Food Chemistry **6** (1981) 1 6

EFFECT OF BOILING ON THE CARBOHYDRATE CONSTITUENTS OF SOME NON-LEAFY VEGETABLES

OYEBIODUN G. LONGE

Department of Animal Science, University of Ibadan, Ibadan, Nigeria

(Received: 16 October, 1979)

ABSTRACT

Losses which occur in the carbohydrate constituents of three non-leafy vegetables—onion (Allium cepa, *L.*), *okra* (Hibiscus esculentus, *L.*) *and eggplant* (Solanum incanum, *L.*)*—when boiled have been determined. Ethanol-soluble sugars, which comprised glucose, fructose and sucrose, and oligosaccharides in the raw samples ranged from 4·9% in okra to 54·9% in onion but were reduced to 0·5% and 8·9% on boiling. Starch content was 2·6% and 9·5% in raw okra and eggplant, respectively but corresponding values in processed samples were 1·3% and 8·6%. Losses in water-soluble polysaccharides ranged from 72·4% and 78·2% in okra and eggplant, respectively to 92·4% in onion. Hemicellulose values of 8·2%, 6·6% and 8·6% for okra, eggplant and onion reduced to 4·5%, 6·2% and 6·7%, respectively on boiling while losses obtained for cellulose were 9%–14·1%. For lignin there was an increase of 2% to 6·2%.*

INTRODUCTION

Although some vegetables, particularly the non-leafy ones, can be eaten raw, in Nigeria they are usually preferred boiled. With the exception of a few vegetables such as onions and peppers which are used in curries, the vegetables are often boiled in water for a few minutes, after which the water is drained off before they are served as such or spiced. The drained water appears syrupy for most vegetables and probably contains carbohydrates since these are one of the major constituents of vegetables (Winsor & Massey, 1959; McConnell & Eastwood, 1974; Ketiku, 1976). McCance & Lawrence (1929) have reported that the NFE, which was thought to represent the most available fraction of carbohydrates, suffers losses when vegetables are cooked in water. Losses of carbohydrate which may occur as a result of such boiling are not known for Nigerian vegetables. The present study was therefore undertaken to provide such information for onion, okra and eggplant

Food Chemistry 0308-8146/81/0006-0001/$02·25

which appear often in Nigerian dishes. Onion and okra are sometimes boiled, drained and eaten with rice but okra is also often served in the cooking water with starchy roots and tubers. Eggplant is often eaten raw but it may be boiled, drained and spiced for serving with starchy tubers.

EXPERIMENTAL

Materials

The okra and eggplant were obtained from the National Horticultural Research Institute, Nigeria, while onions were purchased from the local market. Portions which were not edible—i.e. stalks and scales—were removed and representative samples were taken for moisture analysis. Samples were divided into two lots. One lot was chopped and frozen immediately at −20 °C while the other lot was boiled for the length of times for which they are normally boiled before being served. Boiling was carried out in closed saucepans. Onion was boiled for 15 min while okra and eggplant were each boiled for 20 min. The boiling water was drained off and samples were allowed to cool before freezing at −20 °C. Frozen raw and boiled samples were later freeze-dried, after which they were milled for analysis. Triplicate samples of freeze-dried raw and boiled vegetables were analysed.

Methods

The moisture content of fresh and freeze-dried samples was determined by the methods of the Association of Official Analytical Chemists (AOAC, 1970). Ethanol extracts of sugars were prepared in 80% v/v ethanol and quantitative analysis of the sugars present was accomplished by paper chromatography using ethylacetate–pyridine–water (8:2:1 v/v) as solvent. Sugars were detected by spraying with *p*-anisidine hydrochloride reagent (Mukherjee & Srivastava, 1952).

The oligosaccharides isolated chromatographically in alcohol extract of onion using *n*-butanol–ethanol–water (10:1:2) were hydrolysed to their constituent sugars and determined by the phenol–sulphuric acid method (Dubois *et al.*, 1956). Starch was determined by the glucoamylase method of Thivend *et al.* (1972).

Cell wall constituents (unavailable carbohydrates) were estimated after elimination of soluble sugars by acid hydrolysis (Southgate, 1969) and qualitative investigations of the polysaccharide hydrolysates were carried out as previously described for the ethanol extracts. Sugars identified on paper chromatograms were quantified. Galactose was determined with galactose dehydrogenase and uronic acid by the carbazole method of Dische (1955). Fructose, glucose and sucrose were determined by the enzymic and chemical methods of Johnson *et al.* (1964). Pentoses were determined by the method of Albaum & Umbreit (1947).

RESULTS AND DISCUSSION

The results for the carbohydrates are presented on a dry matter basis. The moisture contents of fresh vegetables was 92·0 %, 92·1 % and 87·1 % for onion, eggplant and okra, respectively.

Ethanol-soluble sugars

On chromatographic analysis, glucose, fructose and sucrose were identified. Oligosaccharides with glucose and fructose as constituent sugars were also identified in the ethanol extract of onion. The spots were higher than that of sucrose and did not correspond to raffinose. The concentrations of the ethanol-soluble sugars are shown in Table 1. The highest total value of 54·9 % was obtained for onion, followed

TABLE 1

CARBOHYDRATE CONSTITUENTS AND LIGNIN OF NON-LEAFY VEGETABLES IN g/100 g SAMPLE (DRY MATTER BASIS)

	Onion		*Okra*		*Eggplant*	
	Raw	*Boiled*	*Raw*	*Boiled*	*Raw*	*Boiled*
Ethanol-soluble sugars:						
Oligosaccharides	6·7	3·1	—	—	—	—
Sucrose	9·0	2·2	3·1	0·1	3·5	0·3
Fructose	25·8	1·4	1·6	0·3	5·7	0·3
Glucose	13·4	3·3	0·2	0·1	1·6	0·03
Starch	—	—	2·6	1·3	9·5	8·2
Water-soluble carbohydrates:						
Glucose	1·8	0·1	1·9	0·9	2·8	0·5
Galactose and galacturonic acid (okra)	—	—	7·1	1·2	0·8	0·2
Fructose	3·9	0·3	—	—	0·5	0·2
Arabinose	—	—	1·2	0·7	—	—
Hemicellulose (hydrolysis products):						
Xylose, Arabinose } Pentose	3·2	3·0	7·2	4·0	4·4	4·2
Glucose	5·4	3·7	0·9	0·5	2·2	2·1
Cellulose	15·0	12·9	19·8	17·0	19·3	17·5
Lignin	1·6	1·7	8·2	8·6	9·9	10·1

by 10·8 % for eggplant and 4·9 % for okra. Fructose was present in highest concentration in all samples and, with the exception of onion, the sucrose content was twice the concentration of glucose. Ethanol-soluble sugars were leached into the boiling water and fructose was the most affected of all sugars. Losses in total soluble sugars were 81·8 %, 90 % and 94 % for onion, okra and eggplant, respectively.

Starch

The concentration of starch expressed as glucose is shown in Table 1 with eggplant having the highest concentration of 9·5 %. No starch was found in onion—a fact which confirmed the reports of Jones & Mann (1963) and Ketiku (1976). Whilst as

much as 50% of the starch was lost as a result of boiling okra, only 9·5% of that in eggplant was affected.

The results for soluble sugars and starch (available carbohydrates) indicate that boiling lowers the proportion of these carbohydrate fractions available when the vegetables are drained before they are consumed. The leached sugars and starch would still be available, however, in cases where the boiling water is not discarded as is sometimes done for okra and onion. McCance & Lawrence (1929) and Southgate & Durnin (1970) have classified these carbohydrate fractions as the most digestible and they more accurately represent available carbohydrates than the classical NFE. Boiling would, however, have resulted in the gelatinisation and swelling of the remaining starch granules, thereby rendering them more susceptible to enzyme degradation during the course of digestion and consequently increasing the availability of the starch. Cooking has already been associated with better utilisation of starches in rats (Michaelis *et al.*, 1978). In this respect it is advantageous to boil the eggplant with a higher starch content and where loss in starch is minimal.

Water-soluble polysaccharides

Results of the qualitative analysis of the water-soluble polysaccharide hydrolysates revealed the presence of fructose and glucose in all samples but, in addition, galactose was also present in okra and eggplant while arabinose was also found in okra. The highest concentration of this polysaccharide occurred in okra (Table 1) and whilst fructose or glucose exist in highest concentration in onion and eggplant, galactose and galacturonic acid were the highest in okra.

The relatively higher value of the water-soluble fraction of okra may be due to mucilages which have been shown to be present in the vegetable (Woolfe, 1977). This might be responsible for the slimy nature of the water extract from this sample. Losses in this fraction were 92·5%, 78·1% and 72·4% for onion, eggplant and okra, respectively. Bacon (1957) has already reported the presence in onion of oligosaccharides containing a mixture of glucose and fructose residues.

Hemicelluloses

Xylose and arabinose constitute the major sugars in the hemicellulose fraction but glucose was also identified as a component sugar in this fraction. Values for pentoses and glucose are presented in Table 1 with onion having 8·6% of the hemicellulose fraction. Only 6·1% of this fraction in the eggplant was lost on boiling in comparison with 22·1% and 45·1% losses recorded for onion and okra, respectively. This is also a reflection of the relative proportion of water-soluble and water-insoluble hemicellulose fractions present in the vegetables.

Cellulose

Cellulose concentration is 15% in onion and approximately 19·0% in okra and eggplant (Table 1). Losses of the cellulose fraction were minimal for the three samples, varying between 9·1% and 14·1%.

Lignin

Onion is very low in lignin compared with the other two samples (Table 1). Lignin content increased by 6·2% in onion but by 2% and 4·8% in eggplant and okra, respectively. Whilst boiling and subsequent drying may have increased the lignin content as a result of the formation of Maillard products (Van Soest, 1965), the lignin values for raw samples could also have been slightly overestimated due to the heat treatment of the samples during the sequential fractionation of the carbohydrates, as suggested by Van Soest (1965). The lignin values obtained presently, however, agree well and are even slightly lower than that obtained for onion by McConnell *et al.* (1974).

Vegetables occur daily in the diets of Africans. Although variations in dietary fibre for boiled, compared with unboiled, samples were very slight, the plant cell wall carbohydrate fractions which constitute the dietary fibre (Trowell, 1972) will represent the major proportion of carbohydrates ingested if the vegetables are consumed without the boiling water. Even though dietary fibre is poorly digested by humans, its importance in Man's diet cannot be underestimated. Dietary fibre increases bulk in the intestine and improves the peristaltic movement of the gut, as well as the water-binding capacity of the contents of the gut (Truelove, 1956; Eastwood, 1973).

Cooking does not affect the water-holding capacity of vegetables (McConnel *et al.*, 1974). Vegetables with high water-holding capacity have laxative effects and this partly explains the low incidence of bowel disorders in Africans (Burkitt, 1973). The cholesterolemic effects of common vegetables in Africans need to be investigated. Gums and mucilagenous forms of dietary fibre have been implicated in the lowering of serum cholesterol in rats (Riccardi & Fahrenbach, 1967). This may, however, depend on the level and type of dietary fibre. Woolfe (1977) reported that plasma cholesterol was unaffected when as much as 3% okra mucilage was fed to rats.

ACKNOWLEDGEMENTS

The author thanks the Director of the National Horticultural Research Institute, Ibadan, Nigeria, for making available some of the samples. Thanks are also due to the University of Ibadan for financing the project described through the Senate Research Grants.

REFERENCES

ALBAUM, H. G. & UMBREIT, W. (1947). Differentiation between ribose-3 phosphate and ribose-5-phosphate by means of the orcin-1-pentose reaction. *J. Biol. Chem.*, **167**, 369–71.

AOAC (1970). *Official Methods of Analysis* (11th edn.) Washington, DC, Association of Official Analytical Chemists.

BACON, J. S. D. (1957). The water-soluble carbohydrates of the onion, *Allium cepa*. *Biochem. J.*, **67**, 5P–6P.

BURKITT, D. P. (1973). Epidemiology of large bowel disease: The role of fibre. *Proc. Nutr. Soc.*, **32**, 145–9.

DISCHE, Z. (1955). *Methods of biochemical analysis* Vol. 2. (Glick, D. (Ed.)), New York, Interscience Publishers Inc., pp. 313 and 38.

DUBOIS, M., GILLES, K., HAMILTON, J. K., REBERS, P. A. & SMITH, F. (1956). Colorimetric method for determinations of sugars and related substances. *Anal. Chem.*, **280**, 350–6.

EASTWOOD, M. A. (1973). Vegetable fibre, its physical properties. *Proc. Nutr. Soc.*, **32**, 137–43.

JOHNSON, G., LAMBERT, C., JOHNSON, D. K. & SUNDERWITH, S. G. (1964). Colorimetric determination of glucose, fructose and sucrose in plant materials using a combination of enzymatic and chemical methods. *J. Agric. Fd Chem.*, **12**, 216–19.

JONES, H. A. & MANN, L. K. (1963). *Onions and their allies*. New York, Interscience Publishers Inc., 63–72.

KETIKU, A. O. (1976). The chemical composition of Nigerian onions (*Allium cepa*, Linn.). *Fd Chem.* **1**, 41–7.

MCCANCE, R. A. & LAWRENCE, R. D. (1929). The carbohydrate content of foods. *Spec. Rep. Ser. Med. Res. Comm.* No. 135.

MCCONNELL, A. A. & EASTWOOD, M. A. (1974). A comparison of methods of measuring fibre in vegetable material. *J. Sci. Fd Agric.* **25**, 1451–6.

MCCONNELL, A. A., EASTWOOD, M. A. & MITCHELL, W. D. (1974). Physical characteristics of vegetable foodstuffs that could influence bowel function. *J. Sci. Fd Agric.* **25**, 1457–64.

MICHAELIS IV, O. E., NACE, C. S. & SZEPESI, B. (1978). Effect of refeeding raw and cooked starches on hepatic enzyme activities of rats. *Br. J. Nutr.* **39**, 85–9.

MUKHERJEE, S. & SRIVASTAVA, H. C. (1952). Improved spray reagent for detection of sugars. *Nature, London*, 169–330.

RICCARDI, B. A. & FAHRENBACH, M. J. (1967). Effect of guargum and pectin on serum and liver lipids of cholesterol-fed rats. *Proc. Soc. Exp. Biol. Med.* **124**, 749–52.

SOUTHGATE, D. A. T. (1969). Determination of carbohydrates in food—Part II: Unavailable carbohydrates. *J. Sci. Fd Agric.* **20**, 331–5.

SOUTHGATE, D. A. T. & DURNIN, J. V. G. A. (1970). Calorie conversion factors. An experimental reassessment of the factors used in the calculation of the energy value of human diets. *Br. J. Nutr.* **24**, 517–35.

THIVEND, P., MERCIER, C. & GUILBOT, A. (1972). Determination of starch with glucoamylase. VII. General carbohydrate methods. In: *Methods in carbohydrate chemistry*. (Whistler, R. L. & BeMiller, J. N. (Eds)). London and New York, Academic Press.

TROWELL, H. (1972). Ischemic heart disease and dietary fibre. *Amer. J. Clin. Nutr.* **25**, 926–32.

TRUELOVE, S. C. (1956). Movements of the large intestine. *Physiol. Rev.* **46**, 457–512.

VAN SOEST, P. J. (1965). Use of detergents in the analysis of fibrous feeds. III. Study of effects of heating and drying on yield of fibre and lignin in forages. *J. Ass. Off. Agric. Chem.* **48**, 785–90.

WINSOR, G. W. & MASSEY, D. A. (1959). The composition of tomato fruit. II—Sap expressed from fruit showing colourless areas in the walls. *J. Sci. Fd Agric.* **10**, 304–7.

WOOLFE, J. (1977). The effect of okra mucilage (*Hibiscus esculentus* L.) on the plasma cholesterol level in rats. *Proc. Nutr. Soc.* **36**, 59A.

Food Chemistry 6 (1981) 7–18

EXTRACTABILITY AND FUNCTIONALITY OF PROTEIN FROM YEAST CELLS GROWN ON CASSAVA HYDROLYSATE

B. ONUMA OKEZIE† & FRANK V. KOSIKOWSKI

Department of Food Science,
Cornell University, Ithaca, New York 14850, USA

(Received: 22 October, 1979)

ABSTRACT

An extraction of protein from two strains of yeast, Candida tropicalis *and* C. utilis, *grown by batch method on cassava hydrolysate was carried out by a combination of mechanical and mild chemical treatment yielding about 71% and 68% protein for* C. tropicalis *and* C. utilis, *respectively. The process resulted in about 75–80% reduction in the nucleic acid level of the freeze dried whole yeast cells of both organisms. The protein concentrates showed a good amino acid profile comparable to egg protein with methionine and tryptophan as the limiting amino acids. The functional characteristics of the two yeast proteins were studied and compared with soy protein concentrate. They showed functional properties possessing very good wettability, emulsion capacity and whippability but poor emulsion stability.*

INTRODUCTION

The resistance of a microbial cell wall to rupture or digestion is one of the major factors that limit the nutritional usefulness and acceptability of single cell protein (SCP). Many investigators have applied various methods to disintegrate and modify the cell wall of microorganisms in order to improve their nutritive and organoleptic quality. Through heat application by cooking and autoclaving, some workers (Cook *et al.*, 1963; Erchul *et al.*, 1968; Kihlberg, 1968) were able to achieve some improvement in the protein value of algal cells. Autolysis has been used to modify yeast cell walls by the action of endogenous enzymes (Hough & Maddox, 1970).

† Present Address: Department of Food Science and Technology, Alabama A & M University, Normal, Alabama 35762, USA.

Food Chemistry 0308-8146/81/0006-0007/$02·25

Plasmolysis and hydrolysis have been employed for the preparation of yeast extracts used as flavouring agents (Acraman, 1966; Kihlberg, 1972).

Akin (1974) reported the extraction of protein from intact *C. utilis* cells by use of aqueous acid in which most of the protein material was in the aqueous acid phase. An alkanol precipitated protein concentrate from the aqueous acid phase was said to possess a better whipping quality than egg albumin.

The importance of protein concentrates or isolates in food systems is often related to their functionality (Mattil, 1971). Labuza *et al.* (1972) studied the functional properties of dry, intact yeast cell protein as adjuncts in bread making while Huang & Rha (1971) investigated the rheological properties of single cell protein concentrate as they relate to dope formation and spinnability.

It was the purpose of this study to extract the proteins of yeasts grown on cassava acid hydrolysate by a combination of mechanical and mild chemical treatments and to note the properties of the extracted proteins for possible application to other foods.

MATERIALS AND METHODS

Preparation of acid hydrolysed cassava medium

Cassava tubers obtained from Puerto Rico, Florida and the Park Avenue Market in New York City were washed, peeled, rewashed and ground in a Hobart grinder.

Ten gramme portions of the peeled, ground cassava were each mixed with 80 ml 0·1 N HCl (one part of ground cassava to eight parts of acid) and autoclaved for 60 min at 121 °C and 5·1 kg/cm^2. The hydrolysed material was cooled to room temperature and then filtered with Whatman No. 42 filter paper.

Thereafter the pH of the cassava hydrolysate was raised to 5·0 with a 5 % solution of ammonium hydroxide, a nitrogen source. After adjustment of the solids of the medium to 5 % it was autoclaved at 5·1 kg/cm^2 for 10 min and cooled.

Disintegration of yeast cells

Freeze dried yeast cells of *Candida tropicalis* and *Candida utilis* grown in our laboratory by batch cultivation were used. The cells were grown on an acid hydrolysed cassava medium (5 % sugar) supplemented with 10 % growth factor composed of mineral salts and yeast extract in a 14 litre New Brunswick fermenter. Eighteen to twenty hour growth inoculum was used to provide an initial cell concentration of 0·2 absorbance reading. Aeration rate was one volume of air per volume of culture medium per minute. Growth was maintained at a temperature of 30 °C and pH of 5·0. The cells were harvested after 60 h. They were separated from the medium by centrifugation at 8000 × g for 10 min using a refrigerated centrifuge (International, Model B-20). The washed cell paste was freeze dried and used.

A 5 % (w/v) suspension of yeast cells in 0·4 % NaOH was disintegrated in a Virtis

homogeniser (Research Equipment Co., Gardner, NY) in the presence of glass beads—Glasperlen 0·25–0·33 mm (VWR Scientific Co., Rochester, NY). The volumetric proportion of cell suspension to glass beads was 1·7 according to Hedenskog *et al.* (1970). During disintegration the homogeniser jar was immersed in an ice-water bath and cell homogenisation or disintegration was carried out at medium speed for 15 min.

Extraction and precipitation of protein

After disintegration, the contents of the homogeniser were transferred to a 500 ml Erlenmeyer flask and incubated for 1 h at 40 °C in an environmental incubator shaker, Model G24, (New Brunswick Scientific Co., New Brunswick, NJ). The incubated mixture was centrifuged at 6000 × g for 10 min with the International refrigerated centrifuge (Model B-20) at 0 °C. After separating the supernatant from the residue, the latter was resuspended in the same volume of either 0·4 % NaOH or water and then extracted a second time. The supernatants were pooled and the volume measured. The residue was then discarded. Aliquots were taken for total nitrogen (Kjeldahl) determinations. The proteins of the supernatants were precipitated with 1 N HCl at pH 4·0–4·5 and then centrifuged at 6000 × g for 60 min for *C. utilis*, which showed difficulty in precipitation at this speed and shorter time. The supernatant was discarded (Fig. 1).

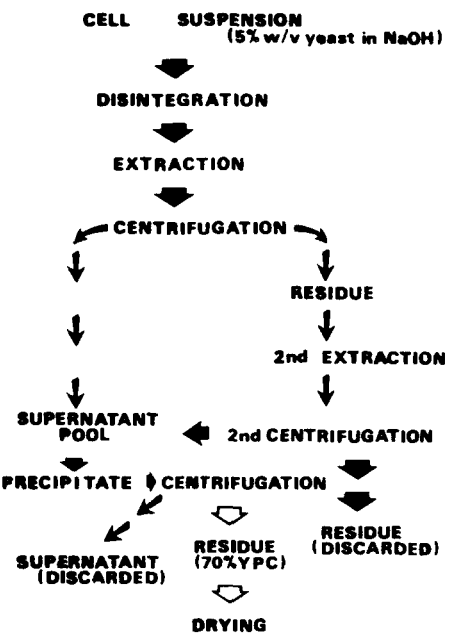


Fig. 1. Schematic flow diagram of the yeast protein extraction and concentration process.

The acid precipitated material (residue) or yeast protein concentrate was freeze dried at 30 °C and 75 cm vacuum using Stokes freeze dryer (F. J. Stokes Co., Philadelphia, PA) and then stored at 4·4 °C until required.

Analysis of yeast protein concentrate

Total Kjeldahl nitrogen was used as a measure of extractability of proteins from the whole yeast cells according to the AOAC method (1970). Extractable protein was measured from the pooled supernatant samples and the precipitable protein was measured with the freeze dried material. Amount of protein recovered was measured as a ratio of the extracted protein to the precipitated protein. All determinations were made in duplicate and averaged.

Amino acids of the freeze dried protein concentrate were analysed according to Moore (1963) and Moore & Stein (1963). Tryptophan was determined by the method of Spies & Chambers (1948, 1949) and total nucleic acid was determined according to Schneider (1945).

Measurement of functional characteristics of the yeast protein concentrates

Functionality studies were carried out with the freeze dried yeast protein concentrates according to the method of Rasekh (1974) with some modifications as described in the respective functional parameter measured. The samples were evaluated for pH, bulk density, wettability, emulsion capacity and stability, whippability, water binding index and water swelling index. The values were compared with those of soy protein concentrate (Promosoy 20/60, Central Soya Co., Chemurgy Division, Chicago, Ill.) In all cases measurements were made in duplicate or triplicate and averaged.

pH: The pH was measured by making a 10 % (w/v) suspension of each sample in distilled water. After mixing in a semi-microblender (Arthur H. Thomas Scientific Co., Philadelphia, PA), the pH was measured with the Zeromatic II pH meter (Beckman Instruments, Inc., Fullerton, Calif.).

Bulk density: A 10 ml graduated cylinder previously tared was gently filled with the protein concentrate and weighed. Bulk density was calculated by dividing the volume (10 ml) by the weight of the sample. Measurements were made in duplicate and averaged and results were expressed on a dry basis.

Wettability: One gramme sample was placed in a 25 ml graduated cylinder with a diameter of 1 cm. A finger was placed over the open end and the cylinder was inverted and clamped at a height of 10 cm from the surface of 500 ml tap water in a 600 ml beaker. The finger was removed, and the time required for the sample to get completely wet was measured with a stop watch. Triplicate measurements were made and the average result taken.

Emulsion capacity: A 1 g sample was mixed with 34 ml of a 3 % NaCl solution in a blender for 30 s. While continuing blending, 30 ml Kraft oil was added at the rate of

10 ml/min and blending continued for an additional 30 s. The sample was transferred to a 50 ml graduated centrifuge tube, was kept in a water bath at 80 °C for 15 min and then centrifuged at 2500 × g for 40 min (International Centrifuge, Universal Model UV, International Equipment Co., Needham Heights, MA). The volume of oil separated from the sample after centrifugation was read directly from the tube. Emulsion capacity was expressed as the amount of oil emulsified and held per gramme of protein concentrate.

Emulsion stability: A 0·5 g sample was dispersed in 12·5 ml distilled water, then 12·5 ml Kraft oil was added at a rate of 12·5 ml/30 s while blending. Each sample was blended in a micro-blender at high speed for an additional 60 s and transferred into a graduated cylinder. Volumetric changes in the foam, oil phase, and aqueous layer were recorded after 0·5, 2, and 6 h. Duplicate measurements were made and averaged.

Whippability (overrun): A 0·5 g sample was blended with 45 ml distilled water at high speed in the blender for 1 min. After blending, the sample was transferred into a 150 ml graduated cylinder. The blender jar was washed with 5 ml distilled water and was then added to the graduated cylinder. For each treatment, two replicates were prepared and blended for 2 and 6 min. Each replicate was duplicated and averaged. The increase in volume after blending was recorded. The per cent overrun was calculated as follows:

$$\% \text{ Overrun} = \frac{\text{Volume after whipping} - \text{volume before whipping}}{\text{Volume before whipping}} \times 100$$

Water swelling and water binding indexes: A 0·5 g sample in duplicate was dispersed in distilled water to a total volume of 5 ml in a graduated centrifuge tube which had previously been dried for 5 h at 100 °C and tared. The dispersed sample was vigorously shaken on a Vortex mixer for about 1 min to assure complete wetting. The sample was allowed to settle at room temperature for 3 h and the volume of the precipitate recorded (swelled volume). The supernatant was carefully separated from the precipitated solids with the aid of a Hamilton syringe, and was gently transferred to a tared aluminium dish, dried overnight (about 18 h) in 100 °C oven and weighed (suspended solids). The weight of insoluble solids was calculated by subtracting the weight of suspended solids from the weight of original sample.

The weight of water bound was measured after drying the insoluble fraction in an oven for 12 h at 100 °C. Functional parameters were calculated as follows:

$$\text{Water swelling index} = \frac{\text{Swelled volume}}{\text{Weight of insoluble fraction}}$$

$$\text{Water binding index} = \frac{\text{Weight of water bound}}{\text{Weight of insoluble fraction}}$$

RESULTS AND DISCUSSION

Extraction and analysis of yeast protein concentrate

In this study, cell wall rupturing was carried out. A homogeniser with glass beads provided a satisfactory method for the cell wall rupture, protein release was accomplished by use of sodium hydroxide and recovery of protein by use of hydrochloric acid.

The recovered protein was calculated as a ratio of the precipitated protein to the extracted protein (Table 1). The amount of total nitrogen extracted from the cells was greatly increased by the use of water for the second extraction, about 92% for *C. tropicalis* and 93% for *C. utilis* compared to 82% and 71%, respectively with NaOH.

TABLE 1

EXTRACTED PROTEIN CONCENTRATE[a] AND NUCLEIC ACID CONTENT OF *Candida tropicalis* AND *Candida utilis* GROWN UNDER BATCH CONDITIONS ON CASSAVA HYDROLYSATE

Organism	*Extracted protein % (supernatant protein)*		*Precipitated protein % (freeze-dried material)*		*Recovered protein % (precipitated protein/ extracted protein)*		*Total nucleic acid*
	2nd extraction with NaOH	*2nd extraction with water*	*2nd extraction with NaOH*	*2nd extraction with water*	*2nd extraction with NaOH*	*2nd extraction with water*	
C. tropicalis	81·9	91·9	70·5	70·5	88·1	76·7	1·2
C. utilis	71·3	92·8	65·7	69·3	92·1	74·7	1·2

[a] N × 6·25.

Candida tropicalis gave a slightly higher value of precipitated protein concentrate of about 71% than *C. utilis* which had about 66–69%. These are within the range of values (65–85%) reported by Newell *et al.* (1975*a*, *b*, *c*) from *C. utilis* and by Robbins *et al.* (1975) from *Saccharomyces fragilis*, but higher than the average value (54%) reported later by Robbins (1976). Vananuvat (1973) obtained 83% from *S. fragilis* while Hedenskog & Ebbinghaus (1972), working with *S. cerevisiae*, obtained a yield of about 64% protein. Such quantitative differences in protein level may be accounted for by species differences.

Both protein concentrates from *C. tropicalis* and *C. utilis* contained considerably lower nucleic acid (NA) of about 1·2% than the whole yeast cells with 5–6%. This amounted to about 75–80% reduction in the NA content of the whole yeast protein. Such a reduction might be due to the heat treatment which could activate the endogenous ribonuclease leading to the degradation of the nucleic acids (Butler *et al.*, 1973). Akin & Chao (1973) working with intact Torula yeast cells were able to reduce the nucleic acid content to less than 2% from about 10·5% using aqueous ammonia and heating to about 90–125°C to induce the decomposition of the NA.

Some individual amino acid levels of the protein concentrates were slightly lower than those of the whole yeast protein (Tables 2 and 3). Such differences in amino acid levels might be due to possible protein denaturation caused by certain extraction steps. According to some investigators (Nash *et al.*, 1971; Samson *et al.*, 1971) an acid environment could cause denaturation of certain proteins. Furthermore,

TABLE 2
AMINO ACID COMPOSITION OF *Candida tropicalis* AND *Candida utilis* PROTEIN CONCENTRATES COMPARED TO WHOLE EGG[a]

Amino acid	*Amino acid g/16 g N*		
	Whole egg	C. tropicalis	C. utilis
Isoleucine	6·77	5·04	4·76
Leucine	7·84	7·63	7·78
Lysine	7·17	7·61	7·19
Methionine	3·43	0·99	1·00
Phenylalanine	5·80	4·33	4·37
Threonine	5·20	4·74	4·75
Tryptophan	1·49	1·31	1·36
Valine	7·41	5·21	5·20
Histidine	2·40	1·83	1·80
Alanine	5·92	5·44	5·50
Arginine	6·70	4·28	4·60
Aspartic acid	7·33	10·15	9·72
Cysteic acid	2·11	0·79	0·86
Glutamic acid	12·37	11·33	11·64
Glycine	3·47	4·50	4·25
Proline	4·16	3·26	3·45
Serine	7·92	4·85	5·05
Tyrosine	3·95	3·75	3·81

[a] From FAO Nutrition Studies No. 24, 1970.

glyceraldehyde-3-phosphate dehydrogenase, constituting about 20% of yeast proteins, has been reported to be denatured by an acid medium due to dissociation into sub-units and thorough disorganisation of the protein conformation (Shibata & Kronman, 1967). Fukushima (1959) also reported that freeze drying caused denaturation of soybean protein due to disulphide bridge formation between molecules. Effects of such protein denaturation seem to be reflected in the reduced amino acid levels.

Based on the amino acid profile of egg protein, methionine appears to be the most limiting essential amino acid of the protein concentrate for both *C. tropicalis* and *C. utilis* (Table 4).

Functional properties of the yeast protein concentrates

Results of the measure of pH, bulk density, wettability, emulsion capacity and whippability are listed in Table 5.

TABLE 3

AMINO ACID COMPOSITION OF *Candida tropicalis* AND *Candida utilis* CELLS GROWN ON CASSAVA HYDROLYSATE BY BATCH METHOD COMPARED TO WHOLE EGG[a]

Amino acid	*Amino acid g/16 g N*		
	Whole egg	C. tropicalis	C. utilis
Isoleucine	6·77	5·25	5·59
Leucine	7·84	7·60	8·10
Lysine	7·17	8·90	8·53
Methionine	3·43	1·40	1·20
Phenylalanine	5·80	4·23	4·42
Threonine	5·20	5·88	5·98
Tryptophan	1·49	1·17	1·28
Valine	7·41	5·67	6·34
Histidine[b]	2·40	2·02	2·13
Alanine	5·92	6·66	6·60
Arginine	6·70	5·00	5·67
Aspartic acid	7·33	11·90	10·39
Cysteic acid	2·11	1·31	1·37
Glutamic acid	12·37	14·94	16·72
Glycine	3·47	4·85	4·71
Proline	4·16	3·32	3·27
Serine	7·94	5·22	5·11
Tyrosine	3·95	3·78	4·08
Total aromatic amino acid	9·75	8·01	8·50
Total sulphur amino acid	5·54	2·71	2·57
Apparent limiting amino acid		tryptophan and methionine	methionine and tryptophan
Protein score	100·0	40·8	35·0

[a] From FAO Nutrition Studies No. 24, 1970.
[b] Essential for children.

TABLE 4

ESSENTIAL AMINO ACIDS AND PROTEIN SCORES OF PROTEIN CONCENTRATES FROM *Candida tropicalis* AND *Candida utilis* COMPARED TO OTHER YEAST PROTEIN SOURCES

Essential amino acids	*Amino acid g/16 g N*				
	C. tropicalis[a]	C. utilis[a]	S. fragilis[b]	S. cerevisiae[c]	Candida *sp.*[d]
Isoleucine	5·04	4·76	3·33	5·7	3·95
Leucine	7·63	7·78	7·25	8·3	8·87
Lysine	7·61	7·19	6·86	8·3	6·45
Aromatic amino acids	8·08	8·18	6·51	9·6	8·64
Sulphur amino acids	1·78	1·86	2·25	1·9[e]	1·49
Threonine	4·74	4·75	3·95	5·6	3·92
Tryptophan	1·31	1·36	0·36	ND[f]	0·96
Valine	5·21	5·20	4·37	6·5	4·30
Apparent limiting amino acid	methionine	methionine	tryptophan	?	sulphur amino acid
Protein score	30	29	21	—	27

[a] From this study.
[b] From Vananuvat (1973).
[c] From Hedenskog & Ebbinghaus (1972).
[d] From Mitsuda *et al.* (1971).
[e] Only methionine.
[f] Not determined.

TABLE 5
FUNCTIONAL PROPERTIES OF *Candida tropicalis* AND *Candida utilis* PROTEIN CONCENTRATE COMPARED TO SOY PROTEIN CONCENTRATE

Functional property	C. tropicalis	C. utilis	*Soy concentrate*
pH[a]	4·4	4·4	6·9
Bulk density (w/v)	0·3	0·3	0·4
Wettability (s)	9·0	10·0	84·0
Emulsion capacity (ml oil/g protein)	11·0	30·0	15·0
Whippability (%)			
after 2 min whipping	7·0	17·0	10·0
after 6 min whipping	6·0	11·0	3·0

[a] 10% solution.

The pH values of the two yeast protein concentrates were the same while the soy concentrate with which the various parameters measured were compared, had a pH value close to neutrality of 6·9. The bulk density of the three concentrates were also similar. The yeast protein concentrates were about eight times more wettable than the soy protein concentrate; the emulsion capacity of *C. utilis* protein concentrate was about two times higher than that of soy concentrate and almost three times that of *C. tropicalis* protein concentrate. A similar pattern was also observed in the whipping capacity of the samples. *Candida utilis* protein concentrate showed a much higher whipping ability of about $1\frac{1}{2}$ times more than soy concentrate and almost 3 times more than *C. tropicalis* protein concentrate after 2 min whipping. Whippability of *C. utilis* decreased by about a third while that of *C. tropicalis* decreased only slightly with increasing whipping time of up to 6 min, indicating a more stable foam than *C. utilis* and soy concentrates. Whippability of soy protein concentrates decreased by about two thirds after 6 min whipping.

In the two yeast protein concentrates the water swelling index was higher than the water binding index (Table 6) and the two indices were slightly lower than that of soy protein concentrate. All the samples had poor emulsion stability. The emulsions

TABLE 6
WATER SWELLING INDEX AND WATER BINDING INDEX OF *Candida tropicalis* AND *Candida utilis* PROTEIN CONCENTRATE COMPARED TO SOY PROTEIN CONCENTRATE

Parameter	C. tropicalis	C. utilis	*Soy concentrate*
Swelled volume (ml) 'A'	2·2	2·4	2·5
Suspended solids (g)	0·1	0·1	0·1
Water bound (g) 'B'	1·8	2·0	2·7
Insoluble fraction (g) 'C'	1·5	0·5	0·5
Water swelling index 'A'/'C'	4·8	5·4	5·5
Water binding index 'B'/'C'	3·9	4·5	5·9

separated into different phases within 30 min after blending. However, the yeast protein concentrates were slightly more stable than the soy protein concentrate (Table 7).

With such comparatively good functional characteristics shown by the yeast protein concentrates, their potential applications in food systems are quite excellent especially with respect to their whippability, emulsifying capacity and wettability.

TABLE 7
EMULSION STABILITY OF *Candida tropicalis* AND *Candida. utilis* PROTEIN CONCENTRATE AND SOY PROTEIN CONCENTRATE

Parameters	C. tropicalis	C. utilis	*Soy concentrate*
		ml	
Foam after 1/2 h	0·0	1·5	3·0
Oil after 1/2 h	18·0	18·0	17·0
Aqueous after 1/2 h	6·0	3·0	3·0
Total volume	24·0	22·5	23·0
Foam after 2 h	0·0	1·5	2·5
Oil after 2 h	16·0	18·0	15·5
Aqueous after 2 h	8·0	3·0	5·0
Total volume	24·0	22·5	23·0
Foam after 6 h	0·0	2·5	2·5
Oil after 6 h	16·0	17·0	15·0
Aqueous after 6 h	8·0	3·0	5·0
Total volume	24·0	22·5	22·5

Products with such good whipping or aerating property could be utilised in the manufacture of confections, whipped toppings, frozen desserts and ice cream. Thus, the yeast protein concentrates could successfully substitute egg white in the making of some of the above products in which it is used. Furthermore, the emulsifying property of the yeast protein concentrates make them potentially useful in meat, ice cream, baking and high protein food industries.

Such potential applications to food industries could enhance the economic use of cassava as a substrate for the production of single cell protein especially among the cassava-dependent cultures.

REFERENCES AND BIBLIOGRAPHY

ACRAMAN, A. R. (1966). Processing brewers' yeast. *Proc. Biochem.*, **1**, 313.

AKIN, C. & CHAO, K. C. (1973). Ammonia extraction of unicellular microorganisms. US Patent 3,775,393.

AKIN, C. (1974). Water-soluble, whippable protein material and process for producing same. US Patent 3,833,552.

ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS. (1970). *Official methods of analysis*. AOAC, Washington, DC.

BOHAK, Z. (1964). N^{ϵ}-(DL-2-Amino-2-Carboxyethyl)-L-Lysine, a new amino acid formed on alkaline treatment of proteins. *J. Biol. Chem.*, **239**, 2878.

BUTLER, G. W., REAY, P. F. & TAPPER, B. A. (1973). Physiological and genetic aspects of cyanogenesis in cassava and other plants. In: *Chronic cassava toxicity*. Lon. Proc. Interdisciplinary Workshop. 65.

CANEPA, A., PIEBER, M., ROMERO, C. & TOHA, J. C. (1972). A method for large reduction of nucleic acid content of yeast. *Biotechnol. and Bioeng.*, **14**, 173.

CASTRO, A. C., SINSKEY, A. J. & TANNENBAUM, S. R. (1971). Reduction of nucleic acid content in *Candida* yeast cells by bovine pancreatic ribonuclease A treatment. *Appl. Microbiol.*, **22**, 422.

COOK, B. B., LAU, E. W. & BAILEY, B. M. (1963). The protein quality of waste grown green algae. *J. Nutr.*, **81**, 23.

ERCHUL, B. A. F. & ISENBERG, D. L. (1968). Protein quality of various algae biomasses produced by a water reclamation pilot plant. *J. Nutr.*, **95**, 374.

FUKUSHIMA, D. (1959). Studies on soybean proteins. *Bull. Agr. Chem. Soc.* (Jap), **23**, 1.

HEDENSKOG, G., MORGREN, H. & ENEBO, L. (1970). A method of obtaining protein concentrate from microorganisms. *Biotechnol. and Bioeng.*, **12**, 947.

HEDENSKOG, G. & EBBINGHAUS, L. (1972). Reduction of nucleic acid content of SCP concentrate. *Biotechnol. and Bioeng.*, **14**, 447.

HOUGH, J. S. & MADDOX, I. S. (1970). Yeast autolysis. *Proc. Biochem.*, **5**(5), 50.

HUANG, F. & RHA, C. K. (1971). Rheological properties of single-cell protein concentrate: Dope formation and its flow behaviour. *J. Food Sci.*, **36**, 1131.

KIHLBERG, R. (1968). Preliminary studies of a *Spirulina* species from an Ethiopian lake as a source of protein for the growing rat. Paper presented at the conference *Preparing Nutr. Protein from Spirulina*. Stockholm.

KIHLBERG, R. (1972). The microbe as a source of food. *Annual Review of Microbiology*, **1602**, 427.

LABUZA, T. P., JONES, K. P., SINSKEY, A. J., GOMEZ, R., WILSON, S. & MILLER, B. (1972). Effect of drying conditions on cell viability and functional properties of single-cell protein. *J. Food Sci.*, **37**, 103.

LINNANE, A. W. & VITOLS, E. (1962). A simple high speed blender for the disintegration of microorganisms. *Biochem. Biophys. Acta.*, **37**, 778.

MATTIL, K. F. (1971). The functional requirements of protein foods. *J. Amer. Oil. Chem. Soc.*, **48**, 1134.

MAUL, S. B., SINSKEY, A. J. & TANNENBAUM, S. R. (1970). New process for reducing the nucleic acid content of yeast. *Nature*, **228**, 181.

MITSUDA, H. B., TONOMURA, F. KAWAI & MACKAWA, T. (1971). Protein isolates from hydrocarbon assimilating microorganisms. In: *Memoirs of the College of Agriculture, Kyoto University*. N. 100 (Food, Sci. & Tech. Series No. 171). Kyoto, Japan.

MOORE, S. (1963). On the determination of cystine as cysteic acid. *J. Biol. Chem.*, **238**(1), 235.

MOORE, S. & STEIN, W. H. (1963). Chromatographic determination of amino acids by the use of automatic recording equipment. In: *Methods in enzymology*. Vol. 6. (Colowick, S. P. & Kaplan, N. O. (Eds.)) Academic Press, New York, 819.

NASH, A. M., KWOLEH, K. W. & WOLF, W. J. (1971). Denaturation of soybean proteins by isoelectric precipitation. *Cereal Chem.*, **46**, 360.

NEWELL, J. A., ROBBINS, E. A. & SEELEY, R. D. (1975*a*). Manufacture of yeast protein isolate having a reduced nucleic acid content by an alkali process. US Patent 3,867,555.

NEWELL, J. A., SEELEY, R. D. & ROBBINS, E. A. (1975*b*). Process of making yeast protein isolate having reduced nucleic acid content. US Patent 3,867,255.

NEWELL, J. A., SEELEY, R. D. & ROBBINS, E. A. (1975*c*). Isolated yeast protein product with intact RNA and a process for making same. US Patent 3,888,839.

NORTHCOTE, D. H., GOULDING, K. J. & HORNE, R. H. (1958). The chemical composition and structure of cell wall of *Chlorella pyrenoidosa*. *Biochem. J.*, **70**, 391.

NOSSAL, P. M. (1953). A mechanical cell disintegration. *Austral. J. Exptl. Biol. Med. Sci.*, **31**, 583.

NOVOTNY, P. (1964). A simple rotary disintegrator for microorganisms and animal tissues. *Nature*, **31**, 583.

OHTA, S., MAUL, S. B., SINSKEY, A. J. & TANNENBAUM, S. R. (1971). Characterisation of heat-shock process for reduction of nucleic acid content of *Candida utilis*. *Appl. Microbiol.*, **22**, 415.

RASEKH, J. (1974). Effect of freeze drying, spray drying and stabilizers on functional characteristics of fish protein concentrate. *J. Milk Food Technol.*, **37**(2), 78.

REHACEK, J., BERAN, K. & BICIK, V. (1969). Disintegration of microorganisms and preparation of yeast cell walls in a new type of disintegrator. *Appl. Microbiol.*, **17**, 462.

ROBBINS, E. A., SUCHER, R. W., SCHULDT, Jr., E. H., SIDOTI, D. R., SEELEY, R. D. & NEWELL, J. A. (1975). Yeast protein isolate with reduced nucleic acid content and process for making same. US Patent 3,887,431.

ROBBINS, E. A. (1976). Manufacture of yeast protein isolate having a reduced nucleic acid content by a thermal process. US Patent 3,991,215.

SAMSON, A. S., CATER, C. M. & MATTEL, K. F. (1971). Preparation and characterisation of coconut protein isolates. *Cereal Chem.*, **48**, 182.

SCHNEIDER, W. C. (1945). Phosphorous compounds in animal tissues. I: Extraction and estimation of deoxypentose nucleic acid and pentose nucleic acid. *J. Biol. Chem.*, **161**, 293.

SHIBATA, Y. & KRONMAN, M. J. (1967). Inactivation of glyceraldehyde-3-phosphate dehydrogenase. *Arch. Biochem. Biophys.*, **118**, 410.

SPIES, J. R. & CHAMBERS, D. C. (1948). Chemical determination of tryptophan. *Anal. Chem.*, **20**(1), 30.

SPIES, J. R. & CHAMBERS, D. C. (1949). Chemical determination of tryptophan in proteins. *Anal. Chem.*, **21**(10), 1249.

VANANUVAT, P. (1973). Production, composition and properties of protein from *Saccharomyces fragilis* grown on crude lactose. Ph.D. Thesis. Cornell University, Ithaca, New York.

WIMPENNY, J. W. T. (1967). Breakage of microorganisms. *Proc. Biochem.*, **2**(7), 41.

ZIEGLER, K., MELCHERT, I. & KURKEM, C. (1967). Nδ-(2-amino-2-carboxy-ethyl)-ornithine, a new amino acid from alkali treated proteins. *Nature*, **214**, 404.

Food Chemistry 6 (1981) 19–40

SAPONINS IN FOOD—A REVIEW

DAVID OAKENFULL

CSIRO Division of Food Research,
PO Box 52, North Ryde, New South Wales 2113, Australia

(Received: 25 October, 1979)

ABSTRACT

Saponins are surface active sterol or triterpene glycosides. They occur in a large number and a wide variety of plants but only about 28 of these are regularly used as food by man. The more commonly eaten of these are soybeans, chick peas, peanuts and spinach. Many different saponins occur, even within a single plant species. Although they can be readily isolated from plant materials by solvent extraction, it is difficult to isolate individual saponins from the crude mixture and this has rarely been achieved. The presence of saponins in plant extracts is readily indicated by their haemolytic activity and ability to form stable foams in aqueous solution, but for unambiguous identification, it is essential to use thin layer chromatography, with as wide as possible a range of spray reagents. Quantitative analysis of saponins is also best achieved by thin layer chromatography. Older methods, such as that based on haemolytic activity, are not reliable. Full chemical structures of some of the food saponins are known, but for the most part information is at present limited to the structure of their aglycones and the identity of the sugars. Although saponins have antibiotic activity and are toxic to fish and insects, they appear to be practically non-toxic to man, remaining within the gastrointestinal tract. Dietary saponins, either isolated or as saponin-containing food plants, lower plasma cholesterol levels in several mammalian species. They are therefore probably important in human diets to reduce the risk of coronary heart disease.

1. INTRODUCTION

Saponins are sterol or triterpene glycosides, mainly of plant origin. They occur in a wide variety of plants, a few of which are used as human food; examples of some more commonly used are soybeans, chick peas, peanuts and spinach. Although

Food Chemistry 0308-8146/81/0006-0019/$02·25

according to source authorities saponins are practically non-toxic to man when taken orally (Merck Index, 1976), they are known to have a number of physiological effects on animals of which the most interesting is that they can lower plasma cholesterol concentrations (Newman *et al.*, 1957; Griminger & Fisher, 1958; Malinow *et al.*, 1977; Oakenfull *et al.*, 1979; Topping *et al.*, 1980). Since it has been suggested that this effect may also occur in humans and that foods rich in saponins may reduce the risk of heart disease (Cheeke, 1976; Oakenfull *et al.*, 1979; Potter *et al.*, 1979) there is a need to identify actual and potential sources of dietary saponins.

It is the purpose of this review to identify those food plants and plant products which contain significant levels of saponins and to survey what is known about the chemical, physical and physiological properties of these materials. The properties of saponins in general are discussed only briefly as background information. There are several recent reviews (Hiller *et al.*, 1966; Basu & Rastogi, 1967; Birk, 1969; Tschesche & Wulff, 1973; Bondi *et al.*, 1973; Cheeke, 1976) and older publications (Kofler, 1927; Ewart, 1931) which provide more detailed information.

2. STRUCTURE AND GENERAL CHARACTERISTICS OF SAPONINS

Saponins are amphiphilic compounds in which sugars (pentoses, hexoses or uronic acids) are linked to a non-polar group (the sapogenin) which may be either a sterol or a triterpene. Most of the sapogenins that have been characterised are triterpenes.

Saponins occur almost exclusively in plants—the only other organisms known to contain saponins are certain species of starfish (Kitagawa & Kobayashi, 1977). They occur in a wide variety of plants with no obvious preponderance in particular species. Only a few of these plants though are used as food by man—possibly because a bitter taste is one of the general characteristics of saponins, although plants can contain other, more powerful, bitter principles.

A very large number of saponins may occur even within a single plant species. Considering soybeans as a typical example, five sapogenins have been identified (Fig. 1) and these are linked to three monosaccharides which may be any three from galactose, arabinose, rhamnose, glucose, xylose or glucuronic acid (Willner *et al.*, 1964). Thus there are $5 \times P_3^6 = 600$ possible structures! Not all of these, of course, need be present but there seems little doubt that there are considerably more than the ten saponins which have so far been separated chromatographically from soybeans (Wolf & Thomas, 1970). The difficulty of separating these complex mixtures and obtaining pure materials is one of the major problems in studying the properties of saponins.

The amphiphilic nature of saponins dominates their physical properties in solution. They are strongly surface active, forming stable foams and acting as emulsifying agents. They generally have a strong haemolytic activity and appear to form micelles in much the same way as detergents (Kofler, 1927). These physical

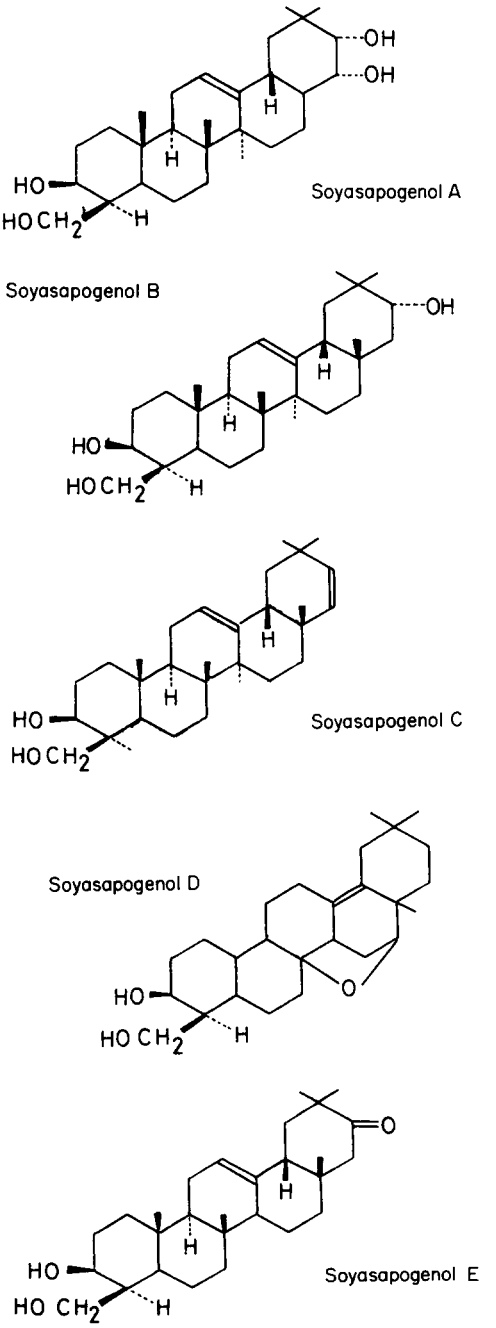


Fig. 1. Soyasapogenols.

properties are exploited in most of the technological uses of saponins—such as in shampoos and carbonated drinks.

3. DETECTION AND ISOLATION OF SAPONINS

Saponins can be isolated from plant materials by extraction with organic solvents. The plant material is first extracted with acetone or diethyl ether, preferably using a Soxhlet extractor to remove lipids, pigments, etc. The solvent is then changed to methanol to give a crude extract containing the saponins. Certain water-soluble saponins can be isolated from this crude extract as the cholesterol complex (see, for example, Walter *et al.*, 1954) but a more general procedure is that described by Kitagawa *et al.* (1976). It is possible though to demonstrate the presence of saponins in the crude extract by thin layer chromatography without proceeding to further purification steps (Kartnig & Ri, 1973).

The presence of saponins is also readily indicated by their haemolytic activity and their ability to form stable foams in aqueous solution. These properties, though, are characteristic of surfactants in general and are not unequivocal for the presence of saponins. They are good indications that saponins may be present but thin layer chromatography is required for more definite identification.

Solvents which have been reported as suitable for developing thin layer plates are summarised in Table 1. A variety of spray reagents can be used which give characteristic colours with saponins, and a few of the more commonly used ones are described in Table 2. These reactions, being characteristic of steroids or triterpenes in general (Lisboa, 1976) are not specific for saponins and it is essential to use several criteria for identification of saponins. The plates can be also sprayed with reagents specific for sugars (such as silver nitrate) and a method has recently been reported in which haemolysis is used to detect saponins on thin layer plates. The plates are

TABLE 1
SOLVENTS SUITABLE FOR THIN LAYER CHROMATOGRAPHY OF SAPONINS ON KIESELGEL

Solvent	*Type of saponin*	*Reference*
Chloroform/methanol/water 65:20–30:10	non-polar	(*Tschesche & Wulff, 1973*)
Chloroform/methanol/water 65:35:10	neutral and non-polar	(*Tschesche & Wulff, 1973*)
Acetic acid/ethanol/water 70:15:15	acid and neutral	(*Tschesche & Wulff, 1973*)
n-Butanol/ethanol/water[a] 1:1:1	acid and neutral	(*Coulson, 1958*)
n-Butanol/ethanol/1 M ammonia 60:13:30·5	polar and acid	(*Wolf & Thomas, 1970*)
n-Butanol/ethanol/15 M ammonia 7:2:5	polar and acid	(*Wolf & Thomas, 1970*)

[a] Developed for paper chromatography.

TABLE 2
SPRAY REAGENTS SUITABLE FOR DETECTING SAPONINS BY THIN LAYER CHROMATOGRAPHY (LISBOA, 1976)

Name	*Composition*	*Conditions*	*Colours*[a]
Carr–Price	Saturated antimony trichloride in chloroform[b]	Heat at 105 °C for 15 min	green–blue–grey
Liebermann–Burchard	30 % Acetic anhydride in 50 % sulphuric acid	Heat at 90 °C for 10 min	green–blue
Vanillin-phosphoric acid	2 % Solution of vanillin in phosphoric acid/ethanol (1:4)	Heat at 120 °C for 10–20 min	grey–blue–mauve
Ekkert	1 % *p*-Anisaldehyde in acetic acid/ sulphuric acid (98:2)	Heat at 90 °C for 10 min	grey–blue–mauve

[a] The colour produced depends on the nature of the sapogenin
[b] This has been used as a dip reagent. It is toxic and suitable precautions should be observed during its use.

coated with a suspension of erythrocytes in gelatin and isotonic sodium chloride. Saponins then appear as white spots on a red background (Smoczkiewicz *et al.*, 1977). Spots which show up by all three of these methods can reasonably be assumed to be saponins.

4. QUANTITATIVE DETERMINATION OF SAPONINS

A useful indication of the saponin content of a sample of plant material—albeit only a lower limit—can be obtained simply by determining the yield of purified saponin, following the procedure of Birk and co-workers (1963) and Applebaum *et al.* (1969). Alternatively, several of the methods used for detection of saponins can be modified to give quantitative information.

A very simple method is based on the foam-forming properties of saponins. A standard volume (e.g. 5 ml) of the saponin solution in 1/15 M dipotassium hydrogen phosphate is shaken for 1 min, in a 25 ml measuring cylinder. The volume of foam remaining in the cylinder after it has stood for a minute is then proportional to the concentration of saponin (O'Dell *et al.*, 1959; Birk, 1969). This method has the disadvantage that it obviously relies on the complete absence of other surfactants and it is not particularly sensitive, being limited to an amount of saponin in excess of about 500 μg.

Various quantitative methods using haemolysis have recently been reviewed by Birk (1969) and the older methods were described in detail by Kofler (1927). These methods rely on the fact that a critical concentration of saponin (the 'haemolytic index'†) is required to lyse erythrocytes. Various amounts of the saponin-containing material are mixed with a suspension of washed erythrocytes in isotonic buffer at pH 7·4. After 24 h the mixture is centrifuged and haemolysis is indicated by the presence

† The 'haemolytic index' is defined as the maximum dilution of saponin (in grammes isotonic salt solution per gramme of saponin) which will cause haemolysis.

of haemoglobin in the supernatant. The minimum amount that will produce haemolysis then gives the saponin concentration—provided that the haemolytic index for that particular saponin, or mixture of saponins, is known. The haemolytic index depends both on the nature of the saponin and the species of animal from which the erythrocytes were obtained, so it is essential to use standards prepared from a purified sample of the saponin, or mixture of saponins, that is being measured.

A very sensitive variant of the haemolysis method has been developed by Kartnig *et al.* (1964). Filter paper discs are wetted with the saponin solution and embedded in a preparation of erythrocytes in gelatin. The diameter of the area over which haemolysis extends within a specified time is then proportional to the concentration of saponin (within a limited range). Again, different calibration curves are needed for saponins of different origin.

Haemolytic methods again have the disadvantage that they rely on the complete absence of other surface-active compounds which may also be haemolytic. Consequently, although very sensitive they are unsuitable for routine testing of unknown plant materials.

Spectrophotometric methods have been reported which exploit the colours produced by reaction of saponins with vanillin (Hiai *et al.*, 1976) or anisaldehyde (Baccou *et al.*, 1977). Although very sensitive, these methods are not suitable for estimating saponins in crude plant extracts since the reactions are not specific and coloured products may be formed from other phytosterols and compounds such as flavanoids which are likely to accompany saponins in such preparations.

A method specifically designed for determining soybean saponins has been described by Gestetner *et al.* (1966). Defatted material (either ground soybeans or soyflour) is refluxed with 0·5 M sulphuric acid in dioxane/water (1:3) to hydrolyse the saponins. The sapogenins that are produced are extracted with diethylether and purified on an alumina column. The concentration of sapogenin in a solution of the purified product can then be determined spectrophotometrically using a modified Liebermann–Burchard reagent (acetic acid/sulphuric acid; 3:2).

Probably the most sensitive and reliable general methods are those based on thin layer chromatography. Quantitative results can be obtained in two ways. The density of the spots obtained with a suitable spray reagent can be measured directly using a densitometer. Alternatively, the saponin spots can be located by using iodine vapour, then scraped off into tubes and treated with concentrated sulphuric acid. The intensity of the brown colour that is produced is then determined spectrophotometrically (Kartnig *et al.*, 1972). The densities of the spots and the intensity of the colours produced by the test samples are then related to the densities and intensities produced from standard solutions of the saponin to provide a measure of the amount present in the unknown sample. Saponins of different origin are likely to produce colours of different intensity so it is again essential to use standards prepared from a purified sample of the saponin that is being measured.

5. FOOD PLANTS WHICH CONTAIN SAPONINS

A search of *Chemical Abstracts* and previous reviews of the literature on saponins revealed only 28 plant species also mentioned in the *Oxford book of food plants* (1969). These are listed in Table 3. Of these, only 17 are used in quantity in any of the world's cuisines. The remainder are herbs and spices which would not contribute significantly to dietary intake of saponins.

The most significant sources of saponins in man's diet are the legumes: soybeans, chick peas, mung beans, peanuts, broad beans, kidney beans and lentils. World production and average *per capita* consumption of some of these are given in Table

TABLE 3
SAPONIN-CONTAINING FOOD PLANTS

Plant	*Saponin content when known (% dry weight)*	*Reference*
Lucerne (*Medicago sativa*)[a]	2–3	(Birk, 1969)
Soybeans	5·6[b]	(Birk, 1969; Smith & Circle, 1972)
Chick peas (*Cicer arietinum*)	3·6	(Applebaum *et al.*, 1969)
Mung beans (*Phaseolus mungo*)	—	(Basu & Rastogi, 1967)
Oats (*Avena sativa*)	0·1–0·3	(Tschesche & Wulff, 1969)
Aubergine (*Solanum melongena*)	—	(Noble, 1948)
Spinach (*Spinacea oleracea*)	—	(Kofler, 1927; Tschesche & Wulff, 1969)
Peanuts	—	(Applebaum *et al.*, 1969)
Amaranthus (*Amaranthus spinosus*)[c]	—	(Kofler, 1927)
Asparagus (*Asparagus officinalis*)	—	(Kawano *et al.*, 1977)
Broad bean (*Vicia faba*)	3·7	(Vinkler & Smyslova, 1967)
Fenugreek	—	(Valoshina *et al.*, 1977)
Kidney beans (*Phaseolus vulgaris*)	—	(Kretsu *et al.*, 1972)
Garlic (*Allium sativum*)	—	(Smoczkiewiczowa & Nitschke, 1975)
Ginseng	—	(Shibata, 1977)
Lentil (*Lens culinaris*)	4	(Applebaum *et al.*, 1969)
Liquorice (*Glycyrrhiza macedonica*)	—	(Shibata, 1977)
Nutmeg (*Myristica fragrans*)	—	(Varshney & Sharma, 1968)
Sarsparilla	—	(Kofler, 1927)
Sugar beet (*Beta vulgaris*)	—	(Birk, 1969)
Sunflower (*Helianthus annus*)	—	(Chirva *et al.*, 1968)
Thyme (*Thymus vulgaris*)	—	(Kofler, 1927)
Tea (*Thea sinensis*)	—	(Kofler, 1927)
Yams (*Dioscorea*)	—	(Kofler, 1927)
Winter squash (*Cucurbita maxima*)	—	(Kofler, 1927)
New Zealand spinach (*Tetragonia expansa*)	—	(Kofler, 1927)
Orache (*Atriplex hortensis*)[d]	—	(Kofler, 1927)
Garden peas	2·5	(Applebaum *et al.*, 1969)

[a] In Australia usually eaten as shoots.
[b] See Table 5.
[c] Used as a spinach in different parts of the tropics (*Oxford book of food plants*, p. 160).
[d] A substitute for spinach or sorrel (*Oxford book of food plants*, p. 160).

TABLE 4
WORLD PRODUCTION AND AVERAGE *per capita* CONSUMPTION OF MAJOR SAPONIN-CONTAINING FOODS

	Soybeans	*Groundnuts*	*Chick peas*	*Sesame seeds*	*Lentils*
Production (1975; 1000 tonnes)[a]	69670	19428	5569	1907	1104
Per capita consumption (g/year, 1972–1974 average)[b]	2·56	1·26	2·48	0·22	—

[a] *Production year book*, Food & Agriculture Organisation of the United Nations, Rome, 1977.
[b] *Provisional food balance sheets*, Food & Agriculture Organisation of the United Nations, Rome, 1977.

TABLE 5
SAPONIN CONTENT OF SOME SOYA BEAN PRODUCTS[a]

Product	% *Saponins (dry weight)*
Whole soybeans	5·6
Soya hulls	2·0
Defatted soya flour	2·2
'Promine-D'[b]	0·3
'G.L. 750'[c]	0·8
'Maxten C'[d]	1·9
'Maxten E'[d]	2·5
Tofu[e]	2·1
Lecithin	2·9

[a] From Potter *et al.*, (1979)
[b] Soyprotein isolate obtained from Central Soya Co. Inc., Illinois, USA.
[c] Soyprotein isolate obtained from Griffith Laboratories Pty Ltd., Victoria, Australia.
[d] Textured soyprotein obtained from Miles Laboratories (Australia) Pty Ltd, Victoria, Australia.
[e] Soybean curd.

4. Soybeans appear to be the major source of dietary saponins. They are used in a great variety of different ways and the various soybean products, including soyprotein isolate† can contain very significant quantities of saponins (see Table 5). The next most significant source of dietary saponins is chick peas which are eaten mainly in the eastern Mediterranean countries, the Middle East, North and East Africa and parts of Asia.

† Saponins have also been found in leaf protein concentrate from lucerne and under some conditions may be at higher concentration than in the original leaf material (Bickoff, 1975; Livingston *et al.*, 1979).

6. THE CHEMISTRY OF FOOD SAPONINS

Soybeans

The sapogenins have been isolated and characterised (see Fig. 1) and the sugars have been identified as rhamnose, galactose, glucose, arabinose, xylose and glucuronic acid. Complete structures of three of the saponins have been determined (Kitagawa *et al.*, 1976). These are soyasaponin I (the major component) and soyasaponins II and III, all of which have soyasapogenin B as the common aglycone. The complete structures are shown in Fig. 2(a), (b) and (c).

Lucerne

Early work identified the sugars glucose, galactose, xylose and rhamnose and showed that soyasapogenols A, B and C were present as aglycones (Walter *et al.*, 1955). The sapogenins are now known to be the five soyasapogenins plus medicagenic acid and hederagenin (Fig. 2(d)). The sugars are the same as those in soybean saponins (Bondi *et al.*, 1973). The structure of one of the saponins has been established (Fig. 2(e)).

Silver beet and sugar beet

The most abundant sapogenin is oleanolic acid (Fig. 2(f)) but there are at least five

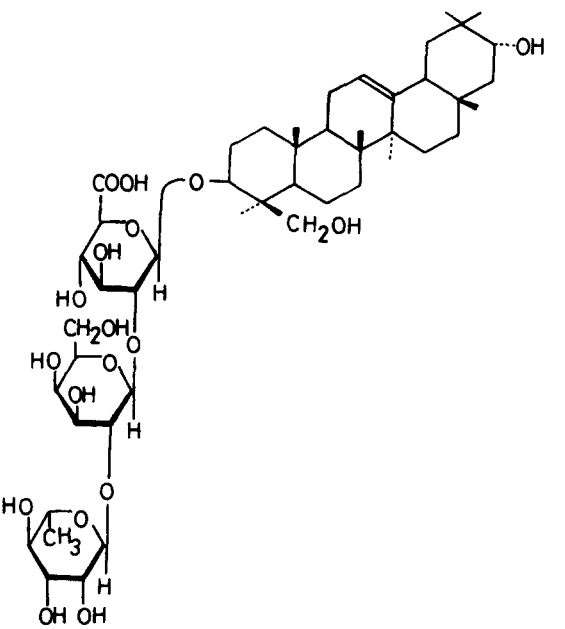


Fig. 2(a). Soyasaponin I.

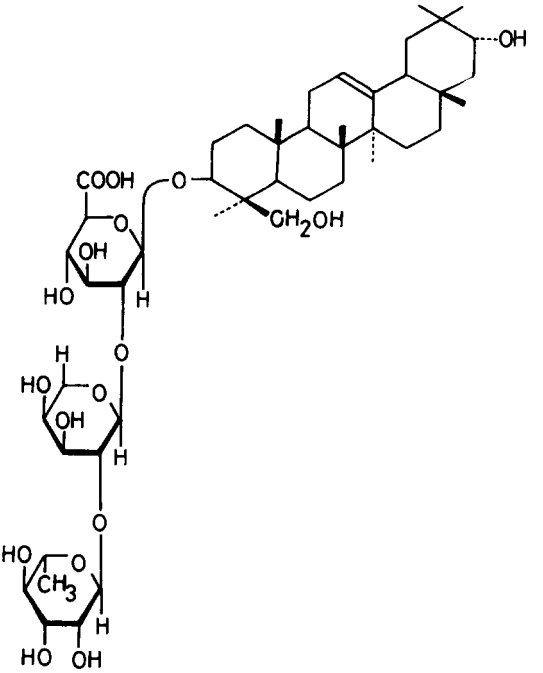


Fig. 2(b). Soyasaponin II.

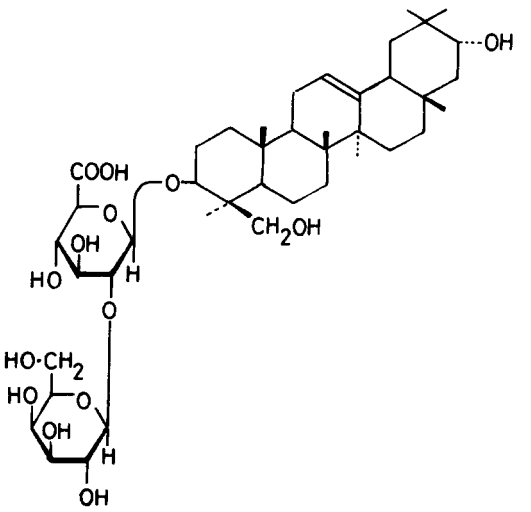


Fig. 2(c). Soyasaponin III.

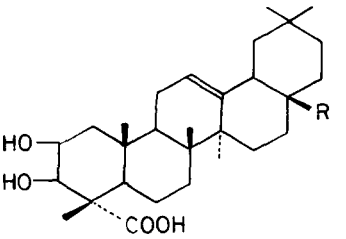


Fig. 2(d). R = COOH, medicagenic acid; R = CH_2OH, hederagenin.

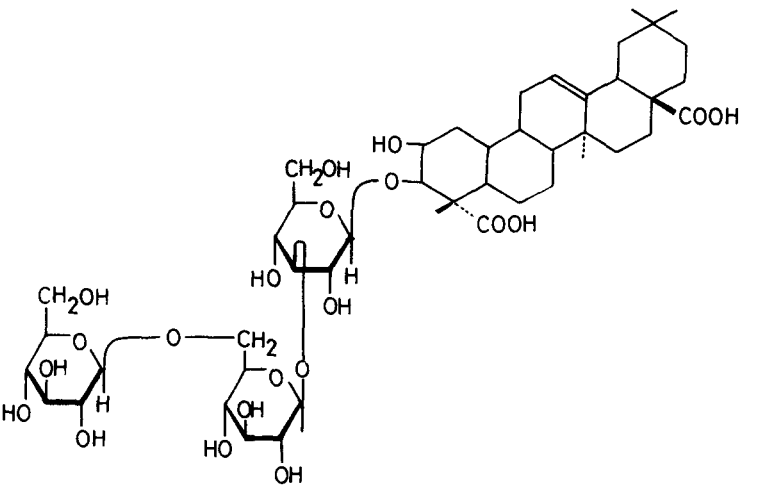


Fig. 2(e). A lucerne saponin.

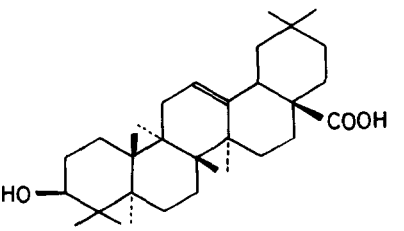


Fig. 2(f). Oleanolic acid.

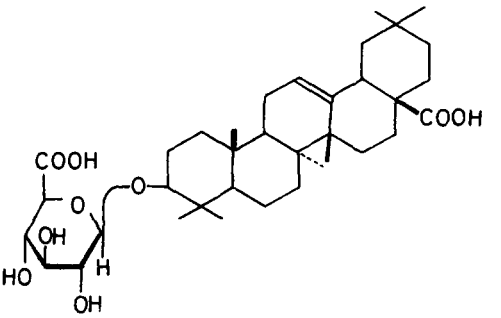


Fig. 2(g). Sugar beet saponin.

others. The structural formula (Fig. 2(g)) of one of the saponins has been established (Wagner & Sternkopf, 1958).

Spinach

Two sapogenins have been identified—oleanolic acid (Fig. 2(f)) and hederagenin (Fig. 2(d))—and structural formulae (Fig. 2(h)) have been reported for two of the saponins (Tschesche & Wulff, 1969).

Phaseolus radiatus (*green gram*)

Not much is yet known about the saponins from this species. Soyasapogenol C (Fig. 1) has been identified and the sugars glucose, arabinose, rhamnose and glucuronic acid (Hiller & Voigt, 1977).

Phaseolus vulgaris (*haricot or kidney bean*)

Five different saponins have been isolated. One sapogenin has been identified (soyasapogenol C) with the sugars, glucose, galactose, arabinose, rhamnose and glucuronic acid (Hiller & Voigt, 1977).

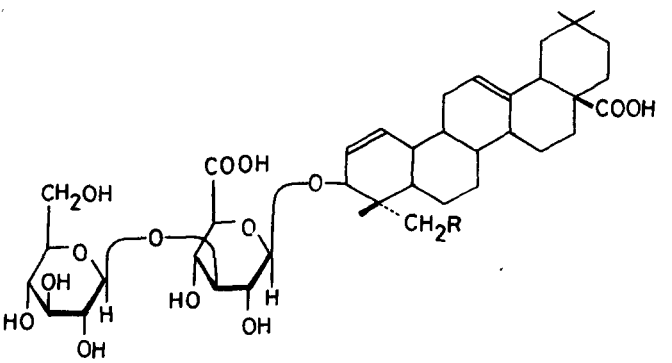


Fig. 2(h). Spinach saponins; R = H or OH.

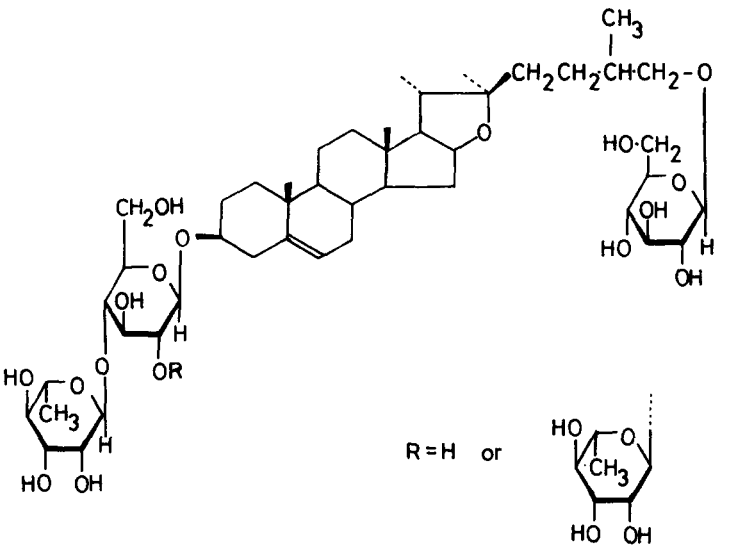


Fig. 2(i). Asparagus saponins.

Asparagus

These are the only steroid saponins known to occur in food plants. The structural formulae of two of them (Fig. 2(i)) have recently been reported (Kawano *et al.*, 1977).

Oats

Two saponins have been reported and their structures investigated but this work is not yet complete (Burkhardt *et al.*, 1964; Tschesche *et al.*, 1973).

Sunflower seeds

Three sapogenins have been identified—soyasapogenols A, B and C (Fig. 1). The sugars that have been identified are glucose, arabinose, xylose and rhamnose (Chirva *et al.*, 1968). No detailed structural information is yet available.

7. BIOLOGICAL AND NUTRITIONAL PROPERTIES OF SAPONINS

Saponins occur in numerous herbal remedies—both oriental and occidental—such as liquorice, sarsaparilla and ginseng (Shibata, 1977). In general they seem to have expectorant and antiinflammatory properties, but probably the most significant property of saponins, considered as dietary components for humans, is their effect on plasma lipid concentrations.

Effect on plasma lipids

It has been known for some time that dietary saponins reduce plasma cholesterol concentrations in animals (Newman *et al.*, 1957; Griminger & Fisher, 1958; Malinow *et al.*, 1977; Oakenfull *et al.*, 1979; Topping *et al.*, 1980). It is generally assumed that, because some saponins form insoluble complexes with cholesterol, saponins combine with cholesterol in the gut and that this prevents or inhibits cholesterol absorption. This explanation seems unlikely though because the cholesterol-lowering effect can be demonstrated with saponins which do *not* complex with cholesterol (Cheeke, 1976). A more likely explanation is that saponins induce adsorption of bile acids by dietary fibre.

Adsorption of bile acids by dietary fibre has been suggested as a general mechanism to explain the cholesterol lowering action of many foods of plant origin (Burkitt & Trowell, 1975). Adsorption would increase the loss of bile acids by faecal excretion and this loss would necessarily be offset by an increased conversion of cholesterol into bile acids in the liver. It has recently been shown, though, that the presence of saponins is required for bile acids to bind to plant fibre preparations *in vitro* (Oakenfull & Fenwick, 1978). Presumably this is because saponins are strongly surface active, but the mechanism of the process has not yet been investigated in detail. It does, however, appear to operate *in vivo* as well as *in vitro* since feeding a purified saponin mixture (commercial 'saponin white') increases the faecal excretion of bile acids in the rat (Oakenfull *et al.*, 1979) and pig (Topping *et al.*, 1980), as well as lowering plasma cholesterol levels.

Most of those plant materials which have a cholesterol-lowering effect in animal feeding trials appear to contain saponins (see Table 6). Carroll (1977) has shown that the hypercholesterolaemia of rabbits on a semisynthetic diet is reduced if the protein component of the diet is of plant, rather than animal, origin. Examination of Carroll's list of proteins (Table 7) shows that those which have the greatest cholesterol-lowering effect are from saponin-containing plants. The effect is thus probably due to saponins, which are likely to have been present as contaminants since samples of soyaprotein isolate and leaf protein from lucerne have been shown to contain saponins (Potter *et al.*, 1979; Bickoff, 1975; Livingston *et al.*, 1979).

The accumulated evidence from feeding trials involving both animals and humans (see Table 6) strongly suggests that saponins (as saponin-containing food plants) could be important in human diets as a means of lowering the plasma cholesterol concentrations. The increase in the incidence of heart disease in Western societies seems to coincide with a decline in the consumption of saponin-rich legumes. From being a major component of the diet of medieval Europe their consumption has dwindled almost to insignificance (Drummond & Wilbraham, 1970). This is illustrated by a comparison of British naval rations for 1745 and 1811 (Table 8). The earlier ration includes oatmeal and 'pease', a mixture of legumes which would contain at least some saponin-containing varieties. The latter diet appears to be saponin-free. Foods containing saponins now make little contribution to the normal Western diet.

TABLE 6
PLANTS OR PLANT PRODUCTS REPORTED TO LOWER PLASMA CHOLESTEROL LEVELS

Plant material (indicates those which are known to contain saponins)*	*Experimental animal*	*Reference*
*Lucerne (*Medicago sativa*)	Rabbit	(Horlick *et al.*, 1967)
*Oats	Chicken	(Fisher & Griminger, 1967)
*Rolled oats	Man	(de Goot *et al.*, 1963)
*Chick peas (*Cicer arietinum*)	Man	(Mathur *et al.*, 1968)
*Mung beans (*Phaseolus mungo*)	Man	
*Green gram (*Phaseolus radiatus*)	Man	
Horse gram (*Dolichus biflorus*)	Man	
Red Gram (*Cajanus cajan*)	Man	
Haricot beans (*Phaseolus vulgaris*)	Man	(Luyken *et al.*, 1962)
Wheat straw	Rabbit	(Moore, 1967)
Rice bran	Rat	
Pectin	Man	(Keys *et al.*, 1961; Jenkins *et al.*, 1975)
Guar gum	Rat	Riccardi & Fahrenbach, 1969)
Guar gum	Man	Jenkins *et al.*, 1975)
Carageenan	Rat	(Riccardi & Fahrenbach, 1969)
*Soybean textured protein	Man	(Sirtori *et al.*, 1977)
*Soybean isolate	Rabbit	(Carroll, 1977; Belton & Truswell, 1978)
*Soybean lecithin	Man	(Simons, 1978)

TABLE 7
EFFECTS OF PROTEINS FROM DIFFERENT SOURCES ON PLASMA CHOLESTEROL LEVELS OF RABBITS FED A CHOLESTEROL-FREE SEMISYNTHETIC BASAL DIET[a]

Source of protein	*Plasma cholesterol (mg/ml)*[b]
Whole egg	235
Skimmed milk	230
Lactalbumin	215
Casein	204
Fish meal	166
Beef steak	160
Pork	110
Egg White	105
Rapeseed flour ('detoxified')	91
Wheat gluten	80
Peanuts[c]	80
Cottonseed	76
Sesame seed[c]	70
Soyprotein isolate[c]	67
Sunflower seed[c]	53
Peas[c]	41
Faba beans[c]	30

[a] From Carroll (1977).
[b] Mean value, 4–6 animals.
[c] Saponin-containing plant.

TABLE 8
BRITISH NAVAL RATIONS

Food	*Daily allowance (oz)*	
	1745	*1811*
Cheese	1·7	1·7
Salt beef	9	4·5
Salt pork	4·6	2·2
Butter	1	1
Suet	nil	0·2
Bread, flour or ship's biscuit	16	19
Oatmeal	5·7	nil
Pease	10·3	nil
Sugar	nil	1
Beer (pints)	8	2

Toxicity of saponins

Before proceeding to feeding trials with humans or recommending saponins as a prophylactic to reduce the risk of heart disease it is clearly essential to know something about both the short term and the chronic toxicity of saponins.

This is a complex problem because saponins are a class of compounds, not a single compound.

The Merck Index (1976) states that saponins are practically non-toxic to man upon oral ingestion. Plant extracts, such as liquorice and sarsaparilla, are permitted as food flavourings in Australia (National Health and Medical Research Council, 1976), Britain (George, 1965) and the USA (George, 1965). Quillaia, a commercial preparation of saponin from quillay bark (*Quillaia saponaria*), is permitted for food use in Britain and 'generally recognised as safe' (GRAS) in the USA (George, 1965). (The levels in use in the USA are given in Table 9 and in Britain quillaia extract is permitted in soft drinks at not more than 200 ppm under the Emulsifiers and Stabilisers in Food Regulations 1975 Act (Statutory Instrument 1975, no. 1486)). There is clearly some uncertainty about the safety of saponins, though, since quillaia is prohibited as a food additive in West Germany (George, 1965) and all saponins

TABLE 9
APPROXIMATE AVERAGE CONCENTRATION OF QUALLAIA SAPONIN USED IN FOODSTUFFS IN THE USA[a]

Foodstuff	*ppm*
Beverages	95
Ice cream, ices	0·12
Candy	18
Syrups	6·8

[a] (National Academy of Sciences, 1965).

are prohibited in foodstuffs in Spain and Morocco (George, 1965). Neither Britain nor the USA has legislation controlling the use of saponins as a class, presumably because of the considerable variation in the toxicity of different saponins.

The oral toxicity of saponins from a number of plant sources is given in Table 10. The lethal dose varies from 25 to 3000 mg/kg body weight. Saponins normally remain within the digestive tract (Birk, 1969) but in severe poisoning gastrointestinal lesions may occur, allowing saponins to enter the blood stream. It is when saponins

TABLE 10
ORAL TOXICITY OF SOME SAPONINS[a]

Source of saponin	*Animal*	*Dose*	*Dosage (mg/kg)*	*Reference*
Sapindus sapindus	mouse	LD	3000	(Spector, 1956)
	mouse	LD	1000	(Spector, 1956)
Agrostemma githago (common corn cockle)	rat	LD_{50}	>50	(Vogel & Marek, 1962)
	rabbit	LD	56–62	(Spector, 1956)
	dog	LD	20–25	(Spector, 1956)
Saponaria vaccaria	mouse	LD_{50}	960	(Abubakirov *et al.*, 1960)
Aesculus hippocastanum (horse chestnut)	rat	LD_{50}	>50	(Abubakirov *et al.*, 1960)
Hedera helix (ivy)	rat	LD_{50}	>100	(Abubakirov *et al.*, 1960)
Gypsophila paniculata	rat	LD_{50}	50	(Vogel & Marek, 1962)
Cyclamen europaeum	rat	LD_{50}	>160	(Vogel & Marek, 1962)
Digitalis purpurea (fox glove)	rat	LD_{50}	>50	(Vogel & Marek, 1962)
	mouse	LD	90	(Spector, 1956)

[a] A condensed version of data compiled by George (1965).

enter the blood stream that damage occurs (the intravenous lethal dose can be one thousandth of the lethal oral dose (George, 1965)). The result is liver damage, haemolysis of red blood cells, respiratory failure, convulsions and coma (Martindale, 1972). Irritation of the gastrointestinal tract by some other cause may increase an animal's susceptibility to saponin poisoning (Ewart, 1931) and it has been suggested that continued ingestion of sublethal doses of saponins can lead to 'corrosion of the intestine' so that saponins eventually enter the blood stream (Solman, 1957). Ewart (1931) advised that people using drastic purgatives or having inflamed intestines or cirrhotic livers should avoid all foods containing saponins, whether of natural or artificial origin.

Effects of sublethal doses have been thoroughly investigated for the saponins from lucerne and soybeans (Lindahl *et al.*, 1957; Heywang *et al.*, 1959; Birk, 1969). Lucerne saponins are probably a cause of bloat in ruminants (Lindahl *et al.*, 1957; Coulson & Davis, 1960) and have been shown to depress the growth rate of rats (Coulson & Evans, 1960) and chicks and reduce egg production by laying hens (Heywang *et al.*, 1959; Birk, 1969). Soybean saponins, however, have no effect on the

growth rate of chicks (Birk, 1969) or other animals (Birk, 1969; Ishaaya *et al.*, 1969). There are also reports which show no adverse effects of consumption of other saponins at sublethal levels:

(1) Ewart (1931) found that guinea pigs could be fed plants rich in saponins for up to ten months without apparent injury. (The plants were *Atalaya hemiglauca* and *Senecio jacobaea*, from Central Australia and New Zealand respectively. They were investigated because they can poison sheep and cattle.)

(2) Oser (1966) fed rats for 12 weeks on control diets and diets containing 0·05 % quillaia saponin or up to 3 % saponin from *Yucca mohavensis* (a Californian desert plant). The saponins had no adverse effects in respect to growth, food utilisation, blood counts, blood glucose, urine analysis or in gross histological findings *post mortem*.

(3) Gaunt *et al.* (1974), in a similar experiment, fed rats for 13 weeks on diets containing up to 4 % quillaia saponin. They found no abnormalities and noted that there was no diarrhoea or other sign of gastrointestinal irritation.

Unfortunately these are all relatively short term studies. One long term toxicity study has recently been reported (Phillips *et al.*, 1979). Quillaia saponin at the level of 0·5 % of the diet had no adverse effects on mice over an 84 week period. Martindale (1972) states that quillaia saponin is too irritant to the gastrointestinal tract to be used internally and most pharmacopoeias advise against the internal use of saponins (George, 1965). The chronic toxicity of saponin would have to be examined further before saponins could be recommended for human consumption as a means of lowering the serum cholesterol level. An exception would be saponins consumed in the form of a traditional foodstuff such as soybeans, consumed in 'normal' quantities.

There seems little doubt, though, that the saponins from soybeans, lucerne, or quillaia are safe for short term human feeding trials at levels of below 50 mg/kg body weight—about 3 g/day.

8. FUTURE RESEARCH

The major problems in saponin chemistry lie in the enormous diversity of naturally occurring saponins. It is clear from the very different properties of the crude mixtures of saponins isolated from different plants that subtle variations in structure give rise to substantial variations in physical, chemical and biological properties. Isolation of pure compounds from a crude extract is very difficult, making it almost impossible to obtain pure compounds in sufficient quantity to investigate their solution chemistry or biological properties. There is thus a need for separation procedures capable of yielding gramme quantities.

Saponins are potentially of great significance in human nutrition since it seems likely that the low saponin content of the typical Western diet may be partly responsible for the high incidence of heart disease in Western countries. For further research in this area it is essential to have reliable quantitative information about the saponin content of various foodstuffs and how this may be affected by processing and cooking. It would then be possible to confirm the results of the earliest recorded human feed trial:

> 'Then said Daniel to Melzar . . . "Prove thy servants I beseech thee ten days and let them give us pulse to eat and water to drink. Then let our countenances be looked upon before thee, and the countenances of the children that eat of the portion of the king's meat". So he consented in this matter and proved them ten days. And at the end of ten days their countenances appeared fairer and fatter in flesh than all the children which did eat the portion of the king's meat'.
>
> (Book of Daniel, Chapter 1.)

REFERENCES

ABUBAKIROV, N. K., KAMBULIN, N. A., KRYZHENKOV, A. N., SULTANOV, T. & EL'KIND, L. A. (1960). Pharmacotoxicological properties of saponin from leaves of *Saponaria vaccaria*. *Spornik Nauch. Trudov. Tashkent Med. Inst.*, **16**, 222. (*Chem. Abst.*, **56**, 2680.)

APPLEBAUM, S. W., MARCO, S. & BIRK, Y. (1969). Saponins as possible factors of resistance of legume seeds to the attack of insects. *J. Agric. Food Chem.*, **17**, 618–22.

BACCOU, J. C., LAMBERT, F. & SAUVAIRE, Y. (1977). Spectrophotometric method for the determination of total steroidal sapogenin. *Analyst*, **102**, 458–65.

BASU, N. & RASTOGI, R. P. (1967). Triterpenoid saponins and sapogenins. *Phytochem.*, **6**, 1249–68.

BELTON, E. A. & TRUSWELL, A. S. (1978). Effect of casein compared with soya-bean protein isolate on plasma cholesterol in the rabbit. *Proc. Nutr. Soc.*, **37**, 12A.

BICKOFF, E. M. (1975). *Welpro: Nutritional evaluation and current FDA status*. Twelfth Technical Alfalfa Conference. United States Department of Agriculture, Berkeley, pp. 110–14.

BIRK, Y., BONDI, A., GESTETNER, B. & ISHAAYA, I. (1963). A thermostable haemolytic factor in soybeans. *Nature*, **197**, 1089–90.

BIRK, Y. (1969). Saponins. In *Toxic constituents of plant foodstuffs* (Liener, I. E. (ed.)). Academic Press, New York, pp. 169–210.

BONDI, A., BIRK, Y. & GESTETNER, B. (1973). Forage saponins. In *Chemistry and biochemistry of herbage* (Butler, G. W. & Bailey, R. W. (eds.)), Vol. 1. Academic Press, New York, pp. 511–28.

BURKHARDT, H. J., MAIZEL, J. V. & MITCHELL, H. K. (1964). Avenacin: an antimicrobial substance isolated from *Avena sativa*. II. Structure. *Biochem.*, **3**, 426–31.

BURKITT, D. P. & TROWELL, H. C. (1975). Refined carbohydrate foods and disease. Academic Press, London.

CARROLL, K. K. (1977). The role of dietary protein in hypercholesterolemia and atherosclerosis. *Lipids*, **13**, 360–5.

CHEEKE, P. R. (1976). Nutritional and physiological properties of saponins. *Nutrition Reports International*, **13**, 315–24.

CHIRVA, V. Y., CHEBOV, L. & LAZUR'EVSKII, G. V. (1968). Sunflower saponins. *Prir. Soedin*, **4**, 140. (*Chem. Abst.*, **69**, 41746.)

COULSON, C. B. (1958). Saponins. I.—Triterpenoid saponins from lucerne and other species. *J. Sci. Food Agric.*, **9**, 281–8.

COULSON, C. B. & DAVIES, T. (1960). Lucerne saponin fractionation and bloat. *Nature*, **188**, 947–8.

COULSON, C. B. & EVANS, R. A. (1960). The effect of saponin, sterols and linoleic acid on the weight increase of growing rats. *Br. J. Nutr.*, **14**, 121–34.

DRUMMOND, J. C. & WILBRAHAM, A. (1970). *The Englishman's food.* Jonathan Cape, London.

EWART, A. J. (1931). *The poisonous action of ingested saponins.* Bulletin No. 50. Council for Scientific and Industrial Research, Melbourne.

FISHER, H. & GRIMINGER, P. (1967). Cholesterol-lowering effects of certain grains and of oat fractions in the chick. *Proc. Soc. Exp. Biol. Med.*, **126**, 108–11.

GAUNT, I. F., GRASSO, P. & GANGOLLI, S. D. (1974). Short-term toxicity of quillaia extract in rats. *Fd Cosmet. Toxicol.*, **12**, 641–50.

GEORGE, A. J. (1965). Legal status and toxicity of saponins. *Fd Cosmet. Toxicol.*, **3**, 85–91.

GESTETNER, B., BIRK, Y., BONDI, A. & TENCER, Y. (1966). Soya bean saponins. VII. A method for the determination of sapogenin and saponin contents in soya beans. *Phytochemistry*, **5**, 803–6.

DE GOOT, A. P., LUYKEN, R. & PIKAAR, N. A. (1963). *Lancet ii*, 303.

GRIMINGER, P. & FISHER, H. (1958). Dietary saponin and plasma cholesterol in the chicken. *Proc. Soc. Exp. Biol. Med.*, **99**, 424–6.

HEYWANG, B. W., THOMPSON, C. R., KEMMERER, A. R. (1959). Effect of alfalfa in saponin on laying chickens. *Poultry Sci.*, **38**, 968–75.

HIAI, S., OURA, H. & NAKAJIMA, T. (1976). Color reaction of some sapogenins and saponins with vanillin and sulfuric acid *Planta Med.*, **29**, 116–22.

HILLER, K., KEIPERT, M. & LINZER, B. (1966). Triterpensaponine. *Die Pharmazie*, **21**, 713–51.

HILLER, K. & VOIGT, G. (1977). Neue ergebnisse in der Erforschung der Triterpensaponine. *Die Pharmazie*, **32**, 365–93.

HORLICK, L., COOKSON, F. B. & FEDEROFF, S. (1967). *Circulation*, **35–36**, 11.

ISHAAYA, I., BIRK, Y., BONDI, A. & TENCER, Y. (1969). Soyabean saponins. IX. Studies of their effect on birds, mammals and cold-blooded organisms. *J. Sci. Fd Agric.*, **20**, 433–6.

JENKINS, D. J. A., LEEDS, A. R., NEWTON, C. & CUMMINGS, J. H. (1975). Effect of pectin guar gum and wheat fibre on plasma cholesterol. *Lancet i*, 1116–17.

KARTNIG, VON TH., GRAUNE, F. J. & HERBST, R. (1964). Zur frage der saponinverteilung bei *Ausculus hippocastanum* während verschiedner keimungs und wachstumsstadien. *Planta Med.*, **12**, 428–39.

KARTNIG, VON TH., RI, C. Y. & WEGSCHAIDER, O. (1972). Untersuchungen über die aufberetung und die stabilität von saponindrogen. *Planta Med.*, **22**, 127–35.

KARTNIG, VON TH. & RI, C. Y. (1973). Dünnschichtchromatographische untersuchungen an den saponin aus cortex quillajae. *Planta Med.*, **23**, 269–377.

KAWANO, K., HIROJI, S. & SAKAMURA, S. (1977). A bitter principle of asparagus. Part II. Isolation and structure of furastanol saponin in asparagus edible shoots. *Agric. Biol. Chem.*, **41**, 1–8.

KEYS, A., GRANDE, F. & ANDERSON, J. T. (1961). Fiber and pectin in the diet and serum cholesterol concentration in man. *Proc. Soc. Exp. Biol. Med.*, **106**, 555–88.

KITAGAWA, I., YOSHIKAWA, M. & YOSIOKA, I. (1976). Saponin and sapogenol. XIII. Structures of three soybean saponins: Soyasaponin I, soyasaponin II and soyasaponin III. *Chem. Pharm. Bull.*, **24**, 121–9.

