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Effect of Restricting the Energy Intake of the Laying Hen, Directly and by Dilution of the Diet, on Egg Production and the Efficiency of Energy Utilisation

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(Accepted for publication November 1971)

An experiment is described in which the daily metabolisable energy (ME) intake of light and medium weight hybrid pullets fed a concentrated diet (ME = 3720 kcal/kg) was restricted directly on an individual hen basis (95, 85 and 75 g/day) and also by dilution of the diet using wood dust as the diluent (13.6, 22.7 and 31.8% wood dust). The concentrated diet and a high-energy diet (ME = 2750 kcal/kg) were also fed *ad libitum*.

Total egg weight and percentage egg production were not significantly different for hens fed the concentrated diet *ad libitum*, at the first two levels of restriction or at the lowest level of dilution.

Both direct restriction and restriction by dilution of the diet resulted in greatly reduced liveweight gains.

Direct restriction of the ME intake resulted in a marked improvement of ME conversion to egg product (Mcal/kg eggs).

Dilution of the diet had a detrimental effect on the ME conversion.

Calculations, based on calorimetric data, of the ME requirement for maintenance and production (eggs plus bodyweight gain) showed that direct restriction resulted in observed ME intakes which, although higher, were closer to the calculated ME requirements than did *ad libitum* feeding. Restriction of ME intake by dilution of the diet resulted in the actual ME intakes being much greater than the calculated requirements, the difference being 70 and 50% of the calculated ME requirements at the 31.8% level of dilution for the light and medium hybrids, respectively.

The possible reasons for the difference between the calculated ME requirements based on calorimetric data and the observed ME intakes obtained in the practical laying trial are discussed.

1. Introduction

The literature relating to the effect of restricting the intake on the utilisation of feed by laying hens has recently been briefly reviewed.[1]

Recent work in this Department has been concerned with the effect of individually restricting the metabolisable energy (ME) intake of caged layers. It was found[1] that when the intake of a concentrated diet (ME = 3550 kcal/kg) was restricted for the laying hen it resulted in small bodyweight gain accompanied by an improved utilisation of ME for egg production. Since limiting the intake of individual hens has a high labour

requirement, or else requires some specially designed equipment, it was decided to investigate the possibility of restricting the ME intake of laying hens by using a diluent in the diet and to compare this with the effect of direct restriction.

Washington workers[2] fed a diet containing 20.2% spruce wood flour to growing birds without noting any adverse effect and they reported a slight growth increase at 28 weeks of age. Guisti[3] found the digestibility of the cellulose of sawdust by chickens to be 2.33%. Davis and Briggs[4] fed white and yellow pine sawdust in a purified diet fed to chicks. They found that 10% sawdust in the diet resulted in the greatest increase in chick growth. At a level of 30% sawdust in the food, growth was retarded and feed conversion efficiency was depressed.

Reid, Hinds and Kurnick[5] found that limitation of the dietary intake of pullets from 9 weeks of age to maturity delayed sexual maturity and reduced growth rate. They used direct restriction of intake and also the addition of bentonite and wood flour to obtain this effect.

Peeler[6] added bentonite to pelleted layers' rations. The birds receiving bentonite had a hen-day production of 71.7% and used 1.70 kg of feed per dozen of 57 g eggs, while for control birds fed the basal diet hen-day production was 72.9% and required 1.75 kg feed per dozen of 57 g eggs. Foster[7] included 5% of clay in a layers' diet and found that adjustment in food intake to changes in the diet had occurred by the time 1 week had elapsed since the dietary change, and the pullets maintained a reasonably constant intake of ME. Ousterhout[8] has briefly reviewed the nutritional effects of clays in feeds and concluded that, in general, for poultry caloric efficiency is improved and also that protein efficiency is enhanced.

In the experiment reported below, three levels of dilution and three levels of restriction of a very high-energy diet were employed to feed caged layers.

2. Experimental

The experiment was started in November 1967 and continued for ten 28-day periods until August 1968.

288 birds, comprising equal numbers of light (Hyline) and medium weight (Hybrid-4) pullets, were housed in single-hen cages with individual feed troughs and communal drinkers and were maintained on a lighting programme of 17 h of light and 7 h of darkness. The birds were housed at 18 weeks, introduced to the experimental diets at 20 weeks and recording commenced at 22 weeks. Records on each pullet for daily egg production and egg weight data were kept for ten 28-day periods. The birds were weighed initially and at the end of each 28-day period.

The pullets of each hybrid strain were randomly divided into eight treatment groups and each group was fed one of the following experiment diets.

1. A high-energy cereal-based diet fed *ad libitum.*
2. A concentrated diet containing 18% arachis oil fed *ad libitum.* This diet was designed so that 85 g of the concentrated diet would contain the same amount of nutrients, including protein, trace elements and vitamins as 110 g of the high-energy diet.

3. Diet 2 restricted to 95 g daily for each individual bird.
4. Diet 2 diluted to contain 13.64% of wood dust and fed *ad libitum*.
5. Diet 2 restricted to 85 g daily for each individual bird.
6. Diet 2 diluted to contain 22.73% of wood dust and fed *ad libitum*.
7. Diet 2 restricted to 75 g daily for each individual bird.
8. Diet 2 diluted to contain 31.82% of wood dust and fed *ad libitum*.

The daily ME requirements of the birds of both breeds for maintenance and production were estimated using the equations derived from the calorimetric work of Waring and Brown.[9, 10]

It was attempted to design the diets in such a way that the birds receiving the diet containing 13.64% of wood dust (diet 4) would eat the same daily quantity of the concentrated dietary component as those being fed 95 g of the concentrated diet (diet 3)

TABLE 1. Composition of the high-energy diet, the concentrated and the diluted diets

Diet No. / Constituent	1 High-energy diet		2 Concentrated diet
Maize meal	59.6		34.5
Ground wheat	10.0		—
Peruvian fish meal (63–65% *CP*)	5.0		10.0
Soyabean meal-extr. (44.5% *CP*)	15.0		26.0
Dried grass meal (16% *CP*)	2.5		2.5
Arachis oil	—		18.0
Ground limestone	6.0		6.0
Steamed bone flour	1.0		2.0
Common Salt	0.5		0.5
[a] Vitamin-mineral supplement	0.4		0.5
Total	100.0		100.0
Dry matter, (%)	88.0		91.6
Crude protein (*CP*), (%)	16.6		21.1
Standard ME (kcal/kg)	2750		3720
Nitrogen-corrected ME (kcal/kg)	2657		3613
Calorie: protein ratio	166		176
Linoleic acid (%)	1.09		4.47
Density (g/ml)	0.536		0.745
Diet no.	4	6	8
Wood dust (%)	13.64	22.74	31.82
Dry matter (%)	92.4	90.5	90.3
Standard ME (kcal/kg)	3240	3000	2790
Nitrogen—corrected ME (kcal/kg)	3160	2930	2750
Crude protein (%)	18.2	16.9	15.5
Calorie:protein ratio	178	177	180
Density (g/ml)	0.665	0.492	0.420
Linoleic acid (%)	2.92	2.40	2.59

[a] The mineral supplement supplied 8×10^6 i.u. vitamin A; 2×10^6 i.u. vitamin D_3; 4 g vitamin B_2; 8 g vitamin E; 6.8 mg vitamin B_{12}; 1 g vitamin K; 20 g nicotinic acid; 8 g panthothenic acid; 0.5 g folic acid; 100 g choline chloride; 20 g Fe; 3 g Co; 100 g Mn; 5 g I, 100 g Zn and 8 g Cu per 1000 kg of high-energy diet. The same supplement was added to the concentrated diet to supply 110/85 times the level of the vitamins present in the high-energy diet.

daily. A similar type of relationship was designed for diets 5 and 6 and diets 7 and 8, respectively.

The high ME level in the concentrated diet was attained using arachis oil, the fatty acids of which had the following percentages: linoleic acid 19.1 %, oleic acid plus stearic acid 65.4 %, palmitic acid 10.9 % and other fatty acids 4.6 %.

The compositions of the diets are given in Table 1. The mean determined values of 3 mixes for crude protein, Ca and P are presented, together with the mean ME content based on two mixes. Metabolisable energy was determined for two mixes of each diet by the total collection method. The first ME values were determined using 4 birds for each treatment. These birds were not from the laying experiment but had been acclimatised to the experimental diets for several weeks. The second set of values were obtained using 4 birds from each treatment at the end of the laying trial. These birds had been on the appropriate treatment for 42 weeks. The density values were determined by loosely filling a beaker with the diet.

Mainly abiche wood dust was used although some mahogany dust was also incorporated into the diets. The average crude fibre content of the wood dust used was 62.8 % and the average crude protein content was 2.2 %. The cereal-based high-energy diet (diet 1) was included merely to indicate how the hens performed on a more normal diet as opposed to the concentrated diet *ad libitum*, directly restricted or restricted by dilution. No major reference is subsequently made to the high-energy diet.

3. Results

The mean results for food intake, egg production, egg weight and efficiency of food conversion are presented in Table 2. The mean data for daily food intake, ME intake, efficiency of ME utilisation and energetic efficiency are presented in Table 3.

3.1. Egg production and egg weight

The percentage egg production is illustrated in Figure 1 and shows that for both breeds production on the concentrated diet fed *ad libitum* was consistently higher than production on the cereal-based high-energy diet. Production on the 75 g level of the concentrated diet [Figure 1(a) and (c)] was fairly consistently lower than that on the *ad libitum*, 95 or 85 g regimes. At the 13.6 % level of dilution the light hybrids [Figure 1(b)] had a lower production than when fed the concentrated diet *ad libitum* except in period 8, while the medium hybrids [Figure 1(d)] gave a higher percentage production on the 13.6 % level of dilution than the concentrated diet from the fifth period until the end of the experiment. Production at the 22.7 % level of dilution was fairly consistently below, and that at the 31.8 % level of dilution always markedly below production at the 13.6 % level.

Percentage egg production was markedly affected by diets ($P < 0.001$) and breeds ($P < 0.05$). There was no significant interaction between diets and breeds. The largest number of eggs produced was on the concentrated diet fed *ad libitum* (diet 2) and at the 95 g and 85 g restricted levels of feeding (diets 3 and 5) and at the lowest level of dilution (diet 4). The percentage production on these 4 diets did not differ significantly at the

TABLE 2. Mean total food intake, egg production and egg weight data for the light and medium hybrids[a]

	Breed	1 High-energy *ad libitum*	2 Concentrated *ad libitum*	3 Concentrated 95 g	4 Concentrated × wood dust (13.64%)	5 Concentrated 85 g	6 Concentrated × wood dust (22.73%)	7 Concentrated 75 g	8 Concentrated × wood dust (31.82%)
No. of hens	L	15	16	19	18	17	19	18	14
	M	19	17	15	17	17	16	16	20
Total food intake (kg)	L	35.01 ± 0.410	30.69 ± 0.606	25.76 ± 0.209	33.23 ± 0.477	23.49 ± 0.237	34.18 ± 0.340	20.99 ± 0.004	34.42 ± 0.432
	M	35.63 ± 0.185	32.6 ± 0.500	26.00 ± 0.271	35.32 ± 0.345	23.66 ± 0.092	35.87 ± 0.154	20.99 ± 0.005	34.94 ± 0.278
"Corrected" total food intake (kg)	L				28.70 ± 0.412		26.41 ± 0.263		23.47 ± 0.295
	M				30.51 ± 0.298		27.72 ± 0.119		23.82 ± 0.190
Total egg weight (kg)	L	12.16 ± 0.489	13.87 ± 0.451	13.28 ± 0.307	12.83 ± 0.310	12.61 ± 0.504	11.65 ± 0.327	11.18 ± 0.404	8.00 ± 0.573
	M	11.82 ± 0.351	13.70 ± 0.336	12.61 ± 0.254	12.54 ± 0.206	12.48 ± 0.238	11.29 ± 0.447	11.25 ± 0.411	6.80 ± 0.395
Food conversion of diet as fed (kg food/kg eggs)	L	2.98 ± 0.188	2.23 ± 0.057	1.96 ± 0.046	2.62 ± 0.067	1.95 ± 0.130	2.97 ± 0.057	1.93 ± 0.089	4.71 ± 0.320
	M	3.07 ± 0.103	2.40 ± 0.056	2.07 ± 0.041	2.69 ± 0.036	1.91 ± 0.037	3.29 ± 0.149	1.92 ± 0.095	5.58 ± 0.292
"Corrected" food conversion (kg food/kg eggs)	L				2.26 ± 0.058		2.29 ± 0.044		3.21 ± 0.218
	M				2.32 ± 0.031		2.54 ± 0.115		3.81 ± 0.199
Total number of eggs (280 days)	B	205.6 ± 4.6	218.8 ± 4.6	210.5 ± 4.6	216.5 ± 4.6	211.5 ± 4.6	195.2 ± 4.6	189.7 ± 4.6	126.5 ± 4.6
Egg production (%)	B	73.5 ± 1.6	78.2 ± 1.6	75.0 ± 1.6	77.4 ± 1.6	75.6 ± 1.6	69.6 ± 1.6	67.7 ± 1.6	45.7 ± 1.6
Mean period mean egg weight (g)	B	58.4 ± 0.63	63.3 ± 0.63	61.8 ± 0.63	60.5 ± 0.63	59.7 ± 0.63	59.1 ± 0.63	59.6 ± 0.65	55.7 ± 0.63
Total egg wt / No. of eggs (g)	L	58.8	62.7	62.2	60.1	59.8	58.7	58.9	58.5
	M	57.7	63.3	61.0	57.0	58.8	59.0	59.4	56.9

[a] Data recorded over a period of 280 days; L = light Hybrid; M = medium Hybrid; B = mean data for both Hybrids.

TABLE 3. Mean daily food and ME intake and ME conversion data

	Breed	1 High-energy *ad libitum*	2 Concentrated *ad libitum*	3 Concentrated 95 g	4 Concentrated + wood dust (13.64%)	5 Concentrated 85 g	6 Concentrated + wood dust (22.73%)	7 Concentrated 75 g	8 Concentrated + wood dust (31.82%)
Daily food intake of diet as fed (g)	L	125.0 ± 1.463	109.6 ± 2.165	92.0 ± 0.748	118.7 ± 1.705	83.9 ± 0.848	122.1 ± 1.215	75.0 ± 0.015	122.9 ± 1.544
	M	127.2 ± 0.661	116.5 ± 1.786	92.9 ± 0.971	126.2 ± 1.234	84.5 ± 0.329	128.1 ± 0.549	75.0 ± 0.017	124.8 ± 0.994
"Corrected" daily food intake (g)	L				102.5 ± 1.473		94.3 ± 0.939		83.8 ± 1.053
	M				108.9 ± 1.065		99.0 ± 0.424		85.1 ± 0.678
Daily ME intake of diet as fed (kcal)	L	344 ± 4.02	408 ± 8.05	342 ± 2.78	385 ± 5.39	312 ± 3.15	366 ± 3.56	279 ± 0.06	343 ± 4.25
	M	350 ± 1.82	433 ± 6.65	346 ± 3.61	409 ± 3.90	314 ± 1.22	384 ± 1.61	279 ± 0.06	348 ± 2.74
[a] Calculated daily ME intake (kcal)	L				381		351		312
	M				405		368		316
ME conversion (Mcal/kg eggs)	L	7.92	8.23	7.22	8.39	6.93	8.80	6.98	12.01
	M	8.29	8.85	7.67	9.13	7.05	9.53	6.94	14.33
[b] Energetic efficiency	L	20.2	19.4	22.2	19.1	23.1	18.2	22.9	13.3
	M	19.3	18.1	20.9	17.5	22.7	16.8	23.0	11.2

[a] Calculated from the ME value for diet 2 assuming that the wood dust has no ME value.

[b] Energetic efficiency $= \frac{\text{gross energy in eggs} \times 100}{\text{ME of food}}$ assuming the gross energy content of egg product to be 1.62 kcal/g.

5 % level. Restriction of the concentrated diet to 75 g caused a significant reduction in the percentage egg production ($P < 0.001$) and dilution of the diet with both 22.7 % wood dust (diet 6) and with 31.8 % wood dust (diet 8) caused a statistically significant reduction ($P < 0.01$ and $P < 0.001$, respectively).

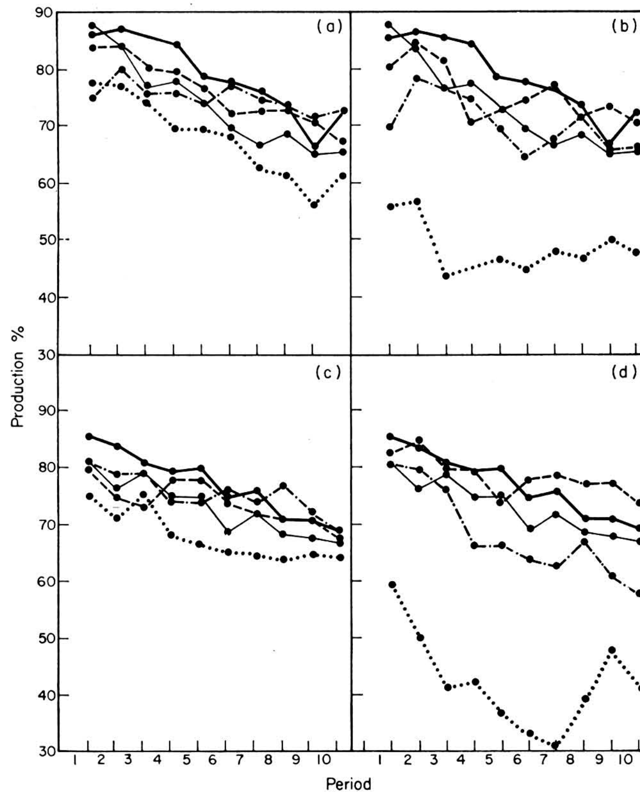

Figure 1. Effect of dietary restriction and dilution on the percentage egg production. (a) and (c) ●————●, Concentrated high-energy *ad libitum*; ●------●, 95 g; ●-·-·-·-●, 85 g; ●······●, 75 g. ●————●, cereal-based high-energy diet *ad libitum*; (a) Hyline (c) Hybrid-4. (b) and (d) ●————●, Concentrated high-energy diet *ad libitum*; ●------●, 13.6% wood dust; ●-·-·-·-●, 22.7% wood dust; ●······●, 31.8% wood dust. ●————●, cereal-based high energy diet *ad libitum*; (b) Hyline (d) Hybrid-4.

The total egg weight data show the same general trends as the values for percentage production. The results for total egg weight showed significant variance heterogeneity between the treatments and consequently the within treatment variances could not be pooled for an analysis of variance. Thus, treatment comparisons were carried out using Welch's test.[11] In the case of the light hybrids this showed, as for percentage production, that the concentrated diet fed *ad libitum*, and at the 95 and 85 g levels of restricted feeding (diets 2, 3 and 5) and diluted with 13.6 % wood dust (diet 4), gave total egg weights which

were not significantly different at the 5% level. Further restriction or dilution caused a highly significant fall in production by the light hybrids. In the case of the medium weight hybrids, the 95 and 85 g levels of restricted feeding significantly reduced total egg weight ($P < 0.01$) and this effect was more marked at the 75 g level ($P < 0.001$). Total egg weight was depressed ($P < 0.01$) at the first level of dilution (diet 4) and further reduced ($P < 0.001$) at the intermediate level (diet 6) and this effect was very marked ($P < 0.001$) for the medium weight hybrids fed the diet containing 31.8% wood dust (diet 8).

Mean period mean egg weight

$$\text{period mean egg weight} = \frac{\text{total weight of eggs weighed per period}}{\text{number of eggs weighed per period}}$$

was significantly affected by dietary treatment ($P < 0.001$) but not by breeds. No significant interaction was found between breeds and diets. The diets giving the highest mean period mean egg weight were the concentrated diet fed *ad libitum* and the 95 g level of restriction. The values for these were not significantly different at the 5% level. Further restriction led to a highly significant ($P < 0.001$) reduction in mean period mean egg weight. Dilution of the diet at the lower level (treatment 4) caused a significant depression of the mean period mean egg weight and this effect was more pronounced ($P < 0.001$) at the intermediate and greatest levels of dilution. The same trends are seen when the mean egg weight is expressed as total egg weight divided by the number of eggs laid.

3.2. Food intake and efficiency of utilisation

Owing to variance heterogeneity in both variables individual variances were calculated for each comparison.

Total food intake is presented as the total intake of the various diets and in the case of the diluted diets also as a "corrected" food intake which gives the intake of the quantity of concentrated diet present in the diluted diets.

The food intake of the concentrated diet fed *ad libitum* was considerably higher ($P < 0.001$) than for the highest restricted level (diet 3) and the intakes of the restricted diets (3, 5 and 7) were fixed in relation to each other by the experimental design. The birds eating the diluted diets (4, 6 and 8) attempted to maintain their nutrient intake by increasing their daily intake above that of the concentrated diet fed *ad libitum* ($P < 0.001$). Although the bulk of material eaten was greater than for the birds on the concentrated diet, the birds fed the diluted diets failed to attain the same intake of energy and other nutrients as the birds on dietary regime 2. This effect was much the same for both the light and medium hybrids, the fall in actual concentrated diet intake being 7, 15 and 26 g for the former and 8, 18 and 33 g for the latter. For the light and medium hybrids this effect on concentrated diet intake was highly significant at the first level of dilution ($P < 0.01$) and for all other cases the effect was very highly significant ($P < 0.001$).

Two figures are presented for efficiency of food utilisation. One of these is the conventional figure based on the intake of diet and the mass of egg product while the other

("corrected" food conversion) is based on the actual intake of the concentrated diet and this gives a better conversion figure than the normal conversion figure for diets 4, 6 and 8. When compared with the concentrated diet fed *ad libitum* food conversion was improved as controlled restriction of the concentrated diet became more severe ($P < 0.001$) in all cases except for the 85 g restricted diet which gave a value not significantly different to that obtained on diet 2. The values obtained, approaching 1.9, are lower than any food conversion figures found by the author in the scientific literature.

The inclusion of wood dust in the diet caused a fall in total egg weight although there was an increase in food intake. This has resulted in a poorer food conversion figure than for diet 2 (all $P < 0.001$) and at the 31.82% level this reached the very poor mean conversion figure of 5.15. When the conversion figure was amended for the wood dust content, the "corrected" conversion figure at the 13.6 and 22.3% level of addition showed no significant difference to the conversion figure obtained for diet 2. At the highest level of wood dust inclusion the detrimental effect on the corrected food conversion was very marked ($P < 0.001$), the average figure being 3.51.

3.3. Metabolisable energy intake and efficiency of utilisation

When the ME values for the diluted diets were determined the values obtained were slightly higher than those calculated by estimating the ME contribution from the concentrated diet present in the mix. The hens fed the concentrated high-energy diet had the very high daily ME intake of 420 kcal.

The ME intake of hens fed the concentrated diet was higher ($P < 0.001$) than the ME intake of birds on any of the other diets. The ME intakes of the restricted diets were

TABLE 4. Initial, final, mean body weight and body weight gain data for light and medium hybrid layers

	Light hybrid—Diet No.							
	1	2	3	4	5	6	7	8
Initial body weight (kg)	1.54	1.57	1.58	1.47	1.50	1.51	1.52	1.57
Final body weight (kg)	1.57	1.98	1.88	1.74	1.69	1.58	1.51	1.40
Mean body weight (kg)	1.58	1.85	1.83	1.67	1.71	1.58	1.60	1.48
Body weight gain (g)	39	281	251	197	202	76	84	−90
	Medium hybrid—Diet No.							
Initial body weight (kg)	2.04	2.06	2.06	2.06	2.17	2.08	1.99	2.07
Final body weight (kg)	1.87	2.71	2.40	2.25	2.11	1.89	1.84	1.73
Mean body weight (kg)	1.94	2.52	2.33	2.21	2.24	1.93	1.99	1.83
Body weight gain (g)	−96	464	269	150	67	−148	−1	−244

directly determined by the degree of restriction of the diet, and so were in the same relative proportions as the feed intake. The statistical differences were as in the case of the feed intake.

For the diluted diets the ME intake fell by approximately 26 g with each increase in the level of dilution.

For both breeds direct restriction of the diet (as opposed to restriction by dilution) resulted in a marked improvement in ME conversion. The treatment mean showed a

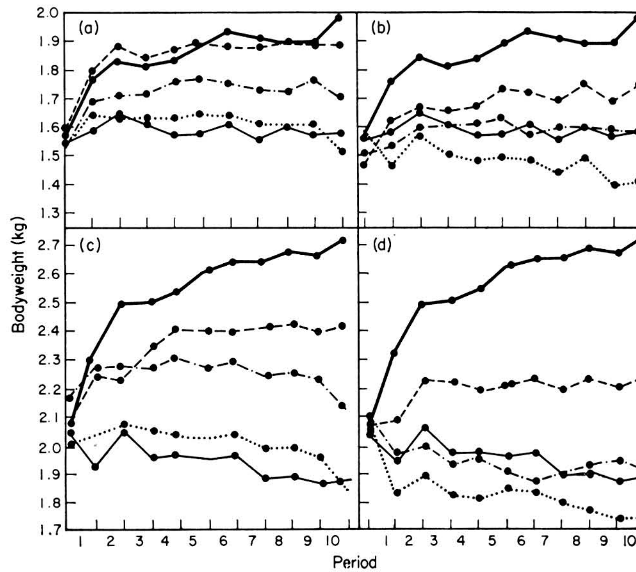

Figure 2. Effect of dietary restriction and dilution on body weight.
(a) and (c) ●———●, Concentrated high-energy diet *ad libitum*; ●------●, 95 g; ●-·-·-·-●, 85 g; ●······● ,75 g. ●———●, cereal-based high-energy diet *ad libitum*; (a) Hyline (c) Hybrid-4
(b) and (d) ●———●, Concentrated high-energy diet *ad libitum*; ●------●, 13.6% wood dust; ●-·-·-·-●, 22.7% wood dust; ●······●, 31.8% wood dust. ●———●, cereal-based high-energy diet *ad libitum*; (b) Hyline (d) Hybrid-4

marked improvement at the 95 and 85 g levels of restriction, but no improvement in ME conversion was obtained by intensifying the degree of restriction from 85 to 75 g.

Although diet 8 (the most dilute diet) had approximately the same ME content as the ordinary high-energy diet (diet 1), the mean ME conversion of diet 8 was 13.2, while for diet 1 it was 8.1.

When ME conversion of the diluted diet was calculated as the ME conversion of the concentrated diet present in the diluted diets, then a considerably higher value was obtained. These values were poorer at all levels of dilution than for the concentrated diet, although this effect may only be significant at the greatest level of dilution.

3.4. Body weight

The initial and final bodyweights, the average bodyweights over the experimental period and the bodyweight gains are presented in Table 4. The mean bodyweights are the arithmetic means of 11 weighings made at 28-day intervals. The bodyweight gain, obtained from this figure and the initial bodyweight, was significantly affected by dietary treatment and by breed. The bodyweights are graphically presented, separately for each breed, and separately for the directly restricted birds and those fed the diluted diets, in Figure 2.

In Figure 2(a) it can be seen that for the Hyline birds there was an initial gain in bodyweight in the first 2 periods and after this bodyweight remained fairly constant. The 95 g level of restriction caused little reduction in bodyweight gain, but the 85 and 75 g levels reduced bodyweight gain to approximately 79 and 28 %, respectively, of the gain on the concentrated diet fed *ad libitum*.

In the case of the Hybrid-4 birds [Figure 2(c)] a much larger bodyweight gain occurred on the concentrated diet fed *ad libitum* and restriction had a very pronounced effect.

TABLE 5. Regression equations of total egg weight, ME conversion and energetic efficiency on daily food intake for the restricted diets and percentage wood dust

Dependent variable Y	Independent variable X	Breed	Regression equation	$F_{(1,2)}$ for regression	% Variation removed by regression
Total egg weight	Daily food intake	L	$Y = 6.1623 + 0.0729X$	12.57 n.s.	86.28
		M	$Y = 7.5384 + 0.0539X$	19.77*	90.81
	% Wood dust	L	$Y = 14.5441 - 0.1734X$	10.94 n.s.	84.55
		M	$Y = 14.5315 - 0.2022X$	9.45 n.s.	82.54
M.E. conversion	Daily food intake	L	$Y = 3.8607 + 0.0386X$	14.36 n.s.	87.77
		M	$Y = 3.1454 + 0.0486X$	68.63*	97.17
	% Wood dust	L	$Y = 7.5544 + 0.1059X$	3.69 n.s.	64.88
		M	$Y = 7.8735 + 0.1517X$	3.40 n.s.	62.98
Energetic efficiency	Daily food intake	L	$Y = 31.6966 - 0.1087X$	14.12 n.s.	87.59
		M	$Y = 32.6847 - 0.1248X$	64.04*	96.97
	% Wood dust	L	$Y = 20.4582 - 0.1735X$	4.35 n.s.	68.48
		M	$Y = 19.2094 - 0.1941X$	4.39 n.s.	68.71

* $= P < 005$; n.s. = non-significant

The first level of restriction reduced bodyweight gain by over 40%, the 85 g level by 85% and at the 75 g level a small loss of bodyweight occurred.

Dilution of the diets had a more marked effect on bodyweight. The increase in the level of dilution caused a decrease in bodyweight gain. For the Hyline birds [Figure 2(b)]

a weight loss occurred only at the greatest level of dilution but for the Hybrid-4 birds, [Figure 2(d)] on average losses of approximately 0.6 and 0.9 g per day occurred at the 22.7 % and the 31.8 % levels of dilution, respectively.

Regression equations are presented for both breeds and both methods of restriction in Table 5 where total egg weight, ME conversion and energetic efficiency are the dependent variables and the independent variables (X) are, in the case of the restricted diets, the daily food intake, and in the case of the diluted diets, the percentage wood dust in the diet.

4. Discussion

The restriction of the feed intake of laying hens is not a new concept. For example, some 30 years ago Temperton and Dudley[12–14] examined the effect of the restriction of food to both growing and laying stock and found that with restriction of intake to a level of about 90 % of the amounts consumed *ad libitum* egg production was fairly well maintained, but at the expense of body weight. There is, however, a lack of data in the literature for egg production, egg weight, feed intake, ME intake and body weight based on results from individually fed modern Hybrid layers consuming high-energy diets and using experimentally determined ME values for individual diets.

The fact that the determined standard ME values (Table 1) for diets 6 and 8 were approximately 56 and 200 kcal/kg higher than the values anticipated from the ME value of diet 2 and the percentage of diluent present would suggest that the wood dust contributed to the ME value directly or indirectly. Guisti[3] reported a digestibility by chickens of 2.33 % for the cellulose fraction of sawdust and Lepp, Harper and Elvehjem[15] suggested that the growth stimulation found in poultry on the inclusion of cellulose in the diet may be due to an effect on the intestinal synthesis of "various known or unknown factors".

The hens in the present experiment showed no major fall in egg production at the 13.6 % level of sawdust. At the 22.7 % level of wood dust a significant ($P < 0.01$) fall occurred. Davis and Briggs[4] found that levels of screened pine sawdust of up to 20 % were tolerated by chicks without ill-effects. The crude fibre content of their sawdust was 60.6 % while the average crude fibre content of the wood dust used in the present experiment was 63 %. In the present experiment the cellulose content of the wood dust was 35.4 % and the lignin content 24.2 %. The results of the present work do not agree with those of Kubota, Lee and Morimoto[16] who found that after about 7 weeks hens on a 7 % crude fibre diet had a much poorer feed intake and egg production than those on diets containing 2.0 and 4.5 % crude fibre.

Calculation of the requirements suggested that at 80 % production a 1.5 kg light Hybrid making no bodyweight gain would require approximately 75 g of the concentreated diet per day, when the requirements were calculated using the equation ME intake (kcal/day) $= 1.86\,E + 115.1\,W$, where $E =$ egg product + change in body weight (g) and $W =$ mean body weight (kg), derived from the calorimetric data of Waring and Brown[10] for the White Leghorn. A similar calculation for the medium Hybrid using a mean equation of $1.92\,E + 90.3\,W$, derived from the data of Waring and Brown[9,10], indicated that for a 2.0 kg hen the daily requirement would be approximately 90 g. Using the equations of Waring and Brown[9,10] the theoretical additional daily ME

required to provide for the maintenance of the observed mean extra bodyweight would be:

Diet	1	2	3	4	5	6	7	8
Additional ME (kcal/bird/day)	5	32	29	23	24	8	9	−11
Additional ME (%)	3	18	16	14	14	5	5	−6

for the light hybrids and for the medium hybrids the theoretical values would be

Diet	1	2	3	4	5	6	7	8
Additional ME (kcal/bird/day)	−10	47	28	15	7	−15	0	−25
Additional ME (%)	−5	22	13	7	3	−7	0	−12

The data indicate that restriction, either directly or by dilution, considerably reduced both the bodyweight gain and the excess energy required to maintain this gain from approximately 20% for both breeds to a figure of even below that required for maintenance of the initial body weight.

However, consideration of the observed energy intakes compared with the theoretical total requirement for both the observed maintenance and production indicates a situation in which each type of restriction gave dissimilar results. The calculated ME requirements for maintenance and egg production for the light hybrids are presented below, the calculated value being based on Waring and Brown's[10] data and the observed egg production and mean bodyweight values. The efficiency data[9, 10] of these workers has been confirmed by Van Es *et al.*[17] using larger numbers of birds under respiration chamber conditions.

Diet	1	2	3	4	5	6	7	8
Calculated ME requirement (kcal/hen/day)	263	307	301	279	281	260	259	199
Observed ME intake (kcal/hen/day)	344	408	342	385	312	366	279	343
Additional ME consumed (% of calculated requirement)	31	33	14	38	11	41	8	72

These data indicate that increasing the degree of restriction by directly limiting intake markedly reduced the excess ME consumed over the theoretical value and at the 75 g level of intake, the calculated and observed values were as close as could reasonably be expected. However, increasing restriction by dilution resulted in a marked increase

in the excess ME consumed over the theoretical value and at the greatest level of dilution the difference was almost 0.75 of the initial theoretical figure. Similar data are presented below for the hybrid 4 birds.

Diet	1	2	3	4	5	6	7	8
Calculated ME requirement (kcal/hen/day)	280	356	328	314	316	275	281	233
Observed ME intake (kcal/hen/day)	350	433	346	409	314	384	279	348
Additional ME consumed (% of calculated requirement)	25	22	5	30	−1	40	−1	49

These data again show that dilution was very effective in the case of restriction by direct limitation, but that increased dilution actually resulted in a large increase in the observed intake compared to the theoretical requirement for the level of egg production and bodyweight gain obtained.

The different effects of restriction and dilution are very readily seen on comparing diets 3 (95 g) and 8 (31.8% wood dust). Although the ME intake is almost identical in both cases, the birds on the former diet had 75% production and a mean weight gain of 260 g while the birds on diet 8 had a production of 46% and a bodyweight loss of 167 g. The reason for the vast difference in the efficiency of utilisation of the ME is not readily obvious.

The improved efficiency of utilisation of ME on directly restricting intake to 95, 85 and 75 g may not merely be due to the deprivation of the bird of excess calories. It could also be due to a lower percentage spillage with a decreasing amount of diet being presented to the bird. An activity factor could also be involved, the bird with less food possibly expending less energy in unproductive activity than the bird with a larger amount of food. Temperature has not been considered in the equations of Waring and Brown,[9, 10] and since the birds were housed in an unheated house during the winter period when ground temperatures fell to −18 °C, this could be a factor in the deviation of the observed from the theoretical values. In addition, the fact that the diets in the present experiment contained 18% arachis oil could lead to a higher requirement than that calculated by Waring and Brown,[9, 10] since experimental results suggest that the ME of high-fat diets is less efficiently used for egg production than the ME of cereal-based diets.[18–21] This is dissimilar to the situation in the cockerel where high-fat diets are used more efficiently for body fat production than are cereal-based diets.[22] The poor utilisation of ME at the high levels of wood dust inclusion could be partially due to high activity, including gut activity, in dealing with a highly fibrous diet of the type used. In addition, the possible presence of toxic factors in the wood dust cannot be ruled out. Also it seems quite likely that the degree of spillage would increase with decreasing density of the diet.

The results of the experiment do show that restriction will give a highly efficient utilisation of ME for production, even at a level only some 66% of the *ad libitum* intake, while only at the lowest level of dilution (13.6% wood dust) was the production maintained without a marked drop in the efficiency of ME utilisation.

In the case of dilution, presumably the decrease in diet density from a maximum of 0.745 g/ml to a minimum of 0.420 g/ml resulted in the birds failing to compensate fully for the effect of dilution by increasing their feed intake. The marked increase in surplus ME usage is probably a function of the spillage, which was noticeably greater for the low-density diets containing wood dust than for the more dense concentrated diet.

The data presented above show a definite relationship between the degree of dilution and the calculated additional ME consumed and in the case of the medium hybrids this relationship is linear. While the food intakes for the three diluted diets were similar it would be expected that the activity associated with prehension should be constant. It would appear, therefore, that other activity, including that associated with the passage of food through the gut, may increase linearly with the fibre content of the diet.

In applying the equations of Waring and Brown[9, 10] it must be borne in mind that these were obtained using hens with a fairly narrow range of egg production and so reservations must be retained when applying their use to a situation of the widely varying productions obtained in this experiment. However, using their equations they did find good agreement between the predicted ME consumption and the actual ME consumption obtained in a laying trial using 576 hens.[23] In addition, these equations assume that the same energy is associated with 1 g of bodyweight as with 1 g of egg product. The nature of the fairly rapid initial weight gain in this experiment was not ascertained, but a considerable proportion of it may be as body fat having a gross energy value of 9.5 kcal/g of dry matter while the gross energy value of dry egg product has been found by Shannon[24] to be 4.9 kcal/g at 22 °C. This suggests that although the equations of Waring and Brown[9, 10] are valid for maintenance and egg production, it would be desirable to initiate work to enable a separate variable for the ME component due to body weight gain to be inserted in the ME intake equation.

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References

1. Jackson, N. *Br. Poult. Sci.* **1970**, 11, 93.
2. Ewing, W. R. *Poultry Nutrition* (W. Ray Ewing, California) 3rd Ed., **1947**, p. 406.
3. Guisti, G. *Clinica. vet., Milano* **1939**, 62, 283.
4. Davis, F.; Briggs, G. M. *Poult. Sci.* **1948**, 27, 117.
5. Reid, B.; Hinds, H. B.; Kurnick, A. A. *Poult. Sci.* **1961**, 40, 1446 (Abstr).
6. Peeler, H. T. cited in *Feedstuffs* **1968**, 40, No. 18.
7. Foster, W. H. *Rec. agric. Res. Minist. Agric. Nth. Ire.* **1968**, 17, 13.
8. Ousterhout, L. E. *Feedstuffs*, **1970**, 42, No. 1, 34.
9. Waring, J. J.; Brown, W. O. *J. agric. Sci., Camb.* **1965**, 65, 139.
10. Waring, J. J.; Brown, W. O. *J. agric. Sci., Camb.* **1967**, 68, 149.

11. Welch, B. L. *Biometrika* **1938**, 29, 350.
12. Temperton, H.; Dudley, F. J. *Emp. J. exp. Agric.* **1941**, 9, 248.
13. Temperton, H.; Dudley, F. J. *Harper Adams Util. Poult. J.* **1944**, 29, 24.
14. Temperton, H.; Dudley, F. J. *Harper Adams Util. Poult. J.* **1945**, 30, 28.
15. Lepp, A.; Harper, A. E.; Elvehjem, C. A. *Poult. Sci.* **1949**, 28, 372.
16. Kubota, D.; Lee, Y. S.; Morimoto, H. *Jap. Poult. Sci.* **1967**, 4, 107.
17. Van Es. *et al. Energy Metabolism of Farm Animals* (E.A.A.P.) Publ. No. 13 **1970**.
18. Weiss, H. S.; Fisher, H. *J. Nutr.* **1957**, 61, 267.
19. Donaldson, W. E. *Poult. Sci.* **1962**, 41, 1060.
20. Waring, J. J.; Addison, R. F.; Brown, W. O. *J. agric. Sci., Camb.* **1968**, 65, 139.
21. Jackson, N.; Kirkpatrick, H. R.; Fulton, R. B. *Br. Poult. Sci.* **1969**, 10, 115.
22. Shannon, D. W. F.; Brown, W. O. *J. agric. Sci., Camb.* **1969**, 72, 479.
23. Brown, W. O.; Waring, J. J.; Addison, R. F. *Progress in Livestock Nutrition* 1962–1967. *Techn. Publ. U.S. Feed Grains Council.* **1967**, p. 77.
24. Shannon, D. W. F. Ph.D. thesis, Queen's University of Belfast **1966**.

J. Sci. Fd Agric. 1972, **23**, 429–440

Changes in Carbohydrates During Growth and Development of Bajra (*Pennisetum typhoides*), Jowar (*Sorghum vulgare*) and Kangni (*Setaria italica*)[a]

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Qualitative and quantitative changes in carbohydrate contents have been studied during the growth and development of bajra (*Pennisetum typhoides*), jowar (*Sorghum vulgare*) and kangni (*Setaria italica*). Besides traces of glucofructosans, sucrose, glucose and fructose are the principal sugars present in the leaf and stem tissues of all stages of growth. As growth proceeds, water-soluble carbohydrates increase in the stem, attaining a maximum value at flowering and initiation of seed formation and thereafter steadily decline reaching a low value at maturation. Cellulose also increases with growth in bajra and jowar stem. This increase is more marked at early stages of growth. Starch content gradually increases with the simultaneous decrease in free and total reducing sugars in the developing grains. Some fructosans are also synthesised in the grains during their formation and presumably serve as intermediates in the synthesis of starch.

