THE TOCOPHEROL CONTENT OF FARM FEEDING-STUFFS

By FRED BROWN

The amounts of the individual tocopherols in some common farm feeding-stuffs have been determined and the presumable vitamin-E potencies of the feeding-stuffs calculated from them. Grass, clover and lucerne contained 10–40 mg. of tocopherol/100 g. of dry matter, and it has been shown that the concentration in grass fell to a low level as the plant matured. Only minute amounts of tocopherols were present in root crops. Barley, maize, oats and wheat contained 1–7 mg. of tocopherols/100 g., but the vitamin-E activity of these crops was lower than the total tocopherol-content might suggest, because of the presence of appreciable amounts of the less active β- and γ-compounds. The bearing of the results on naturally-occurring muscular dystrophy in farm animals is discussed.

Introduction

In a recent review Blaxter & Brown[1] have pointed out that spontaneous muscular dystrophy in sheep and cattle is a problem of considerable practical importance. Thus Willman *et al.*[2] have reported that naturally-occurring muscular dystrophy or ' stiff-lamb disease ' is present in a high proportion of the spring lambs in many States of America and in other countries; the naturally-occurring disease in cattle has been described in detail by Hjärre & Lilleengen[3, 4] and Slagsvold & Lund-Larsen.[5] Willman *et al.*[2] were able to prevent the disease by giving vitamin E to the lambs.

In order to obtain a fuller understanding of the role of vitamin E in the prevention of muscular dystrophy in farm livestock it is important that the vitamin-E activity of common farm feeding-stuffs should be known. The total tocopherol-content of several feeding-stuffs has in fact been determined (see review[1] for list of references), but the figures are of limited value because the four naturally-occurring tocopherols differ considerably in their ability to prevent typical signs of vitamin-E deficiency, such as foetal resorption in the rat or muscular dystrophy in the rabbit.[6] As a consequence, any chemical method for the determination of the vitamin-E content of a foodstuff should distinguish between the individual tocopherols. In this paper estimates of the tocopherols in some common farm feeding-stuffs are recorded. No attempt has been made to survey the vitamin-E content of the numerous varieties of each crop; instead, emphasis has been placed on determination of the tocopherol pattern present.

Experimental

The method used for determining the individual tocopherols has been previously described.[7, 8] It differs from other chemical methods in that the final estimation involves separation of the individual tocopherols by chromatography on filter paper coated with petroleum jelly, with 75% ethanol as the mobile phase. The zones containing the individual tocopherols are extracted with ethanol and the extracted tocopherols are estimated by the $\alpha\alpha'$-dipyridyl–ferric chloride method of Emmerie & Engel.[9] Distinction between the β- and γ-isomers, which have the same R_F value under these conditions, is made possible because the γ-, but not the β-isomer, reacts with sodium carbonate and diazotized *o*-dianisidine to yield a purple dye. The total tocopherol-content of the materials was measured by the Emmerie–Engel method on an aliquot of the concentrate used for chromatography; in effect the total tocopherol-content determined by this method is merely a measurement of the ferric-reducing activity of the concentrate.

Results

The values obtained for the tocopherol contents of the various feeding-stuffs are shown in Tables I and II. For most crops the analyses were straightforward, but a brief description of the more interesting features of the analysis of each group of feeding-stuffs is included below.

Grass and its products

Four species, cocksfoot (*Dactylis glomerata*, S143), meadow fescue (*Festuca pratensis*, S53), perennial rye-grass (*Lolium perenne*, S23) and timothy (*Phleum pratense*, S48) were allowed to grow to maturity and examined at various stages of growth during the season. These grasses were provided by Dr. R. Waite and were representative of the samples recently examined by Waite & Boyd.[10] The only tocopherol found in the grasses was the α-compound, but cocksfoot contained in addition a substance that gave a purple colour with sodium carbonate and diazotized *o*-dianisidine. This substance was not γ- or δ-tocopherol, however,

Table I

Effect of stage of growth of plant on the α-tocopherol contents of four grasses

Date cut (1952)	Average height, in.	α-Tocopherol, mg./100 g. of dry matter	Date cut (1952)	Average height, in.	α-Tocopherol, mg./100 g. of dry matter
Cocksfoot			*Perennial rye-grass*		
16 Apr.	3·5	26·1	17 Apr.	3·8	35·2
23 ,,	4·5	28·9	24 ,,	5·0	28·8
29 ,,	7·2	28·6	6 May	6·3	25·0
6 May	7·2	34·1	13 ,,	6·2	17·9
13 ,,	9·6	34·0	21 ,,	8·3	12·1
26 ,,	19·6	17·6	27 ,,	8·7	13·8
2 June	18·9	13·0	3 June	10·0	11·5
9 ,,	22·7	8·5	10 ,,	10·1	12·4
18 Aug.	32·7	4·0	17 ,,	12·8	13·1
9 Sept.	33·2	2·9	1 July	20·9	7·4
			7 ,,	15·7	8·8
			25 Aug.	27·0	1·6
			15 Sept.	27·9	2·8
Meadow fescue			*Timothy*		
9 Apr.	3·8	30·0	10 Apr.	3·3	21·7
16 ,,	4·2	30·2	17 ,,	3·4	21·7
23 ,,	5·4	26·5	28 ,,	4·8	22·2
5 May	7·9	24·3	5 May	5·8	19·5
20 ,,	13·8	16·6	3 June	16·5	19·1
26 ,,	19·1	10·7	10 ,,	15·6	17·4
2 June	23·9	10·2	17 ,,	17·5	15·1
9 ,,	—	9·6	1 July	20·1	8·8
16 ,,	32·6	9·0	7 ,,	15·0	5·5
18 Aug.	27·2	2·3	25 Aug.	26·4	4·8
8 Sept.	29·1	2·6	15 Sept.	28·4	0·9

since it had an R_F value of 0·02, compared with values of 0·72 and 0·84 for the γ- and δ-compounds respectively.

In view of the importance of grass in the feeding of farm animals, the tocopherol contents of the four grasses were determined at various stages of growth. The results, summarized in Table I, show that the tocopherol content was high in young grass but fell to a low value as the plant matured. One of the major factors contributing to the fall taking place as the plant matures is the concomitant change in the ratio of leaf to stem. Grass stems are poor in tocopherol content, containing only about 2 mg./100 g., whereas leaf contains 20–30 times this quantity.

Determination of the tocopherol contents of grass of a given height (in the present work about 8–10 in.) at different times during the season showed that the amounts present in the four species did not vary to any marked extent. The values are shown in Table II. There was, however, a marked difference in tocopherol content between the species.

The increasing recognition of the value of artificially dried grass as a winter feed for farm animals prompted the determination of the losses in drying the four grasses. Losses of 25–45% were incurred when the freshly cut grasses were dried in a current of air at 105° for 45 minutes under laboratory conditions. These losses are similar to those that Cabell & Ellis[11] found by the rat-assay method on grass samples dried at 60° for 24 hours.

Hay and silage are highly variable products, so that the values reported here can be regarded as typical only of the samples examined. Cocksfoot hay, prepared from grass 12 in. in height, contained approximately 1·8 mg. of α-tocopherol/100 g. of dry matter after four months' storage; after 16 months this value had fallen to 1·3 mg. These figures indicate that losses approaching 90% are incurred during the making of hay and its early storage. Silage-making was found to lead to losses of about 10%; samples of silage prepared from cocksfoot grass 9 in. high contained approximately 30 mg. of α-tocopherol/100 g. of dry matter. It is evident, even from the small number of determinations reported here, that artificial drying and silage-making are much superior to haymaking in preserving the tocopherols of grasses.

Clover, lucerne, kale and other green fodders

All these crops were similar to the grasses in containing α-tocopherol only. In each of

Table II

The tocopherol contents and calculated vitamin-E potencies of feeding-stuffs

Feeding-stuff	Variety	Total tocopherols, mg./100 g. of dry matter	Individual tocopherols, % α-	β-	γ-	δ-	Artifacts *	Calculated vitamin-E potency,† mg./100 g. of dry matter
Grasses (8–10 in.)	Cocksfoot fresh	31·3–36·2	100	0	0	0	0	31–36
	Cocksfoot dried	17·4–24·2	100	0	0	0	0	17–24
	Meadow fescue fresh	18·4–24·3	100	0	0	0	0	18–24
	Meadow fescue dried	12·1–15·0	100	0	0	0	0	12–15
	Perennial rye-grass fresh	30·0–40·0	100	0	0	0	0	30–40
	Perennial rye-grass dried	18·7–23·5	100	0	0	0	0	19–24
	Timothy fresh	18·4–24·9	100	0	0	0	0	18–25
	Timothy dried	10·0–17·2	100	0	0	0	0	10–17
Hay	Cocksfoot	1·3–1·8	100	0	0	0	0	1·3–1·8
Silage	Cocksfoot	30·1–31·0	100	0	0	0	0	30–31
Clover	Wild white fresh	20·2	> 95	0	0	0	< 5	20
	Wild white dried	12·6–13·0	> 95	0	0	0	< 5	12–13
Lucerne	Dupuits, dried	26·4	> 95	0	0	0	< 5	26
	Grimm, dried	28·0	> 95	0	0	0	< 5	28
	Ontario Variegated, dried	27·6	> 95	0	0	0	< 5	28
	Provence, dried	21·8	> 95	0	0	0	< 5	22
Kale	Marrow-stem leaf	78·7	> 95	0	0	0	< 5	79
	Marrow-stem stem	0·9–1·5	> 95	0	0	0	< 5	0·9–1·5
	Thousand-head leaf	37·1	> 95	0	0	0	< 5	37
	Thousand-head stem	1·2–1·6	> 95	0	0	0	< 5	1·2–1·6
Sugar-beet top	Sharpes Klein E leaf	51·2	90	0	0	0	10	47
	Sharpes Klein E petiole	1·7	—	—	—	—	—	—
Mangold	Eldorado	0·8	—	—	—	—	—	—
Fodder beet	Yellow Daeno	1·2	—	—	—	—	—	—
	Red Otofte	0·5	—	—	—	—	—	—
Purple-top swede	Peerless	1·2–1·3	—	—	—	—	—	—
Barley	Bére	5·6–7·1	10	67	0	0	23	1·7–2·1
Oats	Yielder	1·7–3·2	26	63	0	0	11	0·8–1·4
Wheat	Yeoman seed	3·4–3·9	50	20	0	0	30	1·9–2·2
	Yeoman bran	6·9–8·7	14	6	0	0	80	1·1–1·4
Maize	Ground	4·2	10	0	90	0	0	1·2
	Flaked	1·1	10	0	90	0	0	0·3
Beans	Scottish carse	6·6–8·2	9	0	86	0	5	1·9–2·4
Linseed		23·6	0	0	95	0	5	4·7
Coconut meal		0·4	—	—	—	—	—	—
Palm-kernel cake		0·4	—	—	—	—	—	—
Ground-nut meal		0·4–0·5	—	—	—	—	—	—
Fish meal		2·3	100	0	0	0	0	2·3

* The artifacts were assumed to possess no vitamin-E activity

† The vitamin-E potency was calculated by assuming that the biological activities of the individual tocopherols are in the ratio $\alpha : \beta : \gamma : \delta = 100 : 30 : 20 : 1$ (Embree [6])

these materials there was also present a small amount of a substance that had an R_F value of 0·02, reduced ferric chloride and gave a purple colour with sodium carbonate–diazotized *o*-dianisidine. This was presumably the same as the compound found in cocksfoot. Lucerne

was found to be a rich source of α-tocopherol, in confirmation of the estimates by Cabell & Ellis[11] with the rat-assay method. Wild white clover also contained appreciable amounts. The leafy portions of kale and of sugar-beet tops were rich in α-tocopherol, but the stems of kale and the petioles of sugar beet contained only about $\frac{1}{30} - \frac{1}{50}$ of the amounts present in the leaves.

Barley, oats, maize and wheat

The values shown in Table II for these products indicate the importance of using, for the determination of tocopherols, more specific chemical tests than hitherto. The concentrates prepared from wheat seed and wheat bran gave three spots with R_F values 0·50, 0·72 and 0·93 on spraying the developed chromatogram with αα′-dipyridyl and ferric chloride. The spots at R_F 0·50 and 0·72 arose from α- and β-tocopherol respectively, but the spot with R_F 0·93 did not correspond to any of the known tocopherols. The substance giving this spot accounted for 30 and 80% of the ferric-reducing power of the wheat-seed and wheat-bran concentrates respectively. It has been shown previously[8] that a substance with R_F 0·93 provided 9% of the total reducing power of wheat-germ oil.

Concentrates prepared from oats and barley gave chromatograms similar to those of the wheat products. Three spots, with R_F values of 0·50, 0·72 and 0·93, were obtained on the sprayed chromatograms. No spots were obtained when the chromatograms were sprayed with sodium carbonate and diazotized *o*-dianisidine. The spot with R_F 0·93 is probably due to the same compound as that occurring in the wheat products, and that with R_F 0·50 is probably due to α-tocopherol. The substance with R_F 0·72, which accounted for more than 60% of the total tocopherol-content of the concentrates prepared from oats and barley, is of considerable interest. It is not γ-tocopherol, since it does not react with sodium carbonate–diazotized *o*-dianisidine, but it may possibly be β-tocopherol. Preliminary experiments in which the absorption spectrum of the compound and its nitroso-derivative[12] have been examined indicate that this is likely. If these preliminary observations are confirmed, it will be the first record of β-tocopherol occurring in a natural product other than wheat.

