbols of polar lipid fractions: CE — cerebrosides; SG — sterol glycosides; PE — phosphatidyl ethanolamine; PI — phosphatidyl inosite; PC — phosphatidyl choline; LPE — lysophosphatidyl ethanolamine; PS — phosphatidyl serine; LPC — lysophosphatidyl choline; PR — protein-like contamination (at low $R_f$ values); NL — neutral lipid contamination (in the front line)

### *1.1. Conditions of thin-layer chromatography*

*Kieselgel* G was suspended in a 0.01 mole solution of $Na_2CO_3$ to form a 0.5 mm layer, which was activated at 110 °C. Sunflower and rapeseed samples (250 and 500 microgram) were applied on the layer and compared with egg-lecithin (MERCK). The samples were eluted with a mixture of chloroform–methanol–water (65 : 25 : 4). The plates were dried and sprayed with specific reagents (Fig. 1).

The following specific reagents were used: 1. ammonium molybdate–perchloric acid (for phosphatides); 2. ninhydrine (for amino-phosphatides); 3. *Draggendorf*-reagent (for choline phosphatides); 4. orcin-phosphoric acid

(for the component with carbohydrate content) and 5. a chloroform solution of antimony trichlorid (for sterols).

### *1.2. Analysis by gel-chromatography*

Experiments were carried out with a commercial *Sephadex* LH-20 gel (PHARMACIA). In order to ensure better experimental conditions, instead of applying a solvent gradient, we first attempted modification of the column size. In this way, we determined the elution conditions and chromatographic parameters for 3 different columns with lengths: 38, 60 and 112 cm and decreasing inner diameter values (Table 1).

Table 1

*Data obtained on column filled with* Sephadex *LH-20 for gel-chromatographic separation of polar lipids*

| No. | Column height | Inner diameter |
|---|---|---|
| 1 | 38.0 cm | 32 mm |
| 2 | 60.0 cm | 20 mm |
| 3 | 112.0 cm | 10 mm |

*Flow rate conditions*

| Inner diameter mm | Flow rate ml $min^{-1}$ | Linear flow rate ml $cm^{-2} \cdot h^{-1}$ |
|---|---|---|
| 32 | 1.0 | 7.4 |
| 32 | 0.5 | 3.7 |
| 20 | 1.0 | 19.1 |
| 10 | 0.5 | 38.2 |
| 10 | 0.5 | 38.2 |

The columns were filled with a gel previously allowed to swell in the eluent for 24 h (chloroform–methanol 2 : 1 v/v). The flow rate was changed from 0.5 to 1.0 $min^{-1}$, depending on the column applied and the sample examined. Industrial lecithin samples (100 mg) deoiled by the method of PADRUN (1962) were applied to the column and the parameters of separation wer calculated and compared (Table 2).

Five-ml fractions were collected and the refractive indices were recorded by a *Zeiss* refractometer. The fractions were combined according to the peaks indicated by the refractive indices and the components were identified by thin-layer chromatography. This method of separation yielded larger amounts of components, which were determined also by IR spectroscopy

Table 2

*Chromatographic parameters of two different columns*

| | No. I. | No. II. |
|---|---|---|
| h (column diameter) mm | 20 | 10 |
| L (column height) mm | 600 | 1120 |
| $v_t$ (total volume) ml | 185 | 88 |
| $v_o$ (dead volume) ml | 6 | 4 |
| $v_i$ (inner volume) ml | 98.7 | 46.3 |
| $v_m$ (matrix volume) ml | 81.3 | 37.7 |
| $v_e$ (elution volume) ml | 200 | 185 |
| N (number of plates) | 1440 | 2340 |
| EHTP (mm) | 0.41 | 0.48 |
| flow rate, ml $min^{-1}$ | 0.5 | 0.5 |
| cm $h^{-1}$ | 9.55 | 38.2 |
| w (peak width) ml | 20 | 15 |

formulae:

$$v_i = \frac{s_r \cdot d}{\varrho\left(\frac{s_r}{\varrho - 1}\right)} (v_t - v_0) \tag{1}$$

$$v_m = v_t - (v_0 - v_i) \tag{2}$$

$$N = \left[\frac{4\,(v_e - v_0)}{w}\right]^2$$

$$\text{EHTP} = \frac{L}{N}$$

$s_r$(calculated) = 1.8150
$d$ (measured) = 1.2723
$\varrho$ (calculated) = 1.2685

on the basis of data given by WILLIAMS and CHAPMAN (1970) and KATES (1972).

### *1.3. Gas-chromatographic investigations*

The original oils and corresponding lecithin samples as well as the phospholipid components separated from the latter by gel-chromatography were hydrolyzed and converted into methyl esters, then measured under isothermic conditions at 180 °C on a *Chrom*–31 gas chromatograph. The column (2.4 m glass; inner diameter 4.4 mm, with a *Chromosorb* W DMCS support) filled with 10% DEGS (diethylene glycol succinate) stationary phase was mainly suitable for the measurement of saturated and unsaturated fatty acids with 16–20 carbon atoms.

## 2. Results

Industrial samples of sunflower and rapeseed lecithin contained 40–45% oil in addition to polar lipids. Thin-layer chromatographic investigations showed the presence of the following components: phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidyl serine (PS) and phosphatidyl inosite (PI) as well as cerebrosides (CE) and a considerable amount of sterol glycosides (SG). The presence of lysophosphatidyl choline (LPC) and lysophosphatidyl ethanolamine (LPE) probably shows the ripeness of the processed seed but may also be due to hydrolysis during storage. After the lecithin sample was deoiled, the residual neutral lipids — and above the starting line — different protein-like contaminations could be observed by means of various developing reagents. Due to latter contaminations, reproduction of the thin-layer chromatograms, especially quantitative determinations proved to be difficult.

### *2.1. Gel-chromatographic measurements*

Separation with shorter columns gave poor results, therefore these will not be dealt with here. Compared to the 60 cm column, the thinner 112 cm long column gave almost twice as many fractions with much more intensive peaks. Measurements based on the change of the refractive index, were, however, in all cases much affected by the high content of plant pigments (chlorophyll, carotenoids), which caused in the first 10 fractions deviation in the opposite direction and thus, interfered with detection of the elution of lysophosphatides. The sequence of elution for lysophosphatides (according to phosphate substitution) was as follows: LPE, LPC, PC, PE, PS and PI (Fig. 2).

The elution of glycolipids and protein-like substances took place only subsequently, and thus, did not interfere with gel-chromatographic separation.

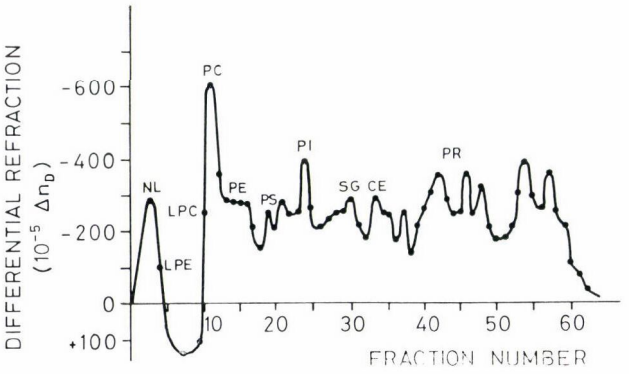


Fig. 2. Gel chromatogram of deoiled rapeseed lecithin detected by differential refraction measurement. Conditions: *Sephadex* LH-20 column 1120×10 mm (details in Table 2, column No. II). Abbreviations as in Fig. 1

A combined scheme of thin-layer chromatograms of fractions, with gel-chromatographic fraction numbers depicted on the abscissa is presented (Fig. 3).

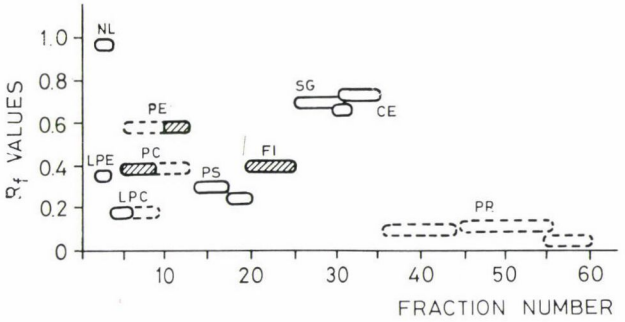


Fig. 3. Combined plot of thin-layer and gel chromatogram [vertical line: $R_f$ values of TLC, horizontal line: fraction numbers in column chromatography (CC)] Conditions: TLC as in Fig. 1, CC as in Fig. 2. Abbreviations as in Fig. 1

Gel-chromatographic fractionation of industrial rapeseed lecithin afforded 14 components. By separation on *Sephadex* LH-20 gel, our primary aim was to analyse mainly phospho- and glycolipids, therefore protein-like substances obtained separately in the final elution fractions have not been studied. Separation on *Sephadex* LH-20 gave better results if these non-lipid substances were previously removed by separation on *Sephadex* G-25. Similar results were obtained by gel-chromatographic investigations of an industrial sample of sunflower lecithin. In this case, separation afforded 12 fractions, although the refractive index pointed to a great number of peaks (Fig. 4).

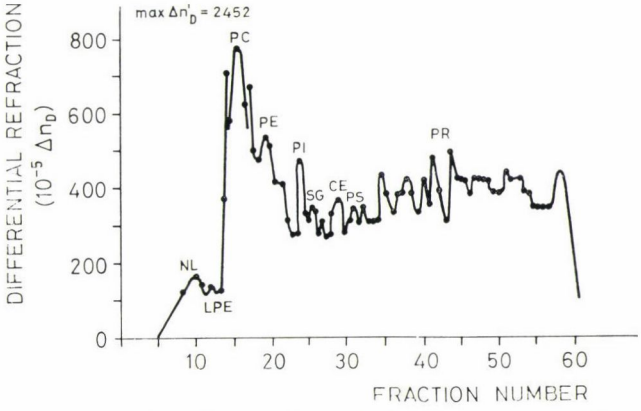


Fig. 4. Gel chromatogram of deoiled sunflower lecithin detected by differential refraction measurement. Conditions: *Sephadex* LH-20 column 1120×10 mm (details in Table 2, column No. II). Abbreviations as in Fig. 1

With identical weights of sunflower samples the differential refractive index showed a value nearly four times as high as that obtained for rapeseed samples, although the fraction weight ratios gave much lower differential values. Probably the weighted fractions were contaminated with gel particles. The higher content in phosphatidyl choline is responsible for the greater number of hydrolysis products (lysophosphatidyl choline) observed in sunflower lecithin analysis. The lysophosphatidyl ethanolamine determined in rapeseed lecithin was, however, not detectable in the sunflower product (Fig. 5).

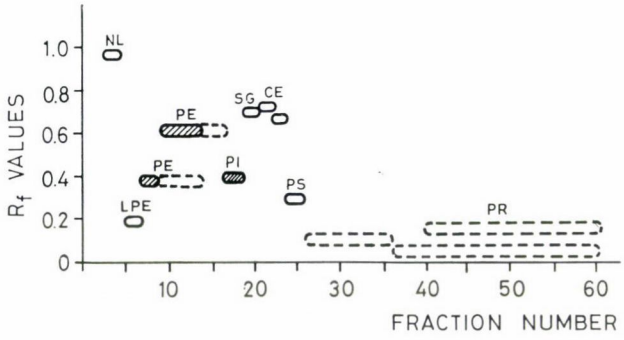


Fig. 5. Combined plot of thin-layer and gel chromatogram [vertical line: $R_f$ values of TLC horizontal line: fraction numbers in column chromatography (CC)]. Conditions: TLC as in Fig. 1, CC as in Fig. 2. Abbreviations as in Fig. 1

The only change in the elution order of components in sunflower lecithin could be observed in phosphatidyl serine, which eluted after the cerebrosides. Both gel and thin-layer chromatographic measurements showed a higher number of protein-like contaminations in sunflower lecithin. At the same time, the marked decrease in pigments has not affected the detection of the first fractions.

### *2.2. Gas-chromatographic measurements of fatty acid composition*

The main component of the fatty acids of the phosphatidyl choline fraction separated from sunflower lecithin was found to be $C_{18:2}$ linoleic acid, which is also characteristic of neutral oil triglycerides. The ratio of saturated $C_{16}$ and $C_{18}$ fatty acids was higher than in triglycerides and the presence of more unsaturated $C_{20}$ components could also be detected (Fig. 6).

Related to sunflower oil triglycerides, a much greater deviation could be observed between the fatty acids of phosphatidyl ethanolamine and those of the choline phosphatide fraction. The ratio of oleic and linoleic acid was

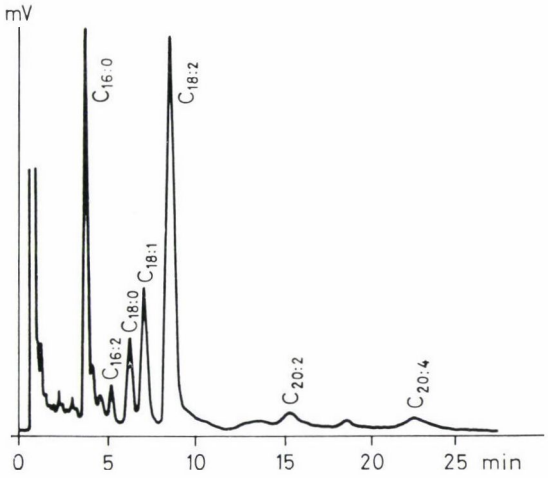


Fig. 6a: Gas chromatogram of fatty acids of phosphatidyl choline fraction fractionated from sunflower lecithin. Stationary phase: 10% DEGS, column temperature: 190 °C. Abbreviations: fatty acid methyl esters are designated as $C_{16:0}$ palmitic acid, $C_{18:1}$ as oleic acid, etc.

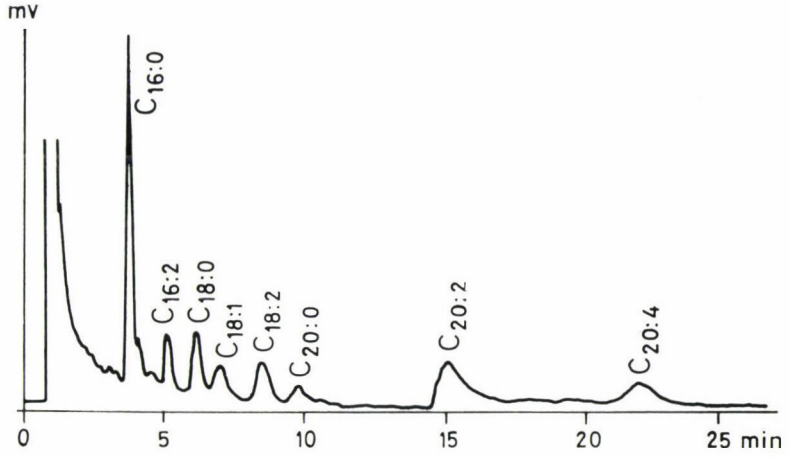


Fig. 6b. Gas chromatogram of fatty acids of phosphatidyl ethanolamine fraction fractionated from sunflower lecithin. Stationary phase: 10% DEGS, column temperature: 190 °C. Abbreviations: as in Fig. 6a

found to be low, while the ratio of saturated and unsaturated fatty acids with carbon atom number $C_{20}$ has markedly increased. Similar results were obtained in the study of fatty acids in phosphatidyl serine and phosphatidyl inosite (Fig. 7).

Compared to the fatty acid composition of rapeseed oil triglycerides, unsaturated $C_{16}$ and $C_{20}$ fatty acids were present in the phospholipid com-

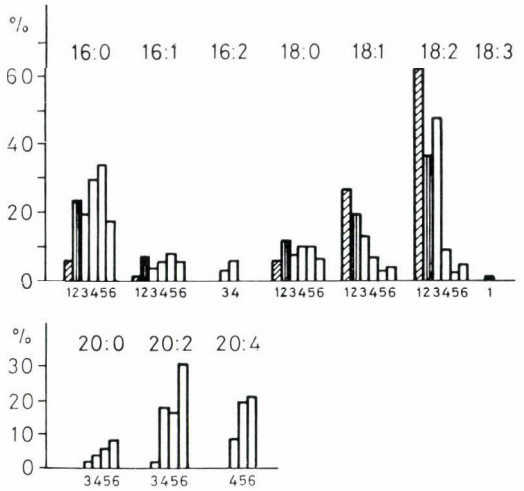


Fig. 7. Fatty acid composition of sunflower oil (1), industrial sunflower lecithin (2) and phospholipid fractions separated by gel chromatography: PC (3), PE (4), PS (5) and PI (6). Abbreviations as in Fig. 1. Fatty acid methyl esters: as in Fig. 6a

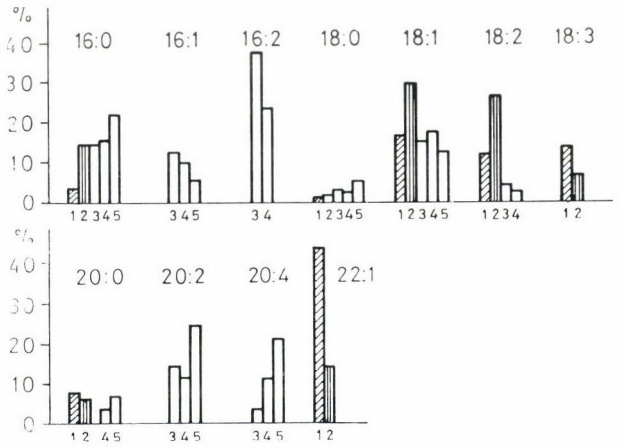


Fig. 8. Fatty acid composition of rapeseed oil (1), rapeseed oil lecithin (2) and phospholipid fractions separated by gel chromatography: PC (3), PE (4) and PS (5). Abbreviations as in Fig. 1. Fatty acid methyl esters: as in Fig. 6a

ponents in measurable amount, while $C_{22:1}$ erucic acid and simultaneously eluting $C_{18:3}$–$C_{20:1}$, characteristic of rapeseed oil could not be detected. In the case of phospholipid groups fractionated from rapeseed lecithin, a gradual decrease could be observed in the ratio of $C_{16:1}$ and $C_{16:2}$ and an increase in the $C_{20:2}$ and $C_{20:4}$ fatty acid ratios has been determined in the sequence: choline, ethanolamine and serine (Fig. 8).

A similar change could be observed for sunflower phospholipids, where the ratio of $C_{20}$ unsaturated fatty acids increased and that of $C_{18:1}$ and $C_{18:2}$ fatty acids were found to decrease. The fatty acid composition of inosite phosphatide has also been measured in sunflower lecithin while it could not be isolated and analysed in rapeseed samples.

## 3. Conclusions

We studied the polar lipids, mainly phospholipids of two samples of industrial lecithin, partly by adapted methods and partly by methods developed by us. No significant difference could be detected on the basis of phosphorus determination. With our qualitative analysis, however, considerable difference could be established. The phosphatidyl ethanolamine content of rapesced lecithin was found to be twice as high as that of sunflower lecithin, while the latter showed higher content of phosphatidyl inosite. The main component in both samples was phosphatidyl choline, which was somewhat higher in the sunflower product, while the phosphatidyl serine content was only about 5% for both samples.

The advantage of the gel-chromatographic method is that in addition to phospholipids, other polar lipids can also be fractionated in one step. Thus, the sterol glycoside content was nearly higher by 50% in rapeseed than in sunflower lecithin, while in the latter sample the amount of cerebrosides was twice as high and the ratio of protein-like substances was 50% higher.

According to our assumption, fractionation of the components took place rather in chromatographic than in molecular weight separation. Thus, in the case of phospholipid components the sequence of elution is probably affected by hydrophilic properties of substitution to the phosphate group (choline, ethanolamine, serine or inosite). The lipophilic character of the phospholipid may be ascribed to the fatty acids. Considerable difference could be determined in the fatty acid composition of phospholipid groups, depending on the phosphate substitution. For both lecithin samples it could be established that, irrespective of the basic material (sunflower or rapeseed lecithin), the fatty acids of choline phosphatide show greatest similarity to the fatty acid composition of triglycerides, while in others also more unsaturated $C_{16}$ and $C_{20}$ fatty acids could be detected.

The presence of $C_{20}$ polyunsaturated fatty acids in the phosphatide fractions of animal tissues has been determined by several authors. Parker and Peterson (1965) found the ratio of $C_{20:4}$ fatty acid to be 6% in rat liver and Eder (1972) showed it to be 9% in bile phosphatides. Only few reports have been published, however, on similar investigations with plant lipids.

VIJAYALAKSHMI and VENKOB RAO (1972) studied only the distribution of $C_{16}$ and $C_{18}$ fatty acids of the phosphatidyl choline fraction of four different oilseed species (cotton, peanut, sesame and mustard). The fatty acid composition of rapeseed lecithin was studied by several authors and there are also available data on $C_{20}$ fatty acids. To the best of our knowledge, however, no such reports have been published so far on the fatty acid composition of the phospholipid components of sunflower lecithin.

The difference in the fatty acid composition of the phospholipid components of sunflower and rapeseed lecithin may probably be due to the genetic properties of the two species. The lack of $C_{22:1}$ in the fatty acids of phospholipids of rapeseed justifies the study of species containing rapeseed oil with low erucic acid content. According to our present knowledge, a relative decrease in phospho- and glycolipids and a considerable increase in triglycerides can be observed during the ripening period of oilseeds, especially in the case of soybeans. Based on the results of biochemical investigations, the formation of triglycerides may be traced back to phospholipids, which points to some contradiction in this field. Similar data, though only partly in agreement with the above, are given by MCKILLICAN and LAROSE (1974), who studied the fatty acid composition of polar and apolar fractions of free lipids (extractable with hexane) and bound lipids (extracted with chloroform-methanol) in their experiments with a Canadian rapeseed species (*Brassica campestris* var. *yellow sarson*). Only 5.7% erucic acid ($C_{22:1}$) was detected in bound polar lipids, while 31–41% was determined for other fractions. Our gel-chromatographic investigations afforded clearer fractions since even the individual phospholipid components could be separated.

The purpose of our further examinations was to compare industrial lecithin samples obtained by the different methods of degumming. Given the quality composition, obtained by gel-chromatographic fractionation, the efficiency of the technology can be determined and compared with results obtained by different methods of hydration.

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Thanks are due to the factories of THE VEGETABLE OIL AND DETERGENT INDRUSTRY in Rákospalota and Győr, Hungary, for providing sunflower and rapeseed lecithin samples and to Mr. J. GALÁCZ (Technical University of Budapest) for his assistance in preparing gas chromatograms.

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# ASSAY INTO THE VOLATILE AMINES IN TOMATOES AND TOMATO PRODUCTS

## PART I.–GAS-CHROMATOGRAPHIC ANALYSIS

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In order to study the changes occurring in the volatile components of tomato flavour during processing, the volatile amines were extracted by steam distillation and collected in dilute hydrochloric acid solution subsequent to alkalinization of the sample. After removal of ammonium chloride from the salt mixture residue of evaporation, the amines were liberated in sealed glass capillaries and re-dissolved in *n*-hexadecane.

The amines were separated by gas chromatography on two different column packs (alkaline *Carbowax* 20 M and *Chromosorb* 103) applying appropriate temperature programs.

To identify the separated components the *enrichment* technique was used.

As a result of the study, raw tomatoes and tomato products were found to contain a great number of amines. The total amount of these compounds is highest in raw tomatoes and decreases during processing.

Of the separated (13–16) components 9 amines could be identified in the three samples: raw tomatoes, tomato purée and powdered tomato. These were: methyl, dimethyl, ethyl, diethyl, propyl, *i*-butyl, butyl, *i*-pentyl and pentyl amine. Experiments are in progress on the amine components that could not be identified hitherto.

During storage and processing the taste and odour substances of the raw materials of the food industry undergo substantial changes. A part of these affects the quality of the product advantageously, the rest disadvantageously. Quality requirements, substantially increasing in recent years, have necessitated a more thorough investigation of factors influencing quality and, within the latter, flavour substances.

The characteristic taste and odour of individual foodstuffs are caused by a great number but small quantity of compounds belonging by their chemical structure to a great variety of types of compounds. Thus their investigation is not a simple task. This is shown also by the fact that though they have been studied for over 100 years, advance has been achieved only since the spreading of gas-chromatographic techniques in the 'sixties.

Compounds of N content play an important part in the taste and odour of some foodstuffs. Of these the non-volatile amino acids, peptides and proteins are important as primary flavour carriers and as precursors of volatile flavour substances (SALUNKHE *et al.*, 1974; BAUMANN & GIERSCHNER, 1974; TRESSL & RENNER, 1976). On the other hand the volatile aliphatic and aro-

matic amines, pyrrole and pyrazine derivatives are components of a number of flavour substances (ASKAR, 1973; MAGA & SIZER, 1973).

This paper wishes to give an account of the results of the study of volatile amines present in ripe raw tomatoes, tomato purée and tomato powder.

A study of the related literature has shown that during the years between 1934 and 1976 at least 33 papers were published on the detection and identification of the volatile flavour substances of tomatoes. The number of compounds described amounts to 285, belonging, by chemical structure, to very different groups of compounds (PETRÓ-TURZA *et al.*, 1977).

Several N-containing flavour compounds were found among the volatile flavour substances of tomatoes, as for instance methional, pyridine, 2-methyl-pyrazine and 2,6-dimethyl-pyrazine by RYDER (1966) and 3-methyl-1-nitro-butane, 3-methyl-3-hydroxy-1-nitrobutane, *i*-valeraldehydoxime and benzyl-cyanide by WOBBEN and co-workers (1974). However, data on aliphatic and aromatic amines were not found in the literature.

The investigation of these compounds may be of interest from several aspects apart from their effect on flavour. They participate in the non-enzymatic browning of fruits and vegetables in the *Maillard* reaction occurring between N-containing compounds and reducing sugars as an effect of heat (ASKAR, 1973). Further, secondary and tertiary amines in biological systems may act as precursors of various nitrosamines in the presence of nitrites. Nitrosamines are considered potential cancerogenic substances occurring in foods and tobacco (RIEDMANN, 1974).

The most probable way of the formation of primary amines is the decarboxylation of amino acids present in foodstuffs, as an effect of enzymes or heat in acid media (ASKAR *et al.*, 1972). Secondary and tertiary amines are the results mainly of alkylation (GOLOVNYA *et al.*, 1969). UDENFRIEND and co-workers (1959) observed the formation of volatile amine compounds in fruits and vegetables in the course of metabolism and an increasing tendency in their quantity during ripening.

In most of the foodstuffs, because of the acidic character of the medium, amines are bound, therefore to liberate them after alkalinization steam distillation is mostly applied (STEIN VON KAMIENSKI, 1957). The amines separated from biological materials by this technique form a relatively uniform fraction and consist, with only a few exceptions, of homologous aliphatic amines (ILERT & HARTMANN, 1972).

In the course of steam distillation the volatile amines are captured in dilute hydrochloric acid thereby being converted into stable amine hydrochloride salts. Chromatography is most suitable to separate and identify the amines. They may be determined directly as salts by thin-layer chromatography (SMITH *et al.*, 1967; KRYLOVA *et al.*, 1971). However several authors

consider the formation of derivatives more convenient (ILERT & HARTMANN, 1972; WIROTAMA & NEY, 1971; JART & BIGLER, 1967).

To study amines in the form of different derivatives gas chromatography was successsfully applied by SEK iand WADA (1974), ISHITOYA and co-workers (1973) and GEJVALL (1974). This method may be used in the direct analysis of volatile bases as shown by COLE and co-workers (1961), ARAD and co-workers (1964) as well as GOLOVNYA and co-workers (1967, 1968, 1969).

In this assay the volatile amines of tomato were studied by gas chromatography.

In the gas-chromatographic analysis of the amine derivatives a problem arose from the fact that all the authors cited developed their methods for the study of model mixtures, where, with known sample quantities, the required amounts of the reagents could be administered. However, the unknown quantity of amine salt mixtures obtained from the samples to be tested could not be quantitatively converted into derivatives because of the impurities present. Therefore direct gas-chromatographic separation and identification of the amines was attempted.

In the case of this method, however, difficulties arise in separation by gas chromatography as shown by the following authors: JAMES and co-workers (1952), BURKS and co-workers (1959), O'DONNELL and MANN (1964) and SZE and co-workers (1963).

Amines are polar compounds therefore they are liable to be absorbed on the support of large active surfaces. As a consequence the peaks become highly asymmetric, furthermore individual amines are eluted with varying retention times. The asymmetry of peaks may cause substantial overlap and thereby render the separation of compounds, eluted one by one, difficult.

These difficulties may be eliminated by the use of specific column packs. The available possibilities are:

– the use of supports of inert surface (LANDAULT & GUIOCHON, 1962);

– the application of a basic organic liquid phase (SZE *et al.*, 1963);

– the treatment of the liquid phase by alkali hydroxide (DECORA & DINNEEN, 1959; SMITH & RADFORD, 1961).

The effect of alkali hydroxide is twofold. It neutralizes the *acidic sites* of the support on the one hand, and affects the migration velocity of the amines on the other, thereby it takes an active part in their separation. The latter effect may be ascribed to the hydrogen bond formation between the the hydrogen atom of the alkali, on the one hand, and the nitrogen of the primary and secondary amines, on the other.

## 1. Materials and methods

### *1.1. Origin and chief characteristics of the tomato samples tested*

Reliable studies on the changes occurring in the volatile amines as an effect of heat treatment are possible only if the raw and heat-treated samples are taken from the same, homogeneous tomato batch. To fulfil this condition samples were purchased in the KECSKEMÉT CANNING FACTORY. The tomato used was a mixed batch of the *Kecskemét* varieties. This was concentrated during 100–120 minutes into a purée of 30% refractive index in a multi-stage continuous *Láng* type system of 15 wagons capacity under a vacuum of 400 mm Hg at 80 °C. The purée was powdered in a *Niro* spray drying apparatus into a tomato powder of 97.7% refractive index at a rate of 800 kg $h^{-1}$, 140 °C inlet temperature and 80 °C outlet temperature.

It should be noted, however that when raw tomato samples of different origin and tomato products were examined significant differences were not found in the qualitative and quantitative composition of the amines.

### *1.2. Extraction of volatile amines from raw tomatoes and tomato products*

The volatile amines were extracted from the samples, after alkalinization, by steam distillation.

From the comminuted and homogenized raw tomatoes 6 000 g were weighed and solid $NaHCO_3$ was added to obtain pH 8.

From the tomato purée 1 260 g, from the tomato powder 392 g were weighed and diluted with distilled water to 6 000 ml, subsequently alkalinization was carried out as with the raw sample. Thus the samples prepared from the three types of material were of identical solids content.

To bind the volatile amines quantitatively 100 ml and 50 ml of 0.1 *N* hydrochloric acid, resp., were introduced into the receiving flask, the gas washing bottle was connected to it and these were cooled by ice-water. Six hundred ml of the distillate were collected and evaporated to dryness under vacuum at temperatures not exceeding 60 °C.

### *1.3. Purification of the amine hydrochloride salt mixture*

The salt mixture obtained by steam distillation of the volatile amines contained, in addition to the amine hydrochlorides, a fair amount of ammonium chloride. The sample was thus substantially diluted and further analysis was difficult. Therefore the salt mixture had to be purified prior to separation by gas chromatography. In order to achieve this the sample was placed on a G4 glass filter and washed through several times with small portions of

absolute alcohol. Amine hydrochlorides dissolve well, ammonium chloride hardly in this solvent. The salt mixture obtained by evaporating the alcoholic solution contained substantially less ammonium chloride and was, therefore, more suitable for further analysis.

### *1.4. Preparation of the sample for gas chromatography*

Because of the reasons discussed in the introduction, direct gas chromatography was chosen for the separation and identification of amines. Prior to the analysis it was necessary to liberate the amines from their salts. The slightly modified method of GOLOVNYA and co-workers (1967, 1968, 1969), was used.

Since amines are highly volatile their liberation from their salts had to be carried out immediately prior to gas chromatography. Six to eight mg of the salt mixture were taken and placed in a 100 mm long glass capillary of 2 mm inner diameter. The solid salt mixture was damped with about 10 $\mu$l distilled water and 50 $\mu$l *n*-hexadecane were layered over it. The content of the capillary was frozen in dry ice, then a piece of a potassium hydroxide tablet was placed over it. The capillary was then sealed with a jet flame and heated for 40 min in a boiling water bath and several times thoroughly shaken. Prior to gas chromatography the content of the capillary was again frozen. After opening the capillary the open end was provided with an elastic polyethylene tube sealed at the farther end. The sample was removed with a *Hamilton* injector syringe through the tube. 15-$\mu$l samples were taken from the organic phase.

To check the method applied, amines were liberated from their salts repeatedly in standard amine hydrochloride samples.

The amine hydrochlorides were prepared as follows: 0.1 ml of analytical grade free base was dissolved in ethanol. The solution was then acidified with *conc.* hydrochloric acid. The acid reaction was checked by pH paper. The solution was then evaporated under vacuum and the solid residue was used to liberate the amines as described above.

### *1.5. Conditions of gas chromatography*

The amines were separated simultaneously on two different column packs in a *Jeol* 1100 and a *Perkin-Elmer* 900 gas chromatograph, respectively.

In the *Jeol* 1100 apparatus a stainless steel column of 3 m length and 3 mm inner diameter was used. The column was packed with *Chromosorb* P support containing 20% *Carbowax* 20 M, treated previously with 2% potassium hydroxide.

Injector temperature ..... 230 °C
Detector temperature ..... 230 °C
Velocity of the nitrogen carrier gas ... 30 ml $min^{-1}$.

Separation was carried out under isothermic conditions at 90 °C for 9 min. The temperature was then raised at a rate of 30 °C $min^{-1}$ to 110 °C and kept at this temperature till all the components were eluted.

In the *Perkin-Elmer* 900 type apparatus a stainless steel column of 3.6 m length and 2 mm inner diameter was used. The column was packed with *Chromosorb* 103.

The temperature of the injector and detector was the same as in the other apparatus and the rate of the carrier gas, too.

The following temperature program was applied: starting temperature 150 °C, for 13 min, raised at a rate of 32 °C $min^{-1}$ to 180 °C final temperature.

The conponents were detected in both cases by FID detector. The separated components were identified by means of an added model amine mixture.

## 2. Results

### *2.1. Gas-chromatographic separation of model amine mixtures*

The optimum conditions of gas chromatography, as described in para· 1.5 were determined by means of a model mixture consisting of 9 amines.

Figure 1 shows the separation of the model mixture on alkaline *Carbowax* 20 M column pack.

As may be seen in the Figure, under the given conditions, methyl and dimethyl amine were eluted together, while the 7 other amines were successfully separated into ideal narrow peaks. The joint peak of methyl and dimethyl amine, however, could not be separated by the variation of the temperature program, either. Therefore it was attempted to separate them on a special new pack, on *Chromosorb* 103, recommended for the separation of amines. The separation of a model mixture, consisting of 9 amines is shown in Fig. 2.

By this method the separation of all 9 components was successful. The appearance of the components followed the same pattern as in the separation on *Carbowax* 20 M, only the sequence of propyl and diethyl amines was reversed.

Although the separation of the 9 amines was quite successful on *Chromosorb* 103, the shape of the peaks was not as ideally narrow as on alkaline *Carbowax* 20 M pack. Therefore gas-chromatographic analysis of the tomato samples was carried out on both column packs.

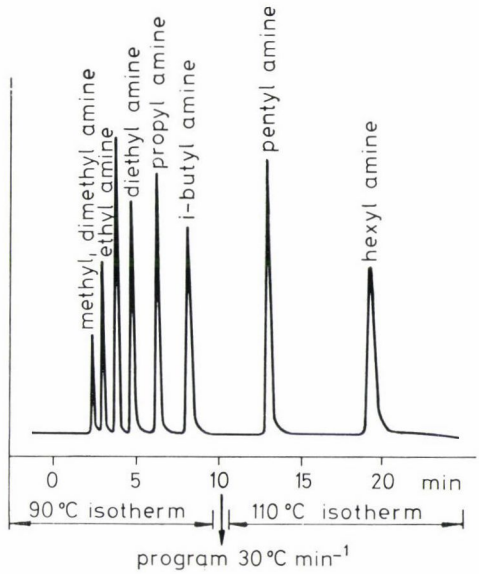


Fig. 1. Separation of a model amine mixture on alkaline *Carbowax* 20 M column pack. Injector temperature: 230 °C; detector temperature: 230 °C; rate of the carrier gas: 30 ml min$^{-1}$. The temperature program and the sequence of amine elution may be seen in the figure

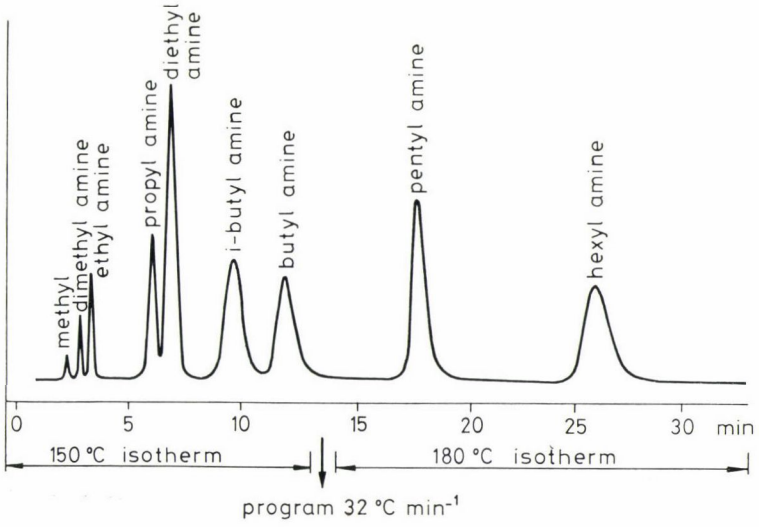


Fig. 2. Separation of a model amine mixture by gas chromatography on *Chromosorb* 103 column pack. For conditions of gas chromatography see Fig. 1

*2.2. Gas chromatography of the tomato samples*

Since the samples used for the extraction of amines were made to contain approximately identical solids content (para. 1.2), the chromatograms derived from them could be directly compared.

The chromatograms obtained under conditions identical to those observed in the model experiments, from raw tomatoes, tomato purée and powdered tomato purée on alkaline *Carbowax* 20 M pack are shown in Fig. 3.

The number of peaks separated from the three samples was almost identical. As regards total quantity of amines the highest amount was found in raw tomatoes, the lowest in tomato powder. Although their quantities differed, ethyl, diethyl, propyl, *i*-butyl, *i*-pentyl and pentyl amines were detected in all three samples. The joint peak of methyl and dimethyl amine appeared also in all the samples.

Of the compounds identified dimethyl amine was the one found in all samples in almost identical and large quantities.

The amount of ethyl amine in the tomato powder was higher than either in the raw tomatoes or in the purée. The same applies to propyl amine, of which only traces were detected in raw tomatoes and in the purée.

The differences in the *i*-butyl and *i*-pentyl amine contents of the three samples were high. Both compounds were found in largest quantity in raw tomatoes. Only traces were detected in the purée, but in the powder their quantity increased. The rest of the identified amines was found in nearly identical amounts in all the three samples.

Beside the identified amines a number of unknown amine compounds appeared in the three samples. These were marked in the chromatogram by numbers 4, 10 and 13, respectively. The quantity of compounds marked 10 and 13 was substantial and showed a significant change during heat treatment therefore the identification of these seems to be the next task.

In parallel to the study of the samples a blank test was also carried out in order to establish whether the reagents used for liberating the amines or the solvent did not contain substances giving peaks in the chromatograms thereby interfering with the analyses. In the chromatograms obtained with the blank some small peaks appeared at the beginning which are apparent also in the chromatograms obtained with the samples. Since these substances were separated unambiguously from the joint peak of methyl and dimethyl amine, eluted first, they did not interfere with the determinations.

Simultaneously with the analyses carried out on *Carbowax* 20 M the samples were separated on *Chromosorb* 103, too. Fig. 4 shows the chromatograms obtained on this column pack.

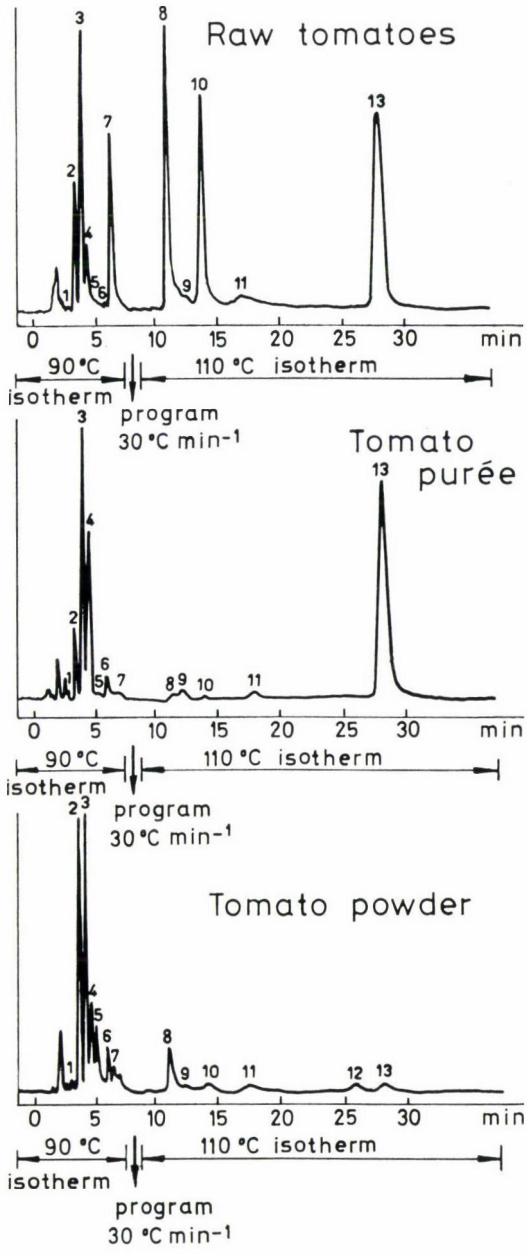


Fig. 3. Separation by gas chromatography of amines extracted from raw tomatoes, tomato puree and powdered tomato adjusted to the same solids content, on *Carbowax* 20 M column pack. For conditions see Fig. 1. The temperature program is seen in the figure. Peaks: 1: methyl or/and dimethyl amine; 2: ethyl amine; 3: diethyl amine; 4: unidentified; 5: propyl amine; 6: unidentified; 7: *i*-butyl amine; 8: *i*-pentyl amine; 9: pentyl amine; 10: unidentified; 11, 12 and 13: unidentified

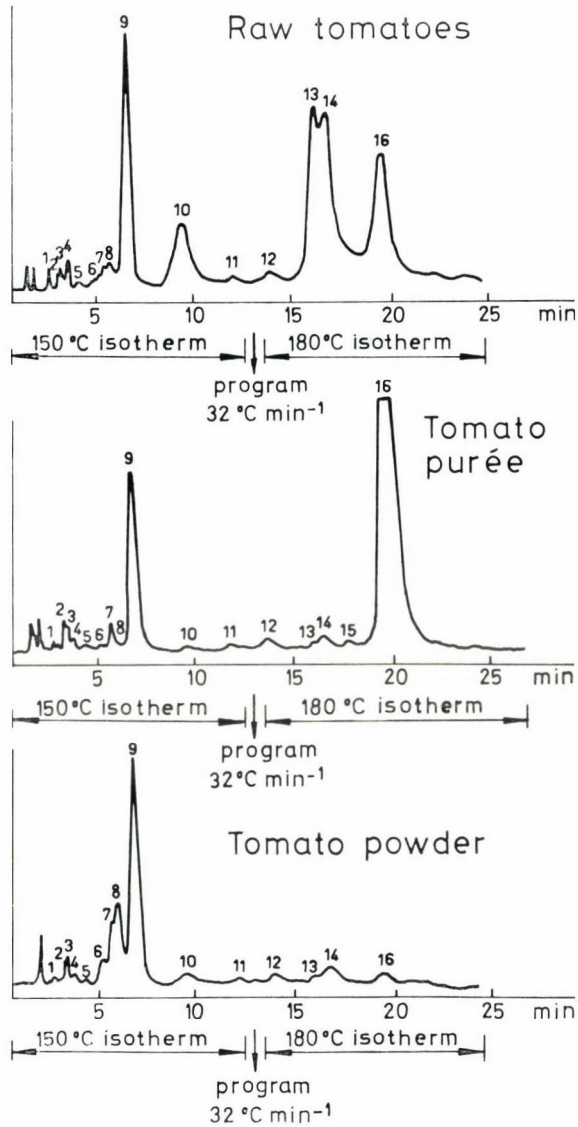


Fig. 4. Separation by gas chromatography of amines extracted from raw tomatoes, tomato purée and powdered tomato adjusted to the same solids content, on *Chromosorb* 103 column pack. For conditions of gas chromatography see Fig. 1. The temperature program is seen in the figure. Peaks: 1: methyl amine; 2: dimethyl amine; 3: unidentified; 4: ethyl amine; 5: unidentified; 6: unidentified; 7: unidentified; 8: propyl amine; 9: diethyl amine; 10: *i*-butyl amine; 11: butyl amine; 12: unidentified; 13: *i*-pentyl amine; 14: unidentified; 15: pentyl amine; 16: unidentified.

The chromatograms thus obtained resembled those obtained on *Carbowax* 20 M, however, methyl and dimethyl were detected in all three samples. Their quantity, similarly to that of ethyl amine was very low.

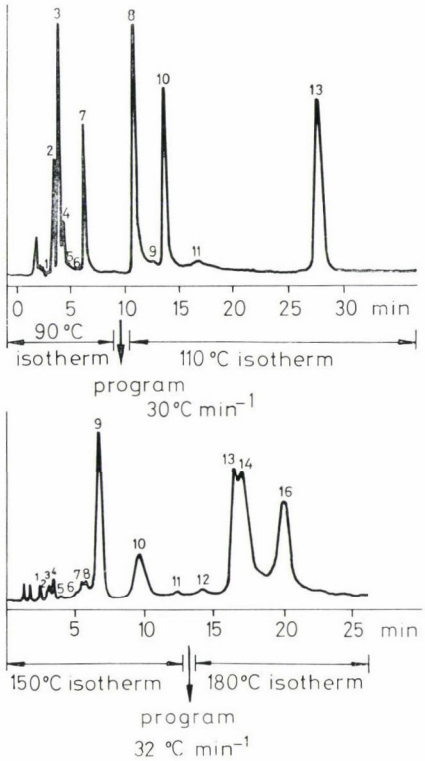


Fig. 5. Separation of amines extracted from raw tomatoes on *rbowax* 20 M (upper part) and on *Chromosorb* 103 column pack (lower part). For conditions of gas chromatography see Fig. 1. Temperature program is seen in the figure. Peaks are as in Fig. 3 and Fig. 4

Methyl, dimethyl and ethyl amine are in the gaseous state at room temperature and therefore extremely volatile. Thus, the method applied in this study is not suitable for their quantitative determination. They may probably be determined accurately in the form of some less volatile derivative. On this column pack butyl amine was also detected in all three samples.

For the sake of comparison the chromatograms of amine compounds as separated on the two different columns are shown in Fig. 5.

It can be seen in the figure that parallel analyses carried out on the two column packs are complementary. On *Chromosorb* 103 the separation of the highly volatile compounds is more successful. On this column methyl and dimethyl amine peaks appeared separately. Further, in the first part of the chromatogram the peaks of some unknown substances, present in minute quantities, showed up. This explains the fact that on *Chromosorb* 103 sixteen, while on *Carbowax* 20 M only 13 peaks could be detected.

The other compounds were better separated on *Carbowax* 20 M. Thus, *i*-pentyl amine and the compound eluted subsequently but not identified, appeared separately.

## 3. Conclusions

The study has shown the presence of a number of amine compounds in raw tomatoes as well as in tomato products. Of the components separated 9 amines were identified in all the samples. As shown by the results the quantity of amines was highest in raw tomatoes gradually diminishing in the course of processing.

The results seem to support the observation of some authors (UDENFRIEND *et al.*, 1959) and experimentally proven by others (GOLOVNYA *et al.*, 1969), namely that amines are formed not only as a result of heat treatment but in the course of biochemical processes as well. Thus they play an important part in the aroma of the raw produce.

The authors of the present study consider it their task, apart from the identification of compounds hitherto unknown, to establish the sensory properties of the separated components in order to be able to establish their effect on the aroma of tomato.

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# FACTORS INFLUENCING KINETIC CONSTANTS OF ENDO-PG FERMENTATION

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Kinetic constants of batch fermentation can be used for planning the continuous culture. Some of the constants determining the beginning and end of the various growth cycles are important factors in determining the right time for changing over to continuous cultivation. Cell and product concentration of the different growth cycles give information about the cell and product concentration of the continuous culture, while $\mu_{max.}$ of the batch determines the maximum value of the dilution rate in the continuous culture.

In the present work the kinetic constants of batch growth and endo-PG synthesis of an *Aspergillus awamori* strain were investigated as functions of concentration of carbon source, the type of inoculum and pH control.

While mycelial yield and growth rate constants ($k$) in the second part of diauxie increased with the increase of carbon source concentration, the highest endo-PG yield was obtained at 6% carbon source concentration, and highest values of the production rate constants ($k_{p_1}$, $k_{p_2}$) at 2%.

The use of vegetative culture as inoculum resulted in a longer first and a shorter second part of the diauxie than that of an *Aspergillus* culture inoculated with spores.

No enzyme synthesis was found in the first part of the diauxie of the culture inoculated with spores, but the use of a vegetative inoculum resulted in a non-growth-related product formation in this part.

In the course of our experiments diauxic growth could not be eliminated by pH control. The mycelial and endo-PG yield of the pH-controlled culture was about 30% higher than the one without pH control.

Kinetic analysis of batch fermentation can give useful information about the type of growth of the culture and of the reproducibility of the fermentation. The growth and production rate constants of batch fermentation, determined by kinetic analysis, can also be used in the planning of continuous culture.

The most important kinetic constants are influenced by many factors. From the most important factors influencing the growth and product yields of microorganisms, the concentration of the carbon source and the type of inoculum, were studied in submerged culture of *Aspergillus awamori*.

In the present study, it was also investigated whether diauxic growth can be eliminated by pH control.

## 1. Materials and methods

### *1.1. Microorganism*

An *Aspergillus awamori* strain was used in the experiments. Ten l of medium were inoculated with 1 000 ml of spore suspension (concentration of spores in the inoculum $10^6$ $ml^{-1}$), or 1 000 ml of a 24-h vegetative culture.

### *1.2. Media*

Extracts of dried sugar beet slices (cossettes; by-product of the sugar industry) were used as media. Pectin was extracted from sugar beet slices with tap water under pressure (30 psi) in an autoclave for 2 h. The extraction of various amounts of dried slices resulted in extracts of 2, 4, 6 and 8 % dry matter content determined with an *Abbe* type refractometer (ZEISS, Jena). The values of the dry matter content are referred to later as "concentration (refr. %) of carbon source" or "concentration of extract", the dry matter content originating from the salts added *(ca.* 0.85 %*)* not being taken into account in the terminology adopted in this article. Thousand ml of the above extract were complemented with $(NH_4)_2SO_4$ 7 g; $KH_2PO_4$ 1 g; $MgSO_4$ 0.6 g. The pH was adjusted to 4.5.

### *1.3. Cultivation*

The cultivation was carried out in a 12-l automatic fermentor (B. BRAUN, Melsungen, F.R.G.). Temperature (30 °C), pH, foam, agitation and aeration were controlled and recorded.

The working volume of the fermentor was 11 l; agitation speed: 700 rpm; the rate of aeration 0.5 l air $l^{-1}$ $min^{-1}$; oxygen transfer rate: 148 mmole $O_2$ $l^{-1}$ $h^{-1}$.

Samples were taken every four h by an automatic sample collector (ensuring a rapid cooling to 4 °C).

### *1.4. Preparation of the mycelia*

Mycelia were removed from the samples by filtration, and after thorough washing and drying to constant weight mycelial yield was determined.

### *1.5. Kinetic analysis*

The growth of the cultures was analysed kinetically according to the method of KONO (1968) and KONO and ASAI (1968, 1971). The kinetic analysis of product formation of the culture of four-phase growth was adapted first time for the analysis of the product formation of diauxie in our previous work (ZETELAKI-HORVÁTH, 1972; ZETELAKI-HORVÁTH & BÉKÁSSY-MOLNÁR, 1973).

The time course of a batch fermentation can be divided into four phases, the induction phase (I), the transient phase (II), the exponential growth phase (III) and the declining phase (IV). From the schematic representation of the rate of growth and the production rate as functions of cell concentration, the kinetic constants can be determined graphically. The slopes of the lines, defined by the data of growth rate, can be expressed as the growth rate constant *(k)*.

### *1.6. Determination of endo-PG activity*

The activity of endo-polygalacturonase (endo-PG) enzyme was determined in an *Ostwald* type viscosimeter in a substrate of sodium polypectate at 50 °C for 60 min (ZETELAKI-HORVÁTH & VAS, 1972).

### *1.7. Chromatography of sugars*

Sugars of the culture filtrate were determined by thin-layer chromatography using a celloluse layer (MN 300, MACHEREY—NAGEL, F.R.G.). Solvent: *n*-butanol : pyridine : water : benzene (5 : 3 : 3 : 1). Developing reagent: 1% *p*-amino-phenol. The quantities of samples applied were 20 and 10 $\mu$l in the case of culture filtrates and that of the 0.5% standard solutions, resp.

## 2. Results

### *2.1. The effect of concentration of the carbon source*

When *Asp. awamori* was cultivated in an extract of sugar beet slices at dry matter contents (DMC) of 2, 4, 6 and 8%, an increase in the mycelial yield was observed with the increase of the carbon source concentration (Fig. 1). The pH was controlled to 3.5.

Maxima of the mycelial yields measured at carbon source concentrations of 2, 4, 6 and 8% were 5.2, 16, 16.6 and 18.8, resp.

The endo-PG yield of the culture increased with the concentration of the carbon source from 2 to 6%. In extract concentrations of 2, 4, 6 and 8% maximum endo-PG yields of 44.2, 86.9, 104.4 and 41.5 l $h^{-1}$ $ml^{-1}$ were found in the 68, 80, 88-hour cultures, resp.

When plotting the data of rates of growth (dx/dt) against mycelial concentration, diauxic growth was obtained in the case of each extract concentration. The specific rates of growth ($k$) of the culture (equivalent to tangents of $\overline{OP_1}$ and $\overline{OP_2}$) at various carbon source concentrations, did not show the same type of change in the first and second part of the diauxic cycle, resp. (Fig. 2, Table 1).

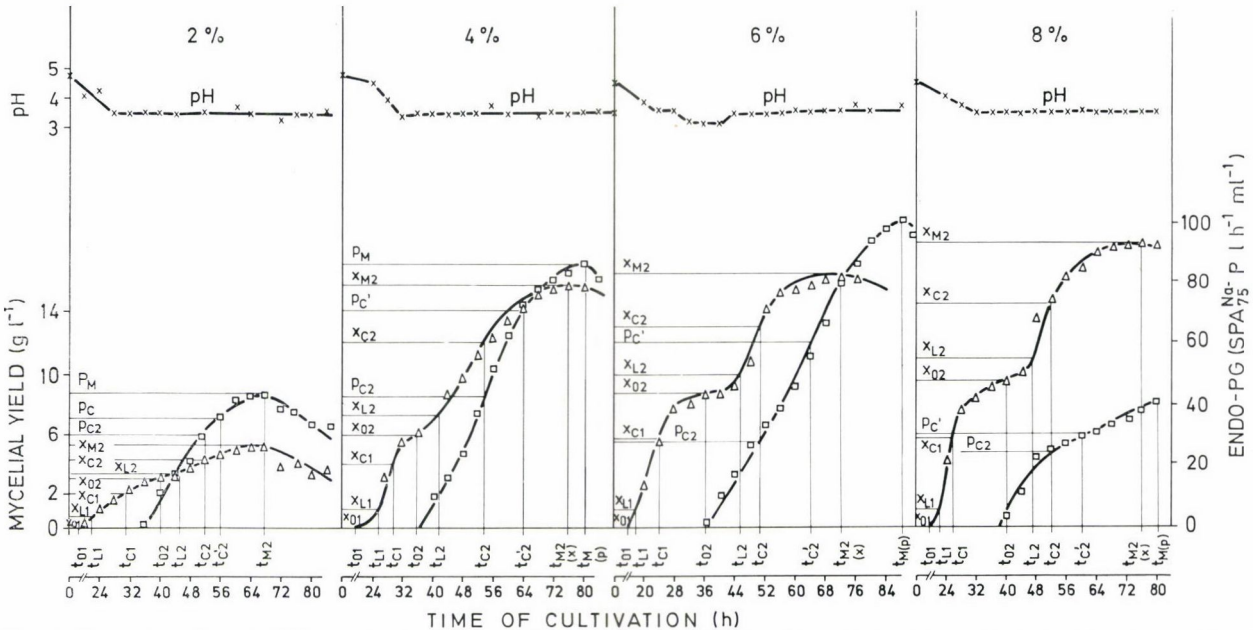


Fig. 1. Growth and endo-PG synthesis of *Aspergillus awamori* as a function of the time of cultivation in media of various dry matter content. (Cultivation conditions: see under paras. 1.2 and 1.3.)

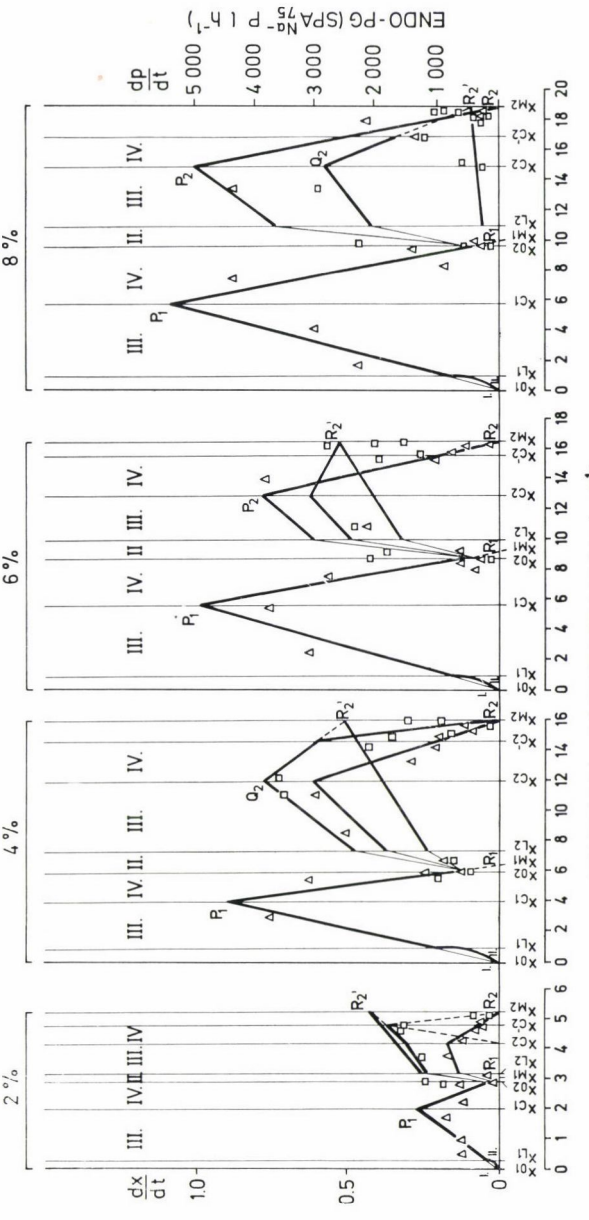


Fig. 2. Rate of growth and endo-PG formation of *Aspergillus awamori* as a function of the mycelial concentration in media of various dry matter contents. (Cultivation conditions: see under paras. 1.2 and 1.3.)