1. Introduction

The dry matter of a plant consists principally of carbohydrates and the stage of its growth is a major factor governing its chemical composition. Though considerable work has been done on the water-soluble carbohydrates of grasses,[1–3] information on the carbohydrates of millets, the stalks of which are used as fodder in India, is scanty. In the present paper, data are presented on the changes in carbohydrates of bajra (*Pennisetum typhoides*), jowar (*Sorghum vulgare*) and kangni (*Setaria italica*) during the normal growth of these forage plants from a young vegetative stage, through inflorescence and seed setting to complete maturation. The carbohydrate changes during the development of grains have also been studied to ascertain the role of fructosans as the possible intermediates in the formation of starch.

2. Material and methods

2.1. Plant material

The following plant species were used as fodder.

(1) Bajra (*Pennisetum typhoides* Buram S. & H.).
  (i) A1/3 (long eared),
  (ii) S.530 (Bristle eared).

[a] Work on bajra and jowar is from the Ph.D. thesis of Dr Rangil Singh and on kangni from the M.Sc. thesis of Mrs Saroj Dua.

(2) Jowar (*Sorghum vulgare* Pers.)
   (i) J.S.263 (white grained, sweet, juicy stem, compact ear),
   (ii) J.S.20 (purple or black grained, non sweet, fine stock lax ear).
(3) Kangni (*Setaria italica* Beauv.).

Genetically pure seeds of these fodder plants were acquired and the plants raised to maturity. A brief account of the field operations and analytical techniques employed follows.

To minimise the error arising from differences in fertility status of the soil, each crop variety was raised in three different fields. At the time of sowing of bajra and jowar, superphosphate and muriate of potash were applied each at the rate of 25 lb[a]/acre of P_2O_5 and K_2O, respectively. Ammonium sulphate at the rate of 50 lb of N/acre was, however, applied in two equal doses, one at the time of sowing and the other as top dressing when the plants were 15 days old. No fertiliser treatment was given to kangni. The crops were sown in rows 2 ft (0.6 m) apart with plants spaced at 1 ft (0.3 m) within the row. Constant care was taken with respect to uniform irrigation and control of weeds.

2.2. Collection of plant samples

Samples were collected at random from the selected alternate rows, second, fourth, sixth etc., the time interval between successive sampling varied from 8 to 10 days for different crops, starting from the growth stage of 30 (bajra and jowar) and 45 (kangni) days after sowing to complete maturation. For germination studies the seedlings were raised in sand in the laboratory. Care was exercised to ensure that the samples collected were representative. In bajra and jowar whole leaf and stem samples were taken at the early stages of growth. However, after the 50-day growth stage after sowing the plants were cut into two halves and the composite samples collected separately for different plant parts like upper (younger), lower (older) leaves and upper, lower stems. In kangni the samples of upper, middle and lower plant parts were collected from the 45-day growth stage after sowing onwards. The grain samples, representing various stages of development, were collected from the tillers which were of uniform growth and had already been tagged at the time of flowering. Samples were always taken at noon to avoid any possible variation due to difference in sampling times (Eagles).[4]

2.3. Preservation and storage of the plant tissues

Fresh samples were dried rapidly in an oven in a forced draught of hot air, ground to 40 mesh and stored in tightly capped glass bottles. At the same time a portion of each fresh sample was preserved in hot ethanol to a final concentration of not less than 80%.

2.4. Qualitative make up of water-soluble carbohydrates in the leaf and stem tissues

Hot water extracts of both dried and ethanol-preserved samples were chromatographed by descending paper partition chromatography as described by Srinivasan and Bhatia.[5] Chromatograms were run with *n*-butanol–acetic acid–water (4:1:5) as the solvent system (organic phase) and the various spray reagents used were: benzidine–trichloroacetic acid,[6] benzidine reagent,[7] urea–metaphosphoric acid,[8] sodium metaperiodate–

[a] Throughout this paper 1 lb = 0.454 kg, 1 acre = 4050 m^2.

benzidine,[9] *p*-anisidine phosphate,[10] naphtha–resorcinol–trichloroacetic acid,[11] silver nitrate reagent[12] and ammonical silver nitrate.[13]

2.5. Quantitative make up of carbohydrates in leaf and stem tissues

2.5.1. Water-soluble carbohydrates

The plant material was extracted free of sugars with boiling hot water and the total water-soluble (t.w.s.) carbohydrates and free-reducing sugars (f.r.s.) were determined from the clarified[14] extract by the techniques employed by Yemm and Willis[15] and Hulme and Narain,[16] respectively. Total non-reducing sugars (n.r.s.) were calculated from the difference in the values of t.w.s. carbohydrates and f.r.s.

2.5.2. Cellulose

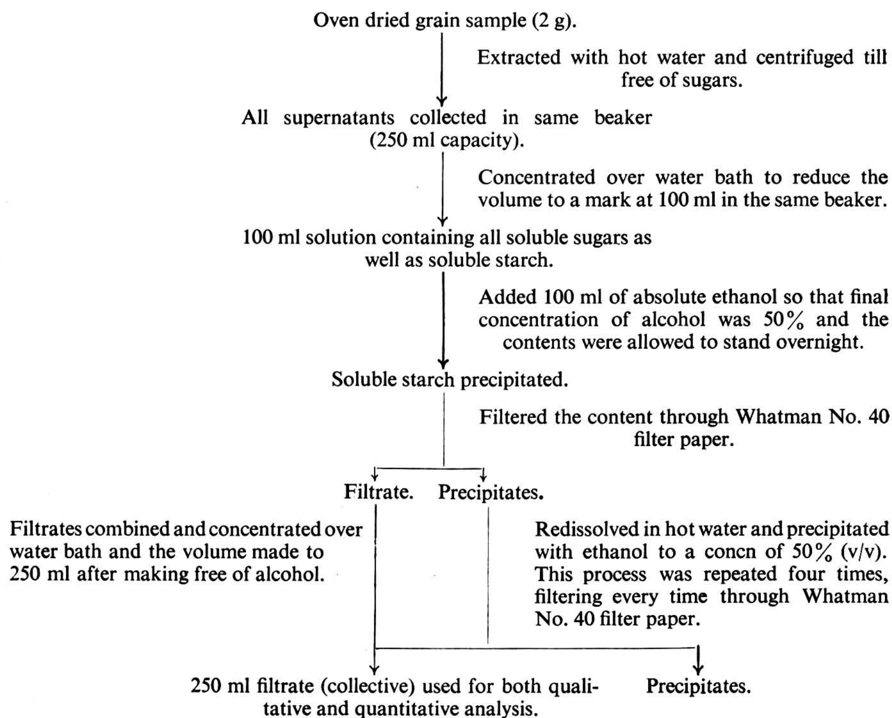
The cellulose content was determined by the technique of Crampton and Maynard.[17]

2.6. Changes in crude protein content

The crude protein content was calculated by multiplying the total nitrogen content of the dried tissues by 6.25. Total nitrogen was determined by the technique of McKenzie and Wallace.[18]

2.7. Carbohydrate changes in maturing grains

For qualitative and quantitative determination of t.w.s. carbohydrates, other than starch, of grain samples the following extraction procedure was adopted.



2.7.1. *Qualitative changes*

Qualitative analysis was done by paper partition chromatography as described.

2.7.2. *Quantitative changes*

2.7.2.1. Total, free reducing and non-reducing sugar

The procedures employed for the determination of total, free reducing and non-reducing sugars were the same as those already described.

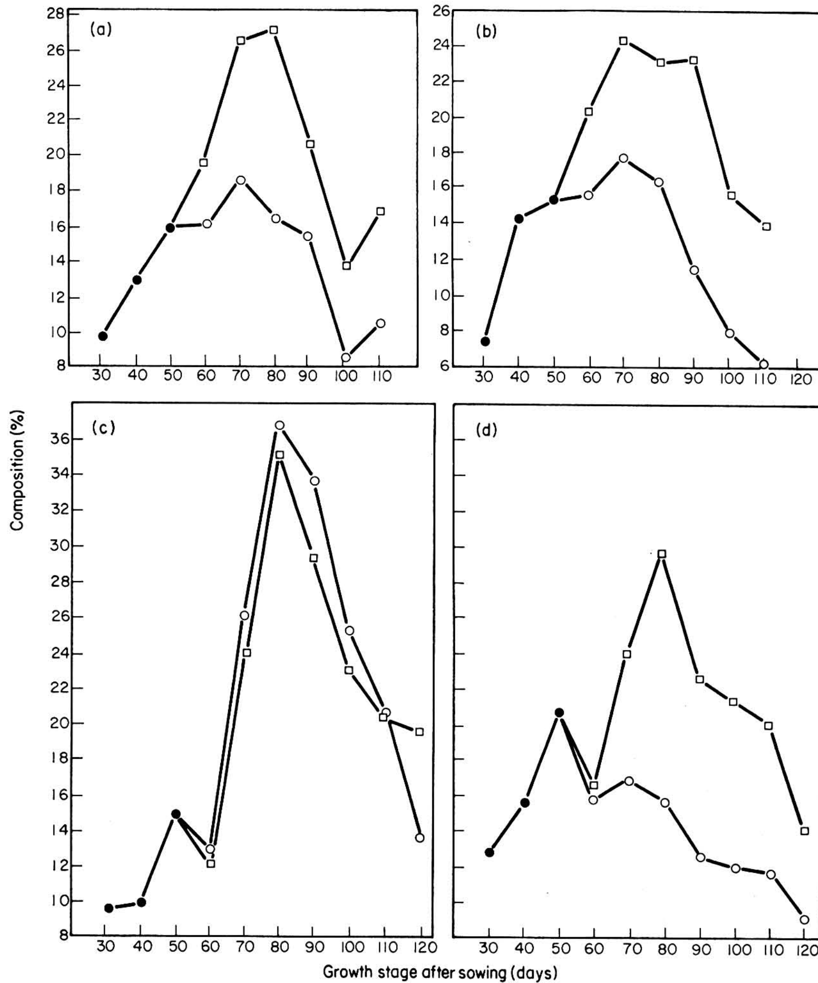


Figure 1. Concentration (% on dry wt basis) of total water-soluble carbohydrates in the stems of: (a) A1/3, (b) S. 530, (c) J.S. 263 and (d) J.S. 20 at different stages of growth. —●—●—, Whole stem; —○—○—, upper stem; □—□—□—, lower-stem.

2.7.2.2. Free and bound glucose, free and bound fructose
Free and bound glucose were determined by the procedure of Klein and Acree[19] from the test extract before and after hydrolysis,[5] respectively. Free and bound fructose were calculated as:

free fructose = free reducing sugars − free glucose,

bound fructose = non-reducing sugars − bound glucose.

2.7.2.3. Starch
The estimation of starch was done by the method of Clegg[20] with the following modifications.

Instead of hot extraction by 80% ethanol the sugars, other than starch, were removed by the extraction procedure of Smith and Grotelueschen.[21] The grain samples were completely freed from other sugars by 65% (v/v) aqueous ethanol extraction.

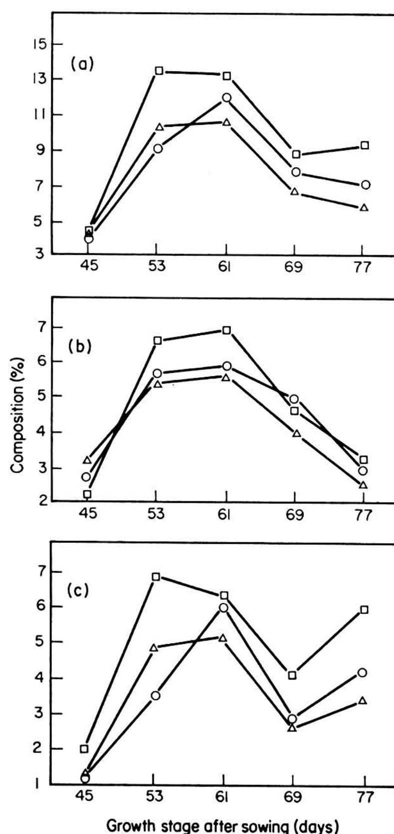


Figure 2. Concentration (% on dry wt basis) of total water-soluble (a) carbohydrates, (b) free-reducing sugars and (c) non-reducing sugars, in various stem parts of kangni at different stages of growth. —○—○—, Upper stem; —□—□—, middle stem; —△—△—, lower stem.

3. Results

3.1. Changes in carbohydrates of leaf and stem tissues at various stages of growth

Sucrose, glucose and fructose were the major sugars detected in all the crops during the entire growth period studied. Traces of starch were also detected when the hot water extract of the leaves was analysed without further treatment. In addition, faint yellow

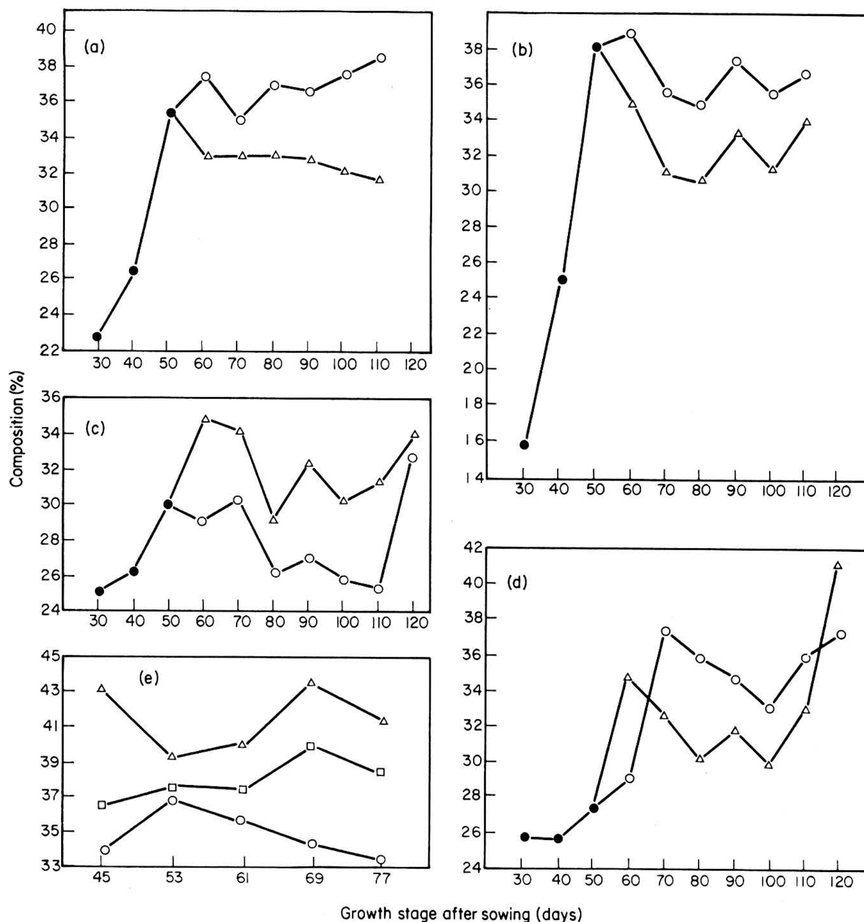


Figure 3. Concentration (% on dry wt basis) of cellulose in the stems of (a) A1/3, (b) S. 530, (c) J.S. 263 (d) J.S. 20 and (e) kangni, at different stages of growth. —●—●—, Whole stem; —○—○—, upper stem; —□—□—, middle stem; —△—△—, lower stem.

coloured spots (with benzidine–trichloroacetic acid) of oligosaccharides with R_F values of 0.088 and 0.061 were noticed in the leaf and stem tissues, respectively, 40 days after sowing in both bajra and jowar. These compounds persisted right up to complete maturation. The intensity of these spots increased with the aging of the plants. Com-

pounds with R_F values higher than 0.40 and 0.91 also appeared after 10 days' growth in bajra and jowar seedlings, respectively. At the same time the fructose spot faded and almost disappeared in jowar seedlings. Those compounds were again detected in both

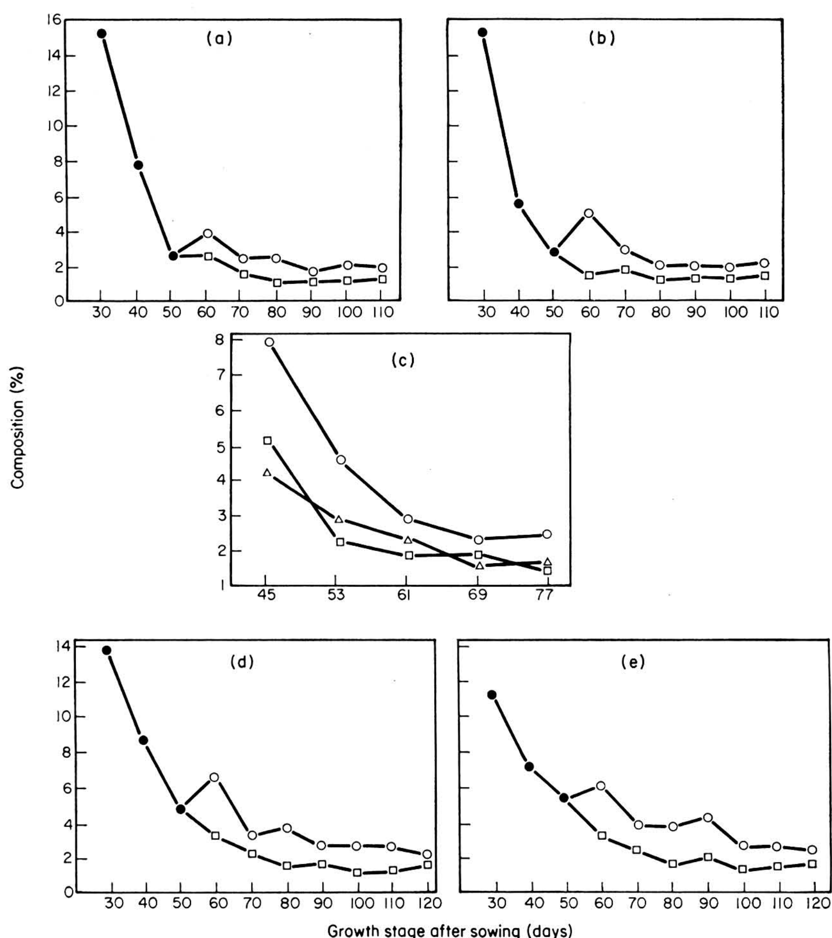


Figure 4. Concentration (% on dry wt basis) of crude protein in the stems of (a) A1/3, (b) S. 530, (c) kangni, (d) J.S. 263 and (e) J.S. 20, at different stages of growth. —●—●—, Whole stem; —○—○—, upper stem; —△—△—, middle stem; —□—□—, lower stem.

the leaf and stem tissues of bajra after 50 days' growth and persisted until maturation stage. No such spots were, however, noticed in jowar after 20 days' growth. In kangni one oligosaccharide spot (R_F between 0 and that of glucose) appeared after 53 days' growth and persisted up to complete maturation of the plant.

Quantitative data on changes in water-soluble carbohydrates at different stages of

growth of bajra, jowar and kangni stems are given in Figures 1 and 2. The water-soluble carbohydrates of leaves remained more or less constant and are, therefore, not reported in this paper.

Variations in cellulose contents of bajra, jowar and kangni stems are given in Figure 3.

3.2. Changes in crude proteins at various stages of growth

Changes in crude proteins of bajra, jowar and kangni stems are given in Figure 4.

The crude protein content decreased with increased growth in all the crops studied.

3.3. Changes in carbohydrates during the development of grains

By chromatography of grain samples at different stages of development, these changes were observed to be very similar in all the crops studied. The presence of sucrose, glucose, fructose and some oligosaccharides was ascertained. The intensity of the fructose spot decreased up to grain maturation. Quantitative data on the changes in the carbohydrate make-up during the development of bajra and jowar grains are presented in Tables 1 and 2 and of kangni in Figure 5.

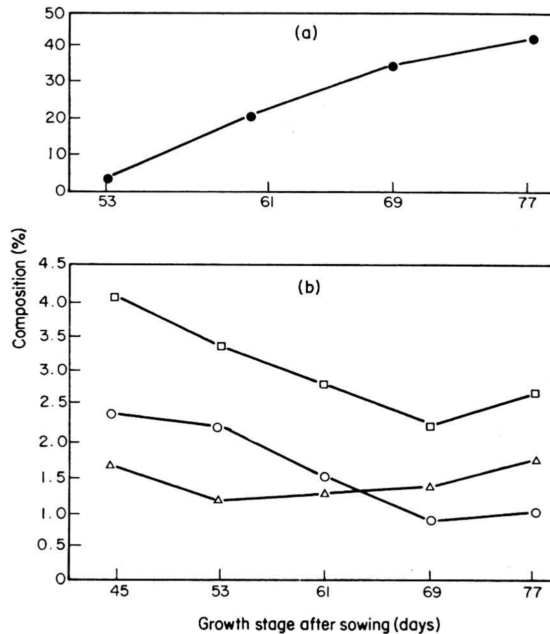


Figure 5. Concentration (% on dry wt basis) of (a) starch, (b) total water-soluble carbohydrates, free-reducing sugars and non-reducing sugars, in kangni grains at different stages of development. —●—●—, Starch; —□—□—, total water-soluble carbohydrates; —○—○—, free-reducing sugars; —△—△—, non-reducing sugars.

TABLE 1. Changes in carbohydrates (%) during the development of bajra grains (values are the average of duplicate estimations from the moisture-free composite samples)

Variety	Growth stage (after sowing) in days	Free reducing sugars	Total water-soluble sugars	Non-reducing sugars	Starch	Free glucose	Free fructose	Bound glucose	Bound fructose
A1/3	70	4.29	5.21	0.92	55.00	2.98	1.31	0.62	0.30
	80	3.82	4.92	1.10	65.05	2.03	1.79	0.43	0.67
	85	3.36	4.33	0.97	65.75	1.63	1.73	0.06	0.91
	90	3.28	4.19	0.91	66.25	1.35	1.93	0.85	0.06
	95	2.99	3.79	0.80	77.50	1.29	1.70	0.06	0.74
	100	3.13	4.33	1.20	82.25	1.39	1.74	0.18	1.02
	110	3.43	5.43	2.10	82.75	2.47	0.96	0.68	1.42
S. 530	70	4.55	5.36	0.81	60.00	2.14	2.41	0.68	0.13
	80	4.34	5.27	0.93	70.50	2.31	2.03	0.06	0.87
	85	3.03	4.35	1.32	73.30	1.74	1.29	0.38	0.94
	90	2.97	3.99	1.01	74.70	1.58	1.39	0.15	0 86
	95	2.62	3.25	0.63	75.25	1.21	1.21	0.34	0.29
	100	2.47	4.46	1.99	85.00	1.69	0.78	1.02	0.97
	110	2.23	4.56	2.33	86.05	1.44	0.79	0.68	1.65

TABLE 2. Changes in carbohydrates (%) during the development of jowar grains (values are the average of duplicate estimations from the moisture-free composite samples)

Variety	Growth (after sowing) stage in days	Free reducing sugars	Total water soluble sugars	Non-reducing sugars	Starch	Free glucose	Free fructose	Bound glucose	Bound fructose
J.S. 263	85	5.02	5.83	0.81	20.00	2.34	2.68	0.42	0.39
	90	4.94	7.61	2.67	33.03	2.67	2.27	1.18	1.49
	95	2.99	6.02	3.03	63.25	1.98	1.01	1.14	1.89
	100	2.93	5.65	2.72	65.70	2.53	0.40	1.32	1.40
	110	2.16	3.89	1.73	71.52	1.86	0.30	1.07	0.66
	120	0.85	2.21	1.36	75.20	0.60	0.25	1.23	0.13
J.S. 20	90	4.51	4.83	0.32	21.25	3.41	1.10	0.18	0.14
	95	4.12	6.24	2.12	32.50	3.11	1.01	0.91	1.21
	100	3.21	4.73	1.52	62.48	2.36	0.85	0.12	1.40
	110	3.09	4.60	1.51	66.31	2.26	0.83	0.67	0.84
	120	2.48	3.75	1.27	73.75	1.80	0.68	0.67	0.60

N.B. In case of J.S. 20 flowering started somewhat later and thus first grain sample was collected at 90-day growth stage.

4. Discussion

The presence of sucrose, glucose and fructose in the leaf and stem tissues, at all stages of growth studied, indicates that these soluble sugars are first formed from the intermediate products of photosynthesis. A rapid hydrolysis of starch for translocation to other tissues may be the reason for its near absence in the leaves.

The oligosaccharides detected in the leaf and stem tissues are glucofructosans which may be temporary storage carbohydrates and which may be hydrolysed to simple sugar units for translocation to the growing points and storage organs. Waite and Boyd[2] reported that in perennial grasses, growing to maturity, fructosans account for the greater part of water-soluble carbohydrates and are the constituents which are more likely to fluctuate with flower initiation and later with seed formation. The compounds with higher R_F values detected in bajra and jowar appear to be some phenolic glycosides. This conclusion is based on their reactions with specific spray reagents. The compounds gave blue coloured spots for phenols when the chromatograms were developed with potassium ferricyanide–ferric chloride reagent[22] and yellow and bright red coloured spots for keto sugars, respectively, with benzidine–trichloroacetic acid[6] and naphtha–resorcinol–trichloroacetic acid.[11] The phenolic compounds are possibly mobilised by way of glycosides (being soluble) and stored in these forms as non-toxic compounds and are liberated again in the growing points to impart resistance to the delicate generative organs against any pathogenic attack. Stafford[23] identified the anthocyanins, apigeninidin, luteolinidin and cyanidin existing as aglycones and acylated and non-acylated glycosides in the first internode of *Sorghum vulgare.*

There is a continuous rise in water-soluble carbohydrates of the stem tissues of bajra, jowar (Figure 1) and kangni (Figure 2) from a very low level at the early stages to a maximum value between the flowering and milk stages (70 to 90 days in bajra and jowar and 53 to 61 days after sowing in kangni). This may be due to the continuous supply of free sugars from leaves which synthesise simple sugars in excess and pass on to the stem, maintaining a relatively low level of these sugars in leaf tissues at all stages of the plant growth. These sugars, thus, accumulate in the stem either as such or in the form of glucofructosans. Towards maturity, there is a decrease in water-soluble carbohydrates. Obviously, once the process of grain formation sets in, the carbohydrate content of stem is determined by balance of two processes: (1) translocation of the carbohydrates synthesised in the leaves to the stem tissues and (2) transport of these carbohydrates from the stem to the grains. Initially, the transport of carbohydrate reserves from the stem is less than the amount translocated from the leaves so that there is a net increase in water-soluble carbohydrates of the stem. With the initiation of grain development a greater demand of water-soluble carbohydrates is made on the stem which cannot be fully met by the active photosynthetic tissue and hence a steady decline in the water-soluble carbohydrates of the stems is observed. Similar observations have been reported by Srinivasan and Bhatia[24] on *Agave Vera Cruz* where it was shown that as the inflorescence axis emerges from the plant, the fructosan content of the tubers declines and this reserve carbohydrate is transported as simple sugars to the upper parts of the axis where again it is stored as fructosans. That flower production has a profound effect on the composition of the plant, causing depletion of the reserve polysaccharides, fructosans, in the stem, has also been reported by Hirst, Mackenzie and Wylam.[25] From the present results, it seems likely that following initiation of flowering there is a massive movement of water-soluble carbohydrates from the stem portions towards the region of grain development.

While in Al/3, S. 530 and J.S. 20 the total water-soluble carbohydrate content is higher in the lower stem portions, the reverse is true in case of J.S. 263 and kangni where

the total water-soluble carbohydrates are preferentially concentrated in the upper stem. We cannot give a reason for it.

The cellulose content (Figure 3) increased rapidly during the period of early growth of bajra and jowar and then the rate of increase slowed down. This can be explained, in part, by the continuous increase in t.w.s. carbohydrates from early growth to flowering. By early milk stage most of the cellulose is already formed. With the initiation of grain formation, the synthesis of cellulose slows down and the available carbohydrates are diverted primarily for the synthesis of grain carbohydrates. Similar observations have been reported by Meyer *et al.*[26] and Stallcup, Roberson and Thurman.[27] An apparent decline in the concentration of cellulose in kangni stem between flowering and milk stage arises possibly from too rapid a rise in the concentration of water-soluble carbohydrates at this stage of growth.

The gradual decline in t.w.s. carbohydrates and free reducing sugars in the developing jowar (Table 2) and kangni (Figure 5) grains indicates that these sugars are used in the synthesis of starch, the concentration of which increases gradually with the aging of the grains. An increase in the t.w.s. carbohydrate and f.r.s. level towards desiccation of grains in A1/3 (Table 1) and kangni (Figure 5) possibly represents the balance of carbohydrates translocated to the grains in excess of the quantity converted into starch. According to Rohrlich and Essner[28] the grain samples of wheat and rye, collected during the 3 to 4 day interval after the end of blooming, showed a steady decrease in glucose and fructose during the first weeks of ripening and later their concentration remained constant. The ears of S. 530, on the other hand, possess long bristles. It is therefore likely that part of the reducing sugars from the grains may be utilised for the building up of cellulose of these developing bristles resulting in a gradual decline in their concentration towards maturity. The results obtained by Johri and Maheshwari[29] and Stoddart[30] from different forages and grasses are quite in accordance with the present findings.

The pattern of changes in bound fructose in bajra and jowar (Tables 1 and 2) and the increase in n.r.s. level in Kangni (Figure 5) during grain development suggest that there may be synthesis of some glucofructosans. This is also in keeping with the observations that on qualitative analysis of kangni grains one oligosaccharide was noticed in the 45 to 53 days' growth stage and later on three such spots appeared with the further maturation of the grains (61 to 77 days). The presence of only traces or total absence of fructose in the matured grains and its appearance during germination indicate that these glucofructosans are possibly synthesized prior to starch formation and act as temporary storage for its final synthesis. Some may even persist in the matured grains. Since the level of starch continued to increase right up to the point of harvest, it can in no way be considered as an indicative of seed ripeness. Continuing polymerisation may occur after the ripening stage (Stoddart).[31]

References

1. Norman, A. G. *Biochem. J.* **1939**, 33, 1201.
2. Waite, R.; Boyd, J. *J. Sci. Fd Agric.* **1953**, 4, 197.
3. Mackenzie, D. J.; Wylam C. B. *J. Sci. Fd Agric.* **1957**, 8, 38.
4. Eagles, C. F. *J. Sci. Fd Agric.* **1967**, 18, 186.
5. Srinivasan, M.; Bhatia, I. S. *Biochem. J.* **1953**, 55, 286.

6. Bacon, J. S. D.; Edelman, J. *Biochem. J.* **1951**, 48, 114.
7. Horrocks, R. H. *Nature, Lond.* **1949**, 164, 444.
8. Wise, C. S.; Dimler, R. J.; Davis, H. A.; Rist, C. E. *Analyt. Chem.* **1955**, 27, 33.
9. Cifonelli, J. A.; Smith, F. *Analyt. Chem.* **1954**, 26, 1132.
10. Mukherjee, S.; Srivastava, H. C. *Nature, Lond.* **1952**, 169, 330.
11. Partridge, S. M. *Biochem. J.* **1948**, 42, 238.
12. Anet, E. F. L. J.; Reynolds, T. M. *Nature, Lond.* **1954**, 174, 930.
13. Partridge, S. M. *Nature, Lond.* **1946**, 158, 270.
14. *Official Methods of Analysis*, A.O.A.C., Washington. **1965**, 10th ed., p. 110.
15. Yemm, E. W.; Willis, A. J., *Biochem. J.* **1954**, 57, 508.
16. Hulme, A. C.; Narain, R. *Biochem. J.* **1931**, 25, 1051.
17. Crampton, E. W.; Maynard, L. A. *J. Nutr.* **1938**, 15, 383.
18. McKenzie, H. A.; Wallace, H. S. *Aust. J. Chem.* **1954**, 7, 55.
19. Klein, G. M.; Acree, S. F. *Bur. Stand. J. Res.* **1930**, 5, 1063.
20. Clegg, K. M. *J. Sci. Fd Agric.* **1956**, 7, 40.
21. Smith, D.; Grotelueschen, R. D. *Crop Sci*, **1966**, 6, 263.
22. Barton, G. M.; Evans, R. S.; Gardner, J. A. F. *Nature, Lond.* **1952**, 170, 249.
23. Stafford, H. A. *Pl. Physiol.* **1966**, 41, 953.
24. Srinivasan, M.; Bhatia, I. S. *Biochem. J.* **1954**, 56, 256.
25. Hirst, E. L.; Mackenzie, D. J.; Wylam, C. B. *J. Sci. Fd Agric.* **1959**, 10, 19.
26. Meyer, J. H.; Weir, W. C.; Jones, L. G.; Hull, J. L. *J. Anim. Sci.* **1957**, 16, 623.
27. Stallcup, O. T.; Roberson, R. R.; Thurman, R. L. *J. Dairy Sci.* **1960**, 43, 867.
28. Rohrlich, M.; Essner, W. *Getreide Mehl.* **1960**, 10, 121 (cf. *Chem. Abstr.* **1961**, 55, 6622).
29. Johri, M. M.; Maheshwari, S. C. *Pl. Cell Physiol.* **1966**, 7, 385.
30. Stoddart, J. L. *Ann. Bot.* **1966**, 30, 311.
31. Stoddart, J. L. *J. agric. Sci., Camb.* **1964**, 62, 67.

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Interpretations of Ultraviolet Absorption in White Wines

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Methods are described for the general analysis of total ultraviolet (u.v.) absorbing constituents of white wines. An elution profile, which is characteristic of the particular wine, is obtained by continuous monitoring of effluent from a dextran gel column at 280 nm. Surprisingly, about half of the total u.v. absorbance in the Riesling wine examined was found to be due to non-phenolic wine constituents, which are separately eluted in the gel column analysis. These are nucleotidic materials, to which little attention has previously been given in oenology.

Data relating to the protein–tannin fraction, and to nucleotidic and phenolic constituents, are directly obtained by recording the wine elution profiles at 265, 280 and 320 nm. The interpretation of the elution curves and the significance and general utility of this new analytical method are discussed.

1. Introduction

Phenolic substances have long been regarded as major factors in wine processing reactions and in the determination of wine quality; the large body of technical literature on this subject has recently been reviewed by Singleton and Esau.[1] From this account it is clear that, while there is much quantitative information on phenolics of white wines, this is of a very non-specific nature, being based upon various colorimetric analyses. Adequate qualitative data are quite generally lacking.

Often, in addition to the the Folin–Denis, vanillin and proanthocyanidin analyses for phenolics, many authors have attempted to evaluate significant factors in wine composition and treatments by use of two-dimensional chromatographic maps of phenolic constituents. However, this approach, which followed the work of Hennig and Burkhardt,[2] is tedious and rather inconclusive because of the number and variety of materials present. Thus it has not been possible to distinguish varietal wines by analytical means, or to recognise small critical changes in phenolic composition, much less relate these to quantitative measures. These deficiencies have been emphasised in recent reports from Flanzy and Aubert,[3] and from Sapis and Ribéreau-Gayon.[4] There is, therefore, an urgent need for new analytical procedures capable of routine operation, which also permit differences in both qualitative and quantitative aspects of chemical composition in wines and juices to be quickly recognised.

Following earlier work with brewing materials,[5] attempts have been made to characterise white wine constituents by dextran gel column analysis,[6,7] but the methods described are laborious and recovery of materials incomplete. Tannin pigments of red

wines are separable from anthocyanins on a Sephadex G-25 gel column[8] and monitoring of pigment eluates provides a "pigment profile" of the wine.[9] We have developed this approach some way towards meeting the above analytical requirements for white wines and find that the method also uniquely provides other information about wine constitution and the nature of u.v. absorption in white wines.

2. Experimental

The wine was made on a 2 gallon scale from *Vitis vinifera* cv. Riesling grapes harvested in the Barossa Valley of South Australia in 1969. As in current commercial practice, the juice was removed rapidly from the pomace after crushing, so that minimal extraction of solids occurred. After addition of SO_2 to 100 parts/million, the fermentation was initiated by a pure yeast culture and allowed to go to completion at 15 °C. After several rackings under CO_2 the wine was filtered and bottled after 10 weeks: the first analyses of this wine were made 6 months after fermentation.

All concentrations of solutions were conducted at under 30 °C in a rotary film evaporator. Nitrogen determinations were by the micro-Kjeldahl procedure.

2.1. Gel column analysis

The column, bed volume 55 × 1.5 cm, was prepared from Sephadex G-25 Fine in 3% aqueous acetic acid (pH 3.6). Solvent flow was set and maintained at 150 ml/h by means of a peristaltic pump. Continuous monitoring of the column eluate (at 265, 280 or 320 nm) was achieved by use of a 2 mm quartz flow cell mounted in the sample beam of a Unicam SP 800A spectrophotometer and the u.v. elution curve was recorded on a multi-range 2 mV recorder at 120 mm/h. Signal expansion up to 25 times was obtained by use of an expansion accessory and the multi-range facility of the recorder. Perfect reproducibility of the elution curve for a particular sample was ensured by use of the same gel bed volume, flow rate and scale expansion; the u.v. profile obtained from a 5 ml aliquot expanded × 10 was identical in resolution and peak heights with those obtained from 10 ml × 5 and 25 ml × 2 expansion. In the latter two cases, as when it was necessary to process larger amounts of material, a concentrate of the wine aliquot (to about 5 ml) was applied to the column.

The standard analytical procedure was to apply a 5 ml wine aliquot, and to use × 10 scale expansion to the recorder. After each analysis, elution was continued for at least 1 h to remove traces of adsorbed materials, when the column was ready for re-use.

Spectra were obtained at elution peaks of the u.v. profile by brief interruption of the elution. The sample was retained in the flow cell by means of a screw clip on the effluent line while the u.v. spectrum was taken, against 3% aqueous acetic acid, on a second recorder with ×10 signal expansion. This procedure in no way affected the u.v. profile of the sample.

2.2. Removal of flavonoids

(a) Saturated neutral lead acetate solution (1 ml) was added to the wine sample (10 ml) and the mixture was then clarified by centrifugation. The u.v. profile of residual

materials corresponding to a 5 ml wine aliquot was measured by gel column analysis of 5.5 ml of the supernatant liquid at 280 nm with ×10 expansion.

For determination of proanthocyanidin content, the precipitate in the above was washed with 2 × 20 ml 50% aqueous methanol, and then stirred with the butanol–HCl reagent (5 ml) for a few minutes. After removal of the precipitate by centrifugation the solution was heated in a boiling water bath for 30 min. The developed pigment was measured in a 10 mm cell at 550 nm. The cyanidin sample used for the calibration line had been purified by gel filtration on Sephadex G-25 in 50% aqueous acetone–HCl.[9]

(b) The wine aliquot (10 ml) was treated with 2 N-HCl (10 ml) and formaldehyde solution (5 ml containing 8 mg/ml), as described by Kramling and Singleton.[10] The mixture was sparged with nitrogen and allowed to stand for 24 h before removal of the slight precipitate by filtration through Celite. The u.v. profile of non-flavonoid wine constituents corresponding to a 5 ml wine aliquot was then obtained by gel column analysis of the above solution (5 ml) with ×25 expansion.

2.3. Chromatography

Fractions from the gel column were examined by cellulose thin layer chromatography (t.l.c.) (Whatman CC41) and also, where appropriate, by gas–liquid chromatography (g.l.c.).

Solvent systems for phenolics were *n*-butanol–acetic acid–water (6 : 1 : 2, v/v (solvent 1)), 2% aqueous acetic acid (solvent 2), and benzene–acetic acid–water (125 : 72 : 3, v/v (solvent 3)). Phenolic constituents were located by u.v. light and u.v./NH_3 and by use of the standard reagents. A new spray reagent for catechins, a 1% solution of picric acid in 95% ethanol, followed by spraying with a 5% solution of potassium hydroxide in 80% ethanol,[11] was found to be sensitive to 1 μg quantities on t.l.c., and is apparently quite specific for their presence.

Amino acids were located on chromatograms in solvent 1 and ethyl acetate–acetic acid–formic acid–water (18 : 3 : 1 : 4, v/v) by use of ninhydrin spray reagent.

Purine and pyrimidine bases were identified in hydrolysates by chromatography in solvent 1 and in iso-propanol–concn hydrochloric acid–water (65 : 20 : 15, v/v solvent 4). The bases were located in u.v. light (254 nm).

For g.l.c., trimethylsilyl derivatives of residues from gel column fractions were prepared in dry pyridine from trimethylchlorosilane and hexamethyldisilazane reagents. A 6 ft (≈ 1.8 m) stainless steel column coated with SE30 was used with a flame ionisation detector, with temperature programming from 100 to 200 °C.

2.4. Hydrolysis of nucleotidic fractions

A 50 ml wine aliquot was reduced to about 5 ml in volume, and the centrifuged concentrate applied to the gel column, as above. The two fractions, V_e 50 to 100 ml and V_e 100 to 150 ml, were collected separately and evaporated to dryness. Each was hydrolysed with 70% perchloric acid (5 ml) at 100 °C for 1.5 h, the cooled mixtures neutralised with aqueous potassium hydroxide and perchloric acid residues removed by filtration of salts from the refrigerated solutions.[12] The filtrates were then concentrated for t.l.c. analysis.

The samples were applied as 10 cm streaks on 20 × 20 cm plates and chromatographed

in solvent 4, alongside reference bases. After extraction from the appropriate areas with concn ammonia–50 % aqueous methanol (1 : 9), the bases were rechromatographed in solvent 1, and finally identified by cochromatography with authentic compounds in solvent 4 and solvent 1.