KITAGAWA, I. & KOBAYASHI, M. (1977). On the structure of the major saponin from the starfish *Acanthaster planci. Tetrahedron Letters*, 859–62.

KOFLER, L. (1927). *Die saponine*, Springer-Verlag, Vienna.

KRETSU, L. G., KMTYA, P. K. & CHIRVA, V. Y. (1972). Structure of *phaseolus vulgaris* saponins. *Khim. Prir. Soedin.* 618–21. (*Chem. Abst.*, **79**, 97962.)

LINDAHL, I. L., DAVIS, R. E., TERTELL, R. T., WHITMORE, G. E., SHALKOP, W. T., DOUGHERTY, R. W., THOMPSON, C. R., VAN ATTA, G. R., BICKOFF, E. M., WALTER, E. D., LIVINGSTON, A. G., GUGGOLZ, J., WILSON, R. H., SIDEMAN, M. B. & DE EDS, F. (1957). Alfalfa saponins: studies on their chemical, pharmacological and physical properties in relation to ruminant bloat. *U.S. Dept. Agr. Tech. Bull. No. 1161.*

LISBOA, B. P. (1976). Thin layer chromatography of sterols and steroids. In *Lipid chromatographic analysis* (Marinetti, G. V. (ed.)). Marcel Dekker. New York.

LIVINGSTON, A. L., KNUCKLES, B. E., EDWARDS, R. H., DE FREMERY, D., MILLER, R. E. & KOHLER, G. O. (1979). Distribution of saponin in alfalfa protein recovery systems. *J. Ag. Food Chem.*, **27**, 362–5.

LUYKEN, R., DE WIJN, J. F., PIKAAR, N. A. & VAN DER MEER, R. (1965). The influence of rolled oats on the cholesterol content of the blood serum. *Voeding*, **26**(5), 229–44.

MALINOW, M. R., MCLAUGHLIN, P., KOHLER, G. O. & LIVINGSTON, A. L. (1977). Prevention of elevated cholesterolemia in monkeys. *Steroids*, **29**, 105–10.

MARTINDALE, (1972). *The extra pharmacopoeia*, 26th edn., Pharmaceutical Press. London, p. 393.

MATHUR, K. S., KHAN, M. A. & SHARMA R. D. (1968). *Br. Med. J. i*, 30–2.
MERCK INDEX (1976). 9th edn., Merck, Rahway, p. 8120.
MOORE, J. H. (1967). The effect of the type of roughage in the diet on plasma cholesterol levels and aortic atherosis in rabbits. *Br. J. Nutr.*, **21**, 202–15.
NATIONAL ACADEMY OF SCIENCES (1965). *Chemicals used in food processing*. NAS, Washington, p. 248.
NATIONAL HEALTH AND MEDICAL RESEARCH COUNCIL (1976). *Approved food standards and approved food additives*. Canberra.
NEWMAN, H. A., KUMMEROV, F. A. & SCOTT, H. H. (1957). Dietary saponin, a factor which may reduce liver and serum cholesterol levels. *Poultry Sci.*, **37**, 42–5.
NOBLE, I. G. (1948). *Anales Acad. Cienc. Med. Fic. y Nat. Habana*, **6**, 261 (cited by Newman *et al.*, 1957).
OAKENFULL, D. G. & FENWICK, D. E. (1978). Adsorption of bile salts from aqueous solution by plant fibre and cholestyramine. *Br. J. Nutr.*, **40**, 299–309.
OAKENFULL, D. G., FENWICK, D. E., HOOD, R. L., TOPPING, D. L., ILLMAN, R. J. & STORER, G. B. (1979). Effects of saponins on bile acids and plasma lipids in the rat. *Br. J. Nutr.*, **42**, 209–16.
O'DELL, B. L., REGAM, W. O. & BEACH, T. J. (1959). Toxic principle in red clover. *Missouri Univ. Agr. Expt. Sta. Res. Bull.*, **702**, 12.
OSER, B. L. (1966). An evaluation of *Yucca mohavensis* as a source of food grade saponin. *Fd Cosmet. Toxicol.*, **4**, 57–61.
Oxford book of food plants (1969). Oxford University Press, London.
PHILLIPS, J. C., BUTTERWORTH, K. R., GAUNT, I. F., EVANS, J. G. & GRASSO, P. (1979). Long-term toxicity studies of quillaia extract in mice. *Fd Cosmet. Toxicol.*, **17**, 23–7.
POTTER, J. D., TOPPING, D. L. & OAKENFULL, D. (1979). Soya products, saponins and plasma cholesterol. *Lancet i*, 223.
RICCARDI, B. A. & FAHRENBACH, M. J. (1969). Effect of guar gum and pectin N.F. on serum and liver lipids of cholesterol-fed rats. *Proc. Soc. Exp. Biol. Med.*, **124**, 749–52.
SHIBATA, S. (1977). Saponins with biological and pharmacological activity. In *New natural products and plant drugs with pharmacological, biological or therapeutical activity* (Wagner, H. & Wolff, P. (eds.)), Springer-Verlag, Berlin, pp. 177–96.
SIMONS, L. A. (1978). The effects of oral lecithin and clofibrate on cholesterol metabolism. *Artery*, **4**, 167–82.
SIRTORI, C. R., AGRADI, E., CONTI, G., MONTERO, O. & GATTI, E. (1977). Soybean-protein diet in the treatment of type II hyperlipoproteinaemia. *Lancet i*, 275–7.
SMITH, A. K. & CIRCLE, S. J. (1972). *Soybeans: chemistry and technology*, Vol. AVI Publishing Co., Westport.
SMOCZKIEWICZ, M. A., NITSCHKE, D. & STAWINSKI, T. M. (1977). Mikrobestimmung von spuren von saponinglykosiden in 'nicht-saponin führen dem' pflanzen material. *Mikrochimica Acta*, **II**, 597–605.
SMOCZKIEWICZOWA, A. & NITSCHKE, D. (1975). Studies on saponins in garlic (*Allium sativum*). *Zesz. Nank. Adad. Ekon. Poznaniu*, Ser. 1, **62**, 43–8 (*Chem. Abst.*, **85**, 59614).
SOLMAN, T. (1957). *A manual of pharmacology*, 8th edn. W. B. Saunders, Philadelphia.
SPECTOR, W. S. (1956). *Handbook of toxicology*, Vol. I. W. B. Saunders, Philadelphia.
TOPPING, D. L., STORER, G. B., CALVERT, G. D., ILLMAN, R. J., OAKENFULL, D. G. & WELLER, R. A. (1980). Effects of dietary saponins on fecal bile acids and neutral sterols, plasma lipids and lipoprotein turnover in the pig. *Am. J. Clin. Nutr.*, **33**, 783–6.
TSCHESCHE, VON R. & WULFF, G. (1969). Triterpenes XXVII. Saponins of spinach (*Spinacea oleracea*). *Justus Liebigs Ann. Chem.*, **726**, 125–35.
TSCHESCHE, VON R. & WULFF, G. (1973). Chemie und biologie der saponine. In *Progress in the chemistry of organic natural products*. (Herz, W., Gnsebach, H. & Kirby, G. W. (eds.)), Vol. 30. Springer-Verlag, Vienna, pp. 461–605.
TSCHESCHE, VON R., JHA, C. & WULFF, G. (1973). Über triterpene XXIX. Zur struktur des avenacins. *Tetrahedron*, **29**, 629–33.
VALOSHINA, D. A., KISELEV, V. P. & RUMYANTSEVA, G. N. (1977). Content of sapogenins and their yield from *Trigonella foenum-graecum* seeds. *Rasit. Resur.*, **13**, 655–7. (*Chem. Abst.*, **88**, 34506.)
VARSHNEY, I. P. & SHARMA, S. C. (1968). Saponins and sapogenins XXII. Chemical investigation of seeds of *Myristica fragrans*. *Indian J. Chem.*, **6**, 474.
VINKLER, G. N. & SMYSLOVA, G. I. (1967). The discovery of a natural auto-antimutagenic system. *Genetika*, 52–6.

VOGEL, G. & MAREK, M. L. (1962). Pharmacology of some saponins. *Arzneimittel-Forsch.*, **12**, 815. (*Chem. Abst.*, **57**, 15752.)

WAGNER, J. & STERNKOPF, G. (1958). Chemical and physiological studies of the saponins of sugar beets. Nahrung, **2**, 338–57.

WALTER, E. D., VAN ATTA, G. R., THOMPSON, C. R. & MACLAY, W. D. (1954). Alfalfa saponin. *J. Am. Chem. Soc.*, **76**, 2271–3.

WALTER, E. D., BICKOFF, E. M., THOMPSON, C. R., ROBINSON, C. H. & DJERASSI, C. (1955). Saponin from ladino clover (*Trifolium repens*). *J. Am. Chem. Soc.*, **77**, 4936–7.

WILLNER, D., GESTETNER, B., LAVIE, D., BIRK, Y. & BONDI, A. (1964). Soya bean saponins. Part V. Soyasapogenol E. *J. Chem. Soc., Suppl.*, *1*, 5885–8.

WOLF, W. J. & THOMAS, B. W. (1970). Thin layer and anion exchange chromatography of soybean saponins. *J. Am. Oil. Chem., Soc.*, **47**, 86–90.

Food Chemistry 6 (1981) 41–45

DETERMINATION OF 5-HYDROXYMETHYLFURFURAL AND CAFFEINE IN COFFEE AND CHICORY EXTRACTS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

ROGER M. SMITH

Department of Chemistry, Loughborough University of Technology, Loughborough, Leicestershire LE11 3TU, UK

(Received: 29 November, 1979)

ABSTRACT

5-Hydroxymethylfurfural and caffeine in instant coffee and coffee/chicory powders have been determined by HPLC on Hypersil (solvent 5% isopropanol/dichloromethane). The former compound is characteristic of chicory and could be used to distinguish between coffee and coffee/chicory mixtures.

INTRODUCTION

Methods for the determination of chicory in coffee/chicory mixtures are hampered by the absence of a readily analysed marker in chicory. Ground coffee beans and chicory roots can be distinguished by microscopic examination (Pearson, 1976) or by measurement of the hot water extracts (Vree & Yeransian, 1973) but these methods cannot be applied to 'instant' powders or coffee extracts.

The concentration of extractable reducing sugars is much higher in roasted chicory (c. 48% as fructose) than in coffee (c. 2%) and a number of methods have used this difference to examine mixtures by paper chromatography of xylose and mannose (Skarka & Radej, 1958), enzymic analysis of fructose and glucose (Promayon *et al.*, 1976) or by spectrophotometric determination of ketoses (Kazi, 1979).

During roasting the carbohydrates are partly converted into 5-hydroxymethylfurfural (HMF), λ_{max} 284 nm, which was found by Smith & Rees (1963) to interfere with the analysis of caffeine, λ_{max} 277 nm, particularly in coffee/chicory mixtures. The HMF could be removed either by treatment with aluminium amalgam (Smith & Rees, 1963; Smith, 1964) or bisulphite (Conacher & Rees, 1964) or the caffeine had to be separated before spectroscopy.

Food Chemistry 0308-8146/81/0006-0041/$02·25

Rees has proposed that HMF could be used to determine chicory but it is reported that in an unpublished study he found that the method was inapplicable because of losses during dry blending of coffee and chicory (Kazi, 1979).

The present work describes a rapid high performance liquid chromatography (HPLC) method for the separation and determination of both HMF and caffeine, and finds a higher concentration of HMF in instant coffee/chicory mixture, which could be used to distinguish it from a coffee powder.

EXPERIMENTAL

Apparatus

HPLC Chromatography: Analyses are carried out using syringe injection on a Shandon column (100 × 5 mm) packed with Hypersil. A Varian Model 4000 gas pressure reservoir pump was used and peaks were detected with a Cecil 2012 variable wavelength detector at 280 nm.

Chemicals

Solvents: Dichloromethane and isopropanol were HPLC grade (Fisons).

Standards: 5-Hydroxymethylfurfural was supplied by Sigma and caffeine was supplied by BDH.

Samples: Instant coffee powder and French coffee (coffee/chicory) were commercially available powders.

Method

Coffee powder (50 mg) was dissolved in cold distilled water (10 ml). Dichloromethane (10 ml) was added and the mixture stirred at intervals over 1 h. (Care had to be taken to avoid the formation of an emulsion). Aliquots (5 μl) of the organic layer were injected on to the Hypersil column and were eluted with 5% isopropanol in dichloromethane at 160 psi.

DISCUSSION

Although a number of previous workers have analysed caffeine and other xanthines in coffee and coffee powder by HPLC, only reverse phase (Smyly *et al.*, 1976) and ion-exchange columns (Murgia *et al.*, 1973; Madison *et al.*, 1976) have been used. However, separation on silica gel has been used to determine caffeine in pharmaceutical preparations, and a number of different solvent systems have been used including 15% isopropanol/dichloromethane, NH_4OH/methanol, or dichloromethane/ethanol/water (Ascione and Chrekian, 1975; Anon, 1978).

In the present study extracts of instant coffee and French coffee powder (51%

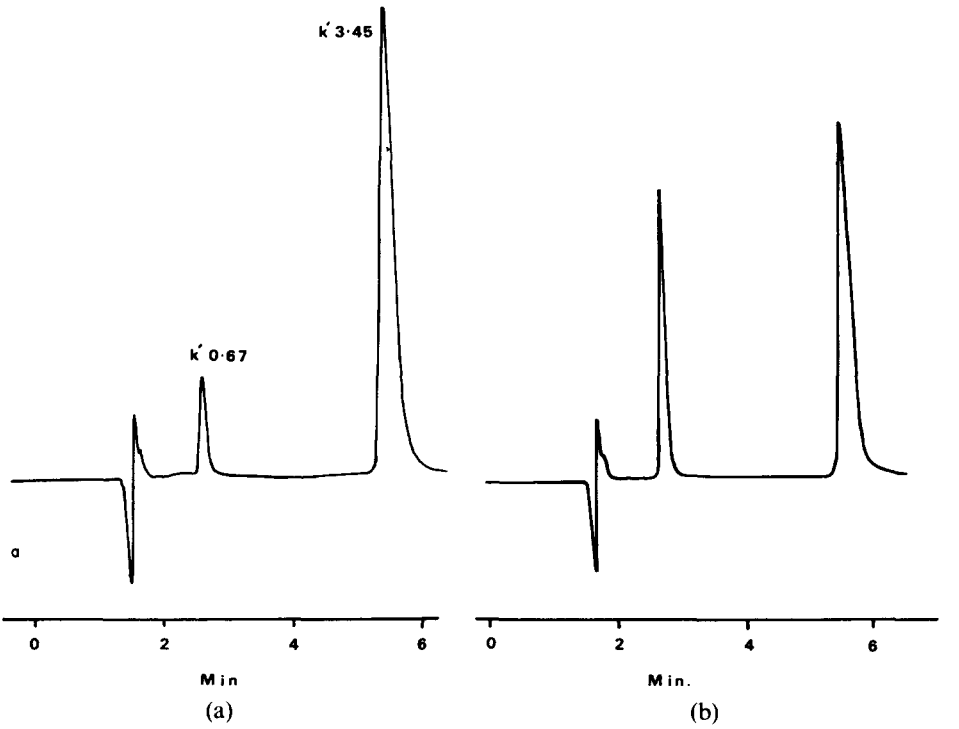


Fig. 1. Chromatograms of extracts of (a) coffee powder and (b) coffee/chicory powder, separated on Hypersil. Solvent 5% isopropanol/dichloromethane. Detector 280 nm.

coffee, 49% chicory) were examined by HPLC on silica gel (Hypersil) (Fig. 1). The two main peaks were identified as HMF ($k' = 0{\cdot}67$) and caffeine ($k' = 3{\cdot}45$) by comparison with authentic samples. The concentration of each component in replicate samples of three brands of instant coffee (two powders and one freeze dried granules) and one brand of French coffee were determined by comparison of peak heights with standard solutions. (Table 1).

Both peaks could also be observed at 254 nm but 280 nm was used as it gave greater sensitivity. Trial runs showed that theophylline was not eluted and that one of the peaks at the solvent front could be furfural, which is reported to be present also in instant coffee as a pentose degradation product (Smith & Rees, 1963).

Examination of the samples using a reverse phase column (ODS Hypersil) showed that HMF was retained even when caffeine was rapidly eluted ($k' = 1{\cdot}3$) and thus it would not have been observed in the previous HPLC studies on coffee.

The determined concentrations of caffeine in the instant coffee powders agree well with the range of values reported by other methods (Anon, 1973; Strahl *et al.*, 1977). As expected from the earlier spectroscopic studies of Smith (1964) the concentration of HMF was much higher in the French coffee, and as a result there is a marked

TABLE 1
DETERMINATION OF 5-HYDROXYMETHYLFURFURAL (HMF) AND CAFFEINE IN INSTANT COFFEE AND COFFEE/CHICORY POWDERS

Instant coffee[a]		% *HMF*	% *Caffeine*	*Ratio (HMF/ Caffeine) × 100*
1 Powder	A	0·079	2·75	2·87
	B	0·076	3·11	2·44
	C	0·076	3·00	2·53
2 Powder	A	0·058	2·92	1·98
	B	0·056	3·13	1·78
3 Freeze dried	A	0·022	3·81	0·57
	B	0·026	4·21	0·62
French coffee[b]				
	A	0·246	2·33	10·55
	B	0·260	2·16	12·04
	C	0·240	2·28	10·53

[a] Replicate samples were run on each powder.
[b] 51 % coffee/49 % chicory.

difference in the HMF/caffeine ratio between the coffee and coffee/chicory mixtures that could be used to distinguish them (Table 1). However, the concentration of HMF determined in the present study is much lower than Rees found in roast chicory (1·2%) and roast coffee (0·1%) (Kazi, 1979) and emphasises that considerable losses had taken place during blending, extraction and drying. Clearly, therefore, although HMF and caffeine can be readily quantified in coffee powders by the present method, the determination of HMF is not suitable as a quantitative method for the determination of chicory in a mixture.

REFERENCES

ANON. (1973). Instant coffee and coffee whiteners, *Which*, **16**, 337.

ANON. (1978). *Preparations for HPLC*, E. Merck, Darmstadt.

ASCIONE, P. P. & CHREKIAN, G. P. (1975). Automated high-pressure liquid chromatographic analyses of aspirin, phenacetin and caffeine. *J. Pharm. Sci.*, **64**, 1029.

CONACHER, H. B. S. & REES, D. I. (1964). The spectrophotometric determination of caffeine in coffee–chicory mixtures: Removal of hydroxymethylfurfural by bisulphite. *Analyst, Lond.*, **89**, 806.

KAZI, T. (1979). Coffee mixtures—spectrophotometric method for the determination of coffee content, *Food Chem.*, **4**, 73.

MADISON, B. L., KOZAREK, W. J. & DAMO, C. P. (1976). High-pressure liquid chromatography of caffeine in coffee. *J. Ass. Offic. Anal. Chem.*, **59**, 1258.

MURGIA, E., RICKARDS, P. & WALTON, H. F. (1973). Liquid chromatography of xanthines, analgesic drugs and coffee. *J. Chromatogr.*, **87**, 523.

PEARSON, D. (1976). *The chemical analysis of foods*, 7th edn. Churchill Livingstone, Edinburgh.

PROMAYON, J., BAREL, M., FOURNY, G. & VINCENT, J. C. (1976). Essais de détermination de la teneur en chicorée dans les melánges solubles de café et de chicorée. *Café Cacao Thé*, **20**, 209.

SKARKA, B. S. & RADEJ, Z. (1958). Proof of adulteration of coffee with substitutes by means of paper chromatography. *Průmysl potravin*, **9**, 484. (*Chem. Abs.* (1959), **53**, 7458.)

SMITH, R. F. (1964). The spectrophotometric determination of caffeine, *Analyst, Lond.*, **89**, 146.

SMITH, R. F. & REES, D. I. (1963). The spectrophotometric determination of caffeine in coffee and tea products with special reference to coffee and chicory mixtures. *Analyst, Lond.*, **88**, 310.

SMYLY, D. S., WOODWARD, B. B. & CONRAD, E. C. (1976). Determination of saccharin, sodium benzoate, and caffeine in beverages by reverse phase high-pressure liquid chromatography. *J. Ass. Offic. Anal. Chem.*, **59**, 14.

STRAHL, N. R., LEWIS, H. & FARGEN, R. (1977). Comparison of gas chromatographic and spectrophotometric methods of determination for caffeine in coffees and teas. *J. Agric. Food Chem.*, **25**, 233.

VREE, P. H. & YERANSIAN, J. A. (1973). Determination of soluble solids in roasted coffee. *J. Ass. Offic. Anal. Chem.*, **56**, 1126.

Food Chemistry 6 (1981) 47–61

INVESTIGATION OF THE CONTENTS OF PHENOLIC AND ALKALOIDAL COMPOUNDS OF GAMMA IRRADIATED POTATOES DURING STORAGE

WILLEM W. A. BERGERS

Institute for Atomic Sciences in Agriculture (ITAL), Keyenbergseweg 6, PO Box 48, 6700 AA Wageningen, The Netherlands

(Received: 4 December, 1979)

ABSTRACT

The contents of phenolic cinnamic acids and coumarins as well as of the glycoalkaloids of gamma irradiated potato tubers have been studied in detail. Gamma irradiation up to 3 kGy had no effect on the glycoalkaloid contents of two potato tuber varieties during a four months storage period. The phenolic compounds behave differently and show a considerable change during storage in potatoes irradiated at the highest dose level, 3 kGy. A time dependent change of phenolic extracts was observed. This change of phenolic compounds could be partly ascribed to the β-glycoside of scopoletin (coumarin, 7-hydroxy-6-methoxy) and was accompanied by a general decrease of chlorogenic acid, the main hydroxy-cinnamic acid of potatoes.

INTRODUCTION

Interest in chemical and biochemical changes in irradiated food began as soon as the possibility of food irradiation on an economic scale was recognised. The reason is twofold, viz. maintenance of good sensoric quality and safety of gamma irradiated food for human consumption. Biochemical changes are important from a toxicological point of view in those commodities known to contain small amounts of naturally occurring toxic compounds. Information on this subject is meagre. One example of the effect of changed biochemical activities due to irradiation leading to accumulation of potential toxic phenolic coumarins in citrus fruit peel is offered by Riov *et al.* (1972). For irradiated potato, several studies of changes of phenolic compounds, especially chlorogenic acid, are described. However, conclusive data on the extent and development of such changes are still lacking (Penner & From, 1972;

Food Chemistry 0308-8146/81/0006-0047/$02·25

Berset & Sandret, 1976). An inhibitory effect of gamma irradiation on wound-induced formation of α-solanine was reported by Wu & Salunkhe (1977), whereas no effect on light-induced glycoalkaloid formation in potato peel was seen by Patil *et al.*, (1971).

The aim of the present investigation was to obtain detailed information about glycoalkaloids and phenolic compounds of irradiated potatoes after prolonged storage.

MATERIALS AND METHODS

Potatoes

Good quality potatoes of Bintje, Eba and Alpha varieties, obtained from the Institute for Storage and Processing of Agriculture Produce at Wageningen, were used throughout this investigation. After harvest, potatoes were stored for some weeks for wound healing and then transferred to a conditioned room with a temperature of about 5 °C and 90 % RH. At least 24 h before irradiation, potatoes were transferred to a conditioned room at 10 °C and 90 % RH and after irradiation these conditions were maintained. No chemical sprout suppressants were used for irradiated or control potatoes.

Gamma irradiation

The tubers were irradiated in lots of 20 kg at the Pilot Plant for Food Irradiation at Wageningen with a ^{60}Co source of 190 kCi in aluminium boxes at dose levels of 100, 500 and 3000 Gy (J kg^{-1}). The dose level of 100 Gy is given at a fixed position at a dose rate of 22 Gy/min and a max/min ratio of 1·16. For 500 and 3000 Gy the transport system was used, resulting in an average dose rate of 100 Gy/min and a max/min ratio of 1·2. Dosimetry for 100 Gy was determined with the Fricke $FeSO_4$ dosimeter and for 500 and 3000 Gy with the perspex dosimeter (Chadwick, 1971).

Chemicals

All standard chemicals were of PA quality; 96 % ethanol for extraction, technical quality; β-glucosidase (5 mg/ml) from Boehringer Mannheim; scopoletin and chlorogenic acid from Sigma; caffeic acid, ferulic acid and paraformaldehyde, reinst, from Merck.

Sampling and general working up procedure

Samples (of 10 potatoes) without visible mechanical and pathological injuries were washed with tap water and cut into symmetrical quarters. Ten quarters were sliced (3–5 mm thickness) and immediately frozen in liquid nitrogen (to avoid any enzymatic activity), stored at −25 °C and freeze-dried. Ten grammes of the powdered lyophilised potato slices were extracted with 100 ml 80 % (v/v) ethanol,

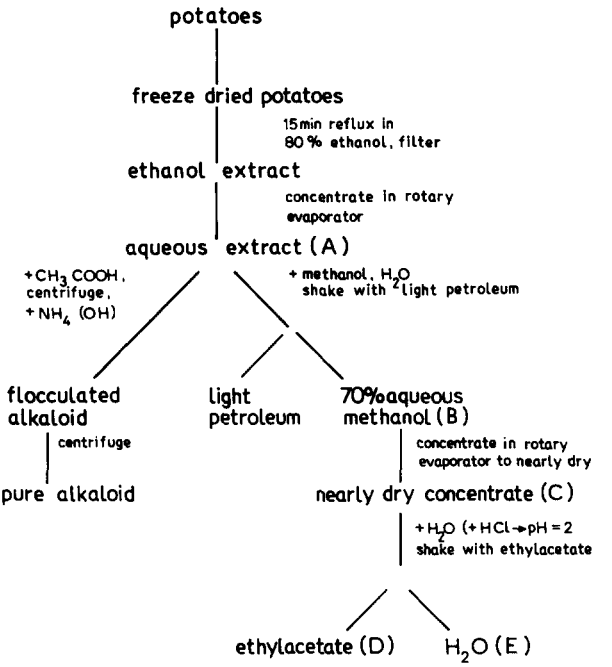


Fig. 1. Outline of the analysis of glycoalkaloids and phenolic compounds.

boiled under reflux for 15 min and filtered while hot by suction. The filtrate was concentrated with the aid of a rotary evaporator on a waterbath at 60 °C to *c.* 10 ml (A). Further preparative steps are described in Fig. 1.

Quantitative assay for alkaloidal compounds

The aqueous extract (A) was acidified with 50 ml 10 % acetic acid and centrifuged for 20 min at 10,000 g. The alkaloids were precipitated from the supernatant by addition of $NH_4(OH)$ to a final pH 10 and heating on a waterbath for 20 min at 70 °C. The precipitated alkaloidal compounds were cooled to 4 °C, followed by separation by centrifugation for 20 min at 10,000 g. The precipitated alkaloids were dissolved in 7 % (w/v) aqueous phosphoric acid and assayed colorimetrically, after addition of a mixture of concentrated phosphoric acid and paraformaldehyde, by measuring the absorbance at 600 nm after 40 min (Bergers, 1980).

Qualitative and quantitative analysis of phenolic compounds

The aqueous extract (A) was made up to 50 ml with 70 % (v/v) methanol and shaken with half its volume of light petroleum (40–60 °C) to remove lipids. UV spectra (250–400 nm) of the methanolic extract (B) were recorded after dilution in 80 % ethanol. Fluorescence intensities were measured after dilution of (B) in 0·066 M

KH_2PO_4/Na_2HPO_4 buffer (pH = 7) with a spectrofluorimeter (excitation wavelength 345–355 nm and emission 445–455 nm).

Further analysis with UV spectra was done by concentrating the methanolic layer (B) to nearly dryness (C), then bringing to 50 ml with distilled water and with HCl to pH 2. Then 50 ml ethylacetate was added for phase partition and from both phases, diluted in 80 % ethanol, (D) and (E), UV spectra (250–400 nm) were recorded (see Fig. 1).

TLC of the methanolic layer (B) was carried out on cellulose plates (Merck). A volume of 50 μl of the extract was applied as a 7 cm long strip on the plates (Desaga microdoser). Development up to about 14 cm was carried out with 10 % acetic acid or BEW (butanol/ethanol/water, 4:1:2·2).

For HPLC, the nearly dry concentrate (C) was dissolved in 50 ml 0·02 M Na_2HPO_4/citric acid, pH 2·85 and centrifuged for 10 min at 3000 g. HPLC runs were performed on a Nucleosil SA column, according to the reversed-phase principle at 40 °C, flow rate at 60 ml/h, UV detection at 310 nm, sample of 10 μl. As a mobile phase a linear gradient of 0·02 M Na_2HPO_4/citric acid at (1) pH 2·85, (2) pH 8·2 was used; programme: (1) 0–2 min, 100 %, followed by 2–27 min decrease; (2) 4 %/min and increase of 4 %/min.

Isolation of scopoletin from irradiated (3 kGy) Eba potatoes

The concentrated extract of 50 g freeze-dried potato powder was made up to 200 ml with 70 % methanol and shaken with 100 ml light petroleum. The methanolic layer was evaporated to near dryness and 200 ml of 0·2 M NaOH/acetic acid buffer (pH 3·6) were added.

This solution was centrifuged for 20 min at 10,000 g and the pellet discarded. The supernatant was shaken three times with 100 ml chloroform, brought to pH 5 with NaOH and freed from chloroform using a rotary evaporator. This extract was incubated at 37 °C after addition of 5 mg β-glucosidase, until no further increase of fluorescence occurred (2 h). The pH was then brought to pH 3·6 with HCl and the solution extracted with four portions of 80 ml chloroform. Because separation of both phases after hydrolysis was very bad, centrifugation was necessary. The combined chloroform layers were dried over $MgSO_4$, evaporated till dryness and the residue dissolved in 5 ml boiling water. From this scopoletin crystallised out on cooling. The collected crystals were dried and weighed (1·5 mg).

RESULTS

Data for the contents of solanidine glycoalkaloids of freeze-dried potato tubers from gamma irradiated and control samples of Alpha and Bintje variety during a four month period are represented in Table 1. These results, analysed by two way analysis of variance, show no effects related to storage time or to irradiation dose.

TABLE 1

SOLANINE CONTENTS OF CONTROL AND IRRADIATED BINTJE AND ALPHA POTATOES DURING SUBSEQUENT STORAGE

	Storage days after gamma irradiation											
	t = 1d		*t = 4d*		*t = 7d*		*t = 14d*		*t = 32d*		*t = 74d*		*t = 125d*	
Bintje														
Control	14·7[a]	(1·73)[b]	12·0	(1·44)	19·2	(0·71)	13·4	(0·61)	14·7	(1·31)	10·0	(1·61)	—[c]	—[c]
0·1 kGy	13·9	(0·53)	16·3	(1·87)	18·2	(1·17)	12·9	(1·68)	11·2	(1·90)	12·3	(1·89)	17·0	(2·19)
0·5 kGy	14·6	(1·19)	8·4	(2·20)	15·4	(1·87)	12·6	(0·61)	15·6	(3·5)	10·4	(1·03)	16·5	(2·41)
3 kGy	16·8	(0·72)	10·1	(3·52)	14·3	(0·40)	13·3	(0·46)	11·2	(3·2)	9·0	(2·87)	17·7	(3·31)
Alpha														
Control	8·0[a]	(0·68)[b]	5·7	(0·62)	7·8	(1·14)	6·9	(1·23)	8·9	(0·14)	7·2	(0·82)	—[c]	—[c]
0·1 kGy	10·2	(1·04)	6·1	(1·18)	11·0	(2·25)	7·2	(1·47)	8·9	(0·21)	8·4	(1·31)	8·5	(0·23)
0·5 kGy	13·5	(0·80)	11·5	(0·40)	14·8	(0·91)	3·8	(0·75)	9·8	(0·29)	8·6	(1·05)	8·0	(1·80)
3 kGy	5·7	(0·26)	9·8	(2·18)	16·2	(0·64)	9·6	(1·94)	6·9	(0·47)	8·0	(2·03)	7·3	(1·46)

[a] Solanine contents (measured as total glycoalkaloid) from 10 symmetrical quarters of potatoes in mg/100 g freeze-dried tissue.

[b] Standard deviation of triplicate chemical analysis.

[c] Discarded because of severe sprouting.

The contents of specific phenolic compounds in irradiated and control potatoes are estimated from UV absorbance spectra of the diluted alcoholic extracts of freeze-dried material. Figure 2 shows the typical absorbance spectra of a potato extract, chlorogenic acid and scopoletin.

When the absorbance at 325 nm is measured in irradiated and control samples an increase for 3 kGy irradiated Eba potatoes is found, which appears to develop

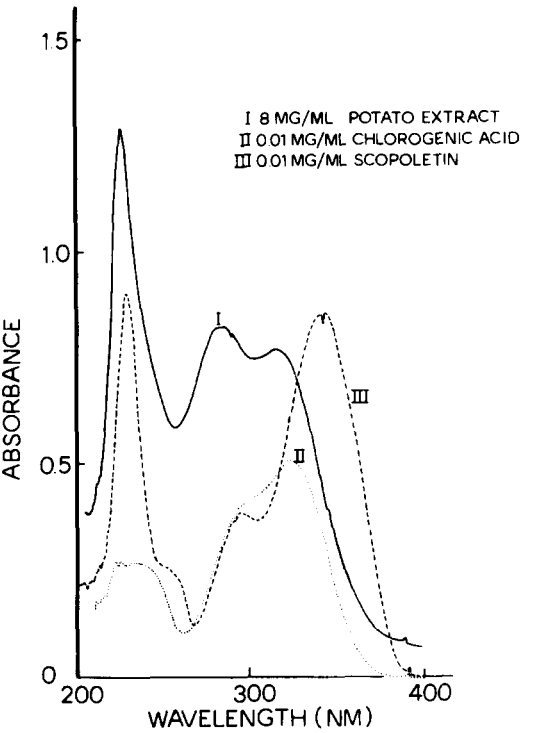


Fig. 2. UV spectra, recorded from 200 to 400 nm, of an alcoholic potato extract, chlorogenic acid and scopoletin.

gradually during the first month in storage (see Fig. 3). In addition to absorbance measurements, the relative fluorescence intensities were determined at wavelengths characteristic for hydroxy-coumarins (see Fig. 4). This showed for both varieties, a pronounced increase of fluorescence for the 3 kGy dose level, which likewise increased during storage and remained elevated during subsequent months.

At phase partition with ethylacetate the relative increase of the 325 nm absorbance in the water phase was greatest for the 3 kGy Eba samples, which indicates an increase of the more water-soluble phenolic compounds (see Fig. 5). Chromatographic analysis was done by TLC and HPLC chromatography of the

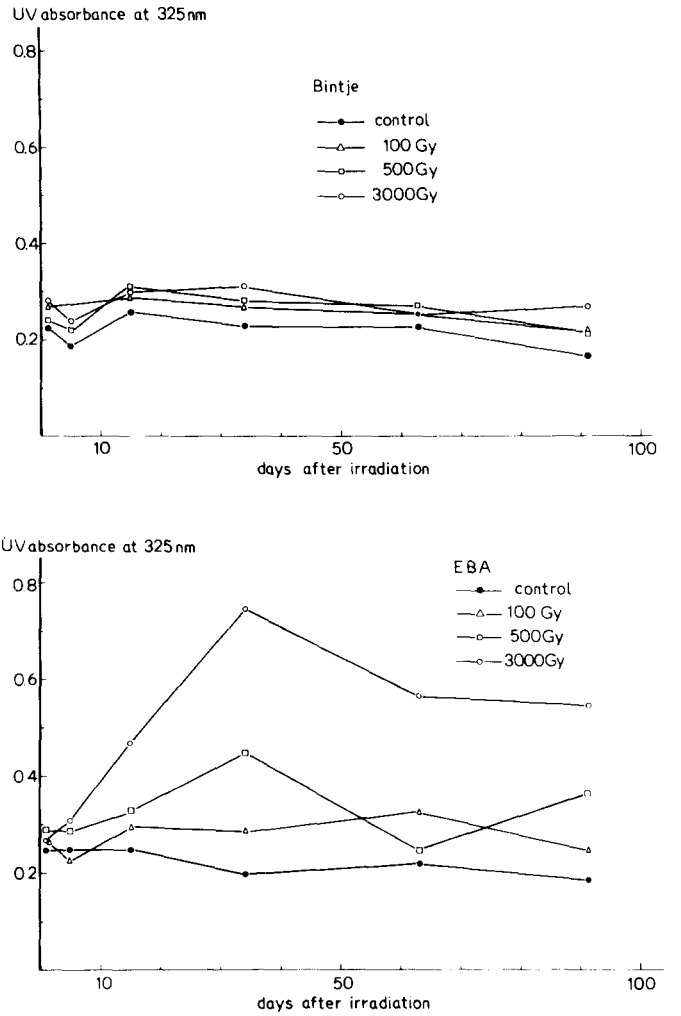


Fig. 3. UV absorbance values at 325 nm of diluted alcoholic extracts (6·7 mg/ml) (mg relative to original freeze-dried potato tissue) for irradiated and control potato tubers of Eba and Bintje variety during storage.

potato extracts. Chlorogenic acid, caffeic acid and occasionally ferulic acid were identified by TLC, see Table 2. The highly fluorescent extracts contained mainly one strongly fluorescent compound which, when converted by β-glucosidase, had the same mobility and fluorescence as scopoletin.

Direct evidence was obtained by isolating and identification after enzymic hydrolysis with β-glycosidase. UV, IR and mass spectra were found to be identical to

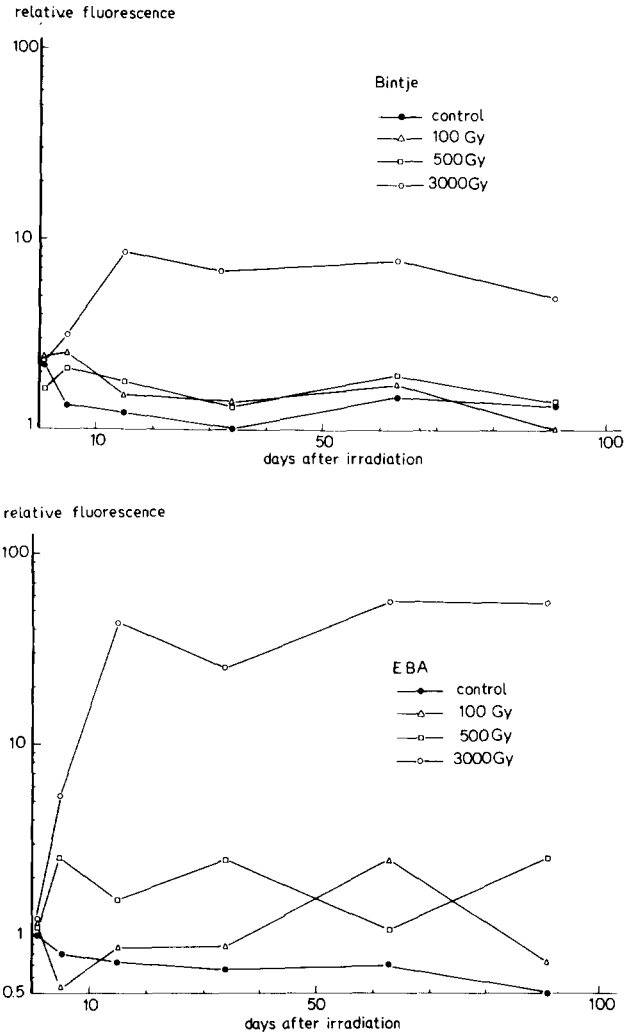


Fig. 4. Relative fluorescence values of diluted alcoholic extracts (8 mg/ml) (mg relative to original freeze-dried potato tissue) in phosphate buffer (pH = 7) for irradiated and control potatoes of Eba and Bintje variety during storage. Excitation wavelength 350 nm, emission 450 nm.

scopoletin. High resolution of phenolic compounds of potato extracts was achieved by reversed-phase chromatography on a HPLC column. By this technique it was found that potato extracts showed only minor changes, besides varietal differences for controls and irradiated potato tubers, up to 0·5 kGy throughout the three month storage period.

These patterns were equal for Eba and Bintje varieties (3 kGy) 1 day after

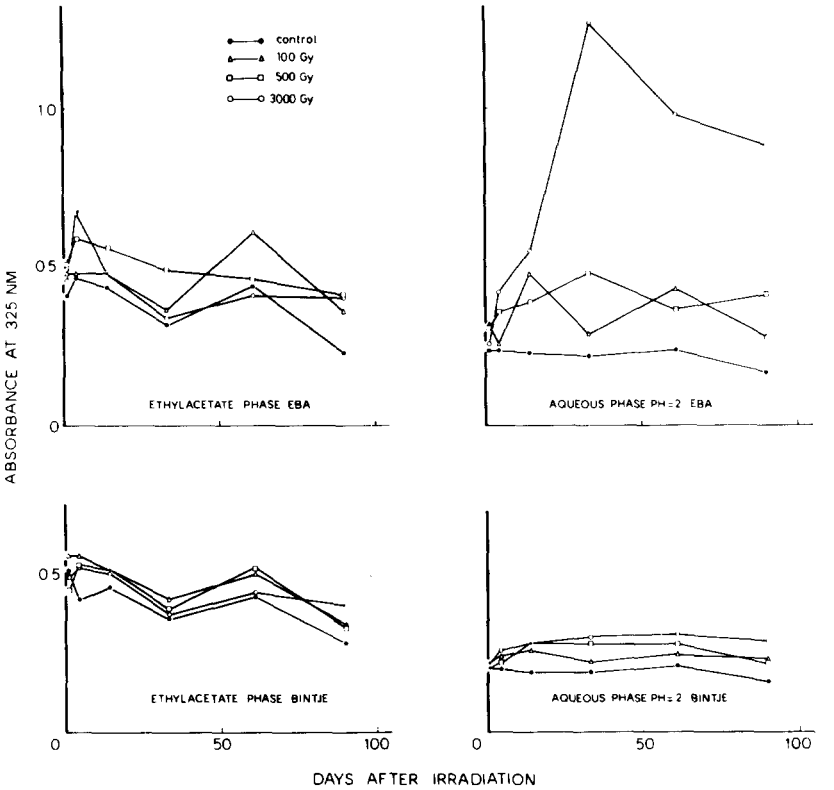


Fig. 5. Relative UV absorbance values at 325 nm of alcoholic extracts of irradiated and control Eba and Bintje potatoes after phase partition between a water-phase (pH = 2) and ethylacetate.

TABLE 2

RF VALUES AND FLUORESCENT COLOURS OF PHENOLIC COMPOUNDS FROM POTATO

Compound	*Rf × 100*		*Fluorescence, excitation 366 nm*	
	10% (v/v) Acetic acid	*BEW*	*Direct*	*After spraying with 1 M NaOH*
Chlorogenic acid	62·73[a]	52	Blue	(Yellow)[b]
Caffeic acid	32·65	79	Blue	(Brownish)[b]
Ferulic acid	37·67	85	Blue	Blue (reddish)[b]
Scopoletin	45	84	Bright blue	Bright blue
Scopoletin-β-glycoside	79	54	Bright mauve	Bright yellow green

[a] *trans*, *cis*-isomers.

[b] colour in visible light.

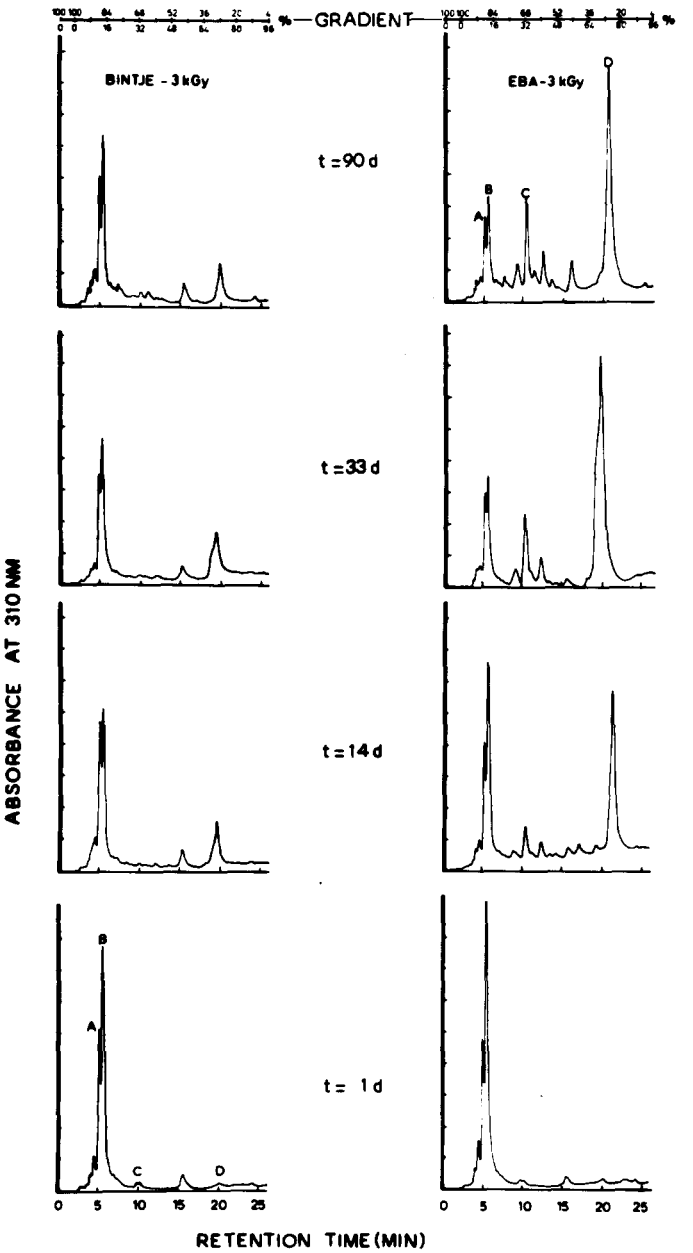


Fig. 6. HPLC chromatograms of alcoholic extracts of Eba and Bintje potatoes irradiated at 3 kGy and sampled 1, 13, 33, and 90 days after irradiation. Peaks A, B and C represent caffeic acid, chlorogenic acid and scopoletin-β-glycoside, respectively. Peak D represents a major unidentified component. As mobile phase a gradient of citric acid/phosphate buffer, pH = 2·85 and 8·2 is used.

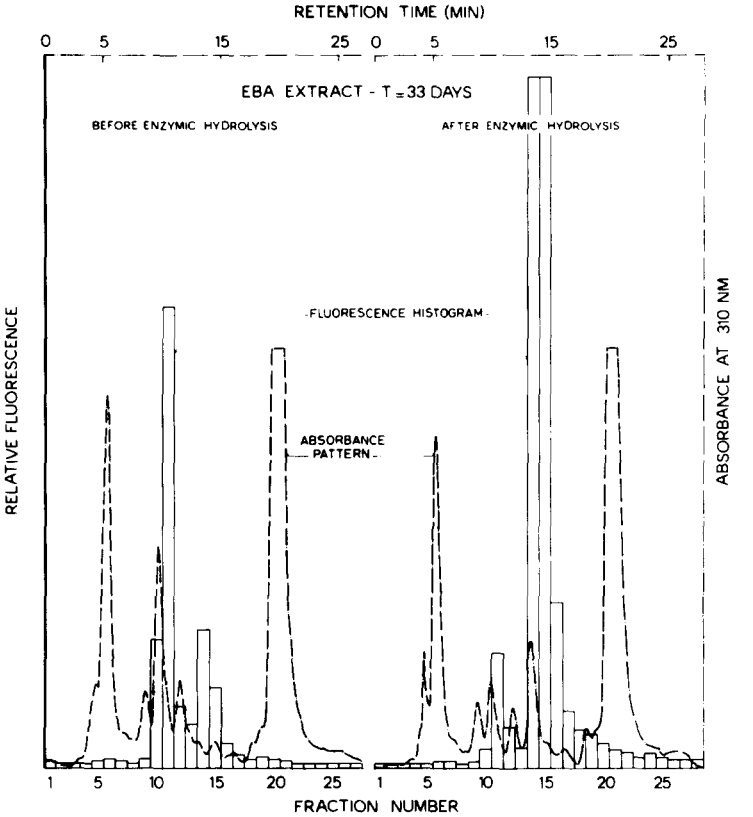


Fig. 7. UV absorbance patterns and fluorescence histogram of HPLC column effluent of a highly fluorescent Eba extract before and after hydrolysis with β-glycosidase. The major peaks represent scopoletin-β-glycoside and scopoletin at retention times of 10·3 and 13·6 min.

TABLE 3

CHLOROGENIC ACID CONTENTS OF IRRADIATED AND CONTROL POTATOES OF BINTJE AND EBA VARIETY (mg/100 g FREEZE-DRIED POTATO TISSUE) DURING STORAGE

	Storage days after gamma irradiation					
	t = 1d	*t = 4d*	*t = 14d*	*t = 33d*	*t = 61d*	*t = 90d*
Bintje						
Control	26	23	24	26	26	17
0·1 kGy	42	35	30	57	39	30
0·5 kGy	30	27	41	41	37	24
3 kGy	45	34	26	24	21	18
Eba						
Control	35	38	33	28	32	30
0·1 kGy	44	40	42	40	44	51
0·5 kGy	46	54	48	62	40	56
3 kGy	46	55	27	12	10	13

irradiation as seen in Fig. 6. This figure also demonstrates the gradual increase of UV absorbing compounds of the 3-kGy treated samples during the first month of storage after irradiation as well as a decline of the chlorogenic acid peak. For Eba this effect is again the most pronounced and the increase of the peak designated as C was due to the accumulation of the β-glycoside of scopoletin. Evidence of this was obtained by fractionating the effluents of the HPLC columns of an enzyme-hydrolysed extract and a non-hydrolysed extract (see Fig. 7). The fluorescence histogram showed two clear peaks changing after enzymic hydrolysis. Retention time of the increasing peak after enzymic hydrolysis corresponds to scopoletin and an increase of overall fluorescent intensity can be observed.

TABLE 4
SCOPOLETIN-β-GLYCOSIDE CONTENTS OF IRRADIATED AND CONTROL EBA POTATO TUBERS DURING STORAGE (mg/100 g FREEZE-DRIED POTATO TISSUE)

	Storage days after irradiation					
	t = 1d	*t = 4d*	*t = 14d*	*t = 33d*	*t = 61d*	*t = 90d*
Control	—[a]	0·81	0·79	1·62	0·43	0·63
0·1 kGy	0·67	0·53	1·52	1·09	3·51	0·54
0·5 kGy	0·70	0·61	—	0·15	1·48	0·25
3 kGy	0·81	—	9·07	28·4	23·3	35·0

[a] No peak detected on HPLC chromatogram.

From the areas of the elution patterns of the HPLC chromatograms the chlorogenic acid of the potato extracts was determined (see Table 3). The data so obtained reflect only minor changes of chlorogenic acid content except for a definite decrease at the highest dose level. Likewise the contents of scopoletin β-glycoside of Eba potatoes have been estimated (see Table 4). For Bintje no clear data could be obtained in this way.

DISCUSSION

The glycoalkaloid contents of irradiated and control potato tubers do not appear to differ after doses up to 3 kGy, during a three month subsequent storage period.

Apparently radiation stress does not affect glycoalkaloids under the conditions studied. The quantitative assay used here is restricted to the determination of glycoalkaloids of solanidine (solanid-5-en) type, although the presence of solanidane glycoalkaloids, which are not detected by the assay, is unlikely (Schreiber *et al.*, 1961). Data of Wu & Salunkhe (1977) of wound-induced solanidine glycoalkaloid formation show dose-dependent inhibition up to 80–90% at 2 kGy,

whilst Patil *et al.* (1971) and the afore-mentioned authors reported no effect of light-induced glycoalkaloid formation in potato peel at 0·1 and 2 kGy. The reason for this may be the well known inhibition of formation of new cells by gamma irradiation, e.g. sprout and wound tuber tissue, which are known to synthesise excessive amounts of glycoalkaloids (Jadhav & Salunkhe, 1975; Fitzpatrick *et al.*, 1977; Ahmed & Müller, 1978). Phenolic compounds behave differently and show a pronounced increase of several specific phenolic compounds, restricted to the highest dose level, 3 kGy, applied. The fluorescent phenolic compound, which accumulated to at least 20–30 times average concentration, was identified as the β-glycoside of scopoletin.

Accumulation of this compound occurs also in virus- and fungus-infected potato tubers (Andreae, 1944; Reppel, 1959; Hughes & Swain, 1960) and their dependency on host control, e.g. the varietal characteristics, has been demonstrated by Clarke (1976). In contrast to fungal-stressed tissue, wound tissue behaves differently. Here chlorogenic acid rather than scopoletin β-glycoside accumulates (Clarke, 1973). From the quantitative data presented here for chlorogenic acid, it appears that only minor changes occur in controls and potatoes irradiated at 0·1 and 0·5 kGy, whilst for the highest dose level a definite decrease during prolonged storage is observed.

Minor changes of polyphenols, e.g. chlorogenic acid have been reported for 0·1–0·5 kGy irradiated potato tubers (Penner & From, 1972; Berset & Sandret, 1976). According to Ogawa & Uritani (1970) the changes of polyphenolic compounds, depend largely on the time in storage between harvest and irradiation; these are greatest only shortly after harvest and are not directly proportional to the dose applied.

However, potatoes used in this investigation had been stored for at least two months before irradiation (now common practice in potato irradiation). For citrus fruits irradiated at 1 kGy and higher, Riov *et al.* (1972) demonstrated a similar accumulation of the β-glycoside of scopoletin and observed a correlation between this accumulation and an increased level of phenylalanine ammonia lyase. They proposed a pathway for biosynthesis of the scopoletin β-glycoside starting from phenylalanine.

For potatoes irradiated at 0·1 kGy, a transient increase of the same enzyme in potato cortex and buds has been demonstrated by Pendharkar & Nair (1975). So for potato tubers the increase of scopoletin β-glycoside likewise may be explained. The change of phenolic compounds reflects a change in metabolic activity. Changes due to direct radiochemical effects can be ruled out in view of the time-dependent change of phenolic compounds and their small concentration in potatoes (Diehl & Schertz, 1975).

Undesirable changes of phenolic compounds in gamma irradiated metabolically active plant foods may be avoided by the use of the lowest possible dose or choosing a relatively unsusceptible variety. For potatoes a dose of 0·1 kGy is sufficient for complete sprout inhibition.

ACKNOWLEDGEMENTS

I am very much indebted to Mr A. van Es, Miss K. J. Hartmans and Mr H. de Ruiter of the Institute for Research on Storage and Processing of Agricultural Produce (IBVL) at Wageningen, for their help in potato storage and HPLC analysis and to Mr C. Landheer of the Organic Chemistry Department of the Agricultural University at Wageningen for help in identification by mass and IR spectra. I also wish to thank Mr J. Davies, Mr D. Kennett and Mr H. Bosman for their assistance in the analytical part of the work. Finally, the author gratefully acknowledges the encouragement and advice of Mr J. G. van Kooy.

REFERENCES

AHMED, S. S. & MÜLLER, K. (1978). Effect of wound-damages on the glycoalkaloid content in potato tubers and chips. *Lebensm.-Wiss. u Technol.*, **11**, 144–6.

ANDREAE, W. A. (1944). The isolation of a blue fluorescent compound scopoletin, from Green Mountain potato tubers, infected with leaf roll virus. *Australian J. Exptl. Biol. Med. Sci.*, **23**, 251–5.

BERGERS, W. W. A. (1980). A rapid quantitative assay for solanidine glycoalkaloids in potatoes and industrial potato protein. *Potato Research*, **23**, 105–10.

BERSET, C. & SANDRET, F. (1976). Sprout radio-inhibition of potato tubers. Comparison of β or γ radiations after-effects on polyphenol content, polyphenoloxidase activity and after-cooking blackening. *Lebensm.-Wiss. u Technol.*, **9**, 85–90.

CHADWICK, K. H. (1971). *Radiation effects and after-effects in the clear polymethylmetacrylate dosimeter.* Thesis, Agricultural Research Report 763. Center for Agricultural Publishing and Documentation, Wageningen, The Netherlands.

CLARKE, D. D. (1973). The accumulation of scopolin in potato tissue in response to infection. *Physiological Plant Pathology*, **3**, 347–58.

CLARKE, D. D. (1976). Host control of scopolin accumulation in infected potato tissue. *Physiological Plant Pathology*, **9**, 199–203.

DIEHL, J. F. (1974). *Qualitative and quantitative changes in the components of irradiated foodstuffs. Suggestions for further analytical studies as a contribution to the evaluation of wholesomeness.* Doc. No. 4097/e. Commission of the European Communities.

DIEHL, J. F. & SCHERTZ, H. (1975). Estimation of radiolytic products as a basis for evaluating the wholesomeness of irradiated foods. *Int. J. appl. Radiat. Isotopes*, **26**, 499–501.

FITZPATRICK, T. J., HERB, S. F., OSMAN, S. F. & MCDERMOTT, J. A. (1977). Potato glycoalkaloids: increases and variations of ratios in aged slices over prolonged storage. *Am. Potato J.*, **54**, 539–44.

HUGHES, J. C. & SWAIN, T. (1960). Scopolin production in potato tubers infected with *Phytophtora Infestans*. *Phytopathology*, **50**, 398–400.

JADHAV, S. J. & SALUNKHE, D. K. (1975). Formation and control of chlorophyll and glycoalkaloids in tubers of *Solanum tuberosum* L. and evaluation of glycoalkaloid toxicity. *Adv. Fd Res.*, **21**, 308–54.

OGAWA, M. & URITANI, I. (1970). Tissue browning of potato tubers induced by gamma irradiation. *Agr. Biol. Chem.*, **34**, 870–7.

PATIL, B. C., SINGH, B. & SALUNKHE, D. K. (1971). Formation of chlorophyll and solanine in Irish potato (*Solanum tuberosum* L.) tubers and their control by gamma radiation and CO_2 enriched packaging. *Lebensm.-Wiss. u Technol.*, **4**, 123–5.

PENDHARKAR, M. B. & NAIR, P. M. (1975). Induction of phenylalanine ammonia lyase (PAL) in gamma irradiated potatoes. *Radiation Botany*, **15**, 191–7.

PENNER, H. & FROM, H. (1972). Studies on the identification of irradiated potatoes II. Determination of chlorogenic acid. *Z. Lebensmittel-Untersuch.-Forsch.*, **150**, 84–7.

REPPEL, L. (1959). Die Beziehungen zwischen scopoletin Gehalt und Virusinfektionen in Blättern und Knollen von *Solanum tuberosum* L. *Planta Med. (Stuttgart)*, **7**, 206–18.

RIOV, J., MONSELISSE, S. P. & GOREN, R. (1972). Stimulation of phenolic biosynthesis in citrus fruit peel by gamma irradiation. *Radiation Res. Rev.*, **3**, 417–27.

SCHREIBER, K., HAMMER, U., ITHAL, E., RIPPENBERGER, H., RUDOLPH, W. & WEISENBORN, A. (1961). Ueber das Alkaloid-Vorkommen verschiedener Solanum-Arten. *Tagungsber. Deut. Akad. Landwirtschaftwiss. Berlin*, **27**, 47–73.

WU, M. T. & SALUNKHE, D. K. (1977). Effect of gamma-irradiation on wound induced glycoalkaloid formation in potato tubers. *Lebensm.-Wiss. u Technol.*, **10**, 141–4.

Food Chemistry 6 (1981) 63–71

TRYPSIN INHIBITOR ACTIVITY IN *VICIA FABA* BEANS

A. RAFIK EL-MAHDY, E. K. MOUSTAFA & M. S. MOHAMED

Food Science Department, Faculty of Agriculture, University of Alexandria, Alexandria, Egypt

(Received: 1 May, 1980)

ABSTRACT

Extracts of mature green, dry and germinated Vicia faba *beans depressed the trypsin activity of casein.* Germination of Vicia faba *beans (for 60 h) lowered the trypsin inhibitor (TI) activity. 0·171M Saline was the most efficient extractant for the TI. Minimal amounts of the TI were extracted in the pH range 4 to 5. The TI of* Vicia faba *beans was undialysable. The inhibitor activity originated in the seeds at the beginning of pod formation and increased with development of maturity. TI was active only towards trypsin and inactive towards papain, rennin and pepsin. Chromatographing* Vicia faba *bean proteins, possessing antitryptic activity, on a column of DEAE-cellulose yielded six peaks, all of which possessed antitryptic activity.*

INTRODUCTION

Information concerning the occurrence of TI in *Vicia faba* beans is both meagre and contradictory. This situation stems from conflicting reports regarding the presence of TI in *Vicia faba* beans. Borchers & Ackerson (1947) and Tannous & Ullah (1968) stated that *Vicia faba* beans contained no TI activity. Conversely, Sohonie & Bhandarkar (1954), Sohonie *et al.* (1958), Nistan (1971), Wilson *et al.* (1972), Warsey *et al.* (1974) and Bhatty (1975) recorded the presence of TI and chymo-TI in *Vicia faba* beans.

The present work was conducted in order to study the presence, behaviour and properties of TI in mature green, mature dry and germinated *Vicia faba* beans.

MATERIALS AND METHODS

Materials

Vicia faba beans, variety Rebaya 40, were secured through the courtesy of the Plant Breeding Department, Ministry of Agriculture. The dry seeds were ground, after cleaning, to pass through a 60 mesh sieve.

Food Chemistry 0308-8146/81/0006-0063/$02·25

Germination was carried out at room temperature (14–18 °C). The whole seeds were steeped for 12 h in distilled water, after which germination lasted for 60 h. The seeds were then dried at 48–49 °C and prepared as already described.

Casein (fat free, 88 % dry matter) was obtained from the BDH Co. Ltd., Great Britain. Double crystallised trypsin was from E. Merck, Germany.

Methods

Nitrogen content was assayed using the micro-Kjeldahl method of the AOAC (1960). Protein content was calculated as N × 6·25.

Extraction of TI from dry (8 %) and green *Vicia faba* beans (16 %) was carried out according to the method of Kakade *et al.* (1969). After the effect of pH on the extraction of TI had been studied, the *Vicia faba* dry beans were extracted with 0·171M saline; the pH of the extraction was adjusted using either sodium hydroxide or hydrochloric acid (0·5M).

Trypsin inhibitor activity was determined using two methods. The first was that of Kunitz (1947) in which the inhibitor was mixed with the trypsin before addition to the substrate (1 % buffered casein, pH 7·6). Proteolytic activity was then determined after incubation at 35 °C for 20 min following the removal of the undigested proteins by trichloroacetic acid (TCA) using the method of McDonald & Chen (1965). Tryptic activity was expressed as liberated tyrosine. The second method was that of Ramirez & Mitchell (1960). This method is similar to that of Kunitz, apart from the following differences: the use of a higher concentration of the substrate (3 % buffered casein), a more basic pH (8·4), direct addition of inhibitor to the reaction mixture containing the trypsin and incubation at 37 °C for 180 min.

TI activity was expressed in two ways. The first was as the reduction in the percentage of casein digested in the presence of the inhibitor. The second was in terms of the tryptic units inhibited per milligramme of inhibitor nitrogen (Kunitz, 1947), tryptic units being defined as the amount of trypsin which produces, in one minute, products of digestion containing one micromole of tyrosine not precipitable by TCA (Greenberg, 1955).

Vicia faba bean extracts were dialysed against tap water at 17–19 °C, distilled water, 0·171M saline, and 0·1M pH 7·6 phosphate buffer at 5–8 °C. After centrifugation in each case, the precipitate was dissolved in the minimum volume of 0·171M saline.

The inhibitor activity towards pepsin, trypsin, papain and rennin was determined. Pepsin and trypsin activities were assessed according to the method of Kunitz (1947). Papain and rennin activities were assayed by means of the procedure of Greenberg (1955).

Diethylaminoethyl (DEAE) cellulose chromatography: The samples were dialysed against pH 7·6 phosphate buffer (0·01M) at 4 °C for 18 h. Fractionation was carried out according to the method of Peterson & Sober (1962). The inhibitory activity of

each fraction was assessed (Kunitz, 1947) and its protein content was determined using the method of Lowry *et al.* (1951) which was calibrated against bovine serum albumin.

RESULTS AND DISCUSSION

Tryptic digestion of casein as affected by extracts of Vicia faba *beans*

The results given in Table 1 were obtained using sodium hydroxide extracts and the inhibitor activity was determined according to the method of Kunitz (1947). The results illustrated in Fig. 1 were obtained by using saline extracts and the inhibitor activity was determined according to the method of Ramirez & Mitchell (1960). From these results it can be deduced that the various forms of *Vicia faba* beans can be arranged on the basis of their inhibitory action in the following decreasing order: dry, mature green and germinated beans. This is in accordance with the results obtained by Hobday *et al.* (1973) who reported that the high levels of trypsin inhibitory activity in the ungerminated seeds of peas, *Pisum sativum*, declined as germination proceeded.

Changes in trypsin inhibitor activity during Vicia faba *bean maturity and storage*

The results shown in Table 2 indicate that the trypsin inhibitor activity increased rapidly following pod formation throughout development and reached a maximum after 120 days of pod formation. Peak inhibitor activity was reached at harvest time. Storage of dry seeds for 14 months did not affect inhibitor activity. Little

TABLE 1

TRYPTIC DIGESTION OF CASEIN AS AFFECTED BY EXTRACTS OF *Vicia faba* BEANS (KUNITZ METHOD)

Types of Vicia faba *bean extract*	Vicia faba *bean nitrogen in reaction mixture* (*mg*)	*Tyrosine liberated*		*Extent of casein digestion* (%)
		Blank (*μg*)	*Treatment* (*μg*)	
Control	0·0	143·15		100·0
Sodium hydroxide extract	0·739	132·83	125·66	87·78
of mature green beans	1·478	116·70	107·97	75·43
(five weeks old)	2·217	85·52	78·25	54·47
	2·957	61·63	54·97	38·41
Sodium hydroxide extract	0·630	137·70	115·49	80·68
of dry beans	1·260	126·35	81·52	56·96
	1·890	115·25	67·44	47·12
	2·522	107·23	48·53	33·91
Sodium hydroxide extract	0·718	138·04	141·45	98·82
of germinated beans	1·435	128·48	122·77	85·77
(60 h)	2·152	118·91	106·52	74·43
	2·871	112·96	94·15	67·12

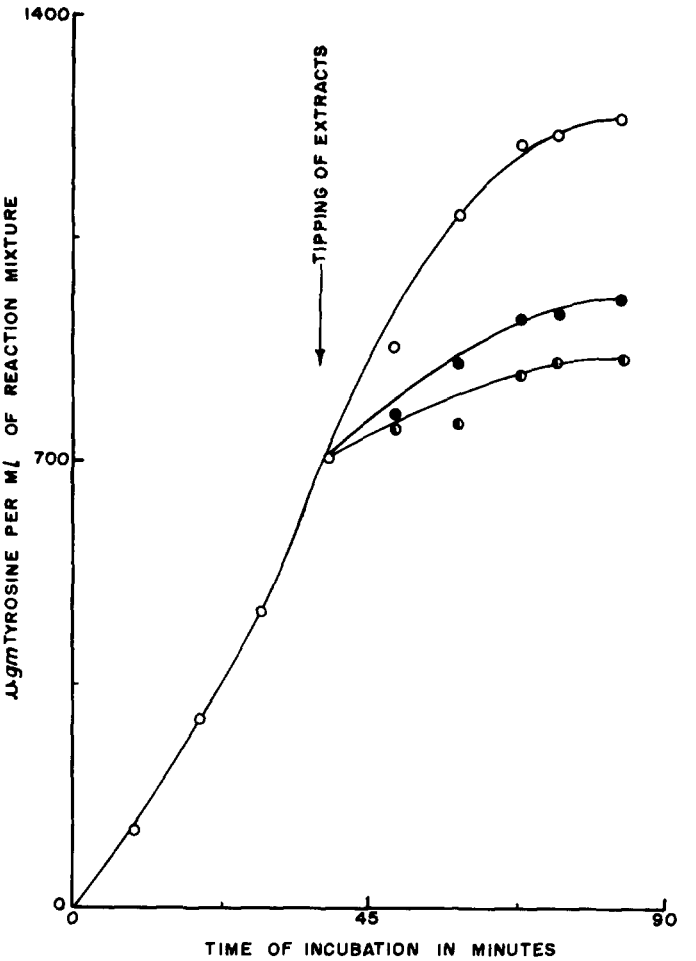


Fig. 1. Progress of trypsin digestion of casein as affected by extracts of *Vicia faba* bean inhibitor, ○ = 'control' casein–trypsin. ◑ = casein–trypsian–dry bean extract. ● = Casein–trypsin–germinated bean extract.

information is available in the literature concerning the changes of trypsin inhibitor during maturity and storage of legume seeds. Sohonie & Bhandarkar (1954) reported that trypsin inhibitor was isolated only from the dry mature seeds of legumes and that it was absent in undeveloped seeds.

Extractability of Vicia faba *trypsin inhibitor*

The results given in Table 3 indicate that saline solutions were the best extractants for the inhibitor, whilst ethyl alcohol (55 % conc.) was the least efficient. Increasing

TABLE 2
TRYPSIN INHIBITOR ACTIVITY[a] OF *Vicia faba* SEEDS AS AFFECTED BY STAGES OF MATURITY AND STORAGE

Stages of maturity (Days)		*Antitryptic activity (Units/mg N* $\times 10^{-3}$*)*
0	'Pod formation'	5
15		25
30		49
45	Mature green seeds	75
60		109
75		137
90		165
105		218
120	Full mature (dry seeds)	265
135	Harvest (dry seeds)	270
570	Storage (dry seeds)	266

[a] Assayed according to the method of Kunitz (1947), using 0·171M saline extracts of the seeds.

the sodium chloride concentration lowered the pH of the extract and consequently reduced the extracted trypsin inhibitor.

Figure 2 shows that minimum amounts of nitrogen were extracted in the pH range 4 to 6 and that these increased on both sides of this range, reaching their maximum at pH values of 1 and 12. The trypsin inhibitor showed a somewhat similar trend except that it had a narrower minimum activity pH range (pH 4–5). This is in accordance with the results of Kunitz (1947) who reported that soybean TI was least soluble at the point of cataphoretic mobility (the isoelectric point) which is at pH 4·5. Thus it may be concluded that the extraction and activity of the trypsin inhibitor from dry

TABLE 3
EXTRACTABILITY OF *Vicia faba* BEAN TRYPSIN INHIBITOR IN DIFFERENT SOLVENTS

Solvent		*pH of the extract*	*Extracted TI (Units/mg N* $\times 10^{-4}$*)*
Distilled water		6·30	2796
Sodium chloride	0·017M	6·00	3659
Sodium chloride	0·171M	5·95	3899
Sodium chloride	0·854M	5·75	3613
Sodium chloride	1·708M	5·65	3416
Sodium chloride	2·562M	5·60	3482
Sodium chloride	3·416M	5·55	2682
Hydrochloric acid	0·05M	2·80	2080
Sulphuric acid	0·025M	3·10	2113
Sodium hydroxide	0·05M	10·50	1138
Ethyl alcohol	55%	6·90	851

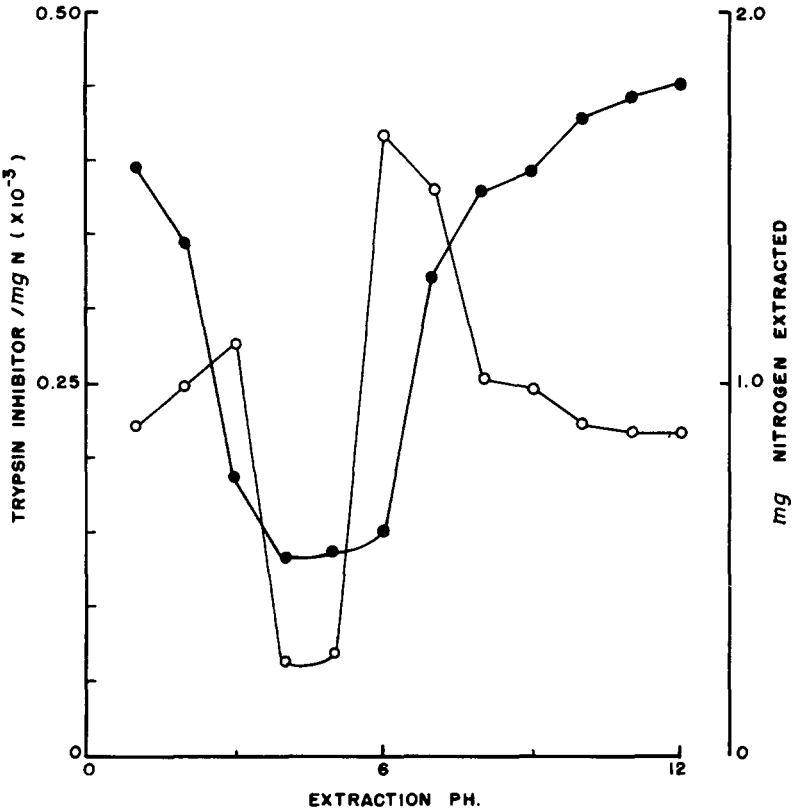


Fig. 2. Extractability of trypsin inhibitor in saline solution as affected by pH. ○ = Trypsin inhibitor units. ● = Nitrogen extracted.

Vicia faba beans is influenced by pH, type and concentration of the extractant and the degree of seed maturity.

Vicia faba *antitryptic activity as affected by dialysis*

The results given in Table 4 show that dialysis for 18 h at 5 °C against saline, phosphate buffer and distilled water reduced the total trypsin inhibitor activity by 3·5 %, 8·7 % and 9 % of that in the control. When the antitryptic activity was assessed in both dissolved precipitates and supernatants it was found that precipitates of distilled tap water and saline possessed 86·9 %, 69 % and 20 % of the antitryptic activity of the control and 95 %, 77 % and 22 % of the total activity after dialysis in each case.

Specificity of Vicia faba *trypsin inhibitor towards animal and plant proteases*

Liener (1969) stated that trypsin inhibitor isolated from double beans (*Faba vulgaris*) has the power to inhibit other proteases. Contrary to this finding, however,

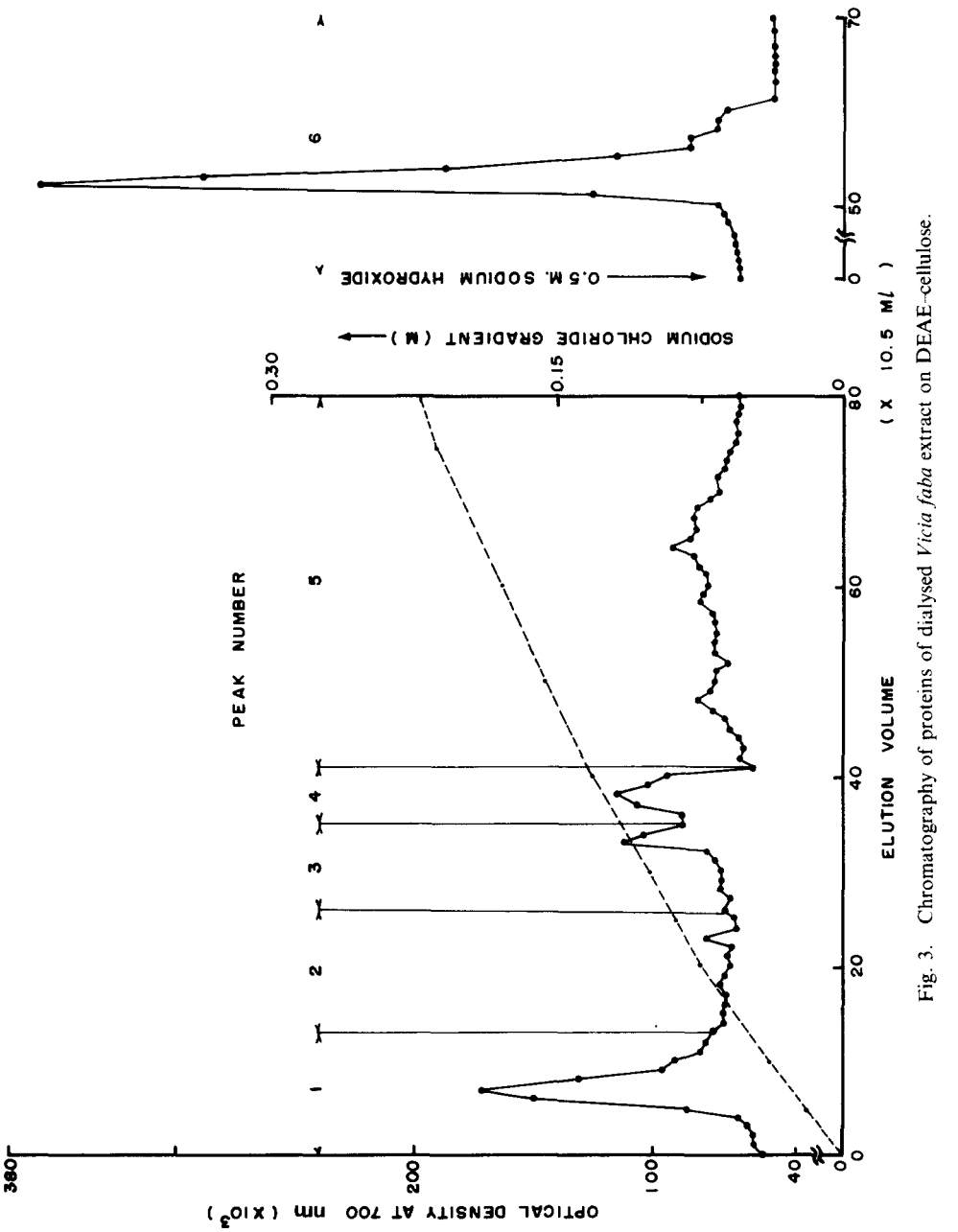


Fig. 3. Chromatography of proteins of dialysed *Vicia faba* extract on DEAE-cellulose.

TABLE 4
EFFECT OF DIALYSIS ON THE TRYPSIN INHIBITOR ACTIVITY[a] OF BEAN EXTRACT

Dialysis treatment	*Antitryptic activity (Units/mg N × 10^{-3})*		*Total*
	Supernatant	*Precipitate*	
Versus distilled water	16	339	355
Versus tap water	81	270	351
Versus 0·171M sodium chloride	277	79	356
Versus 0·1M phosphate buffer pH7·6	376	No precipitate	376
Undialysed extract (control)	390		

[a] Assayed according to Kunitz (1947).

TABLE 5
THE ANTITRYPSIN ACTIVITY OF FRACTIONS OBTAINED BY COLUMN CHROMATOGRAPHY

Peak number	*NaCl gradient* (M)	*Peak volume after concentration (ml)*	*Protein content*		*Antitryptic activity*		
			mg/ml	*% of total protein of combined fractions*	*Units/ml × 10^{-4}*	*Units/mg × 10^{-4} protein*	*% of total antitryptic activity*
1	0·012–0·050	4	0·887	16·13	1417	1598	25·5
2	0·050–0·090	3	0·524	7·16	744	1420	10·2
3	0·090–0·113	3	0·605	8·25	687	1136	9·3
4	0·113–0·130	3	0·623	8·50	632	1015	8·6
5	0·130–0·222	5	1·185	26·94	989	835	22·4
6	0·5M NaOH	7	1·036	32·97	755	729	23·8

the results found in this study showed that *Vicia faba* trypsin inhibitor is highly specific, being without effect on papain, rennin and pepsin. This is in agreement with the results previously reported by Sohonie & Ambe (1955) regarding trypsin inhibitor isolated from field beans (*Dolichos lablab*).

Fractionation of dialysed Vicia faba *protein extract on DEAE*

Fractionation (Fig. 3) shows five major peaks in addition to the large final peak eluted by sodium hydroxide. The six fractions obtained differed markedly from each other in their antitryptic (Table 5) activity. These results are not in agreement with those obtained by Wilson *et al.* (1972) who found that there was little difference between the fractions in their antitryptic activity.

REFERENCES

AOAC (1960). (Association of Agricultural Chemists). *Official Methods of Analysis* (11th edn), Washington, DC.

BHATTY, R. S. (1975). Trypsin inhibitors of faba beans (*Vicia faba* L. var. Minor). 1—Preliminary studies. *Canad. J. Bot.* **53**, 3075–7.

BORCHERS, R. & ACKERSON, C. W. (1947). Trypsin inhibitor: IV—Occurrence in seeds of the leguminosae and other seeds. *Arch. Biochem.* **13**, 291.

GREENBERG, D. M. (1955). Plant proteolytic enzymes. In: *Methods in enzymology*, vol. 2. (Colowick, S. P. & Kaplan, N. O. (Eds)), Academic Press, London and New York, 54.

HOBDAY, S. M., THURMAN, D. A. & BARKER, D. J. (1973). Proteolytic and trypsin inhibitory activities in extracts of germinating *Pisum sativum* seeds. *Phytochem.* **12**, 1041–6.

KAKADE, M. L., SIMONS, N. & LIENER, L. E. (1969). An evaluation of natural versus synthetic substrates for measuring the antitryptic activity of soybean samples. *Cereal Chem.* **46**, 518–26.

KUNITZ, M. (1947). Crystalline soybean trypsin inhibitor: II—General properties. *J. Gen. Physiol.* **30**, 291.

LIENER, I. E. (1969). *Toxic constituents of plant foodstuffs*. Academic Press, London and New York.

LOWRY, O. H., ROSENBROUGH, N. J., FARE, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265.

MCDONALD, C. E. & CHEN, L. L. (1965). The Lowry modification on the Folin reagent for determination of proteinase activity. *Anal. Biochem.* **10**, 175–8.

NISTAN, Z. (1971). *Vicia faba* beans versus soybean meal as a source of protein. *J. Sci. Fd. Agric.* **22**, 252.

PETERSON, E. A. & SOBER, H. A. (1962). Column chromatography of proteins on substituted celluloses. In: *Methods in enzymology*, vol. 5. (Colowick, S. P. & Kaplan, N. O. (Eds)), Academic Press, London and New York, 3–27.

RAMIREZ, J. S. & MITCHELL, H. L. (1960). The trypsin inhibitor of alfalfa. *J. Agric. Fd. Chem.* **8**, 393.

SOHONIE, K. & AMBE, K. S. (1955). Crystalline inhibitors from Indian field bean and double bean. *Nature* **175**, 508.

SOHONIE, K. & BHANDARKAR, A. P. (1954). Trypsin inhibitors in Indian foodstuffs. I—Inhibitors in vegetables. *J. Sci. Ind. Res.* (*India*) **13B**, 50c. (*cf Chem. Abs.* **49**, 534a, 1955).

SOHONIE, K., APTE, U. & AMBE, K. S. (1958). Trypsin inhibitors in Indian foodstuffs: V—Effect of broad bean (*Vicia faba*) and double bean trypsin inhibitors on the growth of rats. *J. Sci. Ind. Res.* (*India*). **17C**, 42. (*cf Chem. Abs.* **52**, 1755c, 1958).

TANNOUS, R. I. & ULLAH, M. (1968). Effect of autoclaving on some nutritional factors in legume seeds. *Proceedings of Fourth Symposium on Human Nutrition and Health in the Near East, Beirut, Lebanon*, p. 61.

WARSEY, A. S., NORTON, G. & STEIN, M. (1974). Protease inhibitors from broad bean: Isolation and purification. *Phytochem.*, **13**, 2481–6.

WILSON, B. J., MCNAB, J. M. & BENTLEY, H. (1972). Trypsin inhibitor activity in field beans (*Vicia faba* L.). *J. Sci. Fd. Agric.* **23**, 679–84.

Food Chemistry **6** (1981) 73–76

BOOK REVIEWS

Annual Reports on Fermentation Processes. Volume 3. Edited by D. Perlman. Academic Press, New York. 1979. 346 pp. Price: US$22·00.

The foreword states that the authors of each chapter were asked to answer the question: 'What are the major developments in the field that were published this past year?' The chapters on Genetics of Industrial Microorganisms, Aeration, Beta-lactam Antibiotics, Antitumour Antibiotics, and Nucleosides and Nucleotides do just this and include extensive bibliographies. Chapters on Cellulase and Biosynthetic Regulation, Single Cell Protein from C_1 Compounds, Microbial Transformations of Steroids, and Mushrooms: Single Cell Protein from Cellulose are more in the nature of general reviews and do not deal only with recently published papers. The chapter on The Use of Economic Analysis of Penicillin G Costs in Establishing Priorities for Fermentation Process Improvement appears to be an original contribution, albeit rather handicapped by the lack of published information on the details and costs of the relevant commercial processes.

The book includes a skimpy index and there are a number of minor printing and grammatical errors, especially in some of the chapters by authors whose native tongue is not English, which might easily have been eliminated by more careful editing. Nevertheless, those chapters that concentrate on the more recent developments do give useful insights into current activities in the fermentation industries.

J. D. OWENS

Food Texture and Rheology. Edited by P. Sherman, Academic Press Inc. Ltd., London. 1979

This book is a collection of papers presented at a symposium, organised on behalf of the International Union of Food Science and Technology, held at Queen Elizabeth

Food Chemistry **6** (1981) 73–76

BOOK REVIEWS

Annual Reports on Fermentation Processes. Volume 3. Edited by D. Perlman. Academic Press, New York. 1979. 346 pp. Price: US$22·00.

The foreword states that the authors of each chapter were asked to answer the question: 'What are the major developments in the field that were published this past year?' The chapters on Genetics of Industrial Microorganisms, Aeration, Beta-lactam Antibiotics, Antitumour Antibiotics, and Nucleosides and Nucleotides do just this and include extensive bibliographies. Chapters on Cellulase and Biosynthetic Regulation, Single Cell Protein from C_1 Compounds, Microbial Transformations of Steroids, and Mushrooms: Single Cell Protein from Cellulose are more in the nature of general reviews and do not deal only with recently published papers. The chapter on The Use of Economic Analysis of Penicillin G Costs in Establishing Priorities for Fermentation Process Improvement appears to be an original contribution, albeit rather handicapped by the lack of published information on the details and costs of the relevant commercial processes.

The book includes a skimpy index and there are a number of minor printing and grammatical errors, especially in some of the chapters by authors whose native tongue is not English, which might easily have been eliminated by more careful editing. Nevertheless, those chapters that concentrate on the more recent developments do give useful insights into current activities in the fermentation industries.

J. D. OWENS

Food Texture and Rheology. Edited by P. Sherman, Academic Press Inc. Ltd., London. 1979

This book is a collection of papers presented at a symposium, organised on behalf of the International Union of Food Science and Technology, held at Queen Elizabeth

College, London, in December 1977. They cover a wide range of topics including: sensory and instrumental methods of texture measurement, the relationship between results obtained by sensory and non-sensory techniques, discussion of specific textural characteristics such as crispness, mouthfeel and spreadability and the rheology of several food products including butter, margarine, instant potato, dough, bread, chocolate mass, cheese and bacon. Papers on the rheology of emulsions, gels and vegetable proteins are also included. The book is well produced and edited with very good diagrams and plates.

Representing as it does the views and experiences of a group of experts actively involved in research in food texture and rheology, this work should be of great interest to both research workers and students engaged in such activities.

J. G. BRENNAN

Developments in Food Science 2. *Proceedings of the Fifth International Congress of Food Science and Technology.* Edited by H. Chiba, M. Fujimaki, K. Iwai, H. Mitsuda and Y. Morita. Co-published by Kodansha Ltd., Tokyo and Elsevier Scientific Publishing Company, Amsterdam–Oxford–New York. 1979.

The Fifth International Congress of Food Science and Technology was held in Kyoto, Japan, in September 1978, under the sponsorship of the International Union of Food Science and Technology (IUFOST). It included two plenary sessions, at the beginning and end of the congress, four supplementary sessions in which some 50 papers were presented by invited speakers, and contributed paper sessions during which some 350 original research papers were presented. This book contains the full texts of the plenary lectures and abridged versions of the papers presented in the supplementary sessions.

The World Food Problem and Meeting the Challenge was the title of the opening address by Emil M. Mrak. He reviewed the many causes of the problem proposed in the literature and some of the numerous suggestions on how to solve it. In particular he discussed the role of food processing and food technology in this context. Professor John Hawthorn spoke to the title 'Everyman and Food Science' at the end of the congress. He indicated some of the ways in which developments in food science and technology have altered the structures of our society and our way of life. He pointed out that, despite a huge increase in demand, the food supply per head has increased substantially over the last decade or so. However, the gap between the developing and the developed countries is increasing. He suggested five ways in which IUFOST could influence this situation and recommended these as a programme for the union. Although poor in financial terms IUFOST is rich in intellectual resources and these should be tapped and used in the task of attempting to improve the lot of the developing countries.

The papers presented at the supplementary sessions are classified under four main

College, London, in December 1977. They cover a wide range of topics including: sensory and instrumental methods of texture measurement, the relationship between results obtained by sensory and non-sensory techniques, discussion of specific textural characteristics such as crispness, mouthfeel and spreadability and the rheology of several food products including butter, margarine, instant potato, dough, bread, chocolate mass, cheese and bacon. Papers on the rheology of emulsions, gels and vegetable proteins are also included. The book is well produced and edited with very good diagrams and plates.

Representing as it does the views and experiences of a group of experts actively involved in research in food texture and rheology, this work should be of great interest to both research workers and students engaged in such activities.

J. G. BRENNAN

Developments in Food Science 2. *Proceedings of the Fifth International Congress of Food Science and Technology.* Edited by H. Chiba, M. Fujimaki, K. Iwai, H. Mitsuda and Y. Morita. Co-published by Kodansha Ltd., Tokyo and Elsevier Scientific Publishing Company, Amsterdam–Oxford–New York. 1979.

The Fifth International Congress of Food Science and Technology was held in Kyoto, Japan, in September 1978, under the sponsorship of the International Union of Food Science and Technology (IUFOST). It included two plenary sessions, at the beginning and end of the congress, four supplementary sessions in which some 50 papers were presented by invited speakers, and contributed paper sessions during which some 350 original research papers were presented. This book contains the full texts of the plenary lectures and abridged versions of the papers presented in the supplementary sessions.

The World Food Problem and Meeting the Challenge was the title of the opening address by Emil M. Mrak. He reviewed the many causes of the problem proposed in the literature and some of the numerous suggestions on how to solve it. In particular he discussed the role of food processing and food technology in this context. Professor John Hawthorn spoke to the title 'Everyman and Food Science' at the end of the congress. He indicated some of the ways in which developments in food science and technology have altered the structures of our society and our way of life. He pointed out that, despite a huge increase in demand, the food supply per head has increased substantially over the last decade or so. However, the gap between the developing and the developed countries is increasing. He suggested five ways in which IUFOST could influence this situation and recommended these as a programme for the union. Although poor in financial terms IUFOST is rich in intellectual resources and these should be tapped and used in the task of attempting to improve the lot of the developing countries.

The papers presented at the supplementary sessions are classified under four main

topic headings: Resources of Food, Safety and Nutritional Aspects of Food, Preservation and Processing of Food, Physical, Chemical and Sensory Properties of Food. The papers vary in length and to some degree in style as might be expected. Each one may be regarded as a mini review of a topic by a specialist. Collectively they represent a very useful contribution to the literature on food science and technology, and should interest students, researchers, food scientists and technologists working in industry.

In the appendix the titles of the research papers are listed under eleven headings and also seven topics discussed at round table meetings.

J. G. BRENNAN

Nutritional Improvement of Food and Feed Proteins. *Advances in Experimental Medicine and Biology*. Volume 10S. Edited by Mendel Friedman. Plenum Publishing Corporation, New York. 1978. xii + 882 pp. Price: $69·50.

This imposing book addresses itself almost entirely to the problem of protein malnutrition and improving the quality, quantity and nutritional availability of foods and feeds.

There are 40 chapters in all dealing with protein from various sources, especially relating to grain quality, amino acid supplementation, fortification, mixed products and one on fibre. Nitrogen retention, nutritional quality and analysis are all extensive areas of coverage and the opening chapter is devoted to RDA for protein in children.

The book in fact represents the proceedings of the Symposium on Improvement of Protein Nutritive Quality of Foods and Feeds, sponsored by the Protein Subdivision of the Division of Agricultural and Food Chemistry of the American Chemical Society, Chicago, Illinois, 29 August–2 September, 1977, with additional invited contributions. At this price it is a most recommendable text to all workers in the field.

G. G. BIRCH

Food and Health: Science and Technology. Edited by G. G. Birch and K. J. Parker. Applied Science Publishers Ltd, London. 1980. xii + 532 pp. Price: £32.

This is a large book containing the papers presented at a Symposium organised by the National College of Food Technology, Weybridge, in 1979. Although it contains a total of 31 papers, it is quite expensive at £32, so it is likely to be bought by libraries and institutions rather than by individuals.

The papers vary widely in scope and their usefulness to the food scientist. For example the first one, by Dr Elsie Widdowson, covers food and health from

topic headings: Resources of Food, Safety and Nutritional Aspects of Food, Preservation and Processing of Food, Physical, Chemical and Sensory Properties of Food. The papers vary in length and to some degree in style as might be expected. Each one may be regarded as a mini review of a topic by a specialist. Collectively they represent a very useful contribution to the literature on food science and technology, and should interest students, researchers, food scientists and technologists working in industry.

In the appendix the titles of the research papers are listed under eleven headings and also seven topics discussed at round table meetings.

J. G. BRENNAN

Nutritional Improvement of Food and Feed Proteins. *Advances in Experimental Medicine and Biology*. Volume 10S. Edited by Mendel Friedman. Plenum Publishing Corporation, New York. 1978. xii + 882 pp. Price: $69·50.

This imposing book addresses itself almost entirely to the problem of protein malnutrition and improving the quality, quantity and nutritional availability of foods and feeds.

There are 40 chapters in all dealing with protein from various sources, especially relating to grain quality, amino acid supplementation, fortification, mixed products and one on fibre. Nitrogen retention, nutritional quality and analysis are all extensive areas of coverage and the opening chapter is devoted to RDA for protein in children.

The book in fact represents the proceedings of the Symposium on Improvement of Protein Nutritive Quality of Foods and Feeds, sponsored by the Protein Subdivision of the Division of Agricultural and Food Chemistry of the American Chemical Society, Chicago, Illinois, 29 August–2 September, 1977, with additional invited contributions. At this price it is a most recommendable text to all workers in the field.

G. G. BIRCH

Food and Health: Science and Technology. Edited by G. G. Birch and K. J. Parker. Applied Science Publishers Ltd, London. 1980. xii + 532 pp. Price: £32.

This is a large book containing the papers presented at a Symposium organised by the National College of Food Technology, Weybridge, in 1979. Although it contains a total of 31 papers, it is quite expensive at £32, so it is likely to be bought by libraries and institutions rather than by individuals.

The papers vary widely in scope and their usefulness to the food scientist. For example the first one, by Dr Elsie Widdowson, covers food and health from

topic headings: Resources of Food, Safety and Nutritional Aspects of Food, Preservation and Processing of Food, Physical, Chemical and Sensory Properties of Food. The papers vary in length and to some degree in style as might be expected. Each one may be regarded as a mini review of a topic by a specialist. Collectively they represent a very useful contribution to the literature on food science and technology, and should interest students, researchers, food scientists and technologists working in industry.

In the appendix the titles of the research papers are listed under eleven headings and also seven topics discussed at round table meetings.

J. G. BRENNAN

Nutritional Improvement of Food and Feed Proteins. *Advances in Experimental Medicine and Biology*. Volume 10S. Edited by Mendel Friedman. Plenum Publishing Corporation, New York. 1978. xii + 882 pp. Price: $69·50.

This imposing book addresses itself almost entirely to the problem of protein malnutrition and improving the quality, quantity and nutritional availability of foods and feeds.

There are 40 chapters in all dealing with protein from various sources, especially relating to grain quality, amino acid supplementation, fortification, mixed products and one on fibre. Nitrogen retention, nutritional quality and analysis are all extensive areas of coverage and the opening chapter is devoted to RDA for protein in children.

The book in fact represents the proceedings of the Symposium on Improvement of Protein Nutritive Quality of Foods and Feeds, sponsored by the Protein Subdivision of the Division of Agricultural and Food Chemistry of the American Chemical Society, Chicago, Illinois, 29 August–2 September, 1977, with additional invited contributions. At this price it is a most recommendable text to all workers in the field.

