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Energy Saving by Membrane Filtration of Process Water of Potato and Wheat Starch Plants*

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ABSTRACT

The results reported here are a first estimation of the energy required by various processes to reduce the COD of effluent from potato and wheat starch plants by recovering the dissolved material. It is clearly shown that membrane filtration can help to minimise the total primary energy input by recovering otherwise wasted substances. With respect to the energy subsidy, most favourable results can be obtained with 60% and 90% COD reduction using coagulation, as well as membrane filtration (hyperfiltration), combined with evaporation, as process stages. According to the energy subsidy ratios, the procedures waste no primary energy and have the advantage of increasing the dry substance yield from the processed raw material.

INTRODUCTION

The production of potato and wheat starch involves substantial losses of water-soluble and partly suspended substances from the raw materials. These losses amount to 20% and 10% of the total dry substances for potatoes and wheat flour, respectively (Table 1).

The 'waste' material consists mainly of proteins, carbohydrates and minerals (Table 2). Part of the potato juice proteins (50%) and diluted

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TABLE 1
Dry Substance Loss and its Possible Recovery in Potato and Wheat Starch Production

Raw material	*Dry substance loss (% of total ds)*	*Recoverable substance (% of ds loss)*
Potatoes	20	20–90
Wheat flour	10	10–90

TABLE 2
Characterisation of the 'Waste' Material

Content	*Potato juice*	*Wheat flour solubles*
Protein (%)	40	25
Coagulable protein (%)	20	8
Carbohydrates (%)	20	40
Minerals (%)	20	6

wheat flour proteins (30%) can be coagulated by heat. The dried coagulates amount to 20% (potatoes) and 8% (wheat flour) of the total 'waste' material.

The remaining solubles and suspended particles can be recovered by concentrating the process water streams. Evaporation alone, or membrane filtration combined with evaporation, are suitable techniques. The process stream volumes depend on the separation techniques used to extract the starch. One-stage centrifugal decantation of potato gratings results in 60%, and double-stage decantation, using water in counter flow, results in 90% separation (Das Neueste aus der Stärkegewinnung, 1979). The resultant potato juice streams contain around 5·0–6·0 and 3·5–4·0% dry substance, respectively. The concentration of the process water stream in gluten–starch separation is around 3% and, using newer process designs it is even slightly higher (Oy Vehnä AB, 1973–1974).

Application of coagulation followed by evaporation recovers up to 90% of the total dry substance losses. Table 3 gives the recovery figures for each stage of juice treatment. The chemical oxygen demand (COD) of the effluents is directly proportional to their dry substance content. Thus, any recovery of substances by coagulation and concentration results in an

TABLE 3
Recovery of Dry Substance Losses in Potato Starch Production in Relation to Juice Treatment

Juice separation (%)*	*Recovery of dry substance losses* (% *of total ds*)		
	Juice treatment		*Total losses waste water*
	Coagulation	*Evaporation*	
0	—	—	20
60	4	9	7
90	6	12	2

* Separation by centrifugal decantation (% of total juice).

equivalent COD reduction. This is extremely important for irrigation and other waste water treatment procedures designed to eliminate pollution.

Irrigation and similar disposal systems for waste water, which use biological digestion, need very little energy input. Energy is mainly required to run the pumps piping the effluent. It is interesting to note that an anaerobic digestion system producing methane may completely offset the energy requirements of operating the treatment facility (Crafts-Lighty *et al.* (1980)). On the other hand, recovery of raw material losses by heating and evaporating the process water streams involves high energy input.

For this reason an analysis was made of energy requirements for potato and wheat starch production in terms of primary energy use and energy costs to recover material losses. This will form a basis for future planning and decision-making in the development of the process.

POTATO AND WHEAT STARCH PROCESS

The processes used for potato and wheat starch production, which were investigated to determine energy requirements in recovering dry substance losses, are outlined in Figs 1 and 2. Both Figures show a membrane filtration stage as a possible extra step to concentrate the waste streams. In each case membrane filtration is followed by steam coagulation of the proteins.

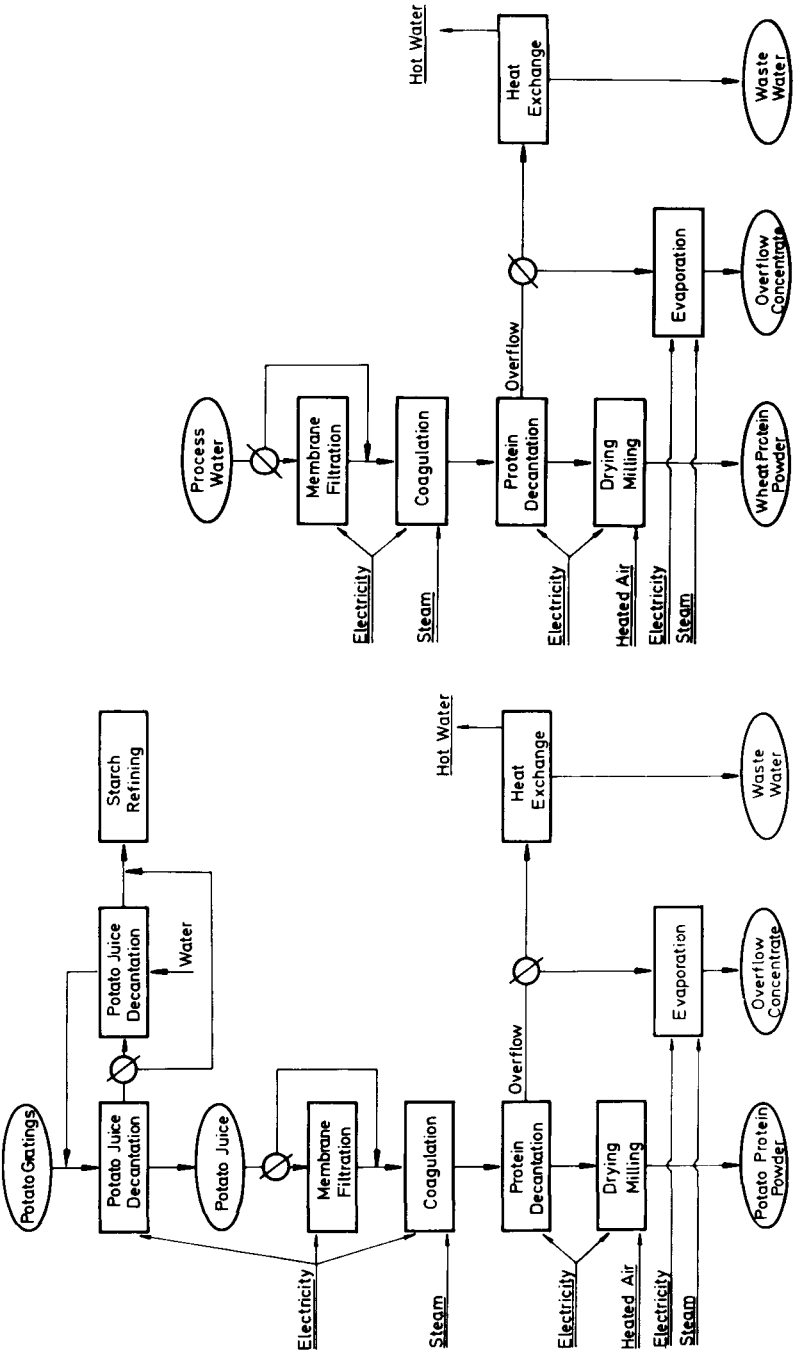

Fig. 1. Simplified flow sheet for potato juice treatment of potato starch plants.

Fig. 2. Simplified flow sheet for process water treatment of wheat starch plants.

Protein coagulation is widely used in potato starch plants and will also be employed in a German wheat starch plant in the near future. Full scale membrane filtration plants will be operated during this season in The Netherlands potato starch industry. In this connection it should be pointed out that membrane filtration was tried without success in a German potato starch plant a few years ago.

The coagulated proteins are flash dried after centrifugal decantation with an Ultrarotor or similar drying equipment which includes a milling facility. After separation of the coagulated proteins, the heated solutions will either be irrigated or evaporated. Prior to irrigation a heat exchanger recovers heat energy for reuse in the process.

METHODS

One of our earlier papers (Meuser & Köhler (1980)) reports the total costs for membrane filtration in comparison with evaporation for the effluents of potato and wheat starch plants. The study examined plants processing 50 tons of potatoes an hour and 3 tons wheat flour an hour. The mass and process water flow of these plants, which are of a typical size for this branch of industry in Germany, were used as a model for this study.

The units of energy used are the megajoule (MJ) and the kilowatt-hour (kWh). The energy consumption is expressed as primary energy input per ton of raw material processed. The use of the term 'primary energy' gives an ultimate basis for the evaluation of energy consumption. The primary energy content of delivered fuels and the factors for their conversion are derived from plant records, which include the average boiler efficiency and steam consumption of the evaporator.

The calculations for the systems and their variations are based on information and measurements derived from potato and wheat starch plants (e.g. Emsland-Stärke, FRG, Stärkefabrik Schrobenhausen, FRG, AKV-Langholt, Denmark and Kröner-Stärke, FRG) which run full- or pilot-scale equipment for coagulation and/or evaporation.

Energy consumption by membrane filtration is calculated according to the figures given by Meindersma (1980). The author has determined that, for a recirculation system, the average energy consumption is 8 kWh/m^3 permeate. For the purpose of this study the figure seems to fit well enough for membrane filtration by ultrafiltration (UF) as well as hyperfiltration (HF). It also takes into account the average flux rates given in Table 4.

According to our own experience (Meuser & Smolnik (1979)), these flux rates might be rather optimistic although Meindersma (1980) has published similar data. He obtained an average permeate flux rate of 21·5 litres $m^{-2} h^{-1}$ at 25 °C while concentrating diluted potato juice from around 4 % to 8 % dry substance content with a hyperfiltration plant of 822 m^2 membrane area. The figure of 18 litres $m^{-2} h^{-1}$ permeate flux rate for wheat starch plant effluents comes from results obtained in a pilot-scale experiment which was carried out with a Wafilin membrane

TABLE 4
Average Flux Rate of Permeate by Membrane Filtration of Potato Juice and Wheat Flour Solubles

Membrane filtration	*Permeate flux rate (litres $m^{-2} h^{-1}$)*	
	Potato juice	*Wheat flour solubles*
UF*	40	—
HF**	20	18

*UF = Ultrafiltration.
**HF = Hyperfiltration.

filtration unit (Boonstra, 1980). The effluent was concentrated by hyperfiltration to 75 % permeate yield.

The calculations start with the centrifugal decantation stage for potato starch production and with the membrane filtration of protein from the coagulation stage for wheat starch production. The last stage of calculated energy input is either that of the evaporation plant or the heat exchangers.

It is assumed possible to recover around 70 % of the heat energy input using heat exchangers. This heat can be recirculated to various process stages. The energy consumption for the evaporation is calculated on a seven-stage evaporator which dissipates 0·19 kg steam (12 bar)/kg evaporated water. The energy uptake of the boiler is quoted as 3·4 MJ of primary energy per kilogram of steam, using natural gas with a primary conversion factor of 1·00. The conversion factor for electrical energy is assumed to be 0·375. This figure deviates considerably from the factor usually given in the literature (Chapman *et al.*, 1974) but, nevertheless, gives, in our opinion, a more precise figure for the effectiveness of German

electricity production (Energiebilanzen der Bundesrepublik Deutschland, 1978).

The analysis neglects the electrical energy used for running defoaming equipment which is an indispensable installation, especially for the membrane filtration of potato juice. It is assumed that this energy input can be balanced out within the whole starch plant system, including the irrigation network, by using an upgraded water piping system. This requires less energy in consequence of membrane filtration.

The recovered materials have an energy content, which is calculated from the average composition of the material and the theoretical energy content of its constituents (e.g. for potato protein powder 15·9 MJ/kg ds and for overflow concentrate 13·4 MJ/kg ds). The water content of the dried coagulate (10 %) and of the concentrate (40 %) are neglected as the materials are considered to be final products. The term 'energy subsidy' expresses the ratio of used primary energy to the energy content of these products.

Expenditure on capital equipment, human labour and some minor material inputs (e.g. sulphuric acid to lower the pH value of the effluents before coagulation, enzymes to lower viscosity of the effluents during evaporation) are also not given any energy equivalent. This may be a correct approach as capital equipment expenditure for the systems with and without membrane filtration equipment is almost the same (Meuser & Köhler, 1980).

Finally, the costs for primary energy input are based on energy prices paid in July, 1980 by one of the starch plants investigated. The money equivalent expressed in DM for 1000 MJ primary energy amounts to DM 8·65 for gas and to 11·05 for electricity giving a cost ratio for gas:electricity of 1·0:1·3.

RESULTS

Tables 5 and 6 show the apportioned energy input for each stage of processing potato juice. The COD reduction amounts to 27 %, 40 %, 60 % and 90 % of potato starch plants' total pollution both with and without the use of membrane filtration. As mentioned before, the COD reduction is almost proportional to the recovery of dry substance losses.

Electrical energy is used at all stages of the system for a variety of functions including decanting the gratings, pumping the juice through the

TABLE 5
Energy Input for the Reduction of Chemical Oxygen Demand in Potato Starch Plants

Process stage	*Energy input (MJ or kWh/t of potatoes)*			
	27% COD reduction		*40% COD reduction*	
	Without UF	*With UF*	*Without UF*	*With UF*
Decantation/potato juice (kWh)	2·7	2·7	5·4	5·4
Membrane filtration/ potato juice (kWh)		2·4		5·2
Coagulation/protein (MJ/kWh)	194 0·6	65 0·2	400 0·9	140 0·3
Decantation/protein (kWh)	1·5	0·5	3·2	1·1
Drying–milling/protein (MJ/kWh)	105 6·0	105 6·0	157 9·0	157 9·0
Heat exchange/overflow (MJ)	−136	−46	−280	−98
Electrical energy input (kWh)	10·8	11·8	18·5	21·0
Thermal energy input (MJ)	163	124	277	199
Total primary energy input (MJ)	266	237	455	401
Primary energy ratio (Gas:electricity)	1·6:1·0	1·1:1·0	1·6:1·0	1·0:1·0
Primary energy savings (%)		11		12

coagulation equipment, decanting the coagulated protein, circulating the air in the drier, running the mill rotor of the drying equipment and piping the effluent through the evaporator. Heat energy input is consumed as steam in the coagulation and evaporation stages and as heated air in the drying stage.

In the cases of 27% and 40% COD reduction (Table 5) most energy is consumed for the drying and milling of the coagulate. The primary energy required to prepare heated air for the system, with and without ultrafiltration, amounts to around 80% and 60% of the total thermal energy input, respectively. It is interesting to note that membrane filtration only slightly increases the electrical energy input into the system. This is mainly due to the fact that less energy is required for piping and decanting the smaller juice volume. The total thermal energy input decreases from 163 MJ to 124 MJ and 277 MJ to 199 MJ, respectively, because of energy input to run the evaporator.

However, this advantage declines if the conversion factor for electricity is considered in the overall energy input calculation. The figures show that at least an 11% primary energy saving is possible with ultrafiltration

TABLE 6
Energy Input for the Reduction of Chemical Oxygen Demand in Potato Starch Plants

Process stage	*Energy input (MJ or kWh/t of potatoes)*			
	60% COD reduction		*90% COD reduction*	
	Without HF	*With HF*	*Without HF*	*With HF*
Decantation/potato juice (kWh)	2·7	2·7	5·4	5·4
Membrane filtration/ potato juice (kWh)		1·8		3·9
Coagulation/protein (MJ/kWh)	194 0·6	95 0·3	400 0·9	211 0·5
Decantation/protein (kWh)	1·5	0·8	3·2	1·7
Drying–milling/protein (MJ/kWh)	105 6·6	105 6·6	178 10·0	178 10·0
Evaporation/overflow (MJ/kWh)	175 2·6	78 1·2	373 5·6	182 2·7
Electrical energy input (kWh)	14·0	13·4	25·1	24·2
Thermal energy input (MJ)	474	278	951	571
Total primary energy input (MJ)	608	406	1194	804
Primary energy ratio (Gas:electricity)	3·5:1·0	2·2:1·0	3·0:1·0	2·5:1·0
Primary energy savings (%)		33		33

followed by heat exchange and irrigation of the effluent in comparison with the system without a membrane filtration stage.

Reduction of COD by 60% or 90% (Table 6) results in much higher primary energy consumption. Thermal energy used in the system with hyperfiltration amounts to about 60% compared with the system without. Again, this figure is lowered by the primary energy used to produce the required electrical energy. This results in a final saving of primary energy input of 33% in favour of the system with hyperfiltration.

In the case of wheat starch plants equipped to reduce COD by 90%, the primary energy input differs markedly between systems with and without a hyperfiltration stage (Table 7). The difference amounts to 68% in favour of the system with hyperfiltration. The major energy-consuming operation in both plant designs is the evaporation stage. Drying and milling of the coagulate consume relatively minor amounts of energy.

Even the electrical energy input is rather small compared with the thermal energy use. The proportions of electrical to thermal energy in terms of primary energy input are: for the system without hyperfiltration, 1:7·3 and, for the system with hyperfiltration, 1:4·2. It must be pointed

TABLE 7
Energy Input for the Reduction of Chemical Oxygen Demand in Wheat Starch Plants

Process stage	*Energy input (MJ or kWh/t of flour)* > 90% *COD reduction*	
	Without HF	*With HF*
Membrane filtration (kWh)		6·7
Coagulation/protein (MJ/kWh)	1261 3·0	348 1·5
Decantation/protein (kWh)	10·0	3·5
Drying/milling (MJ/kWh)	92 5·3	92 5·3
Evaporation/overflow (MJ/kWh)	2123 31·5	589 8·7
Electrical energy input (kWh)	49·8	25·7
Thermal energy input (MJ)	3476	1029
Total primary energy input (MJ)	3954	1275
Primary energy ratio (Gas:electricity)	7·3:1·0	4·2:1·0
Primary energy savings (%)		68

out that the elimination of pollution from potato starch plants with such equipment gives lower energy ratios for the example of 60% and 90% COD reduction. This indicates a notably higher electrical energy demand compared with similar process techniques in wheat starch plants.

Table 8 shows the energy subsidy values for the investigated material recoveries. According to the energy subsidy ratio, energy input exceeds energy output by at least 1·5:1 (60% COD reduction of potato juice) to a maximum of 2·8:1 (90% COD reduction of wheat flour solubles) if no

TABLE 8
Energy Subsidy for the Recovery of Dry Substance Loss in Potato and Wheat Starch Production Using Membrane Filtration

COD reduction (%)	*Energy input (MJ/t raw material)*	*Energy output (MJ/t recovered material)*	*Energy subsidy*	
			With MF	*Without MF*
Potatoes: 27	237	140	1·7	1·9
40	401	210	1·9	2·2
60	406	420	1·0	1·5
90	804	590	1·4	2·1
Wheat flour: 90	1275	1420	0·9	2·8

TABLE 9
Energy Costs for the Reduction of Chemical Oxygen Demand in Starch Plants by Recovering the 'Waste' Materials

Energy	*COD reduction*	*Energy costs (DM/t raw material)*		*Cost savings* (%)
		Without MF	*With MF*	
Gas/electricity	Potato: 27%	2·60	2·30	11·5
Gas/electricity	Potato: 40%	4·40	4·00	9·1
Gas/electricity	Potato: 60%	5·60	3·80	32·1
Gas/electricity	Potato: 90%	10·90	7·50	31·2
Gas/electricity	Wheat flour: 90%	35·30	11·60	67·1

membrane filtration stage is used to reduce COD. Even with the use of membrane filtration, energy input and output is balanced only in cases of 60% and 90% COD reduction of potato juice and wheat flour solubles, as energy subsidy ratios of about 1 indicate.

The calculation of total primary energy use, expressed as energy costs to reduce COD, reflects a cost-saving due to membrane filtration comparable with the figures for primary energy savings (Table 9).

DISCUSSION

The results reported here are a first estimation of the energy required by various processes to reduce the COD of effluent from potato and wheat starch plants by recovering the dissolved material. It is clearly shown that membrane filtration can help to minimise the total primary energy input by recovering otherwise wasted substances. With respect to energy subsidy, most favourable results can be obtained with 60% and 90% COD reduction using coagulation, as well as membrane filtration (hyperfiltration), combined with evaporation, as process stages.

According to the energy subsidy ratios, the procedures waste no primary energy and have the advantage of increasing the dry substance yield from the processed raw material. For this reason these processing techniques are superior to biological treatment of the effluents which do not consume primary energy (Crafts-Lighty *et al.*, 1980). With respect to the energy subsidy ratios, we have to keep in mind that these values would increase slightly if the energy equivalent of the capital expenditure were included.

It is of some interest to apportion the primary energy input to the recovered potato protein. The average yield of potato protein powder is, in the case of 60 % juice decantation, 9·0 kg ds. A total primary energy input of 266 MJ without, and 237 MJ with, ultrafiltration equipment is necessary to recover 27 % of the waste material. This results in an energy input for potato protein powder of 29·6 MJ and 26·3 MJ/kg ds for these respective cases.

Data available in the literature for energy use in wheat gluten production, a similar product to potato protein with respect to protein content, show a value of 31·3 MJ/kg (Crafts-Lighty *et al.*, 1980). This is comparable with the energy input required to recover potato protein from potato juice. Since potato protein powder has a very high nutritional value and is 80 % protein ds, a value of around 35–40 MJ/kg protein total energy input is obtained. This value indicates a low energy requirement in comparison with the production of animal protein which exceeds 200 MJ/kg protein ds. For this reason, the recovery of protein from potato juice remaining from potato starch production should be routine practice.

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Evaluation of Leaf Protein Isolates of Some Egyptian Crops

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ABSTRACT

The in vitro *digestibilities by pepsin, pancreatin and pepsin followed by pancreatin of the leaf protein isolates of four species of Egyptian crops were studied. Bean and cabbage leaf protein isolates were more digestible with pepsin and pancreatin, each used individually, as well as with pepsin followed by pancreatin, than those of tomato and sugar cane. There were only minor variations in the amino acids content of the different leaf protein isolates with the sulphur-containing amino acids being the limiting acids.*

Bean and cabbage leaf protein isolates were superior to those of tomato and sugar cane from the point of view of availability of amino acids.

INTRODUCTION

The amino acid composition of leaf protein concentrate was reported to be as good as, or better than, that of many foodstuffs (Gerloff *et al.*, 1965; Oke, 1971). On the basis of amino acid composition, leaf protein from a wide variety of plant species was considered of high biological value (Akeson & Stahmann, 1965). However, some points remain to be studied before leaf proteins can be successfully introduced into human food. These points are digestibility and nutritive availability of the amino acids of leaf proteins. All the essential amino acids are completely available from certain proteins, especially those of animal origin, while some of

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these amino acids are either absent or unavailable in others, especially those from plant sources. Akeson & Stahmann (1964) stated that *in vitro* methods of protein evaluation were useful in screening new protein foods and processing methods because of their rapidity. This work was carried out to evaluate the digestibility and quality of leaf protein isolates prepared from some Egyptian crops.

MATERIALS AND METHODS

Materials

Leaf protein isolates of bean (*Phaseolus vulgaris*), cabbage (*Brassica oleracea*), tomato (*Lycopersicon esculentum*) and sugar cane (*Saccharum officinarum*) prepared as described elsewhere (Abo Bakr *et al.*, 1982) were used.

Methods

In vitro *digestion of leaf proteins by proteolytic enzymes—Pepsin digests*
Pepsin digests were prepared by the method of Akeson & Stahmann (1964) in which a sample of 500 mg of defatted leaf protein was incubated, with occasional shaking, with 12·5 mg pepsin (1:10,000 ext. hog stomach mucosa, Koch Light laboratories, UK) in 15 ml of 0·1 N HCl for 24 h at 37 °C. Enzyme and sample blanks were run under the described conditions.

In vitro *digestion of leaf proteins by proteolytic enzymes—Pancreatin digests*
The digests of pancreatin (ext. hog pancreas Koch Light laboratories, UK) were prepared by incubating a sample of 100 mg of defatted leaf protein with 4 mg of pancreatin in 7·5 ml phosphate buffer, pH 8, at 73 °C for 24 h. Blanks for enzyme and substrate were also carried out.

In vitro *digestion of leaf proteins by proteolytic enzymes—Pepsin followed by pancreatin digests*
The method of Akeson & Stahmann (1964) was followed. A sample of

100 mg of defatted leaf protein was incubated with 1·5 mg of pepsin in 15 ml 0·1 N HCl at 37 °C for 3 h. This was followed by neutralisation with 7·5 ml phosphate buffer, pH 8. The digestion mixture was incubated for an additional 24 h at 37 °C. Enzyme and sample blanks were prepared under the same conditions. To all digests 2 ml of toluene was added in order to prevent growth of microorganisms (Venkatson & Rege, 1968). At the termination of each digestion, trichloroacetic acid (TCA), 1·6 M, was added to the digest (1:1) which was left for 2 h and then centrifuged, the supernatant being analysed for TCA-soluble nitrogen using the micro Kjeldahl method. Percentage digestion was calculated with respect to the total nitrogen in the sample.

Amino acids determination

Acid and alkaline hydrolyses were carried out separately on 1 g of sample of the defatted isolates according to the method of Block *et al.* (1958).

The monodimensional descending multiple development technique of paper chromatography was employed and the determination of amino acids was carried out according to the method of Mikes (1966). The solvent used was *n*-butyl alcohol:acetic acid:H_2O (144:13:34). For colour development the chromatograms were dipped in 0·25 % ninhydrin solution in acetone containing 1 % acetic acid (Roland & Gross, 1954). The individual spots were cut out and eluted separately using 5 ml of 75 % ethyl alcohol containing 0·25 mg of copper sulphate (Majunders *et al.*, 1956), for 30 min. Standard curves were constructed for each amino acid. Tryptophan was determined according to the method of Miller (1967).

Enzymatically available amino acids estimation

A volume of the pepsin pancreatin digest, prepared as described earlier, was added to five times its volume of 1 % picric acid solution and, after 2 h, the mixture was centrifuged for 30 min at 3500 rpm. The supernatant was passed through a column of an anion exchanger (Amberlite IRA-400) in the chloride form. After rinsing the column with 0·02 N HCl, the combined effluent (150–200 ml) was evaporated to dryness, then dissolved in 10 % isopropyl alcohol and used for the separation and quantitative determination of amino acids by paper chromatography as previously described. Casein was given the same treatment to serve as reference.

RESULTS AND DISCUSSION

In vitro enzymic digestibility of leaf protein isolates

The results in Table 1 indicate that casein was more easily digested by both pepsin and pancreatin than all the four leaf protein isolates. Those of bean and cabbage leaves were more susceptible to the digestion than those of tomato and sugar cane leaves. The digestibility of all four isolates with pancreatin was higher than with pepsin, which was also the case in casein. This may be attributed to both the modes of action and specificities of pepsin and pancreatic enzymes, as well as to the amino acid make up and sequence in the peptides of the leaf isolates.

TABLE 1
In Vitro Enzymic Digestibility of Leaf Protein Isolates and Casein

Protein	*Digestibility (Per cent of total nitrogen)*		
	Pepsin	*Pancreatin*	*Pepsin followed by pancreatin*
Casein	59·7	93·2	100
Bean leaves	53·9	56·4	78·1
Cabbage leaves	43·6	43·4	67·8
Tomato leaves	50·1	52·7	77·6
Sugar cane leaves	40·3	45·3	64·3

The results also show that the digestibility values of all the leaf proteins tested were distinctly higher with pepsin followed by pancreatin than with either pepsin or pancreatin alone. This is because it follows the natural sequence prevailing in the gastro-intestinal tract, whereby proteins are initially exposed to the action of pepsin which first splits the large protein molecules into smaller units which are more easily attacked by the pancreatic proteolytic enzymes. Casein was more digestible with pancreatin and with pepsin followed by pancreatin than the leaf protein isolates, whose digestibilities were higher in the isolates of bean and cabbage leaves than in those of sugar cane and tomato. The results agree with those reported by Buchanan (1969) on wheat leaf proteins.

Amino acids content of leaf protein isolates

Examination of the amino acid pattern of leaf protein isolates (Table 2) indicates the presence of eighteen amino acids including all the essential ones, with lysine, valine, leucine and isoleucine being present in high amounts. The concentrations of glutamic acid, aspartic acid, alanine and arginine were the highest of the non-essential amino acids.

TABLE 2
Total Amino Acids Content of Leaf Protein Isolates

Amino acid	*Leaf protein isolate*			*Reference protein*	
	Bean	*Cabbage*	*Tomato*	*Sugar cane*	*Casein*
	Amino acid per 16 g N				
Lysine	6·8	6·2	6·1	6·8	6·8
Phenylalanine	5·8	5·8	5·7	5·8	5·1
Methionine	2·9	2·3	2·2	2·1	3·3
Threonine	5·0	5·0	5·0	4·9	3·6
Leucine plus Isoleucine	15·6	15·4	15·9	15·6	17·0
Valine	6·0	6·3	6·0	6·15	7·4
Tryptophan	1·9	1·8	1·9	1·6	1·6
Arginine	5·9	6·2	6·1	6·0	4·2
Histidine	2·8	2·8	2·9	2·8	2·6
Tyrosine	4·4	4·2	4·1	4·1	5·7
Cystine	0·9	0·8	0·7	0·9	0·3
Aspartic acid	9·8	8·9	8·2	9·0	6·0
Serine	4·3	4·5	3·8	4·5	5·6
Glutamic acid	12·1	11·5	11·3	11·3	22·8
Proline	5·7	4·6	5·3	4·1	5·9
Glycine	5·8	5·8	5·4	5·4	1·7
Alanine	6·9	7·1	5·6	6·5	2·7

The sulphur-containing amino acids (methionine and cystine) were the lowest in concentration of the essential amino acids. Upon comparing the formation results with those of casein, it can be observed that two essential amino acids—lysine and phenylalanine—were present at the same level. Trytophan and threonine were present at higher levels than in casein. The results obtained agree with reports in the literature which state that no wide variations exist in the amino acid patterns of leaf proteins prepared from different species of leaves (Byers, 1971; Chibnall *et al.*, 1963; Gerloff *et al.*, 1965; Subba Rau *et al.*, 1972).

Enzymatic availability of amino acids from leaf protein isolates

Although the amino acids composition of a protein is a significant parameter in assessing the nutritive value, the degree and rate of release of the amino acids during the digestion process are the most important determinants of the protein quality (Hartman *et al.*, 1967). The data in Table 3 show that proline was absent in the digests prepared from all samples of leaf protein isolates as well as casein, because neither pepsin nor pancreatin was capable of hydrolysing the peptide links involving this amino acid in their composition.

The amino acids produced can be arranged according to the rate of liberation in the following decreasing order: lysine, phenylalanine, leucine and isoleucine, tyrosine, arginine, valine, alanine, serine, threonine, histidine, glutamic acid, glycine, methionine, aspartic acid and cystine. The differences among the four leaf protein isolates tested with regard to the rate of hydrolysis by digestive enzymes were slight.

It will be observed that the sum of the liberated amino acids from bean and cabbage leaf protein isolates is higher than those of tomato and sugar

TABLE 3
Release of Amino Acids by *In Vitro* Pepsin Followed by Pancreatin Hydrolysis of Leaf Protein Isolates

Amino acid	*Leaf protein isolate*			*Reference protein*	
	Bean	*Cabbage*	*Tomato*	*Sugar cane*	*Casein*
	Amino acid per 16 g N				
Lysine	3·2	3·1	2·3	2·4	2·8
Phenylalanine	2·4	2·3	1·6	1·8	2·2
Methionine	0·7	0·6	0·6	0·6	1·0
Threonine	0·8	0·6	0·6	0·7	0·9
Leucine plus Isoleucine	5·6	5·5	4·8	4·7	6·5
Valine	1·3	1·3	1·2	1·1	1·0
Arginine	2·5	2·6	1·8	2·1	2·7
Histidine	0·7	0·7	0·5	0·5	1·6
Tyrosine	2·1	2·1	1·6	1·6	2·3
Cystine	0·2	0·2	0·2	0·2	0·3
Aspartic acid	0·3	0·3	0·2	0·2	0·4
Serine	1·0	0·8	0·8	0·7	1·0
Glutamic acid	0·7	0·6	0·5	0·5	0·6
Glycine	0·5	0·3	0·3	0·2	0·5
Alanine	1·9	1·0	0·8	0·8	0·7

cane. This means that cabbage and bean leaf protein isolates are higher than those of tomato and sugar cane. This, in turn, means that cabbage and bean leaf protein are either more susceptible to proteolysis than the sugar cane and tomato leaf proteins or that the latter isolates may contain inhibitors to the enzymes used. The same trend existed when the total sum of liberated essential amino acids was used as a measure of digestibility, all showing the superiority of the protein of bean and cabbage leaves over that of tomato and sugar cane. The individual essential amino acids released from the leaf protein isolates were nearly as high as those released from the casein (except methionine).

Akeson & Stahmann (1965) studied the amino acids released from several leaf protein concentrates subjected to pepsin followed by pancreatin digestion and found little variation between them in the amount of each amino acid released. The levels of all the essential amino acids released were nearly the same as those released from whole egg and egg white, with the exception of methionine. The availability of each

TABLE 4
Enzymic Availability of Amino Acids of Leaf Protein Isolates

Amino acid	*Leaf protein isolate*			
	Bean	*Cabbage*	*Tomato*	*Sugar cane*
	Individual enzymic liberation of amino acids			
	*Per cent of total amino acids content***			
Lysine	46·8	50·0	37·8	36·2
Phenylalanine	40·6	40·0	28·9	30·3
Methionine	29·5	27·1	29·0	30·1
Threonine	15·6	12·2	11·5	13·4
Leucine plus Isoleucine	35·7	35·6	30·0	30·3
Valine	21·4	20·4	20·4	16·6
Arginine	43·6	46·6	30·0	35·2
Histidine	22·8	23·8	19·0	17·2
Tyrosine	48·4	53·1	42·4	39·0
Cystine	26·4	27·5	28·4	20·4
Aspartic acid	3·4	2·9	2·9	2·5
Serine	22·1	18·6	20·5	15·3
Glutamic acid	5·4	5·0	4·5	4·2
Glycine	7·9	4·4	5·0	4·0
Alanine	17·1	14·1	14·6	11·4

** Obtained by acid hydrolysis.

amino acid in the four isolates prepared in the present study was calculated with reference to its total content (obtained by acid hydrolysis) and the results are given in Table 4. They show that the percentage availabilities of the essential amino acids ranged from 11·5 to 53·1%, being higher in bean and cabbage leaf isolates than in tomato and sugar cane leaf proteins, and with glutamic and aspartic acids having lower values than the other amino acids. The conclusion may be drawn that leaf protein isolates can be considered as rich sources of dietary protein, with methionine as the limiting amino acid.

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Factors Influencing the Caffeine Content of Black Tea: Part 2—The Effect of Production Variables

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ABSTRACT

Caffeine content changed throughout the various stages of black tea production. The large increase during the withering stage was time dependent, but independent of rate and degree of desiccation. The decrease in caffeine levels during fermentation was related to the time and temperature of fermentation. Minor amounts of caffeine were lost by sublimation during the drying process. Tea stored for 6 months contained more caffeine than the freshly manufactured product. The importance of these changes in caffeine levels with respect to the quality and character of the beverage is discussed.

INTRODUCTION

The black tea of commerce is produced from the young shoots harvested from the perennial shrub *Camellia sinensis* (L.) O. Kuntze. There are four distinct stages in the conventional manufacture of the product (Harler, 1963; Eden, 1965). The fresh green leaf is partially desiccated, or 'withered', as the process is termed throughout the industry. The leaf material is then mechanically comminuted and the tissue and cell structure disrupted. During the so-called 'fermentation' phase of manufacture a series of partially controlled enzymic and chemical reactions occur which produce the pigments and taste characteristics associated with the beverage. Fermentation is arrested after a suitable period, and the product is made stable for transit and storage, by drying. Although

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the reactions involved during each stage of manufacture have not been completely characterised, changes in the levels of several important classes of substance have been studied: polyphenols (Roberts, 1962; Sanderson *et al.*, 1976), oxidase enzymes (Takeo, 1966; Cloughley, 1980*a*), amino acids (Choudhurry, 1978), carotenoids (Venkatarishna *et al.*, 1976) and the volatile constituents (Howard, 1978). Since caffeine makes an important contribution to the character of the beverage (Wood & Roberts, 1964; Sanderson *et al.*, 1976; Cloughley, 1982), the present investigation examines the changes in the levels of the alkaloid during the various stages of manufacture. The effect of altering the standard operating procedures during the critical wither and fermentation phases of production is also studied.

MATERIALS AND METHODS

All the leaf used throughout these investigations was manually harvested ('plucked') before 07.00 h from populations of tea bushes cultivated according to established agronomic practices at the Nsuwadzi Tea Research Station. The standard of plucking was that practised on the local tea plantations (Cloughley, 1982). The freshly plucked shoots were transferred to the Research Station pilot-scale production plant, where black tea was manufactured by a standard procedure representative of commercial production in Central Africa.

Standard conditions of manufacture

The leaf material was partially desiccated in an induced current of air at ambient temperature and relative humidity, over a period of 20 h, so that 70 % of initial fresh weight remained. In the tea industry this is referred to as a 70 % wither and the operation is an essential prelude to processing the leaf. Batches (5 kg) of withered leaf were comminuted by a dual processing system widely used in the local industry. The leaf was pre-conditioned in a rotorvane unit and then passed through a series of three machines termed CTC's after their crushing, tearing and cutting action (Werkoven, 1974). The macerated leaf mass (the 'dhool') was spread over a concrete surface to a uniform depth of 2·5 cm and allowed to ferment for 60 min in an air-conditioned room where the temperature varied in the range 22–23 °C. Formal fermentation was arrested by drying the dhool (to

a moisture content of 3 %) in a tray drier designed to simulate the multi-stage models used throughout the industry. Fibre was extracted from the dried leaf and black tea was sieve-sorted to obtain a particle size range approximately corresponding to the commercial grade termed Orange Fannings (Werkoven, 1974). The black tea was stored in perforated bags within the bulk of tea in a standard tea chest for 6 months, this period representing the average time taken for Malawi teas to reach the London auction floors (Cloughley, 1981). In an experiment conducted in February, 1979 samples (100 g) of MT12 and SFS204 were taken after each stage of the standard manufacture and analysed for caffeine. Caffeine from fresh leaf was extracted (Ogutuga & Northcote, 1970) and determined by the method previously described (Cloughley, 1982). All caffeine levels were expressed on a dry mass basis.

In the series of experiments described below the withering and fermentation stages of manufacture were varied with respect to one factor, while the standard conditions were maintained in each other stage.

Withering

Three experiments were devised to vary the degree, the duration and the temperature of the withering operation.

Three batches of freshly plucked leaf of each of the two clones MT12 and SFS204, were withered in separate withering troughs under controlled conditions of air flow and temperature over a period of 18 h so that one batch of each clone was given a 80 %, a 70 %, or a 60 % wither. The ambient temperature and relative humidity of the desiccating air was the same for each and the required rate of water loss was obtained by varying the velocity and volume of the air supply. A fourth batch of each clone from the same harvest was stored in a polythene bag over the same time period. This served as a non-wither control.

A second experiment was conducted on the same clonal leaf and also on Indian hybrid material, where the same degree of wither was achieved over different time periods. A 70 % wither was obtained in 10, 14, 18, 22 and 30 h by manipulating the air supply to the different troughs and taking regular samples to monitor the rate of moisture loss. Batches of fresh leaf were also stored in polythene bags for the same range of time periods.

Batches of SFS204 leaf were given a standard 70 % wither over periods of 8 and 18 h at each of three temperatures—15 ± 1·5 °C, ambient

(21–25 °C) and 35 ± 2 °C. Cooling and heating elements were introduced into the air supply system to maintain the required temperatures.

Fermentation

Batches (5 kg) of comminuted leaf of each of four clones, MT12, SFS371, MFS76 and SFS204, were allowed to ferment in rectangular metal boxes with perforated base plates over a plenum chamber supplying a forced-current of air. The dhool was fermented for periods of 30, 45, 60, 75 and 90 min at ambient temperature (22–25 °C). Samples (100 g) of dhool were also taken at 15 min intervals from each of three boxes of SFS204 fermenting at 15 ± 2, 25 ± 1·5 or 35 ± 2 °C.

RESULTS AND DISCUSSION

Changes in caffeine content during standard manufacture

It can be seen from Fig. 1 that the caffeine levels in both clones undergo marked changes during each stage of tea manufacture and during the

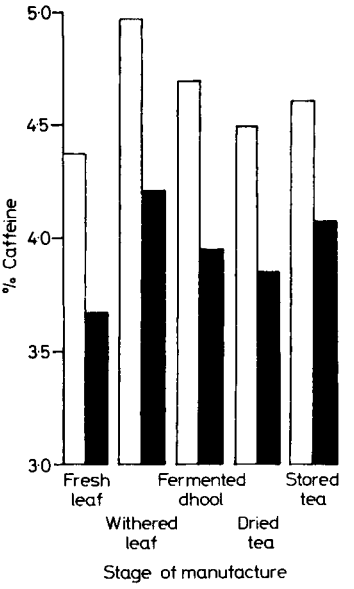

Fig. 1. Effect of each stage of production on caffeine content (% dry weight). Shaded bars represent clone SFS204, unshaded bars, MT12.

subsequent storage of the product. The largest change occurred during the withering phase, where the caffeine content of MT12 and SFS204 increased by 17·8% and 20·6%, respectively, compared with the levels contained in the fresh leaf. During the fermentation and drying stages, however, caffeine levels were progressively reduced. For both clones the magnitude of the reduction during fermentation was greater than the loss introduced by drying. It was also found that caffeine levels had increased after the 6-month storage period, particularly in the tea made from SFS204. The net effect of these changes, including those occurring during the post-manufacture period, was that the caffeine content of the teas made from MT12 and SFS204 was greater than that present in the unprocessed leaf material by about 7·0% and 15·6%, respectively.

The post-harvest increase in caffeine content of tea shoots has been demonstrated (Wood & Chandra, 1955; Sanderson, 1964), but changes occurring in the other phases of commercial manufacture have not been reported previously. It is evident from the present work that the decrease in caffeine levels observed in the fermentation phase and the further decline during drying, taken together, would largely account for the additional caffeine produced during the wither period. The fate of the caffeine lost during fermentation is unknown, but, since the interaction of caffeine with polyphenols has been established (Smith, 1968; Collier *et al.*, 1972), it is likely that the alkaloid is complexed with the fraction of the non-dialysable thearubigin fraction, which becomes insolubilised during fermentation (Millin *et al.*, 1969; Sanderson *et al.*, 1976; Cloughley, 1980*b*). This would have the effect of reducing the proportion of caffeine in solution when the infusion is prepared and thus of hindering the extraction. Clonal differences in the decrease in caffeine content probably reflect genetic differences in the type and molecular weight distribution of the polyphenols.

The relatively minor loss during drying is likely to be caused by sublimation and it is known that caffeine deposits are found on the roofs of commercial driers (Ramaswamy, 1958). Since the temperatures used in the recently introduced fluidised-bed system (Kirtisinghe, 1974) are considerably higher than those in the conventional endless-chain driers, it is likely that relatively greater amounts of caffeine are lost by evaporation. Indeed, preliminary work comparing the two drying systems has shown this to be the case (J. B. Cloughley, unpublished results).

The apparent increase in caffeine levels during storage is related to the loss of the theaflavin class of pigments (Cloughley, 1981). The complex

formed between caffeine and the theaflavins is broken down as the latter is degraded and the free caffeine is then readily available for extraction. The increase in the concentration of uncomplexed caffeine in the tea infusion is likely to contribute to the poor quality of stored tea, since free caffeine has a pronounced bitter taste.

Effects of varying the standard withering procedure

The degree of moisture loss achieved during the standard wither period at ambient temperature has a negligible effect on caffeine content (Table 1). The rate of caffeine production, then, was independent of the degree of physical wither. It can be seen, however, from Fig. 2 that caffeine content is dependent upon wither period. Caffeine levels increased as a linear function of time of post-harvest storage. In preliminary work it was observed that caffeine continued to be formed over a 60-h storage period,

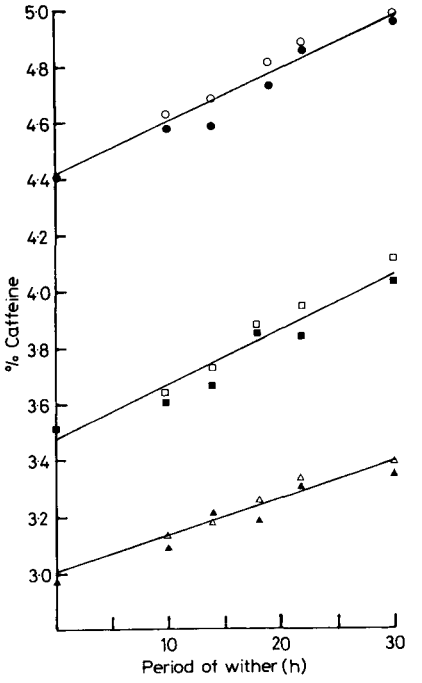

Fig. 2. Effect of time of post-harvest storage on caffeine content (% dry weight). ●, Unwithered leaf of MT12, ○, withered leaf of MT12; ■, Unwithered leaf of SFS204; □, withered leaf of SFS204; ▲, Unwithered leaf of Indian hybrid; △, withered leaf of Indian hybrid.

TABLE 1
Effect of Degree of Wither on Caffeine Content (% Dry Weight) of Black Tea Produced from Two Clones

Wither (%)	*Clone*	
	MT12	*SFS204*
Fresh leaf	4·58	3·72
80	4·65	3·69
70	4·68	3·78
60	4·60	3·85

but such a prolonged wither is remote from commercial practice and in any case would have pronounced deleterious effects on other aspects of tea quality (Sanderson, 1964). For the three varieties of tea shoots considered, the increase in caffeine content over the 30 h wither period was similar—14·3%, 14·0% and 16·5%, respectively, for Indian hybrids MT12 and SFS204.

The effect of temperature on the production of caffeine during the standard storage period of green leaf is shown in Table 2. Higher levels of caffeine were obtained at ambient temperature than at either 15 or 35 °C. The formation reactions are likely to be temperature dependent up to temperatures approaching 35 °C, when thermal denaturation of the enzyme system responsible for the conversion of purine nucleotides to caffeine (Ogutuga & Northcote, 1970) would limit the rate of reaction. It is noteworthy that the caffeine content of the tea withered for 8 h at the elevated temperature is much lower than that produced by standard conditions. The suggestion has been made within the local tea industry that the wither period could be reduced by the use of above ambient air

TABLE 2
Effect of Temperature of Wither on the Caffeine Content (% Dry Weight) of Tea Made from SFS204

	Period (h)	
	8	*18*
Temperature		
15 °C	3·21	3·31
Ambient	3·46	3·66
35 °C	3·34	3·42

TABLE 3
Caffeine Content (% Dry Weight) of Four Clonal Teas as a Function of Fermentation Time

Fermentation time (min)	*Clones*				
	MT12	*SFS371*	*MFS76*	*SFS204*	*Mean*
0	3·46	3·22	3·18	2·90	3·20
30	3·24	2·98	3·14	2·86	3·06
45	3·18	2·95	3·05	2·79	2·99
60	2·99	2·90	2·83	2·81	2·88
75	2·96	2·75	2·81	2·69	2·80
90	2·96	2·60	2·84	2·54	2·74

temperatures. The required physical wither could easily be obtained by this means, but the desirable time-dependent chemical changes, of which caffeine formation is one (Sanderson, 1964), must be allowed to proceed if the full quality and value of the product is to be realised.

Effects of varying the time and temperature of fermentation

There was considerable reduction in caffeine levels throughout the 90-min fermentation time and the magnitude of the rate of loss varied with clone

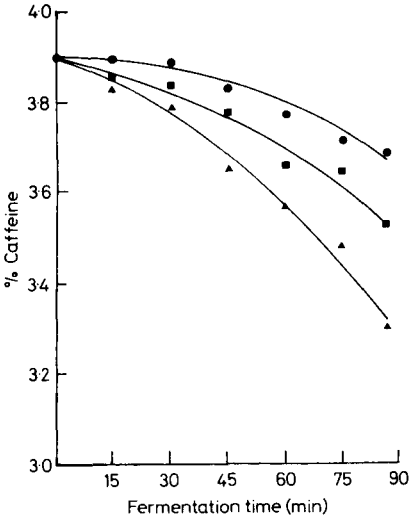

Fig. 3. Effect of the time and temperature of fermentation on caffeine content (% dry weight). ●, 15°C; ■, ambient; ▲, 35°C.

(Table 3). It can be seen from Fig. 3 that the rate of this loss of caffeine during fermentation was temperature dependent. Approximately 14% of the caffeine content of the unfermented leaf had been lost during the 90-min fermentation period at 35 °C compared with only 5·5% at 15 °C. This temperature effect may be related to the increase in the proportion of high molecular weight thearubigins and the concomitant decrease in soluble solids observed during high temperature fermentation (Cloughley, 1980*b*). More caffeine would thus be retained by the insolubilised polyphenol complex associated with the infused leaf and hence the amount of caffeine in the infusion prepared from the tea fermented at high temperature would be lower than that fermented at 15 °C.

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Researches on the Utilisation of the Pigment from *Phytolacca decandra* L. as a Food Colorant: Part 1— Preparation of an Extract Free from Toxic Substances

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ABSTRACT

The problems examined in this paper concern the extraction of the pigment from the berries of the American Pokeberry Phytolacca decandra (americana) *L., which contain a large quantity of red pigment, 'Phytolaccanin', a betacyanin identical to the Betanin of beetroot. A purification procedure was carried out which foresaw the removal of the toxic saponin present in the extract. The extraction was done by pressing juice from berries in two stages followed by separation and washing of residue.*

The raw pigment was purified, after removing the saponin by precipitation with sulphuric acid and following liquid–liquid partition with butanol and diethyl ether. A yield of 87% of phytolaccanin was achieved.

INTRODUCTION

Now that some synthetic red colorants have been banned due to their recognised harmfulness, the use of natural plant pigments is presented as a valid alternative to synthetic ones.

Betanin, E162, also called beetroot red from the name of the plant from which it normally comes, is among the natural pigments accepted by the law in force.

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This pigment, whose structure was elucidated by Wyler & Dreiding (1957, 1959) and Wilcox *et al.* (1965), has been the object of numerous researches concerning its application as a food colourant (von Elbe *et al.*, 1974; von Elbe, 1975; Pasch & von Elbe, 1975, 1979; Adams *et al.*, 1976; Dhillon & Maurer, 1975; Georgakis & Vareitzis, 1976; Kopelman & Saguy, 1977; Savolainen & Kuusi, 1978). The chemistry and applications of betanin were recently reviewed by Harmer (1980).

The berries of *Phytolacca decandra* contain a large quantity of a pigment named 'Phytolaccanin', that has been recognised as identical to the Betanin of beetroot (Wyler & Dreiding, 1961; Piattelli & Minale, 1964). The plant belongs to the Phytolaccaceae family; it is an herbaceous plant, perennial, of a variable height of 1 to 3 m, is common in country areas and is uncultivated. The flowers are whitish and grouped in bunches, turning into fruit in the autumn, forming a fleshy berry of a red–purple colour rich in pigment.

In the berries, especially in the seeds, some saponins, called 'phytolaccatoxins', are present. Their structure was identified by Stout *et al.* (1964) as glycosides of 'phytolaccagenin', a β-amirin triterpenoid derived from oleanolic acid (Boar & Allen, 1973) (Fig. 1).

From research by Johnson (1973) it is known that the toxic effect is due to a series of components in a butanol extract, while others (as the aglycones) are harmless. This research shows, however, that the toxic action is attributed solely to the saponin present in the seeds.

The practice of using the juice of the berries of pokeweed as a food colorant goes back to the end of the last century with the idea of enhancing the colour of red wine, but, since this practice was considered

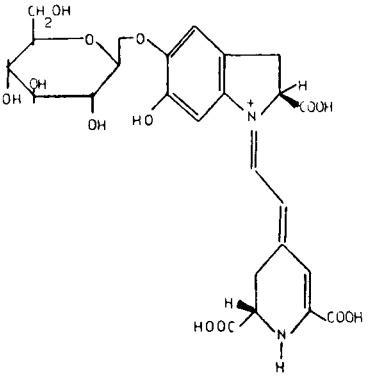


Fig. 1. Phytolaccagenin.

to be adulteration, it was forbidden. The presence of the toxin, however, has prevented the use of pokeweed berries as a food colorant (Clydesdale & Francis, 1976). Driver & Francis (1979*a*) have recently been successful in the preparation of a pigment free from saponin, using a process based on a cold clarification by HCl treatment at pH 3 of pressed juice, followed by separation and liquid–liquid partitions in hexane–acetone, diethyl ether–butanol and petroleum ether until the removal of 97% toxin was achieved.

This purified product was tested for stability and for colouring dessert gels in comparison with Betanin from beetroot (Driver & Francis, 1979*b*).

Russian researchers (Abutalybov *et al.*, 1977) patented a very simple procedure for the preparation of a colouring extract from *Phytolacca* for food use which, however, does not seem to take into account the complete removal of the saponin.

The purpose of the present research is to evaluate the possibility of extracting the pigment of pokeweed berries and of removing the toxic saponin in a simple and accurate way, so as to prepare a safe product suitable as a food colorant.

MATERIALS AND METHODS

Extraction tests

The juice of *Phytolacca decandra* was prepared from mature berries picked in autumn. The clusters were taken off and the berries were frozen and stored at −30 °C. Acidified water at pH 4·5 with citric acid was used as an extracting solution.

The influence on the yield of the pigment extraction of the following operative conditions (Table 1) were considered:

(a) Minimum duration of the contact for the complete extraction of the pigment.
(b) Best ratio solvent volume/berry weight.
(c) Best extraction temperature.
(d) Extraction at three stages.

The berries were mildly pressed to avoid breaking the seeds, then mixed with acidified water at pH 4·5 with citric acid and agitated for the predetermined time at the predetermined temperature.

TABLE 1
Extraction Conditions Examined

Contact time (min)	*Solvent volume: berry weight ratio*	*Temperature (°C)*	*Multistage extraction*
15	2:1	20°	$t = 20$ °C
30	5:1	40°	Extraction time = 3 × 10 min
60	10:1	60°	Ratio, Solvent:berries = 25:1
120	25:1		Stages = 3
180			

Three 5-ml samples were taken and centrifuged for 15 min at 6000 rpm. One millilitre was diluted to the volume corresponding to a concentration of 0·2 g/litre of berries for all the samples.

The pigment concentration was expressed in optical density (OD) at a wavelength of 538 nm.

Removal of the phytolaccatoxins

The procedure is based on the low solubility of the saponins in an acid medium because of the association of carboxylic groups; on the other hand, the phytolaccanin, having an amphoteric behaviour, is soluble in the same pH range. This fact allows the removal of saponin from the juice, as well as other substances present, such as pectins, polyphenols, etc.

The juice extracted was cooled to 5 °C and concentrated H_2SO_4 was added to reach pH 1 and, after 5 mins was centrifuged at 6000 rpm for 15 min; the residue was discarded and to the supernatant was added $Ba(OH)_2$ to pH 5. After the separation of $BaSO_4$ by centrifuging, two liquid–liquid partitions were carried out on the solution in a separating funnel; the first in three stages with butanol (ratio juice: butanol, 1:1) and the second with diethyl ether under the same conditions.

A flow diagram of the extractions and purification procedure is shown in Fig. 2.

The presence of the saponins was evaluated by T.L.C. in the initial juice, in the residue after precipitation with sulphuric acid and in the solution, in the butanolic and diethyl ether phases after partitions and in the final product.

Evaluation of the phytolaccatoxins

(*a*) *Thin layer chromatography*

5 μl of the solution, concentrated ten times at reduced pressure with a rotating evaporator, were loaded on a Silica Gel H chromatoplate (Merck ready to use plates), 0·25 mm thick and developed with chloroform–methanol–water mixture (65:35:10) for 2 h at room temperature.

The saponins were detected by spraying with Liebermann-Burchardt's reagent (Stahl, 1969) and semiquantitatively evaluated according to the size of the spots, in comparison with the standard of saponins prepared from *Phytolacca* as referred to below.

(*b*) *Preparation of the standard saponin*

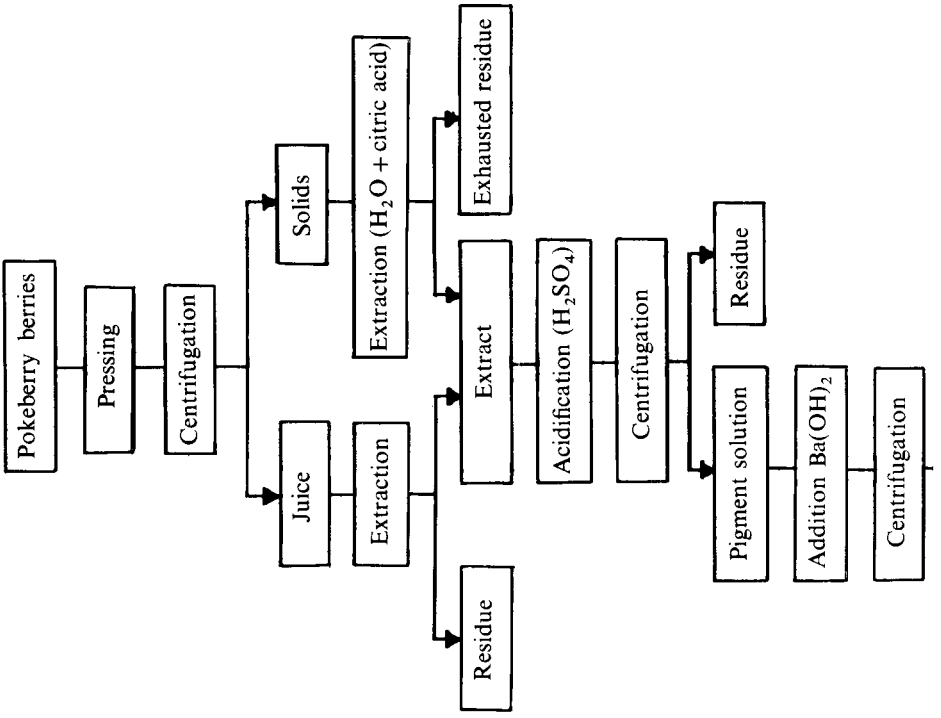
The extraction of phytolaccatoxins from pokeberry seeds was carried out according to the method of Stout *et al.* (1964) which consists of a two-stage extraction in a Soxhlet apparatus first with petroleum ether and then with acetone, followed by precipitation of the saponins with 10% (v/v) H_2SO_4 and chromatography on column of Florex XXS eluting with acetone–ethanol (4:1) and collecting the fractions absorbing at 204–206 nm.

The purification of the saponins recovered was done by means of preparative layer chromatography (PLC).

After concentration to a small volume, the saponin fractions eluted from the Florex XXS column were loaded on a preparative chromatoplate of Silica Gel G, 2 mm thick, and activated at 105 °C for 1 h.

100 μl of the concentrated solution was loaded in continuous lines on each plate. For the detection, 10 μl of the same solution and 10 μl cholesterol alcohol solution (as a detection reference substance) were separately loaded along the start line at the side of the solution being tested.

The chromatogram was developed in about an hour and a half with chloroform–methanol–water (65:35:10). After evaporation of the developing mixture and covering the preparative area of the plate with a glass sheet, the detection area was sprayed with the Liebermann–Burchardt reagent (Stahl, 1969). The saponin spots showed up after heating for 10 min at 100 °C and were identified by their fluorescence under UV light at 366 nm. The bands at the same R_f as those of the reference were scraped off the plate and eluted with the minimum possible amount of ethanol filtering through a sintered glass filter.


Pokeberry berries
Pressing
Centrifugation
Juice
Solids
Extraction
Extraction (H_2O + citric acid)
Residue
Extract
Exhausted residue
Acidification (H_2SO_4)
Centrifugation
Pigment solution
Residue
Addition $Ba(OH)_2$
Centrifugation

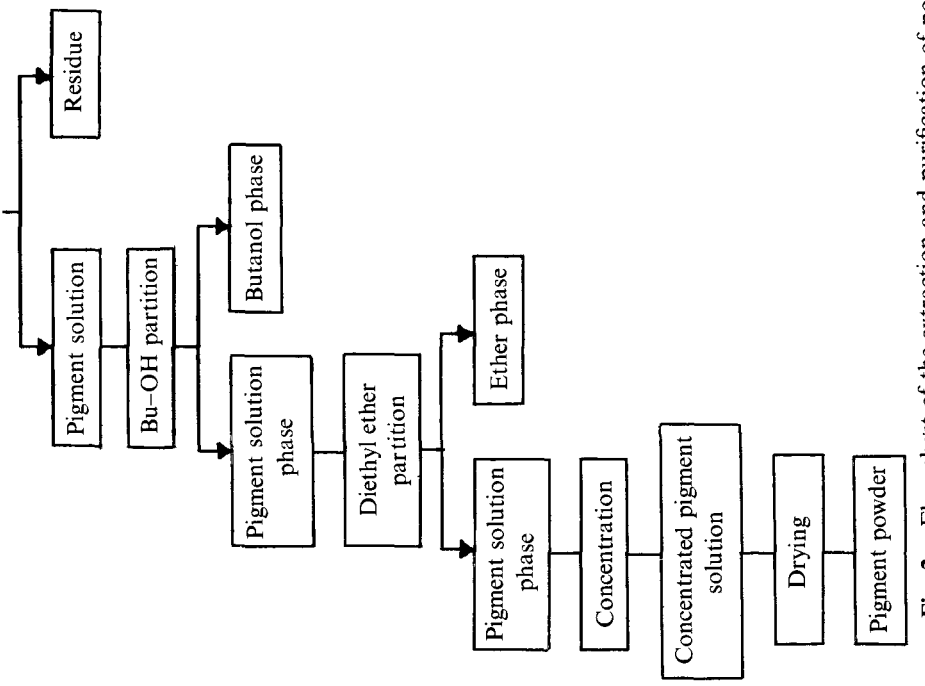


Fig. 2. Flow sheet of the extraction and purification of pokeberry pigment.