3. Results and discussion

3.1. The analytical method

Resolution of u.v.-absorbing materials on gel columns is greatly dependent on adsorptive processes and is therefore much influenced by solvent composition. The system finally chosen, 3 % aqueous acetic acid with Sephadex G-25, gives useful resolution of fractions

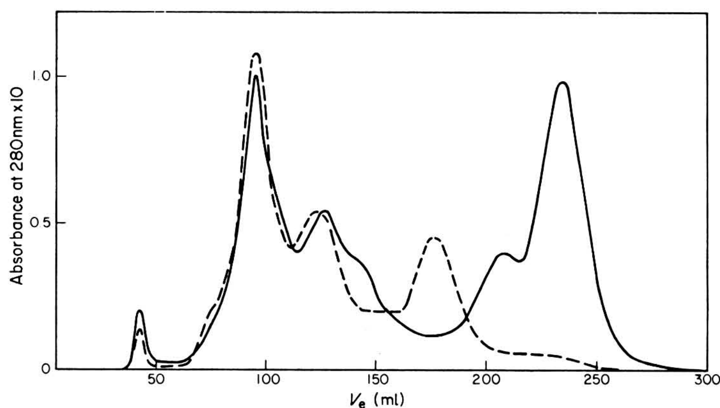


Figure 1. Gel column elution profiles at 280 nm of two young varietal wines (5 ml aliquots). Riesling, ——; Trebbiano, – – –.

at pH 3.6 in reasonable time (2 h) and, with continuous recording of absorbance in the column eluate, the elution curve obtained is quite reproducible as an "ultraviolet profile" of the wine. The ease with which differences in composition between two varietal wines may be detected by this means is illustrated in Figure 1.

Although the elution curves returned to baseline within 300 ml in the analysis of these and many other wines, recovery of u.v. absorbing materials, as determined by the spectrum of total eluates, was generally only 95 to 97 % to this point. This was found to be due to tailing of more strongly adsorbed proanthocyanidin compounds, and recovery was >99 % when elution was continued to 350 ml. In test experiments, such common wine phenolics as *p*-coumaric acid, chlorogenic acid and caffeic acid were eluted as peaks at V_e 188, 190 and 233 ml, respectively, and the u.v. absorbing amino acids phenylalanine, tyrosine and tryptophan at V_e 92, 93 and 136 ml, respectively.

However, flavonols and catechins were strongly adsorbed, emerging as peaks in the range 340 to 360 ml. The retentions of many phenolics in several solvent systems with G-25 gel have been examined by Woof and Pierce,[13] who reported catechins and other

flavonoids to be irreversibly adsorbed. Fortunately, the late recoveries of such compounds from our analytical column appear to be of no consequence, as flavonols are certainly absent from white wines,[14] and we have been unable to detect catechins in any white wines by continued elution past 300 ml or by chromatography. Although the presence of catechins in grape seeds and in unripe juice has been well proven,[1] the literature does not appear to contain any rigorous evidence for their presence in finished wines or even in ripe juice. The term "catechins" has been frequently used in the oenological literature without proof of identity, sometimes to describe phenolics determined by the colorimetric vanillin assay, or even the Folin Denis assay for total phenolics, as in the recent review article of Patschky and Schöne.[15]

Two important features of the gel column method are that artifacts which might arise from prior treatment or concentration of the sample have been avoided by direct application of wine to the column and the fact that the chosen solvent has pH 3.6 which, being average wine pH, is unlikely to lead to any chemical changes during passage down the column. The sensitivity of the method is also worth remarking upon; examination of fractions from the gel column by g.l.c. of trimethylsilyl (TMS) derivatives provided the interesting result that the only compounds detectable in this way were all contained in one fraction (V_e 75 to 112 ml, Figure 1). These were mainly major components such as tartaric, succinic and malic acids and also glycerol, none of which contributes to the u.v. profile. Although many phenolics would not be sufficiently volatile as their TMS derivatives for such analysis, compounds such as caffeic acid are recorded in the elution profile at levels well below those needed for detection by g.l.c.

3.2. Interpretation of the profile

As it seems likely that *all* involatile materials having u.v. absorbance would be important quality factors, the non-selective nature of the analytical method may be regarded as a real advantage. However, because of the complexity of these total materials and the obvious utility of the method, our present aim has been to achieve a general interpretation of the elution curves, which are a measure of total u.v.-absorbing wine components. To this end, we have confined our investigations to a single wine, a 1969 Riesling (Figure 1).

It was first necessary to consider the possibility of interference by u.v.-absorbing wine volatiles, such as the many carbonyl compounds present. Because of the very low molar extinction coefficients (10 to 20) of the simple carbonyls, these could make no contribution to the elution curve, but furfural and 5-hydroxymethylfurfural (ϵ 15 000 and 14 000 at 280 nm) cannot be so easily discounted. Both are eluted as peaks at V_e 90 ml and, if present at say 10 parts/million would add about 15% to absorbance at this point. Furfural has been variously reported as a trace constituent of table wines,[16] but we find that the contribution of added furfural (to 25 parts/million) is easily removed by evaporation of the sample to near dryness at <30 °C; the u.v. profile obtained from solution of the residue in 3% aqueous acetic acid was then identical with that of the original wine. However, 5-hydroxymethylfurfural cannot be removed in this way, and solvent extraction is unsuitable, as some phenolics are also extracted. Fortunately, this aldehyde is generally present only in some sherries, ports and heated table wines;[16, 17] it would rarely be a contributing factor in the u.v. profile of a white wine, and its

presence at levels likely to cause interference is readily detected by colorimetric analysis.[17] Substituted benzaldehydes such as vanillin have equally high ϵ values, and would be eluted in the range V_e 120 to 140 ml. However, they have been only infrequently reported as trace constituents in wines, and t.l.c. investigation of eluate concentrates from this region failed to show any components responding to 2,4-dinitrophenylhydrazine reagent.

In normal circumstances therefore, and certainly in the particular wine examined, there is no significant contribution to u.v. absorption from carbonyl compounds, and the profile is entirely due to involatile constituents.

The first small peak, emerging at the void volume, V_e 44 ml (Figure 1) and having λ_{max} 280 nm, is due to residual wine proteins and associated polymeric phenolic materials; chromatography in solvent 1 and solvent 2 showed no mobile components, but positive responses to both the vanillin and butanol–HCl reagents were obtained. However, the protein content, based upon N analysis of samples isolated from this fraction in preparative column treatments was only 22% and corresponded to 100 mg protein/l. The bulk of the polymeric materials in this first fraction, which totalled 450 mg/l, appears to be pectins and other polysaccharides. Wine proteins, which are frequently responsible for instability problems in white wines, may be estimated by spectral means based upon the above, and these matters will be reported separately.

Most of the amino acids are of course transparent in the u.v. and these, together with residual wine sugars and organic acids, were all found to be eluted fairly early (V_e 90 to 140 ml). However, the very considerable u.v. absorption of other wine constituents eluted in this range (Figure 1) could not be assigned to the few u.v.-absorbing amino acids, none of which were found in this wine. Furthermore, it was known from the work of Woof and Pierce,[13] and from our own observations with test compounds, that all of the likely flavonoids would be eluted late because of adsorption effects. In fact, the simplest phenolic compounds known to occur in wines, the various substituted benzoic acids[18] (vanillic, gallic, syringic, *p*-hydroxybenzoic and protocatechuic acids) were all found to be eluted, in test experiments, within the range V_e 167 to 225 ml. Thus it became evident that almost all u.v.-absorbing wine constituents being eluted up to V_e 150 ml were *non-phenolic*, i.e. only half of the u.v. absorption of this Riesling wine at 280 nm was due to phenolic compounds, and similarly, the phenolic contribution to the u.v. profile of the Trebbiano wine (Figure 1) could not be more than about 30%. These indications are quite contrary to current interpretations of the nature of u.v. absorption in white wines, in which all absorbance has been generally ascribed to phenolics, and have an important bearing on methods in present use for assessment of wine phenolics; they have been supported by a number of experimental observations.

Separation of total phenolics from u.v.-absorbing non-phenolics could not be achieved by prior treatment of the wine. About half of the latter fraction, having peaks at V_e 77, 100 and 126 ml when subsequently put through the gel column, was retained by cation exchange resins (Dowex 50, Amberlite-120, H forms), from which it was eluted, along with amino acids, with 2 N-NH_4OH. However, since no phenolic character was found in any of the wine components eluted from the gel column between V_e 60 and 150 ml (Figure 1), in contrast with those eluted after V_e 150 ml, this entire part of the profile was tentatively assigned to nitrogenous bases; the *in vitro* responses of

fraction concentrates from this part to 1 % aqueous ferric chloride, the most specific test for phenols, were negative, the response to diazo reagent was very weak, and spectral properties (see below) also indicated their non-phenolic character. Chromatography of this total fraction showed many components which absorbed or fluoresced in the u.v.

There are two methods by which flavonoid materials may be more or less specifically removed from a wine. Treatment with salts of heavy metals, such as neutral lead acetate, has long been known to precipitate some phenolics as insoluble chelates. Also, Kramling and Singleton[10] have recently demonstrated that the phenol–formaldehyde reaction may be used to precipitate reactive (i.e. flavonoid) wine components, non-flavonoid phenolics being unaffected. The u.v. profiles of the Riesling wine after each of the above treatments are presented in Figure 2.

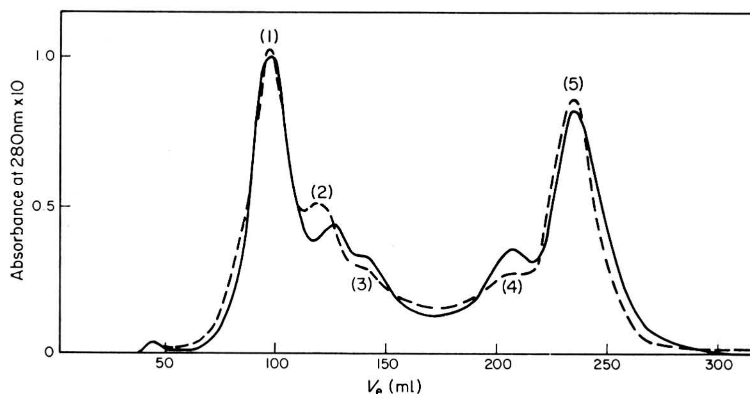


Figure 2. Elution profiles at 280 nm showing residual materials in the Riesling wine (5 ml) after Pb treatment, ——; and after HCHO treatment, – – –. Compare with that of the intact wine (Figure 1).

Comparison with Figure 1 shows that, apart from the large reduction in the protein–tannin peak at V_e 44 ml, there are only slight alterations in the profiles to V_e 150 ml, and there is, in fact, little change overall in either one. The formaldehyde treatment is regarded as the more specific for removal of flavonoids, and the small differences which exist between the intact and treated wines (Figures 1 and 2) are seen to be measures of the contribution of flavonoid constituents to u.v. absorption in the wine, confirming the conclusion of Kramling and Singleton[10] that flavonoids are nearly absent from wines derived from free-run juices. Reactivity to the vanillin reagent was reduced from 30 mg/l (as catechin) to 2 mg/l after formaldehyde treatment.

Analysis of pigments formed from the precipitated lead complex with the butanol–HCl reagent, adapting the procedure of Flanzy and Aubert,[3] gave an apparent pro-anthocyanidin content of only 7 mg/l (as cyanidin, which was identified as the reaction product), the same result being obtained by similar analyses of the untreated wine. Filtrates from the neutral lead acetate and formaldehyde treatments gave negative responses to this reagent.

Further indications of the non-phenolic character of measured materials eluted between V_e 60 and 150 ml are provided by spectra of eluates at various points in the

profile of the formaldehyde treated wine; these are shown in Figure 3. Following the protein–tannin peak at V_e 44 ml, all materials eluted to about V_e 150 ml are seen to have λ_{max} in the range 265 to 275 nm, whereas, beyond this point, the spectra taken are characteristic of cinnamic acid derivatives.

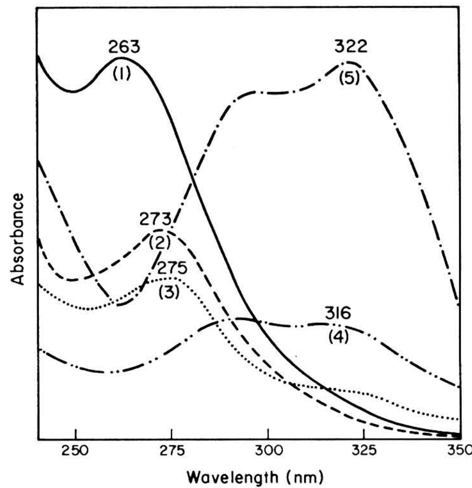


Figure 3. Spectra at elution points 1 to 5 in gel column analysis of HCHO treated wine (cf. Figure 2). Positions of λ_{max} are indicated.

Spectra with absorption maxima in the range noted are typical of nucleotidic materials, i.e. purine and pyrimidine bases, nucleosides and nucleotides. That these are the materials being measured in the first half of the u.v. profile is further supported by Figure 4, in which are illustrated the gel column analyses of a standard nutrient medium

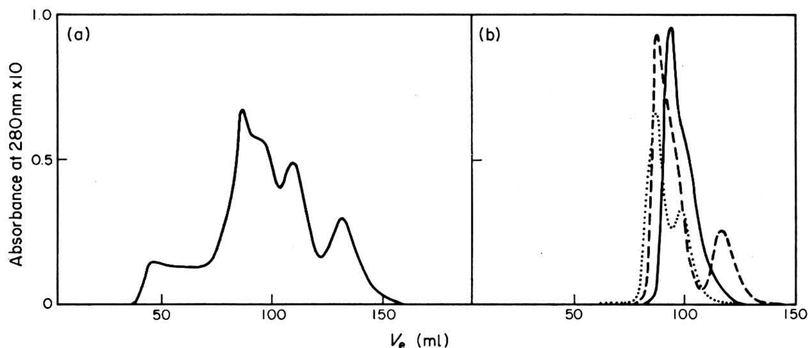


Figure 4. Gel column elution profiles at 280 nm of
(a) 5 ml aliquot 0.6% aqueous nutrient solution ("Bacto-Peptone", Difco Labs).
(b) Purine and pyrimidine bases (4), ——; nucleosides (4), – – –; nucleoside 5′-phosphates (4), ...

(an animal protein hydrolysate) and of test mixtures of authentic nucleotidic compounds. Whereas all known monomeric phenolic constituents of wines emerge after V_e 150 ml, the u.v.-absorbing compounds in these test solutions were all eluted *before* this point, and have elution peaks in the same region as the first main peak of the wine profiles (Figures 1 and 2); treatments with neutral lead acetate and formaldehyde produced no change in the elution profiles of these test solutions.

Many components, which appeared as dark, purple, or yellow spots when viewed on chromatograms under u.v. light (254 nm), were observed in eluates from V_e 50 to 150 ml. The nucleotidic bases guanine, cytosine and uracil were identified in the perchloric acid hydrolysate of the fraction V_e 50 to 120 ml and adenine alone was found in the hydrolysate from the fraction V_e 120 to 150 ml.

The shoulder at 320 nm in the spectrum of materials eluting around V_e 140 ml (fraction 3, Figure 3) suggested the presence of cinnamic acids or derivatives thereof in this fraction. Chromatograms showed free cinnamic acids to be definitely absent from this area, but a major component having the appearance of caffeic acid in u.v. light and with similar R_F in solvent 1 had R_F 0.80 in solvent 2 (caffeic acid 0.35). This material retained the spectral features of fraction 3 and, when rechromatographed in solvent 2, showed blue fluorescing spots at R_F 0.80 and 0.35 to 0.40, the latter being identical with caffeic acid in cochromatography. The likely presence, in this part of the profile, of nitrogenous bases acylated with caffeic acid is inferred from these observations.

The presence of nucleotidic materials in beers, deriving both from the malted barley and from yeast cells during the fermentation, has been well known for many years, and they are regarded as important factors in the stability and flavour characteristics of beer.[12] In contrast, their presence in wines has been scarcely noted, and phenolics have continued to engage the attentions of oenologists in investigations of wine quality factors. Tercelj has reported a number of nucleotidic compounds in wine,[19] and commented that his determinations were complicated by interference from phenolic constituents; this is the only statement of their presence which has appeared to our knowledge and, by inference from beer fermentation studies, they have been presumed to originate from the yeasts during fermentation.[20] However, we have found, in preliminary gel column analyses of grape juices, that these also contain very high contributions, considered in relation to total u.v. absorbance, of nucleotidic materials. It is also relevant to add, in view of the established presence of such compounds in wort and beer, that gel column analysis of these materials has given similar resolutions of u.v.-absorbing components, and that nucleotidic bases were identified in hydrolysates from the early fractions which followed the protein peak.[21]

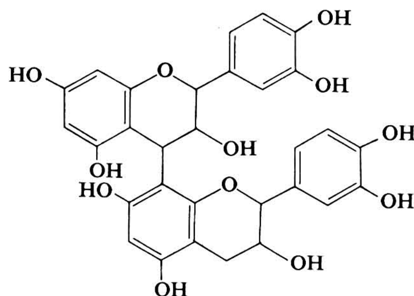
Following the above account, it is obvious that the u.v. absorbance of a wine at 280 nm does not uniquely provide a measure of phenolic content, as is currently assumed,[6] and that the error may be especially large in white wines, which have much lower contents of phenolics than do red wines. Also, the errors inherent in the Folin–Denis and the related Folin–Ciocalteau analyses, commonly used for determination of phenolics in wines and juices,[4] are demonstrated by the fact that such analysis of eluate fractions from the gel column showed *two* broad and approximately equal areas of responsive materials with the mid point at about 170 ml (Figure 2). However, as reported above, phenolics are largely absent from the eluates to 150 ml. This indication of substantial

reactivity by non-phenolic materials with the Folin–Denis reagent was well supported by the finding that the nutrient medium used in the preparation of Figure 4 (a), which resembles the first half of the wine profile both qualitatively and quantitatively, gave an apparent phenolics value of 75 mg/l by this colorimetric analysis.

Many u.v.-absorbing materials eluted after about V_e 150 ml reacted strongly with phenolic reagents, and the spectra of fractions 4 and 5 (Figure 3) indicate the principal components to be cinnamic acid derivatives. Caffeic acid (V_e 236 ml) was identified by chromatography and by g.l.c. of TMS derivatives as the major component of the large peak 5, being responsible for about 80% of absorbance in this region; it was accompanied by the related lactone, aesculetin, identified by cochromatography, and by other minor phenolics.

The major component of the smaller peak 4 (Figure 2) was also a caffeic acid derivative (R_F 0.88 in solvent 1, 0.65 in solvent 2, 0.85 in solvent 3), which could not be identified. Sugars and tartaric acid were sought unsuccessfully in the hydrolysis products from this constituent, the presence of cinnamoyl tartrate esters in wines having been reported by Ribéreau-Gayon.[22] *p*-Coumaric acid and chlorogenic acid, which were both eluted at V_e 188 ml when separately applied to the column, were not found in this Riesling wine.

As part of the phenolic background to the above, proanthocyanidin and vanillin reacting materials were eluted over a wide area of the profile from about V_e 140 ml, reaching a maximum at about V_e 250 ml. The absence of catechins from ripe juices and wines has been previously noted, and it is considered that the flavonoid wine constituents are probably flavan–flavan dimers having structures I, for which many



stereochemical configurations are possible. Weinges, Wild and Kaltenhäuser have pointed to the absence of true leucoanthocyanidins (flavan-3,4-diols) from fruit juices and extracts, and have provided evidence that dimeric proanthocyanidins I are biosynthesised by enzymic dehydrogenation of catechins.[23] The same mechanism and products, occurring during the final growth stages of the berry, appear to be a very likely source of such flavan materials in white wines. Although their content is low, only 7 mg/l by the butanol–HCl assay in this wine, their importance lies in their likely involvement in oxidative browning reactions of white wines; it is significant that the phenolic portion of the profile was found to be quite prone to browning, whereas eluates from the first half, to V_e 150 ml, did not brown on prolonged exposure to air.

4. Summary and conclusions

The u.v. absorbance of this Riesling wine has been shown to be the sum of contributions from several distinct classes of constituents, *viz.* (i) the protein–tannin fraction, (ii) various nucleotidic materials and acylated derivatives thereof, (iii) caffeic acid and derivatives, and (iv) minor flavonoid compounds, in which there is no conjugation with a carbonyl group (as exists in flavones and flavonols).

Some division of materials into the above categories occurs in gel column analysis, and, because of their widely differing spectral characteristics (Figure 3), it follows that a good qualitative assessment of composition, along with a visual presentation of

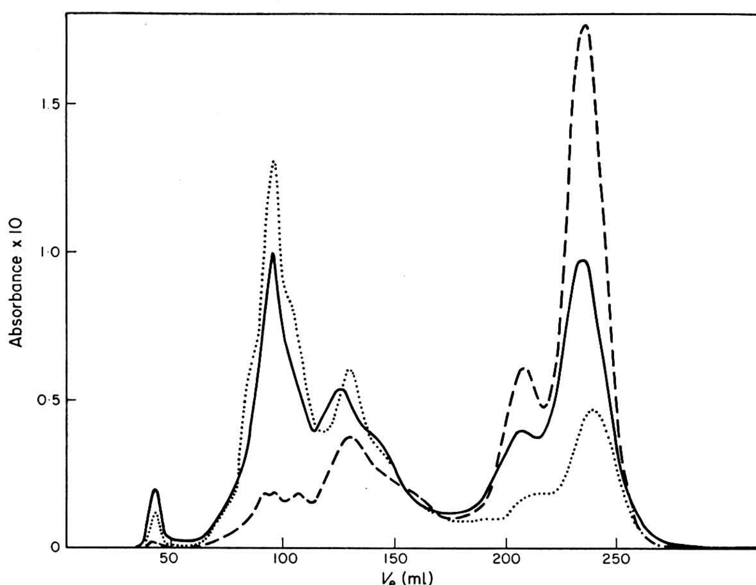


Figure 5. Spectral features of Riesling fractions are indicated by recording the elution profile at 280 nm, ——; 265 nm, ...; 320 nm, – – –.

quantitative features, can be obtained by measuring u.v. profiles at 280, 265 and 320 nm, at which wavelengths absorption is maximised in different parts of the elution curve.

Such data for the Riesling wines are presented in Figure 5. Thus the only discrete fractions having highest optical density at 280 nm were eluted from V_e 40 to 75 ml, corresponding to protein–tannin and possibly polypeptides in the latter portion. As mentioned earlier, proanthocyanidin materials, expected to have the same λ_{max} were eluted over a wide range from V_e 140 ml, and are not evident in Figure 5 against the much larger background of other u.v.-absorbing compounds.

However, using the profile at 280 nm as a reference, the spectral features which distinguish the two major groups of measured constituents are immediately obvious from Figure 5. In the region V_e 75 to 120 ml, $E_{265} > E_{280} \gg E_{320}$ nm, and this is attributed to nucleotidic materials [compare with Figure 4 (b)]. The presence of similar

compounds acylated with cinnamic acids in the region V_e 120 to 150 ml is indicated by the relatively high $E_{320\ nm}$ of this fraction. The mid point in the elution is reached at about V_e 175 ml, where there is considerable overlap of nitrogenous bases with phenolic components. However, from about V_e 200 ml onwards, $E_{320} > E_{280} > E_{265\ nm}$ and most of the absorbance recorded is then due to caffeic acid and derivatives.

If the division of major u.v. absorbing materials is regarded simply as one between nitrogenous bases and caffeic acid, then approximate measures of concentrations may be derived from the curve areas between V_e 75 to 175 ml at 265 nm, and V_e 175 to 275 ml at 320 nm, respectively. For the latter part, using ϵ 13 500 the concentrations of caffeic acid was calculated as 3.6×10^{-4} M or 53 mg/l. The values 11 500 and 500 were chosen as suitable means for ϵ and $E^{1\%}_{1cm}$ of the total nitrogenous bases, and the estimate obtained for their concentration was then 3.9×10^{-4} M, or 85 mg/l. As mentioned earlier, the content of protein, largely responsible for the first small peak, was estimated to be 100 mg/l.

It is proposed that such interpretations of u.v. elution profiles provide a much more accurate and meaningful evaluation of important aspects of wine composition than do the colorimetric and paper chromatographic analyses currently employed. Furthermore, the profiles are objective and reproducible characteristics of the particular wine and can be used to assess qualitative and quantitative differences between wine materials which may be consequent upon varietal or regional origin, and also upon wine treatments. The existence of wide variations in composition due to these first two factors, has in fact long been evident from the observations of Berg on u.v. spectra of white wines,[24] which preceded the many chromatographic investigations of phenolic constituents by other workers.[1] In a survey of 19 varieties of *V. vinifera*, Berg had noted absorption maxima to generally occur in the region 260 to 265 nm, and five types of spectra were observed. The prevalence of this low λ_{max} now strongly suggests that a large part of the u.v. absorbance in most white wines is due not to phenolics but to nitrogenous compounds of nucleotidic origin, as in our Riesling and Trebbiano wines. As such materials are known to have flavour properties,[12] it is possible that they are of more significance than the phenolics in white wine composition, although the latter are certainly involved, as negative quality factors, in browning reactions.

Finally, it is significant that, during the course of investigations of the Riesling wine over 18 months, no change has been observed in the u.v. profiles; it appears that, in the normal absence of oxidative influences, the composition of u.v. absorbing materials in white wines is essentially static. This is in marked contrast to the very dynamic situation which has been shown to exist in the conservation and ageing of red wines.[9, 25]

References

1. Singleton, V. L.; Esau, P. *Phenolic Substances in Grapes and Wine, and their Significance*. Academic Press, New York. **1969**.
2. Hennig, K.; Burkhardt, R. *Am. J. Enol. Vitic.* **1960**, 11, 64.
3. Flanzy, M.; Aubert, S. *Annls Technol. agric.* **1969**, 18 (1), 27.
4. Sapis, J.-C.; Ribéreau-Gayon, P. *Connaiss. Vigne Vin* **1968**, 4, 323.
5. Woof, J. B. *Nature, Lond.* **1962**, 195, 184.
6. Wucherpfennig, K.; Franke, I. *Wein-Wiss.* **1964**, 19 (8), 362.
7. Zakow, D.; Mesrob, B.; Iwanow, T.; Nikowa, Z.; Waltschewska, S. *Mitt. höh. Bundeslehr-u. Vers Anst. Wein-Obst-u. Gartenb.* **1969**, 19, 437.

8. Somers, T. C. *Nature, Lond.* **1966**, 209, 368.
9. Somers, T. C. *Vitis* **1968**, 7, 303.
10. Kramling, T. E.; Singleton, V. L. *Am. J. Enol. Vitic.* **1969**, 20, 86.
11. Tirimanna, A. S. L.; Perera, K. P. W. C. *J. Chromat.* **1971**, 58, 302.
12. Belleau, G.; Buday, A. *Proc. Am. Soc. Brew. Chem.* **1968**, 214.
13. Woof, J. B.; Pierce, J. S. *J. Chromat.* **1967**, 28, 94.
14. Ribéreau-Gayon, P. *C.r. hebd. Séanc. Acad. Sci., Paris,* **1964**, 258, 1335.
15. Patschky, A.; Schöne, H.-J. *Mitt. höh. Bundeslehr-u. VersAnst. Wein-Obst-u. Gartenb.* **1970**, 20, 432.
16. Amerine, M. A.; Joslyn, M. A. *Table Wines*, University of California Press, Berkeley. **1970**, p. 439, 480.
17. Meidell, E.; Filipello, F. *Am. J. Enol. Vitic.* **1969**, 20, 164.
18. Ribéreau-Gayon, P. *C.r. hebd. Séanc. Acad. Sci., Paris,* **1963**, 256, 4108.
19. Tercelj, D. *Annls. Technol. agric.* **1965**, 14, 307.
20. Amerine, M. A.; Joslyn, M. A. *Table Wines*, University of California Press, Berkeley. **1970**, p. 505.
21. Somers, T. C.; Ziemelis, G., **1972**. *J. Inst. Brew.*, in press.
22. Ribéreau-Gayon, P. *C.r. hebd. Séanc. Acad. Sci., Paris,* **1965**, 260, 341.
23. Weinges, K.; Wild, R.; Kaltenhäuser, W. *Z. Lebensmittelunters. u.-Forsch.* **1969**, 140, 129.
24. Berg, H. W. *Fd Res.* **1953**, 18, 399.
25. Somers, T. C. *Phytochemistry* **1971**, 10, 2175.

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A Semi-micro Method for the Determination of Lignin and its Use in Predicting the Digestibility of Forage Crops

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The lignin content of dried grasses can be measured, after prior removal of interfering phenolic materials, by dissolving the residue in 25% acetyl bromide in acetic acid and determining the absorption at 280 nm. The absorption values can also be used to predict the nutritive value of dried grasses, hays and silages.

1. Introduction

Lignin is a complex aromatic polymer which occurs in plant cell walls in close association with cellulose and the hemicellulosic polysaccharides. Johnson, Moore and Zank[1] have published a method for the spectrophotometric determination of lignin in small wood samples whereby the wood, or wood product, is dissolved in a mixture of acetyl bromide in acetic acid and the absorption of the resultant solution is measured at 280 nm. It is this method that has been adapted for dried grass samples.

A knowledge of the nutritive value of a feed is of fundamental importance in animal nutrition experiments. The *in vitro* digestibility technique as devised by Tilley, Deriaz and Terry[2] is probably the most accurate method for indirectly estimating nutritive value but it has disadvantages especially in the length of the operation and the need for a source of rumen liquor. A more rapid method, even if less accurate as a predictor, offers certain advantages for laboratories that do not have the *in vitro* method running continually. Progressive lignification is generally considered to be the major cause of the decline in digestibility as a herbage matures and, of all the grass components that can readily be measured by chemical means, lignin gives the best correlation coefficient with *in vitro* digestibility values.[3–5]

2. Experimental

2.1. Preparation of the samples

Five grasses were used, the diploid and tetraploid strains of S23 and S24 ryegrass and diploid S37 cocksfoot. The grasses were cut at either four or five stages of growth and 12 of these cuts were separated into leaf and leaf sheath–stem fractions. The leaf sheath was included with the stem as they have been found to be closely related with respect to both lignin content and digestibility.[6] All samples were dried within 1 h of cutting in a forced draught oven at 100 to 110 °C for 45 min, ground to pass a 0.7 mm screen and

stored in sealed glass jars at 4 °C. The method of Ellis, Matrone and Maynard,[7] as modified by Waite, Johnston and Armstrong,[8] was used to determine reference lignin contents. Determinations of the *in vitro* digestibility were made by the method of Tilley and Terry[9] as modified by Alexander as McGowan.[10]

2.2. Analytical procedure

Dried grass (approximately 2 g of known ash content) was heated with distilled water (150 ml) at 60 to 65 °C for 30 min with occasional shaking, then filtered hot through a Whatman No. 52 filter paper. The residue was washed thoroughly with water, ethanol, acetone and diethyl ether until no further colour appeared in the filtrate and the preparation was dried at 47 °C overnight. The volumes of the reagents used for washing varied depending on the state of maturity of the grass but did not exceed 200 ml of each. The yield and dry matter content of this crude cell wall preparation were determined and samples (40 to 50 mg) were heated at 70 ± 0.1 °C for 30 min in glass-stoppered test tubes with 5 ml of 25% acetyl bromide in glacial acetic acid. After cooling to 20 °C, the material was transferred to a 250-ml volumetric flask containing 4.5 ml of 2 N-sodium hydroxide in 25 ml acetic acid. Acetic acid was used to wash the residue from the tube and to make the volume up to approximately 200 ml 8 ml of 0.5 M-hydroxylammonium chloride solution was added, the flask was made to the mark with acetic acid, shaken and allowed to stand for at least 1 h to allow the protein sediment to settle before reading the optical density of the solution at 280 nm in 10-mm silica cells. A reagent blank was run with every set of estimations. Fresh reagents were prepared if the reagent blank had an optical density greater than 0.050 units. The absorption values (A) were calculated from the equation:

$$A = \frac{\text{O.D.}_S - \text{O.D.}_B}{c} \quad \text{cm}^{-1}\text{lg}^{-1},$$

where O.D._S = optical density of the sample, O.D._B = optical density of the blank, c = concentration of dry organic matter in the final solution (gl^{-1}).

3. Results

3.1. Effect of preliminary extractions

The work of Johnson, Moore and Zank[1] demonstrated that the method was suitable for measuring the lignin content of extracted woods, but preliminary investigations were necessary to ascertain whether materials similar to those normally found in the grass cell wall would exhibit any absorption in this reagent at 280 nm. The results are given in Table 1. The absorption values obtained from the two cellulose samples and the pectin sample are not significant as will be discussed later.

The scheme, described by Waite and Gorrod,[11] for the comprehensive analysis of grasses which employs successive treatment with azeotropic ethanol–benzene, water at 60 °C and acid pepsin was examined as the final residue is known to be free from low molecular weight phenolic materials and most of the protein. Grass samples were treated in this way and the residue after each extraction was examined by the acetyl bromide procedure. A typical set of results is given in Table 2 and it is clear that the

TABLE 1. The u.v. absorption values (A) at 280 nm of some materials similar to those found in grass cell walls

Material	A ($cm^{-1}lg^{-1}$)
Cellulose powder (Balston's)	0.222
Filter paper (Whatman No. 1)	0.127
Grass hemicellulose (arabino-4-O-methyl-glucuronoxylan)	0.048
Pectin (apple)	0.278
Protein (casein)	0.038

TABLE 2. The effect of extractive treatment on the u.v. absorption values (A) at 280 nm of a grass sample

Material		A $cm^{-1}lg^{-1}$
Dried grass		3.864
after extraction with:		
(a) Ethanol–benzene (1:2)		3.342
(a) Ethanol–benzene (1:2)	and (b) water	2.767
(a) Ethanol–benzene (1:2)	(b) water and (c) acid pepsin	2.748
(a) Acetone	and (b) water	2.720
(a) n-Hexane	and (b) water	2.741
(a) Chloroform–methanol (1:1)	and (b) water	2.677
(a) Chloroform–methanol (2:1)	and (b) water	2.659
(a) Methanol	and (b) water	2.853
(a) Water alone		2.778

In all experiments, the residue after the water extraction was thoroughly washed and dried through ethanol, acetone and ether.

treatment with acid pepsin can be omitted even though it removed about 75% of the protein originally present in the samples. It was noticed that all grass or extracted grass samples left a sediment after being treated with acetyl bromide reagent. By centrifuging off this sediment from a grass with a high protein content, it was found to contain more than 98% of the protein nitrogen originally present in the grass. No other cell wall components could be detected in the suspension.

Complete extraction with boiling ethanol–benzene requires about 18 h, so to speed up the pretreatment of the grass, other solvents were tried which were known to be good extractants for lipids, waxes and phenolic materials. The absorption values obtained from the residues of several such extractions on the same grass sample as above are also given in Table 2. Two other grass samples, the first at a very young stage of growth, the other very mature, gave absorption values of 1.073 and 3.543, respectively, for the residues obtained after ethanol–benzene followed by water extractions while values of 1.038 and 3.591 were obtained from the residues after extraction with water alone, *provided the residues were washed with ethanol, acetone and diethyl ether until no further colour appeared in the filtrate.*

3.2. Effect of duration of heating at 70 °C

Although Johnson, Moore and Zank[1] recommended an acetyl bromide reaction time of 30 min at 70 °C for wood samples, this required confirmation as grass lignins are known to be structurally distinct from wood lignins. The u.v. absorbance from woods at 280 nm rose rapidly for about 10 min, the rate of increase then declined but the absorbance was still rising even after 90 min reaction time. They chose 30 min as the optimum time of reaction because heating beyond this period produced a second

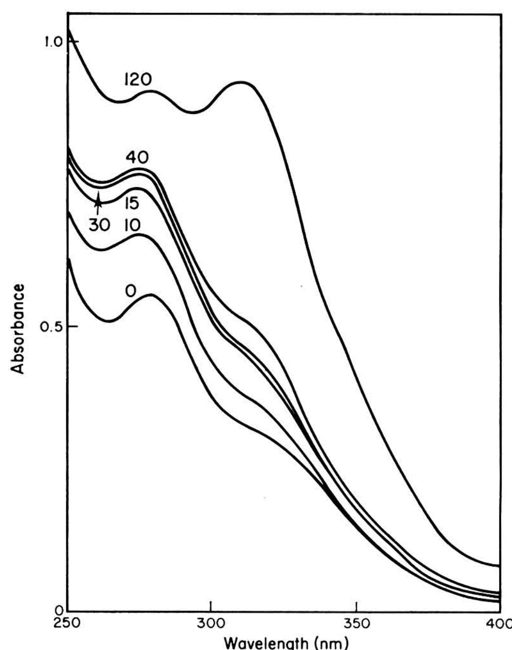

Figure 1. Effect of different acetylbromide reaction times on u.v. spectrum of grass solution heated at 70 °C. Times given against curves are in minutes.

absorption maximum at about 325 nm which they attributed to carbohydrate degradation products. The full spectra, between 250 and 400 nm, of a grass sample heated at 70 °C for various times is shown in Figure 1 and gives some indication of how grass and wood lignins differ on treatment with acetyl bromide. In this grass sample about 75% of the u.v. absorbance at 280 nm was produced rapidly with little or no heating, the intensity increased for about 15 min and then remained fairly steady for a further 25 min. Thereafter, another rapid increase in intensity was noted and the intensity was still increasing even after 2 h. The optimum reaction time was taken as 30 min, to be near the centre of the steady period. The initial rise in absorption at 280 nm was caused by the phenolic chromophore of lignin as it dissolved in the reagent. Grass lignin would appear to be more soluble in the reagent than wood lignins from the intensity of absorption produced without heating. The further increase in intensity after 40 min was due

to a second absorption area with a maximum centred near 310 nm. At 2 h this peak had a greater intensity than the one at 280 nm. As neither purified cellulose or hemicellulose gave this second absorption maximum, we suggest that it is probably produced by acetolysis of the lignin molecule.

3.3. Interpretation of results

To put the results on a quantitative basis should only require the absolute absorptivity of a grass lignin standard. However, attempts so far to prepare a pure grass lignin have been unsuccessful and all the preparations have been contaminated with 5 to 10% carbohydrate which could not be removed by precipitation or other fractionating techniques. The alternative method that has been used entails the analysis of a number of samples for both acetyl bromide absorption value and lignin as measured by Waite, Johnston and Armstrong[8] and calculation of the regression equation. A series of 55 dried grass samples, made up of 31 whole samples, 12 leaf and 12 leaf sheath–stem

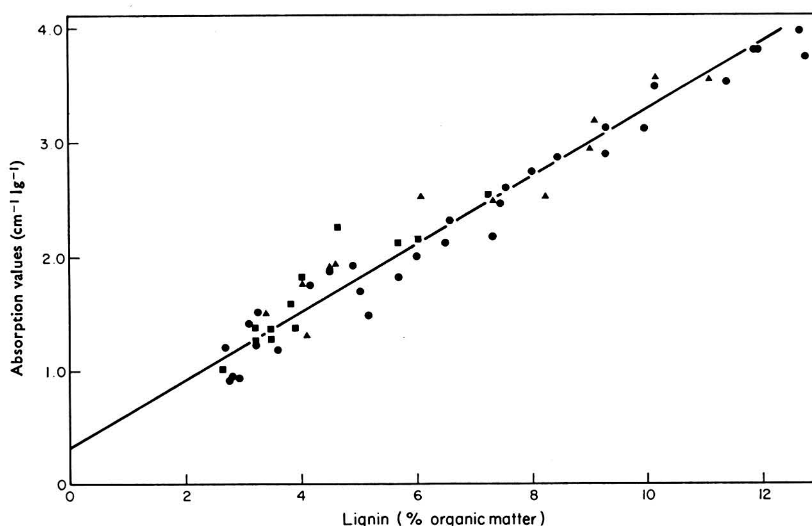

Figure 2. Relationship between acetyl bromide absorption values and lignin content. ●, Whole samples; ▲, leaf sheath–stem samples; ■, leaf samples.

subsamples were analysed in this way. The results shown in Figure 2, gave rise to the regression equation:

$$\text{lignin (\% organic matter)} = 3.36\,A - 1.11,$$

where A = absorption value.

The correlation coefficient (r) for these samples was +0.976 and the standard error of estimate was ±0.66. The acetyl bromide absorption values given in Figure 2 and of all subsequent results are the mean of three determinations, the within sample difference

being less than ±2%, a value which did not show any significant variation at high or low lignin levels.

Holocellulose samples were prepared from 12 of the dried grasses and both acetyl bromide absorption values and lignins were determined. The regression equation for these samples was:

$$\text{lignin (\% organic matter)} = 2.47\,A - 0.36 \qquad r = +0.922.$$

The significant difference between these two regression equations will be discussed later.

The relationship between the lignin absorption values and *in vitro* digestibility of the organic matter was investigated in the dried grass samples already described and also in 19 samples of dried hay and 20 samples of dried silage. The results for all these samples are plotted in Figure 3 and gave rise to the following regression equations.

