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Factors Affecting the Processing of Wara—A Nigerian White Cheese

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(Received: 22 April, 1981)

ABSTRACT

Wara was prepared in the laboratory in the traditional way and factors affecting the processing were investigated. The juice from sodom apple (Calotropis procera), *used for coagulating the curd, showed minimal activities at temperatures below 37°C at pH 6·30. Cold storage for 2 weeks did not seem to affect the enzyme activity. At 40°C the milk clotting time (mct) dropped sharply to 26 min and this levelled out gradually to 1 min at 70–80°C. The mct varied inversely with the amount of the enzyme extract, ranging from 67 min with 0·5 ml to 22 min with 2·5 ml, while yield of wara increased from 28·5% with 0·5 ml to a maximum of 33·7% at 1·5 ml and then dropped to 30·5% with 2·5 ml. Beyond 2 ml of the extract the product had a bitter taste and poor texture whereas, within the range 0·5 to 1·5 ml, it was satisfactory.*

The most dramatic change in the composition when milk was made into wara was the disappearance of the lactose which was reduced from 4·6% to 0·2%. The protein was increased fourfold (3·7–14·8%) and the fat threefold (4·3–13·5%).

INTRODUCTION

Cheesemaking is an ancient art which probably began with the separation of whey from fermented milk, resulting in cheese. Scott (1973) believed that the initial fermentation was due to lactic acid and other acids produced by microorganisms. When such curds are held in salt or brine,

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this results in a little more 'ripening' which gives a more palatable food. The original crude enzyme used for the fermentation is rennet, obtained from the vell (or fourth stomach) of milk-fed young ruminants. However, in Nigeria, plant enzymes are used instead. There are other workers who have used a similar process (Yeshoda, 1941; Singh *et al.*, 1973; Dastur *et al.*, 1948) employing enzyme from *Withania coagulans* berries. Eskin & Landman (1975) used ash gourd (*Bennicasa cerifera*).

In Nigeria white unripened cheese, known as 'wara', is made by the addition of a plant extract (*Calotropis procera*), to the whole milk from cattle. Basically wara provides a means of preserving the whole milk. This paper is an attempt to determine the factors that affect the quality and yield of wara.

MATERIALS AND METHODS

Milk was obtained from cattle of different breeds (Friesian, Ndama, Jersey and White Fulani) in the University of Ife Research Farm at various intervals of time and analysed for its nutrients. Sodom apple leaves (*Calotropis procera*) were also obtained from the same source. The plant was cleaned with plenty of water and the juice extracted with a Webber hydraulic press. In order to eliminate the green pigments (chlorophyll) the juice was centrifuged at 4 °C at 17,000 rpm for 20 min and the supernatant solution obtained was stored in a refrigerator. This was used for the studies of the milk clotting activities (mca) of the enzyme. The following extracts of the plant were also tried.

(i) Acetone extract of the supernatant juice was prepared by a modified method of El-Shibeney *et al.* (1973).
(b) Alcohol extract was prepared by the method of Singh *et al.* (1973).
(c) Sodium chloride extract was also prepared by the method of Singh *et al.* (1973).
(d) Ammonium sulphate extract was prepared by the method of Berkowitz-Hundert *et al.* (1964).

The clotting times were determined using the visual method of Berridge (1954). Milk was adjusted to different pH values (6·6, 6·3, 6·0, 5·7) with 0·1 M NaOH solution and 10 % lactic acid solution. Ten millilitres of each milk sample was placed in an inclined test tube and connected to an electrically driven rotor and placed in a thermostatically controlled water bath.

Each sample was allowed to assume the required temperature by rotating in the water bath at 28 rpm for 5 min. As soon as 0·5 ml of enzyme preparation was added the inclined tube was slowly rotated to observe the change from liquid to solid in the contents of the tube. The clotting time was taken up to the moment when a thin white film appeared on the wall of the tube. The control for these experiments was 10 ml of pasteurised standard milk solution to which no enzyme extract was added or milk solution to which denatured enzyme was added.

For the preparation of wara, varying amounts of the plant extract (10–25 ml) were added to 1000 g of fresh, unpasteurised milk. After about 3–5 min of thorough mixing, a pH of 6·8 at 31 °C was recorded for the mixture. Heating was carried out on a thermostatically controlled hot plate, set to maintain a temperature rise of 1 to 2 °C a minute. Within 22 min (70 °C) pots containing the highest amount of juice extract had started to produce the characteristic white curd of wara. Heating was intensified to enable the curd to release its whey, including some water-soluble components. Cooking was carried out at 95 °C for about 3 min, during which time both texture and flavour were developed. At this stage the pH recorded was 6·40 with a titratable acidity of 0·5% lactic acid. Samples of the curds and whey were taken and stored in the freezer for analysis.

The curds were divided into three parts. One part was left overnight at ambient condition for souring, another was deep-fried in palm oil for about 5 min.

Proximate analysis was done on the samples by the standard method of the AOAC (1970).

RESULTS

The milk used for making wara is usually not standardised. In a case study, a pooled milk from about twenty open-range cows (white Fulani) had the composition shown in Table 1. The proximate contents of fat and protein show an increase of between four- and seven-fold whereas the lactose content was reduced until it was negligible. Information is still lacking on the fate of the vitamins and minerals in the cheese.

The juice was separated by centrifuging at 4 °C at 17,000 rpm for 20 min and the milk clotting activity of the different portions was tested under refrigeration temperatures, room temperature and at 37 °C. Table 2

TABLE 1
The Percent Composition of Wara as it is Eaten (Samples Bought from Five Different Sources)

Sources	*Moisture*	*Fat*	*Protein*	*Ash*	*Lactose*
A	72·2	14·2	12·2	1·08	0·3
B	70·6	14·6	12·7	1·86	0·3
C	68·8	16·1	13·1	1·81	0·2
D	61·6	21·9	14·4	1·80	0·30
E	76·6	11·2	10·5	1·38	0·30
Pooled milk	86·1	4·55	3·90	0·75	4·75

shows that the green leaf juice and the centrifuged juice showed milk clotting activities at 25 °C after 5 h and at 37 °C after 1 h. The sediments and leaves did not exhibit any appreciable activities until after 24 h at 37 °C. It may be suggested that enzyme activities are minimal at temperatures below 37 °C at pH 6·3. It is also apparent that activities are associated with the solution, rather than the sediments of the juice. When the extract was kept at 4 °C for 12 days the enzyme activities showed a similar trend to that shown in Table 2, indicating that the activities were not impaired.

Figure 1 shows the patterns of the milk clotting of enzyme extracts at different temperatures and pH 6·3 of substrate. It was observed that the

TABLE 2
Observed Clots—One Millilitre of Fresh Green Leaf Juice Preparation in 5 ml Pasteurised Standard Milk Protein Solution at pH 6·3 (Average Results in Duplicate)

Enzyme extract preparations	*At 5 °C, After*			*At 25 °C, After*			*At 37 °C, After*		
	1 h	*5 h*	*24 h*	*1 h*	*5 h*	*24 h*	*1 h*	*5 h*	*24 h*
Green juice	=	=	=	=	#	#	+	#	#
Centrifuged juice	=	=	=	=	#	#	#	#	#
Centrifuged sediments	=	=	=	=	+	+	+	+	#
Leaf wash	=	=	=	=	=	+	=	=	#
Control	=	=	=	=	=	=	=	=	=

Heavy clots #
Clots in traces +.
Clots not observed =.
Control—1 ml of boiled juice in 5 ml pasteurised milk solution.

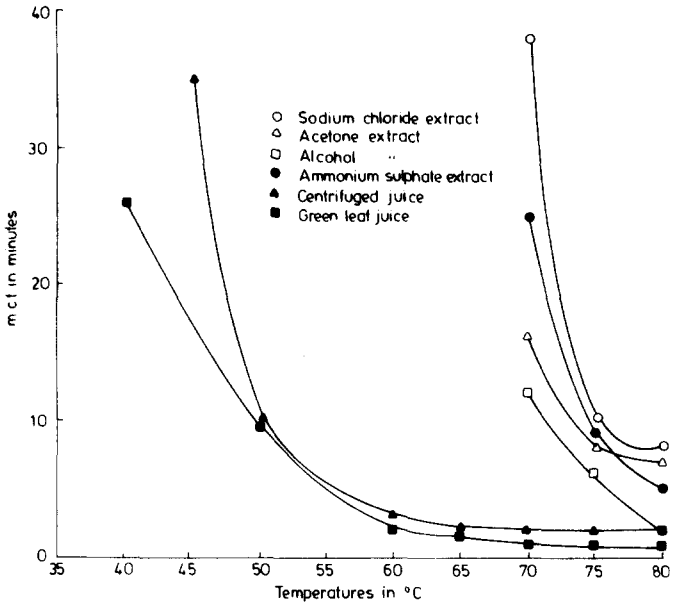

Fig. 1. Effect of temperature on the milk clotting time of sodom apple extracts at pH 6·3 of substrate.

milk clotting time for the green leaf juice dropped sharply to about 26 min at 40 °C, about 9·5 min at 50 °C and 2 min at 60 °C, above which a minimal milk clotting time was effected. At 70–80 °C the recorded mct was 1 min. A similar pattern was observed for the supernatant extract.

At 70 °C and above a sudden drop in the mct was observed for all enzyme extracts—i.e. the sodium chloride extract, 25 min, the acetone extract, 16 min and the alcohol extract, 12 min. At temperatures in the range of 70 °C to 80 °C the mct for the alcohol extract ran closest to that of the original juice extract, followed by that of the ammonium sulphate extract.

It was also noted that the various extraction methods made different impacts on the enzyme activities. It appeared that centrifuging the leaf juice did remove some of the proteases which are active in the temperature range 40 °C to 50 °C. The effect of alcohol treatments on the milk clotting was least severe, followed by that of ammonium sulphate treatments. A significant decrease in enzyme activity was observed by the sodium chloride and acetone treatments.

In Fig. 2(a), (b) and (c) the temperature/mct relationships of the different precipitated enzyme extracts at different substrate pHs are shown. The

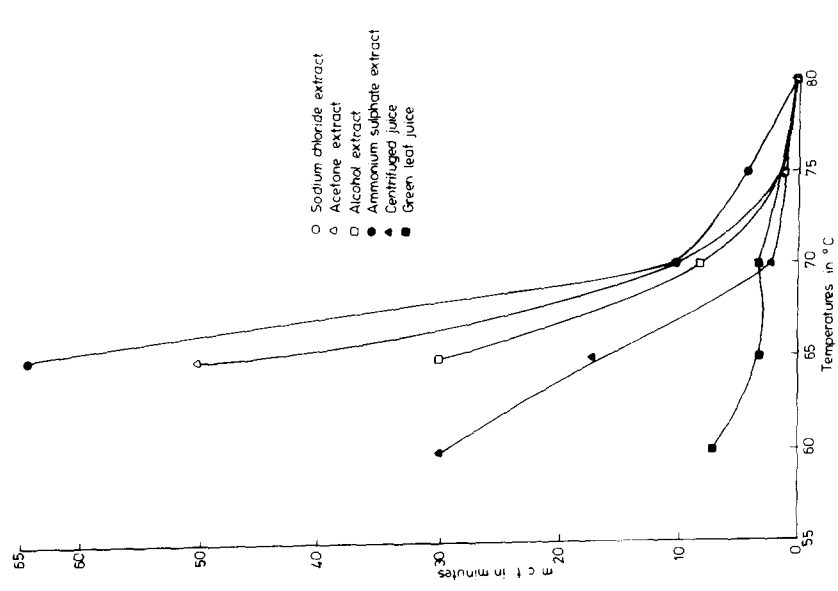

Fig. 2(b). Effect of temperature on the milk clotting time of sodom apple extracts at pH 6·0 of substrate.

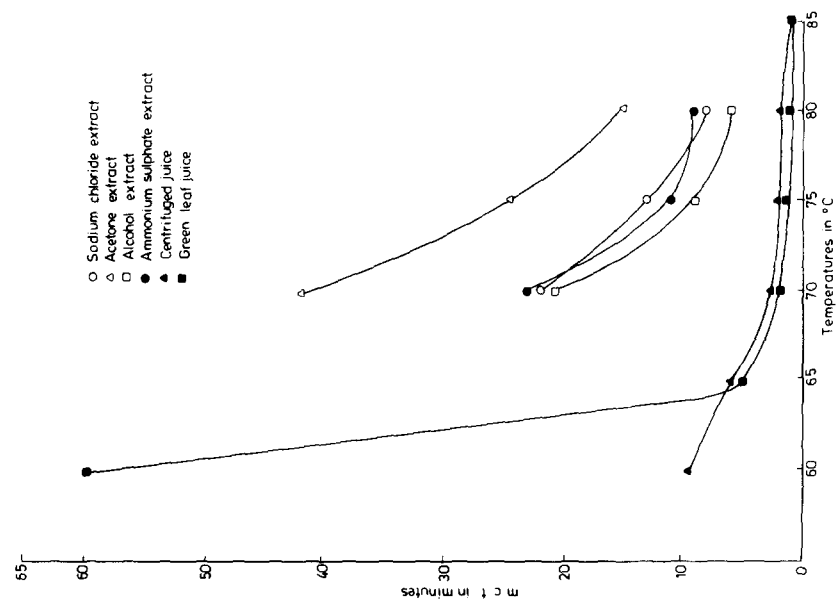

Fig. 2(a). Effect of temperature on the milk clotting time of sodom apple extracts at pH 6·6 of substrate.

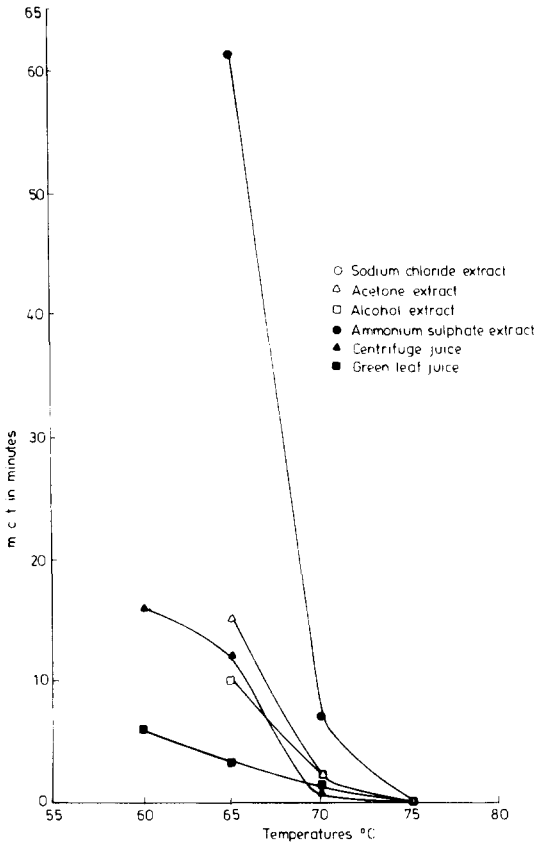

Fig. 2(c). Effect of temperature on the milk clotting time of sodom apple extracts at pH 5·7 of substrate.

mct for all enzyme extracts decreased steeply until they reached a minimum at 80 °C.

Fig. 3 shows the effect of extract concentration on the clotting. The clotting time varies inversely with the concentration, ranging from 67 min with 0·5 ml to 22 min with 2·5 ml. On the other hand, yield of wara increased from 28·7 % with 0·5 ml to a maximum of 33·7 % at 1·5 ml and then dropped to 30·5 % with 2·5 ml (Fig. 4).

The composition of the whey solids at 70–72 °C tended to be constant, irrespective of the concentration of the extract used, except for the protein content which increased from 10·4 % at 0·5 ml to 11·4 % at 2 ml; the total solids also increased from 5·9 % at 0·5 ml to 6·7 % at 2·5 ml (Fig. 5). The

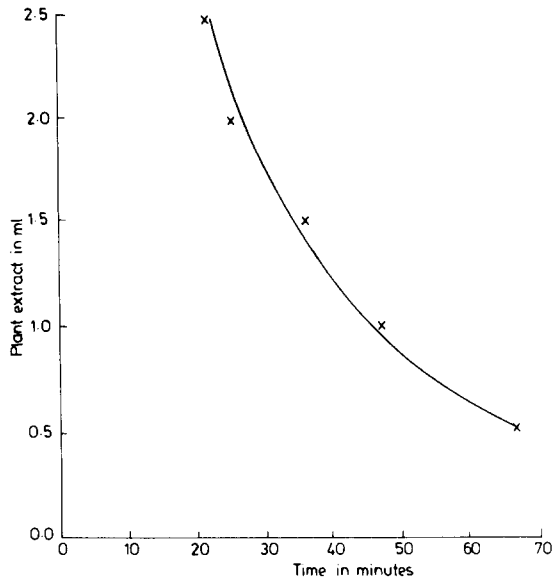

Fig. 3. The volume of sodom apple juice (ml) versus milk clotting time at 70 °C. (Nitrogen content of juice = 0·097 %, total solids of juice = 2·31 %, crude nitrogen on dry basis = 4·2 %.)

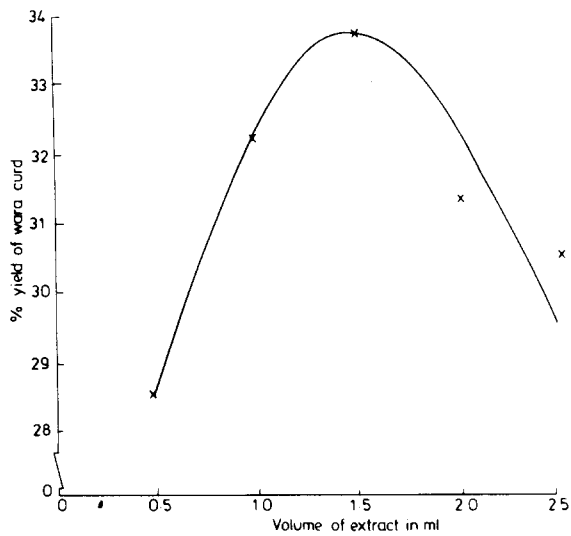

Fig. 4. The volume of sodom apple juice (ml) versus the percentage yield of wara curd at 70 °C.

same effect was observed at 95 °C. The composition of the wara solids is the opposite; the protein content decreased from 48·8 % to 46·6 %; the total solids also decreased, from 30·1 % to 25·1 %. The fat content decreased from 44·3 to 42·3 % (Fig. 6).

Table 1 gives the composition of the pooled milk used for the experiments. Like other analyses it consists mainly of water (87 %) with lactose (4·6 %) as the main carbohydrate component. The Table also

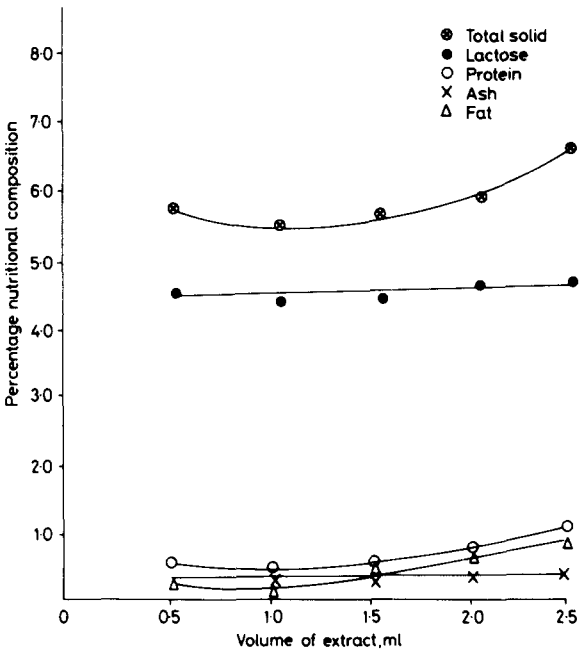

Fig. 5. Percentage composition of whey at 70–72 °C versus extract concentration.

shows the changes in nutrients when milk is made into wara. The most dramatic change is the disappearance of the lactose which, especially with the sour wara, was reduced from 4·6 % to 0·2 %. The protein content was increased fourfold (from 3·6 % to 14·8 %). The fat content was increased threefold (4·3 to 13·5 %), irrespective of the concentration of the enzyme. However, at the highest concentration (2·5 ml), the protein content was decreased (14·8 % at 0·5 ml and 12·9 % at 2·5 ml), as was the fat (13·5 % and 11·6 %, respectively) and total solids (30·6 % and 26·7 %, respectively).

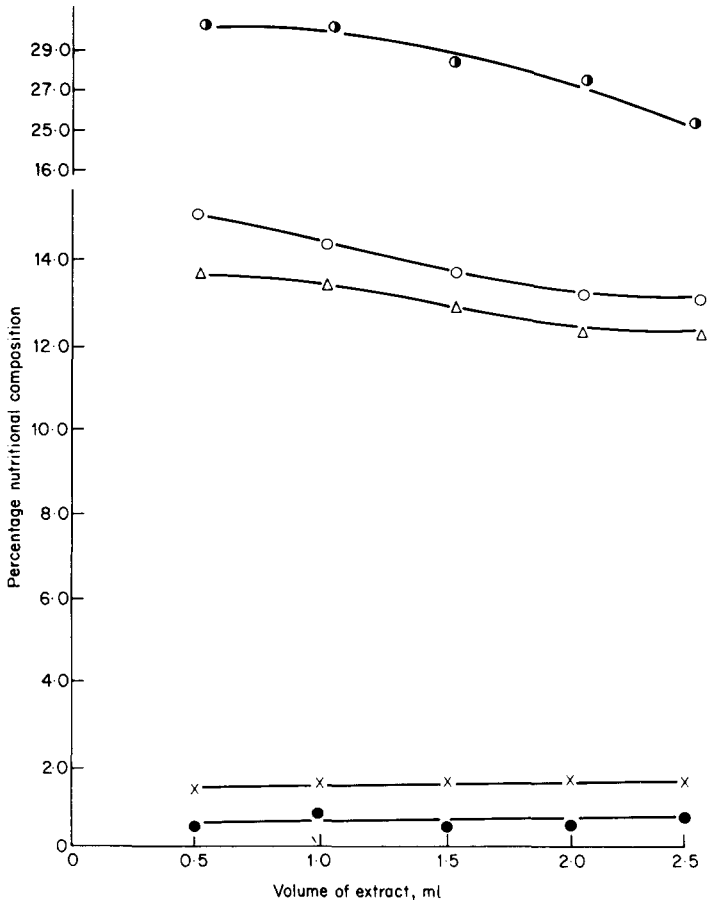

Fig. 6. Percentage composition of wara curd at 70–72 °C versus extract concentration. ◑, Total solid; ○, protein; △, fat; ×, ash; ●, lactose. Extract, M, g % = 4·2 on dry basis.

DISCUSSION

The colour, texture and taste of wara are important quality attributes to consumers and so to establish a range of concentration of plant extracts suitable for production, some trial runs were carried out.

The observations made in this study are that all enzyme preparations from the leaves of sodom apple (*Calotropis procera*) have strongest activities at temperatures above 70 °C and at pH 5·6–6·0. This is in agreement with the findings of other workers such as Richardson *et al.*

(1967), Reps *et al.* (1970). and Vieira De Sa & Manuelo Barbosa (1972) who made some observations on the milk-clotting activities of enzymes other than animal rennet. Reps *et al.* (1970) reported the milk optimum flocculation time for proteases from *Endothea parasitica* and *Byssochlamys fulva* (both fungal rennets) to be at 62–63 °C and 64–66 °C, respectively whilst, in an experiment with cardo extracts, Vieira De Sa & Manuelo Barbosa (1972) observed that the milk clotting time was shortest at 70 °C. Our enzyme preparations from sodom apple exhibited the shortest clotting time at 70–80 °C.

Eskin & Landman (1975) reported pH 5·5 as the optimum for the clotting activity of enzyme from *Benincasa cerifera*, while Berkowitz-Hundert *et al.* (1964) reported pH 5·4 for the strongest activity of enzymes from *Ficus carica* and the seeds of *Ricinus communis*. These workers also observed that the activity decreases steeply with rising pH, as was the case with sodom apple extract.

Our results show a progressive decrease in the mct with increase in the concentration of the crude enzyme at pH 6·6. At a concentration of juice below 0·2 % v/v the mct took over 1 h at 68 °C while it was only 12–13 min at concentrations above 1·3 % v/v. However, at such high concentrations, the cheese curd becomes progressively bitter in taste. Vieira De Sa & Manuelo Barbosa (1972) had earlier reported that increased quantities of vegetable rennet from Cardon (*Cynara cardunculus*) led to a great reduction in clotting time at higher pH, whereas the same increase at pH 5·8 led to only a moderate decrease in clotting time. However, with the enzyme from fig extract, Krishnamurti & Subrahmanyan (1953) observed that for the digestion of aqueous casein solution, no order is followed consistently.

The process of manufacture of wara is very simple and lends itself to easy adaptable modern processing controls, as shown in Fig. 7. The milk coagulator is derived from a local vegetable and it needs no specially prepared starter culture for its milk clotting activity. The duration of the process is short (65 min) compared with the processes for other types of cheese. Therefore more runs of wara can be made per given period of time even with rural preparative techniques.

The cheese curd obtained had a titratable acidity of 0·05 %. However, the titratable acidity of most commercial wara ranges from 0·2 % to 0·27 % and the pH may be as low as 4·7. The increase in acidity may probably be a result of post-processing contamination by the lactic organisms.

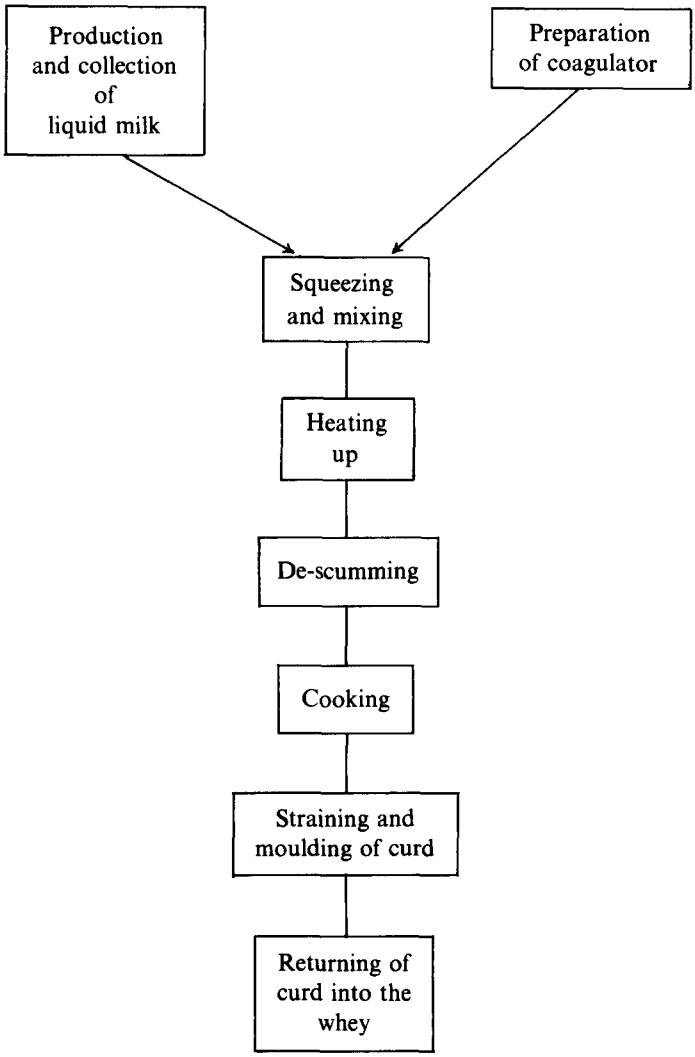

Fig. 7. Process flow chart of household manufacture of warankasi.

In general, the traditional processors do not consider all the necessary process and quality controls, as a result of which the finished products lack the consistent quality attributes which are characteristic of cheese products from conventional processes.

This study has highlighted some of the factors involved in the manufacturing process of wara as it is being practised by village women in Nigeria with an eventual upgrading of the technology by further research.

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Use of Cysteine as a Reducing Agent During Alkali Treatment of Proteins. Biochemical and Nutritional Effects

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(Received: 9 May, 1982)

ABSTRACT

*Alkali treatment of casein, in the presence of 0·1*N *or 0·2*N *sodium hydroxide, at 80°C for 1 h, leads to a loss of amino acids and to the formation of lysinoalanine; in the rat, growth, protein efficiency ratio and nitrogen digestibility are decreased. The addition of cysteine (6·1 g/100 g casein) as a reducing agent during the alkali treatment of casein avoids the formation of lysinoalanine and the harmful biological effects of alkali treatment. Sulphur amino acid (*L*-cysteine or* DL*-methionine) supplementation of treated casein, added after the alkali treatment, is without beneficial effect.*

INTRODUCTION

Lysinoalanine, *N*ε-(DL-2-amino-2-carboxyethyl)-L-lysine, is formed on alkaline treatments (Bohak, 1964) or heat treatments of proteins (Sternberg *et al.*, 1975). Lysinoalanine ingestion induces renal alterations in rat, with the appearance of cytomegalic, polyploid and karyomegalic cells in the proximal tubule (Woodard & Short, 1973, 1977; Woodard *et al.*, 1975; Gould & MacGregor, 1977; Karayiannis *et al.*, 1979). Other species, such as mice, hamsters, rabbits, quail, dogs and monkeys, are not sensitive

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to the nephrotoxic effects of lysinoalanine (De Groot *et al.*, 1976) and it seems that the renal lesions are reversible in rats (Struthers *et al.*, 1978). However, the mode of action of lysinoalanine is not well known and the possible effects in man are still not established. It is thus preferable to avoid or reduce the formation of lysinoalanine during alkali or heat treatments. For this purpose many methods were proposed; namely, the addition of reducing agents during alkali treatment (Friedman, 1975), such as cysteine (Husseini, 1976; Finley *et al.*, 1978; Finley & Kohler, 1979), or the previous acylation of proteins with acetic or succinic anhydrides (Friedman, 1978). On the other hand, the nutritional value of alkali-treated proteins is reduced (De Groot & Slump, 1969; Karayiannis *et al.*, 1979). This may be the result of amino acid destructions (De Groot & Slump, 1969; Provansal *et al.*, 1975), of amino acid isomerisations (Provansal, 1974) and of the formation of unusual compounds, besides lysinoalanine, such as ornithine, ornithinoalanine and alloisoleucine (Provansal, 1974). Consequently, in addition to the nephrotoxicity of lysinoalanine, some essential amino acids may become unavailable.

The aim of the present work was to show the effects of an addition of cysteine, either (first assay) as a reducing agent, before an alkali treatment of casein, in order to avoid the lysinoalanine formation, or (second assay) as a nutritional supplementation, after the alkali treatment, in order to balance the losses of cysteine. The addition of methionine was also assayed and compared with the effects of cysteine addition. In each of these cases, nutritional tests were performed on growing rats.

MATERIALS AND METHODS

Alkali treatment of casein and addition of cysteine

Casein was purchased from Merck (casein nach Hammarsten). Casein solutions, 10 % w/v, were stirred for 1 h at 80 °C in the presence of 0·1 N or 0·2 N sodium hydroxide. Then, after an adjustment to pH 7 with 6 N hydrochloric acid, the treated casein solution was immediately freeze-dried. Other batches of casein were prepared in the same manner with 0·2 N NaOH, but L-cysteine (Merck) was added, at a level of 6·1 g/100 g of protein, before alkali treatment. The added quantity of cysteine was equivalent to the molar content of lysine in casein.

Analytical methods

Amino acid analysis
Proteins were hydrolysed with 6N hydrochloric acid at 110 °C for a period of 24 h under a nitrogen atmosphere. Amino acid analysis was carried out on a Technicon NC 1 autoanalyser, using a single column of Chromobeads A resin (length, 140 cm; diameter, 0·6 cm) maintained at 60 °C.

Nitrogen analysis
Nitrogen content of foods, faeces and urines was determined by the Kjeldahl method, using a Bouat-Crouzet apparatus for distillation.

Diet for animals

The basal diet, provided by UAR (91300 Villemoisson-sur-Orge, France), contained 5 % glucose monohydrate, 70 % wheat starch, 8 % fats (maize and peanut oils, lard), 5 % cellulose, 11 % minerals and 1 % vitamins. The salt mixture contained, in grams per 100 grams of mix: $CaHPO_4$: 43; KCl: 10; NaCl: 10; $MgCl_2$: 5; $MgSO_4$: 5; Fe_2O_3: 3; $FeSO_4 . 7H_2O$: 5; $ZnSO_4 . 7H_2O$: 2; $MnSO_4 . H_2O$: 2·45; $CuSO_4 . 5H_2O$: 0·5; $CoSO_4 . 7H_2O$: 0·004; KI: 0·008 and cellulose up to 100 g. The vitamin mix supplied, per 100 grams of mix, 198 000 IU retinyl acetate, 60 000 IU cholecalciferol, 1·7 g DL-α-tocopherol acetate, 13·6 g choline-HCl, 1 g nicotinic acid, 8 g L ascorbic acid, 1·5 g myoinositol, 0·4 g menadione, 0·2 g thiamine-HCl, 0·15 g riboflavine, 0·1 g pyridoxine HCl, 0·7 g Ca-pantothenate, 50 mg folic acid, 3 mg biotin and 500 mg *p*-amino benzoic acid.

The experimental diets were made up of the basal diet and casein, alkali-treated or not, as the sole protein source. The final protein content (N × 6·25) was always 10 %, except in an experiment of the second assay, where the protein content reached 12 %. Additions of cysteine or methionine are indicated below and in Tables 2 and 4.

Animals and conduct of experiments

Male Sprague–Dawley rats, specific pathogen free, were purchased from IFFA-CREDO breeding (69210 L'Arbresle, France). The animals, weaned and raised to 4 weeks of age at the beginning of experiments, were placed in individual metabolic cages and submitted to a 12-h photoperiod, at a controlled temperature of 25 ± 1 °C. The cages allowed

individual control of food intakes and quantitative collection of faeces and urines separately. Each group contained seven rats. Two series of experiments were performed; the diets and treatments were constituted as follows.

First assay

Group 1: Untreated casein; protein level, 10%.
Group 2: 0·1N NaOH treated casein; protein level, 10%.
Group 3: 0·2N NaOH treated casein; protein level, 10%.
Group 4: L-cysteine added (6·1%) and 0·2N NaOH treated casein; protein level, 10%.

Second assay

Group 5: Untreated casein; protein level, 10%.
Group 6: 0·2N NaOH treated casein; protein level, 10%.
Group 7: 0·2N NaOH treated casein; protein level, 12%.
Group 8: Untreated casein, DL-methionine supplementation: 0·7 g/100 g casein; protein level, 10%.
Group 9: 0·2N NaOH treated casein, DL-methionine supplementation: 1·4 g/100 g casein; protein level, 10%.
Group 10: 0·2N NaOH treated casein, L-cysteine supplementation: 3·35 g/100 g casein; protein level, 10%.

The experimental diets were prepared each day and the rats were fed *ad libitum*. The daily food intake was recorded for each animal, taking account of the dry matter of the refused foods. Rats were weighed every 2 days. The length of assays was 21 days; during the last 7 days, faeces and urines were quantitatively collected. The growth rate in grams per day and the protein efficiency ratio (PER: body weight gain in grams per gram of ingested protein, during 21 days) were calculated. The digestibility (D) of nitrogen and the biological value (BV) were determined according to:

$$D = \frac{I - F}{I} \times 100 \qquad \text{BV} = \frac{I - (F - F_e) - (U - U_e)}{I - (F - F_e)} \times 100$$

where I, F and U represent, respectively the nitrogen intake and the faecal and urinary nitrogen during the last 7 days. Corrections for endogenous faecal (F_e) and urinary (U_e) nitrogen were estimated from the results obtained with animals fed a protein-free diet.

At the end of the experiment, animals were sacrificed with diethyl-ether; kidneys, liver and spleen were rapidly removed and immediately weighed.

RESULTS

Amino acid composition of alkali-treated casein (Table 1)

Alkali treatment leads to a disappearance of cysteine, arginine, serine, lysine and threonine and to the formation of lysinoalanine; the content of lysinoalanine reaches, respectively, 0·7 % and 2·1 % in the 0·1N and 0·2N

TABLE 1
Amino Acid Composition of Casein Before and After Alkali Treatment With or Without Addition of Cysteine

Amino acid as per cent of the sum of amino acids	*Untreated casein* 1	*0·1N NaOH 80°C, 1 h* 2	*0·2N NaOH 80°C, 1 h* 3	*0·2N NaOH 80°C, 1 h with cysteine** 4
Methionine sulphoxide	0·1	0·3	—	0·2
Methionine sulphone	0	0	0	—
Aspartic acid	6·6	7·4	6·5	6·0
Threonine	3·9	3·3	3·2	3·5
Serine	5·2	4·3	3·5	4·7
Glutamic acid	20·8	21·8	21·9	20·2
Proline	10·7	10·3	10·6	9·8
Glycine	1·8	1·8	1·8	1·7
Alanine	2·7	2·8	2·9	2·7
Valine	5·7	5·9	5·9	5·9
1/2 Cystine	0·4	0·1	0·2	7·8
Methionine	2·8	2·5	2·7	2·4
Iso-leucine	5·1	5·1	5·3	4·1
Leucine	8·7	9·5	9·5	8·6
Tyrosine	5·2	5·3	5·4	5·1
Phenylalanine	4·9	5·1	5·0	4·6
Lysinoalanine	0	0·7	2·1	0
Lysine	7·5	7·8	6·9	6·8
Histidine	2·5	2·8	2·8	2·6
Arginine	5·5	4·4	3·8	3·3

* Addition of cysteine before the alkali treatment; 6·1 g L-cysteine/100 g casein.

NaOH-treated casein. Addition of cysteine before the treatment avoids the formation of lysinoalanine and reduces the losses of other amino acids; however, arginine and lysine contents remain at a lower level. According to the pattern of the reference protein (FAO/WHO, 1973) and neglecting the possible isomerisations, sulphur amino acids are the limiting amino acids in untreated and 0·1N NaOH-treated casein, whereas threonine is the first limiting amino acid in the 0·2N NaOH-treated casein, with or without the addition of cysteine.

Nutritional effects of cysteine addition, before treatment (first assay, Table 2)

Mean initial weight of rats is 103 g. The growth is about 4·8 g/day in the control rats fed untreated casein and is the same in the group receiving 0·1N NaOH-treated casein. After 0·2N NaOH treatment, casein permits only a growth of 1 g/day; addition of cysteine before the treatment has a beneficial effect, since growth rate is restored to a value of 6·2 g/day. Moreover, food consumption depressed in group 3, fed alkali treated casein, is significantly enhanced in group 4 and reaches 16·5 g dry matter a day, against 10·3 g/day in the group 3 and 14·3 g/day in the control rats.

TABLE 2
Nutritional Value of Alkali-treated Casein in Rats (First Assay)
(Effect of cysteine addition before alkali treatment)

Group	*Growth (g/21 days)*	*Total dry matter intake (g/21 days)*	*Protein efficiency ratio (21 days)*	*Digestibility*		*Biological value (%)*
				Dry matter (%)	*Nitrogen (%)*	
1	$100 \pm 1{\cdot}9^{a}$	$300 \pm 5{\cdot}0^{a}$	$3{\cdot}37 \pm 0{\cdot}06^{a}$	$88{\cdot}6 \pm 0{\cdot}5^{a}$	$84{\cdot}2 \pm 0{\cdot}7^{a}$	$76{\cdot}7 \pm 1{\cdot}2^{a}$
2	$100 \pm 1{\cdot}8^{a}$	$317 \pm 4{\cdot}0^{ab}$	$3{\cdot}11 \pm 0{\cdot}04$	$90{\cdot}4 \pm 0{\cdot}5^{a}$	$86{\cdot}5 \pm 0{\cdot}7^{a}$	$80{\cdot}2 \pm 0{\cdot}9^{a}$
3	$25 \pm 2{\cdot}0$	$216 \pm 3{\cdot}5$	$1{\cdot}14 \pm 0{\cdot}06$	$89{\cdot}8 \pm 0{\cdot}7^{a}$	$71{\cdot}7 \pm 0{\cdot}6$	$66{\cdot}4 \pm 3{\cdot}1^{b}$
4	$130 \pm 2{\cdot}3$	$346 \pm 12{\cdot}1^{b}$	$3{\cdot}44 \pm 0{\cdot}04^{a}$	$90{\cdot}9 \pm 0{\cdot}4^{a}$	$88{\cdot}8 \pm 0{\cdot}2^{a}$	$74{\cdot}2 \pm 5{\cdot}4^{ab}$

Values are means ±SEM. Means with the same superscript letters are not significantly different ($P < 0{\cdot}01$; Student's *t*-test).
Seven male rats per group; mean initial weight: 103 g.
Group 1: Untreated casein; protein level, 10%.
Group 2: 0·1N NaOH-treated casein; protein level, 10%.
Group 3: 0·2N NaOH-treated casein; protein level, 10%.
Group 4: L-cysteine-added (6·1%) and 0·2N NaOH-treated casein; protein level, 10%.

Protein efficiency ratio is as low as 1·14 in group 3, fed treated casein, and is restored to a value of 3·44 if cysteine is added before the treatment, a value which is not significantly different from that observed in the control group, i.e. 3·37. In group 2, the reduced protein efficiency ratio is related to a slight increase in food consumption. Biological value is only reduced to 66 % in group 3. Dry matter digestibility is not affected by the different treatments, whereas protein digestibility is highly and significantly reduced after a 0·2N NaOH treatment of casein and entirely restored if cysteine is added before alkali treatment.

Variations are shown in the relative organ weights (Table 3), because live weights of animals are quite different. However, it can be observed that ingestion of cysteine-added and alkali-treated casein (group 4) leads to a mild hypertrophy of liver and kidneys, but the relative weights do not exceed 115 % of the control values.

TABLE 3

Relative Organ Weights (grams of organ per 100 g live weight) in Rats Fed Alkali-treated Casein

(Effect of cysteine addition before alkali treatment)

Group	*Final weight of rats (g)*	*Kidneys (g/100 g)*	*Liver (g/100 g)*	*Spleen (g/100 g)*
1	$203 \pm 5{\cdot}7^{a}$	$0{\cdot}72 \pm 0{\cdot}05^{a}$	$4{\cdot}74 \pm 0{\cdot}14^{a}$	$0{\cdot}26 \pm 0{\cdot}01^{ab}$
2	$203 \pm 4{\cdot}5^{a}$	$0{\cdot}73 \pm 0{\cdot}01^{a}$	$4{\cdot}84 \pm 0{\cdot}04^{a}$	$0{\cdot}23 \pm 0{\cdot}01^{ab}$
3	$128 \pm 3{\cdot}4$	$0{\cdot}87 \pm 0{\cdot}03^{b}$	$4{\cdot}99 \pm 0{\cdot}06^{ab}$	$0{\cdot}22 \pm 0{\cdot}01^{b}$
4	$228 \pm 7{\cdot}2$	$0{\cdot}81 \pm 0{\cdot}01^{b}$	$5{\cdot}53 \pm 0{\cdot}05^{b}$	$0{\cdot}27 \pm 0{\cdot}01^{a}$

Values are means ±SEM. Means with the same superscript letters are not significantly different ($P < 0{\cdot}01$; Student's *t*-test). Seven male rats per group; for explanations of treatments, see Table 2.

Nutritional effects of sulphur amino acid supplementation, after alkali treatment (second assay, Table 4)

Mean initial weight of rats is 88 g. The growth is 3·7 g/day in the control rats fed untreated casein; supplementation with 0·7 % methionine leads to a better growth, i.e. 5·0 g/day. At a protein level of 10 % in the diet, treated casein, supplemented or not, does not permit a normal growth, which is between 0·2 and 0·4 g/day. At a 12 % protein level, growth is increased,

TABLE 4
Nutritional Value of Alkali-treated Casein in Rats (Second Assay)
(Effect of sulphur amino acid supplementations)

Group	*Growth (g/21 days)*	*Total dry matter intake (g/21 days)*	*Protein efficiency ratio (21 days)*	*Digestibility*		*Biological value (%)*
				Dry matter (%)	*Nitrogen (%)*	
5	$78 \pm 6{\cdot}0$	$272 \pm 4{\cdot}1$	$2{\cdot}87 \pm 0{\cdot}13$	$89{\cdot}6 \pm 1{\cdot}5^a$	$86{\cdot}3 \pm 0{\cdot}3^a$	$81{\cdot}8 \pm 0{\cdot}7^a$
6	$8 \pm 2{\cdot}0^a$	$146 \pm 3{\cdot}3^a$	$0{\cdot}53 \pm 0{\cdot}14^a$	$87{\cdot}6 \pm 1{\cdot}9^a$	$54{\cdot}4 \pm 0{\cdot}9^b$	$57{\cdot}5 \pm 3{\cdot}4^b$
7	$15 \pm 1{\cdot}4^a$	$155 \pm 2{\cdot}8^a$	$0{\cdot}98 \pm 0{\cdot}08$	$88{\cdot}1 \pm 1{\cdot}6^a$	$70{\cdot}1 \pm 1{\cdot}0$	$58{\cdot}0 \pm 3{\cdot}8^b$
8	$106 \pm 3{\cdot}9$	$312 \pm 3{\cdot}7$	$3{\cdot}51 \pm 0{\cdot}06$	$89{\cdot}3 \pm 1{\cdot}6^a$	$84{\cdot}9 \pm 0{\cdot}4^a$	$79{\cdot}3 \pm 0{\cdot}8^a$
9	$5 \pm 1{\cdot}1^a$	$148 \pm 3{\cdot}9^a$	$0{\cdot}36 \pm 0{\cdot}08^a$	$84{\cdot}9 \pm 1{\cdot}3^a$	$59{\cdot}7 \pm 1{\cdot}0^b$	$54{\cdot}4 \pm 2{\cdot}3^b$
10	$5 \pm 1{\cdot}1^a$	$136 \pm 2{\cdot}8^a$	$0{\cdot}41 \pm 0{\cdot}17^a$	$85{\cdot}1 \pm 1{\cdot}0^a$	$52{\cdot}5 \pm 1{\cdot}1^b$	$55{\cdot}8 \pm 3{\cdot}8^b$

Values are means ±SEM. Means with the same superscript letters are not significantly different ($P < 0{\cdot}01$; Student's *t*-test).
Seven male rats per group; mean initial weight: 88 g.
Group 5: Untreated casein; protein level, 10%.
Group 6: 0·2N NaOH-treated casein; protein level, 10%.
Group 7: 0·2N NaOH-treated casein; protein level, 12%.
Group 8: Untreated casein, methionine supplementation 0·7%; protein level, 10%.
Group 9: 0·2N NaOH-treated casein, methionine supplementation 1·4%; protein level, 10%.
Group 10: 0·2N NaOH-treated casein, cysteine supplementation 3·35%; protein level, 10%.

but not significantly, to 0·7 g/day. Food intake varies between the different groups in the same way as growth. In group 8, after methionine supplementation, food consumption is significantly higher than in the control group. With alkali-treated casein, food intake is always reduced by about half.

Methionine supplementation leads to an increase in the protein efficiency ratio from 2·87 to 3·51. However, sulphur amino acid supplementation remains without effect on alkali-treated casein, whereas an increase in protein level to 12% permits an improvement of the protein efficiency ratio. The biological value remains low in groups fed alkali-treated casein, even after amino acid supplementation, and is only slightly enhanced at a 12% protein level. Dry matter digestibility values are not different between groups; however, protein digestibility is very low with alkali-treated casein and is slightly increased at a 12% protein level. Amino acid supplementation does not modify nitrogen digestibility.

TABLE 5
Relative Organ Weights (grams of organ per 100 g live weight) in Rats Fed Alkali-treated Casein
(Effect of sulphur amino acid supplementation)

Group	*Final weight of rats (g)*	*Kidneys (g/100 g)*	*Liver (g/100 g)*	*Spleen (g/100 g)*
5	$172 \pm 6{\cdot}8$	$0{\cdot}87 \pm 0{\cdot}01^{a}$	$4{\cdot}87 \pm 0{\cdot}14^{ab}$	$0{\cdot}23 \pm 0{\cdot}01^{a}$
6	$95 \pm 4{\cdot}5^{a}$	$1{\cdot}11 \pm 0{\cdot}01^{b}$	$5{\cdot}60 \pm 0{\cdot}10^{a}$	$0{\cdot}25 \pm 0{\cdot}01^{a}$
7	$104 \pm 3{\cdot}4^{a}$	$1{\cdot}15 \pm 0{\cdot}01^{bc}$	$5{\cdot}30 \pm 0{\cdot}06^{ab}$	$0{\cdot}24 \pm 0{\cdot}01^{a}$
8	$201 \pm 5{\cdot}3$	$0{\cdot}81 \pm 0{\cdot}01^{a}$	$4{\cdot}68 \pm 0{\cdot}08^{a}$	$0{\cdot}28 \pm 0{\cdot}02^{a}$
9	$92 \pm 2{\cdot}6^{a}$	$1{\cdot}26 \pm 0{\cdot}02^{c}$	$5{\cdot}40 \pm 0{\cdot}08^{ab}$	$0{\cdot}27 \pm 0{\cdot}01^{a}$
10	$95 \pm 3{\cdot}0^{a}$	$1{\cdot}20 \pm 0{\cdot}01^{c}$	$5{\cdot}00 \pm 0{\cdot}11^{ab}$	$0{\cdot}27 \pm 0{\cdot}01^{a}$

Values are means $\pm$SEM. Means with the same superscript letters are not significantly different ($P < 0{\cdot}01$; Student's *t*-test).
Seven male rats per group; for explanations of treatments, see Table 4.

Ingestion of alkali-treated casein leads to a renal hypertrophy, which is more marked after amino acid supplementation, and to a slight liver hypertrophy (Table 5). Spleen relative weight is not modified by the treatments.

DISCUSSION

Chemical modifications, observed here after alkali treatments of casein, are in good agreement with the results of Provansal (1974) and Provansal *et al.* (1975). Lack of lysinoalanine formation in the presence of cysteine confirms the observations from Husseini (1976), Finley *et al.* (1978) and Finley & Kohler (1979). However, initial values of lysine and arginine content are unable to be recovered after the addition of cysteine. Alkali treatments lead to modifications of the protein structure. In this way, we observed (unpublished results) that, after a G 75 Sephadex gel chromatography, the pattern of elution was the same for untreated casein and for 0·1N sodium hydroxide-treated casein. After a 0·2N sodium hydroxide treatment, a significant peak is present in the area of molecular weight 3000; if cysteine is added before treatment, some peptides of low molecular weight appear.

Neutralisation of sodium hydroxide by hydrochloric acid leads to the

formation of sodium chloride, which is totally present in the food given to rats. After a treatment in the presence of 0·1N or 0·2N sodium hydroxide, supplemental sodium chloride represents from 0·1% to 0·3% in the diet and the final content is 1·2% in the dry matter of the diet. According to Drori (1976), food intake level is depressed in rats only when sodium chloride reaches a level of 2% in the diet. On the other hand, a sodium chloride supplementation assay, performed in our laboratory, showed that food intake is slightly reduced only after 17 days of assay, when sodium chloride was as high as 1·5% in the diet. Thus, in the present experiments, there is no significant risk of sodium imbalance.

Casein, which is treated by 0·1N sodium hydroxide, does not modify the growth, in spite of the presence of lysinoalanine. In fact, according to Slump (1978), lysinoalanine must be released by the action of digestive enzymes and absorbed, in order that toxicity can be expressed. So, in group 2, lysinoalanine may be absorbed in low quantity. In the case of rats receiving casein treated with 0·2N sodium hydroxide (group 3), depression of food consumption and growth may be the consequence of lysinoalanine as well as the presence of inhibitors of trypsin and chymotrypsin, which are found after such alkaline treatments (Possompes *et al.*, 1979; Vimont-Rispoli *et al.*, 1980). It was shown, in our laboratory, that such protease inhibitors are able to depress food consumption in some conditions; young rats seem to be more sensitive. These observations can explain the slight differences between groups 3 and 6, in the two series of experiments. In the latter (group 6) rats were younger and growth, protein efficiency ratio and protein digestibility were all slightly less than in group 3.

Thus, the poor digestibility of alkali-treated proteins, already observed by De Groot & Slump (1969) and Karayiannis *et al.* (1979), is the consequence of crosslinks formation, of amino acid isomerisation and of the presence of trypsin and chymotrypsin inhibitors. Consequently, as we observed (unpublished results) in rats fed 0·2N sodium hydroxide-treated casein, faeces contain peptides and proteins, the molecular weights of which were between 3000 and 30 000. Such peptidic and protein fractions disappear if casein is treated with cysteine before the alkali treatment. The formation of protease inhibitors may be linked to the presence of covalent linkages which appear during alkali treatments; therefore, rigid structures, which are formed, are able to inhibit the serine-enzymes (Laskowski & Kato, 1980).

The good nutritional value of casein, which is alkali-treated in the

presence of a reducing agent, cysteine, suggests that non-peptidic covalent linkages and isomerisations of amino acids are limited in these conditions. De Rham *et al.* (1977) explain the low nutritional value by a loss of amino acids, such as cysteine, which are destroyed during the treatment. Unlike these authors, we cannot observe any improvement of nutritional value by an amino acid supplementation after the treatment. Thus, chemical and structure modifications are probably the main causes of low nutritional value and these modifications must be avoided during treatment.

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Total Volatile Acids Content as a Quality Index of Irradiated Hake (*Merluccius merluccius hubbsii*) Fillets

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(Received: 27 April, 1982)

ABSTRACT

Irradiation of hake (Merluccius merluccius hubbsii) *fillets with 500 krad (5 kGy) of* Co^{60} *gamma rays caused a delay in the formation of volatile acids during storage under refrigeration at 4–5 °C. Loss in organoleptic acceptability could be correlated with the increase in volatile acids both in the unirradiated and irradiated fish stored at 4–5 °C, suggesting that the volatile acids number (VAN) can be a useful tool for the quality evaluation of hake fillets.*

Short chain volatile acids like formic, acetic, iso-valeric and butyric acid have been reported accompanying fish flesh spoilage (Siguressom, 1947; Farber, 1952; Asakawa, 1953*a*, *b*, *c*; Suzuki, 1953*a*, *b*; Bullard *et al.*, 1978; Cooper & Cornforth, 1978). These acids have some influence on the odour and taste of fish foodstuffs (Reineccius, 1979; Venugopal *et al.*, 1981) and are products of several reactions like degradation, peroxidation and autoxidation of lipids and deamination of certain amino acids (Suzuki, 1953*b*; Gardnar, 1975; Curzio & Quaranta, 1982). Asakawa (1953*a*) and Venugopal *et al.* (1981) reported short chain volatile acids in tuna and Indian mackerel, respectively. Lipid distribution in the dark and light muscle of hake is well known and has been shown to be sensitive to degradation during processing of the fish. Irradiation of hake fillets has

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proved to be a good tool for shelf-life extension, when doses in the region of 500 krad (5 kGy) are given. The acceptability period of hake fillets stored at 4–5 °C was observed to be extended three times by irradiation (Curzio & Quaranta, 1982). Production of total volatile acids in hake fillets during storage at refrigeration temperature and its relationship to organoleptic scores of the fish was studied.

Hake came from fishing grounds in the South Atlantic Ocean (latitude 36° to 53° S), where they were caught by commercial vessels. After being kept on ice (three days) the fish were filleted on land and five-fillet blocks (500 g on average) were quick frozen to −30 °C in a plate freezer. They then were packaged in polyethylene and stored under dry ice until irradiation. Half of the batch was irradiated still under dry ice with 500 krad (5 kGy) Co^{60} gamma rays at the rate of 149 krad h^{-1} with a D_{max}/D_{min} ratio of 1·08. Both the irradiated and the control samples, in polyethylene packaging, were cold stored at 4–5 °C.

The determination procedure for total volatile acids was carried out, with slight modifications, according to Venugopal *et al.* (1981): triplicates of approximately 30 g of hake fillet muscle, representative of the whole sample, were homogenised with 90 ml distilled water. To these homogenates 12 ml 1N sulphuric acid (Merck) and 20 ml of 15% phosphotungstic acid (Hopkin and Williams, Ltd) solution were added. These mixes were allowed to stand for 15 min and were filtered to discard the precipitated protein. An aliquot (30 ml) of the filtrates was steam distilled for 15 min and the steam distillate (40 ml) was titrated against 0·01M sodium hydroxide (Merck). Volatile acids number (VAN) is expressed as ml of 0·01M alkali required to neutralise the volatile acids from 100 g of fish muscle.

Sensory evaluation of fish was carried out by a panel of five judges according to the methods described by Quaranta & Perez (1982) and using the nine-point hedonic scale (Miyauchi *et al.*, 1964).

The production of volatile acids during the course of refrigerated storage of irradiated and unirradiated hake fillets is shown in Fig. 1. The VAN value of fresh hake samples was very low and showed increase during storage at 4–5 °C. From Fig. 1 it can be seen that the increase in VAN was rapid in the unirradiated samples as compared with the irradiated ones, so it can be assumed that the exposure of hake to doses of radiation causes retardation of volatile acids production.

The fall in organoleptic score assessed on a nine-point hedonic scale during refrigerated storage of hake fillets was linear (Table 1). As

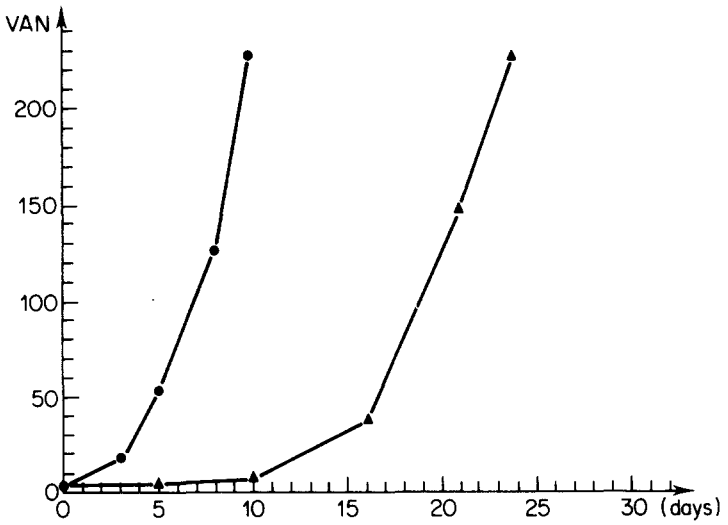

Fig. 1. Volatile acids formed during refrigerated storage of hake; (●) unirradiated, (▲) irradiated (500 krad).

compared with unirradiated fish, irradiated (500 krad (5 kGy)) fish had a longer acceptable shelf-life when stored at 4–5 °C. The panellists were also asked to rank the two products in order of preference (Quaranta & Perez, 1982); significant differences between the unirradiated and irradiated samples were observed after the third day of storage.

Spoilage of fish flesh was observed to be followed by the appearance of formic, acetic and butyric acids in the volatile fraction of the samples (Bullard *et al.*, 1978; Cooper & Cornforth, 1978; Venugopal *et al.*, 1981).

TABLE 1
Organoleptic Score of Irradiated and Control Hake During Storage at 4–5 °C

Storage period (days)	*Organoleptic score*	
	Unirradiated (Slope = −0·784 days^{-1})	*Irradiated (Slope = −0·154 days^{-1})*
0	8·0	8·0
3	5·5	7·0
7	2·5	7·0
12	—	6·5
20	—	5·0
25	—	3·8

Volatile acids production and loss in organoleptic scores of irradiated and unirradiated hake fillets stored under refrigeration were found to be correlated, so the results suggest that VAN can provide a reliable quality index for hake fillets. Some studies reported in the literature (Smith & Alford, 1968; Vyncke, 1970; Mariano & Kaupert, 1973; Curzio & Quaranta, 1982) have indicated that conventional chemical and microbiological indices of fish quality like oxidative rancidity, volatile bases content, volatile reducing substances and total bacterial count are useful for the quality evaluation of irradiated foodstuffs. The results of this experiment indicate also that volatile acids content can be used for freshness assessment of hake fillets.

ACKNOWLEDGEMENTS

Thanks are given to Centro de Investigaciones de Tecnología Pesquera for technical assistance and supply of samples and to Comisión Nacional de Energía Atómica for commissioning the irradiation process. This work was, in part, supported by Comisión de Investigaciones Científicas de la Provincia de Buenos Aires.

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Chemical Composition of Wild Mushrooms Collected From Alexandria, Egypt

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(Received: 16 September, 1982)

ABSTRACT

Fourteen samples of wild mushrooms, collected during the wintertime, were identified as Agaricus campestris, Agaricus rodmani *and* Collybia *sp. Moisture, crude protein, non-protein nitrogen, ether extract, total carbohydrates, crude fibre, vitamin C, ash and minerals (calcium, potassium, sodium, phosphorus, iron, copper and manganese) were determined. In addition, reducing sugars, non-reducing sugars, starch, glycogen and mannitol were determined. Variations in chemical constituents were found to be due to stage of development and type of mushroom.*

The different types of wild mushroom had similar distributions of amino acids. They contained all the essential amino acids in varying amounts.

INTRODUCTION

Mushrooms are plants without chlorophyll, so they are saprophytes and parasites. Mushrooms were known to early Greeks and Romans who divided the mushrooms into edible and poisonous kinds (Louis & Krieger, 1967). Less than twenty-five species of mushroom are widely accepted as food and only a few species have attained the level of an item of commerce (Smith, 1972).

Food Chemistry 0308-8146/83/0011-0031/$03·00

Hayes & Haddad (1976) stated that *Agaricus bisporus* is a relatively good source of protein, potassium, phosphorus, iron, thiamine, riboflavin and niacin. Mushrooms are low in calories, calcium and ascorbic acid and are devoid of vitamin A activity. The main chemical constituents of mushrooms are affected by type (Kurkela, 1972) and stage of development (Motskus, 1973). McConnell & Esselen (1947) reported that fresh mushrooms, *Agaricus bisporus*, contains 0·95% mannitol, 0·28% reducing sugars, 0·59% glycogen and 0·91% haemicellulose. On the other hand, Hammond & Nichols (1976) found that *Agaricus bisporus* had 2·4% glycogen (dry weight) at the immature button stage and 5·8% at the mature flat stage.

Mushroom proteins contain all the essential amino acids which may be influenced by a number of factors (Flegg & Maw, 1977) namely, the type of mushroom, the stage of development, the part of the mushroom sampled and the level of nutrient nitrogen.

No available data were found in the literature concerning wild mushrooms in Alexandria, Egypt. The present work was carried out to collect and identify the wild mushrooms from some locations in Alexandria, and to determine their nutritional value.

MATERIALS AND METHODS

Collection and identification of samples

Fourteen samples were collected from four different locations (three lawns and around salix trees on a farm in Alexandria during the winter (November to March)). Mushrooms at two stages of maturity were collected—button and incompletely open cap.

The wild mushrooms were identified according to Christensen (1965).

Preparation of samples

The freshly collected mushrooms were washed in running water, graded according to stage of development, i.e. button and incompletely open cap, and then dried at 80 °C for 8 h.

The samples were ground to pass through a 60 mesh sieve and stored in Kilner jars at 5 °C.

Analytical methods

Moisture, ether extract, reducing and non-reducing sugars (by the ferricyanide method), ash and phosphorus contents were determined using the standard methods of the Association of Official Analytical Chemists (AOAC, 1975). Vitamin C, crude fibre content and total nitrogen (semi-microKjeldahl method) were estimated as described by Pearson (1972). Non-protein nitrogen was determined according to the method of Kent-Jones & Amos (1967). Starch was hydrolysed by hydrochloric acid and the reducing sugar was determined by Lane and Eynon's method (Pearson, 1972).

Glycogen content was estimated colorimetrically according to the method described by Hassid & Abraham (1955). Mannitol content was determined by the colorimetric method described by Abdel-Akhar *et al.* (1965). Sodium and potassium were estimated in the ash solution using a Corning-EEL flame photometer according to Skoog & West (1963). Calcium, copper, manganese and iron were determined in the ash using a Shimadzv atomic absorption spectrophotometer.

The free, alcohol-soluble sugars were identified chromatographically in different samples according to Hughes *et al.* (1958). The alcohol-insoluble carbohydrates were hydrolysed by 2N sulphuric acid according to Hughes *et al.* (1958) and identified chromatographically as described by Partridge (1946).

Samples were hydrolysed for the determination of amino acids using acid and alkaline methods (Block *et al.*, 1958). The acid hydrolysate was subjected to a monodimensional descending technique of paper chromatography using butanol:acetic acid:water (144:13:34) as solvent according to Mikes (1966). The ninhydrin reagent of Roland & Gross (1954) was used to visualise the amino acids. The spots were identified and eluted using the methods of Majunders *et al.* (1956). The colour intensities of the eluate were measured colorimetrically at 515 nm for all amino acids except proline whose eluate was estimated at 450 nm. Tryptophan was determined colorimetrically in the alkaline hydrolysate, according to the method of Miller (1967).

TABLE 1
Chemical Composition of Various Species of Wild Mushroom (%)

	Development stage	Agaricus rodmani *1*	*2*	*3*	Agaricus campestris *1*	*2*	*3*	*4*	Agaricus campestris *1*	*2*	*3*	Collybia *sp.* *1*	*2*	*3*	*4*
Moisture	a	89·2	89·1	—	90·7	—	89·9	89·6	—	—	—	—	—	—	—
	b	—	—	88·4	87·5	87·1	86·8	86·5	90·1	89·9	89·9	91·2	90·7	90·5	90·4
Crude protein*	a	41·7	43·4	—	34·8	—	36·0	37·0	—	—	—	—	—	—	—
(N × 6·25)	b	—	—	39·8	32·1	32·7	33·0	33·3	38·0	40·5	41·6	28·0	28·9	30·2	30·5
Non-protein	a	0·7	0·9	—	0·5	—	0·6	0·7	—	—	—	—	—	—	—
nitrogen*	b	—	—	0·9	0·6	0·7	0·7	0·7	0·8	1·1	1·1	1·1	0·2	0·3	0·4
True protein*	a	37·5	38·1	—	31·9	—	32·1	32·4	—	—	—	—	—	—	—
(PN × 6·25)	b	—	—	33·8	28·7	28·3	28·5	28·8	33·0	33·8	34·6	27·1	27·7	28·1	28·2
Ether extract*	a	2·2	2·6	—	2·5	—	2·7	2·8	—	—	—	—	—	—	—
	b	—	—	2·3	2·7	2·6	2·9	3·0	2·3	2·0	2·2	4·3	4·0	4·2	4·3
Total carbohydrates*	a	34·0	31·5	—	40·6	—	39·7	38·8	—	—	—	—	—	—	
(by difference)	b	—	—	34·3	43·3	42·6	42·5	42·5	37·8	35·1	33·6	48·7	48·2	46·3	45·9
Crude fibre*	a	11·2	11·5	—	10·8	—	10·5	10·3	—	—	—	—	—	—	—
	b	—	—	12·3	10·5	10·8	10·4	10·2	10·2	10·5	10·6	9·6	9·4	9·6	9·5
Ash*	a	10·2	10·1	—	10·8	—	10·5	10·4	—	—	—	—	—	—	—
	b	—	—	10·4	10·8	10·6	10·5	10·3	10·9	10·8	10·9	9·3	9·3	9·4	9·4
Vitamin C	a	2·5	2·5	—	2·3	—	2·0	2·0	—	—	—	—	—	—	—
(mg/100 g fresh)	b	—	—	2·4	2·2	2·1	1·9	2·0	2·1	2·2	2·2	1·7	1·5	1·4	1·6

a = Button.
b = Open cap.
1—First break. 2—Second break. 3—Third break. 4—Fourth break.
* Calculated on dry weight basis.

RESULTS AND DISCUSSION

Collection and identification of wild mushrooms

The mushrooms were found in abundance (about 500 g samples) on lawns, particularly after heavy rain and when horse manure was added to the lawns. *Agaricus rodmani* was found in one location whilst *Agaricus campestris* (collected from two locations) is the common wild mushroom in Europe and America (Gray, 1977). These two species were collected from lawns and from different locations. The third type was found on (decayed) salix trees on a farm and identified as *Collybia* sp.

Chemical composition of wild mushrooms

Table 1 shows that the moisture content of wild mushrooms varied with the type of mushroom, and with stage of development. The crude protein content of *Agaricus* sp. was higher than that of *Collybia* sp. In addition, it varied widely according to location. Slight variations in crude protein content due to stage of development and number of breaks were noticed. Marked variations were found in the non-protein nitrogen content of various types of wild mushroom.