Table 1

*Kinetic constants of the growth and endo-PG formation of* Aspergillus awamori *when* (Cultures were incubated in 12–l

| Part of diauxie | Carbon source concentration (%) | $t_0$ | $t_L$ | $t_C$ | $t_{Max}$ | $x_0$ | $x_L$ | $x_C$ | $x_M$ |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 2 | 16 | 22.5 | 31 | — | 0.0 | 0.5 | 2.0 | 3.2 |
| 2 | | 40 | 45.5 | 52 | 68 | 2.9 | 3.4 | 4.3 | 5.2 |
| 1 | 4 | 20 | 25.5 | 30 | — | 0.0 | 1.0 | 4.0 | 6.4 |
| 2 | | 36 | 42.5 | 53.5 | 76 | 6.0 | 7.4 | 12.0 | 16.0 |
| 1 | 6 | 16 | 18.0 | 24 | — | 0.0 | 1.0 | 5.7 | 9.1 |
| 2 | | 36 | 45.5 | 50.5 | 72 | 8.8 | 10.0 | 13.0 | 16.6 |
| 1 | 8 | 20 | 22 | 25.5 | — | 0.0 | 1.0 | 5.7 | 10.0 |
| 2 | | 40 | 46.5 | 52 | 76 | 9.6 | 11.0 | 14.5 | 18.8 |

In the second part of the diauxie, the specific rates of growth ($k$) increased with the increase in the concentration of the carbon source. In the first part the highest value of $k$ was obtained in the 4% extract and it decreased later with increasing extract concentration. It can be seen in Fig. 2, that with the increase of the extract concentration, the second part of the diauxie started at an increasingly higher mycelium concentration.

Figure 2 also shows that endo-PG synthesis started only in the second part of the diauxie. According to the positive values of the growth-related and non-growth-related production rate constants, endo-PG formation proved to be both growth and non-growth-related. When the rates of endo-PG formation were plotted against mycelial concentration the growth-related and non-growth-related production rate constants ($k_{p_1}$ and $k_{p_2}$, as measured by the tangents of $\overline{OQ_2}$ and $\overline{OR'_2}$, resp.) were determined.

The value of the growth-related production rate constant ($k_{p_1}$) was the highest at a 2% substrate concentration, then decreased with increasing carbon source concentration. The non-growth-related production rate constant of the endo-PG formation had the highest value (409) at the lowest carbon source concentration. In the 4 and 6% extracts of sugar beet cossettes, its value was about the same, while the lowest value was measured in the 8% extract (Table 1).

### *2.2. The effect of the type of inoculum*

The type of inoculum influences not only the mycelial and the endo-PG yields of the culture, but also many further important kinetic factors: the

*cultivated in sugar beet extract media of different carbohydrate concentration* fermentors at 30 °C; inoculum: spores)

| $k$ | $k_{p_1}$ | $k_{p_2}$ | $p_C$ | $k'_p$ | $p'_C$ | $t'_C$ | $p_M$ | $t_{M/p}$ | $x'_C$ |
|---|---|---|---|---|---|---|---|---|---|
| 0.135 | — | — | 0 | — | — | — | — | — | — |
| 0.041 | 357 | 409 | 29.0 | — | — | — | 44.18 | 58 | — |
| 0.227 | — | — | — | — | — | — | — | — | — |
| 0.050 | 320 | 156 | 43.0 | 0.12 | 70.63 | 64 | 86.89 | 80 | 14.7 |
| 0.173 | — | — | — | — | — | — | — | — | — |
| 0.060 | 238 | 160 | 27.5 | 0.08 | 60.00 | 64 | 104.40 | 88 | 18.0 |
| 0.171 | — | — | — | — | — | — | — | — | — |
| 0.066 | 186 | 21 | 24.0 | 0.10 | 29.00 | 60 | 41.50 | 80 | 16.9 |

beginning and end of the various growth cycles, the duration of the various growth cycles, the beginning of product formation and the time necessary for obtaining maximum yields (Fig. 3 and Table 2).

The lag phase was shorter and the first part of the diauxic cycle longer when vegetative inoculum was used. As can be seen in Fig. 3 maximum mycelial yield was obtained 24 h earlier when, instead of spores, vegetative inoculum was applied and the maximum endo-PG yield appeared 12 h sooner.

In the first part of the diauxic cycle the value of growth rate constant ($k$) was higher (0.227 $h^{-1}$) when spores were used instead of vegetative inoculum (0.160 $h^{-1}$).

Another characteristic of the above two types of inocula is the duration of the declining growth phase in the first part of the diauxie (long in the case of vegetative inoculum and short in the case of spores).

The second part of diauxie was short when vegetative inoculum was used. The duration of the exponential growth phase was about three times longer in the case of spores than in the vegetative culture.

There were also differences in product formation of the cultures inoculated with the various inocula mentioned above. While no endo-PG synthesis was found in the first part of the diauxie when spores were used as the inoculum, endo-PG formation started in the declining growth phase of the first part of diauxie when the culture medium was inoculated with a 24-h vegetative culture.

The values of the growth ($k_{p_1}$) and non-growth ($k_{p_2}$) related production rate constants were higher in the case of spore inocula than those obtained when vegetative inoculum was used.

Table 2

*Kinetic constants of the growth and endo-PG formation of* Aspergillus awamori *when* (Carbon source concentration: 4%; incubation

| Part of di-auxie | Inoculum | $t_0$ | $t_L$ | $t_C$ | $t_M/x$ | $x_0$ | $x_L$ | $x_C$ | $x_M$ |
|---|---|---|---|---|---|---|---|---|---|
| 1 | spores | 20 | 25.5 | 30.0 | — | 0.0 | 1.0 | 4.0 | 6.4 |
| 2 | | 36 | 42.5 | 53.5 | 76 | 6.0 | 7.4 | 12.0 | 16.0 |
| 1 | vegetative culture | 4 | 6.5 | 13.5 | — | 0.3 | 1.2 | 5.0 | 10.8 |
| 2 | | 34 | 40.0 | 44.0 | 52 | 10.4 | 11.4 | 14.0 | 17.0 |

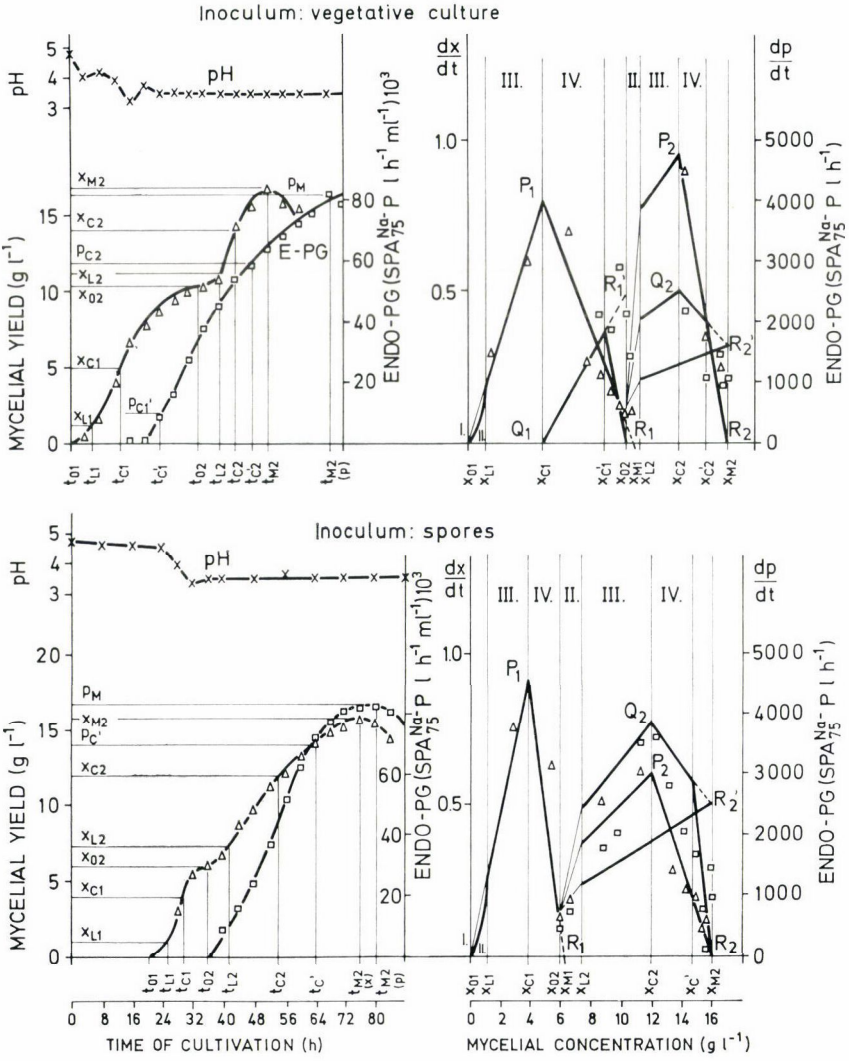


Fig. 3. The effect of the type of inoculum on the kinetic behaviour and on the growth and endo-PG yield of *Aspergillus awamori*. (Cultivation conditions: see under paras. 1.2 and 1.3.)

*inoculated with spores and 24-h vegetative cultures, respectively* at 30°C in 12–l fermentors)

| $k$ | $k_{p_1}$ | $k_{p_2}$ | $pc$ | $k'_p$ | $p'_C$ | $t'_C$ | $p_M$ | $t_{M/p}$ | $x'_C$ |
|---|---|---|---|---|---|---|---|---|---|
| 0.227 | — | — | — | — | — | — | — | — | — |
| 0.050 | 320 | 156 | 43.0 | 0.129 | 70.63 | 64 | 86.89 | 80 | 14.7 |
| 0.160 | 0 | 240 | — | 0.094 | 10.00 | 24 | | | 9 |
| 0.068 | 178 | 94 | 54.0 | 0.075 | 58.86 | 48 | 82.03 | 68 | 15.8 |

In the first part of the diauxic cycle, when the medium was inoculated with vegetative culture, no growth-related product formation was found.

### *2.3. The effect of pH control*

The main purpose of this experiment was to prove that diauxic growth can not be eliminated by pH control. The pH of the medium was adjusted to pH 4.5 and pH control was started only when the pH value of the culture reached 3.5. According to our previous experience, this pH value proved to be optimal for endo-PG production. When pH control started at the beginning of cultivation very poor growth was found together with a much lower production of enzyme (ZETELAKI-HORVÁTH, 1976).

It can be seen in Fig. 4 that the type of growth of culture cannot be influenced by pH control. Diauxic growth occurred in both cultures with or without pH control.

The maximum of the mycelial yield was about 30% higher (12.2 g $l^{-1}$) when cultivation was carried out under pH control. When there was no pH control during cultivation, the growth of the culture was poor, the declining phase of the first part of the diauxie was shorter, and the second part was longer than those in the case of pH control.

There were no significant differences in the values of the growth rate constants of the cultures incubated with or without pH control (Table 3).

The maximal endo-PG yield was much higher (74.8 l $h^{-1}$ $ml^{-1}$) with pH control than without it (51.5 l $h^{-1}$ $ml^{-1}$). The value of the growth-related production rate constant was higher in the case of pH control, while the value of the non-growth-related production rate constant ($k_{p_2}$) and the production rate constant of the final product formation ($k'_p$) were higher when there was no pH control during cultivation.

Table 3

*The effect of pH control on the kinetic constants of the growth and enzyme*
(Inoculum: spores; temperature: 30 °C;

| Part of di-auxie | $t_0$ | $t_L$ | $t_C$ | $t'_C$ | $t_M$ | $x_0$ | $x_L$ | $x_C$ |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | *With pH control** |
| 1 | 18.0 | 22.5 | 25.0 | — | 32.0 | 0.00 | 1.00 | 3.20 |
| 2 | 30.5 | 34.0 | 38.5 | 48 | 66.0 | 4.10 | 4.80 | 6.10 |
| | | | | | | | | *Without pH control* |
| 1 | 16.0 | 22.0 | 24.5 | — | 44.0 | 0.00 | 1.50 | 3.80 |
| 2 | 42.0 | 29.0 | 54.5 | 59 | 66.0 | 7.50 | 8.70 | 10.30 |

* Set at pH 3.5 after the 30th h of fermentation

## 3. Conclusions

Kinetic analysis of the growth, pectolytic enzyme or protein formation of various fungal strains was the subject of our research work for several years.

The occurrence of diauxic growth has been found in pectin and sucrose containing media supplemented with corn-steep liquor in the case of *Aspergillus* strains (ZETELAKI-HORVÁTH & BÉKÁSSY-MOLNÁR, 1975) and in sucrose and glucose media containing corn-steep liquor in the case of *Mucor* and *Rhizopus* strains (ZETELAKI-HORVÁTH *et al.*, 1976; ZETELAKI-HORVÁTH & VAS, 1976), while the above strains grew according to a simple four-stage growth pattern, in synthetic pectin and glucose media not containing corn-steep liquor. Diauxic growth of the above strains was also found in sucrose containing synthetic media.

The occurrence of diauxic growth in media containing corn-steep liquor might be explained by the primary assimilation of the amino acids of the corn-steep in the first part of the diauxic cycle and sugars or pectin in the second cycle. In media containing sucrose without corn-steep liquor, the difference in the rate of glucose and fructose assimilation was assumed to be the reason for diauxie (ZETELAKI-HORVÁTH *et al.*, 1973).

In the course of this work diauxic growth was found in natural pectin medium (extract of sugar beet cossettes), too, where diauxic growth was the consequence of the primary assimilation of sucrose (Fig. 5) being present in the extract at a concentration of 1%.

*synthesis of* Aspergillus awamori *in a 12-l automatic fermentor*
carbon source concentration: 4%)

| $x_M$ | $k$ | $k_{p_1}$ | $k_{p_2}$ | $k'_p$ | $p_C$ | $p'_C$ | $p_M$ | $p_M/_\rho$ |
|---|---|---|---|---|---|---|---|---|
| 4.50 | 0.203 | — | — | — | — | — | — | — |
| 8.30 | 0.056 | 205 | 723 | 0.241 | 6.00 | 32.00 | 51.56 | 70 |
| 7.80 | 0.205 | — | — | — | — | — | — | — |
| 12.20 | 0.048 | 281 | 679 | 0.195 | 31.00 | 50.00 | 74.80 | 70 |

According to the chromatogram, sugars were detectable in the culture filtrate only in the first 24 h of cultivation.

The composition of media (ZETELAKI-HORVÁTH & BÉKÁSSY-MOLNÁR, 1973; ZETELAKI-HORVÁTH *et al.*, 1976; ZETELAKI-HORVÁTH & VAS, 1976) the speed of agitation and scaling up (ZETELAKI-HORVÁTH & BÉKÁSSY-MOLNÁR, 1975) proved to be influencing factors of the values of the kinetic constants and the type of growth as well. In a previous study (ZETELAKI-HORVÁTH, 1977) it was already detected that diauxic growth cannot be eliminated by pH control.

In the present work the effect of carbon source concentration, the kind of inoculum and pH control were examined as influencing factors of the growth and endo-PG formation of *Aspergillus awamori*.

The carbon source concentration of the media has shown different effect on the growth and on the enzyme formation of the culture. The mycelial yield and growth rate constant in the second part of the diauxie increased with carbon concentration from 2 to 8% but the highest endo-PG yield and highest values of production rate constants were found at 6 and 2% carbon source concentration levels, resp.

The use of different inocula resulted in different lengths of the first and second part of the diauxie (Fig. 6) and the beginning of the enzyme synthesis was found at different cycles in the case of spores and vegetative inocula.

Enzyme synthesis in the first part of the diauxie can be explained by the older physiological state of the culture inoculated with a 24-h vegeta-

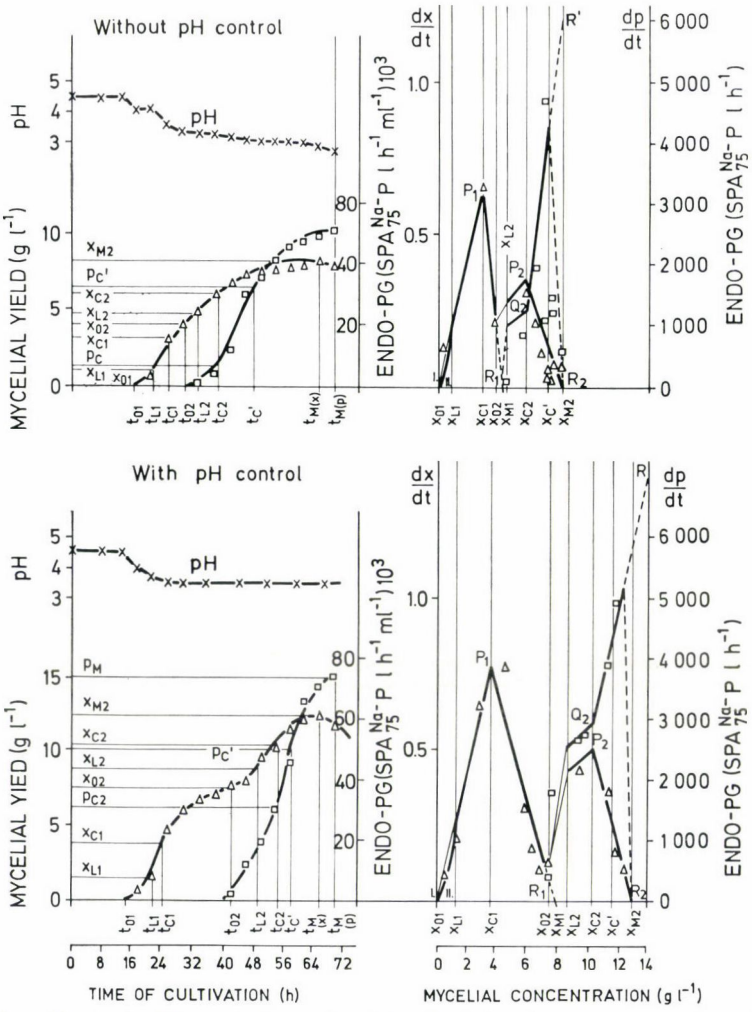


Fig. 4. The effect of pH control on the kinetic behaviour, on growth and endo-PG yield of *Aspergillus awamori*. (Cultivation conditions: see under paras. 1.2. and 1.3)

tive inoculum and with a higher mycelium concentration (Fig. 3) of the culture.

Both constants of the growth and non-growth-related endo-PG formation were higher when spores were used instead of vegetative inoculum (Table 2).

The standard deviation of the kinetic constants of the growth and enzyme formation of 14 cultures was calculated and expressed as per cent of the averages (coefficient of variation; Fig. 7).

In the case of the most important kinetic constants (determining the growth rate and the beginning and end of the various growth cycles) the standard deviation was low. The standard deviation of the growth and non-


Fig. 5. Sugar assimilation of *Asp. awamori* in natural pectin medium (sugar beet extract). Spots (20 $\mu$l) of the diluted (5 ×) 8-, 16-, 32-, 40- and 48-h culture filtrates. In the middle: sucrose, glucose and fructose standard solutions

growth-related production rate constants ($k_{p_1}$ and $k_{p_2}$) proved to be also negligibly low. Only the constants of the final product formation ($p'_C$, $k'_P$) and that of the critical product concentration ($p_C$ in the culture inoculated with spores) gave a higher standard deviation.

pH control increased the mycelial and endo-PG yield of the *Aspergillus awamori* culture highly significantly and had a positive effect on the growth related production rate constant ($k_{p_1}$). The values of the constants of growth rate and the non-growth-related production rate showed no significant differences in cultures with or without pH control.

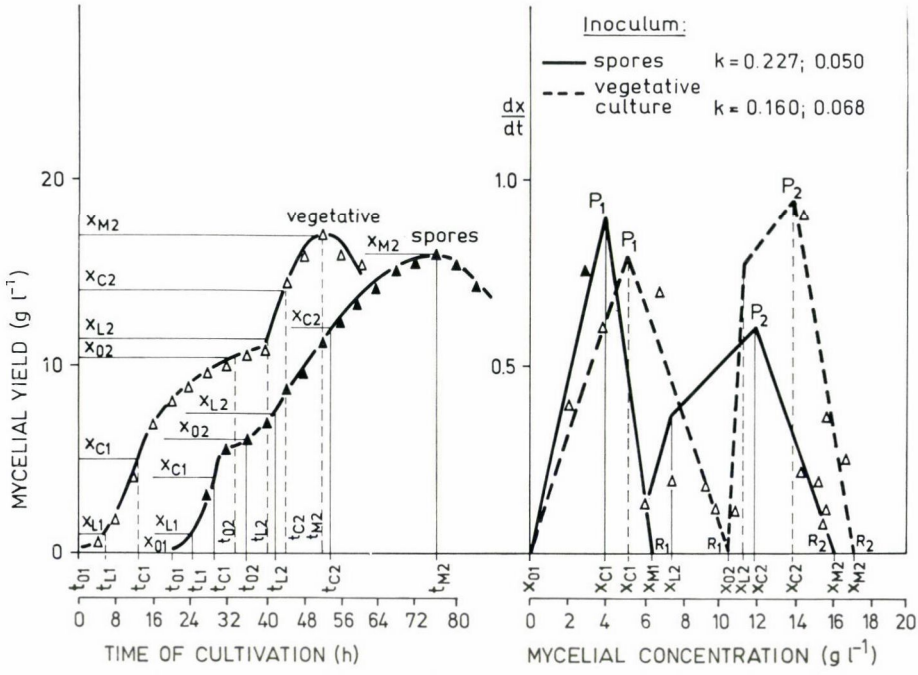


Fig. 6. Comparison of the growth curve and the rates of growth of cultures inoculated with spores and vegetative culture, resp. (Cultivation conditions: see under paras. 1.2. and 1.3.)

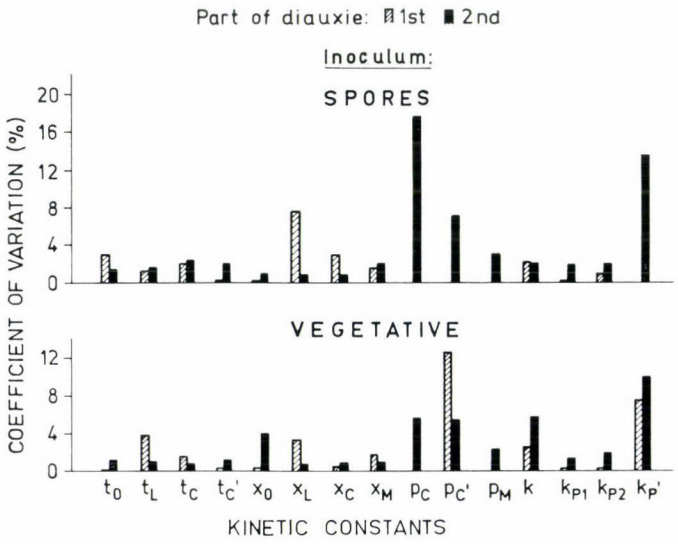


Fig. 7. Coefficient of variation of the kinetic constants of 14 cultures inoculated with spores (no pH control) and vegetative culture with pH control. (Cultivation conditions: see under paras. 1.2 and 1.3.)

### Nomenclature

| | |
|---|---|
| $k$ | growth rate constant ($h^{-1}$) |
| $k_{p_1}$ | production rate constant ($h^{-1}$) |
| $k_{p_2}$ | production rate constant ($h^{-1}$) |
| $k_p$ | production rate constant ($h^{-1}$) |
| $t$ | time, $h$ |
| $x$ | cell 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 $k$, $k_{p_1}$ and $k_{p_2}$ respectively.

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# KINETIC CONSTANTS OF GROWTH FOR USE IN THE DESIGN OF CONTINUOUS CULTURE

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In the present work $\mu_{max}$ was determined from the *Lineweaver-Burk* plot (when the reciprocal of the specific growth rate was plotted against the reciprocal of the carbon source concentration) by means of regression analysis and proved to be 0.214 and 0.077 $h^{-1}$ in the first and the second part of the diauxie, respectively.

Kinetic constants of the batch cultures were accepted as correct ones, if, substituted into the equations of various growth cycles and solved by means of a computer programme, they resulted in data in agreement, within 1.5–2.5%, with the experimental data.

Predicted cell concentrations of the continuous culture were determined by graphic and mathematical methods, using the equations of *Kono*.

Predicted cell concentrations, determined by the above methods, coincide well with the values measured at various dilution rates.

From the most important factors influencing the growth of microorganisms during cultivation the concentration of the carbon source is decisive. The kinetic constants of the batch cultures grown at various carbon source concentrations can be used to design continuous cultivation. *Michaelis* and *Kono* constants were determined from the growth of the *Aspergillus awamori* in batch culture at various carbon source concentrations in a previous work (ZETELAKI-HORVÁTH, 1978). In this article the ranges of dilution rate values suitable for continuous cultivation (using the growth constants $\mu_{max}$ and $k$) and prediction of the cell concentration in the continuous culture (on the basis of the kinetic evaluation of the batch cultures by the method of KONO and ASAI (1968)) are discussed.

## 1. Materials and methods

### 1.1. *Microorganism*

An *Aspergillus awamori* strain was used. Ten l of medium were inoculated with 1000 ml suspension of spores (concentration of spores in the inoculum: $10^6$ $ml^{-1}$).

### *1.2. Media*

As the medium an extract of dried sugar beet slices (cossettes; a by-product of the sugar industry) was used. Pectin was extracted from sugar beet slices with tap water under pressure (30 psi) in an autoclave for 2 h. The extraction of various amounts of dried slices resulted in extracts of 2, 4, 6 and 8 % dry matter content determined by an *Abbe* type refractometer (ZEISS Jena). The values of the dry matter content are referred to later as "concentration (refr. %) of carbon source" or "concentration of extract"; the dry matter content originating from the salts added (*ca.* 0.85 %) was not taken into account in the terminology adapted in this article. Thousand ml of the above extract were complemented with $(NH_4)_2SO_4$ 7 g; $KH_2PO_4$ 1 g; $MgSO_4$ 0.5 g. The pH was adjusted to 4.5.

In the case of continuous cultivation, the batch culture was started in media of 4 % carbon source concentration while the medium given continuously from the 30th h of cultivation contained only 2 % carbon source.

### *1.3. Cultivation*

The cultivation was carried out in a 12-l automatic fermentor (B. BRAUN, Melsungen). Temperature (30 °C), pH, foam, agitation and aeration were controlled and recorded. In the course of continuous cultivation the medium was fed into the fermentor by a membrane pump while the culture was removed continuously by a peristaltic pump (B. BRAUN, Melsungen) or through the inoculation pipe with the help of the effluent air.

The working volume of the fermentor was 11 l (agitation speed: 700 rpm; the rate of aeration 0.5 l air $l^{-1}$ $min^{-1}$; oxygen transfer rate: 148 mmole $O_2$ $l^{-1}$ $h^{-1}$; pH was controlled to 3.5) resulting in a full fermentor in the agitated culture.

Samples were taken in every fourth hour by an automatic sample collector (ensuring rapid cooling to 4 °C). Mycelia were removed from the samples by filtration and, after thorough washing and drying by ventilation, mycelial yield as well as protein and carbohydrate content were determined.

### *1.4. Determination of protein and carbohydrate content*

The protein and carbohydrate content of mycelia was determined by the *biuret* and the *anthrone* method (HERBERT *et al.*, 1971a and 1971b).

### *1.5. Kinetic analysis*

The growth of the batch cultures was analysed kinetically according to the method of KONO (1968) and of KONO and ASAI (1971), while kinetic analysis of the continuous fermentation according to KONO and ASAI (1968).

## 2. Results

### *2.1. The effect of the concentration of carbon source*

*Aspergillus awamori* was cultivated at carbon source concentrations of 2, 4, 6 and 8% to determine the specific growth rate of the culture (Fig. 1).

The pH control started in the 30-h culture and was controlled to 3.5. According to our previous work (ZETELAKI-HORVÁTH, 1976), a pH control to 3.5, from the beginning of the fermentation, resulted in very poor growth together with a much lower enzyme production, while a pH control to 4.5 proved to be advantageous to mycelium synthesis but had also a negative effect on the secondary metabolite (endo-PG) formation.

At various concentrations of the medium the specific rates of growth ($k$) of the cultures as measured by the tangents of $\overline{OP_1}$ and $\overline{OP_2}$ of the first and second part of the diauxic cycle (Table 1) have not changed equally.

Table 1

*Kinetic constants of the growth of* Aspergillus awamori *when cultivated in sugar beet extract media of different dry matter content (DMC)*

| Part of diauxie | DMC (%) | $t_0$ | $t_L$ | $t_C$ | $t_M$ | $x_0$ | $x_L$ | $x_C$ | $x_M$ | $k$ |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | 16 | 22.5 | 31 | — | 0.0 | 0.5 | 2.0 | 3.2 | 0.135 |
| 2 | 2 | 40 | 45.5 | 50 | 68 | 2.9 | 3.4 | 4.3 | 5.2 | 0.041 |
| 1 | | 20 | 25.5 | 30 | — | 0.0 | 1.0 | 4.0 | 6.4 | 0.227 |
| 2 | 4 | 36 | 42.5 | 53.5 | 76 | 6.0 | 7.4 | 12.0 | 16.0 | 0.050 |
| 1 | | 16 | 18.0 | 24 | — | 0.0 | 1.0 | 5.7 | 9.1 | 0.173 |
| 2 | 6 | 36 | 45.5 | 50.5 | 72 | 8.8 | 10.0 | 13.0 | 16.6 | 0.060 |
| 1 | | 20 | 22.0 | 25.5 | — | 0.0 | 1.0 | 5.7 | 10.0 | 0.171 |
| 2 | 8 | 40 | 46.5 | 52 | 76 | 9.6 | 11.0 | 14.5 | 18.8 | 0.066 |

In the first part the highest $k$ value was obtained in the 4% extract, then it decreased with the increase in extract concentration. In the second part of the diauxie the specific rate of growth increased in agreement with the increase of the concentration of carbon source. It can well be seen in Fig. 1 that with the increase of the extract concentration the maximum mycelium concentration of the first part of the diauxie became increasingly higher.

The protein and carbohydrate contents of the mycelia of *Asp. awamori*, harvested from media of various carbon source concentrations were similar (Fig. 2).

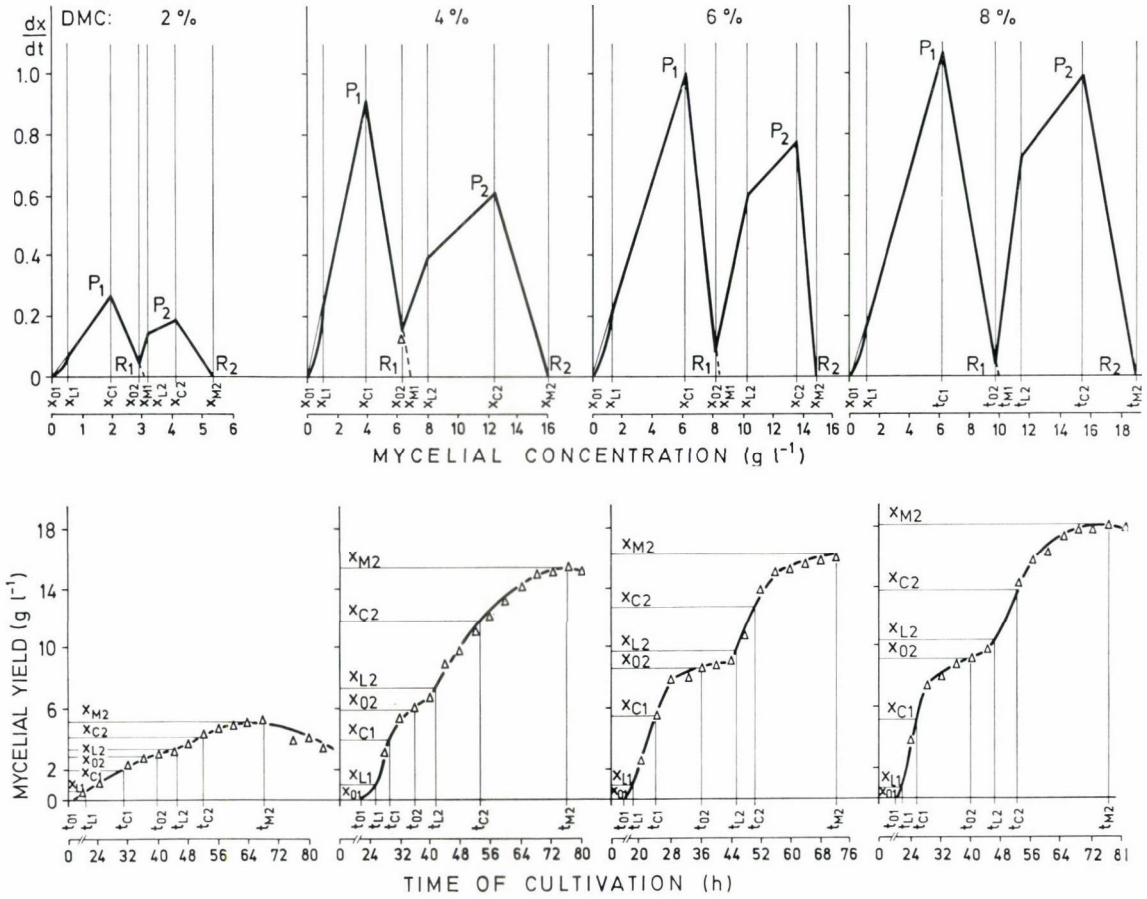



Fig. 1. The growth of *Asp. awamori* as a function of the time of cultivation, and the rate of growth ($dx/dt$) as a function of the mycelial concentration at different carbon source concentrations (DMC). (For cultivation conditions: see paras. 1.2 and 1.3. For symbols see: Nomenclature)

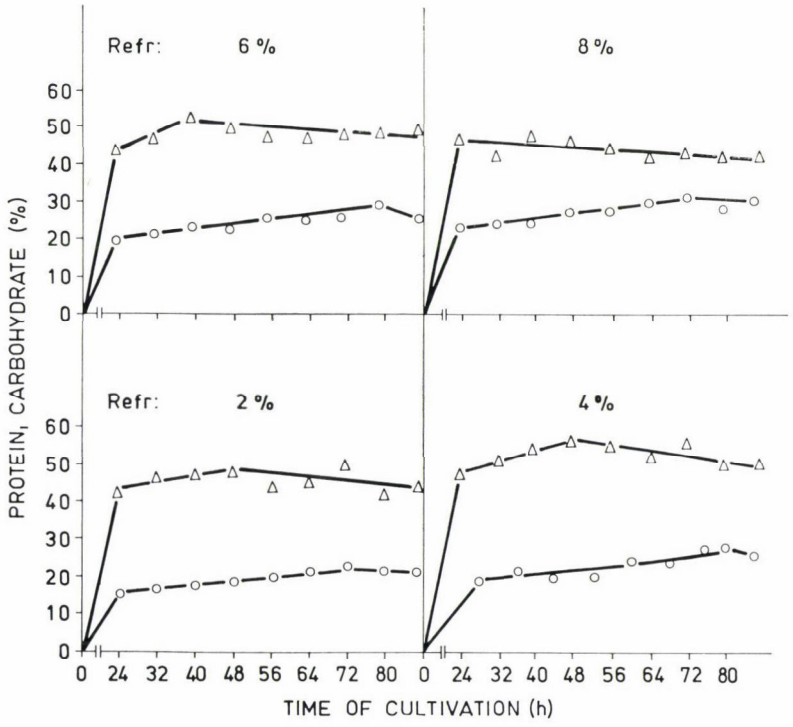



Fig. 2. Protein and carbohydrate content of the mycelium of *Asp. awamori* at different extract concentrations

△: protein ○: carbohydrate

The protein content of the mycelia varied between 40 and 50%, in most extract concentrations. An increase in the N content was observable during the first 40–48 hours, decreasing later till the end of cultivation. The highest values of N content were measured in the 4% extract, where its value had not dropped below 50% until the end of fermentation.

### *2.2. Kinetic constants in the planning of dilution rates in continuous culture*

As the dilution rate cannot be higher than $\mu_{max}$, results of the kinetic analysis of the batch growth serve as very useful keys in determining the approximate limits of dilution rates.

The value of $\mu_{max}$ of the batch culture can be determined by several methods. When $\log_e$ values of the cell concentration ($x$) are plotted against time of cultivation, the tangent of the obtained slope will be equivalent to $\mu_{max}$.

Constants of the enzyme kinetics are also used in the growth kinetics of microorganisms. *Michaelis* constants (AIBA *et al.*, 1965) have been succesfully used for the description of microbial growth. According to MONOD (1949)

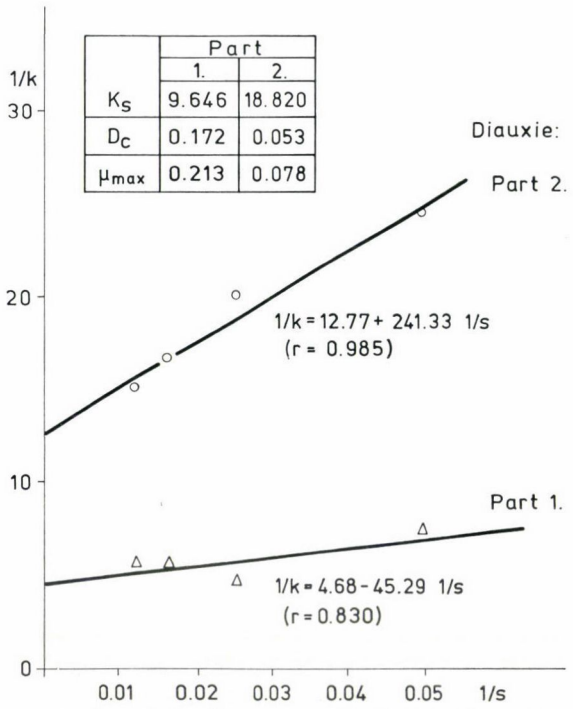



Fig. 3. Graphic representation of the *Lineweaver-Burk* plot; the reciprocal of the growth rate, $1/k$, is plotted against the reciprocal of the substrate concentration, $1/S$, to determine the $K_S$ saturation constant and the maximum growth rate ($\mu_{max}$)

the specific rate of growth ($\mu$) can be determined by the following equation:

$$\mu = \mu_{max} \frac{S}{K_S + S} x \tag{1}$$

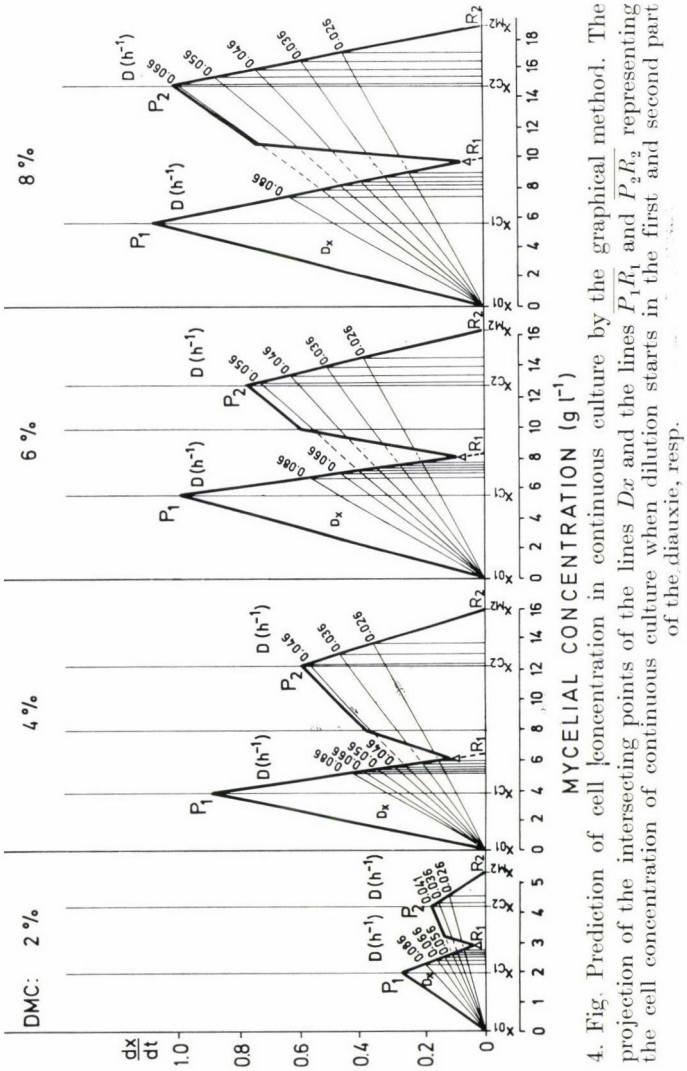
where:

$\mu_{max}$ = maximum growth rate when the substrate is unlimited

$K_S$ = the substrate concentration at which the specific growth rate observed is one-half the maximum value.

The values of $K_S$ and $\mu_{max}$ can be determined graphically from the *Lineweaver-Burk* plots, when the reciprocals of specific growth rate ($k$) are plotted against reciprocals of initial carbon source ($S$) concentration (Fig. 3). *Michaelis* constants ($K_S$ and $\mu_{max}$ values) for the growth of the batch culture of *Aspergillus awamori* were determined in both parts of the diauxie from the *Lineweaver-Burk* plots. (The equations of the slopes were calculated by regression analysis).

In the course of batch cultivation of *Asp. awamori*, the value of $\mu_{max}$ in the first part was about three times that of the second part of the diauxie.




4. Fig. Prediction of cell concentration in continuous culture by the graphical method. The projection of the intersecting points of the lines $Dx$ and the lines $\overline{P_1R_1}$ and $\overline{P_2R_2}$ representing the cell concentration of continuous culture when dilution starts in the first and second part of the diauxie, resp.

The critical dilution rate was the highest when determined from the data of growth rate in the first part of the diauxie (0.172) and about one-third of that when calculated from the second part of the diauxie.

In the case of the saturation constant ($K_S$) the reverse of the above result was obtained. The value of $K_S$ was 1.9 times higher in the second part of the diauxie than in the first part.

### *2.3. Prediction of cell concentration in continuous cultivation*

*2.3.1. Graphic method.* In continuous cultivation, when $x_M \geq x_C$, the growth rate in the declining phase can be used to calculate cell concentration.

In the case of no feed-back, the line ($dx/dt = \mathrm{Dx}$) starts from the origo and intersects somewhere the line described by the equation of the declining phase ($\overline{\mathrm{PR}}$). The projection of the intersecting points on the abscissa indicates the concentration of microorganisms belonging to the given dilution rate (Fig. 4). It can be seen in the Figure that $D$ cannot be more than $k$ of the first part of the diauxie, accordingly the minimum concentration of microorganisms is equivalent to $x_{C_1}$.

The cell concentration of the cultures varies according to the carbon source concentration and to the part of the diauxie where the dilution started from.

*2.3.2. Mathematical method.* Cell concentration in continuous culture was estimated by mathematical calculations of equations 2 and 3.

$$x = \frac{k' x_M}{D - k'} \tag{2}$$

$$k' = \frac{x_C}{x_M - x_C} \tag{3}$$

where:

$x$ = mycelial concentration in the continuous culture
$x_C$ = critical mycelial concentration in the batch
$x_M$ = maximum mycelial concentration in the batch
$D$ = dilution rate

Data of the predicted cell concentration determined by both the graphic and mathematical methods from the data of batch cultivation in various extract concentrations are summarized in Fig. 5. The data of the predicted cell concentration determined by graphic method are lower in the Figure than those determined by the mathematical method. The reason for this fact is that the graphic method can be used only at those dilution rates, the values of which are lower than the value of the specific growth rate ($k$) in the specific part of the diauxie, while in the case of mathematical calculation theoretical values at any dilution rates can be determined.

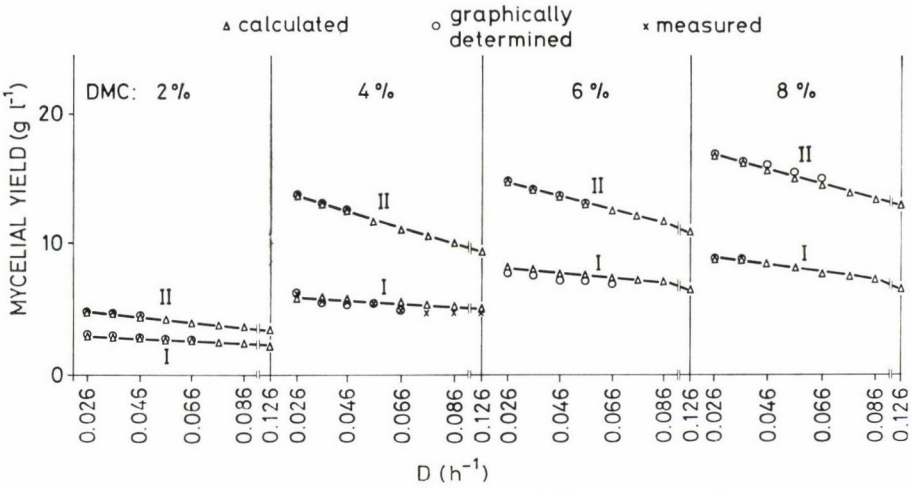



Fig. 5. Comparison of cell concentration predicted by the graphical method and by computation at various carbon source concentrations (DMC) as a function of the dilution rate. Comparison of the steady state cell concentration with the predicted values in 4% dry matter content as a function of the dilution rate

It can be seen in the Figure that predicted cell concentrations determined with both the graphic and computing methods have proven to be the same.

The measured cell concentration in the steady state of the continuous culture of *Aspergillus awamori* was in good agreement with the predicted cell concentration between dilution rates of 0.026 and 0.056 $h^{-1}$. The differences between the real and the predicted values were about 5–8%, but at higher dilution rates these values exceeded 10 %.

As dilution started in the first phase of the diauxic cycle, steady state cell concentration was due to the cell concentration of the declining phase in the first part of the diauxie.

## 3. Conclusions

In the course of batch cultivation of *Aspergillus awamori* the increase in the concentration of the extract of sugar beet cossettes from 2 to 4%, resulted in a more than three-fold increase in mycelial yield. The increase of the extract concentration to 6% resulted in no change of the mycelial yield, but a further increase to 8% increased the mycelial yield by 13%.

The protein and carbohydrate contents of the mycelium as a function of the time of cultivation changed reversely. With the increase in the age of the culture the protein content of the mycelium decreased while its carbohydrate content increased. Protein content was the highest (50–55%) at 4%

and the lowest at 8% carbon source concentration, while the carbohydrate content increased slightly with increase in concentration of the carbon source.

Similarly to our previous work (ZETELAKI-HORVÁTH *et al.*, 1973; ZETELAKI-HORVÁTH & BÉKÁSSY-MOLNÁR, 1973 and 1975, ZETELAKI-HORVÁTH, 1978) diauxic growth was found in natural pectin (extract of sugar beet cossettes) media at each extract concentration tested.

The specific rate of growth in the first and second parts of the diauxie shows no identical change with the increase of the extract concentration. The specific rate of growth was the highest at 4% carbon source concentration in the first part of the diauxie in agreement with the highest protein content of the mycelia. In the second part of the diauxie the specific growth rate increased with the increase in extract concentration.

As 4% carbon source concentration has given the best endo-polygalacturonase yield during 72 h of cultivation in batch culture (ZETELAKI-HORVÁTH, 1978), the batch part of the continuous culture was started with this substrate concentration. A diluting medium of the same concentration was uneconomical in continuous culture, resulting in an endo-PG yield equal to that of the culture grown in a medium of 2% carbon source (ZETELAKI-HORVÁTH, 1977). When a diluting medium of 4% carbon source was used only the mycelial yield increased. This is undesirable in the case of this fermentation because it tends to increase the chances of clogging. According to these results, an extract concentration of 2% proved to be a sufficient diluting medium for continuous endo-PG production by *Aspergillus awamori*.

In the course of this work, changing over to continuous cultivation occurred in the first part of the diauxie when the cell concentration was not too high and the culture was in a better physiological state than in the second part of the diauxie. As cultivation started at 4% extract concentration, the cell concentration of the continuous culture must agree with the cell concentration of the batch culture in the first part of diauxie when an extract concentration of 4% was used. This has been proved by our results.

According to the kinetic analysis of the batch culture the predicted cell concentration, determined by the mathematical and the graphic method of KONO and ASAI (1968), gave identical results. The measured values in the continuous cultivation were in good agreement with the predicted results.

A $\mu_{max}$ value of 0.214 was obtained when determined from the *Lineweaver-Burk* plots for the first part of diauxie of *Aspergillus awamori*, while highest value of the specific growth rates ($k$) of 0.227 $h^{-1}$ was obtained at 4% carbon source concentration according to KONO's method.

**Nomenclature**

| | |
|---|---|
| $k$ | growth rate constant ($h^{-1}$) |
| $t$ | time |
| $x$ | cell 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 the theoretical maximum value of cell and product concentration, respectively. The slope of $\overline{OP}$ represents the value of $k$.

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# EFFECT OF CHLORFLURENOL-METHYL ESTER ON THE TECHNOLOGICAL VALUE OF TOMATOES

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It was found that application of chlorflurenol-methyl ester caused a decrease in the amount of seeds in the fruits of tomatoes. Dry matter, total extract, sugar and acid contents in the control and treated fruits were similar. There were no significant differences in the contents of raw protein, amino acids and $\beta$-carotene.

Chlorflurenol-methyl ester (methyl-2-chloro-9-hydroxyfluorene (9)-carboxylate) belongs to the group of synthetic growth regulators called morphactins.

Based on yield-improving effects and on favourable toxicological and residual data, chlorflurenol is promising for use on cucumbers (Cantliffe *et al.*, 1972; Cantliffe, 1974; Schneider, 1974). Chlorflurenol applied in the form of foliage sprays is very effective in inducing fruit set and in producing yield concentration in tomatoes (Rudich & Rabinowitch, 1974). Small doses of chlorflurenol-methyl ester induce the tomato plant to develop more commercially acceptable fruits with normal colour and shape (Łukasik, 1977). Furthermore, the treatment with fluorene-carboxylic acid derivatives may lead to the development of parthenocarpic fruits in tomatoes, pepper and cucumbers (Schneider, 1964; Łukasik, 1975; Jayakaran, 1973; Robinson *et al.*, 1971).

The purpose of this paper is to compare the content of some chemical compounds in the fruits harvested from plants treated with chlorflurenol-methyl ester and in the fruits obtained from control plants.

## 1. Materials and methods

*Experiment I*

The experiment was carried out on three varieties of tomatoes: *New Yorker*, *Earliest of All* and *Mory 33* grown in the field (Lublin-Felin, 1972). The plants were sprayed with chlorflurenol-methyl ester applied twice, in concentrations of 0.25; 0.5 and 1.0 ppm. Tween 20 was added as a wetting agent

to all spray solutions at a concentration of 0.001%. The first spraying was done at the time when the plants had flower buds on their first cluster (30 May). The second spraying took place after the individual flowers had blossomed on the third or fourth cluster depending on the variety (17 June). The fruits for analysis were harvested fully ripe from the second or third cluster.

*Experiment II*

The experiment was carried out on two varieties of tomatoes: *New Yorker* and *Earliest of All* grown in a greenhouse in Mitscherlich pots (Lublin-Felin, 1974). Chlorflurenol-methyl ester was applied in 0.25 and 0.5 ppm concentrations directly on the single open flowers (soaking). The fruits for analysis were harvested fully matured from the second cluster.

The fruits were analysed with respect to dry matter, total extract, total and reducing sugars, total acids, vitamin C, $\beta$-carotene, raw protein and amino acids.

Dry matter was determined in a desiccator at 105 °C. Total extract was studied by using a refractometer. Sugar content was examined by *Bertrand's* method. Total acids (as citric acid) were determined using titration (CHARLAMPOWICZ, 1966). Vitamin C was examined by *Tilmans'* method and $\beta$-carotene was determined according to *Polish Standards* (PN-71/1 7510). To study the raw protein content *Kjeldhal's* method was applied. The amino acid content was analysed on a Czechoslovakian chromatograph AAA 881 (NEHRING & SCHRÖDER, 1963).

For each analysis 2–4 fruits were used. The data (three or four analyses for each treatment) were compared statistically using *Student's* $t$-test at the 5% level of probability.

## 2. Results and discussion

In all studied varieties of tomatoes chlorflurenol-methyl ester applied in concentrations of 0.5 and 1.0 ppm caused complete or partial parthenocarpy of fruits. Compared to the weight of control seeds decrease in weight of seeds in the fruits harvested from the *New Yorker* and *Earliest of All* varieties treated with chlorflurenol-methyl ester (0.5 ppm) amounted to 1.34 and 2.18%, respectively (Table 2). Less seeds in the fruits is favourable for the processing of tomatoes into concentrates and juices. The formation of seedless fruits of tomatoes under natural growing conditions seems to be promoted especially at low temperatures (MUSEHOLD, 1972). Under the influence of chlorflurenol-methyl ester the development of parthenocarpic fruits is caused by rapidly blocking the natural outward flow of auxin within the ovary (BEYER &

QUEBEDEAUX, 1974). Nevertheless, the parthenocarpy in different varieties of tomatoes treated with chlorflurenol-methyl ester is strongly influenced by genetic and environmental factors. Moreover, higher doses of chlorflurenol-methyl ester caused deformed fruits and the occurrence of blossom-end rot (ŁUKASIK & HUSZCZA, 1974).

As compared to the control, the dry matter and total extract contents of fruits harvested from the plants treated with chlorfurenol-methyl ester in different concentrations underwent small changes only. These changes involved both parthenocarpic fruits and the fruits with small amounts of seeds (Tables 1 and 2). Similarly slight differences in $\beta$-carotene, raw protein and sugar content were observed. Experiments with *Beta vulgaris* var. *altissima* and *Saccharum officinarum* showed also that morphactin treatment did not increase the sugar content. By contrast, in investigations of the fruit quality of *Prunus domestica* an increase of reducing sugar content was observed after the treatment (MERCK *et al.*, cited by SCHNEIDER 1970).

Detailed analyses of amino acid contents in control fruits and in the fruits harvested from the plants treated with chlorflurenol-methyl ester (0.5 ppm) did not show significant differences (Table 3). There were tendencies to increase the vitamin C content in parthenocarpic fruit in the *Earliest of All* variety (Table 1). In the other combinations vitamin C content remained almost at the level of control fruits (Tables 1 and 2).

## 3. Conclusions

The following conclusions are based on the data of the experiments.

- Chlorflurenol-methyl ester caused a significant decrease in seeds in the fruits (Tables 1 and 2).
- Chlorflurenol-methyl ester treatment induced an increase in vitamin C in parthenocarpic fruits of the *Earliest of All* variety (Table 1).
- Under the influence of chlorflurenol-methyl ester, small changes were detected in dry matter and total extracts in treated and in untreated fruits (Tables 1 and 2).
- Application of chlorflurenol-methyl ester produced small fluctuations in the sugar content, total acids, *Kjeldhal* nitrogen, $\beta$-carotene and amino acid (Tables 1, 2 and 3).

Table 1

*Effect of different doses of chlorflurenol-methyl ester applied in the form of foliage sprays on some chemical constituents of the fruits of three tomato varieties*

| Tomato variety | Concentration of chlorflurenol-methyl ester (ppm) | Chemical composition | | | |
|---|---|---|---|---|---|
| | | Total extract % | Total sugar % | Total acids % | Vitamin C mg% |
| New Yorker | 0.00 | 6.40 a | 3.60 a | 0.57 a | 8.00 ab |
| | 0.25 | 6.50 a | 3.80 a | 0.58 a | 10.80 a |
| | 0.50+ | 6.50 a | 3.60 a | 0.56 a | 7.90 ab |
| | 1.00+ | 6.20 a | 3.70 a | 0.58 a | 7.00 b |
| Earliest of All | 0.00 | 7.80 a | 4.00 a | 0.60 a | 6.00 b |
| | 1.00+ | 7.30 a | 3.70 a | 0.54 a | 12.00 a |
| Mory 33 | 0.00 | 6.00 a | 3.60 a | 0.64 a | 12.00 a |
| | 1.00+ | 6.00 a | 3.60 a | 0.57 a | 14.00 a |

Within the columns, only figures followed by different letters differ significantly at the probability level of 5%

+ parthenocarpic fruits

Table 2

*Effect of the different doses of chlorfluerenol-methyl ester applied on the open flowers, on the properties of seeds, on some chemical constituents of the fruits of two varieties of tomatoes*

| Compounds | *New Yorker* | | | *Earliest of All* | |
|---|---|---|---|---|---|
| | Chlorflurenol-methyl ester (ppm) | | | Chlorflurenol-methyl ester (ppm) | |
| | 0.00 | 0.25 | 0.50 | 0.00 | 0.50 |
| Weight of seeds as % of total weight of 10 fruits | 2.64 a | 1.95 b | 1.27 c | 3.20 a | 1.02 b |
| Total extract, % | 4.00 a | 4.00 a | 4.05 a | 5.60 a | 5.60 a |
| Dry matter, % | 4.75 a | 5.00 a | 5.10 a | 6.70 a | 6.60 a |
| Total sugar, % | 3.25 a | 3.03 a | 3.04 a | 4.45 a | 4.46 a |
| Reducing sugars, % | 2.85 a | 2.68 a | 2.77 a | 4.10 a | 4.15 a |
| Total acids, % | 0.43 a | 0.46 a | 0.43 a | 0.46 a | 0.33 a |
| Raw protein, % | 0.43 a | 0.48 a | 0.44 a | 0.71 a | 0.77 a |
| Amino acids in protein, % | 72.70 a | 70.00 a | 64.80 a | 91.00 a | 89.00 a |
| Vitamin C, mg % | 14.40 a | 13.70 a | 13.25 a | 8.30 a | 9.60 a |
| $\beta$-carotene, mg% | 0.36 a | 0.48 a | 0.46 a | 0.34 a | 0.36 a |

Within the columns, only figures followed by different letters differ significantly at the probability level of 5%

Table 3

*Effect of chlorflurenol-methyl ester on the amino acid composition of the proteins (as % of the raw protein) of the* New Yorker *tomato variety*

| Amino acids | Concentration of chlorflurenol-methyl ester (ppm) | |
|---|---|---|
| | 0.00 | 0.50 |
| Lys | 4.9 a | 3.6 a |
| His | — | — |
| $NH_3$ | — | — |
| Arg | 0.8 | — |
| $CySO_3H$ | 1.7 a | 1.5 a |
| Asp | 11.9 a | 10.3 a |
| $MeSO_2$ | 1.2 a | 1.0 a |
| Thr | 3.1 a | 2.8 a |
| Ser | 3.4 a | 3.3 a |
| Glu | 23.4 a | 22.5 a |
| Pro | 2.9 a | 3.5 a |
| Gly | 3.2 a | 2.6 a |
| Ala | 4.7 a | 3.9 a |
| 1/2 Cys | — | — |
| Val | 3.0 a | 2.9 a |
| Met | — | — |
| Ileu | 2.4 a | 2.2 a |
| Leu | 4.4 a | 3.6 a |
| Tyr | — | — |
| Phe | 1.7 a | 1.1 a |

Within the columns none of the figures followed by letter differ a significantly at the probability level of 5%

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# BINDING ENERGY OF BOUND WATER IN FOODSTUFFS

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Data on the heat of binding of bound water in the literature are based either on calorimetric measurements or on calculations by the *Clausius-Clapeyron* equation, the *BET* theory, isosteric sorption heat or other relationships. The critical evaluation of the latter methods has shown that, at temperatures below freezing point, the relationship taking into account the difference in the respective specific heats of bound and non-frozen water or calculation of the isosteric sorption heat yield results nearest to those obtained by calorimetry.

When determining the heat of binding the quantity of bound water has to be standardized. This may be the mono-layer moisture content as determined by the *BET* equation, or the moisture content related to dry matter, calculated on the basis of the non-frozen water at the given temperature. The heat of binding of bound water has to be taken into account on calculating the heat requirement of freeze-drying.