G. G. BIRCH

Food and Health: Science and Technology. Edited by G. G. Birch and K. J. Parker. Applied Science Publishers Ltd, London. 1980. xii + 532 pp. Price: £32.

This is a large book containing the papers presented at a Symposium organised by the National College of Food Technology, Weybridge, in 1979. Although it contains a total of 31 papers, it is quite expensive at £32, so it is likely to be bought by libraries and institutions rather than by individuals.

The papers vary widely in scope and their usefulness to the food scientist. For example the first one, by Dr Elsie Widdowson, covers food and health from

conception to old age with much useful detail and background information for anyone with a general interest in human nutrition. At the other end of the scale are papers on one particular health hazard, such as the last paper of all, by Professor D. Bryce-Smith on lead and brain function in which very little is said about lead in food.

The papers tend to fall into two main groups, one on general considerations in nutrition and health, and the other on technology and processing in relation to food quality and safety. An unusual feature in a nutrition symposium report of this type is the inclusion of a paper on health foods by Mr M. Hanssen, challenging some current practices in food science and technology in the UK.

Many of the contributions have been well researched and will provide useful reference lists for anyone requiring more detailed information, for example Dr R. S. Kirk's paper on erucic acid and a detailed review originating from the MIT on the use of microbial systems for examining the biological activity of foods.

No one person will agree with all the views expressed in these 31 diverse contributions, but at least they may stimulate further interest in food/health issues and assist workers and students in the specific fields covered at the Symposium.

T. GRENBY

Food and Beverage Mycology. Edited by L. R. Beuchat. A.V.I. Publishing Co., Connecticut. 1978, second printing 1979. 527 pp. Price: US$28·00.

This book offers a comprehensive survey of the roles of fungi in the food and beverage industries as spoilage organisms, as useful contributors in food and drink fermentations and as producers of metabolites and biomass. In addition, there are chapters on classification, effects of water availability, mycotoxins, and general methodology.

The fact that it has already been reprinted indicates the need that existed for a general source book on food mycology and the success of this book at meeting that need. Its rapid acceptance is, I am sure, very largely due to the well written and very informative chapters that deal with the roles of fungi associated with specific product types. Certain of the other chapters, while contributing to the comprehensive nature of the book, are less valuable because they deal with topics that are equally well or better reviewed in other publications. Nevertheless, I know of no other book covering the same ground and have no hesitation in recommending it as a source of first recourse for students, food technologists, educators and others wanting information about fungi in the food and beverage industries.

J. D. OWENS

conception to old age with much useful detail and background information for anyone with a general interest in human nutrition. At the other end of the scale are papers on one particular health hazard, such as the last paper of all, by Professor D. Bryce-Smith on lead and brain function in which very little is said about lead in food.

The papers tend to fall into two main groups, one on general considerations in nutrition and health, and the other on technology and processing in relation to food quality and safety. An unusual feature in a nutrition symposium report of this type is the inclusion of a paper on health foods by Mr M. Hanssen, challenging some current practices in food science and technology in the UK.

Many of the contributions have been well researched and will provide useful reference lists for anyone requiring more detailed information, for example Dr R. S. Kirk's paper on erucic acid and a detailed review originating from the MIT on the use of microbial systems for examining the biological activity of foods.

No one person will agree with all the views expressed in these 31 diverse contributions, but at least they may stimulate further interest in food/health issues and assist workers and students in the specific fields covered at the Symposium.

T. GRENBY

Food and Beverage Mycology. Edited by L. R. Beuchat. A.V.I. Publishing Co., Connecticut. 1978, second printing 1979. 527 pp. Price: US$28·00.

This book offers a comprehensive survey of the roles of fungi in the food and beverage industries as spoilage organisms, as useful contributors in food and drink fermentations and as producers of metabolites and biomass. In addition, there are chapters on classification, effects of water availability, mycotoxins, and general methodology.

The fact that it has already been reprinted indicates the need that existed for a general source book on food mycology and the success of this book at meeting that need. Its rapid acceptance is, I am sure, very largely due to the well written and very informative chapters that deal with the roles of fungi associated with specific product types. Certain of the other chapters, while contributing to the comprehensive nature of the book, are less valuable because they deal with topics that are equally well or better reviewed in other publications. Nevertheless, I know of no other book covering the same ground and have no hesitation in recommending it as a source of first recourse for students, food technologists, educators and others wanting information about fungi in the food and beverage industries.

J. D. OWENS

Food Chemistry 7 (1981) 77–80

A RAPID METHOD FOR SELECTIVELY DETERMINING SMALL AMOUNTS OF NIACIN, RIBOFLAVIN AND THIAMINE IN FOODS

GEOFFREY R. SKURRAY

School of Food Sciences, Hawkesbury Agricultural College, Richmond, New South Wales 2753, Australia

(Received: 11 February, 1980)

Niacin, riboflavin and thiamine (as thiochrome) were determined in enzymic hydrolysates of foods by high performance liquid chromatography. Paired ion chromatography, combined with ultraviolet and fluorescent spectroscopy, allowed for highly selective and sensitive detection of the vitamins.

The results of the assays were similar to the more time-consuming manual methods but the accuracy and sensitivity were much higher using fluorescent detection.

INTRODUCTION

The problems associated with the measurement of water-soluble vitamins in foods have been mainly associated with the interference of other compounds in foods and the difficulty of measuring small amounts of vitamins in certain foods.

High performance liquid chromatography has the advantages of selectivity and speed of analysis (Skurray, 1978) but the low sensitivity of ultraviolet detectors is unsuitable for the analysis of processed food containing microgramme quantities of riboflavin and thiamine.

Fluorescent spectroscopy is a sensitive and selective method for determining riboflavin in cereal products (Nobile *et al.*, 1972) and thiamine after converting it to the fluorescent thiochrome (AOAC, 1975).

The aim of the present study was to use the rapidity and selectivity of high performance liquid chromatography in combination with the sensitivity of a fluorescence detector to determine niacin, riboflavin and thiamine in foods.

Fd. Chem. 0308-8146/81/0007-0077/$02·50

MATERIALS AND METHODS

Food samples were digested in duplicate with 0·2M sulphuric acid followed by enzymic digestion with clarase according to the method of Nobile *et al.* (1972).

A 5-ml aliquot of the digest was mixed with 3 ml of 15% NaOH solution and one drop of 1% potassium ferricyanide solution (freshly prepared) to oxidise the thiamine to thiamine thiochrome.

The samples were clarified by passing them through a 0·25 mm filter. A 10-μl aliquot of the sample was applied to a 30 cm × 4 mm micro Bondapak C18 column (Waters Associates) and eluted with degassed 0·2M acetate buffer containing 0·005M heptane sulphonic acid. Recovery samples were prepared by adding known amounts of niacin, riboflavin and thiamine, prior to extraction of the sample.

A flow rate of 1 ml/min was obtained using a Spectra Physics 3500B high performance liquid chromatograph with a 250 nm ultraviolet detector and a Gilson Spectra-Glo fluorescence spectrophotometer with a 390 nm excitation filter and a 475 nm emission filter.

Niacin and riboflavin were also measured by the classical AOAC (1975) methods and thiamine by the method of Nobile *et al.* (1972).

RESULTS AND DISCUSSION

The organic counter ion, heptane sulphonic acid, the cationic niacin and thiamin thiochrome form ion pairs in the mobile phase and the ion-paired complexes partition with the stationary phase. The resulting chromatogram (Fig. 1) indicates that the niacin can be estimated by an ultraviolet detector. However, the sensitivity of fluorescence spectroscopy allows riboflavin and thiamine thiochrome to be detected at the concentrations normally found in foods (10–100 μg/100 g). A recovery of $97 \pm 0{\cdot}5\%$ was determined for internal standards.

Values obtained for thiamine and niacin by chromatography were similar to those determined by the methods of Nobile *et al.* (1972) and the AOAC (1975), respectively (see Table 1). However, the values for riboflavin were lower for the chromatographic method than the AOAC (1975) method, particularly in those foods that contained small amounts of riboflavin (raw herring and white bread).

This may be due to the interference of fluorescent compounds present in foods. Toma & Tabekhia (1979) found a similar correlation between the analysis of rice products by classical methods and chromatography. These workers used an ultraviolet detector at the maximum sensitivity which led to unstable chromatograms. The fluorescence detector used in the present experiments was used at 50% of the maximum sensitivity and the chromatograms were stable and free from interference from ultraviolet absorbing compounds.

The combination of high performance chromatography, ultraviolet and fluorescent

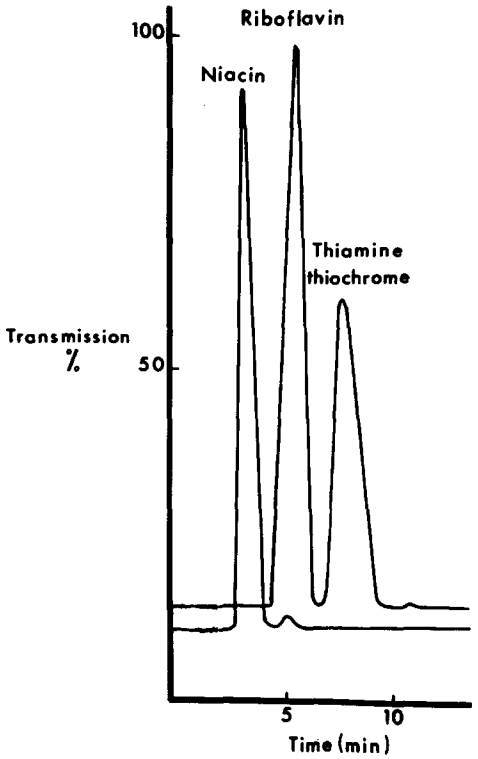


Fig. 1. Chromatogram of niacin (250 nm absorption), riboflavin and thiamine thiochrome (fluorescent absorption) in milk.

TABLE 1
NIACIN, RIBOFLAVIN AND THIAMINE DETERMINATION BY DIFFERENT METHODS

Sample	*Niacin (mg/100 g)*		*Riboflavin (mg/100 g)*		*Thiamine (μg/100 g)*	
	HPLC	*AOAC (1975)*	*HPLC*	*AOAC (1975)*	*HPLC*	*AOAC (1975)*
Bovine muscle (*Longissimus dorsi*)	5·1±0·1	5·4±0·2	0·15±0·01	0·16±0·02	84±1·5	88±3·3
Whole milk	0·13±0·11	0·11±0·01	0·15±0·02	0·20±0·03	32±3	35±2
Raw peas	3·1±0·2	3·4±0·1	0·23±0·04	0·29±0·05	305±11	310±16
Raw herring (Pacific)	4·8±0·3	5·3±0·5	0·09±0·01	0·17±0·03	25±2	24±3
White bread (starch reduced)	1·1±0·1	0·85±0·1	0·05±0·01	0·11±0·02	115±8	110±5
Rolled oats	1·1±0·2	1·4±0·1	0·16±0·03	0·14±0·03	490±11	505±9
Cornflakes (fortified)	12·8±0·8	13·3±0·9	2·5±0·06	2·4±0·09	1100±20	1080±25
Yoghurt (natural)	0·21±0·1	0·21±0·08	0·25±0·03	0·29±0·05	60±4	65±6

detection has the advantage of speed, sensitivity and selectivity in determining niacin, riboflavin and thiamine in foods.

REFERENCES

AOAC (1975). *Official methods of analysis* (12th edn), Association of Official Analytical Chemists, Washington, DC.

NOBILE, S., SAVAGE, V. & HUBER, U. (1972). A new procedure for the determination of thiamine in foods. *Internat. J. Vit. Res.*, **42**, 444–50.

SKURRAY, G. R. (1978). Rapid analysis of foods by high performance liquid chromatography. *Prac. Chem. Eng.*, **31**, 26–9.

TOMA, R. B. & TABEKHIA, M. M. (1979). High performance liquid chromatographic analysis of B-vitamins in rice and rice products. *J. Fd. Sci.*, **44**, 263–5.

Food Chemistry 7 (1981) 81–86

SUGARS IDENTIFIED IN *RAPHIA* PALM WINE

S. I. FAPARUSI

Biochemistry Department, University of Ibadan, Ibadan, Nigeria

(Received: 21 November, 1979)

ABSTRACT

In a study of sugars of Raphia *palm wine, eight palm trees of the* Raphia hookeri *species were tapped. Nine sugars were identified from the palm wine samples. Colorimetric determination of the sugars showed that sucrose, glucose and fructose decreased in concentration during the 22-day period of tapping the* Raphia *palm trees. Three sugars—cellobiose, maltose and xylose—showed slight increases during that period. Three other sugars—rhamnose, arabinose and galacturonic acid—were inconsistent in the days of their appearance. Possible origins of some of the sugars are suggested.*

INTRODUCTION

Two *Raphia* palm trees (*Raphia vinifera* and *R. hookeri*) serve as the chief sources of palm wine (a fermented palm sap) in the swampy regions of southern Nigeria (Faparusi, 1966). The other palm tree supplying palm wine is the oil palm tree (*Elaeis guineensis*), found in the deciduous and derived savanah zones of Nigeria. Unlike the oil palm tree which can be tapped repeatedly, the *Raphia* palm tree is tapped once only in its life before it dies. In addition, a *Raphia* palm tree can be tapped for a period of 18 to 25 days before it dies whilst the oil palm tree is usually tapped for a period of 7 to 9 days before it ceases to produce palm wine.

The best method of tapping a *Raphia* palm tree for its palm wine has been well documented by Tuley (1965).

Previous reports on palm wine have dealt with oil palm wine (Faparusi & Bassir, 1972). This paper is thus limited to palm wine obtained from the *Raphia* palm tree.

The *Raphia hookeri* palm trees employed in this study were located on the banks of streams and rivers in Ode-Ekiti in the Ondo State of Nigeria. Each palm tree was tapped for 22 days, a total of eight palm trees being tapped.

Fd. Chem. 0308-8146/81/0007-0081/$02·50

MATERIALS AND METHODS

The palm wine was collected in a sterilised 10-litre flask. The mouth was corked with non-absorbent cotton wool and the funnel through which the palm juice drops was made to pass through the cotton wool from the taphole to the flask. The wine was always stored in a deep freezer (temperature, $-25\,^{\circ}C$) after collection until it was to be analysed. Daily samples were subjected to qualitative and quantitative analyses of sugars.

(*i*) *Qualitative analyses*

The sugars were separated on chromatographic Whatman No. 1 filter paper in *n*-butanol–acetic acid–water (4:1:1) using a one-way descending method. The chromatograms were usually run for varying periods of 36 to 60 h, depending on the sugars present. After drying the chromatograms in air the sugar spots were located with $AgNO_3$ in ethanol (Faparusi, 1970). Standard sugars were always run along the palm wine specimens for easy identification of the sugar spots.

(*ii*) *Quantitative analyses*

0·01 ml of each sample was spotted in quadruplicate on Whatman No. 1 chromatography paper. The chromatograms were run for periods ranging from 36 h to 60 h. After drying the chromatograms in air one of the quadruplicate strips was cut out and the sugars on it were located with $AgNO_3$ in ethanol as described above. Next, areas containing the spots were marked out and the corresponding areas on each of the remaining triplicates were removed and eluted with 5 ml of distilled water. The amounts of sugars in the eluates were determined colorimetrically following the method described by Dubois *et al.* (1956). The galacturonic acid in the eluate was estimated using the carbazole method (Dische, 1950).

RESULTS AND DISCUSSION

Nine sugars were identified from the *Raphia* palm wine samples. As shown in Table 1, three slow-moving saccharides—xylose, rhamnose and arabinose—were amongst them. Four other sugars—fructose, glucose, maltose and sucrose—obtained from the palm wine samples were earlier shown to be present in oil palm wine (Faparusi & Bassir, 1972).

Two other spots on the chromatograms were identified as those of cellobiose and galacturonic acid with R_g values of 0·40 and 0·20, respectively in *n*-butanol–acetic acid–water (4:1:1). The R_g values of the sugars are also shown in Table 1. Rhamnose and xylose were identified on chromatograms run for 36 h. Glucose, fructose and arabinose were identified on chromatograms run for 48 h, whilst maltose, sucrose, cellobiose and galacturonic acid separated well on chromatograms run for 60 h.

TABLE 1
SUGARS IDENTIFIED IN *Raphia* PALM WINE

Sugars	R_g *in BuOH–AC–H_2O (4:1:1)*	*Concentration ranges (mg per 100 ml)*
L-Arabinose	1·15	×
D-Xylose	1·30	0·30–0·50
L-Rhamnose	1·60	×
D-Fructose	1·10	0·70–0·90
D-Glucose	1·00	0·85–1·30
Maltose	0·50	0·45–0·80
Sucrose	0·65	0·50–1·40
Cellobiose	0·40	0·10–0·30
Galacturonic acid	0·20	×

× = Variable.
AC = Acetic acid.

Figure 1 shows the variation of concentrations of some sugars when the *Raphia* palm trees were tapped for 22 days. Only sucrose, maltose, glucose and fructose were obtained in palm wine samples obtained on the first day of tapping. Other sugars were obtained from samples obtained later. Xylose was first identified in palm wine samples obtained from about the ninth day, whilst cellobiose was obtained after the first eleven days.

Rhamnose, arabinose and galacturonic acid are not shown in Fig. 1 because their appearance in the palm wine samples was rather inconsistent. Sometimes, one of them could be identified in palm wine samples for a period of 3 to 6 days which were not always consecutive. As discussed later, these three sugars are products of the

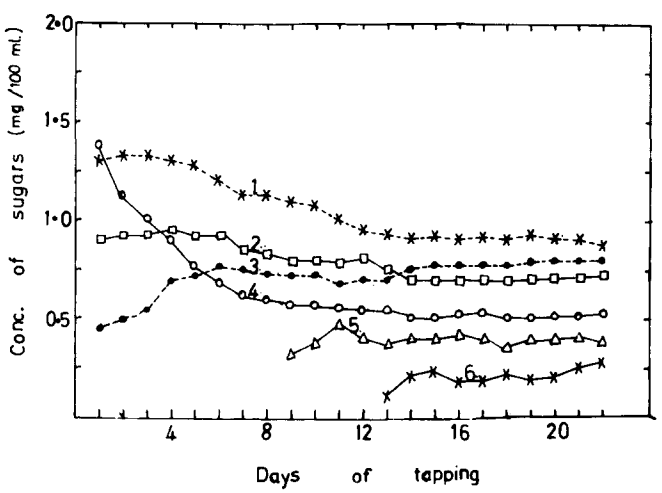


Fig. 1. Variation of sugar concentrations during the tapping period of a *Raphia* palm tree. (1) Glucose. (2) Fructose. (3) Maltose. (4) Sucrose. (5) Xylose. (6) Cellobiose.

hydrolysis of polysaccharides, probably by the microorganisms. Every day the tapholes are cleared of their microorganisms by shaving off pieces of tissue. The efficiency of daily clearance would vary and this would result in a variation in the daily microbial population in the tapholes. Thus the capability of microorganisms to hydrolyse polysaccharides to the three sugars would vary. From the list of sugars identified, it is possible to classify them into three categories. First are those that were obtained from all the palm wine samples. Fructose, glucose, maltose and sucrose belong to this group. Excepting the maltose, the other three sugars have similarly been identified in oil palm wine (Faparusi & Bassir, 1972).

The second group of sugars are those which were identified towards the middle of the tapping period. Xylose and cellobiose belong to this group.

The third group consists of those substances which appeared irregularly for a few days. Galacturonic acid, arabinose and rhamnose are in this category. Fructose, glucose and sucrose are common plant sugars; sucrose has been identified in sieve tube exudates (Zimmerman, 1957) and constituents of other palm trees—for example, *Cocos nucifera* and *Phoenix silvestris* (Gopinathan, 1962).

Other sugars are known to be constituents of plant polysaccharides. For example, maltose is a product of starch hydrolysis, cellobiose being similarly obtained from cellulose. Galacturonic acid can be obtained from a pectin. Xylan is a structural polysaccharide of plant tissues usually associated with cellulose which, on hydrolysis, is converted to xylose (Whistler, 1950); some plant xylans also contain L-arabinose units.

A variety of microflora were identified in the analyses of the taphole. These microflora included such yeasts as *Candida* spp., *Torulopsis* spp., *Saccharomyces cerevisiae*, *Pichia pastoris*, *P. silvestris*, *Trichosporon fermentans*, *Endomycopss fasciculata* and *Hansenula anomala*. Some bacteria also isolated from the taphole included species of *Bacillus*, *Lactobacillus*, *Micrococcus*, *Pediococcus* and *Streptomyces*. These microorganisms are capable of hydrolysing the previously mentioned plant polysaccharides to produce some of the sugars in the palm wine. As shown in Fig. 1, three sugars decreased in concentration during the 22-day period of tapping of the palm trees. Sucrose decreased from about 1·8 mg to around 0·6 mg per 100 ml within the first seven days before remaining almost constant throughout the rest of the period. Glucose decreased steadily from around 1·3 mg to 0·9 mg per 100 ml; similarly, fructose decreased from 0·95 mg to 0·7 mg per 100 ml. The initial sharp drop in the level of sucrose is usually reflected in the change of taste of the palm wine from sweet to sharp. With the exception of initial small increases, the concentrations of cellobiose and xylose were constant throughout the period of their identification. Maltose increased during the first six days from 0·45 mg to 0·75 mg per 100 ml; thereafter, the concentration remained almost constant throughout the rest of the period. The initial increase could probably have been due to the preferential metabolism of other sugars by the microorganisms in the tapholes. However, with less of the preferential sugars being available for metabolism,

coupled with the increase in the microflora population in the tapholes, part of the maltose could be metabolised as it is produced from polysaccharide hydrolysis. A considerable decrease in sucrose concentration could be due to its preferential usage by the microorganisms. Hestrin (1953) has shown that yeasts could metabolise sucrose faster than glucose through metabolic routes other than hydrolysis. Such routes as phosphorolysis (Hestrin, 1953), transglucosidation and transfructosidation have been demonstrated by Bacon (1954).

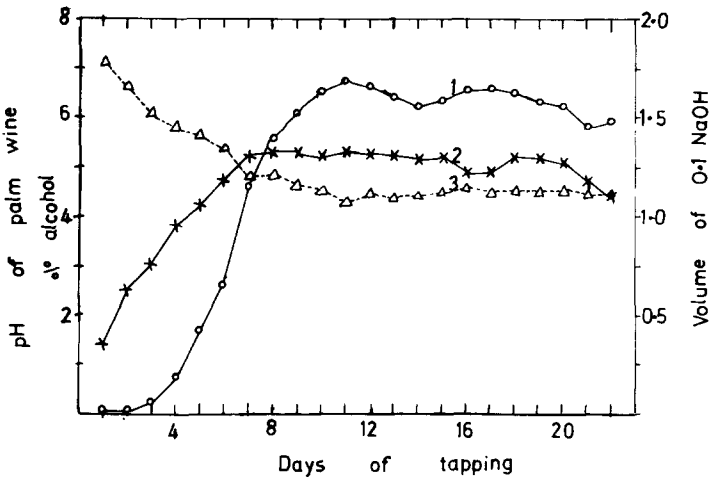


Fig. 2. Variation of alcohol, titratable acids and pH during the period of tapping a *Raphia* palm tree. (1) Titratable acids. (2) Alcohol. (3) pH of palm wine.

Figure 2 shows variations of alcohol and titratable acids during the period of tapping the *Raphia* palm trees. The acids were titrated against 0·1N NaOH using phenol red as the indicator. Alcohol increased from 1·4% to about 5·3% on the eighth day, whilst the titratable acids increased from an equivalent of 0·05 ml to 1·7 ml of 0·1N NaOH on the eleventh day of the tapping period. The concentrations of alcohol and the acids remained constant throughout the rest of the period. Similarly, the pH of palm wine fell from around 7·1 to 4·3 on the eleventh day, after which there was no further fall in pH value. The alcohol and acids contribute to the potency and the sharp taste of palm wine.

REFERENCES

BACON, J. S. D. (1954). The oligosaccharides produced by the action of yeast invertase preparation on sucrose. *Biochem. J.*, **57**, 320–8.

DISCHE, Z. (1950). A modification of the carbazole reaction of hexuronic acids for the study of polyuronides. *J. Biol. Chem.*, **183**, 489–94.

DUBOIS, M., GILES, K. A., HAMILTON, J. K., REBERS, P. A. & SMITH, F. (1956). Colorimetric method for determination of sugars and related substances. *Analyt. Chem.*, **28**, 350–6.

FAPARUSI, S. I. (1966). *A biochemical study of palm wine from different varieties of* Elaeis guineensis. Ph.D. Thesis, University of Ibadan, Nigeria.

FAPARUSI, S. I. (1970). Sugar changes during the preparation of Burukutu beer. *J. Sci. Fd. Agric.*, **21**, 79–81.

FAPARUSI, S. I. & BASSIR, O. (1972). Factors affecting the quality of palm wine. 1. Period of tapping a palm tree. *West Afric. J. Biol. & Appl. Chem.*, **15**, 17–23.

GOPINATHAN, N. (1962). Sugars in palm juices. *Chem. Abstr.*, **56**, 13256.

HESTRIN, S. (1953). Non-oxidative and non-proteolytic enzymes. Glycosidases. *Ann. Rev. Biochem.*, **22**, 85–106.

TULEY, P. (1965). How to tap a *Raphia* palm, *Nigerian Field*, **XXX**, 120–32.

WHISTLER, R. (1950). Xylan *Adv. Carbohyd. Chem.*, **5**, 269–90.

ZIMMERMAN, H. M. (1957). Translocation of organic substances in trees. Part 1. The nature of sugars in the sieve tube exudate of a tree, *Plant Physiol.*, **32**, 288–91.

Food Chemistry 7 (1981) 87–94

CHANGES IN VOLATILE FLAVOUR COMPONENTS OF SOYBEANS DURING ROASTING

H. KATO, Y. DOI, T. TSUGITA, K. KOSAI, T. KAMIYA & T. KURATA*

Department of Agricultural Chemistry, The University of Tokyo, Bunkyo-ku, Tokyo, Japan

(Received: 2 January, 1980)

ABSTRACT

Volatile concentrates were prepared from whole soybeans, roasted at 200°C for 10, 20 and 30 min, by steam distillation under reduced pressure followed by ether extraction, and analysed by gas chromatography and gas chromatography–mass spectrometry, in comparison with the volatile concentrate from raw soybean flour.

Major flavour components of raw soybean, such as n-*hexanol, 1-octen-3-ol and* n-*hexanal, decreased during the course of roasting, but the rate of this decrease was not rapid, especially between 10 and 20 minutes' roasting. During the roasting period, alkylated pyrazines, oxygenated furans, oxygenated pyrroles and phenols were formed or increased markedly. On the other hand, sensory evaluation showed that a flavour change to 'desirable' from 'beany' or 'objectionable' occurred between 10 and 20 minutes' roasting. These results suggest that roast flavour masks the 'beany' flavour in soybean.*

INTRODUCTION

Soybean is one of the most important sources of vegetable oil and protein and is receiving increased attention because of its low cost and high nutritive value. Although soybeans have been used for human consumption in some countries in Asia, there are considerable limitations to their use in other parts of the world. The most significant factor responsible for such limitations is probably the characteristic soybean flavour. Numerous investigations have been carried out on the flavour of

* Present address: Department of Food and Nutrition, Ochanomizu University, Bunkyo-ku, Tokyo, Japan.

Fd. Chem. 0308-8146/81/0007-0087/$02·50

raw soybean and a variety of soybean products and Maga (1973) has reviewed the subject.

Raw soybean has beany, bitter and astringent flavours. On heat treatment, however, the typical soybean flavour is generally observed to decrease, although protein solubility also decreases and colour darkens at higher temperatures. Wilkens & Lin (1970) reported on the volatile flavour components of deep fat-fried soybeans, which have a pleasant odour described as 'roasted nut' or 'peanut butter-like'. Roasted soybean flour—called Kinako in Japanese—has traditionally been consumed in Japan and has a desirable flavour without any beany odour or bitter taste. Kawamura (1967) analysed the composition of Kinako and showed that the amount of reducing sugars increased twice on roasting of the soybean. Miyata *et al.* (1977) analysed the headspace vapour of roast soybean flour and identified several carbonyl compounds. However, a detailed analysis of Kinako flavour has not yet been carried out. This paper deals with the changes in the volatile components of soybeans during the roasting process.

MATERIAL AND METHODS

Soybeans and preparation of roasted soybean flour

The soybeans used were grown in the United States of America and imported to the Ajinomoto Company, Japan, for the production of soybean oil in 1978.

Whole soybeans (200 g) were put into a pear shaped flask previously heated to 200 °C with a heating mantle and equipped with a stirrer to avoid non-uniform heating of soybeans and a thermometer to measure their surface temperature. The flask was connected to a rotary evaporator and gently exhausted with the aid of an aspirator to remove the excess steam generated from the soybeans during roasting. Roasting at 200 °C was carried out for exactly 10, 20 or 30 min. The roasted sample was taken out of the flask immediately after roasting and ground in a coffee mill to obtain finely powdered soybeans.

Preparation of whole volatile concentrate

Roasted soybean flour, prepared from 400 g of raw soybeans, was steam distilled under reduced pressure (20–29 mm Hg) at 44 °C water bath temperature for 3 h to collect approximately 750 ml of distillate in a cold-trap with ice and sodium chloride (< -10 °C). The distillate was saturated with sodium chloride and then extracted with diethyl ether (750 ml). The organic layer was concentrated under atmospheric pressure at 36–39 °C to obtain the whole volatile concentrate of roasted soybean flour. Raw soybeans were also powdered and treated similarly for a comparison of their volatile composition.

In order to compare the relative amount of each volatile component, an internal standard (IS-1) was added before steam distillation in the form of an ethereal

solution of heptadecane ($C_{17}H_{36}$, 91·5 mg). In order to determine the absolute amount of each volatile component, a second internal standard (IS-2)—100 μl of an ethereal solution of docosane ($C_{22}H_{46}$, 1 mg/ml)—was added to the whole volatile concentrate and the mixture was concentrated again to approximately 100 μl.

Gas chromatography (GC)

One microlitre of whole volatile concentrate, obtained as described above, was injected by means of a microsyringe into a Shimadzu Model 5A gas chromatograph equipped with a flame ionisation detector. The conditions were as follows. Column: glass capillary column (0·28 mm × 60 m) coated with Carbowax 20M. Carrier gas: nitrogen (1·5 ml/min, split ratio of 1:15·3). Column temperature, 60–190 °C (4 °C/min). Injection port and detector temperature: 200 °C.

Gas chromatography–mass spectrometry (GC–MS)

Whole volatile concentrate (1 μl) was similarly analysed by means of an Hitachi Model M-50 mass spectrometer connected with an Hitachi Model 063 gas chromatograph. The conditions were as follows. Ionisation voltage: 25 eV. Ion source temperature: 200 °C. Column: as described above for GC analysis. Carrier gas: helium (inlet pressure, 0·2 kg/cm^2). Column temperature: 60–170 °C (3 °C/min). Injection port temperature, 200 °C.

Identification of volatile components

Each volatile component was identified by comparing its GC retention time with that of an authentic specimen and also by comparing its mass spectrum, recorded by means of GC–MS, with that of an authentic specimen or the reported spectrum.

Estimation of relative amount of each volatile component

The peak area of each volatile component on the gas chromatogram of the whole volatile concentrate was measured by means of a Shimadzu Model Chromatopack E1A integrator, connected with the Shimadzu gas chromatograph described above. The relative amount of each volatile component was estimated from the peak area ratio of the component to the peak area of the first internal standard, heptadecane.

RESULTS AND DISCUSSION

Soybean temperature and weight during roasting

Figure 1 shows the increase in soybean temperature and the decrease in soybean weight during roasting at 200 °C. After 10 min at 200 °C, soybean temperature was about 110 °C, after 20 min it was approximately 150 °C and after 30 min it rose to around 175 °C. The weight loss was remarkable between 10 and 20 minutes' roasting at 200 °C. After 20 min, soybean weight had decreased to around 175 g from 200 g

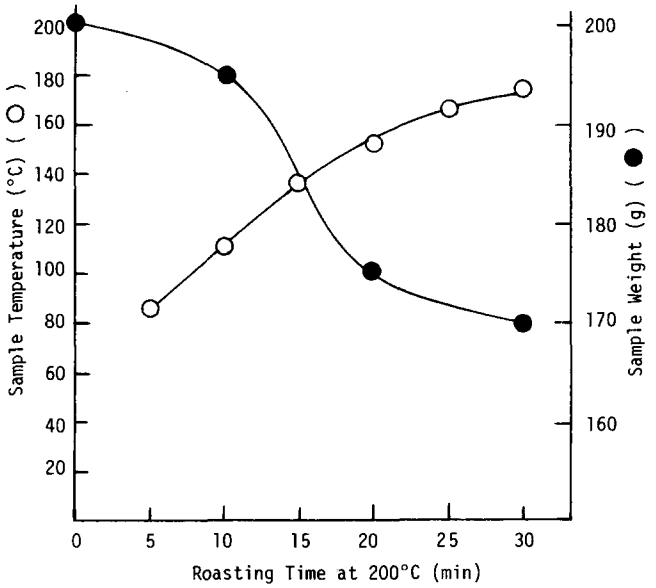


Fig. 1. Changes in sample temperature and weight of soybeans during roasting in a heating mantle at 200 °C.

and, after 30 min, it was approximately 170 g. Kawamura (1967) reported that the moisture contents of raw soybean and roasted soybean flour (Kinako) were about 10 % and 6 %, respectively—that is, moisture loss was only around 4 % (8 g/200 g). Accordingly, the results shown in Fig. 1 indicate that decomposition of soybean components, in addition to evaporation of water, occurs—remarkably—above 110 °C.

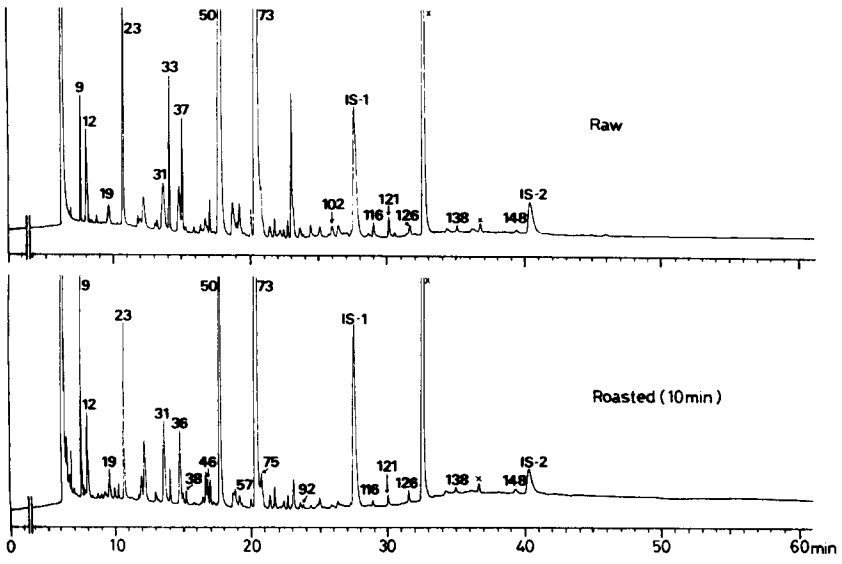
Organoleptic evaluation of roasted soybean flour

Raw and 10-, 20- and 30-min roasted soybean flours were examined organoleptically by several panels at the authors' laboratory and described as follows: raw soybean flour, 'characteristic green' and 'beany flavour'; 10-min roasted soybean flour, 'raw soybean-like' and 'soybean curd-like', 'not favourable to eat'; 20-min roasted soybean flour, 'Kinako-like', 'a pleasant flavour', 'slightly coloured'; 30-min roasted soybean flour, 'coffee-like' or 'roasted barley-like', 'a pleasant flavour different from Kinako', 'dark coloured'.

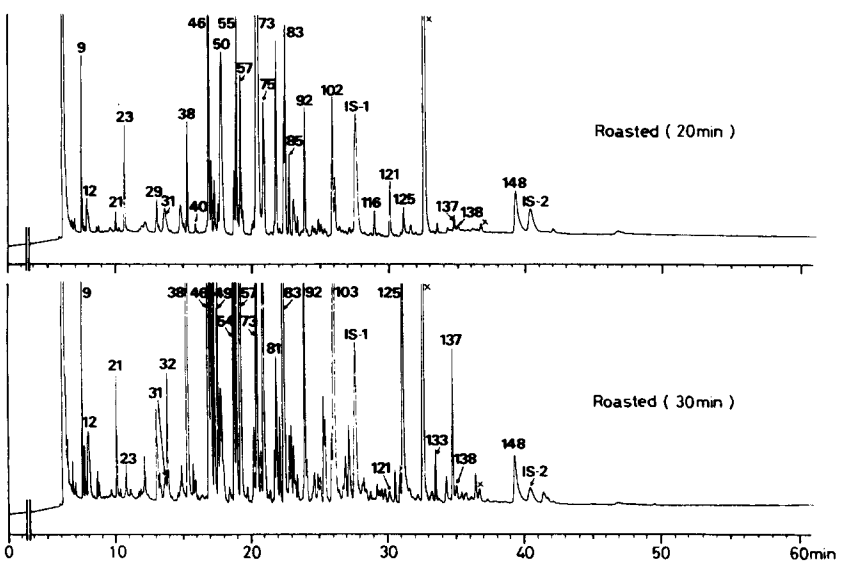
From the above results it is considered that a striking change of flavour occurs also between 10 and 20 minutes' roasting.

Volatile components identified in roasted soybean flour

Each whole volatile concentrate had the odour of the soybean flour from which it was derived. Figure 2 shows the gas chromatograms of the whole volatile



(a)



(b)

Fig. 2. (a) and (b) Gas chromatograms of whole volatile concentrates obtained from raw and roasted soybean flour. IS-1: *n*-alkane, $C_{17}H_{36}$. IS-2: *n*-alkane, $C_{22}H_{46}$. × : impurity.

concentrates. About 150 peaks were observed and 48 volatile components were identified, as shown in Table 1.

Changes of volatile components during roasting

Table 1 also shows the relative amount of each component of raw and roasted soybean flours, calculated from the ratio of the peak area of the component to that of the internal standard (IS-1 in Fig. 2). Peak number in this Table corresponds to that in Fig. 2. The determination of the absolute amount of each volatile component is not presented here, the investigation being underway.

As shown in Table 1, the major volatile components in powdered raw soybean were *n*-hexanol, 1-octen-3-ol and *n*-hexanal. The result is in accordance with the findings of Fujimaki *et al.* (1965) and of Arai *et al.* (1967) except for 1-octen-3-ol. Although both 1-octen-3-ol and *n*-heptanol had the same retention time under the gas chromatographic conditions used, peak 73 was found to contain 1-octen-3-ol as the main component with a little contamination of *n*-heptanol indicated from the GC–MS data. Badenhop & Wilkens (1969) stated that 1-octen-3-ol was formed enzymatically during soaking of soybeans. On the other hand, Mattick & Hand (1969) reported that a typical soy flavour was associated with ethyl vinyl ketone and stated that the ketone was not present in the intact raw soybean but was formed enzymatically during maceration of soybeans. In the present experiment, however, ethyl vinyl ketone was not detected although there were considerable amounts of 1-octen-3-ol.

Volatile components associated with beany flavour, such as aliphatic aldehydes and alcohols, generally decreased with increasing roasting time. However, the reductions of *n*-hexanol, 1-octen-3-ol and *n*-hexanal were not remarkable between 10 and 20 minutes' roasting, as shown in Table 1. This fact indicates that the change to desirable flavour from beany or objectionable flavours does not depend on the disappearance of these components and suggests that volatile components newly produced on roasting may be able to mask the beany flavour.

Some volatile components, such as benzaldehyde, phenyl-acetaldehyde, deca-2,4-dien-1-al and three ketones, showed complicated behaviour during the roasting period, indicating that the differences between the formation rate and the disappearance rate of these components are critical.

Furans, pyrroles and pyrazines were not detected in raw soybean flour; neither were phenols, except for 4-vinylguaiacol which was only present in trace amounts. These heterocyclic or aromatic compounds were newly formed on roasting and increased during the course of roasting. Volatile phenolic compounds would be formed through degradation of the corresponding phenolic acid (Steinke & Paulson, 1964; Fiddler *et al.*, 1967). Oxygenated furan compounds, such as furfural, 5-methylfurfural and furfuryl alcohol, could be degradation products of sugars and pyrazine compounds are well known as products of the reaction between amino acids and reducing sugars. However, a part of the pyrazines would be produced by

TABLE 1

QUANTITATIVE CHANGES OF IDENTIFIED COMPOUNDS IN WHOLE VOLATILE CONCENTRATE OF SOYBEAN DURING ROASTING

Peak No.	Compound	Raw		Roasting at 200°C for 10 min		20 min		30 min
Alcohols								
12	Ethanol	0·17		0·21		0·11		0·33
19	*n*-Propanol	0·03		0·08		0·02		0·03
26	*n*-Butanol	0·05		0·09	↓	0·01		0·01
31	*iso*-Amylalcohol	0·30		0·31	↓	0·19	↓	0·14
36	*n*-Pentanol	0·25		0·24		0·19		0·18
50	*n*-Hexanol	5·20	↓	1·37		1·22	↓	0·64
73	1-Octen-3-ol	3·98		6·88		4·20	↓	1·94
73′	*n*-Heptanol							
90	*n*-Octanol	0·04	↓	0·02		0·01	↓	t
103	Furfurylalcohol	—		0·01	↑	0·24	↑	3·23
104	*n*-Nonanol	0·06		0·02		0·04		0·01
126	Benzylalcohol	0·04		0·03		0·04		0·03
Aldehydes								
15	*n*-Pentanal	0·02		0·01		0·02		0·04
23	*n*-Hexanal	1·22	↓	0·31		0·26	↓	0·08
30	*n*-Heptanal	0·02		t		0·03		0·02
33	*trans*-2-Hexenal	0·34	↓	0·06	↓	0·01		0·01
41	*n*-Octanal	t		t		0·01		0·01
(76	Furfural)							
82	*n*-Decanal	t		0·01		0·02	↑	0·23
85	Benzaldehyde	0·05		0·03	↑	0·23		0·15
92	5-Methylfurfural	—		0·01	↑	0·45	↑	0·95
102	Phenylacetaldehyde	0·05	↓	0·01	↑	0·49	↓	0·22
116	Deca-2,4-dien-1-al	0·11	↓	0·04	↑	0·29	↓	0·05
121								
Ketones								
29	2-Heptanone	0·02		0·02	↑	0·11		0·08
37	3-Octanone	0·28	↓	0·02		0·04	↓	0·01
40	2-Octanone	0·02		0·01		0·03	↑	0·07
Pyrazines								
32	Pyrazine	—		—		—	↑	0·33
38	Methylpyrazine	—	↑	0·03	↑	0·30	↑	6·98
46	2,5-Dimethyl-	—	↑	0·05	↑	1·33	↑	5·09
47	2,6-Dimethyl-	—	↑	0·05	↑	0·21	↑	2·43
48	Ethyl-	—		0·01	↑	0·16	↑	1·09
49	2,3-Dimethyl-	—		—	↑	0·08	↑	0·64
54	2-Ethyl-3-methyl-	—		—	↑	0·21	↑	1·36
55	2-Ethyl-5-methyl-	—	↑	0·05	↑	0·69	↑	1·31
57	Trimethyl-	—	↑	0·03	↑	0·54	↑	2·03
72	2,6-Diethyl-	—		—	↑	0·05	↑	0·28
75	2,6-Dimethyl-3-ethyl- and Furfural	—	↑	0·04	↑	0·68	↑	3·32
81	2,5-Diethyl-3-methyl-	—	↑	0·05	↑	0·66		0·52
Phenols								
125	Guaiacol	—		t	↑	0·11	↑	2·01
148	4-Vinylguaiacol	t		0·02	↑	0·38		0·40
150	4-Vinylphenol	—		—		t		t
Pyrroles								
83	1-Acetylpyrrole	—		0·02	↑	0·64	↑	11·18
133	2-Acetylpyrrole	—		t	↑	0·03	↑	0·14
137	2-Formylpyrrole	—		t	↑	0·06	↑	0·44
Others								
8	Ethyl formate	t		0·01		0·02		0·03
9	Ethyl acetate	0·28		0·34		0·32		0·49
21	Toluene	t		0·02		0·05	↑	0·27
138	γ-Nonalactone	0·02		0·01		0·03		0·07

Value is the ratio of the peak area of each compound to that of the internal standard, $C_{17}H_{36}$.
↓ or ↑ show a distinct decrease or increase during each 10-min roasting period.
t Trace. — Not detected.

dry-thermal degradation of protein (Kato *et al.*, 1972). On the other hand, soy protein isolates produced neither furfurals nor pyrazines on wet heat treatment (Qvist & von Sydow, 1974).

Of the heterocyclic compounds identified, alkylpyrazines are considered to be important volatile components for roast flavour (Wang *et al.*, 1969; Buttery *et al.*, 1971). Koehler *et al.* (1971) reported odour threshold levels of some alkylpyrazines and showed that, of the compounds examined, monoethylmonomethylpyrazine had the lowest odour detection threshold level. Of the pyrazines listed in Table 1, 2-ethyl-3-methyl- and 2-ethyl-5-methyl-pyrazines are present in significant amounts and may be important in the masking of beany flavour. The detailed mechanism of masking and interactions between volatile components are now being studied in the authors' laboratory.

REFERENCES

ARAI, S., KOYANAGI, O. & FUJIMAKI, M. (1967). Studies on flavour components in soybean. Part IV. Volatile neutral compounds. *Agric. Biol. Chem.*, **31**, 868–73.

BADENHOP, A. F. & WILKENS, W. F. (1969). The formation of 1-octen-3-ol in soybeans during soaking. *J. Amer. Oil Chemists Soc.*, **46**, 179–82.

BUTTERY, R. G., SEIFERT, R. M., GUADAGNI, D. G. & LING, L. C. (1971). Characterization of volatile pyrazine and pyridine components of potato chips. *J. Agric. Food Chem.*, **19**, 969–71.

FIDDLER, W., PARKER, W. E., WASSERMAN, A. E. & DOERR, R. C. (1967). Thermal decomposition of ferulic acid. *J. Agric. Food Chem.*, **15**, 757–61.

FUJIMAKI, M., ARAI, S., KIRIGAYA, N. & SAKURAI, Y. (1965). Studies on flavour components in soybean Part I. Aliphatic carbonyl compounds. *Agric. Biol. Chem.*, **29**, 855–63.

KATO, H., HAYASE, F. & FUJIMAKI, M. (1972). Major volatile components formed from casein during roasting. *Agric. Biol. Chem.*, **36**, 951–9.

KAWAMURA, S. (1967). Sugars and other components of Kinako or parched full-fat soybean flour. *Eiyo to Shokuryo*, **20**, 174–6.

KOEHLER, P. E., MASON, M. E. & ODELL, V. (1971). Odour threshold levels of pyrazine compounds and assessment of their role in the flavour of roasted foods. *J. Food Sci.*, **36**, 816–18.

MAGA, J. A. (1973). A review of flavour investigations associated with the soy products, raw soybeans, defatted flakes and flours and isolates. *J. Agric. Food Chem.*, **21**, 864–8.

MATTICK, L. R. & HAND, D. B. (1969). Identification of a volatile component in soybeans that contributes to the raw bean flavour. *J. Agric. Food Chem.*, **17**, 15–17.

MIYATA, Y., SHIOTA, Y. & NISHIBORI, K. (1977). Flavour components of roast soybeans (Part 1). Gas chromatograms of headspace vapour. *Kaseigaku Zasshi*, **28**, 42–7.

QVIST, I. H. & VON SYDOW, E. C. F. (1974). Unconventional proteins as aroma precursors. Chemical analysis of the volatile compounds in heated soy, casein, and fish protein model systems. *J. Agric. Food Chem.*, **22**, 1077–84.

STEINKE, R. D. & PAULSON, M. C. (1964). The production of steam-volatile phenols during the soaking and alcoholic fermentation of grain. *J. Agric. Food Chem.*, **12**, 381–7.

WANG, P-S., KATO, H. & FUJIMAKI, M. (1969). Studies on flavour components of roasted barley. Part III. The major volatile basic compounds. *Agric. Biol. Chem.*, **33**, 1775–81.

WILKENS, W. F. & LIN, F. M. (1970). Volatile components of deep fat-fried soybeans. *J. Agric. Food Chem.*, **18**, 337–9.

Food Chemistry 7 (1981) 95–104

CHANGES IN THE CARBOHYDRATE CONSTITUENTS OF CHICKPEA AND GREENGRAM DURING GERMINATION

T. V. JAYA & L. V. VENKATARAMAN

Protein Technology Discipline,
Central Food Technological Research Institute, Mysore 570013, India

(Received: 15 January, 1980)

ABSTRACT

A decrease in the concentration of total carbohydrates was observed during 96 hours' germination of chickpea and greengram. However, whilst the starch and oligosaccharide levels decreased, the reducing sugars increased. Starch was the major carbohydrate and about 50% of it was hydrolysed after 96 hours' germination. The amylose proportion of the starches increased during germination. Starches from germinated chickpea and greengram had better swelling power and solubility, but their intrinsic viscosity was lower. Changes occurred in the concentration of hemicelluloses during germination but their composition did not alter. The above-mentioned changes, occurring during germination, appear to have a favourable effect on the nutritional value of chickpea and greengram.

INTRODUCTION

Legumes contain large amounts of carbohydrates, varying from 55 to 60%. The composition of carbohydrates varies with the legume, although starch is the major constituent (Nigam & Giri, 1961; Pant & Tulsiani, 1968). They contain oligosaccharides (raffinose, stachyose, melibiose, verbascose, etc.) which have been associated (Rackis *et al.*, 1970; Calloway *et al.*, 1971) with flatus formation. The legumes contain other water-insoluble polysaccharides (hemicelluloses, celluloses, etc.) which may also be responsible for flatus and interfere with digestion (Wagner *et al.*, 1976; Booher *et al.*, 1951).

In many parts of India and far east Asia, legumes (chickpea, soybean, greengram and others) are consumed in germinated form. Germinated legumes are believed to

Fd. Chem. 0308-8146/81/0007-0095/$02·50

be nutritionally superior because they are more easily digested and have a higher vitamin content (Subbulakshmi *et al.*, 1976; Chen *et al.*, 1975). During germination, the reserve carbohydrates are broken down to simpler sugars due to the formation and activation of hydrolytic enzymes (Varner, 1965; Hsu *et al.*, 1973). However, the pattern of reserve carbohydrate hydrolysis, during the early stages of germination, varies with the legume (Matheson & Saini, 1977).

This paper describes an investigation into changes in the qualitative and quantitative distribution of the carbohydrates of chickpea and greengram during the early stages of germination. The properties of the starches isolated from both germinated and ungerminated chickpea and greengram are also described.

MATERIALS AND METHODS

Legumes and germination

Chickpea and greengram (*Cicer arietinum* and *Phaseolus aureus*) were soaked in water for 4 h and germinated in the dark on moist vermiculite at between 25 and 27 °C. The seedlings were harvested at 48-h and 96-h intervals, freeze-dried and ground to a fine powder. Enzyme activities were determined on freshly harvested seedlings.

Estimation of starch and sugars

Starch and total sugars were estimated as glucose equivalents (McCready *et al.*, 1950) and reducing sugars were determined using 3,5-dinitro salicyclic acid (Bernfeld, 1954). Pentosans were precipitated as the phloroglucinol derivatives and estimated gravimetrically (AOAC, 1970).

Extraction, fractionation and identification of ethanol-soluble sugars

The ethanol-soluble sugars were extracted from the legume flour by repeated shaking with 70% ethanol and the extracts were pooled. The extractions were repeated until the final extract showed a negative test for sugars. The ethanol was evaporated from the pooled extracts under vacuum at 40 °C, then deionised by shaking the extract with Dowex 50 (H^+ form, 200 to 300 mesh) and concentrated under vacuum. A known volume of the concentrated extract was adsorbed on a carbon–celite (1:1) column and sugars were eluted with different concentrations of alcohol (up to 30%) as suggested by Whistler & Be Miller (1962). The eluted sugars were then concentrated and further separated and identified using descending paper chromatography. Oligosaccharides were separated on Whatman No. 3 paper by developing the chromatogram for 4 h using propanol–ethanol–water (7:1:2). Monosaccharides were separated by developing the chromatogram for 12 h using an ethyl acetate–pyridine–water system (8:2:1).

An attempt was made to identify unknown sugars by partial acid and enzymic

hydrolysis and also by determining their specific rotation using a Highler standard polarimeter.

Hemicelluloses, celluloses and lignins

Hemicelluloses, celluloses and lignin components of both germinated and ungerminated chickpea and greengram were obtained as suggested by Whistler & Wolfrom (1968). The hexose and pentose composition of hemicelluloses A and B was determined after acid hydrolysis, using paper chromatography.

Estimation of amylase and diastase activities

Amylase activity was estimated by the method suggested by Young & Varner (1959) and expressed as the milligrammes of maltose formed after incubation at 37 °C for 10 min. Diastase activity was estimated by the AOAC (1961) procedure and expressed as milligrammes of maltose released from 10 g of seeds at 30 °C for 60 min.

Isolation of starches from germinated and ungerminated chickpea and greengram

Starch was isolated from the samples using the method of Schoch & Maywald (1968). The protein from the starch was removed as far as possible. The purified starch was tested for protein, starch, amylose and moisture.

Characterisation of the starches

The solubility and swelling properties of the starches were determined after gelatinising them in water at different temperatures (Leach *et al.*, 1959).

The gelatinisation temperature of the starches was studied by the microscopic method suggested by Schoch & Maywald (1968), making use of the birefringence property of starch (under polarised light). The temperatures necessary for a 2, 50 and 98 % loss of birefringence were determined.

Viscosity measurements were made using an Oswald viscometer with a viscometric constant of 0·04375 at 30 °C. The flow time of water at 30 °C was found to be 183·4 s. The intrinsic viscosity (η) was calculated as follows:

$$\frac{\eta_s}{\eta_0} - \mathrm{I} = \eta_{sp}$$

$$(\eta_{sp}/C) \text{ millilitres g}^{-1} = \text{viscosity number.}$$

When the viscosity number is plotted against C, the y intercept gives the intrinsic viscosity (η).

Where η_0 is the flow time of the solvent (0·5M potassium hydroxide), η_s is the flow time of the starch solution, C is the concentration of the starch solution and η_{sp} is the specific viscosity.

RESULTS

Carbohydrate composition of germinated and ungerminated chickpea and greengram

The total carbohydrates in the legumes studied decreased when germinated for either 48 or 96 h. Reducing sugars increased at the expense of starch and non-reducing sugars (Table 1). A slight increase in the level of pentosans was also found.

TABLE 1

CARBOHYDRATE COMPOSITION OF GERMINATED AND UNGERMINATED CHICKPEA AND GREENGRAM[a] (GRAMMES PER 100 g OF SEEDS)

Legume	*Germination (h)*	*Total carbohydrates*	*Soluble carbohydrates*			*Pentosans*	*Starch*
			Total	*Reducing*	*Non-reducing*[b]		
Chickpea	0	61·2	14·9	2·2	12·7	4·7	40·5
	48	60·0	18·3	5·7	12·6	5·0	36·0
	96	52·9	21·9	12·5	9·4	5·1	25·2
Greengram	0	61·6	20·0	2·5	17·5	3·1	38·3
	48	52·5	21·9	8·7	13·2	3·2	27·0
	96	44·0	23·5	10·8	12·7	4·0	16·0

[a] Estimations based on chemical analysis.
[b] Expressed as the difference between total and reducing sugars.

Sugar distribution in germinated and ungerminated chickpea and greengram

The sugars present in the 70 % ethanol extract of the samples are shown in Table 2. The monosaccharides were found to be mainly glucose, fructose and galactose. The concentrations of monosaccharides and raffinose were higher in ungerminated greengram than in ungerminated chickpea. Sucrose and verbascose were the major oligosaccharides found in the ungerminated seeds. The monosaccharide concentration increased considerably in both chickpea and greengram during germination whilst stachyose and verbascose disappeared.

Three of the sugars present in chickpea and greengram could not be identified. However, the unknown I (R_f value, 2·3) is probably a pentose. Unknown II (R_f value, 0·83) afforded glucose, galactose and fructose upon partial acid hydrolysis. Its specific rotation in water is $[\alpha]_D^{28} = +86{\cdot}5°$. Unknown III ($R_f$ value, 0·11) has a specific rotation of $[\alpha]_D^{28} = +165{\cdot}6°$. Hydrolysis with α-galactosidase (isolated from germinating groundnuts and purified) of III afforded verbascose, stachyose, raffinose and sucrose with some galactose and is, presumably, an even higher molecular weight member of the raffinose series of oligosaccharides.

Hemicelluloses, celluloses and lignins

The hemicelluloses in germinated and ungerminated chickpea and greengram were separated into A and B components (Whistler & Wolfrom, 1968). Both components contained pentoses and hexoses. However, hemicellulose A had more

TABLE 2

PATTERN OF 70% ETHANOL-SOLUBLE SUGARS IN GERMINATED AND UNGERMINATED CHICKPEA AND GREENGRAM COTYLEDONS[a] (GRAMMES PER 100 g OF SEEDS)

Sugars	*Chickpea*			*Greengram*		
	Duration of germination (h)					
	0	*48*	*96*	*0*	*48*	*96*
Rhamnose	—	—	—	—	—	1·46
Unknown I (R_f 2·3)[b]	1·16	—	—	—	1·33	1·01
Fructose	0·46	1·10	1·26	1·49	3·99	2·29
Glucose Galactose	0·62	4·59	11·23	1·00	3·39	6·05
Sucrose	2·92	9·14	6·91	1·97	7·16	8·35
Unknown II (R_f 0·83)[b]	1·05	1·08	0·96	2·83	1·33	0·91
Melibiose	0·35	0·57	0·61	1·49	1·06	0·79
Raffinose	1·44	0·78	1·02	2·60	2·66	1·21
Stachyose	2·58	1·08	—	2·83	—	—
Verbascose	3·80	—	—	3·46	—	—
Unknown III (R_f 0·11)[b]	0·54	—	—	2·37	1·06	1·04

[a] Quantified after resolving and purifying by the paper chromatographic technique.
[b] The R_f values expressed are in reference to sucrose in the butanol–ethanol–water system.

hexoses and hemicellulose B had more pentoses (Table 3). During germination, hemicelluloses A and B decreased in greengram, whilst in chickpea A they decreased but in B increased. To further characterise these hemicelluloses, the fractions were partially hydrolysed with hydrochloric acid and the hydrolysates were spotted on paper. The chromatograms were developed using the (12 h) butanol–acetic acid–water solvent system. Hemicellulose A (an acidic glucan) consisted of glucose (major) and galacturonic acid with traces of cellobiose. Hemicellulose B was highly heterogeneous and was made up of xylose, arabinose, glucose, galactose and galacturonic acid. The composition of the hemicelluloses of the two legumes was similar. Germination did not alter the composition of the hemicellulose constituents of the two legumes.

TABLE 3

HEMICELLULOSES, CELLULOSES AND LIGNINS IN GERMINATED AND UNGERMINATED CHICKPEA AND GREENGRAM[a] (GRAMMES PER 100 g OF SEEDS)

Legume	*Germination (h)*	*Total*	*Hemicelluloses*						*Celluloses*[b]	*Lignins*
			A			*B*				
			Hexoses	*Pentoses*	*Total*	*Hexoses*	*Pentoses*	*Total*		
Chickpea	0	3·62	1·90	0·06	1·96	0·30	1·36	1·66	1·16	0·23
	48	3·11	1·63	0·05	1·68	0·27	1·16	1·43	0·72	0·25
	96	3·56	1·47	0·04	1·51	0·12	1·93	2·05	0·71	0·34
Greengram	0	6·13	2·97	0·06	3·03	0·50	2·60	3·10	0·26	0·08
	48	4·81	1·93	0·04	1·97	0·48	2·36	2·84	0·44	0·20
	96	2·78	1·55	0·05	1·60	0·41	0·77	1·18	0·45	0·30

[a] Constituents reported were assayed by chemical methods.
[b] Expressed as glucose.

Characteristics of starches of germinated and ungerminated chickpea and greengram

A marked reduction in the starch content was observed during germination of chickpea and greengram. The amylose contents of chickpea and greengram (ungerminated) were 35 and 21 % of the total starch respectively. The amylose content of the total starch increased during germination of both chickpea and greengram.

The starches isolated from these legumes were 89 % pure and contained about 0·6 % protein and 8 % moisture.

Swelling power and solubility

A progressive increase in swelling and solubility was observed for both the legumes up to 80 °C (Fig. 1). Germination improved the swelling power and solubility of both chickpea and greengram starches. Starches from germinated and ungerminated greengram had better swelling power and higher solubility than chickpea starches.

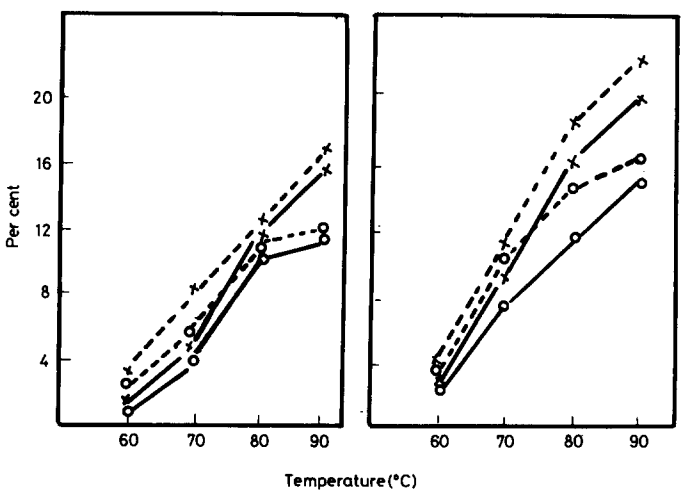


Fig. 1. Solubility and swelling power of chickpea (left) and greengram (right) starches. —○—○—, ungerminated; — × — × —, germinated for 48 h; solid line: per cent solubles; dashed line, swelling power.

Gelatinisation temperature

The initial, intermediate and final gelatinisation temperatures of starches from germinated and ungerminated chickpea and greengram are shown in Table 4. Gelatinisation began at 50·5 °C for the starches from both ungerminated chickpea and ungerminated greengram. Gelatinisation began at lower temperatures for starches obtained from germinated chickpea and greengram up to the intermediate stage. The final gelatinisation temperatures were the same.

TABLE 4

TEMPERATURES OBSERVED FOR THE INITIAL (2%), INTERMEDIATE (50%) AND FINAL (98%) LOSS OF BIREFRINGENCE IN STARCHES

Starch	*Gelatinisation temperatures* (°C)		
	Initial (2%)	*Intermediate* (50%)	*Final* (98%)
Ungerminated chickpea	50·5	63	70
Germinated chickpea	49·5	62	70
Ungerminated greengram	50·5	67	74
Germinated greengram	40·0	66	74

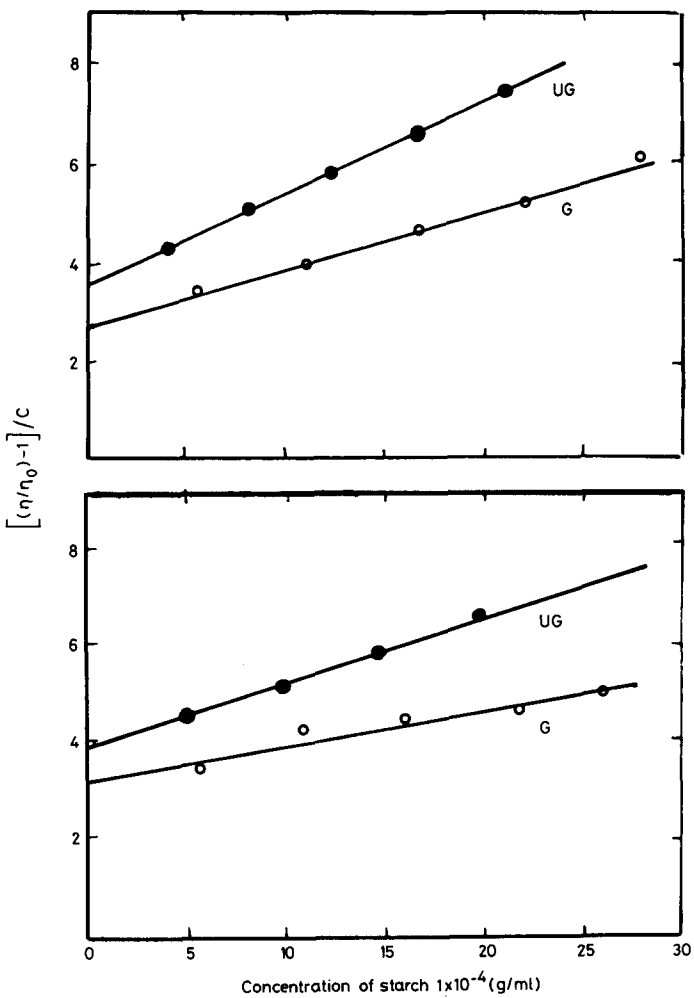


Fig. 2. Intrinsic viscosity of chickpea (top) and greengram (bottom) starches in 0·5M potassium hydroxide at 30°C. UG—ungerminated; G—germinated for 48 h.

Intrinsic viscosity

A plot of the viscosity of starches from germinated and ungerminated chickpea and greengram as a function of concentration is shown in Fig. 2. The y intercept gives the viscosity (η). A decrease in the apparent viscosity was observed for 'germination-modified' starches compared with corresponding ungerminated ones and this decrease was more pronounced in the case of chickpea.

Activities of amylases and diastase

The activities of amylase and diastase increased in both chickpea and greengram

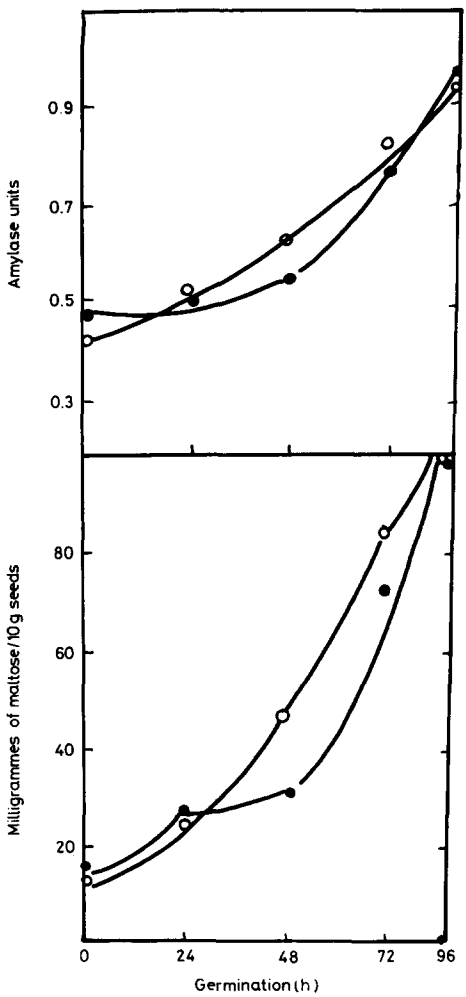


Fig. 3. Activity of amylase (top) and diastase (bottom) in germinating chickpea and greengram. —●—●—, chickpea; —○—○—, greengram.

as germination progressed (Fig. 3). The activities of amylase and diastase were higher in greengram at 24 and 48 h germination but the level of activity was the same in both legumes at 72 h germination.

DISCUSSION

Germination results in a marked reduction of total carbohydrates, coupled with qualitative alterations in the make up of individual constituents. During the early phases of germination, it appears that the oligosaccharides are hydrolysed to simpler sugars and starch hydrolysis by amylases may be initiated at slightly later stages of the germination period (Pridham, 1958; Pazur *et al.*, 1962; Fernandez & Nicholas, 1976; Matheson & Saini, 1977). Generally, reports on germination studies have been limited to changes in reducing and non-reducing sugars and starch content (Nigam & Giri, 1961; Ganesh Kumar & Venkataraman, 1976; Subbulakshmi *et al.*, 1976).

Germination has a considerable influence on the concentration and characteristics of starches. The initial reduction in total starch and the apparent increase in amylose during germination is attributed to selective enzymic degradation of amylopectin by amylase and phosphorylases (Koller *et al.*, 1962). The changes in the gelatinisation properties and the lowering of the viscosity of starches during germination may be due to the enzymic breakdown of amylopectin resulting in the formation of linear fragments. As the amylose/amylopectin ratio is altered during germination, it is expressed in a corresponding alteration in the physical properties of the starch.

Some of the changes in the concentration of oligosaccharides, hemicelluloses, amylose, amylopectin, etc., brought about by germination, may have a favourable impact on the digestibility, the flatus-forming property and other nutritional qualities of these legumes.

ACKNOWLEDGEMENT

The authors are grateful to Dr D. B. Wankhede for his valuable suggestions and one of the authors (T.V.J.) wishes to thank the Council for Scientific and Industrial Research (India) for the research grant.

REFERENCES

AOAC (1961). *Official methods of analysis of the Association of Agricultural Chemists* (9th edn), Washington, DC, USA.

AOAC (1970). *Official methods of analysis of the Association of Agricultural Chemists* (11th edn), Washington, DC, USA.

BERNFELD, P. (1954). In: *Methods in enzymology*, Vol. 1, 149.
BOOHER, L. E., BEHAN, I. & MCMEANS, E. (1951). Biological utilization of unmodified and modified starches. *J. Nutr.*, **45**, 75.
CALLOWAY, D. H., HICKEY, C. A. & MURPHY, E. L. (1971). Reduction of intestinal gas forming properties of legumes by traditional and experimental food processing methods. *J. Food Sci.*, **36**, 251.
CHEN, L. H., WELLS, C. E. & FORDHAM, J. R. (1975). Germinated seeds for human consumption. *J. Food Sci.*, **40**, 1290.
FERNANDEZ, J. T. & NICHOLAS, G. (1976). Starch degradation in the cotyledons of germinating lentils. *Plant Physiol.*, **58**, 618.
GANESH KUMAR, K. & VENKATARAMAN, L. V. (1976). Studies on the *in vitro* digestibility of starch in some legumes before and after germination. *Nutr. Rep. Internatl.*, **13**, 115–24.
HSU, S. H., HODLOY, H. H. & HYMOWITZ, T. (1973). Changes in the carbohydrate contents of germinating soybean seeds. *Crop Sci.*, **13**, 407.
KOLLER, D., MAYER, A. M., MAYBER, A. P. & KLEIN, S. (1962). Seed germination. *Ann. Rev. Plant Physiol.*, **13**, 437.
LEACH, H. W., MCCOWEN, L. D. & SCHOCH, T. J. (1959). Structure of starch granules. I. Swelling power and solubility patterns of various starches. *Cereal Chem.*, **36**, 534.
MATHESON, N. K. & SAINI, H. S. (1977). Polysaccharides and oligosaccharides changes in germinating lupin cotyledons. *Phytochem.*, **16**, 59.
MCCREADY, R. M., GUGGOLZ, J., SILVIERA, V. & OWENS, H. S. (1950). Determination of starch and amylose in vegetables—Application to peas. *Anal. Chem.*, **22**, 1156.
NIGAM, V. N. & GIRI, K. V. (1961). Sugars in pulses. *Can. J. Biochem. Physiol.*, **39**, 1847.
PANT, R. & TULSIANI, D. R. P. (1968). Total soluble carbohydrates and reducing substances of some leguminous seeds. *Curr. Sci.*, **37**, 74.
PAZUR, J. H., SHADKSHARASWAMY, M. & MEIDELL, G. E. (1962). The metabolism of oligosaccharides in germinating soybean (*Glysine max.*). *Arch. Biochem. Biophys.*, **99**, 78.
PRIDHAM, J. B. (1958). Occurrence and metabolism of oligosaccharides in broad beans (*Vicia faba*), *Nature (London)*, **182**, 1687.
RACKIS, J. J., SESSA, D. J., STEGGERDA, F. R., SHIMIZU, R., ANDERSON, J. & PEARL, S. L. (1970). Soybean factors relating to gas production by intestinal bacteria. *J. Food Sci.*, **35**, 634.
SCHOCH, T. J. & MAYWALD, E. C. (1968). Preparation and properties of various legume starches. *Cereal Chem.*, **45**, 568.
SUBBULAKSHMI, G., GANESH KUMAR, K. & VENKATARAMAN, L. V. (1976). Effect of germination on the carbohydrates, proteins, trypsin inhibitors, amylase inhibitors and hemaglutinins in horsegram and mothbeans. *Nutr. Rep. Internatl.*, **13**, 19.
SWAIN, R. R. & DEKKER, E. E. (1966). Seed germination studies. II. Pathways for starch degradation in germinating pea seedlings. *Biochem. Biophys. Acta*, **122**, 87.
VARNER, J. E. (1965). In: *Plant biochemistry*, Academic Press, New York.
WAGNER, J. R., BECKER, R., GUMBMANN, M. R. & OLSON, A. C. (1976). Hydrogen production in the rat following ingestion of raffinose, stachyose and verbascose and oligosaccharides-free residue. *J. Nutr.*, **106**, 466.
WHISTLER, R. L. & BE MILLER, J. N. (1962). In: *Methods in carbohydrate chemistry*, Vol. I (Whistler, R. L. & Wolfrom, M. L. (Eds)), Academic Press, New York.
WHISTLER, R. L. & WOLFROM, M. L. (1968). In: *Methods in carbohydrate chemistry*, Vol. V (Whistler, R. L. & Wolfrom, M. L. (Eds)), Academic Press, New York.
YOUNG, J. L. & VARNER, J. E. (1959). Enzymic activity of germinating pea seeds, *Arch. Biochem. Biophys.*, **84**, 71.

Food Chemistry 7 (1981) 105–108

TRYPTOPHAN CONTENT OF VEGAN AND OMNIVOROUS ONE-DAY MIXED FOOD DIET SAMPLES

BABOO M. NAIR & RICKARD ÖSTE

Department of Applied Nutrition, Chemical Centre, University of Lund, Box 740, S-220 07 Lund, Sweden

&

INGRID ANDERSSON

Institution of Physiological Chemistry, Chemical Centre, University of Lund, Box 750, S-220 07 Lund, Sweden

(Received: 31 January, 1980)

ABSTRACT

The tryptophan contents of a series of one-day food samples of vegan and omnivorous subjects are determined. Trytophan contents are higher in the vegan diets, especially when calculated per unit weight of protein.

INTRODUCTION

Trytophan is one of the essential amino acids for human beings. Various metabolic products of tryptophan are important for the normal functioning of the brain. It is also a precursor for the synthesis of niacin in the body. The joint FAO/WHO ad hoc committee on energy and protein requirements estimates the requirement of tryptophan for human adults as 3·5 mg/kg body weight per day (FAO/WHO, 1973). The tryptophan content of many food constituents is rather low. Moreover, the availability of tryptophan to the organism is reduced by the Maillard reaction whilst food products are undergoing heat treatment (Dworschak & Hegedus, 1974).

Most of the protein amino acids can be determined by ion exchange chromatography or gas chromatography after hydrolysis. Tryptophan is destroyed during acid hydrolysis and very often amino acid analysis data lack values for tryptophan. As a consequence, data on the intake of tryptophan by various groups of the population are not available. The present paper deals with the results of

Fd. Chem. 0308-8146/81/0007-0105/$02·50

tryptophan analyses of a series of 1-day mixed food diet samples collected in connection with an extensive nutritional and health survey, using a simple spectrofluorometric method developed in the authors' laboratory (Öste *et al.*, 1976).

MATERIALS AND METHODS

Food samples

The vegan diets were collected from six individuals born before 1928. Three of them were females and the other three males. The omnivorous diets were collected in connection with another nutrition survey. Everything consumed during a given day was duplicated as exactly as possible and collected in a 2-litre milk carton. In addition a description of the foods was also submitted along with the samples.

Sample preparation

The duplicate 1-day mixed food diets were homogenised in an Ultra Turrax stainless steel homogeniser and the fat was extracted with chloroform. The fat free suspension was lyophilised and ground to a fine powder before further analysis.

Enzymic hydrolysis

Samples of about 10 mg protein were incubated, rotating end to end, over a period of 24 h at 37 °C with 1 mg of papain (2x crystallised suspension from Sigma Chemical Co., St Louis, Mo.) in 5 ml of freshly prepared incubation mixture. The incubation mixture contained 8M urea, 0·005M thioglycollic acid, 0·002M EDTA and 0·1M sodium borate and was adjusted to pH 7·6. After incubation the urea solution was separated from the insoluble residue by centrifugation (6000 rpm for 20 min).

Fluorimetric measurements

The apparatus used was an Aminco Bowman spectrofluorimeter equipped with an Aminco photomultiplier photometer. The excitation wavelength was 288 nm and the emission wavelength, 348 nm. Two millilitres of a 8M urea solution containing 0·1M sodium borate buffer adjusted to pH 7·6 were transferred to a quartz cell. The blank fluorescence was adjusted to zero. An aliquot of 100 μl from the sample previously incubated as above was added and mixed thoroughly. The fluorescence was measured again and the tryptophan content was calculated.

Table 1 shows the tryptophan content of a series of one-day mixed food samples collected from vegans as well as omnivores. The vegan diets do not include milk or eggs. As can be seen from Table 2, the protein contents of the omnivorous diets and vegan diets are almost the same. However, both vegan males and non-vegan males consume significantly higher levels of protein per day than the vegan females and omnivorous females. Daily intakes of tryptophan among vegans (0·61 g/day for females and 1·11 g/day for males) are higher than among omnivores (0·44 g/day for

TABLE 1

TRYPTOPHAN CONTENT OF ONE-DAY MIXED FOOD SAMPLES OF VEGAN AND OMNIVOROUS DIETS

	Tryptophan content (g/day)			
	Vegan		*Omnivorous*	
	Females	*Males*	*Females*	*Males*
	0·47	0·86	0·33	0·30
	0·60	1·04	0·34	0·32
	0·53	1·53	0·31	0·33
	0·86	1·10	0·28	0·34
	0·49	0·81	0·57	0·74
	0·33	0·64	0·32	0·30
	0·49	0·71	0·51	0·27
	0·58	0·76	0·34	0·27
	0·78	0·71	0·57	0·48
	0·89	0·69	0·55	0·56
	0·81	0·71	0·44	0·73
	0·66	0·70	0·37	0·50
Mean	0·62	0·86	0·40	0·44
Standard deviation	0·17	0·25	0·10	0·17
Range	0·33–0·89	0·70–1·53	0·31–0·57	0·27–0·56
Median	0·61	1·11	0·44	0·51

TABLE 2

PROTEIN CONTENT OF ONE-DAY MIXED FOOD SAMPLES OF VEGAN AND OMNIVOROUS DIETS

	Protein content (g/day)			
	Vegan		*Omnivorous*	
	Females	*Males*	*Females*	*Males*
	26·7	48·6	42·4	50·6
	35·5	49·0	48·8	46·3
	32·0	68·9	47·4	32·2
	39·4	49·7	45·9	50·9
	30·4	39·3	40·1	74·9
	20·8	41·2	26·5	31·5
	39·4	47·8	40·4	46·3
	31·9	44·9	27·5	49·2
	44·1	40·2	36·7	45·1
	48·3	50·1	45·4	40·6
	56·8	54·3	48·9	51·0
	40·8	49·1	44·9	35·2
Mean	37·2	48·6	41·2	47·8
Standard deviation	9·8	7·8	7·6	10·7
Range	20·8–48·3	40·2–68·9	27·5–48·9	31·5–74·9
Median	34·6	54·5	38·2	53·2

TABLE 3
TRYPTOPHAN CONTENT OF ONE-DAY MIXED FOOD SAMPLES OF VEGAN AND OMNIVOROUS DIETS (PROTEIN BASIS)

	Tryptophan content (mg/g of protein)			
	Vegan		*Omnivorous*	
	Females	*Males*	*Females*	*Males*
	17·6	17·7	7·7	5·9
	16·9	21·2	7·1	6·9
	16·5	22·2	6·6	6·3
	21·8	22·1	6·1	6·7
	16·1	20·6	14·2	9·9
	15·8	15·5	12·5	9·5
	12·4	14·9	12·5	5·9
	18·2	16·9	12·4	5·5
	17·6	17·7	13·1	12·7
	18·4	13·8	12·2	13·8
	14·3	13·1	9·0	14·4
	16·2	14·3	8·2	14·2
Mean	16·8	17·5	10·1	9·3
Standard deviation	2·3	3·3	2·9	3·5
Range	14·3–21·8	13·1–22·2	6·1–14·2	5·9–14·4
Median	18·1	17·7	10·2	10·1

females and 0·51 g/day for males). Further, the vegan diets seem to contain more tryptophan per gramme of protein (18·1 mg/g of protein for females and 17·7 mg/g of protein for males) than the omnivorous diets (10·2 mg/g of protein for females and 10·1 mg/g of protein for males) (Table 3).

The National Research Council's Food and Nutrition Board (1974) recommends a daily dietary allowance of 3 mg of tryptophan per kilogramme of body weight for adults. All diets analysed—both vegan and omnivorous—contain the necessary amounts of tryptophan to guarantee that these daily requirements are satisfied.

REFERENCES

DWORSCHAK, E. & HEGEDUS, H. (1974). Effect of heat treatments on the nutritive value of proteins in milk powder, *Acta Alim., Acad. Sci. Hung.*, 3, 337.

FAO/WHO (1973). *Energy and protein requirements*. Report of a Joint FAO/WHO Ad hoc Expert Committee. WHO Techn. Rep. Ser. 522.

ÖSTE, R., NAIR, B. M. & DAHLQVIST, A. (1976). A simple method for determination of tryptophan in food samples, *J. Agric. Food Chem.*, 24–6.

Food Chemistry 7 (1981) 109–116

THE PREPARATION OF PROTEIN CONCENTRATES FROM *SYMPHYTUM ASPERRIMUM* (DONN) (RUSSIAN COMFREY) FOR NON-RUMINANT FEEDS AND HUMAN FOODS

L. G. PLASKETT

Biotechnical Processes Ltd, Hillsborough House, Ashley, Nr. Tiverton, Devon EX16 5PA, Great Britain

(Received: 2 January, 1980)

ABSTRACT

Extracts were prepared from fresh Russian Comfrey plants by successive extractions with water and NaOH. Almost 55% of crop dry matter and 69% of crop crude protein were extracted. The extracts were then acidified to produce precipitates that contained 21·7% of crop dry matter and 52·6% of crop crude protein. The crude protein in these precipitates was found to be over 90% true protein; it is suggested that such concentrates may prove to be valuable as non-ruminant feeds. The solid residues, containing 11–12% of crude protein, may be usable as feed for beef cattle. Large quantities (33%) of crop dry matter remain in the supernatants following precipitation at low nitrogen levels (6·9%) and would probably be usable for microbial protein production following enrichment with a nitrogen source.

INTRODUCTION

Record yields of leaves and stems from *Symphytum asperrimum* (Donn) in the region of 300 tonnes per annum per hectare (wet weight) in East Africa and New Zealand and up to 150 tonnes per annum (wet weight) per hectare in the United Kingdom have given this plant an outstanding reputation as a prolific producer (Hills, 1976*a*). At a mean dry matter content of 12·5% the United Kingdom figure represents 18·75 tonnes per annum per hectare of dry matter. This compares favourably with the usual yields of dry matter from grass, although, in practice, allowance must be made for mean commercial yields to fall well below the best experimental yields. Another factor favouring Russian Comfrey is its high content of crude protein in the dry matter, reported to be up to almost 35% (Hills, 1976*b*),

Fd. Chem. 0308-8146/81/0007-0109/$02·50

although commonly rather lower than this at around 25%. Coincidence of maximum yield with maximum crude protein would yield 6·5 tonnes of crude protein per annum per hectare. If even half of this protein productivity could be realised in commercial practice, it would exceed by a wide margin the protein yield from good grassland at, say, 1·8 tonnes per hectare. Notwithstanding the fact that Russian Comfrey is expensive to plant and that mean commercial yields are not established, it was of interest to establish whether protein concentrates could be produced in good yields from Russian Comfrey for non-ruminant feed by methods likely to prove practicable and economic.

Furthermore, Russian Comfrey has been implicated as a possible cause of hepatotoxicity in farm animals because closely related species contain pyrrolizidine alkaloids (Long, 1976). Although Russian Comfrey has been widely fed to stock without apparent harm, it might prove beneficial in future to use the protein of this productive species after separation or partial separation from the alkaloid fraction. A suitable protein concentrate would provide a low-alkaloid, low-fibre feed to compete in temperate agriculture with soya meal and fishmeal and, subject to further purification and decolorisation, a food-grade protein.

A problem that had to be faced at the outset was the impossibility of expressing juice from Russian Comfrey by the methods commonly employed for leaf protein production from grass or lucerne. Juice expression is prevented by the large quantities of mucilage or gum that exude from the tissues when they are macerated; this difficulty has necessitated the addition of relatively large volumes of water to the macerated leaf, mixing thoroughly before extraction and, finally, recovering the extracted solids from the resulting dilute liquor.

MATERIALS AND METHODS

Materials—Russian Comfrey

Russian Comfrey was obtained from growers in Devon who were members of the Henry Doubleday Research Association and was supplied in batches of approximately 1 kg for each experiment. Some of the plants were flowering when cut. The work was performed during September and October, 1978 and the crop was low in protein content, presumably on account of lack of manure and the imminent end of the growing season.

Frozen Russian Comfrey was obtained in August, 1977 directly from the Henry Doubleday Research Association; it was significantly lower in dry matter content and richer in crude protein.

Materials—Chemicals

Chemicals were ordinary reagent grade obtained from British Drug Houses Limited, except for the sulphuric acid used in Kjeldahl digestions, which was Analar.

Semi-micro Kjeldahl determination for total nitrogen

A sample (50–800 mg; depending upon its expected nitrogen content) was heated in a 50-ml Kjeldahl flask together with 4 ml of concentrated sulphuric acid and one catalyst tablet containing 1 g Na_2SO_4 and 0·05 g Se. Heating was performed on an electrothermal Kjeldahl digestion rack and was continued for 8 h after the digestion mixture cleared. After cooling the digest was made up to 25 ml and 1 ml or 2 ml samples were taken for distillation in a Markham Still, using 5 % boric acid solution, for collecting the ammonia for subsequent titration with N/20 HCl.

Determination of true protein

A sample of material was stirred for 48 h at ambient temperature with a convenient volume of 4 % trichloroacetic acid and then filtered. A portion of filtrate and the residue were then subjected to the Kjeldahl nitrogen determination as a measure of non-protein nitrogen (filtrate) and the true protein nitrogen (residue). The method was based upon that of Lexander *et al.* (1970).

Dry matter determination

The bulk of the moisture was driven off from the sample on a hot plate at 60 °C; drying was afterwards completed in a vacuum desiccator.

Extraction of Russian Comfrey

Leaves and stems were passed through a 'Kenwood' power mincer set for fine cutting and either collected in a bowl exposed to the air or allowed to fall from the mincer directly into boiled water containing a trace of sodium metabisulphite. When exposed to the air the minced material darkened rapidly but it remained green after dropping directly into water.

In either case the minced material was then stirred with three times its own weight of water containing sodium metabisulphite. In the case of minced material that had been dropped directly into water, a gradual browning occurred during stirring. A very viscous mass was obtained after approximately 30 min of stirring; this was separated into juice (aqueous extract I) and solid fractions by passing it through a 'Kenwood' juice extractor. The solid residue was weighed and the weight of juice that had been removed replaced by adding more water: after a further 30 minutes of mixing the material was de-juiced again, yielding aqueous extract II plus a solid residue. Finally, the residue from preparing aqueous extract II was weighed and extracted with three times its own weight of 0·3 % NaOH solution at 50 °C with stirring for 30 min; the alkaline extract was separated by pressing through muslin.

EXPERIMENTAL AND RESULTS

Extraction

Some attempts were made to employ frozen material for extraction, but freezing and thawing brought about entirely altered extraction properties whereby juice was

expressible without prior addition of water. This juice amounted to 34% of crop weight. Since freezing and thawing produced antefactual extraction characteristics and would not be a practical step in any commercial process, the use of frozen material was discontinued.

Quantitative results of a typical extraction are given in Table 1. The starting material contained 14·3% of dry matter and crude protein amounted to 16·7% of the dry matter. The first and second aqueous extracts were not analysed separately;

TABLE 1

DISTRIBUTION OF TOTAL WEIGHT, DRY MATTER AND CRUDE PROTEIN DURING EXTRACTION OF RUSSIAN COMFREY WITH WATER (X2) AND NaOH SOLUTION (X1)

	Weight (*g*)	*Dry matter* (%)	*Dry matter* (*g*)	*Crude protein* (*g*)	*Crude protein* (N × 6·25) *as % of dry matter*
Minced Russian Comfrey	785	14·3	112·5	19·67	17·5
Added water (first extraction)	2355	—	—	—	—
Residue after first extraction	798	—	—	—	—
Juice (aqueous extract I)	2342	—	—	—	—
Added water (second extraction)	2342	—	—	—	—
Residue after second extraction	550	12·3	67·5	10·03	14·8
Juice (aqueous extract II)	2590	—	—	—	—
Combined aqueous extracts I and II	4845	1·03	49·9	9·64	19·3
Added NaOH solution	1650	0·30	5·0	—	—
Residue after NaOH extraction	415	12·4	51·6	6·12	11·9
Juice (NaOH extract)	1785	0·89	15·9	3·91	24·6*

* 35·9% of the extracted dry matter (total dry matter minus dry matter added as NaOH).

as expected, the combined aqueous extracts were dilute (only about 1% dry matter) and, at 19·3% crude protein in the dry matter, were only slightly enriched with regard to protein content. The solid residue from the extraction at 14·8% crude protein in dry matter showed a corresponding slight diminution from the crude protein level of the starting material. A further drop in the crude protein content of the residue was affected by the subsequent extraction with NaOH solution (to 11·9% in the dry matter). The NaOH extract itself contained dry matter that was 24·6% crude protein; subtraction of the dry matter that had been added as NaOH showed that the NaOH-extracted dry matter was 35·9% crude protein, making this by far the highest concentration of protein that was accomplished during the extraction stages.

Coagulation of protein from extracts

The combined first and second aqueous extracts could not be coagulated upon heating.

To test the effect of acidification, various additions of HCl were made to the combined extracts between 0 and 100 millimoles per litre.

In the case of the NaOH extracts, these were first neutralised with HCl and then a further 50 millimoles of HCl per litre were added. The results are given in Table 2.

In the acidification of aqueous extracts, the use of 50 millimoles of HCl per litre gave the optimum recovery of nitrogen in the precipitate, even though a small improvement of solids recovery was noted at only 25 millimoles of HCl per litre.

TABLE 2

DISTRIBUTION OF DRY MATTER AND CRUDE PROTEIN DURING ACID COAGULATION OF AQUEOUS AND NaOH EXTRACTS OF RUSSIAN COMFREY

Coagulation conditions	*Whole extracts*		*Precipitates*		*Precipitates*		*Crude protein as % of whole extract crude protein*
	Dry matter (g/litre)	*Crude protein (g/litre)*	*Dry matter (g/litre of original extract)*	*Crude protein (g/litre of original extract)*	*Dry matter (% of whole extract)*	*Crude protein (% of precipitate dry matter)*	
Combined aqueous extracts plus 25 millimoles of HCl per litre	10·30	1·99	3·99	1·41	38·7	35·3	70·8
Combined aqueous extracts plus 50 millimoles of HCl per litre	10·30	1·99	3·85	1·48	37·4	38·4	74·4
Combined aqueous extracts plus 100 millimoles of HCl per litre	10·30	1·99	3·51	1·28	34·1	36·4	64·3
Neutralised NaOH extract plus 50 millimoles of HCl per litre	9·68	2·37	4·86	1·81	50·2	37·2	76·4

It should be noted that acidification at 50 millimoles of HCl per litre is twice as effective at precipitating protein as it is at precipitating dry matter, so that the precipitate is about twice as concentrated as the whole extract with respect to its protein content. This leaves a high percentage (62·6 %) of the original extract dry matter still in solution after acidification, containing only about a quarter (25·6 %) of the original extract nitrogen. Therefore, the crude protein in the dry matter of the supernatant liquor after coagulation is low (6·9 %) and is likely to consist mainly of non-protein nitrogen.

Adding to the aqueous extracts even as little as 10 millimoles of HCl per litre produced precipitation, but the precipitate was difficult to separate.

Upon standing at ambient temperature for 12–24 h, a precipitate formed spontaneously in the aqueous extracts. Unlike the acid precipitates, which were distinctly brown in colour, the spontaneously formed precipitates were green,

leaving a dark brown supernatant. These precipitates were also difficult to separate completely and hence spontaneous precipitation would not be a suitable process step. It is likely that enzymic reactions are involved since spontaneous precipitation does not occur after heating the extract to 90 °C.

Several experiments were run employing methanol or *n*-butanol as precipitating agents, with or without acidification. Addition of an equal volume of *n*-butanol to the extracts caused precipitation, leaving a still very brown supernatant. The use of *n*-butanol tended to give rise to very stable emulsions; when separated, neutral *n*-butanol extracts were clear green and acid *n*-butanol extracts were dark brown. In view of the success achieved with acidification in the absence of solvents and the observation that neither methanol nor *n*-butanol caused any further precipitation in the supernatants remaining after acid coagulation, these solvent processes were not developed further. It is possible, however, that they could eventually be employed to produce decolorised protein concentrates for human consumption. The most effective decolorisation was achieved by precipitating the protein at a pH between 1·0 and 2·0 in the presence of *n*-butanol, as under these conditions the colouring matter partitioned mainly into the *n*-butanol layer, although several hours at ambient temperature were needed to maximise the separation of coloured material from protein.

Processing of frozen Comfrey

In cases where frozen material was thawed and juice expressed, the dry matter content of the juice was approximately 2·5 %, amounting to just over 8·0 % of the total crop dry matter; its crude protein content, at 22·5 % in the dry matter, did not represent a concentration of protein compared with the whole crop (25·1 % crude protein in the dry matter) and was not readily coagulable from the juice either by heating or acidification.

True protein content of acid-coagulated protein concentrates

The trichloracetic acid soluble component of the nitrogen in the acid coagulated protein concentrates (after the addition of 50 millimoles of HCl per litre) was found to be 5·6 % of the nitrogen content of the whole coagulum; this corresponds to 94·4 % of the nitrogen in the coagulum being present as true protein.

DISCUSSION

Gumminess is an inherent characteristic of minced or macerated Russian Comfrey and no way has been found to overcome this barrier to extraction processes except by adding water and thereby diluting the juice. The results obtained are quite unlike those obtained during the production of leaf protein from grass or lucerne, which are compared with the present results in Table 3. From this Table it is clear that when

Russian Comfrey is extracted as described above, the extraction differs from leaf protein extraction from lucerne (expression of juice) mainly in the respect that (a) a much greater quantity of soluble solids is extracted, a high proportion of which are non-protein and (b) a greater effectiveness exists at the coagulation stage in separating protein from non-protein components.

TABLE 3
COMPARISON OF AQUEOUS EXTRACTION OF RUSSIAN COMFREY WITH TYPICAL RESULTS OBTAINED FROM LUCERNE EXTRACTION (CONSENSUS OF LITERATURE)[a]

	Russian Comfrey	*Lucerne*
Crop crude protein as % of crop dry matter	16·7[b]	20
Dry matter in juice/aqueous extract as % of crop dry matter	44·4	20
Crude protein in juice as % of crop crude protein	49·0	45·5
Coagulable protein as % of juice protein	74·4	60
Coagulable dry matter as % of juice dry matter	37·4	50
Crude protein as % of coagulable dry matter	38·4	60

[a] See, in particular, Wilkins (1976).
[b] Would normally be approximately 25% in a well-manured crop.