γ-Tocopherol contributed 90% of the ferric-reducing activity of the maize concentrate applied to the chromatogram, and it has only one-fifth of the biological activity of the α-compound; the remaining 10% was due to α-tocopherol. Consequently the vitamin-E potency of maize is only 28% of what it would be if all the tocopherol present were α-tocopherol.

Miscellaneous

Mangolds, fodder beet and swedes contained such small quantities of substances that reduce ferric chloride that their individual tocopherol-patterns were not examined. Groundnut meal, palm-kernel cake and coconut meal were similarly low in total tocopherol-content and were not further examined. Linseed was a rich source of tocopherols, but more than 95% of the total reducing power was due to the γ-compound. Beans were also found to be rich in total tocopherols, although low in vitamin-E potency (see Table II); this confirms the observation of Harris, Quaife & Swanson.[13]

Discussion

The chemical determination of the total tocopherol-content of a food is of limited practical value, because the individual tocopherols vary widely in biological potency. The results shown in Table II, giving the proportions of the individual tocopherols present, have been used to compute the vitamin-E activities of the feeding-stuffs in terms of α-tocopherol, and these are given in the last column. For this the relative vitamin-E activities of the four naturally-occurring tocopherols have been taken to be[6]: α, 100; β, 30; γ, 20; δ, 1. It will be noted that the total tocopherol-content of some feeds corresponds with the vitamin-E activity, but for a number of others there is considerable divergence. It is possible to estimate by the method described here the vitamin-E activity of individual feeds and hence the potency of mixed rations.

Estimates of the vitamin-E potency of rations give incomplete information on whether the rations contain sufficient vitamin E to prevent the onset of deficiency signs. As pointed out elsewhere,[1] the unsaturated fatty acid content of the ration considerably modifies the requirement of animals and birds for vitamin E, and it is possible that other dietary ingredients have similar effects. An example of these dietary interactions of unknown nature can be inferred from the results published by Whiting, Willman & Loosli[14] on stiff-lamb disease. These workers gave to pregnant ewes two rations containing approximately equal quantities of total tocopherols. One ration consisted of clover and lucerne hays and cull beans and the

other of oat and grass hays, corn silage and a grain mixture. Ewes given the former ration produced lambs that developed muscular dystrophy, but the disease did not occur in lambs on the latter ration. The production of dystrophy was associated with a low tocopherol-content of the ewes' milk, and Whiting *et al.*[14] thought this was due to the low proportion of α-tocopherol in the clover, lucerne hays and beans. The analyses presented in Table II suggest that the so-called deficient diet probably contained more α-tocopherol than the other, since clover and lucerne contain α-tocopherol only, whereas the β- and γ-compounds form a large proportion of the tocopherols present in oats and maize. The contention of Whiting *et al.* therefore appears to be incorrect. The results of their experiment, however, emphasize the important interrelationship between vitamin E and other dietary factors.

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The Hannah Dairy Research Institute
Kirkhill, Ayr

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References

[1] Blaxter, K. L. & Brown, F., *Nutr. Abstr. Rev.*, 1952, **22**, 1
[2] Willman, J. P., Loosli, J. K., Asdell, S. A., Morrison, F. B. & Olafson, P., *J. Anim. Sci.*, 1945, **4**, 128
[3] Hjärre, A. & Lilleengen, K., *Virchows Arch.*, 1936, **297**, 565
[4] Hjärre, A. & Lilleengen, K., *Nord. med. Tidskr.*, 1936, p. 472
[5] Slagsvold, L. & Lund-Larsen, H., *Norsk VetTidsskr.*, 1934, **46**, 529
[6] Embree, N. D., *Annu. Rev. Biochem.*, 1947, **16**, 323
[7] Brown, F., *Biochem. J.*, 1952, **51**, 237
[8] Brown, F., *Biochem. J.*, 1952, **52**, 523
[9] Emmerie, A. & Engel, C., *Rec. Trav. chim. Pays-Bas*, 1938, **57**, 1351
[10] Waite, R. & Boyd, J., *J. Sci. Fd Agric.*, 1953, **4**, 197
[11] Cabell, C. A. & Ellis, N. R., *J. Nutr.*, 1942, **23**, 633
[12] Quaife M. L., *J. biol. Chem.*, 1948, **175**, 605
[13] Harris, P. L., Quaife, M. L. & Swanson, W. J., *J. Nutr.*, 1950, **40**, 367
[14] Whiting, F., Willman, J. P. & Loosli, J. K., *J. Anim. Sci.*, 1949, **8**, 234

CONNECTIVE TISSUE OF MEAT. II.*—Determination of Hydroxyproline

By L. C. BAKER, L. H. LAMPITT and K. P. BROWN

The density of colour produced in Neuman & Logan's method for the estimation of hydroxyproline was measured in a Hilger Spekker absorptiometer. On any one occasion satisfactory duplication was obtained with aliquots of the same solution of hydroxyproline, and a smooth curve resulted when concentration was plotted against colour density. However, from time to time the level of the curve fluctuated slightly, and therefore the colour should be developed in standard solutions when any ' unknowns ' are being estimated, as recommended by Neuman & Logan. The density of the colour was depressed to some extent by amino-acids present in the solution (e.g. in the form of protein hydrolysate) when the ratio of amino-acid to hydroxyproline was about 50 to 1 or greater.

Introduction

In 1950 Neuman & Logan[1] published a method for the determination of hydroxyproline and applied it to the acid hydrolysates of proteins. The method depends upon the oxidation of hydroxyproline by hydrogen peroxide, in alkaline solution in the presence of copper ions, to give an unknown compound which, after acidification, develops a red colour on heating

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with Ehrlich's reagent (*p*-dimethylaminobenzaldehyde in *n*-propanol). The density of the colour so developed is greater than that developed in the presence of isatin in the method described by McFarlane & Guest[2] upon which the Neuman & Logan technique is based.

Neuman & Logan[1] found that commercial gelatin and collagen prepared from mammalian hide, tendon and bone had an average hydroxyproline content of 13·4%, a figure which they state is unique among proteins. It was further found that tyrosine was the only other amino-acid 'commonly encountered in protein hydrolysates' which gave a colour under the conditions of the method; it developed 1·5% of the colour developed by an equal weight of hydroxyproline. (Pure tryptophan gave 0·7% of the colour developed by an equal weight of hydroxyproline, but humin formation during hydrolysis of the protein eliminated this possible source of error.)

In a later paper the same authors[3] applied the method to the determination of collagen and elastin in tissues, the high concentration of hydroxyproline in collagen making the method particularly applicable to tissues containing only a small amount of collagen, such as muscle (meat).

It appeared to the present authors desirable to compare the results obtained by their modification of the Lowry, Gilligan & Katersky[4] method[5] with those to be obtained using the Neuman & Logan technique.

Experimental

Instruments used

In the method of Neuman & Logan,[1] the amount of hydroxyproline in the unknown solution, which should be between 5 and 15 μg./ml., is determined by measuring the optical density (extinction) of the coloured solution developed from it, referred to a standard curve prepared at the same time from three standard solutions containing respectively 5, 10 and 15 μg. of hydroxyproline/ml. Using the photoelectric colorimeter described by Evelyn[6] and a No. 540 filter, Neuman & Logan found extinctions of approximately 0·15, 0·30 and 0·45 for these standard solutions. In this instrument the cell holding the coloured solution is a soft-glass test-tube 7 in. long and $\frac{7}{8}$ in. in internal diameter; this tube is supported in a plastic sleeve in which are two windows 1 in. × $\frac{7}{16}$ in. arranged diametrically opposite to one another to allow light to pass through. This arrangement gives a light path of approximately 2 cm., but clearly the curved surfaces through which the light enters and leaves the cell prohibit the determination of a molecular extinction coefficient.

In the work to be described measurements of colour density were made with a Hilger Spekker absorptiometer, by means of the 1-cm. cell, holding approximately 7 ml. of liquid. The dimensions of the cells for this instrument are such that the maximum length of light path obtainable with the 10 ml. of coloured solution produced in the Neuman & Logan[1] method is 1 cm., approximately half that used by those workers.

Relationship between colour density and concentration of hydroxyproline over the range 5–50 μg./ml

In order that the extinction measured should be in the region of maximum sensitivity, it was necessary to use solutions of hydroxyproline approximately double the strength of those used by Neuman & Logan; the colour production over the range of concentration of hydroxyproline of 5–50 μg./ml. was therefore studied. By means of a Unicam spectrophotometer, the wavelength of maximum absorption of light by the coloured solution was found to be 560 mμ, whereas Neuman & Logan[1] imply that they found it to be 550 mμ. An Ilford Spectrum Yellow-green filter No. 605 (transmission 530–575 mμ) was therefore used in the Spekker absorptiometer.

It was proved to be essential to prepare the Ehrlich reagent from *p*-dimethylaminobenzaldehyde recrystallized in the way recommended by Neuman & Logan[1] and redistilled *n*-propyl alcohol (the fraction boiling in the range 96–98°) in order to obtain solutions of a red hue and of the necessary degree of stability, otherwise the solutions had an orange-red hue and faded rapidly. Under these conditions the optical densities of the coloured solutions developed from aliquots of the same standard solution of hydroxyproline were in reasonable agreement, and a smooth curve was obtained relating optical density and concentration, although increment of colour per unit of hydroxyproline decreased slightly with rise of concentration. Nevertheless, throughout this work, although on any one occasion a smooth curve relating concentration to colour density has been

observed from time to time, the variation increasing with increasing hydroxyproline concentration. For this reason aliquots of three different standard solutions have been included with any 'unknowns' for colour development, as recommended by Neuman & Logan. The results upon which these conclusions are based are given in Table I, and the extreme variation in the level of the curve (Expts. 4 and 6) is illustrated in Fig. 1.

Table I

Relationship between concentration of hydroxyproline and colour density

	Extinction of hydroxyproline solutions						
Wt. of hydroxyproline, μg. ..	5	10	15	20	30	40	50
Expt. No. 1	0·106	0·190	0·269	0·319	0·461	0·562	—
	0·096	0·175	0·270	—	0·461	0·572	—
,, ,, 2	0·098	0·185	0·257	0·329	0·455	0·565	0·640
	0·100	0·188	0·240	0·324	0·459	0·570	0·635
,, ,, 3	0·082	0·165	0·237	—	0·406	0·554	0·635
	0·085	0·163	0·235	0·315	0·439	0·548	0·658
,, ,, 4	0·080	0·167	0·238	0·290	0·401	0·499	0·590
	0·081	0·160	0·231	0·291	0·400	0·494	0·570
,, ,, 5	0·092	0·183	0·237	0·306	0·439	0·538	—
	0·089	0·170	0·240	0·317	0·431	0·539	—
,, ,, 6	0·098	0·186	0·259	0·329	0·459	0·590	
	0·099	0·190	0·258	0·339	0·461	0·575	

Detailed study of the technique

The method was applied to acid hydrolysates of the aqueous autoclave-extract of the residue of connective tissue left after extracting raw, chopped meat with 0·1N-sodium hydroxide solution (cf.[5]); the duplication of the results obtained on aliquots of the same hydrolysate was sometimes poor, and agreement between different determinations on the same sample of meat was often unsatisfactory. The effects of varying the conditions of colour production on the extinction of the final coloured solution were therefore studied.

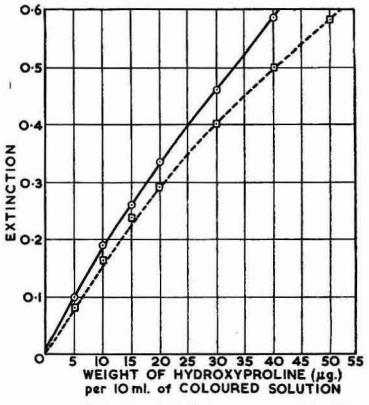

FIG. 1.—*Relationship between concentration of hydroxyproline and colour density*

The procedure in the method published by Neuman & Logan[1] is to add to 1 ml. of solution containing the hydroxyproline, 1 ml. of 0·01M-copper sulphate solution, 1 ml. of 2·5N-sodium hydroxide solution (at which stage the concentration of sodium hydroxide, 0·83N, is sufficient to prevent the separation of a visible colloidal precipitate of cupric hydroxide) and 1 ml. of 6% hydrogen peroxide solution. At this stage a yellow-brown colour develops, and a brown precipitate containing copper peroxide separates. The suspension is shaken occasionally, and after five minutes the solution is heated in a water bath at 80° for a further five minutes to destroy peroxide present in excess; during this heating a black precipitate normally settles out (cupric hydroxide). The solution is cooled in an ice–water mixture and acidified by addition of 4 ml. of 3N-sulphuric acid, with shaking; 2 ml. of Ehrlich's reagent is then added and the whole is heated in a water bath at 70° for 16 minutes to develop the red colour. Finally the coloured solution is cooled in water and its extinction measured. The authors do not suggest any particular limitation of the time elapsing between completion of colour development and the measurement of colour density, but they do state that the colour given by tyrosine may be differentiated from that given by hydroxyproline by its bronze hue, its much greater stability and its greater solubility in amyl alcohol; this statement implies that the colour

given by hydroxyproline is not entirely stable. Various factors have been studied in some detail.

