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An Examination of the Volatile Compounds Present in Cooked Bramley's Seedling Apples and the Changes they Undergo on Processing[a]

H. E. Nursten and M. L. Woolfe[b]

Atkin-Thompson Laboratory, Procter Department of Food and Leather Science, The University, Leeds LS2 9JT, England

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An efficient, yet relatively inexpensive combined gas chromatography–mass spectrometry–odour port unit has been used to follow the changes in the volatiles of cooking apples during cooking. Since headspace samples contained insufficient volatiles for mass spectrometric identification, they were isolated by a Likens and Nickerson extractor, the pentane extract being concentrated before examination. The volatiles were collected during successive intervals from the same cooking sample. Alcohols were prominent throughout the exaggerated cooking time of 6.5 h and were accompanied by varying amounts of hexanal, phenylacetaldehyde, damascenone and farnesenes. In the early stages, several esters were present in amounts sufficient for identification, but no evidence of their later evolution and of the accompanying apple odours was obtained. 2-Furfural, benzaldehyde, 5-methyl-2-furfural and 2,4-decadienal were not detected initially, but contributed increasingly later, 2-furfural finally becoming the predominant volatile.

A taste panel established a highly significant difference between cooked apple prepared fresh from Bramley's stored at +5 °C for 5 months and blast-frozen precooked apple, stored for 5 months at −20 °C and made originally from the same batch of Bramley's. The former was preferred as being less acid and having more apple flavour. Instrumental comparison of extracts prepared by the above methods showed that precooked frozen apple stored for only 1 week contains relatively higher concentrations of esters and of *n*-hexanal than after 5-months' storage, when traces of the decadienal were found. The sensory and instrumental evaluations thus accord with one another.

1. Introduction

There are four reasons for the work described here. First, a sophisticated system of combined gas chromatography–mass spectrometry (g.c.–m.s.) had been set up and evaluated,[1] but since it had been designed for the investigation of food volatiles the next step is to test its effectiveness in this respect. Second, there is great interest in the flavour changes which occur during the processing of foods. Third, our colleagues are much concerned with precooked frozen foods,[2] particularly for hospital catering. Fourth, fruits contain relatively high concentrations of volatile constituents and therefore tend to be the most suitable subject for initial study.

[a] Presented in part at the 3rd International Congress of Food Science and Technology, Washington, D.C. on August 11th, 1970.

[b] Present address: Spillers Ltd, Research and Technology Centre, Station Road, Cambridge CB1 2JN.

These reasons led to the choice of cooked Bramley's Seedling apples for investigation. A large change of flavour becomes apparent on cooking and apple sauce is a widely used product. Whilst flavour studies on eating apples are numerous, a summary of the compounds identified having been compiled recently by Flath *et al.*[3], no investigation of Bramley's Seedling apples has been published.

Although headspace sampling is the ideal method, the concentration of volatiles is generally too small for mass spectral identification. The major problem in examining cooked apples is the large percentage of water vapour present. It is often advantageous in such cases to extract the volatiles into a non-polar solvent and the Likens and Nickerson system of concurrent distillation and extraction, developed for the investigation of hop oils[4] and since used very successfully, for instance, in the study of the flavour of cooked carrots[5, 6] and of green peppers,[7] seemed attractive. The Likens and Nickerson extractor[4] was found to be extremely efficient in concentrating high-boiling esters and sesquiterpene hydrocarbons from ng/g levels. Using pentane, the efficiencies of recovery for these compounds were all 78% or higher for a 1-h distillation, most compounds giving a recovery of over 90%.[4] It is not known how unfavourable the method of extraction can be, especially for very polar compounds.

The relationship of the extract to the headspace has not been investigated, but the great advantage of the extractor is that a relatively large quantity of foodstuff can be quickly processed with the minimum of solvent. Hence, artifacts from the solvent are kept to a minimum.

2. Experimental and results

2.1. Instrumentation

A Philips PV4000 Research gas chromatograph was coupled to an Edwards High Vacuum International E606 fast-scanning mass spectrometer as already described.[1]

2.2. Likens and Nickerson extractor

The apparatus used followed the original design,[4] but was modified in two ways according to the recommendation of Land.[5] First, a ground-glass joint, B19/26, was added at the top of the aqueous distillate side. Normally it was stoppered, but the stopper was removed at intervals to smell the aqueous distillate in order to follow the boiling process. Second, the diameter of the solvent distillate arm was reduced to increase the vapour velocity, thus preventing access of aqueous vapours. In addition the solvent and aqueous distillate arms were sealed into the central body of the apparatus at an angle. As the two vapour streams swirled around the central condenser, they created a cyclone effect, which aided mixing of the vapours before condensation. Both solvent and aqueous distillate arms were lagged with asbestos tape.

2.3. Concentration of the pentane extract

The advantage of the Likens and Nickerson extractor is that it can be placed on flasks of up to 10-l; thus a large quantity of foodstuff can be processed. In fact several large lots can be processed consecutively with one charge of pentane.

Before use, the pentane (B.D.H. Analar) was distilled at about 100 ml/h through a 61 cm × 2.5 cm glass column, filled with 1.6-mm stainless steel Dixon gauze rings, the first and last quarters of the distillate being discarded.

The charge of pentane employed in a specific distillation was such (25 to 45 ml) as could readily be concentrated with a microdistillation apparatus without any intermediate concentrating steps. The microdistillation apparatus consisted of a pear-shaped flask (25 ml) with two necks; the pentane extract was introduced intermittently through one neck and fractionation occurred on a 25 cm × 1.3 cm column, filled with 1.6-mm glass Fenske helices and fitted to the other neck. A still head on top of the column led the distillate to a cold finger and permitted the reflux ratio to be controlled. Normally, one third of the condensate was drawn off. The flask was heated by means of a water bath on a stirrer–hot plate.

An extract would typically be concentrated as follows: after codistillation with a foodstuff, the pentane extract is left in the refrigerator with anhydrous sodium sulphate (B.D.H. Analar, 1 g) for only one hour. The solution is then filtered off and fed directly into the 25-ml pear-shaped flask, if the volume is small, or periodically from a 5-ml separating funnel, if it is larger. The volume of the extract is reduced to about 3 ml. During the distillation the level of the water in the heating bath was kept just below that of the pentane extract. The temperature of the water-bath is usually 45 °C at the beginning of distillation and is raised to just under 50 °C by the end. The 3 ml of pentane concentrate are transferred with a little washing of distilled solvent into a specially constructed 5-ml pear-shaped flask in which the extract is further concentrated until the final volume is 0.1 to 0.2 ml. The concentrate is transferred by means of a 100-μl syringe to a specially prepared Pyrex glass vial, which is then sealed and stored in the refrigerator for analysis.

2.4. Blank run of distillation and concentration

1.3 l of boiled distilled water were poured into a 2-l flask. The Likens and Nickerson head was placed on top and 30 ml of purified pentane were placed in a flask under the solvent arm. The water (Simmerstat setting 4 to 5) and pentane (setting 2) were boiled for 1.5 h.

The pentane extract was concentrated to 0.1 ml as described above. A 1.0-μl sample was injected on a 19.1 m × 0.5 mm porous layer open tubular (P.L.O.T.) column coated with 1% Carbowax 20M on Celite 545, 120 to 150 mesh, containing 25% Pyrex glass, which was programmed from 70 to 190 °C at 3 deg. C/min. All the effluent was led into the F.I.D. No significant peaks at ×100 attenuation were observed other than the solvent peak.

2.5. Analysis of cooked Bramley's

2.5.1. Preparation of extract

Peeled, cored and diced Bramley's (1.9 kg) were held in salt water to prevent enzymic browning until heated in the Likens and Nickerson extractor with 2 l of boiled-out distilled water, 45 ml of pentane being used. The apple had to be heated slowly, since foaming was a major problem. When all the apple had broken down, after about an hour, the foaming subsided. Extraction was continued for a further 2 h.

Previous essences had precipitated a white solid after concentration and cooling in ice. The white solid readily dissolved again on warming. Long-chain fatty acids, which would be steam-distilled and extracted by the pentane, were suspected, and a potassium disc prepared from about 2 mg of the solid confirmed this by giving strong bands at 2920, 2850 and 1708 cm^{-1}. A weak band at 3005 cm^{-1} suggested the presence of unsaturation. The 1200 cm^{-1} region made a contribution from palmitic acid likely.

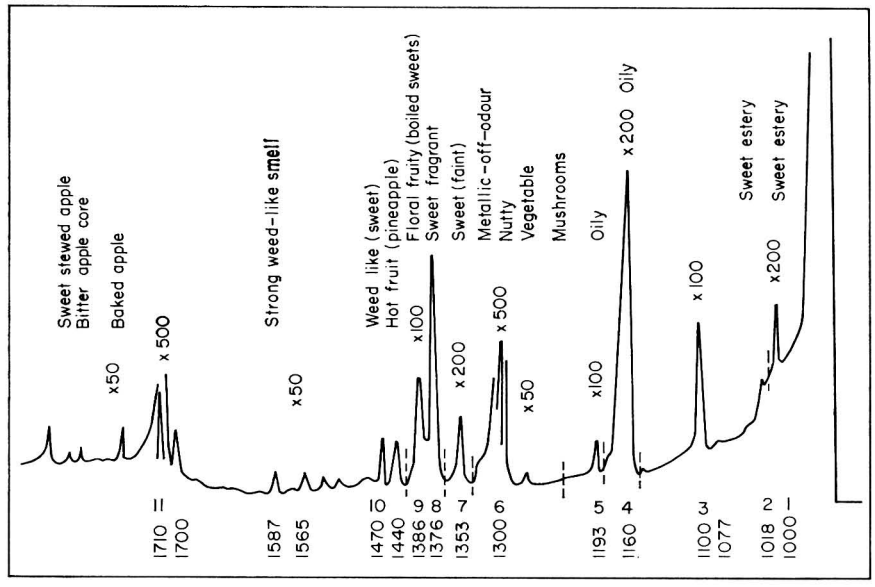

Figure 1. Analysis of cooked Bramley's extract on Carbowax 1540 P.L.O.T. column. The attenuations, retention indices and odour assessments are indicated.

2.5.2. Analysis

1.0-μl samples of the extract were injected on the gas chromatograph on each of two P.L.O.T. columns. One column was coated with Carbowax 1540; Figure 1 shows typical results. The other column was coated with Silicone rubber SE-30; Figure 2 shows results obtained for this column.

The conditions were as follows.

2.5.2.1. Gas chromatograph

Column, 18.5 m × 0.5 mm, coated with 1% Carbowax 1540 on 150- to 200-mesh Celite 545 containing 35% Pyrex glass. Carrier gas: helium at 2.8 ml/min. Initial temperature = 50 °C. Programme rate = 3 deg. C/min. Final temperature = 150 °C.

Column, 26 m × 0.5 mm, coated with 2% SE-30 on 120- to 150-mesh Celite 545 containing 30% B-37 Pyrex glass. Carrier gas: helium at 2.8 ml/min for mass spectrometry, at 1.7 ml/min for odour assessment. Both columns had injection temperature = 200 °C, detector temperature = 230 °C. Hydrogen pressure = 8 lb/in^2 [a]. Air pressure = 10 lb/in^2.

[a] Throughout this paper 1 $lb/in^2 \simeq 6890\ N/m^2$.

2.5.2.2. Mass spectrometer

Operating pressure: ion source pressure = 4.7 to 2.0×10^{-6} Torr., analyser head pressure = 2.7 to 1.8×10^{-7} Torr. Connecting line temperature = 175 °C. Ion source housing temperature = 160 °C. Scan rate = 2.8 max/5. Span = max. Multiplier voltage = 3.5 kV I_{beam} = 50 μA. I_{fil} = 2.4 A. I_{box} = 0.01 μA. An *m/e* 27 trace[1] was obtained for runs on both columns and 3-s scans at a u.v. recorder chart speed of 12.7 cm/s (5 in/s).

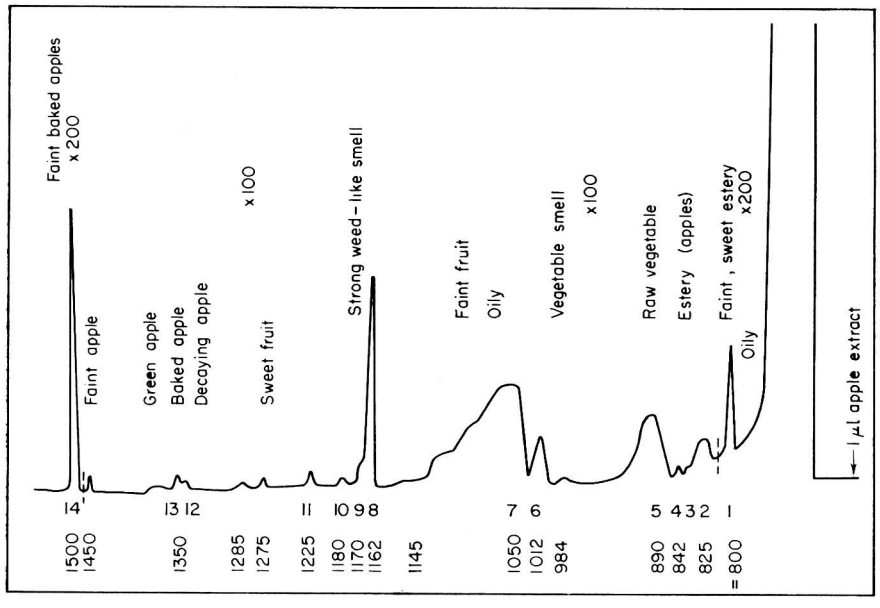

Figure. 2. Analysis of cooked Bramley's extract on SE-30 P.L.O.T. column. The attenuations, retention indices and odour assessments are indicated.

2.6. Extracts of cooked Bramley's obtained at different intervals of a cooking period

It was found that altering the cooking time changes the proportions of certain compounds in the extract. Hence, by heating apples for different periods, one should be able to determine which compounds are present in fresh diced apple and which are produced by heat processing. As already discussed, the most direct way of examining the volatiles at any instant is to take headspace samples.

2.6.1. Headspace samples

Peeled, cored Bramley's (0.55 kg) were diced into a 2-l flask. The flask was fitted with a glass tap and a rubber septum (Subaseal, W. Freeman and Co. Ltd, Barnsley). 50 ml of boiled-out distilled water were added and the flask placed in a hot water bath at 80 °C for 15 min. A 10-ml vapour sample was taken and injected on the 19.1 m × 0.5 mm P.L.O.T. column coated with 1% Carbowax 20M, using interrupted carrier gas flow and temporary cooling of the adapter holding the column to the injector. The apples

were boiled for 15 min with the glass tap open to prevent build up of pressure. The flask was then placed back in the 80 °C water bath with the tap closed and after 5 min another 10 ml vapour sample removed. Four further samples were taken in a similar manner, each after a further 15-min interval of boiling with the tap open. Figure 3 shows chromatograms (a), (b), (c) and (d) for boiling times of zero, 0.5 h, 1 h and 1.25 h, respectively. After the experiment, the flask was cleaned, boiled-out distilled water (500 ml) added and a 10-ml sample of vapour removed at 80 °C. It showed no significant peaks compared with the apple headspace.

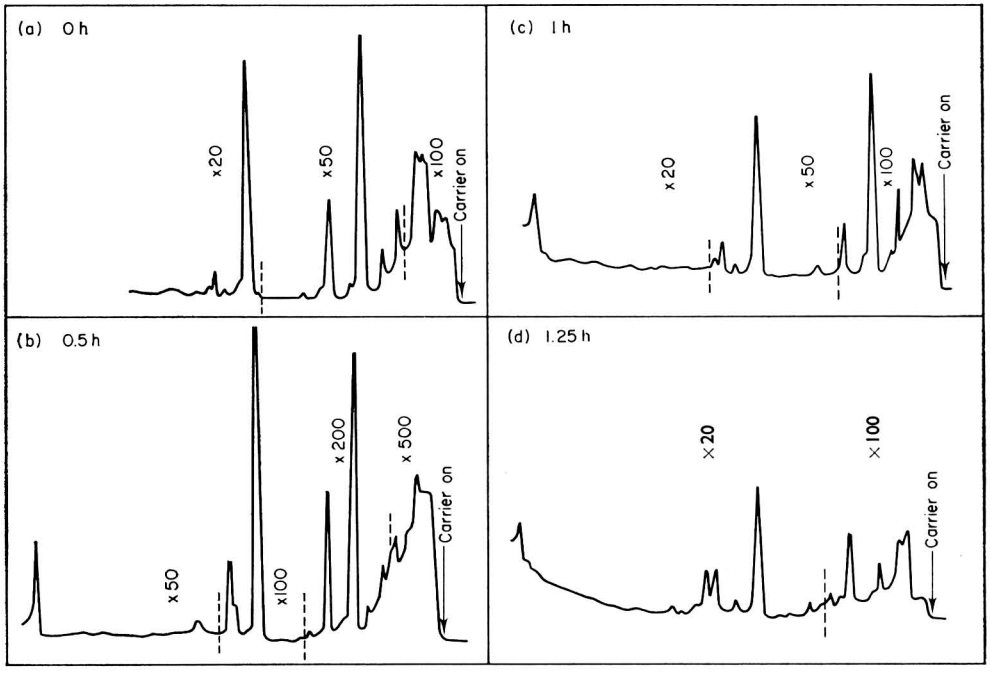

Figure 3. Vapour samples of cooked Bramley's on Carbowax 20M P.L.O.T. column: (a) at the start; (b) after 0.5 h boil; (c) after 1 h boil; (d) after 1.25 h boil.

The headspace does show differences in the gas chromatographic peaks both qualitatively and quantitatively as the boiling time progresses. There is also a general similarity between the headspace and the pentane extracts. Unfortunately, the headspace concentrations are too small for mass spectral identification. The 100-fold increase in vapour sample required to give a sufficient concentration would be difficult, as shown previously.[8]

The length of boiling of the Bramley's is far in excess of that used in domestic preparation, which takes about 10 min. The exaggeration was deliberate for three reasons. The first, a minor one, is that heat transfer inside a round-bottom flask is not as good as in a cooking pan. The second is that, since the nose is sensitive to very subtle changes, a longer cooking time was intended to exaggerate the changes which occur in

the domestic process. The third reason is that a longer time allows more volatiles to be collected, making instrumental identification feasible. Care was taken in long heating times not to burn the apples, which might give rise to other reactions such as caramelisation of sugars.

As confirmation of the identity of peaks in the vapour samples was needed, it was decided to prepare extracts. In order to emphasise the changes which are occurring and to allow better differentiation between compounds produced by the heating process and those of the fresh apple, fresh pentane was used for each successive boiling interval.

2.6.2. Pentane extracts of cooked apple

Peeled and cored Bramley's (1.62 kg) were diced into a 10-l flask and boiled-out distilled water (2 l) was added. The Likens and Nickerson extractor was placed on the flask and extraction with pentane started.

The apples were boiled for a total period of 6.5 h, during which the pentane was changed five times. Whenever the pentane was being replaced the Likens and Nickerson extractor was tilted to remove the pentane layer as completely as possible. The sequence of events and the aroma of each pentane extract is given below.

Flask no.	Period of boiling	Aroma of extract
1	To apple boiling	Fresh green apple (faint)
2	0 h to 0.5 h	Fresh and green apple with slight cooked apple aroma
3	0.5 h to 1.5 h	Freshness almost gone—cooked flavour a little better
4	1.5 h to 4 h	Sweet, malty, slight caramel
5	4 h to 6.5 h	Overcooked apple, very sweet and malty

The extracts were concentrated as described before. Each concentrate was stored under refrigeration in specially made Pyrex vials whilst awaiting analysis. The five extracts were analysed by combined g.c.–m.s. Conditions are given below. The extract from flask 1 was found not to contain enough sample for analysis. This would be expected since, prior to boiling, the quantity of volatiles available for extraction by pentane vapour would be very small. Figure 4 shows a comparison of the chromatograms obtained from flasks 2 to 5, respectively. Mass spectra, taken of those peaks which are numbered in Figure 4(a), are detailed in Table 1.

2.6.2.1. Gas chromatography

The 19.1 m × 0.5 mm P.L.O.T. column coated with 1% Carbowax 20M. Carrier gas: helium (a) 2 ml/min for odour assessment (b) 3 ml/min for mass spectral analysis. Initial column temperature = 70 °C; programme rate = 3 deg. C/min; final temperature = 190 °C; detector temperature = 230 °C; injection temperature = 210 °C.

2.6.2.2. Mass spectrometry

Conditions were as above. Multiplier voltage was used at 3.5 and 4 kV, depending on size of peak and background. A trace of m/e 27 was also taken.

2.7. Investigation of the changes in flavour of precooked frozen apple on storage

On the basis of the previous analyses, it was hoped that a sufficient number of the compounds identified would be significant in the aroma of cooked apple to enable changes in flavour with storage or freezing times to be followed.

The apples (3.75 kg) were cored, peeled and boiled with water (750 ml) inside a steam-jacketed boiler for 3 min. After cooking, the apple pulp was portioned (750 g) into standard trays (240 mm × 240 mm × 40 mm 14-gauge aluminium with loose-fitting lid). Half the trays were treated in a blast freezer at −25 °C for 1.5 h. The remaining trays were cooled relatively slowly to 0 °C overnight, then placed in a deep-freeze at −20 °C. When both samples were at −20 °C (after half a day), they were sealed in polyethylene (120-gauge) and returned to the deep-freeze.

After 1 week, one pack of deep-frozen precooked apple (i.e. that initially cooled to 0 °C) was boiled for 1.5 h in a flask fitted with the Likens and Nickerson extractor. The pentane extract was concentrated, stored as described previously and examined after 5 months (see section 2.7.3).

2.7.1. Taste panel comparison of precooked apple frozen in different ways

After 3.5 months' storage, the flavour of deep-frozen apple was compared with that of blast-frozen apple. One pack of each was reheated in a forced hot-air oven (Elektro-helios Ltd, London) at 180 °C for 25 min, i.e. under hospital reheating conditions.

Samples of the two packs of apples were given to a panel of 10 people and the declared control differences test[9] was used. The taster was presented with three samples, labelled "standard", B and C, respectively. The taster was asked to taste the "standard" first, then B and C last. B and C were scored independently on a five-point scale, the degree of difference from the "standard" being assessed. The scale ranged from a score of 5 (same as "standard") to 0 (extremely different from "standard"). One of the samples, B or C, was always the same as the "standard" and so acted as the hidden control.

A correlated t-test[9] was used to assess the significance of the difference between the two packs of apples. In this taste panel two combinations of the possible four of presenting the "standard", B and C were given to each of the 10 panel members. The score of the hidden control was subtracted from the score of the test sample.

A frequency distribution chart was constructed and the results are shown below.

Difference between the hidden control and the test sample (d):	+2	+1	0	−1	−2	−3
Frequency of occurrence:	0	9	6	4	1	0

Total number of differences (n): 20

Mean difference score ($\bar{d}$): $d/n = +0.15$

$$\therefore \quad t = \frac{\bar{d}}{\sqrt{\dfrac{\sum d^2 - (\sum d)^2/n}{n(n-1)}}} = \frac{0.15}{\sqrt{16.55/380}} = 0.8$$

Degrees of freedom ($n - 1$): 19

From the Table[10] for a panel of 20 people, 19 degrees of freedom and a level of significance of 5%, t must be greater than 2.09.

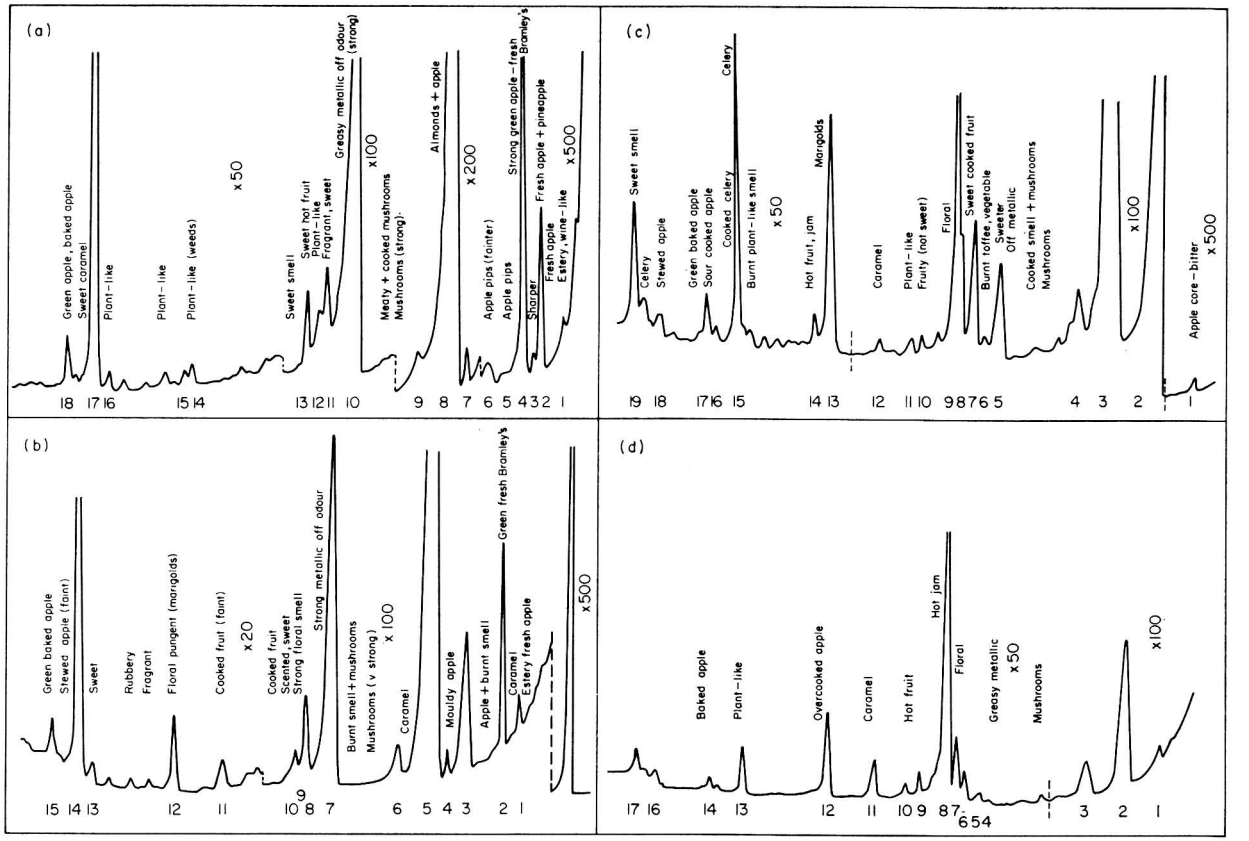

Figure 4. Analysis of successive extracts of cooking Bramley's on Carbowax 20M P.L.O.T. column: (a) after first 0.5 h boil (flask 2); (b) after next 1 h boil (flask 3); (c) after next 2.5 h boil (flask 4); (d) after final 2.5 h boil (flask 5). The important peaks are numbered for identification through Table 1, the numbers for a specific peak not necessarily corresponding on successive diagrams. The attenuations and odour assessments are also indicated.

TABLE I

Gas chomatographic data, mass spectra and identities of components of pentane extracts of cooked Bramley's Seedling apples

Figure 4(a) or reference compound				Figure 4(b)		Figure (4c)		Figure 4(d)		Figure 1 or ref. cpd.		Figure 2 or ref. cpd.		With boric acid precolumn peak no.	Comments
Peak no. or name	R.I.	Mass spectrum: *m/e* values in order of decreasing intensity	m.w.	Peak no.	R.I.	Peak No.	R.I.	Peak no.	R.I.	Peak no.	R.I.	Peak no.	R.I.		
1	915	57 29 43 42 41 45 31 30 39 75 74 102 73 72	102												There is pentane in the spectrum
Ethyl propionate	920	57 29 45 31 30 43 75 74 102 73	102												
2	995	43 41 71 29 88 42 45 60 73 39 61 89 70 101	116	1	995					1	1000	1	800	1	
Ethyl butyrate	998	43 29 71 41 39 88 45 60 42	116								1022		775		
3	1002	43 29 41 42 57 71 39 45 88 102 60 85 73 74 61 87 56 55 115 116	130							2	1018	3	838		Underlined *m/e* values occur in preceding peak 2
Ethyl 2-methylbutyrate	1007	29 41 57 39 102 45 74 85 56 55 87 73 115	130												
4	1030	44 43 56 41 39 29 45 42 57 55 72 58 82 71 67		2	1028										
n-Hexanal	1030	44 43 41 56 39 29 57 45 42 72 55 58 82 67 71	100												
5	1075	43 41 71 39 29 42 89 60												2	Weak spectrum. Isopropyl butyrate and isobutyrate have different spectra and R.I.s
n-Propyl butyrate	1080	43 41 71 42 39 89 60 31 61 73 101	130												
6	1102	31 41 56 43 42 29 39 55 57 33 72		3	1100	2	1100	2	1100	3	1100	2	825		
1-Butanol	1100	31 56 41 43 42 29 33 39	74										822		
7	1142	41 55 83 69 97 98 51 54		4	1140										Spectrum not unlike that of hexenal
8	1170	41 57 56 29 31 43 39 55 70 42 (69 83 98)		5	1168	3	1165	3	1167	4	1160	5	890		Values in brackets are not present in corresponding spectra of other essences.
2-Methyl-1-butanol	1165	41 57 56 29 31 70 55 39 42 43	88												
trans-2-Hexenal	1172	41 29 39 42 55 57 69 83 43 53 70 98	98											3	The spectrum of 3-methyl-1-butanol does not fit
9	1220	42 41 29 31 55 70 57 43		6	1220					5	1193				
1-Pentanol	1215	42 41 31 29 55 43 70 57	88								1198				
10	1315	43 56 41 42 31 55 29 69 57 84		7	1315	5	1314			6	1300	7	1050		
1-Hexanol	1320	56 43 42 41 31 55 29 69 39 84	102								1300		1050		
11	1356	57 41 29 39 67 82 31 43 71 44 55 69		8	1356	7	1357			7	1353	6	1012		
cis-2-Hexenol		57 41 67 82 29 43 39 55 56 31 ref. 12	100												
trans-2-Hexenol		57 29 41 43 82 67 42 39 56 55 ref. 12	100												
12	1365	43 56 41 42 29 71 55 69 57 89 84 39										10	1180		
n-Hexyl butyrate	1360	43 56 41 71 42 29 89 84 55 39 69 57	172												
13	1385	43 41 29 57 56 42 103 55 85 84 69 74										11	1225		
n-Hexyl 2-methylbutyrate	1390	43 29 41 57 56 42 103 85 55 84 69 74	186												
14	1560	91 39 65 120 51 92 29 63	120	12	1560	13	1560	12	1562						
Phenylacetaldehyde	1555	91 39 65 120 51 29 63 50 41	120												
15	1570	43 56 29 41 55 84 69 117 61 99 71 39 57										12	1350	6	
n-Hexyl *n*-hexanoate	1572	43 56 29 41 42 84 55 117 61 99 39 69 71 60	200												

16	1672	41 93 69 67 55 119 107 91 56 161 204	204	13	1674										Weak spectrum
cis,trans-α-Farnesene		almost identical with *trans,trans*, but 119 stronger ref. 13													
17	1695	41 55 93 69 43 29 79 39 107 123 119 81 (135 161 189 204)	204	14	1697	15	1700	13	1700	11	1710	14	1500	7	Values in brackets are minor peaks. Modified by boric acid
trans,trans-α-Farnesene		41 93 69 55 107 79 91 119 43 81 77 105 (123 135 161 189 204) ref. 13	204												
18	1732[b]	69 41 121 105 91 77 79 190 175	190	15	1732[b]	17	1730[b]	14	1730[b]			13	1357		
Damascenone	1747	69 41 121 39 27 105 190 91 29 120 77 79 51 175 53 122	190												
Damascenone		69 121 41 105 190 91 39 120 77 175 79 122 70 ref. 13	190												
Unknown		131 41 55 56 39 42 75 67 69 101 103 132				6	1330	5[a]	1332						
Unknown		43 69 41 55 29 45 72 101 59 57 56 39 111 (157 170 128)		9[a]	1375	8	1375	7	1375	8	1376	8	1162	4[a]	Not affected by boric acid. Values in brackets are minor peaks
		39 96 95 29 38 67 37		10	1375	9	1380			9	1386	4	842	4[a]	
2-Furfuraldehyde	1385	39 96 95 29 38 37 67	96								1381		847		
Unknown		45 59 101 57 43 29 41 67 81 69 83				10	1430	9	1433	10	1470	9[a]	1170		Possibly an ether
		77 51 39 106 105 50	106			11	1455	10	1455						
Benzaldehyde	1460	77 51 50 106 105 52 39 78 38	106												
		53 110 109 39 43 29 51 38 37	110			12	1500	11	1502						
5-Methyl-2-furfuraldehyde	1500	53 110 109 39 29 43 51 50 38 81	110												
Unknown		39 41 133 148 69 50 105 77 79 91				14	1590								*m/e* 105, 91, 77 suggest aromatic hydrocarbon
		81 41 42 29 83 67 54 53 95 55 123 (152)	152			16	1715[b]								Value in brackets very weak
trans,cis-2,4-Decadienal		81 41 83 67 55 95 82 68 54 79 53 (152) ref. 7	152												
trans,trans-2,4-Decadienal		81 41 67 55 83 82 95 54 42 53 68 (123 152) ref. 6, 7	152												Values in brackets not in intensity sequence
Unknown		43 41 55 117 71 88 89 61				18	1800[b]	16	1800[b]						Multiple peak on g.c., yet simple spectrum
Unknown		43 41 71 55 39 117 42 45 89 70 94 95 81 60 (168 141 123)				19	1841[b]	17	1842[b]						Values in brackets are minor peaks
Unknown		43 55 98 29 42 39 67 71 73						6	1367						

[a] = present in.
[b] = extrapolated.