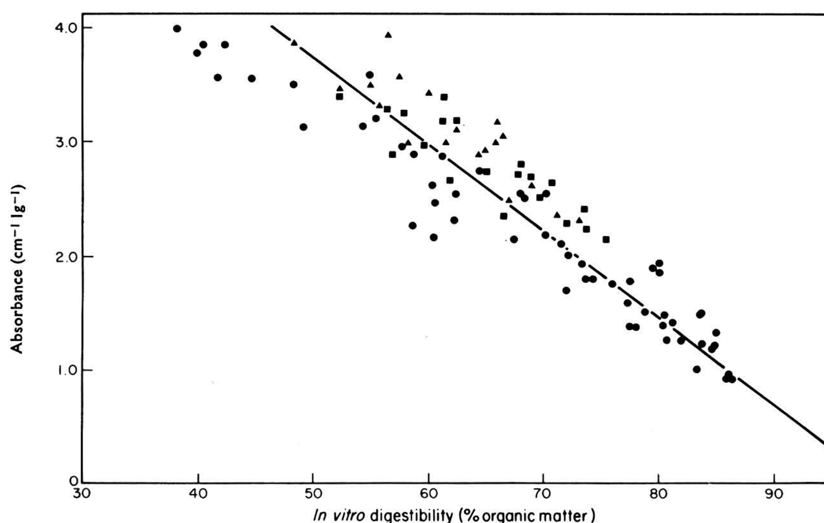

Figure 3. Relationship between acetyl bromide absorption values and *in vitro* digestibility. ●, Dried grasses; ▲, hays; ■, silages.

All samples: digestibility = 99.75 − 13.34 A, $r = -0.923$

dried grasses: digestibility = 102.47 − 15.54 A, $r = -0.965$

hays: digestibility = 100.75 − 12.54 A, $r = -0.882$

silages: digestibility = 104.97 − 14.31 A, $r = -0.867$.

For the same population of dried grasses, the *in vitro* digestibility and lignin content, as determined by Waite, Johnston and Armstrong,[8] were related by the equation:

$$\text{digestibility} = 96.61 - 4.49\,L \quad r = -0.953,$$

where L = lignin (% organic matter).

For the hay and silage samples, the *in vitro* digestibility and lignin content, as determined by the method of Deriaz,[12] were related by the equations:

hays: digestibility $= 89.09 - 3.52\,L$, $r = -0.703$

silages: digestibility $= 86.98 - 3.21\,L$, $r = -0.815$

where $L =$ lignin (% organic matter).

4. Discussion

The preliminary investigations demonstrated that low molecular weight phenolic materials, as was expected, gave very strong absorption maxima in the region of 280 nm when brought into solution by the acetyl bromide reagent and so interfered with the estimation of lignin. However, these materials could readily be removed by solvent extraction to yield crude cell wall preparations which were sufficiently pure to be used to determine lignin. Cell wall polysaccharides gave very low absorption values (Table 1). By using the derived regression equation for holocelluloses, the filter paper would appear to contain no lignin while the cellulose powder would have a content of around 0.2%. Indeed the cellulose powder was shown to contain a small amount of lignin by using the method of Waite, Johnston and Armstrong.[8] The somewhat high value obtained from apple pectin is not really significant when it is remembered that grasses contain only about 2% pectin.[8] Proteins were expected to cause the greatest interference as they may contain high proportions of the aromatic amino acids which do absorb strongly in the region of 280 nm. Three experiments indicated that such interference did not occur. Acid casein, a protein mixture containing approximately 12.5% by weight of the aromatic amino acids, gave virtually no absorption value. Digestion of the crude cell walls with the proteolytic enzyme pepsin removed more than 75% of the protein from different samples but the absorption value of a typical sample after this treatment was only reduced by 0.7%. The insoluble residue that remained after the acetyl bromide reaction accounted for more than 98% of the protein nitrogen from the original sample and consisted almost entirely of protein indicating that all other cell wall components were soluble in the reagent. It is relevant to comment here on some of the problems associated with the determination of lignin by the method of Ellis, Matrone and Maynard.[7] There are numerous reports[13, 14] that lignin is partly soluble in mineral acid probably as a result of degradation. Grass samples can have very high protein contents and, under acidic conditions, insoluble residues can result from condensation reactions between protein and carbohydrate degradation products. Even the use of proteolytic enzymes in preliminary hydrolysis does not remove all the protein[7, 15] and it is customary to adjust the values obtained for lignin to correct for this nitrogenous impurity. The use of detergents has also been suggested by Van Soest[16] but they too cannot solubilise all the protein and are reported to form colloidal suspensions of lignin which are lost on filtration.[17]

Although grass lignins are chemically distinct from wood lignins by possessing *p*-hydroxycinnamyl residues as well as the coniferyl and sinapyl moieties found in wood lignins, the full u.v. spectra of the acetyl bromide reaction products are similar in having an absorption maximum in the region of 280 nm and the experimental conditions used

for determining lignin in wood can be used also for grass lignin. The absorption maximum probably arises from both the free and bound phenolic elements that make up the lignin polymer.

The two regression equations obtained when twelve of the dried grass samples were compared with the holocelluloses derived from them were significantly different and suggest that the residual lignin in these holocelluloses had a different structure from the whole lignin. The absorption was higher in the holocellulose per unit of lignin which suggests that more free phenolic groups are present per molecular unit. This is consistent with the belief that cross-linkages in the lignin network are broken down prior to complete dissolution of lignin during the preparation of a holocellulose.

All of the results given previously are based on concentration of ash free sample. If the ash value is not known, an accurate estimation of lignin can still be made although a different regression equation to the one given previously would have to be used, namely:

$$\text{lignin (\% dry matter)} = 3.37\,A - 1.05,$$

where A = absorption value of dry matter. The correlation coefficient (r) for these samples was +0.977 and the standard error of estimate was ±0.61.

In schemes that have been proposed for the analysis of animal feeds, much emphasis has been placed on high predictability of nutritive value. *In vivo* techniques are impractical on a large scale while the *in vitro* determination of Tilley and Terry[9] and Alexander and McGowan,[18] although giving a very good prediction of nutritive value, is still lengthy to perform. Many authors have used chemical parameters, the two most successful being fibre and lignin. Van Soest[16] found that the correlation coefficients between digestibility and acid-detergent fibre, crude fibre and detergent lignin were –0.81, –0.75 and –0.78, respectively. Mowat, Kwan and Winch,[20] using Van Soest's method showed that if grasses and legumes were separated the correlation coefficients between digestibility and lignin could be as high as –0.94 and –0.95.

When the *in vitro* digestibility and acetyl bromide lignin contents of 94 grass samples comprising 55 oven dried grasses, 19 hays and 20 grass silages were compared a correlation coefficient of –0.923 was obtained. Separation of the results into individual populations improved the correlation coefficient for the dried grasses to –0.965. This is little different from the correlation coefficient between digestibility and lignin as measured by the method of Waite, Johnston and Armstrong,[8] for the same samples but was obtained in a much shorter time. The individual hay and silage populations had reduced correlation coefficients of –0.882 and –0.867, but these values were higher than those obtained by comparing digestibility and lignin as measured by the method of Deriaz,[12] namely –0.703 and –0.815. The correlation coefficient for the silage values is the lowest obtained but these analyses were carried out after drying the wet silage by heat and it may be noted that Alexander and McGowan[20] showed that fresh undried silages gave a much better correlation coefficient between *in vivo* and *in vitro* digestibilities than did heat dried silages. The dried hay and silage samples had been contributed by another laboratory and had necessitated subsampling bulks of forage after the *in vitro* digestibility and lignin determinations had been made.

The method described offers distinct advantages over other methods of estimating

lignin and digestibility in that, in addition to the simplicity of the method and the relatively short working time involved, the determinations can be made on milligram quantities. Indeed, the sample size can be reduced to 10 mg, with corresponding reduction in the reagents used, without loss of accuracy but with a higher standard error. This should be useful in measuring lignin content or predicting the digestibility of material removed from different parts of the digestive system of experimental animals. The accuracy of the method is enhanced if the regression equation employed is derived from similar types of material to the experimental samples, and to this end it is intended to determine relationships given by legumes and grass–legume mixtures.

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References

1. Johnson, D. B.; Moore, W. E.; Zank, L. C. *TAPPI* **1961**, 44, 793.
2. Tilley, J. M. A.; Deriaz, R. E.; Terry, R. A. *Int. Grassld Congr.* **1961**, 533.
3. Oh, Hi Kon; Baumgardt, B. R.; Scholl, J. M. *J. Dairy Sci.* **1966**, 49, 850.
4. Sullivan, J. T. *Agron. J.* **1962**, 54, 511.
5. Van Soest, P. J. *J. Anim. Sci.* **1964**, 23, 838.
6. Johnston, M. J.; Waite, R. *J. agric. Sci., Camb.* **1965**, 64, 211.
7. Ellis, G. H.; Matrone, G.; Maynard, L. A. *J. Anim. Sci.* **1946**, 5, 285.
8. Waite, R.; Johnston, M. J.; Armstrong, D. G. *J. agric. Sci., Camb.* **1964**, 62, 391.
9. Tilley, J. M. A.; Terry, R. A. *J. Br. Grassld Soc.* **1963**, 18, 104.
10. Alexander, R. H.; McGowan, M. *J. Br. Grassld Soc.* **1961**, 16, 275.
11. Waite, R.; Gorrod, A. R. N. *J. Sci. Fd Agric.* **1959**, 10, 317.
12. Deriaz, R. E. *J. Sci. Fd Agric.* **1961**, 12, 152.
13. Pew, J. C. *TAPPI* **1957**, 40, 553.
14. Pearl, I. A.; Busche, L. R. *TAPPI* **1960**, 43, 961.
15. Czerkawski, J. W. *Br. J. Nutr.* **1967**, 21, 325.
16. Van Soest, P. J. *J. Ass. off. agric. Chem.* **1963**, 46, 829.
17. Porter, P.; Singleton, A. G. *Br. J. Nutr.* **1971**, 25, 3.
18. Alexander, R. H.; McGowan, M. *J. Br. Grassld Soc.* **1961**, 16, 275.
19. Mowat, D. N.; Kwan, M. L.; Winch, J. F. *Can. J. Pl. Sci.* **1969**, 49, 499.
20. Alexander, R. H.; McGowan, M. *J. Br. Grassld Soc.* **1969**, 24, 195.

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Physical and Chemical Changes in Developing Strawberry Fruits

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Strawberry fruits of the variety Red Gauntlet were harvested at 7 day intervals after petal fall. Changes in fruit weight, percentages survival on the plant, chlorophyll, carotenoid and anthocyanin, titratable acid, pH of extracts and sugar content were measured. Also changes in soluble and total pectic polysaccharides in alcohol-insoluble residues of harvested fruits were followed during development.

Fruit growth was not exponential and in later stages of growth the falling survival rate was correlated to fruit softening. Net synthesis of chlorophyll and carotenoid pigments took place up to 28 days and anthocyanin synthesis commenced 28 to 35 days after petal fall. The sugar content of berries increased with time and titratable acid concentrations increased slightly during development, falling in ripening fruits.

The specific viscosity of soluble pectic polysaccharides fell from 28 days after petal fall. There was net synthesis of polyuronide but not neutral polysaccharide during the development of fruits and the amount of insoluble pectic polysaccharide became small and relatively constant compared with the amount of soluble polysaccharide by 21 days after petal fall. Fruits undergoing senescence lost almost all their insoluble pectic polysaccharides.

The developmental processes taking place in growing fruits, especially with respect to changes in cell wall structure, and the relevance of results to fruit storage are discussed.

1. Introduction

The storage life and quality of strawberry fruits are governed by their condition at harvest. It is known that immature fruits store well compared with mature fruits as they can ripen during storage. The strawberry, unlike the apple, does not exhibit a respiration climacteric. However, it does enter a period of rapid ripening shortly before harvest and the changes taking place up to and including this period are not well established.

The changes which take place in cell wall polysaccharides during the ripening of strawberries have been partially established.[1,2] Gizis[3] has shown that certain enzymes which have been related to the breakdown of polyuronide are present in strawberry tissue at harvest. Other workers have produced quantitative measurements of red pigments,[4–7] titratable acid[4,7,8] and percent soluble solids[4,7] present in partially ripe and ripe fresh and frozen strawberries.

The above changes apply only to ripe or nearly ripe fruits; they have not been

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established in relation to berry development and it is in an attempt to do this that a preliminary investigation into preharvest changes in strawberry fruits has been undertaken.

2. Experimental

2.1. Fruit source and sampling

Maiden year strawberry plants of the variety Red Gauntlet, set out in rows of 64 individual plants, were used as a source of fruit. It was necessary to label 75 flowers at petal fall for each sampling date, with 7 sampling dates, each 7 days apart plus an initial sample at petal fall. For each sample, 25 flowers at petal fall were labelled on each of three consecutive days. Several flowers on one plant were labelled depending on the numbers at petal fall, and consecutive plants in the rows were labelled for successive sampling dates to spread samples across the whole plot. It was usual to avoid labelling flowers on the same plant with different picking dates although this did happen on occasions. (Petal fall was taken to be the point at which one or more petals had fallen or could be easily dislodged from the flower.)

Sample fruits were divided into diseased and sound fruits. Among the diseased fruits those which had failed to develop or were lost due to some other factor were noted before they were discarded. The numbers of sound fruits were noted, their total weights recorded and mean weights calculated. Fruit was then subjected to analysis.

2.2. Fruit analysis

The weights of samples for analysis varied according to the mean weight of fruits. However, previous experiments had shown that, for acid and sugar analysis, the maximum sample weight giving a linear extraction curve at a constant volume was 50 g, thus samples were kept under 50 g or thereabouts, if possible. This was found to be an adequate weight for pigment analysis and cell wall preparations.

2.3. Titratable acid and sugar

Acids and sugars were extracted by cutting a known weight of fruits into approximately 0.2 to 0.4 cm thick slices and refluxing them for 30 min in 500 ml of distilled water (350 ml in the 7-day sample). The extract was filtered hot, allowed to cool and 75 ml titrated with 0.1 N-sodium hydroxide solution to a pH of 8.2 to estimate the acid content. The pH of the solution was also noted.

The sugar content was estimated by appropriately diluting a sample of the filtrate and estimating its sugar content in terms of sucrose using an automated sulphonated α-naphthol system.[9]

2.4. Pigments

The anthocyanins were extracted by macerating samples at 2 °C in 2 volumes of 1% HCl in methanol in a MSE Atomix homogeniser for 0.5 min at half speed and 1 min at full speed. Samples were then filtered under suction and washed twice with 20 to 30 ml of 1% HCl in MeOH. Filtrates were made up to a suitable volume and their optical density at 510 nm (minus that at 600 nm to correct for background absorbance) measured on a Beckman DBGT recording spectrophotometer. In the 42- and 49-day samples

when mean berry weight reduced numbers of berries sampled, opposite quarters of fruits were taken to increase numbers sampled. Anthocyanin content of fruits was calculated as pelargonidin-3-monoglucoside (molar absorptivity 36 000).[10]

Chlorophyll and carotenoid pigment were extracted by macerating berries at 2 °C in 4 volumes of acetone in the MSE homogeniser as for anthocyanin extraction. The slurry was filtered under suction, washed three times with 20 to 30 ml of 80 % acetone and made up to a convenient volume. Chlorophyll concentration was estimated directly by measuring optical density at 645, 652 and 663 nm and 700 nm for background contaminants.[11] Carotenoids were separated from the acetone extract and estimated as described by Knee.[12]

2.5. Cell wall preparation and analysis

Alcohol-insoluble residues (a.i.r.) of samples were prepared from the residue left after anthocyanin extraction. The residue was washed with large volumes of ethanol to remove any residual C_{15} glycosides and then refluxed for 30 min in ethanol. This preparation was then filtered under suction in a weighed sintered glass funnel (porosity 3), washed with ethanol and ether and left to air dry for at least 72 h. The dry weight of the a.i.r. was noted and it was stored in stoppered jars.

Soluble pectic polysaccharides were estimated by extracting 100-mg samples of the a.i.r. for 24 h with 25 ml of 0.05 M-ethylenediaminetetra-acetic acid (EDTA), 0.1 M-Na_2HPO_4 (pH 6.9), containing a few drops of toluene at 20 °C. Samples were then filtered and the viscosity of 10-ml aliquots measured against a water blank using a "U" tube viscometer in a water bath at 35 °C. Residues were then washed and the filtrates made up to a constant vol. Samples were taken for analysis of the polyuronide and neutral polysaccharide content.

In a separate experiment the soluble pectic polysaccharides were extracted from 100-mg samples of the a.i.r. as above. The residue obtained from the extraction contained the insoluble pectic polysaccharides, which were extracted with a pectinase enzyme using the procedure described by Knee.[13] All filtrates were analysed for polyuronide and neutral polysaccharide content and the sum of the soluble and insoluble pectic polysaccharides was taken to represent the total pectin content of the cell walls.

The polyuronide content of samples was estimated as anhydrouronic acid equivalents using an automated modification of the carbazole system reported by Bitter and Muir.[14] Neutral polysaccharide, measured as anhydroglucose units, was estimated using the same method as that for sugar analysis.[9,13]

3. Results

3.1. Fruit weight and percentage survival on the plant

Figure 1 shows the increase in mean weight and the decrease in percentage survival of fruits with time. The percentage survival fell sharply 35 to 42 days after petal fall, shortly before fruit growth ceased. Growth was not exponential but was relatively rapid in early development and became only slightly less rapid up to the 42-day sample, after which growth ceased.

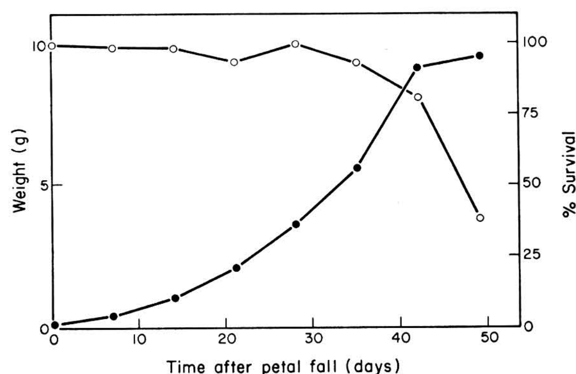

Figure 1. Mean weight and survival rate during the development of Red Gauntlet strawberry fruits. ●, Mean wt; ○, survival rate.

3.2. Soluble sugar and titratable acid

Soluble sugars (Table 1) increased steadily during development and ripening, and did not decrease during senescence. Similarly, titratable acid (Table 1) increased during

TABLE 1. Changes in sugar and acid content, pH and a.i.r. in developing strawberry fruits

No. of days after petal fall	Mean fruit (g)	Sugar content (mg/fruit)[a]	Acid content (mg/fruit)[b]	pH of extract	Wt of alcohol insoluble residue (mg/fruit)
0	0.10	—	—	—	24
9	0.38	9	2.7	4.6	35
14	1.05	31	6.7	4.3	96
21	2.09	53	15.5	3.6	118
28	3.63	122	30.5	3.4	175
35	5.59	284	46.1	3.3	161
42	9.15	443	69.5	3.3	176
49	9.50	551	51.0	3.7	217

[a] as sucrose.
[a] as anhydro citric acid.

development but was less in overripe fruits. The pH of extracts behaved similarly, becoming relatively stable at pH 3.6 to 3.3 from the 21-day sample. The 49-day sample, which had a low acid content, had a correspondingly high pH.

3.3. Pigment composition of fruits

The chlorophyll content of fruits fell rapidly until 28 to 35 days after petal fall on a per g fresh weight basis. Carotenoid content fell less rapidly but both pigments reached very low levels in ripe fruits (Figure 2). However, on the basis of amount per fruit (Figure 3) synthesis of both pigments continued until 21 to 28 days after petal fall.

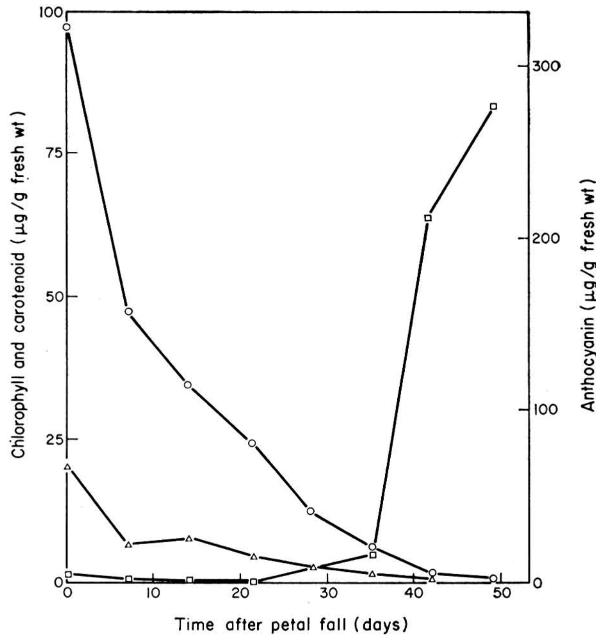

Figure 2. Changes in pigment concentration per unit fresh weight in developing strawberry fruits. o, Chlorophyll; △, carotenoid; □, anthocyanin.

Anthocyanin production was very low until 35 days after petal fall when chlorophyll and carotenoid synthesis had ceased. It then increased very rapidly reaching at least 75% of the final anthocyanin concentration in about 7 days.

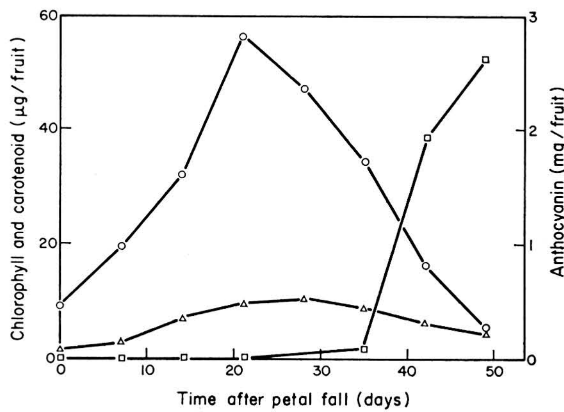

Figure 3. Changes in pigment concentrations per fruit in developing strawberry fruits. o, Chlorophyll; △, carotenoid; □, anthocyanin.

3.4. Cell wall changes

The quantity of a.i.r. decreased on a per g fresh-weight basis and increased on a per fruit basis during the development and ripening of fruits (Table 1). Thus there was net synthesis of polysaccharides during growth, but this did not keep pace with cell expansion.

The specific viscosity was directly related to the concentration of polyuronide per

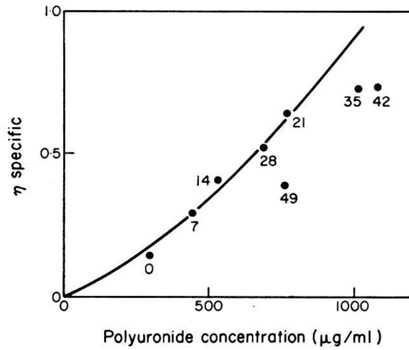

Figure 4. Relationship between specific viscosity and polyuronide concentration in water soluble extracts of alcohol insoluble residues of developing fruits. Subscript numbers refer to days from petal fall.

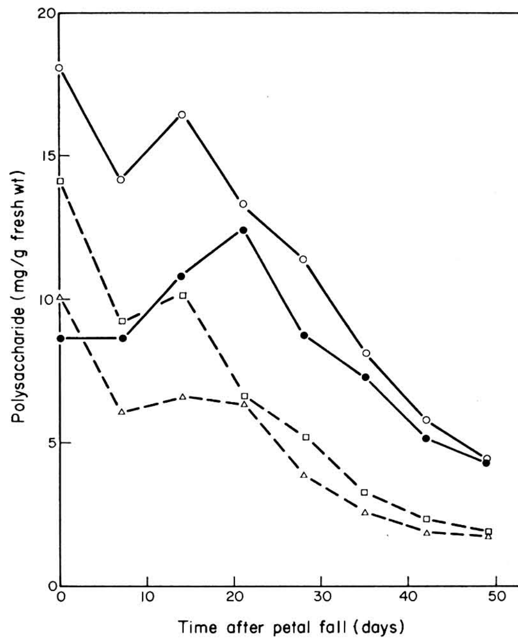

Figure 5. Changes in total and soluble pectic polysaccharides per unit fresh weight during fruit development. ○, Total polyuronide; ●, water soluble polyuronide; □, total neutral polysaccharide; △, water soluble neutral polysaccharide.

ml of buffer soluble extract up to the 28-day sample (Figure 4). The 35- and 42-day samples fell below the values that would be expected for the concentration of polyuronide present and indicated that some breakdown of polyuronide to lower molecular weight compounds had taken place. The 49-day sample also lay below the expected value and fruits were considered to be undergoing rapid autolysis.

The results show overall synthesis of polyuronide in fruits up to the 42-day sample (Figure 6), but neutral polysaccharide was not synthesised in any quantity after the 21- and 28-day samples. Net synthesis of cell wall polysaccharides ceased 42 days after petal fall from which time fruits underwent autolysis.

It can be seen (Figures 5 and 6) that in all samples the neutral polysaccharide represents only a small fraction of the pectin.

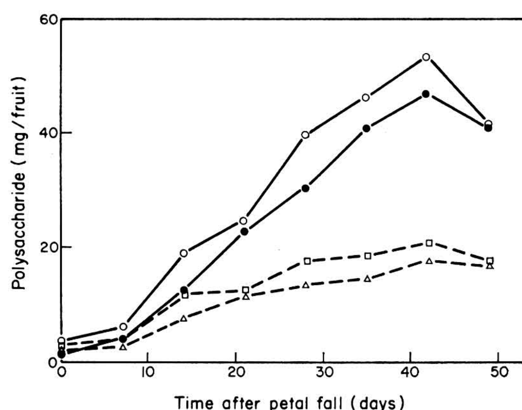

Figure 6. Changes in total and soluble pectic polysaccharides per fruit during fruit development. ○, Total polyuronide; ●, water-soluble polyuronide; □, total neutral polysaccharide; △, water-soluble neutral polysaccharide.

The quantities of buffer-soluble polysaccharides were considerably below the values for total pectic polysaccharides up to the 14-day sample (Figure 5). From 21 to 42 days after petal fall however they represented a large proportion of the total pectic polysaccharides present; the insoluble polysaccharides constituted a small proportion from about 20 days and in the 49-day sample practically all were soluble, which was consistent with observed breakdown of the tissues.

4. Discussion

4.1. Quantitative comparisons and cell wall extraction procedures

Published results refer mainly to the composition of half-ripe and fully ripe strawberries. Quantities of titratable acids and the pH of extracted tissue recorded in the above work agree with published data.[4,7,8] Soluble sugars are slightly lower than published results, but as these are expressed as per cent soluble solids this is to be expected.

The carotenoid contents of ripe samples are slightly higher than those given by Galler

and Mackinney.[15] However, both carotenoid and chlorophyll results are of the same order as those previously obtained by the author (unpublished data). The anthocyanin concentrations agree with Blundstone's[5] results for under-ripe and half-ripe fruits, but not for fully ripe fruits. Considerably more anthocyanin is present in ripe fruits than that quoted by Blundstone for the same variety. Calculated values for cyanidin-3-monoglucoside content of fruits fall within the limits of results reported for frozen fruit.[4]

The figures quoted for the quantity of a.i.r. per fruit in ripening fruits are of the same order as those quoted by Neal[1] for different varieties. It is generally found that a portion of the polyuronide in fruits and other plant tissues can be made soluble only by degradative treatments. While this portion may be chemically distinct, there is no chemical basis for a subdivision of the soluble pectin by extraction with reagents such as water, calgon or EDTA. EDTA phosphate at pH 6.9 was found to be the most efficient extractant of soluble polysaccharides and was therefore adopted for routine use.

4.2. Fruit development

The first 7 to 10 days of development after petal fall is probably a phase of cell multiplication, after which a steady increase in the size of fruits accompanied by an increase in cell wall materials takes place. This is considered to be a period of cell expansion within the fruit.

Chlorophyll and carotenoid production ceases 20 to 30 days after petal fall and the decline in the concentration in these pigments after this time indicates the degeneration of plastids in the fruit cells. Anthocyanin is not produced in any quantity until 35 days after petal fall and the time between the cessation of green and yellow pigment production and the commencement of red pigment production corresponds to the white stage of development.

It has already been noted that the soluble polysaccharides reach a relatively constant level when compared to total pectic polysaccharides about 20 days after petal fall. Also the specific viscosity begins to fall below the expected value between the 28- and 35-day samples. Thus it can be said that the white stage represents the point in fruit development when the physiology is turned over to the ripening process.

Only small quantities of insoluble polyuronide and neutral polysaccharide are left in the cell wall from 20 to 40 days after petal fall. However, during this time considerable softening of the fruits occurs. It can be seen from the results that there is a gross synthesis of polyuronide in this time and little of the insoluble pectin is liberated. It has been suggested[1] that there is a pectic fraction which becomes water-soluble during maturation but this is not supported by the above results: this may be due to the different extraction methods used. Thus, it is proposed that a change in the cell wall polysaccharide takes place in such a way that the uronide polymers become rearranged to allow more plasticity in the walls of the fruit. Neal[1] proposes that chelation of the calcium ions which are thought to form crosslinks between carboxyl groups of polyuronide chains may lead to softening of tissue, and also suggests that esterification of the carboxyl groups would prevent such cationic links forming. Pectin methylesterase has been reported in fruits,[1,3] and this, if active during ripening, would tend to reverse the processes postulated by Neal.

The lowering of the specific viscosity of the 35- and 42-day samples supports published data[3] and suggests enzymic breakdown of the soluble polyuronide chains. Neal[1] failed to detect polygalacturonase activity in ripe strawberry fruits. However, Gizis[3] reports its activity in ripe fruits and also that this enzyme and others present have no activity on the insoluble fraction of cell wall polyuronide.

There is a continuous developmental process during the growth and ripening of strawberry fruits and, although it can be said that there is a definite point in growth when the physiology of the fruit is turned over to the ripening process, there is no distinct point during ripening when fruit can be said to be at its optimum in relation to fruit storage. After this physiological change in the fruit visual redness can be correlated with the ripening and softening of fruits. Also as fruits ripen and become softer they become more susceptible to disease and this is reflected in the lower survival rate of ripe fruits. It is therefore necessary to pick fruit to be stored as soon after the white stage as possible, bearing in mind that it must be commercially ripe when brought out of storage.

The respiration of fruits and associated metabolic processes are suppressed by the low temperatures used in their storage. However, such conditions cannot be expected to slow down processes such as the softening of cell walls to the same degree, thus fruit stored in a ripe condition can be expected to deteriorate quickly.

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References

1. Neal, G. E. *J. Sci. Fd Agric.* **1965**, 16, 604.
2. Wade, P. *J. Sci. Fd Agric.* **1964**, 15, 51.
3. Gizis, E. J. The Isolation and Characterisations of the Pectic enzymes and the Pectic substances of the North West Strawberry, **1964**, Ph.D. Thesis, Oregon State University.
4. Wrolstad, R. E.; Putman, T. P.; Varseveld, G. W. *J. Fd Sci.* **1970**, 35, 448.
5. Blundstone, H. A. W. A quantitative survey of the anthocyanins of red fruits, **1969**, Tech. Bull. No. 17, Fruit and Vegetable Preservation Research Association, Chipping Campden, Gloucestershire.
6. Blundstone, H. A. W.; Crean, B. A. The pigments of red fruits, **1966**, Fruit and Vegetable Preservation Research Association, Chipping Campden, Gloucestershire.
7. Robinson, W. B.; Lee, F. A.; Slate, G. L.; Pederson, C. S. Chemical Composition and Freezing Adaptability of Strawberries, **1947**, Bulletin No. 726, New York State Agricultural Experiment Station, New York.
8. Hulme, A. C.; Wooltorton, L. S. C. *Chemy. Ind.* **1958** (22), 659.
9. Devor, A. W. *J. Am. chem. Soc.* **1950**, 72, 2008.
10. Wrolstad, R. E.; Hildrum, K. I.: Amos, J. F. *J. Chromatog.* **1970**, 50, 311.
11. Holden, M. In *Chemistry and Biochemistry of Plant Pigments*, (T. W. Goodwin, ed.) Academic Press, London. **1965**, p. 461.
12. Knee, M. *J. exp. Bot.* **1972**, 23, 184.
13. Knee, M. *J. Sci. Fd Agric.* **1971**, 22, 371.
14. Bitter, T.; Muir, H. M. *Analyt. Biochem.* **1962**, 4, 330.
15. Galler, M.; Mackinney, G. *J. Fd Sci.* **1965**, 30, 393.

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Determination of Pectic Substances in Plant Material

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(Accepted for publication May 1971)

Pectic substances have been extracted from plant material (a pasture legume, *Stylosanthes humilis*) by homogenisation with a solution of ammonium oxalate and oxalic acid and subsequently hydrolysed with polygalacturonase. The galacturonic acid produced was determined by a modified carbazole reaction. The enzymic hydrolysis step may be omitted by relying on the acid in the carbazole reaction partially to hydrolyse the pectic substances, but in doing so the sensitivity of the method is reduced sevenfold. Attempts to carry out the hydrolysis with sulphuric acid before the carbazole reaction gave incomplete hydrolysis and probably decarboxylation. Attempts to extract the pectic substances with alkali gave low values, probably because of incomplete extraction.

1. Introduction

In our studies of the digestion of polysaccharides by ruminants a specific method was required for the analysis of pectic substances in plant materials. The carbazole method has been extensively used for this purpose and frequently the acid of the carbazole reagent has been relied upon to hydrolyse the polymer to galacturonic acid which produces the chromophore.[1,2] We were concerned to avoid interference from other uronic acids which might be produced from our plant materials in this type of treatment and also to achieve maximum sensitivity in our analysis by ensuring complete conversion of pectic substances to galacturonic acid. It is well known that polyuronides are resistant to acid hydrolysis and undergo decarboxylation. We have therefore investigated the acid hydrolysis step and have also sought an alternative in the use of a polygalacturonase for the hydrolysis.

Polygalacturonase is an α-(1,4)-polygalacturonide glycanohydrolase (E.C. 3.2.1.15)[3] and the purified enzyme from a fungal source has been shown to produce complete conversion of the polymer to galacturonic acid.[4] This type of enzyme has frequently been utilised in structural studies of pectic substances[5–7] and has also been used in analysis,[8–10] with subsequent rather complex procedures for the determination of galacturonic acid. We have investigated the extent to which the enzymic hydrolysis may most simply and effectively be combined with a subsequent carbazole analysis. Dische has shown[11] that by careful control of the carbazole analysis conditions the reaction may be made very much more sensitive for galacturonic acid than for any other uronic acid. In this respect the carbazole reaction may be regarded as the most appropriate method of analysis of uronic acids for use with pectic substances, since all

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other colorimetric methods and also the decarboxylation methods distinguish much less effectively between galacturonic and the other uronic acids.[12] We have reinvestigated the conditions used by Dische[11] with the particular aim of using them to follow up the enzymic hydrolysis.

2. Experimental

2.1. Carbazole reaction

83% sulphuric acid (6 vol. concentrated sulphuric acid (May and Baker "Pronalysis", 97.5 to 100% w/w) plus 1 vol. distilled water) (5.4 ml) was cooled to 4 °C with ice–water in pyrex (6 in × 1 in) tubes. Standard solutions (0.4 ml) containing 0 to 250 μg galacturonic acid (B.D.H.) were added to the cooled acid and the mixture vigorously agitated. The tubes were next brought to room temperature with running tap water and then immersed in a water-bath at 60 °C ± 0.1 deg. for exactly 1.5 min before cooling in tap water. A 0.1% (w/v) solution of carbazole (B.D.H., twice recrystallised from benzene) in ethanol (0.2 ml) was added next and the mixture shaken. The tubes containing the reaction mixture were then incubated in a water-bath at 30 °C ± 0.5 deg. for 1 h. The absorbance of the coloured solutions was then immediately measured at 530 nm.

Blank determinations contained all of the carbazole reagents except for galacturonic acid solution, which was replaced with distilled water. A plot of absorbance against galacturonic acid concentration gave a straight line for the above range of concentrations.

2.2. Acid hydrolysis of purified pectic acid

Pectic acid, isolated from the stems of *S. humilis* by Dr M. Alam, was prehydrolysed with 0.1 N-sulphuric acid for 4 h at 100 °C to remove neutral sugars. Its isolation and chemical structure have been described previously.[14] In an attempt to complete the conversion of the pure pectic acid to galacturonic acid before the carbazole reaction a two-stage hydrolysis was used.

2.2.1. Primary hydrolysis

Primary hydrolysis was carried out at 30 °C at a concentration of 20 mg pectic acid/ml 72% sulphuric acid[13] for times ranging from 0.5 to 6 h. Water was then added rapidly at room temperature in the ratio of 28 ml to 1 ml of 72% sulphuric acid to terminate the primary hydrolysis step.

2.2.2. Secondary hydrolysis

To investigate the effect of time in the secondary step, in a series of experiments, primary hydrolysis was conducted for 1 h at 30 °C and, after dilution as above, was followed by secondary hydrolysis at 100 °C for varying times ranging from 0 to 5 h.

2.3. Enzymic hydrolysis of purified pectic acid

Pure pectic acid (5 mg) was dissolved in 0.1 M-sodium acetate buffer (10 ml, pH 4.4 to 4.5), polygalacturonase (SIGMA Chemical Co. U.S.A., fungal origin; 1 mg) was added followed by a few drops of toluene and the reaction mixture incubated at 30 °C

while samples were removed at intervals up to 24 h for analysis by the carbazole method. The enzyme was deactivated after removal of each sample by heating (100 °C for 1 to 2 min).

2.4. Effect of oxalate ion on enzymic hydrolysis of pectic acid

Pure pectic acid (5 mg) in 0.1 M-sodium acetate buffer (10 ml, pH 4.4 to 4.5) containing ammonium oxalate (B.D.H., 25 mg) and oxalic acid (B.D.H., 25 mg) was incubated with polygalacturonase (1 mg) for 24 h at 30 °C, and aliquots removed at intervals to determine the extent of hydrolysis.

2.5. Extraction of plant pectic substances

2.5.1. With alkali

Fresh, finely cut plant material (stem and leaf separately, approximately 0.5 g) was homogenised (Quickfit homogeniser operated at 2000 r.p.m.) in 0.5 N-sodium hydroxide (10 ml), neutralised with 0.5 N-sulphuric acid and centrifuged. The supernatant solution was divided into three measured portions for the following treatments which preceded analysis for pectic acid (as polygalacturonic acid) by successive enzymic hydrolysis and carbazole reaction under the conditions described above.

(a) No treatment (control).
(b) Charcoal treatment (to remove interfering inhibitors).
(c) Mild prehydrolysis treatment (to remove neutral sugars from pectic acid).

In sample (b) charcoal (15 mg/ml) was added, shaken at room temperature for 10 min, and then centrifuged. In sample (c) the solution was made to 0.1 N-sulphuric acid by addition of 10 N-acid and heated 4 h at 100 °C. The hydrolysate was then neutralised with sodium hydroxide (10 N).

2.5.2. With ammonium oxalate/oxalic acid

Finely cut *S. humilis* (approximately 0.5 g) was homogenised with successive 10 ml portions of an aqueous solution of ammonium oxalate and oxalic acid (0.25% with respect to each) in a Virtis "45" homogeniser at 40 000 r.p.m. for 5 to 10 min initially at room temperature. Before the first homogenisation the suspension was heated to 100 °C for 2 min to deactivate enzymes and then cooled. The resultant homogenates were boiled under reflux for 1 h then centrifuged and analysed for pectic acid as above.

3. Results and discussion

3.1. The carbazole reaction

The conditions used were those described by Dische[11] as giving the highest relative specificity for galacturonic acid, but we have found that in the relatively mild acid conditions utilised in this analysis a high degree of control over the total thermal history of the reaction mixture is essential if the analysis is to be reproducible. The initial treatment at 60 °C for 1.5 min is intended to convert a fixed proportion of the galacturonic acid to 5-carboxy-2-formylfuran which is subsequently reacted with

carbazole to produce the chromophore. The latter reaction occurs relatively slowly and Dische[11] has recommended that the absorbance of the solution be measured after 1 h at room temperature. We have found it necessary to control the temperature more accurately during this development of colour and the results shown in Figure 1 also

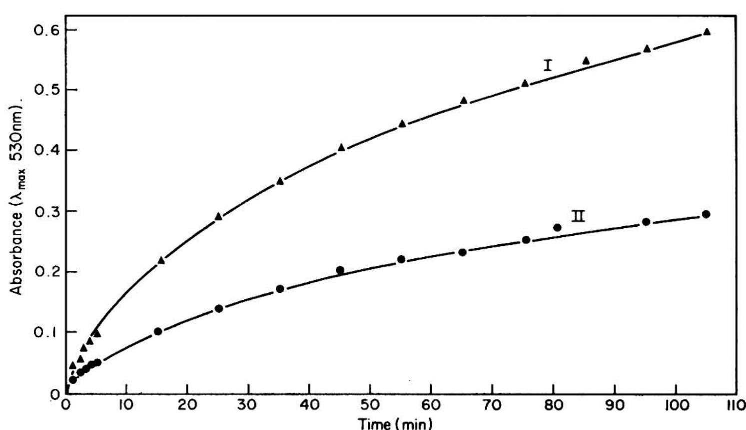

Figure 1. Time dependence in the carbazole reaction. Galacturonic acid solution, I, 160 μg/0.4 ml; II, 80 μg/0.4 ml.

demonstrate the importance of accurate control of time in this phase of the reaction. In this respect an important experimental difficulty arises since in processing large batches of analyses it will obviously be difficult to read the absorbance of each sample at exactly 1 h after addition of carbazole. We have found it necessary therefore to note the exact time at which each sample was placed in the spectrophotometer and to apply a factor based on the slope of the curves in Figure 1 at 1 h to correct the absorbance to that pertaining at 1 h. This factor (F) was 0.00672 min^{-1} when used in the expression

$$A\text{ (corr.)} = \frac{A\text{ (obs.)}}{1 + F(t - 60)}$$

where A (corr.) = corrected absorbance at 60 min and A (obs.) = absorbance at time t min.