(a) Effect of strength of copper sulphate

McFarlane & Guest[2] state that the concentration of copper sulphate affects the colour produced when isatin is allowed to react with the solution of oxidized hydroxyproline. In order to determine whether this copper-concentration effect applied to the Neuman & Logan technique, a series of experiments were carried out with varying concentrations of copper. The results (Table II) show that a reduction in copper concentration below that stated by the authors, leads to a decrease in colour density and an increase in copper concentration to a slight rise in colour density.

Table II

Effect of concentration of copper sulphate on colour density

	Extinctions					
Wt. of hydroxyproline, μg. ..	5	10	15	20	30	40
0·003M-$CuSO_4$	0·073 0·082	0·152 0·164	0·227 0·230	0·282 0·286	0·400 0·380	0·495 0·486
0·01M- ,, ...	—	0·188	—	0·335	0·460	0·561
0·02M- ,, ...	—	0·194	—	0·343	0·470	0·572
0·05M- ,, ...	—	0·191	—	0·349	0·480	0·588

(b) Effect of strength of sodium hydroxide solution and of impurities in it

The colour was developed in a number of aliquots of the same standard solution, containing 20 μg. of hydroxyproline/ml., with sodium hydroxide solution of increasing strength and also sodium hydroxide solution to which sodium carbonate had been added. The results obtained (Table III) show that the maximum colour density was produced with 2·5N-sodium hydroxide solution, the intensity of colour falling with both weaker and stronger alkali. It was noted that as the alkali used became weaker, more cupric hydroxide was precipitated initially, more copper peroxide was formed, and the evolution of gas during oxidation was less vigorous. The presence of carbonate did not affect colour development unless contamination was gross.

Table III

Influence of the concentration of sodium hydroxide on colour density

Normality of NaOH soln...	0·5	1·0	1·5	2·0	2·5	3·0	3·5	4·0	5·0
Extinction	0·239	0·34	0·371	0·381	0·371	0·340 0·344	0·330 0·340	0·314 0·313	0·299 0·289

(c) Effect of strength of hydrogen peroxide

McFarlane & Guest[2] used 1 ml. of 6% hydrogen peroxide for the oxidation of much larger quantities of hydroxyproline, 200–1600 μg., than those used by Neuman & Logan,[1] 5–15 μg., or, in these experiments, 5–50 μg. The colour was therefore developed in a number of aliquots containing 40 μg. hydroxyproline/ml., with different strengths of hydrogen peroxide. The results (Table IV) show that the maximum colour was developed with 1·8% peroxide.

Table IV

Influence of the strength of hydrogen peroxide on colour density

Strength of H_2O_2. %	0·6	1·8	3·0	4·2	6·0
Extinction	0·545 0·562	0·640 0·640	0·620 0·625	0·560 0·560	0·480 0·482

(d) Temperature of oxidation with hydrogen peroxide

Results given in Table V show that there is no significant difference between the optical densities of coloured solutions prepared when the oxidation is carried out at 10° or at 25°.

(e) Rate of fading of colour

The rates of fading of standard solutions containing 15, 30 and 40 μg. of hydroxyproline/ml. were followed and the results are plotted in Fig. 2, from which it will be seen that the stronger the colour the more rapid the fading; in 30 minutes a solution of optical density

Table V

Influence of oxidation temperature on colour density

Wt. of hydroxyproline, μg. . .	5	10	15	20	30	40	50
Extinction at 10°	0·080	0·175	0·219	0·318	0·459	0·564	0·660
,, ,, 25°	0·074	0·158	0·229	0·320	0·459	0·548	0·660

about 0·58 lost roughly 4% of its colour, whereas weaker solutions faded by only about 1·5% in the same time.

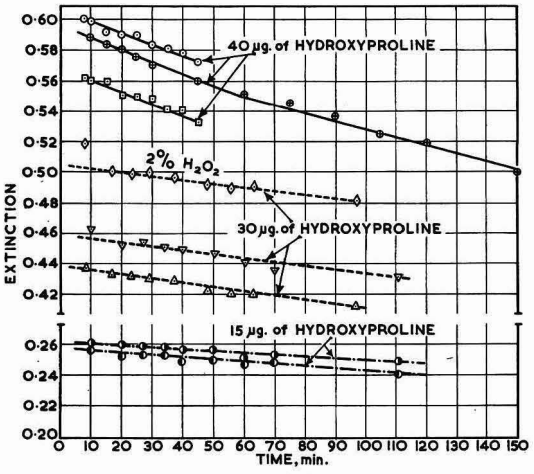

FIG. 2.—*Rate of fading of the coloured solution*

(f) Effect of added amino-acids

Neuman & Logan found that hydroxyproline was completely recovered when it was submitted to their conditions of hydrolysis, either alone or in the presence of an amino-acid mixture whose composition was such as would be derived from collagen, omitting hydroxyproline; the ratio of the weight of amino-acid mixture to weight of hydroxyproline was the same as that in collagen, namely about 8 to 1.

It is important to note, however, that when Neuman & Logan submitted hydroxyproline to acid hydrolysis in the presence of about 37 times its weight of casein or cattle haemoglobin, recovery of the hydroxyproline was not quite complete; they suggested that this was due to the reaction of the hydroxyproline with some substance liberated during the hydrolysis.

These findings have been substantiated, but, as shown in Tables VI, VII, VIII and IX, the ratio of the weight of amino-acid (other than hydroxyproline) to hydroxyproline is the factor that influences the development of colour. When this ratio is 50–100 to 1 the optical density of the coloured solution is appreciably lowered, whether the hydroxyproline is hydrolysed with the protein or whether it is added to the protein hydrolysate.

(i) *With a ratio of amino-acid to hydroxyproline up to* 50 *to* 1.—The results shown in Table VI were obtained by hydrolysing (i) sufficient of a solution of Bacto Difco gelatin (total nitrogen 15·8, moisture 12·3 and ash 2·2%) to give a final solution containing 40 μg. of hydroxyproline/ml. and (ii) sufficient of the gelatin solution containing added hydroxyproline to give a final solution containing 40 μg. of hydroxyproline/ml., of which half was added as hydroxyproline. Colour was developed in 1 ml. of each of these solutions and also in 1 ml. of (iii) a standard solution containing 40 μg. of hydroxyproline and (iv) a solution of the gelatin hydrolysate (i) to which hydroxyproline was added containing 40 μg. of hydroxyproline in all, of which half was due to hydroxyproline added as such.

To obtain the results given in Table VII a mixture of amino-acids was prepared in the

proportions found in collagen, but omitting hydroxyproline and proline (found to be contaminated with hydroxyproline); 0·17 g. of this mixture was dissolved in 100 ml. of water.

Table VI

Influence of amino-acids on colour density

	Hydroxyproline soln. (iii)	Gelatin hydrolysate + hydroxyproline (iv)	Hydrolysate of gelatin & hydroxyproline (ii)	Gelatin hydrolysate (i)
Ratio amino-acid/hydroxyproline ..	—	4 : 1	4 : 1	8 : 1
Extinction	0·590 0·590	0·589 0·580	0·589 0·580	0·590 0·584

Table VII

Influence of amino-acids on colour density

	Ratio amino-acid/ hydroxyproline	Extinction With 2% H_2O_2	Extinction With 6% H_2O_2
0·5 ml. of amino-acid soln. }	—	0·009 0·010	0·009 0·010
,, ,, water }			
,, ,, amino-acid soln. }	42 : 1	0·321 0·323	0·296 0·290
,, ,, hydroxyproline soln. }			
,, ,, water }	—	0·348 0·350	0·310 0·298
,, ,, hydroxyproline soln. }			

These results show that, with the standard strength of 6% hydrogen peroxide, the presence of 42 times as much amino-acid as hydroxyproline results in a slight reduction of colour production, of the same order as that found by Neuman & Logan.[1] When 2% hydrogen peroxide is used, the colour produced by hydroxyproline alone is significantly higher than that obtained with 6% hydrogen peroxide, but the presence of the other amino-acid results in a greater lowering of the optical density. This is an argument in favour of using 6% hydrogen peroxide, as recommended by Neuman & Logan.

(ii) *Preparation of a sample of muscle protein.*—This sample was prepared in order that aliquots of hydrolysate made from it could be used to determine whether it contained any hydroxyproline and whether the presence of muscle-protein hydrolysate influenced the colour produced from hydroxyproline.

Care was taken to ensure that the muscle protein was free from connective tissue. A sample of beef was freed from obvious fat and skin; approximately 250 g. was minced and then treated in a blender with 7% lithium chloride solution, about 2½ l. being used. After standing overnight the suspension was centrifuged in 250-ml. bottles. A layer of fat on the surface, in which some fibrous material was entrained, was held back while the main bulk of viscous liquid was decanted through glass wool, leaving a jelly-like layer at the bottom of the bottle. This jelly was mixed with more lithium chloride solution in the blender, centrifuged and the extract decanted off. The bulked extracts, which were free from any suspended matter, were diluted with water and heated to 80° to coagulate the protein. When the coagulated protein had settled, the slightly cloudy supernatant liquid was decanted and the residue washed with water, once by stirring and centrifuging and then on a Buchner funnel until free from chloride. The protein was freeze-dried and the dry product powdered in a mortar.

(iii) *With a ratio of amino-acids (in the form of muscle-protein hydrolysate) to hydroxyproline of approximately* 100 *to* 1.—In some of the techniques investigated for determining collagen in meat (see Part III, to be published) considerable amounts of non-collagenous protein are dissolved and the ratio of the weight of amino-acids other than hydroxyproline to weight of hydroxyproline is of the order of 100 to 1.

Muscle protein (0·2 g.) was hydrolysed in a sealed tube with 5 ml. of 6N-hydrochloric acid for 8 hours at 25 lb./sq. in. pressure, neutralized and diluted to 50 ml. The colour was developed in two series of solutions; in one series 0·5 ml. of water, and in the other 0·5 ml. of hydrolysate, was added to 0·5 ml. of a series of standard solutions of hydroxyproline, with the results shown in Table VIII.

Table VIII

Influence of amino-acids on colour density

Wt. of hydroxyproline added, μg.	0	10	20	30	40
Extinction when water is added	0·005	0·169	0·330	0·488	0·590
	—	0·183	0·330	0·498	0·594
Extinction when hydrolysate is added	0·028	0·150	0·260	0·410	0·531
	0·024	0·150	0·260	0·408	0·494

These results show that the colour developed by hydroxyproline is markedly depressed when amino-acids in the form of protein hydrolysate are present in the proportion of about 100 parts of hydrolysate amino-acid to 1 part of hydroxyproline. It is clear that the colour developed by muscle-protein hydrolysate alone is negligible.

(iv) *With a ratio of amino-acids (in the form of muscle-protein hydrolysate) to hydroxyproline of approximately* 1200 *to* 1.—Muscle protein (0·5 g.) was hydrolysed in a sealed tube with 10 ml. of 6N-hydrochloric acid, neutralized and diluted to 27·5 ml. With both 2% and 6% hydrogen peroxide the colour was developed in (*a*) 1 ml. of hydrolysate, (*b*) 1 ml. of standard solution containing 15 μg. of hydroxyproline, and (*c*) 1 ml. of (*a*) plus 15 μg. of hydroxyproline added with the 0·01M-copper sulphate solution (see Table IX).

Table IX

Influence of amino-acids on colour density

	(*a*) Hydrolysate	(*b*) 15 μg. of hydroxyproline	(*c*) Hydrolysate + hydroxyproline
Extinction, with 2% H_2O_2	0·096	0·280	0·191
,, ,, 6% H_2O_2	0·113	0·251	0·191

Although the hydrolysate developed reasonable colour the extinction of the standard solution to which hydrolysate was added was much less than the extinction of the standard alone. Furthermore, it should be noted that with pure hydroxyproline (*b*) the 2% peroxide gave a higher extinction, whereas when hydrolysate was also present (*c*) the same result was obtained with 2% and 6% peroxide; this confirms that the depression of the extinction effected by hydrolysate is lower when 6% peroxide is used than when 2% peroxide is used.

(v) *Effect of increasing the strength of copper sulphate in the presence of protein hydrolysate.*—The protein hydrolysate prepared in the way described in section (iv) (4 ml.) was diluted to 15 ml., and the colour was developed in three series of solutions. Each series consisted of 0·5 ml. of the diluted hydrolysate, and 0·5 ml. of standard solutions containing 10, 20, 30 and 40 μg. of hydroxyproline; the colour was developed in one series with 0·01M-, in the next with 0·02M-, and in the last with 0·05M-copper sulphate solution. The ratio of the weight of amino-acids to weight of hydroxyproline in the experiment was approximately 100 to 1 (see Table X).