Therefore, with the panel used, there was no significant difference between the two packs of precooked frozen apple.

2.7.2. Taste panel comparison of blast-frozen cooked apple with freshly cooked apple

After 5-months' storage, a comparison of blast-frozen cooked apple with freshly cooked apple from the same original batch of Bramley's stored for the same length of time at +5 °C was made. Ideally, the comparison should have been between precooked frozen apple stored for 5 months and unstored apples freshly cooked, but this is impossible if the apples are to be of the same batch. Care was taken in the preparation of the cooked apple to achieve the same consistency and texture as in the reheated frozen apple.

Samples of the two forms of cooked apple were given to a panel of six, this time all the four possible combinations of the two forms as "standard", B and C were presented. The result was assessed as before.

Difference between control and test sample (d):	−4	−3	−2	−1	0	+1	+2
Frequency of occurrence:	1	1	14	5	2	1	0

Total number of differences (n): 24

Mean difference score ($\bar{d}$): −1.625

$$\therefore \quad t = \frac{\bar{d}}{\sqrt{\dfrac{\sum d^2 - (\sum d)^2/n}{n(n-1)}}} = \frac{-1.625}{\sqrt{13.63/552}} = \underline{10.41}$$

Degrees of freedom ($n - 1$): 23

From the Table[10] for a panel of 6 people, 23 degrees of freedom and significance at a 0.1 % level, t must be greater than 3.80.

The above result therefore implies a highly significant difference between cooked apple prepared from fresh Bramley's stored at +5 °C for 5 months and blast-frozen precooked apple stored for 5 months at −20 °C.

2.7.3. Analysis of precooked frozen apple

Since a significant difference between the two forms of cooked apple had been detected by the tasters, it became of interest to see whether this difference could be detected by the analytical techniques developed earlier.

The remaining pack of deep-frozen precooked apple stored for 5 months was boiled for 1.5 h in a flask fitted with the Likens and Nickerson extractor. The pentane extract was concentrated as before and injected on the 19.1 m × 0.5 mm P.L.O.T. column coated with 1 % Carbowax 20M. An extract previously prepared from deep-frozen precooked apple stored for one week only, which had been kept in its glass vial for 5 months, was also injected on the same column under virtually identical conditions. Figure 5 shows the result of the analyses and odour descriptions. The numbered peaks were scanned and their position marked on the m/e 27 trace. By comparison with the mass spectra given in the interpretation of results, some of the peaks could be identified as in Tables 2 and 3.

3. Interpretation of results

The procedure for identification of gas chromatographic peaks was usually as follows. The mass spectrum is checked either from its molecular ion or its major fragmentation ions against spectra given in Cornu and Massot.[11] One of three situations commonly arises.

First, a published spectrum may agree exactly with the unknown. This occurs rarely, for the reasons discussed elsewhere.[8] The processes of fragmentation are not as fundamental as, say, those giving rise to infrared, ultraviolet or n.m.r. spectra; they vary with the geometry, temperature or type of ion source, as well as the ion source energy. By and large compounds do give the same fragments, but the relative abundance of the fragments varies from one spectrometer to another, even with the same model of spectrometer. More disturbing still even on the same machine the abundances can alter from day to day. Since the Edwards mass spectrometer is voltage scanning, the mass spectra suffer from mass discrimination to some degree.

The second case is that in which most of the fragments in the unknown spectrum agree with those in the reference spectrum but, when the fragments are arranged in order of abundance, several reversals in the order become apparent in comparison with the reference spectrum. The wisest course of action is to run the reference compound on the Edwards machine to produce a reference spectrum under similar conditions. Even so, peak reversals have to be accepted in interpreting unknown spectra, but all the characteristic fragmentation ions in the reference spectrum should be present.

The third case tends unfortunately to be most frequent: there is no reference spectrum. Here, only a tentative structure can be proposed by considering the fragment ions and the molecular ion, if it is present.

Retention indices can be useful in eliminating alternative structures. Gas chromatographers usually put forward the identity of a peak by agreement of retention on two or more columns with phases of different polarities. In the following interpretations, provided the mass spectrum is in agreement with the reference spectrum and there is available at least one retention index and this is in agreement with the reference compound to within ± 5 R.I. units, when chromatographed under the same conditions, the identification is considered positive.

Most of the conclusions drawn from the interpretation of the mass spectra obtained have been included in Table 1, which is based primarily on the spectra recorded for flask 2 of the prolonged cooking experiment but draws in the results for the other flasks and those obtained for the runs illustrated in Figures 1 and 2. Unless otherwise indicated, the reference spectra given have been recorded on authentic compounds run on the g.c.–m.s. unit under similar conditions. Where gas chromatographic peaks on different runs were equated this is based on substantial correspondence of their mass spectra but only rarely on their complete identity.

The following additional points need to be made, starting with peaks from flask 2.

Peak 3 is of interest, since here a small peak sitting on the tail of a much greater one has been identified by largely disregarding m/e values due to the preceding compound.

Peak 8 is identified as due to a mixture, the minor component being traced through the presence of m/e values additional to those of the major compound.

Peak 11 was identified as 2-hexen-1-ol through the work of Honkanen *et al.*,[12] authentic reference compounds not being available. It is not possible to decide between the isomers on the basis of the spectra obtained here but previous work[3] points to the *trans*-isomer.

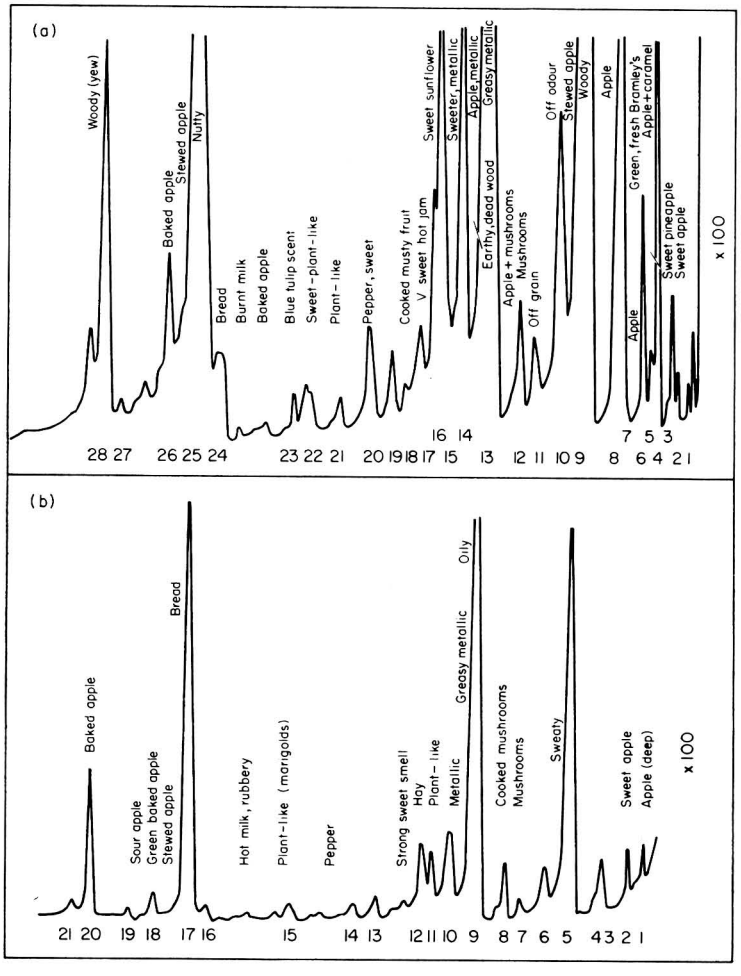

Figure 5. Analysis of extract from precooked frozen Bramley's on Carbowax 20M P.L.O.T. column: (a) stored frozen for 1 week; (b) stored frozen for 5 months. The important peaks are numbered for identification through Tables 2 and 3. The odour assessments are indicated. Attenuation ×100.

Peak 16 possesses a spectrum similar to that of peak 17. Murray[13] has published the mass spectrum of a compound which he had isolated from the wax coating of Granny Smith and Delicious apples, as well as from quinces and pears, and to which he assigned the structure of *trans,trans*-α-farnesene. Peak 17 is due to this compound. Murray[13]

has also examined another isomer with an identical mass spectrum, except for an increase in *m/e* 119. He assigned the structure of *cis,trans*-α-farnesene to it and peak 16 appears to be due to it.

Peak 18 gave a mass spectrum very close to that recently published by Demole *et al.*[14] for damascenone, 2,6,6-trimethyl-1-*trans*-crotonyl-1,3-cyclohexadiene, a compound discovered by them to be an important component of Bulgarian rose oil. Even more recently, Demole and Berthet[15] have identified the same compound in Burley

TABLE 2. Pentane extract of deep-frozen precooked apple after 1 week's storage [see Figure 5(a)]

Peak no.	Identity
1	Ethyl acetate
2	Ethyl propionate
3	3-Methylbutanal
4	Ethyl butyrate
5	Ethyl 2-methylbutyrate
6	*n*-Hexanal
7	*n*-Propyl butyrate
8	1-Butanol
9	2-Methyl-1-butanol
10	1-Pentanol
11	Unknown
12	Unknown
13	1-Hexanol
14	*trans*-2-Hexen-1-ol + unknown [cf. Figure 4(c), peak 6]
15	*n*-Hexyl butyrate + unknown, m.w. 170 [cf. Figure 4(c), peak 8]
16	*n*-Hexyl 2-methylbutyrate + 2-furfuraldehyde
17	Unknown
18	Unknown [cf. Figure 4(c), peak 10]
19	Benzaldehyde
20	Unknown
21	Unknown
22	*n*-Hexyl hexanoate + phenylacetaldehyde
23	Unknown, m.w. 148 [cf. Figure 4(c), peak 13]
24	*cis, trans*-α-Farnesene
25	*trans, trans*-α-Farnesene
26	Damascenone
27	Unknown, m.w. 220
28	Unknown [cf. Figure 4(c), peak 19]

tobacco and therefore expect it to be widely distributed, particularly in essential oils of plants, fermented and dried in air and so likely to be rich in carotenoid oxidation products. Damascenone is described as having an exceptionally powerful and characteristic odour.[14] An authentic sample[16] confirmed the close correspondence of the mass spectrum and gave a retention index 15 units greater.

Turning to the additional peaks from flask 4, its peak 8 has not been identified. It gave a complicated spectrum which suggests a mixture, since 170 and 157 are only 13 *m/e* units apart, but exactly the same spectrum was obtained from peaks on two other

gas chromatographic columns, which makes inhomogeneity unlikely. The behaviour is that of a non-polar compound.

Peak 11 gave a spectrum suggesting a heptanol, but its behaviour on a silicone column indicates it to be non-polar; hence possibly an ether.

Peak 16 can be identified as 2,4-decadienal, the mass spectral details given by Buttery *et al.*[6,7] making it slightly more likely to be the *trans,trans*-isomer.

TABLE 3. Pentane extract of deep-frozen precooked apple after 5 months' storage [see Figure 5(b)]

Peak no.	Identity
1	Ethyl butyrate
2	*n*-Hexanal
3	*n*-Propyl butyrate
4	1-Butanol
5	2-Methyl-1-butanol
6	1-Pentanol
7	Unknown
8	Unknown, m.w. 112
9	1-Hexanol
10	Unknown + unknown [cf. Figure 4(c), peak 6] + trace 2-hexen-1-ol
11	*n*-Hexyl butyrate + trace unknown, m.w. 170 [cf. Figure 4(c), peak 8]
12	*n*-Hexyl 2-methylbutyrate + 2-furfuraldehyde
13	Benzaldehyde
14	Unknown, m.w. 136
15	*n*-Hexyl hexanoate + phenylacetaldehyde
16	*cis, trans*-α-Farnesene
17	*trans, trans*-α-Farnesene
18	Damascenone + trace 2,4-decadienal
19	Unknown
20	Unknown, m.w. 220 (probably a mixture)
21	Unknown [cf. Figure 4(c), peak 19]

4. Discussion

4.1. Performance of the combined gas chromatography–mass spectrometry unit

The first reason for this work was to test the effectiveness in investigating food flavours of a novel system for combined gas chromatography–mass spectrometry.

The performance of the unit has in fact been very satisfactory indeed, a whole range of essences having been examined and a large number of mass spectra recorded. The spectra were clear and the background of column bleed and residual gases has been very low. Chromatograms of essences obtained by monitoring *m/e* 27 compare very well with those of the F.I.D., little time lag being experienced for compounds eluted at 50 to 190 °C.

4.2. Volatiles of cooked Bramley's and changes in them on prolonged cooking

Preliminary work (Figures 1 and 2) with the volatiles of cooked Bramley's allowed retention data, odour assessments and some mass spectra to be recorded. It demonstrated the feasibility of basing an investigation of the volatile constituents of cooking

apples on Likens and Nickerson's extractor.[4] To attempt our second objective, namely, to follow the way in which food volatiles change on processing, samples were required sequentially after different cooking times. Headspace sampling (Figure 3) did indeed show that changes were taking place in the volatiles, but the amounts which could be introduced into the gas chromatograph in this way were not large enough to permit mass spectral identification. By means of the Likens and Nickerson extractor, sufficient amounts of the volatiles were obtained (Figure 4) from all except flask 1, which contained the volatiles given off only during the heating-up period. The results are given in Table 1, which also includes those for the preliminary work.

Flask 2, containing the volatiles extracted during the first 0.5-h boil, led to a good chromatogram, which allowed 18 compounds to be identified. Of these, 14 have been previously reported[3,17] but in eating varieties of apples. Damascenone and phenylacetaldehyde have not previously been found in apples. The two α-farnesenes are known apple constituents, but of the coating.[13]

Flath *et al.*[18] showed that the main components directly associated with Delicious apple aroma are ethyl 2-methylbutyrate, *n*-hexanal and 2-hexenal. All three components are present in flask 2, which after all represents almost a fresh Bramley's aroma. The odour assessment of the large *n*-hexanal peak was in fact very green, fresh Bramley's. Very small underlying peaks may well have modified this assessment, for chromatography of authentic *n*-hexanal did not give as pleasant a note. Phenylacetaldehyde has an aroma of sweet honey or clover, which has also been described as violets, rose perfume and lilac.[19]

The two principal peaks of flask 2 essence were due mainly to 2-methyl-1-butanol and 1-hexanol. Although alcohols are prominent they contribute very little to the aroma since they possess high threshold values. On the other hand, all the esters identified will contribute to the aroma. Ethyl butyrate, ethyl 2-methylbutyrate and *n*-hexyl 2-methylbutyrate, in particular, have a very sweet aroma resembling apple. Other significant esters may well be hidden under the larger peaks.

The odour assessments illustrate again the greater sensitivity of the nose for some substances compared with instruments. In Figure 4(a), at an R.I. of about 1300, there elutes a very strong smell of mushrooms which appears on all odour assessments. After the 1-hexanol peak there is a very strong metallic off-odour. Close examination of these areas on the recorder traces revealed no significant departures from the baseline. Peak 18 has an aroma of baked apple but authentic damascenone,[16] although having a persistent camphoraceous and fruity odour, was not detectable by smell at the odour port of the gas chromatograph. Murray[13] describes α-farnesene as having a strong attractive apple-like odour, but this is not borne out by odour assessments on peak 17, which is the third largest peak from flask 2 essence.

As the time of heating is increased the chromatographic picture changes markedly.

The most significant change during the heating process is the rapid disappearance of the esters, only ethyl butyrate surviving into flask 3 (Figure 4). It and *n*-hexanal are still present, though reduced in amount, and contribute an apple note. All the esters are absent from flask 4.

The five alcohols are evolved throughout the first 4-h boiling, by which time they have more or less boiled off. It is worth noting that 1-butanol clearly reached a maximum in flask 4.

The two farnesenes are also evolved at least throughout the first 4-h boiling the concentration falling progressively. Although they are relatively high-boiling compounds one would have expected them to boil off faster and this leads to a suspicion of the presence of a precursor.

The four aldehydes form two pairs: the two hexenals are lost within the first 0.5-h boiling, but *n*-hexanal and phenylacetaldehyde are evolved throughout, the former falling off progressively, but the latter being present in largest amount in flask 4. A third group of aldehydes, 2-furfural, benzaldehyde, 5-methylfurfural and a dienal, is absent from flask 2. The two furfurals are evolved after the first 0.5 h, furfural becoming the predominant constituent in flask 5, whereas the other two are evolved only after 1.5 h. 2-Furfural, 5-methyl-2-furfural and benzaldehyde all have almond odours.

This lag in volatile production is most interesting. The two furfurals can be taken as circumstantial evidence for sugar degradation, benzaldehyde for hydrolysis of cyanogenic glycosides and the dienal possibly for lipid oxidation; all of these processes would require time to occur.

Apart from some free sugars, which are usually hexoses and so would lead mainly to 5-hydroxymethyl-2-furfural, flavonol glycosides would hydrolyse to give further sugars including pentoses and methylpentoses. These could give rise to furfural and 5-methyl-2-furfural. Ascorbic acid, of which Bramley's contain substantial quantities (up to 30 mg/100 g),[20] could well be a major source of the furfurals. A further possible source of sugars is pectin and hemicellulose.

The absence of 5-hydroxymethyl-2-furfural from the extracts, when apple juice contains 0 to 18 mg/l,[21] is at first surprising, but it appears not to partition into pentane.

The occurrence of phenylacetaldehyde has been noted in other heated foods.[7, 22] Its production is most likely to be due to Strecker degradation of phenylalanine, which is present in Bramley's.

The production of a very small amount of benzaldehyde is interesting. Its formation by Strecker degradation is doubtful, for phenylglycine is not a common amino acid. It more probably originates in the hydrolysis of the cyanogenic glycoside, amygdalin, present in the pips. It might be produced by breakdown of phenylacetaldehyde and has been reported as a product of sucrose caramelisation.

The formation of 2,4-decadienal in heated foods is well-known, but this dienal has also been isolated from bell peppers under relatively mild conditions.[7] Its formation is attributed to oxidation of long-chain unsaturated fatty acids, such as linoleic and linolenic acids. The double bonds would be expected to be *cis*, yet rearrangement seems to occur readily to the more stable *trans* form. Dienals have very potent aromas; *trans*-2,*cis*-6-nonadienal is the main contributor to cucumber odour.[23] It seems unlikely that decadienal accounts for the sour cooked-apple aroma of flask 4, peak 16.

Damascenone is evolved throughout the exaggerated cooking time, but decreases in amount, suggesting that it was present originally and was not being formed during the process. If this is so, it may be a true metabolite rather than a carotenoid breakdown product as suggested by Demole and Berthet.[15]

Several of the identifications made above were supported by the results of subtractive gas chromatography with a precolumn of boric acid,[24, 25] which selectively reacts with primary and secondary alcohols. Thus peaks due to 1-butanol, 2-methyl-1-butanol,

1-hexanol and 2-hexanol disappeared or were greatly reduced in intensity, but that due to furfuraldehyde was left unchanged. Peak 8, flask 4, is not an alcohol because it is unchanged by boric acid, but *trans,trans*-α-farnesene is chemically affected by boric acid although it is not an alcohol, there being a large increase in *m/e* 107 in the mass spectrum. The peaks surviving a boric acid precolumn are indicated in Table 1.

4.3. Changes in flavour of precooked frozen apple on storage

Although a taste panel was unable to distinguish deep-frozen from blast-frozen precooked Bramley's after 3.5 months' storage, it established a highly significant difference between cooked apple prepared fresh from Bramley's stored at +5 °C for 5 months and blast-frozen precooked apple, stored for 5 months at −20 °C and made originally from the same batch of Bramley's. Comments from almost every panel member indicated that the freshly cooked apple had a pleasant apple taste and less acidity, whereas the precooked frozen apple had a much blander, flatter and generally weaker apple taste and more acidity.

Such a marked flavour difference should be assessable instrumentally. A comparison was therefore made between an extract of precooked frozen apple stored for 1 week (and stored for 5 months in the form of the extract) and stored for 5 months. Chromatograms run under virtually identical conditions are reproduced in Figure 5 and the mass spectral identifications made are listed in Tables 2 and 3.

From these data it is apparent that the precooked frozen apple stored for only 1 week contains relatively higher concentrations of the esters and of *n*-hexanal, which, on the basis of the work done with Delicious apples,[7] accounts nicely for the taste panel's down-grading of the precooked frozen apple stored 5 months as such.

That esters and other low-boiling constituents should be lost on longer storage is not surprising. In addition, traces of the decadienal had appeared, suggesting that lipid oxidation had occurred during longer storage to a greater extent than during the short cooking time.

It should be noted that several new components appeared in the extract of precooked frozen apple (stored for 1 week), which had not been observed in the investigation of cooked apples, although there is little difference in the odour descriptions. Among the additional components, ethyl acetate and 3-methylbutanal were identified.

Acidity played a prominent part in the taste panel's assessment, but has not been considered further here. Its effects would have to be taken into account in any more far-reaching investigation, as would several other factors.

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Composition of the Aqueous Extracts of Rapeseed Meals

M. T. Lo and D. C. Hill

Department of Nutrition, University of Guelph, Guelph, Ontario, Canada
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Aqueous extracts of rapeseed meals were separated into four fractions by Sephadex (G25) column chromatography. Fraction I was found to be rich in protein as judged by nitrogen and amino-acid content and u.v. absorbance at 280 nm. Fraction II contained glucosinolates and peptide-like materials. Fractions III and IV contained only trace amounts of amino acids and nitrogen. Further separation of fraction IV into three components was achieved by chromatography on silicic acid. Two of the components were shown, by paper chromatography and u.v. absorbance scanning, to be rich in caffeic acid and chlorogenic acid, respectively.

1. Introduction

In recent years, a number of methods have been described for preparing rapeseed flour or protein concentrates from rapeseed or rapeseed meals.[1–3] Water extraction of the ground seed or the meal is apparently an essential step in these methods to obtain a light-coloured bland product low in glucosinolates. However, water extraction usually results in a 20% or more removal of dry matter and 20 to 22% of the original nitrogen content of the seed. The nature of the extracted material is not known but is thought to be a mixture of protein and non-protein nitrogenous material including the glucosinolates and non-nitrogenous substance possibly polyphenolic acids.

This paper describes a fractionation of aqueous extracts of rapeseed meal using gel filtration and a preliminary study of the composition of the fractions.

2. Experimental

2.1. Material

Commercial *Brassica napus* and cultivar Bronowski meals, prepared by a prepress–solvent procedure, were obtained from Western Canada. The two meals were chosen because of their marked difference in glucosinolate content. *Brassica napus* is relatively high in glucosinolate content, whereas Bronowski is very low.

Samples of the rapeseed meals (100 g) were extracted with 800 ml of boiling water in a water-bath for 10 min with occasional stirring. The water-meal slurry was then centrifuged at 16 000 **g** for 20 min at 25 °C and the supernatant was collected. The residue was re-extracted with another 800 ml of boiling water and the two supernatants were pooled and freeze-dried.

2.2. Chemical analysis of dried extract

Moisture, ash, nitrogen, crude fibre, calcium and phosphorus were determined by A.O.A.C. procedures.[4] Amino-acid content was determined by ion exchange using an automatic Technicon Amino-Acid Analyzer. Hydrolysates were prepared as described previously.[5] Glucosinolate content was determined by measuring the HSO_4^- released through enzymic hydrolysis.[6] Non-protein nitrogen was determined by the method of Becker, Milner and Nagel.[7]

2.3. Gel filtration

Sephadex G25 [Pharmacia (Canada) Ltd] was suspended in about 1000 ml of water (100 g of dry gel), allowed to stand for 24 h and then packed in a Pharmacia laboratory column (K25/100). A 0.5-g sample of dried aqueous extract, dissolved in 5 ml of water, was applied to the top of the column. The solution was allowed to enter the gel and the column was eluted with 0.05% NaCl solution using a flow rate of 1 ml/min. The effluent was collected in 10-ml fractions by an automatic fraction collector (Buchler Instruments, Inc.). The absorbance of each fraction was measured with a spectrophotometer (SP 500 Series 2, UNICAM) at a wavelength of 280 nm. The fractions, making up the individual peak-areas, were combined and scanned for u.v. absorbance spectra (SP 800B UNICAM). Nitrogen and glucosinolate distribution in the fractions were determined on pooled 50-ml fractions (5 collections).

2.4. Partition chromatography

Partition on silicic acid as described by Hasegawa, Johnson and Gould[8] was employed. Dry silicic acid[9] (12 g) was mixed with 6 ml of 0.5 N-H_2SO_4 in a mortar. The resulting free-flowing powder was slurried in 80 ml of solvent B (cyclohexane–chloroform, 1:9) and added to a glass column, 1.2 × 20 cm, to form a packed column 15-cm long.

A 100-mg sample (freeze-dried material of fraction IV, Figure 1) was exhaustively extracted with ethanol and the ethanol extract filtered through Whatman No. 1 paper and evaporated to dryness *in vacuo*. The dried material was mixed thoroughly with 1 g of silicic acid (acidified with 0.5 ml of 0.5 N-H_2SO_4) and the mixture transferred to the top of the column. A plug of glass wool was placed in the top of the column to prevent disturbance to the surface during addition of the eluating solvents. The prepared column was eluted at a flow rate of approximately 1 ml/min with a mixture of different proportions of solvent B and solvent C (2-methyl-2-propanol–chloroform, 2:3) as follows. Eluation was started with 50 ml of B, followed successively by 40 ml of B and 5 ml of C; 40 ml of B and 10 ml of C, 35 ml of B and 15 ml of C, 30 ml of B and 20 ml of C; 40 ml of B and 60 ml of C and finally 30 ml of B and 70 ml of C. The effluent was collected in 5-ml fractions and the absorbance of each was measured at a wavelength of 320 nm. The pooled 5-ml fractions making up each of the peak areas were scanned for u.v. absorbance and examined by paper chromatography for identification of component compounds. The solvent system[10] used for developing the paper chromatograph was *n*-butanol–acetic acid–water (4:1:5) and spots were detected by u.v. fluorescence.

Authentic compounds of caffeic acid and chlorogenic acid (Nutritional Biochemicals, Cleveland, Ohio) were chromatogrammed separately and together on silicic acid columns

to determine their elution times and the eluted fractions, pooled for each peak, were examined for u.v. absorbance and chromatographed on paper.

3. Results and discussion

The results of the chemical analysis of the dried aqueous extracts and the original meals are given in Table 1. The higher dry-matter content of the aqueous extract of *B. napus* meal compared with that of Bronowski cultivar may be attributed to a greater amount of glucosinolate contained in the former meal. Although the total protein (N × 6.25) content of the two aqueous extracts were comparable, there was a difference

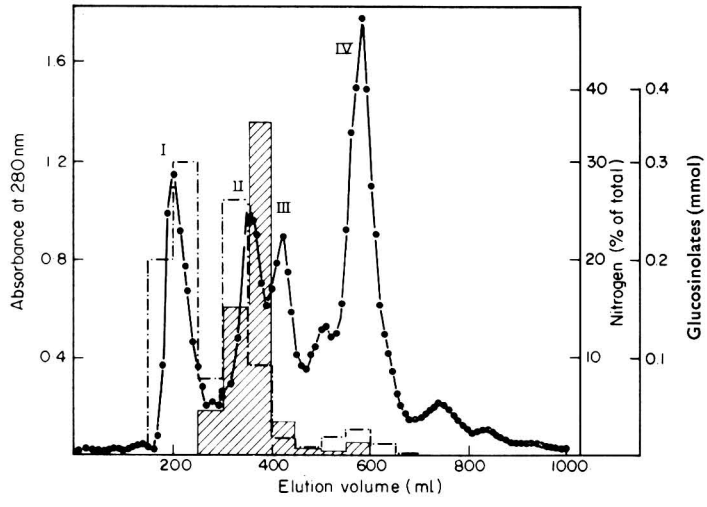

Figure 1. Chromatograph of an aqueous extract of *B. napus* meal on Sephadex G25. Column 100 × 2.5 cm; eluant 0.05% NaCl; 10-ml fractions were collected; nitrogen and glucosinolates were determined on a pooled sample of 5 fractions, with glucosinolate reported as mmol/g of dried material applied to the column; fractions I, II, III and IV are indicated.
—●—●—, Absorbance at 280 nm; ·—·—, nitrogen (% of total); shaded area, glucosinolates.

in the amount of trichloroacetic acid precipitatable protein. On the basis of nitrogen data it appears that about 14 to 18% of the original meal nitrogen was water-soluble in this experiment and at least 47% of this nitrogen was non-protein nitrogen.

A typical chromatogram obtained from the aqueous extract of *Brassica napus* meal by gel filtration is shown in Figure 1. Based on the u.v. absorbance at 280 nm this aqueous extract yielded four major fractions consisting of the elution volumes 160 to 250 ml, 260 to 380 ml, 390 to 460 ml and 500 to 700 ml. Fraction I contained 50% of the total nitrogen recovered from the column and fraction II, 37% of nitrogen. Glucosinolates quantitatively recorded as mmol/50 ml of eluate were distributed between fractions II and III, with the major portion concentrated in fraction II. Fraction IV contained only trace amounts of nitrogen and glucosinolates despite its strong absorbance at 280 nm.

TABLE 1. Chemical composition of rapeseed meals and their dried aqueous extracts (%)

	Dry matter (Freeze-dried)	Ash	Ca	P	Crude fibre	Moisture	Glucos-inolates[b]	Protein (N × 6.25)		
								Precipitable[c]	Soluble[c]	Total
Aqueous extract										
Bronowski	20.1[a]	11.4	0.3	0.4	1.7	4.0	132	13.2	11.8	25.0
B. napus	27.2[a]	11.6	0.1	0.7	1.5	4.3	600	10.8	13.1	24.2
Original meal										
Bronowski	100	6.8	0.6	1.0	14.0	7.5	28	—	—	—
B. napus	100	6.7	0.5	1.1	13.4	8.7	180	—	—	—

Results are average of three determinations.

[a] of original meal.

[b] μmol/g.

[c] A weighed portion of dried extract was redissolved in H_2O and the protein precipitated with trichloroacetic acid added to give a concentration of 0.8 M.

Gel filtration of extracts from the cultivar Bronowski also yielded four fractions, fraction I and fraction II contained 70 and 20%, respectively, of the total nitrogen recovered from the column; and the total glucosinolate found in the extract from the Bronowski meal was about 10% of that from *Brassica napus*.

The results suggest that gel filtration can successfully separate water-soluble proteins from low molecular weight nitrogenous materials including glucosinolates and from non-nitrogenous substance. Janson[11] has recently reported the isolation of several protein fractions from rapeseed by gel filtration.

TABLE 2. Total amino acids (mg) found in fractions I, II and III obtained by gel filtration[a] of the aqueous extract of rapeseed meals

Fraction	Bronowski			*B. napus*		
	I	II	III	I	II	III
1. Aspartic acid	2.57	1.40	0.05	2.22	0.51	0.04
2. Threonine	2.19	0.45	0.02	2.01	0.37	0.01
3. Serine	1.74	0.46	0.01	1.63	0.19	0.01
4. Glutamic acid	9.36	3.87	0.06	8.28	1.37	0.04
5. Proline	3.60	0.60	0.03	3.00	0.21	0.03
6. Glycine	2.82	0.78	0.04	2.45	0.29	0.02
7. Alanine	2.34	0.77	0.03	1.94	0.28	0.02
8. Cystine	1.89	0.34	0.02	1.50	0.15	0.02
9. Valine	3.31	0.79	0.05	2.37	0.12	0.04
10. Methionine	1.44	0.40	0.01	0.87	0.18	0.01
11. Isoleucine	1.82	0.45	0.02	1.47	0.18	0.02
12. Leucine	2.88	0.60	0.03	2.30	0.24	0.03
13. Tyrosine	1.65	0.53	0.04	1.32	0.54	0.03
14. Phenylalanine	1.89	1.71	0.03	1.44	0.63	0.03
15. Histidine	1.90	0.49	0.02	1.79	0.21	0.01
16. Lysine	4.46	0.90	0.03	3.84	0.37	0.03
17. Arginine	2.72	0.45	0	2.42	0	0
Total	48.58	14.99	0.49	40.85	5.84	0.39

Results are the average of four determinations.