3.2. Effect of prehydrolysis of pectic acid on the carbazole reaction

The pectic acid which we have utilised in our calibration experiments has been subjected to a mild hydrolysis to remove most of the arabinose, galactose and rhamnose units originally present. It was therefore essentially a pure α-1,4-galacturonan and in all subsequent references to analysis of pectic acid we shall imply analysis for galacturonan. This approach was taken to ensure that accurate weights of galacturonan (free from neutral sugars) were available for calibration experiments. We have, however, confirmed previous claims[12] that neutral sugars do not significantly interfere with the carbazole analysis.

Application of our standard carbazole analysis to the pectic acid gave the usual

chromophore (Figure 5, curve I). This might be envisaged as arising both from a partial hydrolysis of the pectic acid to galacturonic acid under the conditions of analysis, and also from acid-catalysed elimination reactions at the reducing end group of a galacturonan. However, the colour developed from pectic acid was only 14% of that produced by an equal weight of galacturonic acid (Figure 2).

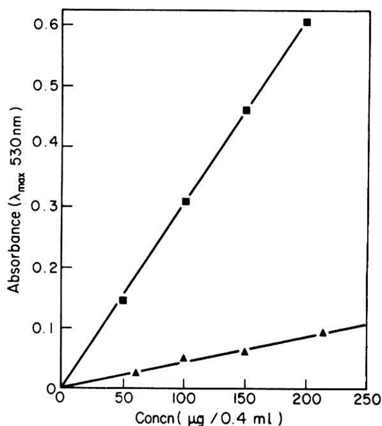

Figure 2. Sensitivity of the carbazole reaction for galacturonic acid and pectic acid. —▲—▲—, Pectic acid; —■—■—, galacturonic acid.

In order to improve the sensitivity of the analysis we have investigated the possibility of hydrolysing the pectic acid before carbazole analysis. Pectic acid is insoluble in dilute mineral acids and attempted hydrolysis in such a medium would obviously be inefficient. It is soluble, however, in 72% sulphuric acid and after at least 0.5 h at 30 °C such a solution gives no precipitate on dilution with water. Figure 3 (curve I) shows that the

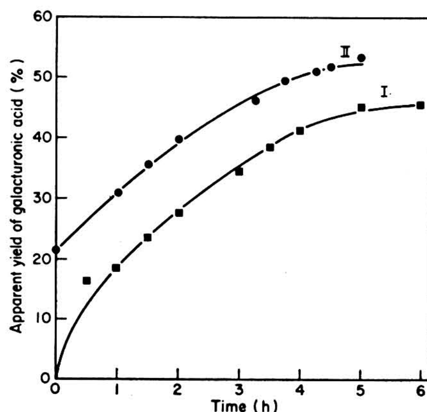

Figure 3. Prehydrolysis of pectic acid before the carbazole reaction. —■—■—, Primary hydrolysis; —●—●—, secondary hydrolysis.

apparent yield of galacturonic acid from pectic acid, measured by subsequent carbazole reaction, continues to increase in 72% sulphuric acid at 30 °C up to 5 to 6 h but at this stage the yield is only 45%. After such a primary hydrolysis for 1 h, a secondary hydrolysis at 100 °C after dilution of the sulphuric acid to 0.9 N gave an apparent yield of galacturonic acid of 50% at 5 h (Figure 3, Curve II). The results in Figure 3 indicate very limited advantage in prehydrolysis with acid during pectic acid analysis. The low apparent conversions may be due both to incomplete hydrolysis and to decarboxylation.

Since prehydrolysis of the pectic acid with sulphuric acid was unsatisfactory we investigated the use of a fungal polygalacturonase. This enzyme in acetate buffer rapidly hydrolysed the pure pectic acid to galacturonic acid and Figure 4 shows that

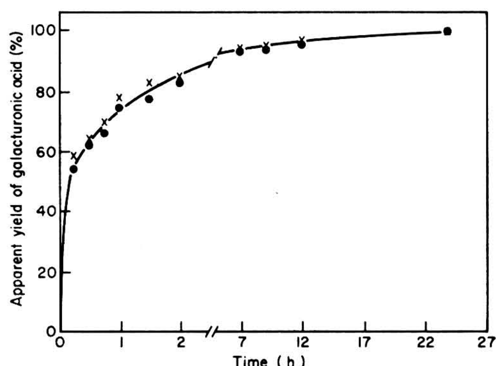

Figure 4. Enzymic hydrolysis of pectic acid, —x—x—, In absence of oxalate ion; —●—●—, in presence of oxalate ion.

the conversion, measured by subsequent carbazole reaction, was approximately 100% after 24 h. In subsequent work we required the presence of oxalate ion in order to extract pectic acid from the plant and Figure 4 also shows this ion does not significantly affect the hydrolysis. In order to investigate the possibility that neutral sugars covalently bonded to the pectic acid *in vivo* might interfere with the action of the enzyme, we carried out a similar hydrolysis on the intact pectic acid isolated by ammonium oxalate extraction of *S. humilis*.[14] This material gave an apparent yield of 73% of galacturonic acid and this figure agrees quite well with the previous value[14] of 82% based on equivalent weight determinations and including 6% of esterified uronic acid. We conclude, therefore, that the presence of neutral sugar substituents does not significantly affect the action of the enzyme.

3.3. Extraction of pectic substances from plant material

We have assumed that any attempt to hydrolyse pectic substances *in situ* in the plant before analysis by the carbazole method would be unlikely to proceed to completion. Solubilisation and extraction of the pectic substances were therefore necessary. Sodium hydroxide was used to extract several samples of stem and leaf as described in the experimental section and results are summarised in Table 1. These show that the results are not affected by either mild hydrolysis (to remove neutral sugars) nor by

charcoal treatment (to remove enzyme inhibitors). The pectic acid contents shown in Table 1, however, were considerably lower than those anticipated from our previous work on *S. humilis*[15] and we believe that the alkali extracted only a small proportion of

TABLE 1. Apparent pectic acid content by alkaline extraction

Treatment after extraction[a]	Plant sample	Pectic acid (% of dry wt)	
		Stem	Leaf
None	(i)	3.40	2.44
(Control)	(ii)	3.96	2.68
	(iii)	[b]1.52	2.28
	(iv)	2.88	2.04
Mild prehydrolysis	(i)	3.32	2.00
	(ii)	3.52	2.32
	(iii)	[b]1.28	2.20
Charcoal	(iv)	2.80	1.60

[a] See experimental section.
[b] In this experiment the stems used were older than those used in all other experiments.

the total pectic substance. Furthermore, the spectrum of the solution obtained from the carbazole reaction on the enzyme-hydrolysed alkaline extract is given in Figure 5 (curves II and III) and shows interference from a chromophore at 460 nm which was not given by pure pectic or galacturonic acids. Presumably the new chromophore originates in materials other than pectic acid, extracted from the plant by the alkali.

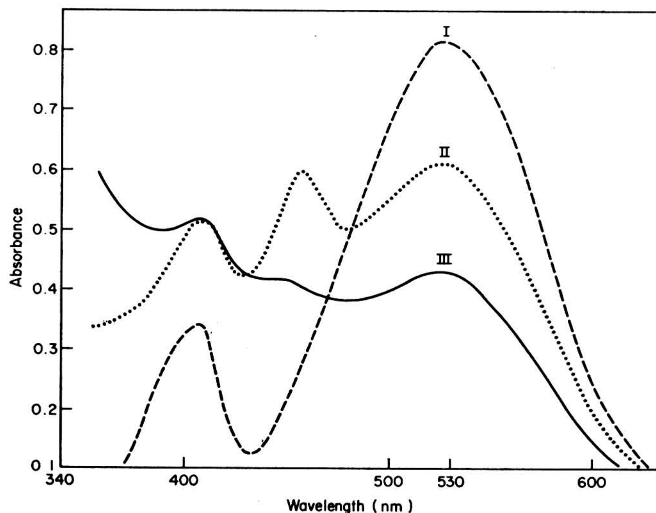

Figure 5. Absorption spectra of carbazole reaction mixtures. I, Galacturonic acid, crude and purified pectic acid; II, stem pectic acid extracted with alkali; III, leaf pectic acid extracted with alkali.

Since alkali was shown to be unsatisfactory as an extractant a solution of ammonium oxalate and oxalic acid was used.[16] The results of a series of extractions of *Stylosanthes* hay with the oxalate solution was shown in Figure 6 and indicate that most of the extractable pectic acid is removed in the first two extractions.

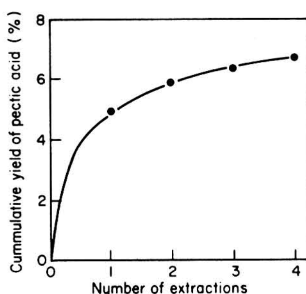

Figure 6. Successive oxalate extractions of pectic acid from *Stylosanthes humilis* hay.

The recommended sequence of procedures for pectic acid analysis in the plant is therefore as follows:

(i) homogenise and boil at least twice with oxalate solution,
(ii) centrifuge extract and hydrolyse with polygalacturonase,
(iii) determine galacturonic acid by carbazole reaction.

Values of 10 to 12% have typically been obtained by application of the above procedures to fresh *S. humilis* stems and these figures represent extractable galacturonan. We know that the pectic substance of this plant contains about 20% neutral sugars,[14] so that the extractable pectic substance is about 12.5 to 15% of the plant dry weight. This figure will err on the low side to the extent that galacturonan remains unextracted by the oxalate solution. We have, however, found that only a small proportion of galacturonic acid is present in hemicelluloses obtained from this plant after prior rigorous extraction with oxalate.[17] Any pectic substance not extracted with oxalate would most probably be subsequently isolated with the hemicelluloses and would reveal its presence through the observation of galacturonic acid in an examination of the hemicellulose compositions. We conclude therefore that the oxalate extraction of galacturonan, as described, approaches completion and that the above values for pectic substance in the plant are realistic.

Acknowledgements

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References

1. Bitter, T.; Muir, H. M. *Analyt. Biochem.* **1962,** 4, 330.
2. Sadaji Furutani; Yutaka Osajima. *Kyushu Daigaku Nogakubu Gakugai Zasshi* **1965,** 22, 35. (*Chem. Abstr.* **1966,** 64, 3948a.)
3. Phaff, H. J. In *Methods in Enzymology,* Vol. 8 (E. J. Neufeld and V. Ginsburg, eds). Academic Press, New York. **1966,** p. 636.
4. McCready, R. M.; Saegmiller, C. G. *Archs Biochem. Biophys.* **1954,** 50, 440.
5. Aspinall, G. O.; Gestetner, B.; Molloy, J. A.; Uddin, M. *J. chem. Soc.* (C), **1968,** 2554.
6. Biswas, A. E.; Rao, C. V. N. *Indian. J. Chem.* **1969,** 7, 592.
7. Jones, J. K. N.; Reid, W. W. *J. chem. Soc.* **1954,** 1361; and **1955,** 1890.
8. Albersheim, P.; Bonner, J. *J. biol. Chem.* **1959,** 234, 3105.
9. McCready, R. M.; McComb, E. A. *Analyt. Chem.* **1952,** 24, 1630.
10. McComb, E. A.; McCready, R. M. *Analyt. Chem.* **1952,** 24, 1986.
11. Dische, Z. *J. biol. Chem.* **1950,** 183, 489.
12. Montreuil, J.; Spik, G. In *Microdosage des Glucides, Méthodes Colorimetriques de dosage des glucides totaux.* Faculté des Sciences de Lille, Lille, France. **1963,** p. 59.
13. Adams, G. A. In *Methods in Carbohydrate Chemistry,* Vol. 5 (R. L. Whistler, ed.). Academic Press, New York. **1965,** p. 186.
14. Alam, M.; Richards, G. N. *Aust. J. Chem.* **1971,** 24, 1041.
15. Pang Way, C.; Richards G. N. *Aust. J. Chem.* **1971,** 24, 1049.
16. Bishop, C. T. *Can. J. Chem.* **1955,** 33, 1521.
17. Blake, J. D. unpublished results.

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Relationship between Free Glutathione Content and Quality Assessment Parameters of Wheat Cultivars (*Triticum aestivum* L.)

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Freshly milled flour samples from ten cultivars of bread wheat were analysed for free reduced glutathione (GSH) and oxidised glutathione (GSSG) by an enzymic method. Dough rheological properties for each flour were assessed by standard Brabender techniques. Flour quality differences were also measured by Pelshenke fermentation and Zeleny sedimentation techniques.

Reduced glutathione levels were negatively correlated with oxidised glutathione levels, Farinogram mixing tolerance, Extensogram resistance, Pelshenke time and Zeleny volume. The ratios of oxidised to reduced glutathione (GSSG:GSH) were positively correlated with all dough and flour quality parameters, except Extensogram extensibility.

It is concluded that there are differences in the endogenous contents of reduced and oxidised glutathione between flour samples from ten cultivars of wheat. These differences may contribute significantly to variations in the quality assessment parameters established between cultivars in this study.

1. Introduction

Previous work by Frater and Hird[1] and Hird[2] on the reaction of reduced GSH and oxidised GSSG glutathione with dough prepared from heterogeneous commercial flour samples, indicated that both forms change the rheological properties of the dough. Increasing concentrations of GSH and GSSG added to dough altered its consistency as shown by decreased mixing tolerance and resistance to extension. GSH was about three times more effective than GSSG at diminishing these properties of dough. These changes are considered to be indirect evidence of thiol–disulphide exchange reactions between both GSH, GSSG and protein disulphide bonds and thiol groups, respectively.

More recently, commercial flour samples from Victorian "fair average quality" wheat have been analysed for endogenous levels of low molecular weight thiols and disulphides, in particular, GSH and GSSG, by Hird, Croker and Jones.[3] They concluded that the levels of GSH and GSSG found in commercial flours were large enough to influence the rheological properties of dough. Jones and Carnegie[4] have reported that GSSG is the only acidic disulphide peptide which is both normally present in flour and also has marked dough rheological activity.

As it is known that flour samples from different cultivars of wheat with similar protein contents can have different flour quality and dough rheological properties, it could

be expected that there might be a relation between these properties and levels of endogenous glutathione. This paper reports on the endogenous levels of free GSH and GSSG found in flour samples milled from ten spring wheat cultivars (*Triticum aestivum* L.). The quantitative importance of free glutathione is discussed in relation to Brabender, Pelshenke and Zeleny parameters, used to asses the flour quality and dough rheological properties of the cultivars studied.

2. Experimental

2.1. Field experiment

During the season 1969 to 1970, ten cultivars of wheat (*T. aestivum* L.) were grown at Longerenong Agricultural College in a randomised block experiment, replicated four times.

2.2. Cultivars

The ten cultivars selected for study were: Emblem, Falcon, Gabo, Stockade, Timgalen, Wren, Olympic, Insignia, Pinnacle and Mexico 120. All the cultivars selected are spring wheats with white kernels. The first five listed tend to have harder kernels than the others. The gluten properties vary from strong to medium-strong except for the last three cultivars, which have weak glutens.

2.3. Milling

Composite grain samples for each cultivar were prepared from the four field replicates. The grain was conditioned to 13.5% moisture content and milled on a Brabender Quadrumat experimental mill. Each sample was milled immediately before the glutathione determinations.

2.4. Enzymic estimation of reduced and oxidised glutathione

The glyoxalase method described by Davidson and Hird[5] was used for the determinations of free GSH and GSSG. The standard reagents GSH, GSSG, glutathione reductase III and $NADPH_2$ type III were obtained from Sigma Chemical Co.

2.5. Preparation of flour extracts for enzymic estimation

The extracts on freshly milled flour samples were prepared by the method described by Hird, Croker and Jones.[3] Recovery of GSSG (0.1 μmol) added to the extract ranged from 78 to 98%.

2.6. Estimation of dough rheological properties

Dough rheological properties of cultivars were determined by standard Brabender Farinograph and Extensograph techniques. Percentage water absorption and mixing resistance (b.u.) were recorded from Farinograms. Triplicate Extensograms on 150 g dough samples were produced for each cultivar after 45, 90 and 135 min rest periods, respectively. Dough resistance (b.u.) and extensibility (mm) were calculated from Extensograms rested 135 min only. The ratios of Extensogram resistance to Extensogram extensibility (r.e.r.) were then calculated.

Table 1. Analytical and quality assessment properties of ten cultivars of wheat (*T. aestivum* L.)

Cultivar	Flour protein content (%)	Ash content (%)	Water absorption (% at 500 b.u.)	Farinogram mixing tolerance (m.t.)	Extensogram[a] extensibility (e.e.)	Extensogram[a] resistance (e.r.)	Ratio e.r.:e.e. (r.e.r.)	Pelshenke (Pel.)	Zeleny (Zel.)
Stockade	11.9	0.53	64.8	415	202	229	1.13	78	46
Wren	11.7	0.49	55.5	420	214	292	1.36	79	45
Timgalen	13.7	0.55	62.2	405	211	209	0.99	53	38
Emblem	11.7	0.58	64.4	420	178	249	1.39	80	41
Gabo	12.6	0.58	64.5	430	195	253	1.29	56	40
Falcon	13.0	0.52	66.0	410	201	216	1.07	53	37
Olympic	11.5	0.44	56.0	400	200	256	1.28	60	45
Insignia	11.6	0.41	59.8	390	183	186	1.02	49	39
Pinnacle	12.4	0.39	58.7	360	224	140	0.63	56	31
Mexico 120	10.9	0.40	58.0	350	165	140	0.85	32	25

[a] Doughs pulled at 135 min.
Flours were conditioned to 12% moisture and results calculated on wet basis.

2.7. Pelshenke and Zeleny flour quality assessment

Triplicate Pelshenke[6] and Zeleny[7] tests, which are standard wheat breeders' tests for rapidly assessing flour quality, were performed on each cultivar.

3. Results

3.1. Analytical, Brabender, Pelshenke and Zeleny characteristics of wheat cultivars

Table 1 lists the flour protein, ash, water absorption, Brabender, Pelshenke and Zeleny data for each of the cultivars. These measurements have been listed in an order determined by the ranking of cultivars on their GSH contents, as shown in Table 2. Variations in the Brabender, Pelshenke and Zeleny data between cultivars are unrelated to protein percentage levels of the flour samples.

Table 2 lists the levels of free GSH and GSSG determined enzymically in flour

TABLE 2. Free glutathione measurements on ten cultivars of wheat (*T. aestivum* L.)

Cultivar	Reduced glutathione (μmol/g flour) (GSH)	Oxidised glutathione (μmol/g flour) (GSSG)	Total glutathione equivalents (TG)	Glutathione ratio GSSG:GSH (GR)	Recovery of 0.1 μmol GSSG added to extract
Stockade	0.034	0.030	0.094	0.88:1.0	92%
Wren	0.039	0.037	0.113	0.95:1.0	88%
Timgalen	0.045	0.036	0.117	0.80:1.0	84%
Emblem	0.051	0.047	0.145	0.92:1.0	97%
Gabo	0.054	0.028	0.110	0.52:1.0	90%
Falcon	0.069	0.024	0.117	0.35:1.0	78%
Olympic	0.077	0.039	0.155	0.51:1.0	98%
Insignia	0.072	0.022	0.116	0.31:1.0	93%
Pinnacle	0.088	0.014	0.116	0.16:1.0	86%
Mexico 120	0.132	0.015	0.162	0.11:1.0	78%

samples from the ten cultivars. The data are mean values from duplicate millings, extractions and determinations. GSH levels vary from 0.034 to 0.132 μmol/g flour and GSSG levels from 0.014 to 0.047 μmol/g flour. Total glutathione equivalents (TG) vary from 0.094 to 0.162 μmol/g flour and the ratio of oxidised to reduced glutathione GSSG:GSH (GR) varies from 0.11 to 0.95.

Table 3 compares the correlation coefficients between Brabender, Pelshenke and Zeleny data and free glutathione levels for the ten cultivars. GSH is negatively correlated at $P = 0.01$ with Farinogram mixing tolerance (m.t.), Pelshenke (Pel.), Zeleny sedimentation volume (Zel.) and at $P = 0.05$ with Extensogram resistance (e.r.) and GSSG. GSH is not significantly correlated with Extensogram extensibility (e.e.).

Free GSSG is positively correlated at $P = 0.01$ with e.r. and r.e.r. and at $P = 0.05$ with m.t., Pel. and Zel. It is not significantly correlated with e.e. The ratio, GSSG:GSH (GR) is positively correlated at $P = 0.01$ with m.t., e.r., Pel., Zel. and at $P = 0.05$ with r.e.r. It is not significantly correlated with e.e.

TABLE 3. Correlation coefficient matrix for free glutathione and quality assessment data on ten cultivars of wheat (*T. aestivum* L.)

Data code[a]	m.t.	e.e.	e.r.	r.e.r.	Pel.	Zel.	GSH	GSSG	GR	TG
m.t.	1.00	0.18	0.89**	0.85**	0.69*	0.83**	−0.87**	0.73*	0.78**	−0.44
e.e.		1.00	0.18	−0.16	0.35	0.31	−0.47	0.03	0.21	−0.59
e.r.			1.00	0.94**	0.73*	0.87**	−0.74*	0.82**	0.79**	−0.18
r.e.r				1.00	0.64	0.77**	−0.60	0.83**	0.72*	0.02
Pel.					1.00	0.79**	−0.80**	0.71*	0.85**	−0.37
Zel.						1.00	−0.84**	0.74*	0.77**	−0.39
GSH							1.00	−0.66*	−0.86**	0.68*
GSSG								1.00	0.85**	0.10
GR									1.00	−0.31
TG										1.00

[a] For notation see Tables 1 and 2.
*Correlation coefficient significant at $P = 0.05$.
**Correlation coefficient significant at $P = 0.01$.

4. Discussion

The significant negative correlations between free GSH levels and the quality assessment parameters provide evidence suggesting that GSH concentration may be a genetically controlled factor contributing to protein quality differences between the ten wheat cultivars studied. These results may help to elucidate more specifically the inter-relationship between thiol groups and differences in mixing "stability" and stretching "stiffness" between wheat cultivars described by Jardine, Moss and Mullaly.[8] In the present study, those cultivars which produce wheaten flour with relatively lower concentrations of free GSH have better flour and dough rheological properties than those with relatively higher GSH concentrations.

Increasing concentrations of free GSH in wheaten flour possibly facilitates an increase in the rate of breakdown of a protein disulphide network present in dough. The probable effect of this cleaving process is to increase the fluidity of the dough and, consequently, to lessen resistance to forces associated with:

(a) the pressure of carbon dioxide trapped within a Pelshenke doughball;
(b) mixing action of the blades in the Farinograph bowl;
(c) the pulling action of the descending Extensograph hook.

The range of GSSG concentrations found in this study is similar to that reported by Hird, Croker and Jones[3] and is small compared with the range of GSH concentrations. The finding that GSSG and the ratio GSSG:GSH are positively correlated with most quality assessment parameters shown in Table 3 is a statistical reflection of the negative correlations between those properties and GSH levels. It is suggested that the rheological influence of GSH and GSSG levels on dough specimens for each cultivar studied may depend on the concentration of protein disulphides and protein thiols, respectively; consequently on the rates of thiol–disulphide exchange reactions.

It is possible that the negative correlation found between GSH and GSSG may also exist between protein thiol and protein disulphide levels within the cultivars studied. Unpublished personal data of total SS:SH ratios for a limited number of Australian wheat cultivars indicates that higher total SS:SH ratios are a characteristic of varieties with better dough rheological properties.

On the evidence presented here, it is feasible that routine measurements of GSH and GSSG:GSH levels may provide the wheat breeder with a biochemical technique to assess the potential flour quality and dough rheological properties of cultivars and crossbred progenies. It is possible that optimum GSH and GSSG:GSH levels could be defined for the kind of cultivars required and utilised in wheat breeding programmes.

Finally, total glutathione equivalents (TG), shown in Table 3, are negatively but not significantly correlated with all quality assessment data. Of the various dough rheological parameters studied in this investigation, Extensogram extensibility (e.e.) is the only one found not to be significantly correlated with any free glutathione determinations. In terms of glutathione levels, e.e. does not appear to be a variety-dependent parameter.

5. Conclusions

It is concluded that there are differences in the endogenous contents of reduced and oxidised glutathione between flour samples from ten cultivars of wheat. These differences

may contribute significantly to variations in the Brabender, Pelshenke and Zeleny results found between the cultivars studied.

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References

1. Frater, R.; Hird, F. J. R. *Biochem. J.* **1963**, 88, 100.
2. Hird, F. J. R. *Proc. Int. Wheat Genet. Symp. Hereditas Suppl.* **1966**, 2, 29.
3. Hird, F. J. R.; Croker, I. W. D.; Jones, W. L. *J. Sci. Fd Agric.* **1969**, 19, 602.
4. Jones, I. K.; Carnegie, P. R. *J. Sci. Fd Agric.* **1969**, 20, 60.
5. Davidson, B. E.; Hird, F. J. R. *Biochem. J.* **1964**, 93, 232.
6. Pelshenke, P. *Getreide Mehl Brot* **1948**, 19, 119.
7. Zeleny, L. *Cereal Chem.* **1947**, 24, 465.
8. Jardine, R.; Moss, H. J.; Mullaly, J. V. *Aust. J. agric. Res.* **1963**, 14, 603.

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Fatty Acid Composition of Eight Species of Indian Marine Fish

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Tropical fish oils were found to be relatively saturated. The species examined showed wide variations in myristic acid (2 to 11.3), palmitic (20 to 35) stearic (7 to 16), $C_{18:1}$ (7.9 to 24), $C_{20:5}$ (1 to 11) and $C_{22:6}$ (2 to 10). Mussel lipids and lipids of mullet contained very high percentages of odd numbered fatty acids.

1. Introduction

Considerable data[1–7] are available on the fatty acid composition of the lipids present in cold water fish species, while very little published literature is available on the types and composition of fatty acids present in lipids of warm water species. The present communication aims at filling this gap in our knowledge and reports in detail on the fatty acids present in the body oil of 6 species of fish, one sample of liver oil and the lipids of the shell fish. A comparative study was also undertaken of the fatty acid composition of black and white muscle in the fish seer.

2. Experimental

Lipids were quantitatively extracted from the fish samples with a chloroform–methanol mixture (2:1 v/v) by the method of Bligh and Dyer.[8] The chloroform extracts were washed twice following the washing procedure of Folch, Lees and Sloane Stanley.[9]

2.1. Preparation of methyl esters

The lipids were saponified by the official methods of the American Oil Chemists' Society. The fatty acids recovered were esterified with methanol–HCl reagent. Addition of a drop of 2,2′-dimethoxypropane was found to give very rapid and quantitative conversion of fatty acids to methyl esters.

2.2. Gas–liquid chromatography

Gas–Liquid chromatographic analyses were carried out on a gas-chromatograph F and M model 1609, equipped with a flame ionisation detector and a Honeywell strip chart Recorder (3 mV). A stainless steel column, 6 ft × 3/16 in (≈0.9 m × 4.8 mm), packed with Chromosorb W (45 to 60 mesh) coated with 15% DEGS was used. Nitrogen was used as the carrier gas.

The operating conditions were as follows.

Column temperature, 200 °C; injection port temperature, 300 °C; detector port temperature, 300 °C; nitrogen, 120 ml/min; hydrogen, 35 ml/min; air, 350 ml/min.

Methyl esters of fish oils diluted in chloroform were injected with a Hamilton 10-μl microsyringe. Two analyses were carried out, 3.5 μl at 8 × 1000 attenuation and 2 μl at 4 × 100. Results given are those at 4 × 1000 attentuation. Reference standards were injected at 2 × 1000 attenuation.

2.3. Reference standards

2.3.1. Unsaturated fatty acid methyl esters

The following National Institutes of Health standards were used. Methyl 11-octadecenoate (99 %), methyl 12-octadecenoate (96.6 %), methyl nervonate ($C_{24:1}$, :99 %), methyl-5,8,11,14,17-eicosapentaenoate (94.6 %), methyl petroselinate ($C_{18:1}$, :99 %), methyl erucate ($C_{22:1}$, :99 %), methyl 11-eicosaenoate (99 %) and methyl 4,7,10,13,16,19-docosahexaenoate (96.4 %) purity based on g.l.c. analysis, prepared by Hormel Institute (courtesy of the National Institutes of Health, U.S.A.).

TABLE 1. Lipid content of fish species analysed

Common name	Species	Lipid content (g/100 g wet muscle)
1. *Lactarius*	*Lactarius lactarius*	5.2
2. *Caranx*	*Caranx kalla*	2.2
3. Malabar sole	*Cynoglossus semifasciatus*	2.0
4. Seer black		18.0
white	*Scomberomorous guttatum*	13.0
5. Tuna	*Katzuwonus pelamis*	0.8
6. Mullet	*Mugil passia*	3.0
7. Mussels	*Mytilus edulis*	2.7

TABLE 2. Fatty acid composition of eight

Name of species	$C_{12:0}$	$C_{13:?}$	$C_{14:0}$	$C_{14:1}$	$C_{15:0}$	$C_{15:1}$	$C_{16:0}$	$C_{16:1}$	$C_{17}{}^{a}{}_{:0}$	$C_{17:1}$	$C_{18:0}$
1. *Lactarius*	—	—	5.2	0.6	1.0	0.1	35.5	8.0	1.3	0.6	11 3
2. *Caranx*	2.1	0.6	8.7	0.3	1.1	0.6	33.8	5.7	1.7	0.7	15.1
3. Sole	—	—	9.6	0.5	1.6	—	32.3	9.0	2.8	0.8	14.0
4. Seer white	0.3	0.1	10.4	0.4	1.5	—	26.0	11.2	3.1	1.8	9.3
black	—	—	11.3	0.4	1.0	—	28.0	10.7	2.0	1.9	9.8
5. Tuna, white	0.1	0.1	2.0	0.9	1.4	—	30.5	2.5	2.1	1.0	16.6
6. Mullet	—	0.2	6.9	4.6	1.1	—	28.3	10.6	1.3	5.2	8.5
7. Ray liver oil	—	—	3.6	0.3	1.0	+	33.7	15.5	2.1	1.3	10.1
8. Mussels	—	—	9.1	3.4	—	—	30.5	9.3	2.2	2.0	7.0

[a] Combines critical pair $C_{16:2}$ and $C_{17:0}$.

2.3.2. Saturated fatty acid methyl esters

C_{12}, C_{13}, C_{14}, C_{16}, C_{17} and C_{18} A. G. Fluka Switzerland.

Fatty acids of unknown samples were identified by comparison with the retention times of reference standards. Fatty acids whose standards were unavailable were identified tentatively. The peak areas were determined by triangulation and corrected to give weight per cent composition by the procedure of Ackman and Sipos.[10] The probable errors are for major components <5%, low to medium range components <10% and up to 50% of the minor components.

3. Results and discussion

Lipid contents of fish species analysed are given in Table 1. The fatty acid composition of the lipids of 6 species of marine fish, one liver oil and one shell fish are listed in Table 2. These species are of much commercial importance to India. The data clearly confirm our earlier observations[11,12] that tropical fish oils are relatively saturated. The important fatty acids showing wide variations among the species examined are myristic acid, from 2 to 11.3; palmitic acid, 20 to 35; stearic acid, 7 to 16; $C_{18:1}$, 7.9 to 24; $C_{20:5}$, 1 to 11; $C_{22:6}$, 2 to 10.

Another outstanding feature is that the completely saturated acids range from 46.1 to 62.5% of the total fish oils. The main peculiarities of these oils are the high content of stearic acid and generally low content of long chain polyenes. Tuna lipids recorded the highest amount of stearic acid, namely 16.6%.

Odd numbered acids C_{15}, C_{17} are present in most species examined. Mussel lipid showed the highest level of C_{17} acids, $C_{17:0}$ 2.2% and $C_{17:1}$, 2%. Mullet lipid contained C_{15}, C_{17} and C_{19} odd numbered acids, $C_{17:1}$ recording the maximum amount of 5.2%. Preponderance of odd numbered fatty acids in the body oil of mullet has been reported by a number of workers[1,6,7]. Both mullet oil and mussel lipid contained very low levels of $C_{18:1}$ and high levels of $C_{16:1}$ acid. *Caranx*, *Lactarius*, sole and tuna lipids contained docosahexaenoic acid to a comparatively lesser extent. In tuna lipids, this acid is found to be replaced by eicosa pentaenoic acid, which is present to the extent of 10.2%. In most species of fish examined, $C_{20:5}$ was predominant, with a corresponding decrease

species of marine lipids—carbon numbers

$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{18:4}$	C_{19}	$C_{20:1}$	$C_{20:2}$	$C_{20:3}$	$C_{20:4}$	$C_{20:5}$	$C_{22:1}$	$C_{22:4}$	$C_{22:5}$	$C_{22:6}$	$C_{24:1}$
24.2	1.6	0.3	0.4	—	0.2	—	—	0.1	4.4	0.2	0.5	0.5	4.0	—
15.5	1.4	0.7	1.5	—	0.4	—	—	0.2	5.0	—	—	0.9	4.0	—
19.0	1.5	0.7	4.7	—	—	—	—	—	1.5	—	—	0.1	2.0	—
15.0	2.4	1.5	0.9	—	2.0	0.4	0.4	0.3	3.5	0.5	—	0.5	8.2	0.3
13.6	3.6	1.6	0.7	—	1.0	—	0.4	0.2	3.2	—	—	0.1	10.5	—
24.0	0.6	0.2	0.7	—	0.9	—	—	0.9	10.2	—	1.1	1.0	3.2	—
8.7	—	1.6	2.0	2.9	1.5	1.0	—	—	3.0	0.3	0.3	1.0	9.5	1.5
22.5	1.8	0.4	0.9	—	0.3	—	—	0.2	2.5	—	0.4	0.2	3.1	—
7.9	3.9	0.6	5.7	—	0.5	—	—	3.6	4.3	—	0.7	0.8	8.5	—

in $C_{22:6}$ concentration. This should be characteristic of most tropical species of fish, although no earlier data are available. The metabolic pathways affecting polyunsaturated fatty acids in marine organisms are apparently the same as in mammals.[13, 14] It is well known that phytoplankton produce the higher polyunsaturated fatty acids characteristic of fish oils [13, 15], as well as the shorter fatty acids, 18:2 ω 6, 18:3 ω 6 and 18:3 ω 3. These latter acids are usually found in fish oils to a lesser degree for they are further converted by zooplankton into highly unsaturated fatty acids of chain length C_{20} and C_{22}.[13] Fish were shown to be capable of the same synthesis, if necessary[14, 16] It has been pointed out by Lovern[17] that the fatty acid composition of stored fat of fish is affected by feeding habits, sexual maturity and spawning. Earlier studies have shown that at lower temperatures $C_{22:6}$ ω 3 is more essential than $C_{20:5}$ ω 3^6.[18] Rieser *et al.*[6] also showed that conversion of 18:2 ω 6 and 18:3 ω 3 acids to longer chain, more unsaturated fatty acids in the mullet is depended on environment. No such data are available on tropical species. The significance of high amounts of 18:0 and 18:1 ω g acids observed in warm waters cannot be correlated with any of these observations. Recently Kemp and Smith[19] showed that raising the adaptation temperature by 20 °C halved the percentage of $C_{20:1}$, $C_{20:4}$ and $C_{22:6}$ fatty acids in the lipids of goldfish intestinal mucosa. Fatty acid composition of the black and white muscle of seer is almost identical, except that the dark muscle is slightly richer in $C_{14:0}$ and $C_{22:6}$ acids.

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References

1. Gruger, E. H., Jr.; Nelson, R. W.; Stansby, M. E. *J. Am. Oil Chem. Soc.* **1964**, 41, 662.
2. Ackman, R. G.; Eaton, C. A.; Ke, P. J. *J. Fish. Res. Bd Can.* **1967**, 24 (12), 2563.
3. Ackman, R. G.; Burgher, R. D. *Can. J. Biochem. Physiol.* **1963**, 41, 2501.
4. Ackman, R. G.; Sipos, J. C. *J. Fish. Res. Bd Can.* **1964**, 21 (4), 841.
5. Ackman, R. G.; Sipos, J. C. *Comp. Biochem. Physiol.* **1964**, 15, 445.
6. Reiser, R.; Stevenson, B.; Kayama, M.; Choudhury, R. B. R.; Wood, D. W. *J. Am. Oil Chem. Soc.* **1963**, 40, 507.
7. Sen, N.; Schlenk, H. *J. Am Oil Chem. Soc.* **1964**, 41, 241.
8. Bligh, E. G.; Dyer, W. J. *Can. J. Biochem. Physiol.* **1959**, 37, 911.
9. Folch, J.; Lees, M.; Sloane Stanley, G. H. *J. biol. Chem.* **1957**, 226, 497.
10. Ackman, R. G.; Sipos, J. C. *J. Am. Oil Chem. Soc.* **1964**, 41, 377.
11. Gopakumar, K.; Rajendranathan Nair, M. *Indian J. Biochem.* **1967**, 4, 229.
12. Gopakumar, K.; Rajendranathan Nair, M. *Fish Technol.* **1966**, 3, 21.
13. Kayama, M. *Bull. Jap. Soc. scient. Fish.* **1964**, 30, 647.
14. Kayama, M.; Tsuchiya, Y.; Nevenzel, J. C.; Fulco, A.; Mead J. F. *J. Am. Oil Chem. Soc.* **1963**, 40, 499.
15. Ackman, R. G.; Jangaard, P. M.; Hoyle, R. J.; Brockerhoff, H. *J. Fish. Res. Bd Can.* **1964**, 21 (4), 747.
16. Ackman, R. G. *J. Fish. Res. Bd Can.* **1964**, 21 (2), 247.
17. Lovern, J. A. *A. Rev. Oceanogr. mar. Biol.* **1964**, 2, 169.
18. Kayama, M.; Tsuchiya, Y.; Mead, J. F. *Bull. Jap. Soc. scient. Fish.* **1963a**, 29, 452.
19. Kemp, P.; Smith, M. W. *Biochem. J.* **1970**, 117, 9.

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Amino Acid Pattern of the Egyptian Apricot Fruits (Hamawy)

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Apricot fruits were subjected to acid hydrolysis and the amino acids were separated and quantitatively determined by paper chromatography. The analysis revealed the presence of 19 amino acids, predominantly glutamic acid, aspartic acid, histidine, arginine and tyrosine. The results also demonstrated that the concentrations of cystine, methionine and hydroxyproline were relatively small.

1. Introduction

Apricot, *Prunus armeniaca*, Hamawy variety is the most important stone fruit, which is widely cultivated in Egypt. Most of the previous studies were concerned with the type and concentrations of carbohydrates,[1-4] organic acids[5] and carotenoids[4,6] of the apricot fruit. Several recent literature reports indicate that virtually no data are available concerning the amino acid content of the apricot fruits (Hamawy variety). The purpose of the present work is to study the amino acid patterns, since they play an important role in nutrition and in the darkening of apricot products during processing and subsequent storage.

2. Materials and methods

2.1. Sampling

One kg of fresh apricot fruit (*Prunus armeniaca*) of Hamawy variety, grown in Bahareya Oasis was used in this study. The stones were removed and the fruits were blended and kept for analysis.

2.2. Methods of analysis

2.2.1. Determination of the total amino acids

A known weight of the edible portion was hydrolysed with HCl (6 N) for 24 h at 110 °C, according to the Khan and Baker method.[7] The HCl was eliminated by evaporation under vacuum to near dryness and finally made up to 10 ml with isopropyl alcohol solution (10%). Portions were spotted on Whatman No. 1 filter paper and two dimensional ascending chromatograms were carried out using the buffered method given by Levy and Chung,[8] and using *n*-butanol–acetic acid–water (12:3:5 v/v) as the first solvent for 24 h at 20 °C, followed by the *m*-cresol–phenol mixture (2:1) in presence of borate buffer (pH 8.3) for 30 h.

After development with the alcoholic ninhydrin reagent (0.2%) and the identification

of the amino acids, their quantitative estimation was carried out cutting the coloured dotted areas of each individual amino acid and eluting with aqueous ethanol (75% containing copper nitrate). The concentrations were determined colorimetrically at 510 nm.

The proline content was located with the isatin reagent (0.2% in acetone) and eluted with acetone–water mixture (2:1). The colour was determined at 570 nm.

2.2.2. Estimation of cysteine and methionine[9]

A quantity of the sample was weighed in a conical flask and then placed in an ice bath and cooled to 0 °C. Cold performic acid reagent was added to the sample and oxidation was allowed to continue at 0 °C for 16 h. The solution was evaporated under vacuum at 30 to 35 °C, after which it was hydrolysed by the HCl solution (6 N) for 24 h. The hydrochloric acid was removed by repeated concentration in a rotatory evaporator and the hydrolysate was finally made up to a known volume with isopropyl alcohol 10% and cysteine and methionine were determined by the paper chromatography technique above.

2.2.3. Determination of tryptophan[10]

The tryptophan content was determined colorimetrically after alkali hydrolysis with barium hydroxide solution (14%) for 22 h.

All the amino acids were calculated from prepared standard curves.

3. Results and discussion

This investigation was carried out to determine the amino acid contents of apricot fruits since data on the amino acid content and biological value are commonly used to assess nutritive quality of the protein foods.

Apricot protein content, which was 6.65% as shown in Table 1 was subjected to acid hydrolysis.