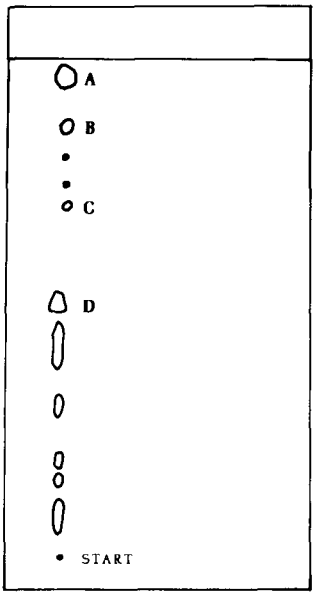


Fig. 3. TLC of saponins from pokeberries: A, B = saponin aglycons; C = xylosyl–phytolaccatoxin; D = glucosyl–xylosyl– phytolaccatoxin.

The different fractions extracted were examined spectrophotometrically, then concentrated and re-chromatographed for further purification.

The chromatogram obtained is shown in Fig. 3.

The separation obtained conforms with the description given by Johnson (1973); in particular, R_f values for the xylosyl–phytolaccatoxin and for glucosyl–xylosyl–phytolaccatoxin, the two components attributed the greatest toxicity, were 0·53 and 0·73, respectively, against 0·50 and 0·68 quoted by him.

(c) Determination of the phytolaccanin

To evaluate the pigment yield, the phytolaccanin present in the berries and in the final product was evaluated by spectrophotometry. The solution of the pigment extracted by acidified water with citric acid at pH 4·5 was appropriately diluted and read at 538 nm in a 1 cm long cuvette. From the OD the concentration was calculated by using $E^{1\%}_{1cm}$ 538 nm = 1120 (Wyler *et al.*, 1959).

(d) Analytical determinations on the juice

Dry matter, ash, free acidity, total sugar and total nitrogen contents were determined according to the official methods of the Italian Ministry of

Agriculture and Forestry (1961), total polyphenols according to Singleton & Rossi (1965) and total lipids according to Bligh & Dyer (1959).

RESULTS

Extraction tests

Figure 4 shows the effect of the different conditions on the extraction. The following observations were made.

(a) With regard to the extraction time, almost all the pigment was obtained after 30 min. The same time was used in successive tests.

(b) The best solvent:berry ratio seems to be 2:1. In fact, the slight improvement of the yield obtainable with a higher ratio does not compensate for an eventual loss of pigment that could take place over the longer time necessary to evaporate large amounts of solvent, during the concentration of the extract.

(c) The increase in temperature does not improve the yield; on the contrary, it tends to lower it, because heating may give rise to a decay of the pigment.

(d) The extraction in three stages gave an OD of 0·530 equal to that obtained from a single stage: OD = 0·527.

Removal of the toxins

The semiquantitative evaluation of toxic saponin on TLC showed that, with the procedure adopted, about 80% of the total triterpenoids was

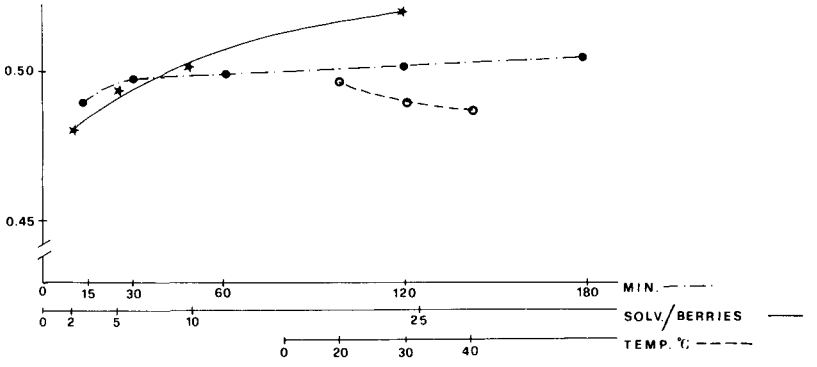


Fig. 4. Extraction conditions tests. Ordinate:absorbance.

TABLE 2
Compositions of *Phytolacca decandra* Berries and Pigment Concentrate

	Berry	*Concentrate*
pH	5·9	5
Dry matter (DM) (%)	18	70
Ash (% DM)	5·2	28·0
Free acidity (% DM) (as citric acid)	1·2	0·9
Total sugars (% DM)	36·7	49·6
Total polyphenol (% DM)	4·7	0·2
Total lipids (% DM)	15·0	—
Total nitrogen (Kjeldhal) (% DM)	2·6	Traces
Phytolaccanin (% DM)	1·4	12·3

removed by precipitation with sulphuric acid, and the remaining 20 % was further extracted by the butanol phase. The removal of the saponins was therefore complete after the second stage of the process.

Table 2 shows the composition of the juice of the pokeweed berries and of the obtained pigment concentrate. The final yield of pigment was 87 %. The product can be stored as a concentrate of 70 ° Bx in a refrigerator at 5 °C without discoloring or can be dehydrated and vacuum sealed.

DISCUSSION

(1) From the results obtained, all the extraction conditions tested gave nearly the same yield of pigment. In fact, most of the pigment is found to be dissolved in the juice and so there are not any limiting phenomena of diffusion through the cell walls.

This fact is supported by observing the extraction time; within 15 min, an almost exhaustive recovery of the pigment was reached. This is of practical notable interest as the chromophore appears to be more easily extractable in respect to that of beetroot red, where the quantity found is lower.

The use of high solvent volume:berry weight ratios allows one to obtain a high yield, getting a more complete exhaustion of the skins of the berries. However, excessive volumes of the extract make the successive

stages of processing more laborious, with the danger of losing some of the pigment during the concentration process.

The best technique for the exhaustion of the material without turning to a large amount of solvent seems to be the centrifuging of the berry skins after the berries have been broken followed by washing with the extracting mixture.

Regarding the extraction temperature, it is interesting to see that it has no influence on the diffusion of the pigment; instead, it activates the bleaching of the chromophore, accelerating the disappearance of the colour.

(2) The quantitative elimination of saponins from the juice was obtained by precipitation of the largest part with concentrated sulphuric acid followed by their complete removal by partition with butanol. The extraction and purification process shown in Fig. 2 therefore seem proposable.

With the toxicological problem resolved, the extraction of the phytolaccanin from the berries of *Phytolacca decandra* for food use can be considered as a practical application, since the procedure does not require a complicated extraction technique and separation of the substances present in the juice. In fact Table 2 shows that the pigment is present in high concentration in the juice and this does not give rise to serious problems of enrichment since for practical use, it is sufficient to have a harmless product even if it is not chemically pure.

Moreover, the commercial products of beetroot red contain, on a dry weight basis, a maximum of 1% Betanin (Pasch *et al.*, 1975) or a maximum of 8% if obtained by enrichment after removing sugars by aerobic fermentation (Adams *et al.*, 1976). For these reasons the phytolaccanin can also be used parallel to the Betanin of beetroot. Aspects of the use and behaviour of the product obtained will be examined in the second part of this paper.

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Polyhydroxy Flavonoid Antioxidants for Edible Oils. Structural Criteria for Activity

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ABSTRACT

The antioxidant properties of a series of polyhydroxy flavonoids and related compounds have been evaluated. The results have been correlated with the structures of the compounds concerned, which have been shown to function mainly as primary antioxidants. The ability of some such compounds to form complexes with copper has been demonstrated from a study of UV spectra and is probably a contributing factor to the stabilising effects of such compounds.

Antioxidant activity is favoured by a multiplicity of phenolic hydroxyl groups and depends critically on the co-operation of the 4-carbonyl with either the 3- or the 5-hydroxyl groups. Dihydro-flavones are slightly more active than the corresponding flavones.

INTRODUCTION

Polyhydroxy flavonoid substances are recognised as of importance in conferring stability towards autoxidation on the lipids of vegetable tissues (Herrmann, 1976; Hudson & Mahgoub, 1980, 1981). Several attempts have been made to relate their antioxidant activities to chemical structure (e.g. Simpson & Uri, 1956, 1961; Mehta & Seshadri, 1959; Crawford *et al.*, 1961; Letan, 1966*a*, *b*). Pratt (1976) reviewed and further developed these studies, but despite this considerable volume of data the correlation between antioxidant activity and chemical structure remained far from clear. In part this appears to be due to the use by different workers of

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different methods of assessment, different substrate systems and different concentrations of active antioxidants. The situation is further confused by the fact that many of the more active polyhydroxy flavonoids are bimodal in function, possessing both primary and secondary (synergistic) antioxidant activity, and these two effects are not always clearly distinguishable from each other.

We considered that it was important to clarify the situation since it might then be possible to select appropriate natural antioxidants of the flavonoid class for use in food systems, and to draw attention to directions in which even more active compounds, embodying both primary and secondary functions, might be sought.

The studies here reported quantify both primary and secondary antioxidant properties in a restricted but strictly comparable series of flavonoids. Secondary activity is related to metal-complexing capacity through UV spectrophotometric studies. Trace metals act as pro-oxidants in natural glyceride oils. Hence, our studies centred around the formation of complexes with the most active of these, copper.

MATERIALS AND METHODS

Materials

Lard, used as a substrate for all the stability tests, was donated by Messrs Scot Bowyers Ltd., Trowbridge, Wilts, Great Britain. It was unrefined, had not been chemically processed and was free from added antioxidants.

The less accessible flavonoid and related compounds, 3- and 5-hydroxy flavones, fisetin, luteolin, fustin, eriodictyol and butein, were obtained from Apin Chemicals Ltd., Cardiff, Wales, Great Britain.

The tocopherol used for the evaluation of synergistic effects was DL-α-tocopherol, kindly donated by Roche Products Ltd.

Methods

UV spectra were determined in ethanolic solution, using a Unicam SP800 recording spectrophotometer.

Induction periods, as a measure of stability towards autoxidation, were determined in a Metrohm Rancimat. This is an automated instrument that has recently been developed for the evaluation of the stability of oils and fats. Operating within the range 100–140 °C, it continuously monitors the

conductivity of an aqueous solution from the volatiles emerging from heated test material through which is passed a controlled air stream. After a classical induction period, the conductivity of the aqueous solutions increases rapidly as a result of increasing concentrations of dissolved volatile acids.

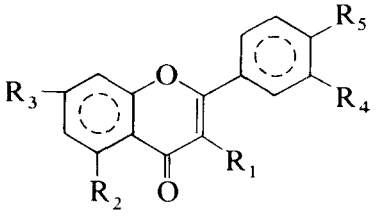
Synergism was quantified by the method of Bishov and Henick (1972), using the formula:

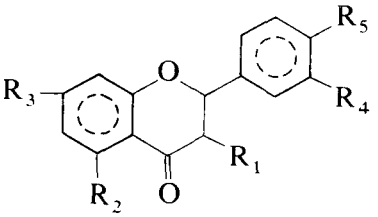
$$\% \text{ Synergism} = 100\left[\frac{(I_M - I_L) - (I_A - I_L) - (I_S - I_L)}{(I_M - I_L)}\right]$$

Where: I_L = Induction period of the substrate (in this case, lard).
I_A = Induction period of substrate plus primary antioxidant.
I_S = Induction period of substrate plus synergist.
I_M = Induction period of substrate plus primary antioxidant plus synergist.

RESULTS AND DISCUSSION

The following flavonoids and related substances were studied.



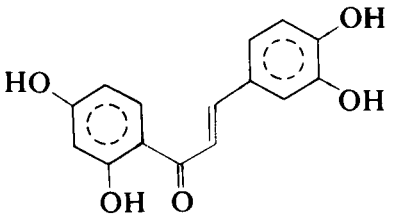


Flavones

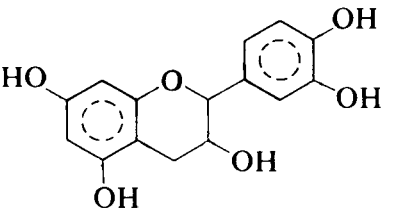
3-Hydroxyflavone:
$R_1 = OH, R_2 = R_3 = R_4 = R_5 = H$
5-Hydroxyflavone:
$R_2 = OH, R_1 = R_3 = R_4 = R_5 = H$
Fisetin:
$R_1 = R_3 = R_4 = R_5 = OH, R_2 = H$
Quercetin:
$R_1 = R_2 = R_3 = R_4 = R_5 = OH$
Luteolin:
$R_2 = R_3 = R_4 = R_5 = OH, R_1 = H$
Quercitrin:
$R_2 = R_3 = R_4 = R_5 = OH, R_1 = ORha$

Flavanones

Fustin:
$R_1 = R_3 = R_4 = R_5 = OH, R_2 = H$
Taxifolin:
$R_1 = R_2 = R_3 = R_4 = R_5 = OH$
Eriodictyol:
$R_2 = R_3 = R_4 = R_5 = OH, R_1 = H$



Chalcone
Butein



Flavan
Catechin

Spectrophotometry

UV and visible spectra in ethanolic solutions were recorded both for the flavonoids and for solutions containing equimolar concentrations of flavonoids and Cu^{++} ions. By comparing λ_{max} and ε values in the absence and presence of Cu, it was possible to determine whether or not complexes were being formed. Characteristic spectral data are shown in Table 1. Only λ_{max} values above 280 nm are quoted since lower maxima relate only to the isolated aromatic rings, not to interactions between the rings or to complex formation.

All the flavonoids listed exhibit strong maxima in the range 328–402 nm. The absence of such a maximum in catechin shows that these are due to the interaction of the 4-carbonyl with the adjacent aromatic ring, enhanced, in the flavones butein and quercitrin, by conjugation with the 2:3-double bond.

Ligand complex formation with Cu^{++} is monitored by (1) a fall in ε at 700 nm, the maximum for the blue solvated Cu^{++} ion and (2) a shift in the principal λ_{max} of the flavonoids. The flavones (and butein) show a bathochromic shift of from 56 to 76 nm due to ligand formation involving the co-operation of Cu^{++}, 4-CO and 3-OH or 5-OH. The flavanones, on the other hand, show a hypsochromic shift of from 28–51 nm. Evidently, in both cases, as observed by the abolition or major reduction in absorbance at 700 nm, complexes must have been formed, although they appear to be of different structures. Typical spectra, for quercetin and taxifolin, are shown in Figs 1 and 2.

Ligand formation with the 3-OH and 5-OH flavones is weak, as there is appreciable survival of absorption at 700 nm: of the two, 3-hydroxy flavone forms a stronger complex. With catechin there is little complex formation, owing to the absence of CO: the minor fall in absorbance at

TABLE 1
Spectrophotometric Data for Flavonoid Compounds and their Copper Complexes

Flavonoid compound	*In ethanol*				*1:1 Cu complex* in ethanol*			
(1) *Flavones* (F)	λ_{max}	*Abs*	*Molarity*	ε	λ_{max}	*Abs*	*Molarity*	ε
3-Hydroxy F	306	1·20	10^{-4}	12 000				
	344	1·62	10^{-4}	16 200	414	1·00	5×10^{-5}	20 000
					700	0·76	10^{-2}	76
5-Hydroxy F	335	0·36	5×10^{-5}	7200	410	0·28	5×10^{-5}	5600
					700	0·98	10^{-2}	98
Fisetin	320	0·62	5×10^{-5}	12 400				
	362	1·16	5×10^{-5}	23 200	438	0·92	5×10^{-5}	18 400
					700	—	—	—
Quercetin	297	0·92	$1·5 \times 10^{-4}$	6133				
	378	1·70	$1·5 \times 10^{-4}$	11 133	434	1·12	5×10^{-5}	22 400
					700	—	—	—
Luteolin	294	0·84	5×10^{-5}	16 800				
	350	1·64	5×10^{-5}	32 800	414	1·11	5×10^{-5}	22 200
					700	—	—	—
(2) *Flavanones*								
Fustin	285	0·58	5×10^{-5}	11 600				
	338	1·24	5×10^{-5}	24 800	287	0·84	5×10^{-5}	16 800
					700	—	—	—
Taxifolin	292	0·94	5×10^{-5}	18 800				
	330	1·25	5×10^{-5}	25 000	300	1·16	5×10^{-5}	23 200
					700	—	—	—
Eriodictyol	287	0·71	5×10^{-5}	14 200				
	328	1·12	5×10^{-5}	22 400	300	1·06	5×10^{-5}	21 200
					700	—	—	—
(3) *Others*								
Butein	386	1·52	5×10^{-5}	30 400	460	0·96	5×10^{-5}	19 200
					700	—	—	—
Quercitrin	402	0·81	5×10^{-5}	16 200	418	0·98	5×10^{-5}	19 600
Catechin	282	0·60	10^{-4}	6000	700	1·3	10^{-2}	130
*Copper cyclohexyl butyrate	—	—	—	—	700	1·96	10^{-2}	196

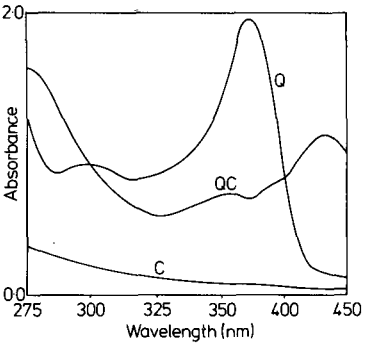


Fig. 1. UV absorption spectra of quercetin (Q, $1{\cdot}5 \times 10^{-4}$ M), its 1:1 Cu complex (QC, 5×10^{-5} M) and copper cyclohexyl butyrate (C, 10^{-4} M).

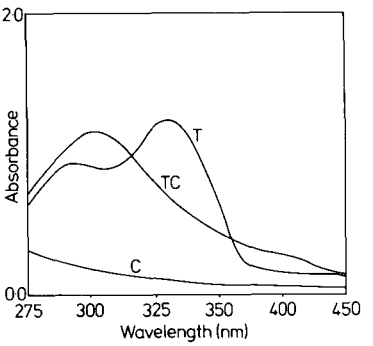


Fig. 2. UV absorption spectra of Taxifolin (T, 5×10^{-5} M), its 1:1 Cu complex (TC, 5×10^{-5} M) and copper cyclohexyl butyrate (C, 10^{-4} M).

TABLE 2

Effect of Added Flavonoid Compounds on the Stability of Lard at 100°C (Means of Duplicate Determinations)

Flavonoid compound	*At $2{\cdot}3 \times 10^{-4}$ M concentrations*			*At $2{\cdot}3 \times 10^{-3}$ M concentrations*		
	Induction periods (h)		*% Synergism*	*Induction periods (h)*		*% Synergism*
	Alone	*With 0·01% α-tocopherol*		*Alone*	*With 0·01% α-tocopherol*	
3-Hydroxy flavone	1·4	17·0	4	1·4	19·7	18
5-Hydroxy flavone	1·4	15·7	—	1·4	17·3	5
Fisetin	8·5	24·8	5	39·3	52·6	—
Quercetin	7·1	26·7	18	36·9	43·6	—
Luteolin	4·3	19·2	—	16·7	32·9	4
Fustin	6·7	27·8	23	81·5	72·4	—
Taxifolin	8·2	22·6	—	52·3	68·4	1
Eriodictyol	6·7	24·2	11	45·6	74·8	19
Butein	14·0	26·0	—	47·7	55·9	—
Catechin	1·6	14·4	—	2·8	15·5	—
Quercitrin	1·9	14·2	—	9·8	23·7	—

The induction period for lard without any additives was 1·4 h, and for lard with 0·01 % of added α-tocopherol, 16·4 h.

700 nm is probably due to interaction of Cu^{++} with the 3′:4′-vicinal hydroxy groups. Quercitrin also shows some absorption at 700 nm, which must be due to complex formation being confined to the 5-OH group.

Antioxidant effects

Induction periods of the flavonoids, as additives to lard at 100 °C at the $2{\cdot}3 \times 10^{-4}$ M and $2{\cdot}3 \times 10^{-3}$ M levels, are shown in Table 2. These data determine the extent of primary antioxidant activity. Since we are also concerned with secondary (synergistic) activity, in a second series of experiments α-tocopherol was added at approximately its optimum level of 0·01 %. Resulting induction periods are also included in Table 2. Synergism can be observed in several cases, when calculated by the method of Bishov & Henick (1972). From these data the following conclusions may be drawn.

3- and 5-hydroxy flavones have no primary antioxidant activity but, as could have been anticipated from their complex formation with Cu^{++}, there are signs of synergistic properties, especially with the former.

The polyhydroxy flavones, fisetin, quercetin and luteolin, show good primary antioxidant activity. Fisetin and quercetin, which contain the 3-OH group, are better than luteolin, which relies only on the 5-OH group. Quercitrin shows a significant synergistic effect with α-tocopherol at the lower level.

In the flavanones, activity of a similar order of magnitude to that of the flavones is evident. Taxifolin, especially at the higher level, is distinctly superior to its unsaturated analogue, quercetin, and eriodictyol to its corresponding analogue, luteolin. The superiority of fustin over fisetin is equally clear. As was shown from spectrophotometric studies, these compounds must form metal complexes of a different kind from those formed by flavones.

Catechin shows a very low order of primary antioxidant activity and no synergism. Quercitrin, in which, as compared with its aglycone, quercetin, the 3-OH group is not available for complex formation, still shows some primary activity, but no synergism.

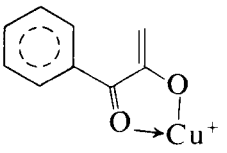
Butein, which can be regarded as an open-chain analogue of luteolin, is highly active. Presumably this is due to CO and 2-OH interaction. The 2-OH group is equivalent to the 5-OH of luteolin but, since butein is a much more flexible molecule, any complex formed will be subject to less strain and will therefore be more stable than the complex derived from luteolin.

CONCLUSIONS

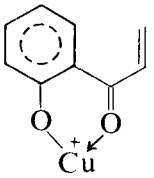
The data provided, taken with those of previous workers, indicate that optimum antioxidant activity in flavonoids is associated with the following structural features:

(1) Multiple phenolic hydroxyl groups, especially the 3′:4′-dihydroxy configuration.
(2) A 4-CO group.
(3) A free 3-OH group in preference to a 5-OH group, or 3- and 5-OH simultaneously present.

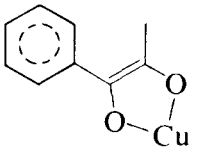
The forms of the various copper complexes are probably as follows:



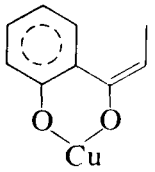
3-Hydroxy flavones



5-Hydroxy flavones



3-Hydroxy flavanones



5-Hydroxy flavanones

Flavones on this basis form ligands with Cu^{++} which are responsible for the bathochromic effect on their UV spectra. However, the complexes formed with the flavanones (dihydroflavones) arise from enediol or enolic groups. Such enediol complexes, produced by taxifolin and fustin, and the enolate produced by eriodictyol, account for the hypsochromic effect on the UV spectra of these flavanones. It is of interest to note that the enediol structure is reminiscent of that of ascorbic acid and that strong complexes can result from the acidic character of the hydroxyl groups involved.

It is not easy to relate our data precisely to those of earlier workers because of differences in test methods, substrates, antioxidant concentrations and temperatures. Some workers (e.g. Mehta & Seshadri, 1959) emphasise the need for a 2:3-double bond for antioxidant activity, but we have found the flavanones to be broadly at least as effective as the

flavones. Again, several reports (Simpson & Uri, 1956, 1961; Mehta & Seshadri, 1959; Letan, 1966*a*,*b*) stress the importance of the 3-hydroxy group. Whilst agreeing with previous work which showed that glycosylation (quercitrin versus quercetin) reduced activity, we noted in at least one case (eriodictyol) that absence of the 3-hydroxy group was not inimical to activity, in agreement with Crawford *et al.* (1961).

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Enzymatic Activities of Maxilact

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ABSTRACT

Nineteen enzymatic activities of Maxilact 20.000 have been assayed using the API ZYM micromethod. Some of them have been found to be positive and these have been studied in more detail. Hydrolysis of lactulose by the action of β-galactosidase is slower than that of lactose. Proteolytic activity has been found in both the soluble and insoluble fractions of Maxilact. Optimum temperature and pH are 45 °C and 7, respectively. The action of Maxilact on casein did not alter β-casein, but degraded α_s-casein to give a peptide with electrophoretic mobility similar to that produced by the action of rennet. A weak lipolytic activity on tributyrine was also detected.

INTRODUCTION

The development during the last few years of commercial processes for the isolation of β-galactosidase (lactase) from microbial sources has increased the possibilities of the utilisation of lactose. The hydrolysis of lactose results in several changes in its properties such as increase in solubility and sweetness. This allows its use as a sweetener and permits the preparation of a non-crystallising high solids concentrate for use in ice cream and other foods.

Commercial β-galactosidase preparations isolated from the yeast *Kluyveromyces lactis* (Maxilact®) are used in milk products to hydrolyse lactose. However, these preparations can also be active on other

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substrates which contain a β-galactosylic bond such as lactulose (4-*O*-β-D-galactopyranosyl-D-fructose). This sugar is obtained from lactose in yields higher than 80% (Hicks & Parrish, 1980). Lactulose could be further hydrolysed, resulting in a mixture of monosaccharides with a 50% content of fructose, giving a sweetness level higher than that of hydrolysed lactose.

A method of accelerating cheese ripening has been developed which involves the previous addition of Maxilact to milk (Thompson & Brower, 1976). Using this technique, it has been observed that there is an increase in proteolysis. Although some authors have attributed these effects to a higher availability of glucose for the active microorganisms (Weaver & Kroger, 1978) it could also be due to the presence in Maxilact of some types of proteolytic enzymes (Hemme *et al.*, 1978; Marschke & Dulley, 1978). The presence of contaminating proteolytic activity in Maxilact has recently been confirmed (Marschke *et al.*, 1980).

Considering the importance of the enzymatic action of Maxilact on cheese ripening, as well as its possible use for the hydrolysis of disaccharides other than lactose, we have studied some of the enzymatic activities of Maxilact such as the specific proteolytic action on caseins and hydrolytic action on lactulose.

MATERIALS AND METHODS

The lactase preparation used was Maxilact® 20.000 batch 154 (Gist-Brocades NV Delft, The Netherlands) which is a partly purified β-galactosidase from *Kluyveromyces lactis*. It was used in all experiments dissolved in potassium phosphate, pH 7.

Assay of activities

The API ZYM (API System: La Balue-les-Grottes 38390 Montalieu-Vercieu, France) system has been used. This is a semi-quantitative micromethod system designed for research that allows the study of nineteen enzymatic reactions (Schmidt *et al.*, 1979). The system consists of a strip composed of twenty microtubes, the bottom of which forms a support especially designed to contain the enzymatic substrate and buffer. Two drops of 1·0 g/litre of Maxilact solution were added to each cupule of the strip and incubation was carried out for 6 h at 45 °C. After

incubation one drop of each API ZYM reagent was added and colour was developed after 5 min.

β-Galactosidase activity

Solutions of 5% of lactulose or lactose containing 250–500 mg/litre of Maxilact were made with phosphate buffer. These solutions were immersed in a waterbath at 32 °C and samples were taken several times and diluted 1:10 with methanol. One millilitre of the methanolic solution was mixed with 1 ml of 0·05% β-phenylglucoside solution and 1 ml of 0·05% inositol solution. The mixture was evaporated under vacuum at room temperature and converted to trimethylsilyl (TMS) derivatives using *N*-(trimethylsilyl) imidazol as reported previously (Martinez-Castro & Olano, 1980). TMS derivatives of carbohydrates were analysed by gas–liquid chromatography (GLC)—with a 881 Perkin-Elmer apparatus fitted with two 3 m × 3·2 mm stainless steel columns packed with 3% OV-17 on Chromosorb W-HP 80-100 mesh held isothermally at 185 °C for 5 min and then programmed from 185 to 255 °C at 4 °C/min. Areas were measured as the product of the peak height and mean base. Response factors were calculated with several mixtures of pure standards. Retention times (RRT) were relative to the α-lactose peak and measured at 255 °C.

Proteolytic activity

Total proteolytic activity on casein was measured by a modification of the method of Kunitz (1947). Two millilitres of enzyme solution containing 2·0 g/litre were added to 2 ml of 1% (w/v) of casein in phosphate buffer and incubated for 20 h at the required temperature. The reaction was stopped by adding 12 ml of 5% trichloroacetic acid (TCA). After thorough mixing and standing for 1 h the solution was filtered. The TCA-soluble nitrogen level was estimated by the method of Lowry *et al.* (1951) and compared with a control in which TCA was added to the casein before the addition of enzyme solution.

The specific action on caseins was followed by polyacrylamide-gel electrophoresis at pH 8·4 as reported previously (Ramos *et al.*, 1977). Relative mobilities were calculated against the migration of the marker dye Bromophenol Blue, including the length of gel before and after standing with amido Black 10 B as proposed by Weber & Osborn (1969).

Quantitative measurements were by densitometry with a Chromoscan MK-II (Joyce Loebl Co., Gateshead, Great Britain). Quantities of α_s and β-casein in the gels were referred to the percentages found in the control.

Lipolytic activity

The production of free fatty acids (FFA) was estimated after incubating the following mixture for several days at 30 °C: 4 ml of a solution of 10 g/litre of Maxilact in distilled water; 5 ml of a 10 % (w/v) emulsion of tributyrin (Fluka) in a 10 % arabic gum solution and 5 ml of a 0·044 M $CaCl_2$ solution. The solutions were adjusted to pH 7 before mixing and 0·1 ml of a 0·1 % thimerosal sodium salt (ethylmercurythiosalicylic acid sodium salt) solution was added to each sample. Two controls were carried out simultaneously. In one the enzyme solution was replaced by water; in the other the emulsion substrate was replaced by water. After incubation, 20 ml of 1:1 alcohol:ether were added to each sample and the solutions were titrated with 0·05 N NaOH.

RESULTS AND DISCUSSION

Screening of activities

Table 1 shows the activities obtained for the nineteen enzymatic determinations from the API ZYM micromethod. A value ranging from 0 to 5 was assigned to the colours developed according to the colour chart of the method. Zero corresponds to a negative reaction and 5 to a reaction of maximum intensity. Although β-galactosidase activity was the main enzymatic activity found in Maxilact, other activities such as β-glucosidase, α-galactosidase, phosphatase alkaline, phosphoamidase and leucine arylamidase, as well as different lipolytic activities, were also detected. All these activities were found by Schmidt *et al.* (1979) in fifteen strains of *Kluyveromyces lactis* isolated from cheese. It is probable that these activities account for the acceleration observed in the ripening of cheeses made with Maxilact-treated milk.

β-Galactosidase activity

The action of Maxilact was assayed on two substrates, lactose and

TABLE 1
Enzymatic activities of Maxilact

No	*Enzyme Assayed for*	*Substrate*	*pH*	*Activity mark*
1	Control			
2	Phosphatase alkaline	2-naphthyl phosphate	8·5	<0·5
3	Esterase (C4)	2-naphthyl butyrate	6·5	<0·5
4	Esterase lipase (C8)	2-naphthyl caprylate	7·5	<0·5
5	Lipase (C14)	2-naphthyl myristate	7·5	0
6	Leucine arylamidase	L-leucyl-2-naphthylamide	7·5	<0·5
7	Valine arylamidase	L-valyl-2-naphthylamide	7·5	0
8	Cystine arylamidase	L-cystyl-2-naphthylamide	7·5	0
9	Trypsin	*N*-benzoyl-DL-arginine-2-naphthylamide	8·5	0
10	Chymotrypsin	*N*-glutaryl-phenyl-alanine-2-naphthylamide	7·5	0
11	Phosphatase acid	2-naphthyl phosphate	5·4	0
12	Phosphoamidase	naphthol-AS-BI-phosphodiamide	5·4	0·5
13	α-Galactosidase	6-Br-2-naphthyl-αD-galactopyranoside	5·4	0·5
14	β-Galactosidase	2-naphthyl-βD-galactopyranoside	5·4	5
15	β-Glucoronidase	naphthol-AS-BI-βD-glucuronate	5·4	0
16	α-Glucosidase	2-naphthyl-αD-glucopyranoside	5·4	0
17	β-Glucosidase	6-Br-2-naphthyl-βD-gluco-pyranoside	5·4	0·5
18	*N*-Acetyl-β-glucosaminidase	1-naphthyl-*N*-acetyl-βD-gluco-saminide	5·4	0
19	α-Mannosidase	6-Br-2-naphthyl-αD-manno-pyranoside	5·4	0
20	α-Fucosidase	2-naphthyl-αL-fucopyranoside	5·4	0

lactulose, at enzymatic concentrations of 250 and 500 mg/litre. As expected, the higher concentration of Maxilact gave the faster hydrolysis of both disaccharides. Lactose was hydrolysed up to 80 % in about 1 and 2·5 h for 500 and 250 mg/litre of Maxilact, respectively. Total hydrolysis of lactose required 2·5 and 5·0 h for 500 and 250 mg/litre of Maxilact, respectively. These results are in agreement with previously reported work (Bouvy, 1974; Toba & Adachi, 1978; Burvall *et al.*, 1979).

Figure 1 shows the effect of Maxilact on a 5 % lactulose solution. Hydrolysis of lactulose was slower than that of lactose. 250 mg/litre of enzyme hydrolysed 40 % of the disaccharide in 7·5 h whereas 500 mg/litre

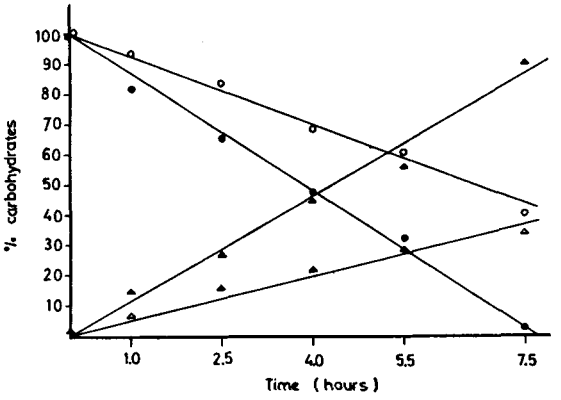

Fig. 1. Hydrolysis of a 5 % lactulose solution with two different levels of Maxilact. Open symbols: 250 mg/litre. Closed symbols: 500 mg/litre. Circles: lactulose. Triangles: monosaccharides.

hydrolysed nearly 100 % in the same time. The concentration of galactose present in the monosaccharide fraction was 12 % lower than that of fructose. Hydrolysis of disaccharides by β-galactosidase is accompanied by the formation of oligosaccharides having a β-galactosidic moiety. Toba & Adachi (1978) have identified up to six disaccharides and some trisaccharides during enzymatic hydrolysis of lactose; thus, the formation of galactosyl–galactosides and galactosyl–fructosides can be expected in the hydrolysates of lactulose. Several peaks appearing in the chromatograms of lactulose hydrolysate (Fig. 2) can be assigned to oligosaccharides. Their relative retention times are given in Table 2; values for lactose hydrolysate are given for comparison purposes. The main oligosaccharide peaks appearing in the chromatograms of lactulose hydrolysates had different retention times from those of lactose hydrolysates.

TABLE 2
Relative Retention Times of Disaccharide Peaks in Chromatograms from Lactulose and Lactose Hydrolysates

	Relative retention times							
Lactulose	0·795	1·135[a]	1·306	1·557	1·785	1·819	2·518	
Lactose	0·766	1·000[b]	1·323[c]	1·590	1·614	1·785	1·877	2·022

[a] Lactose
[b] α-Lactose
[c] β-Lactose

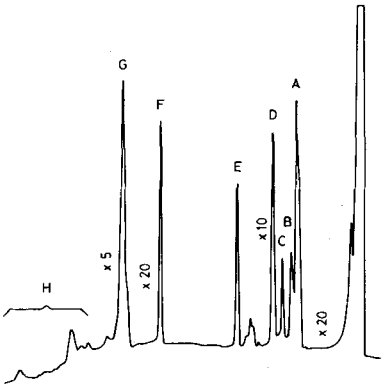

Fig. 2. Chromatogram of the TMS derivatives of free neutral carbohydrates of a lactulose hydrolysate. A: fructose; B, C, D: γ-, α- and β-galactose; E and F: internal standards (*m*-inositol and phenyl β-glucoside), G: lactulose, H: oligosaccharides.

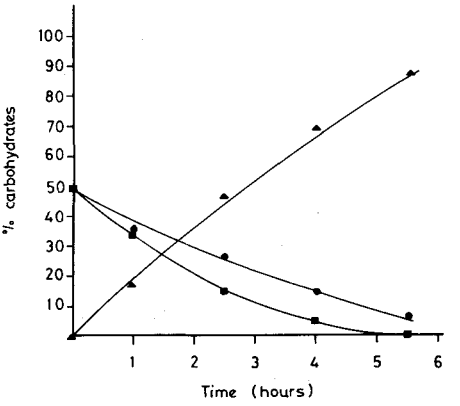

Fig. 3. Enzymic hydrolysis of an equimolecular mixture of lactose and lactulose (5 % disaccharide solution) with 500 mg/litre of Maxilact. Circles: lactulose, squares: lactose, triangles: monosaccharides.

Because of the difficulty of obtaining lactulose in a pure state, most of the lactulose preparations used in the industry are mixtures containing lactose. In practice it is important to know the behaviour of Maxilact in mixtures with lactose. Figure 3 shows the hydrolysis of a 1:1 mixture of lactose: lactulose. Although lactose was hydrolysed faster than lactulose, as was found during the hydrolysis of each disaccharide in separate experiments, the presence of lactulose resulted in a decrease of the rate of hydrolysis of lactose.

Since both disaccharides have a β-galactosidic moiety they are competitive substrates for the enzyme.

Proteolytic activity

Maxilact 20.000 is a yellowish white powder containing insoluble material; some authors (Dahlqvist *et al.*, 1977; Hemme *et al.*, 1979) observed that centrifugation or filtration of Maxilact solutions did not significantly alter the β-galactosidase activity. Nevertheless, we have found some proteolytic activity in both soluble and insoluble fractions. When 50 mg of Maxilact were ground in a tissue homogeniser with 25 ml of phosphate buffer, the soluble fraction showed about 75 % of the total proteolytic activity. The insoluble fraction retained a weak proteolytic

activity after exhaustive grinding and washing with the buffer as shown by electrophoresis. In the successive assays a complete homogenisate, including all material from Maxilact, was used.

When casein was incubated with several concentrations of Maxilact from 3 to 20 h, proteolysis was not detected after 3 h by the TCA-soluble nitrogen method, but electrophoretic analysis showed that 75% of α_s-casein disappeared, and a new band with 22% relative intensity appeared in the gels. Probably the protease action starts by splitting casein to give some insoluble peptides which are degraded to non-protein nitrogen afterwards.

The inhibition effect of cystein, ethylene diamine tetra-acetic acid (EDTA), mercaptoethanol, KCl, NaCl, Na_2SO_4, and K_2SO_4 at 10^{-2} M concentration has been assayed. The percentage of inhibition of the proteolytic activity was 65% for EDTA, 45% for cystein and 30% for mercaptoethanol. The other assayed substances did not show any inhibition effect.

Effect of temperature

The optimum temperature was estimated by using the assay method in the range 30–55 °C. The maximum activity was around 45 °C. 30 °C and 55 °C gave 20 and 64% of the maximum of activity, respectively as shown in Fig. 4. Heat stability was measured after incubation at 45, 50 and 55 °C for 60 min. at pH 7. The remaining activity was 75, 95 and 77%, respectively; 15 min at 80 °C or 5 min at 100 °C completely inactivated the enzyme.

Effect of pH

Proteolytic activity of Maxilact was found in the pH range 6–8 with a maximum around pH 7, as shown in Fig. 5. Below pH 4 and above pH 9 this activity is completely lost. Marschke *et al.* (1980) also found a wide range of pH for the proteolytic activity of Maxilact 40.000 with the maximum around pH 6 and a similar value for Maxilact 20.000.

Action on caseins

Figure 6 shows the electrophoretic patterns of casein after incubation at 45 °C for 20 h at pH 8·1 with Maxilact (a) and the control (b). β-casein was

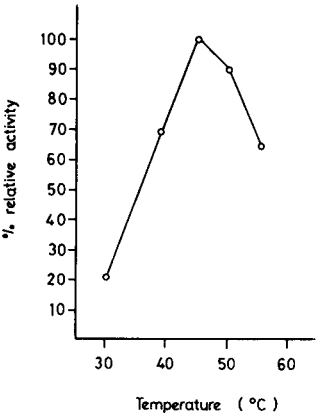

Fig. 4. Effect of incubation temperature on proteolytic activity of Maxilact. (20 h at pH 7.)

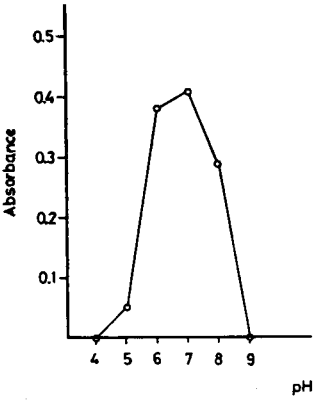

Fig. 5. Effect of pH on proteolytic activity of Maxilact. Incubation at 45 °C during 20 h.

unaltered but clearly a breakdown of α_s-casein can be observed, giving rise to a new band with higher mobility (0·85) than that of α_s-casein (0·78). α_s-Casein degraded by the action of rennet also gives a band with the same electrophoretic mobility (0·85). This band has been identified and designated as α_{s_1}I by Hill *et al.* (1974) and Creamer & Richardson (1974) and originates from the cleavage of the 23—24 bond. Other proteases as pepsin or impurities in acid phosphatase from wheat germ also give rise to this band (Hill *et al.*, 1974).

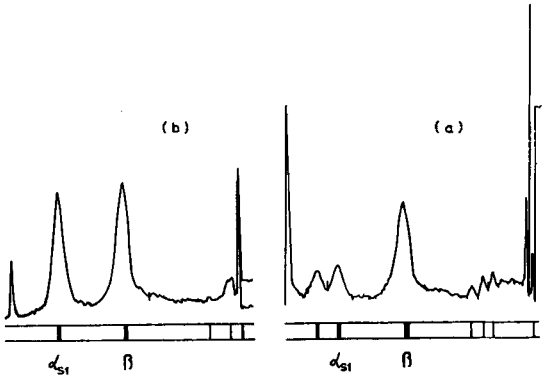

Fig. 6. Densitometric curves and schematic diagrams of casein after incubation with Maxilact (a) and control (b) at 45 °C for 20 h, pH 8·1.

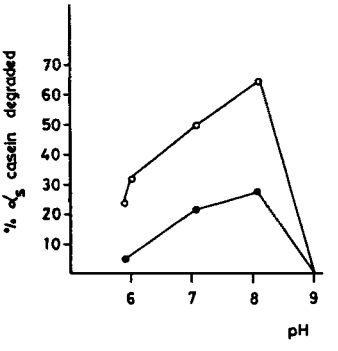

Fig. 7. Effect of pH on degradation of α_s-casein. Incubation at 45 °C for 3 h (●), and 20 h (○).

The peptide formed from α_s-casein by the action of Maxilact could be more susceptible to the further action of cheese starters than unaltered casein, contributing to the ripening acceleration in cheese made with hydrolysed milks.

Degradation of α_s-casein was studied in the pH range 5·8–9 and the results are shown in Fig. 7.

Lipolytic activity

After an incubation period of 1 day there was little or no detectable increase of free fatty acids (FFA). After 2 or 3 days, 5 or 7·5 μmol of FFA per millilitre of emulsion were formed, respectively.

ACKNOWLEDGEMENT

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Colour Stability of Shisonin, Red Pigment of a Perilla (*Perilla ocimoides* L. var. *crispa* Benth)

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ABSTRACT

The colour stability of shisonin, a red pigment isolated from the leaves of a perilla (Perilla ocimoides *L. var.* crispa *Benth*), *was studied in model systems. At pH less than 6, the absorbance of shisonin in solution decreases with increase in pH. The degradation of shisonin in buffers at both pH 3 and 4 followed first-order reaction kinetics. Fructose, sucrose, metal cations* Cu^{++} *and* Fe^{+++}, *hydrogen peroxide, oxygen and daylight affected the degradation rate of shisonin, while glucose, lactose, glycine and leucine had essentially no effect on the degradation rate.*

INTRODUCTION

Much attention has recently been paid to the use of natural pigments in food as permissible colorants. This interest has arisen due to the growing restrictions on the use of a number of artificial colouring compounds.

The leaf of a perilla, 'Shiso' (*Perilla ocimoides* L. var. *crispa* Benth), has been extensively used to give an appetising red colour and additional flavour to 'Umeboshi' (Japanese plum pickles), 'Shibazuke' (Japanese red-dyed pickles) or 'Benishoga' (Japanese red-dyed ginger) since ancient times in Japan. A chemical study of the red pigment was first made by Kondo (1931). Kuroda & Wada (1936) isolated from the perilla leaves pigments called shisonin A and B as chlorides. Amorphous shisonin B chloride was confirmed to be a compound of shisonin A chloride and *p*-coumaric acid. Takeda & Hayashi (1964) gave the structure of shisonin as 3-(*p*-coumaroyl-glucosido)-5-glucoside of cyanidine. Recently, Goto *et*

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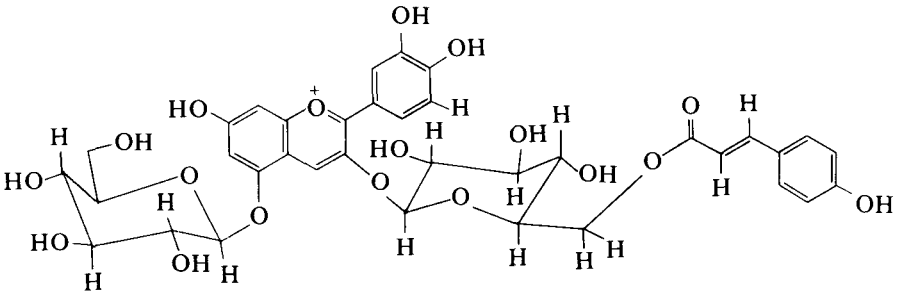
al. (1978) (by means of a ^{1}H–NMR spectrum) presented the complete stereo structure of shisonine as cyanidine 3-(6-*O*-*trans*-*p*-coumaroyl-β-D-glucoside)-5-(β-glucoside), as follows:



Although a large number of similar pigments are found in other plants (Francis, 1977), shisonin from the perilla leaf is one of the best sources of the red pigment as a food colorant. Nevertheless, few studies are reported in the literature concerning the stability of shisonin (Miyauchi, 1975). It has generally been recognised that the stability of colour during the preparation of red pigmented foods coloured by natural pigments is affected by several factors: pH, light, oxygen, bacterial degradation, metals and other chemicals (Mashakis *et al.*, 1957; Daravingas & Cain, 1968; Palamidis & Marhakis, 1975; Calrei & Francis, 1978).

The purpose of this study was to investigate how some chemical and physical factors affect the colour stability of shisonin in model systems.

EXPERIMENTAL

Isolation of shisonin

Fresh 'Shiso' leaves were stirred with 1 % HCl–methanol for 24 h at 5 °C. The red coloured extracts were filtered under suction. The filtrates were concentrated to half volume in a rotary evaporator at 40 °C. Lead acetate (isomolar amounts to HCl) was added and the white lead chloride, precipitated at 1–2 °C, was removed by centrifugation at 3000 rpm for 15 min. Shisonin was then precipitated by gradual addition of lead acetate, separated by centrifugation (3000 rpm for 15 min) and dissolved in 5 % HCl–methanol. The crude shisonin chloride was precipitated by the addition of ethyl ether. The pure shisonin chloride was obtained by preparative paper chromatography on Toyo No. 526 filter paper using

BuOH: AcOH: H_2O (4:1:2, v/v) as the developing solvent, as described by Kuroda & Wada (1936). The paper chromatogram gave a main spot of shisonin at R_f 0·65 and other minor spots at R_f 0·50, 0·39, 0·35 and 0·26. The combined anthocyanin spots (R_f: 0·65) were treated with 0·5% HCl–methanol and extracts were evaporated *in vacuo* below 40 °C to dryness and then further dried over KOH. Although some trials for the crystallisation of shisonin chloride or picrate were unsuccessful, paper chromatographically pure shisonin chloride was obtained in a shiny violet coloured amorphous state.

Preparation of shisonin solution

McIlvain's citric–phosphate buffer (0·1 M) solutions with pH values of 2, 3, 4, 5, 6 and 7 were prepared. Buffer of pH 2 was prepared by adding 1N HCl to the McIlvain's buffer. Shisonin solutions were prepared by adding 1 ml of 0·05% (w/v) shisonin chloride in 0·1 M citric acid solution to 19 ml of each buffer. Changes in the pH of the buffer caused by increasing temperature were not corrected. The shisonin solution was introduced immediately into vials with teflon-lined screw caps and was flushed with nitrogen gas to limit oxygen. The vials were sealed and placed in a thermostatically controlled bath (±0·1 °C) for heat treatment. Temperature of the solutions in the vials were within 1 °C of that expected in less than 3 min. Vials were drawn out periodically and cooled immediately in tap water to room temperature. Subsequently, the visible spectrum of the solution was recorded using a Hitachi–Perkin Elmer type 139 UV–Vis spectrophotometer. Based on the visible spectrum of the solution, the colour intensity after the treatment was measured using an Hirama type 6B photometer.

Influence of additives and other substances

Selected metal cations (Cu^{++} and Fe^{+++}), carbohydrates (glucose, fructose, lactose and sucrose) and other substances (glycine, leucine, hydrogen peroxide) at several concentrations were examined for their effect on the rate of bleaching of shisonin solutions at 90 °C. The effect of oxygen on the rate of fading was measured in buffered shisonin solution (5 ml) in a test tube (20 ml). Air in the head space of vials was replaced with oxygen. After sealing with a screw cap, the reactor was maintained at 90 °C in a water bath and shaken for the time tested.

Effect of light on the bleaching was determined on the solution at pH 3. Samples introduced into 20 ml vials to be exposed to daylight were placed outside a window for direct exposure to daylight for 5 or 10 h, and the colour intensity of exposed samples was compared with the control in a vial wrapped with aluminium foil.

RESULTS AND DISCUSSION

Absorption spectra

Visible spectra (350–650 nm) were obtained for shisonin solutions at each pH value, as shown in Fig. 1. Shisonin shows peaks at 514 nm, 515 nm, 518 nm and 520 nm, for pH 2, 3, 4 and 5, respectively; the absorption maximum shifted towards a longer wavelength, and the absorbance decreased with an increase in pH. At pH 7, absorption maximum shifted to 590 nm.

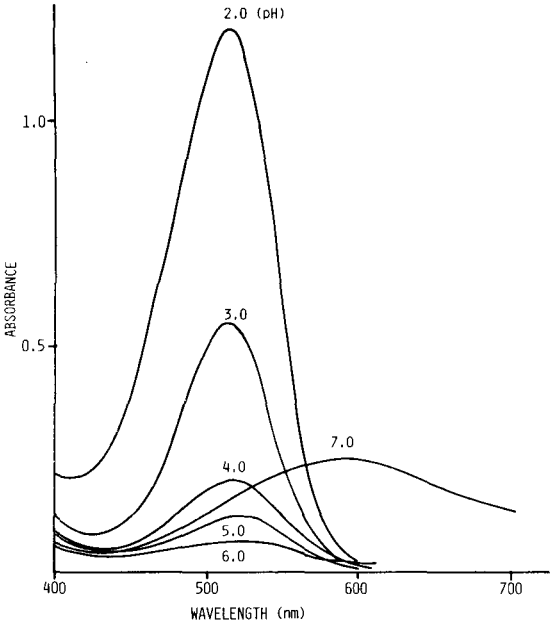


Fig. 1. Visible spectra of shisonin at pH 2, 3, 4, 5, 6 and 7. Shisonin concentration; 2 mg/100 ml buffer.

Colour stability of shisonin

(a) Effect of temperature

When the solutions of shisonin were heated for different lengths of time, the colour intensity diminished and no shift of absorption maximum was observed, as shown in Fig. 2. Figure 3 shows the change of absorbance of shisonin solutions heated at 98 °C, at pH 3, 4 and 5. The absorbance decreased with increasing heating time and was finally equilibrated except at pH 5·0. In the case of pH 5, the apparent increase in colour intensity with the lapse of time above 3 h was due to the formation of a browning pigment. This browning substance was characterised by the monotonous decrease of the transmittance toward the UV region. The change of colour was studied by calculating the percentage of retained absorbance at 515 nm (would-be retained shisonin) after heating as:

$$\%R = \frac{\text{absorption at } x \text{ time}}{\text{absorption at 0 time} - \text{absorption at } \infty \text{ time}} \times 100$$

where $\%R$ = per cent of the retained absorption; absorption at ∞ time = the absorption when no further change could be detected at 98 °C (15 h).

For each temperature tested at pH 3, the percentage ($\%R$) versus time generated a straight line when plotted on a semilogarithmic scale (Fig. 4), thus verifying that shisonin degradation obeys first-order kinetics. Plots of the data obtained at pH 4 differed only in the slope of the curves. The slopes of the lines indicate that the degradation rate can be expressed in

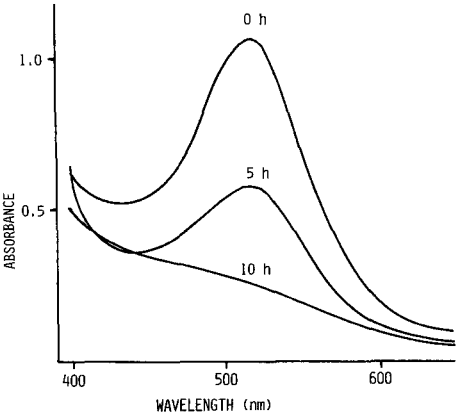


Fig. 2. Changes of spectra of shisonin at pH 4 at 98 °C. Shisonin concentration: 10 mg/100 ml buffer.

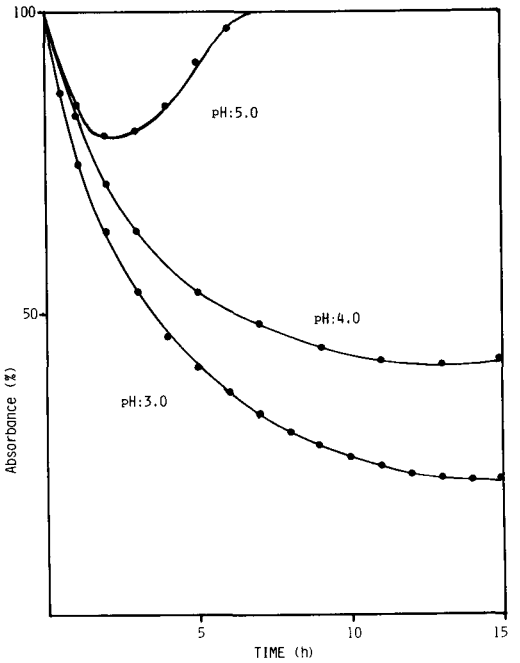


Fig. 3. Time course of the change in colour intensity of shisonin at pH 3, 4 and 5 at 98 °C.

terms of half-life, $T_{1/2}$ (shown in Table 1). $T_{1/2}$ values were calculated from the regression equation as follows:

$$\ln 50 = ax + b$$

The degradation rate at pH 5 cannot be estimated because the concentration of a browning pigment was built up during the heat treatment. These data indicate that the stability of shisonin at pH 3 is slightly

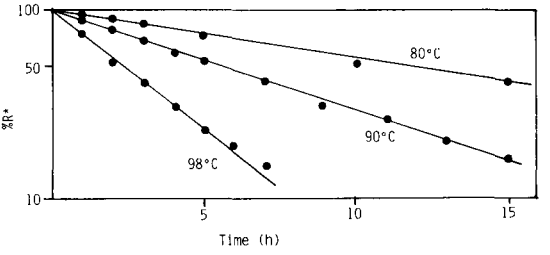


Fig. 4. Degradation rates for shisonin at 80 °C, 90 °C and 98 °C at pH 3.

$$\%R = \frac{\text{Absorption at } x \text{ time}}{\text{Absorption at 0 time} - \text{absorption at } \infty \text{ time}} \times 100$$

TABLE 1
Half-Life Values for Shisonin at pH 3 and 4 at 80 °C, 90 °C and 98 °C

pH \ *Temperature* (°C)	*98*	*90*	*80*
3·0	140 (min)	340	760
4·0	120	220	580

greater than that at pH 4. When the shisonin in buffers of pH 3, 4 and 5 was stored for 10 days at 5 °C, no detectable reduction in absorbance was observed.

(*b*) *Effect of additive*
First-order reaction kinetics permitted reaction rates to be expressed in terms of half-life ($T_{1/2}$) values. Table 2 lists the effects of the additives on the $T_{1/2}$ value of shisonin. pH values were checked and adjusted to the desired pH by the addition of 0·1 M citric acid or K_2HPO_4, if necessary, before the experiment.

TABLE 2
Effect of Selected Additives on the Half-life Values for Shisonin at pH 3 and 4 at 90 °C

Additive \ *pH*	3·0	4·0
Control	350 (min)	220 (min)
Glucose (5%)	315	185
Lactose (5%)	320	190
Fructose (5%)	55	35
Fructose (2%)	130	65
Sucrose (5%)	145	105
Cu^{++} (100 ppm)	185	90
Cu^{++} (500 ppm)	40	30
Fe^{+++} (100 ppm)	285	190
Fe^{+++} (500 ppm)	125	110
Glycine (2%)	345	185
Leucine (2%)	350	195
Glycine (2%) + Glucose (2%)	300	160
H_2O_2 (100 ppm)	140	100
H_2O_2 (500 ppm)	65	35

Five per cent glucose and lactose added to the buffer had essentially no effect on the $T_{1/2}$ value of shisonin, while the addition of fructose and sucrose decreased the $T_{1/2}$ values markedly. The $T_{1/2}$ value was nearly inversely proportional to the fructose concentration, suggesting that the rate of shisonin degradation obeyed first-order kinetics with respect to the initial concentration of fructose. The degradation process of shisonin in the presence of fructose or sucrose could not be discovered in the present study. These results suggest that the addition of fructose or sucrose to foods coloured with shisonin should be avoided in view of the colour destabilisation.

Added ferric ion caused a decrease in the $T_{1/2}$ value of shisonin and cupric ion caused an even larger degradation to form brown coloured precipitant. One might conclude from the results that these metal ions act as catalysts for the degradation of shisonin. Pasch & von Elbe (1979) also suggested the catalytic effect of these ions for the degradation of betanin.

Amino acids, glycine or L-α-leucine had no effect on the rate of degradation at pH 3, while causing a slight increase in the rate of degradation at pH 4. Added glucose with glycine had no effect on the degradation rate at pH 3 but had a slight effect at pH 4. Small amounts of hydrogen peroxide had a marked effect on the decrease of $T_{1/2}$ values, this decrease being inversely proportional to the concentration of hydrogen peroxide. No protective effect for the degradation of shisonin was observed for the additives tested in this study.

(c) Influence of light or oxygen

The sensitivity of shisonin to degradation by light was studied by storing shisonin solutions at pH 3 under nitrogen with or without exposure to daylight for 5 and 10 h. Exposure to daylight for 5 and 10 h increased the degradation by 28 % and 51 %, respectively. The presence of oxygen in the head space of vials decreased the $T_{1/2}$ value at 90 °C, pH 3 (2·5 h versus 4·1 h). These results demonstrated the sensitivity of shisonin to oxygen and light and the need to protect products containing shisonin against long exposure to air or light.

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Book Review

Introduction to Multidimensional Scaling. Edited by S. Schiffman, M. Lance Reynolds and Forrest W. Young. Academic Press, New York, 1981. xvi + 413 pp. Price: $29.50.

Multidimensional scaling (MDS) is a somewhat specialised and daunting prospect for a work-a-day chemist. However, for those engaged in the relationship between objective chemical measurement and the sensory quality of food this book is clearly a prominent and authoritative text. It contains several chapters under three main headings: 'Basic Concepts', 'Method' and 'Applications and Theory'. Repeated examples of foods, blends, flavours and chemicals are of direct interest to readers of this journal and the book explains, in full detail, how MDS works and what it will do. References are complete, but not excessive, at the end of each chapter and the book as a whole is notable for its sound mathematical approach to the subject. Although solidly psychophysical, the book has many satisfying aspects for the food and flavour chemist. In one study, for example, the structures of fourteen musks undergoing similarity ratings are illustrated. The problem of individual differences in subjective psychophysical judgements can be adequately allowed for in the MDS method and the book, especially in view of its modest price, is certainly recommendable to food chemists concerned with taste, flavour, odour and texture.

G. G. Birch

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Announcement

GUMS AND STABILISERS FOR THE FOOD INDUSTRY

An international conference on 'Gums and Stabilisers for the Food Industry', with special emphasis on the applications of hydrocolloids, is to be held from 11–15 July, 1983; it has been organised by the North East Wales Institute.

All aspects of the legislation, characterisation and utilisation relating to, and the new technological developments in, the food hydrocolloids field will be covered.

Authors are invited to submit abstracts of papers (not more than 250 words); the papers should be on the following aspects of hydrocolloids (polysaccharides or proteins)—characterisation, utilisation (both product and end-use areas and food processing effects), legislation and toxicology.

Further information may be obtained from: The Conference Secretariat, Research Division, The North East Wales Institute, Kelsterton College, Connah's Quay, Deeside, Clwyd CH5 4BR, Wales, Great Britain.