Table X

Effect on colour density of increasing concentration of copper sulphate in the presence of added amino-acid

Wt. of hydroxyproline added, μg.	Extinctions 0·01M-$CuSO_4$	0·02M-$CuSO_4$	0·05M-$CuSO_4$
10	0·170	0·179	0·191
20	0·290	0·315	0·324
30	0·407	0·423	0·444
40	0·480	0·513	0·552

If these results are compared with those in Table II for hydroxyproline in pure solution, it will be noted that the depressing effect which protein hydrolysate in considerable excess exercises on the development of colour is diminished by use of stronger copper sulphate solution. Expressed in another way, it may be said that the increase in colour obtained with stronger copper sulphate is slight in pure solutions of hydroxyproline, and more marked when considerable excess of protein hydrolysate is present.

Summary

1. The dimensions of the cells available for the Hilger Spekker absorptiometer used for measurement of the colour density limited the length of light path, and it was therefore desirable to employ standard solutions of hydroxyproline approximately double the strength of those used by Neuman & Logan.

2. Over the range 5–50 μg. of hydroxyproline/ml. (i.e. per 10 ml. of coloured solution) reasonable agreement was obtained on any one occasion between the densities of coloured solutions developed from aliquots of the same standard solution, and a smooth curve resulted when colour density was plotted against concentration. Nevertheless, from time to time the level of the standard curve fluctuated a little, and therefore it is advisable to include three standard solutions with each set of 'unknowns', as recommended by Neuman & Logan.

3. The density of the coloured solution developed from hydroxyproline was affected: (*a*) by variation in the concentration of copper sulphate, (*b*) by variation in the concentration of hydrogen peroxide, (*c*) by the presence of amino-acids other than hydroxyproline. When the concentration of copper sulphate was increased from 0·01M. to 0·05M. the density of colour given by hydroxyproline alone was scarcely affected, but in the presence of other amino-acids an appreciable increase in colour density resulted.

When the concentration of hydrogen peroxide used was reduced from 6% to 2%, a marked increase in colour density was noted for solutions of hydroxyproline alone, but when other amino-acids were present the increase was less.

When other amino-acids (as a mixture or in the form of protein hydrolysate) were present in the ratio of amino-acid to hydroxyproline of 8 to 1, the colour density was not affected; a slight decrease in colour density was noted at a ratio of 50 to 1, which became more marked at ratios of 100 to 1 and 1200 to 1.

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The Lyons Laboratories
Hammersmith Road
London, W.14

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References

[1] Neuman, R. E. & Logan, M. A., *J. biol. Chem.*, 1950, **184,** 299
[2] McFarlane, W. D. & Guest, G. H., *Canad. J. Res.*, 1939, [B] **17,** 139
[3] Neuman, R. E. & Logan, M. A., *J. biol. Chem.*, 1950, **186,** 549
[4] Lowry, D. H., Gilligan, D. R. & Katersky, E. M., *J. biol. Chem.*, 1951, **139,** 795
[5] Lampitt, L. H., Baker, L. C. & Brown, K. P., *J. Sci. Fd Agric.*, 1952, **3,** 367
[6] Evelyn, K. A., *J. biol. Chem.*, 1936, **115,** 63

THE VITAMIN-B GROUP IN WHITE FISH MEAL

By H. PRITCHARD and D. R. WRAIGE

Samples of standard white fish meal, typical of that produced on a large scale for animal feeding in this country, have been tested for their content of eight members of the vitamin-B complex and the average figures obtained compared with those widely scattered throughout the literature. It was found that white fish meal will supply, to rations for animal feeding, significant quantities of the known members of the vitamin-B group, in addition to protein of high biological value.

White fish meal, as defined in the Fertiliser and Feeding Stuffs Act, 1926, has become such an important ingredient in animal feeding-stuff that the entire British production, amounting to over 75,000 tons annually, is easily absorbed in this way.

The meal, widely regarded as a source of protein of high biological value, was shown by

Sherwood & Crouch[1] to produce more rapid growth and require less total feed when 6% was added to a ration than if none was included; other protein-supplements, such as groundnut and linseed meals, were not so good. In a metabolism experiment carried out on rats, du Toit & Smuts[2] showed that when white fish meal was compared with other sources of animal protein, the following biological values were obtained:

White fish meal	94	Meat meal	67
Crayfish meal	81	Meat and bone meal	67
Fish meal	71		

In pig feeding, barley was found by Hughes & Ittner[3] to give most efficient results when fed with fish meal; the addition of fish solubles to the meal as a source of animal-protein factor produced only slight improvement in its efficiency, indicating that the maximum value was being derived by the animal from the source of protein.

Besides possessing protein with biological value higher than that from most sources, white fish meal contains other factors, probably members of the vitamin-B group, which expanding knowledge of nutrition is throwing into prominence. A good deal of the evidence concerning the presence of these latter substances has been derived from animal-feeding experiments. With poultry, for example, several workers[4, 5] showed that diets supplemented with fish meal produced improvements in hatchability, owing, it was shown later,[6] to its contribution of cobalamin (vitamin B_{12}).

Figures published in various countries for different members of the vitamin-B group in fish meal have been obtained in estimations carried out abroad on the types of meal produced in their own locality. Owing to the variation in source of raw material and methods of drying, such figures can be taken only as a rough guide to those to be expected in material produced in Great Britain. Here the largest supply of white fish meal is derived from drying the head, backbone and flaps of white fish, such as cod and haddock, after the fillets have been removed for human consumption. The main sources of the fish are Iceland, Bear Island and the White Sea. The bulk of the white fish meal produced in this country is prepared in steam-heated driers of special construction, and the drying operation is carried out as soon as the trawler discharges its cargo. Thus the treatment is gentle and a minimum of destructive action on the various nutrients is allowed to occur.

In order to establish accurately the level at which certain members of the vitamin-B group occur in British-made white fish meal, samples typical of the bulk of material produced in this country were subjected to laboratory examination by microbiological assay.

To obtain as representative a series as possible, the products of the largest fish-meal factory in Britain were selected for examination. Samples were drawn from the works, representing a whole day's output, and several were bulked, so that the average samples represented the output of meal from material brought in from summer and winter fishing. The vitamins examined and the means of assay are given in Table I.

Table I

Vitamin	Test organism	Method
Thiamine	*Lactobacillus fermentum* 36	Sarett & Cheldelin[7]
Riboflavin	*L. helveticus*	Snell & Strong[8]
Nicotinic acid	*L. arabinosus* 17/5	Snell & Wright[9]
Pantothenic acid	,, ,,	Hoag, Sarett & Cheldelin[10]
Pyridoxine	*Neurospora sitophila*	Stokes *et al.*[11]
Cobalamin	*L. leichmannii* 313	Skeggs *et al.*[12]
Inositol	*Saccharomyces carlsbergensis*	Jones[13]
Choline	*N. crassa* (choline-less mutant)	Horowitz & Beadle[14]

For the assay of each of the vitamins listed in Table I, the meals were prepared for examination according to the individual behaviour and characteristics of the vitamin being estimated. The means used were as follows:

Thiamine was extracted from the fish meals by digestion with taka-diastase and papain; an acetate buffer of pH 4·5 was used.

Riboflavin results have been found in this Laboratory to be slightly higher when the meals have been steamed than when autoclaved for half an hour with 0·1N-hydrochloric acid, and steaming was used in the present tests.

Nicotinic acid was extracted by autoclaving the meals for 15 to 20 minutes at 15-lb./sq. in. pressure with N-hydrochloric acid solution.

Pantothenic acid is unstable to acid and alkali, and the extraction was carried out in a similar manner to that of thiamine, enzymically at pH 4·5.

Pyridoxine was extracted from the meal by the method of Morris, Herwig & Jones.[15] The sample was autoclaved at 10-lb./sq. in. pressure for one hour.

Cobalamin was extracted in the manner found least destructive in this Laboratory, namely to steam the sample with an acetate buffer of pH 5·0 for half an hour. In the type of assay used for this vitamin, treatment with cyanide did not enhance the figure.

Inositol assays were carried out on a sample which had been autoclaved with 5N-hydrochloric acid at 10-lb./sq. in. pressure for one hour.

Choline was extracted by autoclaving for two hours at 10-lb./sq. in. pressure in 3% sulphuric acid; it was separated on a Permutit column.

In every case cited above, after extraction had been completed, the pH of the solution was restored to that of the corresponding assay-medium. The solutions were made up to a known volume and centrifuged, and the clear supernatant liquor was diluted to the level necessary for assay.

The two average samples, carefully made up to represent the average fish meal produced at two different periods in the year, namely about June and November, were subjected to assay for the complete range of the vitamins listed in Table I.

The results obtained are given in Table II.

Table II

Vitamin-B potencies (μg./g.) of average samples of white fish meal produced in bulk in a factory in Great Britain

	Nov.–Dec.	May–June
Moisture	9·56*	10·32*
Thiamine	3·9	2·5
Riboflavin	13·5	5·9
Nicotinic acid	46·0	43·7
Pantothenic acid	11·8	5·55
Pyridoxine	11·2	2·69
Cobalamin	0·12	0·12
Inositol	—	230
Choline	—	4000

* Per cent.

It was evident that the potency of some of the vitamins was lower during May and June than in November and December, and the results obtained on a range of samples of white fish meal from various sources produced during these periods were therefore collected. Statistical conclusions are recorded in Table III.

Table III

Results (expressed in μg./g.) obtained on white fish meal from various sources

	Winter		Summer			
	No. of samples	Standard deviation of means	No. of samples	Standard deviation of means	Significance of diffs.	Coeff. of variance
Riboflavin	3	13·5 ± 2·3	4	5·95 ± 1·8	Signif. at $P = 0.05$	35
Nicotinic acid	3	46·4 ± 3·1	3	43·9 ± 1·9	Nil	10
Pantothenic acid	3	11·1 ± 1·0	5	5·56 ± 0·6	Signif. at $P = 0.01$	22
Cobalamin	3	0·12 ± 0·023	3	0·12 ± 0·01	Nil	25

It is apparent from the restricted number of tests carried out that the contents of riboflavin and pantothenic acid tend to be lower in white fish meal produced during the summer than in that produced during the winter. It is intended, however, to examine more samples to obtain further evidence on this point. Pyridoxine was assayed only on the average samples, but a sample of the May and June average was sent to another Laboratory, which reported 2·8 μg. of pyridoxine per gram. In all cases, however, a fair quantity of each of the vitamins was found to be present.

Comparison with results from other sources

It is to be expected that a product such as white fish meal, so widely produced and used in animal nutrition, would have been examined by other interested workers. The figures in

the literature, however, indicate that the examinations have been carried out piecemeal, and no complete set of potencies has been found.

The results recorded in the present paper are of value chiefly because the assays have been conducted on meals produced in Britain and are therefore typical of what can be expected in the material used in provender milling in this country.

In Table IV the figures available in the literature have been compared with those found in the present work.

Table IV

Published values (μg./g.) for various B-group vitamins in white fish meal

Vitamin	Av. figures from Table II	Published values
Thiamine	3·2	2·4–4·8[16]
Riboflavin	8·7	2·67[17]
		3·4–8·1[18]
		5·6–8·6[19]
		12·0[20]
		8·0[21]
Nicotinic acid	44·8	50–90[22]
		6·0[21]
Pantothenic acid	8·67	6·0[21]
Pyridoxine	6·94	20[23]
Cobalamin	0·12	0·06–0·12[16]
Inositol	230	310[24]
Choline	4000	3290[25]
		4450[26]

The figures obtained in the present work agree quite well with those in the literature, except in the case of pyridoxine, where the figure quoted by Henderson *et al.*[23] was based on fish muscle; the influence of quantities of bone and skin, which are dried into the fish meal, has not been taken into account. The figure for nicotinic acid given by Peck[21] is unaccountably low.

Contribution to animal needs

The contribution made to animal needs for the various B-group vitamins by white fish meal, at a level of 10% in the ration, can be assessed from the potencies given in Table II and the data for animal needs published in a previous paper.[27] It is evident that white fish meal, as well as containing content of protein of high biological activity, contributes to the diet large quantities of certain members of the vitamin-B group. Nearly all requirements for riboflavin, nicotinic acid and pyridoxine would be supplied to beasts, and more than a quarter of all requirements to poultry, when fish meal is fed at this level.

It is difficult to assess the amount of inositol or choline required by various animals, as the utilization of both these vitamins is influenced by other factors in the diet; choline, for example, can be replaced partly by methionine. Nevertheless, both inositol and choline appear to be at a sufficiently high level in fish meal to contribute greatly to the daily needs of animals.

Finally, the amount of cobalamin required by the animal will vary according to the type of protein with which it has to deal. More would be required to utilize a ration high in vegetable protein than to utilize one containing adequate quantities of animal protein such as would be supplied by 10% white fish meal, and it is evident that the meal contains enough for this purpose.