In the course of cryogenic freezing foodstuffs are exposed to extremely low temperature (*i.e.* to −196 °C, in liquid nitrogen) and after freeze-drying the residual moisture content (1–3 %) is substantially lower than after conventional drying.

A common feature of the two technologies is that a higher proportion of the moisture content is affected than by conventional methods of freezing and drying. In consequence a renewed interest arises as regards the problem of "bound" water, involving the moisture content not freezing at reduced temperatures, the amount of "bound" water and the binding energy of this water.

## 1. Review of related literature

Data in the literature related to the binding energy fall into two groups. One group contains data calculated on the basis of the *Clausius–Clapeyron* equation, the *BET* theory or other relationships. The other group contains data obtained by calorimetry. In the majority of these data binding energy and heat of binding are treated as one and the same concept. Where differentiation is applied this is indicated.

*1.1. Calculations*

Some calculation methods recently proposed for the calculation of the binding energy of bound water are described below. Their critical review is given in Section 2.

Implying that "at the temperature $t$ the work of the osmotic forces involved in removing 1 kg bound water is equal to the binding energy of bound water at the given temperature" RYUTOV (1976) suggests the following equation for the determination of the binding energy of bound water:

$$E_t = L_t \frac{-t}{273 \cdot 15} \text{ kJ kg}^{-1} \tag{1}$$

where $L_t$ = melting heat of ice at $t$ °C, kJ kg$^{-1}$
$t$ = temperature, °C ($t_c > t > t_e$)
$t_c$ = cryoscopic temperature, °C
$t_e$ = eutectic temperature, °C.

Both GINZBURG (1976) and RYUTOV (1976) suggest *Rebinder*'s equation to calculate binding energy:

$$E_t = RT \ln \frac{p_v}{p_w}, \text{ kJ kg}^{-1} \tag{2}$$

where $p_v$ = saturated vapour pressure of the free water
$p_w$ = equilibrium vapour pressure above the material of moisture content $W$
$R$ = gas constant
$T$ = temperature, $K$.

Considering that, in their opinion, in freezing at a given temperature the bound water is at equilibrium with the concentrated solution and with the ice

$$p_w = p_i$$

where $p_i$ = saturated vapour pressure above the ice.

According to RIEDEL (1961) the heat of binding

$$E_t = E_0 + \int_{T_1}^{T_2} (c_w - c_{bw})\, dT \tag{3}$$

where $E_0$ = adsorption heat determined by calorimetry at 0 °C, kJ kg$^{-1}$ °C$^{-1}$
$c_w$ = specific heat of the free water, kJ kg$^{-1}$ °C$^{-1}$
$c_{bw}$ = specific heat of the bound water, kJ kg$^{-1}$ °C$^{-1}$
$c_w$ = 4.2177 + 0.01878 $t$, kJ kg$^{-1}$ °C$^{-1}$ (below 0 °C)
$c_{bw}$ = 1.672 + 0.01162 $t$, kJ kg$^{-1}$ °C$^{-1}$ (below 0 °C).

On the basis of the *BET* theory many authors (IMRE, 1974; IGLESIAS & CHIRIFE, 1976c) have applied the following relationship in the determination of the adsorption heat:

$$Q = RT \ln C \tag{4}$$

where $C$ = the constant of the *BET* equation.

IGLESIAS and CHIRIFE (1976a) suggest for the calculation of isosteric heat from the sorption isotherms as measured at two different temperatures, the following relationship:

$$Q = R\,[(T_1 T_2)\,(T_2 - T_1)]\,[\ln\,(a_{w2}/a_{w1})] \tag{5}$$

where $Q$ = heat of binding at temperature $\dfrac{T_1 + T_2}{2}$, kJ kg$^{-1}$

$a_{w2}$ = water activity $(p_u/p_v)$ at temperature $T_2$

$a_{w1}$ = water activity at temperature $T_1$.

*1.2. Measurements*

In recent years several methods of measurement were worked out in relation to drying and particularly to freeze-drying for the determination of heat of binding.

Heat effect of heat of binding

$$Q = Q_u - Q_v \tag{6}$$

where $Q_u$ = heat of vaporisation related to the material of a given moisture content

$Q_v$ = heat of vaporisation of free water.

The drying heat requirement of starches of different moisture contents was determined by OLENEV and CHIZHOV (1973) by electrocalorimetry at +41 °C. The results obtained are shown in Fig. 1.

They found that depending on the kind of starch, the heat of vaporisation becomes higher than the heat of vaporisation of pure water at +41 °C (2.345 MJ kg$^{-1}$) below a moisture content of 4.24 – 0.84 g per g solids.

Below 0.16–0.20 g moisture content per g solids the evaporation heat increases substantially.

On the basis of the heat of binding 4 groups of moisture content were arbitrarily established with corn starch. These are shown in Table 1.

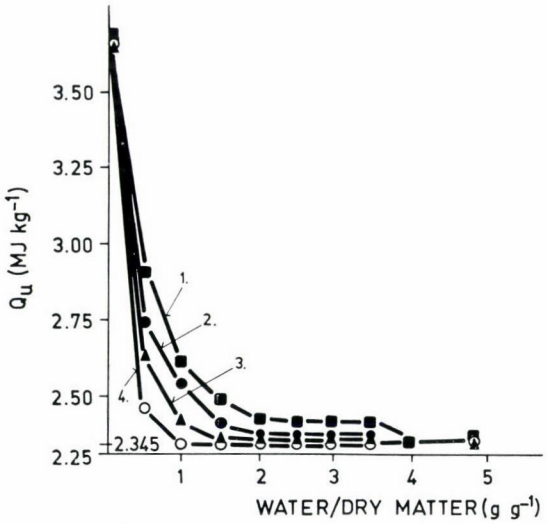


Fig. 1. Specific heat of vaporization of starch pastes. 1: Corn; 2: potato; 3: modified potato; 4: waxen corn

Table 1

*Distribution of water in corn starch paste according to heat of binding*

| Specific heat of vaporisation, $r$ MJ kg$^{-1}$ | Heat of binding of water kJ kg$^{-1}$ | Group and state of water |
|---|---|---|
| r = 2.345 | $E_t = 0$ | I free water |
| $2.345 < r \leq 2.500$ | $0 < E_t \leq 155$ | II slightly bound |
| $2.500 < r \leq 3.000$ | $155 < E_t \leq 655$ | III strongly bound |
| $r > 3.000$ | $E_t > 655$ | IV very strongly bound |

The amount of water not frozen between —6 and —30 °C was also determined in starch (Table 2).

Table 2

*The amount of non-frozen water in various starch samples*

| Starch | Quantity of non-frozen water ($g\,g^{-1}$ dry matter) | | | |
|---|---|---|---|---|
| | —6 °C | —10 °C | —18 °C | —30 °C |
| Potato | 0.42 | 0.34 | 0.33 | 0.29 |
| Corn | 0.44 | 0.35 | 0.31 | 0.30 |
| Modified potato | 0.45 | 0.36 | 0.32 | 0.28 |

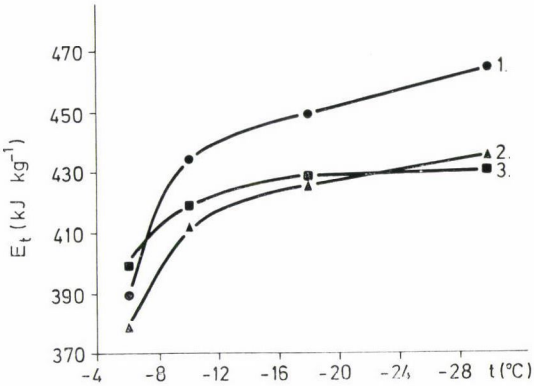


Fig. 2 Heat of binding of the non-frozen water in different starch pastes. 1: Potato; 2: corn; 3: modified potato

On the basis of Table 2 and Fig. 1 the binding energy of the amount of water not frozen in starch paste was also determined at various temperatures (Fig. 2).

KAUCHESHVILI and co-workers (1972) describe the results of determinations of the binding energy of bound water in the course of freeze-drying.

Of these determinations those obtained on freeze-drying raw beef in the form of spherical shells are shown in Fig. 3.

The heat requirement till the complete sublimation of the total amount of ice is 2 759–2 800 kJ kg$^{-1}$. When the moisture content reaches 0.25–0.33 g per g dry matter the heat requirement slightly decreases. However, after further reduction of the moisture content a significant increase in the heat requirement is apparent reaching 7 106 kJ kg$^{-1}$ or even higher values at 0.01 g per g dry matter. Calculated values of the heat of binding are shown in Fig. 4.

## 2. Discussion of calculations and results

In the course of freezing and in the sublimation phase of freeze-drying foodstuffs represent a system in which water is present in various forms, such as

- frozen,
- free, non-frozen
- non-frozen, bound (by different energies).

It is known further that with the decrease of temperature the quantity of frozen water increases to a certain limit and in parallel the amount of non-frozen, free water decreases.

Presumably, the quantity of bound water, if characterized by a monolayer moisture content on the basis of the *BET* equation, increases with the decrease in temperature (IGLESIAS & CHIRIFE, 1976b).

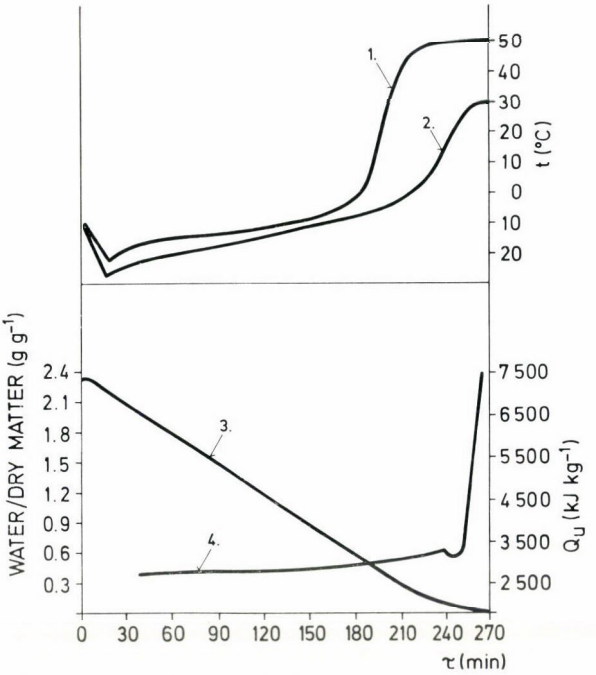


Fig. 3. Freeze-drying process of meat. 1: Temperature of the inner surface of meat; 2: temperature of the outer surface; 3: moisture content; 4: specific sublimation or evaporation heat

On the basis of the determinations described it may be established that the heat of binding is measurable (*e.g.* in corn starch paste) at a sufficiently high moisture content (4.24 g per g dry matter) and may reach considerable values at low moisture contents. Heat of binding is highly dependent on the properties of the foodstuff, *i.e.* there are substantial differences in the heats of binding of starch pastes obtained from different plants.

### *2.1. Calculation of heat of binding from the parameters of water and ice*

The calculation methods as suggested by various authors may be accepted for the determination of binding energy or heat, only if the results obtained are near to those obtained by measurement.

The results, related to beef, of the determinations carried out by KAUCHESHVILI and co-workers (1972) as cited above, and data as measured and calculated by RIEDEL (1961) again in relation to beef, were taken into account. Data of RIEDEL contain relationships between moisture content, water activity, bound water, non-frozen free water and temperature.

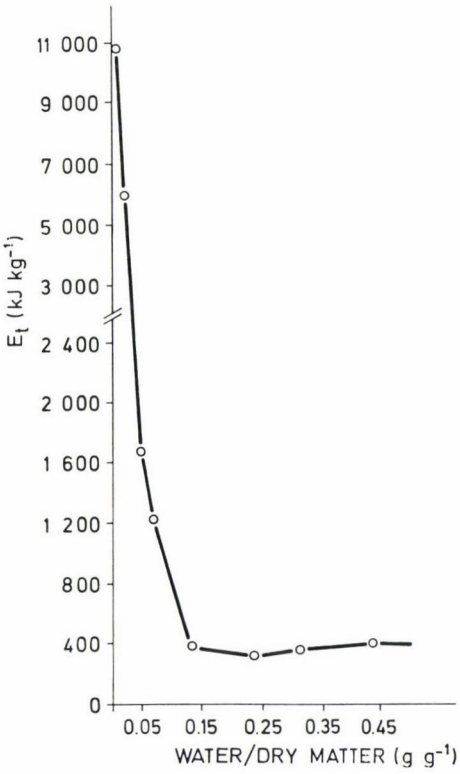


Fig. 4. Heat of binding as a function of moisture content in meat during freeze-drying

The relationships as demonstrated in equations (1) and (2) show that the binding energy of bound water deduced from the physical parameters of water and ice are independent of the characteristics of the foodstuff, thus data deduced from them are identical for every foodstuff.

Heat of binding

$$\Delta H = \Delta\mu + T\Delta S \tag{7}$$

where $\Delta\mu$ = change of the chemical potential
$\Delta S$ = change in entropy.

Equations (1) and (2) do not take into account member $T\Delta S$ in equation (7) and thus indicate the reversible work of water binding, instead of the energy of water binding.

The change of binding energy with temperature may be calculated by equation (3).

$$E_t - E_0 = \int_{T_1}^{T_2} (c_w - c_{bw})\, \mathrm{d}T \tag{8}$$

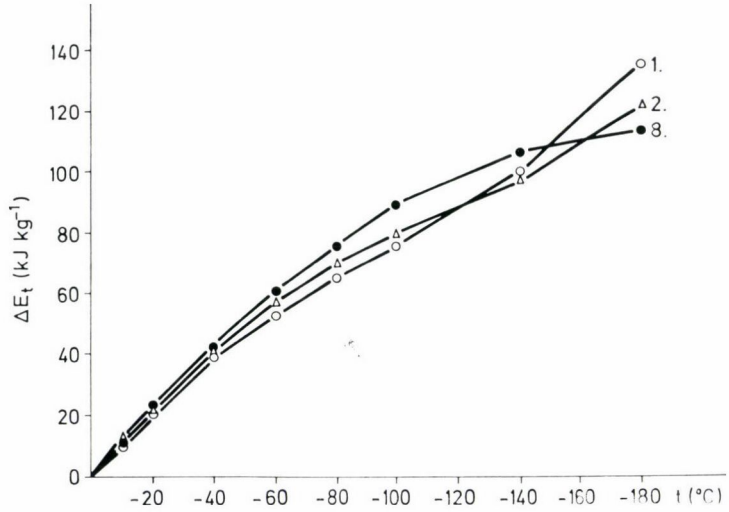


Fig. 5. Changes in the binding energy of bound water as a function of temperature, calculated by equations (1), (2) and (8)

Table 3

*Change in the binding energy of bound water as a function of temperature*

| Temperature °C | Binding energy of bound water (kJ kg$^{-1}$) according to equation | | |
|---|---|---|---|
| | (1) | (2) | (8) |
| —1 | 1.2 | 1.2 | 1.2 |
| —5 | 6.8 | 7.0 | 6.2 |
| —10 | 11.5 | 11.8 | 12.0 |
| —20 | 21.8 | 22.8 | 23.8 |
| —40 | 39.4 | 42.1 | 44.8 |
| —60 | 53.5 | 58.0 | 63.1 |
| —80 | 65.7 | 70.7 | 78.7 |
| —100 | 77.1 | 80.7 | 91.6 |
| —140 | 102.5 | 98.8 | 109.1 |
| —180 | 139.3 | 124.7 | 115.9 |

The results obtained by equations (1), (2) and (8) as shown in Table 3 and Fig. 5 are in agreement, in spite of the fact that correlation (1) is based on the change of freezing heat, (2) on the vapour pressure of water and ice and (8) on the specific heat of free and bound water.

*2.1.1. The melting heat of ice.* For the calculation of the temperature-dependent melting heat of ice, the following relationship in equation (1) was suggested by RYUTOV:

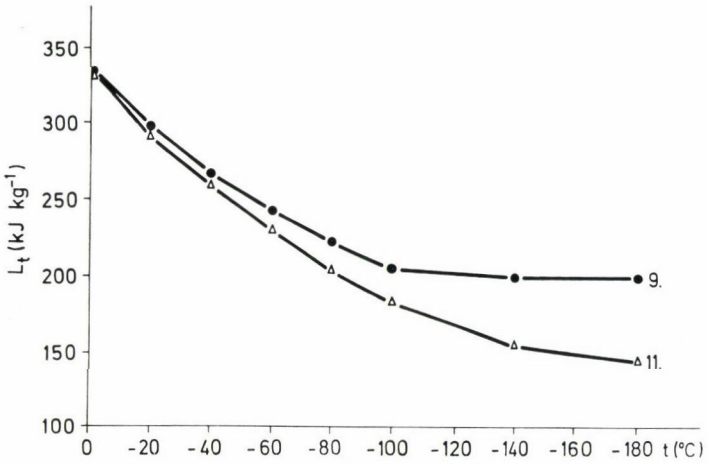


Fig. 6. Changing of the melting heat of ice as a function of temperature, calculated by equations (9) and (11)

$$L_t = 334.19 + 1.926t + 0.0069t^2 \text{ kJ kg}^{-1} \tag{9}$$

Melting heat may be calculated more adequately from equations:

$$\frac{\mathrm{d}L}{\mathrm{d}T} = c_v - c_i \tag{10}$$

$$L_t = L_0 + \int_{T_1}^{T_2} (c_v - c_i)\,\mathrm{d}T \tag{11}$$

$$c_i = 2.114 + 0.0071t \text{ kJ kg}^{-1} \text{ °C}^{-1} \tag{12}$$

where $c_i$ = specific heat of ice.

As may be seen in Fig. 6 decreasing values are obtained by the relationship (9) only up to $-140$ °C, hereafter a slight increase takes place, which, however, is not possible.

*2.2. Calculation of the binding energy taking into account the individual properties of foodstuffs*

Equation (3) accounts in member $E_0$ for the individual characteristics of foodstuffs. RIEDEL (1961) determined calorimetrically the sorption heat $E_0$ and obtained

$$E_0 = 502.4 \text{ kJ kg}^{-1}$$

with beef freeze-dried to 2% moisture content and subsequently rehydrated to 0.14 g moisture content per g dry matter. In the knowledge of $c_w$ and $c_{br}$

the binding energy may be calculated by equation (3) at temperatures below 0 °C.

Equation (4) accounts for the individual characteristics of the foodstuffs, too. To calculate the constant C in the *BET* equation the following known relationship was used:

$$\frac{a_w}{(1-a_w)\,x} = \frac{1}{x_m \cdot C} - \frac{(C-1)}{x_m \cdot C}\,a_w \tag{13}$$

$$S = (C-1)\,|\,x_m C$$

$$I = 1\,(x_m C)$$

where $a_w$ = water activity
$x_m$ = monolayer moisture content, g $g^{-1}$ dry matter
$x$ = moisture content belonging to the straight section of the isotherm and different from $x_m$, g $g^{-1}$ dry matter
$S$ = slope
and $I$ = intercept in the coordinate system $a_w\,|\,(1-a_w)\,x, a_w$

Since the isotherms were not published in the above cited work of RIEDEL (1961), only the water activity values of meat samples of various moisture contents it seemed more expedient to use the following relationships, pertinent to the monolayer values, and determine the constant $C$ from the water activity $(a_w)_m$ of the monolayer as calculated for different temperatures:

$$\frac{(a_w)_m}{1-(a_w)_m} = \frac{1}{C}\,[1 + (C-1)\,(a_w)_m] \tag{14}$$

$$(a_w)_m = \frac{-1 \pm \sqrt{C}}{C-1} \tag{15}$$

or

$$C = \left[1 - \frac{1}{(a_w)_m}\right]^2$$

In calculating the isosteric heat of sorption on the basis of equation (5) again the water activity of the monolayer moisture content of meat as calculated for different temperatures, was taken into account. The given temperatures $t$ are the mean values of $t+5$ °C and $t-5$ °C.

It is known from the work (Figs. 3 and 4) of KAUCHESHVILI and coworkers (1972) that the binding energy of water in beef varies between 360 and 1000 kJ $kg^{-1}$ at moisture content values of 0.44–0.08 g $g^{-1}$ dry matter. The temperature of measurements varied between +25 °C and +50 °C.

The heat of binding of the bound water and of the monolayer moisture content of beef as calculated by equations (3), (4) and (5), are shown in Table 4.

Table 4

*Heat of binding of bound water and of monolayer moisture content in beef*

| Temperature °C | Heat of binding of monolayer moisture content ($g\,g^{-1}$ dry matter) | Heat of binding of bound water (kJ $kg^{-1}$) according to equation | | |
|---|---|---|---|---|
| | | (3) | (4) | (5) |
| 0 | 0.08 | 502.4 | 178.2 | 477.2 |
| —20 | 0.10 | 478.6 | 186.8 | 449.0 |
| —40 | 0.12 | 457.6 | 182.2 | 420.7 |
| —60 | 0.137 | 439.2 | 195.6 | 405.9 |
| —80 | 0.174 | 423.7 | 215.4 | 375.5 |
| —100 | 0.200 | 420.8 | 231.4 | 341.5 |
| —140 | | 333.3 | | |
| —180 | | 386.5 | | |

On analysis of the data in the Table it may be seen that equations (3) and (5) lead to nearly identical results and these closely approximate the heats of binding as measured at substantially higher temperatures.

Results obtained by the *BET* equation (4) were considerably lower. IGLESIAS and CHIRIFE (1976c) arrived at similar conclusions when comparing for 29 different foodstuffs (fruits, chicken, fish, dairy products, vegetables, spices), the values obtained by the *BET* equation to the isosteric heat values of water vapour sorption in the moisture content range of 0.01–0.32 g $g^{-1}$ dry matter as calculated from the sorption isotherms plotted at +25 °C and +45 °C, resp., and related to 35 °C.

The value of isosteric heat of binding proved to be 2 to 7 times higher than the value obtained by the *BET* equation.

The "net" isosteric sorption heat values calculated by the above authors differed highly depending on whether caluculated on the basis of desorption or adsorption isotherms. The "net" isosteric sorption heat values generally increased steadily with decreasing moisture content and reached, in some of the foodstuffs, the value of 68.5 kJ $mol^{-1}$ (3 800 kJ $kg^{-1}$).

According to VARSÁNYI (1977) it has been realized recently that it is unfounded to lend such an energetic interpretation to the *BET* equation constant and thus equation (4) has no reasonable basis.

Therefore, to calculate the binding energy of bound water, it is expedient to use the relationships (3) and (5).

### *2.3. Definition of the quantity of bound water*

In order to be able to use any of the calculation methods suggested for the determination of heat of binding the quantity of bound water has to be defined. One value may be the monolayer moisture content ($x_m$) calculated from water activity – moisture content data by the *BET* equation. Another value of the quantity of bound water may be obtained from the amount related to the dry matter content ($x$) of the water not frozen upon freezing at a given temperature.

If the value of water activity related to $x_m$ and $x$ is known at two different temperatures, equation (5) may be used for the calculation of the heat of binding. If the value of the heat of binding is known for any temperature below 0 °C it may be calculated for any other temperature below 0 °C by equation (3).

### *2.4. The significance of heat of binding in freeze-drying*

The binding energy of bound water plays an important role in the desorption section of freeze-drying when the ice had sublimed from the product under freeze-drying and the water non-frozen and bound by different energies is being evaporated.

The heat requirement of freeze-drying is, with some omissions, as follows:

$$Q_1 = \frac{W_i}{100}(r_m + L_m) + \frac{W_b}{100}(r_m + E_m), \text{ kJ kg}^{-1} \tag{16}$$

where $Q_1$ = heat requirement of freeze-drying, kJ kg$^{-1}$
$W_i$ = percentage of water (W) turned into ice
$W_b$ = percentage of non-frozen, bound water
$W$ = moisture content % of the mass of foodstuff
$W = W_i + W_b$
$r_m$ = mean value of the heat of vaporization in the temperature range $t_{se}$–$t_f$
$L_m$ = mean value of the heat of melting of ice in the temperature range $t_{se}$–$t_{cr}$
$E_m$ = mean value of the binding energy of non-frozen bound water in the temperature range $t_{se}$–$t_f$
$t_{se}$ = starting temperature of freeze-drying
$t_{cr}$ = cryoscopic temperature
$t_f$ = final temperature of freeze-drying.

*

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# STUDIES ON THE FINE STRUCTURE OF WHEAT GLIADIN

## PART I.–GENERAL CHARACTERISTICS OF GLIADIN FRACTIONS. ANALYSIS OF THE HIGH MOLECULAR WEIGHT FRAGMENTS OBTAINED BY PAPAIN HYDROLYSIS

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A gliadin fraction (G-2) prepared from commercial wheat flour by gel chromatography was analysed. The gross amino acid composition, the quality and quantity of N-terminal amino acids as well as the amino acid composition of fragments obtained by polyacryl amide disc gel electrophoresis were determined.

The G-2 fraction was degraded by papain enzyme and the amino acid composition, quantity and quality of the N-terminal amino acids of polypeptide fragments of higher molecular weight (5–25 thousand) thus obtained, were determined. The number of amino acid molecules of these high molecular weight polypeptide sections and the number of some selected amino acid molecules [Glutamic acid (GLU), Glutamine (GLN), Proline (PRO) and amino acids with hyrophobic branched chains,] processed in a computer, permitted drawing conclusions as to the probable distribution of these amino acids in recurring sequence sections.

It was established that the higher molecular weight fractions obtained in the course of papain hydrolysis contained accumulated -GLU(GLN)-PRO monotonous sequence sections.

In recent years, research on wheat gliadin has shown a certain slackening. It is true that new methods of fractionation have been introduced and the fractions thus obtained were studied, however these investigations did not contribute substantially to the knowledge of gliadin (BOOTH, 1970; VARGA, 1973). The main problem was that new methods and their combination did not result in the separation of a homogeneous polypeptide either. Some encouraging results were obtained by the combination of gel electrophoresis and isoelectric focussing (WRIGLEY, 1972), however, these permitted of the deduction of some qualitative conclusions only.

Thus, beside the refining of the fractionation techniques and the application of new methods of separation, the analysis by other techniques of protein fractions obtained by group fractionation seems expedient. As to the technique to be used the gross amino acid composition of the protein to be studied and the results of partial hydrolyses, hitherto known, are informative.

The apparent heterogeneity of wheat protein fractions may be traced back to the heterogeneity of the terminal groups, since no significant difference has been detected in the amino acid composition of the polypeptide chain. Thus, the heterogeneity of the terminal groups is a consequence of

the different numbers of amino acids in the polypeptide chain. This assumption is borne out by electrophoretic analysis of proteins obtained by group fractionation, further by some results obtained by the combination of disc-gel electrophoresis with immunochemical reactions (NIMMO & SULLIVAN, 1967). These studies have shown all the gliadin fractions obtained by disc-gel electrophoresis to give a precipitation zone with gliadin antiserum. This may be explained only if each fraction contains a polypeptide chain section of identical structure.

The high gross glutamic acid, proline and aspartic acid contents, amounting to more than 50% of the total amino acid content, are probably not evenly distributed within the chain. This assumption is reinforced by some results of partial hydrolysis, where in certain peptide fractions the majority of hydrophoric acids appeared while GLU and PRO were missing (YERMA & MCCALLA, 1966).

On the basis of the above said it was decided to use papain in studies on the structure of gliadin fractions. Papain is a protease enzyme of plant origin. The analysis of model peptides has shown that it does not attack peptide fractions containing poly GLU(GLN).

Thus, it seemed suitable for the production of peptides or fragments of differing character.

## 1. Materials and methods

### *1.1. Materials*

*1.1.1. Gliadin fractions.* Second fraction in the chromatogram on *Sephadex* G-100 gel obtained by the method of *Osborn* from BL 112 wheat flour (max. ash: 1.12%) (Fig. 1). Mol weight: 30 000 (20 to 40 thousand). Protein content related to dry matter (*Kjeldahl* method): 98.2%. Degree of amidification: 85%. Symbol: G-2.

*1.1.2 Papain.* NUTRITIONAL BIOCHEMICALS Co. (Cleveland, Ohio) product. Cysteine with added EDTA-di-Na salt. Activity: 10 U $mg^{-1}$.

*1.1.3. Cation exchange raisins. Dowex* $50 \times 2$ and *Dowex* $50 \times 8$. Products of SIGMA CHEMICAL Co. (USA) Mesh: 100–200.

*1.1.4. Dextran gel. Sephadex* G-15 *Sephadex* G-75 and G-100 (PHARMACIA FINE CHEMICALS, Uppsala, Sweden). Grain: 40–120 $\mu$m.

*1.1.5. Reagents used in gel electrophoresis.* Polyacryl amide (PAG) set of reagents (REANAL Composition) analytical grade. Glacial acetic acid, 96% (ERDŐKÉMIA, Hungary), analytical grade. Urea (REANAL, Hungary), analytical grade.

*1.1.6. Polygram.* Kieselgel (ready-made Kieselgel layer, MACHEREY-NAGEL, Co., F.R.G.) and *Fixion (Dowex* 50×8) of Na-cycle, cation exchange thin-layer (CHINOIN, Hungary).

*1.2. Methods*

*1.2.1. Preparation of gliadin Fraction G-2.* Wheat flour was extracted with petroleum ether and butanol to obtain gluten. The gliadin fraction was extracted from the air-dried gluten with 70% alcohol. This fraction was purified by repeated precipitation and re-dissolution (2×), then fractionated on *Sephadex* G-100 gel, swollen in 0.1 *M* acetic acid. Ten ml were applied containing 300 mg protein in 10 ml 0.1 *M* acetic acid. The second fraction was collected, precipitated by dialysis and dried at room temperature.

*1.2.2. Determination of the protein content.* The *micro-Kjeldahl* method was used. A protein sample of 20 mg was wet-ashed in 5 ml *conc.* $H_2SO_4$ and two drops of $H_2O_2$, for 80 min. Then it was diluted fourfold with distilled water and the $NH_3$ determined. The protein content was obtained by multiplying by 5.7.

*1.2.3. Determination of the gross amino acid composition.* An amount of 50 mg gliadin preparation was hydrolyzed in 5 ml *conc.* HCl, in a sealed ampoule, under $N_2$ atmosphere at 105±1 °C for 32 h. The hydrolysate was filtered, evaporated to dryness on a *Rotadest* apparatus and taken up in a citrate buffer of pH 2.2. The amino acids were determined in an AAA 831 (MIKROTECHNA, Czechoslovakia) type analyser applying a 2-column technique.

Tryptophan was determined from a separate sample of 20 mg after maceration with $Ba(OH)_2$ at 105 °C for 32 h. The filtered and evaporated sample was run on a *Fixion* plate. The tryptophan (TRP) was detected with ninhydrin and the quantity was determined in the *Vitatron* densitometer, by comparing the spot with a known TRP spot.

*1.2.4. Determination of the N-terminal amino acids.* The N-terminal amino acids in the gliadin fraction were determined by the method developed by the author (VARGA, 1967). The dinitrophenyl (DNP) protein was hydrolyzed with 5.6 *N* HCl at 105 ± 1 °C during 18 h in a sealed ampoule under $N_2$ atmosphere.

The ether-soluble DNP amino acids were separated on *Polygram* layer by two-dimensional technique. Quantitative evaluation was carried out directly on the layer with a *Chromoscan* densitometer, by comparing the spot with that of known amounts of DNP amino acids.

The water-soluble DNP derivatives were separated by one-dimensional, repeated running technique.

*1.2.5. Disc polyacryl amide gel electrophoresis (PAG) of the gliadin fraction.* Separation was carried out in an acid system (pH 3.5) with 10% acryl amide gel concentration, using a spacer gel layer. Both the gel and electrode buffer contained 4 *M* urea. The buffer was prepared with $\beta$-alanine and glacial acetic acid. When the disc electrophoresis was finished the protein fractions were fixed in 10% trichloro acetic acid solution (TCA) and dried with *Coomassie Brillant Blue* 250 (C.B.B. 250). (The dye consisted of 1 ml of the aqueous 1% solution of C.B.B. 250 and 19 ml 12.5% TCA solution). The method is advantageous because only the protein zones become coloured. Quantitative evaluation was carried out directly in the gel rods with the *Chromoscan* densitometer at 620 nm.

*1.2.6. Gross amino acid composition of fractions $\omega$, $\gamma$, $\beta$, $\alpha$, obtained by gel electrophoresis.* The gel disc containing the desired fraction was cut out (after fixing with TCA) then hydrolyzed with *conc.* HCl in an ampoule at $105 \pm 1$ °C for 32 h, under $N_2$ atmosphere. The fractions were filtered, evaporated to dryness, dissolved in pH 2.2 citrate buffer and the amino acids were determined in an amino acid analyser.

*1.2.7. Enzymatic hydrolysis of the gliadin fraction and separation by gel chromatography of the degradation products.* Portions of 300 mg were hydrolyzed with papain. These were dissolved in 0.2 *N* KOH ($\sim$ 50 ml). Their pH was adjusted to 7.2 with 1% acetic acid. A 2% papain solution protected with 2 ml NaCN was used to dissolve the protein. Degradation was carried out at 37 °C during 24 h, under $N_2$ stream. Subsequently again 1 ml of the enzyme solution was added and the hydrolysis continued. After a total of 48 h degradation, the pH of the hydrolysate was adjusted to 9.0 (0.5 *N* KOH), hydrolysis was stopped by boiling. It was then condensed in a rotating vacuum evaporator at 25 °C and converted into a 0.1 *M* solution with acetic acid ($\sim$15 ml). The dialysate was desalted (*Dowex* $50 \times 4$ cation exchanger $H^+$ column, the amino acids were eluted with 7 *N* $NH_4OH$) and the amino acids determined in an analyser.

The concentrated peptides were dissolved in 0.1 *M* acetic acid and fractionated on *Sephadex* G-15 gel. The fractions were detected in the LKB system in UV absorption units.

## 2. Results and conclusions

### *2.1. The amino acid composition of gliadin*

The distribution of fractions obtained by chromatography on *Sephadex* G-100 gel from a gliadin preparation prepared by the method of *Osborne* is shown in Fig. 1.

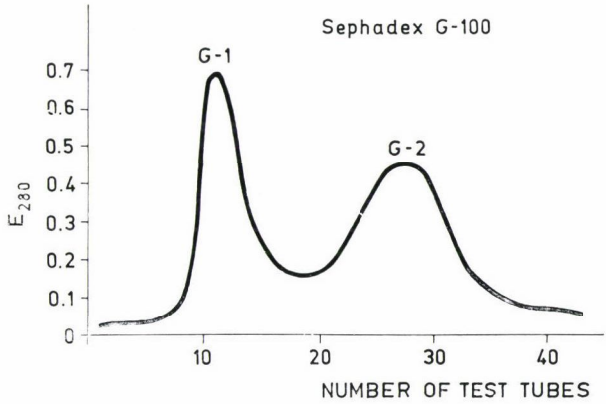


Fig. 1. Gel-chromatographic elution diagram of gliadin dissolved in 0.1 *M* acetic acid. Column dimensions: 4×35 cm. Elution volumes: 10 ml per test tube

Further investigations were aimed at Fraction G-2. The fraction, as is shown in the figure, represents ~65% of the total gliadin. The protein content of Fraction G-2 was found to be 98.2%, determined according to *Kjeldahl* and the result multiplied by a factor of 5.7.

The gross amino acid composition of Fraction G-2 is shown in Table 1.

### *2.2. N-terminal amino acids in gliadin*

Data related to the N-terminal amino acids are given in Table 2.

As it may be seen in the Table, valine (VAL) and leucine (LEU) form about 50% of the total of N-terminal amino acids. It will be shown later that these form the terminal groups of the polypeptides characteristic of gliadin.

### *2.3. Electrophoretic properties of the gliadin fraction*

The results of the disc gel electrophoresis of the gliadin fraction are illustrated in Fig. 2.

The electrophoresis, carried out in an acid medium in the presence of urea resulted in 10 fractions, discernible by the naked eye. The majority of the fractions is $\gamma$-gliadin (~ 60–70%).

The gross amino acid compositions of fractions $\omega$, $\gamma$, $\beta$, $\alpha$ is given also in Table 1.

### *2.4. Amino acid composition of the fractions separated by gel electrophoresis*

As it can be seen the $\alpha$-gliadin fraction differs in its amino acid composition from the other fractions.

Table 1

*Gross amino acid composition of Fraction G-2 and of the characteristic fraction groups separated by disc-gel electrophoresis from G-2*
(g amino acid in 100 g protein, related to dry matter)

| Amino acids | Fraction G-2 | Fractions separated by gel electrophoresis from G-2 | | | |
|---|---|---|---|---|---|
| | | $\omega$ fractions | $\gamma$ fractions | $\beta$ fractions | $\alpha$ fractions |
| LYS | 0.35 | — | 0.30 | 0.25 | 0.30 |
| HIS | 1.80 | 0.70 | 1.90 | 1.80 | 1.90 |
| ARG | 3.20 | 2.80 | 3.20 | 2.80 | 4.20 |
| ASP | 2.40 | 2.40 | 1.80 | 2.50 | 3.10 |
| THR | 1.80 | 1.60 | 1.80 | 2.20 | 2.50 |
| SER | 5.10 | 5.00 | 5.40 | 4.80 | 4.70 |
| GLU | 37.40 | 40.60 | 38.60 | 36.30 | 30.80 |
| PRO | 13.20 | 11.50 | 13.60 | 10.80 | 11.00 |
| CYS | 2.60 | 0.80 | 0.90 | 3.10 | 4.20 |
| GLY | 2.50 | 2.10 | 2.50 | 2.60 | 2.05 |
| ALA | 2.40 | 2.60 | 2.30 | 2.50 | 3.00 |
| VAL | 3.80 | 3.60 | 3.40 | 4.60 | 4.40 |
| MET | 1.30 | 1.50 | 1.20 | 1.00 | 1.10 |
| ILE | 5.50 | 5.80 | 5.30 | 5.40 | 5.20 |
| LEU | 8.40 | 8.60 | 8.20 | 7.90 | 9.10 |
| TYR | 0.80 | 0.90 | 0.80 | 1.10 | 1.40 |
| PHE | 6.40 | 6.00 | 6.80 | 6.70 | 7.40 |
| TRP | 1.10 | 1.05 | 0.80 | 1.00 | 1.30 |

Table 2

*N-terminal amino acids in gliadin Fraction G-2*

| N-terminal amino acids | DNP-amino acid $\mu$g (in 20 mg DNP-protein) | N-terminal amino acid, $\mu$g / 1 g protein | N-terminal amino acid, $\mu$mole / 1 g protein | N-terminal amino acids $\mu$mole rounded up to integer / 1 g protein |
|---|---|---|---|---|
| ASP | 8.2 | 181.0 | 1.35 | 1 |
| GLU | 15.0 | 350.0 | 2.38 | 2 |
| SER | 6.0 | 150.0 | 1.05 | 1 |
| THR | 8.5 | 179.0 | 1.44 | 1 |
| GLY | 4.0 | 63.0 | 0.88 | 1 |
| ALA | 7.5 | 129.0 | 1.10 | 1 |
| VAL | 45.3 | 919.0 | 8.12 | 8 |
| LEU | 12.0 | 261.7 | 1.70 | 2 |
| PHE | 7.0 | 173.0 | 1.07 | 1 |
| HIS | 20.4 | 651.0 | 4.21 | 4 |

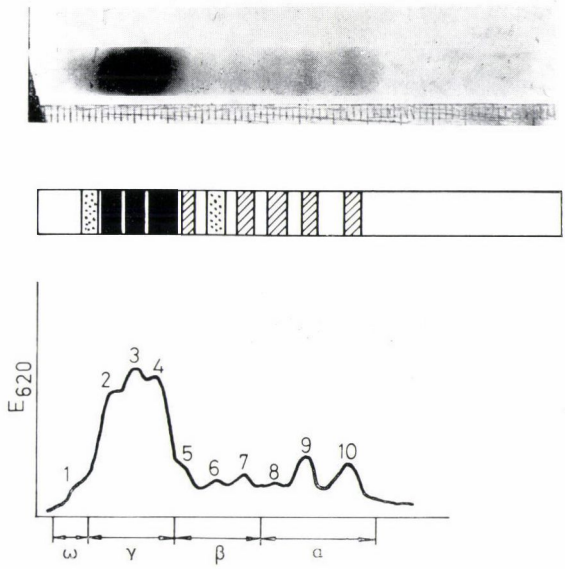


Fig. 2. Polyacryl amide disc electrophoresis and electrophoretogram of Fraction G-2

The different character of the polypeptides is apparent in the greater quantity of basic amino acids and in the lower quantity of GLU. A higher amount of the sulfur containing cystine (CYS) is also characteristic. This fact (also characteristic of $\beta$-fractions) points to the intermolecular —S—S— bonds of these fractions. Presumably this proves also the more globular character of these fractions.

### *2.5. Analysis of the products of hydrolysis by papain*

Fraction G-2 was dialysed subsequent to hydrolysis by papain. The peptides were then separated on *Sephadex* G-15 gel column. The liberated amino acids thus separated were concentrated and determined in the analyser. Results are shown in Table 3.

The free amino acids formed *ca.* 3% of the original protein. The large quantity of hydrophobic amino acids is striking (ALA and VAL are present in above 22%). The quantity of GLY was also found substantial. This tendency is an evident consequence of the degradation mechanism of papain, since this enzyme attacks amino acids with hydrophobic branched chains. The larger quantities of the above mentioned amino acids seem a proof of the higher incidence of splitting sites beside these amino acids, at the same time of the location of these amino acids near to chain ends or to one another.

The distribution in the papain hydrolysate is illustrated by the elution diagram in Fig. 3.

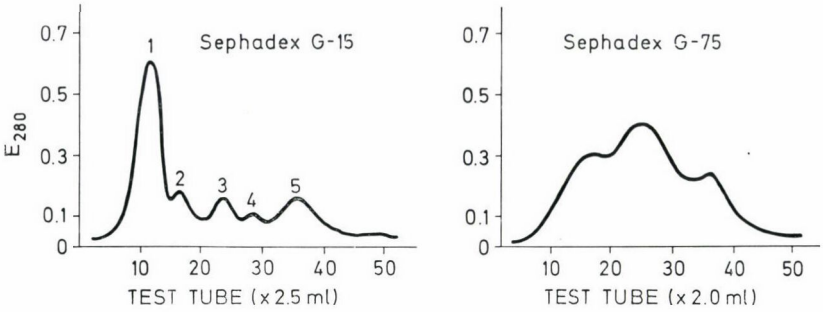


Fig. 3. Fractionation by gel chromatography of the papain hydrolysate of Fraction G-2. Column dimensions: 60 × 1.5 cm. Eluent: 0.1 *M* acetic acid. Further separation of the 1st fraction obtained on *Sephadex* G-15 from papain hydrolysate of Fraction G-2. Column dimensions: 50 × 1.5 cm. Eluent: 0.1 *M* acetic acid

Table 3

*Free amino acids separated by dialysis from papain-degraded Fraction G-2*
(Percentage of total amino acid content)

| Amino acid | Percentage | Amino acid | Percentage |
|---|---|---|---|
| HIS | 1.7 | VAL | 7.1 |
| ARG | 4.6 | MET | 1.2 |
| ASP | 5.5 | ILE | 4.1 |
| THR | 2.4 | LEU | 8.4 |
| SER | 5.3 | TYR | 1.0 |
| GLU | 19.1 | PHE | 4.8 |
| PRO | 4.8 | CYS | 3.1 |
| GLY | 12.3 | | |
| ALA | 15.2 | | |

As may be seen, the polypeptide sections above a molecular weight of 1500–2000 (number of members 10–15) amount to 50% (G-21). The number of separated peptide fractions is four (G-22, G-23, G-24, G-25). This fractionation occurs not necessarily according to molecular weight, peptides with aromatic ring containing amino acids exert an effect on it.

The fraction containing excluded polypeptide sections (G-21) was further degraded on *Sephadex* G-75 gel and the separation of three fractions could be observed (G-211, G-212, G-213) (see Fig. 3). The molecular weights of individual fractions varied between 5 and 25 thousand. The major part had a molecular weight of 20,000.

The gross amino acid composition of the fractions and the qualitative and quantitative relations of the N-terminal amino acids are illustrated in Figs. 4 and 5.

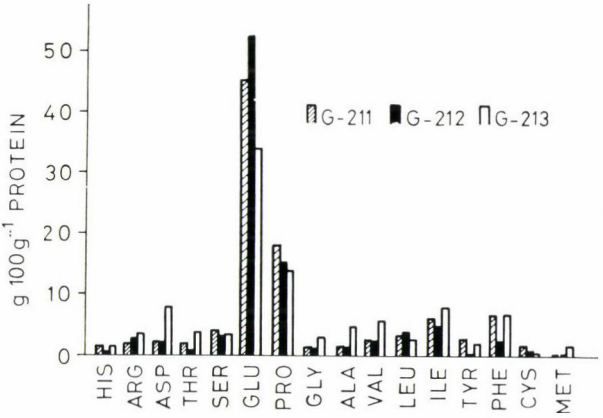


Fig. 4. Gross data of the amino acid composition of fractions obtained on G-75 gel from Fraction G-21; (g amino acid per 100 g protein dry matter)

It is strikingly apparent in the column diagram of Fig. 4 that the first two fractions have an increased GLU content (45 and 52%, resp.). The PRO content is also high, particularly in the first fraction. The quantity of the amino acids of hydrophobic character, such as alanine (ALA), VAL, LEU and isoleucine (ILE), showed a diminishing tendency. Taking into account that in the first and second fractions the pooled quantity of GLU and PRO amounts to 63 and 67%, resp., it is evident that within the polypeptide chain they must be placed side by side, bound together. On determining the numbers of molecules from the molecular weights and data of amino acid composition of the fractions, the most probable distribution of the above mentioned amino acids within the polypeptide chain may be obtained by means of a computer program, for a statistically recurrent chain section (EWART, 1973).

The calculation is carried out on the basis of the following correlation

$$P = \frac{\sum_{j=1}^{j=\frac{n}{S}} (-1)^{j+1} \cdot C_j^{N-n+1} \cdot C_{n-jS}^{N-jS}}{C_n^N}$$

where P = probability
N = number of amino acids in the polypeptide
n = number of the selected amino acids
S = probable number of amino acids (2,3,4, . . . . etc.) in the statistically recurrent chain sections
C = number of repetitions, combinations.

$$C_n^N = \frac{N!}{n!\,(N-n)!}$$

The results obtained by computer pertinent to gliadin Fraction 2 are shown in Table 4.

Table 4

*Different probabilities of GLU(GLN), GLU(GLN)PRO and hydrophobic amino acids in recurrent sequence sections of the polypeptide chain*

| Amino acid grouping | Number of molecules in the amino acid group in the polypeptide chain of *320 members* | Probability of occurrence if the number of amino acids selected in the recurrent sequence section is | | | | |
|---|---|---|---|---|---|---|
| | | 2 | 3 | 4 | 5 | 6 |
| GLU(GLN) | 100 | 1.000 | 0.999 | 0.901* | 0.477 | 0.172 |
| GLU(GLN) PRO | 144 | 1.000 | 1.000 | 0.999 | 0.978* | 0.787* |
| Hydrophobic and other amino acids | 76 | 1.000 | 0.978* | 0.542 | 0.156 | 0.037 |

* Probability value co-ordinated to the still probable number of amino acid members

It may be seen in the Table that in the recurrent polypeptide sequence sections there are at least 4 GLU(GLN) + 1 PRO, or 3 hydrophobic branched-chain or other amino acids. Presuming the statistical character of the distribution two GLU, or one GLU and one PRO are located side by side in each chain section. Computer data related to the fractions separated on G-75 gel of papain-degraded polypeptide chain sections of higher molecular weight, are summarized in Table 5.

It is evident from the data that in the recurrent chain sections, beside 5–6 GLU(GLN), 2–3 PRO and 2 hydrophobic or other amino acids may be present. This in turn proves the presence, in chain sections of 9–11 members, of accumulated glutamine (glutamic acid)-proline units. If the recurrent sequence sections are not of random distribution there is a high probability of poly-GLU—PRO chain sections.

The N-terminal data related to the three gliadin fractions as mentioned above, are represented on the column diagram in Fig. 5. The diagram shows unambiguously the presence in every fraction of ALA, VAL, and LEU N-terminals and these are decisive quantitatively as well. A significant amount of GLU is present in Fraction 3.

By comparing these data with those obtained by the computer the structure of gliadin appears to be as follows:

— at the N-terminals of polypeptide sections hydrophobic and other amino acids (but no GLU and PRO) are located;
— fractions of higher molecular weight (G-211 and G-212) contain with high probability accumulated GLU and PRO containing monotonic sequence

Table 5

*Distribution probability of fractions separated on Sephadex G-75 from the higher molecular weight polypeptide sections in papain-hydrolyzed Fraction G-2*

Gliadin Fraction G-211

| Amino acid group | Number of amino acid molecules in the polypeptide chain of *192 members* | Probability of occurrence if the number of selected amino acids is in the recurrent sequence section | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| GLU(GLN) | 78 | 1.000 | 1.000 | 0.979* | 0.734 | 0.388 | 0.168 | 0.067 | — | — |
| GLU(GLN)PRO | 116 | 1.000 | 1.000 | 1.000 | 1.000 | 0.999 | 0.933* | 0.770 | 0.558 | 0.267 |
| Hydrophobic ond other amino acids | 38 | 0.999* | 0.721 | 0.194 | 0.037 | — | — | — | — | — |

Gliadin Fraction G-212

| Amino acid group | Number of amino acid molecules in the polypeptide chain of *103 members* | Probability of occurrence if the number of selected amino acids is in the recurrent sequence section | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| GLU(GLN) | 53 | 1.000 | 1.000 | 0.996 | 0.911* | 0.684 | 0.354 | 0.184 | 0.089 | — |
| GLU(GLN)PRO | 73 | 1.000 | 1.000 | 1.000 | 1.000 | 0.999 | 0.980 | 0.910* | 0.799 | 0.637 |
| Hydrophobic and other amino acids | 16 | 0.935* | 0.258 | 0.036 | — | — | — | — | — | — |

Gliadin Fraction G-213

| Amino acid group | Number of amino acid molecules in the polypeptide chain of *77 members* | Probability of occurrence if the number of selected amino acids is in the recurrent sequence section | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| GLU(GLN) | 23 | 0.999 | 0.875* | 0.326 | 0.092 | 0.023 | — | — | — | — |
| GLU(GLN)PRO | 45 | 1.000 | 1.000 | 0.999 | 0.957* | 0.778 | 0.523 | 0.312 | 0.121 | — |
| Hydrophobic and other amino acids | 21 | 0.999* | 0.756 | 0.237 | 0.060 | 0.013 | — | — | — | — |

* Probability value co-ordinated to the still probable number of amino acid members

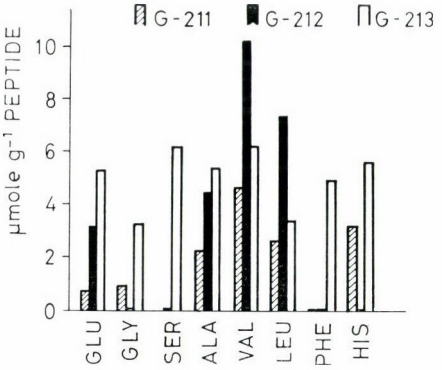


Fig. 5. N-terminal amino acids of fractions obtained on *Sephadex* G-75 gel from the Fraction G-21 (μmole per g peptide)

sections. The resistance shown to degradation by papain also proves the above;

– since the molecular weight of Fractions G-211 and G-212 is around 15 and 25 thousand the peptide sections containing non-degraded accumulated GLU—PRO groupings come from the inside of the original peptide chain. Otherwise, from the original units of 30 000 molecular weight, sections of such high molecular weight could not have resisted splitting;

– amino acids of hydrophobic branched-chains accumulated in peptides of lower molecular weights which in their turn were located nearer to the ends of the polypeptide chains.

Forthcoming publications will contain the results obtained by investigations on peptides of low member number formed during papain degradation.

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# CONTRIBUTIONS TO THE DEVELOPMENT OF SENSORY EVALUATION METHODS

## PART I. — RECOGNITION TESTS WITH AQUEOUS SODIUM CHLORIDE SOLUTIONS OF DIFFERENT CONCENTRATION

F. KULCSÁR, ZS. FALUSI and J. KOVÁCS

(Received June 10, 1977, revision received April 2, 1978, accepted May 26, 1978)

An assay was made to establish how many different salt concentrations may be recognized by panelists by memory, after a single tasting. At the same time three difference tests were compared. It was found that if the difference between two concentrations is at least 0.03% the panelists are capable to recall five concentrations. The study has shown that recognition of concentrations by memory is not as accurate as by difference tests.

In the evaluation of foods sensory analysis plays an important part. In the last 10–15 years, many authors have published comprehensive reviews on the bases, problems and methods of sensory analysis (AMERINE *et al.*, 1965; VAJDA, 1969a, b; TILGNER, 1971; KIERMEIER & HAEVECKER, 1972; TAKAGI, 1973; HARRIES, 1973; SARDINI, 1973).

The authors all agree as regards the rapid development of analytical methods, and of the theory of sensory evaluation in recent years. Several trends in the development evolved.

The results of sensory analyses can realistically be interpreted only by mathematical statistical methods. Important mathematical statistical tests were developed to eliminate the uncertainity factor in the sensory evaluation methods (ÖRSI, 1972; KOCHAN & LENDVAI, 1974; ÖRSI & KOCHAN, 1975).

The methods of analysis made a significant progress, too. Of these, the various difference tests, described in the comprehensive works proved to be the most sensitive: tetrad test of RENNER and RÖMER (1973) and limit dilution method of TILGNER (1962).

Partly to replace, partly to complement sensory evaluation instrumental test methods were also developed (SYDOW, 1971). Determination of the shape, the number and size of various components and the determination of colour in certain products may be efficiently executed by instrumental measurement instead of organoleptic methods. However, the sensory evaluation of taste and odour may, presumably for long, not be replaced by aroma determination methods.

The capability for sensory evaluation and the improvement of this capability by special training has also been the subject of thorough investi-

gation (TELEKI-VÁMOSSY, 1974; MOLNÁR & FALUSI, 1977). On the basis of results achieved in many countries, including Hungary, the selection and training of persons capable of sensory evaluation are specified in standards. The aim of this study was to establish the taste sensitivity of judges who — according to HUNGARIAN STANDARD (1976) possess the capability of tasting and have certain experience in sensory evaluation. In selecting panel numbers and in their training, care is taken to establish their taste threshold, their capacity of confounding taste, however, little attention is paid to their capability of discerning differences in concentration. A low taste threshold value and the capability of recognizing tastes form the precondition only of being suitable for evaluation. The task in practical taste testing is to recognize grades of characteristic tastes by memory.

The capacity to recognize concentration is important in view of standardization, too. At present sensory qualities are judged by scoring tests. Foods are judged by comparison to a "theoretically best food", since most foods cannot be produced in that quality. The poorest part of the scoring tests is that describing taste and odour. It is very difficult to describe the taste effects sensed and their changes with decrease of the quality. To achieve a more accurate description, the faculty to assess by memory several taste concentrations after appropriate training is a great asset.

We plan to start with the study of basic tastes and then, in the knowledge of basic correlations, to advance to the analysis of mixed flavours. In the present paper our study into the recognizing ability for salty taste is reported. The sensory response to salty taste has been the subject of a great number of investigations (JOHANSSON *et al.*, 1973).

Since this sensory effect is the function of NaCl concentration only, it is relatively easy to study it. We tried to establish the number of salt concentrations the panelists are capable to recognize by memory, after a single experimental experience. In parallel to the concentration recognizing tests, the faculties of the judges were studied by difference tests as well.

## 1. Materials and methods

The test panel consisted of five members. In selecting them they were put to a preliminary test as described in an earlier paper (MOLNÁR & FALUSI, 1977) and were found to exhibit the lowest threshold value.

### *1.1. Preparation of the solutions*

Solutions were prepared according to HUNGARIAN STANDARD (1976). Analytical grade NaCl, dried at 105 °C for an hour, was dissolved in water

previously boiled for 10 min and filtered. The model solutions thus prepared were kept in brown reagent bottles.

*1.2. Procedure*

The panel members, separated from one another, received the solutions in coded bottles and tasted them in spoons.

*1.3. Memory tests*

Panel members could get acquainted with the solutions by tasting them several times on the day prior to the test. The solutions were marked: mild, medium, strong and stronger. On the next day the solutions were presented to the members in coded reagent bottles one by one and they had to identify them into the above categories.

The memory tests were carried out first with three, four and finally with five solutions of different concentrations. The concentrations were selected to include salty taste from mild to very intense (0.02–0.14%).

In the 1st series, the concentrations were 0.02, 0.08 and 0.14%, marked mild, medium and strong, resp. The samples were understood on a single occasion and then examined on six different occasions.

In the 2nd experimental series, the concentrations were 0.02, 0.05, 0.08 and 0.14%, marked very mild, mild, medium and strong, resp. These samples were understood by the panel at two different occasions.

In the 3rd experimental series, the concentrations were 0.02, 0.05, 0.08, 0.11 and 0.14%, marked very mild, mild, medium, strong, very strong, resp.

*1.4. Paired comparison*

The members received sample pairs and had to decide which of the two was more salty. The concentrations of the solutions were between 0.10 and 0.20%.

*1.5. Triangle tests*

In the triangle test, two identical and one different samples are presented simultaneously and the judge is asked to indicate the odd sample. Here the same concentrations were applied as in the memory tests.

*1.6. Tetrad test*

The members received 4 solutions. One was served as the standard. They had to decide which one (or two) of the solutions was of the same

concentration as the standard. The same salt concentrations were applied as in the previous tests.

## 2. Results

The results of the three experimental series of memory tests are given in Table 1. As can be seen, the number of correct answers, when 3 or 4 salt concentrations had to be differenciated, always exceeds the number necessary to reach 99% statistical significance. In the case of 5 test samples the probability level was somewhat lower (96%).

Table 1

*Memory tests with salt solutions and the mathematical statistical evaluation of the results*

| Date Sept. 1976 | Number of concentrations to be recognized | Experimental series | Number of correct judgements | Number of correct judgements required at P = 5% | Error of first kind (%) |
|---|---|---|---|---|---|
| 6. | 3 | 10 | 9 | 7 | 0.036 |
| 7. | 3 | 10 | 8 | 7 | 0.340 |
| 8. | 3 | 10 | 8 | 7 | 0.340 |
| 10. | 3 | 10 | 8 | 7 | 0.340 |
| 13. | 3 | 8 | 8 | 6 | 0.015 |
| 14. | 3 | 10 | 8 | 7 | 0.340 |
| 15. | 4 | 10 | 9 | 6 | 0.003 |
| 16. | 4 | 10 | 8 | 6 | 0.042 |
| 17. | 5 | 10 | 5 | 4 | 3.280 |

The error of first kind calculated on the basis of the binomial distribution shows the probability of error in our conclusions. The acceptable limit of error P = 5%.

The judgements received in these three series were evaluated from the aspect of individual salt concentrations. The frequency of mistaken judgements is illustrated in Fig. 1. It is interesting to note that the ratio of mistaken judgements was highest with medium concentrations and the recognition of mild and strong solutions worked at much higher probability levels.

The difference tests served to check the recognizing ability if judges examined tastes by memory instead of a direct comparison with a standard. The results are shown in Table 2. It may be seen that the difference methods permit of differentiation between smaller differences in concentrations than the memory tests. Thus, if the aim is to identify by memory as many concentrations as possible, reducing the differences in concentrations is not permissible.