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Effects of Different Levels of Palm Oil and Sulphur in Cassava-Based Diets

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(Received: 5 January, 1982)

ABSTRACT

The effects of different levels of palm oil and sulphur on the nutritive value of a cassava leaf protein diet was investigated using rats. With 40% cassava and no palm oil, the protein efficiency ratio (PER) and net protein utilisation (NPU) were very low (0·3 and 23·8, respectively) but the true digestibility was the same as that of the casein control (95·0%). With 5% palm oil there was no improvement in PER but both the NPU and biological value (BV) were increased appreciably (38·1 and 40·3, respectively). The best result was obtained with 10% palm oil, with which the PER was more than doubled (0·7) and there were similar increases in both the NPU and BV (46·6 and 48·9, respectively). Cassava at the 40% level was supplemented with various sources of sulphur—i.e. methionine, sodium thiosulphate, elemental sulphur, sodium sulphate and ferrous sulphate. With methionine, the PER, compared with the control (0·7), was more than trebled (2·2) and the NPU and BV were also significantly increased (72·6 and 83·3, respectively). Elemental sulphur gave a PER of 1·8 whilst sodium sulphate and ferrous sulphate gave 1·9. The lowest PER was obtained from sodium thiosulphate (1·1). The importance of palm oil and sulphur donors in cassava-based diets is discussed.

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INTRODUCTION

The many ways in which the body detoxifies cyanide have been reviewed by Oke (1973). The most important pathway is through the enzyme rhodanese which uses sulphur from sulphur donors to convert cyanide (CN) to thiocyanate (SCN). This enzyme is concentrated mainly in the liver but occurs also in the kidney, spleen and other organs of the body (Oke, 1969). The sulphur donor is mainly methionine which is the first limiting amino acid in cassava-based diets. Cyanide in cassava has been implicated as the cause of tropical ataxic neuropathy (TAN) in cassava-eating areas of the tropics (Oshuntokun, 1973) and the detoxification product, thiocyanate (SCN), as the cause of goitre (Ekpechi, 1973; Delange *et al.*, 1973). Oshuntokun (1969) found that the blood of patients with TAN contained very little or no methionine owing to its use for detoxification. It therefore appears that there are two main ways of supplying sufficient methionine for its normal functions in the body.

(a) By furnishing another source of sulphur for the detoxification process and thus sparing methionine.
(b) By supplying a good source of protein, very rich in methionine, so that the excess can be used for detoxification.

This is very important, especially in the livestock industry, if cassava is to replace maize as a source of energy. However, cassava, unlike maize, is virtually devoid of ether extract. It was at first thought that the beneficial effect of fats or oils added to cassava was due to the essential fatty acids (EFA) contributed to the diet, and also the higher caloric density of the fat-containing diet. Subsequently it was found that powdered starch produced ulcerogenic effects upon the gastric mucosa of animals (Henry, 1965) and this could be avoided by the addition of fats and oils which not only improved the physical characteristics of the diet but also the palatability and the absorption or retention of other nutrients. Thus, 3% of corn oil was found to improve gain and efficiency of pigs whether in the presence or absence of methionine supplementation, especially at higher levels of cassava in the diet (Shimada *et al.*, 1971). Hew & Hutagalung (1972), using a 50% cassava ration, were able to increase the gain in weight of swine significantly by supplementation with 0·2% methionine, but 0·4% had no further effect except with the addition of palm oil. They concluded that there might be a reaction between fatty acids and methionine.

An attempt was therefore made in this investigation to establish the effect of various levels of local palm oil and sources of sulphur on the nutritive value of cassava-based diets in rats.

MATERIALS AND METHODS

Cassava chips were kindly supplied by Dr S. K. Hahn of the International Institute for Tropical Agriculture, Ibadan, Nigeria (to whom we are very grateful). Leaf protein concentrate (LPC) was extracted from *Amaranthus caudatus* by the method of Davys & Pirie (1969).

Eighty male albino rats of the Wistar strain, weaned at 23–24 days, were obtained from our disease-free stock and reared on a commercial stock diet (Pfizer Livestock Feed, Ltd, Nigeria) until they were 30–31 days old and weighed 50–60 g. They were weighed to the nearest 0·1 g and allocated on the basis of weight and litter origin to eight groups of ten rats each. The rats were individually housed in a battery of wire cages with facilities for separate faecal and urinary collection.

The composition of the diets is shown in Table 1. The LPC and casein were added to the test diet and control rations at the expense of maize starch, to give 10 g crude protein per 100 g diet. The diets were each initially mixed in a 10-litre plastic bucket and then in a powered

TABLE 1
Composition of the Diets
(Percentage dry weight basis)

Ingredients	*Diet*							
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
Maize starch	80	70	40	35	30	25	20	15
Cassava	—	—	40	40	40	40	40	40
Palm oil	10	10	—	5	10	15	20	25
Leaf protein concentrate (as protein N × 6·25)	—	—	10	10	10	10	10	10
Casein (as protein N × 6·25)	—	10	—	—	—	—	—	—
Mineral mix*	4	4	4	4	4	4	4	4
Vitamin mix*	1	1	1	1	1	1	1	1
Cellulose powder	5	5	5	5	5	5	5	5

* Umoh & Oke (1974).

TABLE 2
Composition of the Diets Used in the Trial Comparing Sources of Sulphur
(Percentage dry weight basis)

Ingredients	*Diet*							
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
Palm oil	10	10	10	10	10	10	10	10
Vitamin mix*	1	1	1	1	1	1	1	1
Mineral mix*	1	1	1	1	1	1	1	1
Non-nutritive cellulose	5	5	5	5	5	5	5	5
LPC (as protein N × 6·25)	—	10	10	10	10	10	10	10
Cassava	—	—	40	40	40	40	40	40
Maize starch	80	70	29·85	29·85	29·85	29·85	29·85	29·85
Sulphur source	—	—	0·15% Methionine	0·15% +0·25% Lys.	Elemental sulphur	Sodium thiosulphate	Sodium sulphate	Ferrous sulphate

* Umoh & Oke (1974).

'Kenwood chef' domestic food mixer for 15–20 min. Homogeneity of the diet was checked by determining the N content of each diet in duplicate immediately after mixing and in the middle of the experimental period.

One group of the rats was given the N free basal diet (diet 1, Table 1), the second group was placed on the casein control diet and the remaining six groups were randomly allocated to the test rations containing 0–25 % palm oil. In another series of experiments a similar procedure was used except that the cassava level was kept at 40 %, palm oil at 10 % and different sources of sulphur (methionine, elemental sulphur, sodium thiosulphate, sodium sulphate and ferrous sulphate), were used at the 0·15 % level. The composition of the ration is shown in Table 2. All the diets and water were offered *ad libitum*. Throughout the experimental period, the animal house temperature varied between 26 °C and 29 °C with a mean at 27·5 °C.

The weights of the animals were recorded every other day throughout the experiment. The first 3 days were regarded as an acclimatisation period during which no records were kept of food consumption and no collection of faeces was made. Collection of faeces was made daily for the last 10 days of the feeding experiment. The faeces of individual rats were pooled, dried at 75 °C for 3 days and ground to powder for faecal nitrogen determination. Daily records of food consumption were kept for the last 10 days of the experiment.

For the determination of digestibility, an additional precaution was taken with the faecal collection and food consumption. Nitrogen was determined on the dried ground faecal samples of each rat by the semi-micro distillation apparatus of Markham (1942). The true digestibility (TD) was calculated by the balance-sheet method of Mitchell (1923–24).

For the determination of the protein efficiency ratio (PER), net protein utilisation (NPU) and biological value (BV), the rats in each group were weighed to the nearest 0·1 g on the last 10 days of the 13-day dietary treatment and killed with chloroform. The food intake in the last 10-day period was measured and this value and the determined crude protein content of the diet were used to calculate the amount of protein consumed during the test. The PER was calculated from these results using the formula given by the United States National Academy of Sciences/ National Research Council (1963).

The carcasses of the rats were dried in a hot air circulation oven at 85 °C, after incisions were made into the skull, thoracic and body cavities. The dried carcasses were digested for N determinations by a modified

TABLE 3
Energy Values of the Various Rations (kg/g)

Ration	*Energy value (kg/g)*
Basal diet	21·0
Casein (Control)	20·8
40% Cassava + 0% palm oil + 10% LPC	19·4
40% Cassava + 5% palm oil + 10% LPC	20·8
40% Cassava + 10% palm oil + 10% LPC	20·8
40% Cassava + 15% palm oil + 10% LPC	21·0
40% Cassava + 20% palm oil + 10% LPC	22·2
40% Cassava + 25% palm oil + 10% LPC	23·9

method of Rippon (1959), the modification being that it was not found necessary to autoclave the carcass slurry. Complete disintegration occurred when the carcass was first pounded in a porcelain mortar and treated with 100 ml concentrated sulphuric acid followed by 100 ml distilled water. The resultant dark brown solution was made up to 250 ml and 25 ml duplicate portions were taken for N determination. The NPU values were calculated using the original equation of Miller & Bender (1955). The BV was computed by dividing NPU by TD (Bender & Haizelden, 1957).

The energy contents of the rations were determined, using a ballistic bomb calorimeter, by the method of Miller & Payne (1959) (Table 3).

RESULTS

The results are shown in Table 4. With the diet containing 40% cassava in the absence of palm oil, the TD was not affected (95%) but the NPU was

TABLE 4
Performance of the Wistar Albino Rats on the Various Test and Control Rations
(Mean values ± SD)

Treatment	*Number of rats*	*Protein intake*	*Weight gain/loss*	*PER*	*TD*	*NPU*	*BV*
Basal diet	10	0·6 ± 0·0	−10·5 ± 0·9	—	—	—	—
Casein	10	9·2 ± 1·8	22·2 ± 0·8	2·4 ± 0·1	95·3 ± 1·1	73·9 ± 1·5	77·6 ± 2·2
40% Cassava + 0% palm oil + 10% LPC	10	4·7 ± 0·3	1·4 ± 0·1	0·3 ± 0·1	95·0 ± 1·5	23·8 ± 1·9	25·0 ± 2·0
40% Cassava + 5% palm oil + 10% LPC	10	7·3 ± 0·4	2·5 ± 0·4	0·3 ± 0·1	94·8 ± 1·8	38·1 ± 1·7	40·3 ± 2·5
40% Cassava + 10% palm oil + 10% LPC	10	7·8 ± 0·4	5·7 ± 0·1	0·7 ± 0·1	95·4 ± 1·4	46·6 ± 1·0	48·9 ± 1·7
40% Cassava + 15% palm oil + 10% LPC	10	6·3 ± 0·5	4·7 ± 0·6	0·7 ± 0·0	96·1 ± 1·0	48·1 ± 1·1	49·7 ± 1·5
40% Cassava + 20% palm oil + 10% LPC	10	5·7 ± 0·7	4·2 ± 0·7	0·7 ± 0·1	96·4 ± 0·7	42·9 ± 1·4	44·5 ± 1·7
40% Cassava + 25% palm oil + 10% LPC	10	5·8 ± 0·9	4·1 ± 0·7	0·7 ± 0·1	94·6 ± 1·6	43·1 ± 2·1	45·6 ± 2·0
40% Cassava + 10% palm oil* + 0·15% Methionine + 10% LPC (as protein)	8	7·4 ± 0·7	15·5 ± 2·5	2·1 ± 0·2	87·4 ± 5·0	72·6 ± 0·8	83·3 ± 5·0
40% Cassava + 10% palm oil/ + 0·15% Methionine + 0·25% Lysine**	8	15·9 ± 2·1	49·4 ± 4·8	3·1 ± 0·3	86·9 ± 2·1	80·1 ± 0·5	92·1 ± 1·1

* Umoh & Oke (1977).
** Umoh *et al.* (1978).

TABLE 5

Protein Consumption and the Performance of the Rats on the Sulphur Supplemented LPC–Cassava Starch Mixture (Mean values ± SD)

Treatment	*Number of rats*	*Protein consumption*	*Weight gain/loss*	*PER*	*TD*	*NPU*	*BV*
Basal diet	10	0·9 ± 0·0	−9·3 ± 0·6	—	—	—	—
Maize starch + LPC	10	13·5 ± 0·5	23·2 ± 1·5	1·7 ± 0·1	88·6 ± 1·8	65·8 ± 0·9	74·4 ± 0·5
40% Cassava + LPC	10	7·8 ± 0·4	5·7 ± 0·4	0·7 ± 0·1	85·0 ± 1·4	46·6 ± 1·0	48·9 ± 1·7
40% Cassava + LPC + 0·15% Meth.*	10	10·1 ± 0·3	21·3 ± 0·9	2·2 ± 0·2	87·4 ± 3·1	72·6 ± 0·4	83·3 ± 3·3
40% Cassava + LPC + 0·15% Meth. + 0·25% Lys.**	10	15·9 ± 1·3	49·4 ± 2·8	3·1 ± 0·2	83·3 ± 2·7	75·7 ± 0·7	92·1 ± 1·1
40% Cassava + LPC + elemental sulphur	10	10·5 ± 0·2	18·9 ± 1·1	1·8 ± 0·1	90·3 ± 2·1	67·3 ± 1·5	74·5 ± 2·5
40% Cassava + LPC + sodium thiosulphate	10	9·8 ± 1·0	11·3 ± 0·5	1·1 ± 0·2	95·2 ± 3·4	50·5 ± 0·9	53·0 ± 1·6
40% Cassava + LPC + sodium sulphate	10	9·2 ± 0·2	17·8 ± 1·5	1·9 ± 0·1	93·3 ± 3·3	69·2 ± 0·8	74·2 ± 1·1
40% Cassava + LPC + ferrous sulphate	10	9·5 ± 0·9	18·0 ± 1·1	1·9 ± 0·3	91·2 ± 2·1	70·3 ± 1·9	77·1 ± 2·3

* Umoh & Oke (1977).

** Umoh *et al.* (1978).

considerably reduced (23·8) compared with casein and this resulted in a very low BV of 25 %. When palm oil was added at the 5 % level there was a slight improvement in NPU (38·1) and BV (40·3) but the PER remained the same (0·3). At the 10 % level the PER was more than doubled (0·7) and there was a considerable improvement in both NPU (46·6) and BV (48·9). A subsequent gradual increase to 25 % did not have any further effects on the different parameters.

Table 3 shows that the rations were virtually isocaloric. As shown in Table 5, supplementation with 0·15 % methionine raised the PER to 2·2, the NPU to 72·6 and the BV to 83·3. On the other hand, when elemental sulphur was used as the sulphur donor, the PER gave 1·8 which is still higher than the control without sulphur (0·7) but not as good as methionine. The digestibility was slightly improved (90·3) but the NPU (67·3) and BV (74·5) were inferior to that of the diet with methionine. Sodium thiosulphate was less effective still, with a PER of 1·1. Again, the digestibility was improved (95·2) but the NPU (50·5) and BV (53·0) were much lower than those of the diet with methionine. Sodium sulphate and ferrous sulphate were very similar, both giving a PER of 1·9 and very close NPU and BV values.

DISCUSSION

Fat is a concentrated form of energy and so, in such an experiment, ensuring that the diet is isocaloric is imperative before any justifiable conclusion can be drawn, especially as addition of fats to nutritionally adequate diets sometimes produces slight increases in growth with improvement in feed efficiency. In this experiment the different rations were fairly isocaloric and so some direct comparison is possible. Palm oil is chosen mainly because it is the local oil available and also traditionally it is used as an antidote to cyanide toxicity.

Apart from increasing the digestibility and improving the BV and NPU of the diet, palm oil doubled the PER (from 0·3 to 0·7). After the 10 % level it did not seem to have any further effect on the PER, BV, TD or NPU, which is in agreement with the results of Hew (1975) who reported a faster rate of weight gain from the addition of palm oil up to 8 % to cassava sago diet, beyond which a plateau occurred. When the cassava level is low (10–24 %) it appears that the level of oil does not matter. Thus, Devendra & Hew (1977) found, at this level of cassava, that the addition

of palm oil ranging from 0–30 % did not affect the rate of weight gain and feed efficiency and carcass characteristics, but suggested a maximum level of 5 % for pigs.

There are a few explanations which could be advanced for the observed beneficial effect of palm oil. The results in Table 4 show that the presence of 5 % palm oil increased the protein (feed) intake from 4·7 g to 7·3 g and the maximum intake was 7·8 g at the 10 % palm oil level, after which the intake started to drop again. Thus, palatability is increased by the presence of palm oil. Another explanation may be the provision of essential fatty acids, although this may be provided by other components of the ration. It therefore appears that improved palatability may be the main factor.

Not all sulphur-containing compounds can act as sulphur donors to rhodanese. In fact, compounds such as sodium sulphide, dithiobiuret and cystine were found to act as inhibitors and their action has been explained on the basis of blocking of the enzyme rhodanese so that it cannot combine with thiosulphate (Saunders & Himwich, 1948). On the other hand, Sorbo (1951) did not obtain any inhibition with cysteine alone and has established the mechanism for the reaction so that sulphate, thiosulphate and other sulphydryl compounds can react with the active group (i.e. an active disulphide group rather than an —SH group) in the enzyme. With no sulphur donor in Table 5, PER was 0·7 but when methionine was used as the sulphur donor the PER was more than trebled (2·2) and there were also significant increases in the NPU and BV. It was only the TD that was not affected by the sulphur donor.

Elemental sulphur seems to be effective as a substitute for methionine in the detoxification of cyanide with a PER of 1·8 which is slightly higher than even the LPC ration without cassava (PER 1·7). Better still, sodium sulphate and ferrous sulphate gave PER values of 1·9 so that sulphates in general seem to be equally effective. What was surprising was that sodium thiosulphate, which might be anticipated to be the most reactive, was the least reactive (PER 1·1). If it is assumed, according to Sorbo (1951), that the mechanism of the reaction is by hydrocyanolysis of the first disulphide compound formed by the action of thiosulphate on rhodanese followed by splitting off of sulphite and the formation of thiocyanate, as in the reaction of hexathionate with cyanide (Foss, 1950), then the detoxification should be facilitated more by thiosulphate than sulphates.

Since, apart from the need for methionine for protein synthesis, it is also needed for the supply of SO_4^{2-} by oxidation to meet the specific

requirements of the body, it is possible that the addition of sulphate as a source of sulphur helps to spare methionine for the supply of this anion and so results in improvement of the ration, resulting in a weight gain (18·9 g) that is very close to that of methionine supplementation (21·3). Barrett *et al.* (1977) have also found sulphate to be effective in the detoxification of cyanide when given to rats as KCN. However, the weight gain and the urinary and plasma thiocyanate levels were much higher in the methionine supplementation than in the potassium sulphate. They also showed that the sulphur in the sulphate ion actually took part in the detoxification process by using ^{35}S K_2SO_4 and ^{35}S methionine.

Our results are very similar to those of Job (1975) who reported a PER value of 2·09 for a cassava-based control diet for pigs that was improved significantly to PER 2·39 by supplementation with 0·2 % methionine and also with 0·2 % elemental sulphur (PER 2·32). However, 0·785 % sodium thiosulphate did not give a significant increase (PER 2·22). It therefore appears that elemental sulphur and sulphate ion can also act as sulphur donors for detoxifying cyanide in monogastric animals.

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Changes in the Protein Complex of Wheat Dough Affected by Soybean 11 S Globulin: Part 2—The Interactions of Soybean 11 S Globulin with Gluten Proteins

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ABSTRACT

The interactions between soy 11 S globulin and protein fractions of wheaten doughs were examined using a multistep extraction method followed by molecular sieving and characterisation of the fractions obtained. The soy 11 S globulin preparation was coupled with fluoresceine isothiocyanate to permit tracing of this protein in the complexes formed. In model experiments the average molecular weights of globulin/prolamine complexes were determined, the dispersion of light on the protein molecules being measured. The formation of high molecular weight complexes of soy 11 S globulin and prolamine was found. A shift between albumin/globulin and gluten fractions was also observed, which resulted in an increase in the contents of low molecular weight fractions dispersible in pyrophosphate buffer and acetic acid.

Most of the investigations concerning the enrichment of bread with soybean proteins deal with technological problems. The documentation of the chemically induced changes in the dough–protein complex is rather limited and a number of questions are still awaiting answers. The data reported by Jakubczyk et al. (1973), Matthews (1972), Pollock & Geeds (1960a, b) do not explain the character of the interactions between soy proteins and gluten systems and give only inadequate information on the changes of molecular structure or aggregation/disaggregation phenomena occurring in the proteins of enriched dough. The results presented in

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our previous study (Lampart-Szczapa & Jankiewicz, 1982, encouraged us to continue the experiments to explain the rôle played by soybean 11 S globulin treated as a model soy protein in modifying the gluten matrix of wheat dough. In this paper the effects of soybean 11 S globulin on the fractional distribution of the dough–protein complex and average molecular weights of the fractions are presented.

MATERIALS AND METHODS

In the study laboratory flours obtained from wheat grain of the Grana and Mironowska varieties were used. Their properties were described in the first part of this paper (Lampart-Szczapa & Jankiewicz, 1982). The soybean 11 S globulin used in the experiments was isolated from defatted soy meal according to the slightly modified procedure of Wolf *et al.* (1962). The preparation was purified as described by Catsimpoolas *et al.* (1967). To prevent aggregation of the globulin preparation the protein dispersion was stabilised with 2-mercaptoethanol. Just before their use the protein dispersions were concentrated in the course of dialysis against buffered polyethyleneglycol (molecular weight 20 000), pH and ionic strength were adjusted and 2-mercaptoethanol removed by filtration on Sephadex G-100 columns under conditions excluding oxygen uptake. For part of the experiments the globulin was coupled with fluoresceine isothiocyanate as described by Rinderknecht (1962) to enable the tracing of the incorporation of such modified protein into separate gluten fractions using a fluorescence technique.

The gliadin preparation used in the experiment was obtained by fractionating BDH commercial gliadin on columns filled with Sephadex G-100. The second fraction eluted was taken for further studies.

Both the soy globulin and gliadin preparations were characterised by a molecular sieving technique—soy globulin additionally by an electrophoretic technique to demonstrate the degree of their homogeneity.

As shown in Figs 1 and 2, the soybean 11 S globulin preparation was homogeneous whereas the gliadin one contained small quantities of polymerisation products.

In the course of the experiments the fractional distribution of the protein complex in dough enriched with soybean 11 S globulin coupled with fluoresceine isothiocyanate was analysed (Table 1).

The multistep extraction procedure and characterisation of the protein

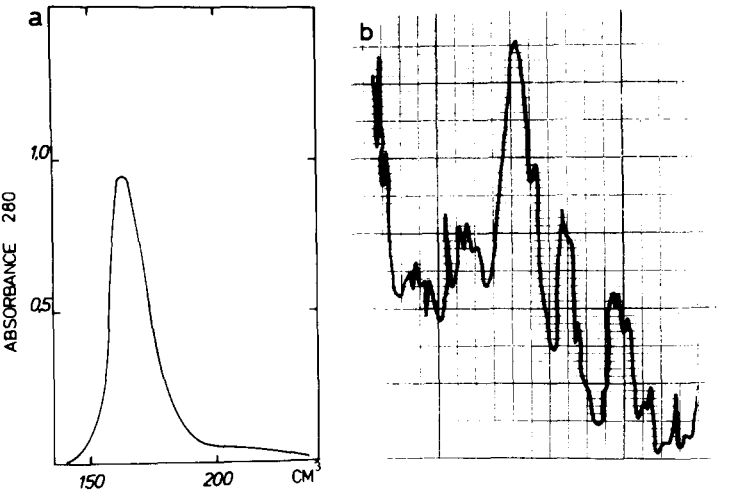

Fig. 1. The characteristics of the preparation of soybean 11 S globulin. (a), Eluogram. 20 mg protein. Column—Pharmacia K 26/95, Sephadex G100, phosphate buffer, pH 7·6, $\mu = 0{\cdot}5$, 0·01 M 2-mercaptoethanol; flow, 30 cm^3/h. (b) Electrophoregram. 0·5 mg protein. Polyacrylamide gel—Cyanogum 41, 6%, 130 V, 60 mA, 4 h, 18 °C.

dispersions by molecular sieving and fluorescence measurements for the fractions eluted were applied. (See Lampart-Szczapa & Jankiewicz, 1982.)

In a separate experiment a model system for studying the interactions between soybean 11 S globulin and gliadin was designed. The dispersions of globulin and gliadin proteins were mixed together (in the ratio of 1:4 in

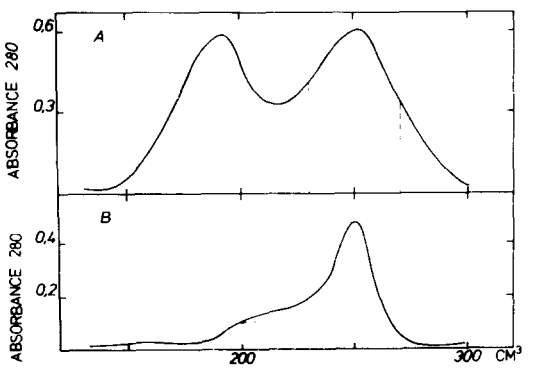

Fig. 2. The characteristics of the gliadin preparations. A. Crude preparation. B. Purified fraction used in experiments. 30 mg protein. Column—Pharmacia K 26/95, Sephadex G-100, acetate buffer, pH 3·25, $\mu = 0{\cdot}07$; flow, 30 cm^3/h.

TABLE 1
The Contents of the 11 S Soybean Globulin Preparation in the Experimental Doughs*

Wheat variety	*Addition of 11 S soybean globulin preparation*				
	Level of 11 S globulin addition	*In per cent of dry substance of flour*	*In per cent of protein contents*		
			Proteins dispersible in acetic acid	*Gliadins*	*Total proteins*
Grana	0	—	—	—	—
	I	0·37	5·3	7·8	2·7
	II	0·55	7·9	11·6	4·1
	III	1·10	15·8	23·3	8·2
Mironowska	0	—	—	—	—
	I	0·29	5·0	7·1	2·5
	II	0·44	7·5	10·7	3·8
	III	0·88	15·0	21·5	7·5

* Globulin coupled with fluoresceine isothiocyanate.

amounts corresponding to the addition of 4% of soy product to wheat flour, i.e. level II of globulin addition to the dough) in buffered media of programmed pH and ionic strength covering the ranges occurring in the bread dough. The changes of average molecular weights of the protein complexes were determined using the technique of measurement of light dispersed on protein molecules (Piechowiak *et al.*, 1975). The mixtures of globulin and gliadin proteins were kept for 2 h at room temperature prior to analysis.

RESULTS AND DISCUSSION

The laboratory flours used in this study were characterised technologically (Lampart-Szczapa & Jankiewicz, 1982) and represented examples of wheat flour of good (Mironowska) and medium (Grana) technological properties.

The preparation of soybean 11 S globulin was homogeneous, as shown in Fig. 1. The physico-chemical properties of the preparation corresponded to those described by other authors (Eldridge & Wolf, 1967; Hill & Braidenbach, 1974; Wolf, 1972). The preparation of gliadin contained small quantities of polymerisation products (Fig. 2). Similar impurities of the gliadin purified repeatedly on Sephadex columns were reported previously (Hlynka, 1964; Jankiewicz, 1968; Preston & Woodbury, 1976). These impurities are related to the dynamic balance between gliadin forms of different degrees of polymerisation.

The incorporation of soybean 11 S globulin into different protein fractions extracted from enriched dough was demonstrated by fluorescence measurements (Table 2). The changes of fluorescence of protein dispersions resulting from the increasing quantities of globulin preparation introduced into the dough system indicated the incorporation of that protein into gluten proteins. In the case of glutenin-like proteins dispersible in sodium hydroxide, the incorporation was especially intense. The fluorescence data obtained for protein dispersions in the pyrophosphate extract were highly correlated with globulin additions (Fig. 3). The increase in fluorescence of gluten proteins dispersed in acetic acid was relatively lower for the highest globulin addition, especially in the case of wheat variety of better technological properties (Mironowska). For gluten proteins dispersed in sodium hydroxide the fluorescence data demonstrated similar values in the case of globulin addition levels I and II

TABLE 2
The Effects of Modified 11 S Soybean Globulin Addition* on the Fluorescence of Proteins Extracted from Experimental Doughs

Wheat variety	*Levels of 11 S globulin addition*	*Fluorescence of protein dispersion calculated as fluoresceine isothiocyanate (ng)*		
		In 1 cm^3 of protein extract	*On the basis of 0·0028 mg N/cm^3*	
			Determined	*Corrected***
	Dispersions in 0·01M pyrophosphate buffer, pH 7			
Grana	0	8·45	1·37	—
	I	54·79	9·29	7·92
	II	72·81	13·00	11·63
	III	126·64	21·83	20·46
Mironowska	0	10·15	1·69	—
	I	45·65	8·30	6·61
	II	57·97	10·74	9·05
	III	109·36	19·53	17·84
	Dispersions in 0·05M acetic acid, pH 3·1			
Grana	0	138·60	6·57	—
	I	356·52	14·06	7·49
	II	442·12	16·62	10·05
	III	611·18	21·37	14·80
Mironowska	0	106·50	4·60	—
	I	180·46	9·68	5·08
	II	247·74	11·87	7·27
	III	373·42	15·89	11·29
	Dispersions in 0·01M sodium hydroxide			
Grana	0	2 308·00	276·53	—
	I	2 871·79	652·68	376·15
	II	4 383·74	687·65	411·12
	III	4 265·73	1 398·60	1 122·07
Mironowska	0	2 564·00	211·84	—
	I	4 220·28	594·41	382·57
	II	3 882·12	617·72	405·88
	III	7 974·94	1 340·33	1 128·49

* Coupled with fluoresceine isothiocyanate.
** Fluorescence of control doughs subtracted.

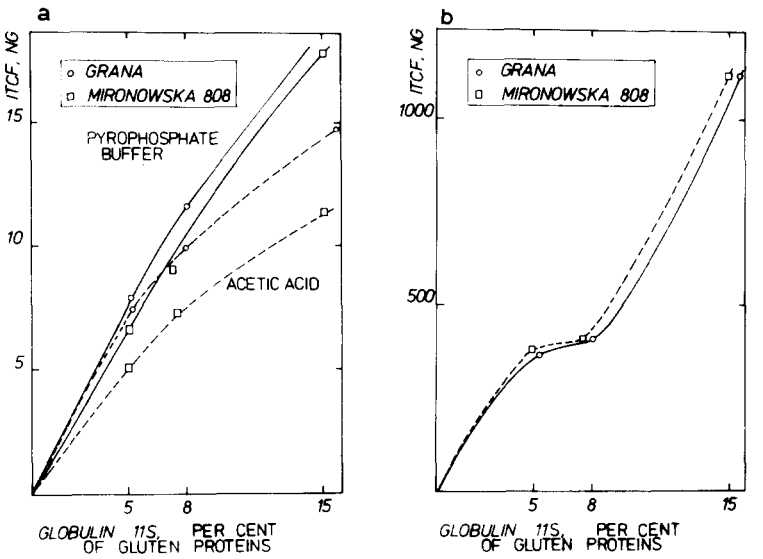

Fig. 3. The changes of fluorescences (measured for the protein solutions of 0·028 mg N/cm^3 concentration; the solutions as stated in Table 2) of protein extracts obtained from wheat dough enriched with the soybean 11 S globulin preparation (soybean 11 S globulin coupled with fluoresceine isothiocyanate). (a) Pyrophosphate buffer, acetic acid; (b) sodium hydroxide.

and a high increase corresponding to level III (Fig. 3). The varietal effect was evident.

The results obtained in the experiments suggest participation of soybean 11 S globulin in the formation of highly aggregated protein complexes showing lowered dispersibility, or even insolubility, under experimental conditions.

More detailed information was gained after fractionation of the protein dispersions described above. The measurements of fluorescence of fractions eluted from Sephadex columns demonstrated rather limited incorporation of soybean 11 S globulin into albumin and globulin proteins dispersible in pyrophosphate buffer (Fig. 4) whereas, in the case of gluten proteins dispersible in acetic acid, most of the globulin preparation added was identified in glutenin-like fractions of highest molecular weight (Fig. 5). The changes registered for glutenin-like fractions were more intense for the Grana variety, which can be explained in terms of the lower tolerance of 'weaker' wheat to modifying agents as is

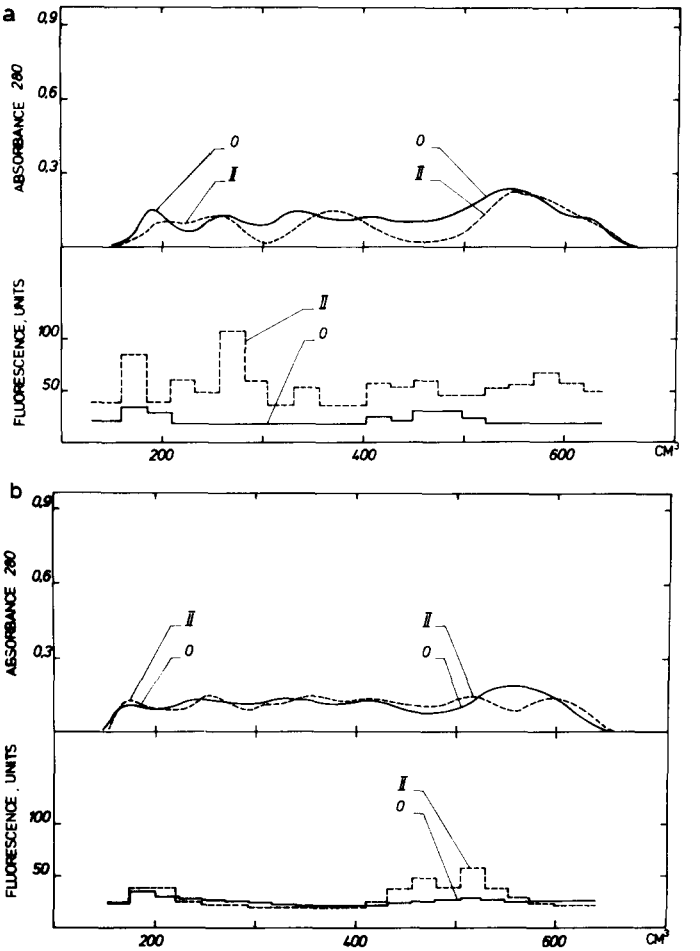

Fig. 4. The effects of addition of soybean globulin to wheat dough on fractional composition of proteins dispersible in pyrophosphate buffer. (a) Grana variety. (b) Mironowska variety. The soybean 11 S globulin was added as stated in Table 1, level II, 20 mg protein. Column—Pharmacia K 26/95, Sephadex G_3 100, 0·01 M pyrophosphate buffer, pH 7; flow, 30 cm^3/h.

well known in cereal technology (Hlynka, 1964; Jankiewicz, 1975; Jankiewicz *et al.*, 1974). The highly labile protein complex of wheat dough is easily modified by changes of the medium or of any additions. Changes of pH or ionic strength especially, result in advanced aggregation or disaggregation of the system (Gasiorowski & Jankowski, 1967; Hlynka, 1964; Jankiewicz, 1975). The conformational changes of protein mol-

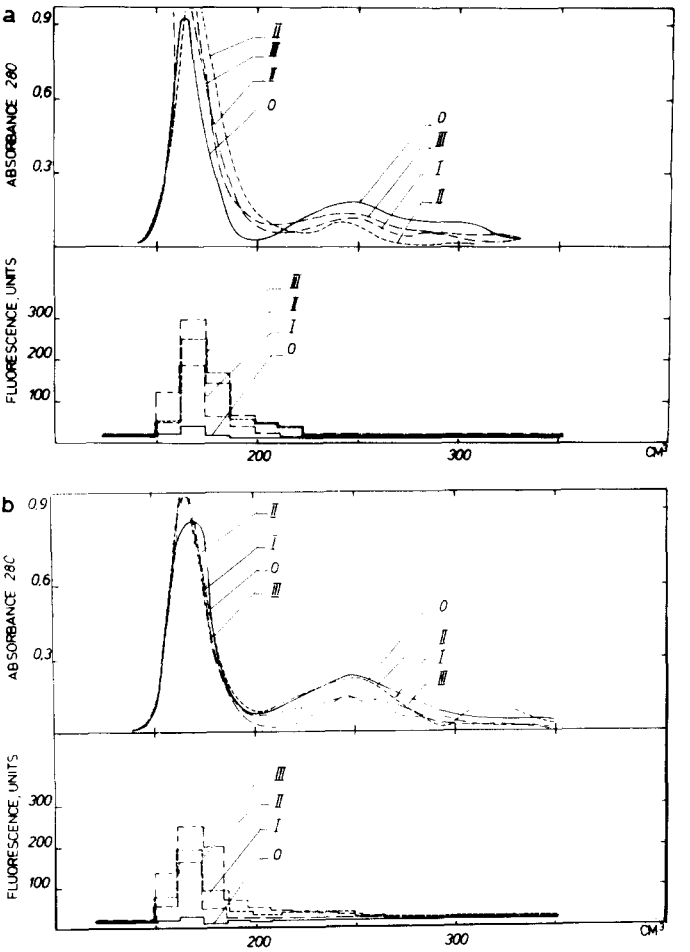

Fig. 5. The effects of addition of soybean 11 S globulin to wheat dough on fractional composition of proteins dispersible in acetic acid. (a) Grana variety. (b) Mironowska variety. The soybean 11 S globulin was added as stated in Table 1, levels I, II and III, 30 mg protein. Column—Pharmacia K 26/95. Sephadex G100, 0·05M acetate buffer, pH 4·1; flow, 30 cm^3/h.

ecules cannot be avoided and should be taken into account in any experiment (Jankiewicz & Kaczkowski, 1972).

Therefore, in a separate study, the rôle of pH and ionic strength changes in the formation of soybean 11 S globulin/gliadin aggregates was characterised. The behaviour of soybean 11 S globulin in different media (pH and ionic strength) has been described by other authors

(Catsimpoolas *et al.*, 1969; Hatsiya *et al.*, 1972*a*, *b*; Ishino & Okamoto, 1975; Megen, 1974; Okubo & Shibasaki, 1974; Wu & Inglett, 1974). In buffered solutions adjusted to pH 7 and ionic strength $\mu = 0{\cdot}5$ the monomeric form of soybean 11 S globulin prevails.

The lowering of ionic strength to about $\mu = 0{\cdot}1$ stimulates aggregation of that protein, whereas further lowering of ionic strength leads to dissociation of complexes formed. To prevent oxidation changes the authors recommended the use of 2-mercaptoethanol in concentrations of 0·01 M as a protective agent.

In our experiments this reagent was removed from protein dispersion to prevent its destructive effect on gliadin, used as a model cereal protein (Jankiewicz & Kaczkowski, 1972) for interaction studies.

Some interference due to oxidation reactions in the results obtained was obvious in this case. The average molecular weights calculated for soybean 11 S globulin preparation in selected medium changed in this study (Table 3) as reported in the literature. The dissociation effect typical of a medium of low ionic strength was less evident however, probably due to the changes of oxidative character. The highest aggregation of soybean 11 S globulin was demonstrated for ionic strength $\mu = 0{\cdot}1$. The average molecular weights calculated for gliadin under the same conditions as for soybean 11 S globulin were affected by pH and ionic strength changes. The lowest values were found for pH 5·5 and ionic strengths of $\mu = 0{\cdot}01$ and $\mu = 0{\cdot}05$. Increased pH and ionic strength resulted in the aggregation of that protein.

The behaviour of a mixture of soybean 11 S globulin with gliadin was very different from that found for each of these proteins. The increase in average molecular weights was especially intense for pH 6 and 7 and ionic strength $\mu = 0{\cdot}01$, as well as for pH 7 and ionic strength $\mu = 0{\cdot}05$ or pH 5 and ionic strength $\mu = 0{\cdot}5$. The results obtained provide additional evidence for the formation of the aggregates from gluten and soy proteins in conditions typical of wheat dough. In most cases the average molecular weights calculated for the system were much higher than those determined for separate components. The conditions typical of wheat dough (pH 6, $\mu = 0{\cdot}05$–$0{\cdot}10$) stimulated the aggregation of protein complexes.

The results presented in this study demonstrate the lability of a protein complex of the wheat dough enriched with soy protein preparation and an aggregation phenomenon changing the technological properties of the system.

TABLE 3
The Effects of pH and Ionic Strength of Experimental Doughs on Molecular Weights* of 11 S Soybean Globulin and Gliadin Preparations and Products of their Interactions

Protein	*pH of buffer solutions***	*Molecular weights (Daltons) Ionic strength (μ)* 0·01	0·05	0·10	0·50
Globulin 11 S	5·0	44 000	45 000	1 727 000	233 000
	5·5	676 000	143 000	606 000	858 000
	6·0	231 000	—	1 240 000	518 000
	7·0	400 000	1 100 000	2 857 000	400 000
Gliadin	5·0	150 000	105 000	97 000	180 000
	5·5	70 000	68 000	80 000	130 000
	6·0	215 000	80 000	100 000	196 000
	7·0	200 000	200 000	186 000	180 000
Gliadin + globulin 11 S	5·0	94 000	88 000	556 000	10 000 000
	5·5	678 000	172 000	338 000	1 250 000
	6·0	9 000 000	2 500 000	3 500 000	707 000
	7·0	8 000 000	20 000 000	2 000 000	1 502 000

* The average molecular weight calculated from measurements of light dispersed on protein molecules.
** pH 5·0, 5·5, 6·0—acetate buffer; pH 7·0—phosphate buffer.

CONCLUSIONS

(1) Evidence that soy globulins mostly interact with gluten proteins of dough, forming glutenin-like complexes of high molecular weight, has been presented.
(2) This reaction results in the lowering of the dispersibility of gluten proteins in acetic acid. The formation of aggregates indispersible under normal conditions is highly probable.
(3) The formation of the aggregates consisting of soy globulin and gluten proteins is counterbalanced by disaggregation of part of the gluten proteins. The disaggregation products cause slight increase in the contents of pyrophosphate and acetic acid dispersible protein fractions of wheat flour as evidenced by comparison of the nitrogen and fluorescence distributions for the extracts analysed (Parts 1 and 2 of this study).

(4) The gluten protein complex reacts with soy globulin with varying intensities according to varietal breadmaking potential. Weak wheat is less tolerant to the modifying effect of soy globulin than wheat of good technological quality.

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Polyhydroxy Flavonoid Antioxidants for Edible Oils. Phospholipids as Synergists

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ABSTRACT

The synergistic effects of phosphatidyl ethanolamine and phosphatidyl choline in enhancing the antioxidant properties of some polyhydroxy flavonoids in lard at 100–140°C have been investigated. Phosphatidyl ethanolamine is very effective in all cases, especially when used at concentrations upwards of 0·1%, with flavonoids at levels of 0·007% to 0·07%. Phosphatidyl choline has little synergistic activity.

Possible causes of the synergism are discussed. It is concluded that the presence in the synergist molecule of a strongly acid, proton generating function is of importance.

INTRODUCTION

The polyhydroxy flavones, which are of widespread natural occurrence, have been shown to be important components of vegetable tissues by virtue of their pronounced effect in stabilising the lipid fractions against autoxidation (Herrmann, 1976; Hudson & Mahgoub, 1980). However, the unexpectedly high stability of isolated vegetable structural lipids could not be accounted for purely on the basis of the presence of primary antioxidants. In a preliminary communication (Hudson & Mahgoub, 1981) we showed that it appeared to be due to the simultaneous presence of primary antioxidants and a group of synergists, essentially phospholipids.

The antioxidant properties of the polyhydroxy flavones, and some related compounds, have been described by various authors during the

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past twenty-five years, although there have been conflicting findings as to details. In a recent communication (Hudson & Lewis, 1983) we reported the results of an investigation of the effects of eleven relevant compounds in model systems. We were able to relate antioxidant efficiency to chemical structure and our general conclusion was that polyhydroxy flavonoids functioned mainly as *primary* antioxidants, i.e. that, since they did not exhibit more than a very minor degree of synergism with α-tocopherol, they could not justifiably be claimed to exhibit the bimodal function sometimes ascribed to them (Pratt, 1976).

The synergism which we had observed in a very few cases between the polyhydroxyflavone, quercetin, and phospholipids, reinforced the above view, but it seemed necessary to establish it more firmly. The present paper reports synergistic effects obtained by the co-operation of phosphatidyl ethanolamine with a range of polyhydroxy flavonoids in model systems.

MATERIALS AND METHODS

Materials

Lard, used as a substrate for all the stability tests, was kindly donated by Messrs Scot Bowyers Ltd, Trowbridge, Wilts, Great Britain. It was unrefined, had not been chemically processed and was free from added antioxidants.

Seven primary antioxidants, i.e.

Quercetin	3:5:7:3^1:4^1-Pentahydroxy flavone
Fisetin	3:7:3^1:4^1-Tetrahydroxy flavone
Luteolin	5:7:3^1:4^1-Tetrahydroxy flavone
Taxifolin	3:5:7:3^1:4^1-Pentahydroxy flavan(4)one
Fustin	3:7:3^1:4^1-Tetrahydroxy flavan(4)one
Eriodictyol	5:7:3^1:4^1-Tetrahydroxy flavan(4)one
Butein	3:4:2^1:4^1-Tetrahydroxy chalcone

were selected for investigation. Quercetin was purchased from Koch-Light Laboratories, Ltd, Colnbrook, Bucks, Great Britain, and taxifolin from Sigma London Chemical Co. Ltd, London, Great Britain. The less accessible flavonoids, and butein, were purchased from Apin Chemicals Ltd, Cardiff, Wales, Great Britain.

1:2-Dipalmitoyl-*sn*-glycerol-3-phosphoethanolamine and the analogous choline derivative were synthetic compounds purchased from Sigma London Chemical Co. Ltd who also supplied egg and soyabean phosphatidyl ethanolamines as isolated from natural sources and stated to be 98% pure.

Methods

Induction periods (IP's) were determined as previously described (Hudson & Lewis, 1983) in the Metrohm Rancimat. Data shown in Tables 1 to 4 are means of duplicate determinations, which very rarely differ from the mean value by more than $\pm 5\%$. Synergism was calculated by the method of Bishov & Henick (1972) using the formula:

$$\% \text{ Synergism} = \frac{100[(I_M - I_L) - (I_A - I_L) - (I_S - I_L)]}{(I_M - I_L)}$$

Where: I_L = Induction period of the substrate (in this case, lard).
I_A = Induction period of the substrate + primary antioxidant.
I_S = Induction period of the substrate + synergist.
I_M = Induction period of the substrate + primary antioxidant + synergist.

The component fatty acids of egg and soyabean PE were determined by gas chromatographic analysis of the methyl esters, using a Pye 104 chromatograph at 190°C, on a 12% PEGS column.

RESULTS AND DISCUSSION

The synergistic effects of 1:2-dipalmitoyl-*sn*-glycerol-3-phosphoethanolamine (PE) and -choline (PC)

In order to investigate the relationship between synergistic activity, as measured by the extension of the induction period (IP), and the relative proportions of synergist to primary antioxidant, a fixed level of primary antioxidant, known from previous studies to show an appreciable protective effect, was maintained whilst PE and PC levels were varied over a wide range. The level of primary antioxidant was $2{\cdot}3 \times 10^{-3}$M with PE or PC at from 0·25 to 4·0 times this molarity. It was thought that this

TABLE 1

Synergism Between Dipalmitoyl Phosphatidyl Ethanolamine (PE) and High Levels of Polyhydroxy Flavonoids in Lard at 140 °C

Flavonoids	*Molar ratios of PE* to flavonoid. Flavonoids at* $2{\cdot}3 \times 10^{-3}$ M										
	0:1·0	*0·25:1·0*		*0·5:1·0*		*1·0:1·0***		*2·0:1·0*		*4·0:1·0*	
	IP (h)	*IP (h)*	*% Syn*	*IP (h)*	*% Syn*	*IP (h)*	*% Syn*	*IP (h)*	*% Syn*	*IP (h)*	*% Syn*
Quercetin	2·0	2·9	32	4·3	54	6·9	71	16·6	87	19·1	88
Fisetin	2·5	ND		4·5	45	8·4	70	18·9	86	ND	
Luteolin	1·2	ND		1·7	29	3·6	66	11·5	89	ND	
Taxifolin	2·7	3·4	21	5·2	48	8·9	69	16·9	83	20·0	85
Fustin	2·8	ND		5·2	46	8·6	67	20·4	86	ND	
Eriodictyol	1·9	ND		2·6	26	3·8	49	11·5	83	ND	
Butein	4·2	ND		4·3	1	5·3	19	16·0	73	ND	

* IP's for PE alone in lard at 140 °C were 0·13, 0·14, 0·17, 0·23, 0·33 and 0·59 h for molarities of 0, 0·25, 0·5, 1·0, 2·0 and $4{\cdot}0 \times 2{\cdot}3 \times 10^{-3}$, respectively.

** Expressed in familiar terms this is about 0·16 % dipalmitoyl phosphatidyl ethanolamine and 0·07 % quercetin.

ND = Not determined.

range might afford data that could provide a clue as to the mechanism of the synergistic effect. In our earlier paper (Hudson & Lewis, 1983) the IP's were determined at 100 °C, but once it was found that synergistic effects were very pronounced and the induction periods were prolonged, the test temperature was raised for convenience to 140 °C.

Results for PE are shown in Table 1 and for PC in Table 2. It is apparent from Table 1 that the effects of all the primary antioxidants are substantially enhanced by the presence of PE. As the molar proportion of PE to primary antioxidant rises to 2:1 the relationship is very roughly linear. Beyond 2:1 there is still an increase in IP but it becomes proportionately smaller. The order of antioxidant activity of the individual polyhydroxy flavones is hardly affected by the presence of synergist. As had already been noted in our previous paper, those flavonoids which incorporate a 3-hydroxy group are more effective stabilisers than those, i.e. luteolin and eriodictyol, in which only a 5-hydroxyl group is present.

The data presented in Table 2 show that PC is much inferior, as a synergist, to PE. Some synergism can be observed for quercetin but none at all for taxifolin. For taxifolin a definite antagonistic or pro-oxidant effect is apparent.

In our earlier studies it had been shown that the polyhydroxy flavonoids were much less effective at a low concentration ($2{\cdot}3 \times 10^{-4}$M, approximately 0·007%) than at the higher level already discussed. It therefore seemed important to find out whether, at the lower levels of flavonoids, PE could still function as a useful synergist. The results of these studies are shown in Table 3, with levels of synergist (0·5 to $2{\cdot}0 \times 2{\cdot}3 \times 10^{-3}$M dipalmitoyl phosphatidyl enthanolamine) that had already

TABLE 2

Synergism Between Dipalmitoyl Phosphatidyl Choline (PC) and Quercetin and Taxifolin in Lard at 140 °C

Flavonoids	*Molar ratios of PC* to flavonoid. Flavonoids at* $2{\cdot}3 \times 10^{-3}$M								
	0:1·0	*0·25:1·0*		*0·5:1·0*		*1·0:1·0*		*2·0:1·0*	
	IP(h)	*IP(h)*	*% Syn*	*IP(h)*	*% Syn*	*IP(h)*	*% Syn*	*IP(h)*	*% Syn*
Quercetin	2·0	3·4	42	3·3	40	2·0	—	2·8	31
Taxifolin	2·7	2·2	—	2·0	—	1·0	—	1·4	—

* IP's for PC alone in lard at 140 °C were 0·13, 0·16, 0·15, 0·10, 0·10 h for molarities of 0, 0·25, 0·5, 1·0 and $2{\cdot}0 \times 2{\cdot}3 \times 10^{-3}$, respectively.

TABLE 3

Synergism Between Dipalmitoyl Phosphadtidyl Ethanolamine (PE) and Low Levels of Polyhydroxy Flavonoids in Lard at 100 °C

Flavonoids	*Molar ratios of PE* to flavonoid. Flavonoids at* $2{\cdot}3 \times 10^{-4}$M						
	0:1·0	*5·0:1·0*		*10·0:1·0*		*20·0:1·0*	
	IP (h)	*IP (h)*	*% Syn*	*IP (h)*	*% Syn*	*IP (h)*	*% Syn*
Quercetin	7·1	8·3	11	24·3	72	31·6	77
Fisetin	8·5	ND		27·1	69	30·0	71
Luteolin	4·3	ND		12·5	66	13·7	66
Taxifolin	8·2	9·9	15	13·9	39	14·8	40
Fustin	6·7	ND		16·3	59	23·0	69
Eriodictyol	6·7	ND		14·5	53	17·3	58
Butein	14·0	ND		29·7	53	33·5	57

* IP's for PE alone in lard at 100 °C were 1·4, 1·8, 2·2 and 2·7 h for molarities of 0, 0·5, 1·0 and $2{\cdot}0 \times 2{\cdot}3 \times 10^{-3}$, respectively.

been shown to perform successfully with the higher levels of polyhydroxy flavonoids (Table 1).

At the lower levels of flavonoids the synergistic effects are still clearly apparent, albeit not below the molar proportion of 10:1 for PE: flavonoid. In order to compare IP's at 100 °C (Table 3) with those at 140 °C (Tables 1, 2 and 4) the latter need to be multiplied by approximately 16. With these reservations and allowing for the much lower primary antioxidant activity of flavonoids at $2{\cdot}3 \times 10^{-4}$M as compared with $2{\cdot}3 \times 10^{-3}$M, the synergistic efficiency of PE is equally evident at both levels.

Variation of synergistic effect with fatty acid composition of phosphatidyl ethanolamine

Since phosphatidyl ethanolamines can either be extracted in a 'pure' form from natural sources or prepared synthetically, it was of interest to compare natural egg and soyabean PE with synthetic dipalmitoyl PE. The results are shown in Table 4, which also includes the results of component fatty acid analyses of the synergists.

Not surprisingly, the fully saturated synthetic dipalmitoyl PE is the most effective synergist for both quercetin and taxifolin. Egg PE is apparently much better than soyabean PE, despite its content of the

TABLE 4

Synergism Between Phosphatidyl Ethanolamines (PE) of Different Origins at 2:1 Molar Ratios with Quercetin and Taxifolin in Lard at 140 °C

Flavonoids	*Source of PE**									
	No PE	*Egg PE*			*Soyabean PE*			*Dipalmitoyl PE*		
	IP (h)	*IP (h)*	*% Syn*	*Fatty acids*	*IP (h)*	*% Syn*	*Fatty acids*	*IP (h)*	*% Syn*	*Fatty acids*
Quercetin	2·0	10·3	81		3·1	37		16·6	87	
Taxifolin	2·7	8·2	67		2·8	4		16·9	83	
Fatty acids										
16:0				20·6			26·6			100·0
18:0				33·5			3·2			—
18:1				24·2			6·5			—
18:2				14·7			57·1			—
18:3				0·2			6·6			—
20:4				6·8			—			—

* IP's for PE alone in lard at 140 °C, derived from egg and from soyabean oil, and synthetic dipalmitoyl PE, at $2 \times 2{\cdot}3 \times 10^{-3}$ M were 0·23, 0·12 and 0·33 h, respectively, and for lard alone 0·13 h.

tetraenoic acid, arachidonic acid (20:4). However, the overall unsaturation of soyabean PE is much greater than that of egg PE and this probably determines its inferiority as a synergist, whether as a result of oxidative instability or through other reactions.

CONCLUSIONS

Phosphatidyl ethanolamine (PE) and phosphatidyl choline (PC) have negligible activity as antioxidants *per se* in a lard substrate. However, PE, especially in the form of 1:2-dipalmitoyl-*sn*-glycerol-3-phosphoethanolamine, is a very effective synergist when used in conjunction with polyhydroxy flavonoids at levels of the order of 0·1% or more. The greater the concentration of primary antioxidant, the greater is the effect observed. On the other hand, under the same conditions, PC is comparatively ineffective.

The mechanism of the synergistic action is by no means clear. It is commonly suggested that phospholipids function by 'sequestering' trace metals and so reducing their pro-oxidant activity. Some support for this view has been provided by Lunde *et al.* (1976), who showed that metal ions could be transferred from an aqueous to a non-polar medium when the latter contained dissolved phospholipids. Our studies, however, have shown that the concentrations of PE required to show significant synergism are of a much higher molarity than would be needed to interact with the few parts per million at which trace metals are, at most, present in natural oils and fats.

Alternative views are that antioxidant synergists are substances capable of either (a) releasing protons and thus bringing about the rapid decomposition of hydroperoxides without generating free radicals (e.g. Tai *et al.*, 1974) or (b) effecting the regeneration of primary antioxidants and so prolonging their useful life (e.g. Brandt *et al.*, 1973). Such mechanisms might compete successfully with the normal free radical decomposition of hydroperoxides which promotes the propagation reaction. Other well recognised synergists, such as citric and phosphoric acids, may well act in the same way, although they are at a disadvantage in comparison with phospholipids in having very low oil solubility. If proton generation is a key factor in synergism the inferiority of PC relative to PE can be explained: PC cannot act as a proton donor since, at neutral pH, it occurs exclusively in the form of a zwitterion.

Finally, the work of Jewell & Nawar (1980) is relevant to this topic. These authors studied the oxidation of 1:2-dipalmitoyl-*sn*-glycerol-3-phosphoethanolamine by air at 150 °C, with particular reference to the pyrolysis products. The absence of aldehydes was ascribed to their reaction with the free amino group, and indeed condensation products of an appropriate kind were identified in the product at the end of the oxidation. The conditions used were similar to those obtaining in the Rancimat, especially when it was run at 140 °C. Undoubtedly at such temperatures phospholipids undergo chemical changes quite apart from the oxidative degradation of their fatty acid moieties, but such changes apparently do not inhibit their synergistic activity.

Further investigations are needed in order to throw more light on these aspects.

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Enhancement of Soft Cheese Flavour Using Animal Lipase Preparations

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ABSTRACT

Domiati cheese was made from milk incorporated with certain animal lipase preparations—i.e. Cacordase and Capalase L—at concentrations of 0·02% and 0·04% of each preparation. The addition of lipases to cheese milk at different concentrations had a slight effect on the main chemical composition of the cheese. The treatment enhanced the flavour development and the flavour intensity of 4-week-old cheese made from lipase-treated milk was more pronounced than that of an 8-week-old control cheese. After 8 weeks of pickling, a rancid flavour was developed in cheese made from milk treated with higher amounts of lipase (0·04% of milk weight).

INTRODUCTION

Domiati cheese is considered to be the main national soft cheese produced in Egypt. The development of desirable flavour in such cheese requires several months, particularly when the cheese is made from pasteurised milk. Recently, a great effort has been directed towards the acceleration of cheese ripening and enhancing the development of the characteristic flavour of cheese. Liebich *et al.* (1970) suggested that free fatty acids (FFA) are the basis of cheese flavour and that the characteristic aroma is, in particular, due to the ratio of FFA to other volatile components.

Acceleration of milk fat hydrolysis by the incorporation of certain

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lipase preparations into either cheese milk or curd has been applied in cheese making as a useful controlled method to enhance the development of cheese flavour (Richardson & Nelson, 1967; Richardson *et al.*, 1971; Roberts, 1971; Kosikowski, 1975; Peppler, 1975; Jolly & Kosikowski, 1978; Abdel Salam *et al.*, 1979; Sood & Kosikowski, 1979).

The aim of the investigation reported in this paper was to evaluate the development of Domiati cheese flavour as it is affected by the incorporation into cheese milk of certain animal lipase preparations—i.e. Cacordase (kid goat–lamb) and Capalase L (lamb pregastric).

EXPERIMENTAL

Materials

Fresh buffalo's and cow's milks were obtained from the herd of the Faculty of Agriculture, Zagazig University, Egypt. Commercial liquid calf rennet and good quality salt were used in cheese making. Two commercial lipase preparations—Cacordase (kig goat–lamb) from the Dumman World Trade Co., Wisconsin, USA and Capalase L (lamb pregastric) from Dairy Land Food Laboratories, Waukesha, were used.

Cheese making

Domiati cheese was made from a mixture of 1:1 buffalo's milk and cow's milk.

Cheese milk was heated to 165 °F (73·8 °C) for 15 s, immediately cooled to 100 °F (37·7 °C) and salt was then added at a level of 8 % of milk weight. Salted milk was then divided into five parts. The milk of the first four parts was treated with either Cacordase or Capalase L at concentrations of 0·02 % or 0·04 % of each preparation, whilst the milk of the fifth part was left as a control. The cheese making process was completed as described by Fahmi & Sharara (1950).

The resultant cheeses were pickled in their own drained whey and stored at room temperature (20–25 °C) for 8 weeks.

Analytical methods

Cheese samples were analysed for moisture, fat, salt, total nitrogen, soluble nitrogen and amino acid-N as described by Ling (1963). Cheese

fat acidity was estimated as described by Abdel Kader (1971) whilst the method of Harper (1953) was adopted for the determination of free volatile fatty acids with chain lengths of C_2, C_3, C_4, C_5 or higher.

Cheese scoring

The organoleptic properties of cheese samples were examined according to the scoring sheet proposed by El Koussy *et al.* (1970) with maximum score points of 60 for flavour and 40 for body and texture.

RESULTS AND DISCUSSION

Organoleptic evaluation

It is evident from Table 1 that Domiati cheese made from milk treated with either Cacordase or Capalase L showed, after 4 weeks of pickling, excellent flavour and highly acceptable consistency. Moreover, the flavour intensity was more pronounced at this stage of ripening than in control cheese pickled for 8 weeks. Cheese consistency was improved in the different treatments during the period of pickling. However, rancid flavour was detected in cheese made from milk treated with both lipase preparations at concentration of 0·04 % after 8 weeks of pickling. In the light of this evidence, the addition of both lipase preparations to cheese milk at a concentration of 0·02 % could be recommended to accelerate the flavour development of Domiati cheese.

TABLE 1
Organoleptic Properties of Domiati Cheese Made from Lipase-treated Milk

Pickling period (weeks)		*Control*	*Cacordase*		*Capalase L*	
			0·02 %	*0·04 %*	*0·02 %*	*0·04 %*
Fresh	Flavour (60)	38	44	45	42	44
	B & T (40)	30	32	33	32	34
4	Flavour (60)	42	50	53	52	55
	B & T (40)	32	36	35	35	36
8	Flavour (60)	46	56	40	55	42
	B & T (40)	34	37	36	36	36

B & T: Body and texture.