Notwithstanding the latter effect, coagulated protein from Russian Comfrey contains a lower percentage of crude protein than coagulated protein from lucerne. At present, no information is available concerning the non-protein components in the coagulum from Russian Comfrey; if they include a considerable amount of energy sources that are available to non-ruminants, then the protein concentrates may represent a very good non-ruminant feed which would be more balanced in protein and energy than is found in protein concentrate from lucerne. However, if the non-protein components are mainly polyphenols and non-available carbohydrates, this could not apply.

Without doubt, the gumminess of Russian Comfrey is a disadvantage in extraction. The extraction process is slow, presumably because protein and the smaller diffusible molecules are entrained in gum. This idea of entrainment in gum is supported by the finding that the aqueous and NaOH extracts exhibit the same ratio of precipitable to non-precipitable nitrogen whereas, in the absence of gum, these would tend to extract at their own characteristic rates.

In Russian Comfrey of high total nitrogen content more proteinaceous precipitates can be expected. In the present work, when frozen and thawed material having a crude protein content of 25·1% was subjected to juice expression followed by NaOH extraction, acidification of the NaOH extract yielded a coagulum of 42·9% crude protein. The increase is limited, probably because, in crop plants generally, fertilisation with nitrogen increases non-protein nitrogen levels more than the levels of true protein.

The residues obtained after NaOH extraction contained 11–12% crude protein in

the dry matter and hence would be useful feeds for beef cattle. Increased manuring may increase this value also.

Approximately a third of the crop dry matter remains in soluble form in the supernatant liquors remaining after coagulation. Such a substantial by-product would need to be utilised in a commercial process and may be suitable for yeast or mycelial protein production by processes that have already been evaluated for the 'whey' from lucerne leaf protein production (Worgan & Wilkins, 1977). The supernatant liquors from Russian Comfrey were shown to support fairly rapid growth of *Penicillium* spp. This fraction can also be expected to contain the allantoin which is thought to be responsible for at least some of the curative properties of Russian Comfrey (Titherley, 1976) and a concentrate of allantoin might be obtainable from it.

Further work is required to define more fully the parameters for optimal protein extraction, to project the economics of a commercial process and to test the protein concentrates as potential feed or food ingredients.

ACKNOWLEDGEMENT

A research grant from the Henry Doubleday Research Association is gratefully acknowledged, as is the contribution of ideas and information relating to Comfrey from their Director, Mr Lawrence D. Hills.

REFERENCES

HILLS, L. D. (1976*a*). *Comfrey, past, present and future*, Faber & Faber, p. 46.

HILLS, L. D. (1976*b*). *Comfrey, past, present and future*, Faber & Faber, p. 89.

LEXANDER, K., CARLSSON, R., SCHALEN, V., SIMOSSON, A. & LUNDBORG, T. (1970). Quantities and qualities of leaf protein concentrates from wild species and crop species grown under controlled conditions, *Ann. appl. Biol.*, **66**, 193.

LONG, D. B. (1976). In: *Comfrey, past, present and future*, Faber & Faber, p. 229.

TITHERLEY, A. W. (1976). In: *Comfrey, past, present and future*, Faber & Faber, p. 239.

WILKINS, R. J. (1976) (Ed.). *Green crop fractionation*, British Grassland Society and British Society of Animal Production.

WORGAN, J. T. & WILKINS, R. J. (1977). In: *Green crop fractionation* (Wilkins, R. J. (Ed.)), British Grassland Society and British Society of Animal Production.

Food Chemistry 7 (1981) 117–124

THE DETERMINATION OF THE CHOLESTEROL CONTENT IN READY-TO-SERVE FOODS BY GAS–LIQUID CHROMATOGRAPHY

J. R. VAN DELDEN, J. L. COZIJNSEN & P. FOLSTAR

Agricultural University, Department of Food Science, de Dreijen 12, 6703 BC Wageningen, The Netherlands

(Received: 5 February, 1980)

ABSTRACT

*A method is described for the determination of the amount of cholesterol in various types of ready-to-serve foods. The food samples were freeze-dried before analysis. The lipids were extracted from the freeze-dried foods (a) with petroleum ether (40–60°C) in a Soxhlet apparatus or (b) with a mixed solvent system of chloroform:methanol:water in a Waring blender. The lipids were saponified and the unsaponifiable fraction was recovered by extraction with ether. The amount of cholesterol was measured by gas–liquid chromatography without prior derivatisation. Cholesteryl-*n*-butyrate was used as the internal standard. The results obtained by extraction methods (a) and (b) were not significantly different from each other.*

INTRODUCTION

In recent years the role of cholesterol in food and nutrition has been increasingly emphasised. Official recommendations concerning the acceptable daily intake of cholesterol have accelerated the need for a precise method of determining cholesterol (Nederlandse Voedingsraad, 1978).

Recently it was stated that the determination of the cholesterol content in multicomponent foods may lead to special problems. Because these foods contain a variety of ingredients of both animal and vegetable origin, complete extraction of the cholesterol is sometimes difficult (Sweeney & Weihrauch, 1976). The official method of the AOAC for the determination of cholesterol in multicomponent foods, which is based on the method of Punwar (1975, 1976) includes extraction of the lipids from the sample with a mixed solvent system of chloroform:methanol: water and saponification and extraction of the unsaponifiable fraction with benzene.

Fd. Chem. 0308-8146/81/0007-0117/$02·50

The sterols are analysed as trimethylsilyl (TMS) ethers by gas–liquid chromatography (GLC); TMS-cholesterol is determined using 5-α-cholestane as an internal standard (Anon., 1976).

In our experience the extraction procedure with the mixed solvent system is quite laborious; moreover, troublesome emulsions are often formed. In order to avoid the disadvantages mentioned we have studied another extraction method. In this cholesterol was extracted from freeze-dried ready-to-serve mixed dishes with petroleum ether (40–60 °C) in a Soxhlet apparatus. The lipids were saponified and the unsaponifiable fraction was obtained using the official Dutch method for the analysis of lipids (NNI, 1961). The cholesterol content was determined by gas–liquid chromatography without prior derivatisation using cholesteryl-*n*-butyrate as an internal standard. The results were compared with those obtained by extraction with a mixed solvent system.

METHODS

Materials

Ready-to-serve frozen meals were bought from local grocers.

Preparation of the meals

The meals were freeze-dried, homogenised in a Waring blender (model 32 BL 80) and canned under nitrogen.

Moisture content of the freeze-dried meals

This was defined and determined as the loss on drying to constant weight (1–1·5 h) at 105 °C.

Extraction of the lipids from the freeze-dried meals

(a) Between 10 and 25 grammes of the freeze-dried sample were weighed into a 33 × 118 mm extraction thimble (Schleicher and Schüll No. 603). The sample was extracted for 3 or 6 h in a Soxhlet apparatus with petroleum ether (40–60 °C). (All reagents used were pro analysis.) Next, the solvent was evaporated in a rotating vacuum evaporator (Büchi, Type R) at 40 °C. In case the fat content had to be determined the residue was dried at 105 °C for 1 h. (b) Between 5 and 10 grammes of the freeze-dried sample were extracted with a mixed solvent system of chloroform: methanol:water using the official AOAC method for multicomponent foods (Anon., 1976). In case the fat content had to be determined the residue was dried for 1 h at 105 °C.

Isolation of the unsaponifiable fraction

This fraction was obtained by the official Dutch method (NNI, 1961) as follows. Two grammes of residue were saponified with 25 ml of 1N ethanolic sodium

hydroxide solution for 1 h by refluxing. This solution was poured into a separating funnel using 50 ml of distilled water. Next, the unsaponifiable fraction was obtained by extraction with three portions of 50 ml of ether each. The combined ether fractions were washed with 20 ml of distilled water until the ether fraction was alkali-free, as indicated by phenolphthalein. Next, the solvent was evaporated in a rotating vacuum evaporator at 30 °C. Finally, the unsaponifiable fraction was dissolved into 5 ml of a standard solution of 100 mg of cholesteryl-*n*-butyrate in 50 ml of hexane.

Conditions for gas–liquid chromatography

Three microlitres of the unsaponifiable fraction in hexane were analysed by GLC using a Carlo Erba Fractovap 2300 gas chromatograph equipped with a flame ionisation detector coupled with an Infotronics CRS 304 computing integrator. The analysis was carried out on a 90 × 0·2 cm glass column with 5 % SE-52 on GasChrom Q 100–120 mesh (SE-52 = 5 % phenyl, methyl silicone, Hewlett Packard, catalogue No. 85-37715; GasChrom Q, Chrompack, catalogue No. 1955). The oven temperature was 240 °C. The temperature of both the injection port and the detector was 275 °C. The carrier gas was nitrogen at a rate of 20 ml/min. The peaks in the gas chromatogram were identified by gas–liquid chromatography–mass spectrometry (GC–MS) using a combined system of a Pye 204 gas chromatograph and a VG Micromass 7070 P mass spectrometer.

EXPERIMENT

Interpretation of the chromatogram

Under the conditions of the experiment the retention times of cholesterol and cholesteryl-*n*-butyrate were 11·9 and 31·1 min, respectively. The efficiency of the column, described in terms of the number of its theoretical plates (N), amounted to 1600 plates per metre for the cholesterol peak at a carrier gas rate of 20 ml/min; N was calculated from $5{\cdot}54\ (t'/w')^2$ where t' is the adjusted retention time and w' is the width at half-height. Figure 1 shows an example of a chromatogram obtained from an infants' meal (ingredients, according to the manufacturer's description, beef, apple sauce and beans). Cholesterol and cholesteryl-*n*-butyrate were identified by comparison of the retention times with those of known reference compounds as well as by their mass spectra. Moreover, the mass spectral analysis indicated that the peaks at 11·9 and 31·1 min were almost exclusively cholesterol and cholesteryl-*n*-butyrate, respectively. The study of any possible interference by these peaks with peaks of other unsaponifiable components will be the subject of further work.

Linearity and reproducibility

The response linearity of cholesterol under the experimental conditions was determined by plotting the ratio of the peak areas (cholesterol–cholesteryl-*n*-butyrate) against the ratio weights (cholesterol–cholesteryl-*n*-butyrate) with various

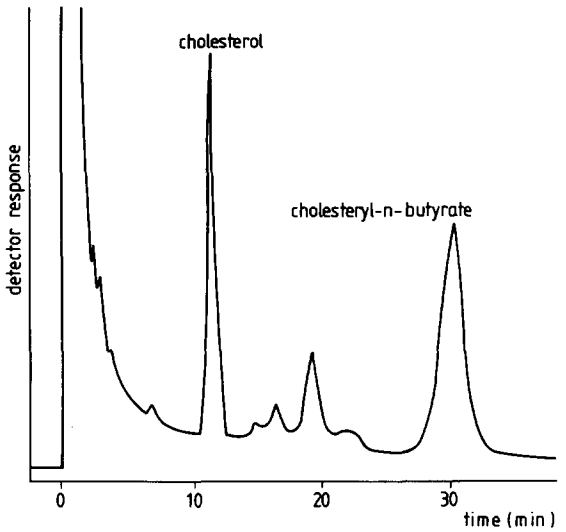


Fig. 1. Example of a gas chromatogram of an infants' meal.

amounts of cholesterol and a fixed amount of cholesteryl butyrate. The results are given in Fig. 2. It was found that the response is linear between 1 and 20 μg of cholesterol using 6 μg of the internal standard.

The reproducibility of the response was tested by injecting 3 μl of mixtures of cholesterol and cholesteryl-*n*-butyrate in hexane nine times each. The amount of cholesterol was varied whilst the amount of cholesteryl-*n*-butyrate was kept constant. The response factor was calculated as:

$$\frac{\text{weight of cholesteryl-}n\text{-butyrate} \times \text{area of cholesterol}}{\text{weight of cholesterol} \times \text{area of cholesteryl-}n\text{-butyrate}}$$

The results are summarised in Table 1. From this Table it can be concluded that the

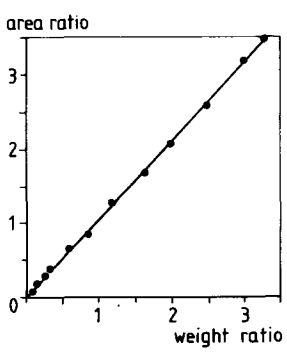


Fig. 2. Response linearity of cholesterol:area ratio (cholesterol/cholesteryl-*n*-butyrate plotted against weight ratio (cholesterol/cholesteryl-*n*-butyrate) with various amounts of cholesterol and a fixed amount of cholesteryl-*n*-butyrate.

TABLE 1
RESPONSE FACTORS AND VARIATION COEFFICIENTS OF CHOLESTEROL[a]

Amount of cholesterol (μg)	*Average response factor* (n = 9)	*Variation coefficient* (%)
0·50	1·23	5·0
1·50	1·09	1·2
3·00	1·05	1·2
6·00	1·05	1·5
9·00	1·06	2·0
12·00	1·06	2·2
15·00	1·06	2·4
18·00	1·05	2·2
21·00	1·06	2·5

[a] In all determinations 6 μg of cholesteryl-*n*-butyrate were used as an internal standard. The injection volume was 3 μl.

response factor was constant between 1·5 and 21 μg of cholesterol. The average response factor, as calculated from all determinations over the whole range of concentrations of cholesterol, amounts to 1·06.

Extraction of cholesterol from foods

Different types of ready-to-serve multicomponent meals were extracted according to two different methods, as already described. Both the fat content and the cholesterol content of each meal were determined. The cholesterol content of the meal was calculated by use of the following formula

$$X = \frac{c.p.M.}{R.I.S.}$$

where: X = milligrammes of cholesterol in the meal, c = area of the cholesterol peak, p = milligrammes of the internal standard, M = dry weight of the meal, R = response factor (= 1·06), I = area of the peak of the internal standard and S = dry weight of the sample.

The results of the determinations are summarised in Table 2. It can be concluded that there are no significant differences between the extraction methods: even an extraction of 3 h with petroleum ether (40–60 °C) in a Soxhlet apparatus gives results equal to those obtained by mixed solvent extraction or extraction for 6 h with petroleum ether (40–60 °C) in the Soxhlet apparatus.

DISCUSSION

In this study it was found that both mixed solvent extraction with chloroform: methanol:water in a Waring blender and an extraction with petroleum ether (40–60 °C) in a Soxhlet apparatus give the same results for cholesterol and fat

TABLE 2

EFFECT OF THE EXTRACTION METHODS ON CHOLESTEROL[a] AND FAT CONTENTS OF DIFFERENT TYPES OF FREEZE-DRIED READY-TO-SERVE FOODS

Meal	*Weight of the meal* (*g*)	*Weight of the meal after freeze-drying* (*g*)	*Moisture content of the freeze-dried meal* (% *w*/*w*)	*Cholesterol* (*mg*) *in one meal*			*Fat* (% *w*/*w*) *in one meal*		
				Petroleum ether (*40–60°C*) *extraction in Soxhlet apparatus*		*Chloroform: methanol: water extraction in Waring blender*	*Petroleum ether* (*40–60°C*) *extraction in Soxhlet apparatus*		*Chloroform: methanol: water extraction in Waring blender*
				3 h	6 h		3 h	6 h	
Fried rice with egg, bacon and meat (from a local restaurant)	925	428	3·00	455·1	448·0	454·2	14·25	14·72	14·87
Macaroni with ham and cheese (frozen)	268	175	3·10	39·5	38·0	36·7	11·13	11·37	11·44
Pizza primavera (frozen)	413	190	2·84	42·2	39·9	38·7	21·19	21·28	21·35
Pizza grandioso (frozen)	445	196	3·38	47·4	49·4	45·2	24·26	24·52	25·23
Hot pot with red cabbage and mashed potatoes (frozen)	569	113	6·35	104·9	103·7	100·4	17·00	17·43	17·19

[a] All values are average values from three determinations.

TABLE 3
CHOLESTEROL AND FAT CONTENTS OF READY-TO-SERVE FOODS[a]

Meal	*Weight of the meal (g)*	*Weight of the meal after freeze-drying (g)*	*Moisture content of the freeze-dried meal (% w/w)*	*Cholesterol[b] (mg)*	*Fat[b] (% w/w)*
Carrot with mashed potatoes, onions and beef (frozen)	583	128	3·70	92·0	16·90
Hashed meat with red cabbage and mashed potatoes (frozen)	753	181	3·10	197·0	22·80
Chicken with apple sauce and potatoes (frozen)	520	116	4·90	65·0	4·70
Fried rice with grilled meat peanut sauce (frozen)	538	212	4·20	163·0	19·80
Minced meat with peas, carrots and mashed potatoes (frozen)	633	195	3·70	137·0	22·50
Beef with red kidney, beans and apple sauce (infants' meal, canned)	138	31	6·30	12·0	11·50
Beef with butter-beans and potatoes (infants' meal, canned)	248	51	2·40	19·0	14·70
Chicken with mixed vegetables and rice (infants' meal, canned)	252	40	4·80	4·0	1·80

[a] All values are average values from three determinations.
[b] Cholesterol and fat were obtained by extraction with petroleum ether (40–60 °C) in a Soxhlet apparatus for 3 h.

contents in ready-to-serve multicomponent foods which were freeze-dried before analysis.

Cholesterol was determined by GLC without prior derivatisation. It was found that the response showed good linearity and reproducibility. This result is in agreement with that of Tuinstra *et al.* (1975) who showed that derivatisation of cholesterol before GLC is superfluous because adsorption due to the uncovered active sides of the support does not take place as long as silanised supports (in our case GasChrom Q) are used.

The study of the method reported in this paper will be the subject of further work.

Examples of the application of the method in other types of multicomponent foods are given in Table 3.

ACKNOWLEDGEMENTS

The authors wish to thank Professor Dr W. Pilnik (Agricultural University, Laboratory of Food Chemistry, Wageningen, The Netherlands) for his stimulating interest in this work, Mr P. Peters (Van Schuppen Chemie B.V., Veenendaal, The Netherlands) for a gift of cholesteryl-*n*-butyrate and for valuable discussions, Dr M. A. Posthumus (Agricultural University, Laboratory of Organic Chemistry, Wageningen, The Netherlands) for GC–MS studies and several students for assistance during their laboratory class period in food chemistry.

REFERENCES

ANON. (1976). 'Changes in methods: 43.B05'. Cholesterol in multicomponent foods. *J.A.O.A.C.*, **59**(2), 481.

NEDERLANDSE VOEDINGSRAAD (1978). Aanbevolen hoeveelheden energie en voedingsstoffen. *Voeding*, **39**(11), 325.

NNI (1961). Nederlandse Norm NEN 1046. Nederlands Normalisatie Instituut, Rijswijk, The Netherlands.

PUNWAR, J. K. (1975). Gas–liquid chromatographic determination of total cholesterol in multicomponent foods. *J.A.O.A.C.*, **58**(4), 804.

PUNWAR, J. K. (1976). Collaborative study for the comparison of two methods for the determination of total cholesterol in multicomponent foods. *J.A.O.A.C.*, **59**(1), 46.

SWEENEY, J. P. & WEIHRAUCH, J. L. (1976). Summary of available data for cholesterol in foods and methods for its determination. *C.R.C. Crit. Rev. Food and Nutr.*, **8**, 131.

TUINSTRA, L. G. M. TH., ROOS, J. B. & VERSNEL, A. (1975). Adsorptie van sterolen tijdens de gaschromatografische scheiding en detectie. *De Ware(n) Chemicus*, **5**, 18.

Food Chemistry 7 (1981) 125–138

PURIFICATION, CRYSTALLISATION AND CHARACTERISATION OF CARBOXYPEPTIDASE FROM WHEAT BRAN

HIRONORI UMETSU, MASAMI ABE, YOSHIHARU SUGAWARA & TAKAO NAKAI

Food Research Laboratory, Pentel Co. Ltd, Yoshi-io, Soka, Saitama 340, Japan

&

SINGO WATANABE & EIJI ICHISHIMA

Department of Agricultural Chemistry, Tokyo Noko University, Fuchu, Tokyo 183, Japan

(Received: 27 February, 1980)

ABSTRACT

A carboxypeptidase was purified and crystallised from wheat bran. Disc gel electrophoresis at pH 4·0 and ultracentrifugal analysis revealed that the enzyme was essentially homogeneous. The sedimentation constant and isoelectric point were determined to be 6·3 S and 6·0, respectively. The molecular weight of the enzyme was estimated to be 118,000 by a gel filtration method. The enzyme liberated carboxyl terminal amino acid residues from a wide range of N*-substituted dipeptides and tripeptides which contain* L*-proline. It had a pH optimum at pH 4·0 for* Z*-Glu-Tyr* (Z*-benzyloxycarbonyl). The* K_m *and* k_{cat} *values for* Z*-Glu-Tyr at pH 4·0 and 30°C were 0·19 mM and 20* s^{-1}*, respectively. The enzyme hydrolysed* Z*-Gly-Pro-Leu-Gly-Pro and bradykinin sequentially at pH 4·0 from their carboxyl terminal amino acid residues. The enzyme activity was completely inhibited by DFP.*

ABBREVIATIONS

Z-, benzyloxycarbonyl; *Bz-*, benzoyl; DFP, diisopropylfluorophosphate; EDTA, ethylenediaminetetraacetic acid; PCMB, *p*-chloromercuribenzoic acid. The abbreviated designation of amino acid residue denotes the L-form.

Fd. Chem. 0308-8146/81/0007-0125/$02·50

INTRODUCTION

Carboxypeptidases from various plant tissues have the ability to liberate basic, acidic and neutral amino acid residues, including that of proline, from the carboxyl terminal amino acid of polypeptides (Zuber, 1968; Visuri *et al.*, 1969; Carey & Wells, 1972; Ihle & Dure, 1972; Kubota *et al.*, 1973; Matoba & Doi, 1975). These enzymes from plants have an optimum pH from 5 to 6 and possess active serine residues. Preston & Kruger (1976, 1977) purified and characterised two carboxypeptidases from germinated wheat. These enzymes had molecular weights of 55,000 and 61,000, a pH optimum of 5·2 for *Z*-Phe-Ala and were considered to play a role in protein hydrolysis during storage.

In the present paper, the method of large-scale purification from wheat bran of a new carboxypeptidase with a molecular weight of 118,000 is described, as are its enzymic properties.

MATERIALS AND METHODS

Enzyme source

Wheat bran from bread and common wheat (*Triticum aestivum* L.) was used as a primary source.

Materials

Z-Phe-Ala, Gly-Gly-Gly, Gly-Phe-Phe and Phe-Phe-Phe were purchased from Sigma Chemicals Co. *Z*-Pro-Pro was purchased from Bachem Inc. All other *N*-substituted dipeptides and bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) were obtained from the Protein Research Foundation, Osaka.

Sephadex G-150 was obtained from Pharmacia Fine Chemicals Co. The dimensions of the column used for gel filtration were 80 cm × 2·0 cm diameter. CM-cellulose (CM-32) was obtained from the Whatman Co. Ampholine and the electrofocusing column (110 ml) from LKB-Producter, AB, and hollow fibres from the Asahikasei Co. Ltd, Tokyo. Silica gel G was purchased from Wako Pure Chemicals Ltd, Osaka.

Assay of carboxypeptidase

The peptide *Z*-Glu-Tyr was used for the detection of carboxypeptidase activity. The reaction mixture had the following composition: 0·5 ml substrate (10^{-3}M) dissolved in 0·05M acetate buffer (pH 4·0) and 0·5 ml enzyme dissolved in the same buffer. After incubation at 30 °C for 20 min, the reaction was stopped by the addition of 1 ml of freshly prepared ninhydrin reagent (Cooking & Yemm, 1954) and 3 ml of 0·5M citrate buffer (pH 5·0) followed by heating at 100 °C for 15 min. The mixture was cooled in an ice water bath for 10 min and the extent of hydrolysis was measured

at 570 nm. The zero-time sample served as a blank. A solution of L-tyrosine (10^{-4}M) was used as a standard. Results were expressed as katals (Florkin & Stotz, 1973). One katal is the amount of enzyme required to liberate 1 M tyrosine a second from *Z*-Glu-Tyr at pH 4·0 and 30 °C.

Protein determination

Protein concentrations were estimated from the absorbance at 280 nm with the Shimazu model UV-150-02 double-beam spectrophotometer.

Purification of the carboxypeptidase from wheat bran

A flow chart for the isolation of carboxypeptidase from wheat bran is shown in Fig. 1.

Wheat bran was extracted with 50 litres of 0·6M acetic acid solution with stirring for 15 h at 12 °C. The insoluble material was removed by filtration and the filtrate was brought to 60% ammonium sulphate saturation by the addition of solid ammonium sulphate. The resulting precipitate was collected by centrifugation at 4000 × *g* and stored at 4 °C as a crude enzyme preparation.

Three hundred grammes of the crude enzyme precipitates were added to 300 ml of ammonium sulphate solution containing 73·5 g of solid ammonium sulphate. The

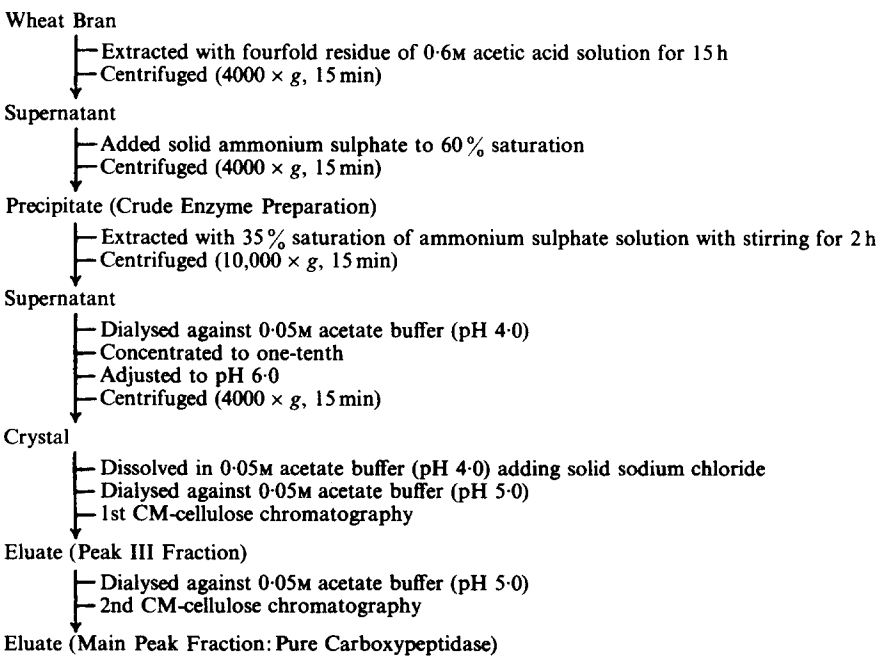


Fig. 1. Flow chart for the isolation of carboxypeptidase from wheat bran.

suspension was stirred for 2 h and the active supernatant was collected by centrifugation.

The supernatant was dialysed against to 0·05M acetate buffer (pH 4·0) and concentrated to one-tenth of its original volume by means of ultrafiltration with hollow fibres. In this procedure the resulting enzyme solution became slightly turbid because a small amount of the enzyme was crystallised. When the pH of the enzyme solution was adjusted to 6·0 with 0·1N sodium hydroxide solution, crystallisation took place immediately. After the suspended solution had stood overnight, the crystalline enzyme was collected by centrifugation. The enzyme crystals are insoluble in 0·05M acetate buffer (pH 6·0) so they were washed three times with a small volume of 0·05M acetate buffer (pH 6·0). The crystals were suspended in 0·05M acetate buffer (pH 4·0) and dissolved by adding a small quantity of solid sodium chloride and stirring.

The dissolved enzyme preparation was chromatographed on a CM-cellulose column (1·5 cm × 30 cm) using a linear gradient from 0·05M to 0·5M acetate buffer (pH 5·0). The major active peak (fraction III) was rechromatographed on a CM-cellulose column (1·5 cm × 30 cm) using a linear gradient from 0·1M to 0·5M acetate buffer (pH 5·0).

All operations of the purification procedure were performed at 4 °C. For all purification steps, carboxypeptidase activity was assayed with *Z*-Glu-Tyr as the substrate at pH 4·0.

Disc gel electrophoresis

Disc gel electrophoresis was performed according to the methods of Reisfeld *et al.* (1962) in 0·35M β-alanine-acetic acid buffer (pH 4·5) at a constant current of 4 mA per tube for 90 min. Protein was stained with Coomassie Brilliant Blue R-250 and carbohydrate was stained with periodic acid-Schiff reagent by means of the method of Zacharius *et al.* (1969).

Ultracentrifugation

Ultracentrifugation was carried out with a Spinco model E analytical ultracentrifuge. The sedimentation constant was measured at two protein concentrations of 0·18% and 0·34% in 0·05M acetate buffer (pH 4·0).

Estimation of molecular weight

The molecular weight was estimated from the elution volume on Sephadex G-150 using 0·05M acetate buffer pH 4·0 (Andrews, 1964). The columns were calibrated for estimating molecular weight by using γ-globulin, bovine serum albumin, egg albumin and β-lactoglobulin as standard proteins. The sodium dodecyl sulphate–polyacrylamide gel method of Weber & Osborn (1969) was also employed to determine molecular weight. A 6 mA current was applied per tube and the average time per run was 5 h. Gels were stained with Coomassie Brilliant Blue R-250. Proteins of known molecular weights served as reference markers.

Electrofocusing

Electrofocusing was carried out according to the method of Vesterberg & Svensson (1966) in a 110 ml column for 60 h at 300 V and 4 °C.

Amino acid analysis of carboxypeptidase

The purified enzyme was hydrolysed by the method of Moore & Stein (1963). Amino acids were analysed with a JEOL model JLC-6AH amino acid analyser. Tryptophan was determined spectrophotometrically according to the method described by Bencze & Schmid (1957). The number of residues of amino acids was calculated for a molecular weight of 118,000. The carbohydrate content was determined by the method of Dubois *et al.* (1956).

Thin layer chromatography

The solvent system of thin layer chromatography with Silica gel G was phenol: water:aq. NH_3 (100:20:0·5 by volume).

Relative rates of peptide hydrolysis by carboxypeptidase

The reaction mixture had the following composition: 0·5 ml of each substrate (2×10^{-3}M) dissolved in 0·05M acetate buffer (pH 4·0) and 0·5 ml of the enzyme dissolved in the same buffer.

Release of C-terminal amino acid residues from peptides

Z-Gly-Pro-Leu-Gly-Pro (20 μmol) was incubated with 36·5 nkat (216 μg) of the enzyme at 30 °C in 4 ml of 0·05M acetate buffer (pH 4·0). Bradykinin (0·76 μmol) was incubated with 3·76 nkat (22·2 μg) of the enzyme at 30 °C in 4 ml of 0·05M acetate buffer (pH 4·0). After the scheduled time periods, 0·5 ml of the mixture was withdrawn and the enzymic reaction was stopped by the addition of 0·5 ml of 0·4M trichloroacetic acid solution. The stopped reaction mixture was applied to the automatic amino acid analyser, JEOL model JLC-6AH.

Activation and inhibition studies

The enzyme (12 μg) was preincubated with chemical reagent in 0·5 ml of 0·05M acetate buffer (pH 4·0) at 30 °C for 20 min. In the case of DFP and monoiodoacetic acid, the preincubation periods were 40 min and 18 h, respectively. After preincubation, enzymic activity was determined with *Z*-Glu-Tyr as a substrate. When the chemical reagent interfered with ninhydrin coloration, the value of the absorbance at 570 nm was corrected.

RESULTS AND DISCUSSION

Purification of carboxypeptidase from wheat bran

The extraction of the enzyme from crude enzyme preparations was used, together with an ammonium sulphate solution, as the initial step in the purification of the

carboxypeptidase. In this step a large amount of gluten-like protein was removed, such removal facilitating the subsequent purification procedure. Crystallisation was applied as the second purification step. In the crystallisation procedure, a large amount of the coloured substance was removed and the specific activity of the enzyme increased about 40-fold. Crystallisation was the most effective procedure for removing coloured substances and increasing specific activity with good yield. The third step of the purification was CM-cellulose chromatography (Fig. 2). Four active

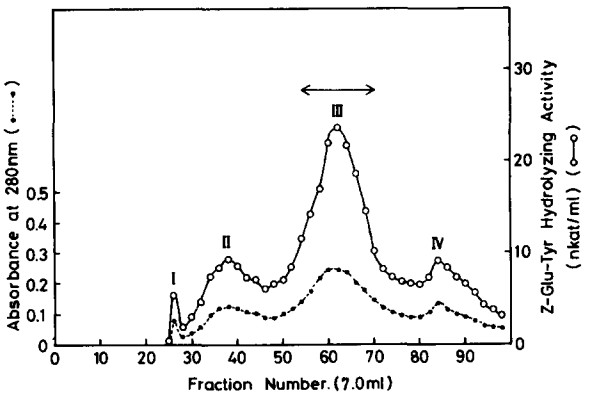


Fig. 2. Elution pattern of carboxypeptidase from wheat bran on CM-cellulose (see text for details); ●---●, absorbance at 280 nm; ○——○, activity on *Z*-Glu-Tyr. Active peaks are designated I, II, III and IV. Arrow indicates pooled fractions.

peaks were eluted and the major active peak (carboxypeptidase III fraction) was collected for rechromatography on CM-cellulose. The main active peak was collected as the purified enzyme preparation of carboxypeptidase III. The nature of the other three enzymes (fractions I, II and IV) has not been investigated in detail, although the isoelectric points of these enzymes were determined to be 4·8, 5·5 and 6·5. We consider they are carboxypeptidases, because they possess *Z*-Glu-Tyr hydrolysing activity and their mixed crystal is octahedral, like the crystal of the purified carboxypeptidase III. These four enzymes may be analogous in molecular structure.

A small sample of the enzyme was dialysed against distilled water and then freeze dried. The material obtained was completely dissolved in 0·05M acetate buffer (pH 4·0). A solution containing 1 mg of the enzyme per millilitre gave an absorbance of 1·96 at 280 nm. The specific activity for carboxypeptidase of the purified enzyme was 0·169 kat per kilogramme of enzyme, resulting in a 540-fold increase compared with the crude extract. A hundred and thirty milligrammes of the purified enzyme were obtained from 12·5 kg of the wheat bran. The purification of a typical enzyme preparation is shown in Table 1.

TABLE 1
PURIFICATION OF CARBOXYPEPTIDASE FROM WHEAT BRAN

Fraction	*Total protein* (A_{280})	*Total activity* (*nkat*)	*Specific activity* ($nkat/A_{280}$)	*Recovery* (%)
Crude extract	350000	58000	0·16	100
Extract from $(NH_4)_2SO_4$ precipitate	11000	23800	2·15	40·9
Crystallisation	203	16700	82·3	28·8
1st CM-cellulose	85·8	7350	85·6	12·7
2nd CM-cellulose	63·8	5500	86·3	9·5
Crystallisation	—	—	86·4 (0·169 kat/kg)[a]	—

[a] Calculated from the datum of $A^{0.1\%}_{1cm}$ at 280 nm = 1·96.

The crystals of the purified enzyme were prepared by the method described above. The crystal form of the purified enzyme was octahedral (Fig. 3).

Homogeneity of the enzyme preparation

Disc electrophoresis patterns of the purified enzyme at pH 4·0 are shown in Fig. 4. The enzyme behaved as a single component with either a low or a high protein concentration. The band of protein staining is equal to that of carbohydrate staining in mobility. The carboxypeptidase from wheat bran must be a glycoprotein.

The purified enzyme behaved as a single component in analytical ultracentrifugation (Fig. 5). The sedimentation constant gave a value $S_{20,w}$ of 6·3 S.

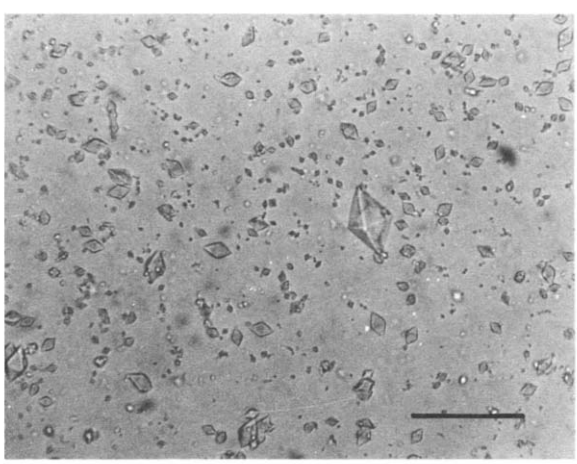


Fig. 3. Crystal of the purified carboxypeptidase from wheat bran. Bar indicates 20 μm.

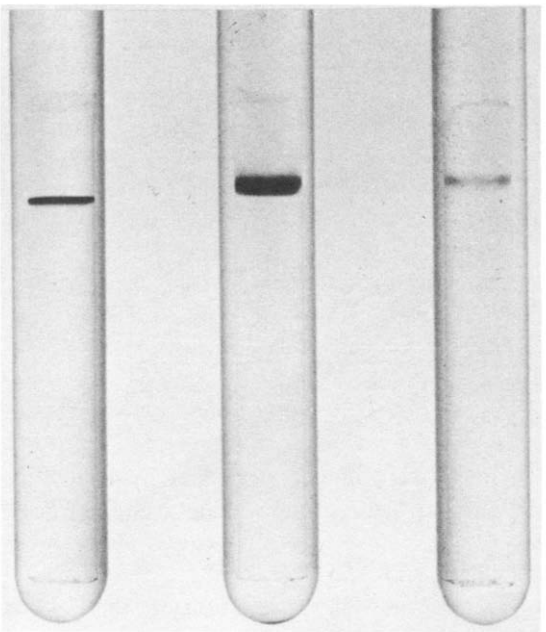


Fig. 4. Disc gel electrophoretograms of carboxypeptidase. Slots A and B stained with Coomassie Brilliant Blue, slot C stained with periodic acid–Schiff reagent.

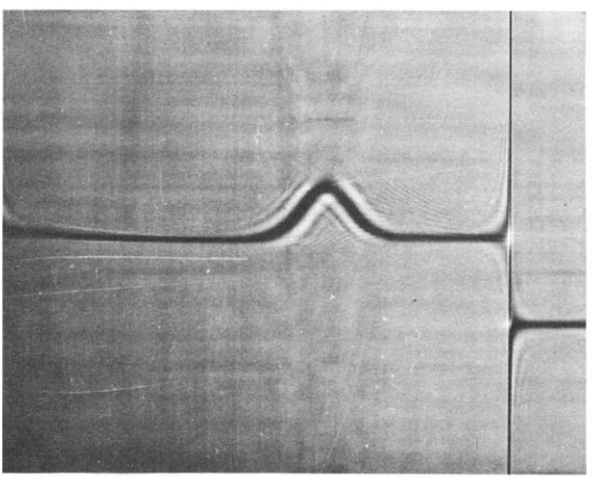


Fig. 5. Sedimentation pattern of pure carboxypeptidase: 0·34 % protein, 23 °C, 64,000 rpm. Exposure taken after 40 min.

Physical and chemical properties of the carboxypeptidase from wheat bran

The molecular weight was estimated from the elution volume on Sephadex G-150. Assuming a linear relationship between log molecular weight and V_e/V_o, the calculated molecular weight of the enzyme was 118,000. This value is in the probable range for a globular protein with $S_{20,w}$ 6·3 S.

Two protein bands with a molecular weight of 35,000 and 25,000 were detected in SDS–polyacrylamide gel electrophoresis. When the enzyme was not treated with 1 % 2-mercaptoethanol, a single band of 58,000 molecular weight was observed in SDS–polyacrylamide gel electrophoresis. This indicates that the enzyme from wheat bran is tetrameric.

Electrofucusing was performed by loading 0·13 mg of the enzyme in a focusing column. The isoelectric point of the enzyme was determined to be 6·0.

Table 2 shows the results of the amino acid analysis of the enzyme. The amino acid composition of carboxypeptidase from wheat bran was very similar to that of germinated cottonseed (Ihle & Dure, 1972) and different from that of *Citrus natsudaidai* (Kubota *et al.*, 1974) in its proline, glycine and tryptophan content. The enzyme from wheat bran was obviously different from that of fungus, *Phymatorichum omnivorum* (Boston & Prescott, 1968), carboxypeptidase Y (Hayashi, 1976), carboxypeptidase A (Bargetzi *et al.*, 1963) and B (Cox *et al.*, 1962) in lysine, arginine, glycine, alanine and methionine content.

TABLE 2
AMINO ACID COMPOSITION OF CARBOXYPEPTIDASE FROM WHEAT BRAN

Amino acid	*Protein (g/100 g)*	*Calculated residues (MW 118,000) (No.)*
Lys	2·07	17
His	3·71	28
Arg	6·52	44
Asp	9·76	87
Thr	5·65	56
Ser	5·67	64
Glu	7·49	60
Pro	4·79	49
Gly	5·08	80
Ala	6·09	81
Cys (half)	2·08	17
Val	5·48	55
Met	1·08	9
Ile	3·20	29
Leu	7·45	67
Tyr	7·25	47
Phe	4·33	31
Trp	5·85	34
Total	——	855
Glc *N*	3·75	25
Other hexoses	8·70	57

Enzymic properties of the carboxypeptidase

The optimum pH of the carboxypeptidase with *Z*-Gly-Tyr was estimated at pH 4·0. Carboxypeptidases obtained from plants are most active at pH 5·0 to 6·0; for example, the enzyme from barley has an optimum pH at 5·2 for *Z*-Phe-Ala (Visuri *et al.*, 1969), that from germinated wheat at pH 5·2 for *A*-Phe-Ala (Preston & Kruger, 1977), that from *Citrus* fruits at pH 5·6 for *Z*-Leu-Phe (Zuber, 1968) and that from *Phaseolus vulgaris* at pH 5·6 for *Z*-Gly-Leu (Shaw & Wells, 1967). In this point, this enzyme differs from previously isolated carboxypeptidases of plants and resembles that from moulds such as *Aspergillus* (Ichishima, 1972) and *Penicillium* (Yokoyama *et al.*, 1974; Jones & Hofmann, 1972).

Values of K_m and K_{cat} in the hydrolysis of *Z*-Glu-Tyr were calculated from Lineweaver–Burk plots. The K_m and K_{cat} values at pH 4·0 and 30 °C were 0·19 mM and 20 s^{-1}, respectively.

Table 3 shows the relative rates of hydrolysis of a series of peptides by the enzyme. When the penultimate amino acid residue from the carboxyl terminal amino acid residue was phenylalanine or tyrosine, the hydrolysis rate of the substrate was high. If the penultimate residues had acidic side chains the hydrolysis was moderately high. When glycine or proline was in the penultimate position, the release of the terminal amino acids was slow. A change in the penultimate position of the carboxyl terminal from phenylalanine or tyrosine to glutamic acid and from glutamic acid to glycine or proline led to a marked decrease in the hydrolysis rate. The liberation of proline from *Z*-Phe-Pro indicated that the enzyme broke the imidopeptide bond. Although only limited data are available on the effect of the carboxyl terminal

TABLE 3
RELATIVE RATES OF PEPTIDE HYDROLYSIS BY CARBOXYPEPTIDASE FROM WHEAT BRAN

	Peptide	*Relative activity*
(1)	*N*-Substituted peptide	
	Z-Phe-Ala	1760
	Z-Phe-Tyr	560
	Z-Phe-Pro	24
	Z-Tyr-Glu	600
	Z-Glu-Tyr	100
	Z-Glu-Phe	95
	Z-Gly-Phe	27
	Z-Gly-Leu	15
	Bz-Gly-Lys	2
	Z-Gly-Pro	Trace
	Z-Gly-Pro-Leu-Gly-Pro	2
	Z-Pro-Pro	0
(2)	Tripeptide	
	Phe-Phe-Phe	250
	Gly-Phe-Phe	56
	Gly-Gly-Gly	Trace

Rate of hydrolysis of *Z*-Glu-Tyr = 100.

residues, the rate of hydrolysis was less influenced by the amino acid residues occupying the carboxyl terminal than the penultimate position. Thus it may be concluded that the penultimate amino acid to the carboxyl terminal residue in *N*-substituted dipeptides mainly affects the rate of hydrolysis by the enzyme. The same results were obtained from carboxypeptidases of *Aspergillus* (Ichishima, 1972) and French bean leaves (Carey & Wells, 1972). The carboxypeptidase from wheat bran scarcely hydrolysed *Z*-Gly-Pro but more easily split off the Gly-Pro bond on a long chain substrate, *Z*-Gly-Pro-Leu-Gly-Pro. It is assumed that the third or succeeding amino acid residues from the carboxyl terminal residue were involved in binding between the enzyme and the substrate.

The enzyme hydrolysed Gly-Phe-Phe and Phe-Phe-Phe well. Phenylalanine liberated from carboxyl terminal residue in Gly-Phe-Phe was detected by the TLC method. However, Gly-Gly-Gly is a poor substrate.

TABLE 4

RELEASE OF CARBOXYL TERMINAL AMINO ACID RESIDUES FROM BRADYKININ[a] BY CARBOXYPEPTIDASE FROM WHEAT BRAN

Incubation time	*Amino acid released (μmole per μmole substrate)*				
	Gly	*Ser*	*Pro*	*Phe*	*Arg*
10 min	0	0·016	0·022	0·066	0·974
20 min	0	0·026	0·040	0·100	0·987
1 h	0·009	0·095	0·100	0·280	0·993
3 h	0·018	0·372	0·378	0·742	0·997
8 h	0·028	0·867	0·875	1·642	1·000

[a] Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg.

Tables 4 and 5 show the results of the action of the purified carboxypeptidase on bradykinin and *Z*-Gly-Pro-Leu-Gly-Pro at pH 4·0. In bradykinin, the carboxyl terminal, arginine, was quickly released. Release of the second residue, phenylalanine, from the carboxyl terminal of bradykinin was relatively slow because of the presence of proline in the third position. The third residue, proline, was liberated slowly and release of the fourth residue, serine, was nearly equivalent to that of the proline. These observations agree with the results obtained from the hydrolysis of a series of *N*-substituted dipeptides. The enzyme released proline from the carboxyl terminal residue of *Z*-Gly-Pro-Leu-Gly-Pro at a rather slow rate. Release of the second residue (carboxyl terminal glycine) then occurred but from the third position leucine was liberated slowly. In this way it is confirmed that the enzyme liberated amino acids successively from the carboxyl terminal residues of peptides.

The diluted enzyme preparation was stored at 4 °C and pH 2 to 9·5. It was stable in the pH range 4 to 8 for at least six months. Freezing and thawing of the enzyme

TABLE 5
RELEASE OF CARBOXYL TERMINAL AMINO ACID RESIDUES FROM *Z*-Gly-Pro-Leu-Gly-Pro BY CARBOXYPEPTIDASE FROM WHEAT BRAN

Incubation time	*Amino acid released (μmole per μmole substrate)*		
	Leu	*Gly*	*Pro*
20 min	0	0·005	0·013
1 h	0	0·009	0·035
3 h	0·003	0·041	0·089
9 h	0·022	0·127	0·234
27 h	0·094	0·277	0·538

solution destroyed the enzymic activity almost completely. No loss of activity took place on freeze drying of the diluted enzyme solution in citrate buffer.

The effects of some chemical reagents on the enzymic reaction rate at pH 4·0 have been examined (Table 6). Many reports show that serine carboxypeptidases (EC 3.4.16.1) from plants and microorganisms in general have the serine active site inhibited by DFP (Ihle & Dure, 1972; Hayashi *et al.*, 1973; Matoba & Doi, 1975). The carboxypeptidase of wheat bran is also completely inhibited by DFP and considered to have the essential serine. The enzyme was partially inhibited by PCMB and not inhibited by monoiodoacetic acid, 2-mercaptoethanol and EDTA. Triton X-100 and Brij 35 activated 50% and 23% of the enzyme activity, respectively, whilst sodium dodecyl sulphate completely inhibited the enzyme activity.

TABLE 6
EFFECTS OF CHEMICAL REAGENTS ON THE ACTIVITY OF CARBOXYPEPTIDASE FROM WHEAT BRAN

Reagent	*Concentration (mM or %[a])*	*Residual activity (%)*
Monoiodoacetic acid	2·5	94
	0·25	100
PCMB	1·0	65
	0·1	77
2-Mercaptoethanol	5·0	99
	0·5	104
EDTA	5·0	98
	1·0	102
DFP	11·5	0
Triton X-100	0·5[a]	146
	0·2[a]	147
Brij 35	0·5[a]	123
Sodium dodecyl sulphate	0·1[a]	0
	0·01[a]	0
None	—	100

[a] The concentrations of reagent are expressed as % w/w.

The effects of metal ions on the enzymic activity have been examined. The enzyme was not affected at all by Pb^{2+}, Cu^{2+}, Co^{2+}, Ca^{2+} and Zn^{2+} at 1 mM and 10 mM concentrations. It was also not inhibited by Hg^{2+} at 1 mM concentration which is known to react with sulphydryl and carboxyl groups. Sulphydryl groups may not be essential for enzyme activity.

Preston & Kruger (1976, 1977) reported the purification of two carboxypeptidases from germinated wheat, as well as their properties. The molecular weights and isoelectric points of these enzymes are 55,000 and 61,000, 5·60 and 5·68, respectively, whereas those of the wheat bran carboxypeptidase are 118,000 and 6·0. Germinated wheat carboxypeptidases resemble wheat bran carboxypeptidase in their ability to liberate most amino acid residues from the carboxyl terminal residues of polypeptide chains but are apparently different with regard to molecular weight, isoelectric point and optimum pH.

REFERENCES

ANDREWS, P. (1964). Estimation of molecular weights of proteins by Sephadex gel-filtration, *Biochem. J.*, **91**, 222–32.

BARGETZI, J-P., SAMPATH KUMAR, K. S. V., COX, D. J., WALSH, K. A. & NEURATH, H. (1963). The amino acid composition of bovine pancreatic carboxypeptidase A, *Biochemistry*, **2**, 1468–74.

BENCZE, W. L. & SCHMID, K. (1957). Determination of tyrosine and tryptophan in proteins, *Anal. Chem.*, **29**, 1193–6.

BOSTON, J. D. & PRESCOTT, J. M. (1968). Some physical properties of a fungal carboxypeptidase, *Arch. Biochem. Biophys.*, **128**, 88–94.

CAREY, J. N. & WELLS, J. R. E. (1972). Phaseolain, *J. Biol. Chem.*, **247**, 5573–9.

COOKING, E. C. & YEMM, E. W. (1954). Estimation of amino acids by ninhydrin, *Biochem. J.*, **58**, xii.

COX, D. J., WINTERSBERGER, E. & NEURATH, H. (1962). Bovine pancreatic procarboxypeptidase B. II. Mechanism of activation, *Biochemistry*, **1**, 1078–82.

DUBOIS, M., GILLES, K. A., HAMILTON, J. K., REBERS, P. A. & SMITH, F. (1956). Colorimetric method for determination of sugars and related substances, *Anal. Chem.*, **28**, 350–6.

FLORKIN, M. & STOTZ, E. H. (1973). Units of enzyme activity. In: *Comprehensive biochemistry*, Vol. 13 (Florkin, M. & Stotz, E. H. (Eds)), Amsterdam, Elsevier, 26–7.

HAYASHI, R., MOORE, S. & STEIN, W. (1973). Serine at the active center of yeast carboxypeptidase, *J. Biol. Chem.*, **248**, 8366–9.

HAYASHI, R. (1976). Carboxypeptidase Y. In: *Methods in enzymology*, Vol. 45B (Lorand, L. (Ed.)), New York, San Francisco and London, Academic Press, 568–87.

ICHISHIMA, E. (1972). Purification and characterization of a new type of acid carboxypeptidase from *Aspergillus*, *Biochem. Biophys. Acta*, **258**, 274–88.

IHLE, J. N. & DURE, L. S. (1972). The developmental biochemistry of cottonseed embryogenesis and germination: I. Purification and properties of a carboxypeptidase from cotyledons, *J. Biol. Chem.*, **247**, 5034–40.

IHLE, J. K. & DURE, L. S. (1972). The developmental biochemistry of cottonseed embryogenesis and germination: II. Catalytic properties of the cotton carboxypeptidase, *J. Biol. Chem.*, **247**, 5041–7.

JONES, S. R. & HOFMAN, T. (1972). Penicillocarboxypeptidase-S, A non-specific SH-dependent exopeptidase, *Can. J. Biochem.*, **50**, 1297–1310.

KUBOTA, Y., SHOJI, S., FUNAKOSHI, T. & UEKI, H. (1973). Carboxypeptidase CN: I. Purification and characterization of the enzyme from exocarp of *Citrus natsudaidai* Hayata, *J. Biochem.* (Tokyo), **74**, 757–70.

KUBOTA, Y., SHOJI, S., FUNAKOSHI, T. & UEKI, H. (1974). Carboxypeptidase CN: II. Stability and some chemical and kinetic properties, *J. Biochem.* (*Tokyo*), **76**, 375–84.

MATOBA, T. & DOI, E. (1975). Substrate specificity of carboxypeptidase from water melon, *J. Biochem. (Tokyo)*, **77**, 1297–303.

MOORE, S. & STEIN, W. (1963). Chromatographic determination of amino acids by the use of automatic recording equipment. In: *Methods in enzymology*, Vol. 6 (Colowick, S. P. & Kaplan, N. O. (Eds)), New York and London, Academic Press, 819–31.

PRESTON, K. R. & KRUGER, J. E. (1976). Purification and properties of two proteolytic enzymes with carboxypeptidase activity in germinated wheat, *Plant Physiol.*, **58**, 516–20.

PRESTON, K. & KRUGER, J. (1977). Specificity of two isolated wheat carboxypeptidases, *Phytochem.*, **16**, 525–8.

REISFELD, R. A., LEWIS, U. T. & WILLIAMS, D. E. (1962). Disc electrophoresis of basic proteins and peptides on polyacryamide gels, *Nature*, **195**, 281–3.

SHAW, D. L. & WELLS, J. R. E. (1967). A unique amino acid sequence around a reactive serine residue in peptidase from French beans and yeast, *Biochem. J.*, **104**, 5c.

VESTERBERG, O. & SVENSSON, H. (1966). Isoelectric fractionation, analysis and characterization of ampholytes in natural pH gradients: IV. Further studies on the resolving power in connection with separation of myoglobin, *Acta Chem. Scand.*, **20**, 820–34.

VISURI, K., MIKOLA, J. & ENARI, T. M. (1969). Isolation and partial characterization of a carboxypeptidase from barley, *Eur. J. Biochem.*, **7**, 193–9.

WEBER, K. & OSBORN, M. (1969). The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis, *J. Biol. Chem.*, **244**, 4406–12.

YOKOYAMA, S., OOBAYASHI, A., TANABE, O., SUGAWARA, S., ARAKI, E. & ICHISHIMA, E. (1974). Production and some properties of a new type of acid carboxypeptidase of *Penicillium* molds, *Appl. Microbiol.*, **27**, 953–60.

ZACHARIUS, R. M., ZELL, T. E., MORRISON, J. H. & WOODLOCK, J. J. (1969). Glycoprotein staining electrophoresis on acrylamide gels, *Anal. Biochem.*, **30**, 148–52.

ZUBER, H. (1968). Reinigung und Eigenschaften der Carboxypeptidase aus Citrusfrüchten, *Hoppe-Seyler's Z. Physiol. Chem.*, **349**, 1137–52.

Food Chemistry 7 (1981) 139–145

A RAPID METHOD FOR THE DETERMINATION OF PENTOSANS IN WHEAT FLOUR

S. G. DOUGLAS

Flour Milling and Baking Research Association, Chorleywood, Rickmansworth, Herts WD3 5SH, Great Britain

(Received: 3 March, 1980)

ABSTRACT

A rapid and reproducible method is presented for the determination of pentosans in wheat flour.

Flour (approximately 5 mg) is added to 2 ml of water followed by 10 ml of a solution of glacial acetic acid, hydrochloric acid, phloroglucinol and glucose in a stoppered boiling tube.

The boiling tube is placed in a water bath for twenty-five minutes and the absorbance of the resulting solution measured at 552 nm and 510 nm.

The absorbance of interfering sugars differs very little at these two wavelengths but is considerable for the pentosans. Accordingly, the pentosans may be estimated, even when an excess of interfering sugars is present, by subtracting the absorbance at 510 nm from that at 552 nm.

INTRODUCTION

A rapid and reproducible method was required to determine the pentosan content of a large number of samples of wheat flour as part of a research project at the Flour Milling and Baking Research Association.

Pentosans (polypentosides) are five membered sugar-residue polymers of the general formula $(C_5H_8O_4)n$. They compete with starch and protein for the available water in a bread or cake matrix and so can influence product quality. Pentosans are also an important constituent of dietary fibre (Southgate, 1976). The traditional Tollens method of analysis of pentosans (Fraser *et al.*, 1956) utilises the hydrolysis of the pentosans to furfural (2-furancarboxyaldehyde, $C_5H_4O_2$) by 12% hydrochloric

Fd. Chem. 0308-8146/81/0007-0139/$02·50

acid. The furfural is distilled at a controlled rate and determined colorimetrically with orcinol/iron reagent (Fraser *et al.*, 1956) or by gas–liquid chromatography (GLC). This, however, is a time-consuming method, so the direct colour reaction of Dische & Borenfreund (1957) and the modifications of Cracknell & Moye (1970) were investigated.

EXPERIMENTAL

Samples of commercial flour were sieved and separated into various fractions which were then examined for pentosan content using a modified Cracknell & Moye (1970) method. The results obtained were compared with those found using both the Tollens/orcinol iron method and the Tollens/GLC method.

Extracting solution

Acetic acid AR, glacial, 110 ml.
Hydrochloric acid AR, concentrated 2 ml.
Phloroglucinol, 20% w/v in ethanol 5 ml.
Glucose, 1·75% w/v aqueous 1 ml.

Method

Flour (*ca.* 4·5–5·5 mg) was weighed into a stoppered glass tube and 2 ml of water, followed by 10 ml of the freshly prepared extracting solution, were added. The tubes were placed in a vigorously boiling water bath for 25 min, cooled rapidly in flowing cold water and the absorbance at 552 nm and 510 nm measured. The tubes were shaken twice during the heating in the water bath.

The percentage of pentosans in the flour was calculated by subtraction of the reading at 510 nm from that at 552 nm and comparison of the results with a calibration curve.

A distilled water blank and standards of D-(+)-xylose made up in distilled water were placed in the water bath alongside the flour samples and their absorbances at 552 nm and 510 nm were recorded at the same time as those of the samples. Standards were always treated concurrently with the samples.

Standards and samples were always read as soon as possible after cooling as a gradual loss in colour was noted on standing. The colour of the solutions faded approximately 20% over a 60-min period.

Construction of calibration curve

D-(+)-xylose, 0·1 g, was taken and made up to 100 ml in distilled water in a volumetric flask (solution A).

Ten millilitres of solution A were diluted to 100 ml in a volumetric flask, giving 0·01 g D-(+)-xylose per 100 ml (solution B).

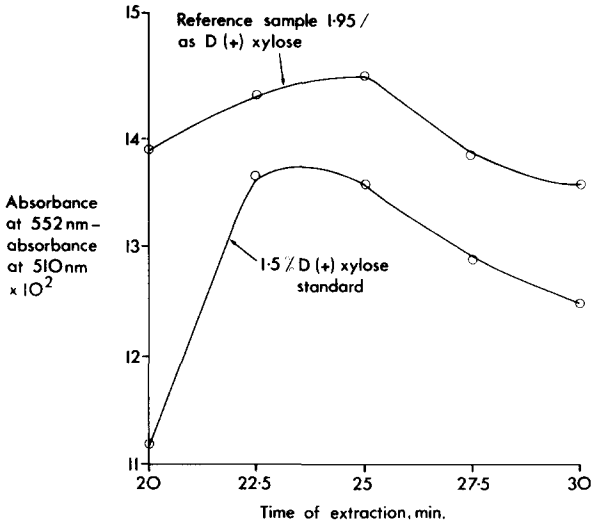


Fig. 1. Optimisation of reaction time.

Aliquots of 0·5, 1·0, 1·5 and 2·0 ml solution B were made up to 2·0 ml with distilled water and treated as described above. Reaction time was first optimised (Fig. 1).

RESULTS AND DISCUSSION

Reagents and blanks

Typical ultraviolet and visible spectra of samples and standards are shown in Figs 3, 4 and 5 along with a typical calibration (Fig. 2).

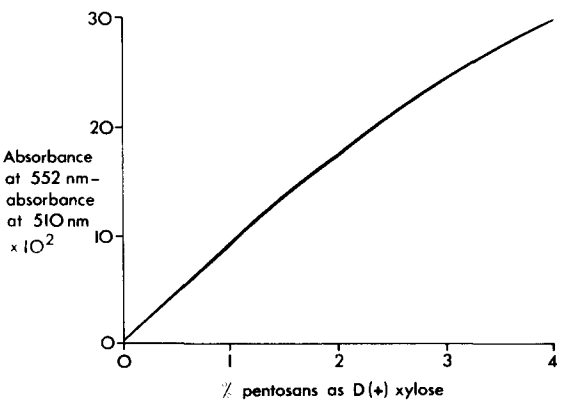


Fig. 2. Typical calibration curves.

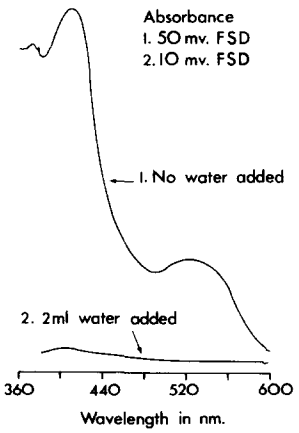


Fig. 3. Reagent blanks.

The importance of adding distilled water to the samples is apparent from the spectra in Fig. 3. The reagent blank without added water was red/orange in colour, whereas the reagent blank containing water was colourless.

The effect of this red/orange colour was to impart a negative value to the pentosan concentration of the sample because the absorbance at 510 nm was greater than that at 552 nm (Fig. 3). The minimum amount of water required to negate this effect was found to be 1·5 ml, but typically 2 ml of distilled water were added to each sample.

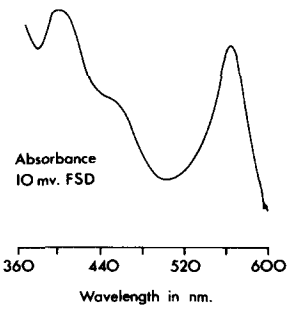


Fig. 4. Typical scan of a flour sample.

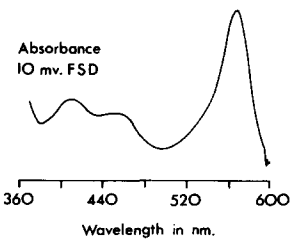


Fig. 5. Typical scan of a D-(+)-xylose standard.

Dische & Borenfreund (1957) reported that cholesterol grade acetic acid was required for the extracting solutions, but in the present study AR grade acetic acid was found to be adequate. The cholesterol grade acetic acid was used because of impurities in the normal AR acid causing a large peak at 440 nm. It may be seen from Fig. 3 that no such peak was found after adding distilled water to the reagent blank. However, the use of freshly opened acetic acid is advised, as nearly empty bottles of glacial acetic acid were found to impart a red colour to the extracting solution, even before its addition to the samples.

Reaction time

Twenty-five minutes was found to be the optimum reaction time in the water bath (Fig. 1). This is slightly longer than previously suggested (Dische & Borenfreund, 1957; Fraser *et al.*, 1956), probably due to the extra water added to the samples.

Precision of method

Duplicate results of six samples of flour analysed concurrently for pentosan content are given in Table 1.

Identical samples were also analysed in duplicate for pentosan content on consecutive days and the mean values of both days' results are given in Table 2.

The calibration curves constructed simultaneously with the samples on day 1 and day 2 were identical.

TABLE 1
TYPICAL RESULTS OF SAMPLE DUPLICATES PROCESSED CONCURRENTLY

Sample No.	% *Pentosan* (*as* D-(+)-*xylose*)	
1	1·85	1·85
2	1·95	1·65
3	2·25	2·20
4	1·80	1·85
5	1·90	1·90
6	1·55	1·65

TABLE 2
DUPLICATE RESULTS FOR MEAN OF SIX SAMPLES PROCESSED ON CONSECUTIVE DAYS

Sample No.	% *Pentosan* (*as* D-(+)-*xylose*)	
	Day 1	*Day 2*
1	1·85	1·93
2	1·80	1·95
3	2·23	2·15
4	1·83	1·90
5	1·90	1·83
6	1·60	1·63

TABLE 3
COMPARISON OF PROPOSED METHOD (B) WITH TOLLENS' METHOD (A)

Sample No.	% *Pentosan* (*as* D-(+)-*xylose*)	
	Method A	*Method B*
A	2·04, 2·14 mean = 2·09	1·74, 1·69 mean = 1·72
B	2·53, 2·56 mean = 2·55	1·95, 1·84 mean = 1·90
C	1·20	0·96
D	1·65	2·16
E	2·25	2·30
F	1·67	1·60
G	1·25	1·50

TABLE 4
COMPARISON OF PROPOSED METHOD (B) WITH DISTILLATION/GLC METHOD (C)

Sample No.	% *Pentosan* (*as* D-(+)-*xylose*)	
	Method C	*Method B*
X	1·26	1·40, 1·45, 1·25 mean = 1·37
Y	2·33	2·20, 2·10, 2·15 mean = 2·15

Comparison with Tollens' procedure

Seven flours were analysed for pentosan content using the controlled rate distillation/orcinol iron method (Method A) and the method described above (Method B). The comparison of the results is given in Table 3.

Pentosan levels were also determined in reference samples used for the distillation/GLC method (Method C). The comparison of results is shown in Table 4.

The 2·33% pentosan GLC standard was examined using the Tollens/orcinol iron method and found to give a result of 2·25% of pentosan.

The range of pentosan concentrations determined is not very wide (*ca.* 1·0%–3·0% as D-(+)-xylose). However, this is the range of pentosan concentrations found in cake and biscuit flours. A preliminary investigation of bran samples gave reproducible results in the expected range; for example, 5·88, 6·06, 5·85 and 6·03% pentosan for quadruplicate samples of wholemeal flour.

The taking of replicate representative samples of bran is not easy with a sample weight of 5 mg unless the sample is finely ball-milled. The sample weight may easily be doubled but a larger sample weight than 10 mg of high bran content products will produce too highly coloured solutions. Thus, a pre-digestion and dilution is needed, followed by the colour development with phloroglucinol as above.

Possible interfering substances in the sample

Starch, glucose and maltose were investigated as possible interfering substances. Weighed amounts were processed in the same way as the flour and the results are given in Table 5.

TABLE 5

Interfering substance	*Absorbance (552–510 nm)*	*% Pentosan equivalent as D-(+)-xylose for a 5 mg sample of interfering substance*
Starch	+1·1	+0·01
Glucose	−3·7	−0·15
Maltose	−1·2	−0·01

CONCLUSIONS

The proposed modified method provides a rapid and reproducible means for the determination of pentosans in wheat flour. The method has been shown to be in satisfactory (±0·1%) agreement with the traditional Tollens method at low levels (*ca.* 1·5%) of pentosan as D-(+)-xylose and below, but gives lower results than expected at higher levels (*ca.* 2%) of pentosan as D-(+)-xylose (see Fig. 6).

The results given in Tables 1 and 2 suggest an overall figure for the precision of the

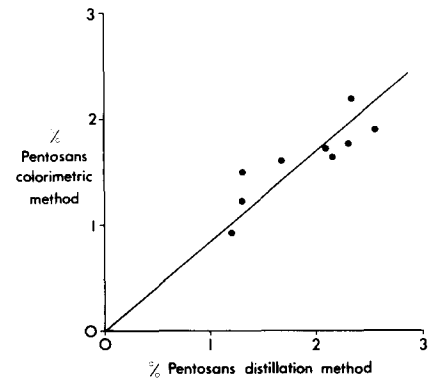


Fig. 6. Comparison of distillation versus colorimetric methods.

method of $\pm 0{\cdot}1\,\%$ pentosan at the $2\,\%$ pentosan level in wheat flour, which will probably be acceptable for most purposes.

Cracknell & Moye (1970) and Dische & Borenfreund (1957) give no indication of the precision of the direct colorimetric method, but results on flours and starches ranked these materials in the same order as the Tollens method; the method is also reported to be more accurate than the Tollens distillation method, one disadvantage of which is the instability of furfural. In the proposed method expensive grades of acetic acid are not needed in the reagent to obtain an acceptable blank and a completely colourless blank is obtained when water is added at the extraction stage.

The limitations of the method are the problem of sampling, discussed above, and, as with many colorimetric methods of analysis, colour fading. However, it was found here that the advantages of the method—rapidity, simplicity of operation and reproducibility—far outweighed the disadvantages.

ACKNOWLEDGEMENTS

The author would like to thank Dr D. Folkes of the Lord Rank Research Laboratories, High Wycombe, for supplying the reference samples for Method C, Dr B. G. Osborne for help and encouragement and Mr N. Gore for technical assistance.

REFERENCES

CRACKNELL, R. L. & MOYE, C. J. (1970). A colourimetric method for the determination of pentosans in cereal products. *Proc. 20th Ann. Conf. R.A.C.I. Canberra*, 17–20 August, 67.77.

DISCHE, Z. & BORENFREUND, E. (1957). A new colour reaction for the determination of aldopentose in presence of other saccharides. *Biochim. Biophys. Acta*, **23**, 639–42.

FRASER, J. R., BRANDEN-BRAVO, M. & HOLMES, D. C. (1956). The proximate analysis of wheat flour carbohydrates. *J. Sci. Fd Agric.*, **7**, 577–89.

SOUTHGATE, D. A. T. (1976). *Determination of food carbohydrates*, London, Applied Science Publishers Ltd.

Food Chemistry 7 (1981) 147–150

Short Communication

Peroxidase and Catalase Activity in Carrot

INTRODUCTION

Peroxidase and catalase, which are widely distributed in plants, are considered to have an empirical relationship to off-flavours in some vegetables (Svensson, 1977). A reduction of the activity of these enzymes in carrot, cauliflower and French beans by heat increased the storage stability of these vegetables (Baardseth, 1978).

Peroxidase appears to be the most heat stable enzyme in plants. For this reason peroxidase activity is widely used as an index of blanching. It has been generally accepted that if peroxidase is inactivated it is quite unlikely that other enzymes will be active. However, complete inactivation of peroxidase has been shown not to be necessary for quality preservation in frozen vegetables (Bøttcher, 1975*a*,*b*; Baardseth, 1978). It has been accepted as a general rule in the food industry that if there is activity of peroxidase, no catalase activity should be detected.

The aim of the present study was to determine the range of the specific activities of peroxidase and catalase in one variety of carrot grown at five different geographical locations and to test the correlations between peroxidase, catalase, protein and weight of the carrot.

MATERIALS AND METHODS

Materials

Carrots (*Daucus carota*, var. *Nantes Duke*) were grown at five different geographical locations and harvested in two or three replicates. They were harvested at the same stage of maturity but their size and weight differed.

Fd. Chem. 0308-8146/81/0007-0147/$02·50

Methods

Catalase, peroxidase, heat inactivation and pH optima were determined as previously described (Baardseth & Slinde, 1979). Protein was calculated on a fresh weight basis, as described by Lowry *et al.* (1951).

RESULTS AND DISCUSSION

The average activity of peroxidase (0·27 ΔA per min per milligramme of protein) and catalase (0·82 ΔA per min per milligramme of protein) (Table 1), the pH optima and the heat inactivation pattern were similar to those determined earlier (Baardseth & Slinde, 1979). From the standard deviation (Table 1) of the peroxidase ($\pm$0·10) and catalase ($\pm$0·32) activities it can be seen that the variations in specific activities are relatively large. The peroxidase and catalase activities were therefore measured in

TABLE 1

PEROXIDASE AND CATALASE ACTIVITIES (ΔA PER MIN PER MILLIGRAMME OF PROTEIN) IN CARROTS HARVESTED AT FIVE DIFFERENT LOCATIONS AND IN TWO OR THREE REPLICATES

	Peroxidase ΔA per min per milligramme of protein			*Catalase ΔA per min per milligramme of protein*		
	Replicate			*Replicate*		
Location	*a*	*b*	*c*	*a*	*b*	*c*
I	0·22	0·32	0·28	0·51	0·63	0·67
II	0·12	0·18		0·58	0·43	
III	0·17	0·37		0·86	1·01	
IV	0·18	0·48		1·59	1·19	
V	0·31	0·35	0·30	1·06	0·50	0·77
$\bar{x}$		0·27			0·82	
SD		0·10			0·32	
n		12			12	

SD = Standard deviation.

forty-two carrots with weights from 40 to 500 g. No correlation was found between weight and protein content, weight or protein and enzyme activity (Fig. 1) or between peroxidase and catalase activity. This is also in agreement with other experiments on the same carrot variety as used in this study, where no significant difference in peroxidase activity could be detected if they were harvested six weeks before normal harvesting or stored for 20 weeks at 0 °C and a relative humidity of 86 % (sampling every week). However, in these experiments there was a wide range in specific activities (Baardseth, 1976).

In conclusion, the food industry has to be aware of the great differences in the activity of peroxidase and catalase when measuring the residual activity. On the other hand, the heat treatment has to be sufficient so that other deteriorating

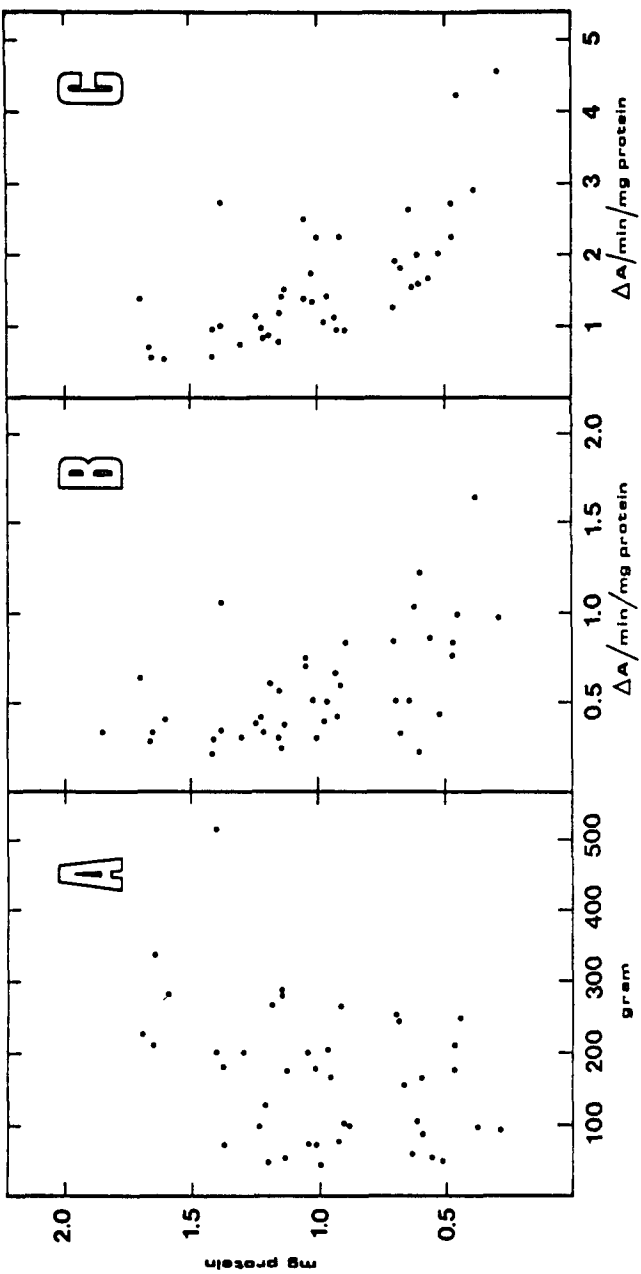


Fig. 1. Relationship between protein content and (A) weight of carrot ($r = 0{\cdot}37$), (B) peroxidase activity ($r = -0{\cdot}68$) and (C) catalase activity ($r = -0{\cdot}55$). The correlation coefficient between peroxidase and catalase was found to be $r = 0{\cdot}58$.

enzymes are inactivated. Since complete inactivation of peroxidase for quality preservation is not necessary (Baardseth, 1978; Bøttcher, 1975*a*,*b*) other degradative enzymes related to quality which are more heat sensitive should be sought.

REFERENCES

BAARDSETH, P. (1976). *Analysis of lipid and peroxidase activity in carrot during maturation and storage.* NINF Report No. 27, 1–7. (In Norwegian.)

BAARDSETH, P. (1978). Quality changes of frozen vegetables. *Food Chem.*, **3**, 271–82.

BAARDSETH, P. & SLINDE, E. (1980). Heat inactivation and pH optima of peroxidase and catalase in carrot, swede and Brussels sprouts. *Food Chem.*, **5**, 169–74.

BØTTCHER, H. (1975*a*). The enzyme content and the quality of frozen vegetables. I. Residual peroxidase activity. *Nahrung*, **19**, 173–9.

BØTTCHER, H. (1975*b*). The enzyme content and the quality of frozen vegetables. II. Effects on the quality of frozen vegetables. *Nahrung*, **19**, 245–53.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–73.

SVENSSON, S. G. (1977). Inactivation of enzymes during thermal processing. In: *Physical, chemical and biological changes in food caused by thermal processing.* (Høyem, T. & Kvåle, O. (Eds)), Applied Science Publishers Ltd, London. Chapter 12, 202–17.

(Received: 26 November, 1979)

PERNILLE BAARDSETH & ERIK SLINDE,
Norwegian Food Research Institute,
PO Box 50, N-1432,
Aas-NLH, Norway

Food Chemistry 7 (1981) 151–154

Short Communication

The Fatty Acid Composition of Some Tubers Grown in Nigeria

The fat content of some common tubers grown in Nigeria was determined and the distribution of fatty acids in them was measured by gas–liquid chromatography (GLC). The fat content was low (< 1·2%) in all species but the peel contained more fat than the pulp. Oleic acid (35–68%) and palmitic acid (21–39%) were the principal fatty acids, but arichidic acid was present in small amounts (< 3%) in all samples. The other fatty acids were present only in minor quantities, except for the higher levels in the peel.

INTRODUCTION

Tubers form a very important group of foodstuffs in Nigeria. Yams are very popular in the south. They are boiled and either eaten with stew or mixed with palm oil and made into a pottage. The most popular of the yam dishes, however, is pounded yam. In some areas, only boiled yam is pounded whereas, in others, yam is pounded with boiled cocoyam to improve the texture of the final product. Both sweet and Irish potatoes are common in northern Nigeria. They are either boiled or fried and eaten with stew. The sweet potato is sometimes eaten raw without peeling. Carrots are also largely grown in the north but are widely used as a dessert, either boiled or raw. Cassava is generally made into 'Gari' which is popular throughout the country. During preparation the tubers are peeled and the peel is fed to animals in most rural areas.

Tubers contain a high proportion of starch (Oyenuga, 1968) and only contribute a small amount of fat. However, their fatty acid composition is not published. Therefore, the fat content and fatty acid composition were determined for the following tubers with and without the peel: water yam (*Dioscorea alata*), white yam (*D. rotunda*), yellow yam (*D. esculenta*), sweet and Irish potatoes (*Ipomoea batatas*), cassava (*Manihot utilissima*), cocoyam (*Xanthosoma saqittifolium* Schitt) and carrot (*D. carrota*).

Fd. Chem. 0308-8146/81/0007-0151/$02·50

EXPERIMENTAL

Extraction of fats

The tubers were peeled, grated and dried in an oven between 100 °C and 150 °C. The tissue was then ground and a known weight of the powder was extracted using the Soxhlet method (Pearson, 1976) with diethyl ether as the solvent.

Hydrolysis of the fat and esterification of the fatty acids

The rapid method for the preparation of fatty acid methyl esters for gas chromatographic analysis described by Metcalfe & Schmidt (1960) was employed.

Gas–liquid chromatographic analysis of the fatty acid esters

A Packard 419 (FID) chromatograph was employed, using a column containing 5% SE-30 on Chromosorb GAW, DMSC, 80/100 mesh. The temperature was programmed between 150 °C and 275 °C. Rate of temperature rise was 1 °C/min. Identification of the peaks was carried out using internal standards and the proportions calculated from ratios of the weights of peaks cut from photocopies of chromatograms.

DISCUSSION

The results presented in Table 1 show that the tubers contained very little fat but that there was a wide distribution of fatty acids in the fats. It was found that the peel contained more fat than the pulp. This was very pronounced in sweet potato where the percentage of fat in the peel was 4·22 as compared with 0·47 in the pulp. It was also found that boiling removed some of the fat from yams.

Palmitic acid (C_{16}:0) was the most abundant of the saturated acids and was present in all the samples, with proportions ranging from 21·6% in the sweet potato peel to 39·4% in yellow yam. All the other saturated acids present were so only in small proportions. The sweet potato peel, apart from having a higher proportion of fat, also had higher proportions of both lauric and capric acids than the pulp. Only the potato species (sweet and Irish) contained stearic acid, the peel having a greater proportion than the pulp. Behenic acid (C_{22}:0) was found only in the peel. Oleic acid (C_{18}:1) was the most abundant unsaturated acid and accounted for between 49·1% and 68·3% of the total fatty acids component of the various tubers examined. It was found that the tubers provided very little of the essential fatty acids. When present they formed the minor components. All three yam species examined contained between 2% and 2·9% palmitoleic acid, which was completely absent in all other tubers.

TABLE 1

PERCENTAGE FATTY ACID COMPOSITION OF OILS OF SOME NIGERIAN TUBERS

Sample	*Per cent fat*	*Saturated*							*Unsaturated*				
		$C_{10}:0$	$C_{12}:0$	$C_{14}:0$	$C_{16}:0$	$C_{18}:0$	$C_{20}:0$	$C_{22}:0$	$C_{16}:1$	$C_{18}:1$	$C_{18}:2$	$C_{18}:3$	$C_{20}:4$
Cassava	1·21	—	—	—	34·8	—	2·4	—	—	62·7	—	—	—
Cocoyam (red)	0·49	—	—	0·7	29·2	—	0·7	—	1·4	67·8	0·3	—	—
Cocoyam (white)	0·39	—	—	—	33·2	—	1·5	—	—	65·3	—	—	—
Cocoyam (peel)	1·14	—	—	—	34·5	—	7·8	2·7	—	55·1	—	—	—
Yam (white)	0·19	—	—	0·2	36·1	—	1·5	—	2·0	57·8	1·7	0·7	—
Yam (boiled)	0·16	—	—	—	37·7	—	1·1	—	2·9	57·1	0·9	0·3	—
Yam (yellow)	0·18	—	1·6	1·6	39·4	—	2·4	—	2·4	49·1	0·5	0·5	2·6
Water yam	0·36	—	—	—	35·0	—	3·0	—	2·9	58·9	0·3	0·3	—
Potato (sweet)	0·47	0·9	5·4	—	32·3	6·5	2·2	—	—	52·0	0·2	0·7	—
Potato (sweet) (peel)	4·22	3·5	14·1	2·5	21·6	13·2	6·0	3·5	—	35·1	—	0·6	—
Potato (Irish)	0·14	—	—	1·4	27·9	8·8	3·0	—	—	55·1	—	1·9	1·9
Carrot	1·4	—	1·8	—	24·6	—	2·0	—	1·5	68·3	—	1·8	—

REFERENCES

GANONG, W. F. (1975). *Review of medical physiology*, San Francisco, California, Lange Medical Publications, 223–9.

METCALFE, L. D. & SCHMIDT, A. A. (1960). The rapid preparation of fatty acid esters for gas chromatographic analysis, *Anal. Chem.*, **33**, 363–4.

OYENUGA, V. A. (1968). *Nigeria's foods and feeding-stuffs*, Ibadan Univ. Fac. of Agric. Techn. Bull. No. 1 (3rd ed.), 27.

PEARSON, D. (1976). *The chemical analysis of foods*, New York, Churchill Livingstone, 14.

O. O. P. FABOYA,
Chemistry Department,
University of Ibadan,
Ibadan,
Nigeria.

(Received: 13 December, 1979)

Food Chemistry 7 (1981) 155–158

BOOK REVIEWS

Qualitative Analysis of Flavor and Fragrance Volatiles by Glass Capillary Gas Chromatography. By Walter Jennings and Takayuki Shibamoto, Academic Press, New York. 1980. vii + 472 pp. Price: $39·00.

This book consists of a 26-page review of analytical considerations and four Tables.

The review is concise and instructive. It covers most aspects: GC systems, retention indices, selective detectors, ancillary reactions, reaction gas chromatography, GC–MS, and even a little on HPLC–GC. There are 102 references. The first Table lists the compounds alphabetically and gives their retention indices on WCOT columns, one (0·28 mm × 50 m) coated with methyl silicone OV-101, admixed with 1% Carbowax 20M to reduce tailing, the other (0·2 mm × 80 m) coated with Carbowax 20M. Two further Tables list the same data, but in increasing order of retention indices, first on OV-101, then on 20M. The last Table comprises the normalised mass spectra determined with an Hitachi RMU-6M and a Datalizer 002B, the more prominent lines being marked with their m/e values. Name, formula, molecular formula and weight, and retention indices are also given.

The retention indices of 1262 compounds are included, with mass spectra of 701.

All those interested in the identification of flavorants and perfumes will wish to possess a copy of this book, reference to which will undoubtedly prove valuable from time to time, but it covers less than half the known flavorants and the data need to be cast in computerised form to yield their full benefits.

HARRY E. NURSTEN

Developments in Food Packaging—1. Edited by S. J. Palling. Applied Science Publishers Ltd, London. 1980. xv + 192 pp. Price: £18·00.

This book comprises seven chapters. Five deal with developments in packaging materials and containers. The remaining two are concerned with developments in food technology and international legislation relevant to the packaging of foods.

Food Chemistry 7 (1981) 155–158

BOOK REVIEWS

Qualitative Analysis of Flavor and Fragrance Volatiles by Glass Capillary Gas Chromatography. By Walter Jennings and Takayuki Shibamoto, Academic Press, New York. 1980. vii + 472 pp. Price: $39·00.

This book consists of a 26-page review of analytical considerations and four Tables.

The review is concise and instructive. It covers most aspects: GC systems, retention indices, selective detectors, ancillary reactions, reaction gas chromatography, GC–MS, and even a little on HPLC–GC. There are 102 references. The first Table lists the compounds alphabetically and gives their retention indices on WCOT columns, one (0·28 mm × 50 m) coated with methyl silicone OV-101, admixed with 1% Carbowax 20M to reduce tailing, the other (0·2 mm × 80 m) coated with Carbowax 20M. Two further Tables list the same data, but in increasing order of retention indices, first on OV-101, then on 20M. The last Table comprises the normalised mass spectra determined with an Hitachi RMU-6M and a Datalizer 002B, the more prominent lines being marked with their m/e values. Name, formula, molecular formula and weight, and retention indices are also given.

The retention indices of 1262 compounds are included, with mass spectra of 701.

All those interested in the identification of flavorants and perfumes will wish to possess a copy of this book, reference to which will undoubtedly prove valuable from time to time, but it covers less than half the known flavorants and the data need to be cast in computerised form to yield their full benefits.

HARRY E. NURSTEN

Developments in Food Packaging—1. Edited by S. J. Palling. Applied Science Publishers Ltd, London. 1980. xv + 192 pp. Price: £18·00.

This book comprises seven chapters. Five deal with developments in packaging materials and containers. The remaining two are concerned with developments in food technology and international legislation relevant to the packaging of foods.

J. D. Martin is the author of the chapter entitled 'Metal Containers and Closures'. He outlines developments in tinplate, tin-free steel and aluminium and in three- and two-piece containers made therefrom. He also covers closures for metal and glass containers. J. H. Briston writes about 'Rigid Plastics Packaging'. He discusses the various techniques used to produce rigid plastic containers and the characteristics of the many plastic materials available. The uses for such containers in the food industry are also indicated. 'Flexible Plastics Packaging' is the title of the chapter contributed by R. R. Goddard. This covers briefly the flexible materials available, methods of production and fabrication and developments in packaging machinery. Packaging requirements for various groups of foods are outlined and current methods and materials used are mentioned. D. G. Osbourne contributes a chapter on 'Glass' in which he outlines the properties of this material, methods of manufacturing glass containers and applications for such containers in the food and beverage industries.

'Paper and Board Developments' are discussed by W. A. Jones. The applications for such materials are outlined, and commercial and environmental issues discussed. M. C. Philip writes on 'Food Technology to the Year 2000'. He discusses likely developments in the production, harvesting or slaughter and processing of meat and poultry, fish, cereals, fruit and vegetables and the search for new sources of protein. Under the heading of 'processing' he deals with energy requirements, aseptic packaging, irradiation and applications for micro-processors. The final chapter, by M. A. Andrews, is concerned with 'International Legislation'. Legislation relating to packaging in the UK, the EEC and the USA is summarised and that relating to metals and plastic monomers is covered in some detail. Environmental issues associated with the packaging industry are discussed, as are the problems associated with the assessment of the safety of materials used for food packaging.

This work fills a long-standing gap in the literature available to technical personnel working in the food industry and students of food science and technology at all levels. There are some excellent books available on packaging in general, but no recent UK publication dealing specifically with foods. It may be less useful to those working in the packaging industry as it assumes a knowledge of food processing and preservation, rather than including a chapter to cover these subjects.

J. G. BRENNAN

Fats and Oils: Chemistry and Technology. Edited by R. J. Hamilton and A. Bhati. Applied Science Publishers Ltd, London. 255 pp. Price: £24·00.

This book is based on the symposium on 'Recent Advances in the Chemistry and Technology of Fats and Oils', held in December, 1979, and comprises 11 chapters by various authors on different aspects of fats and oils.

J. D. Martin is the author of the chapter entitled 'Metal Containers and Closures'. He outlines developments in tinplate, tin-free steel and aluminium and in three- and two-piece containers made therefrom. He also covers closures for metal and glass containers. J. H. Briston writes about 'Rigid Plastics Packaging'. He discusses the various techniques used to produce rigid plastic containers and the characteristics of the many plastic materials available. The uses for such containers in the food industry are also indicated. 'Flexible Plastics Packaging' is the title of the chapter contributed by R. R. Goddard. This covers briefly the flexible materials available, methods of production and fabrication and developments in packaging machinery. Packaging requirements for various groups of foods are outlined and current methods and materials used are mentioned. D. G. Osbourne contributes a chapter on 'Glass' in which he outlines the properties of this material, methods of manufacturing glass containers and applications for such containers in the food and beverage industries.

'Paper and Board Developments' are discussed by W. A. Jones. The applications for such materials are outlined, and commercial and environmental issues discussed. M. C. Philip writes on 'Food Technology to the Year 2000'. He discusses likely developments in the production, harvesting or slaughter and processing of meat and poultry, fish, cereals, fruit and vegetables and the search for new sources of protein. Under the heading of 'processing' he deals with energy requirements, aseptic packaging, irradiation and applications for micro-processors. The final chapter, by M. A. Andrews, is concerned with 'International Legislation'. Legislation relating to packaging in the UK, the EEC and the USA is summarised and that relating to metals and plastic monomers is covered in some detail. Environmental issues associated with the packaging industry are discussed, as are the problems associated with the assessment of the safety of materials used for food packaging.

This work fills a long-standing gap in the literature available to technical personnel working in the food industry and students of food science and technology at all levels. There are some excellent books available on packaging in general, but no recent UK publication dealing specifically with foods. It may be less useful to those working in the packaging industry as it assumes a knowledge of food processing and preservation, rather than including a chapter to cover these subjects.

J. G. BRENNAN

Fats and Oils: Chemistry and Technology. Edited by R. J. Hamilton and A. Bhati. Applied Science Publishers Ltd, London. 255 pp. Price: £24·00.

This book is based on the symposium on 'Recent Advances in the Chemistry and Technology of Fats and Oils', held in December, 1979, and comprises 11 chapters by various authors on different aspects of fats and oils.

Chapters 1 and 2 deal with two main analytical techniques. The former details the various chromatographic procedures (GLC, TLC, HPLC) involved in the analysis of fats and oils, whilst the latter provides a very useful and easy to understand description of the theory and use of NMR in fat determination. Both these chapters would be of interest to workers in chemical or food laboratories.

Chapters 3, 4 and 5 describe the chemistry of oxygenated sugars, glycerides and prostaglandins, respectively and are important not only for their scientific content, but also to make the reader realise that fats and oils do include more than just the simple glycerides.

Chapters 6 and 7 deal with extraction and processing techniques and provide up to date descriptions of these processes—again very useful reading. Following on from this, Chapter 8 describes the use of fats and oils in the confectionery industry and Chapter 10 the problems which can arise with fats in the food industry. These include adulteration and rancidity.

An unusual Chapter 9 makes fascinating reading and describes how fats and oils may be chemically modified during their production in animals by changing the feed. This contrasts with the more usual modifications after extraction. The final chapter deals with the importance of the palm oil industry.

In common with many books of this type the reader can always see other topics which should have been included. However, there is enough information in the book to make it well worth purchasing by anyone working with fats and oils or by libraries in universities and in the food and related industries. At £24·00 the book is rather expensive, however. At the end of each chapter useful references are provided to enable further details of particular aspects to be obtained.

MALCOLM W. KEARSLEY

Developments in Meat Science. Vol. 1. Edited by Ralston Lawrie, Applied Science Publishers Ltd, London. 1980. 254 pp. 37 Figures. 26 Tables. Price: £17·00.

This book represents the first volume discussing pertinent developments in meat science. It is divided into nine different chapters authored by experts in the field. Chapter 1 is written by Dr D. B. Crighton of the University of Nottingham and reviews the hormonal controls involving reproduction and growth in meat animals. Of particular interest is his discussion of hormonal effects upon growth and carcass quality, especially the section dealing with natural gonadotrophins and the possibility of producing immunisation against specific steroids in order to achieve non-surgical castration. Chapter 2, by Dr J. R. Bendall of the UK Meat Research Institute, discusses electrical stimulation of intact hide-on carcasses, dressed carcasses and sides. He not only discusses such important points as voltage, pulse rate and duration of stimulation, but points out the positive aspects of the practice

Chapters 1 and 2 deal with two main analytical techniques. The former details the various chromatographic procedures (GLC, TLC, HPLC) involved in the analysis of fats and oils, whilst the latter provides a very useful and easy to understand description of the theory and use of NMR in fat determination. Both these chapters would be of interest to workers in chemical or food laboratories.

Chapters 3, 4 and 5 describe the chemistry of oxygenated sugars, glycerides and prostaglandins, respectively and are important not only for their scientific content, but also to make the reader realise that fats and oils do include more than just the simple glycerides.

Chapters 6 and 7 deal with extraction and processing techniques and provide up to date descriptions of these processes—again very useful reading. Following on from this, Chapter 8 describes the use of fats and oils in the confectionery industry and Chapter 10 the problems which can arise with fats in the food industry. These include adulteration and rancidity.

An unusual Chapter 9 makes fascinating reading and describes how fats and oils may be chemically modified during their production in animals by changing the feed. This contrasts with the more usual modifications after extraction. The final chapter deals with the importance of the palm oil industry.

In common with many books of this type the reader can always see other topics which should have been included. However, there is enough information in the book to make it well worth purchasing by anyone working with fats and oils or by libraries in universities and in the food and related industries. At £24·00 the book is rather expensive, however. At the end of each chapter useful references are provided to enable further details of particular aspects to be obtained.

MALCOLM W. KEARSLEY

Developments in Meat Science. Vol. 1. Edited by Ralston Lawrie, Applied Science Publishers Ltd, London. 1980. 254 pp. 37 Figures. 26 Tables. Price: £17·00.

This book represents the first volume discussing pertinent developments in meat science. It is divided into nine different chapters authored by experts in the field. Chapter 1 is written by Dr D. B. Crighton of the University of Nottingham and reviews the hormonal controls involving reproduction and growth in meat animals. Of particular interest is his discussion of hormonal effects upon growth and carcass quality, especially the section dealing with natural gonadotrophins and the possibility of producing immunisation against specific steroids in order to achieve non-surgical castration. Chapter 2, by Dr J. R. Bendall of the UK Meat Research Institute, discusses electrical stimulation of intact hide-on carcasses, dressed carcasses and sides. He not only discusses such important points as voltage, pulse rate and duration of stimulation, but points out the positive aspects of the practice

including reduced shrinkage, improved colour, prevention of cold-induced toughness and its usefulness in speeding up throughput. Chapter 3, written by A. Cuthbertson of the Meat and Livestock Commission of the UK, discusses the rationale and economic advantages for hot processing of meat. He concludes that hot processing may increase total value by some 18%. Chapter 4, by Dr O. G. Gadian of the University of Oxford, reviews the potential for using nuclear magnetic resonance to study the various chemical and physical properties of meat.