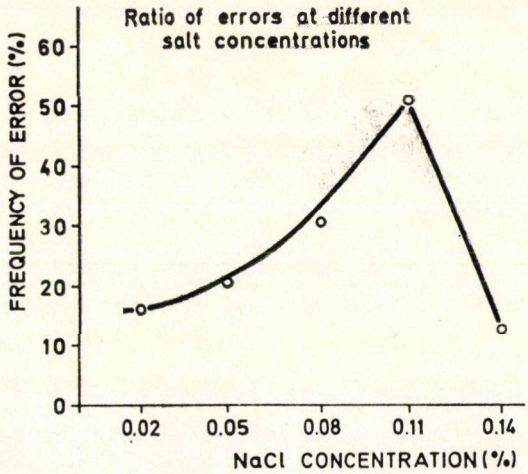


Fig. 1. Ratio of errors at different salt concentrations. The ratio of errors in recognizing individual concentrations by memory in the concentration range of 0.02–0.14% NaCl

Comparing the three differentiation methods, the paired comparison is recommended for use in the selection and preparation of tasters for some more intricate tests because of its simplicity, of the ratio of errors in these experiments and of its popularity.

Table 2

*Results of memory and difference tests of salty taste and their mathematical statistical evaluation*

| Method of analysis | Range of differences in concentrations (%) | Number of tests | Number of correct judgements | Number of correct judgements required at P = 5% | Error of first kind (%) |
|---|---|---|---|---|---|
| Memory test | 0–0.045 | 10 | 7 | 7 | 1.9 |
| | 0.045–0.09 | 32 | 31 | 14 | $4 \cdot 10^{-12}$ |
| | 0.09 | 6 | 6 | 5 | 0.14 |
| Paired comparison | 0.005 | 25 | 16 | 18 | $11.5 \cdot 10^{-2}$ |
| | 0.01 | 25 | 22 | 18 | $8 \cdot 10^{-3}$ |
| | 0.02 | 25 | 22 | 18 | $8 \cdot 10^{-3}$ |
| | 0.03 | 25 | 25 | 18 | $3 \cdot 10^{-6}$ |
| | 0.05 | 25 | 25 | 18 | $3 \cdot 10^{-6}$ |
| Triangular test | 0.05 | 30 | 26 | 15 | $2 \cdot 10^{-7}$ |
| Quadrangular test | 0.02 | 5 | 3 | 3 | 3.5 |
| | 0.03 | 10 | 9 | 5 | $8 \cdot 10^{-5}$ |
| | 0.05 | 12 | 11 | 5 | $3 \cdot 10^{-6}$ |

## 3. Conclusions

As it is seen from the experiments with aqueous sodium chloride solutions, experienced tasters are capable of differentiating between five salt concentrations if the difference between concentrations is at least 0.03%. Difference tests enable differentiation between samples with concentration differences as low as 0.01%. The ratio of error was highest with medium salt concentrations. To explain this phenomenon further investigations are necessary.

The results show that in test standards, where difference tests cannot be applied, it is not advised to present more than five concentrations, because panel members usually cannot recognize more.

*

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# CHANGES IN THE PROPERTIES OF LARD AS AN EFFECT OF RANDOM INTERESTERIFICATION

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This study is concerned with the interesterification of lard under varied conditions (catalyst 0.2% metallic sodium or 0.5% sodium methoxide; temperature 90, 100, 110, 120, 130, 150 °C; time: 1, 2 and 3 h, resp.). According to the results, the reaction occurs in the presence of 0.5% sodium methoxide at 90 °C during 60 min and is, therefore, extremely useful in the manufacture of edible fats and margarines.

As an effect of interesterification the complete melting point of lard was reduced from 45.8 °C to 40.8–42.7 °C and its dilatation curve became less steep. The distribution of triglyceride groups both according to number of carbon atoms and to saturation of the fatty acid components, changed. On testing distribution according to number of carbon atoms a significant change was found in the following two groups: the quantity of the triglyceride group with 52 carbon atoms in the three fatty acid components, interesterified with sodium methoxide diminished by 21.4%, while that of the group of 54 carbon atoms increased by 13.8%. The triglyceride structure turned more heterogeneous.

After interesterification the consistency of the fats became harder and the loose crystal structure, consisting of large crystals, more dense, as established by penetration tests. The margarine base made of interesterified lard improved, the slope of its dilatation curve decreased and its structure became microcrystalline.

It is an important task of the vegetable oil industry to produce fats suitable for very different nutritional and technical requirements. The characteristics of a fat are functions of two factors, the fatty acid composition and the triglyceride composition. Therefore the fat satisfying a definite requirement has to be suitable from the aspect of both factors.

The appropriate composition is achieved by the industry since almost 100 years by two methods: hydrogenation and blending. In recent years a third method of processing became generally used in practice abroad, *i.e.* interesterification (GROS & FEUGE, 1949; PERÉDI, 1955b).

In the course of this process the various esters (triglycerides) react through their alkyl and alkoxy groups and thus a triglyceride structure different from the original is formed.

This reaction permits of further development of the mixtures of both natural and hydrogenated fats in order to improve their consistency in the rheological sense. A number of fats are either too mellow or too brittle or become crystalline or granulous during storage unsuitable for consumption

or for use in the baking or confectionary industries. These unfavourable properties may be eliminated by interesterification. It is estimated that nearly one million tons are processed in this way per year all over the world (DGF, 1973).

The reaction is easy to carry out. The glycerides of the selected fat mixture are made to react at a relatively low temperature (0–100 °C) within a short period in the presence of an alkali-alcoholate or an alkali metal catalyst.

The mechanism of the reaction is ionic and occurs by mesomerism in two steps: first the catalyst is additioned and then the anion of the catalyst and the alkoxy group of the ester are exchanged (NAUDET, 1976). (Fig. 1.)

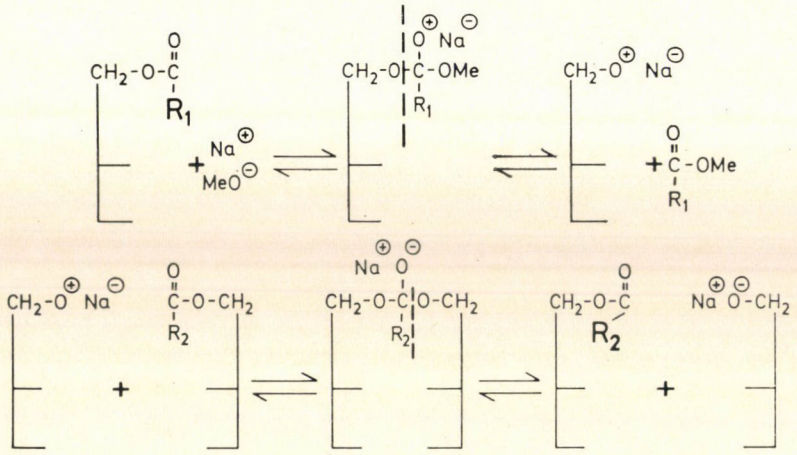

Fig. 1. Reaction mechanism of interesterification

As an effect of the second step the catalyst has changed, however, it remains active and capable of reacting. Under the influence of oxygen, always present, the fat reacts with metallic sodium to form diacyl peroxide and sodium methoxide and the reaction proceeds as above (GAUGLITZ & MALINS, 1960).

Thus the reaction is bimolecular and therefore its equilibrium is a function of concentration proportions (DGF, 1973). It does not require substantial energy input, the equilibrium constant of the individual reaction equals about 1. This in its turn means that the result follows statistical laws and may be calculated by combinatorial methods. Thus, the main problems of interesterification are touched upon: the triglyceride composition of individual fats and the extent of change affected by interesterification.

As regards the triglyceridé composition of fats, several theories are known: completely "even" distribution (HILDITCH, 1956), "random" distribution (HILDITCH, 1956) and the "partial random" distribution (KARTHA, 1953). According to investigations carried out hitherto, none of them is of general validity. Complying with the laws of biogenesis a variety of compositions may exist, however, the partial random distribution is the most frequent.

Since the final result of interesterification approximates random composition the final result of the reaction may be inferred if the original composition is known (BAILEY, 1951).

The calculation requires thorough work (PERÉDI, 1955a), since in the fat formed according to random distribution many glycerides may be present, as many as may be formed by $k$ classes of variations from $n$ elements. In the case of $n$ kinds of fatty acids the number of variations equals:

$$\frac{n^3 - n^2}{2}$$

An extensive change may be expected as an effect of interesterification in fats the composition of which differed highly from the random composition, if the reaction is carried out in the homogeneous phase non-directed (PERÉNYI, 1952; 1955b; JÁKY & PERÉDI, 1951).

By the method called directed process (ECKEY, 1954) the triglyceride composition of almost every fat may be altered. In the course of this process a brittle state is induced in which the components of high melting point separate and thus the equilibrium conditions and the composition of the residual fluid fraction will differ from that of the original fat.

The aim of this study was to interesterify in a homogeneous way the most important fat in Hungary, the lard, to make it suitable for use in margarine. Lard is used all over the world as a basic material of baking fats and margarines. However its well-known granulous, loose structure, the narrow range of plasticity cause innumerable difficulties in its use in the baking industry. In fats utilizing lard large, soft crystals are formed during storage interfering with the taste and consistency of the product. In batters it is difficult to beat it up, it does not shorten the pastry, the volume of products prepared with it is smaller as compared to those made with vegetable fat (HOERR & WAUGH, 1955).

LUTTON and co-workers (1962) attribute these unsuitable properties of lard, the trend to form crystal conglomerates, to the triglycerides containing two saturated fatty acid, particularly to those containing palmitic acid in the 2-position.

These are mainly the SPO (stearo-palmito-oleo) triglycerides. These triglycerides form about 25% of lard. The crystals appear in beta formation

in a trifold length of fatty acid chain ($\beta'$–3). Since the composition of the fat is not random, the effect of interesterification may be improved: it causes a significant reduction in the quantity of triglycerides of detrimental effect and produces a more advantageous structure, similar to that produced by vegetable oils.

## 1. Materials and methods

To achieve our aim the random interesterification process was applied in this study. Normal slaughterhouse lard was used. Characteristics of this lard are given in Table 1.

Prior to interesterification the lard was exposed to the removal of free fatty acids and water. Metallic sodium (0.2%) or sodium methoxide (0.5%) was used as a catalyst. The fat was made to react in the presence of $N_2$ gas in a flask provided with a mixer at temperatures between 90 and 150 °C during periods of 1 to 3 h.

Table 1

*Characteristics of natural lard*

| | | |
|---|---|---|
| Acid number | 0.07 | (HUNGARIAN STANDARD, 1973 a) |
| Moisture content, % | 0.015 | (HUNGARIAN STANDARD, 1974) |
| Soap content, % | 0.000 | (HUNGARIAN STANDARD, 1973 b) |
| Complete melting point, °C | 45.8 | (KAUFMANN, 1958) |
| Solidification point, °C | 27.2 | (HUNGARIAN STANDARD, 1973 c) |

Fatty acid composition (w%)
(HUNGARIAN STANDARD, 1973 d)

| | | | |
|---|---|---|---|
| $C_{14}$ | 1.0 | $C_{18}$ | 13.8 |
| $C_{16}$ | 23.5 | $C_{18:1}$ | 46.8 |
| $C_{16:1}$ | 2.1 | $C_{18:2}$ | 11.3 |
| $C_{17}$ | 0.2 | $C_{18:3}$ | 0.4 |
| $C_{17:1}$ | 0.2 | $C_{20}$ | 0.7 |

On completion of the reaction the catalyst was inactivated by the addition of dilute solution of acetic acid. The fat was washed with hot water and dried with anhydrous sodium sulphate.

The effect of the reaction was studied by several methods.

The following characteristics were established:

— complete melting point,
— solidification curve,

— dilatation curve,
— triglyceride composition according to number of carbon atoms,
— triglyceride composition according to saturation degree,
— penetration value,
— microscopic picture of fat crystals,
— free cholesterol content.

Utility tests were also carried out: the samples were used to prepare margarine base and pastry and their properties were studied.

The complete melting point was determined by KAUFMANN's method (1958).

Solidification of the fats was tested by the method used in the standard on the solidification point of fatty acids (HUNGARIAN STANDARD, 1973 c) and plotted as a temperature-time diagram.

Dilatation curves were established according to the HUNGARIAN STANDARD, 1973 c)

Determination of triglyceride groups according to number of carbon atoms was carried out by gas chromatography at programmed temperature (KUKSIS, 1965), on a *Pye Unicam* 104 apparatus with a double flame ionization detector.

Conditions of analysis: column: glass tube of 60 cm length, 3 mm diameter; packing: 5% OV–101 on Gas Chrom Q (80–100 mesh). Temperature: thermostat 260–330 °C, 3 °C $min^{-1}$, evaporator 380 °C, detector 360 °C. Carrier gas: 60 ml $min^{-1}$ nitrogen.

For separation of triglycerides according to their degree of saturation and for their quantitative determination a combined thin-layer and gas chromatographic method (MARINETTI, 1967) was used. The triglycerides grouped according to the degree of saturation of their fatty acid components were purified by thin-layer chromatography then fractionated on Kieselgel G layer impregnated with 10% $AgNO_3$. The solvent was chloroform. The fractions were visualized in UV-light with 2.7-dichloro-fluoresceine. The quantity and fatty acid composition of the fractions were determined by gas-chromatography (HUNGARIAN STANDARD, 1973 d) subsequent to elution interesterification and the addition of $C_{17}$ methylester as internal standard. The quantities of triglyceride groups were calculated on the basis of the above.

The yield value of fats and margarines was determined by penetration measurements. Results were expressed as g per $cm^2$ (HAIGHTON, 1959). A conical penetrometer according to *Sommer* and *Runge* was used in the tests.

The change in the crystal structure of the fats was observed with a *Zeiss Amplival* polarization microscope (PERÉDI & SZUNGYI, 1975). The observations were recorded on transparency.

To determine the cholesterol content, thin-layer chromatography was applied. Lard contains appr. 0.19% free cholesterol which is transformed by interesterification into cholesterol-fatty acid ester. By appropriate thin-layer technique this process may be followed and thus the extent of interesterification established (DGF, 1976).

The composition of the fat-base of margarine was as follows: lard 40%, coconut oil 10%, hardened rapeseed oil (melting point 38 °C) 35%, rapeseed oil 15%.

A laboratory mixer was used to beat 25% sugar, 14% fat, 15% egg, 15% milk and 31% flour for cakes.

The analytical results were evaluated by the following equation:

$$x = \bar{x} \pm ts_{\bar{x}}$$

where $x$ = probable value in case of $P = 95\%$
$\bar{x}$ = average
$t$ = 2.318 for $n - 1 = 8$, calculated at 95% confidence limit
$s_{\bar{x}}$ = standard error

Results were rounded up taking into account the accuracy of the methods.

## 2. Results and conclusions

The following catalyst concentrations were applied in interesterification: 0.2% metallic sodium or 0.5% sodium methoxide. Temperatures were as follows: 90, 100, 110, 120, 130 and 150 °C, time: 1, 2 and 3 h, resp.

Results have shown the reaction to occur at 90 °C within 1 h, thus higher temperatures and longer reaction periods were not needed.

However, great care has to be taken to remove all foreign matter, particularly water and free fatty acids since these inactivate the catalyst.

The results are grouped according to the method of analysis.

### *2.1. Complete melting point*

As an effect of interesterification the complete melting point of the fat decreased. While the crude lard became transparent at 45.8 °C, the interesterified lard did so at 40.8–42.7 °C. Interesterification was carried out at 90, 100, 110 °C for 1, 2 and 3 h, resp., in the presence of 0.5% sodium

methoxide. The average decrease of nine interesterifications amounted to 3.8 °C (Table 2).

Under conditions examined the complete melting point of the interesterified fat was 42.0 ± 0.5 °C at a probability level of 95%.

Table 2

*Change in the complete melting point upon interesterification*

| Sample | Complete melting pont, °C |
|---|---|
| Natural lard | 45.8 |
| Interesterified lard 1 | 42.7 |
| 2 | 41.8 |
| 3 | 40.8 |
| 4 | 42.2 |
| 5 | 42.4 |
| 6 | 42.4 |
| 7 | 42.4 |
| 8 | 41.8 |
| 9 | 41.2 |

$\bar{x} = 42.0$ °C; $s_{\bar{x}} = 0.21$, $x = 42.0 \pm 0.5$ °C.

## 2.2. *Solidification curves*

As an effect of interesterification, the solidification curves underwent a substantial change (Fig. 2).

Two maxima are detectable on the solidification curves of untreated lard. This is a consequence of the crystallization within a narrow temperature range of triglycerides of almost identical composition. As shown by their solidification curves, the interesterified samples do not contain great amounts of these triglycerides. The differences between the solidification curves of the samples interesterified at 90, 100 and 110 °C were not significant. The time of treatment did not affect the result either.

## 2.3. *Dilatation curves*

The effect of interesterification on the dilatation curves was excessive. Between 0 and 35 °C the quantity of solid matter substantially decreased, the fat became more balanced. This permits of the conclusion that the

triglycerides present in natural lard were transformed into other triglycerides (Fig. 3).

Changes as observed at 15, 20, 25, 30 and 35 °C, resp., are shown in Table 3.

The respective dilatation values of the interesterified fats measured at identical temperatures showed surprisingly low differences among themselves, while the overall average decrease from the original value is great, *e.g.* at 15 °C 251 mm³ per 25 g. The standard deviations of values are low, *e.g.* the expected dilatation value of interesterified fats at 15 °C was 499 ± 9.5 mm³ per 25 g fat at a probability level of 95%.

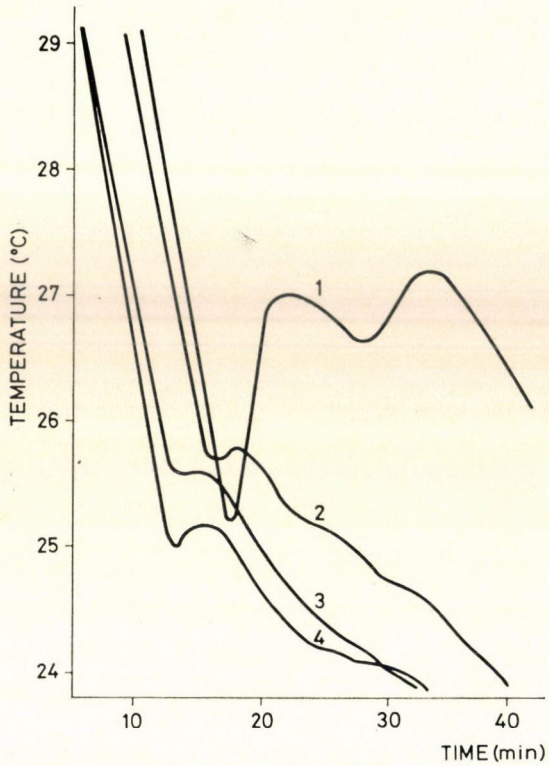


Fig. 2. Solidification curves of natural and interesterified lard
1. Natural lard; 2. lard interesterified at 110 °C (0.5% sodium methoxide, 1 h); 3. lard interesterified at 90 °C (0.5% sodium methoxide, 1 h); 4. lard interesterified at 100 °C (0.5% sodium methoxide, 1 h). Measurements were taken at 1-min intervals

### *2.4. Triglyceride composition according to number of carbon atoms*

The triglyceride composition was highly affected by the reaction. The triglyceride composition of the original fat different from random is transformed and approximates the calculated value of composition (Table 4).

The difference is particularly great with the groups containing C 52 and C 54. The quantity of triglycerides containing a single palmitic acid (C 52) substantially decreased and the group containing only fatty acids with 18 carbon atoms (C 54) substantially increased (Fig. 4).

Changes in values of the different triglyceride groups, on the basis of nine interesterifications are shown in Table 5 and Fig. 5.

These values prove also how little the difference is between the compositions of interesterified fats and how they differ from the composition of the original fat.

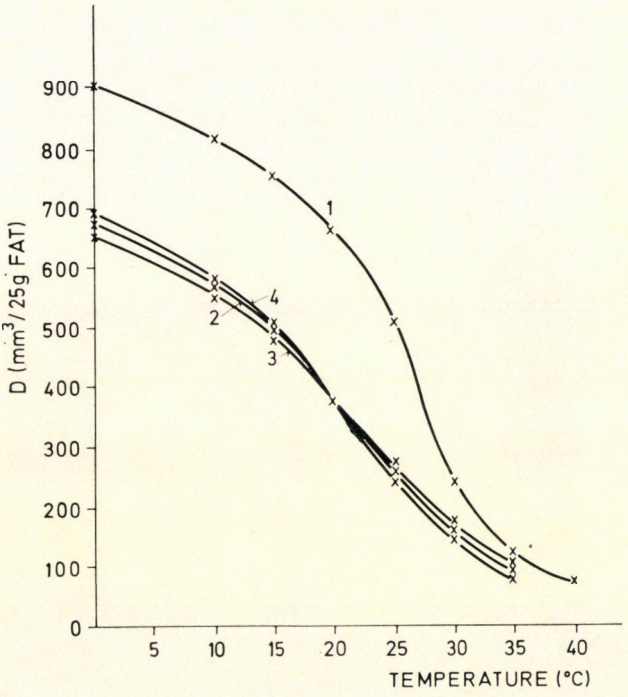


Fig. 3. Dilatation curves of natural and interesterified lard. 1. Natural lard; 2. lard interesterified at 90 °C (0.5% sodium methoxide, 1 h); 3. lard interesterified at 100 °C (0.5% sodium methoxide, 1 h); 4. lard interesterified at 110 °C (0.5% sodium methoxide, 1 h)

### *2.5. Triglyceride composition according to saturation*

The structure modifying effect of interesterification is proven by this test (Table 6).

In the course of interesterification the proportion of the four triglyceride groups shifted in the direction of random distribution. The amount of trisat-

Table 3

*Change in dilatation values upon interesterification*

| Sample | DILATATION mm³ per 25 g | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 15 °C | $\Delta D$ (15 °C) | 20 °C | $\Delta D$ (20 °C) | 25 °C | $\Delta D$ (25 °C) | 30 °C | $\Delta D$ (30 °C) | 35 °C | $\Delta D$ (35 °C) |
| Natural lard | 750 | — | 660 | — | 519 | — | 239 | — | 124 | — |
| Interesterified | | | | | | | | | | |
| lard 1 | 514 | −236 | 411 | −249 | 273 | −246 | 183 | −56 | 112 | −12 |
| 2 | 498 | −252 | 376 | −284 | 257 | −262 | 173 | −66 | 107 | −17 |
| 3 | 500 | −250 | 368 | −292 | 264 | −255 | 174 | −65 | 107 | −17 |
| 4 | 490 | −260 | 380 | −280 | 261 | −258 | 164 | −75 | 93 | −31 |
| 5 | 483 | −267 | 359 | −301 | 254 | −265 | 139 | −100 | 100 | −24 |
| 6 | 483 | −267 | 379 | −281 | 251 | −268 | 160 | −79 | 84 | −40 |
| 7 | 498 | −252 | 375 | −285 | 258 | −261 | 164 | −75 | 100 | −24 |
| 8 | 516 | −234 | 389 | −271 | 267 | −252 | 175 | −64 | 103 | −21 |
| 9 | 510 | −240 | 375 | −285 | 257 | −262 | 172 | −67 | 101 | −23 |
| $\bar{x}$ | 499 | | 379 | | 260 | | 167 | | 101 | |
| $s_{\bar{x}}$ | 4.1 | | 4.8 | | 2.3 | | 4.2 | | 2.8 | |
| $x$ | 499 ± 9.6 | | 379 ± 11.2 | | 260 ± 5.3 | | 167 ± 9.8 | | 101 ± 6.4 | |

Table 4

*Distribution by number of carbon atoms of triglyceride groups in natural and interesterified lard*

| Number of carbon atoms[a] | Triglyceride composition (Mol%) | | | |
|---|---|---|---|---|
| | Natural | Lard interesterified with | | Calculated values[b] |
| | | metallic sodium[c] | sodium methoxide[d] | |
| C 46 | 0.7 | 0.8 | 0.6 | 0.3 |
| C 48 | 2.5 | 4.7 | 4.4 | 3.4 |
| C 50 | 13.8 | 19.1 | 18.6 | 15.6 |
| C 52 | 62.8 | 40.6 | 41.4 | 42.8 |
| C 54 | 19.0 | 32.9 | 32.8 | 36.0 |
| C 56 | 1.2 | 1.9 | 2.2 | 1.9 |

Note: [a] Number of carbon atoms is understood to mean the sum of the number of carbon atoms in the three fatty acid components

[b] Results were obtained by calculation of probability, assuming complete random distribution in positions 1,2 and 3 of the triglycerides:
A, B, C glycerides mol% = $6 \times (A)\ (B)\ (C) \times 10^{-4}$
A, B, B glycerides mol % = $3 \times (A)\ (B)^2 \times 10^{-4}$
A, A, A glycerides mol% = $(A)^3 \times 10^{-4}$
where A, B, C are the mole percentages of the different fatty acids.

[c] 0.2% metallic sodium, 1 h, 100 °C

[d] 0.5% sodium methoxide, 1 h, 100 °C

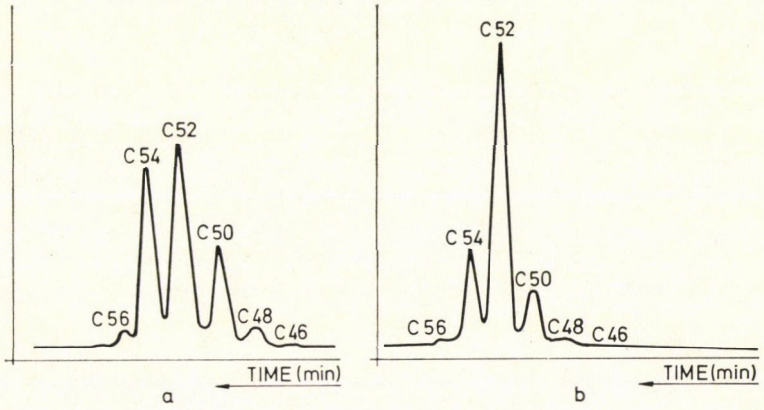


Fig. 4. Chromatograms of triglyceride groups of the natural and interesterified lard according to number of carbon atoms

a. lard interesterified (0.5% sodium methoxide, 90 °C, 1 h); b. natural lard. Column packed with 5% OV-101 on Gas Chrom Q (80–100 mesh). Carrier gas 60 ml $min^{-1}$ nitrogen. Injection temperature 180 °C, thermostat temperature programmed: 260–330 °C rate: 3 °C $min^{-1}$, detector temperature 360 °C.

C 46–C 56 = The number of carbon atoms of the three fatty acid components

Table 5

*Changes induced by interesterification (mol%) in the triglyceride composition according to number of carbon atoms*

| Sample | $C_{50}$ | | $C_{52}$ | | $C_{54}$ | |
|---|---|---|---|---|---|---|
| | % | Δ% | % | Δ% | % | Δ% |
| Natural lard | 13.8 | | 62.8 | | 19.0 | |
| Interesterified lard[e] | | | | | | |
| 1 | 17.8 | +4.0 | 41.8 | −21.0 | 34.5 | +15.5 |
| 2 | 17.6 | +3.8 | 42.0 | −20.8 | 33.6 | +14.6 |
| 3 | 17.8 | +4.0 | 42.1 | −20.7 | 32.9 | +13.9 |
| 4 | 17.9 | +4.1 | 41.9 | −20.9 | 33.2 | +14.2 |
| 5 | 18.1 | +4.3 | 42.5 | −20.3 | 32.7 | +13.7 |
| 6 | 18.8 | +5.0 | 42.5 | −20.3 | 31.1 | +12.1 |
| 7 | 17.9 | +4.1 | 42.2 | −20.6 | 33.0 | +14.0 |
| 8 | 17.8 | +4.0 | 41.3 | −21.8 | 34.2 | +15.2 |
| 9 | 17.7 | +3.9 | 41.8 | −21.0 | 32.9 | +13.9 |
| $\bar{x}$ | 17.9 | | 42.0 | | 33.1 | |
| $s_{\bar{x}}$ | 0.12 | | 0.12 | | 0.33 | |
| $x$ | 17.9 ± 0.27 | | 42.0 ± 0.29 | | 33.1 ± 0.76 | |

Note: $e$ = temperature: 90–100–110 °C, catalyst: 0.5% sodium methoxide, time: 1–2–3 hours. C 50–C 54 = The number of carbon atoms in the three fatty acid components. Δ% = The difference between the percentage values of original fat and interesterified fat. For method of calculation see Note in Table 4

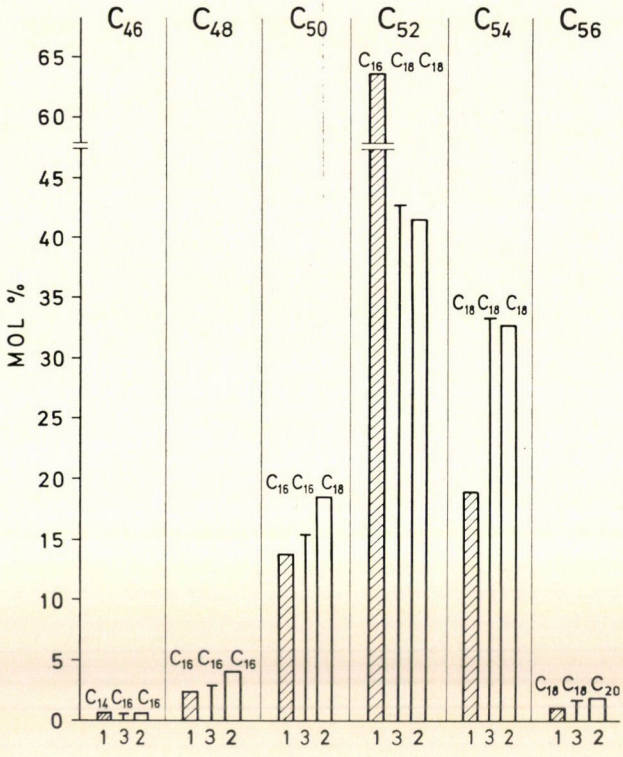


Fig. 5. Changes in values of the different triglyceride groups on the basis of nine interesterifications. 1. Natural lard; 2. interesterified lard*; 3. calculated values.
*Note:* * = 0.5% sodium methoxide, 90–100–110 °C, 1–2–3 h.
C 46–C 56 = The number of carbon atoms of three fatty acid components

urated and tri-unsaturated glycerides increased. The quantity of mono-saturated and di-saturated glycerides decreased and the composition became similar to the random (Fig. 6).

### *2.6. Penetration values*

Data hitherto given, such as reduction of melting point, changes in the solidification curve, decrease of the solid part seem leading to the conclusion that the fat is softened by interesterification. However, the penetration values point to just the opposite, the fats became harder (Fig. 7).

Hardening is probably due to the change in the crystal structure. As mentioned earlier, the large crystal conglomerates, undesirable in lard, consist of $\beta'$-3 primary crystal cells. The more heterogeneous triglyceride structure, formed as an effect of interesterification promotes the formation of $\beta'$-2

primary cells, arranged in a crystal-lattice consisting of many small crystals. Thereby as the specific surface of the crystals increases they bind more fluid components on their surface and the fat becomes more plastic.

Table 6

*Quantities of triglyceride groups according to their degree of saturation in natural and interesterified lard*

| | Triglyceride composition (Mol%) | | | |
|---|---|---|---|---|
| | Natural lard | Lard interesterified with | | Calculated value |
| | | metallic sodium (1) | sodium methoxide (2) | |
| $S_3$ | 1.7 | 5.2 | 7.1 | 6.4 |
| $S_2O$ | 27.1 | 23.3 | 24.8 | 24.0 |
| $SO_2$ | 32.7 | 28.4 | 21.3 | 30.0 |
| $S_2L$ | 7.7 | 6.4 | 4.9 | 4.8 |
| $O_3$ | 7.6 | 11.9 | 7.4 | 12.5 |
| SOL | 13.0 | 12.4 | 17.3 | 12.0 |
| $O_2L$ | 4.2 | 6.2 | 11.2 | 7.6 |
| $SL_2$ | 1.3 | 3.5 | 1.3 | 1.2 |
| $OL_2$ | 4.7 | 2.7 | 4.7 | 1.5 |
| Total: | | | | |
| $S_3$ | 1.7 | 5.2 | 7.1 | 6.4 |
| $S_2U$ | 34.8 | 29.7 | 29.7 | 28.8 |
| $SU_2$ | 47.0 | 44.3 | 39.9 | 43.2 |
| $U_3$ | 16.5 | 20.8 | 23.3 | 21.6 |

*Note:* S = saturated fatty acids, O = oleic acid, L = linoleic acid + linolenic acid, U = unsaturated acids (O+L). $U_3$ = tri-unsaturated triglycerides, $SU_2$ = mono-saturated-di-unsaturated triglycerides, $S_2U$ = disaturated–mono-unsaturated triglycerides, $S_3$ = tri-saturated triglycerides. For method of calculation see Note in Table 4.

## *2.7. Changes in the crystal structure*

Changes in the crystal structure after a 6-week storage period at 20 °C, are illustrated in Figs. 8 and 9 (×250). As it may be seen, crystals in the original fat form large heaps, while the interesterified fat retains its microcrystalline system during a longer storage period.

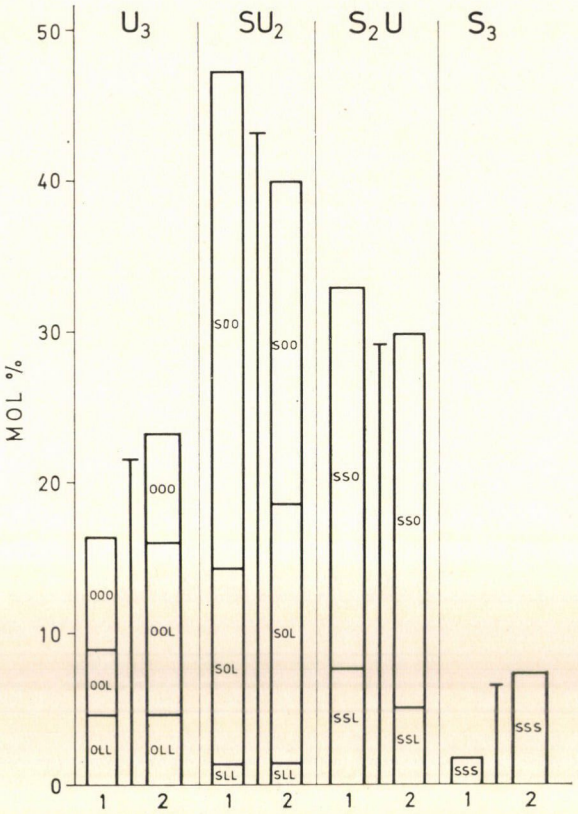


Fig. 6. Distribution of triglycerides according to saturation.
1. Natural lard; 2. lard interesterified (0.5% sodium methoxide, 90 °C, 1 h) S = saturated fatty acids, O = oleic acid, L = linoleic and linolenic acids, U = unsaturated fatty acids (O+L). $U_3$ = tri-unsaturated triglycerides, $SU_2$ = mono-saturated – di-unsaturated triglycerides, $S_2$ = disaturated – mono-unsaturated triglycerides, $S_3$ = tri-saturated triglycerides

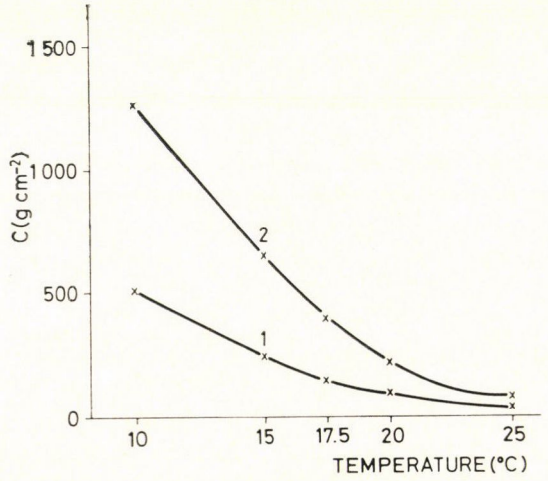


Fig. 7. Penetration value (C) as a function of temperature (T).
1. Natural lard; 2. lard interesterified (0.5% sodium methoxide, 90 °C, 1 h)

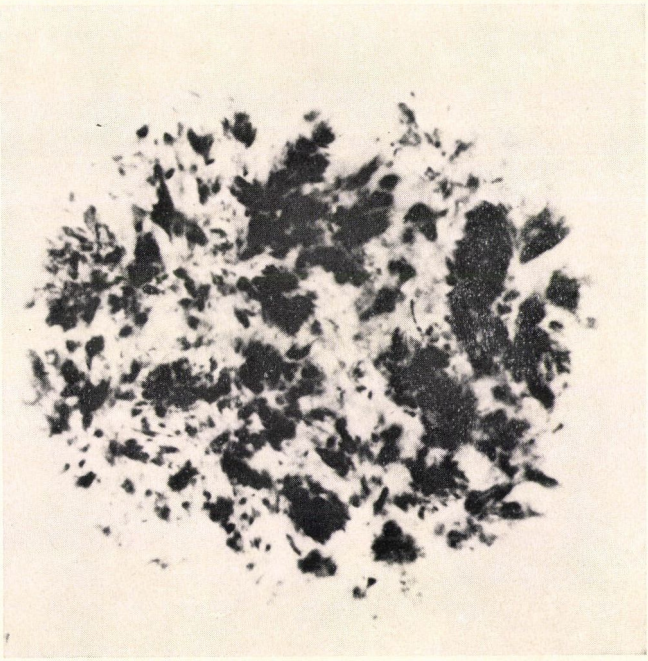

Fig. 8. Crystal structure of natural lard

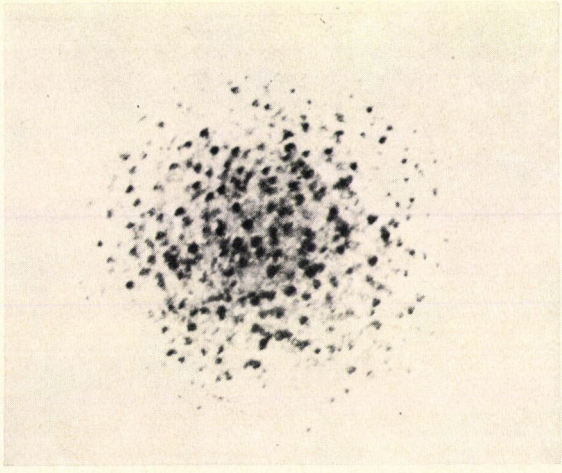

Fig. 9. Crystal structure of interesterified lard

### *2.8. Free cholesterol content*

In contrast to the original fat, the interesterified fat did not contain cholesterol, which proves again the probability of structural change (Fig. 10).

### *2.9. Margarine preparation test*

A substantial difference was found between the quality of the original and interesterified fat. The finer crystal structure of the interesterified fat made itself feel in the margarine base. Even after a long storage period a perfectly smooth shortening of good consistency could be prepared with this fat.

### *2.10. Baking test*

As shown by BAILEY (1951), HOERR and WAUGH (1955) the disadvantageous crystal structure of lard affects cakes baked with it. In mixtures used for cake baking only a low amount of air can be included and this is dispersed in large bubbles, too.

Cake mixtures utilizing interesterified fat, however, as previously shown by HOERR and WAUGH (1955), have also been found capable of incorporating more and better dispersed air. On baking these will have a larger volume and finer crumb.

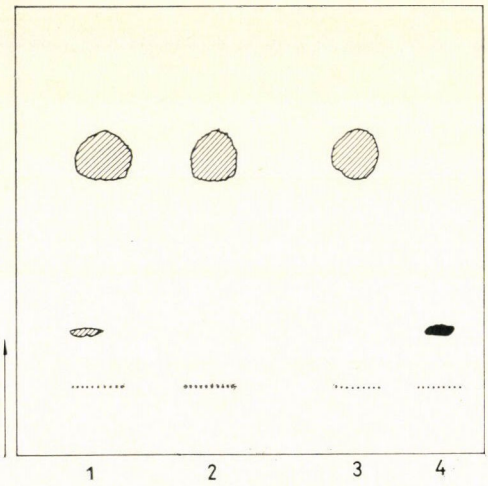


Fig. 10. Detection of sterols. Thin-layer chromatogram of natural lard and interesterified lards.
1. Natural lard; 2. lard interesterified (0.5% sodium methoxide, 90 °C, 1 h); 3. lard interesterified (0.2% metallic sodium, 90 °C, 1 h); 4. cholesterol. Layer: Kieselgel 60, solvents: 1. Petroleum ether : diethylether: glacial acetic acid (70 : 30 : 1). 2. Diethylether: petroleum ether: glacial acetic acid (70 : 30 : 1)

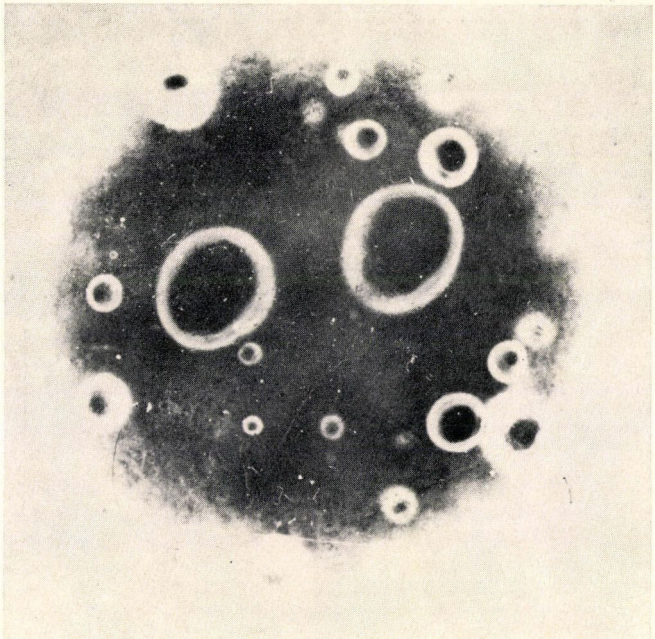

Fig. 11. Microscopic picture of pastry, made with natural lard, prior to baking (magnification 250 ×)

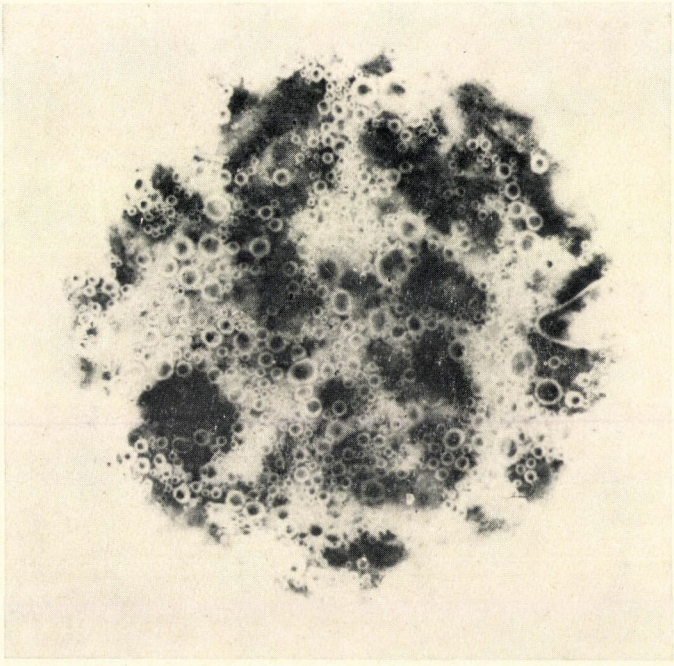

Fig. 12. Microscopic picture of pastry, prepared with interesterified lard, prior to baking (magnification 250 ×)

According to experiences gained in the course of this study margarines and shortenings prepared with interesterified fat were better than those prepared with the original lard, not only from the rheological aspect but for their baking properties as well (Figs. 11 and 12).

It may be concluded that the lard can be interesterified by a relatively simple technique. The product thus obtained is of better physico-chemical characteristics than the original fat.

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# RADIATION EFFECTS ON ACTIVITY AND STORAGE STABILITY OF ENDO-POLYGALACTURONASE

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In the practical application endo-polygalacturonase (endo-PG), is an enzyme disrupting the pectic materials between the cells of plant tissues without attacking cell walls.

In the field of application the demand on sterility is serious. This is why radiation decontamination of the endo-PG preparation (pilot product of the CENTRAL FOOD RESEARCH INSTITUTE) was attempted and followed by the determination of its storage stability.

Irradiation of the enzyme was carried out in powdered form, under aerobic conditions with a $^{60}Co$ radiation source. Doses of 5 and 10 kGy (0.5 and 1.0 Mrad) gamma rays decreased the initial viable cell count ($2.6 \cdot 10^7$ $g^{-1}$) of the preparation by 4 and 6 orders of magnitude, resp. The survival level showed no significant differences when the preparation was irradiated with doses of 5, 10 and 20 kGy, while the dose of 40 kGy resulted in a practically germ-free preparation ($\leq 7$ $g^{-1}$).

The loss of endo-PG activity, found as a result of irradiation, was compensated by better storage stability of the irradiated samples. The use of the doses of 5, 10, 20 and 40 kGy resulted in a 6.4, 24, 30 and 40% decrease in the activity of the enzyme. On the other hand, the preparations treated with 5, 10, 20 and 40 kGy proved to be stable, and showed only a slight loss in their respective activity, compared to the 18% activity loss of the control.

Endo-polygalacturonase (endo-PG) can disrupt plant tissues without attacking cell walls. The enzyme decomposes the pectin-containing materials between the cells resulting in a suspension consisting of tissue particles smaller than 0.25 mm.

The concentration of the resulting suspension may be varied in the course of enzyme treatment, by the quantity of buffer or water given together with the enzyme to the prepared (grated) plant tissue in accordance with the final aim of enzyme treatment: vegetable creams or concentrates, baby food, vegetable juices, or cocktails consisting of various vegetables, or mixtures of different fruits and vegetables.

In the field of application of endo-PG enzyme the demand on sterility is very much emphasized. The use of the enzyme is not permitted if the viable cell count of the preparation exceeds a certain tolerance value. This is why the reduction of the viable count of the preparation was attempted by the use of gamma irradiation.

Ionizing radiation, of great interest from the point of view of radiobiology and food preservation, has been used in the case of various enzymes

for a long time (MARPLES & GLEW, 1958; BAKER & GOLDBLITH, 1961; VAS, 1962; BRUSTAD *et al.*, 1966; GIOVANNOZZI-SERMANNI *et al.*, 1969; VAS, 1969; WINSTEAD & REECE, 1970; LYNN, 1971; RADOLA & DELINCÉE, 1972; DIEHL, 1975).

## 1. Materials and methods

### *1.1. Enzyme preparation*

The enzyme preparation was produced at the FERMENTATION DEPARTMENT of the CENTRAL FOOD RESEARCH INSTITUTE, Budapest, by 72-h submerged cultivation of an *Aspergillus awamori* strain. The enzyme was precipitated from the fermentation broth by methanol.

The activity of endo-PG and endo-PMG of the preparation proved to be 4400 and 12 l $h^{-1}$ $g^{-1}$, respectively, while its macerase activity was 3.42 l $h^{-1}$ $g^{-1}$. The dry matter content of the technical preparation: 90%; protein content: 33%; carbohydrate content: 21%; amino acid composition: alanine, aspartic acid, serine, glutamic acid, leucine, glycine, tyrosine and valine.

### *1.2. Determination of enzyme activities*

Activities of *endo-PG* and *endo-PMG* were determined in an *Ostwald* type viscosimeter using sodium polypectate (SIGMA CHEMICAL WORKS, St. Louis, USA) and *Pomosin* pectin (POMOSIN GmbH, FRG), resp. and were expressed as specific pectolytic activity ($SPA_{75}$ l $h^{-1}$ $g^{-1}$), indicating that quantity of substrate the viscosity of which can be reduced by 75% by one gramm of enzyme during one h of incubation at 50 °C (ZETELAKI-HORVÁTH & VAS, 1972).

*Macerase activity* was determined by an instrumental method (ZETELAKI-HORVÁTH, 1974) on potato tissue disks (variety: *Rózsa*) of standard thickness and diameter and was characterized by the reciprocal of the time necessary for a predetermined degree of disruption of the tissue by the enzyme.

### *1.3. Irradiation of the enzyme preparation*

The powdered endo-PG preparation was irradiated in sealed polyethylene bags under aerobic conditions at 24 °C in the MICROBIOLOGICAL DEPARTMENT of the CENTRAL FOOD RESEARCH INSTITUTE, Budapest, using a $^{60}Co$ radiation source (Type: RH-$\gamma$-30), with doses of 5, 10, 20 and 40 kGy, resp.

*1.4. Determination of viable count*

Viable count was determined by the Most Probable Method in Nutrient broth (Oxoid, CM 67).

## 2. Results

*2.1. Temperature- and pH-optimum of the enzymes*

The effect of *temperature* on the endo-PG was investigated in the range of 20–80 °C at intervals of 10 °C. As a result of this work, 50 °C proved to be optimal for the activity of all the three enzyme components (endo-PG, endo-PMG and macerase of the preparation (Fig. 1).

The activity of endo-PG showed a very sharp optimum at 50 °C. The decrease or increase in temperature by 10 °C resulted in 37 and 30% decrease in endo-PG activity, resp.

In a temperature range of 30–50 °C the activity of endo-PMG and macerase changed only to a small extent but the increase of temperature to 60 °C resulted in a considerable decrease in the activity of endo-PMG. As macerase activity was determined on a substrate of potato tissue, incubation temperatures higher than 50 °C could not be tested because of the undesirable change in the consistency of the tissue.

The effect of *pH* on the activity of the investigated endo-PG preparation was tested in a pH range of 2.5–8.0 at intervals of 0.5 pH value. In contrast to the common temperature optimum of the three enzyme components of the preparation, their pH optima were different. Maximum endo-PG activity was measured at a pH value of 4.5, while endo-PMG and macerase activities showed maxima at pH 3.5 (Fig. 2).

The activity of the endo-PMG and macerase enzyme components at the other investigated pH values showed no common change. The decrease of the optimal pH by 0.5 resulted in a 60% decrease in the endo-PMG activity while the macerase activity decreased only by 18%. The increase of the pH value from 4.0 to 4.5 decreased the endo-PMG activity by 38%, but the activity of the macerase enzyme remained unchanged.

*2.2. Irradiation of endo-PG by gamma rays*

*2.2.1. Effect of irradiation on the activity of endo-PG.* Endo-PG activity of the preparation was investigated after irradiation with different doses of gamma rays (Fig. 3).

It was found that irradiation with 10, 20 and 40 kGy resulted in a 23, 30 and 40% inactivation of the endo-PG enzyme. As these losses in activity

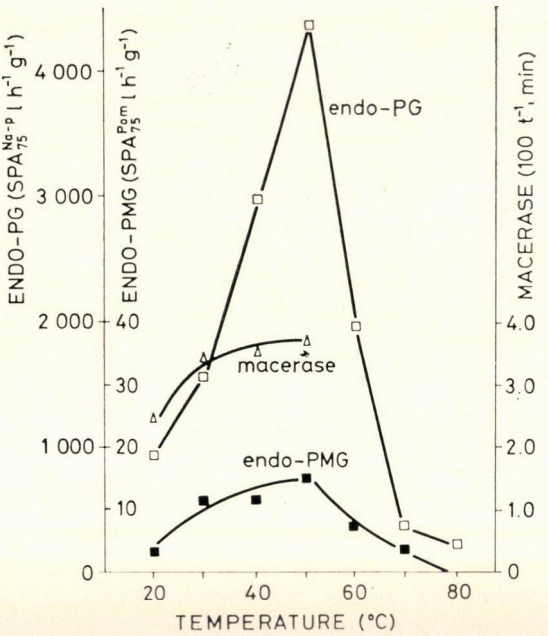



Fig. 1. Activity of endo-PG, endo-PMG and macerase of an endo-PG enzyme preparation (No. PG-225) as a function of temperature.
(Activities of the enzyme were measured after a one hour incubation period at a pH value of 4.5)

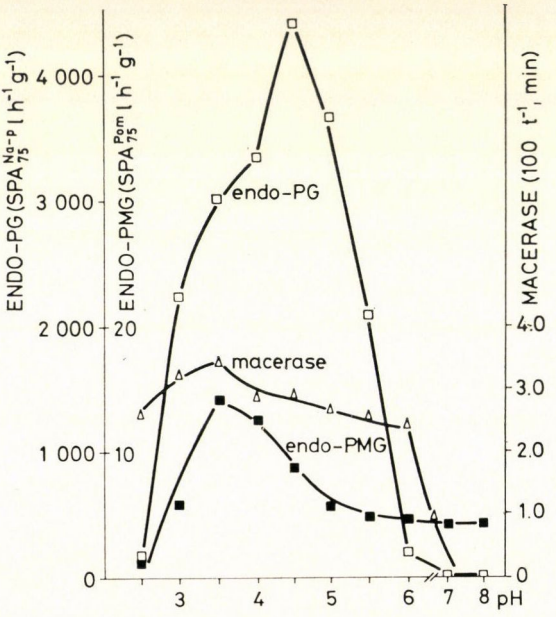



Fig. 2. Activity of endo-PG, endo-PMG and macerase of an endo-PG enzyme preparation (No. PG-225) as a function of the pH.
(Activities of the enzyme were measured after an incubation period of 1-h at a temperature of 50 °C)

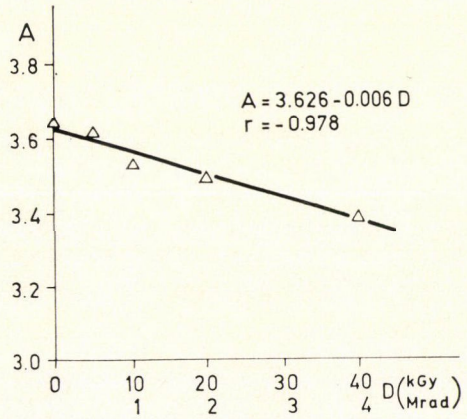



Fig. 3. Logarithm of percentage residual endo-PG activity *(A)* as a function of radiation dose *(D)*. (The enzyme was irradiated in powdered form at room temperature. Activity before irradiation ($SPA_{75}^{Na\text{-}P}$: 4400 l $h^{-1}$ $g^{-1}$)

were rather high, further irradiation was carried out so that the above doses were complemented with a lower one, 5 kGy. The decrease in the endo-PG enzyme activity as a result of a dose of 5 kGy proved to be considerably lower (6.5%) than in the case of higher doses.

*2.2.2. Effect of gamma rays on the viable cell count of the enzyme preparation.* Sterility is a basic demand in application. The final phases of technology of enzyme production cannot result in a sterile preparation. The viable count of the investigated technical endo-PG preparation proved to be $4.8 \cdot 10^{-7}$ $g^{-1}$. It was attempted to decrease this by gamma irradiation (Fig. 4).

Five and 10 kGy decreased the initial count of the preparation by 4 and 6 orders of magnitude. The survival level was about the same (42 and 32 $g^{-1}$) when the enzyme preparation was irradiated with 10 and 20 kGy gamma rays, while the enzyme samples irradiated with 40 kGy proved to be practically sterile (viable count $\leq 7$ $g^{-1}$).

*2.2.3. Storage stability of the irradiated enzyme preparations.* Endo-PG preparations irradiated by different doses of gamma rays were stored at a temperature of 5–8 °C for a period of 2.5 years. Endo-PG activity of the preparation irradiated at various dose levels were tested at certain intervals during storage (Fig. 5).

In the first 20 weeks of storage the activity of both irradiated and non-irradiated control samples decreased to a rather large extent. The highest loss of activity was measured in the case of the non-irradiated control sample. The activity loss of the samples irradiated with doses of 20 and 40 kGy was also high, doses of 10 and 5 kGy gamma rays resulted in a considerably lower damage to endo-PG activity.

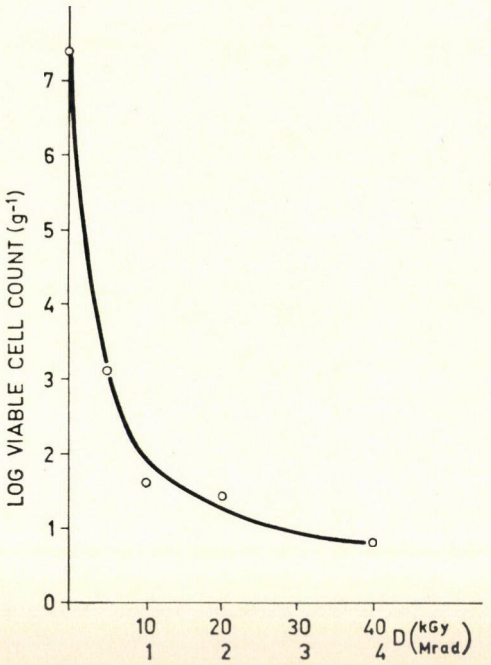



Fig. 4. Logarithm of viable cell count of the enzyme preparation as a function of the radiation dose *(D)*. (The enzyme was irradiated in powdered form at room temperature)

An unexpected phenomenon was observed in the course of this experiment. The activity of the control sample showed a decreasing tendency throughout the storage period while in the case of the irradiated samples, after a 40-week storage period the activity began to increase again.

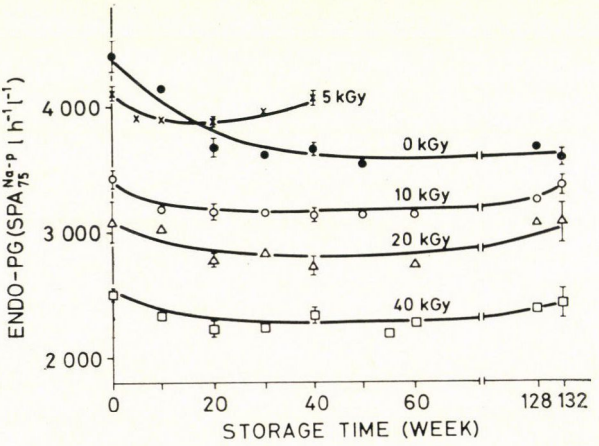
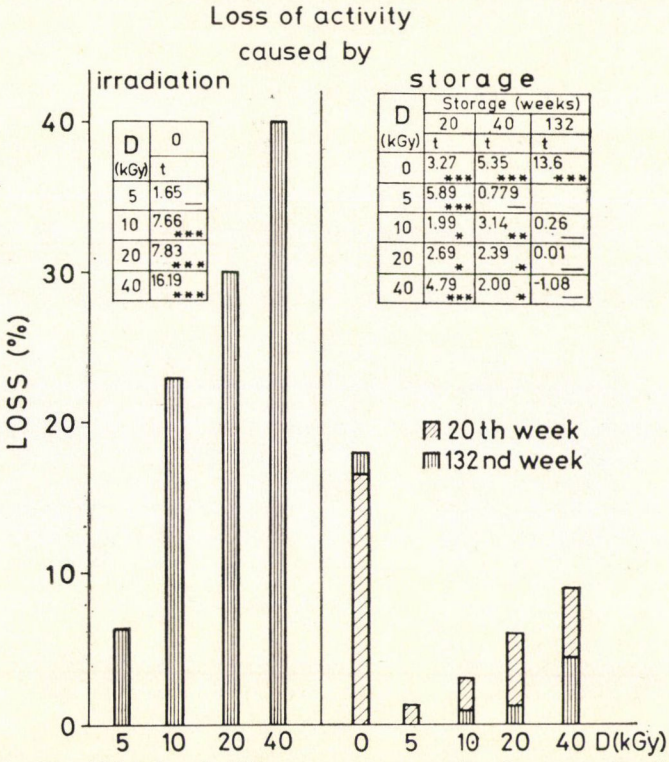



Fig. 5. Stability of the irradiated and non-irradiated samples as a function of the storage period. (Temperature of storage: 5–8 °C)




| D (kGy) | 0 |
|---|---|
| | t |
| 5 | 1.65 — |
| 10 | 7.66 *** |
| 20 | 7.83 *** |
| 40 | 16.19 *** |

| D (kGy) | Storage (weeks) 20 | 40 | 132 |
|---|---|---|---|
| | t | t | t |
| 0 | 3.27 *** | 5.35 *** | 13.6 *** |
| 5 | 5.89 *** | 0.779 — | |
| 10 | 1.99 * | 3.14 ** | 0.26 — |
| 20 | 2.69 * | 2.39 * | 0.01 — |
| 40 | 4.79 *** | 2.00 * | -1.08 — |

Fig. 6. Activity loss (%) of endo-PG enzyme as a result of gamma irradiation and loss of activity of the irradiated and control samples during storage. (Symbols: — not significant, * significant, ** highly significant, *** very highly significant)

## 3. Conclusions

The reduction of the viable cell count of the various enzyme preparations prior to their application in the food industry is advisable. Ionizing radiation is often used for this purpose (LOTT & FRANK, 1973). In this work endo-PG preparation was irradiated with $^{60}Co$ gamma rays using doses of 5 and 10 kGy when the viable cell count ($10^7$ $g^{-1}$) of the enzyme preparation decreased to $1.8 \cdot 10^3$ and $4.2 \cdot 10^1$, resp. This result is similar to those obtained by VAS & PROSZT (1965), LEUCHTENBERGER & RUTTLOFF (1976) and DELINCÉE *et al.*, (1975) who produced germ-free pectolytic and proteolytic enzyme preparations, resp., by the use of 10 kGy. In connection with the inactivation of the enzyme our results were somewhat different. The above authors found no inactivation when enzyme preparations were irradiated with 10 kGy, while a 23% inactivation of the endo-PG preparation, investigated in this study, was found as a result of the same dose. The extent of inactivation could be significantly decreased (from 23 to 6.4%) when only the half (5 kGy) of the above dose was applied (Fig. 6).