No published data were found on the protein content of *Agaricus rodmani* and *Collybia* sp.; values of crude protein and non-protein nitrogen contents of *Agaricus campestris* obtained in this study are in agreement with those reported by Szymczak (1972), Motskus (1973) and Zhuk *et al.* (1975).

Location, stage of development and number of flushes had no marked effect on the ether extract, crude fibre and ash contents of wild mushrooms. The crude fibre and ash contents of collected *Agaricus campestris* are similar to those reported by Hayes & Haddad (1976). The vitamin C content of the collected mushrooms was relatively low.

The data in Table 2 shows that reducing sugars increased with increasing age and number of flushes, in the studied species of mushrooms. Variations in non-reducing sugars content were similar to those reported for reducing sugars. The reducing sugars and non-reducing sugars were highest in *Agaricus campestris* and lowest in *Collybia* sp. These values are higher than those reported by Szymczak (1972).

No relationship was noticed between the starch content and location for *Agaricus campestris*. It decreased with increasing number of flushes.

TABLE 2
Carbohydrate Contents of Various Species of Wild Mushroom
(% on dry weight basis)

	Development stage	Agaricus rodmani			Agaricus campestris				Agaricus campestris			Collybia *sp.*			
		1	*2*	*3*	*1*	*2*	*3*	*4*	*1*	*2*	*3*	*1*	*2*	*3*	*4*
Reducing sugars	a	5·2	6·4	—	8·0	—	8·4	9·3	—	—	—	—	—	—	—
	b	—	—	9·1	9·2	9·4	9·6	9·8	6·8	6·9	7·3	4·5	4·8	5·7	5·8
Non-reducing sugars	a	5·2	5·4	—	6·3	—	6·8	7·5	—	—	—	—	—	—	—
	b	—	—	7·9	7·0	7·6	7·9	7·9	5·4	5·8	6·4	4·0	4·3	4·9	5·0
Starch	a	7·9	6·2	—	10·9	—	9·7	8·8	—	—	—	—	—	—	—
	b	—	—	6·5	13·1	12·3	9·3	8·5	12·7	12·5	11·7	23·8	21·6	21·3	17·2
Glycogen	a	12·2	12·0	—	5·3	—	5·3	5·1	—	—	—	—	—	—	—
	b	—	—	12·9	6·8	6·4	6·0	6·9	7·2	7·7	7·7	10·6	11·0	11·3	11·5
Mannitol	a	11·9	12·2	—	12·7	—	12·6	12·6	—	—	—	—	—	—	—
	b	—	—	11·3	12·0	11·8	15·6	12·3	9·4	8·5	8·5	5·4	5·8	5·5	5·8

a = Button.
b = Open cap.
1—First break. 2—Second break. 3—Third break. 4—Fourth break.

TABLE 3
Minerals Content of Various Species of Wild Mushroom
(On a dry weight basis)

	Development stage	Agaricus rodmani			Agaricus campestris				Agaricus campestris			Collybia *sp.*			
		1	*2*	*3*	*1*	*2*	*3*	*4*	*1*	*2*	*3*	*1*	*2*	*3*	*4*
Calcium (%)	a	0·20	0·18	—	0·25	—	0·26	0·22	—	—	—	—	—	—	—
	b	—	—	0·26	0·23	0·28	0·26	0·24	0·20	0·21	0·23	0·28	0·30	0·31	0·31
Potassium (%)	a	4·4	4·4	—	4·6	—	4·6	4·6	—	—	—	—	—	—	—
	b	—	—	4·5	4·9	4·9	4·9	4·8	4·3	4·3	4·3	5·1	5·1	5·1	5·1
Sodium (%)	a	0·68	0·70	—	0·86	—	0·88	0·91	—	—	—	—	—	—	—
	b	—	—	0·73	0·93	0·95	0·96	0·98	1·30	1·36	1·38	0·18	0·20	0·25	0·27
Phosphorus (%)	a	1·48	1·50	—	1·52	—	1·53	1·56	—	—	—	—	—	—	—
	b	—	—	1·62	1·50	1·55	1·53	1·51	1·58	1·62	1·59	0·83	0·87	0·84	0·84
Iron (mg/100 g)	a	16·8	19·0	—	18·3	—	20·8	22·0	—	—	—	—	—	—	—
	b	—	—	23·6	20·5	24·0	26·2	28·4	17·8	19·2	22·0	32·0	36·2	39·3	42·9
Copper (mg/100 g)	a	0·32	0·38	—	0·60	—	0·52	0·58	—	—	—	—	—	—	—
	b	—	—	0·34	0·55	0·58	0·56	0·58	0·44	0·42	0·48	0·70	0·78	0·72	0·77
Manganese (mg/100 g)	a	0·21	0·23	—	0·42	—	0·44	0·45	—	—	—	—	—	—	—
	b	—	—	0·26	0·42	0·46	0·46	0·47	0·34	0·35	0·38	0·12	0·15	0·16	0·13

a = Button.
b = Open cap.
1—First break. 2—Second break. 3—Third break. 4—Fourth break.

Glycogen content seems to be a specific characteristic of the type of mushroom, which is not affected markedly by environmental conditions.

Collybia sp. had the lowest amount of mannitol content. No marked relationship was noticed between the mannitol content and number of flushes or stage of maturity.

Qualitative analysis of carbohydrates by paper chromatography showed that galactose was the main sugar present. Traces of ribose, arabinose and rhamnose were detected. Moderate concentrations of glucose were present in *Agaricus campestris* and *Collybia* sp., but not in *Agaricus rodmani*. These results are in agreement with those obtained by Hughes *et al.* (1958).

Minerals

The mineral contents of various species of wild mushrooms are given in Table 3. *Agaricus* sp. had higher amounts of phosphorus, sodium and manganese than *Collybia* sp. The values of calcium, potassium, iron and copper for *Collybia* sp. were higher than those for *Agaricus* sp. The locality seems to have an effect on the potassium and calcium contents of wild mushrooms. No relationship between phosphorus and copper content and locality, stage of maturity and number of flushes was noticed. The sodium and iron content seem to increase with increasing number of flushes and stage of development.

Values of calcium, iron, copper and manganese for *Agaricus campestris* obtained in this study are in agreement with those reported in the literature (Maggioni *et al.* 1968; Kurkela, 1972; Charlampowicz *et al.*, 1973), whilst values of potassium, sodium and phosphorus are higher than those reported by Maggioni *et al.* (1968) and Charlampowicz *et al.* (1973).

Amino acid content

The results in Table 4 show the presence of eighteen amino acids, including all the essential ones, in the different species of wild mushrooms. It is clear that *Agaricus rodmani*, *Agaricus campestris* and *Collybia* sp. had similar distributions of amino acids as well as similar relative orders of magnitude.

The results show that these wild mushrooms are primarily deficient in lysine, phenylalanine, methionine, valine and arginine when compared

TABLE 4
Amino Acid Content of Various Species of Wild Mushrooms (g/16 g Nitrogen)

Amino acid	Agaricus rodmani		Agaricus campestris		Collybia *sp.*
	Button	*Open cap*	*Button*	*Open cap*	*Open cap*
Iso-leucine + leucine	8·2	8·8	7·8	7·7	7·3
Lysine	4·4	4·6	4·7	4·0	5·7
Methionine	3·3	3·4	1·6	2·4	2·0
Cystine	0·8	0·8	0·9	0·8	1·2
Phenylalanine	4·5	3·4	4·7	3·8	4·0
Tyrosine	5·9	6·7	4·7	4·8	4·8
Threonine	4·6	4·8	3·9	4·4	4·4
Tryptophan	10·8	12·2	10·4	10·8	11·4
Valine	4·5	4·3	4·7	4·5	4·0
Arginine	4·4	4·1	4·8	4·7	5·0
Histidine	1·9	1·5	2·4	1·6	2·0
Alanine	6·0	6·2	6·3	5·4	6·3
Aspartic acid	8·5	8·3	8·6	7·9	10·1
Glutamic acid	16·7	17·4	18·4	19·2	20·2
Glycine	4·4	3·7	3·9	3·7	3·0
Proline	7·9	6·2	7·8	6·8	6·3
Serine	4·5	4·5	4·6	4·8	5·1

with egg protein. The results for amino acid contents of *Agaricus campestris* obtained in the present work are in agreement with those obtained by Hughes *et al.* (1958), Mlodecki *et al.* (1958), Riewe (1971) and Hayes & Haddad (1976).

Gino (1965) found that *Agaricus campestris* had low contents of tryptophan, methionine and cystine.

ACKNOWLEDGEMENT

The authors wish to thank Dr A. M. Kamer for his assistance in classification of the wild mushrooms collected during this work.

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The Contribution of Tropical Chillies to Ascorbic Acid Consumption

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(Received: 30 September, 1981)

ABSTRACT

Eight common types of African chillies readily available in the markets, were examined for their ascorbic acid content. These included two species of Capsicum annum *L., both the fresh and the dry varieties (Ata-rodo and Tatase). Other types studied included the fresh and dry varieties of two species of* Capsicum frutescens *L. (Sombo and Ata wewe). The ascorbate value in the raw samples ranged from 4·6 ± 0·3 % in the dry sample of one of the varieties of* Capsicum frutescens *L. (dry Sombo) to 55·1 ± 1·5 % in the fresh variety of one of the* Capsicum frutescens *L. species (fresh Ata wewe).*

After cooking for a 25-min period there was a loss of vitamin C of between 66 % and 98·3 % in chillie sauce made from fresh and dry chillies, respectively. Considering that an adult consumes 240 g of fresh chillie sauce a day, this provides (despite cooking loss) about 80 % of the ascorbate requirement. This contribution is quite appreciable and should be recognised in practice since the conventional sources of vitamin C (fruits and vegetables) are often either expensive or not available.

INTRODUCTION

The contribution of chillies, and the various dishes made from them, to nutrient consumption, especially of vitamin C, has not been sufficiently

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emphasised despite the large quantity used in making various African sauces, soups and dishes.

A combination of a number of chillies forms the base of a sauce or soup and the addition of other items such as onions, tomatoes, vegetables, curry powder, etc., depends on individual taste. The use of fresh chillies is more common in the wet season while dry peppers are more in use in the dry season (ICNND, 1967). The excess production of chillies during the wet season is blanched and dried for use in the dry season. From the literature it is clear that quite an appreciable amount of ascorbic acid is lost in the preparation of vegetables (Krehl & Winter, 1950; Bender, 1966; Duckworth, 1960; Fafunfo & Bassir, 1977; Ajayi *et al.*, 1980; Keshinro & Ketiku, 1979). The contribution of chillies to vegetable soups therefore needs to be evaluated.

MATERIAL AND METHODS

Chillie samples and treatment

Eight different types of fresh and dry chillies were studied. There are two varieties of *Capsicum annum* L., both available in the dried and fresh states (Ata-rodo and Tatase) and two varieties of *Capsicum frutescens* L. species, both available in the fresh and dried state (Sombo and Ata-wewe). These are the most common types of chillies used for chillie sauces in tropical countries. Each chillie sample was macerated and the vitamin C content evaluated. Different recipe mixtures of these chillie samples were made prepared as per the domestic practice. Each recipe was cooked for a period of 25 min. Recipe combinations and quantity of fresh and dry chillies used for chillie sauce were:

Combination 1

Capsicum annum L.	(C)	160 g
Capsicum annum L.	(A)	60 g
Capsicum frutescens L.	(G)	60 g

Combination 2

Capsicum annum L.	(C)	160 g
Capsicum annum L.	(A)	60 g
Capsicum frutescens L.	(E)	60 g

Combination 3

Capsicum annum L.	(C)	160 g
Capsicum annum L.	(E)	60 g
Capsicum frutescens L.	(G)	60 g

Two-hundred grams of water were blended into each of Combinations 1 to 3 before cooking.

Combination 4

Capsicum annum L.	(C)	70 g
Capsicum annum L.	(A)	30 g
Capsicum frutescens L.	(G)	30 g

Combination 5

Capsicum annum L.	(C)	70 g
Capsicum annum L.	(A)	30 g
Capsicum frutescens L.	(E)	30 g

Combination 6

Capsicum annum L.	(C)	70 g
Capsicum annum L.	(E)	30 g
Capsicum frutescens L.	(G)	30 g

Three-hundred grams of water were blended into each of Combinations 4 to 6 before cooking. The quantity of each sample that made up the recipe combination and the cooking period were predetermined. The ascorbic acid content was analysed at each stage of the preparation and after every subsequent reheating of 5 minutes' duration.

Analytical procedure

The vitamin C value was determined using Roe's (1954) method as presented by the Association of Vitamin Chemists (1966). This method estimates the 'total' ascorbic acid value in food materials. It involves the oxidation of ascorbic acid (AA) to dehydroascorbic acid (DHAA) which is facilitated by carefully adding two drops of bromine. In the case of excess bromine, nitrogen gas was passed through the sample solution until dissolved bromine was expelled. The DHA was subsequently transformed to diketogluconic acid (DKGA) after coupling with 2,4-dinitrophenylhydrazine to give a red coloured osazone. The absorbances developed by

TABLE 1
Ascorbic Acid and Moisture Content of Some Common African Chillies

	Local name	*English description*	*Botanical name*	*Moisture content %*	*Mean ascorbic acid content mg/100 g*
A	Ata-rodo (fresh)	Round shape	*Capsicum annum* L.	86·1 ± 1·3	43·0 ± 4·0
B	Ata-rodo (dry)	Round shape	*Capsicum annum* L.	8·8 ± 1·0	5·0 ± 0
C	Tatase (Fresh)	Bell shape	*Capsicum annum* L.	89·9 ± 4·0	43·9 ± 3·4
D	Tatase (Dry)	Bell shape	*Capiscum annum* L.	12·4 ± 2·0	5·3 ± 0·9
E	Sombo (Fresh)	Long thin shape	*Capsicum frutescens* L.	74·3 ± 3·2	54·7 ± 7·7
F	Sombo (Dry)	Long thin shape	*Capsicum frutescens* L.	9·8 ± 1·7	4·6 ± 1·1
G	Ata wewe (Fresh)	Tiny short shape	*Capsicum frutescens* L.	74·2 ± 3·1	55·1 ± 4·5
H	Ata wewe (Dry)	Tiny short shape	*Capsicum frutescens* L.	12·3 ± 2·3	5·7 ± 1·0

the samples were estimated using pure ascorbic acid as the standard measured at 520 nm.

RESULTS AND DISCUSSION

All the fresh samples had considerable amounts of vitamin C, as shown in Table 1. The dry chillies proved to be a poor source of ascorbic acid and this must have been due to the blanching and drying preservation process.

In the dry season, when more dried chillies were used in making chillie sauces, the ascorbic acid content of the dry raw ingredients was 0·06 mg/g which is 80% lower than in the fresh raw ingredient. There was a reduction of 98·3% of vitamin C during the 25 min heat treatment. No trace of ascorbic acid was left in the sauce at the end of the first reheating period of 5 min (Tables 2 and 3). The contribution of ascorbate in chillie sauce made from raw dry chillie was also confirmed in an ICNND survey (ICNND, 1967) but other items added, such as onion and tomatoes, made the vitamin C value of such sauce even more recognisable. Only 44% of the ascorbate content of the raw ingredients was retained after 25 min of heat treatment. Since sauces are usually heated for about 5 minutes' duration before consumption, this procedure is responsible for the loss of

TABLE 2
Changes in Ascorbic Acid Content During the Preparation of Chillie Sauce
(Using fresh raw chillies)
Milligrams of Vitamin C as in Combination 1 (380 g Sauce)

Recipe combination number	*Raw ground ingredient (280 g)*	*Approximate quantity of cooked chillie sauce (380 g)*	*First reheating (362 g)*	*Second reheating (350 g)*
1	82·2 ± 6·0 (0·29)	39·0 ± 1·4 (0·10)	11·0 ± 1·0 (0·03)	Nil
2	84·2 ± 6·8 (0·30)	37·2 ± 1·4 (0·10)	12·6 ± 1·0 (0·03)	Nil
3	83·8 ± 4·9 (0·30)	35·6 ± 1·4 (0·09)	10·8 ± 1·8 (0·03)	Nil
Grand mean	83·4 ± 6·9 (0·30)	37·3 ± 1·4 (0·10)	11·5 ± 1·8 (0·03)	Nil

Figures in parentheses are the ascorbic acid estimation in milligrams per gram of chillie sauce.

TABLE 3
Change in Ascorbic Acid Content During the Preparation of Chillie Sauce (Using raw dry chillies)
Milligrams of Vitamin C as in Chillie Sauce Combinations 4–6 (250 g sauce)

Recipe combination number	*Dried raw ingredients (130 g)*	*Cooked sauce (250 g)*	*First reheating (225 g)*
4	7·8 ± 0·8 (0·06)	3·0 (0·001)	Nil
5	6·9 ± 0·8 (0·05)	2·5 (0·001)	Nil
6	7·9 ± 1·1 (0·06)	2·9 (0·001)	Nil
Grand mean	7·5 ± 1·1 (0·06)	2·8 (0·001)	Nil

Figures in parentheses are the ascorbic acid estimation in milligrammes per gram chillie sauce.

70 % of the vitamin C left after the initial cooking. At the end of the second reheating period, there was no trace of vitamin C left in the chillie sauce.

Considering that most vegetables lose between 80 % and 100 % of their vitamin C content during cooking (Fafunfo & Bassir, 1977; Keshinro & Ketiku, 1979) and the rising cost of fruits (Oke, 1966), sauces made from fresh chillies can contribute appreciably to ascorbate consumption provided reheating is discouraged and a generous consumption practised.

If as much as 240 g of fresh chillie sauce is consumed per person per day (Keshinro, 1980) this will contribute about 80 % of the daily ascorbate requirement. The ascorbate content of the sauce can be further improved by the addition of other condiments such as tomatoes, onions, etc.

Conclusively, chillies should be recognised as one of the major sauces of ascorbic acid, particularly for those parts of the world that consume quite large amounts.

ACKNOWLEDGEMENT

The authors wish to express their gratitude to Professor Omololu, who provided the facilities for this study, and also the field and laboratory staff of the Department of Human Nutrition, University of Ibadan.

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New Products From Wheat

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(Received: 24 May, 1982)

ABSTRACT

The present paper describes a new way of processing wheat, which yields quite new products to be used for technological purposes. The new process offers great advantages, both from the nutritional and the technological points of view.

INTRODUCTION

The wheat flour obtained by conventional methods is poor in vitamins and minerals and is therefore usually enriched with these. It is also low in fibre content, and a present trend is to increase the fibre content of the flour for baking. At the same time, even the white flour is too bulky for the preparation of, for example, solutions with a high concentration of nutrients.

Food Chemistry 0308-8146/83/0011-0051/$03·00

By extraction of the wheat, rather than grinding, we have obtained low-bulk solutions containing not only most of the protein and the partly hydrolysed starch of the wheat grain, but also the vitamins from the bran, which gives us new possibilities for the technological use of wheat. It can, for example, be mixed with milk to give a drink with a low lactose content but a high nutrient content.

The bran fraction obtained in the same process is a much more pure and tasty product than the bran obtained by milling and it can also, with certain precautions, be obtained with a very low content of phytate. This bran is already commercially available for pharmaceutical purposes.

MATERIALS AND METHODS

Preparation of wheat extract and washed bran

Patent has been granted (Swedish Patent No. 7711742-2, Plavia AB, Box 19051, S-254 84 Helsingborg, Sweden).

Laboratory scale

To 3 litres of water at 50 °C was added 0·5 g of protease ('Neutrase', Novo AB, Copenhagen, Denmark) and 1·5 kg of wheat, which had previously been coarsely crushed. After 1 h at 50 °C essentially all of the protein had been solubilised. Then 0·1 g of α-amylase ('BAN 120 L', Novo) was added, and the temperature increased to 75 °C for 2 h. After that it was again cooled to 60 °C, and 0·5 g of β-amylase ('Fungamyl 1600', Novo) was added. The temperature was kept at 60 °C for up to 12 h (depending on the degree of starch hydrolysis wanted).

The mixture was filtered and the bran washed repeatedly with water, which was then added to the filtrate. The bran was then dried.

The filtered extract was either concentrated into a syrup or spray-dried. These processes must be performed under mild conditions to avoid Maillard reactions.

Pilot plant scale

Under corresponding conditions, but widely varying the amounts and kinds of enzymes used, as well as the treatment times, larger batches (100 kg of wheat) were prepared in an industrial pilot plant. To some batches only protease was added, utilising the amylolytic enzymes of the wheat grains for the process.

Chemical analysis

Protein

Nitrogen was assayed by the Kjeldahl method and the amount of protein calculated as 5·7 × N for wheat and 6·38 × N for milk.

The amino acid composition was analysed by gas chromatography after acid hydrolysis (Gehrke *et al.*, 1968; Nair, 1977). The chemical score value was calculated using the FAO/WHO reference protein for the calculation.

The true digestibility (TD), the biological value (BV) and the net protein utilisation (NPU) were measured on growing rats, as described by Eggum (1973), except that the vitamins and minerals were added in the amounts described by Forsum *et al.* (1973), and maize starch was used as a carbohydrate base in the diets.

Carbohydrates

The amounts of low-molecular carbohydrate components (glucose, maltose, maltotriose, etc., as well as fructose and sucrose) were determined by gas–liquid chromatography as trimethyl-silyl ethers (glucose, fructose, maltose, isomaltose and sucrose) according to Theander & Åman (1976) and high pressure liquid chromatography (maltotriose and higher oligosaccharides up to DP 8). Conditions: A Waters ALC-202 high pressure liquid chromatograph equipped with a Model 6000A pump and a Model 660 solvent flow programmer was used, μ Bondapak/Carbohydrate 30 cm × 4 mm inside diameter column, H_2O/CH_3CN (35/65) as solvent, flow rate of 2–4 ml/min and an RI detector.

Starch was determined by a modified enzymatic method as described by Larsson *et al.* (1979). Cellulose, hemicellulose, acidic polysaccharides (polyuronides) and Klason-lignin, as well as the detergent fibre residues, according to Van Soest were determined as described by Theander & Åman (1980).

Dextrose equivalents were determined by the oxidation of aldehyde end groups with hypoiodite according to Willstätter & Schudel (1918).

Fat

Fat analysis was performed by petroleum ether extraction and gravimetry. The individual fatty acids were analysed by gas chromatography. These analyses were made by the laboratory of Karlshamns Oljefabriker AB.

Vitamins
The vitamins were assayed by Ernaeringskemisk Afdelning at Statens Levnedsmiddelinstitut, Søborg, Denmark.

Minerals
The minerals were assayed by atomic absorption at the Department of Analytical Chemistry, University of Lund, Sweden.

RESULTS AND DISCUSSION

Wheat extract

The wheat extract was obtained either as a light brown syrup (about 30 % water) with a pleasant malty smell and taste, or as a slightly coloured spray-dried powder (about 2 % water). The composition of the dry matter is shown in Table 1. The extract is completely soluble in water.

TABLE 1
Composition of a Batch of the Wheat Extract
(The figures are given as per cent dry matter)

	Wheat extract
Carbohydrate	87 %
Protein	11·5 %
Fat	0·5 %
Ash	1·8 %

Carbohydrates
The carbohydrates of the extract were hydrolysis products of starch plus sugars originally present in the wheat. The degree of hydrolysis could be varied within wide limits, and thereby also the properties of the extract (Fig. 1 and Table 2).

Extracts with a low degree of starch hydrolysis were most suitable for spray-drying, and extracts with a high degree of hydrolysis were best for syrup preparation. If a syrup was prepared from an extract with a low degree of starch hydrolysis, retrogradation occurred on storage.

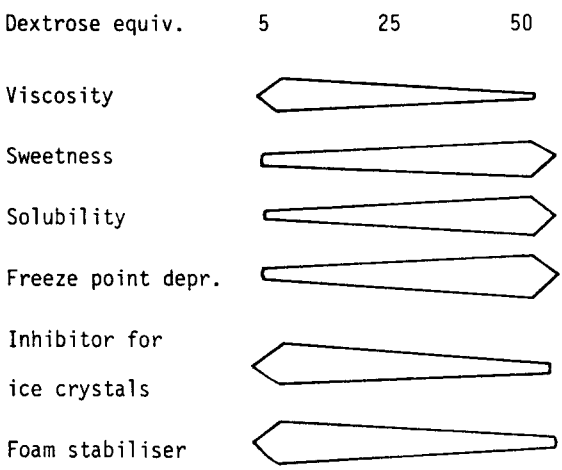

Fig. 1. Properties of the wheat extract (in syrup form) as related to the degree of hydrolysis of the starch.

Protein

The degree of hydrolysis of the protein was kept low in order to avoid the bitter taste of free amino acids. The results of the analysis of the one batch before and after acid hydrolysis showed that 6·6 % of the total amino acids were present in free form in the extract.

Lysine is the limiting amino acid in wheat, and is also the limiting amino acid of the wheat extract protein. The protein present in the bran of the wheat has a higher lysine content than that of the endosperm. If the enzyme treatment efficiently solubilises the bran protein, the protein of the extract may (like whole grain flour) have a higher biological value than

TABLE 2
Carbohydrate Composition of a Strongly Hydrolysed Batch of Wheat Extract

	Per cent of total carbohydrate
Glucose	3
Maltose	60
Maltotriose	19
Higher maltosaccharides	15
Isomaltose, sucrose, fructose	<2

ordinary flour. On the other hand, Maillard reactions during the extraction and evaporation may decrease the lysine content—and thus the biological value—of the protein. An example of amino acid recovery is given in Table 3. In this pilot plant experiment the amino acid analysis indicated a lysine loss of only about 10% (compare below).

TABLE 3
Amino Acid Composition of the Protein in One Batch of Wheat Extract (Spray-dried) and the Wheat from Which it was Prepared

	Amino acid (g/16 g N)		
	Crushed wheat	*Wheat extract*	*Recovery (%)*
Alanine	4·6	4·1	88
Valine	6·8	5·1	75
Glycine	5·1	4·5	89
Iso-leucine	4·1	3·9	96
Leucine	8·2	7·7	94
Proline	12·4	12·7	103
Threonine	3·6	3·2	89
Serine	7·5	7·2	97
Phenylalanine	5·2	5·2	100
Aspartic acid	6·2	5·4	87
Glutamic acid	38·5	37·5	98
Lysine	2·8	2·5	89

The lysine loss may, however, very well be larger than indicated by chromatographic analysis of the amino acids. Some of the first intermediates in the Maillard reaction will, although the lysine is not biologically available, be converted back into lysine when acid hydrolysis is performed for the chromatography. Therefore, nitrogen balance experiments were performed on rats.

At very low lysine content in the diet there seems to be a compensating metabolic mechanism in the rat, due either to a slow endogenous lysine synthesis or to reduced catabolism of lysine from degraded tissue protein (Bender, 1973). In the absence of lysine a biological value of about 40 will be found, provided that the other essential amino acids are present in sufficient amounts. It is therefore possible that a nitrogen balance study with a protein so low in lysine as wheat protein may underestimate the

lysine lost during processing. For this reason we performed the nitrogen balance studies with mixtures of wheat extract and milk powder. The proportions were such that 65% of the nitrogen originated from the wheat extract and 35% from the milk. In such a mixture lysine will still be the limiting amino acid, but the BV and NPU values will be higher (Table 4). Losses of available lysine were clearly revealed by this procedure, as seen from the Table.

TABLE 4

Nitrogen Balance Studies With Two Spray-dried Batches of Wheat Extract and the Crushed Wheat from Which the Extracts Were Prepared

(In batch A there were no measurable losses of lysine, but in batch B about 25% of the available lysine had been lost. This was later found to be caused by too high a temperature in the spray-drying process. In these experiments the wheat preparations were mixed with milk (35% of the nitrogen) to increase the lysine content (see text).)

	Batch A		*Batch B*	
	Crushed wheat plus milk	*Wheat extract plus milk*	*Crushed wheat plus milk*	*Wheat extract plus milk*
TD (±SD)	91·1 ± 0·70	91·5 ± 2·18	91·3 ± 1·04	90·3 ± 1·49
BV	74·9 ± 1·86	71·5 ± 3·69	74·1 ± 2·65	66·5 ± 1·41
NPU	68·3 ± 2·09	65·4 ± 2·91	67·7 ± 2·74	60·1 ± 1·85

Batch A of Table 4 showed no significant loss of available lysine, but in Batch B there was a loss of 25%, in spite of the fact that this batch was identical with the one in Table 3, where the amino acid analysis only indicated a 10% loss. Therefore, the final evaluation of a process like this must be performed with biological assay, rather than amino acid analysis alone.

Nitrogen balance was also studied without milk protein supplementation (Table 5). This is a different batch from those shown in previous Tables.

The results show that the preparation of the wheat extract can be carried out without losses in the nutritional value of the protein, but it is important to watch the process used carefully in order to avoid overheating in any of the steps. Due to the contribution of the more lysine-rich proteins from the bran, the BV of carefully prepared wheat extract is higher than in ordinary white wheat flour.

TABLE 5

Nitrogen Balance Studies with One Batch of Spray-dried Wheat Extract and the Crushed Wheat from Which This Extract was Prepared. The Results do not Indicate Losses of Available Lysine. No Milk was Added in This Experiment

	Wheat extract	*Crushed wheat*
TD (±SD)	86·9 ± 3·67	89·8 ± 2·45
BV	53·3 ± 1·95	53·9 ± 2·87
NPU	46·3 ± 1·25	48·0 ± 3·58

Vitamins

The amounts of some vitamins in one batch of the wheat extract, and the crushed wheat from which it was prepared, are shown in Table 6. Thiamine and niacin are eluted from the bran during the process and recovered in the extract. The slightly lower recovery of thiamine may be due to heat inactivation. The tocopherols are fat soluble and only partly recovered in the extract.

TABLE 6

The Amounts of Some Vitamins in One Spray-dried Batch of the Wheat Extract and the Corresponding Crushed Wheat. The Water-soluble Vitamins are Recovered in the Extract

	Crushed wheat	*Wheat extract*
Dry weight (%)	92·3	96·1
Thiamin (μg/g dry weight)	3·14	3·38
Niacin (μg/g dry weight)	44	55
Tocopherols (μg/g dry weight)	30·2	13·4

Minerals

The amounts of some minerals recovered in the same experiment are seen in Table 7. Only a fraction of the minerals are recovered in the syrup. The minerals apparently are so strongly bound to the bran (see below) that they are only partly removed by the washing procedure. This is particularly true for zinc and iron.

TABLE 7

The Amounts of Some Minerals in One Spray-dried Batch of the Wheat Extract and the Corresponding Crushed Wheat. The Minerals are only Partly Recovered in the Extract

	Crushed wheat	*Wheat extract*
Dry weight (%)	92·3	96·1
Zinc (μg/g dry weight)	41·2	14·6
Iron (μg/g dry weight)	61·8	11·5
Calcium (mg/g dry weight)	0·83	0·38
Phosphorus (mg/g dry weight)	3·6	1·7

Technological use of wheat extract

The wheat extract has a wide potential for use in different kinds of foods and drinks, especially since its properties can be varied by changing the degree of starch hydrolysis.

One especial use will be mentioned here because of its value from a nutritional point of view—the mixture of wheat extract with milk. Such mixtures are good in taste, and there is a strongly complementary effect by the nutrients in the milk and the wheat extract, respectively. When the ingredients are used in suitable proportions, the lactose content of the product will be low, and this is of great interest due to the widespread occurrence of lactase deficiency (for a review, see Dahlqvist, In press). This enzyme deficiency limits the use of milk for support feeding programmes as well as for malnutrition treatment. If wheat extract is mixed with milk powder in such proportions that half of the protein comes from the wheat and half from the milk, this will reduce the lactose content of the powder to between 25% and 30% of that in the milk powder (depending on whether skim milk powder or full fat milk powder is used). This is due to the higher protein content of the milk. This reduced lactose content can probably be tolerated by lactase-deficient subjects. For comparison it can be mentioned that lactose-hydrolysed milk, now available in Japan, has 75% of its lactose hydrolysed. The remaining 25% is regarded as harmless. Since the wheat extract has a low fat content, extra fat can also be added to the mixture, which will further decrease the relative lactose content.

When wheat protein and milk protein are mixed in appropriate proportions, the complementary action of the essential amino acids will give the mixture a high nutritional value (Table 8).

Not only the amino acids, but also the vitamins have a complementary

TABLE 8

Chemical Score of the Proteins in Wheat, Milk and a Mixture Containing 50% of Each Protein. In Spite of the Low Chemical Score of the Wheat Protein by Itself, the Mixture has a High Chemical Score.

	Chemical score		
	Wheat protein	*Milk protein*	*Protein mixture (50:50)*
Iso-leucine	82	132	107
Leucine	83	141	112
Lysine	46	133	90
Phenylalanine plus tyrosine	120	162	141
Threonine	62	105	84
Valine	86	130	108
Total score	46	100	84

The values for methionine, cysteine and tryptophan are not given.

effect in the wheat extract–milk mixture (Table 9). Iron and ascorbic acid will, however, have to be given from other sources. The mixture should also be fortified with vitamin A.

By mixing wheat extract and milk, a product can thus be obtained which could make a good nutritional support in many situations, and at

TABLE 9

Nutrient Density for Some Vitamins and Minerals in Wheat Extract and Full Fat Milk, Respectively. The Values have been Calculated According to Hansen (1973), so that a Value of 1·0 Indicates that the Content of the Nutrient in Question per Energy Unit is Just Sufficient to Cover our Need.

	Nutrient density	
	Wheat extract	*Milk (full fat)*
Vitamin A	0	1·34
Vitamin B_1 (thiamine)	1·33	0·90
Vitamin B_2 (riboflavin)	0·44	3·69
Niacin	1·50	0·15
Ascorbic acid	0	0·38
Fe	0·19	0·12
Ca	0·11	2·99

TABLE 10
Composition of One Batch of Washed Bran

	Per cent dry matter
Ash	6·1
Ethanol-extractives (non-carbohydrates)	7·1
Low-molecular carbohydrates (mainly glucose, maltose, maltotriose)	0·6
Starch	1·1
Cellulose	13·9
Hemicellulose	37·1
Acidic polysaccharides	2·3
Klason-lignin	10·2
NDF-van Soest	74·2
ADF-van Soest	22·1

the same time be better tolerated than ordinary milk. Clinical experiments are in progress with this mixture to evaluate both the tolerance for it in lactase-deficient subjects and its nutritional value.

Washed bran

The washed bran obtained in the process is a much more pure product than the bran obtained from the mills. The starch content is very low (Table 10). The washed bran has a better taste than ordinary bran and is excellently suited both for technological and for pharmaceutical use.

It can be obtained with a very low content of phytate.

TABLE 11
The Amount of Some Minerals in One Batch of Washed Bran and the Corresponding Crushed Wheat (*cf.* Table 7)

	Washed bran	*Crushed wheat*
Dry weight (%)	93·7	92·3
Zinc (μg/g dry weight)	166	41·2
Iron (μg/g dry weight)	253	61·8
Calcium (mg/g dry weight)	5·8	0·83
Phosphorus (mg/g dry weight)	8·4	3·6

The minerals are strongly bound to the bran and therefore to a large percentage recovered in the washed bran fraction (Table 11). A remarkably high Ca concentration is found in the bran, about seven times that in the wheat. It is probable that Ca has been absorbed from the water during the washing of the bran.

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The Vitamin C Content of Some Kiwifruits (*Actinidia chinensis* Planch., Variety Hayward)

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(Received: 14 June, 1982)

ABSTRACT

Skin, outer pericarp, inner pericarp and core, respectively comprised 4·8, 43·7, 45·0 and 6·5% of a mean whole kiwifruit weight of 111·3 ± 1·3 g. Kiwifruit weights ranged from 99·0 to 125·3 g. Vitamin C content per 100 g edible flesh ranged from 37·8 to 53·6 mg with means of 43·7 ± 1·7 mg for total vitamin C, 41·9 ± 1·5 mg for ascorbic acid (AA) and 1·7 ± 0·4 mg for dehydroascorbic acid (DHA). Vitamin C contents were found to be at least half previously reported contents, possibly due to losses during storage and transportation from New Zealand. Total vitamin C concentrations per 100 g of skin, outer pericarp, inner pericarp and core were 41·7 ± 3·1 mg, 42·9 ± 2·0 mg, 45·5 ± 2·3 mg and 42·3 ± 2·6 mg, respectively. Outer and inner pericarp contained similar concentrations of both AA with 40·7 ± 1·7 mg and 42·7 ± 2·1 mg, and DHA with 2·1 mg and 2·8 ± 0·7 mg per 100 g tissue, respectively. Skin and core contained lower concentrations of AA with 28·1 ± 2·4 mg and 31·1 ± 2·2 mg, and correspondingly higher contents of DHA with 13·5 ± 2·4 mg and 11·2 ± 1·3 mg per 100 g tissue, respectively. Whole fruit weight correlated with DHA concentration in both skin ($r = -0·644$) *and core* ($r = -0·693$).

INTRODUCTION

Since the introduction of *Actinidia chinensis* seed to New Zealand in 1904, the commercial growing of kiwifruits has expanded and spread to many

Food Chemistry 0308-8146/83/0011-0063/$03·00

countries. Several varieties are cultivated but the larger fruits of the Hayward variety comprise the majority of the kiwifruit crop grown worldwide (Lawes, 1979). Although kiwifruits are normally consumed as fresh fruit, studies have demonstrated that, for example, Californian grown kiwifruits can be successfully canned or frozen (Beutel *et al.*, 1976; Wildman & Luh, 1981). Kiwifruits are also grown on a large scale in several European countries (Youssef & Bergamini, 1979), and yet it is only in recent years that the fresh kiwifruits have started to become popular in the United Kingdom.

It has been known for some years that kiwifruits contain significant amounts of vitamin C (Randoin & Boisselot, 1945), and Table 1 shows some examples of the reported ascorbic acid (AA) content of several kiwifruit varieties. It is seen that these contents are higher than the typical mean vitamin C contents of such fruits as grapefruits, oranges, strawberries and lemons (40, 50, 60, and 80 mg vitamin C per 100 g edible flesh, respectively), and are more similar to the contents of blackcurrants and

TABLE 1
Ascorbic Acid Contents of Some Varieties of Kiwifruit

Kiwifruit variety	*Ascorbic acid content (mg/100 g edible flesh)*	*Reference*
Abbott	106–137	Sozzi *et al.* (1976)
	96–123	Bauckmann (1977)
	170	Youssef & Bergamini (1979)
Bruno	153–295	Bauckmann (1977)
	212	Youssef & Bergamini (1979)
Hayward	160–200	Gunther *et al.* (1970)
	150–200	Coggiatti (1971)
	57	Benk (1972)
	105	Beutel *et al.* (1976)
	122–138	Sozzi *et al.* (1976)
	78–203	Bauckmann (1977)
	126	Youssef & Bergamini (1979)
	101	Wildman & Luh (1981)
Monty	103–118	Sozzi *et al.* (1976)
	135–160	Bauckmann (1977)
	238	Youssef & Bergamini (1979)
Unnamed	252–376	Randoin & Boisselot (1945)
	58–141	Matheson (1951)
	250–380	Benk (1970)

guavas (both 200 mg vitamin C per 100 g edible flesh), (Paul & Southgate, 1978). Clearly, the inclusion of kiwifruits in the diet could significantly contribute to the United Kingdom recommended daily intake of 30 mg vitamin C. However, it is difficult to account for the wide ranges of AA contents shown in Table 1 due to the lack of data in the reference work. The determined AA contents of any fruits will be influenced by many factors such as environmental conditions, degree of maturity, handling, storage and method of analysis. AA content might be expected to vary with fruit size and variety, and yet it is not possible to draw any overall conclusion from the quoted results.

One objective of this work was to analyse a sample of kiwifruits from a supermarket and to assess the vitamin C content of kiwifruits after transportation from New Zealand. In addition, none of these reported studies included the equally antiscorbutic DHA, nor the distribution of vitamin C within the fruit. Beutel *et al.* (1976) stated that a greater concentration of AA had been found near the skin compared with other parts of the kiwifruit but no figures or details were given. A second objective of this work was therefore to determine the distribution of both AA and DHA in the ripe kiwifruit.

MATERIALS AND METHODS

Grade 1 kiwifruits of the Hayward variety were obtained from a supermarket during September, 1981 as supplied by Auckland Export Ltd. (Grower C881), New Zealand. The fruits had a weight range of 99·0 to 125·3 g and were stored at 4 °C until required. The kiwifruit components were separated with the aid of a scalpel for fresh and dry weight determinations. Although tissue losses were negligible when removing the skin, some small loss ($<2\%$) was unavoidable when separating the inner pericarp from the core (see Fig. 1). The fresh weights of the edible flesh and inner pericarp were calculated thus: weight of edible flesh = (weight of whole fruit) – (weight of skin), weight of inner pericarp = (weight of whole fruit) – (weights of outer pericarp, skin and core).

Dry solids weight was determined by drying the kiwifruit parts to constant weight at 95 °C in a circulated-air oven. As a further characteristic of the fruits used, the cell sap (juice) of the edible flesh was expressed using a pestle and mortar, and the percentage refractometric solids was measured at 20 °C using an Abbé refractometer calibrated with sucrose

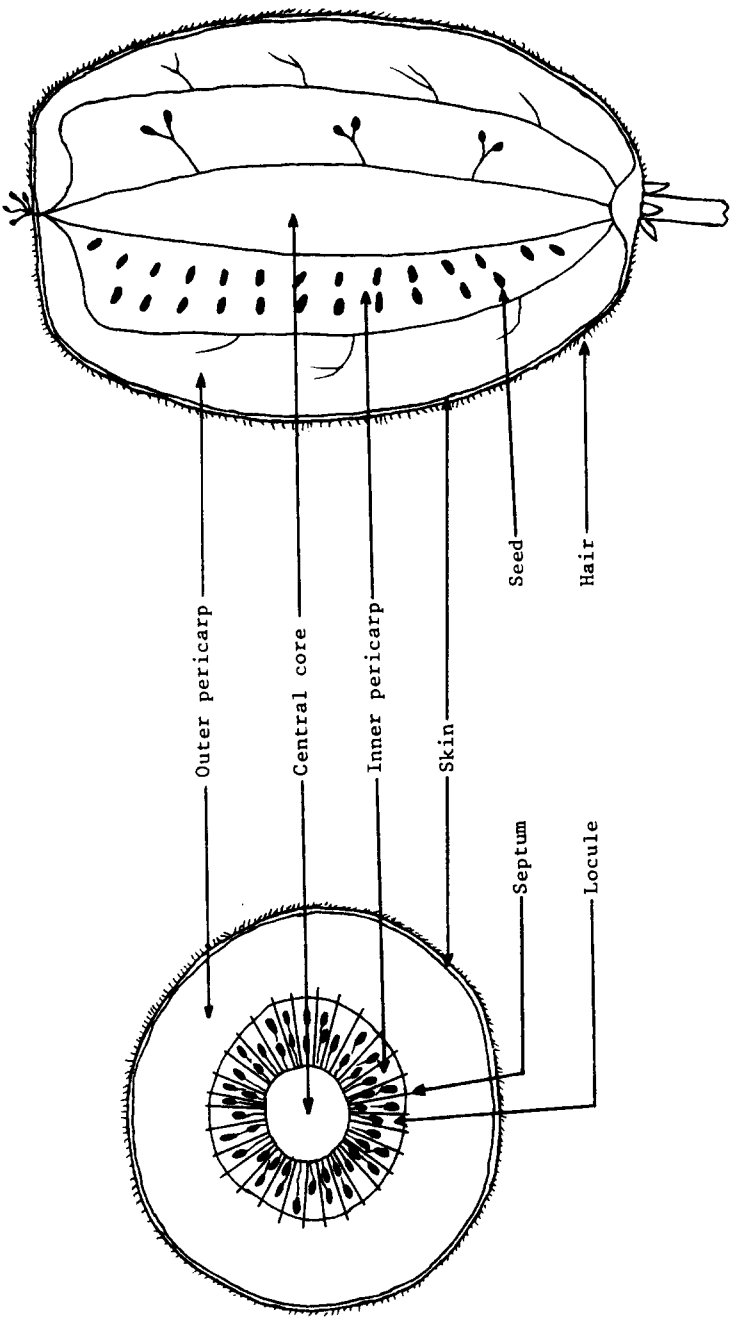

Fig. 1. Diagrammatic representation of transverse and longitudinal sections through a kiwifruit (after Hopping, 1976).

solutions. The results are shown in Table 2 and ranged from 16·3 to 20·0% with a mean of 17·7 ± 0·3% refractometric solids expressed as sucrose. Kiwifruit weight did not correlate with refractometric solids content.

The reagents used for the determination of vitamin C were prepared in the standard manner described by the AOAC (1980). The kiwifruit parts

TABLE 2
Fresh Weights and Refractometric Solids Contents of Edible Flesh Cell Sap of Eleven Kiwifruits

	Fresh weight (g)		*Edible flesh cell sap concentration per cent refractometric solids at 20°C as sucrose*
	Whole fruit	*Edible flesh**	
	99·18	94·45	17·5
	102·18	97·31	17·3
	104·07	99·11	16·9
	105·86	100·81	16·3
	107·67	102·53	18·3
	108·11	102·95	18·0
	110·65	105·37	17·1
	111·38	106·07	17·0
	111·99	106·65	20·0
	116·15	110·61	18·9
	125·28	119·30	17·0
Means	109·32	104·11	17·7
Standard error of means	2·16	2·05	0·3

* Based on edible flesh proportion of whole fruit of 95·23%. (Mean of twenty-four kiwifruits from Tables 3 and 4.)

were separated with the aid of a scalpel dipped in extracting solution, and these were immediately submerged in 50 ml extracting solution to minimise enzymic oxidation at the cut surfaces. Vitamin C was extracted by first cutting up the tissues and then macerating for 1 min with 60 ml ice cooled 3% metaphosphoric–8% acetic acid solution in a 125 ml dreschel bottle, using an L2R Silverson tubular mixer (head diameter 16 mm) prior to centrifuging at 1000 *g* for 4 min. The supernatant was filtered through

muslin and made up to 200 ml except for whole edible flesh samples which were made up to 250 ml with extracting solution. For determinations on the whole edible flesh, the edible flesh was divided and each half was extracted and centrifuged separately, the supernatants finally being added together. AA was determined by titration with 2,6-dichlorophenol-indophenol dye, and total vitamin C (AA plus DHA) by the fluorimetric method described by AOAC (1980). The DHA present was calculated by difference.

RESULTS AND DISCUSSION

The fresh and dry weights of two samples of kiwifruits were investigated first. Twelve kiwifruits (mean weight = 110·2 ± 1·8 g) were separated into skin and edible flesh and the fresh and dry weights of these components are shown in Table 3. The weight of edible flesh ranged from 94·1 to 111·4 g with a mean of 105·0 ± 1·8 g, and represented a mean proportion of 95·3 % of the whole fruit. The weight of skin ranged from 4·5 to 6·2 g with a mean of 5·2 ± 0·1 g, and represented a mean proportion of 4·7 % of the whole fruit. Dry weight of the edible flesh ranged from 14·3 to 19·4 g with a mean of 16·9 ± 0·4 g, representing a mean dry solids content of 16·1 % of the edible flesh.Dry weight of the skin ranged from 1·2 to 1·5 g with a mean of 1·4 g, constituting a mean dry solids content of 27·1 % of the skin. Dry weight of the edible flesh correlated with whole fruit weight ($r = +0{\cdot}736$) and edible flesh weight ($r = +0{\cdot}725$) where r throughout is the product moment correlation coefficient significant at the 95 % confidence level.

A further twelve kiwifruits (mean weight 111·3 ± 1·3 g) were separated into the four parts skin, outer pericarp, inner pericarp and core (see Fig. 1). The fresh and dry weights of these parts are shown in Table 4. The mean fresh weights of edible flesh and skin were very similar to the first sample as shown in Table 3. Outer pericarp weight ranged from 36·4 to 58·5 g with a mean of 48·7 ± 1·6 g representing 43·7 % of the whole fruit. Inner pericarp weight ranged from 43·7 to 54·9 g with a mean of 50·0 ± 1·2 g representing 45·0 % of the whole fruit. Core weight ranged from 5·4 to 8·5 g with a mean of 7·2 ± 0·3 g representing 6·5 % of the whole fruit. Mean skin dry weight was also very similar to that shown in Table 3, having a dry solids content of 26·9 %. Mean dry weights of the outer pericarp, inner pericarp and core were 7·3 ± 0·2 g, 8·4 ± 0·3 g and

TABLE 3
Fresh Weights and Dry Solids Weights of the Edible Flesh and Skin of Twelve Kiwifruits

	Fresh weight (g)			*Dry solids weight (g)*		
	Whole fruit	*Edible flesh*	*Skin*	*Whole fruit**	*Edible flesh*	*Skin*
	98·95	94·13	4·82	15·66	14·32	1·34
	101·60	95·43	6·17	17·44	15·91	1·53
	102·32	97·19	5·13	16·60	15·26	1·34
	109·17	104·64	4·53	18·02	16·81	1·21
	109·57	103·85	5·72	19·43	17·93	1·50
	111·46	106·45	5·01	18·62	17·27	1·35
	111·82	106·98	4·84	18·38	16·96	1·42
	114·51	109·57	4·94	20·24	18·82	1·42
	114·96	109·60	5·36	17·04	15·67	1·37
	115·31	109·73	5·58	19·77	18·24	1·53
	116·35	111·22	5·13	18·20	16·72	1·48
	116·59	111·38	5·21	20·77	19·38	1·39
Means	110·22	105·01	5·20	18·35	16·94	1·41
Standard error of means	1·77	1·79	0·13	0·44	0·43	0·03
Per cent proportion of fresh weights	100·00	95·27	4·72	16·65	16·13	27·12

* Calculated from component parts.

TABLE 4
Fresh Weights and Dry Solids Weights of the Skin, Outer Pericarp, Inner Pericarp and Core of Twelve Kiwifruits

	Fresh weight (g)						*Dry solids weight (g)*			
	Whole fruit	*Edible flesh*	*Skin*	*Outer pericarp*	*Inner pericarp*	*Core*	*Skin*	*Outer pericarp*	*Inner pericarp*	*Core*
	104·69	99·11	5·58	36·44	54·44	8·23	1·45	5·68	9·31	1·39
	105·92	101·22	4·70	44·21	49·13	7·88	1·34	6·61	8·71	1·33
	107·25	101·90	5·35	48·89	44·52	8·49	1·52	7·55	7·96	1·46
	107·86	103·15	4·71	47·78	47·68	7·69	1·26	6·50	7·31	1·22
	109·80	104·92	4·88	48·20	51·19	5·53	1·30	7·30	8·50	0·88
	110·60	104·66	5·94	55·18	44·08	5·40	1·50	7·85	6·40	0·85
	112·42	108·05	4·37	46·88	54·56	6·61	1·24	7·65	9·66	1·10
	113·56	107·02	6·54	45·51	54·86	6·65	1·61	6·80	9·19	1·04
	113·98	108·97	5·01	48·71	52·42	7·84	1·40	7·31	8·51	1·29
	114·54	109·15	5·39	49·86	51·70	7·59	1·54	7·98	9·59	1·29
	115·00	109·03	5·97	58·51	43·67	6·85	1·51	8·77	7·17	1·12
	119·72	113·91	5·81	53·74	52·04	8·13	1·59	7·78	8·61	1·32
Means	111·28	105·92	5·35	48·66	50·02	7·24	1·44	7·32	8·41	1·19
Standard error of means	1·27	1·21	0·19	1·63	1·20	0·30	0·04	0·24	0·30	0·06
Per cent proportion of fresh weights	100·00	95·18	4·81	43·73	44·95	6·51	26·92	15·04	16·81	16·44

1·2 ± 0·1 g, respectively. The percentage dry solids content of these latter three parts was therefore similar to about 16% dry solids. Whole fruit weight correlated with outer pericarp fresh weight ($r = +0{\cdot}650$) and with outer pericarp dry weight ($r = +0{\cdot}678$). This suggests that increasing fruit weight is due primarily to an increase in the solids content of the outer pericarp of the kiwifruit. Such an increase might be expected as a result of the build up of starch and sugars during maturation.

Samples of kiwifruits were then analysed for vitamin C content. The vitamin C content of ten kiwifruits is shown in Table 5. Total vitamin C content ranged from 37·8 to 53·6 mg with a mean of 43·7 ± 1·7 mg total vitamin C per 100 g edible flesh. AA content ranged from 36·6 to 49·1 mg with a mean of 41·9 ± 1·5 mg AA per 100 g edible flesh. DHA content ranged from 0·2 to 4·5 mg with a mean of 1·7 ± 0·4 mg DHA per 100 g edible flesh. These values for vitamin C content are considerably lower than the typical values shown in Table 1. Although it is not always clearly stated, most of these reported studies seem to have used freshly harvested fruits. However, Benk (1972) used kiwifruits as marketed in Germany,

TABLE 5
Fresh Weights and Vitamin C Contents of the Edible Flesh of Ten Kiwifruits

	Fresh weight (g)		*Vitamin C (mg/100 g of edible flesh (fresh weight))*		
	Whole fruit	*Edible flesh**	*Total*	*AA*	*DHA*
	105·09	100·08	46·49	46·32	0·17
	106·88	101·78	53·60	49·11	4·49
	109·28	104·07	50·23	47·46	2·77
	110·16	104·91	37·83	36·63	1·20
	110·46	105·19	45·61	44·86	0·75
	111·08	105·78	39·13	37·75	1·38
	111·34	106·03	41·07	38·16	2·91
	113·18	107·78	44·19	41·88	2·31
	115·70	110·18	39·76	38·75	1·01
	120·06	114·33	38·90	38·51	0·39
Means	111·32	106·01	43·68	41·94	1·74
Standard error of means	1·35	1·28	1·68	1·46	0·43

* Based on edible flesh proportion of whole fruit of 95·23%. (Mean proportion of twenty-four kiwifruits from Tables 3 and 4.)

presumably from New Zealand, rather than as grown locally. As these results are of the same order as reported by Benk (57 mg AA per 100 g edible flesh), it seems possible that these relatively lower vitamin C contents may be due at least in part to the storage conditions and transportation time involved during the export chain. Such losses could be evaluated by further studies on the vitamin C content of kiwifruits from the time of harvesting, throughout storage and transportation, to the time of sale. Even so, these kiwifruits would still constitute quite a good alternative source of vitamin C in the diet.

A second sample of ten kiwifruits was separated into the four major components and the vitamin C content determined in each of the parts (see Table 6). There is a similarity of mean total vitamin C concentration in each part of the fruit, being $41{\cdot}7 \pm 3{\cdot}1$ mg, $42{\cdot}9 \pm 2{\cdot}0$ mg, $45{\cdot}5 \pm 2{\cdot}3$ mg, $42{\cdot}3 \pm 2{\cdot}6$ mg total vitamin C per 100 g skin, outer pericarp, inner pericarp and core, respectively. The outer pericarp and inner pericarp also contained a similar mean concentration of AA, having $40{\cdot}7 \pm 1{\cdot}7$ mg and $42{\cdot}7 \pm 2{\cdot}1$ mg AA per 100 g of tissue, respectively. Both parts contained correspondingly small concentrations of DHA, the outer pericarp and inner pericarp having $2{\cdot}1 \pm 0{\cdot}6$ mg and $2{\cdot}8 \pm 0{\cdot}7$ mg DHA per 100 g of tissue, respectively. Finally, the skin and core had a lower mean AA concentration of $28{\cdot}1 \pm 2{\cdot}4$ mg and $31{\cdot}1 \pm 2{\cdot}2$ mg per 100 g of tissue, respectively, and a correspondingly higher DHA concentration of $13{\cdot}5 \pm 2{\cdot}4$ mg and $11{\cdot}2 \pm 1{\cdot}3$ mg per 100 g of tissue, respectively. The DHA concentrations ranged from 29·5 to 2·3 mg per 100 g skin, and 15·6 to 3·7 mg per 100 g of core. Computing the mean vitamin C content of the edible flesh from the concentrations in the three components shown in Table 6 gives values per 100 g of edible flesh of 47·0 mg total vitamin C, 43·7 mg AA and 3·3 mg DHA. These values are slightly higher than those shown in Table 4, but within experimental error this might be expected because the vitamin C content of edible flesh in Table 4 was based on two extractions (i.e. the two halves of the edible flesh) whereas the vitamin C content computed from Table 6 is based on three extractions (i.e. outer pericarp, inner pericarp and core) and three extractions would give a slightly enhanced recovery of the vitamin in the edible flesh.

Although no other correlation between whole fruit weight and vitamin C concentration was found, whole fruit weight did correlate with DHA in both the skin ($r = -0{\cdot}644$) and the core ($r = -0{\cdot}693$). The correlation is not high but suggests that as whole fruit weight increases, the concentration of DHA in the skin and core decreases. It is interesting that the

TABLE 6
Vitamin C Contents of the Skin, Outer Pericarp, Inner Pericarp and Core of Ten Kiwifruits

	Fresh weight whole fruit	*Vitamin C (mg/100 g fresh weight of tissue)**											
		Skin			*Outer pericarp*			*Inner pericarp*			*Core*		
	(g)	*Total*	*AA*	*DHA*	*Total*	*AA*	*DHA*	*Total*	*AA*	*DHA*	*Total*	*AA*	*DHA*
	105·40	39·84	18·74	21·10	36·82	34·09	2·73	46·90	41·68	5·22	42·42	27·70	14·72
	109·75	32·95	18·37	14·58	35·19	34·80	0·39	42·73	41·21	1·52	49·44	33·89	15·55
	111·20	56·64	27·10	29·54	45·22	41·81	3·41	38·02	34·37	3·65	30·39	16·71	13·68
	111·72	44·32	31·84	12·48	40·93	39·46	1·47	37·22	36·92	0·30	43·60	30·26	13·34
	111·91	41·26	26·58	14·68	53·82	47·18	6·64	56·88	49·68	7·20	54·32	39·23	15·09
	111·91	33·09	27·14	5·95	40·46	38·39	2·07	54·12	51·91	2·21	37·86	30·18	7·68
	112·72	40·04	26·38	13·66	42·56	41·53	1·03	43·67	41·33	2·34	32·43	25·89	6·54
	113·87	57·66	44·53	13·13	52·67	50·96	1·71	46·25	43·86	2·39	45·21	36·17	9·04
	114·29	43·27	35·45	7·82	43·58	43·46	0·12	52·66	51·78	0·88	52·15	39·38	12·77
	117·71	27·56	25·27	2·29	37·42	35·61	1·81	36·80	34·19	2·61	34·73	31·07	3·66
Means	112·05	41·66	28·14	13·52	42·87	40·73	2·14	45·53	42·69	2·83	42·26	31·05	11·21
Standard error of means	1·01	3·06	2·44	2·44	1·99	1·73	0·59	2·28	2·10	0·65	2·62	2·15	1·31

* Based on the following tissue proportions of the whole fruit. Skin, 4·81 %; outer pericarp, 43·73 %; inner pericarp, 44·95 %; core, 6·51 %. (Mean proportions of twelve kiwifruits from Table 4.)

skin and core should also apparently contain higher proportions of DHA. It is known that high concentrations of vitamin C are often found in rapidly metabolising tissues, particularly where changes in the cell wall are occurring, as in cell division, and it is possible that the decreasing DHA concentration is related to detailed changes occurring in the skin and core tissues as the fruit matures. Some of the gross structural changes are discussed by Hopping (1976) but there is no indication of any obvious relationship to either DHA or AA concentration. A similar observation was made with peas (Selman & Rolfe, 1979) over a much wider weight and maturity range. There the DHA concentration in the skin decreased with increasing pea maturity and it was postulated that this could be related to catabolic pathways involved in the cell wall breakdown and resorption which is known to occur in the inner layers of the pea skin over the maturity range studied. It is possible that similar cellular changes may occur in the core of the kiwifruit during a certain stage of maturation; for example, in the outer hypodermal layer of the outer pericarp next to the skin. Further studies on the vitamin C concentration of kiwifruits, coupled with a detailed histological study over a wide range of maturity, might help to elucidate a part of the major functions of AA and DHA in the fruit.

ACKNOWLEDGEMENT

The author is grateful to Mr J. Moreland of Safeway Foodstores Ltd., Loughborough, Leics., Great Britain.

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Book Reviews

Food Carbohydrates. Edited by D. R. Lineback and G. E. Inglett, AVI Publishing Co. Inc., Westport, Connecticut, USA. 1982. xii + 494 pp. Price: $49·50.

This book is the proceedings of an IFT/IUFOST joint symposium held in June, 1981 and contains chapters on, 'Modern methods of analysis of corn derived sweeteners' (Bernetti), 'Corn syrup selections in food applications' (Strickler), 'Taste receptor origin of sweetness' (Beidler), 'Fructose—A regulatory perspective' (Frattali), 'Polyols: Chemistry and application' (Emodi), 'Sucrose and health' (Bollenback), 'Lactose and the sugars of honey and maple. Reactions, properties and analysis' (Doner and Hicks), 'Sugar dehydration reactions' (Feather), 'The influence of food carbohydrates on dental caries' (DePaola) 'Carbohydrates, sweet taste and obesity: Changing consumption patterns and health implications' (Drewnowski, Gruen and Grinker), 'Health implications of food carbohydrates: Heart disease and diabetes' (Reiser), 'Lectins: carbohydrate-binding proteins in search of a function' (Goldstein), 'Current concepts of starch structure' (Hood), 'Starch derivatives and their use in foods' (Smith), 'Food applications of gums' (Glicksman), 'Dietary fibre in health and disease' (Kritchevsky), 'Dietary fibre analysis—Concepts and problems' (Dintzis), 'Effect of preparative procedure on the evaluation of *in vitro* indigestible residue (Dietary fibre)' (Rasper), 'Analysis of polysaccharides' (Aspinall), 'Protein–polysaccharide interactions' (Cherry), 'Hydrocolloid interactions with

starches' (Christianson), 'Polysaccharide conformation in solutions and gels' (Dea) and 'Metabolism and mutagenic effects of Maillard reaction products' (Perkins, Becher, Genthner and Martin).

Of the twenty-three chapters, twenty-one are contributed by American authors, one is from the UK and one from Canada. This surprising imbalance for a IUFOST symposium cannot pass without note. However, the Editors have made an attempt to cover a very big subject by a formidable array of chapters and, on this, they are to be congratulated.

Needless to say, chapters vary in their profundity and literature coverage but most contain new applications for those involved in both the industrial and nutritional areas. Beidler's chapter on taste receptor origin of sweetness is succinct and useful but one wonders at the omission of further chapters on, say, dihydrochalcone sweeteners and the excellent work on sugar structure and sweetness carried out by Hodge, Dick and others at USDA, Peoria. Some of the newer developments in Europe, such as the non-metabolisable carbohydrates (polydextrose) and hydrogenated glucose syrups are either ignored or barely mentioned.

Overall, the book is important and well-presented. Its price is standard and its contents cannot be ignored.

G. G. Birch

AVI Food Products Formulary Series. Volume 4. Fabricated Foods. Edited by M. J. Inglett and G. E. Inglett. AVI Publishing Co. Inc., Westport, Connecticut, USA. 1982. xi + 146 pp. Price: $38·00.

This useful little book is a collection of industrial food formulas. It is succinct, easy to read, with each formula listed simply in pounds or percentage. Where appropriate, a very brief and simple procedure is also included under each heading. Industrial additives and ingredients are asterisked with the address of source (including zip code!).

The book is very recommendable to food scientists interested in product development and its price is quite acceptable.

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Chemical Methods for Measuring Changes in Freeze Stored Fish: A Review

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(Received: 12 April, 1982)

ABSTRACT

Chemical methods and procedures for measurement of changes in freeze-stored fish are reviewed.

A variety of lipid extraction methods, protein and amino acids assay procedures, trimethylamine production and enzymatic activity evaluation are discussed.

With chemical methods only it would be impossible to determine whether a sample had changed or not. Parallel chemical and sensory methods are suggested for quality evaluation.

INTRODUCTION

Physical, chemical and organoleptic changes observed in eating quality and other properties during freeze storage of fish are of great commercial importance. Those changes due to poor freezing and storage conditions, bad quality packaging or unknown previous cold storage history can be detected with some ease in the frozen fish and also in the thawed and cooked state (Banks, 1962). The flavour of cooked fish may show some

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'cold store' effects and the arising toughness and dryness often make frozen fish less acceptable (Mills, 1975). The development of oxidative rancidity is an additional problem with fatty fish.

Although many changes can be readily detected, some are very difficult to quantify. This topic has been reviewed by Soudan (1968) and Gould & Peters (1971).

Because of differences in fish from different species and changing characteristics of fishing grounds (Love, 1964), and partly due to seasonal variations in the composition of fish muscle (MacCallum *et al.*, 1968; Castell & Bishop, 1973), a universally accepted method for measuring changes, in even one attribute, has so far proved elusive (Mills, 1975).

Many of the reactions assayed in chemical analysis of frozen fish muscle have different temperature coefficients; thus it may happen that a particular method for measuring changes at one temperature may not be applicable or give poor reliability in another set of storage conditions.

This review includes those methods that have been used in measurement of chemical parameters like amino acids changes, denaturation of proteins, trimethylamine production, free fatty acid content and enzyme activity in freeze stored fish.

LIPIDS

Although the free fatty acid content is not used as an estimate of freeze storage changes, the nature and composition of free fatty acids produced in freeze stored fish muscle have been extensively examined (Olley *et al.*, 1962; Hanson & Olley, 1964; Olley & Duncan, 1965; Ackman, 1967; Anderson & Ravesi, 1969; Castell, 1971; Vorobeva & Sukhanova, 1971*b*; Wood *et al.*, 1969; Mills, 1975).

A colorimetric approach to assaying free fatty acids was obtained by measuring the colour development of copper salts and cobalt salts in soaps of long chain free fatty acids dissolved in chloroform (Ayers, 1956; Duncombe, 1963; Itaya & Ui, 1965; Novack, 1965; Schimmel & Knobil, 1970). Although the amount of total lipids does not suffer important changes during freeze storage of fish muscle (Peters *et al.*, 1968), the content of free fatty acids increases due to lipid hydrolysis (Dyer & Fraser, 1959; Bligh, 1961; Bligh & Scott, 1966).

Production of carbonyls from lipids and development of rancidity are other changes that have been observed. Estimations of carbonyls were achieved by Tokunaga (1964*a*, *b*); he observed a slight increase during storage in the frozen state. Altufeva *et al.* (1970) arrived at similar results.

Oxidative rancidity in fat-containing frozen fish can be measured by means of the thiobarbituric acid (TBA) test. The possibilities of direct determination of TBA values in trichloroacetic acid extracts instead of distillates of fish seem promising (Yu & Sinnhuber, 1957; Sinnhuber & Yu, 1958; Tarladgis *et al.*, 1960; Vyncke, 1970, 1974). Other kinds of chemical evaluation of rancidity in fatty fish have been presented, where peroxide values were measured and the Kreis test was applied (Banks, 1937, 1945).

Rancidity measurement by peroxide determination under anaerobic conditions seems to be the most sensitive method to evaluate oxidative changes in fatty fish muscle (Banks, 1966).

PROTEINS

The most useful method that has been applied in the analysis of freeze stored fish quality changes is measurement of the extractable proteins (Mills, 1975). Denaturation of fish proteins is the cause of an observed decrease in the amounts of soluble proteins extracted with neutral salt solutions (Reay, 1933; Reay, 1934; Reay & Kuchel, 1936; Dyer *et al.*, 1950). The mechanisms of the changes that are achieved in fish proteins during freezing have been widely reported in the literature (Dyer, 1951; Duerr & Dyer, 1952; Love, 1958; Connell, 1960*a*, *b*; Dyer & Dingle, 1961; Sawant & Magar, 1961; Connell, 1962*a*, *b*; Connell, 1968).

The method of neutral salt solution extraction has proved to be useful, but shows a great scattering of values—the difficulty is that they vary seasonally and with storage temperature (Love, 1956; Luijpen, 1957; Ironside & Love, 1958; Castell & Bishop, 1973). This fact may be connected with the phenomenon that pH has some influence on fish protein denaturation (Kelly, 1967). It has been demonstrated that acceptability of frozen fish can be predicted more accurately considering simultaneously pH and extractable protein content (Cowie & Little, 1966). The rate of freezing has been reported by Love (1956, 1958) to influence protein extractability. This was also studied by Nikkila (1957), Nishimoto & Tanake (1960) and Love (1962*b*).