Chapter 5 is written by Dr I. F. Penny of the Meat Research Institute of the UK and discusses the conditioning of meat from the standpoint of improvement during ageing. Dr Penny discusses both the structural and the enzymic changes that occur and explains them in the light of present knowledge of the myofibrillar proteins and the action of indigenous muscle proteases. Chapter 6 covers the important topic of reclamation and upgrading of abattoir waste protein into human food and is written by Dr R. H. Young of the Tropical Products Institute, London. Chapter 7 is written by Dr R. H. Locker of the Meat Industry Research Institute of New Zealand and covers the development of several cured products produced from lamb. Chapter 8 covers the problem of volatile nitrosamine formation and possible means of reducing its level in meat products. It is written by Dr C. L. Walters of the British Food Manufacturing Industries Research Association. Chapter 9, by Anne L. Ford and Dr Robert J. Park of the CSIRO Meat Research Laboratory in Australia, reviews the area of odours and flavours in meat. The authors point out that, contrary to the beliefs of many in the meat industry, breed, sex and age appear to have little influence upon meat flavour, although diet has been shown to play an important role.

The depth of discussion and the practical approach of the authors, coupled with an extensive literature review, should make this book a valuable reference for both the practical worker in the industry and for the meat scientist.

A. M. PEARSON

Food Chemistry 7 (1981) 159–168

CHEMICAL CHANGES IN CASEIN HEATED WITH AND WITHOUT D-GLUCOSE IN THE POWDERED STATE OR IN AN AQUEOUS SOLUTION

HIROMICHI KATO, MITSUO MATSUMURA* & FUMITAKA HAYASE

Department of Agricultural Chemistry, The University of Tokyo, Bunkyo-ku, Tokyo, Japan

(Received: 31 January, 1980)

ABSTRACT

Chemical changes in casein, heated either in the presence or the absence of D-*glucose at 50 or 75°C in a powdered state at 75% relative humidity (RH), or in an aqueous solution, were investigated. During heating, colour development, the formation of polymerised products and severe decomposition of amino acid residues occurred. In the powder reaction system, mainly basic amino acids decomposed whereas, in the solution system, all amino acids were uniformly decomposed. In addition, small amounts of free amino acids and low molecular weight peptides (below MW 500) were detected, these amounts being greater in the solution system, suggesting that non-enzymic cleavage of peptide linkage occurred. These changes were observed both in the absence and in the presence of glucose but glucose promoted such changes.*

INTRODUCTION

Protein in foods suffers complicated chemical changes during storage, processing and cooking, especially through interaction with reducing sugars or Maillard reactions. These changes are involved in cross-link formation and the decomposition of amino acid residues and have been widely investigated from both the nutritional loss and food safety viewpoints (Lea & Hannan, 1950; Tanaka *et al.*, 1975; Erbersdobler *et al.*, 1976; Hurrell & Carpenter, 1976, 1977; Mori & Nakatsuji, 1977; Bender, 1978).

* Present address: Nippon Roche Co. Ltd, Chiyoda-ku, Tokyo, Japan.

Food Chemistry 0308-8146/81/0007-0159/$02·75

In previous papers, the authors reported on the chemical changes in casein heated above 120 °C, with emphasis on the racemisation of amino acid residues (Hayase *et al.*, 1975, 1979). In the study described in this paper casein was heated either in the presence or the absence of D-glucose at a lower temperature (50 or 75 °C) in the powdered state (75 % RH), or in aqueous solution, and the decomposition pattern of amino acid residues, in addition to the formation of free amino acids and low molecular weight peptides in the heated casein, was investigated.

MATERIALS AND METHODS

Preparation of reaction systems

Hammarsten casein (Merck) was used as a model protein. Casein was dissolved at 50 °C in water adjusted to pH 7·0 with 0·1N NaOH. In order to inactivate any residual protease activity in the protein preparation, the casein solution was heated at 80 °C for 20 min, precipitated at pH 4·7 and the precipitates washed with water. Thus, treated casein was redissolved in water neutralised by the addition of 0·1N NaOH, then freeze dried and kept in a freezer at −20 °C until used. D-Glucose (Kanto Kagaku Co. Ltd, Tokyo) was used without further purification. A casein–glucose mixture was prepared by freeze drying a 2 % solution of casein containing the amount of D-glucose equivalent to the free amino acid groups in the casein (casein: glucose, 88·5: 11·5 w/w). In the powder system experiments, casein alone and casein–glucose mixture were stored at 50 or 75 °C and at 75 % RH for various periods. In the solution system experiments, however, aqueous solutions of 8 % casein alone and 8 % casein plus glucose, in the same ratio as described above, were heated at 75 °C for various periods.

Determination of colour development

The heated casein and casein–glucose mixtures were suspended at pH 8·0 and Pronase, a complex enzyme preparation from *Streptomyces griseus* (Kaken Kagaku Co. Ltd, Tokyo), was added to digest the insoluble substances. Next, the absorbance at 420 nm was measured, according to the method of Clark & Tannenbaum (1970).

Polyacrylamide gel electrophoresis

Disc SDS-polyacrylamide gel electrophoresis was performed at pH 7·0 using the method of Weber & Osborn (1969). Electrophoresis was carried out in the presence of 0·1 % SDS at 8 MA per gel for 3·5 h, 10 % acrylamide gel being used. A sample was diluted to 0·1 % casein and applied to each gel.

Preparation of low molecular weight peptide fraction

Heated casein or a casein–glucose mixture (3 g) was added to 100 ml of water and stirred for 2 h. The treatment was repeated, one more portion of water (100 ml) being

added, then the solution was filtered. Trichloroacetic acid (TCA), 30 % w/v solution, was added to the filtrate up to 10 % of the TCA concentration. The TCA-soluble fraction was extracted with diethyl ether and the aqueous layer was neutralised. Half of the resulting solution was evaporated and the free amino acids were analysed. The remaining solution was filtered with an Amicon TCF 10 membrane to obtain the fraction below 500 MW. This membrane-filtrate was designated a low molecular weight peptide fraction.

Amino acid analysis

Determination of free amino acids and amino acid composition in heated casein and the low molecular weight peptide fraction was carried out by means of an Hitachi Amino Acid Analyser KLA-5. Hydrolysis was carried out by heating at 110 °C for 20 h in 6N HCl.

RESULTS AND DISCUSSION

Colour development

Colour development in casein during heating at 50 or 75 °C, with and without glucose, in the powder or solution systems, is shown in Table 1. In the presence of glucose, casein was very brown, especially in the powder reaction system at RH 75 %, although some colour development was also observed in the case of casein alone. Labuza *et al.* (1976) showed that a casein–glucose model system stored at 35 °C has a browning maximum at 0·82 water activity (a_w). Generally, maximum browning is observed between 0·3 and 0·7 a_w, depending on the type of food (Eichner, 1975). Accordingly, it is reasonable to assume that browning of casein–glucose in a powder

TABLE 1
COLOUR DEVELOPMENT IN CASEIN HEATED WITH AND WITHOUT GLUCOSE IN THE POWDERED STATE OR IN AN AQUEOUS SOLUTION

Sample	*Reaction system*	*Heat treatment* (°C)	*Days*	*Absorbance at 420 nm (1 g casein/100 ml)*
Casein	Powder	50	7	0·030
			21	0·035
		75	7	0·040
			21	0·052
	Solution	75	7	0·067
			21	0·090
Casein–glucose	Powder	50	7	0·260
			21	0·295
		75	7	2·205
			21	2·310
	Solution	75	7	0·200
			21	0·730

system proceeds much faster than that in a solution system. However, Table 1 shows that, in the absence of glucose, an 8% casein solution browned faster than casein powder.

Disc SDS-polyacrylamide gel electrophoretic pattern

Figures 1 and 2 show the disc polyacrylamide gel electrophoretic patterns of casein heated with and without glucose in the powder and solution systems, respectively. In every case, a high molecular weight polymerised protein band

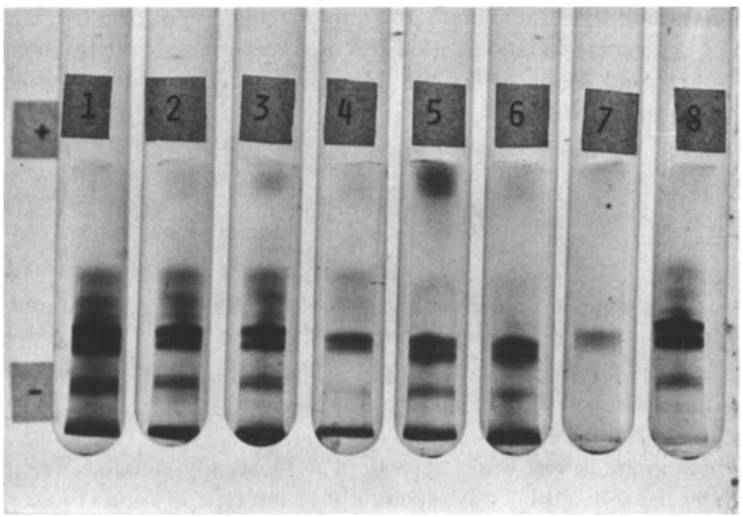


Fig. 1. Disc SDS-polyacrylamide gel electrophoresis of heated casein and casein–glucose mixture in the powder system. 1. Casein at 50 °C for 7 days. 2. Casein at 50 °C for 21 days. 3. Casein at 75 °C for 1 day. 4. Casein at 75 °C for 3 days. 5. Casein–glucose at 50 °C for 3 days. 6. Casein–glucose at 75 °C for 3 h. 7. Casein–glucose at 75 °C for 6 h. 8. Unheated casein (control).

appeared at the starting point of the gel. Mohammad *et al.* (1949) reported that the molecular weight increased between two and three times when ovalbumin was heated at 53 °C in the presence of glucose in aqueous solution. In the Maillard reaction, the ε-amino group of the lysine residue in the protein is known to react with glucose to form cross-linking (Hurrell & Carpenter, 1977). In the present experiment, since casein alone also polymerised during heating, as shown in Figs 1 and 2, the cross-link formation between the ε-amino group of the lysine residue and the carboxyl or amide group (Hurrell & Carpenter, 1976) and the involvement of a phosphoric acid residue in casein would be possible, in addition to Maillard-type cross-linking.

On heating the casein alone in the solution system at 75 °C for 7 days, two bands, different from the two main bands of native casein, were observed (Fig. 2).

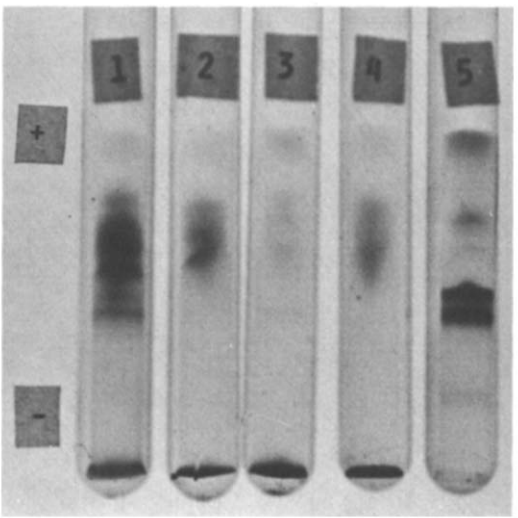


Fig. 2. Disc SDS-polyacrylamide gel electrophoresis of heated casein and casein–glucose mixture in the solution system. 1. Casein at 75 °C for 7 days. 2. Casein at 75 °C for 21 days. 3. Casein–glucose at 75 °C for 7 days. 4. Casein–glucose at 75 °C for 21 days. 5. Unheated casein (control).

Decomposition pattern of amino acid residues

Figure 3 shows the changes in the amino acid composition of casein during heating with and without glucose in the powder system. Basic amino acids were extensively decomposed even in the case of casein alone. The degree of decomposition was 26–40 % after 21 days at 50 °C in casein, reaching 46–76 % after 3 days at 50 °C in the casein–glucose mixture. Of three basic amino acids studied, lysine was most rapidly decomposed. Tanaka *et al.* (1975) also reported that binding of glucose to egg albumin occurred mainly in the early stages of browning at 35 °C and at 68 % RH.

Figure 4 shows changes in the amino acid composition of casein during heating with and without glucose in the solution system. In this case, all amino acid residues were uniformly decomposed, the presence of glucose promoting such decomposition. Compared with the powder reaction system, the decomposition of basic amino acids was suppressed but that of neutral and acidic amino acids was promoted in the solution system. In the casein–glucose mixture, the degree of decomposition of all amino acids exceeded 30 % after 21 days at 75 °C.

From the above results, a difference in the decomposition pattern of amino acids between the powder and solution systems is noticeable. In the solution system, the Maillard reaction would be suppressed because of its higher water activity (Eichner, 1975), whereas other ionic reactions seem to be promoted.

Formation of free amino acids and low molecular weight peptides

Although no free amino acids were detected in the TCA-soluble fraction of the

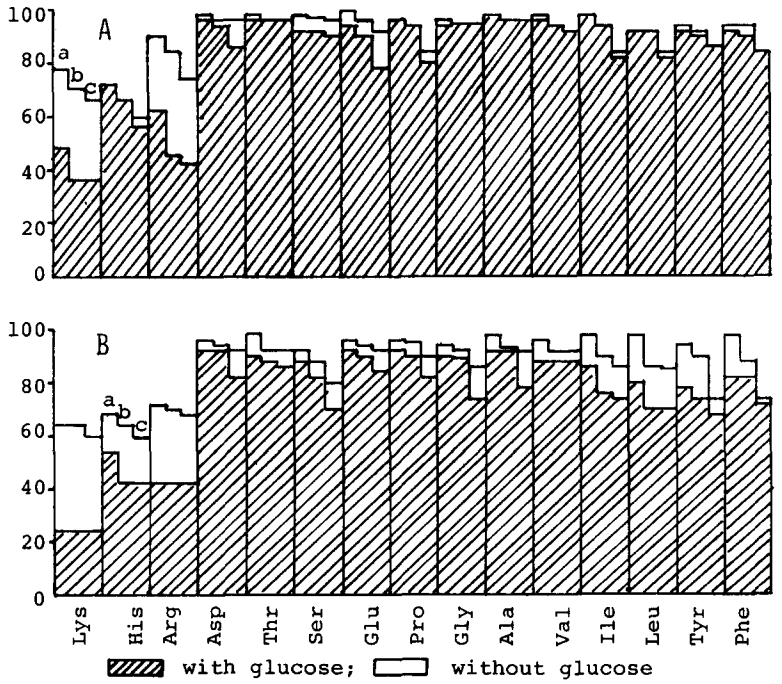


Fig. 3. Changes in amino acid composition of casein on heating with and without glucose for (a) 3 days, (b) 7 days and (c) 21 days in the powder system. A: Heated at 50 °C. B: Heated at 75 °C. The amount of each amino acid in casein before heating is represented as 100. Met, Cys and Trp were not determined.

Fig. 4. Changes in amino acid composition of casein on heating with and without glucose at 75 °C for (a) 7 days and (b) 21 days in the solution system. The amount of each amino acid in casein before heating is represented as 100.

TABLE 2

FREE AMINO ACIDS[a] OCCURRING IN CASEIN HEATED WITH AND WITHOUT GLUCOSE IN THE POWDER REACTION SYSTEM

	Without glucose				*With glucose*			
	50°C		*75°C*		*50°C*		*75°C*	
	7 Days	*21 Days*	*7 Days*	*21 Days*	*7 Days*	*21 Days*	*7 Days*	*21 Days*
Asp	0·02	0·05	0·36	0·06	0·02	0·09	0·19	1·08
Thr	—	0·04	—	0·02	—	0·08	—	0·15
Ser	0·08	0·25	1·15	0·22	0·08	0·25	0·28	1·53
Glu	—	—	0·07	—	—	—	0·19	0·19
Gly	0·16	0·18	0·97	0·43	0·18	0·34	0·64	0·24
Ala	0·06	0·07	0·54	0·27	0·09	0·15	0·88	1·12

[a] 10^{-7} mole per gramme of casein.
—, not detected.
Lys, His, Arg, Pro, Val, Ile, Leu, Tyr and Phe were not detected.

native casein before heating, small amounts of free amino acids were detected after heating. Table 2 shows the formation of several free amino acids on heating casein with and without glucose in the powder system. The free amino acids detected were aspartic acid, threonine, serine, glutamic acid, glycine and alanine; no other amino acids were liberated. After 21 days at 75°C in the casein–glucose system, the total amount of free amino acids was no more than 4 mg% of the weight of the original

TABLE 3

FREE AMINO ACIDS[a] OCCURRING IN CASEIN HEATED WITH AND WITHOUT GLUCOSE IN THE SOLUTION SYSTEM

	Without glucose 75°C		*With glucose 75°C*	
	7 Days	*21 Days*	*7 Days*	*21 Days*
Lys	0·08	0·56	0·34	0·60
His	0·07	0·34	0·04	0·36
Arg	0·34	—	0·26	0·31
Asp	0·08	0·26	0·08	0·56
Thr	0·04	0·09	0·03	0·07
Ser	0·03	0·84	0·02	0·92
Glu	0·53	0·12	0·18	0·65
Pro	0·22	0·87	0·07	0·58
Gly	0·45	0·51	0·18	0·35
Ala	0·28	0·41	0·27	0·56
Val	—	0·03	0·56	0·10
Ile	0·05	0·22	0·04	0·19
Leu	0·12	0·33	0·11	0·24
Tyr	—	0·06	—	0·04
Phe	—	0·06	—	0·10

[a] 10^{-7} mole per gramme of casein.
—, not detected.

casein. On the other hand, fifteen different amino acids were detected in the solution system, as shown in Table 3.

Figures 5 and 6 show the amino acid composition of a low molecular weight peptide fraction (below MW 500) of casein, heated with and without glucose in the powder and solution systems, respectively. The amount of small peptides increased with heating time. In the case of the powder system, the amount of small peptides in the presence of glucose, which was calculated as approximately 0·25 % of the original casein, was less than that in the absence of glucose (Fig. 5). It was thought that, in the powder system, glucose reacted with peptides liberated from casein so that the amount was reduced. On the other hand, in the aqueous solution, the amount of peptides in the presence of glucose was larger than that in the absence of glucose after 21 days at 75 °C. The total amount of small peptides formed from the casein–glucose mixture heated at 75 °C for 21 days was calculated at around 0·7 % of the original casein. In the solution system, due to the dilution effect, peptides did not react so readily with glucose, so that the residual amounts were greater (Fig. 6).

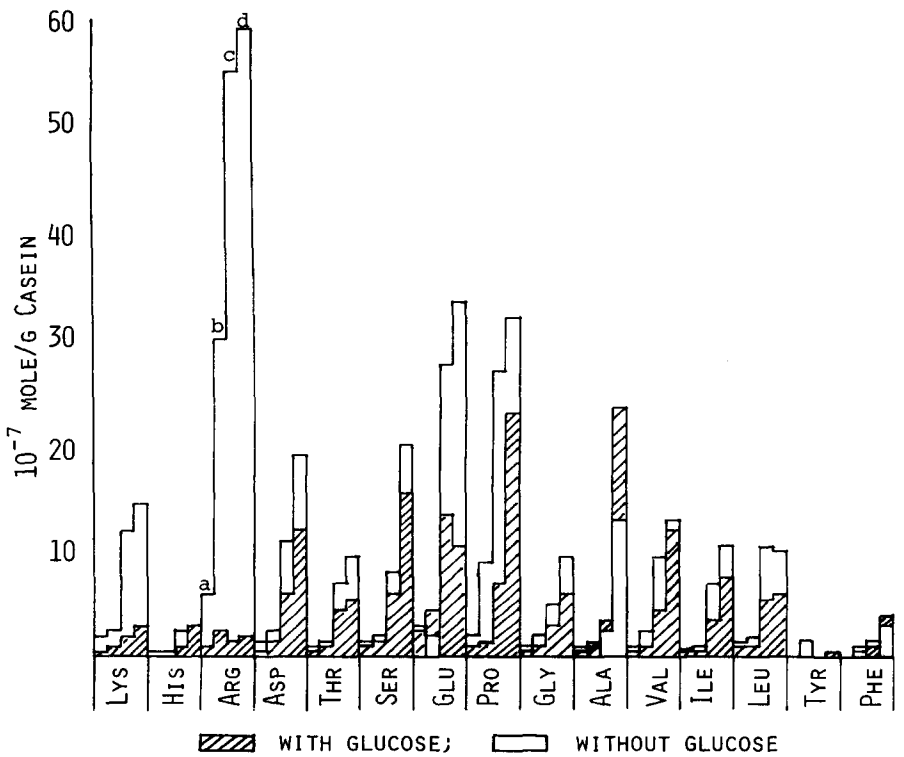


Fig. 5. Amino acid composition of low molecular weight peptides (below MW 500) obtained from casein heated with and without glucose in the powder system. a, at 50 °C for 7 days; b, at 50 °C for 21 days; c, at 75 °C for 7 days; d, at 75 °C for 21 days. Met, Cys and Trp were not determined.

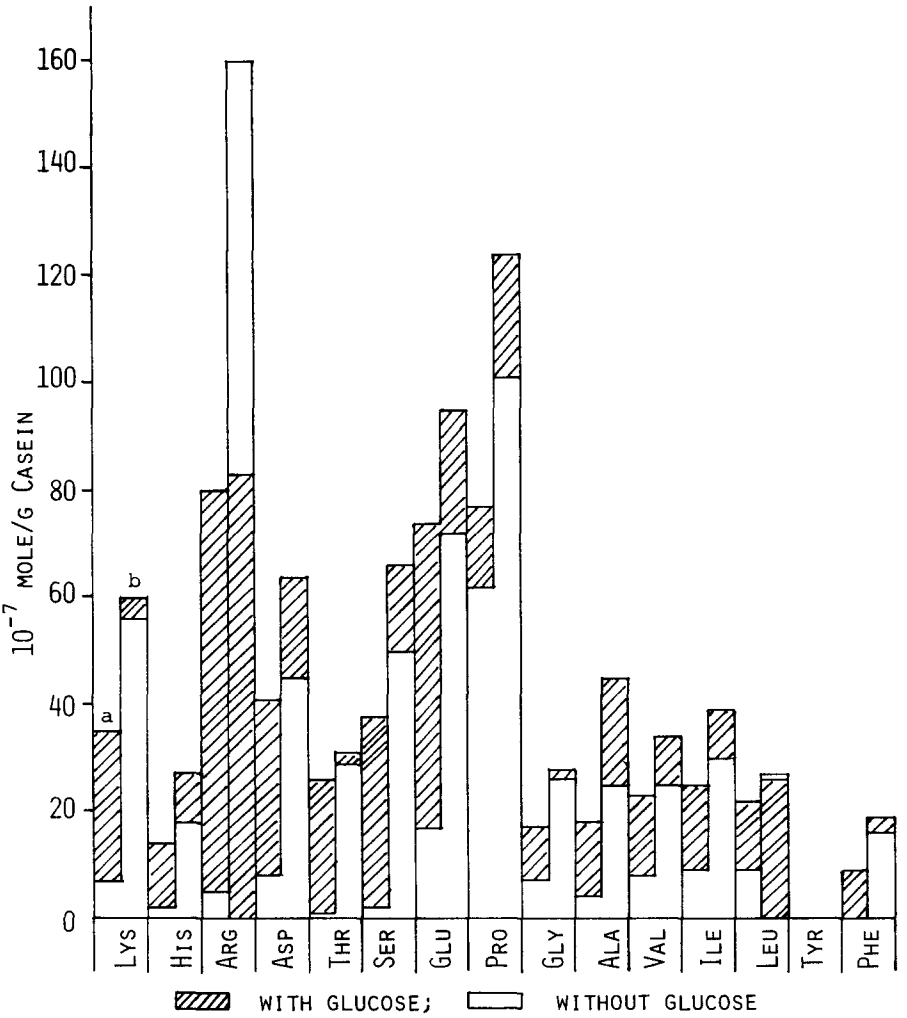


Fig. 6. Amino acid composition of low molecular weight peptides (below MW 500) obtained from casein heated with and without glucose in the solution system. a, at 75 °C for 7 days; b, at 75 °C for 21 days.

The mechanism of formation of these low molecular weight products from casein is unknown. The possibility of the reactivation of heat-inactivated proteases cannot be ruled out. However, there are some reports on the non-enzymic cleavage or hydrolysis of the peptide linkage. Chuyen *et al.* (1973) reported the liberation of free amino acids in the reaction of tri- and tetra-peptides with glyoxal at 100 °C. Hashiba (1975) described the formation of glycine in a Maillard reaction between glucose and

di- or tri-glycine. It is suggested that the results obtained in the present model experiments support the occurrence of non-enzymic cleavage or hydrolysis of peptide linkages in casein molecules during heat treatment and that glucose promotes the reaction. As already mentioned, in the aqueous solution system, all amino acid residues were decomposed (Fig. 4). From the results shown in Figs 4, 5 and 6, it could be postulated that new *N*-terminal amino acids are produced by non-enzymic cleavage or hydrolysis and that some of them decompose through reaction with glucose. Furthermore, in food processing or cooking, proteins should be considered a source of amino acids and low molecular weight peptides, which may be important flavour substances as well as precursors of volatile components.

REFERENCES

BENDER, A. E. (1978). *Food processing and nutrition*, Academic Press, London, 59–79.

CHUYEN, N. V., KURATA, T. & FUJIMAKI, M. (1973). Formation of *N*-[2(3-alkylpyrazin-2-on-1-yl)acyl]-amino acids or -peptides on heating tri- or tetra-peptides with glyoxal, *Agric. Biol. Chem.*, **37**, 1613–18.

CLARK, A. V. & TANNENBAUM, S. R. (1970). Isolation and characterization of pigments from protein–carbonyl browning systems, *J. Agric. Fd. Chem.*, **18**, 891–4.

EICHNER, K. (1975). The influence of water content on non-enzymic browning reactions in dehydrated foods and model systems and the inhibition of fat oxidation by browning intermediates. In: *Water relations of foods* (Duckworth, R. B. (Ed)), Academic Press, London, 417–34.

ERBERSDOBLER, H., COLE, D. J. A., BOORMAN, K. N., BUTTERY, P. J., LEWIS, D., NEALE, R. J. & SWAN, H. (1976). Amino acid availability. In: *Protein metabolism and nutrition* (Cole, D. J. A. *et al.* (Eds)), Butterworths, London.

HASHIBA, P. (1975). A glucose–diglycine condensation product participating in oxygen-dependent browning, *J. Agric. Fd. Chem.*, **23**, 539–42.

HAYASE, F., KATO, H. & FUJIMAKI, M. (1975). Racemization of amino acid residues in proteins and poly(L-amino acids) during roasting, *J. Agric. Fd. Chem.*, **23**, 491–4.

HAYASE, F., KATO, H. & FUJIMAKI, M. (1979). Racemization of amino acid residues in casein roasted with glucose and/or methyl linoleate, *Agric. Biol. Chem.*, **43**, 2459–65.

HURRELL, R. F. & CARPENTER, K. J. (1976). Nutritional significance of cross-link formation during food processing. In: *Protein crosslinking* (Friedman, M. (Ed)), Plenum Press, London, 225–38.

HURRELL, R. F. & CARPENTER, K. J. (1977). Maillard reaction in foods. In: *Physical, chemical and biological changes in food caused by thermal processing* (Hotem, T. & Kvale, O. (Eds)), Applied Science Publishers Limited, London, 168–84.

LABUZA, T. P., WARREN, R. M. & WARMBIER, H. C. (1976). The physical aspects with respect to water and nonenzymatic browning. In: *Protein crosslinking* (Friedman, M. (Ed)), Plenum Press, London, 379–418.

LEA, C. H. & HANNAN, R. S. (1950). Studies of the reaction between proteins and reducing sugars in the dry state, *Biochem. Biophys. Acta*, **5**, 433–54.

MOHAMMAD, A., FRAENKEL-CONRAT, H. & OLCOTT, H. S. (1949). Browning reaction of proteins with glucose, *Arch. Biochem.*, **24**, 157–78.

MORI, B. & NAKATSUJI, H. (1977). Utilization in rats of ^{14}C-L-lysine-labelled casein browned by the amino–carbonyl reaction, *Agric. Biol. Chem.*, **41**, 345–50.

TANAKA, M., LEE, T. C. & CHICHESTER, C. O. (1975). Effect of browning on chemical properties of egg albumin, *Agric. Biol. Chem.*, **39**, 863–6.

WEBER, K. & OSBORN, M. (1969). The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis, *J. Biol. Chem.*, **244**, 4406–12.

Food Chemistry 7 (1981) 169–174

SOME CHEMICAL AND ORGANOLEPTIC ASSESSMENT STUDIES ON THE STORAGE CHARACTERISTICS OF THE WEST AFRICAN LONG CROAKER (*PSEUDOTOLITHUS TYPUS*)

S. S. BOSSEY

Nigerian Institute for Oceanography and Marine Research, PMB 12729, Victoria Island, Lagos, Nigeria

&

S. O. TALABI*

UN/FAO Andre Mayer Research Project, Torry Research Station, 135 Abbey Road, Aberdeen AB9 8DG, Scotland, Great Britain

(Received: 2 February, 1980)

ABSTRACT

Icing has been found to extend the storage life of Pseudotolithus typus *for 22 days. Changes in the TVB, TMA and FFA were investigated as quality indices. Lipid and protein nitrogen contents were monitored. Organoleptic assessment methods were also employed in determining quality changes. The latter appeared to be more reliable than the chemical indices. Changes in lipids and protein nitrogen levels, although inconsistent, were indicative of a leaching process in the gutted fish. The effect of gutting on the storage quality of iced* P. typus *was shown to be marginal.*

INTRODUCTION

Fish is a highly perishable commodity and environmental conditions in the tropics predispose it to a high rate of spoilage. According to Jones & Disney (1976), spoilage of tropical fishes often occurs within 24 h, although, when iced, they could keep for an appreciable length of time. In a previous consideration of this subject, Disney *et al.* (1975) highlighted the need to investigate the use of ice, pointing out that tropical

* Present address: Nigerian Institute for Oceanography and Marine Research, Victoria Island, Lagos, Nigeria.

Food Chemistry 0308-8146/81/0007-0169/$02·75

countries with an interest in the use of chilling techniques should arrange for ice storage trials to be carried out on potentially important species at the onset of development programmes.

So far, such trials have been relatively rare and include those of Amu & Disney (1973) which showed that some West African fish species will keep in ice for between 20 and 26 days. Watanabe (1961) showed that the Brazilian snapper would keep for between 11 and 16 days according to the work of Crawford & Finch (1968). Ice storage trials by Velankar & Kamasastra (1956) showed that some Indian fish species would keep in ice for periods ranging from 7 to 45 days.

The lack of information on the ice storage characteristics of commercially important Nigerian fish species has made it difficult to encourage the development of the industry in this direction. At present, only a few fishing companies in Nigeria make use of ice to preserve fish. The use of ice by artisanal fishermen and fish retailers is almost non-existent. This work therefore attempts to determine the ice storage characteristics of *Pseudotolithus typus*—one of the most commercially important Nigerian marine fish species.

MATERIALS AND METHODS

Pseudotolithus typus were caught by the Research vessel 'Kiara', off the Lagos coastal area using granton trawl nets. Immediately after the catch, one batch of forty fish was gutted, washed in clean seawater and immediately iced. The ungutted batch, also containing forty fish, was also washed in clean seawater and iced. Both batches were then stored in insulated containers and placed in a cold room set at +2 °C. The ice was replenished frequently. Analyses were carried out on the first day of storage and subsequently every three or four days on manually filleted skin-on samples of the fish.

Chemical analyses

Trimethylamine (TMA) and total volatile bases (TVB) were analysed by the Conway microdiffusion technique of Beatty & Gibbons (1937). Percent protein nitrogen of the fillet was determined by means of the Kjel-Foss automatic protein nitrogen analyser. Total lipid determination on 100 g of minced fillets was carried out by the method of Bligh & Dyer (1959) and was followed by the determination of the free fatty acids (FFA) by the method employed by Bligh & Scott (1966), on cod muscle. In all cases, duplicate analyses were also carried out.

Organoleptic assessment

A five-strong panel was briefly trained on the organoleptic assessment of fresh fish. Samples from the stored fish were then assessed as follows.

Two fillets of fish from each batch were used, care being taken to include the

muscle tissue from the belly flap, as well as from the dorsal region, of the fish. Cooking was by steaming in 2 % brine for 15 min and samples were presented to the tasters on white flat plates.

Parameters employed by the judges were as follows.

(a) Characteristics of uncooked fillets:
  (i) Skin condition
  (ii) Odour
  (iii) Texture
(b) Characteristics of cooked fillets:
  (i) Odour
  (ii) Flavour
  (iii) Texture

Scores were on a numerical scale of 1 (lowest) to 10 (highest) quality.

RESULTS AND DISCUSSION

Data on the effect of storage period on the lipid content, free fatty acids, TMA, TVB and percent protein nitrogen are presented in Table 1. With an increase in the storage time, the total protein nitrogen changed inconsistently. The TVB increased in both gutted and ungutted fish throughout the storage period. Although the rates of increase appear similar in both treatments up to the fourteenth day, a higher level was obtained in the ungutted fish. The patterns of TMA production in the two treatments were rather erratic but the final level was higher in the gutted than in the ungutted fish. The lipids in both treatments fell inconsistently. There was a gradual accumulation of FFA up to the seventh day of storage. Subsequently, the FFA appeared to gradually diminish in quantity until the eighteenth day, after which there was again a build up, with greater accumulation occurring in the ungutted fish.

Assessment of the quality of fish stored in ice by means of chemical methods has been exhaustively investigated. Nevertheless, wide variations in experimental procedures and conditions have rendered impossible a comparison of the conclusions reached by various workers. The merits of most of the techniques have also been questioned. Thus, recent studies on tropical marine species have shown TMA and TVB to be of little value as quality indices in ice fish studies (Amu & Disney, 1973). Earlier, Farber (1965), in his review of freshness tests, showed that TMA was not a sensitive, reliable or reproducible index of spoilage. In contrast, studies on temperate fish species by Beatty & Gibbons (1937), Boury & Schvinle (1932; 1935) and Dyer (1945) showed that TMA estimation was a reliable quality index. TVB, as a spoilage index, has also been employed in assessing the quality of

TABLE 1
CHANGES IN THE PERCENT LIPID, FFA, TMA AND TVB AND PROTEIN IN *Pseudotolithus typus* STORED IN ICE

	Lipid content (%)		*Free fatty acid of lipid (%)*		*TMA mg N/100 g of fish*	
	Gutted fish	*Ungutted fish*	*Gutted fish*	*Ungutted fish*	*Gutted fish*	*Ungutted fish*
1	0·8	1·6	1·4	1·6	0·2	0·16
4	0·5	1·5	1·5	2·4	0·6	0·7
7	0·4	0·5	2·2	2·9	0·9	0·87
11	0·6	1·4	2·2	2·1	0·9	1·0
14	0·3	1·0	0·8	1·0	1·4	1·0
18	0·4	0·4	0·4	0·4	1·2	1·5
21	0·6	0·5	1·1	0·8	0·8	1·0
25	0·2	0·3	1·8	2·4	1·0	1·4

	TVB mg N/100 g		*Total protein (%)*	
	Gutted fish	*Ungutted fish*	*Gutted fish*	*Ungutted fish*
1	20·1	26·8	19·7	15·5
4	13·4	20·1	19·6	19·4
7	16·8	18·8	10·7	19·5
11	20·2	26·2	16·0	17·4
14	23·5	23·5	16·6	17·9
18	26·9	20·3	14·1	17·0
21	26·9	33·6	15·4	17·5
25	33·6	60·5	14·9	17·5

several temperate fish species and Farber (1965) suggested 30 mg N/100 g fish as the maximum limit of acceptability.

After the third day of ice storage there was some degree of linearity in the trend of the TVB results. In spite of this linearity, it is doubtful if this index could be used as a quality control parameter. Nevertheless, if the TVB maximum of 30 mg N/100 g of fish reported for temperate fish species by Farber (1965) was taken, it can then be suggested that the values of 26·88 mg/100 g for gutted—and 33·60 mg/100 g for ungutted—fish represent the time limit of acceptability. These levels correspond to the twenty-first day in ice in this fish.

The situation in respect of the TMA was not very different. The erratic pattern of the TMA data obtained in both gutted and ungutted fish in the first seven days of storage was nevertheless in line with the findings of Herzerberg *et al.* (1976) on *Sardinella aurita*. This observation appeared to show that the TMA index might not be suitable in the assessment of quality changes in iced *P. typus* and indeed confirms the earlier work of Amu & Disney (1973) on some other West African fish species.

Changes in the lipid and protein nitrogen levels in the two treatments were included in this study in an attempt to examine the extent of change and as a basis for future work on the spoilage matter in this fish. As Table 1 shows, lipid changes appear to be very erratic and may be due to the effect of storage on the extractability

of the lipid by the method employed. However, the results show that more protein nitrogen and lipids were retained in the ungutted fish, presumably because there was less leaching.

Results presented in Tables 2(A) and 2(B), giving average scores for appearance, smell, taste and texture of the raw and cooked fish, appear to have a consistent trend. The standard deviation of the data became rather high at the later stage of storage. In spite of this, the trend is obvious. With more training, the panel would probably

TABLE 2(A)
TASTE PANEL SCORES OF UNCOOKED FILLETS OF ICE STORED CROAKERS

Day	*Treatment*	*Skin characteristics (appearance)*	*SD**	*Odour*	*SD*	*Texture*	*SD*
0	—	8·8	0·4	8·8	0·4	8·4	1·3
4	Gutted	8·3	0·9	7·3	1·0	8·0	0·8
	Ungutted	8·0	0·8	7·5	0·6	7·3	1·5
7	Gutted	7·8	0·5	7·5	1·0	7·3	1·0
	Ungutted	8·0	0·4	7·3	0·5	7·5	0·6
11	Gutted	8·5	0·6	7·8	0·5	6·7	1·3
	Ungutted	8·5	0·6	7·5	0·6	7·0	1·4
14	Gutted	7·3	1·0	6·7	0·6	7·3	0·6
	Ungutted	7·5	1·0	7·3	0·6	6·3	0·6
18	Gutted	7·8	0·9	6·5	0·6	6·8	0·5
	Ungutted	7·5	0·9	6·3	1·3	6·5	0·6
21	Gutted	6·8	0·9	6·0	0·8	6·0	0·8
	Ungutted	6·8	0·9	5·3	0·5	5·5	0·6
24	Gutted	5·0	1·8	5·7	1·2	5·0	2·6
	Ungutted	5·0	1·7	5·0	1·7	4·0	1·7

* Standard deviation.

TABLE 2(B)
TASTE PANEL SCORES OF COOKED FILLETS OF ICE STORED CROAKERS

Day	*Treatment*	*Odour*	*SD**	*Flavour*	*SD*	*Texture*	*SD*
0	—	7·6	0·5	8·4	0·5	8·0	0·7
4	Gutted	8·0	0·8	8·0	0·8	6·5	0·6
	Ungutted	7·5	0·6	6·8	0·5	6·8	1·3
7	Gutted	7·5	1·2	8·0	0·4	8·0	0·3
	Ungutted	7·0	0·8	7·8	0·5	7·5	0·6
11	Gutted	7·0	0·8	7·8	1·0	7·3	0·5
	Ungutted	7·5	0·6	7·8	1·0	7·0	0·8
14	Gutted	6·7	1·0	6·7	0·6	7·0	0·6
	Ungutted	6·0	0·6	6·0	1·2	6·7	1·0
18	Gutted	7·5	1·3	7·3	0·5	6·8	0·5
	Ungutted	7·5	0·6	6·8	1·0	7·0	0·8
21	Gutted	6·8	1·0	7·3	0·5	7·0	0·5
	Ungutted	7·0	0·3	7·8	0·5	6·8	0·5
24	Gutted	5·3	1·5	5·7	1·2	5·3	0·6
	Ungutted	4·3	2·3	4·0	2·0	4·3	1·2

* Standard deviation.

have provided lower standard deviations. This observation appears to be consistent with the results of Amu & Disney (1973) in which such assessments were found to be of limited value because of variations arising from the assessment of different individuals. When a score of 6 was taken as the minimum level of acceptability, icing appeared to have extended the storage life of the fish for 22 days. This, indeed, appears to be consistent with the previously discussed indication that the TVB index showed that the fish would keep in ice for at least 21 days. Furthermore, it can be seen from the data that any advantage derived from gutting was marginal.

CONCLUSIONS

By extending the storage life of the fish *Pseudotolithus typus* through the use of ice, it should be possible to store and transport it over a considerable distance from the coast. The organoleptic assessment results have served to show that there is some potential in the use of this simple method in the quality assessment of this fish. It is equally essential that an economic appraisal of the use of ice for the preservation of this fish be thoroughly investigated.

REFERENCES

AMU, L. & DISNEY, J. G. (1973). Quality changes in West African marine fish during iced storage, *Tropical Science*, **15**, 125–38.

BEATTY, S. A. & GIBBONS, N. E. (1937). The measurement of spoilage in fish, *J. Fisheries Board of Canada*, **3**, 77–91.

BLIGH, E. G. & DYER, W. J. (1959). A rapid method of total lipid extraction and purification, *Canadian J. Biochem. and Physiology*, **37**, 911–17.

BLIGH, E. G. & SCOTT, M. A. (1966). Lipids of cod muscle and the effect of frozen storage, *J. Fish Res. Bd. Canada*, **23**, 1025–36.

BOURY, M. & SCHVINLE, J. (1932). L'Alteration du poison. I. Rev. trav. office Science Tech. peche maritimes, **5**, 311–13.

CRAWFORD, L. & FINCH, R. (1968). Quality changes in albacore tuna during storage on ice and RSW, *Food Technology*, **22**, 1280.

DISNEY, J. G., COLE, R. & JONES, N. R. (1975). Considerations in the use of tropical fish species. In: *Fishery products* (Kreuzer, R. (Ed.)), Fishing News (Books), Surrey, 329–37.

DYER, W. J. (1945). Amines in fish: Colorimetric determination of TMA as the picrate salt, *J. Fisheries Research Board of Canada*, **6**, 351–8.

FARBER, L. (1965). In: *Fish as food* (Vol. IV). (Borgstrom, G. (Ed.)), Academic Press, New York and London.

HERZERBERG, A., PASTEUR, R. & ROSSEMBLATT, M. (1976). Keeping quality and freshness determination of *Sardinella aurita*. *Conference Proceedings: Handling Processing and Marketing of Tropical Fish*, 83–8.

JONES, N. R. & DISNEY, J. G. (1976). Technology in fisheries development in the tropics, *Conference Proceedings: Handling, Processing and Marketing of Tropical Fish*, 27–31.

VELANKAR, N. K. & KAMASASTRA, P. V. (1956). The bacterial flora, trimethylamine and total volatile nitrogen of fish muscle at 0°C (in ice), *Indian J. Fish*, **3**, 269–89.

WATANABE, K. (1961). *Spoilage in iced Pescadafognette from South Brazilian fishing grounds*, Inst. Ocean., University S. Paulo Contr., p. 163.

Food Chemistry 7 (1981) 175–179

THE GROSS CHEMICAL COMPOSITION OF ANTARCTIC KRILL SHELL WASTE

MARIAN NACZK, JÓZEF SYNOWIECKI & ZDZISŁAW E. SIKORSKI

Department of Food Preservation and Technical Microbiology, Technical University Politechnika Gdanska, Poland

(Received: 6 March, 1980)

ABSTRACT

Krill, a small shrimp-like crustacean, is actually being exploited commercially in the Antarctic waters. In order to evaluate the offals generated during the processing of krill to food and feed products, as commercial sources of chitin, three kinds of shell waste were analysed. Depending upon the method of krill processing applied the shell waste contained 49–60% of proteinaceous material. The content of crude chitin in the dried deproteinised shells ranged from 34 to 49%. About 60% of the salt mixture impregnating the shells was phosphates and carbonates of calcium and magnesium.

INTRODUCTION

The Antarctic krill is a small, shrimp-like crustacean which is classified by fishermen as large when its total length is from about 45 to 55 mm and its weight from 0·7 to 1·1 g. The utilisable resources of these crustaceans are estimated by various specialists to range from 100 to 150 million tons yearly. The average fishing yield of a factory trawler during the 100–120 days' season in the Antarctic waters is actually from 40–60 tons a day, although the fluctuation of catches is very high, as the maximum can reach 60 tons for each 15 minutes of trawling.

Containing about 14% crude protein and 4% fat, krill meat is considered by many specialists to be a rich potential source of nutrients for farm animals and for direct human consumption. There are several commercial propositions on how to make different food products from this raw material (Fig. 1). However, the economy of commercial krill fisheries will, of course, depend not only on the efficiency of catching and use of the edible parts, but also on the practical possibilities of full utilisation of all the inedible constituents of these crustaceans. Chitin, which is one

Food Chemistry 0308-8146/81/0007-0175/$02·75

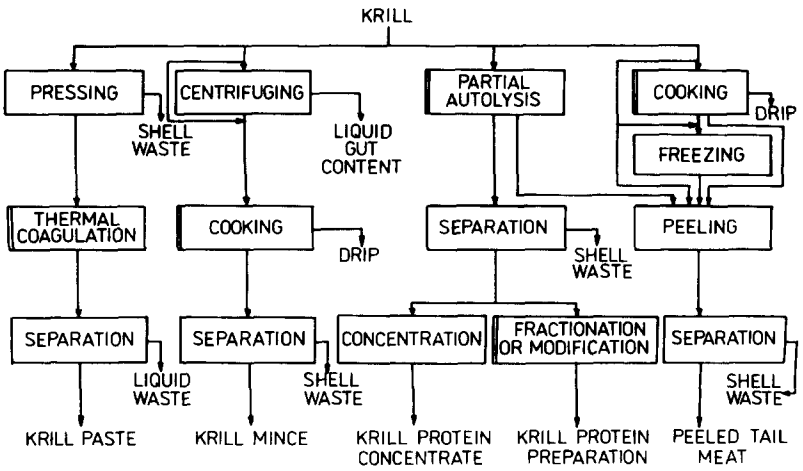


Fig. 1. General flow sheet of krill processing.

of the main components of the shell, can find several applications in the industry and in analytical chemistry (Muzzarelli, 1977). Thus it is important to know the composition of krill offals, which can be used for the production of chitin and chitosan.

MATERIALS AND METHODS

Three kinds of krill offal, separated in the course of semi-commercial processing from the edible parts, were analysed—i.e. offal from raw krill, offal from raw krill dried in an atmosphere of combustion gases and offal from cooked krill.

The water content of the material was determined by oven drying at 105 °C, the content of total nitrogen by the Kjeldahl method, the content of lipids by Soxhlet extraction with ligroin and by the Folch method and total ash by mineralisation at 550 °C.

The content of the shell was determined in a quantity of material, corresponding to 5 g of dry matter, by digesting the accompanying proteins in 100 cm^3 5 % KOH for 2 h at 100 °C, with intermittent mixing, separating the shells using a G-1 Schott filter, washing with five portions of 200 cm^3 of distilled water followed by three portions of 25 cm^3 of acetone and drying to a constant weight at 105 °C.

The content of chitin was determined in approximately 1 g of deproteinised shell by digesting in 20 cm^3 of 3 % HCl at room temperature with continuous mixing for 2 h, separating the chitin on a G-1 filter, washing with distilled water and acetone and drying to a constant weight at 105 °C.

TABLE 1

THE GROSS CHEMICAL COMPOSITION OF KRILL OFFAL

Kind of offal	*Total nitrogen % of dry material*	*Chitin nitrogen*	*Nitrogen in various extracts*				*Lipids*		*Ash*	*Deproteinised shell*
		% of total nitrogen					*% of dry material*			
			$NaHCO_3$	*KCl*	*SDS*	*KOH*	*Soxhlet*	*Folch*		
Raw	8 ± 1·1	10·6 ± 5·7	34·5 ± 8	7·6 ± 1·7	9·9 ± 5·2	36 ± 4·9	4·8 ± 1·6	6·7 ± 1	28·2 ± 5·3	44·5 ± 7·5
Raw dried	7 ± 0·3	19·2 ± 0·2	29 ± 11·9	4·9 ± 0·2	6·8 ± 2·3	42 ± 11	5·7 ± 2·4	—	29·8 ± 3·9	47·2 ± 1
Cooked	9·4 ± 1·6	6·6 ± 2·8	5·1 ± 2·2	3·6 ± 1	4·8 ± 1·5	79·9 ± 5·2	8·8 ± 1·3	20 ± 2·5	17·1 ± 7·1	21·3 ± 6·1

All data are mean values of four determinations in 2–6 samples of offal, ± standard deviations.

The content of CaO, MgO, P_2O_5, CO_2 and other oxides in the deproteinised shell was determined using classical gravimetric methods.

A crude characterisation of the forces responsible for the binding of proteins to the chitinous structure was obtained by selective extractions using aqueous solutions of 0·02M $NaHCO_3$ for 2 h under refrigeration, followed by 0·75M KCl for 2 h at 20 °C and pH 7·5, 1 % SDS for 12 h at 20 °C and pH 7·5 and 5 % KOH for 2 h at 100 °C.

RESULTS

The content of total nitrogen and of different protein fractions extracted with various agents (Table 1) both depend on the type of shell waste and on the maturity of the krill. The data given in Table 1 indicate that for purposes of chitin production the most promising raw material is the raw krill offal, containing the least proportion of firmly bound proteins, which can be removed only by extraction with hot alkali and is rich in shell material. This raw offal is used in Poland for the semi-commercial production of chitin and chitosan of very high quality (Brzeski *et al.*, 1980). The somewhat high content of easily solubilised protein fractions in dried offal was probably caused by enzymic hydrolysis of the proteins before drying.

The content of crude chitin in the dry, deproteinised krill shells ranged from 34 % to 49 %, depending on the extent of impregnation of the material with mineral salts, which is different at various stages of the animal's development. The average concentration of crude chitin in the deproteinised shell was 40·2 ± 5·2 % and was similar to that reported by Yanase (1975), calculated on the basis of *N*-acetylglucosamine determinations. The crude chitin, separated from krill offal according to the procedure described above, contained, on the average, 6·3 ± 0·2 % nitrogen and 0·5 ± 0·25 % ash.

The lipid content was fairly independent of the kind of offal.

The content of mineral salts depends very significantly on the age of the krill

TABLE 2
MINERALS IN RAW DRIED KRILL OFFAL

Component	*Content in % of dry matter*	
	Offal	*Shell*
CaO	6·22 ± 0·11	13·02 ± 0·24
MgO	0·88 ± 0·12	1·85 ± 0·26
P_2O_5	3·83 ± 0·03	8·03 ± 0·07
CO_2	2·80 ± 0·15	5·87 ± 0·31
R_2O_3*	8·12 ± 0·16	17·0 ± 0·33

* Oxides of cations precipitated by NH_4OH in the form of hydroxides.
Mean values of four determinations ± standard deviations.

animals, as older specimens have a more calcified shell. The main components of the mixture of salts are the phosphates and carbonates of calcium and magnesium (Table 2).

REFERENCES

BRZESKI, M., MIECZKOWSKA, M., STOLZ, H., WOJTASZ, A., SOWA, K. & NEUGEBAUER, W. (1980). Technologia otrzymywania chityny i chitosanu. Paper presented at the Seminar on Chitin and Chitosan, Sea Fisheries Institute, Gdynia.

MUZZARELLI, R. A. A. (1977). *Chitin*, Pergamon Press Ltd.

YANASE, M. (1975). Chemical composition of the exoskeleton of Antarctic krill, *Bull. Tokai Reg. Fish. Res. Lab.*, **83**, 1.

Food Chemistry 7 (1981) 181–187

EFFECT OF COOKING ON THE NUTRITIVE VALUE OF KOKO/KOSAI—A TRADITIONAL BREAKFAST MEAL OF THE HAUSAS IN NORTHERN NIGERIA

O. A. OSINUBI* & O. U. EKA†

Department of Biochemistry, Ahmadu Bello University, Zaria, Nigeria

(Received: 17 March, 1980)

ABSTRACT

The effect of cooking on the nutritive value of koko/kosai was assessed by chemical analysis. There was significant loss in the proximate composition due to cooking. In addition, losses of some mineral elements and vitamins were observed. The losses in potassium, iron, zinc and phosphorus were found to be 23%, 6·7%, 25% and 13·3%, respectively.

Losses of vitamins B_1, B_2, C and carotene were found to be 20%, 46%, 37% and 9·6%, respectively.

The amino acid pattern of the koko/kosai was only slightly affected by the traditional method of cooking employed. Some suggestions and recommendations are made on how to retain most of the nutrients when cooking the meals.

INTRODUCTION

The nutritional importance of a foodstuff in a diet depends on the composition of the raw foodstuff, the amount that is usually consumed and the extent to which nutrients are destroyed or lost during preparation of the diet.

Most Nigerian foods have to be prepared and cooked before they can be eaten. A lot of food preparation in Nigeria involves boiling, frying or roasting. At each stage of the preparation some of the nutrients may be discarded or destroyed while in some cases toxic substances may be discarded (Oke, 1965).

Some investigators have reported losses incurred during the preparation of some

* Department of Biochemistry, College of Medicine, University of Lagos, Nigeria.
† Medical School, University of Calabar, Calabar, Nigeria.

Food Chemistry 0308-8146/81/0007-0181/$02·75

Nigerian foods (Malik, 1967; Umoh, 1973; Umoh & Bassir, 1977; Eka & Hobbs, 1978; Eka, 1978). The present studies were carried out to evaluate the effect of the traditional methods of cooking on the nutrient status of koko/kosai which is commonly eaten as a breakfast meal by the Hausas in northern Nigeria.

It was hoped that the findings might provide a good scientific basis for advising the peasantry in northern Nigeria about the best ways of cooking in order to minimise nutrient losses.

EXPERIMENTAL

Preparation of the meals for analysis

The meals investigated were koko (guinea corn pap) and kosai (bean cake). The meals were prepared in the laboratory according to the methods described by Eka & Edijala (1972), Eka & Kay (1977) and Eka (1978).

The ingredients in koko include guinea corn, red pepper, dried ginger and cloves. In the preparation of the koko, the guinea corn, together with water, is formed into a thin paste. This is strained through a sieve into a container and set aside for at least 12 h to sediment and ferment. The clear supernatant is decanted off and the thick sediment is collected and mixed with sufficient cold water to form a thick paste. The thick paste is then added to a suitable amount of boiling water with constant stirring and cooked for 3 to 5 min to form a thick viscous paste (pap). Sugar or salt is added (if desired) and the pap is eaten along with kosai (bean cake).

The ingredients in kosai include bean (cowpea) paste, onions, salt and fresh red pepper. In the preparation of the kosai the cowpeas are cleaned and washed and then soaked in cold water overnight (12–15 h) using about two volumes of water. The softened and dehusked beans are ground, using a grinding stone or a mechanical grinder, to obtain a paste. To the bean paste is added a small amount of guinea corn flour (binding material), minced onions, salt and pepper and the whole is mixed in a mortar or container to the desired consistency. The enriched paste is then divided into balls (40–60 g) which are deep-fried using groundnut oil to which potash has been added.

Standard koko and kosai were prepared in the laboratory. The proportion of ingredients used was established by household survey work. The standard meals were prepared in the laboratory by a Hausa woman using the data established during the survey.

The koko and kosai were homogenised and mixed in the proportions usually consumed by the individuals of a household. The raw forms were similarly treated. The mixing was carried out using a food blender (Braun Multimix Mx. 32). Portions of both raw and cooked food samples so treated were dried immediately at 60–70 °C using an air circulating oven. The resulting material was ground into a fine powder, passed through a 40 mesh sieve (AOAC, 1965) and stored in screw-capped bottles.

Portions of the raw and cooked meal samples were freeze-dried for the determination of vitamins.

Sample analysis

The methods of treatment of samples and analysis were those recommended by the Association of Official Agricultural Chemists (AOAC, 1965) and the Association of Official Analytical Chemists (AOAC, 1975).

The ash and organic matter were determined by incineration of a known weight of the sample until ash was obtained. The organic matter was obtained by subtracting the weight of the ash from the weight of the dry material.

The lipid was estimated by exhaustive extraction of a known weight of fried sample with petroleum ether (boiling point, 60–80 °C) using a Soxhlet apparatus. The protein was determined by the macro-Kjeldahl method (AOAC, 1975). The carbohydrate content was obtained by difference; that is, subtracting the total protein and lipid from the organic matter. Crude fibre was estimated by the AOAC method (AOAC, 1965).

Mineral element composition was determined using an atomic absorption spectrophotometer. Phosphorus was determined by the molybdovanadate method of the AOAC (AOAC, 1975). Vitamins B_1 and B_2 and carotenes were estimated by the standard method (AOVC, 1966) and vitamin C was determined by Evered's method, (Evered, 1960). The amino acid analysis was carried out using an automatic amino acid analyser (Beckman 120 C). The procedure followed was that described by Oyeleke (1977).

RESULTS

Changes in the nutrient composition of the koko/kosai during the traditional method of preparation were measured from the standard cooked and raw-mixed meal (which contained the prepared ingredients in the same proportion as the cooked meal). The results are shown in Tables 1 to 5.

Losses were observed and the differences between the raw and the cooked forms of

TABLE 1
PROXIMATE COMPOSITION OF MEAL SAMPLES (MEAN* ± STANDARD DEVIATION)

Sample	*Moisture (g/100 g wet weight)*	*Ash (g/100 g)*	*Crude protein (dry weight)*	*Petroleum ether extract (dry weight)*	*Crude fibre (dry weight)*	*Carbohydrate (dry weight)*
Koko/kosai						
Raw	66·68 ± 0·04	1·89 ± 0·00	11·17 ± 0·19	45·39 ± 0·17	0·74 ± 0·01	40·83 ± 0·13
Cooked	70·08 ± 0·02	1·88 ± 0·00	10·46 ± 0·15	43·00 ± 0·10	0·68 ± 0·01	43·98 ± 0·28

* Mean of five determinations.

TABLE 2
MINERAL ELEMENT CONTENT OF SAMPLES (mg/g DRY MATTER) (MEAN ± STANDARD ERROR)

Sample	*K*	*Na*	*Ca*	*Mg*	*Zn*	*Cu*	*Fe*	*P*
Koko/kosai								
Raw	2·0 ± 0·01	3·92 ± 0·03	0·46 ± 0·04	0·80 ± 0·01	0·12 ± 0·001	0·01 ± 0·002	0·15 ± 0·003	4·42 ± 0·01
Cooked	1·57 ± 0·02	3·92 ± 0·04	0·39 ± 0·01	0·71 ± 0·04	0·09 ± 0·003	0·01 ± 0·004	0·14 ± 0·0	3·83 ± 0·01

TABLE 3
VITAMIN CONTENT OF THE MEAL (mg/100 g DRY WEIGHT) (MEAN ± STANDARD DEVIATION)

Sample	B_1	B_2	*C*	*Carotene*
Koko/kosai				
Raw	6·11 ± 0·11	1·99 ± 0·01	10·50 ± 0·50	130·00 ± 5·00
Cooked	4·89 ± 0·12	1·08 ± 0·08	6·60 ± 0·01	117·50 ± 2·50

TABLE 4
AMINO ACID COMPOSITION OF THE MEAL IN MILLIGRAMMES PER GRAMME OF FOOD SAMPLE (MEAN OF TWO DETERMINATIONS)

Amino acid	*Raw koko/kosai*	*Cooked koko/kosai*
Apartic acid	10·25	9·17
Serine	5·25	4·56
Glutamic acid	18·91	17·91
Proline	4·07	4·14
Glycine	2·88	2·76
Alanine	3·75	3·80
Histidine	2·87	2·71
Arginine	6·81	6·58
Threonine	7·21	7·07
Cysteine (half)	0·00	0·00
Methionine	1·09	1·00
Valine	6·25	5·46
Iso-leucine	4·17	4·25
Leucine	12·17	10·92
Tyrosine	8·10	7·10
Phenylalanine	6·20	5·10
*Tryptophan	0·93	0·84
Lysine	4·50	3·77
Organic N (mg) content in the food sample	17·57	16·74
Calculated N (mg) from recovered amino acid	15·92	14·77
% recovery	90·61	88·23

* Determined chemically using ferric chloride reagent.

TABLE 5
ESSENTIAL AMINO ACID COMPOSITION (mg/g N) OF THE MEAL, COMPARISON WITH FAO/WHO PROVISIONAL PATTERN (FAO/WHO, 1973)

Amino acid	*Raw koko/kosai*	*Cooked koko/kosai*	*FAO*
Iso-leucine	237	254	250
Leucine	693	652	440
Lysine	256	225	340
Methionine + Cysteine	62	60	220
Phenylalanine + Tryrosine	814	729	380
Threonine	410	422	250
Tryptophan	53	50	60
Valine	356	326	310

the meal in terms of the lipid, protein and ash were found to be significant with values of $P < 0{\cdot}01$, $P < 0{\cdot}05$ and $P < 0{\cdot}01$, respectively.

The meal showed some significant losses of potassium, iron, zinc and phosphorus (Table 2) which were found to be 23 %, 6·7 %, 25 % and 13·3 %, respectively. Losses were also observed in all the vitamins studied. These were significant at $P < 0{\cdot}005$ for vitamins B_1 and B_2, $P < 0{\cdot}025$ for carotenes and $P < 0{\cdot}01$ for vitamin C. The losses were 20 % for vitamin B_1, 46 % for vitamin B_2, 37 % for vitamin C and 9·6 % for carotene (Table 3).

Each of the amino acids, with the exception of *iso*-leucine, showed a decrease in the cooked meal (Table 4). The essential amino acids were compared with the FAO/WHO provisional pattern (Table 5). The cooked and raw meals were found to be deficient in methionine and cysteine, lysine and tryptophan. Phenylalanine, tyrosine, threonine and leucine content were found to be high. Valine and *iso*-leucine compared favourably with FAO/WHO provisional patterns.

The calorie content of the cooked meal was 605 % and of the raw meal, 616 %. The net protein calorie percentage was 6·90 % for the cooked meal and 7·20 % for the raw meal.

DISCUSSION

The apparent increase observed in the percentage of total carbohydrate in the cooked meal is probably due to the losses reported for the other nutrients. The decrease in lipid and crude protein appears to be rather too slight to be of nutritional significance. The losses of the vitamins can be attributed to the leaching out of the fat-soluble vitamins into the cooking oil and to the high temperature reached, as well as to open pan cooking (Malik, 1967). The reduction in the vitamins due to the heat treatment may be explained by the fact that the vitamins are heat labile. The potash used during the preparation of the meal makes the cooking medium alkaline and,

under such conditions, there may be losses of vitamins B_1 and B_2 and some other vitamins (Lee, 1958; Bender, 1966; Davidson *et al.*, 1975). The losses of some of the vitamins may also be due to the presence of oxygen (Harris & Von Leescke, 1960) and of traces of copper and iron in the meal (Bender, 1966).

The decrease in the amino acid content during preparation may be attributed to the heat treatment and possibly also to loss into the cooking oil. The loss could also be due to a combination of one or more of the amino acids in linkages that are not easily hydrolysed during digestion.

The cooked and raw meals were found to be deficient in methionine, cysteine, lysine and tryptophan. Cereals are mainly deficient in lysine but rich in methionine (Baptist & Perera, 1956; Kakade, 1974). Some of the lysine content of the meals was contributed by the other ingredients present in the food—for example, cowpeas. It is interesting to note the level of lysine obtained by the combination of the plant proteins used in preparing the meal. For this reason the staple diet of the Hausas is likely to give a well balanced mixture of essential amino acids when a sufficient quantity of each ingredient is used. The large proportion of sorghum (guinea corn) in the meal contributed greatly to its high leucine content (Haikerwal & Mathieson, 1971).

The koko/kosai may meet the minimum value of 6 NDP cals % recommended for adults but requires augmentation by increasing the level of kosai in order to meet the minimum value of 8 NDP cals % recommended for infants (Olayide *et al.*, 1972). The meal is adequate in most of the mineral elements. Iron is rather high in the food and would meet the recommended daily allowance of 12 mg/100 g dry matter (Oke, 1972). A part of the iron content must have arisen by contamination from metal containers used at certain stages of the preparation. The actual amount of these minerals available to the consumer may depend on the limitation of absorption caused by phytic acid or oxalic acid present in the food (Okoh & Eka, 1978).

CONCLUSIONS

A knowledge of the traditional method of preparation and the effect of cooking on the nutrient composition of this meal allows the following recommendations and suggestions to be made.

(1) Potash should not be added to the cooking oil used during the preparation of the meal.
(2) The volume of the cooking oil used in frying the kosai should be reduced and the oil left over after frying should be used in preparing stew or other meals.
(3) The proportion of kosai to koko should be high.
(4) Vegetables and fruits should be taken with the meal in order to supply the necessary vitamins, e.g. vitamin C.

ACKNOWLEDGEMENTS

The authors are grateful to Mr L. Lawal for helping in the analysis of some of the mineral elements and also to the Board of Research, Ahmadu Bello University, Zaria, for financial assistance in carrying out the work.

REFERENCES

ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS (AOAC). (1965). *Methods of analysis.* (10th edn.), Washington, DC.

ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS (AOAC). (1975). *Methods of analysis.* (12th edn.), Washington, DC.

ASSOCIATION OF VITAMIN CHEMISTS (AOVC). (1966). *Methods of vitamin assay.* (3rd edn.), New York, London, Sydney, Interscience Publishers.

BAPTIST, N. G. & PERERA, B. P. (1956). Essential amino acids of some tropical cereal millets. *Brit. J. Nutr.*, **10**, 334.

BENDER, A. E. (1966). Nutritional effects of food processing. *J. Fd. Techn.*, **1**, 261.

DAVIDSON, S., PASSMORE, R., BROCK, J. F. & TRUSTWELL, A. S. (1975). *Human nutrition and dietetics.* (6th edn.), Livingstone, English Lang. Book Soc. and Churchill.

EKA, O. U. (1978). Chemical evaluation of nutritive value of soya paps and porridges, the Nigerian weaning foods. *Fd. Chem.*, **3**, 199.

EKA, O. U. & EDIJALA, J. K. (1972). Chemical composition of some traditionally prepared Nigerian food. *Nigerian J. Sci.*, **6**, 177.

EKA, O. U. & HOBBS, C. (1978). Effect of cooking on nutritive value of *Danwake. Nigerian Jour. Science*, **12**, 6.

EKA, O. U. & KAY, T. (1977). Chemical evaluation of the nutrient status of soya bean meals prepared using traditional methods. *Samaru. Agric. Newsletter*, **19**, 76.

EVERED, D. F. (1960). Determination of ascorbic acid in highly coloured solutions with *N*-Bromosuccinimide. *Analyst*, **85**, 515.

FAO/WHO (1973). *Energy and protein requirements.* Report of a Joint FAO/WHO *Ad Hoc* Expert Committee Wld. Hlth. Org. Techn. Rep. Series No. 522.

HAIKERWAL, M. & MATHIESON, A. R. (1971). Protein content and amino acid composition of sorghum grain. *Cereal Chem.*, **48**(6), 690.

HARRIS, R. E. & VON LEESCKE, H. (1960). *Nutritional evaluation of food processing.* New York, Wiley.

KAKADE, M. L. (1974). Biochemical basis for the differences in plant protein utilisation. *J. Agric. Fd. Chem.*, **22**(4), 550.

LEE, F. A. (1958). The blanching process. *Adv. Fd. Res.*, **8**, 63.

MALIK, Z. R. (1967). Nutritional change in some Nigerian Foods during dietary preparation and their effects on mammalian body. *PhD Thesis*, University of Ibadan, Nigeria.

OKE, O. L. (1965). Chemical studies on some Nigerian foodstuffs: 'Lafun'. *W. Aft. J. Biol. Chem.*, **8**(3), 53.

OKE, O. L. (1972). A nutrition policy for Nigeria, *World Rev. Nutr. Dietetics*, **14**, 1.

OKOH, P. N. & EKA, O. U. (1978). Effect of traditional methods of preparation on the nutrient status of Fura-Nono. *Savana*, **7**(1), 67.

OLAYIDE, S. O., OLATUNBOSUN, D., IDUSOGIE, E. O. & ABIAGOM, J. D. (1972). *A quantitative analysis of food requirements, supplies, and demands in Nigeria, 1965–1985.* Paper 19, Federal Ministry of Agriculture, Lagos, Nigeria.

OYELEKE, O. (1977). Assessment of the nutritive value of two varieties of sorghum grain, *Sorghum bicolor* (L), *MSc Thesis*, Ahmadu Bello University, Zaria, Nigeria.

UMOH, I. B. (1973). Changes in the nutritive values of some Nigerian chiefs after cooking by certain south eastern Nigerian traditional methods, *PhD Thesis*, University of Ibadan, Nigeria.

UMOH, I. B. & BASSIR, O. (1977). Nutrient changes in some traditional Nigerian foods during cooking. Part 1: Vitamin changes. *Fd. Chem.*, **2**, 155.

Food Chemistry 7 (1981) 189–193

BIOCHEMICAL CHANGES IN GREEN SWEET PEPPERS DURING STORAGE AT DIFFERENT TEMPERATURES

DOMENICA TONELLI, ENRICO GATTAVECCHIA & ROLANDO BUDINI

Chemical Institute 'G. Ciamician', University of Bologna, Via Selmi 2, 40126 Bologna, Italy

(Received: 17 March, 1980)

ABSTRACT

The effect of temperature on the levels of moisture, ascorbic acid, proteins and phenolic compounds in green sweet peppers (cvs. Sumo and Quadrato d'Asti) was investigated after seven days' storage at a relative humidity of 85–90%. Chemical composition did not differ appreciably after storage at 10°C, whereas changes were found after storage at 20°C and 30°C. Therefore, 10°C was the most favourable storage temperature for peppers although, at 20°C, retention of quality was adequate in terms of texture and appearance.

INTRODUCTION

The determination of storage conditions for the maintenance of the high quality of fruit and vegetables before sale is most interesting in the agricultural–economic field. Minimum losses in quality are desirable in post-harvest ripening. In the last few years considerable efforts have been made to determine changes occurring in the main constituents during the storage of several agricultural products and, as a result, significant progress has been made in assessing and controlling quality.

Sweet peppers, although economically important, have received comparatively little attention. The chemical composition associated with ripening and the effects of storage temperature have been investigated to some extent (Awasthi *et al.*, 1976; Shorikova *et al.*, 1977; Saimbhi *et al.*, 1977; Kozukue & Ogata, 1972; Shukla *et al.*, 1975; Ivanova *et al.*, 1976). However, no information is available about the biochemical changes occurring in pepper fruits after harvest.

Food Chemistry 0308-8146/81/0007-0189/$02·75

The aim of the investigation reported in this paper was to study some compositional changes that take place during the storage of sweet peppers. In this regard ascorbic acid, proteins, phenolic compounds and moisture were taken into consideration. Temperatures of 20 °C and 30 °C were chosen to simulate unfavourable conditions occurring in non-refrigerated transport. Our work was undertaken to verify whether experimental injuries sustained for a short period led to produce deterioration resulting in appreciable variations in chemical composition.

MATERIALS AND METHODS

Materials

Peppers (*Capsicum annum* cvs. Quadrato d'Asti and Sumo) were supplied from the CRIOF (Centro Sperimentale Conservazione e Trasformazione degli Orto florofrutticoli), University of Bologna. They were picked at the mature green stage in August. Some fruits were immediately examined for compositional analysis and some were kept for storage. They were held for 7 days in three plywood cabinets at 10 °C, 20 °C and 30 °C and a 85–90 % relative humidity. The temperature was maintained within $\pm 0{\cdot}5$ °C. Fifty randomly selected fruits from each group were analysed. They were cut into small pieces and, after the seeds had been removed, were rapidly comminuted in a high-speed blendor. A sub-sample was taken for each determination. All chemicals were analytical grade and were used as received. All solutions were prepared using doubly distilled water.

Compositional analyses

Fresh or stored homogenised samples were dried in a vacuum oven at 70 °C for 3 h to determine the moisture content (AOAC, 1975). Ascorbic acid was evaluated using the indophenol dye procedure as reported by Gielfrich & Bernard-Griffiths (1975), modified by changing the concentration of aqueous metaphosphoric acid from 4 % to 6 % to obtain a better deproteinisation. This procedure was used because it is simple and used extensively for quality control determinations. Furthermore, this analytical method leads to results for ascorbic acid content similar to those obtained by the 2,4-dinitrophenylhydrazine method, as reported by Matthews & Hall (1978). The extraction technique described by Tonelli *et al.* (1980) was used for protein determination. The method of Lowry *et al.* (1951) was followed to quantify the protein contents and bovine serum albumin was used as a standard. A procedure based on the formation of the Prussian Blue complex was developed to estimate the level of phenolic compounds. A 5 g sample was immediately treated with 2M aqueous HCl in a 1/10 (w/v) ratio and the resulting mixture was heated for 30 min in a 95 °C waterbath. The cooled slurry was filtered through a filter paper into a 250 ml flask.

The residue was rinsed with sufficient water to bring the extract to volume. The diluted solution (3 ml) was transferred to a cuvette of 1 cm path length. Next, 200 μl of 0·008M $K_3Fe(CN)_6$ was added and immediately followed by the addition of 200 μl of 0·1M $FeCl_3$ in 0·1M HCl. The absorbance measurements were made after 5 min at 700 nm with a Perkin Elmer Model 124 spectrophotometer against blanks of identical composition in which the pepper extract was omitted. The incubation temperature was 23 °C ± 0·05 °C. The total phenol content was determined, using catechin as the standard, by constructing a calibration plot for different amounts of catechin. The absorbance at 700 nm was linear with concentrations of catechin up to 10^{-4}M/litre. This colorimetric estimation for total phenols is affected by ascorbic acid. Therefore, the optical density caused by ascorbate is to be subtracted from that obtained by means of the procedure described above. The interference due to ascorbic acid was estimated with a standard curve constructed in the same way as for catechin.

RESULTS AND DISCUSSION

The results of the chemical analyses of fresh and stored peppers are shown in Tables 1 and 2. Fruits of the investigated cultivars differed slightly in chemical composition. Moreover, the results indicated that the effect of storage temperature was quite similar for both varieties. No significant difference in chemical composition was found between fresh peppers and fruits stored at 10 °C. Therefore, this temperature proved to give the best storage stability in this investigation, in agreement with the findings of Ivanova *et al.* (1976). Tables 1 and 2 show that protein, ascorbic acid and phenolic compounds levels increased during storage at 20 °C. These results demonstrated, in agreement with those of Saimbhi *et al.* (1977), that pepper fruits ripened normally during storage at 20 °C. The protein content increased even when the fruits were held at 30 °C. If ripening is considered as a phase of enhanced synthesis (Hulme, 1972), the above observations suggest that ripening capacity is retained even during storage at 30 °C. On the other hand, in such conditions pepper fruits exhibited about the same content of phenolic compounds but were lower in ascorbic acid level compared with those stored at 20 °C. The effect of a higher temperature may possibly result in a higher activity of oxidising enzymes, especially phenolases. The oxidation of the phenolic compounds leads, in turn, by reaction with the intermediate quinones, to the oxidation of ascorbic acid (Monties, 1966); it may explain the slight loss of ascorbic acid occurring when peppers were stored at 30 °C. Storage at high temperature also resulted in a significant decrease in the moisture content. The water content of many food products has an important effect on their market quality requirement. Peppers proved to be highly susceptible to moisture loss, resulting in a reduced yield of saleable commodity due to softening and wilting.

TABLE 1
CHEMICAL COMPOSITION OF 100 g OF FRESH OR STORED PEPPERS (cv. Quadrato d'Asti)

Analysis	*Fresh peppers*			*Peppers stored at 10°C*			*Peppers stored at 20°C*			*Peppers stored at 30°C*		
	Mean	*SD*	(N)*	*Mean*	*SD*	(N)	*Mean*	*SD*	(N)	*Mean*	*SD*	(N)
Moisture (%)	92·8	0·10	(4)	93·5	0·11	(4)	92·5	0·14	(4)	91·0	0·12	(4)
Proteins[a] (mg)	44·1	1·54	(5)	45·2	1·37	(5)	54·1	1·89	(5)	63·8	2·05	(5)
Total phenols[b] (mg)	7·4	0·31	(5)	10·2	0·51	(5)	34·1	1·45	(5)	38·4	1·63	(5)
Vitamin C (mg)	60·3	2·03	(4)	65·2	1·98	(4)	94·5	3·86	(4)	85·3	3·45	(4)

* Standard deviation from mean evaluated from *N* observations.
[a] Calculated as albumin serum bovine equivalent.
[b] Calculated as catechin equivalent.

TABLE 2
CHEMICAL COMPOSITION OF 100 g OF FRESH OR STORED PEPPERS (cv. Sumo)

Analysis	*Fresh peppers*			*Peppers stored at 10°C*			*Peppers stored at 20°C*			*Peppers stored at 30°C*		
	Mean	*SD*	(N)*	*Mean*	*SD*	(N)	*Mean*	*SD*	(N)	*Mean*	*SD*	(N)
Moisture (%)	92·6	0·11	(4)	93·2	0·09	(4)	92·6	0·12	(4)	91·2	0·09	(4)
Proteins[a] (mg)	45·6	1·79	(5)	46·8	1·64	(5)	56·1	1·32	(5)	64·3	1·89	(5)
Total phenols[b] (mg)	9·6	0·58	(5)	10·4	0·45	(5)	38·2	1·87	(5)	40·4	1·51	(5)
Vitamin C (mg)	58·7	1·75	(4)	60·5	2·12	(4)	90·9	2·87	(4)	82·1	2·57	(4)

* Standard deviation from mean evaluated from *N* observations.
[a] Calculated as albumin serum bovine equivalent.
[b] Calculated as catechin equivalent.

ACKNOWLEDGEMENTS

This work was supported in part by the Italian National Research Council (CNR) (Contract 78.00206, Finalised Project 'Containers') and published with CNR permission.

REFERENCES

AOAC (1975). Moisture. In: *Official Methods of Analysis of the Association of Official Analytical Chemists* (12th edn.), Washington, DC, p. 394.

AWASTHI, D. N., GHILDIYAL, P. C. & JOSHI, S. (1976). Ascorbic acid content and its correlation with the age and size of developing fruits of some chilli varieties, *Prog. Hortic.*, **7**(4), 15–18.

GIELFRICH, M. L. & BERNARD-GRIFFITHS, M. A. (1975). Observations sur le dosage de la vitamine C, *Sci. Agron. Rennes*, 53–9.

HULME, A. C. (1972). Proteins of fruits. Their involvements as enzymes in ripening. Review, *J. Food Technol.*, **7**, 343–71.

IVANOVA, E. I., NACHEVNAYA, I. I. & BELYAKOVA, E. V. (1976). Storage of vegetable and cucurbitaceous crops at different temperatures. *Sb. Nauch. Tr. VNII*, 3–4, 53–9 (In Russian) (In *Hortic. Abstr.*, **47** (1977), 575).

KOZUKUE, E. G. & OGATA, K. (1972). Physiological and chemical studies of chilling injury in pepper fruits, *J. Food Sci*, **37**, 708–11.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent, *J. Biol. Chem.*, **193**, 265–75.

MATTHEWS, R. F. & HALL, J. W. (1978). Ascorbic acid, dehydroascorbic acid and diketogulonic acid in frozen green peppers, *J. Food Sci.*, **43**, 532–4.

MONTIES, B. (1966). Nature and chemical properties of the major polyphenols of apples, *Ann. Physiol. Veg.*, **8**, 49–73.

SAIMBHI, M. S., KAUR, G. D. & NANDPURI, K. S. (1977). Chemical constituents in mature green and red fruits of some varieties of chilli, *Qual. Plant.-Plant Foods Hum. Nutr.*, **27**, 171–5.

SHORIKOVA, YU, G., YAKOULEVA, L. A. & ISAGULYAN, E. A. (1977). Polyphenols of sweet peppers, *Fiziol. Biochim. Kul't Rast.*, **9**, 183–9.

SHUKLA, F. C., SAIMBHI, M. S. & SINGH, H. (1975). Postharvest losses in moisture and ascorbic acid contents of some chilli varieties under ordinary storage and dehydration, *Indian Food Packer*, **29**(6), 20–3.

TONELLI, D., BUDINI, R., GATTAVECCHIA, E. & GIROTTI, S. (1980). Extraction techniques of protein from vegetal tissue. Protein content of the ripening tomato fruit, *Annali di Chimica*, **70**(1–2), 43–7.

Food Chemistry 7 (1981) 195–201

EFFECTS OF BENZYLAMINOPURINE ON MANGO FRUIT RIPENING

C. PASSERA & P. SPETTOLI

Universita' degli Studi di Padova, Istituto di Chimica Agraria e Industrie Agrarie, Via Gradenigo 6, 35100 Padova, Italy

(Received: 1 May, 1980)

ABSTRACT

The total and reducing sugars, organic acid and free amino acid contents of the edible portions of mango (cv. Boribo) were determined in unripe and ripe fruits, both treated and untreated with benzylaminopurine (BA).

Total and reducing sugar contents were increased and organic acids and free amino acid contents were decreased after ripening in both cases. Benzylaminopurine delayed ripening and the composition of the ripe fruit was different from that of the untreated control.

INTRODUCTION

Mangoes and other tropical fruits suffer from severe shelf-life problems as a result of post-harvest disease, insect infestation and premature ripening (Beyers *et al.*, 1979).

Several chemicals, including growth regulators, have been used to control ripening, to improve the colour and to reduce losses during storage (Subramanyam *et al.*, 1975). Maleic hydrazide and 2,4,5-trichloroethylphosphonic acid delayed the ripening process but did not alter the chemical composition or quality (Krishnamurthy Shantha & Subramanyam, 1970), while succinic acid, 2,2-dimethylhydrazide, ethephon and chloromequat improved fruit quality in terms of carotenoid content (Krishnamurthy Shantha & Subramanyam, 1973; Subramanyam & Sebastian, 1970).

The main effect of the synthetic growth regulator, benzylaminopurine (BA), is concerned with its ability to delay the phenomena linked to the senescence of leaves of different plant species, such as the degradation of chlorophyll (Osborne & McCalla, 1961; Persona *et al.*, 1957), RNA and DNA (Gunning & Barkley, 1963;

Food Chemistry 0308-8146/81/0007-0195/$02·75

Osborne, 1962) and protein (Richmond & Lang, 1957; Tavares & Kende, 1970; Passera & Albuzio, 1975, 1978).

The purpose of this study was to determine the changes in the concentration of total and reducing sugars, organic acids and free amino acids which occur during the ripening of mango fruits and the effect induced on such constituents by treatment with benzylaminopurine.

MATERIALS AND METHODS

Freshly picked, unripe olive green mangoes (cv. Boribo) were supplied by a farm in Genale, Somaliland. Fruits of uniform size and of the same age were divided into three batches, each of ten pieces. A batch of fruits was immediately analysed, while the other two batches were held for 6 h in 8 litres of distilled water or in 8 litres of 2×10^{-4}M benzylaminopurine solution. Thereafter, fruits were blotted by filter paper and stored at room temperature (28–22 °C day/night) until they softened. Physical changes—for example, colour—were evaluated visually and texture was noted by pressing the fruit with the fingers. The skin colour became yellow, the pulp turned orange and the flesh ripening was uniform in all fruits. Differences in colour and flavour were not observed between BA treated and untreated fruits. The inedible parts (skin, seed) were removed. The edible portions of all ten fruits were combined, chopped into small pieces, mixed thoroughly and aliquots of 25 g were homogenised for 5 min in 100 ml of 80% ethanol. Homogenates were centrifuged and the supernatants collected. Pellets were extracted with 20% ethanol and recentrifuged. Ethanolic extracts were combined and concentrated to 5 ml *in vacuo* at 45 °C. The concentrate, diluted with distilled water to 50 ml, was passed through a column (1×10 cm) of IR-120 resin, 100–200 mesh, in H^+ form and then through a column of IRA-400 resin in $HCOO^-$ form. The amino acids were eluted from IR resin with 120 ml of 2N NH_4OH and organic acids were eluted from IRA-400 resin with 120 ml of 4N HCOOH (Ferrari *et al.*, 1963). The percolate of anionic resin contained sugars. The eluates of both columns were evaporated to dryness *in vacuo* at 45 °C. Amino acids were dissolved in Na–citrate buffer, pH 2·87, and analysed by means of a Technicon amino acid analyser. Organic acids and sugars were dissolved in anhydrous pyridine.

Gas–liquid chromatography was used to identify and quantitate sugars in fruits, with erythritol as the internal standard and hexamethyldisilazane (HMDS) and trifluoroacetic acid (TFA) (Pierce Chemical Co.) as the silylating agents. One millilitre of the pyridine solution containing sugars and erythritol was added to 0·9 ml of HMDS and 0·1 ml of TFA mixture and allowed to react for 15 min (Bosi, 1973). One microlitre of silylated mixture was injected into the gas chromatograph. A Varian Model 1840-1 with a flame ionisation detector was used. The carrier gas

(nitrogen) flow rate was 35 ml/min. A stainless steel JXR 3% column, 2 m × 3 mm internal diameter, was used. Column temperature was programmed to increase from 100 °C to 215 °C at a rate of 6 °/min, then to remain at 275 °C with a detector temperature of 300 °C and the injector also at 275 °C.

Organic acids were tested by GLC. Silylation of 1 ml of pyridine solution containing organic acids and erythritol was carried out by adding 0·25 ml of HMDS and 0·10 ml of TFA and allowing the reaction to proceed for 15 min at 60 °C. Two microlitres of silylated solution were used for chromatographic analysis. The other conditions were as follows: column, JXR 3%; injector and detector temperature, 200 and 250 °C, respectively; column temperature programmed from 85 °C to 250 °C at a rate 6 °/min: nitrogen carrier flow, 35 ml/min.

RESULTS

Mango fruits, treated and untreated with BA, ripened at 7 and 5 days after harvest, respectively.

During ripening, a drastic increase in total sugars was observed: in the unripe mangoes total sugars were 4% whereas, in ripe fruits, they were 14% of the fresh weight. At picking, reducing sugars were higher than sucrose, but at ripening the latter increased at a much faster rate. BA did not affect the level of total sugars but it did enhance the content of sucrose and decreased that of reducing sugars (Table 1).

TABLE 1
TOTAL AND REDUCING SUGAR CONTENTS (% FRESH WEIGHT) IN UNRIPE AND RIPE MANGO FRUITS TREATED AND UNTREATED WITH BENZYLAMINOPURINE (BA)

Sugar	*Unripe (at harvest)*	*Edible ripe*	
		Control	*BA*
α-Glucose	1·33	2·26	1·77
β-Glucose	0·87	1·32	0·80
Fructose	0·65	1·34	0·53
Sucrose	1·22	9·56	11·30
Total	4·07	14·48	14·40

Organic acids decreased from 766 mg/100 g (fresh weight) in the green fruits to 498 and 230 mg/100 g (fresh weight) in the ripe fruits treated and untreated with benzylaminopurine, respectively. Citric, pyruvic and oxalic acids were identified in unripe mangoes. Of these, citric acid was predominant but its level decreased at ripening. Succinic and malic acids were also present in the fruits treated with BA. In addition, citric acid in BA-treated mangoes was higher than in untreated ones (Table 2).

TABLE 2
ORGANIC ACID CONTENTS (mg/100 g FRESH WEIGHT) IN UNRIPE AND RIPE MANGO FRUITS TREATED AND UNTREATED WITH BENZYLAMINOPURINE (BA)

Acid	*Unripe (at harvest)*	*Edible ripe*	
		Control	*BA*
Oxalic acid	1·75	2·00	1·25
Pyruvic acid	0·22	1·00	0·12
Succinic acid	traces	traces	4·00
Malic acid	—	—	16·00
Citric acid	765·00	227·50	477·50
Total	766·97	230·50	498·87

Seventeen amino acids were identified in ripe and unripe fruits, only four (arginine, alanine, glutamic and aspartic acids) being present at high concentrations. The ripening process was associated with a decrease of all the free amino acids, except alanine, ornithine and glycine, which increased, and threonine, which was unchanged. Arginine, aspartic and glutamic acids were the main factors in determining the decrease of the amino acid level (Table 3).

The content of amino acids was reduced by the BA treatment. Moreover, BA treatment affected alanine enhancement during ripening.

TABLE 3
FREE AMINO ACIDS (mg/100 g FRESH WEIGHT) IN UNRIPE AND RIPE MANGO FRUITS TREATED AND UNTREATED WITH BENZYLAMINOPURINE (BA)

Amino acids	*Unripe (at harvest)*	*Edible ripe*	
		Control	*BA*
Aspartic	7·54	0·12	0·26
Threonine	1·06	1·04	0·92
Serine	1·73	1·46	1·46
Glutamic	9·91	2·07	3·53
Proline	1·82	1·45	0·53
Glycine	0·17	0·52	0·26
Alanine	7·56	14·96	10·95
Valine	0·96	0·50	1·02
Iso-leucine	0·23	0·18	0·13
Leucine	0·30	0·14	0·12
Tyrosine	0·36	0·28	0·41
Fenylalanine	0·75	0·12	0·12
γ-Amino-*n*-butyric acid	0·18	0·10	0·12
Ornithine	0·20	1·97	1·69
Lysine	1·36	0·83	0·38
Histidine	0·50	0·34	0·22
Arginine	33·53	26·34	12·36
Total	68·16	52·42	34·72

DISCUSSION

The ripening of mango fruits involves several metabolic activities resulting in colour changes of the skin of the edible portion, as well as the development of a sweet taste and a pleasing flavour. These alterations are accompanied by changes in chemical composition.

During the ripening of Boribo mango fruits, the free amino acid content decreases, while the number of amino acids is unchanged. These results differ from those found in Alphonso mango where ripening induces a reduction of the free amino acid content, some amino acids remaining and some disappearing (Subramanyam *et al.*, 1975). The observed fall in the concentration of free amino acids is consistent with the increase in protein synthesis found at ripening (Passera & Mohamed, 1978; Hansen, 1970). Changes in the total number of amino acids were also observed in some Pakistani mango varieties (Elahi & Khan, 1973).

γ-Aminobutyric acid, one of the most representative amino acids in the Alphonso cultivar, is present in small amounts in Boribo mango. Furthermore, unlike the Alphonso mango, the γ-aminobutyric acid level of the Boribo cultivar does not increase throughout the ripening period. Therefore, if the relationship among the oxo-acids and enzyme activities suggested for Alphonso mango is valid (Krishnamurthy & Subramanyam, 1971), γ-aminobutyric acid must be metabolised as soon as it is synthesised.

It has been reported that carbon from γ-aminobutyric acid goes very rapidly into the intermediates of the carboxylic acid cycle (Inatomi & Slaughter, 1971).

The arginine level, which also decreases during ripening, is related to the glutamic acid metabolism (Bryan, 1976). In addition, the arginine content is decreased by BA.

The increase of alanine, also observed in the ripening of Alphonso mango (Krishnamurthy *et al.*, 1973) may be interpreted as being due to a higher availability of pyruvate, because this oxo-acid may regulate the synthesis of alanine (Bryan, 1976). Since, during ripening, the activity of the malic enzyme increases (Krishnamurthy *et al.*, 1971), the lower increase of alanine and the higher content of malic acid of BA-treated fruits support the hypothesis that the phytoregulator inhibits malic enzyme activity.

The BA treatment also affects succinic and citric acid contents, presumably because the phytoregulator increases substrate entering the tricarboxylic acid cycle (Moore & Miller, 1972). This is supported by the fact that the TCA enzymes are stimulated by BA (Servettaz *et al.*, 1976).

Many of the enzymes present during the ripening of fruits possess hydrolytic activity—for example, the enzymes involved in the degradation of starch (Rhodes & Wooltorton, 1967). In mango, while invertase and amylase activities increase, the level of starch falls (Mattoo & Modi, 1969). This finding is in agreement with our results since hexose and non-reducing sugars increase at ripening. BA affects the ratios among sugars but not their total content, although kinetins are known to be

involved in the mobilisation of starch reserves (Berridge & Ralph, 1971) and in promoting amylase activity (Gepstain & Ilan, 1970).

REFERENCES

BERRIDGE, M. V. & RALPH, R. K. (1971). Kinetin and carbohydrate metabolism in Chinese cabbage. *Plant Physiol.*, **47**, 562–7.

BEYERS, M., THOMAS, A. C. & VAN TONDER, A. J. (1979). γ-irradiation of subtropical fruits. 1. Composition Tables of mango, papaya, strawberry, and litchi fruits at the edible-ripe stage. *J. Agr. Food Chem.*, **27**, 37–42.

BOSI, G. (1973). Mèthode rapide pour la determination par chromatographie en phase gazeuse des glicides du nectar: technique de prèlèvament du nectar et de prèparation des èthers triméthylsilyles en présence d'eau. *Apidologie*, **4**, 57–64.

BRYAN, J. K. (1976). Amino acid biosynthesis and its regulation. In: *Plant biochemistry*. (3rd edn.) (Bonner, J. & Varner, J. E. (Eds)), New York, San Francisco, London, Academic Press, 525–60.

ELAHI, M. & KHAN, N. (1973). Physicochemical changes in some Pakistani mango varieties during storage ripening. *J. Agr. Food Chem.*, **21**, 229–31.

FERRARI, G., PASSERA, C. & CULTRERA, R. (1963). Ricerche sul meccanismo di sintesi degli amminoacidi nei vegetali. Nota I: Il cammino del C^{14} nella formazione degli amminoacidi in Chlorella vulgaris. *Ric. sci.*, **33**(II-B), 181–8.

GEPSTAIN, S. & ILAN, I. (1970). A possible action of kinetin on amylase activity in cotyledons of *Phaseolus vulgaris*. *Plant Cell Physiol.*, **11**, 819–22.

GUNNING, B. E. S. & BARKLEY, W. K. (1963). Kinin-induced directed transport and senescence in detached leaves. *Nature*, **199**, 262–5.

HANSEN, E. (1970). Proteins. In: *Food science and technology*. Vol. 1 (Hulme, A. C. (Ed.)), London, New York, Academic Press, 147–57.

INATOMI, K. & SLAUGHTER, J. C. (1971). The role of glutamate decarboxylase and γ-aminobutyric acid in germinating barley. *J. Expt. Bot.*, **22**, 561–71.

KRISHNAMURTHY, SHANTHA & SUBRAMANYAM, H. (1970). Effect of malic hydrazide and 2,4,5-trichlorophenoxypropionic acid on ripening and quality of mango fruit. *Pestic. Sci.*, **1**, 63–5.

KRISHNAMURTHY, SHANTHA, PATWARDHAN, M. V. & SUBRAMANYAM, H. (1971). Biochemical changes during ripening of the mango fruit. *Phytochemistry*, **10**, 2577–81.

KRISHNAMURTHY, SHANTHA & SUBRAMANYAM, H. (1973). Pre- and post-harvest physiology of mango fruit: A review. *Trop. Sci.*, **15**, 167–93.

MATTOO, A. K. & MODI, V. V. (1969). *Proc. Int. Conf. Subtrop. Fruits*, London, p. 111.

MOORE, JR., T. S. & MILLER, C. O. (1972). Effects of cytochinins on the respiration of soybean callus tissue. *Plant Physiol.*, **50**, 594–8.

OSBORNE, D. J. (1962). Effect of kinetin on protein and nucleic acid metabolism in *Xanthium* leaves during senescence. *Plant Physiol.*, **37**, 595–602.

OSBORNE, D. J. & MCCALLA, D. R. (1961). Rapid bioassay for kinetin and kinin using senescing leaf time. *Plant Physiol.*, **36**, 219–21.

PASSERA, C. & ALBUZIO, A. (1975). Effetto della benzilaminopurina sulle reazioni di fissazione della CO_2 in foglie di orzo. *Agrochimica*, **XIX**, 480–90.