Other growth-factors have been found by some workers to be present, in addition to those mentioned in this paper. Combs *et al.*[28] found that when two rations fed to chicks—one group of rations deriving its supplementary protein chiefly from soya-bean meal, and the other from 2% white fish meal—were analysed for all the known members of the vitamin-B group and the amino-acids known to be needed for chicks, the results indicated that none of these factors was responsible for the added growth-stimulus resulting from the addition of 2% fish meal in the ration. It is likely, therefore, that other nutritional factors will be discovered in white fish meal.

Conclusion

In the typical white fish meals examined for their content of eight members of the vitamin-B group, the levels of potency found were such as would make significant contributions to the needs of livestock.

The figures obtained were, in the main, well within the range of those previously published, except for pyridoxine, where the only figure available was that calculated from work by Henderson *et al.*[23] on fish muscle.

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13 Hamilton Square
Birkenhead

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References

[1] Sherwood, R. M. & Crouch, J. R., *Texas agric. Exp. Sta. Bull.* No. 588, 1940, p. 3
[2] du Toit, B. A. & Smuts, D. B., *Onderstepoort J. vet. Sci.*, 1941, **16**, 191
[3] Hughes, E. H. & Ittner, N. R., *Calif. agric. Exp. Sta. Bull.* No. 661, 1942, p. 3
[4] Bird, H. R., Rubin, N., Whitson, D. & Hayner, S. K., *Poultry Sci.*, 1946, **25**, 285
[5] Lindstrom, R. G., Moore, P. R., Petersen, C. F. & Wiese, A. C., *Poultry Sci.*, 1949, **28**, 464
[6] Coates, M. E., *Brit. J. Nutr.*, 1952, **6**, 335
[7] Sarett, H. P. & Cheldelin, V. H., *J. biol. Chem.*, 1944, **155**, 153
[8] Snell, E. E. & Strong, F. M., *Industr. Engng Chem. (Anal.)*, 1939, **11**, 346
[9] Snell, E. E. & Wright, L. D., *J. biol. Chem.*, 1941, **139**, 675
[10] Hoag, E. H., Sarett, H. P. & Cheldelin, V. H., *Industr. Engng Chem. (Anal.)*, 1945, **17**, 602
[11] Stokes, J. L., Larsen, A., Woodward, C. R. & Foster, J. W., *J. biol. Chem.*, 1943, **150**, 17
[12] Skeggs, H. R., Nepple, H. M., Valentik, K. A., Huff, J. W. & Wright, L. D., *J. biol. Chem.*, 1950, **184**, 211
[13] Jones, A., *Analyst*, 1951, **76**, 588
[14] Horowitz, N. H. & Beadle, G. W., *J. biol. Chem.*, 1943, **150**, 325
[15] Morris, S., Herwig, G. & Jones, A., *Analyst*, 1949, **74**, 37
[16] Robinson, F. A., ' The Vitamin B Complex ', 1951 (London : Chapman & Hall)
[17] Schormuller, J., *Z. Unter. Lebensmitt.*, 1939, **77**, 1
[18] Coulton, T. J. & Bird, H. R., *Poultry Sci.*, 1941, **20**, 3
[19] Smith, P. H. & Kuzmeski, J. W., *Mass. agric. Exp. Sta. Control Service Circ.*, 8 June, 1942
[20] Fairbanks, B. W. & Hamilton, T. S., *N. Amer. Vet.*, 1942, **23**, 575
[21] Peck, L., *Feedstuffs*, 1948, (July), p. 3
[22] Hale, E. B., Davies, G. K. & Baldwin, H. R., *J. biol. Chem.*, 1942, **146**, 565
[23] Henderson, L. M., Weisman, H. A. & Elvehjem, C. A., *J. Nutr.*, 1941, **21**, 589
[24] Davidson, A. L., private communication, 1952
[25] Engel, R. W., *J. Nutr.*, 1943, **25**, 441
[26] McElroy, L. W., Rigney, H. A. & Draper, H. H., *Sci. Agric.*, 1948, **28**, 268
[27] Pritchard, H. & Wraige, D. R., *J. Sci. Fd Agric.*, 1952, **2**, 74
[28] Combs, G. E., Heuser, G. F. & Norris, L. C., *Poultry Sci.*, 1948, **27**, 238

THE ESTIMATION OF VITAMIN-E ACTIVITY BY PAPER CHROMATOGRAPHY

By P. W. RUSSELL EGGITT and L. D. WARD

Paper chromatography has recently been applied to the separation and estimation of the individual tocopherols. The present communication draws attention to errors that are likely to arise if a solution of ' Vaseline ' petroleum jelly in ethyl ether is used to impregnate the filter paper in order to achieve phase-reversal. The use of liquid paraffin B.P., dissolved in light petroleum, not only overcomes these objections, but enables the elution after chromatography to be simplified. Losses of tocopherols are minimized by chromatography in an inert atmosphere. The modified technique, which is described in detail, has consistently given recovery figures of 95%, or better, when tested with mixtures of synthetic tocopherols.

Brown[1] has recently applied reversed-phase paper chromatography to separating and determining the individual tocopherols in vegetable oils. This advance in technique is of considerable interest to manufacturers of animal feeding-stuffs, because the importance of vitamin E in the nutrition of livestock is becoming increasingly apparent. Clear differentiation between the tocopherols themselves and interfering substances is essential if a reliable estimate of the biological potency of a feeding-stuff is to be obtained by chemical means.

It was reported by Brown that some destruction of the tocopherols occurred during his chromatogram runs and that, as a 16-hour development period was necessary for adequate separation of the γ- and δ-compounds, recovery figures of the order of 80% were the best obtainable. We have found that this can be much improved by simple modifications, so that the resulting method promises considerably increased accuracy for the chemical estimation of vitamin-E activity.

Brown used Whatman No. 1 paper, coated by dipping it in a 2·5% (w/v) solution of 'Vaseline' petroleum jelly in ethyl ether and then allowing the solvent to evaporate in the air.[2] Our work has shown that the ether must be peroxide-free to prevent losses of tocopherols, even though the paper be used for chromatography some time after preparation. An extreme instance that can be cited is one in which papers prepared from an ether solution of petroleum jelly had been allowed to stand in the light for some weeks. These were spotted with alcoholic solutions of the tocopherols and eluted as soon as the alcohol had evaporated. Even with a short period of contact, almost all the tocopherol was lost, apparently owing to oxidation. We suggest that the use of ethyl ether is best avoided: moreover, petroleum jelly is much more easily dispersed in 40–60 light petroleum than in ether and its change does not affect the performance of the papers.

A second difficulty arises from the use of 'Vaseline' as the phase-reversing agent. Even the white grade contains impurities that give a colour with the Emmerie & Engel[3] reagents. After a run they are concentrated in a broad band behind the solvent front: although they are not detected on spraying with ferric chloride and $\alpha\alpha'$-dipyridyl, they raise the blank value for the paper, particularly near the region occupied by δ-tocopherol. This blank, varying along the length of the paper after a run, means that an unspotted control strip must be used to give the true blank reading corresponding to the 'tocopherol zones' cut from an adjacent chromatogram. Fig. 1 shows the result obtained by determining the blank colour produced by 1½-in. sections, cut from a 1-in.-wide strip of paper impregnated with 'Vaseline' after developing with 75% alcohol. Impurities giving a similar effect have also been found in some batches of Whatman No. 1 filter paper; these can be removed by Soxhlet extraction

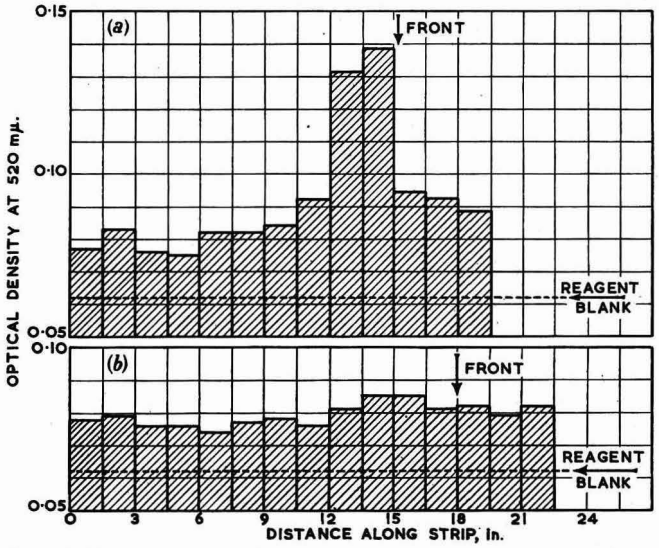

FIG. 1.—*Emmerie–Engel values for 1½-in. sections along prepared paper strips after development with 75% ethyl alcohol*

(*a*) *Whatman No. 1 paper treated with a 2½% solution of petroleum jelly in ethyl ether (maximum variation equivalent to 6·3 μg. of tocopherol)*

(*b*) *Alcohol-extracted paper treated with a 3% solution of liquid paraffin B.P. in light petroleum (maximum variation equivalent to 1·1 μg. of tocopherol)*

with absolute alcohol before treating with the phase-reversing agent. Other batches of the filter paper have not required this treatment.

We have examined a number of possible alternatives to petroleum jelly and have finally adopted liquid paraffin B.P. This is dispersed in light petroleum (boiling range 40–60°) for impregnating the strips. Concentrations of 2½ to 5% (w/v) may be used and some control of the R_F values can be exercised by this means. Other liquid paraffins, with widely different viscosities, have been tried, and the viscosity has been found to influence the R_F values. We have finally chosen a 3% solution of liquid paraffin B.P., which is a considerable improvement on petroleum jelly. The difficulty with the varying blank is almost eliminated, as indicated in Fig. 1; the speed of the solvent front during a run is increased, allowing a shorter time to be used; the separation of the tocopherol spots is improved; the tocopherol zones can be quantitatively eluted with cold alcohol; and the paraffin solution is more convenient to prepare. Typical R_F values of the tocopherols, obtained with paper impregnated with a 3% solution of liquid paraffin B.P. in light petroleum, and with 75% alcohol as the developing solvent, are: α-, 0·24; β-, 0·48; γ-, 0·48; δ-, 0·65. These figures illustrate the good separation of the three spots obtained with an overnight run, as the solvent front moves some 31 cm. past the spot origin in 16 hours; the distance depends on the ambient temperature.

The previously published technique involved the elution of the tocopherol zone on a chromatogram with hot ethyl alcohol after location by spraying an adjacent strip. This removed some petroleum jelly, and benzene was therefore added to clear the solution, which was filtered before colour development by the Emmerie & Engel method. With liquid paraffin the tocopherol can be quantitatively eluted with cold alcohol to give a clear solution and filtration is also unnecessary, thus removing a rather tedious step.

Finally Brown found no advantage in carrying out the chromatography in nitrogen or other non-oxidizing atmosphere, whereas under our conditions such a precaution is essential for quantitative working. Tocopherol spotted as an alcoholic solution on a paper treated with liquid paraffin is unstable in air, losses of up to 40% occurring on overnight storage. These losses are considerably reduced by keeping the paper in the chromatography tank in the aqueous-alcohol vapour (12% loss), but for complete stability the air must be displaced with nitrogen. The tocopherol can be recovered quantitatively from papers stored in nitrogen for much longer periods.

The following practical details of the modifications used in this laboratory may be of interest. The β- and γ-tocopherols are estimated together (their biological values are reported to be not markedly different) unless recourse is made to the method of Weisler *et al.*[4] for the separate determination of the γ-tocopherol. We obtain recoveries of the tocopherols of about 95% after chromatography for 16 hours, with quantitative recovery from a spot on the paper out of reach of the solvent front.

Experimental

Reagents

Ethyl alcohol, absolute.—redistilled from potassium hydroxide and potassium permanganate.
Light petroleum (40–60°) *A.R.*—redistilled.
Liquid paraffin B.P.
Ferric chloride (*hydrated*) *A.R.*—0·2% in absolute alcohol.
αα′-Dipyridyl.—0·5% in absolute alcohol.

Separate solutions of ferric chloride and αα′-dipyridyl of the same concentration, but dissolved in glacial acetic acid, are best used for spraying the papers used for spot location. The acetic acid reagents make this test much more sensitive, an observation due to M. L. Quaife (unpublished).

Chromatography

Individual strips of Whatman No. 1 paper 1 in. wide are used for each sample. These are passed through a 3% (w/v) solution of liquid paraffin B.P. in light petroleum (40–60°) and the solvent is allowed to dry.

The paper to be used should first be tested for interfering impurities by running an untreated strip in the chromatography tank and measuring the variations in blank readings obtained with 1½-in. sections along its length, using the Emmerie & Engel technique. Some batches of paper are best extracted before use in a Soxhlet with absolute alcohol.

Descending chromatography is preferred, although it is perhaps less convenient than the ascending method, as the separation of the tocopherols is better in a given time. It is somewhat difficult to construct a glass trough for the developing solvent and polythene has been found to be satisfactory. A suitable length of wide-bore polythene tube is plugged at each end with polythene rod and a slot cut along the tube with a scalpel to form the trough. The plugs may be sealed in by pressing the ends on a smooth-surfaced electric hot-plate.