The decrease in solubility of extractable proteins as a protein denaturation indicator may have some relationship with the lipid content of fish muscle, as reported by Dyer & Morton (1956), Love (1962*a*), Connell (1968) and Ackman (1967).

Volatile basic nitrogen is another measure of denaturation of proteins. Various methods have been assayed in the determination of volatile base

development during frozen storage of fish fillets. The most used are those methods described by Lucke & Geidel (1935), Antonacopoulos (1968) and Vyncke (1969).

TRIMETHYLAMINE PRODUCTION

Because under frozen storage conditions of temperature and humidity there is little or no bacterial growth, negligible trimethylamine production from trimethylamine oxide due to microbiological action is detected (Castell *et al.*, 1971). So, measurement of trimethylamine content in fish muscle can be used as an index of the pre-freezing history of samples (Dyer *et al.*, 1962), because trimethylamine oxide is hydrolysed during storage producing formaldehyde and dimethylamine, as reported by Connell & Howgate (1968, 1969). Measurement of dimethylamine has been proposed by Castell *et al.* (1970) as an indicator of cold storage deterioration. However, this method of measurement of dimethylamine and formaldehyde has some limits in its application because many investigators have reported that there is no increase in content of these chemicals during frozen storage of lemon sole, plaice, halibut, wolf-fish, redfish and certain shellfish (Amano & Tozawa, 1967; Yamada & Harada, 1969; Castell *et al.*, 1971).

AMINO ACIDS

Many studies have been carried out on changes in amino acid concentration in freeze stored fish (Partmann, 1969; Vorobeva & Sukhanova, 1971*a*; Danolow *et al.*, 1973), but in general this method is not considered useful for the determination of changes due to freezing. It was found that alterations in relative amino acid concentrations may be expected after freezing, while the total amount of free amino acids present remains constant.

ENZYMATIC ACTIVITY

It was suggested by Gould (1964) that enzyme properties may help to reveal quality changes in frozen stored fish. α-Glycerophosphate-dehydrogenase and malic enzyme have been described as those with greatest potential profits.

A method for measuring deterioration through determination of fibre

contractility in fish muscle has been presented by Partmann (1955). More studies and results of application on frozen fish muscle have been reported by Partmann (1970) and Yamanaka & Mackie (1971). This method is based on the measurement of changes in adenosine triphosphatase activity during frozen storage, the difficulty being that it is temperature dependent (Connell, 1960*b*).

Determination of dehydrogenase activity in fish muscle allows frozen fish to be distinguished from fresh (Ciani & Salerni, 1964); aldolase activity (Connell, 1966) can be used as a reliable method to evaluate deterioration in fish after freezing.

CONCLUSIONS

A great number of a wide variety of methods for the measurement of chemical and quality changes in frozen stored fish can be found in the literature, but due to a variety of complicating factors, such as seasonal changes, pH, pre-freezing history, size and fishing ground characteristics, it is difficult to find one method that gives good results for commercial or research application. According to Mills (1975), 'the very fact that so many tests have been proposed and developed is indicative of the complex nature of the problems that exist' and this seems to hold for chemical evaluation of the changes occurring during frozen storage of fish.

It must be concluded that by chemical methods alone it would be impossible to determine whether a sample had changed or not. So, additional experiences with non-sensory methods will be necessary to determine the acceptability and marketability of a given product.

Due to the complexity of the changes achieved in freeze-stored fish and the not well-determined relationship between sensory and non-sensory assays, it cannot be concluded that one test or even a combination of tests will predict all the characteristics of the sample after some period of storage.

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The Free and Total Folate Activity in Some Commonly Available Tropical Foodstuffs

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(Received: 11 March, 1982)

ABSTRACT

The free and total folate activities of common tropical food materials, as purchased in the market place, were measured microbiologically. The values obtained can be used to provide information on the folate content of the diet in relation to food consumption, particularly during pregnancy when folate deficiency can occur.

INTRODUCTION

Most foods contain various forms of folic acid in mixed foods and since man cannot synthesise the pteridine ring structure of this vitamin (Hoppner *et al.*, 1972), the main source of it is from food intake.

Folic acid occurs in the free form which is readily absorbed from the gut (Anderson *et al.*, 1960) and in conjugated forms from which free folate may be released by enzymic treatments (Butterworth *et al.*, 1969). Microbiological analysis of this vitamin after conjugase treatment gives a measure of the total folate content of the food, although not necessarily that biologically available to man.

Folate in foods may be partially or wholly destroyed by processing or storage and in particular large losses may occur during preparation and

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cooking of the food, especially by leaching of water-soluble vitamins into cooking water. Also, heat treatment and oxidation destroy folate (Herbert, 1963; Hurdle, 1967). Folic acid metabolism in relation to anaemia, particularly in pregnancy, has been extensively studied, (Hibbard, 1962). Hence it is of vital interest to examine the folate activities of some commonly available tropical food materials, to provide data for use in the compilation of diets to boost folic acid intake.

MATERIALS AND METHODOLOGY

Materials

Individual samples of each type of food material were thoroughly mixed to give a representative composite sample. Except for the fresh leafy vegetables which were analysed as soon as possible after purchase, all samples were freeze dried prior to storage.

Analytical procedure

The moisture content of each representative sample was measured by subtracting, from the original weight, the weight after lyophilisation.

Free and total folic activity were determined microbiologically using a method described in 'Analytical Microbiology', (Kavanna, 1963) except that samples were extracted in phosphate ascorbate buffer solution at 15 lb pressure (121 °C) for 15 min. Ascorbic acid was added to the sample before extraction because folic acid is known to be more stable in the presence of vitamin C (Hurdle, 1967). Difco folic acid casein assay medium was used and the samples were made up to appropriate volume, filtered and assayed.

Amylase buffer solution (1 %) was added to the starch samples (for enzymic reaction to take place) with some ascorbic acid to stabilise the folic acid activity. The starch samples were heated at 40 °C for 2 h in a shaking water bath. The samples were then re-sterilised for 5 min at 15 lb pressure (121 °C). This procedure helps in the digestion of the starch content and allows for easy release of the folic acid content.

Lactobacillus casei NCDO 243 was employed as the test organism since it is accepted as giving broadly the same response to free folates as higher animals.

TABLE 1
The Microbiological Estimation of the Free and Total Folate Activities in Some Commonly Available Tropical Foods

Local name of raw food materials	*Scientific name*	*English name*	*Moisture content (%)*	*Free folate content (mg/100 g)*	*Total folate content (mg/100 g)*
Vegetables					
Tete	*Amaranthus* L. sp.	Greens	83·7 ± 4·0	50·1 ± 8·2	61·0 ± 5·7
Sokoyokoto	*Celesia argentea* linn	or	85·7 ± 6·1	66·8 ± 8·9	101 ± 9·8
Gbure	*Talanum triangulare* J.	spinach	92·2 ± 4·1	25·6 ± 8·8	54·2 ± 7·9
Igbagba	*Selanum Gillo raddi* L.		88·4 ± 2·9	44·5 ± 8·2	64·4 ± 7·4
Ewedu	*Cochorus oliturius* L.	Artichokes	89·8 ± 4·6	59·0 ± 4·0	68·0 ± 7·7
Ila	*Hibiscus esculentum* L.	Lady's finger/Okra	89·7 ± 2·4	24·8 ± 6·3	76·9 ± 4·4
Ewe Gbaguda			87·8 ± 1·6	34·9 ± 5·7	66·2 ± 5·2
Ishapa tutu	*Hibiscus* sp.	Fresh fruit vegetable fruit	88·3 ± 2·8	29·4 ± 4·6	51·9 ± 8·1
Ishapa gbigbe	*Hibiscus* sp.	Sun-dried vegetable	6·6 ± 1·5	4·6 ± 1·1	7·8 ± 1·5
Iroko			89·6 ± 4·3	30·4 ± 5·6	68·7 ± 3·9
Nuts and legumes					
Ewa funfun		Black eye beans		26·4 ± 6·8	99·0 ± 9·8
Ewa Pupa		Red beans		25·0 ± 4·5	103·6 ± 9·9
Ewa Suresure		Brown beans		29·8 ± 6·2	112 ± 8·7
Ewa Dudu		Black		24·9 ± 5·5	110 ± 7·7
Epa (Tutu)		Groundnut (Raw)		28·3 ± 9·0	78·8 ± 8·6
Egusi		Melon seed		41·5 ± 6·6	66·8 ± 7·4
Iru	*Parkia filicoidea* Welw	Locust beans		8·8 ± 3·1	12·7 ± 4·5
Ogiri		Fermented melon seed		4·9 ± 2·3	7·8 ± 2·2
Spices					
Ata Tutu	*Capsicum* sp.	African ripe red pepper (Fresh)	86·9 ± 5·5	16·8 ± 2·1	26·3 ± 7·7
Ata Gbigbe		African ripe red pepper (Dry)		20·6 ± 3·4	32·2 ± 6·1

TABLE 1—*contd.*

Local name of raw food materials	*Scientific name*	*English name*	*Moisture content (%)*	*Free folate content (mg/100 g)*	*Total folate content (mg/100 g)*
Roots and tubers					
Isu Ibo	*Dioscorea cayenensis* L.	Yellow yam	71·9 ± 3·8	24·3 ± 3·8	38·7 ± 5·7
Isu Funfun	*Dioscorea cotundata* Pior	White yam	73·4 ± 4·1	22·6 ± 6·5	30·3 ± 3·5
Ewura	*Dioscorea alata* L.	Water yam	80·6 ± 3·5	20·5 ± 4·1	29·8 ± 6·3
Koko	*Colocosia esculenta* sp.	Cocoyam	70·0 ± 3·8	18·7 ± 5·4	24·3 ± 3·7
Esuru	*Dioscorea dumelorium* Pax	Cluster yam	73·6 ± 4·8	20·1 ± 3·8	25·8 ± 3·4
Gbaguda	*Manileut utilissime* Pohu sp.	Cassava	78·6 ± 6·3	9·2 ± 2·6	16·3 ± 2·9
Odunkun	*Ipomoea batatas* Pior	Swect potato	79·0 ± 3·1	30·6 ± 6·2	50·6 ± 3·9
Potato	*Dioscorea bulbifera* sp.	Potato	79·3 ± 4·8	10·6 ± 3·3	14·4 ± 1·1
Cereals					
Iresi	*Oryza sativa* L.	Rice		11·9 ± 3·3	24·8 ± 3·4
Agbado (Tutu)	*Zea mays* L.	Fresh corn	69·9 ± 5·1	23·9 ± 2·5	31·6 ± 7·1
Oka baba (pupa)		Red millet		6·8 ± 2·1	15·3 ± 2·9
Oka baba (funfun)		White millet		7·8 ± 1·9	18·7 ± 3·8
Jero/Dawadawa		Sorghum variety		6·9 ± 2·3	14·9 ± 2·7
Fruits					
Osam		Orange	89·3 ± 1·2	10·1 ± 0·9	15·4 ± 1·8
Osan		Tangerine	88·2 ± 4·3	12·2 ± 2·3	18·3 ± 1·1
Ibepe		Pawpaw	92·4 ± 2·4	3·5 ± 1·1	5·6 ± 1·9
Mangoro		Mango	82·8 ± 2·2	2·6 ± 1·5	7·3 ± 1·7

Gurova	Guava	86·8 ± 2·2	7·7 ± 1·9	11·0 ± 2·1
Opon Oyinbo	Pineapple	84·8 ± 4·3	7·3 ± 2·2	12·1 ± 1·8
Osan Kikan	Grapefruit	87·3 ± 5·2	5·4 ± 0·9	10·3 ± 1·3
Osan Wewe	Lime	85·6 ± 3·9	4·6 ± 1·1	6·1 ± 1·7
Osan Wewe Kikan	Lemon	84·5 ± 5·1	5·3 ± 1·3	6·6 ± 1·2
Ogede Omini	Banana	77·3 ± 5·8	8·3 ± 2·4	15·3 ± 4·2
Ogede Paranta	Banana	75·5 ± 6·3	8·8 ± 1·9	14·8 ± 2·7
Ogede agbagda	Plantain	73·6 ± 4·9	9·1 ± 3·6	16·3 ± 2·8
Pear	Avocado pear	81·2 ± 6·5	9·8 ± 6·6	21·8 ± 4·5
Ubepa	Local pear	83·3 ± 7·1	10·6 ± 3·8	24·8 ± 3·9
Meat products				
Ifun*	Offals*	75·1 ± 3·2	40·8 ± 6·5	72·8 ± 5·4
Eran Elede	Pork lean meat	68·7 ± 4·8	Trace	Trace
Eran Malu	Cow lean meat	71·2 ± 5·8	Trace	Trace
Eran Adie	Poultry meat	69·7 ± 5·7	Trace	Trace
Sea Foods				
Eja Olora	Fatty fish	70·3 ± 3·9	3·8 ± 1·2	11·3 ± 2·8
Eja	Fish	79·8 ± 4·6	4·3 ± 1·0	15·6 ± 1·8
Ede	Crayfish	65·8 ± 6·8	6·6 ± 0·9	11·8 ± 2·9
Akan	Crab	70·6 ± 4·1	5·9 ± 1·6	10·8 ± 3·3
Eggs				
Eyin Agriculture	Cage eggs		8·8 ± 2·3	16·3 ± 2·8
Eyin Adie	Free-range eggs		10·2 ± 1·9	24·8 ± 3·9

* Offals is a mixture of the following: intestine, stomach, kidney, liver and heart.

RESULTS AND DISCUSSION

Fresh vegetables, various tropical roots and tubers are very good sources of folate. However, it must be borne in mind that appreciable losses may occur during preparation and cooking of the food material (Cheldelin *et al.*, 1943; Schweigert *et al.*, 1946; Hurdle, 1967; Schroeder, 1971; Hoppner *et al.*, 1972).

Dried vegetables, various lean meats, including poultry, sea foods and fruits had low contents of folic acid (Table 1). This confirms the findings of Hurdle *et al.* (1968) that folic acid is more stable in fresh food samples in the presence of ascorbic acid, and these fresh vegetables are confirmed to be good sources of vitamin C (Keshinro & Ketiku, 1979). Since most fruits are consumed raw, a sufficient quantity can provide an appreciable amount of the daily folate requirement.

More than 60% of a normal Nigerian diet is made up of roots and tubers (Keshinro, 1980). Since a four-year-old child can consume up to 400 g of roots and tubers (cooked) at a single meal (Keshinro, 1980) they could supply an appreciable percentage of the daily folate requirement provided the folic acid loss is curtailed to the barest minimum during cooking.

ACKNOWLEDGEMENTS

I acknowledge the valuable help given me by the Laboratory of the Government Chemist, London, and its staff in providing me with facilities and assistance during my attachment to the institution. My gratitude also goes to the Commonwealth University Commission for financial assistance during my programme in the United Kingdom.

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Chemical Changes During the Ripening of Domiati Cheese Made From Dried Milk

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(Received: 27 April, 1982)

ABSTRACT

The ripening of Domiati cheese made from fresh and dried milk was investigated. Nitrogen fractions were determined, the proteinaceous fraction of cheese was analysed on Sephadex G-100 and the free fatty acids were determined by gas–liquid chromatography. The nitrogen fractions from fresh milk cheese had higher nitrogen concentrations than those from cheese made from dried milk. The elution pattern of the proteinaceous fractions and the pattern of free fatty acids of fresh and dried milk cheese were similar. However, the small molecular weight N fractions and the concentration of free fatty acids were higher in the former cheese than the latter.

INTRODUCTION

Domiati cheese is considered to be the most popular indigenous soft cheese in Egypt. The increasing demand for this type of cheese conflicts with a marked shortage in the fresh milk supply. Several studies have been carried out to solve this problem by the use of dried milk in cheese making (Hamdy *et al.*, 1970; Hofi *et al.*, 1970; Mahran *et al.*, 1970; Abdel Kader, 1971; El Sadek *et al.*, 1974; Omar, 1977). Most of these studies were mainly concerned with the technological problems, e.g. slow coagulation, weak curd and slow drainage of the whey.

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It is generally recognised that protein—and, in some cases, fat—breakdown is a good index of the progress of ripening and the products of these processes may contribute to background taste and provide a pool of flavour precursors. However, there is little information on changes in cheese protein and the formation of free fatty acids during the ripening of Domiati cheese made from reconstituted milk. Therefore, the present study was carried out to obtain information on these changes.

MATERIALS AND METHODS

Materials

Fresh cow's milk was obtained from the herd of the Faculty of Agriculture, Zagazig University, Egypt. Skimmed and whole dried milks were purchased from the Misr Food and Milk Company. Commercial calf liquid rennet and good quality dairy salt were used in cheese making.

Cheese making

Skimmed and whole dried milks were reconstituted in water at a ratio of 1:8. Domiati cheese was made from fresh cow's milk and reconstituted milks as described by Fahmi & Sharara (1950). The fat percentage of the cheese milk in each case was adjusted to about 4% by the addition of cream where necessary. Resultant cheese was pickled in salted whey (about 10% NaCl) for 2 months at room temperature (20–25°C). Treatments were conducted in triplicate.

Cheese analysis

Compositional analysis of cheese
Cheese samples were analysed when fresh and after 2 months of pickling for moisture, fat, acidity and total nitrogen contents, as described by Ling (1963).

Nitrogen distribution
Water-soluble nitrogen (SN) and non-protein nitrogen (NPN) were determined periodically by the semi-microKjeldahl method, as described by Ling (1963). Peptide nitrogen and amino acid nitrogen were estimated as given by Stadhauders (1959).

Gel filtration of cheese protein

The proteinaceous fractions were extracted from the cheese samples (1 and 2 months old) as described by Tokita & Mesono (1968). Ten millilitres of the extract were loaded onto a column (70 × 2·4 cm) of Sephadex G100 (Pharmacia Ltd, Sweden), previously prepared and equilibrated with 0·033 M sodium acetate buffer (pH 7·0). The columns were eluted at 40 ml/h with the same buffer. Fractions of 10 ml were collected and monitored at 280 nm. The fractions of each peak were collected together and analysed for nitrogen content and expressed as a percentage of the total nitrogen of the original sample. The molecular weight of the fractions of each peak was calculated as given by Determan (1968).

Preparation of the samples for gas–liquid chromatography

The sodium soaps of the free fatty acids were prepared from the 2-months-old cheese samples according to the method of Kuzdzal & Kuzdzal-Savoie (1966). Methyl esters of the free fatty acids were prepared as described by Kuzdzal-Savoie & Kuzdzal (1967). Methyl esters were separated in a Pye Unicam series 104 gas chromatograph (Pye Unicam, Cambridge, Great Britain) equipped with a dual flame ionisation detector. Columns 3·6 m long and of 2 mm inner diameter were used with 80–100 mesh silanised Chromosorb W carrier coated with 10% polyethylene glycol adipate as a stationary phase. Temperature programming at a rate of 5 °C/min was applied in the range of 130–180 °C. The temperature of the injection port was 200 °C and that of the detector, 300 °C. Carrier gas flow (He) was adjusted to 35 ml/min. Chart speed was 5 mm/min. Peak areas were calculated by multiplying the peak height at the maximum by the width of the peak at half of its height. Results are expressed as milligrams per 100 g of cheese.

RESULTS AND DISCUSSION

The gross chemical composition of cheese

Table 1 shows that Domiati cheese made from reconstituted skimmed or whole dried milk differed from control cheese made from fresh milk in having higher moisture, higher salt, lower fat and lower total nitrogen contents. However, small differences were observed in the gross chemical composition of Domiati cheese made from skimmed or whole dried milk.

TABLE 1
Gross Chemical Composition of Domiati Cheese Made from Dried Milk

Composition	*Pickling period (weeks)*	*Fresh milk cheese*	*Skimmed dried milk cheese*	*Whole dried milk cheese*
Moisture (%)	Fresh	62·4	64·6	64·9
	(8)	55·2	59·5	58·3
Salt (%)*	Fresh	10·9	13·1	14·1
	(8)	14·2	19·6	19·5
Fat (%)*	Fresh	42·5	41·0	41·3
	(8)	46·9	44·4	45·6
Total nitrogen (%)	Fresh	2·62	2·36	2·20
	(8)	2·46	2·12	2·09

* Results are given on a dry matter basis.

The results obtained could be attributed to the effect of heat treatment on milk proteins and fat during the drying process with decreases, as reported, in the protein and fat retention (Theo *et al.*, 1970) that enhances the penetration of salt into the cheese (Peters & Knoop, 1974). The general trend of the results agrees with those of Hamdy *et al.* (1970) and Hofi *et al.* (1970).

TABLE 2
Nitrogen Fractions of Domiati Cheese Made from Dried Milk

Fractions of nitrogen (% of TN)	*Pickling period (weeks)*	*Fresh milk cheese*	*Skimmed dried milk cheese*	*Whole dried milk cheese*
SN*	Fresh	10·4	8·15	9·11
	(8)	19·6	17·2	18·4
NPN**	Fresh	3·96	3·01	3·14
	(8)	9·66	8·12	8·53
Peptide N	Fresh	1·19	0·92	0·87
	(8)	4·01	2·18	2·95
Amino acid N	Fresh	0·54	0·39	0·35
	(8)	3·96	2·44	2·36

* Soluble nitrogen compounds.
** Non-protein nitrogen.

Nitrogen distribution of the protein degradative products

Table 2 shows that protein degradation took place in fresh milk cheese at a relatively higher rate than in cheese made from skimmed or whole dried milk. The percentages of SN, NPN, peptide N and amino acid in the former cheese were higher than that in the latter at each period of ripening. These results could be due to the higher salt contents of the dried milk cheeses (Table 1) which reduce the proteolytic activity of rennet (Fox & Walley, 1970; Noomen, 1978). The high salt content might also reduce the growth and activity of lactic acid bacteria which are considered to be essential contributors to protein degradation (Exterkate, 1975).

Gel filtration of cheese protein on Sephadex G-100

The elution pattern of cheese protein on Sephadex G-100 is illustrated in Fig. 1 and Table 3. Three major fractions (I, II and III) were obtained from 1-month-old cheese samples made from fresh or dried milks. The molecular weights of these fractions were 200 000, 55 000 and 30 000 for fractions I, II and III, respectively. The percentage of the area of the low molecular weight fraction (fraction III) was higher in fresh milk than in dried milk cheese. After 2 months of ripening, an additional fraction (fraction IV) was obtained in the elution pattern of cheese protein extracted from fresh and dried milk cheese. The percentage of the area of this fraction (of total elution pattern area) was also higher in fresh milk cheese. In the light of the above-mentioned evidence it could be concluded that protein degradation took place in cheese made from fresh milk at a higher rate than in dried milk cheese.

Free fatty acids

Table 4 shows that the pattern of the free fatty acids extracted from fresh or dried milk cheeses was similar. However, the fresh milk cheese showed higher concentrations of the free volatile and non-volatile fatty acids compared with the dried milk cheese. Meanwhile, small differences were observed in the concentrations of the individual free fatty acids of Domiati cheese made from skimmed or dried milks. The results indicate that the hydrolysis of fat in Domiati cheese made from fresh milk was higher than in cheese made from dried milk. This was in agreement with the results obtained by Poznanski *et al.* (1978).

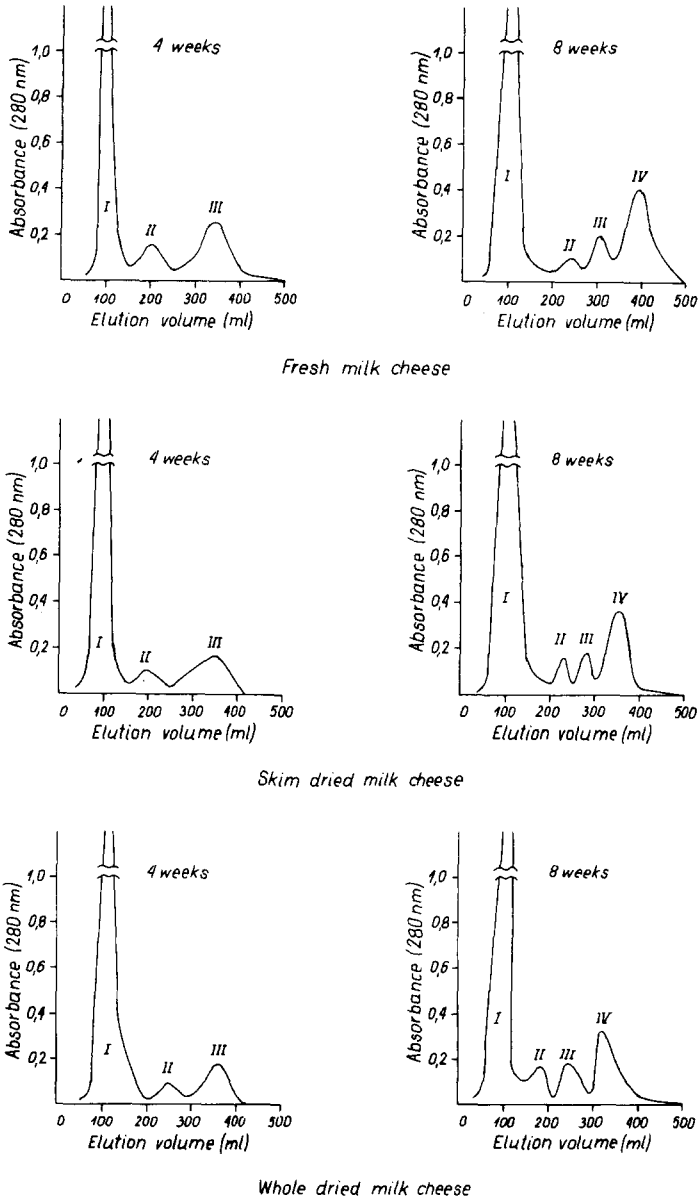

Fig. 1. Sephadex G100 elution diagram of Domiati cheese protein.

TABLE 3
Fractionation on Sephadex G100 of Soluble Protein Fractions from Domiati Cheese

Total elution pattern area		*Cheese manufactured from*					
		Fresh milk		*Skimmed dried milk*		*Whole dried milk*	
		Age in weeks					
		4	*8*	*4*	*8*	*4*	*8*
Fraction I	Molecular weight[a] ($\times 10^3$)	200	170	200	170	200	170
	Nitrogen (%)	1·53	0·41	1·59	0·42	1·43	0·44
	Per cent of total area	58·6	1·86	67·6	19·8	65·0	21·0
Fraction II	Molecular weight[a] ($\times 10^3$)	55	40	65	40	65	40
	Nitrogen (%)	0·72	0·27	0·48	0·31	2·52	0·34
	Per cent of total area	27·4	11·2	20·3	14·5	23·7	16·2
Fraction III	Molecular weight[a] ($\times 10^3$)	30	20	30	20	30	20
	Nitrogen (%)	0·37	0·19	0·29	0·17	0·25	0·13
	Per cent of total area	14·0	7·11	12·1	8·12	11·3	6·19
Fraction IV	Molecular weight[a] ($\times 10^3$)	—	10	—	10	—	10
	Nitrogen (%)	—	1·59	—	1·22	—	1·18
	Per cent of total area	—	64·9	—	57·6	—	56·7

[a] Molecular weight was estimated as the point equivalent to maximum height of the individual peaks.

TABLE 4
Free Fatty Acids from 8-week-old Domiati Cheese Made from Fresh Milk, Skimmed Dried Milk and Whole Dried Milk (mg/100 g of Cheese)

Fatty acids	*Cheese from*		
	Fresh milk	*Skimmed dried milk*	*Whole dried milk*
C_2	1·44	Trace	0·73
C_4	0·34	Trace	0·29
C_6	0·78	Trace	0·49
C_8	1·90	Trace	0·92
C_{10}	4·35	3·90	3·35
C_{12}	6·20	5·20	4·11
C_{14}	11·9	11·2	10·0
C_{16}	33·1	29·4	23·6
C_{18}	6·16	5·93	6·09
$C_{18:1}$	12·9	19·5	14·3
$C_{18:2}$	1·38	1·27	2·39
$C_{18:3}$	0·59	Trace	Trace

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Stability of Calcium Salts of Phosphorylated Sucrose in Fluids of the Digestive Tract

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(Received: 23 July, 1982)

ABSTRACT

Calcium salts of phosphorylated sucrose, in addition to having cariostatic properties, can serve as a source of calcium and phosphorus and partly satisfy the nutritional requirements of an organism. It has been proved that two such preparations, commercial Australian 'Anticay' and a Czech product, have similar properties. Calcium present in these preparations is dissociated partly in saliva and totally in gastric juice. Phosphorus is released only in the duodenum and small intestine. Calcium salts of phosphorylated sucrose are not hydrolysed by invertase.

INTRODUCTION

Synthetic esters of sucrose and phosphoric acid in the form of their calcium salts are used as food additives. They are mainly used as cariostatic agents (Colonial Sugar Refining Co., 1959, 1964*a*, 1965*a*) and for technological advantages, e.g. in the production of breakfast cereals (Stauffer Chemical Company, 1966). Owing to their chemical composition they can serve as a suitable form of calcium and phosphorus supply. Calcium salts of phosphorylated sucrose are produced by technology described in the patent literature (Colonial Sugar Refining Co., 1964*b*, 1965*b*, 1968, 1969*a*, *b*, *c*, 1970*a*, *b*; Wheatstone, 1972, etc.). The product is not one compound, but a mixture of several esters of sucrose, mainly monophosphate, diphosphate, cyclic phosphate and

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phosphoric acid esterified with two molecules of sucrose. Inorganic phosphates are the normal components of these preparations and are mainly responsible for the cariostatic effect. The preparation widely used is known under the commercial name 'Anticay'. A preparation with a similar chemical composition to Anticay was prepared on a laboratory scale in the Department of Organic Technology of the Prague Institute of Chemical Technology (Ranný, 1976). The authors used this opportunity to compare some biochemical properties of both preparations. It should be noted that the first commercial preparation of calcium salts of phosphorylated sucrose (called Hesperonal) had already been introduced on the market by Merck after the procedure described by Neuberg & Pollak (1910).

EXPERIMENTAL

Materials

Calcium salts of phosphorylated sucrose (Ca–PSa) were prepared in the Department of Organic Technology (Ranný, 1976). Anticay was a commercial product (Sugar Refining Company Ltd). The gastric and duodenal juices were kindly provided by the Institute of Experimental Medicine of the Thomayer Hospital in Prague-Krč. The intestinal mucosa was obtained from pork and mice intestine in our laboratory. Trypsin, pancreatin (pancreatic amylase) were from Merck, Darmstadt, pepsin from Léčiva, Prague, and chymotrypsin from Reanal, Budapest. The other chemicals used were supplied by Lachema, Brno.

Methods

Determination of the availability of calcium and phosphate ions in the fluids of the digestive tract after dissolution of Ca–PSa

Sixty milligrams of each preparation (Ca–PSa or Anticay) were dissolved in 5 ml of the appropriate medium (saliva, gastric and duodenal juices, mucosa homogenate, distilled water, 0·16 M HCl and artificial gastric juice) and equilibrated to 37 °C. Samples (1 ml) were taken at appropriate time intervals and the concentrations of calcium and phosphate ions were determined by the commercial test of Lachema, Brno. The samples of saliva, gastric and duodenal juices were collected from a few healthy people. The artificial gastric juice (780 ml 0·1 M KH_2PO_4 and 90 ml 2 M

HCl were adjusted with distilled water to 6 litres) was prepared according to Stoilov (1977). Intestinal mucosa was homogenized both in 0·1 M Tris-HCl buffer (pH 7·2) and in 0·1 M $NaHCO_3$ (pH 8·3).

Separation of Ca–PSa hydrolytic products by TLC
Glass plates (20 × 20 cm) were coated with Kieselgel (nach Stahl, Merck) in 0·02 M acetate buffer, pH 5·0 (30 g/60 ml) and 0·25 mm thick layers were prepared. Neutralized samples (2 μl) were applied on the activated plates which were developed in ethylacetate: 65 % isopropanol = 65:35 to a distance of 16 cm. The separated substances were detected with orcinol reagent (Stahl, 1969): 1 g $FeCl_3$ in 100 ml 10 % H_2SO_4 and 6 % orcinol in ethanol mixed in the ratio 10:1 just before using.

Assay of enzymatic activities
Activities of pepsin, trypsin and chymotrypsin were assayed by the methods described by Keil & Šormová (1959). Activity of amylase was examined according to Bergmeyer (1974). The uptake of CO_2 by *Saccharomyces cerevisiae* was measured using the Warburg apparatus (Kleinzeller, 1964).

RESULTS AND DISCUSSION

Dissociation of Ca–PSa in model media

Ca–PSa is dissolved well in distilled water except for a small proportion of insoluble calcium phosphate. Calcium originating from Ca–PSa is well dissociated immediately after dissolving. The same situation can be followed when Ca–PSa preparations are dissolved in 0·16 M HCl (approximately the concentration of HCl in gastric juice) (Table 1). On the contrary when the artificial gastric juice is used a part of the calcium released is bound to phosphate present in this model medium.

Phosphate ions are not released either in distilled water or in 0·16 M HCl or in the artificial gastric juice.

Availability of calcium and phosphate ions after dissolution of Ca–PSa in saliva in vitro
The course of calcium release into ion form in time dependence is shown in Table 1. Immediately after dissolution of the preparation about 30 % of

TABLE 1
Changes in the Concentration of Available Dissociated Calcium in Human Saliva, Gastric Juice and 0·16 M HCl after Dissolution of Ca–PSa and Incubation at 37 °C

Medium	*Hours of incubation*					
	0	*0·5*	*1*	*1·5*	*2*	*3*
	Available calcium in dissociated form (of amount in dissolved Ca–PSa)					
Distilled water	86·0	86·2	86·5	87·0	86·5	86·2
0·16 M HCl	85·6	85·8	86·0	85·6	86·6	86·5
Saliva	31·0	50·3	53·9	51·6	52·0	50·0
Gastric juice	64·8	68·1	79·4	81·1	83·5	86·0

calcium is available in the dissociated form. In the following half hour of incubation the amount of calcium ions increases to about 50 % of calcium content in the preparation dissolved. This stepwise increase of calcium ion concentration in time course is in relation to the rate of Ca–PSa dissolving in the viscous medium of saliva. The reason for the limited amount of calcium available from Ca–PSa in saliva probably lies in the fact that saliva is, under normal circumstances, partly saturated with calcium (Ganong, 1973) and therefore only a part of the calcium ions can be released into solution. Of course, phosphate also naturally occurs in saliva and can bind part of the calcium ions as well. The total amount of calcium available from Ca–PSa in saliva depends on the amount of Ca–PSa dissolved and the properties of saliva (mainly the concentrations of calcium and phosphate ions). Table 1 represents an average because a combined sample of saliva was used for these experiments.

Breakdown of ester bonds was not found after dissolving Ca–PSa in saliva (under experiments *in vitro*) or during the following incubation at 37 °C, in spite of the fact that some phosphatase activity is supposed to be present in human saliva according the literature (Šantavý, 1975). It is possible to assume that in saliva only a part of the calcium present in Ca–PSa (or Anticay) is available in dissociated form while all phosphorus remains bound.

Availability of calcium and phosphate ions after dissolution of Ca–PSa in gastric juice in vitro

The availability of calcium in gastric juice is evident from Table 1. As in saliva, the greatest dissociation of calcium occurs immediately after

dissolution. In the course of the following incubation the amount of dissociated calcium is then further increased. After 3 h of incubation about 90 % of the calcium present in the preparation can be found in the dissociated form. This 90 % of the calcium present in Ca–PSa represents all of the calcium bound to sucrose. The rest of the calcium forms insoluble—and therefore unutilizable—calcium phosphate.

The ester bonds of phosphoric acid present in the compounds of Ca–PSa (or Anticay) are not hydrolysed so well in gastric juice. There are two reasons for this. First, phosphatases do not occur in gastric juice. Secondly, its acidity is not sufficient for the hydrolytic breakdown of ester bonds. This was proved in model experiments using different solutions of HCl. Ca–PSa kept in 0·2 M HCl at 40 °C for 30 h remained untouched (Fig. 1, spot 5). The hydrolysis of Ca–PSa in 1 M HCl at 100 °C for 30 min

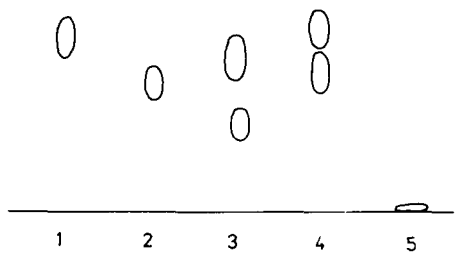

Fig. 1. Separation of hydrolysis products of Ca–PSa by TLC 1. Glucose. 2. Fructose. 3. Ca–PSa hydrolysed in 1 M HCl at 100 °C for 30 min. 4. Sucrose left in 0·2 M HCl at 40 °C for 30 h. 5. Ca–PSa left in 0·2 M HCl at 40 ° for 30 h.

caused its breakdown to two compounds which are not glucose and fructose but some phosphorylated monosacharides. This finding shows high resistance of ester bonds to hydrolysis in the acid environment. The results obtained indicate that ester bonds of Ca–PSa can be split only by the action of phosphatases in distal parts of the digestive tract.

Stability of Ca–PSa in duodenal juice

Because of the finding that all calcium bound in the preparation of Ca–PSa is present in gastric juice in available dissociated form there was no reason to investigate its dissociation in the media of the intestines. We suppose that calcium transferred to the soluble form in the stomach is mainly absorbed in this part of the digestive tract. The general view on the resorption and utilization of calcium *in vivo* will be given in a subsequent paper by means of the preparation labelled with radionuclide ^{45}Ca

(Rauch *et al.*, 1983). The time course of the hydrolytic breakdown of ester bonds is shown in Fig. 2. About 15% of esterically bound phosphorus was released into inorganic phosphate form. A similar course of hydrolysis was demonstrated both for Australian Anticay and the Czech product. This finding is in accordance with the literary data that the activity of alkaline phosphatase in intestines is quite low compared with other tissues (Hasselgren *et al.* 1978).

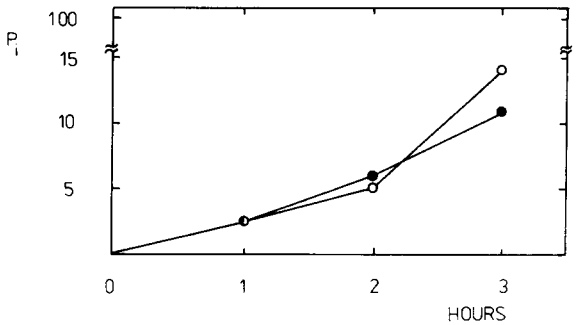

Fig. 2. Hydrolytic breakdown of Ca–PSa in duodenal juice. ○ = Anticay, ● = Cs–PSa (Czech preparation). P_i = Per cent of released inorganic phosphate from Ca–PSa.

Stability for Ca–PSa in the medium of the small intestine

For technical reasons the stability of Ca–PSa in the small intestine was investigated in the appropriate parts of mice and pork gut. Owing to the variability of intestinal content, the pH of the media was adjusted to pH 7·2 or 8·3. The hydrolysis of ester bonds of phosphoric acid is shown in Fig. 3 using pork intestinal mucosa. The course of hydrolysis in the

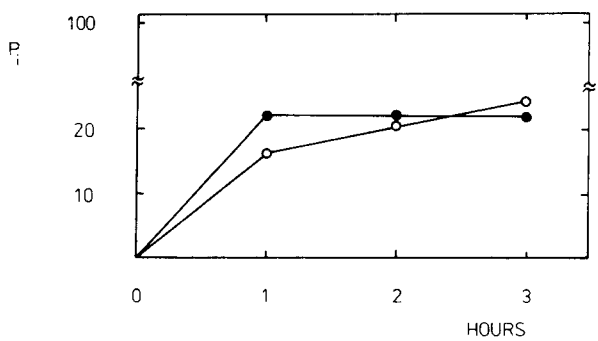

Fig. 3. Hydrolytic breakdown of Anticay in the medium of the small intestine. ● = pH 7·2, ○ = pH 8·3.

medium prepared from mice intestinal mucosa was similar. It is also evident (Fig. 3) that there are no differences in the rate of ester bond hydrolysis at each pH tested which roughly represent the usual pH of intestinal contents. The hydrolysis of ester bonds of Ca–PSa is caused by the intestinal phosphatases which were proved to be present in this part of the intestinal tract (Fishman, 1974; Hasselgren *et al.*, 1978). Intestinal phosphatases do not change their activities markedly in the pH range studied. The degree of Ca–PSa hydrolysis in the small intestine seems to be higher than in the duodenum. The real amount of hydrolysed bonds of phosphoric acid will, however, be affected *in vivo* by a number of scarcely predictable factors. Also, *in vivo* the splitting of ester bonds is a continuous process while *in vitro* it is evaluated separately in duodenal juice and intestinal mucosa. It can be supposed, however, that total hydrolysis of Ca–PSa will not be achieved and therefore a part of the phosphorus will not be utilized in the organism. An important advantage of the Ca–PSa (or Anticay) preparation can be seen in the fact that ions of calcium and phosphorus are released step by step. The release of phosphorus in the intestinal part of the digestive tract avoids the formation of insoluble calcium phosphate in the stomach and therefore the utilization of both inorganic ions is better than when they are applied in the form of their soluble inorganic salts. This finding indicates the advantages of Ca–PSa which can partly supply the calcium and phosphorus needs of an organism when this compound is added to food as a cariostatic agent. Ca–PSa can, therefore, as well as its protective function against dental decay, fulfil a further important rôle in nutrition. These aspects—and, in addition, there may be some technological ones—makes it possible to apply Ca–PSa to food. The simultaneous utilization of calcium and phosphorus from Ca–PSa makes this cariostatic agent nutritionally advantageous.

Effect of Ca–PSa preparation on enzymes of the digestive tract

The effect of Ca–PSa on the activity of important enzymes of the digestive tract was investigated. On the basis of repeated experiments it can be confirmed that the activities of pepsin, trypsin and chymotrypsin are not influenced by the presence of Ca–PSa (or Anticay) in concentrations up to 2%. The situation is a little more complex in the case of saliva and pancreatic amylase. The activity of both enzymes is affected by change of pH or calcium ion concentration. The concentration of dissociated calcium ions is dependent not only on the concentration of dissolved salts

of phosphorylated sucrose, but on the pH value and phosphate concentration as well.

From the technological point of view it was interesting to know if the investigated preparations of phosphorylated sucrose are split by the action of invertase. The results demonstrated in Fig. 4 show that Ca–PSa is not hydrolyzed by invertase. The breakdown of the preparation (in the

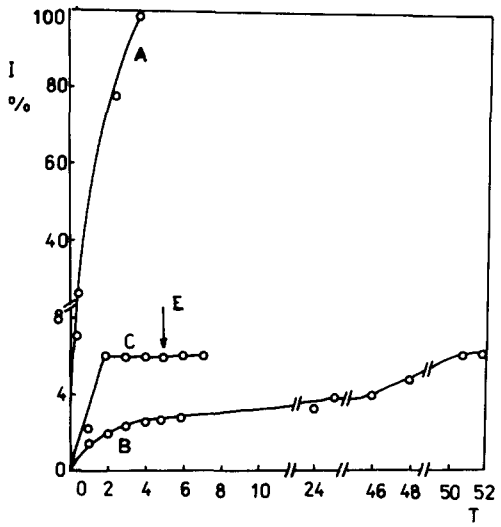

Fig. 4. Effect of invertase on Ca salts of phosphorylated sucrose. I = Per cent of sucrose inversion. T = reaction time (h). A = hydrolysis of sucrose (control). B = hydrolysis course of Ca–PSa preparation (the same conditions as at A). C = hydrolysis course of Ca–PSa at ten times higher activity of invertase. E = second addition of invertase, the same activity as at C.

demonstrated case, 6 %) corresponds to the concentration of free sucrose present in the Ca–PSa used in the experiment. Under comparable conditions the hydrolysis of sucrose (curve A) was terminated in 3 h, 40 min. The small amount of sucrose in Ca–PSa is hydrolysed in the course of 52 h. This very slow rate of hydrolysis is probably caused by the competitive inhibition of Ca–PSa. One hundred times higher concentration of invertase remarkably accelerates the rate of hydrolysis. Hydrolysis was completed in 2 h. However, the amount of hydrolyzed preparation remained unchanged. Similar results were achieved when Ca–PSa contained lower amounts of free sucrose, as well as with Anticay (1 % of sucrose). In all cases the amount of preparation split by the action

of invertase corresponds to the amount of free sucrose present in the preparation.

These observations indicate that the preparations of phosphorylated sucrose cannot be further metabolized without dephosphorylation. The dephosphorylation can be performed by different phosphatases. However, in the digestive tract this can occur only in its intestinal part. The assumption that Ca–PSa cannot be metabolized without dephosphorylation was verified using cells of *Saccharomyces cerevisiae* (baker's yeast). Under comparable conditions the uptake of CO_2 was measured. Free sucrose and phosphorylated sucrose (intact and treated with invertase) were used alternatively as substrates. The amount of CO_2

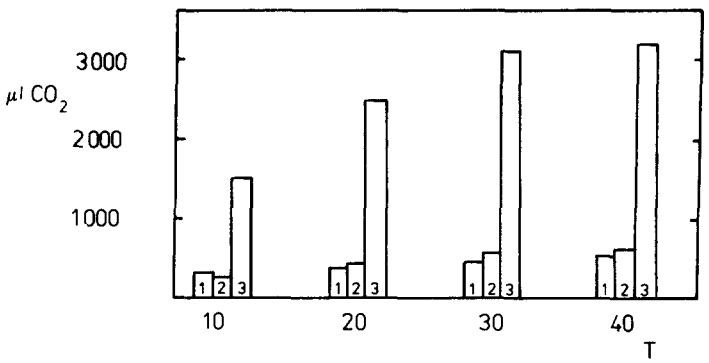

Fig. 5. CO_2 released by yeast in the presence of different substrates. 1. Ca–PSa intact. 2. Ca–PSa treated with invertase. 3. Sucrose. T = time of respiration (min).

released was about 15 % of that found with free sucrose. The rate of CO_2 uptake under these conditions corresponds to the amount of endogenous substrates of yeast cells and free sucrose present in tested preparation of Ca–PSa. (Fig. 5). This finding allows the assumption that Ca–PSa cannot serve as a nutrient for yeast until it is dephosphorylated. These experiments with baker's yeast confirmed the results obtained with isolated invertase. In addition, they proved that Ca–PSa cannot accelerate dough ripening and remain in bakery products unchanged and available in its original form.

CONCLUSIONS

Ca–PSa, which is, in many countries, used as a cariostatic agent, can, as well as this main rôle, support the organism with calcium. The

experiments, performed *in vitro*, prove that calcium bound to phosphorylated sucrose is well dissociable and therefore good presumptions are given for its absorption in organisms. Firm phosphorus bonds assist calcium absorption because the formation of insoluble calcium phosphate is avoided. Partial decomposition of ester bonds of phosphoric acid in the duodenum and intestines indicates the possibility of an adequate utilization of phosphorus in organisms also.

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Effect of Ascorbate on an Isolated Mitochondrial Fraction During Ageing of Swede (*Brassica napus* var. *napobrassica*)

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(Received: 22 October, 1982)

ABSTRACT

The average sedimentation coefficient ($\bar{S}$-value) of swede mitochondria was 8300 ± 400 S in a hypotonic 0·25 M *sucrose buffer, when malate dehydrogenase was used as the marker enzyme. A differential centrifugation procedure giving optimal recovery of the mitochondria has been worked out. Transmission electron micrographs of the isolated mitochondrial fraction show well preserved, but somewhat swollen, mitochondria. The fraction contained approximately 4% plastoglobuli and/or peroxisomes. The respiratory control ratio (RCR; state 3: state 4 respiration) was found to be 3·2 ± 0·6. Ascorbic acid equilibrates with the respiratory chain and both the endogenous and ascorbic acid-stimulated respiration increased throughout the storage year. Ascorbic acid contributed to the non-enzymatic cytochrome* c *reducing activity in plant extracts.*

INTRODUCTION

The swede (*Brassica napus* var. *napobrassica*) can be stored for considerable lengths of time with only slight changes in flavour and colour

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(Berg, van den & Lentz, 1977; Baardseth, 1978). In some fresh vegetables the presence of certain deteriorative enzymes can change the quality within days or weeks (Svensson, 1977). The most important enzymes which are claimed to cause development of off-flavour and off-odour are peroxidase, catalase (Talburt & Smith, 1967) and lipoxygenase (for review, see Galliard, 1975). The swede contains relatively high activities of catalase and peroxidase, while phenolase and lipoxygenase were not detected by Baardseth & Slinde (1980). The absence of lipoxygenase and phenolase may, in part, explain the long storage life of swedes.

The swede contains 25 mg ascorbic acid per gram of protein (Paul & Southgate, 1978). This high amount of ascorbic acid can be an efficient antioxidant and also function as an endogenous substrate to the respiratory chain, as well as contributing to a low redox potential within the swede. In an attempt to elucidate the long storage stability of swede we have compared the ascorbic acid respiration of an isolated mitochondrial fraction from swede when harvested and after 1 year of storage.

Isolation of plant mitochondria is complicated by the toughness of the tissue and inadequate isolation procedures (Jackson & Mooe, 1979). By using the method of optimal mitochondrial recovery (Slinde *et al.*, 1974) an isolation procedure for swede mitochondria has been established.

MATERIALS AND METHODS

Determination of average sedimentation coefficient ($\bar{S}$-value)

The swede (*Brassica napus* var. *napobrassica* var. Bangholm) used in the experiments was obtained locally at harvest and stored for 1 year at 0 °C with 95–100 % relative humidity as described by Berg, van den & Lentz (1977). The swede was washed, peeled, cut and homogenized (Ultra-Turrax TP 18/10, Janke and Kunkel KG) in an equal amount (w/v) of 0·25 M sucrose, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid), 1 mM EDTA, pH 7·4, adjusted with KOH. The homogenate was centrifuged at a centrifugal effect of $10 \times 10^7 \text{ min}^{-1}$ to remove nuclei and other heavy cellular fragments (Slinde *et al.*, 1975). The supernatant was termed '100 % homogenate'. Determination of the $\bar{S}$-value (average sedimentation coefficient) was performed as previously described (Slinde & Flatmark, 1973). Ten-millilitre samples of the '100 % homogenate' were transferred to matched

centrifuge tubes, diluted with buffer to give $R_{min} = 6{\cdot}2$ cm, and subjected to centrifugation at different time integrals of rpm^2. Total time integrals of rpm^2 were determined by an integrator with a precision of 3 % (Slinde et al., 1974). The sedimentation experiments were carried out using the swinging-bucket HB-4 rotor of a Sorvall RC2-B centrifuge. All operations were carried out at 4 °C.

Respiratory rates

Oxygen consumption was determined with a Clark oxygen electrode (Yellow Springs Instr. Biological Oxygen Monitor).

Enzymatic assay

Malate dehydrogenase (EC 1.1.1.37) activity was determined according to Bergmeyer *et al.* (1974). Catalase activity was measured at 230 nm using hydrogen peroxide as substrate (Bergmeyer *et al.*, 1974; Baardseth & Slinde, 1980).

Protein

Protein was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as standard.

Morphological examinations

Fixations for electron microscopy were performed in 1 % followed by 2·5 % glutaraldehyde, and post-fixed in 1 % osmium tetroxide. Specimens were dehydrated and embedded in Epon. The blocks were sectioned on a LKB Ultrotome III, and ultrathin sections (50 nm) stained in uranyl acetate followed by lead citrate, were studied in a Philips EM 300.

RESULTS

Sedimentation and morphology of the mitochondria

Malate dehydrogenase was used as the mitochondrial marker enzyme. Figure 1 shows the sedimentation behaviour of the malate dehydrogenase

activity as a function of the centrifugal effect. The $\bar{S}$-value was found to be 8300 ± 400 S. The convergence level was reached when 23 % of the total malate dehydrogenase activity was sedimented. For optimal recovery (Slinde *et al.*, 1975) of swede mitochondria the fractionation procedure shown in Table 1 has been established using the determined $\bar{S}$-value.

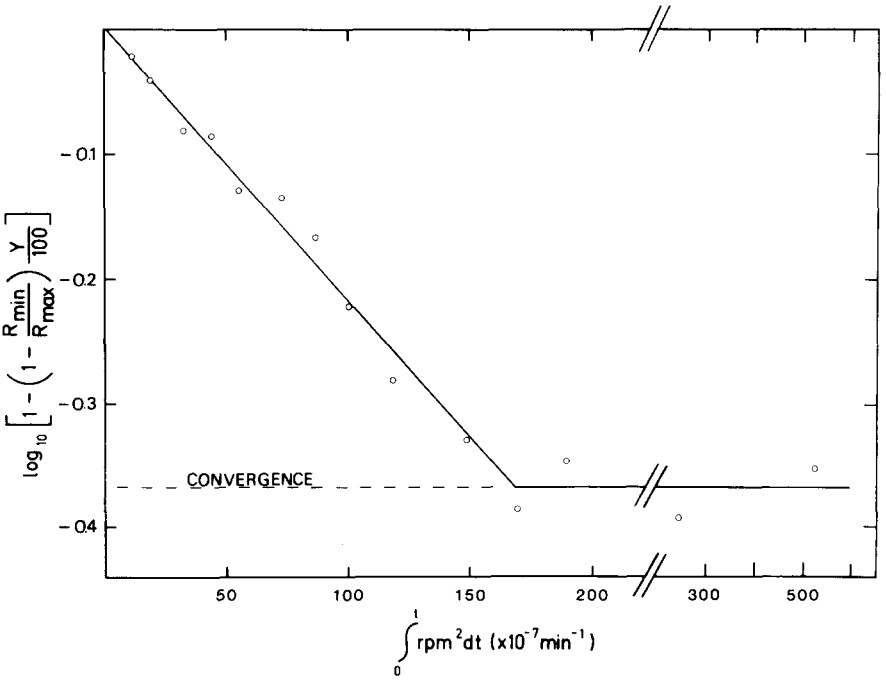
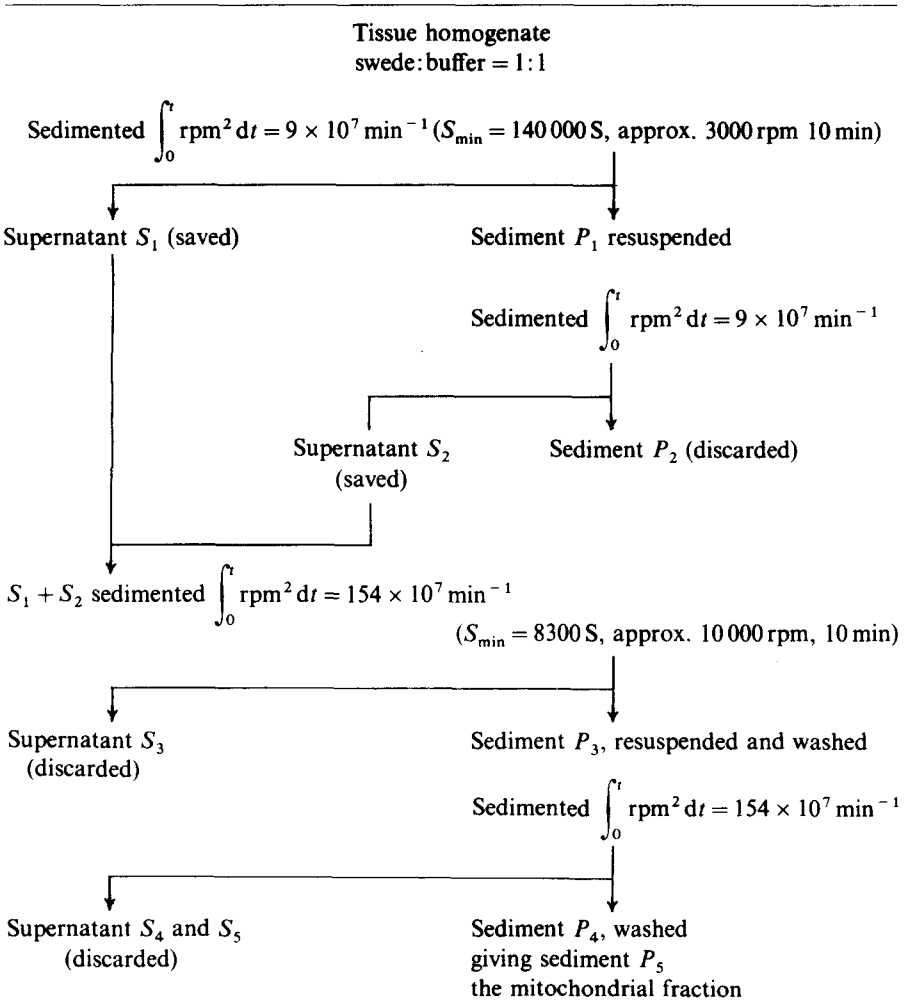


Fig. 1. Determination of the average sedimentation coefficient of swede mitochondria. The protein concentration was 0·63 mg/ml, and malate dehydrogenase was used as the marker enzyme. The average sedimentation coefficient was determined from the slope of the curve, $\bar{S} = 8300 \pm 400$ S. For details see text.

Electron microscopic studies (Fig. 2) revealed that the isolated mitochondrial fraction was somewhat heterogeneous and 3·7 % of the particles were identified as plastoglobuli and/or peroxisomes. Peroxisomes could be identified when the crystal core was present, but this was often lacking. The fraction was, however, rich in catalase activity, which is localized to the peroxisomes of, for example, differentiating potato tuber tissue (Brinkman & Sminia, 1977).

TABLE 1

Procedure for the Optimal Recovery of Swede Mitochondria ($\bar{S} = 8300S$) by Differential Centrifugation Using the HB-4 Rotor of the Sorvall RC2-B Refrigerated Centrifuge ($R_{min} = 6{\cdot}2$ cm and $R_{max} = 14{\cdot}4$ cm). Medium; 0·25 M Sucrose, 10 mM HEPES, 1 mM EDTA, pH 7·4 and 4 °C



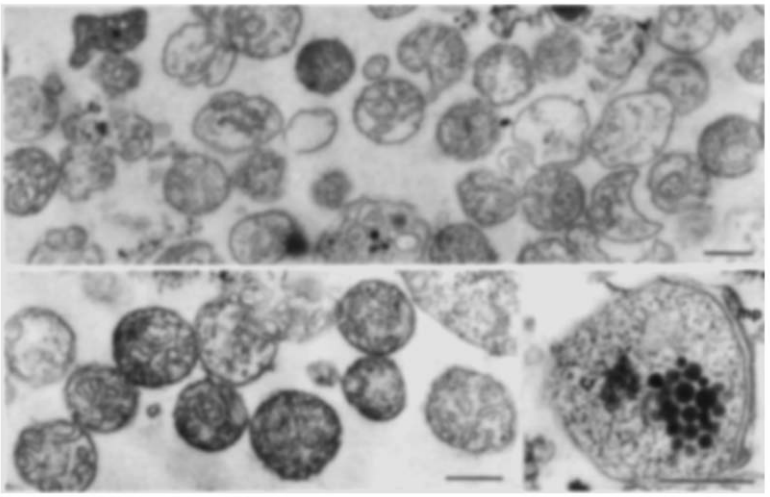


Fig. 2. Transmission electron micrographs of the isolated mitochondrial fraction. (A) General view, (B) intact mitochondrial, (C) peroxisome with crystalloid (cr) and marginal plate (mp). The bars represent 0·5 μm.

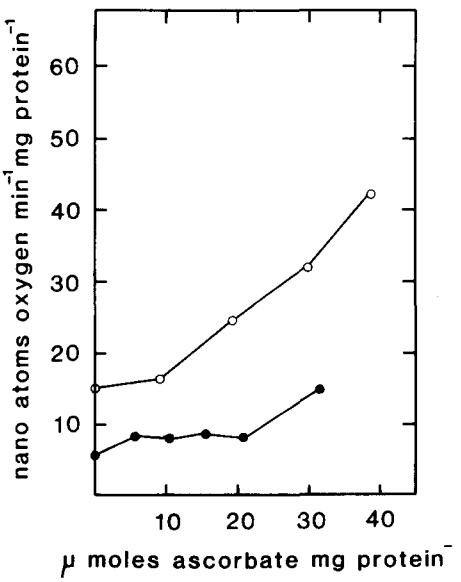


Fig. 3. Respiratory rates in the presence of ascorbate in 50 mM glucose, 175 mM sucrose, 10 mM HEPES, 5 mM $MgCl_2$, 5 mM KH_2PO_4, at pH 7·4 of a newly isolated harvested mitochondrial population (●) and a mitochondrial population isolated from swede that had been stored 1 year (○).

Respiratory properties

The electron micrographs show swollen (Fig. 2(A)) as well as intact (Fig. 2(B)) mitochondria. The state 3 respiration in the presence of ADP was rather high (Jackson & Moore, 1979) as compared with the state 4 respiration with succinate as substrate (conditions as in Fig. 3), giving respiratory control ratios (RCR) of $3{\cdot}2 \pm 0{\cdot}6$ (state 3:state 4). When ascorbic acid was used as substrate, no respiratory control was observed when ADP was added. It was found that the endogenous respiration increased slightly throughout the storage year, and a higher respiratory rate was also observed when ascorbic acid was added. Figure 3 shows a comparison between newly harvested swede and swede stored for 1 year. Mitochondria from newly harvested swede have a low endogenous respiration and are only slightly stimulated by the addition of ascorbic acid. After storage of swede for 1 year the endogenous respiration is higher and ascorbic acid stimulates the respiration. The results have been similar for two storage periods.

DISCUSSION

Swede has very good storage properties (Berg, van den & Lentz, 1977; Baardseth, 1978). Both the peroxidase and catalase activities and the peroxidase/catalase ratio are relatively high (Baardseth & Slinde, 1980).

The respiratory properties of the isolated mitochondrial fraction (Fig. 3) indicate that the redox level of the respiratory chain was influenced by the endogenous amount of ascorbic acid since the respiration of mitochondria from stored swede was stimulated when ascorbic acid is added (Fig. 3). The endogenous respiration increased during the storage period, indicating that other substrates, e.g. fatty acids, were activated to keep a satisfactory redox level in the swede.

Plant extracts generally contain a non-enzymatic endogenous cytochrome *c* reducing activity. We have confirmed this in swede. At present, the nature of this reducing activity is not known, but it has been mentioned that the activity could be due to flavin nucleotides or chlorogenic acid, although the molecular weight of the compounds is not known (Pohl & Wiermann, 1981). However, ascorbic acid is a general reducing agent of cytochrome *c* (Minnaert, 1961). Thus, ascorbic acid present in a plant extract may give reduction of cytochrome *c*.

The mitochondrial fraction isolated in the present study is well defined (Fig. 1, Table 1). The amount of plastoglobuli/peroxisomes represents a problem in the interpretation of the polarographic traces. It is known that peroxisomal enzymes participate in the β-oxidation of fatty acids in mammalia (Lazarow & DeDuve, 1976). An interaction between these two organelles is therefore likely also in plants. Generally, ascorbic acid does not stimulate respiration significantly unless a membrane permeable redox mediator is added. However, when the mitochondria are isolated in a slightly hypotonic solution, as in this study, ascorbic acid equilibrates with the respiratory chain.

In conclusion, our results indicate that the intracellular level of ascorbic acid may influence the storage stability of swedes.

ACKNOWLEDGEMENT

The authors thank Ms Grethe Enersen for technical assistance.

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Quantitative Separation of Species in Fish Mixtures by Multivariate Analysis of Electrofocused Protein Bands

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(Received: 28 July, 1982)

ABSTRACT

Water-soluble proteins from cod and saithe and from mixtures of the two species were analysed by electro-focusing on polyacrylamide gels. Ten protein bands could be quantified by measuring peak heights on the corresponding densitometer traces. By employing multiple linear regression analysis, the composition of mixtures in the range 10–90% cod could be determined.

INTRODUCTION

It has been shown that fish species may be distinguished by electrophoretic analysis of extracts of the flesh proteins (Mackie, 1980). Methods of zone electrophoresis and isoelectric focusing were compared for the identification of raw, cooked and canned fish products.

Isoelectric focusing provides the greatest resolution of proteins and gives distinguishable patterns for even closely related species of fish (Mackie, 1980).

The quantitative determination of cod/saithe mixtures has been studied by electrophoresis (Hume & Mackie, 1980). This method was based on the measurement of two species-specific zones in gel electrophoresis. Good results were obtained provided that peak areas for the single species were determined by extrapolation from the cod/saithe mixtures instead of from

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100% samples. This demonstrates that the peak areas for the two peaks used were not strictly linearly related to the mixture composition.

The quantitative analyses of binary meat mixtures were studied by means of isoelectric focusing of aqueous extracts (Kaiser *et al.*, 1980). Semiquantitative results were obtained based on the measurement of two peaks on the densitograms. Calibration plots were non-linear, displaying, in most instances, S-shaped curves.

Quantitative analyses based on measurements of only two peaks per sample have thus met with limited success.

In the present work ten peaks were measured for each sample densitogram, and quantitative results were calculated by multiple linear regression.

EXPERIMENTAL

Materials

Cod (*Gadus morhua*) and saithe (*Pollachius virens*) were brought fresh to the laboratory. Fillets cut from the flesh were homogenized and mixtures of cod and saithe were prepared in the range 10–90% cod. Homogenates from single species and mixtures were frozen and kept at −20°C for 2 days. Samples were then allowed to thaw at room temperature. Aliquots of the drippings were applied to the electrofocusing gel, after suitable dilution if necessary.

Iso-electric focusing

This was carried out on LKB Ampholine 1804-101 polyacrylamide gels in the pH range 3·5–9·5 using the LKB 2117 Multiphor according to the recommendations of the supplier.

The following solutions were used:

Fixing solution:	Add 57·5 g trichloroacetic acid and 17·25 g sulphosalicylic acid to 500 ml distilled water.
Destaining solution:	Mix 500 ml ethanol and 160 ml acetic acid and dilute to 2 litres with distilled water.
Staining solution:	Dissolve 0·460 g Coomassie Brilliant Blue R-250 in 400 ml destaining solution. Use only once.

Preserving solution:	Add 40 ml of glycerol to 400 ml of the destaining solution.
Anode–electrode solution:	1 M H_3PO_4
Cathode–electrode solution:	1 M NaOH

The LKB 2103 power supply was set to power $P = 30$ W, voltage $U = 1500$ V and current $I = 50$ mA. Time for the experiment was 1·5 h. After completion of the isoelectric separation, the PAG plate was fixed for half-an-hour. It was then washed in the destaining solution for 5 min and was stained for 10 min at 60° in the staining solution. The PAG plate was destained with several changes of the destaining solution until the background was clear. The plate was placed in the preserving solution for an hour. It was then placed on a clean glass plate to dry at room temperature overnight. Finally, the gel surface was covered with a protective plastic sheet.

Densitometry and data analysis

A Pye-Unicam SP 8-100 spectrophotometer equipped with a densitometer accessory was used. Operating parameters were: densitometer

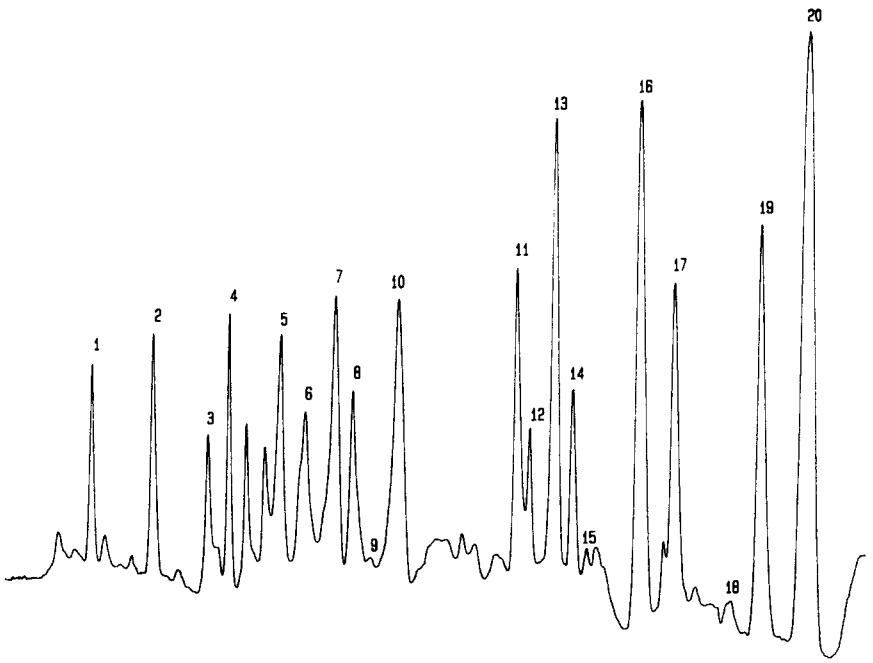

Fig. 1. Densitometer trace of the electrofocused gel of a mixture of cod and saithe containing 30% cod.

speed, 0·2 mm/s; chart speed, 3 cm/min; slit width, 0·2 mm and wavelength, 520 nm.