PASSERA, C. & ALBUZIO, A. (1978). Benzylaminopurine induced changes in photosynthesis and photorespiration of barley plants. *Biologia Plantarum (Praha)*, **20**(4), 274–80.

PASSERA, C. & MOHAMED, MUCTAR, ALI (1978). Variazione della composizione chimica durante la maturazione del mango. *Rivista di Agricoltura Subtropicale e Tropicale*, **LXXII**, 119–24.

PERSONA, C., SAMBOSKI, D. J. & FORSYTH, F. R. (1957). Effect of benzimidazole on detached wheat leaves. *Nature*, **180**, 1294–5.

RICHMOND, A. E. & LANG, A. (1957). Effect of kinetin on protein and survival of detached *Xanthium* leaves. *Science*, **125**, 650–1.

RHODES, M. J. C. & WOOLTORTON, L. S. C. (1967). The respiration climateric in apple fruits. The action of hydrolitic enzymes in peel tissue during the climateric period in fruit detached from the tree. *Phytochemistry*, **6**, 1–12.

SERVETTAZ, O., CORTESE, F. & LONGO, C. P. (1976). Effect of benzyladenine on some enzymes of mitochondria and microbodies in excised sunflower cotyledons. *Plant Physiol.*, **58**, 569–72.

SUBRAMANYAM, H. & SEBASTIAN, K. (1970). Effect of succinic acid 2,2-dimethylhydrazide on carotene development in Alphonso mango. *Hort. Sci.*, **5**(3), 160–1.

SUBRAMANYAM, H., KRISHNAMURTHY SHANTA & PARPIA, H. A. B. (1975). Physiology and biochemistry of mango fruit. In: *Advances in food research*. (Chichester, C. O., Mrak, E. M. & Stewart, G. F. (Eds)), New York, San Francisco, London, Academic Press, 223–305.

TAVARES, J. & KENDE, H. (1970). The effect of 6-benzylaminopurine on protein metabolism in senescing corn leaves. *Phytochemistry*, **9**, 1763–9.

Food Chemistry 7 (1981) 203–217

A BRIEF REVIEW OF THE RÔLE OF CARBOHYDRATE IN RELATION TO CORONARY HEART DISEASE, STRESS, NEUROTRANSMITTERS AND BRAIN FUNCTION

L. F. GREEN

12, Broad Reaches, Ludham, Great Yarmouth, Norfolk NR29 5PD, Great Britain

&

I. WINCKLER

53, Cambridge Crescent, Teddington, Middlesex TW1 1BDX, Great Britain

(Received: 6 May, 1980)

ABSTRACT

Food is mostly composed of protein, fat and carbohydrate. Carbohydrates are divided into four main groups: polysaccharides, disaccharides, monosaccharides and pentoses, but certain other rare carbohydrates are mentioned. The digestion process is described and the difference of sucrose from other carbohydrates in yielding fructose as one of its metabolic products is shown to be suspected as contributing to coronary heart disease.

The importance of D-*glucose in maintaining physical efficiency is well established, but mental acuity is shown also to be carbohydrate linked. The relationship between carbohydrate ingestion, stress and brain serotonin levels is discussed. The need for interdisciplinary studies to unravel the complexities of such relationships is emphasised and some of the particular problems in connection with them are mentioned.*

INTRODUCTION

The major components of most foods are: protein, built from amino acids, of the general formula $R.CH(NH_2)COOH$, linked together by peptide bonds but of extremely complex structure; fats, such as palmitic acid, $CH_3(CH_2)_{14}COOH$, stearic acid, $CH_3(CH_2)_{16}COOH$ and oleic acid, $CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$,

Food Chemistry 0308-8146/81/0007-0203/$02·75

and carbohydrates. Carbohydrates are frequently denigrated because they are alleged to contribute nothing but energy and replace the intake of more essential ingredients, but they *are* a necessary part of human nutrition and affect metabolism more profoundly than might at first be suspected. Carbohydrates can be divided into four groups as follows.

Group 1—The polysaccharides; large molecules comprised of hexose polymers, such as starch and glycogen $(C_6H_{10}O_5)_n$ which occur in vegetable foods and animal residues, respectively. Although represented by the foregoing conventional formula, these differ considerably, depending on their source. There are also the hydrolysates of starch (glucose syrups) made by the action of acid and/or enzymes, which are complex mixtures ranging from very high molecular mass saccharides to the simple monosaccharide, D-glucose. The proportion of constituents in such hydrolysates depends on the conditions and mode of hydrolysis.

Group 2—The disaccharides $(C_{12}H_{22}O_{11})$ containing two component monosaccharides, of which the most common are sucrose (Fig. 1), occurring in the sugar cane and in sugar beet, lactose (Fig. 2), occurring in milk, and maltose (Fig. 3) which is formed by the malting of barley starch.

Group 3—This group comprises the monosaccharides $(C_6H_{12}O_6)$ of which the most important are D-glucose (Fig. 4) and D-fructose (Fig. 5) occurring in fruit and vegetables (Shallenberger, 1974*a*).

Group 4—made up of the pentoses $(C_6H_{10}O_5)$ such as D-ribose (Fig. 6) and 2-deoxy-D-ribose (Fig. 7); these do not substantially exist in their free forms in foods. The pentoses do, however, occur in nucleic acids—for example, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA)—which are macromolecules with molecular masses of millions; in high energy compounds such as adenosine

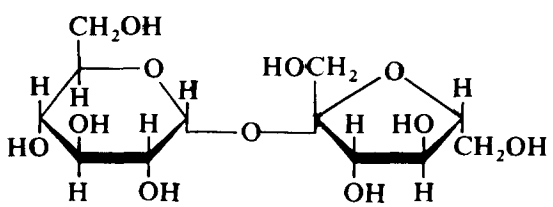


Fig. 1. Sucrose; β-D-fructofuranosyl-α-D-glucopyranose (Pigman & Horton, 1970*a*).

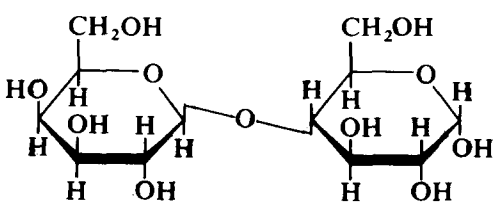


Fig. 2. Lactose; 4-O-β-D-galactopyranosyl-α-D-glucopyranose (Pigman & Horton, 1970*b*).

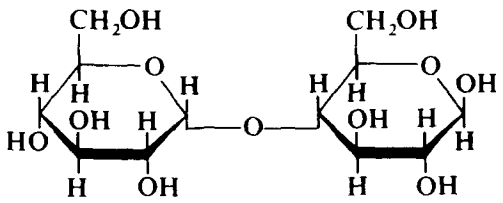


Fig. 3. Maltose; 4-O-α-glucopyranosyl-β-D-glucopyranose (Pigman & Horton, 1970*c*).

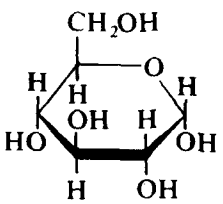


Fig. 4. D-glucose; α-D-glucopyranose (Pigman, 1957*a*).

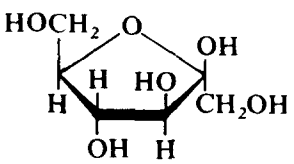


Fig. 5. D-fructose; β-D-fructofuranose (Pigman, 1957*b*).

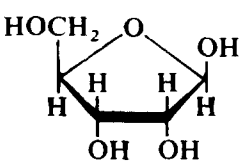


Fig. 6. D-ribose; β-D-ribofuranose.

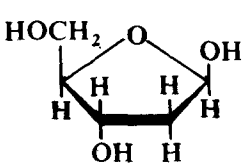


Fig. 7. 2-Deoxy-D-ribose; 2-deoxy-β-D-ribofuranose.

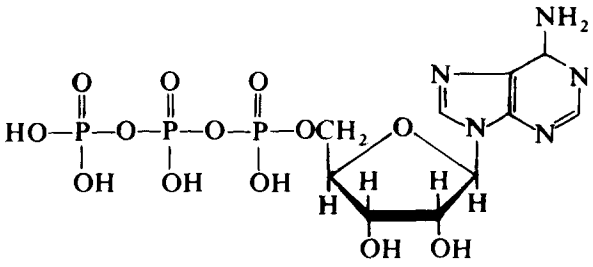


Fig. 8. Adenosine triphosphate (ATP).

triphosphate (ATP) (Fig. 8), although its carbohydrate contribution to the diet is negligible; and in certain polysaccharides such as the pentosans of fruit and gums.

Certain other carbohydrates occasionally find their way into the human diet. For example, the disaccharide, α-α-trehalose (α-D-glucopyranosyl-α-D-glucopyranoside), containing two glucose units, found in young mushrooms. The tetrasaccharide, stachyose, *O*-α-D-galactopyranosyl-(1 → 6)-*O*-α-D-galactopyranosyl-(1 → 6)-*O*-α-D-glucopyranosyl-(1 → 2)-β-D-fructofuranoside (Shallenberger, 1974*b*) is ingested in 'Japanese artichokes', but cannot be hydrolysed by intraluminal or intestinal enzymes and absorbed, and therefore passes to the lower small intestine and colon, where it is fermented by bacteria to yield two- and three-carbon fragments, hydrogen and carbon dioxide (Cristofaro *et al.*, 1974).

The digestion of carbohydrates starts off in the mouth, where salivary α-amylase is mixed with incoming food, thereby initiating the conversion of starch and dextrins. The digestion continues in the stomach until the hydrochloric acid present destroys the amylase activity and substitutes acid hydrolysis for enzyme splitting. The stomach contents then empty into the small intestine where enzyme hydrolysis again supervenes. Pancreatic α-amylase continues the splitting process, but final hydrolysis to monosaccharides takes place in the wall of the small intestine. Specific processes occur (Gray, 1975): the hydrolysis of sucrose by the enzyme sucrose-α-dextrinase yields D-glucose and D-fructose:

$$\underset{\text{Sucrose}}{C_{12}H_{22}O_{11}} + H_2O \rightarrow \underset{\text{D-glucose}}{C_6H_{12}O_6} + \underset{\text{D-fructose}}{C_6H_{12}O_6}$$

whereas the hydrolysis of lactose, by means of the enzyme β-galactosidase, gives D-glucose and D-galactose (Fig. 9):

$$\underset{\text{Lactose}}{C_{12}H_{22}O_{11}} + H_2O \rightarrow \underset{\text{D-glucose}}{C_6H_{12}O_6} + \underset{\text{D-galactose}}{C_6H_{12}O_6}$$

A third enzyme, α-glucosidase, hydrolyses maltose to two molecules of D-glucose:

$$\underset{\text{Maltose}}{C_{12}H_{22}O_{11}} + H_2O \rightarrow 2\underset{\text{D-glucose}}{C_6H_{12}O_6}$$

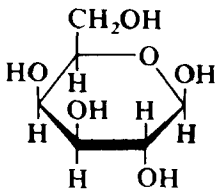


Fig. 9. D-galactose; β-D-galactopyranose.

The complete hydrolysis of starch and glucose syrups gives D-glucose only. Thus, it is significant that sucrose is the only major sugar to yield D-fructose as one of its products of hydrolysis. However, a new variety of glucose syrup has been developed in which about half of the D-glucose present has been converted enzymatically to D-fructose so that, to all intents and purposes, invert sugar is formed, i.e. an approximately equal mixture of D-glucose and D-fructose. When ingested, the D-fructose present must certainly follow a similar metabolic pathway to that derived from the intake of sucrose. (It should be noted that the term 'glucose syrup', as used in this paper, does not include such fructose-containing syrups.)

CLINICAL SIGNIFICANCE

Because of the different metabolic pathways necessary for the utilisation of D-glucose and D-fructose, it has become increasingly certain that the type of carbohydrate ingested may be of importance, and much clinical investigation has been undertaken since the early work of Bennett & Dodds (1925), Butterfield *et al.* (1964) and Dodds *et al.* (1959).

D-Glucose is rapidly absorbed, resulting in an increase in blood sugar concentration and, thereby, a release of insulin. The function of insulin is to increase the uptake of glucose in the muscles and liver. In consequence, the early rise in post-prandial blood sugar concentration is followed by a gradual return to normal blood sugar values.

In contrast, both D-fructose and D-galactose require transformation in the liver to glucose or glycogen, which serves as a reserve for D-glucose, before they are available as utilisable sources of energy. D-glucose is 'the only hexose known to exist in the free state in the fasting human body' (Davidson *et al.*, 1972*a*). The greater proportion of it is stored in the liver and the muscles as glycogen, but a highly significant portion is in the blood.

CORONARY HEART DISEASE

D-Glucose may be converted to fat and be stored in fat depots, and can lead to elevated serum triglyceride levels (Fredrickson *et al.*, 1937; Ahrens *et al.*, 1961).

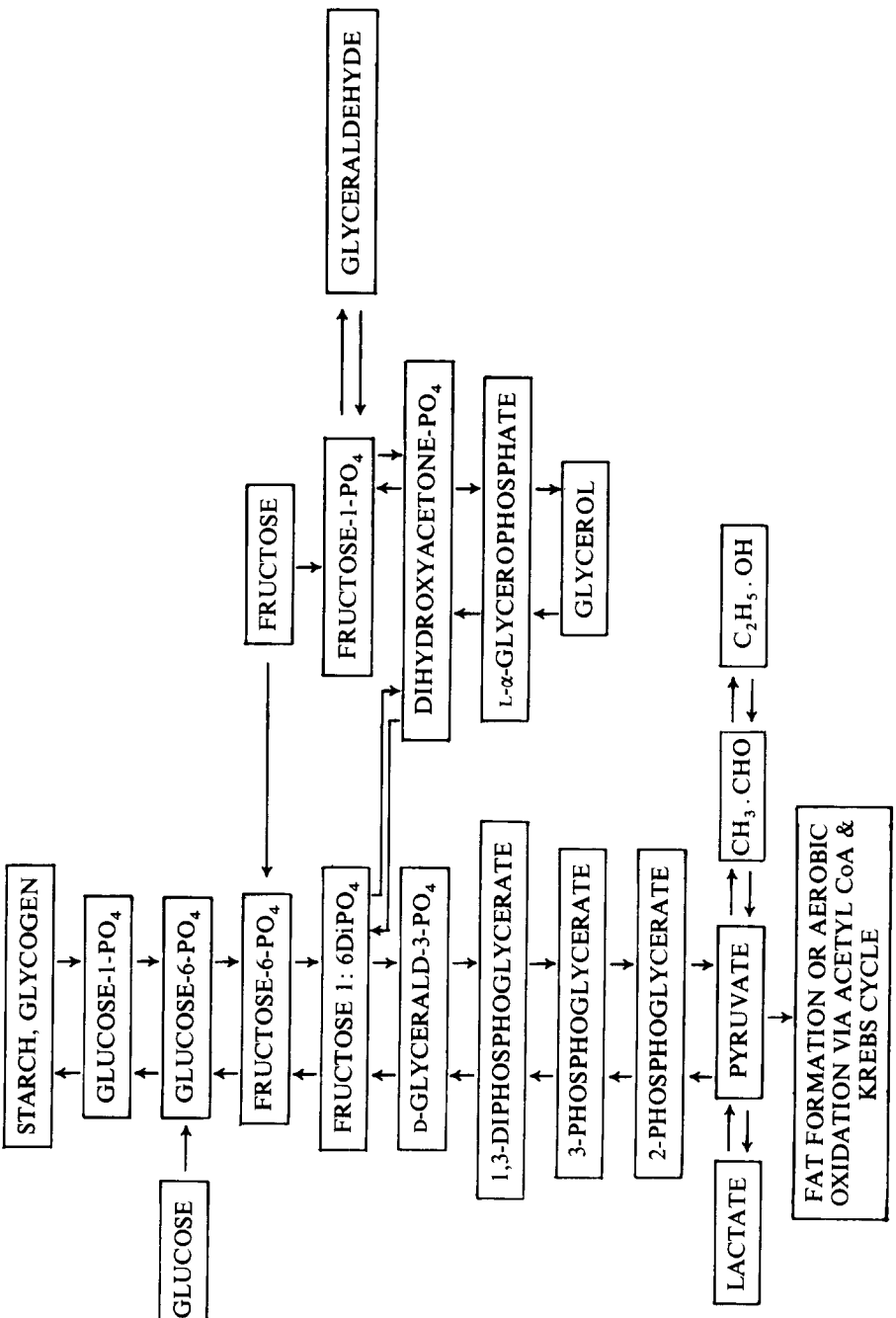


Fig. 10. Metabolic pathway of starch, glycogen, glucose and fructose.

D-fructose appears to raise serum triglyceride levels more than D-glucose (Macdonald, 1967), perhaps because D-glucose needs to be converted to D-glucose-6-phosphate and then to D-fructose-6-phosphate before being convertible to glycerol phosphate, glycerol and thence to fat (Fig. 10).

These considerations have been taken to imply that sucrose is implicated in the development of coronary heart disease (Yudkin, 1957; Yudkin, 1963; Yudkin, 1964; Yudkin, 1971) although it is fair to say that Yudkin's theories have been strongly criticised (Keys, 1971; Walker, 1971). D-fructose—and hence sucrose—appears, then, to produce different lipid effects from D-glucose, and it may not be too extravagant to suggest that it also produces different neurological effects.

D-Glucose is dissipated in all tissues of the body to yield 3·75 kcal (15·69 kJ)/g energy compared with protein at 9·3 kcal (38·91 kJ)/g and fat at 4·1 kcal (17·15 kJ)/g (McCance & Widdowson, 1960). Blood glucose is the sole fuel of the brain (except in starvation situations) and the central nervous system, the former requiring about 144 g per diem (Bell *et al.*, 1972), and recent investigations have indicated that D-glucose may play a very important rôle additional to that of providing energy and the build-up of glycogen. 'Nearly one-fifth of the total basal metabolism takes place in the brain. Nervous tissue seems to have no important store or reserve of energy and is, therefore, immediately dependent on the level of glucose in the blood for its continued activity. If the glucose in the blood is suddenly reduced . . . changes in the nervous activity quickly follow. However, in starvation, the brain can utilise other fuels' (Davidson *et al.*, 1972*b*) such as carbohydrate provided by protein conversion and keto-acids, particularly β-hydroxybutyric acid (Davidson *et al.*, 1972*c*), and some investigators are becoming concerned with the effect of use or restriction of use of the provision of D-glucose in these rôles, upon human behaviour (Brooke, 1973; White, 1978).

STRESS AND MENTAL ACUITY

There are a large number of areas in which the rapidity of response to external stimuli and, therefore, brain function, is extremely important, if not vital. There is a whole gradation of efficiency levels in almost every daily job of work, which, at some point, becomes uneconomical. Some idea of these considerations can be seen in a complex diagram given by Passot (1971). Some of the areas in which carbohydrate and, in particular, D-glucose, have been suggested as playing an important part are as follows.

Accidents in factories. There are 300,000 accidents reported each year in factories and on construction sites (Davies, 1972). Evidence has been offered that ingestion of a glucose syrup drink reduced the accident rate in the first morning shift at a foundry (Brook, 1973).

Motor racing. The levels of noradrenaline, adrenaline, free fatty acids,

triglycerides and cholesterol have been studied in motor-racing drivers (Taggart & Carruthers, 1970) because, in their view, this sport provided extremely emotional and aggressive situations with adrenaline release, whereas anger caused noradrenaline release (Carruthers, 1971). Adrenaline is antagonistic to insulin because 'it increases cyclic AMP (adenosine monophosphate) in the liver, this activates lactate formation and promotes lipolysis in adipose tissue. It inhibits insulin secretion' (Wright, 1971). Hence, factors studied by Taggart and Carruthers may be related to carbohydrate ingestion.

Car driving. Creatinine, 11-hydroxycorticosterol and catecholamine urinary secretions have been studied in 'normal' subjects and in others with coronary heart disease (Bellit *et al.*, 1969). 'Normal' heart rates were increased by car driving in busy streets and maximal lipid response was triggered by relatively low plasma catecholamine levels which were attained during driving in traffic (Taggart *et al.*, 1969). Again, a blood glucose level elevated by the ingestion of carbohydrate may affect such factors.

Heart attack. A link has been suggested between 'stress' and 'atheroma' (Taggart & Carruthers, 1970).

Accidents. Car and lorry accidents may be related to low blood glucose levels (Christian, 1972). There is little evidence whether or not long distance lorry drivers, especially on M-roads, suffer from 'stress', but many drivers eat spasmodically and may embark on their monotonous journeys with low blood glucose levels. On the other hand, van drivers, especially during town deliveries, must be subjected to high 'stress'. Accidents and fatigue in small sailing boats have been discussed (Bennet, 1972) and it has been reported that meals are often irregular and infrequent due to the many exigencies of sailing.

A relationship appears to exist between the ingestion of carbohydrate (and hence elevated blood glucose) and the ability to cope with stressful situations. In a number of sports: canoeing (Green & Bagley, 1970); cycling (Brooke *et al.*, 1975); football (Muckle, 1973) and badminton (Green & Bagley, 1974), it has been shown that the ingestion of carbohydrate, carefully regulated in relation to energy output, can improve the quality of performance in the sport under study, but it has been suspected that a 'raised blood glucose level may benefit neural function in the physical work performance' (Brooke *et al.*, 1975). Mental acuity is important in many fields of sports activity and 'the capacity to perform certain skilled tasks associated with sports may be enhanced by supplementation of glucose . . .' (White, 1978); to secure an immediate response to the starter's gun; to determine at what point a long-distance runner requires to put on that extra spurt; to shoot accurately at the goal; to fire accurately at the bull's-eye after a long ski-run in pentathlon events—to name a few; but there are many daily occupations where mental acuity is equally—if not more—important, and where the dangers of error are enormous. In a recent study, a relationship between the type of carbohydrate ingested and the ability to answer questions was established (Birch, 1974; Birch *et al.*, 1977). It is not

yet apparent where, exactly, D-glucose fits into the process of nerve-impulse transmission, other than as an energy source, but there is a growing accumulation of information concerning brain tryptophan ($C_{11}H_{12}N_2O_2$) and serotonin ($C_{11}H_{10}N_2$, 5-hydroxytryptamine) levels and their relationship with carbohydrate ingestion. Mental depression has been said to be associated with a deficit in brain serotonin (Lapin & Oxenkrug, 1969; Coppen, 1972).

NEUTROTRANSMITTERS

It has been shown that elevated serum tryptophan levels result when rats are fed carbohydrate (Fernstrom & Wurtman, 1971). About 90% plasma tryptophan is bound to albumin and the remainder is present in the free form. Free plasma tryptophan levels have been shown to affect brain tryptophan and serotonin levels (Wurtman & Fernstrom, 1972) and L-tryptophan has been used in the treatment of anxiety states, although its use, even in large doses, as an antidepressant, has been disappointing, perhaps because of the rapid breakdown of about 90% in the body to nicotinic acid (Young & Sourkes, 1974), which is not beneficial as an antimanic agent. Tryptophan is one of the essential amino acids; it cannot be synthesised by the brain or by any other tissue. It must be provided by food which, on average, provides 0·5 to 2·0 g per diem. In order to gain access to the brain, it is suggested (Fernstrom, 1977) that it must compete with the other neutral amino acids of the plasma: leucine $(CH_3)_2 . CH . CH_2 . CH(NH_2)COOH$, *iso*-leucine $(CH_3)(C_2H_5)CH(NH_2)COOH$, valine $(CH_3)_2CH . CH(NH_2)COOH$, phenylalanine $C_6H_5 . CH_2 . CH(NH_2)COOH$ and tyrosine, $HO . C_6H_4 . CH_2 . CH(NH_2)COOH$, for binding to a specific carrier protein; so that its rate of entry into the brain is a function of the ratio of tryptophan to neutral amino acids concentrations (Wurtman & Fernstrom, 1974, 1975). Procedures which raise serum tyrosine levels reduce the amount of tryptophan in the brain, and an increase in serum tryptophan or leucine inhibits tyrosine uptake by the brain (Guroff, 1962). Hence, any agent which favours a higher tryptophan/neutral amino acids ratio ought to favour brain serotonin synthesis.

The mammalian brain is a complex organ containing many distinct populations of cells, including neurons. Each neuron contains distinct regions which possess particular functions: the dendrites, the cell body (protein synthesising portion), the axon and the boutons (the transmitting portions). Within the neuron, the flow of information is polarised from the dendrites through the cell body and the axon to the boutons. The junction across which the boutons of one neuron transmit signals to the dendrites of a second neuron is known as the synapse, and certain compounds (the neurotransmitters) are responsible for the transmission of signals across the synaptic junction. Currently, four compounds are recognised as brain neurotransmitters: acetylcholine $(CH_3)_3NH . CH_2 . CH_2 . O . COCH_3$, dopamine (Fig. 11), norepinephrine or noradrenaline (Fig. 12) and serotonin (Fig. 13). Recent

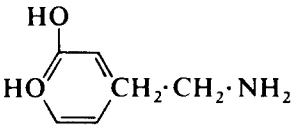


Fig. 11. Dopamine.

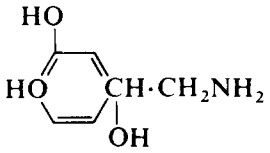


Fig. 12. Noradrenaline (norepinephrine).

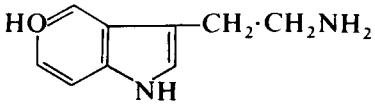


Fig. 13. Serotonin.

work has shown that the last compound is profoundly affected by the consumption of foods, especially carbohydrate. As this depends on its ability to stimulate insulin secretion and to depress plasma fatty acids, different carbohydrates, in particular D-glucose and D-fructose, may well exert different effects according to their ability to alter insulin and fatty acid levels.

Rats, injected with insulin, showed raised brain tryptophan and brain serotonin levels (Fernstrom & Wurtman, 1971). The consumption of almost any meal is followed by the release of insulin, so that eating could be a major indirect stimulus of serotonin synthesis. When fasting rats were fed a carbohydrate meal to which 18% protein had been added, plasma tryptophan rose to a higher level than that induced by carbohydrate alone, but there was no additional rise in brain tryptophan or serotonin levels (Fernstrom & Wurtman, 1973).

L-tryptophan follows two distinct metabolic routes:

1. L-tryptophan → nicotinic acid

through many intermediate stages (Fig. 14). It is potentiated by a rate-limiting, haem (iron-protoporphyrin IX)-dependent enzyme, tryptophan pyrollase.

2. L-tryptophan → serotonin (Fig. 15)

It has been suggested that D-glucose inhibits the action of tryptophan pyrollase in the first route, thus leading to increased serotonin formation (Badaway & Evans, 1976). D-fructose does not inhibit the action, whilst sucrose is intermediate in its effect.

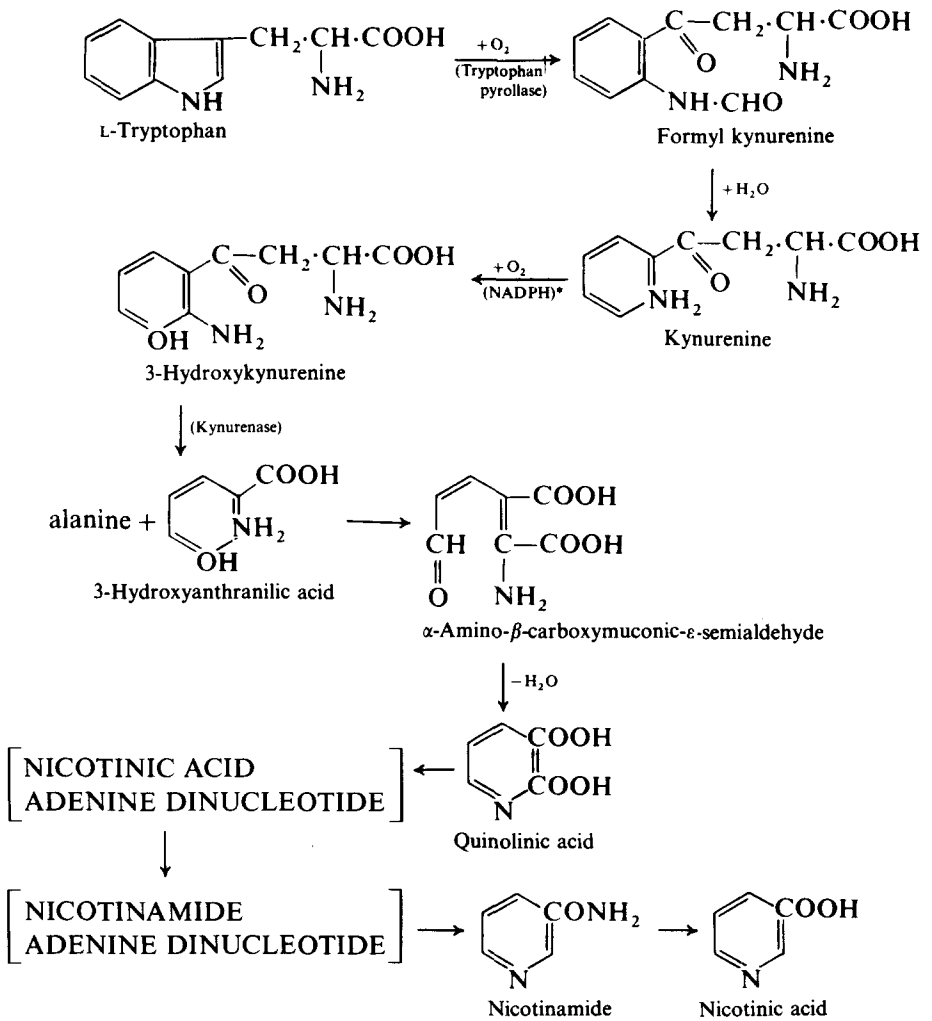


Fig. 14. Conversion of L-tryptophan to nicotinic acid.* NADPH = reduced nicotinamide-adenine-dinucleotide-phosphate.

The second route, in its final reaction, is catalysed by aromatic-L-amino acid decarboxylase, producing serotonin; thus, brain tryptophan is the precursor of brain serotonin.

Many workers have demonstrated the essential rôle of serotonin in the regulation of mood, and the implication of serotonin in many anxiety states such as depression, schizophrenia and anorexia nervosa, in alcoholism and in chronic drug addiction has been reported. The value of the serotonin precursor, tryptophan, as an

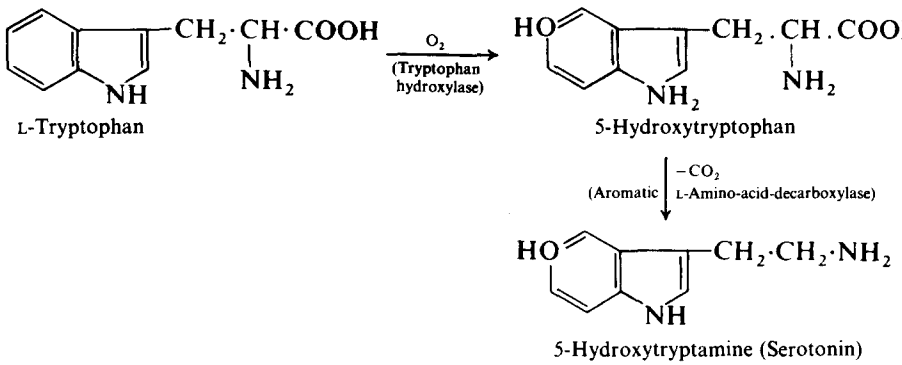


Fig. 15. Conversion of L-tryptophan to serotonin.

antimanic and antidepressant has already been mentioned. It has been assumed that total levels of a neurotransmitter in the brain are related to the amounts actually present within the nerve endings, waiting to be released when the neuron depolarises. Variations in the amount released presumably indicate the amount of information crossing the synapse. These changes could, therefore, modify the traffic through the brain network and be reflected as changes in response. The rate of synthesis of brain serotonin is governed by the local concentration of tryptophan which, in turn, is governed by the rate of transport of tryptophan across the blood/brain barrier. The use of D-glucose may also provide a second mechanism for an enhanced synthesis of serotonin. Tryptophan is unique in being the only circulating amino acid that is protein bound and it is in this form that it is available to the brain. Most of the plasma-free tryptophan is removed via the liver by the quantitatively more important kynurenine pathway (see route 1 above). Free fatty acids are known to compete with tryptophan for the binding sites on the protein molecule. However, as a result of the anti-lipolytic function of insulin released by the ingestion of a glucose load, the concentration of free fatty acids decreases and more tryptophan binds to protein.

SOME PROBLEMS

The literature provides many isolated studies spread through a large number of disciplines, but the rôle of carbohydrate in mental alertness and proper functioning appears to be established. Co-operative studies of a multidisciplinary nature are required to further this extremely important area of investigation, to unravel the complex inter-relationship of the many hormones and blood chemicals involved. This work will be concerned with 'normal' states, as well, because these must be the bases for comparison. To establish these is, itself, a much more difficult task than is, at first, apparent. Published figures may not be applicable because they themselves

may have been obtained without due appreciation of the difficulties, but also because results obtained by one analytical method may not always coincide with those obtained by another. It is important that like be compared with like. Further, it is not always appreciated that almost all physiological values are subject to Circadian rhythms; that is, 'normal' values may vary between certain limits, depending on time. The rhythm may be short or long, but it is extremely important to have knowledge of the rhythm of the particular parameter under study, otherwise conclusions can be hopelessly astray. In addition, there is little appreciation of the effects of exercise on physiological values. How often subjects hurry along a corridor or up or down stairs to the place of sampling! It may be very important to allow subjects a rest period before sampling commences and for initial samples to consist of three, interspersed by, say, 15 min, and for these three to be within a given small range before experimentation proper may commence. The very act of taking samples can, itself, engender stress symptoms, especially when subjects are unaccustomed to, say, intravenous blood sampling (Green, 1972). In experiments set up to study the effects of external stress, there can be over-stress, which can be dangerous to human volunteers, and there is the possibility of results being invalidated due to emotional reactions between the experimental subject and the experimenter. Possessed of adequate samples taken under satisfactory conditions, there are many analytical problems to be solved, for although there are well established chemical methods for some parameters, there is none for others, or those that do exist are not adequately precise and appropriate for this type of investigation. These are but a few of the problems associated with the proper assessment of the ability of subjects to cope with stressful situations, but the chemist's training is such that he can bring a balanced judgement to work. He *must* be aware of—and able to control—those aspects of the investigation which will affect his results. In these days of increasing pace and stress, it is important to be able to relate food ingested with chemical, physical, psychological and neurological phenomena.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the very helpful contributions to this paper made by Mr M. A. Ford, Beecham Products, Coleford, Gloucs.

REFERENCES

AHRENS, E. H., HIRSCH, S., OETTE, K., FARQUHAR, J. W. & STEIN, Y. (1961). Carbohydrates—Induced and fat-induced lipemia. *Trans. Ass. Amer. Physicians*, **74**, 134–6.

BADAWAY, A. A-B. & EVANS, M. (1976). The regulation of rat liver tryptophan pyrollase activity by reduced nicotinamide-adenine dinucleotide (phosphate). Experiments with glucose and nicotinamide. *Biochem. J.*, **156**, 381–90.

BELL, C. H., DAVIDSON, J. N. & EMSLIE-SMITH, D. (1972). *Textbook of physiology and biochemistry* (8th edn.), 316, London and Edinburgh, Churchill Livingstone.
BELLIT, S., ROMAN, L. & KOSTIS, J. (1969). The effects of automobile driving on catecholamines and adrenocorticol excretion. *Am. J. Cardiol.*, **24**, 365–8.
BENNET, G. (1972). *Br. med. J. Letter* (10th June), p. 657.
BENNET, T. I. & DODDS, E. C. (1925). The oral administration of large quantities of glucose. *Lancet*, i, 429–32.
BIRCH, G. G. (1974). Dietary carbohydrate and intellectual performance. *Biochem. Soc. Trans., 44th Meeting*, 300–1.
BIRCH, G. G., COWELL, N. D. & CHARLES, G. A. (1977). Effects of dietary carbohydrate on intellectual performance. *Proc. Nutr. Soc.*, **36**, 25A.
BROOKE, J. D. (1973). Carbohydrates and human performance. In: *Molecular structure and function of food carbohydrate* (Birch, G. G. & Green, L. F. (Eds)), London, Applied Science, 235–63.
BROOKE, J. D., DAVIES, G. J. & GREEN, L. F. (1975). The effects of normal and glucose syrup work-diets on the performance of racing cyclists. *J. Sports Med.*, **15**, 257–65.
BROOKE, J. D., TOOGOOD, S., GREEN, L. F. & BAGLEY, R. (1975). Factory accidents and carbohydrate supplements. *Proc. Nutr. Soc.*, **32**, 94A.
BUTTERFIELD, W. J. H., SARGEANT, B. M. & WHICHELOW, M. J. (1964). The metabolism of human forearm tissues after ingestion of glucose, fructose, sucrose or liquid glucose. *Lancet*, i, 574–7.
CARRUTHERS, M. (1971). 'What do you eat?' or 'What's eating you?' *New Scientist*, **49**, 542–4.
CHRISTIAN, M. G. (1972). *Br. med. J. Letters* (29th April), p. 295.
COPPEN, A. J. (1972). Indoleamines and affective disorders. *J. Psychiatr. Res.*, **9**, 163–71.
CRISTOFARO, E., MOTTU, F. & WUHRMANN, J. J. (1974). Involvement of the raffinose family of oligosaccharides in flatulence. In: *Sugars in nutrition* (Sipple, H. L. & McNutt, K. W. (Eds)), 321, New York, London, Academic Press Inc.
DAVIDSON, S., PASSMORE, R. & BROCK, J. F. (1972*a*). Carbohydrates. In: *Human nutrition and dietetics* (34th edn), London, Churchill Livingstone, p. 34.
DAVIDSON, S., PASSMORE, R. & BROCK, J. F. (1972*b*). Carbohydrates. In: *Human nutrition and dietetics* (34th edn), London, Churchill Livingstone, p. 39.
DAVIDSON, S., PASSMORE, R. & BROCK, J. F. (1972*c*). Carbohydrates. In: *Human nutrition and dietetics* (34th edn), London, Churchill Livingstone, p. 237.
DAVIES, SIR J. (1972). *Financial Times* (June 17).
DODDS, E. C., FAIRWEATHER, F. A., MILLER, A. L. & ROSE, C. F. M. (1959). Blood sugar response of normal adults to dextrose. *Lancet*, i, 485–8.
FERNSTROM, J. D. (1977). Effects of the diet on brain neurotransmitters. *Metabolic clin. Exp.*, **26**, 207–23.
FERNSTROM, J. D. & WURTMAN, R. J. (1971). Brain serotonin content: Increase following ingestion of carbohydrate diet. *Science*, **174**, 1023–5.
FERNSTROM, J. D. & WURTMAN, R. J. (1973). Correlations between brain tryptophan and plasma neutral amino-acid levels following food consumption in rats. *Life Science*, **13**, 517–24.
FREDRICKSON, D. S., LEVY, R. I. & LEES, R. S. (1937). Fat transport in lipoproteins—An integrated approach to mechanisms and disorders. *New Eng. J. Med.*, **276**, 34–281.
GRAY, G. M. (1975). Carbohydrate digestion and absorption—Role of the small intestine. *New Eng. J. Med.*, **292** (Part 23), 1225–30.
GREEN, L. F. (1972). Blood glucose measurements in the field trials. *Br. J. Sports Med.*, **6**, 116.
GREEN, L. F. & BAGLEY, R. (1970). Ingestion of a glucose syrup drink during long distance canoeing. *Br. J. Sports Med.*, **6**, 125–8.
GREEN, L. F. & BAGLEY, R. (1974). The effects of a glucose syrup drink during exercise. *Br. J. physical Education* (July), xiii.
GUROFF, G. (1962). Studies on aromatic amino-acid uptake by rat-brain *in vivo*. *J. Biol. Chem.*, **237**, 803–6.
KEYS, A. (1971). Sucrose in the diet and coronary heart disease. *Atherosclerosis*, **14**, 193–202.
LAPIN, I. P. & OXENKRUG, G. F. (1969). Intensification of the central serotoninergic processes as a possible determinant of the thymoleptic effect. *Lancet*, i, 132–6.
MACDONALD, I. (1967). Interrelationship between the influence of dietary carbohydrates and fats on fasting serum lipids. *Am. J. clin. Nutr.*, **20**, 345–51.
MCCANCE, R. A. & WIDDOWSON, E. M. (1960). In: *The composition of foods*, London, HMSO.
MUCKLE, D. S. (1973). Glucose syrup ingestion and team performance in soccer. *Br. J. Sports Med.*, **7**, 340–3.

PASSOT, J. (1971). In: *The measured stress and strain in measurement of man at work* (Singleton, W. D., Fox, J. G., & Winfield, D. (Eds)), London, Taylor & Francis, 27.
PIGMAN, W. (1957*a*). In: *The carbohydrates* (1st edn.), New York, Academic Press, 91.
PIGMAN, W. (1957*b*). In: *The carbohydrates* (1st edn.), New York, Academic Press, 95.
PIGMAN, W. & HORTON, D. (1970*a*). In: *The carbohydrates* (2nd edn.), Vol. IIA, New York, Academic Press, 101.
PIGMAN, W. & HORTON, D. (1970*b*). In: *The carbohydrates* (2nd edn.), Vol. IIA, New York, Academic Press, 104.
PIGMAN, W. & HORTON, D. (1970*c*). In: *The carbohydrates* (2nd edn.), Vol. IIA, New York, Academic Press, 107.
SHALLENBERGER, R. S. (1974*a*). Occurrence of various sugars in foods. In: *Sugars in nutrition* (Sipple, H. L. & McNutt, K. W. (Eds)), New York and London, Academic Press Inc., 74 and 75.
SHALLENBERGER, R. S. (1974*b*). Occurrence of various sugars in foods. In: *Sugars in nutrition* (Sipple, H. L. & McNutt, K. W. (Eds)), New York and London, Academic Press Inc., 72.
TAGGART, P. & CARRUTHERS, M. (1970). Endogenous hyperlipidaemia induced by emotional stress of racing driving. *Lancet*, **i**, 363–6.
TAGGART, P., GIBBONS, D. & SOMERVILLE, W. (1969). Some effects of motor car driving on the normal and abnormal heart. *Amer. med. J.*, **4**, 130–4.
WALKER, A. R. P. (1971). Sugar intake and coronary heart disease. *Atherosclerosis*, **14**, 137–52.
WHITE, J. A. (1978). Energy and athletic performance. *Br. Nutr. Found. Nutr. Bull. 24*, **4**(6), 394–401.
WRIGHT, S. (1971). In: *Applied physiology* (12th edn.) (Keele, E. E. & Neil, E. (Eds)), London, Oxford University Press, 492.
WURTMAN, R. I. & FERNSTROM, J. D. (1972). Perspectives in neuropharmacology (Snyder, S. B. (Ed)), Oxford University Press, 143.
WURTMAN, R. I. & FERNSTROM, J. D. (1974). Effects of the diet on brain neurotransmitters. *Nutr. Rev.*, **32**, 193–200.
WURTMAN, R. I. & FERNSTROM, J. D. (1975). Control of brain monoamine synthesis by diet and plasma amino acids 1, 2, 3. *Am. J. clin. Nutr.*, **28**, 638–47.
YOUNG, S. N. & SOURKES, T. L. (1974). Antidepressant action of tryptophan. *Lancet*, **ii**, 897–8.
YUDKIN, J. (1957). Diet and coronary thrombosis, hypothesis and fact. *Lancet*, **ii**, 155–62.
YUDKIN, J. (1963). Nutrition and palatability with special reference to obesity, myocardial infarction, and other diseases of civilisation. *Lancet*, **i**, 1335–8.
YUDKIN, J. (1964). Dietary fat and dietary sugar, in relation to ischaemic heart disease and diabetes. *Lancet*, **ii**, 4–5.
YUDKIN, J. (1971). Sugar consumption and myocardial infarction. *Lancet*, **i**, 296–97.

Food Chemistry 7 (1981) 219–225

SULPHITE ADDUCTS OF CYCLIC MONOTERPENE KETONES

TAKAYUKI SHIBAMOTO, YOSHIYUKI ENOMOTO* & TAKUYA AKIYAMA*

Department of Environmental Toxicology, University of California, Davis, California 95616, USA

(Received: 8 May, 1980)

ABSTRACT

Structures of sulphite addition products of some cyclic monoterpene ketones with Na_2SO_3 and/or $NaHSO_3$ were studied. The conjugated enones reacted with Na_2SO_3 and gave 1,4-addition products. The non-conjugated compounds did not react with Na_2SO_3. $NaHSO_3$ made radical additions to non-conjugated olefinic bonds. On the other hand, ketones having no olefinic bond did not form addition products. 1,4-addition products underwent a reverse reaction when alkali was added but the radical addition product was stable towards alkali and did not undergo reverse reaction.

INTRODUCTION

The 1,2-addition reaction of $NaHSO_3$ to carbonyl compounds, used to isolate and purify monoterpene ketones from essential oils, has long been an object of study (Gubareva, 1948; Ishikawa & Shinra, 1956). The bisulphite addition products of monoterpene ketones on aldehyde have also been studied by several researchers (Norton *et al.*, 1968; Johnson & Jones, 1978).

A ketone yields a sulphite addition product when combined with Na_2SO_3 and $NaHSO_3$. The addition product is separated from the oil and then treated by alkali to reform the original ketone (Okuda, 1968). Johnson & Jones (1978) reported the structural assignments of the bisulphite adducts of α,β-unsaturated monoterpene aldehydes. They observed that the sulphite addition occurred at a conjugated double bond as well as at a carbonyl group. The isolated double bond, however, requires the presence of an oxidant. There have been no detailed descriptions of the structures of sulphite addition products of cyclic monoterpene ketones. In the study reported in

* Present address: Ogawa and Co. Ltd, 6-32-9 Akabanenishi, Kita-Ku, Tokyo, Japan.

Food Chemistry 0308-8146/81/0007-0219/$02·75

this paper we investigated the structure of the sulphite addition products of cyclic monoterpene ketones and their behaviour in an alkali solution.

EXPERIMENTAL

Materials

Dihydrocarvone (2), pulegone (3), piperitone (4), piperitenone (5), and menthone (6) were purchased from Nippon Terpene Chemical Co. Ltd and *l*-carvone (1) was obtained from Ogawa & Co. Ltd. Na_2SO_3, $NaHSO_3$ and $NaHCO_3$ came from Nakarai Chemicals Ltd. All organic chemicals were purified to over 95% by distillation before use.

Infrared spectra were taken on a Jasco IR-S spectrometer; samples were run as nujol paste. NMR spectra were run on a Jeol JNM-FX 100 nuclear magnetic resonance spectrometer using 1% tetramethyl silane as an internal standard.

Experiment 1: Reaction of l-*Carvone (1) with* Na_2SO_3

A solution containing 5 g of (1) (0·033 mol), 8·4 g of Na_2SO_3 (0·067 mol) and 5·6 g of $NaHCO_3$ (0·067 mol) as buffer in 38 ml of H_2O was stirred for 7 h at room temperature. The pH of the reaction mixture changed from 8 to 9. After a small amount of unreacted material was removed with ethyl ether, water was distilled off by vacuum distillation. The residue was dried to a solid in a desiccator; 22 g of white solid was obtained. The solid (10 g) was dissolved in 50 ml of hot ethanol and undissolved materials were filtered off. The solvent was removed from the filtrate *in vacuo* to yield a white powder which was subsequently recrystallised from ethanol (5 g): IR 1690–1710 (saturated six membered cyclic ketone), 1180 (SO_2), 900 cm^{-1} (terminal vinyl); NMR (CD_3OD) δ 1·30 (d, 3H, $J = 7$ Hz, methyl), 1·77 (s, 3H, olefinic methyl), 4·80 (s, 2H, vinyl). The doublet at δ 1·30 changed to a singlet in D_2O. This white powder was identified as (7) (Fig. 1).

Experiment 2: Reaction of (1) with Na_2SO_3 *and* $NaHSO_3$

An aqueous solution (7 ml) of $NaHSO_3$ (3·4 g, 0·033 mol) was added dropwise to the stirred solution of (1) (5 g, 0·033 mol), Na_2SO_3 (2·5 g, 0·02 mol) and H_2O (18 ml) over a 4-h period at 70–75 °C, and stirring was continued for an additional 3 h at the same temperature. The pH of the reaction solution changed from 10 to 9. The reaction mixture was treated by the same procedure as in Experiment 1 and 7 g of white powder was obtained. The powder was identified as (7) by IR and NMR.

Experiment 3

The experiment was conducted in the same manner as Experiment 2 except for the amount of aqueous solution (14 ml) of $NaHSO_3$ (6·9 g, 0·066 mol) used (twice that of Experiment 2). The pH of the reaction solution changed from 10 to 7. The reaction

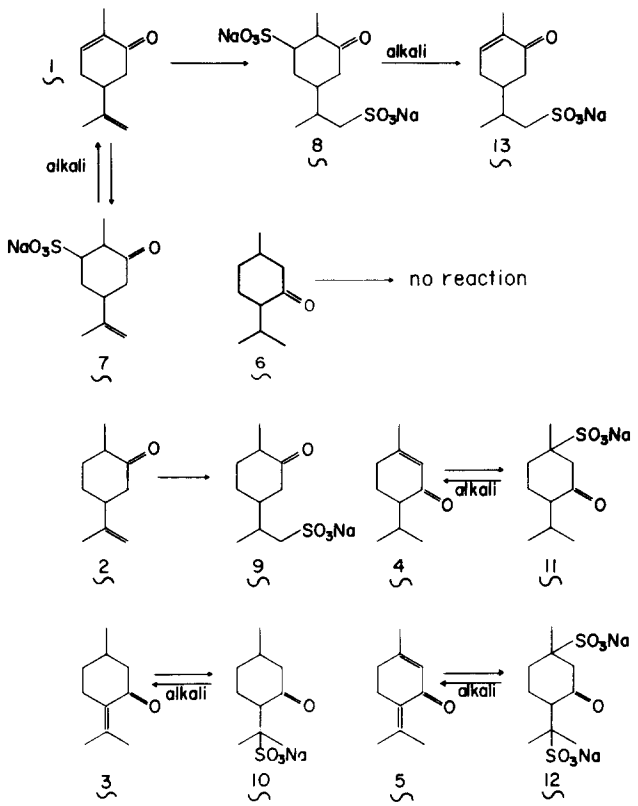


Fig. 1. Formation of the sulphite addition products from the following monoterpene compounds: (1) *l*-Carvone. (2) Dihydrocarvone. (3) Pulegone. (4) Piperitone. (5) Piperitenone. (6) Menthone.

mixture was treated as in Experiment 1 and yielded 8 g of white powder: NMR (CD_3OD) δ 1·13 (d, 3H, methyl), 1·28 and 1·30 (two d, 3H, methyl). The doublets at δ 1·13, 1·28 and 1·30 were changed to singlets by D_2O. The white powder was identified at (8) (Fig. 1).

Experiment 4: Reaction of (1) with $NaHSO_3$

Compound (1) (5 g, 0·033 mol) was added to an aqueous solution (26 ml) of $NaHSO_3$ (13·7 g, 0·132 mol) and the solution was stirred for 7 h at 70–75 °C. The pH of the reaction solution remained 4·5. The reaction mixture was treated by the same procedure as in Experiment 1 and 8 g of white powder was obtained. This powder was identified as (8) (Fig. 1).

The other ketones ((2), (3), (4), (5) and (6), Fig. 1) were reacted with Na_2SO_3 and/or $NaHSO_3$ by the same experimental procedure used for (1). The experimental conditions and results are shown in Table 1.

TABLE 1
EXPERIMENTAL RESULTS OF THE REACTION OF KETONES WITH Na_2SO_3 AND/OR $NaHSO_3$

Ketone	*Experiment 1*		*Experiment 2*		*Experiment 3*		*Experiment 4*	
	Product	*Yield*	*Product*	*Yield*	*Product*	*Yield*	*Product*	*Yield*
(1)	(7)	100[a]	(7)	140	(8)	160	(8)	160
(2)	[b]		(9)	90	(9)	120	(9)	120
(3)	(10)	40	(10)	120	(10)	100	(10)	114
(4)	(11)	124	(11)	120	(11)	124	(11)	138
(5)	(12)	120	—		(12)	86	(12)	140
(6)	[b]		[b]		[b]		[b]	

[a] $\frac{\text{The amount of the adduct produced}}{\text{The amount of the ketone used}} \times 100$.

[b] No reaction.

Reaction of sulphite addition products with NaOH

Each addition product (5 g) was added to 6% NaOH solution (4 mol) and the solution was stirred for 30 min at room temperature. The reaction mixture was extracted with three 10-ml portions of ethyl ether. The extract was dried over anhydrous $MgSO_4$ and the solvent was removed. The oil obtained was analysed using gas chromatography, NMR, and IR.

RESULTS AND DISCUSSION

The monoterpene ketones used for this study and their sulphite addition products are shown in Fig. 1. The NMR data of sulphite products are listed in Table 2. The reaction conditions and results of the experiments are summarised in Table 1.

Na_2SO_3 reacted with the conjugated enone (1) and gave a 1,4-addition product but did not react with the vinyl group of (1). The other ketones showed the same results as (1). That is, Na_2SO_3 reacted with the ketones which possess a conjugated enone (e.g. (1), (3), (4) and (5), Fig. 1) and gave 1,4-addition products, but did not react with the ketones which do not have a conjugated enone (for example, (2) and (6), Fig. 1). The reaction mechanism of (1) with Na_2SO_3 and $NaHSO_3$ is shown in Scheme 1.

First, Na_2SO_3 reacts with the conjugated enone of (1) to give the addition product (7) and NaOH. This is a reversible reaction. Thus, if the NaOH formed is neutralised with $NaHSO_3$, Na_2SO_3 is produced in the reaction mixture and the 1,4-addition reaction progresses smoothly. When equimolar amounts of $NaHSO_3$ to (1) are used (refer to Experiment 2), all $NaHSO_3$ is consumed by reaction with the NaOH formed. In this case, the reaction is over after this first step, which gives product (7). On the other hand, when twice molar amounts of $NaHSO_3$ to (1) are used, the excess $NaHSO_3$ remains in the reaction mixture, reacts with a vinyl group of (7) (anti-Markownikoff type radical addition, Kharasch *et al.*, 1938; Kharasch *et al.*, 1939;

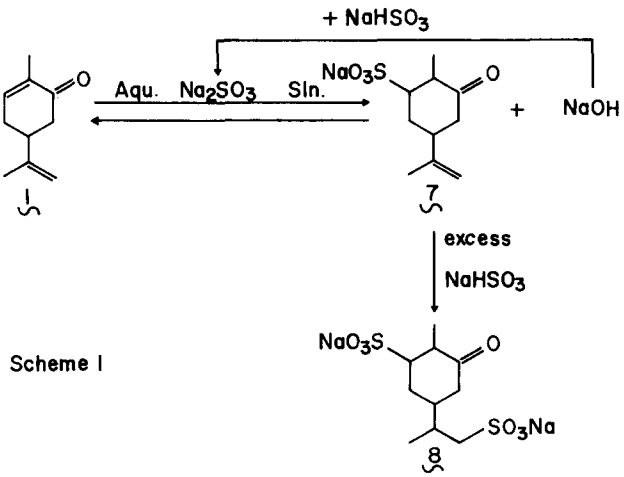
TABLE 2
NMR DATA OF SULPHITE ADDITION PRODUCTS

Compound	*Spectral data*
(7)	δ 1·30 (3H, d, J = 7 Hz, —CH_3^a), 1·77 (3H, s, $=\overset{|}{C}—CH_3^a$), 1·8–3·3 (7H, m, aliphatic CH_2 and CH), 4·8 (2H, s, $\diagup C=CH_2^a$).
(8)	δ 1·16 (3H, d, J = 7 Hz, CH_3^a—CH—CH_2SO_3Na), 1·28, 1·30 (3H, two d, J = 7 Hz, —CH_3^a), 1·6–3·6 (10H, m, aliphatic CH_2 and CH).
(9)	δ 0·97 (3H, d, J = 6 Hz, —CH_3^a), 1·11 (3H, d, J = 7 Hz, $CH_3^a—\overset{|}{C}H—CH_2SO_3Na$), 1·2–3·3 (11H, m, aliphatic CH_2 and CH).
(10)	δ 0·96, 1·01 (3H, two d, J = 6 and 7 Hz, —CH_3^a), 1·42, 1·45 [6H, two d, J = 2 Hz, —$C(CH_3^a)_2$—SO_3Na], 1·4–3·4 (8H, m, aliphatic CH_2 and CH).
(11)	δ 0·86, 0·88, 0·93 [6H, three d, —$CH(CH_3^a)_2$], 1·26, 1·32 (3H, two s, —CH_3^a), 1·4–3·1 (8H, m, aliphatic CH_2 and CH).
(12)	δ 1·29, 1·33 (3H, two s, —CH_3^a), 1·43, 1·46 [6H, two s, —$CH(CH_3^a)_2SO_3Na$], 1·5–3·2 (7H, m, aliphatic CH_2 and CH).
(13)	δ 1·13 (3H, d, J = 7 Hz, $CH_3^a—\overset{|}{C}H—CH_2SO_3Na$), 1·71 (3H, s, $=\overset{|}{C}—CH_3^a$), 1·9–3·2 (3H, m, aliphatic CH_2 and CH), 6·70–6·97 (1H, m, $\diagup C=CH^a—$).

[a] The protons used to identify the position of sulphite addition.

Norton *et al.*, 1968) and gives product (8). $NaHSO_3$ contributes both 1,4-addition and radical addition in the case of Experiment 4. In the case of (2), since (2) does not react with Na_2SO_3, $NaHSO_3$ alone contributes to the reactions of Experiments 2 and 3, as well as Experiments 1 and 4.

The one ketone which does not possess an olefinic bond (6) does not form any addition products under the conditions used for this experiment, indicating that 1,2-addition to a carbonyl group may not occur easily.



Scheme I

Compound (1) was recovered from (8) with alkali. Compound (13), formed by the loss of sulphite from the conjugated enone, was recovered from (8), but not from (1), by the procedure described in the experimental section. Compounds (3), (4) and (5) were recovered from (10), (11) and (12), respectively, with alkali, but (2) was not recovered from (9).

CONCLUSIONS

The results indicate that 1,4-addition sulphite compounds undergo a reverse reaction easily when alkali is added but that radical addition sulphite compounds are stable towards alkali such as NaOH. Even so, the sulphite addition reaction has, for many years, been used for the purification of carbonyl compounds. The reaction does not occur or an original carbonyl compound is not recovered in some cases. It is necessary to exercise great care when using this reaction for the purification of monoterpenes or for the isolation of monoterpenes from an essential oil.

REFERENCES

GUBAREVA, M. A. (1948). Bisulfite compounds of aldehydes and ketones. *Zhur. Obschchie Khim.*, **18**, 238.

ISHIKAWA, K. & SHINRA, K. (1956). The relation between the dissociation constants and structures of the addition products of aliphatic carbonyl compounds. III. Dissociation constants of some addition products of methyl ketones. *J. Chem. Soc. Japan*, **77**, 275.

JOHNSON, T. J. & JONES, R. A. (1978). The chemistry of terpenes-VIII, characterization of the bisulphite adducts of α,β-unsaturated aldehydes by NMR spectroscopy. *Tetrahedron*, **34**, 547.

KHARASCH, M. S., MAY, E. M. & MAYO, F. R. (1938). Peroxide effect in the addition of reagents to unsaturated compounds. (XVII). Addition of bisulfite. *J. Org. Chem.*, **3**, 175.

KHARASCH, M. S., SCHECK, R. T. E. & MAYO, F. R. (1939). Peroxide effect in the addition of reagents to unsaturated compounds. (XXIII). Reaction of styrene with bisulfite. *J. Amer. Chem. Soc.*, **61**, 3092.

NORTON, C. J., SEPPI, N. F. & REUTER, M. F. (1968). Alkanesulfonate synthesis. I. Ion catalysis of sulfite radical-ion addition to olefins. *J. Org. Chem.*, **33**, 4185.

OKUDA, O. (1968). *Koryo Kagaku Soran*, Hirokawa Publishing Co., Tokyo, 905–45.

Food Chemistry 7 (1981) 227–235

EGGPLANT POLYPHENOL OXIDASE: PURIFICATION, CHARACTERISATION AND PROPERTIES

MOHAMED HASSAS ROUDSARI, ALICE SIGNORET & JEAN CROUZET

Centre de Génie et Technologie Alimentaires, Université des Sciences et Techniques de Languedoc, 34060—Montpellier Cédex, France

(Received: 11 May, 1980)

ABSTRACT

The isolation and purification of polyphenol oxidase from purple eggplant (Solanum melongena *L.*) *is described. A 15-fold purified preparation has been obtained with 15% yield by a procedure involving* $(NH_4)_2SO_4$ *precipitation, DEAE cellulose chromatography and Sephadex G 100 gel filtration.*

The enzyme has an optimum pH of 6·4 and a molecular weight of 79 000 ± 5000.

Inhibition studies with sodium diethyl dithiocarbamate and potassium cyanide and dialysis against the latter show that the enzyme requires copper.

Eggplant polyphenol oxidase exhibits only a catecholase activity and presents a great affinity for catechol and caffeic acid. This latter compound is no doubt its natural substrate.

These results show that conversely to tomato, where a polyphenol oxidase activity was tightly bound to the peroxidase, in the case of eggplant there is a true polyphenol oxidase enzyme.

INTRODUCTION

Polyphenol oxidase is widely responsible for enzymatic browning in vegetables and particularly in eggplant (*Solanum melongena* L.). Notwithstanding the extent of this reaction during handling, storage and processing of the eggplant, this enzyme has only been dealt with in a few studies.

The activity of polyphenol oxidase was compared for three varieties of eggplant in relation to enzymatic browning (Flick *et al.*, 1977, 1978). There are relatively few studies concerning purification and characterisation of the enzyme (Knapp, 1961,

Food Chemistry 0308-8146/81/0007-0227/$02·75

1965; Rhoades & Tai-lei Chen, 1968; Ramaswamy & Rege, 1975), no doubt due to difficulties encountered during the purification process.

The purpose of this work was to obtain an enzyme preparation sufficiently purified so that its main properties might be studied.

MATERIAL AND METHODS

Vegetables and reagents

Purple eggplants were purchased in Montpellier market and stored in a cold room at 4 °C to await use (two to three days maximum). Reagents used in this study were all of analytical grade.

Preparation of crude extract

All steps of the procedure described below were carried out at 4 °C.

Previously chilled eggplants were peeled, cut into pieces and homogenised in a Waring Blendor in the presence of 200 mM MacIlvaine buffer (pH 7·5) containing insoluble polyvinyl pyrrolidone (Polyclar AT). One-hundred-and-sixty-six millilitres of buffer and 5·4 g of Polyclar were used for each 100 g of tissue. Preliminary studies have shown that in these conditions the measured activity was maximum. The homogenate was centrifuged at 18 000 *g* for 30 min and the supernatant filtered through a Buchner funnel. The filtrate so obtained is the crude extract.

Measurement of enzyme activity

All determinations were carried out at 25 °C. Between 50 and 100 μl of enzymatic extract were rapidly added to a solution of 100 mM MacIlvaine buffer (pH 6·35), 100 mM catechol.

Generally, polyphenol oxidase activity was determined by spectrophotometry at 420 nm according to Joslyn & Ponting (1948). The total volume of the reaction mixture was 2·5 ml. One unit of polyphenol oxidase activity was defined as a change of 10^{-4} absorbance units a second; thus, the enzymatic activity was expressed in ΔA_{420} nm 10^{-4}/s.

In some cases—namely, substrate specificity studies—oxygen uptake was measured using a Clark type oxygen electrode (Mitsuda *et al.*, 1967). The total volume of the reaction mixture was 1·9 ml. The enzymatic activity was expressed in micromoles of O_2 consumed per minute.

Protein determination

Proteins were quantitatively analysed according to the method of Lowry *et al.* (1951) using bovine serum albumin as a standard, either directly or after precipitation with trichloracetic acid if $(NH_4)_2SO_4$ were present. In the fractions eluted after chromatographic separations proteins were estimated by determining absorbance at 278 nm.

Gel electrophoresis

Acrylamide gel electrophoresis (acrylamide 5%) was carried out according to Davis (1964) with a tris glycine buffer, pH 8·3. Proteins were stained with Coomassie blue and enzymatic activity was revealed using dopamine (200 mM) in 100 mM McIlvaine buffer, pH 6·35, as substrate.

Molecular weight determination

The apparent molecular weight was determined by gel chromatography according to the method of Laurent & Killander (1964). A column of Sephadex G 100 (2·5 × 100 cm) was equilibrated with 100 mM McIlvaine buffer, pH 7·5, and calibrated with cytochrome C (12 500), chymotrypsinogen (25 000), ovalbumin (45 000) and bovine serum albumin (67 000 monomeric and 134 000 dimeric).

Dialysis against KCN

The partially purified enzymatic preparation was dialysed three times against 100 mM McIlvaine buffer, pH 7·4, containing KCN in concentrations from 10^{-6} to 10^{-2} M, then three times against the buffer in order to eliminate the excess KCN. The dialyses were performed for 4 h at 4 °C.

RESULTS

Partial purification of eggplant polyphenol oxidase

All steps of the procedure described below were carried out at 4 °C.

First step: Ammonium sulphate precipitation: The enzyme precipitated from the crude extract in the fraction included between 30% and 70% of saturation in $(NH_4)_2SO_4$. Under these conditions 87% of proteins were eliminated and 60% of activity recovered.

The precipitate was isolated by centrifugation and dissolved in 5 mM McIlvaine buffer, pH 7·5. The elimination of $(NH_4)_2SO_4$ was carried out by gel filtration on Sephadex G 25 (column Pharmacia K 26/100) equilibrated with the same buffer.

Second step: Ion exchange chromatography on DEAE cellulose: The fractions containing the activity were pooled together and applied on a DEAE cellulose column (2 × 60 cm) previously equilibrated with a 25 mM McIlvaine buffer, pH 7·5.

The column was first washed with 100 ml of this buffer and developed with a linear gradient elution (200 ml of 30 mM–200 ml of 80 mM buffer). The absorbance at 278 nm and polyphenol oxidase activity were determined on each fraction (Fig. 1).

Third step: Gel filtration on Sephadex G 100: The fractions containing the activity were pooled together, concentrated by ultrafiltration in an Amicon cell fitted with a Diaflo membrane PM 30 and layered on the surface of a Sephadex G 100 (column Pharmacia K26/100) equilibrated with a 100 mM McIlvaine buffer, pH 7·5. The elution pattern is shown in Fig. 2.

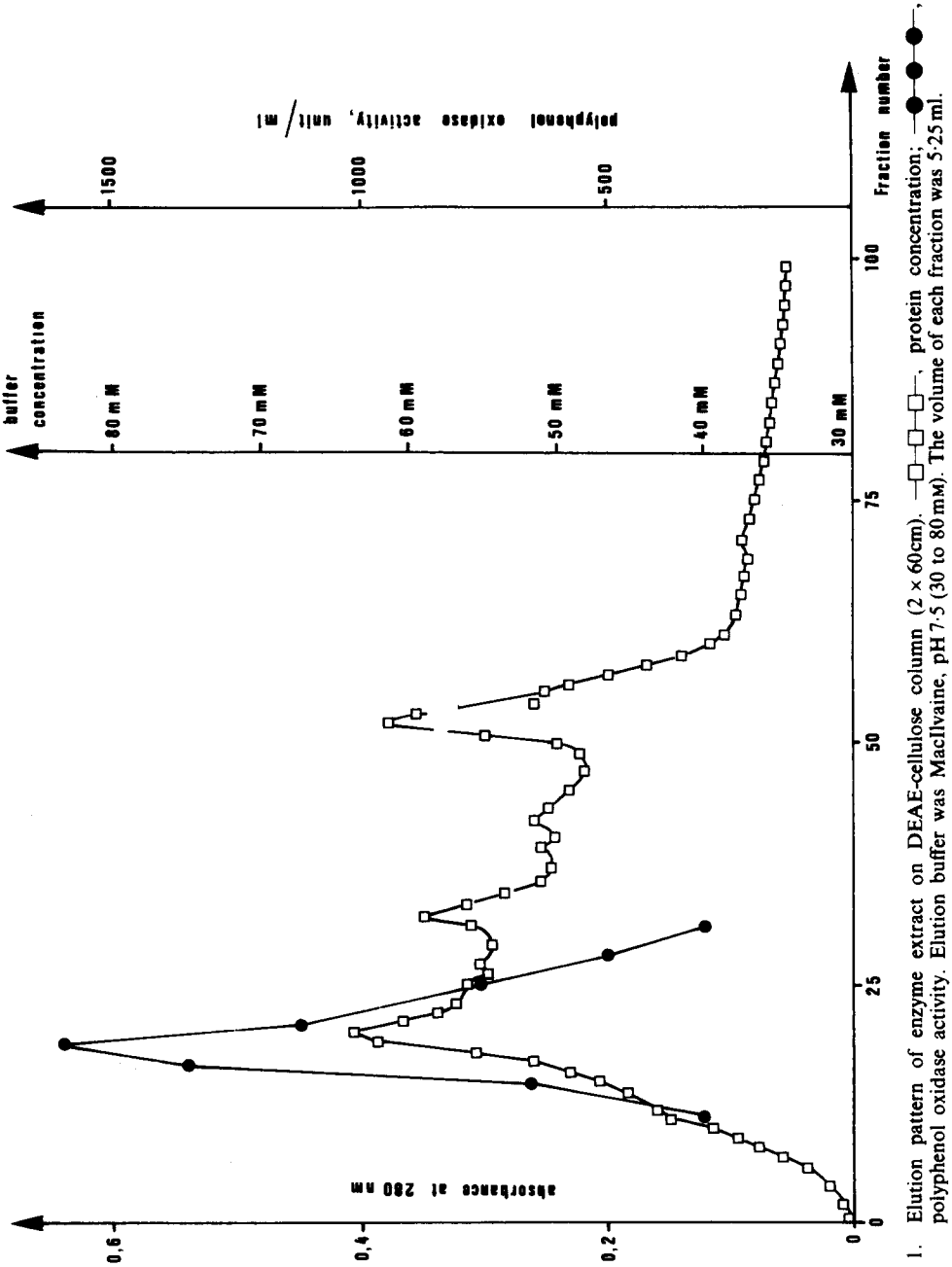


Fig. 1. Elution pattern of enzyme extract on DEAE-cellulose column (2 × 60cm). —□—□—, protein concentration; —●—●—, polyphenol oxidase activity. Elution buffer was Mac Ilvaine, pH 7·5 (30 to 80 mM). The volume of each fraction was 5·25 ml.

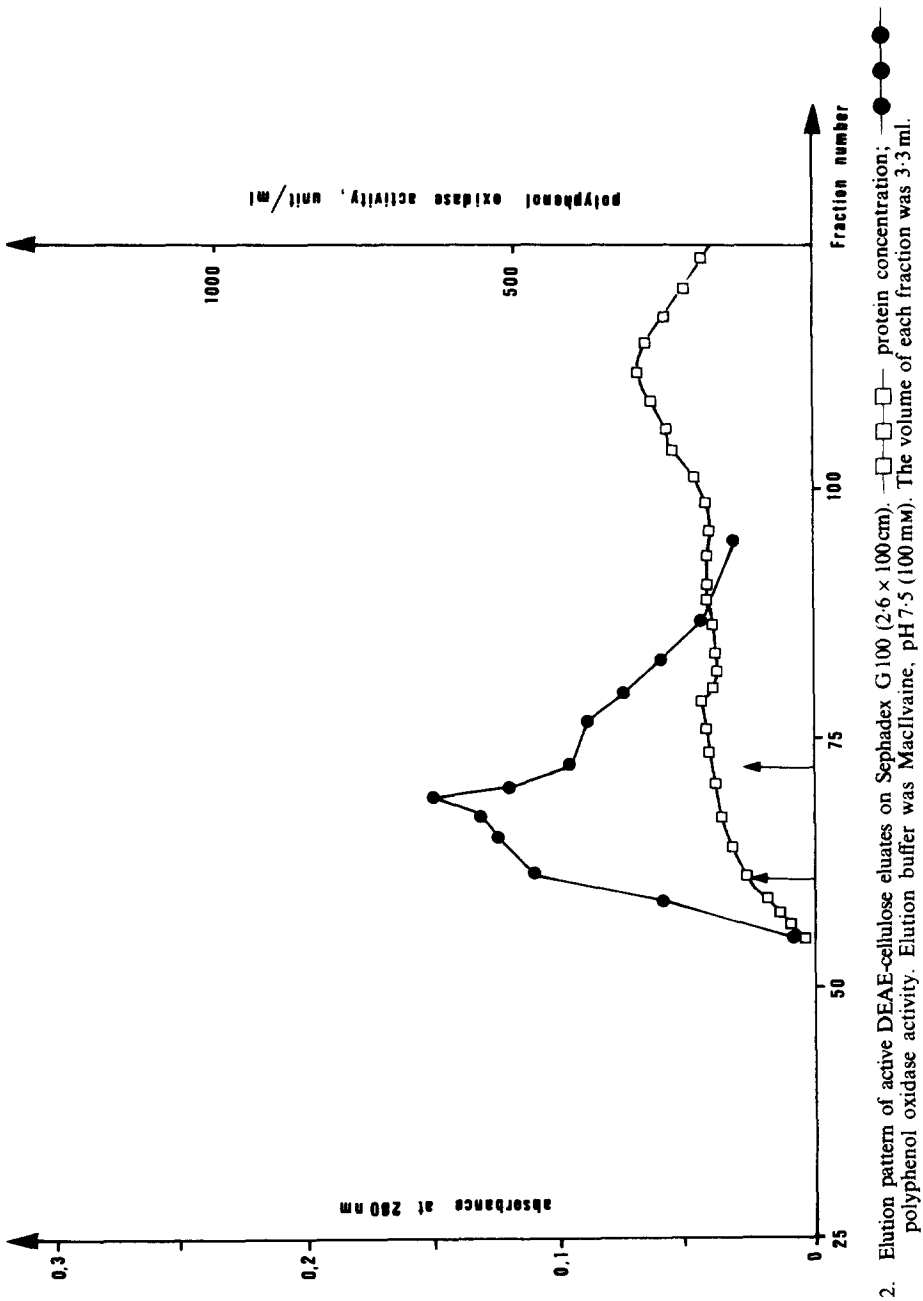


Fig. 2. Elution pattern of active DEAE-cellulose eluates on Sephadex G 100 (2·6 × 100 cm). —□—□—□— protein concentration; —●—●— polyphenol oxidase activity. Elution buffer was MacIlvaine, pH 7·5 (100 mM). The volume of each fraction was 3·3 ml.

The preparation obtained from the fractions corresponding to the polyphenol oxidase activity peak (61 to 73) exhibited only one band in polyacrylamide gel electrophoresis after activity was revealed.

The results of a typical purification are shown in Table 1. The eggplant polyphenol oxidase was purified 15-fold from the crude extract with a yield of 15%. The best specific activity obtained was 14 333 $\Delta A_{420}\, 10^{-4} s^{-1}$ per milligramme of protein.

TABLE 1
PURIFICATION OF EGGPLANT POLYPHENOL OXIDASE

Step	*Total proteins (mg)*	*Total activity (unit)*	*Specific activity (unit/mg)*	*Fold purification*	*Yield (%)*
Crude extract	400	381060	952	1	100
Ammonium sulphate fractionation (30–70%) saturation	82·5	171000	2072	2	45
DEAE-cellulose	56	126500	2253	2·37	33·2
Sephadex G 100	3·8	55556	14333	15	14·6

Optimum pH

The optimum pH is about 6·4 with catechol as substrate. The following buffers were used: citric acid–sodium phosphate, 50 mM (pH 4·0–6·5); sodium phosphates, 50 mM (pH 5·8–7·8); tris-HCl, 50 mM (pH 7·0–9·0); glycine–sodium hydroxide, 50 mM (pH 8·6–10·4).

Molecular weight

The apparent molecular weight estimated by gel filtration was 79 000 ± 5000.

Action of inhibitors

KCN and diethyldithiocarbamate (DIECA) at concentrations varying respectively from $2{\cdot}26 \times 10^{-4}$ to $2{\cdot}6 \times 10^{-6}$ M and from 5×10^{-4} to 5×10^{-6} M act as competitive inhibitors of eggplant polyphenol oxidase. K_i values obtained according to Dixon (1953) were found equal to 10^{-4} M for KCN and 2×10^{-3} M for DIECA.

Dialysis against KCN

The data obtained for enzymatic inactivation after dialysis against several concentrations of KCN are given in Table 2. The values obtained for activity after addition of $CuSO_4$ to the dialysed enzyme are also shown in this Table. The results are expressed in per cent of residual activity relative to the non dialysed enzyme.

TABLE 2
DIALYSIS OF EGGPLANT POLYPHENOL OXIDASE AGAINST KCN

KCN concentration M	*Residual activity (%)* *After dialysis*	*After addition of $CuSO_4$*
10^{-2}	7·5	78·2
10^{-3}	18·5	60·4
10^{-4}	25·7	62·3
10^{-6}	83·7	78·2
0	100	92·5

TABLE 3
SUBSTRATE SPECIFICITY OF EGGPLANT POLYPHENOL OXIDASE

Substrate	K'm (mM)	*Relative rate* (%)
Catechol	7·0	100·0
Me-4 Catechol	222·0	12·7
3-4 Dihydroxybenzoic acid	17·2	7·6
Caffeic acid	8·3	312·5
Dopamine	66·6	141·0
(+)Catechin	15·1	45·9
Chlorogenic acid	55·0	0·38
Guaiacol	277·0	1·25
m-Cresol	27·0	1·6
p-Cresol	185·0	1·6
Resorcinol	1000·0	2·5
Hydroquinone	182·0	1·8
Pyrogallol	114·0	48·0

Substrate specificity

The values of Michaelis constants of the enzyme for different phenolic substrates: monophenols, diphenols and different phenolic compounds, are given in Table 3. In this case kinetics studies were carried out by determining oxygen uptake as a function of time. These determinations were carried out for only one oxygen concentration and, moreover, the saturation of the medium can vary according to the nature of the first substrate used. Under these conditions the values obtained for Michaelis constants must be considered as apparent. The maximum rate values relative to catechol are also given in Table 3.

DISCUSSION

Some difficulties are generally encountered during purification of polyphenol oxidases of plant origin (Mayer & Harel, 1979). In the case of eggplant these difficulties have been pointed out by Rhoades & Tai-lei Chen (1968) and Ramaswamy & Rege (1975) and no doubt explain the relatively weak values

obtained in our work for the yield and purification factor. However, the degree of purification obtained was judged sufficient for studying enzymic characteristics.

The value of 6·4 obtained for the optimum pH with catechol as substrate differs distinctly from those noted by Rhoades & Tai-lei Chen (1968) and Ramaswamy & Rege (1975) who found, respectively, 7·0 and 6·8 with the same substrate. These differences are probably connected with the degree of purity of the preparations used.

The inhibitory effect of reagents such as KCN or DIECA shows that eggplant polyphenol oxidase is a metallo-enzyme. The results obtained for K_i, $K_i \simeq 10^{-4}$M, compared with the values reported by Signoret & Crouzet (1978*b*) for tomato enzyme (eggplant and tomato belong to the same botanic family) may indicate that the two enzymes differ through the nature of the metal.

The results obtained by dialysis of the eggplant enzyme against KCN show a decrease of activity from 20 % to 90 % in connection with the cyanide concentration. Sixty per cent or more of initial activity was recovered by the addition of $CuSO_4$. Conversely, no loss of polyphenol oxidase activity was observed for the tomato enzyme (Signoret & Crouzet, 1978*b*).

From these results it can be inferred that eggplant enzyme isolated with the above procedure is a copper enzyme, i.e. a true polyphenol oxidase. Moreover, it has been possible to separate polyphenol oxidase and peroxidase activities of eggplant by gel chromatography on Sephadex G 100 (Hassas Roudsari, unpublished results). Conversely, different proofs (Signoret & Crouzet, 1978*a*,*b*) suggest a peroxidase in tomato acting as a polyphenol oxidase for the oxidation of phenolic substrates.

Molecular weights of higher plant polyphenol oxidases estimated from results obtained by gel filtration or acrylamide gel electrophoresis cover a wide range (Mayer & Harel, 1979). On the other hand, polyphenol oxidase exhibits a multiplicity largely attributed to polymeric forms. However, the value obtained for eggplant polyphenol oxidase (79 000) agrees with values of 60–70 000 frequently cited in the literature.

The substrate specificity results show that the eggplant enzyme reacts rapidly with caffeic acid, dopamine and catechol and fairly rapidly with (+)-catechin which is generally found among polyphenol oxidase substrates. Our results do not agree with these obtained by Ramaswamy & Rege (1975). These authors report that activity is more important with catechol than with caffeic acid and above all find rather considerable activity with chlorogenic acid. It must be pointed out that in this work, enzyme activity was measured by following the increase of absorbance at 400 nm, whatever substrate they might have used, and that determinations were performed on crude extract.

Relative activities found for monophenols, *p*- or *m*-diphenols or triphenols are generally very weak. Under these conditions it can be assumed that eggplant polyphenol oxidase only has a catecholase activity exclusive of any cresolase activity.

Eggplant polyphenol oxidase has a good affinity for catechol and caffeic acid. The natural substrate of the enzyme is certainly this latter compound or one of its derivatives (Ramaswamy & Rege, 1975).

All the results reported in the present work are consistent with the occurrence of a true polyphenol oxidase enzyme in eggplant fruit, conversely to tomato fruit.

REFERENCES

DAVIS, B. J. (1964). Disc electrophoresis. II Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.*, **121**, 404–27.

DIXON, M. (1953). Determination of enzyme-inhibitor constants. *Biochem. J.*, **55**, 170–1.

FLICK, JR., G. J., ORY, R. L. & ST ANGELO, A. J. (1977). Comparison of nutrient composition and of enzyme activity in purple, green and white eggplants. *J. Agric. Food Chem.*, **25**, 117–20.

FLICK, JR., G. J., BURNETTE, F. S., AUNG, L. H., ORY, R. L. & ST ANGELO, A. J. (1978). Chemical composition and biochemical properties of Mirlitons (*Sechium edule*) and purple, green and white eggplants (*Solanum melongena*). *J. Agric. Food Chem.*, **26**, 1000–5.

JOSLYN, M. A. & PONTING, J. D. (1948). Acid ascorbic oxidation and browning in apple tissue extracts. *Arch. Biochem.*, **19**, 47–63.

KNAPP, F. W. (1961). Browning enzymes of eggplant. *Proc. Fla. State. Hort. Soc.*, **74**, 256–9.

KNAPP, F. W. (1965). Some characteristics of eggplant and avocado polyphenolases. *J. Fd Sci.*, **30**, 930–6.

LAURENT, T. C. & KILLANDER, J. (1964). A theory of gel filtration and its experimental verification. *J. Chromatog.*, **14**, 317–30.

LOWRY, O. H., ROSENBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurements with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–7.

MAYER, A. M. and HAREL, E. (1979). Polyphenol oxidases in plants. *Phytochemistry*, **18**, 193–215.

MITSUDA, H., YASUMATO, K., YAMAMOTO, A. & KUSANO, T. (1967). Study on soybean lipoxygenase. *Agric. Biol. Chem.*, **31**, 117–18.

RAMASWAMY, S. & REGE, D. V. (1975). Polyphenoloxidase of *Solanum melongena* and its natural substrate. *Acta Alimentaria*, **4**, 355–65.

RHOADES, J. L. & TAI-LEI CHEN, T. (1968). The preparation and properties of eggplant polyphenol oxidase. *Proc. La Acad. Sci.*, **31**, 121–5.

SIGNORET, A. & CROUZET, J. (1978*a*). Activités polyphénol oxydasique et peroxydasique du fruit de la tomate (*Lycopersicum esculentum*). Purification et quelques propriétés. *Agric. Biol. Chem.*, **42**, 1871–7.

SIGNORET, A. & CROUZET, J. (1978*b*). *Activités polyphénol oxydase et peroxydase de la tomate*. Assemblée générale du Groupe Polyphénols, Nancy.

Food Chemistry 7 (1981) 237–238

BOOK REVIEWS

Food Packaging Materials—Aspects of Analysis and Migration of Contaminants. By N.T. Crosby. Applied Science Publishers Ltd, London, 1981. Price: £14·00.

This book comprises eight chapters as follows: Chapter 1—Introduction. A good summary of food spoilage and control mechanisms is presented. The general functions of packaging and the packaging requirements of various groups of foods are briefly outlined. Chapter 2—Plastic Packaging Material. The author briefly outlines the chemical structure, method of manufacture and important properties of polyolefins, non-ethylenic thermoplastics and copolymers. Laminates are briefly discussed, as are other constituents of plastics—i.e. residues of polymerisation and materials added as processing aids. Chapter 3—Determination of Monomers. The advantages and limitations of different methods of sampling the environment and analysing the samples for vinyl chloride monomer are discussed. Personal monitoring systems and the checking of effluent samples for this monomer are also briefly covered. Theoretical and practical aspects of headspace gas chromatography for measuring vinyl chloride monomer levels in polymers and foods are presented. The measurement of levels of other monomers is discussed. Chapter 4—Toxicological Aspects. Problems associated with evaluating the toxicity of compounds are discussed. The principles of toxicological testing are outlined, including the determination of acute, sub-acute and chronic toxicity. Extrapolation of animal data to man is briefly discussed. So also are epidemiological and other human studies. The results of toxicity studies on vinyl chloride, vinylidene chloride, acrylonitrile and styrene are summarised. Chapter 5—International Legislation. Legislation relating to the composition of packaging materials used for food is summarised. This includes the UK, the USA, the EEC countries and other countries. Chapter 6—Migration—Theoretical Aspects. Theoretical principles involved in the migration of compounds from a packaging material into food, by diffusion or absorption mechanisms, are outlined. The use of models to represent migration under different conditions is discussed. Chapter 7—Migration—Experimental Determination. Food simulants are discussed with emphasis on the selection of fat simulants. Methods for the determination of global migration are

Food Chemistry (7) (1981)—
Printed in Great Britain

described and some of the problems encountered with such tests are discussed. Specific migration, the study of the movement of single chemical compounds, is briefly discussed. This usually involves the use of model compounds or radiochemically labelled compounds. Some of the problems involved in this type of testing are indicated. Chapter 8—Other Food Contact Materials. Some problems specific to the use of tinplate, stainless steels, aluminium, pewter, ceramics, glass, papers, boards and rubber materials in contact with food are indicated. The problem of lead pollution is discussed.

The book represents an up-to-date account of this important topic by an author with direct experience in the field. It should be of use to scientists and technologists working in both the food and packaging industries and to students preparing to enter these industries or research establishments concerned with such topics.

J. G. BRENNAN

Natural Colours for Foods and Other Uses. Edited by J. N. Counsell. Applied Science Publishers Ltd, London, 1981. 167 pp. Price: £13·00.

Although a reasonably priced book, it is, at first sight, difficult to see at which part of the market this book is aimed since it describes many different aspects of the use of natural colours, from foods to cosmetics. The book describes the proceedings of a conference held by Roche Products Ltd, in October 1979 on natural colours.

Chapters 1 and 2 deal basically with the nature and importance of colour and provide different aspects of the topic of colour from that which we normally consider.

Chapters 3, 4 and 5 describe the importance and role of colour in foods, cosmetics and hospital pharmacy, three quite contrasting areas. For the food technologist it is an insight into the problems these other industries face.

Chapter 6 deals quite comprehensively with carotenoids whilst a single chapter—Chapter 7—is left to deal with 'some other' natural colours. Not a very satisfactory state of affairs. A chapter on legislative aspects of natural colours completes the book.

After reading the book one is left with the feeling that it is a classic example of opportunity missed in the sense that so much could have been said yet so little was said. This is inevitable, however, when trying to deal with a wide ranging subject in a short space of time. The references provided at the end of each chapter do alleviate the problem to a certain extent, although these are often rather thin on the ground.

Undoubtedly the book will find a place on the market but I feel this will be more for the emotive value of food colours rather than for the book's content.

M. W. KEARSLEY

described and some of the problems encountered with such tests are discussed. Specific migration, the study of the movement of single chemical compounds, is briefly discussed. This usually involves the use of model compounds or radiochemically labelled compounds. Some of the problems involved in this type of testing are indicated. Chapter 8—Other Food Contact Materials. Some problems specific to the use of tinplate, stainless steels, aluminium, pewter, ceramics, glass, papers, boards and rubber materials in contact with food are indicated. The problem of lead pollution is discussed.

The book represents an up-to-date account of this important topic by an author with direct experience in the field. It should be of use to scientists and technologists working in both the food and packaging industries and to students preparing to enter these industries or research establishments concerned with such topics.

J. G. BRENNAN

Natural Colours for Foods and Other Uses. Edited by J. N. Counsell. Applied Science Publishers Ltd, London, 1981. 167 pp. Price: £13·00.

Although a reasonably priced book, it is, at first sight, difficult to see at which part of the market this book is aimed since it describes many different aspects of the use of natural colours, from foods to cosmetics. The book describes the proceedings of a conference held by Roche Products Ltd, in October 1979 on natural colours.

Chapters 1 and 2 deal basically with the nature and importance of colour and provide different aspects of the topic of colour from that which we normally consider.

Chapters 3, 4 and 5 describe the importance and role of colour in foods, cosmetics and hospital pharmacy, three quite contrasting areas. For the food technologist it is an insight into the problems these other industries face.

Chapter 6 deals quite comprehensively with carotenoids whilst a single chapter—Chapter 7—is left to deal with 'some other' natural colours. Not a very satisfactory state of affairs. A chapter on legislative aspects of natural colours completes the book.

After reading the book one is left with the feeling that it is a classic example of opportunity missed in the sense that so much could have been said yet so little was said. This is inevitable, however, when trying to deal with a wide ranging subject in a short space of time. The references provided at the end of each chapter do alleviate the problem to a certain extent, although these are often rather thin on the ground.

Undoubtedly the book will find a place on the market but I feel this will be more for the emotive value of food colours rather than for the book's content.

M. W. KEARSLEY

Food Chemistry 7 (1981) 239–247

BLUE–GREEN ALGAE AS A SOURCE OF PROTEIN†

YOUNG R. CHOI* & PERICLES MARKAKIS

Department of Food Science and Human Nutrition,
Michigan State University, East Lansing, Michigan 48824, USA

(Received: 11 February, 1980)

ABSTRACT

Anabaena flos-aquae, *an N-fixing and photosynthesising blue–green alga, was grown in defined liquid media. Although this organism grew well in autotrophic solution, it rendered a higher yield in a medium containing 1% glucose. A good procedure for extracting the protein from the cells was heating them in 3* N *HCl at 95°C for 10 min, adjusting the pH to 11 with 3* N *NaOH after cooling, letting them stand at room temperature for 1 h, centrifuging out the debris and exposing the supernatant to 15,000 international lux of fluorescent lamp light for 10 h. The treated solution contained 80% of the nitrogen of the cells and it had a pale yellow colour. The protein of the solution was further fractionated into a pH-4-insoluble fraction and an acetone-insoluble fraction. Both fractions were easily digested by pepsin* in vitro *and were deficient in sulphur-containing amino acids. The amino acid scores of the fractions were 74 and 60, respectively.*

INTRODUCTION

Blue–green algae (BGA) are primitive micro-organisms of particular interest because they can both photosynthesise and fix atmospheric nitrogen (Wolk, 1973). They are thought to contribute significantly to sustaining the productivity of rice fields—a common habitat of these organisms. Their indigestible cell wall with the

* Present address: Food Protein Research and Development Centre, Texas A & M University, College Station, Texas 77840, USA.

† This paper has been assigned Michigan Agricultural Experiment Station Journal Article No. 9319.

Food Chemistry 0308-8146/81/0007-0239/$02·75

surrounding mucilage, their colour and strange flavour make them rather unattractive as human food. Should it be possible, however, to prepare a protein extract of good sensory quality from these organisms, their economic significance might increase. The purpose of the work described in this paper was to study the growth of BGA under controlled conditions and the extraction of a potentially useful protein fraction from these organisms.

MATERIALS AND METHODS

Growth of the algae

An axenic culture of *Anabaena flos-aquae*, obtained from the Botany Department of Michigan State University, was grown in the Kratz and Myers basal medium (Kratz & Myers, 1955). This is a solution of salts essentially free from nitrogen and carbon. Both these elements are provided to the organism by the air that is bubbled through the solution. In an effort to study the effect of other sources of nitrogen and carbon on the growth of these algae the following modifications of the basal medium (BM) were used: (a) BM + sodium nitrite, 100 mg/litre; (b) BM + sodium nitrate, 100 mg/litre; (c) BM + peptone, 50 mg/litre; (d) BM + glucose at five levels, 0·5, 1·0, 1·5, 2·0 and 2·5% and (e) urea, 100 mg/litre.

Sixteen litres of each medium were sterilised at 100 °C for 1 h in a five-gallon flask. After inoculation, the medium was aerated continually with compressed air passing through a sterilising cotton filter at the rate of 2·5 litres (at 1 atm) a second. Five vertical fluorescent tubes delivering 15,000 IL (international lux) of light at the closest point of the flask surface were used. Although the light tubes were arranged on a plane rather than cylindrically around the flask, the air-agitation of the flask contents was sufficient to sustain a vigorous growth of the algae. The temperature of the growth medium was approximately 30 °C. To follow the progress of algal growth, periodically, 10 ml of medium were withdrawn through a tube permanently inserted through the stopper of the flask. The cells of the 10 ml sample were centrifuged down at 10,000 × *g*, washed twice with water, dried and weighed. For the study of the algal protein, the cells were harvested when the concentration reached a maximum of about 260 mg of dry matter per litre.

An alternative method of estimating cell concentration was to measure the absorbance of the washed cells at 420 nm after resuspending them in 10 ml of distilled water. The precision of this measurement was increased by inserting between the cuvette and the cuvette holder a piece of Whatman 3 MM filter paper which had been dipped in paraffin oil and drained overnight (opal paper).

Absorption spectra

A Bausch and Lamb 505 spectrophotometer was used to obtain the absorption spectra of cell suspensions and protein isolates.

Decolorisation

Bleaching of the green colour was attempted on both the intact cells and the protein extracts. (a) The cells were washed, suspended in water, the pH of which was roughly adjusted to several levels (7, 8, 9, 10, 11 and 12) by means of 0·1 N NaOH, the suspensions were transferred into 15 ml ampoules and the ampoules were sealed and exposed to 15,000 IL of fluorescent light for 10 h. (b) The protein-containing extracts were bleached as described in the next section.

Protein extraction and fractionation

After establishing with a crude extract that the proteins of BGA were least soluble near pH 4·0 and most soluble in the pH range 10·0 to 12·0, the following three extraction and fractionation procedures were tried.

(*a*) *Acid pretreatment:* In this procedure the cells were suspended in 3 N HCl and heated at 95 °C for 10 min. The pH of the suspension was then adjusted to 11·0 with 3 N NaOH and left at room temperature for one hour. Centrifugation (10,000 × *g* for 20 min) followed, which resulted in a supernatant (protein extract) and a precipitate (cell debris). The extract was poured in Petri dishes and exposed to 15,000 IL fluorescent light for 10 h. The pH of the extract was then adjusted to 4·0 with 1 N HCl. A second centrifugation (50,000 × *g*, 20 min) resulted in a precipitate and a supernatant. The precipitate was dialysed, freeze-dried and named 'protein isolate A'. The supernatant was concentrated under reduced pressure and mixed with excess acetone; a new precipitate was formed, which was dialysed, freeze-dried and named 'protein isolate B'.

(*b*) *Alkaline pretreatment:* This procedure differed from the previous one only in that the cells were suspended and heated in 0·125 N NaOH instead of 3 N HCl; the subsequent treatment was the same. The final fractions were named 'protein isolate C' and 'protein isolate D'. The two procedures are summarised in Fig. 1.

(*c*) *Mechanical treatment:* In this procedure the cells were suspended in 0·125 N NaOH and subjected to the shearing effect of a device called Polytron (marketed in the USA by Brinkman Co., New York). The macerate was centrifuged at 10,000 × *g* for 20 min and the fractionation proceeded as in Fig. 1 after the corresponding centrifugation step. The final fractions were called 'protein isolate E' and 'protein isolate F'.