[a] 0.5 g of dry aqueous extract was dissolved into 5 ml of water and then applied to a G25 Sephadex column.

Calculation of the total dry matter and nitrogen recovered in the eluate from the sample (0.5 g) placed on the column were about 120 to 130 and 90% respectively. The greater than 100% recovery of dry matter can logically be attributed to the accumulation of NaCl from the eluting solution.

When the four fractions were freeze-dried the colour of the dried material ranged from pale white for fraction I to greenish–yellow for fraction IV. The fractions were powdery in consistency except for fraction II which was gummy.

The amounts of amino acids eluted in fractions I, II and III are given in Table 2. Of the total amino acid obtained, 76 and 87% were located in fraction I for Bronowski extract and *B. napus* extract, respectively, and 23 and 12% in fraction II. Only 0.8% of the total amino acid was found in fraction III. These results suggest that fraction I

contained its nitrogen largely in the form of protein and fraction II as low molecular weight nitrogenous material, probably peptides. The small amount of amino acids found in fraction III indicates that the rapeseed meals did not contain large quantities of free amino acids.

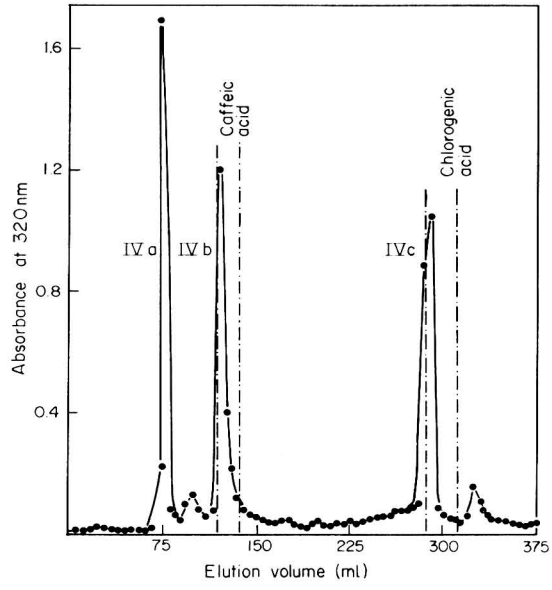

Figure 2. Silicic acid chromatography of an ethanol extract of fraction IV (*B. napus*, Figure 1). Chlorogenic acid and caffeic acid were chromatogrammed separately.

Preliminary scanning from 250 to 350 nm of the four fractions obtained from the gel filtration indicated a shifting of a maximum absorbance at 260 to 280 nm to 320 to 330 nm progressively for fractions I to IV. This would be the expected result if fraction I was rich in protein and fraction IV contained considerable amounts of polyphenolic compounds.

Results of partition chromatography of fraction IV on silicic acid are shown in Figure 2. Three components are evident, IVa, IVb and IVc. The latter two have essentially the same retention times as caffeic acid and chlorogenic acid, respectively (the location of peaks for authentic samples of the two compounds are indicated by dotted lines).

The components IVa, IVb and IVc were subjected to u.v. scanning (Figure 3). Comparable patterns to caffeic acid and chlorogenic acid were obtained for components IVb and IVc, respectively. Wavelengths of the u.v. absorption peaks and R_F values obtained from paper chromatography are summarised in Table 3. In view of the similarity of the u.v. spectra and R_F values for components IVb and IVc and authentic compounds of caffeic acid and chlorogenic acid it is reasonable to conclude that these two components are caffeic acid and chlorogenic acid, respectively. Component IVa

has not been identified. Its u.v. spectrum was quite different from those of caffeic acid and chlorogenic acid; although by paper chromatography its R_F value was similar to that of caffeic acid.

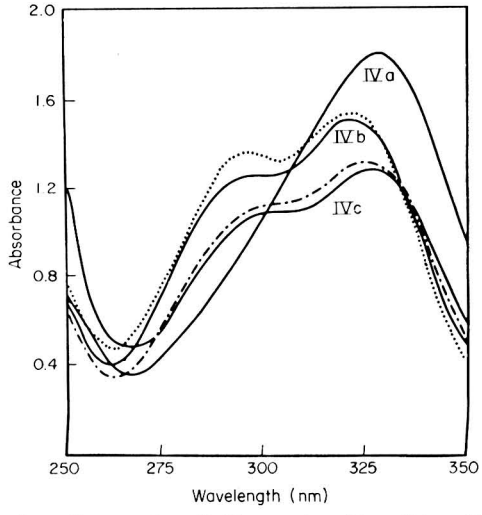

Figure 3. Ultraviolet absorption spectra of chlorogenic acid, caffeic acid and components IVa, IVb and IVc obtained by silicic acid chromatography.
——, Components IVa, IVb and IVc; —·—·, chlorogenic acid; ·····, caffeic acid.

TABLE 3. R_F values and ultraviolet absorption peaks of caffeic and chlorogenic acids and of components IVa, IVb, IVc

Sample	R_F values	Approximate u.v. absorption peaks (nm)	
		minor	major
Caffeic acid	0.81	292	324
Chlorogenic acid	0.61	295	325
Component IVa	0.81	—	328
Component IVb	0.80	292	324
Component IVc	0.60	295	325

It has been suggested that the green colour of protein isolates obtained from sunflower seed is associated with the presence of chlorogenic acid in the seed.[14, 15] Possibly this compound and also caffeic acid may be concerned in a coloration which is frequently observed to develop during the preparation of protein isolates from rapeseed.

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Effects of Interesterification and Fatty Acid Supplementation on the Digestibility of Tallow in Milk Replacers for Calves

Rosemary K. Hamilton and A. M. Raven[a]

Agricultural Chemistry Department, Queen's University, Belfast, N. Ireland and Chemical and Animal Nutrition Research Division, Ministry of Agriculture for Northern Ireland

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The effects of interesterification and fatty acid supplementation on the nutritive value of homogenised fats in milk replacers for calves were studied in two digestibility experiments, each of a 6 × 6 latin square design. Using a level of 20% added fat, the mean apparent digestibilities of the total dietary lipids were: butterfat, 96.5%; interesterified butterfat, 95.5%; tallow, 88.7%; interesterified tallow, 93.1%; tallow supplemented with trimyristin and triolein by interesterification, 92.3%; tallow supplemented with trimyristin and triolein by simple admixture, 89.9%. The high digestibility of butterfat is not dependent apparently on any specific non-random distribution of its constituent fatty acids. The digestibility of the tallow was lower and interesterification just failed to give a significant improvement. The inclusion of additional myristic and oleic acids had no significant effect.

Using a level of 15% added fat, the mean apparent digestibilities of the total dietary lipids were: tallow, 79.7%; interesterified tallow, 89.2%; interesterified, reduced stearic acid, tallow 87.9%; interesterified, reduced stearic acid, added butyric acid, tallow, 96.3%; interesterified, added butyric acid, tallow 94.7%; tallow admixed with tributyrin, 84.0%. The inclusion in tallow of butyric acid by a process involving interesterification resulted in substantial increases in digestibility to values similar to those obtained for butterfat and interesterified butterfat in the first experiment. The improvement was not achieved by simple admixture of tributyrin with tallow. Reduction of the stearic acid content in tallow to a level similar to that in butterfat had no significant effect on fat digestibility.

The lower digestibility of tallow than of butterfat was due mainly to lower digestibility of palmitic and stearic acids and a higher percentage of the latter acid in tallow. Modifications which effected an improvement in the digestibility of tallow generally did so by improving the digestibility of these acids.

1. Introduction

One of the major expenses in rearing calves is the feeding of whole milk and, with the intensification of calf-rearing in recent years and the growing need to minimise feed costs, the use of milk replacers for early feeding has become increasingly necessary. The increase in calf production and a world surplus of fats have stimulated research to overcome problems which occurred with early attempts to use added fat in milk substitutes, and milk replacers containing added fat are now being produced and increasingly used. It should be appreciated that, at this stage, the young milk-fed calf

[a] Present address: Advisory and Development Service, West of Scotland Agricultural College, Auchincruive, Ayr, Scotland.

functions essentially as a monogastric animal[1-3] and thus the constituents of any liquid diet fed to the calf should be susceptible to digestion by the enzymes secreted by the calf.

Most commercially produced milk replacers at the present time are based on separated milk powder and dried whey with the addition of a source of fat. An ideal type of fat should be one with a high digestibility giving results as good as or better than those given by butterfat and yet should be cheap and readily available. Beef tallow fulfils the last two requirements in being readily available following the expansion of the meat industry and costing about half as much as most vegetable oils. Tallow, however, has a mean digestibility of about 86%,[4-7] a full 10% lower than that of butterfat[7-14] and this puts a limitation on the amount of it which can be satisfactorily included in high-fat milk replacers.

Work on the composition and structure of butterfat and tallow has shown that the three main differences between them are: (i) their contents of stearic acid, butterfat containing 12 to 15% and tallow 20 to 25% by weight, (ii) their contents of short chain fatty acids, butterfat containing about 10% by weight of fatty acids with carbon number below 12 whereas tallow contains virtually none and (iii) their triglyceride structure. It is likely that one or a combination of these factors is responsible for the higher digestibility of butterfat.

Work with chicks[15-17] and rats[18] has shown that the digestibility of a fat by these species is related to its content of long-chain saturated acids and can be improved by the addition of unsaturated and medium-chain fatty acids. It has also been reported[19] that low digestibilities of tallow, lard and rapeseed oil in milk-fed lambs are related to a high stearic acid content. With regard to a possible effect of short-chain acids, veal calves fed filled milk containing butyrated lard, prepared by an ester interchange reaction and having approximately the same butyric acid content as butter, have given weight gains as good as those by calves on a diet containing butterfat and significantly better than those on a diet containing natural lard.[20] Evidence that triglyceride structure, in particular the position of palmitic acid in triglyceride molecules, is a factor in determining fat digestibility has been obtained in studies with pigs,[21] chicks[15] and human infants.[22, 23]

In the present investigation, four types of modifications to beef tallow were examined; (i) interesterification of the triglyceride structure, (ii) supplementation with additional oleic and myristic acids, (iii) reduction of the content of stearic acid and (iv) inclusion of short-chain fatty acid in the form of butyric acid.

2. Experimental

2.1. Design of experiments

Two balance trials were carried out each of a 6 × 6 latin square design. Each experimental period consisted of a 4-day preliminary feeding period followed by a 6-day collection period.

2.2. Diets

Milk replacers were prepared by incorporation of the test fats, at a level of 20% in experiment 1 and 15% in experiment 2, by homogenisation with liquid-separated milk

to give a fat globule size of 2 to 4 μm in diameter followed by spray-drying. The dietary fats were prepared by Price's Chemicals Ltd, Bromborough, Cheshire and in each experiment the various modified fats were made from the same batch of butterfat or of tallow as required. Interesterified fats were prepared by interesterification of the fats concerned in hexane for 6 h under nitrogen using a sodium methoxide catalyst (1%). Complete randomisation of the triglycerides is achieved under these conditions.[24] The fats used in each milk replacer (m.r.) were as follows:

Experiment 1

- m.r. 19 Butterfat.
- m.r. 20 Interesterified butterfat.
- m.r. 21 Tallow.
- m.r. 22 Interesterified tallow.
- m.r. 23 Interesterified supplemented tallow, prepared by interesterification of a mixture of 80% tallow, 10% glyceryl trimyristate and 10% glyceryl trioleate, by weight.
- m.r. 24 Tallow admixed with supplementary triglycerides, prepared by simply mixing 80% tallow, 10% glyceryl trimyristate and 10% glyceryl trioleate, by weight.

Experiment 2

- m.r. 25 Tallow.
- m.r. 26 Interesterified tallow.
- m.r. 27 Tallow with stearic acid content reduced to a level similar to that in butterfat, prepared by hydrolysis of the fat, partial removal of stearic acid and re-esterification of the remaining fatty acids.
- m.r. 28 Tallow with reduced stearic acid content and added butyric acid, prepared by interesterifying 90% of the reduced stearic acid tallow with 10% of glyceryl tributyrate.
- m.r. 29 Tallow with added butyric acid, prepared by interesterifying 90% of the same batch of tallow as used in treatment m.r. 25 with 10% of glyceryl tributyrate.
- m.r. 30 Tallow with added butyric acid, prepared by simply admixing 90% of the same batch of tallow as used in treatment m.r. 25 with 10% of glyceryl tributyrate.

The milk replacers were fed by bucket twice a day at 9.00 a.m. and 5.00 p.m. to young Friesian bull calves initially about one week old at the start of each experiment. For each feed 290 g of milk replacer were reconstituted with 2 l of luke warm water to give a liquid "filled" milk containing 12.5% of dry matter. Each feed was supplemented with 0.94 g of a dry vitamin supplement providing levels of 5280 i.u. vitamin A, 1320 i.u. vitamin D_3 and 22 mg vitamin E (Dl-α-tocopheryl acetate) per kg of dry milk replacer. 5 ml of a solution of B vitamins and 5 ml of a solution of mineral supplements were also added. The compositions of the B vitamin and mineral solutions are given in Table 1. When each feed was consumed, a further 1 l of water was added to the bucket to ensure complete intake of any feed residues.

TABLE 1. Compositions of the supplementary solutions of B vitamins and minerals

Supplement	Amount	Amount supplied/kg dry matter of milk replacer
Vitamin B complex		
Choline chloride	100 g	1.764 g
Nicotinic acid	1.0 g	17.6 mg
Calcium pantothenate	1.0 g	17.6 mg
Thiamine hydrochloride	300 mg	5.29 mg
Riboflavin	300 mg	5.29 mg
Pyridoxine hydrochloride	300 mg	5.29 mg
Folic acid	30 mg	0.529 mg
Biotin	10 mg	0.176 mg
Cobalamine	3 mg	0.053 mg
Mineral supplements		
Magnesium as $MgCl_2 \cdot 6H_2O$	142.2 g	300 mg Mg
Iron as $FeSO_4 \cdot 7H_2O$	19.8 g	70 mg Fe
Manganese as $MnSO_4 \cdot 4H_2O$	3.2 g	10 mg Mn
Copper as $CuSO_4 \cdot 5H_2O$	1.11 g	5 mg Cu
Cobalt as $CoSO_4 \cdot 7H_2O$	0.08 g	0.3 mg Co
Iodine as KI	0.04 g	0.5 mg I

2.3. Sampling of faeces and urine

Total collections of faeces and urine were made during the balance periods. A constant proportion of the weight of wet faeces from each animal was retained and stored in a deep-freeze at −20°C. The volume of urine was measured each day and one twentieth retained and bulked. 50 ml of 5% oxalic acid was added to each bucket at the commencement of each days' collection to prevent loss of ammonia.

2.4. Analysis of diets, faeces and urine

Nitrogen determinations were made on the composite wet samples of faeces and bulked samples of urine immediately after the end of each balance period and the dry matter contents of the faeces ascertained. All other analysis of faeces was carried out on composite samples of dried, milled faeces. The dry matter and crude protein (N × 6.25) contents in the milk replacers and faeces were determined by the usual methods of proximate analysis. The determinations of total lipid in both the milk replacers and faeces were made by the British Standards Institution method[25] for dried milk. This method involves treatment with hydrochloric acid before extraction of the lipids and thus enables any fatty acids present as soaps to be converted to free fatty acids and therefore extracted.

Individual fatty acids were determined by methylating the lipids extracted from the diets and faeces using 0.025 N-sodium methoxide in methanol[26] and separating and estimating the methyl esters by gas–liquid chromatography. In the first experiment, the instrument used was a Pye Series 104 dual-column gas chromatograph with flame ionisation detector. The liquid stationary phase was commercially prepared polyethylene glycol adipate (10%) supported on 100/120 mesh diatomite C (Pye Unicam).

The carrier gas was oxygen-free nitrogen with a flow rate of 45 ml/min and the esters were eluted at 180 °C. Peak areas were estimated using a Hewlett–Packard integrator. In the second experiment, the instrument used was a Hewlett–Packard dual column research gas chromatograph with flame ionisation detector. The liquid stationary phase was diethylene glycol succinate (6%) supported on high-performance AW-DMCS Chromosorb W (Hewlett–Packard). The carrier gas was oxygen-free nitrogen and the esters were eluted from the column at a temperature programmed from 140 to 170 °C at a rate of 4 deg. C/min and with an upper-limit period of 5 min. Peak areas were estimated using the Hewlett–Packard integrator, identified by comparison of their retention times with those of a standard mixture of methyl esters and converted to percentages of individual fatty acids using factors derived from standard mixtures of methyl esters.

The percentage digestibility and nitrogen retention results were subjected to angular transformation prior to analysis of variance in order to obtain maximum precision with the statistical analyses. In experiment 2 the use of a balanced latin square design enabled the mean results to be adjusted for residual effects.

3. Results

3.1. Experiment 1

The chemical composition of the milk replacers, digestibilities of dry matter, fat and crude protein and nitrogen retentions are given in Table 2. The mean apparent digestibility of the dry matter was very high for each treatment and highest by a small percentage for the diet containing butterfat (m.r. 19). The butterfat itself was well absorbed having a mean apparent digestibility of 96.5% which compares well with values obtained by other workers.[7–14] The transformed results indicate that the mean digestibility of the fat on the interesterified butterfat treatment was not significantly different from that on the natural butterfat treatment. The mean digestibility of 88.7% for tallow was slightly higher than values obtained by other workers[4–7] but significantly lower than that of the butterfat ($P < 0.01$). Although the mean digestibility of 93.1% obtained for the interesterified tallow was higher than that for the untreated tallow, the transformed results indicated that the difference in digestibility just failed to achieve significance at $P < 0.05$. The mean fat digestibilities for the two supplemented tallow treatments, m.r. 23 and m.r. 24 were only a little higher than the value for the tallow treatment m.r. 21 and appreciably lower than those obtained for both butterfat and interesterified butterfat.

The crude protein digestibilities were very high, comparing favourably with the values for the protein of whole milk reported by other workers[9, 10, 13, 14] and there were no significant differences between treatments. The values obtained for mean percentage nitrogen retention were not as high as expected, particularly for the two butterfat treatments, in comparison with values ranging from 40 to 70% determined for diets containing butterfat by various workers[3, 5, 10, 13, 14, 27] and the only significant difference obtained ($P < 0.05$) was between treatments m.r. 19 and m.r. 24.

Very small quantities of fatty acids with carbon number 12 or less were found in the faeces and these acids were assumed to be virtually 100% digested. The fatty acid

TABLE 2. Chemical composition of milk replacers and mean digestibility and nitrogen retention data in experiment 1

	Diet						
	Type of fat						
	m.r. 19 Butterfat	m.r. 20 Interesterified butterfat	m.r. 21 Tallow	m.r. 22 Interesterified tallow	m.r. 23 Interesterified supplemented tallow	m.r. 24 Tallow admixed with supplementary triglycerides	Standard error of a mean
Chemical composition of milk replacer							
Fat, % in dry matter	21.1	19.1	20.2	20.3	21.0	20.5	—
Crude protein, % in dry matter	28.6	28.2	28.3	28.1	27.6	28.0	—
Mean apparent digestibility, %							
Dry matter	96.4	95.4	94.7	96.2	96.1	95.6	—
Fat	96.5	95.5	88.7	93.1	92.3	89.9	—
Crude protein	95.4	93.1	94.5	95.7	95.6	95.3	—
Nitrogen retention, %	34.5	37.3	36.7	38.0	41.0	42.2	—
Transformed means							
Dry matter digestibility	79.2	77.9	77.1	79.0	78.9	78.4	1.08
Fat digestibility	79.7	78.6	71.7	75.8	74.5	72.9	1.82
Crude protein digestibility	77.8	75.3	76.7	78.3	78.1	77.9	1.18
Nitrogen retention	35.9	37.5	37.3	37.9	39.8	40.5	1.44

compositions of the dietary fats and the mean digestibilities of the principal long-chain fatty acids are given in Table 3.

The mean apparent digestibility values for the four fatty acids in the butterfat and tallow treatments compare well with those obtained by Raven[3] for similar treatments. The results show that the differences in digestibility between the dietary fats may be accounted for mainly by differences in the digestibilities of stearic and palmitic acids. The mean apparent digestibility of stearic acid in tallow was significantly lower than its mean digestibility in butterfat ($P < 0.01$) and both interesterified butterfat and interesterified tallow ($P < 0.05$). Although the two supplemented tallow treatments were associated with higher digestibilities of stearic acid than occurred in tallow, the increases did not achieve significance. The mean digestibility of palmitic acid in tallow was also significantly lower than its mean digestibility in butterfat ($P < 0.01$). Although the various modifications of tallow were each associated with some improvement in digestibility of palmitic acid none of the differences attained significance.

Digestibility differences between treatments for myristic acid and oleic acid were not so evident. Although the mean apparent digestibility of myristic acid in tallow was lower than the values determined for all other fats, the only significant difference was that between the butterfat and tallow treatments ($P < 0.05$). Mean apparent digestibilities of oleic acid were all high with no significant differences between treatments except for a surprisingly low value on treatment m.r. 24.

3.2. Experiment 2

The chemical compositions of the milk replacers, digestibilities of dry matter, fat and crude protein and nitrogen retentions are given in Table 4. The mean apparent dry matter digestibility of 92.4% for the tallow treatment was a little lower than the value of 94.7% obtained for the tallow treatment in experiment 1. The digestibility of the dry matter was significantly higher on the treatments containing fats with butyric acid added by interesterification (m.r. 28 and m.r. 29) than on the tallow treatment (m.r. 25), but the differences obtained on the other treatments were not significant.

The differences in the mean apparent digestibility values for fat were greater than those for the dry matter digestibilities. The value of 79.7% obtained for the tallow treatment was lower than the value of 88.7% obtained for the tallow treatment in experiment 1. Although the mean apparent digestibility for the interesterified tallow treatment (m.r. 26) was appreciably higher than that for the untreated tallow treatment (m.r. 25), the increase as in experiment 1 failed to achieve significance. However, the digestibility of the fat of treatment m.r. 29 was significantly higher ($P < 0.05$) than that of treatment m.r. 30, indicating a substantial and significant improvement from interesterification when the tallow contained an addition of glyceryl tributyrate. The mean digestibilities of 96.3% and 94.7% obtained for the fats containing butyric acid included by interesterification (m.r. 28 and m.r. 29) were satisfactorily high and similar to the value of 96.5% obtained for butterfat in experiment 1. The transformed results indicate that the digestibilities of the fats of treatments m.r. 28 and m.r. 29 were significantly higher than that of the tallow treatment m.r. 25 ($P < 0.01$). The results for treatment m.r. 30 show the importance of the method of incorporation of butyric acid into tallow, since simple admixture of tributyrin with tallow gave a

TABLE 3. Fatty acid composition of dietary fats and mean digestibility data in experiment 1

	Diet						
	Type of fat						
	m.r. 19 Butterfat	m.r. 20 Interesterified butterfat	m.r. 21 Tallow	m.r. 22 Interesterified tallow	m.r. 23 Interesterified supplemented tallow	m.r. 24 Tallow admixed with supplementary triglycerides	Standard error of a mean
Fatty acid composition, weight %							
4:0	3.3	3.5	—	—	—	—	—
6:0	1.9	1.8	—	—	—	—	—
8:0	1.2	1.0	—	—	—	—	—
10:0	2.7	3.0	—	—	—	—	—
12:0	2.9	3.1	2.3	3.1	1.7	1.7	—
14:0	12.0	11.5	3.7	4.5	11.8	12.2	—
16:0	25.7	25.3	24.9	23.5	21.3	20.6	—
16:1	1.6	2.0	2.4	2.3	1.9	2.7	—
18:0	13.5	12.9	26.5	25.6	22.2	21.5	—
18:1	30.7	29.9	33.9	31.9	33.8	33.5	—
Mean apparent digestibility of fatty acids, %							
14:0	98.3	95.2	93.3	97.6	96.5	96.7	—
16:0	96.1	94.5	85.3	92.1	92.1	91.5	—
18:0	92.4	91.3	79.7	90.3	88.3	88.9	—
18:1	98.3	97.6	97.1	95.7	97.7	91.6	—
Transformed means							
14:0	82.9	78.6	76.1	81.5	79.8	80.7	2.03
16:0	79.2	77.2	69.0	74.5	72.2	74.5	2.13
18:0	75.0	73.8	64.9	72.8	68.0	72.0	2.65
18:1	83.6	82.1	81.4	80.9	81.9	75.2	1.83

TABLE 4. Chemical composition of milk replacers and mean digestibility and nitrogen retention data in experiment 2

	Diet						
	Type of fat						
	m.r. 25 Tallow	m.r. 26 Interesterified tallow	m.r. 27 Interesterified, reduced stearic acid, tallow	m.r. 28 Interesterified, reduced stearic acid, added butyric acid, tallow	m.r. 29 Interesterified, added butyric acid, tallow	m.r. 30 Tallow admixed with tributyrin	Standard error of a mean
Chemical composition of milk replacer							
Fat, % in dry matter	17.3	16.0	16.1	15.2	17.5	15.3	—
Crude protein, % in dry matter	28.7	28.9	29.0	28.9	28.4	28.7	—
Mean apparent digestibility adjusted for residual effects, %							
Dry matter	92.4	95.3	93.9	96.4	96.3	92.7	—
Fat	79.7	89.2	87.9	96.3	94.7	84.0	—
Crude protein	92.0	94.7	93.1	95.0	92.2	95.6	—
Nitrogen retention adjusted for residual effects, %	35.2	34.2	27.9	37.9	30.7	37.4	—
Transformed means							
Dry matter digestibility	74.6	77.5	76.3	79.1	79.1	75.2	1.44
Fat digestibility	64.5	70.9	71.5	78.9	77.6	68.2	2.87
Crude protein digestibility	73.6	76.7	74.7	77.1	73.8	77.8	1.76
Nitrogen retention	36.4	35.8	31.9	38.0	33.7	37.7	2.16

mean digestibility which although rather higher (non-significantly) than the value obtained for untreated tallow, was substantially less than the values obtained for the two interesterified butyrated tallow treatments. Indeed, almost half of the increase in digestibility on treatment m.r. 30 can be attributed to the addition of 10% of a highly digestible triglyceride. In addition, two out of the six calves showed some reluctance to drink the milk replacer in which this fat was incorporated. The digestibility of the fat of treatment m.r. 27 compared with that of treatment m.r. 26 clearly shows that reduction of stearic acid content in the fat did not improve digestibility.

As in experiment 1, the mean apparent crude protein digestibilities were all high, with no significant differences between treatments. Again, the mean percentages of nitrogen retained were low, although the figure of 36.5% for the tallow treatment found in experiment 2 is similar to the value of 36.7% obtained in experiment 1. There were no significant differences between treatments.

The fatty acid compositions of the dietary fats and the digestibilities of myristic, palmitic, stearic and oleic acids were determined as in experiment 1 and are given in Table 5. The results show that the lower mean apparent digestibilities of tallow and interesterified tallow in experiment 2 as compared with experiment 1 were associated with a general lowering of the mean apparent fatty acid digestibilities in experiment 2. Again, the major differences were seen in the mean apparent digestibilities of the two long-chain, saturated fatty acids, palmitic and stearic. The transformed results indicate that the digestibilities of stearic acid for the two interesterified butyrated tallow treatments m.r. 28 and m.r. 29 were significantly higher ($P < 0.01$) than the digestibility for the tallow treatment m.r. 25. The mean digestibility of stearic acid for treatment m.r. 29 was also significantly higher than its digestibility for the interesterified tallow treatment m.r. 26 and the tallow admixed with tributyrin treatment m.r. 30 ($P < 0.05$). Although the digestibility of stearic acid for the interesterified tallow treatment was substantially greater than its digestibility for the untreated tallow treatment, the difference just failed to achieve significance. The mean digestibilities of palmitic acid showed similar differences to those of stearic acid, the digestibility for treatment m.r. 29 being significantly higher than that for treatment m.r. 25 ($P < 0.01$) and that for treatment m.r. 30 ($P < 0.05$). As with stearic acid, the higher digestibility of palmitic acid for the interesterified tallow treatment than for the untreated tallow treatment failed to achieve significance.

The differences in the mean apparent digestibility values for myristic acid were not as large as those for stearic and palmitic acid. None achieved significance, although the difference between the digestibilities of myristic acid for treatments m.r. 25 and m.r. 29 just failed to do so. The mean apparent digestibility values for oleic acid were all low in comparison with the values determined in experiment 1. The digestibilities for treatments m.r. 28 and m.r. 29 were significantly higher than those for treatments m.r. 25 and m.r. 30.

4. Discussion

Randomisation of the fatty acids in the triglyceride molecules by interesterification had little or no effect on the digestibility of butterfat, indicating that there is no specific triglyceride structure responsible for the high digestibility of butterfat. In contrast, the

TABLE 5. Fatty acid composition of dietary fats and mean digestibility data in experiment 2

	Diet						
	Type of fat						
	m.r. 25 Tallow	m.r. 26 Interesterified tallow	m.r. 27 Interesterified, reduced stearic acid, tallow	m.r. 28 Interesterified, reduced stearic acid, added butyric acid, tallow	m.r. 29 Interesterified, added butyric acid, tallow	m.r. 30 Tallow admixed with tributyrin	Standard error of a mean
Fatty acid composition, weight %							
4:0	—	—	—	11.2	6.1	10.9	—
14:0	3.0	2.8	3.3	3.2	2.8	2.7	—
16:0	21.7	20.7	15.1	13.6	22.4	21.5	—
16:1	3.7	3.4	4.6	4.2	3.5	3.7	—
18:0	19.5	20.8	13.0	10.7	23.0	20.8	—
18:1	32.0	28.0	44.0	44.6	30.7	30.3	—
Mean apparent digestibility of fatty acids, adjusted for residual effects, %							
14:0	90.4	91.8	92.8	95.7	97.1	91.0	—
16:0	81.9	87.6	89.0	93.3	96.5	88.3	—
18:0	70.4	83.4	82.7	90.7	95.2	83.5	—
18:1	89.5	93.1	94.6	98.3	96.9	90.0	—
Transformed means							
14:0	72.0	73.4	74.4	78.0	80.2	72.5	2.79
16:0	64.8	69.3	70.6	75.0	79.2	70.0	2.71
18:0	57.0	65.9	65.4	72.3	77.3	66.1	3.51
18:1	71.0	74.7	76.5	82.5	79.9	71.5	2.81

results of both experiment 1 and experiment 2 suggest that some improvement in the digestibility of tallow was effected by the randomisation of the triglyceride fatty acids by interesterification. However, the differences in digestibility obtained were not significant. In considering the effect of interesterification on the digestibility of tallow, it is interesting to compare the results of the various supplemented tallow treatments. Incorporation of supplementary fatty acids into tallow by interesterification will, of course, result in the randomisation of the arrangmeent of triglyceride fatty acids already present in the fat and the results show that for each treatment in which tallow was supplemented with additional fatty acids by interesterification, myristic and oleic acids in experiment 1 and butyric acid in experiment 2, the mean apparent digestibility value was higher than for the corresponding treatment in which tallow was supplemented by admixture only. Furthermore, the results in experiment 2 did show that interesterification of tallow containing an addition of glyceryl tributyrate effected a significant improvement in fat digestibility. One possible effect of randomisation of the fatty acid arrangement of tallow is that it may result in the production of greater quantities of amphiphilic species such as 2-mono-olein on hydrolysis by pancreatic lipase, which could enhance the micellar solubilisation of the less polar products of lipolysis. Alternatively, it may result in a greater proportion of the long-chain, saturated acids being located in the 2-position and consequently greater production of monoglycerides of these acids on lipolysis. Mattson and Volpenhein[28] have shown that, in rats, palmitic acid was better absorbed as the monoglyceride than as the free acid.