The tocopherols in absolute alcohol, and free from sterols or carotenoids, are spotted on the papers with an Agla micrometer syringe dispensing 5–10 μl. containing 20–30 μg. of each tocopherol. Several applications may be made, if necessary, the alcohol being allowed to evaporate each time before adding more solution. No more than 10 μl. may be applied in a single spotting.

The chromatograms are developed overnight (16 hours) in an atmosphere of nitrogen saturated with the vapour of 75% alcohol used as the developing solvent. Exposure of the papers to the air is kept to a minimum. A strip spotted with a known mixture of tocopherols is run between duplicate test-strips and is sprayed with the Emmerie & Engel reagents (dissolved in acetic acid) when the run is complete. With this as a guide the tocopherol zones can be marked on the test strips placed alongside. It is useful to have a control spot on each strip near the bottom and out of reach of the solvent front, as this should be quantitatively recovered.

Elution and colour-development

As quickly as possible after removing from the nitrogen 1½-in. sections are marked on the strips, each incorporating a tocopherol zone. Similar blank sections are cut from the same strip or from equivalent positions on a specially-run unspotted strip. A blank is also required below the solvent-front for the control spot.

Each section is cut into eight equal fractions with very sharp dissecting scissors, to keep the number of cellulose fibres distributed in the elution liquid to a minimum. The papers are then dropped into a 50-ml. conical flask (B14 ground-glass neck and stopper) containing 3 ml. of absolute alcohol, and allowed to stand at room temperature in the dark for ½–1 hour. Without prior filtration 0·5 ml. of the ferric chloride and then 0·5 ml. of dipyridyl solution are added; the solutions are well mixed and decanted from the paper fragments into a spectrophotometer cell. The red colour is measured at a wavelength of 520 mμ 2 minutes after the dipyridyl pipette is blown out. Bacteriological pipettes are convenient for the rapid addition of the Emmerie & Engel reagents. It is important that the colour development and measurement be carried out in dim artificial light, as the reaction is very photosensitive.

With this total volume of 4 ml. and with the Uvispek spectrophotometer at 520 mμ, a slit width of 0·1 mm., 1-cm. cells, and alcohol in the reference cell, the α-tocopherol content is given by multiplying the optical density, less that of the blank, by 100·2; this is useful, in that the instrument is made virtually direct-reading merely by moving the decimal point.

The two minutes allowed for the Emmerie & Engel reaction permits almost the maximum colour-development with α-, β- and γ-tocopherols, but care in exact standardization is required with δ-tocopherol with which the colour develops more slowly.[5]

Acknowledgments

We wish to thank Mr. R. J. Ward (Dunn Nutritional Laboratory, Cambridge), for providing γ- and δ-tocopherols and for his interest in the work, and also the Directors of Spillers Ltd., for allowing us to publish this note.

Spillers Ltd. Central Laboratory
Station Road
Cambridge

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References

[1] Brown, F., *Biochem. J.*, 1952, **51**, 237; 1952, **52**, 523
[2] Winteringham, F. P. W., Harrison, A. & Bridges, R. G., *Nature, Lond.*, 1950, **166**, 999
[3] Emmerie, A. & Engel, C., *Rec. Trav. chim. Pays-Bas*, 1938, **57**, 1351
[4] Weisler, L., Robeson, C. D. & Baxter, J. G., *Analyt. Chem.*, 1947, **19**, 906
[5] Stern, M. H. & Baxter, J. G., *Analyt. Chem.*, 1947, **19**, 902

THE INFLUENCE OF PIPERONYL BUTOXIDE AND DDT ON THE DETERMINATION OF THE PYRETHRINS CONTENT OF COMMERCIAL SPRAY-INSECTICIDES BY THE A.O.A.C.* AND THE SEIL METHODS

By G. J. WARREN

The estimation of total pyrethrins either by the A.O.A.C.* method of analysis or the Seil method yields high results in the presence of the synergist piperonyl butoxide, or DDT. Both these compounds, on hydrolysis with alcoholic potassium hydroxide, produce acidic substances which, being apparently only slightly soluble in light petroleum and non-volatile in steam, do not affect appreciably the estimation of pyrethrins I, but, being soluble in ether, give falsely high results for pyrethrins II by either method.

Introduction

The A.O.A.C.* method, as given in the 7th edition, 1950, makes no provision for ' blank ' determinations on the alcoholic N-sodium hydroxide solution used for hydrolysis, though as much as 50 ml. or more may be needed, depending on the presence of other materials. According to the B.P.C. 1949, however, a ' blank ' should always be run and the titration figures deducted from those obtained for the actual pyrethrins determination.

Another cause of possible variation in the pyrethrins content of commercial spray-insecticides is the base oil used, generally kerosene or deodorized kerosene. Titration figures are obtained for both these oils after boiling with alcoholic 0·5N-potassium hydroxide and proceeding according to the proper methods. Here again the pyrethrins II figure is the higher.

Experimental

In order to determine the magnitude of the errors introduced by these various materials, the following mixtures were analysed both by the A.O.A.C. and the Seil methods:

(1) Blank of 20 ml. of alcoholic 0·5N-KOH soln.
(2) Blank of 40 ml. of alcoholic 0·5N-KOH soln.
(3) 100 ml. of deodorized kerosene (spray base) + 20 ml. of alcoholic 0·5N-KOH soln.
(4) 100 ml. of crude kerosene + 20 ml. of alcoholic 0·5N-KOH soln.
(5) 5 g. of piperonyl butoxide in 100 ml. of spray base + 40 ml. of alcoholic 0·5N-KOH soln.
(6) 1 g. of piperonyl butoxide in 100 ml. of spray base + 40 ml. of alcoholic 0·5N-KOH soln.
(7) 4 g. of pure *pp'*-DDT in 100 ml. of spray base + 40 ml. of alcoholic 0·5N-KOH soln.
(8) 4 g. of technical DDT in 100 ml. of spray base + 40 ml. of alcoholic 0·5N-KOH soln.
(9) 1 g. of technical DDT + 1 g. of piperonyl butoxide in 100 ml. of spray base + 40 ml. of alcoholic 0·5N-KOH soln.

The kerosenes were drawn from normal deliveries of the commercial materials.

The piperonyl butoxide was the commercial material containing not less than 80% of piperonyl butoxide (butyl 3 : 4-methylenedioxy-6-propylbenzyl diethylene glycol ether).

The pure *pp'*-DDT was prepared by recrystallization from alcohol and had m.p. 109°.

The technical DDT contained 80% of the *pp'*-compound.

Determination

A.O.A.C. method.—The B.P.C. 1949 modification of this method was employed, in which hydrochloric acid is used instead of sulphuric acid for the liberation of the pyrethrins I; this saves an additional filtration, with its possible sources of loss. Otherwise the official method was followed.

Seil method.—The only modification of the original Seil method was in the use of N-sulphuric acid for the liberation of the pyrethrins I before steam distillation; 1 ml. in excess was employed for the 200 ml. of filtrate used. Steam distillation was conducted as rapidly as possible and between 20 and 30 minutes were required to reduce the volume to between 15 and 20 ml.

* Association of Official Agricultural Chemists (Washington)

Results

Table I gives the actual titration figures obtained, in ml. of 0·01M-potassium iodate solution for pyrethrins I (A.O.A.C.), and in ml. of 0·02N-sodium hydroxide solution for pyrethrins II (A.O.A.C.), and for both pyrethrins I and II (Seil). They represent the mean of three determinations in each case.

Table I

Blanks	A.O.A.C. Titration figures		Seil Titration figures	
	Py.* I, ml. 0·01M-KIO_3	Py. II, ml. 0·02N-NaOH	Py. I, ml. 0·02N-NaOH	Py. II, ml. 0·02N-NaOH
Alcoholic 0·5N-KOH, 20 ml.	0·1	0·38	0·2	0·4
Alcoholic 0·5N-KOH, 40 ml.	0·1	0·7	0·33	0·7
Alcoholic 0·5N-KOH, 20 ml. / Spray base, 100 ml.	0·1	0·87	0·23	0·78
Alcoholic 0·5N-KOH, 20 ml. / Kerosene, 100 ml.	0·15	0·55	0·45	0·85
Alcoholic 0·5N-KOH, 20 ml. / Piperonyl butoxide, 5 g. / Spray base, 100 ml.	0·47	2·75	0·52	3·45
Alcoholic 0·5N-KOH, 40 ml. / Piperonyl butoxide, 1 g. / Spray base, 100 ml.	0·28	1·58	0·3	1·8
Alcoholic 0·5N-KOH, 40 ml. / DDT (pure), 4 g. / Spray base, 100 ml.	0·10	2·2	0·23	1·8
Alcoholic 0·5N-KOH, 40 ml. / DDT (technical), 4 g. / Spray base, 100 ml.	0·10	5·2	0·3	4·18
Alcoholic 0·5N-KOH, 40 ml. / DDT (technical), 1 g. / Piperonyl butoxide, 1 g. / Spray base, 100 ml.	0·25	2·35	0·3	2·57

* Py. = Pyrethrins

The titration figures for the pyrethrins II determination are quite high with either method in the presence of piperonyl butoxide and DDT, both pure *pp'*- and technical, and would represent an appreciable amount of the total pyrethrins present. Thus a spray containing 0·1% of total pyrethrins and 4% of technical DDT would give a titration figure of roughly 14·5 ml. of 0·02N-sodium hydroxide in the pyrethrins II determination by the Seil method, instead of 10·5 ml. for the pyrethrins II content alone. It will be noticed that the base oil and the alcoholic potassium hydroxide contribute to the errors.

Some indication of the differences is given in Table II, where definite amounts of a 25% pyrethrum extract were estimated in the presence of piperonyl butoxide, technical DDT, and both together, in solution in spray base. The effect of deducting the titration figures of Table I from those actually obtained, where appropriate, is also shown. The same samples of pyrethrum extract, piperonyl butoxide, technical DDT and spray base were used for each test, and the means of three determinations in each case are recorded. Percentages of pyrethrins I and II are calculated by means of the usual factors.

It is clear that by ignoring the effect of either the synergist or the DDT, or both, errors of the order of 16% of the total pyrethrins can be introduced.

Discussion

When examining a commercial spray-insecticide containing pyrethrins it is quite easy to run an alcoholic potassium hydroxide blank at the time of making the test, but it is quite another matter to have at hand samples of the spray base, DDT and other materials used in the formulation in order to run blanks on these. It would appear, therefore, that some method of isolating the pyrethrins before determination is needed, as it is highly probable that the other ingredients will vary considerably in composition from batch to batch and between different manufacturers, so that no general factor for deduction for these materials could be employed. The separation

Table II

A.O.A.C. method

	No allowance for blanks					Allowing for blanks				
	Py. I		Py. II		Total	Py. I		Py. II		Total
	Titration, ml. 0·01M-KIO_3	Found, %	Titration, ml. 0·02N-NaOH	Found, %	py., %	Titration, ml. 0·01M-KIO_3	Found, %	Titration, ml. 0·02N-NaOH	Found, %	py., %
25% py. extract, 1 g. Alcoholic 0·5N-KOH, 20 ml.	22·33	15·9	24·76	11·6	27·50	22·23	15·8	24·38	11·4	27·2
25% py. extract, 1 g. Pure DDT, 4 g. Spray base, 100 ml. Alcoholic 0·5N-KOH, 40 ml.	23·76	16·9	27·33	12·8	29·7	23·66	16·9	25·13	11·7	28·6
25% py. extract, 1 g. Technical DDT, 4 g. Spray base, 100 ml. Alcoholic 0·5N-KOH, 40 ml.	23·23	16·5	28·02	13·1	29·6	23·12	16·5	22·82	10·7	27·2
25% py. extract, 0·4 g. Alcoholic 0·5N-KOH, 20 ml.	8·9	15·8	10·4	12·2	28·0	8·8	15·7	10·02	11·7	27·4
25% py. extract, 0·4 g. Piperonyl butoxide, 1 g. Spray base, 100 ml. Alcoholic 0·5N-KOH, 40 ml.	8·68	15·5	13·92	16·3	31·8	8·4	15·0	12·34	14·4	29·4
25% py. extract, 0·4 g. Piperonyl butoxide, 1 g. Technical DDT, 1 g. Alcoholic 0·5N-KOH, 40 ml. Spray base, 100 ml.	8·50	15·1	13·23	15·5	30·6	8·25	14·7	10·88	12·7	27·4

Table II (contd.)