TABLE 1. Moisture and total protein contents of the Egyptian apricot fruits (Hamawy variety)

Chemical analysis	%
Moisture[a]	83.94
Total protein[b]	6.65

[a] Moisture was determined at 70 °C under vacuum.
[b] Total protein was estimated by Kjeldahl method.

After the identification of the amino acids, each individual amino acid was quantitatively determined and the concentrations were calculated as mg/g nitrogen as illustrated in Table 2.

TABLE 2. Total amino acids content of the Egyptian apricot fruits (dry basis)

Amino acids	mg/100 g	mg/g nitrogen
Aspartic acid	180	187
Glutamic acid	280	292
Serine	75	78
Glycine	68	71
Threonine	44	46
Alanine	86	90
Tyrosine	88	92
Valine	40	42
Leucine and isoleucine	65	68
Phenylalanine	35	36
Lysine	56	58
Arginine	94	100
Histidine	101	105
Methionine	24	25
Tryptophan	50	52
Hydroxyproline	18	19
Proline	42	44
Cysteine	20	21

From these results, it is quite certain that apricot fruits contain 19 amino acids. Glutamic acid and aspartic acid are the predominant amino acids, followed by histidine, arginine, tyrosine, alanine, serine, glycine, leucine and isoleucine, lysine, tryptophan, threonine, valine, proline and phenylalanine. The values for hydroxyproline showed that it was present in smaller amounts. On the other hand, some of the essential amino acids such as histidine, arginine, the leucines, lysine and tryptophan were found in large quantities when compared with the other amino acids presented in Table 2. From the same table it can be seen that apricot fruits are poor sources for the sulphur-containing amino acids (cysteine and methionine). It is interesting to note from these findings that most of the essential amino acids were found in low levels. However, the daily eating of this common fruit, together with various other protein sources may meet the essential amino acids in the proportion required.

References

1. Jacob, M. B. *The Chemistry and Technology Food and Food Products* Vol. 1. Interscience Publishers, New York. **1951**.
2. Ash, A. S. F.; Reynolds, T. M. *Aust. J. Chem.* **1955**, 8, 444.
3. Joslyn, M. A. *Methods of Food Analysis, Applied to Plant Products* Academic Press, New York. **1950**.
4. Sarhan, M. A. I. Studies on the production of Apricot Juice M.Sc. Thesis, Cairo University. **1970**.
5. Souty, M.; Andre, P.; Poggi, A. *Industrie aliment. Agricoles* **1969**, 3, 391.
6. Sandana, J. C.; Ahmed, B. *J. scient. ind. Res.* **1949**, 8 2, 35.
7. Khan, N. A.; Baker, B. E. *J. agric. Fd Chem.* **1955**, 3, 853.
8. Levy, A. L.; Chung, D. *Analyt. Chem.* **1953**, 25, 396.
9. Jamalian, J.; Pellet, P. L. *J. Sci. Fd Agric.* **1968**, 19, 378.
10. Lombard, J. H.; Delange, D. J. *Analyt. Biochem.* **1965**, 10, 2601.

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The Potassium Requirement of Crops Grown in Rotation

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A 5-year trial measured the effect of varying rates of potassium fertiliser applied in combination with two rates of nitrogen to a rotation: potatoes, kale, barley, grass cut for conservation and wheat. Residual values of potassium applied during the rotation were measured on a crop of spring wheat planted in the sixth year.

Crops grown in the rotation responded increasingly to potassium as follows: kale, barley, grass, wheat, potatoes. The higher rate of potassium applied (706 lb/acre[a] K in 5 years) was justified by the total value of crops harvested; this return was derived mainly from potatoes and wheat.

The higher rate of potassium applied was insufficient to maintain the soil in K balance (applications less removal in crops) when the higher rate of nitrogen was used. Soil analysis indicated no residues from the lower rate of K applied but the higher rate raised available K levels appreciably. The wheat crop grown after 5 years under the rotation showed residues from the lower rate of K applied equivalent to 55 lb/acre fresh K but no additional residue from the higher rate.

1. Introduction

Fertiliser recommendations are mainly based upon the results of field experiments measuring responses to applied nutrients by individual crops, but adherence to standard recommendations[1] may sometimes result in a loss of potassium from the soil over a period of time. Some of this deficit may be made good by release of K from the soil, which can be considerable,[2–4] but long-term work on different soils is required to measure the effects of changes in K balance on the cropping potential of soils.The object of the experiment described here was to measure the effects on yield of crops grown in rotation of run-down of soil K by including a treatment receiving no K, and of build-up by comparing generous applications with a more conservative policy, and to measure K effects at two levels of N.

2. Experimental

The experiment was laid down in Spring 1965 on the farm of the Cheshire College of Agriculture on a gently undulating site with a variable soil including gley podzol of the Reaseheath series and gleyed brown earth of the Stockbridge series.

The order of crops in the rotation and rates of K and N fertilisers applied are shown

[a] Throughout this paper 1 lb ≈ 0.45 kg, 1 acre ≈ 0.405 ha.

in Table 1. Each phase of the rotation was represented by one series of plots in each season, N being applied to whole plots of these series and potassium to subplots. Subplots measured 45 × 40 ft[a], large enough to allow working with normal farm

TABLE 1. Fertilisers applied to crops grown in rotation

Treatment	lb N/acre (ammonium nitrate 34.5% N) n_1	n_2	lb K/acre (muriate of potash 60% K_2O) k_0	k_1	k_2	lb P/acre (triple supers)
Potatoes	84	168	0	112	224	58
Kale	67	134	0	37	74	29
Barley	0[a]	45	0	28	56	22
Ryegrass	45	90	0	37	74	73
	(For each of 4 cuts, 1st application in seedbed)					(in seedbed)
Wheat	45	90	0	28	56	22
Total	376	797	0	353	706	204

[a] 45 and 90 lb N applied to barley in 1965 to 1966.

machinery, working always along the length of the plots to minimise spread of fertilisers between treatments. All fertilisers were applied broadcast by hand. Sub samples for determination of dry matter and K content were taken at harvest from 1966 onwards. Sampling of cereals and grass presented no problems and good agreement was obtained between duplicates, but results with potatoes and, particularly, with kale were sometimes erratic.

Establishment and growth of crops was satisfactory, with the following exceptions. No yields were recorded from wheat in 1965 or from barley in 1965 and 1966 due to severe lodging. As a result N dressings for barley were reduced from 1967.

Kale was sown very late in 1966 and yielded poorly. No kale was sown in 1969, though fertilisers were applied. Grass was sown late in 1969 and established with difficulty suffering from weed competition and only one cut could be recorded.

The potato crop planted late in 1969 was virtually a failure.

Leaf and soil analysis confirmed suspected Mg deficiency in cereals in 1967 and 5 cwt[b]/ acre kieserite was applied to the whole area in January 1968.

It was originally intended that the experiment should continue for at least 10 years, but supervision difficulties from 1969 made this impracticable and it was decided to crop the whole area in 1970 with spring wheat to measure residual effects of fertilisers applied to the rotation. Fresh K dressings at 55 lbK/acre were applied by machine to half series, so that the residual effects could be measured in the presence and absence of fresh K. Uniform dressings of 22 lb and 45 lb N per acre were applied in the seedbed and as a top dressing, respectively.

[a] Throughout this paper 1 ft ≈ 0.3 m.
[b] Throughout this paper 1 cwt ≈ 51 kg.

3. Results

3.1. Yields of crops grown in rotation

Conclusions can be based only on the following crops; grass, kale and potatoes 1965, 66, 67 and 68; wheat 1966, 67, 68 and 69; barley 1967, 68 and 69. The yields have been subjected to statistical analysis using as an estimate of "error" the treatment × season interactions. Individual annual yields are given in Appendix Table A1; mean yields and effects are summarised in Table 2.

TABLE 2. Mean yields and responses by crops grown in rotation (cwt/acre)

Crop	Mean yield	Effects				
		$k_1 - k_0$	$k_2 - k_1$	S.E.	$n_2 - n_1$	S.E.
Potatoes	282	89	33	35.2	33	8.7
Ryegrass	78.7	5.8	2.6	3.9	15.5	1.7
Wheat grain	30.0	5.6	2.1	2.7	−3.3	2.3
Wheat straw	37	19	9	6.7	1	3.6
Barley grain	36.8	0.2	2.7	2.6	4.4	2.3
Barley straw	28	2	1	6.8	4	0.6
Kale	428	19	1	30.5	67	36

3.2. Potatoes

K and N both increased yield, the K response increasing with lapse of time. The K response increased sharply in 1967 when, for the first time, potatoes were planted on land which had previously carried grass.

3.3. Wheat

Wheat showed large responses to K, averaging 7½ cwt/acre/year ($k_2 - k_0$). The N rates chosen were too high under the conditions of the experiment, extra N depressing the yield except in 1966. There was no significant N × K interaction, but there are indications that it was negative and quite large in those seasons where high N was most damaging. Straw yields were available from three seasons only; K increased straw yield, all other effects being negligible.

3.4. Barley

None of the treatment effects reached the level of significance in respect of grain yield. Apparent responses of 4 cwt grain to 45 lb/acre N and of 3 cwt to the higher rate of K were recorded. N increased straw yield by 4 cwt/acre.

3.5. Kale

Experimental error was high and no treatment effects were significant.

3.6. Grass

Grass was more responsive to N than other crops and only moderately responsive to K, 8 cwt dry matter per acre per annum when K was applied at the higher rate. The variation in fertiliser effects between cuts has been examined using data for the years 1966 to 1969, see Table 3,

TABLE 3. Grass: mean fertiliser response per cut, seasons 1966 to 1968 (cwt per acre dry matter)

Cut	Mean yield			Effects		
		$k_1 - k_0$	$k_2 - k_1$	S.E.	$n_2 - n_1$	S.E.
June	14.0	1.9	1.2	0.88	3.5	1.17
July	21.5	2.3	0.6	0.88	5.1	1.17
August	18.1	0.7	Nil	0.88	1.0	1.17
September	20.8	1.8	Nil	0.88	6.7	1.17
Annual total	74.4	6.7	1.8	1.78	16.3	2.34

Both N and K effects were small in the third cut taken at the beginning of August; K effects were largest in the first cut of the year and N was most effective at the last cut, taken, usually, at the end of September. It is possible that Mg deficiency limited response to K in 1967; visual symptoms were seen on barley, though grain yields did not appear to suffer, and available Mg was low on the series carrying grass.

3.7. Potassium uptake in crops

Uptake data are available from all crops harvested from 1966 onwards. Table 4 lists mean K removals in recorded crops and it is seen from this that removals could exceed

TABLE 4. Average K uptakes by crops (derived from crops for which full data are available) (lb K per acre)

Crop			Treatment			
	n_1k_0	n_1k_1	n_1k_2	n_2k_0	n_2k_1	n_2k_2
Grass (1966, 67, 68)	144	223	257	156	262	337
Potatoes (1966, 67, 68)	90	168	192	88	182	216
[a]Kale (1966, 68)	202	206	230	142	159	226
Wheat (1967, 68, 69)	20	29	47	18	30	36
Barley (1967, 68, 69)	23	27	30	27	28	33
Total for rotation	479	653	756	431	661	848
K applied	—	353	706	—	353	706
Balance	−479	−300	−40	−431	−308	−142

[a] 1967 crop, which yielded poorly, excluded.

total applications in the rotation even at the higher rate of K application, particularly where much N is used.

3.8. Soil analysis

Available K and Mg were determined by routine methods in use by N.A.A.S. in samples taken from all plots in March of 1966, 67, 68 and 69. Results for available K are shown in Table 5. There was no effect of treatment on available K in the first 2 years. In 1968 and 69, while the k_1 treatment did not raise soil K above that of the control plots, k_2 increased it by about 20 parts/million. Increasing N application tended to reduce available K (by 6 parts/million in 1969). Available soil Mg was not affected by treatment. The effect of 5 cwt/acre kieserite applied in January 1968 was short lived,

available soil Mg being 196 parts/million shortly after Mg was applied and 69 parts/million only a year later, indicating considerable leaching.

TABLE 5. Effect of treatment on soil potassium

Year	Mean of n_1 and n_2 treatments parts/million K K treatment k_0	k_1	k_2	S.E.
1966	40.1	46.9	47.1	3.07
1967	51.8	50.3	49.5	4.30
1968	47.3	49.4	68.0	1.92
1969	51.0	51.3	70.0	2.34

3.9. Residual test

The difficulties experienced in 1969 resulted in an anomalous situation with respect to the comparison between series, by omission of the kale crop, and the lower offtakes of K in grass (an unrecorded amount in weeds removed from the plots plus one cut only of grass) and in potatoes. This may have resulted in yield variation between series greater than would normally have been expected had cropping been normal in 1969. Establishment of wheat was uneven, particularly on series 2 where it followed barley, leading to ingress of weeds. That there would be great yield differences between series was evident in observations from an early stage in the growth of the crop, and preceding crop had a far greater effect on the yield of wheat than any fertiliser treatment.

The yield data were statistically analysed, comparing treatment effects with those treatment × series interactions which were relatively small. For testing the effects of fresh K and residual N, all the appropriate treatment × series interactions were used with, respectively, 4 and 8 degrees of freedom. For testing effects of residual K and its interactions with fresh K and residual N, it was found that interactions of series with residual K and residual N × K interaction were large, consequently these were excluded from the estimate of "error" which was made up from the remaining series × treatment interactions (16 d.f. in all).

TABLE 6. Residual test. Spring wheat 1970, mean yields and effects (cwt/acre grain at 85% dry matter)

Series and preceding crop	Mean yield	Fresh K 55 lb k/acre	$k_1 - k_0$	Residual effects $k_2 - k_0$	$N \times K^a$	$n_2 - n_1$
1. Wheat	20.7	4.0	2.7	3.1	−5.6	−1.7
2. Barley	19.9	0.6	−0.6	−2.6	6.4	−1.0
3. Potatoes	29.8	1.8	8.0	3.2	4.6	1.0
4. Ryegrass	24.0	1.0	2.7	2.5	7.2	−0.4
5. Fallow[b]	34.5	3.3	−1.1	4.5	−0.6	−5.9
Mean	25.8	2.1	2.3	2.1	2.4	−1.6
S.E.		0.62	0.65		0.92	0.69

[a] $n_2 - n_1 \times k_2 - k_0$.
[b] Fertilisers applied for kale 1969, but no crop planted.

Main features of the results are shown in Table 6. Fresh K increased yield by approximately 2 cwt/acre grain, residual K increased yield but the response varied greatly from series to series and also according to rate of N applied to the rotation. There was, surprisingly, no interaction between residual and fresh K. The very large N × K interaction on series 4 was due to the low yield of the n_1k_2 plots, an area which had consistently yielded below expectation in the past but this was the only case where what appeared to be abnormal behaviour in the 1970 crop had any historical precedent.

4. Discussion

Costing the K applied at 2.66 p per lb K (£25 per ton for muriate of potash, 60% K_2O) and valuing potatoes at £12, wheat and barley at £25, grass dry matter at £25 and fresh kale at £1.25 per ton, the application of 353 lb K/acre (cost £9.39) to the five crops yielded £61 margin; applying a further 353 lb K gave an additional margin of £22 but this came almost entirely from potatoes and wheat. Kale did not respond and though grass responded up to the higher rate applied, the increase from the second increment was small, about 2½ cwt d.m., which would not quite cover the cost of the extra fertiliser. A better financial return might have been obtained by using the higher rate of K for potatoes and wheat only and applying the lower rate for the less responsive crops, or by a different distribution of the same total amount of K among the crops of the rotation. To apply the greater part of the K requirement of the rotation for the most responsive crops would agree with the findings of Clarke and Smith[5] in experiments to measure the residual effects of fertilisers.

The contrast between wheat and barley is interesting. It is usual to generalise advice for P and K manuring of cereals[1] but most of the data upon which recommendations are based come from experiments on spring barley. The effect of K removal by a preceding grass crop on the K response of wheat is well known.[6] In this experiment barley followed kale which was as exhaustive of soil K as grass and it is suggested that the K requirements of wheat and barley differ.

Table 4 shows that even when the higher rate of K was applied, there could have been a nett loss of K from the soil if N was applied generously, demonstrating the difficulty of maintaining soil K in balance when the rotation includes extractive crops which are cut and removed. The experimental rotation with grass and kale both cut and removed was particularly extractive and is not likely to be found in practice, where, if kale were grown, it would not occupy as much as one fifth of the area under crops and might, more likely, be fed off in the field so that its K content would be returned to the soil. Had kale been excluded from the rotation, there would have been a small positive K balance even at the high rate of N.

This experiment demonstrated that it is difficult to maintain soil K under cut grass, a problem to which attention has been drawn before.[7-9] K was inefficiently used since applying increasing dressings had a large effect in increasing K content of herbage and a relatively smaller effect in increasing yield. From 1966 to 1969, applying 147 lb K/acre at the high rate of N increased grass yield by 8.7 cwt d.m. per acre and K uptake by 106 lb; applying a further 147 lb increased K uptake by 75 lb and yield by only 2.8 cwt/acre, while K removals in grass still exceeded additions by 43 lb/acre/annum. Table 7 shows that fertiliser K was less efficiently used in late cuts.

This suggests that K for grass should be applied early and is contrary to previous findings on the same farm when K applied in summer and autumn was particularly effective,[10] but it seems that in this experiment K utilisation by the rotation as a whole would have been improved by applying less than the higher rate to grass, applying it for the early cuts only, and increasing applications for the following wheat and potatoes to restore the balance in the soil.

TABLE 7. Effect of time of cutting on efficiency of utilisation of K by grass

	Effect of potassium ($k_2 - k_1$): mean of n_1 and n_2 treatments	
	Yield cwt d.m./acre	K uptake lb/acre
Early cuts	6.0	69
Late cuts	2.5	78

Only the higher rate of K fertiliser increased available K in the soil above the level in the control plots and the differences in K balance between treatments were not well reflected in soil analysis. Soluble K did not decline in the period 1966 to 1969 on plots receiving no K fertiliser, though the crops harvested had removed from 300 to 400 lb K/acre. This suggests considerable release of K from soil minerals and Dr M. A. Jones of the Department of Soil Science, University of Newcastle-upon-Tyne, who examined samples from the site, reported that the fine sand fraction of the soil contained 10% feldspar which could release significant amounts of K. Some K originating from lower levels in the soil might have been accumulated in the surface by decaying roots.

The results obtained with the wheat crop planted in 1970 to test residual values were inconclusive because of the large effect of cropping sequence on yield and response to residual K. Soil analysis results suggested that useful residues from applied K would only be found at the higher level of treatment, though it must be emphasised that a full cropping year had elapsed between taking the soil samples and the planting of wheat in 1970. However, though there was an indication of useful K residues from the lower rate applied to the rotation, there was no further response to residual K above that level. This is surprising because wheat grown in the rotation had been responsive to K.

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Appendix

TABLE A1. Annual yields at varying rates of potassium (mean of n_1 and n_2) and mean effect of N (cwt/acre)

Crop	Year	Treatment k_0	k_1	k_2	$n_2 - n_1$
Potatoes (total tubers)	1965	243	300	332	37
	1966	284	282	318	56
	1967	164	335	397	25
	1968	156	287	290	16
	Mean	212	301	334	33
	1969	146	106	175	−63
Ryegrass (d. m.)	1965	87.4	90.7	95.7	13.3
	1966	80.7	77.3	85.0	13.1
	1967	54.2	72.7	64.4	15.6
	1968	73.3	78.1	84.3	20.3
	Mean	73.9	79.7	82.3	15.5
	1969 (1 cut)	15.7	17.3	15.7	4.9
Wheat grain (85% d.m.)	1966	27.3	35.2	36.1	0.3
	1967	32.6	39.9	43.4	−0.2
	1968	22.0	26.3	26.8	−9.6
	1969	20.3	23.3	26.8	−3.7
	Mean	25.6	31.2	33.3	−3.3
Wheat straw (85% d.m.)	1967	34	54	66	8
	1968	27	46	50	−1
	1969	15	16	24	−4
	Mean	25	38	47	1
Barley grain (85% d.m.)	1967	38.3	42.8	43.2	4.8
	1968	44.2	40.5	40.9	1.7
	1969	25.3	25.1	31.5	8.1
	Mean	35.9	36.1	38.8	4.4
Barley straw	1967	24	34	33	5
	1968	37	39	38	5
	1969	16	13	17	3
	Mean	26	28	29	4
Kale (fresh weight)	1965	499	521	563	99
	1966	135	149	140	21
	1967	456	551	497	−6
	1968	572	515	540	152
	Mean	415	434	435	67

References

1. N.A.A.S. Advisory Papers No. 4, Ministry of Agriculture, Fisheries and Food. **1967**.
2. Williams R. J. B.; Cooke, G. W.; Widdowson, F. V. *J. agric. Sci., Camb.* **1963**, 60, 353.
3. Widdowson, F. V.; Penny, A.; Williams, R. J. B. *J. agric. Sci., Camb.* **1967**, 68, 293.
4. Briget's Experimental Husbandry Farm Report, Ministry of Agriculture, Fisheries and Food. **1962**, 9.
5. Clarke, R. T.; Smith, C. W. R. *Expl. Husb.* **1965**, 12, 15.
6. Widdowson, F. V.; Penny, A.; Williams, R. J. B. *J. agric. Sci., Camb.* **1963**, 61, 397
7. Hopper, M. J.; Clement, C. R. *Int. Conf. Soil Sci.* Comm. II & IV, **1966**, Aberdeen 237.
8. Johnston, A. E. Potassium Institute Ltd, 1st Colloquium **1971**, 135.
9. Clement, C. R. Potassium Institute Ltd, 1st Colloquium **1971**, 93.
10. Gething, P. A. *Expl. Husb.* **1966**, 13, 72.
11. Jones, M. A. Private communication. **1971**.

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Influence of Banded Fertiliser on the Chemical Environment Surrounding the Band

II. Effect on Soil-solution Cations, Cation–Anion Balance and Solution Phosphorus

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The influence of several banded fertilisers on soil solution content of Ca, Mg, K, P and on cation–anion balance at two distances from the band was studied. Treatments consisted of various combinations of monocalcium phosphate (MCP), KCl, NH_4NO_3, $(NH_4)_2HPO_4$ and urea applied on a nutrient element basis of 22.4 or 67.2 kg/ha.

Ammonium nitrate or KCl, supplied separately, produced the highest solution concentrations of Ca (up to 1200 parts/million) and Mg (up to 500 parts/million). Solution concentration of Ca and Mg increased over a 32-day incubation time near treatments containing urea. Treatments containing $(NH_4)_2HPO_4$ initially reduced solution Ca and Mg contents only when applied at 67.2 kg/ha. All treatments not containing K had similar but smaller effects on solution K than they did on Ca and Mg. Highly significant correlations were obtained between total mequiv. of Ca + Mg and NO_3 for treatments containing urea or $(NH_4)_2HPO_4$, while low or non-significant correlations resulted from NH_4NO_3 treatments. Treatments containing $(NH_4)_2HPO_4$ and MCP yielded soil solution P concentrations as high as 1000 parts/million. Urea combined with $(NH_4)_2HPO_4$ or MCP drastically reduced solution P concentrations.

1. Introduction

A previous paper[1] discussed the influence of banded fertiliser on soil pH and solution nitrogen content near the band. Results indicated that pH near banded fertiliser can be changed substantially. The pH of the soil solution 0.9 to 2.5 cm from the band was as low as 4.20 from a NH_4NO_3 + MCP + KCl treatment and as high as 8.15 from urea + KCl. Large pH changes would be expected to significantly affect soil cations by favouring the formation of various compounds. All treatments containing N initially yielded high solution concentrations of NH_4-N, which would displace cations from the exchange complex. Heavy broadcast applications of NH_4NO_3 or $(NH_4)_2SO_4$ have been shown to substantially lower pH and exchangeable K in the surface 46 cm of soil.[2] Banded fertilisers should produce similar results but in a smaller volume of soil. However, little is known of the magnitude or extent to which banded fertilisers influence soil cations. This paper describes the effect of banded fertilisers on solution contents of Ca, Mg and K and on cation-anion balance. Effect on solution P content also is described.

TABLE 1. Influence of several carriers of N, P and K on the concentration of Ca, Mg and K in soil solution (parts/million) at varying distances from the fertiliser band[a]

	Incubation time (days)	Each nutrient applied at the rate of 22.4 kg/ha						Each nutrient applied at the rate of 67.2 kg/ha					
		Ca		Mg		K		Ca		Mg		K	
		Inner core[b]	Outer core[b]	Inner core[b]	Outer core[b]	Inner core[b]	Outer core[b]	Inner core[b]	Outer core[b]	Inner core[b]	Outer core[b]	Inner core[b]	Outer core[b]
NH_4NO_3	4	867	667	393	312	48	26	1170	1092	1491	453	59	52
	8	763	658	368	327	32	23	830	900	394	429	47	45
	16	793	665	397	353	23	20	966	1020	387	411	20	19
	32	693	575	385	325	12	12	1228	1130	533	499	46	46
Urea	4	150	317	79	122	38	33	157	250	110	112	22	27
	8	563	488	308	253	39	22	150	157	89	141	14	42
	16	913	692	442	362	22	17	112	517	102	239	39	36
	32	555	478	303	260	12	12	1058	1030	536	465	30	30
MCP	4	117	117	72	30	13	13	703	397	240	198	20	19
	8	94	87	78	43	9	9	647	393	282	233	19	16
	16	147	143	96	80	9	9	550	445	200	186	13	13
	32	137	113	83	82	11	7	517	312	228	217	17	17
$(NH_4)_2HPO_4$	4	117	250	67	98	45	30	50	140	19	101	39	33
	8	107	282	92	132	27	19	62	215	48	150	38	29
	16	283	437	217	222	24	19	125	315	62	144	43	43
	32	657	560	413	303	23	11	233	478	227	260	32	26
KCl	4	592	397	299	194	540	119	1127	857	334	257	2181	371
	8	452	358	210	172	341	90	797	808	394	394	1126	501
	16	288	283	147	143	212	87	697	734	277	290	650	342
	32	315	277	137	142	121	68	625	655	267	264	393	256
NH_4NO_3 + MCP	4	805	650	413	330	45	26	1030	927	378	390	52	46
	8	653	530	338	267	32	20	1091	887	442	417	49	49
	16	627	557	320	300	23	19	1020	953	366	341	50	49
	32	840	512	408	258	7	9	1006	940	415	409	43	43
Urea + MCP	4	168	327	82	147	22	15	60	100	23	63	39	27
	8	305	433	173	227	33	22	75	145	6	63	26	25
	16	730	580	427	322	30	14	147	420	39	175	53	34
	32	687	487	370	255	7	6	560	662	305	364	62	67

NH_4NO_3 + KCl	4	1646	908	795	463	616	152	1114	1305	449	530	1574	890
	8	1110	822	542	398	378	153	1150	1210	459	498	1221	712
	16	797	647	412	340	252	116	1471	1428	564	544	803	483
	32	662	842	353	403	130	84	1217	1240	528	641	523	403
Urea + KCl	4	312	585	237	317	650	105	97	340	137	220	2157	365
	8	538	612	323	333	486	110	125	333	107	183	1139	524
	16	1007	847	473	423	307	129	175	360	141	234	838	484
	32	750	697	400	372	189	104	1116	1195	546	514	542	396
MCP + KCl	4	513	387	300	205	346	53	1143	953	422	409	1546	451
	8	387	357	248	190	298	66	991	920	402	408	1076	404
	16	287	305	165	143	214	85	910	785	355	327	702	379
	32	217	220	122	118	124	68	625	620	271	278	422	329
$(NH_4)_2HPO_4$ + KCl	4	208	445	177	240	435	49	68	447	19	247	1589	433
	8	198	429	177	227	391	107	153	473	95	239	1261	553
	16	377	532	288	282	302	120	197	543	126	262	797	437
	32	600	591	378	323	182	94	342	608	199	349	630	438
NH_4NO_3 + MCP + KCl	4	1133	858	535	462	606	106	1686	1678	568	611	1913	641
	8	1113	838	473	408	476	156	1163	1250	479	500	1343	686
	16	850	798	398	387	291	136	1131	1236	468	535	907	505
	32	798	762	377	538	169	107	1121	1175	461	487	520	406
Urea + MCP + KCl	4	360	570	230	317	646	108	90	307	39	231	1957	264
	8	435	580	282	321	439	121	128	325	28	171	1307	514
	16	870	857	482	425	333	133	124	505	13	248	916	477
	32	885	703	485	397	165	104	727	1033	383	493	737	471
Check	4	115		47		9		261		122		13	
	8	116		58		13		275		116		14	
	16	130		60		10		300		147		22	
	32	122		73		9		354		184		13	

[a] Average of three replicates.
[b] Inner core was bounded by radii of 0.95 to 2.5 cm from the centre of the band; outer core was bounded by radii of 2.5 to 3.8 cm from the centre of the band.

2. Experimental

The various aspects of soil and treatment preparation, soil sampling and solution extraction have been discussed in a previous publication. In brief, a laboratory simulation of a fertiliser band 46 cm long and surrounded in cross section by 7 cm of soil was prepared. Two rates of application of each fertiliser material were used, equivalent to 22.4 and 67.2 kg/ha of each nutrient (N, P or K) contained in the material. After various equilibration periods, soil cores bounded by radii of 0.95 to 2.5 cm (inner core) and 2.5 to 3.8 cm (outer core) were obtained with the use of an extraction apparatus. The soil solution in these cores was extracted with pressure. A more detailed account of the experimental techniques has been published previously.[1]

The soil solution content of Ca and K was determined with a Beckman model D.U. flame spectrophotometer and Mg was determined with a Jarrell–Ash atomic absorption unit. Phosphorus was determined photometrically with ammonium molybdate and stannous chloride.[3]

3. Results and discussion

3.1. Effect on solution cations

The effect of various sources and combinations of N, P and K, applied at several rates, on soil solution content of Ca, Mg and K is shown in Table 1.

Of the single fertiliser salt treatments, NH_4NO_3 and KCl were most effective in producing and maintaining high solution concentrations of Ca and Mg. Ammonium nitrate was more effective, however, since it maintained the soil solution 0.10 to 0.50 pH unit lower than KCl. This would favour dissolution of various Ca and Mg compounds, such as dolomite (present in this soil). In addition, nitrification of NH_4NO_3 produced twice the number of anions as KCl which would aid cation diffusion away from the fertiliser band. Combining NH_4NO_3 and KCl yielded higher initial Ca and Mg contents than any other treatment.

Application of urea (alone or in combination) at 22.4 kg/ha resulted in increasing solution Ca and Mg contents for 16 days after which the contents decreased. When applied at 67.2 kg/ha, solution cation content remained low for the first three sampling times and then increased to very high levels at 32 days. The initial pH near treatments containing urea was higher than for any other treatment.[1] This lowered Ca and Mg solubility. As the pH decreased with time Ca and Mg solubility increased, resulting in high solution Ca and Mg concentrations.

Solution Ca and Mg contents, as shown in Table 1, were generally diminished by treatments containing $(NH_4)_2HPO_4$, particularly when applied at 67.2 kg/ha. In addition, the exchangeable Ca content for the inner core (data not shown) was initially reduced to 3.3 mequiv. compared to 6.7 mequiv. for the check. It was still low, 5.8 mequiv., at 32 days. Formation of calcium phosphate probably accounts for the low solution and exchangeable Ca level. Calcium was displaced from the exchange by the NH_4 ion and was then rapidly removed from solution as various calcium phosphate compounds formed.

Monocalcium phosphate, applied alone or combined with other fertiliser materials at 22.4 kg/ha, had little influence on solution Ca or Mg contents (compare check levels at

foot of Table 1). However, application of MCP at 67.2 kg/ha greatly increased solution Ca and Mg contents. Combination of NH_4NO_3 and KCl with MCP tended to decrease the Ca and increase the Mg in soil solution as compared to when these salts were applied alone. The combination of MCP with urea considerably reduced the Ca and Mg content as compared to when MCP was not supplied. Apparently a combination of high pH (due to urea) and P concentration (from MCP) favoured the formulation of relatively insoluble Ca and Mg phosphates.

Generally, the various treatments not containing K had about an equal effect on solution K content as they did on solution Ca and Mg content. However, K accounts for a small part of the total solution cation content and therefore, makes the K results appear unimpressive. Solution K contents near treatments containing KCl were significantly influenced by the accompanying material. Combining other fertilisers with KCl generally resulted in higher contents (particularly for the 16- and 32-day sampling times) than when KCl was supplied alone. Monocalcium phosphate tended to initially depress solution K contents which may have been due to conversion of K to a less soluble form. Other investigations have shown that when KCl is associated with MCP the initial reaction products that form are relatively insoluble Ca-K phosphates.[4,5] These products would eventually disappear as more insoluble Ca phosphates form, but they could initially reduce the amount of K in the fertiliser solution.

All treatments exerted more influence on solution cation content at the high than low application rate. However, the threefold increase in application was rarely matched with a similar increase in solution cation content. Apparently the quantity of Ca, Mg or K dissolved or displaced at the low rate represented a rather easily available form of these cations and at the high rate, a more difficultly available form.

3.2. Cation–anion balance

Previous work[1] and data presented in this paper indicated that increases or decreases in solution NO_3 over the four sampling times were accompanied by similar fluctuations in solution Ca and Mg. These relationships were further investigated by use of correlation and regression analyses. The results for the low rate of application (22.4 kg/ha) presented in Table 2 show that the correlations between mequiv. Ca + Mg and mequiv. NO_3 were highly significant for all treatments containing urea. With these treatments, variations in the mequiv. of NO_3 accounted for 67 to 98% of the variation in the mequiv. of Ca and Mg. Correlations were somewhat lower with $(NH_4)_2HPO_4$ and NH_4NO_3, especially when combined with MCP or KCl. The data are not presented for the high rate of application (67.2 kg/ha) but the correlations were generally lower than for the low rate of application.

Plotting mequiv. of Ca + Mg against mequiv. of NO_3 helps explain previously discussed data concerning pH and N,[1] and solution cation content. For example, urea hydrolysis resulted in initially high pH levels and low NO_3 contents since little NH_4 had been nitrified at the 4-day sampling time. Also the high pH favoured formation of relatively insoluble Ca and Mg compounds resulting in low solution concentrations of Ca and Mg. As the pH decreased with time (due to nitrification), the concentration of NO_3, Ca and Mg increased. The increases in Ca + Mg and NO_3 were nearly proportional with time so the data were well represented by a linear regression equation.

TABLE 2. Effect of various sources of banded fertilisers on the linear regression equations and correlation coefficients between the mequiv. of Ca + Mg and the mequiv. of NO_3 in soil solution in the inner and outer core samples over the four sampling periods[a]

Treatment[b]	Regression equation[c]	Corr. coeff.[d]	Coeff. of determination
NH_4NO_3	$Y = 0.30 + 0.99X$	0.82**	0.672
Urea	$Y = 1.54X - 15.00$	0.99**	0.980
$(NH_4)_2HPO_4$	$Y = 1.20X - 3.40$	0.95**	0.903
NH_4NO_3 + MCP	$Y = 36.20 + 0.40X$	0.69*	0.476
Urea + MCP	$Y = 1.37X - 15.5$	0.90**	0.810
NH_4NO_3 + KCl	$Y = 1.90X - 17.00$	0.94**	0.884
Urea + KCl	$Y = 18.80 + 1.09X$	0.95**	0.903
$(NH_4)_2HPO_4$ + KCl	$Y = 15.70 + 0.89X$	0.78*	0.608
NH_4NO_3 + MCP + KCl	$Y = 40.80 + 0.75X$	0.75*	0.560
Urea + MCP + KCl	$Y = 23.30 + 0.98X$	0.82**	0.672

[a] Samples were collected at 4, 8, 16 and 32 days after application of the fertilisers.
[b] Each fertiliser nutrient (N, P or K) was applied at a rate of 22.4 kg/ha.
[c] Y = mequiv. Ca + Mg; X = mequiv. NO_3.
[d] ** = significant at 1 % level; * = significant at 5 % level.

Similarly, with treatments containing $(NH_4)_2HPO_4$, Ca and Mg were initially removed from solution through formation of Ca and Mg phosphates, but with time the NH_4 was nitrified, and the NO_3 content increased and pH decreased.

3.3. Solution phosphorus

The solution P content near banded P fertiliser is initially high but decreases rapidly with time as it equilibrates with soil and forms insoluble P compounds. Soil pH and the concentration of other ions in solution considerably influence the amount of P that is maintained in solution and the conversion to less available forms.

The highest solution P content was achieved when $(NH_4)_2HPO_4$ or MCP was supplied alone (Table 3). Application rates of 67.2 kg/ha resulted in solution concentrations as high as 1000 parts/million. Other investigations have shown that combining NH_4NO_3 and/or KCl with MCP or dicalcium phosphate (DCP) increases P solubility and availability to plants as compared to supplying MCP or DCP alone.[6,7] However, in this study the combination of NH_4NO_3 or KCl with MCP or $(NH_4)_2HPO_4$ decreased the solution P concentration. The effect was generally greater for the outer sampling core. These salts increased the solution Ca and Mg concentrations which in turn enhanced the formation of Ca and Mg phosphates. However, KCl apparently had more influence on solution P levels than Ca and Mg. The solution P content was moderately reduced by NH_4NO_3 but drastically reduced by KCl (compare MCP + NH_4NO_3 and MCP + KCl treatments).

As expected, combining urea with MCP drastically reduced the solution P content. The urea initially increased pH which favoured the formation of insoluble Ca phosphates, thereby removing P from solution. Solution P increased with time to the 16-day sampling period and then decreased (22.4 kg/ha rate, inner core). This coincides with the decrease in pH with time for the treatments containing urea.[5] The optimum pH for P

TABLE 3. Effect of various P sources, supplied alone or combined with N and/or K (applied at the rate of 22.4 and 67.2 kg/ha) on the P content in the soil solution (parts/million)[a]

Treatment	Sample position[b]	First experiment (22.4 kg/ha) Sampling time[b]				Second experiment (67.2 kg/ha) Sampling time[c]			
		4	8	16	32	4	8	16	32
MCP	Inner core	184	178	67	46	1000	725	289	147
	Outer core	1.0	6.3	4.7	5.0	280	181	136	43
$(NH_4)_2HPO_4$	Inner core	334	334	211	136	1094	792	341	222
	Outer core	2.0	9	14	10.6	172	171	113	99
NH_4NO_3 + MCP	Inner core	193	168	80	35.8	840	741	480	164
	Outer core	<0.1	1.5	2.7	2.2	108	174	130	76
Urea + MCP	Inner core	16	30	73	21.3	327	83	53	46
	Outer core	<0.1	<0.1	<0.1	<0.1	7	5	12	35
MCP + KCl	Inner core	65.6	91	41.4	26.5	495	307	208	127
	Outer core	0.3	2.1	0.5	1.8	288	42	70	37
$(NH_4)_2HPO_4$ + KCl	Inner core	184	196	135	75.7	914	298	207	152
	Outer core	<0.1	5.5	7.0	3.5	140	72	80	72
NH_4NO_3 + MCP + KCl	Inner core	113	113	57	55.6	1092	457	272	135
	Outer core	<0.1	0.5	<0.1	3.0	203	128	84	77
Urea + MCP + KCl	Inner core	10.8	16.2	51.3	33.7	107	86	58	42
	Outer core	<0.1	0.1	0.1	0.3	0.7	1	2.5	21

[a] Average of three replicates.
[b] Inner core is for the soil bounded by radii of 0.95 to 2.5 cm and outer core is for t he soil sample bounded by radii of 2.5 to 3.8 cm from the centre of the band.
[c] Days after start of experiment.

solubility was probably reached between 8 and 16 days. By 32 days, pH had attained a level favouring Fe- and $AlPO_4$ formation, thereby accounting for the decrease in solution P at the end of the experiment. Application of urea at 67.2 kg/ha resulted in a decrease in P with time in the inner core and an increase in soluble P in the outer core. The total pH change for the outer core was twice that for the inner core which would again account for the results.

Application rate greatly influenced solution P content. For the inner core the rate increase generally tripled P content. Much larger increases (often several hundred times) were noted in the outer core. This large increase was probably related to the soil phosphate fixing capacity. Phosphorus rarely diffuses far in soil since it rapidly forms insoluble Fe, Al and Ca phosphates. More P was supplied at the high rate than could be fixed in the inner core with the result that large amounts of P diffused into the outer core.

4. Conclusions

This study shows that recommended rates of row fertiliser can drastically change the solution concentration of major soil cations (Ca, Mg and K) up to 3.8 cm from the centre of the band. The single fertiliser salt treatments, NH_4NO_3 and KCl, produced the highest solution concentrations of Ca and Mg. Treatments containing $(NH_4)_2HPO_4$ generally decreased solution Ca and Mg contents, presumably due to formation of Ca and Mg phosphates. Only when applied at 67.2 kg/ha did MCP increase Ca and Mg contents. Generally, the various treatments (not containing K) had about an equal effect on solution K content as they did on solution Ca and Mg content.

Highly significant correlations were obtained between mequiv. of Ca + Mg and NO_3 for treatments containing urea or $(NH_4)_2HPO_4$. For these treatments, 60 to 98 % of the variation in Ca + Mg content was accounted for by a change in NO_3 content. Somewhat lower correlations were observed with treatments containing NH_4NO_3, presumably due to the relative lack of change in Ca, Mg and NO_3 content with time.

Solution P concentrations as high as 1000 parts/million were found near treatments containing $(NH_4)_2HPO_4$ and MCP. Combination of urea with MCP or $(NH_4)_2HPO_4$ drastically reduced the solution concentration of P. On the basis of these results, urea should not be band applied with $(NH_4)_2HPO_4$ or MCP.