Considering the storage stability of the above samples, the endo-PG activity of the non-irradiated control samples showed a decreasing tendency during storage at 5–8 °C, reaching a very highly significant (18%) inactivation after a 2.5-year storage period. In contrast to this the irradiated samples showed significant loss in activity only in the first part of the storage period (20–40 weeks) but these losses were not significant after 132 weeks of storage (Figs. 5 and 6). The sample irradiated with 5 kGy, having still only a shorter storage life, proved to be different from the samples treated by higher doses, for its 40-week endo-PG activity did not differ significantly from the value of its 0-week activity. The average standard deviation of the 0, 5, 10, 20 and 132 week endo-PG samples was found to be 3.8%. BACHMAN and co-workers (1977) also gave account of the activity increase of gamma irradiated amylolytic and pectolytic enzyme preparations when 5 to 15 kGy doses were used, but they found this increase under a considerably shorter (12-week) storage period.

According to our results the use of a dose of 5 kGy can be recommended for the radiation decontamination of endo-PG preparations for the following reasons:

— The decrease in activity after irradiation was the least at this dose level.

— The reduction of viable cell count by four orders of magnitude is satisfactory since the air contamination of the industrial preparations (when filtration and drying of the enzyme preparation are carried out in closed industrial equipment) is expected to be considerably lower than that of the pilot plant product used in the present experiments. This dose is likely to be sufficient also due to the fact that the microbiological quality requirement for the products of the canning industry (in the field of application of endo-PG) is $10^2$ $g^{-1}$.

Gamma irradiation has the additional advantage of its use resulting in a preparation free from chemical residues.

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# KINETIC ANALYSIS OF BATCH FERMENTATION OF YOGHURT

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Batch yoghurt fermentations were carried out at 40, 45, and 50 °C, inoculum concentrations of 1, 3 and 5%, resp., and in two parallels each. The growth curves of the two microorganisms, *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, forming the microbial flora of yoghurt fermentation were plotted simultaneously. The growth curves were used to construct the growth rate curves and to determine that kinetic constant, which is most important for transforming the batch process into a continuous one, *i.e.* the specific growth rate coefficient *(k)*. Grouping the growth rate coefficients according to temperature, quantity of inoculum and kind of microorganism they were evaluated by analysis of variance.

The $k$ value belonging to *Str. thermophilus* in identical fermentations proved to be significantly higher than that belonging to *Lb. bulgaricus*. The $k$ values belonging to both bacteria were significantly higher at 45 °C than at 40 °C. Because of intense morphological changes occurring at 50 °C the $k$ value at this temperature was not determined. No significant difference was found between fermentations started with 3 or 5% inoculum. Using 1% inoculum the growth curves were of diauxic character. The $k$ value calculated from the first phase of these fermentations was significantly higher than in the case of fermentations with 3 or 5% inoculum.

The well-known advantages of continuous fermentation processes as against batch fermentation indicate the study of the possibility of transforming the batch fermentation of yoghurt, this important dairy product, into a continuous one.

In order to develop a continuous fermentation process one has to proceed from the kinetic constants of the batch process. Therefore the aim of this study was to investigate batch fermentation.

The optimum parameters of traditional yoghurt manufacture are well-known, technology in Hungary is making use of them. Literature of the dairy industry provides plenty of related references (PETE, 1957; KURMANN, 1967, 1969; HUMPHREYS & PLUNKETT, 1969; BAKE, 1971; DALHUISEN, 1974).

Yoghurt is manufactured in Hungary from cow's milk using *Streptococcus thermophilus* and *Lactobacillus bulgaricus* as starters.

The fat content of the milk to be used is set accurately and the milk is pasteurised. To ensure good consistency, the milk is pasteurised at 85–90 °C with a retention time of 5–10 min. The milk is then cooled to 45 °C and let in the tank for inoculation. An inoculum of 3–5% is added and the milk thoroughly mixed.

The inoculum is a 1 : 1 or at most 2 : 1 mixture of *Str. thermophilus* and *Lb. bulgaricus* cultures. The starter culture is made of good quality milk mixture. This is heated to 90–95 °C and pasteurized for 15–30 min. The milk cooled to 45 °C is then inoculated with 3–5% culture and kept at a temperature of 43–45 °C for 2–3 h, till the setting in of coagulation. It is then cooled with water and ripened in a cold chamber for 24 h. A satisfactory culture is of 40–50 ° Soxhlet-Henkel (SH) and the ratio of cocci to rods of 1 : 1 or 2 : 1. The proportion of *Str. thermophilus* increases if

— ripening is finished at a low acid number (26–28 °SH),
— the incubation temperature is low (37 °C),
— the quantity of inoculum used is low.

In all other cases *Lb. bulgaricus* prevails.

The milk inoculated with yoghurt culture is then filled into plastic bottles, sealed and ripened in a water bath of 45 °C or in a ripening chamber for 2–3 h. In order to prevent over-acidification coagulation has to be interrupted on reaching the isoelectric point, pH 4.7 or 28–32 °SH. Thus the yoghurt is rapidly cooled in two steps. In the first step, within 60–80 min it is cooled below 18 °C to interrupt growth. In the subsequent 60–90 min the yoghurt is cooled to 5–12 °C in order to limit enzyme activity.

Post-ripening in the cold chamber has to last at least 12 h. During this time the process of acidification stops, the consistency of yoghurt solidifies and the taste and smell of the product become fuller. The degree of acidity of a satisfactory yoghurt is 36–40 °SH.

The most modern yoghurt manufacturing technique applied in Hungary, in the BUDAPEST DAIRY PLANT may be considered a continuous process, however fermentation itself is not continuous. The essence of the process is that the mass culture is continuously injected through a dosing pump into the appropriately prepared milk, circulating in the pipe system. The inoculated milk is then automatically portioned into plastic containers, incubated and cooled. Insofar as the production of the mass culture is continuous, continuous yoghurt manufacture may be considered resolved.

In recent years several papers were published on the conversion of yoghurt manufacture into a continuous process (BOTTAZZI *et al.*, 1971; JÄHNERT & HEBERLE, 1972; LELIEVELD, 1976). At the same time some processes were patented (ATANASSOV & ANDREJEV, 1970; LIPATOV *et al.*, 1970; SCHULER, 1971) of which the most characteristic is the process patented by SCHULER (1971) in the German Federal Republic. In this technology mother and mass culture are not needed in the manufacture of yoghurt and the ripening period is reduced to 1–1.5 h. To prepare solid yoghurt the sterilized milk is inoculated with the yoghurt culture in a tank and is ripened under steady mixing till

a pH of 5.6 is reached. At this point the draining of the acidified milk and the adding of fresh milk of 45 °C is started. Care should be taken to maintain the dilution rate at a level ensuring a pH of 5.6 and a temperature of 45 °C all the time. The milk distributed into plastic containers is ripened for a further 1–1.5 h at the given temperature to reach a pH of 4.7. Hereafter the containers are cooled. A drawback of the process is that if, because of some trouble in the dilution rate, the pH of the milk in the tank is reduced below 4.7 (the isoelectric point of casein) and then distributed into containers, it never forms a solid coagulate and the whole amount is wasted.

In order to achieve safe production it seems more expedient to transform the inoculum fermentation (mass culture) into a continuous process. Should some trouble occur in inoculum fermentation this will not entail the spoilage of the whole amount of milk. At the same time the fermentor volume requirement is also lower. Thus the aim of this study was to determine the parameters needed to the transformation of inoculum fermentation into a continuous process.

In the development of continuous fermentation systems the kinetic constants of batch fermentation serve as starting points thus the kinetics of batch fermentation formed the subject of the first phase of the investigations. Since the aim of the study was to determine the parameters of continuous fermentation, the batch experiments had to be carried out under conditions of continuous fermentation (identical fermentor, mixing, etc.). However, under these conditions yoghurt of solid consistency was not formed and a product of acceptable sensory quality did not issue from this mass culture fermentation.

The effect of the inoculum quantity derived from mass culture fermentation upon the specific growth rate coefficient was studied at 3 different temperatures (40, 45, 50 °C) with 3 different quantities of inoculum at each level (1, 3, 5%). In order to be able to evaluate results by mathematical statistical methods at each temperature–inoculum pair 2 parallel fermentations were carried out.

## 1. Materials and methods

### *1.1. Yoghurt culture*

The yoghurt culture applied was a mixture of *Str. thermophilus* and *Lb. bulgaricus*, maintained in the laboratory of the CONTROL STATION FOR MILK PRODUCTS. The culture was maintained by transferring it twice weekly into freshly sterilized milk. The cultures were incubated at 45 °C for 2–2.5 h then stored at +5 °C.

### *1.2. Fermentor*

A flask with 3 necks of 1.5 l useful capacity was used as the fermentor. This was thermostated in a *Wassermann* water bath to $\pm$ 0.3 °C accuracy. The milk was stirred with an electromotor driven inflected glass rod at 200 rpm. The apparatus is illustrated in Fig. 1.

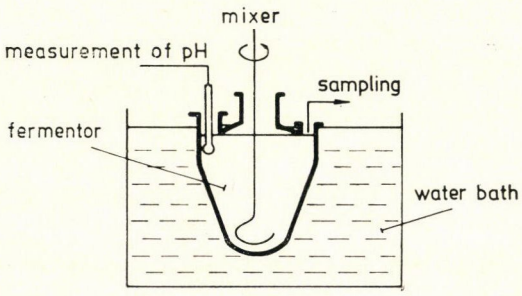


Fig. 1. Experimental equipment for batch-fermentation of yoghurt

### *1.3. Measurement of pH*

The pH was measured on *Seybold* GPB and *Radelkisz* OP 205 type instruments with continuously immersed combined glass electrodes.

### *1.4. Measurement of acidity*

The *Soxhlet–Henkel* method, generally applied in the dairy industry, was used. The titre is understood to mean the number of ml of a 0.25 *N* NaOH solution required to neutralize 100 ml milk using phenolphtalein indicator and is marked °SH. Procedure: 20.0 ml milk with 0.2 ml alcoholic phenolphtalein solution added is titrated with 0.1 *N* NaOH solution to pale pink. The °SH is obtained by multiplying the alkaline solution used, by 2.

### *1.5. Cell count determination*

Since in this study of the kinetics of yoghurt fermentation the cell count of two microorganisms had to be determined side by side, taking advantage of the difference in their morphology, the method of *Breed* was applied (DEMETER, 1952).

On the strength of preliminary investigations the samples were diluted prior to application to contain about 40–60 cells per microscopic field. Because of the statistical fluctuation of the counted cells due to their Poisson-type distribution, the total sum of counted units has to reach

a value of 300 at each point of measurement, otherwise the relative error of the mean value cannot be reduced below 15% with 95% confidence (CASSEL, 1965). Thus, both cocci and bacilli were counted in 3 squares and 3 fields of vision per each square. The average cell count per field was computed on the basis of cell counts enumerated in the 9 fields, taking into account the dilution. The cell count per ml was calculated by multiplying the cell count per field with the microscope factor $8.85 \cdot 10^5$.

*1.6. Determination of the specific growth rate coefficient* (k)

*1.6.1. Analysis of growth and product formation.* The average cell count per ml and the pH of the fermentation broth were plotted v. time. The method used for the enumeration of cells gives reliable results only up to the isoelectric point of casein, because beyond the isoelectric point the precipitated casein flakes interfere with counting. The growth curve was plotted at pH 4.8. On plotting the curves best fitting the points of measurement growth and product formation curves issue.

*1.6.2. Calculation of growth and product formation rate curves.* The difference quotients belonging to various points of time were calculated from the fitted curves of growth and product formation. These were then plotted as functions of cell density or product concentration.

*1.6.3. Determination of the* k *value.* The $k$ value represents the growth rate coefficient belonging to the exponential phase of growth. In this phase the equation of growth rate is the following:

$$\frac{dx}{dt} = k \cdot x$$

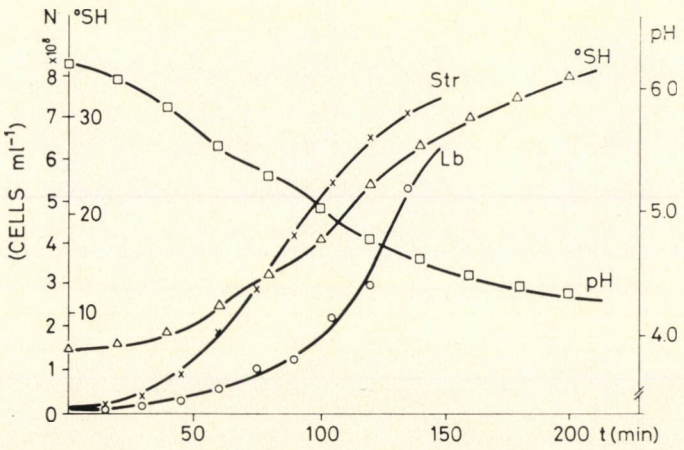


Fig. 2. Yoghurt fermentation at 45 °C with 3% inoculum. Growth and product formation curves

This is the equation of a straight line starting from the origin of the growth rate curve. The $k$ value was determined by regression analysis. This process is illustrated by utilizing the data of the fermentation at 45 °C with 3% inoculum. The data are given in Table 1. The growth and product formation curves are presented in Fig. 2, while the growth and product formation rate curves in Figs. 3 and 4.

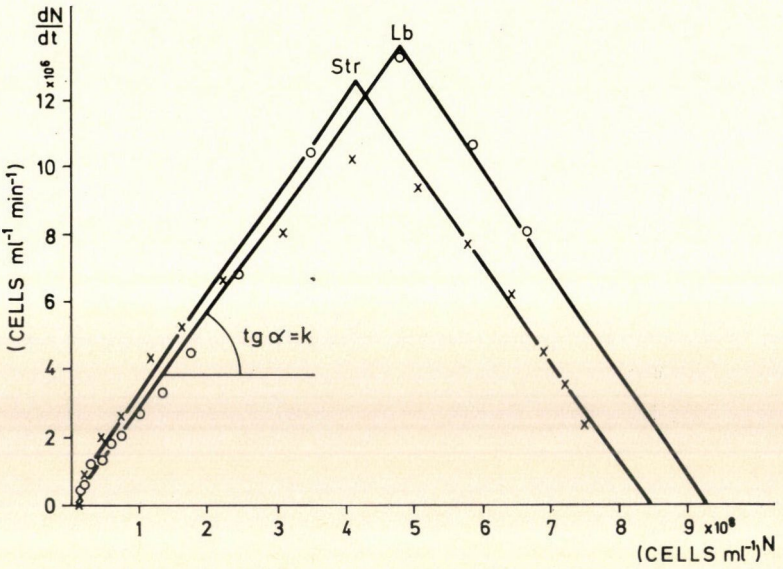


Fig. 3. Yoghurt fermentation at 45 °C with 3% inoculum. Product formation rate curve

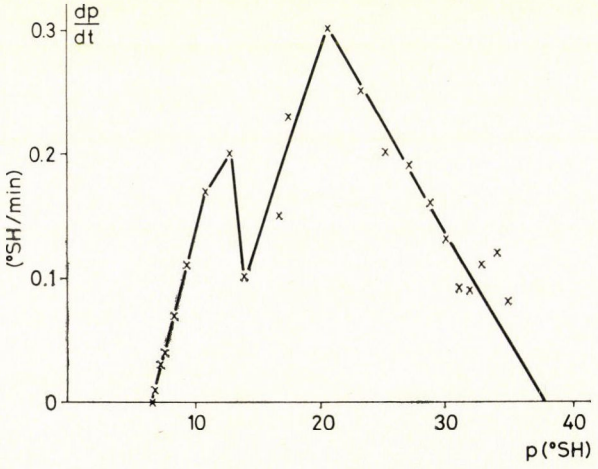


Fig. 4. Yoghurt fermentation at 45 °C with 3% inoculum. Growth rate curves

Table 1

*Data of yoghurt fermentation at 45 °C with 3% inoculum*

| t (min) | Str. thermophilus | | Lb. bulgaricus | | pH | p (° SH) | $\frac{dp}{dt}$ |
|---|---|---|---|---|---|---|---|
| | N (cell $ml^{-1}$) | $\frac{dN}{dt}$ | N (cell $ml^{-1}$) | $\frac{dN}{dt}$ | | | |
| | ($\times 10^6$) | | | | | | |
| 0 | 9 | 0.0 | 9 | 0.0 | 6.23 | 6.7 | 0.00 |
| 10 | 12 | 0.3 | 9 | 0.0 | 6.19 | 6.8 | 0.01 |
| 20 | 22 | 1.0 | 11 | 0.2 | 6.11 | 7.1 | 0.03 |
| 30 | 44 | 2.2 | 16 | 0.5 | 6.01 | 7.5 | 0.04 |
| 40 | 75 | 3.1 | 27 | 1.1 | 5.89 | 8.2 | 0.07 |
| 50 | 119 | 4.4 | 40 | 1.3 | 5.73 | 9.3 | 0.11 |
| 60 | 172 | 5.3 | 57 | 1.7 | 5.56 | 11.0 | 0.17 |
| 70 | 239 | 6.7 | 78 | 2.1 | 5.43 | 13.0 | 0.20 |
| 80 | 319 | 8.0 | 104 | 2.6 | 5.32 | 14.0 | 0.10 |
| 90 | 420 | 10.1 | 137 | 3.3 | 5.25 | 15.5 | 0.15 |
| 100 | 513 | 9.3 | 181 | 4.4 | 5.07 | 17.8 | 0.23 |
| 110 | 589 | 7.6 | 250 | 6.9 | 4.93 | 20.8 | 0.30 |
| 120 | 650 | 6.1 | 354 | 10.4 | 4.82 | 23.3 | 0.25 |
| 130 | 695 | 4.5 | 487 | 13.3 | 4.73 | 25.3 | 0.20 |
| 140 | 730 | 4.5 | 593 | 10.6 | 4.65 | 27.2 | 0.19 |
| 150 | 752 | 2.2 | 673 | 8.0 | 4.58 | 28.8 | 0.16 |
| 160 | | | | | 4.53 | 30.1 | 0.13 |
| 170 | | | | | 4.48 | 31.0 | 0.09 |
| 180 | | | | | 4.43 | 31.9 | 0.09 |
| 190 | | | | | 4.40 | 33.0 | 0.11 |
| 200 | | | | | 4.36 | 34.2 | 0.12 |
| 210 | | | | | 4.32 | 35.0 | 0.08 |

## 2. Results

### *2.1. Fermentations at 40 °C*

Fermentations at 40 °C, carried out with 1 and 3% inoculum, resp., were diauxic in character. Using 5% inoculum diauxie was not observed. Diauxie is illustrated in Figs. 5, 6 and 7, based on data of the fermentation with 1% inoculum. In determining the specific growth rate coefficient only the first step of the diauxie was taken into account.

The specific growth rate coefficients as functions of inoculum are given in Table 2.

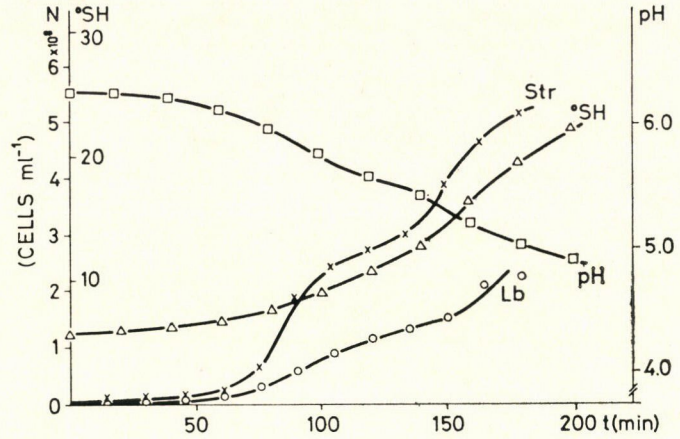


Fig. 5. Yoghurt fermentation at 40 °C with 1% inoculum. Growth and product formation curves

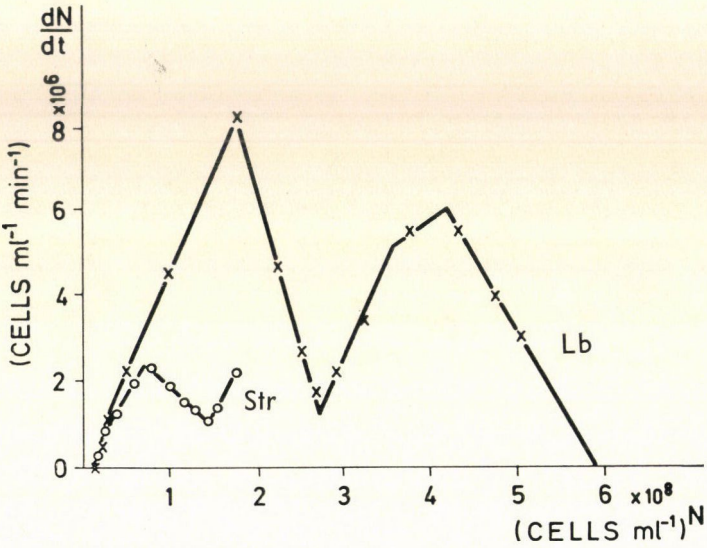


Fig. 6. Yoghurt fermentation at 40 °C with 1% inoculum. Growth rate curves

*2.2. Fermentations at 45 °C*

Diauxie was observed in fermentation with 1% inoculum, only. These fermentations are shown in Figs. 2, 3 and 4, used to illustrate the method of evaluation. Both fermentations at 40 and 45 °C, were healthy, morphological changes in the microbial flora were not observed. The specific growth rate coefficients are given in Table 3.

Table 2

*Specific growth rate coefficients of fermentations at 40 °C*

| Inoculum (%) | Str. thermophilus k ($min^{-1}$) | Lb. bulgaricus k ($min^{-1}$) |
|---|---|---|
| 1 | 0.0450* | 0.0354* |
| | 0.0352 | 0.0310* |
| 3 | 0.0400* | 0.0300* |
| | 0.0280 | 0.0227 |
| 5 | 0.0312 | 0.0286 |
| | 0.0294 | 0.0259 |

* Growth rate coefficient calculated from the first phase of the diauxic growth curve

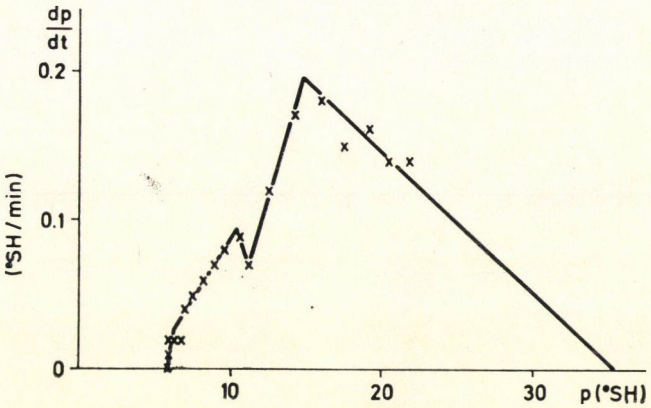


Fig. 7. Yoghurt fermentation at 40 °C with 1% inoculum. Product formation rate curve

Table 3

*Specific growth rate coefficients of fermentations at 45 °C*

| Inoculum (%) | Str. thermophilus k ($min^{-1}$) | Lb. bulgaricus k ($min^{-1}$) |
|---|---|---|
| 1 | 0.0432* | 0.0422* |
| | 0.0500* | 0.0376* |
| 3 | 0.0337 | 0.0305 |
| | 0.0300 | 0.0277 |
| 5 | 0.0386 | 0.0280 |
| | 0.0432 | 0.0276 |

* Growth rate coefficient calculated from the first phase of the diauxic growth curve

### 2.3. *Fermentations at 50 °C*

In fermentations at 50 °C, about 60–70 min after the initial healthy growth, a strong morphological change could be observed. Individual *Str. thermophilus* cells shrank to about half of their original size (1 $\mu$m). In the *Lb. bulgaricus* cells a very strong volution granulation could be observed. The growth and product formation curves of a fermentation started with 3% inoculum are shown in Fig. 8.

Because of the degeneration symptoms and short fermentation period the specific growth rate coefficients were not calculated.

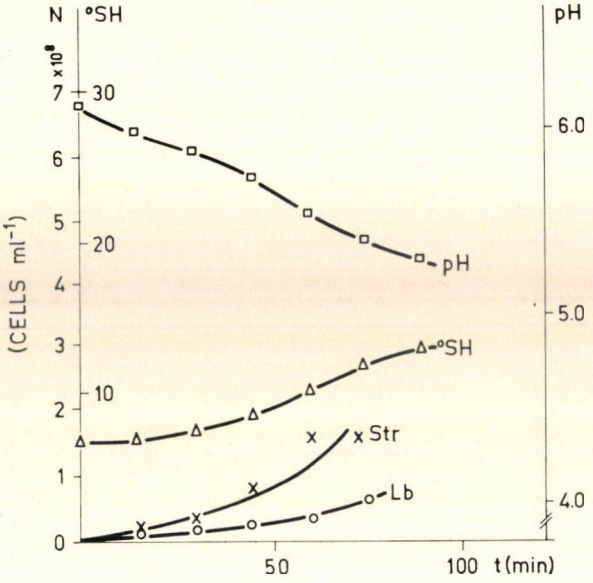


Fig. 8. Yoghurt fermentation at 50 °C with 3% inoculum. Growth and product formation curves

## 3. Conclusions

The specific growth rate coefficients of fermentations, carried out at different temperatures and with different inocula are summed up in Table 4. These were used in the analyses of variance.

The homogeneity of the standard deviations between parallels was proven by Bartlett's test and the data were evaluated by analysis of variance. The results of calculations, aimed at the existence of an exceptional treatment, are given in Table 5. On comparing the calculated $F$ value with the critical value a significant difference was found between treatments. A further analysis of this is given in Table 6.

Table 4

*Summary data of specific growth rate coefficients of batch yoghurt fermentations*

| Temperature (°C) | Inoculum (%) | Str. thermophilus k ($min^{-1}$) | Lb. bulgaricus k ($min^{-1}$) |
|---|---|---|---|
| 40 | 1 | 0.0450 | 0.0354 |
| | | 0.0352 | 0.0310 |
| | 3 | 0.0400 | 0.0300 |
| | | 0.0280 | 0.0227 |
| | 5 | 0.0312 | 0.0286 |
| | | 0.0294 | 0.0259 |
| 45 | 1 | 0.0432 | 0.0422 |
| | | 0.0500 | 0.0376 |
| | 3 | 0.0337 | 0.0305 |
| | | 0.0300 | 0.0277 |
| | 5 | 0.0386 | 0.0280 |
| | | 0.0432 | 0.0276 |

Table 5

*Variance table of* k *values*

| Source of variance | SQ | DF | $S^2$ | F | $F_{95}$ | $F_{99}$ |
|---|---|---|---|---|---|---|
| Total | 0.00114 | 23 | | | | |
| Between treatments | 0.00092 | 11 | $8.36 \cdot 10^{-5}$ | 4.57** | 2.8 | 4.3 |
| Between parallels | 0.00022 | 12 | $1.83 \cdot 10^{-5} = S_0^2$ | | | |

** Difference significant at the 99% probability level

Table 6

*Breakdown of the variance of* k *values*

| Source of variance | SQ | DF | $S^2$ | F | $F_{95}$ | $F_{99}$ |
|---|---|---|---|---|---|---|
| Between treatments | 0.00092 | 11 | | | | |
| Effect of microorganism | 0.00026 | 1 | $26 \cdot 10^{-5}$ | 14.2** | 4.8 | 9.3 |
| Effect of temperature | 0.00010 | 1 | $10 \cdot 10^{-5}$ | 5.46* | 4.8 | 9.3 |
| Effect of inoculum | 0.00043 | 2 | $21.5 \cdot 10^{-5}$ | 11.7** | 3.9 | 6.9 |
| Residue | 0.00013 | 7 | $1.85 \cdot 10^{-5}$ | 1.01 | 3.0 | 4.8 |
| Parallels | | 12 | $1.83 \cdot 10^{-5}$ | | | |

* Difference significant at the 95% level
** Difference significant at the 99% level

On the basis of the $F$ values as found in the table of variances, the difference between the specific growth rate coefficients belonging to different microorganisms or different inocula, was significant at the 99% probability level, while that belonging to different temperatures was significant at the 95% probability level.

At the given temperature the growth rate coefficient of *Str. thermophilus* is significantly higher at the 99% probability level than that of *Lb. bulgaricus*.

As regards the effect of temperature the growth rate coefficients at 45 °C were significantly higher at the 95% probability level than those at 40 °C.

The effect of the amount of inoculum was determined on the basis of the lowest significant difference (SD) between sums of treatments.

$$SD = t_{95} \cdot \sqrt{2 \cdot n \cdot s_0^2}, \text{ where}$$

$t_{95}$ = *Student*'s $t$ value belonging to the 95% probability level with a degree of freedom equal to that of $s_0^2$, $t_{95} = 2.18$;
$n$ = number of parallels within treatments to be compared (8);
$s_0^2$ = variance of the $k$ values of parallel tests, $s_0^2 = 1.83 \cdot 10^{-5}$.
After substitution SD = 0.037 $min^{-1}$.

| Inoculum (%) | Sum of treatment ($min^{-1}$) |
|---|---|
| 1 | 0.3196 |
| 3 | 0.2426 |
| 5 | 0.2525 |

On comparing the difference between sums of treatment with SD it may be seen that fermentations started with 1% inoculum had a significantly higher specific growth rate coefficient than fermentations with 3 or 5% inoculum. Between the latter 2 inoculum quantities difference was not observed. Since fermentations started with 1% inoculum were of diauxic character the determination of the growth rate coefficient was not unambiguous, therefore these data were not taken into account on calculating the parameters of a continuous process. Accordingly the growth rate coefficients required to calculate the dilution rates applicable in a continuous process are summarized in Table 7.

The points of view applied in selecting the temperature and dilution rate suitable for use in the continuous process and the results of experimental continuous fermentations will be given in a forthcoming paper.

Table 7

*95% confidence intervals of the mean values of the specific growth rate coefficients in batch yoghurt fermentations*

(Only data of fermentations started with 3 or 5% inocula were taken into account)

| Temperature (°C) | Str. thermophilus k ($min^{-1}$) | Lb. bulgaricus k ($min^{-1}$) |
|---|---|---|
| 40 | $0.0321 \pm 0.0068$ | $0.0268 \pm 0.0068$ |
| 45 | $0.0364 \pm 0.0068$ | $0.0284 \pm 0.0068$ |

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# ENERGY REQUIREMENTS FOR AERATION AND AGITATION IN THE PRODUCTION OF SOME PECTIC ENZYMES ON THE LABORATORY SCALE

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A substantial part of the fermentation costs derives from the energy consumption of agitation and aeration. It was attempted to determine the optimal agitation and aeration conditions for the endo-polygalacturonase (endo-PG) production of *Aspergillus awamori* in submerged culture. Endo-PG yield was investigated as a function of the $O_2$ transfer rate in the range of 26–179 mmol $l^{-1}$ $h^{-1}$ (ensured by agitation speeds of 460–940 rpm and aeration rates of 0.5–1.5 l $l^{-1}$ $min^{-1}$). An $O_2$ transfer rate of 49 mmol (corresponding to an agitation speed of 460 rpm and an aeration rate of 1.0 l $l^{-1}$ $min^{-1}$) proved to be optimal for the production of endo-PG enzyme. This $O_2$ transfer rate was found optimal also for the production of unit amount of endo-PG (sufficient to decompose 75% of 1 $m^3$ pectin solution) by the lowest power consumption (0.0079 kWh) and for the production of the highest endo-PG yield (126 $m^3$ $h^{-1}$) by unit power consumption (1 kWh).

Endo-PMG activity of the culture was less by three orders of magnitude than that of endo-PG. Mycelial yield was also poor as a result of the carbon source (pectin inducer) used in the nutrient medium.

As a consequence of the low yields, energy requirements for the production of unit endo-PMG and mycelia were higher than in the case of endo-PG.

The progress of the fermentation industry in the last decades was remarkable. The yields of antibiotic and enzyme fermentations have multiplied as a consequence of the development of technologies and apparatuses and high-standard genetic and microbiological work.

In this industry economy is a question of great importance where good yields are accepted only in case the production is undoubtedly economical. A considerable part of the cost of a fermentation product is derived from the power requirements for agitation and aeration. The value of $O_2$ transfer rate can give information about the efficiency of agitation and aeration, for its value increases with the increase of the agitation speed and of aeration rate.

In the present work the above important factors, influencing to a great extent the economy of the fermentation product, were analysed, to determine optimal aeration and agitation parameters.

## 1. Materials and methods

### *1.1. Microorganism*

An *Aspergillus awamori* (No. 2 of the collection of the FERMENTATION DEPARTMENT of the CENTRAL FOOD RESEARCH INSTITUTE, Budapest) strain was used for the experiments.

*1.2. Cultivation conditions*

Cultivation was carried out in 10-l glass fermentors each containing 5.9 l of the medium. This volume was inoculated with 600 ml of a 24-h vegetative culture. The temperature of incubation was 28 °C. Optimal agitation (460, 700 and 940 rpm) and aeration conditions (0.5, 1.0 and 1.5 $l\,l^{-1}\,min^{-1}$) were determined in natural pectin medium (extract of sugar beet slices, osettes) with a dry matter content of 4%.

Oxygen transfer rates (determined by the method of COOPER and coworkers, 1944) under various conditions of agitation and aeration are summarized in Table 1.

*1.3. Determination of enzyme activities*

*1.3.1. Endo-polygalacturonase (endo-PG) and endo-polymethylgalacturonase (endo-PMG).* Activity of endo-PG and endo-PMG was determined in an *Ostwald* type viscosimeter using Na-polypectate (SIGMA CHEMICAL WORKS, USA) and *Pomosin* pectin with a degree of esterification of 70% (POMOSIN GmbH, FRG) as the substrates (ZETELAKI & VAS, 1972).

*1.4. Determination of optimal agitation and aeration conditions*

Power consumed for the production of a unit quantity of product, as well as quantity of product produced by unit power consumption were investigated as a function of the following factors:

— speed of agitation
— rate of aeration
— period of cultivation.

The other variables were fixed during the experiment.

*1.4.1. Power consumption of agitation.* In the course of the experiments, agitators of six fermentors were driven by a common driving shaft. The power of the agitation of the six fermentors at various speeds of agitation are also given in Table 1. Data were measured in $A$ and converted into $W$ according to the following equation:

$$P_{(W)} = 3I \cdot U \cdot \cos \varphi$$

where:

I = current intensity, A
U = voltage
$\cos \varphi$ = power factor

*1.4.2. Power consumption of aeration.* From the air flow (l $h^{-1}$) of one fermentor and from the average pressure (atm) of the air, the power consumption of aeration was calculated as follows:

— *air-stream* in the case of maximal air consumption:
W (1 fermentor) = 594 l $h^{-1}$
W (6 fermentors) = 3564 l $h^{-1}$ = $0.99 \cdot 10^{-3}$ $m^3$ $s^{-1}$

— *air pressure:*

p = 1.75 atm = 0.177 MPa

— *net power:*

W p = $0.99 \cdot 10^{-3}$ $m^3$ $s^{-1}$ $17.15 \cdot 10^4$ N $m^{-2}$ = 169.8 W = 0.1698 kW

Assuming 70% efficiency, a

— *gross power* of 0.242 kW was obtained.

Power of the agitation and aeration as a function of $O_2$ transfer rate is depicted in Fig. 1.

Data in the Figure clearly show that the increase of aeration rate resulted in a considerable increase in power requirement.

*1.4.3. Power input on the products.* The power consumed for the production of a unit quantity of mycelium or enzyme and the quantity of mycelium and enzyme produced by unit power consumption were calculated from the measured electrical values and from the time of cultivation.

Table 1

*$O_2$ transfer rate and power consumption of agitation and aeration in 10-l KUTESZ fermentors*

| $O_2$ transfer rate (mmol $l^{-1}$ $h^{-1}$) | Agitation | | Aeration | | |
|---|---|---|---|---|---|
| | rpm | kW/6 ferm | 1 $l^{-1}$ $min^{-1}$ | 1 $h^{-1}$/1 ferm | kW/6 ferm |
| 26 | 460 | 0.436 | 0.5 | 198 | 0.080 |
| 49 | 460 | 0.436 | 1.0 | 396 | 0.159 |
| 60 | 460 | 0.436 | 1.5 | 594 | 0.238 |
| 85 | 700 | 0.452 | 0.5 | 198 | 0.080 |
| 93 | 940 | 0.470 | 0.5 | 198 | 0.080 |
| 100 | 700 | 0.452 | 1.0 | 396 | 0.159 |
| 126 | 940 | 0.470 | 1.0 | 396 | 0.159 |
| 146 | 700 | 0.452 | 1.5 | 594 | 0.238 |
| 179 | 940 | 0.470 | 1.5 | 594 | 0.238 |

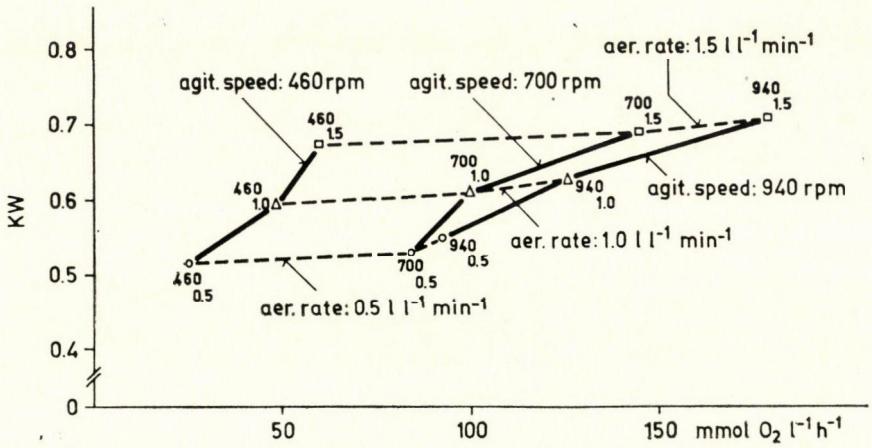


Fig. 1. Power requirements for agitation and aeration in six 10-1 KUTESZ fermentors as a function of $O_2$ transfer rate

## 2. Results

### *2.1. Enzyme production as a function of the $O_2$ transfer rate*

Oxygen transfer rate, as produced by various agitation and aeration conditions, was chosen to investigate the common effect of the latter factors.

Endo PG-and endo-PMG of the filtrate of the 48-, 60-, and 72-h *Aspergillus awamori* cultures as functions of $O_2$ transfer rate were plotted in Fig. 2. The above ages of cultures are of importance from the point of view of economy when determining the optimal time of the harvest.

The highest endo-PG concentration ($SPA_{75}^{Na\text{-}p}$: 115 l $h^{-1}$ $m^{-1}$) was measured in the filtrate of the 60-h culture at an $O_2$ transfer rate of 49 mmol $l^{-1}$ $h^{-1}$ (corresponding to an agitation speed and aeration rate of 460 rpm and 1.0 l $l^{-1}$ $min^{-1}$, resp.). Endo-PG yields measured at an $O_2$ transfer rate of 60 mmol in the 60- and 72-h cultures were about the same. A further increase in the $O_2$ transfer rate resulted in a decrease in the activity of endo-PG.

The highest endo-PMG activity was measured at the $O_2$ transfer rate of 60 mmol at each of the above cultivation periods but its value increased with the age of the culture ($SPA_{75}^{Pom}$: 0.57; 0.82 and 1.75 l $h^{-1}$ $ml^{-1}$). The further increase in the $O_2$ transfer rate resulted in a remarkable decrease in enzyme activity.

The mycelial yield of the culture showed a maximum at an $O_2$ transfer rate of 60 mmol $l^{-1}$ $h^{-1}$ and an increasing trend from 85 to 179 mmol $l^{-1}$ $h^{-1}$.

*2.2. Enzyme produced by unit power consumption*

The *endo-PG* yield produced by unit power consumption changed considerably both as a function of the $O_2$ transfer rate and of the period of cultivation, resp. (Fig. 3).

Highest endo-PG yields ($SPA_{75}^{Na\text{-}p}$: 126 and 81 $m^3\ h^{-1}$) produced by a unit power consumption were measured in the 60- and 48-h cultures

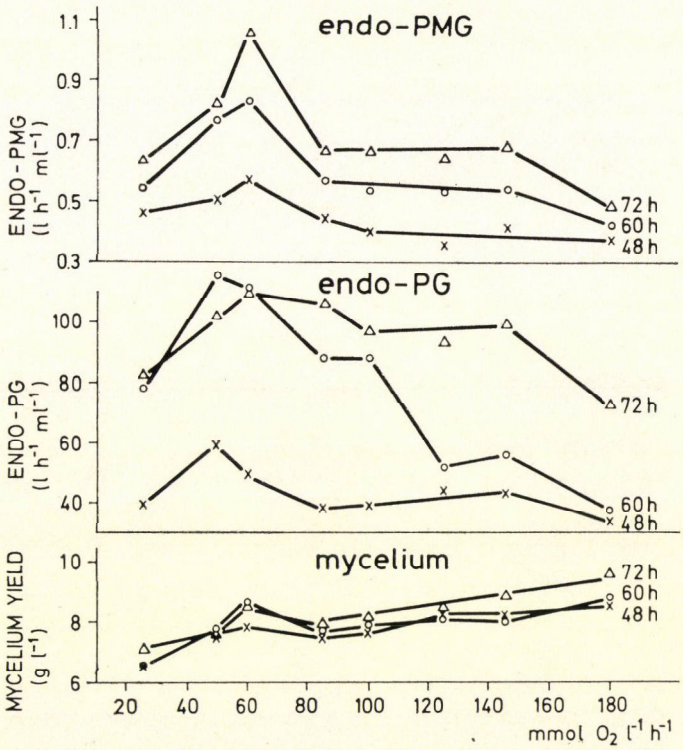


Fig. 2. Mycelial and enzyme (endo-PG, endo-PMG and macerase) yields in 48-, 60- and 72-h *Asp. awamori* cultures as a function of $O_2$ transfer rate. (Cultivation condition: see para. 1.2)

at an $O_2$ transfer rate of 49 mmol $l^{-1}\ h^{-1}$, while in the 72-h culture the highest endo-PG yield ($SPA_{75}^{Na\text{-}p}$: 107 $m^3\ h^{-1}$) was obtained at an $O_2$ transfer rate of 85 mmol. At an $O_2$ transfer rate of 49 mmol 1 $h^{-1}$ the endo-PG yield was not remarkably lower.

The maxima of the *endo-PMG* yields ($SPA_{75}^{Pom}$: 0.837 and 0.865 $m^3\ h^{-1}$) produced by unit power consumption were measured at $O_2$ transfer rates of 49 and 60 mmol in the 60- and 72-h cultures.

### *2.3. Power input on unit quantity of enzyme*

The power input on a unit product was also calculated under various aeration and agitation conditions, *i.e.* at various $O_2$ transfer rates and at different ages of the culture.

The lowest power input on the production of a unit amount of *endo-PG* ($m^3\ h^{-1}$) proved to be 0.0079 kWh (Fig. 4) in the 60-h culture at an $O_2$ transfer rate of 49 mmol $l^{-1}\ h^{-1}$.

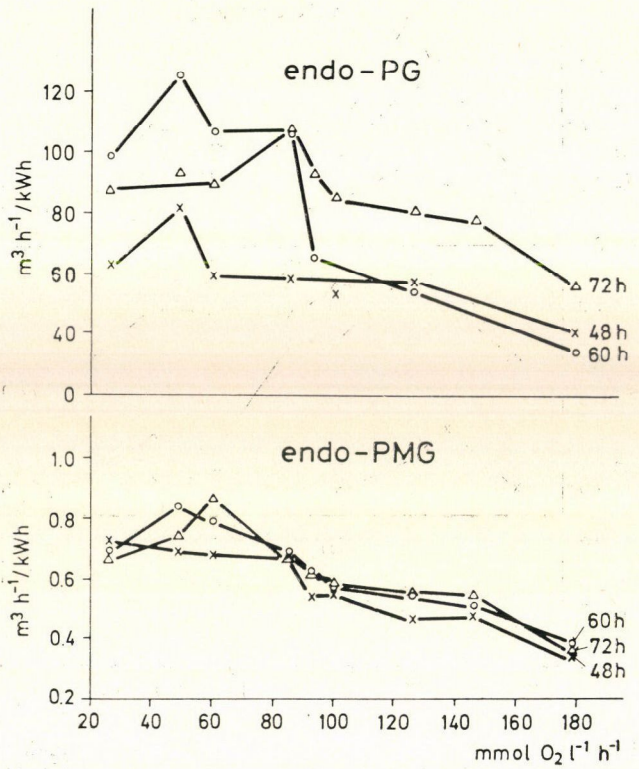


Fig. 3. Endo-PG and endo-PMG yields produced by unit power consumption as a function of $O_2$ transfer rate

The lowest power inputs on the production of unit *endo-PMG* ($m^3\ h^{-1}$) in the 60- and 72-h cultures were 1.194 and 1.156 kWh (Fig. 4) at $O_2$ transfer rates of 49 and 60 mmol $l^{-1}\ h^{-1}$.

One kg of mycelium was produced by the lowest power consumption (79 kWh) in the 48-h culture at an $O_2$ transfer rate of 93 mmol $l^{-1}\ h^{-1}$ (Fig. 5).

A summary of the results is presented in Tables 2 and 3.

Table 2

*Power consumption of the production of endo-PG, endo-PMG enzymes and mycelia of* Aspergillus awamori *culture*

| Agitation speed rpm | Aaration rate $l l^{-1} min^{-1}$ | Oxygen transfer rate mmol $l^{-1}$ $h^{-1}$ | Power consumption per unit amount of | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Endo-PG | | | Endo-PMG | | | Mycelium | | |
| | | | $m^{-3}$ h $kWh^{-1}$ | | | | | | | | |
| | | | Growth period (h) | | | | | | | | |
| | | | 48 | 60 | 72 | 48 | 60 | 72 | 48 | 60 | 72 |
| 460 | 0.5 | 26 | 0.016 | 0.0101 | 0.0115 | 1.371 | 1.449 | 1.498 | 96.38 | 121.41 | 134.11 |
| 460 | 1.0 | 49 | 0.012 | 0.0079 | 0.0107 | 1.436 | 1.195 | 1.336 | 93.95 | 117.43 | 142.80 |
| 460 | 1.5 | 60 | 0.017 | 0.0093 | 0.0112 | 1.450 | 1.254 | 1.156 | 105.03 | 119.29 | 144.86 |
| 700 | 0.5 | 85 | 0.017 | 0.0093 | 0.0093 | 1.465 | 1.431 | 1.492 | 87.46 | 106.40 | 124.35 |
| 940 | 0.5 | 93 | 0.014 | 0.0152 | 0.0108 | 1.824 | 1.596 | 1.601 | 79.04 | 108.75 | 120.73 |
| 700 | 1.0 | 100 | 0.019 | 0.0107 | 0.0117 | 1.834 | 1.744 | 1.704 | 97.77 | 118.26 | 137.47 |
| 940 | 1.0 | 126 | 0.017 | 0.0185 | 0.0124 | 2.169 | 1.836 | 1.798 | 92.04 | 117.94 | 136.83 |
| 700 | 1.5 | 146 | 0.022 | 0.0139 | 0.0127 | 2.017 | 1.981 | 1.849 | 102.85 | 131.85 | 144.00 |
| 940 | 1.5 | 179 | 0.025 | 0.0289 | 0.0178 | 2.705 | 2.606 | 2.679 | 101.13 | 129.12 | 112.79 |

□ optimal results
— near optimal results

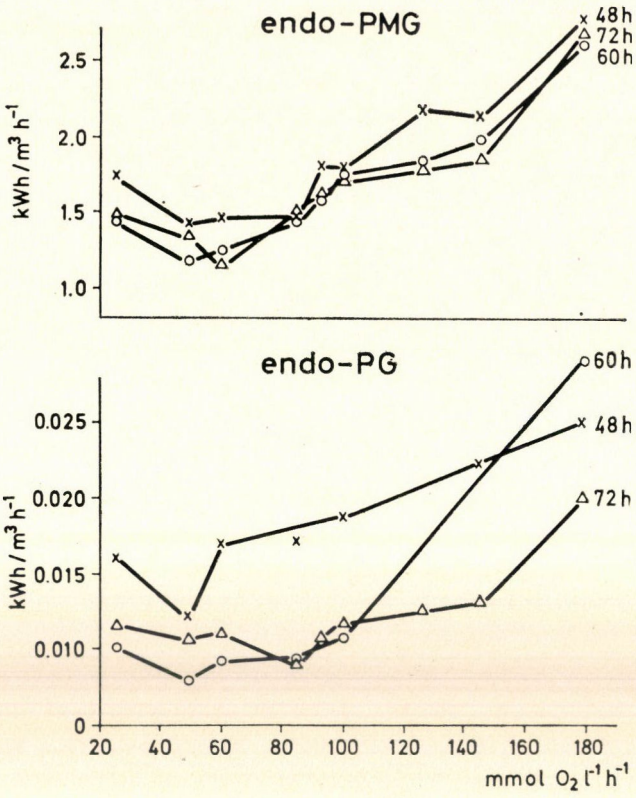


Fig. 4. Power input on unit quantity of endo-PG and endo-PMG enzyme as a function of $O_2$ transfer rate

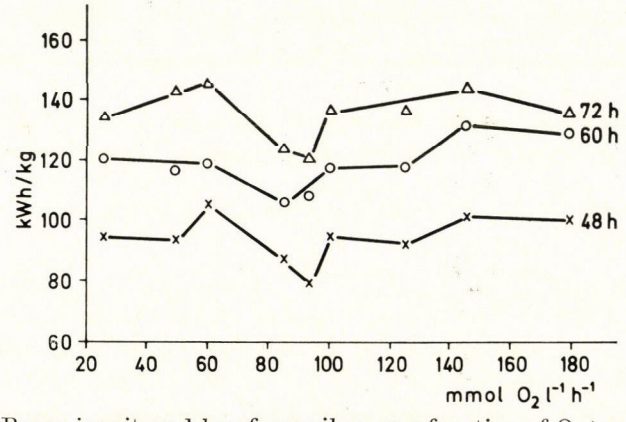


Fig. 5. Power inpuit on 1 kg of myceilum as a function of $O_2$ transfer rate

Table 3

*Optimal conditions* (time of cultivation, $O_2$ transfer rate, agitation and aeration) *for the endo-PG, endo-PMG and mycelial synthesis of* Aspergillus awamori

| Product | Time of cultivation (h) | $O_2$ transfer rate (mmol $l^{-1}$ $h^{-1}$) | Agitation (rpm) | Aeration ($l$ $min^{-1}$) | $m^3h^{-1}$ $kWh^{-1}$ | kWh $m^{-3}h$ |
|---|---|---|---|---|---|---|
| E-PG | 60 | 49 | 460 | 1 | 126 | 0.0079 |
| E-PMG | 72 | 60 | 460 | 1.5 | 1.194 | 0.865 |
| | | | | | $g \cdot kWh^{-1}$ | kWh $kg^{-1}$ · |
| Mycelium | 48 | 180 | 940 | 1.5 | 12.7 | 79 |

## 3. Conclusions

In the course of this work a certain relationship between $O_2$ transfer rate and enzyme yield of the culture was found. As $O_2$ transfer rate is a result of the agitation and aeration conditions (increasing with the increase of agitation speed and of aeration rate), it can be used in itself for the evaluation of the effect of the above two variables (AIBA *et al.*, 1965; TAKEI *et al.*, 1975).

Power requirement (increases also with the increase of the agitation and aeration intensity) of enzyme production was investigated as a function of $O_2$ transfer rate. It was found that power input of the maximal aeration rate (1.5 l $l^{-1}$ $min^{-1}$) proved to be approximately the half of that of the minimum agitation speed (460 rpm) in this experiment.

Endo-PG yield increased with the increase of the power of agitation at the lowest power input of aeration. At higher power input of aeration the increasing power input of agitation resulted in a negative effect on the enzyme yield (Fig. 6).

In contrast to the above results, TAKEI and co-workers (1975) found no correlation between protease yield and power requirement of agitation in a *Streptomyces* culture.

When the enzyme yield of the 60- and 72-h *Asp. awamori* cultures was examined as a function of $O_2$ transfer rate, the maxima of endo-PG and endo-PMG yields were obtained at a lower (49 and 60 mmol l $h^{-1}$, resp.) range of oxygen transfer rates, tested in this work. Highest endo-PG yield of the 72-h culture (somewhat lower than the 60-h maximum) was measured at $O_2$ transfer rate of 60 mmol $O_2$ $l^{-1}$ $h^{-1}$ and nearly the same at 85 mmol $l^{-1}$ $h^{-1}$, while only the 73% of this yield was produced at 26 mmol. These results were in agreement with our previous results when constitutive and inductive endo-PG formation of an other *Asp. awamori* strain was tested in 100 l fermentor (ZETELAKI-HORVÁTH & BÉKÁSSY-MOLNÁR, 1975). According

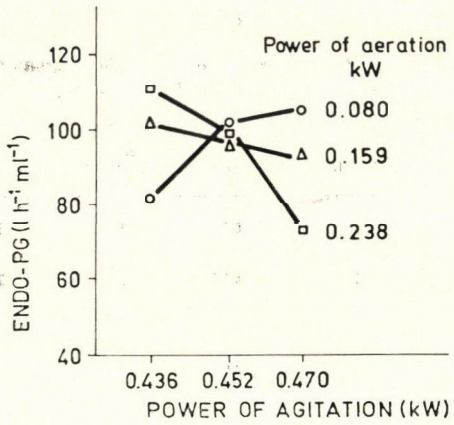


Fig. 6. Endo-PG yield as a function of power of agitation

to the above results a low range of $O_2$ transfer rate of 40–85 mmol $l^{-1}$ $h^{-1}$ seems to be optimal for the production of endo-PG (a hydrolase enzyme), while for the production of an oxydative enzyme (glucose oxidase) a demand for higher $O_2$ transfer rate was found. An $O_2$ transfer rate over 180 mmol could still increase the glucose oxidase yield of *Asp. niger* (ZETELAKI & VAS, 1968; ZETELAKI, 1970).

Power consumption for the production of unit ($m^3$ $h^{-1}$) endo-PG and mycelium (kg) were calculated too, though endo-PMG (if present at all) can be considered only as an accompanying enzyme of endo-PG, and cultivation conditions were chosen to be optimal for the endo-PG synthesis and not for the mycelial yield.

The lowest power inputs (0.0079 and 1.156 kWh) on the production of a certain quantity (sufficient to decompose the 75% of 1 $m^3$ substrate) of endo-PG and endo-PMG were measured at $O_2$ transfer rates of 49 and 60 mmol, resp.

The highest mycelial yield produced by unit power consumption was 12.7 g at an $O_2$ transfer of 18 mmol $l^{-1}$ $h^{-1}$ in the 48-h culture.

The maxima of endo-PG (126 $m^3$ $h^{-1}$) and endo-PMG (0.84 $m^3$ $h^{-1}$) yields produced by unit power input (1.0 kWh) were also found at $O_2$ transfer rates of 49 and 60 mmol $l^{-1}$ $h^{-1}$.

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# STUDY OF THE OXIDATIVE STABILITY OF VEGETABLE OILS BY MEANS OF THE DERIVATOGRAPH

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Oxidative changes in fats and oils during storage can be estimated by the determination of oxidative stability and dissolved oxygen content. Oxidative stability is usually investigated by accelerated stability tests, following forced oxidation. The dissolved oxygen content of fats and oils can be measured by the use of membrane-covered polarographic sensors.

A novel method has been developed for the thermoanalytical investigation of the oxidative stability of vegetable oils using a derivatograph. The aim of our experiments was to find optimum experimental conditions for examining the oxidative stability and the oxidation state. Sunflower and rapeseed oils at different states of oxidation were studied. The oxidation process was evaluated by highly reproducible thermogravimetric (TG) and derivative thermogravimetric (DTG) measurements.

It has been found that the oxidative stability of vegetable oils can be determined under isothermal conditions by means of the derivatograph. The new method is suitable also for routine quality control and, combined with the measurement of dissolved oxygen, it facilitates the fast evaluation of the storability of oils.

The autoxidative deterioration of edible fats and oils is not merely an economic problem of the food industry. Apart from the loss caused by rancidification, the nutritional value of the products also decreases as a result of the destruction of essential fatty acids and vitamins. The oxidative stability of an oil or fat is one of its most important properties and advance information on its susceptibility towards rancidity allows the determination of the quality.

As is well-known, the most important cause of the oxidative deterioration of lipids is the dissolution of oxygen from air and its subsequent reaction with the unsaturated glycerides. Consequently, the storability of edible oils is determined by the oxidative stability and dissolved oxygen content.

A number of methods have been developed to estimate or predict the keeping time, *i.e.* the actual room temperature stability. Most of these procedures are based on the fact that the samples are subjected to conditions accelerating the normal oxidation process. Oxidative stability of the products is characterized by the length of the induction period of the autoxidation process (PARDUN, 1969; THOMPSON, 1966). The simple, fast, and direct method for the determination of the dissolved oxygen content in fats and oils is provided by an electrochemical procedure using membrane-covered polarographic sensors (BECKER & NIEDERSTEBRUCH, 1966).

There is a possibility to estimate the oxidative changes of fats and oils and their tendency to further deterioration by studying the thermo-oxidative behaviour by thermal analysis, a widely used method for the investigation of ageing processes.

So far only a few papers have been published on the application of thermoanalytical methods to evaluate the oxidative stability of fats and oils. CROSS (1970) used the differential scanning calorimeter (DSC) to characterize different oils and shortenings. The exothermic enthalpy change was detected under isothermal conditions during the rapid oxidation process, which follows the induction period. Good correlation was found between the results obtained by standard tests and DSC data. American researchers (NIESCHLAG *et al.*, 1974; HASSEL, 1976) suggest thermogravimetric (TG) and pressure differential calorimetric (PDC) methods for the estimation of oil stability, using both static (isothermal) and dynamic programs (*i.e.* determination of the changes as a function of temperature).

Since autoxidation results change both in enthalpy and weight, complex thermoanalytical methods can be employed to study the process. Investigation of the autoxidative change and oxidative stability (storability) of fats and oils by complex thermoanalytical methods had not been published before our experiments. Our aim was to develop a simple, fast method suitable for routine evaluation of the storability of edible oils using a derivatograph. Our previous studies on the thermo-oxidative behaviour of vegetable oils are published elsewhere (BUZÁS *et al.*, 1977; 1978).