TABLE 2
Main Chemical Composition of Domiati Cheese Made from Lipase-treated Milk

Composition	Pickling period (weeks)	Control	Cacordase		Capalase L	
			0·02%	0·04%	0·02%	0·04%
Moisture (%)	Fresh	59·8	60·9	61·1	60·0	60·2
	8	50·1	52·5	52·9	52·0	53·0
Fat (%)*	Fresh	44·3	43·6	43·4	44·0	43·6
	8	54·1	50·8	50·9	51·0	51·0
Salt (%)*	Fresh	13·5	14·0	14·0	13·9	13·9
	8	10·1	10·5	11·0	10·4	10·3
Total nitrogen (%)*	Fresh	4·78	4·76	4·72	4·74	4·80
	8	4·42	4·40	4·38	4·40	4·44
Acidity**	Fresh	0·23	0·27	0·30	0·28	0·30
	8	1·52	1·82	1·94	1·70	1·88

* Results are calculated on a dry matter basis.
** Results are expressed as lactic acid percentage.

Main chemical composition of cheese

A study of the data shown in Table 2 could lead one to conclude that cheese made from milk treated with both lipase preparations showed slightly higher moisture and acidity and a lower fat content (on a dry basis) than control cheese. On the other hand, cheese made from lipase-treated milk did not exhibit marked differences from controls in either salt or total nitrogen contents. Abdel Salam *et al.* (1979) showed that the addition of certain animal lipase preparations to Ras cheese milk did not affect the main chemical composition of the resultant cheese.

Soluble nitrogenous compounds and cheese fat acidity

From the results representing the soluble nitrogen and amino acid-N (Table 3) it can be seen that the degree of protein degradation in cheese made from lipase-treated milk was slightly higher than that of control cheese during the pickling period. This can be attributed to the contamination of such lipase preparations with proteases (Jolly & Kosikowski, 1978).

TABLE 3
Soluble Nitrogen at pH 4·6, Amino Acid-N and Cheese Fat Acidity of Domiati Cheese Made from Lipase-treated Milk

	Pickling period (weeks)	*Control*	*Cacordase*		*Capalase L*	
			0·02%	*0·04%*	*0·02%*	*0·04%*
Soluble N*	Fresh	8·51	8·78	8·90	8·70	8·80
	4	15·6	18·2	18·6	17·2	18·0
	8	19·2	23·0	23·7	21·5	22·2
Amino acid N*	Fresh	0·80	0·82	0·82	0·80	0·80
	4	1·10	1·20	1·22	1·18	1·18
	8	1·50	2·10	2·40	1·96	2·22
Cheese fat acidity**	Fresh	0·40	0·50	0·56	0·50	0·52
	4	0·58	1·00	1·20	0·80	1·16
	8	0·86	1·60	2·20	1·30	1·86

* Results are expressed as percentage of total N.
** Results are expressed as millilitres of 0·1N NaOH per 10 g of cheese fat.

With regard to cheese fat acidity, it is evident from the results obtained (Table 3) that the addition of both lipase preparations to cheese milk stimulated the hydrolysis of cheese fat. This was more remarkable with the Cacordase preparation. Also, a further increase in cheese fat acidity was observed in cheese made from milk treated with the higher concentration (0·04%) of both lipase preparations. Several investigators have shown that lipolysis in cheese was accelerated by adding lipase preparations to cheese milk or curd (Richardson & Nelson, 1967; Richardson *et al.*, 1971; Peppler, 1975).

Free volatile fatty acids

From the results recorded in Table 4 it can be seen that neither acetic nor propionic acids were markedly affected. This could be explained on the basis that the origin of these acids in cheese is supposed to be the fermentation of lactose, as well as the decarboxylation and deamination of amino acids through specific metabolic pathways (Kosikowski & Mocquot, 1958; Nakae & Elliott, 1965*a*, *b*). On the other hand, the concentrations of butyric acid and acids with chain lengths of C_5 and greater were found to be higher in cheese made from lipase-treated milk,

TABLE 4
Free Volatile Fatty Acids of Domiati Cheese Made from Lipase-treated Milk (Millilitres of 0·01N NaOH per 100 g of cheese)

Pickling period (weeks)	*FFA**	*Control*	*Cacordase*		*Capalase L*	
			0·02%	*0·04%*	*0·02%*	*0·04%*
Fresh	C_2	2·00	2·20	2·20	2·00	2·40
	C_3	0·50	0·46	0·50	0·48	0·46
	C_4	1·00	1·80	2·00	1·40	1·60
	C_5 and higher	4·00	5·20	5·60	5·00	5·40
	Total	7·50	9·66	10·3	8·88	9·86
4	C_2	2·40	2·60	2·80	2·60	2·60
	C_3	0·50	0·50	0·60	0·58	0·58
	C_4	2·20	3·40	4·60	3·00	4·00
	C_5 and higher	5·80	10·8	12·0	9·10	11·2
	Total	10·9	17·3	20·0	15·3	18·4
8	C_2	3·00	3·40	3·50	3·30	3·40
	C_3	0·60	0·60	0·68	0·56	0·60
	C_4	4·00	6·10	8·20	5·90	7·20
	C_5 and higher	6·50	16·0	22·0	13·0	19·6
	Total	14·1	26·1	34·4	22·8	30·8

* Free fatty acids.

probably due to the greater fat hydrolysis by such lipase preparations. Also, a greater accumulation of acids of C_4 chain length and higher was observed in cheese made from Cacordase-treated milk. It is clear also that the addition of lipases to cheese milk reduced the time required for the development of free fatty acid formation. Thus, the level of total free fatty acids in 4-week-old cheese made from milk with added 0·02% lipase preparation was higher than that of control cheese pickled for 8 weeks.

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Polycyclic Aromatic Hydrocarbons in Grilled Meat Products—A Review

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ABSTRACT

Current efforts to detect and quantify carcinogenic polycyclic aromatic hydrocarbons (PAH) in the environment include analyses of foods. Grilled foods have been under scrutiny for almost 20 years. In this review reports concerning PAH contamination of grilled meat products are discussed. It is evident that the level of contamination varies considerably, primarily due to the extent of fat pyrolysis which has taken place. If proper precautions against fat pyrolysis are taken, and out-of-the-ordinary fuels such as crumpled paper or cones are not used, the PAH contamination is reduced substantially. Furthermore, if the relatively low consumption of grilled meat products is taken into consideration public concern over possible health risks due to grilling appears unwarranted.

INTRODUCTION

The carcinogenicity and ubiquity, at very low concentrations, of polycyclic aromatic hydrocarbons (PAH) have made these substances a challenge to analytical chemists. A great number of such compounds have been identified and quantified in virtually all segments of our environment; the literature on PAH in foods has been reviewed by Lo & Sandi (1978). Seppilli & Scassellati Sforzolini (1963) were the first to detect carcinogenic PAH's in grilled meat.

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TABLE 1

Mean Concentrations Reported for Carcinogenic Polycyclic Aromatic Hydrocarbons (PAH) in Charcoal Grilled Meat Products (μg/kg)

Sample	*B(a)P*[1] *I*	*B(b)F*[1] *II*	*B(a)A*[1] *III*	*Chrysene IV*	*I:II:III:IV*	*Number of samples*	*Analytical method*[2]	*Reference*
Sausages ('Rostbratwürsten')	<0·5					4	Capillary GLC, mass fragmentography	Binnemann (1979)
Beef	21·5					6	TLC, fluorescence spectroscopy	Doremire *et al.* (1979)
Pork	29·3					6		
Lamb	10·5					6		
Turkey	ND[3]					6		
Steak	0–2·0		1·0–8·0		1:—:3·5:—	?	HPLC, uv and/or fluorescence spectroscopy	Panalaks (1976)
Pork chops	5·0		ND			1		
Chicken	0·5		ND			1		
Steak	1·5		1		1:—:0·7:—	1	HPLC	
	3		1		1:—:0·3:—		GLC, flame ionisation	
Hamburger	30	50	39		1:1·7:1·3:—	1	HPLC	
	20	30	50		1:1·5:2·5:—		GLC	
Frankfurters	8		3		1:—:0·4:—	1	HPLC	
	5		2		1:—:0·4:—		GLC	
Chinese sausages	30	25	60		1:0·8:2·0:—	1	HPLC	
	25	30	40		1:1·2:1·6:—		GLC	
Sausages ('Kulmbacher Bratwurst')	0·06	0·15	0·6	0·3	1:2·5:10:5·0	2	TLC, *in situ* fluorescence spectroscopy	Tóth & Blaas (1973)
Sausages ('Coburger Bratwurst')	15·2	13·3			1:0·9:—:—	1		
Roast beef	0·8	0·7	0·8	1·2	1:0·9:1·0:1·5	1		
Bacon, fat ('Schweinebauch')	12·6	9·9	4·6	10·1	1:0·8:0·4:0·8	1		
Sausages ('Thüringer Bratwurst')	10	2·3	19		1:0·2:1·9:—	3	TLC, ultraviolet and fluorescence spectroscopy	Fritz (1973)

Pork steak ('Schweinerücken')								
Fat	7	1·3	11		1:0·2:1·6:—	3		
Lean	4·2		8·7		1:—:2·1:—	3		
Roast beef	0·7		3·3		1:—:4·7:—	3		
Sausages ('Coburger Bratwurst')	10·7	9·5	17·7		1:0·9:1·7:—	1		Fábián (1968)
Hamburger, fat	2·6		2·7	1·7	1:—:1·0:0·7	1	PC, ultraviolet spectroscopy	Lijinsky & Ross (1967)
Hamburger, lean	ND		ND	ND		1		
Hamburger, frozen	11·2		4·5	3·2	1:—:0·4:0·3	1		
Pork chop	7·9		8·2	6·3	1:—:1·0:0·8	1		
Chicken	3·7		3·2	2·2	1:—:0·9:0·6	1		
Sirloin steak	11·1		10·3	9·3	1:—:0·9:0·8	1		
T-bone steak	50·4		31·0	25·4	1:—:0·6:0·5	1		
Ribs	10·5		3·6	2·2	1:—:0·3:0·2	1	PC, ultraviolet and fluorescence spectroscopy	Lijinsky & Shubik (1965)
Steak	5·8		1·4	0·6	1:—:0·2:0·1	1		
Steak	8		4·5	1·4	1:—:0·6:0·2	1		Lijinsky & Shubik (1964)
Pork chops	<0·2		<5			2	HPLC, ultraviolet and fluorescence spectroscopy	Fretheim (unpublished results)
Pork 'bones' ('fleskebein')	<0·2		<4			2		
Beefsteak on the bone	<0·1		<3			1		
Lamb cutlets	<0·2		<2			2		

[1] Abbreviations for PAH's:
B(a)P = Benzo(a)pyrene.
B(b)F = Benzo(b)fluoranthene.
B(a)A = Benz(a)anthracene.
Formulae and relative carcinogenicities are given in Fig. 1.

[2] Abbreviations for analytical methods:
GLC = Gas–liquid chromatography.
TLC = Thin layer chromatography.
HPLC = High performance liquid chromatography.
PC = Paper chromatography.

[3] ND = Not detected.
Blank spaces imply not investigated.

This review restricts itself to the contamination of grilled meat products by PAH for two reasons:

(1) Grilling of foods is increasing in many countries, including Norway, and it is felt appropriate to collect available information on possible ill effects from grilling.
(2) It has been reported that the levels of contamination by these compounds in grilled products can be influenced both by the type of raw material used and by the procedure and/or the apparatus employed.

In addition to bringing together the available data it is the aim of this review to evaluate whether the situation calls for some kind of action by regulatory agencies.

ANALYTICAL DATA

Table 1 summarises the analytical results reported for meat products grilled over charcoal. It can be seen that, with few exceptions, the researchers have, broadly speaking, established similar relative proportions between the PAH's of the various samples, whereas the respective levels determined differ significantly. The assertion that the absolute concentration of PAH is, in general, subject to greater variations than the relative concentration of the individual compounds (Tóth & Blaas, 1973) is, therefore, supported. It should be realised, however, that the observed differences in concentration levels may stem both from actual differences in the products analysed and from lesser or greater systematic errors or shortcomings of the analytical procedures employed. A brief evaluation of the various methods in question is, therefore, called for here.

ANALYTICAL PROCEDURES

The analysis of PAH is constantly being improved, the latest contribution with regard to foods being the paper by Crosby *et al.* (1981). The following review is limited to methodology employed in work quoted in Table 1.

Clean-up

By and large all the cited researchers have employed similar steps in their procedures for obtaining a final solution of purified PAH, liquid–liquid extraction and column chromatography being the essential steps employed. There are differences, however, both in choice of solvents and chromatographic materials, as well as in whether or not the meat samples are hydrolysed in an initial step. The importance of these differences can only be evaluated in terms of figures on recovery.

Early workers (Lijinsky & Shubik, 1964, 1965; Lijinsky & Ross, 1967; Fábián, 1968) did not provide recovery data, but Lijinsky & Shubik (1964) comment that, due to losses, the concentrations they report are minimum values. Fritz (1973) reported about 80% recovery of B(a)P (abbreviations are explained in the footnotes to Table 1) whilst Grimmer & Hildebrandt (1967) (whose method was employed by Tóth & Blaas (1973)) obtained recoveries ranging from 87% to 98% for the PAH's listed in Table 1. In my own work, using the polycarbonate extraction procedure of Potthast & Eigner (1975), recoveries were estimated to be about 80% (unpublished results).

Panalaks (1976) and Binnemann (1979) hydrolysed their samples prior to extraction. By employing internal standards they obtained average recoveries of 104% and 105%, respectively, in their determinations of B(a)P. Doremire *et al.* (1979) did not report on recoveries obtained.

In summary, recovery does not appear to be a cause for serious concern when evaluating reported PAH concentrations. It must be pointed out, however, that, except for the two workers who employed an initial step of hydrolysis (Panalaks, 1976; Binnemann, 1979), the reported recoveries do not include losses due to incomplete extraction from the solid meat samples. Nevertheless, the data quoted in Table 1 do not suggest that this difference in clean-up has had any major effect on the PAH levels determined.

Separation and determination

There can be no doubt that capillary gas–liquid chromatography–mass fragmentography (GC–MF) is the method of choice for the determination of known carcinogenic PAH's. As pointed out by Binnemann (1979), the reproducibility, specificity and sensitivity of this method makes it preferable. The other methods are innately inferior in terms of

separation power, and if separation is incomplete and subsequent determination insufficiently specific, the resulting values may be too high. Particularly if checks on recovery have not been included, reports on surprisingly high concentrations must be viewed with some scepticism.

Panalaks (1976) provided a comparison of the accuracy of HPLC and GLC (not capillary) in these analyses by subjecting his PAH extracts to determination by both methods (Table 1). Values obtained for low concentrations differ by up to 50 % but his results suggest that, within this range, reported values are accurate.

EVALUATION OF REPORTED PAH CONCENTRATIONS

Table 1 indicates that the level of PAH contamination may vary from undetectable amounts to maximum values in the order of 50 μg/kg in the case of B(a)P. The high value of 50·4 μg/kg reported by Lijinsky & Ross (1967), is not supported by data on recovery and represents one sample only. However, other analyses in the same investigation yielded much lower values, indicating that the levels were, in fact, very different. Similarly, Panalaks (1976) found relatively high concentrations in two of his samples and low concentrations in others, in his case supported by checks on recovery. It appears, therefore, that the wide range of concentrations reported do reflect real differences in levels of contamination, and the question arises: What are the reasons for these differences?

FACTORS INFLUENCING THE LEVEL OF CONTAMINATION

Effect of the sample's fat content

As early as 1964, Lijinsky and Shubik stated: 'The most likely source of the polynuclear hydrocarbons is the melted fat which drips on the hot coals and is pyrolysed at the prevailing high temperature. The polynuclear hydrocarbons in the smoke are then deposited on the meat as the smoke rises'. This statement was based on their observation that no nitrogen-containing polycyclic compounds could be detected in extracts of the grilled meats whereas carbazoles and acridines are very much in

evidence in pyrolysates of nitrogen-containing raw materials. Lijinsky & Shubik (1965) determined the contamination of both lean meat (steak) and fat meat (ribs) as a result of charcoal grilling (Table 1). The increased PAH levels of grilled ribs were taken as support of the above fat pyrolysis hypothesis. Subsequent work by Lijinsky & Ross (1967), Fritz (1973), Tóth & Blaas (1973), and Doremire *et al.* (1979; see Table 2) has provided further support, so there can be no doubt that fatty meats yield grilled products with higher levels of PAH contamination than lean meats.

TABLE 2
Effect of Fat Content (Adjusted) on the Benzo(a)pyrene Content of Charcoal Grilled Ground Beef Patties
(Mean value of six determinations (Doremire *et al.*, 1979))

Percentage fat	15·0	19·5	29·8	39·1
Benzo(a)pyrene conc. ($\mu g/kg$)	16·0	22·8	30·9	121·0

Effect of variations in the grilling process

Masuda *et al.* (1966) grilled fish in an electric broiler and found only minimal amounts of PAH to be produced; a gas grill yielded somewhat higher levels. Investigations by Lijinsky & Ross (1967) showed that the more severe the heat treatment (extended exposure, closeness to heat source), the higher the resulting concentrations of PAH. If dripping of melted fat onto the heat source was prevented, as in electric grilling or in other grills of appropriate design, no carcinogenic PAH's were detected in the grilled products. The latter prerequisites for attaining minimal PAH levels have been confirmed by other workers (Grimmer & Hildebrandt, 1967; Tóth & Blaas, 1973; Lintas & De Matthaeis, 1979). Thus, it has been indisputably demonstrated that the grilling procedure has a definite influence on the extent to which grilled products are contaminated by PAH.

Effect of various fuels

Fábián (1968), Fritz (1973), Tóth & Blaas (1973), and Binnemann (1979) have investigated the effect on contamination of grilling over fuels other than charcoal. The unambiguous conclusion to be drawn from their work is that charcoal is preferable; hardwood and crumpled paper, as well as

pine or fir cones, all lead to increased levels of PAH contamination. As pointed out by Tóth & Blaas (1973), the difference probably arises from the fact that, whereas charcoal smoulders with hardly any emittance of smoke, the other fuels produce more or less sooty smoke. PAH's from the smoke are assumed to settle on the goods being grilled.

TOXICOLOGICAL EVALUATION OF THE PAH CONTAMINATION

Generally speaking, the severity of any toxic contamination arises from the toxicity, as well as the concentration, of the contaminating compounds. PAH toxicity rests with the acknowledged potency of some PAH's as carcinogens (Fig. 1) and will not be commented on further here.

The question is whether or not the concentrations reported in Table 1 constitute a cause for alarm. To the author's knowledge the only well defined guideline for answering that question is provided by the West German meat regulations which state that the edible portion of smoked meat products shall contain no more than 1 μg/kg of B(a)P. By this standard most of the products in Table 1 are unfit for human consumption. It must be realised, however, that this West German

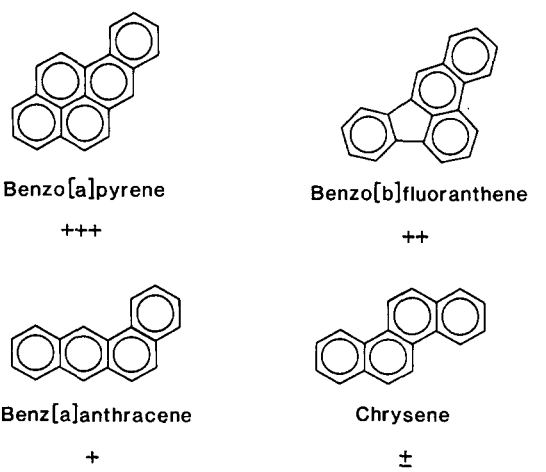

Fig. 1. Structural formulae and relative carcinogenicities of polycyclic aromatic hydrocarbons included in Table 1. ± Uncertain or weakly carcinogenic. + Carcinogenic. ++, +++ Strongly carcinogenic. (Committee on Biologic Effects, 1972; *cf.* IARC Working Group on the Evaluation of the Carcinogenic Risk of Chemicals to Man, 1973).

regulation is more or less arbitrary. It was found that proper processing procedures yielded smoked products containing less than 1 μg/kg of B(a)P (Tóth & Blaas, 1972), so this limit was introduced to rid the market of black-smoked products containing high levels (up to 55 μg/kg) of B(a)P. The latter products were deemed undesirable since their relatively high PAH content was suspected to be capable of possibly having ill effects on the health of consumers. Nevertheless, as pointed out by Lo & Sandi (1978), there is no evidence that any PAH compound can cause cancer in man via the oral route, especially not at the concentrations in question.

Another point is the relative importance of grilled products as compared with other foodstuffs. Data by Fritz (1971), based on analyses and consumption patterns in East Germany, place PAH intake due to eating of grilled foods at about 0·25 % of the total. Undoubtedly, grilling is much more common in, for example, the USA, but even a twenty-fold relative increase only brings grilled food PAH up to 1/20 of the total. This evidence that the PAH intake from grilled foods is a minor problem is supported by the conclusions drawn by Grimmer & Hildebrandt (1967), among others.

There is, however, a different aspect of the issue to be considered. Elmenhorst & Dontenwill (1967) found that grilling of 1 kg of bacon produced smoke containing 1·29 mg B(a)P, as well as other PAH's. To what extent does such smoke contaminate the atmosphere for people working at commercial grills?

CONCLUSIONS

Analytical works over the past decade have substantiated the early findings of Lijinsky and his coworkers (1964, 1965, 1967): PAH contamination of grilled foods is primarily due to pyrolysis of melted fat; practical measures that bring about a reduction in fat pyrolysis reduce PAH concentrations to very low levels. The possibilities include limiting grilling to lean meat, avoiding excessive heating, or keeping melted fat away from the source of heat. Thus, electric grilling is preferable as far as PAH is concerned. Furthermore, German workers have shown that solid fuels other than charcoal are unsuitable due to the PAH content of the smoke they produce.

It appears that no more research is needed to support Lijinsky's

original conclusions or to determine the general level of contamination caused by grilling over charcoal. The remaining issue is one for governmental authorities. Are traces of PAH in charcoal grilled foods a reason for concern? The present author suspects not. On the other hand, however, a ban on (commercial) grill designs that do not prevent fat pyrolysis is conceivable.

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The Effect of Sterols on the Oxidation of Edible Oils

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ABSTRACT

The effect of sterols on the oxidation of a triglyceride mixture, similar in composition to olive oil, has been studied at 180°C. Δ^5-Avenasterol and fucosterol are effective as antioxidants, whilst other sterols, including cholesterol and stigmasterol, are ineffective. The antioxidant effect of Δ^5-avenasterol increases with concentration in the range 0·01% to 0·1%.

An hypothesis is presented to explain the effectiveness of the sterols as antioxidants. It is concluded that lipid free radicals react rapidly with sterols at unhindered allylic carbon atoms. Isomerisation leads to a relatively stable allylic tertiary free radical, which is slow to react further, and this interrupts the autoxidation chain.

INTRODUCTION

Vegetable oils containing polyunsaturated fatty acids oxidise rapidly at elevated temperatures. This results in a reduction in the essential fatty acid content and darkening and polymerisation of the oil. Common anti-oxidants, including butylated hydroxyanisole (BHA) or tocopherols, retard oxidation at ambient temperatures but they are ineffective at retarding oxidation at the high temperatures (180 °C) required for frying food (Ramel *et al.*, 1965). Methyl polysiloxanes are added to vegetable oils to retard oxidation in heated oils (Martin, 1953). It has also been

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claimed that triterpene alcohols and hydrocarbons (Boskou & Katsikas, 1979), or certain sterols (Sims *et al.*, 1972; Boskou & Morton, 1976) retard the deterioration of oils during prolonged heating. It is believed that an ethylidene group in the side chain of the sterol is required for this antioxidant effect (Sims *et al.*, 1972).

This work is directed towards gaining a better understanding of the effect of sterol structure and concentration on the rate of oxidation of a vegetable oil. The effect of added sterols on the drop in iodine value of a model triglyceride mixture has been studied during oxidation at 180 °C. The triglyceride mixture studied was similar in fatty acid composition to olive oil, and was free of unsaponifiable matter, which might exhibit synergistic effects with added sterols. Changes in iodine value, which accompany the oxidation of oils at elevated temperatures (Waltking & Zmachinski, 1970), have been confirmed by refractive index and fatty acid determinations.

MATERIALS AND METHODS

Materials

BHA, cholesterol and trioleylglycerol, prepared from technical oleic acid, were purchased from BDH Chemicals Ltd. The fatty acid composition of the triglyceride mixture was found to be 76% oleic acid, 8% saturated fatty acids (stearic, palmitic, myristic), 7% palmitoleic acid, 6% linoleic acid with traces of other fatty acids. Stigmasterol and α-tocopherol were purchased from the Sigma Chemical Company.

Fucosterol and Δ^5-avenasterol were isolated in the laboratory from brown algae (*Fucus vesiculosus*) (Heilbron *et al.*, 1934) and green algae (*Ulva lactuca*) (Gibbons *et al.*, 1968), respectively. The electron impact mass spectra of the sterols were in agreement with reported spectra (Bergman *et al.*, 1965; Gibbons *et al.*, 1968). The sterols appeared pure by GLC under the conditions commonly used for analysing sterol mixtures (Boskou & Morton, 1975).

Methods

Samples of the triglyceride mixture (technical trioleylglycerol) (50 g) containing additives were heated at 180 ± 5 °C for 8 h a day with cooling

to room temperature at night. Aliquots (5 g) were removed after 24 h, 48 h, 72 h total heating time and the samples were stored under nitrogen in a refrigerator until required for analysis. A sample of trioleylglycerol without additives was heated as a control in each experiment.

Iodine values are the mean of duplicate determinations by Wij's method, according to the British Standards specification. Gas chromatographic analysis was performed on methyl esters prepared according to Brockerhoff (1965). A 2 m column of DEGS (10% on Chromosorb W, 80–100 mesh) was used at 180 °C for the analysis. Antioxidant activity was calculated in terms of the protective index (PI) used by Sims *et al.*, 1972:

$$PI = \frac{\text{Change in iodine value of a control sample}}{\text{Change in iodine value of a sample containing additives}}$$

PI > 1 indicates that the additive has an antioxidant effect.

RESULTS AND DISCUSSION

Δ^5-Avenasterol and fucosterol are both effective at minimising the fall in iodine value during heating at 180 °C (Fig. 1). Stigmasterol, cholesterol,

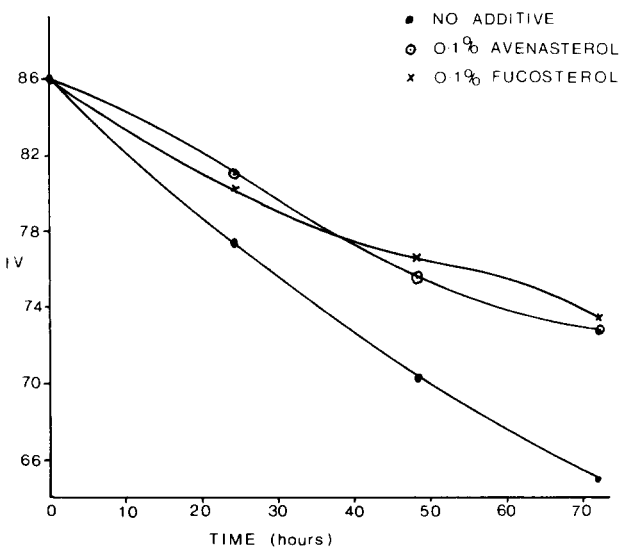


Fig. 1. The Iodine Value (IV) of trioleylglycerol during heating at 180 °C in the presence or absence of additives.

TABLE 1
Protective Indices (PI) of Additives During the Heating of Technical Trioleylglycerol at 180°C

Additive	*Concentration* (%)	*PI after heating for:* 24 h	48 h	72 h
α-Tocopherol	0·02	0·97	1·01	1·01
BHA	0·02	0·96	1·04	1·00
Cholesterol	0·1	0·96	1·12	1·12
Stigmasterol	0·1	0·95	1·11	1·09
Fucosterol	0·1	1·52	1·71	1·66
Δ^5-Avenasterol	0·1	1·78	1·69	1·59
Δ^5-Avenasterol	0·05	1·16	1·19	1·09
Δ^5-Avenasterol	0·01	1·10	1·09	1·07

BHA and α-tocopherol are relatively ineffective as antioxidants, having PI values $< 1·2$, whilst Δ^5-avenasterol and fucosterol have PI values $> 1·5$, when present at a concentration of 0·1 % (Table 1). The magnitude of the antioxidant properties of Δ^5-avenasterol can be appreciated when one considers the effect on the essential fatty acid content of the oil (Fig. 2). The time at 180°C for a 50% reduction in linoleic acid content in the

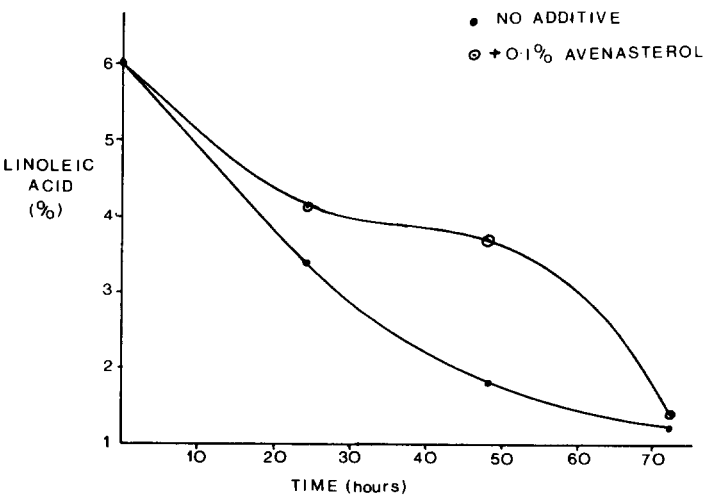
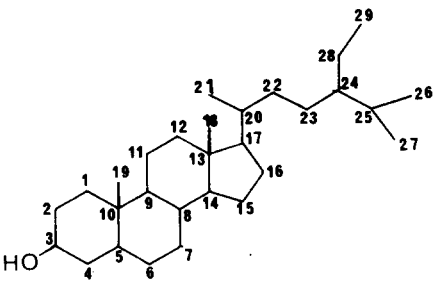


Fig. 2. The linoleic acid content of trioleylglycerol during heating at 180°C in the presence or absence of Δ^5-avenasterol.

absence of sterols is approximately 28 h whilst, in the presence of 0·1 % Δ^5-avenasterol, the time increases to approximately 59 h.

The effectiveness of Δ^5-avenasterol as an antioxidant increases with concentration in the range 0·01–0·1 %. Δ^5-Avenasterol is present in olive oil at <0·02 % (Itoh *et al.*, 1973) and it is clear that the sterol has a very slight antioxidant effect at this concentration in oil heated at 180 °C. However, synergistic effects with other unsaponifiable components may occur in olive oil.

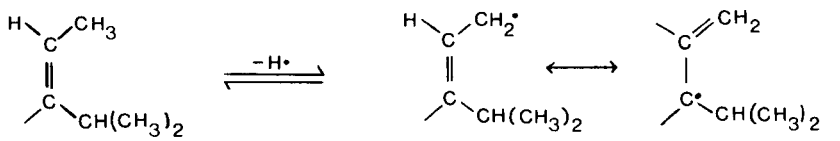
Previous work (Sims *et al.*, 1972) has shown that the order of effectiveness as antioxidants in safflower oil at 180 °C is vernosterol > Δ^7-avenasterol > fucosterol (Fig. 3) whilst spinasterol, ergosterol, lanosterol, β-sitosterol, stigmasterol and cholesterol have no significant



STEROL	DOUBLE BONDS
Vernosterol	8, 14, 24(28)
Δ^5-Avenasterol	5, 24(28)
Fucosterol	5, 24(28)
Stigmasterol	5, 22
Δ^7-Avenasterol	7, 24(28)

Fig. 3. The structure of plant sterols.

antioxidant activity. The 4-methylsterol, citrostadienol, is also effective as an antioxidant. These observations can be explained by the hypothesis that sterols with a structure that allows them to react rapidly with lipid free radicals to form relatively stable free radicals are effective as antioxidants. Relatively stable free radicals interrupt the triglyceride autoxidation chain reaction. The antioxidant effect is greatest when free radical formation from the sterol is relatively rapid due to the presence of unhindered hydrogen atoms on an allylic carbon atom, and when the free radical thus formed can isomerise to a tertiary free radical (as shown below), which is known to be relatively stable (Nonhebel & Walton, 1974).



Thus, sterols with an ethylidene group in the side chain are most effective as antioxidants, but a further antioxidant effect may arise from the presence and position of one or more endocyclic double bonds. Vernosterol has an antioxidant effect arising from the rapid formation of free radicals at C-29, but slower formation of free radicals at C-11 or C-16, which are stabilised by delocalisation over two double bonds, also contributes to the antioxidant activity of the sterol. Thus, vernosterol is more effective than sterols with one endocyclic double bond; for example, fucosterol.

Stigmasterol does not have significant antioxidant activity, despite being able to form tertiary free radicals at the allylic carbon atoms C-20 and C-24. Presumably the rate of loss of hydrogen atoms at tertiary carbon atoms is slow because of steric hindrance to the approach of a free radical.

Future work is intended to investigate whether differences exist in the antioxidant effects of Δ^5 and Δ^7 sterols, and also to study whether synergistic effects occur in the antioxidant activity of sterols.

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Technical Note

Occurrence of Aflatoxins in Some Egyptian Food Crops Collected From Two Coastal Regions

ABSTRACT

Two-hundred-and-forty-one samples representing cereals, legumes, oil seeds and nuts were collected from two coastal regions in Egypt. Mouldy samples were collected from lots stored under different conditions. Forty-eight per cent ((116 samples) of the samples gave bright greenish-yellow fluorescence, whereas 8% (19 samples) contained aflatoxins. Hazel nut, soybean and cottonseed meal samples were free from aflatoxins whilst white corn, yellow corn, peanuts and almonds had relatively higher concentrations of aflatoxins than other crops. Aflatoxin B_1 was present in all contaminated samples whilst G_2 was found only in peanuts. Aflatoxin G_1 was found only in yellow corn, paddy rice and peanut samples. High concentrations of aflatoxins were found in mouldy samples of cereals and legumes which are popular in Egyptian diets. The high concentrations of aflatoxins found in contaminated samples may be due to the method of sampling and to unfavourable storage conditions.

INTRODUCTION

Although it has been shown that some of the most important plant foods are susceptible to contamination by aflatoxins, little work has been published on the presence of aflatoxins in Egyptian foods. Aflatoxins have been detected in maize, barley, rice and wheat in several countries. Hesseltine (1974) and Gergis *et al.* (1977) detected low levels of aflatoxins in a few samples of corn, lentils, faba beans, fenugreek, peanuts and cotton cake in Egypt.

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The Mediterranean coastal regions of Egypt are characterised by a rainy winter and a humid summer. The primitive methods of storage of food crops by farmers in villages or by merchants and processors in towns and cities may encourage mould growth and the production of aflatoxins. Therefore, the work described in this paper was carried out to investigate the presence of aflatoxins in mouldy food crops collected from two coastal governorates (Alexandria and Al-Behera).

EXPERIMENTAL

Two-hundred-and-twenty-seven samples were collected from eight food crops common in the Egyptian diet and generally stored under unfavourable conditions. Fourteen samples were also collected from soybeans (whose cultivation is expanding), cottonseed meal (which is generally used as an animal feed) and from almonds and hazel nuts to represent the major imported nuts. The number of samples examined from each crop is shown in Table 1.

One-kilogram samples were collected during the period from

TABLE 1

Distribution of Collected Samples Among Crops and Number of Positive Samples for Bright Greenish Yellow Fluorescence (BGYF) and for Aflatoxins

Agricultural commodities	*Number of samples collected*	*Number of positive samples for BGYF*	*Number of positive samples for aflatoxins*
White corn	49	36	4
Peanuts	45	33	5
Paddy rice	39	22	3
Faba beans	30	6	1
Wheat	26	3	1
Cottonseed	18	7	1
Yellow corn	16	4	2
Cottonseed meal	7	—	—
Lentils	4	2	1
Almonds	3	2	1
Soy beans	3	1	—
Hazel nuts	1	—	—
Total	241	116	19

November, 1978 to April, 1979, from lots stored by different means—for example, in closed storage rooms, on the roofs of farm houses, in mud silos and in jute sacks. All collected samples showed mouldiness, mechanical damage or insect damage. Screening for bright greenish yellow fluorescence (BGYF) under ultraviolet light (365 nm) was used to select suspected seeds or seed fractions (after crushing the seeds) for detection of aflatoxins (Shotwell *et al.*, 1974). Thin layer chromatography was used for the determination of aflatoxin compounds in the samples which were positive for BGYF (AOAC, 1975).

RESULTS AND DISCUSSION

The results shown in Table 1 indicate that aflatoxins were found only in 7·88 % of the total samples and in 16·3 % of BGYF positive screened samples. Although the collected samples were chosen from mouldy and damaged lots in the stores, the numbers of aflatoxin contaminated samples were small. Although Shotwell *et al.*'s (1974) results indicated that all BGYF positive corn samples contained aflatoxins, our results showed that quite a low percentage of BGYF positive samples of the main field crops (white corn, peanuts, paddy rice, wheat, faba bean and cottonseeds) contain aflatoxins. This difference reflects the significance of agronomic variables. Moulds other than *Aspergillus flavus* may be present on agricultural commodities and only about 5 % of *Aspergillus flavus* strains produce aflatoxin under certain environmental conditions (Goldblatt, 1972). Due to Egyptian climatic conditions and agricultural practices, most agricultural food crops have a low moisture content after harvesting.

Different crops varied greatly with regard to percentage of aflatoxin positive samples. Almonds, lentils and yellow corn showed the higher percentages (13 % to 33 %) followed by peanuts, white corn (11 % and 8 %), paddy rice (7·7 %), cottonseed (5·5 %), wheat (3·8 %) and faba beans (3·3 %). No aflatoxins were detected in cottonseed meal, soy beans or in one sample of hazel nut. White corn, rice, lentils, wheat and faba beans constitute the major stable items in the Egyptian diet (FAO, 1977).

The concentration of the different types of aflatoxins present in the positive samples are given in Table 2. Aflatoxin B_1 was found in all positive samples, whilst G_2 was found only in two samples of peanuts. G_1 was only found in peanuts, yellow corn and paddy rice. Aflatoxins B_1, B_2,

TABLE 2
Concentration of Aflatoxins in the Positive Samples of Various Crops

Commodity	*Type and quantity of aflatoxin (ppb)*				
	B_1	B_2	G_1	G_2	*Total*
White corn	3607·1	4256	9020	ND	16883
	10450·0	5481	ND	ND	15931
	7103·5	4326	ND	ND	11429
	1429·1	577	ND	ND	2006
Peanuts	5871·4	5567	10458	148	22072
	1387·5	1758	4347	ND	7492
	1227·7	1433	2558	ND	5219
	810·0	ND	294	ND	1104
	90·9	375	150	363	979
Paddy rice	498·9	ND	ND	ND	499
	360·0	ND	32·5	ND	393
	285·0	ND	ND	ND	285
Faba beans	125·0	12·5	ND	ND	138
Wheat	1387·5	101·6	ND	ND	1489
Cottonseed	489·6	ND	ND	ND	490
Yellow corn	612·1	547	530	ND	1689
	378·8	ND	434·1	ND	813
Lentils	72·1	ND	ND	ND	72·1
Almonds	5566·6	ND	ND	ND	5567

ND = Not detected.

G_1 and G_2 were detected in cereals (Hesseltine, 1974). Gergis *et al.* (1977) reported 6·3, 3·1, 3·2, 5·8–7·0, 5·4–8·0, and 9·7–11·7 ppb of total aflatoxins (mainly B_1 and B_2) in stored corn, lentils, faba beans, fenugreek, peanuts and cottonseed cake samples, respectively. The higher concentrations of aflatoxins reported in this paper may be attributed to bad storage conditions and to collecting mouldy samples. It was observed that samples containing high aflatoxin concentrations were stored for long periods under open storage conditions. Storage conditions and facilities should therefore be given due attention, whether on a household or a commercial basis. Shotwell *et al.* (1974) found that levels of aflatoxin ranged from 3 to 84 000 ppb in contaminated corn kernels, whilst one kernel had 207 000 ppb of aflatoxin B_1. The aflatoxin B_1 content of some contaminated peanut kernels ranged from 10 000 to 750 000 ppb whilst the aflatoxin B_2 content ranged from 8000 to 250 000 ppb (Lee *et al.*, 1968).

The results show that aflatoxins were present in high concentrations in the mouldy samples of main food crops. Many processors and consumers do not discriminate against mouldy commodities.

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Technical Note

Amino Acid Composition of Two Italian Durum Wheat Varieties and Their Semolina

ABSTRACT

Two Italian durum wheat varieties and their semolina were analysed for protein content and amino acid composition. There was a difference in protein content between the two varieties. The concentration (in protein) of lysine, methionine and phenyl alanine was equal in both varieties. A large difference was found in glutamic acid and valine. A slight difference was found in other amino acids. There was a difference in the concentration of amino acids between wheat and its semolina. The biological value was equal in both varieties. The difference in results is attributed to the varietal difference.

INTRODUCTION

Several studies were concerned with fractionation of protein in maturing wheats by column chromatography and electrophoresis. Pomeranz *et al.* (1966) studied the amino acid composition of two wheat varieties and the flour milled from these two varieties and Kohler & Palter (1967) studied some methods for amino acid analysis of wheat products.

Eggum *et al.* (1979) derived a regression equation for the biological value of maize protein as an example of a cereal crop. The equation is based on lysine which is frequently the first limiting amino acid in cereal grains.

The present study compares the amino acid composition and the biological value of two Italian durum wheat varieties and their semolina.

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MATERIALS AND METHODS

Two Italian durum wheat varieties, Capeiti and Creso, were obtained from the farm of Cerealicoltura, Rome, Italy.

Semolina was milled samples of wheat ground in a ball mill to a fine homogeneous powder.

Acid hydrolysis was used in the preparation of a sample for amino acid analysis. Test tubes were narrowed after the addition of 40 mg of sample (dry-weight basis) and 4 ml of 6N hydrochloric acid were added. The tubes were placed in a dry-ice alcohol bath and sealed under vacuum. The contents of the tubes were hydrolysed for 22 h at 110 °C and then filtered through a fritted disc funnel. The filtrate was evaporated to dryness three times under partial vacuum and diluted with 0·2N sodium citrate buffer, pH 2·2. The samples were stored at −20 °C until required for analysis. The hydrolysates were practically clear, indicating little, if any, formation of humin. Amino acid analyses were carried out by the ion exchange column chromatography method of Spackman *et al.* (1958) on a Beckman Model 121 Amino Acid Analyser.

The procedure of Moore (1963) was used to determine cystine and methionine. Tryptophan was determined according to the method of Concon (1975). Nitrogen was determined by the microKjeldahl method (AACC, 1969). The biological value was calculated according to the technique of Eggum *et al.* (1979).

RESULTS AND DISCUSSION

Table 1 shows the amino acid composition of two Italian durum wheat varieties and their semolina. The results are the average of duplicate determinations. There is a slight difference in the protein content (0·2%) between the Capeiti variety and the Creso variety.

The concentrations in wheat protein of lysine, methionine and phenyl alanine are the same in both varieties and there is a slight difference between the varieties in histidine, aspartic acid, threonine, serine, proline, alanine, cystine, *iso*-leucine, leucine, tyrosine and tryptophan. The greatest difference is in glutamic acid and valine. The difference in results is attributed to the varietal difference.

Milling of wheat into semolina reduces the concentration of lysine, histidine, arginine, aspartic acid, threonine, glycine, alanine, valine

TABLE 1
Amino Acid Composition of Two Italian Durum Wheat Varieties and Their Semolina

	Capeiti variety		*Creso variety*	
	Wheat	*Semolina*	*Wheat*	*Semolina*
Protein (%, dry basis)	12·4	12·0	12·6	12·1
	g of amino acid/100 g protein			
Lysine	2·70	1·90	2·70	2·00
Histidine	2·30	2·10	2·20	2·10
Ammonia	4·10	4·10	4·00	4·20
Arginine	4·70	4·00	3·90	3·20
Aspartic acid	5·01	3·80	5·30	4·10
Threonine	3·00	2·70	3·30	2·90
Serine	4·80	4·91	4·60	4·70
Glutamic acid	30·0	34·5	34·0	35·6
Proline	9·50	12·6	9·70	12·1
Glycine	4·01	3·30	5·01	3·60
Alanine	3·50	2·71	3·70	3·10
Cystine	2·30	2·41	2·40	2·50
Valine	4·80	4·36	3·20	4·30
Methionine	1·66	1·90	1·70	1·95
Iso-leucine	3·90	3·90	4·10	4·10
Leucine	6·80	7·00	7·10	7·30
Tyrosine	3·01	2·86	2·60	2·20
Phenyl alanine	4·63	4·75	4·70	4·80
Tryptophan	1·50	1·10	1·40	1·10
Biological value (%)	63·6	56·4	63·6	57·3

tyrosine and tryptophan. Concentrations of serine, proline, glutamic acid, cystine, methionine, leucine and phenyl alanine are higher in semolina than in wheat proteins. There is no significant change in *iso*-leucine concentration.

Pomeranz *et al.* (1966) and Shoup *et al.* (1966) (quoted from Pomeranz *et al.*) found that milling of wheat into flour reduced the concentration of lysine, arginine, aspartic acid, threonine, glycine and alanine, but that concentrations of glutamic acid and proline were higher in flour than in wheat proteins. There were no significant changes in concentrations of histidine, serine, valine, *iso*-leucine, leucine, tyrosine and phenyl alanine.

The concentration of ammonia depends primarily on the extent of cleavage during acid hydrolysis of amide groups from glutamine and asparaginine, although substantial amounts of ammonia may also result from the breakdown of certain amino acids, notably serine and threonine.

The concentration of ammonia was equal in wheat and semolina of the Capeiti variety but was high in semolina of the Creso variety. Pomeranz *et al.* (1966) found that ammonia was higher in flour than in wheat proteins.

The major variations in the reported values in the literature and the present study are due to varietal differences and variations in methods of analysis (Kohler & Palter, 1967).

The development of the computerised formulation of feeds and the necessity of supplying nutritionally balanced foods to a growing population have increased the need for more precise and reliable data on the amino acid composition of wheat products. The biological value % depends on the concentration of lysine in the protein. It is preferable to produce wheat varieties high in lysine. This value was equal in both wheat varieties and differed slightly in the semolina.

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Book Reviews

Developments in Food Proteins—1. Edited by B. J. F. Hudson. Applied Science Publishers, London and New Jersey. x + 335 pp. 1982. Price: £35·00.

This book is one of the Developments Series introduced by Applied Science Publishers with the objective of bringing together the latest trends and developments in a specific field of study and publishing them within six months of their being written. In a rapidly changing field this is a laudable concept and the specific field chosen for this book—Developments in Food Proteins—is, without doubt, an area that has undergone much activity in the last few years. Many technological developments have taken place in the use of food proteins in recent years and the book includes coverage of a number of the most important aspects.

The Editor and publishers, in their choice of the areas to be included, have covered basic chemical, biochemical and physico-chemical advances in food proteins through to some commercial and political considerations of the uses of food proteins. This is a rather wider concept than is normally used; however, it makes an interesting approach. The nine chapters cover: modification of food proteins; application of scanning calorimetry; use of ultrafiltration; milk proteins in formulated foods; analysis of novel proteins in meat products; vegetable proteins in large-scale catering and leaf protein as human food and algae—a future source of protein.

Inevitably in a book of this type there is variation in the style between

the various contributors and some chapters are more easily read than others. On the one hand there are reviews covering wide areas, such as the first chapter, which reviews chemical and enzymatic modification of food protein and includes some 440 references, and the fourth chapter, which reviews the use of milk proteins in formulated foods, whilst, on the other hand, specific aspects are dealt with in chapters 2 and 5 where the application of scanning calorimetry to the study of protein behaviour in foods and the analysis of novel protein in meat products are covered.

Possible future trends are included, especially in respect of potential new protein sources; for example in Chapter 8—'Towards Leaf Protein as a Human Food', and Chapter 9—'The Algae—A Future Source of Protein'.

There was a tendency in some chapters to include too much basic information on techniques; such information would be covered in more detail elsewhere and it is important in a book of this nature to have emphasis on new developments and applications.

The book contributes a useful updating on recent progress in some of the food protein areas and will be of particular interest to food science and technology students and those scientists and technologists involved in food research and development.

G. Cheeseman

Meat and Meat Products. Factors Affecting Quality Control. Edited by N. R. P. Wilson, Applied Science Publishers, London and New Jersey, 1981. 207 pp. Price: £16·00.

This book is written primarily for the meat technologist and proceeds to cover all aspects of meat and meat product manufacturing in a logical sequence from the live animal to the final cleaning of the meat plant. Chapters are presented on Animal Health in Relation to the Quality of Meat; Transportation and Handling of Livestock Prior to Slaughter; Abattoirs and Meat Plants; Handling of Meat; Microbiological Quality Control and Standards; Meats for Processing; Curing; Sausages and Other Comminuted Meat Products; Other Processed Meats and Meat Plant Cleaning.

The book is very 'readable' and is full of useful and relevant information; it contains very little 'padding'. It is clearly presented, although perhaps would have benefited from a larger number of tables

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The book is very 'readable' and is full of useful and relevant information; it contains very little 'padding'. It is clearly presented, although perhaps would have benefited from a larger number of tables

and illustrations than it has (15 and 13, respectively) as better use of photographs of animals, meat plant and equipment could have emphasised points made in the text to advantage.

This is a relatively minor criticism, however, as the text is comprehensive and detailed and obviously draws on a wealth of first hand experience from its several contributors (E. J. Dyett, R. B. Hughes and C. R. V. Jones). This is reflected in the practical, rather than theoretical, style of the book, thus making it of greater interest to the meat technologist than the meat scientist.

The book should be essential reading for students of meat technology and will also be of considerable interest to experienced managers and technologists involved with the manufacture of meat products. Students of food chemistry and food science will also find the book valuable background material.

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Simultaneous Adjustment of pH and Total Acidity in Wine

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ABSTRACT

An ion exchange procedure coupled with the use of $KHCO_3$ can be employed simultaneously to adjust the acidity and the pH of wines to predetermined values. The ion exchange procedure is used to deacidify a calculated portion of the wine whilst the $KHCO_3$ treatment is used primarily to adjust pH. The calculations employed take into consideration the buffer capacity of the wine and the stoichiometry of acid-base neutralisation phenomena. The acidity may be reduced 50% without adversely affecting the wine and the procedure is both commercially practical and economically feasible.

INTRODUCTION

A sour (acid) taste component is a common characteristic of wine flavour. In dry (low sugar) white wine, or wines of low tannin content, it is the only discernible taste. However, a continuing problem with many wines is an overabundant acid taste.

To better understand the acid taste characteristic, the individual acids found need to be considered. A number of different acids are found in wine, but malic and tartaric acids predominate. Those found in small amounts are citric, lactic, succinic and phosphoric. All of these acids are classified as *weak* acids. That is, they do not dissociate completely in

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aqueous solution. Instead, an equilibrium is established between the undissociated acid, HA, and the free ions, H^+ and A^-.

$$HA \rightleftharpoons H^+ + A^-$$

and the weak acid has the dissociation constant, K, or:

$$K = \frac{[H^+][A^-]}{[HA]}$$

Total acidity is measurable by titration with a standard base. By definition, pH is the negative logarithm of $[H^+]$ or $1/\log[H^+]$, and measurable as such.

As a rough practical approximation, only 1 % of the acids in wine are dissociated. Thus [HA] is almost a hundred times greater than $[H^+]$. On the other hand, whilst the magnitude of the sour taste response is due to both [HA] and $[H^+]$ (Harvey, 1920; Amerine *et al.*, 1965), the sour taste in wines is ten times greater for $[H^+]$ than for [HA] (Plane *et al.*, 1980). Therefore, as [HA] in wines is almost a hundred times greater than $[H^+]$, the sour taste is mainly due to undissociated acid. This has led to the development of an empirical acid taste index, I_a (Plane *et al.*, 1980) as follows:

$$I_a = \text{Titratable acidity (g tartaric/litre)} - \text{pH}$$

Although the index may seem mathematically untenable, it is, in part for the reasons given above, a valid wine quality parameter and correlates well with subjective estimates of wine sourness.

Because the acid taste is largely due to HA, microbiological ('malo-lactic' fermentation) and physical (tartrate precipitation) methods are employed to effect a reduction in total acids. These methods are imprecise, however. Chemical methods, such as the use of ion exchange resins (Mattick & Gogel, 1980) and acid neutralisation and precipitation with potassium bicarbonate (*Official J. Europ. Comm.*, 1977; Mattick *et al.*, 1980) are more precise, but consequent alteration of pH is not easily controlled.

Potassium bicarbonate acts by two mechanisms to reduce the acidity. The bicarbonate ion is a base which can neutralise acids by the removal of H^+:

$$KHCO_3 + HA \rightleftharpoons K^+ + A^- + HCO_3^- + H^+$$

$$HCO_3^- + H^+ \rightarrow H_2CO_3 \rightarrow H_2O + CO_2$$

Further, the K^+ will precipitate as potassium bitartrate (KHTa), removing even more acidity:

$$K^+ + A^- \rightarrow KA\downarrow \qquad \text{if } A^- \text{ is } HTa^-$$

The addition of $KHCO_3$ will raise the pH of the wine, and this limits the amount of acid that may be neutralised. For practical purposes, the pH should not be raised higher than 3·6 in order to preserve colour and maintain bacteriological stability. However, if the pH of the wine is below 3·6, subsequent precipitation of HTa^- causes the pH to fall to 3·5 or lower. This is caused by the precipitation of the bitartrate ion in a region where, on average, more than one proton occurs on each tartrate species. As can be seen in Fig. 1, the point where there is one proton per species is just above pH 3·5. The curves shown in Fig. 1 apply only to water.

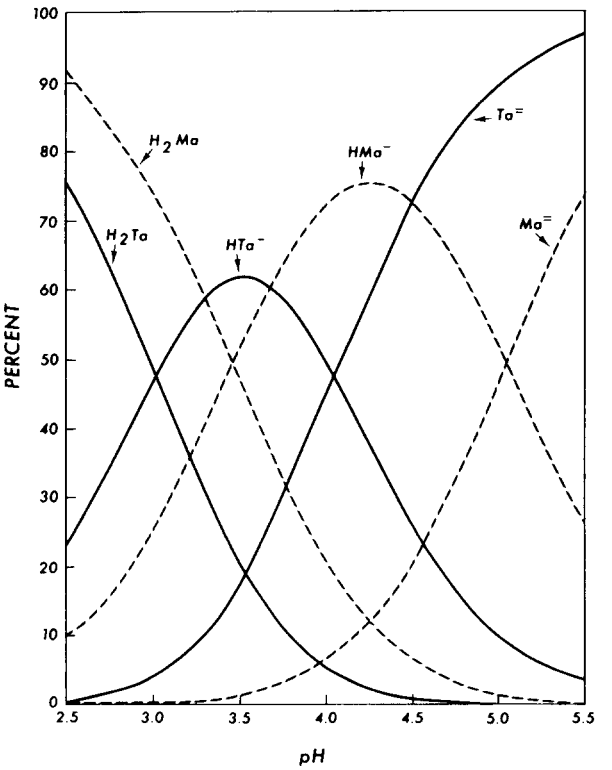

Fig. 1. Relative concentrations of tartaric and malic ionic species in water at various pH values.

In the lower dielectric medium of wine, all species will be less ionised and the curves are displaced to a higher pH with the maximum of HTa^- occurring at 3·65 (Berg & Keefer, 1958). Below this point, precipitation of HTa^- releases some H^+ from H_2Ta (which exceeds the tartrate ion, $Ta^=$). However, above pH 3·7, precipitation causes a rise in pH, since $Ta^=$ outweighs H_2Ta, and precipitation of HTa^- results in the net removal of H^+ from solution.

This paper describes a simultaneous adjustment of both wine pH and total acidity. It appears to be commercially applicable to the production of acceptable wines, and also economically feasible. The principle employed may have application to other non-proteinaceous liquid foods with excessive acidity.

PROCEDURE

Ion exchange

A strong cation column (Duolite C-25D), a sulphonated copolymer of styrene and divinyl benzene, and a weak anion column (Duolite A-7D), an epoxyamine where the principal functional groups are secondary amines, were employed in these studies. The former is used in the acid form and the latter in the free base form. The free amine is the reaction site and does not involve an exchange of OH^-. Therefore, in the regeneration of the base resin, regeneration should be carried out to pH 9. Furthermore, the free base resin has poor salt-splitting ability and it is necessary to first exchange the wine cations for H^+ and then to convert the salts to acids. The acids are then removed by reaction with the amines.

Titration with $KHCO_3$

The titration of a wine with 1 M $KHCO_3$ and, for comparative purposes, 1 M NaOH, is shown in Fig. 2. The salient feature of each curve is the long linear rise in pH with addition of base. The advantage of using $KHCO_3$ over NaOH is that the former extends the linear portion of the curve to pH 5·0. The slope of the linear portion of the titration curve is the buffer 'value' of the wine. The reciprocal of the slope is the buffer capacity (equivalents/litre/pH unit). The buffer capacity of a wine can be

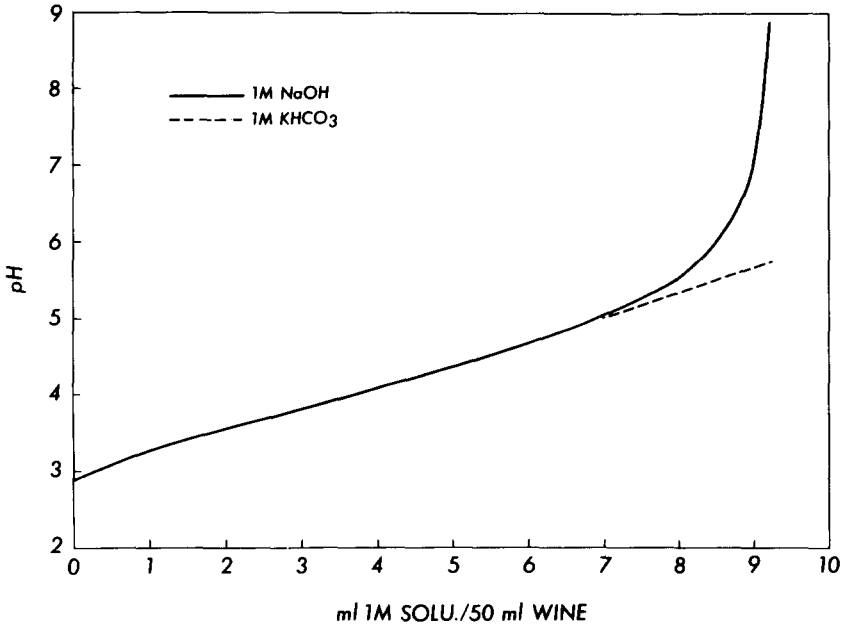

Fig. 2. Titration of a wine with 1M NaOH and 1M $KHCO_3$.

calculated by determining the initial pH of the wine, and then titrating 50 ml to pH 5·0 with 1M NaOH.

$$\text{Buffer capacity (Eq/litre/pH)} = \frac{1000\ (\text{Equivalents of NaOH})}{V_{\text{wine}}(5{\cdot}0 - \text{Initial pH})}$$

Since this is a linear function, the pH can be changed to any predetermined value with $KHCO_3$ as follows:

$KHCO_3$ (g/litre) to add

$$= \text{Buffer capacity} \times 100 \times (\text{Desired pH} - \text{Initial pH})$$

By titrating 50 ml of wine with 1M NaOH to pH 5·0, the maximum amount of $KHCO_3$ that may be safely added can be determined. pH 3·5 is employed, rather than pH 3·6, to allow a safety factor.

Maximum $KHCO_3$ (g/litre) to add

$$= \frac{2{\cdot}0 \times \text{ml NaOH} \times \text{N NaOH} \times (3{\cdot}5 - \text{Initial pH})}{(5{\cdot}0 - \text{Initial pH})}$$

The reduction in acidity in grams per litre of tartaric acid experienced would be between 0·83 [$KHCO_3$ (grams per litre) added] and 0·98 [$KHCO_3$ (grams per litre) added]. Familiarity with the type of wine, the tartrate and potassium concentration, and the pH makes this prediction more precise.

RESULTS

Ion exchange

As shown in Fig. 3, the addition of an ion exchanged wine (initial total acid content, 1·02) in amounts from 0 to 40 %, back to the untreated wine results in a progressive linear decrease in total acidity, malic and tartaric acids. As shown in Fig. 4, the decrease in potassium and sodium is also

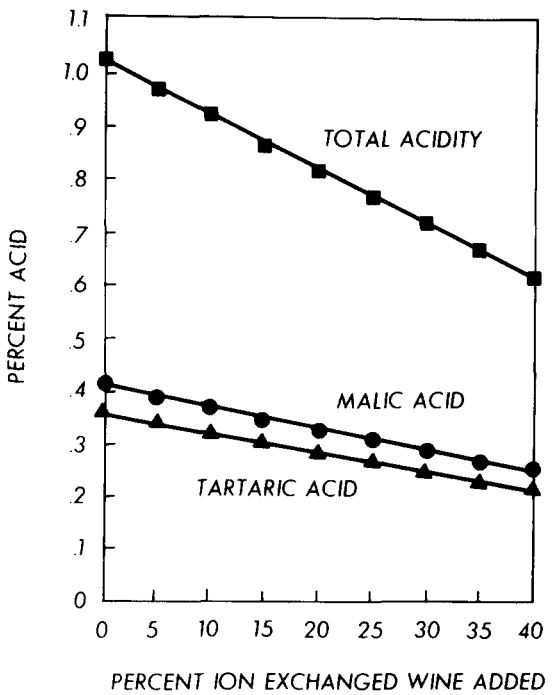

Fig. 3. Effect of the addition of cation and anion exchanged wines on the total acidity and malic and tartaric acid.