Peak heights were measured manually for 20 peaks on the densitometer traces (Fig. 1). Peak areas for the same peaks were calculated electronically by feeding the analog output from the spectrophotometer to a Spectra-Physics SP4000 data system for chromatography.

Linear regression calculations and cluster analysis were carried out on a Hewlett Packard 9825 desk-top computer. Cluster analysis was performed as previously described (Bøe & Gjerde, 1980).

RESULTS AND DISCUSSION

Choice of variables

As expected, the automatic integration of peak areas was difficult due to the ill-defined baseline (see Fig. 1). Peak heights were measured manually from a subjectively drawn baseline. The last peak on the densitogram was, in all cases, the greatest, and was relatively constant provided that similar amounts of protein were applied to the gels. Two types of normalization of the densitometer traces were studied. Peak 20 was taken as an internal standard and its area or height was set equal to 100 and all other peaks were expressed relative to this peak. In the second type of normalization the sum of all 20 peak heights or areas was set equal to 1000.

The reproducibility of peak values was studied by measuring ten samples of saithe and nine samples of cod. Peaks in Fig. 1, in addition to those 20 that were quantitatively evaluated, were, in all cases, classified as non-reproducible by visual comparison of the corresponding densitometer traces. For the remaining 20 peaks the following criterion of reproducibility was laid down: if, for a given peak, the maximum value is greater than twice the minimum value for more than one fish of a given species, the peak is classified as non-reproducible. The results obtained by applying this criterion are given in Table 1. It can be seen that peak heights are clearly superior to peak areas. Of the 20 peaks measured, 17 are reproducible for saithe and 15 for cod if peak 20 is used as the internal standard. If we demand reproducibility for both species at the same time, the number of peaks is reduced to ten.

The peak heights measured for the 19 samples of cod and saithe are

TABLE 1
Number of Reproducible* Areas or Peak Heights of a Total of 20, for 10 Saithe and 9 Cod Samples.
Variable normalized on:

	Sum of areas = 1000	*Area of peak 20 = 100*	*Sum of peak heights = 1000*	*Peak height, peak 20 = 100*
Saithe	8	12	16	17
Cod	11	10	14	15
Saithe and cod	4	5	9	10

* Non-reproducible: for more than one sample of cod or saithe, the maximum value is greater than twice the minimum value.

given in Table 2. Peaks numbers 4, 6, 7, 9, 11, 12, 13, 14, 18 and 20 were reproducible and were used in subsequent analyses. It was found that the greatest variability was due to differences in staining and in the amounts of protein applied to the gels. For best results these variables should be controlled as closely as possible.

Cluster analysis

The purpose of the cluster analysis is to study if the 19 fish samples can be classified into two groups based on their protein patterns. The analysis is based on calculations of pairwise distances between objects in a 10-dimensional space, each dimension corresponding to one protein peak. In this treatment the distance between objects is inversely proportional to their mutual similarity. The result of the cluster analysis is presented in graphical form as a dendrogram. Figure 2 shows the dendrogram resulting from the analysis based on Euclidean distances as a similarity index. A very similar result was obtained using correlation coefficients.

The figure may be seen as a graphical representation of the reproducibility within classes, and the separation between classes. It can be seen that cod and saithe samples are grouped into two homogeneous classes which are clearly separated from each other.

It was therefore decided that the quantitative separation of cod/saithe mixtures could be based on average values of peak heights for 10 samples of saithe and 9 samples of cod.

TABLE 2

Peak Heights Measured on Densitometer Traces of Electrofocused Gels for Samples of Saithe and Cod

Species	*Sample No.*	*Peak No.*																			
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*
Saithe	1	570	415	55	505	385	255	845	80	205	150	495	150	540	325	85	710	405	140	80	1 340
	2	480	270	45	415	370	200	625	35	145	95	410	100	430	270	90	530	310	115	55	1 000
	3	500	255	5	455	320	225	580	90	140	120	330	120	460	250	110	520	295	95	80	1 080
	4	635	486	260	540	455	250	805	135	145	550	495	310	465	490	220	630	360	120	600	1 375
	5	515	355	35	480	380	175	600	50	125	180	440	125	235	260	160	550	300	140	180	1 130
	6	680	360	15	485	505	270	665	70	180	180	525	175	600	445	112	660	360	180	90	1 095
	7	665	460	130	545	350	295	535	120	110	470	505	195	630	340	100	600	350	115	300	1 190
	8	640	300	20	595	335	200	780	80	110	170	630	140	460	270	110	725	440	145	135	1 195
	9	685	290	30	740	405	165	830	90	170	160	715	120	470	335	125	760	440	130	140	1 330
	10	495	310	20	700	285	144	778	30	115	100	495	100	546	220	170	625	300	130	60	1 075
Cod	11	60	60	540	95	325	280	190	535	80	590	285	470	462	268	75	90	63	110	775	1 185
	12	50	40	540	135	370	265	147	515	65	650	228	485	370	220	74	92	45	125	880	1 092
	13	100	150	805	285	640	550	115	740	60	890	450	815	600	550	260	60	60	210	1 180	1 780
	14	150	180	870	200	290	725	190	587	90	700	310	760	620	565	270	90	65	100	1 180	1 555
	15	45	45	600	120	85	400	145	405	30	525	135	520	365	328	85	80	60	100	845	1 020
	16	40	45	610	155	65	510	200	545	30	647	240	501	472	330	90	50	75	160	910	1 260
	17	45	85	690	225	208	512	190	776	75	734	320	600	385	350	160	90	120	115	910	1 140
	18	85	125	840	255	425	460	175	765	70	935	285	790	705	650	355	90	80	180	995	1 545
	19	185	295	805	210	370	355	130	860	60	815	400	645	750	700	400	55	55	160	1 060	1 670

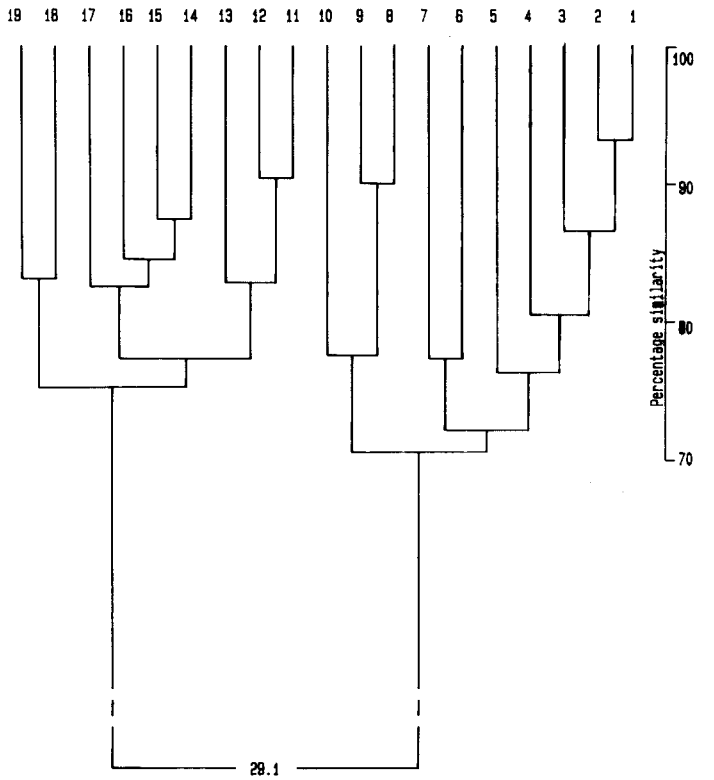

Fig. 2. Dendrogram of cod and saithe based on data for 10 peaks from Table 2. Peak 20 was used as the internal standard.

Quantitative separation of mixtures

Samples of cod and saithe used for the preparation of cod/saithe mixtures were not among those included in Table 2. Peak heights for two sets of mixtures covering the range 10–90 % cod are given in Table 3.

The mixtures were treated as unknowns and their composition was calculated based on the known protein patterns of the classes of cod and saithe established above.

The quantitative separation of cod/saithe mixtures is based on the general linear model (eqn. (1):

$$y = A_0 + A_1X_1 + A_2X_2 \tag{1}$$

TABLE 3
Relative Peak Heights Measured on Densitometer Traces of Mixtures of Cod and Saithe

Per cent cod	*Peak No.* 4	6	7	9	11	12	13	14	18	20
10	40·98	8·72	45·11	1·50	41·35	6·77	62·78	14·66	10·90	100·00
30	39·58	17·97	48·14	5·08	41·53	25·42	72·03	14·66	7·97	100·00
50	33·83	16·92	36·84	2·26	23·68	35·56	55·64	7·89	9·02	100·00
70	22·71	19·52	23·51	7·57	31·27	33·27	43·82	14·34	5·58	100·00
90	13·83	23·40	22·98	4·26	21·28	48·40	43·19	15·96	6·38	100·00
10	47·08	29·53	51·75	0·58	42·40	8·19	65·50	16·08	7·60	100·00
30	37·27	20·91	42·68	1·61	37·53	25·20	68·63	29·49	10·19	100·00
50	27·90	18·45	37·77	0·86	40·34	35·62	72·10	21·46	10·73	100·00
70	17·65	13·24	31·99	1·10	24·63	36·03	72·79	31·25	15·44	100·00
90	10·33	10·33	18·33	0·67	22·67	38·00	70·33	24·33	16·38	100·00
0*	46·37	18·55	59·66	12·27	42·63	12·83	41·29	27·05	11·20	100·00
100*	13·57	33·45	12·67	4·65	21·43	45·79	38·22	31·45	10·31	100·00

* Mean values from Table 2.

where: y = cod/saithe mixture composition (peak pattern) $y = (y_1, y_2, .. y_{10})$; A_0 = constant correction term; A_1 = cod parameter; A_2 = saithe parameter; and

X_1 = cod composition (peak pattern)
X_2 = saithe composition (peak pattern)
$X = (X_1, X_2, \ldots X_{10})$

Results obtained by using eqn. (1) are discussed below, but first a simpler version of the equation is presented.

(*i*) *Simple linear regression*

If it is assumed that the water-soluble proteins from cod behave independently of proteins from saithe, as determined by electrofocusing of cod/saithe mixtures, this corresponds to $A_0 = 0$ and $A_1 + A_2 = 1$ in eqn. (1). Equation (1) is thus transformed into eqn. (2):

$$y - X_2 = A_1(X_1 - X_2) \tag{2}$$

The parameter A_1 is, under the given assumptions, identical to the fraction of cod in the cod/saithe mixture. A_1 can be found by the least squares method, in which case eqn. (2) corresponds to simple linear regression without constant term. Results calculated by eqn. (2) are given in Table 4. It can be seen that there is only a fair agreement between true

TABLE 4
Analysis of Cod/Saithe Mixtures. Percentage of Cod

Actual	*Calculated by*			
	Linear regression		*Multiple linear regression*	
	Calculated	*SD*	*Calculated*	*SD lower–upper limit*
10	8	14·8	10	13·1 – 14·8
30	19	16·0	25	9·7 – 15·3
50	48	13·7	48	0·8 – 11·7
70	63	9·5	62	2·3 – 7·1
90	81	8·7	80	5·6 – 7·8
10	5	14·6	10	14·0 – 15·9
30	31	13·5	36	4·5 – 11·9
50	45	16·3	50	0·2 – 14·0
70	62	17·5	65	6·4 – 15·9
90	78	18·5	79	12·9 – 18·2

SD = Standard deviation.

and calculated compositions. Calculated percentages are, on average, 15·9 relative per cent lower than the true percentages. In particular, the mixtures containing 10% and 30% cod are not reliably determined by eqn. (2). The failure may be associated with the assumption of additivity of cod and saithe proteins, but may also be connected with the general least squares framework underlying the linear regression model (eqn. (2)). The least squares method presupposes that the uncertainty in the independent variable $(X_1 - X_2)$ is less than the uncertainty in the dependent variable $(y - X_2)$. It cannot be assumed that this requirement is fulfilled for the range of mixtures studied.

A better model is therefore sought and may be arrived at by the following argument.

(ii) Multiple linear regression

Synthetic mixtures of cod and saithe may be calculated, based on the pure components, by eqn. (2). Calculated mixtures (y) may then be compared with actual mixtures by correlation analysis using calculated mixtures as the independent variable. The desired composition is then defined as the calculated mixture that gives the highest correlation coefficient. This formulation leads to the general linear regression line (eqn. (3)):

$$y = mx + b \tag{3}$$

where: y = actual cod/saithe mixture composition; x = calculated cod/saithe mixture; m = slope of line; b = constant term.

From eqn. (2):

$$x = A_1(X_1 - X_2) + X_2$$

Therefore: eqn. (3) becomes

$$y = m(A_1(X_1 - X_2) + X_2) + b = mA_1X_1 + m(1 - A_1)X_2 + b$$

$$= P_1X_1 + P_2X_2 + b \tag{4}$$

Equation (4) is equivalent to eqn. (1) and corresponds to multiple linear regression of y on two independent variables, X_1 and X_2. The cod and saithe compositions, X_1 and X_2, represent mean values of 9 and 10 samples, respectively and therefore are more accurate than the actual cod/saithe composition measured on one sample. Equation (4) is therefore a better model than eqn. (2) and would be expected to give better results. The parameters P_1 and P_2 are calculated by multiple linear regression and the cod fraction, A_1, can then be found:

$$A_1 = P_1/m = P_1/(P_1 + P_2) \tag{5}$$

Results calculated from eqn. (5) are given in Table 4. From this Table it is seen that the agreement between true and calculated compositions is better than previously. Calculated compositions are, on average, 4·7 relative per cent lower than true compositions.

Upper and lower limits for the standard deviations of A_1 given in Table 4 were calculated according to Green & Margerison (1978), and correspond to independence of P_1 and P_2 and total correlation ($r^2 = 1$) between P_1 and P_2, respectively. As given above, $P_1 + P_2$ equals m, the slope of the regression line of actual composition against calculated composition. $P_1 + P_2$ was found in the range 1·05–1·10 with a mean value of 1·07. Thus, P_1 and P_2 are strongly correlated and the actual standard deviation of A_1 is expected to lie close to the lower limit and is significantly better than the standard deviation obtained by using eqn. (2).

In conclusion, it has been shown that quantitative separation of species in raw fish mixtures can be carried out by multiple linear regression on patterns of water-soluble proteins.

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Effect of Cooking on Tryptophan, Basic Amino Acids, Protein Solubility and Retention of Some Vitamins in Two Varieties of Chick Pea

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(Received: 9 June, 1982)

ABSTRACT

Egyptian and Italian varieties of chick pea were analysed chemically to study their total constituents. The two varieties were cooked for 1 and 2 h and autoclaved for 1 h at 121°C. Their constituent basic amino acids, tryptophan, thiamin, pyridoxin and niacin, were determined.

There were differences in the chemical constituents between the two varieties. Cooking decreased lysine, histidine and arginine, the effect being highly significant. Tryptophan decreased, the effect being significant. In addition, protein solubility decreased with increased cooking time.

The vitamin content also decreased with increased cooking time. It is preferable to cook chick pea in an autoclave at 121°C for not more than 1 h to minimise losses in vitamins and amino acids.

INTRODUCTION

The effect of heat on the nutritive quality of plant proteins has attracted the attention of many investigators. The nutritional availability of amino acids might be influenced by the treatment of protein. Simple controlled heating improves the nutritional value of seed protein, but amino acids,

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especially lysine, cystine and arginine, are destroyed when heating is severe (Fincher, 1958; Gonzalez *et al.*, 1960).

Miller *et al.* (1973) found a loss of vitamin in legumes resulting from processing, where heating decreased the vitamin content by a variable amount ranging from 25% to 77%.

The study described in this paper had been carried out to compare the effect of cooking on tryptophan, basic amino acids (of the limiting amino acids), protein solubility and the retention of thiamin, pyridoxine and niacin in two different varieties of chick pea.

MATERIALS AND METHODS

Chick pea (*Cicer arietinum*) (2 kg) was obtained from the Ministry of Agriculture, Cairo, Egypt. The Italian variety was obtained from Professor C. Galoppini, Pisa University, Italy. Random samples (100 g each) were cooked according to Hulse *et al.* (1977). Undecorticated raw and dry cooked seeds were ground in a hammer mill to pass through a 40 mesh sieve. Crude protein and protein hydrolysate were prepared according to Gonzalez *et al.* (1960). The basic amino acids were eluted according to the procedure of Moore *et al.* (1958) on a Beckman Model 121 Amino Acid Analyser. Tryptophan was determined according to Sodek *et al.* (1975). Moisture, nitrogen, total sugars, glucose, ash, crude fibre, niacin and oil contents were determined by the methods of the Association of Official Analytical Chemists (1965). Nitrogen solubility was determined by the method of Lawhon *et al.* (1972). Thiamin and pyridoxine were determined according to Osborne & Voogt (1978). Statistical analysis was carried out according to the methods of Ostle (1964).

RESULTS AND DISCUSSION

The results are the average of two determinations. Table 1 shows the chemical constituents of two varieties of chick pea. The protein content of the Egyptian variety (23·1%) was lower than that of the Italian variety (26·1%). The percentages of total sugars, glucose, oil, ash and crude fibre

TABLE 1
Per Cent Composition of Chick Pea

Constituents	*Egyptian variety*		*Italian variety*	
	Flour	*Crude protein*	*Flour*	*Crude protein*
Protein (N × 6·25)	23·2	56·3	26·1	60·2
Total sugars	3·4	9·5	4·3	12·4
Glucose equivalent of reducing materials produced from mild acid hydrolysis	46·6	12·8	44·9	9·3
Oil	5·3	—	6·1	—
Ash	3·3	7·6	3·1	7·2
Crude fibre	6·4	—	3·8	—

differed slightly between varieties. These differences may be attributed to varietal characteristics, region of cultivation and environmental factors.

Data for tryptophan and basic amino acids of raw and cooked chick pea of both varieties are listed in Table 2. Slight differences were found in the amino acid contents of the two varieties. The method of cooking affected the amino acid content. The reduction of lysine after autoclaving 1 h at 121 °C did not exceed 3 % in both varieties. Cooking for 2 h in the atmosphere reduced lysine 5·2 % in the Egyptian variety and 7·2 % in the

TABLE 2
Effect of Cooking on Tryptophan and Basic Amino Acids of Chick Pea

Identification	*Tryptophan*	*Lysine*	*Histidine*	*Arginine*
	(Grams of amino acid per 16 grams of nitrogen)			
		Egyptian variety		
Control	1·10	6·80	2·90	10·40
Cooked 1 h	1·10	6·70	2·80	10·00
Cooked 2 h	1·01	6·40	2·60	9·30
Autoclaved 1 h at 121 °C	1·10	6·60	2·80	9·10
		Italian variety		
Control	1·15	6·90	2·80	11·6
Cooked 1 h	1·10	6·80	2·70	11·1
Cooked 2 h	1·01	6·35	2·63	10·6
Autoclaved 1 h at 121 °C	1·11	6·66	2·74	10·5

TABLE 3
Effect of Cooking on Protein Solubility of Chick Pea

Variety	*Control*	*Cooked 1 h*	*Cooked 2 h*	*Autoclaved 1 h at 121°C*
	Nitrogen solubility in 0·02 N NaOH (%)			
Egyptian	98	59	56	58
Italian	98	61	57	61

Italian variety. Martinez & Frampton (1958) found a reduction of 3 % in the lysine content after autoclaving cottonseed meal for 2 h at 121 °C; they also found a reduction of 15 % in the lysine content of peanut meal after the same treatment. Gonzalez *et al.* (1960) found a reduction of 3 % in the lysine content after autoclaving chick pea at 121 °C for 1 h. Statistical analysis showed a highly significant difference in the lysine content of both varieties of cooked chick pea.

Cooking affected tryptophan, the effect being significant in the Egyptian variety and highly significant in the Italian variety. Histidine and arginine were affected by cooking, the effect being highly significant in both varieties.

Protein solubility was affected by cooking procedure, as shown in Table 3. Chick pea is used for food by a large part of the world's

TABLE 4
Effect of Cooking on Vitamin Content of Chick Pea

Identification	*Vitamin content (mg/100 g dry weight)*		
	Thiamin	*Pyridoxin*	*Niacin*
		Egyptian variety	
Control	0·69	0·71	1·58
Cooked 1 h	0·59	0·68	1·48
Cooked 2 h	0·54	0·61	1·45
Autoclaved 1 h at 121 °C	0·58	0·62	1·46
Average cooking water (μg/ml)	1·11	0·31	2·41
		Italian variety	
Control	0·86	0·61	1·74
Cooked 1 h	0·61	0·56	1·46
Cooked 2 h	0·54	0·41	1·21
Autoclaved 1 h at 121 °C	0·60	0·55	1·45
Average cooking water (μg/ml)	1·42	0·36	3·14

population because it is a potential source of a high quality protein. The nutritional availability of amino acids might be influenced by the treatment of protein. Simple controlled heating improves the nutritional value of the protein but amino acids, especially lysine, histidine and arginine, are destroyed when heating is severe.

The vitamin values for raw and cooked chick pea are shown in Table 4. There were considerable variations in these values between cooking processes. Statistical analysis showed a significant difference between the vitamin contents of the two varieties. It is preferable to cook chick pea in an autoclave at 121 °C for not more than 1 h to minimise the losses in vitamins and amino acids. In addition, the cooking water can be used with effect because of its vitamin content.

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Calcium Distribution in Rat after *Per Os* Administration of ^{45}Ca-Salts of Phosphorylated Sucrose

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(Received: 23 July, 1982)

ABSTRACT

The incorporation of calcium from ^{45}Ca labelled calcium salts of phosphorylated sucrose and $^{45}CaCl_2$ was compared in vivo. *It is possible to assume that the incorporation of ^{45}Ca after* per os *application of ^{45}Ca-salts of phosphorylated sucrose is about 5–15% lower in comparison with $^{45}CaCl_2$. This difference in calcium incorporation is explained by the presence of insoluble (and therefore unutilizable) $^{45}Ca_3(PO_4)_2$ in the preparations of calcium salts of phosphorylated sucrose. The maximal incorporation of radioactivity can be found in bones 24–48 h after receipt, depending on the preparation applied. The rate of calcium incorporation into teeth is slower and the maximum is reached 72 h after* per os *application of any preparation tested. It was proved that calcium is transported from bones to teeth before its excretion from the organism. Calcium incorporation into organs is very low (about 3% of applied radioactivity). Almost all calcium incorporated in these tissues is excreted from the organism in the course of 3 days. The results indicate that calcium salts of phosphorylated sucrose could be used as a suitable support for calcium fortification in selected foodstuffs.*

INTRODUCTION

It is proposed that calcium salts of phosphorylated sucrose can be used for various purposes. They are effective fertilizers in foliar applications.

Food Chemistry 0308-8146/83/0011-0145/$03·00

Sugar phosphate esters have been found to penetrate leaf tissue much better than inorganic phosphate (Colonial Sugar Refining Co., 1967). Due to their cariostatic properties and their content of calcium and phosphorus they can find wide application for the fortification of foodstuffs and fodder. The multiple rôle of calcium salts of phosphorylated sucrose favours their application against other calcium compounds. The availability of calcium in the fluids of the digestive tract was described in our previous paper on the basis of *in vitro* experiments (Káš *et al.*, 1983).

The aim of this study was to compare calcium utilization from the tested compound and that of $CaCl_2$ using a ^{45}Ca label.

EXPERIMENTAL

Chemicals

Radionuclide ^{45}Ca (code RCa-3) in the form of calcium chloride was supplied by the Institute of Nuclear Research (The Radioisotope Production and Distribution Centre, Swierk, Otwock, Poland). The liquid scintillator, Aquasol, and the tissue solubilizer, Protosol, were obtained from NEN Chemicals, Boston, USA. The other chemicals used were from Lachema, Brno, Czechoslovakia.

Experimental animals

White male rats, mean weight 170 g, were purchased from fy. Velaz, Lysolaje near Prague.

Methods

Preparation of ^{45}CaO

To 5 ml of the radioactivity labelled commercial $^{45}CaCl_2$ (total radioactivity, 370 MBq) inactive calcium chloride (2·0 g) was added as a support. Calcium ions were then precipitated under intensive stirring by adding 5% sodium carbonate until alkaline pH was reached. The precipitate of calcium carbonate was decanted and then dried at 105 °C. The dried calcium carbonate was parched in an electric oven at 900 °C for 1 h. A yield of 1·7 g of ^{45}CaO with a total radioactivity of 320 MBq was obtained.

Preparation of ^{45}Ca labelled calcium salt of phosphorylated sucrose (^{45}Ca-PSa)

Sucrose (4·3 g) was dissolved in 20 ml of distilled water in an Erlenmeyer flask (50 ml). Under permanent stirring 1·7 g ^{45}CaO was then added. The reaction was started by the dropwise addition of 1·9 g $POCl_3$ and carried out at 1–4 °C. Four volumes of ethanol were slowly added to the reaction mixture under intensive stirring. After precipitation and product sedimentation, the supernatant was poured off and the product was extracted four times with 75 % ethanol to remove $CaCl_2$. The washed product was filtered and dried in the open air. ^{45}Ca-PSa, weighing 2·4 g, with a total radioactivity of 24 MBq, was obtained.

Preparation of $^{45}CaCl_2$ solution for experiments

It was necessary, for the intended metabolic studies, to have preparations of $^{45}CaCl_2$ and ^{45}Ca-PSa with the same radioactivity. For this purpose inactive $CaCl_2$ was added to commercial $^{45}CaCl_2$ to obtain both solutions ($^{45}CaCl_2$ and ^{45}Ca-PSa) with the same specific radioactivity.

Administration of ^{45}Ca-PSa to rats per os

The appropriate amount of ^{45}Ca-PSa (corresponding to 40, 160, 200 and 400 kBq radioactivity) was administered to the stomachs of experimental rats in the form of water solution. The animals were then kept in metabolic cages, produced by Kavalier, Sázava, Czechoslovakia. The animals were supplied with Larsen diet and water *ad libitum*. For each experiment six animals were used. Organs were removed immediately after the rats were killed and processed as described.

Measurement of ^{45}Ca radioactivity

Samples of tissue (50 mg) were disintegrated directly in the cuvettes used for scintillation measurements and 0·5 ml of Protosol was added. The cuvettes were well closed with polyethylene plugs and their contents heated at 55 °C for 2 h. If the solution was still coloured, 0·2–0·5 ml of 30 % H_2O_2 was added and the cuvette was further heated at 55 °C for 30 min. After cooling, 0·1 ml of glacial acetic acid and 10 ml of liquid scintillator Aquasol were added. The contents of the cuvettes were well mixed and left to equilibrate for 1 h before radioactivity was measured. The radioactivity of ^{45}Ca was measured on a Packard-Tricarb apparatus.

The samples of bones and teeth were solubilized by the addition of 0·1 ml concentrated $HClO_4$ and 0·4 ml 30 % H_2O_2 per 50 mg of tissue.

The solubilization was performed in a water bath at 60 °C until complete. (The method of solubilization was slightly modified according to Mahin & Lofberg, 1966.) After cooling, 10 ml of scintillator Aquasol was added and radioactivity was measured in the same way as for the other samples.

Because the samples of faeces were not well solubilized by this procedure, the radioactivity of dried faeces was measured in an infinite layer by means of a Geiger-Müller counter.

RESULTS AND DISCUSSION

Secretion of ^{45}Ca in faeces and urine after *per os* application of ^{45}Ca-PSa and $^{45}CaCl_2$

All ^{45}Ca, unincorporated and incorporated into organs, originating from the dissociable calcium compounds is excreted from the organism during 2–3 days (Tables 1 and 2). In the first two days calcium is excreted more

TABLE 1

^{45}Ca Excretion in Faeces and Urine of Experimental Rats After *Per os* Application of ^{45}Ca-PSa

Time after ^{45}Ca application (h)	*Sample*	*Applied dose of ^{45}Ca (kBq)*			
		400	*200*	*160*	*40*
		Excreted ^{45}Ca radioactivity (kBq)			
18	Faeces	272·13	158·12	114·78	14·64
	Urine	4·11	6·12	0·98	0·10
42	Faeces	54·74	42·96	33·97	13·45
	Urine	1·14	1·48	0·40	1·25
66	Faeces	14·20	7·12	2·14	0·31
	Urine	0·63	0·30	0·09	0·08
90	Faeces	2·37	2·02	0·64	0·06
	Urine	0·39	0·09	0·29	0·04
114	Faeces	0·43	1·63	0·26	0·02
	Urine	0·29	0·14	0·08	0·01
Total		350·43	219·68	156·63	29·96

The values are the arithmetic means of six determinations corresponding to the number of animals in each experimental group.

TABLE 2

^{45}Ca Excretion in Faeces and Urine of Experimental Rats after *Per os* Application of $^{45}CaCl_2$

Time after ^{45}Ca application (h)	*Sample*	*Applied dose of ^{45}Ca (kBq)*			
		400	*200*	*160*	*40*
		Excreted ^{45}Ca radioactivity (kBq)			
18	Faeces	246·17	172·43	108·21	18·64
	Urine	5·28	5·64	1·47	0·28
42	Faeces	52·74	38·96	26·31	3·04
	Urine	3·32	1·12	0·63	0·98
66	Faeces	11·93	8·31	3·78	0·47
	Urine	0·71	1·28	0·16	0·12
90	Faeces	1·89	2·38	1·25	0·16
	Urine	0·31	0·84	0·23	0·04
114	Faeces	0·74	1·94	0·38	0·09
	Urine	0·42	0·31	0·11	0·05
Total		323·51	223·21	142·53	23·87

The values are the arithmetic means of six determinations corresponding to the number of animals in each experimental group.

than 90% in faeces. Higher excretion (about 5–15%) was found during this period when ^{45}Ca-PSa was applied (compare Tables 1 and 2). This finding can be explained by assuming that ^{45}Ca-PSa also contains undissociable $^{45}Ca_3(PO_4)_2$ which is not utilizable and therefore is excreted from the organism. This idea is supported by the results presented in Table 3. The sample of faeces after *per os* application of ^{45}Ca-PSa was (after measurement of its radioactivity) extracted with 5% trichloroacetic acid. In this way all dissociable ^{45}Ca was removed. The radioactivity of acid extract and non-extractable residue was then measured. The non-extractable residue of faeces from the animals to which ^{45}Ca-PSa was administered contained more ^{45}Ca than that from the animals fed with $^{45}CaCl_2$. The difference represents about 10% of the original radioactivity and most probably corresponds to $^{45}Ca_3(PO_4)_2$ present in the preparation of ^{45}Ca-PSa. The mutual comparison of the radioactivity found in the stomachs and the small intestines of experimental animals after application of ^{45}Ca-PSa and $^{45}CaCl_2$ supports this explanation (Table 4). More ^{45}Ca was found in the experimental animals fed with

TABLE 3
^{45}Ca Distribution in Faeces 18 h after *Per os* Application of ^{45}Ca-PSa and $^{45}CaCl_2$

Applied compound	*^{45}Ca-PSa*	*$^{45}CaCl_2$*
Sample	*Total radioactivity of the sample (kBq ± SD)*	
Original faeces	43·9 ± 2·8	42·2 ± 2·4
Acid extract of faeces	34·1 ± 2·2	37·2 ± 1·8
Non-extractable residue	8·2 ± 0·4	3·9 ± 0·6

^{45}Ca-PSa. No radioactivity was found in lipid extracts (chloroform: methanol = 1:1) of intestinal contents and faeces.

Similar amounts of radioactivity are excreted in faeces and urine in both experimental groups (Tables 1 and 2). The ^{45}Ca incorporated into the organs is excreted in the course of 3 days in relation to the rate of metabolic turnover in that organ. ^{45}Ca is replaced stage by stage by inactive Ca from the accepted fodder. It will be demonstrated later that the major part of this radioactivity originated from ^{45}Ca excreted from bones and teeth.

TABLE 4
Total Radioactivity of ^{45}Ca in Stomach and Small Intestine after *Per os* Application of ^{45}Ca-PSa and $^{45}CaCl_2$

Time after ^{45}Ca application (h)	*Sample*	*Applied dose of ^{45}Ca (kBq)*			
		400	*200*	*160*	*40*
		^{45}Ca radioactivity (kBq)			
6	^{45}Ca-PSa	200	125	70	25
	$^{45}CaCl_2$	180	95	60	20
24	^{45}Ca-PSa	8	3	2	0
	$^{45}CaCl_2$	4	1	0	0
48	^{45}Ca-PSa	1	0	0	0
	$^{45}CaCl_2$	0	0	0	0

Incorporation of ^{45}Ca into the individual tissues after *per os* application of ^{45}Ca-PSa and $^{45}CaCl_2$

More than 90 % of ^{45}Ca from the incorporated calcium was transferred into bones and teeth after *per os* application of either ^{45}Ca-PSa or ^{45}Ca-Cl_2. The incorporation of ^{45}Ca was investigated in the leg (tibia) and in the incisor (teeth) of rats. The total amount of incorporated ^{45}Ca was about 5–15 % lower when the ^{45}Ca-PSa preparation was applied (compared with $^{45}CaCl_2$). The time dependence of ^{45}Ca incorporation into bones and teeth at different amounts of applied radioactivity is shown in Table 5 (for ^{45}Ca-PSa) and Table 6 (for $^{45}CaCl_2$). The lower incorporation of ^{45}Ca from ^{45}Ca-PSa in relation to $^{45}CaCl_2$ is probably caused by the presence of unutilizable $^{45}Ca_3(PO_4)_2$ in the preparation of ^{45}Ca-PSa, as noted earlier.

The time course of ^{45}Ca incorporation into bones differs from that of teeth. The highest incorporation of ^{45}Ca into bones was found 48 h after application of ^{45}Ca-PSa or earlier (after 24 h) when $^{45}CaCl_2$ was used. On the other hand, the highest radioactivity in teeth was reached 72 h after *per os* application of both tested preparations. These differences can be

TABLE 5

^{45}Ca Incorporation into Bones and Teeth after *Per os* Application of ^{45}Ca-PSa

Time after ^{45}Ca application (h)	*Sample*	*Applied dose of ^{45}Ca (kBq)*			
		400	*200*	*160*	*40*
		^{45}Ca incorporation into 1 g of sample (kBq)			
6	Bones	14·75	8·46	6·96	4·12
	Teeth	11·73	10·34	7·12	1·63
24	Bones	18·38	12·36	7·36	4·96
	Teeth	14·12	11·34	8·63	2·40
48	Bones	28·36	12·63	7·12	3·26
	Teeth	18·86	11·12	8·86	1·93
72	Bones	24·16	9·44	4·18	4·14
	Teeth	22·63	12·34	9·16	3·02
96	Bones	18·45	5·36	4·32	2·36
	Teeth	16·43	11·63	9·06	2·53
Total		187·87	105·02	72·77	30·35

TABLE 6
^{45}Ca Incorporation into Bones and Teeth after *Per os* Application of $^{45}CaCl_2$

Time after ^{45}Ca application (h)	*Sample*	*Applied dose of ^{45}Ca (kBq)* 400	200	160	40
		^{45}Ca incorporation into 1 g of sample (kBq)			
6	Bones	20·46	12·96	11·37	3·46
	Teeth	14·37	9·47	5·38	1·14
24	Bones	33·12	16·86	12·14	3·96
	Teeth	17·96	10·15	7·12	2·09
48	Bones	29·78	11·96	9·16	5·16
	Teeth	19·37	11·36	8·96	3·63
72	Bones	27·13	12·46	4·87	2·36
	Teeth	20·96	14·78	8·12	3·35
96	Bones	19·86	8·16	2·13	2·34
	Teeth	17·47	8·74	7·75	3·12
Total		220·48	116·63	75·00	30·61

explained by the different manner of ^{45}Ca incorporation into bones and teeth. The amount of ^{45}Ca incorporated into teeth increases gradually, but is slower than that into bones. The amount of incorporated radioactivity in teeth decreases in the direction from the fang to the tip (Table 7). The highest incorporation of ^{45}Ca in teeth is reached when all

TABLE 7
Statistical Evaluation of ^{45}Ca Incorporation into Important Tissues after *Per os* Application of ^{45}Ca-PSa and $^{45}CaCl_2$ to Ten Animals

Applied compound	*^{45}Ca-PSa*	*$^{45}CaCl_2$*
Examined tissue	*^{45}Ca incorporation into 1 g of tissue (kBq)*	
Bone (tibia)	130 ± 21	146 ± 18
Whole tooth (incisor)	77 ± 9	80 ± 11
Tip of the tooth	23 ± 4	28 ± 5
Liver	0·34 ± 0·07	0·42 ± 0·09

TABLE 8
^{45}Ca Incorporation into Organs after *Per os* Application of ^{45}Ca-PSa

Time after ^{45}Ca application (h)	*Sample*	*Applied dose of ^{45}Ca (kBq)*			
		400	*200*	*160*	*40*
		^{45}Ca incorporation into 1 g of tissue (kBq)			
6	Liver	0·72	0·51	0·33	0·09
	Kidney	0·86	0·60	0·32	0·10
	Heart	0·64	0·38	0·19	0·10
	Lung	1·34	1·09	0·72	0·20
	Spleen	0·57	0·40	0·35	0·14
	Muscle	1·12	1·11	0·40	0·20
	Total	5·25	4·09	2·31	0·83
24	Liver	0·68	0·62	0·36	0·16
	Kidney	0·77	0·54	0·31	0·23
	Heart	0·62	0·35	0·22	0·08
	Lung	1·42	1·12	0·56	0·24
	Spleen	0·68	0·43	0·24	0·13
	Muscle	1·02	1·15	0·63	0·24
	Total	5·19	4·21	2·32	1·08
48	Liver	0·32	0·23	0·18	0·11
	Kidney	0·41	0·35	0·26	0·12
	Heart	0·29	0·15	0·09	0·05
	Lung	0·94	0·63	0·39	0·20
	Spleen	0·21	0·26	0·08	0·03
	Muscle	0·77	0·72	0·33	0·17
	Total	2·94	2·34	1·33	0·68
72	Liver	0·15	0·08	0·07	0·06
	Kidney	0·22	0·18	0·12	0·08
	Heart	0·11	0·04	0·06	0·02
	Lung	0·35	0·30	0·21	0·16
	Spleen	0·04	0·06	0·04	0·01
	Muscle	0·28	0·22	0·23	0·09
	Total	1·15	0·88	0·73	0·42
96	Liver	0·04	0·02	0·01	0·01
	Lung	0·12	0·06	0·05	0·03
	Muscle	0·09	0·05	0·04	0·03
	Total	0·25	0·13	0·10	0·07

TABLE 9
^{45}Ca Incorporation into Organs after *Per os* Application of $^{45}CaCl_2$

Time after ^{45}Ca application (h)	*Sample*	*Applied dose of ^{45}Ca (kBq)*			
		400	*200*	*160*	*40*
		^{45}Ca incorporation into 1 g of tissue (kBq)			
5	Liver	0·81	0·52	0·30	0·16
	Kidney	0·89	0·72	0·38	0·12
	Heart	0·62	0·36	0·10	0·11
	Lung	1·59	1·15	0·92	0·33
	Spleen	0·56	0·42	0·32	0·18
	Muscle	1·38	1·06	0·53	0·35
	Total	5·85	4·23	2·55	1·25
24	Liver	0·76	0·60	0·37	0·15
	Kidney	0·78	0·64	0·36	0·22
	Heart	0·64	0·28	0·18	0·10
	Lung	1·72	0·82	0·62	0·26
	Spleen	0·68	0·46	0·28	0·08
	Muscle	1·23	0·98	0·72	0·24
	Total	5·81	3·78	2·53	1·05
48	Liver	0·28	0·26	0·18	0·13
	Kidney	0·48	0·33	0·28	0·13
	Heart	0·24	0·16	0·12	0·08
	Lung	1·08	0·72	0·42	0·16
	Spleen	0·26	0·23	0·10	0·06
	Muscle	0·89	0·73	0·42	0·15
	Total	3·23	2·43	1·52	0·71
72	Liver	0·14	0·13	0·11	0·08
	Kidney	0·28	0·14	0·10	0·06
	Heart	0·13	0·08	0·06	0·03
	Lung	0·42	0·31	0·18	0·14
	Spleen	0·08	0·04	0·03	0·02
	Muscle	0·27	0·23	0·14	0·12
	Total	1·32	0·93	0·62	0·45
96	Liver	0·08	0·03	0·01	0·01
	Lung	0·10	0·11	0·06	0·05
	Muscle	0·08	0·07	0·05	0·04
	Total	0·26	0·21	0·12	0·10

unabsorbed radionuclides of calcium are excreted from the organism. These results indicate that ^{45}Ca must be redistributed in the rat organism from bones to teeth.

The incorporation of ^{45}Ca into organs was also investigated (Tables 8 and 9). It is notable that the amount of incorporated radioactivity did not exceed 3 % of the total radioactivity applied. The maximum radioactivity in the organs investigated was found 6 h after *per os* application of both preparations. In the course of 3 days almost all incorporated ^{45}Ca is excreted. The total incorporated radioactivity in the case of ^{45}Ca-PSa application is also lower (5–15 % in comparison with $^{45}CaCl_2$). The reason for this is the same as that for the incorporation of ^{45}Ca into bones and teeth.

The incorporation of ^{45}Ca into organs decreases in the order: lung, muscle, kidney, liver, heart and spleen. Remarkable differences were found among the individual tissues; e.g. about 2–3 times higher incorporation of ^{45}Ca was found in lung compared with spleen.

CONCLUSIONS

The results presented in this paper indicate that calcium salts of phosphorylated sucrose can serve as a suitable support of calcium. Calcium utilization from this compound is only a little lower than that of the well dissociable inorganic salt, $CaCl_2$. Calcium salts of phosphorylated sucrose have some evident advantages; among these are better acceptability, cariostatic properties and technological characteristics.

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Conference Report

INFOODS CONFERENCE

A small international planning conference on the topic of 'An International Network of Food Data Systems' was held on 30 January–5 February, 1983, at the Rockefeller Conference and Study Center, Bellagio, Italy. It was sponsored through the Food, Nutrition and Poverty Subprogramme of the United Nations University and supported by various US government agencies, private foundations and the food industry. Representatives from FAO, WHO, IUNS and IUFOST participated in the discussions. The purpose was to explore the needs for, and current limitations of, food composition data bases, especially in the international context.

The conference focused on the design and scope of an organisation to be called INFOODS (International Network of Food Data Systems) which would promote international participation and co-operation in the acquisition and interchange of quality data on the nutrient composition of foods, beverages and their ingredients in forms appropriate to meet the needs of government agencies; nutrition scientists; health and agriculture professionals; policy makers and planners; food producers, processors and retailers; consumers.

The conference identified the more important aspects of INFOODS to include: (1) a network of regional data centres; (2) an organisational/administration framework for various expert task forces; (3) the generation of special international data bases; and (4) a general and

specific resource for persons and organisations interested in food composition data on a worldwide basis.

Moreover, the conference agreed that INFOODS should take the responsibility of initiating, directing and co-ordinating efforts in the following areas: (a) development of international criteria for judging the quality of data on food composition; (b) codification of existing sources of useful data on food consumption; (c) promotion of the generation and dissemination of new data on the composition of foods, beverages and their ingredients; (d) facilitation of the access, retrieval and interchange of food composition data on a worldwide basis.

An interim committee was formed, to start to implement the recommendations and programme of work arising out of the conference. Dr Vernon R. Young was elected as chairman of the committee and Dr William Rand, executive secretary. Persons interested in contributing to, and/or obtaining help from, INFOODS are urged to contact either of the above at Room 56-331, Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 USA (Tel. (617) 253-5801).

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Announcements

'PROFITABILITY OF FOOD PROCESSING—1984 ONWARDS—THE CHEMICAL ENGINEERS' CONTRIBUTION', 10–12 April 1984, Bath, Great Britain

In conjunction with the Annual Meeting of the Institution of Chemical Engineers in the Spring of 1984, the South Western Branch will be holding a three-day symposium at Bath on the contribution of chemical engineering to the profitability of food processing.

Contributions are invited from those concerned with production methods, equipment design and research in the field of food processing. These should include (but are not necessarily restricted to) papers which highlight one or more of the following topics in particular and their impact on profitability: yield, minimisation of waste, minimisation of rejection rate, energy conservation, heat and mass transfer, packaging and distribution, hygienic design and sanitation, mathematical modelling and optimisation, and value engineering.

The symposium will probably be structured under the following subject headings:

(a) *Materials handling and preparation*, e.g. measurement of quantity and quality, control and instrumentation;
(b) *Processing*, e.g. unit operations including extraction, heat transfer, mixing, blending, comminution, drying and evaporation;
(c) *Preservation*, e.g. sterilisation, refrigeration, drying and radiation;

(d) *Finished product handling*, e.g. packaging, warehousing and distribution;
(e) *Food in the future*, e.g. engineering aspects of novel foods, single cell protein, fish farming and alternative raw materials.

Titles and synopses of proposed papers or enquiries concerning relevance should be sent to Miss Jane Ellis, or Mrs Gillian Nelson, Conference Section, The Institution of Chemical Engineers, 165–171 Railway Terrace, Rugby CV21 3HQ, Great Britain (Tel: 0788-78214, Telex 311780).

GUMS AND STABILISERS FOR THE FOOD INDUSTRY. 2. APPLICATIONS OF HYDROCOLLOIDS

The above conference will be held at the North E Wales Institute of Higher Education on 11–15 July 1983. Any enquiries concerning the conference should be made to the Conference Secretariat, Research Division, The North E Wales Institute, Kelsterton College, Connah's Quay, Deeside, Clwyd CH5 4BR, Great Britain.

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Hydroxy Isoflavones as Antioxidants for Edible Oils

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(Received: 18 August, 1982)

ABSTRACT

The antioxidant properties of some naturally occurring mono-, di- and tri-hydroxy isoflavones have been evaluated. Determination of induction periods in lard at 100°C showed that genistein and prunetin were active, and 4',6,7-trihydroxy-isoflavone marginally so, but the other isoflavones were inactive.

Apigenin, the flavone analogue of genistein, was inactive but the activity of the other flavone examined, luteolin, was marked.

Pronounced synergism was evident when genistein and 4',6,7-trihydroxy-isoflavone were supplemented with phosphatidyl ethanolamine.

INTRODUCTION

The naturally occurring isoflavones are to be found mainly in the germinating seeds of the family *Leguminosae*, being much less widespread than the better known flavones. They have been observed to have oestrogenic, anti-fungal, anti-haemolytic and antioxidant properties and are therefore of food significance, being widely consumed in such commodities as soyabeans and lentils.

Since we have already reported on the antioxidant properties of the chemically very closely related flavones (Hudson & Lewis, 1983*a*) and on synergistic interactions between flavones and phospholipids (Hudson &

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Lewis, 1983*b*) it seemed appropriate to extend these studies to the isoflavones. Such studies, it was hoped, would also assist us in drawing conclusions regarding the relationships between antioxidant activity and chemical structure.

MATERIALS AND METHODS

As substrate for the oxidation studies, pure dried rendered lard, free from additives and not chemically processed, was used.

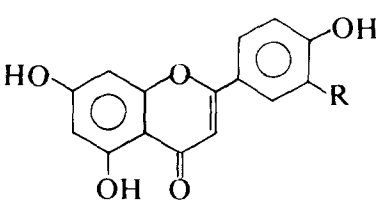
The isoflavones, and the two flavones used for comparison, were purchased from Apin Chemicals Ltd, Cardiff, Wales.

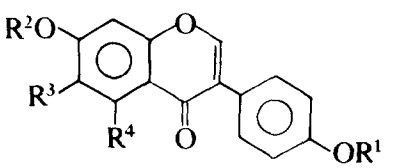
The phosphatidyl ethanolamine was synthetic sn-glycerol phosphatidyl ethanolamine dipalmitate (Sigma, London, Ltd).

Induction periods were determined automatically in the Metrohm Rancimat, which was briefly described in our earlier publication (Hudson & Lewis, 1983*a*).

RESULTS AND DISCUSSION

Table 1 shows induction periods corresponding to various levels of addition of two flavones and six isoflavones to lard at 100 °C. The structures of these compounds are shown in (I) and (II).





(I) *Flavones*
Apigenin, R = H
Luteolin, R = OH

(II) *Isoflavones*
Daidzein, $R^1 = R^2 = R^3 = R^4 = H$
Formononetin, $R^1 = CH_3$, $R^2 = R^3 = R^4 = H$
Genistein, $R^1 = R^2 = R^3 = H$, $R^4 = OH$
4′,6,7-Trihydroxy-isoflavone, $R^1 = R^2 = R^4 = H$, $R^3 = OH$
Biochanin A, $R^1 = CH_3$, $R^2 = R^3 = H$, $R^4 = OH$
Prunetin, $R^1 = R^3 = H$, $R^2 = CH_3$, $R^4 = OH$

TABLE 1
Primary Antioxidant Effects of Isoflavones and Related Flavones. Induction Periods in Lard at 100°C(h)

Isoflavone or flavone	*Antioxidant concentration (%)*					
	None	*0·005*	*0·01*	*0·025*	*0·05*	*0·10*
Daidzein	1·4	1·45	1·4	1·4	1·4	1·4
Formononetin	1·3	1·3	1·3	1·3	1·3	1·3
Genistein	1·35	1·6	1·8	2·0	2·6	3·5
4′,6,7-Trihydroxy-isoflavone	1·3	1·3	1·3	1·3	1·35	1·6
Biochanin A	1·3	1·3	1·3	1·4	1·3	1·3
Prunetin	1·3	1·3	1·4	1·8	2·2	2·6
Apigenin	1·3	1·3	1·4	1·3	1·3	1·3
Luteolin	1·3	3·6	6·3	11·1	12·7	29·0

It will be noted that under the test conditions three isoflavones and one flavone have no antioxidant activity at up to the high concentration of 0·1 %, that a further isoflavone has only marginal activity, and that two, genistein and prunetin, have distinct, if weak, activity. The second flavone, luteolin, has marked activity.

Table 2 shows induction periods of combinations of the synergist, phosphatidyl ethanolamine, itself a very weak antioxidant, with the two isoflavones that have attracted the most interest, genistein, the dominant isoflavone of soyabeans, and 4′,6,7-trihydroxy-isoflavone which has been identified in the fermented soya product, tempeh, but not in unfermented products (Gyorgy *et al.*, 1964). Using the method of Bishov & Henick (1972) to calculate synergistic efficiency, there is marked synergism between the two components in the case of 4′,6,7-trihydroxy-isoflavone, but in the case of genistein the synergism seems to be dependent on the concentration of primary antioxidant. There is a suggestion that it operates only at low genistein levels unless the phosphatidyl ethanolamine concentration is correspondingly raised. This feature is probably relevant to the stability of the various legume preparations to which antioxidant properties have been attributed (Pratt, 1972; Hammerschmidt & Pratt, 1978; Pratt & Birac, 1979) since phospholipids are present in soyabean preparations.

In vegetable products both flavones and isoflavones are predominantly present in the form of *O*-glycosides, which themselves have some antioxidant activity. Through the action of endogenous enzymes, in the

TABLE 2
Synergism between Isoflavones and Phosphatidyl Ethanolamine. Induction Periods in Lard at 100°C(h)

Primary antioxidant	*Concentration* (%)	*Phosphatidyl ethanolamine* (%)	*Induction period*	*Synergism* (%)
None	—	None	1·3	—
	—	0·05	1·5	—
	—	0·15	1·8	—
Genistein	0·025	None	2·0	—
	0·05	None	2·6	—
	0·10	None	3·5	—
	0·025	0·05	3·3	55
	0·025	0·15	4·7	65
	0·05	0·05	3·2	21
	0·05	0·15	8·1	74
	0·10	0·05	3·5	−9
	0·10	0·15	9·6	67
4′,6,7-Trihydroxy-isoflavone	0·025	0·15	4·0	81
	0·05	0·15	4·6	83
	0·10	0·15	5·0	78

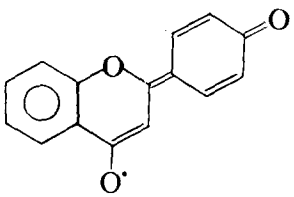
course of fermentation, or under other processing conditions, these are hydrolysed to the more active aglycones. Considering only the aglycones, our results enable us to draw some conclusions regarding the relationships between structure and antioxidant activity in the isoflavones.

In the isoflavones it is clear that both the 4′- and the 5-hydroxy groups are needed for significant activity, as in genistein. Even 4′,6,7-trihydroxy-isoflavone is, at best, only marginally active. The need for a 5-hydroxy group implies the need for an interaction by hydrogen bonding with the 4-carbonyl. This also emerged in the case of the flavones (Hudson & Lewis, 1983*a*) but the 3-hydroxyl, in the case of the flavones, was more effective. The isoflavones cannot incorporate this last kind of interaction.

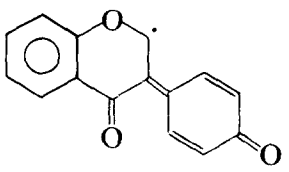
The comparison between apigenin, which is inactive, and genistein is particularly instructive, since the only difference between them is in the point of attachment of ring B to the chroman ring. In the case of apigenin, marked activity appears on insertion of the 3′-hydroxy group, giving the catechol configuration in luteolin, which is well recognised as important for really effective antioxidant activity (quercetin, taxifolin, etc.). It is

interesting that the 3′,4′,5,7-tetrahydroxy-isoflavone, orobol, found rarely in the *Papilionoidae*, and not known to occur in food legumes, could be predicted to be a very effective antioxidant under our test procedure.

Primary antioxidants function through their ability to form resonance stabilised free radicals with quinonoid structures. If it is assumed that the 4′-hydroxy group and the 4-carbonyl are minimum requirements for such stabilisation to be possible in flavones and isoflavones, the canonical forms corresponding to the initial 4′-phenoxy radicals will be (III) and (IV), respectively:



(III)



(IV)

In the case of (IV) the 4-carbonyl remains intact and can presumably still interact with the 5-hydroxy, if present, whereas in (III) the 4-carbonyl loses its normal functionality. This may be an explanation of the superior antioxidant activity of genistein as compared with apigenin. Of course, once the 3′-hydroxy is also introduced as in the more effective flavones, various other free radical forms can be postulated and these considerations will not necessarily apply.

It is difficult to compare our data on antioxidant activity with those of previous workers because their test methods have relied on quite different criteria from ours. Pratt and co-workers (Pratt, 1972; Hammerschmidt & Pratt, 1978; Pratt & Birac, 1979) determined relative rates of coupled oxidation of β-carotene and linoleic acid emulsions. Under these conditions 4′,6,7-trihydroxy-isoflavone appears to be as effective as quercetin, and considerably better than genistein, in inhibiting oxidation. Judged by anti-haemolytic activity (Gyorgy *et al.*, 1964) it was equal to α-tocopherol and much better than genistein. Judged by ability to inhibit the activity of lipoxygenase (Naim *et al.*, 1976), genistein was active but much less so than quercetin. In their ability to inhibit oxidation of retinol acetate in the presence of methyl linolenate (Ikehata *et al.*, 1968) genistein and daidzein were slightly active but considerably less so than 4′,6,7-trihydroxy-isoflavone, which was twice as active as orobol. All these tests, unlike ours, involved complex systems, partly in aqueous media.

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Changes in the Chemical Constituents of New Zealand Grapefruit During Maturation

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(Received: 18 August, 1982)

ABSTRACT

New Zealand grapefruit were harvested at intervals throughout the 1978 and 1981 seasons and their juices analysed for several chemical constituents. The pH ranged from 3·0 to 3·8, the total soluble solids from 10·3 to 14·4 °Brix, the titratable acid from 0·92 to 2·56 g citric acid/100 ml, the Brix:acid ratio from 4·2 to 14·1, and the ascorbic acid from 33·2 to 38·3 mg/100 ml. Total flavonoids ranged from 626 to 1470 mg/litre, naringin from 528 to 743 mg/litre and limonin from 4·7 to 20·2 mg/litre. There was considerable variation in the concentration of the bitter constituents between the two seasons. The results obtained are compared with other published figures and their implications for juice processors and consumers are discussed.

INTRODUCTION

Although New Zealand is situated climatically at the very southern limit for citrus culture, the citrus industry has expanded steadily over the past

decade with the total citrus crop doubling from 14 045 tonnes in 1974 to 28 647 tonnes in 1980 (Anon., 1981). While only 20 % of the citrus crop is processed, New Zealand grapefruit (hereafter referred to as NZGF) accounts for over half of this tonnage, making it the most important citrus fruit from a processor's point of view.

NZGF is a natural hybrid of obscure origin with tangelo characteristics (Hodgson, 1967). Although limited information is available on the composition of NZGF juices (Dawes, 1970; Robertson, 1975), there have been no published reports on the nature of the bitter compounds in NZGF or the way in which their concentration might change during maturation. The study reported here was undertaken to provide such information.

Bitterness in citrus fruits and products is primarily caused by two groups of compounds—flavonoids and limonoids (Kefford & Chandler, 1970). Flavonoid bitterness is due mainly to naringin and has been studied most frequently with respect to grapefruit products. Naringin occurs in low concentrations in the juice of mature grapefruit, and in higher concentration in segment membranes, core and peel (Sinclair, 1972).

Limonoid bitterness is associated with Navel oranges and grapefruit. Limonin, the major limonoid, causes delayed bitterness in the juice from early season to mid-season Washington Navel, Australia Valencia and Israel Shamouti oranges, as well as from Marsh grapefruit. Limonoid bitter principles generally decrease with maturity of the fruit (Maier *et al.*, 1977).

EXPERIMENTAL

Raw material

The NZGF used in this study were of the Golden Special variety propagated on trifoliata (*Poncirus trifoliata*) rootstock in the Massey University orchard, Palmerston North. The trees were planted in 1975. During both the 1978 and 1981 seasons, six trees were selected at random from the orchard and at intervals from early June or July until late October, two fruit were picked from each tree. The selected fruits were judged to be of average size and colour for the tree at that harvest and picking was evenly distributed around the outside of the trees.

Juice was extracted from the fruit with a mechanical hand-reamer and screened through a double layer of cheese-cloth to remove gross suspended matter. Where necessary juice was pasteurised by heating to, and maintaining, a temperature of 95 °C for 5 min and then cooling.

Common juice parameters

The pH was determined using a combination electrode connected to a Triac pH meter with digital output. Prior to measurements being made the instrument was calibrated with buffers at pH 3·2 and 7·0. Total soluble solids (TSS) were measured by refractometer and expressed as degrees Brix (°B). Titratable acidity was determined by titration of the juice against NaOH (0·1 M) using phenolphthalein (0·5 %w/v) as an indicator. Results are expressed as grams of citric acid per 100 ml of juice. Ascorbic acid was determined spectrophotometrically using 2,6 dichlorophenolindophenol (Pearson, 1976).

Bitter compounds

Total flavonoid content was determined during the 1978 season using the Davis test (Davis, 1947). It has been shown by Hagen *et al.* (1966) and Tatum *et al.* (1972) that the total flavonoid content, as determined by the Davis test, is approximately 2·1 times the naringin concentration.

During the 1981 season the naringin concentration was determined using the thin layer chromatographic–spectrophotometric method of Tatum & Berry (1973*a*).

Limonin was analysed by a method involving thin layer chromatography (TLC) and densitometry which combined, with slight modifications, the separation procedure of Chandler (1971) and the visualisation procedure of Tatum & Berry (1973*b*). Quantitation was based on measurement of the heights of densitometric peaks corresponding to the standard and unknown limonin spots on the same TLC plate using a Shimadzu CS-910 TLC scanner. This analytical procedure does not distinguish between limonin and its non-bitter precursor which is generally believed to be limonoic acid A-ring lactone (Maier *et al.*, 1977). The precursor is extracted from the juice and converted into limonin during preparation of the extract for chromatography.

RESULTS AND DISCUSSION

The changes in the chemical constituents of NZGF juice during maturation are summarised in Table 1. With the exception of ascorbic acid, all the parameters measured changed significantly during maturation. The increases in pH and TSS and decreases in titratable acidity parallel very closely the trends reported by others (Sinclair, 1972; Robertson, 1975).

The ascorbic acid concentration reported here is within the range of 25 to 60 mg/100 ml reported by Nagy (1980) for a wide variety of grapefruit grown in various parts of the world. The figures are marginally but significantly higher than those reported earlier by Robertson (1975) for NZGF juice (mean value of 29·0 mg/100 ml), possibly because the juice analysed in this present study was extracted immediately after harvesting compared with a delay of 3 days in the earlier study. Recently, Albach *et al.* (1981*a*) reported constant values of ascorbic acid in early and mid-season grapefruit, a trend which is evident in the results in Table 1. Albach also reported a 3-year mean of ascorbic acid in grapefruit juice (primarily the Ruby Red variety) of 31·3 mg/100 ml; this compares with 36·9 in the present study.

Care is needed when comparing the naringin values in Table 1 with other published results since many authors erroneously equate the total flavonoid concentration as determined by the Davis method with naringin concentration. Fisher (1976) developed a far more specific HPLC procedure for determining naringin and this method was used by Dougherty & Fisher (1977) and Ting & McAllister (1977). They determined the naringin contents of Florida grapefruit juice and reported ranges of 152–711, 240–903 and 123–442 mg/litre and averages of 310, 456 and 285 mg/litre respectively. Comparison of these values with those presented in Table 1 indicates that NZGF juices contain significantly higher concentrations of naringin (1981 average 650 mg/litre) than do Florida grapefruit juices. On the other hand, the total flavonoid contents of NZGF juice (1978 average 1153 mg/litre) fall within the range of values reported by Maurer *et al.* (1950) but outside that reported by Dougherty & Fisher (1977) for USA grapefruit juice; namely, 130–1880 and 277–910 mg/litre respectively.

Although the data are not strictly comparable, it is clear that flavonoid contents were higher in 1981 than in 1978. Also, there was a more than doubling of concentration of total flavonoids in 1978 compared with a

TABLE 1
Changes in the Chemical Constituents of NZ Grapefruit Juice During Maturation

Picking date	*pH*	*Total soluble solids (°Brix)*	*Titratable acidity (g citric acid/100 ml)*	*Brix:acid ratio*	*Ascorbic acid (mg/100 ml)*	*Total flavonoids (mg/litre)*	*Naringin (mg/litre)*	*Limonin (mg/litre)*
11 July 1978	3·1	10·3	1·83	5·7	ND[a]	626	ND	17·9
2 August 1978	3·4	11·6	1·47	7·9	ND	980	ND	18·6
22 August 1978	3·6	12·2	1·08	11·3	ND	1 060	ND	17·4
13 September 1978	3·8	12·6	0·94	13·4	ND	1 420	ND	20·2
3 October 1978	3·7	12·1	0·96	12·6	ND	1 470	ND	12·4
25 October 1978	3·7	12·9	0·92	14·1	ND	1 362	ND	11·8
2 June 1981	3·0	10·8	2·56	4·2	ND	ND	743	7·5
22 June 1981	3·1	11·6	2·40	4·8	37·8	ND	615	8·8
13 July 1981	3·1	12·1	2·12	5·7	36·5	ND	575	6·1
3 August 1981	3·2	12·8	2·08	6·2	37·8	ND	655	5·7
24 August 1981	3·3	13·6	1·86	7·3	38·3	ND	662	4·8
24 September 1981	3·4	14·4	1·84	7·8	37·8	ND	528	4·8
5 October 1981	3·4	13·5	1·63	8·3	37·3	ND	670	5·3
26 October 1981	3·5	13·5	1·31	10·3	33·2	ND	735	4·7

[a] Not determined.

relatively constant concentration of naringin in 1981; these two processes are not mutually exclusive. The steady increase in total flavonoid concentration during a season does not appear to have been observed previously, although the reverse does: Maurer *et al.* (1950) reported a decrease in juice naringin concentration as the season progressed with a slight rise towards the end of the season. In contrast, Kesterson & Hendrickson (1953) and Albach *et al.* (1981*b*) observed no significant decrease in juice naringin concentration until late in the season.

The actual concentrations of limonin are quite different in the two seasons, with values in 1978 about three times those in 1981. Moreover, for the 1978 season the changes in limonin content are rather abrupt with an almost constant level of limonin for 2 months, followed by a sharp drop over a period when the 1981 values remain relatively constant. It has been well established that the limonin content of citrus fruit tissues decreases with advancing maturity (Chandler *et al.*, 1976; Albach *et al.*, 1981*a*). For example, Chandler *et al.* reported that the limonin content in the juice of Navel oranges grown on trifoliata rootstock fell approximately from 22 ppm on 24 April to 12 ppm on 25 June to 6 ppm on 25 August. From published data it appears that rates of disappearance of limonin vary markedly with the crop for both horticultural and climatic reasons. For example, Mansell & McIntosh (1980) reported that there was no relationship between limonin, Brix, acid and Brix:acid ratio in grapefruit juice samples from three State Test Houses in Florida.

The Florida Department of Citrus (Anon., 1975) has specified that a Grade A grapefruit juice, processed outside the period for peak quality, must contain less than either 5 mg/litre limonin or 600 mg/litre naringin. European workers (Buffa & Bellenot, 1962) have suggested that a naringin content of 300–700 mg/litre is necessary for the characteristic grapefruit flavour, otherwise the juice is too insipid or too bitter. In both of the above cases naringin content is as determined by the Davis test and is thus strictly speaking the total flavonoid content.

While these figures can only be viewed as guidelines because of the recognised effects of acidity and sweetness on subjective responses to bitterness (Kamen *et al.*, 1961), they do suggest that none of the juice extracted from NZGF during the 1978 or 1981 seasons could be regarded as acceptable by consumers. Citrus processors in New Zealand have recognised this fact for many years and marketed NZGF juice either in combination with other fruit juices or diluted with sugar and water as a fruit drink.

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An Enzymic Method for the Determination of Starch in Meat Products

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(Received: 18 June, 1982)

ABSTRACT

The starch content of meat products is determined as glucose by an enzyme-hydrolytic method using thermo-stable α-amylase and amyloglucosidase for hydrolysis. The method is compared with a polarimetric and an acid-hydrolytic method.

The recovery in various cooked and uncooked sausages containing up to 12% starch ranged from 92 to 102% of the starch content estimated from the recipe. The average coefficient of variation was 2%. The presence of lactose did not influence the starch determination. In a collaborative study an average of 103% of the estimated starch value was recovered. The random and systematic errors were of equal size, each amounting to a maximum of 8% of the starch content of the sausages.

The application of the method for food inspection purposes is discussed. The method is considered to be suited for starch analysis in meat products.

INTRODUCTION

The determination of starch in meat products is based on various principles, i.e. optical rotation of polarized light (Ewers, 1908; Grossfeld, 1921; Ulmann & Richter, 1962) or hydrolysis followed by various specific (Ruttloff *et al.*, 1966; Gutmann, 1970) or non-specific (McCready *et al.*, 1974; AOAC, 1980) carbohydrate analyses. For hydrolysis, acid (Lind, 1977) or enzymes (Beutler, 1978; Haissig & Dickson, 1979; Ettel, 1981) may be used.

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Skimmed milk powder containing approximately 50 % lactose is often an ingredient in various meat products. In analytical procedures applying non-specific hydrolysis, the glucose part of the lactose molecule will interfere, resulting in too high apparent starch levels. Thus, hydrolysis of lactose should be prevented by using conditions specific for the glucosidic bonds of starch.

In the present study an enzymic method for starch determination in the presence of lactose is reported, using results reported in Hahnefeld *et al.* (1975) to achieve hydrolysis and the procedure by Lind (1977) for glucose determination. The method has been compared with a polarimetric and an acid-hydrolytic method of starch determination. Further, a collaborative study has been performed.

MATERIALS AND METHODS

Sources of starch

Potato flour and isolated wheat starch of Norwegian origin were used. Perfectamyl D12, a native potato starch, was obtained from Avebe, Veedam, The Netherlands. White Starch Super M12, an acid treated potato starch, originated from Kartoffelmelcentralen AMBA, Herning, Denmark.