Several methods of altering the proportion of fatty acids of relatively lower digestibility than others in tallow were investigated. The addition of a quantity of highly digestible fatty acids to tallow would be expected to have a beneficial effect on the digestibility of the fat as a whole, simply by replacing some of the long-chain, saturated fatty acids which are less-well digested. However, the presence of the supplementary fatty acids, myristic and oleic, did not seem to have any appreciable effect on the digestion of the long-chain, saturated acids or the fat as a whole. The method of removal of long-chain saturated fatty acids used in experiment 2 proved successful in reducing the content of both stearic and palmitic acids in the fat of treatment m.r. 27. However, when the results for this treatment are compared with those for the interesterified ordinary tallow treatment, m.r. 26, it is evident that the reductions did not significantly improve either the digestibilities of the acids concerned or the fat as a whole. This result was unexpected in view of the detrimental effect of a high proportion of long-chain, saturated fatty acid in a dietary fat on its digestibility observed in several different species.[15–19, 29]

The most successful chemical modification to beef tallow in this series of experiments was the incorporation of butyric acid into the fat by interesterification which effected a considerable improvement in the digestibility of tallow. The digestibilities of the two butyrated tallow treatments m.r. 28 and m.r. 29 were, in fact, comparable to digestibility values determined for butterfat and this is in accordance with the findings of Hopper, Gardner and Johnson[20] who achieved weight gains for veal calves on a diet containing tributyrin lard which equalled those of calves on a diet containing butterfat. It is interesting to consider how the overall improvement in fat digestibility may have been achieved. A direct comparison of treatments m.r. 25 and m.r. 26 shows that in experi-

ment 2, the interesterification of tallow was associated with an improvement in digestibility of 9.5%. The further improvement in fat digestibility from 89.2% on treatment m.r. 26 to 94.7% on treatment m.r. 29 suggests that the total improvement in the digestibility of tallow on incorporation of 6.1% of butyric acid by interesterification was not due solely to a combined effect of interesterification and inclusion of a very highly digestible fatty acid, but that the inclusion of butyric acid in the fat also enhanced the digestibility of the other fatty acids of tallow and hence of the fat as a whole. The digestibility of the remaining 93.9% of interesterified tallow in treatment m.r. 29 was calculated to be 5.2% greater than that of the interesterified tallow in treatment m.r. 26. This suggestion is supported by the results for the mean apparent digestibilities of palmitic and stearic acids. The digestibility of palmitic acid increased from 87.6% in interesterified tallow to 96.5% in butyrated tallow. Similarly, the digestibility of stearic acid increased from 83.4% in interesterified tallow to 95.2% in butyrated tallow. In conclusion, it is evident that a combination of supplementation with glyceryl tributyrate and interesterification was necessary to achieve a high digestibility for tallow comparable to that for butterfat.

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A Study on Iraqi Date Extract: "Dibis" as Substrate for Mycological Fat Production

Kaiser Naguib, I. A. Al-Sohaily and A. S. Al-Sultan

Biology Department, College of Science, Baghdad University, Iraq
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A date extract known locally in Iraq as "dibis" was used as substrate for mycological fat formation by *Penicillium lilacinum*, *P. soppii* and *Aspergillus nidulans*. The three fungi were grown on dibis medium at three concentrations. Carbohydrates readily disappeared but this was accompanied by poor fungal growth and low yields of fat.

Fungal growth on dibis media enriched with asparagine led to heavy mycelial yields together with higher fat contents. Both economic and fat coefficients rose considerably. It is concluded that nitrogen deficiency in dibis media is at least one of the major factors against good fungal growth and high fat formation.

1. Introduction

The potential capacity of many fungi to produce fat encouraged several workers to try to apply these fungi to produce fat from cheap carbohydrate sources. Thus, Nadson and Konokitina[1] used potatoes. Fink *et al.*[2, 3] used milk whey, molasses and sulphite liquor. Damm[4] also used molasses. Sweet potatoes were used by Kaibara,[5] Ikeda[6] and Naguib and Louis.[7]

Extensive studies on the fat-forming capacities of fungi were also carried out. A survey of a number of fungi (40 species) was carried out by Woodbine, Gregory and Walker.[8] Many fungi were found to have high fat-forming capacity particularly *Penicillium* and *Aspergillus* species.

In the present investigation, a product obtained from Iraqi dates known locally as "dibis" was used. Dibis is already used as a foodstuff rich in sugar content. It is produced on a large scale and partly used for local consumption as food and for industry and partly for export. It was thought, therefore, to use dibis as a carbohydrate substrate for three fungi to find how far this source of carbohydrate can be used for mycological fat production.

The nitrogen content of dibis ranges from 0.1 to 0.2%, a value too low to support good fungal growth. Therefore, supplementing dibis with an external source of nitrogen was also attempted.

2. Materials and methods

The date extract or dibis used as substrate is obtained from the "Zohdy" variety of date palm and registered in the market as AA product. Dibis is produced by cooking dates in steam for 6 to 12 h. The product is then decanted and concentrated at 60 °C

under low pressure until the water content drops to about 25 to 28%. Dibis is a brown liquid similar to molasses in appearance and viscosity. It is very sweet in taste due to the presence of high amounts of soluble sugars amounting to 65 to 67%. About 60% of the sugars are in the form of monosaccharides, the rest being disaccharides.

2.1. Fungi

The fungi used in the present study were *Penicillium lilacinum* Thom., *P. soppii*, Zaleski and *Aspergillus nidulans* Eidam obtained from Centraalbureau voor Schimmelcultures, Baarn, Holland. They were subcultured every 2 weeks and were grown on slopes for sporulation using Dox's agar medium.

2.2. Composition of media and cultural conditions

Media used in the present work were made by dissolving the weighed amount of dibis in hot water which was then cooled and other ingredients added when required and finally made up to volume. Three media were prepared at three dibis concentrations, namely, 7.5, 15 and 30% by weight. The media were then distributed in 250-ml conical flasks each receiving 50 ml of medium. Flasks were plugged and autoclaved at 10 lb (4.54 kg) for 15 min. After cooling, the flasks were inoculated with a spore suspension prepared from one-week-old slant cultures. The culture flasks were incubated at 25 °C and after 10 days triplicate sets of cultures were withdrawn for study.

2.3. Methods of analysis

The fungal mats were separated by filtration, they were dried and the fat contents were determined. Drying and fat estimation were carried out as previously given by Woodbine *et al.*[8]

The filtrate was made up to volume and analysed for its total carbohydrate content. This was done after the manner described by Said and Naguib[9] by a modified Schaffer–Hartmann's method after acid hydrolysis.

3. Results

3.1. Fungal growth and fat formation on dibis medium

The three fungi were grown for 10 days on unsupplemented dibis media at three concentrations, namely, 7.5, 15 and 30%. The pH value of the perpared media was found to be around 5.5.

The dry weight and the fat content were determined for the fungal mats, while the total carbohydrate content in terms of glucose was determined for the culture filtrates as well as for the initial samples.

3.1.1. Total carbohydrate uptake and dry weights of fungal mats

The mean total carbohydrate uptake, the mean dry weights and the economic coefficients are given in Table 1.

The uptake of carbohydrates from the culture media by the three fungi increased with increase in dibis concentration with no appreciable differences between these fungi. But the dry weight of fungal mat increased with increase in dibis concentration in the case of two fungi only, namely, *P. soppii* and *A. nidulans*. In *P. lilacinum*, on the

other hand, the dry weight dropped in 30% concentration even below that in 7.5% concentration, though with much higher carbohydrate uptake.

These results are clearly reflected in the economic coefficients. The economic coefficient reported for ordinary growth conditions lies between 20 and 25. The values reported in the Table seem to be comparatively low, since they range between 2.6 and 14.1. Moreover, the change in the economic coefficient with increase in concentration differed with the fungus used. In the case of *P. lilacinum* there was a gradual decrease with increase in concentration, while in *P. soppii*, it increased with increase in concentration. In *A. nidulans*, the intermediate concentration gave the lowest economic coefficient.

3.1.2. Fat content of the fungal mats

The mean fat content of the fungal mats, the fat percentage as well as the fat coefficients are given in Table 2.

Table 2 clearly shows that very low fat contents were obtained in mats of the three fungi used and in the three dibis concentrations, in spite of the high carbohydrate supply in the culture media. The biggest yield of fat was obtained by *A. nidulans* on 30% dibis, but this constituted only 4% of the dry mycelium. On the other hand, a relatively high fat percentage was obtained by *A. nidulans* on 15% dibis medium, i.e. 19% and by *P. soppii* on 7.5% dibis medium, i.e. 19.3% but, owing to the high consumption of carbohydrates in these two cases, the fat coefficients were 0.4 and 1.6, respectively. Fat coefficients were calculated from the weight of fat produced in g/100 g of sugar consumed. The fat coefficient under suitable conditions for fat formation in synthetic media could be raised up to 17 with *P. lilacinum*,[10] to 12.5 with *P. soppii*[11] and to 14.5 with *A. nidulans*.[12]

The depressed capabilities of the present fungi for fat formation may therefore be ascribed to some difficulties in the dibis media and consequently dibis as it is, cannot support good growth and high fat formation.

3.2. Fungal growth and fat formation on dibis medium supplemented with asparagine

The presence of very low nitrogen level (about 0.1% N) in dibis was thought to be a big handicap to good fungal growth and can at least partly account for the low mycelial yields and the subsequent low fat contents obtained in the previous experiment. In the present experiment, the dibis medium was enriched by a nitrogen source in the form of asparagine. Asparagine was added at the concentration of 100 mg of N/100 ml of medium, in order to give a level that has been found to be favourable for fat formation by several investigators such as Heide,[13] Naguib and Walker[14] and Naguib and Saddik.[15] The dibis was added at the three concentrations used in the previous experiment so that three different C:N ratios were established. The C:N ratio was found to be one of the major factors affecting fat formation.[14, 16] The media in this experiment were adjusted to pH 7 which is more favourable for fat formation.

3.2.1. Total carbohydrate uptake and dry weights of fungal mats

The mean total carbohydrate uptake, the mean dry weights of the fungal mats as well as the economic coefficients are given in Table 3.

TABLE 1. Mean total carbohydrate uptake, mean dry weight of fungal mats and the economic coefficient in three dibis concentrations

	Dibis concentration								
	7.5%			15%			30%		
	Carbohydrate uptake[a]	Dry wt[b]	Economic coefficient[c]	Carbohydrate uptake[a]	Dry wt[b]	Economic coefficient[c]	Carbohydrate uptake[a]	Dry wt[b]	Economic coefficient[c]
Initial	5.380			10.800			19.580		
Penicillium lilacinum	4.804	675	14.06	9.778	760	7.77	17.196	448	2.60
P. soppii	4.764	376	7.80	9.936	833	8.38	18.059	1733	9.60
Aspergillus nidulans	4.494	257	5.71	9.697	419	4.33	17.852	1109	6.23

[a] Carbohydrate uptake is expressed in g of glucose/100 ml of medium.
[b] Dry weight is expressed in mg/100 ml of medium.
[c] Economic coefficient is expressed in g of dry mycelium/100 g of sugar consumed.

TABLE 2. Mean fat content in fungal mycelium and the fat coefficient in three dibis concentrations

	Dibis concentration								
	7.5%			15%			30%		
	Total fat (mg/100 ml)	Fat (%)	Fat coefficient[a]	Total fat (mg/100 ml)	Fat (%)	Fat coefficient	Total fat (mg/100 ml)	Fat (%)	Fat coefficient
Penicillium lilacinum	91	13.5	1.9	78	10.3	0.8	40	9.0	0.2
P. soppii	60	16.0	1.3	160	19.3	1.6	293	16.9	1.6
Aspergillus nidulans	19	7.4	0.4	361	8.6	0.3	449	4.1	0.3

[a] Fat coefficient is expressed as g of fat/100 g of sugar consumed.

Results of carbohydrate uptake do not show any appreciable rise in the presence of asparagine over those obtained in the previous experiment. On the contrary, uptake was suppressed in some cases, particularly in the case of *P. lilacinum*. In spite of this, the development of fungal mycelium was very high in all dibis concentrations used and by the three fungi studied. This was eventually reflected in very high values for the economic coefficient; the carbohydrate uptake being largely utilised for cellular synthesis. In this respect, *P. soppii* was superior to both *P. lilacinum* and *A. nidulans* in accumulation of mycelium, though *A. nidulans* gave the highest economic coefficient. It can also be noted that, in the three fungi, the economic coefficient decreased with increase in dibis concentration.

3.2.2. Fat content of the fungal mats

The mean total fat content, the fat percentage in mycelium and the fat coefficients are given in Table 4.

On the whole, larger amounts of fat could be produced in the presence of asparagine, compared with those obtained in its absence; the strongest fat producer being *P. soppii*. This can also be shown through the high fat coefficients. Nevertheless, most values obtained are still below those obtained under favourable conditions for fat formation.[17, 18]

Though a high C:N ratio in the culture medium is known to be more conducive to fat formation in fungal mycelium, in the experiments reported here this was not always the case. The intermediate dibis concentration gave the highest total fat and fat percentage in the case of *P. soppii* and *A. nidulans*. With *P. lilacinum*, the lowest concentration was the most favourable for fat formation as it was for growth.

4. Discussion

Results presented in this investigation clearly show that the three fungi used and in particular *P. lilacinum* gave poor growth on the three dibis concentrations, namely, 7.5, 15 and 30%. This was accompanied by high consumption of carbohydrates. Consequently, the economic coefficients were low. It seems therefore that carbohydrates that disappeared from culture media were not properly utilised. A high rate of respiration under these conditions may partly account for the big loss of carbohydrates that were not utilised in cellular synthesis.

With regard to fat formation, the fungal mats grown on dibis media at all concentrations used unexpectedly had very low fat contents ranging between 4.1 and 19.3% by weight. Using synthetic media favourable for fat formation, Murray and Walker[11] obtained a fat yield amounting to 35 to 40% of the dry mycelium with *P. soppii*, Naguib and Walker[19] obtained 53% with *P. lilacinum* and Naguib[20] obtained 46% with *A. nidulans*.

Owing to this very poor fat formation and the very high carbohydrate consumption, the fat coefficients were very low ranging between 0.2 and 1.9.

In addition to its low nitrogen content, dibis is also acidic in reaction. Asparagine was therefore added to enrich the dibis medium with nitrogen and the pH was adjusted to 7. Asparagine was found to be superior to ammonium or nitrate for fat promotion

TABLE 3. Mean total carbohydrate uptake, mean dry weight of fungal mats and the economic coefficient in dibis media supplemented with asparagine

	Dibis concentration								
	7.5%			15%			30%		
	Carbohydrate uptake[a]	Dry wt[b]	Economic coefficient[c]	Carbohydrate uptake[a]	Dry wt[b]	Economic coefficient[c]	Carbohydrate uptake[a]	Dry wt[b]	Economic coefficient[c]
Initial	5.120			10.850			19.980		
Penicillium lilacinum	3.652	1587	43.5	4.464	1333	29.9	3.800	1086	28.6
P. soppii	5.120	1824	35.6	0.280	3086	30.1	15.430	4500	29.2
Aspergillus nidulans	3.250	1676	51.5	6.48	2990	46.1	8.990	3646	40.5

[a] Carbohydrate uptake is expressed in g of glucose/100 ml of medium.
[b] Dry weight is expressed in mg/100 ml of medium.
[c] Economic coefficient is expressed in g of dry mycelium/100 g of sugar consumed.

TABLE 4. Mean fat content in fungal mycelium and the fat coefficient in dibis media supplemented with asparagine

	Dibis concentration								
	7.5%			15%			30%		
	Total fat (mg/100 ml)	Fat (%)	Fat coefficient[a]	Total fat (mg/100 ml)	Fat (%)	Fat coefficient[a]	Total fat (mg/100 ml)	Fat (%)	Fat[a] coefficient
Penicillium lilacinum	217	13.7	5.94	117	8.75	2.62	103	9.45	2.71
P. soppii	351	19.3	6.86	641	20.7	6.23	580	12.9	3.77
Aspergillus nidulans	130	7.8	4.00	413	13.8	13.8	343	9.4	3.82

[a] Fat coefficient is expressed as g of fat/100 g of sugar consumed.

using *A. nidulans*.[15] Several workers such as Prill, Wenck and Peterson[21] using *A. fischeri*, Blinc and Bojec[22] using *Mucor mucedo*, Ikeda[23] using *A. candidus* and Garrido and Walker[17] using *A. nidulans* have shown that a pH around neutrality is more suitable for fat formation.

The economic coefficients were found to rise in all fungi studied with the addition of asparagine alone at pH 7 from values ranging from 2.6 to 14 to values ranging from 28.6 to 51.6, with much heavier mycelial yields. *A. nidulans* was the fungus most affected but the greatest amounts of mycelium were obtained by *P. soppii* at the highest dibis concentration. Asparagine had therefore been involved in active building up of cellular material. As might be expected, appreciable amounts of fat were produced by the three fungi; the highest amount being obtained by *P. soppii* on the 15% dibis concentration. The general trend in fat percentage did not markedly change by addition of asparagine but the fat coefficient rose considerably reaching 20 times in some cases. However, fat accumulation did not show a direct response to the general increase in C:N ratio in dibis media from 7.5% to 30% dibis concentration, *P. lilacinum* showed the best fat formation on 7.5% dibis concentration, while *P. soppii* and *A. nidulans* showed maximum fat formation at 15%.

It is concluded therefore that nitrogen deficiency in dibis media is probably the major factor against good mycelial growth and that together with other factors it accounts for the low fat yield.

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Comparison of Acid-induced Conformation Changes Between 7 S and 11 S Globulin in Soybean Seeds

I. Koshiyama

Noda Institute for Scientific Research, Noda, Noda-shi, Chiba-ken, Japan
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The acid-induced conformation changes between 7 and 11 S globulin, the major storage proteins in soybean seeds, were compared by ultraviolet difference spectra, ultracentrifugation and optical rotatory dispersion. Maximum denaturation occurred at approximately pH 2 in both and dissociation of the proteins into subunits and unfolding of the polypeptide chains were observed simultaneously. However, both proteins showed apparent differences in their readiness to undergo acid-induced denaturation. The differences were particularly remarkable in the presence of 0.1 ionic strength sodium chloride.

1. Introduction

7 and 11 S globulin[a] are the most important components of the major proteins in soybean seeds (glycine max). Of late years, Fukushima[1] has shown that there are no appreciable differences in the internal structures of the two proteins. However, more recently Saio, Kamiya and Watanabe[2] indicated that there were significant differences between the physical properties of tofu-gels prepared from the crude 7 and 11 S components of defatted soybean meal, i.e. tofu-gel from the latter was much harder than that from the former, and that the differences were caused mainly by the number of sulphydryl groups contained in both.[3] Therefore, it is very interesting in practical and fundamental investigations of soybean proteins to compare the physical and chemical properties of pure preparations of 7 and 11 S globulin.

As to the acid-induced conformation changes, it has been shown that the sedimentation patterns of pure 7 S globulin are affected by the ionic strength of sodium chloride.[4] On the other hand, Wolf *et al.*[5] have shown that 11 S globulin is converted into a slowly settling component as the result of dissociation into subunits (polypeptide chains) at low pH and low ionic strength using the cold-insoluble fraction (c.i.f.) of soybean proteins and suggested that the dissociation of the protein affected by pH and ionic strength was due to forces of electrostatic repulsion between the subunits.

In this paper a further direct comparison of acid-induced conformation differences between the two proteins is made with the effect of ionic strength using pure preparations by ultracentrifugation, ultraviolet difference spectra and optical rotatory dispersion (o.r.d.).

[a] 7 and 11 S globulin were given the names of γ-conglycinin and glycinin, respectively, by Catsimpoolas.[6]

2. Experimental

2.1. Materials

7 S globulin was prepared according to the method described in previous papers.[7,8] 11 S globulin was purified from c.i.f. by gel filtration with Sephadex G100 and G200 as described previously.[9]

2.2. Methods

2.2.1. Sedimentation analysis

Ultracentrifugal analysis was performed with a Hitachi UCA-1 centrifuge at 51 000 rev./min and 20 °C.

2.2.2. Ultraviolet difference spectra

Difference spectra were measured with a Hitachi model ESP-2 recording spectrophotometer. The measurements were made using a protein concentration of 4.16×10^{-6} M for 7 S and 3.05×10^{-6} M for 11 S globulin. Molar absorptivity differences were calculated on the basis of 180 000 m.w. for 7 S[10] and 322 000 for 11 S globulin.[9]

2.2.3. Optical rotatory dispersion (o.r.d.)

O.r.d. measurements were made using a Japan Spectroscopic model o.r.d./u.v.-5 recording spectropolarimeter equipped with a xenon arc lamp and a photomultiplier.[9,11] For measurements in the visible and near-ultraviolet regions, a quartz cell with a light path length of 50 mm was used. The protein concentrations were 0.34% for 7 and 11 S globulin in 0.01 N-HCl and in 0.01 N-HCl containing 0.1 M-NaCl; 0.37 and 0.34% for native 7 and 11 S globulins, respectively, in 0.5 μmolar standard buffer[a] without 2-mercaptoethanol; and 0.42 and 0.28% for denatured 7 and 11 S globulins, respectively, in 0.01 M-Tris–HCl buffer containing 8 M-urea, pH 8.20.

In the ultraviolet region, a quartz cell with a light path length of 1.0 mm was employed with half the concentrations of those used in the visible and near-ultraviolet regions. The mean residue weight, M_0, of 107.4 calculated from the amino-acid composition[10] for 7 S and of 114.1 from that[12] for 11 S globulin were used and the absorption wavelength associated with the rotation, λ_c, was assumed to be 212 nm.

3. Results and discussion

3.1. Ultraviolet difference spectra

Difference spectra obtained when 7 and 11 S globulin solutions at different pH values were measured against solutions of identical concentration of native proteins in 0.5 μmolar standard buffer without 2-mercaptoethanol are shown in Figures 1(a) and 2(a), respectively. As shown in Figure 1, the gradual development of peaks at 280 to 281 and 288 to 289 nm demonstrated the increasing extent of acid denaturation for 7 S globulin, but the peak at 291 to 292 nm due to tryptophan groups,[14–16] of which there are 3 mol in a mol of 7 S,[10] could not be found. This result suggested that the

[a] Potassium phosphate buffer (0.0325 M-K_2HPO_4, 0.0026 M-KH_2PO_4, 0.01 M-2-mercaptoethanol and 0.4 M-NaCl, pH 7.60, ionic strength of 0.5)[13] was the 0.5 μmolar standard buffer.

tryptophan residues were located on the surface of the protein molecule. In 11 S globulin a shoulder at 291 to 292 nm, in addition to the peaks at 280 to 281 and 286 to 287 nm which represented both tryptophan and tyrosine groups,[14-16] appeared in proportion to acid denaturation.

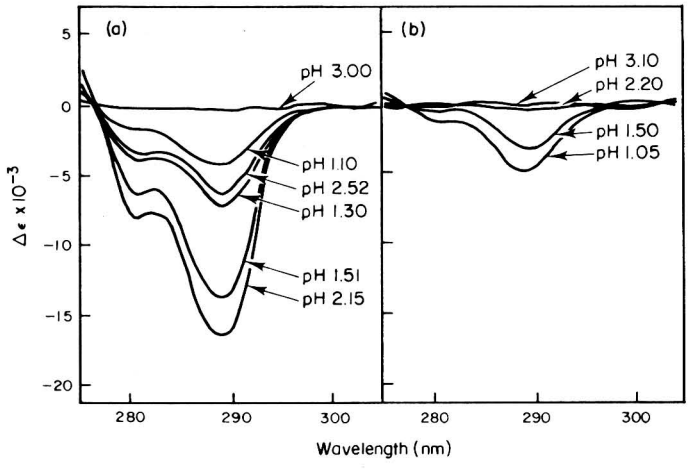

Figure 1. Acid-induced ultraviolet spectra difference of 7 S globulin. The protein was treated for 3 h in the indicated pH solutions with 0.01 M-CH_3COONa–HCl buffer (a) and in buffer containing 0.1 M-NaCl (b).

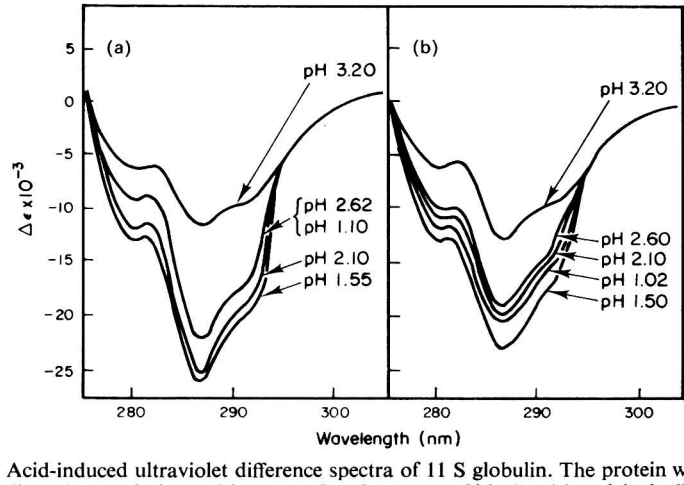

Figure 2. Acid-induced ultraviolet difference spectra of 11 S globulin. The protein was treated for 3 h in the indicated pH solutions with 0.01 M-CH_3COONa–HCl buffer (a) and in buffer containing 0.1 M-NaCl (b).

Judging from the values for molar absorptivity difference, maximum denaturation occurs at approximately pH 2 for both proteins. Further decrease of pH below this value resulted in reduction of the $\Delta\epsilon$ values in both proteins together. These results agreed well with that of Catsimpoolas, Wang and Berg[17] for 11 S globulin. This "red

shift" may be explained by the possible formation of associated structures of unfolded polypeptide chains (subunits) through hydrogen bonding of uncharged carboxylate groups and such an association may cause partial "reburying" of chromophore groups expressed as a "red shift" as suggested by Catsimpoolas *et al.*[17]

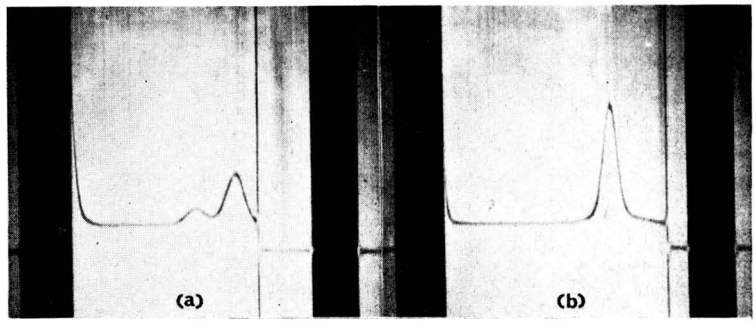

Figure 3. Effect of ionic strength on the acid-induced dissociation of 7 S globulin. The protein was treated overnight in 0.01 N-HCl (a) and in 0.01 N-HCl containing 0.1 M-NaCl (b). Photographs were taken after 40 min at 51 200 rev/min and 20 °C. Direction of sedimentation is from right to left.

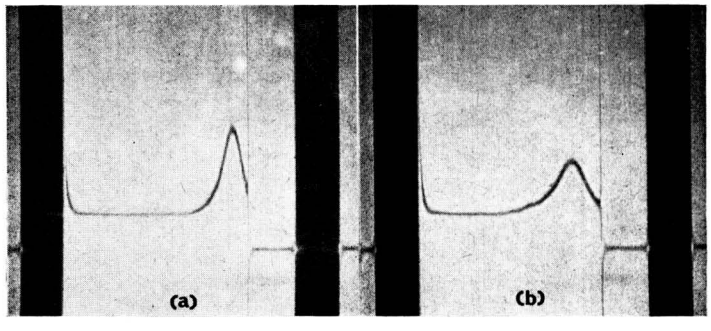

Figure 4. Effect of ionic strength on the acid-induced dissociation of 11 S globulin. The protein was treated overnight in 0.01 N-HCl (a) and in 0.01 N-HCl containing 0.1 M-NaCl (b). Photographs were taken after 40 min at 51 200 rev/min and 20 °C. Direction of sedimentation is from right to left.

However, the characteristic "denaturation blue shift" of 7 S globulin observed at low ionic strength [Figure (a)] was much smaller in the presence of 0.1 ionic strength sodium chloride as shown in Figure 1(b). Even at pH 2, at which the $\Delta\epsilon_{max,}$ value was given in low ionic strength, the exposure of tyrosine groups could be scarcely found. On the other hand, the effect was small for 11 S as shown in Figure 2(b). Rather, it may safely be said that denaturation is not affected very much.

3.2. Ultracentrifugation

Sedimentation behaviour for 7 and 11 S globulin at pH 2 (in 0.01 N-HCl), at which maximum exposure of the chromophore groups in the ultraviolet difference spectra of both was observed, is illustrated in Figures 3(a) and 4(a), respectively. Both proteins were apparently converted into slowly sedimenting components having the

sedimentation constant ($s_{20,w}$) of 1.92 S for 7 S and 2.48 S for 11 S, but dissociation of 7 S was incomplete after overnight treatment in 0.01 N-HCl as compared with 11 S. The faster sedimenting dissociated component from 7 S shown in Figure 3(a), having the sedimentation constant of 5.47 S, almost disappeared as the concentration of the slower sedimenting component increased after prolonged treatment for 12 days.

The effects of ionic strength on the ultracentrifugal patterns of both proteins by addition of 0.1 M-NaCl to 0.01 N-HCl are shown in Figures 3(b) and 4(b). As shown in Figure 3(b), 7 S kept a sedimentation pattern of native protein having $s_{20,w}$ value of approximately 7 S. On the other hand, the effect was scarcely found in 11 S as shown in Figure 4(b), though the sedimentation pattern changed to a slightly slower sedimenting component ($s_{20,w}$ = 3.75 S) than that in 0.01 N-HCl.

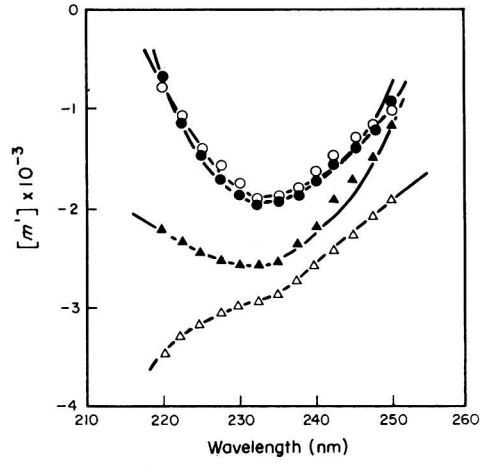

Figure 5. Ultraviolet o.r.d. curves of 7 S globulin in 0.5 μmolar standard buffer (—○—○—); 0.01 M Tris–HCl buffer containing 8 M-urea, pH 8.20 (—△—△—); 0.01 N-HCl (—▲—▲—); and 0.01 N-HCl containing 0.1 M-NaCl (—●—●—).

3.3. Optical rotatory dispersion

The acid-induced denaturation behaviour of the two proteins was further compared by o.r.d. The o.r.d. curves of 7 and 11 S globulin derived under various conditions in ultraviolet region between 220 and 250 nm were compared in Figures 5 and 6, respectively. As shown in Figure 5, 7 S in 0.01 N-HCl still slightly showed a broad Cotton trough near 233 nm. This result may indicate that the unfolding of the gross structure is incomplete. However, in case of 11 S globulin, the Cotton effect was completely lost in 0.01 N-HCl and, consequently, the acid-induced o.r.d. curve came fairly near to that of denatured protein in 8 M-urea solution as shown in Figure 6. This may be nothing but almost complete unfolding corresponding to that induced with 8 M-urea.

On the other hand, addition of 0.1 M-NaCl to 0.01 N-HCl apparently gave stability to the gross structure of 7 S, i.e. the o.r.d. curve agreed well with that of native protein (Figure 5), whereas the effect for 11 S globulin was scarcely observed, as shown in Figure 6.

The same results were also obtained as those in the visible and near-ultraviolet regions between 300 and 600 nm. The resulting data treated by Moffitt–Yang's equation[18, 19] are shown in Table 1. If the values of a_0 are assumed to represent the degrees of unfolding in the gross structure of the proteins, the a_0 value of 7 S in 0.01 N-HCl may indicate incomplete unfolding and that in 0.01 N-HCl containing 0.1 M-NaCl may

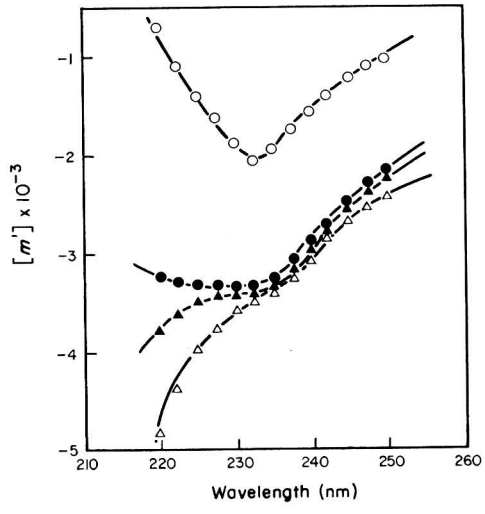

Figure 6. Ultraviolet o.r.d. curves of 11 S globulin in 0.5 μmolar standard buffer (—○—○—); 0.01 M-Tris–HCl buffer containing 8 M-urea, pH 8.20 (—△—△—); 0.01 N-HCl (—▲—▲—); and 0.01 N-HCl containing 0.1 M-NaCl (—●—●—).