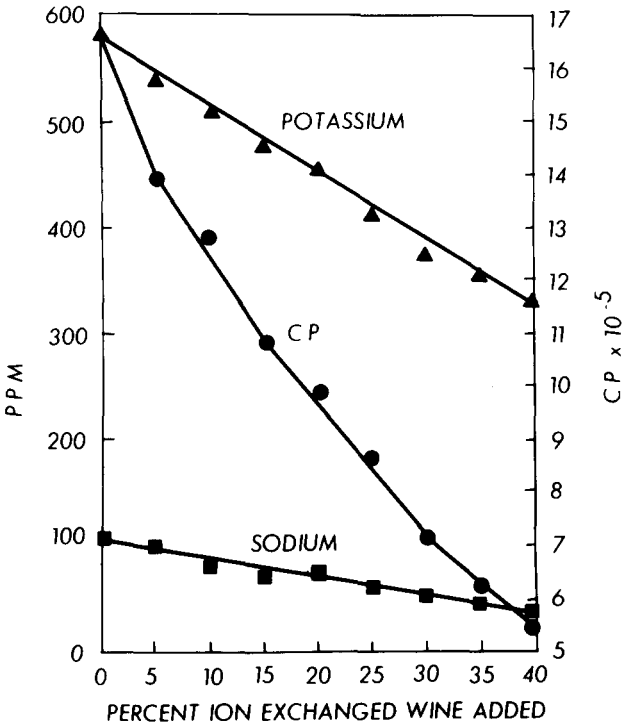

Fig. 4. Effect of the addition of cation and anion exchanged wines on the potassium and sodium concentration and the concentration product (CP).

linear, but the concentration product (CP), calculated from the pH, tartrate and potassium concentrations, is curvilinear, as expected. CP is used as an index of a wine's 'tartrate stability'.

An example, whereby it was desired to reduce, by ion exchange, the total acidity of a white wine (1·24% acid, as tartaric) to 0·70% is given in Table 1. Calculations showed that 44% (1·24 – 0·70/1·24) needed to be

TABLE 1
Analysis of a White Wine Before and After Ion Exchange Treatment

	pH	*Total acidity (%)*		*Tartrate (g/100 ml)*	*Potassium (ppm)*	*Sodium (ppm)*	*CP (× 10^{-5})*
		Observed	*Calculated*				
Original wine	2·85	1·24	—	0·30	590	37	12·1
Treated	2·84	0·71	0·70	0·17	340	20	3·9

treated. This amount was exchanged and blended with the original wine in the ratio 44:56. The important point to be noted is that the pH was not altered.

Studies have shown that the ion exchange method can be used to reduce the acidity of wine by 50 % before any deleterious effects can be noticed.

Simultaneous adjustment of total acidity and pH

The information needed to simultaneously adjust the pH and the total acidity of a wine is given in Table 2.

Using the following calculations, the quantity of $KHCO_3$ (g/litre) and the amount of wine to be ion exchanged can be determined as follows. Calculate $KHCO_3$ (g/litre) to raise the pH to a predetermined value (A) from the expression:

$$A = \frac{10^2 \times \text{ml}_{\text{NaOH}} \times \text{N}_{\text{NaOH}} \times (\text{Desired pH} - \text{Initial pH})}{V_{\text{wine}} \text{ titrated } (5{\cdot}0 - \text{Initial pH})}$$

Since the addition of the $KHCO_3$ to raise the pH will lower the acidity, the acidity of the wine with the addition of $KHCO_3$ (B) will be calculated:

$$B = \text{Initial acidity (g/litre tartaric acid)} - 0{\cdot}75(A)$$

The fraction of the wine to be ion exchanged (C) is then calculated:

$$C = \frac{(B) - \text{desired acidity (g/litre tartaric acid)}}{(B)}$$

Since all cations and acids will be removed by the ion exchange, it is not necessary to add the $KHCO_3$ to that fraction. Therefore, we can reduce the amount of $KHCO_3$ to be added to the unexchanged portion.

TABLE 2
Information Needed for the Simultaneous Adjustment of pH and Total Acid

(1)	Information to be determined
	(a) Initial pH
	(b) ml 1M NaOH to titrate 50·0 ml wine to pH 5·0
	(c) Total acidity in g/litre tartaric acid
(2)	Finished product
	(a) Desired pH
	(b) Desired total acidity in g/litre tartaric acid

g $KHCO_3$/litre to be added to the unexchanged portion $= A(1 - C)$. In practice, the method takes advantage of the acid reduction by $KHCO_3$ and ion exchange and further uses the $KHCO_3$ for pH adjustment. If the pH of the unexchanged portion of the wine is adjusted, the addition of the exchanged portion will not change the pH. Thus, a simultaneous adjustment of low pH, high acid wine may be accomplished.

A white wine (variety, Seyval Blanc) with an acidity of 12·6 g/litre calculated as tartaric acid and a pH of 2·96 was to be adjusted to an acidity of 7·0 g/litre and pH 3·30. Fifty millilitres of wine required 6·16 ml of 1 M NaOH to titrate the wine to pH 5·0. The calculations are listed below:

$$\text{g } KHCO_3\text{/litre} = A$$

$$= \frac{10^2(6{\cdot}16)(1{\cdot}0)(3{\cdot}30 - 2{\cdot}96)}{50(5{\cdot}0 - 2{\cdot}96)} = 2{\cdot}05 \text{ g } KHCO_3\text{/litre}$$

$$\text{Acidity after } KHCO_3 = B = 12{\cdot}60 - 0{\cdot}75(2{\cdot}05) = 11{\cdot}06$$

$$\text{Fraction to be exchanged} = C = \frac{11{\cdot}06 - 7{\cdot}0}{11{\cdot}06} = 0{\cdot}367$$

g $KHCO_3$/litre finished wine $= 2{\cdot}05(1 - 0{\cdot}367) = 1{\cdot}30$ g $KHCO_3$/litre finished ion. The wine was treated and a summary is shown in Table 3.

TABLE 3
Summary of Example of Treated Wine by the Simultaneous Adjustment of pH and Total Acid

		1 litre of wine	*100 gal (378·54 litres) of wine*
Volume exchanged		367 ml	36·7 gal
Volume unexchanged		633 ml	63·3 gal
Amount $KHCO_3$ added		1·30 g	108 lb or 492·1 g
Analysis	*Initial*	*Adjusted*	
		Immediately after adjustment	*1 month at 30°F*
pH	2·96	3·32	3·31
Total acidity	12·6	7·2	7·1

The additional drop in pH or acid was not observed in this example since the amount of potassium and tartrate removed rendered the wine stable. A wine stable to tartrate deposition will allow the acid and pH to be adjusted to $\pm 0{\cdot}2$ g/litre and $\pm 0{\cdot}02$, respectively.

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Chemical Composition of *Tetracarpidium conophorum* (Conophor Nut)

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ABSTRACT

Conophor nut was analysed and shown to contain 40% free lipid, 24% protein and a very low level of non-protein nitrogen on a dry weight basis. Over two-thirds of the fatty acids is linolenic acid and 80% poly-unsaturated fatty acids. The sugar content (sucrose) is only 2% of the nut but the carbohydrate fraction consists mainly of unidentified polysaccharide(s).

INTRODUCTION

Tetracarpidium conophorum (Muell Arg) Hutch & Delta is a tropical climbing shrub. It is grown in Nigeria for its fruits which bear some oil-rich nuts. The only known use of the nuts is as a snack. The crop has a great potential as a source of food because it is very prolific. Gunstone *et al.* (1947) and Hilditch & Seavell (1950) have shown that the shelled nut contains over 60% linolenic acid and have concluded that the oil is technically superior to linseed oil as a drying oil. The present work was undertaken to study the chemical composition of the nut as a first step towards developing it as a source of food.

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EXPERIMENTAL

Preparation of materials

The nuts were collected fresh from areas in the Oyo and Ondo States of Nigeria. After drying at 70 °C for 1 h, the nuts were either hand shelled or shelled in a mechanical sheller. Some nuts were cooked before shelling and then dehydrated in an air oven. The dried samples were milled in a disc mill and then placed in sealed cellophane bags kept in a freezer at −20 °C until required for analysis.

Chemical analysis

Proximate analysis
The moisture content was determined in an air oven at 100 °C. Ash was determined in a muffle furnace and fibre as described by the AOAC (AOAC, 1975). Free lipids were extracted in a Soxhlet apparatus and determined as described by the AOAC (AOAC, 1975). Total lipid was determined by acid hydrolysis. Nitrogen was determined by the micro-Kjeldahl method. The percentage nitrogen was converted to crude protein by multiplying by 6·25.

Analysis of carbohydrate fraction

Total soluble sugar was determined by the phenol–sulphuric acid method of Dubois *et al.* (1956). Hydrolysable carbohydrate was determined by the method of the AOAC (1975); 1 g of dried material was hydrolysed with hydrochloric acid, neutralised and the sugar formed determined by Benedict's quantitative method.

Thin layer chromatography of sugars
The sugars were extracted by 80 % ethanol. The extract was subjected to separation by TLC plates coated with phosphate buffered silica gel G (Stahl, 1969) using ethyl acetate as the solvent system. The sugars were revealed by spraying the plates with α-naphthol. The plates were then air dried and later sprayed lightly with 90 % H_2SO_4. They were then heated in an oven maintained at 100 °C. The sugars yielded purple spots.

Analysis of the lipid fraction

The iodine value was determined by the Wijs method (AOAC, 1975) and the saponification value according to AOAC procedures. The refractive index was determined using an Abbe refractometer.

Fatty acid analysis

The fatty acid methyl esters were prepared as follows. About 1 g of oil was saponified with 20 ml 5% methanolic KOH for 30 min. The medium was neutralised with conc. H_2SO_4. Excess conc. H_2SO_4 was added to a level of 5% based on the weight of the mixture. The mixture was refluxed for a further period of 30 min. After the addition of water, fatty acid methyl esters were extracted with hexane and dried over sodium sulphate. The fatty acids were analysed by GLC using a Pye 104 gas chromatograph under the following conditions. Column: glass; stationary phase: DEGS 10%; support: 100–200 mesh Diatomite CAW; carrier gas: nitrogen; inlet pressure: 20 psig; oven temperature: 190 °C; detector temperature: 210 °C; detector: hydrogen flame ionisation; sensitivity: 1×10^{-9} A.

RESULTS AND DISCUSSION

A hundred unshelled nuts weighed 950 g. The ratio of the weight of the shell to the edible portions of the nut is 28 to 72, based on nuts weighing 500 g. The composition of the nut is given in Table 1. The free lipid

TABLE 1
Proximate Composition of *T. conophorum*

	Per cent on wet weight basis	*Per cent on dry weight basis*
Moisture	59·5	—
Fat	17·1 (free)	50·0 (free and bound)
Ash		2·8
Protein		23·5
Fibre		5·9
Nitrogen free extractive (by difference)		17·8

content of the nut on a dry weight basis is about 40 %. The protein content is 24 % on a dry weight basis. The apparent nitrogen free extractives are low.

Carbohydrate fraction

The results are presented in Table 2. The total sugar content was about 2 %. However, the acid hydrolysable carbohydrate was fairly high. There is no evidence of the presence of starch in the nut. The iodine stain test was negative. Microscopic examination showed that starch granules were not present in the material. The nature of the polysaccharide is unknown. Thin layer chromatography of the ethanolic extract revealed that sucrose was the only detectable sugar. The apparent reducing sugar, as determined by Benedict's volumetric method, may be an artifact.

TABLE 2
Composition of the Carbohydrate Fraction of *T. conophorum* on a Dry Weight Basis

Reducing sugar	0·5 %
Total sugar	2·3 %
Acid hydrolysable carbohydrate	7·2 %

Lipid fraction

The oil is pale yellow and clear. It was noted that the solvents used for extracting the lipids should be removed at low temperatures or under inert atmosphere to prevent the oil charring to a brown colour. The oil with the best colour was extracted from the boiled seeds. The characteristics of the oil are shown in Table 3. The iodine value is very high. As shown in Table 4, linolenic acid constitutes 66 % of the fatty acids. Polyunsaturated fatty acids account for over 80 % of the fatty acids in the oil. The figures agree with those reported by Hilditch & Seavell (1950).

TABLE 3
Characteristics of the Oil of *T. conophorum*

Iodine value	204
SG	0·932 5
Refractive index at 28·5 °C	1·470 8

TABLE 4
GLC of the Fatty Acid Methyl Esters of Oil Extracted from Conophorum
(% of total methyl esters)

Palmitic acid	1·3
Stearic acid	2·3
Oleic acid	14·4
Linoleic acid	15·8
Linolenic acid	66·3

Protein fraction

After defatting, the resulting cake is grey in colour and has a characteristic conophor nut flavour. This residue has about 35% protein content. The non-protein nitrogen was 9·2% of the total nitrogen. These qualities suggest that the cake may be of value in supplementing low protein foods.

CONCLUSIONS

Conophor nut oil has a high polyunsaturated fatty acid content and one of the highest levels of linolenic acid among plants. The protein content of the defatted nut is about 40%. This nut should be studied further with respect to its use in foods.

ACKNOWLEDGEMENTS

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The Stability Constant for the Interaction Between Bovine Serum Albumin and Magnesium *N*-Phenyl-8-aminonaphthalenesulphonate

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ABSTRACT

A close examination of some previously reported data for the binding of N*-phenyl-8-aminonaphthalenesulphonate to bovine serum albumin shows that the suggested 3:1 stoichiometry of ligand to protein with an average equilibrium constant in the region of* $10^6 M^{-1}$ *does not fully explain all the binding characteristics. Data and calculations are presented in this paper which demonstrate that simple fluorescence measurements on mixtures of protein and ligand do not give unambiguous results. Our results are more consistent with a stoichiometry greater than 3:1, perhaps including additional interactions leading to the formation of non-fluorescent species and decreasing the activity of the ligand in solution. Three binding sites, however, appear to bind the ligand more strongly than the rest.*

INTRODUCTION

The interaction between *N*-phenyl-8-aminonaphthalenesulphonate (ANS) and bovine serum albumin (BSA) or certain other proteins leads to the formation of a product in which the fluorescence of the ANS is enhanced by a factor of approximately 200 (Weber & Laurence, 1954; Stryer, 1965). This behaviour has attracted considerable interest as a result of the belief that the induced fluorescence is a function of the apolar nature of the environment of the binding site (Stryer, 1965; Jonas &

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Weber, 1971; Feinstein & Felsenfeld, 1975) and supported by the observation that the fluorescence of ANS and related compounds is a function of the dielectric constant of the medium in which they are dissolved (Weber & Laurence, 1954; Stryer, 1965; McClure & Edelman, 1966). Apart from its use for the quantitative analysis of serum proteins, the fluorescence behaviour of bound ANS and related compounds has been used to follow conformational changes in proteins (Edelman & McClure, 1968). In the case of binding to BSA the most recent evidence suggests that a complex with a stoichiometry of 3 moles ligand to 1 mole protein is formed (pH 7) and that the average binding constant (Ma *et al.*, 1973; Naik *et al.*, 1975) is of the order of $10^6 M^{-1}$. Earlier reported work, however, suggested a stoichiometry of 5:1 (Weber & Young, 1964; Daniel & Weber, 1966).

Assuming a stepwise addition of ligand (L) to protein (P):

$$P + L \rightleftharpoons PL \qquad K_1 = [PL]/[P][L] \tag{1}$$

$$PL + L \rightleftharpoons PL_2 \qquad K_2 = [PL_2]/[PL][L] \tag{2}$$

$$PL_2 + L \rightleftharpoons PL_3 \qquad K_3 = [PL_3]/[PL_2][L] \tag{3}$$

Constants K_1, K_2 and K_3 have been reported as $2{\cdot}9 \times 10^6 M^{-1}$, $6{\cdot}1 \times 10^5 M^{-1}$ and $5{\cdot}6 \times 10^5 M^{-1}$, respectively (Naik *et al.*, 1975) and $3{\cdot}02 \times 10^6 M^{-1}$, $6{\cdot}92 \times 10^5 M^{-1}$ and $1{\cdot}58 \times 10^5 M^{-1}$, respectively (Ma *et al.*, 1973), all measurements being made at 25 °C. The former workers used the method of continuous variation to confirm the stoichiometry as 3:1. The experiments were carried out under the conditions [ANS] + [BSA] = $10^{-5} M$ with the maximum of the continuous variation curve appearing at a ligand mole fraction of 0·76. It is not difficult to calculate the theoretical continuous variation curve given the experimental equilibrium constants and the concentrations. If the initial amounts of BSA and ANS are a and b, respectively and the amounts of the complexes PL, PL_2 and PL_3 present at equilibrium are $(x - y)$, $(y - z)$ and z, respectively then, from the law of mass action expression for each equilibrium step:

$$x^2 - \left(a + b - y - z + \frac{1}{K_1}\right)x + ab - ay - az + \frac{y}{K_1} = 0 \quad \text{from step 1} \tag{4}$$

$$y^2 - \left(b - z + \frac{1}{K_2}\right)y + bx - xz - x^2 + \frac{z}{K_2} = 0 \quad \text{from step 2} \tag{5}$$

$$z^2 - \left(b - x + \frac{1}{K_3}\right)z + by - xy - y^2 = 0 \quad \text{from step 3} \tag{6}$$

By initially setting x, y and z to zero and successively evaluating these quantities from eqns (4), (5) and (6), iteration leads to converging values for the unknowns under conditions of the continuous variation experiments. The amount of bound ligand, which is presumed to be proportional to the fluorescence intensity of the complex, is then $x + y + z$. The results of our own calculation around the maximum point on the continuous variation curve are shown in Fig. 1. It is immediately apparent that the theoretical maximum lies at a ligand mole fraction of 0·67 which suggests an apparent stoichiometry close to 2:1, whereas the observed stoichiometry was 3:1 by this method.

The theory of continuous variation plots has been considered in detail by Likussar & Boltz (1971) but only for single step reactions of the type:

$$mP + nL \rightleftharpoons P_mL_n \tag{7}$$

where the expected result of a maximum at the stoichiometric composition is found. When parallel or consecutive reactions are involved,

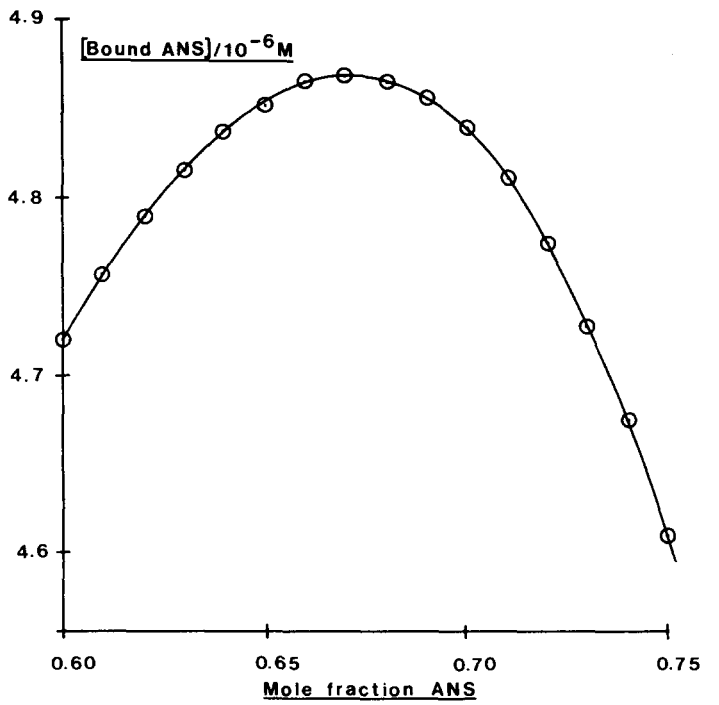

Fig. 1. Part of continuous variation curve computed for the BSA–ANS reaction with $K_1 = 2{\cdot}9 \times 10^6 M^{-1}$, $K_2 = 6{\cdot}1 \times 10^5 M^{-1}$, $K_3 = 5{\cdot}6 \times 10^5 M^{-1}$ and [ANS] + [BSA] = 1×10^{-5}M. Stoichiometry = 3:1 (ANS:BSA).

this theoretical value is only obtained when the equilibrium constants for each step are very large (probably greater than $10^7 M^{-1}$ in our case). If indeed a series of consecutive additions of ligand to protein is taking place, for the reaction in question, with an average binding constant (taken over equilibria with similar binding constants) of $10^6 M^{-1}$ or less, then it is necessary to postulate a stoichiometry greater than 3:1.

The object of this investigation is to consider the correspondence between the observed continuous variation curves and those predicted theoretically from derived equilibrium constants and to comment on the discrepancies between the observed stoichiometric ratios.

EXPERIMENTAL

Preparation of magnesium *N*-phenyl-8-aminonaphthalenesulphonate

The sodium salt of ANS is uncrystallisable and was converted to the easily crystallised magnesium salt. Magnesium nitrate (5 g) was added to a solution (5 %w/v, aqueous) of the sodium salt of ANS (Eastman Kodak Ltd) and the mixture brought to the boil. After cooling, the precipitate was collected, decolorised with charcoal and recrystallised three times from hot water. The product was dried over phosphorus pentoxide under reduced pressure. Microanalysis gave: C = 61·96 %, H = 4·01 %, N = 4·6 %, S = 10·33 % and Mg = 3·88 %. Expected for $Mg(C_{16}H_{12}NSO_3)_2$: C = 61·94 %. H = 3·87 %, N = 4·52 %, S = 10·35 % and Mg = 3·87 %.

Purification of bovine serum albumin

Bovine serum albumin was obtained from Sigma Chemicals Ltd. Since the protein may contain variable amounts of lipid and fatty acid impurity, it was decided to subject it to the purification suggested by Chen (1967). The protein (7 g) was dissolved in water (70 ml) at room temperature. Acid washed activated charcoal (3 g) was added and the pH of the solution lowered to 3·0 by the addition of hydrochloric acid (0·2 M). The solution was placed in an ice bath and stirred for 1 h. The charcoal was removed by centrifuging at 38 000 × *g* for 40 min at 2 °C. The clarified solution was brought to pH 7·0 by the addition of sodium hydroxide, dialysed and lyophilised. The product was subjected to disc gel electrophoresis using 6 % acrylamide/5 % bisacrylamide gels in pH 8·8 Tris-glycine buffer at 200 V

for 45 min. Gels were stained with naphthalene black when the protein was found to run as a single component. Before purification the protein gave rise to at least five components distinguishable by this technique.

Fluorescence measurements

All fluorescence measurements were made using a Perkin Elmer 204 spectrofluorimeter (1 cm silica cells) in phosphate buffer (0·1 M, pH 6·8) with the buffer used as a reference. Unless otherwise stated, measurements were made at an excitation wavelength of 380 nm and emitted light was observed at 467 nm.

Solutions of ANS and BSA were prepared by weight on the basis of an average molecular weight of 69 000 for BSA. However, because of the hygroscopic nature of BSA concentrations were checked spectrophotometrically at 280 nm assuming a value of $E^{1\%}_{1\,cm} = 6{\cdot}60$. In most cases solutions prepared by weight gave the correct absorbance. Continuous variation curves were constructed from fluorescence measurements on solutions containing [ANS] + [BSA] = 10^{-5} M at mole ratios of 0 to 1 in steps of 0·1 and 0·05 around the maximum. For calculation of equilibrium constants fluorescence emission was measured for solutions containing [BSA] = 2×10^{-6} M and [ANS] = $0{-}2 \times 10^{-5}$ M. The absorbance of all solutions subjected to fluorescence measurement was found at 380 nm.

THEORY

Correction of fluorescence data

The light passing into the fluorescing solution is absorbed by the bound ligand and also by the free ligand and the protein. Emission takes place only as a result of interaction with the bound ligand. For light travelling in a medium containing an absorbing species, the intensity (I) at a distance, x, into that medium is related to the intensity (I_o) incident on that medium by:

$$I = I_o \exp(-\sum k_n x) \tag{8}$$

where k_1, k_2, ... are constants characteristic of the absorbing species.

Since the fluorescence intensity is proportional to the amount of light absorbed by the fluorescent species:

$$\frac{dI_f}{I} = -k_f dx \tag{9}$$

where dI_f/I is the fractional loss of intensity due to absorption by the fluorescent species and k_f is the corresponding constant. In the presence of other absorbing species, I is the value of the intensity before light enters the element dx; that is:

$$\frac{dI_f}{I_o \exp(-\sum k_n x)} = -k_f dx \tag{10}$$

Integrating between the limits $x = 0$ and $x = x$ and using the fact that fluorescence intensity (F) is proportional to the reduction in excitation intensity due to the fluorescent species (i.e. $I_o - I_f$), the result:

$$F = \varphi I_o \frac{k_f}{\sum k_n} \{1 - \exp(-\sum k_n x)\} \tag{11}$$

is obtained where φ is the quantum yield. Expressing in terms of absorbance:

$$F = \varphi I_o \frac{A_f}{A}(1 - 10^{-A}) \tag{12}$$

where A_f and A are the absorbance of the fluorescing species and the total absorbance of the solution at the wavelength of excitation, respectively.

Since the quantum yield is not known, only the quantity $\varphi I_o A_f$ can be computed. However, since A_f is expected to be directly proportional to concentration and hence $\varphi I_o A_f$ is also proportional to concentration, this quantity was used in place of fluorescence intensity.

Determination of equilibrium constants

This is illustrated for three stepwise equilibria as described by eqns (1) to (3). By taking the formula derived by Naik *et al.* (1975) for this system relating the average number ($\bar{n}$) of bound ligand molecules per protein molecule to the concentration of free ligand:

$$\bar{n} = \frac{K_1[L] + 2K_1K_2[L]^2 + 3K_1K_2K_3[L]^3}{1 + K_1[L] + K_1K_2[L]^2 + K_1K_2K_3[L]^3} \tag{13}$$

Dividing both sides by [L] and integrating results in:

$$\int_0^{[L]} \frac{\bar{n}}{[L]} d[L] + Q = \ln (1 + K_1[L] + K_1K_2[L]^2 + K_1K_2K_3[L]^3) \quad (14)$$

The integral of $\bar{n}/[L]$ may be determined graphically from the experimental data and Q is a constant to allow for errors introduced in the integration at small values of [L]. Expressing in exponential form:

$$\exp \int_0^{[L]} \frac{\bar{n}}{[L]} d[L] = A + B[L] + C[L]^2 + D[L]^3 \quad (15)$$

where A, B, C and D are constants. When $Q = 0$, that is, when there is no error of integration, $A = 1$. Under normal circumstances with $Q \neq 0$, then $K_1 = B/A$, $K_1K_2 = C/A$, $K_1K_2K_3 = D/A$. In practice, the coefficients A, B, C and D were determined by polynomial regression of numerically integrated values on [L].

It can be shown that the same treatment is applicable to stoichiometries other than 3 with a similar result to eqn. (15) with the exception that the degree of polynomial is equal to the stoichiometry.

RESULTS AND DISCUSSION

Microanalysis data for the magnesium salt of ANS and gel electrophoresis of BSA suggest that the reactants were of acceptable quality. This specification for ANS, however, does not exclude the possibility of traces of highly fluorescent impurities and therefore some means of establishing spectroscopic purity was required. Since emission of light in fluorescence always takes place from the same energy level, the emission spectrum is expected to be independent of the energy of the exciting radiation. Emission spectra were therefore recorded between 420 and 560 nm at a variety of excitation wavelengths, for buffered aqueous solutions of ANS (10^{-5} M). Maximum emission was found to occur at 520 nm and the position of this maximum was found to be independent of the energy of excitation. Moreover, when the fluorescence intensity observed at each wavelength for one value of excitation energy was plotted against the corresponding fluorescence intensity at another excitation energy, straight lines passing through the origin were obtained. This is illustrated in Fig. 2 and suggests that the solution contains only one fluorescent species or a number of species with the same excitation

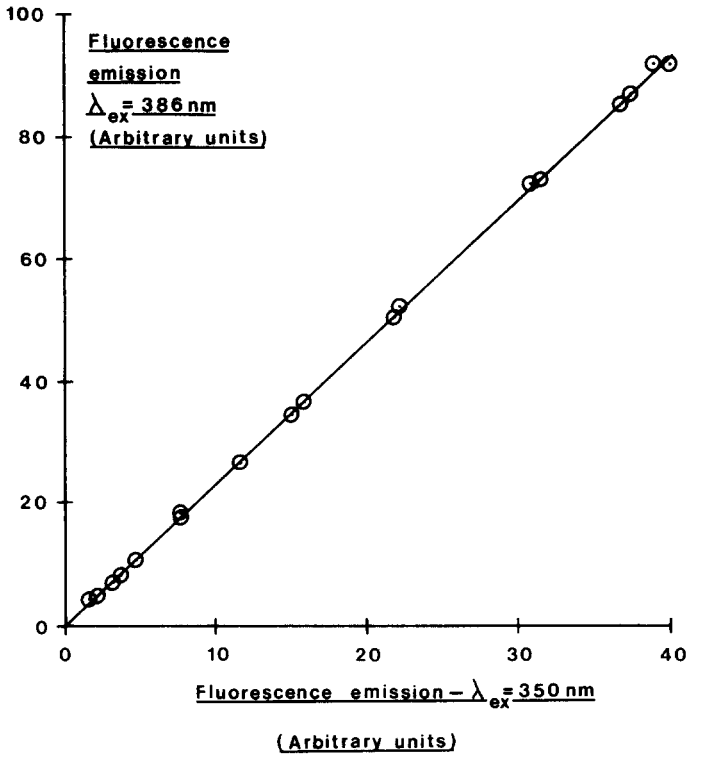

Fig. 2. Graph of emission intensity for an excitation wavelength of 386 nm as a function of emission intensity at an excitation wavelength of 350 nm. Intensities plotted as pairs at each wavelength of emission taken from spectra recorded between 440 and 600 nm.

characteristics. Since the last possibility is remote the product was considered to be spectroscopically pure. A similar result was observed for mixtures containing buffered mixtures of BSA and ANS except that the maximum of emission, whose position was unaffected by the relative amounts of the two reactants present, occurred at 467 nm.

A continuous variation curve for the ANS–BSA mixtures is shown in Fig. 3. The result is consistent with previously reported work (Naik *et al.*, 1975) showing a maximum at an ANS mole fraction of 0·75 and therefore suggesting a stoichiometry of 3:1 for the interaction. The results of fluorescence measurements on mixtures of the reactants containing a constant amount of protein (2×10^{-6}M) and a variable amount of ANS to a maximum mole ratio of 10:1 (ANS:BSA) are shown in Table 1, together with values of $\varphi I_o A_f$ calculated according to eqn. (12). The previously

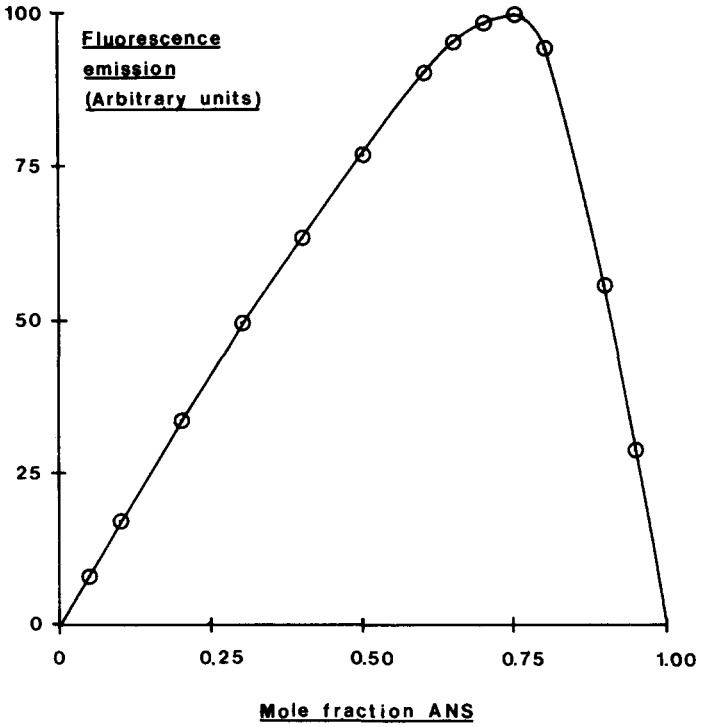

Fig. 3. Continuous variation curve obtained experimentally for ANS–BSA mixtures with [ANS] + [BSA] = 1×10^{-5}M, pH 7.

reported analyses of such equilibrium data have relied on experimental determination of the average number of moles of ANS bound per mole of protein at each measurement and similarly the method adopted here is dependent on this quantity being accurately calculable or determinable experimentally. In terms of the quantity $\varphi I_o A_f$ used in place of concentration of bound ANS in this work, the average number of moles ANS bound per protein molecule is:

$$\bar{n} = \frac{[\mathrm{L}]_{\mathrm{bound}}}{[\mathrm{P}]_{\mathrm{total}}} = \frac{A_f^*}{(A_f^*)_{\max}} n \tag{16}$$

where n is the stoichiometry of the reaction, A_f^* denotes $\varphi I_o A_f$ and $(A_f^*)_{\max}$ is the value of A_f^* corresponding to complete binding of the protein, i.e. the value derived from the fluorescence intensity in the presence of a large excess of ligand. Our results show, however, that at the highest concentration of ANS (2×10^{-5}M), the emission intensity

TABLE 1

Experimental Data for the Addition of ANS to BSA (2×10^{-6}M) with Calculated Values of Emission Intensity After Correction for Absorbance Due to Non-fluorescent Species According to Equation (12)

$[ANS]/10^{-6}$M	*Fluorescence emission**	*Absorbance at 380 nm*	$\varphi I_o A_f$†	*Predicted*† $\varphi I_o A_f$
0·5	5·5	0·004	2·40	—
1·0	10·5	0·007	4·60	4·34
1·5	15·5	0·009	6·80	—
2·0	20·5	0·012	9·03	8·85
3·0	30·0	0·018	13·30	13·21
4·0	38·0	0·024	16·96	17·26
5·0	46·0	0·030	20·67	20·94
6·0	53·0	0·036	23·96	24·21
7·0	59·0	0·042	26·88	27·07
8·0	63·5	0·047	29·10	29·56
9·0	69·5	0·052	32·03	31·71
10·0	73·0	0·058	33·87	33·57
11·0	75·5	0·065	35·30	35·20
12·0	77·5	0·070	36·44	36·61
13·0	79·5	0·074	37·55	37·86
14·0	81·5	0·078	38·67	38·96
20·0	88·0	0·108	43·17	43·63

* Arbitrary units.

† See text.

expressed as A_f^* is still increasing and the use of concentrations of ANS sufficiently high to ensure complete binding of BSA is limited by the need for large corrections due to absorbance. It is also apparent that simple extrapolation of our fluorescence-concentration data is not possible with any degree of certainty. Previous workers (Daniel & Weber, 1966; Ma *et al.*, 1973; Naik *et al.*, 1975) have attempted to resolve this difficulty by calibrating their spectrofluorimeter with solutions containing an excess of BSA but it was found in this investigation that significant conclusions about the behaviour of the BSA–ANS system could be deduced by simply observing the variation of the calculated equilibrium constants as a function of the limiting value of A_f^*.

In order to calculate equilibrium constants the treatment of data suggested by eqn. (15) was applied for stoichiometries of 3, 4 and 5. The $(A_f^*)_{max}$ value was set at arbitrary levels greater than, or equal to, the

highest experimental measurement and [L] values calculated for each data point for each assumed stoichiometry. When integrated data were fitted to 3rd, 4th and 5th degree polynomials in [L], an apparently perfect fit, independent of choice of $(A_f^*)_{max}$ to at least a value of 75, the limit of our trial values, was obtained in all cases. An example, typical of all results, is shown for $(A_f^*)_{max} = 60$ for stoichiometries of 3 and 4, in Fig. 4. In order to test the overall accuracy of these calculations which require several operations on the data, the experimental results for A_f^* were predicted from the derived equilibrium constants using eqns (4), (5) and (6). In the cases tried (3:1 stoichiometry) predicted values were in excellent agreement with experimental data, a typical set of results, obtained for $(A_f^*)_{max} = 60$ and $K_1 = 3{\cdot}43 \times 10^5 M^{-1}$, $K_2 = 3{\cdot}22 \times 10^5 M^{-1}$, $K_3 = 3{\cdot}88 \times 10^4 M^{-1}$, being shown alongside experimental values in Table 1.

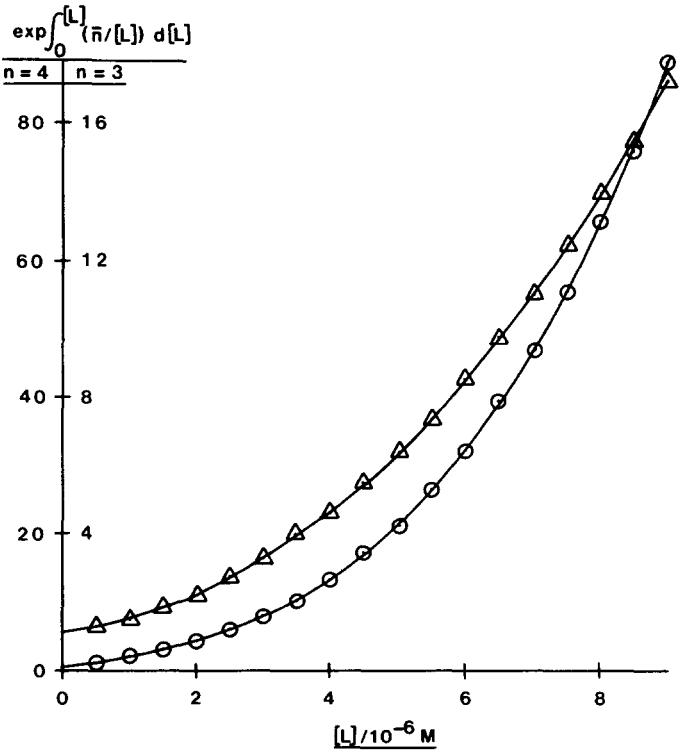

Fig. 4. Graphs of $\exp \int_0^{[L]} (\bar{n}/[L])\, d[L]$ as a function of [L], calculated for stoichiometries (ANS:BSA) of 4:1 and 3:1, for the addition of ANS to BSA (2×10^{-6} M). Points from experimental data denoted by ⊙ for $n = 4$ and △ for $n = 3$. Curve is drawn through points predicted from corresponding polynomial regression.

Considering first the results obtained using the calculation for a 3:1 stoichiometry, it is found that sensible values for coefficients of regression are obtained for all values of $(A_f^*)_{max}$ tried, the magnitude of these coefficients decreasing as expected with increasing $(A_f^*)_{max}$. The largest coefficients are obtained when the maximum value is set to the last experimental point and calculated equilibrium constants are $K_1 = 1{\cdot}84 \times 10^6 M^{-1}$, $K_2 = 8{\cdot}92 \times 10^4 M^{-1}$ and $K_3 = 1{\cdot}08 \times 10^6 M^{-1}$. It is interesting to note that our data have also frequently given values for K_3 as being greater than those for K_2, suggesting that the treatment of the data is not capable of resolving correctly the contributions made by steps 2 and 3 of the reaction or that additional complexation is taking place to yield complexes of higher stoichiometry and distorting the contributions from K_2 and K_3. The value of K_1 compares very favourably with values of $2{\cdot}94 \times 10^6 M^{-1}$ and $3{\cdot}02 \times 10^6 M^{-1}$ reported elsewhere (Ma *et al.*, 1973; Naik *et al.*, 1975). The predicted continuous variation curve using our values for the equilibrium constants offers no improvement over that predicted in the Introduction.

Considering next the results obtained assuming a 4:1 stoichiometry in the calculation, the highest equilibrium constants found were for an $(A_f^*)_{max}$ value of 50 giving $K_1 = 1{\cdot}04 \times 10^6 M^{-1}$, $K_2 = 4{\cdot}96 \times 10^5 M^{-1}$, $K_3 = 5{\cdot}77 \times 10^5 M^{-1}$ and $K_4 = 6{\cdot}02 \times 10^4 M^{-1}$. Reducing the value of $(A_f^*)_{max}$ below 50 appeared to give a large intercept and negative values for some of the coefficients, as did high values (e.g. 75). Using the values for the constants given above to predict a continuous variation curve by the method already applied to a 3:1 stoichiometry, but with the addition of a fourth step, the maximum point on the curve lies at an ANS mole fraction of 0·71. This result is in better agreement with the observed maximum of 0·75. However, when theoretical and observed continuous variation curves are superimposed, normalised to each other at an ANS mole fraction of 0·75, considerable differences between the data still exist. It is interesting to note, however, that of the four constants, three are within a factor of approximately 2 of each other whereas the fourth is an order of magnitude lower, confirming the held belief of three relatively strong binding sites. The last statement applies to constants derived at other values of $(A_f^*)_{max}$. For example, at a value of 55 the derived constants are $1{\cdot}13 \times 10^6 M^{-1}$, $2{\cdot}83 \times 10^5 M^{-1}$, $4{\cdot}88 \times 10^5 M^{-1}$ and $1{\cdot}85 \times 10^4 M^{-1}$, respectively and at a value of 60 they are $8{\cdot}21 \times 10^5 M^{-1}$, $2{\cdot}76 \times 10^5 M^{-1}$, $3{\cdot}33 \times 10^5 M^{-1}$ and $8{\cdot}62 \times 10^3 M^{-1}$, respectively.

Although this technique was applied to the calculation involving a 5:1 stoichiometry, the precision of the data was insufficient for sensible values for the constants to be derived.

A previously reported method of determining the equivalent to the $(A_f^*)_{max}$ value in terms of fluorescence is based on the observation that graphs of the reciprocal of fluorescence intensity as a function of the reciprocal of total ligand concentration at constant substrate concentration appear linear and may be extrapolated to infinite ligand concentration. By expressing the binding of ligand in terms of the Scatchard equation (Scatchard, 1949):

$$\frac{\bar{n}}{[L]} = K_d(n - \bar{n}) \tag{17}$$

where K_d is an average dissociation constant, it can be shown that:

$$\frac{1}{F} = \frac{1}{F_{max}} + \frac{1}{F_{max}K_d}\frac{1}{[L]_T} \tag{18}$$

where F represents the fluorescence intensity of the mixture at a particular total ligand concentration $[L]_T$ and F_{max} is the value of fluorescence intensity assuming the protein in the mixture to be fully complexed. By the reasoning used in this paper, F and F_{max} may be replaced by A_f^* and $(A_f^*)_{max}$, respectively. An essential assumption in the derivation of this equation is that:

$$\frac{1}{1 + K_d[L]_T}\alpha \ll 1 \tag{19}$$

where α is the fraction of ligand bound. This may arise either as a result of $K_d[L]_T$ being large or α being small. Thus, linearity of the reciprocal plot is expected when stable complexes are produced or at high concentrations of added ligand. Such a plot for the reaction in question gave rise to some curvature but extrapolation of the high concentration portion of the curve suggests $(A_f^*)_{max}$ values in the region of 75. Since the values of the calculated equilibrium constants fall with increasing $(A_f^*)_{max}$, the result suggests somewhat weaker complexes than reported above and the discrepancy in the maximum of the continuous variation plots becomes greater.

CONCLUSION

The results and calculations reported here show that there is no unique solution to the determination of equilibrium constants for the BSA–ANS reaction from fluorescence measurements on solutions at various molar ratios of the reactants. The main limitation is the ability to unequivocally determine the amount of ANS bound as the fluorescent complex, but even if this could be achieved, there does not appear to be a single stoichiometry which satisfies the data. Our results, however, allow the following conclusions to be drawn:

(1) In order for the stoichiometry of the reaction to appear as 3:1 by the method of continuous variation it is necessary for either the equilibrium constants (K_1, K_2, K_3) to be at least an order of magnitude greater than those obtained here and in previous work (Ma *et al.*, 1973; Naik *et al.*, 1975), or for the stoichiometry to be greater than 3:1. Our results, calculated for a stoichiometry of 4:1, lead to better continuous variation curves but large discrepancies still exist.

(2) The treatment of data neglects other interactions which may not lead to fluorescent products but which will, nevertheless, reduce the activity of ANS in solution. The true amount of free ligand may therefore be overestimated when calculated from initial concentrations and fluorescence intensities or concentration of fluorescent products.

(3) It is reasonable to conclude that three relatively stable complexes are formed and that any fluorescent complexes formed additionally are somewhat more dissociated.

(4) The treatment of the data has assumed that the ligand molecules bound to BSA all fluoresce with the same quantum efficiency. This is a reasonable assumption since it was found that the emission spectrum is unchanged as the molar ratio of reactants is varied and it is known that the quantum yield of fluorescence is related to λ_{max} for the emission. However, the applicability of this assumption in this system needs to be rigorously verified.

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Formation of Oligosaccharides by β-Galactosidase from *Streptococcus thermophilus*

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ABSTRACT

The β-galactosidase of Streptococcus thermophilus *exhibited substantial glycosyl transferase activity during the hydrolysis of lactose in milk. The new sugars formed accounted for 25% of the total sugars when the lactose had been 94% hydrolysed. Sixty per cent of the new sugars were identified as allolactose and 30% as 6-O-β-D-galactopyranosyl-D-galactose. No other disaccharides, trisaccharides or higher oligosaccharides were detected. The ratio of free glucose to free galactose during hydrolysis was as high as two to one.*

INTRODUCTION

Enzymatic hydrolysis of lactose occurs by a transgalactosidic mechanism (Wallenfels & Malhotra, 1961). The galactose moiety of a β-galactoside is transferred to an acceptor containing an hydroxyl group. The most common acceptor in aqueous solutions is water. Consequently, it is expected that one molecule of lactose yields one molecule of glucose and one of galactose. However, the acceptor molecule can also be another substrate with hydroxyl groups. Therefore, when lactose and β-galactosidase are present, it is possible to have new glycosidic bonds formed

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between individual carbohydrates, resulting in oligosaccharides (including new disaccharides) of varying molecular weights and composition (Huber *et al.*, 1976).

The β-galactosidase from *Streptococcus thermophilus* appears promising for commercial hydrolysis of milk lactose (Somkuti & Steinberg, 1979; Greenberg & Mahoney, 1981; Ramana Rao & Dutta, 1981). Toba *et al.* (1981) reported that this enzyme formed several oligosaccharides when acting on lactose and identified some of the products; however, no quantitative data were given so the significance of this transferase activity is not clear.

The purpose of this research was to study the proportions of glucose, galactose and oligosaccharides (defined as all sugars in the system other than glucose, galactose and lactose) formed by hydrolysis and transferase action when the β-galactosidase of *S. thermophilus* was used to hydrolyse lactose in milk.

MATERIALS AND METHODS

Materials

The β-galactosidase from *S. thermophilus* was prepared as described by Greenberg & Mahoney (1981). Skim milk powder was a gift from the Yankee Milk Co-operative (Springfield, MA, USA). 6-*O*-β-D-Galactopyranosyl-D-galactopyranose and 6-*O*-β-D-galactopyranosyl-D-glucopyranose (allolactose) were a gift from Professor T. Toba (Toboku University, Sendai, Japan). All other chemicals were of reagent grade quality or better.

Hydrolysis of lactose in milk

Reconstituted skim milk was prepared by making a 10% (w/v) solution with the skim milk powder. The solution was stored at 2 °C with 0·02% NaN_3 as a preservative. The enzyme was assayed for activity at 37 °C by a discontinuous method (Mahoney *et al.*, 1975) using 2·2 mM *o*-nitrophenyl-β-D-galactopyranoside (ONPG) in 0·1 M potassium phosphate pH 7·0, containing 0·1 mM $MnCl_2$, 0·5 mM dithioerythritol (DTE) and 0·02% NaN_3. One ONPG unit of enzyme activity was the amount of enzyme needed to liberate 1 μmol of *o*-nitrophenol per minute under the conditions described.

Approximately 3000 ONPG units of β-galactosidase were added to 100 ml of reconstituted skim milk at 37 °C. Two millilitre samples were taken at various times and added to 40 μl of 4N HCl. The samples were then deproteinised with 0·4 ml 10%(w/v) $ZnSO_4.7H_2O$ and 65 μl 4N NaOH. The amount of glucose present was determined by the Glucostat procedure (*Worthington Enzyme Manual*, 1972) and those samples containing approximately 25%, 50%, 75%, 84% and 94% of the maximum amount of glucose possible were stored frozen for further analysis.

Paper chromatography

The descending paper chromatography technique of McFarren *et al.* (1951) was used to separate the sugars before quantitation. The solvent system was ethyl acetate, pyridine, water (2·5:1·0:3·5, v/v/v). A 10-μl syringe was used to apply the sample, 2 μl at a time, such that 50 μg to 200 μg of the monosaccharides or oligosaccharides to be analysed were applied. For the chromatograms that were analysed for glucose and galactose, the paper (Whatman No. 1) was developed for 36 h. For the oligosaccharides, the paper was developed for 60 h. The chromatograms were dried every 24 h to allow a fresh solvent front to descend through the paper.

Each piece of paper was spotted five times with the same sample and developed. The outer two lanes were cut out and stained by a silver nitrate method (Trevelyan *et al.*, 1950) as modified by Nordin (1981). The chromatogram was dipped in a dish containing acetone and silver nitrate (10 ml of saturated nitrate per litre of acetone). The chromatogram was then dipped in 0·5% potassium hydroxide in methanol. As black spots developed, the chromatogram was dipped in 10% sodium thiosulphate and 0·15% sodium metabisulphate and allowed to dry. The two stained outer lanes were placed next to the unstained lanes and used to identify the positions of the sugar spots in the unstained lanes.

Quantitative analysis of sugars

The unstained 'spots' were cut out and a point fashioned on one edge of the 'spot'. The sugar was then eluted with 2 ml of water. The eluted sugar was analysed by the phenol–sulphuric acid method of Dubois *et al.* (1956), using half of the volumes recommended. The samples were compared

with standard curves of glucose, galactose and lactose where appropriate. The oligosaccharide fraction was compared with the lactose standard curve.

Each fraction was analysed four times (three lanes on four chromatograms) in duplicate; the standard deviation of each sugar was then computed. The recoveries of glucose, galactose and lactose from pure solutions subjected to deproteinisation, paper chromatography and elution were determined.

RESULTS

Typical chromatograms of separated reaction products are shown in Figs 1 and 2. The glucose and galactose, which are well separated in this system (Fig. 1), migrate much further than any of the other sugars. No sugars

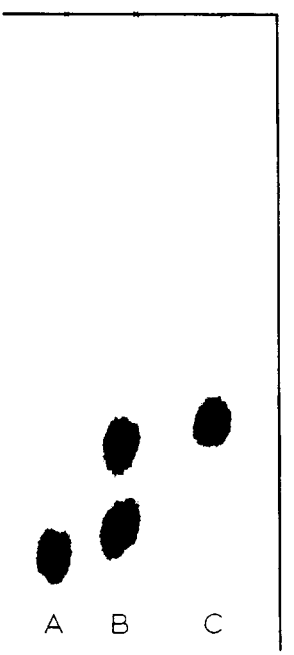

Fig. 1. The separation of glucose and galactose by paper chromatography. A, glucose; B, sample from milk; C, galactose.

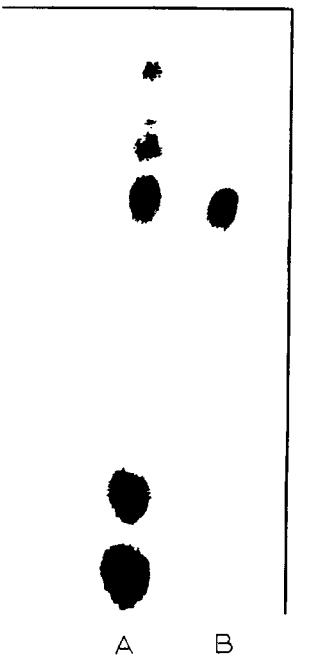

Fig. 2. The separation of lactose and transferase products by paper chromatography. A, sample from milk; B, lactose.

were observed in between galactose and lactose but there were oligosaccharides between lactose and the origin (Fig. 2). The recovery of glucose, galactose and lactose was 90% ± 3·0%, 91% ± 2·5% and 89% ± 6·1%, respectively.

The appearance of reaction products with time is shown in Fig. 3. Oligosaccharides appeared as soon as the monosaccharides were liberated, whilst glucose was liberated faster than galactose, indicating that some of the galactose was being used to synthesise the oligosaccharides.

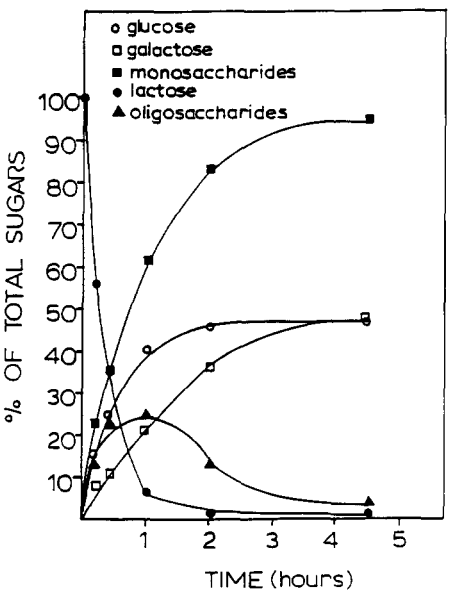

Fig. 3. Profile of the rates of utilisation of lactose and the formation of products in milk with time at 37°C.

The peak concentration of transferase products was reached after about 1 h, when they accounted for 25% of all the sugars present. Thereafter, the concentration of oligosaccharides declined, presumably because they were being hydrolysed more rapidly than they were being formed.

The values obtained for the concentration of each sugar species during the reaction are shown in Table 1. The ratio of glucose to galactose reached a maximum value of more than two whilst there was still about 35% lactose remaining and before the oligosaccharide level reached its maximum. The greatest absolute difference in glucose and galactose

TABLE 1

The Distribution of Sugars During Hydrolysis of Lactose in Milk by *S. thermophilus* β-Galactosidase at 37 °C[a]

Time (*h*)	*Lactose* (%)	*Glucose* (%)	*Galactose* (%)	*Glucose/galactose ratio*	*Oligosaccharides* (%)	*Glucose/galactose ratio in oligosaccharides*[b]
0·17	55·7 ± 0·44	14·6 ± 2·0	8·7 ± 0·44	1·67	13·1 ± 0·54	0·36
0·42	35·3 ± 1·8	25·0 ± 0·85	11·4 ± 1·3	2·19	22·7 ± 1·7	0·26
1·00	6·16 ± 0·84	40·2 ± 3·6	21·3 ± 1·8	1·89	24·8 ± 1·8	0·21
2·00	1·74 ± 1·25	46·4 ± 6·6	37·1 ± 2·7	1·25	13·8 ± 0·95	0·18
4·50	1·17 ± 0·98	46·7 ± 1·9	47·5 ± 3·4	0·98	3·61 ± 0·16	—[c]

[a] Average of four determinations, plus and minus half the range.

[b] The amount of each monosaccharide was calculated as the total monosaccharide resulting from lactose hydrolysis minus the free monosaccharide.

[c] Not determined because the values were not significantly different.

concentrations was observed when almost all (94%) of the lactose had been hydrolysed and the oligosaccharide level was at a maximum, although by that time (1 h) the glucose/galactose ratio had fallen slightly.

The ratio (calculated) of glucose to galactose in the oligosaccharides fell during the course of the reaction. This could be because oligosaccharides containing glucose—such as allolactose—were hydrolysed at a faster rate than those containing only galactose.

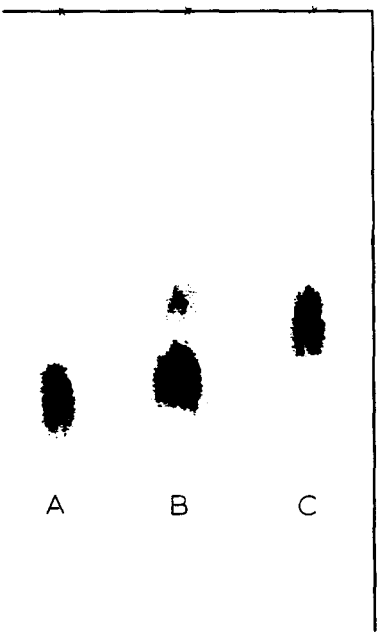

Fig. 4. Paper chromatography of the transferase products after separation from lactose. A, allolactose; B, sample from milk; C, 6-*O*-β-D-galactopyranosyl-D-galactose.

The identity of the major oligosaccharides formed is indicated in Fig. 4. The oligosaccharides readily detectable after 1 h of reaction co-chromatographed with allolactose and 6-*O*-β-D-galactopyranosyl-galactopyranose. Quantitation of the sugars showed that about 60% of the total oligosaccharides detected were allolactose and about 30% were the digalactose product. Thus, about three-quarters of the sugars in the oligosaccharides were presumed to be galactose which is in good agreement with the calculated values in Table 1. No oligosaccharides, other than the two disaccharides above, were detected in the reaction mixture.

DISCUSSION

The β-galactosidase of *S. thermophilus* exhibits substantive transferase activity during the hydrolysis of lactose in milk, with new products forming up to 25% of the total sugars. Allolactose was the major transferase product and comprised 60% of the oligosaccharides when the latter reached their highest level.

Huber *et al.* (1976) showed that β-galactosidase from *Escherichia coli* had even higher transferase activity when the enzyme acted on 0·5M lactose (17%w/v). They found that the maximum levels of both allolactose and of other oligosaccharides containing three or more sugar units each reached about 25% of the total sugars present. Thus, the combined transferase products (at their maximum concentrations) formed about 50% of all the sugars. They also found that oligosaccharide formation was reduced when the starting lactose concentration was lowered. The very high values that they reported may be due primarily to the fact that they used a much higher starting lactose concentration than is present in milk.

Roberts & Pettinati (1957) also reported that the level of oligosaccharide formation was dependent on the starting lactose concentration, when using β-galactosidase from *Saccharomyces fragilis*. With 35% lactose, they found 44% of the total sugars were oligosaccharides whilst, with 5% lactose, they found 22% oligosaccharides, which is very similar to our results. Burvall *et al.* (1979) determined oligosaccharide formation using β-galactosidase from *Saccharomyces lactis* in the form of a commercial product, 'Maxilact'. Using milk as the substrate they found that the maximum level of oligosaccharides was about 5% of the total sugars. However, this value did not include disaccharides other than lactose, so the total transferase activity may be much higher.

Toba *et al.* (1981) used a cell-free extract of a different strain of *S. thermophilus* from that used by us to hydrolyse 20% lactose and demonstrated the formation of several transferase products including allolactose, 6-*O*-β-D-galactopyranosyl-D-galactose, 2-*O*-β-D-galactopyranosyl-D-glucose and tri- and higher oligosaccharides. We were not able to detect any products other than the allolactose and the 6-*O*-β-D-galactopyranosyl-D-galactose, probably because, at the much lower lactose concentration, the amounts of these other compounds were too low for detection by our system. Huber *et al.* (1976) also reported that at low lactose levels (0·05M and less) oligosaccharides with three or more

sugar units were not formed in sufficient quantity to be detected by either gel filtration or gas–liquid chromatography.

The major transferase product that we detected was allolactose. Considering the quantities formed—15% of the total sugars was allolactose when 94% of the lactose had been hydrolysed—this may be of some nutritional significance, since allolactose is hydrolysed at only one-tenth of the rate for lactose in the human intestine (Burvall *et al.*, 1980). If the *S. thermophilus* enzyme is to be used to produce low-lactose milk for those suffering from hypolactasia, it is preferable to allow the hydrolysis to proceed essentially to completion as it is only then that all the oligosaccharides are broken down.

The large difference in glucose and galactose concentrations during the reaction has implications for those wishing to follow the reaction by analysis of the products. The 'degree of hydrolysis' of lactose is often estimated by the determination of glucose and by then assuming that a mole of glucose represents a mole of lactose hydrolysed. At higher lactose concentrations, the value for glucose will tend to overestimate the amount of lactose remaining but will give a fairly good estimate of the total number of glycosides remaining to be hydrolysed. At lower lactose concentrations, the glucose value underestimates the remaining glycosides. Determination of galactose and use of the same assumption leads to an even worse overestimate of the amount of lactose remaining and also to overestimation of the remaining glycosides.

The degree of lactose hydrolysis cannot be calculated by the determination of a single monosaccharide, except where the former approaches 100%. However, for many applications the functional properties of the product depend mostly on the total monosaccharides produced and these can be determined as reducing sugars. The number of glycosides remaining as lactose plus oligosaccharides can be calculated by difference when the original lactose concentration is known. In view of the above, when analysing β-galactosidase activity, it may be more useful to determine the monosaccharides produced as a percentage of the maximum rather than the percentage lactose hydrolysed.

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Effect of Cooking on the Nutritive Value of Common Food Plants of Tanzania: Part 1—Vitamin C in Some of the Wild Green Leafy Vegetables

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ABSTRACT

The vitamin C (ascorbic acid) content of fresh and cooked material of sixteen varieties of wild leafy vegetables was estimated. In fresh leaves, the vitamin varied from 204 mg/100 g fresh weight in Moringa oleifera *to 0·7 mg in* Emilia javanica.

When 20 mg samples were cooked in 400 ml of water, the vitamin C content decreased considerably. Losses observed ranged from 98·5% (Moringa oleifera) *to nil* (Emilia javanica). *Smaller losses of the vitamin were observed when vegetable samples were cooked in 100 ml of water.*

Since most leafy vegetables are cooked prior to consumption, it is recommended that the vegetables are cooked in small amounts of water for short periods to minimise loss of vitamin C and that the cooking water be consumed if no bitter compounds are present.

INTRODUCTION

Although fruits are rich in vitamin C, recent research has shown that some green leafy vegetables are an equally good source of vitamin C. In Tanzania, the symptoms of vitamin C deficiency have been rare due to fairly good consumption of fruits. However, due to the increasing price of fruits, people with low incomes are unable to consume them in sufficient

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quantity. If this situation continues in Tanzania, there might be some cases of scurvy in the future.

Several reports indicate the presence of vitamin C in green leafy vegetables (Oke, 1966; Keshinro & Ketiku, 1979; Ifon & Bassir, 1979). Unfortunately, however, most of the vegetables are eaten in the cooked state and it is universally believed that vitamin C is reduced by cooking (Krehl & Winters, 1950; Gordon & Noble, 1964; Oke, 1967; Fafunso & Bassir, 1976; Imbamba, 1977; Keshinro & Ketiku, 1979; Ajayi *et al.*, 1980).