Meat sausages

Sausage batters were formulated to contain 9 % meat protein (beef), 20 % fat, 2 % skimmed milk powder, 1·8 % salt, spices and various levels of starch. When altering the starch content, a constant proportion between the remaining ingredients was achieved by adjusting the amount of water added. The batter was stuffed into casings, each sausage weighing about 100 g. The sausages were analysed in the uncooked state or were heated in a water bath until they reached a core temperature of 80 °C prior to analyses.

Enzyme-hydrolytic starch determination

Samples were prepared as described by Lind (1977). Homogenized samples (10·00 g) were mixed with 30 ml citrate (0·05 M)-phosphate (0·1 M)

buffer (pH 5·0) in a 100-ml beaker. To the sample/buffer mixture 0·5 ml buffer-diluted α-amylase solution, Termamyl L60 (Novo Industri A/S, Bagsvaerd, Denmark) (1:100 v/v) was added, followed by thorough mixing with a glass rod. The beaker was subsequently covered with aluminium foil and autoclaved at 105 °C (0·5 atm) for 10 min.

After autoclaving, the mixture was allowed to cool to 60 °C prior to the addition of 0·15 ml amyloglucosidase, NOVO 150 (Novo Industri A/S, Bagsvaerd, Denmark) and homogenization with an Ultra-Turrax (Type 18/10, Janke & Kunkel IKA-Werk, Staufen, W. Germany) for 1 min. After 1 h in a water bath at 60 °C, the sample was quantitatively transferred to a 100-ml volumetric flask, using hot water for rinsing. The sample was cooled to room temperature and diluted to 100 ml. An aliquot was filtered and diluted 1:100 with distilled water. This solution was used for glucose determination and further estimation of the starch content using glucose oxidase, peroxidase and *o*-dianisidine hydrochloride according to the procedure given by Lind (1977). Buffer, instead of homogenized sausage, was analysed as a reagent blank.

Acid-hydrolytic starch determination

Starch determination using sulphuric acid for hydrolysis, followed by enzymic glucose determination was performed according to the procedure given by Lind (1977).

Polarimetric starch determination

Polarimetric starch determination was done according to a modified version of the Ewers' procedure (Ewers, 1908). One part homogenized sample, mixed with two parts 8 % (w/v) ethanolic KOH, was heated on a boiling water bath for 45 min. The solution was filtered through asbestos by water suction. The starch was washed on the filter with 90 % ethanol and dissolved in an appropriate volume of 25 % HCl before filtering through kieselguhr. Exactly 30 min after the addition of HCl, the rotation of the solution was measured in a 0·05° Zeiss Kreis polarimeter (Oberkochen, West Germany) using a 2 dm cell. A specific rotation of $[\alpha]_D = +200$ was used for calculating the starch content (Zelenka & Šašek, 1966).

Collaborative study

The collaborative study was carried out in accordance with recommendations given by Youden (1975). Twenty-one of twenty-five invited laboratories participated in the collaborative study. Each laboratory was provided with a detailed analytical description, the reagents needed for the analysis and three unknown frozen samples of meat sausages (A, B, C) containing various levels of starch. Each sample was to be analysed once in separate series. Prior to the analyses, the participants were asked to analyse a fourth unknown sample until reproducible results were obtained within a given interval (3·1–3·6 % starch). The expected starch content of the samples was stipulated from the starch content of the potato flour. This content was determined by the method under investigation. The starch content was corrected for a background value of 0·21 % starch equivalents originating from glycogen and glucose of the sausage ingredients.

RESULTS AND DISCUSSION

Evaluation of the method

The starch content of sausages containing various amounts of potato flour was determined by the enzyme-hydrolytic and polarimetric methods (Table 1). When corrections were made for glucose equivalents

TABLE 1
Starch Content of Sausages as Determined by the Enzyme-Hydrolytic and Polarimetric Methods

	Starch content (g/100 g)					
Added	*Enzyme-hydrolytic method*			*Polarimetric method*		
	$\bar{x}$	s_d	n	$\bar{x}$	s_d	n
0	0·25[a]	0·01	3	0·13[a]	0·05	2
1·58	1·44	0·04	9	1·48	0·04	8
3·15	3·07	0·12	9	3·18	0·08	8
12·00	11·50	0·45	2	11·89	0·19	2

[a] Used for correcting starch content determined by respective method.

of the starch-free sausages, recovery and precision agreed well between the two methods. At all levels the enzymically determined starch content was 97 % of that determined by the polarimetric method. The recovery will be slightly influenced by the choice of specific rotation used when calculating the starch content (Zelenka & Šašek, 1966).

In various cooked and uncooked sausages analysed by the enzyme-hydrolytic method, the recovery of added starch ranged from 92 to 102 % of the amount estimated from the recipe (Table 2). The highest recovery

TABLE 2
Starch Content of Cooked and Uncooked Sausages as Determined by the Enzyme-Hydrolytic Method

Treatment of sausage	*Starch content (g/100 g)*			
	Added	*Determined*[a]		
		$\bar{x}$	s_d	n
Uncooked	3·18	3·23	0·01	2
	8·00	7·58	0·21	2
	12·00	11·00	0·00	2
Cooked	3·27	3·28	0·05	2
	8·29	7·63	0·20	2
	12·22	11·56	0·04	2

[a] Corrections made for background (0·25 g/100 g).

was at the lowest starch level. Heat treatment of the sausages did not seem to influence recovery as long as corrections were made for changes in dry matter during cooking (Table 2). The average coefficient of variation of all sausage samples (Tables 1 and 2) was 2 %. Furthermore, the starch content of three native starches and one partially hydrolyzed starch analysed by the enzyme-hydrolytic method agreed well with dry matter analyses and starch contents given by the producers (Table 3).

All sausages analysed contained 2 % skimmed milk powder, representing approximately 1 % lactose. The lactose content did not interfere in the starch determination (Tables 1 and 2). Thus, the actual hydrolytic conditions did not cause any degradation of lactose. This was further verified by the lack of response of the enzyme-hydrolytic treatment to pure lactose and skimmed milk powder (Table 3). The effect of acid hydrolysis on these same products can be seen from Table 4. The apparent starch content of the sausages was too high due to the lactose content,

TABLE 3
Starch Content of Various Sausage Components as Determined by the Enzyme-Hydrolytic Method

Component	*Dry matter* (*g/100 g*)	*Starch content* (*g/100 g*)		
		$\bar{x}$	s_d	n
Potato flour	86·7	83·0	3·12	19
Wheat starch	88·0	82·7	3·59	9
Perfectamyl D12	88·6[a]	87·8	2·23	2
White starch M12	90·3[b]	85·2	0·26	3
Skimmed milk powder	—	<0·05	—	2
Lactose	—	<0·20	—	2
Glucose-1-phosphate	—	0·00	—	2
Glucose-6-phosphate	—	0·00	—	2

[a] 87·9% carbohydrates given by the producer.
[b] 12% water, 0·3% ash, 0·08% protein given by the producer.

whereas the starch content of potato flour corresponded well with that determined by the enzyme-hydrolytic method (Table 3). Thus, the interference from lactose in the starch determination demonstrates that acid hydrolysis cannot be used when analysing lactose-containing products.

With all methods based on hydrolysis of starch followed by glucose determination, corrections must be made for the endogenous glucose equivalents of the meat. Any free glucose can be determined by omitting the hydrolysis step from the analytical procedure. However, glucose in

TABLE 4
Starch Content of Sausages and Various Sausage Ingredients as Determined by the Acid-Hydrolytic Method

Sample	*Starch content* (*g/100 g*)		
	$\bar{x}$	s_d	n
Potato flour	83·4	2·10	10
Skimmed milk powder	20·9	0·53	2
Sausage			
Cooked (3·27% starch added)	3·86	0·12	4
Uncooked (3·18% starch added)	3·86	0·13	8

glycogen will interfere even when specific conditions are used for starch hydrolysis. In the present study the overall glucose equivalents of the starch-free batters corresponded to a starch content of 0·25 % (Table 1). Glucose 1-phosphate and glucose-6-phosphate did not respond to the analytical conditions (Table 3) and are thus not included in this background value. The corresponding meat blank given by Lind (1977) was 0·15 %. Results from Swift *et al.* (1960) give an average of 0·53 % glycogen in beef 2–3 h after slaughtering while Scheper *et al.* (1978) report data representing an average of 0·18 % glucose equivalents from glucose and glycogen of pork 24 h post mortem. Thus, from these data the endogenous glucose from the meat ingredients can be corrected for when the meat content of a product is known. Undesirable influence from glucose equivalents of the meat is thereby prevented.

Collaborative study

The average starch recovery of the samples subjected to the collaborative study was 103 % of the expected values (Table 5).

TABLE 5
Starch Content of Meat Sausages Subjected to a Collaborative Study in Twenty Laboratories

Sample	*Starch content (g/100 g)*		
	Expected	*Detected*	
		$\bar{x}$	s_d
A	2·36	2·41	0·17
B	5·76	5·95	0·75
C	3·58	3·74	0·26

Most laboratories participating in the collaborative test supplied their analytical results to the closer area around the expected values, although some were more distant (Figs 1 and 2). One laboratory was excluded from the presentation due to extremely deviating results (Youden, 1975).

The pattern made by the points of this type of chart demonstrates the types of error involved in the analytical procedure. If the scattering of points is caused by random errors only, the points should be divided equally among the four quadrants made up by the lines representing the average value of each sample (Youden, 1975). Systematic errors will cause

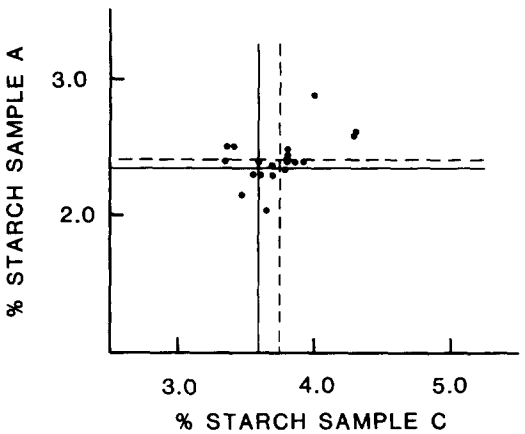

Fig. 1. Starch content of samples A and C as obtained by the enzyme-hydrolytic method. (——) Starch added. (– – –) Starch determined. Average of twenty laboratories.

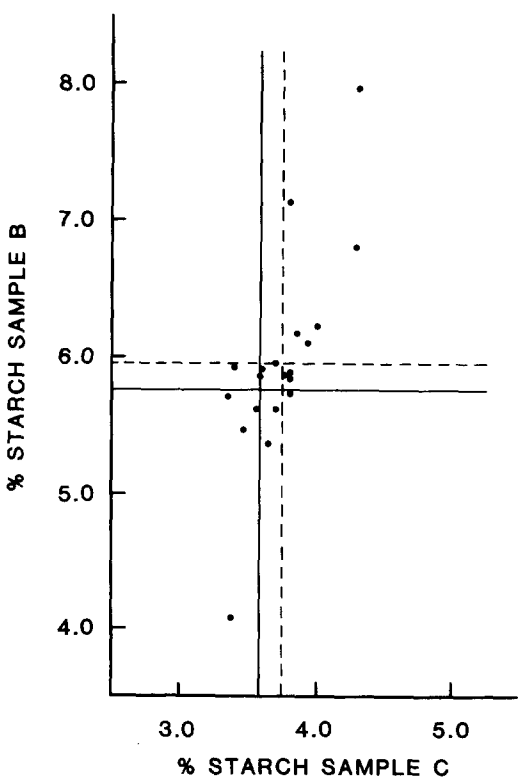

Fig. 2. Starch content of samples B and C as obtained by the enzyme-hydrolytic method. (——) Starch added. (– – –) Starch determined. Average of twenty laboratories.

the results to be too high or too low and will create a pattern where the points gather along a diagonal with an angle of 45 °C to the horizontal axis. The more ellipsoid the pattern appears, the greater the influence of the systematic error. The points in Figures 1 and 2 show a tendency towards an ellipsoid pattern, indicating that both systematic (s_b) and random (s_r) errors contributed to the final results.

The standard deviation, s_d (Table 5), of the data distribution reflects the combined effects of these two types of error. Calculations showed that the systematic and the random errors were of equal size (Table 6).

TABLE 6
Errors Related to Starch Analysis in the Collaborative Study

Pair of samples	*Standard deviation,* s_d	*Random error* s_r	*Systematic error* s_b
AC	0·27	0·16	0·15
BC	0·68	0·41	0·38

At the lower starch level, the random error corresponded to 5·4 % of the average starch content. The random error is a measure of how successfully repeat determinations can be done in the same laboratory under the same conditions. It is likely, however, that each laboratory, upon prolonged application, will reduce its random error to the level (2·0 %) obtained when testing the method prior to the collaborative study. Thus, the method appears to be well suited for the analysis of starch in meat products. The systematic error, being a measure of the displacement that may be encountered between laboratories, is less likely to decrease with practice. With the finding of a systematic error of 5 to 8 % of the starch content, however, the method also proves to be applicable for general purposes like food inspection analyses.

ACKNOWLEDGEMENTS

The skilful technical assistance of Ms L. Kaaber is gratefully acknowledged. The author is grateful to Dr H. Russwurm and Professor H. K. Dahle for their help in the planning and administration of the collaborative study and to the following laboratories for their

participation: Department of Food Hygiene, The Veterinary College of Norway; Government Quality Control of Processed Fruits and Vegetables; Government Vitamin Institute; Norwegian Meat Research Laboratory; The Norwegian Potato Industries Laboratory; NOVO Industri A/S, Denmark; The Official Norwegian Quality Control Institute for Canned Fish Products; Research Laboratory of the Norwegian Canning Industry; State Institute of Consumer Research; The Municipal/Regional Food Inspection Units of Asker and Baerum, Bergen, Drammen, Eidsvoll, Haugesund and Karmøy, Nordfjordeid, Sandefjord, Stavanger, Strømmen, Trondheim, Ålesund.

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Substrate Specificity of Alkaline Proteinase from *Aspergillus sojae*, a Soy Sauce Producing Fungus

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ABSTRACT

The specificity of highly purified alkaline proteinase (EC 3.4.21.14) from Aspergillus sojae *was investigated with oxidized insulin A and B chains. Hydrolysis of the oxidized insulin A chain was observed at three peptide bonds* ($CySO_3H^{A11}$—Ser^{A12}, Tyr^{A14}—Gln^{A15}, Glu^{A17}—Asn^{A18}). *Hydrolysis of the oxidized insulin B chain was mainly observed at one peptide bond* (Leu^{B15}—Tyr^{B16}) *and additionally at four others* (Glu^{B13}—Ala^{B14}, Ala^{B14}—Leu^{B15}, Tyr^{B16}—Leu^{B17}, Val^{B18}—$CySO_3H^{B19}$).

Hydrolysis of angiotensin was observed at the Tyr^4—Ile^5 *bond. Hydrolysis of proangiotensin was observed at the* Tyr^4—Ile^5 *and* Phe^8—His^9 bonds.

INTRODUCTION

Soy sauce (Fukushima, 1978, 1980; Ogawa & Fujita, 1980; Young & Wood, 1974) is the product of a complex fermentation in which a mixture of soybean and wheat flour is incubated with *Aspergillus sojae* or *Aspergillus oryzae* and the resulting mass is placed in brine (23% NaCl solution). Soy sauce has occupied a place of honor as a condiment in

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Oriental cuisine since time immemorial and is now finding its way into the occidental kitchen (Young & Wood, 1974).

The alkaline proteinase (EC 3.4.21.14) isolated from *Aspergillus sojae* (Hayashi *et al.*, 1967*a*,*b*) is an important hydrolyzing enzyme in soy sauce fermentation. This is a DFP-sensitive enzyme with a molecular weight similar to that of pancreatic proteinases (Hayashi *et al.*, 1970).

The partially purified preparation of alkaline proteinase from *Aspergillus sojae* (Hayashi *et al.*, 1967*b*) seemed to be homogeneous by free boundary electrophoresis at pH 4·0 and 7·8 and by paper electrophoresis at pH 4·0 and 7·8, and the sedimentation pattern showed it to be monodisperse. However, the presence of a major band and three minor bands was noted on disc gel electrophoresis at pH 9·4.

In the present study, we obtained a further purified preparation of the alkaline proteinase by preparative polyacrylamide disc gel electrophoresis. The specificity of the proteinase was investigated, therefore, using the oxidized insulin A and B chains, angiotensin and proangiotensin. The results are discussed in relation to the bond specificities of the other serine proteinases.

MATERIALS AND METHODS

Materials

Crystalline bovine insulin (Lot 57590) was purchased from Fluka AG, Buchs SG, Switzerland. Human angiotensin (formerly designated angiotensin II) and proangiotensin (formerly designated angiotensin I) were supplied by the Protein Research Foundation, Osaka, Japan.

Alkaline proteinase assay

Activity at pH 7·5 was assayed according to the method described Hayashi *et al.* (1967*b*), except that a reagent (a mixture of 0·33 M CH_3COOH, 0·22 M CH_3COONa and 0·11 M CCl_3COOH) was used to stop the enzymatic reaction. One katal (symbol kat) of the alkaline proteinase is defined as the amount of enzyme which yields the color equivalent of 1 ml of tyrosine per second with the Folin-Ciocalteau phenol reagent, using casein as a substrate at pH 7·5 and 30 °C, according to the enzyme nomenclature of Florkin & Stotz (1973) and the IUPAC and IUB recommendations (1972).

Protein concentration

Protein concentrations were usually estimated from the absorbance at 280 nm by the factor $E^{1\%}_{1\,cm} = 8{\cdot}98$ (Hayashi *et al.* 1967*a*) using a Hitachi Model 101 spectrophotometer.

Polyacrylamide disc gel electrophoresis

Polyacrylamide disc gel electrophoresis was performed at 3 °C with the standard pore formulation (Gabriel, 1971) at pH 9·4 and 2 mA constant for the gel.

Preparative polyacrylamide disc gel electrophoresis

Preparative polyacrylamide disc gel electrophoresis was performed at pH 9·4, 40 mA and 3 °C according to the previously published method (Shuster, 1971).

Oxidation of insulin

Oxidation of insulin was performed as described by Craig *et al.* (1961). Oxidized insulin A and B chains were prepared as described by Griffin *et al.* (1966). The oxidized insulin A and B chains were determined to be pure by *N*-terminal analysis with 1-fluoro-2,4-dinitrobenzene (Frankel-Conrat *et al.*, 1955) and amino acid analysis.

Hydrolysis of oxidized insulin A and B chains

The oxidized insulin A chain (2 μmol, 5·1 mg) was dissolved in 10 ml 0·02 M phosphate buffer (pH 7·5) and then 1·44 nkat (10^{-9} kat) of the alkaline proteinase was added to the solution at a ratio of 2084:1 (mol insulin A chain per mol alkaline proteinase). The mixture was incubated at 30 °C for 90 min.

The oxidized insulin B chain (2 μmol, 7 mg) was dissolved in distilled water and the pH was adjusted to 7·5 with 1 M NH_4OH; 3·2 nkat of the alkaline proteinase was added to the solution at a ratio of 2674:1.

One drop of concentrated HCl was added to inactivate the enzyme. The samples of hydrolysates were stored at −20 °C and the frozen digest was lyophilized.

Hydrolysis of angiotensin and proangiotensin

Angiotensin (1·98 μmol, 2 mg) was dissolved in 10 ml of distilled water, the pH was adjusted to 7·5 with 1 M NH_4OH, and 7 nkat of the alkaline proteinase was added to the solution. The mixture was incubated at 30 °C for 3 h.

Proangiotensin (1·54 μmol, 2 mg) was dissolved in 10 ml of distilled water adjusted to pH 7·5 with 1 M NH_4OH, and incubated at 30 °C with 7 nkat of the alkaline proteinase for 3 h.

Separation of peptides from the enzymatic digest

The freeze-dried digest was then dissolved in 50 μl of 1 M NH_4OH and was separated on Toyo filter paper (60 × 60 cm) in the first dimension by high voltage paper electrophoresis and in the second dimension by paper chromatography by a method previously described (Tanaka *et al.*, 1977).

The letters A and B refer to peptides isolated and purified from the oxidized insulin A and B chains, respectively.

Amino acid analysis

Freeze-dried peptides were dissolved in 2 ml 5·7 M HCl including 5 μl of 2-mercaptoethanol and one drop of 5% phenol, and were hydrolyzed at 110 °C for 24 h. Hydrolysates of peptides were examined with a Hitachi amino acid analyzer, Model 835–30.

Determination of N-terminal amino acid of peptides

The N-terminal amino acids of the purified peptides from digests of the oxidized insulin A and B chains were determined by the DNS method (Gray, 1972; Morse & Horecker, 1966). The DNS-amino acids were identified by polyamide layer sheet (7·5 × 7·5 cm) with the solvent system: 1·5% HCOOH, and benzene–acetic acid (9:1, v/v).

Determination of C-terminal amino acid of peptides

The C-terminal amino acids of the purified peptides were determined with 0·1 nkat of *Penicillium janthinellum* acid carboxypeptidase (Yokoyama *et al.*, 1975*a*,*b*, 1981) at pH 3·7 and 30 °C.

TABLE 1
Amino Acid Compositions of Peptides Obtained from the 90-min Digest of Oxidized Insulin A Chain by *Aspergillus sojae* Alkaline Proteinase at pH 7·5

Amino acid	*Peptides (residues/molecules)*				
	A-1	*A-2*	*A-3*	*A-4*	*A-5*
$CySO_3H$		4·67(4)	3·61(3)	1·03(1)	
Asp		1·64(2)		1·96(2)	
Ser		2·32(2)	1·18(1)		0·95(1)
Glu	1·92(2)	3·64(4)	2·05(2)		
Gly		1·00(1)	1·00(1)		
Ala		1·39(1)	1·13(1)		
Val		2·00(2)	1·62(2)		
Ile		0·77(1)	0·65(1)		
Leu	1·00(1)	1·97(2)			1·39(1)
Tyr		1·72(2)		1·00(1)	1·00(1)
N-Terminus	Glx	Gly	Gly	Asx	Ser
C-Terminus	Glu		$CySO_3H$		Tyr
Suggested segment	15–17	A-chain	1–11	18–21	12–14
Recovery (μmol)	0·162	0·031	0·369	0·254	0·179

Recovery of peptides

The recoveries (μmol) of peptides in Tables 1 and 3 were calculated from the amino acid composition.

Relative rates of hydrolysis

The relative rates of hydrolysis at the $CySO_3H^{A11}$—Ser^{A12} in the oxidized insulin A chain, Leu^{B15}—Tyr^{B16} in the oxidized insulin B chain and Tyr^{4}—Ile^{5} in the proangiotensin were respectively calculated from the peptides recovered.

RESULTS AND DISCUSSION

The highly purified preparation of *Aspergillus sojae* alkaline proteinase migrates as a single band on disc gel electrophoresis at pH 9·4 and the result is shown in Fig. 1. The highly purified preparation of alkaline proteinase had no exopeptidase activities; its specific activity was 113 nkat/kg of enzyme.

TABLE 2

Amino Acid Compositions of Peptides Obtained from the 10-min Digest of Oxidized Insulin B Chain by the Alkaline Proteinase from *Aspergillus sojae*

Values in parentheses denote the theoretical number of residues of a given amino acid in the peptide

Amino acid	*Peptides (residues/molecules)*							
	B-1	*B-2*	*B-3*	*B-4*	*B-5*	*B-6*	*B-7*	*B-8*
$CySO_3H$	1·04(1)	1·01(1)			2·03(2)	1·00(1)	0·78(1)	0·59(1)
Asp	1·00(1)	1·00(1)			1·36(1)		1·00(1)	
Thr					1·00(1)	0·96(1)		1·24(1)
Ser	0·93(1)	0·95(1)			1·06(1)		1·02(1)	
Glu	2·04(2)	2·05(2)			2·65(3)	1·40(1)	1·17(1)	1·38(1)
Pro					1·03(1)	1·11(1)		1·34(1)
Gly	1·14(1)	1·20(1)			3·56(3)	2·13(2)	1·47(1)	2·17(2)
Ala	1·07(1)			1·00(1)	1·89(2)	0·85(1)	1·05(1)	1·23(1)
Val	1·83(2)	1·88(2)	1·00(1)		? (3)	1·27(1)	1·96(2)	0·91(1)
Leu	3·04(3)	2·10(2)	1·05(1)	1·07(1)	3·79(4)	1·14(1)	1·70(2)	1·00(1)
Tyr			0·82(1)		1·65(2)	1·17(1)		1·73(1)
Phe	0·85(1)	0·78(1)			2·88(3)	1·73(2)	0·35(1)	1·78(2)
Lys					1·00(1)	0·39(1)		1·42(1)
His	1·91(2)	1·87(2)			2·75(2)		1·21(2)	
Arg					1·08(1)	0·52(1)		0·71(1)
Recovery (μmol)	0·115	0·069	0·043	0·270	0·394	0·032	0·057	0·036
N-Terminus	Phe	Phe	Tyr	Ala		Leu		Tyr
C-Terminus	Leu	Glu				Ala	Ala	Ala
Suggested segment	1–15	1–13	16–18	14–15	B chain	17–30	1–14	16–30

TABLE 3

Amino Acid Compositions of Peptides Obtained from Angiotensin and Proangiotensin by the Alkaline Proteinase from *Aspergillus sojae*

Amino acid	*Peptides (residues/molecules)*				
	Angiotensin		*Proangiotensin*		
	a-I	*a-II*	*p-I*	*p-II*	*p-III*
Asp	1·00(1)		1·00(1)		
Pro		1·13(1)		1·40(1)	
Val	0·87(1)		0·81(1)		
Ile		0·89(1)		0·66(1)	
Leu					1·00(1)
Tyr	1·03(1)		1·09(1)		
Phe		1·00(1)		1·00(1)	
His		0·86(1)		1·23(1)	0·70(1)
Arg	1·07(1)		1·13(1)		
Recovery (μmol)	0·76	0·68	1·03	0·32	0·65
Suggested segment	1–4	5–8	1–4	5–8	9–10

Fig. 1. Disc gel electrophoresis of the alkaline proteinase from *Aspergillus sojae* at pH 9·4 gel.

The rates of hydrolysis of oxidized insulin A and B chains by the alkaline proteinase were examined and the results are shown in Fig. 2.

The four peptides obtained in 90 minutes' incubation with the oxidized insulin A chain were isolated and purified as described. Table 1 lists the peptides, their amino acid compositions and recoveries. All of the peptides were readily identifiable from the known sequence of the oxidized insulin A chain. The N-terminals and C-terminals of peptides isolated were

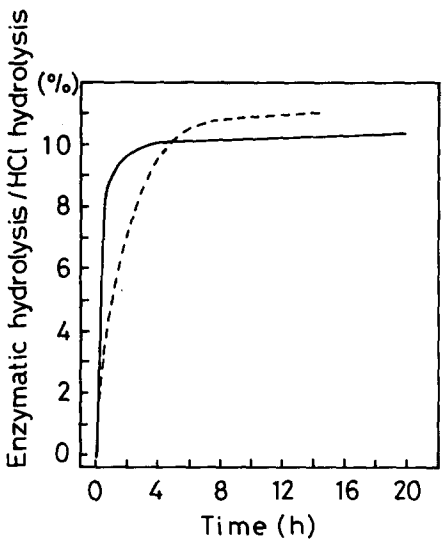

Fig. 2. Time course for the hydrolysis of oxidized insulin A and B chains by the alkaline proteinase from *Aspergillus sojae*. Increase in amino groups was determined by the ninhydrin method. The reaction mixture contained 2 μmol of A or B chains in 10 ml solution at 30 °C. Aliquots (500 μl) were withdrawn at various time intervals. Total free amino groups were determined with the hydrolysates of 6 N HCl at 110 °C for 24 h -----, oxidized insulin A chain, ——, oxidized insulin B chain.

identified as shown in Table 1. Hydrolysis of the oxidized insulin A chain was observed at three peptide bonds ($CySO_3H^{A11}$—Ser^{A12}, Tyr^{A14}—Gln^{A15}, Glu^{A17}—Asn^{A18}). A summary of the initial cleavage sites of the oxidized insulin A chain by the alkaline proteinase is shown in Fig. 3.

For the hydrolysis of the oxidized insulin B chain the reaction proceeds almost linearly for about 5–10 min; thereafter a reduction is seen in the reaction rate. Figure 2 shows an unusually restricted specificity of the alkaline proteinase towards the oxidized insulin B chain. The seven peptides obtained in 10 minutes' incubation with the oxidized insulin B

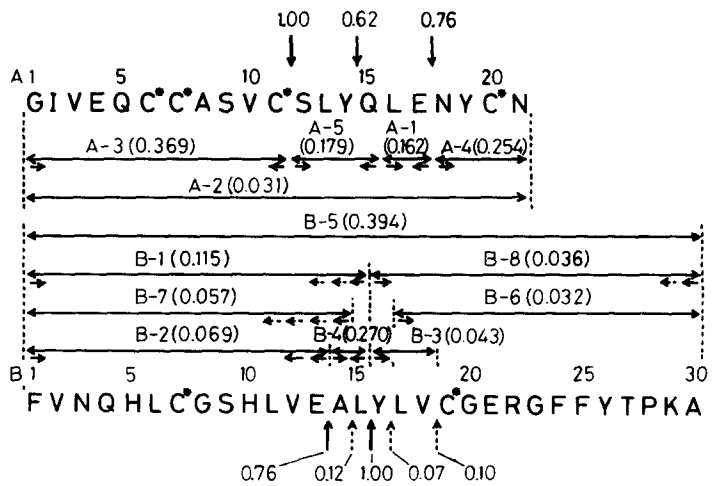

Fig. 3. Summary of the specificity of the alkaline proteinase from *Aspergillus sojae* towards the oxidized insulin A and B chains at pH 7·5 and 30 °C. The enzymatic digest of the chains was directly separated as described in the text. Abbreviations of amino acids follow the alphabetical system. C* indicates cysteine sulfonic acid. Values in parentheses denote the uncorrected recovery (μmol) of peptide (↔). Perpendicular arrows (↓, ↑ or ↑) indicate the bond split and the relative rate of hydrolysis of A 11–12 bond and B 15–16 bond, respectively, the degree of hydrolysis being as follows: ↑ > ↑. Horizontal arrows indicate residues removed by the following procedures: → identified by the DNS-method; ← amino acid released with *Penicillium janthinellum* acid carboxypeptidase (Yokoyama *et al.*, 1975*a*,*b*, 1981), the degree of hydrolysis by the acid carboxypeptidase being as follows: ← > ←.

chain were isolated and purified. Table 2 lists the peptides and their amino acid compositions and recoveries. The strong spots were B-1 and B-4. N-terminals of B-1, B-2, B-3, B-4, B-6 and B-8 were identified. C-terminals of B-1, B-2, B-6, B-7 and B-8 were identified (Table 2). The results in Fig. 3 show that the alkaline proteinase primarily hydrolyzed at one peptide bond in the oxidized insulin B chain, the Leu^{B15}—Tyr^{B16}, and additionally at four others (Glu^{B13}—Ala^{B14}, Ala^{B14}—Leu^{B15}, Tyr^{B16}—Leu^{B17} and Val^{B18}—$CySO_3H^{B19}$).

When angiotensin was digested with the alkaline proteinase for 3 h, the products could be resolved into two spots by high-voltage paper electrophoresis at pH 6·5 and paper chromatography. The digestion products of angiotensin were subjected to amino acid analysis. The results are shown in Table 3. The peptide bond that had been hydrolyzed by the alkaline proteinase was Tyr^4—Ile^5 bond in angiotensin.

When proangiotensin was digested with the alkaline proteinase for 3 h, the three peptides were obtained. The results are shown in Table 3. The peptide bonds that had been hydrolyzed by the alkaline proteinase were the Tyr^4—Ile^5 bond and the Phe^8—His^9 bond. In this experiment, we found the characteristic small dipeptide His—Leu, p—III, in the hydrolysate of proangiotensin. A summary of the specificity of the alkaline proteinase for angiotensin and proangiotensin is shown in Table 4.

TABLE 4

Hydrophobicities of Side Chain of Amino Acid Residues Adjacent to those Involved in the Bond to be Split by the Alkaline Proteinase from *Aspergillus sojae*

Arrow indicates position of the splitting site. The relative hydrolysis rate of oxidized insulin A and B chains was calculated from the peptide recovered.

Substrate	*Amino acid residues adjacent to those involved in the bond to be split by the enzyme* S_2 S_1 S'_1 S'_2 / $—P_2—P_1—P'_1—P'_2—$	*Hydrophobicities of side chain of amino acid residues* Δgt *(kcal/mol)*	*Relative rate of hydrolysis*
Oxidized insulin A chain	–V–C–S–L– (11|12)	–1·5–?–(–)0·3–1·8–	1·00
	–L–E–N–Y– (17|18)	–1·8–0–0·01–2·3–	0·76
	–L–Y–Q–L– (14|15)	–1·8–2·3–0·1–1·8–	0·62
Oxidized insulin B chain	–A–L–Y–L (15|16)	–0·5–1·8–2·3–1·8–	1·00
	–V–E–A–L– (13|14)	–1·5–0·6–0·5–1·8–	0·76
	–E–A–L–Y– (14|15)	–0·6–0·5–1·8–2·3–	0·12
	–L–V–C–G– (18|19)	–1·8–1·5–?–0–	0·09
	–L–Y–L–V– (16|17)	–1·8–2·3–1·8–1·5–	0·07
Proangiotensin	–V–Y–I–H– (4|5)	–1·5–2·3–3·0–0·5–	1·00
	–P–F–H–L– (8|9)	–2·6–2·5–0·5–1·8–	0·72

Spadari *et al.* (1974) showed that the serine proteinase from *A. oryzae*, aspergillopeptidase B, cleaved essentially the bond Leu^{15}—Tyr^{16} in the oxidized insulin B chain at 0 °C, pH 10·2 for 30 minutes' incubation (Fig. 4). Two minor peptides, B1–B16 and B17–B30, were present only in 4–5 % amounts. The initial cleavage site at the Leu^{15}—Tyr^{16} bond in the oxidized insulin B chain with the alkaline proteinase from *A. sojae* was similar to that reported on work on aspergillopeptidase B (Spadari *et al.*, 1974) and thermomycolin (EC 3.4.21.14, thermomycolase) from *Malbrancheu pullchella* var. *sulfurea*, a thermophilic fungi imperfecti, at pH 9·0, 45 °C for 5 min (Stevenson & Gaucher, 1975). However, *A. sojae* alkaline proteinase could not split the Phe^{24}—Phe^{25} and Gln^{4}—His^{5} bonds, which are known minor cleavage sites with thermomycolin.

The highly restricted specificity and initial cleavage site at the Leu^{15}—Tyr^{16} bond in the oxidized insulin B chain with the serine alkaline

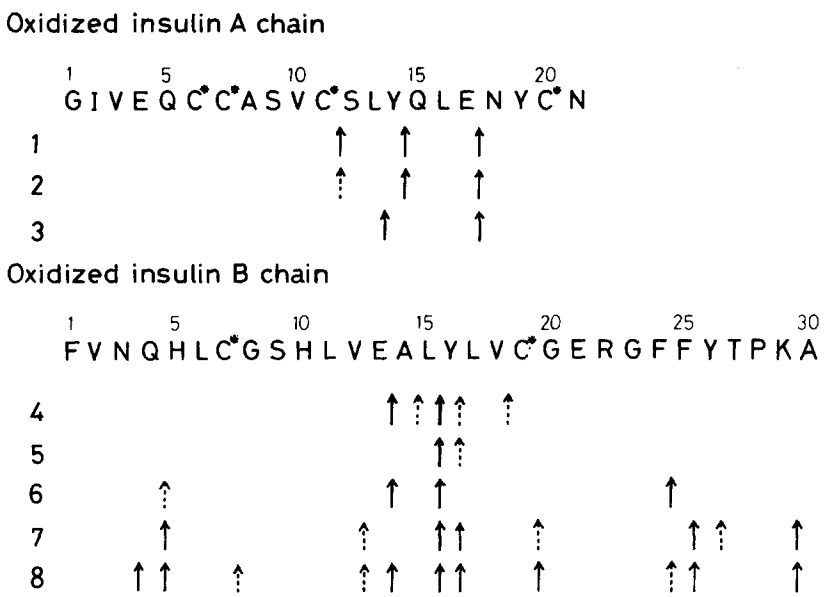

Fig. 4. Hydrolysis of oxidized insulin A and B chains by various alkaline serine proteinases. 1. *Aspergillus sojae* alkaline proteinase, pH 7·5, 30 °C, 90 min. 2. Aspergillopeptidase B, pH 10·2, 0 °C, 30 min. (Spadari *et al.*, 1974). 3. Thermomycolin, pH 7·0, 25 °C, 5 min. (Stevenson & Gaucher, 1975). 4. *Aspergillus sojae* alkaline proteinase, pH 7·5, 30 °C, 10 min. 5. Aspergillopeptidase B, pH 10·2, 0 °C, 30 min. (Spadari *et al.*, 1974). 6. Thermomycolin, pH 9·0, 45 °C, 5 min. (Stevenson & Gaucher, 1975). 7. *Aspergillus oryzae* alkaline proteinase, pH 9, 25 °C, 2 h. (Morihara & Tsuzuki, 1969). 8. *Aspergillus flavus* alkaline proteinase, pH 8·5, 37 °C, 1 h. (Turkova & Mikes, 1970).

proteinases from *A. sojae*, *A. oryzae* (Spadari *et al.*, 1974), and *Malbrancheu pullchela* var. *sulfurea* (Stevenson & Gaucher, 1975) are much narrower specificities than those previously reported from *A. oryzae* (Morihara & Tsuzuki, 1969) and *A. flavus* (Turková & Mikeš, 1970). It is notable that the presence of *Aspergillus* acid carboxypeptidase (Ichishima & Arai, 1973) activity in both alkaline proteinase preparations from *A. oryzae* and *A. flavus* still remains a possibility.

Staphylococcus aureus protease (Drapeau, 1976) has high affinity for the Glu^{13}—Ala^{14} bond. *A. sojae* alkaline proteinase cleaves the same bond. However, the *A. sojae* proteinase could not split the Glu^{21}—Arg^{22} bond, which is the known cleavage site with *S. aureus* proteinase.

In the present study, residues in peptides and the corresponding subsites in the enzyme are numbered according to Schechter & Berger (1967).

Angiotensin or proangiotensin has been used to a very limited extent as a proteinase substrate. However, while the alkaline proteinase cleaves the Tyr^4—Ile^5 bond in angiotensin, the enzyme cleaves the Tyr^4—Ile^5 and Phe^8—His^9 bonds in proangiotensin. The results suggest that susceptibility to the alkaline proteinase does not require the presence of an amino acid residue at the P'_3 position. The specificity of the alkaline proteinase, as observed with the release of the dipeptide His^9—Leu^{10} from the C-terminus, resembles the specificity of the angiotensin converting enzyme (EC 3.4.15.1, dipeptidyl carboxypeptidase).

The effect of the hydrolysis rate of side chains in the amino acid residue of the substrates was elucidated in the present experiments. Hydrophobicities (Tanford, 1962; Nozaki & Tanford, 1971) of the side chain of amino acid residues adjacent to those involved in the bonds to be split by the alkaline proteinase from *A. sojae* are indicated in Table 4. The peptide bonds which have a hydrophobic amino acid such as leucine, valine, phenylalanine and tyrosine in the P_2 and P'_2 positions are preferentially cleaved by the alkaline proteinase from *A. sojae*. These results show that the S_2–P_2 and S'_2–P'_2 interactions might be important in increasing enzyme–substrate affinity and turnover rate. It will be noted that, for substrates of the type P_2–P_1–P'_1–P'_2, a change in the P_2 or P'_2 position from hydrophobic amino acid to hydrophilic amino acid leads to a substitution in the P_1 or P'_1 position from hydrophilic amino acid to hydrophobic amino acid as shown in Table 4. The present results show that the specificity of the alkaline proteinase for the S_2—P_2 interaction resembles that of papain (EC 3.4.22.2).

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Food Chemistry **11** (1983) 201–216

GC/MS and Sensory Analysis of Volatiles from Three Cultivars of Capsicum

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(Received: 2 November, 1982)

ABSTRACT

Volatiles from three cultivars of Capsicum annuum*—Jalapeño, Anaheim and Fresno—were analyzed by GC (gas chromatography) and by an experienced panel of judges. The Jalapeño volatiles were further measured by MS (mass spectroscopy) and by sniffing of the GC eluate. Agitation of the chopped, fresh fruit by swirling of the container increased nasal irritation, which interfered with descriptive analysis of the aroma. Without agitation, varietal differences were more apparent. Discriminating terms included 'rose' for Jalapeño > Fresno > Anaheim, 'grassy' for Anaheim > Fresno and Jalapeño and 'garbanzo beans' for Anaheim > Fresno and Jalapeño. All varieties possessed aromas described as green, green-vegetable, floral and apple. GC and GC/MS analyses indicated the presence of esters, alcohols, ketones and pyrazines which have been described previously as having green, fruity and floral aromas. Several monoterpenes and sesquiterpenes were tentatively identified. 'Green-like' terms were used almost exclusively when sniffing of the GC eluates from Jalapeño. 'Green pepper' frequently was used to describe volatiles in the retention index range where the 2-alkyl-3-methoxypyrazines would be expected to elute. Absence of floral- or apple-like aromas, similar to those perceived in the fresh fruit, may have been due to separation of compounds which interact to create these qualities, or to changes attributable to the extraction procedures.*

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INTRODUCTION

Fruit of the genus *Capsicum*, both fresh and dried, are used extensively in tropical and semi-tropical regions of the world as spices and as staples in the diet. Capsicums are used primarily for their contributions to pungency and to color, but the various varieties do have distinctive aromas (Heath, 1981). Capsaicinoids, the pungent principles in capsicum, have been studied in detail (Maga, 1975; Polesello & Pizzocaro, 1976; Todd *et al.*, 1977; Salzer, 1977). In contrast, the other volatile flavour compounds of this species have received limited attention. Buttery *et al.* (1969*b*) reported the presence of *trans*-beta-ocimene, limonene, methyl salicylate, linalool and many other compounds in *C. annuum* var. *grossum*, Sendt (green bell pepper). Also isolated was 2-methoxy-3-isobutylpyrazine, which possessed a characteristic bell pepper aroma and was reported to have an absolute odor threshold of two parts per 10^{12} in water. Preliminary volatile analysis of an unidentified variety of chili pepper indicated the presence of this same compound.

According to Haymon & Aurand (1971), the neutral volatile fraction of *C. frutescens* (Tabasco peppers) yielded 125 peaks, of which 24 were identified, including 4-methylpentyl-2-methylbutanoate, 3-methylpentyl-3-methylbutanoate, and 4-methylpentyl-4-methyl pentanoate. That the authors did not detect 2-isobutyl-3-methoxypyrazine in that isolate is not surprising since this compound would have been extracted in the acidic fraction.

Utilizing headspace techniques, Murray & Whitfield (1975) isolated several 2-alkyl-3-methoxypyrazines, including 2-isopropyl-, 2-isobutyl-, and 2-sec-butyl-3-methoxypyrazine, in *C. annuum* var. *grossum* and red *C. frutescens*. The predominant compound in both species was 2-isobutyl-3-methoxypyrazine. Huffman *et al.* (1978) reported the occurrence of 2-isobutyl-3-methoxy pyrazine in a disease-resistant Jalapeño cultivar, J100, mostly in the outer wall of the fruit. Although no analysis was reported for other volatile flavor compounds, the investigators concluded that 2-isobutyl-3-methoxypyrazine was the major compound responsible for Jalapeño flavor. Takahashi *et al.* (1979) reported hexenal, pulegone and seven hydrocarbons in the essential oil of *C. annuum fasciculatum*.

Whereas previous investigations have been concerned with hedonic (like–dislike) responses to Jalapeño peppers (Weisenfelder *et al.*, 1978; Saldana & Meyer, 1981), no reports are available on the aroma quality of these and related fruit. Recent studies have noted that, in addition to

acquiring a liking for the intensity and character of the pungency of capsicum, people typically like the widely varying aromas and flavors of the many varieties of the fruit (Rozin & Schiller, 1980; Rozin *et al.*, 1981, 1982).

The present experiments were undertaken to investigate the relationship between the volatile constituents and aroma characteristics of three commercially important California capsicums.

PROCEDURES

Cultivars studied were *Capsicum annuum* varieties of Anaheim, Jalapeño and Fresno, grown under identical cultural practices in Sacramento, California. Peppers were harvested both green and mature, on alternate days. For sensory evaluation and for isolation of volatiles, the fruit was washed and cut into small pieces (*ca.* 1–1·5 cm^2), after removal of the stem, calyx and seeds.

Sensory analysis

Approximately 30 g of cut fruit were served at room temperature (20° ± 1 °C) in black, coded wine glasses (volume, 250 ml) covered with watchglasses. A panel of six judges (21–26 years of age), all of whom had extensive previous experience in descriptive sensory analysis, devoted four group sessions to the development of descriptors and corresponding references for the three capsicum samples (Table 1). These terms were included on the evaluation forms and judges were required to acquaint themselves with the corresponding odor references prior to each evaluation session. Evaluations were made at room temperature in isolated booths illuminated with red lights to disguise visible differences among samples. At each session, four samples were presented in randomized order: the three varieties plus a replicate of one variety as an internal check on judge reproducibility. Each sample was presented in duplicate, so that judges could alternate between duplicates to allow headspace re-equilibration in previously sniffed glasses.

Using a 10-point odor intensity scale for each descriptor, judges evaluated the samples under two conditions: (1) perceived aroma characteristics without agitating the wine glass and (2) after vigorously swirling the wine glass for 5 s.

TABLE 1
Descriptors and References Used in Sensory Analysis of the Aroma of Fresh Capsicum

Descriptor	*Reference*
Tomato leaf	Tomato leaf
Grassy	Fresh grass clippings, macerated in water
Green	*Cis*-3-hexen-l-ol, 10% in water
Fresh green bean	Fresh green beans, macerated in water
Canned green bean	Canned green beans, macerated in water
Cucumber	Fresh cucumber, sliced and finely chopped
Garbanzo bean	Canned garbanzo beans, crushed
Rose	Red rose petals macerated in water
Floral	Phenylethyl acetate, 10% in ethanol
Fruity	Mixture of: γ nona lactone *Cis*-3-hexen-l-ol Isoamyl acetate 2,6-dimethyl 2-heptenal Ethyl levulinate Ethyl hexadienoate Ethyl butyrate Ethanol
Apple	Golden Delicious apple, chopped
Citrus	Citrus diethyl acetal, 10% in ethanol
Cidery	Lady Lee Apple Juice, diluted 3:1 with water
Eucalyptus	Eucalyptus leaves, chopped
Piney	Pine needles, chopped
Cedar wood oil	Cedar wood oil
Irritating	None

Sample preparation

Extracts of fresh peppers were prepared for GC and GC/MS analyses using a simultaneous stream distillation-extraction apparatus designed by Nickerson & Likens (1966) and modified by Maarse & Kepner (1970). Peppers were prepared as described previously. Placed in a 3-liter distillation flask with 375 ml of distilled water were 375 g of the fruit, a magnetic stirring bar and 10 mg of tristearin (Eastman-Kodak Co., Lot No. A5B). The solvent flask was filled with 10 ml pentane (Matheson, Coleman & Bell, Insecticide Grade, Lot No. 7G08) and the center arm was charged with sufficient pentane and water to allow these to return to their respective flasks. The solvent and distillation flasks were heated in a 55 °C

water bath and a 130 °C paraffin oil bath, respectively. Distillation was continued for 2 h after the onset of turbidity in the center arm. The extract was collected and concentrated to 0·2 ml under a stream of nitrogen that had been purified by passage through a mixture of molecular sieves 5A and 13X. Samples were stored in Teflon capped vials at −20 °C until analyzed.

Gas chromatographic analysis

Extracts were analyzed on a Hewlett-Packard 5711A gas chromatograph equipped with a highly linear glass inlet splitter and adapted to WCOT glass capillary columns. Inlet and detector temperatures were 250 °C. Detector gas flow rates were optimized at 30 ml/min nitrogen (makeup), 30 ml/min hydrogen and 300 ml/min air.

Two columns were used; specific conditions of analyses and pertinent characteristics of each column were:

(1) 42·6 m × 0·22 mm inside diameter, coated with SP 2100. Column efficiency was determined at 100 °C, with helium at an average linear carrier gas velocity of 16·7 cm/s and a split ratio of *ca.* 1:100. Under these conditions, the column exhibited 97 600 effective theoretical plates for a compound with a partition ratio (k) of 7·0.

(2) 28·2 m × 0·28 mm inside diameter column identified as Carbowax 20 M. Comparison of retention indices of knowns on this column with those found in the literature (Kepner, 1974) exhibited some discrepancies, which may relate to the fact that the Carbowax liquid phases are not a single molecular species, but a mixture of polyethylene glycol fragments of a specified average, and unspecified range, of molecular weights. Column efficiency was determined at 100 °C, with an average linear carrier gas velocity (helium) of 18·0 cm/s and with the split ratio adjusted to *ca.* 1:100. The column had 86 400 effective theoretical plates for a compound with $k = 6·4$. In the remainder of this paper, this column will be referred to as the polar phase column.

Kovats retention indices (Kovats, 1965) were determined for the unknown compounds in each extract. Isothermal retention indices were determined on the SP 2100 column at 30 °C, 80 °C, 130 °C, 180 °C and 220 °C. Temperature programmed chromatograms were obtained on the SP 2100 column with oven temperature held at 30 °C for 8 min and then

programmed to 200 °C at 1 °C/min and held. The flow rates and split ratios were the same as described previously.

Gas chromatography/mass spectrometry

For GC/MS analysis, a Hewlett/Packard model 5985A quadropole mass spectrometer was interfaced with a Hewlett/Packard 5840A gas chromatograph. Utilizing splitless injection (Grob & Grob, 1974), 0·2 μl of Jalapeño extract was coinjected with a 0·8 μl 'solvent plug' of pentane. The eluate was split 1:1 to a flame ionization detector and the mass spectrometer via glass and platinum–iridium connections. The analysis was performed on a 30-m SE-54 column, temperature programmed from 45 °C to 270 °C at 11 °C/min. Mass spectra were taken every second over the *m/e* range 45 to 450 with an ionization voltage of 70 eV. When an impure spectrum was suspected, selective ion chromatograms were obtained. If possible, a scan was taken at another point and/or a neighboring spectrum was subtracted to obtain a mass spectrum of a single compound.

Gas chromatographic eluate sniffing

A Packard model No. 427 GC equipped with a thermal conductivity detector (TCD) was used for eluate sniffing. Injector and detector blocks were set at 250 °C with the thermistor temperature at 400 °C. A 6 ft, 1/8 in inside diameter stainless steel column packed with 10% SP 2100 on 100/120 Chromosorb W AW was used. Helium carrier gas flow rate was adjusted to 15 ml/min for both the reference and actual column. Each run employed 5 μl of Jalapeño extract isolated as described previously. Isothermal runs at a number of temperatures indicated that oven temperatures of 50 °C and 130 °C eluted the greatest number of compounds which could be detected by sniffing at the exit port.

The exit port was modified for sniffing using an apparatus similar to that described by Spencer (1977). Five judges from those who participated in the descriptive analyses were introduced to eluate sniffing by evaluating the two chromatographic regions of interest twice before their results were included. The judges developed aroma descriptors and corresponding references were obtained. During final evaluations, judges referred to the list of thirty-one aroma descriptors. They evaluated each chromatographic region twice with runs lasting approximately 20 min. Retention

indices were calculated for each descriptor mentioned. Common descriptors with similar retentions were pooled.

RESULTS AND DISCUSSION

Identification of volatile constituents

Figures 1(a), (b) and (c) represents FID chromatograms for the Jalapeño, Fresno and Anaheim extracts, respectively. Compounds identified are listed in Table 2. The Jalapeño extract embraced the widest range of volatiles and was analyzed by GC/MS. Compounds in the other extracts were correlated on the basis of precisely determined retention indices (Yabumoto *et al.*, 1977; Spencer *et al.*, 1978). Identifications as shown in Table 2 are supported by reference spectra (Haymon & Aurand, 1971;

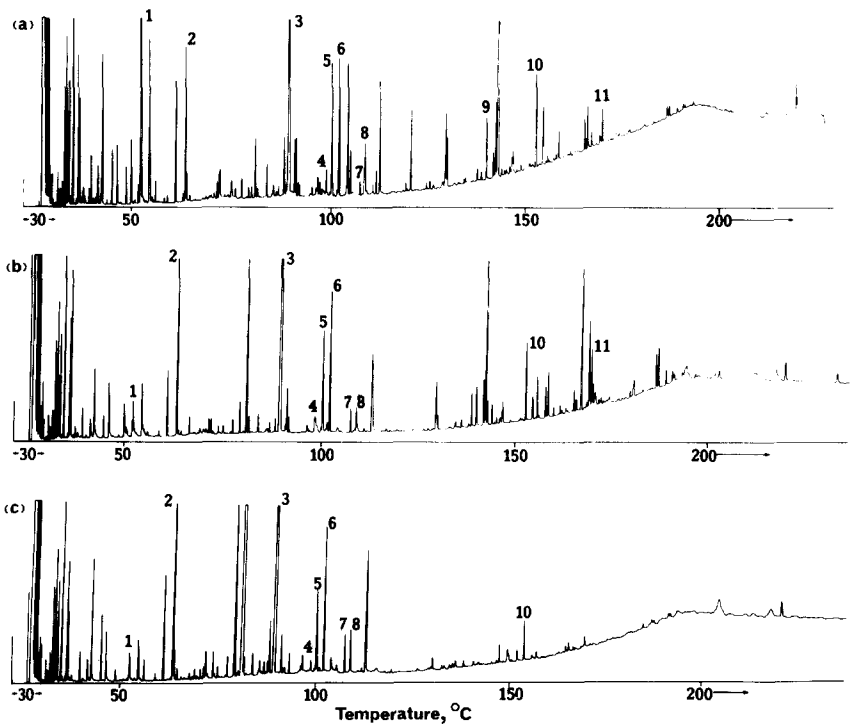

Fig. 1. Representative chromatograms of capsicum extracts on an SP 2100 column. (a) Jalapeño. (b) Fresno. (c) Anaheim. See text for chromatographic details.

TABLE 2
Retention Indices and Criteria of Identification of Compounds Isolated from Capsicum Extracts

Compound	*Peak number*	*SP 2100 known*	*SP 2100 literature*	*SP 2100 Jalapeño*	*SP 2100 Fresno*	*SP 2100 Anaheim*	*Polar known*	*Polar Jalapeño*	*Polar Fresno*	*Polar Anaheim*	*Criteria of identification*
Cis-3-hexen-l-ol	1	846[1]	849	851	849	852	1 323[3]	1 320	1 318	1 318	MS[5], RI[6], PE[8]
Benzaldehyde	2	936[1]	946	938	936	937	1 468[3]	1 468	1 471	1 465	MS, RI, PE
Linalool	3	1 090[1]	1 090	1 088	1 086	1 088	1 483[3]	1 482	1 481	1 481	MS, RI, PE
2-sec-butyl-3-methoxypyrazine	4	1 160[2]	—	1 162	1 157	1 160	1 465[3]	1 464	1 463	1 462	MS, RI, PE, NSD[9]
2-isobutyl-3-methoxypyrazine	5	1 168[2]	—	1 171	1 166	1 168	1 487[3]	1 488	1 492	1 488	MS, RI, PE, NSD
Methyl salicylate	6	1 190[2]	1 180	1 188	1 186	1 187	1 752[4]	1 754	1 756	1 753	MS, RI, PE
Cis-3-hexenyl isopentanoate	7	1 220[2]	—	1 218	1 219	1 220	—	—	—	—	MS, RIS[7], PE
n-hexyl isopentanoate	8	1 226[2]	1 229	1 227	1 225	1 226	—	—	—	—	MS, RIS, PE
β-ionone	9	1 485[4]	1 481	1 487	—	—	1 889[4]	1 885	—	—	MS, RI, PE
Hexadecane	10	1 601[4]	1 600	1 601	1 600	1 599	1 600[3]	1 600	1 599	—	MS, RI, PE
Heptadecane	11	1 701[4]	1 700	1 701	1 700	—	1 700[4]	1 700	1 702	—	MS, RI, PE

[1,2,3,4] Retentions of known and unknown at 80 °C, 130 °C, 140 °C and 180 °C, respectively.
[5] Mass Spectrometry.
[6] Retention indices on dissimilar liquid phases.
[7] Retention indices on single liquid phase.
[8] Peak enrichment.
[9] Nitrogen selective detector.

Kennet *et al.*, 1977; Stenhagen *et al.*, 1974), Kovats retention indices on dissimilar liquid phases and peak enrichment, unless otherwise noted. All spectra in this study were obtained from a single scan of the mass to charge ratio (*m/e*) range under study.

Spectral data were obtained on a few compounds not listed in Table 2. While insufficient for identification, the data indicated structural features that permitted tentative conclusions to be drawn. Mass spectral patterns consistent with the presence of a hexyl hexanoate isomer, three hexyl pentanoate isomers and several monoterpenes and sequiterpenes were observed in certain multicomponent fractions, but the degree of resolution was too poor to permit definitive characterization. In addition, a spectrum matching that of nona-2-ene-4-one was obtained. The *trans* isomer rarely occurs naturally, but has been reported in green bell peppers (Buttery *et al.*, 1969*b*). A compound of similar retention characteristics occurs in the extracts, but it is not possible to specify the isomer from these data. Another spectrum showed good agreement with hexyl butyrate-isobutyrate spectra, but comparisons of retention indices failed to confirm the presence of either of these compounds in the extracts.

The published aroma descriptors of the compounds identified in capsicum extract (Table 3) are similar to descriptive terms described herein. Spectral evidence indicated the presence of a hexylbutyrate isomer, three other hexyl pentanoate isomers, a hexyl hexanoate isomer

TABLE 3
Representative Aroma Descriptors of Compounds Identified in Capsicum Extracts

Compound	*Descriptor*	*Source*
Cis-3-hexen-l-ol	Green, grassy	Arctander (1969)
Benzaldehyde	Bitter almond	Arctander (1969)
Linalool	Floral	Fenaroli (1975)
2-sec-butyl-3-methoxypyrazine	Green bell pepper	Parliament & Epstein (1973)
2-isobutyl-3-methoxypyrazine	Green bell pepper	Buttery *et al.* (1969*a*)
Methyl salicylate	Wintergreen-like	Fenaroli (1975)
Cis-3-hexenyl isopentanoate	Sweet green, apple-like	Arctander (1969)
n-hexyl isopentanoate	Fruity, similar to unripe apple	Arctander (1969)
β-ionone	Violet-like, fruity and woody	Fenaroli (1975)
Hexadecane		
Heptadecane		

and six sequiterpenes. The unidentified esters may contribute to green or fruity aromas (Arctander, 1969). Sesquiterpenes and monoterpenes, while generally less potent than compounds with functional groups, may also contribute to aroma character. The aroma descriptors for this group of compounds are varied and possible contributions cannot be assessed without more conclusive identification. The use of aroma descriptors to hypothesize the contribution of a single compound to an aroma composite may be misleading due to blending, masking, synergism and other changes in perceived character, which may take place upon mixing of compounds.

The compounds identified in the extracts and the terms used in descriptive analysis indicated that the aroma of the cultivars under study was not attributable to any one family of compounds. The assumption that the 2-alkyl-3-methoxypyrazines are solely or predominately responsible for aromas in all capsicum (Huffman *et al.*, 1978) may be erroneous.

Descriptive aroma analysis

Means and standard deviations for the sensory descriptors, 'overall aroma' and 'irritating' are shown in Fig. 2 for three varieties of capsicum evaluated with and without swirling the container. Although individual judges responded differently to three varieties (as indicated in the analysis of variance by the significant interaction of judges by varieties), judges as a group were consistent across replications. Application of Fisher's LSD to treatment means established that overall aroma intensity and degree of nasal irritation between the Jalapeño and the Fresno varieties did not differ significantly, but were greater ($p < 0{\cdot}001$) than that of the Anaheim variety.

Agitating the samples increased both sensory attributes, especially the nasal irritation, possibly due to the release of capsaicinoids into the headspace of the container. Although the size and hydrogen bonding capability of the capsaicinoids would suggest that they have only limited volatility, apparently an extremely low headspace concentration can elicit the nasal pain response. The capsaicinoids stimulate free nerve endings in the nasal and oral cavity, indicating that the trigeminal, or fifth cranial nerve, can modify perception of odor intensity. In a study of patients with surgically induced, unilateral destruction of the trigeminal nerve, Cain (1974) observed consistently lower perceived odor magnitudes with the

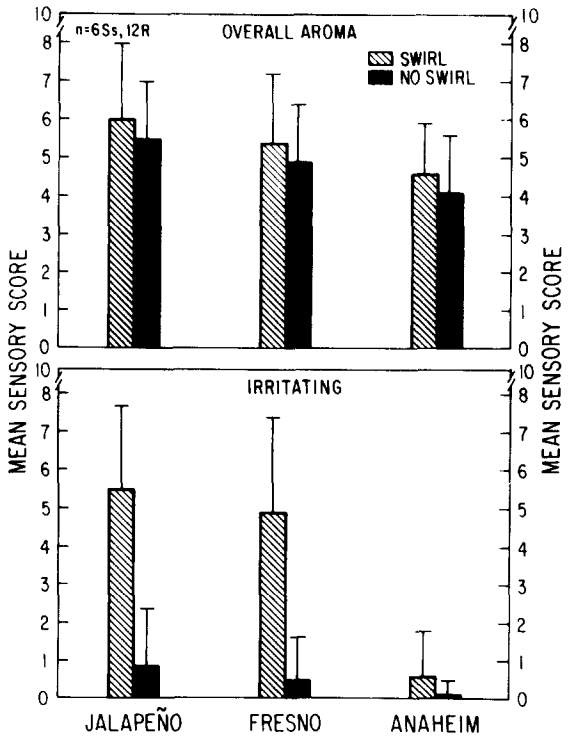

Fig. 2. Means (six subjects, twelve replications) and standard deviations for intensity of overall aroma and of irritating characteristic of three varieties of capsicum, with and without swirling of the container.

ablated nostril than with the normal, suggesting interaction between perception of odor and pain stimuli (Cain, 1974; Cain & Murphy, 1980). The impact that capsaicin irritation may have had on the perceived intensity of individual aromas in this study is unknown, but should be considered as a possible influence.

The remaining aroma descriptors were analyzed separately (Fig. 3). Varieties differed significantly ($P < 0{\cdot}01$) under the non-agitated condition, but were not significant sources of variation when the samples were agitated, as established by analysis of variance. The difficulty experienced by judges in discriminating the three varieties when samples were agitated may be attributable to the impact of the capsaicinoids which some judges felt completely dominated the other attributes of the sample.

Variation in judge response was reflected by the relatively large standard deviations about the means, attributable to variation in the use

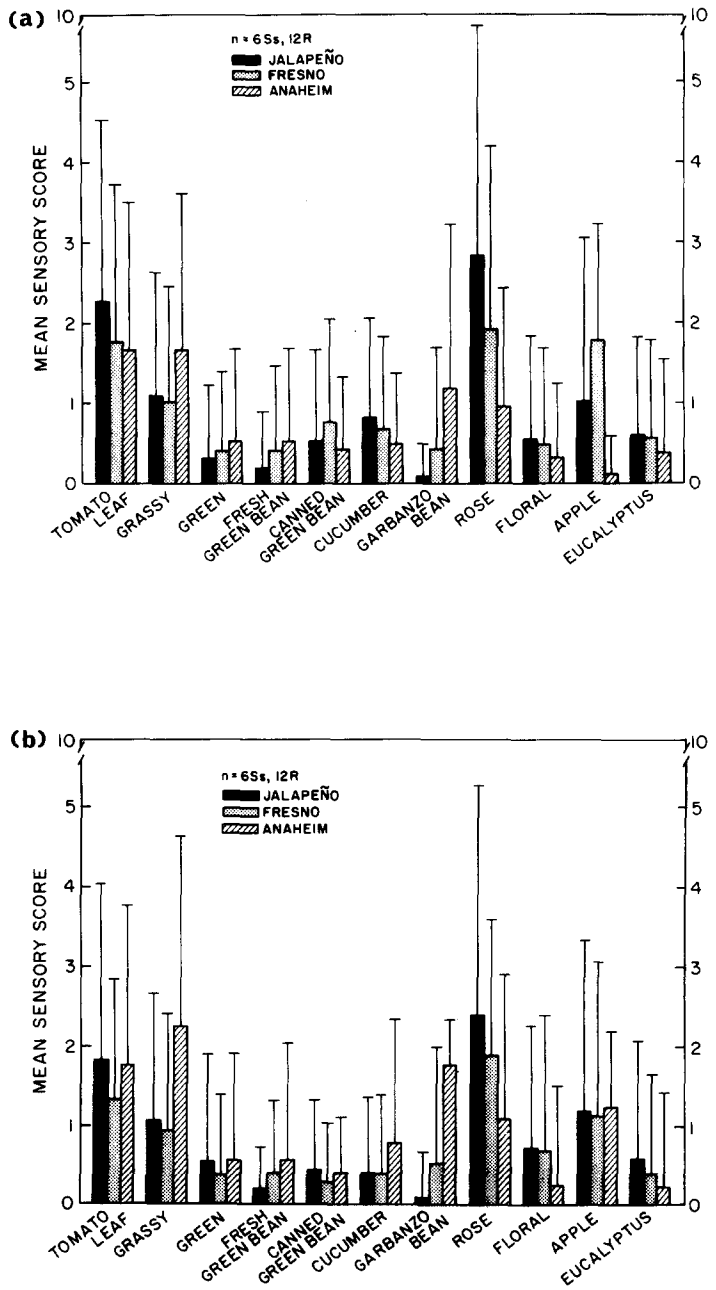

Fig. 3. Means (six subjects, twelve replications) and standard deviations for aroma intensity for eleven descriptors for three varieties of capsicum. (a) Without swirling of the container. (b) Swirling the container.

of similar terms, to differential sensitivity and/or to the aroma complexity of the headspace mixture. Several compounds have been reported to have similar odors, e.g. all of the following have been described as being 'green', or 'green vegetable' like in character: cis-3-hexen-1-ol (Furia & Bellanca, 1975), 2-sec-butyl-3-methoxypyrazine (Murray *et al.*, 1975), 2-isobutyl-3-methoxypyrazine (Murray *et al.*, 1975) and 2-isobutyl-3-methoxypyrazine (Takken *et al.*, 1975). These compounds were identified in extracts from the capsicum studied herein and may be responsible for the frequent use of green descriptors. The compound 2-sec-butyl-3-methoxypyrazine was described herein as resembling cooked celery, in contrast to Murray *et al.* (1970) who described this compound as possessing a strong pea-like aroma, while Parliament & Epstein (1973) reported that it possessed a bell pepper aroma and Cronin & Stanton (1976) felt the aroma was similar to that of raw carrot.

The Anaheim variety was rated significantly higher than the other varieties in intensity of grassy aroma (both conditions), fresh green bean (agitated) and garbanzo bean (non-agitated) aromas. The Jalapeño and Anaheim varieties differed significantly for the descriptor 'garbanzo bean' (agitated). The Fresno and Anaheim varieties differed significantly in 'floral' (agitated) aroma. The most significant difference among all varieties was for the descriptor 'rose' (non-agitated). The presence of the 'rose', 'floral' and 'apple' aromas suggest that compounds other than the 2-alkyl-3-methoxypyrazines may be important contributors to aroma of these capsicums. Previously, Keller *et al.* (1981) reported that volatiles from fresh Jalapeño concentrate had a pleasant, floral aroma, as well as an appreciable bell pepper character.

Table 4 lists results of sniffing tests conducted on specific retention index zones, and frequency of mention for descriptors used at 50° and 130 °C. Only descriptors used two or more times are included. Most terms describe aromas which are green or vegetable-like in character, consistent with many of the compounds identified and discussed earlier. The aromas of 2-sec-butyl-3-methoxypyrazine and 2-isobutyl-3-methoxypyrazine have been described as green bell pepper in character (Parliament & Epstein, 1973; Takken *et al.*, 1975). The green pepper descriptor was used most frequently at 130 °C in the 1170–1199 retention index range. This range corresponds with the retention indices at the same temperature on a WCOT SP 2100 column.