TABLE 1. Optical rotatory dispersion data calculated from Moffitt–Yang's equation for 7 and 11 S globulin in 0.5 μmolar standard buffer, 0.01 M-Tris-HCl buffer containing 8 M urea, pH 8.20, 0.01 N-HCl and 0.01 N-HCl containing 0.1 M-NaCl

	7 S			11 S		
	a_0	b_0	λ_c (nm)	a_0	b_0	λ_c (nm)
Native	−246	−38	226	−246	−33	223
8 M-urea[a]	−600	0	216	−556	−5	215
0.01 N-HCl[a]	−454	−6	219	−542	0	217
0.01 N-HCl + 0.1 M-NaCl[a]	−279	−27	225	−510	−3	217

[a] Overnight treatment.

be almost equal to that of the native protein assuming the a_0 values in 8 M-urea and the 0.5 μmolar standard buffer for complete unfolding and native protein, respectively. However, the a_0 value of 11 S in 0.01 N-HCl coincided fairly well with that in 8 M-urea. Even if ionic strength increased, considerable unfolding of the gross structure was observed from the a_0 value. The same results were obtained from the dispersion constant, λ_c, determined by the one-term Drude equation.[18, 19]

The results from ultraviolet difference spectra, ultracentrifugation and o.r.d. suggested that acid-induced conformation changes of both proteins were not only due to dissociation of the proteins into subunits by electrostatic repulsion of charged groups but also due to destruction of the gross structures and unfolding of the polypeptide chains. In other words, it might be impossible to dissociate both proteins into the subunits without unfolding of their internal structures. The same result was also observed in the treatments of 7 S^{20} and 11 S globulin[17] with urea or guanidine hydrochloride.

However, there were apparent differences in the readiness of acid-induced denaturation between 7 and 11 S globulin. In particular, the differences were remarkable at higher ionic strengths. This suggested that the strength of the internal binding forces which combined the subunits or kept the overall structures were different between 7 and 11 S globulins. Considered from another viewpoint, the amino-acid group participating in the formation of the binding forces were presumed to be different for the two proteins.

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Influence of Glucosinolates and a Tentative High-molecular Detrimental Factor on the Nutritional Value of Rapeseed Meal

E. Josefsson and L. Munck

Division of Chemistry, Swedish Seed Association, Svalöf, Sweden
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Feeding experiments with mice suggest that high-molecular weight compounds in rapeseed meal without direct relationship to glucosinolates have a detrimental effect on the nutritional value of the meal. This effect is precluded by treatment with ethanol or upon heating. Ethanol extraction improves the nutritional value of the residue as a result of the removal of non-essential nitrogenous compounds.

The nutritional value of the seed meal of the cultivar Bronowski was superior to that of the high-glucosinolate meal. A freeze-dried ethanol extract of the high-glucosinolate cultivar Sv Rigo was toxic to mice, especially when fed in combination with myrosinase, in contrast to ethanol extract from the low-glucosinolate cultivar Bronowski. A decrease of the glucosinolate content to a level below that of Bronowski did not effect the growth response of mice.

1. Introduction

Seed meals of *Brassica napus* L. and *B. campestris* L. contain 40 to 45% crude protein in the dry matter.[1] This protein has an amino-acid composition that is favourable for both food and feed.[2] But the use of the meal as a feed for non-ruminants is limited by its content of glucosinolates.[3] On hydrolysis by the native enzyme system myrosinase, the glucosinolates produce isothiocyanates or oxazolidinethiones, which evoke toxic effects in the animals.[3]

By extracting with water, with water in buffer solutions, or with organic solvents, it has been possible to remove the glucosinolates from the meal.[4–7] The extracted meals have generally displayed high nutritional values in feed tests[4–6] (Appelqvist and Munck unpublished results). Since the extractions cause losses of protein and would be expensive to carry out on a commercial basis, attempts are being made to reduce the glucosinolate content by plant breeding. As a result of a study of the glucosinolate content in various species and cultivars it was found that the cultivar Bronowski contains very low amounts of glucosinolates.[8] This cultivar is now widely used in plant breeding programmes to combine a high yield with a low-glucosinolate content.

It should not be taken for granted, however, that a low-glucosinolate content in a cultivar improves its value as a feed. Other products causing toxicity might be produced instead of glucosinolates. Therefore, seed meals of Bronowski and a cultivar with a

high-glucosinolate content were treated in various ways and compared as protein feeds for mice. The nutritional value of the meal of a breeding line with a still lower glucosinolate content than Bronowski was also studied. Furthermore, the effects of various technical treatments of the meals were tested.

2. Experimental

2.1. Preparation of meals

Seed of a high quality was obtained from the Oil Crops Division, Swedish Seed Association, Svalöf.

Whole seeds were heat-treated after adjustment of their moisture content to about 8%. During the heat treatments the seeds were kept in a closed, rotating vessel. The seeds were defatted with hexane in steel tubes according to Troëng,[9] filtered and washed with petroleum ether in Büchner funnels.

Meals prepared from non-heat-treated seeds were treated or extracted with ethanol. For ethanol treatments, 300 g of rapeseed meal was mixed with 3 l of 80% ethanol at room temperature. The mixture was stirred for 1 h. Then, the solvent was removed by evaporation *in vacuo* at 30 °C. For ethanol extractions, 300 g of rapeseed meal was mixed with 1.75 l of 80% ethanol either at room temperature or at 60 °C. The mixture was stirred for 30 min. After filtering in a Büchner funnel, the extracted meal was re-extracted twice with 1.40 l of 80% ethanol. Finally, the meal was washed in the Büchner funnel with 2 l of 80% ethanol. The ethanol extract was diluted with water and freeze-dried.

Myrosinase was prepared as described by Appelqvist and Josefsson.[10]

2.2. Analytical methods

Glucosinolate content was determined according to the method of Appelqvist and Josefsson[10] and myrosinase activity was estimated according to Schwimmer.[11]

2.3. Feeding experiments

Growth studies on CBA female and male mice (feeding period 14 or 20 days), Swiss × CBA female and male mice (feeding period 14 days) as well as specific pathogen-free NMRI male mice (feeding period 11 days) were performed mainly according to Munck.[12] For the dietary composition see Table 1.

3. Results

The results of the preliminary experiments in Table 2 show that the mice decreased in weight when fed with high-glucosinolate meal that had not been heat-treated. On the other hand, the mice gained in weight, although slowly, when fed on untreated low-glucosinolate meal (experiments I and II). Experiment III in the same Table shows that heat treatment of the seed greatly improved the nutritional value of the two meals, but the weight gains were significantly higher for the mice fed with low-glucosinolate meal.

Three rapeseed meals with different glucosinolate levels were included in the experiment in Table 3. According to this Table, a further decrease of the glucosinolate content below the level of Bronowski did not improve the growth of mice.

Table 4 shows that even as weak a heat treatment of the seed as 60 °C for 15 min improved the Bronowski meal and that higher temperatures resulted in further improvements. An ethanol treatment of non-heat-treated meal increased the nutritional value

TABLE 1. Dietary composition

A. Synthetic diet
   (1) Sample protein equivalent to 100 g of N × 6.25
   (2) Margarine, 50 g
   (3) Wheat starch, dry, 475 g
   (4) Salt mixture, 50 g[a]
   (5) (a) Vitamin mixture, 2 g[b]
   (b) 120,000 i.u. vitamin A, 60,000 i.u. vitamin D
   (c) 4 ml of vitamin E (3%)
   (d) Choline chloride, 2.5 g
   (e) Inositol, 1.5 g
   (6) Cellulose, add to 1000 g

B. Control diet
   (1) 950 g of barley meal
   (2) 50 g of salt mixture
   (3) 1 g of vitamin mixture; vitamins A, D, E; choline chloride, 1.5 g

Vitamins were dissolved in water at room temperature and mixed into 50 g of starch or barley meal and dried at 35 °C. All other components were mixed dry.

[a] 29.3 g of table salt, 81.7 g of KH_2PO_4, 12.0 g of $MgSO_4$, 80.1 g of $CaCO_3$, 5.7 g of $FeSO_4 \cdot 7\,H_2O$, 0.94 g of $MnSO_4 \cdot 2\,H_2O$, 0.06 g of $ZnCO_3$, 0.1 g of $CuSO_4$, 0.005 g of $CoCl_2$.

[b] Biotin, 0.1 g; folic acid, 0.25 g; vitamin K, 2.5 g; pyridoxal phosphate, 2.5 g; aneurin, 5.0 g; riboflavin, 5.0 g; nicotinamide, 10.0 g; Ca pantothenate, 20.0 g; vitamin B_{12}, 1000 γ; aerosil, 10.65 g; inositol, 50.0 g.

considerably, but less feed was consumed than when the meal had been extracted with ethanol. An addition of freeze-dried ethanol extract from Bronowski meal to a control diet, resulting in a glucosinolate level that was higher than in the diets with Bronowski meal, did not effect the weight gains, feed intakes or protein efficiency ratio (p.e.r.) values.

In the experiments presented in Table 5, all diets containing low-glucosinolate meal or extract from that meal were superior to the corresponding diets with a high-glucosinolate meal. Treatment with 80% ethanol increased the nutritional value of the two meals. Ethanol extraction at room temperature improved the meals as compared with ethanol treatment, although the effect on the Bronowski meal was statistically significant only with regard to the feed consumption. When the ethanol solution used for the extraction had a temperature of 60 °C more glucosinolates were removed from the high-glucosinolate meal and the myrosinase activity disappeared completely. The nutritional value of this meal was also increased considerably. Addition of freeze-dried extract of Rigo meal to the control diet decreased the nutritional value of this diet.

This effect was more prominent when myrosinase was added together with the ethanol extract. No significant effects were found on adding freeze-dried ethanol extract of Bronowski to the control diet with or without myrosinase.

TABLE 2. Weight gains of mice fed rapeseed meal with a high- (Rigo) or a low- (Bronowski) glucosinolate content

Experiment no.	Source of protein	Feeding period (days)	Weight gain (g)
I	Rigo, no heat treatment	14	−3.2 ± 0.2
	Bronowski, no heat treatment	14	+2.7 ± 0.4
II	Rigo, no heat treatment	20	−1.4 ± 0.2
	Bronowski, no heat treatment	20	+5.6 ± 0.4
III	Rigo. The seed was heat treated at 90 °C for 15 min before defatting	20	+8.9 ± 0.2
	Bronowski. The seed was heat treated at 90 °C for 15 min before defatting	20	+10.6 ± 0.2

Each value is the average for 6 female (experiment I) or 8 male (experiments II and III) mice (CBA) ± S.E.

TABLE 3. Weight gains, feed intakes and p.e.r. value of mice fed rapeseed meals with various glucosinolate content and without heat treatment

Source of protein	mg/g Dry meal Oxazolidine-thiones	Isothio-cyanates	Weight gain (g)	Feed intake (g)	p.e.r. value
Control (barley meal)	—	—	9.6	48.0	1.49
Rigo	11.2	3.7	—[a]	—	—
Bronowski	1.9	0.3	3.8	31.2	0.95
Sv 69/1023, selected line from Bronowski	0.1	0.1	3.6	30.1	0.92

Each value is the average for 5 male mice (Swiss × CBA).
[a] All animals died within 8 days.

The experiments reported in Table 6 were made in order to find the optimal time and temperature combination for heat treatment of low-glucosinolate meal at a constant moisture content. The differences between treatments were small and not significant, although there was a tendency for a greater weight gain and higher p.e.r. values when meals treated at 100 °C were included in the diets compared with meals treated at 90 °C. The meal treated with 80% ethanol produced lower gains than any of the heat-treated meals.

4. Discussion

The superiority of heat-treated Bronowski seed meal as compared with commercial rapeseed meals has been demonstrated by Salmon[13] for turkeys, by Lo and Hill[14] for mice and by Oliver, McDonald and Opuszyńska[15] for rats. When Bell, Youngs and

TABLE 4. Weight gains, feed intakes and p.e.r. values of mice fed rapeseed meal, cv. Bronowski, with various treatments

Source of protein	mg/g Diet		Myrosinase activity (% of untreated Bronowski meal)	Weight gain (g)	Feed intake (g)	p.e.r. value
	Oxazolidine-thiones	Isothio-cyanates				
Control (barley meal)	—	—	—	8.3	44.9	1.39
Bronowski, no treatment	0.30	0.12	100	2.2	28.8	0.64
Bronowski, heat-treated at 60 °C	0.30	0.12	82	3.7	33.4	0.95
Bronowski, heat-treated at 70 °C	0.30	0.12	127	4.3	36.0	0.99
Bronowski, heat-treated at 80 °C	0.30	0.12	57	6.0	40.0	1.21
Bronowski, heat-treated at 90 °C	0.30	0.12	5	6.9	42.1	1.39
Bronowski, treated with 80% ethanol	0.26	0.03	0	5.9	38.6	1.26
Bronowski, extracted with 80% ethanol	0.08	0.01	0	7.2	42.6	1.24
Control (barley meal) with addition of freeze-dried ethanol extract from Bronowski meal	0.42	0.18	0	8.1	43.9	1.39

Heat treatments were performed for 15 min on seed with 8% moisture. Each value is the average of 5 female mice (Swiss × CBA).

TABLE 5. Weight gains, feed intakes and p.e.r. values of mice fed glucosinolates of various levels

Source of protein	mg/g Diet		Myrosinase activity (% of untreated Rigo meal)	Weight gain (g)	Feed intake (g)	p.e.r. value
	Oxazolidine-thiones	Isothio-cyanates				
Control (barley meal)	—	—	—	10.8 ± 0.6	47.4 ± 1.2	1.72 ± 0.07
Rigo, no treatment	2.36	1.19	100	—[a]	—	—
Rigo, treated with 80% ethanol	2.03	0.77	15	−0.9 ± 0.6	23.4 ± 1.6	—
Rigo, extracted with 80% ethanol at room temp.	0.95	0.38	3	3.9 ± 0.3	33.4 ± 1.0	1.00 ± 0.06
Rigo, extracted with 80% ethanol at 60 °C	0.66	0.37	0	8.5 ± 0.8	40.7 ± 1.5	1.78 ± 0.12
Control + extract from Rigo	1.95	1.28	0	6.8 ± 0.4	36.0 ± 1.0	1.45 ± 0.06
Control + extract from Rigo + 0.4% myrosinase	1.95	1.28	100	2.3 ± 0.2	28.2 ± 1.0	0.64 ± 0.07
Bronowski, no treatment	0.19	0.09	51	4.9 ± 0.6	33.8 ± 1.0	1.30 ± 0.14
Bronowski, treated with 80% ethanol	0.11	0.02	0	8.5 ± 0.5	41.3 ± 0.9	1.68 ± 0.07
Bronowski, extracted with 80% ethanol at room temp.	0.08	0.00	0	10.0 ± 0.7	45.5 ± 1.7	1.87 ± 0.10
Bronowski, extracted with 80% ethanol at 60 °C	0.04	0.04	0	9.7 ± 0.5	42.9 ± 0.9	1.93 ± 0.06
Control + extract from Bronowski	0.15	0.09	0	10.0 ± 0.7	45.8 ± 1.4	1.67 ± 0.09
Control + extract from Bronowski + 0.4% myrosinase	0.15	0.09	100	10.3 ± 0.7	45.6 ± 1.2	1.75 ± 0.09

Each value is the average of 8 male mice (Spf/NMRI) ± S.E. Myrosinase was added dry.
[a] All animals died within 8 days.

Downey[16] compared various heat-treated *Brassica* meals containing glucosinolates of various types and at several levels as feed for mice they found the Bronowski meal to be superior to meals containing higher amounts of glucosinolates. When conducting feed experiments with meal and protein concentrate of Bronowski that had not been heat-treated, Lo and Hill[14] obtained poor weight gains with mice. They suggested that this was due to release of isothiocyanates and oxazolidinethiones through enzymic hydrolysis of glucosinolates, although the glucosinolate content of Bronowski was low. The experiment in Table 3, using a meal with a lower glucosinolate content than Bronowski meal,

TABLE 6. Weight gains, feed intakes and p.e.r. values of mice fed rapeseed meal, cv. Bronowski, with various treatments

Source of protein	Weight gain (g)	Feed intake (g)	p.e.r. value
Control (barley meal)	12.1 ± 0.49	52.1 ± 1.0	1.77 ± 0.05
Bronowski, heat-treated for 5 min at 90 °C	10.4 ± 0.81	46.7 ± 1.3	1.88 ± 0.10
Bronowski, heat-treated for 15 min at 90 °C	9.3 ± 0.55	46.3 ± 1.2	1.65 ± 0.08
Bronowski, heat-treated for 60 min at 90 °C	10.3 ± 0.45	47.5 ± 1.0	1.80 ± 0.06
Bronowski, heat-treated for 5 min at 100 °C	10.8 ± 0.28	47.0 ± 1.4	1.88 ± 0.04
Bronowski, heat-treated for 15 min at 100 °C	10.6 ± 0.34	45.7 ± 1.5	2.02 ± 0.05
Bronowski, heat-treated for 60 min at 100 °C	11.6 ± 0.63	49.2 ± 1.3	1.99 ± 0.08
Bronowski, treated with 80% ethanol	8.4 ± 0.20	43.0 ± 1.8	1.64 ± 0.05

The myrosinase was completely inactivated in all samples. Each value is the average of 8 male mice (Spf/NMRI) ± S.E.

and the experiment in Table 5, when a control diet was supplied with myrosinase and with glucosinolates to a level approximately equal to that of the diets with Bronowski meal, clearly indicate, however, that isothiocyanates and oxazolidinethiones released from Bronowski meal do not effect its nutritional value appreciably.

Still, the glucosinolates of Bronowski meal might have an effect, since VanEtten *et al.*[17] have shown that they can yield not only isothiocyanates and oxazolidinethiones but also nitriles, which are highly toxic.[18] The nitrile formation can be precluded e.g. by dry heating the meal.[17] Formation of nitriles as the main explanation of the poor nutritional value of untreated Bronowski meal is contradicted, however, because a decrease of the glucosinolate content to a still lower level did not effect the nutritional value of the meal (Table 3).

On the other hand, other factors than glucosinolates may be responsible for the low nutritional value of untreated Bronowski meal. The experiments with ethanol extracts in Table 5 indicate that there are no low-molecular compounds in Bronowski meal with deleterious effects acting without the presence of enzymes. The possible presence of high-molecular compounds with deleterious effects in rapeseed meals does not seem to have been explored. Such compounds might exist in rapeseed as well, as they exist in soya bean, although their effects in the former might have been obscured by more salient effects of glucosinolates in high-glucosinolate cultivars. Recently, Staron[19] reported that a fraction of water-soluble proteins from rapeseed meal was toxic to mice.

Finally, the possible effects of the various treatments of the rapeseed samples will be briefly discussed.

(i) In the seed meal of the cultivar Rigo, the presence of compounds with a *low molecular weight*, extractable with 80% ethanol, had a great negative influence on the nutritional value when the adequate enzyme was present. As shown in Table 5, the ethanol extract of Rigo meal with addition of myrosinase caused a pronounced growth inhibition when added to a standard diet. Most likely this effect was caused by oxazolidinethiones and isothiocyanates released by the myrosinase. Untreated Rigo meal was, however, more toxic than ethanol extract with addition of myrosinase, which might be an effect of a production of nitriles from glucosinolates in this meal. Extracts from Bronowski did not exhibit these effects and the relatively low growth on unheated meal turns the interest to the nutritional value of high-molecular compounds in this cultivar.

(ii) Heat treatment, as well as ethanol treatment, may have effects on *high-molecular compounds* in the meal.

(a) Myrosinase is destroyed as well as enzymes that release nitriles from glucosinolates. In the present experiments, this seems to have been important only for Rigo meal.
(b) There may be a denaturation of specific, toxic, high-molecular compounds that possibly exist in rapeseed meal. This possibility should be considered for low- as well as high-glucosinolate meal.
(c) There may also be less specific positive effects due to a generally better digestibility of denaturated protein, which has to be studied in specific tests.

(iii) The greater improving effect of ethanol extraction of meals low in glucosinolates as compared with only ethanol treatment might be explained by a selective extraction of nitrogenous compounds. Generally, the protein content of the meals (according to the Kjeldahl method) was increased from about 45% to about 49% by the ethanol extraction. Amino acids accounted for approximately 85% of the crude protein of the ethanol-treated meals and for almost 100% of the extracted meals. The percentage distribution of individual amino acids was approximately the same in the two types of meals. Although the various diets contained the same amounts of crude protein, the variation in total amino-acid content of the protein was not taken into consideration. Thus, this variation might have had some influence on the nutritional value of the meals. The ratio between content of amino acids in ethanol-treated Bronowski meal when compared with the ethanol-extracted meal (Table 5) was 1:1.18 for lysine, 1:1.15 for methionine and 1:1.12 for isoleucine (isoleucine and methionine are the limiting amino acids in rapeseed meal).[2] This difference is accentuated by an increased food intake of the diet containing the ethanol-extracted meal as compared with the diet with the ethanol-treated meal. It is likely that these differences can explain the difference in nutritional value between ethanol-treated and ethanol-extracted Bronowski meals.

(iv) The treatments of the meals also might cause negative effects on the availability of lysine and methionine in the meals. From experiments on heat treatment of barley seed (85 °C, 24 h) at various water contents, Munck[20] concluded that most lysine was destroyed at 10 to 15% water content. This is in agreement with Miller, Carpenter and

Milner,[21] who investigated a system containing cod muscle and glucose. In both experiments, the conclusion was that another limiting amino acid, probably methionine, was destroyed at higher water contents (30 to 40%). Short treatments comparable with the heat treatments in the present paper caused no significant negative effects in the barley feeding experiments (lysine limiting). It is not likely that methionine is affected in heat treatment of rapeseed meal at 90 to 100 °C for 1 h at the low water content of 8%. Thus, if barley and rapeseed react similarly—which is not self-evident—the negative effects of the heat treatments should be small. This is in agreement with the results in Table 6.

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A Study on the Composition of Garlic Skins and the Structural Features of the Isolated Pectic Acid

A. F. Abdel-Fattah and M. Edrees

Laboratory of Microbiological Chemistry, National Research Centre Dokki, Cairo and El-Nasr Company for Dehydrating Agricultural Products, Cairo, Egypt

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An investigation of the composition of garlic skins showed the presence of proteins, lipids, lignin, mannitol, pectin and polysaccharides. Garlic skins are rich in pectin (27%), combined rhamnose (11.42%) and galactose (5.6%). Periodate oxidation studies of pectic acid showed the absence of 1,3-linked residues and branch points and indicated a linear galacturonan of 1,4-linked D-galacturonic acid. Chromatographic analyses were carried out on the hydrolysis products of pectic acid, which had been oxidised by periodate plus bromine, and also on those obtained after reduction of the periodate-oxidised pectic acid.

1. Introduction

Until now no detailed studies have been carried out on the constituents of garlic skins. Recently, Abdel-Fattah and Khaireldin[1] have reported the isolation of pectin from garlic skins. They have found that ammonium oxalate is more efficient than dilute HCl solution in extracting garlic pectin of high methoxyl content (7.89%).

The present work was undertaken to investigate the composition of garlic skins. Although lipids, lignin, mannitol and sugar components of garlic polysaccharides were among the constituents investigated, more emphasis was placed on pectic acid. This study may lead to a better use of garlic skins. It may also give useful information on the structural features of garlic pectic acid.

2. Experimental

2.1. Specimen

Garlic (*Allium sativum*) skins were obtained from El-Nasr Co. for Dehydrating Agricultural Products. Before use they were freed from any foreign substance.

2.2. Analytical methods

2.2.1. Total ash

Ashing of garlic skins was carried out at 800 °C.

2.2.2. Crude protein

Organic N was determined by Kjeldahl's method and multiplied by 6.25.

2.2.3. Total lipids

Lipids were isolated by extraction, under reflux, with ethyl acetate or *n*-hexane, each for 12 h. In either case, the filtered extract was evaporated to dryness under reduced pressure at 40 °C and the dry residue represented the total lipid content.

2.2.4. Total sterols

The lipid material isolated with ethyl acetate was refluxed with 1 N-ethanolic KOH (5 ml/g of lipid) and benzene (50 ml) for 2 h. Thereafter, water (2 vol.) was added and the unsaponified fraction was extracted by shaking with ether (4 vol.). The ethereal extract was evaporated to dryness and the residue obtained represented the unsaponified fraction. The total sterol content of the residue was then determined according to the Liebermann–Burchard colour reaction method.[2]

2.2.5. Lignin

This was determined according to the method of Ritter, Seborg and Mitchell.[3]

2.2.6. Mannitol

Mannitol was determined titrimetrically by direct periodate oxidation using the milled garlic skins and titration of the liberated iodine with standard sodium thiosulphate solution.[4] It was also determined gravimetrically by extraction with boiling 85% ethanol for 24 h. The alcoholic extract was filtered, concentrated and then treated with cold acetone. After cooling for several days mannitol crystallised out. After isolation by centrifugation, the m.p. of crystalline mannitol and a mixed m.p. were determined. It was also detected chromatographically using ethyl acetate–pyridine–acetic acid–water (5:5:1:3, v/v)[5] as solvent and Dedonder reagent[6] as detecting agent.

2.2.7. Quantitative determination of sugar components in garlic skins hydrolysate

Garlic skins were hydrolysed in 2 N-H_2SO_4 for 16 h in a boiling water bath. The hydrolysate was then chromatographed on Whatman No. 3MM paper using the solvent *n*-butanol–pyridine–water (6:4:3, v/v)[7] and aniline phthalate as spray reagent.[8] The unstained area corresponding to the position of each sugar was cut off and eluted with a definite volume of water. Reaction with orcinol[9] was used for the determination of arabinose and xylose while galactose, glucose and rhamnose were determined by reaction with L-cysteine–sulphuric acid.[10, 11]

2.2.8. Preparation and determination of pectin and pectic acid

Pectin was prepared from garlic skins by extraction with 0.3% ammonium oxalate solution at 90 °C for 1 h and the extract was treated with one vol. of HCl-acidified ethanol.[1] The isolated pectin was dissolved in water, repeatedly precipitated as before and finally dried and weighed. The isolated material was also determined colorimetrically by the method of Bitter and Muir.[12]

Pectic acid was prepared by saponifying the pectin with dilute NaOH followed by precipitation with HCl. The uronic acid content of the pectic acid was determined colorimetrically according to the method of Bitter and Muir.[12]

2.2.9. Hydrolysis of pectic acid

Pectic acid (0.51 g) was hydrolysed in 1 N-H_2SO_4 (100 ml) at 100 °C for 16 h and the hydrolysate, after removal of sulphate with $BaCO_3$, was chromatographed on Whatman No. 1 paper using *n*-butanol–pyridine–water (6:4:3, v/v) as solvent and aniline phthalate as detecting agent.[8]

2.2.10. Oxidation of pectic acid with nitric acid

A portion of the neutralised pectic acid hydrolysate was oxidised with nitric acid according to Heyne and Whistler[13] and the m.p. of the crystallised product as well as the mixed m.p. with authentic mucic acid were determined.

2.2.11. Oxidative hydrolysis of pectic acid

Oxidation of pectic acid (0.53 g) with bromine (0.5 ml) in the presence of hydrobromic acid (20 ml, 7.5%) was performed according to Heidelberger and Goebel.[14] The m.p. of the crystallised product as well as the mixed m.p. with authentic mucic acid were determined.

2.2.12. Preparation of the dibutyl ester of the pectic acid oxidation product

This was done according to the method of Carson[15] using the crystallised products obtained by oxidation and oxidative hydrolysis of pectic acid as well as authentic mucic acid. The m.p. of each product as well as mixed m.p. with authentic dibutyl mucate were determined.

2.2.13. Preparation of the acetate derivative of the dibutyl ester

This was achieved according to the method of Abdel-AKher and Smith[16] using the two dibutyl ester preparations under investigation and also authentic dibutyl mucate. The m.p. of the product and mixed m.p. were determined.

2.2.14. Periodate oxidation of pectic acid under controlled conditions

This was performed by dispersing 0.5476 g (dry basis; uronic acid content: 96.8%) in 200-ml of cold acetate buffer, pH 3.8, followed by the addition of 200 ml of 0.05 M-$NaIO_4$ and the reaction mixture was left, with occasional shaking, in the dark at 2°C for 10 days. During that period 5-ml aliquots were withdrawn at definite time intervals and the consumed periodate was determined according to the method of Fleury and Lange.[17]

2.2.15. Chromatographic analysis of the periodate–bromine oxidised pectic acid

Oxidation of the pectic acid was achieved by treating a suspension (3 g/100 ml of water) with sodium metaperiodate (15.8 g/100 ml) and allowing the mixture to stand at room temperature for 24 h with continuous stirring. The periodate-oxidised pectic acid was then precipitated with 750 ml of *t*-butyl alcohol, filtered, dissolved in water and dialysed for 24 h. Thereafter, 15 g of strontium sulphate and 5 ml of bromine were added and the mixture was immediately stirred for 24 h at room temperature (25 °C). After removing excess bromine by aeration, the reaction mixture was treated with 60 ml of 6 N-H_2SO_4 and the precipitate removed by filtration. The filtrate was then dialysed for 3 days,

concentrated by pervaporation and hydrolysed in 0.05 N-H_2SO_4 for 36 h at 100 °C. After removal of sulphate with barium hydroxide, the hydrolysate was chromatographed using the solvents listed in Table 3. Detection of the spots was achieved with aniline–xylose and aniline phthalate as spray reagents.[8]

2.2.16. Preparation of the acid potassium salt of D-(–)*tartaric acid*

This was performed according to the method of Levene and Kreider.[18] The optical rotation of the product was also determined.

2.2.17. Reduction of the periodate-oxidised pectic acid and chromatographic analysis of the hydrolysed product

After the controlled oxidation of the pectic acid which by periodate had reached completion, the reaction mixture was treated with ethylene glycol, dialysed overnight and then treated with cation exchange resin Lewatit S 100 (H^+). Sodium borohydride (0.5 g in 20 ml of water) was then added and the solution was left for 24 h at 2 °C. After dialysis overnight, the solution was treated with cation exchange resin (H^+) and pervaporated until its volume reached 100 ml. Sulphuric acid was then added until its strength reached 2 N and the solution was heated for 10 h in a boiling water bath. The sulphate ions were removed with barium carbonate and by filtration; the filtrate was again treated with cation exchange resin (H^+) and concentrated by pervaporation. The sirupy hydrolysate was chromatographed on Whatman No. 1 paper with the solvents listed in Table 4. Detection of the spots was achieved with aniline phthalate and Dedonder reagents.

3. Results and discussion

3.1. Composition of garlic skins

As recorded in Table 1, pectin was found to constitute 26 to 27% of garlic skins. No significant difference was found between the amount of pectin determined gravimetrically or colorimetrically.

Chromatographic investigation of the acid hydrolysate of garlic skins indicated the presence of arabinose, xylose, rhamnose, glucose and galactose or their polymers. Rhamnose formed the major part of the total sugar content. The presence of high amounts of rhamnose in the skins of *Allium cepa*[19] suggests that this sugar may be a characteristic component of *Allium* species.

Mannitol was also found in garlic skins. This was shown by its direct determination in the skins and by isolation with boiling ethyl alcohol (85%). The crystallised mannitol was identical in m.p. and R_F value with authentic material.

Garlic skins were also found to contain appreciable amounts of proteins (7.76%). They are thus more promising as a source of proteins than onion skins which are poor in their content of proteins (0.43%).[19]

Lipids constituted a minor component of garlic skins. Extraction with ethyl acetate gave a higher yield (0.43%) than that with *n*-hexane (0.37%). Total sterols were found to make up about 62% of the unsaponified lipid fraction.

3.2. Structural features of the garlic pectic acid

Acid hydrolysis of pectic acid and chromatography of the hydrolysate revealed the presence of only galacturonic acid. Oxidation of pectic acid hydrolysate with nitric acid or oxidative hydrolysis of pectic acid with bromine in the presence of hydrobromic acid resulted in the production of mucic acid which was identical in m.p. with an authentic sample. Similarly, the dibutyl ester of the produced mucic acid was identical in m.p. (145 °C) with authentic dibutyl mucate. Furthermore, the acetate derivative of the

TABLE 1. Percentage composition of garlic skins

Ash	9.91
Crude protein	7.76
Total lipids:	
n-hexane	0.37
ethyl acetate	0.43
Unsaponified lipid fraction	0.21
Total sterols	0.13
Lignin	1.61
Mannitol:	
periodate	3.11
ethyl alcohol	2.00
Pectin:	
gravimetrically	27.00
colorimetrically	26.11
Arabinose	1.80
Xylose	1.34
Rhamnose	11.42
Glucose	1.94
Galactose	5.60

TABLE 2. Periodate oxidation of pectic acid under controlled conditions

Time (days)	1	2	3	4	5	6	7	8	9	10
Periodate reduced (mol/anhydro-galacturonic acid unit)	0.455	0.578	0.641	0.771	0.834	0.897	0.927	0.977	0.999	0.999

dibutyl ester showed a m.p. and mixed m.p. (112 °C) which were also identical with an authentic sample. These results suggest that the garlic pectic acid under investigation is composed of galacturonic acid residues.