Chemical analysis

Moisture, crude protein (% N × 6·25), crude fat and ash were determined by AOAC methods (AOAC, 1975). Amino acid analysis was performed on 22 h and 72 h acid hydrolysates using a Beckman Model 121 automatic amino acid analyser. Methionine and cysteine were determined after performic acid oxidation (Lewis, 1966). Tryptophan was measured by the Spies procedure (Spies, 1967). Total nucleic acid was estimated by the method of Gale & Folks (1953). Protein digestibility, or rather N-digestibility, was determined *in vitro* as follows. Two hundred

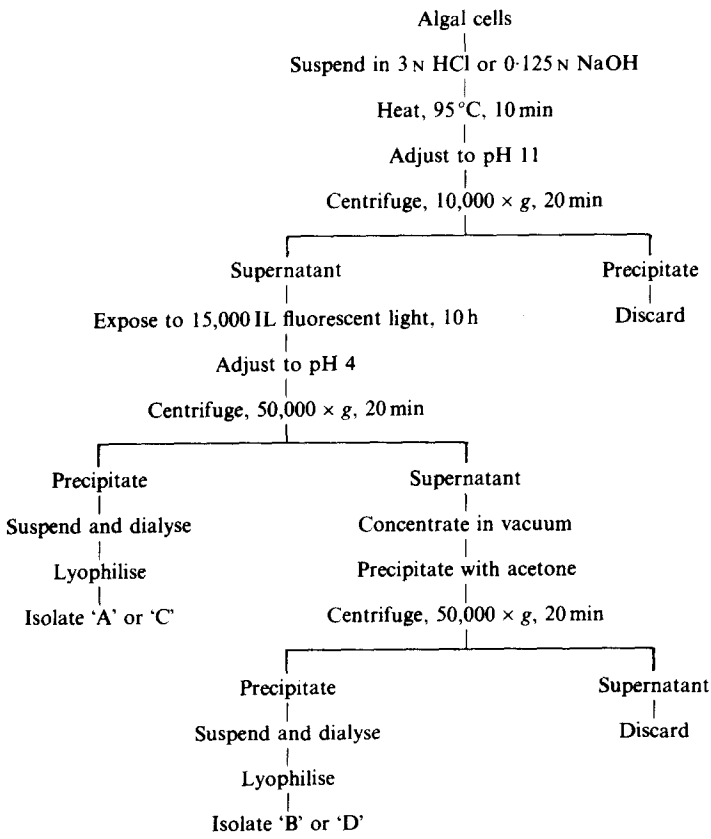


Fig. 1. Preparation of blue–green algal protein isolates ('A' and 'B' after 3 N HCl pretreatment; 'C' and 'D' after 125 N NaOH treatment).

milligrammes of protein isolates, whose cells or casein (for reference) were suspended in 50 ml of 0·2 M KCl–HCl buffer, pH 1·8; four drops of toluene and 10 mg of pepsin (Sigma Chemical Co.) were added to the suspension, which was then incubated at 38 °C. Periodically during 16 h, 5 ml of reaction mixture were transferred into 5 ml of 20% trichloroacetic acid (TCA) and the precipitate was centrifuged out. The nitrogen in the supernatant was determined by the micro-Kjeldahl method.

RESULTS AND DISCUSSION

Algal growth

The growth of BGA in a basal medium followed the typical S-curve of microbial growth in general. Maximum cell concentration was observed on the eleventh day,

when 250–260 mg of dry algae per litre were obtained. The cell concentrations observed after 11 days of growth on media containing additional (to atmospheric N_2) sources of nitrogen are shown in Table 1. Sodium nitrite and sodium nitrate depressed the yield in cells, while urea and peptone did not affect the yield substantially. Table 1 also shows the crude protein content of the cells. The sodium salts of nitrous and nitric acid resulted in lowering the protein content. This may be associated with the greater production of mucilage (carbohydrates) observed around the cells grown in the presence of these salts.

TABLE 1
YIELD AND PROTEIN CONTENT OF *A. flos-aquae* CELLS HARVESTED AFTER ELEVEN DAYS OF GROWTH IN MEDIA CONTAINING VARIOUS SOURCES OF NITROGEN

N-source	*Conc. of N-source (mg/litre)*	*Yield in dry cells (mg per litre of medium)*[a]	*Protein (% N × 6·25), dry basis*[a]
Control (atm. N_2)	—	256	63
Sodium nitrite	100	140	44
Sodium nitrate	100	155	50
Urea	100	263	63
Peptone	50	245	63

[a] Average of six values (three batches × two determinations) not differing more than 6% from the mean.

Addition of glucose to the growth medium up to 1% (10 g/litre) increased the cell yield. Further increase in glucose concentration, however, suppressed the growth of BGA (Fig. 2). Apparently, BGA are not obligate autotrophs. These data confirm those of Kiyohara *et al.* (1960) and Khoza & Whitten (1971) who have shown that BGA can use sources of carbon other than CO_2.

Decolorisation

The absorption spectrum of a cell suspension of *A. flos-aquae* shows three major peaks at approximately 430, 630 and 680 nm. The 430 nm peak may be attributed to chlorophyll and carotenoids, the 630 nm peak to phycocyanin and the 680 nm peak again to chlorophyll.

The pH of the suspending liquid affects the absorption spectrum of the cells both before and after the decolorising treatment. When the cells were exposed to 15,000 IL of fluorescent lamp light for 10 h at pH 12, the colour of the cells was reduced to pale yellow; the 680 and 630 nm maxima disappeared and the 430 nm peak was greatly reduced. When the same illumination treatment was applied at pH 7, considerable blue–green colour was discernible on the cells and both the 630 and 430 nm peaks were strong (Fig. 3). At pH levels between 7 and 12 intermediate degrees of bleaching were observed. The decolorisation of the extracts was more complete than that of the cells. An almost white powder was obtained when the

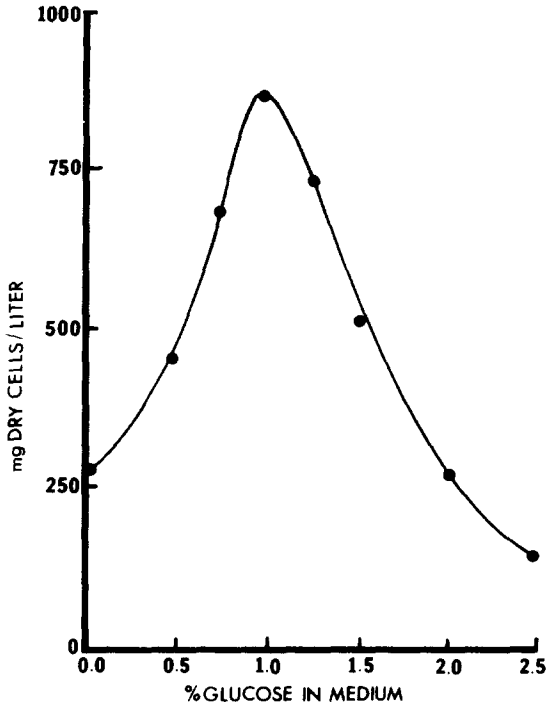


Fig. 2. *A. flos-aquae* cell concentration after 11 days of growth in basal medium containing varying amounts of glucose.

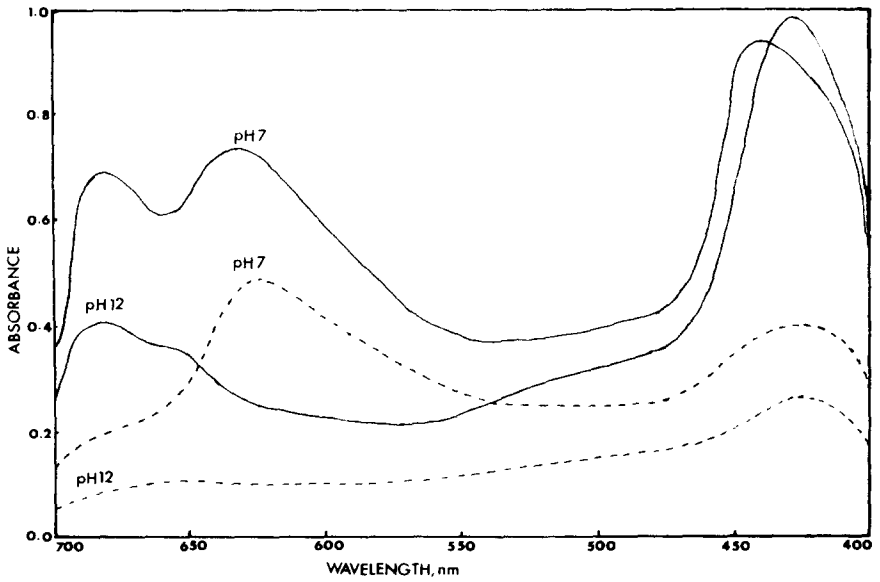


Fig. 3. Absorption spectra of *A. flos-aquae* cells exposed to 15,000 lL light for 10 h at pH 7 and 12. Solid line: before exposure; broken line: after exposure.

decolorised protein extracts were dried. It is interesting that photolysis of both the cells and the protein extract resulted not only in degreening, but also in removal of the unpleasant odour, typical of these algae.

Composition

The protein content of *A. flos-aquae* cells has already been shown in Table 1. The algae grown in basal medium were also found to contain, on a dry basis, 12·2% lipids, 4·2% ash and 4·0% nucleic acid. The amino acid composition of the cells, after acid hydrolysis, appears in Table 2. The same table shows the amino acid composition of protein isolates 'A' and 'B', along with the 1973 FAO/WHO

TABLE 2
AMINO ACID COMPOSITION OF *A. flos-aquae* CELLS AND PROTEIN ISOLATES 'A' AND 'B' (GRAMMES OF AMINO ACID/100 g PROTEIN)

Amino acid	*Algae*	*Protein isolate 'A'*	*Protein isolate 'B'*	*1973 FAO/WHO provis. pattern*
Alanine	7·6	7·6	9·2	—
Arginine	9·3	9·5	7·2	—
Aspartic acid	11·6	14·8	14·1	—
Cysteine	0·6	0·9	0·8	3·5
Methionine	1·7	1·7	1·3	
Glutamic acid	11·6	13·2	14·1	—
Glycine	4·1	4·1	4·9	—
Histidine	1·8	ND	ND	—
Iso-leucine	5·5	6·7	6·2	4·0
Leucine	8·8	10·9	9·4	7·0
Lysine	5·1	4·6	5·2	5·5
Phenylalanine	4·8	4·8	4·2	6·0
Tyrosine	5·5	ND	ND	
Proline	3·4	3·7	4·3	—
Serine	4·1	4·8	5·6	—
Threonine	5·8	5·6	6·6	4·0
Tryptophan	1·6	0·8	0·7	1·0
Valine	8·2	7·3	7·1	5·0

ND = not detected.

TABLE 3
NITROGEN RECOVERY FROM ALGAL CELLS BY VARIOUS EXTRACTION METHODS

Extraction method	*Extracted-N as % of cell-N*	*Isolate-N, A, C or E as % of cell-N*	*Isolate-N, B, D or F as % of cell-N*	*Total isolate-N as % of cell-N*
NaOH pretreatment, heating and photolysis	70·1 ± 1·2[a]	12·5 ± 0·3	43·1 ± 1·5	55·6
HCl pretreatment, heating and photolysis	80·1 ± 2·1	29·1 ± 1·9	35·1 ± 1·0	64·2
Mechanical extraction (no heating, no photolysis)	41 ± 0·7	26·2 ± 0·6	10·3 ± 0·4	36·5

[a] Means and standard errors of three replicates × three determinations.

provisional amino acid scoring pattern (FAO/WHO, 1973). Apparently, the algal protein and the isolates are deficient in sulphur-containing amino acids. The amino acid score for the total algal protein is 66, for isolate 'A' it is 74 and for 'B' it is 60. Worth noticing is the destruction of histidine and tyrosine during extraction and fractionation. The protein recovery after extraction and fractionation by the procedures used in this study is shown in Table 3. The mechanical extraction method resulted in the lowest yield (36·5 %) in terms of protein isolates. Extraction with HCl pretreatment, heating and photolysis gave the highest yield (64·2 %) and NaOH pretreatment with heating and photolysis resulted in an intermediate yield (55·6 %).

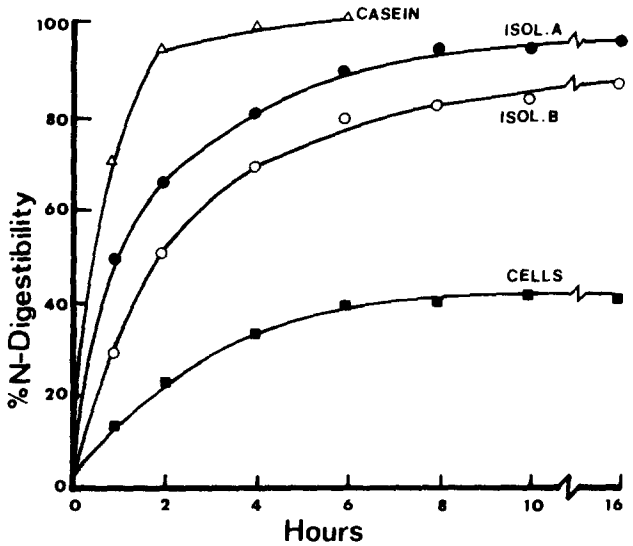


Fig. 4. Nitrogen digestibility of casein, isolates 'A' and 'B', and whole cells of *A. flos-aquae* by pepsin *in vitro*.

As the protein isolates obtained by the acid pretreatment method were in high yield and almost white, they were subjected to amino acid analysis, the results of which have already been discussed, and to the pepsin digestibility test. The results of the latter test are shown in Fig. 4, and suggest that isolates 'A' and 'B' are easily digested by pepsin *in vitro*, although more slowly than casein, and that whole cells of *A. flos-aquae* are not well digested under these conditions.

REFERENCES

AOAC (1975). *Methods of analysis*, Association of Official Analytical Chemists, Washington, DC.
FAO/WHO (1973). *Energy and protein requirements*, Food and Agriculture Organization, Rome.

GALE, E. F. & FOLKS, J. P. (1953). The assimilation of amino acids by bacteria. 14. Nucleic acid and protein synthesis in *Staphylococcus areus*. *Biochem. J.*, **53**, 483.

KRATZ, W. A. & MYERS, J. (1955). Nutrition and growth of several blue–green algae, *Amer. J. Bot.*, **42**, 282.

KHOZA, T. & WHITTEN, B. A. (1971). Heterotrophic growth of blue–green algae. *Arch. Mikrobiol.*, **79**, 280.

KIYOHARA, T., FUJITA, Y., HATTORI, A. & WATANABE, A. (1960). Heterotrophic culture of a blue-green algae. *J. Gen. Appl. Microbiol.*, **8**, 165.

LEWIS, O. A. M. (1966). Short ion-exchange column method for the estimation of cystine and methionine, *Nature*, **209**, 1239.

SPIES, J. R. (1967). Determination of tryptophan in proteins, *Anal. Chem.*, **39**, 1412.

WOLK, C. P. (1973). Physiology and cytological chemistry of blue–green algae, *Bacter. Reviews*, **37**, 37.

Food Chemistry 7 (1981) 249–256

THE ENZYMIC DEACYLATION OF *p*-NITROPHENYL ESTERS AND PHOSPHATIDYLCHOLINE IN *VICIA FABA MINOR*

H. M. HENDERSON,* D. L. SHAMBROCK* & N. A. M. ESKIN†
**Department of Food Science, †Department of Foods and Nutrition, University of Manitoba, Winnipeg, Manitoba R3T 2N2, Canada*

(Received: 20 February, 1981)

ABSTRACT

Two enzymic deacylating activities have been detected in crude extracts of small fababeans—the hydrolysis of p-*nitrophenyl fatty acyl esters and that of phosphatidylcholine. Kinetic studies demonstrated differences between the two activities with respect to substrate inhibition and to the effects of pH, Triton X-100, calcium chloride and mercuric chloride. The sequential activity of phospholipases* A_1 *and* A_2*, and lysophospholipase, in fababeans is postulated, and some similarity is indicated between the hydrolysis of* p-*nitrophenyl palmitate and fababean alkaline lipase. The significance of results from this study is discussed in relation to earlier work on lipid-degrading enzymes in fababeans.*

INTRODUCTION

The small fababean, or horse bean, *Vicia faba* L. var. *minor* (Peterm.) Beck, is widely cultivated in many countries, including the majority of the member-states of the European Economic Community, and is an increasingly important special crop in the Canadian Prairies. Processed fababeans become rancid very soon after tissue disruption, presenting storage and acceptability problems. The rapid development of rancidity is due to the relatively high proportion of unsaturated fatty acids present in the lipid fraction, especially linoleic acid which accounts for 55·1% of the component fatty acids in fababean lipids (Hinchcliffe *et al.*, 1977). Eskin & Henderson (1974*a*, *b*) obtained two fractions of fababean lipoxygenase, which showed markedly differing responses to calcium ions, and both of which appeared to exhibit a high degree of specificity towards free polyunsaturated fatty acids, especially linoleic acid. However, the greater amount of fababean fatty acids occurs

Food Chemistry 0308-8146/81/0007-0249/$02·75

in esterified form, as triacylglycerols and phospholipids which, respectively, account for 30% and 66% of fababean lipids. Dundas *et al.* (1978). demonstrated an active fababean lipase, while Atwal *et al.* (1979) isolated and characterised phospholipase D from fababeans.

The purpose of the investigation reported in this paper was the detection and biochemical characterisation in fababeans of an enzymic system releasing fatty acids from phospholipids. During the course of this investigation it was also found that fababean extracts hydrolysed several *p*-nitrophenyl fatty acyl esters.

EXPERIMENTAL

Source material

Small fababeans (*Vicia faba* L. var. *minor* cv. Diana) were obtained from the Department of Plant Science, University of Manitoba. The beans were harvested in 1976 and subsequently stored at 4 °C.

Protein determination

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

Enzyme preparation

Crude extracts were prepared from dehulled fababean acetone powder and were employed for kinetic studies since attempts at partial purification did not result in an increase of specific enzyme activity.

Enzyme assays

The hydrolysis of *p*-nitrophenyl esters was determined by continuous spectrophotometric measurement at 400 nm of the *p*-nitrophenol released in 10 min at 25 °C (Galliard, 1971). The change in A_{400} was related to a standard curve for *p*-nitrophenol measured at the pH of the incubation. Incubation mixtures (total volume of 3·0 ml in 1-cm glass cuvettes) contained *p*-nitrophenyl ester (0·67 mM) and Triton X-100 (1·33 mg/ml) in 0·1 M diethanolamine–HCl buffer, pH 8·5, and an enzyme preparation (8·0 mg of protein per millilitre). Control experiments demonstrated that negligible non-enzymic hydrolysis of the substrate occurred at pH 6–9. One unit of enzyme activity was equivalent to one micromole of *p*-nitrophenol released per minute at 25 °C.

The hydrolysis of phosphatidylcholine (1,2-diacyl-*sn*-glycero-3-phosphorylcholine, PC) was determined by measuring the decrease in acyl ester content at the end of a 10-min incubation period at 25 °C. Acyl ester content was determined by a modification (Renkonen, 1961) of the hydroxamate method (Snyder & Stephens, 1959). Employing a modification of the method of Galliard (1970), incubation

mixtures (total volume of 2·4 ml) contained 0·05–1·00 mM of PC (0·10–2·00 microequivalents of acyl ester per millilitre) and Triton X-100 (4 mg) in 0·1 M acetate buffer, pH 5·6, and 1·0 ml (10 mg of protein per millilitre) of an enzyme preparation. Incubation was stopped by boiling, and after cooling, 3·0 ml of chloroform and 6·0 ml of methanol were added to each mixture to form the monophasic mixture of Bligh & Dyer (1959). After drying of the chloroform layer the acyl ester determination was carried out. The A_{530} values were related to a standard curve for methyl palmitate and indicated the number of microequivalents of ester remaining after hydrolysis. One unit of enzyme activity was equal to one microequivalent of ester hydrolysed in 10 min at 25 °C.

RESULTS

Hydrolysis of p-*nitrophenyl esters*

A linear relationship between enzyme concentration and *p*-nitrophenol released was evident with respect to all determinations carried out. Initial reaction velocities were determined at pH 7·5 and 8·5 in the presence of various concentrations of each of the following *p*-nitrophenyl ester substrates: acetate, laurate, myristate, palmitate and stearate. The assay could not be carried out at pH 7·5 with *p*-nitrophenyl stearate owing to the instability of the emulsion. The enzyme activity was found to be inhibited by higher substrate concentrations, with optimal activity occurring in the substrate concentration range of 0·34 mM for *p*-nitrophenyl laurate (pH 8·5) to 1·0 mM for *p*-nitrophenyl palmitate (pH 8·5). The hydrolysis of *p*-nitrophenyl acetate was linear with respect to substrate concentrations up to at least 0·7 mM. The corresponding Lineweaver–Burk plots required extrapolation of the linear portions to enable apparent K_m values to be calculated for each substrate (Table 1).

In each case, the demonstrated maximum rate of hydrolysis was lower than the theoretical maximum velocity calculated from the Lineweaver–Burk plots, meaning

TABLE 1

CALCULATED VALUES FROM LINEWEAVER–BURK PLOTS FOR THE HYDROLYSIS OF *p*-NITROPHENYL ESTERS

Substrate	*pH*	V_{max} (*theoretical*) (*units per mg of protein*)	K_m (*mM p-nitrophenyl ester*)
p-Nitrophenyl acetate	7·5	10·00	0·59
	8·5	16·67	1·43
p-Nitrophenyl laurate	7·5	0·418	0·07
	8·5	0·704	0·67
p-Nitrophenyl myristate	7·5	0·400	0·50
	8·5	0·435	0·22
p-Nitrophenyl palmitate	7·5	0·308	0·63
	8·5	0·333	0·29
p-Nitrophenyl stearate	8·5	1·111	1·82

that full enzyme saturation with substrate could not be attained without causing substrate inhibition, as shown in Figs 1 and 2 for *p*-nitrophenyl palmitate (PNP).

The enzyme was active within a pH range of 6·5 to 9·0, with the optimum at pH 8·5. The optimum temperature was 37 °C and the enzyme was completely inactivated by exposure to 75 °C for 2 min. The hydrolysis of PNP was markedly optimal in the presence of 2 mg/ml of Triton X-100, was activated by calcium chloride up to a concentration of 3·0 mM and was inhibited non-competitively by mercuric chloride, with approximately 40 % of the original activity inhibited by 1·0 mM $HgCl_2$ and complete inhibition in the presence of 5·0 mM $HgCl_2$.

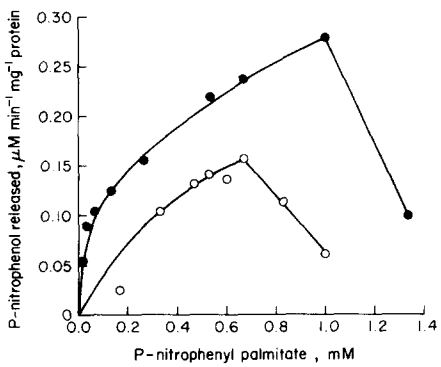


Fig. 1. Effect of *p*-nitrophenyl palmitate concentration upon enzyme activity at pH 7·5 (○) and 8·5 (●).

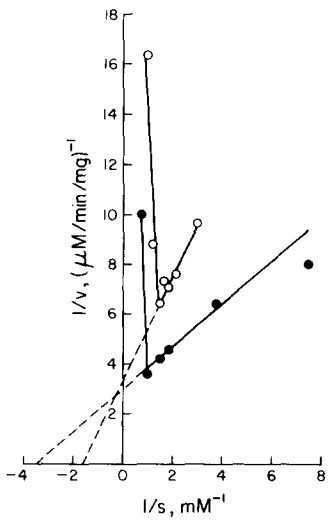


Fig. 2. Lineweaver–Burk plots of the effect of *p*-nitrophenyl palmitate upon enzyme activity at pH 7·5 (○) and 8·5 (●).

Hydrolysis of PC

A linear relationship was established between enzyme concentration and microequivalents of acyl ester content of PC hydrolysed. The substrate-inhibition effect was not demonstrated and K_m values for PC were 0·18 mM (pH 5·6) and 0·72 mM (pH 7·5). The enzyme was active at pH 4·5–8·5, with the primary optimum at pH 5·6 and a possible secondary optimum at pH 7·5. There was a slight optimal activity in the presence of 0·83–2·50 mg/ml of Triton X-100, and concentrations of Triton up to 6·66 mg/ml were found to be inhibitory. The enzyme was not affected by calcium chloride up to 3·0 mM and was slightly inhibited by mercuric chloride, with 2 % of the original activity inhibited by 1·0 nM $HgCl_2$ and 6 % by 5·0 mM $HgCl_2$.

DISCUSSION

The detection and characterisation of two enzymic deacylating activities in *Vicia faba minor*—the hydrolysis of *p*-nitrophenyl fatty acyl esters and the decrease in acyl ester content of PC—has been achieved. Crude extracts had to be used in this study owing to purification difficulties, possibly due to the close association of enzyme activity with a major protein component (Galliard, 1971). Therefore it is not shown whether both reactions are caused by a single low-specific enzyme (Galliard, 1971) or by separate lipid acylhydrolases (Hasson & Laties, 1976*a*). The question of enzyme specificity resulted in the study of the two activities in fababeans described in this paper.

The hydroxamate method was used in the determination of PC hydrolysis, in the manner employed by Galliard (1970) and by Shepard & Pitt (1976*a*, *b*). It is suggested that the decrease in acyl ester content of PC fababeans may be due to the sequential activities of phospholipases A_1 and A_2, and lysophospholipase. Such phospholipases have been demonstrated in other plant tissues, especially in potatoes (Galliard, 1970; Galliard & Dennis, 1974; Hirayama *et al.*, 1975; Hasson & Laties, 1976*a*, *b*; Shepard & Pitt, 1976*b*). In this study, it was found that glycerophosphate, which contains a phosphoric ester linkage but no acyl linkages, gave a negative result with the hydroxamate method. Therefore the measured decrease of ester content of PC was indicative of deacylation at positions C_1 and/or C_2 and did not involve phospholipase C- or D-type phosphodiesterase activities, even though the latter is present in fababeans (Atwal *et al.*, 1979). The occurrence in fababeans of up to three phospholipid acylhydrolases is suggested since the hydroxamate method does not distinguish among the specificities of these three enzymes.

Substrate inhibition was demonstrated with long-chain *p*-nitrophenyl fatty acyl esters, but not with *p*-nitrophenyl acetate or with PC. Such inhibition was demonstrated for the potato enzyme by Galliard (1971) who suggested that it may be due to interaction between the enzyme and the long-chain fatty acid components of lipids. The K_m values are relative with respect to enzyme–substrate affinities and are apparently due to substrate solubility since the enzymic reaction occurs at the micelle–water interface in an emulsion system. Our K_m values for PC compare with corresponding values from potato of 1·7 mM (Hirayama *et al.*, 1975) and 0·54 mM (Matsuda & Hirayama, 1979).

The pH optimum of 8·5 for the hydrolysis of PNP was identical to that of the fababean alkaline lipase of Dundas *et al.* (1978) and similar to values obtained for the potato enzyme by Galliard (1971) in the presence of Triton X-100. However, the pH optimum for PC hydrolysis was 5·6, similar to corresponding values for the potato enzyme (Galliard, 1970; Matsuda & Hirayama, 1979), suggesting the possibility that the hydrolysis of PNP and of PC in fababeans may not be catalysed by a single enzyme. Heat stability studies indicated the relative sensitivity towards heat of PNP hydrolysis compared with that of fababean lipoxygenase (Eskin &

Henderson, 1974*a*), supporting the view that the hydrolytic enzymes are somewhat more heat-sensitive than lipoxygenase, as shown in potatoes (Galliard & Rayward-Smith, 1977). A relatively mild heat-treatment may therefore be adequate for the control of enzymic lipid degradation in stored processed fababeans.

Results from this study have indicated the differing effects of Triton X-100, of calcium ions and of mercuric chloride, on the hydrolysis of PNP and of PC in fababeans. It is not strictly necessary to use a detergent for emulsifying PC, and the inhibitory effect of Triton could have been due to a dilution effect, resulting in the decreased availability of PC to the enzyme, or to an alteration in the characteristics of the mixed phospholipid–detergent micelles (Dennis, 1973). Activation by calcium ions could not be demonstrated in the case of PC hydrolysis, as sufficient calcium ions for maximal activity may have been already present in the crude extracts. Mercuric chloride inhibition of PNP hydrolysis is comparable to that of fababean lipase (Dundas *et al.*, 1978) and may indicate the participation of sulphydryl groups in the maintenance of enzymic structural integrity.

A sequential hydrolytic and oxidative enzymic pathway is proposed, by which fababean endogenous lipids may be degraded upon tissue disruption, incorporating our previous lipid enzyme studies (Fig. 3).

The pathway indicates the specificity of fababean lipoxygenase towards free fatty acids (Eskin & Henderson, 1974*a*) and, in some respects, resembles that proposed by Galliard *et al.* (1977) for disrupted tomato fruits. It is suggested that the phospholipid acylhydrolases may be distinct from the fababean alkaline lipase shown to be active towards triacylglycerols (Dundas *et al.*, 1978), which latter enzyme has some properties in common with the hydrolysis of *p*-nitrophenyl esters.

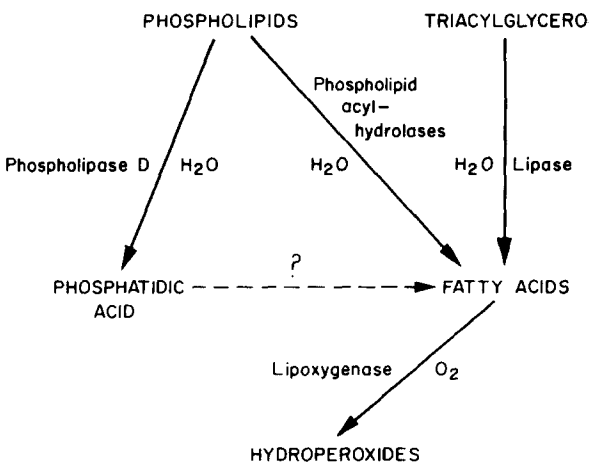


Fig. 3. Proposed pathway for the enzymic degradation of endogenous lipids upon disruption of *Vicia faba minor* tissues.

Possible supporting evidence from this study is that porcine pancreatic lipase was very slightly active towards PC at pH 5·6 and 7·4. We have been unable to convincingly demonstrate with fababean extracts the production of free fatty acids from phosphatidic acid, but a very low phosphatase activity has been identified (Atwal and Eskin, unpublished work). The subject of continuing studies is the determination of the enzymic pathways by which, upon fababean tissue disruption, PC could be completely degraded into its five component parts.

ACKNOWLEDGEMENT

Financial assistance from the Natural Sciences and Engineering Research Council, Canada is gratefully acknowledged.

REFERENCES

ATWAL, A. S., ESKIN, N. A. M. & HENDERSON, H. M. (1979). Isolation and characterization of phospholipase D from fababeans. *Lipids*, **14**, 913–17.

BLIGH, E. G. & DYER, W. J. (1959). A rapid method of total lipid extraction and purification, *Can. J. Biochem. Physiol.*, **37**, 912–17.

DENNIS, E. A. (1973). Kinetic dependence of phospholipase A_2 activity on the detergent Triton X-100. *J. Lipid Res.*, **14**, 152–9.

DUNDAS, D. G. A., HENDERSON, H. M. & ESKIN, N. A. M. (1978). Lipase from *Vicia faba minor*. *Fd. Chem.*, **3**, 171–8.

ESKIN, N. A. M. & HENDERSON, H. M. (1974*a*). Lipoxygenase in *Vicia faba minor*. *Phytochem.*, **13**, 2713–16.

ESKIN, N. A. M. & HENDERSON, H. M. (1974*b*). A study of lipoxygenase from small faba beans. *Proc. IV Int. Congress Food Sci. and Technol.*, **1**, 263–9.

GALLIARD, T. (1970). The enzymic breakdown of lipids in potato tuber by phospholipid- and galactolipid-acyl hydrolase activities and by lipoxygenase. *Phytochem.*, **9**, 1725–1734.

GALLIARD, T. (1971). The enzymic deacylation of phospholipids and galactolipids in plants. Purification and properties of a lipolytic acyl-hydrolase from potato tubers. *Biochem. J.*, **121**, 379–90.

GALLIARD, T. & DENNIS, S. (1974). Phospholipase, galactolipase and acyl transferase activities of a lipolytic enzyme from potato. *Phytochem.*, **13**, 1731–5.

GALLIARD, T. & RAYWARD-SMITH, M. P. (1977). Distribution and heat inactivation of lipid-degrading enzymes in potato tuber. *J. Food Biochem.*, **1**, 351–9.

GALLIARD, T., MATTHEW, J. A., WRIGHT, A. J. & FISHWICK, M. J. (1977). The enzymic breakdown of lipids to volatile and non-volatile carbonyl fragments in disrupted tomato fruits. *J. Sci. Fd. Agric.*, **28**, 863–8.

HASSON, E. P. & LATIES, G. G. (1976*a*). Separation and characterization of potato lipid acylhydrolases. *Plant Physiol.*, **57**, 142–7.

HASSON, E. P. & LATIES, G. G. (1976*b*). Purification and characterization of an A type phospholipase from potato and its effect on potato mitochondria. *Plant Physiol.*, **57**, 148–52.

HINCHCLIFFE, C., MCDANIEL, M., VAISEY, M. & ESKIN, N. A. M. (1977). The flavour of fababeans as affected by heat and storage. *Can. Inst. Food Sci. Technol. J.*, **10**, 181–4.

HIRAYAMA, O., MATSUDA, H., TAKEDA, H., MAENAKA, K. & TAKATSUKA, H. (1975). Purification and properties of a lipid acyl-hydrolase from potato tubers. *Biochim. Biophys. Acta*, **384**, 127–37.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–75.

MATSUDA, H. & HIRAYAMA, O. (1979). Purification and properties of a lipolytic acyl-hydrolase from potato leaves. *Biochim. Biophys. Acta*, **573**, 155–65.

RENKONEN, O. (1961). A note on spectrophotometric determination of acyl ester groups in lipids. *Biochim. Biophys. Acta*, **54**, 361–2.

SHEPARD, D. V. & PITT, D. (1976*a*). Purification of a phospholipase from *Botrytis* and its effects on plant tissues. *Phytochem.*, **15**, 1465–70.

SHEPARD, D. V. & PITT, D. (1976*b*). Purification and physiological properties of two lipolytic enzymes of *Solanum tuberosum*. *Phytochem.*, **15**, 1471–4.

SNYDER, F. & STEPHENS, N. (1959). A simplified spectrophotometric determination of ester groups in lipids. *Biochim. Biophys. Acta*, **34**, 244–5.

Food Chemistry 7 (1981) 257–266

THE EFFECT OF HEAT ON CABBAGE AND BRUSSELS SPROUT PEROXIDASE ENZYMES

KATHRYN M. MCLELLAN and DAVID S. ROBINSON
Procter Department of Food Science, University of Leeds, Leeds LS2 9JT, Great Britain

(Received: 28 February, 1980)

ABSTRACT

Preparations of soluble, ionically bound and covalently bound peroxidases extracted from cabbage and Brussels sprout have been obtained. The effect of heating each fraction at 60°C, 65°C, 70°C and 75°C on peroxidase activity has been studied. Biphasic inactivation kinetics were observed in all cases, except for Spring cabbage ionically bound peroxidases, which were also found to be less heat stable. Heat inactivation energies for the inactivation of the peroxidases in each fraction have been calculated. Following inactivation, incubation at 30°C allowed regeneration of some of the inactivated peroxidase enzymes in the case of each of the three soluble fractions, but only Brussels sprout ionically bound peroxidases showed detectable regeneration.

INTRODUCTION

Peroxidase is one of the most heat stable naturally occurring enzymes present in vegetables and fruit. Consequently, the presence or absence of peroxidase activity is now widely used for the estimation of the effectiveness of blanching procedures in the food industry. Several authors have reported that the initial heat inactivation of peroxidase enzymes is rapid, followed by a much slower inactivation period, and have concluded that the heat inactivation process is biphasic (Yamamoto *et al.*, 1962; Adams, 1977, 1978; Lee & Hammes, 1979).

Although it seems likely that the biphasic heat inactivation and regeneration processes might be due to the presence of peroxidase isoenzymes (Shannon *et al.*, 1966) with different sensitivities to heat, Adams (1977), who studied the heat inactivation characteristics of his isolated isoenzyme preparations from horseradish, still obtained biphasic plots.

Food Chemistry 0308-8146/81/0007-0257/$02·75

Also, it has been known for a long time that following heat inactivation of peroxidase some regeneration of the enzyme can occur during subsequent storage of many blanched vegetables. Further, the activity of the regenerated enzyme has been shown to correlate with the production of off-flavours (Esselen & Anderson, 1956; Burnette, 1977). Adams (1977) has reported that regeneration of peroxidase is non-linear with time and Tamura & Morita (1975), who used spectral methods to study heat inactivation of peroxidase, suggested that reversible inactivation was due to reassociation of the heat displaced prosthetic haem group with the protein portion of the enzyme.

Recently, Gkinis & Fennema (1978), who studied the changes in the activity of peroxidase enzymes during low temperature storage of green beans, reported the presence of three distinct peroxidase fractions. Soluble peroxidases were extracted with low ionic strength buffer solutions, while higher ionic strength buffers were required to extract an ionically bound peroxidase fraction; a covalently bound peroxidase fraction was only extracted after treatment of the homogenate with pectinase or cellulase enzymes. The present paper reports the study of the heat inactivation and regeneration properties of the soluble peroxidases, ionically bound peroxidases and, where present, covalently bound peroxidase enzymes in some samples of cabbage and Brussels sprout.

EXPERIMENTAL

Reagents

o-Dianisidine was obtained from Koch-Light Laboratories Ltd, Colnbrook, Bucks, Great Britain, and purified horseradish peroxidase from Worthington Biochemical Corps., Freehold, New Jersey. All other chemicals were obtained in the Analar grade from BDH Chemicals Ltd, Poole, Great Britain.

Samples

Brassica oleracea var. capitata (whole hearted cabbage), *Brassica oleracea* var. gemmifera (Brussels sprouts) and Spring cabbage were purchased in a local market in October, January and June, respectively.

METHODS

Fractionation of peroxidases

Ten grammes of inner leaves from whole hearted cabbage, Spring cabbage or Brussels sprouts were roughly shredded and then homogenised for 3 min in 80 cm^3 ice-cold 0·01 M phosphate buffer, pH 6·0, using a blender. The resultant suspension was filtered through a double layer of muslin, divided into four 20 cm^3 portions and

the homogenate centrifuged. All centrifugations were carried out at 15,000 *g*, 4 °C for 20 min. The supernatant fluids, defined as the soluble peroxidase fraction, were collected and retained for assay. A 1 in 10 dilution of this fraction in 0·01 M phosphate buffer, pH 6·0, was found to be necessary in order to obtain suitable spectrophotometric measurements during the enzymic assay.

The remaining residue in each centrifuge tube was resuspended in 20 cm^3 of ice-cold water, centrifuged and the resulting supernatant discarded. The washing procedure was repeated a second time. This was considered sufficient, since the supernatant obtained from a third washing contained less than 0·005 % of the soluble peroxidase activity. Each portion of washed residue was resuspended in 20 cm^3 of ice-cold 1 M NaCl and the homogenate centrifuged. The supernatant fluid, defined as the ionically bound peroxidase fraction, was retained for assay. A 1 in 2 dilution of this fraction in water was found to be necessary in order to obtain suitable spectrophotometric measurements during the enzymic assay.

The residue in each test tube was resuspended in 20 cm^3 of ice-cold 1 M NaCl, centrifuged and the resulting supernatant discarded. The washing procedure was repeated a second time to ensure the complete removal of ionically bound peroxidases. The supernatant from a third washing contained no detectable peroxidase activity. Each portion of the residue was resuspended in 5 cm^3 of ice-cold 0·01 M phosphate buffer, pH 6·0. The whole homogenate was retained for assay as the covalently bound peroxidase fraction.

Inactivation of peroxidases

Some 0·2 cm^3 of the extracts containing one of the peroxidase fractions was placed in Pyrex tubes (15 × 1·6 cm, wall thickness, 1·8 mm) and subjected to various temperatures (60 °C, 65 °C, 70 °C and 75 °C) for different periods of time up to 10 min. After heat treatment the extracts were rapidly cooled in iced water and held at −18 °C until required for enzymic assay. Duplicate samples were used for each time/temperature treatment.

Initially it was found difficult to obtain reproducible results for each time/temperature treatment, especially in the case of Brussels sprout peroxidases. However, this problem was overcome by soaking all glassware overnight in Decon 75, washing thoroughly and rinsing in distilled water before use.

Regeneration of peroxidases

Some 0·2 cm^3 of the extract containing one of the peroxidase fractions was placed in Pyrex tubes (15 × 1·6 cm, wall thickness, 1·8 mm) and heat inactivated.

Heat treatments at 75 °C for periods of 5, 2 and 2 min were found to inactivate 75–85 % of the soluble, ionically bound and covalently bound peroxidase fractions respectively, except in the case of Spring cabbage ionically bound peroxidases when 2 min at 70 °C was sufficient. For regeneration, the samples were then held at 30 °C for different periods of time. Total regeneration was measured after 2·5 h at 30 °C.

Following the required treatment, the samples were cooled in iced water and held at −18 °C until required for assay. Triplicate samples were studied for each regeneration time period.

Enzyme analysis

Peroxidase activity was estimated using the method described by Burnette & Flick (1977) with the following modifications. The reaction mixture contained 2·6 cm^3 0·01 M phosphate buffer at pH 6·0, 0·1 cm^3 0·8 % H_2O_2 in 0·01 M phosphate buffer at pH 6·0 and 0·2 cm^3 enzyme extract. The enzymic reaction was started by the addition of 0·1 cm^3 of 1 % (w/v) *o*-dianisidine in methanol and the initial reaction rate measured at 460 nm using a Unicam SP1800 ultraviolet spectrophotometer with a Unicam AR25 linear recorder at room temperature.

Using purified horseradish peroxidase, the detection limit for this assay was found to be 0·5 ng cm^{-3}.

RESULTS

Three peroxidase fractions were extracted from both whole hearted and Spring cabbage, which were distinguishable by their binding properties. The soluble, ionically bound and covalently bound peroxidase fractions constituted 92·3 %, 7·7 % and (5×10^{-4}) %, respectively of the total peroxidase activity extracted from whole hearted cabbage, as is shown in Table 1, when estimated at room temperature. The relative abundances of the three peroxidase fractions extracted from Brussels sprouts were 91·9 % soluble, 8·1 % ionically bound, with only a trace of covalently bound peroxidase when assayed at room temperature. The two vegetables, therefore, seem to possess similar relative amounts of the two major peroxidase fractions. However, for whole hearted cabbage the fraction of total peroxidase activity made up by covalently bound enzymes was approximately ten-fold that found in Brussels sprout extracts. However, since it was found necessary to remove certain fibrous material by filtration before extracting the various peroxidase fractions, it is possible that some bound activity was not detected.

Figures 1 to 3 show the effect on peroxidase activity of heating samples of whole

TABLE 1

RELATIVE ACTIVITIES OF PEROXIDASE IN CABBAGE AND BRUSSELS SPROUT EXTRACTS

Fraction	*Cabbage*	*Brussels sprout*
	$OD_{460} \cdot min^{-1} \cdot (ml\ extract)^{-1}$	
Soluble	19·9	18·7
Ionically bound	1·65	1·65
Covalently bound	0·01	0·0012

hearted cabbage extracts containing either soluble, ionically bound or covalently bound peroxidases. It can be seen that, in each case, the enzyme was inactivated to an extent dependent on both the temperature and the length of exposure to that temperature. The curves are hyperbolic, indicating that the inactivation process is at least biphasic. Comparison of Fig. 1 with Figs 2 and 3 suggests that the soluble peroxidase fraction contained a greater proportion of heat stable enzymes than the bound peroxidase fractions. Figures 4 and 5 show the results of similar heat

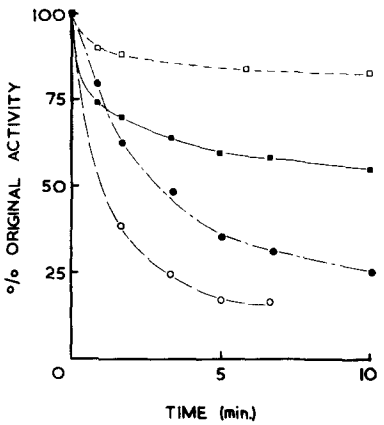


Fig. 1. Heat inactivation of soluble peroxidases from whole hearted cabbage. Scale: □------□ Heating at 60°C. ■——■ Heating at 65°C. ●– – –● Heating at 70°C. ○——○ Heating at 75°C.

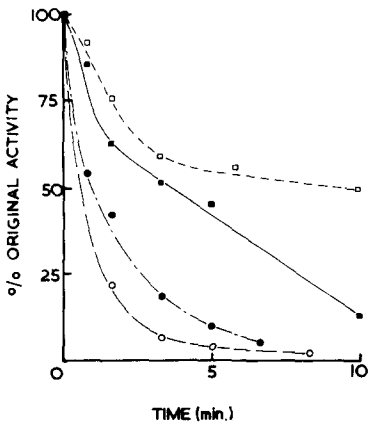


Fig. 2. Heat inactivation of ionically bound peroxidases from whole hearted cabbage. Scale: □------□ Heating at 60°C. ■——■ Heating at 65°C. ●– – –● Heating at 70°C. ○——○ Heating at 75°C.

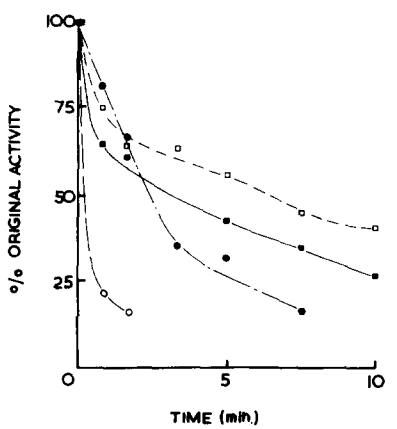


Fig. 3. Heat inactivation of covalently bound peroxidases from whole hearted cabbage. Scale: □------□ Heating at 60°C. ■——■ Heating at 65°C. ●– – –● Heating at 70°C. ○——○ Heating at 75°C.

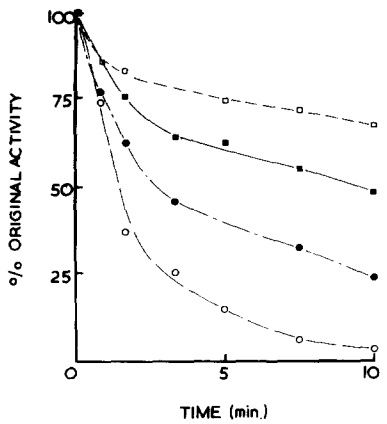


Fig. 4. Heat inactivation of soluble peroxidases from Spring cabbage. Scale: □------□ Heating at 60 °C. ■——■ Heating at 65 °C. ●– – –● Heating at 70 °C. ○——○ Heating at 75 °C.

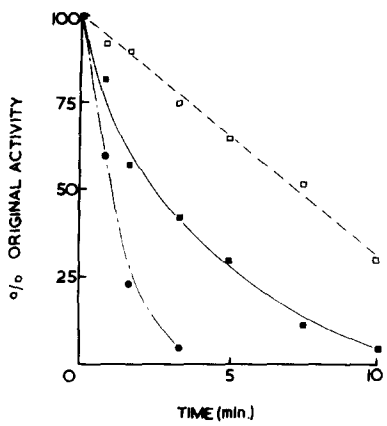


Fig. 5. Heat inactivation of ionically bound peroxidases from Spring cabbage. Scale: □------□ Heating at 60 °C. ■——■ Heating at 65 °C. ●– – –● Heating at 70 °C. ○——○ Heating at 75 °C.

treatments applied to the extracts from Spring cabbage. The shapes of the curves in Fig. 4 again indicate biphasic inactivation of soluble peroxidases and the stability to heat was comparable with that shown in Fig. 1 for whole hearted cabbage soluble peroxidases.

However, as shown in Fig. 5, the extracted ionically bound peroxidases from Spring cabbage were considerably less heat stable than any of the other peroxidases

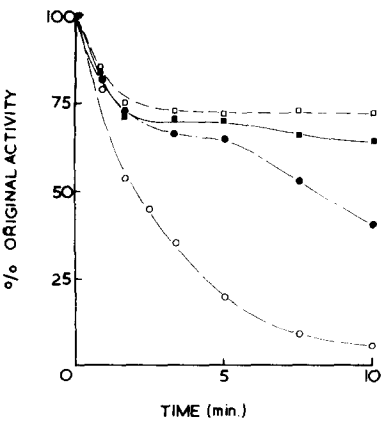


Fig. 6. Heat inactivation of soluble peroxidases from Brussels sprouts. Scale: □------□ Heating at 60 °C. ■——■ Heating at 65 °C. ●– – –● Heating at 70 °C. ○——○ Heating at 75 °C.

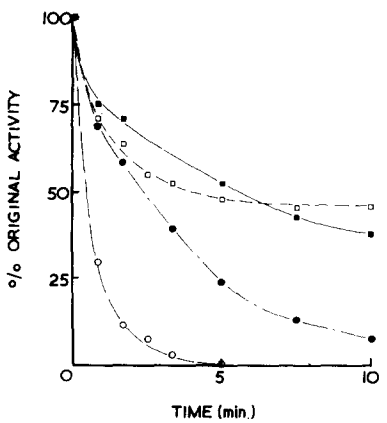


Fig. 7. Heat inactivation of ionically bound peroxidases from Brussels sprouts. Scale: □------□ Heating at 60 °C. ■——■ Heating at 65 °C. ●– – –● Heating at 70 °C. ○——○ Heating at 75 °C.

extracted from either whole hearted or Spring cabbage. It should also be noted that the shapes of the curves in Fig. 5 are more linear.

The heat inactivation curves for peroxidase enzymes present in extracts of soluble and ionically bound peroxidase fractions obtained from Brussels sprouts are shown in Figs. 6 and 7, respectively. Again, the degree of heat inactivation of peroxidase enzymes increased with the temperature and the length of exposure to that temperature. All plots for heat inactivation of Brussels sprout peroxidases indicate a biphasic process and the soluble peroxidase enzymes appeared to be more heat stable than the extracted ionically bound peroxidase enzymes.

The results shown in Figs 1 to 7 were used for calculating the heat inactivation energies (E_a) of peroxidase enzymes. For this purpose rate constants were calculated for the heat inactivation of peroxidases in samples treated at specified temperatures. The rate constants were obtained from the gradient of a plot of ln (% original activity) against the period of time of exposure to heat. These plots were found to be biphasic, but the second, longer phase corresponding to heat stable peroxidase, which formed a straight line when plotted logarithmically, was used for the calculation of each rate constant. Arrhenius plots of ln (rate constant) against $1/T$ were then prepared (Laidler, 1958) (Fig. 8) and the gradients of these straight line

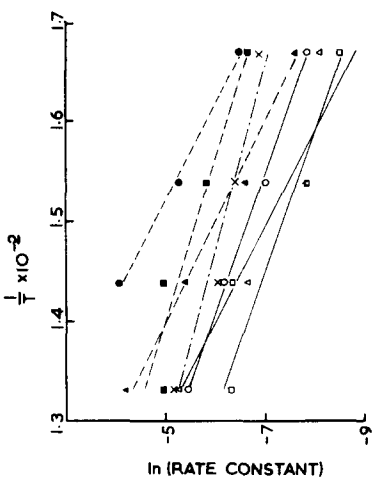


Fig. 8. Arrhenius plots for the inactivation of peroxidases. Scale: □——□ Cabbage, soluble. ■------■ Cabbage, ionically bound. ○——○ Spring cabbage, soluble. ●------● Spring cabbage, ionically bound. △——△ Brussels sprout, soluble. ▲------▲ Brussels sprout, ionically bound. ×——× Cabbage, covalently bound.

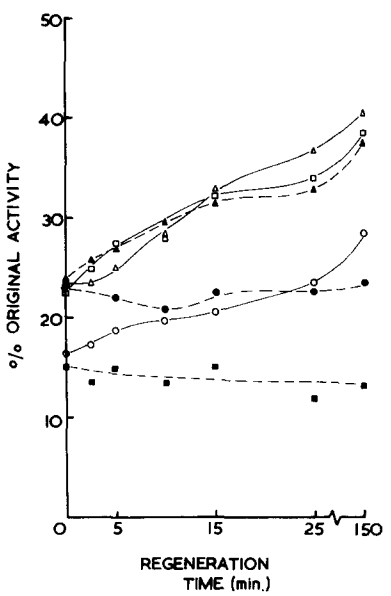


Fig. 9. Regeneration of peroxidases. Scale: □——□ Cabbage, soluble. ■------■ Cabbage, ionically bound. ○——○ Spring cabbage, soluble. ●------● Spring cabbage, ionically bound. △——△ Brussels sprout, soluble. ▲------▲ Brussels sprout, ionically bound.

TABLE 2
E_a FOR THE INACTIVATION OF PEROXIDASES
(calculated from Arrhenius plots)

Fraction	*Whole hearted cabbage*	*Spring cabbage* E_a ($kJ\,mole^{-1}$)	*Brussels sprout*
Soluble	6·51	5·92	7·77
Ionically bound	5·25	10·0	8·52
Covalently bound	3·99	—	—

plots used in the calculation of E_a values shown in Table 2. The calculated energy of heat inactivation (Table 2) of the soluble peroxidases extracted from whole hearted cabbage was greater than the E_a value calculated for bound peroxidases from the same source. The heat inactivation energies for soluble and ionically bound peroxidases present in the Brussels sprout extracts were similar (Table 2), and were somewhat greater than the E_a values for respective soluble and ionically bound peroxidases present in the extracts of whole hearted cabbage. Also, although the E_a values, 6·51 and 5·92 kJ mole^{-1}, for peroxidase enzymes present in the soluble peroxidase fraction from whole hearted cabbage and Spring cabbage, respectively, were similar, the E_a value for ionically bound peroxidases extracted from Spring cabbage was 10·0 kJ mole^{-1}, twice the value calculated for the corresponding whole hearted cabbage samples.

Shown in Fig. 9 is the regeneration of peroxidase activity at 30 °C for extracts of the soluble and ionically bound peroxidases. The soluble peroxidases from whole hearted cabbage, Spring cabbage and Brussels sprouts reacted similarly to the treatment—in each case approximately 12 % of the original activity of the samples was observed to regenerate over a 25-min period and a further 4 % over the following 2 h when assayed at room temperature. However, extracts of the ionically bound peroxidases obtained from the two vegetables reacted very differently when assayed at room temperature insofar as Brussels sprout ionically bound peroxidases regenerated 10 % of the original activity over the first 25 min and a further 7 % over the following 2 h, whereas extracts of whole hearted and Spring cabbage ionically bound peroxidases showed no significant regeneration over a period of 2·5 h. The total regeneration over the 2·5-h period considered was approximately 16–17 % of the original activity, for the soluble peroxidases and for Brussels sprout extracts of the ionically bound peroxidases.

DISCUSSION

The development of off-flavours during the storage of frozen vegetables is an important consideration in the food industry. While certain vegetables such as peas and green beans appear to store successfully, the storage of other vegetables is less

satisfactory. Frozen Brussels sprouts, for example, do not seem to store very successfully even following conventional blanching procedures. For this reason it is interesting to consider any peculiarities of Brussels sprout peroxidases which may add to the knowledge of this vegetable.

Several authors have found the heat inactivation of peroxidases to be a biphasic process (Yamamoto *et al.*, 1962; Adams, 1977, 1978; Lee & Hammes, 1979) and the results from the present investigation further substantiate these observations. Yamamoto *et al.* (1962) suggested two possible explanations; either there are two species of peroxidase with different heat sensitivities, or a protective mechanism is operating. Peroxidases are known to exist as isoenzymes which can be divided into two distinct groups according to both their physical and catalytic properties (Shannon *et al.*, 1966; Kay *et al.*, 1967). Adams (1977) therefore considered that isoenzymes with different heat sensitivities might offer an explanation for the biphasic inactivation kinetics. However, when Adams (1977) tested the response to heat treatment of his isolated isoenzymes he reported that the inactivation curves were still biphasic and thus concluded that the occurrence of isoenzymes could not easily explain the kinetics of inactivation.

The present study investigated the possibility that the different peroxidase fractions might also have different heat sensitivities. While the heat sensitivities of the fractions were indeed found to be significantly different in each of the vegetables considered, the various fractions still showed biphasic kinetics of inactivation when studied individually, with the exception of the Spring cabbage ionically bound peroxidase fraction (Fig. 5). The latter fraction also showed a relatively high heat inactivation energy (Table 2), while whole hearted cabbage and Spring cabbage soluble peroxidases showed very similar characteristics. The variation in the properties of the ionically bound fractions from these vegetables is clearly shown by the shape of the inactivation plots in Figs 2 and 5 and by the activation energies in Table 2. Therefore, it seems that the presence of different species of peroxidase with varying heat sensitivity, as suggested by Yamamoto *et al.* (1962) is not a simple distinction. Perhaps some complex picture, taking into account both the isoenzymes and the different physical states of peroxidase enzymes, might explain the occurrence of biphasic inactivation plots.

Returning to the possibility of some peculiarity of Brussels sprout peroxidase which might explain the problems of storage of this vegetable, it is interesting that Brussels sprouts do not appear to have any covalently bound peroxidase. Gkinis & Fennema (1978) extracted all three fractions from green beans. Cabbage was shown to have each of the three fractions in the present investigation. Whether or not this is significant requires further study.

A second property of Brussels sprout peroxidases which may be important regarding the quality loss in this vegetable during storage is the ability of the ionically bound fraction to regenerate following heat inactivation. This ability of Brussels Sprouts may well be significant in any quality deterioration which could be

attributed to peroxidases. Neither whole hearted cabbage nor Spring cabbage ionically bound peroxidases showed any significant regeneration of activity during 2·5 h at 30 °C. The soluble peroxidase fractions from each of the three vegetables showed comparable degrees of regeneration over the same time period.

It is also interesting to note that the activation energies for the heat inactivation of the two fractions of peroxidases found in Brussels sprouts (Table 2) are much higher than E_a's calculated for the corresponding fractions extracted from cabbage. This suggests that Brussels sprout peroxidases are more heat stable. It can also be seen that Brussels sprout soluble peroxidase and Brussels sprout ionically bound peroxidase have similar E_a values.

ACKNOWLEDGEMENT

The authors thank the Science Research Council for the award of a Postgraduate Research Studentship to Kathryn M. McLellan.

REFERENCES

ADAMS, J. B. (1977). The kinetics and mechanism of thermal inactivation of peroxidase in buffer and vegetable systems with reference to the high temperature–short time processing of foods. *Campden Food Preservation Research Association Tech. Bull. No. 34.*

ADAMS, J. B. (1978). The inactivation and regeneration of peroxidase in relation to the high temperature–short time processing of vegetables. *Inst. of Food Sci. and Tech. Proceedings*, **11**(2), 71–80.

BURNETTE, F. S. (1977). Peroxidase and its relationship to food flavour and quality: A review. *J. Food Sci.*, **42**, 1–6.

BURNETTE, F. S. & FLICK, G. J. (1977). Comparison of three assays for peroxidase in the Blue Crab. *J. Food Protection*, **40**, 854–6.

ESSELEN, W. B. & ANDERSON, E. E. (1956). Thermal destruction of peroxidase in vegetables at high temperatures. *Fd. Research*, **21**, 322–5.

GKINIS, A. M. & FENNEMA, O. R. (1978). Changes in soluble and bound peroxidases during low temperature storage of green beans. *J. Food Sci.*, **43**, 527–31.

KAY, E., SHANNON, L. M. & LEW, J. Y. (1967). Peroxidase isoenzymes from horseradish roots. II. Catalytic properties. *J. Biol. Chem.*, **242**, 2470–3.

LAIDLER, K. J. (1958). *The chemical kinetics of enzyme action.* Oxford University Press.

LEE, Y. C. & HAMMES, J. K. (1979). Heat inactivation of peroxidase in corn-on-the-cob. *J. Food Sci.*, **44**, 785–7.

SHANNON, L. M., KAY, E. & LEW, J. Y. (1966). Peroxidase isoenzymes from horseradish. I. Isolation and physical properties. *J. Biol. Chem.*, **241**, 2166–72.

TAMURA, Y. & MORITA, Y. (1975). Thermal denaturation and regeneration of Japanese-radish peroxidase. *J. Biochemistry*, **78**, 561–71.

YAMAMOTO, H. Y., STEINBERG, M. P. & NELSON, A. I. (1962). Kinetic studies on the heat inactivation of peroxidase in sweet corn. *J. Food Sci.*, **27**, 113–19.

Food Chemistry 7 (1981) 267–271

DIGESTIBILITY OF CARBOHYDRATES OF RAW AND COOKED BENGAL GRAM (*CICER ARIETINUM*), GREEN GRAM (*PHASEOLUS AUREUS*) AND HORSE GRAM (*DOLICHOS BIFLORUS*)

VIJAYA KHADER* & S. VENKAT RAO

Discipline of Biochemistry and Applied Nutrition,
Central Food Technological Research Institute, Mysore, India

(Received: 29 April, 1980)

ABSTRACT

In vitro digestion with pancreatin, preceded by pepsin, increased the rate of liberation of maltose from soaked Bengal gram, Green gram and Horse gram on blending first in water prior to cooking instead of cooking first before blending. However, the faecal bulk and caecal volume were not significantly different when the legumes were fed to rats after cooking as such, or cooking prior to, or after, blending. The results indicate that increasing the rate of digestion of carbohydrates may not be of value in reducing the flatulence caused by these legumes.

INTRODUCTION

Legumes are well known as inducers of flatulence in experimental animals and human subjects (Hellendoorn, 1969; Steggerda & Dimmick, 1966; Hedin, 1962; Steggerda, 1967). This property of legumes is a drawback to their wider use in infant and geriatric foods. The hypothesis advanced for the causation of flatulence is that the carbohydrates escaping digestion and absorption in the small intestine may be subjected to bacterial attack in the large intestine, resulting in the production of gases such as carbon dioxide, hydrogen and methane.

Kon *et al.* (1971) reported that cells of cooked legume products were very resistant to breakage and a high percentage survived common blending and digestive processes. They reported that blending raw beans before cooking resulted in a considerable increase in the rate of liberation of maltose as compared with beans

* Present address: Postgraduate Department of Food and Nutrition, Sardar Patel University, Vallabh Vidyanagar, Gujarat, India.

Food Chemistry 0308-8146/81/0007-0267/$02·75

cooked first and then ground. Subba Rao & Desikachar (1964) observed that the caecal volume and faecal bulk of rats fed legume diets were distinctly greater than those of rats fed a casein diet, due possibly to indigestible residues.

Since blending raw beans before cooking improved the digestibility of starch, it was of interest to study whether such a treatment would also affect the faecal bulk and caecal volume of rats fed three common legumes—Bengal gram, Green gram and Horse gram. The studies reported in this paper deal with *in vitro* and *in vivo* experiments on the affect of blending, before and after cooking the legumes, on carbohydrate digestibility, as determined by enzyme digestion, faecal bulk and caecal volume.

MATERIALS AND METHODS

Materials

Bengal gram dhal (split legume), Green gram dhal, whole Horse gram and casein used in the experiments were obtained from a local market.

Methods

Blended and cooked legumes: The samples were prepared as follows for *in vitro* digestibility. (1) By blending the legume soaked overnight. (2) Legumes were soaked overnight, cooked for periods ranging from 20 to 50 min and blended for 5 min in a Waring blender in the case of Green gram and Bengal gram and for 8 min in the case of Horse gram. (3) Bengal gram and Green gram were soaked overnight, blended for 5 min and cooked for 5 min while the soaked whole Horse gram was blended for 8 min and cooked for 20 min. For studies on faecal bulk and caecal volume, the samples were prepared as described above except that the samples obtained by means of method (1) were cooked for 20–50 min instead of being blended. The cooked samples were dried in a current of air at 60 °C overnight and ground to a fine powder before incorporation into the diets.

Analytical methods: In vitro digestibility with pancreatin, preceded by pepsin, was investigated. The *in vitro* digestibility was determined from the quantity of reducing sugar (expressed as maltose) liberated by pancreatin, preceded by pepsin (Kon *et al.*, 1971). In all cases, the final concentration of beans in the slurry was 2 % by weight, as this amount contained about 1 % starch. Two millilitres of legume slurry and 3 ml of pepsin (Difco) solution made up as 2 mg/ml of glycine–HCl buffer, pH 2, were incubated at 37 °C for 2 h. The pH of the samples was then adjusted to 7·0 with NaOH. Next, 5 ml of pancreatin (Merck), made up to contain 5 mg/ml of 0·05 M phosphate buffer, pH 7·0, were added and incubated at 37 °C. One millilitre samples were withdrawn for analysis at the end of 1, 30 and 60 min. The maltose released was estimated by the dinitrosalicylic acid method (Bernfeld, 1955). From the maltose

values obtained, the increase in the per cent of maltose liberated due to blending before or after cooking was calculated.

Faecal bulk and caecal volume: Ten groups of adult male rats (strain Wistar), weighing 230–250 g, eight per group, were allotted according to a completely randomised design and fed diets containing legumes subjected to various treatments, or a casein diet at a 15 % protein level. The diets were otherwise similar in composition to that used by Venkat Rao *et al.* (1971). Faeces were collected for a period of 10 days, after a preliminary period of 6 days (Subba Rao & Desikachar, 1964). The faecal samples were preserved in 2 % oxalic acid and dried at 100 °C for determination of faecal bulk. The rats were killed at the end of 16 days and caecal volumes were measured by displacement of water in a graduated cylinder (McCall *et al.*, 1962). The results were statistically analysed by the analysis of variance method appropriate for completely randomised design and differences were tested for significance by means of Duncan's new multiple range test at the 5 % level (Duncan, 1955).

RESULTS AND DISCUSSION

In vitro *digestibility of carbohydrates*

In the case of all three legumes blended after soaking overnight, the rate of maltose liberation was low on digestion with pancreatin, preceded by pepsin (Table 1). However, when the gram was cooked and blended, a marked increase in the rate of liberation of maltose by the enzymes was observed. A further marked increase in

TABLE 1

In Vitro DIGESTIBILITY OF CARBOHYDRATES OF BENGAL GRAM, GREEN GRAM AND HORSE GRAM BY PANCREATIN PRECEDED BY PEPSIN

Materials	*Milligrammes of maltose liberated from 2 g of material* Time (*min*)		*Increase in the percentage of maltose liberated due to blending and cooking* Time (*min*)	
	1	*60*	*1*	*60*
Bengal gram				
Soaked and blended	1109	1287	—	—
Soaked, cooked and blended	1214	1607	10	25
Soaked, blended and cooked	1604	2071	45	61
Green gram				
Soaked and blended	1036	1286	—	—
Soaked, cooked and blended	1143	1714	10	33
Soaked, blended and cooked	1393	2107	35	64
Horse gram				
Soaked and blended	1107	1357	—	—
Soaked, cooked and blended	1393	1679	27	24
Soaked, blended and cooked	1750	2179	58	61

the rate of liberation of maltose was observed from the sample blended first before cooking. This may be due to an increase in the breakage of the cell walls in the case of blended and cooked samples, with consequent release of more cell contents, resulting in the increase in the rate of liberation of maltose observed by Kon *et al.* (1971) in the case of California white beans.

Faecal bulk and caecal volume

Blending prior to, or after, cooking did not show any significant difference in the faecal bulk or caecal volume of rats fed on the three legumes (Table 2). The faecal

TABLE 2

THE EFFECT OF BLENDING PRIOR TO, OR AFTER, COOKING IN THE LEGUMES ON THE FAECAL BULK AND CAECAL VOLUME IN ADULT RATS

Diet	*Faecal bulk*† (*g/day*)	*Caecal volume*† (*ml*)
Bengal gram		
Soaked and cooked	$1{\cdot}66^{a}$	$5{\cdot}89^{ab}$
Soaked, cooked and blended	$1{\cdot}70^{a}$	$6{\cdot}22^{ab}$
Soaked, blended and cooked	$1{\cdot}71^{a}$	$6{\cdot}24^{ab}$
Green gram		
Soaked and cooked	$1{\cdot}51^{a}$	$5{\cdot}39^{b}$
Soaked, cooked and blended	$1{\cdot}27^{a}$	$5{\cdot}38^{b}$
Soaked, blended and cooked	$1{\cdot}51^{a}$	$6{\cdot}11^{ab}$
Horse gram		
Soaked and cooked	$3{\cdot}32^{b}$	$5{\cdot}98^{ab}$
Soaked, cooked and blended	$3{\cdot}12^{b}$	$6{\cdot}85^{a}$
Soaked, blended and cooked	$3{\cdot}32^{b} \pm 0{\cdot}14$	$6{\cdot}48^{ab} \pm 0{\cdot}41$
Casein*	$0{\cdot}56^{c} \pm 0{\cdot}15$ (69 df)	$3{\cdot}06^{c} \pm 0{\cdot}44$ (69 df)
Standard error of the mean (69 df)	$\pm 0{\cdot}04$	$\pm 0{\cdot}41$

† Values not sharing same superscript letters are significantly different.
* The average is based on seven values only.
df = Degrees of freedom.

bulk and caecal volume of rats fed on casein diets were considerably lower than those fed on the legumes. The results indicate that although there was a marked increase in the *in vitro* digestibility of carbohydrates of the three legumes as a result of blending and cooking, this treatment did not result in any improvement in the *in vivo* digestibility of carbohydrates as observed from data on faecal bulk and caecal volume.

Cooking Chick pea, Cow pea and Green gram was reported to considerably increase the *in vitro* digestibility by α-amylase (Ganesh Kumar & Venkataraman, 1976). The cooked diets also showed significantly lower flatus compared with the corresponding values of raw diets (Venkataraman & Jaya, 1975). However, Hellendoorn (1969) reported that cooking beans for longer or shorter periods of time

did not alter their flatulence activity. Shurpalekar *et al.* (1973) also reported, from experiments on *in vitro* and *in vivo* studies in rats, that the flatus-inducing capacity of legumes is correlated with the digestibility of the carbohydrates. Green gram, which had the most digestible carbohydrate, produced the least amount of flatus, whilst Bengal gram and Red gram, the least digestible, produced the maximum amount of flatus. However, the results obtained in the present experiment indicate that attempts to increase the rate of digestion of carbohydrates alone may not be of value in reducing the flatulence activity of legumes.

ACKNOWLEDGEMENT

The authors wish to thank Miss D. Rajalakshmi for help in the statistical analysis of the data.

REFERENCES

BERNFELD, P. (1955). Amylases α and β. In: *Methods in enzymology* (Colowick, S. P. & Kaplan, N. O. (eds)), Vol. I. Academic Press, New York, pp. 149–50.

DUNCAN, D. B. (1955). Multiple range and multiple *F* tests, *Biometrics*, **11**, 1–42.

GANESH KUMAR, K. & VENKATARAMAN, L. V. (1976). Studies on the *in vitro* digestibility of starch in some legumes before and after germination, *Nutr. Rep. Int.*, **13**, 115–24.

HEDIN, P. A. (1962). Gastrointestinal gas production in rats as influenced by some animal and vegetable diets, sulfiting and antibiotic supplementation, *J. Nutr.*, **77**, 471–6.

HELLENDOORN, E. W. (1969). Intestinal effects following ingestion of beans, *Food Technol.*, **23**, 795–800.

KON, S., WAGNER, J. R., BECKER, R., BOOTH, A. N. & ROBBINS, D. J. (1971). Optimizing nutrient availability of legume food products, *J. Food Sci.*, **36**, 635–9.

MCCALL, M. G., NEWMAN, G. E. & VALBERG, L. S. (1962). Enlargement of the caecum in the rat in iron deficiency, *Br. J. Nutr.*, **16**, 333–8.

SHURPALEKAR, K. S., SUNDARAVALLI, O. E. & DESAI, B. L. M. (1973). Effect of cooking and germination on the flatus inducing capacity of some legumes. Paper presented at the Meeting on Nutritional Aspects of Common Beans and other Legume Seeds as Animal and Human Foods, Ribeirao Preto, Brazil, 6–9 November, 1973.

STEGGERDA, F. R. (1967). Physiological effects of soybean fractions. In: *Proceedings of an International Conference on Soybean Protein Foods, Peoria, Ill., USA*, 94–9.

STEGGERDA, F. R. & DIMMICK, J. F. (1966). Effects of bean diets on concentration of carbon dioxide in flatus, *Am. J. Clin. Nutr.*, **19**, 120–4.

SUBBA RAO, P. V. & DESIKACHAR, H. S.R. (1964). Indigestible residue in pulse diets. *Ind. J. Expt. Biol.*, **2**, 243–4.

VENKATARAMAN, L. V. & JAYA, T. V. (1975). Gastrointestinal gas production in rats fed on diets containing germinated legumes, *Nutr. Rep. Int.*, **12**, 387–98.

VENKAT RAO, S., KURIEN, S., ARUNA, N. G., NARAYANASWAMY, D., RAJALAKSHMI, D. & SWAMINATHAN, M. (1971). Blood amino acid studies in the weanling rat on diets containing raw and autoclaved soybean, *Nutr. Rep. Int.*, **3**, 189–98.

Food Chemistry 7 (1981) 273–288

FUNCTIONAL PROPERTIES OF PROTEINS: POSSIBLE RELATIONSHIPS BETWEEN STRUCTURE AND FUNCTION IN FOAMS

JOHN E. KINSELLA

Institute of Food Science,
Cornell University, Ithaca, NY 14853, USA

(Received: 11 June, 1980)

ABSTRACT

Proteins via interactions with other components in food systems perform several desirable functions which are related to their structure and physico-chemical properties. In this paper the nature of food foams, the possible relationships between the molecular structure and the foaming capacity of different proteins, and factors affecting these are discussed.

INTRODUCTION

The increasing cost of production of animal and avian proteins, reflecting the spiralling cost of energy and feed, the limited area of available arable land and a growing world population, may compel the food industry to use greater amounts of plant, and perhaps yeast, proteins. Functional properties, cost and nutritive value are the principal criteria that will determine the adoption and acceptability of proteins from these sources.

While nutritional value is ultimately very important in considering proteins as food ingredients, the physico-chemical characteristics and interactions of proteins with other components in foods determine the usefulness and success of protein ingredients in food systems. These, collectively referred to as functional properties, are important in influencing the processing, preparation and quality attributes of foods (Kinsella, 1978). The intrinsic properties of a protein are governed by the content and disposition of amino acids, molecular size, shape, conformation, net charge and protein/protein interactions. However, even though the properties of a single component are significant, it is the manner in which they interact with other components—for example, water, proteins and lipids—in foods, that ultimately

Food Chemistry 0308-8146/81/0007-0273/$02·75

determines their functionality and application(s). Thus, in most food systems the overall behaviour or final quality attributes are due to the aggregate effects of several interactions between various components, e.g. protein, water and lipids in emulsions or protein, water and air in foams, as these are affected by pH, ions, etc. Proteins perform a variety of functions and each of these may depend on different molecular features or interactions (Kinsella, 1979). Information correlating the structure of proteins with specific functions in foods is limited, probably because of the diversity of composition, structure and conformation of food proteins. In this paper the molecular properties which affect surface properties and foaming capacity of some food proteins are discussed.

FOOD FOAMS

The behaviour of proteins at interfaces—i.e. air/water, oil/water—is of fundamental significance in food systems, such as foams and emulsions. Foams—for example, whipped toppings, whipped creams, souffles, mousses, bavoroises, meringues, angel-food cakes, leavened breads, ice creams, etc.—are formed by the entrapment of air which is enveloped by protein films. The air cells impart body and smoothness, ensure uniform rheological properties and facilitate dispersion of flavours. The lightness and mouth-feel of foams are universally enjoyed and foams, perhaps because of the large surface area and the volatilisation of flavours into the air vacuoles, seem to maximise the perception of flavours. Surface active proteins control aeration and texture by facilitating the uniform distribution of fine air cells throughout the food matrix.

Egg-white is the most widely used foaming/whipping agent. Gelatins, casein, milk and whey proteins, gluten, soy proteins and hydrolysed derivatives of these are also used. While foaming capacity is an important criterion, the stability of foams in the presence of other food ingredients (salts, surfactants, etc.) at different pH values and when subjected to heating is equally important. For most food foams, particularly where heating is involved, egg-white is the foaming agent of choice because it retains its foaming properties, i.e. capacity and stability when subjected to heat.

Foam formation and structure

Food foams, which are composed of gas (air), liquid (water) and surface active agent (protein), exhibit high viscosity, low density, high surface area and high surface energy. For optimum foam formation the surfactant should be soluble in the liquid phase and be capable of rapid migration and orientation to form an interfacial film around nascent gas bubbles. During formation the gas bubbles should have sufficient residence time within the liquid phase to allow the formation of an interfacial film and prevent coalescence of these transient bubbles. When air bubbles enter the liquid they tend to coalesce to minimise surface exposure, but the presence

of an appropriate surfactant minimises coalescence by forming an interfacial film barrier. In the absence of surfactant, bubbles tend to burst instantaneously because of the high surface tension of water. Appropriate surfactants stabilise the bubble by forming a cohesive deformable film which ideally resists excessive localised thinning. Furthermore, repulsion between the adjacent surfactant layers—i.e. disjoining pressure—prevents thinning of the aqueous film between contiguous bubbles. The surfactant layer should possess film elasticity such that applied stresses which tend toward localised thinning are rapidly counterbalanced by restoring forces generated partly by a local increased surface tension gradient (Kitchener & Cooper, 1959; Sebba, 1975).

In a sparging system the newly encapsulated bubble must have sufficient kinetic energy to penetrate the surface layer of the surfactant solution and burst through, despite the repulsion between the foam film and the surface film (Sebba, 1975). As they emerge from the liquid phase, foam bubbles move toward each other until they almost touch. They remain separated by a very thin aqueous film which is stabilised by the disjoining forces between the surfactant films. The pressure pulling the bubbles together (Laplace capillary pressure) reflects the tendency of the liquid surface of the capillary aqueous phase to expose minimum surface area between the bubbles. The Laplace forces, which increase with propinquity, tend to cause coalescence. However, these are counteracted by double-layer repulsive forces which increase as the films approach closely, thereby retarding further thinning of the capillary phase.

The latter forces may, in turn, be opposed by Van de Waals attractive forces between surfactant films which, at a critical distance, tend to thin the intervening aqueous layer. Thus, these opposing forces cause foams to be thermodynamically unstable. In time, the lamellar liquid tends to drain and when the space between films decrease to *ca.* 50 Å the molecular attractions cause film coalescence which results in gradual collapse of the foam.

Drainage occurs because of gravity and low surface tension. Gravity flow is very much dependent upon the viscosity of both the capillary fluid *per se* and the surface of the interfacial film material exposed to the aqueous phase. High viscosity is desirable to prevent gravity deformation of the film and to hold the liquid column within the lamella. Drainage is also directly influenced by the bulk density and viscosity of the lamellar fluid. Agents which increase viscosity—for example, sucrose—enhance foam stability. The spaces at the intersection of foam lamellae—i.e. plateau borders—have curved boundaries which create a negative pressure within the enclosed space. This tends to retard drainage from the lamellae even against gravity (Sebba, 1975).

Foams are metastable systems. The liquid between the lamellae tends to drain with time, gas diffusion occurs from the small to large bubbles and the film tends to thin and become fragile. This results in unstable packing of the bubbles within the foam matrix; rearrangement (repacking) occurs and the associated shocks cause

localised rupture. Thus a newly formed foam is usually composed of undistorted spherical bubbles. As initial drainage occurs, packing occurs and metastable foams with planar or slightly curved lamellae of reasonably uniform thickness are formed. In time, further thinning occurs under disjoining pressures and polyhedral bubbles with pentagonal dodecahedra shapes are formed (Davis & Rideal, 1963). The contiguous surfaces are planar and the liquid between the planar interfaces is held against gravity by capillary forces, by its increased viscosity due to structuring by the viscous film surface and by the negative pressure at the plateau borders.

Surface viscosity is a major factor contributing to the stability of foam lamellae. In addition to reducing drainage, the viscosity of the surface and bulk liquid minimises the destructive effects of shocks. High surface viscosity and high film yield values are correlated with strong foams because they reflect strong cohesion between the film forming molecules (Cumper, 1953). Films with high viscosity and possessing viscoelasticity form more stable foams than highly viscous but rigid films which tend to be brittle. Viscoelasticity allows the foam film to respond to stresses by expansion and compression of area where necessary (Brady & Ross, 1944). The capacity of the interfacial surfactant to adapt and reinforce a stressed area is also important. A stress causes depression in the foam bubble which expands the surface in that area with concomitant thinning and weakening of the surfactant film. In a viscous but fluid film, surfactant molecules flow into this thinned area of higher surface tension and reinforce it (Marangoni effect). If the surfactant is too viscous because of the slow rate of diffusion of the film material (Davis & Rideal, 1963), rupture and leakage may ensue when the film becomes too thin (< 50 Å).

Although high surface viscosity is desirable for foam stabilisation, it is not desirable during initial foam formation where surfactant solubility, mobility (i.e. migration to the interface) and molecular flexibility are required. Certain proteins, because they can change viscosity by unfolding and by molecular interaction, are excellent for the formation and stabilisation of foams.

PROTEINS AND FOAMING

Protein-based foams depend upon the intrinsic molecular properties of the protein being used. Thus, amino acid sequence and disposition; molecular size, shape, conformation and flexibility; surface polarity, charge, hydrophobicity, etc., all influence foaming behaviour in food systems. These, in turn, are affected by processing history and by the physical and chemical environment in which the protein is being used. Understanding the particular molecular properties accounting for optimum foaming characteristics should enable the food scientist to select appropriate proteins and also to predict the surfactant properties of food proteins as their molecular characteristics are described.

In a protein foaming system three sequential stages are involved. Initially the

soiuble globular proteins diffuse to the air–water interface, concentrate and reduce surface tension; then some unfolding of polypeptides occurs at the interface with concurrent reorientation (polar moieties toward the water) and, finally, interactions between the polypeptides occur to form a continuous film. This may be accompanied or followed by some denaturation and coagulation. The ability to rapidly diffuse to the interface, reorient and form a viscous film without excessive aggregation or coagulation is critical for the formation of protein based foams (Cumper, 1953) but to ensure foam stability the film must possess intermolecular cohesiveness (viscosity) and a certain degree of elasticity to permit localised contact deformation. Proteins vary immensely in their capacity for foam formation and foam stabilisation. This reflects the different physical properties of the proteins which determine their capacity to perform the functions required for foaming (Table 1).

TABLE 1
FACTORS AFFECTING FORMATION AND STABILITY OF PROTEIN FOAMS

Solubility
Rate of diffusion to interface and adsorption
(Molecular properties)
Rate of unfolding (hydrophobicity)
Reorientation of polypeptides
Association of polypeptides to facilitate formation of a viscous surface film with elasticity
Balance of intrapeptide attractions and repulsions
Tendency to aggregate without excessive surface coagulation
Surface charge and hydration

These reflect size, composition, structure, conformation, compactness, rigidity, charge intramolecular forces operating in the protein molecules as affected by protein concentration, ions, pH, temperature, agitation and other components (lipids, surfactants, sugars).

Protein films

Because formation and rheological properties of protein films are related to foaming properties, data obtained from studies of protein adsorption at surfaces is useful in evaluating foaming characteristics (Sebba, 1975; Davis & Rideal, 1963; Cumper, 1953; Brady & Ross, 1944; Benjamin *et al.*, 1975; Bull, 1938; Phillips *et al.*, 1975; Bull, 1972). In model systems proteins rapidly adsorb at an interface to form a surface film in less than 0·8 s (Bull, 1938). Bull (1972) estimated that a surface layer of ovalbumin (surface concentration 1·8 mg/m^2) adsorbed and denatured from a 0·03% protein solution in 0·36 s. Benjamin *et al.* (1975) estimated diffusion rates ranging from 3·3 to 0·7 $\times$ 10^{-10} m^2 s^{-1} for several proteins with β-casein being adsorbed more rapidly than κ-casein, serum albumin or lysozyme. At low bulk protein concentrations adsorption is apparently diffusion controlled. At high protein concentrations or in stirred systems adsorption is extremely rapid. In general, it is assumed that diffusion is rapid enough to ensure that a bubble passing

through a solution of surface active protein will become enveloped by a film of the surfactant (Cumper, 1953).

Greater surface activity of proteins is reflected in a more rapid rate of adsorption, as indicated by an increase in surface pressure (Π) with time and with protein concentration. For example, at similar concentrations, β-casein is more surface active than κ-casein, serum albumin and, as shown in Fig. 1, much more so than lysozyme (Benjamin *et al.*, 1975; Phillips *et al.*, 1975). This reflects not only the rate of diffusion of the native protein to the interface, but also its molecular 'flexibility'. There is an energy barrier to adsorption associated with the penetration of protein molecules into the interface and to conformational rearrangements at the interface as a film is formed. Unfolding and partial surface denaturation involve a co-operative transition in the tertiary structure of the protein when it reaches the interface, followed by a rearrangement of molecules in the film to attain the lowest free energy conformation. The latter is affected by the manner of surface packing of protein molecules in the film (Phillips *et al.*, 1975).

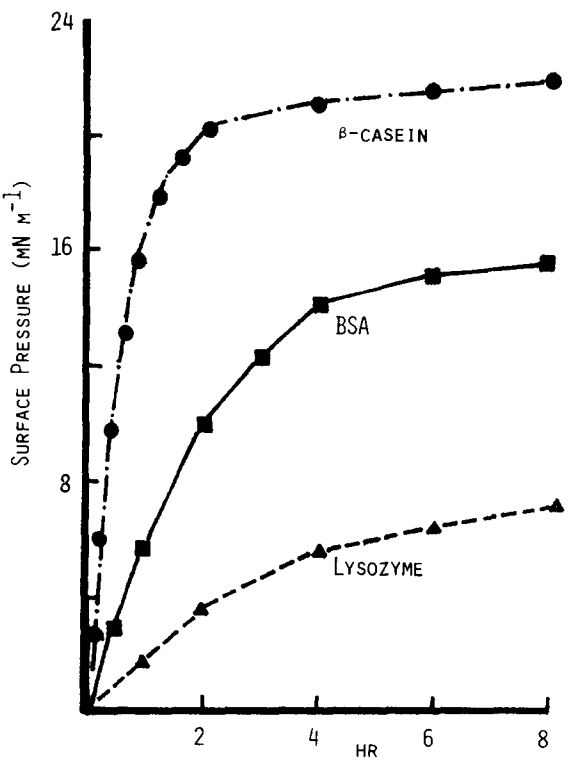


Fig. 1. Relative rates of adsorption as reflected in surface pressure of β-casein, bovine serum albumin (BSA) and lysozyme (from Graham & Phillips, 1975).

The surface concentrations (Γ) of proteins increase with concentration in the subphase. The surface pressure is markedly affected by surface concentration although the magnitude varies significantly with different proteins because of their different molecular composition and properties. Generally, surface pressure increases with surface concentration to a critical level above which little further change occurs, e.g. at the same surface concentration (1·5 mg/m^2) κ-casein and β-casein showed surface pressures of 5 and 15 mN m^{-1}, respectively (Benjamin *et al.*, 1975), i.e. β-casein was a more effective surfactant. Similar data have been obtained with bovine serum albumin and lysozyme (Phillips *et al.*, 1975) which reflect the structural properties of these molecules.

The β-casein adsorbs rapidly even at very low concentrations (0·01 mg/100 ml) and spreads rapidly to form a film. At low concentrations and low surface pressures there is ample area for each molecule (>38 Å/residue) to extend and spread in the plane of the interface with very few loops protruding into either phase. As surface concentration and pressure is increased (around 7 mN m^{-1}) folding and looping of the β-casein becomes pronounced. Monolayer coverage forming a film of 60 Å thickness of tightly packed folded molecules ($>7{\cdot}7$ Å^2/residue) is complete when the concentration is 0·1 mg/100 ml. Further adsorption does not occur because of charge repulsion and steric factors until substrate concentration is 1 mg/100 ml when multilayer formation occurs (Phillips *et al.*, 1975).

Lysozyme adsorbs at a slightly slower rate than β-casein and surface pressure is much less than β-casein at any given surface concentration, reflecting very limited unfolding of the lysozyme. At high surface concentrations, lysozyme can form a film although it retains extensive structural regions, i.e. it does not undergo surface denaturation (Phillips *et al.*, 1975). Above a certain bulk concentration ($>$ 1 mg/100 ml), multilayers of lysozyme accumulate. These multilayers may be regarded as gelled protein layers whose rheological properties depend upon the degree of crosslinking (disulphide linkages) and intermolecular interactions. Lysozyme multilayer films are more ordered, as reflected in greater viscosity, resistance to shear and lower compressibility than β-casein films (Phillips *et al.*, 1975).

In surface films of protein the mean interfacial area per residue ranges from 14 to 17 Å^2 for unfolded polypeptides and 4 to 15 Å^2 for closely packed conformations or compressed films in which the side chains are perpendicular to the surface (Birdi, 1973). As surface pressure increases, looping of the polypeptide chain occurs so that higher surface concentration is possible. In such films all the residues are not in the interface, but only segments of the loops enter the interface.

The surface active properties of proteins are very much influenced by the content and disposition of hydrophobic residues, molecular configuration, extent and nature of intermolecular bonds. Based on the content of polar and apolar amino acids, Hatch (1965) showed that the molar ratio of polar:apolar amino acids in proteins ranged from 1 to 3. Birdi (1973) found no correlation between surface

unfolding and molecular weight or helix content of proteins, but observed a fair relationship with the polar:apolar ratio. Proteins with low ratios ($<1{\cdot}3$) unfolded completely at an interface, with the more hydrocarbon-like residues oriented away from the water. Kato & Nakai (1979) showed a close correlation between 'hydrophobicity' and surfactant properties of several proteins, i.e. BSA, β-lactoglobulin, κ-casein and ovalbumin.

Horiuchi *et al.* (1973) found some relationships between content of hydrophobic amino acids and surface activity of proteins, but there were exceptions. However, they reported good correlation between surface hydrophobicity and surface activity. Thus protein molecules with hydrophobic regions on their surface adsorbed more readily (e.g. β-casein) at interfaces and were difficult to desorb. Heat denaturation ('partial') of certain proteins increased their hydrophobicity and enhanced their surface activity. The effect of heat treatment was more pronounced in the case of highly structured rigid proteins, e.g. lysozyme (Kato & Nakai, 1979).

When a protein spreads on an air/water surface the molecule apparently unfolds with sidechains becoming oriented according to their polarity. Unfolding is apparently facilitated by the presence of hydrophobic domains in the surface regions. However, certain proteins at interfaces can retain their α-helix structure, at least in part, and the amount of structure remaining depends upon surface pressure, i.e. available space for unfolding, β-lactoglobulin, for example (Malcolm, 1975).

Protein foams

Relationships between film forming behaviour and foaming properties of proteins have been demonstrated (Cumper, 1953; Horiuchi *et al.*, 1973; Graham & Phillips, 1975; Mita *et al.*, 1977, 1978). Graham & Phillips (1975) compared the interfacial adsorption behaviour and foaming properties of three pure proteins with different tertiary structures—i.e. β-casein, a largely non-structured flexible random coil, the highly ordered rigid, inflexible, globular lysozyme, and the less ordered, more flexible, globular bovine serum albumin. Under standard conditions and at several protein concentrations the β-casein rapidly adsorbed at the surface to attain stable film pressure (Π); albumin took much longer to reach constant Π, which was lower than with β-casein, and the lysozyme adsorbed very slowly (Fig. 1). The relative foaming abilities (time to reach half maximum volume) were 4, 12 and over 30 min for casein, albumin and lysozyme, respectively.

The surface adsorption behaviour revealed that proteins showing rapid adsorption, i.e. quick build-up of surface pressure, rapidly formed foams of large volumes, e.g. β-casein compared with lysozyme (Fig. 2). This is because the β-casein, being a loose, flexible molecule, can rapidly adsorb at the air/water interface of new bubbles, reduce the interfacial free energy and form a continuous film around the air bubble (Graham & Phillips, 1975). When the rate of formation and stabilisation of new air cells is greater than the rate of collapse, the volume of the foam increases.

The rate of migration of the proteins to the newly formed interface of nascent

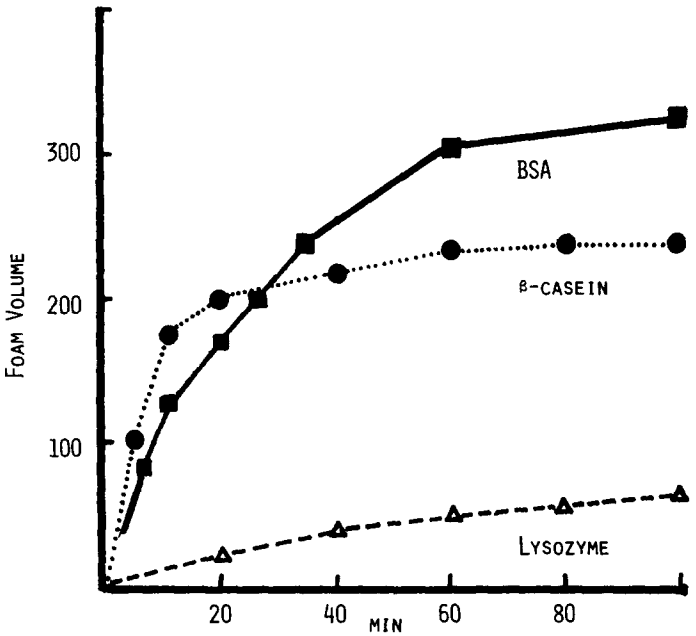


Fig. 2. Relative foaming capacities expressed as foam volume of β-casein, bovine serum albumin (BSA) and lysozyme (from Graham & Phillips, 1975).

bubbles is important, but inherent surface activity and the ability of the protein to unfold and reorient at the interface and engage in molecular adhesion is critical for foam formation. Thus, whilst β-casein and lysozyme migrate to the interface at roughly equal rates, β-casein, because of its molecular flexibility (random coil), unfolds, adsorbs and spreads rapidly at the interface, thus facilitating foam formation. The globular proteins arrive at the interface as rapidly, but because of their inflexible structure, polarity and compact shape (e.g. lysozyme), less of the molecule occupies the surface, i.e. Π does not build up as rapidly. Furthermore, because of their structural rigidity, unfolding and spreading apparently occur quite slowly so that foam formation takes longer. The foam bubbles are smaller, of finer texture and creamier, but more stable than the coarser, rapidly-formed foam as obtained with β-casein (Graham & Phillips, 1975) (Fig. 2).

Foam stability

Whilst the capacity to foam is important, the ability of a protein to stabilise a foam is also critical. Proteins differ in their capacity to stabilise foams and, ironically, some of the properties desired for facile foam formation, i.e. molecular flexibility, do not ensure stability while molecular characteristics which convey foam stability, i.e. intermolecular interactions and cohesiveness, are not compatible with

facile foam formation. The foam stabilities, i.e. half-life (irrespective of initial foam volume) of β-casein, albumin and lysozyme were 15, 32 and >200 min, respectively, i.e. the coarser, large bubbled β-casein foams collapsed rapidly whereas lysozyme formed very stable fine foams (Graham & Phillips, 1975). The physical requirements for stability in a protein foam are similar to those of any foam. The interfacial film should be structurally stable and relatively impermeable to the entrapped air; at a critical distance contiguous bubbles should be repelled to minimise coalescence; the outward projecting polar polypeptide loops or segments should retain the lamellar liquid against gravity and the protein in the film should possess both rigidity and flexibility to withstand local shocks while permitting sufficient mobility of component molecules to strengthen localised stress thinning.