Descriptors similar to the rose, floral and apple terms mentioned in the descriptive analysis of the cut fruit were not noted during exit port sniffing. The floral and fruity nuances may be absent because: (a) the

TABLE 4
Frequency of Mention of Aroma Descriptors for Retention Indices at Oven Temperatures of 50° and 130°C

Retention index	*Descriptor*	*Frequency*	*Retention index*	*Descriptor*	*Frequency*
	Oven temperature of 50°C			*Oven temperature of 130°C*	
713	Buttery	4	851	Oily	2
764	Varnish	2	851	Piney/green	4
780	Buttery	2	867	Cooked potato	3
828	Oily	2	887	Cooked potato	3
847	Burnt green pepper	2	925	Buttery	2
856	Green	2	1056	Green/mint	2
860	Green/grassy	2	1071	Grassy	2
862	Piney	2	1074	Green	3
874	Green/grassy	3	1078	Green pepper	2
880	Grassy	2	1094	Green pepper	2
885	Green	2	1121	Burnt hot dog	2
896	Green	2	1170	Green pepper	4
899	Skunky	2	1170	Evergreen/piney	2
			1179	Green pepper	3
			1183	Green pepper	4
			1183	Cucumber	3
			1199	Green pepper	3
			1244	Cooked vegetable	3
			1249	Grassy	2
			1294	Green pepper	3
			1317	Green pepper	3

notes may be attributable to interaction (i.e. blending, synergism, etc.) of two or more compounds in the headspace (separation of these compounds would eliminate the floral and fruity components of the aroma composite); (b) the character of the responsible compound(s) may change as a function of concentration; (c) extraction may radically alter the concentration of a compound (the concentration of an eluting compound is constantly changing and consequently the aroma character may change also); (d) judges may not have sniffed the eluate at the critical moment or may have had difficulty in articulating a descriptor for an aroma that was changing in character.

ACKNOWLEDGEMENTS

A major portion of this paper is from a dissertation submitted in partial fulfillment of the requirements for the MS degree in Food Science by R. L.

Chitwood. We are grateful to Professor P. G. Smith of the UCD Department of Vegetable Crops for assistance in obtaining and identifying the capsicums.

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Mineral Composition, Ionisable Iron and Soluble Zinc in Malted Grains of Pearl Millet and Ragi

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(Received: 22 October, 1982)

ABSTRACT

Grain samples of nine varieties of pearl millet (P. typhoides) *and six varieties of ragi* (E. coracona) *were analysed before and after malting for total ash, phosphorus, phytin phosphorus, calcium, magnesium, iron, zinc, manganese, copper and chromium contents. Pearl millet alone varietal differences were significant for iron, manganese and chromium contents. Significant nutrient losses in malting were, in pearl millet: iron and manganese, 40%; copper, 30%, and phosphorus, 25% and, in ragi: calcium, 40%; zinc, 30% and copper 25%. As judged by an* in vitro *method, the availability of iron and zinc in millets improved several fold on malting. The values for ionizable iron (mg per 100 g of raw and malted grains) were 0·64 and 2·70 in pearl millet and 0·29 and 2·98 in ragi. Soluble zinc contents per 100 g of raw and malted grains, respectively were 2·04 and 5·25 mg in pearl millet and 2·15 and 3·24 mg in ragi. Reduction in phytin phosphorus on malting of these millets partly explained the improved availability of iron and zinc.*

INTRODUCTION

In terms of acreage and grain production, pearl millet (*Pennisetum typhoides*) and ragi (*Eleusine coracana*) are the two important millet crops of India. These two millets, being staple foods, are the main source of energy and protein in the daily diets of large segments of the

population. One of the practices of consumption of these foodgrains involves malting or germination. Because of its excellent growth-promoting properties and high calorific value, the nutritional significance of malted ragi as an infant food is well recognised (Chandrasekhara & Swaminathan, 1953). In the present investigations, the effect of malting on the mineral and trace element composition of both ragi and pearl millet is studied. Further, an attempt has been made to evaluate, by using *in vitro* methods, the availability of iron and zinc before and after the malting of these two millets.

MATERIALS AND METHODS

Grain samples of nine varieties of pearl millet and six of ragi were obtained from Millet Breeder, Andhra Pradesh Agriculture University, Hyderabad. All the samples were cleaned to remove extraneous matter and were washed thoroughly with glass distilled water to remove adhering impurities. The samples were then dried at 50 °C in an oven to a constant weight. About 25 g of the sample was soaked in water for 18 h. The soaked grains were germinated for 72 h at 28 °C and then dried at 50 °C in an oven. By gentle rubbing in lint cloth, the radicle was removed and the malted grain samples were obtained. This procedure for preparing malted grains was essentially the same as that described by Malleshi & Desikachar (1979). Both raw and malted grains were ground in a stainless steel Wiley mill to pass through a 40 mesh sieve before analysis.

The samples were analysed for total ash (500 °C), calcium and phosphorus by AOAC (1960) methods. Iron, manganese, copper, zinc and chromium were estimated by atomic absorption spectrophotometry (Varian Techtron Model AAS 1000). Molybdenum was determined only in raw grains following the colorimetric procedure of Sandell (1959). Phytin-phosphorus in raw and malted grains was estimated by the procedure of Makower (1970). For the estimation of available iron content, an *in vitro* technique described by Narasinga Rao & Prabhavathi (1978) was used. In this method, the food sample was digested with pepsin-hydrochloric acid at pH 1·3 for 90 min. The supernatant from the digested sample was quantitatively separated, neutralised to pH 7·5 and analysed for ionisable iron and total soluble zinc content. Expressed as per cent of total iron in the sample, the ionisable iron content at pH 7·5 reflects the available iron content.

RESULTS AND DISCUSSION

Whole and malted grain composition

The mineral and trace element contents of the raw grains of pearl millet showed that varietal differences were significant (CV >25%) for iron, manganese and chromium (Table 1). In ragi varieties, values for all the inorganic nutrients varied within narrow ranges and varietal differences were not significant. The values for different nutrients in the two millets were within the normal ranges reported in the literature (Deosthale *et al.*, 1970; Deosthale *et al.*, 1977; Variano-Marston & Hoseney, 1980). Of the two millets, ragi was rich in calcium, manganese and molybdenum whilst pearl millet contained high amounts of iron, copper and zinc.

Malting had altered the mineral and trace element composition of both millets. In pearl millet, about 40% of iron and manganese, 30% of copper and 25% of phosphorus were lost on malting. In ragi, malted grain was poorer in calcium by 40%, zinc, 30% and copper, 25%. The losses on malting were 20% or less for the other nutrients. Studies on food legumes had shown that mineral and trace element losses were mostly during initial soaking in water and negligible during germination (Udayasekhara Rao, 1981). In the present study, in the preparation of malted grains, the vegetative portion—that is, the coleoptile and rootlets—was discarded. Consequently, there was a dry matter loss of 11% in pearl millet and 6% in ragi. This probably was partly responsible for the observed nutrient losses on malting of these millets.

Available iron

The ionisable iron content of pearl millet was 0·64 mg per 100 g which was more than twice the value of 0·29 mg per 100 g in ragi (Table 2). Ionisable iron, expressed as per cent of total iron, is an index of the bioavailability of food iron (Narasinga Rao & Prabhavathi, 1978). Only 9·0% of the total iron was ionisable in ungerminated pearl millet and only 7·4% in ragi. Compared with the ungerminated grains the ionisable iron content was significantly higher in malted grains. Malting increased the ionisable iron in pearl millet fourfold and in ragi tenfold. In malted ragi, 88% of the iron was ionisable whilst in pearl millet it was about 74%. Thus, the bioavailability of iron was between eight- and twelvefold higher in malted than in ungerminated grains.

TABLE 1
Mineral Composition of Whole and Malted Grains of Pearl Millet and Ragi
(*Values are Mean ± SE*)

	Ash (g%)	*mg per 100 g*				*μg per g*				
		Phosphorus	*Magnesium*	*Calcium*	*Iron*	*Zinc*	*Manganese*	*Copper*	*Molybdenum*	*Chromium*
Pearl millet (9)*										
Whole grain	1·8 ± 0·04	379 ± 10·5	137 ± 10·2	46 ± 1·2	8·0 ± 0·75	31·4 ± 2·00	11·5 ± 0·92	10·6 ± 0·58	0·69 ± 0·058	0·23 ± 0·019
Malted grain	1·4 ± 0·03	285 ± 11·1	110 ± 7·9	41 ± 1·1	3·7 ± 0·21	26·4 ± 1·85	7·0 ± 0·66	6·7 ± 0·88	—	0·20 ± 0·017
Ragi (6)										
Whole grain	2·4 ± 0·03	320 ± 3·4	137 ± 2·4	398 ± 10·5	3·9 ± 0·10	23·3 ± 1·01	54·9 ± 1·44	4·7 ± 0·21	1·02 ± 0·030	0·28 ± 0·014
Malted grain	1·9 ± 0·09	264 ± 1·9	115 ± 2·1	207 ± 9·1	3·4 ± 0·08	16·5 ± 0·67	46·0 ± 1·27	3·6 ± 0·11	—	0·26 ± 0·018

* Number of varieties analysed.

TABLE 2

Ionisable Iron, Soluble Zinc and Phytin-Phosphorus Contents of Whole and Malted Grains of Pearl Millet and Ragi

(*Values are Mean ± SE*)

	Ionisable iron at pH 7·5		*Soluble zinc at pH 7·5*		*Phytin-phosphorus*	
	mg/100 g	*Per cent of total iron*	*µg/g*	*Per cent of total zinc*	*mg/100 g*	*Per cent of total phosphorus*
Pearl millet (9)*						
Whole grain	0·64 ± 0·026	9·0 ± 0·33	0·653 ± 0·038	2·04 ± 0·055	172 ± 2·1	45·5 ± 0·93
Malted grain	2·70 ± 0·141	73·5 ± 0·94	5·25 ± 0·403	19·8 ± 0·335	69 ± 1·7	24·4 ± 0·69
Ragi (6)						
Whole grain	0·29 ± 0·022	7·4 ± 0·41	2·15 ± 0·281	9·04 ± 0·198	132 ± 2·3	41·2 ± 1·09
Malted grain	2·98 ± 0·084	88·3 ± 0·41	3·24 ± 0·125	19·7 ± 0·210	88 ± 2·9	33·3 ± 0·97

* Figures in parentheses indicate number of varieties.

Soluble zinc

Only 2% and 9% of total zinc in the raw grains of pearl millet and ragi, respectively was in the soluble form. In malted grains, about 20% of the zinc was soluble at pH 7·5 in the pepsin–HCl digest (Table 2). There is no experimental evidence yet to suggest that the soluble zinc, as estimated in the present study, is an index of availability of food zinc. If it is, as in the case of iron, then the present study would indicate that malting has a beneficial effect on the availability of zinc in pearl millet and ragi.

Phytin phosphorus

Reduction in phytin phosphorus during the germination of wheat (Pomeranz, 1971) and some legumes (Belavady & Banerjee, 1953; Gad *et al.*, 1982) has been well documented. In the present study the phytin-phosphorus content of 172 mg per 100 g in ungerminated pearl millet was reduced to 69 mg per 100 g in the malted grain (Table 2). The reduction in phytin-phosphorus on malting of ragi was also significant (from 132 mg to 88 mg). Since phytate has been implicated as an interfering factor in the availability of divalent metal ions (Reinhold *et al.*, 1975) the results of the present study suggested that decrease in phytin-phosphorus on malting of pearl millet and ragi probably had a beneficial effect on iron and zinc availability.

As a staple food in the diet, pearl millet, as well as ragi, could be considered an important contributor to meeting the dietary requirements of most of the nutrients studied. The studies further suggested that, although malting had resulted in nutrient losses, the availability of iron—and possibly of zinc—as judged by the *in vitro* method, was significantly enhanced.

ACKNOWLEDGEMENTS

We thank Dr P. G. Tulpule, Director, National Institute of Nutrition, for his keen interest in the study and Messrs M. P. C. Vijayaraj and M. M. Khan for their excellent technical assistance.

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Food Chemistry **11** (1983) 225–229

Book Reviews

The Chemistry of Heterocyclic Flavouring and Aroma Compounds. Edited by G. Vernin. Ellis Horwood, Chichester, 1982. 375 pp. Price: £32.50.

This book has chapters on 'Heterocyclic aroma compounds precursors' (Garnero), 'Heterocyclic aroma compounds in foods: occurrence and organoleptic properties' (Vernin and Vernin), 'Mechanisms of formation of heterocyclic compounds in Maillard and pyrolysis reactions' (Vernin and Parkanyi), 'General synthetic methods for heterocyclic compounds used for flavourings' (Vernin and Shaffei), 'Computer application of non-interactive program of simulation of organic synthesis in Maillard reaction: a proposition for new heterocyclic compounds for flavours' (Barone and Chanon), 'Recent techniques in the analysis of heterocyclic aroma compounds in foods' (Peyron), 'Mass spectrometry of heterocyclic compounds used for flavouring' (Vernin and Petitjean), and 'The legislation of flavours' (Vernin).

The book is characterised throughout by its truly chemical approach to the subject. It is very well referenced and makes extensive use of structural diagrams, tables of homologues and reaction mechanisms. In this respect the food applications occupy a smaller proportion of the book and, although there are some qualitative structure/activity relationships, quantitative structure/activity relationships are confined to a few threshold values. The final chapter is an exception, dealing with the toxicity and legislation of some hundreds of compounds on the CoE and FEMA lists and concluding with 10 important references.

Overall the book is highly factual but lacks rather in critical appraisal. Nevertheless it is of immense value as a reference work for scientists in this field of endeavour. It is reasonably priced and its accurate chemical content makes it highly recommendable.

G. G. Birch

Dictionary of Nutrition and Food Technology. 5th Edn, A. E. Bender. Butterworths, London, 1982. 309 pp. £15.

The fifth edition of this important dictionary has 250 new terms and 350 of the entries have been revised. Obsolete terms are retained for obvious reasons. An extensive bibliography of some 180 books has been listed under separate headings and the general style of the dictionary is the short and clear answer to the meaning of a word. The dictionary concludes with some useful tables of recommended allowances, energy values of average food portions and protein contents.

The reasonable price of this dictionary makes it highly recommendable to all.

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Food Proteins. Edited by P. F. Fox and J. J. Condon. Applied Science Publishers Ltd, London, 1982. 361 pp. Price: £38.00.

This book contains the papers presented at the symposium held at Cork in 1981 to mark the opening of the new food science building. The editors claim that the scope of the symposium is unique and indeed with 19 contributors covering a very wide spectrum of interest this claim may well be true. However, it could be considered that the breadth of coverage and variety in treatment the topics received distracts from the usefulness of the book as a specialist review. Some topics may have received more coverage than justified in relation to overall balance: the importance of the relationship between structure and functional properties of food proteins is recognised with comprehensive coverage which compares with the similarly extensive review on the Irish food protein industry, which although of interest would have much less universal relevance. A few chapters are relatively short or lack sufficient critical appreciation of current research and future developments.

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The book does contain some excellent topics whilst a few treatments do not reach such a relatively high standard. The potential purchaser has, therefore, to judge whether the overall presentation provides useful and interesting information and whether any areas of specialist interest have been covered comprehensively and include current information. Considering the number of topics covered, the index did seem rather limited. The book would be of interest to food scientists and technologists, students and teachers and indeed may find general interest to a wider audience concerned with agriculture, food and nutrition.

G. C. Cheeseman

Developments in Dairy Chemistry—1. Edited by P. F. Fox. Applied Science Publishers Ltd, London, 1982. 409 pp. Price: £44.00.

This book continues the 'Developments' series of review books summarising current knowledge in many areas. *Developments in dairy chemistry—1* covers a wide field but nonetheless a good selection of topics has been made covering the chemistry, structure, properties and behaviour of the milk proteins, achieving a comprehensive treatment.

Fundamental molecular composition, physico-chemistry and biosynthesis of milk proteins are dealt with in the first four chapters. They are each concise, but detailed, reviews of current knowledge in their areas and contribute a useful up-to-date source of reference information.

The structure and physical-chemical characteristics of individual bovine milk proteins and the structure and colloidal properties of casein micelles are comprehensively reviewed. Problems of interspecies homology, an area of continuing interest as more information on composition, structure and non-bovine milk proteins is obtained, are highlighted and attention is focused on the many problems remaining before the process of milk protein synthesis is understood.

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described and the factors affecting the heat stability of milk assessed. Age gelation of sterilized milk and the changes brought about by the action of natural milk enzymes during storage on the properties of milk protein are discussed, and the most recent information on compositional identity of many of the enzyme-derived fractions of casein is included.

Milk proteins constitute 20–30 % of our total dietary protein intake, which indicates their importance in our diet, and the nutritional aspects of these proteins are reviewed.

The last three chapters deal with the manufacture and use of milk products. The technical aspects are covered concisely and an understanding of the processes is helped by the inclusion of a generous number of diagrams.

Overall, this is an excellent book, which has brought together in a concise and for the most part easily readable manner, the current knowledge on milk proteins. It covers detailed fundamental information as well as more practical commercial development and, although many of the topics may have been covered in greater detail elsewhere, this book brings them together for current assessment. The book would be primarily of interest to the dairy chemistry specialist, food science student and those engaged in dairy research and development, but it also would be a useful reference book to many concerned with the scientific aspects, education and advice in agriculture and food generally.

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theoretical and experimental aspects and are entitled basic concepts, structure and stability of electrocratic colloids, macromolecular adsorption and colloid stability, the oil–water interface and emulsion stability and adsorption of proteins. In a chapter on experimental methods the determination of particle size is discussed in detail and covers light scattering and small-angle neutron scattering techniques and ultracentrifugation. The use of microelectrophoresis in the study of colloidal systems is also discussed. In another chapter the rheology of colloidal systems, especially particulate dispersions, is treated in some detail. A case study of the colloidal aspects of milk is presented and in a final chapter, concerning colloids in food processing, the structure and properties of selected foods are described. The foods include cream, butter, ice-cream, margarine, salad cream, mayonnaise, meat emulsions and cake batters.

The book is well presented with good diagrams and clear captions. Each chapter ends with a useful list of general and specific references. There are few printing errors. It is aimed at research workers in industry and academia and also at undergraduate students in chemistry, biochemistry, food science and technology. There should be sections in this book to interest all such persons but to fully appreciate the more fundamental aspects a fairly strong mathematical background is necessary.

J. G. Brennan

Food Chemistry **11** (1983) 231

Editorial

'The Chemistry of Physiologically Active Compounds in Foods and Feedingstuffs' was the title of a Symposium held on 26 October 1982 at the Scientific Societies Lecture Theatre, New Burlington Place, London W1, UK and organized by the Food Chemistry Group of the Royal Society of Chemistry. It comprises the seventh in this series to be published in *Food Chemistry*. Professor Bell's introductory survey makes an excellent review of the papers and he rightly emphasizes the need for suitable identification tests and quantitative methods of determination of the toxins; but two aspects of the subject could, perhaps, also be emphasized: how western food processing, or its absence in some instances, may affect the presence and physiological effect of the toxins and, secondly, the development of new, gimmicky foods (e.g. fried potato skins) in which changes in composition are little understood.

It has been said that all foodstuffs are comprised of protein, fat and carbohydrate in one proportion or another. This is an oversimplification for trace elements, toxins, processing by-products, microbial infection, etc., can cause such apparently simple mixtures to be highly suspect. It is a token of human resistance that probably the vast majority of people are never, or only slightly, affected by such menaces.

L. F. Green

Food Chemistry **11** (1983) 233–234

Foreword

E. A. Bell

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Physiologically active compounds in human food and animal feeds can originate in many different ways. Some such compounds occur naturally in the plants and animals that we use for food. Some are the products of chemical reactions which take place during cooking or processing, some are toxins synthesised by contaminating micro-organisms, and some, again, find their way into food from the environment in which it is prepared or from materials in which the food is packed. The potential toxicity of numerous plant species to man and his grazing animals is well known. Even some of those species which are used as crop plants may contain toxins that have to be removed or destroyed during food preparation. Other potentially toxic species have been rendered harmless by human endeavour during the course of domestication. The successive selection of toxin-low varieties over countless generations, with man, horses, cows and sheep providing biological assays, has made it possible to transform highly toxic wild forms to more acceptable toxin-low domesticated varieties.

While some toxins may be removed or destroyed during food processing, others may be formed. It is therefore essential that we understand not only the nature and stability of natural toxins, but also the nature, stability and origins of the synthetic ones. Similarly, we must appreciate the dangers of animal and plant foods becoming contaminated with moulds or bacteria either before or after processing.

In all our different approaches to these problems we have a common need—the need for reliable analytical techniques for the identification and quantitative determination of the toxins.

If we can analyse natural populations of plants in the laboratory, we can identify toxin-low individuals without the need for biological assay and use these individuals for the development of toxin-low varieties. If we can determine the concentration of synthetic toxins produced during the processing of food, then we can determine the condition for synthesis of these compounds and avoid them. If we can identify mycotoxins or, indeed, inorganic contaminants at low concentration, then we can not only identify a potential hazard before it cause a problem but we can determine how and when such contamination arises.

With improved analytical techniques the chemist can provide the botanist, the plant breeder and the food industry with the information they require to improve old, and develop new foods most efficiently. It is essential, however, that the resolution of these problems should be undertaken on an interdisciplinary basis.

Food Chemistry **11** (1983) 235–247

Glycoalkaloids in Potatoes

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(Received: 25 January, 1983)

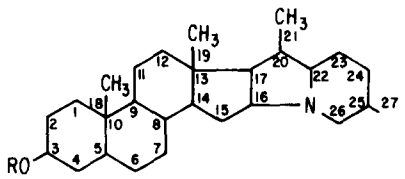
ABSTRACT

The glycoalkaloids present in the cultivated potato plant might result in their being unacceptable by present-day food standards, if that vegetable were introduced today. The alkaloids are derived biosynthetically from cholesterol. The range of glycoalkaloids found in nature is given. Two main methods of determining these alkaloids have been developed, enabling a general screening procedure to be employed by growers. The advent of protoplast techniques has opened up the possibility of many new species and they present the chemist with new problems. There can be a conflict between the requirement of pest- and disease-resistant potatoes and those with low levels of glycoalkaloids. Damage and microbial infection to potatoes may cause glycoalkaloids to develop. In the end, the consumer must be the final arbiter.

INTRODUCTION

In the one-hundred-fifty years since glycoalkaloids were first isolated from potatoes, these compounds have been the subject of much controversy and concern. If potatoes were introduced as a new food product today in the United States or the United Kingdom, there is some question as to whether they would be acceptable by the food safety standards of both countries because of their glycoalkaloid content. Numerous reviews on the chemistry (Prelog & Jeger, 1953; Schreiber,

1968; Ripperger & Schreiber, 1981) and biochemistry (Roddick, 1974; Jadhav & Salunhke, 1975) and physiological activity (Zitnak, 1977; Jadhav *et al.*, 1981) have appeared. The emphasis of this review will deal with the current status of research on glycoalkaloids, particularly in relation to food safety. To put this work in the proper context, a brief summary of glycoalkaloid chemistry is presented.



I R = H

I_a R = H, Δ^5 UNSAT.

I_b R = H Δ^5 UNSAT., 23 O-C-CH_3 (O)

II R = H

II_a R = H, Δ^5 UNSAT.

Although glycoalkaloids are found in other genera (*Lycopersicon* and *Cestrum*), we will restrict our discussion to those found in *Solanum* species, emphasizing the glycoalkaloids of the cultivated potato plant, *S. tuberosum*. The aglycones, or alkaloid portion, of *Solanum* glycoalkaloids contain the steroidal nucleus and are, in fact, biosynthetic products of cholesterol (Heftmann *et al.*, 1967). The two most common skeletal types occurring in *Solanum* are the solanidanes (I) and spirosolanes (II). The alkaloids are usually found in plant tissue as

TABLE 1
Composition of Glycoalkaloids

Glycoalkaloid	*Aglycone*	*Aglycone structure*	*R carbohydrates*
α-Solanine	Solanidine	I_a (20S, 22R, 25S)	Solatriose
α-Chaconine	Solanidine	I_a (20S, 22R, 25S)	Chacotriose
Solasonine	Solasodine	II_a (22R, 25R)	Solatriose
Solamargine	Solasodine	II_a (22R, 25R)	Chacotriose
Tomatine	Tomatidine	II (22S, 25S)	Lycotetraose
Solacauline	Soladulcidine	II (22R, 25R)	Polyatriose
α-Solamarine	Tomatidenol	II_a (22S, 25S)	Solatriose
β-Solamarine	Tomatidenol	II_a (22S, 25S)	Chacotriose
Demissine	Demissidine	I (20S, 22R, 25S)	Lycotetraose
Leptine I	Acetylleptinidine	I_b (20S, 22R, 25S)	Chacotriose
Leptine II	Acetylleptinidine	I_b (20S, 22R, 25S)	Solatriose
Commersonine	Demissidine	I (20S, 22R, 25S)	Commertetrose

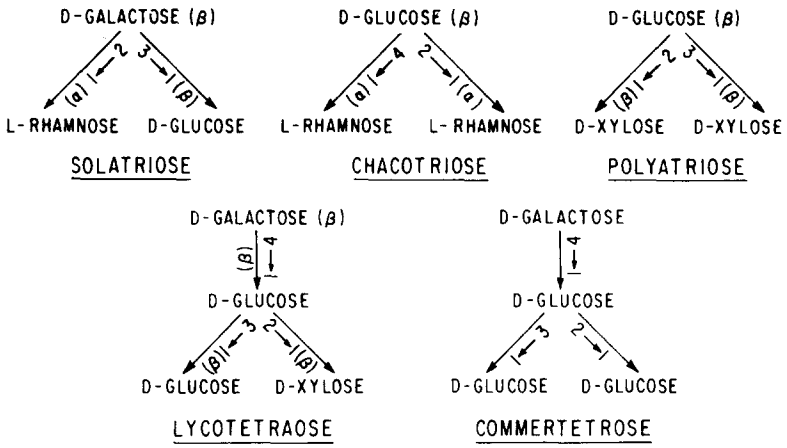


Fig. 1. Carbohydrate moieties of glycoalkaloid.

glycosides (derivatized at the 3,β-hydroxyl), thus the terms glycoalkaloids or steroidal glycoalkaloids. A partial listing of glycoalkaloids containing the solanidane or spirosolane aglycone is given in Table 1. The carbohydrate structures are shown in Fig. 1. As mentioned, the alkaloids are biosynthetically derived from cholesterol and divergence of the biosynthetic path to the solanidane or spirosolane structure occurs at a relatively late stage (Fig. 2); therefore, it is not surprising that either one

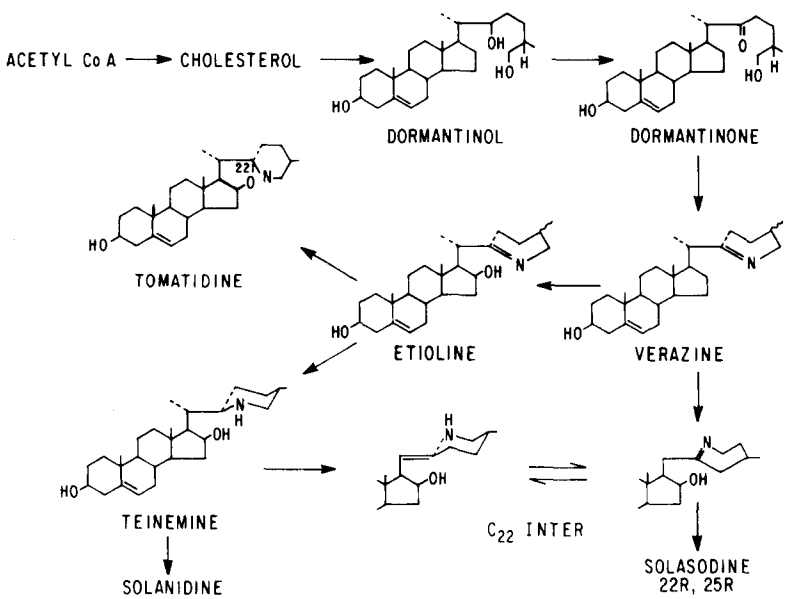


Fig. 2. Biosynthesis of glycoalkaloid aclycone.

or both ring structures are found in a particular species. Although the structure of the carbohydrate moiety of the glycoalkaloid may be of minor interest from a chemical or chemataxonomic standpoint, it may be of considerable biological significance, particularly with respect to toxicity. Thus, chemical characterization, of necessity, must include analysis of the carbohydrate portion of the glycoalkaloid. Table 2 illustrates the type of diversity of glycoalkaloids one can find in nature, which contain the same aglycone but differ in carbohydrate structure.

TABLE 2
New Solasodine Glycoalkaloids

Glycoalkaloid	*Carbohydrate*[a]	*Species*	*Reference*
	3-1 2-1 2-1		
Solardixine	-Gal–Glu–Glu–Rham	*S. lactiniatum* *S. khasianum*	Bite *et al.* (1969)
Solasurine	-Glu–Rham	*S. elaeagnifolium* *S. aviculare*	Seth & Chatterjee (1968–1971)
Solashabanine	Gal, 3 Glu, Rham	*S. lactiniatum*	Bite *et al.* (1972)
Solaradinine	Gal, 4 Glu, Rham	*S. lactiniatum*	Bite *et al.* (1972)
Solapersine	Gal, Glu, 2 Xyl	*S. persicum*	Novuzov *et al.* (1975)
Solatifoline	Glu, Gal, Rham	*S. platanifolium*	Puri (1975)

[a] Glu = Glucose.
Gal = Galactose.
Rham = Rhamnose.
Xyl = Xylose.

STRUCTURE OF GLYCOALKALOIDS

The chemical characterization of glycoalkaloids has been greatly facilitated by the use of NMR and mass spectrometry. In many cases, complete characterization of the aglycone can be accomplished by Nuclear Magnetic Resonance NMR (^{13}C primarily). NMR assignments for the more common *Solanum* alkaloids have appeared in the recent literature (Badeglia *et al.*, 1977; Weston *et al.*, 1977). Assignments for the carbons in the carbohydrate moiety are ambiguous and, therefore, of less utility in characterizing this portion of the molecule (Mahato *et al.*, 1980). Glycoalkaloids are quite amenable to permethylation analysis for carbohydrate characterization. We have developed a modified Hakomori procedure for such an analysis (Herb *et al.*, 1975). The glycoalkaloid

(1–2 mg) is permethylated, using dimsyl sodium and methyl iodide. After destruction of the excess dimsyl sodium with water, the permethylated glycoalkaloid is extracted with benzene rather than chloroform. Since the permethylated glycoalkaloid, because of the steroidal aglycone, partitions completely into the benzene layer and dimethyl sulfoxide does not, the permethylated compound can be isolated without the problem of dealing with contamination from solvent or reagent. In the usual manner, the permethylated glycoalkaloid is hydrolyzed, and the partially methylated sugars are reduced and then acetylated. Gas chromatography/mass spectrometry (GC/MS) of these derivatives (using OV-225 SCOT columns) is generally sufficient for unambiguous characterization. Using this technique, we have been able to characterize a series of glycoalkaloids containing the commeratetrose glycoside (with solanidine, tomatidine, or demissidine as the aglycone).

GLYCOALKALOID ANALYSIS

A considerable effort in the chemistry of glycoalkaloids has been devoted to developing analytical methods for glycoalkaloid determination in plants and plant products. There have been two main aspects in the development of analytical methodology: (a) the development of rapid, sensitive methods for the determination of the total glycoalkaloid content (TGA) of market potatoes and (b) a general method for glycoalkaloid determination (both concentration and composition) in *Solanum* species that are potential breeding material. The need for rapid TGA analysis reflects concern about the variability of glycoalkaloid concentration in commodities such as potatoes. Environmental conditions in the field can significantly affect glycoalkaloid levels in potatoes (Sinden & Webb, 1974) (Table 3). Methods have been developed (Table 4) that enable potato growers and marketers to determine glycoalkaloid levels in a general screening procedure, or on a spot basis in suspect samples. These methods ultimately rely on colorimetric titrations, and the degree of accuracy one obtains depends on the extent of purity of the glycoalkaloid fraction. Most of the colorimetric assays, e.g. phenol–sulfuric acid method, are relatively non-specific and therefore the purer the glycoalkaloid fraction, the more accurate the results; impurities, with these methods, tend to give abnormally high results. The thin layer chromatographic method (TLC), at best, can only be considered semi-quantitative. More sophisticated

TABLE 3
Variation of Glycoalkaloid Concentration with Location

Location	*mg/100 g fw*[a]	*Location*	*mg/100 g fw*[a]
Parma, ID	20·5	Lexington, KY	10·2
Fairhope, Al	16·0	Ithaca, NY	9·8
Cheyenne, WY	13·3	Riverhead, NY	9·5
Charleston, SC	11·3	Prosser, WA	7·8
Salisbury, MD	10·7	Beltsville, MD	7·3
Presque Isle, ME	10·6		

[a] fw = fresh weight.

chromatographic procedures, less accessible to non-chemists, have recently been developed; for example, Bushway *et al.* (1979) have used high pressure liquid chromatography (HPLC) for the analysis of solanine and chaconine in a variety of potato products. A protocol, from the standpoint of food safety, would most likely involve using one of the 'quick and dirty' methods to determine if a sample is equivalent to, or exceeds, acceptable glycoalkaloid levels (*ca.* 20 mg/g fresh weight in potatoes) followed by one of the more accurate methods if acceptable levels are exceeded.

The methods summarized in Table 4 are satisfactory for plant and plant products of known and invariable glycoalkaloid composition. This

TABLE 4
Rapid TGA Analytical Methods

Isolation	*Quantitation*	*Specificity*	*Reference*
Extraction/precipitation	Colorimetric (H_2CO—H_2SO_4)	+ + +	Rooke *et al.* (1943)
Extraction/precipitation	Colorimetric ($SbCl_3$)	+ +	Bretzloff (1971)
Extraction/hydrolysis	Colorimetric (BrpH Br_2)	+ + +	Fitzpatrick & Osman (1974)
Extraction/hydrolysis	Colorimetric (BrThy BLUE)	+ + +	Coxon *et al.* (1979)
Extraction/TLC	Colorimetric ($SbCl_3$)	+ +	Cadle *et al.* (1979)
Extraction/precipitation	Colorimetric	+ +	Dabbs & Hilton (1953)

is the case for most market potatoes which are derived from the species, *S. tuberosum*. However, in the last 50 years there has been an attempt on the part of potato breeders to develop natural disease and pest resistance by introducing uncultivated *Solanum* species into breeding programmes. For example, a clone, USDA x-9656, which is a hybrid of *S. tuberosum* and *S. demissum*, was introduced in the 1940's for resistance against the late-blight pathogen, *Phytophthora infestans*. Today, many other *Solanum* species are being considered for breeding, because of the resistance that these species exhibit towards pests and microorganisms. With the advent of protoplast techniques, a whole new vista of producing hybrids of incompatible species is now also possible. This presents a new problem to the chemist with regard to glycoalkaloid analysis; even though we may know the glycoalkaloid composition of the parents, we cannot accurately predict the glycoalkaloid composition of the individual progeny (Sanford & Sinden, 1972; McCollum & Sinden, 1979). Not only will there be variable composition with respect to the glycoalkaloids found in the parents, there is also the possibility of finding glycoalkaloids not present in either parent. For this, and other reasons, too complicated to discuss in this brief review, a more inclusive method for determining glycoalkaloid levels and composition is required. A gas chromatographic method for determining glycoalkaloid composition was developed in our laboratory that requires permethylation and high temperature GC (Herb *et al.*, 1975). HPLC methods, while simpler to carry out, are not capable of the same degree of resolution as GC. TLC analysis, in conjunction with GC, can be an effective means of characterizing glycoalkaloids in an unknown sample. Using this procedure, we have analyzed the glycoalkaloids in a number of species of interest to the breeders (Table 5). The analysis of four clones of *S. acauli* demonstrates the variability in composition one may observe in a single species (the possibility that *S. acauli* is not a pure species, but a hybrid, could account for this variation).

Quantitation of TGA in species other than *S. tuberosum* may also require analytical methods different from the ones discussed above. For many of the 'fast' analytical methods, one must precipitate the glycoalkaloid fraction. Since most glycoalkaloids are insoluble in aqueous base, this can be easily accomplished. The few that are soluble under these conditions must be measured by other techniques; however, one cannot assume that a particular glycoalkaloid can be quantitatively precipitated from an aqueous plant extract because it is insoluble in base. A recent experience is illustrative. We have developed a method for

TABLE 5
Glycoalkaloids of Selected Solanum Species

Species	*β-Chaconine*	*α-Chaconine*	*Glycoalkaloid α-Solanine*	*Solamarines*[a]	*Demissine*	*Tomatine*
S. ajanhuiri[b]	3·5[c]	39·0	57·3			
S. curtilobum		34·8	46·4	5·3	13·4	
S. stenotonum	5·5	69·8	24·7			
S. juzepczukii		14·0	37·8	7·7	40·4	
S. acaule 1[d]					95·5	
S. acaule 2					62·1	30·9
S. acaule 3					88·2	11·6
S. acaule 4					64·0	34·0

[a] Combined value for α- and β-solamarine.
[b] All species are cultivated except for *S. acaule*.
[c] Values represent per cent of total glycoalkaloids.
[d] Four clones of species *S. acaule* were analyzed.

measuring TGA that does not require precipitation (Fitzpatrick & Osman, 1974). This method is based on titrating the free aglycone after hydrolysis of the glycoalkaloid. In collaboration with the Beltsville Agricultural Research Center, we wished to determine the TGA of a plant extract that was known to contain only demissine and solanine, two glycoalkaloids that are insoluble in aqueous base. Using our method, we obtained values twice that from methods that required precipitation. We subsequently showed that the glycoalkaloids were only partially precipitated (about 50%) using standard techniques that, under most circumstances, result in complete precipitation. Although we do not know what causes this phenomenon, the fact that solubility can be affected by other constituents in the plant extract must be considered when attempting TGA analysis of *Solanum* species other than *S. tuberosum*.

GLYCOALKALOIDS AND HUMAN HEALTH

The degree of effort that has been expended on developing analytical techniques is a reflection of the concern about toxicity of these compounds, and their presence in our food supply. The potato industry in the United States has used, as a guideline, a maximum of 20 mg TGA/100 g fresh weight of potato tuber for glycoalkaloid content. How this figure was determined, and the historical controversy about just how toxic glycoalkaloids are, is beyond the scope of this paper. Although experimental toxicity studies with animals, particularly mice (Nishie *et al.*, 1971, 1975) would indicate that these compounds are relatively non-toxic, empirical data concerning cases of potato poisoning in humans are sufficient cause for the potato industry to be concerned about glycoalkaloid levels. A recent review summarizes present knowledge of glycoalkaloid toxicity (Roddick, 1982); however, since human toxicity data for glycoalkaloids are lacking, as is usually the case in these circumstances, the controversy will continue.

Since breeders can produce potatoes that contain little or no glycoalkaloids, are we really dealing with a non-issue? Unfortunately, the question is not that simple to answer. As previously mentioned, breeders are interested in developing plant lines that are resistant to pests and disease. Glycoalkaloids have been shown to be effective in the control of certain pests (Tingey *et al.*, 1978; Raman *et al.*, 1979). Kuhn & Gauhe

(1961) isolated the leptine glycoalkaloids (23-acetylsolanidine aglycone) from the species *S. chacoense*, which is resistant to the Colorado potato beetle (CPB) (Stürckow & Löw, 1961). In recent field tests (Sinden *et al.*, 1980), glycoalkaloid composition and concentration were shown to be factors in CPB resistance. In an attempt to incorporate CPB resistance, *S. chacoense* was used in a breeding programme that eventually lead to the Lenape cultivar (Akeley *et al.*, 1968). This variety was never released because of reports of illness due to consumption of these potatoes (Zitnak & Johnston, 1970). Therefore, although studies have shown that glycoalkaloids are a vector in resistance to pests, the presence of these compounds in the edible portion of the plant at toxic levels precludes the use of these compounds as means of resistance. A more complete understanding of the biosynthetic control mechanism in the plant is a prerequisite to any resistance programme based on glycoalkaloids.

The appearance of new potato products may present a problem vis à vis glycoalkaloids. In the United States, fried potato skins have become somewhat popular in restaurants. The TGA distribution in the potato tuber is shown in Table 6. The greatest concentration of glycoalkaloids is

TABLE 6
Distribution of Glycoalkaloids in Tubers

Tuber part	*mg/100 g Dry weight*	*Part = % of tuber*
Skin	64	2
Peel (Skin and outer cortex)	68–75	11
Flesh	21	90

found just below the skin; therefore, these products can contain high levels of glycoalkaloids. Producers of this commodity can take precautions to ensure low TGA levels in these products.

De novo synthesis of glycoalkaloids after harvesting is a potential hazard in potatoes. There is much evidence to suggest that mechanically damaged (Wu & Salunhke, 1976) and light-exposed tubers (which usually have green areas due to high chlorophyll content (Jadhav & Salunhke, 1975)) contain abnormally high concentrations of glycoalkaloids. Our analysis of market potatoes that were damaged (Fitzpatrick *et al.* 1978) did not indicate glycoalkaloid concentrations at hazardous levels. Injury

to tubers can also result in the synthesis of glycoalkaloids that are not normally found in the healthy tuber. Shih & Kuc̀ (1974) have reported the presence of the solamarines in Kennebec slices after incubation; these compounds are not found in undamaged, healthy tubers.

In conclusion, the potato industry has been well aware of the health problems related to potato glycoalkaloids and, in general, it has tried to limit glycoalkaloid levels even though there may be some advantage to having high levels of these compounds present in the plant. As with most produce, there is no effective means of monitoring potatoes in the market place that may contain high TGA because of mishandling or microbial infection; the consumer must use judgment in using foods of questionable quality. For example, potato tubers that appear to be contaminated or green (as mentioned, chlorophyll levels correlate with glycoalkaloid levels) should be discarded. The potato grower should be responsible for growing low glycoalkaloid potatoes, the distributor for delivery of a good product, but the consumer is still the final arbiter.

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Glucosinolates and their Breakdown Products in Cruciferous Crops, Foods and Feedingstuffs

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(Received: 25 January, 1983)

ABSTRACT

Almost one hundred glucosinolates are distributed in plants, primarily amongst members of the Cruciferae. Their presence in the genus Brassica *is of major concern in any consideration of their effect in animal feedingstuffs and human foods. Following a discussion of the structure of glucosinolates and the nature of the various products formed on their enzymically induced hydrolysis, the factors affecting the qualitative and quantitative content of glucosinolates and hydrolysis products in plants, foods and feedingstuffs are considered. The major physiologically active compounds derived from glucosinolates are isothiocyanates, nitriles and oxazolidine-2-thiones and their major effects are described, particular consideration being given to the goitrogenic and anti-carcinogenic activities of some of these products. The need for detailed examinations of the chronic and subchronic effects of glucosinolates and their products, of the effect of processing on the content of these compounds and of their fate in humans, animals and poultry is also emphasised.*

INTRODUCTION

The pungency which is characteristic of mustard, cress and radish is dependent upon the hydrolysis of glucosinolates and on the nature and amount of the products thus formed (Tookey *et al.*, 1980; Fenwick *et al.*, 1983). The typical flavours of brassica vegetables, such as cabbage and

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swede, are more complex but again glucosinolate hydrolysis products make a significant contribution (MacLeod, 1976). In addition to possessing such desirable sensory properties, these products elicit a variety of physiological responses in animals and man. It is appropriate here to consider the extent, and limitations, of our present knowledge of these subjects, following a general discussion of the chemistry and biochemistry of this novel class of sulphur-containing anions.

The importance of cruciferous plants has, of course, long been recognized, initially for their curative and medicinal properties and latterly for culinary uses. Carbonized mustard seed has been unearthed in China and dated to *c.* 4000 BC, and cruciferous crops have been referred to in Sanskrit, Chinese, Greek, Roman and Arabic manuscripts. Pythagoras (*c.* 530 BC) and Hippokrates (*c.* 400 BC) being just two authors who have described the medicinal properties of mustard juice (oil). In Europe, the Middle Ages may be seen as the period of rapid expansion and spread in the cultivation of cruciferous (cole) crops (Fenwick *et al.*, 1983). As an indication of the present economic significance of these crops, global production of cabbage exceeded 32 million tonnes in 1978 and in Japan alone the annual consumption of radish is some 5 million tonnes. Rapeseed is now the world's fourth most important oilseed of commerce with a current production of over 11 million tonnes. The development of newer cultivars with reduced glucosinolate content has been a major factor in the expansion of this crop in recent years.

OCCURRENCE

Glucosinolates appear to be limited to certain families of dicotyledenous angiosperms, occurring predominantly (although not exclusively) in the order Capparales *sensu* Cronquist or Taktajan which constitutes the Capparaceae, Moringaceae, Resedaceae, Tovariaceae and Cruciferae (Kjaer, 1974). Since the present paper is concerned primarily with the rôle of these compounds in food and feedingstuffs, emphasis will be placed upon the latter family and, in particular, upon the genus *Brassica*, including cole crops (*Brassica oleracea* spp.), condiments (*B. juncea*), oilseeds (*B. napus* and *B. campestris*) and forage crops. Not all glucosinolates occur within the Cruciferae and perhaps only 15–20 have been found within the genus *Brassica*, their qualitative and quantitative composition depending upon such factors as species, age of plant and part examined (Tookey *et al.*, 1980; Fenwick *et al.*, 1983).

STRUCTURE

Ninety-one glucosinolates have recently been recorded and, with a single exception, all are considered to possess the same basic skeleton (Fig. 1, I) (Ettlinger & Lundeen, 1956). In general, the structure of glucosinolates has been extrapolated from that of their hydrolysis products (see below). X-Ray crystallographic analysis has confirmed the side chain, R, and O-sulphonate groups to possess the Z-(anti) configuration (Marsh & Waser, 1970). Differences between glucosinolates depend upon the chemical nature of the side chain and this also has a significant effect upon the ultimate products of hydrolysis. Glucosinolates usually possess a β-D-thioglucose grouping, the only exception having an additional sinapic acid moiety esterified at the 6 position (Linschied *et al.*, 1980).

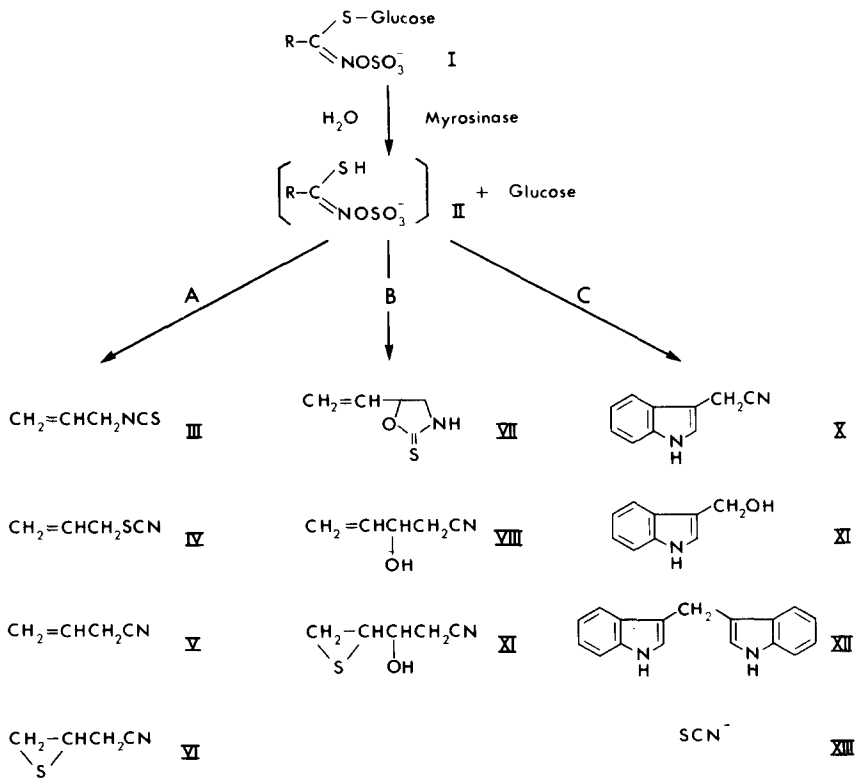

Fig. 1. Schematic representation of the enzymatic decomposition of (A) sinigrin; 2-propenyl glucosinolate, (B) progoitrin; 2-hydroxy-3-butenyl glucosinolate, and (C) glucobrassicin; 3-indolylmethyl glucosinolate.

GLUCOSINOLATE HYDROLYSIS

As indicated above, the physiological responses encountered when brassicas are fed to animals and humans are due to the properties of the hydrolysis products. This hydrolysis is catalysed by an enzyme, myrosinase (thioglucoside glycohydrolase EC 3:2:3:1), which, although found in all plants containing glucosinolates, is separated from this substrate in the intact tissue. Thus, cellular disruption is necessary for glucosinolate hydrolysis to occur (Björkman, 1976). The primary product of the hydrolysis is an unstable aglucone (Fig. 1) which decomposes further to a range of products. The ultimate products are dependent upon the chemical structure of the glucosinolate sidechains, the conditions of the hydrolysis and the presence of any cofactor(s). Thus, the balance of products formed, and their sensory and physiological properties, are dependent upon the nature and extent of the hydrolysis and upon the composition of the original glucosinolate mixture.

In passing it should be noted that thioglucosidase activity has been found in other organisms including fungi (Reese *et al.*, 1958), *Aspergillus niger*, *Aspergillus sydowi* and *Enterobacter cloaceae* (Ohtsuru & Kawatani, 1979) and mammalian (Goodman *et al.*, 1959) and human (Oginsky *et al.*, 1965) bacteria. The latter are clearly significant when intact glucosinolates are ingested, but very little is known about the fate of these compounds under such conditions.

The most common product of myrosinase hydrolysis is an isothiocyanate formed from the aglucone via a Lossen rearrangement. Such compounds are responsible for the desirable pungency referred to above. Very occasionally, organic thiocyanates may be formed, although the exact mode of formation remains unclear. Thiocyanates lack the desirable pungency properties of the isomeric isothiocyanates and have been implicated in off-flavours in milk and meat from animals consuming the weeds *Thlaspi arvense* (stinkweed) and *Coronopus didymus* (landcress) (Whiting *et al.*, 1958; Walker & Gray, 1970).

Under certain circumstances the aglucone may yield a nitrile, rather than an isothiocyanate (Tookey *et al.*, 1980). This tendency is enhanced at acid pH and so may be of particular concern during the preparation and storage of such products as pickled cabbage, coleslaw and sauerkraut (Daxenbichler *et al.*, 1980). Certain vegetables (cabbage and swede) and oilseeds (*Crambe abyssinica*) have been shown to possess a protein (epithiospecifier protein) which lacks enzymatic activity but which, in

conjunction with ferrous ion, facilitates the insertion of a sulphur atom into a terminally unsaturated glucosinolate sidechain (Tookey, 1973; Petroski & Tookey, 1982).

Isothiocyanates formed from glucosinolates possessing a β-hydroxyl group are themselves unstable and spontaneously cyclize to the oxazolidine-2-thione. Other products, such as a cyanohydroxyepithioalkane or cyanohydroxyalkene, may also be formed and the multiplicity of detrimental effects associated with these hydrolysis products, together with the ubiquity of the parent glucosinolate in the genus *Brassica* (Fenwick *et al.*, 1983) are of particular concern.

Glucosinolates containing an indolic nucleus commonly occur and may be abundant in certain brassicas (Gmelin & Virtanen, 1961; 1962). At pH 7, thiocyanate ion is formed together with products possessing the indole nucleus. The former compound, which is a known goitrogen, may also be formed from *p*-hydroxybenzyl glucosinolate, mainly found in white mustard seed (*Sinapis alba*).

CONTENT OF GLUCOSINOLATES

The glucosinolate content of a plant is dependent upon its ontogenetic state, the part examined and the agronomic factors associated with its growth. The total glucosinolate content of selected brassicas is shown in Fig. 2. More extensive data have recently been published elsewhere (Tookey *et al.*, 1980; Fenwick *et al.*, 1983). More significant in many cases, however, is the content of the individual glucosinolates since this determines the sensory and physiological properties of the product.

Whereas total glucosinolate content may be conveniently determined by the measurement of the glucose released after myrosinase treatment, methods for the determination of individual glucosinolate content are more complex (McGregor *et al.*, 1983). Individual glucosinolates may be measured directly by high performance liquid chromatography or, after suitable volatilization, by gas chromatography. Alternatively, they may be determined indirectly by independent measurement of their isothiocyanate, nitrile, oxazolidine-2-thione and thiocyanate ion hydrolysis products. Detailed reviews of these methods have appeared elsewhere (Olsen & Sørensen, 1981; McGregor *et al.*, 1983).

Recently, advances in analytical methodology have enabled the individual glucosinolate contents of many common vegetables. e.g.

GLUCOSINOLATE*			
TOTAL		INDIVIDUAL	
RED CABBAGE (VanEtten *et al.*, 1980)	410–1 090	6–102	2-PROPENYL
		22–143	3-CH_3SO-PROPYL
		150–390	4-CH_3SO-BUTYL
		155–330	INDOLE
WHITE CABBAGE (VanEtten *et al.*, 1980)	330–840	35–590	2-PROPENYL
		46–270	3-CH_3SO-PROPYL
		0–144	4-CH_3SO-BUTYL
		51–510	INDOLE
SAVOY CABBAGE (VanEtten *et al.*, 1980)	470–1 240	0–160	2-PROPENYL
		70–420	3-CH_3SO-PROPYL
		1–40	4-CH_3SO-BUTYL
		300–526	INDOLE
CHINESE CABBAGE (Daxenbichler *et al.*, 1979)	170–1 360	0–250	3-BUTENYL
		13–275	4-PENTENYL
		3–194	2-HYDROXY 3 BUTENYL
		4–257	5-CH_3SO-PENTENYL
		22–320	2-PHENYLETHYL
		97–547	INDOLE
BRUSSELS SPROUTS (Heaney & Fenwick, 1980*a*)	600–3 900	110–1 560	2-PROPENYL
		30–500	3-BUTENYL
		40–990	2-HYDROXY 3 BUTENYL
		220–1 110	INDOLE
RUTABAGA (SWEDE) (Carlson *et al.*, 1982)	260 (mean)	220–670	2-HYDROXY 3 BUTENYL
		37–380	4-CH_3S-BUTYL
		4–330	5-CH_3S-PENTYL
		0–110	5-CH_3SO-PENTYL
		5–470	2-PHENYLETHYL
		70–570	INDOLE

* μg/g fresh weight, calculated as potassium salt.

Fig. 2. Major glucosinolates of fresh brassica vegetables.

cabbage, Chinese cabbage, cauliflower, Brussels sprouts and swede, to be determined. A compilation of these, and other data, has recently been made (Fenwick *et al.*, 1983). Detailed investigations by workers at the Northern Regional Research Centre of the United States Department of Agriculture (USDA) have identified eleven glucosinolates in cabbage

(VanEtten *et al.*, 1976; 1980). Both white and savoy types contain mainly glucosinolates possessing 3-carbon side chains whereas those possessing 4-carbon aglucones predominate in red cabbage. Further investigations on Chinese cabbage (Daxenbichler *et al.*, 1979) revealed predominantly glucosinolates containing 5-carbon side chains. The range of individual glucosinolate contents for the major components in a variety of brassicas is shown in Fig. 2.

Agronomic factors, e.g. moisture, soil type and fertilizer application, are known to exert a significant effect on glucosinolate content. Stress factors tend to increase glucosinolate content, presumably because of the increased amino acid and carbohydrate pools necessary for glucosinolate biosynthesis (Freeman & Mossadeghi, 1973: MacLeod & Nussbaum, 1977). The application of sulphate fertilizer, as might be expected, leads to an increase in glucosinolate content (Josefsson, 1970). The effect of nitrogenous fertilizer is less clear cut, with increasing application generally leading to a decrease in total glucosinolate content (Trzebny, 1967). Recent work, however, indicates that individual glucosinolates may be affected in widely differing ways, with indole glucosinolates being relatively unaffected by nitrogen application over the range 0–250 kg/ha (Heaney *et al.*, 1983). Such variation as described above would therefore be expected to produce a considerable effect on the flavour strength of individual *Brassica* cultivars.

In a detailed analysis of the total glucosinolate contents of Brussels sprouts grown at five sites in the UK, Heaney & Fenwick (1980*a*) found wide site/site variation for an individual cultivar. Unexpectedly, the relative contribution of the individual glucosinolates to the total was little affected by this site effect. It was suggested (Heaney & Fenwick, 1980*b*) that the pattern of *relative* glucosinolate abundance might serve as an aid to cultivar identification in this vegetable. Whilst there are indications of similar behaviour in other brassica vegetables (K. Sones, R. K. Heaney and G. R. Fenwick, unpublished) none has yet been found to exhibit the degree of cultivar specificity noted in Brussels sprouts.

DIETARY INTAKE OF GLUCOSINOLATES IN HUMANS

Any meaningful calculation of dietary intake of glucosinolates is subject to considerable qualification. Not only because of the wide variation in

glucosinolate contents of different species, different cultivars or even the same cultivar from different sites, but because of the wide variation in intake of cruciferous crops between groups of different ethnic origin and socio-economic level. The only published data is from Canada and has produced an average daily intake of 7·9 mg glucosinolate per person per day (Mullin & Sahasrabudhe, 1978). This figure is undoubtedly an underestimate because of limitations of the analytical method, and because the authors omitted from their calculation any contribution from the ubiquitous mustard paste. A more realistic figure might be suggested as 12–16 mg glucosinolate per person per day. In addition, possible 'indirect' sources (see below) were not considered. The above calculation was based upon data indicating an average dietary intake (ADI) of cruciferous crops in the Canadian population examined of approximately 18 g per person per day. About 20 % of the total was made up of processed cabbage (coleslaw and sauerkraut). Dietary data on the UK for 1979 indicates the ADI of cauliflower, cabbage, Brussels sprouts and turnips to be 20, 11, 9 and 5·5 g, respectively (Anon, 1980). A detailed study is presently under way to calculate the glucosinolate ADI in the UK; from results already obtained (K. Sones, R. K. Heaney and G.R. Fenwick, unpublished data) it is evident that the figure obtained will be significantly higher than that found in Canada.

In addition to these direct sources, milk and poultry products provide an indirect source of glucosinolate hydrolysis products via the feeding of forage brassicas or rapeseed meal. Thiocyanate ion (< 10 mg/litre; Piironen & Virtanen, 1963), hydroxynitriles (2·9 mg/litre; Papas *et al.*, 1979*a*) and oxazolidine-2-thione (50–100 μg/litre; Peltola, 1965) have been measured in milk and there is some evidence for the occurrence of the latter in egg yolk, (see Fenwick & Curtis, 1980*a*). Glucosinolate breakdown products have not, however, been found in the meat of cattle fed meal from *Crambe abyssinica* (VanEtten *et al.*, 1977), at least in amounts > 1 ppm, the detection limit of the methods employed.

It should be emphasized that it is the glucosinolate breakdown products which enter the body from these indirect sources, as is the case when condiments and uncooked crucifers are consumed. Cooking, however, inactivates most, if not all, of the endogenous myrosinase and so ingestion of such products leads, mainly, to the intake of intact glucosinolates. Very little is known about the extent and nature of the *in vivo* metabolism of glucosinolate in humans and animals and the area is worthy of further investigation.

EFFECT OF PROCESSING ON GLUCOSINOLATE CONTENT

Detailed investigations into the effect of processing on glucosinolate content are very few. Effects on the flavour of cooked, frozen and dehydrated brassicas have been reported by MacLeod (1976). The presence of relatively large amounts of 2-propenyl nitrile in cooked, processed cabbage was attributed to a non-enzymatic or thermal process (MacLeod & Macleod, 1970). This intriguing suggestion does not appear to have been closely followed up. Recently, Srisangnam *et al.*, (1980*b*) have attributed to the same compound the objectional odour found in rehydrated freeze-dehydrated cabbage. Various attempts have been made to improve the flavour of processed brassica vegetables by adding myrosinase to replace that destroyed by processing (see MacLeod 1970). Although some improvement in flavour was noted, the sought-after 'fresh cooked' characteristics were not apparent.

In the Far East, large quantities of radish and other crucifers are consumed, mainly after processing. This may take the form of drying, salting or fermenting. Kjaer *et al.* (1978) have examined the effect of processing Japanese radish. The vegetable was fermented after drying or salting and the volatiles analysed by combined gas chromatography–mass spectrometry. Isothiocyanates and nitriles were present in the early stage of processing but disappeared thereafter. The rates of disappearance of these volatiles varied from batch to batch and, not unexpectedly, salted and dried products behaved differently. Disulphides and oxidized disulphides formed the majority of possible isothiocyanate-derived volatiles.

In Western countries the most common brassica fermentation product is sauerkraut. Daxenbichler *et al.*, (1980) have examined this product, which accounts for nearly 15% of the total cabbage consumed in the United States. After 14 days' fermentation, no intact glucosinolates could be detected, the major product being 1-cyano-3-methylsulphinyl-propane (1·6–2·5 mg/100 g sauerkraut) and accounting for approximately half of the parent glucosinolate. Thiocyanate ion (0·9–1·7 mg/100 g) was also detected but no products corresponding to the other major cabbage glucosinolate, 2-propenyl-, could be detected.

Recent work (Srisangnam *et al.*, 1980*b*; Fenwick *et al.*, 1983) on cabbage and Brussels sprouts has shown that considerable ($< 30\%$) losses of glucosinolates occur during cooking. This loss is mainly due to the 'leaching out' of these compounds into the cooking liquor. Some

decomposition (perhaps 10% of the total content) does occur and indole glucosinolates seem most affected in this respect. This presumably occurs during cooking and before the enzyme is completely inactivated. A comprehensive investigation of this situation using modern techniques of analysis of both volatile hydrolysis products and glucosinolates, allied to sophisticated sensory analysis, is clearly needed.

Condiment mustard is prone to oxidation and certain continental type preparations contain sulphur dioxide to inhibit both this process and subsequent discoloration of the product. It is possible for the pungent 2-propenyl isothiocyanate to react with sulphur dioxide to form 2-propenylaminothiocarbonyl sulphonate. Not only does this reaction lead to a loss of pungency, but decomposition of the involatile sulphonate to 2-propenyl thiol and related sulphides is associated with the development of a garlic-like off-odour on storage (Griffiths *et al.*, 1980; Frijters *et al.*, 1981). There are reports that the addition of sulphur dioxide to horseradish powder has a detrimental effect on flavour (Weber, 1964). Preliminary experiments have shown that the reaction of 2-propenyl isothiocyanate with sulphur dioxide is much faster than that of 2-phenylethyl isothiocyanate, the other major component of horseradish essence. This presumably leads to the reduction in odour mentioned above (P. G. Jones and G. R. Fenwick, unpublished results).

The presence of glucosinolates in crops intended for animal consumption causes serious problems (Clandinin & Robblee, 1981; Thomke, 1981), leading mainly to goitrogenicity and poor palatability. Levels of glucosinolates in the meal from European-type rapeseed may vary from 40–70 mg/g, significantly higher than that from conventional varieties of summer rapeseed grown in Canada. Numerous processes have been described for the 'detoxification' (i.e. removal of glucosinolates) of such meal (Maheshwari *et al.*, 1981) but over the last decade advances by plant breeders in various countries have led to the commercial availability of seed-yielding meal containing approximately 10 mg glucosinolates per gram.

Rapeseed is grown primarily for its oil content and processing includes heat treatment prior to removal of the oil. This inactivates myrosinase and means that subsequent breakdown of glucosinolates when the meal is consumed must be due to bacterial or chemical activity *in vivo*. Lanzani *et al.* (1974) have found traces of 1,3-thiazolidine-2-thione following the breakdown of 2-hydroxy-3-butenyl glucosinolate by sheep rumen fluid. A 4-hydroxylated oxazolidine-2-thione has been identified as a metabolite

of this same glucosinolate in the rat (Michajlovskij & Langer, 1971). Papas *et al.* (1979*a*) have argued that the absence of measurable amounts of oxazolidine thione in the milk from cattle fed rapeseed meal points to a difference in glucosinolate metabolism in this animal compared with that occurring in plants. In laying hens hydroxynitriles are more readily formed from rapeseed glucosinolates than oxazolidine-2-thione in all areas of the digestive tract (Smith & Campbell, 1976).

PHYSIOLOGICAL EFFECTS OF GLUCOSINOLATE HYDROLYSIS PRODUCTS

With two exceptions, considered below, the main effects are conveniently considered on the basis of the various hydrolysis products which may be formed on glucosinolate breakdown. A more comprehensive report of these effects has recently been published (Fenwick *et al.*, 1983).

Effects of isothiocyanates

Naturally occurring isothiocyanates possess a range of antifungal (Virtanen, 1962), antibacterial (Dornberger *et al.*, 1975) and antimicrobial (Zsolnai, 1966) activities which are probably the basis of the use of brassicas in folk medicine. Zsolnai (1966) has suggested that the activity of isothiocyanates against gram positive organisms is due to their binding to sulphydryl sites. A similar explanation has been offered for the inhibitory action of benzyl isothiocyanate against papain (Tang, 1974). Kojima & Ogawa (1971) have studied the effect of isothiocyanates on yeast cultures. All inhibited the oxygen uptake of the yeast, the most active being 2-propenyl isothiocyanate. Such properties would be advantageous in such 'fresh' products as coleslaw.

Lichtenstein *et al.* (1964) have identified 2-phenyl ethyl isothiocyanate (present in root vegetables) to be a potent insecticide and it is probable that insecticidal effects found in extracts of garden cress and radish are also due to isothiocyanates.

Considerable work has been carried out on the activity of glucosinolates and their hydrolysis products toward field insects and pests. The former may function as feeding stimulants in the large white butterfly (David & Gardiner, 1966), feeding deterrants in the pea aphid (Nault & Styer, 1972) and ovipositing-inducing substances (Nair *et al.*, 1976).

Although glucosinolates have been claimed to act as attractants for the weevil (Matsumoto, 1970) and the flea beetle (Queinnec, 1967) it seems more likely that it is the volatile hydrolysis product which is involved in plant finding. Evidence for the rôle of both glucosinolates and isothiocyanates in host selection and ovipositing has been produced by Nair & McEwen (1976). The pungency of the isothiocyanates may also explain, in part, the insect-repellent properties of certain glucosinolates. Finch (1978) has described detailed studies on the rôle of glucosinolate hydrolysis products as attractants for the cabbage root fly. The main factors to emerge were that those plants lacking glucosinolates yielding volatile products on hydrolysis, failed to stimulate ovipositing and that there was a relationship between ovipositing and attraction stimuli. Based upon such work, chemical traps have been developed for the control of pest damage in developing and mature brassica crops.

The pungency of the isothiocyanate hydrolysis products is the primary reason for the palatability problems encountered when animals and poultry are fed rations containing high glucosinolate rapeseed meal; this problem has been largely overcome with the availability of the newer, low glucosinolate, rapeseed varieties.

Effects of nitriles

Tookey *et al.* (1980) have reported considerable data which indicate that nitriles are the most toxic of the normal glucosinolate hydrolysis products. The acute toxicities of (S)-1-cyano-2-hydroxy-3-butene and 1-cyano-2-hydroxy-3,4-epithiobutane were 170 and 178 mg/kg, respectively (VanEtten *et al.*, 1969), the toxicity of the latter being diminished by polymerization reactions. Significant decreases in organ weight have recently been shown to occur during long-term feeding trials in rats in which up to 300 ppm of (S)-1-cyano-2-hydroxy-3,4-epithiobutanes were fed. Dose-dependent lesions were noted in the liver, pancreas and, especially, kidney, (Gould *et al.*, 1980). These results confirm earlier work in which a crude nitrile fraction from *Crambe abyssinica* proved toxic to rats at 0·2% of the ration and produced liver and kidney lesions at half that level (VanEtten *et al.*, 1969). The massive liver haemorrhage and associated mortality sometimes encountered when laying hens are fed rapeseed meal (Fenwick & Curtis, 1980*b*) has been suggested to be due to hydroxynitriles, individually (Brak & Henkel, 1978) or in combination with intact glucosinolates (Papas *et al.*, 1979*b*).

This has not, however, been unequivocally established. Josefsson & Uppström (1976) concluded that the poor growth of mice fed raw rapeseed meal was due to the presence of nitriles which were preferentially formed from the parent glucosinolates by a large molecular weight component.