The studies of the controlled oxidation of pectic acid by periodate revealed that the oxidation reached completion after the reduction of about 0.999 mol of periodate/anhydrogalacturonic acid unit (Table 2). Thus, almost complete cleavage of D-galacturonic acid residues was achieved on periodate oxidation of the pectic acid, indicating the absence of 1,3-linked residues and branch points.

Chromatographic analysis of the periodate–bromine oxidised pectic acid hydrolysate revealed the presence of D-(−)tartaric acid (levo-L-tartaric acid) and oxalic acid (R_F values are recorded in Table 3). The presence of D-(−)tartaric acid was further confirmed by isolating its acid potassium salt which showed $(\alpha)_D^{30} = -24°$ (0.24% in water). In this respect, Levene and Kreider[18] found $(\alpha)_D^{26} = -22°$ (0.204%) for the L-potassium acid tartrate.

TABLE 3. R_F values of the hydrolysis products of the oxidised pectic acid

Solvent	Hydrolysis products D-(−) Tartaric acid	Oxalic acid
Ethyl acetate–acetic acid–water (3:1:1, v/v)[20]	0.38	0.54
n-Butanol–acetic acid–water (4:1:5, v/v)[8]	0.20	0.70
n-Butanol–formic acid–water (10:2:15, v/v)[8]	0.19	0.37

TABLE 4. R_F values of the hydrolysis products obtained from the reduced periodate-oxidised pectic acid

Solvent	Hydrolysis products Threitol	Glycerol
Ethyl acetate–acetic acid–water (3:1:3, v/v)[20]	0.36	0.24
Ethylmethylketone–acetic acid–water (9:1:1, v/v) saturated with boric acid[21]	0.83	0.66
Ethyl acetate–pyridine–water (10:4:3, v/v)[20]	0.79	0.57
n-Butanol–pyridine–water (6:4:3, v/v)[7]	0.75	0.56

No galacturonic acid residues were found in the hydrolysate of the periodate–bromine oxidised pectic acid. This result provides additional evidence that complete cleavage of D-galacturonic acid was achieved by the controlled oxidation of pectic acid with periodate. The detection of D-(−)tartaric acid and the fact that garlic pectin rotates the plane of polarised light to the right[1] confirm the presence of α,1-4-linked D-galacturonic acid residues in the pectic acid of garlic skins.

Reduction of the periodate-oxidised pectic acid followed by hydrolysis provided substantial evidence of the constitution of pectic acid by the detection of threitol (R_F values of the products are recorded in Table 4). In addition, the absence of galactose among the hydrolysis products confirms the aforementioned results.

Acknowledgement

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Molybdenum in Black Shales and the Incidence of Bovine Hypocuprosis

I. Thomson, I. Thornton and J. S. Webb

Applied Geochemistry Research Group Imperial College of Science and Technology, London, England
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Stream sediments were sampled systematically in nine areas totalling 1100 sq. miles[a] underlain in part by marine black shales ranging in age from Ordovician to Cretaceous. Regional patterns of molybdenum in the sediment were related to the underlying bedrock. Soils developed from the black shale parent material together with associated pasture herbage in eight of the areas contained above normal concentrations of molybdenum (>3 parts/million).

Detailed studies in four of the areas confirmed the high molybdenum content of stream sediment, rock, soil and pasture. Examples are shown of the importance of both glacial drift and of specific soil factors such as pH in the interpretation of geochemical reconnaissance data for agriculture. Clinical hypocuprosis in cattle is already recognised over parts of two of the molybdenum-anomalous areas. In the other two, copper deficiency disorders are not recognised but may well occur at a sub-clinical level. It is suggested that appreciable acreages of the United Kingdom underlain by marine black shales may be enriched in molybdenum and be potentially limiting to livestock performance.

1. Introduction

Regional geochemical surveys of some 6000 sq. miles in England, Wales and Ireland have indicated patterns of anomalously high levels of molybdenum in stream sediment samples collected at a mean density of one sample per sq. mile.[1–4] These patterns have been related to corresponding anomalies in the molybdenum content of the local rocks, soils and herbage. In each of these surveys, the principal bedrock sources of the molybdenum proved to be marine black shales, which are known to contain a variable assemblage of elements in high concentrations, commonly including molybdenum. Rocks of this type are widespread in England and Wales. The total outcrop area in which they may occur is in the order of 6000 sq. miles (Figure 1).[5–7]

Excess molybdenum in the diet of grazing cattle reduces the utilisation of dietary copper and over a period of months may lead to the reduction of copper reserves in the liver and deficiency symptoms in the animal. An encouraging degree of correlation has been found between the stream sediment patterns and the incidence of clinical molybdenum-induced hypocuprosis in cattle. Furthermore, the results of blood copper analysis have indicated that geochemical reconnaissance may assist in delineating

[a] Throughout this paper 1 sq. mile = 2.589 km².

suspect areas in which the disorder may occur at sub-clinical level.[8–10] Subsequent copper supplementation trials have shown a marked response in both live weight gain and physical appearance in cattle showing no clinical symptoms of hypocuprosis.[10]

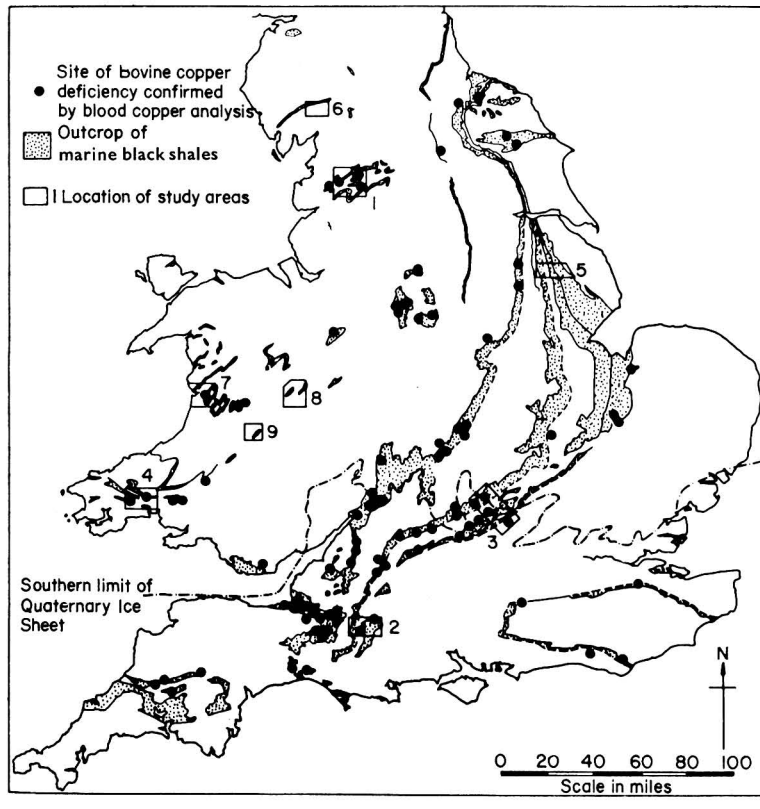

Figure 1. Location of study areas in relation to the principal outcrops of marine black shales showing the known distribution of bovine copper deficiency in localities underlain by black shale. (Information from Agricultural Development and Advisory Service and veterinary surgeons; geology after Institute of Geological Sciences.)

Conditioned copper deficiency in cattle has been reported from a number of localities underlain by marine black shales (Figure 1) and, in a few cases, related to the molybdeniferous nature of the bedrock, soil and pasture. On the other hand, large areas remain in which no clinical disorders in livestock have been recognised but in which, in the light of the geology and the results of previous geochemical investigations, sub-clinical disorders are possible.

The purpose of the present study, initiated in 1968, was to examine the possible extent of this particular problem and the benefit that might be expected to accrue to the agricultural economy from a national geochemical reconnaissance survey.

2. Experimental

Earlier surveys in England and Wales have been carried out over Carboniferous black shales of Namurian age in Derbyshire and North Staffordshire[3] and the entire outcrop of the Lower Lias from the Dorset coast in the south northwards to Yorkshire.[4] The present study incorporated geochemical reconnaissance of nine study areas totalling 1100 sq. miles, each in part underlain by black shales varying in age from Ordovician and Silurian in south and central Wales to Jurassic and Lower Cretaceous in central and southern England (Figure 1). Some of the more extensive outcrops were covered at more than one locality. The resulting patterns for molybdenum and copper were compared with the known distribution of bovine hypocuprosis, using information obtained from the Veterinary Investigation Service and private veterinary practitioners in the respective areas. All reported cases had been previously confirmed by blood analysis. More detailed geochemical studies were carried out in four areas where dairy or livestock farming predominated.

The reconnaissance was based on sampling the active sediment of tributary drainage (maximum catchment area of 10 sq. miles) at a mean density of one sample per sq. mile. Unless stated otherwise, the soil data refer to samples collected at a depth of 12 to 18 in[a]; samples of the topsoil were taken from 0 to 6 in. Pasture herbage was sampled during July and August, and consisted almost entirely of mixed grasses. In those few samples in which clover was present the amount was considered too small to affect the molybdenum content to any significant degree.

Stream sediment, rock and soil samples were analysed spectrographically for 12 elements including copper and molybdenum.[11] Molybdenum in herbage was determined colorimetrically[12] and copper by atomic absorption.

3. Results

In all but one of the nine study areas, molybdeniferous patterns were detected in the stream sediments associated geographically with the outcrop of black shales (Table 1). Anomalous values range up to maxima of 3 to 60 parts/million Mo compared to <2 parts/million in adjoining streams. Clinical bovine copper deficiency is recognised in parts of the Bicester and Bowland Forest areas but has not been recorded elsewhere.

The results of follow-up studies carried out in four of these areas are summarised below.

3.1. Bowland Forest (area 1, Figure 1)

The area is underlain by Carboniferous limestones, sandstones and shales. Dark grey to black marine shales occur interbedded with limestones and sandstones in the Bowland Shale Group (Figure 2). This formation is geologically equivalent to the Clare Shales of Co. Limerick and the Edale Shales of Derbyshire, which are known to be molybdeniferous and associated with extensive nutritional disorders.[2,3] Glacial deposits of locally-derived boulder clay form an extensive cover on all but the highest ground. Soils are poorly drained throughout due to both high rainfall and the impervious nature

[a] Throughout this paper 1 in $= 2.54 \times 10^{-2}$m.

of the many soil parent materials. Molybdenum-induced copper deficiency has been recognised in the vicinity of Chipping in the west, where clinical symptoms are observed in herds grazing pastures over the Bowland Shales.[13]

Molybdeniferous stream sediments ranging from 3 to 60 parts/million Mo delineate broad areas around Chipping and Newton Fell (Figure 2). The distribution of these patterns indicates that the source of molybdenum lies in the Bowland Shale Group and

TABLE 1. Magnitude of anomalous molybdenum patterns detected by stream sediment reconnaissance (Location of study areas indicated in Fig. 1)

Study area	Location in Figure 1	Black shale formation	Mo stream sediment anomaly: Mo content of stream sediment parts/million	Mo stream sediment anomaly: Area of Mo anomaly (sq. miles)
Bowland Forest Lancashire, Yorkshire	1	Bowland Shale Group (Carboniferous)	3–60	35
Shaftesbury Dorset, Somerset, Wiltshire	2	b. Kimmeridge Clay (Jurassic)	3–13	22
		a. Oxford Clay (Jurassic)	3–6	3
Bicester Buckinghamshire, Oxfordshire	3	Oxford Clay (Jurassic)	3–8	8
Meidrim West Carmarthenshire	4	Black Dicranograptus shales (Ordovician)	3–30	5
Market Rasen Lincolnshire	5	c. Black shales in Lower Cretaceous	3	2
		b. Kimmeridge Clay (Jurassic)	3–4	3
		a. Oxford Clay (Jurassic)	3–5	4
Kendal Westmorland	6	Stockdale Shales and Lower Brathay Flags (Silurian)	<2	0
Machynlleth Cardiganshire	7	Lower Llandovery and also thin black shale bands in Mid Llandovery (Silurian)	3–8	7
Shelve Shropshire	8	Rorrington Shale Group (Ordovician)	3–4	1
Rhayader Radnorshire	9	Black shales in Caradoc/Llandeilo sediments (Ordovician)	3–6	2

this has been confirmed in the follow-up studies (Table 2). South-westerly extensions of the anomalies onto other rocks in part reflect glacial smearing of Bowland Shale material in the direction of ice movement. The absence of any extensive molybdenum pattern in the northeast is believed to be due to the presence of exotic glacial deposits which almost completely mask the underlying Bowland Shale in this part of the study area.

The results of rock, soil and herbage analyses are summarised in Table 2. Bedrock samples show maximum values of 40 parts/million Mo in organic-rich black shale

horizons in the Bowland Shale Group. All other rocks contain 2 parts/million or less. Traverses across the area confirm that molybdeniferous soils with up to 85 parts/million Mo are confined to residual parent material derived from the black shale or to glacial drift containing black shale debris. Residual soils and glacial drift derived from other

TABLE 2. Range and mean molybdenum content of stream sediment, rock, soil and herbage and copper content of herbage in the four detailed study areas.

Area and source rock	Mo (parts/million) Stream sediment	Mo (parts/million) Rock	Mo (parts/million) Soil (12 to 18 in depth)	Cu (parts/million) Pasture herbage	Cu (parts/million) Pasture herbage	Bovine copper deficiency
Bowland Forest						
Black shale (Bowland Shale)	3–60	13 <2–40 (26)[a]	12 <2–85 (190)	2.9 0.8–7.2 (33)	7.2 5.0–17.5 (33)	Recognised in part of area
Other rocks (Carboniferous grey shales, limestones and sandstones)	<2	<2 <2–2 (10)	<2 <2–4 (89)	0.9 0.7–1.0 (5)	8.5 6.3–10.3 (5)	
Shaftesbury						
Black shale (Kimmeridge Clay and Oxford Clay)	3–13	12[b] (1)	4 <2–40 (105)	2.4 0.6–6.4 (35)	9.9 6.5–16.0 (35)	Not recognised
Other rocks (Jurassic calcareous clays, limestones and cretaceous sandstones)	<2	<2 <2 (9)	<2 <2–5 (70)	0.7 0.2–2.4 (20)	7.6 4.0–13.5 (20)	
Bicester						
Black shale (Oxford Clay)	3–8	5 2–14 (15)	3 <2–8 (33)	1.7 1.0–2.4 (8)	6.8 4.5–11.0 (8)	Recognised in local districts infertility reported
Other rocks (Jurassic calcareous clays, limestones and boulder clay)	<2	<2 <2 (13)	<2 <2 (122)	0.7 0.1–1.3 (37)	7.4 4.5–11.0 (37)	
Meidrim						
Black shale (Dicranograptus shales)	3–30	4 <2–7 (23)	7 <2–30 (66)	1.5 0.1–3.8 (32)	7.2 5.0–17.5 (32)	Not recognised
Other rocks (Ordovician grey shales, grits, flags)	<2	<2 <2 (13)	<2 <2–5 (63)	0.9 0.3–2.6 (15)	8.5 6.3–10.3 (15)	

[a] Number of samples in parenthesis.
[b] Kimmeridge Clay only: Oxford Clay, not exposed.

rocks invariably carry <4 parts/million Mo. The molybdenum content of pasture, which varies up to 7.2 parts/million, tends to be higher on molybdeniferous soils, though uptake is markedly influenced by local soil conditions—maximum availability being recorded at very poorly drained sites and minimum uptake from those soils which are better drained and/or iron-rich (Figure 3).[14] Surprisingly, no significant correlation was observed in this area between relative uptake and pH, which ranged from 3.8 to 6.8 in the topsoil.

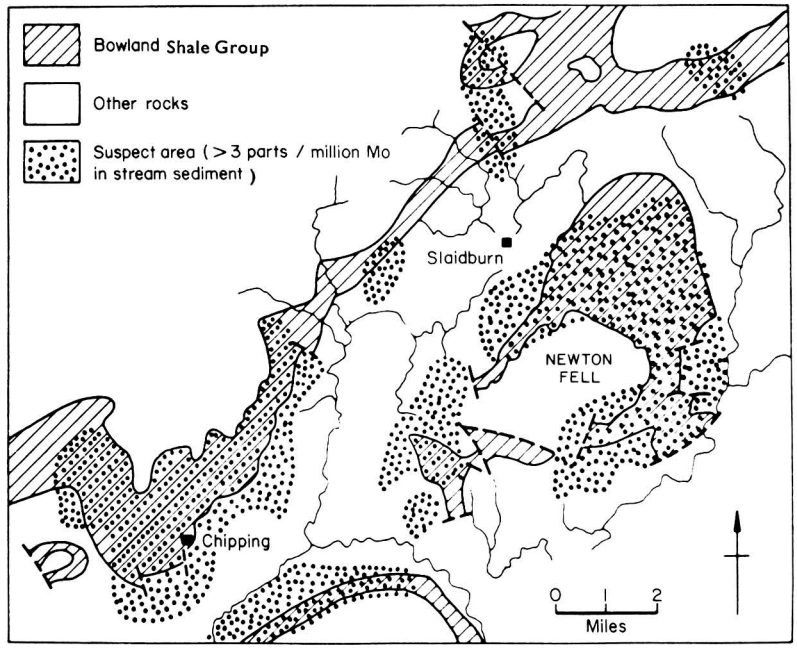

Figure 2. Outcrop of Bowland Shale and distribution of molybdenum in stream sediment of tributary drainage in the Bowland Forest area. (Geology after Institute of Geological Sciences.)

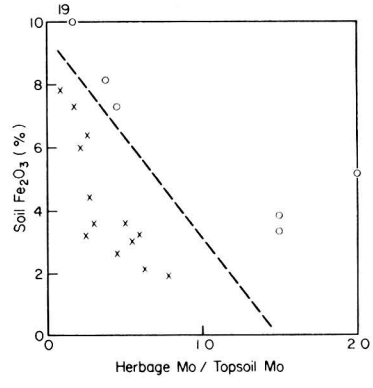

Figure 3. Relative uptake of molybdenum by pasture herbage in relation to the total iron content of molybdenum anomalous soils (0 to 6 in) developed from Bowland Shale material. (x) Moderate—poorly drained soils, (o) very poorly drained soils.

3.2. Shaftesbury (area 2, Figure 1)

The geology comprises clays, limestones and sandstones of Jurassic and Cretaceous age (Figure 4). Black shales are developed in the Lower Oxford Clay and in the Kimmeridge Clay, both of which contain bituminous horizons.[15] Except for river alluvium,

soil parent material is entirely residual; noncalcareous surface-water gleys are developed on the clays and better drained soils on the limestone and sandstone areas. No hypocuprosis has been reported.

Stream sediment reconnaissance revealed a major anomaly with values rising to 13 parts/million Mo on the Kimmeridge Clay and two smaller anomalies up to 6 parts/million on the Oxford Clay north and south of Wincanton. Elsewhere, the content was normal at <2 parts/million Mo (Figure 4).

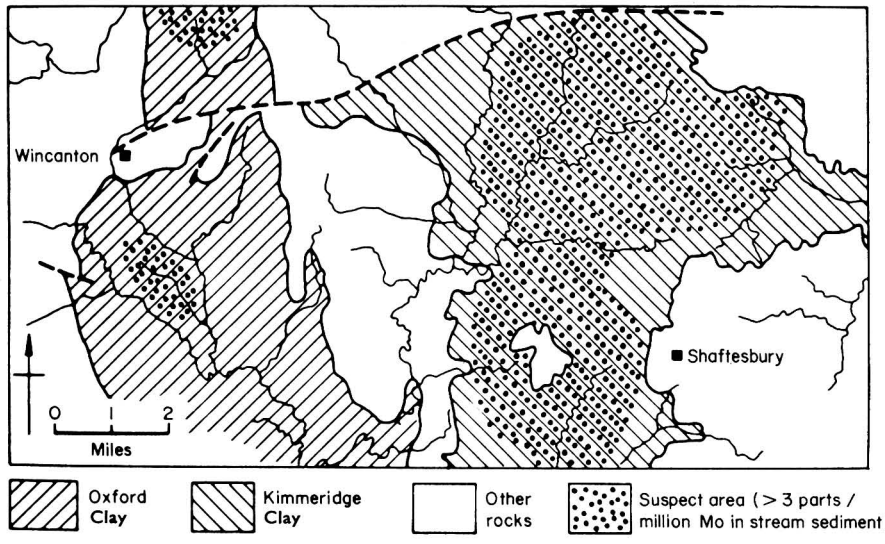

Figure 4. Simplified geology and distribution of molybdenum in stream sediment of tributary drainage in the Shaftesbury area. (Geology after Institute of Geological Sciences.)

Bedrock is very poorly exposed but the one sample of Kimmeridge Clay from within the main stream sediment anomaly contained 12 parts/million Mo compared to <2 parts/million from other rocks in background areas (Table 2).

Soil traverses showed concentrations of molybdenum up to 20 parts/million within the stream sediment anomaly on the Kimmeridge Clay and up to 16 parts/million in the anomalous areas delineated on the Oxford Clay. Elsewhere, apart from one sample containing 5 parts/million Mo, the soils were background at <2 parts/million. Maximum values for pasture (up to 6.4 parts/million Mo) were detected on anomalous soils compared to 2.4 parts/million from control sites. The mean value of 2.4 parts/million Mo for samples within the stream sediment anomaly is significantly higher than the background mean of 0.7 parts/million ($P > 0.001$), the difference being similar to that observed in the Bowland Forest area. Relative uptake tends to increase with topsoil pH over the range 5.3 to 7.8 and also with organic carbon content (Table 3).[14]

3.3. Bicester (area 3, Figure 1)

The survey area lies in the Vale of Aylesbury where the geology consists of Jurassic and Cretaceous clays, limestones and sandstones, overlain in the north by a thin veneer of

exotic boulder clay (Figure 5). The main development of black shales occurs in the Lower Oxford Clay, though some are also found in the overlying formations.

Soil-type broadly reflects the geology, with surface-water gleys in the clay vales and on drift-covered areas, whereas well-drained soils are typical on the limestone and sandstone. Bovine hypocuprosis has been confirmed at a number of localities and infertility is widespread. As shown in Figure 5, stream sediment reconnaissance indicated two anomalous areas underlain by Oxford Clay around Marsh Gibbon (up to 5 parts/million Mo) and near Steeple Claydon (up to 8 parts/million). Elsewhere molybdenum was <2 parts/million.

Samples of black shale from the Oxford Clay within the stream sediment anomaly contained 2 to 14 parts/million. Mo compared with <2 parts/million in other rocks (Table 2). Anomalous levels of molybdenum in soils rising to 8 parts/million are mainly found within the areas on the Oxford Clay delineated by the stream sediment reconnaissance. Isolated soil values up to 5 parts/million over the Kimmeridge Clay and on

TABLE 3. Relationship between relative uptake of molybdenum by pasture herbage, soil organic carbon content and soil pH

Soil organic carbon (%)	<4.0		4.0–6.6	
Soil pH	5.8–6.8		5.6–6.7	6.8–7.8
Herbage Mo[a]	0.18–0.84	(11)	0.47–0.94 (9)	1.10–2.30 (5)
Topsoil Mo	0.53		0.75	1.66

[a] Range and mean values, no. of samples in parenthesis.

alluvium may be attributed to thin black shale bands (which are known to occur in this formation) and Oxford Clay detritus, respectively. Soils developed on other rocks and on the glacial drift are normal at <2 parts/million. In contrast with the two previous areas, the molybdenum content of the pasture herbage growing on molybdeniferous soils shows relatively less difference from that of pasture on normal soils. There is, however, a marked tendency towards values of the order of 2 parts/million Mo in samples from the anomalous areas.

3.3. Meidrim (area 4, Figure 1)

The geology comprises Ordovician and Silurian grits and shales overlain unconformably in the south by red marls of the Old Red Sandstone formation (Figure 6). Black shales occur at many levels in the Ordovician, the most characteristic being in the Dicranograptus shales.[16] Apart from small patches of boulder clay found throughout the area, the overburden is dominantly residual with poorly drained soils developed on the Ordovician and Silurian, and deep freely-drained loams on the Old Red Sandstone.

Immature soils are commonly found on the steep slopes of the deeply incised river valleys. No hypocuprosis has been reported.

Stream sediment reconnaissance revealed a pattern of 3 to 30 parts/million Mo in streams draining Dicranograptus black shales between Meidrim and Llanglydwen, contrasting with <2 parts/million elsewhere (Figure 6). The anomaly may be depressed or even completely suppressed in some catchments where molybdenum-rich black shale material in the stream sediment is heavily diluted by detritus containing normal levels of

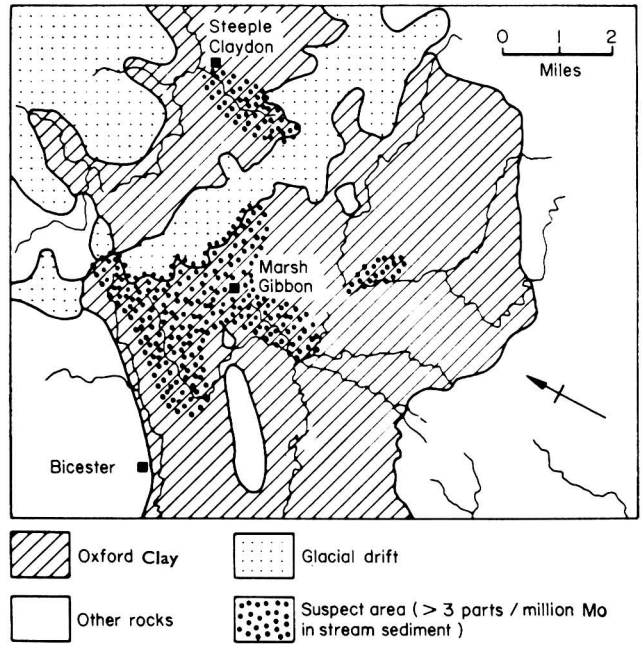

Figure 5. Simplified geology and distribution of molybdenum in stream sediment of tributary drainage in the Bicester area. (Geology after Institute of Geological Sciences.)

molybdenum. No abnormal concentrations of molybdenum were recorded in the sediment of streams draining the Dicranograptus formations south of St. Clears where the sequence is largely represented by gritty shales and limestones.

The analytical data summarised in Table 2 show that the highest concentrations of molybdenum occur in the Dicranograptus black shale and in residual soil derived from this rock. Molybdeniferous soils (2 to 10 parts/million) are also encountered on river alluvium containing black shale debris. The molybdenum content of mixed herbage from pastures on molybdeniferous soils also tends to be slightly higher than in comparable samples from surrounding background areas, though the difference between the mean values is not statistically significant, ($P = 0.05$), despite the fact that topsoil values up to 14 parts/million Mo were recorded. Within the anomalous soils, the molybdenum content of the herbage is broadly related to soil reaction with uptake

increasing over the observed pH range of 4.0 to 7.0 (Figure 7).[14] The limiting effect of acid pH (4.0 to 5.5 being common in black shale soils) may well be responsible for the relatively low content of herbage molybdenum recorded in the anomalous area.

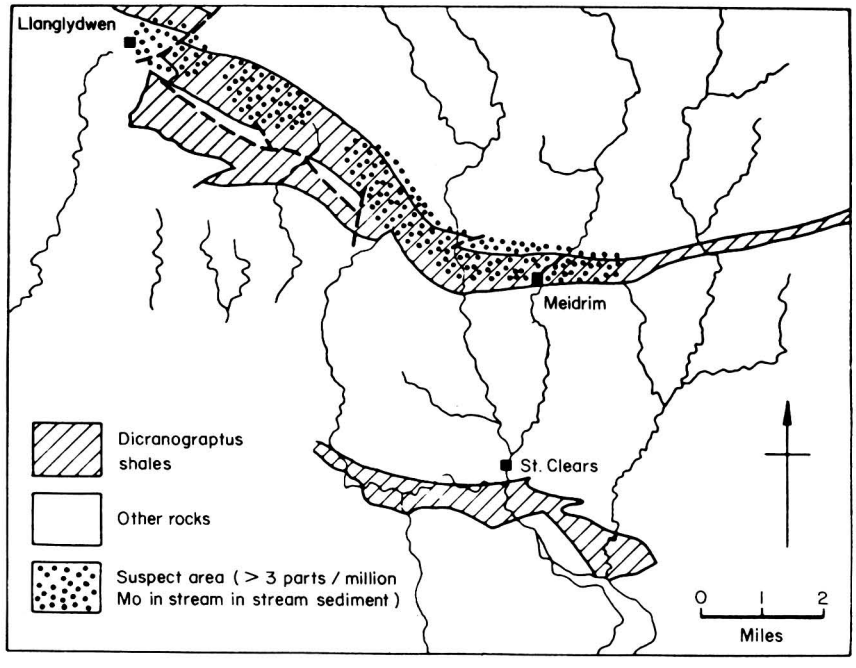

Figure 6. Outcrop of Dicranograptus shales and distribution of molybdenum in stream sediment of tributary drainage in the Meidrim area. (Geology after Institute of Geological Sciences.)

4. Discussion

4.1. Agricultural significance of the geochemical data

Excess amounts of molybdenum in the diet of grazing cattle interfere with normal copper absorption and utilisation to produce an "induced" or "conditioned" deficiency in the animal.[17] Both the molybdenum and copper contents of herbage are therefore important in determining the nutritional status of pasture.

Workers in Ireland provisionally set the potentially toxic content of molybdenum in herbage dry matter at 5 parts/million.[18] In England and Wales, the Agricultural Research Council has tentatively placed the copper requirements of cattle at 10 parts/million in the herbage dry matter.[19] It has been suggested that where herbage is 10 parts/million or less, only small amounts of molybdenum would be needed to cause a conditioned copper deficiency.[20] Forage containing over 5 parts/million Mo has been related to peat scours and molybdenosis in cattle in the United States,[21] while in New Zealand pasture containing 10 parts/million Mo has been shown to be toxic if the copper content is >10 parts/million, and 3 to 10 parts/million Mo to be harmful if

copper is <10 parts/million.[22] Copper deficiency in cattle, described as peat scours, has been found on pasture containing 2 to 4 parts/million Mo in parts of south-west England.[23]

In the light of these observations the higher concentrations of molybdenum detected in pasture herbage in the present study, particularly in the Bowland Forest and Shaftesbury areas, could be associated with induced copper deficiency, especially where the copper content is below 10 parts/million (Table 2). The results also indicate that molybdeniferous soils and herbage are most likely to be encountered in the suspect molybdeniferous areas delineated by stream sediment reconnaissance. This does not, of

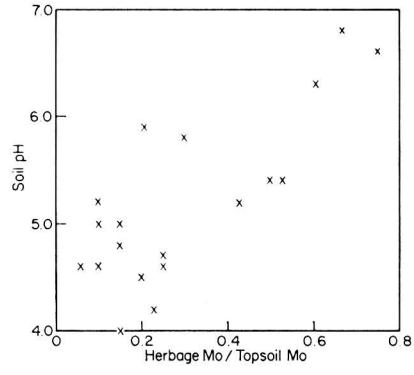

Figure 7. Relative uptake of molybdenum by pasture herbage in relation to the pH of molybdenum anomalous soils (0 to 6 in) developed from Meidrim shale material ($r = +0.900$. $P < 0.001$).

course, imply that *all* soils within the stream sediment anomalies are necessarily molybdeniferous, nor that areas of molybdeniferous soil (too small to be detected by geochemical reconnaissance) will not be found in catchments where the stream sediment values are in the background range.

In the Bowland Forest area, the molybdenum and copper status of herbage sampled east of Slaidburn is comparable to that associated with clinical copper deficiency near Chipping.[13] The whole 35 sq. miles of the stream sediment anomaly must therefore be regarded as suspect; this represents a considerable extension of the area of conditioned copper deficiency previously recognised.

Molybdenum and copper in herbage on parts of the Kimmeridge and Oxford Clay soils in the Shaftesbury area fall within the range associated with copper deficiency in Bowland Forest. However, at present there is no record of clinical disorders in this area. Local veterinary practitioners consider animal production in the clay vales to be below that which could reasonably be expected and have blamed poor quality forage and a high incidence of parasite infestation. It seems possible that poor production may also be due in part to dietary trace element imbalance, particularly within the suspect molybdeniferous catchments totalling 25 sq. miles delineated by the stream sediment reconnaissance.

The Bicester stream sediment anomalies covering 8 sq. miles are less intense than those recorded in either of the preceding areas. Herbage contents are correspondingly

lower. Nevertheless, values in excess of 2 parts/million have been recorded within the anomalies and, at places, outside where there could be molybdeniferous soils associated with thin bands of black shale too small to be detected by stream sediment sampling. These results confirm and extend previous indications. At present, bovine hypocuprosis has been recorded on a number of farms, including two within the stream sediment anomaly; infertility is widespread. It seems probable that excess molybdenum could be implicated and further work is planned following discussions with the Ministry of Agriculture.