Seil method

	No allowance for blanks					Allowing for blanks				
	Py. I		Py. II		Total	Py. I		Py. II		Total
	Titration, ml. 0·02N-NaOH	Found, %	Titration, ml. 0·02N-NaOH	Found, %	py., %	Titration, ml. 0·02N-NaOH	Found, %	Titration, ml. 0·02N-NaOH	Found, %	py., %
25% py. extract, 1 g. Alcoholic 0·5N-KOH, 20 ml.	18·03	14·9	26·52	12·4	27·3	17·83	14·7	26·12	12·2	26·9
25% py. extract, 1 g. Pure DDT, 4 g. Spray base, 100 ml. Alcoholic 0·5N-KOH, 40 ml.	17·73	14·6	28·33	13·2	27·8	17·50	14·4	26·53	12·4	26·8
25% py. extract, 1 g. Technical DDT, 4 g. Spray base, 100 ml. Alcoholic 0·5N-KOH, 40 ml.	17·65	14·5	30·20	14·1	28·6	17·35	14·5	26·02	12·2	26·7
25% py. extract, 0·4 g. Alcoholic 0·5N-KOH, 20 ml.	7·2	14·9	10·61	12·4	27·3	7·0	14·4	10·21	12·0	26·4
25% py. extract, 0·4 g. Piperonyl butoxide, 1 g. Spray base, 100 ml. Alcoholic 0·5N-KOH, 40 ml.	7·23	14·9	14·03	16·4	31·3	6·93	14·3	12·23	14·3	28·6
25% py. extract, 0·4 g. Piperonyl butoxide, 1 g. Technical DDT, 1 g. Alcoholic 0·5N-KOH, 40 ml. Spray base, 100 ml.	7·35	15·2	13·37	15·6	30·8	7·05	14·5	10·80	12·6	27·1

of DDT from pyrethrins by means of an alumina column has been described by Pownings,[1] and the method is satisfactory for the DDT. The pyrethrins, however, require considerable elution, and in the presence of piperonyl butoxide both these materials are adsorbed by the alumina; their separation has, so far, resisted the usual solvents and mixtures of solvents used for elution.

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Jeyes' Sanitary Compounds Co. Ltd.
Richmond Street
London, E.13

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Reference

[1] Pownings, R. F., *J. Coun. sci. industr. Res. Aust.*, 1945, **18**, (2)

THE ELECTRODIALYTIC REMOVAL OF INORGANIC MATERIAL FROM ORGANIC MARINE PRODUCTS

By J. C. DUNBAR and T. J. MITCHELL

A. Seaweed.—By means of a three-compartment cell with ceramic diaphragms, suspensions of dried ground *Laminaria cloustoni* stipe were electrodialysed to remove inorganic material. The total-ash content of the seaweed was reduced from 33·4 to 7·4% in 30 minutes, representing a removal of 78% of the ash. Tests were also made on dried, ground frond, and a much smaller removal was effected. The total-ash content was reduced from 28 to 22·8% in 30 minutes, representing a removal of about 18% of the ash. Duplicate tests were made to determine the amount of alkali and chlorine recovered and the energy required. The following figures were obtained (per lb. of dry seaweed): alkali recovered, 0·053 lb.; chlorine recovered (estimated), 0·056 lb.; energy required, 3·03 kw.

B. Fish albumen.—A three-compartment cell, with diaphragms of cellulosic material, was used for tests to reduce the ash content of fish albumen. It was found possible to reduce the ash content from 7·7 to 4·0% in 90 minutes, representing a removal of 52·3% of the inorganic material.

The fact that not all the inorganic material is removed in either A or B is tentatively attributed to the presence of a certain proportion of it in an insoluble form such as calcium alginate in seaweed and calcium citrate in fish albumen. Electrodialysis of calcium alginate and calcium citrate gave results which supported this view.

Introduction

A. Seaweed

The importance of seaweed as a source of various chemicals has been recognized for some considerable time. Kelp-burning to obtain iodine, and the recovery of potash have long been practised. Among the organic materials obtainable from seaweed are laminarin, fucoidin, mannitol and alginic acid. Of these, alginic acid and its salts have been extensively utilized in recent years, the two main processes for extraction being the Le Gloahec–Herter process[1] and Green's cold process.[2]

Electrokinetic treatment of seaweed has been more limited in its use. A Russian patent[3] described a systematic scheme for the consecutive separation of iodide, bromide, chloride and

alginic acid, and the eventual recovery of alkali and cellulosic material. A Norwegian patent[4] detailed a system in which alginic acid was continuously produced from seaweed by electrolytic means. Actual figures for yields and electrical energy requirements, however, seem to be lacking, and the present work is intended to give some data of this kind regarding the removal of inorganic material from seaweed.

B. Fish albumen

The production of fish albumen was first developed in Germany and has been taken up in Great Britain. The product is intended as a substitute for egg albumen, and the latter part of this work deals with attempts to reduce the ash content of the material.

A. Seaweed

Material

The seaweed used in this investigation was *Laminaria cloustoni* which had been dried and ground to pass a 1·6-mm. screen. Separate samples of stipes and fronds were used.

Analysis of seaweed

Methods

The methods recommended by Black[5] were used.

Moisture content.—This was determined by drying a 5-g. sample in an electric oven at 100° for six hours.

Total ash content.—The method of dry ashing was used, 1 g. of the material being heated, at dull redness, to constant weight in a silica crucible.

Insoluble ash content.—Seaweed (1 g.) was heated in a silica crucible at dull redness for 30–45 minutes, allowed to cool and extracted with four 5-ml. portions of hot water, the clear liquor being decanted off after each extraction through an ashless filter-paper, and the bulk of the ash being finally transferred to the paper. The solid on the filter paper was washed with four 5-ml. portions of boiling water, transferred to the crucible again and heated at dull redness to constant weight.

Soluble ash content.—This was obtained by difference.

Results

The results of analysis of the materials are shown in Table I. The combustible material represents the organic content of the seaweed. The constitution of the organic material in seaweed varies according to the season of the year in which it is harvested,[5] and, as the material used was harvested in November, the organic content of the fronds was greater than that of the stipes.

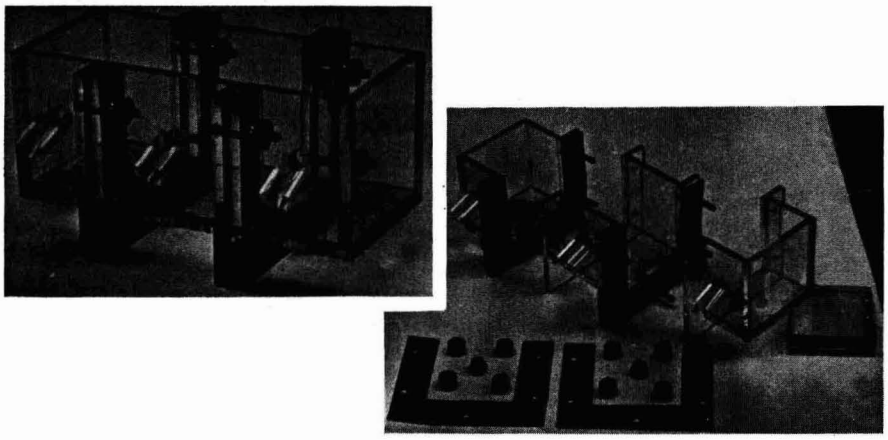
Table I

	Percentage composition				
	Moisture	Total ash*	Insoluble ash*	Soluble ash*	Combustible material*
Stipes	7·9	33·4	9·1	24·3	66·6
Fronds	6·4	28·0	13·0	15·0	72·0

* Dry basis

Apparatus

The cell used for the electrodialysis of the suspensions is shown assembled and in sections in Figs. 1 and 2. It is constructed of ¼-in. Perspex sheet in three sections to facilitate the insertion of suitable diaphragms. The end compartments are 3 in. high, 3 in. wide and 2 in. long, and the centre compartment is a 3-in. cube. A circular hole, ⅜ in. in diameter, is cut in the centre of the base of each section for ease in draining the individual compartments. A similar hole is cut in the centre of the front face of each section, from which slopes a semicircular trough. These overflow outlets enable a constant level to be maintained in each compartment. The end sections are flanged along the inner edge and the centre section along both edges. These flanges are fitted with rubber gaskets to give a watertight joint. The construction of the cell permits easy removal of the diaphragms for examination and cleaning.



FIGS. 1 (*left*) and 2 (*right*).—*The cell used for electrodialysis, assembled and in section*

The diaphragms are ceramic plates 4 in. × 4 in. × $\frac{1}{8}$ in. with a pore-volume of 33·2% and a pore-diameter of 7·2 μ. Their permeability, expressed as the volume in ml. passed by 100 sq. cm. per hour at 20° and at a pressure of 10 cm. of water, is 285 ml.

The electrodes are made of $\frac{1}{4}$-in. carbon sheet, $2\frac{3}{4}$ in. wide × 3 in. long, fitted with brass clips so that they can be fastened to the side of the cell and thus suspended at any distance from the diaphragms as required.

The electrical circuit is shown in Fig. 3, the potential being controlled by the two variable resistances, R_1 and R_2, to give any applied voltage between 0 and 250 V. The rheostats are wired as potential dividers with an overall resistance of 75 Ω and a current rating of 4A. In the circuit are two voltmeters (0–40 and 0–300 V) and three ammeters (0–0·5, 0–5 and 0–20 A), the ammeters measuring the current flowing through the shunted section of the rheostats.

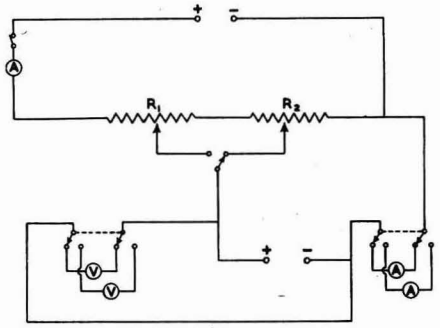


FIG. 3.—*Electrical circuit for electro-osmosis*

Experimental

The experimental work falls into two sections, (*a*) the electrodialysis of stipes and (*b*) the electrodialysis of fronds.

(*a*) *Electrodialysis of stipes*

In all the following tests, 5·000 g. (4·605 g. dry weight) of dried, milled stipes were placed in the centre compartment of the cell and 200 ml. of distilled water was added. The cathodes and anode sections were filled with distilled water to the overflow level. The centre compartment was stirred throughout each test. The electrodes were $\frac{1}{4}$ in. from the diaphragms in all cases.

The first three tests were a preliminary investigation of the effect of different voltages on the separation of the soluble inorganic material.

*Test A*1.—On applying a potential difference of 40 V across the cell, it was observed that the resistance dropped slowly and steadily over a period of 50 minutes before remaining fairly constant. The current was switched off after 60 minutes. During the electrodialysis the changes in pH of the liquids in the various compartments were noted. The centre section retained its original acidity throughout, the anode and cathode compartments becoming distinctly acid and alkaline respectively. The cathode section was washed out with 5N-hydrochloric acid and the amount of alkali chlorides present was determined by evaporation of the

recovered solution. The cathode liquid was tested qualitatively for magnesium and calcium, but no traces were found. The anode compartment was analysed qualitatively and found to contain a large quantity of chloride ions and also traces of sulphate. The alkali chlorides in the cathode section account for 10% of the original seaweed, on a dry basis.

Test A2.—In this test, a potential of 50 v was applied. The resistance was very slowly broken down and attained a constant value after 45 minutes. The contents of the cathode section were estimated as before. The contents of the centre compartment were evaporated to dryness and analysed by the procedure adopted for the original seaweed analysis. No traces of organic material were noted in the cathode section.

Test A3.—An initial potential difference of 100 v was applied in this test. The temperature of the centre compartment rose rapidly to 40° in 10 minutes and the resistance dropped rapidly. The potential was then reduced to 50 v, the temperature and the resistance remaining constant from this time. The test was continued for a further 20 minutes, making 30 minutes in all.

Test A4.—A continuous-flow system was fitted to the cathode compartment in an attempt to improve the separation by removing the ions from the cathode section, thus preventing an ionic ' back pressure '. A potential difference of 50 v was maintained throughout the test. A constant resistance was obtained after 30 minutes, but the value was greater than that obtained in the previous tests. The test was run for 50 minutes in all.

Tests A5 and A6.—Two final tests were carried out under identical conditions. A potential difference of 100 v was applied for 20 minutes, at the end of which time the potential was reduced to 50 v and the test terminated after a further 10 minutes. To obtain more detailed analyses of the materials transferred to the cathode and anode sections, both compartments were washed out with distilled water and analysed volumetrically. The cathode solution was titrated with hydrochloric acid and the alkali content determined as potassium. The anode solution was titrated with sodium hydroxide solution to obtain the total acidity. The hydrochloric acid present was then determined in the neutral solution by titration with silver nitrate, with potassium chromate as indicator. The difference in the estimations was due to a trace of sulphate ions.

The results obtained are shown in Tables II and III.

Table II

Test No.	Alkali chloride in cathode section	Material recovered from centre compartment		
		Total ash	Soluble ash	Insoluble ash
A1	10·0*	—	—	—
A2	13·3	25·0	17·2	7·8
A3	7·8	7·4	1·9	5·5
A4	—	19·4	13·5	5·9
A5	—	13·2	5·8	7·4
A6	—	12·6	5·4	7·2
Original material		33·4	24·3	9·1

* All figures refer to percentage based on original material

Table III

Additional data from tests A5 and A6

	% of original material	
	Test A5	Test A6
Recovered material from centre section	89·0	88·8
Total alkalinity of cathode section (as K)	5·15	5·47
Total acidity of anode section (as HCl)	1·96	1·96
Hydrochloric acid content of anode section	1·60	1·60
Sulphuric acid content of anode section	0·28	0·28

The first three tests show that the best separation was obtained by applying a high potential to break down the initial resistance. This reduced the time required to obtain satisfactory separation. The effect of having a continuous flow of distilled water through the cathode section was small. The best results were obtained in test A3, and this is attributed to a gradual clogging of the cathode diaphragm with precipitated solid, which might be either inorganic or organic in nature.