The stability of foams reflects the rheological properties of interfacial films of proteins (Buckingham, 1972). Protein foams with films are generally least stable. Such protein films show low shear resistance, low viscosity, low dilational modulus and are compressible. They form relatively unstable foams because polypeptide interactions are weak, e.g. β-casein (Graham & Phillips, 1975). In contrast, films of albumin form thicker adhering cohesive layers of protein that resist compression and rupture. Lysozyme forms films that are resistant to mechanical or gravitational deformation because the molecules in the film pack closely and strongly associate to form viscous incompressible rigid films which retard drainage by viscous flow of the liquid from the lamella (Graham & Phillips, 1975).

Thus, for foam stabilisation, protein molecules should, upon reorientation at the interface as foam is formed, associate strongly to form continuous intermolecular polymers enveloping the air bubble. Hydrogen bonding, electrostatic and hydrophobic interactions are important for this intermolecular cohesion (Mita *et al.*, 1977, 1978; MacRitchie, 1970). Limited surface denaturation is required to impart viscosity and rigidity to the interfacial film for foam stabilisation, but excessive denaturation leading to coagulation and aggregation destabilises the foam. A balance of these effects is required. In addition, the ability to respond rapidly to localised changes in the interfacial film area is important to stability—i.e. the capacity of the film to spread to areas of thinning film is important.

The importance of shear viscosity in determining foam stability has been demonstrated for several proteins: gluten, β-casein, lysozyme, bovine serum albumin and cytoplasmic protein from clover (Graham & Phillips, 1975; Mita *et al.*, 1977, 1978; Buckingham, 1972; MacRitchie, 1970; Buckingham & Reid, 1974). Proteins which form viscous films with high shear viscosity tend to form more stable foams. Nevertheless, high surface viscosity is not the only determinant of film stability because excessive rigidity prevents the surface film from adapting to localised shocks (Cumper, 1953). The surface yield stress of the film is also a determinant and Buckingham (1972) showed that the strength of foams made from BSA was influenced more by surface yield stress than surface viscosity. Viscoelasticity enables the interfacial film to adapt in response to localised stress and

subsequently restore upon removal of the stress. Larger, folded, disulphide linked polypeptide loops—e.g. glutenin films—possess good viscoelasticity (Mita *et al.*, 1977, 1978).

Thus, the surfactant protein must concurrently perform at least two functions to permit film formation; that is, reduce surface (interfacial) tension of the liquid and form structural, continuous, cohesive films around the air vacuoles. The latter function is facilitated by some degree of surface denaturation, which accentuates protein–protein interaction and enhances cohesive forces between the proteins in the film capsule. However, complete denaturation is undesirable, because it results in membrane fragility and foam collapse. When foam is first formed, the liquid tends to drain away at a rate dependent upon viscosity and gravity. Eventually, as the bubbles approach the outer, more hydrophilic, surfaces of the film in the aqueous lamellar phase, they tend to repel each other via charge effects. These are counteracted by surface energy of the intervening water layer and miscellaneous weak attractive forces. In time, with agitation and further leakage, bonds (hydrogen and ionic) are formed between contiguous bubbles and aggregation of the protein film begins with progressive destabilisation of the foam. The bubbles decrease in number but increase in size until, eventually, the foam collapses.

Factors affecting foaming

Several factors, in addition to the protein *per se*, affect the foaming properties of proteins—protein concentration, pH, temperature, salt, sugars and lipids. The volume and stability of foams tend to increase with protein concentration and foams formed with higher concentrations of foaming protein are finer, denser and usually more stable because of the thicker interfacial films.

The pH of the dispersing medium markedly affects foaming, particularly foam stability, by its direct effects on net charge and conformation of the protein. Mita *et al.* (1977, 1978) showed that surface tensions of wheat proteins were lowest in the neighbourhood of their isoelectric points (pH 6·5–7·5). Furthermore, gluten monolayers showed that the highest viscosity and surface rigidity occurred around the isoelectric pH of 7·5. Both foam volume and foam stability (half-life) of foams stabilised by glutenin were maximum in the isoelectric region where the rate of bubble coalescence was minimum. At the isoelectric point, where electrostatic attractions are maximum, proteins assume a compact state and, provided coagulation does not occur, more protein adsorbs at the interface, resulting in maximal reduction of surface tension. At this pH the adsorbed films of glutenin are thickest and viscosity and elasticity tend to be highest because of extensive electrostatic bonding between molecules in the film layer (Mita *et al.*, 1977, 1978).

Films of clover cytoplasmic protein show sharp maxima for surface viscosity and yield stress at their isoelectric pH (5·5) which is close to the pH for maximum foam stability (Buckingham & Reid, 1974). The available data reflect the importance of electrostatic interactions on the rheological properties of interfacial films and show a

close relationship between these properties and foam stability. The limiting values for viscosity, rigidity and foaming capacity of proteins occur at, or near, the isoelectric point when interpeptide electrostatic interactions are near maximum (Cumper, 1953; Buckingham, 1972). Generally, protein films, provided the proteins do not coagulate, show maximum rheological properties at the isoelectric pH. The films are deformable but compact and tough because of maximum electrostatic adhesion. However, while maximum stability occurs in the isoelectric range of proteins, minimum solubility is usually observed in the same region and for foam formation reasonable solubility of the protein is desirable to enable surface adsorption to occur. Many proteins easily coagulate in the pI region. The presence of coagulated protein reduces film stability and foam life because they reduce film strength. Hence, although film strength is maximum at pI because of extensive interaction, concurrent coagulation may reduce foam stability. Most food foams are formed outside the isoelectric pH range of the proteins used.

Heat treatments and temperature affect foaming via their effects on protein structure and viscosity of the aqueous phase. Limited heating, which induces partial unfolding of globular proteins without causing thermal coagulation, facilitates foam formation, e.g. heated egg white forms better foams with greater numbers of smaller bubbles and increased rigidity than does the unheated native protein (Cumper, 1953). Heating BSA or lysozyme markedly increased the rate of their surface adsorption and stable surface pressures were increased by 20% and 250%, respectively, compared with the native proteins. The surface concentration was also increased and the foaming capacity of these heated proteins was enhanced (Phillips *et al.*, 1975; Graham & Phillips, 1975). Heating apparently facilitates film formation and foaming, but while foam volumes were enhanced, the stability of these foams (bovine serum albumin) was reduced compared with the native proteins (Graham & Phillips, 1975). Apparently the greater the rigidity of native globular proteins, the greater the improvement caused by limited heating. Increasing the temperature of gluten dispersions from 20 to 40 °C decreased the surface tension, i.e. from 46 to 38 (dyne/cm) but also significantly reduced foam volume (Mita *et al.*, 1977, 1978). In this case the higher temperature may have decreased foam stability by causing coagulation and by decreasing viscosity (MacRitchie, 1970; Boyd *et al.*, 1973).

Ions which affect solubility and conformation also influence the foaming properties of proteins. Salt decreased foaming of egg white by causing increased leakage and decreased stability (Cumper, 1935). Conceivably, the salt, via a salting, in effect reduced the surface denaturation necessary to impart the required rheological properties. Different ions, via their effects on the structure of water and protein conformation, may vary in their effects on foaming properties. Obviously ionic strength of the medium affects foaming properties because ions may reduce the coulombic forces between polypeptide chains and modify stability. Sodium chloride reduced surface viscosity and rigidity of protein films but increased spreading rate (Cumper, 1953), i.e. it weakened interpeptide attractions and for certain proteins

increased foam volume (larger bubbles). Although it may reduce film strength, and thereby impair stability, salt may lessen the amount of coagulation and reduce the rate of destabilisation so that the net effect is an overall increase in foam volume. For many proteins increasing ionic strength tends to result in better foams with finer bubbles, particularly for pH regions outside the maximum foaming range (Cumper, 1953).

Polyhydroxy compounds—for example, sucrose—tend to enhance foam stability, apparently by increasing the viscosity of lamellar water and thereby retarding drainage. The foaming properties of egg albumin, a glycoprotein, may in part be due to the capacity of the glyco-residues (which presumably are exposed to the lamellar water) to structure this water and retard drainage.

Surfactant amphiphilic lipids (monoglycerides, phospholipids) destabilise foams. Conceivably, these disrupt the interfacial film by causing the hydrophobic surface of the protein in the film to reorient and thereby weaken protein:protein interactions.

Ageing improves foam stability and this is related to the knowledge that surface viscosity and rigidity of protein films at an air/water interface increase with time (Cumper, 1953). Buckingham & Reid (1974) reported that both the viscosity and yield stress of cytoplasmic proteins increased with time. Significantly, the time taken to reach limiting values of viscosity and yield stress was markedly affected by pH being significantly lower in the isoelectric pH region where maximum values were obtained after approximately 20 min. The strength of foam formed from cytoplasmic protein also demonstrated its limiting value close to this pH which was attained after an ageing period of 15–20 min (Buckingham, 1972). Thus, the ageing phenomenon probably reflects the gradual increase in intermolecular associations in the interfacial film as the polypeptides find their least constrained configurations. These obviously involve electrostatic interactions which are most facilely formed in the isoelectric region. In addition, accretion of extra protein may result in thicker, stronger interfacial films which enhance stability.

Proteins

Proteins of different origins vary immensely in their foaming properties, reflecting their differences in composition, conformation, structural rigidity and their interactions with other compounds and the immediate environment.

Egg white is the pre-eminent foaming protein used in the food industry and has been successful because it possesses the capacity to form uniform foams of good volume whilst ensuring foam stability in the presence of other compounds, and also upon heating. This reflects the unique combination of proteins with different physical properties that occur in egg white. Ovalbumin, comprising over 60% of egg-white, is a glycoprotein (45,000 dalton) that is relatively easily denatured by heat and surface adsorption. It readily forms films, particularly after heat treatment. Ovalbumin readily forms foams, but alone requires longer whipping times than other egg white protein components (Palmer, 1972). Prior heat treatment enhances

the foaming properties of ovalbumin (Cumper, 1953). This presumably is due to thermally induced unfolding, i.e. 'loosening' of the globular molecule, which facilitates more rapid formation of a cohesive film around the air bubbles. Heating enhances foam formation although it may reduce somewhat the stability of ovalbumin foams.

The globulins are a heterogeneous group accounting for approximately 8% of egg-white proteins. These can foam rapidly to form large volume foams. They improve the foaming characteristics of ovalbumin and they stabilise foams following thermal coagulation—for example, in angel cakes (Palmer, 1972). The lysozyme component, unless heated, has inferior foaming properties but does form viscous interfacial films which impart superior foam stability to egg-white foams. Ovamucin, which accounts for around 2% of egg-white, stabilises foams formed during a short whipping time (Palmer, 1972). The ovomucoids, (glycoproteins) amounting to 12% of egg white, are extremely heat stable. These, because of resistance to thermal coagulation, may enhance the stability of egg-white foams by imparting flexibility and molecular mobility—i.e. viscoelasticity—to the films during heating. In addition, the 'glyco' moieties of these glycoproteins probably structure and stabilise the lamellar water, thereby retarding drainage and coalescence. In practice, relatively long whipping times (6–8 min) enhance the foaming properties of egg white (smaller bubbles, more dense foams, with greater stability and less drainage) by causing greater unravelling of ovalbumin molecules, forming thicker lamellae composed of the various proteins, and allowing the 'ageing' phenomenon, i.e. greater intermolecular polymerisation. Too long whipping times (>10 min) tend to cause excessive coagulation of ovalbumin which aggregates and destabilises the foam. The inclusion of sugar tends to retard coagulation and drainage from the formed foams. Acid or acid salts increase the stability of egg-white foams (Palmer, 1972), presumably by improving their rheological properties via enhanced electrostatic interactions.

Waniska & Kinsella (1979) reported that the foaming properties (capacity, strength and stability) of ovalbumin were related to protein concentration, reached limiting values around pH 4·0 (slightly below the pI) and were enhanced by salt (0·3M), particularly in the isoelectric pH region. Conceivably, the salt reduced aggregation in the isoelectric region of the ovalbumin where electrostatic interactions are maximum. This permitted the formation of a denser, more compact, interfacial film with superior viscosity and elasticity.

The foaming properties of other food proteins are not so well characterised. Cumper (1953) was one of the first researchers to report the basic properties of protein-based foams using model systems with egg albumin, insulin and trypsin. More recently, Mita *et al.* (1977, 1978) characterised and reviewed some of the foaming properties of gluten which are important in foods. Bread, during proofing and breadmaking, for example, has to entrap gas bubbles and expand as a foam-like structure. To facilitate gas entrapment, the gluten should have a low surface free

energy at its interface so that the evolving gas can expand. Gluten forms dense stable foams, the stability of which was maximum at pH 7·5, i.e. in the isoelectric range of gluten. Gluten monolayers possessed the highest viscosity and surface rigidity in this pH range. This may be attributed to the compact nature of the proteins and extensive electrostatic interactions which result in a closely packed surface layer or film. Gluten foams tend to be more stable than egg albumin (Mita *et al.*, 1977, 1978) and this in part is due to their strength, e.g. most protein films collapse at pressures around 20 whereas gluten films require 32 dyne cm^{-1} (Tschoegel & Alexander, 1960). This has been attributed to the much greater degree of hydrogen bonding in gluten foams between the large number of glutamine residues. These enhance the cohesiveness between the polypeptides in the protein film. Mita *et al.* (1977, 1978) showed that of the gluten proteins, glutenin had much superior foaming properties (volume, stability) compared with gliadin or gluten itself, e.g. glutenin formed bubbles that were 10- and 30-fold more stable than those from gluten or gliadin, respectively. This may be related to the large glutenin molecules, which are polymers composed of disulphide linked polypeptides exceeding one million daltons. Since surface viscosity is related to the degree of polymerisation of the molecules in the surface layer (Flory, 1940), glutenin forms a more viscous film than gliadin. The extensive intermolecular disulphide bonding in glutenin may also impart elasticity to the films. Reduction of the disulphide linkages significantly reduces the foaming and stability characteristics of glutenin foams (Mita *et al.*, 1977, 1978), presumably by reducing polymer size, viscosity and also the elasticity of the glutenin films.

Beer foams have been intensively studied because they impart desirable visual and 'mouthfeel' quality attributes to this beverage. Beer foam is essentially a proteinaceous foam containing proteins and glycoproteins, ranging in molecular weights from 10,000 to 150,000. No one has characterised these proteins. Foaming varies and is affected by other compounds (humulone, cations) and is impaired by lipid materials (Schultze *et al.*, 1976).

Proteins from soy, especially when fractionated, hydrolysed or modified, have reasonably good foaming properties (Kinsella, 1979). Many commercial preparations of foaming or whipping proteins are blends of proteins (egg white, caseins, gelatin, gluten) and/or partially hydrolysed proteins (gluten, gelatin, soy, milk proteins). Gunther (1979) has recently reviewed this topic.

Horiuchi *et al.* (1973) measured the hydrophobicity of gelatin, partially pepsinolysed ovalbumin, gluten and soy proteins. Foam stability did not show a relationship with the composite content of hydrophobic amino acids, e.g. partially hydrolysed soy, gluten and ovalbumin with 33, 35 and 38% hydrophobic amino acids showed relative foam stabilities of 17, 341 and 100, respectively. However, they reported a good correlation between surface hydrophobicity and foam stability and a linear relationship between foam stability and extent of surface adsorption which was directly related to surface hydrophobicity (Horiuchi *et al.*, 1973).

The phenomenon of foaming reflects various molecular properties of proteins and

provides an example of structure-functional relationships as they affect a quality attribute of foods. These reflect the physical and chemical interactions of the component molecules. While there is copious information available concerning the chemical and physical properties of major food components *per se*, knowledge of physical and chemical interactions between the various components is limited. Continued research to elucidate the physical and chemical basis of these interactions is necessary.

REFERENCES

BENJAMIN, J., FEIJTER DE J., EVANS, M. T., GRAHAM, D. E. & PHILLIPS, M. C. (1975). *Farad. Discussions of Chem. Soc.* **59**, 218.

BIRDI, K. S. (1973). *J. Coll. Interfac. Sci.* **43**, 545.

BOYD, J. J., MITCHELL, J., IRONS, L., MUSSELWHITE, P. & SHERMAN, P. (1973). *J. Coll. Interfac. Sci.* **45**, 478.

BRADY, A. & ROSS, S. (1944). *J. Am. Chem. Soc.* **66**, 1348.

BUCKINGHAM, J. H. (1972). Ph.D. Thesis, University of Wellington, N.Z.

BUCKINGHAM, J. H. & REID, C. S. (1974). *Rev. Rural Sci.* **1**, 65.

BULL, J. (1938). *J. Biol. Chem.* **123**, 17.

BULL, H. H. (1972). *J. Coll. Interfac. Sci.* **41**, 305.

CUMPER, C. W. (1953). *Trans Farad. Soc.* **49**, 1360.

DAVIS, J. T. & RIDEAL, E. K. (1963). *Interfacial phenomena*, Academic Press, New York.

FLORY, P. J. (1940). *J. Am. Chem. Soc.* **62**, 1057.

GRAHAM, D. & PHILLIPS, M. (1975). *Foams* (Ackers, R. J. (Ed)), Academic Press, London, 237.

GUNTHER, R. C. (1979). *J. Am. Oil Chem. Soc.* **56**, 345.

HATCH, F. T. (1965). *Nature*, **206**, 777.

HORIUCHI, T., FUKUSHIMA, D., SUGIMATO, M. & HATTORI, T. (1973). *Food Chem.* **3**, 35.

KATO, A. & NAKAI, S. (1979). *Con. Inst. Food Sci. Tech. J.*

KINSELLA, J. E. (1978). *Crit. Rev. Food Sci. and Nutr.*, **10**, 147.

KINSELLA, J. E. (1979). *J. Am. Oil Chem. Soc.* **56**, 242.

KITCHENER, J. A. & COOPER, C. F. (1959). *Quart. Reviews*, **13**, 71.

MACRITCHIE, F. (1970). *J. Macromol. Sci. Chem.* **A4**, 1169.

MALCOLM, B. R. (1975). *Applied chemistry at protein interfaces.* Advances in Chem. Series. (Gould, R. F. (Ed)), Am. Chem. Soc., Washington, DC, p. 338.

MITA, T., ISHIDO, F. & MATSUMOTO, H. (1977). *J. Coll. Interfac. Sci.* **59**, 172; *J. Coll. Interfac. Sci.* (1978) **64**, 143.

PALMER, H. H. (1972). *Food theory and applications* (Paul, P. & Palmer, H. (Eds)) John Wiley and Sons, New York, 538.

PHILLIPS, M. C., EVANS, M. T., GRAHAM, D. E. & OLDANI, D. (1975). *Colloid and Polym. Sci.* **253**, 424.

SCHULTZE, W., HERWIG, W., GLY, W. H. & CHICOYE, E. (1976). *Am. Soc. Brew. Chem. J.* **34**, 181.

SEBBA, F. (1975). *Colloidal dispersion and micellar behavior.* (Mittal, K. K. (Ed)), ACS Sym. Series No. 9, Washington, DC.

TSCHOEGEL, N. W. & ALEXANDER, A. (1960). *J. Coll. Interfac. Sci.* **15**, 155.

WANISKA, R. D. & KINSELLA, J. E. (1979). *J. Food Sci.* **44**, 1398.

Food Chemistry 7 (1981) 289–303

EFFECTS OF LENGTH OF FROZEN STORAGE, COOKING AND HOLDING TEMPERATURES UPON COMPONENT PHOSPHOLIPIDS AND THE FATTY ACID COMPOSITION OF MEAT TRIGLYCERIDES AND PHOSPHOLIPIDS

J. O. IGENE,* A. M. PEARSON & J. I. GRAY

Department of Food Science and Human Nutrition, Michigan State University, East Lansing, MI 48824, USA

(Received: 14 October, 1980)

ABSTRACT

Fatty acid profiles of triglycerides and phospholipids, and the levels of component phospholipids, were determined in beef and dark and light chicken meat at 0, 8 and 13 months of frozen storage at −18°C and again after cooking and holding at either 4°C or −18°C for 48 h. There was a significant decline in the amount of PE (phosphatidyl ethanolamine) and PC (phosphatidyl choline) during frozen storage, but the decline was much greater upon cooking. PE and PC were also more stable following cooking upon holding at −18°C than at 4°C. Only minor changes occurred in the fatty acid profiles of the triglycerides during either freezer storage of the raw meat or subsequent storage after cooking. The drippings collected upon cooking contained largely triglycerides whereas PE was essentially absent, indicating that it was bound to the membranes. The increased proportion of PE in cooked meat, coupled with its susceptibility to oxidation, indicated that it may play a key role in the autoxidation of cooked meat.

INTRODUCTION

The control of lipid oxidation in meat products has become increasingly important with the consumption of pre-cooked meat items for both institutional and home use. Igene & Pearson (1979) have presented evidence that phospholipids are the major contributors to the development of warmed-over flavour (WOF) in meat from different species of animals. In another study, Igene *et al.* (1979*b*) have shown that

* Present address: Department of Food Science and Technology, University of Maiduguri, PMB 1069, Maiduguri, Nigeria.

Food Chemistry 0308-8146/81/0007-0289/$02·75

meat held at 4 °C for 48 h following cooking is more susceptible to the development of off-flavour than similar samples held at −18 °C for 48 h. Younathan & Watts (1960) have reported that the development of off-flavours in cooked meat is generally due to the autoxidation of tissue lipids. The present investigation was undertaken to evaluate the relative changes in the fatty acid composition of the triglycerides and total phospholipids as influenced by frozen storage, cooking and subsequent holding of the cooked meat at either 4 °C or −18 °C for 48 h. It was also the objective of the study to determine the stability of the phospholipid components, especially phosphatidyl ethanolamine (PE) and phosphatidyl choline (PC), during freezer storage, cooking and subsequent holding at 4 °C or −18 °C.

MATERIALS AND METHODS

Meat and treatments

Beef *Longissimus dorsi* muscle and chicken light meat (breasts) and dark meat (thighs) were prepared, packaged and frozen as outlined previously by Igene *et al.* (1979*b*). After 0, 8 and 13 months of frozen storage at −18 °C, the meat was ground and prepared for analysis. Duplicate 100 g samples were removed and used for total fat extraction by the procedure of Folch *et al.* (1957). Thereafter, each duplicate sample was separated into the triglycerides and phospholipids using the method of Choudhury *et al.* (1960) as explained by Igene *et al.* (1979*b*). Details of the separation of the phospholipids, which was carried out by thin layer chromatography, and the quantitation of the phospholipid components using the phosphorus assay of Rouser *et al.* (1966) were published earlier by Igene *et al.* (1979*b*).

Cooking and analysis of drippings

The meat was cooked in sealed retortable pouches by placing in boiling water and heating to an internal temperature of 70 °C. In the studies on the composition of drippings, the pouches were opened and the drippings collected and measured. The lipids were extracted from both the drippings and the cooked meat and separated into triglycerides and phospholipids as previously described. Similar samples of the cooked meat were held in unsealed pouches at either 4 °C or −18 °C for an additional 48 h, after which they were analysed as before. The component phospholipids in the drippings were separated by thin layer chromatography and identified by comparing with a known standard mixture of phospholipids.

Gas–liquid chromatographic analysis of fatty acid methyl esters

The fatty acid composition of the triglycerides and phospholipids from beef and chicken dark and white meat was determined by converting a composite mixture of the phospholipids and triglycerides from the duplicate samples to methyl esters of their component fatty acids using the boron trifluoride–methanol procedure

described by Morrison & Smith (1964). All results reported here represent the means of duplicate determinations. Gas chromatographic analyses of the fatty acid methyl esters were performed using a Perkin-Elmer model 900 gas chromatograph equipped with a flame ionisation detector. The column, 2 m × 2 mm (inside diameter) stainless steel, was packed with 10% (w/w) diethylene glycol succinate (DEGS) on Supelcoport (Supelco, Inc., Bellafonte, PA, USA). The column temperature was set at 185 °C, the injection port at 220 °C and the detector at 250 °C. The carrier gas was helium and the flow rate was maintained at 35 ml/min, while hydrogen gas and air were adjusted to 30 and 285 ml/min, respectively.

Qualitative identification of the emerging peaks was carried out by comparing retention times with those of standard fatty acid methyl esters (Applied Science Laboratory Inc., State College, PA, USA). Peak areas were calculated quantitatively as the product of peak height and width at half height. Results were expressed as percentage of the total area.

Statistical analyses

Analysis of variance was carried out using a Control Data Corporation (CDC) 7500 computer. Significance of treatment means was determined using Tukey's test for multiple comparisons, as outlined by Steel & Torrie (1960).

RESULTS AND DISCUSSION

Fatty acid composition of triglycerides

Changes in the fatty acid composition of the beef triglycerides during frozen storage and cooking are shown in Table 1. Although total unsaturation decreased slightly during frozen storage for 8 and 13 months, the difference in unsaturation was not large and is probably unimportant. Similarly, small differences in unsaturation between the raw frozen and cooked meat were also recorded. These small changes in the total unsaturation reflected the stability of the saturated, mono- and dienoic fatty acids during frozen storage and cooking.

The fatty acid composition of the triglycerides extracted from chicken dark meat is presented in Table 2. Total unsaturation in the frozen raw meat gradually increased during frozen storage, the levels being 69·47, 71·81 and 73·29% at 0, 8 and 13 months, respectively. However, the percentages of total unsaturation in the cooked meat held either at 4 °C or −18 °C remained relatively constant, indicating that the fatty acids in the triglycerides of chicken dark meat were not greatly altered during cooking and subsequent storage.

While the percentage of total unsaturated fatty acids slightly increased during frozen storage of raw chicken dark meat, the reverse was the case during frozen storage of raw chicken white meat, with the values changing from 75·53 to 71·03% at 0 and 13 months, respectively (Table 3). Since the differences between the raw

TABLE 1

CHANGES IN THE FATTY ACID COMPOSITION OF BEEF TRIGLYCERIDES DURING FROZEN STORAGE AND COOKING[a]

Fatty acids	*0 Months*			*8 Months*			*13 Months*		
	Fresh meat	*Cooked and stored*		*Frozen meat*	*Cooked and stored*		*Frozen meat*	*Cooked and stored*	
		+4°C	*−18°C*		*+4°C*	*−18°C*		*+4°C*	*−18°C*
12:0	—	—	—	—	—	—	—	—	—
14:0	—	—	—	—	—	—	—	—	—
14:1	5·76	3·76	4·16	3·44	4·03	4·01	3·77	3·48	3·16
15:0	1·87	1·60	2·00	0·70	0·64	0·92	0·99	0·93	0·56
15:1	0·49	0·72	0·50	0·35	0·48	0·33	0·47	0·30	0·42
16:0	27·99	30·04	28·30	33·48	33·82	33·40	28·24	28·35	33·59
16:1	4·94	5·04	5·83	5·28	5·56	6·02	4·14	3·87	3·65
16:2	1·10	0·96	0·82	0·70	1·29	1·34	1·13	0·77	0·71
17:0	0·83	0·80	0·83	0·53	0·48	0·67	0·94	0·58	0·63
18:0	14·54	14·39	13·98	13·05	13·53	12·70	16·31	16·94	14·13
18:1	40·50	40·45	40·23	41·41	39·05	39·26	41·99	42·69	41·05
18:2	1·98	1·44	2·46	1·06	1·12	1·35	1·13	1·36	1·26
18:3ω6	—	—	—	—	—	—	0·14	—	—
18:3ω6	—	00·80	0·89	—	—	—	0·56	0·46	0·84
20:1	—	—	—	—	—	—	0·19	0·27	—
% Sat.	45·23	46·83	45·11	47·76	48·47	47·69	46·48	46·80	48·91
% Mono	51·69	49·97	50·72	50·48	49·12	49·62	50·56	50·61	48·28
% Di and Mono-	3·08	3·20	4·17	1·76	2·41	2·69	2·96	2·59	2·81
% Total unsat.	54·77	53·17	54·89	52·24	51·53	52·31	53·52	53·20	51·09

[a] Calculated as percentage of total fatty acids. Each value represents the mean of duplicate determinations.
Drip and drippings were collected and thoroughly mixed with the ground samples before removing duplicates for analysis. Thus, values shown represent total losses during freezing and/or cooking.

TABLE 2

CHANGES IN THE FATTY ACID COMPOSITION OF CHICKEN DARK MEAT TRIGLYCERIDES DURING FROZEN STORAGE AND COOKING[a]

Fatty acids	*0 Months*			*8 Months*			*13 Months*		
	Fresh meat	*Cooked and stored*		*Frozen meat*	*Cooked and stored*		*Frozen meat*	*Cooked and stored*	
		+4°C	*−18°C*		*+4°C*	*−18°C*		*+4°C*	*−18°C*
14:1	0·68	0·72	0·61	0·89	0·90	0·62	0·81	0·90	0·78
15:0	—	—	—	—	—	—	—	—	—
15:1	—	—	—	—	—	—	—	—	—
16:0	25·26	23·82	26·15	22·22	22·25	21·30	21·04	23·88	21·18
16:1	5·04	5·18	5·44	6·03	6·07	5·81	4·07	4·62	4·11
16:2	—	—	—	0·47	0·43	0·35	—	0·16	0·26
17:0	—	0·38	0·38	0·30	0·48	0·13	—	—	—
18:0	5·26	4·77	5·78	5·67	5·75	5·63	5·67	5·87	8·13
18:1	41·68	44·63	40·79	40·78	39·90	41·81	42·78	43·17	38·98
18:2	20·63	19·38	19·24	22·34	22·35	22·72	23·72	19·74	23·95
18:3ω6	0·26	0·08	0·18	—	0·27	0·18	0·29	0·20	0·29
18:3ω3	1·18	1·04	1·43	1·30	1·60	1·45	1·40	1·23	1·62
20:1	—	—	—	—	—	—	—	—	0·35
20:4	—	—	—	—	—	—	0·22	0·23	0·35
% Sat.	30·53	28·97	32·31	28·19	28·48	27·06	27·71	29·75	29·31
% Mono	47·40	50·53	46·84	47·70	46·87	48·24	47·66	48·69	44·22
% Di	20·63	19·38	19·24	22·81	22·78	23·07	23·72	19·90	24·21
% Poly	1·44	1·12	1·61	1·30	1·87	1·63	1·91	1·66	2·26
Total Unsat.	69·47	71·03	69·69	71·81	71·52	72·94	73·29	70·25	70·69

[a] Calculated as percentage of total fatty acids. Each value represents the mean of duplicate determinations.
Drip and drippings were collected and thoroughly mixed with the ground samples before removing duplicates for analysis. Thus, values shown represent total losses during freezing and/or cooking.

TABLE 3

CHANGES IN THE FATTY ACID COMPOSITION OF CHICKEN WHITE MEAT TRIGLYCERIDES DURING FROZEN STORAGE AND COOKING[a]

Fatty acids	*0 Months*			*8 Months*			*13 Months*		
	Fresh meat	*Cooked and stored*		*Frozen meat*	*Cooked and stored*		*Frozen meat*	*Cooked and stored*	
		+4°C	*−18°C*		*+4°C*	*−18°C*		*+4°C*	*−18°C*
14:0	—	—	—	—	—	—	—	—	—
14:1	0·98	0·66	0·68	0·41	0·54	0·45	0·85	0·87	0·82
15:0	—	—	—	—	—	—	—	—	—
15:1	0·52	1·05	0·43	—	0·32	—	0·37	0·39	0·33
16:0	18·84	18·40	17·85	22·04	18·25	19·73	22·21	22·00	19·78
16:1	4·31	4·15	4·21	5·23	4·45	4·21	4·27	5·24	4·09
16:2	—	—	—	0·42	0·36	0·41	0·37	0·28	0·22
17:0	—	—	—	0·34	0·36	0·36	0·27	0·37	0·16
18:0	7·63	6·50	7·12	6·42	6·93	6·88	6·49	6·88	6·95
18:1	43·17	43·35	42·15	41·84	44·37	43·89	37·32	39·12	38·39
18:2	22·59	21·67	23·67	22·18	22·29	22·53	24·68	21·83	25·88
18:3ω6	0·26	0·33	—	0·28	0·25	0·18	0·30	—	0·33
18:3ω3	1·70	1·70	1·08	0·84	1·88	1·36	1·71	1·75	1·96
20:1	—	1·39	2·16	—	—	—	0·09	0·17	—
20:2	—	0·80	0·65	—	—	—	—	—	—
20:3	—	—	—	—	—	—	0·21	0·30	—
20:4	—	—	—	—	—	—	0·86	0·80	1·09
% Sat.	26·47	24·90	24·97	28·80	25·54	26·97	28·97	29·25	26·89
% Mono	48·98	50·60	49·63	47·48	49·68	48·55	42·90	45·79	43·63
% Di	22·59	22·47	24·32	22·60	22·65	22·94	25·05	22·11	26·10
% Poly	1·96	2·03	1·08	1·12	2·13	1·54	3·08	2·85	3·38
Total unsat.	73·53	75·10	75·03	71·20	74·46	73·03	71·03	70·75	73·11

[a] Calculated as percentage of total fatty acids. Each value represents the mean of duplicate determinations.
Drip and drippings were collected and thoroughly mixed with the ground samples before removing duplicates for analysis. Thus, values shown represent total losses during freezing and/or cooking.

TABLE 4

CHANGES IN THE FATTY ACID COMPOSITION OF BEEF PHOSPHOLIPIDS DURING FROZEN STORAGE AND COOKING[a]

Fatty acids	*0 Months*			*8 Months*			*13 Months*		
	Fresh meat	*Cooked and stored*		*Frozen meat*	*Cooked and stored*		*Frozen meat*	*Cooked and stored*	
		+4°C	*−18°C*		*+4°C*	*−18°C*		*+4°C*	*−18°C*
12:0	—	—	—	—	—	—	—	—	—
14:0	—	—	—	—	—	—	—	—	—
14:1	1·46	0·72	1·85	2·52	1·27	2·42	1·84	2·03	2·10
15:0	0·52	—	0·82	0·50	0·67	0·55	0·53	0·34	0·71
15:1	1·16	1·86	2·06	0·95	0·53	0·62	0·62	0·30	0·98
16:0	18·49	18·73	20·03	27·73	22·65	25·27	26·87	23·93	23·13
16:1	3·61	3·67	4·33	5·29	4·00	5·59	3·32	3·38	3·58
16:2	0·74	0·25	0·41	0·76	0·60	0·62	0·71	0·85	0·46
17:0	0·92	0·84	0·62	0·76	0·80	0·91	0·70	0·81	0·75
18:0	12·95	12·83	13·19	12·60	14·51	13·26	13·50	16·56	13·76
18:1	33·44	34·81	35·66	35·92	36·98	38·07	37·41	36·80	35·57
18:2	10·52	12·66	10·10	5·29	7·19	5·39	6·81	6·39	8·95
18:3ω6	0·37	—	—	—	1·20	0·78	—	—	0·13
18:3ω3	1·29	0·68	0·91	0·57	—	—	0·70	1·35	0·59
20:1	—	—	—	0·44	0·67	0·28	—	0·20	0·16
20:2	0·69	0·21	0·25	0·44	0·47	0·17	0·30	0·25	0·39
20:3	2·77	1·92	1·73	0·76	1·60	1·14	1·07	1·08	1·46
20:4	8·51	9·32	6·59	3·02	6·33	3·87	4·56	4·83	6·25
20:5	0·76	—	—	—	0·53	—	—	—	0·18
22:2	—	—	—	—	—	—	—	—	—
22:3	—	—	—	—	—	—	—	—	0·13
22:4	0·88	0·42	0·63	—	—	0·38	0·59	0·24	0·39
22:5ω6	—	—	—	1·57	—	0·06	—	—	0·33
22:5ω3	0·92	1·08	0·82	0·88	—	0·62	0·47	0·66	—
22:6	—	—	—	—	—	—	—	—	—
% Sat.	32·88	32·40	34·66	41·59	38·63	39·99	41·60	41·64	38·35
% Mono	39·67	41·06	43·90	45·12	43·45	46·98	43·19	42·71	42·39
% Di	11·95	13·12	10·76	6·49	8·26	6·18	7·82	7·49	9·80
% Poly	15·50	13·42	10·68	6·80	9·66	6·85	7·39	8·16	9·46
Total unsat.	67·12	67·60	65·34	58·41	61·37	60·01	58·40	58·36	61·65

[a] Calculated as percentage of total fatty acids. Each value represents the mean of duplicate determinations.
Drip and drippings were collected and thoroughly mixed with the ground samples before removing duplicates for analysis. Thus, values shown represent total losses during freezing and/or cooking.

chicken white and dark meats were slight and not in the same direction, they were probably not significant. The levels of total unsaturation in frozen chicken white meat were slightly less than the values in cooked white meat, although the amount of unsaturated fatty acids in the latter also slightly declined during frozen storage. The results showed that only minor changes occurred in the fatty acid composition of the triglycerides during frozen storage and cooking of either beef, chicken dark or white meat. Thus, the results are in agreement with the work of Chang & Watts (1952), who have shown that only slight changes occurred in the fatty acid composition of triglycerides during cooking of meat.

Fatty acid composition of phospholipids

The fatty acid composition of beef phospholipids is presented in Table 4. Significant changes occurred in the unsaturated fatty acids of the phospholipids during frozen storage. Total unsaturation in the raw tissues declined during frozen storage, the levels being 67·12, 58·41 and 58·40% at 0, 8 and 13 months, respectively. This decrease in unsaturation may indicate the occurrence of lipid oxidation during frozen storage, especially up to 8 months. This hypothesis is supported by the results of a previous study by Igene *et al.* (1979*b*) who reported significant increases in TBA numbers in frozen raw beef after 8 and 13 months of storage.

The fatty acid composition for the chicken dark meat phospholipids is presented in Table 5. Most of the changes in unsaturation during frozen storage and cooking were due to alterations in the levels of $C_{18:1}$, $C_{18:2}$, $C_{20:4}$ and $C_{22:4}$ fatty acids. Whereas the relative proportion of monoenoic fatty acids increased significantly in the raw meat during frozen storage, the level of polyenoic fatty acids declined markedly over the same period. The dienoic fatty acids were relatively unchanged. Most of the changes in the fatty acid profile of chicken white meat phospholipids (Table 6) occurred in the polyenoic fatty acids. The PUFA's were less stable in cooked meat held at 4 °C for 48 h than in that cooked and held at −18 °C for 48 h. The instability of the PUFA's seems to verify their involvement in the development of rancidity during frozen storage or in the development of WOF in cooked meat, as suggested by others (Younathan & Watts, 1960; Watts, 1962; Love & Pearson, 1971; Igene *et al.*, 1979*b*). In addition, El-Gharbawi & Dugan (1965) reported that oxidation of dried beef tissue lipids seems to occur in two stages—the phospholipids are oxidised first and the neutral fat autoxidises later. They also reported that loss of the unsaturated fatty acids was more pronounced in the phospholipid fraction than in the neutral fat during storage.

Total phospholipids

As shown in Table 7, the concentration of total phospholipids consistently declined during the frozen storage of raw meat—a fact which substantiates the results of Davidkova & Khan (1967) and of Lee & Dawson (1976) upon frozen

TABLE 5

CHANGES IN THE FATTY ACID COMPOSITION OF CHICKEN DARK MEAT PHOSPHOLIPIDS DURING FROZEN STORAGE AND COOKING[a]

Fatty acids	*0 Months*			*8 Months*			*13 Months*		
	Fresh meat	*Cooked and stored*		*Frozen meat*	*Cooked and stored*		*Frozen meat*	*Cooked and stored*	
		+4°C	*−18°C*		*+4°C*	*−18°C*		*+4°C*	*−18°C*
12:0	—	—	—	—	—	—	—	—	—
14:0	—	—	—	—	—	—	—	—	—
14:1	0·14	0·17	0·16	—	—	0·22	—	—	0·28
15:0	—	—	—	0·54	0·86	—	—	—	—
15:1	0·37	0·57	0·31	—	—	0·88	—	—	0·23
16:0	15·60	16·52	16·44	12·63	18·44	15·65	17·99	23·27	19·85
16:1	1·05	1·03	0·79	1·61	2·20	2·88	2·57	2·00	2·15
16:2	—	0·23	0·17	0·21	0·39	0·19	0·34	0·28	0·23
17:0	—	—	—	0·27	0·43	0·51	—	0·38	—
18:0	18·16	21·03	20·83	18·19	18·44	15·51	12·47	17·27	15·31
18:1	20·43	19·74	19·34	23·94	26·07	28·53	31·75	26·27	29·48
18:2	21·51	19·22	20·11	21·83	20·95	23·26	23·63	18·00	18·82
18:3ω6	0·37	0·23	0·35	0·24	0·12	0·19	0·28	0·13	0·35
18:3ω3	0·41	0·43	0·53	0·88	0·45	0·64	1·21	0·88	0·68
20:1	—	—	0·26	0·13	1·08	—	0·19	—	0·23
20:2	—	—	—	0·96	0·97	1·42	—	—	—
20:3	0·53	0·52	0·57	0·44	—	0·22	0·34	0·75	0·60
20:4	17·41	18·88	15·28	13·86	8·44	8·97	7·90	8·76	10·43
20:5	—	—	—	—	—	—	—	—	—
22:3	—	—	—	0·29	—	—	—	—	—
22:4	1·23	0·57	1·84	1·12	0·60	0·42	0·42	0·88	0·74
22:5ω6	—	—	—	0·21	—	—	—	—	—
22:ω3	2·79	0·86	0·33	0·24	—	—	—	—	—
22:6	—	—	2·69	2·41	0·56	0·51	0·91	1·13	0·62
% Sat.	33·76	37·55	37·27	31·63	38·17	31·67	30·46	40·92	35·16
% Mono-	21·99	21·51	20·86	25·68	29·35	32·51	34·51	28·27	32·37
% Di-	21·51	19·45	20·28	23·00	22·31	24·87	23·97	18·28	19·05
% Poly	22·74	21·49	21·59	19·69	10·17	10·95	11·06	12·53	13·42
Total unsat.	66·24	62·45	62·73	68·91	61·83	68·33	69·54	59·08	64·84

[a] Calculated as percentage of total fatty acids. Each value represents the mean of duplicate determinations.
Drip and drippings were collected and thoroughly mixed with the ground samples before removing duplicates for analysis. Thus, values shown represent total losses during freezing and/or cooking.

TABLE 6

CHANGES IN THE FATTY ACID COMPOSITION OF CHICKEN WHITE MEAT PHOSPHOLIPIDS DURING FROZEN STORAGE AND COOKING[a]

Fatty acids	*0 Months*			*8 Months*			*13 Months*		
	Fresh meat	*Cooked and stored*		*Frozen meat*	*Cooked and stored*		*Frozen meat*	*Cooked and stored*	
		+4°C	*−18°C*		*+4°C*	*−18°C*		*+4°C*	*−18°C*
12:0	—	—	—	—	—	—	—	—	—
14:0	—	—	—	—	—	—	—	—	—
14:1	0·65	0·15	—	—	—	—	—	—	—
15:0	—	—	0·26	—	—	—	—	—	—
15:1	2·01	0·57	0·61	0·95	0·67	1·58	—	—	—
16:0	14·26	18·25	20·45	19·98	20·86	17·85	20·61	22·18	20·13
16:1	0·70	0·84	1·02	1·14	0·94	0·85	0·79	0·80	0·83
16:2	—	0·23	—	0·19	0·27	0·28	—	0·23	0·08
17:0	—	—	—	0·43	0·36	0·45	—	0·17	—
18:0	14·67	14·78	14·82	13·13	16·36	14·91	17·98	14·84	18·80
18:1	21·44	21·99	21·90	19·26	25·40	22·59	21·17	20·93	19·46
18:2	16·30	17·78	16·44	14·27	15·37	13·56	16·61	18·47	16·70
18:3ω6	2·85	0·23	—	—	—	—	—	—	—
18:3ω3	—	0·57	0·15	0·14	0·27	9·55	0·28	0·60	0·50
20:1	—	—	—	—	—	—	—	—	—
20:2	0·39	0·34	0·38	4·56	1·35	0·76	—	0·23	—
20:3	0·79	1·45	1·23	0·48	0·81	0·68	0·51	0·91	0·67
20:4	17·23	15·77	16·34	11·09	13·85	13·44	16·89	15·70	15·47
20:5	—	0·44	—	0·36	—	—	0·42	—	0·37
22:3	1·96	1·59	—	—	—	—	—	—	—
22:4	0·52	0·28	1·17	0·52	0·45	0·45	1·61	1·19	1·50
22:5ω6	—	—	—	—	—	1·02	0·79	—	0·42
22:5ω3	1·46	0·95	0·64	11·65	1·02	—	0·31	1·02	0·83
22:6	4·77	3·79	4·59	0·95	2·02	2·03	2·04	2·73	4·24
% Sat.	28·93	33·03	35·53	33·54	37·58	33·21	38·59	37·19	38·93
% Mono	24·80	23·55	23·53	21·35	27·01	25·02	21·96	21·73	20·29
% Di	16·69	18·35	16·82	19·02	16·99	14·60	16·61	18·93	16·78
% Poly	29·58	25·07	24·12	26·09	18·42	27·17	22·84	22·15	24·00
Total unsat.	71·07	66·93	64·47	66·46	62·42	66·79	61·41	62·81	61·07

[a] Calculated as percentage of total fatty acids. Each value represents the mean of duplicate determinations.
Drip and drippings were collected and thoroughly mixed with the ground samples before removing duplicates for analysis. Thus, values shown represent total losses during freezing and/or cooking.

TABLE 7

EFFECT OF LENGTH OF FROZEN STORAGE, COOKING AND HOLDING TEMPERATURE FOLLOWING COOKING UPON THE STABILITY OF INDIVIDUAL PHOSPHOLIPID COMPONENTS IN BEEF, CHICKEN DARK MEAT AND CHICKEN WHITE MEAT (COMBINED)[a]

Phospholipids[b]	*Storage temperature*	*Storage time (months)*[e]		
		0	*8*	*13*
LPC	Fresh/frozen	$1{\cdot}25 \pm 0{\cdot}69^{f}$	$1{\cdot}43 \pm 0{\cdot}52^{f}$	$1{\cdot}93 \pm 0{\cdot}66^{f}$
	Cooked/4 °C[c]	$1{\cdot}54 \pm 0{\cdot}67^{f}$	$1{\cdot}80 \pm 0{\cdot}39^{f}$	$1{\cdot}09 \pm 0{\cdot}56^{i}$
	Cooked/−18 °C[d]	$1{\cdot}91 \pm 0{\cdot}71^{f}$	$1{\cdot}37 \pm 0{\cdot}85^{f,i}$	$1{\cdot}14 \pm 0{\cdot}24^{i}$
SP	Fresh/frozen	$2{\cdot}34 \pm 0{\cdot}59^{f}$	$1{\cdot}88 \pm 0{\cdot}88^{f,g}$	$1{\cdot}45 \pm 0{\cdot}60^{g}$
	Cooked/4 °C[c]	$1{\cdot}69 \pm 0{\cdot}54^{f}$	$1{\cdot}74 \pm 0{\cdot}57^{f}$	$1{\cdot}87 \pm 0{\cdot}78^{f}$
	Cooked/−18 °C[d]	$1{\cdot}95 \pm 0{\cdot}66^{f}$	$1{\cdot}67 \pm 0{\cdot}54^{f}$	$0{\cdot}92 \pm 0{\cdot}59^{g}$
PE	Fresh/frozen	$6{\cdot}68 \pm 1{\cdot}08^{f}$	$4{\cdot}89 \pm 0{\cdot}80^{h}$	$3{\cdot}77 \pm 0{\cdot}64^{i}$
	Cooked/4 °C[c]	$2{\cdot}55 \pm 1{\cdot}06^{g}$	$2{\cdot}64 \pm 1{\cdot}03^{g,j}$	$2{\cdot}58 \pm 0{\cdot}63^{g}$
	Cooked/−18 °C[d]	$4{\cdot}09 \pm 1{\cdot}85^{g}$	$3{\cdot}69 \pm 0{\cdot}99^{g,i}$	$3{\cdot}17 \pm 0{\cdot}60^{g}$
PC	Fresh/frozen	$16{\cdot}70 \pm 0{\cdot}99^{f}$	$14{\cdot}39 \pm 1{\cdot}94^{g}$	$12{\cdot}21 \pm 1{\cdot}82^{h}$
	Cooked/4 °C[c]	$12{\cdot}89 \pm 0{\cdot}73^{h}$	$11{\cdot}46 \pm 1{\cdot}61^{i}$	$10{\cdot}80 \pm 2{\cdot}09^{i}$
	Cooked/−18 °C[d]	$15{\cdot}48 \pm 0{\cdot}99^{g}$	$11{\cdot}60 \pm 2{\cdot}84^{i}$	$12{\cdot}23 \pm 2{\cdot}40^{h}$
TP	Fresh/frozen	$29{\cdot}26 \pm 1{\cdot}92^{f}$	$24{\cdot}17 \pm 2{\cdot}82^{g}$	$22{\cdot}39 \pm 1{\cdot}45^{g}$
	Cooked/4 °C[c]	$24{\cdot}85 \pm 0{\cdot}98^{g}$	$20{\cdot}52 \pm 2{\cdot}00^{h}$	$20{\cdot}62 \pm 2{\cdot}01^{h}$
	Cooked/−18 °C[d]	$26{\cdot}76 \pm 1{\cdot}84^{i}$	$21{\cdot}25 \pm 3{\cdot}38^{h}$	$22{\cdot}28 \pm 2{\cdot}29^{h}$

[a] Each mean value represents twelve determinations, four each for beef, chicken dark meat and white meat. Values are expressed as milligrammes of phosphorus per gramme of phospholipid.
[b] Abbreviations for phospholipids are: LPC = lysophosphatidyl choline; SP = sphingomyelin; PE = phosphatidyl ethanolamine; PC = phosphatidyl choline; TP = total phospholipid phosphorus.
[c] Meat was cooked and held at 4 °C for 48 h.
[d] Meat was cooked and held at −18 °C for 48 h.
[e] Means with the same superscripts in the same column and line for a single phospholipid component are not significantly different at $P < 0{\cdot}05$.

storage of raw chicken. This is also in agreement with an earlier report of Zipser *et al.* (1962) who showed that a progressive loss in total phospholipids occurred during refrigerated storage of mullet tissues. Analysis of variance showed that total phospholipids were significantly ($P < 0{\cdot}05$) higher in raw than in cooked meat. Total phospholipids also tended to be higher in the cooked meat held at −18 °C for 48 h following cooking, in comparison with that held at 4 °C.

Phospholipid components in the drippings

Analysis of the phospholipids recovered from the drippings of cooked beef is shown in Fig. 1. Samples A and C represent drippings from beef, while B is from a standard mixture of known phospholipid components. Phosphatidyl ethanolamine (PE) was completely absent in the cooking drippings, which suggests that it is more tightly bound to the membrane than phosphatidyl choline (PC) or other phospholipid components. Thus, the relative proportion of PE in the meat would increase during cooking and would be expected to play an important role in the oxidation of cooked meat because of its reactivity to atmospheric oxygen (Corliss & Dugan, 1970: Tsai & Smith, 1971).

The fatty acid composition of the triglycerides and phospholipids in the drippings

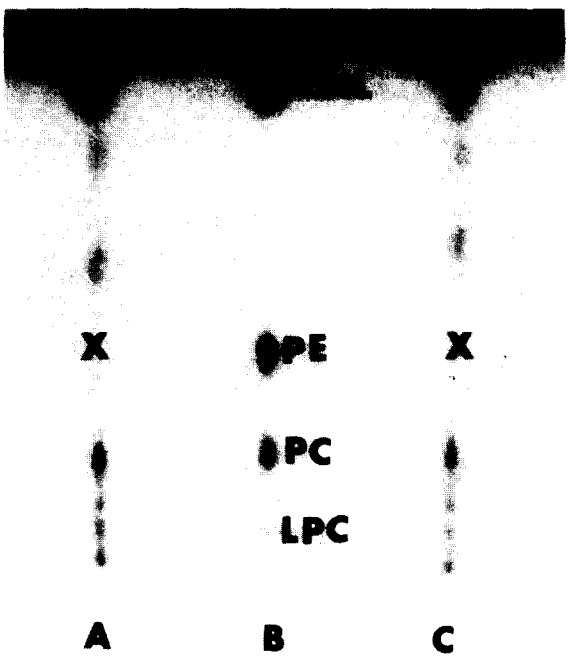


Fig. 1. Thin layer plate showing composition of phospholipid components in beef drippings. A and C show total phospholipids from beef drippings. B shows standard mixture of phospholipids containing LPC (lysophosphatidyl choline), PC (phosphatidyl choline), PE (phosphatidyl ethanolamine) and CHL (cholesterol). × indicates the positions where PE is missing from the drippings.

from beef and from chicken dark meat are presented in Table 8. The levels of monoenoic fatty acids in the chicken dark meat drippings were similar to those found in beef, both in the triglyceride and phospholipid fractions. However, much higher levels of dienoic fatty acids were recovered in the drippings from chicken dark meat than from beef. It is noteworthy that extremely low levels of polyenoic fatty acids (0·36 and 2·15%) were found in both the triglycerides and phospholipids from chicken dark meat drippings.

The low levels of PUFA's in the phospholipids of the drippings verify the fact that the more highly unsaturated phospholipid components are firmly held by the membranes. Similar results were reported earlier by Giam & Dugan (1965) who also observed that the cooking of meat did not liberate any appreciable quantity of the more 'tightly bound lipids'—that is, those which were not extracted with petroleum ether in a Soxhlet apparatus. These results lend support to the postulation of Janicki & Appledorf (1974) that cooking of meat results in greater losses of saturated fat because C 18:1 and C 18:2 fatty acids are more likely to be involved as structural components and as components of phospholipids. Consequently, it can be concluded that cooking results in a higher proportion of PUFA's in cooked meat.

TABLE 8
FATTY ACID COMPOSITION OF THE TRIGLYCERIDES AND PHOSPHOLIPIDS OBTAINED IN THE COOKED DRIPPINGS FROM BEEF AND CHICKEN DARK MEAT[a]

Fatty acid	*Beef drippings*		*Chicken dark meat drippings*	
	Triglycerides	*Phospholipids*	*Triglycerides*	*Phospholipids*
12:0	—	—	1·16	—
14:0	—	—	1·52	0·69
14:1	6·03	2·07	—	—
15:0	1·79	0·95	—	—
15:1	0·45	0·30	—	—
16:0	34·21	27·43	26·92	21·93
16:1	5·86	4·96	6·51	6·85
16:2	1·34	0·71	—	—
17:0	0·89	0·59	—	1·60
18:0	12·96	16·37	6·37	11·05
18:1	34·91	41·86	40·52	43·40
18:2	1·34	3·27	15·63	10·65
18:3	—	—	0·22	0·23
18:3	0·22	0·59	0·14	1·28
20:1	—	0·47	1·01	1·71
20:2	—	—	—	—
20:3	—	—	—	0·64
20:4	—	0·43	—	—
% Saturated	49·85	45·34	35·97	35·27
% Monoenoic	47·25	49·66	48·04	51·96
% Dienoic	2·68	3·98	15·63	10·62
% Polyenoic	0·22	1·02	0·36	2·15
Total unsaturation	50·15	54·66	64·03	64·73

[a] Calculated as percentage of total fatty acids. Each value represents the mean of duplicate determinations.

This, plus the fact that cooking releases a significant amount of non-haem iron from bound haem pigments (Igene *et al.*, 1979*a*) makes cooked meat extremely susceptible to lipid oxidation.

Composition and stability of component phospholipids

The concentrations of lysophosphatidyl choline (LPC), sphingomyelin (SP), PE, PC and total phospholipids were measured in both the raw and cooked meat during frozen storage. The data representing combined mean values from beef, chicken dark meat and white meat are presented in Table 7.

The concentration of LPC in raw meat comprised 4·27, 4·90 and 6·60 % of initial total phospholipids at 0, 8 and 13 months, respectively. Although the results were not statistically significant ($P < 0·05$), there appeared to be proportionately more LPC at the end of 13 months freezer storage than in the original raw meat. However, the level of LPC in fresh tissue seems to be quite variable. For instance, Davidkova & Khan (1967) reported a value of 1·5 % in fresh chicken tissues, while Keller & Kinsella (1973) and Lee & Dawson (1976) reported values of 0·6 and 6·8 % for LPC,

respectively. The increasing concentration of LPC (Table 7) during frozen storage may be indicative of some phospholipase activity. Several researchers (Awad *et al.*, 1968; Braddock & Dugan, 1972) have also reported increasing levels of LPC during frozen storage of meat.

The initial level of PC in fresh tissue at zero time comprised 57·07 % of the total phospholipids. This value is in good agreement with those reported for PC by Peng & Dugan (1965) and Keller & Kinsella (1973). The concentration of PC in raw meat was found to decline during frozen storage, with the major change occurring between 0 and 8 months of frozen storage. This significant decrease in PC can explain the apparent increase in LPC during frozen storage of the raw meat. Levels of PC consistently decreased in the cooked meat held at 4 °C, while there was not a consistent decline in the cooked meat held at − 18 °C. Results showed that the lowest concentrations of PC were found in the cooked meat held at 4 °C for 48 h, regardless of the length of time in frozen storage.

PE also decreased significantly ($P < 0·05$) in the raw meat at both 8 and 13 months of frozen storage (Table 7). On average, PE also decreased in the cooked meat held at − 18 °C for 48 h following cooking, although the decline was not statistically significant. The initial concentration of PE in the fresh tissue (6·68 mg of phosphorus per gramme of phospholipid) comprised 22·83 % of the total phospholipids. This was in good agreement with the values reported by Davidkova & Khan (1967) and Keller & Kinsella (1973).

The level of PE was significantly ($P < 0·01$) higher in the fresh/raw frozen than in the cooked meat following subsequent storage, regardless of the length of time in freezer storage. However, PE was relatively higher in the cooked meat held at − 18 °C for 48 h than in similar cooked meat held at 4 °C for 48 h. The lower level of PE in meat held at 4 °C may be due to a higher rate of oxidation of the polyunsaturated fatty acids of PE (Tables 4 and 5).

ACKNOWLEDGEMENTS

Michigan Agricultural Experiment Station Journal Article No. 9054. This investigation was supported in part by the National Science Foundation under Research Grant ENG 76-04591. The authors acknowledge the assistance of T. H. Coleman and R. A. Merkel in furnishing the poultry meat and beef samples, respectively.

REFERENCES

AWAD, A., POWRIE, W. D. & FENNEMA, O. (1968). Chemical deterioration of frozen bovine muscle at −4 °C. *J. Food Sci.*, **33**, 227–35.

BRADDOCK, R. J. & DUGAN, JR., L. R. (1972). Phospholipid changes in muscle from frozen stored Lake Michigan Coho salmon. *J. Food Sci.*, **37**, 426–9.

CAMPBELL, A. D. & TURKKI, P. R. (1967). Lipids of raw and cooked ground beef and pork. *J. Food Sci.*, **32**, 143–7.

CHANG, I. C. L. & WATTS, B. M. (1952). The fatty acid content of meat and poultry before and after cooking. *J. Am. Oil Chem. Soc.*, **29**, 334–8.

CHOUDHURY, R. B. R. & ARNOLD, L. K. (1960). The determination of the neutral oil content of crude vegetable oil. *J. Am. Oil Chem. Soc.*, **37**, 87–8.

CORLISS, G. A. & DUGAN, JR., L. R. (1970). Phospholipid oxidation in emulsions. *Lipids*, **5**, 846–53.

DAVIDKOVA, E. & KHAN, A. W. (1967). Changes in lipid composition of chicken muscle during frozen storage. *J. Food Sci.*, **32**, 35–7.

EL-GHARBAWI, M. I. & DUGAN, JR., L. R. (1965). Stability of nitrogenous compounds and lipids during storage of freeze-dried raw beef. *J. Food Sci.*, **30**, 817–22.

FOLCH, J., LEES, M. & STANLEY, G. H. S. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.*, **226**, 497–509.

GIAM, I. & DUGAN, JR., L. R. (1965). The fatty acid composition of free and bound lipids in freeze-dried meats. *J. Food Sci.*, **30**, 262–5.

IGENE, J. O., KING, J., PEARSON, A. M. & GRAY, J. I. (1979*a*). Influence of heme pigments, nitrite and non-heme iron on development of warmed-over flavor (WOF) in cooked meat. *J. Agr. Food Chem.*, **27**, 838–42.

IGENE, J. O., PEARSON, A. M., MERKEL, R. A. & COLEMAN, T. H. (1979*b*). Effect of frozen storage time, cooking and holding temperature upon extractable lipids and TBA values of beef and chicken. *J. Anim. Sci.*, **49**, 701–7.

IGENE, J. O. & PEARSON, A. M. (1979). Role of phospholipids and triglycerides in warmed-over flavor development in meat model systems. *J. Food Sci.*, **44**, 1285–90.

JANICKI, L. L. & APPLEDORF, H. (1974). Effect of broiling, grill frying and microwave cooking on moisture, some lipid components and total fatty acids of ground beef. *J. Food Sci.*, **39**, 715–17.

KELLER, J. D. & KINSELLA, J. E. (1973). Phospholipid changes and lipid oxidation during cooking and frozen storage of raw ground beef. *J. Food Sci.*, **38**, 1200–4.

LEE, W. T. & DAWSON, L. E. (1976). Changes in phospholipids in chicken tissues during cooking in fresh and reused cooking oil and during frozen storage. *J. Food Sci.*, **41**, 598–600.

LOVE, J. D. & PEARSON, A. M. (1971). Lipid oxidation in meat and meat products—A review. *J. Am. Oil Chem. Soc.*, **48**, 547–9.

MORRISON, W. R. & SMITH, L. M. (1964). Preparation of fatty acid methylesters and dimethylacetals from lipids with boron fluoride-methanol. *J. Lipid Res.*, **5**, 600–8.

PENG, C. Y. & DUGAN, JR., L. R. (1965). Composition and structure of phospholipids in chicken muscle tissues. *J. Am. Oil Chem. Soc.*, **42**, 533–6.

ROUSER, G., SIAKOTOS, A. M. & FLEISCHER, S. (1966). Quantitative analysis of phospholipids by thin layer chromatography and phosphorus analysis of spots. *Lipids*, **1**, 85–6.

STEEL, R. G. D. & TORRIE, J. H. (1960). *Principles and procedures of statistics*, McGraw-Hill Book Co., Inc., New York.

TSAI, L. S. & SMITH, L. M. (1971). Role of the bases and phosphoryl bases of phospholipids in the autoxidation of methyl linoleate emulsions. *Lipids*, **6**, 196–202.

WATTS, B. M. (1962). Meat products. In: *Symposium of Foods: Lipids and Their Oxidation*. (Schultz, H. W., Day, E. A. & Sinnhuber, R. O. (Eds)), AVI Publ. Co., Westport, CT, 202–14.

YOUNATHAN, M. T. & WATTS, B. M. (1960). Oxidation of tissue lipids in cooked pork. *Food Res.*, **25**, 538–43.

ZIPSER, M. W., DUPONT, J. & WATTS, B. M. (1962). Extraction of lipids from oxidizing mullet. *J. Food Sci.*, **27**, 135–8.

Food Chemistry 7 (1981) 305–310

OXIDATION OF METHIONINE: EFFECT OF ASCORBIC ACID AUTOXIDATION

ANDERS AKSNES & LEIF REIN NJAA

Government Vitamin Institute,
Directorate of Fisheries, N-5013, Bergen, Norway

(Received: 1 June, 1980)

ABSTRACT

Free methionine is oxidised in solutions containing ascorbic acid. For this oxidation to take place, molecular oxygen is required and trace amounts of metal ions catalyse the reaction. Our results indicate that ascorbic acid must autoxidise before reacting with methionine. Catalase, histidine and scavengers of hydroxyl radical (benzoic acid and ethanol) inhibit the reaction. The superoxide anion is not involved in the reaction as superoxide dismutase did not affect the oxidation rate. Dehydroascorbic acid also oxidises methionine, but at a much slower rate than ascorbic acid. In the presence of ascorbic acid, the main product in the reaction is methionine sulphoxide, but other products are also formed. Peptide-bound methionine is oxidised to a much lesser extent than free methionine.

INTRODUCTION

In connection with studies on the biological availability of methionine sulphoxide (Gjøen & Njaa, 1977), we have been interested in studying the effect on methionine of model systems containing naturally occurring oxidising compounds. One such system is autoxidising ascorbic acid. It shows reducing, as well as oxidising, properties and was recently commented on in connection with the destruction of vitamin B_{12} (Hogenkamp, 1980). Deng *et al.* (1978) pointed out that ascorbic acid mixed with fish flesh showed antioxidant, as well as pro-oxidant, properties.

In the presence of molecular oxygen, ascorbic acid autoxidises to dehydroascorbic acid, especially in the presence of catalytic amounts of metal ions (Lyman *et al.*, 1937). Solutions containing autoxidising ascorbic acid are proposed to generate the strong oxidants, hydrogen peroxide, hydroxyl radical and superoxide anion, and

Food Chemistry 0308-8146/81/0007-0305/$02·75

the strong reducing agent, ascorbic free radical (Orr, 1967; Hogenkamp, 1980). Several enzymes are inactivated in the presence of ascorbic acid (Orr, 1967; Westall *et al.*, 1976; Brown *et al.*, 1978) and orange and lemon juices, which contain relatively large amounts of ascorbic acid, are reported to contain more than 50 % of their free methionine in the sulphoxide form (Kefford & Chandler, 1970).

In view of the widespread use of ascorbic acid in food and drinks, as well as in vitamin tablets, it seemed warranted to study the effect of ascorbic acid autoxidation on methionine oxidation.

MATERIALS AND METHODS

Catalase and superoxide dismutase were obtained from Sigma (St. Louis, Missouri, USA), ascorbic acid from Merck (Darmstadt, West Germany) and dehydroascorbic acid from Koch-Light (Buckinghamshire, Great Britain).

Total methionine was determined by its thioether group with an iodoplatinate reagent after reduction with $TiCl_3$. Unoxidised methionine was determined without the reduction step and methionine sulphoxide was obtained by difference as methionine sulphone is not reduced with $TiCl_3$ (Njaa, 1980). Proteins and peptides were hydrolysed for 10 h at 120 °C in $Ba(OH)_2$ (Slump & Schreuder, 1969) before total and unoxidised methionine were determined. Chromatographic analyses of the reaction products were performed in a Technicon Amino Acid Analyser (Model NC-2P) using resin C and ninhydrin as reagent. Methionine was also determined microbiologically with *Pediococcus cerevisiae* (ATCC 8042) (Gjøen & Njaa, 1977); methionine sulphoxide was fully active for the test organism. The reaction mixtures contained initially 0·1 mM methionine and 0·2 mM ascorbic acid or dehydroascorbic acid, and the incubations were performed at room temperature in 0·05M phosphate buffer, pH 6·0. Cu^{2+} was added as $CuSO_4$.

RESULTS AND DISCUSSION

Incubation of methionine with ascorbic acid for 4 days resulted in a 50 % decrease of unoxidised methionine, as measured by the iodoplatinate method (Fig. 1). The reaction was stimulated by 10 μM Cu^{2+} and was inhibited by 10 μM EDTA. Under anaerobic conditions no reaction occurred. There was no significant decrease in methionine when ascorbic acid was incubated with proteins (Table 1), probably because the methionine residues are buried in the interior of the proteins. Glycyl-DL-methionine, casein hydrolysate, methionine ethylester, and acetylmethionine showed minor decreases in methionine. Reduction with $TiCl_3$ resulted in total methionine values practically equal to the original values, indicating that the oxidation of these compounds proceeded only to the sulphoxide stage.

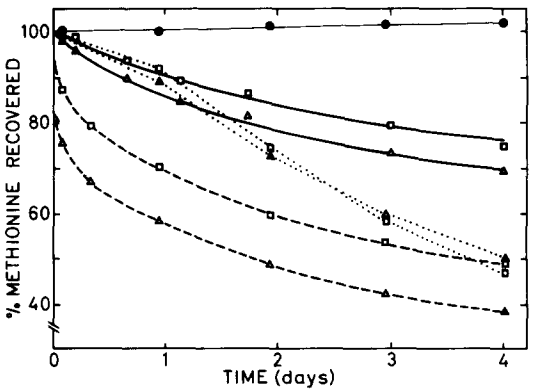


Fig. 1. Effect on methionine (0·1 mM) in solutions containing 0·2 mM ascorbic acid (– – –), 0·2 mM dehydroascorbic acid (·····) or 0·2 mM H_2O_2 (——) in the absence (□) and presence (△) of 10 μM Cu^{2+} and in the case of dehydroascorbic acid, in the presence of 10 μM EDTA (●).

When the reaction in the presence of 10 μM Cu^{2+} was followed by ninhydrin, there was a rapid decrease in colour development (about 30%) during the first 4 h, followed by a much slower decrease (total about 35% after 4 days). In the absence of added Cu^{2+}, the reaction was significantly slower and was completely inhibited by 10 μM EDTA. Chromatography of a similar reaction mixture showed a total loss of ninhydrin colour of 38%. This mixture contained, by the iodoplatinate method, 34% and 53% of the initial amount as unoxidised and total methionine, respectively. On the chromatogram methionine accounted for 32% of the initial ninhydrin colour, methionine sulphoxide for 13%, ammonia for 14·5% and homocysteic acid for 2·5%. The sum of methionine and methionine sulphoxide is

TABLE 1

EFFECT ON FREE METHIONINE, PEPTIDE BOUND METHIONINE, METHIONINE ETHYLESTER AND ACETYLMETHIONINE IN A SOLUTION CONTAINING ASCORBIC ACID

	% loss	*% loss after reduction*
L-methionine	66	47
Glycyl-DL-methionine	16	4
L-methionine ethylester	9	0
Acetylmethionine	6	3
Casein hydrolysate	9	0
Casein	−1	0
Ovalbumin	1	2

The methionine assays were performed in hydrolysates of samples incubated for 4 days under aerobic conditions. The concentration of ascorbic acid was 5·7 mM, that of methionine *ca.* 2·7 mM and that of Cu^{2+}, 10 μM.

also in good agreement with that found for methionine determined microbiologically (50 % decrease) in the same reaction mixture. Incubation of methionine sulphoxide with ascorbic acid under the same conditions also showed a peak on the amino acid analyser, coinciding with homocysteic acid.

The results indicate several degradation products of methionine and that methionine sulphoxide is a possible intermediate. The amounts of methionine and methionine sulphoxide determined on the amino acid analyser do not add up to the amount determined by the iodoplatinate method after reduction with $TiCl_3$. There may therefore be other reducible products than methionine sulphoxide present in which deamination has occurred. This effect of ascorbic acid on α-amino groups is also reported for other amino acids and proteins (Richheimer, 1975). The appearance of homocysteic acid would indicate a demethylation reaction.

The oxidation of methionine seems to proceed by two independent reactions. During the first 2–4 h, the oxidation was rapid and catalysed by added Cu^{2+}. The second reaction was much slower and showed no detectable change in reaction rate when the initial added Cu^{2+} concentration was varied from 0 to 200 μM. No precautions were taken to remove contaminating metal ions and, as the reaction was completely inhibited when 10 μM EDTA was added after 3·5 h, these contaminating metal ions seem to have a catalytic effect in the slow reaction. There was no significant change in rate of the slow reaction when Cu^{2+} was added after 3·5 h.

The reaction between methionine and dehydroascorbic acid was slow, completely inhibited by 10 μM EDTA, and there was no change in rate of methionine decrease in the presence of added Cu^{2+} (Fig. 1). Therefore, this reaction cannot account for the initial rapid loss of methionine. As the effects of EDTA and Cu^{2+} were similar to those observed with ascorbic acid when these were added 3·5 h after the start of the reaction, dehydroascorbic acid may be responsible for the slow loss of methionine.

Autoxidation of ascorbic acid was followed by the decrease in the absorbance at 265 nm (Puget & Michelson, 1974) and was found to be nearly complete (98 %) after half an hour and 2·5 h in the presence and absence of added Cu^{2+}, respectively. The initial rapid loss in methionine seems, then, to be associated with the autoxidation of ascorbic acid, and intermediates formed in this autoxidation are probably responsible. To obtain a clue to this question, incubations were carried out in the presence of proposed scavengers of hydrogen peroxide, superoxide anion, hydroxyl radical and singlet oxygen. The effect on methionine was inhibited to a great extent by histidine, benzoic acid, ethanol, or catalytic amounts of catalase (Fig. 2). No inhibition was observed in the presence of superoxide dismutase and a mixture of superoxide dismutase and catalase showed the same inhibitory effect as with catalase alone. Bovine serum albumin showed no inhibition of the oxidation, indicating that the effect of catalase is catalytic.

The inhibitory effect of catalase indicates that H_2O_2 is involved in the oxidation of methionine. The reaction between methionine and H_2O_2 in the presence or absence of added Cu^{2+} under the same conditions as described for ascorbic acid (Fig. 1), is much

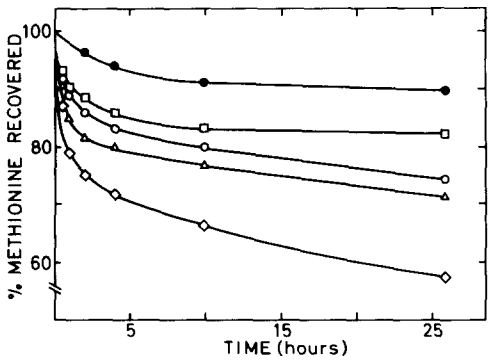


Fig. 2. Reaction of methionine (0·1 mM) in a solution of ascorbic acid (0·2 mM) and Cu^{2+} (10 μM) (◇), and the effect of 1·0 mM ethanol (△), 1·0 mM benzoic acid (○), 1·0 mM histidine (●), or 10 units/ml catalase (□).

slower, however, and the inhibitory effect of catalase can, for this reason, not be explained by an inhibition of the direct reaction between methionine and H_2O_2. We therefore assume that H_2O_2 in the presence of either ascorbic acid or its autoxidation products is involved in the production of hydroxyl radical (Orr, 1967; Richheimer, 1975). Because benzoic acid and ethanol were inhibitory, this radical seems to be involved in the oxidation (Neta & Dorfman, 1968). On the other hand, superoxide anion is not involved as superoxide dismutase had no effect on the reaction.

The distinct inhibitory effect of histidine shown in Fig. 2 may probably be explained by its proposed scavenging of singlet oxygen (Matheson *et al.*, 1975). As the specificity of histidine is not clear and other specific scavengers are not referred to in the literature (Krinsky, 1979) we conclude that it cannot be excluded as a potent oxidant of methionine.

The present results show that free methionine may be oxidised to methionine sulphoxide or destroyed in solutions containing autoxidating ascorbic acid. Hydrogen peroxide and dehydroascorbic acid, which are formed in the autoxidation, cannot account for the initial rapid decrease in unoxidised methionine. This part of the oxidation process seems to be caused by hydroxyl radical or possibly singlet oxygen. According to Shechter *et al.* (1975), free methionine and methionine in peptides or in denaturated proteins are oxidised by different oxidising agents to about the same extent, and Cuq *et al.* (1978) report that methionine in casein is easily oxidised by mild oxidants. This does not seem to be the case with oxidation due to autoxidising ascorbic acid (Table 1). However, the finding that hydrogen peroxide, hydroxyl radical and singlet oxygen are probably formed in autoxidising ascorbic acid may explain why many enzymes are inactivated by ascorbic acid. The complicated reactions and the many products formed from methionine when incubated with autoxidising ascorbic acid make this system

unsuitable as a model system for specific oxidation of methionine to methionine sulphoxide.

The importance of our findings for food and food processing have to be elucidated as ascorbic acid is used as an additive to food, both as an antioxidant and to improve the baking characteristics of wheat flour (Mair & Grosch, 1979). In the latter case, ascorbic acid must autoxidise to dehydroascorbic acid to perform the improving effect in dough and will thus be available for methionine oxidation.

ACKNOWLEDGEMENT

This work was supported by The Norwegian Fisheries Research Council (NFFR).

REFERENCES

BROWN, F. C., HARRALSON, J. D. & ZAWAD, J. S. (1978). Denaturation of proteins by ascorbic acid: Effects on dopamine-β-hydroxylase, *Neurochem. Res.* **3**, 357–66.

CUQ, J-L., BESANCON, P., CHARTIER, L. & CHEFTEL, C. (1978). Oxidation of methionine residues of food proteins and nutritional availability of protein-bound methionine sulphoxide, *Fd. Chem.* **3**, 85–102.

DENG, J. C., WATSON, M., BATES, R. P. & SCHROEDER, E. (1978). Ascorbic acid as an antioxidant in fish flesh and its degradation, *J. Fd. Sci.* **43**, 457–60.

GJØEN, A. U. & NJAA, L. R. (1977). Methionine sulphoxide as a source of sulphur-containing amino acids for the young rat, *Br. J. Nutr.* **37**, 93–105.

HOGENKAMP, H. P. C. (1980). The interaction between vitamin B_{12} and vitamin C, *Am. J. Clin. Nutr.* **33**, 1–3.

KEFFORD, J. F. & CHANDLER, B. V. (1970). Nitrogen compounds. In: *The chemical constituents of citrus fruits*, Academic Press, New York, 49–57.

KRINSKY, N. I. (1979). Biological roles of singlet oxygen. In: *Singlet oxygen* (Wasserman, H. H. & Murray, R. W. (Eds)). Academic Press, New York, 597–641.

LYMAN, C. M., SCHULZE, M. O. & KING, C. G. (1937). The effect of metaphosphoric acid and some other inorganic acids on the catalytic oxidation of ascorbic acid, *J. Biol. Chem.* **118**, 757–64.

MAIR, G. & GROSCH, W. (1979). Changes in glutathione content (reduced and oxidised form) and the effect of ascorbic acid and potassium bromate on glutathione oxidation during dough mixing, *J. Sci. Fd. Agric.* **30**, 914–20.

MATHESON, I. B. C., ETHERIDGE, R. D., KRATOWICH, N. R. & LEE, J. (1975). The quenching of singlet oxygen by amino acids and proteins, *Photochem. Photobiol.* **21**, 165–71.

NETA, P. & DORFMAN, L. M. (1968). Pulse radiolysis studies. XIII. Rate constants for the reaction of hydroxyl radicals with aromatic compounds in aqueous solutions, *Adv. Chem. Ser.* **81**, 222–30.

NJAA, L. R. (1980). A method for determination of unoxidised and total methionine in protein concentrates, with special reference to fish meals, *Br. J. Nutr.* **43**, 339–48.

ORR, C. W. M. (1967). Studies on ascorbic acid. I. Factors influencing the ascorbate-mediated inhibition of catalase, *Biochemistry*, **6**, 2995–3000.

PUGET, K. & MICHELSON, A. M. (1974). Iron containing superoxide dismutases from *luminous bacteria*, *Biochimie*, **56**, 1255–67.

RICHHEIMER, S. L. (1975). *Reactions of proteins and amino acids with ascorbic acid and hydrogen peroxide*, MS thesis Stanford University, USA.

SHECHTER, Y., BURSTEIN, Y. & PATCHORNIK, A. (1975). Selective oxidation of methionine residues in proteins, *Biochemistry*, **14**, 4497–503.

SLUMP, P. & SCHREUDER, H. A. W. (1969). Determination of tryptophan in foods, *Anal. Biochem.* **27**, 182–6.

WESTALL, F. C., THOMPSON, M. & ROBINSON, A. B. (1976). Degradation of encephalitonic protein in aerobic ascorbic acid solutions, *Experientia*, **32**, 848–9.

Food Chemistry 7 (1981) 311–315

Technical Note

Isolation and Tentative Identification of a new Constituent of Bourbon Vanilla Bean Extract

ABSTRACT

Separation of a fragrant 5-piperidone compound, containing three methyl groups and also of methylbenzoate from bourbon vanilla bean extract was achieved by liquid chromatography and by gas–liquid partition chromatography, respectively.

Commercial vanilla extract is prepared from the cured, shredded bean by extraction with aqueous ethanol and is used extensively in the food industries. The principal constituent, vanillin, whose structure was first determined by Reimer, accounts for about 0·18 to 0·29 % of a typical extract. Small amounts of other compounds have been identified via derivatives, including acetaldehyde (Bohnsack, 1967), diacetyl, furfural, benzaldehyde, 2,5-methylfurfural, anisaldehyde, benzaldehyde acetophenone and acetic-, iso-butyric-, benzoic- and vanillic acids. *p*-Hydroxybenzyl-alcohol (Pratt & Dubitte, 1969) has been identified by TLC. Other compounds found and identified are: *p*-hydroxybenzaldehyde (Bohnsack, 1967), *p*-hydroxybenzyl-methylether, *p*-hydroxy *m*-methoxycinnamic acid (ferulic acid), caprylic-, capric-, formic-, malic- and oxalic acids, *p*-cresol and guaiacol. In addition, Gnadinger (1925) prepared an oil by extraction with chloroform which contains a pleasant-smelling alcohol, still unidentified, and a cinnamic acid ester. The search for components, present in small amounts, is hampered by the degrading effect of air and heat on the very labile compounds in the extract. For instance, steam distillation of the original bean or its extracts results in a volatile oil, afflicted with a disagreeable burnt odour, which has no resemblance to the aroma of the original bean. For this reason, we sought a less destructive, milder method for the separation of labile compounds. This note describes such a mild procedure involving a prior partial separation of crude ethanolic vanilla extract with ether, followed by careful concentration of the ethereal

Food Chemistry 0308-8146/81/0007-0311/$02·75

extract and subsequent chromatography on alumina or silica gel. A detailed description of the technique is presented in the experimental section.

EXPERIMENTAL

Materials

All solvents used in liquid chromatography were checked for purity on TLC and purified. *n*-Hexane was passed through a silica column; acetone was distilled from $KMnO_4$; ether, methanol, benzene and ethylacetate were distilled; spectrograde chloroform containing about 0·75 % ethanol was used as obtained. Activated silica gel (Grace, Davison Chemical) grade 923, 100/200 mesh and neutral alumina (Camag) were employed for column chromatography. Analysis of mass spectra were obtained on an Hitachi Perkin-Elmer RMU-6 coupled to a gas chromatograph. Infrared spectra were obtained on a Perkin-Elmer model 457 spectrophotometer. NMR spectra were obtained on a Varian A-60 spectrometer using tetramethylsilane as an internal reference. Bourbon vanilla beans (third quality, furnished by Zink and Triest, Philadelphia, Pa, USA) were employed.

Preparation of alcoholic and ether extracts

Bourbon beans (300 g, 25 % moisture) were shredded in a Waring Blender and extracted with 3 litres of 40 % ethanol in a closed flask on a waterbath at 50 °C for 8 h with occasional shaking of the flask. The cooled extract was filtered through a filter cloth, the residue was pressed and the pressings were added to the extract (A). 500 ml of A were extracted in a separatory funnel with three portions of purified ether. A portion of this extract (100 ml) was concentrated at room temperature and under reduced pressure (aspirator) to a small volume until the vanillin began to crystallise. The concentrated extract (AA) was freed from vanillin by filtering and dried on about 10 g of neutral alumina in air at room temperature, alumina (B).

Column chromatography with alumina

A 9 cm high, 2 cm inner diameter, column was prepared with *n*-hexane and neutral alumina. On top of this column a layer of washed and dried sand was deposited (*ca.* $\frac{1}{2}$ cm). Two grammes of alumina (B) were distributed on top of this layer and then capped with another layer of sand; this procedure was adopted to minimise disturbing the alumina (B) during development of the column.

The column was eluted with a 7:3 mixture of hexane and chloroform, whereupon a weak, light-coloured fluorescence in long-wave UV light, appeared. Development continued with the same solvents (1:1) and with increasing proportions of chloroform. The band was collected; TLC indicated the presence of two components with relatively high R_f. Elution with chloroform gave no further development. The column was then eluted with benzene, followed by benzene and

ethylacetate with growing proportions of the latter; no further development could be detected. Elution with ether produced residual vanillin; a new strong light-coloured fluorescent band appeared at the top of the column. Further development with ether was unsuccessful in eluting any compounds. Therefore, acetone in increasing amounts was added; only vanillin in diminishing amounts was eluted. Acetone and water (50:1) resulted in movement of the fluorescent band which slowly eluted from the column. TLC indicated two components; the minor spot was identified as vanillin. A small volume of the effluent was carefully evaporated, giving a residue with a minty camphoraceous odour. The column was finally eluted with acetone and water (10:1). The effluent was gently evaporated to obtain a small amount of an oily residue, along with a small amount of water and acetone. The low melting, pale yellow volatile residue was dried over $CaCl_2$ in a desiccator.

TABLE 1
MASS SPECTRUM OF NEW COMPOUND

Mass (*e*)	*Intensity* (%)	*Base* (*mm*)	*Mass* (*e*)	*Intensity* (%)	*Base* (*mm*)
44	100	135	44	100	135
37	6·6	9	62	2	3
38	30·3	41	70	5·9	8
39	34·8	47	72	6·3	8·5
40	100	135	78	6·3	8·5
41	20	27	80	3·7	5
42	100	135	82	3·7	5
43	100	135	83	36·2	49
44	100	135	84	52·9	71·5
45	43·7	59	85	5·5	7·5
46	14·7	20	86	6·5	9
50	2·2	3	99	68	92
51	9·6	13	100	8·1	11
52	13·3	18	102	100	135
53	4·4	6	103	13·3	18
54	18·5	25	106	2·9	4
55	7·4	10	118	6·3	5·6
56	64	87	124	2·9	4
57	45·9	62	133	3·7	5
58	36·3	49	139	5·1	7
			140	1·4	2

The infrared spectrum (neat) showed major bands at 3500 cm^{-1} (NH) and 1700 cm^{-1} (CO). The mass spectrum is presented in Table 1. The parent peak at 139 and the P + 1 at 140 indicated the formula $C_8H_{13}NO$ which, coupled with the IR and NMR data, indicated the compound to be most consistent with the structures:

2,4,4-trimethyl-5-piperidone-2-ene or 3,4,4-trimethyl-5-piperidone-2-ene.

Confirmation of the new compound by silica chromatography

A 26 cm long, 2 cm inner diameter, silica gel column was prepared with *n*-hexane.

A layer of sand was added to the top of the column and a few grammes of silica gel, upon which 2 ml of extract AA had been deposited and dried, were then added to the top of the column and capped again with sand. The column was developed with a mixture of chloroform and *n*-hexane (72:28). A light coloured fluorescent band developed and moved down. Monitoring of effluent was done by TLC using 10:1 hexane–acetone (two development passes); visualisation by iodine in UV showed six components. Development was continued with ever increasing amounts of chloroform until only traces of vanillin were visible on TLC. The solvent was then changed to chloroform with increasing amounts of acetone added until the proportion of the solvents reached 1:1. TLC indicated the original spot becoming strongly light fluorescent in UV light and showing one more high R_f spot. The acetone proportion was increased until only acetone was used. The solvent was then changed to acetone and methanol (80:20); the effluent from this last mixture was collected and subjected to fractional distillation using a waterbath. The TLC checks of the distillate showed a strong light fluorescence at low R_f. A larger amount (about 200 ml) of the distillate was carefully evaporated in air. The identical oily residue with the minty fragrance obtained before remained in the beaker. The IR, NMR and mass spectra were identical to those obtained previously.

Presence of methylbenzoate in bourbon vanilla extract

Separation of the components of the extract was accomplished by GLPC, (10 in × 1/8 in 9% Carbowax 20 m on a 80/100 Chromosorb column) with direct passage to the mass spectrometer which allowed identification of peak No. 6 as methylbenzoate (Table 2).

TABLE 2
MASS SPECTRUM OF METHYLBENZOATE (PEAK NO. 6)

Mass (*e*)	*Intensity* (%)	*Base* (*mm*)	*Mass* (*e*)	*Intensity* (%)	*Base* (*mm*)
105	100	283·5	73	7·7	15·2
40	2·8	8·2	74	14·8	42
41	19	54	75	4·4	12·5
42	8	22·8	76	5·4	15·5
43	39	102	77	64	181·5
44	23·4	66·5	78	6·8	19·5
45	34·7	98·5	81	3·1	9
46	7	20	83	5·2	15
50	13	37	85	6·6	18·8
51	26·1	74	91	4·5	13
55	14·4	41	105	100	283·5
56	5·4	15·5	106	10·9	31
57	15	44	121	1·4	4
59	20·8	59	109	2·8	8
69	7·7	22	127	2·8	8
70	5·3	15·2	136	42·3	120
71	10·9	31	137	4·2	12
72	3·8	10·8	138	0·7	2

RESULTS

Using the chromatographic technique, we have succeeded in obtaining a new fragrant component from Bourbon vanilla bean extract. This compound, obtained by elution from alumina with acetone, has a camphoraceous minty odour, a burning taste and the appearance of a yellow oil. The infrared spectrum indicated the presence of -NH (3500 cm^{-1}) and a carbonyl group (1700 cm^{-1}). The mass spectrum showed a parent peak at 139, which, upon comparison with mass and isotopic abundance Tables, suggested the formula $C_8H_{13}NO$. The NMR spectrum (C_6H_6) was relatively uncomplicated, showing a six proton singlet at 71 Hz, suggestive of gem dimethyl groups, a sharp three proton singlet at 102·5 Hz, indicating a vinyl methyl, a two proton singlet at 131 Hz (—CH_2—), a broad one proton peak at *ca.* 210 Hz, which disappeared upon shaking with D_2O (—NH—), and a broad one-hydrogen signal at 360 Hz (—C—CH—). Taken together, the data are best accommodated by the two isomeric structures postulated.

The broadness of the vinylic proton appears to be most consistent with the second structure, but final confirmation must await synthesis.

ACKNOWLEDGEMENT

The authors thank Mr Martin Cohen for the initial interpretation of the infrared and mass spectra.

REFERENCES

BOHNSACK, H. (1971). *Riechstoffe, Aromen, Koerperpflege*, **27**, 125.
BOHNSACK, H. (1967). *Riechstoffe, Aromen, Koerperpflege*, **17**(4), 133–5.
GNADINGER, C. B. (1925). *J. Ind. Engn. Chem.*, **17**, 303.
PRATT, Y. & DUBITTE, J. (1969). *Anal. Falsif. Expert. Chem.*, **62**, 688.
REIMER, K. Be. 9,423 (Ger).

ERIC FEYERTAG & ROBERT HUTCHINS,
Department of Chemistry,
Drexel University, Philadelphia, Pennsylvania, 19104, USA

(Received: 26 March, 1980)

Food Chemistry 7 (1981) 317-318

BOOK REVIEW

Developments in Food Analysis Techniques—2. Edited by R. D. King. Applied Science Publishers Ltd, London. 1980. x + 323 pp. Price: £20·00.

The publishers have provided a useful service by introducing their Developments Series of books on various aspects of science and technology, written by groups of workers expert in their subject. Fortunately, techniques in food analysis were included as a subject and this volume is the second in its series. The first volume consisted of contributions on vitamins, water, nitrogen and protein, carbohydrates, GLC, HPLC, enzymatic methods, ion selective electrodes and AAS. This volume continues in the attempt to describe recent developments and trends in a further series of techniques and subjects.

The first chapter is claimed by J. G. Brennan to be a brief review of developments in food texture measurement but, in fact, turns out to be a more comprehensive up-to-date account of this broad subject. The section on instrumental methods is covered thoroughly in great detail but it is doubtful whether the lengthy tables of sensory texture profiling techniques are appropriate to this volume. Four pages are devoted to frankfurters and two to vanilla cookies. There follows a chapter by the Editor giving a brief introduction to methods used for the determination of the colour of foods and a useful review of methods for the extraction, separation and identification of synthetic and natural colouring matters. This chapter risks Gallic scorn by stating that blindfolded people have difficulty in distinguishing between red and white wines by taste!

A somewhat academic review on fluorimetric methods by J. W. Bridges is timely since the technique is becoming more widely used in food contaminant work, and another by W. J. Olsman and C. Hitchcock on the detection and determination of vegetable proteins, in particular soya protein, in meat products is the most comprehensive and up-to-date review available.

Chapter 4 is a reminder from E. C. Apling of the almost overlooked usefulness of the optical microscope. He praises its instant usability by any food analyst

Fd. Chem. (7) (1981)—
Printed in Great Britain

compared with the expertise and delays demanded by many of the modern black boxes. The chapter gives succinct practical advice and reviews the more modern applications of the instrument to quantitative analysis.

The advice of real experience given by C. Hitchcock and E. W. Hammond on the determination of lipids in foods probably justifies the purchase of the book alone. Their title is misleading since the chapter covers not only the Unilever experiences of the newer GLC, TLC and HPLC techniques in fatty acids and triglyceride analysis, but also the determination of rancidity and oil-soluble vitamins. The chapter is full of sound and practical advice which new or inexperienced workers should heed.

The only major area of criticism of the book is that a rather excessive third of it is devoted to the chapter on food texture measurement. In all other respects, apart from the inevitable somewhat variable standard of presentation by the various authors, it is recommended as a compact and well-produced useful addition to the food laboratory bookshelf as an up-to-date account of six further analytical subjects.

R. S. KIRK

Food Chemistry 7 (1981) 319

ANNOUNCEMENT

'DIETARY FIBRE—AN INTERNATIONAL SYMPOSIUM'

The National College of Food Technology, Weybridge, Surrey, Great Britain, is the venue for an international symposium on 'Dietary Fibre' which will take place there on 29–31 March, 1982. The aim of the symposium is to cover all the main scientific aspects of the definition, use and effects of dietary fibre.

Dr D. A. T. Southgate of the Food Research Institute, Norwich, will give the keynote lecture on 'Testing the Fibre Hypothesis'. Other eminent speakers will include Drs Burkitt, Eastwood, Leeds, Hamilton, Selvendran, Hill, Apling and Coates. The proceedings of the symposium will once again be published as a book by Applied Science Publishers.

Further information may be obtained from: The Secretary, National College of Food Technology (University of Reading), St. Georges Avenue, Weybridge, Surrey KT13 0DE, Great Britain.

Food Chemistry 0308-8146/81/0007-0319/$02·75

Food Chemistry 7 (1981) 321–323

INDEX TO VOLUME 7

(*Figures in parentheses are issue numbers*)

FOOD CHEMISTRY

An International Journal

This journal is concerned mainly with the chemistry and biochemistry of foods and chemical and biochemical changes occurring in foods. It is also concerned with sensory and nutritional properties of food within a chemical or biochemical framework. The journal provides a vehicle for the widest possible dissemination of knowledge in food chemistry, mainly as research papers and reviews.

Research papers must be factual and original and must not have been published elsewhere, though papers whose biological or technological aspects have already been published may be accepted if previously unpublished chemical information and conclusions are now included. The following subjects are among those open for discussion: pure and applied food chemistry; structural changes in molecules during the processing and storage of foods; chemical aspects of nutrition, physiology and health; chemical quality control in food chemical engineering and technology; direct effects on food of the use of agrochemicals; chemistry relating to sensory aspects; chemical additives; chemical toxins; chemical analysis of food; food biochemistry; and chemistry relating to microbiological aspects.

Type of contributions Research papers; Review articles; Book reviews; Conferences and meetings; Short communications; Letters to the Editor

Submission of papers Papers should be sent to the Editors (Dr G. G. Birch and Mr L. F. Green, c/o National College of Food Technology, St. Georges Avenue, Weybridge, Surrey, England), to members of the Editorial Board for submission to the Editors, or to the publishers.

Special regulations for readers in the USA This journal has been registered with the Copyright Clearance Center, Inc., 21 Congress Street, Salem, MA 01970, USA. Consent is given for copying of articles for personal or internal use, or for the personal or internal use of specific clients. This consent is given on the condition that the copier pay through the Center the per-copy fee stated in the code on the first page of each article for copying beyond that permitted by Sections 107 or 108 of the US Copyright Law. If no code appears in an article, the author has not given broad consent to copy and permission to copy must be obtained directly from the author. All articles published prior to 1979 may be copied for a per-copy fee of US$2.50, also payable through the Center. This consent does not extend to other kinds of copying, such as for general distribution, resale, advertising and promotion purposes, or for creating new collective works.

Subscriptions Four issues per volume (16·5 x 24 cm). Subscription orders should be addressed to:

APPLIED SCIENCE PUBLISHERS LTD, Ripple Road, Barking, Essex, England

LIST OF MEMBERS OF EDITORIAL BOARD