Lüthy *et al.* (1980) have found 1-cyano-2,3-epithiopropane to have weak mutagenic activity when screened against *Salmonella typhimurium* TA100 and TA1535, presumably a consequence of its alkylating ability. The authors recommended that the possible carcinogenic properties of this compound be determined in long-term mammalian feeding trials. In view of the biological and toxicological properties associated with this class of compound, and their ready formation from glucosinolates present in common brassica vegetables (Tookey *et al.*, 1980), more detailed evaluation of their acute and chronic toxicity is needed.

Effects of oxazolidine-2-thione

By far the most important properties of oxazolidine-2-thiones is the anti-thyroid activity mentioned below. The 5-vinyl analogue, potentially present in most brassicas, is much less toxic than nitriles possessing an LD_{50} of approximately 1300 mg/kg (VanEtten *et al.*, 1969). Despite structural similarity to several known teratogens, this compound has not been found to be active in this respect in rats (Khera, 1977).

Whilst the rôle of glucosinolate breakdown products in the flavour of cruciferous plants and crops is not within the remit of this paper (see MacLeod, 1976), mention should be made of the intense bitterness of 5-vinyloxazolidine-2-thione (Fenwick & Griffiths, 1981), which causes problems of consumer acceptance of frozen Brussels sprouts and contributes to the palatability problems encountered in rapeseed meal.

Recently, Nishie & Daxenbichler (1982) have investigated the effect of short-term dosage of (R)-5-vinyloxazolidine-2-thione (obtained from *Crambe* seed meal) on rat livers. Treatment increased relative liver weight and in some enlarged livers increased mitosis occurred, resulting in an increased number of hepatocytes. The authors suggested that a reinvestigation of the mitogenic property of this compound was needed and, if proved positive, should be followed by examination of its possible carcinogenicity. Independently, Pearson *et al.* (1983) have found evidence of a mitogenic effect in poultry fed rapeseed meal containing (S)-5-vinyloxazolidine-2-thione.

The feeding of rapeseed meal to brown egg laying strains of chicken has long been known to cause fishy off-flavours and has limited the use of this excellent source of home-produced protein in the UK. It is now evident that the taint, due to levels of trimethylamine of approximately 1 ppm in the egg, is primarily due to the inhibitory action of 5-vinyloxazolidine-2-thione on hepatic trimethylamine oxidase (Fenwick & Curtis, 1980*b*). Since the levels, albeit reduced, of 2-hydroxy-3-butenyl glucosinolate in the meal of low glucosinolate rapeseed cultivars can also form sufficient oxazolidine-2-thione to inhibit this enzyme, and cause taints, in susceptable birds the most effective solution would seem to be that of selective breeding of the chicken (Butler *et al.*, 1982).

GOITROGENICITY

Thiocyanate ion, isothiocyanates and oxazolidine-2-thiones have all been shown to be goitrogenic (Langer & Greer, 1977) and the feeding of forage brassicas and oilseed meals, which are potential sources of these compounds, is associated with thyroid enlargement (hypothyroidism). The rôle of diet, and in particular the involvement of brassica products, in human goitre is much less clear; Greer (1962) has pointed out that only 4% of human goitre cannot be attributed to the effects of insufficient iodine in the diet. Even in regions of natural iodine deficiency, a convincing relationship between intake of cruciferous vegetables and goitre has yet to be established.

Thiocyanate ion, which exerts its effect by competing with iodine for thyroidal transport and thus decreases iodide uptake and hormone formation, is now known to be the factor responsible for cabbage goitre, originally reported by Chesney *et al.* (1928). The effect is only evident in iodine-deficient diets. The goitrogenic effect of isothiocyanates may be attributed to their *in vivo* metabolism to thiocyanate ion (Langer & Greer, 1977), and, as such, is alleviated by dietary iodide supplementation. Langer (1966) has shown that when rats were fed on an iodine-deficient ration containing levels of thiocyanate ion, 2-propenyl isothiocyanate and 5-vinyloxazolidine-2-thione equivalent to those found in a cabbage diet, the weight gain, decreased uptake of iodine by the thyroid and histological changes in the thyroid were similar to those expected from the cabbage diet fed *ad libitum*.

In contrast to the behaviour of thiocyanate ion, oxazolidine-2-thiones

are considered to exert their anti-thyroid effect by interfering with the synthesis of thyroid hormones. An important feature of this behaviour is that this effect is not alleviated by dietary iodide (Langer & Greer, 1977). Langer & Michajlovskij (1969) have shown that as little as 40 μg of 5-vinyloxazolidine-2-thione administered to rats for 20 days could produce a significant increase in thyroid size. The lowest single dose suppressing iodothyronine synthesis was 20 μg, and a corresponding dose of 80 μg was necessary to suppress radio-iodine uptake.

More recently, Elfving (1980) has reported thyroxine synthesis in the rat to be inhibited at a daily dose level of 1 μg and goitre to be produced at a daily dose level of 5 μg over 3 weeks. Commenting on these and other results, the author reported 5-vinyloxazolidine-2-thione to be one of the most potent antithyroid substances known.

It is against this background that suggestions have been made that endemic goitre in several parts of the world arises, in part at least, from brassica-derived goitrogens being transmitted in milk. There has been considerable work done on this problem, particularly in relation to goitre in Tasmania and Finland, and detailed reviews have been presented elsewhere (VanEtten & Wolff, 1973; Langer & Greer, 1977; Elfving, 1980). Clements & Wishart (1956) suggested that endemic goitre in Tasmania was due to an active goitrogen transmitted through the milk of cattle foraging on brassicas. Virtanen *et al.* (1963) have cast doubt upon the validity of the radio-iodine experiments cited by Clements and Wishart. It appears that iodine uptake by the human thyroid gland is much affected by variation in the iodine supply, and iodine had been periodically supplied in schools in Tasmania for prophylaxis of goitre. Later work (Peltola, 1965) suggests that the radio-iodine test itself may not be sensitive enough for the detection of goitrogen-induced endemic goitre. Milk from areas of endemic goitre in Finland has been shown to contain 5-vinyloxazolidine-2-thione levels of 50–100 μg/litre. Rats fed such milk developed enlarged thyroids. Radio-iodine tests indicate that a single dose of 50 mg is needed to produce a clear thyrostatic effect in man (Vilkki *et al.*, 1962) and between 25–50 mg can cause a significant decrease in thyroidal radio-iodine uptake (Langer *et al.*, 1971).

These workers (Vilkki *et al.*, 1962; Langer *et al.*, 1971) have also demonstrated the effect of oxazolidine-2-thione in man. Only a small proportion (0·1 %) of the dose necessary to reduce thyroidal radio-iodine uptake was needed to cause a significant thyrostatic effect.

The thiocyanate content of milk obtained from cattle fed brassicas

(Piironen & Virtanen, 1963) was greater (5–8 mg/litre) than that in controls (2 mg/litre), but since Vilkki *et al.*, (1962) have demonstrated that a dosage of 200–1000 mg is needed to inhibit radio-iodine uptake in man, it seems unlikely that such milk will produce serious thyroidal abnormality in man.

In summarising all the available evidence, Elfving (1980) has concluded that naturally occurring 5-vinyloxazolidine-2- thione must be considered as one of the aetiological factors causing endemic goitre. The reduction of glucosinolate content of brassica forage crops and oilseed meals should, however, reduce the significance of this situation.

STIMULATION OF DETOXIFICATION ENZYMES

Certain aromatic and indolic glucosinolates hydrolysis products have been found to inhibit the neoplastic effects of carcinogens when administered, prior to the carcinogen, in the diet or by oral intubation. Benzyl and 2-phenylethyl isothiocyanates inhibit 7,12-dimethyl-benzanthracene-induced mammary tumour development and 3,4-benzpyrene-induced lung and forestomach tumour in mice (Wattenberg, 1979*a*). Benzyl thiocyanate was active only against mammary neoplasia. Benzyl isothiocyanate has also been found to reduce the incidence of 3,4-BP or 7,12-DMBA-induced pulmonary adenoma in mice (Wattenberg, 1979*a*). Whilst the effects of other isothiocyanates have not been studied in detail, phenyl isothiocyanate apparently behaves similarly to the other aromatic compounds, but 2-propenyl isothiocyanate is without effect on the development of 7, 12-DMBA-induced pulmonary adenoma (Wattenberg, 1979*b*).

The feeding of cruciferous vegetables to rodents has been shown to stimulate aryl hydrocarbon hydroxylase activity, (Wattenberg *et al.*, 1975) enhance the *in vivo* detoxification of the carcinogens aflatoxin B_1, polybromobiphenyl (Stoewsand *et al.*, 1978) and 1,2-dimethyl hydrazine (Srisangnam *et al.*, 1980*a*) as well as stimulating enzymes which 0-dealkylate phenacetin, and 7-ethoxy coumarin, hydroxylate hexobarbital (Pantuck *et al.*, 1976), enhance glutathione-S-transferase activity of the forestomach (Sparnins & Wattenberg, 1981) and induce mixed functional oxidase activity (Babish & Stoewsand, 1975). Dietary Brussels sprouts and cabbage in human subjects have been shown to stimulate the metabolism and excretion of antipyrin and phenacetin (Pantuck *et al.*,

1979). These effects may be viewed as resulting from a co-ordinated response of detoxification processes.

Loub *et al.* (1975) have shown that the compounds responsible for some, if not all of the above effects are indole-3-carbinol (XI), indole-3-acetonitrile (X) and 3,3′ diindolylmethane (XII) (see Fig. 1). Furthermore these compounds have been shown to inhibit 3,4-BP-induced forestomach neoplasia and pulmonary adenoma in rats (Wattenberg, 1979*b*). Before the results of the animal studies reported here are extrapolated to humans, epidemiological studies are clearly necessary. However, the data already acquired do offer the intriguing possibility of brassicas being grown and used for their therapeutic value.

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Nitrosamines in Beverages

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(Received: 25 January, 1983)

ABSTRACT

Traces of nitrosodimethylamine (NDMA) have been reported in many types of beer but are not normally found in beverages (other than those derived from malt) consumed in the UK. A range of control methods is now in use, and these have led to marked reductions in NDMA; the chemical and biochemical processes involved in NDMA formation in malt, and in the treatments which can minimise this, are discussed. Analytical methods for the measurement of NDMA and other volatile nitrosamines are now well established and can give high quality analytical data. Analysis of non-volatile nitrosamines at trace levels presents some novel problems; these are discussed along with some approaches to their solution. Data from a few samples illustrate some of the various questions which may require further consideration.

INTRODUCTION

The presence of traces of volatile *N*-nitrosamines in foods—particularly cured meats—has been known for some time (Scanlan, 1975; Sen *et al.*, 1979). More recently, there have been reports of the presence of these compounds in beer and in some other beverages (Spiegelhalder *et al.*, 1979; Sen *et al.*, 1980). Primarily the reports have concerned *N*-nitrosodimethylamine (NDMA) at levels of a few micrograms per

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kilogram, but *N*-nitrosopyrrolidine (NPyr) has also been reported; usually the level is less than that of NDMA in the same sample.

Further investigations have shown that the NDMA and NPyr are derived almost entirely (and possibly exclusively) from the malt used in the production of beer (Wainwright *et al.*, 1982). Other products are derived from malt, and volatile nitrosamines from this source have been found in vinegar and in whisky. On the other hand, the malt extract used widely in the food industry appeared to be free of NDMA (D. J. McWeeny, C. Crews and R. C. Massey, unpublished data); presumably this evaporates along with much of the water when the extract is concentrated under reduced pressure. There have been no substantial reports of NDMA being found in other grain-derived alcoholic beverages in amounts comparable with those in beverages based on malted barley; grape-based beverages (e.g. wine and brandy) also seem to be virtually NDMA free. However, whilst there have been reports that small amounts of NDMA can be found in dried milk produced in North America (Havery *et al.*, 1982), and very low levels have been found in pasteurised liquid milk, neither of these findings has been confirmed in studies of the same commodities produced in the UK (D. J. McWeeny, C. Crews and R. C. Massey, unpublished, 1981).

Reports of the presence of NDMA in beer began to circulate in 1978 and were rapidly confirmed in various countries around the world. Levels up to 70 μg/kg were reported. There is no evidence that NDMA had been widely present at these levels for any substantial period before that time. Examination of beer from different sources, and of the ingredients used at each location, quickly revealed that malt was the major source of NDMA, and, in particular, that malt dried on kilns direct-fired with natural gas was frequently found to contain relatively high levels of NDMA. In the UK and Western Europe the advent of relatively cheap supplies of natural gas from the North Sea had led to the conversion of some existing kilns to gas-firing and to the choice of gas as the fuel for new kilns commissioned during the mid- and late-1970's. The purpose of this paper is to consider some of the chemical factors underlying these observations and to discuss the ways in which these provided a basis for measures which have successfully reduced NDMA levels; the development of analytical procedures for monitoring the effect of these measures will be described. Our understanding of the chemistry underlying the palliative measures is limited and the implications of this in terms of the need for further work will be discussed.

CHEMISTRY OF NDMA FORMATION

N-nitrosamines arise from the interaction of a secondary amine with a nitrosating species; they may also be formed through nitrosative dealkylation of a tertiary amine. In its simplest form, reaction of a secondary amine (in its unprotonated form), with an alkali metal nitrite in aqueous solution, leads to *N*-nitrosamine formation in good yields; the reaction is pH dependent, and, depending on the pK_a of the secondary amine, the rate is often maximal around pH 3. The reaction has been extensively studied in relation to food systems and particularly with respect to cured meat products. A variety of factors which affect this simple mechanism has been identified; for example, (i) certain phenols undergo *C*-nitrosation to form nitroso-phenols which can then catalyse *N*-nitrosation, (ii) thiols can undergo *S*-nitrosation to form nitrosothiols which can *trans*-nitrosate to form *N*-nitrosamines, (iii) the solubility of nitrogen oxides in a lipid phase can lead to enhanced nitrosation of relatively non-polar amines (McWeeny, 1982).

By contrast, relatively little is known about the nitrosative dealkylation of tertiary amines. Smith & Loeppky (1967) have established the features of the mechanism in simple systems and shown that the reaction is pH dependent. Only weakly acid conditions are required; under strongly acid conditions reaction is slow, and this is the basis for the widely used method for distinguishing tertiary amines from primary and secondary amines. The extent to which the mechanism is modified by conditions in food systems has not been studied in depth.

In the absence of detailed information about nitrosation mechanisms operating during the 'kilning' stage of malt production, the practical approach in dealing with the NDMA in malt production was to try to adjust the gas-burning systems to correspond to those which were associated with low NDMA levels from oil- or anthracite-fired kilns. The combustion systems differed in two respects:

(i) Conventional natural gas burners produce a very hot flame in which atmospheric nitrogen can be converted to nitrogen oxides; in turn, these can act as nitrosating agents. Nitrogen fixation is strongly temperature dependent and can become significant in the air: natural gas flame.
(ii) Natural gas is a 'clean' fuel and has a very low sulphur content as compared with oil and anthracite fuels.

The influence of the first factor might be eliminated if means of reducing the flame temperature could be devised. This is largely an engineering problem and not appropriate for discussion in detail here, but it can be reported that gas burners have now been produced which are capable of giving nitrogen oxides (NO_x) in concentrations indistinguishable from those in the intake air. However, there are control problems in the use of these burners and, in any event, the ambient NO_x levels in some urban environments have now been shown to be capable of giving measurable levels of NDMA in malt. This latter observation indicates that even the use of indirectly heated air (via a heat exchanger) cannot be guaranteed to give low NDMA malt.

Alternative approaches based on reducing the level of NDMA through the control of the amine precursor have been widely studied. Although dimethylamine is present in germinated barley it does not seem likely to be the source of NDMA. Lowering the surface pH of the barley to 2·5 before kilning can completely inhibit NDMA formation (Ladish, 1981); if dimethylamine was the NDMA precursor this treatment would accelerate, rather than retard, the nitrosation. The observed pH dependence effects indicate the possible involvement of a nitrosative de-alkylation reaction. Studies on NDMA formation in malt indicate that the precursors are bio-synthesised during germination (Mangino *et al.*, 1982). The possible rôle of two tertiary amines (hordenine and gramine) which are known to be formed during germination has been studied (Mangino *et al.*, 1982; Wainwright *et al.*, 1982). Additional circumstantial evidence pointing to these compounds comes from the observation by Wainwright *et al.*, (1982) that removal of the rootlets (in which the two amines are known to be concentrated) reduces the NDMA levels considerably. The hypothesis that one or both of these amines is an NDMA precursor has been successfully applied in some of the palliative measures which have been adopted; e.g. it is known that the addition of about 100 ppm potassium bromate to the barley during germination severely restricts the development of rootlets, and tests show that it also leads to a useful reduction in NDMA levels. More direct evidence of their involvement as NDMA precursors comes from chemical studies in which it has been shown that NDMA can be formed from these and similar compounds (Mangino *et al.*, 1982). After heating malt roots with NO_x, a compound thought to be *C*-nitrohordenine was isolated (Wainwright *et al.*, 1982); this could arise from atmospheric oxidation of the initial nitrosation product (*C*-nitroso hordenine), but a direct nitration route analogous to

that favoured at low NO_x concentrations for the formation of *N*-nitramines might perhaps be an alternative (Challis, *et al.*, 1982). C-nitrohordenine is stable when heated alone at 90 °C but gives 90 % yield of NDMA when heated with NO_x at this temperature for 64 h (Fig. 1). Although the definitive evidence that these reactions operate in germinated barley during kilning is still lacking, the knowledge that the reactions can occur and that ample amounts of the precursor are present make it a reasonable hypothesis.

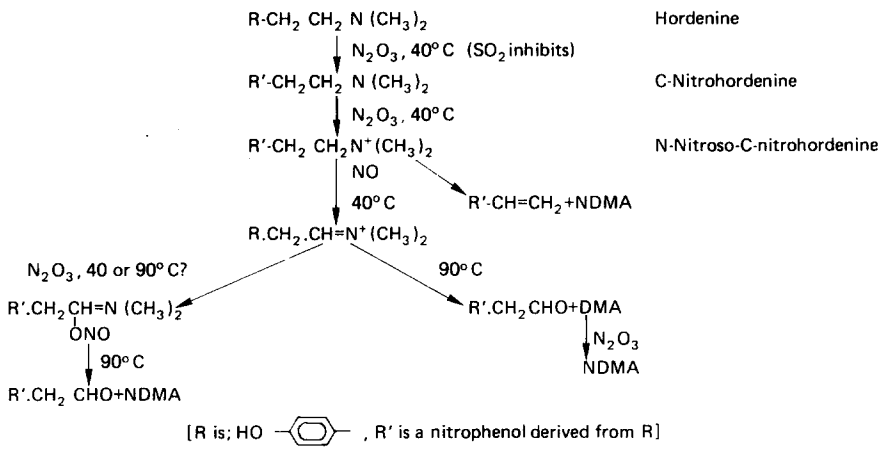

Fig. 1. Possible routes to NDMA formation of hordenine. (After Wainwright, 1981).

Control of NDMA formation by reducing the concentrations of the nitrosating agent and the amine precursor has been considered above; the rôle of SO_2 as an inhibitor will now be discussed. In practical terms the effect is simple; the incorporation of SO_2 into the combustion gases (either by injecting SO_2 or more usually by burning elemental sulphur) can give a dramatic reduction in NDMA levels. The chemical basis of this effect is not fully understood. Sulphur dioxide and/or sulphites are not normally considered to be good nitrosation inhibitors as such, and some indirect effects seem indicated. One mechanism appears to be that the surface pH of the malt is lowered and it can be shown that this reduces the rate of nitrosation (i.e. other studies show that spraying acid on to the germinated barley before kilning has a similar effect). This may be taken as an indication that NDMA formation involves nitrosative de-alkylation of a tertiary amine rather than direct nitrosation of a secondary amine.

However, the magnitude of the SO_2 effect is greater than can be accounted for simply as a pH effect. Another possibility is that nitrosyl sulphuric acid is formed (as in the 'chamber process' for sulphuric acid production). However, there is no satisfactory experimental evidence that (i) nitrosyl sulphuric acid formation occurs to a significant extent under the dilute conditions existing in kilning gases and (ii) nitrosyl sulphuric acid is relatively ineffective as a nitrosating species. Both these requirements must be met if the hypothesis is to be taken seriously.

It has been shown that SO_2 can prevent the formation of an intermediate during the early stages of kilning and that this intermediate reacts to form NDMA during the later, higher temperature stage; SO_2 does not inhibit this later stage but NO_x does seem to be required (O'Brien, *et al.*, 1980; Wainwright *et al.*, 1982). The fact that SO_2 inhibits the first nitrosative stage, but not the second stage, tends to indicate that it does not exert its inhibitory action by combining with NO_x, e.g. to form nitrosyl sulphuric acid. For the present, the chemical basis for the effectiveness of sulphuring in ensuring low NDMA levels in malt is only partially explainable. One possibility currently under investigation is that, before nitrosation occurs, the phenolic precursor undergoes some other reaction, and that the selective inhibitory action of SO_2 is upon this preliminary reaction.

ANALYTICAL METHODS

N-Nitrosodimethylamine

When the NDMA in malt problem emerged it immediately generated a requirement for a lot of data about NDMA levels, but the accuracy requirement was not great. As the nature of the problem and the means of controlling it emerged, the requirement changed somewhat. The requirement now is for a method which can be used in specifications for raw materials, products and process control; methods must be rapid, suitable for routine use, highly specific and highly accurate, e.g. the Analytical Methods Committee of the Institute of Brewing set the target for analytical quality as a coefficient of variation of 10 % at 1 or 2 μg/kg. A number of methods have been published but all the commonly used ones employ a gas chromatographic separation using the Thermal Energy Analyser as a detector (Fig. 2). This works upon the principle that the GC effluent is passed directly into a catalytic pyrolysis tube at reduced

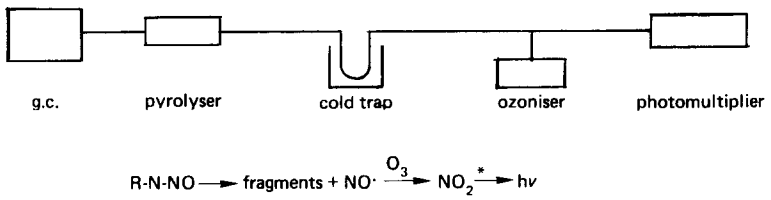

Fig. 2. Principles of GC–IEA detection of volatile N-nitrosamines.

pressure under conditions which selectively cleave the N—NO bond. Pyrolysis products other than NO are condensed in a cold trap and the NO is converted to NO_2^* in a stream of ozone. The photon released on return to ground state is detected by a suitable photomultiplier tube. The instrument is highly specific for nitrosamines when used as a GC detector—it is also highly sensitive, i.e. 30 pg is quite detectable. Detection of 0·1 μg/kg can be achieved by chromatographing a 5 ml aliquot of a 100 μl concentrate prepared from a 10 g sample, i.e. only a 100-fold concentration is required during sample preparation. Because the detector is so specific, the selectivity requirement during the sample work-up does not have to be great; if samples are inadequately cleaned up before analysis, the limit is set by degradation in GC column performance due to repeat injections rather than by detector performance. A method based upon distillation from an alkaline liquid paraffin slurry of the sample, dichloromethane extraction of the distillate, and concentration of the extract has the advantage of being readily applicable to a wide range of sample materials (Fine, *et al.*, 1975).

Alternative procedures which avoid the rather cumbersome and messy features of this procedure, but retain the distillation/extraction/concentration concept, have been devised and proved successful for a range of foods (Goodhead & Gough, 1975). For work on beer, spirits or aqueous extracts of malt more specific methods have now been developed; initially these relied on pouring the sample down an adsorbent column and then eluting the NDMA in dichloromethane; this provided a simple means of removing the water and the more polar components of the sample (Gough & Fine, 1979; Hotchkiss, 1981). More recently, methods (e.g. Long, 1982, Method 2) which are based on a simple liquid: liquid extraction with dichloromethane have been tested; these can give extremely good data, but some users complain of problems with emulsion formation at the liquid: liquid extraction stage, and with loss of GC column performance due to the relatively large amount of extraneous

material in the final concentrate. The methods do have the advantage of requiring relatively simple equipment and only relatively unskilled operators for the sample work-up. In all cases it is necessary to avoid formation of NDMA during the analytical procedure—this is usually achieved by adding sodium sulphamate, sodium azide or hydrazine sulphate to destroy any nitrosating agents early in the procedure; the efficacy of these steps can be further checked by adding another amine to the sample and examining the GC trace for evidence of its nitroso-derivative. Care must also be taken to correct for recovery—this is often done by means of nitroso-dipropylamine (NDPA) added as an internal standard, and in this case it is necessary to establish that there is no differential retention of the less volatile NDPA, particularly at the concentration stage.

Collaborative trials using prescribed methods have been conducted on malt and on beer by AOAC, ASBC and IOB. Some of the results are summarised in Table 1; they indicate considerable success in developing methods for use at the μg/kg level. The quality of the data is remarkably good when compared with that for methods for other substances at this level.

Typical levels encountered in beer produced in the UK are now around 0·4 μg/kg and this indicates that levels in ale/lager malts are usually below

TABLE 1

Summarised Data for some Recent Collaborative Trials on NDMA Analysis in Beer and Malt

Organiser	*Matrix*	*Method*	*Number of labs.*	*Outliers excluded*	*Mean conc. (μg/kg)*	*CV %*
AOAC	Beer	Atm. Dist.	13	2	0·55	15
			13	2	5·22	13
ASBC	Beer	(i) Vac. Dist.	7	0	1·54	14
			7	0	4·60	8
		(ii) Celite/DCM	6	0	1·46	7
			8	0	4·17	14
ASBC	Malt	(i) Vac. Dist.	7	0	3·34	14
			8	0	8·20	21
		(ii) Celite/DCM	6	0	3·38	17
			7	0	8·07	18
IOB	Malt	Wort/DCM	8	0	2·80	14
			8	0	6·87	11

5 μg/kg. This is a 'target' level for some commercial purposes, and the trials indicate that a coefficient of variation around 15% can be achieved at this level. It is noteworthy that, whereas only 4 years ago, volatile nitrosamine analysis was a highly specialised procedure which only two laboratories in the UK were equipped to carry out, this quality of data is now being achieved in many industrial laboratories with relatively simple and rapid procedures suitable for routine use and requiring a comparatively small input of skilled manpower.

Non-volatile Nitrosamines

As indicated above, methods for the analysis of volatile nitrosamines by GC–TEA procedures are now well established; by contrast, methods for non-volatile nitrosamines are not readily available. One general approach is to derivatise the polar groupings on a non-volatile nitrosamine, thereby increasing its volatility to a point at which a GC–TEA chromatographic procedure can be adopted. Necessarily, this approach has its limitations when the chemical nature of the non-volatile nitrosamines is unknown. The application of HPLC techniques provides wider possibilities, but has accompanying problems associated with the TEA detector. First, it is not specific to *N*-nitrosamines when used in this way; there are a number of non-volatile nitrogen-containing compounds which will give a TEA response. Some of these have been identified in a study of a wide range of compounds (Table 2) by C. L. Walters (unpublished data 1982); the extent to which these compounds may exist in beverages is not known, but if they are present they will give a TEA response and it will be necessary to adopt additional confirmatory tests for nitrosamines. Secondly, there are major compatibility problems between the TEA and the HPLC solvents commonly used for polar compounds. Solvents containing more than a trace of water have seriously adverse affects on the base line stability of the TEA, and the loss of sensitivity is very substantial, e.g. two orders of magnitude. Also the inorganic buffers and ion-pairing reagents which might be used for HPLC of polar/ionisable compounds have an adverse and cumulative effect on the performance of the catalytic pyrolysis tube. Thirdly, the use of HPLC on a routine basis is hampered by the problem that after a relatively short time the cold traps become blocked by frozen HPLC solvent.

One partial solution to these problems is to use a reverse-phase micro-bore HPLC system (Massey, *et al.*, 1982); the flow of aqueous solvent is

TABLE 2
Thermal Energy Analyser Response to some Organic Compounds (molar response relative to NDMA)

Class	*Compound*	*Response*
N-nitrosamine	*N*-nitrosodimethylamine	1·00
N-nitrosamide	*N*-ethyl *N*-nitrosourea	0·02
N-nitrososulphonamide	*N*-methyl *N*-nitroso toluenesulphonamide	0·17
N-nitramine	*N*-nitrodimethylamine	0·40
S-nitroso	*S*-nitrosotriphenylmethanethiol	0·53
C-nitro	nitrobenzene	0·01
	2-nitropropane	0·41
O-nitro	ethyl nitrate	1·10
O-nitroso	ethyl nitrate	0·70

Response $\ll 0{\cdot}01$ obtained from: cyclohexanone oxime, imidazole, azobisisobutyronitrile, pyrrole, azoxyanisole, furan, thiophene, linoleic acid, crotonaldehyde and methylvinylketone.

quite small and this can be mixed with a much larger flow of acetone before the introduction to the TEA pyrolyser. In this way an ionic nitrosamine N-nitroso *N'*,*N'*dimethylpiperazinium iodide has been successfully chromatographed on a 1 mm bore ODS column by reverse phase ion-pair chromatography using 0·1M ammonium heptanesulphonate in methanol: water (70:30). The column flow rate was 20 μl/min; acetone make-up rate prior to TEA was 2 ml/min. There is still some loss of detector sensitivity with this method, but a more severe limitation may be the 0·5 μl sample volume. If a useful amount of the analyte is to be injected then the sample clean-up/concentration for micro-bore HPLC procedures may have to be much more extensive than those described for GC–TEA.

An alternative approach has been used for studies on N-nitroso amino acids in beer (Massey *et al.*, 1982). Sample clean-up is achieved by adsorption on to a 'Preptube' (Thermo-Electron Corporation, Waltham, MA., USA), elution with ethyl acetate, evaporation and re-dissolving in acetone; the procedure gives 90 % recovery for *N*-nitrosoproline in beer and a detection limit of 1 μg/kg. Using the technique, four samples of beer, each containing approximately 1 μg/kg NDMA (0·014 μmol/kg) were examined. *N*-nitrosoproline levels ranged from 0·01 to 0·26 μmol/kg and the abundance of an unidentified neutral N-nitrosamine ranged from

0·01 to 0·25 μmol/kg. The data are shown in Table 3. This Table includes a measure of the total *N*-nitrosamine content of the samples. This is determined by a procedure based on that of Walters *et al.*, (1978), in which the sample is dissolved in ethyl acetate, nitric oxide is released from nitrite and other nitrosating agents by adding acetic acid before adding a HBr/acetic acid mixture which liberates NO from N-nitrosamines; the NO is swept into the ozonolysis chamber of a TEA and measured in the usual way. In all four samples the total nitrosamine figure is significantly

TABLE 3
N-nitrosamine Content of Beer

Sample	*Concentration (μmol/kg)**			
	N-*nitroso-dimethylamine*	N-*nitroso-proline*	*Major neutral* N-*nitrosamine*	*Total* N-*nitrosamine*
1	0·014	0·162	0·25	17·4
2	0·012	0·052	0·17	6·0
3	0·014	0·040	0·01	1·1
4	0·012	0·012	0·01	0·2

* Assuming stoichiometric release of NO from all *N*-nitrosamines; 0·014 μmol/kg is equivalent to 1·0 μg/kg NDMA.

greater than that from the major GC and HPLC peaks—but the magnitude of the discrepancy varies considerably. Clearly, the relative abundance of the components responsible for the total nitrosamine figure must vary widely. The HBr/acetic acid method is not entirely specific for nitrosamines; it is known to respond to nitrothiols, nitrolic acids and some aliphatic nitroso compounds. There is little information about the existence (or otherwise) of these compounds in food and there must therefore be some uncertainty about the specific interpretation of the data; the general conclusion remains the same, i.e. that there can be major differences in the amount of releasable NO in beers with closely similar NDMA levels.

For GC–TEA procedures the amount of material accumulating in the cold traps is relatively small; for HPLC–TEA methods, the whole of the solvent is retained in the traps and, unless special steps are taken, it is impracticable to work on a routine basis. Trap temperatures, which cause the solvent to solidify, will lead to rapid 'blocking' of the gas flow. Some improvement can be achieved by choosing a combination of solvent and

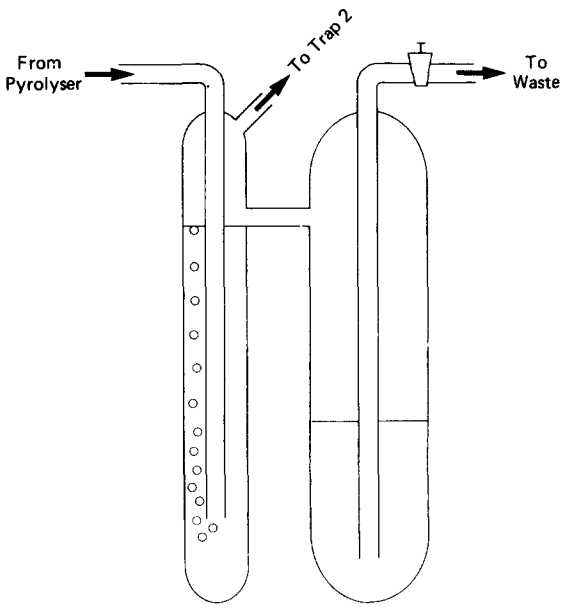

Fig. 3. High capacity, low deadspace solvent trap for use with HPLC–TEA (diagrammatic).

trap-temperature which keeps the condensed solvent in the liquid phase. Modifications to the trap design, utilising an overflow system, can then provide traps with a large capacity but a low apparent volume (Fig. 3); in combination with a switching system a pair of these traps can provide the means of condensing the solvent indefinitely in an HPLC–TEA system without interruption (Massey and McWeeny, to be published).

GENERAL DISCUSSION

The amount of information about nitrosamines in beverages—and how to measure them—has grown enormously in the last 4 years; much of the information relates to NDMA. This reflects two facts: (i) that NDMA is known to be a potent carcinogen in experimental animals, (ii) that there is an instrument which allows it to be measured relatively easily, but with high sensitivity and specificity. The concentration of effort which these influences have produced has left a number of other aspects of the problem relatively unexplored. Now that the NDMA-in-beverages

problem is largely under control and with the development of more generally applicable analytical methods for *N*-nitrosamines, there may be more opportunity to look at the problem in a wider context.

If the data on 'total nitrosamines' accurately reflect the total concentration of nitrosamines, it becomes important to assess whether these unknown nitrosamines are likely to represent a hazard to man. In-chemical terms they are similar because of their common possession of an N—NO grouping. However, the carcinogenicity of NDMA is related to something different—its ability to promote alkylation of genetic material. There have been extensive studies made of the relative potency of a wide range of nitrosamines as carcinogens but these have not produced any simple rules which can be used accurately to predict potency (Lijinsky, 1982). In an event, the need still seems to be for the chemist to identify and quantify nitrosamines before a toxicological assessment can be made.

The alternative strategy would be to attempt to minimise the abundance of these compounds. The very limited amount of data presented earlier in relation to beer (see Table 3) does indicate that there may be ways of doing this. In this context one can consider the chemistry of the measures currently in use for controlling NDMA. Clearly, the overall level of nitrosamines in malt beverages is likely to be reduced if kilning is conducted under 'low NO_x' conditions, e.g. with reliable 'low NO_x' burners or indirect heating used in conjunction with low ambient NO_x levels. The other control measures have been rather more specifically targeted on NDMA production, and their effects on other nitrosamines are not easy to predict, thus:

(i) Bromate treatment of the germinating barley is aimed at suppressing development of the site at which the likely amine precursor of NDMA is generated—we have no information about its effects on the abundance of other nitrosatable amines in germinating barley.

(ii) Lowering the pH is known to be useful in reducing the rate of nitrosative de-alkylation of tertiary amines, such as hordenine, but it might also be expected to enhance nitrosation of secondary amines.

(iii) The effect of using SO_2 is less easy to predict and will depend on the chemical mechanism through which it operates. First, if its effectiveness relies on pH reduction the situation is the same as (ii) above. Secondly, sulphur dioxide is not normally regarded as an

inhibitor of secondary amine nitrosation, but if its effect is to interfere with nitrosative de-alkylation of tertiary amines in some general way, then its efficacy will depend on the contribution these amines make to 'total nitrosamine' formation. Thirdly, if its effect is related to the phenolic moiety of hordenine, it will have little effect on the formation of other nitrosamines. Fourthly, if its effect is on the NO_x in the combustion gas (e.g. through the formation of a product with low nitrosating power, (Wainwright, 1981) rather than on the amine) then SO_2 may exert a broad-ranging effect on nitrosamine formation.

Answers to questions of this nature are now potentially available to the chemist and biochemist. Now that the urgent action to remove NDMA from beverages has been taken, it may be time to consider some of these subsidiary questions, the need to answer them, and the ways of doing so.

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Food Chemistry **11** (1983) 289–307

Mycotoxins in Dairy Products

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(Received: 25 January, 1983)

ABSTRACT

Certain fungi produce chemical substances that cause toxic symptoms when food containing them is ingested by man or animals. These compounds are referred to as mycotoxins. Mycotoxins may contaminate dairy products by moulds growing on them, or by the carry-over of mycotoxins occurring in animal feedstuffs ingested by dairy cattle. An example of the first mentioned category is sterigmatocystin, a carcinogenic mycotoxin sometimes occurring on hard cheese. An example of the second category is aflatoxin M_1, a compound strongly suspected to be carcinogenic, which often occurs in milk. Due to the fact that processing of milk does not decrease the aflatoxin M_1 content, aflatoxin M_1 occurs in various dairy products. Sensitive methods of analysis for the determination of mycotoxins in dairy products have been developed in the last 10 years, most of them are based on TLC- or HPLC-separation procedures, followed by fluorimetric measurement.

The most fundamental way to tackle the problem of mycotoxin contamination of dairy products is to prevent fungal growth on the dairy products or, in the case of carry-over of mycotoxins, in the crop before, during and after harvest. If measures to prevent fungal growth and mycotoxins production are not taken or fail, one can sometimes resort to physical or chemical methods to eliminate mycotoxins.

INTRODUCTION

Mycotoxins are secondary metabolites of fungi which may exert toxic, carcinogenic, mutagenic, teratogenic and oestrogenic effects. Although some fungi, such as the ergot-producing *Claviceps purpurea*, have been known for centuries because of their high and acute toxicity, it was only after the discovery of the aflatoxins in 1960 in the United Kingdom, that research showed that a large number of other fungus species could form other, and equally important, toxic secondary metabolites. At present, over 150 different mycotoxins are known, varying in structure as shown in Fig. 1, where examples are given of some well known and less well known mycotoxins.

The presence of mycotoxins in dairy products may be the result of:

(1) The contamination of the milk caused by contamination of the feedingstuff consumed by the cow.
(2) Direct fungal contamination of the dairy product which may result in the formation of mycotoxins. Cheese is a product especially sensitive to mould during one of the stages of its preparation or during storage of the product.

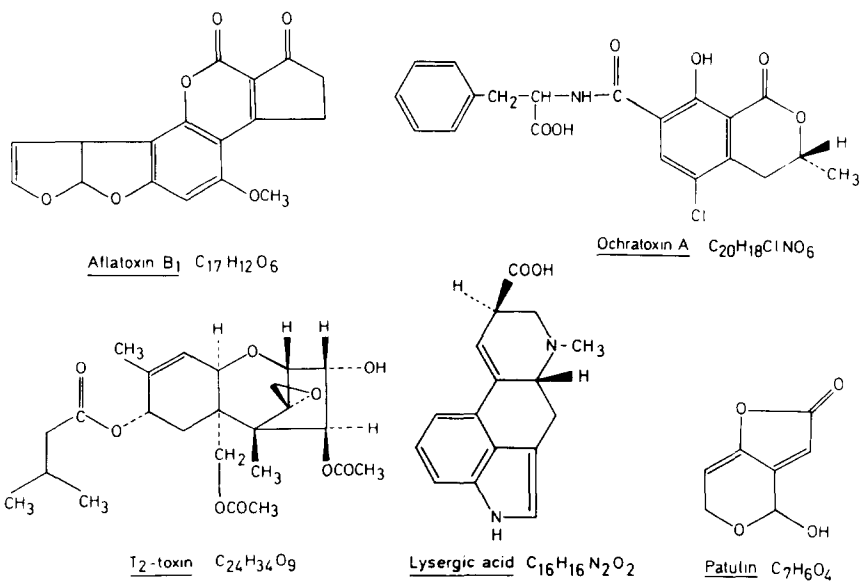
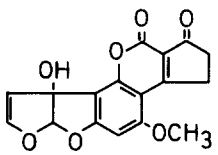


Fig. 1. Chemical structures of some mycotoxins.

AFLATOXIN M_1

The first mentioned possibility is of major importance as it was found that aflatoxin B_1, the most notorious of the mycotoxins, is converted by the dairy cow into its 4-monohydroxy derivative, aflatoxin M_1 (Fig. 2), which appears in the milk (Allcroft & Carnaghan, 1963). Experiments with cows have shown that about 1–4 % of the ingested aflatoxin B_1 can be recovered in the milk as aflatoxin M_1. From experimental work with rainbow trout,



aflatoxin M_1

Fig. 2. Chemical structure of aflatoxin M_1.

there are indications that aflatoxin M_1 is carcinogenic, although probably less potent than aflatoxin B_1 (Canton *et al.*, 1975; Sinnhuber *et al.*, 1974). Hsieh (1982) conducted a chronic study with two groups of fifty male Fisher rats which were continuously fed diets containing 50 μg M_1/kg and 50 μg B_1/kg, respectively, for 18 months. At the end of this period, multi-hepatocarcinomas were found in 100 % of the B_1-group, whereas no evidence of neoplasm was found in any of the M_1-group. Despite this, the question as to whether aflatoxin M_1 must be considered as a carcinogen is difficult to answer, as more extensive studies than those mentioned have not been possible, because of the lack of sufficient pure material. On the basis of its structural resemblance to aflatoxin B_1, toxicologists consider it possible that aflatoxin M_1 has carcinogenic properties. Therefore, the presence of this compound is considered to be undesirable in food. In consequence, some countries have enacted legal measures to control M_1 contamination of milk and milk products. From an international inquiry (Schuller *et al.*, 1983) it became clear that, until 1981, only the Netherlands, Switzerland and the United States had established tolerance levels for M_1 in milk at 0·1, 0·01–0·05 and 0·5 μg/kg, respectively. However, in various other countries the feasibility of establishing tolerances for M_1 contamination of milk has been discussed. Because of the undesirability of M_1 in food, much scientific attention has been focused on the development of methods to detect aflatoxin M_1, on the occurrence of this toxin in dairy products, on the effects of processing on

M_1 and on ways of eliminating the toxin if prevention of its occurrence has failed.

ANALYSIS FOR AFLATOXIN M_1

In an analytical procedure, several steps can be distinguished (Fig. 3). Sampling and sample preparation are not problems, because aflatoxin M_1 is homogeneously distributed in the milk. The extraction and clean up, however, need much more attention. Aflatoxin M_1 is a semi-polar component extractable with chloroform. Lipids and other substances

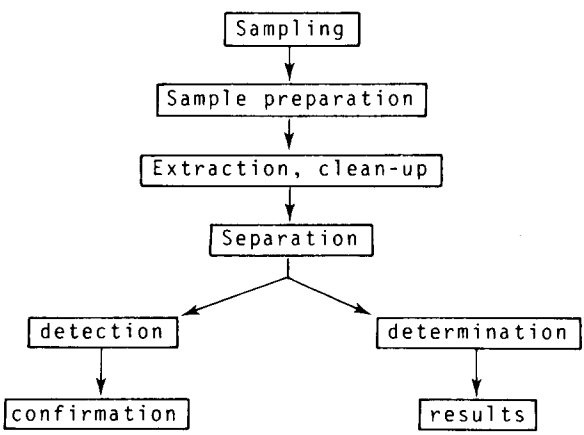


Fig. 3. Analytical procedure for mycotoxin analysis.

that are co-extracted, and which may interfere with the final detection, are removed by either column chromatography or liquid–liquid extraction, or both. Usually, a final extract in chloroform is obtained. Since estimation of aflatoxin M_1 is usually based upon its characteristic intensive blue-fluorescence when irradiated with ultraviolet light, other fluorescent substances, which are co-extracted together with aflatoxins, may interfere with the estimation. To separate aflatoxin M_1 from the matrix, chromatographic techniques are normally used. Both one- and two-dimensional thin layer chromatography (TLC), as well as high performance liquid chromatography (HPLC), with normal phase and reversed phase systems, are used. Once M_1 is separated, its quantity can be determined. When TLC is applied, the plate is irradiated with

longwave ultraviolet light. Aflatoxin M_1 fluoresces blue and the intensity of fluorescence can be used as a measure of its quantity. The determination of the intensity of fluorescence can be done visually or, more precisely, densitometrically.

Depending on the desired limit of detection, one- or two- dimensional TLC is applied. In European countries there is a tendency to establish tolerances for M_1 at very low levels, 0·1 μg/kg or even less. In such cases, two-dimensional TLC should be the method of choice. Two-dimensional TLC is carried out as follows (see Fig. 4): an aliquot of the sample extract

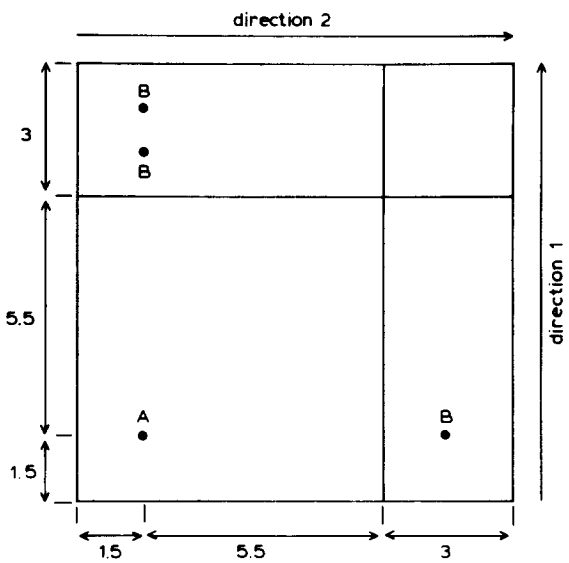


Fig. 4. Schematic of thin layer chromatogram for two-dimensional chromatography. Direction 1: Diethylether–methanol–water (94 + 4·5 + 1·5, lined tank) Direction 2: Chloroform–acetone–methanol (90 + 10 + 2, unlined tank). Measures are in centimetres.

is spotted at A and known amounts of aflatoxin M_1 standard are spotted at B. The plate is then developed in directions 1 and 2, respectively, using two different solvent systems. Figure 5 illustrates the separation pattern of an extract of milk, prepared according to the method of Stubblefield (1979), after two-dimensional TLC separation. The milk sample was contaminated at a level of 0·1 μg/kg. Detection and quantifying of aflatoxin M_1 in the extract can be carried out by visual or densitometric comparison of the intensities of fluorescence of the M_1 spots from sample and standards.

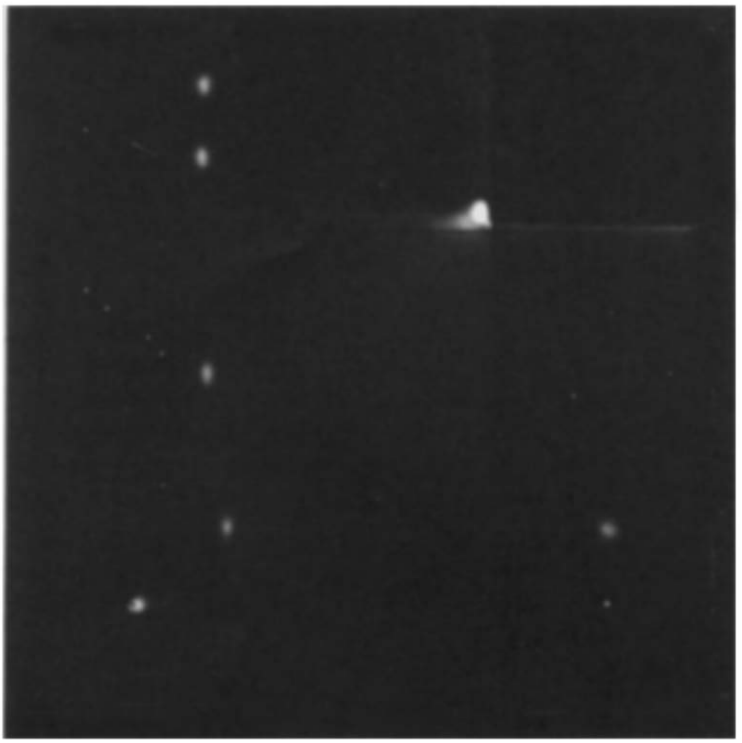


Fig. 5. Two-dimensional separation of milk extract contaminated with aflatoxin M_1 (0·1 μg/litre), prepared according to the method of Stubblefield (1979).

In spite of the elaborate clean up and separation techniques, interfering compounds, which have similar fluorescent and chromatographic behaviour as aflatoxin M_1, exist. In order to minimize the possibility of false-positives, the identity of aflatoxin M_1 in positive samples has to be confirmed. The most reliable method for this purpose is high resolution mass spectroscopy (MS). MS, in combination with thin layer chromatography, however, is rather time consuming, and not every laboratory is equipped with this sophisticated type of apparatus. Therefore, simple chemical techniques are preferred. These techniques do not offer the same absolute certainty as MS, but they practically exclude false-positives. An example of such a confirmatory test is a method developed by Trucksess (1976) and modified by van Egmond *et al.* (1978). In that procedure, the presumed M_1 spot from the extract, as well as one of the standard spots (developed in direction 2), are derivatized on the TLC plate by

superimposing a drop of trifluoroacetic acid–hexane on each of these spots after two-dimensional TLC, followed by heating the plate at 75 °C to allow the reaction to occur. The exact chemical nature of the reaction product which is formed is not yet known. If the reaction is carried out in a test tube, the M_1 hemiacetal is formed, resulting from the acid-catalyzed addition of water to the terminal furan ring (Fig. 6). However, on the TLC

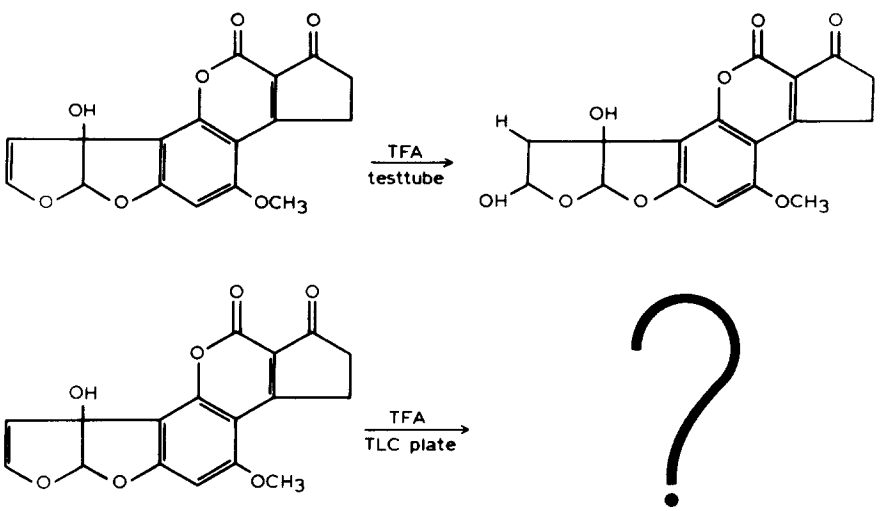


Fig. 6. Reaction of aflatoxin M_1 by treatment with trifluoroacetic acid.

plate, another reaction occurs as the main product; it has a different R_f value to the M_1 hemiacetal. Like the M_1 hemiacetal, the M_1 derivative formed on the TLC plate has strong fluorescent properties, as well as a specific lower R_f value than M_1, and use is made of the latter characteristic in the confirmatory test. After the reaction, the plate is developed once more, the M_1 derivative from the sample and the standard are examined to determine if they have moved the same distance and show the same colour of fluorescence. The result of this test can be seen in Fig. 7.

Methods based on quite different principles from the chromatographic techniques are the immuno-assays. The immuno-assays are still in an early stage of application for mycotoxin research. Some publications about Enzyme Linked Immuno Sorbent Assay (ELISA) and Radio Immuno Assay (RIA) have appeared (Pestka *et al.*, 1981). Although promising, the immuno-assays are not yet able to compete fully with the chromatographic procedures.

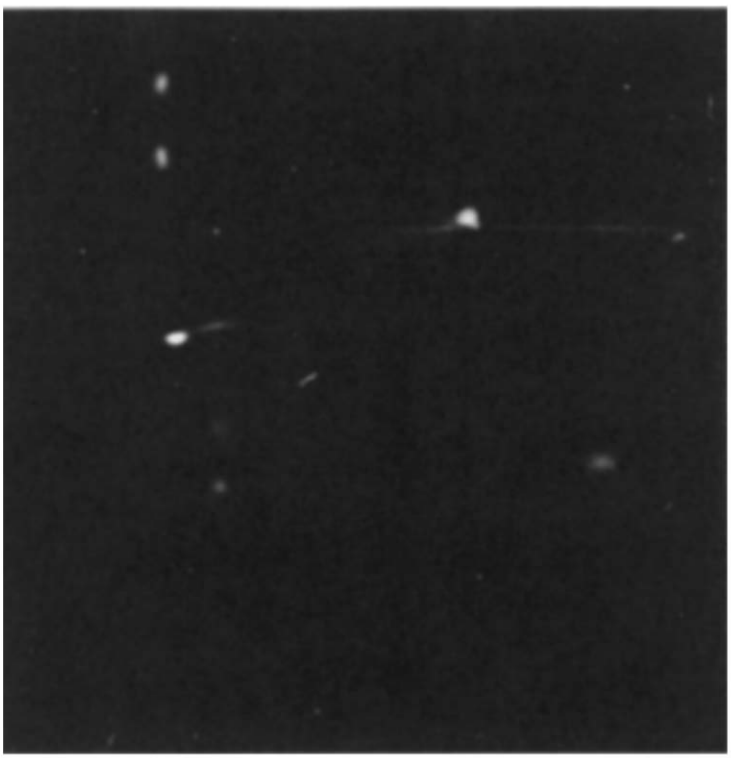


Fig. 7. Result of a confirmatory test applied to an extract of milk containing aflatoxin M_1.

OCCURRENCE OF AFLATOXIN M_1

The availability of analytical methods has enabled the occurrence of aflatoxin M_1 in dairy products to be investigated. In several countries within the European Community milk is monitored routinely for aflatoxin M_1. As an example, the results of a survey of 105 samples of milk, collected in the Netherlands in the winter period of 1981 (van Egmond *et al.*, 1982*a*) are presented in Fig. 8, as a histogram, with class intervals of 0·03 μg/litre. In 84 of 105 samples, aflatoxin M_1 could be detected in levels ranging from 0·015 to 0·09 μg/litre. The mean M_1 content in raw, as well as in heat-treated, milk, was 0·03 μg/litre, the median M_1 content for both types of milk was also 0·03 μg/litre.

The results of a similar survey, carried out in 1972, are presented in

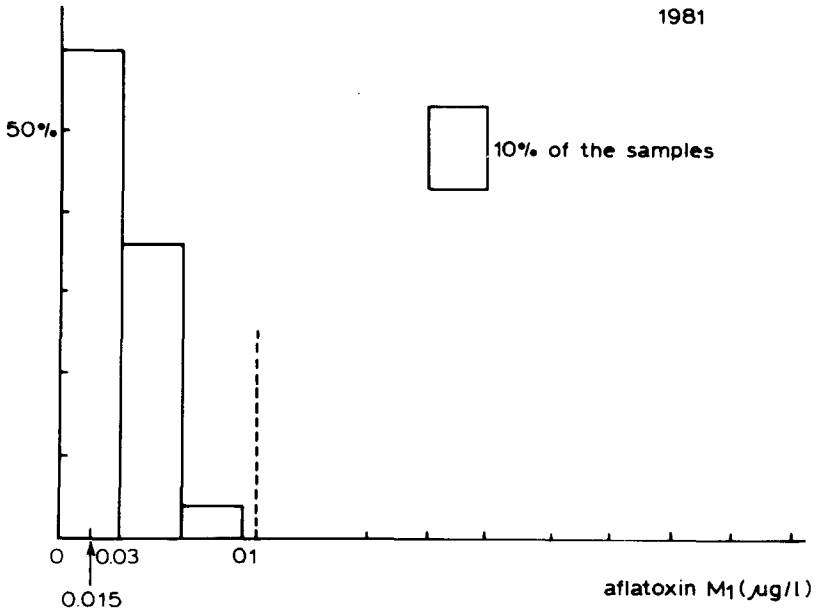


Fig. 8. Frequency distribution of M_1 concentrations in samples of Dutch consumption milk (1981).

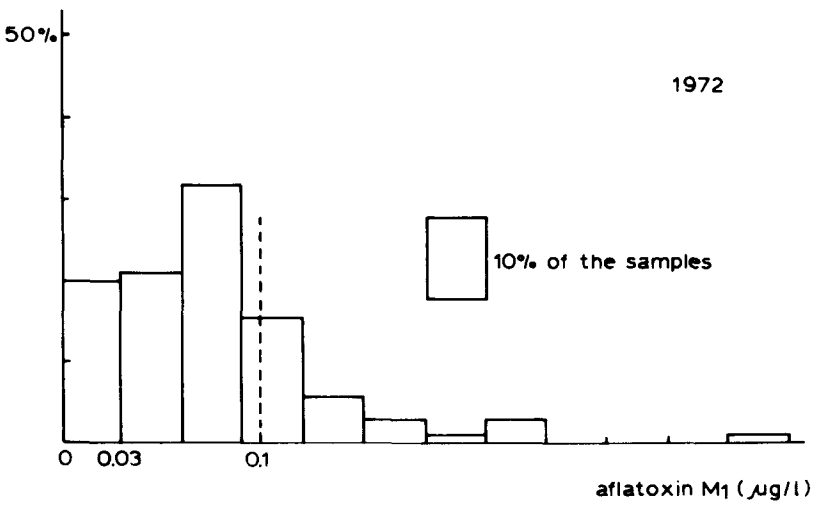


Fig. 9. Frequency distribution of M_1 concentrations in samples of Dutch consumption milk (1972).

Fig. 9. In 1972 *ca.* 18% of the samples had an M_1 content of 0·1 μg/litre, the Dutch action level; in 1981 none of the samples had an M_1 content exceeding this level. This significant decrease in the M_1 contamination is probably the effect of the EEC Directive (Anon., 1974) on permitted levels for undesirable substances and products in feedingstuffs, which came into force in 1976, a year in-between the two surveys. In this directive, a maximum concentration of 20 μg aflatoxin B_1/kg supplementary feedingstuff for dairy cattle is tolerated. Recent data from France, another EEC member state, showed that comparable M_1 levels were found in French retail milk (Fremy *et al.*, 1982). In countries where feedingstuff regulations are more stringent, i.e. in Switzerland, lower M_1 levels occur in the milk.

EFFECTS OF PROCESSING ON AFLATOXIN M_1

The frequent occurrence of aflatoxin M_1 in milk has raised the question: what happens if such contaminated milk is processed in the normal manner by the dairy industry? There have been several investigations into this subject, one of which was carried out in our Institute in close co-operation with the Netherlands Institute for Dairy Research (van Egmond *et al.*, 1977). In that study, milk, naturally contaminated with M_1, was either pasteurized or sterilized, and yogurt and cheese were prepared. The results of the study are presented in Fig. 10. In that Figure the length of the bars represents the M_1 content found. The aflatoxin M_1 content of milk is not reduced by heat treatments like pasteurization and sterilization, nor was any reduction noticed in the M_1 content during the preparation of yogurt and cheese. It is of interest to note that the M_1 concentration in cheese was about four times higher than in the milk from which it was prepared. Accordingly, the M_1 concentration in the whey was proportionally lower. Furthermore, the various yogurt-cultures and cheese-starters did not influence the M_1 content.

More recent studies were carried out by Brackett & Marth (1982) who studied the fate of M_1 throughout the ageing period of Cheddar cheese, and noticed a concentration effect of M_1 to a much greater extent than we found with our Gouda cheese. Brackett and Marth mentioned several possible reasons to explain the effect. One is that proteolysis and lipolysis, which occur during the ageing process, could result in changes within the cheese, which allow recovery of more or less toxin in the

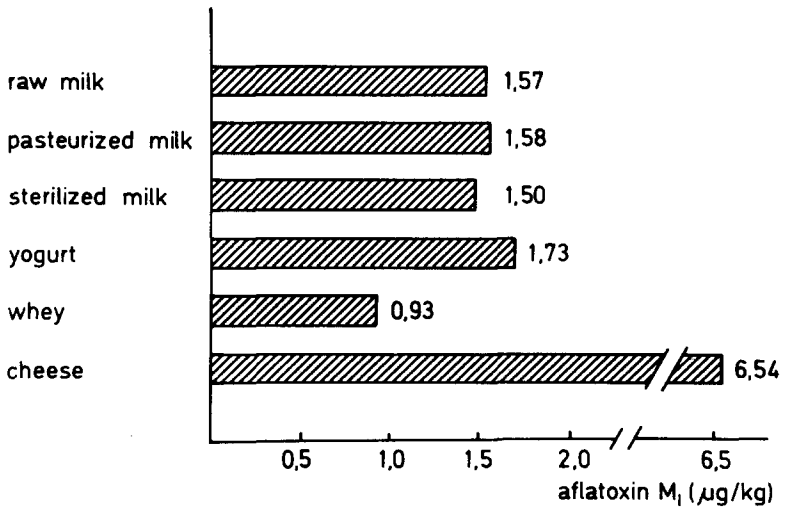


Fig. 10. The effect of processing on the aflatoxin M_1 content of milk and milk products.

analysis. If aflatoxin M_1 is bound to casein, as is suggested, cleaving of proteins or an increase in free fatty acids could act to change this binding. It can be assumed that aflatoxin M_1 is not covalently bound, since it can be extracted. One way M_1 could be bound is by hydrophobic interactions with hydrophobic areas of the caseins. If this is true, one could explain how binding might be affected by the ageing or processing of Cheddar cheese. Proteolysis could cleave proteins in a way that allows less hydrophobic areas of the casein micelle to exist. Lipolysis results in the release of fatty acids which might, in turn, exert a detergent effect on the hydrophobically bound M_1. This might allow the toxin to be more readily retrieved by extraction.

Although there are differences in the results of the various studies on the effect of processing on M_1, they tend to come to the same conclusion: processing of milk does not destroy aflatoxin M_1, an observation which is of considerable practical importance. It gives rise to the question: what possibilities, then, exist to eliminate M_1 from milk?

ELIMINATION OF AFLATOXIN M_1

Applebaum *et al.* (1982*a*, 1982*b*) have investigated several possibilities to eliminate or inactivate M_1 in milk. One method involves chemical

treatment with potassium sulphite; another used hydrogen peroxide in combination with riboflavin and a third method uses bentonite to adsorb the toxin. Although M_1 seems to be reduced by 45–98 %, these methods are not really applicable for the dairy industry, at least not at this moment, since as yet nothing is known about the biological safety and the nutritional and functional properties of the treated products; moreover, the costs of the processes may be considerable.

If the destruction of aflatoxin M_1 in milk cannot be readily achieved, then M_1 can only be excluded from milk by prevention or elimination of B_1 from the feed. So, attention should be directed to the prevention of aflatoxin formation in agricultural commodities, which is fundamentally a problem of good agricultural practice and the curtailment of fungal growth in storage. Mould contamination can occur during development, harvest, storage, shipment and processing. If measures to prevent mould growth and aflatoxin formation are not taken or fail, one can resort to detoxification. Several techniques have been proposed for detoxifying commodities containing aflatoxin. One of the techniques, the ammoniation technique, is used in practice already. Research has shown (Lee *et al.*, 1974) that treatment of aflatoxin B_1 with ammonia opens the lactone-ring of the molecule and the ammonium salt of the resulting hydroxyacid is formed (see Fig. 11). In order to avoid reclosure of the lactone ring, the reaction should be conducted at an elevated temperature and pressure,

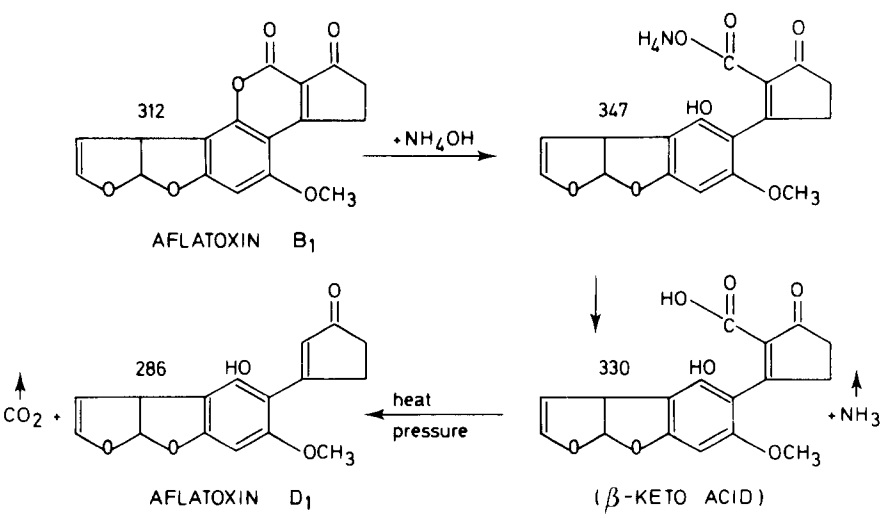


Fig. 11. Decomposition of aflatoxin B_1 by treatment with ammonia (Lee *et al.*, 1974).

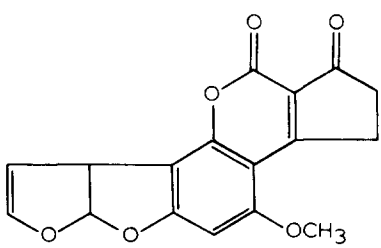
causing the decarboxylation of the β-keto acid to the so-called aflatoxin D_1. Although this reaction occurs in a test tube, in reality the situation is more complicated. Aflatoxin B_1 binds irreversibly to substrates when treated with weak bases and only a small amount of aflatoxin D_1 is formed under those conditions. This phenomenon has been noted for corn (Beckwith *et al.*, 1975). In a variant of the procedure, developed in the USA for the decontamination of cottonseed (Price *et al.*, 1982), elevated temperature and pressure are not applied; however, no reclosure of the lactone ring occurs. Perhaps here also the opened B_1-molecule is irreversibly bound to matrix-substances, as is the case with corn. In practice it has been shown that 95–98% of the aflatoxin present in feedstuffs is decomposed. The ammoniation procedure is already used for corn, peanutmeal and cottonseed. In the Netherlands, decontaminated peanutmeal from Senegal is offered for sale at an attractive price.

OTHER MYCOTOXINS IN DAIRY PRODUCTS

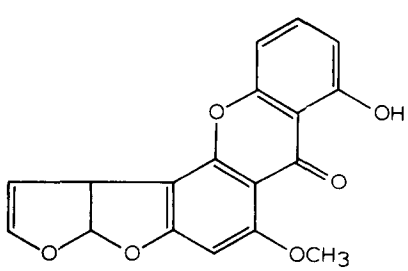
Although there have been studies into the carry-over of some mycotoxins other than aflatoxins; for instance, ochratoxin A, zearalenone (Shreeve *et al.*, 1979), T-2 toxin (Robinson *et al.*, 1979) and sterigmatocystin (Kraus, 1978), they are rare, and the outcome of these studies does not present a reasonable cause for concern. Another threat, however, is the contamination of dairy products by mycotoxins, caused by direct fungal contamination.

Among the dairy products, cheese is the only product really susceptible to fungal growth (although products like milk powder may become mouldy if stored under exceptionally bad conditions and, in that case, mycotoxins may be formed). Cheese is a product which can become mouldy intentionally or accidentally. Moulds that are intentionally present on cheese, such as the *Penicillium* species on the French Camembert and Roquefort cheeses, may produce mycophenolic acid, penicillic acid and roquefortine while colonizing the cheeses. However, these compounds should not be classified as either extremely toxic or carcinogenic, since there are no data avilable to suggest carcinogenic properties. Since the amounts in which these metabolites may occur in cheese are not alarming, it may be concluded that the mould-ripened cheeses do not present a threat to human health, at least not as far as mycotoxins are involved.