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References

1. Isensee, A. R.; Walsh, L. M. *J. Sci. Fd Agric.* **1971**, 22, 105.
2. Abruna, F.; Pearson, R. W.; Elkins, C. B. *Proc. Soil Sci. Soc. Am.* **1958**, 22, 539.
3. *C. A. Black, Methods of Soil Analysis, Pt. 2, Chemical and Microbiological Properties*, (Black C. A.; Evens, D. D.; White, J. L.; Ensminger, L. E.; Clark, F. E., eds.) Amer. Soc. Agron., Madison, Wis. 1043. **1965**.
4. Bouldin, D. R.; Lehr, J. R.; Sample, E. C. *Proc. Soil Sci. Soc. Am.* **1960**, 24, 464.
5. Tyler, K. B.; Broadbent, F. E.; Kondo, V. *Agric. J., Vict. B.C.* **1958**, 50, 626.
6. Bouldin, D. R.; Sample, E. C. *Proc. Soil Sci. Soc. Am.* **1958**, 22, 124.
7. Starostka, R. W.; Hill, W. L. *Proc. Soil Sci. Soc. Am.* **1955**, 19, 123.

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Nutritional Effects of Including Different Levels and Sources of Protein in Milk Replacers for Calves

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Two digestibility and nitrogen balance experiments were carried out with groups of young calves given high-fat milk replacers reconstituted with water and fed in liquid form. In the first experiment six different milk replacers with crude protein contents ranging from 12.7 up to 27.6% in the dry matter were compared, the differences in protein content being brought about by adjustments to the level of separated milk protein used in the replacers. The apparent digestibility of organic matter was very high on all the treatments and unaffected by level of protein. Although the crude protein fraction showed an increase in apparent digestibility with increase in level of protein, its true digestibility was estimated to be virtually 100% on all the treatments. Retention of nitrogen expressed as a percentage of total intake remained fairly constant for diets containing from 18.8 to 27.6% of crude protein and the weight of nitrogen retained increased with each successive increase in level of dietary protein.

In the second experiment the effects of replacing half of the milk protein in a control milk replacer by different forms of animal protein were studied. The true digestibilities of the total dietary protein on the treatment containing only separated milk protein and on the other treatments containing partial substitution by dried delactosed whey, dried blood, white fish meal and meat meal were found to be 94.1, 90.9, 85.4, 91.1 and 86.6%, respectively. Their biological values were 74.7, 70.3, 69.1, 66.0 and 65.5%, respectively. A sixth treatment in which the meat meal was provided with an enzyme supplement did not effect any significant change in either digestibility or biological value. Retentions of nitrogen expressed as percentages of intake were significantly lower on all the treatments containing partial replacement of separated milk protein by the other sources of protein than on the control treatment.

1. Introduction

In order to achieve both efficient feed utilisation and minimal risk of digestive upsets, milk replacers for young calves have to be formulated from a limited range of individual food commodities which calves can digest efficiently. Consequently, such milk replacers usually consist of milk products such as separated milk and dried whey, fats such as tallow and vegetable oils, and mineral and vitamin supplements. The use of fat[1] and of protein[2] in milk replacers has been recently reviewed. In the case of protein the present high cost of separated milk powder has stimulated a need to critically examine

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both the total requirement for protein and the nutritive value of possible alternative sources which might be used as partial replacements for separated milk protein.

The present paper describes digestibility and nitrogen balance experiments undertaken to investigate the effect on nutritive value of milk replacers of both alteration in the level of protein and partial substitution of separated milk protein by alternative sources of animal protein.

2. Experimental

2.1. Diets

The milk replacers (m.r.) studied are shown in Table 1 together with analytical data on their chemical composition. High-fat milk powder B was prepared by homogenising the requisite quantities of stabilised butterfat containing 3.3% of added lecithin and liquid separated milk to produce a "filled" milk containing 45% of fat in the dry matter, followed by spray drying. Butterfat was used as the source of fat because previous work had shown other fats to be of lower digestibility accompanied by a tendency for some reduction in protein digestibility,[3] and it was wished to avoid any similar effect in the present experiment. Amendments to the level of protein in milk replacers, m.r. 1 to 6, were made by interchange of milk protein and glucose. Since reductions in the level of separated milk protein were accompanied by corresponding reductions in the levels of lactose and of mineral constituents supplied by the separated milk, additional lactose, dicalcium phosphate and sodium chloride were added in order to maintain similar levels of lactose and mineral constituents in all the milk replacers. High-fat milk powder T was prepared by homogenising the requisite quantities of stabilised tallow containing 3.3% of added lecithin and liquid separated milk to produce a "filled" milk containing 50% of fat in the dry matter, followed by spray drying. Different forms of animal protein were provided by replacing 30% of separated milk powder, which supplied half of the total protein in the control milk replacer (m.r. 7), by sufficient of each alternative source of protein coupled with adjustments to the level of glucose necessary to maintain the overall proximate composition of the diets. The percentages of crude protein in the dry matter of the sources of protein being compared were separated milk powder 34.5%, dried delactosed whey 27.8%, dried blood 90.0%, white fish meal 72.3% and meat meal 79.5%.

Chlorotetracycline was included in each milk replacer at a level of 88 mg per kg of diet. Dry vitamin supplements were also added to supply 5280 i.u. of vitamin A, 660 i.u. of vitamin D_3 and 11 mg of vitamin E per kg of diet. Determinations of dry matter were made on all diets before commencement of the experimental periods, and the amounts equivalent to 283.5 g of dry matter (the amount per feed) calculated. Immediately before feeding, which occurred twice daily at 9 a.m. and 5 p.m., the calculated allowance of powder was reconstituted with 2 l of warm water to give a liquid "filled" milk containing 12.4% of dry matter. 5 ml of a solution of a mineral mixture prepared according to the formula of Blaxter and Wood[4] and 5 ml of a solution of B vitamins prepared as previously described,[5] were added to each feed. After the consumption of the milk replacer, a further 0.5 l of water was added to the bucket to ensure complete intake of any feed residues.

TABLE 1. Composition of the milk replacers

Experiment no.				1					2			
Treatment no.	m.r.1	m.r.2	m.r.3	m.r.4	m.r.5	m.r.6	m.r.7	m.r.8	m.r.9	m.r.10	m.r.11	m.r.12
Percentage composition												
Spray-dried separated milk powder	8.0	16.6	25.2	33.8	42.4	51.0	45.0	15.0	15.0	15.0	15.0	15.0
High-fat milk powder B	46.0	45.8	45.6	45.4	45.2	45.0						
High-fat milk powder T							30.0	30.0	30.0	30.0	30.0	30.0
Glucose	21.0	17.6	14.2	10.8	7.4	4.0	25.0	17.0	43.0	40.0	41.5	41.5
Lactose	21.5	17.2	12.9	8.6	4.3	0.0						
Delactosed whey								38.0				
Dried blood									12.0			
White fish meal										15.0		
Meat meal											13.5	13.5
Proteolytic enzyme supplement												+
Dicalcium phosphate	2.70	2.16	1.62	1.08	0.54	0.00						
Sodium chloride	0.80	0.64	0.48	0.32	0.16	0.00						
Antibiotic, vitamin and trace mineral supplements	+	+	+	+	+	+	+	+	+	+	+	+
Chemical composition (% of dry matter)												
Organic matter	94.0	94.0	93.9	93.9	93.8	93.5	94.1	87.7	94.4	93.9	94.2	94.2
Crude protein	12.7	15.4	18.8	21.7	24.5	27.6	20.1	20.8	20.8	21.6	20.7	20.7
Fat	21.9	22.0	21.6	21.6	22.1	22.6	15.3	15.6	17.0	17.4	17.2	17.2
Total carbohydrates	59.4	56.6	53.5	50.6	47.2	43.3	58.7	51.3	56.6	54.9	56.3	56.3
Ash	5.97	5.96	6.07	6.08	6.23	6.51	5.92	12.26	5.61	6.10	5.79	5.79

2.2. Plan of experiments

Two digestibility and nitrogen balance experiments were carried out, each being a 6×6 latin square design experiment with six calves. The calves were housed in conventional metabolism crates when about 3 to 4 days old and the required series of digestibility trials and nitrogen balances carried out. Each balance consisted of a preliminary feeding period of 5 days followed by a collection period (faeces and urine) of 6 days. The calves were weighed at the beginning and end of each period. Their mean liveweights during experiment 1 and experiment 2 were both 41 kg. All the animals remained in good condition throughout the balance periods, except for one animal in experiment 1 which injured itself between periods 3 and 4 and had to be removed from the remainder of the experiment. In no instance was any food refused.

2.3. Collection and analysis of samples

Daily 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 refrigerator. Likewise, a definite fraction of each day's output of urine was taken and bulked. 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 faeces.

Before chemical analysis the samples of dried faeces were milled to pass through a $\frac{1}{32}$ inch sieve. The dry matter, crude protein ($N \times 6.25$) and ash constituents in the milk replacers and dried 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[6] method 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. The percentages of total carbohydrates were obtained by summating the percentages of crude protein, total lipid and ash in the dry matter and subtracting from 100.

Samples of the diets used in experiment 2 were also subjected to determination of *in vitro* digestibility by the pepsin–hydrochloric acid method.[7]

3. Results

3.1. Experiment 1

It is evident from the results given in Table 2 that the digestibility of the organic matter remained fairly constant for all the diets, which had widely differing crude protein contents ranging from 12.7 up to 27.6%. Although the results for the digestibility of the lipid and carbohydrate fractions of the dry matter likewise remained fairly constant, the results for crude protein show a progressive increase with level of crude protein. However, the results represent apparent digestibility and it is of interest to consider whether any improvement occurred in the true digestibility of dietary protein. Statistical examination of the relationship between total faecal nitrogen excretion in

TABLE 2. Mean digestibility and nitrogen metabolism data in experiment 1

Treatment no.	m.r.1	m.r.2	m.r.3	m.r.4	m.r.5	m.r.6	Standard error of a mean	Significance of treatment differences[a]
Apparent digestibility								
Organic matter (%)	95.4	95.5	95.3	94.6	95.6	95.5	0.78	n.s.
Crude protein (%)	82.9	84.1	85.3	88.9	91.8	92.3	1.52	**
Total lipid (%)	93.5	94.1	95.4	90.2	92.3	92.2	2.26	n.s.
Total carbohydrate (%)	98.6	99.1	98.8	98.9	99.1	99.2	0.28	n.s.
Nitrogen metabolism								
Nitrogen intake (g/day)	11.5	14.0	17.1	19.7	22.3	25.1	—	—
Nitrogen retention (g/day)	4.47	5.57	8.26	9.25	11.22	11.92	0.81	***
Nitrogen retention (% of intake)	38.8	39.8	48.4	47.0	50.4	47.6	3.72	n.s.

[a] In Tables 2 and 3; *** indicates significant at $P<0.001$, ** significant at $P<0.01$, * significant at $P<0.05$ and n.s. not significant.

grams (Y) and percentage of crude protein in the diet (X) gave a regression equation of

$$Y = 13.832 - 0.058X,$$

with no significant effect of protein level on faecal nitrogen excretion. Although there is likely to be considerable error in using this equation to extrapolate from the minimum protein level used in this experiment to one of zero intake, it is worth noting that such an extrapolation indicates a value for metabolic faecal nitrogen of 4.1 g/kg dry matter intake compared with the value of 2.5 g/kg dry matter intake suggested by a Working Party organised by the Agricultural Research Council.[8] The evidence that dietary level of protein had very little influence on faecal excretion of nitrogen indicates that the protein in all the diets had a true digestibility of virtually 100%.

The retention of nitrogen expressed as a percentage of the total intake remained fairly constant for the diets containing 18.8 up to 27.6% of crude protein. Thus increases in the level of crude protein were accompanied by increases in the weight of nitrogen retained daily.

3.2. Experiment 2

In this experiment the percentages of crude protein in each of the diets were very similar, and therefore permit comparison of their apparent digestibilities. The results given in Table 3 indicate that the use of the alternative sources of protein to separated milk protein were all associated with some reduction in digestibility of the total dietary protein. The least reductions occurred with delactosed whey and white fish meal, and the mean values for these treatments (m.r. 8 and m.r. 10) were not significantly different from that of the solely separated milk protein treatment (m.r. 7). However, the lower values for the treatments containing dried blood (m.r. 9) and meat meal (m.r. 11) were significantly lower than that of treatment m.r. 7 ($P < 0.05$) and it is clear that the use of the enzyme supplement in treatment m.r. 12 did not effect any improvement in the digestibility of the meat meal protein. It is also of interest that the mean digestibility of the crude protein increased from 76.3% in period 1 to 85.3% in period 6. Previous work with fats has shown that the digestibility of some alternative fats to butterfat also improves with increasing age of calves.[9]

The true digestibilities and biological values of the total crude protein in each diet were calculated using the recommended factors[8] of 2.5 g/kg dry matter intake for metabolic faecal nitrogen and 0.20 g/day/kg liveweight$^{0.73}$ for endogenous urinary nitrogen. As all the protein on treatment m.r. 7 was from separated milk and its true digestibility of 94.1% was appreciably less than the value of about 100% to be expected from the results of experiment 1, some reservation must again be expressed about the recommended factor for calculating metabolic faecal nitrogen. In order for the true digestibility on treatment m.r. 7 to be 100% the factor for metabolic faecal nitrogen would have to be 4.4 g/kg dry matter intake, which agrees fairly closely with the estimate of 4.1 g/kg from the results of experiment 1. Although an error in the assumed value for metabolic faecal nitrogen would effect the true digestibility results and biological values it would make little difference to the relative values of the various treatments. As with apparent digestibility, the true digestibilities of the protein on the

TABLE 3. Mean digestibility and nitrogen metabolism data in experiment 2

Treatment no.	m.r.7	m.r.8	m.r.9	m.r.10	m.r.11	m.r.12	Standard error of a mean	Significance of treatment differences
Apparent digestibility								
Organic matter (%)	93.0	92.6	89.8	90.7	90.3	89.4	0.72	**
Crude protein (%)	86.2	83.4	77.9	83.8	79.0	77.5	2.02	*
Total lipid (%)	78.6	79.9	68.3	73.6	74.0	70.2	2.99	n.s.
Total carbohydrates (%)	98.7	98.3	98.6	98.3	97.8	98.0	0.41	n.s.
True digestibility								
Total crude protein (%)	94.1	90.9	85.4	91.1	86.6	85.1	2.01	*
Pepsin/HCl digestible protein	98.0	97.6	93.6	91.0	92.8	93.6	—	—
Nitrogen metabolism								
Nitrogen intake (g/day)	18.2	18.9	18.9	19.6	18.8	19.0	—	—
Nitrogen retention (g/day)	8.40	7.30	6.40	7.34	6.27	6.45	0.40	**
Nitrogen retention (% of intake)	46.0	40.0	35.1	37.5	33.3	34.1	1.89	**
Biological value of crude protein (%)	74.7	70.3	69.1	66.0	65.5	67.6	1.70	*

treatments containing partial substitution of separated milk protein were all lower than on treatment m.r. 7, the values on the dried blood and meat meal treatments being significantly lower ($P < 0.05$).

The use of less digestible sources of protein in treatments m.r. 9 to 12 was associated with lower digestibility of fat than on treatment m.r. 7 but the differences obtained just failed to reach significance. Carbohydrate digestibility was evidently not affected.

The biological values of the protein of treatments m.r. 8 to 12 were all lower than that of the solely separated milk protein treatment m.r. 7, the values on the white fish meal and meat meal treatments being significantly lower at $P < 0.01$, that on the blood meal treatment at $P < 0.05$ and that on the dried whey treatment just failing to attain significance.

The mean nitrogen retentions, expressed as percentages of intake, were significantly lower on all treatments m.r. 8 to 12 than on treatment m.r. 7 (the dried blood and meat meal treatments at $P < 0.001$, the white fish meal treatment at $P < 0.01$ and the dried whey treatment at $P < 0.05$).

4. Discussion

It is evident that increase in level of separated milk protein over the wide range from 12.7 to 27.6% of the diet has little or no influence on its true digestibility which for the particular dietary composition used in experiment 1 was maintained at virtually 100%. Furthermore, the weight of nitrogen retained daily increased with each successive increase in level of dietary protein so if maximum growth of lean body tissue is desired there would appear to be good justification for having a level of dietary protein as high as 27% in high-fat milk replacers similar to those used in experiment 1. Since body gain by the young calf consists largely of protein and associated water, increases in nitrogen retention should be accompanied by increases in rate of liveweight gain, particularly as the calorific value per kg of gain is reduced by the deposition of protein plus water in preference to fat. Roy[2] recently calculated the crude protein contents required in milk replacers for calves at various liveweights and gaining at different rates. For calves of 40 kg liveweight, similar to the mean liveweights of the calves used in the present experiments he estimated the minimum crude protein contents required in the dry matter of high energy milk replacers to be 21.0% for maintenance + 0.5 kg gain/day and 27.0% for maintenance + 1.0 kg gain/day showing that the requirements increase appreciably with increase in rate of liveweight gain.

The nutritional value of the alternative sources of protein to separated milk protein needs to be assessed both in terms of digestibility and biological value. The partial replacement of separated milk protein by dried delactosed whey could affect overall protein digestibility as a result of reduced curd formation in the abomasum[10] or because of denaturation occurring during processing of the whey.[11] In describing such denaturation Roy[11] has shown that it is associated with a reduction in ionisable calcium, poor clotting ability by rennet and reduced digestibility, but no effect on the biological value of the protein. A further possible influence on protein digestibility is the high content of mineral matter in delactosed whey because the addition of minerals simulating whey ash has been reported to increase overall incidence of diarrhoea.[12] The delactosed

whey used in the present trial had a total ash content of 24.2% in the dry matter and its use in treatment m.r. 8 gave a total ash content of 12.26% in the dry matter of that diet which was approximately double the levels present in the other diets. This high ash content led to a correspondingly reduced organic matter content and since dry matter intakes on all the treatments were the same it is evident that treatment m.r. 8 supplied a lower intake of total organic matter and also of digestible organic matter. This lower energy intake would, presumably tend to lower the biological value and overall efficiency of nitrogen retention on treatment m.r. 8 compared with the other treatments. In spite of the possible ways in which the use of dried delactosed whey may result in some reduction in nutritive value the results for treatment m.r. 8 compared with treatment m.r. 7 indicate that its use in the present experiment did not significantly affect either digestibility of crude protein or biological value, so any effects which may have occurred had only minor influence on these criteria of nutritive value. Overall retention of nitrogen, expressed as percentage of intake, was however significantly reduced. In comparing the amino acid composition and nutritive value of the total milk proteins with that of the whey protein fraction Porter[13] concluded that the latter is better endowed with total sulphur amino acids and has a superior nutritive value. The use of undried liquid delactosed whey added to liquid separated milk and fat prior to spray drying should minimise denaturation and may give better results than dried delactosed whey.

Turning to the other sources of protein it is evident that the fish meal protein had the highest digestibility. The calculated true digestibility of 91.1% for the total protein on treatment m.r. 10 compared with that of 94.1% on treatment m.r. 7 suggests that the fish meal protein was about 6% lower in digestibility than the separated milk protein. Huber and Slade[14] found that the digestibility of the crude protein in fish flour averaged about 10% less than that of separated milk protein. They also found that live weight gains were similar for groups of calves containing up to 40% of their dietary protein as fish flour, provided by 11.7% fish flour in the diet, but that live weight gains were significantly depressed at 60%. The proteins from the dried blood and the meat meal were of lowest digestibility in the present experiment and the enzyme supplement did not effect any improvement in the digestibility of the meat meal protein. The significantly lower biological values of the total protein on treatments containing fish meal, dried blood and meat meal than on the treatment containing only separated milk protein indicate less efficient utilisation of the amino acids absorbed from the diets containing these alternative sources of protein, presumably because the balance of amino acids absorbed from these sources was less satisfactory for the synthesis of body protein. When all the results are considered it is evident that the most striking effects were brought about by the inclusion of dried blood and meat meal. The use of each of these sources of protein resulted in important and significant depressions in digestibility of organic matter and crude protein, and in overall nitrogen retention.

It is noteworthy that lower mean digestibilities of crude protein were in all cases except the dried whey diet associated with lower fat digestibility. In fact a significant correlation coefficient of +0.529 ($P < 0.001$, $n = 34$) was obtained between the digestibilities of crude protein and fat. The use of alternative fats of lower digestibility in place of butterfat in liquid diets for calves has likewise been found to reduce protein digestibility.[3]

Although the pepsin–HCl digestible protein results did indicate lower digestibility of the protein on treatments m.r.9 to 12 than on treatment m.r. 7, they did not succeed in ranking the alternative protein treatments in accordance with their actual mean digestibilities. The procedure can not therefore be recommended as a valid screening test for alternative sources of protein.

An overall assessment of the nutritive value of the different sources of protein should include both true digestibility and biological value. The product of these two values indicates the utilised protein on each treatment and when the results are expressed relative to a value of 100 for treatment m.r. 7, values of 91, 84, 86, 81 and 82 are obtained for treatments m.r. 8, m.r. 9, m.r. 10, m.r. 11 and m.r. 12, respectively. These relative values do not, however, entirely represent the nutritional value of different sources of protein because they do not take into account the digestibility of other chemical fractions supplied by the protein foods concerned or the effect of lower digestibility of protein on the digestibility of other dietary constituents.

Acknowledgements

The author wishes to thank L. E. Pritchitt and Co. Ltd, Newtownards, Co. Down, for the spray-dried separated milk powder and high-fat milk powders. Thanks are also expressed to Mr C. A. Billington and Mr R. J. Murphy for valuable technical assistance, and to Mr C. Weatherup for advice on the statistical analyses.

References

1. Raven, A. M. *J. Sci. Fd Agric.* **1970**, 21, 352.
2. Roy, J. H. B. *J. Sci. Fd Agric.* **1970**, 21, 346.
3. Raven, A. M.; Robinson, K. L. *Br. J. Nutr.* **1960**, 14, 135.
4. Blaxter, K. L.; Wood, W. A. *Br. J. Nutr.* **1952**, 6, 1.
5. Raven, A. M.; Robinson, K. L. *Br. J. Nutr.* **1959**, 13, 178.
6. British Standards Institution, *Methods for the Chemical Analysis of Dried Milk* London. B.S. 1743. **1951**.
7. Knowles, F.; Watkin, J. E. *A Practical Course in Agricultural Chemistry* Macmillan and Co. Ltd, London, **1937**.
8. *The Nutrient Requirements of Farm Livestock. No.* 2. *Ruminants, Technical Reviews and Summaries* Agricultural Research Council, London. **1965**.
9. Raven, A. M.; Robinson, K. L. *J. Soc. Dairy Technol.* **1964**, 17, 5.
10. Gorrill, A. D. L.; Thomas, J. W. *J. Nutr.* **1967**, 92, 215.
11. Roy, J. H. B. *Vet. Rec.* **1964**, 76, 511.
12. Bush, L. J.; Schuh, J. D.; Tennille, N. B.; Waller, G. R. *J. Dairy Sci.* **1963**, 46, 703.
13. Porter, J. W. G. *J. Soc. Dairy Technol.* **1966**, 19, 3.
14. Huber, J. T.; Slade, L. M. *J. Dairy Sci.* **1967**, 50, 1296.

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Volatile Constituents of Horseradish Roots

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Essences of the volatile components of five samples of horseradish root, three fresh and two of dried flakes, have been prepared and examined by gas chromatography (g.c.) and combined gas chromatography–mass spectrometry (g.c.–m.s.). At least 17 compounds are involved. Five of these have been rigorously identified by comparison with authentic compounds, synthesised where necessary, through mass spectra and retention data and the identities of three others have been tentatively established. The compounds concerned are methyl, ethyl, isopropyl, 2-butyl, allyl, 4-pentenyl and 2-phenethyl isothiocyanate and allyl thiocyanate. The constituents of the five essences were assessed semi-quantitatively by g.c. and definite differences found. These were reinforced by examination of the headspace vapours over the coarsely grated root.

Additional attention was devoted to the allyl isothiocyanate–thiocyanate interconversion and to the odour assessment of the volatile horseradish components.

1. Introduction

It has been well established that the principal pungent compounds of horseradish, *Armoracia lapathifolia* Gilib., are allyl and 2-phenethyl isothiocyanate.[1–3] However, there are also a number of isolated and seemingly unsubstantiated reports concerning minor components, such as phenylpropyl isothiocyanate,[4] methylthiopentyl isothiocyanate[5] and diallyl sulphide.[6] 2-Butyl isothiocyanate has been frequently reported as a subsidiary acrid component of the related wasabi,[7–9] the Japanese horseradish, *Wasabia japonica* Matsum. Japanese workers interested in "Wasabiko", a processed Japanese horseradish product, have identified tentatively on the basis of retention data methyl and 3-butenyl isothiocyanates as acrid minor components of horseradish and of wasabi.[7]

There has also been some speculation concerning the degradation of allyl isothiocyanate both on steam distillation of horseradish to form carbonyl sulphide, carbon disulphide and hydrogen sulphide,[10] and in processed horseradish products on prolonged storage to produce allylamine and allyl cyanide.[11] However, no evaluation has been reported of the significance of these various minor components and degradation products in the aroma of horseradish.

The presence of allyl thiocyanate in systems where the corresponding isothiocyanate

is predominant is well known,[12] and in the case of *Lepidium sativum* L. the benzyl thiocyanate found has been shown to be formed by an enzymic mechanism.[13] It has also been recognised that the isothiocyanate ⇌ thiocyanate rearrangement occurs in the case of the allyl group and can be brought about by heat in such systems as processed mustard oil.[14] However, allyl thiocyanate has not been reported as occurring in horseradish volatiles or shown to be present in commercial horseradish oils.

That different varieties of horseradish have different aroma qualities which exist over and above the pungency is well recognised. In particular, Hungarian horseradish and cultivated horseradish from some other areas have a fine odour which is preferred by the manufacturer of processed horseradish products.

In consequence, this study has two objectives: first, to establish rigorously the identity of the principal components of horseradish aroma and, second, to explain in chemical terms why the aroma of one variety should differ from that of another.

2. Experimental

2.1. Raw material

Five samples were available: roots of English horseradish, grown in East Anglia in a mineral (i.e. sandy, upland) or a peaty (i.e. dark, alluvial) soil, roots of Hungarian horseradish and samples of commercially flaked dried Hungarian and Japanese horseradish. The fresh roots were stored wrapped in polyethylene and the dried samples in air-tight bottles, both at 4 to 6 °C.

2.2. Preparation of essence

As in the U.K. horseradish is normally eaten in a prepared but uncooked form, steam distillation with the inherent risks of artifact production is not an appropriate method of preparing an essence. Headspace analysis is the most direct method for identifying compounds and was later used for assessing the more volatile constituents. However, in view of the small quantities involved and of the interest in higher-boiling components, headspace examination is not suitable for combined (g.c.–m.s.) analysis. Hence, solvent extraction was chosen to prepare an essence, the components of which were to be separated and identified. The solvents used were redistilled on a fractionating column, the ether having been first treated for the removal of peroxides by the method of Vogel.[15]

Freshly washed, peeled and coarsely grated horseradish (4 g) was packed into a small Soxhlet thimble and extracted with solvent (10 ml pentane, 4 ml ether) for 2 h. The contents of the thimble were replaced by fresh material (4 g) and the extraction continued for a further 2 h. This procedure was carried out two more times. The extract was then reduced to 1 ml by removal of the solvent on a small fractionating column. Where a more concentrated essence was required, the essence, contained in a glass vial with a long open neck, was carefully warmed and allowed to evaporate to 30 to 50 μl.

The flaked, dried samples of horseradish were reconstituted, prior to extraction, by shaking in a stoppered flask with excess of water at 30 °C for a period of 17 h. Before weighing out, the reconstituted horseradish was dried externally on filter paper and the coarse shreds finely chopped by hand.

2.3. Gas chromatography

2.3.1. Flame ionisation detector

The essences were analysed by g.c. under the following conditions.

Column: 70 ft (21.3 m) glass capillary P.L.O.T.[16]
Coating: 0.8% w/v Carbowax 20M solution in methylene chloride containing 0.04% Atpet-80 to reduce tailing.
Carrier gas: nitrogen, 2 ml/min.
Detector oven 200 °C, injector 160 °C.
Programme: isothermal for 10 min at 90 °C, then increasing at 4 deg. C/min to 180 °C.
Sample size: 0.5 μl.

From the chromatograms seven peaks were found to be common to all the essences and a semi-quantitative comparison was made on the basis of peak areas, assuming the peaks to be triangular and the area to equal height × width at base/2. Where peaks are attenuated, the area is multiplied by the attenuation factor. A further assumption is that the detector response is not only linear, but equal for all the compounds involved. The results are given in Table 1. The first set of figures gives the area of each peak of the essence in arbitrary but consistent units. The percentage given is a fraction of the total area represented by the seven major peaks.

TABLE 1. Gas chromatographic peak areas (f.i.d.) of the main components of horseradish essences

Type of horseradish	Peak 1	Peak 2 2-butyl isothio-cyanate	Peak 3 allyl isothio-cyanate	Peak 4 allyl thio-cyanate	Peak 5 4-pentenyl isothio-cyanate	Peak 6	Peak 7 2-phenethyl isothio-cyanate
English (mineral	0.12	1.10	21.75	0.95	0.24	0.60	15.9
soil)	0.3%	2.7%	53.5%	2.3%	0.6%	1.5%	39.2%
English (peaty soil)	0.10	0.52	20.6	0.74	0.25	0.55	14.25
	0.3%	1.4%	55.7%	2.0%	0.7%	1.5%	38.4%
Hungarian	0.16	0.26	26.4	1.28	2.01	1.85	20.4
	0.3%	0.5%	48.4%	2.5%	4.0%	3.7%	40.5%
Dried flaked	0.05	0.07	8.85	0.32	0.14	0.42	10.34
Hungarian	0.25%	0.35%	44.3%	1.6%	0.7%	2.1%	51.3%
Dried flaked	Trace	Trace	15.25	1.07	1.00	0.24	16.1
Japanese	—	—	45.4%	3.2%	3.0%	0.7%	47.8%

2.3.2. Electron capture detector

As an aid to identification of the components present, the essences were chromatographed under the same conditions as above but with an electron capture detector (e.c.d.). The flame ionisation detector (f.i.d.) gives approximately a mass response to most organic compounds, whereas the e.c.d. responds only to certain types of compounds. It has a high sensitivity of response to compounds containing halogen atoms, conjugated carbonyl groups and a range of sulphur compounds, but poor response to

the majority of organic compounds. The ratio of response of e.c.d. to f.i.d., ϕ, has been used as a parameter in the identification of sulphur compounds.[17] In the present context the e.c.d. has provided useful additional information to that of identification by mass spectrometry.

The essences were run under the following conditions:

e.c.d. source: Ni^{63}, 10 mCi. Pulse space:150 μS. Column flowrate:2 ml/min. Purge flowrate: 60 ml/min.

Peaks 3 and 4 were found to give a high response on e.c.d., the ratios of response, ϕ, being 40 and 400 respectively.

2.3.3. Gas chromatography–mass spectrometry

Mass spectra of the seven peaks were obtained from an English horseradish (mineral soil) essence. The gas chromatographic conditions were as previously, except that the carrier gas was helium and the flowrate 4.5 ml/min. The mass spectrometer operating conditions were as follows:

Ion source pressure: 3.0×10^{-6} Torr. Connecting line: 180 °C. Top hat: 160 °C. Multiplier voltage: 3.0 kV. I_{beam}: 50 μA. I_{fil}: 2.4A. I_{box}: 0.01 mA. Scan: 3 s for *m/e* 16 to 300. U.v. recorder chart speed: 5 in/s.

Standard compounds were prepared (see later). The mass spectra of the horseradish peaks are given below alongside those of the standard compounds. The peaks in each spectrum are given in order of decreasing intensity, the appropriate background spectrum having been subtracted first.

2.3.3.1. Peak 1 m.w. = 78

76 38 37 40 26 32 28 44 78 49 25.

Unknown—the spectrum has some peaks in common with that of carbon disulphide[18] (m.w. = 78: 76 32 44 78 38 28 77 64 34 14), but this compound gives a very low f.i.d. response and would be eluted more quickly so as to be totally obscured by the solvent.

2.3.3.2. Peak 2 m.w. = 115

29 41 27 56 57 39 44 115 86 55 60 72 42.

Laboratory prepared 2-butyl isothiocyanate gave: m.w. = 115

29 41 27 56 57 28 115 39 86 26 55 60 59 42 32.

2.3.3.3. Peak 3 m.w. = 99

41 99 39 72 27 26 40 38 45 37 71 32 59 98 100.

Allyl isothiocyanate (Hopkin & Williams Ltd.) gave: m.w. = 99

41 99 72 39 38 27 40 26 45 37 42 44 46 58 34.

2.3.3.4. Peak 4 m.w. = 99

41 99 39 27 72 38 40 37 45 32 71 46 58 59 67.

Laboratory prepared allyl thiocyanate gave: m.w. = 99

41 99 39 27 26 72 38 45 44 40 46 37 25 59 58.

2.3.3.5. Peak 5 m.w. = 127

41 27 29 39 67 70 99 72 55 53 85 126 127 42 60.

Laboratory prepared 4-pentenyl isothiocyanate gave: m.w. = 127
41 27 39 29 67 70 72 53 55 26 99 40 85 126 127.

2.3.3.6. Peak 6 m.w. possibly = 185

43 91 41 44 27 39 29 163 42 70 57 71 55 56 51.

Unknown—by comparison with available reference spectra[29] it was considered that this compound was not an isothiocyanate.

2.3.3.7. Peak 7 m.w. = 163

91 163 39 27 65 51 105 77 92 41 50 79 26 72 103.

Laboratory prepared 2-phenethyl isothiocyanate gave: m.w. = 163
91 163 39 27 65 105 51 77 92 50 79 41 72 63 26.

2.3.4. Retention indices

Retention indices were determined for both the standards and for the unknown components of the horseradish essences.

Two columns were used:

1. a 73 ft (22.25 m) P.L.O.T. column coated with 0.8% w/v Carbowax 20M (+Atpet 80–0.04%),
2. a 71 ft (21.64 m) P.L.O.T. column coated with 0.5% w/v silicone elastomer S.E. 30.

Carrier gas: nitrogen at 2 ml/min.

F.i.d. chromatographic runs were temperature programmed at an increase of 2 deg. C/min unless stated otherwise from a starting temperature of 75 °C.

At least three chromatographic runs were made in each instance to obtain retention ndices (r.i.), which were reproducible to within five units.

The results are given in Table 2.

TABLE 2. Retention data for horseradish volatiles and appropriate reference compounds

	Carbowax 20M			Silicone elastomer S.E. 30	
Reference compound		Horseradish		Reference compound	Horseradish
Name	r.i.	Peak no.	r.i.	r.i.	r.i.
Isopropyl isothiocyanate	1136		1137	829	
Ethyl isothiocyanate	1180		1185	812	
Methyl isothiocyanate	1183		1185	751	
2-Butyl isothiocyanate	1217	2	1220	929	930
Allyl isothiocyanate	1300	3	1308	900	902
Allyl thiocyanate	1386	4	1388	1065	1065
4-Pentenyl isothiocyanate	1469	5	1470	1089	1085
2-Phenethyl isothiocyanate	2117	7	2119	1510	1515

As, according to the chromatogram on Carbowax 20M, the methyl, ethyl and isopropyl isothiocyanates appear to be present in very low concentration in all the essences and as these substances are eluted rapidly from the S.E. 30 column, their peaks were expected to be obscured by the solvent on the latter column.

2.4. Laboratory preparations of standards

2.4.1. Methyl, ethyl, isopropyl and 2-butyl isothiocyanates

These isothiocyanates were prepared by the same general method from the corresponding amine or ammonium hydrochloride salt.

To a solution of thiophosgene (0.018 mol) in chloroform (25 ml) was added with stirring a solution of the amine (0.018 mol) in water (7 ml). Then slowly with continued stirring, 1 N-sodium hydroxide (39.5 ml) was added dropwise. The chloroform layer was separated straight away, dried with anhydrous calcium chloride and the solvent removed on a rotary evaporator. The residue was distilled.

Methyl isothiocyanate distilled at atmospheric pressure and the fraction b.p. 114 to 115 °C (lit. 119 °C/760 mm)[19] was collected. On cooling it gave a white crystalline solid; it was noted to be a very powerful lachrymator with a smell resembling horseradish.

Ethyl isothiocyanate distilled at atmospheric pressure and the fraction b.p. 130 to 132 °C (lit. 132 °C/760 mm)[19] was collected. The product was a yellow oil, extremely pungent with a slightly garlicky aroma.

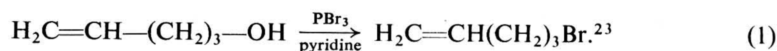
Isopropyl isothiocyanate distilled at atmospheric pressure and the fraction b.p. 137 to 137.5 °C (lit. 137 °C/760 mm)[19] was collected. It was pungent, but the aroma was not distinctive.

2-Butyl isothiocyanate was distilled under vacuum and the fraction b.p. 54 °C/13 mm (lit. 47 °C/11 mm)[20] collected. The product was a colourless oil with a faintly acrid but characteristic, leafy-green odour.

2.4.2. Allyl thiocyanate[21]

Potassium thiocyanate (12.1 g) in dimethyl sulphoxide (50 ml) was cooled to 0 °C in ice. Allyl bromide (7 ml) was added dropwise with shaking, the temperature being kept at 0 °C. When the addition had been completed, the flask was transferred to a water bath at 30 °C and shaken for 1 h. The fine white precipitate of potassium bromide formed was filtered off and the solution extracted with diethyl ether (20 ml). This ether extract of allyl thiocyanate was not purified further and was used for chromatographic analysis. It was noted to have a garlicky–horseradishy aroma, but was not pungent.

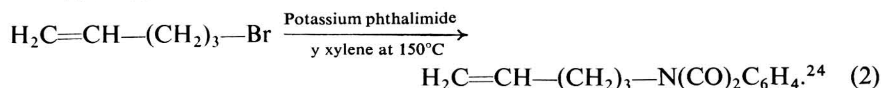
2.4.3. 4-Pentenyl isothiocyanate[22]



$$H_2C{=}CH{-}(CH_2)_3{-}OH \xrightarrow[\text{pyridine}]{PBr_3} H_2C{=}CH(CH_2)_3Br.^{23} \qquad (1)$$

4-Penten-1-ol (5 g, Koch-Light Ltd) in pyridine (1.3 g) was stirred at −25 to −30 °C in an ethanol–dry ice bath, whilst phosphorus tribromide (6.55 g) was added dropwise over about 30 min. The mixture was then distilled and the fraction, b.p. 128 to 130 °C at atmospheric pressure, collected. The distillate was washed twice with water, then with

dilute sodium hydroxide, and was dried over anhydrous calcium chloride overnight. Yield: 49% (70%).[22]



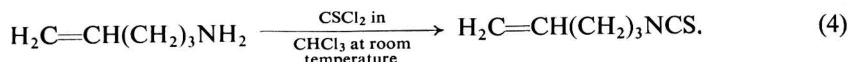
$$H_2C{=}CH{-}(CH_2)_3{-}Br \xrightarrow[\text{y xylene at 150°C}]{\text{Potassium phthalimide}} H_2C{=}CH{-}(CH_2)_3{-}N(CO)_2C_6H_4.^{24} \quad (2)$$

Potassium phthalimide (5.43 g) was mixed with dry xylene (7.7 ml) and 5-bromo-1-pentene (4.21 g) and the mixture stirred and refluxed at an oil bath temperature of 150 °C. The product was cooled, mixed with water (12.9 ml) and 2 N-sodium hydroxide (2.6 ml) and extracted three times with ether. After drying the extract overnight with anhydrous sodium sulphate, ether and xylene were removed on a rotary evaporator. The residue was distilled under vacuum to give a colourless oil, b.p. 174 °C/8 mm (163 °C/2 mm)[22] in a yield of 40% (75%).[22]

$$H_2C{=}CH(CH_2)_3N(CO)_2C_6H_4 \xrightarrow[\text{EtOH 100°C}]{\text{Hydrazine}} H_2C{=}CH(CH_2)_3NH_2.^{22} \quad (3)$$

The phthalimide (2.43 g) and hydrazine hydrate (0.625 g) in ethanol (14 ml) were refluxed for 1 h. The suspension was digested with 6 N-HCl (46.7 ml) at 100 °C for a further hour, and concentrated on the rotary evaporator to about 20 ml. It was then made strongly alkaline with sodium hydroxide, salt was added to saturation and the amine was extracted with ether. The extract was dried with potassium hydroxide pellets and the ether removed by fractional distillation.

Yield of 4-pentenylamine = 81% (83%).[22]



$$H_2C{=}CH(CH_2)_3NH_2 \xrightarrow[\text{CHCl}_3\text{ at room temperature}]{\text{CSCl}_2\text{ in}} H_2C{=}CH(CH_2)_3NCS. \quad (4)$$

Thiophosgene (1.04 g) in chloroform (12.5 ml) was stirred with 4-pentenylamine (0.8 g) in water (3.5 ml). 1 N-Sodium hydroxide (19.7 ml) was added dropwise to the stirred solution and the chloroform layer separated and dried. The solvent was removed on the rotary evaporator under vacuum, 4-pentenyl isothiocyanate being obtained as a faintly yellow oil, b.p. 78 °C/13 mm (75 °C/12 mm. Yield 35.5%).[22]

2.4.4. 2-Phenethyl isothiocyanate[25]

Phenethylamine (18.9 ml) and triethylamine (63 ml) in sodium-dried ether (75 ml) were treated dropwise with stirring at −5 °C with carbon disulphide (9 ml). The mixture was then kept in a stoppered flask in a refrigerator until the following day, when, at −5 to −10 °C, phosphorus oxychloride (14 ml) in dry ether (15 ml) was added dropwise with stirring and the mixture again left overnight. The solution was decanted from the precipitate and filtered. The precipitate was washed twice with ether by vigorous shaking and the ether washings combined. They were washed with aqueous sodium bicarbonate and dried with anhydrous sodium sulphate. The ether was removed on a rotary evaporator and the product distilled, the fraction b.p. 134 to 136 °C/5 to 6 mm (143 to 145 °C/12 mm. Yield 55%)[25] being collected.