Vitamin C is essential to the human body to prevent scurvy and has been shown to reduce the severity of stress, heart diseases and cancer (Anderson, 1977). Local communities should be educated to recognise (a) the importance of vitamin C, (b) that green leafy vegetables are a cheap source of vitamin C, (c) that a lot of vitamin C is lost during traditional methods of cooking and (d) that there is a need to modify the cooking process, thereby retaining greater amounts of the vitamin without sacrificing palatability and appearance.

In Tanzania, to the authors' knowledge, there seems to have been no pertinent research work undertaken so far on the effect of traditional cooking on the nutritive value of food plants. The present work is aimed at determining the content of vitamin C in some wild green leafy vegetables, as well as the effect of cooking.

MATERIALS AND METHODS

Leaves from sixteen vegetable varieties were collected in the morning between 8 a.m. and 9 a.m. and carried to the laboratory in polythene bags. Tender leaves (just mature) were used for analysis. Leaves from each variety were divided into four triplicated 20 g samples. One sample was kept in a hot air oven at 102 °C until constant weight was obtained in order to determine the moisture content. A second sample was used for the estimation of vitamin C. The third and fourth samples were used for the determination of vitamin C in vegetables cooked in two volumes of boiling water: (a) 'excess water' using 400 ml for 20 g and (b) 'little water' using 100 ml for 20 g except for *L. cornuta* where 200 ml was needed. Leaves were completely covered only when 400 ml was used. In both cases, aluminium vessels were used which were covered whilst boiling. The amount of water used for (b) was just sufficient, i.e. no water remained after the leaves were sufficiently cooked.

Vitamin C was estimated according to the methods of the AOAC (1975). The material was extracted with 5% metaphosphoric-ethylene diamine tetraacetic acid solution. A measured amount of the above extract, after filtration, was titrated with 2,6-dichlorophenol indophenol and a persistent pink colour for 30 s was considered to be the end point. Freshly prepared standard ascorbic acid solution was used for calculating the ascorbic acid content in the extract.

RESULTS AND DISCUSSION

Of the sixteen species studied in this project the fresh leaves of *M. oleifera* were found to be the richest in vitamin C (204 mg/100 g) content, followed by *A. graecizans* (127 mg/100 g). An FAO report (1968) gives data on vitamin C for five of the sixteen plants examined by us. After adjusting the FAO data to the moisture content of these plants of this region, the contents of vitamin C (FAO report) for *M. oleifera*, *P. oleracea* and *T. portulacastrum* more or less agree with those of the present paper, whilst those of *B. alba* and *G. gynandra* are very low. *A. gangetica*, *E. javanica*, *P. peruviana* and *V. cinerea* have negligible amounts of the vitamin. Fresh leaves of the other plants have reasonable amounts of vitamin C (Table 1).

The moisture content of the leaves varied from 71·6% in *A. graecizans* to 93·0% in *P. oleracea*.

Boiling the leaves with excess water resulted in a remarkable loss of the vitamin (varying from 98·5 to 18·3%) in almost all the leaves except in those where the vitamin content in fresh leaves is very low. *M. oleifera*, *P. plebeium*, *J. insularis*, *T. portulacastrum*, *A. spinosus*, *P. oleracea*, *L. cornuta*, *P. salicifolium* and *A. graecizans* are the worst affected, in that order, where the losses are 98·5%, 97·1%, 97·1%, 96·7%, 96·5%, 96·4%, 94·5%, 88·8%, and 86·2%, respectively. Of those plants with significant amounts of vitamin C in their fresh leaves, only *G. gynandra* retained a fairly high amount (81·7%) of the vitamin. Morton (1967) reported the loss of about 80% from the leaves of Balsam-pear when they are parboiled and washed two or three times to reduce the bitterness. In *B. alba*, Keshinro & Ketiku (1979) recorded the loss of 19·6% when parboiled with excess water for 5 min and then cooled for 10 min.

Even when the leaves are boiled with little water, there is a loss in the vitamin content although it is less than that which occurs with boiling in excess water. This loss varies from 96·7% to 5·3%. Of those plants with

TABLE 1
Vitamin C (mg/100 g fresh material) in Fresh and Cooked Wild Leafy Green Vegetables

Botanical name of plant	*Per cent moisture content*	*Vitamin C mg/100 g material*				*Per cent of vitamin C lost*	
		*FAO**	*Fresh*	*Cooked I*	*Cooked II*	*Cooked I*	*Cooked II*
Amaranthus graecizans	71·6 ± 1·04	—	127·0 ± 1·60	17·5 ± 0·47	41·8 ± 1·24	86·2	67·0
Amaranthus spinosus	80·0 ± 1·04	—	63·2 ± 0·65	2·2 ± 0·10	33·9 ± 0·61	96·5	46·4
Asystasia gangetica	82·0 ± 1·44	—	3·4 ± 0·06	1·5 ± 0·00	1·5 ± 0·00	55·9	55·9
Basella alba	90·0 ± 0·74	52·0	76·6 ± 0·76	19·8 ± 0·15	50·6 ± 0·52	74·2	33·9
Brassica juncea	90·6 ± 1·38	—	42·2 ± 0·40	5·9 ± 0·17	20·5 ± 0·55	86·1	51·4
Emilia javanica	90·4 ± 1·27	—	0·7 ± 0·00	0·7 ± 0·00	0·7 ± 0·00	0·0	0·0
Gynandropsis gynandra	80·6 ± 1·85	19·1	78·0 ± 1·30	63·7 ± 1·04	73·9 ± 0·72	18·3	5·3
Justicia insularis	75·0 ± 1·38	—	57·2 ± 0·81	1·7 ± 0·10	1·9 ± 0·06	97·1	96·7
Launaea cornuta	85·0 ± 1·59	—	24·4 ± 1·05	1·3 ± 0·06	1·6 ± 0·10	94·5	93·5
Moringa oleifera	75·4 ± 1·50	172·0	204·0 ± 1·18	3·0 ± 0·10	29·7 ± 0·51	98·5	85·4
Physalis peruviana	86·0 ± 2·31	—	1·9 ± 0·06	1·5 ± 0·00	1·5 ± 0·00	21·1	21·1
Polygonum plebeium	82·0 ± 1·21	—	58·4 ± 0·85	1·7 ± 0·06	35·6 ± 0·57	97·1	39·1
Polygonum salicifolium	87·0 ± 1·19	—	36·7 ± 0·46	4·1 ± 0·11	16·0 ± 0·10	88·8	56·4
Portulaca oleracea	93·0 ± 1·31	43·2	46·8 ± 0·53	1·7 ± 0·06	2·7 ± 0·11	96·4	94·2
Trianthema portulacastrum	83·6 ± 1·27	40·3	46·1 ± 0·75	1·5 ± 0·10	4·2 ± 0·21	96·7	90·9
Vernonia cinerea	85·2 ± 1·41	—	1·7 ± 0·00	1·1 ± 0·00	1·1 ± 0·00	35·4	35·4

Fresh = Uncooked leaves. Cooked I = Leaves boiled with excess water. Cooked II = Leaves boiled with little water.
* After adjusting to the moisture content as per the present paper.
— Indicates no data.

higher amounts of the vitamin in their fresh leaves, *J. insularis*, *P. oleracea*, *L. cornuta*, *T. portulacastrum* and *M. oleifera* lost major amounts (96·7%, 94·2%, 93·5%, 90·9% and 85·4%, respectively). Only in four species was the retention of vitamin C more than 50%—*G. gynandra*, *B. alba*, *P. plebeium* and *A. spinosus* (94·7%, 66·1%, 60·9% and 53·6%, respectively). Ajayi *et al.* (1980) found that the vitamin C losses of blanched vegetable leaves varied from 62·2% to 93·1%.

The present work clearly indicates the loss of vitamin C during cooking, such a loss being greater when the leaves are cooked with excess water. The results are in general agreement with those of other workers (Fafunso & Bassir, 1976; Imbamba, 1977; Ifon & Bassir, 1979; Keshinro & Ketiku, 1979; Ajayi *et al.*, 1980) although they are not exactly comparable due to the differences in cooking methods.

Typically, Tanzanian women boil the vegetables in a slight excess of water which is then discarded. The percentage of vitamin C lost by this method is higher than by the other method of boiling using very little water (Table 1). Analyses for vitamin C in the cooking water were not performed in the present work. However, Krehl & Winters (1950), Keshinro & Ketiku (1979) and Ajayi *et al.* (1980) found appreciable amounts of the vitamin in the cooking water. Therefore, it is recommended that the cooking water be taken along with cooked vegetable.

When leafy vegetables are cooked the content of vitamin C decreases due to (a) its easy water solubility and (b) its loss through oxidation during cooking. However, more vitamin can be saved if the cooking is carried out with little water and for as short a time as possible without sacrificing palatability. Bitter leaves (e.g. *L. cornuta*) must, of course, be boiled with excess water and the excess water discarded to remove the bitter principals.

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A Sensitive and Selective Method for the Determination of Reducing Sugars and Sucrose in Food and Plant Material by High Performance Liquid Chromatography

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ABSTRACT

A method is described for the determination of reducing sugars and sucrose in food and plant material. Sugars are extracted with hot water and the extract is clarified with neutral lead acetate, deionised, treated with ethanol and filtered. A plain silica column, which is continuously modified by an amine dissolved in the mobile phase, is used for chromatography. Reducing sugars are determined, after post-column reaction with tetrazolium blue, by the resulting absorbance at 550 nm. Sucrose is determined indirectly as glucose after hydrolysis by β-fructosidase. Accuracy and precision are comparable with those of existing methods whilst the limitations of other methods, such as short column life and low detector sensitivity and selectivity, have largely been overcome. The method is particularly suitable for samples having low sugar content and/or high concentrations of interfering material.

INTRODUCTION

The accurate determination of individual sugars in food is of some importance, not only as a means of obtaining detailed information regarding carbohydrate composition but also in view of current interest in nutritional aspects and the possible influence of individual sugars on the properties of food and related products.

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A wide range of methods of analysis of sugars is available comprising various physical (chromatographic and polarimetric), chemical, colorimetric and enzymatic procedures (Lee, 1978). In general, chromatographic methods are the most useful for the determination of individual sugars in food and other samples where a number of different sugars may be present. Gas–liquid chromatography (GLC) and, more recently, high performance liquid chromatography (HPLC) have been used extensively for carbohydrate analysis. HPLC offers a number of advantages over GLC (Conrad & Palmer, 1976); for example, derivatisation prior to chromatographic analysis is unnecessary.

Development of a high performance liquid chromatographic method may be considered in terms of three aspects—sample preparation, chromatography and detection and quantitation. A number of recent HPLC methods for the determination of sugars in food and food products have utilised simple pre-treatment of liquid samples or extracts involving guard columns (Hurst *et al.*, 1979), mini-column cleanup (Dunmire & Otto, 1979) or C-18 cartridges (Richmond *et al.*, 1981). Sugars were separated on commercial carbohydrate columns or bonded phase packing materials and detected by refractive index. An alternative sample preparation procedure involving clarification and deionisation of extracts was described by Dean (1978).

Difficulty was experienced with a number of samples analysed in this laboratory, however, particularly those in which the concentration of interfering material was large in relation to carbohydrate concentration. Extracts were not always sufficiently concentrated or free from interfering material for accurate quantitation of sugars to be possible when refractive index detection was used, even after extensive cleanup, and bonded phase column packing materials gave a short useful column life.

Greatly improved sensitivity and selectivity in detection with minimal loss in resolution may be obtained by utilising post-column reactor systems (Deelder *et al.*, 1978; Frei & Scholten, 1979). A number of reagents have been used for the detection of sugars on this basis (D'Amboise *et al.*, 1980). The method described in this paper utilises a plain silica column which is continuously modified by a polyfunctional amine dissolved in the mobile phase (Aitzetmüller, 1978) and post-column reaction with tetrazolium blue (D'Amboise *et al.*, 1980; Noel *et al.*, 1979). Reaction with the amine modifier used for chromatography is negligible under experimental conditions. Reducing sugars—and also sucrose—can be determined.

EXPERIMENTAL

Reagents

Analytical grade reagents were used without further purification with the exception of acetonitrile (chemically pure grade) which was redistilled before use. Arabinose, fructose, galactose, maltose, ribose, sorbose, xylose and tetrazolium blue were from Merck. Cellobiose, mannose, melibiose and rhamnose were from Eastman. Glucose, lactose and sucrose were from BDH. β-Fructosidase (β-D-fructofuranoside fructohydrolase EC 3.2.1.26) was from Boehringer-Mannheim. HPLC amine modifier I was obtained from NATEC, Hamburg.

Extraction of sugars

Finely ground material (1–10 g depending on sugar content) was weighed into a 100 ml volumetric flask. Sufficient calcium carbonate was added to neutralise acidity. Sugars were extracted with water (80 ml at 80–90 °C for 1 h in a water bath). The extract was cooled to room temperature and made up to volume. Samples such as molasses, honey and fruit juice were diluted prior to clarification.

Clarification of extract

Saturated neutral lead acetate (AOAC, 1975; sufficient to complete precipitation) was added to 20 ml of extract and the solution was made up to 50 ml. A copious precipitate formed with some extracts but generally this settled within a few minutes to leave a clear supernatant.

Deionisation

A mixed bed resin (Bio-Rad AG 501-X8) was used. The anion exchange component was converted from the hydroxide to the bicarbonate form before use in order to minimise absorption of sugars (Dean, 1978). 1 g of air dried resin was added to 5 ml clarified extract and the solution was allowed to stand for 30 min with occasional swirling. Two millilitres of deionised solution were made up to 10 ml with ethanol and filtered (0·6 μm membrane filter, Schleicher & Schüll, Dassel, West Germany). The filtered solution was used for chromatography.

Hydrolysis of sucrose

As sucrose cannot be determined directly when tetrazolium blue is used for detection, an indirect method was used involving hydrolysis of sucrose by β-fructosidase. The following procedure was used.

Twenty millilitres of unclarified sugar extract were added to 25 ml acetate buffer (0·1M, pH 4·65) in a 50 ml volumetric flask, 4 mg β-fructosidase was added and the solution was incubated for 1 h at 20–25 °C. Neutral lead acetate was added and the solution was made up to volume, deionised and prepared for chromatography as explained previously.

Sucrose was determined as glucose by difference between the total glucose content after hydrolysis and the free glucose content.

$$\% \text{ Sucrose} = 1{\cdot}90\ (\% \text{ total glucose} - \% \text{ free glucose})$$

Chromatography

A schematic diagram of the apparatus used for sugar analysis is shown in Fig. 1. Milton-Roy minipumps were used to pump the reagent and mobile phase. The column (25 cm × 0·4 cm) was packed with silica (Li Chrosorb Si 60 5 μm (Merck)) using the balanced density method (Asshauer & Halasz, 1974). A small pre-column (10 cm × 0·4 cm, Li Chrosorb Si 60 10 μm) was used to saturate the mobile phase with silica. Acetonitrile: water (75:25 or 80:20) containing 0·01 % HPLC amine modifier I

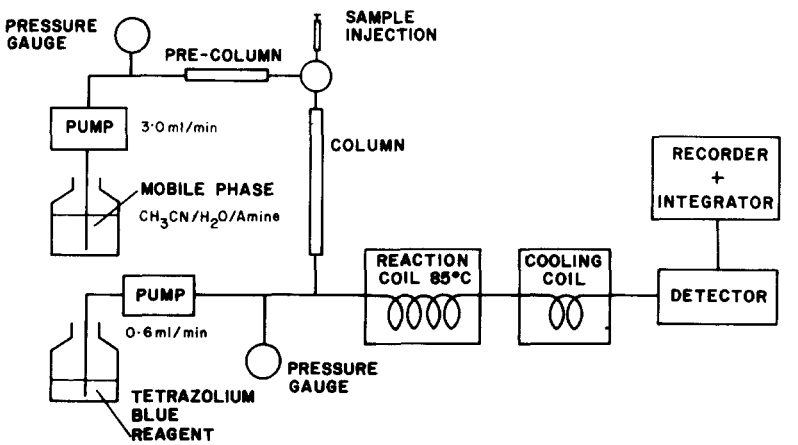

Fig. 1. Schematic diagram of sugar analyser.

(Aitzetmüller, 1978) was used as the mobile phase. Samples were injected into the column by means of a high pressure loop injector fitted with a 10 μl sample loop. The mobile phase and tetrazolium blue reagent were filtered (0·6 μm membrane filter) prior to chromatography.

Detection and quantitation

Sugars were detected after reduction of tetrazolium blue in a stainless steel reactor of the type described by D'Amboise *et al.* (1980), connected to the column outlet. Absorbance at 550 nm was monitored by a UV III detector (LDC). The reactor was flushed out daily with 50 % ethanol after use. Chromatograms and peak areas were recorded on a Hewlett-Packard Model No. 3390A integrator.

The conditions for post-column reaction were optimised with regard to repeatability and reproducibility of determinations, speed of analysis and sensitivity of detection. The following conditions, which were found to be satisfactory for most purposes, were used for subsequent analyses.

Flow rates: Mobile phase, 3·0 ml/min; reagent, 0·6 ml/min; reaction temperature, 85 °C. Reagent composition: tetrazolium blue, 0·1 % in NaOH 0·36M/ethanol 50 %. Dimensions of reactor: length, 4·5 m (including 1 m cooling coil); coil diameter, 4·5 cm; internal diameter of tubing 0·5 mm.

A refractive index detector (Waters model R 401) was used to determine capacity values of the sugars and a few chromatograms of samples were run using this detector for comparison.

RESULTS AND DISCUSSION

Sample preparation

Various solvents and extraction procedures (single and multiple) were investigated. It was found that a single extraction with hot water was adequate for most purposes. Extraction with 80 % ethanol (AOAC, 1975) requires an additional evaporation stage in order to ensure efficient clarification. For this reason, it was found to be more convenient to add ethanol to solutions after deionisation in order to precipitate polysaccharides and non-carbohydrate material not removed by previous treatment. Only one filtration per sample was required immediately

before chromatography. Filtration and/or centrifugation at intermediate stages was found to be unnecessary, except in a few cases where large quantities of starting material were used for the determination of sugars present in very low concentrations.

Minor changes in sugar concentration were found to occur at the deionisation stage, even after treatment of the resin with carbon dioxide. Chromatograms were obtained of a standard before and after treatment with the resin, as described for the samples, in order to correct for this.

Calibration of chromatographic data

Calibration plots for peak areas of standards were obtained and regression equations and correlation coefficients were determined. Regression data and detection limits for some common sugars are listed in Table 1. Correlation coefficients were valid for monosaccharides in the

TABLE 1
Regression Equations,[a] Correlation Coefficients[a] and Detection Limits[b] for Some Common Sugars

Sugar	*A*	*B*	*Correlation coefficient*	*Detection[b] limit (ng)*
Xylose	310·457	10 840 300	0·999 867	9
Fructose	19 137·7	21 065 800	0·999 125	6
Glucose	−1 357·93	16 861 600	0·999 840	11
Maltose	4 381·78	5 614 910	0·999 449	40
Lactose	−3 287·36	4 432 440	0·999 615	55

[a] $Y = A + BX$, peak area versus concentration (g/100 ml).
[b] Values were determined for signal:noise ratio of 2:1.

concentration range 0·001 % to 0·08 % and for disaccharides in the range 0·005 % to 0·08 %. Background noise caused erratic results for disaccharides at concentrations below 0·005 % while the fructose plot became non-linear at concentrations above 0·08 %.

Retention data

Capacity (k') values are listed in Table 2 for a number of reducing sugars and sucrose for both 75:25 and 80:20 acetonitrile:water ratios. In

TABLE 2
k' Values for Reducing Sugars and Sucrose

	k' *value*	
	75% CH_3CN	*80% CH_3CN*
Rhamnose	1·17	1·50
Ribose	1·28	1·63
Xylose	1·71	2·40
Arabinose	2·09	2·88
Fructose	2·33	3·46
Sorbose	2·35	3·55
Mannose	2·83	4·42
Glucose	3·27	5·32
Galactose	3·62	5·81
Sucrose	5·15	10·1
Cellobiose	6·93	14·2
Maltose	7·04	14·5
Lactose	8·37	17·3
Melibiose	9·53	21·0
Maltotriose	14·9	—

general, the relative *k'* values are similar to those obtained using NH_2-bonded phase columns (Binder, 1980). A chromatogram of some of these sugars is shown in Fig. 2.

Evaluation of the method

The precision of the method was evaluated by multiple determinations on a single sample. As no suitable reference method was available, the accuracy of the method was assessed on the basis of recovery of sugars added to the sample prior to extraction.

Sub-samples were taken from a finely ground freeze-dried mango sample and were subjected to extraction of sugars, clarification, de-ionisation and chromatographic analysis. The results are summarised in Table 3.

The experiment was repeated using sub-samples of the same mango powder to which maltose (approximately 20% by weight) and sucrose (approximately 50% by weight) were added prior to extraction of sugars. The former sugar was added to check recovery and the latter to investigate whether any inversion of sucrose occurred during extraction.

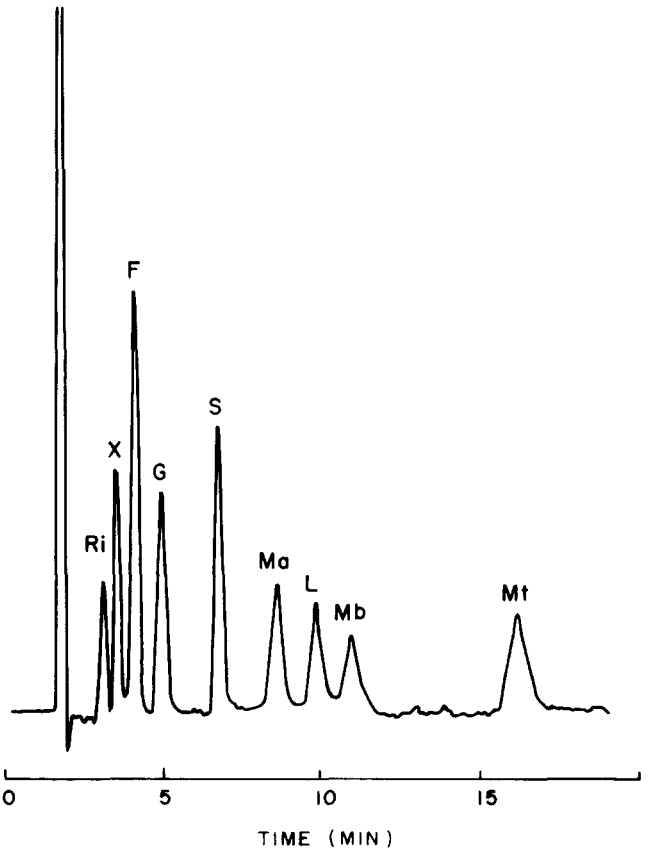

Fig. 2. Chromatogram of ribose (Ri), xylose (X), fructose (F), glucose (G), sucrose (S), maltose (Ma), lactose (L), melibiose (Mb) (all 1 %w/v) and maltotriose (Mt) (2 %w/v). Detector: refractive index, attenuation 16 ×.

TABLE 3
Sugar Analysis Data (Mango Powder)

Sugar	*Number of determinations*	*Mean content (g/100 g)*	*Coefficient of variation (%)*
Fructose	6	27·5	2·96
Glucose	6	21·8	2·77
Sucrose	6	24·0	3·46

TABLE 4
Sugar Analysis Data (Mango Powder with Added Sugars)

Sugar	*Number of determinations*	*Mean content (g/100 g)*	*Coefficient of variation (%)*	*Per cent recovery*	*Coefficient of variation (%)*
Fructose	6	28·1	2·24		
Glucose	6	22·6	1·96		
Maltose	6			98·2	5·14

The results, which are summarised in Table 4, indicate that losses of sugars are minimal and that inversion of sucrose is negligible under experimental conditions.

Coefficients of variation for fructose and glucose (on the basis of six chromatographic determinations on a single extract) were 1·88 and 1·91, respectively. Similar values were obtained for standards, indicating that variation is due partly to chromatographic data and partly to sample preparation.

Recoveries of other sugars were also investigated and results for molasses are given in Table 5.

TABLE 5
Recovery Data for Sugars Added to Molasses

Sugar	*Xylose*	*Fructose*	*Glucose*	*Maltose*	*Sucrose*
Recovery %	94·9	99·1	98·1	99·3	97·9
	94·5	100·6	98·3	93·3	95·9

Sugar analysis data for a variety of other samples are listed in Table 6. Data are included for samples having very high concentrations of interfering material, such as coffee and roasted chicory. The superiority of detection utilising tetrazolium blue over refractive index detection for samples of this type is illustrated in Fig. 3, where chromatograms obtained using both detection methods are shown for a chicory extract. An additional concentration stage was required after clarification and deionisation in order to obtain a sufficiently concentrated extract for refractive index detection. It can be seen that, even after extensive

TABLE 6
Sugar Analysis and Recovery Data for Various Samples

Sample	*Sugar content*[a]								*Sugar recovery*[b]		
	Xylose	*Fructose*	*Glucose*[c]	*Maltose*	*Lactose*	*Sucrose*[d]	*Rhamnose*	*Arabinose*	*Mannose*	*Xylose*	*Maltose*	*Sucrose*
Pure coffee		0·17	0·13			0·19	0·09	0·68	0·07		102·1	
(extract, spray dried)		0·16	0·12			0·21	0·10	0·66	0·07		102·9	
Chicory root		1·24	0·27			0·25				93·3	102·3	
(roasted, extract)		1·22	0·26			0·36				94·0	98·1	
Chicory root	0·02	1·07	0·17			5·70						
(fresh)	0·02	1·08	0·20			6·33					96·3	
Chicory root		0·99	0·26	0·30		3·90						
(dried)		0·87	0·22			3·57					98·8	
Chicory root	0·63	4·87	2·02			1·62					107·2	
(roasted)	0·60	4·73	1·98			1·44					102·8	
Grapefruit juice		2·21	1·99			0·57						
(unsweetened)		2·11	1·97			0·74					100·0	
Honey		37·40	29·58	4·31		2·55						
		36·44	29·75			2·22					97·0	
Molasses		4·80	3·51			29·34					94·5	
		4·90	3·52			30·53					97·9	
Milk powder		0·11	0·23		25·28	19·23					95·5	100·2
		0·13	0·24		26·20	20·37					98·7	96·1

[a] Values listed are grams of sugar (excluding added sugars) per 100 g sample (g/100 ml sample in the case of grapefruit juice).
[b] Values listed are percentages of theoretical recoveries of added sugars.
[c] An unidentified component (probably galactose) eluted as a shoulder on the glucose peak in the coffee extract.
[d] Sucrose figures for chicory samples were calculated assuming that no glucose is formed by hydrolysis of inulin.

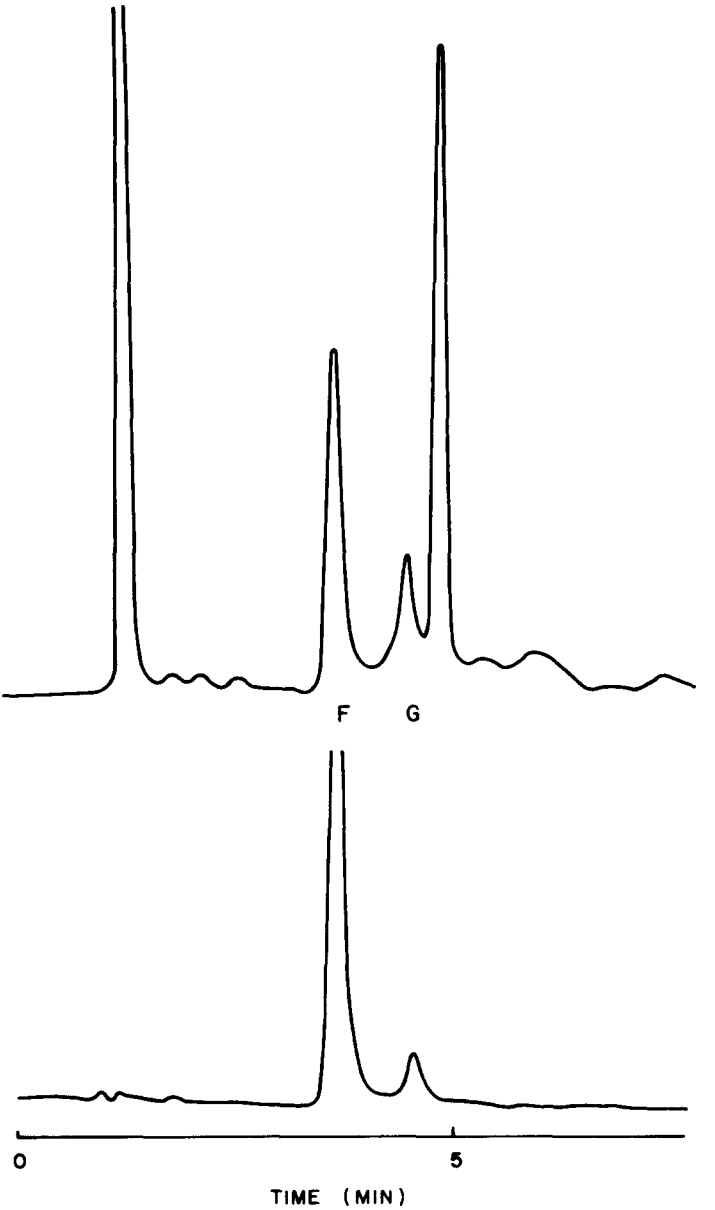

Fig. 3. Chromatograms of a chicory extract. Comparison of refractive index detection (upper chromatogram) and detection utilising tetrazolium blue (lower chromatogram). In addition to fructose (F) and glucose (G) a number of unidentified components elute close to glucose and make accurate quantitation difficult when refractive index detection is used but no interference occurs when tetrazolium blue is used for detection.

cleanup, some diffuse background remains and a major unidentified component elutes close to glucose. Accurate identification and quantitation of sugars is therefore difficult whereas no such problems were encountered when tetrazolium blue was used for detection.

The post-column reaction conditions used for the detection of sugars are different from those recommended by D'Amboise *et al.* (1980) but offer the advantages of more rapid analysis and decreased consumption of reagent without loss in sensitivity.

The main limitation of the method is that non-reducing sugars cannot be determined directly. The method has, however, been successfully adapted for the analysis of sucrose. It should be possible to determine other non-reducing sugars of importance in food analysis by utilising similar enzymatic hydrolysis procedures; for example, raffinose, which is hydrolysed by β-fructosidase to fructose and melibiose.

Determination of sucrose as glucose formed by enzymatic hydrolysis results in fewer possible interferences through hydrolysis of other sugars than determination of sucrose as fructose. Interference may occur in a few specific cases; for example, molasses contains a small proportion of ketoses which would be hydrolysed by β-fructosidase to fructose and glucose. Interferences of this type are usually minor, however, and would not generally be a problem in food analysis.

Inulin, a major component of chicory, was found to be hydrolysed, at least partially, by the enzyme preparation to fructose. Inulin also contains a small proportion of glucose attached to a fructan chain as a non-reducing end group linked as in sucrose (Aspinall, 1970). It is possible that a small quantity of glucose may be formed by hydrolysis of this linkage by β-fructosidase but this would occur only if the fructan chain were completely hydrolysed as substituted β-fructofuranosyl residues are not attacked by the enzyme (Bergmeyer & Bernt, 1974). The presence of small quantities of sucrose in the chicory extract, determined using tetrazolium blue detection (Table 6), was also shown by refractive index detection.

The method is reasonably rapid. Monosaccharides can be analysed in less than 10 min and disaccharides in less than 15 min. The time required for sample preparation is comparable with that required for chromatography if a large number of samples are processed simultaneously. A column life of over 6 months has been obtained. This covered method development and also routine analysis including a number of particularly dirty samples, and the pre-column only had to be replaced infrequently during this period. Accuracy and precision are comparable with those of

existing methods while sensitivity and selectivity are superior to most. The apparatus and materials used are relatively inexpensive and the method is suitable for analysis of a wide range of food and related materials, particularly those having low carbohydrate content and high concentrations of interfering material.

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Effects of Post-harvest Handling on Quality Attributes of Tomatoes in the Nigerian Marketing System

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ABSTRACT

Changes in ascorbic acid and other quality attributes of fresh ripe tomatoes transported from the production areas in northern Nigeria to an urban wholesale market in south-western Nigeria were investigated. Reduced ascorbic acid concentration was lower, by as much as 35%, and dehydroascorbic acid concentration higher in damaged tomatoes relative to the undamaged control. Damaged tomatoes had a higher pH but lower total solids, soluble solids and titratable acidity than the undamaged fruits. pH and titratable acidity, as per cent citric acid, ranged from 4·06 and 0·453, respectively for undamaged tomatoes to 4·34 and 0·330, respectively for fruits with signs of bacterial and fungal rots. Total solids and soluble solids ranged from 6·12% and 5·32%, respectively for undamaged fruits to 5·39% and 4·34%, respectively for bruised tomatoes.

INTRODUCTION

There is a dearth of information on post-harvest losses in fruits and vegetables in developing countries. The few data available are largely limited to field surveys that are not carried out by a described methodology and therefore are difficult to interpret (NAS, 1978). There is, therefore, the need for better estimates of post-harvest losses in

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perishable fruits and vegetables in developing countries as a first step towards devising appropriate measures to reduce these losses.

Substantial losses, at times up to 100%, were observed in a preliminary survey of post-harvest losses of perishable fruits and vegetables in western Nigeria (Olorunda & Aboaba, 1978). These losses occurred at different points in the delivery system, right from the farm gate to the urban markets. Whilst quantitative food loss estimates are important, loss of food quality through changes in the composition of essential nutrients also deserves attention. The objective of this study is to critically evaluate the effects of post-harvest handling on the chemical quality attributes of fresh tomatoes in the Nigerian marketing system.

MATERIALS AND METHODS

Fresh tomatoes, mainly of the 'Ronita' cultivar, transported from the production areas in Kano state in northern Nigeria to the 'Shasha' wholesale market in Ibadan in south-western Nigeria, were obtained from typical marketing agents as the trucks reached the market. The produce, packed in bamboo or cane baskets, was transported in open non-refrigerated trucks over a distance of over 1000 km, the transit period being approximately 3 days.

On each sampling date, about 20 kg of tomatoes, representative of a truck load, were examined for damage and then classified into two broad categories—marketable and unmarketable—based on the severity of the damage. The marketable tomatoes were re-examined and classified into three groups, according to the nature of the predominant type of damage, as follows. (1) Mechanical damage due to cuts and bruises. (2) Physiological damage due to wilting and over-ripening. (3) Pathological damage due to bacterial and fungal rots. A 500-g sample of ripe fruits was taken from each class and analysed for reduced ascorbic acid, total ascorbic acid, total solids, soluble solids, pH and titratable acidity. Undamaged ripe tomatoes, free of defects, served as controls.

Analytical procedures

Reduced ascorbic acid was determined spectrophotometrically by the indophenol method (Ruck, 1969). Total ascorbic acid was estimated by first converting dehydroascorbic acid to reduced ascorbic acid with

homocysteine (Hughes, 1956). Dehydroascorbic acid content was obtained by difference. Total solids were determined by drying in a vacuum oven at 68 °C and soluble solids were measured with a Bausch and Lomb Abbe desk refractometer. pH was measured directly on a Metrohm–Herisau pH meter, model E-520. Titratable acidity was determined by titration with standard 0·1N NaOH to an end point of pH 8·1 and expressed as per cent citric acid.

RESULTS

Only 50 % of the tomatoes transported from northern Nigeria were free of defects by the time they reached the 'Shasha' wholesale market in Ibadan, south-western Nigeria (Table 1). Almost 20 % of the tomatoes were considered unmarketable due predominantly to mechanical damage. Another 30 % of the tomatoes were slightly damaged, but were still

TABLE 1
Per Cent Distribution of Various Types of Damage in Tomatoes Transported from Northern to Southern Nigeria*

Nature of damage	*% of total fruits sampled with damage*
Marketable fruits	
Undamaged	51·0 ± 4·6
Mechanical	9·4 ± 4·2
Physiological	17·7 ± 6·3
Pathological	4·8 ± 0·5
Unmarketable fruits	17·1 ± 3·3

* Values presented are the means and standard deviations over five sampling days.

considered marketable. In this case, wilting and over-ripeness were the most common defects (Table 1).

The changes in ascorbic acid in tomatoes transported from northern to southern Nigeria are presented in Table 2. Total ascorbic acid (TAA) and reduced ascorbic acid (RAA) levels were lower in damaged than in undamaged tomatoes, especially in tomatoes with signs of bacterial and fungal rots (pathological damage) in which TAA and RAA levels were

TABLE 2
Ascorbic Acid Changes in Damaged and Undamaged Tomatoes*

Ascorbic acid (mg/100 g fresh weight)	*Undamaged (control)*	*Nature of damage*		
		Mechanical	*Physiological*	*Pathological*
Total ascorbic acid (TAA)	21·8 ± 0·72	18·0 ± 1·31	19·2 ± 1·02	15·1 ± 0·52
Reduced ascorbic acid (RAA)	19·6 ± 0·70	14·9 ± 1·52	16·2 ± 0·92	12·5 ± 0·91
Dehydroascorbic acid (DHAA)	2·25 ± 0·11	3·09 ± 0·29	2·98 ± 0·24	2·54 ± 0·50
$\frac{\text{RAA}}{\text{DHAA}}$	8·7	4·8	5·4	4·9

* Values presented are the means and standard deviations over five sampling days.

lower by 30% and 35%, respectively, relative to the undamaged tomatoes. Dehydroascorbic acid (DHAA) levels were lowest in undamaged tomatoes. The ratio of RAA to DHAA decreased from approximately 9:1 in undamaged tomatoes to approximately 5:1 in damaged tomatoes.

Total and soluble solids were slightly lower in damaged tomatoes than in the undamaged control (Table 3). There was an increase in pH and a 20–27% reduction in titratable acidity in damaged, relative to undamaged, tomatoes (Table 3).

TABLE 3
Total Solids, Soluble Solids, pH and Titratable Acidity of Undamaged and Damaged Tomatoes*

Chemical quality attribute	*Undamaged (control)*	*Nature of damage*		
		Mechanical	*Physiological*	*Pathological*
Total solids (%)	6·12 ± 0·26	5·39 ± 0·44	5·64 ± 0·27	5·41 ± 0·38
Soluble solids (%)	5·32 ± 0·79	4·34 ± 0·56	4·58 ± 0·33	4·48 ± 0·57
pH	4·06 ± 0·06	4·26 ± 0·09	4·24 ± 0·11	4·34 ± 0·09
Titratable acidity (% citric acid)	0·453 ± 0·06	0·358 ± 0·06	0·363 ± 0·05	0·330 ± 0·04

* Values presented are the means and standard deviations over five sampling days.

DISCUSSION

Ineffective packaging and rough handling during loading and unloading of trucks are presumably responsible for the high incidence of mechanical damage in tomatoes transported from northern to southern Nigeria. The use of bamboo or cane baskets for the packaging of tomatoes is not conducive to efficient handling or suitable for modern systems of transportation. At heavier loading capacities of trucks, damage to contents from the rough sides and sharp edges of the baskets occurs. Inner linings with dried leaves, when used, are usually ineffective in preventing damage.

The increase in the proportion of dehydroascorbic acid (DHAA) to reduced ascorbic acid (RAA) in damaged tomatoes relative to the undamaged controls suggests that ascorbic acid oxidation is faster in damaged tomatoes. Exposure of the tomatoes to high tropical temperatures in open, non-refrigerated trucks, over a relatively long transit period of about 3 days, might have accelerated the conversion of reduced ascorbic acid to the oxidised forms, especially in damaged tomatoes. This represents a significant loss in nutritional quality since DHAA has only 75% of the antiscorbutic activity of RAA (Mills *et al.*, 1949) and diketogulonic acid has no antiscorbutic activity at all.

Since it is the general practice in Nigeria to pack tomatoes at different stages of ripeness, including green fruits, in the same container, it is conceivable that premature ripening, triggered by in-transit damage, is responsible for the lower total solids, soluble solids and titratable acidity of damaged tomatoes relative to the undamaged control. Soluble solids of tomatoes have been reported to increase from the mature-green stage, reaching the highest point at the table-ripe stage (Craft & Heinze, 1954; Winsor *et al.*, 1962). Hicks *et al.* (1976) observed that the titratable acidity of Florida MH-1 tomatoes increased with ripeness although other investigators have reported a decline in the titratable acidity of tomatoes during ripening (Bisogni *et al.*, 1976).

In conclusion, apart from the high quantitative losses previously reported (Olorunda & Aboaba, 1978), poor post-harvest handling also results in substantial loss of vitamin C in tomatoes in the Nigerian marketing system. The introduction of improved packaging and more efficient transportation methods, as well as the organisation of the marketing and distribution channels to avoid unnecessary delays, would go a long way towards reducing these losses.

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Technical Note

Selenium Content of Food Consumed by Egyptians

The selenium content of foodstuffs conventionally consumed in Egypt was determined by the hybrid technique and atomic absorption spectrometry (Perkin-Elmer Model 300). The results are listed in Table 1. In comparison with the data obtained in the USA (Morris & Levander, 1970; Schroeder *et al.*, 1970), in Canada (Arthur, 1972; Thompson *et al.*, 1975), in Germany (Oelschlaeger & Menke, 1969; Ackermann & Bergner, 1972) and in Japan (Yasumoto *et al.*, 1976), the data generally agree well with our results, with the exception of corn and rice. The selenium levels were found to be low in corn and rice. In the USA the selenium level was more than 0·16 ppm in corn (Ferretti & Levander, 1974) and 0·30 ppm in rice (Morris & Levander, 1970).

Of the foodstuffs conventionally consumed in Egypt, wheat, barley, broad beans, lentil, garlic and fish contained relatively high concentrations of selenium.

A considerable difference was found between white flour and whole flour from wheat and corn. Lentil (peeled or unpeeled) demonstrated the same relationship. The previous data suggest that the refining process might be removing some of the selenium present in the peel.

The average amount of selenium in the Egyptian diet was calculated from the data of the present investigation and data previously published by other investigators and the *per capita* daily intake of foodstuffs in Egypt. The results are listed in Table 2. The daily intake of foodstuffs in Egypt is approximately 1200 g, providing about 3270 kcal of energy (about 13 682 Joules) and containing about 90 g protein, including 13 g of animal protein and 77 g of plant protein (Ministry of Agriculture, Department of Economic Research of Plant Production, Egypt, 1977).

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TABLE 1
Selenium Content of Various Foodstuffs

Foodstuff	*Selenium content (ppm)*
Raw wheat	0·50 ± 0·02
Wheat flour (high extraction, 85–93%)	0·47 ± 0·03
Wheat flour (low extraction, 68–72%)	0·43 ± 0·03
Raw white corn	0·02 ± 0·01
White corn flour	0·01 ± 0·01
Raw yellow corn	0·03 ± 0·02
Yellow corn flour	0·02 ± 0·02
White rice (polished)	0·20 ± 0·02
Broad beans	0·50 ± 0·04
Lentil	0·60 ± 0·04
Lentil (peeled)	0·56 ± 0·04
Chick pea	0·32 ± 0·03
Egyptian lupin	0·31 ± 0·02
Fenugreek	0·29 ± 0·04
Barley	0·41 ± 0·05
Sesame	0·38 ± 0·05
Onion	0·02 ± 0·01
Garlic	0·52 ± 0·03
Potatoes	0·03 ± 0·01
Sweet potatoes	0·05 ± 0·01
Sugar	0·02 ± 0·02
Glucose	0·01 ± 0·01
Cottonseed oil	0·06 ± 0·02
Beef (meat)	0·22 ± 0·04
Lamb (meat)	0·19 ± 0·05
Chicken	0·19 ± 0·02
Fish (Bolty)	0·40 ± 0·07
Eggs	0·09 ± 0·02
Milk	0·07 ± 0·02

The selenium intake was calculated to be approximately 195 μg per capita per day (Table 2). The value obtained agreed fairly well with the reported average intake in the USA of 60–150 μg (Schroeder *et al.*, 1970), in Canada of 113–200 μg (Thompson *et al.*, 1975), and in Japan of 208 μg (Yasumoto, 1976) and is above the dietary requirement for selenium of 63–120 μg per day (National Academy of Science, USA, 1971). Obviously, enormous individual variations are possible, depending upon dietary habits and also upon the content of individual foodstuffs.

TABLE 2
Selenium Intake from Foodstuffs

Foodstuff	*Intake of food** (*g per day per capita*)	*Selenium content* (*ppm*)	*Selenium intake* (*μg per day per capita*)
Wheat	290	0·45	130·50
White corn	223	0·01	2·23
Yellow corn	45	0·02	0·90
Rice	90	0·20	18·00
Broad bean	15	0·50	7·50
Lentil	4	0·58	2·32
Other pulses	3	0·30	0·90
Onion	35	0·02	0·70
Potatoes	38	0·03	1·14
Vegetables	23	0·02**	0·46
Fruits	137	0·03**	4·11
Sugar	62	0·02	1·24
Glucose	3	0·01	0·03
Oils	29	0·06	1·74
Meats	19	0·22	4·18
Chicken	9	0·19	1·71
Fish	13	0·40	5·20
Eggs	4	0·09	0·36
Milk	162	0·07	11·34
Total	1 204		194·56

* Quoted from data of Department of Economic Research of Plant Production, Ministry of Agriculture, Egypt (1977).
** Quoted from Morris & Levander (1970), Schroder *et al.* (1970), Thompson *et al.* (1975) and Treptow *et al.* (1978).

The principal sources of selenium in the Egyptian diet are wheat (130 μg), rice (18 μg), milk (11 μg), broad beans (8 μg) and fish (5 μg).

The major sources of selenium in Canadian foods are wheat (98 μg), poultry (24 μg), pork (21 μg) and fish (17 μg) (Thompson *et al.*, 1975). In the Japanese diet they are fish (78 μg), rice (59 μg), soybean (14 μg) and wheat (13 μg) (Yasumoto *et al.*, 1976).

Because the average diet in Egypt is rich in selenium, the possibility of a deficiency, at least in adults, appears to be remote; wheat (as bread and other baked products) constitutes the major item of the Egyptian diet.

The calculation shows that the average Egyptian diet contains approximately 0·16 ppm selenium. The human dietary requirement for selenium is likely to be in the range of 0·1 to 0·2 ppm in food (National

Academy of Science, USA, 1971). Animal experiments have indicated that diets containing more than 5 ppm selenium can be harmful (Tinsley *et al.*, 1967).

The chemical forms of selenium that occur in Egyptian foods and its biological availability remain to be investigated. It has been stated that the selenium in fish is much less active than that in chickens (Morris & Levander, 1970; Cantor *et al.*, 1975). Therefore, the total selenium content of foods may not be a valid indicator of their nutritional value.

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Technical Note

Thin Layer Chromatographic (TLC) Analysis of Malic and Lactic Acids*

INTRODUCTION

The presence or absence of malic acid in wines is frequently used as an index to monitor the progress or signal the completion of a malolactic fermentation.

Although several analytical methods using gas chromatography and enzymatic procedures may be used for qualitative and quantitative determinations of malic acid, these methods are frequently beyond the requirements of wineries or winemakers who have available only minimal instrumentation and technical personnel. Because of the latter limitations, many winemakers rely heavily upon paper chromatography as a convenient method for routine analyses. This paper describes a simple, economical and rapid means of determining malic and lactic acids.

Paper chromatography of organic acids and its application to wine analyses has been used successfully for many years (Lugg & Overell, 1948; Dölle, 1958; Ribéreau-Gayon & Peynaud, 1958; Ingraham & Cooke, 1960), one of the most extensively used procedures being that described by Kunkee (1968). This method is quite suitable for the qualitative evaluations of malic and lactic acids; however, a prolonged period of development (8 to 16 h), is frequently required to achieve acceptable resolution. To decrease the required time for development we

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found that the use of thin layer chromatography (TLC) and a change in the pH indicator reduced development time of the Kunkee (1968) procedure to 45–60 min with no loss in resolution.

MATERIALS

(1) Formic acid, *n*-butanol, bromophenol blue (sodium salt).
(2) Pre-coated TLC plastic sheets, microcrystalline cellulose (without fluorescent indicator). MC/B, Manufacturing Chemists, Inc., 2909 Highland Avenue, Cincinnati, Ohio 45212, USA.
(3) Micropipettes (10 μl), disposable.
(4) Developing jar (85 × 130 cm).
(5) Jar lid, petri plate top or small flexible plastic bag affixed to jar with a rubber band.
(6) Standard acids, malic and lactic.
(7) Electric hair dryer.
(8) Volumetric flasks (100 ml).
(9) Separatory funnel (250 ml).

PROCEDURE

Developing solvent

To a separatory funnel containing 100 ml distilled water, add 0·1 g bromophenol blue. Next, add 100 ml *n*-butanol and 10 ml formic acid. Shake the mixture for 2 min then allow it to stand undisturbed, preferably overnight. Remove and discard the lower aqueous phase. The organic phase (yellow colour) is used as the developing solvent.

Various quantities of developer can be prepared if the concentration ratios of components are maintained. The developer, placed in a sealed separatory funnel under a well-ventilated hood, has been stored for 2 weeks without loss of effective resolution.

Standard acid solutions

Prepare 0·5 % malic and 0·5 % lactic acid standards. Small aliquots, e.g. 1·0 ml of the stock solutions, may be stored in a freezer and are readily available for future determinations.

Preparation of chromatogram

(a) TLC sheets (6·5 × 10 cm). Six sheets can be conveniently cut from one standard 20 × 20 cm sheet.
(b) Using a soft lead pencil draw a baseline perpendicular along the shortest dimension of the paper located 1 cm from the edge.
(c) Superimpose five pencilled reference marks on the baseline. Allow 1 cm distance from each side of the paper. Take care and do not penetrate the cellulose layer of the sheet.
(d) Apply small amounts of the test samples from a micropipette (10 μl) to the respective reference markers. Two 10 μl-applications are recommended for the unknown wine samples; 10 μl (50 μg) for the standard acid solutions. The hair dryer is used to facilitate drying.
(e) When the spots are dry, place the sheets in the developing jar containing the developing solvent (3 to 5 mm in depth). Cover the jar with a petri plate lid or use flexible plastic film secured to the jar by a rubber band.
(f) After 45–60 min at room temperature, remove the developed sheets and air dry in a well-ventilated hood. Heat (hair dryer) may again be used to reduce drying time.
(g) Yellow acid spots appear on a blue background, and greater colour contrast can be obtained by quickly passing the chromatogram over ammonium hydroxide vapours. However, overexposure to alkaline fumes may reduce the clarity of the acidic spots.

EVALUATION

From the line of origin measure the migratory distance (mm) of the acidic spots and the solvent front. The ratio of the distance travelled (acidic spots/solvent front) provides a decimal value (R_f) which is a convenient means of recording data.

DISCUSSION

As may be seen in Table 1, the R_f values for malic and lactic acids remain quite constant throughout the three time intervals that were examined.

TABLE 1
TLC R_f Values of Malic and Lactic Acids at Various Time Intervals

	Time of solvent migration (min)		
	30	*45*	*60*
Standards Malic	0·56	0·60	0·60
Lactic	0·84	0·81	0·83
Samples Wine	0·54 (malic)	0·57	0·58
Wine	0·84 (lactic)	0·82	0·83
Solvent migration (mm)	50	62	78

Although discrete separations of the acidic components were observed in the shortest run (30 min), the extended development times provide greater assurance that the components will separate sufficiently, thereby avoiding artifacts arising from superimposition of the acids.

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Differential Scanning Calorimetry in Food Research—A Review*

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ABSTRACT

Differential scanning calorimetry (DSC) has gained remarkable popularity in thermal studies of foods and their components following the development of instrumentation of sufficient sensitivity. DSC is rapid, facile and capable of supplying both thermodynamic (heat capacity, enthalpy and entropy) and kinetic data (reaction rate and activation energy) on protein denaturation. Calorimetric studies have also provided a better insight into the order–disorder transition processes of granular starch and other gelling polysaccharides. DSC can be used to characterise mixtures of polymorphic forms of fats as well as to evaluate hydrogenation and various tempering regimes for their effectiveness in bringing about desired polymorphic changes. Calorimetry has also been employed to examine the physical state and properties of water in foodstuffs.

INTRODUCTION

Differential scanning calorimetry (DSC) is a thermoanalytical technique for monitoring changes in physical or chemical properties of materials as a function of temperature by detecting the heat changes associated with such processes. In DSC, the measuring principle is to compare the rate of heat flow to the sample and to an inert material which are heated or cooled at the same rate. Changes in the sample that are associated with

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absorption or evolution of heat cause a change in the differential heat flow which is then recorded as a peak. The area under the peak is directly proportional to the enthalpic change and its direction indicates whether the thermal event is endothermic or exothermic. The method is only one of a family of related techniques, the principal of which are DTA (differential thermal analysis), TG (thermogravimetry) and DSC. Several textbooks (Porter & Johnson, 1968, 1970, 1974; Mackenzie, 1970, 1972; Weidemann, 1972) and reviews (Ladbrooke & Chapman, 1969; Sturtevant, 1972; Gill, 1974; Privalov, 1974; Brennan, 1976) covering the fundamental concepts, instrumentation and general applications of calorimetry exist.

Improvements in the sensitivity of commercially available calorimeters during the last 15 years have made the DSC a popular tool for investigating the thermodynamic properties of food components. The aim of this review is to summarise some of the main results obtained to date in thermal studies of food systems. The discussion is by no means exhaustive, but rather attempts to highlight the applications of DSC in studies of heat-induced transformations and interactions among food components as well as its potential use in product development and quality control.

PROTEINS

Classical calorimetry of protein denaturation

Heat denaturation of small globular proteins is generally considered reversible in high yield, provided that the reaction is carried out under conditions preventing aggregation, i.e. dilute solution and far from the isoelectric point. This allows indirect thermodynamic evaluation of the process by applying equilibrium thermodynamics and assuming a two-state model, i.e. A (native) $\rightarrow B$ (denatured). Under these conditions and using any property sensitive to the state of protein, one can determine the equilibrium constant, K, of the process and subsequently the standard enthalpy change, ΔH°, from the van't Hoff equation:

$$\Delta H^\circ = RT^2 \frac{\mathrm{d} \ln \mathrm{K}}{\mathrm{d}T} \tag{1}$$

The standard free energy change, ΔG°, and the standard entropy change, ΔS°, may be then obtained from eqns (2) and (3), respectively:

$$\Delta G^\circ = -RT \ln K \tag{2}$$

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \tag{3}$$

Numerical estimates of the above thermodynamic parameters are of considerable significance in understanding the molecular aspects of the denaturation reaction (Tanford, 1968).

A number of studies on protein denaturation have been made employing such indirect methods (Scott & Scheraga, 1963; Brandts, 1964; Hermans & Acampora, 1967) without, however, any means of assessing the validity of the assumption that denaturation can be presented as a two-state transition. Only recent advances in the sensitivity of instrumentation have allowed direct calorimetric studies (Jackson & Brandts, 1970; Tsong *et al.*, 1970; Biltonen *et al.*, 1971; Privalov, 1974) and made possible a decision concerning the correctness of the two-state concept by comparing ΔH_{cal} with the ΔH° obtained from equilibrium studies. The equivalence of the enthalpies would be strong evidence in favour of the two-state, 'all-or-none' character of the process. In this context, calorimetry has the unique advantage of providing not only the calorimetric enthalpy, ΔH_{cal} (from the area of the heat absorption), but also the effective enthalpy of the process (from the sharpness of the transition and using the van't Hoff equation) (Jackson & Brandts, 1970; Privalov & Khechinashvili, 1974).

Privalov & Khechinashvili (1974) have studied the thermal properties of five small compact globular proteins by DSC in very dilute solutions (0·05–0·5%) under slow heating rates (1 °C/min). The temperature dependence of the denaturation enthalpy, ΔH, was determined from changes in ΔH at different conformational stabilities of the proteins induced by changes in the pH and assuming minor changes in the ionisation enthalpies. The thermodynamic data indicated that alteration in the pH itself had indeed no effect on the specific heat capacity or enthalpy of denaturation which validated the adopted experimental approach. The main findings of this study are summarised below.

(1) The initial specific heat capacities, C_p, of these proteins were very similar. The typical dependence of C_p on temperature at different pHs is illustrated in Fig. 1. With increase in temperature, C_p increases linearly up to the temperature at which denaturation heat absorption starts.

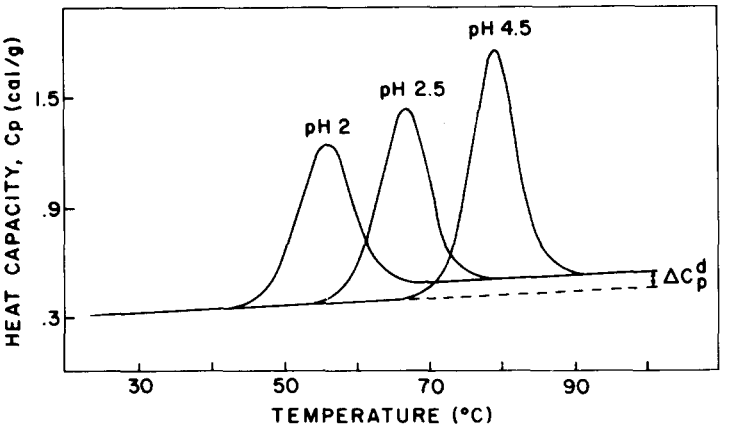

Fig. 1. Temperature dependence of partial specific heats of lysozyme at different pH values. (Adapted from Privalov, 1974.)

(2) Denaturational changes in specific heat capacities, ΔC_p^d (Fig. 1), do not vary with temperature, but the value is different for each protein.

(3) Specific denaturation enthalpy, Δh, increases linearly with temperature (Fig. 2) and the slope of the line is equal to ΔC_p. Similar trends are also seen in the published data of Brandts (1964) and Jackson & Brandts (1970). The plots of Δh versus temperature are convergent and all intersect at 110 °C. At this temperature hydrophobic interaction becomes negligible so that the Δh at 110 °C corresponds to the enthalpy of disruption of hydrogen-bonding only. Analysis of all the thermodynamic functions (ΔH, ΔS and ΔG) led to the conclusion (Privalov, 1974, 1979) that the denaturation enthalpy for small globular proteins represents the composite result of two main effects: (a) The negative heat associated with the disruption of hydrophobic bonds, i.e. the negative heat (heat evolved) of ordering water in 'clathrate' structures around exposed non-polar groups (Brandts & Hunt, 1967). This negative contribution increases with a decrease in temperature. (b) The positive contribution of disruption of hydrogen bonds which depends little on temperature.

(4) The deviation of the ratio $\Delta H_{cal}/\Delta H_{eff}$ from unity was slight ($1{\cdot}05 \pm 0{\cdot}03$) and, therefore, the denaturation can, in fact, be regarded as a co-operative transition between two states. The same conclusion was also reached by Jackson & Brandts (1970) and Biltonen *et al.* (1971) who reported good agreement between van't Hoff and direct calorimetric estimates of ΔH for chymotrypsinogen denaturation.

The kinetic parameters for denaturation of proteins can be also

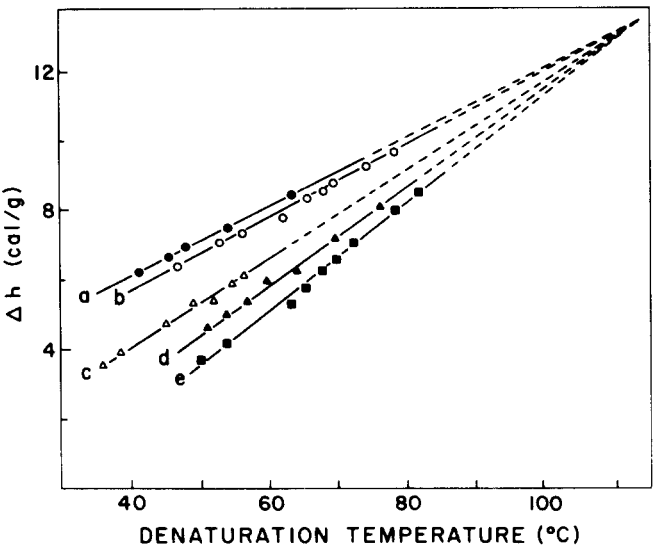

Fig. 2. Temperature dependence of the specific enthalpy of denaturation. (a) Ribonuclease A. (b) Lysozyme. (c) α-Chymotrypsin. (d) Cytochrome c. (e) Metmyoglobin. (Adapted from Privalov & Khechinashvili, 1974.)

determined from DSC curves. The vertical displacement from the base line, at any temperature, is proportional to the rate of heat flow into the sample, dH/dT, and consequently is a measure of the reaction rate. Several methods have been developed to determine the rate constants (Borchardt & Daniels, 1957; Kissinger, 1957; Beech, 1969). The activation energy may then be calculated from the Arrhenius plot of ln K versus $1/T$.

In practical DSC work both protein concentration (5–20%) and heating rates (5–20 °C/min) are quite high in order to resemble actual processing conditions. Under these conditions, however, denaturation becomes an irreversible process since extensive intermolecular interactions are favoured and aggregation of the unfolded protein molecules immediately proceeds. In contrast to denaturation, which is connected with intensive heat absorption, aggregation is generally considered as an exothermic process. Therefore, it becomes more difficult to interpret ΔH_{cal} values quantitatively, since they represent the net product of a positive (denaturation) and a negative (aggregation) contributor. In addition to protein concentration, other parameters such as pH, ionic strength and tertiary and quaternary structure of the protein can also

affect the observed ΔH_{cal} values by their influence on protein conformational stability (Hermansson, 1978, 1979*a*; Privalov, 1979). Nevertheless, since the energies involved in aggregation are low (Donovan & Ross, 1973), it is still possible to interpret the calorimetric data of protein denaturation using the above-described concepts of classical calorimetry.