## 1. Materials and methods

Sunflower and rapeseed oils being the most important edible oils in Hungary were chosen as experimental models. Due to the high essential fatty acid content sunflower oil (average iodine number 127–136) is more sensitive to autoxidation than rapeseed oil (average iodine number 94–106). In order to study the effect of autoxidation on the thermooxidative behaviour, we compared fresh and aged oils. Aged samples were obtained from fresh oils either by aeration at 100 °C for two days, or by storing at room temperature for six months. Rancidity of the samples was characterized by peroxide values (POV) determined according to the standard iodometric test.

| Sapmle | POV (mval oxygen per kg oil) | Sample | POV (mval oxygen per kg oil) |
|---|---|---|---|
| Sunflower oil | | Rapeseed oil | |
| fresh | 1.0 | fresh | 2.0 |
| stored at room temperature | 12.0 | stored at room temperature | 15.0 |
| oxidized at 100 °C | 120.0 | oxidized at 100 °C | 90.0 |

Investigations have been carried out by means of the derivatograph (manufactured by MAGYAR OPTIKAI MŰVEK, Budapest), using photoregistration. Considering the results of previous studies (BUZÁS *et al.*, 1977; 1978), isothermal conditions proved to be suitable for evaluating storability and detecting autoxidative changes. Temperature was raised rapidly (in 10 min) to the reaction temperature and thermogravimetric (TG), derivative thermogravimetric (DTG) and differential thermoanalytical (DTA) curves were studied as a function of time.

For the study of both oxidative and thermal changes, samples were dispersed as a thin film on a ceramic block (of *c.* 2 cm diameter and 3 cm height), which was a fire-brick with a considerably large surface (1 $m^2$ $g^{-1}$ measured by argon adsorption) placed on a Pt plate sample holder. It has been proved that this sample holder can be heated up to 1600 °C without changing its specific surface. (A similar block was the reference material for DTA measurements.) Investigations were carried out under air flow (20 l $h^{-1}$). Sample weight was 400–450 mg.

The aim of our experiments was to determine the optimum conditions for the study of the oxidative stability and oxidation state.

## 2. Results

Figures 1 and 2 present the thermoanalytical curves of fresh, stored and aged sunflower oils. 98 °C was first chosen for the reaction temperature, similarly to the standard stability test. In this case remarkable differences could be observed between the thermooxidative behaviour of fresh and aged samples.

Evaluation of the TG curves was based on classical methods. The technique of following the oxidation of edible oils by weighing samples at intervals has been used for a long time. The method has been standardized for the investigation of the initial stages of autoxidation by OLCOTT & EINSET (1958). According to this procedure, the length of the induction period is taken as the time elapsed from the beginning of the experiment to the point the samples have gained 0.4% in weight.

As it can be seen, the oxygen uptake of oils at 98 °C is too fast to distinguish between fresh and slightly oxidized samples. In order to model autoxidation more properly and to find optimum conditions for the study of the initial phase, we have lowered the temperature. At 90 °C and especially at 80 °C, greater differences can be found in the length of the induction periods, the slope of the TG curves and the time of TG, DTG and DTA maxima. The changes occurred still within a reasonable time.

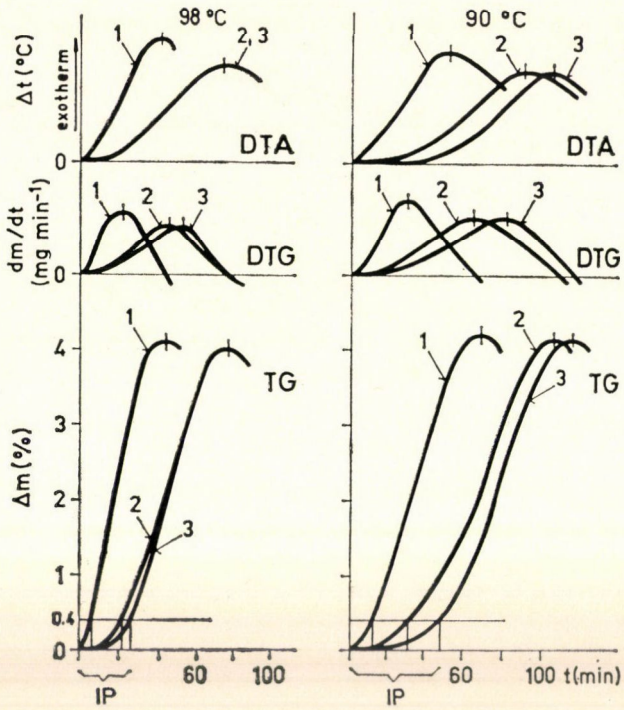


Fig. 1. Thermoanalytical curves of sunflower oils at 98 and 90 °C. IP: induction period, dm/dt: rate of weight change; 1: aged (POV = 120; 2: stored (POV = 12); 3: fresh (POV = 1)

Fresh, stored and aged rapeseed oils were investigated in the same way (Fig. 3). Rapeseed oil is more resistant to oxidation due to its fatty acid composition, therefore its oxygen uptake was slower than that of sunflower oil. In contrast to sunflower oil, with rapeseed oil no measurable weight increase could be determined within 3–3.5 hours, when heated at 80 °C.

## 3. Conclusions

According to the results, oxidative stability of the oils can be characterized by the following data:

— length of induction period (IP, min)
— time of maximum weight gain ($TG_{max}$, min)
— time of maximum rate of weight increase ($DTG_{max}$, min)
— time of maximum enthalpy change ($DTA_{max}$, min)

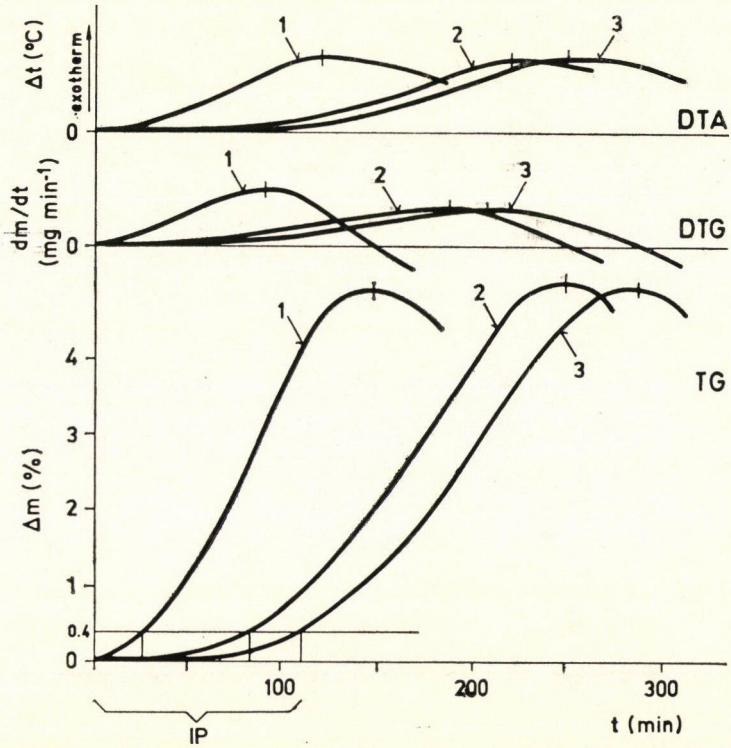


Fig. 2. Thermoanalytical curves of sunflower oils at 80 °C (For legend see Fig. 1)

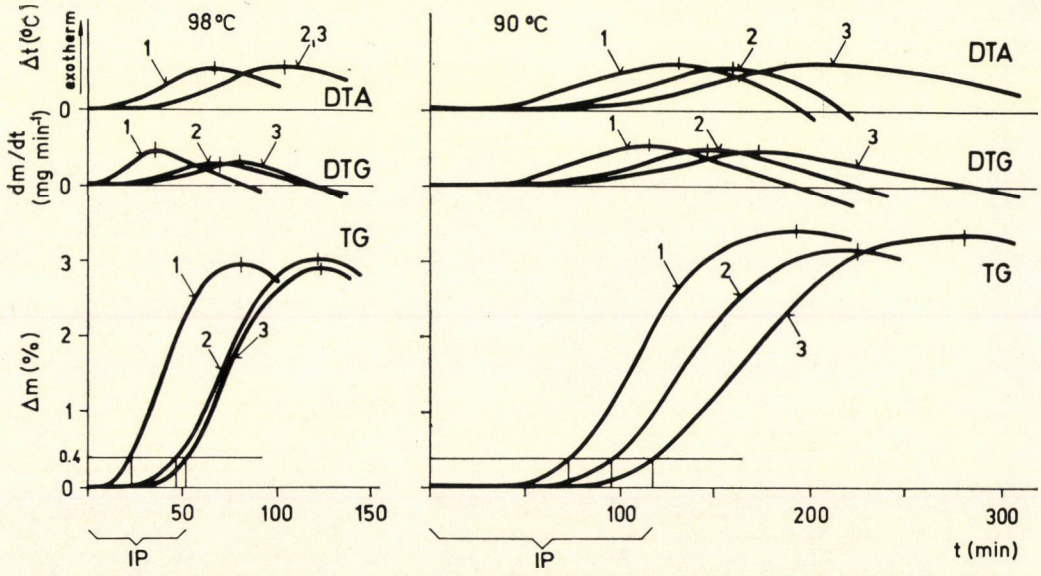


Fig. 3. Thermoanalytical curves of rapeseed oils at 98 and 90 °C. 1: aged (POV = 90.0): 2: stored (POV = 15.0); 3: fresh (POV = 2.0) For further legend see Fig. 1

The total weight gained ($\Delta m$, %) depends practically on the experimental conditions.

In practice, the determination of small differences is generally required. Consequently, sunflower oils can be feasibly studied at 80 °C, while 90 °C was found to be suitable for the evaluation of the oxidation state (storability) of rapeseed oils.

The thermooxidative behaviour of the two kinds of oils can be readily compared at 90 °C, as it is shown in Fig. 4. The difference between the two kinds of oils can be observed: the aged rapeseed oil of high POV oxidized considerably slower than fresh sunflower oil. This was shown not only by the length of the induction periods but also by the slope of the TG curves and by the time required to reach the highest oxidation rate, *i.e.* the DTG maxima. It can also be seen that under the same experimental conditions the maximum weight increase depends only on the type of the oil.

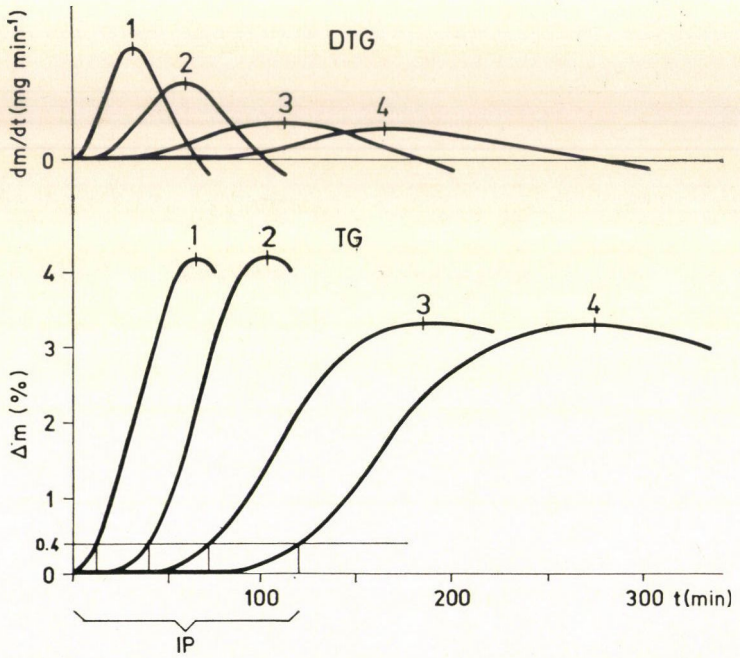


Fig. 4. TG and DTG curves of sunflower and rapeseed oils at 90 °C. 1: sunflower, aged; 2: sunflower, fresh; 3: rapeseed, aged; 4: rapeseed, fresh. (For further legend see Figs. 1 and 2)

Good reproducibility of the measurements was ensured by the high accuracy of weighing. The standard deviation of the data (calculated from the results of 11 measurements) was as follows (coefficient of variability):

| | |
|---|---|
| Induction period | ± 4.4 % |
| Time of $DTG_{max}$ | ± 5.3 % |
| $DTA_{max}$ | ± 3.4 % |
| $TG_{max}$ | ± 3.4 % |

The results were supported by the behaviour of bottled products held in the dark at room temperature. Oxidative changes were followed by the determination of the dissolved oxygen content and the standard iodometric peroxide value. The dissolved oxygen content of the oils was determined by means of a RADELKISZ (Hungary) product dissolved oxygen meter, *Aquacheck*, OH-501. Figure 5 shows that the rate of dissolved oxygen consumption is much higher for sunflower oil than for rapeseed oil. Hundred percent saturation corresponds to about 40 ppm oxygen (BECKER & NIEDERSTEBRUCH, 1966), which may be sufficient to cause flavour reversion. After storing for three months characteristic off-flavour of the samples could be detected.

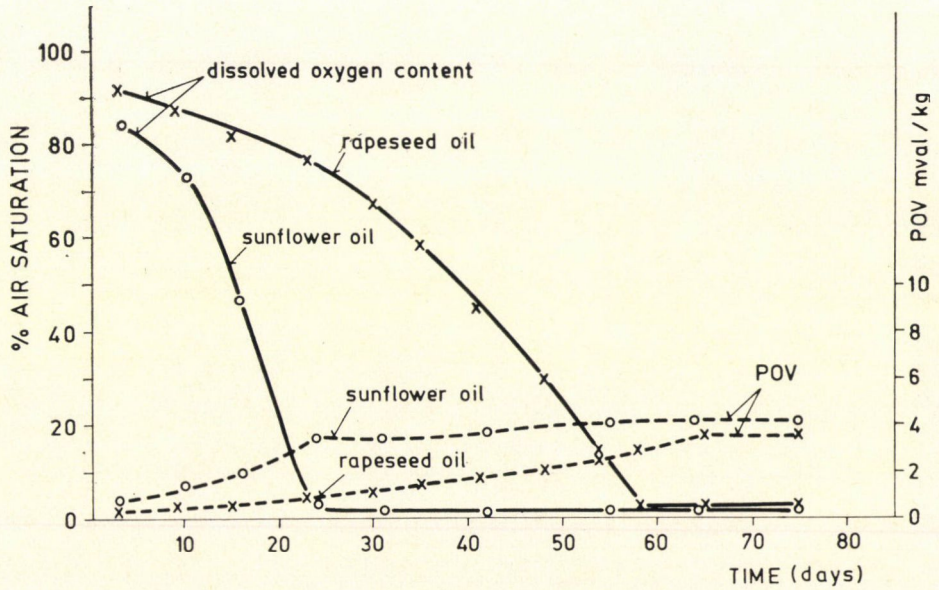


Fig. 5. Autoxidation of bottled oils held in the dark at room temperature

In summary, we may conclude that the oxidative stability of edible oils can be evaluated by means of the derivatograph under isothermal conditions, by modelling the oxidation processes. Oxidative changes can be followed quantitatively with TG and DTG curves, while DTA measurements show exothermic enthalpy changes.

This new method is suitable also for industrial quality control, therefore, we wish to extend our investigations to the study of correlation between this procedure and standard methods.

*

The paper was presented at the Scientific Colloquium organized by the Central Food Research Institute on 24th of September, 1976.

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# DOSE LIMITS *VERSUS* DOSE RANGE

## SUGGESTIONS FOR AN UNAMBIGUOUS TERMINOLOGY IN FOOD IRRADIATION

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(Received November 14, 1977)

In the field of food irradiation, as in industrial irradiation in general, the term "dose range" is often used rather loosely to describe widely differing intervals of absorbed doses. This indiscriminate usage has resulted in considerable confusion, and it is the purpose of this proposal to help clarify the situation.

Indeed, the term dose range is frequently used colloquially for what are, in fact, dose limits, instead of for the interval between two limits. It would reduce the confusion that has arisen in food irradiation if, in formal definitions, the term dose range is avoided: it is the various dose limits themselves that should be defined and specified.

## 1. Delimitation of the problem

### *1.1. Dose measurement*

As the measurement of absorbed dose[a] itself has a certain error, repeated determination of the dose in the same dose meter necessarily leads to a series of different values, which establishes the dose uncertainty of the method of determination, the data, in most cases, showing a *Gaussian* distribution (Fig. 1). The corresponding tolerance limits of dose are calculated as usual by the use of *Student*'s $t$ value. Since, with any acceptable dose meter, the scatter caused by the experimental error is relatively small and, in fact, is usually negligible in comparison with the variations caused by many other factors under industrial conditions, this particular uncertainty is of minor importance.

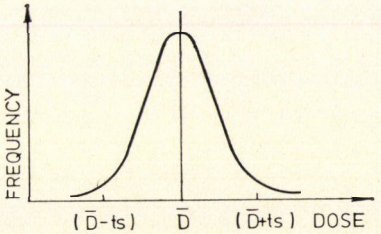


Fig. 1. Frequency distribution of dose measurement data, reflecting experimental error of the dosimetric method. (For nomenclature see Section 2)

[a] The term dose as used in this text will always refer to absorbed dose.

### *1.2. Dose measurement in the industrially irradiated product*

Of decisive practical significance in the irradiation plant is the variation of doses that can be observed under industrial conditions, as determined by a number of equivalent dose meters placed in various parts of the containers filled with the product to be irradiated. The distribution of doses measured under these conditions depends mainly on design and operating parameters of the plant, as well as on the absorption properties of the product. This dose distribution, which is normally determined during the commissioning of the plant and process, also defines the variation in the expected positions of the minimum and maximum doses. The dose uncertainties in the product depend, among others, on the apparatus and on the product bulk density (BD) as described by OWEN (1963), CHADWICK (1973; 1974), CHADWICK and co-workers (1974) and MCLAUGHLIN (1977).

The mean minimum and mean maximum doses measured in the product, their respective standard deviations ($s$) and tolerance factors ($k$) for a given probability level will define the practical limits in the irradiation plant (Fig. 2):

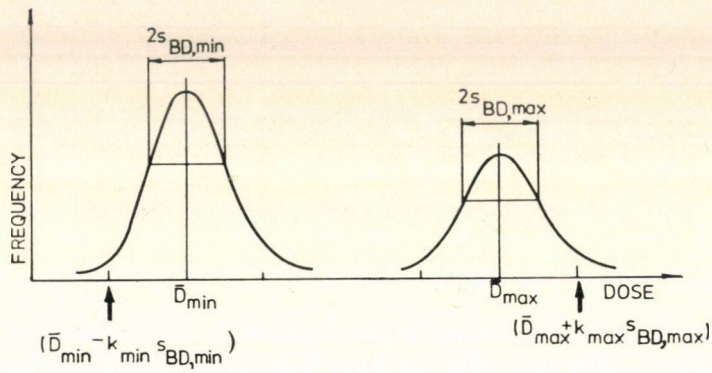


Fig. 2. Frequency distribution of dose absorbed in the product treated in an irradiator showing variations in dose, mainly due to variation in bulk density (BD), as measured at the two regions of the product where the maximum and minimum values of absorbed dose are likely to occur. (For nomenclature see Section 2)

$$\bar{D}_{\min} - k_{\min}\, s_{\mathrm{BD,\,min}}$$

and

$$\bar{D}_{\max} + k_{\max}\, s_{\mathrm{BD,\,max}}$$

The tolerance factor replaces the $t$ value in the estimation of *practical dose limits*. It determines which fraction $\beta$ of the product packages will at the most receive a treatment below or above the limits at a certain probability level P based on a number of measurements N. It should be emphasized that these practical dose limits are based on *measured values*, using the best dosimetry data available.

### *1.3. Technological dose limits*

It is also useful to distinguish measured dose limits (Section 1.2) from the interval of doses within which a given product can be expected to receive a technologically useful treatment. This interval is prescribed by experience and has a range of values below which no technologically valuable change occurs and above which detrimental alterations of technological properties of the product may ensue. This may be defined as the technological range of doses having limits $D_{t,\,min}$ and $D_{t,\,max}$. In contrast to the "quantities" discussed in Sections 1.1 and 1.2, the limiting values of the latter range are not *measured quantities*, *i.e.* not measured on the spot and on the occasion by dosimetry. They are quantities defined on the basis of previous technological experience with the given product.

### *1.4. Legal dose limits*

Finally, we propose to identify and distinguish two further limits of dose in food irradiation which we call the *legal dose limits*, to be defined by the appropriate regulatory body in law. These limits $D_{l,\,min}$ and $D_{l,\,max}$ are defined rigidly, without tolerance. Typically, the phraseology is "No part of the product shall receive" doses less than the legal minimum or more than the legal maximum dose.

## 2. Quantities considered

$\bar{D}$ is mean value of N measurements with a given dose meter

$s$ is the standard deviation of N measurements of dose with a given dose meter

$t$ is *Student*'s $t$ value for the N measurements at a probability level of P

$\bar{D}_{min}$, $\bar{D}_{max}$ are the mean minimum and maximum doses determined by a suitable sampling of calibrated dose meter readings throughout a production run

$s_{BD,\,min}$, $s_{BD,\,max}$ are the measured standard deviations of the minimum and maximum absorbed doses resulting from fluctuations in the density (BD) of the product

$k_{min}$, $k_{max}$ are the tolerance factors for the measured minimum and maximum absorbed doses, resp., at a given level of probability, P, with given numbers of measurements of the minimum and maximum, $N_{min}$ and $N_{max}$, resp.

$D_{t,\,min}$ is the technological minimum dose in the irradiation of a given food item based on food irradiation experience, *i.e.* that minimum

dose which is expected to guarantee the technologically desired effect (*e.g.* sprout inhibition, radappertization, *etc.*)

$D_{t,\,max}$ is the technological maximum dose, *i.e.* that dose above which, for reasons of marketability, the product is considered to be technologically unacceptable. This maximum is defined on the basis of subsequent storage and distribution requirements, *etc.* and is chosen to ensure that the food is sensorically acceptable to the consumer (*e.g.* suitable appearance, taste, smell, *etc.*)

$D_{l,\,min}$ is the legal minimum dose, *i.e.* the minimum absorbed dose in all the products as required in law

$D_{l,\,max}$ is the legal maximum dose, *i.e.* that dose above which the product is defined as being legally tainted. This definition is based on doses used and found to be safe in wholesomeness experiments.

## 3. Relations of the quantities

Based on legal and food technological considerations, it is obvious that the legal minimum dose should be higher than, or equal to, the technological minimum dose:

$$D_{l,\,min} \geq D_{t,\,min} \tag{1}$$

Since the technological and legal definitions consider entirely different factors and aspects, the legal maximum dose can be less than, equal to, or greater than, the technological maximum dose:

$$D_{l,\,max} \begin{matrix} < \\ = \\ > \end{matrix} D_{t,\,max} \tag{2}$$

### *3.1. General case*

For the general case, the following relations can be established:

Minimum dose limits:

$$D_{t,\,min} \leq D_{l,\,min} \leq (\overline{D}_{min} - k_{min}\, s_{BD,\,min}) \tag{3}$$

Maximum dose limits:

$$D_{l,\,max} \geq (\overline{D}_{max} + k_{max}\, s_{BD,\,max}) \leq D_{t,\,max} \tag{4}$$

bearing in mind relation (2) above. Figure 3 shows a possible interrelationship.

### *3.2. Ideal case*

For the ideal case (Fig. 4), the following relations hold:

$$(\overline{D}_{min} - k_{min}\, s_{BD,\,min}) = D_{l,\,min} = D_{t,\,min}$$

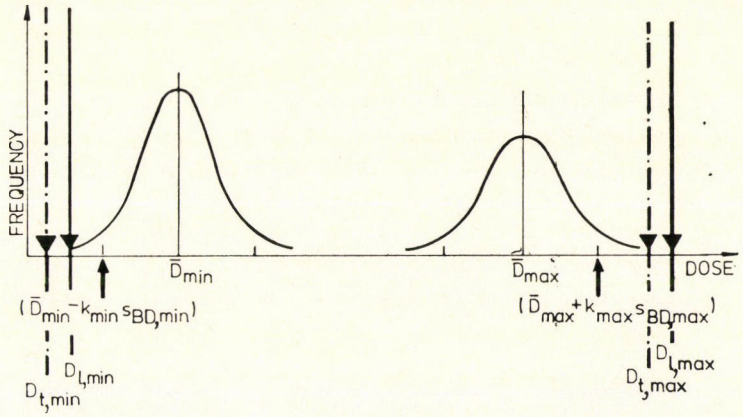


Fig. 3. Interrelationship of technological and legal dose limits, as well as dose distribution in the product. The *general* case. (For nomenclature see Section 2)

Maximum dose limits:

$$(\bar{D}_{max} + k_{max}\, s_{BD,\ max}) = D_{l,\ max} = D_{t,\ max}$$

### *3.3. A practical consideration*

In view of the fact that $(D_{max} - D_{min})$ or the uniformity ratio, $U$ $(= D_{max}/D_{min})$, are sometimes fixed for a given process by adjusting plant operating parameters and product configurations, for economic reasons the value of $(D_{min} - k_{min}\, s_{BD,\ min})$ may be set accordingly during the commissioning of the plant so that it equals the legal minimum dose:

$$(\bar{D}_{min} - k_{min}\, s_{BD,\ min}) = D_{l,\ min}$$

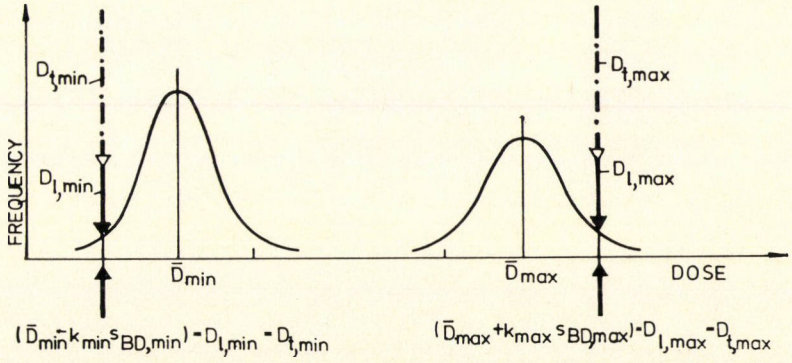


Fig. 4. Interrelationship of technological and legal dose limits and dose distribution in the product. The *ideal* case. (For nomenclature see Section 2)

### *3.4. A plea*

The term effective dose, $D_{eff}$, is often used, and misused, in texts on food irradiation. It means different things to different people and we suggest that it be dropped.

## 4. The next steps

It is believed that the change of emphasis away from *dose range* to *dose limits* will result in clearer terminology of the whole field of food irradiation. We hope that this suggestion will find favour with regulatory bodies, it being, we feel, of considerable practical importance. Comments on the above proposals are sought from all those familiar with, and interested in, the problem.

On the first version of this paper (communicated to *Food Irradiation Newsletter*, Vol. 1, 1977, No. 3, pp. 28–33) two such comments have already been received and may be cited here: (a) BRYNJOLFSSON and WANG: Dose limits (*Food Irradiation Newsletter*, Vol. 2, 1978, No. 2) and (b) VAS, LADOMERY and BECK: Use of dose limit specifications in regulations on food irradiation (*Food Irradiation Newsletter*, Vol. 2, 1978, No. 2).

If our suggestions, after suitable modification, are accepted, only one quantity will have to be decided upon, *i.e.* the value of the probability level, P, to be taken into account when calculating appropriate tolerance factors.

This article did not aim to discuss the significance of the chosen probability level which will, in turn, influence the magnitude of the $t$ and $k$ values. A discussion of the probability level will be the subject of a separate communication.

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# STUDY OF CUCUMBERS IN A MILD PICKLE PACKAGED AND STORED IN DIFFERENT WAYS

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The aim of the study was to determine the changes occurring during storage in pickled cucumbers packaged in plastic pouches, on the one hand, and the extent of damage caused by the cucumbers in the packaging material, on the other. The model substance used in the experiments was cucumber in a mild pickle. This was packaged in sealable pouches made of regenerated cellulose film coated on both sides with a poly(vinylidene chloride) emulsion. The pouches were stored at +3, +20 and +37 °C for 36, and 78 days, resp.

In the course of the study changes in the consistency and sensory qualities of the product and in the strength of the packaging material were tested.

It was concluded that pickled cucumbers packaged in pouches approached the quality of the bottled cucumbers. The strength parameters of the polymer film changed during storage, but this did not limit its use as a packaging material.

In modern packaging the use of flexible plastic materials or materials combined with plastic steadily increases. One of the possible uses is the packaging of pickles. This possibility is the more advantageous since in this packaging the weight ratio of product and wrapping material is favourable, the price of the plastic wrapping is economical in comparison to the bottle and it is a one-way packaging method. However, the storage stability of these goods is said to be limited.

There are but very few data in the literature on the keeping quality of pickles, in particular of pickled cucumbers packaged in plastic film. SACHAROV and GRIFFIN (1970) treat the subject only from the aspect of packaging regardless of the changes to be expected in the keeping quality of cucumber. They recommend polyamide and polyethylene combination or pliofilm for the purpose. Several other authors (MARTINELLI, 1969; ANON, 1969; NIEBOER, 1970) treat the subject in a general manner suggesting only various packaging materials but omitting the problem of changes during processing and storage. KARDOS and SZENES (1972) in their manual give only an outline of the interaction of packaging and product quality of pickled cucumbers.

Since data were not available in the literature it was considered important to investigate the changes in pickled cucumber stored in pouches made of plastic films under various conditions in comparison to pickled cucumbers bottled in the traditional form.

The investigations followed two directions. Changes in the cucumber during storage, on the one hand, and changes in the strength characteristics of the packaging material, on the other, were studied.

## 1. Materials and methods

### *1.1. Materials*

*Pickled cucumber.* Small-sized cucumbers, heat treated according to the formula $\frac{20\text{–}40\text{–}30 \text{ min}^*}{85 \text{ °C}}$ in 5-kg jars (MÉM STANDARD, 1959) were used. The length of cucumbers was 7–9 cm, their diameter 2.5–3.5 cm.

*Packaging foils.* For packaging the cucumbers a regenerated cellulose film (35 g $m^{-2}$), coated on both sides with poly(vinyl chloride)–poly(vinylidene chloride) copolymer (PVdC) layer formed from an aqueous emulsion in 0.010 mm thickness, was used. The film was made into pouches of $200 \times 240$ mm by heat sealing. The SCS (Cello Bisaran) film was the product of W. R. GRACE A/S (Denmark).

### *1.2. Methods*

*1.2.1. Packaging, heat treatment and storage.* Three hundred g of cucumbers previously exposed to the above heat treatment, were placed in jars of about 0.5 l filled up with 150 g brine and closed by the *Neophoenix* system. These served as the control-lot.

The plastic film pouches were filled with 300 g cucumbers and 320 g brine to cover them completely. The jars and pouches were filled up with the brine of the 5-kg jars of the starting material. The pouches were closed by heat sealing.

The jars and the pouches were heat treated in the same way in open vats at 85 °C. The heat treatment consisted of a 20-min period of temperature increase, 15 min at 85 °C and a 15-min cooling period. Twenty minutes were applied to bring the temperature to 85 °C, because both the cucumbers and brine were cold. The retention period was 15 min because the cucumbers had been heat treated previously.

The products, thus exposed to a second heat treatment in the new packaging, were stored in a thermostat at +37 °C and 65% relative humidity (RH), at room temperature (+20 °C on the average) at 57% RH and in a cold chamber of +3 °C and 77% RH for 36, 65 and 78 days, resp., in accordance with trade requirements.

* *i.e.*: heated at 85 °C for 40 min, come-up time: 20 min, cooling time: 30 min

*1.2.2. Consistency.* The consistency of the pickled cucumbers was determined with a LABOR penetrometer (Hungary). Measurements (in units of 0.1 mm) were carried out at room temperature with a cone [of standard mass (150 ± 0.25 g) and length (43.2 ± 0.25 mm) and an inclination angle of 30°] after a penetration time of 5 sec.

Ten cucumbers of each sample were measured. Each cucumber was tested in 3 places and the results were averaged. Samples were selected at random.

*1.2.3. Sensory tests.* For the sensory test an average of 5 samples was prepared of cucumbers packaged and stored under different conditions and tested by a panel of 10–12 skilled judges. The maximum score for each characteristic tested (colour, taste, odour and consistency) was 7. The results were ranked according to KRAMER's method (1956).

*1.2.4. Tensile strength and elongation at break.* To establish the mechanical properties of the packaging material its tensile strength and elongation at break were measured, according to the HUNGARIAN STANDARD (1952). Ten-mm wide strips were cut from each sample in machine and transverse directions. The fixing distance between samples was 100 mm.

An FMPW type 500 apparatus of VEB WERKSTOFF PRÜFMASCHINEN (GDR) was used for the measurement under conditions laid down in HUNGARIAN STANDARD (1974). The measuring limit was adjusted to 1 kN (100 kp), the read-off accuracy for tensile strength was 2 N (0.2 kp) and for elongation at break 0.5%.

*1.2.5. Bursting strength.* In order to be able to establish the probable resistance to inner tension effected by improper storage and the extent of mechanical stress resisted, the bursting strength of the packaging material was established.

A KORPU–BAAR type apparatus (Holland) was used with test samples of 10×10 cm. The test period of increasing stress was 10–15 sec. The fixing diameter in the instrument was 50 mm and the read-off accuracy 2 Pa (0.2 kp $m^{-2}$).

*1.2.6. Strength of heat sealing.* Since pouches will be produced most probably by means of heat sealing the heat sealing efficiency of the film was tested. Pouches were made on a HERBOR type apparatus (Hungary). These were then cut into pieces of 15 mm in machine and transverse directions. Measurements were carried out under conditions specified in HUNGARIAN STANDARD (1974). The samples thus obtained were fixed in the apparatus so as to have the heat seal exactly at the middle of the fixing distance.

The FMPW 500 type apparatus of VEB WERKSTOFF PRÜFMASCHINEN (GDR) was used for the measurements with a fixing distance of 100 mm, the measuring limit adjusted to 1 kN (100 kp) at an accuracy of 2 N (0.2 kp) and 0.5%, resp.

*1.2.7. Evaluation of results.* Mathematical statistical methods were used to evaluate results. To establish the comparability of results the $F$ test, and to compare the groups, analysis of variance or *Student*'s $t$ test were applied (SVÁB, 1967). The results of sensory tests were evaluated by KRAMER's ranking method (1956).

## 2. Results

The results of the study are discussed in two separate chapters. In the first chapter the changes occurring in the cucumbers packaged and stored under different conditions, in the second chapter the changes in the properties of the packaging material, are discussed.

### *2.1. Changes in the properties of cucumbers*

The consistency and the sensory quality of the cucumbers packaged in jars and in plastic pouches was studied as a function of storage time and conditions of storage. Their results are as follows.

*2.1.1. Changes in consistency.* The changes of consistency as measured by the penetrometer, are illustrated in the histogram in Fig. 1. The height of the columns shows the average value of 10 measurements, expressed in degrees of penetration. The bars in the columns stand for the double of the value of the standard deviation.

As it may be seen in the Figure, the consistency of cucumbers, particularly those packaged in plastic pouches, changed substantially during storage.

The consistency of cucumbers stored for 36 days at 37 °C was lower for both those bottled and packaged in plastic pouches than prior to the second heat treatment. The softening of the cucumbers packaged in pouches was particularly high since the penetration value increased from 36.2 to 62.6. The consistency of samples stored at 20 °C and 3 °C, resp., resembled that of the original sample packaged in 5–l jars.

The consistency of samples stored for 65 days as expressed in penetrometer values, was nearly the same for all the samples independent of the temperature of storage and packaging material.

The consistency of samples stored for 78 days was similarly of the same penetrometer value. Thus the packaging material caused no difference in the quality of the product.

In order to be able to evaluate the results more accurately they were compared by *Student*'s $t$ test. Samples were compared to the original material and to cucumbers packaged and heat treated, but not stored yet.

The results, illustrated in Fig. 1, show that samples packaged in jars and stored at 37 °C for 36 days were highly significantly, those packaged in plastic pouches and stored under the above conditions were very highly significantly, softer than the samples prior to storage. The samples stored in pouches at 20°C for 78 days were significantly harder than originally. All other samples did not differ from the sample heat treated but not yet stored.

When compared to the original product, the samples stored in pouches for 36 days differed slightly, those stored for 65 days more substantially. Cucumbers stored both in jars and in pouches for 78 days at 3 °C were significantly harder than the original material.

*2.1.2. Changes in the organoleptic quality.* The samples packaged in jars and in pouches and stored under different conditions for 36 and 78 days, were tested by a panel at the INSTITUTE OF FOOD TECHNOLOGY AND MICROBIOLOGY OF THE UNIVERSITY of HORTICULTURE, Budapest.

Results for samples stored for 36 days are given in Table 1, results for those stored for 78 days are shown in Table 2.

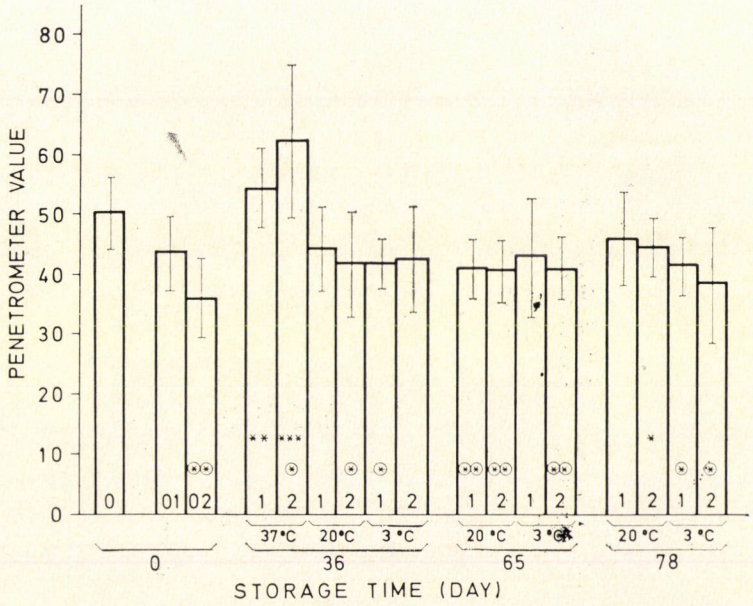


Fig. 1. Consistency values of pickled cucumbers stored for various periods at different temperatures in jars (1) and in plastic pouches (2) as established by penetrometer measurements at room temperature; bars indicate standard deviation
0: Original heat treated product; 01: bottled product after second heat treatment; 02: pouch-packed product after second heat treatment.
Results of *Student*'s *t* test: * significantly differing from the original product (O) at the 95% probability level; ** highly significantly differing from the original product (O) at the probability level of 99%; * significantly differing from the product repacked and given second heat treatment but prior to storage (01 and 02, resp.) at the 95% probability level; ** highly significantly differing from the repacked and second time heat treated product prior to storage (01 and 02, resp.) at the 99% probability level

Table 1

*Results of the sensory test of pickled cucumbers packaged in jars or in pouches and stored for 36 days at different temperatures. Evaluation according to rank sums*

| Packaging material | Temperature of storage (°C) | Average scores | | | | | Rank sums on the basis of | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | colour | odour | taste | consistency | total score | colour | odour | taste | consistency | total score |
| Jar | 37 | 5.7 | 6.3 | 6.1 | 6.4 | 24.5 | 39.5 | 30.0 | 28.5 | 33.5 | 28.0 |
| Plastic | 37 | 5.7 | 6.2 | 6.0 | 6.1 | 24.0 | 33.5 | 33.5 | 29.0 | 41.0 | 35.5 |
| Jar | 20 | 5.9 | 6.0 | 5.5 | 6.1 | 23.5 | 29.0 | 38.5 | 43.0 | 38.5 | 42.0 |
| Plastic | 20 | 6.1 | 6.2 | 5.5 | 6.2 | 24.0 | 25.0 | 33.5 | 41.5 | 36.0 | 38.0 |
| Jar | 3 | 5.9 | 5.7 | 5.7 | 6.6 | 23.9 | 33.5 | 45.5* | 36.5 | 28.5 | 36.0 |
| Plastic | 3 | 5.0 | 6.4 | 5.9 | 6.4 | 23.7 | 49.5** | 29.0 | 31.5 | 32.5 | 33.5 |

* The product is significantly better or worse if the rank sum is lower than 25 or higher than 45 (P = 5%), resp.
** The product is highly significantly better or worse if the rank sum is lower than 22 or higher than 48 (P = 1%), resp.

Table 2

*Result of the sensory test of pickled cucumbers packaged in jars or in pouches and stored for 78 days at different temperatures. Evaluation according to rank sums*

| Packaging material | Temperature of storage (°C) | Average scores | | | | | Rank sums on the basis of | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | colour | odour | taste | consistency | total score | colour | odour | taste | consistency | total score |
| Jar | 20 | 6.2 | 6.4 | 6.5 | 6.0 | 25.1 | 19.5 | 24.0 | 23.5 | 29.5 | 25.0 |
| Plastic | 20 | 5.5 | 6.1 | 5.9 | 5.5 | 23.0 | 34.0** | 31.0 | 32.0* | 33.0* | 34.0** |
| Jar | 3 | 6.2 | 6.5 | 6.4 | 6.7 | 25.8 | 19.0 | 23.5 | 22.5 | 17.0 | 18.0 |
| Plastic | 3 | 5.6 | 6.6 | 6.6 | 6.7 | 25.5 | 30.5 | 21.5 | 22.0 | 18.5 | 22.5 |

* The product is significantly better or worse if the rank sum is lower than 19 or higher than 31 (P = 5%), resp.
** The product is highly significantly better or worse if the rank sum is lower than 17 or higher than 33 (P = 1 %), resp.

The colour of the sample stored for 36 days at 3 °C in pouches was highly significantly worse than the colour of the sample stored in jars. The odour of the sample in pouch was also significantly worse than that of the sample bottled.

The colour of the samples packaged in pouches and stored at 20 °C for 78 days, was highly significantly, the taste and consistency significantly, worse than that of the samples packaged in jars. The total score of the pouch-packaged samples was highly significantly lower than that of the bottled samples, in other cases the difference was not significant.

### *2.2. Investigation of the packaging material*

The packaging material was studied in view of the requirements of the food industry to establish whether it was suitable for use in packaging pickled cucumbers, whether the cucumbers or their brine caused a change detrimental to the plastic material. The mechanical properties and sealing strength of the plastic pouches used for packaging and storage of cucumbers were tested. The pouches to be tested were washed first in tap water, then with distilled water free of acid. Residual drops of water were soaked up with filter paper. The pouches were then cut into suitable pieces and dried in a drying oven for 48 h at 70 °C.

*2.2.1. Mechanical properties.* Pouches containing pickled cucumbers and stored at 3, 20 and 37 °C for 36 and 65 days, resp., were tested for any change in their tensile strength in machine and transverse directions and bursting strength. Tensile and bursting strengths were expressed in MPa (kp $cm^{-2}$), while the elongation at break in %. The average results of measurements ($\bar{x}$) and the standard deviations ($\pm s$) are summarized in Table 3.

The results given in the table were exposed to analysis of variance in order to establish the effect of heat upon the packaging material stored for different periods. The results of the analysis of variance are given in Table 4.

As it may be seen in the table the tensile strength of the packaging material measured in machine direction differed highly significantly from the initial value after 36 days storage as an effect of increasing temperature. After 65 days the difference was very highly significant. The change in the values of tensile strength measured in the transverse direction was not so explicit. The change was significant only in the samples stored for 65 days.

The differences with increasing storage temperature in elongation at break measured in machine direction were highly significant after 36 days and significant after 65 days. The elongation at break values measured in transverse direction suffered a very highly significant change as caused by storage at high temperature after 36 days, while after 65 days the difference was not significant.

Table 3

*Average values ($\bar{x}$) and standard deviations (s) of the tensile strength and elongation at break as measured in machine direction* (M) *and transverse direction* (T) *on 10 test samples each and of the bursting strength as measured on 5 test samples each of the plastic film used for packaging pickled cucumber and stored for different periods at different temperatures*

| Storage temperature (°C) | Storage period (days) | Tensile strength | | | | Elongation at break (%) | | | | Bursting strength MPa | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | M | | T | | M | | T | | | |
| | | $\bar{x}$ | ±s | $\bar{x}$ | ±s | $\bar{x}$ | ±s | $\bar{x}$ | ±s | $\bar{x}$ | ±s |
| – | 0 | 39.15 | 1.765 | 34.75 | 0.418 | 20.9 | 2.92 | 34.3 | 5.35 | 0.35 | 0.017 |
| 3 | 36 | 31.16 | 1.293 | 33.96 | 1.742 | 18.6 | 8.25 | 9.6 | 2.41 | 0.33 | 0.024 |
| | 65 | 23.66 | 0.829 | 33.69 | 2.408 | 32.8 | 8.67 | 15.0 | 1.25 | 0.32 | 0.019 |
| 20 | 36 | 27.06 | 1.308 | 28.56 | 1.892 | 10.0 | 2.13 | 5.2 | 1.32 | 0.35 | 0.037 |
| | 65 | 27.36 | 2.290 | 31.66 | 1.639 | 22.6 | 7.71 | 13.6 | 2.27 | 0.33 | 0.036 |
| 37 | 36 | 33.16 | 5.637 | 32.23 | 3.492 | 16.8 | 3.33 | 5.6 | 1.58 | 0.31 | 0.052 |

In the values of bursting strength significant change was not caused by storage at high temperatures.

*2.2.3. Changes in the seal strength of the packaging material.* The tensile strength and elongation at break of the seal was measured at machine direction and transverse direction with 6 test samples each. Results of the measurements are given in Table 5.

Table 4

*Comparison by the* F *and* t *tests of the tensile strength and elongation at break values as measured in machine* (M) *and transverse* (T) *direction on 10 samples each and the bursting strength values as measured on 5 test samples each of plastic film used for packaging pickled cucumbers and stored at different temperatures for different periods*

| Storage period (days) | Storage temperature (°C) | Comparative test | Property studied | | | | |
|---|---|---|---|---|---|---|---|
| | | | Tensile strength | | Elongation at break | | Bursting strength |
| | | | M | T | M | T | |
| 36 | 2<br>20<br>37 | F | 8.25** | 2.89 | 7.37** | 17.7*** | 2.58 |
| 65 | 2<br>20 | t | 4.80*** | 2.20* | 2.78* | 1.71 | 0.78 |

Difference:

* significant at the 95% probability level,
** highly significant at the 99% probability level,
*** very highly significant at the 99.9% probability level.

Table 5

*Average values* ($\bar{x}$) *and standard deviations* (s) *of tensile strength and elongation at break values as measured in machine* (M) *and transverse* (T) *direction on 6 test samples each of the plastic film used for packaging pickled cucumber in order to establish the strength of heat seal and the comparison by* F *test of the variations of the measurements and by* t *test the averages of the measurements*

| | Tensile strength (MPa) | | Elongation at break (%) | |
|---|---|---|---|---|
| | M | T | M | T |
| $\bar{x}$ | 21.90 | 22.94 | 12.3 | 13.3 |
| $\pm s$ | 1.87 | 1.34 | 2.9 | 3.4 |
| F | 1.93 | | 1.40 | |
| t | 1.103 | | 0.543 | |

Data in the table show that the strength of the seal was not affected by the direction of the film on sealing. Significant differences in the tensile strength and elongation at break values were not observed in either direction.

## 3. Conclusions

It appears from the results that the consistency of the pickled cucumbers, whether packaged in plastic pouches or in jars, changed to about the same extent (appr. 5%) when stored at +20 or +3 °C for 36, 65 or 78 days, resp. The consistency of the pickle in plastic pouches, stored at +37 °C for 36 days deteriorated by about 15% compared to that of bottled pickles (Fig. 1).

Sensory tests have shown the colour, odour and consistency of the pouch-packed cucumbers, stored at 20 °C for 78 days to be significantly poorer than that of the bottled pickles (Tables 2, 3). In all other cases differences due to the packaging material could not be observed.

The results of the study permit of the conclusion that plastic pouches may be used for the packaging of cucumbers in mild pickle.

The tensile strength, elongation at break and bursting strength values of the plastic film show a general reduction in mechanical strength even if this tendency is not quite consistent. However, the extent of deterioration in the packaging material did not affect the safety of its use for this purpose.

It seems from the tests on seals that the direction of the plastic material need not be taken into account in the preparation of the pouches.

Data on the differences between the tensile strength of the film and seal strength show that the latter ensures safe packaging of goods of the given gross weight.

The study permits of the general conclusion that regenerated cellulose film coated on both sides with PVdC emulsion is suitable for use in consumer packs of pickled cucumbers.

*

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# COMPARATIVE STUDIES ON THE INDUSTRIAL HYDROGENATION OF RAPESEED OILS OF TRADITIONAL AND OF REDUCED ERUCIC ACID CONTENT

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In recent years a comprehensive study into the cultivation of rape varieties with reduced erucic acid content was launched in Hungary. The hydrogenation process of oils obtained from varieties *IR-1* and *Wipol*, grown on a large scale, was compared to the hydrogenation of traditional rapeseed oil. The refractive index, slip point, fatty acid composition, trans isomer content, were studied as functions of hydrogenation period in the course of fat manufacture of low and high slip point.

The experiments have shown the saturation and isomerization process to occur in a similar way as in traditional rapeseed oils. The use of fresh catalyst is favourable for the saturation reactions, while in the presence of used catalyst the rate of geometrical isomerization is higher and that of saturation slower.

The slip point raising effect of erucic acid, present in traditional rapeseed fats in about 50% by forming long-chain saturated and isomeric fatty acids during hydrogenation, is counterbalanced: (a) in fats hydrogenated to a high slip point by the higher amount of saturated fatty acids: (b) in fats with lower slip point by the higher proportion of geometric isomers.

Hydrogenated vegetable oils form a substantial part of fats used for food purposes. In Hungary rapeseed oil is an important raw material of the so-called "manufactured" edible fats, *e.g.* margarine, shortening. On the nutritional and biological role of the main fatty acid component of rapeseed oil, that is on erucic acid, research is carried out all over the world. In monodiet animal feeding experiments with rapeseed oil the erucic acid was found to be enhanced in the heart of the test animal and to cause deleterious biological changes. These changes were considered to be due to long-chain fatty acids, mainly to erucic acid (APPELQUIST & OHLSON, 1972; CONACHER & PAGE, 1972). As a consequence the majority of countries where rape is grown is engaged in improving and cultivating rape with zero or low erucic acid content.

In recent years similar experiments were started in Hungary as well to produce rape varieties of reduced erucic acid content and to use the seeds grown in largescale experiments for industrial processing. The RESEARCH INSTITUTE FOR THE VEGETABLE OIL AND DETERGENT INDUSTRY participated not only in the cultivation experiments but also in the processing of the new varieties and in the processing of the oil thus obtained. In the course of these experiments the hydrogenation of the oils obtained from the rape variety *Wipol*, grown in large scale experiments in 1975 and from variety *IR-1*,

grown in 1976, was compared with the hydrogenation process of the traditional rape variety.

The hydrogenation of vegetable oils is a technique known and applied since the beginning of this century. However, the reaction kinetics of this process is not yet sufficiently elucidated. The partial hydrogenation of triglycerides is an extremely intricate, simultaneously and successively occurring series of reactions, accompanied by the adsorption and desorption of the reacting components and of the products, and saturation and isomerization reactions (ALBRIGHT, 1973). Some of the double-bonds of the unsaturated fatty acids becomes saturated with hydrogen, a part of the remaining double-bonds undergo isomerization or an alteration of their geometrical configuration or of their position. In the course of the reaction the isomerized double-bonds become saturated, too, or take part in further isomerization. Changes in the relative reaction rates of saturation and isomerization have a significant effect on the properties of the hydrogenated product (ALBRIGHT *et al.*, 1970).

The saturation and isomerization reactions occurring on the surface of the catalyst may be followed only with the complete application of the analytical characteristics (ALBRIGHT & WISNIAK, 1962; ALLEN, 1968; NAKAZAWA *et al.*, 1956). In earlier papers we reported on the changes of the analytical characteristics in the course of industrial hydrogenation of sunflower seed and soybean oils. Relations between individual characteristics, selectivity of hydrogenation, were investigated (KURUCZ *et al.*, 1974). It was established that in the case of identical conditions of reaction and identical kinds of oil the relationship of slip point and refractive index, of iodine number and refractive index, of trans isomer content and refractive index may be described by a close mathematical correlation. On the basis of these relationships it is possible to gain more information on a process by measurement of an analytical characteristic.

In the course of hydrogenation, a mixture continuously changing in its composition is studied and this is an extremely difficult task. This fact is reflected in the literature on this subject where many contradictions are encountered. Our observations are in accordance with results in recent literature (ALLEN & COVEY, 1970; PATTERSON, 1973).

## 1. Materials and methods

The industrial hydrogenation processes were carried out with three rapeseed oils of different erucic acid content. The fatty acid composition is shown in Table 1. Variety *Wipol* of reduced erucic acid content was improved in Poland, variety *IR-1* in Hungary.

Table 1

*Fatty acid composition of rapeseed oils of traditional and of reduced erucic acid content, respectively*

| Fatty acid component | Per cent of total fatty acids | | |
|---|---|---|---|
| | Varieties | | |
| | *Wipol* | *IR-1* | Traditional |
| C 16 | 3.2 | 3.3 | 2.7 |
| C 16 : 1 | 0.1 | 0.2 | 0.4 |
| C 18 | 0.9 | 1.4 | 0.9 |
| C 18 : 1 | 32.8 | 49.0 | 13.7 |
| C 18 : 2 | 16.5 | 17.4 | 14.0 |
| C 18 : 3 | 8.1 | 8.4 | 9.6 |
| C 20 | 0.4 | 0.9 | 0.9 |
| C 20 : 1 | 14.3 | 10.7 | 8.0 |
| C 22 : 1 | 23.7 | 8.7 | 51.8 |

Since in margarine manufacture basic fats of different solidity are required the three oils were hydrogenated from low slip point (28–30 °C) to high slip point (43–45 °C) and these processes were investigated. Conditions of hydrogenation are given in Table 2 and the characteristics of the final products in Table 3.

The oils were hardened in a *Normann* equipment of batch operation in batches of 6 000 kg each. The temperature was 180–200 °C. The *Unichema* P type catalyst contained 20–25% nickel. To fats of slip points above 40 °C, fresh, active catalyst, to fats of slip points about 28–30 °C, used catalyst were applied.

Table 2

*Conditions of rapeseed oil hydrogenation*

| Rape variety | Serial number of hardening | Temperature (°C) | Quantity and character of catalyst (kg) | Reaction period (min) | Slip point expected (°C) |
|---|---|---|---|---|---|
| Traditional variety | 1 | 180–186 | 25 U + 10 F | 60 | 28–30 |
| | 2 | 180–192 | 25 F | 60 | 43–45 |
| *Wipol* | 3 | 170 | 50 U | 60 | 28–30 |
| | 4 | 190 | 25 F | 60 | 43–45 |
| *IR-1* | 5 | 200 | 60 U + 5 F | 80 | 28–30 |
| | 6 | 200 | 25 F | 70 | 43–45 |

F = fresh, U = used

Table

*Characteristics of*

| Serial number of hydrogenation | $n_D^{40°C}$ | Slip point (°C) | Trans (%) | Fatty acid | | |
|---|---|---|---|---|---|---|
| | | | | C 16 | C 18 | C 18 : 1 |
| 1. | 1.4627 | 28.5 | 60.7 | 3.6 | 2.6 | 23.7 |
| 2. | 1.4622 | 27.0 | 70.6 | 3.9 | 1.9 | 47.7 |
| 3. | 1.4616 | 29.8 | 73.1 | 3.6 | 4.1 | 64.5 |
| 4. | — | 44.4 | 43.6 | 3.5 | 9.1 | 20.6 |
| 5. | — | 45.0 | 40.0 | 3.8 | 21.7 | 39.1 |
| 6. | — | 45.1 | 42.3 | 4.1 | 26.3 | 47.7 |

The fatty acid composition was determined in accordance with HUNGARIAN STANDARD (1973) after transesterification in a methyl alcohol-benzene-sulphuric acid mixture by gas chromatography in a *Pye Unicam* 104 apparatus.

The total quantity of the isolated trans isomer fatty acids was determined by infra-red spectroscopy, IUPAC method, on the basis of the absorption maximum at 970 $cm^{-1}$, in *Zeiss* UR 20 spectrophotometer (A.O.C.S., 1970).

The slip point and refractive index were determined according to HUNGARIAN STANDARDS (1974 and 1966). The theoretical iodine number was calculated on the basis of the fatty acid composition.

The Hungarian standard methods comply essentially with those given in the manual of COCKS and REDE (1966).

## 2. Results

The changes in the analytical characteristics were followed by the analysis of samples taken every 10 min.

For hydrogenation to low slip points, the analytical characteristics of traditional rapeseed oil and of the varieties *Wipol* and *IR-1* are shown in Fig. 1. The Figure illustrates changes in the main fatty acid components, in the quantity of the trans isomer fatty acids and in the slip points as a function of time.

Rapid saturation of linolenic acid, an increased hydrogen uptake with the formation of $C_{18:1}$ fatty acids and a highly intense trans isomer formation is characteristic of the hydrogenation of all three rapeseed oil varieties. Saturated fatty acids were formed during hydrogenation only in very low proportion.

*the final products*

| composition (%) | | | | | | Theoretical iodine number |
|---|---|---|---|---|---|---|
| C 18 : 2 | C 18 : 3 | C 20 | C 20 : 1 | C 22 | C 22 : 1 | |
| 8.1 | — | 1.0 | 8.9 | 1.8 | 50.7 | 81.3 |
| 5.3 | 0.8 | 1.0 | 16.0 | 0.4 | 23.4 | 85.1 |
| 6.1 | 0.7 | 0.8 | 10.8 | 1.0 | 8.4 | 85.1 |
| 3.4 | — | 2.6 | 6.8 | 13.3 | 40.7 | 60.7 |
| 0.9 | — | 4.2 | 9.7 | 4.3 | 16.3 | 57.0 |
| 1.8 | — | 3.9 | 7.8 | 2.6 | 5.8 | 57.0 |

In the case of hydrogenation to a high slip point the chief fatty acid components, the formation of trans isomer fatty acids and the slip points are shown in Fig. 2.