2.5. Headspace analysis of horseradish

Headspace analysis enables the more volatile compounds which would be lost in preparing an essence to be detected and it also gives a more accurate picture of the pattern of volatiles as sensed by the nose.

The fresh roots of horseradish were washed, peeled and coarsely grated. A sample (25 g) was placed in a 100-ml round-bottomed flask and sealed with a tap and rubber septum fitting.[26] Samples of commercially flaked, dried horseradish (15 g) were placed

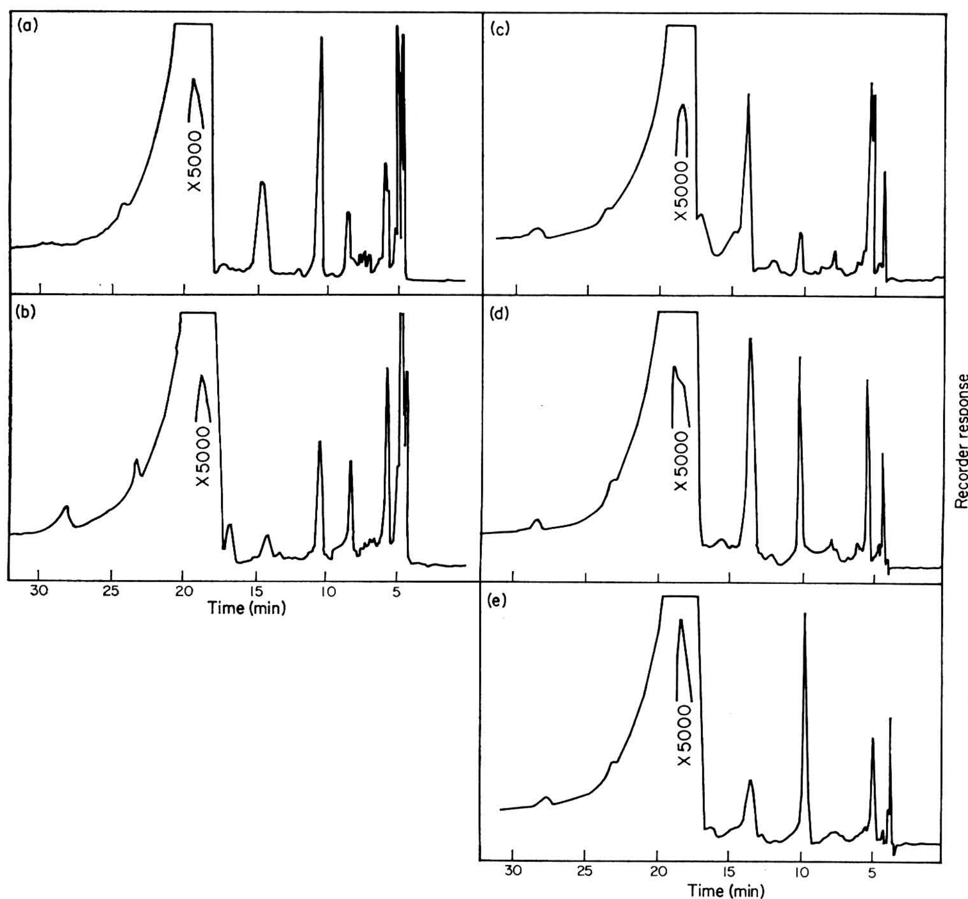


Figure 1. Chromatograms of vapour samples (20 ml) taken from the headspace above (a) reconstituted dried Hungarian horseradish; (b) reconstituted dried Japanese horseradish; (c) fresh English horseradish (mineral soil); (d) fresh English horseradish (peaty soil); (e) fresh Hungarian horseradish. Column 71 ft (21.64 m) P.L.O.T., coated with 0.8% Carbowax 20M; temperature programmed from 70 °C at 2 deg. C/min; flow rate, 2 ml/min nitrogen; f.i.d. attentuation ×500.

in similar flasks and distilled water (50 ml) added. The flasks were shaken in a constant temperature bath at 30 °C, vapour samples for analysis being withdrawn at various intervals.

In order to use a P.L.O.T. column for vapour sample analysis, a U-bend was placed in the gas stream immediately in front of the column and was immersed in a small Dewar flask containing liquid nitrogen. Vapour (20 ml) was injected using a 5 ml gas-tight

syringe and, when the oven had reached operating temperature, the Dewar flask was removed to start the run. By this means the components of the vapour sample were flushed onto the column as a sharp slug, rather than dispersed in a large volume of air.

A 71 ft (21.64 m) P.L.O.T. column, coated with 0.8% w/v Carbowax 20M + 0.04% Atpet 80, was used at a nitrogen flowrate of 2 ml/min. The runs were programmed from 70 °C at 2 deg. C/min.

Changes with time in the peak heights of the components relative to one another for each particular type of horseradish were noted. Also, at the same interval of time from grating or reconstitution, both qualitative and quantitative differences in the headspace chromatograms between different horseradish types were apparent. Those at 50 min are illustrated in Figure 1.

2.6. Odour evaluation

With a splitting system fitted before the detector, the column effluent could be sniffed

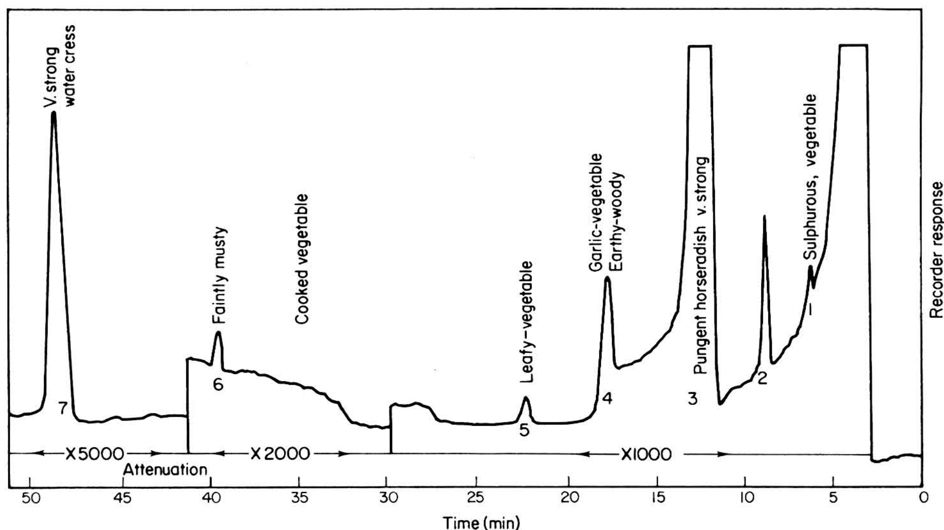


Figure 2. Chromatogram of Soxhlet essence of English horseradish (mineral soil). Column 70 ft (21.3 m) P.L.O.T., coated with 0.8% w/v Carbowax 20M; temperature, initially 90 °C (10 min), then programmed at 4 deg C/min; flowrate 2 ml/min nitrogen. Aromas observed at the sniffer are recorded at the corresponding positions on the f.i.d. chromatogram.

and an odour assessment made of the various peaks in the essences as they were eluted.

With the essences the only really distinctive aromas observed were those of the two major components, allyl and 2-phenethyl isothiocyanate. Various other, leafy-green, vegetable-like aromas were detected (see Figure 2), but these smells were not sufficiently characteristic to be ascribed to a particular variety of horseradish.

In comparing the headspace analyses, although the chromatograms were different, the differences were not reflected by corresponding differences in aromas, i.e. where a peak was present in one sample but not in another, that peak was not found to have any

significant odour. However, in the first group of peaks emerging from the column, despite a general putrid vegetable background, varying in intensity from one variety to another, there was also noted to be present a characteristic "sweet sickly" aroma. This aroma was particularly strong in the two dried samples and a similar less intense aroma was present in the fresh varieties. Fresh Hungarian horseradish did not appear to have the vegetable-like background aroma found with the early peaks in the other samples.

In order to study the early peaks more closely, some headspace analyses were also run on a 130 ft (39.62 m) P.L.O.T. column coated with Carbowax 1540 (0.8% w/v). Runs were made isothermally at 50 °C with a column flowrate of 2 ml/min of nitrogen. This gave a better separation of these early peaks and led to a more accurate allocation of aromas to certain peaks in this region.

2.7. Study of the isothiocyanate–thiocyanate system

Isothiocyanates can be distinguished from thiocyanates by their i.r. spectra. To characterise the allyl thiocyanate prepared, its i.r. spectrum was therefore determined between sodium chloride plates and compared with that of allyl isothiocyanate (Hopkin & Williams Ltd). The results are given in Table 3.

TABLE 3. Characteristic infrared absorption for allyl thiocyanate and isothiocyanate

Compound	Absorption between 2000 and 2500 cm^{-1}	Assignment	
Allyl thiocyanate	2151 cm^{-1}, strong	2140 cm^{-1}, strong, thiocyanate —C≡N stretch[27]	
Allyl isothiocyanate	2091 cm^{-1}, very strong; 2166 cm^{-1}, strong	2050–2150 cm^{-1}, very strong; 2170–2221 cm^{-1} strong	asymmetric stretching of —N=C=S[28]

It is clear from the absorptions in the 2100 cm^{-1} region that one sample contains predominantly allyl thiocyanate and the other predominantly the isothiocyanate. However, when the laboratory prepared allyl thiocyanate was analysed by gas chromatography, two peaks were observed, the additional one having the same retention time as allyl isothiocyanate. From the peak areas the mixture contained 24% allyl isothiocyanate and 76% allyl thiocyanate. As the i.r. spectrum of allyl thiocyanate gave no indication of the presence of any allyl isothiocyanate, rearrangement seems to be occurring during the gas chromatographic analysis either on the column or in the heated injection port. Rearrangement on the column is also suggested by the shape of the peaks in the chromatogram, tailing occurring from the allyl isothiocyanate to the allyl thiocyanate peak.

2.8. Allyl thiocyanate in horseradish

Kirk, Black and Mustakas[14] have indicated that the heating of an essential oil leads to an equilibrium mixture of allyl isothiocyanate and allyl thiocyanate. They demonstrated

such an equilibrium to exist between isothiocyanate and thiocyanate in both synthetic and natural systems. Irrespective of whether the isothiocyanate or the thiocyanate had been synthesised, the product after equilibration at 100 °C consisted of a mixture with the thiocyanate present in 7.7 to 9.9% concentration.

It would seem from this work that it is impossible to obtain either the thiocyanate or the isothiocyanate in a form completely free of its isomer. On this basis it was thought desirable to determine the thiocyanate: isothiocyanate ratios for a number of sources of allyl isothiocyanate. These are given in Table 4.

Table 4. Allyl isothiocyanate: thiocyanate ratios

Source of mixture	Average composition (%) Allyl isothiocyanate	Allyl thiocyanate
"Horseradish oil", commercial	92.7	7.3
Essence of Soxhlet extraction of fresh English horseradish (mineral soil)	95.8	4.2
Steam distillate of fresh English horseradish (mineral soil)	89.5	10.5
Allyl isothiocyanate (Hopkin & Williams Ltd)	93.0	7.0

The compositions were calculated from the peak areas of the chromatograms, run isothermally at 90 °C, assuming that only two components are present and that the detector is equally sensitive to both.

If the isothiocyanate is sufficiently heat labile to rearrange on heating at 100 °C, the question arises to what extent does rearrangement occur either on the capillary column or in the heated injection port.

There is no easy way of examining for rearrangement in the injection port, as, when it is unheated, peaks tend to spread out and become broad with tailing. However, rearrangement on the column was examined by injecting a sample of the mixture, trapping out the allyl isothiocyanate into a P.L.O.T. U-tube and then re-running the trapped isothiocyanate. The resultant chromatogram showed only a single peak in the isothiocyanate position, no peak at the thiocyanate position being present. This was repeated a number of times with the same results, which provided conclusive evidence that rearrangement was not occurring at 90 °C on the column.

3. Discussion

Five components of horseradish aroma have been identified by mass spectrometry and gas chromatography retention data, and these compounds have been shown to be common to several different types of horseradish.

Of the five components, the major two, allyl and 2-phenethyl isothiocyanate, have been generally recognised as the principal pungent compounds of horseradish. 2-Butyl isothiocyanate has also been previously identified in wasabi[9] and Japanese horseradish,[7,8] but by u.v. spectrometry and paper and g.l.c. 4-Pentenyl isothiocyanate has

not been reported before as a component of horseradish, but was originally identified in the seed cake of rape.[22]

The literature concerning isothiocyanate–thiocyanate rearrangements makes the presence of allyl thiocyanate in horseradish not unexpected. However, it had not been previously detected, possibly because gas chromatographic resolution in earlier work had not been sufficient to separate the isomers. Altogether, it does not seem to have been generally recognised that any heat processed allyl isothiocyanate-containing oil, e.g. commercial horseradish oil, will inevitably contain a proportion of allyl thiocyanate.

Retention data alone provide some evidence for the presence of small amounts of methyl, ethyl and isopropyl isothiocyanate in horseradish. The substances responsible for a further number of peaks present in the essences were not identified, but they did not appear to make a significant contribution to the odour.

Alcohols, aldehydes, ketones and a range of sulphur compounds are commonly found in the volatiles of vegetable foodstuffs. It is therefore somewhat unusual that virtually only isothiocyanates have been detected in horseradish. However, the isothiocyanates of horseradish are present in such large amounts compared with the common volatiles of other foodstuffs, that with the degree of concentration employed here, only the isothiocyanates could be detected. In other words, sixteen grams of horseradish suffice for the extraction of 1 ml of essence, whereas in most other investigations of vegetables a number of kg is used in the preparation of a few microlitres of essence (both essences still containing substantial amounts of extracting solvent).

The exact significance in the overall aroma of horseradish of each of the compounds identified is difficult to assess. The pungent, lachrymatory allyl isothiocyanate is clearly the most important component and, when it is lost below its natural level in cabbages, the product appears abnormally flat and dull in flavour.[30] The other major component present in almost equivalent amounts is 2-phenethyl isothiocyanate and this compound is known to have the very distinctive aroma of fresh water cress. It perhaps gives one a tingly sensation, but does not appear to be pungent or lachrymatory. Despite the high proportion of 2-phenethyl isothiocyanate present in horseradish and its distinctive aroma, no note characteristic of this isothiocyanate is evident on smelling freshly grated horseradish, i.e. this compound is either masked or blends with others present so as to lose or change its aroma qualities.

An explanation of the differences in the aroma qualities between the varieties could be simply due to changes in the proportions of the two major odour compounds. A small change in the proportions could well be sufficient to alter significantly the overall aroma effect. However, it is possible that variations in the minor isothiocyanates could be significant.

Although isothiocyanates have aromas which characterise them as a group of compounds, each is sufficiently distinctive to be identifiable above the general pungency, sharpness and lachrymatory qualities. For example, 2-butyl isothiocyanate has a slight acridness, but nevertheless has a distinctive leafy-green aroma. It has an oiliness about its smell, but with a certain vegetable-like quality. 4-Pentenyl isothiocyanate is also an isothiocyanate as characterised by its acridness, but it has a thinner, lighter, more fragrant quality about its aroma, though also perhaps a slight leafiness. Allyl thiocyanate has a strong and characteristic aroma, very garlic-like in quality, perhaps similar to the

corresponding isothiocyanate but lacking any pungency or lachrymatory properties. These distinctive qualities of the minor components must be expected to exert some effect on the overall aroma quality.

On the basis of the work reported here, the explanation for the greater desirability of the Hungarian horseradish could lie in one or both of the following factors.

1. It contains a significantly greater proportion of 4-pentenyl isothiocyanate.
2. The major differences in the odour assessments on the headspace vapours of the samples examined lay in a group of early peaks. A sickly sweet aroma was detected in this region, varying in intensity and possibly being associated with different notes, against a general background of cooked cabbage to putrid vegetable aromas. The compounds responsible are likely to be volatile sulphur compounds, which would be largely lost in essence preparation. For the Hungarian horseradish, the sickly sweet odour and the vegetable-like background were less pronounced and allowed a more aromatic note considerable prominence.

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References

1. Stoll, A.; Seebeck, E. *Helv. chim. Acta* **1948**, 31, 1439.
2. Isaac, O.; Kohlstaedt, E. *Arch. Pharm., Berl.* **1962**, 295, 165.
3. Stahmann, M. A.; Link, K. P.; Walker, J. C. *J. agric. Res.* **1943**, 67, 49.
4. Heiduschka, A.; Zwergal, A. *J. prakt. Chem.* **1931**, 132, 201.
5. Winter, A. G. *Hippokrates* **1957**, 28, 695.
6. Guillaume A.; Shajik, A. *Tunis. med.* **1951**, 39, 951.
7. Kojima, M.; Akahori, Y.; Ichikawa, I.; Mochizuki, M. *J. Ferment. Technol., Osaka* **1966**, 44, 177.
8. Kojima, M.; Ichikawa, I. *J. Ferment. Technol., Osaka* **1969**, 47, 263.
9. Nagashima, Z. *J. agric. Chem. Soc. Japan* **1954**, 28, 119.
10. Schildknecht, H.; Rauch, G. *Z. Naturf.* **1962**, 17b, 800.
11. Weber, F. E. *Univ. Microfilms* No. 64-8465, p. 160, or *Dis. Abst.* **1964**, 25, 1142.
12. Gmelin, R.; Virtanen, A. I. *Acta chem. scand.* **1959**, 13, 1474.
13. Virtanen, A. I.; Saarivirta, M. *Acta chem. fenn.* **1962**, B35, 102.
14. Kirk, L. D.; Black, L. T.; Mustakas, G. C. *J. Am. Oil Chem. Soc.* **1964**, 41, 599.
15. Vogel, A. I. *Practical Organic Chemistry* Longmans, London. **1957** 3rd edition, 163.
16. Cronin, D. A. *J. Chromat.* **1970**, 48, 406.
17. Hartmann, H.; Oaks, D.; Dimick, K. P. *Analyt. Chem.* **1964**, 36, 1560.
18. Cornu, A.; Massot, R. *Compilation of Mass Spectral Data and* 1*st Supplement* Heyden, London. **1966** and **1967**.
19. Kojima, M.; Akahori, Y.; Ichikawa, I. *J. agric. Chem. Soc. Japan* **1968**, 42, 185.
20. Kjaer, A.; Friis, P. *Acta chem. scand.* **1962**, 16, 936.
21. Yoneda, S.; Kitano, H.; Fukui, K. *J. chem. Soc. Japan* **1962**, 65, 1816.
22. Kjaer, A.; Jensen, R. B. *Acta. chem. scand.* **1956**, 10, 1356.
23. LaForge, F. B.; Green, N.; Gerodorff, W. A. *J. Am. chem. Soc.* **1948**, 70, 3707.
24. Kharasch, M. S.; Fuchs, C. F. *J. org. Chem.* **1944**, 9, 359.
25. Martin, D.; Beyer, E.; Gross, H. *Chem. Ber.* **1965**, 98, 2425.
26. Gilliver, P. J., personal communication.
27. Lieber, E.; Rao, C. N. R.; Ramachandran, J. *Spectrochim. Acta* **1959**, 13, 297.
28. Svatek, E.; Zahradnik, R.; Kjaer, A. *Acta chem. scand.* **1959**, 13, 442.
29. Kjaer, A. *Acta chem. scand.* **1963**, 17, 2143.
30. McLeod, A. J. *Flavour Ind.* **1970**, 1, 665.

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Food and Microbiology Groups Symposium September 1971

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A Rapid, Inexpensive Counting Technique using Agar Droplets

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This miniature pour plate technique is finding rapid acceptance in our laboratories for enumeration of total viable counts on foods, fabric or skin, for disinfectant testing and research work on pure cultures. At least three times as many samples per day can be processed, compared with the standard pour plate method. It possesses most of the advantages common to miniature methods (speed, economy, short incubation time) but few of the disadvantages (needing extra skill, causing eye fatigue, or requiring separate handling to other analyses which may be carried out). The technique can be used entirely manually, but its full value is realised when two simple aids are employed.

Plating out is done in standard Petri dishes, but each dilution is plated out as a row of five 0.1-ml droplets. Plating can be done entirely manually using disposable capillary pipettes (Harshaw Chemicals Ltd, Daventry, Northants), but preferably using the specially designed foot operated Diluted/Dispenser. With this, polypropylene drinking straws (Hygienic Drinking Straws Co. Ltd, Fishponds, Bristol) may be used. Decimal or centimal dilutions are made in bottles of 9 ml melted agar, transferring 1.0 or 1.0 ml, respectively. Dilution may be done entirely manually but is facilitated by the Diluter/Dispenser.

Three centimal dilutions suffice for a completely unknown sample (10^2 to 10^9 organisms/g), but for most samples two dilutions, i.e. two rows of five droplets, are adequate. By placing four rows in the dish and four in the lid, four samples can be plated out in one Petri dish. Diluting and plating a sample takes 40 to 50 s if the Diluter/Dispenser is used. Sample numbers need be written once only, and dilution factors need not be written since the order of concentrations in the droplet rows is self evident.

Incubation time is shorter than for the standard pour plate, e.g. 24 instead of 48 h. The small colonies are counted using a bench lens, stereomicroscope or the specially designed Projection Viewer which throws a bright image of a droplet, magnified to standard Petri dish size, onto a screen in front of the technician. Colonies can be counted easily, even in the presence of considerable quantities of food debris. From one to five droplets are counted—we generally try to count at least 50 colonies. The accuracy is excellent; there is usually no significant difference between droplet and standard pour plate counts. In a few instances, where aeration is important, the droplet technique will give a higher figure.

Unlike many other miniature methods this technique integrates easily with other standard analyses made at the same time, e.g. *Staphylococcus aureus* by spread plates, or coliform counts by plate or MPN methods. In fact, when the Diluter/Dispenser is being used these conventional bacteriological methods are also facilitated. The technique is very easily learned and

quickly preferred by most technicians when the two aids are available. Savings in manipulative time, Petri dishes, media and pipettes alone justify use of the technique, for in most laboratories the cost of the two aids is recovered several times in a year. However, reduced incubation time may be the most important feature for many laboratories. Other features such as reduced demand for incubator space and portability may not always be noticed.

Dye Reduction Tests on Meat Products

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Enzymic reduction of dyes has been investigated by several workers[1-9] as a rapid means of assessing microbial activity in meat products.

Various suggested procedures have been applied to practical examination of meat and meat products and have proved useful for the testing of cooked meats. A rapid test has been devised for assessing the bacteriological condition of cooked cured meats using resazurin. 20 ml of resazurin solution (0.0001 %) are added to a plastic pouch containing 100 g of sliced product and the contents are kneaded for about 20 s. The fluid is then transferred from the pouch to a glass bottle, incubated for 20 min at 37 °C and the colour assessed on a 7 point scale, as in milk testing.

A significant correlation has been found between colour and bacterial load, as expressed by a colony count at 30 °C. About 600 samples have been tested by the technique with counts ranging from 100 to 100 million/g. The complete colour range of the resazurin, from blue to colourless, was obtained.

The method is much less useful with raw meats owing to the high enzymic activity of the meat itself. Anomalies have also been encountered with some cured meats where high reducing activity appears to have been associated with the use of ascorbate as one of the curing ingredients.

References

1. Proctor, B. E.; Greenlie, D. G. *Fd Res.* **1939**, 4, 441.
2. Straka, R. P.; Stokes, J. L. *Fd Technol.* **1957**, 22, 412.
3. Wells, F. E. *Fd Technol.* **1959**, 24, 584.
4. Walker, H. W.; Coffin, W. J.; Ayres, J. C. *Fd Technol.* **1959**, 24, 578.
5. Saffle, R. L.; May, K. N.; Hamid, H. A.; Irby, J. D. *Fd Technol.* **1961**, 11, 15.
6. Wilhelmsen, J. B. 11th European Research Workers Belgrade **1965**.
7. Baker, K. R. *Fd Mf.* **1966**, 41, 4.
8. Losonczy, S. *Husipar* **1969**, 18, 4, 161.
9. Bradshaw, N. J.; Dyett, E. J.; Herschdoerfer, S. H. *J. Sci. Fd Agric.* **1961**, 4, 341.

Gases in Blue Cheeses

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The principal organism of ripening of blue cheeses is *Penicillium roqueforti*. *In vitro* experiments reported in the literature show this organism to be active at very low levels of O_2 in a N_2 atmosphere, but inhibited by increasing CO_2 concentrations. The conclusion drawn from

these observations, that the contribution of the skewering operation in blue cheeses was to lower the CO_2 in cheeses to a level permitting mould activity, was subjected to a study.

A gas chromatograph using a thermal conductivity detector system was used to estimate concentrations of carbon dioxide, nitrogen and oxygen in the cheeses throughout their ripening life. From 10 days after manufacture the gas composition within the cheese was $CO_2 - 20\%$ v/v, $N_2 - 80\%$ v/v, $O_2 - 0\%$ v/v.

The level of CO_2 remains constant throughout the pre-piercing period in spite of a slow diffusion of this gas from the cheese as the result of the difference in internal and external gas concentration.

After piercing the level of CO_2 drops sharply to 9% v/v, whilst the level of oxygen rises to approximately 10%. Blueing, rise in pH and rise in free amino acids follow within a few days of piercing. It has been shown that in unpierced cheeses made from curd seeded with spores, the levels of O_2 and CO_2 remained virtually unchanged until natural cracks developed in the cheese coat due to loss of moisture.

Cheese pierced in an atmosphere of CO_2 and nitrogen did not blue owing to the lack of a supply of oxygen.

The proposition appears to be justified that the piercing step supplies the oxygen required by the blueing organism and reduces the CO_2 level to establish a ratio of $CO_2:O_2$ within the cheese suitable for the activity of the organism *P. roqueforti*.

Viability of *Staphylococcus aureus, Salmonella typhimurium* and *Salmonella senftenberg* Heated and Recovered on a Solid Medium of Controlled Water Activity

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The effect of water activity (A_w) of the heating and recovery medium on the heat resistance of *Staphylococcus aureus*, *Salmonella typhimurium* and *Salmonella senftenberg* was studied.

The solid medium heat resistance method employed involved heating, by immersion, of surface inoculated agar plates and recovery of survivors *in situ*, thus eliminating post-heating dilution and plating and approaching the conditions encountered by bacteria in heat treated foods.

The organisms were heated at 55 °C on a basal medium of tryptone soya agar (A_w 0.997). The A_w of the medium was adjusted to lower levels with glycerol, NaCl or sucrose. The main disadvantage of the heating technique was that heat resistance studies could only be carried out at A_w levels permitting growth on the heating/recovery medium. The minimum A_w levels were as follows: *S. aureus*; 0.93/glycerol, 0.90/NaCl or sucrose, *S. typhimurium* and *S. senftenberg;* 0.945/glycerol and 0.98/NaCl or sucrose. Below these A_w levels no visible growth was observed under the conditions of these experiments.

Heat resistance of *S. aureus* and *S. typhimurium* increased as the A_w of the medium decreased: the percentage survival passed through a maximum at an A_w level which was related to the solute used. For instance, *S. aureus* exhibited some relationship between maximum heat resistance and the tolerance of the unheated culture to reduced A_w for a particular solute. Such a relationship could be used to predict the response of a given organism to the modified solute content of a food. The maximum survival of *S. aureus* heated at 55 °C for 12 min occurred at a higher A_w with glycerol (A_w 0.97) than with NaCl (0.92) or sucrose (0.90). These maxima on solid media differed from those obtained when *S. aureus* was heated at 55 °C for 5 min in suspension and plated on media of controlled A_w: the maximum survival was at A_w 0.997, viability being reduced to one quarter at A_w 0.98 for sucrose and NaCl. With glycerol the same decrease in viability occurred at A_w 0.93.

The maximum survival of both *S. typhimurium* and *S. aureus* heated at 55 °C occurred at the same A_w (0.97) with glycerol. This maximum was not affected by the duration of heating.

As a contrast, heat resistance of *S. senftenberg* 775W at 55 °C was virtually unaffected by reduction in the A_w of the heating/recovery medium.

Viability Studies on Ascopores and Vegetative Cells of *Saccharomyces cerevisiae* Exposed to Microwaves at 2450 MHz

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In an attempt to distinguish between thermal and postulated non-thermal effects of microwaves, apparatus was designed whereby the thermal energy generated on positioning yeast cell suspension (5-ml aliquots, concentration adjusted to about 2×10^5 cells/ml) in a microwave field, of variable intensity, was preferentially absorbed by hexane. Hexane was chosen as the heat transfer medium on the basis of its low dielectric constant and freezing point, together with its intermediate thermal conductivity value. Cell suspensions were contained within an oscillating double shell pyrex heat exchanger, through which a flow of hexane from a refrigerated tank (held at − 30 °C) was maintained, in order to prevent the overall suspension temperature from approaching the thermal death points (t.d.p.), of the cells. In a control experiment employing conventional heating, the t.d.p. of vegetative cells and ascopores were found to approximate to 4 min at 56 and 60 °C, respectively. The heat exchanger was incorporated in a waveguide coupled to a variable power microwave generator, equipped with precision timer controls. It was found that at a power level of 40 watts and without the coolant being cycled, a total loss of viability was observed after exposures of 30 and 40 s for vegetative cells and spores, respectively, the recorded suspension temperature in each case exceeding the t.d.p. With cooling, at the same power level, total loss of viability was observed after 8 and 12 min for vegatative cells and spores, respectively, but in neither case did the recorded suspension temperatures exceed 33 °C. The result is attributed to super heating at the microthermal level and the mechanism and implications of this effect are discussed.

Studies on Kenkey, a Ghanaian Cereal Food

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Kenkey is a fermented maize dumpling, and a staple food of a large part of the people of Ghana. In the preparation of Kenkey the maize grain is first cleaned by winnowing and then washed. It is then soaked for 1 to 2 days at about 30 °C, mixed into a dough with water and left to undergo spontaneous fermentation for 2 to 3 days. The dough is then divided into two equal parts. One is made into a slurry with more water and boiled into a thick porridge called "Aflata". The other part is mixed back, once the Aflata has cooled. The dough is then divided, scaled and shaped into balls which are wrapped in leaves. They are then immersed in water and boiled in large iron pots. The final product is referred to as Kenkey.

There are many varieties of Kenkey. Some contain additives such as sugar, salt or sweet potato. Some made from whole meal are dark in colour, some made from high extraction flour are white. Some are wrapped in plantain leaves, others in maize sheafs. Shape and method of wrapping also differ widely.

Microbiological aspects

Fermentation proceeds in several stages. Initially *Aspergillus* and *Penicillium* spp. predominate, but soon decline to reappear later. *Lactobacteria* multiply rapidly after about 9 hours of fermentation reaching a peak between 24 to 36 hours. Wild yeasts appear on the surface of the dough after the first day. Together with *Lactobacteria*, these yeasts form the predominant micro-organisms during the later phase of fermentation.

Nutritional aspects

Table 1 shows that the proximate composition of Kenkey is similar to that of the original maize. Hence there is little protein loss due to processing. Previous work in this laboratory has shown that the same is true for Koko, but not for Ogi, where considerable protein losses occur.

By analogy with Ogi and Koko it is probable that water soluble vitamins and trace elements are lost during processing. Investigations in this laboratory are now centred on the effect of the Kenkey making process on calcium and phytic acid levels. Maize is especially low in calcium but high in phosphorus. Since there is evidence that phytic acid may interfere with calcium metabolism in man (the obligatory enrichment of wheat flour with chalk in the U.K. is based on this premise) it is essential that the role of the interaction in maize Kenkey be established.

TABLE 1. Effect of Kenkey processing on proximate composition (% dry basis)

	Original maize	Soaked maize	Steeping water	Fermented dough	Kenkey	Cooking water
Moisture	15.0	32.4		51.0	64.5	
Total solids	85.0	67.6	0.4	49.0	35.5	1.3
Crude protein	10.7	11.4	0.2	11.7	11.6	0.2
Crude fat	4.9	4.0	Trace	1.5	1.4	Trace
Ash	1.5	1.5	Trace	1.5	3.1	n.d.
Crude fibre	1.7	1.8	Trace	1.7	1.7	Trace
Carbohydrate	81.2	81.3	n.d.	83.6	83.2	n.d.

n.d., not determined

The Antibacterial Action of Spices and its Nutritional Implications

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Spices have historically occupied an important role in the diet of most cultures. They are highly prized for their flavour and are traditionally credited with a wide range of pharmacological and preservative properties. Studies on a variety of spices have shown that they do in fact exhibit antibacterial properties, but most previous workers do not consider this effect of importance at dietary levels.[1]

During the course of nutritional studies in Ethiopia we have studied the antibacterial activity of local spices, and evaluated the effect of this on the nutritional status of the population.

Two mixtures of spices are used locally to flavour the stew, or wat, that is eaten at almost every meal. Chow is a mixture of 15 spices based on *Capsicum frutescens* and has a highly pungent flavour. It is added to the wat at a level of 11%. Alternatively, Alicha is added at a level of 2%, giving the wat a much milder flavour. This is eaten far less often than wat containing Chow.

The antibacterial activity of each mixture was assessed by incorporating it into agar at various levels and measuring the growth of a known inoculum. The method has been described elsewhere.[1]

Both spices were found to exhibit strong antibacterial action at normal dietary levels. Chow was active against Gram-positive and Gram-negative bacteria concerned in food poisoning and spoilage. Alicha was more active weight for weight, but had a narrower spectrum, exhibiting far less activity against Gram-negative species. It was particularly ineffective against *Escherichia coli*, and Chow was relatively ineffective against *Klebsiella aerogenes*.

The antibacterial activity was found to be stable to heating at 120 °C for 30 min. It was not affected by the action of dilute acids or alkalis.

In a series of feeding experiments on rats it was observed that the faeces of rats fed Chow exhibited similar antibacterial action to the spice and the magnitude of this was related to the amount of spice fed.

With heavy inocula (10^5 to 10^6 organisms per plate) significant growth of *Staphylococcus aureus* did occur on plates containing Chow. However, when this happened the characteristic pungent odour of the spice was replaced by a sickly, sweet smell. This would render the wat totally unpalatable.

It seems likely that spices play an important role in preventing food spoilage and bacterial food poisoning, both by their antibacterial effect and by indicating the presence of viable organisms.

The presence of antibacterial substances in the gut is known to exert both beneficial and harmful effects on health and nutritional status.[2] Despite the high level of consumption of spices in Ethiopia, the prevalence of avitaminosis B is negligible[3] nor has avitaminosis K been reported.[3] On the other hand, preliminary studies on Ethiopian infants suggest that the spice may exert a significant effect in the prevention of gastroenteritis.

Changes in diet towards western patterns, and especially the changes in infant feeding patterns that are encouraged in Ethiopia, minimise the opportunity for the spices to exert a protective effect and the implications of this are disturbing.

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References

1. Rivers, J. P. W.; Hill, A. W. *Proc. Nutr. Soc.* **1971**, 30, 77A.
2. Hötzel, D.; Barnes, R. H. *Vitams. Horm.* **1966**, 24, 115.
3. I.C.N.N.D. Nutritional Survey in Ethiopia **1959**.

Enumeration of certain Lactic Acid Bacteria from Wiltshire Bacon-curing Brines

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Methods for the enumeration of lactobacilli, leuconostocs and pediococci were studied using samples of bacon-curing brine from three factories. A selective plating method used successfully with a wide range of other sources, e.g. human sources,[1] silage[2] and cheese,[3] proved to be unsatisfactory as either no or very few colonies developed.[4] This method involves the use of a nutritionally adequate selective plating medium, e.g. a Tween-containing medium of pH value 5.4 incorporating 0.2M acetate buffer; the double-layer plate method or incubation in an atmosphere of either $N_2 + CO_2$ or $H_2 + CO_2$ may be used. The reason for the unsuitability of this technique for bacon-curing brine was that the bacteria were sensitive to the selective agent acetate.[4]

For bacon-curing brines Keddie's medium[5] pH value 5.4, without added acetate, was found to be a satisfactory selective plating medium. However, it was necessary to use an atmosphere of $H_2 + CO_2$ for incubation to provide strictly anaerobic conditions. Otherwise other bacteria, i.e. micrococci, were able to develop because the selective action of the medium was reduced by the omission of acetate and the use of $N_2 + CO_2$ did not provide the strictly anaerobic conditions necessary to prevent their development. It was essential to prevent the growth of micrococci as they inhibited the development of lactic acid bacteria. A comparison of different temperatures of incubation in the range 17 to 37 °C resulted in the routine use of 25 °C for up to 6 days. Dilutions were prepared in Tween broth and plates were inoculated on the surface by the drop plate method which gave higher colony counts than the pour plate method. By using the procedure outlined colony counts of 10^2 to10^6/ml were obtained. Few colonies developed on Tween agar medium containing 5% (w/v) or more added NaCl.

A provisional identification of colonies was made by examining three features in Tween semi-solid agar cultures inoculated directly from the isolation medium, i.e. morphology, catalase activity and homofermentative or heterofermentative ability. Results were checked on representative cultures after purification. The colonies examined comprised homofermentative lactobacilli and leuconostocs in similar proportions; only one colony appeared to be that of a pediococcus.

Lactic acid bacteria form a very minor part of the flora of Wiltshire bacon-curing brines and as such are probably of little significance. However, this work illustrates the principle that a selective enumeration and isolation procedure evolved for use with specific sources is not necessarily suitable for work with the same type of micro-organism from a different source.

References

1. Rogosa, M.; Mitchell, J. A.; Wiseman, R. F. *J. Bact.* **1951**, 62, 132.
2. Keddie, R. M. *Proc. Soc. appl. Bact.* **1951**, 14, 157.
3. Mabbit, L. A.; Zielinska, M. *J. appl. Bact.* **1956**, 19, 95.
4. Varnam, A. H.; Grainger, J. M. *J. appl. Bact.* **1970**, 33, iii.
5. Harrington, W. F; McCance, M. E. *Laboratory Methods in Microbiology*, Academic Press, London, and New York. **1966**, p. 264.

Water Uptake in Beans

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"Hardshell" in leguminous seeds, a condition described as a failure of seeds to imbibe water within a reasonable time, is a problem of long standing with many conflicting views put forward.

A close examination under the binocular microscope of a random batch of beans reveals a population of large, round beans with a micropyle in the open condition and a much smaller population of small, flat beans with a micropyle in the closed condition.

The water uptake of intact seeds and "naked" cotyledons of harricot beans (*Phaseolus vulgaris* sp.) was investigated at 25, 50, 75 and 100 °C.

The half-times of water uptake by intact seeds possessing a micropyle in the open condition are reduced by a factor of two when compared with water uptake by the corresponding "naked" cotyledons. This factor is temperature independent and points to the fact that in large intact seeds the structural features responsible for allowing the water to enter through the seedcoat are also temperature independent.

The half-times of uptake of water of intact small seeds are reduced by a factor of 10 below 50 °C and by a factor of 5 at all temperature above 50 °C when compared with water uptake by the corresponding "naked" cotyledons.

The difference in the behaviour of intact small seeds as compared to the intact large ones is that in the former seeds, the micropyle is in a closed condition, whilst in the latter it is in the open condition. Once a temperature of 50 °C has been reached, the micropyle of the small seed opens to its maximum size. It is speculated that the dimensions of the micropylar openings of large and small seeds are of the ratio of 2.5.

Blocking techniques using araldite revealed that the micropyle is the most significant water inlet channel into the seed, though the testa and parahilum provide channels of alternative water entry through at lower diffusion rates.

Measurements of rates of diffusion through "naked" cotyledons show them to be temperature dependent and inversely dependent upon the linear dimensions of the cotyledon.

Effect of Cultural Conditions on Proteases Produced by *Pseudomonas* Spp.

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Two psychophilic and two mesophilic *Pseudomonas* spp. were grown at 5, 25 and 35 °C with and without aeration. Protease activity was followed by a casein precipitation technique.

A scheme was devised for the isolation of enzyme systems produced and cellulose acetate electrophoresis was employed to determine the heterogeneity of the systems. Species and strain differences in the number of enzyme components were demonstrated which were influenced by the conditions of growth.

Model Systems in Heat Resistance Studies

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Variations in the environment, with respect to pH, salts, inhibitory agents and food constituents, have important practical implications relative to the resistance of bacterial cells, spores and enzymes in foods which undergo heat processing. Changes in the heat resistance of thermophilic and mesophilic bacterial spores and of exo-enzyme systems were shown to occur as a result of the use of model systems of heating menstrua.

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Although unnecessary standardisation is not desired, and due allowance for the type of subject matter must be made, papers submitted to the Journal should conform as far as possible to the following pattern:

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Acknowledgements.

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Spore Research 1971

edited by A. N. Barker
Department of Bacteriology, The University, Leeds, England
G. W. Gould
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March 1972, xiv+370 pp., approx. £5.75

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T. Wall and Sons (Ice Cream) Limited, London, England

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