Another matter is the accidental occurrence of moulds on cheeses. Cheese can easily become mouldy during the ripening process in warehouses, and after cutting and slicing during storage in shops or at home. Northolt *et al.* (1980) found that various toxin-producing *Aspergilli* and *Penicillia* could be isolated from cheeses ripening in warehouses. Fortunately, the aflatoxin producers, *Aspergillus flavus* and *A. parasiticus*, were seldom found, which confirmed the findings of others. However. *Aspergillus versicolor*, a species known to be capable of producing sterigmatocystin, seemed to be present frequently. Sterigmatocystin has a chemical structure somewhat resembling that of aflatoxin B_1 (see Fig. 12). Aflatoxin B_1 contains a coumarin ring, sterigmatocystin has a xanthone ring fused to the furan system. Like aflatoxin B_1, sterigmatocystin has carcinogenic properties, although less extreme (Anon., 1976). The presence on cheese of *A. versicolor* required the development of a method of analysis for sterigmatocystin to enable the occurrence of sterigmatocystin in Dutch hard cheese to be



Aflatoxin B_1 $C_{17}H_{12}O_6$



Sterigmatocystin $C_{18}H_{12}O_6$

Fig. 12. Chemical structures of aflatoxin B_1 and sterigmatocystin.

determined. A procedure was developed in which two-dimensional thin layer chromatography (see Fig. 13) is applied to achieve a separation on the plate which enables the analyst to detect concentrations of sterigmatocystin in cheese as low as 5 μg/kg (van Egmond *et al.*, 1980).

The availability of the new method allowed Northolt *et al.* (1980) to conduct a survey of ripening cheeses from warehouses. Of those cheeses

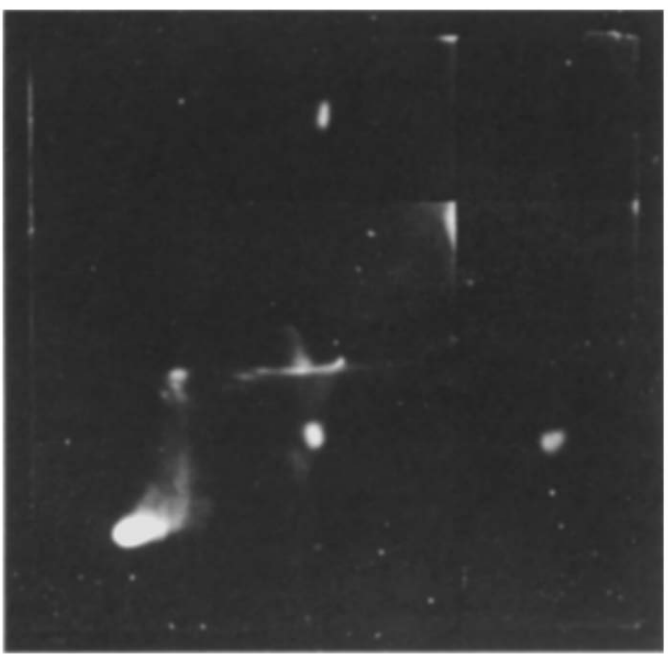


Fig. 13. Two-dimensionally developed thin layer chromatogram of cheese extracts contaminated with sterigmatocystin.

which were found to be mouldy with *Aspergillus versicolor*, a part of the outer 1 cm thick layer was analyzed for sterigmatocystin. From a total of 39 cheeses with ages varying from less than 2 months to more than 7 months, nine were found positive for sterigmatocystin. The positive samples originated from all categories, the levels found in the 1 cm thick outer layer ranged from 5 to 600 μg/kg.

The fact that sterigmatocystin was found in commercial cheeses led to questions about the migration and the stability of this toxin in cheese, once it had been formed. Consequently, the distribution of sterigmatocystin over the outer layer of six naturally contaminated Gouda

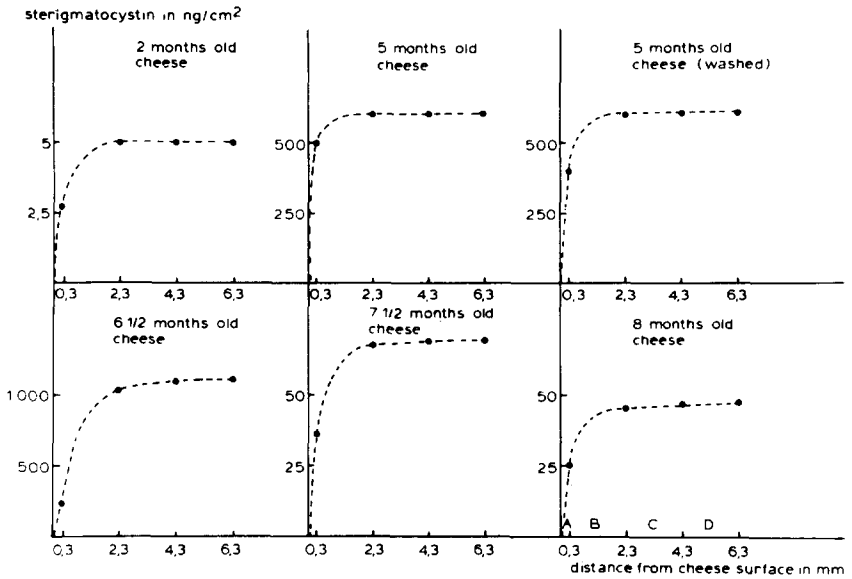


Fig. 14. Distribution of sterigmatocystin in rind and cheese layers of some cheeses of various age. N.B. The amount of sterigmatocystin is expressed in different scales.

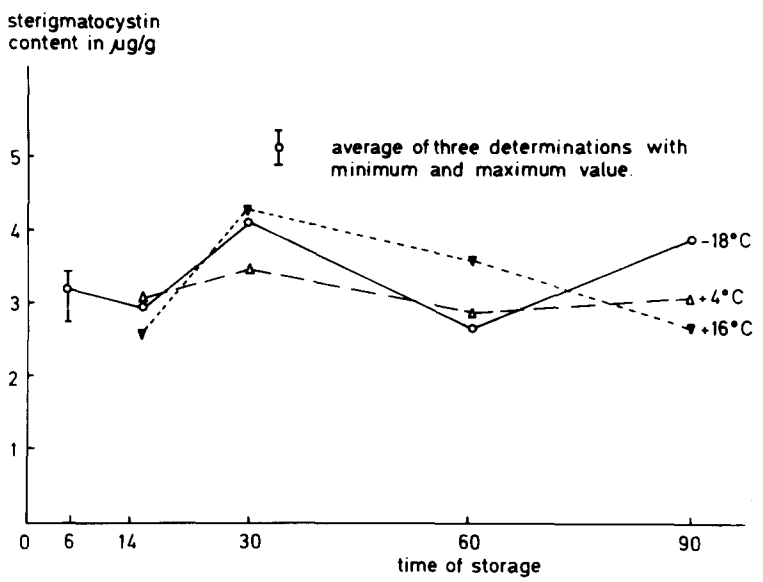


Fig. 15. Stability of sterigmatocystin in cheese during 90 days of storage at different temperatures.

cheeses, varying in age from 2 to 8 months, was investigated (van Egmond, 1982*b*). It appeared that the concentration of sterigmatocystin decreased rapidly from the outside to the inside. After 6 mm no sterigmatocystin could be detected, as is illustrated in Fig. 14. In addition, the stability of sterigmatocystin in cheese was followed over a period of 3 months at freezer, refrigerator and warehouse temperatures (van Egmond, 1982*b*). Figure 15 clearly illustrates that sterigmatocystin in cheese is stable for 3 months at various temperatures.

The cause of contamination of cheeses with *A. versicolor* and factors influencing fungal growth and sterigmatocystin production are currently being investigated in order to enable the formulation of preventive measures. The first results of these studies, however, have shown that the matter is very complex and unpredictable, so that research into this problem is continuing.

CONCLUSION

This introduction to mycotoxins in dairy products has been far from complete. Because of the restrictions in the scope of this paper, attention has been focused mainly on two mycotoxins, aflatoxin M_1 and sterigmatocystin. M_1 represents the group of mycotoxins entering the dairy product indirectly; sterigmatocystin is an example of a mycotoxin resulting directly from fungal contamination of the dairy product. Both mycotoxins are more or less suspected carcinogens and therefore deserve special attention. Although many aspects about the behaviour and significance of mycotoxins in dairy products are yet unknown or unclear, the concerted efforts in the recent past have made it possible to detect them in very low amounts. These developments offer a good starting position for further research and routine investigations on mycotoxins in dairy products.

ACKNOWLEDGEMENTS

The author thanks Mr A. E. Buckle, Agricultural Development and Advisory Service, Ministry of Agriculture, Fisheries and Food, Derby, Great Britain; Professor C. J. Mirocha, Department of Plant Pathology, University of Minnesota, St. Paul, USA and Dr P. L. Schuller,

Laboratory for Chemical Analysis of Foodstuffs, National Institute of Public Health, Bilthoven, The Netherlands, for their criticism and helpful suggestions during preparation of the manuscript.

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Haemagglutinins (Lectins) in Beans

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(Received: 25 January, 1983)

ABSTRACT

Many toxins, together with some indigestible sugars, occur in legumes. The toxins vicine and convicine are amongst those which may cause an acute haemolytic anaemia, whilst a neurotoxin can cause spastic paralysis of the lower limbs. Adequate cooking mostly destroys such toxins; lectins are amongst the responsible toxins when the legumes are not properly cooked. Toxins are assessed in haemagglutinin units using erythrocytes from certain animals. In vivo *and* in vitro *toxicities have been measured and the effects of heat on lectins determined. The importance of these findings is enhanced by the spread of western food technology to communities eating a high proportion of legumes compared with the western world.*

INTRODUCTION

Despite their content of toxins, legumes have been consumed for thousands of years. Over 4000 years ago Esau sold his birthright for bread and a potage of lentils, and the first recorded assay of protein quality is in the first chapter of the Book of Daniel where a diet of lentils was found to be as good, judged clinically, as 'king's meat'.

Legumes botanically include the entire family of Leguminosae, but the Food and Agriculture Organisation excludes groundnuts and soybeans. Gastronomically we tend to class groundnuts as nuts, and to deal

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separately with soybeans which are grown mainly as a source of oil and of protein for animal feed. That leaves the common food terms such as peas, beans, lentils, grams and dhals. There are legumes eaten in the wet form (65–90% water) such as fresh garden peas (*Pisum sativum*) and beans consumed fresh in the pod such as French beans (*Phaseolus vulgaris*) and those eaten after drying (10–13% water) such as dried peas (*Pisum sativum*), chick peas or Bengal gram (*Cicer arietinum*), haricot beans (*Ph. vulgaris*), and lentils (*Lens culinaris*) which store well and are the common legumes of commerce.

There seems to be no scientific distinction between peas and beans and in the textbook phrase 'peas, beans and pulses', the word pulse refers to the dried form. This is a Hindi word and there is no equivalent in other languages; in French the term is simply *legume sèc*.

A startling number of toxins are found in legumes: (1) several trypsin inhibitors; (2) a number of amylase inhibitors; (3) cyanogenetic glycosides; (4) haemagglutinins or lectins in a variety of forms; (5) polyphenols and (6) alkaloids (Jaffé, 1980). In addition, there are indigestible sugars including stachyose, raffinose and verbascose.

The broad bean, *Vicia faba*, contains toxins which affect only certain individuals. Favism is an acute haemolytic anaemia induced in genetically sensitive people who lack the enzyme glucose-6-phosphate dehydrogenase in their red blood cells. The toxins responsible include vicine and convicine, and the defect is said to affect some 100 million people, mainly in the Mediterranean and Middle East countries, being rare or virtually absent in Northern Europe. (A possible problem loomed when textured vegetable protein (TVP) was prepared from field bean, *Vicia faba*, in Great Britain in the middle 1970's, since there are now many such Mediterranean peoples living in this country. However, the unreliability of this crop in Great Britian led back to the use of soya for TVP.)

One other specific problem is lathyrism, a spastic paralysis of the lower limbs caused by a neurotoxin in the Kesari dhal, *Lathyrus sativus*. Normally it is eaten only in small amounts when it grows together with the wheat crop but, since it is extremely resistant to drought, it can predominate under such adverse weather conditions. Recently, there has been a problem in parts of India where the crop has been intentionally, although illegally, grown because of its high yield.

Despite all these toxins there are few problems apart from those specific ones mentioned, probably because they are mostly destroyed by adequate cooking. However, a new problem has arisen in Great Britain in recent

years due to the consumption of raw or incompletely cooked beans. This may well be an unrecognised problem elsewhere.

The first outbreak of food poisoning was reported by the Public Health Laboratory Service in 1976 (*British Medical Journal*, 1976). Eight schoolboys and an adult ate raw kidney beans, *Phaseolus vulgaris*, which had been soaked in water for a day. All became acutely nauseated between 1 and $1\frac{1}{2}$ h later and began to vomit, followed by diarrhoea. None of the usual bacterial pathogens was isolated from the faeces or vomit, nor could any bacterial or mould contamination be identified in an unopened packet of beans; no arsenic, mercury, lead or cyanide was detected, and the authors concluded that the effect must have been due to a natural toxin.

This report suggested to us that the responsible toxins were likely to be lectins, and subsequent investigation proved this to be correct. Seven outbreaks involving 43 persons were linked to haemagglutinins present in uncooked beans (Noah *et al.*, 1980).

Subsequently, 25 outbreaks involving 100 persons have been reported but, as described below, information volunteered by members of the public suggests a total reaching 355 outbreaks involving 870 persons.

The raw beans are hard and brittle but become soft enough to eat when soaked for a few hours. As described later, incompletely cooked beans appear to have been the cause of more outbreaks than raw beans.

Stillmark (1889) was the first to describe a phytohaemagglutinin, namely ricin, in the castor bean and to show that it was capable of agglutinating human and animal erythrocytes. He reported 112 cases of accidental poisoning due to the ingestion of the castor bean or its products and showed that the toxin was destroyed by heat. Subsequent reports include toxic effects of runner beans (Faschingbauer & Kofler, 1929).

In 1948 the population of Berlin was supplied with 'flaked beans' which led to a severe outbreak of food poisoning. This was apparently due to incomplete processing, since the toxin could be destroyed by further cooking (Griebel, 1950).

METHODS

Samples of beans

Locally purchased beans were soaked in three parts of tap water for 16–18 h at room temperature. If they were to be cooked, the soaking water

was discarded and the beans cooked in fresh water for 30 min at 100 °C in an open pan. Cooking times were measured from the time of addition of beans to boiling water.

Estimation of haemagglutinin activity

Standard trypsinated rabbit erythrocytes were prepared according to the method of Liener (1955). Samples of dried, powdered beans were extracted with 0·9 % saline and the extracts diluted twofold serially from 1 to 512 in a series of ten test tubes. Two millilitres of standard trypsinated red blood cell suspension were added to each tube which was left at room temperature for 2 h. As suggested by Marquardt *et al.* (1975), the cells were resuspended for 10 min prior to reading.

One haemagglutinin unit (HU) is arbitrarily defined as the level of test solution which causes 50 % of the standard suspension of red cells to sediment, as noted by 50 % decrease in the absorption at 620 nm measured in a spectrophotometer CE 303 (Cecil Instruments).

The procedure involves twofold dilution stages and an error of 0·02 in the measured absorbance can result in an error of one dilution stage, so assay values can be in error by up to about 30 %.

Erythrocytes from rabbit, rat, cow and man may be used in this type of assay and they vary in their reaction to the lectins, so serving to differentiate them, but it is not established how these relate to the toxicity of the original beans.

Animals and diets

Rats of the Hooded Lister strain of the Queen Elizabeth Colony were used at 28 days of age. The basic diet was composed of corn starch, arachis oil, a complete vitamin and mineral mixture and solka floc as a source of fibre; the beans were added at the expense of the starch.

RESULT AND DISCUSSION

Lectin content of beans

Of nine types of legume tested only the three varieties of *Phaseolus vulgaris*—the red and white kidney beans and the rose coco bean—

contained large amounts of lectins relative to the amounts found in the other types examined (see Table 1).

Soaking overnight (16–18 h) removed varying amounts of lectins so that it would not be possible to estimate the amount left in beans eaten raw if they had been soaked first. The garden pea, for example, lost 65 % of its lectin content, while the three samples of red kidney beans lost between 20 % and 70 %.

TABLE 1
Lectin Content of a Number of Legumes (Figures rounded off)

Name	*HU* per gram (dry weight)*	*Removed after soaking in water 18 h (%)*
Red lentils (*Lens esculenta*)	7 700	22
Green lentils (*Lens esculenta*)	1 800	19
Garden peas (*Pisum sativum*)	5 100	65
Yellow split peas (*Lens culinaris*)	1 600	11
Green split peas (*Lens culinaris*)	1 000	11
Black eye beans (*Vigna sinensis*)	1 000	100
Red kidney beans (*Phaseolus vulgaris*):		
Sample 1	53 000	66
Sample 2	44 000	29
Sample 3	37 000	22
White kidney beans (*Phaseolus vulgaris*):		
Sample 1	43 500	36
Sample 2	17 000	18
Rose coco beans (*Phaseolus vulgaris*)	39 000	18

* HU = Haemagglutinin unit.

In vivo toxicity

Precipitation of red blood cells, as observed *in vitro*, cannot explain the *in vivo* effects, since red cell agglutination would be immediately fatal. Consequently, it is not certain that the *in vitro* test is measuring toxicity. The likelihood that this is so was indicated by analysis of one sample which had been found to cause vomiting and diarrhoea in fifteen persons.

This sample had been eaten raw (after soaking) and was subsequently stored at a low temperature for about 3 weeks. After that time it was tested for agglutination and found to contain 19 000 HU/g.

Further evidence was sought by feeding both red and white kidney beans to experimental animals. Diets containing different amounts of raw beans, ranging from 10 to 80% (2·5 to 20% protein), were fed to rats for 14 days. Table 2 shows that, at the two higher levels of 80% and 60% beans, most of the rats died within 7 days on both red and white kidney beans. All died within 14 days at levels of 20% (5% bean protein) and above; only those fed 10% beans survived, even though this was a diet supplying only 2·5% protein.

TABLE 2
Toxic Effect of Raw Kidney Beans (Three rats per group)

Experiment A—Differing protein levels

Diet		*Mortality*			
Content of raw beans (g/kg)	*Protein level (g/kg)*	*White beans*		*Red beans*	
		7 days	*14 days*	*7 days*	*14 days*
800	200	3	—	3	—
600	150	3	—	1	3
400	100	1	3	2	3
200	50	1	3	0	3
100	25	0	0	0	0

Experiment B—Constant protein level 200 g/kg (Five rats per group)

Diet (g/kg)		*White beans*		*Red beans*	
Raw beans	*Cooked beans*	*Total weight change (g)*	*Mortality*	*Total weight change (g)*	*Mortality*
150	50	—	5	—	5
100	100	—	5	—	5
50	150	—	5	—	5
25	175	+18·3	0	+17·0	0
0	200	+50·0	0	+56·4	0

When the animals were fed isonitrogenous diets at 20% protein, using cooked beans to make up the protein level, again all the rats died when the diet included 5% protein from raw beans, red and white (20% raw beans in diet) and survived and gained weight on 2·5% raw bean protein. This weight gain was less than on cooked beans alone, indicating some toxicity at this low dietary level. Pusztai *et al.* (1975) showed that the addition of raw beans to a diet containing 5% casein depressed appetite, growth and protein quality.

Heat destruction of lectins

Three samples of red beans, two samples of white and one of rose coco beans were added to boiling water and boiled for 2, 5, 10 or 20 min.

Table 3 shows that the amounts and stability of the lectins varied. In one sample of red beans all the agglutinating power was destroyed after 2 min, while in another sample only 62 % was destroyed after 10 min and a small amount remained after 20 min. The lectins in the two samples of white beans differed slightly in stability—the agglutinating power was destroyed after 2 min in one sample, but a small amount remained after 5 min in the second sample.

TABLE 3
Haemagglutinin Content (units per gram)

Kidney beans	*Raw HU/g sample*	*Soaked % HU removed*	*Cooking 100°C*			
			2 min	*5 min*	*10 min*	*20 min*
			% HU destroyed			
Red (1)	53 000	66	—	—	62	93
Red (2)	44 200	29	93	97	100	100
Red (3)	36 700	22	100	100	100	100
White (1)	43 500	36	100	100	100	100
White (2)	17 000	18	89	93	100	100
Rose Coco	39 400	18	—	—	—	100

Inadequate cooking

One case of food poisoning was reported in five persons following the consumption of red beans which had been cooked in what is termed a slow cooker for a period of 5 h. There was no precise evidence of the temperature, but the device is said to cook at about 80 °C. Since complete destruction of the toxins could be affected in a few minutes at 100 °C, it was surprising to find that the beans could still be toxic after 5 h even at 80 °C. If the rate of chemical reactions doubles for each 10 °C rise in temperature, then 10 min at 100 °C is equivalent to about 40 min at 80 °C, or 80 min if the temperature had been at 70 °C.

This observation suggested the possibility that there was a temperature threshold for destruction of the toxin, which was examined by heating

samples of beans at 70, 80, 90 and 100 °C, and measuring agglutinating properties.

The results are shown in Fig. 1 and Table 4. They are replicated three times on the sample shown in Fig. 1, and repeated on four additional samples of red beans, as shown in Table 4.

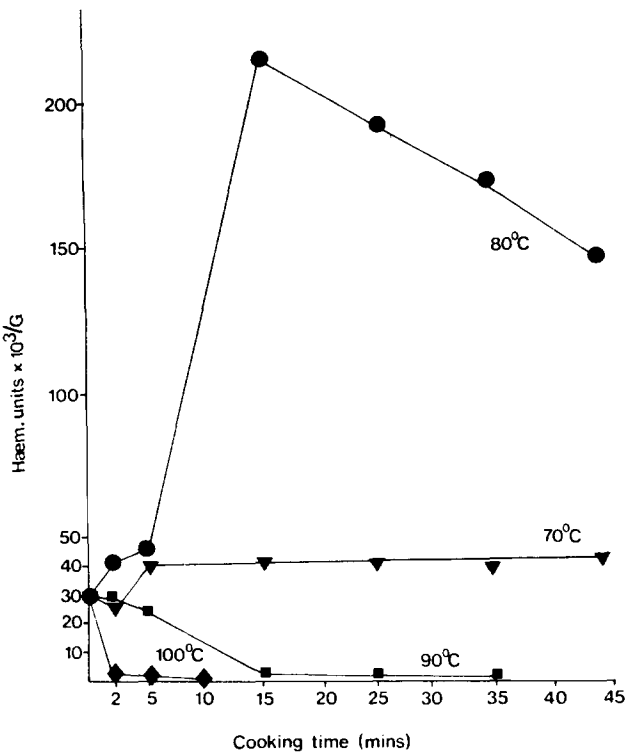

Fig. 1. Change in lectin content at different temperatures.

The expectation of a slower rate of destruction of lectins at 90 °C was verified. At 80 °C there was a marked increase in agglutinating power, reaching a level seven times as high as in the raw beans after 15 min, then falling slowly.

At 70 °C there was a 50 % increase in agglutinating power which was unchanged by continuing heating at 70 °C for the remainder of the 45-min period of the experiment.

When publicity about the toxicity of uncooked beans was broadcast in

a series of television programmes (BBC, 1981) 355 letters were received reporting illness in 870 persons.

Of the outbreaks reported, 93 % had occurred in the home, one-third from beans either consumed raw with a salad (presumably after soaking to make them soft enough to eat) or the individual had eaten a 'few' beans, number unspecified, after soaking and before cooking them. The smallest number of uncooked beans reported to cause sickness was four.

TABLE 4

Haemagglutinin Content of Five Samples of Red Kidney Beans (*Ph. vulgaris*) Raw and Heated for 20 min at 70 °C or 80 °C.
(No activity was left after this time when heated at 100 °C)

Sample number	*Raw (unsoaked)*	*Raw (soaked)*	*70 °C*	*80 °C*
1	28 000	—	35 000	215 000
2	14 000	11 000	18 000	19 000
3	17 500	14 000	35 000	35 000
4	44 000	28 000	45 000	68 000
5	70 500	55 000	41 500	102 500

The remaining two-thirds of the outbreaks followed consumption of mixed dishes that were variously reported as having been simmered or cooked in stews or casseroles; half of these mentioned the use of 'slow cookers'.

It is clear that a large cooking vessel heated in an oven will not reach the temperature of the oven for many hours so that it is likely that many of these beans had suffered an *increase* in toxicity through being heated at a temperature of possibly 70–80 °C.

The 24 outbreaks from restaurant and refectory meals were mostly described as *chili con carne*, again presumably underheated or eaten raw with salads.

It has been suggested (Etzler, 1979; Jaffé, 1980) that ingested lectins cause their ill-effects by combining with the carbohydrate moiety of substances present in the cells lining the surface of the small intestine, and cause non-specific interference with absorption. It is possible that the increase in toxicity on heating at 80 °C may be due to the breakdown of a large molecule into a number of smaller molecules which are also toxic.

GENERAL COMMENT

About 800 out of 2660 plant species examined contain agglutinating activity (Allen & Brilliantine, 1969).

Jaffé (1980) quotes more than 200 *Phaeolus* species that have been examined. In the present work, red and green lentils, garden peas, green and yellow split peas and black eye beans were found to have relatively small amounts of lectins, less than 5000 units per gram compared with 40 000 to 50 000 in the three types of *Phaseolus vulgaris* examined.

Most legumes that have been found to agglutinate red blood cells contain a number of lectins which can be classified according to their specificity in agglutinating blood from different animal species—rat, rabbit, cow and man (Jaffé *et al.*, 1974). It is not clear which of the different types of lectins may be toxic to man on ingestion, and in the present work only rabbit erythrocytes were used to detect their presence.

Of the legumes, possibly the garden pea, *Pisum sativum*, is the one most commonly eaten raw in Great Britain and, so far as is known, no problems have arisen. The other types of legumes which may be eaten raw are the mung bean and the alfalfa bean which are sprouted, and again there does not appear to be any problem in their case. The problem so far appears to be limited to the beans with some 50 000 HU/g. However, since as few as 'four or five' beans have been reported to cause nausea and vomiting in some individuals, there may be differing *in vivo* toxicities not revealed by the *in vitro* assay.

One variety of *Phaseolus vulgaris*, Dwarf French beans, was assayed for lectin content in the immature wet state and found to contain less than 1000 HU/g. When left to mature on the plant until the pods dried and shrivelled, and the beans hardened, they were found to contain similar amounts to those reported here for the purchased dried beans, so it appears that the toxin is formed during the final maturation period and that there is no harm from consuming raw, wet beans in the pod.

Pusztai *et al.* (1979) have shown the great variety in amounts and types of lectins that can be found in thirteen cultivars of kidney beans, and the present work shows their variable stability to heat.

Infant Feeding

Korte (1972) found residual lectins in (incompletely) cooked meals composed of mixtures of maize and beans in a Tanzanian village, and this

clearly indicates that care must be taken in the preparation, in particular of infant foods prepared from beans. When cooked at temperatures below 93 °C, the food caused diarrhoea. Szperl-Seyfried (1951) found that heating an extract of beans at 75 °C for 1 h did not destroy its toxicity.

The spread of western food technology to other communities could create a problem, since much of this technology leans towards 'instant' mixes composed of uncooked or partially cooked powders which are simply mixed with hot water and 'do not require cooking'. We have found (unpublished) that roller-drying of a wet mixture of cowpeas, milk powder and rice flour did not destroy trypsin inhibitors, so it is not likely that roller-drying provides adequate heat treatment for the complete destruction of lectins.

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Food Allergies Associated with Cereal Products

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ABSTRACT

Intolerance reactions to cereal proteins have been frequently reported in patients with allergic and gastrointestinal disease, although not all the reactions may be mediated by an immunological mechanism (e.g. coeliac disease). The isolation and chemical identification of allergens and 'toxic fractions' from cereal proteins is reviewed along with the current views on the mechanism of coeliac disease.

INTRODUCTION

Cereal grains may be regarded as the seeds of domesticated grasses, and they have formed the basis of the human diet since the birth of civilization. In the United Kingdom, they represent approximately 25% of the daily protein intake (National Food Survey Committee, 1980) which is derived mainly from bread and bakery products. As with other common food protein sources such as cow's milk and eggs, a small number of people ingesting cereal proteins may develop adverse

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reactions. In this review we shall examine the evidence for allergic reactions to ingested cereal proteins and the nature of the allergenic components. We shall compare the immunological response obtained in food allergy to that obtained in coeliac disease (wheat gluten-induced enteropathy) and those conditions which result from occupational exposure to cereal grain products.

CEREAL PROTEIN CHEMISTRY

The bulk of the cereal grain consists of the endosperm which is composed of starch (70–75%) and proteins (7–15%), as well as lipids and other minor components. There have been several reviews and reports relating classification of cereal protein to their functionality (e.g. Kent, 1969; Shukla, 1975; Daniels & Frazier, 1977). In these reports, the emphasis is very largely on wheat, reflecting its importance as a cereal food and particularly in breadmaking.

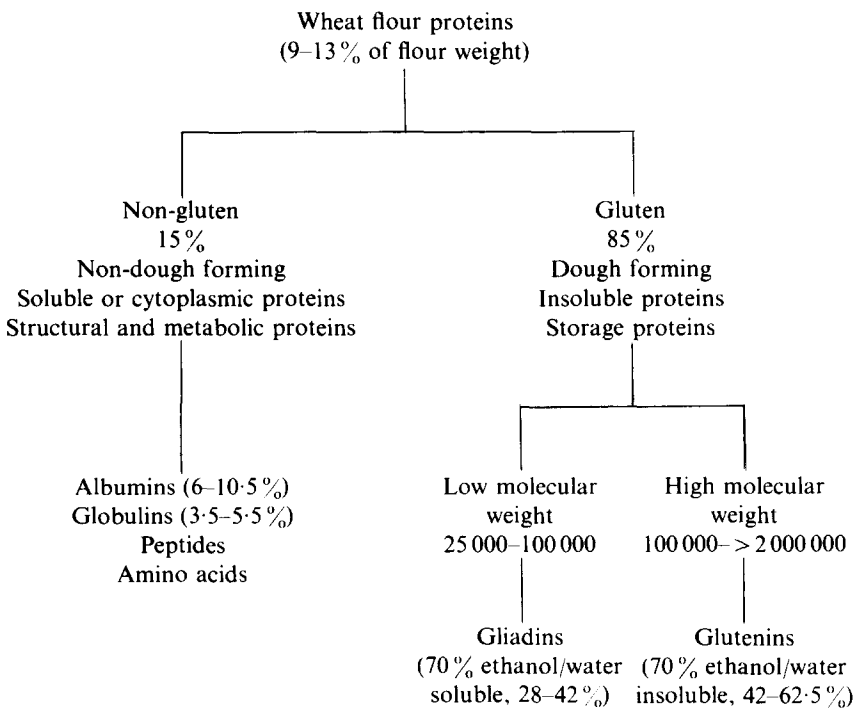

Fig. 1. Osborne classification of wheat proteins.

The classification of cereal proteins is made in terms of either biological function or solubility criteria. In terms of biological function, we can define structural proteins which help to hold the component parts of the cell wall together, metabolic proteins such as enzymes which, at germination, break down lipids and carbohydrates, and the storage proteins which act as a nitrogen supply for the developing embryo.

Modern methods of fractionation and classification of cereal proteins are based on the early work of Osborne (1907). This classification (Fig. 1) is still widely used today and is based on solubility criteria in water and alcohol. It has formed the basis of many fractionations to identify the antigenic and allergenic components of cereals in allergic and gastrointestinal disease.

FOOD ALLERGY RESULTING FROM INGESTION OF CEREAL PROTEINS

There is still considerable controversy as to the importance and incidence of food allergy, particularly in atopic dermatitis, eczema, and bronchial asthma (Aas, 1978; Soothill, 1979; May, 1980; Taylor, 1980). The clinical features of food allergy may be observed in the gastrointestinal tract, skin, respiratory system or other organs, and symptoms may occur immediately or many hours after ingestion of the offending food. Although food allergy has normally been associated with children, there is increasing evidence that adults may also show adverse reactions. The exact mechanism of food allergy remains uncertain and, because of the considerable variation in clinical symptoms and time course of the reaction, it is likely that more than one mechanism exists. In discussing food allergy resulting from cereals, we shall consider mainly those reactions resulting from the formation and presence of specific IgE or reaginic antibodies. These antibodies bind to tissue mast cells and circulating basophils through specific receptors. When two molecules of IgE (Immunoglobulin E) are bridged by an allergen molecule, a series of biochemical pathways is initiated which results in the release of pharmacological mediators and the consequent clinical changes.

The exact incidence of food allergy resulting from ingestion of cereals is unknown, and is complicated by the presence of inhalent allergies and by cross-reactions between cereals and grass pollen allergens (Wraith *et al.*, 1979). However, it is clear that at least some suspected wheat intolerance

reactions are true allergies, and may be improved by elimination diets (Rowe, 1972).

Wheat-specific IgE antibodies have been detected in patients with suspected food allergy to cereals in several studies (Hoffman & Haddad, 1974; Wraith *et al.*, 1979; Sutton *et al.*, 1982). However, it is clear that in certain normal individuals (Goodwin, 1982) (Table 1), as well as in patients with no suspected wheat protein intolerance (Aas, 1978; Wraith *et al.*, 1979), specific IgE antibodies to wheat and other cereal proteins may also be detected.

TABLE 1

Incidence of Elevated Rice, Wheat Grain and Grass Pollen—Specific IgE in a Normal Adult Population (from Goodwin, 1982)

Allergen	*Number of sera tested*	*Number of sera with elevated level of specific IgE antibody*	
		*1·5–2 × mean uptake**	*≥ 2 × mean uptake*
Rice	99	7	4
Wheat grain	96	1	1
Grass pollen	99	0	9

* Mean uptake calculated for all sera tested for a particular allergen.

Several possible explanations have been put forward for the presence of specific IgE antibodies in normal individuals and in food allergic patients with no suspected wheat intolerance. Hoffman & Haddad (1974) suggested that, at least in some of the patients they had investigated, the close proximity of their homes to a flour mill may have resulted in an inhalant allergy to cereal proteins. In such patients, ingestion of cereal products has no effect, and this has been confirmed in subsequent studies (Aas, 1978; Baldo & Wrigley, 1978).

Secondly, the close taxonomic relationship between the cereals and the common grasses (Fig. 2) has suggested to many authors that some, if not all, the detectable wheat-specific IgE may be due to cross-reactions with grass-pollen-specific IgE (Hoffman, 1975; Aas, 1978; Wraith *et al.*, 1979). In addition, immunochemical analyses of wheat extracts have shown the presence of at least forty-four antigenic components, some of which cross-react with antigens present in grass pollen extracts (Blands *et al.*,

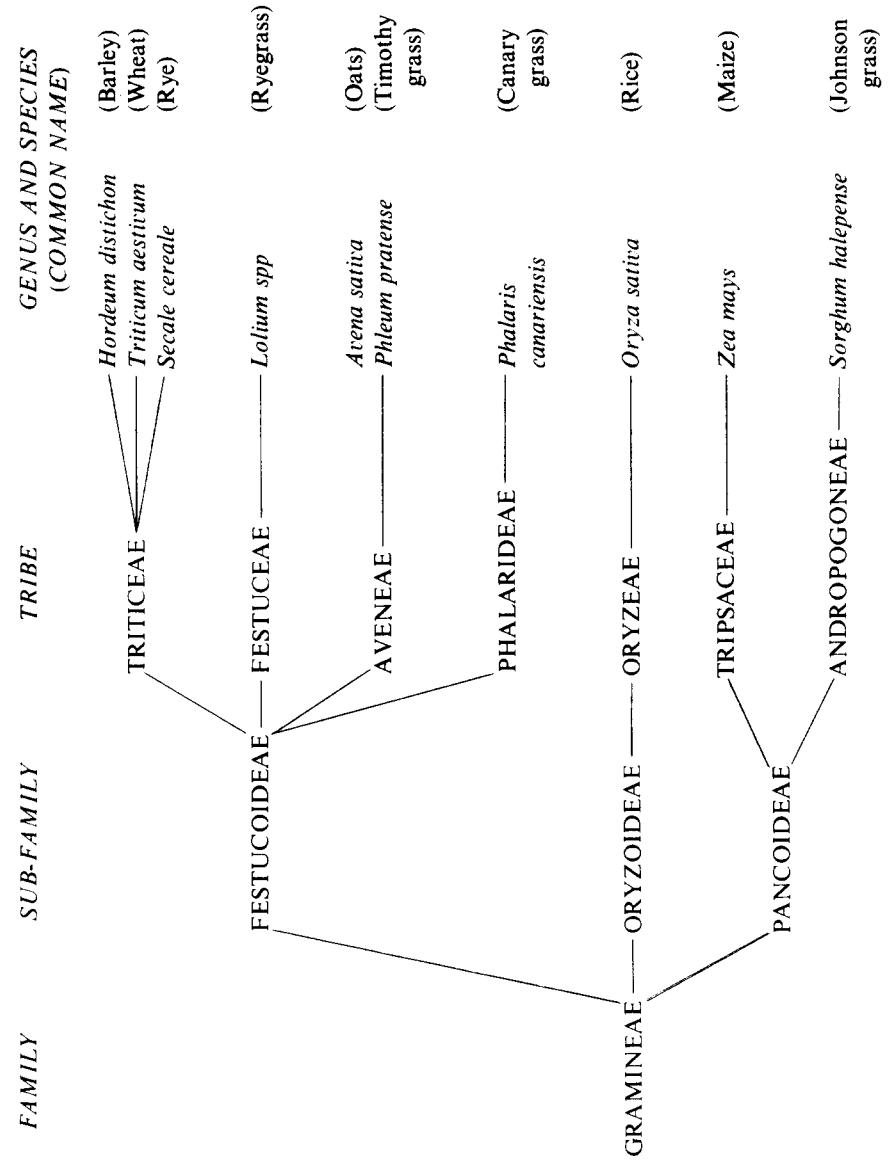

Fig. 2. Taxonomic relationship between cereal grains and some common grasses.

1976). Some sera, however, may contain antibodies against cereal proteins alone, and it is likely that this is the result of ingestion rather than inhalation.

A third possibility is that the observed IgE antibody in both normal and asymptomatic patients is due to direct dietary stimulus, and evidence for such a mechanism in normal individuals ingesting soya protein has recently been described (Goodwin, 1982).

In patients in whom cereal-specific IgE antibodies have been detected, only a limited number of studies have been carried out to determine the extent of cross-reactivity between the cereals and the nature of the allergenic component. Hoffman (1975) observed considerable cross-reactivity among the cereal proteins in both patients with and those without overt symptoms of allergic reactivity to cereals, and in particular a strong cross-reaction was observed between corn and rice. It is interesting to note that in our studies (Goodwin, 1982) and those of Aas (1978), specific IgE antibody to rice (considered to be of low allergenicity) has been frequently detected in asymptomatic patients and normal subjects. More recently, Baldo *et al.* (1982) and Sutton *et al.* (1982) also observed considerable cross-reactivity between wheat, rye and barley, using sera from patients with asthma and eczema.

Using isolated wheat fractions, Hoffman (1975) identified the albumin and globulin fractions as binding the most IgE from the sera of his patients, while gliadin and gluten bound little IgE antibody. Sutton *et al.*, (1982) confirmed the importance of the globulin fraction and also identified the glutenins as being important allergens in their patients with asthma and eczema. The albumins, however, were not effective in binding IgE, and the conflict with the results obtained by Hoffman (1975) may be the result of the inclusion of patients with inhalant allergies in the earlier study.

ASSOCIATION BETWEEN CEREAL PROTEINS AND OTHER DISORDERS

There have been an increasing number of reports in recent years of therapeutic responses to exclusion diets, particularly those excluding cereal proteins, in patients with various disorders. Although these are described as cases of food allergy, there is little evidence for an immunological mechanism.

It has been suggested that there is an increased incidence of schizophrenia in coeliacs (Dohan, 1970), and an association between gluten and schizophrenia (Ross-Smith & Jenner, 1980). Baldwin (1979), however, after studying the relationship between schizophrenia and various illnesses, considered that there was little evidence for a relationship with coeliac disease. Grant (1979) has shown that exclusion of wheat and other foodstuffs from the diet results in an improvement in patients with migraine symptoms.

Further clinical studies, as well as laboratory studies, are necessary before any firm conclusions can be drawn as to the effect of cereal proteins in these conditions.

COELIAC DISEASE

The first clear description of coeliac disease or gluten-sensitive enteropathy was made by Samuel Gee in 1888 (Gee, 1888). He also made the far-sighted comment that 'if the patient can be cured at all it must be by means of diet'. The first evidence that this was the case did not appear until 1950, when Dicke (1950), working in Holland, observed that wheat was the harmful agent, and that its withdrawal from the diet resulted in clinical improvement. Subsequent investigations showed that the damaging component of wheat was confined to the protein fraction (Dicke *et al.*, 1953, van de Kamer *et al.*, 1953).

Paulley (1954) observed that villous atrophy was a characteristic of small intestinal biopsy specimens obtained from patients with coeliac disease, and with the introduction of peroral biopsy techniques, it was shown that the villous architecture returned progressively towards normal when gluten was withdrawn from the diet, and recurred if it were re-introduced (Doniach & Shiner, 1957; Rubin, 1960).

These observations have since been confirmed in numerous studies and have led to a definition of the disease as suggested, for example, by Booth (1975), 'a condition in which there is an abnormal jejunal mucosa which improves morphologically when treated with a gluten-free diet and which again shows abnormalities when gluten is reintroduced'.

There are two main hypotheses for the mechanism of coeliac disease (reviewed in Kasarda, 1978; Asquith & Haeney, 1979). In the first mechanism, it is suggested (Frazer, 1956; Townley *et al.*, 1973) that a

specific mucosal peptidase deficiency allows accumulation of harmful amounts of a toxic partial gluten digest. This hypothesis has been modified to suggest that an abnormal, rather than deficient, peptidase is present, which catalyses the formation of a toxic gluten peptide (Yeomans, 1974; Cordone *et al.*, 1975). The second theory suggests that reaction to the disease is the outcome of an immunological response such as an Arthus-type immune complex reaction, or cell-mediated delayed hypersensitivity to wheat gluten in the jejunal mucosa. As support for this theory, many immunological changes in coeliac patients have been presented (Fig. 3). An additional hypothesis suggests

Humoral immune response	*Cell-mediated immune response*	*Mucosal and pathological changes*
Antibodies to dietary proteins including gluten	Impaired lymphocyte PHA response	Lesion resembles Arthus reaction
Antibodies to reticulin	Impaired lymphocyte cytotoxicity	Infiltrates of immunologically competent cells
Increased serum IgA and decreased serum IgM	Positive *in vitro* lymphocyte transformation test and leucocyte migration inhibition tests with gluten fractions	Decreased IgA- and increased IgM-containing plasma cells
Circulating immune complexes	Increased incidence of HLA-B8 and HLA-DW3	Gluten-specific antibodies in jejunal juice

Fig. 3. Summary of observed immunological changes in coeliac disease.

that gluten-induced damage depends on the material acting as a lectin (Weiser & Douglas, 1976). Either or both of these theories may be correct and, particularly in the case of the immunological changes, it is uncertain whether they are the cause or a result of the disease.

Rôle of humoral immunity in coeliac disease

There are a number of reports concerning humoral immunity in coeliac disease. Total serum IgG (Immunoglobulin G) levels and IgM

(Immunoglobulin M) levels tend to be low, and this may, in part, be explained by loss of immunoglobulin into the bowel lumen (Jarnum *et al.*, 1970). Impaired synthesis of IgM has been implicated (Brown *et al.*, 1969). IgA levels are often increased in untreated disease and fall towards normal during gluten withdrawal (see Asquith & Haeney, 1979).

Specific antibodies to gluten and other dietary antigens have been measured in coeliac disease (Taylor *et al.*, 1961; Ferguson & Carswell, 1972; Haeney *et al.*, 1982). High titres of serum antibody to gluten (or gliadin) have been detected by a variety of techniques (Taylor *et al.*, 1961; Ferguson & Carswell, 1972; Kieffer *et al.*, 1981; Unsworth *et al.*, 1981) and tend to fall towards normal levels with treatment. Antibodies to gluten and other dietary antigens, which may be of the IgG or IgA (Immunoglobulin A) class (Kieffer *et al.*, 1981; Unsworth *et al.*, 1981; Jewell & Truelove, 1972; Ferguson, 1976) but generally not the IgE class may also be found, but in lower titres, in patients with other gastrointestinal diseases and in healthy subjects. These findings have frequently been interpreted as indicating that anti-gluten antibodies are of little fundamental importance in coeliac disease, but merely the result of an (ill-defined) leakiness of a damaged intestine.

Evidence for the involvement of an Arthus reaction in coeliac disease has been obtained from the histological changes occurring in the intestine following *in vivo* gluten challenge (Shiner & Ballard, 1972; Anand *et al.*, 1981). Circulating immune complexes have been found in active coeliac disease (Doe *et al.*, 1973; Mowbray *et al.*, 1973; Jewell, 1979) and dermatitis herpetiformis (Mowbray *et al.*, 1973; Hall *et al.*, 1980), although it is not known what antigen they contain. Also, total haemolytic complement and complement component C3 have been shown to fall in coeliac patients following *in vivo* challenge with gluten (maximally 1–8 h after challenge) (Doe *et al.*, 1975; McNeish *et al.*, 1975).

However, it is clear that the presence or absence of antibody to gluten may be less important than the type of antibody formed (e.g. IgG subclass) or the composition of the immune complex (formed in antigen or antibody excess or at equivalence). We have recently reported preliminary findings (Rawcliffe *et al.*, 1982) that an IgG anaphylactic antibody (IgG S-TS, short-term sensitizing (Parish, 1981)) may be found to gluten in a proportion of coeliac patients, but not at all in inflammatory bowel disease where there is also damage to the intestinal mucosa. Such an antibody could be involved in an initiating event in the production of an Arthus reaction.

FRACTIONATION OF WHEAT GLUTEN TO IDENTIFY TOXIC FRACTIONS

There have been several studies carried out to identify the toxic fraction of wheat gluten responsible for coeliac disease (reviewed by Asquith & Haeney, 1979; Rawcliffe, 1981).

Wheat gluten, obtained by the Osborne fractionation scheme outlined in Fig. 1, is subsequently fractionated by one of two procedures. In the first procedure, described by Frazer *et al.* (1959), wheat gluten is partially digested with pepsin and trypsin to give a water-soluble product: Frazer's Fraction III. This fraction retains the damaging properties associated with the starting wheat gluten. In the second type of procedure, fractionation is obtained by 70% ethanol/water extraction of crude gluten to yield the soluble (gliadin) and insoluble (glutenin) fractions.

A detailed fractionation scheme, starting from Frazer's Fraction III, has been carried out over a number of years by the group working at the Radcliffe Infirmary in Oxford. Using Frazer's Fraction III as the starting material, three fractions, A, B and C, are obtained by ultrafiltration

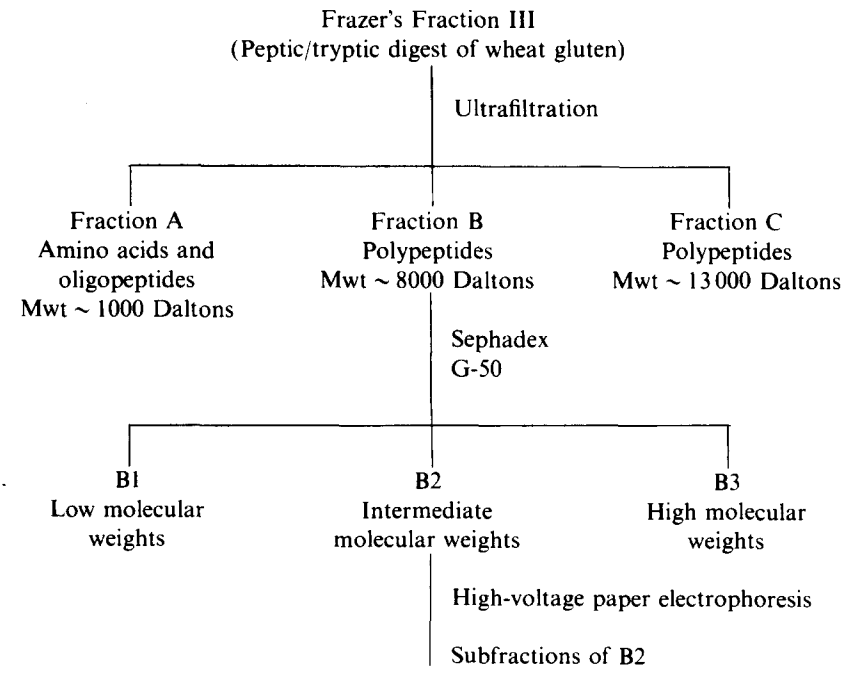

Fig. 4. Fractionation of Frazer's Fraction III by ultrafiltration and gel-filtration.

(Dissanayake *et al.*, 1974) (Fig. 4). The intermediate polypeptide fraction B was further fractionated by Sephadex G-50 chromatography (Offord *et al.*, 1978), and then further again by high-voltage paper electrophoresis.

Assessment of the toxicity of the fractions has been made by feeding (Dissanayake *et al.*, 1974; Rawcliffe, 1981), although negative results following oral challenge with small amounts of material must be regarded with caution (Rawcliffe, 1981). Alternative approaches have been to measure intestinal absorptive function or evidence of immunological reactivity; skin tests (Anand *et al.*, 1977), *in vitro* organ culture, lymphocyte transformation (Sikora *et al.*, 1976) and leucocyte migration inhibition tests and specific antibody measurements. A summary of toxicity tests and immunological assays on these wheat gluten fractions is given in Table 2. Similar tests by other workers using gliadin and gluten fractions have been included for completeness.

Although toxicity is observed mainly with the intermediate polypeptide fraction B, inconsistent and contrasting results have been obtained in immunological assays (Rawcliffe, 1981). Our recent studies, using a

TABLE 2
Summary of Toxicity and Immunological Tests on Wheat Gluten Fractions

Fraction	*Toxicity by feeding*	*Results obtained in immunological assays*		
		Skin tests	*Lymphocyte transformation/ leucocyte migration*	*Antibody** formation*
Frazer's Fraction III	Toxic	+	+	+
Gluten Fraction A	Non-toxic	Not done	Not done	±
B	Toxic	+	Not done	+
B1	Non-toxic	+ +	−	+
B2	(Toxic)*	+ + +	(+)*	+
B3	Toxic	+ +	−	+ +
C	Toxic	Not done	Not done	+ +
Gliadins	Toxic	−	+	+
Glutenin	(Toxic)***	Not done	Not done	Not done

* This fraction was originally described as toxic and producing a positive lymphocyte transformation response, but these results have not been confirmed in subsequent studies (Rawcliffe, 1981).

** Assessed using micro-ELISA technique (unpublished findings).

*** Some reported toxicity for the glutenin fraction may have been due to contamination with gliadins.

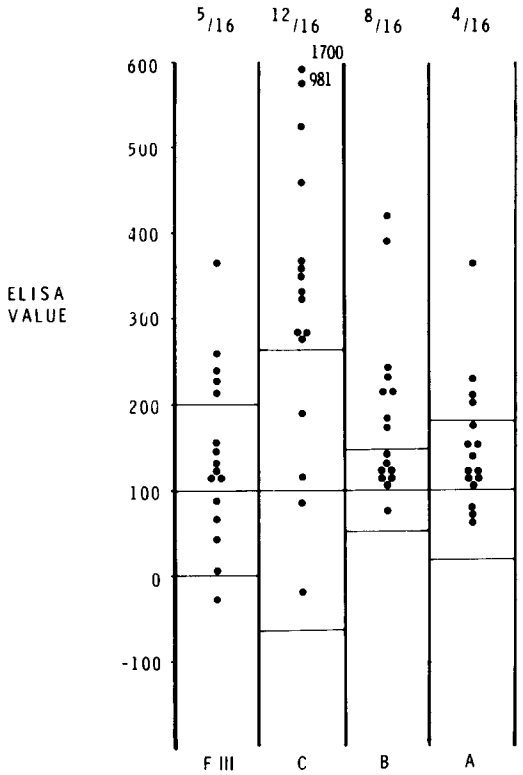

Fig. 5. IgC antibodies detected to toxic and non-toxic fractions of wheat gluten by micro-ELISA tests.

micro-ELISA technique (Fig. 5), show that IgG antibody against toxic (B and C) as well as non-toxic (A) fractions may be demonstrated in the sera of coeliacs. IgG antibodies are most frequently observed to the intermediate and high molecular weight fractions B and C, and the best differentiation between normals and coeliacs is obtained using fraction C. This may, however, merely reflect the improved binding of the antibody to the macromolecular antigen in the assay rather than any increased antigenic potential.

ALLERGIC DISEASE ASSOCIATED WITH THE INHALATION OF CEREAL GRAINS AND PROTEINS

Although occupational allergic disease in bakers and grain handlers is the result of inhalation of cereal products, we shall briefly review both the

clinical evidence and immunological studies designed to identify the allergenic components of the grain. Allergic rhinitis and asthma, following inhalation of flour dust, is perhaps the oldest occupational allergic disease. Ramazzini, in the early eighteenth century, described shortness of breath and urticaria in grain workers (Ramazzini, 1713), and Roman bakers are known to have worn masks to protect them from the flour. Clinical and epidemiological studies over a number of years (Schwartz, 1947; Stresemann, 1967; Popa *et al.*, 1970; Hendrick *et al.*, 1976; Jarvinen *et al.*, 1979; Editorial, 1981; Tse & Raghuprasad, 1982) have shown the incidence of baker's asthma to range from 2% to 50% depending on the population investigated. Allergic rhinitis normally precedes the development of asthma and atopic subjects are more likely to develop the disease than non-atopics.

Examination of sera from patients with baker's asthma has shown the presence of cereal-specific IgE antibodies. Of forty different antigens detected in wheat flour, Blands *et al.* (1976) identified three major allergens, although they were not isolated or characterised. Subsequent studies Baldo & Wrigley (1978) showed that the water-soluble proteins and, in particular, the albumin components of flour, were important allergens, and later studies (Baldo *et al.*, 1980) showed there to be considerable cross-reaction with taxonomically closely related cereals such as rye. In the latter study, it was suggested that the bran layers of cereal grains were at least as allergenic as flour. The response to inhaled cereal proteins thus differs considerably from that observed in food allergic reactions, where the globulin and glutenin fractions are considered important allergens (Sutton *et al.*, 1982).

In addition to cereal allergens, contaminants of the grain such as weevils, mites, fungal spores and thermophilic bacteria may provide allergenic material and contribute to the pathogenesis of the allergic disease (Tse & Raghuprasad, 1982) and the activation of complement by grain dust by non-immunological mechanisms may induce an inflammatory process in the lungs of sensitized and non-sensitized individuals (Olenchock *et al.*, 1978).

CONCLUSIONS

In conclusion, cereal proteins may induce adverse reactions such as food allergy and coeliac disease in a small number of individuals. The fractions responsible for IgE antibody formation, in both baker's asthma and food

allergy, are mainly the water-soluble albumins and globulins, while the insoluble gluten fractions are toxic and antigenic in patients with coeliac disease. The difference in the response to these fractions may reflect their solubility in mucous membranes (e.g. albumins in baker's asthma) as well as their relative stability to digestion in the gastrointestinal tract.

Although the immune responses obtained in patients with these conditions are not mutually exclusive, it is likely that further work into the nature of the antigenic/allergenic response in cereal protein intolerance will lead to a better understanding of food intolerance in general.

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Toxicological Aspects of Food Safety

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(Received: 25 January, 1983)

ABSTRACT

The safety-in-use of a food chemical in terms of human exposure is based on relevant information on the chemistry of the material and its biological effects in experimental animals. In the UK the information required is contained in the MAFF memorandum of 1965 on the procedure for submissions on food additives and methods of toxicity testing. The submission should provide, in addition to particulars of the composition, purity and commercial usage of a compound, toxicity data obtained from animal studies. Details of these investigations in animal models which include acute, short-term and chronic studies will be described. More recently the DHSS has set guidelines for the mutagenicity testing of food chemicals. In the USA further refinements have been incorporated into safety evaluation studies. These include exposure in utero *of animals to test compounds, multigeneration studies, extension of long-term studies to 20% survival of animals and compliance with Good Laboratory Practice (GLP) requirement. Thus, the investigative work required by regulatory authorities for the clearance of a food chemical can take up to three years at a cost in excess of £300 000 at present value. Clearly, in the present economic climate the high and escalating cost of obtaining approval for new food chemicals is bound to discourage product development.*

In addition, growing concern over the chemical contaminants in the human environment, many of which have direct or indirect impact in human food supply, has generated a long life of substances on which safety evidence is insufficient.

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In the light of these logistic and economic constraints, alternative approaches are indicated for assessing the safety of chemicals in the human environment. Some of the alternative schemes proposed will be discussed.

One of the inevitable consequences resulting from the inexorable rise in world population has been a growing demand on food production, and the additional impact of urbanisation has accentuated problems associated with food distribution and supply. Food scientists and technologists, by developing new and innovative methods of preservation, formulation and processing of food products, have considerably ameliorated the latter problem. Thus, processed foods constitute an important component of the human diet.

Processed foods contain a wide variety of chemical additives to achieve specific technological objectives (Table 1). Clearly, it is imperative that these chemical additives, at the levels encountered in the diet, must not endanger human health.

TABLE 1
Types of Chemical Additives used in Foods for Human Consumption

1. Antioxidants	7. pH control agents
2. Colours	8. Preservatives
3. Emulsifiers	9. Processing aids
4. Flavours	10. Solvents
5. Humectants	11. Stabilizers
6. Lubricants	12. Sweeteners
Miscellaneous: Anticaking agents	
Curing agents	
Bleaching and improving agents	

The safety of a food additive in terms of human exposure is assessed by the careful evaluation of a variety of relevant information on the material including its chemical composition and toxicity data in experimental animals. In the UK the information required for the safety evaluation of food additives is contained in the memorandum issued by the Ministry of Agriculture, Fisheries and Food in 1965 (MAFF, 1965). In addition, this memorandum recommends procedures for toxicity tests. Guidelines for mutagenicity test procedures on food chemicals were given by the DHSS

in 1979 (DHSS, 1979). Toxicity tests on food chemicals have been further elaborated by procedures introduced in the USA. These include *in utero* exposure of animals to a test compound, multigeneration studies and extending chronic feeding studies to 20% survival of experimental animals. An additional requirement is that toxicity studies should comply with the FDA code for Good Laboratory Practice (GLP) (FDA, 1978). The various tests are summarised in Table 2.

TABLE 2
Information Required and Guidelines Prescribed for the Safety Assessment of Food Chemicals

UK recommendations (MAFF, 1965)
Composition, purity, commercial usage

Animal toxicity data:

Type of study	*Species*	*Information derived*
ACUTE	Rat, mouse, non-rodent	LD_{50} dose
SUB-ACUTE (90-days)	Rat, mouse, non-rodent	No effect level
CHRONIC (2 years)	Rat, mouse, non-rodent	Carcinogenicity; effect on fertility; other chronic effects.

Other information: metabolism, evidence of human effects and mutagenicity studies.

Guidelines on mutagenicity studies (DHSS, 1979).

Recommendations by Food and Drug Administration, USA (FDA, 1978).
1. *In utero* exposure to test compounds.
2. Multigeneration studies.
3. Chronic studies extended to 20% survival of animals.
4. Compliance with Good Laboratory Practice (GLP) requirements.

Toxicity studies require the collaborative efforts of specialists in various aspects of toxicology from a wide range of disciplines, including Biology, Chemistry, Pathology and Statistics. In addition, facilities such as animal accommodation and special equipment and instrumentation are required for conducting these studies. The cost of undertaking the conventional toxicity studies on a food chemical is approximately £300 000, and time for completion is 3–4 years. At the present time about 300 food additives in use await toxicological assessment.

Furthermore, there is growing concern over chemical contaminants in

the human environment, many of which have direct or indirect impact in human food supply. These contaminants, examples of which are shown in Table 3, can gain access to human foods at the production stage, during storage and processing, and from food packagings. In addition, there is a variety of potentially toxic substances occurring naturally in foods.

TABLE 3
Some Chemical Contaminants in Human Processed Foods

1. Production:	agrochemicals; pesticides; metals; nitrite, nitrate; polycyclic aromatic hydrocarbons, etc.
2. Storage:	mycotoxins (aflatoxin, etc.); pest infestation; ethylene oxide, etc.
3. Processing:	filtering aids; lubricants; mould-releasing agents.
4. Packaging:	lacquers; metals; adhesives; plastics chemicals-monomers, solvents, etc.

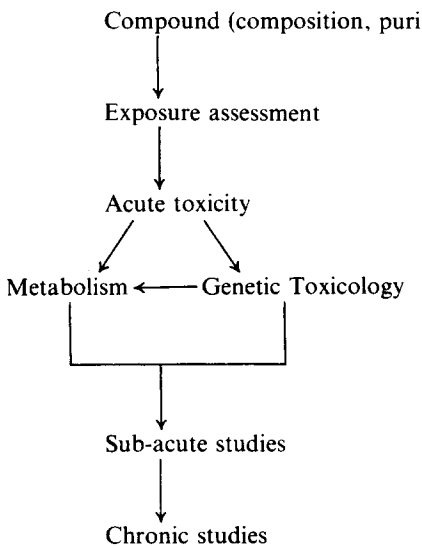
Thus, the long list of chemicals in the human diet, besides industrial chemicals, drugs and pesticides awaiting toxicological assessment, far exceeds the testing facilities and limited technical and specialist resources available. In the light of these logistic and economic constraints, alternative approaches have been explored for assessing the safety of chemicals to man. An additional impetus in this direction is the growing disquiet among toxicologists on the scientific validity of some of the current testing procedures, the problems associated with interpretation of animal findings and the extrapolation of this data to man.

The three alternative approaches considered (Table 4) are the Frawley (1967) proposals for food packaging additives, the scheme based on structure–activity relationships suggested by Cramer *et al.* (1978), and the 'Decision-Tree' approach recommended by the Food Safety Council (1978) in the USA. The main deficiencies relating to the Frawley proposals are that little account is taken of other genetic or chronic effects likely to result from long-term exposure of animals to chemicals, and, if studies were extended to investigate such effects, the demand for animal studies would not be reduced. Furthermore, difference in species sensitivity to a test compound may provide anomalous findings difficult to fit into the general scheme. The chief problem associated with the scheme proposed by Cramer *et al.* is the lack of adequate information relating chemical structures to biological activity, thereby limiting the range of applicability.

The 'Decision-Tree' approach, based on a systematic and sequential progression of investigations, has much to commend it in the safety

TABLE 4
Alternative Approaches for Safety Assessment

1. Proposals for Food Packaging Additives (Frawley, 1967)
   Clear for use compounds at 0·2% level—
   IF—1. Not carcinogenic
   2. No-effect dose not less than 1 g/kg body weight
   3. Migration from packaging into food not more than 0·1 ppm.
2. Estimation of toxic hazard (Cramer *et al.*, 1978).
   Toxicity studies on compounds to be designed on the basis of chemical structure/biological activity relationship.
3. System for Food Safety Assessment (Food Safety Council, 1978).
   Scheme based on a 'Decision-Tree' approach along the following lines (simplified):



assessment of food chemicals. However, cognizance must be taken of certain problems and deficiencies associated with each of the sequence of investigative steps. First, the various short-term tests for investigating carcinogenic and mutagenic effects of chemicals are by no means infallible and are subject to considerable experimental and interpretative problems. In the absence of a definitive short-term test for genetic effects, recourse is taken to employing a battery of tests. The results of the tests can be conflicting, leading to difficulties in the interpretation of findings. Secondly, metabolic studies, conducted to enable the design of appropriate animal studies in terms of species selection and definition of

dosing regimen, must take account of the influence of various factors on the absorption, metabolism, disposition and excretion of orally ingested food chemicals and the relevance of these modifying factors on the biological effects produced in experimental animals.

The rôle of metabolism is clearly of prime importance in the study of toxicology of chemicals, and a brief survey of some of the salient aspects of metabolic processes may be pertinent. Food chemicals may be subjected to metabolism by the gastro-intestinal juices, microflora which reside in the gastro-intestinal tract and the small intestinal mucosa prior to being absorbed in the body. The gastro-intestinal juices contain hydrolytic enzymes capable of degrading some esters, peptides and emulsifiers used as food additives to their component acids and alcohols, amino acids and monoglycerides, respectively. The gut microflora possess a wide range of metabolic capability and can either effect the detoxification of some contaminants such as the conversion of the toxic methyl mercury compounds to inorganic mercury, or lead to the formation of potentially toxic metabolites of food additives. Thus, for example, the azo colours Brown FK and Red 2G are degraded to form triaminobenzene and aniline, respectively, and cyclamates are metabolized to cyclohexylamine. Two noteworthy features of gut flora are, first, the ability of dietary factors and ingested chemicals to modify the microbial population and their metabolic profile, and, secondly, important differences in the metabolic capability of gut flora in experimental animal species and in man.

The small intestinal mucosa and internal organs in the body are replete with enzyme systems capable of mediating biotransformation reactions on food chemicals and contaminants. By far the most important organ involved in the metabolism of foreign chemicals is the liver, and the hepatic compartment of particular significance is the endoplasmic reticulum which can be isolated in a pure form by the differential centrifugation of liver homogenates. This hepatic component, referred to as the microsomal fraction, contains a number of metabolizing enzymes participating in the degradation of a wide variety of chemicals, including drugs, pesticides, food additives and contaminants (Table 5). The activities of microsomal enzymes may be divided broadly into two types of reaction. Phase I reactions lead to the conversion of compounds to polar, water-soluble products and Phase II reactions mediate the formation of conjugates of compounds or their metabolites with glucuronic acid, sulphate, glutathione or amino acid to yield even more

TABLE 5
Environmental Chemicals Metabolized by Liver Microsomal Enzymes—Examples

DRUGS:	Barbiturates; paracetamol; antibiotics, etc.
FOOD ADDITIVES:	BHT and phenolic antioxidants; benzoates and *p*-hydroxybenzoates; biphenyl, etc.
PESTICIDES:	DDT and halogenated compounds; carbamates, paraquat, etc.
INDUSTRIAL CHEMICALS:	Carbon tetrachloride; vinyl chloride; acrylonitrile; phthalates, etc.
Miscellaneous:	Aflatoxin and mycotoxins; TCDD; polycyclic aromatic hydrocarbons, etc.

water-soluble products, thereby facilitating their clearance and excretion from the organism. Phase I reactions are mediated by a group of enzymes, collectively referred to as mixed function oxidases, dependent on the haemoprotein cytochrome P-450.

Metabolic reactions on food chemicals and contaminants lead either to the formation of toxic intermediates or detoxification products, and furthermore, these reactions can occur either simultaneously or sequentially on a foreign compound. Thus, the biological effects produced are determined by the net result of bioactivation and detoxification enzymic processes acting on a compound. Factors known to influence the interplay of these two processes—and hence the toxicity of compounds—include dosage, mode and frequency of treatment, age, sex and species of

TABLE 6
Species Differences in Metabolic Disposition and Toxicity—Examples

Compound	*Toxic effect*	*Species*[a]	*Causes*
Degraded carrageenan	Bowel ulcers	Guinea-pig (+) Rat (−)	Gut absorption
Orange RN	Methaemoglobin in red cells	Rat (+) Ferret (−)	Microflora metabolism and formation of aniline
Coumarin	Liver injury	Rat (+) Mouse (−) Baboon (−)	Difference in hepatic metabolic pattern

[a] +, Positive effect; −, no effect.

animal, nutrition and dietary factors, hormonal imbalance and genetic deficiencies.

Additionally, differences in biological effects in animal species may, in certain cases, be due to qualitative differences in the metabolism of compounds. Some examples of species differences ascribable to rate of absorption, rate of metabolism or route of metabolism of food chemicals are shown in Table 6.

TABLE 7
Other New Areas of Research in Toxicology

Experimental
1. Study of chemical structure–metabolism–biological activity–species difference relationships.
2. Development of sensitive diagnostic tests for chemically induced chronic injury in experimental animals and in man.
3. Development of *in vitro* test systems to replace animal studies.

Other areas: 1. Epidemiology.
2. Risk/benefit assessment.

Other areas in toxicological research meriting further investigation are shown in Table 7. Clearly, the need for a determined and concerted effort into basic research in toxicology is bound to benefit not only the food industry, enabling a rational approach in the design of new additives, but also provide a sound and realistic basis for assessing the safety of food chemicals to man and his progeny.

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