Bovine copper deficiency has not been reported in the Meidrim area. On the other hand, herbage molybdenum and copper contents point to the possibility of sub-clinical or latent problems particularly within the 5 sq. miles delineated by the geochemical anomalies. Over 2 parts/million Mo have been found in herbage on soils with >5 parts/million Mo and pH <5.5; if such pastures were limed, molybdenum uptake would be expected to rise and thereby increase the likelihood of livestock disorders.

4.2. Regional implications

The rock formations associated with molybdenum stream sediment anomalies described above maintain similar lithological characteristics for considerable distances beyond the study areas. Similar associations have also been recorded at a number of other localities in England and Wales (Table 1 and previous work).[3,4,24] It is therefore possible that appreciable areas underlain by rocks known to contain black shale horizons as indicated in Figure 1, may prove to be molybdeniferous.

The southern limit of glaciation is shown in Figure 1. With the exception of river alluvium, the soils south of this line are dominantly residual in origin and can generally be expected to reflect in their composition the metal content of the underlying bedrock. To the north, the rocks may be covered by more or less extensive areas of glacial deposits. Here, the compositional relationship between rock and soil is liable to be modified by the presence of boulder clay and other types of drift. With some important exceptions where the drift is exotic and may bear no relationship to the underlying bedrock, the bulk of the glacial cover is of local origin, being derived from and retaining the chemical characteristics of nearby bedrock. In these latter circumstances the patterns of molybdeniferous soil (and stream sediment) may extend beyond the boundaries of the parent molybdeniferous bedrock as a result of glacial "smearing" in the direction of ice-movement. On the other hand, when the glacial deposits have been derived from a distant source, the metal content of the soil parent material may be very different from that of the underlying rocks.

Mention has been made of the previous reconnaissance of the Lower Lias, the outcrop of which extends well into the area affected by glaciation.[4] Of the 1200 sq. miles underlain by this formation, the suspect molybdeniferous catchment area as indicated by stream sediment sampling was 350 sq. miles, or about 25%. While the present survey areas cannot be considered as being representative of the distribution of molybdeniferous black shales over the country as a whole, the results suggest that a similar percentage may usefully be applied as a tentative working guide to the possible extent of suspect molybdeniferous soils. Accepting these provisos, the potential problem areas could therefore aggregate some 1500 sq. miles or nearly 1 000 000 acres,

consistent with previous estimates.[25] The possibility of latent or sub-clinical deficiencies over an area of this order of magnitude could be of considerable economic significance, particularly in view of the substantial improvement obtained by copper supplementation previously recorded in following-up the results of an earlier stream sediment reconnaissance.[10]

Acknowledgements

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Changes in the Composition of Ovomucin During Liquefaction of Thick Egg White: The Effect of Ionic Strength and Magnesium Salts

D. S. Robinson and J. B. Monsey

A.R.C. Food Research Institute, Colney Lane, Norwich NOR 7OF, England
(Accepted for publication April 1972)

Addition of small amounts of either sodium chloride or magnesium salts to thick egg white inhibited the natural thinning processes. Chemical analyses of the ovomucin complex and analytical ultracentrifugation of fully reduced derivatives obtained from thick egg white incubated at 37 °C showed that either 0.01 M-magnesium salt or 0.03 M-sodium chloride also reduced the losses of the β-ovomucins which normally occur during liquefaction of thick egg white. A possible mechanism by which magnesium and sodium chloride retard egg white liquefaction is discussed in relation to the known effect of these substances on ovomucin–lysozyme interactions. A proposed commercial application of the findings is outlined.

1. Introduction

In the previous publication (Robinson and Monsey 1972)[1] we showed that both the β-ovomucins[2] and the carbohydrate moieties of α-ovomucin[2] were modified to more soluble forms during the natural thinning of samples of thick egg white. Although these results indicate that either chemical or enzymic attack on the ovomucins may be a cause of egg white liquefaction, the gel property of thick egg white may be due not entirely to just ovomucin polymers. For instance, even though the evidence is mainly circumstantial, a number of workers (Hawthorne[3], Cotterill and Winter,[4] Brooks and Hale[5,6] and Kato, Nakamura and Sato[7]) have proposed that interactions between ovomucin and lysozyme molecules determine the rigidity of the thick egg white gel. Hawthorne[3] postulated that during egg white liquefaction lysozyme and ovomucin complex in such a way so as to change the physical state of the ovomucin molecules and destroy the gel structure, whereas the other workers[4–7] have proposed that a lysozyme–ovomucin complex is responsible for the rigidity of the gel and that dissociation of this complex initiated by increases in the pH value of egg white during the storage of whole shell eggs causes liquefaction of thick egg white. As we[8] have shown that lysozyme can react stoichiometrically with soluble derivatives of ovomucin *in vitro* to form insoluble "salt-like" complexes and that this reaction is influenced by the pH value, the ionic strength and the concentrations of bivalent cations (Robinson),[9] it is relevant to test by experiment the effect of these variables on the natural thinning of thick egg white and the composition of ovomucin.

This paper is concerned with the effect of various amounts of sodium chloride and salts of magnesium at a constant pH value on the rate of liquefaction of thick egg white and the effect of these additives on the changes in the composition of ovomucin[1] which occur during the natural liquefaction of thick egg white.

2. Experimental

Separations of thick egg white obtained from newly laid Thornber 404 hens, manipulations under aceptic conditions, induced natural thinning of homogenised thick egg white at 37 °C, preparations and reduction of purified ovomucin complex, analytical ultracentrifugations and chemical analyses were as described previously.[1] Various amounts of either dry sodium chloride, magnesium chloride, magnesium acetate, magnesium bromide, magnesium sulphate or magnesium thiocyanate were dissolved in 25 ml of thick egg white prior to incubation at 37 °C. Preparations of purified ovomucin complex were obtained from homogenised samples (100 ml) of newly laid thick egg white, which had been held at 37 °C for 20 h and thick egg white which had been held at 37 °C for 20 h in the presence of either 0.01 M-magnesium acetate or 0.03 M-sodium chloride. Attempts to test the effect of other divalent metal cations such as calcium, zinc, cadmium and mercury which also affect interactions between lysozyme and ovomucin complex[9] were unsuccessful due to the precipitation of egg white proteins by these cations.

3. Results

Owing to the presence of two phases and the retention of air bubbles in the test samples of thick egg white, it was not possible to determine reliable relationships between shear rate and shear stress by viscometry. However, the amount of gel that remained after 20 h at 37 °C is shown clearly in the photographs (Figure 1) as the length of column which retained air bubbles. After measuring the heights of the gel columns recorded on photographic prints it was found that the addition of either 0.01 M-magnesium acetate ($I = 0.03$) or 0.15 M-sodium chloride ($I = 0.15$) appreciably inhibited the natural thinning of thick egg white (Tables 1 and 2). Also, as shown in Tables 1 and 2, 0.0025 M-magnesium acetate ($I = 0.0075$) and 0.015 M-sodium chloride ($I = 0.015$) reduced the natural thinning processes. The addition of more than either 0.01 M-magnesium acetate or 0.15 M-sodium chloride as well as preventing liquefaction also caused immediate contraction of the thick egg white gel as shown in Figure 1, tubes 4, 5, 6 and 12. The pH value of 8.40 for the samples of homogenised thick egg white was only affected slightly by the presence of small amounts of sodium chloride and magnesium acetate. Similar tests carried out with either magnesium bromide, magnesium chloride, magnesium iodide, magnesium sulphate or magnesium thiocyanate showed that these salts also inhibited the natural thinning of thick egg white when $I_{\text{Mg salt}} = 0.03$.

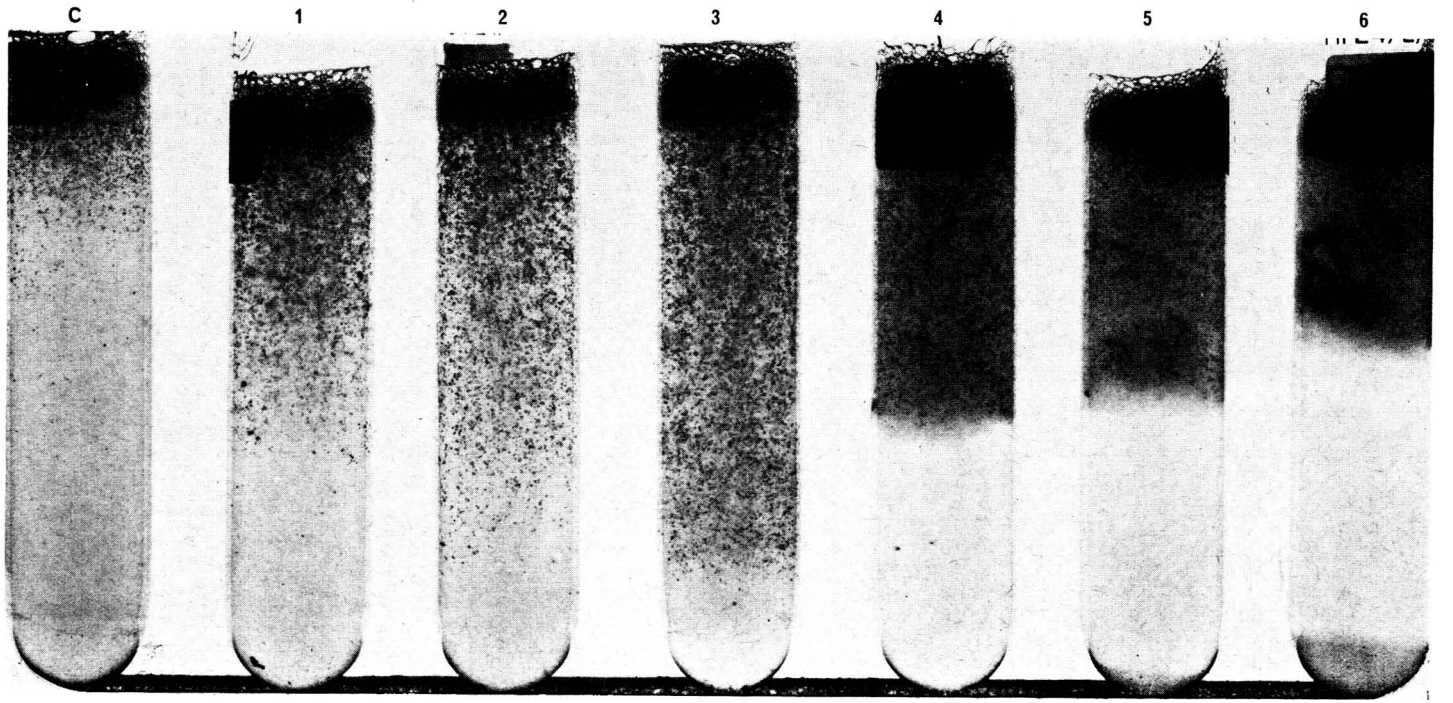
Preparation of ovomucin complex from the samples of thick egg white held at 37 °C in the presence and absence of either magnesium acetate or sodium chloride showed that smaller amounts of ovomucin complex were in general obtained from the liquefied samples of thick egg white (Table 3). However, there is no apparent explanation for the

extremely low yield of ovomucin complex from the sample of egg white to which sodium chloride had been added. Preliminary analysis for sialic acid of preparations of ovomucin complex obtained from samples of thick egg white which had been incubated at 37 °C in the presence of 0.01 M-magnesium acetate for various intervals of time showed that these preparations contained a greater proportion of *N*-acetylneuraminic acid than the preparations of ovomucin complex obtained from samples of thick egg white to which no magnesium had been added (Figure 2). More detailed analyses (Table 4) for monosaccharides of the preparations of ovomucin complex showed that thinning induced at 37 °C for 20 h resulted in the isolation of an ovomucin complex which contained significantly smaller amounts of *N*-acetylgalactosamine, galactose and *N*-acetylneuraminic acid and thus confirmed our previous results.[1] In the presence of 0.01 M-magnesium acetate and to a smaller extent in the presence of 0.03 M-sodium chloride these losses of carbohydrate were appreciably reduced. Also, a greater proportion of mannose was present in the preparations of ovomucin complex obtained from samples of thick egg white liquefied at 37 °C to which inorganic salts had not been added (Tables 4 and 5). The effect of 0.01 M-magnesium acetate and to a smaller extent the effect of 0.03 M-sodium chloride on preventing the loss of the *N*-acetylgalactosamine, galactose and sialic acid residues relative to mannose is shown in Table 5. For purposes of comparison these values reported previously[1] for the ratio of residues of monosaccharides/mol of mannose present in purified preparations of α- and β-ovomucins are also given in Table 5. As shown in Table 6, the molar ratio of Glu + Asp to Thr + Ser present in the preparations of ovomucin complex increased as thinning of the thick egg white progressed and again confirmed our previous finding and also showed that both magnesium acetate and sodium chloride reduced the extent of the loss of threonine and serine from ovomucin complex during natural thinning induced at 37 °C. For purposes of comparison those values reported previously[1] for the ratio of Glu + Asp to Thr + Ser in purified preparations of α- and β-ovomucins are also given in Table 6.

Sedimentation velocity experiments (Figure 3) in the presence of 5 M-guanidine hydrochloride with the fully reduced and alkylated derivatives of the preparations of ovomucin complex showed that the preparations of ovomucin complex obtained from thick egg white incubated in the presence of either 0.01 M-magnesium acetate or 0.03 M-sodium chloride contained proportionally more of the faster sedimenting (5.7 S) β-ovomucins than the analogous ovomucin samples obtained from thinned thick egg white without added salts. The relative amounts of the α- and β-ovomucins (2.4 and 5.7 S, respectively) present in the preparations of ovomucin complex were determined as described previously[1] and are given in Table 7.

4. Discussion

Although the apparent viscosities of the samples of incubated thick egg white to which inorganic salts were added could not be measured, a visual assessment of the rigidity of the thick egg white gel, although somewhat subjective and arbitrary, has allowed us to compare the effect of various amounts of either sodium chloride or salts of magnesium on the natural thinning of thick egg white induced by incubation at 37 °C.



(a)

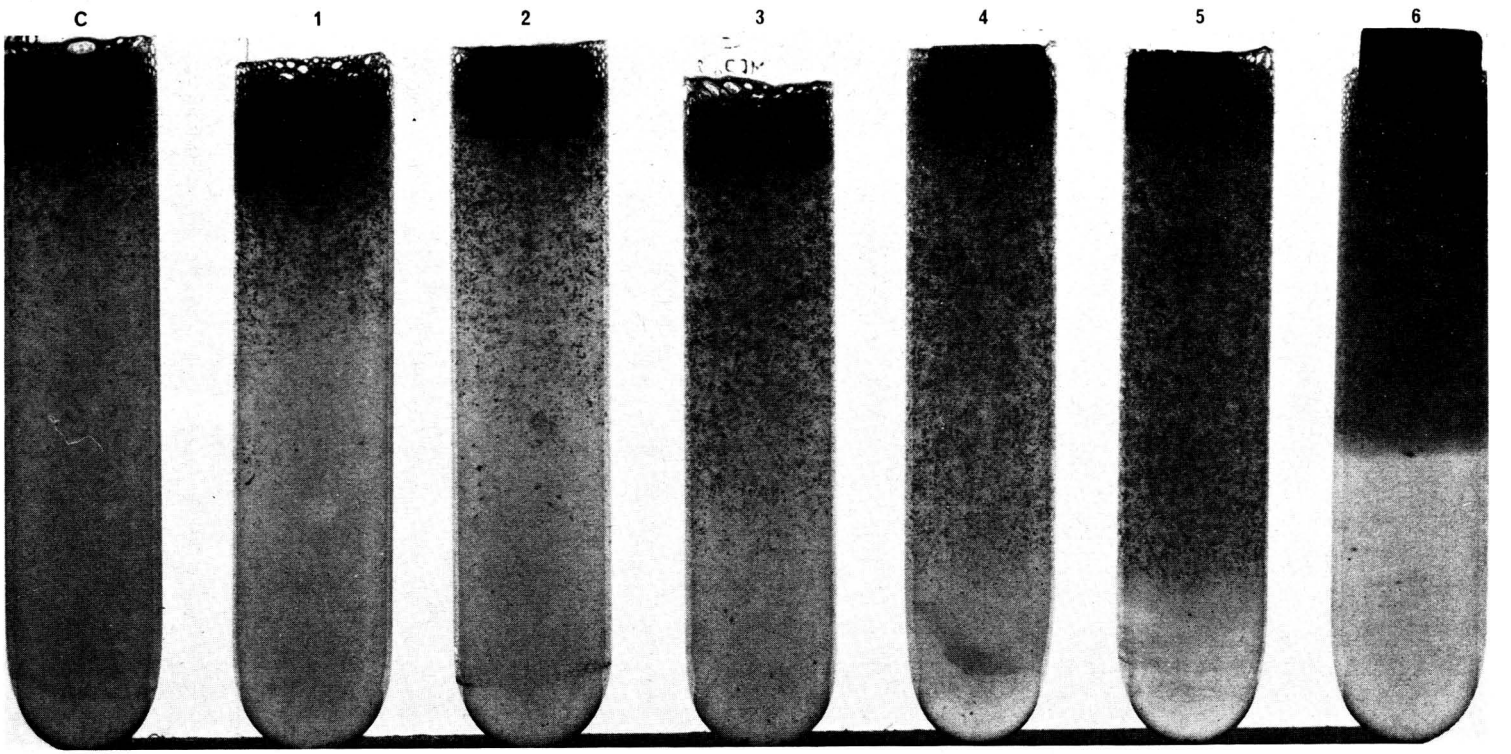


Figure 1. The effect of inorganic salts on the thinning of thick egg white at 37 °C. (a) Magnesium acetate: C = 0, (1) = 0.0025 M, (2) = 0.005 M, (3) = 0.01 M, (4) = 0.025 M, (5) = 0.05 M, (6) = 0.10 M.
(b) Sodium chloride: C = 0, (1) = 0.0075 M, (2) = 0.015 M, (3) = 0.03 M, (4) = 0.075 M, (5) = 0.15 M, (6) = 0.30 M.

TABLE 1. Inhibition by magnesium acetate of the liquefaction of homogenised thick egg white

Concentration of added magnesium acetate (mol)	Height of gel column after 20 h at 37 °C (cm)	pH value at 22 °C
0	2.5	8.44
0.0025	5.0	8.41
0.005	6.0	8.41
0.010	7.2	8.33
0.025	5.2	8.22
0.050	4.5	8.13
0.100	3.8	8.01

TABLE 2. Inhibition by sodium chloride of the liquefaction of homogenised thick egg white

Concentration of added sodium chloride (mol)	Height of gel column after 20 h at 37 °C (cm)	pH value at 22 °C
0	2.5	8.44
0.0075	3.8	8.40
0.015	5.0	8.38
0.03	6.0	8.39
0.075	7.2	8.36
0.150	7.4	8.34
0.30	5.2	8.24

TABLE 3. Effect of liquefaction of thick egg white on the yield of ovomucin complex

	Amount of dry ovomucin complex obtained/100 ml of thick egg white (mg)
Newly laid thick egg white	142
Thick egg white held at 37 °C:	
1. in the presence of 0.01 M-magnesium acetate;	151
2. in the presence of 0.03 M-sodium chloride;	89
3. no added inorganic salts.	113

The results given in this paper, which show that the loss of the β-ovomucins and the induced thinning of thick egg white can be retarded by either very small amounts of added magnesium salts or the addition of sodium chloride directly to homogenised thick egg white, indicate that the reduced rate of thinning in the presence of added magnesium ion or additional salt might have resulted from a decreased interaction

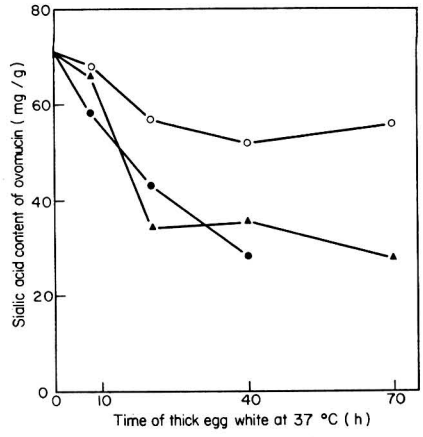


Figure 2. The effect of incubation of thick egg white at 37 °C on the sialic acid content of ovomucin complex.
Ovomucin complex from thick egg white which contained magnesium ($I = 0.03$) held at 37 °C, —○—○—; ovomucin complex from thick egg white which contained sodium chloride ($I = 0.03$) held at 37 °C, —●—●—; ovomucin complex from thick egg white without additive held at 37 °C, —▲—▲—

TABLE 4. The composition of ovomucin complex

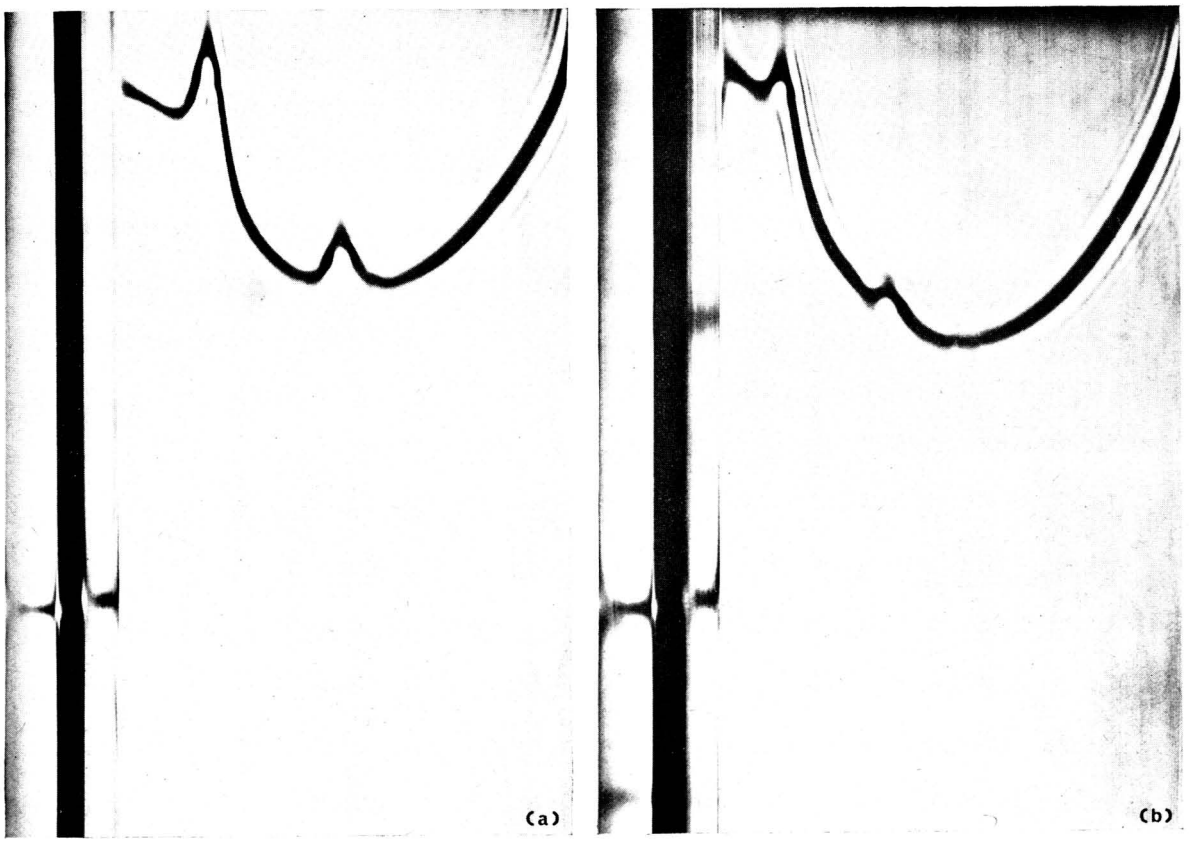
	g/100 g				mol/10^5g of sample			
	(1)	(2)	(3)	(4)	(1)	(2)	(3)	(4)
Protein[a]	57.7	57.3	80.6	85.8				
N-acetylglucosamine	8.6	8.6	7.7	7.4	39.3	38.9	34.9	33.8
N-acetylgalactosamine	4.0	3.2	2.2	1.8	18.1	14.5	10.1	8.3
Galactose	8.5	6.4	5.8	4.6	47.2	35.5	32.2	25.5
Mannose	2.7	2.9	2.9	3.6	15.0	16.1	16.1	20.0
Total sialic acid (Warren)[15]	7.1	5.7	4.3	3.5	23.0	18.5	13.9	11.2
Sulphate	1.8	2.1	1.1	1.4	18.7	21.9	11.5	14.6

[a] From amino-acid analysis.
(1) Ovomucin complex from newly laid thick egg white.
(2) Ovomucin complex from thick egg white which contained magnesium acetate ($I = 0.03$) held at 37 °C for 20 h.
(3) Ovomucin complex from thick egg white which contained sodium chloride ($I = 0.03$) held at 37 °C for 20 h.
(4) Ovomucin complex from thick egg white without additive held at 37 °C for 20 h.

TABLE 5. Residues/mol of mannose in ovomucin complex

	(1)	(2)	(3)	(4)	α-ovomucin[a]	β-ovomucin[a]
N-acetylglucosamine	2.6	2.4	2.2	1.7	1.2	2.2
N-acetylgalactosamine	1.2	0.9	0.6	0.4	0.1	1.7
Galactose	3.1	2.2	2.0	1.3	0.4	4.7
Sialic acid	1.5	1.2	0.8	0.5	0.1	2.0
Sulphate ion	1.2	1.4	0.7	0.7	0.3	1.2
Total	9.6	8.1	6.3	4.6	2.1	11.8

[a] From Robinson and Monsey.[2]
(1) (2) (3) (4) as Table 4.


(a)
(b)

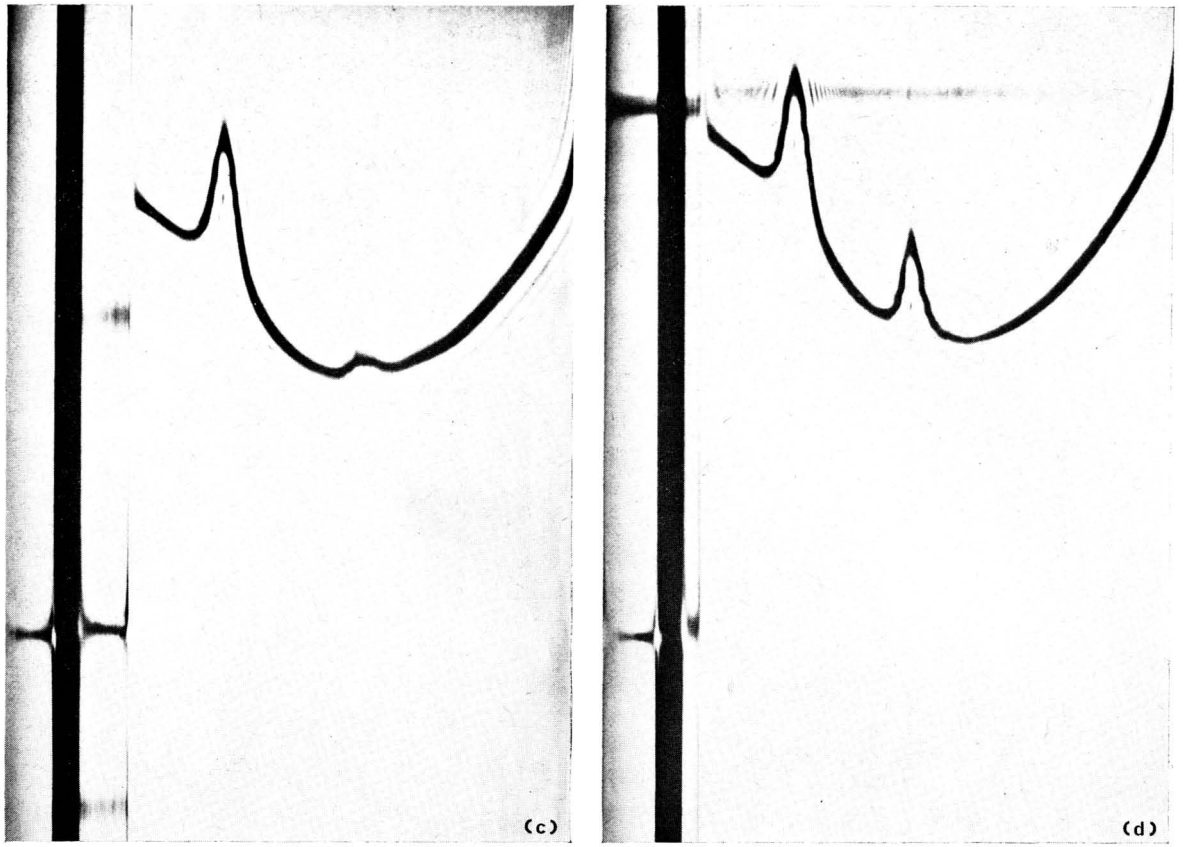
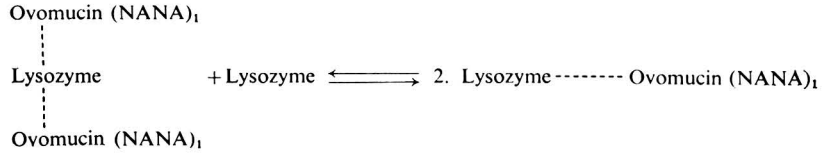


Figure 3. Sedimentation-velocity experiments with reduced ovomucin complex (0.5% w/v) in 5 M-guanidine hydrochloride prepared from thick egg white held at 37 °C in the presence of (a) 0.01 M magnesium acetate; (b) in 0.03 M-sodium chloride; (c) no added inorganic salts and (d) prepared from newly laid thick egg white.

between ovomucin and lysozyme which magnesium ions and sodium chloride are known to cause[8,9]. Therefore, it is possible that the dissociation of a lysozyme–ovomucin complex by excess lysozyme as proposed by Robinson[9] and shown as follows:



may represent the first essential reaction during the natural thinning of thick egg white.

As barium, calcium, zinc, cadmium and mercuric ions caused precipitation of the egg white proteins we cannot establish whether there is a cationic series of bivalent cations for the inhibition of egg white thinning. Further, as all the magnesium salts

TABLE 6. Ratio of $\frac{\text{Asp} + \text{Glu}}{\text{Thr} + \text{Ser}}$ in ovomucin complex

Sample	μmol
(1)	0.97
(2)	1.25
(3)	1.32
(4)	1.37
α-ovomucin[a]	1.38
β-ovomucin[a]	0.58

[a] From Robinson and Monsey.[2]
(1) (2) (3) (4) as Table 4.

TABLE 7. Relative amounts of α- and β-reduced ovomucins present in the preparations

Sample	% of total peak area	
	α-reduced	β-reduced
(1)	65.9	34.0
(2)	73.7	26.2
(3)	86.0	13.9
(4)	91.2	8.7

(1) (2) (3) (4) as Table 4.

tested caused maximum inhibition of thinning when I added Mg salt = 0.03, there does not appear to be an anionic series. Therefore, the results of the present experiments do not permit the ionic nature of the functional groups and the types of bonding present in the proposed ovomucin–lysozyme complex to be defined. However, *in vitro* experiments[8,9] with solutions of reduced ovomucin complex have indicated that the interactions between lysozyme and ovomucin are mainly electrostatic and may involve two basic groups of the lysozyme molecule and one sialic acid residue of the ovomucin complex.

On the other hand our results do not exclude a more specific role for the reduction of egg white thinning by magnesium, namely the inhibition of specific enzymes capable of degrading the oligosaccharide moieties of the ovomucins. However, although magnesium is an inhibitor of β-galactosidase obtained from *Escherichia coli*,[10] the presence in egg white of a similar enzyme has not been reported and therefore any proposal that the formation of the soluble forms of ovomucins produced when egg white is held at 37 °C, is a result of enzymic action must await further investigation.