Two stages may be observed in hydrogenations illustrated in Fig. 2. In the first phase, along with the formation of oleic acid, the saturation of linoleic and linolenic acids is characteristic. The formation of the saturated fatty acids occurs in the second phase. Curves with maxima are characteristic of the formation of trans isomer fatty acids. In the first phase of hydrogena-

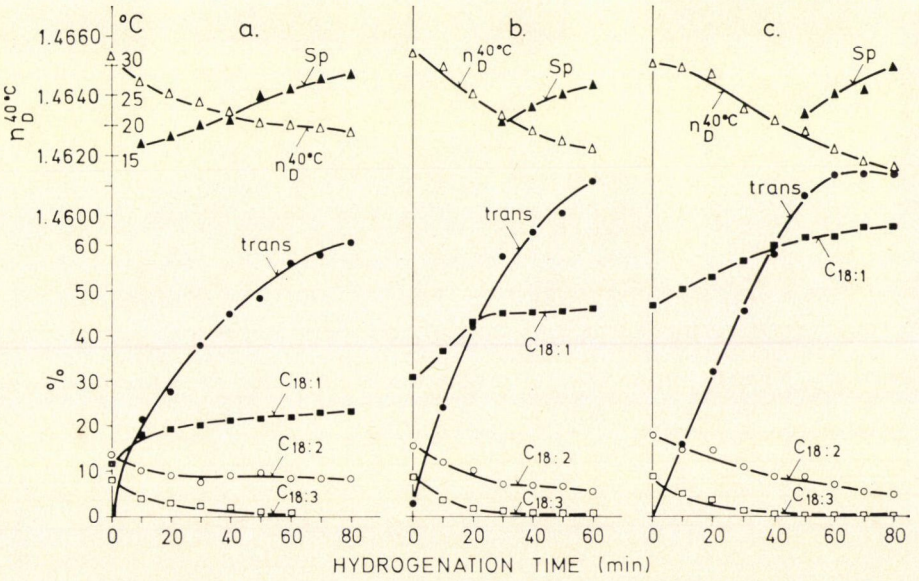


Fig. 1. Analytical characteristics *vs.* reaction time in hydrogenations to low slip points a. No. 1 traditional rapeseed oil; b. No. 3 *Wipol* rapeseed oil; c. No. 5 *IR-1* rapeseed oil

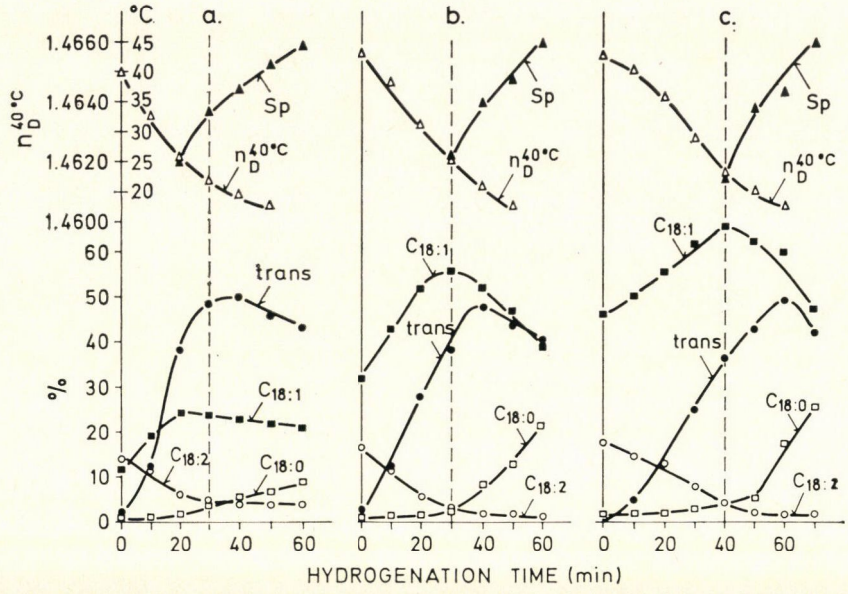


Fig. 2. Analytical characteristics *vs.* reaction time in hydrogenations to high slip points a. No. 2 traditional rapeseed oil; b. No. 4 *Wipol* rapeseed oil; c. No. 6 *IR-1* rapeseed oil

tion, geometrical isomers are formed at high intensity, while after reaching a maximum in the second phase their quantity gradually declines because of saturation.

## 3. Conclusions

In the course of the hydrogenation of traditional rapeseed oils of traditional and reduced erucic acid content the partial processes are similar, even if the extent is different, due to the differences in their composition as illustrated in Figs. 1 and 2.

In hydrogenations to low slip points the factory applies catalysts which had been used several times previously. The utilization of a catalyst of lower activity reduces the rate of saturated fatty acid formation to a minimum, increases at the same time the quantity of geometrical isomers forming simultaneously with partial saturation reactions, *i.e.* increases the selectivity of the reaction. Comparing the fatty acid composition of the oils with traditional erucic acid content with those of reduced erucic acid content it may be seen that the slip point increasing effect of long-chain fatty acids is compensated not by the formation of saturated fatty acids, but by the higher amount of geometrical isomers. The total quantity of the geometrical isomers is higher by about 10–15%. The melting points of fatty acids, taking

an important part in the formation of low slip point, are: *cis* $C_{18:1}$ 16.3 °C; *trans* $C_{18:1}$ 43.7 °C; *cis* $C_{22:1}$ 33.5 °C; *trans* $C_{22:1}$ 60 °C. On the basis of the above it is evident that the development of geometrical isomers in rapeseed oils of reduced erucic acid content resembles the hydrogenation of sunflower seed and soybean oils. Thus in rapeseed oils of low slip point and reduced erucic acid content hardening is due to $C_{18:1}$ isomers similarly to soybean and sunflower oils.

Hydrogenations to a high slip point (Fig. 2) fall into two phases from the aspect of reaction kinetics. The two phases are shown in the figure by a broken line. In the first phase the poly-unsaturated fatty acids are converted into monoenes, linolenic acid becomes saturated and the quantity of linoleic acid is reduced to 2–3%. Formation of saturated fatty acids is at minimum. Beside monoene formation at an average rate of 0.65% per min, the formation of geometrical isomers is also significant at a rate of 1% per min. Thus, beside the partial saturation of polyenes, geometrical isomerization takes also place and in parallel the *cis* monoene present in the oil is partly converted into *trans* isomer.

The formation of saturated fatty acids is characteristic of the second phase. In consequence of the initial fatty acid composition in products of high slip point a significant difference appears in the saturated fatty acid distribution in rapeseed oils of traditional and reduced erucic acid content.

While in the traditional rapeseed oil 46.7% behenic acid is formed (related to the total amount of saturated fatty acids), this value is 12.6 with variety *Wipol* and 6.9 with *IR-1*. At the same time in fats of reduced erucic acid content the quantity of the total saturated fatty acids is higher by 5–10% and this compensates for the lower behenic acid content in the production of higher slip point.

The total amount of geometrical isomers is about 40% with all three rapeseed oils. The difference is in the quality of the *trans* isomer fatty acids, since the traditional rapeseed oil contains a larger amount of *trans* $C_{22:1}$ fatty acid.

The relationships between analytical characteristics apply for the rapeseed oils of lower erucic acid content, too. This is shown in Fig. 3 where the relationship between refractive index and slip point is illustrated in hydrogenations to lower slip points.

To describe the relationships in exact mathematical terms the analysis of several series is necessary. However, it may be seen in the figure that the constants of the relationship are influenced beside the reaction conditions by the rape variety, too.

Straight lines *a*, *b* and *c* are nearly parallel, thus, a change of identical extent in the slip point, *e.g.* from 25 °C to 30 °C, causes identical change in the refractive index. On the other hand, to identical slip points belong

different refractive indices in the three rapeseed oils. In the traditional rapeseed oil, to a slip point of 25 °C belongs $n_D^{40} = 1.4632$, while in *Wipol* oil $n_D^{40} = 1.4625$ and in *IR-1* oil $n_D^{40} = 1.4623$.

Our next aim is to establish the mathematical relationships for the different varieties on the basis of several hydrogenation series.

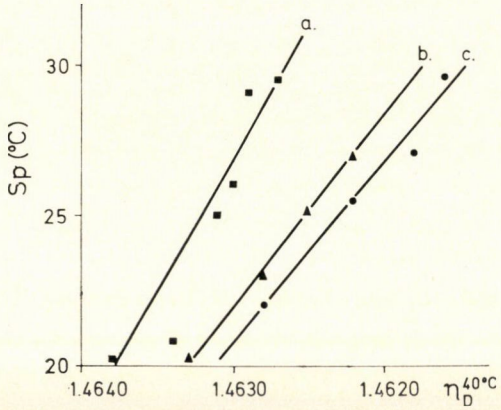


Fig. 3. Relationship between the refractive index and the slip point in hydrogenations to low slip points
a. Traditional rapeseed oil; b. *Wipol* rapeseed oil; c. *IR-1* rapeseed oil

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# EFFECTS OF FOOD PRESERVATIVES AND ANTIOXIDANTS ON COLONY FORMATION BY HEATED CONIDIA OF *ASPERGILLUS FLAVUS*

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Conidia of *Aspergillus flavus* were observed to undergo reversible injury upon exposure to heat. The pH (3.5, 5.5 or 8.0) of standard potato dextrose agar (PDA) recovery medium did not affect colony formation by heated or unheated conidia. Injured conidia had an increased sensitivity to sodium chloride (10%), potassium sorbate (25 ppm), sodium benzoate (150 ppm), calcium and sodium propionates (2000 ppm), and sulfur dioxide (50 ppm). Butylated hydroxyanisole, at 100 ppm, inhibited colony formation by unheated and heated conidia at about the same rate, whereas butylated hydroxytoluene, propyl gallate, and nordihydroguaiaretic acid were without effect at a concentration of 1000 ppm in PDA recovery medium. Sensitivity to antimycotics and butylated hydroxyanisole was enhanced in PDA at pH 3.5 as compared to pH 5.5.

Injury and subsequent death or recovery of bacterial cells exposed to adverse environments have been observed for many years. Researchers have shown that sublethal injury (stress) is a result of structural or metabolic damage and that death is more likely to occur when cells are exposed to sub-optimal environments during the recovery period. Resistance to injury or death is affected by cultural conditions to which bacterial cells are exposed before chemical or physical assault as well as to the nature and severity of the assault. The fact that repair of injured cells is known to depend greatly on cultural conditions used for recovery requires that special consideration be given to composition of enrichment broths and plating agars used for enumeration of microorganisms in foods.

The bulk of research in the area of injury and recovery of microorganisms has been directed toward bacteria. Only recently have investigators recognized that fungal cells can be injured upon exposure to adverse environments. Mazur and Schmidt (1968) hypothesized that the major cause of injury in frozen cells of *Saccharomyces cerevisiae* occurs when intracellular ice is formed and recrystallized during slow warming. Tanaka and Miyatake (1975) reported that fermentation products play an important role in the injury of cells of *S. cerevisiae* in frozen dough. In later studies alcohols with more than three carbon atoms and the coexistence of wheat flour and ethanol were observed to induce injury in frozen bakers' yeast (Tanaka *et al.*, 1976).

Yeasts exhibit sublethal injury upon exposure to heat. Nelson (1972) reported that heated cells of *S. cerevisiae*, *Kluyveromyces lactis*, and *Candida*

*utilis* were more sensitive to sub-optimal pH than were control cells. TSUCHIDO *et al.* (1972) demonstrated that sorbic acid markedly inhibited protein synthesis and respiratory activity of thermally injured cells of *C. utilis*. The ability of cells of *S. cerevisiae* to recover from thermal treatment has been attributed to possible utilization of endogenous reserves (STEVENSON & RICHARDS, 1976), whereas the extent of injury and recovery of several other species of yeasts was influenced substantially by utilizable sugars in suspending media (HAGLER & LEWIS, 1974).

ADAMS and ORDAL (1976) noted that heated conidia of an aflatoxigenic strain of *Aspergillus parasiticus* had increased sensitivity to sodium chloride in recovery media. The number of injured conidia increased as the heating time continued. STEVENSON and GRAUMLICH (1978) recently reviewed injury and recovery of yeasts and molds.

Antioxidants are added to a variety of foods to retard the development of oxidative rancidity. Because of their phenolic structure, several antioxidants also have antimicrobial activity. Butylated hydroxyanisole (BHA) is lethal to *Staphylococcus aureus* and *Escherichia coli* (CHANG & BRANEN, 1975; SHIH & HARRIS, 1977), *Vibrio parahaemolyticus* (ROBACH *et al.*, 1977), and *Salmonella typhimurium* and *Aspergillus parasiticus* (CHANG & BRANEN, 1975). Nordihydroguaiaretic acid (NDGA) is inhibitory to *S. cerevisiae* (KAUFMANN & AHMED, 1967) whereas both NDGA and propyl gallate are inhibitory to *E. coli* (SHIH & HARRIS, 1977). To the authors' knowledge, no reports are available on the effects of antioxidants on heat-treated conidia of *Aspergillus flavus*.

Experiments described in this paper were conducted to determine first if conidia of *A. flavus* would undergo heat injury, and secondly what response heated conidia have to common preservatives and antioxidants used by the food industry.

## 1. Materials and methods

### *1.1. Organism*

*Aspergillus flavus* NRRL 3517 was grown on potato dextrose agar (PDA pH 5.5, Difco, Detroit, MI, USA) plates at 29 °C for 7 to 10 days. Conidial suspensions were prepared by flooding the surface of the culture with sterile 0.05% Tween-80 and gently rubbing with a glass rod. The suspension was filtered through sterile glass wool to remove hyphal fragments and diluted in 0.1 *mM* potassium phosphate buffer (pH 7.0) to yield ca. $10^8$ colony-forming units (cfu) per ml. Conidia were subjected to test conditions immediately after harvesting.

*1.2. Exposure of conidia to heat*

Ten milliliters of conidial suspension were transferred to 250-ml Erlenmeyer flasks containing 90 ml of 0.1 *mM* phosphate buffer (pH 7.0) adjusted to 50, 53, 55, or 58 °C by submerging in a New Brunswick Aquatherm waterbath shaker (New Brunswick Scientific Co., New Brunswick, NJ, U.S.A.). After agitating at 150 rpm for periods up to 140 min, aliquots were withdrawn, diluted in phosphate buffer, and plated in the appropriate recovery medium using the pour-plate technique.

*1.3. Recovery media*

Potato dextrose agar served as a basal recovery medium for all experiments. In addition to PDA, in certain experiments plate count agar (PCA, Difco) was also used as a recovery medium. Adjustments to pH 3.5 and 8.0 were achieved by adding sterile 10% tartaric acid and 12*M* NaOH, respectively, to sterile media. Sodium chloride (10 and 20%, w/v) and sucrose (20 and 40%) were added to PDA before sterilizing, followed by adjustment to pH 3.5. Potassium sorbate, sodium benzoate, calcium and sodium propionate, and sodium bisulfite were dissolved in sterile water, whereas butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate, and nordihydroguaiaretic acid (NDGA) were dissolved in 95% ethanol before adding to liquefied PDA (pH 3.5 and 5, 43 to 46 °C) to give graded concentrations ranging to 1000 ppm just prior to pouring. Control PDA plating media contained no added preservatives or antioxidants. Effect of ethanol on colony formation by *A. flavus* was determined. Conidia that had not been exposed to elevated temperatures served as controls.

*1.4. Incubation and enumeration procedures*

Poured plates were inverted and incubated at 29 °C, generally for 4 to 5 days, before colonies were counted. In those tests where added chemicals retarded the rate of colony formation and growth, incubation time was extended to a maximum of 16 days. Observations on the rate of colony formation and growth on PDA containing NaCl and sucrose were made at daily intervals up to 11 days.

Data presented represent means of three or more independent trials performed in duplicate.

## 2. Results and discussion

Since conidia of some strains of aspergilli vary in resistance to heat inactivation (Doyle & Marth, 1975), rates of inactivation of *A. flavus* NRRL 3517 at 50, 53, 55, and 58 °C were determined (Fig. 1). The *D* values were

calculated from the straight-lined portions of each curve and from these data the $z$ value was determined to be 11 °F. Strain 3517 exhibited somewhat greater tolerance to heat than did strains of *A. parasiticus* and *A. flavus* studied by DOYLE and MARTH (1975). In the course of investigation we also observed that 20- to 25-day-old conidia were more resistant to heat than were younger conidia, and because of this age effect, 7- to 10-day-old conidia were chosen for examination in subsequent tests. Our observations on increased heat resistance of older conidia are in conflict with DOYLE and MARTH (1975). They noted that $D$ values for conidia of two strains of *A. parasiticus* and one

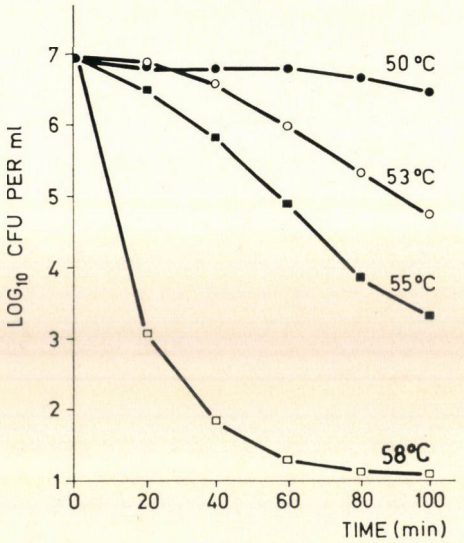


Fig. 1. Heat inactivation of conidia of *A. flavus*. Heated conidia were plated on PDA (pH 3.5)

strain of *A. flavus* decreased in 20-day-old cultures as compared to 10-day-old cultures. However, they also found that the rate of production of conidia and the growth substrate on which strains were cultured also greatly influenced the tolerance to heat, thus possibly explaining the differences from results reported here.

There has been some indication that acidified (pH 3.5) media often used to enumerate yeasts and molds in foods may be inferior to media adjusted to neutral or alkaline pH. KOBURGER (1971) showed that regardless of the acid employed to reduce the pH of PDA to 3.5, none of the acidified media recovered as many yeasts and molds from a variety of 32 food samples as did PDA at pH 7.0 containing 100 ppm each of chloramphenicol and chlortetracycline. FLANNIGAN (1974) reported that viable counts of yeasts and molds in barley were lower when PDA and malt extract agar were acidified to pH 3.5 as compared to pH 5.6. On the other hand, STEVENSON and RICHARDS

(1976) stated that differences in counts from heat-injured *S. cerevisiae* plated on acidified and non-acidified PCA and PDA were not affected by pH, but rather by nutritional differences. With knowledge of these reports, unheated and heated (53 °C) conidia of *A. flavus* were plated on PDA at pH 3.5, 5.5, and 8.0 to determine their response to the reaction of the recovery media. Repeated trials failed to show any significant differences in numbers of colonies formed on PDA at pH 3.5, 5.5 and 8.0 by conidia after comparable times of exposure to heat. It may be that certain types of propagules, *e.g.* hyphal fragments or spores, of some fungi are more sensitive to media pH than are others. The response of a wide variety of types of propagules and genera naturally occurring in foods is not known; however, data presented here indicate that perhaps acidified PDA (pH 3.5) may be suitable for enumerating conidia of *A. flavus* in such foods. Several additional strains of *A. flavus* should be tested to confirm this observation.

Having selected 53 °C as the temperature at which conidia of *A. flavus* would be exposed in subsequent experiments before plating on PDA (pH. 3.5), tests were conducted to determine whether injury could be detected. The classical two-media recovery system was used for this purpose. Both sucrose and sodium chloride were examined as stress chemicals when added to acidified PDA recovery medium. Although injury was not detected using the sucrose system, a portion of viable heated conidia had a decreased tolerance to sodium chloride (Fig. 2), indicating that heat injury had occurred. Injured conidia were evident after 40 min and increased with heating time. ADAMS and ORDAL (1976) reported similar observations on a somewhat more heat-tolerant strain of *A. parasiticus*.

In addition to a decreased number of colonies being formed by heated conidia of *A. flavus* on PDA containing 10% sodium chloride, the rate of development of colonies was also substantially retarded (Fig. 3). For example, after 4 days of incubation, $8.4 \times 10^1$ cfu per ml of heated conidial suspension had formed colonies >4 mm in diameter on acidified PDA containing 10% sodium chloride as compared to $1.0 \times 10^6$ cfu per ml plated on PDA without added sodium chloride. The latter count represents nearly all of the viable cfu per ml present (compared to 12 days of incubation), whereas the count on the sodium chloride medium after 4 days was <1% of that observed after 12 days of incubation. The rate of colony development by unheated conidia plated on acidified PDA containing sodium chloride was also impaired as compared to its control (PDA without sodium chloride), although not as severely as that of the heated conidia. ADAMS and ORDAL (1976) found that the growth pattern of thermally injured conidia of *A. parasiticus* was different from that of unheated conidia. A greater time span required for the appearance of all of the colonies in the heated suspension was attributed to a variation in the degree of injury among individual conidia. This is in agreement with

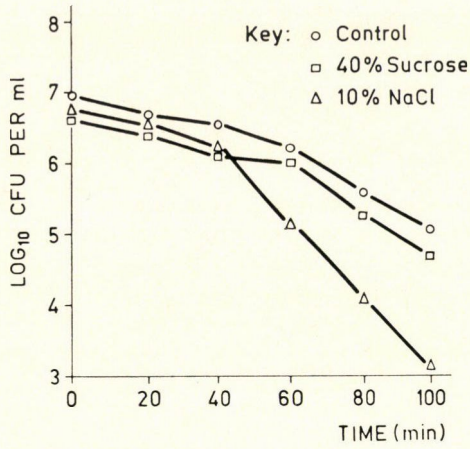


Fig. 2. Colony formation by conidia of *A. flavus* heated at 53 °C and plated on PDA (pH 3.5) containing 40% sucrose (w/v) and 10% sodium chloride

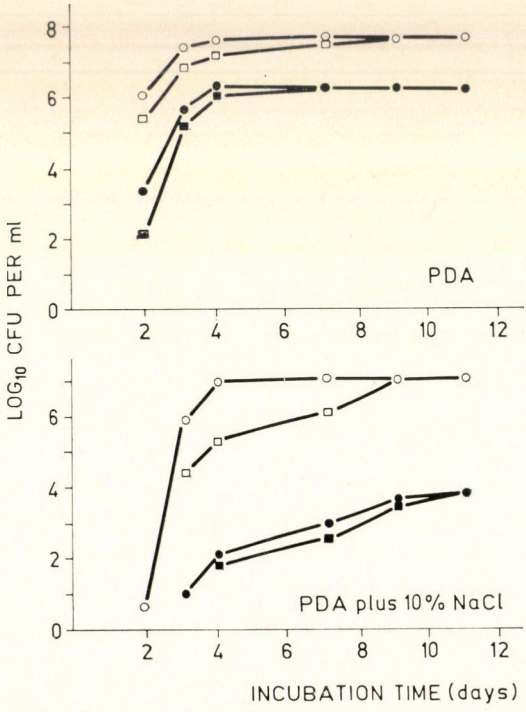


Fig. 3. Rate of colony development by unheated (open symbols) and heated (53 °C for 100 min, closed symbols) conidia of *A. flavus* plated on PDA (pH 3.5) and PDA (pH 3.5) containing 10% sodium chloride. Circles represent total colonies and squares represent colonies > 4 mm in diameter

observations reported here on *A. flavus*. Once colonies are visible on PDA containing sodium chloride, the rate of development is similar, regardless of whether conidia had been exposed to heat. This indicates that a permanent genetic alteration, at least one that would be expressed phenotypically, does not occur in the heat-injured conidia of *A. flavus*.

In a discussion of treatments related to food processing that may induce cell injury, BUSTA (1976) pointed out that there is no reason to believe that exposure of some microorganisms to various preservatives could not cause cellular damage. There is also no reason to believe that injured cells might not be more susceptible to the inhibitory action of common food preservatives than would uninjured cells. To test this hypothesis, several antimycotic compounds were added to PDA (pH 3.5 and 5.5) recovery media on which unheated and heated conidia of *A. flavus* were plated. Potassium sorbate (Fig. 4) and sodium benzoate (Fig. 5) inhibited colony formation by unheated conidia slightly, the effect being somewhat greater at pH 3.5. A marked decrease in tolerance to these preservatives was noted for heated

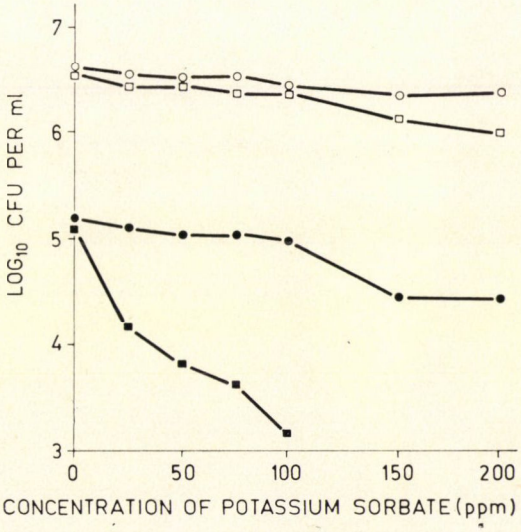


Fig. 4. Effects of potassium sorbate on colony formation by unheated (open symbols) and heated (53 °C for 100 min, closed symbols) conidia of *A. flavus* plated on PDA (pH 5.5, circles; pH 3.5, squares)

conidia plated on PDA at pH 3.5; injury of conidia was detected in PDA with as low as 25 ppm of potassium sorbate and 150 ppm of sodium benzoate. Similar trends were noted in PDA supplemented with calcium propionate (Fig. 6), although both unheated and heated conidia were tolerant to higher concentrations as compared to potassium sorbate and sodium benzoate. Injury was detected when heated conidia were plated on PDA (pH 3.5)

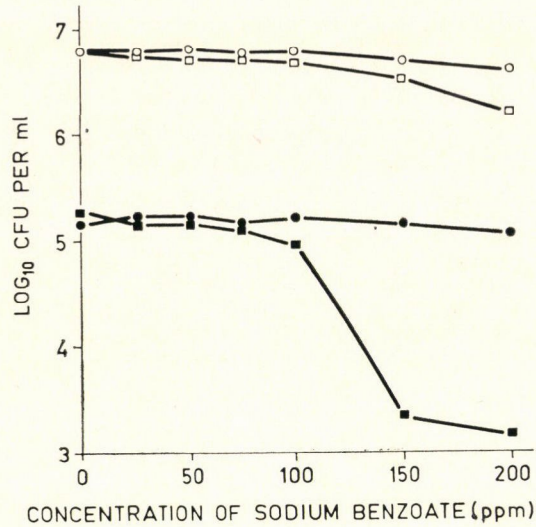


Fig. 5. Effects of sodium benzoate on colony formation by unheated (open symbols) and heated (53 °C for 100 min, closed symbols) conidia of *A. flavus* plated on PDA (pH 5.5, circles; pH 3.5, squares)

containing 2000 ppm of calcium propionate. The number of heated conidia forming colonies was reduced from $10^5$ per ml (PDA containing no calcium propionate) to zero by plating on PDA (pH 3.5) containing 3000 ppm. The number of cfu per ml was reduced by only 1.6 logs when unheated conidia were plated on the same media. Results from experiments using sodium propionate were similar to those shown in Fig. 6; however, sodium propionate,

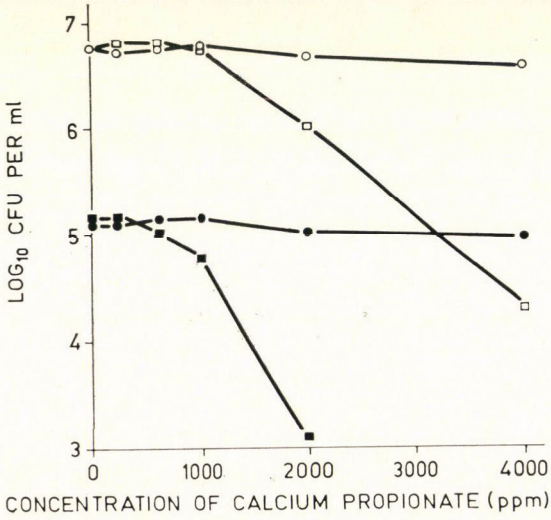


Fig. 6. Effects of calcium propionate on colony formation by unheated (open symbols) and heated (53 °C for 100 min, closed symbols) conidia of *A. flavus* plated on PDA (pH 5.5, circles; pH 3.5, squares)

at the same concentrations, did not appear to inhibit colony formation to the extent observed for calcium propionate.

TSUCHIDO *et al.* (1972) studied the effects of sorbic acid on heat-treated cells of *C. utilis*. The acid did not affect leakage of cellular soluble material or recovery of salt tolerance of heat-treated cells. However, sorbic acid did inhibit protein synthesis and respiratory activity in heated cells during the recovery period. Considering data on conidia of *A. flavus*, it is tempting to suggest that repair mechanisms after heat treatment may be similar to those observed for *C. utilis*.

The effects of sulfur dioxide on conidia of *A. flavus* are shown in Fig. 7. The inhibitory effects were greater at pH 3.5 than at pH 5.5 for both unheated and heated conidia. Heated conidia had a decreased tolerance to sulfur dioxide at concentrations as low as 50 ppm (pH 3.5), providing further evidence of injury.

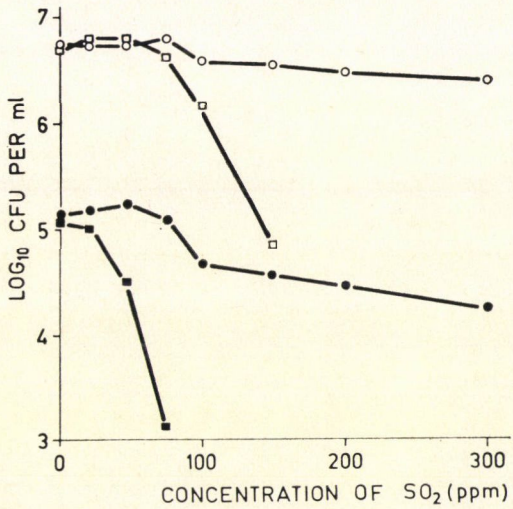


Fig. 7. Effects of $SO_2$ on colony formation by unheated (open symbols) and heated (53 °C for 100 min, closed symbols) conidia of *A. flavus* plated on PDA (pH 5.5, circles; pH 3.5, squares)

The antimycotic activity of salts of sorbic, benzoic, and propionic acids has been known for some time to be enhanced at pH values favoring undissociation. This was shown for unheated conidia plated on PDA at pH 3.5 and 5.5 containing calcium propionate and sulfur dioxide in the present study, and would undoubtedly hold true for potassium sorbate and sodium benzoate at higher test concentrations. The point to be made from data reported here is that the detrimental effects of the antimycotics tested are magnified when conidia to which they are exposed are heat-injured. It is important to note also that these enhanced detrimental effects occur at

concentrations of preservatives considerably lower than those sometimes used by the food industry. Thus, although the increased sensitivity of conidia of *A. flavus* and perhaps other molds to certain preservatives is beneficial in prolonging shelf life, enumeration of stressed conidia in the presence of such preservatives may be hindered.

Tests to determine antimycotic and stress effects of food antioxidants revealed that BHT (up to 1000 ppm), propyl gallate (1000 ppm), and NDGA (1000 ppm) were not detrimental to colony formation by unheated or heated conidia of *A. flavus* plated on PDA (pH 3.5 or 5.5). Butylated hydroxyanisole was inhibitory at concentrations greater than 200 ppm, regardless of pretreatment of conidia or pH of PDA (Fig. 8). Activity was enhanced at pH 3.5 as compared to pH 5.5, but the extent of inhibition was similar for unheated and heated conidia, indicating that the heat-damaged site or repair mechanism was not adversely affected by BHA. The increased antimycotic activity of

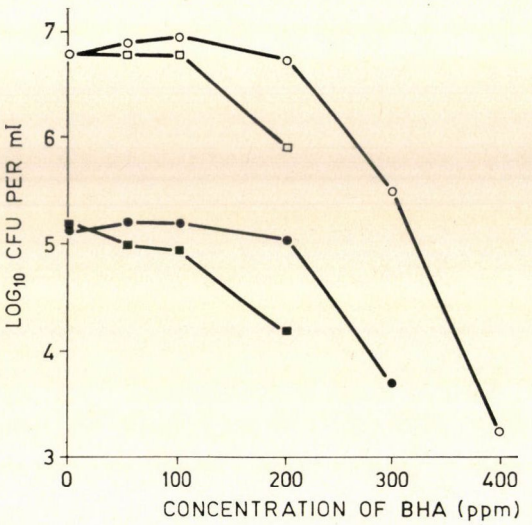


Fig. 8. Effects of butylated hydroxyanisole on colony formation by unheated (open symbols) and heated (53 °C for 100 min, closed symbols) conidia of *A. flavus* plated on PDA (pH 5.5, circles; pH 3.5, squares)

BHA at pH 3.5 may have been due to chelation of iron or other minerals involved in normal metabolic processes. The concentration of BHA observed to inhibit growth of *A. flavus* was similar to that reported by CHANG and BRANEN (1975) for *A. parasiticus*. They reported that 50 ppm of BHA slightly decreased mycelial growth and aflatoxin production, and 250 ppm or greater prevented all growth and aflatoxin production.

Reports on heated cells of yeasts indicate that the availability of glucose in recovery media may play an important role in the repair process. HAGLER

and LEWIS (1974) demonstrated that glucose was detrimental to heat-damaged yeasts. MEYER (1975), on the other hand, reported that glucose had no effect on a *Candida* sp. after exposure to elevated temperatures. We conducted experiments in which unheated and heated (up to 140 min at 53 °C) conidia of *A. flavus* were plated on PDA and PCA (contains 0.1% glucose as compared to 2.0% in PDA), both at pH 3.5 and 5.5. Results from eight independent trials showed that the number of cfu per ml of test suspension was similar, regardless of the type or pH of recovery medium. This is in contrast to data reported by STEVENSON and RICHARDS (1976) indicating that PCA was superior to PDA for recovering heat-injured *S. cerevisiae*. Structure and physiology of yeast cells and conidia are considerably different, however, and repair mechanisms may not be entirely comparable.

The addition of dyes and antibiotics (JARVIS, 1973; MOSSEL *et al.*, 1975; JARVIS, 1978) to media used for enumerating yeasts and molds has been promoted as a way to control fungal colony development and bacterial growth. We examined the effects of rose bengal and several antibiotics on colony formation by unheated and heated (100 min at 53 °C) conidia of *A. flavus* on PDA (pH 3.5) recovery medium. Chloramphenicol and chlortetracycline (up to 200 ppm, separately and in combination), streptomycin (100 ppm), oxytetracycline (100 ppm), and kanamycin (100 ppm) had no apparent effect on the number of colonies formed. Rose bengal (100 ppm), oxgall (1.0%), and Botran$^{R}$ (2,6-dichloro-4-nitroaniline, 200 ppm) likewise did not inhibit colony formation by viable unheated or heated conidia of *A. flavus*.

The increased sensitivity of conidia of *A. flavus* to *sodium chloride* and several common food preservatives may have practical implications to the food processor. The extent of inactivation of heat-damaged conidia may depend upon the presence of these compounds during heat processing and/or the time elapsed between heat treatment and exposure to the compounds. The synergistic effect of preservatives and heat on inactivation of conidia of *A. flavus* has yet to be demonstrated, but experiments are in progress in our laboratory to explore this possibility.

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# Abstracts

of papers presented at the

## CONFERENCE ON FOOD MICROBIOLOGY

organized by the

MICROBIOLOGY SECTION OF THE HUNGARIAN SCIENTIFIC SOCIETY FOR FOOD INDUSTRY

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## FOOD MICROBIOLOGICAL ACTIVITIES OF INTERNATIONAL ORGANIZATIONS

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Issuing regulations on food microbiology is the task of individual countries. Methods of analysis (routine methods) are set up by each country. However, in the case of foods marketed through international channels of trade the recommendations of international organizations, international standards (reference methods) have to be taken into account. For this reason Hungarian standards contain, in addition to the local methods of microbiology, also those accepted internationally. A substantial difference may exist between the two kinds of methods. Routine methods are not suitable for the analysis of export goods. The difference between the two methods may be illustrated by the ISO method for Salmonella detection.

Sampling, the preparation of the sample, types of analytical methods, the detection of microorganisms causing food poisoning are topics of primary concern to international organizations. The World Health Organization held a consultation on the the processing of data of food-borne diseases with a view to prevention and protection.

Microbiological control activities are advanced in Hungary, thus their results may be useful in the work of the international organizations.

# SIGNIFICANCE OF THE WORK OF THE MICROBIOLOGY SECTION OF MÉTE (HUNGARIAN SCIENTIFIC SOCIETY FOR FOOD INDUSTRY) IN THE DEVELOPMENT OF THE FOOD INDDUSTRY

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This scientific session was organized to celebrate the 15th anniversary of the Microbiology Section of this Society.

The Microbiology Section is a central functional organ of MÉTE with the task of organizing the co-operation of microbiologists working in different branches of the industry, in different fields of plant control, research and educational institutes, in order to promote the solution of their common and specialized problems.

The main task of the Microbiology Section is to contribute to the development of food science and to co-operate in the establishment of standards for the sanitary conditions of food production and commercialization, and in their control. These activities promote the prevention of financial losses induced by microbiological spoilage, they further the manufacture of products of high quality in the interest of the consumer and the steadily increasing food export.

The Microbiology Section contributes to the development of food microbiology in the following ways:

– It organizes, in co-operation with other sections, lectures and meetings to discuss common problems related to technology, microbiology and quality.

– To promote the practical solution of various problems of different industrial branches, the Section sets up working groups, *e.g.* the working groups on methodology, and on mycotoxins, which have been functioning for several years.

– The Section edits a Food Microbiology Series in order to improve work efficiency of industrial microbiologists and technologists.

– It organizes study tours within Hungary and abroad, invites distinguished foreign experts for mutual exchange of experiences and in order to intensify co-operation.

– To awake the interest of university and college students, the Society invites participation in scientific competitions organized every 3 years.

Thus, the Microbiology Section fulfils an important task in the development of food science and in its practical applications.

# MICROBIOLOGICAL RESEARCH IN HUNGARY, RESULTS ACHIEVED AND FUTURE TASKS

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Of the eight decades of Hungarian food industrial microbiology certainly the last 25–30 years were the most successful from the aspect of both quality and quantity. After a short survey of the research results achieved in the field of the distilling, brewing, wine, tobacco, milk, baking, canning, beverage, meat, refrigeration, sugar and cereal industries the author points out as the main tasks of future research, work on the production, by propagation of microorganisms, of protein and amino acids from hydrocarbons, fats or cellulose (by enzymic or microbial hydrolysis), the production of alcohol by fermentation, the production of enzyme preparations for use in the food industries and the optimization of fermentation. As regards protection against microorganisms the further development of physical methods seems desirable. In the field of plant hygiene, microbiology of the raw materials and additives, research into the system of on-line control, diagnostics of the most important microorganisms, investigation of the occurrence of mycotoxins, development and unification of analytical methods and the development of microbiological standards seem the most important research tasks for the near future. As regards marketing of foods, microbiological and sanitation problems of the catering industry deserve treatment of high priority. In planning and evaluating microbiological research aspects of economic and technological feasibility deserve attention.

# MICROBIOLOGICAL CONTROL IN THE HUNGARIAN FOOD INDUSTRY AND ITS RELATION TO SANITATION

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In Hungary microbiological control is carried out at two levels. Food inspection is concerned mainly with the acceptability and wholesomeness of food. In this respect pathogenic and facultative pathogenic microorganisms possibly present in foods play a decisive role.

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The control activity of food sanitation inspectorates forms a basis for judging the sanitary conditions of technological processes and to take appropriate steps.

The enforcement of sanitary requirements in technological processes is the task of the manufacturing plant, thus, the plants themselves have to carry out extensive microbiological tests. The aim of these tests is to serve with data to the sanitary evaluation of technological procedures and to the microbiological evaluation of the product. In Hungary special attention is paid to the microbiology of beverages, dried vegetables, seasonings, to the methodology of the analysis of certain canned goods and to microbiological product evaluation.

## MICROBIOLOGY IN THE HUNGARIAN FOOD CONTROL INSTITUTES

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The task of checking the microbiological condition of foods of vegetable and fruit origin was assigned to the food control institutes.

Work is carried out on three different lines:

1. Unification and modernizing of *analytical methods* on the basis of comparative tests in co-operation with the laboratories of the various branches of the industry.

2. Assessment of the level of microbiological contamination in the products and carrying out storage tests, in order to establish realistic *limit values*.

3. Elaboration of the view-points of microbiological control by on-line tests, in order to develop a complete quality control network (from the raw material to the consumer).

The two latter tasks are closely related to the specific profile of the institutes. The professional co-ordination of the microbiological tasks of the institutes is the duty of the Ministry through the Department of Animal Health and Food Hygiene of the Ministry of Agriculture and Food.

A further task of the Centre is to promote international and Hungarian standardization. The Centre is responsible for initiating basic and higher training in food microbiology and exerting patronage over it.

Microbiological work in food control is closely related to quality control within the various branches of industry, inasmuch as one of its basic tasks is to promote internal industrial control. The microbiological activity of the Centre finds professional support in the scientific work of various research institutes (Central Food Research Institute, Institute of Nutrition) and

universities. The assistance of MÉTE (Hungarian Scientific Society for Food Industry) in organizing collaborative tests, scientific conferences and in publishing technical literature deserves special attention.

## ACCEPTANCE SAMPLING SYSTEMS APPLIED IN THE MICROBIOLOGICAL CONTROL OF FOODSTUFFS

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Frequently the microbiological quality of food is assessed by measurable characteristics (indicator cell count, enzyme tests). However, generally applied systems treat also these characteristics as attributes. This means not only loss of information but the conversion into attribute is at the same time arbitrary.

Conversion into attribute would be justifiable if the effect or frequency of the measured characteristic would change abruptly at its boundary. Data supporting this idea were not found in the related literature, neither investigations of the author could prove the occurrence of such abrupt changes.

The efficiency of methods of qualification may be checked by operating characteristic curves which determine the probability of acceptance as a function of the value of the characteristic as measured. In the experience of the author microbiological measuring values, after appropriate transformation, are of normal distribution, thus, the operating characteristic may be determined as such, too.

Materials of low microbial counts (*e.g.* canned goods) deserve special attention.

## RECENT RESULTS ABOUT THE CONTROL OF MICROBIAL SPOILAGE OF FOOD BY IONISING RADIATION

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Papers published from 1973 to 1977 in the field of radiation control of microbiological spoilage are reviewed, grouping the subjects according to the type of the process and the food treated. Various laboratories from 33 countries have recently published data on the subject matter. Radurization of dates, prepackaged vegetables, grains of high moisture content, bread,

various meats and meat products was reported. The most wide-spread research activities could be observed in the field of radurization of fishes and marine products (shellfish, shrimps). Radiation decontamination of dry food ingredients (enzyme preparations, protein preparations, starch, spices) and that of cork stoppers were studied in various laboratories. Radappertization research on several animal-protein foods made a remarkable progress and the minimal dose requirements are well established. Combination of radiation treatment with other antimicrobial agents (salt, preservatives, heat, etc.) was investigated by many laboratories. Foods involved in these investigations were bread, several tropical and subtropical fruits, apple juice, groundnuts, fish fillets and shrimps, but a considerable part of the data relate to model systems. A better understanding of the synergistic effect will require additional knowledge and the continuation of long-range research and development in the field of combined treatments are recommended.

## MICROBIOLOGY AND SANITATION IN THE HUNGARIAN REFRIGERATION INDUSTRY

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Since only a smaller part of the products of the refrigeration industry are cooked or blanched and the major part is not exposed to heat treatment, attending to manufacturing and personal hygiene has been of great importance.

A network of sanitation has been developed simultaneously with the setting up of laboratories in the cold stores. Sanitary specifications, rules of cleaning were evolved.

Systematic microbiological testing of quick-frozen products was started about 10 years ago. Initially, only the indicator microflora was assessed. Later, by permission of the Ministry of Agriculture and Food the tests were extended over pathogenic bacteria, too.

A sanitary schedule up to 1980 was developed and its execution has been continually checked. The strict sanitary rules of the German Federal Republic have been introduced into a number of cold stores.

# MICROBIOLOGICAL METHODS APPLIED IN THE REFRIGERATION INDUSTRY

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In 1968 a laboratory for microbiological control was set up within the Central Laboratory for Quality Control of the industry. From then on microbiological control was gradually introduced into the plants.

In view of the increasing demand at home and in order to be able to satisfy export requirements the tests for pathogenic bacteria became necessary in the industry. Ministerial Rule 23.341/73 established the necessity of testing for the following microorganisms:

*E. coli* and coliforms, total viable cell count, *Clostridia*, *Staphylococcus aureus* coag. positive, *Salmonellae*, *Enterococci*, moulds and fungi, psychrotolerant microorganisms, and protein-degrading microorganisms.

In 1974 the Central Laboratory for Quality Control was granted permission by the Ministry of Agriculture and Food to test for pathogens and thus this has been the subject of study since 1975.

# ASSESSMENT OF THE MICROBIOLOGICAL QUALITY OF QUICK-FROZEN FRUIT AND VEGETABLES IN THE COLD STORE OF THE CO-OPERATIVE FARM, NAGYRÉDE

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Microbiological data of the three most important products from the aspect of both home market and export (green peas, raspberries and cubes of tomato-shaped paprika), were evaluated .These three quick-frozen products were tested for cell counts of mesophilic and protein-degrading bacteria, staphylococci, coliform bacteria and moulds. The results obtained during 3 years (about 1000 data) were processed by statistical methods.

The aim was to assess the microbiological quality of these products. The frequency distribution of cell counts within batches was of the normal type in relation to all three products. Thus, data were suitable for further mathematical processing. The Bartlett test has shown deviations within batch to be very low, 0.3 on the average as expressed in log units. Thus, batches may be considered homogeneous from the point of view of cell count.

The yearly average values were compared by simple analysis of variance. The $F$ test has shown that the yearly levels of cell count did not differ significantly.

On the basis of deviations within a batch and of specified normatives, the internal standards within the plant were calculated at confidence levels of 84 and 95%, resp. The average values, apart from two cases, were found to be well below the norms within the plant. Thus, the cold storage has proven to ensure adequate microbiological quality.

The level of contamination found in this study was satisfactory. The low deviations in cell counts within batches and between batches have shown the production technology to be steady and sanitary specifications strictly adhered to.

## MICROBIOLOGICAL QUALITY OF REFRIGERATED PRODUCTS DURING STORAGE

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The aim of this study was to follow up the contamination level of products of the refrigeration industry during storage under different conditions:

in the cold store at −20 °C
in the display cabinet at −5 °C
in the refrigerator at +5 °C
at room temperature of +20 °C.

To characterize the microbiological quality the mesophilic aerobic cell count and the coliform count of 10 kinds of convenience foods and ready-to-cook dishes and of one blanched vegetable (spinach) were determined.

A smell characteristic of spoilage was observed in convenience foods and ready-to-cook dishes at a microbial count of $10^8$ $g^{-1}$.

In storage at +20 C° and +5 °C the cell count of the products investigated did not exceed the critical limit value as specified in sanitary regulations, within the period guaranteed, which is 6 h at +20 °C and 1 day at +5 °C.

There was no change in the microbiological state of the products stored at −20 °C for 12 months.

The number of coliforms decreased at −5 and −20 °C while increased at +5 and +20 °C in spinach.

Storage life of the products was found to be dependent not only on the initial cell count, but also on the composition of the sample. It may be stated that products of the refrigeration industry, the initial cell count of which did not exceed the permitted level, were of appropriate quality during the period guaranteed.

## MICROBIOLOGICAL STUDY OF POTATOES PEELED AND STORED UNDER DIFFERENT CONDITIONS

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In classifying foods their microbiological state is of decisive importance. The microbiological state is determined by two factors: the character of the microorganisms present and the cell count.

The aim of this study was to find the storage temperature and the packaging method at which potatoes peeled and treated with antioxidant ($SO_2$) could be most advantageously stored.

Fresh and stored potatoes were peeled then dipped into a solution of 0.2% $SO_2$ for 3 min. These were stored in parallel with peeled but untreated potatoes as the control. Samples were packed in polyethylene bags (0.04 mm) or in flexible PVC film of 0.015 mm thickness and stored at 2.1–3.1, 17.7–21.5 or 23–26 °C, resp. The dilution assay technique was used to determine mesophilic aerobic bacterial count.

The most probable viable cell count was evaluated by means of *Hoskins'* table.

Fresh peeled potatoes exhibited the highest storage stability when placed on tray and packed in PVC film, and stored at 2.1–3.1 °C.

This sample could be kept for 17 days without spoilage. At temperatures about 10 °C even treatment with $SO_2$ did not prevent spoilage. The cell count exceeded $10^8$ $g^{-1}$.

The use of the two kinds of packaging material did not affect the samples in different ways.

Untreated potatoes had a storage life of 13 days under refrigeration. When treated with $SO_2$ their storage life was extended over 17 days.

Stored potatoes when peeled and treated with $SO_2$ had a storage life of 15 days.

## MICROBIOLOGICAL ASPECTS OF THE MANUFACTURE OF DISTILLERS', BAKERS' AND FEED YEASTS

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Problems of microbiological character in all plants belonging to the Trust of Distilling Enterprises are dealt with by this Research Institute. Products of these plants are as follows: distiller's, baker's and feed yeast,

different brandies, alcoholic and alcohol-free beverages, starch and derivatives and vinegar. The paper presented informs on the activities in relation to these products, particularly to the first three of them. The activities may be grouped as follows:

– A stock collection is maintained at the Institute consisting mainly of yeasts (strains of *Saccharomyces cerevisiae*, 26 strains of *Torulopsis* and 37 strains belonging to the *Candida* genus), further of moulds (129 strains altogether) and of a few bacterial strains.

– The characteristics of the yeast strains are studied under conditions similar to those present in plants. Methods are developed for their classification.

– The producton lines are tested at various phases of production, including equipment and adjuncts, beginning with the raw material through the semi-products, right through to the product in order to find the main points of infection.

– Suggestions are made as to the elimination of centres of infection.

Finally concrete examples are given of the microbiological condition and quality of the products as affected by measures taken in view of modernizing industrial equipment and up-dating technologies.

## THE EFFECT OF DETERGENTS COMBINED WITH DISINFECTANTS UPON THE MICROBIAL CONTAMINATION IN YEAST FACTORIES

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Since disinfectants generally used contain chlorine and are therefore corrosive, it is desirable to seek for disinfectants of different type.

In order to protect the equipment of the newly built yeast factory the introduction of a disinfectant of a new type was necessary. In selecting a new type of disinfectant or detergent the main aspects to be taken into account are the existing conditions in the plant.

In the above yeast factory a recirculating mechanical cleaning system is operated, and a separate disinfection system is not provided for. Thus it is expedient to apply a detergent combined with disinfectant in a single operation. It is suggested to use a liquid mixture of detergents and disinfectants because it suits better factory conditions than agents in the solid state.

Taking into account the aspect of economy, it is advised to use an up-to-date Hungarian product. On the basis of the above considerations the following products were given a trial; Ultra Vill, Ultra bottle cleaner, Unidest.

The effect of the combined detergents was tested in the laboratory with *Saccharomyces cerevisiae* and mesophilic aerobic bacteria isolated from the mash. Solutions of 1 and 2% in experimental treatment periods of 5, 10, 15 and 20 min at 40, 50, 60 and 70 °C, resp., were applied. Subsequent to the laboratory tests an industrial scale experiment was carried out with 1% solutions of Ultra Vill and Ultra bottle detergent. Results have shown the Ultra bottle detergent to be more effective because: it has a definite germicidal effect; it cleans satisfactorily; the fermentor is bright subsequent to cleansing; the solution itself may be used several times; it saves labour, work time and energy. Due to intensive foaming Ultra Vill is not suitable for use in factory.

## CORRELATION BETWEEN MICROBIOLOGICAL QUALITY, STORAGE STABILITY AND CHEMICAL COMPOSITION OF BAKERS' YEAST

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The quality of baker's yeast, its leavening capacity depends largely on its microbiological purity. Commercial yeast may be infected more or less with "wild" yeasts. The contaminants may be detected and identified by traditional methods of microbiology. By our new method of lipid analysis the industrially propagated cell mass was shown to contain none or very low quantities of poly-unsaturated $C_{18}$ and $C_{20}$ fatty acids, while wild yeasts contained substantial amounts (20–30% of total fatty acids) as proven by gas chromatography. To increase the sensitivity of the gas chromatographic method for detecting the polyene fatty acid composition the cell mass was microbiologically enriched in wild yeasts. Commercial bakers' yeast was propagated in the laboratory under different conditions of aeration and the polyene fatty acid composition, as a specific index of the microbial contamination, was established.

The following observations were made:

– at a low rate of aeration pure bakers' yeasts proliferated, while the growth of the wild yeasts was inhibited by the low oxygen concentration and the alcohol content;

– at medium or high rates of aeration, wild yeasts proliferated more rapidly and the culture became enriched.

## NEW EXPERIMENTAL METHOD FOR THE DETERMINATION OF THERMAL DEATH PARAMETERS OF MICROORGANISMS

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The basic principle of the classical methods for the study of thermal death is that the coefficient of thermal death rate or the decimal reduction time is determined at several temperatures and the results are plotted to establish the thermal death curve from which the appropriate parameters may be calculated ($z$, $Q_{10}$). However, in the case of survival curves the relationship between the logarithm of viable cell count and time is not always linear. There are transient stages in the curve which render the determination of the coefficient of death rate uncertain. A further problem may be caused by the deviation resulting from differences in experimental media, when measurements are carried out at different temperatures.

The essence of the new method, eliminating the above described drawbacks, is that the death curve may be plotted on the basis of a single series of measurements carried out in a single system. The temperature in the tank containing the thoroughly mixed microbial suspension is continually changed with time and measured. Taking samples at predetermined intervals the viable cell count belonging to a given point of time is determined and its logarithm is plotted *vs* time. The value of the coefficient of death rate changes at different temperatures, thus, the slope of the survival curve changes from point to point in accordance with the temperature at the given moment.

The decimal reduction time of the moment may be calculated from the differential quotient with time of the survival curve. If also the momentary temperature is known, the logarithm of the decimal reduction time may be plotted against temperature and thus, the death curve may be established. According to measurements hitherto carried out the values obtained for $k$, $D$, $z$, and $Q_{10}$ were similar to those obtained by traditional methods. However, measurements require less time, they are simpler and the deviation of results is also smaller.

## INCREASING THE NUTRITION-BIOLOGICAL VALUE OF YEAST PROTEIN BY MUTATION TECHNIQUE

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Yeast protein is deficient in sulfur-containing amino acids, the combined quantities of methionine and cysteine are below the level laid down as desirable by FAO/WHO. In consequence the utilization of other essential

amino acids is also reduced, since utilization follows the limiting amino acid. Complementation of proteins in animal feeds by certain amino acids (methionine, lysine) may be considered general practice. However, in this case it should be taken into account that amino acids not bound are utilized only to 30–40% because of the differing rate of absorption.

The aim of these experiments was to produce yeast mutants enriched in methionine. The following yeast strains were examined: *Candida*, *Rhodotorula*, *Saccharomyces*. Two kinds of mutagenic agent were applied: nitrite and UV light. The death curves of the original strain in the presence of both mutagenic agents were established. In the knowledge of these, conditions of treatment were selected to cause death of 2–3 orders of magnitude. Treatment with nitrite was carried out according to the method of Schlegel and Bowien. After selecting the mutant, it was propagated on an enriched medium for its complete development. Since yeast cells rich in methionine do not exhibit auxotrophic deficiency, the customary operations of selecting could not be applied with success.

Taken as the starting point that cells containing more methionine require more sulfate it was tried to select mutants by transplanting colonies to media poor and rich in sulfate using the velvet technique. To increase the efficiency of mutant selection assays were made with methionine antagonist, methionine homologues and fungicidal antibiotics (Nystatin, Canesten). It was found that the mutant and the original strain possessed different sensitivities towards fungicidal antibiotics. A difference was observed in the vitamin U stimulating effect, too. However, with a methionine antagonist (nor-leucin) the expected result was not achieved. An addition of nor-leucin, even at a concentration of 0.5% did not cause inhibition in growth. Only at a nor-leucin concentration as high as 1% could we observe a prolongation of lag phase in the parent strain culture and mutant culture as well.

## THE MICROFLORA OF FOODS

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Many foodstuffs contain a certain amount of viable microorganisms. In most of the descriptions this microbial flora is mentioned mechanically under the term "viable cell count" or "total germ count" and the composition is left out of consideration.

The composition of the population of the foodstuffs is rather complex and the results obtained by analysis depend to a great extent on the applied method.

The population found in milk, dairy products and beverages is described. The population falls into three groups of microorganisms: 1. indigenous, 2. incidental and 3. foreign.

Detection of species was carried out on the basis of the methodology developed by Polónyi. In milk and dairy products *Streptococci* (*Str. lactis*) may be considered specific, while in beverages yeasts. *Micrococci*, apparently indifferent, could be found abundantly in all the products investigated. The milk-agar of Polónyi was unsuitable for the isolation and cultivation of *Lactobacilli*.

## MICROBIOLOGICAL INVESTIGATION OF FOOD PRODUCTS IN COUNTY HAJDÚ-BIHAR

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The continuous control of food production and marketing units in County Hajdú-Bihar and in Debrecen itself is ensured. Sanitary inspectors take samples on their inspection tours of plants and commercial units. In the more important food processing plants (*e.g.* Powdered milk factory, Berettyóújfalu; meat processing plant, milk processing plant) the representative of the Food-bacteriology Laboratory of the Station for Epidemiology and Hygiene carries out expert sampling. Bacteriological testing of food products is continuous and systematic. The bacteriology laboratory is responsible for the control of the following branches of the industry: dairy, meat, confectionary, canning, soda-water and noodle industries. The products of these branches are also tested in the commercial network, after transport and storage.

The results of the tests carried out in the last 5 years were tabulated. Data of bacteriological purity in the factory and in the commercial network were compared. The products mostly objected to were those of the dairy, meat and confectionary industries. Of the goods sampled in commercial establishments most objectionable were meat products, canned goods and noodles.

An account is given of the experiences gained in the course of investigating certain branches of the food industry and these are related to the production in individual years. The help rendered to individual branches on the basis of bacteriological tests is described.

# NEW DATA AND VIEW-POINTS TO THE JUDGEMENT OF THE MICROBIOLOGICAL CONDITION OF CERTAIN COLD DISHES

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The production of cold dishes for the catering trade increases from year to year in Hungary. Thus, these food items, valuable from the nutritional aspect, gain importance as staple foods. Since, however, most of their ingredients, first of all the native proteins and carbohydrates, are extremely prone to spoilage and form a very good medium for the growth of microorganisms, to keep them under strict microbiological control becomes more and more important.

Around the sixties, when the microbiological control of these products started, the sanitary condition of the products and the producing plants were judged on the basis of empirical counts. In the possession of a high number of data and by the use of mathematical statistical methods, first in 1974 it became possible to suggest the establishment of objective sanitary limits for these products on the basis of their indicator flora.

Since 1974 further 1 800 samples were analysed and more than 7 500 data obtained. The results related to three types of cold dishes (salads with vinegar, or with mayonnaise and jellied products).

On the basis of mean cell counts and, taking into account standard deviations, suggestions are presented as to the cell count limit values of acceptance *(m)* and rejection *(M)* and in the case of individual lots to the maximum cell count *(c)* in the range just acceptable *(m—M)*.

The results of microbiological tests, carried out in the framework of the sampling program, permit of the following conclusions:

1. the production hygiene of the given product is *unobjectionable* if none of the sample elements taken within the framework of the sampling program exceeds, in relation to any of the indices, value $m$;
2. it is *tolerable* if the number of elements between $m$ and $M$ does not exceed number $c$, but none of them reaches or exceeds value $M$ in relation to any of the indices;
3. production hygiene is *objectionable* if the result related to a single or more sample elements, even in relation to a single index, reaches or exceeds value $M$.

# COMPARATIVE STUDY OF MICROBIOLOGICAL TECHNIQUES FOR THE CONTROL OF CLEANING AND DISINFECTION

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Four methods used in the control of the efficiency of cleaning and disinfection in food plants were compared. Tests were carried out on 4 different surfaces with 2 strains. Surfaces were infected with the dilutions of 18–24 h broth cultures of *Saccharomyces cerevisiae* and *Escherichia coli*. Samples were taken from the infected rubber, tile, concrete and glass surfaces by Ten Cate agar-sausage, swabbing, Rodac plating and rinsing techniques. From the samples the number of recovered cells was established. In the knowledge of the recovered and the contaminating cell count a ratio of recovery, or percentage of recovery was calculated. The great number of results was evaluated by two-aspect analysis of variance subsequent to the Bartlett test. With the Ten Cate agar-sausage technique cell recovery from 3 surfaces fluctuated between 19 and 32%. The evaluation has shown the technique not to be sufficiently precise and reproducible. Combined with swabbing the technique proved to be sufficiently reliable.

By the Rodac plating technique, cell recovery was between 28 and 69%. Using yeasts for infection recovery reached 28%, for bacteria it was 63%. The same technique gave a recovery value of 69% on rubber surface. It seems that recovery on smooth elastic surface is higher by the Rodac method than by the Ten Cate technique. Reproducibility and accuracy of the two methods is similar. Because of the higher recovery ratio the Rodac method is considered suitable for the control of cleaning and disinfection.