Food proteins

In attempting to relate the quality properties of cooked meat with the denaturation of the contractible myofibrillar proteins (actin and myosin), many investigators have studied the thermostability of isolated proteins and extrapolated their findings to the whole muscle (Hamm, 1977). There is an inherent limitation, however, associated with such an experimental approach. The behaviour of the proteins might not be the same in the intact tissue and in the isolated preparations. On the other hand, a technique such as DSC has an obvious advantage because it is capable of studying the thermal properties of meat proteins in their natural state. Quinn *et al.* (1980) have used DSC to monitor the changes in heat stability of beef proteins during processing of meat into sausage batter. The thermogram of ground beef muscle showed three endotherms at 60 °C, 66 °C and 83 °C. Assignment of these transitions to the denaturation of myosin, sarcoplasmic proteins and actin was based on the calorimetric studies of Wright *et al.* (1977) on purified muscle proteins. The thermoprofile of a typical Wiener batter, however, revealed only a single transition at 72 °C, indicative of decreasing heat stability of the proteins. When they further investigated the effect of various processing treatments on the thermoprofile of the proteins, they found that salt, at concentrations used in processed meats, is responsible for such changes in the thermal stability. The effect of heat treatment at various water contents on myoglobin was reported by Hägerdal & Martens (1976). At water contents below 30% the transition temperature increased and a linear relationship was found between ΔH and water content which suggested that only part of the protein underwent denaturation. This interpretation was also consistent with the trend in solubility of the heat-treated samples.

Donovan *et al.* (1975) have studied the heat denaturation of egg white and its component proteins by DSC. The denaturation endotherms of the individual proteins indicated that the thermostability was of the order ovalbumin > lysozyme > conalbumin. Lowering the pH from 9·0 to 7·0 or adding Al^{3+} to the egg white increased the denaturation temperature of

conalbumin by 4 and 12 °C, respectively. Furthermore, addition of sucrose (conc. 10%) increased the stability of all three proteins (endotherms were shifted 2 °C to higher temperatures), presumably due to the suppressive effect of this solute on water activity. These findings have a direct bearing in establishing appropriate processing conditions for stabilising egg white proteins during pasteurisation. The irreversible transformation of the ovalbumin into its more heat-stable form, *S*-ovalbumin, was further investigated (Donovan & Mapes, 1976). Formation of *S*-ovalbumin during storage of eggs at elevated temperatures is responsible for the poor performance of egg whites in angel cake formulations. Since the denaturation temperature of *S*-ovalbumin is 8 °C greater than that of ovalbumin, the relative amounts of these proteins are readily determined from a DSC thermogram and consequently the quality of stored or processed egg white can be evaluated.

DSC thermograms of 10% whey protein dispersions showed a low temperature small endotherm (α-lactalbumin) at pH ≥ 4·0 and a high temperature large endotherm (β-lactoglobulin) at pH 2–9 (Hermansson, 1979*a*). When the calorimetric experiments were carried out in the presence of 0·2M NaCl, only slight increases in the denaturation temperatures were seen which implies that salt has little influence in the maintenance of conformational stability of whey proteins. The thermal behaviour of β-lactoglobulin in aqueous solutions was also examined by De Wit & Swinkels (1980) using DSC. These workers concluded that heat denaturation of β-lactoglobulin is of the first order in the range 65–72 °C and that, above this temperature range, the denaturation kinetics change.

In addition to nutritional quality, the functional characterisation of vegetable proteins is very important in evaluating the performance of a protein ingredient in a food system. Protein functionality is not only determined by the physico-chemical properties of the protein raw material, but also by the processing conditions during protein isolation, as well as by interactions with other non-protein components. Processing conditions (temperature, pH, ionic strength, water content) play a dominant rôle in functionality because of their impact on the conformational state (i.e. degree of denaturation) of protein isolates. Accordingly, DSC was used to determine the amount of native protein in soy (Hermansson, 1979*b*; Armstrong *et al.*, 1979) and faba bean (Murray *et al.*, 1981) protein isolates. These studies showed that the transition enthalpy changes can be interpreted as a measure of the proportion of protein that has not been denatured during the preparation of the isolate.

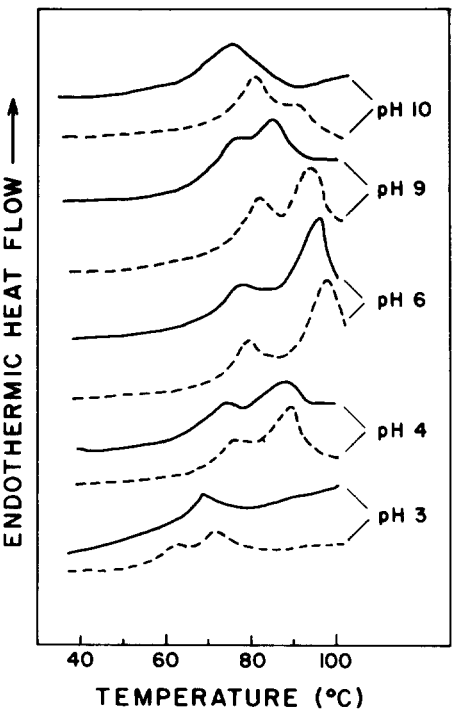

Fig. 3. DSC thermograms of 10% soy protein dispersions in distilled water (——) or 0·2M NaCl (– – –) at pH 3–10. (Adapted from Hermansson, 1978.)

Thus, if a protein isolate is already partly denatured, the ΔH will decrease, and if it is completely denatured no endothermic transition will appear. In view of the detrimental effect of heat treatment on protein solubility (Kinsella, 1979), the DSC tracing appears more useful as a quick test to determine the severity of processing conditions and the solubility characteristics of a protein isolate than measurement of the nitrogen solubility index (NSI).

The heat denaturation of soy proteins was studied by DSC in concentrated dispersions (10%), where interactions between protein molecules are favoured and protein gelation occurs (Hermansson, 1978). Two peaks were observed at pHs between 4 and 9 (Fig. 3), corresponding to the denaturation of 7S (low temperature endotherm) and 11S (high temperature endotherm) globulin. As expected, greater thermostability was seen in the isoelectric region (pH 4–5) where the net charge is low. As one moves away from this pH region the transition temperature decreases considerably. Furthermore, although no actual ΔH values were reported,

the peak areas also seem to decrease outside the isoelectric region. The above trends in the thermal patterns are similar to those reported by Privalov & Khechinashvili (1974) for small globular proteins heated at low protein concentrations. It appears, therefore, that aggregation phenomena do not cause any qualitative changes in the thermodynamics of the denaturation reaction. The stabilising role of salt in maintaining the tertiary and quaternary structure of soy proteins is also evident from the increase in the transition temperatures, particularly at any given pH value outside the isoelectric region (Fig. 3).

Protein interactions

Various physico-chemical methods, such as gel filtration, affinity chromatography, fluorescence polarisation and ultracentrifugation, have been employed in studies of protein interactions. DSC is also capable of detecting an interacting system if there is a significant change in the thermal properties of the species involved. Donovan and his co-workers conducted a series of investigations on the association complexes of several proteins with small molecules or other proteins (Donovan & Ross, 1973; Donovan & Ross, 1975*a*, *b*; Donovan & Beardslee, 1975). Table 1 lists the temperatures, enthalpies and activation energies for denaturation of the interacting species and their complexes. The thermal stability of avidin (a minor protein of egg albumen), as seen by both transition temperature and denaturation enthalpy, increased markedly when biotin was bound to this protein. Donovan & Ross (1973) suggested that in addition to the enthalpy of binding, large heat capacity differences between native and denatured avidin (with or without bound biotin) may substantially contribute to this remarkable increase in ΔH. Similarly, association of trypsin with either trypsin inhibitor or ovomucoid gave thermograms in which the characteristic peaks of the interacting species were no longer present, being replaced by a new higher temperature peak of the complex. Kinetic treatment of the denaturation data suggested that thermal denaturation of protein–protein complexes is not rate-limited by their dissociation (Donovan & Beardslee, 1975). Interestingly, calorimetry on the ovalbumin–lysozyme system, an interaction complex that is readily detected by fluorescence polarisation (Nakai & Kason, 1974) and sedimentation equilibrium (Howlett & Nichol, 1973), failed to demonstrate that such association takes place. Only two peaks were observed at temperatures characteristic of the components involved. This

TABLE 1

Denaturation Temperatures (Temperature of Peak Maximum, T_d), Transition Enthalpies (ΔH_d) and Activation Energies (E_a) of Proteins and Protein Complexes

Protein or complex	T_d (°C)	ΔH_d (*kcal/mol*)	E_a (*kcal/mol*)
Avidin[a]	85	298 ± 10	90
β-Trypsin[b]	72	194 ± 10	63
Soybean trypsin inhibitor (STI)[b]	76	110 ± 5	69
Ovomucoid[b]	79	152 ± 6	74
Conalbumin[c]	63	320[d]	—
Avidin–biotin complex[a]	131	1 065 ± 30	—
β-Trypsin–STI complex[b]	88	313 ± 13	67
β-Trypsin–ovomucoid complex[b]	81	350 ± 14	77
Diferric-conalbumin[c]	83	630[d]	—
Aluminium-conalbumin[c]	72	400[d]	—
Dicupric-conalbumin[c]	68	330[d]	—

[a] Heating rate 12·5 °C/min, pH 6·84. Reported transition temperatures are those obtained by extrapolation to a heating rate of 0 °C/min (Donovan &|Ross, 1973).
[b] Heating rate 10·0 °C/min, pH 6·7 (Donovan & Beardslee, 1975).
[c] Heating rate 10·0 °C/min, pH 7·5 or 8·3 (Donovan & Ross, 1975*a*).
[d] Average precision ± 10 kcal/mol.

may be related to the very small association constant of the complex which is indicative of a very weak interaction. Increases in the heat stability of conalbumin on binding Cu^{2+}, Al^{3+} (Donovan & Ross, 1975*a*) and Fe^{3+} (Donovan & Ross, 1975*b*) were also observed by DSC. The order of increasing heat stability of the metal ion complexes of conalbumin was $Cu^{2+} < Al^{3+} < Fe^{3+}$. Tsalkova & Privalov (1980) have recently reported calorimetric studies on troponin C (the calcium-binding component from skeletal muscle). The denaturation data suggested that the structure of troponin C consists of two independent co-operative blocks, the thermal stability of which is largely dependent on the Ca^{2+} concentration. Both structures are stabilised in the presence of Ca^{2+} and thus their transition endotherms shift towards higher temperatures.

CARBOHYDRATES

The gelatinisation of starch is of primary importance in the food industry because of its impact on the texture of starch-based foods. A number of

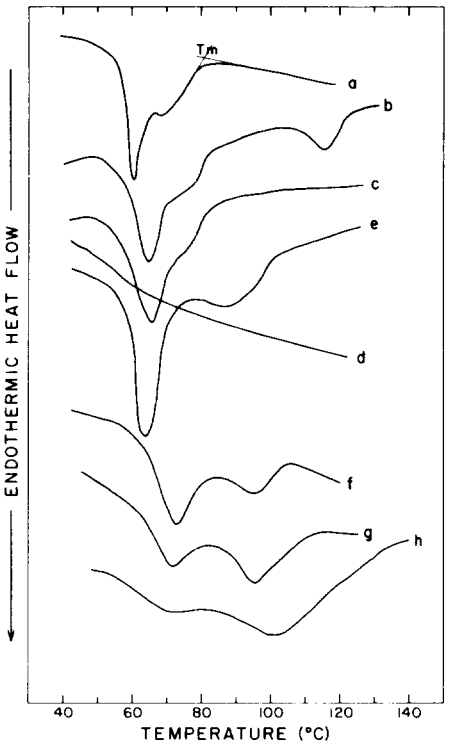

Fig. 4. DSC thermograms of starches (all samples were defatted except (b)). (a) Potato; (b) corn; (c) corn; (d) gelatinised corn; (e) smooth pea; (f) acid-modified smooth pea (5·1% lintnerised); (g) acid-modified smooth pea (9·6% lintnerised); (h) acid-modified smooth pea (20·2% lintnerised). Per cent concentrations of starch (w/w) from top to bottom were: 46·3, 46·9, 47·2, 48·4, 47·5, 47·8, 47·9, 47·6.

methods to follow the gelatinisation process have been devised, based on turbidity, swelling, solubility, absorption of dyes, X-ray diffraction, birefringence, enzymic digestibility, NMR, light scattering and DSC. DSC is particularly well suited to investigate the heat-induced phase transitions of starch/water systems because it is capable of studying these processes over a wide range of temperatures and moisture contents.

Typical DSC thermograms for several starches are illustrated in Fig. 4. Two endothermic transitions are observed for defatted native starches when heated at intermediate water contents, i.e. 45–50% w/w starch: water (Donovan, 1979; Biliaderis *et al.*, 1980; Von Eberstein *et al.*, 1980; Eliasson, 1980). These transitions are not exhibited by gelatinised starches (Fig. 4d). The influence of water content on the appearance of

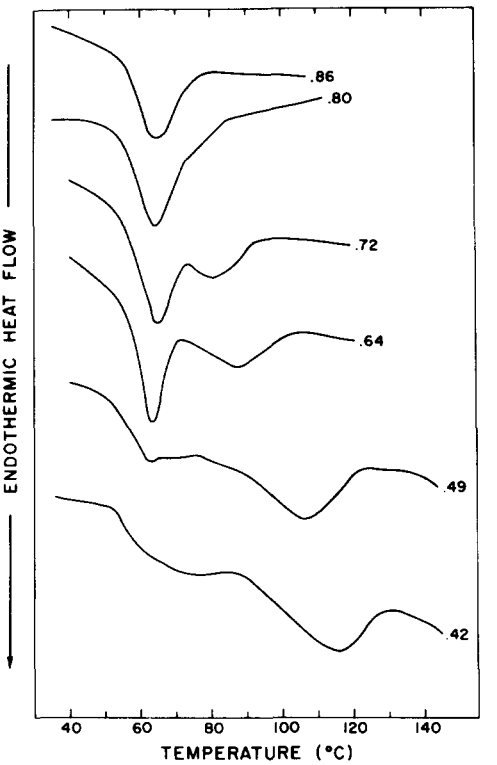

Fig. 5. DSC thermograms of smooth pea starch heated at different water concentrations; numerals represent volume fraction of water. Per cent concentrations of starch (w/w) from top to bottom were: 19·0, 26·6, 37·0, 45·6, 60·8 and 67·5. (Adapted from Biliaderis *et al.*, 1980.)

these two endotherms is shown in Fig. 5. With an excess of water, only a single endotherm is observed. However, as the ratio of starch/water increases, the second endotherm begins to develop at higher temperatures and becomes predominant at low water contents. Although the first transition occurs at constant temperature there is a progressive shift of the second endotherm towards higher temperatures as the water content decreases. This observation, and the well known semi-crystalline character of the starch granule, led to a theoretical treatment of the experimental data by employing equations that characterise other polymer systems (Donovan, 1979; Biliaderis *et al.*, 1980). The concluding temperatures (*Tm*, Fig. 4a) of the thermograms were plotted against the volume fraction of water (i.e. ratio of the volume of water to the total volume of

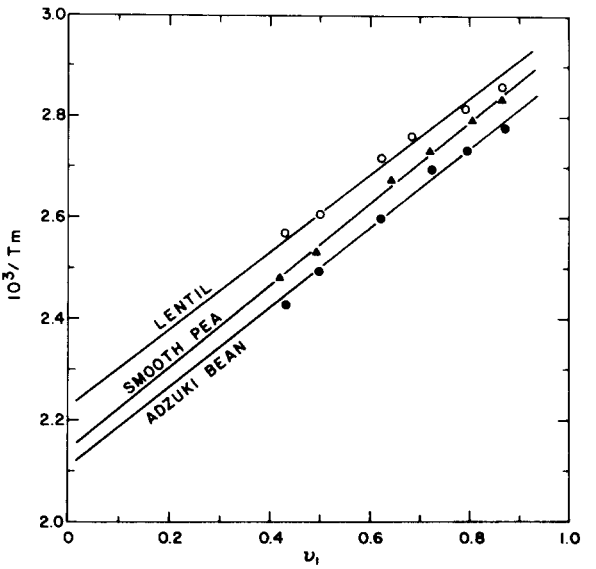

Fig. 6. Plots of $1/Tm$ (K) vs. v_1 for three starches. (Adapted from Biliaderis *et al.*, 1980.)

starch plus water) according to the Flory–Huggins equation (Flory, 1953):

$$\frac{1}{Tm} - \frac{1}{Tm^\circ} = \left(\frac{R}{\Delta Hu}\right)\left(\frac{Vu}{V_1}\right)(v_1 - X_1 v_1^2) \tag{4}$$

where R is the gas constant, ΔHu is the fusion enthalpy per repeating unit (glucose), Vu/V_1 is the ratio of the molar volume of the repeating unit to that of the diluent (water), Tm (K) is the melting point of the diluent–polymer mixture, Tm° (K) is the true melting point of the undiluted polymer, v_1 is the volume fraction of the diluent and X_1 is the Flory interaction parameter. Ideally, $X_1 = 0$ and thus eqn. (4) gives a linear relation between v_1 and $1/Tm$. Data plotted according to eqn. (4) show good agreement for the dependence of Tm on v_1 with the Flory–Huggins theory (Fig. 6); this suggests that starch gelatinisation may indeed be treated like a melting transition of a semi-crystalline synthetic polymer. The appearance of two endothermic transitions at intermediate moisture contents was further interpreted as representing two distinct mechanisms by which granule disorganisation takes place (Donovan, 1979; Biliaderis *et al.*, 1980). First, at high water contents, swelling upon hydration and increased chain motions upon heating in the amorphous parts of the starch granule destabilise the system and cause the melting of starch

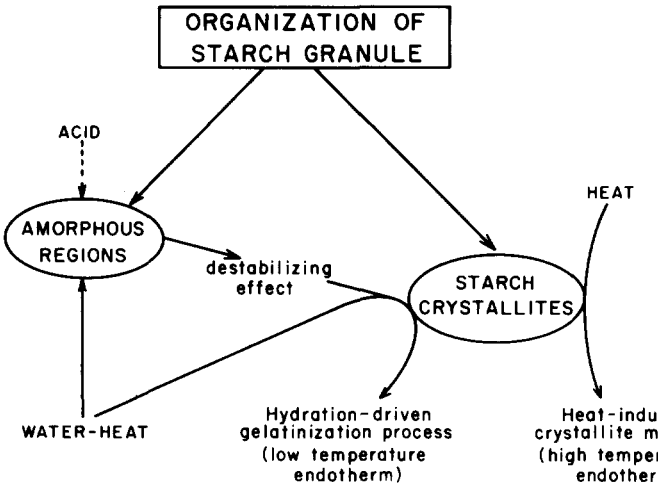

Fig. 7. Schematic representation of phase transition mechanisms of granular starch.

crystallites at low temperatures (first endotherm); a process known as gelatinisation. Secondly, in more concentrated starch suspensions, this destabilising effect is reduced due to limited amounts of water present and, therefore, 'true-melting' of the starch crystallites occurs at higher temperatures (second endotherm). The destabilising action exerted by the non-crystalline parts of the granule was further demonstrated from thermograms of acid-treated granular starches (Biliaderis *et al.*, 1980). Acid treatment (i.e. lintnerisation) of the starch selectively cleaves the amorphous regions of the granule. Thus, upon progressive lintnerisation the obtained thermograms (Fig. 4f, g, h) are similar to those of the native starch heated at low water contents (Fig. 5) which implies that the destabilising effect is indeed associated with the non-crystalline areas of the granule. The overall transition mechanism is illustrated in Fig. 7. In addition to these two irreversible endothermic transitions, a third reversible endotherm, at yet higher temperatures (Fig. 4b), has been reported (Kugimiya *et al.*, 1980; Von Eberstein *et al.*, 1980; Eliasson, 1980). This transition, the *Tm* of which is also dependent on the ν_1 of water (Eliasson, 1980; Donovan & Mapes, 1980), was interpreted as a disordering process of amylose–lipid complexes. Thus, defatted or lipid-free starches do not exhibit this endotherm. A calorimetric method for determining the amylose content of starches based on the formation and melting of amylose–lysolecithin complex was recently proposed by Kugimiya & Donovan (1981).

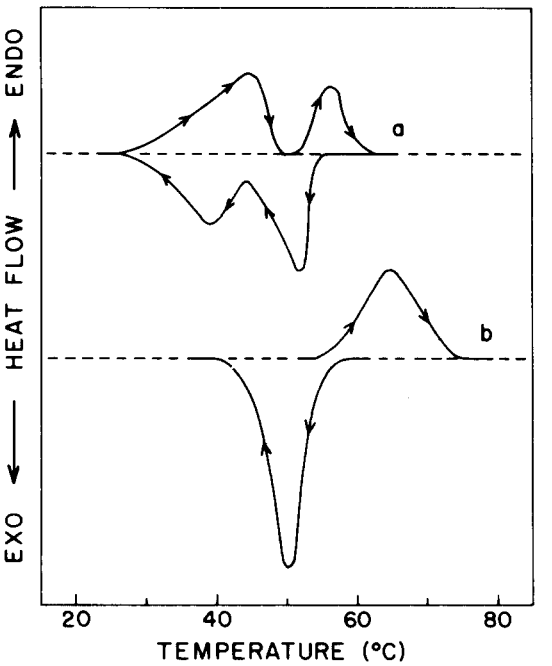

Fig. 8. Heating and cooling scans of ι-carrageenan (a) and κ-carrageenan (b). (Adapted from Morris *et al.*, 1980.)

Although the transition enthalpy for gelatinisation is significantly affected by the heating rate, starch damage and water content (Stevens & Elton, 1971; Wootton & Bamunuarachchi, 1979*a*, *b*; Donovan, 1979; Von Eberstein *et al.*, 1980), ΔH values in the range of 2·5–5·0 cal/g are usually reported by most workers. The depressing effect of sucrose on the gelatinisation of wheat starch, as seen by increase in transition temperature (Jacobsberg & Daniels, 1974; Donovan, 1977; Wootton & Bamunuarachchi, 1980) as well as by lowering in ΔH (Wootton & Bamunuarachchi, 1980) was also demonstrated calorimetrically.

Another important process that has a profound effect on the rheological characteristics of starch pastes is retrogradation (i.e. recrystallisation of the starch molecules). Retrogradation, an exothermic process, is responsible for the firming, shrinkage and syneresis of aged starch gels. A close relationship was found between the ageing of wheat starch gels as measured by endothermic peak height and the staling of bread as measured by crumb firmness at storage temperatures of $-1°$, $10°$ and $21\,°C$ (Colwell *et al.*, 1969). Although the transition temperatures do not

change upon ageing of starch gels (Colwell *et al.*, 1969), there is a time-dependent increase in the ΔH values (Von Eberstein *et al.*, 1980).

Thermally induced order→disorder transitions in other polysaccharide systems are accompanied by appreciable enthalpy changes (Reid *et al.*, 1974). Morris *et al.* (1980) have recently employed DSC to probe the temperature course of such transitions in carrageenan gels. This approach offered valuable insight into the gelation mechanism. In the presence of K^+ ions, which have been shown by light scattering to induce aggregation, the heating and cooling scans of ι-carrageenan (Fig. 8a) showed two distinct molecular processes; one without hysteresis and one (higher temperature endotherm) with significant hysteresis. The temperature course of this second process is close to that observed for κ-carrageenan (Fig. 8b) which is obtained only under aggregating conditions (i.e. presence of K^+). On the basis of this evidence and in agreement with the trend in the ΔH values, they interpreted these transitions as isolated double helix–coil and aggregated double helix–coil, respectively. Similar DSC studies on gelatin (Petrie & Becker, 1970) and other thermally reversible gels (Haas *et al.*, 1970) have been reported.

LIPIDS

In investigations of the physical properties of lipids, thermal analysis has long proven to be a powerful technique. Rek (1972) and Ladbrooke & Chapman (1969) have reviewed the applications of calorimetry in studying the phenomenon of polymorphism in fats. Polymorphism, the existence of more than one crystalline modification of the same substance, is frequently encountered in the lipid field. For instance, most triglycerides have been found to exist in three forms, α, β' and β, which, in this order, display increasing thermodynamic stability, melting point, heat of fusion and melting dilatation. Furthermore, these modifications have characteristic X-ray diffraction patterns and infra-red spectra and can also be confirmed by DSC. Thus, on the curves of Fig. 9, the low temperature endotherms correspond to the melting of the α-form which is followed by an exothermic effect, due to crystallisation into the β'-form, and an endothermic peak at the melting point of the β'-form. Similar heat effects are seen for the β'–β transition upon further heating (Lavery, 1958; Ladbrooke & Chapman, 1969). However, the lack of an exothermic peak for the α–β' or β'–β transitions does not always imply that the fat is

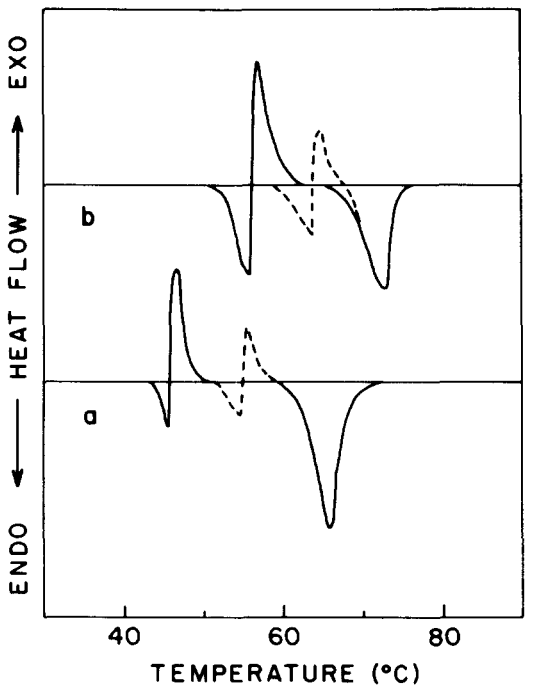

Fig. 9. DTA curves for fully saturated C_{16} (a) and C_{18} (b) monoacid triglycerides. (Adapted from Ladbrooke & Chapman, 1969.)

already in the β' or β modification, respectively. Apparently, crystallisation is a kinetic phenomenon and, therefore, if the rate of these transitions is slow, only incomplete transformation takes place during the heating cycle in the calorimeter (Rek, 1972). Recent DSC studies of the effect of chain length and unsaturation on the polymorphism of monoacid triglycerides demonstrated the presence of at least two intermediate endotherms (β'_1, β'_2) between those of α- and β-forms (Hagemann *et al.*, 1972). Hagemann *et al.* (1975) further reported that the melting point and ΔH_{fusion} for the β-form of both the *trans*- and *cis*-positional isomer series of octadecenoic acid alternate depending on double bond position; an even position correlated with higher transition temperatures and higher ΔH_{fusion}. Heats of fusion in the magnitude of 45–50 cal/g for saturated monoacid triglycerides were reported (Hampson & Rothbart, 1969). DSC was also used to examine the polymorphism of individual saturated triglycerides of the 18-n-18 and 16-n-16 series, where n is an even-carbon saturated fatty acid of 2 to 18 carbons in length (Lovegren & Gray, 1978; Gray & Lovegren, 1978). With mixed chain triglycerides, like

those of natural or hydrogenated fats, more complex forms of polymorphism are possible.

When two triglycerides are mixed the properties of the mixture are described by a binary phase diagram. DSC is the most convenient method for determining the temperatures of the phase boundaries (Perron *et al.*, 1969). The thermal behaviour of such mixtures may deviate considerably from that of the individual components (Barbano & Sherbon, 1978). DSC has also found wide applications in the blending of vegetable oils (Haighton & Hannewijk, 1958; Hannewijk & Haighton, 1958; Berger & Akehurst, 1966), evaluation of fractionated and hydrogenated fats derived from palm oil (Rossell, 1975; Jacobsberg & Ho, 1976), polymorphism and tempering of cocoa butter (Chapman *et al.*, 1971; Huyghebaert & Hendricks, 1971) or mixtures of cocoa butter with 2-oleodipalmitin and 2-elaidodipalmitin (Gray *et al.*, 1976), melting behaviour of hydrogenated hard butters (Marcus & Puri, 1978), the effect of processing conditions on the consistency of butter fat (Sherbon & Dolby, 1972; Sherbon, 1974) and oxidative stability of oils (Cross, 1970; Sliwiok & Kowalska, 1972). Furthermore, Kawamura (1979, 1980) using DSC isothermal analysis, found that crystallisation kinetics of palm oil resembles that of high polymers and that transformations to more stable crystalline forms (α–β'–β) take place at the early stages of the isothermal crystallisation.

A DSC technique has been used to determine solid–liquid ratios in fats (Bentz & Breidenbach, 1969; Miller *et al.*, 1969; Walker & Bosin, 1971) and it was found comparable with dilatometry (SFI) and wide-line NMR methods. Briefly, this method is based on measuring the heat of fusion for that portion of the fat which is solid. Besides the fact that this technique is faster than dilatometry, DSC offers a flexible means for tempering the fat at different temperature regimes prior to the determination, as well as a thermal 'fingerprint' of the fat. The latter is of importance in distinguishing between two fats with identical SFI values.

Emulsifiers heated in water at a temperature above the so-called Krafft point, where melting of the hydrocarbon chains takes place, form liquid-crystalline mesophases. First, a lamellar or neat structure is formed which, upon further heating, may transform to hexagonal or cubic structures (Ladbrooke & Chapman, 1969; Krog, 1975). A 'dispersion phase' has also been recognised in monoglyceride–water systems by adding water to the lamellar phase. Emulsification ability, as well as interactions between protein–lipid and amylose–lipid components, are

enhanced when the emulsifier is added either in an aqueous lamellar or dispersion form (Krog, 1977). On cooling below the Krafft point, the hydrocarbon chains recrystallise and a gel structure is formed. DSC can detect the heat changes associated with the above phase transitions (e.g. gel–lamellar) and thus can be used to determine the phase diagrams (Wilton & Friberg, 1971), for quality control purposes and for determining factors which increase the range of existence of lamellar and dispersion phases in monoglyceride–water systems.

WATER IN FOOD SYSTEMS

Although a concise definition of the term 'bound water' is nearly impossible, the concept of free and bound water is of great significance with regard to texture, chemical deterioration and microbial stability of foods. One definition of bound water is that it is the unfreezable water; this is assumed to be water which due to its strong interactions with other constituents is unable to crystallise on cooling. Thus, among several methods available for studying water binding, DSC has been extensively used to determine the amount of unfreezable water in foodstuffs. Briefly, this technique measures the heat required to melt the water fraction that remains frozen when the system is cooled to $-50\,^{\circ}C$. When water contents are sufficiently low that only unfreezable water is present, there is no DSC peak. Using samples of different water contents it is possible to determine the point at which all of the remaining water is unfreezable. A typical plot of such data is given in Fig. 10; the amount of unfreezable water corresponds to the intercept of the line with the water content-axis (Simatos *et al.*, 1975). Reported estimates of unfreezable water range from 0·2–0·5 gram of water per gram of solids for typical foods and their components (Davies & Webb, 1969; Parducci & Duckworth, 1972; Fennema, 1977; Wootton and Bamunuarachchi, 1978).

The use of lower freezing temperatures and calorimeters with greater temperature control during cooling and heating revealed that a fraction of the unfreezable water is in fact in a glassy or vitreous state (Parducci and Duckworth, 1972; Simatos *et al.*, 1975). The glass transition temperature (endotherm G, Fig. 11) depends on the cooling rate. If prior cooling is rapid in relation to diffusion and crystallisation of water, the interstitial fluid has a low concentration of solutes and forms a glassy phase at a low temperature. If prior cooling is slow, however, the glass

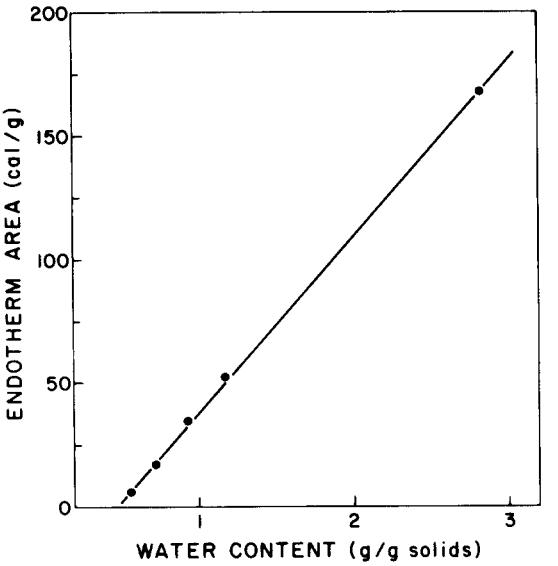

Fig. 10. Typical plot of the melting peak area (freezable water) measured by DSC versus total water content. (Adapted from Ladbrooke & Chapman, 1969 and Simatos *et al.*, 1975.)

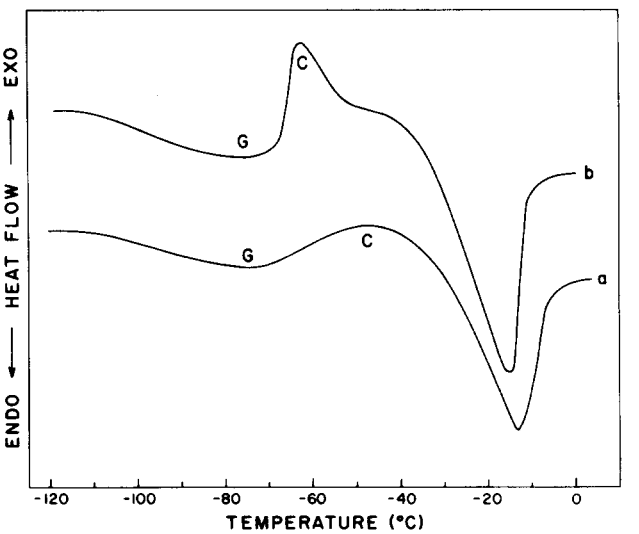

Fig. 11. Thermoprofiles for beef muscle (a) (0·44 g water/g dry solids) and egg white (b) (0·52 g water/g dry solids). Scans performed with a heating rate of 2 °C/min after cooling to −150 °C at a rate of 22 °C/min. (Adapted from Simatos *et al.*, 1975.)

transition occurs at a higher temperature independent of the solute concentration. This implies that the water content of the glassy phase is constant (Simatos *et al.*, 1975; Hardman, 1978). An exothermic effect (peak C, Fig. 11) is also observed after the glass transition which corresponds to the crystallisation of a fraction of vitreous water (Ladbrooke & Chapman, 1969; Simatos *et al.*, 1975).

Several workers have also reported results on water binding obtained by vapour abstraction methods (Hoyer & Birdi, 1968; Karmas & DiMarco, 1970*a*, *b*, *c*; Bushuk & Mehrotra, 1977). A delay in the removal of water, as well as an increase in the transition enthalpy (heat of vaporisation), were generally interpreted as an indication of stronger binding of water to a substrate. Such interpretations, however, must be made with caution. First, desorption DSC curves show non-specific broad endotherms and thus the problem of interpolating the base-line becomes difficult. Secondly, the process of vapour removal is largely dependent on the mass transfer conditions (physical state of material, vapour pressure and nature of the atmosphere in the DSC cell, etc.) Desorption calorimetric data of practical importance related to the sorption of water by dry milk and whey powders were reported by Berlin *et al.* (1971). Storage of these products under high relative humidity ($RH \geq 50\%$) induced lactose crystallisation (amorphous lactose–α-lactose monohydrate) which was detected by a single endothermic dehydration peak at 135 °C. Therefore, DSC appears to be a useful probe to monitor the physical state of lactose in whey and milk powders.

Results reported by Simatos *et al.* (1975) demonstrated the potential applications of DSC for kinetic studies of sublimation, as well as measuring the latent heat of sublimation—both important for calculations involving the freeze-drying process. Koga & Yoshizumi (1977) have investigated the thermal behaviour of rapidly frozen water–alcohol mixtures and whisky samples at various ethanol concentrations. The DSC thermograms revealed eight transitions over the entire concentration range. The ΔH of every peak remarkably changed at 40 % and 60 % ethanol content, suggesting that structural alterations in the mixture take place at these concentrations. Their calorimetric data also showed that a stronger interaction between ethanol and water occurs in aged whisky than in water–ethanol mixtures. Furthermore, DSC measurements of the freezing process showed that the process obeyed first order kinetics in the concentration range of 0–75 % ethanol and that the freezing parameters (freezing temperature, ΔH and kinetic constant) of

distilled spirits coincide with those of simple water–ethanol mixtures (Koga & Yoshizumi, 1979).

CONCLUSIONS

DSC is a useful investigative tool for studying various heat-related phenomena in foods and their components by monitoring the associated changes in enthalpy. Its ability to study these processes under the dynamic temperature conditions occurring during processing, as well as to provide both thermodynamic and kinetic data, constitute the main advantages of this technique. However, since changes in enthalpy are non-specific, the use of other complementary methods may be required to understand the physical nature of the phenomena observed.

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Varietal Differences in the Fatty Acid Composition of Oils from Cowpea (*Vigna unguiculata*) and Limabean (*Phaseolus lunatus*)

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ABSTRACT

The fatty acid composition of the extracted oils from ten varieties of cowpea, eighteen varieties of limabean and two of soyabean were determined by gas–liquid chromatography. All the seeds, with the exception of soyabean, were poor sources of oil. The major fatty acids in the cowpea and limabean varieties were linoleic ($C_{18:2}$), palmitic ($C_{16:0}$) and linolenic ($C_{18:3}$) acids. In the soyabeans, linoleic and oleic ($C_{18:1}$) acids were the major fatty acids. Traces of gadoleic ($C_{20:1}$), erucic ($C_{22:1}$) and lignoceric ($C_{24:0}$) acids were obtained in Cowpea Kano 1696, Farv-13 and Nigeria B_7 and in limabean TPL 2 and TPL 187. Considerable variability was encountered among the different fatty acids in the different varieties of cowpea and limabean. This is indicated by the differences in fatty acid composition and the high coefficient of variation obtained for ether extract (28·6 and 46·2% in cowpea and limabean, respectively), lauric acid (100 and 50%, respectively), oleic acid (35·1 and 34·7%, respectively), myristic acid (30 and 30·8%, respectively) and behenic acid (39·4 and 30·4%, respectively). Some of these differences may be genetic.

INTRODUCTION

Legumes, especially cowpeas and limabeans, can be considered to be very important in Nigerian diets because they are grown and used as staple food commodities. Their meals are the most important sources of protein and other essential nutrients.

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Because of the growing importance of these legumes to Nigerian agriculture, chemical investigations have mainly centred on their protein and anti-nutritional constituents. A general lack of information on their fatty acids has necessitated the present systematic evaluation of these important nutrients. Knowledge about the fatty acid pattern of foodstuffs is important in view of the observed influence of dietary fatty acid quality on the development of certain diseased conditions (Scheig, 1968). Moreover, in-depth elucidation of these constituents is of importance to plant breeding programmes since such a compositional analysis allows genetically controlled changes in the composition of the seed. As a first step, we have undertaken screening of different varieties of cowpea (*Vigna unguiculata*) and limabean (*Phaseolus lunatus*) cultivated in the same environment for possible variation in total lipid content and fatty acid composition. The study also provides a basis for comparison with the soyabean, the fatty acids of which have been extensively studied.

EXPERIMENTAL

Materials

Ten varieties of cowpea, eighteen of limabean and two of soyabean seeds were obtained from the National Cereals Research Institute and the International Institute of Tropical Agriculture, both in Ibadan, Nigeria, about 6 months after harvesting. The seeds were finely ground in a laboratory mill to a particle size of 0·5 mm and dried at 50 °C to constant weight.

Methods

The powdered samples were extracted in 20 g portions in a Soxhlet extractor for 20 h with redistilled ethyl ether, previously rendered peroxide free, and the total lipid was determined by gravimetry (AOAC, 1975). About 100 mg of the extracted fat was methylated (Metcalf & Schmidt, 1960) with methanol in the presence of boron trifluoride (BF_3) in benzene solution. The methyl esters were extracted with petroleum ether. After removal of the solvent in nitrogen atmosphere, the esters were stored with drops of benzene at -20 °C until analysed by gas–liquid chromatography (usually within a week).

Gas–liquid chromatography

The resolution of the various fatty acids was effected with a Varian Aerograph Series 2100 gas chromatograph, on a steel column, 10 ft × $\frac{1}{4}$ in outer diameter, packed with 10% stabilised diethylene glycol succinate (DEGS), on Chromosorb 100/120 mesh. The chromatograph was equipped with a flame ionisation detector (FID) and attached to a Hewlett-Packard integrator, Model 3380A. The operating conditions were as follows: attenuation, 4; range, 10^{-10}A/mV; mode, isothermal; column temperature, 170°C; injector port temperature, 250°C; detector port temperature, 250°C; carrier gas, helium at 38 ml/min; ancillary gases, hydrogen at 35 ml/min and compressed air at 350 ml/min; chart speed, 0·5 in/min.

Methyl esters of the legume oils diluted in benzene were injected with a Hamilton 10-μl microsyringe. Two analyses were carried out, 3·5 μl at 8 × 1000 attenuation and 2 μl at 4 × 1000. Results given are those at 4 × 1000 attenuation.

For the correct identification of the component acids in each sample, a standard (Hormel) mixture of methyl esters was run through the same conditioned column under identical conditions. This enabled the correct identification of the fatty acid esters of the test samples. Percentage composition of each fraction was evaluated from the peak area of individual components, measured by mechanical integrator. They were identified by their retention times compared with those of the reference standards and corrected to give weight per cent composition by the procedure of Ackman & Sipos (1964). Quantitative results with the reference standards agreed with the stated composition data with a relative error, less than 10% for *major* components (> 10% of the total mixture) and less than 5% for the *minor* components (< 10% of the total mixture).

RESULTS AND DISCUSSION

Total fat content of the ten cowpea and eighteen limabean varieties and the fatty acid patterns in the extracted fat samples are presented in Tables 1 and 2. Fatty acid distribution in the two legume types did not show many differences except that the limabeans contained a greater concentration of unsaturated fatty acids than the cowpea oils. However,

TABLE 1

Percentage Fatty Acid Composition in Different Varieties of Cowpea

Cowpea varieties	*Total ether extract (%)*	*12:0*	*14:0*	*14:1*	*16:0*	*16:1*	*18:0*	*18:1*	*18:2*	*18:3*	*20:0*	*20:1*	*22:0*	*22:1*	*24:0*	*Percentage saturated fatty acids*	*Percentage polyunsaturated fatty acids*
Igbira	1·88	0·02	0·19	—	22·5	0·56	5·98	3·25	35·2	16·1	2·00	—	4·26	—	—	34·9	56·3
Samaru Local	1·63	—	0·18	—	26·7	0·36	5·87	5·03	30·9	23·3	2·18	—	4·59	—	—	39·5	55·1
Kano 1696	1·54	0·14	0·21	0·14	23·0	0·41	5·53	8·32	33·3	16·1	2·02	—	3·99	0·37	2·31	27·2	53·6
Blackie	1·25	0·02	0·26	—	30·0	0·23	6·35	9·92	32·4	9·12	2·63	—	8·75	—	—	48·0	42·6
Adzuki	2·01	0·02	0·18	—	22·5	—	5·99	10·86	30·3	19·7	2·25	—	6·58	—	—	37·5	51·7
Farv-13	2·22	0·02	0·16	0·07	23·9	—	4·30	12·85	24·8	23·3	2·31	0·03	6·52	0·01	2·09	38·6	48·5
West Bred	2·45	—	0·15	—	22·7	—	3·98	8·30	35·9	22·6	1·22	—	3·41	—	—	31·5	60·3
Ife Brown	2·88	0·09	0·33	—	30·6	—	5·48	7·55	28·4	14·0	2·16	—	9·98	—	—	48·7	43·8
Prima	1·37	0·04	0·21	—	23·1	—	4·71	7·28	35·6	22·8	1·69	—	4·33	—	—	34·6	58·2
Nigeria B_1	2·36	0·15	0·15	0·08	26·5	—	6·61	6·36	30·2	20·6	2·46	0·02	4·16	0·14	1·89	41·8	51·2
Mean	1·96	0·06	0·20		25·1		5·48	7·98	31·7	18·8	2·09		5·66	—	—	38·2	52·1
Standard deviation	0·56	0·06	0·06		3·13		0·88	2·80	3·54	4·79	0·40		2·23	—	—	6·76	5·84
Coefficient of variation (%)	28·57	100·00	30·00		12·5		15·77	35·08	11·17	25·52	19·04		39·4	—	—	17·7	11·2

TABLE 2
Total Fat and Fatty Acid Composition of Different Limabean Varieties (%)

Limabean varieties	*Accession numbers*	*Total ether extract (%)*	*12:0*	*14:0*	*14:1*	*16:0*	*16:1*	*18:0*	*18:1*	*18:2*	*18:3*	*20:0*	*20:1*	*22:0*	*22:1*	*24:0*	*Percentage saturated fatty acids*	*Percentage polyunsaturated fatty acids*
TPL 1	Acc. 64024	1·62	—	0·42	—	23·1	0·31	6·43	7·34	40·6	18·4	1·21	—	0·92	—	—	32·1	60·3
TPL 2	Acc. 64065	2·70	0·06	0·38	—	25·5	0·52	7·44	7·44	33·5	19·5	1·54	0·10	1·08	0·35	2·00	37·8	53·9
TPL 3	Acc. 64068	1·09	0·03	0·28	—	22·3	1·91	5·61	6·67	36·4	22·1	1·40	—	1·09	—	—	30·7	60·7
TPL 4	Acc. 64006	1·09	0·05	0·28	—	22·7	—	4·95	6·92	41·5	13·7	1·40	—	1·09	—	—	30·3	62·7
TPL 5	Acc. 64053	1·61	0·06	0·64	—	22·0	—	7·61	7·38	38·1	20·5	1·59	—	1·07	—	—	33·0	59·7
TPL 6	Acc. 64016	0·81	0·02	0·40	—	21·2	1·19	7·77	6·68	36·6	21·4	1·77	—	1·12	—	—	31·2	61·0
TPL 7	Acc. 64028	1·48	—	0·18	—	15·7	—	4·79	18·3	45·9	11·2	1·18	—	0·59	—	—	22·4	69·3
TPL 8	Acc. 64027	1·35	—	0·24	—	13·8	0·40	3·49	7·77	47·9	20·8	4·53	—	0·37	—	—	22·4	69·4
TPL 9	Acc. 64033	1·56	0·03	0·28	—	18·6	0·86	4·70	9·81	43·6	18·8	1·83	—	1·01	—	—	26·5	62·9
TPL 10	Acc. 64032	2·67	0·05	0·56	—	21·8	1·23	5·14	7·31	39·7	18·2	1·25	—	0·93	—	—	29·7	61·8
TPL 11	Acc. 64022	2·99	0·01	0·40	—	23·8	0·64	7·73	5·95	37·9	19·3	1·79	—	0·74	—	—	34·5	58·9
TPL 13	Acc. 64020	0·51	0·01	0·44	—	21·5	0·56	5·84	9·49	35·2	15·7	1·32	—	1·53	—	—	30·6	59·3
TPL 14e	Acc. 64064	2·85	0·02	0·38	—	19·6	0·30	6·11	10·7	41·3	18·1	1·25	—	0·77	—	—	28·1	60·9
TPL 17	Acc. 64026	1·36	0·02	0·48	—	21·7	0·49	7·25	10·3	39·4	16·6	1·33	—	0·75	—	—	31·5	57·6
TPL 183	Acc. 64017	1·23	0·04	0·59	—	22·8	0·28	7·24	9·28	40·3	14·3	1·58	—	1·01	—	—	33·2	57·2
TPL 187	Acc. 64095	1·26	0·06	0·34	0·26	22·2	0·63	5·71	9·79	42·2	16·3	1·20	0·05	0·82	1·26	0·16	30·1	57·0
TPL 304	Acc. 64096	1·13	—	0·36	—	23·0	0·51	4·87	6·75	43·1	18·5	1·17	—	0·63	—	—	30·1	62·7
TPL 071-33	Acc. 65005	1·16	0·04	0·34	—	21·8	1·56	6·02	14·7	36·0	16·5	1·10	—	0·51	—	—	29·9	53·9
Mean		1·58	0·04	0·39	—	21·3	0·63	6·04	9·03	40·0	17·8	1·58	—	0·89	—	—	30·2	60·5
Standard deviation		0·73	0·02	0·12		2·82	0·53	1·26	3·13	3·78	2·83	0·77	—	0·27	—	—	3·74	4·17
Coefficient of variation (%)		46·2	50·0	30·8	—	13·3	84·1	20·9	34·7	9·46	15·93	48·7	—	30·3	—	—	12·4	6·89

TABLE 3
Total Fat and Fatty Acid Composition in Two Soyabean Varieties (%)

Soyabean varieties	*Total ether extract (%)*	*12:0*	*14:0*	*14:1*	*16:0*	*16:1*	*18:0*	*18:1*	*18:2*	*18:3*	*20:0*	*20:1*	*22:0*	*22:1*	*24:0*	*Percentage saturated fatty acids*	*Percentage polyunsaturated fatty acids*
GE 104	26·6	—	0·10	—	9·71	0·68	3·10	27·3	43·8	4·86	2·09	—	—	—	—	15·1	53·7
GE 109	21·6	—	0·10	—	11·0	0·14	2·86	23·4	55·3	5·13	2·08	—	—	—	—	16·1	60·5

when compared with the soyabean varieties (Table 3), the soyabean oils were distinctly different from the cowpea and limabean oils in both total fat and fatty acid contents. While the mean total fat contents in the cowpea and limabean varieties were 1·96 and 1·58 %, respectively, the two soyabean varieties contained an average of 24·1 % of total fat. Interestingly, linoleic acid was the major fatty acid in the three legume types and their total unsaturated fatty acids did not differ to any great extent. But while the cowpea and limabean oils gave ranges of 27·2–48·7 % and 22·4–37·8 % of saturated acids, respectively, the soyabean oils contained only an average of 15·6 % saturated fatty acids. The level of saturation was comparatively low due primarily to the low content of palmitic and stearic acids and the complete absence of lauric and behenic acids. Further, fatty acids above C_{20} were also completely absent from the soyabean lipids while the major fatty acids in descending order of abundance were essentially linoleic and oleic acids. Among the cowpeas and limabeans, the order was linoleic—palmitic—linolenic acids except in limabeans TPL 6, TPL 8 and TPL 9, where it was linoleic—linolenic—palmitic acids.

Among the different varieties of both cowpeas and limabeans, the fatty acid compositions showed distinct varietal differences. This fact is illustrated by the high coefficient of variation obtained and the rather wide range of the most prominent fatty acids in the different oils: notably 3·25–12·9 % cowpea oleic acid, 5·95–18·3 % limabean oleic acid, 24·8–35·9 % cowpea linoleic acid, 9·12–23·3 % cowpea linolenic acid and 11·2–22·1 % limabean linolenic acid. While legumes are generally known to contain numerous minor acids with fourteen to eighteen carbons (Kuemmel, 1964), the presence of gadoleic ($C_{20:1}$), behenic ($C_{22:0}$), erucic ($C_{22:1}$) and lignoceric ($C_{24:0}$) acids was a rather unusual characteristic. Limabean varieties TPL 2 and TPL 187 and cowpeas Kano 1696, Farv-13 and Nigeria B_7 are particularly noteworthy as those legumes contain trace amounts of erucic acid, which is known to be toxic (Downey *et al.*, 1969). It is possible that there exist in cowpea and limabean legumes differences in the genetic control of oil quality. Putt *et al.* (1969) have shown that, in flax and safflower, the composition of oil is controlled by the genotype of the seed, rather than by the genotype of the maternal plant. Assuming that the same holds for legume seeds, it may be expected that because of the inherent heterozygosity of all legumes (Kuemmel, 1964), differences in fatty acid patterns are bound to exist in the different legumes and the different varieties of the same legume types. The present

results are, however, too limited to permit any generalisation to be made from them.

It is important to note that, since cowpea and limabean oils are very low in total fat, the composition has very little effect on the total fatty acid contents of human and animal diets. In most cases, the oils used in cooking these legume meals in Nigeria are more important in the diet than that present in the cowpea and limabean seeds.

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Sulphite Mediated Destruction of β-Carotene: The Partial Characterisation of Reaction Products

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ABSTRACT

Sulphite species will catalyse the destruction of β-carotene in homogeneous solution at pH 5·7 in the presence of Mn^{2+}, oxygen and glycine. The products are highly oxygenated, containing from 10 to 15 oxygen atoms per β-carotene molecule. There is no evidence of extensive fragmentation or polymerisation of the carotenoid. The products appear to be widely hydrogen bonded and retain some conjugation of double bonds.

INTRODUCTION

The oxidation of sulphite species by air is known (Hayon *et al.*, 1972) to involve free radical intermediates such as $^{\cdot}HSO_3$, $^{\cdot}OH$, $HO_2^{\cdot}$ and possibly $^{\cdot}SO_3^-$ and it is expected that these radicals would be capable of rapid interaction with unsaturated compounds and particularly polyenes. It is common to find mixtures of polyenes (e.g. carotenoids) and sulphite species in foods (e.g. dehydrated vegetables, beverages containing carotenoids) and although the generally accepted rôle of sulphite in such systems includes that of an antioxidant, recently Peiser & Yang (1979) reported the sulphite mediated destruction of β-carotene (as measured by absorbance at 454 nm) in the presence of oxygen. The system reported contained glycine (20 mM), Na_2SO_3 (1 mM), β-carotene (7·4 mM) and Mn^{2+} (50 μM) in 76% ethanol at pH 9·2. Under these conditions over

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90% of the carotenoid was destroyed in 15s, the loss occurring concurrently with aerobic oxidation of sulphite. This reaction was effectively inhibited by free radical scavengers: α-tocopherol 1,2-dihydroxybenzene-3,5-disulphonic acid and butylated hydroxytoluene. A mechanism involving the sulphite free radical was suggested as follows:

$$Mn^{2+} + O_2 \longrightarrow Mn^{3+} + O_2^-$$

$$Mn^{3+} + SO_3^{2-} \longrightarrow Mn^{2+} + SO_3^-$$

$$\beta\text{-carotene} + O_2^- + H^+ \longrightarrow \beta\text{-carotene}^{\cdot} + H_2O_2$$

$$\beta\text{-carotene} + SO_3^- \longrightarrow \beta\text{-carotene}^{\cdot} + H^+ + SO_3^{2-}$$

$$\beta\text{-carotene}^{\cdot} + O_2 \longrightarrow \beta\text{-carotene—O—O}^{\cdot}$$

Other possibilities for the loss of β-carotene include the well known free radical induced isomerisation (Fischer, 1968; Huang *et al.*, 1968); sulphur dioxide can, for example, be used to isomerise natural rubber (Huang *et al.*, 1974). It is interesting to note, however, that the effect of sulphitation on the isomerisation of lycopene is that of inhibition (Miers *et al.*, 1958). Sulphonation at the double bonds involving a mechanism similar to that proposed by Kharasch *et al.* (1939*a*, *b*) and other reactions typical of free radical interactions with olefinic bonds which can lead to polymerisation, elimination and fragmentation, are also possible.

Here we describe the reaction reported by Peiser & Yang (1979) at a more realistic pH with regard to food and comment on the nature of the reaction products.

EXPERIMENTAL

General procedure adopted for carrying out the reaction

All reaction mixtures contained buffer (borax at pH 9·2 and acetate at pH 5·7) at a final concentration of 0·025M, manganous chloride (52 μM) and glycine (21 mM), the solvent being 76% aqueous ethanol. All-*trans* β-carotene (BDH Chemicals Ltd) was added as a solution in chloroform (0·22% w/v), and final concentrations in the reaction mixture ranged from 80 to 500 μM. To start the reaction the buffered solution containing β-carotene (9·5 ml) was mixed with a solution of sodium metabisulphite (2 mM, 0·5 ml). The progress of the reaction was followed by transferring the reaction mixture to a 1 cm silica cell and the absorbance due to β-

carotene was measured at 454 nm as a function of time. The concentration of sulphite species was determined by the method of Humphrey *et al.* (1970) using 5,5′-dithiobis-(2-nitrobenzoic acid).

Preparation of reaction product fractions

The reaction products could be extracted with petroleum spirit and recovered by evaporation at 25 °C under reduced pressure. Recoveries were determined gravimetrically. Crystals could be separated from the reaction mixture by adding water until an organic layer separated out, followed by a further addition of water of twice the volume of the organic layer. The aqueous part of the solution was then frozen, the ice crushed and washed with chloroform until the washings were clear. When the ice was melted and excess chloroform removed under reduced pressure, crystals separated out. The products were subjected to thin layer chromatography on silica gel G with petroleum spirit (boiling point, 90–100 °C): benzene (1:1) and chloroform: methanol (3:2) as solvent and also to column chromatography on Sephadex LH20 (2·5 × 50 cm) with ethanol as the eluting solvent (0·4 ml/min). The column effluent was fractionated and absorbance of the fractions measured at 280 and 360 nm, where the products were found to absorb.

Properties of reaction products

All samples of reaction product prepared as described above, as well as combined fractions corresponding approximately to each chromatographic peak, were subjected to microanalysis. The chromatographic fractions were also subjected to molecular weight determination by vapour phase osmometry in chloroform. Infra-red spectra were recorded from KBr discs.

In order to investigate the acid–base properties of the products, the reaction mixture was acidified with HCl to pH < 1 and the products extracted with petroleum spirit (boiling point, 40–60 °C). After washing with water the solvent was removed at 25 °C under reduced pressure. The product was dissolved in a mixture of chloroform:ethanol:water (15:63:22) and titrated with sodium hydroxide (0·01 M) using a pH meter with a combined glass–calomel electrode. All experiments were supported with controls containing either no β-carotene or sulphite ion, as appropriate.

RESULTS

The previously reported study (Peiser & Yang, 1979) of this reaction was at pH 9·2 and therefore of little use for predicting the importance of the sulphite mediated destruction of β-carotene in foods. It was therefore decided to carry out the reaction at pH 5·7 and 9·2 and the results, comparing the rates of loss of carotenoid and of sulphite species, are shown in Table 1. It is immediately apparent that the reaction is somewhat slower at the lower pH and is associated with a slower rate of loss of sulphite. It is nevertheless a rapid reaction on the time scale of food processing operations, all the β-carotene being lost in approximately 1 min. Under the same conditions but with no added sulphite there was no detectable change in β-carotene concentration over this time scale, as was the case when butylated hydroxytoluene (30 mM) was present in sulphited mixtures. To take into account the possible adventitious addition of a catalytic impurity in the sodium metabisulphite or other reactant, a mixture of all reactants other than β-carotene was allowed to undergo oxidation before addition of the carotenoid. The lack of any effect under these conditions confirmed the need for oxidation of the sulphite species in the reaction mechanism at both pH values used. It may therefore be concluded that, in principle, the reaction between sulphite species and β-carotene takes place at the pH of food systems.

The products from reactions carried out at both pH values were quantitatively extracted from the reaction mixtures using petroleum spirit (boiling point, 40–60 °C) and showed a maximum absorbance at 360 nm in this solvent. The result therefore represents a considerable shift in

TABLE 1

Loss in β-Carotene and Sulphite with Time at pH 5·7 and 9·2. Initial Concentrations: β-Carotene = 80 μM; Sulphite = 100 μM

Time(s)	*Relative β-carotene concentration*		*Relative sulphite concentration*	
	pH 5·7	*pH 9·2*	*pH 5·7*	*pH 9·2*
0	1·00	1·00	1·00	1·00
10	0·76	0·15		
20	0·51	0·10	0·55	0·00
30	0·26	0·07		
40	0·12	0·05	0·10	0·00
50	0·09	0·05		

absorbance maximum from the position due to β-carotene at 454 nm. The ratio E_{360}/E_{454} for the product was 2·5 compared with a value of 0·2 for β-carotene. The crystalline product isolated showed absorbance only at 284 nm.

Microanalysis of total reaction products showed less than 0·3% S (pH 5·7) and less than 0·5% S (pH 9·2) with no nitrogen and a large amount (22–25%) of unaccountable mass presumably due to oxygen. These microanalyses also gave residue and the reported values are given after the subtraction of the contribution due to this residue. The microanalysis of β-carotene samples after being subjected to a control experiment in which no sulphite species had been added, but which had been put through an identical procedure to the above samples, gave C = 88·9%, H = 10·6% (required for $C_{40}H_{56}$: C = 89·6%, H = 10·4%). Similarly, the crystalline product isolated contained no significant amount of sulphur or nitrogen but had an oxygen content (by difference) of 37%. Infra-red spectroscopy showed a broad intense band at 3500 cm^{-1} characteristic of H-bonded OH for both total products and the crystalline substance.

Thin layer chromatography showed the products to be immobile in petroleum spirit/benzene whereas β-carotene ran under these conditions at $R_f = 0·82$. In contrast, when chloroform/methanol was used as the solvent the products ran as a single spot at $R_f = 0·92$ with β-carotene at $R_f = 0·78$. The possible polar nature of these products suggested by infra-red spectroscopy is supported by this observation. No C=O stretching was observed. Titration of reaction products showed an increase in apparent pH value of the solution from 3·5 to 12·5 with an endpoint at pH 8·5. The equivalent weight of the acid was calculated to be 1600. A control experiment in which a reaction mixture containing no β-carotene was subjected to extraction and titration showed no buffering capability. In the case of the titration of the crystalline product an acid with pK 7·0 (in 22% H_2O) was observed with an equivalent weight of 590. The crystalline product therefore shows a larger effect due to ionisable hydrogen atoms.