The recent findings of Sauveur[11] who showed that the amount of both magnesium and calcium present in egg white decreased during thinning induced by storage of whole shell eggs and his proposal that electrostatic interactions between the bivalent metal cations and the egg white proteins are important, is compatible with our proposal that magnesium ion and salt inhibit interactions of ovomucin–lysozyme complex with excess lysozyme. Also the fall in osmotic pressure during storage as shown by Sauveur,[11] while perhaps reflecting a loss of cations from egg white, may also have been caused by a dissociation of an expanded network of ovomucin–lysozyme polymers which would be expected to make a greater contribution to osmotic pressure than the sum of the components of a dissociated network of polymers (Laurent and Ogston).[12]

Our studies reported here and previously[1] and the results of Sauveur[11] show that the concentrations of inorganic salts and their interactions between proteins present in egg white may afford a key factor for the prevention of egg white thinning. As only small amounts of any magnesium salt need be added to egg white to reduce the natural thinning then the addition of such substances to bulk separated newly-laid egg white could be beneficial to industry. On a molar basis the magnesium ion is 15 times more effective than the sodium ion (Tables 1 and 2) for preventing liquefaction of thick egg white. As the natural concentration of magnesium ion in egg white is approximately 4 mM[13,14] and as a further addition of only 10 mM are required to prevent thinning of thick egg white, then any procedure which results in a 3- to 4-fold increase in the concentration of magnesium ion in egg white should reduce the deterioration of egg white during handling. Furthermore, as egg white also contains approximately 65 mmol of sodium, 24 mmol of potassium and 2 mmol of calcium[13,14] then if the concentration of any or all of these elements could be increased either by feeding higher levels of these elements or by selective breeding of hens which secrete more inorganic salts into the white, then the natural thinning of the thick egg white fraction which occurs first during the plumping of the egg in the shell gland of domestic fowl and second during the storage of newly laid eggs may be reduced.

Acknowledgements

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Immunochemical Behaviour of the Proteins of the Orange

P. Cantagalli, V. Forconi, G. Gagnoni and J. Pieri

Laboratorio Provinciale di Igiene e Profilassi, Reparto Medico Micrografico e Reparto Chimico, Siena, Italy

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Gel filtration of the proteins isolated from orange juice showed three fractions. The two with higher molecular weight were used to prepare rabbit immunosera. By testing the immunosera with concentrated orange and lemon juice substantial differences between the proteins of these two citrus fruits were demonstrated. Our research has also shown that these sera can be used for analysis of commercial products.

1. Introduction

Despite numerous efforts to devise a method of testing the citrus fruit juice content of commercial products no completely satisfactory method has been found. In fact methods based on the analysis of amino acids,[1–5] phosphates[6] and phospholipids[7] are of limited value because of the lack of specificity of these components.

In this paper we describe the fractionation of orange juice proteins and the use of partially purified protein preparations to obtain specific orange antisera.

2. Experimental

2.1. Protein extraction

850 ml of the juice of commercial oranges were neutralised to pH 7 with ammonia and dialysed for 5 days at 4 °C. After centrifugation at 14 000 **g** ammonium sulphate was added to 2 M concentration. The sediment was allowed to settle for 12 h and then collected by centrifugation at 14 000 **g**. The sediment was resuspended in 50 ml of distilled water and dialysed to eliminate NH_4^+. After measurement of the proteins (mg 0.259/ml) the liquid was freeze-dried.

2.2. Gel filtration on Sephadex

About 20 mg of proteins in 20 ml of buffer, pH 7.4 (0.008 M-$Na_2HPO_4 \cdot 2H_2O$, 0.001 M-KH_2PO_4, 0.137 M-NaCl, 0.005 M-KCl) were centrifuged to eliminate the precipitated pectin and then filtered through a column (50 × 4 cm) of Sephadex G100 in the same buffer. The effluent was collected with a fraction collector in fractions of about 10 ml each and the absorbance at 215 and 225 nm was estimated on a Beckmann DU spectrophotometer. The difference between the absorbances at 215 and 225 nm was calculated.[8] The fractions containing proteins were collected, dialysed and freeze-dried.

2.3. Determination of molecular weights

Andrews' method[9, 10] was used to determine the molecular weights of the protein fractions separated through Sephadex using standard gamma globulin, albumin and ribonuclease.

2.4. Discontinuous analytical electrophoresis on polyacrylamide gel

The protein samples, 100 – 400 μl of 1% w/v solution in phosphate buffer, pH 7.8 (0.003 M-Na_2HPO_4, 0.0004 M-KH_2PO_4), were loaded on the top of the gel in 11% (w/v) sucrose. The acrylamide concentration was 7.5% (w/v) and the solutions were as described in the instruction sheets (Shandon Scientific Company, London) for a gel system buffer, pH 9.5; no ferricyanide was present in the gel. The runs were performed in 8 × 0.7 cm i.d. glass tubes in a vertical apparatus (Canalco Industrial Corporation, Rockville, Md., U.S.A.), according to the methods of Ornestein[11] and of Davis,[12] by applying 5 mA/tube for 90 min. The staining was carried out overnight with aniline blue-black (0.5% w/v) in 7.5% (by vol.) acetic acid and excess dye was removed by applying 12.5 mA/gel in the same acetic acid solution. Bromophenol blue mobility in the system was assumed to be 1.

2.5. Preparation of antisera

Four-month-old rabbits weighing about 2 kg were used. After testing them to make sure that they had no antibodies for citrus fruit proteins, they were inoculated with a solution containing 0.5 mg/ml of the proteins in the following fashion: (i) 2 ml of the proteins solution in 2 ml of Freund's complete adjuvant were injected subcutaneously; (ii) 15 days later, 2 ml of the protein solution was injected subcutaneously and (iii) 15 days later, the same dose was administered intraperitoneally. Seven days after the last inoculation blood samples were collected and the serum was used for the immunological test.

2.6. Immunodiffusion analysis

The immunodiffusion analyses were carried out on 1% agarose gel on microscope slides.[13] The diameter of the well for the antiserum was 8 mm and that of the wells for

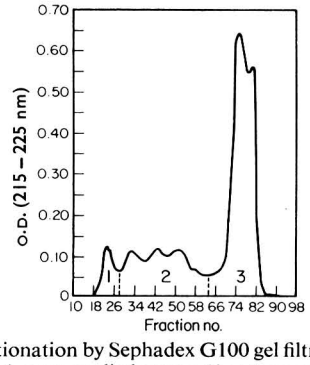

Figure 1. Orange proteins fractionation by Sephadex G100 gel filtration. 20 mg of proteins, dissolved in 20 ml of phosphate buffer pH 7.4, were applied onto a 50 × 4 cm column of Sephadex G100.

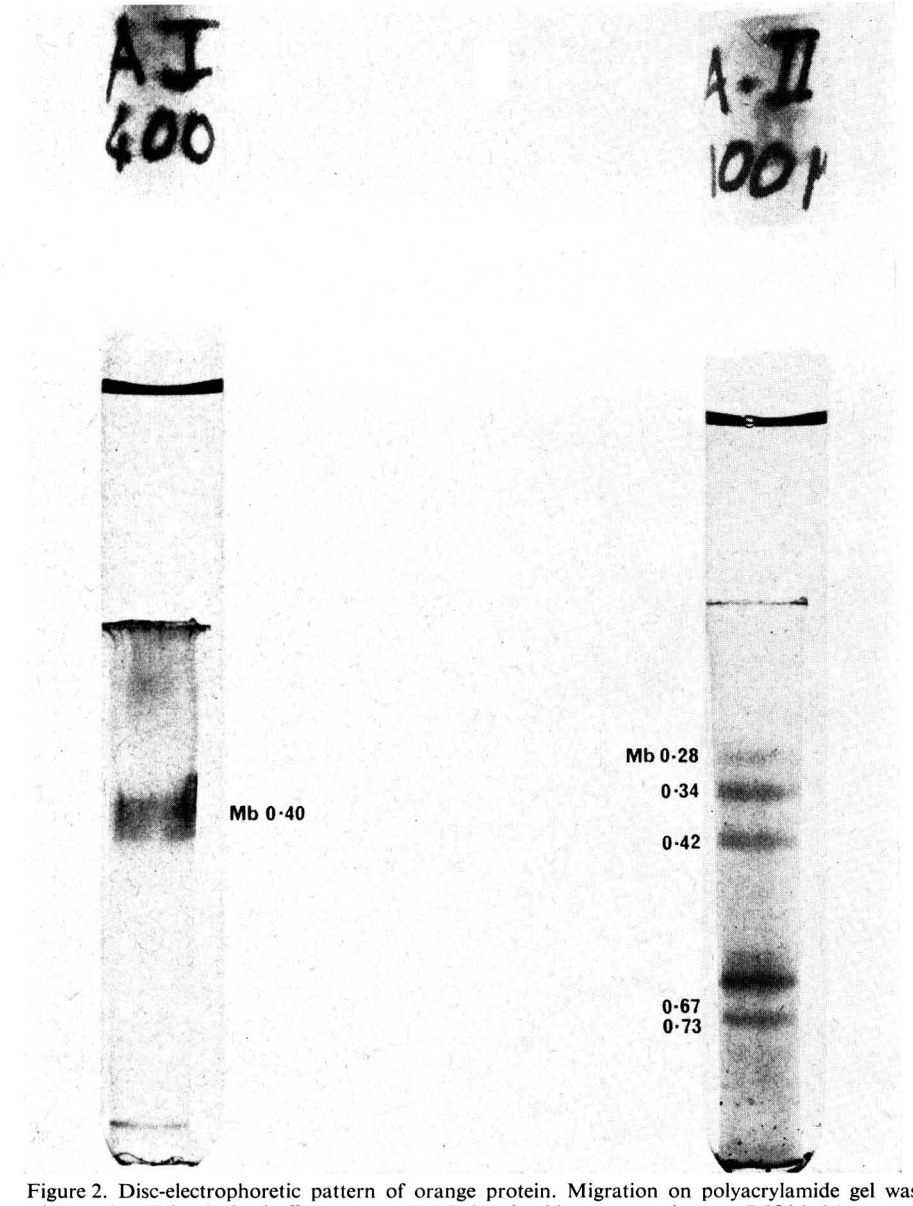

Figure 2. Disc-electrophoretic pattern of orange protein. Migration on polyacrylamide gel was carried out in a Tris–glycine buffer system, pH 9.5. Acrylamide concentration was 7.5% (w/v).

the antigen was 6 mm. The antigens were prepared as follows: the raw and pasteurised natural orange juices and the commercial orange juice were concentrated ten times after dialysis and freeze-drying; the products based on orange juice (12%) were concentrated one hundred times after dialysis and freeze-drying. The antigen–antibody

precipitates were stained with Coomassie Blue (0.1% solution in a mixture of 50% methanol, 10% acetic acid and 40% distilled water) and rinsed in a mixture of 50% methanol, 10% acetic acid, 10% glycerine and 30% distilled water.

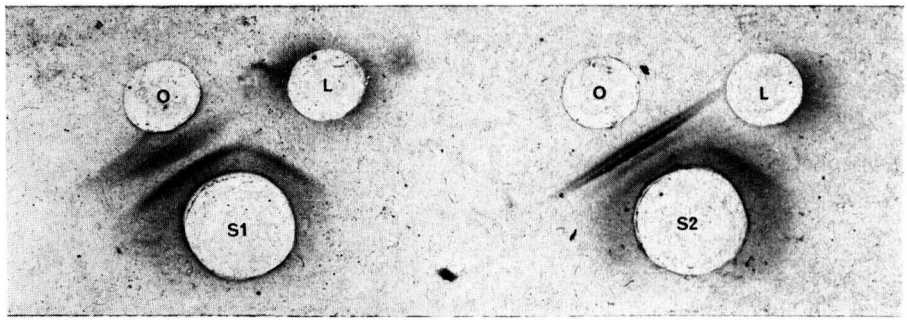

Figure 3. Ouchterlony plate showing precipitation patterns between orange antiserum fraction 1 (S1) and orange antiserum fraction 2 (S2) with orange extract (O) and lemon extract (L).

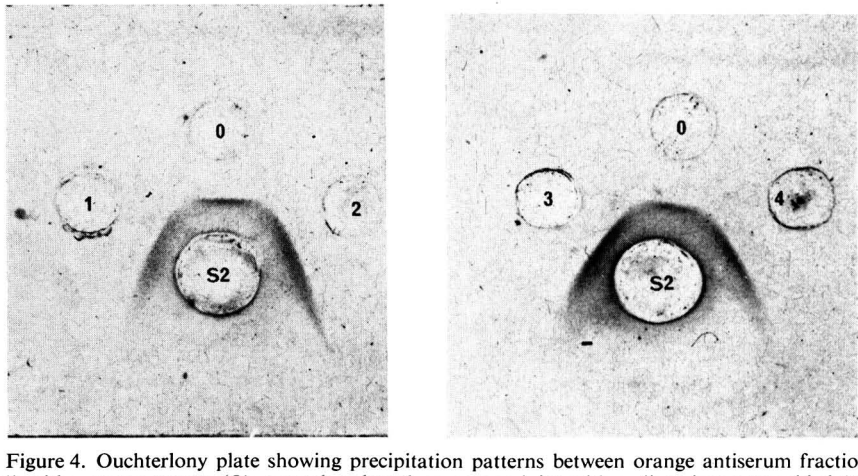

Figure 4. Ouchterlony plate showing precipitation patterns between orange antiserum fraction 2 (S2) with: orange extract (O), a product based on orange juice with sodium benzoate added (1), a product based on a pasteurised orange juice (2) and two pasteurised commercial orange juices (3) and (4).

3. Results

The fractionation of the proteins of the orange on Sephadex G100 is shown in Figure 1. Protein fractions 1 (m.w. 200 000) and 2 (m.w. 100 000 to 50 000), which, respectively, make up 11.5 and 32.4% of the filtered proteins, were used for the preparation of the antisera. Fraction 3 was not used for immunisation because of its low molecular weight.

The electrophoresis analysis of fraction 1 presents a wide band with anodic migration of Mb 0.40, whereas that of fraction 2 presents five distinctly separated, anodic bands of Mb 0.28, 0.34, 0.42, 0.67, 0.73 (Figure 2).

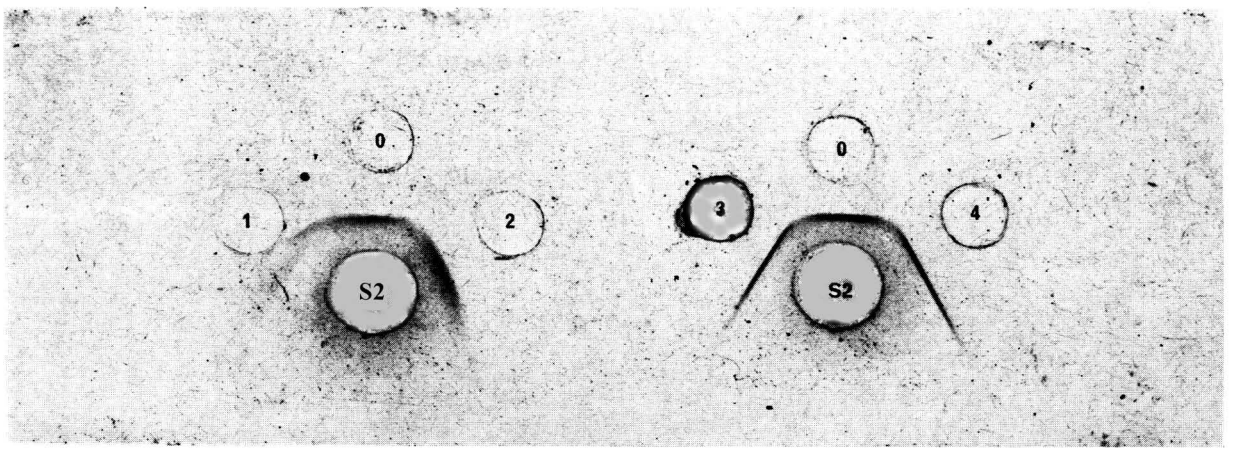

Figure 5. Ouchterlony plate showing precipitation patterns between orange antiserum fraction 2 (S2) with: orange extract (O) at room temperature and heated to 70 °C for 60 min (1), 80 °C for 60 min (2), 90 °C for 30 min (3) and 100 °C for 10 min (4).

The orange antisera obtained by immunising rabbits with fractions 1 and 2 were tested with the total protein extract of orange and lemon juice (Figure 3). The antibodies for fraction 1 reacted with at least three proteins of the orange extract and one of the lemon extract. The antibodies for fraction 2 reacted with at least four of the orange proteins and only one of the lemon's. The orange antisera fraction 2 was also tested with the processed orange juice (Figure 4) and with natural orange juices heated to various temperatures for various periods (Figure 5).

4. Discussion

From the above results it can be seen that it is possible to test the orange juice content of products with the immunological method. In fact, we have shown that pasteurisation does not alter the protein fraction of orange juice and that other substances which are usually added to commercial orange juices (ascorbic acid, dyes, citric acid) do not interfere with the immuno assay because they can be easily eliminated by dialysis.

Because of the specificity of immunological analysis it is surer than the methods based on analysis of the amino acids, phosphorus or phospholipid content. Furthermore, a large number of samples can be tested with a small quantity of serum without any special apparatus, making it practical for routine testing of commercial orange juices.

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Formation of Methional and Methanethiol from Methionine

T. Wainwright, J. F. McMahon and J. McDowell

Research Laboratory, Arthur Guinness Son & Co. (Dublin) Ltd
St. James's Gate, Dublin 8, Ireland
(Accepted for publication April 1972)

Methanethiol is formed from methionine and sulphite in a reaction catalysed by iron or manganous ions. Methionine sulphoxide and dimethyl disulphide are other products of the reaction. Methional [3-(methylthio)propional] is postulated as an intermediate in the reaction.

1. Introduction

Whilst studying mercaptan formation by brewery yeasts it was observed that relatively large amounts of mercaptan and methional were formed chemically.[1] This paper reports the conditions involved.

2. Experimental

2.1. Reaction system

Maximum mercaptan formation occurred with 500 μmol of methionine, 50 μmol of potassium metabisulphite and 10 μmol of ferrous sulphate in a final volume of 100 ml. After incubation for 2 h, without shaking, at 24 °C the reaction was stopped by addition of 100 μmol ethylenediamine tetra-acetate.

2.2. Mercaptan determination

Five ml of 2 M-HCl were added and the flask, immersed in a water bath at 70 °C, was aspirated with oxygen-free nitrogen (75 ml/min for 2 h). H_2S was removed by bubbling the volatiles through 0.25 M-zinc acetate; mercaptan and disulphide were then trapped by mercuric acetate and measured colorimetrically.[2]

Results are expressed in terms of the amount of methanethiol which would give the equivalent colour under the same displacement conditions.

In some experiments a mercuric chloride trap (which absorbs mercaptans but not disulphides) was used before the mercuric acetate. The mercaptan trapped in 5 ml of 5% (w/v) mercuric chloride was estimated colorimetrically after addition of 3 ml of 5% (w/v) mercuric acetate before the *p*-amino-*N*-dimethylaniline sulphate and ferric chloride. This showed that the major product was mercaptan and not disulphide.

2.3. Chromatography

A flame photometric detector specific for sulphur compounds was used for gas chromatography of 0.5-ml vapour samples. Two columns were used in order to distinguish between ethanethiol and dimethyl disulphide.[3] Methanethiol and ethanethiol were detected on an FEP column, 8 m × 2 mm internal diameter, packed with 5% polyphenyl ether + 0.05% phosphoric acid on Teflon, 40 to 60 mesh. Dimethyl disulphide was detected on a glass column, 1 m × 2 mm internal diameter, packed with 20% by weight of an equal mixture of GE-SF 96 and Embaphase on acid-washed Chromasorb W, 100 to 120 mesh.

Spots were located by ninhydrin on paper chromatograms run 18 h in a *tert*-butanol–butan-2-one–formic acid–water (40:30:15:15) ascending solvent system.[4]

TABLE 1. Mercaptan formation from methionine and from methional in 100-ml volumes of distilled water, brewery worts and 5% w/v glucose

Substrate	Medium	Metabisulphite (50 μmol)	Ferrous sulphate (10 μmol)	Mercaptan (μmol)
Methionine (500 μmol)	Distilled water	−	−	0
Methionine (500 μmol)	Distilled water	+	+	3.2
Methionine (500 μmol)	Brewery worts	−	−	0
Methionine (500 μmol)	Brewery worts	+	+	0
Methionine (500 μmol)	5% w/v glucose	−	−	0
Methionine (500 μmol)	5% w/v glucose	+	+	2.3
Methional (100 μmol)	Distilled water	−	−	0.4
Methional (100 μmol)	Distilled water	+	+	2.8
Methional (100 μmol)	Brewery worts	−	−	1.5
Methional (100 μmol)	Brewery worts	+	+	1.0
Methional (100 μmol)	5% w/v glucose	−	−	0.2
Methional (100 μmol)	5% w/v glucose	+	+	2.4

3. Results

3.1. Reaction in distilled water

Methanethiol, dimethyl disulphide and methionine sulphoxide were identified as reaction products from methionine. Metabisulphite replaced sulphite, but sulphide and sulphate were inactive. Fe^{2+}, Mn^{2+} and Fe^{3+} were ten times as active as Cu^{+} and Cu^{2+}; other metals tested were inactive.

Methional gave a higher yield of mercaptan than methionine. This reaction occurred without added metal ions but was inhibited by EDTA, whilst Fe^{2+} ions increased the yield.

When methionine was replaced by ethionine, ethanethiol rather than methanethiol was formed.

3.2. Reaction in complex media

Mercaptan was formed from methional when distilled water was replaced by brewery wort, but not from methionine. 5% w/v of glucose decreased the mercaptan yield from methionine but had little effect with methional (Table 1).

3.3. Incubation time and pH

Mercaptan formation increased with time up to 2 h but there was little further increase at 18 h. Maximum yield is at pH 3.0, with little activity above pH 5.

3.4. Effect of oxidising and reducing agents

Methionine is degraded by peroxide or by certain metal ions in the presence of ascorbic acid.[5] When ascorbic acid was added to the present model system, or when the ferrous ion or sulphite was replaced by peroxide, the yield of mercaptan was negligible.

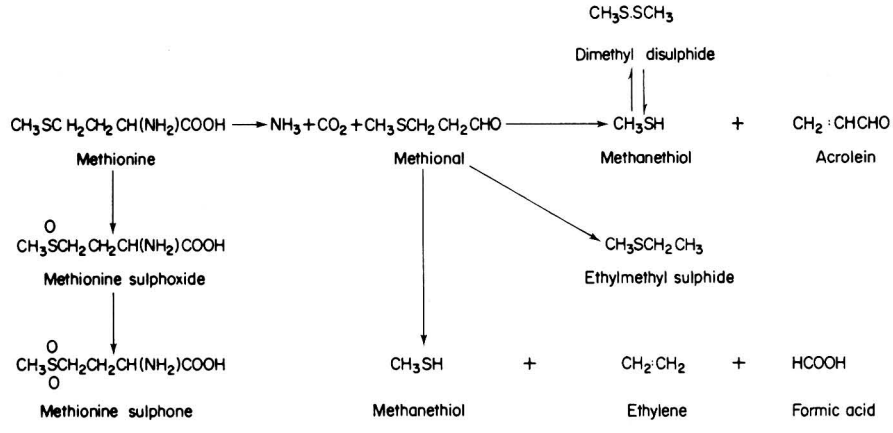

Figure 1. Reaction products of methionine.[5,7,12]

4. Discussion

Methionine is oxidised or decomposed under a variety of conditions but the details of the reactions are still uncertain. Figure 1 depicts the reaction products and suggested pathways for their formation.

Cuprous, or cupric ions plus ascorbate, decompose methionine in phosphate buffer at pH 6.8 and 30 °C in air to give ethylene.[5] Ferrous ions have up to 60% of the activity of cuprous ions if hydrogen peroxide is added. Acrolein and volatile sulphur compounds are also detected as reaction products.

Methional, but not methionine, is converted to ethylene by sulphite and manganous ions provided peroxidase is present.[6] Ferrous ions will not substitute for manganous ions, but peroxide will. However, when methionine instead of methional is treated with manganous ions and sulphite, methionine sulphoxide is formed in almost 85% yield rather than methional and its degradation products.[7]

Methionine is decomposed by heating with carbonyl compounds or by the action of light,[8-10] volatile sulphur compounds being among the products. A special case, in which the carbonyl compound is pyridoxal, is connected with formation of iron sulphide and methanethiol in canned foods.[11]

In the flavin-catalysed degradation of methionine to ethylene, caused by light, metal ions such as Fe^{2+} inhibit.[12] The formation of methanethiol and dimethyl disulphide was shown by gas–liquid chromatography. The optimum pH for ethylene formation was 8.5, with little activity below pH 6.

In the present system, conditions for maximum mercaptan production differ from those described for formation of ethylene or methionine sulphoxide. Ferrous ions are the most effective, the optimum pH is considerably lower and peroxide and ascorbate inhibit.

Methionine is decarboxylated by ferrous ions to methional.[13] Methional is then decomposed by ferrous ions and sulphite to give mercaptan. The fact that methionine, unlike methional, is not decomposed in brewery wort suggests that the reaction from methionine to methional does not occur in wort.

Methionine occurs naturally in many foods and is sometimes added e.g. to make fodder yeast more nutritious. Sulphite is used for blanching, as a preservative and traces often remain in sterilised equipment. Since methional and other volatile sulphur compounds have a marked effect on the organoleptic qualities of many foods[14–18] their formation from methionine and sulphite at room temperature could be important in off flavours of several foodstuffs.

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Pectinesterase Activity of some Iraqi Dates at Different Stages of Maturity

H. A. Al-Jasim and K. S. Al-Delaimy

Department of Food and Dairy Technology, College of Agriculture
University of Baghdad, Abu-Ghraib, Iraq
(*Accepted for publication March 1972*)

Changes in the pectinesterase (PE) activity in four Iraqi date varieties at different stages of maturity were followed. The enzyme activity was determined by titration of the carboxylic groups released by the hydrolysis of pectin. The results obtained show that there was an increase in PE activity in these varieties with ripening. The greatest increase occurred during the period when the fruit had passed the yellowish stage and turned soft. This increase in the enzyme activity was then followed by a general decrease as the fruit matured and ripened on the tree. The data also indicate that there was a qualitative difference in the behaviour of these varieties.

1. Introduction

There is much more information available in the literature on the changes in pectic substances during ripening of fruits than on enzymes catalysing these changes. Undoubtedly, these changes are of considerable importance in the softening of fruits.

Pectinesterase (PE) is one of the enzymes involved in these changes and is widely distributed in the tissue of most higher plants.[1] This enzyme catalyses the hydrolysis of ester bonds of pectic substances to methyl alcohol and pectic or pectinic acid. Weurman[2] studied the pectinesterase activity in Doyenne Boussoch pears. He noticed that PE activity on a fresh weight basis reached a maximum in the early stages of development and decreased markedly thereafter. Dennison, Hall and Nettles[3] reported that PE activity was low in tomatoes until the fruits reached the mature green stage, at which time the activity rose sharply until about two days after the fruits began to change colour. Jacquin[4] observed an increase in PE activity in apples and pears with ripening. In Bartlett pears, Nagel and Patterson[5] found that PE activity of pears increased during maturation, while it decreased in relation to fresh weight and protein content.

This investigation was concerned with the change of PE activity throughout maturation of four Iraqi date varieties.

2. Experimental

2.1. Sampling and analyses

Date samples were obtained from the palm trees of the experimental station of the College of Agriculture in Abu Ghraib. The four varieties used in this study were:

Zahdi, Khadraawi, Khastaawi and Berbin. Only one tree of each variety was used for sampling. The fruits were picked from bunches of the tree at different positions at the time of sampling. Fifty fruits of each variety were used.

2.2. Preparation of enzyme extract

The perianths and seeds were removed and date meat was cut into small pieces and mixed well. Samples (50 g) were mixed with 100 ml of 1.5 M-NaCl solution and blended (Waring blender) for 3 min. The pH of this slurry was adjusted to 7 using 0.1 N-sodium hydroxide solution. The slurry was left in the refrigerator (2 to 3 °C) for 60 min and then filtered through No. 2 Whatman filter paper in a Buchner funnel with a slight vacuum. 100 ml of filtrate was collected from each sample and used as the crude extract for the enzyme assay.

2.3. Assay of PE activity

PE activity was determined at 30 °C by titrating the free carboxylic groups produced on de-esterification of pectin, with 0.02 N-NaOH solution. The reaction mixture consisted of 20 ml of 1% (w/v) apple pectin (250 grade B.D.H.) and 20 ml of enzyme extract. The pH of the mixture was maintained within the range 7.0 to 7.5 by the use of a Beckman Zeromatic pH meter. Immediately after mixing the enzyme extract and the substrate, the pH was adjusted to 7.5 and then a measured vol. of the dilute alkali was added to keep the pH within the given range. The reaction mixture was continuously agitated by a magnetic stirrer. The reaction time was 10 min. The activity of the crude enzyme was expressed as COOH μequiv./min/g of fresh fruit. The enzyme assays were carried out in duplicate on the one extract and the average value reported; in the majority of cases the replicates gave identical results.

3. Results and discussion

The terms Khimri, Khalaal, Rutab and Tamar are used in Iraq to denote stages in ripening.[6] Khimri is the green stage of the fruit. Khalaal, the next stage, is the stage of turning of the skin from green to yellow, pink or red. Rutab, is characterised by darkening of the skin from the colour of Khalaal to brown or to nearly black or to green in green group as Khadraawi. With those four basic terms some other descriptive words such as small green, mature green, yellowish green, mature yellow, soft, 50% soft and dry were used to indicate the closest possible description of the fruit at the date of sampling.

Table 1 shows the PE activity of Zahdi, Berbin, Khadraawi and Khastaawi dates during maturation and ripening. It is clear from the Table that PE activity is lower in the dates than in other fruits, e.g. Sharabi apples,[7] probably due to the lower protein content of dates.

The data indicate a similar pattern in that all varieties exhibit a maximum build-up of PE activity in the 50% soft stage to be followed by a general decrease in the dry stage. Qualitative differences are obvious, however, in the behaviour of the respective varieties. Thus Zahdi and Khastaawi show a fairly constant activity until the yellowish green stage where a sudden rise occurred which continued until the fruit became 50%

TABLE 1. Pectinesterase activity of four Iraqi date varieties during ripening

Date of sampling	Stage of ripening	Activity COOH μequiv./min/g of fresh fruit Zahdi	Berbin	Khadraawi	Khastraawi
26 June 1969	Small, green (Khimri)	0.54	0.40	0.39	0.42
9 July 1969	Mature green (Khimri)	0.55	0.59	0.40	0.42
23 July 1969	Yellowish green (Khalaal)	0.56	0.60	0.69	0.45
7 August 1969	Mature yellow (Khalaal)	0.92	0.67	0.72	0.76
20 August 1969	50% soft (Rutab)	1.15	0.95	0.95	0.85
17 September 1969	Dry (Tamar)	0.90	0.72	0.90	0.80

soft. In Berbin there is a marked increase in activity in the second week of assay to be followed by an almost constant activity period for about a month. The greatest rise in activity occurs, again, between the mature yellow and 50% soft stage. The behaviour of Khadraawi is typical. The initial increase in activity occurs in the yellowish green stage and remains constant until the fruit is mature yellow. This is followed, again, by a sharp increase to the 50% soft stage. The decrease in activity after the 50% soft stage also appears to depend on the variety; Zahdi and Berbin showing a considerable drop while Khadraawi and Khastaawi show practically no change at all.

It may be concluded from the foregoing that there is an increase in PE activity of dates with ripening. The greatest part of this increase occurs during the period that the fruit has passed the yellowish stage and turns soft. This might well be due to the inactivation of a possible inhibitor—tannins for example—at this stage of maturity. The varietal differences in the decrease in activity from the 50% soft stage to the dry stage need further investigation by following the changes at shorter intervals. Such a decrease has already been noticed by Weurman in tree ripened pears.[2]

Further properties of the date pectinesterase are under study.

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Title.—This should be concise and explanatory of the purpose of the paper. Where a series of related papers is submitted each individual paper should have the same general heading, followed by a series number and title of the part.

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Results.—These should be presented concisely, using tables or illustrations for clarity. Adequate indication of the level of experimental error and the statistical significance of results should be given. The number of illustrations and graphical chemical formulae used must be kept to a minimum and only in exceptional cases will tables *and* graphs derived from them be accepted for publication.

Authors should distinguish clearly between main and subsidiary headings.

Discussion.—In general, the discussion and interpretation of results should follow their presentation, in a separation section.

Conclusions.—These should not merely repeat preceding sections.

Acknowledgements.

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Manuscripts

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(*b*) Tabulated matter should be clearly set out and the number of columns in each table should be kept as low as possible.

(*c*) Drawings of figures must be drawn on plain white paper or board or on to tracing paper, not larger than 20 × 30 cm. Lines are to be drawn in Indian ink; *all lettering and numerals and all points on graphs* must be inserted *clearly and lightly* in blue pencil and *not in ink*. The author's name and the title of the paper should be written on the back of each drawing. Legends and captions should be typed on a separate sheet.

(*d*) Half-tone photographs should be included only when essential. They must be printed on contrasting glossy paper and have sharp outlines. The size should be such that, when the print is reduced to the normal size for reproduction (12 × 17 cm maximum), the detail is still clear. (It is inevitable that some loss of clarity will occur during the preparation of the block and subsequent printing.)

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