The recovery by the swabbing technique on rubber, tile and concrete surfaces contaminated with yeasts amounted to 15–35%. From the surface of bottling jars the recovery was 58%. When bacteria were used for contamination the recovery from rubber and tile surfaces was nearly the same, from concrete it was 63%. Swabbing requires more equipment and work than Rodac plating or the Ten Cate technique. In practice it may be used for the study of highly contaminated surfaces or for controlling the efficiency of disinfection.

In the course of this study the rinsing technique proved to be the most sensitive and most reliable. The equipment and work requirement of this method is lower than that of the swabbing technique. The Rodac plating technique is suggested for use in Hungary.

# MICROBIOLOGICAL INVESTIGATION OF SMALL-SCALE NOODLE MANUFACTURE WITH SPECIAL REFERENCE TO THE STAPHYLOCOCCUS COUNT

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Small-scale noodle manufacturing plants may be divided into two groups, those utilizing machinery, and those relying completely on manual work.

Samples studied throughout three years were the products of four plants of different technical level. Mesophilic aerobic microorganisms, coliforms, *Escherichia coli*, mould and *Staphylococcus aureus* were enumerated during the three years in the plants.

On the basis of data obtained, taking into account the international specifications related to the microbiological quality of noodles (ICMSF) and the regulations on the Hungarian Ministry of Health and Ministry of Food and Agriculture (1977) the internal microbiological normatives for each plant were established.

Experiences have shown the necessity of adhering to the following considerations in small-scale noodle manufacture:

– Strict observance of regulations on hygiene of the Hungarian Ministry of Agriculture and Food 6/1972. Cleaning and disinfection has to be carried out in every work shift with an efficient detergent.

– Reduction of the resting time of the noodle mixture to half an hour –one hour. When the dough is ready start drying immediately. Within one shift several batches of dough shall be mixed.

– Machines not manufactured for use in the food industry should be removed immediately. All machines should be cleaned and disinfected every 4 hours.

A further aim of this study was to test five internationally accepted media for the detection of *Staphylococcus aureus* as to their sensitivity, evaluability, storage capacity and price. The five nutrient media were: tryptone glucose yeast broth containing 10% NaCl, phenolphthalein-diphosphate-polymixin medium, egg-yolk azide medium, Baird-Parker medium and TPEYA. The egg-yolk azide medium proved to be equally sensitive as that specified by ISO and in the Hungarian Standard (MSZ 3640-74) for decisive test, in detecting *Staphylococcus aureus* in noodles. It is at the same time of good keeping quality and relatively cheap. Its use may be suggested for informative routine tests.

# COMPARATIVE STUDY OF NUTRIENT MEDIA FOR THE DETECTION OF COLIFORM BACTERIA AND MOULDS IN BUTTER

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The study was carried out according to the directives of CMEA (Council for Mutual Economic Assistance) laid down in the minutes of the Moscow Conference, 1973.

Nutrient media for the detection of coliforms in butter were compared according to the following pattern:

- Brilliant green–bile–lactose broth, at 30 °C, for 48 h;
- brilliant green–bile–lactose broth, at 37 °C for 48 h;
- Klimmer medium at 37 °C and for 24 h.

First a slightly and a highly infected butter sample were investigated, in 10 parallels each.

On evaluating the results by the sign test it was found that there was no significant difference in the coliform count of cultures obtained by the three methods compared.

In the next experimental series butter samples taken from 20 different batches were examined by the three methods. Results were evaluated again by the sign test. No significant difference was found in the coliform count between the methods.

Thus, the three methods may be used equally well with samples of high or low coliform counts.

Two nutrient media were compared in the determination of moulds in butter. In the first experimental series the colony counts obtained on two different nutrient media, from butter samples originating from bacthes of different contamination, were compared. The colony counts obtained on potato-agar were in every case more scattered than those obtained on malt agar. However, the difference was significant only in a single case.

To gain a more thorough insight into the differences between the mould counts on the two media, samples of 20 different butter batches were investigated.

Results were evaluated by the $t$ test of paired data of average colony counts and by multifactorial analysis of variance on the square roots of colony counts. No significant difference was found between the two nutrient media by the $t$ test, because the differences in the mould colony counts of the different butter samples cancelled each other.

Analysis of variance has shown significant differences between samples, however, due to interaction between media and butter samples (and/or their mould population) the colony count was higher some times on the potato agar, at other times on the malt agar.

# MICROBIOLOGICAL TESTS IN THE DAIRY INDUSTRY

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The microbiological tests made on the technological line — as a result of their function — have an important role in the quality of dairy products.

The aim of these investigations is to control the technological opertions, to detect possible sources of contamination and defects and their elimination from the processing line. In a narrow sense this means the microbiological analysis of samples taken at predetermined points of the production line. In a wider sense studies comprise all the factors which may turn — directly or indirectly — into a source of contamination or may influence the quality of the end-product. The analysis of food additives, the control of measurable characteristics of technology and other relating factors, is also an important task.

To the first group belong tests requiring less specified training and producing rapid results (based on enzymic reactions) of indicative character, which can be carried out at the site of production. To the second group belong microbiological methods requiring higher professional training, more time and a well-equipped laboratory. These are the true (quantitative) microbiological tests. Methods belonging to the first group are primarily of preventive character. The role of the analytical methods pertaining to the second group is to explore the causes of hidden defects and give more exact information.

According to its extent and depth inspection is to be classified as follows:

- daily routine tests with the aim of prevention,
- tests in view of a preappointed important aim,
- general hygienic control repeated at fixed periods.

The various aspects of control activity are interconnected and it is necessary that those of a wider scope should comprise all the factors and methods of the routine tests. In organizing the control activity analyses of preventive character require primary attention, since in the case of a rapidly perishable product, such as pasteurized milk, by the time the results of quantitative microbiological tests are available, the product is consumed. However, in the case of products of a longer storage life, too, tests aimed at the prevention of faults are most important, due to economic and other causes.

Well-organized on-line control ensures safety in relation to hygiene, improves the quality of the products and extends their storage life. It assists to reveal the defects in the production line and to eliminate them and thereby it ensures an even and more efficient production. It affects advantageously the discipline of technology and improves the hygienic approach of employees.

## COMPARATIVE EVALUATION OF TEST METHODS FOR THE MICROBIOLOGICAL AND ENZYMIC QUALIFICATION OF FRESH MILK

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In several countries the microbiological quality of raw milk on receipt in the factory is taken into account. This problem has arisen in Hungary, too. To carry out the test appropriate methods are necessary.

Indices used to characterize the microbiological condition and keeping quality of milk (cell count, reducing capacity, protein stability) are varied and a uniform picture cannot be gained from the literature. The variably close paired correlation between individual properties shows that the results of different tests represent a mixture of the various components of quality.

In order to have a good survey of the problem several hundreds of milk samples of different quality were tested. The total viable cell count, 6 types of reduction tests and 5 types of protein coagulation (clot-on-boiling) tests were carried out. The relationship between the above characteristics and the initial acidity was established. The number of the quality components carrying independent information and the relation of these factors to the characteristics measured was investigated by factor analysis. The results obtained using an R-20 type computer of the Mosonmagyaróvár Knitwear Factory, are as follows:

– Initial acidity was distinct from the system formed by the other characteristics.

– The remaining 12 characteristics may be developed from the linear combination of 8 quality components. Out of these 8, four participate in the construction of several characteristics. Thus only these 4 quality components stand for connection between the characteristics. The other 4 carry only surplus information otherwise inexpressible. However, this surplus amounts only to 10–15% of the total change. The 4 connected components evaluated on the basis of factor weights, are as follows:

1. Tendency to coagulate at body temperature. This is best approximated by a boiling test subsequent to incubation at 37 °C.

2. Tendency to coagulate in the chilled state. This is best approximated by a boiling test subsequent to incubation at 4 °C.

3. Capacity for rapid reduction. This is well characterized by the rezazurine test after 30 min of incubation.

4. Slow reducing capacity. This is in fairly close relation to the complete discoloration of the methylene blue or the rezazurine reagent and to none of the other characteristics. The slow reducing capacity is less significant than the three other factors.

On the basis of these investigations the establishment of three characteristics seems necessary in order to characterize the technical hygienic condition of milk. Thus, the results of boiling tests after incubation at 37 and 4 °C and of the 30 min rezazurine test enable the determination of the other characteristics with not more than 10–15% uncertainty. A classification method on the basis of the 3-factor system is under development.

## ANALYSIS OF FACTORS INFLUENCING LYSOZYME ACTIVITY

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Egg-white lysozyme inhibits the development of the vegetative forms of Clostridia causing butyric acid blowing in hard cheese, by dissolution of certain components of the cell wall. Literature data on the efficiency of lysozyme differ.

Uncertainty in this field may be caused according to the investigations of the author, by the fact that lysozyme may be bound not only to bacterial cells but also to the proteins present in milk. The lysozyme of high iso-electric point (pH 10.5) has a positive charge in a cheese medium of about 6.5 pH, while cheese proteins have a negative charge. It was found that only about 10–15% of the lysozyme goes into whey forming about 90% of the product in cheese manufacture, while in the relatively low amount of cheese is the rest of lysozyme and may be liberated by the addition of acid.

In determining lysozyme activity difficulty is caused by the circumstance that the clarification of the suitable dead bacterial suspension is not in direct relation to either time or the enzyme substrate ratio. The curves of clarification may be interpreted only if it is asumed that the decrease of turbidity occurs in two steps.

# DATA FOR CHARACTERIZING THE BACTERIOPHAGE CONTAMINATION OF SEMIHARD CHEESE MANUFACTURING PLANTS AND FOR CHANGES IN CHEESE QUALITY AS EFFECTED BY BACTERIOPHAGE INFECTION

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Bacteriophage contamination may cause irregular acidification in cheese manufacture and thereby impairment of cheese quality. The bacteriophage contamination in semi-hard cheese manufacturing plants was studied.

It was shown in the study that titre of phages in the cheese whey varied between 10 and $10^{10}$ $ml^{-1}$. The characteristic titre amounted to $10^{7}$–$10^{8}$ $ml^{-1}$.

Phages detected in whey displayed a wide lysis spectrum. 62% of the phages affected *Str. cremoris*, 33% *Str. lactis*, 3% *Str. diacetilactis* and 2% *Leuc. citrovorum*.

Thirty three % of the enriched phages impaired acidification in *Str. lactis* cultures, 18% in *Str. cremoris* cultures, 6% in *Str. diacetilactis* cultures, while 43% were capable of affecting a wide range of mixed cultures. 14 cultures from the single-strain, and 7 cultures from the starters of our Institute were not susceptible to the phages isolated from the plants.

This may provide an explanation for the fact that, inspite of the high level of contamination in the plants, a severe irregularity of acidification is rarely encountered. The most sensitive strain was susceptible to 9, the most sensitive starter to 10 types of phages.

To study the morphology of the isolated phages electron-microscopic analysis was applied. The isolated phages possessed isometric or elongated heads. The diameter of the heads of phages with isometric heads varied between 53–66 nm. Their tail structure was contractible or non-contractible The tail length of phages with contractible tail was 180 nm, while non-contractible tails were of 160 nm length. At the end of the contractible tail structure a terminal base-plate of greater diameter than that of the tail, with pins on it, was to be observed. Phages with elongated head were of 40 × 60 nm head size, and their tail of 80 nm length was non-contractible.

# PREPARATION AND APPLICATION IN THE DAIRY INDUSTRY OF CONCENTRATED, FREEZE-DRIED CULTURES OF LACTIC ACID BACTERIA

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The rapid technical development of recent years and increased quality requirements made it inevitable to use, instead of liquid cultures (of high labour and material requirement, maintained in multi-stage industrial processes) concentrated cultures of good keeping quality which, due to their high cell density and activity are suitable to simplify culturing, and to make it more safe and controllable as well as to produce uniform culture and thereby a more even final product.

The aim of this work was to select a technique for the concentration of a butter culture from *Str. lactis*, *Str. cremoris*, *Str. diacetilactis* and *Leuc. citrovorum* lactic acid bacteria, for freeze-drying this and for storing the powdered culture, to make this powdered culture concentrate suitable for the direct inoculation of milk in the mass acidifying vat.

The first task was to find the optimum age of the butter culture from the aspect of concentration and lyophilization. It was found that, among the cultures in the log phase, at the end of the log phase, beginning of the stationary phase, in the stationary phase and at the beginning of the declining phase, the cells being in the stationary phase suffered the sligthest damage.

Concentrated cultures in the stationary phase were frozen by freezing rates of 0.027, 1.70 and 5.90 °C $s^{-1}$, resp. The best result was obtained with the freezing rate of 4.90 °C $s^{-1}$.

The protective effect of the following compounds was studied: Gelatine+ +$Na_3$citrate+Na-glutamate+saccharose, Saccharose+$Na_3$citrate, 7.5% lactose solution, skim-milk reconstituted for 10% solids content from powdered skim-milk. It was found that the butter culture could be freeze-dried most efficiently in a saccharose-$Na_3$ citrate medium.

The freeze-dried concentrates were stored at −25, +5 and +25 °C, resp. At the end of the storage period, highest survival and activity as well as aroma development were found with cultures stored at −25 °C.

The freeze-dried cultures were tested in semi-industrial scale experiments. Using an inoculum of 2 g powdered culture processed according to the above for 100 litre milk, the mass acidifier obtained at 23 °C during 18–20 h clotting time had identical parameters to those obtained in good quality by the traditional method.

# COMPARISON OF ALKALINE PHOSPHATASE TESTS OF MILK AND DAIRY PRODUCTS BY COLORIMETRY

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Phosphatase tests of milk carried out under identical conditions, using different buffers and the same substrate or the same buffer and different substrates, were evaluated by colour measurement.

In an earlier study it was found that the sensitivity of the alkaline phosphatase test can be increased by increasing the volume or the quantity of the sample, or by the proportionate increase of the concentration of the reagents in the final reaction or by the proportionate reduction of their volume.

Of the following buffers: $Na_2CO_3$—$NaHCO_3$, KOH—$KHCO_3$, $Ba(OH)_2$—$H_3BO_3$, veronal Na—NaOH, $NH_4Cl$—$NH_4OH$, ethanol amine—HCl—dicyclohexamine, applying the same substrate the latter two buffers proved to be the best. The $NH_4Cl$-$NH_4OH$ buffer, specified in the Soviet Standard had the best stability.

The substrates tested were: phenyl, phenolphthalein, *p*-nitrophenyl, fluorescein, alpha-naphtylphosphate, *o*-cresolphthalein, tropeolin, cresol red, *o*-naphtholphthalein, thymol blue, thymolphthalein, bromothymol blue phosphate. The samed lot of milk was use as the substrate.

A *Momcolor* S objective tristimulus apparatus was applied for purposes of comparison. The color reactions were related to the $\Delta$E value of phosphatase-negative milk. The $\Delta$E values needed for comparison were calculated on the small computer TPA-I.

Color reactions in the order of decreasing evaluability: *o*-cresolphthalein, cresol red, phenolphthalein, thymolphthalein, bromothymol blue, tropeolin with alkali, alpha-naphthol, Fast Violet B, Fast Red KL, Diazolblue, *p*-nitrophenol-alkali, alpha-naphthol, Fast Red 2GL, Fast Blue BB, Fluorescein-alkali, alpha-naphthol with Fast Garnet GBC.

The reaction of the phenol-2.6 dibromochinone chlorimide could not be evaluated under this experimental conditions.

Colorimetry is a means by which the subjective qualitative data could be converted into objective quantitative values and with the aid of the models better substrates could be designed.

# THE ROLE AND SIGNIFICANCE OF MICROBIOLOGY IN ENOLOGY AND THE MICROBIOLOGICAL CONTROL IN WINE PRODUCTION

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Microbiology in enology, the bases of which were laid down by Pasteur, is in close connection with every phase of wine production and treatment. Its importance in theory and practice is supported by the fact that the International Office for Viticulture and Enology has formed a Sub-committee of Wine Microbiology in 1965. This is expected to treat such basic problems as unification of methods of wine microbiology, setting up an international wine-yeast catalogue, investigating the products of the metabolism of yeasts and lactic acid bacteria, production of dried wine yeast, etc.

In Hungary the Research Institute for Viticulture and Enology has been engaged since 1901 in research of wine microbiology. The following subjects were studied:

- wine yeasts and their application,
- ensuring biological stability to wine,
- microbiological control of enology,
- analysis of microbiological processes in the course of white and red wine processing on large scale.

The 87 strains of the wine-yeast collection ensure under every circumstance the sound fermentation of musts. The Institute places about 9–10 thousand portions of yeast per annum at the disposal of the customers.

Based on the detection of yeast strains causing turbidity and on the study of the tolerance to heat and fungicides, it is possible to ensure the biological stability of wines by means of chemical and physical processes. Recent research has shown the role played by certain technical operations in developing the geranium flavour in wine. Taking this into account, it is possible to eliminate this undesirable flavour.

An overall system of microbiological control of the process of wine making has been developed. This includes the raw materials, vinification, fermentation, treatment of wine, bottling, bottled wine, adjuncts and by-products. All larger plants have set up microbiological laboratories and the control of bottled wine is general.

Technological and analytical development makes it both necessary and possible to thoroughly investigate the processes occurring during wine production and treatment and to revaluate the results obtained earlier. The study of the biochemical properties of yeasts and lactic acid bacteria, of the products of their metabolism (acetaldehyde, $H_2S$, $SO_2$ formation), of the

inactivation of enzymes, the effect of heat treatment, is necessary in order to be able to develop the complex technology of white and red wine production and thereby improve their quality.

## THE MICROBIAL POPULATION OF WINE AS AFFECTED BY TECHNOLOGICAL OPERATIONS

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In the Central Hungarian wine cellars 80.4% of the total production is marketed in bottles. The bottling of young, fresh wines, containing some sugar and perhaps little alcohol becomes increasingly difficult because of the tightening of sanitary specifications. Chemical agents have to be gradually eliminated and replaced by physical procedures. However, it is possible to manufacture a stable product by strict technology under appropriate microbiological specifications.

In this study the microbiological state of wine was followed throughout the cellar operations right through to bottling. The selected batches differed in their sugar content and in consequence in the potassium sorbate content added as preservative.

The sugar content of *Zöldszilváni* was 21.4 g $l^{-1}$ and the potassium sorbate 200 mg $l^{-1}$. *Ezerjó* contained 3.7 g $l^{-1}$ sugar and 132 mg $l^{-1}$ potassium sorbate. Potassium sorbate was not added to *Oporto rose* containing 0.8 g $l^{-1}$ sugar.

The results permitted of the following conclusions: within 24 h upon removal of the clarifying agent the cell count is not reduced. but after some days a 90% reduction may be observed. Filtering through kieselguhr may effect reduction by another order of magnitude. Cell count is affected by the sugar content, even in the presence of potassium sorbate. This was proven by the fact that the reduction of cell count was much slower in *Zöldszilváni* than in *Ezerjó* or *Oporto rose*.

The cell count of wine when transferred from the cellar to the bottling plant should be kept at the level of $6 \times 10^1$–$2 \times 10^2$ cell $ml^{-1}$. The contamination level of the final product is decisively affected by the quality of the filtration, by the efficiency of washing and the sanitary condition of the filling tubes. It was found by mathematical statistical analysis of the data of the filling operation that under the given conditions the viable cell count of the samples examined did not exceed the permitted level of $10^1$ cell $l^{-1}$ (using potassium sorbate as a preservative).

# MICROBIOLOGY OF THE IN-BOTTLE FERMENTED CHAMPAGNE

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To take into account microbiological considerations is an important task in every branch of the food industry, thus in the manufacture of champagne, too. Microbiological spoilage is due mostly to yeasts. There are no standards, nor specifications to show the degree of contamination just permissible and the value at which spoilage is inevitable.

In this study the microbiological quality of in-bottle fermented champagne as affected by individual steps of the production technology, was examined. The effect of different fermentation temperatures was investigated and it was found that to ferment in a cellar at temperatures above 15 °C was dangerous. In the final processes the product may become contaminated, therefore it is possible and necessary to apply cleaning and disinfection measures in order to ensure storage stability.

Champagne fermented in the bottle must remain stable for 12 months, without sedimentation. It was shown in storage experiments at different temperatures that the stability of the product is not affected by the temperature at an initial cell density of $10^1$–$10^3$ $ml^{-1}$, except in the case when the champagne contains wild yeasts. When the conditions for the growth of yeasts turn unfavourable, wild yeasts may proliferate. When, due to deficiencies in the technology, the champagne became contaminated with *Candida valida*, *Pichia membranaefaciens*, *Cryptococcus laurentii* and *Rhodotorula rubra*, the latter organisms proliferated at every storage temperature and caused, without fermentation, undesirable turbidity. Stability depends mainly on the population of the final product.

The material, personal and organizational conditions of the adherence to sanitary specifications must be ensured. An overall cleaning and disinfection plan has to be developed and strictly adhered to, under microbiological control.

## MICROBIOLOGICAL STUDY OF CIDER PRODUCTION WITH SPECIAL REFERENCE TO THE COMPOSITION OF THE YEAST POPULATION

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Of fruit wines the cider with its agreeable flavour and aroma plays an important role in some countries. In Hungary, apples or the by-products of apple processing (peel, core) serve as the raw material.

The aim of this study was to characterize the microbiological state of the phases of cider fermentation. An assay was made to elucidate, by microbiological analysis of the samples taken, the changes occurring in the composition and quantity of the yeast population during fermentation.

Of the great number of yeasts isolated from the pressed apple juice several would have been able to ferment the juice. However, the spontaneous fermentation may have resulted in an uneven cider containing perhaps unpleasant flavour and aroma substances. The fermentation can be controlled by the inoculation of the juice with *Saccharomyces cerevisiae*, which suppresses the natural yeast population.

On the basis of the microbiological study of the production phases suggestions were made in order to improve the efficiency of production and the quality of the product.

## MICROBIOLOGICAL TESTS IN VARIOUS PHASES OF PRODUCTION IN THE PANNONIA BREWERY

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In large-scale brewing, because of its complexity and dimensions, microbiology is of much greater importance than it is in small-scale beer production.

The increasing requirements on quality and shelf-life entail a certain degree of production-directing role of microbiology. Up-to-date factory microbiology is responsible for the continuously good quality of the product.

This purpose is severed by systematic on-line testing of the raw materials, the equipment, the semi-finished and finished products.

Tests were extended over the microbiological control of water, compressed air, inoculum and diatomaceous earth. Analysis of the inoculum is of special importance.

Selective media were used to detect Lactobacilli and wild yeasts.

To promote the biological cleanliness of production equipment a well-organized and systematic control was introduced. The shape and dimensions of the equipment determine not only the method of cleaning and disinfection, but at the same time the possibilities of control as well.

In addition to the generally used plate pouring technique the agar-sausage method of Ten Cate was introduced to enable the discovery of the critical points of large storage vats, too.

In the control of semi-finished products, great attention is paid to the detection of groups of bacteria most dangerous for the these products. In view of the storage stability of the final product the strains endangering this and the fecal indicator flora characteristic of the sanitary condition of production, are examined.

A further task of the laboratory is to inform the management of the results of the control tests and to help in the development of preventive measures.

## MICROBIOLOGICAL LABORATORIES IN THE CANNING INDUSTRY

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Laboratory work in the field of microbiology of food canning has been introduced in Hungary more than 20 years ago. Microbiological laboratories operate in every canning factory. The total of their staff amounts to 56 of which 20 possess university degrees, 18 are technicians, 11 laboratory assistants and 7 auxiliary personnel. Their activities fall in the following fields: sanitary inspection 12%, on-line checking 28%, analysis of spoiled goods 16%, product checking 44%. During 1976 a total of 65 950 microbiological samples were examined.

Up-to-date microbiological checking is possible, however, to improve efficiency, the methods of on-line monitoring are being reorganized and unified.

The new system of inspection is developed on the basis of experiences gained in Hungarian laboratories and in international practice, first of all on the recommendations of the FAO/WHO Codex Alimentarius.

# MICROBIOLOGICAL CHARACTERISTICS OF HAND- AND MACHINE-HARVESTED SNAP BEANS

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On-line tests in the course of processing hand- and machine-harvested snap beans were carried out on the 3000 kg $h^{-1}$ capacity bean processing line of the Paks Canning Factory. The harvesting machine was of type FZB.

Judged by physical parameters the quality of beans harvested by machine was satisfactory. Of the harvested beans 1.8% was in clusters, 6.6% pods broken into pieces above 2 cm, 0.5% pods broken into pieces below 2 cm and 0.4% extraneous matter.

Phase tests were carried out on 5 occasions in two parallel samples by the dilution test. Results were evaluated by two-variable analysis of variance.

The total aerobic viable cell count and the aerobic spore count was found to be independent of the method of harvesting. Contamination with anaerobic spores was higher in the machine-harvested beans than in the hand-harvested ones. The spore count of the beans was of the order of $10^1$.

A significant increase in cell count was observed upon manual filling of the beans and through the adding of brine of relatively high contamination. The brine contained some aerobic spores and the spore count was increased by blanching. The latter is probably due to the fact that at the time of the study sufficient raw material was not available and after the frequent stops cleaning was superficial and spore centres could be formed in the blancher. By keeping the temperature of the brine continuously at 90 °C it is possible to achieve a reduction in the number of spores and viable cells. The keeping quality of the batches tested was found satisfactory. The spoilage of individual samples could be traced back to faulty closing.

The results of the study have shown that upon selecting a bean variety suitable for machine-harvesting, by proper use of the harvesting machines, by strict adherence to specifications of technology and hygiene canned snap beans of good quality may be produced.

# DETERMINATION OF THE STERILIZATION REQUIREMENT IN THE CANNING INDUSTRY BY CALCULATION OF A HEAT TREATMENT EQUIVALENT

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The concept of the sterilization equivalent, the principle of its establishment have been known in the microbiology of canning for several decades.

The aim of this study was the development of a practical method for the establishment of a sterilization equivalent on the basis of measurable characteristics of the microbiological state or at least relate the equivalent to these characteristics.

On the basis of the sterilization equivalent the survival rate as determined in laboratory fractionated heat treatment experiments (phase tests, cell counts) and in industrial heat processing (spoilage by survivors) may be considered as the values of one and the same function. Thus, the sterilization equivalent suitable for the dimensioning of the operation may be equally well determined either by appropriate on-line tests or by sterilization experiments.

Equivalent of the microbiological state

$$A = (N_0 + 2m + \log G - \log r)D_0$$

where $N_0$ logarithm of the initial cell count per gramm of the fraction most probably surviving (in this case some spores)

$D_0$ decimal reduction time of the above fraction at selected temperature, $t_0$

$G$ net content of the canned product

$r$ tolerated percentage of spoilage by survivors.

Equivalent of the technological process:

$$A = \int_0^x 10^{\frac{t-t_0}{z}} \, dx$$

where $t$ temperature; $x$ time, temperature history of the product

$t_0$ selected temperature identical with the former

$z$ temperature constant, characteristic of the most probably surviving fraction.

In the development to a practical method for the establishment of the sterilization requirement verification of a temperature constant $z = 20$ °C substantially differing from the generally accepted one (10 °C), was of decisive importance.

A method, partially based on on-line microbiological tests, was developed for the heat treatment of canned peas ($A$: min, $z$: 20 °C, $t_0$: 100 °C) and was confirmed biometrically. It was shown that safe production ($r = 0.1\%$) may be achieved by an $A$-equivalent heat treatment of 150–300 min, depending on the initial cell count.

The technique proved suitable for the establishment of the heat requirement of other vegetable products, too. The temperature constant $z = 20$ °C may be used for snap beans ($A$ = 70–90 min, pH = 5.5) and for a three-component vegetable mixture ($A$ = 90–100 min, pH = 5.5–5.8).

In establishing the sterilization requirement of other, mainly medium acid vegetables, production safety may be achieved by the parallel utilization of two equivalents, on the basis of the empirical data below:

| Product | pH | z = 20 °C A ($t_0$ = 100 °C) | z = 10 °C $F_0$ ($t_0$ = 121.11 °C) |
|---|---|---|---|
| | | equivalent, min. | |
| Snap beans + acid | 4.5 | 60–80 | 2–4 |
| Snap beans + acid | 4.0 | 40–50 | 0.2–2 |
| Cauliflower + acid | 4.7 | 28–30 | 0.3–0.4 |
| Tomato | 4.2–4.5 | 2–10 | 0.003–0.01 |
| Pickles | 3.5–4.2 | 1.5–2 | 0.001 |

the latter data serve only for purposes of orientation)

This exact method as developed for establishing heat requirement permits of process-optimization inasmuch it enables the selection of the most suitable operation of processes of different temperature history but identical sterilization equivalents.

## HEAT TREATMENT OF CANNED AND BOTTLED FRUITS AND VEGETABLES IN A TUNNEL PASTEURIZER

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Heat penetration was measured in ten products of different kind and size in a tunnel pasteurizer of the *Stiels* system. Parallel to this test heat penetration values in the same products were measured in batch pasteurizing tanks.

Measurements were carried out with an *Ellab* thermo-electric thermometer. The $F_0$ value was determined by the use of a planimeter in tracing the boundary line of the area below the thermal death curve.

To prove the validity of the experiments an incubation test was also applied. By means of measurements and calculations it was possible to compare the results of heat treatment in the two equipments.

On the basis of the above the following conclusions were drawn: as compared to the batch-type pasteurizer, $F_0$ values found were similar and sometimes higher.

The desired core temperature may be attained by increasing the various stages of the heat treatment by 2–4 °C.

The fluctuation in core temperature was slight, while that of the heating space fluctuated by 0.5–1.5 °C.

Cans, and jars, drifting along in the equipment received the same heat treatment. The shift in the appearance of the marked units amounted to a maximum of 2 to 8 min. Heat treatment equivalents required for pickled products ($F_0 = 0.002$–$0.04$) and for bottled fruit ($F_0 = 0.1$–$0.4$) were attained with greater accuracy and safety in the tunnel pasteurizer than in the batch pasteurizing tanks.

## ANALYSIS OF ON-LINE CONTROL OF CERTAIN PRODUCTS OF THE CANNING INDUSTRY

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The aim of this study was to compare the sanitation level of the products of several years on the basis of the average values of mesophilic aerobic cell counts and to evaluate the effect of different technological operations on the cell count.

To achieve our aim we evaluated by means of mathematical statistical methods the results of on-line tests in the canning industry from the year 1972 through 1976. Control tests were carried out at the following points of the line: raw material, washing efficiency, cell count reducing effect of blanching; microbial population of brine and sugar solution used in filling the cans and jars; cell count in the product prior to closing the jar or can. The residual cell count in the product after heat treatment was separately evaluated.

In selecting the product to be tested the aim was to select one belonging to a group of products and manufactured on a large scale. Products more thoroughly studied were: bottled mixed fruit, green peas, snap beans, lecsó (slices of green paprika in tomato puree), tomato puree.

The investigation has shown for individual products the point in the manufacturing process where in view of the quality of the final product a severe control is necessary.

## UNIFICATION OF METHODS FOR THE MICROBIOLOGICAL CONTROL OF BEVERAGES

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Unification of methods for establishing the microbiological quality of vegetables and their standardization lags far behind of those of animal foods. The selection of methods suitable for routine tests, efficient and reproducible, has been started 4 years ago within the Working Group for Methodology of Microbiology.

In 1977 the development of the method for determining the yeast count, the mesophilic aerobic cell count in beverages has been completed. In this work 7 laboratories, some industrial ones, others belonging to food control institutes, have participated. Of the 10 home-made and foreign media of different composition the acidified yeast extract-glucose medium (pH 4.5; yeast extract 0.05%, glucose 2.0%) was selected for the final test. This medium proved to be simple and efficient. It is of interest to note that there was no significant difference between the yeast counts obtained on 8 of the 10 different media.

There was no significant difference between the three methods applied: MPN, plating and membrane filter. However, in the case of low yeast concentration ($10–10^3$ $ml^{-1}$) the counts obtained by the MPN method were significantly higher than those obtained by other methods. Incubation was carried out at 30 °C for 48 h, complying with international standards. No significant difference was found between counts of 48 or 72 h incubation. The standard deviation of the method within laboratory amounted to 0.2, while that between laboratories to 0.3, corresponding to the usual value of microbiological counting methods.

# STUDY OF FACTORS AFFECTING THE MICROBIOLOGICAL KEEPING QUALITY OF CARBONATED SOFT DRINKS

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The keeping quality of soft drinks is substantially affected by the composition of the basic compounds, the carbonate content, the method by which the food was processed and the storage temperature. The initial cell count depends largely on the sanitary conditions of the plant.

In order to be able to determine the keeping quality, the cell count limit value indicating spoilage — related to the different types of soft drinks, — was established. A hedonic scale of 20 scores was used in sensory evaluations and the yeast count served as index of the microbiological state. A close correlation was detected between yeast count and sensory value. Above a cell count of $10^5$ $l^{-1}$ the sample was found unacceptable.

In beverages of satisfactory carbonation (6 g $l^{-1}$) an initial reduction of cell count is followed by a rest period, after which cell growth becomes similar to that observed in non-carbonated beverages. The period of adaptation takes 10–15 days.

On studying beverages with different initial cell counts it was found that shelf-life is ensured by yeast counts below 1 $ml^{-1}$.

The study of the effect of storage temperature on keeping quality has shown that under refrigeration, products of relatively high initial cell count may be kept 1.5–2 times longer than at room temperature.

# MICROBIOLOGICAL CONDITIONS IN THE BEVERAGE PLANTS OF THE TRUST OF BREWERY INDUSTRIES

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Beverages prepared from Cola concentrate and the aromatic substances of citrus fruits belong to the most popular alcohol-free drinks in Hungary. The demand for these beverages of characteristic flavour and rich in aromatic substances is on steady rise and this rise has to be followed by the product volume.

In the plants of the Trust of Brewing Industries, such beverages are manufactured since 1971. The production line was started with Pepsi Cola, and it was followed in 1976 by beverages based on the extracts of citrus fruits. As far as the aspect of technique and technology is concerned, the plants differ substantially. Because of differences in their location (next to a brewery, or established independently) the environmental conditions differ, too. These circumstances determine the microbiological characteristics of the plant, and, thereby, the cell count of the beverage.

The subject of these investigations was to establish the viable cell count in Pepsi Cola and citrus fruit-based beverages on samples sent in, with special reference to mesophilic bacteria and yeasts.

Our determinations were carried out by the membrane-filter technique using a 0.45 $\mu$m mesh cellulose-nitrate filter. Trypcasin-glucose-yeast extract (TGY) agar was used to determine the total viable cell count, while yeast count was established on acidified yeast extract-glucose agar (pH = 4.5).

The correlation between the microbiological characteristics of beverages produced at different technological levels and under different conditions are summed up in Table 1.

Table 1

*Results of the microbiological study of beverages*

| | Products of beverage plants | | | |
|---|---|---|---|---|
| | located next to a brewery | | | located independently |
| | Lemon | Orange | Pepsi Cola | Golden bridge Orange |
| Number of samples | 18 | 15 | 52 | 21 |
| Total viable mesophilic count 1 $dl^{-1}$ | 38 | 72 | 23 | 90 |
| Yeast count 1 $dl^{-1}$ | 7 | 25 | 20 | 10 |
| Characteristics of production line | Seitz: 15 000 bottles or 30 000 bottles per h | | | LF 10 1 000 bottles per h |

As may be seen from the results, the vicinity of a brewery has not affected significantly the microbiological contamination of beverages if the hygienic requirements have been attented to on both, the brewery and the beverage bottling sides. The technological level affects the microbiological quality of beverages, but much less than expected.

## MICROBIOLOGICAL INVESTIGATION OF COCA-COLA AND STAR BEVERAGES

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The Trust of the Alcohol Industry produces about 180 million bottles of beverages per year. The major part of these is formed by Coca-Cola and Star, both manufactured from imported basic materials. These products were exposed to methodological and storage tests.

To establish the microbiological quality of the beverages two methods are applied at present: the membrane filtration and the plating technique. The comparability of the results obtained by the two methods was studied in model experiments carried out with a *Saccharomyces cerevisiae* strain. The following characteristics were varied in four experimental series: cell count in the model suspension, mesh of the membrane filter, sample quantity used for filtering. For determining the yeast cell count a mesh of 0.8 $\mu$m proved to be more expedient than the 3.0 $\mu$m mesh filter. Using the 0.8 $\mu$m filter and samples of varied cell count and different quantities, the difference in results of the two methods was shown to be not significant.

Experiments were carried out to preserve fruit-based beverages by chemical agents. The aim of these experiments was to find the appropriate preservatives and the quantity which would ensure a three-month storage life, as specified in Hungarian regulations, without interfering with the sensory quality. The preservatives tested were: Na-benzoate, Na-sorbate and the combination of the two. Various amounts of the preservatives were added to the beverage and the bottles were stored at room temperature and under refrigeration. Three months storage life was found to be ensured by 0.6 g $l^{-1}$ benzoate, or 0.4 g $l^{-1}$ sorbate or 0.2 g $l^{-1}$ benzoate plus 0.15 g $l^{-1}$ sorbate. Sensory evaluation has shown the combination of benzoate and sorbate to affect least the sensory qualities of beverages.

## MICROBIOLOGICAL AND HYGIENIC MONITORING IN THE SZOB FRUIT PROCESSING PLANT

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During the last five years regular studies have been carried out in the Szob Fruit Processing Plant. This is an account of the microbiological study of water used in the plant, of various stages of the line and of the final products.

The bacteriological investigation of water was carried out according to the Hungarian Standard (MSZ 22901-71). In testing various phases of production and the final product experiences gained in Hungary and abroad were taken into account. Results were evaluated by mathematical statistical methods.

The quality of water of varied origin used in the plant was not satisfactory as shown in microbiological tests (Fig. 1).

However, after turning to good account some of the suggestions of the authors an improvement was observed in the quality of water.

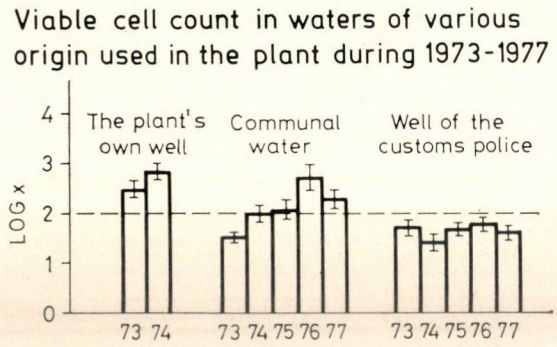


Fig. 1.

The phase tests have shown that microorganisms contaminating the basic materials and additives had opportunity to proliferate at certain stages of production, however, if specifications of hygiene and technology were strictly adhered to during syrup boiling and bottling, the final product was still of good microbiological quality (Table 1).

Table 1

| Sample | Yeast count |
|---|---|
| Granulated sugar | $5.7 \cdot 10^1$ 10 $g^{-1}$ |
| Dye | $10^3$ $g^{-1}$ |
| Starch syrup | $10^1$ $ml^{-1}$ |
| Decanted raspberry juice | $1.5 \cdot 10^2$ $ml^{-1}$ |
| Filtered raspberry juice | $10^2$ $ml^{-1}$ |
| Boiled raspberry juice | 0 $ml^{-1}$ |
| Final product | 0 $ml^{-1}$ |

The microbiological quality of the final product was generally satisfactory, though certain fluctuation could be observed during the years studied (Fig. 2).

The substantial improvement in 1977 in the quality of the products is due mainly to the installation of an automatic bottle washing machine.

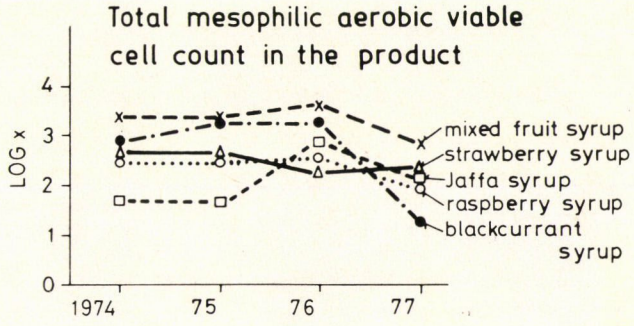


Fig. 2.

## MICROBIAL POPULATION OF MINERAL WATERS AS A FUNCTION OF $CO_2$ CONTENT AND STORAGE TIME

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Microorganisms isolated from two mineral waters of different original temperature (68 and 42 °C, resp.), which suffered secondary contamination during bottling, were inoculated into bottled mineral water. A part of the inoculated bottles contained no $CO_2$, another part 4 g $l^{-1}$ $CO_2$ and the third part 8 g $l^{-1}$ $CO_2$. For inoculation suspensions of different cell density were used. During storage at predetermined intervals samples were taken and the cell counts in the samples of different $CO_2$ content were determined. The bottles were stored at identical temperatures.

Results have shown that in bottles without $CO_2$ microorganisms proliferated rapidly, and independently of the initial cell count. In bottles containing 8 g $l^{-1}$ $CO_2$, microorganisms were destroyed within a few days even at high cell densities. In bottles with 4 g $l^{-1}$ $CO_2$ when cell counts were low, the population was soon destroyed. At high cell density, however, 4 g $l^{-1}$ $CO_2$ did not prevent spoilage.

The study permits of the conclusion that the storage stability of mineral waters, in the case of non-aseptic filling, depends highly on the quantity of $CO_2$ in the water. If the cell count is low, a lower $CO_2$ concentration is sufficient to ensure stability, while with high cell density higher $CO_2$ concentration is necessary.

# A STUDY OF MICROORGANISMS IN THE COURSE OF GROWING, HARVESTING AND PROCESSING OF SWEET PEPPER

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The inner side of the pericarp of the paprika pods becames contaminated by bacterial, yeast and mould cells already on the plant. This occurs at an early stage of development of the pods.

The study of the microscopic sections of the apex of pods has shown that some of them opened because of the drying of the style. By spraying the paprika plants systematically with fungicides prior to harvesting spoilage during after-ripening and storage may be reduced by about 20–25%.

Spoilage of the pods may be effected on the plant by injecting them with a suspension of microorganisms. Pods on the plant resist spoilage more efficiently than in the harvested state. Injured pods spoil more rapily than the unimpaired ones. Even if a single bacterial strain is used for injection spoilage is always caused by the association of a variety of microorganisms. As a consequence of environmental influences one of the strains becomes dominant and causes a characteristic form of spoilage.

In the course of spoilage the pericarp becomes thin and deteriorates. Within the pod on the pericarp bacteria and fungi proliferate and this reduces the quality of the ground paprika. During spoilage the useful components of the pericarp decrease while products of metabolism causing taste deterioration increase.

During storage of the pods their microbiological condition deteriorates. Storage conditions in their present state increase contamination of the pod surface and on the inside of the pericarp microorganisms proliferate. The microbiological state of the raw pods determines the quality of the semi-product and thereby that of the ground paprika, as well.

By washing the pods in the course of processing their contamination may be reduced. By drying, the number of vegetative cells may be substantially reduced. The microflora of the product is not significantly affected by further processing.

From spoiled paprika *Rhizopus nigricans*, *Aspergillus niger*, *Aspergillus elegans*, *Penicillium* strains, *Rhizopus oryzae*, *Mucor*, *Circinella*, *Cladosporium* strains were isolated.

The microbiological condition of ground paprika depends on the year of cultivation, on the climatic conditions during storage and after-ripening, and on the method of storage. The quality grades of ground paprika do not mean differences in their microbiological quality. Between samples belonging

to adjoining quality grades the difference in viable cell count, spore count, coliforms, *Escherichia coli*, moulds or yeasts is non-significant as has been proved by analysis of variance.

Ground paprika obtained from pods not stored, nor after-ripened is of significantly higher microbiological quality than the product of the traditional method.

## COMPARATIVE STUDY ON THE MICROBIAL-COUNT-REDUCING EFFECT OF TREATMENT WITH ETHYLENE OXIDE AND GAMMA-RAYS IN GROUND PAPRIKA

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Seasonings, thus ground paprika, too, are contaminated by various microorganisms. The storage life, and sanitary state of products of the canning and meat processing industries, of cold dishes is influenced, endangered by such seasonings.

In recent years the demand for microbiologically pure seasonings, thus for ground paprika as well, increased. The introduction of technological processes for the reduction of microbial contamination of seasonings without impairing their volatile aroma substances, is indicated.

In this study the microbial count reducing effects of treatment with ethylene oxide and ionizing radiation were compared.

Cell count reduction by ethylene oxide was carried out in *Degesch*'s gas chambers (ethylene oxide: 600 g per $m^3$, treatment period: 6 h, at 25 °C with a gas mixture of 10% $CO_2$ and 90% ethylene oxide).

Radiation treatment was carried out in a $^{60}Co$ panoramic radiation source. The samples were treated with 5, 9.3 and 11 kGy, resp. A storage period of 6 months was applied. Results were evaluated by two-component analysis of variance.

It was found that both treatment with ethylene oxide and with 5 kGy reduced mesophilic aerobic cell count to the same extent (by 2.5 orders of magnitude). By the use of higher doses a more extensive reduction could be achieved, however, for practical reasons, this is indicated only in very exceptional cases. Spores of the microorganisms showed similar resistance to both ethylene oxide and radiation treatment.

In the treated samples coliform and *E. coli* I were not detected. Treatment with ethylene oxide did not reduce the mould count, whereas treatment with 5 kGy brought about a reduction. The colouring substance of ground paprika was practically not affected by the treatments.

Treatment with a radiation dose of 5 kGy caused a similar cell count reduction of 2.5 orders of magnitude as ethylene oxide, including the reduction of mould spores. Thus, irradiation is suitable for the reduction of cell count in closed packages under exclusion of subsequent contamination.

## AN ASSAY INTO THE REDUCTION OF CELL DENSITY IN DRIED ONIONS BY TREATMENT WITH GAMMA-RAYS OR ETHYLENE OXIDE

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The cell count in dried onions as affected by gamma-radiation (3.7–9.8 kGy), by treatment with ethylene oxide or as a function of storage time, was investigated. It was found that treatment with 3.7 and 4.7 kGy was sufficient to obtain a product of low cell count. A dose of 4.7 kGy reduced the initial cell count by 2–3 orders of magnitude. Yeast counts were sufficiently reduced, too. The yeast count was reduced by treatment with 3.7 kGy to values below 10 $g^{-1}$. The number of aerobic spores was also reduced to 10 $g^{-1}$ or below. Considering that the international norm for the spore count is 100 $g^{-1}$ a product of adequate quality may be achieved by irradiation.

Treatment with ethylene oxide approximated the results achieved by irradiation with 4.7 kGy.

The difference in cell count between samples taken from various parts of the sack of onions was not significant, for neither of the treatments.

The mesophilic aerobic viable cell count in dried onions was further reduced in the course of storage, because cells damaged by irradiation perished. This phenomenon was not observed in onions treated with ethylene oxide.

## CONTAMINATION OF DRIED VEGETABLES DURING PROCESSING AND STORAGE

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The increase in production, its proportion in Hungarian export and the growing home demand necessitate increased attention to the quality of dried vegetables.

An important feature of the quality specifications are the bacteriological requirements. These are more and more exacting both at home and abroad.

Every phase of the production of dried onion slices, cubes of tomato-shaped paprika and celery was exposed to thorough microbiological tests. In the samples taken at critical points of production the mesophilic aerobic cell count, the spore count, aerobic spore, anaerobic spore, coliform, *Escherichia coli* mould and yeast counts were determined. Results were evaluated by mathematical statistical methods.

The plant normatives for the vegetable products studied were determined at 84 and 95% probability level. By means of these internal plant standards it is possible to establish the percentage at which these products comply with the quality requirements of the trade.

Microbiological control of the production of dried vegetables is an inevitable task and a condition of the development of the export market. It is not sufficient to analyse only the final product because by this only the given state of affairs is recorded and this helps only in deciding whether the given product corresponds to the requirements or not. It is necessary to follow production throughout every stage and thereby ensure the possibility of preventive measures. Satisfactory product can be achieved only by this procedure.

Prior to final packing and distribution the dried vegetables are stored for some time. Since these products are not sterile — they contain a high number of viable cells in an inactive state — it seemed desirable to follow up changes in their microbial population during the storage period. During storage under sanitary conditions increase of the population was not observed, rather some decrease.

## RADIATION EFFECTS ON ACTIVITY AND STORAGE STABILITY OF ENDO-POLYGALACTURONASE

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(see article on pp. 299—307 of this issue)

## STUDY OF THE MICROBIAL POPULATION IN MEAT PRODUCTS OF REDUCED NITRATE CONTENT

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The reduction of the quantity of nitrate, depending on the extent of reduction and on the product, may cause a shift in the microflora of a meat product and this may cause spoilage. In order to prevent possible spoilage it was desirable to investigate changes in the microbial population.

Three series of dry sausage ripening experiments were carried out. Products containing 600 mg $kg^{-1}$ nitrate and no added nitrate were prepared. During the ripening period samples were taken every week and exposed to chemical and microbiological analysis. The latter consisted in determining the total viable cell count, the viable spore count, the homo- and heterofermentative *Lactobacillus* count, and the coliforms. In the course of chemical analysis the nitrite and nitrate content, the pH, moisture content, water activity, nitroso and total pigment content and the percentage of reddening were determined.

The investigations have shown that products manufactured without added nitrate contained detectable amounts of both nitrite and nitrate. Apart from the differences in the quantity of nitrite and nitrate and the percentage of reddening no other differences could be detected by the chemical and microbiological methods applied. Sensory tests have shown the product manufactured without added nitrate to be of slightly different taste. However, signs of spoilage could not be detected, thus it is possible to produce dry sausage without the addition of nitrate, only certain parameters of production had to be adhered to.

## THE NECESSITY OF VIRUS DIAGNOSTICS IN THE MEAT INDUSTRY

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Abnormality in the water binding capacity and consistency of beef was studied throughout several years. Apart from generally known factors it was observed that meat considered suitable on the basis of bacteriological macroscopic tests did not form mixtures (pulp) of appropriate quality. The meat product made of these mixtures were crumbly and did not bind water properly.

It was found that the beef-cattle giving such meat suffered from epidemic cough and were slaughtered in feverish condition. Viruses RS and PI-3 caused a feverish condition in which pyrogen substances were liberated and these inhibited water binding and thereby the development of the proper consistency in the course of industrial meat processing.

In the years 1974–1976 it was possible to investigate the processing of meat originating from animals treated with antibiotics and untreated and uncomplicated, put through bacteriological tests.

Regulations on meat control valid at present do not contain restrictions as to the marketing or processing of meat from animals suffering from viremic fever.

Meat control laboratories are not obliged to carry out virus tests, though the disease mentioned as an example may be easily detected in histological sections by syncytia and cytoplasmic inclusions. Other viremic diseases may cause deterioration of consistency in meat products.

Suitable and simple methods for the detection of the viremic condition are available. It is suggested to include these in the supplementary meat tests or at least test their suitability.

## CONCENTRATION AND PURIFICATION OF VIRUSES AND MACROMOLECULES WITH POLYETHYLENE GLYCOL

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The combined polyethylene glycol (PEG) precipitation-PEG reverse gradient method is suitable for the isolation, concentration and purification of macromolecules and viruses. It may be used to concentrate suspensions of large volumes *(e.g.* 15 litre) in a short time by simple means.

The principle of the method, based on the work of McClendon, Polson, Yamamoto, Clark and others, is surveyed with special reference to the isolation of different viruses and enzymes.

The practical application and experiences are detailed on the basis of the work carried out by the authors.

Mycobacteriophages were precipitated from lysates by $PEG_{6000}$ and NaCl applied in concentrations from 6 to 9% and 0.5 *M*, respectively. The precipitates obtained were purified by pelleting in a PEG reverse gradient.

The efficiency of the method was 75–95%.

## MICROBIOLOGICAL PROBLEMS OF SUGAR MANUFACTURE. USE OF VARIOUS DISINFECTANTS AND THEIR MECHANISM OF ACTION

I. Tóth-Zsiga

*Hungarian Scientific Society for Food Industry, H-1361 Budapest, Pf. 5.*

Iodophor preparations of various names (Iosan, Wescodyne, Rapidyne) were tested as disinfectants. Solutions of 0.5; 0.8; 1.0 and 1.5% resp., were used with stored and washed sugar beets. The mathematical statistical evaluation of results has shown that by disinfecting the washed beets not only the processing losses were reduced but the sugar content of molasses was lower and thus the white sugar yield increased.

A prerequisite of the efficiency of disinfection during storage is the cleanliness of the beet surface (free from weed and soil).

## A STUDY OF MOULD CONTAMINATION IN MIXED FEEDS

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The mould contamination of seven mixed feed samples and of two basic materials was investigated on four nutrient media: Czapek-Dox, Sabouraud, Beer-agar and Salt-agar.

The aim of the study was not only to determine the mould count but to compare the methods of analysis and to find the most sensitive one. Sensitivity of the medium was determined on the basis of the mould count.

There was but a slight difference between mould counts as established on the four different nutrients. Thus all four nutrients are suitable to determine cell count.

When the same experiment was carried out with pure cultures of the isolated microorganisms the results were similar. However, further experiments are desirable to compose selective nutrient media for the separation of various mould strains. This concept is supported by the fact that taxonomically different strains showed different composition.

Finally, it is suggested to unify the Sabouraud medium because this was the one that gave the highest mould counts and the largest variety of moulds. The preparation of the medium is simple.

## MICROBIOLOGY OF THE BAKING INDUSTRY

Zs. Havas-Fekete, A. Bozó and E. Aranyi

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Aerobic spore-forming bacteria causing ropiness, in bread, as well as moulds contaminate the dough by way of the flour. However, insufficiently sanitary production lines may cause secondary contamination in bread. Moulds are killed by baking. Production hygiene has a decisive effect on the bacterial count in bread after baking. The time of spoilage, as was shown in these experiments, depends on the initial cell count. In the case of identical products under identical storage conditions those of higher initial spore count ($10^5$ $g^{-1}$) were spoiled three days earlier than the products of lower initial spore of $10^3$ $g^{-1}$.

Products of the same type in different plants suffered different types of spoilage-stickiness or ropiness in various plants are due to "local microbial populations".

In breads wrapped in plastic film moulding set in within 2–3 days at room temperature. This indicates the necessity of appropriate production hygiene.

The mesophilic cell count in the spoiled products exceeded $10^7$ $g^{-1}$ in every case. It was found that bakery products of an initial cell density of not higher than $10^3$–$10^4$ $g^{-1}$ could be kept safely to the end of the specified storage time at room temperature, presumed that sanitary conditions were observed.

## MICROBIOLOGICAL QUALITY CHARACTERISTICS OF TOBACCO PRODUCTS

D. Kántor

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The microbial population of tobacco products as consumer goods was rarely investigated. This is because the products suffer spoilage only when the relative humidity of the storage space exceeds 75% and the moisture content of the product is more than about 14–15% corresponding to 65–70% ERH.

In this study the complete selection of Hungarian tobacco products was investigated. The microbial population of product groups differed, depending on the quality of the tobacco used, the technology applied and the physico-chemical properties of the product. The mesophilic aerobic cell

count was about $10^5$–$10^6$ $g^{-1}$, the yeast count $10^2$–$10^4$ $g^{-1}$ and the mould count $10^2$–$10^4$ $g^{-1}$. At this levels of contamination sensory degradation was not observed.

When comparing Hungarian products to foreign products the latter were found to have lower cell counts.

The majority of the isolated yeast strains were identified as *Penicillium* and *Aspergillus*, typical of store room populations. However, strains characteristic of the field population, such as *Alternaria*, *Cladosporium* and *Fusarium* were also found.

On the basis of the strains found the possibility of the products being contaminated with mycotoxins cannot be excluded.

The storage experiments have shown the physical chemical parameters of tobacco to be responsible for the microbiological condition of the product. In experiments simulating marketing conditions deterioration of quality was not observed. This was proven also by the sensory characteristics. In the course of the study the problem of the heterogeneity of the cell counts arose.

Climatic conditions in the tobacco distribution stores were kept under continuous control. The temperature in the storage rooms was between 4.5 and 23.5 °C and the relative humidity between 55% and 73%. The rate of marketing was about 2–3 months. Since the slow equilibration of moisture content is characteristic of tobacco products, the extreme climatic parameters compensate one another. Thus, the spoilage of tobacco products with standard moisture content can be excluded in the stores of the Hungarian Tobacco Industry.

# BOOK REVIEW

## D. A. A. Mossel: **Microbiology of Foods. Occurrence, Prevention and Monitoring of Hazards and Deterioration.**

The University of Utrecht. Faculty of Veterinary Medicine, Utrecht, 1977. 165 pages

Professor Mossel is one of the most widely-known microbiologists. This book is a revised edition of his monograph of similar titles which appeared in 1975 in the series CRC Critical Reviews in Environmental Control and in the edition of the University of Utrecht, respectively. This relatively small-sized book deals with a tremendous amount of information accumulated in the widely ramified areas of food microbiology; there are more than 2000 literature references in the book but they are dealt with in an expert-like and critically analytical manner which lend themselves to easy and good reading.

Major chapters of the book: Diseases of Microbial Origin Transmitted by Food; The Mechanism of Microbial Deterioration of Foods; The Control of Microbial Quality of Foods; The Microbiological Monitoring of Foods. The new edition deals with the literature up to June 1977 and it is conveniently supplemented with a detailed subject-matter index as well. As every good technical book, this work provides not only information but it conveys an outlook as well and it can therefore be profitably used as a university textbook, too. The approach to modern food microbiology with due consideration given to the ecological and quantitative aspects is a guiding principle throughout the whole book. It may therefore be recommended to research workers, quality controllers, technologists and university students concerned with microbiological issues in food manufacture, control and utilization.

J. Farkas

# ERRATA

In the paper covering pp. 155 to 166 of Vol. 7, No. 2 of this periodical *(Optimization of the composition of the medium for the production of milk-clotting enzyme of microbial origin in submerged culture* — by A. ERDÉLYI and E. KISS), misprints distorting the sense occurred. The corrections are as follows:

On page 156, para 1.2.
replace: 48 mold by: 48 h old
On page 157, Table 1, column 2
replace: $(O_{oi}, K)$ by: $(x_{oi}, K)$
On page 160, in the title of para 2.1.
replace: of complete factorial design by: of multi-factor design
On page 163, only the significant members of the four equations are to be set in boldface, as seen below:

with $CaCO_3$

$$\hat{y} = \mathbf{355} - \mathbf{126}x_1 + \mathbf{60}x_2 + \mathbf{137}x_3 - 14x_1x_2 + 25x_1x_3 + 5x_2x_3 + \mathbf{46}x_1x_2x_3: \quad (15)$$

$$\hat{y} = \mathbf{323} - \mathbf{210}x_1 + \mathbf{39}x_2 \times \mathbf{83}x_3 - 11x_1x_2 - 12x_1x_3 + 7x_2x_3 + \mathbf{14}x_1x_2x_3: \quad (16)$$

with $Ca(NO_3)_2$

$$\hat{y} = \mathbf{400} - \mathbf{302}x_1 + 32x_2 + \mathbf{60}x_3 - 25x_1x_2 - 31x_1x_3 - 7x_2x_3 + 16x_1x_2x_3: \quad (17)$$

$$\hat{y} = \mathbf{223} - \mathbf{188}x_1 + 0x_2 + \mathbf{68}x_3 - 31x_1x_2 - \mathbf{67}x_1x_3 + 5x_2x_3 - 14x_1x_2x_3: \quad (18)$$

On page 164, Fig. 6, numbers below the 4th and 5th columns:

| | | | | | |
|---|---|---|---|---|---|
| replace: | 1.35 | 1.35 | by: | 1.15 | 1.15 |
| | 3.50 | 3.50 | | 3.75 | 3.75 |

A kiadásért felel az Akadémiai Kiadó igazgatója. Műszaki szerkesztő: Botyánszky Pál
A kézirat nyomdába érkezett: 1978. VI. 19. — Terjedelem: 14,5 (A/5) ív, 56 ábra

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