Successful resolution of reaction products was achieved on alkylated dextran (Sephadex LH20) with ethanol as the eluting solvent. A typical chromatogram of total reaction products is shown in Fig. 1 where absorbances at 280 and 360 nm are indicated. Molecular weights determined by vapour phase osmometry are shown at the corresponding points on the chromatogram. The crystalline product was found to

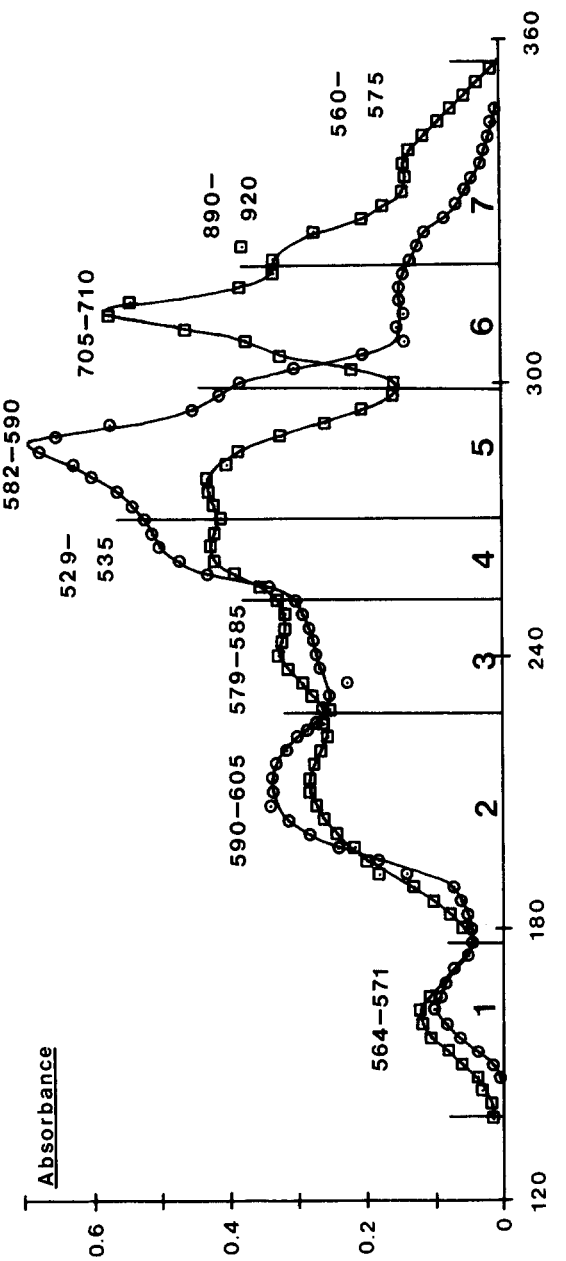

Fig. 1. Elution profile for the separation of reaction products using Sephadex LH20 with ethanol as the solvent. Molecular weights determined, by vapour phase osmometry, for the components shown are indicated. Absorbances at 280 nm denoted by —⊙—, and at 360 nm denoted by —⊡—. Fraction numbers relate to microanalysis data shown in Table 2.

TABLE 2
Microanalysis of Chromatographic Fractions. Fraction Numbers Identified in Fig. 1

Fraction	*Empirical formula (atoms per carbon atom)*	
	H	*O*
1	1·53	0·37
2	1·59	0·28
3	1·56	0·27
4	1·54	0·24
5	1·56	0·26
6	1·59	0·30
7	1·62	0·36

correspond mainly to the early fractions (to an elution volume of 300 ml) whereas the remaining components in the mother liquor gave the remainder of the chromatogram on analysis. Individual fractions recovered after column chromatography were also subjected to microanalysis. No residue was found after the analyses and the amounts of C, H and O (by difference), expressed as atoms per carbon atom are shown in Table 2. The fractions are numbered according to Fig. 1. It is evident that the C—H composition of the products is constant and that all fractions contain considerable amounts of oxygen, the number of atoms combined per original β-carotene molecule being 10–15 over the range of reported values.

DISCUSSION

The results clearly indicate the formation of highly polar, H-bonded products with a high degree of oxygenation. No significant amount of sulphur or nitrogen was incorporated into the reaction products; the addition of one sulphur atom per β-carotene molecule would lead to a sulphur content in the region of 5 % whereas the observed amounts were consistently less than 0·5 %. The amount of oxygen incorporated is comparable with that recently found (Teixeira Neto *et al.*, 1981) for autoxidation of β-carotene; manometric measurement showed that up to 7 moles of O_2 were consumed per mole of carotenoid, equivalent to 14 atoms of the element.

Although the final degree of oxidation is similar to that observed in

autoxidation, an apparent difference between the corresponding products is the lack of any clear carbonyl stretching observed by infra-red spectroscopy in this work (Ouyang *et al.*, 1980). It is likely, therefore, that the oxygen exists mainly in the form of hydroxyl groups and possibly also in the form of stable peroxides. It was not possible to resolve the contributions from the latter since the infra-red characteristics of peroxides involve minor features (Bellamy, 1962). The intense absorption of the products in the ultraviolet region of the spectrum suggests that the products still have extensive conjugation within the hydrocarbon chain and the possibility of enolic OH is strongly implied. The ability of the products to form salts with metal ions is possible, as suggested by the residue remaining after microanalysis of extracts from solutions at pH 5·7 or pH 9·2, and also by the pH titration behaviour of the free acids. Molecular weight determinations suggest little fragmentation or polymerisation. Assuming an average molecular weight in the region of 600, the average number of ionisable groups per β-carotene molecule is approximately 0·4 for the whole products mixture, whereas the equivalent weight of the acid in the crystalline product suggests that this has an average of one acid group per molecule. The products running later on the chromatogram therefore seem to be the less acidic components.

The Sephadex LH20 medium used for the separation of reaction products can lead to separations on the basis of adsorption, gel filtration and/or partition. It is evident that for this system in ethanol, the mechanism is not that of molecular weight discrimination.

The reaction reported here was carried out in homogeneous solution to facilitate the study of products. However, if the feasibility of this reaction in a food system is to be considered it must be capable of taking place in a heterogeneous system and this possibility is, at present, being investigated. However, encouraging results have recently been published by Lizada & Yang (1981) on sulphite induced lipid peroxidation in emulsified linoleic acid in the range pH 4–6. Linoleic acid hydroperoxides, formed in this reaction, were identified and specific adducts involving sulphite were also reported.

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Ultrathin-layer Isoelectric Focusing of Partially Purified Peroxidase from Tomato Fruit

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ABSTRACT

Partially purified peroxidase from tomato fruits was obtained by gel filtration on Ultrogel AcA 34 of a pH 5·5 extract prepared by homogenising the fruits with 0·4M McIlvaine buffer containing 0·2% Triton-X 100, subsequent centrifugation at 2000 × g and ultrafiltration of the supernatant. This fraction, with a molecular weight of 43 000 daltons, showed an optimum activity of pH 5·3 with guaiacol as the hydrogen donor, a K_m for H_2O_2 of 0·8 mM at optimum guaiacol concentration, a K_m of 9·0 mM for guaiacol at optimum H_2O_2 concentration and competitive cyanide inhibition with a K_i of 1·0 μM. Ultrathin-layer isoelectric focusing in 50 μm polyacrylamide gels resulted in the separation of eight peroxidase isozyme bands, detected mainly in the range pH 2 to 4. Parallel experiments carried out with horseradish peroxidase (Boehringer, Mannheim) exhibited the main enzyme activities in the range pH 7 to 9.

INTRODUCTION

Peroxidases are considered to contribute to changes in the flavour, texture and colour of raw and processed foods (Burnette, 1977; Haard,

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1977). In most cases, a deteriorative aspect—for example, 'off-flavour'—is indicated (Sessa & Anderson, 1981). On the other hand, the results of flavour research work carried out on tea or tobacco show that, during processing, enzymic pathways also have an enhancing effect. Of all the enzymes involved peroxidases are supposed to play the most important role (Sanderson, 1975; Enzell, 1981). This argument arises from knowledge about the aroma composition of these foods (Sanderson, 1975; Enzell, 1981) and from the fact that peroxidases exhibit only low substrate specificity with regard to electron donors. In addition to the general peroxidation reactions, peroxidases can also catalyse oxidation, catalatic and hydroxylation reactions (Haard, 1977). Thus, substances from different chemical classes can be changed by the action of peroxidases (Sessa & Anderson, 1981).

Recently, these considerations have been integrated into problems of the biogenesis of flavour components in tomato fruits and processed tomato products (Signoret & Crouzet, 1978), and some characteristics of peroxidase from tomato fruits have been investigated (Signoret & Crouzet, 1978; Kokkinakis & Brooks, 1979; Jen *et al.*, 1980). The work reported in this paper was intended to widen our knowledge about the properties of the enzyme from ripe tomato fruits.

MATERIALS AND METHODS

Fruits

Ripe red tomato fruits (varieties not specified) were used.

Preparation of an enzyme extract

Four hundred grams of tomato fruits were homogenised for 2 min in a Braun mixer with 400 ml of 0·4M potassium phosphate–citric acid (McIlvaine) buffer, pH 5·5, containing 0·2% Triton-X 100. The homogenate was filtered through four layers of cheesecloth, and the crude extract (280 ml) was centrifuged for 10 min at 2000 × *g*. Fifty millilitres of the supernatant (250 ml) were concentrated tenfold under nitrogen (3 bar) in an Amicon ultrafiltration cell using a Kalle PA 20 membrane. All steps were carried out at 2 °C.

Gel filtration

Two-and-a-half millilitres of the ultrafiltered extract was loaded onto a column (2·5 × 95 cm) of Ultrogel AcA 34 (LKB) in the eluting 0·1 M McIlvaine buffer, pH 5·5. Elution was carried out under a flow rate of 15 ml/h using a Vario-perpex II pump (LKB). Seven millilitre fractions were collected using an Ultrorac II system (LKB). The eluates were monitored for protein absorbance at 280 nm by an LKB Uvicord S spectrophotometer and also assayed for peroxidase activity (see below). Eluates with peroxidase activity (fractions 47–55) were pooled. For ultrathin-layer focusing it was necessary to repeat the gel filtration step four times. The collected peroxidase fractions were concentrated by ultrafiltration on a Kalle PA 20 membrane subjected to 3 bar of nitrogen. In all, this way, a 150-fold concentration was achieved. All steps were carried out at 2 °C.

Ultrathin-layer isoelectric focusing

Reagents

Acrylamide (recrystallised), *N*,*N*′-methylenebisacrylamide (recrystallised) and ammonium persulphate came from Serva (Heidelberg). Methacryloxypropyltrimethoxysilane was available as Polyfix 1000 from Desaga (Heidelberg). Glass plates, polyester films and spacer strips were all from Desaga (Heidelberg). Several batches of analytical grade pH 2–11 'Servalyt' and technical grade 'Servalyt T' carrier ampholytes were obtained from Serva (Heidelberg). All other reagents were analytical grade materials from Merck (Darmstadt).

Silanisation

Mylar D polyester films, 175 μm thick (300 × 125 mm) (Desaga, Heidelberg), were pretreated by immersing in a 6N NaOH solution for 15 min at room temperature, with occasional shaking. After rinsing with tap water and distilled water the films were air-dried. The alkali pretreated films were silanised by immersion into a 0·2% ethanol–water solution (1:1, w/v) of Polyfix 1000. The films were dried for 20 min at 80 °C.

Cover films were prepared by treating 100 μm Mylar D polyester films (300 × 125 mm) (Desaga, Heidelberg) with 6N NaOH for 15 min, as already described, but without silanisation. After washing with water and air-drying, the hydrophilised films were stored.

Preparation of the gels

Five per cent T and 3 % C gels (Hjerten, 1962) containing 3 % (w/v) carrier ampholytes were prepared. In order to obtain 10 ml of polymerisation liquid, sufficient for two gels, the following solutions were mixed: 5·65 ml of twice distilled water, 1·7 ml of 30 % solution of acrylamide, 1·5 ml of a 1 % solution of *N,N'*-methylenebisacrylamide, 0·75 ml of the carrier ampholyte and 0·8 ml of a 1·5 % solution of ammonium persulphate. The polymerisation mixture was deaerated by shaking for 30 s in the vacuum from a water jet pump. For the preparation of 50 μm gels the 'flap technique' described by Radola (1980) was applied.

Isoelectric focusing

Ultrathin-layer focusing was carried out in a Desaphor flat-bed apparatus equipped with a 1200/200 power supply (Desaga, Heidelberg). A cooling solution of 4 °C was circulated through the apparatus from a LKB Multitemp.

At the ends of the gel 1 cm electrode strips of MN 866 cellulose paper (Macherey and Nagel, Düren) were placed as an extension of the gel layer. The strips were soaked in the following electrode solutions: at the cathode, 2M ethylenediamine containing 0·025 arginine and 0·025M lysine; at the anode, 0·025M aspartic and 0·025M glutamic acid. The electrodes were laid on the strips and covered with a 4 mm glass plate to prevent the layer from drying out.

The samples were applied by means of a 10 μl Eppendorf syringe using a silicone rubber strip (Desaga, Heidelberg). The application strips were slightly greased with silicone oil DC 200 (Radola, 1980).

The following focusing conditions were used. The 12 cm gels were prefocused for 30 min at 400 V and after the sample application the focusing was continued for 30 min at 400 V. Finally, the voltage was raised to 800 V for 30 min, and 1000 V for 60 min. The pH gradient was determined at 4 °C by direct measurement on the gel surface at 0·5–1 cm intervals using a flat membrane glass electrode (Desaga, Heidelberg) and a digital pH meter (Knick, Berlin).

Staining

Immediately after focusing the gel was transferred to a 20 % (w/v) solution of trichloroacetic acid in water. Shaking for 10 min was sufficient to wash out the carrier ampholytes. The gel was then rinsed for 30 s with a methanol–water–glacial acetic acid mixture (25:65:10, v/v) to remove

trichloroacetic acid. Staining was carried out with a freshly prepared 0·05% solution of Coomassie Blue R (Serva, Heidelberg) in the methanol–water–glacial acetic acid mixture (25:65:10, v/v). Destaining was achieved using the same solvent mixture.

Visualisation of peroxidase activity was carried out with the cellogel print technique. A cellogel strip (Serva, Heidelberg) was soaked in 0·3M McIlvaine buffer, pH 5·5, and dried. The strip was then immersed in a substrate solution containing 1% *o*-dianisidine hydrochloride and 1% urea peroxide in 100 ml distilled water. The excess of substrate solution was separated by a strip of filter paper and the cellogel strip was rolled onto the surface of the gel. Zones of peroxidase activity were detected as green–brown bands within 1–4 min.

Peroxidase assay

A rapid peroxidase filter paper test was performed according to Delincée & Radola (1972).

Peroxidase activity was assayed by measuring the slope from the initial increase in absorbance at 470 nm caused by oxidation of guaiacol to tetraguaiacol in the presence of H_2O_2 (Bergmeyer & Gawehn, 1974). The assay mixture (3·2 ml) contained 2 ml 0·1M McIlvaine buffer, pH 5·5, x ml enzyme solution, $3-(2+x)$ ml twice distilled water, 0·1 ml of 0·2M guaiacol and 0·1 ml of 3 mM H_2O_2. The measurements were initiated by the addition of x ml of column effluents. Reference blanks contained all reagents except enzyme. A Shimadzu spectrophotometer UV-110-02 was used. One unit of enzyme activity was defined as catalysing the oxidation of 1 μM guaiacol/min in the above system in 1 cm path length cuvettes at 22–24 °C, based on the absorption coefficient for tetraguaiacol of 26 600 mol^{-1} at 470 nm.

Thin-layer gel chromatography

Thin-layer gel chromatography was carried out with Sephadex 200 superfine (Pharmacia) according to Radola (1968*a*,*b*). Samples, each of 20 μl (protein content about 0·5%), were applied using the Desaga applicator.

Kinetic studies

K_m values were determined from Lineweaver–Burk plots measured at pH 5·3 with 0·5 mM H_2O_2 and 7 mM guaiacol, respectively.

Potassium cyanide inhibition was performed over the range of 10^{-7} to 10^{-3}M/litre. Activity assays were carried out after pre-incubation of cyanide for 5 min. The reference blank consisted of all reagents except KCN and H_2O_2. The K_i value was determined from the plot 1/v versus cyanide concentration (Dixon, 1953).

pH optimum

The pH optimum of tomato peroxidase was determined over the range pH 4·0–8·0 with the following buffer solutions: 0·1M McIlvaine buffer; 0·1M potassium monobasic and dibasic phosphates. A digital pH meter (Knick, Berlin) was used.

Protein determination

Protein determinations were carried out using the Lowry method (Lowry *et al.*, 1951) with bovine albumin (Serva, Heidelberg) as the standard.

RESULTS AND DISCUSSION

Partial purification of tomato peroxidase

Homogenisation of ripe tomato fruits with 0·4M McIlvaine buffer, pH 5·5, containing 0·2% Triton-X 100, and subsequent gel filtration on Ultrogel AcA 34 (LKB) of the ultrafiltered supernatant of the 2000 × *g* centrifugation provided a partially purified peroxidase preparation. Table 1 summarises the results of the purification steps.

Recently, Kokkinakis & Brooks (1979) described the occurrence of 'soluble' and 'bound' forms of peroxidase in tomato fruits, and these authors demonstrated that the 'soluble' form occurs particularly in unripe green fruits. In our experiments, ripe red fruits were used, and only 50% of total peroxidase activity was obtained applying McIlvaine buffer, pH 5·5, alone; after detergent (Triton-X 100) was added, 85–100% of the total enzymic activity could be solubilised. From the findings shown in Table 1, the ultrafiltration resulted in a low increase in peroxidase activity with a nearly 50% loss of proteins. The PA 20 membrane retains proteins (>20000 daltons); thus, some low molecular weight proteins and peptides must have filtered through. The gel filtration step (Fig. 1) led to a

TABLE 1
Partial Purification of Peroxidase from Tomato Fruits

Fraction	*Volume* (*ml*)	*Protein* (*mg/ml*)	*Total protein* (*mg*)	*Activity* (*units/ml*)	*Specific activity* (*units/mg*)	*Total activity* (*units*)	*Recovery* (%)
Crude extract	250	2·2	550	1·3	0·59	325	100
Ultrafiltration	5	10·3	257[a]	11·9	1·15	298[a]	92
Gel filtration (Fractions 47–55)[c]	63	0·07	44[b]	0·4	5·7	252[b]	76

[a] Values calculated from 25 ml total ultrafiltrate obtained (see 'Material and Methods' section).
[b] Values calculated in view of total volume of 250 ml crude extract (see 'Material and Methods' section).
[c] See Fig. 1.

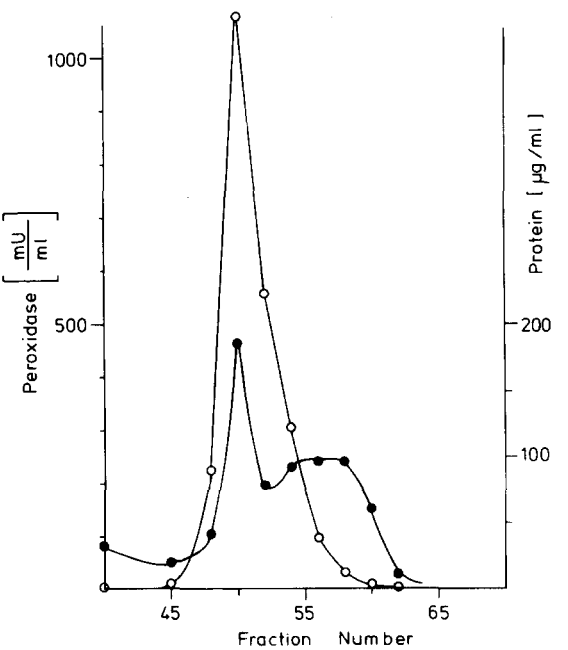

Fig. 1. Part of the elution profile of partially purified peroxidase from tomato fruits on Ultrogel AcA 34. The pooled fractions 47–55 were used for further investigations. Experimental data are described in the 'Material and Methods' section. ○—○ Peroxidase activity (mU/ml). ●—● Protein concentration (µg/ml).

tenfold purification of peroxidase. This partially purified enzyme showed an RZ-value (i.e. absorbance ratio, 403:280 nm) of 0·6, indicating that some impurities were present in the preparation.

Properties

Molecular weight
Thin-layer gel chromatography showed a molecular weight of 43 000 daltons. Molecular weights of peroxidases from different sources ranged from 30 000 to 60 000 daltons (Srivastava & van Huystee, 1977).

pH optimum
The pH optimum of tomato peroxidase was found to be 5·3. Jen *et al.* (1980) reported a similar value for the enzyme from green tomato fruits, whereas Signoret & Crouzet (1978) detected a pH optimum of 6·6. pH optima in the range 5·5 to 6·0 have also been found for various plant peroxidases, e.g. tobacco (Mäder *et al.*, 1977) and soybean (Sessa & Anderson, 1981). Reed (1966) pointed out that the pH optimum depends on the structure of the hydrogen donor used.

Stability
The well known high stability of plant peroxidases to heat inactivation (Reed, 1966) was confirmed for peroxidase from tomato fruits. After 20 minutes' heating at 70 °C, about 30 % of the enzyme activity was inactivated. Total inactivation resulted after thermal treatment for 12 min at 90 °C. Similarly to the pH optimum, the results of thermal inactivation vary according to the substrate used (Nebesky *et al.*, 1950), the ionic strength of the buffer (Wilder, 1962) and the molecular species (Srivastava & van Huystee, 1977; Nessel & Mäder, 1977).

Enzyme kinetics

Classical kinetic studies were performed on our isolated peroxidase fraction, examining the effect of varying the concentrations of guaiacol and H_2O_2 on the enzyme activity. Substrate concentration analyses via Lineweaver–Burk plots exhibited a K_m of 0·8 mM for H_2O_2 at optimum guaiacol concentration and a K_m of 9·0 mM for guaiacol at optimum H_2O_2 concentration.

When the influence of cyanide was studied, peroxidase was strongly

inhibited. The kinetics examined via a Dixon plot (Dixon, 1953) showed competitive inhibition. The inhibition constant, K_i, was determined to be 1·0 μM (pH 5·3). For horseradish peroxidase K_i values of 3·3 μM (pH 6) and 1·3 μM (pH 5) with *o*-dianisidine as the hydrogen donor were reported (Fridovich, 1963). For soybean peroxidase Sessa & Anderson (1981) found a K_i of 0·15 μM (pH 5·5), indicating that the enzyme from this source is more sensitive to cyanide inhibition than the horseradish or tomato peroxidase.

Ultrathin-layer isoelectric focusing

For ultrathin-layer isoelectric focusing the gel filtration step was repeated four times, and the collected peroxidase active fractions were concentrated by ultrafiltration. With ultrathin-layer isoelectric focusing in the pH range 2–11 we observed twenty-two protein bands after staining with Coomassie Blue (Fig. 2).

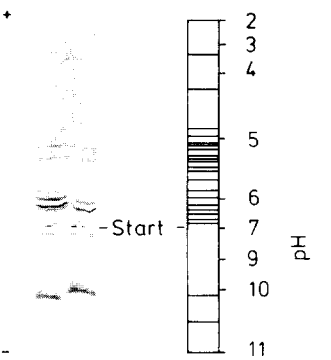

Fig. 2. Ultrathin-layer isoelectric focusing in the pH range 2–11 of partially purified peroxidase from tomato fruits. Staining, Coomassie Blue. Experimental data, see 'Material and Methods' section.

Staining the enzyme with the cellogel technique in the pH range 2–11, six peroxidase isozymes were detected. The highest enzymic activity was noted in the pH range 2–3 where the peroxidase bands appeared within 30 s after staining. The low activities detected at pH 4–5 and 9–10 were only visualised after staining for 4 min. Figure 3 shows the zymograms of peroxidase from tomato fruits compared to the results obtained with a commercial horseradish peroxidase (Boehringer, Mannheim).

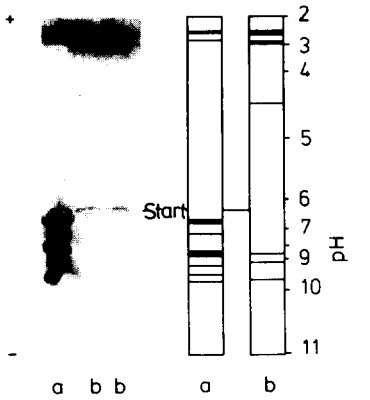

Fig. 3. Ultrathin-layer isoelectric focusing in the pH range 2–11 of horseradish peroxidase (a) and partially purified peroxidase from tomato fruits (b). Staining, peroxidase substrate. Experimental data, see 'Materials and Methods' section.

The zymograms indicate that the anodic bands were evident both for peroxidase from tomato fruits and horseradish. Nevertheless, for the horseradish enzyme the main activities were observed in the pH range 7–9 (Fig. 3).

As the anodic enzyme activities were separated at the limit of the ampholyte system used (pH 2–11), we tried to obtain better differentiation by applying an ampholyte range of pH 2–4. The results of ultrathin-layer isoelectric focusing in the pH range 2–4 are shown in Fig. 4.

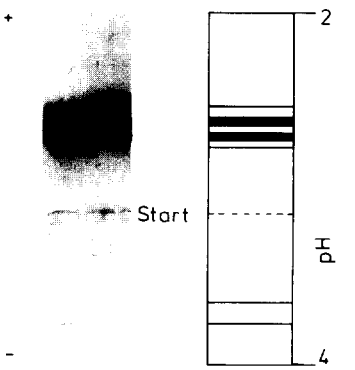

Fig. 4. Ultrathin-layer isoelectric focusing in the pH range 2–4 of partially purified peroxidase from tomato fruits. Staining, peroxidase substrate. Experimental data, see 'Material and Methods' section.

In addition to the two bands with the main enzymic activities depicted in Fig. 3, two further isozymes were noted. Thus, in all, eight isozymes of peroxidase from tomato fruits were detected. Apparently, the number of peroxidase isozymes depends on maturity. Ku *et al.* (1970) found six isozymes in green tomatoes, but eight peroxidases in ripe fruits. Eight isozymes were also detected by Drawert & Görg (1975) in their study of different tomato varieties.

Further work should involve preparative isoelectric focusing to isolate the different isozymes and to study them in detail.

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Compositional Differences in Some Limabean (*Phaseolus lunatus*) Varieties

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ABSTRACT

Eighteen varieties of limabean (Phaseolus lunatus) *were analysed for their proximate chemical composition, mineral constituents and amino acid composition. The crude protein levels were remarkably similar except for that of TPL 071-33 (25·9%), which was higher than for most of the other varieties. Ether extract was low in all varieties, with a percentage coefficient of variation of 46·2%. Potassium was the most abundant mineral element, ranging from 550 mg/100 g in TPL 4 to 2380 mg/100 g in TPL 14e. Phosphorus and magnesium contents were also appreciably high in all varieties while calcium was the poorest major mineral element. Methionine and cystine were the most limiting amino acids and showed some degree of intervarietal differences, their coefficients of variation (CV) being 19·0 and 12·2%, respectively. Other amino acids which showed varietal differences included serine (CV 18·2%), glutamic acid (CV 15·4%), proline (CV 14·4%), glycine (CV 14·5%) and alanine (CV 14·4%). Limabean TPL 2 was deficient in histidine whilst trytophan was adequate in all varieties but deficient in TPL 2, TPL 6, TPL 13 and TPL 187. Glutamic acid, followed by aspartic acid, levels were generally the highest in each of the limabean varieties. The implications of these findings are fully discussed.*

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TABLE 1
General Appearance of Limabean Varieties

Accession numbers	*Variety names*	*Seed colour*	*Seed coat texture*	*Seed shape*
Acc. 64924	TPL 1	Mottled grey	Smooth	Kidney-shaped
Acc. 64065	TPL 2	Coffee brown; brown-eyed	Glossy	Round oval
Acc. 64068	TPL 3	Mottled wine; brown-eyed	Smooth	Round-oval, cowpea-like
Acc. 64006	TPL 4	Mottled black brown; grey-eyed	Glossy	Round oval
Acc. 64053	TPL 5	Black	Glossy	Flat; kidney-shaped
Acc. 64006	TPL 6	Mottled dark wine with white spots	Smooth	Flat; kidney-shaped
Acc. 64028	TPL 7	Grey with irregular black and brown strips; grey-eyed	Glossy	Round-oval, cowpea-like
Acc. 64027	TPL 8	Mottled grey	Glossy	Flat; kidney-shaped
Acc. 64033	TPL 9	Black; brown-eyed	Smooth	Flat; kidney-shaped
Acc. 64032	TPL 10	White	Smooth	Flat; kidney-shaped, broader than cowpea
Acc. 64022	TPL 11	Brown with black strips and white dots	Glossy	Round-oval
Acc. 64020	TPL 13	White	Smooth	Flat; kidney-shaped, much bigger than cowpea
Acc. 64064	TPL 14e	Coffee brown, tending towards black	Glossy	Round-oval
Acc. 64026	TPL 17	Brown and black mottles on a grey background	Smooth	Flat; kidney-shaped
Acc. 64017	TPL 183	Grey with coffee-brown mottles, brown-eyed	Smooth	Round-oval
Acc. 64035	TPL 187	Mottled wine; brown-eyed	Glossy	Round-oval
Acc. 64095	TPL 304	Mahogany-brown with grey strips	Glossy	Very flat and broad
Acc. 65005	TPL 071-33	Grey with irregular purple, brown and white mottles, wine-eyed	Smooth	

INTRODUCTION

Limabean has been shown to compare favourably with soyabean in terms of its protein production potentials (Luse, 1979). It serves as a popular and useful condiment in soup preparation and sometimes as a major protein component of diets.

Considerable phenotypic differences exist among varieties in terms of seed size, shape and colour which may have implications for the nutrient composition of such varieties. Plant breeders have accepted improved nutrient composition as a valid breeding objective and are now releasing crop varieties more resistant to pests and microbial infection, as well as with improved nutrient composition. These varieties must be tested first chemically and then biologically for their overall nutritional characteristics in addition to the individual nutrient levels for which they were selected. The plant breeders also require a constant feedback on crops of improved nutrient composition for incorporation into their breeding programmes.

Extensive breeding studies with several legumes in Nigeria have resulted in the availability of a number of improved varieties which combine such desirable characters as early maturity, high-harvest index and good yield potentials. Very little is, however, known about the nutritional value of these varieties and there has been no screening of genetic stocks for protein content and quality. Presently, there are more than 100 accessions and 800 different breeding lines of cultivated limabean varieties available in Nigerian research institutes. Within these materials, it is possible that a limabean genotype with superior protein quality already exists. The studies reported here are part of a series of systematic investigations which have been designed to biochemically evaluate the different varieties of limabean cultivated in Nigeria for food.

MATERIALS AND METHODS

Eighteen varieties of limabeans were employed in this study. The shape, colour, texture and accession numbers of these varieties are given in Table 1. The seeds were obtained from the International Institute of Tropical Agriculture and the National Cereals Research Institute, Moor Plantation, both in Ibadan, Nigeria. They were ground in a laboratory mill to a particle size of 0·5 mm.

ANALYTICAL METHODS

Chemical composition

Proximate constituents were determined by the methods of the Association of Official Analytical Chemists (AOAC, 1975). Mineral constituents were determined by first wet-ashing the samples with a mixture of nitric acid, perchloric acid and sulphuric acid, followed by flaming in a Perkin-Elmer atomic absorption spectrophotometer 200, using different lamps. The phosphovanadomolybdate method was used for the estimation of phosphorus (AOAC, 1975).

Amino acids

The amino acids contents were determined by the method of Moore & Stein (1954) as modified by Bidmead & Ley (1958).

About 100 mg of each sample was hydrolysed in duplicate with 10 ml of 6N HCl in a sealed pyrex tube which was previously flushed with nitrogen before hydrolysing the samples in an air-draught oven at 110 °C for 24 h. Small quantities of 2-mercaptoethanol (0·5 ml per litre of 6N HCl) were usually added to the samples to improve the recovery of the amino acids, particularly methionine.

The amino acids were separated by means of the column chromatographic technique, using the automated Technicon Model TSM Sequential Analyser. Cystine was determined in duplicate as cysteic acid by the method of Moore (1963) while tryptophan was chemically determined by the method of Miller (1967).

RESULTS AND DISCUSSION

Proximate chemical composition

The chemical compositions of the limabean varieties are presented in Table 2. Results obtained show that the protein, crude fibre and NFE contents of the different varieties were similar. The narrow range of the relative measure of dispersion, as indicated by the coefficient of variation for these parameters, shows this fact. Ether extract showed greater variation, ranging between 0·51 in TPL 13 and 2·99 in TPL 11. This

TABLE 2
Proximate Composition of Limabean Varieties, Expressed as Percentage Dry Matter

Limabean varieties	*Accession numbers*	*Dry matter* (%)	*Crude protein*	*Ether extract*	*Crude fibre*	*Total ash*	*Nitrogen-free extract*
TPL 1	Acc. 64024	92·9	23·6	1·62	5·36	3·99	65·4
TPL 2	Acc. 64065	92·7	22·4	2·70	6·65	5·29	63·0
TPL 3	Acc. 64068	92·2	24·0	1·09	6·23	5·53	63·2
TPL 4	Acc. 64006	91·7	22·9	1·09	5·86	5·15	64·3
TPL 5	Acc. 64053	93·1	21·7	1·61	5·48	4·62	65·6
TPL 6	Acc. 64008	93·0	23·5	0·81	4·86	5·54	65·3
TPL 7	Acc. 64028	93·4	22·1	1·48	5·45	5·18	65·8
TPL 8	Acc. 64027	93·0	22·6	1·35	5·79	4·21	66·0
TPL 9	Acc. 64033	92·9	22·0	1·56	5·38	4·89	66·2
TPL 10	Acc. 64032	93·7	21·0	2·67	4·93	3·79	67·6
TPL 11	Acc. 64022	91·9	21·9	2·99	5·36	5·60	64·2
TPL 13	Acc. 64020	94·1	23·5	0·51	4·85	5·95	65·2
TPL 14e	Acc. 64064	88·3	20·6	2·85	5·68	4·61	66·3
TPL 17	Acc. 64026	86·0	21·0	1·36	5·45	5·16	67·0
TPL 183	Acc. 64017	91·0	22·0	1·23	5·84	5·16	65·8
TPL 187	Acc. 64095	91·0	22·2	1·26	5·76	5·66	65·1
TPL 304	Acc. 64096	88·8	22·7	1·13	6·08	6·37	63·7
TPL 071-33	Acc. 65005	83·3	25·9	1·16	5·56	4·34	63·1
Mean		91·3	22·4	1·58	5·58	5·06	65·2
Standard deviation		2·91	1·39	0·73	0·47	0·69	1·39
Coefficient of variation (%)		2·30	6·20	46·20	8·42	13·64	2·13

TABLE 3
Mineral Composition of Limabean Varieties (mg/100 g)

Limabean varieties	*Accession numbers*	*Phosphorus*	*Calcium*	*Magnesium*	*Potassium*	*Sodium*	*Manganese*	*Iron*	*Copper*	*Zinc*
TPL 1	Acc. 64024	462	65·0	160	1 000	24·0	1·4	5·0	1·2	3·7
TPL 2	Acc. 64065	375	63·0	145	1 550	24·0	1·5	5·5	1·4	3·1
TPL 3	Acc. 64068	380	58·0	108	1 750	24·0	1·6	5·5	1·3	3·9
TPL 4	Acc. 64006	343	85·0	155	550	21·0	1·8	5·5	1·5	1·7
TPL 5	Acc. 64053	339	65·0	218	2 130	20·0	2·1	6·0	1·3	5·2
TPL 6	Acc. 64008	480	77·0	170	1 850	24·0	1·9	5·0	1·2	3·0
TPL 7	Acc. 64028	410	73·0	218	2 050	26·0	2·2	7·0	1·4	6·5
TPL 8	Acc. 64027	316	77·0	180	1 450	23·0	2·0	4·5	1·1	2·4
TPL 9	Acc. 64033	480	70·0	180	2 050	20·0	2·5	6·5	1·4	4·0
TPL 10	Acc. 64032	400	79·0	190	2 050	26·0	1·8	7·5	1·4	4·4
TPL 11	Acc. 64022	318	69·0	160	1 700	24·0	1·8	7·5	1·2	4·1
TPL 13	Acc. 64020	395	81·0	190	2 100	27·0	2·0	8·0	1·2	5·4
TPL 14e	Acc. 64064	420	70·0	180	2 380	21·0	1·9	6·5	1·4	4·5
TPL 17	Acc. 64026	420	90·0	150	2 280	22·0	2·2	6·0	1·2	6·4
TPL 183	Acc. 64017	360	70·0	190	2 230	24·0	2·5	6·3	1·3	4·7
TPL 187	Acc. 64095	360	80·0	200	2 200	28·0	2·0	5·5	1·2	4·0
TPL 304	Acc. 64096	320	90·0	190	2 280	20·0	2·0	6·4	1·2	5·2
TPL 071-33	Acc. 64005	360	70·0	190	2 130	22·0	2·1	5·9	1·3	4·1
Mean		385	74·00	176	1 874	23·3	1·95	6·12	1·29	4·24
Standard deviation		52·4	9·04	27·0	482	2·43	0·3	0·95	0·11	1·25
Coefficient of variation (%)		13·6	12·2	15·3	25·7	10·42	15·4	15·5	8·53	29·5

variability (coefficient of variation, 46·2%), is not of great nutritional significance, since the levels are so low as not to contribute significantly to the fatty acid needs of the consuming population. Also, the slight variations noticed in protein levels may not be due to actual differences in true protein but could be due to variable levels of cyanogenetic glycosides and free HCN which have been shown to be present to varying extents in different varieties of limabean (Butler & Conn, 1964).

Mineral composition

The individual mineral composition of the limabean varieties, together with their mean values, standard deviations and percentage coefficients of variation are shown in Table 3. Of all the mineral constituents, potassium was the most abundant, ranging from 550 mg/100 g in TPL 4 to 2380 mg/100 g in TPL 14e. Phosphorus ranged between 316 and 480 mg/100 g and the values for this element, except in TPL 8, TPL 11 and TPL 304, are higher than in the locust bean seed, green gram, pigeon pea and kidney bean (Oyenuga, 1968). There was, however, a low calcium content in all the limabean varieties when compared with soyabean (Markley, 1950), peanut meal (Woodroof, 1969) and pigeon pea (Oyenuga, 1968). With the exception of TPL 8, TPL 1 and TPL 6, the levels of iron were higher than the levels encountered in many legumes, particularly those of cowpea and green gram (Oyenuga, 1968). It would appear from these results that limabeans would constitute a valuable source of the major elements and of Fe, Zn, Mn and Cu, particularly in diets for humans, in which no special provision is made for the supply of these vital nutrients.

Amino acids

The amino acids composition of the limabean varieties are shown in Table 4. All varieties used in this study had relatively high non-essential and essential amino acids, with the exception of cystine and methionine. The methionine contents of limabean TPL 183 and TPL 14e were slightly higher than those of all the other varieties. Histidine was particularly low in TPL 2 while tryptophan appeared to be deficient in TPL 1, TPL 4, TPL 5, TPL 6, TPL 11, TPL 183 and TPL 304 but adequate in all other varieties. Lysine levels varied between 5·60 in TPL 183 and 7·81 g·16 gN in TPL 14e, with a coefficient of variation of 6·78%.

TABLE 4

Total Amino Acid Composition of Limabean Varieties (g/16 g N)

Amino acids	*TPL 1*	*TPL 2*	*TPL 3*	*TPL 4*	*TPL 5*	*TPL 6*	*TPL 7*	*TPL 8*	*TPL 9*	*TPL 10*	*TPL 11*
Lysine	7·01	6·62	7·05	6·83	7·19	6·34	6·65	6·67	6·55	6·85	6·45
Histidine	3·59	2·21	3·18	3·34	3·51	3·84	3·57	3·08	3·22	3·67	3·36
Arginine	6·61	6·82	6·22	6·78	6·42	6·94	6·21	6·56	6·18	6·28	7·01
Aspartic acid	12·8	11·3	12·9	11·8	12·2	14·2	12·1	12·8	12·5	11·3	12·5
Threonine	5·61	3·84	4·03	4·45	3·44	4·33	4·30	4·06	4·16	4·64	3·93
Serine	5·08	5·38	6·24	5·50	4·37	6·52	5·14	4·88	5·28	4·08	4·94
Glutamic acid	18·7	16·0	16·9	21·4	19·3	16·0	20·0	22·8	18·6	18·4	19·9
Proline	4·85	3·69	4·03	4·95	5·40	5·44	4·74	3·66	4·55	4·25	4·81
Glycine	4·17	4·27	4·46	4·62	6·68	5·12	5·83	5·56	5·10	5·79	4·91
Alanine	6·37	7·53	6·38	4·88	5·43	4·97	5·96	5·03	5·89	5·31	4·45
Cystine	0·98	1·31	0·98	1·08	0·82	1·23	1·16	1·07	1·06	0·77	1·12
Valine	6·25	6·37	5·42	6·71	6·33	5·84	5·22	5·19	5·54	5·67	5·67
Methionine	1·32	1·05	1·38	1·58	1·06	1·48	1·09	1·18	1·50	1·41	1·34
Isoleucine	6·37	4·88	5·88	6·32	4·98	5·49	5·66	5·18	6·30	4·92	5·33
Leucine	9·04	7·34	8·24	8·44	8·55	8·44	8·72	8·24	8·72	8·27	7·04
Tyrosine	4·72	5·04	4·94	3·73	4·82	3·88	3·86	5·84	4·75	3·97	4·85
Phenylalanine	6·85	7·68	8·07	8·24	7·46	7·08	7·16	7·42	7·25	5·42	6·99
Tryptophan	1·44	1·24	1·40	1·44	1·35	1·20	1·38	1·45	1·50	1·45	1·50

TABLE 4—*contd.*

Amino acids	*TPL 13*	*TPL 14e*	*TPL 17*	*TPL 183*	*TPL 187*	*TPL 304*	*TPL 071-33*	*TAO reference standard*	*Mean*	*Standard deviation*	*Percentage coefficient of variation*
Lysine	6·86	7·81	6·93	5·60	6·42	6·94	7·24	4·2	6·78	0·46	6·78
Histidine	3·03	3·95	3·58	2·56	3·72	3·08	2·86	2·4	3·30	0·45	13·6
Arginine	8·28	6·11	6·70	6·29	6·73	6·44	5·99	2·0	6·59	0·52	7·89
Aspartic acid	11·6	11·9	12·2	11·5	11·6	11·8	12·8	—	12·2	0·74	6·06
Threonine	3·83	4·21	4·47	5·38	3·76	4·50	4·28	2·8	4·29	0·53	12·4
Serine	4·50	7·16	4·33	4·08	3·67	6·08	5·88	—	5·17	0·94	18·2
Glutamic acid	21·5	16·7	19·3	28·8	17·7	19·2	18·4	—	19·4	2·98	15·4
Proline	5·04	3·87	3·56	3·49	5·00	4·89	5·03	—	4·51	0·65	14·4
Glycine	4·24	6·42	5·16	4·74	6·03	4·74	5·32	—	5·18	0·75	14·5
Alanine	4·61	6·03	5·25	5·49	6·89	5·22	5·46	—	5·62	0·81	14·4
Cystine	1·08	1·04	1·02	1·12	1·12	1·13	1·08	2·0	1·07	0·13	12·2
Valine	5·75	5·95	5·41	6·02	4·41	6·21	5·88	4·2	5·77	0·54	9·36
Methionine	1·23	1·93	1·46	2·02	1·72	1·32	1·43	2·2	1·42	0·27	19·0
Isoleucine	4·89	6·11	5·83	6·10	5·73	4·94	5·86	4·2	5·60	0·54	9·64
Leucine	8·94	11·2	8·77	8·38	8·54	8·12	8·42	4·8	8·52	0·82	9·62
Tyrosine	4·95	5·33	4·50	4·41	3·74	4·28	4·42	—	4·56	0·58	12·7
Phenylalanine	6·85	9·49	8·11	8·62	6·45	8·03	7·94	2·8	7·51	0·90	12·0
Tryptophan	1·04	1·41	1·50	1·48	1·08	1·40	1·38	1·4	1·39	0·14	10·1

The observation of sulphur amino acid deficiency in legumes has been consistently reported (Elias *et al.*, 1963; Jaffe, 1949) and an explanation is still lacking. The data presented here, when compared with target values for the rat (Coates *et al.*, 1969) and the FAO (1970) reference amino acid pattern, show a relative deficiency in the sulphur-containing amino acids, cystine and methionine. Tryptophan also appeared as the second limiting amino acid.

Because the phenotypic expression of seed protein and amino acid contents of beans have been found to vary, depending on the genotype and several environmental factors (Rutger, 1970; Leleji, 1971), it was hoped that this study would uncover a variety or strain of limabean with higher cystine and methionine contents than has heretofore been reported. This expectation was not realised although the varieties of limabeans studied represent less than one-fifth of the different accessions of the improved varieties of limabean available in Nigeria.

It was, however, observed that there were overall dissimilarities and varietal differences in the amino acid composition of the different limabean varieties and the standard deviation values from a mean value exceeded 10% for most amino acids except lysine (CV, 6·78%), arginine (CV, 7·89%), aspartic acid (CV, 6·06%), valine (CV, 9·36%), isoleucine (CV, 9·64%) and leucine (CV, 9·62%). The variability in methionine is remarkable (CV, 19·0%) and this may be of potential importance for breeding studies which could be exploited in not only selecting agronomically acceptable varieties but also in breeding for improved protein quality. Kelly (1971) has indicated that real genetic differences in at least one form of methionine, as measured microbiologically, do exist and might be exploited. Results regarding variability in methionine should, however, be interpreted with caution because of the problem associated with the quantification of the sulphur amino acids after hydrolysis (Schram *et al.*, 1953).

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Lipid Changes During Germination of Fenugreek Seeds (*Trigonella foenum-graecum*)

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ABSTRACT

Fenugreek seeds were germinated in the dark for 96 h. Total lipid extracts were prepared and found to decrease on germination. Ultraviolet, visible and infra-red spectra were estimated for the lipids of ungerminated and germinated fenugreek seeds. Free fatty acids (FFA), total chlorophyll and carotenoid pigments increased greatly after germination. On the other hand, triglycerides, phospholipids and unsaponifiable matter decreased. Determination of individual phospholipids showed that phosphatidylcholine (PC) and phosphatidylethanolamine (PE) constitute about 67% of the total phospholipids of ungerminated seeds. After germination PC and PE decreased whilst phosphatidic acid (PA) and phosphoglyceric acid (PG)—degradation products of phospholipids by phospholipases—increased. The fatty acid composition showed that the total unsaturated fatty acids decreased whilst the total saturated fatty acids increased on germination. The fatty acids 18:2 and 18:3 were the most abundant acids in the lipids of the ungerminated seeds and fell after germination from 41·2% to 31·8% and 23·2% to 14·4%, respectively. The minor constituent fatty acids 20:0, 22:0 and 20:1 increased by 3·3-, 3·0- and 7·8-fold, respectively after germination.

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INTRODUCTION

Fenugreek (*Trigonella foenum-graecum* L.) is an erect annual herb of the Legumino family indigenous to western Asia and southeastern Europe. It has long been cultivated in the Mediterranean area, in India and in North Africa (Rosengarten, 1969).

The seeds of fenugreek contain about 7·8 % oil which is a golden-yellow colour. The oil has a disagreeable odour and a bitter taste.

Egyptian fenugreek oil was studied by Shahat (1947). He found that the ranges of constants obtained from the analysis of seven samples of fenugreek oil were 115–116 for the iodine value and 178–183 for the saponification value. He also studied the fatty acid composition using the distillation of esters. The fatty acids consist of 33·7 % linoleic, 35·1 % oleic and 13·8 % linolenic acids. Badami & Kalburgi (1969) analysed the component fenugreek fatty acids using reversed phase column chromatography. They also detected hexadecanoic acid. Baccou *et al.* (1978) studied the fatty acid composition of fenugreek oil from different countries using gas–liquid chromatography. They found that the percentages of linoleic and linolenic acids differ according to place and conditions of cultivation of the plant. They also found that the oil had marked drying properties.

The information concerning the changes in storage lipids, primarily triglycerides and phospholipids, as well as the changes in fatty acid composition after germination of fenugreek seeds, is meagre. Thus, the study described below was undertaken to investigate the changes in lipid classes during germination of fenugreek seeds.

MATERIALS AND METHODS

Materials

Seed type

The fenugreek seeds of the Geiza 2 variety were purchased from a local market at Alexandria.

Germination of seeds

The method used was that described by Bates *et al.* (1977), where the seeds were germinated in the dark at 30–35 °C for 96 h. At the end of the germination period, sprouts were dried in an air oven at 40 °C.

Preparation of samples
Ungerminated and germinated dried seeds were ground in a Wiley laboratory mill to pass through 40-mesh sieve. The ground samples were stored in jars at 5 °C until analysed.

Methods

Moisture content was determined by AOAC (1975) method. Free fatty acids (FFA) and unsaponifiable matter were determined according to the AOCS (1966) methods. Total lipid extract was prepared using the method described by El-Sebaiy *et al.* (1980).

Separation and quantification of lipid classes
Lipid classes were separated using thin layer chromatography (TLC) on silica gel G (0·5 mm) plates by the method of Kates (1972) using petroleum ether:diethyl ether:acetic acid (70:30:2, v/v/v) as the solvent system. The localisation and identification of lipid classes was accomplished by using a sulphuric acid–formaldehyde spray reagent (Rouser *et al.*, 1970) and standards. The separated, visualised and identified lipid classes were quantified by scanning at 700 nm using a dual wavelength chromatogram scanner (Shimadzu CS-190). In addition, the triglycerides were determined gravimetrically after being separated by using preparative thin layer (1 mm) plates coated with silica gel G. The bands of triglycerides were scraped and eluted using chloroform:methanol:ether (1:1:1, v/v/v). Total and individual phospholipid phosphorus was quantified using the method described by El-Sabaiy *et al.* (1980).

Fatty acid analysis
Aliquots containing 10–30 mg of the total lipid extract were esterified according to Hilditch & Williams (1964). The completeness of fatty acid esterification was monitored by TLC using 1% ether in benzene as solvent, the conversion being greater than 95%. The methyl esters of fatty acids were analysed using a Shimadzu GC 45 gas chromatograph. Operating conditions for the diethylene glycol–succinate column (15% of Chromosorb WAW 80–100 mesh, 4 m long and 4 mm inner diameter) were as described by Baccou *et al.* (1978).

Assay of carotenoid pigments and chlorophyll
The carotenoid pigments were determined by the following method. The

ground sample (40 mesh) was homogenised with 12% ethanolic potassium hydroxide solution (1:5, v/v). The homogenate was extracted with 50 ml petroleum ether, washed until alkali-free, filtered and evaporated to 10 ml final volume. The total carotenoid pigments were determined colorimetrically at 440 nm in the final extract. The thin layer chromatographic procedure of Vinkler & Kiszel-Richter (1972) was used for the fractionation of the individual carotenoids. The bands of carotenoids were scraped, eluted with methanol, evaporated by a stream of nitrogen gas and the fractions were redissolved in petroleum ether. The absorbance of each fraction was measured using a spectrocolorimeter at 451 nm (Spekol, Carl Zeiss, Jena). The percentage of each individual carotenoid was calculated according to the method of Goodwin (1965).

Total chlorophyll was determined by the colorimetric method described by the AOAC (1975).

Spectral analysis

Visible and ultraviolet (UV) spectra were estimated according to the method of Kates (1972) in absolute alcohol and chloroform, respectively using a Pye-Unicam SP 8000 spectrophotometer. The infra-red (IR) spectrum was measured by means of a Shimadzu IR-400 spectrophotometer using the potassium bromide pellet method (Kates, 1972).

RESULTS AND DISCUSSION

Total lipids

An 18% decrease in the dry weight was observed after 96 h of germination. This result was previously reported by Mayer & Paljakoff-Mayber (1963) for germinated seeds other than fenugreek. They reported that a net loss of dry weight occurs as a result of oxidation and breakdown of stored compounds.

As shown in Table 1, the extraction of ungerminated fenugreek by a chloroform–methanol mixture yielded 7·37% total lipids, expressed on a dry weight basis. This value is in the range mentioned by Shahat (1947), who reported 7% for Egyptian fenugreek, by Sauvarie & Baccou (1976) who found 8%, by Elmadfa & Kuhl (1976) who reported 8·5%, and by Baccou *et al.* (1978) who reported a range between 5·5 and 7·5% for different varieties of fenugreek seeds from different locations of the Mediterranean

TABLE 1
Effect of Germination on Total Lipid and Lipid Classes of Fenugreek Seeds

	Ungerminated	*Germinated*
Total lipid (% of dry matter)	7·37	4·80
Unsaponifiable matter (% of total lipid)	9·07	6·85
Free fatty acids (% of total lipid)	1·22	2·41
Lipid classes (% of total lipid)[a]		
Total phospholipids	6·05 (7·21)	4·21 (4·70)
Monoglycerides	0·13	6·99
Diglycerides	5·49	7·18
Sterols	1·25	14·3
Free fatty acids	1·59	4·27
Triglycerides	68·6 (66·4)	51·5 (56·7)
Hydrocarbons + sterol esters + pigments	17·0	11·6

[a] Values in parentheses are obtained by the colorimetric method for phospholipids and the gravimetric method for triglycerides.

area. It seems that the percentage of total lipids differs according to the location and the conditions of cultivation.

Germination of fenugreek seeds for 96 h decreased the total lipid content by 34·9 % with respect to ungerminated seeds. A more realistic picture can be obtained when the total lipid is recalculated taking into consideration the loss in dry weight after germination. The loss in lipid content was 46·3 %. The decrease in total lipid content after germination was in agreement with that found by other workers; Watt & Merril (1963) for germinated mung beans and soybeans, Zimmerman & Klosterman (1965) for germinated flaxseed and Fordham *et al.* (1975) for germinated peas and beans.

Carotenoid pigments and chlorophyll

Spectral analysis (Table 2) shows that during germination of fenugreek seeds new species of carotenoids are synthesised which possess absorption maxima at 410 and 430 nm. The original carotenoid has maximum absorption at 247 nm.

Chlorophyll pigments with maximum absorption at 610 nm originate after germination. The infra-red spectra of germinated seeds are identical and no change in the spectra can be detected as a result of germination.

TABLE 2
The Spectral Characteristics of Ungerminated and Germinated Fenugreek Lipids

	Ungerminated	*Germinated*	*Probable compounds*
UV (nm)	247	247	Carotenoids
Visible	—	410	Carotenoids
(nm)	—	430	Carotenoids
	452	—	Carotenoids
	—	610	Chlorophyll
	670	670	Chlorophyll

These observations are verified by the determination of carotenoid pigments and chlorophyll as shown in Table 3. The ungerminated seeds contain appreciable amounts of carotenoid pigments compared with other pulses, roots and green feeds. The yellowish colour of the seeds may in part be attributed to the presence of carotenoid pigments. Aitken & Hankin (1970) reported values of 1 μg/g for pigeon peas, 16 μg/g for field peas, 3·4 μg/g for maize seeds, 336 to 898 μg/g for carrots and 125 to 263 μg/g for potatoes. Perennial ryegrass had 370 μg/g and yellow lupin leaves, 358 to 758 μg/g.

Total carotenoids increased significantly on germination to reach six-fold compared with the ungerminated seeds. Cryptoxanthin, which constitutes the major carotenoid pigment of the ungerminated seeds,

TABLE 3
The Carotenoid Pigments and Total Chlorophyll of Ungerminated and Germinated Fenugreek Seeds

Pigment	*Ungerminated*		*Germinated*	
	mg/g	*% of total*	*mg/g*	*% of total*
β-Carotene	0·12	5·6	9·66	77·6
Cryptoxanthin	1·95	91·2	0·90	7·26
Lutein + zeoxanthin	0·07	3·16	1·88	15·1
Total carotenoids[a]	2·13	100	12·4	100
Total chlorophyll (μg/g)	9·89		132	

[a] Total carotenoid pigments were determined as β-carotene.

decreased on germination to reach less than half its original content. On the other hand, β-carotene, which is a nutritional component as the precursor of vitamin A, and lutein plus zeoxanthin increased, after 96 h of germination, about 80- and 27-fold compared with their original contents, respectively. No available data are found in the literature concerning the effect of germination on the carotenoid pigments of fenugreek seeds.

Germination of fenugreek seeds for 96 h increased the total chlorophyll content to reach 13-fold its initial value in the ungerminated seeds.

Lipid classes

Lipid classes were estimated after charring on TL chromatoplates using the scanning method. At the same time and for the sake of comparison, phospholipids and triglycerides were determined by other specific methods, as already mentioned. As shown in Table 1, the triglycerides constituted the main class of total lipids present in the ungerminated seeds. After germination only about 80 % of the original triglycerides remained. This result is in agreement with that mentioned by Zimmerman & Klosterman (1965) who found that, after 90 h of germination, only 47 % of the original amount of triglycerides remained in the germinated flaxseeds. On the other hand, germination increased mono- and diglycerides. Diglycerides increased by 30 % of the original amount whilst monoglycerides increased about 54-fold. It may be concluded that during germination triglycerides are hydrolysed into mono- , diglycerides and free fatty acids.

A similar decrease was observed in the total phospholipids after germination, which amounted to 30 % (or 35 %) of the original amount, depending on the method of determination. Hardman & Crombie (1958) found a similar decrease in the phospholipids of water melon seeds after germination in the dark, whilst no change occurred when germination was conducted in the presence of light. On the other hand, Zimmerman & Klosterman (1965) observed an increase in the amount of phospholipids in germinated flaxseed after 72 h.

Free fatty acid content increased greatly, comprising about 2·41 % of the total lipids in the seedling. This result coincided with that found by Zimmerman & Klosterman (1965) for flaxseed, who found that FFA increased greatly, comprising 10 % of the total lipids in the seedling after 90 hours' germination. The increase in free fatty acid levels in the

germinating tissues suggested that lipase activity was greater than that of the enzymes involved in the oxidative breakdown of the fatty acids and resulted in net accumulation of free fatty acids.

The unsaponifiable matter of the total lipid decreased from 9·1% to 6·9% after germination. The unsaponifiable matter in the total lipid of ungerminated fenugreek is much higher than that mentioned by Shankarcharya & Natarajan (1972) who reported 4%, and Baccou *et al.* (1978) who reported 3%. The high percentage of unsaponifiable matter may be attributed to the method which was used for the preparation of the total lipid extract.

Individual phospholipids

The percentage of individual phospholipids is given in Table 4. Phosphatidylcholine and phosphatidylethanolamine constitute the major phospholipids present, amounting to 67·6% of the total. Germination reduced the two phospholipids by 32·7% of the original content. The concentration of phosphatidic and phosphoglyceric acids present showed an increase. This increase may be attributed to the breakdown of phosphoglycerides by the action of phospholipases.

From these results it may be concluded that the phospholipids of germinated fenugreek seeds are transformed to other constituents which are required during the germination process.

TABLE 4
Phospholipids of Fenugreek Seeds as Affected by Germination

Phospholipid	*Phospholipid as per cent of lipid phosphorus*	
	Ungerminated	*Germinated*
Phosphatidyl serine (PS)	5·6	9·8
Lysophosphatidyl choline + phosphatidyl inositol (LPC + PI)	9·5	15·9
Sphingolipid (SL)	12·2	14·0
Phosphatidylcholine (PC)	44·1	30·1
Phosphatidylethanolamine (PE)	23·6	15·5
Phosphatidic acid (PA)	2·4	8·5
Phosphoglyceric acid (PG)	2·6	6·2
Lipid phosphorus (mg/100 g)	274	179

Fatty acid composition

Table 5 shows that the total saturated fatty acids comprise 19·1 % of the total ungerminated lipid. Palmitic acid was 10·8 %. Total unsaturated fatty acids amounted to 81·8 %. Linoleic acid is the highest (41·2 %) followed by linolenic (23·2 %) and then oleic (15·9 %) acids. This composition of fatty acids puts fenugreek oil in the drying oil category.

The percentage of linolenic acid differs significantly from the results previously given by Shahat (1947) of 13·8 % for Egyptian fenugreek oil as well as those of Badami & Kalburgi (1969) of 13 % and of Zafar *et al.* (1975) of 7 % for Indian grains. Our results compared well with the ranges of fatty acids mentioned by Baccou *et al.* (1978) for fenugreek oils from different varieties and origins. Hilditch & Williams (1964) found that temperature and atmosphere are the principal factors accounting for variations, especially in linolenic acid.

The fatty acid composition of triglycerides with regard to fatty acids 16:0, 18:0 and 18:1 varied significantly after the course of germination. It was observed that the saturated acid, 16:0, increased after germination while the unsaturated acid, 18:1, decreased. The 18:2 fatty acid fell from

TABLE 5
Effect of Germination on the Fatty Acid Composition of Fenugreek Lipid

Fatty acid	*Fatty acid %*	
	Ungerminated	*Germinated*
Saturated		
Myristic C14	0·2	0·0
Palmitic C16	10·8	14·5
Stearic C18	5·1	4·7
Arachidic C20	2·1	6·9
Behenic C22	0·9	2·7
Total saturated	19·1	32·8
Unsaturated		
Oleic C18:1	15·9	10·3
Linoleic C18:2	41·2	31·8
Linolenic C18:3	23·2	14·9
Gadoleic C20:1	0·8	6·2
Erucic C22:1	9·7	4·7
Total unsaturated	81·8	67·9

41·2 to 31·8% whilst the 18:3 fatty acid decreased from 23·2 to 14·9% in the germinated seeds. It therefore appears that the specificity of the fenugreek lipase was to produce somewhat larger amounts of 18:3 and 18:2 in the free form. This was due to the fact that 18:3 and 18:2 were the most abundant acids in the lipids of the ungerminated seeds and therefore showed a large change. These findings agreed with those mentioned by Zimmerman & Klosterman (1965) for germinating flaxseed. These authors found that the content of the major fatty acid, 18:3, fell from 44·7 to 37·8% during the first 18 h of germination and this was accompanied by a large increase in the 18:3 content of free fatty acids.

The minor constituent fatty acids, 20:0, 22:0 and 20:1, increased significantly by 3·3-, 3·0- and 7·8-fold, respectively. As stated by Zimmerman & Klosterman (1965), the increase in these minor acids was possibly due to the fact that the triglycerides were in a dynamic state and were continually being hydrolysed to glycerol and free fatty acids and resynthesised into new species of triglycerides.

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Research papers must be factual and original and must not have been published elsewhere. 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

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