The figure obtained for the hydrochloric acid content of the anode compartment is only useful for the determination of the small sulphuric acid content by difference, and as a guide to the quantity of chlorine held in solution at the anode. The chlorine liberated at the anode during electrolysis could not be determined without using an enclosed anode compartment. It may, however, be estimated by difference, as the original weight of the stipes minus the sum of the weights of the recovered material, the alkali content of the cathode section and the sulphate.

On this basis, the chlorine liberated, expressed as a percentage of the original material, was 5·6% in test A5 and 5·5% in test A6.

The power requirements for these two tests were estimated graphically.

	Test A5	Test A6
Power required per lb. of dry seaweed	2·93 kw.	3·13 kw.
Alkali recovered per lb. of dry seaweed	0·052 lb.	0·055 lb.
Estimated recoverable chlorine per lb. of dry seaweed ..	0·056 lb.	0·055 lb.

(*b*) *Electrodialysis of fronds*

Three tests were carried out to find the effect of different potentials. The tests and subsequent analyses were carried out in a similar manner to tests A1–A3, and the results are shown in Table IV.

Test B1.—The duration of this test was 35 minutes at a potential difference of 50 v. The residue obtained on evaporation of the contents of the cathode section contained traces of organic material, which resulted in a high figure for the alkali-chloride content. The organic content was not identified.

Test B2.—This was a short test of 10 minutes' duration and was carried out at a potential difference of 100 v. The resistance dropped slowly at first and remained constant after eight minutes.

Test B3.—The final test on fronds was carried out at a potential difference of 100 v. until the resistance became steady after 10 minutes. The potential was then reduced to 50 v. and the test continued for a further 20 minutes. The contents of the anode section were examined qualitatively and found to contain a large concentration of chloride ions and a trace of sulphate ions. The material recovered from the cathode section again contained a trace of organic material.

Table IV

Test No.	Alkali chloride in cathode section	Recovered material from centre section		
		Total ash	Soluble ash	Insoluble ash
B1	6·2*	23·4	10·5	12·9
B2	3·0	24·2	11·0	13·2
B3	6·1	22·8	9·6	13·2
Original fronds		28·0	15·0	13·0

* All figures refer to percentage based on original material

B. Fish albumen

Material

The fish albumen used was a sample of a commercial product.

Analysis of fish protein

Moisture content (as supplied), 10%
Ash content, 7·7%
Protein content, 76·1% (Kjeldahl N × 6·25)

A considerable number of tests were made before a suitable method of ashing was found. The method adopted was to carbonize the material in a platinum crucible supported in an iron crucible over a Meker burner. The carbonized material was then heated slowly in a muffle furnace to 800°, taking three hours to raise the temperature from 300 to 800°, so that the material could swell gradually. The material was finally ashed by heating at 800°.

Apparatus

The cell used for electrodialysis is shown in Fig. 4. It is made of glass and has three sections. The centre section is made from glass tubing of internal diameter 1·5 in. and length, 4 in.; it has inlet and outlet holes, 0·75 in. in diameter, at the top and bottom respectively, the lower one being closed with a rubber bung during operation. The outer compartments are also constructed from glass tubing of internal diameter 2 in. and length 5 in. These sections also have inlet and outlet holes through which a current of water may be passed during electrodialysis. Two pairs of outer sections were used, the first having nickel and the second platinum electrodes. The electrodes are fused into the outer sections in glass sheaths, the nickel electrodes on tungsten-wire leads and the platinum electrodes on platinum wire. Diaphragms are held in position over the ends of the centre section by means of two rubber bungs 2 in. in diameter, the outer sections being fixed securely in position on these bungs.

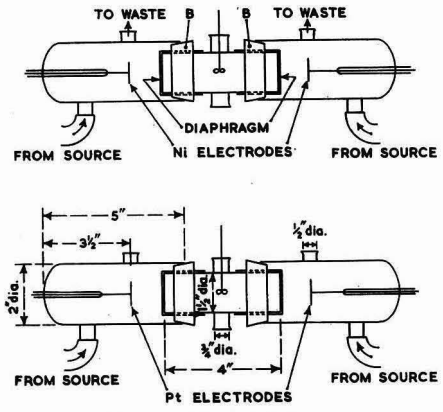


FIG. 4.—*Electrodialytic cells*

A mechanical stirrer which passes through the upper opening of the centre section provides agitation for the suspension.

The diaphragms found suitable for use with the cell were of Cellophane, and regenerated cellulose in a form used commercially as sausage skin.

The electrical circuit used was identical with that described in the preceding section of the paper.

Experimental

In all the tests made, 4% (w/v) solutions were used; the solutions were made by mixing 4 g. of the fish albumen into a cream in a mortar, transferring the material to a beaker, adding the bulk of the water and stirring for 60–90 minutes. The solution was then transferred to the centre section of the electrodialytic cell and the total volume made up to 100 ml.

After electrodialysis, the contents of the centre section were washed into a beaker, evaporated to dryness on a steam bath and finally ashed by the method already described.

In the initial tests the applied potential was kept constant, but in later runs it was decided to limit the current in the cell to 60 mA to avoid heating effects.

All the experimental data and results from the 10 tests made are given in Table V. The first six tests were made with the nickel-electrode cell and tests 7–10 with the platinum-electrode cell.

Calcium alginate

In an attempt to explain the incomplete nature of the electrodialysis, tests were made on calcium alginate and calcium citrate to find out whether or not calcium would migrate to the cathode section from these relatively insoluble salts.

1 g. of alginic acid was dissolved in 750 ml. of 1% sodium carbonate solution, and the resultant solution mixed with an excess of 10% of calcium chloride solution to precipitate calcium alginate. This precipitate was allowed to settle, and was then filtered and washed free of excess calcium, the wash water being tested with ammonium oxalate. The washed calcium alginate was suspended in 100 ml. of distilled water in the centre section of the glass electrodialytic cell, the outer sections were filled with distilled water and a potential of 235 V was applied. Readings of current were taken every 10 minutes for one hour and samples withdrawn at similar periods from the cathode section. These samples were tested with ammonium oxalate for the presence of calcium. The current rose slowly from an initial value of 10 mA to a final value of 40 mA. At the end of one hour, a few drops of 10% calcium chloride solution were introduced to the centre compartment. After five minutes the current had risen to 60 mA and after a further five minutes to 100 mA.

The approximate concentrations of calcium present in the various samples were obtained by comparison with standard precipitates obtained from solutions of calcium chloride of various concentrations.

Table V

Experimental data from tests 1–10

Test No.	Diaphragm	Water in outer sections	Applied potential, V	Initial current, mA	Final current, mA	Duration of tests, min.	Ash content, % of electrodialysed material	Remarks
1	C*	TC‡	200	5	5	60	7·1	
2	S†	,,	,,	25	25	60	6·9	
3	C	DC§	,,	15	10	60	6·8	
4	S	TC	,,	10	10	60	6·8	
5	C	DS‖	,,	250	275	35	—	Presence of nickel in centre compartment shown by test with dimethyl glyoxime
6	S	,,	,,	20	100	50	—	As in test 5
7	C	,,	200–95	10	60	60	5·74	Applied potential reduced after 40 minutes to maintain current constant at 60 mA
8	S	,,	,, ,,	10	60	60	5·99	Applied potential reduced after 25 minutes to maintain current constant at 60 mA
9 (1)	C	,,	200–90	10	60	60	4·03	After 60 minutes the test was interrupted and the outer sections washed out and refilled with distilled water. The test was then continued for a further 30 minutes
(2)	C	,,	200–135	20	60	30		
10 (1)	S	,,	200–95	10	60	60	5·2	Procedure as in test 9
(2)	S	,,	200–115	10	60	30		

* C = Cellophane
† S = Cellulose (sausage skin)
‡ TC = Tap-water circulated
§ DC = Distilled water circulated
‖ DS = Distilled water static

A similar test was made with calcium citrate; in this case 2 g. of the solid were suspended in 100 ml. of distilled water in the centre section of the cell, and electrodialysis was carried out for one hour. The current rose from an initial value of 5 mA to a final value of 40 mA, the potential being 235 V throughout. The results are shown in Table VI.

Table VI

Time, min.		0	10	20	30	40	50	60	65	70
Concn. of calcium in cathode section, p.p.m.	Calcium alginate	0	—	6	10	12	15	20*	50	90
	Calcium citrate	0	—	2	10	20	70	100	—	—

* Calcium chloride added after 60 minutes

It will be seen that a very small amount of the total calcium present is removed by electrodialysis. With calcium alginate, after one hour, the approximate amount removed is of the order of 1–2%, and with calcium citrate about 4–5%. The effect of introducing calcium in solution is clearly shown by the rapid increase in the rate of migration on adding a small amount of calcium chloride solution, as was done at the 60-minute mark in the first test. The rate of migration is increased from about 4 mg./hr. to about 84 mg./hr. The calcium of calcium citrate is removed more readily than that of calcium alginate, but in neither case, concentrations being taken into account, is there any comparison with the effect of calcium in solution.

Conclusions

A reasonably satisfactory separation of the soluble inorganic material from seaweed stipes can be obtained by electrodialysis, using a three-compartment cell with ceramic diaphragms. With fronds, a small quantity of organic material migrates to the cathode section.

With fish protein, it was found possible to effect a fairly considerable reduction in the inorganic content by electrodialysis through Cellophane diaphragms. The failure of the method

when water was circulated through the outer compartments is unexpected, the constant flow of water apparently keeping the resistance at a high value.

In both aspects of the investigation, the removal of the inorganic matter, although considerable, is not complete and, since the tests carried out on calcium alginate and calcium citrate demonstrate clearly the very great differences in rates of electrodialysis of soluble and relatively insoluble calcium salts, it is suggested that this may be due partly to a proportion of the inorganic material being present in an insoluble form, as calcium alginate in seaweed and as calcium citrate in fish albumen.

Acknowledgments

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Technical Chemistry Department
Royal Technical College
Glasgow

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References

[1] Le Gloahec, V. C. E. & Herter, J. R., U.S.P. 2,188,092 (1938)
[2] Green, H. C., U.S.P. 2,036,934 (1936)
[3] Florenskii, P. A., Litvinov, R. N. & Bryantsev, N. Ya., U.S.S.R. Pat. 51,091 (1937)
[4] Kolle, E., Norweg. Pat. 64,468 (1942)
[5] Black, W. A. P., *J. Soc. chem. Ind., Lond.*, 1948, **67**, 165

THE ELECTRODEPOSITION OF POWDERED INORGANIC MATERIALS FROM SUSPENSION IN ORGANIC LIQUIDS

By J. C. DUNBAR and T. J. MITCHELL

Variables affecting the electrodeposition of basic magnesium carbonate and calcium carbonate from suspension in organic liquids have been investigated. Temperature has little effect. Deposition increases linearly, in most cases, with voltage, time, concentration and electrode surface. An unusual effect has been noted with high concentrations of materials containing a hydroxyl group, the amount of deposit increasing very rapidly with increasing concentration. With different organic liquids as suspending media, the amount of deposit increases with increasing dielectric constant in the series ethyl acetate, *n*-amyl alcohol, *n*-butyl alcohol, *n*-propyl alcohol, ethanol and methanol. With acetone and nitrobenzene little or no deposition is obtained, whereas *cyclo*hexanone gives a result in agreement with the first series where magnesium carbonate is deposited, but gives no appreciable deposit with calcium carbonate. In certain cases a break occurs in the relationships when the deposit is heavy, possibly because the weight of the deposit causes a partial breaking-off. The mechanical strength of the deposit appears to depend upon the conditions of deposition. It has also been shown that the presence of small amounts of water or of acid has no apparent effect, but larger amounts cause, in the first case, an increasingly wet deposit and in the second, complete inhibition. The purity of the suspending medium can affect the action greatly. Heavy currents obtained during tests on calcium carbonate suspended in methanol are thought to be due to a reaction between these substances.

Introduction

Observations on electrophoretic deposition were made by Harsanyi,[1] Hansgirg,[2] Egyesült Izzólámpa és Villamossági R.T.,[3] Patai & Tomaschek,[4] N.V. Philips Gloeilampenfabrieken[5, 6] and de Boer, Hamaker & Verwey.[7]

The electrodeposition of powdered inorganic materials from suspension in organic liquids has been described by Hamaker,[8] who obtained deposits of barium, strontium and magnesium

carbonates, magnesium and aluminium oxides and calcium fluoride from suspension in methanol, ethanol, acetone and mixtures of these. He derived an expression relating the amount of deposit to the concentration, the electrical field, the electrode surface, the time and a constant depending upon the chemical constitution of the suspension. A rapid decrease in current was observed in some cases while deposition was proceeding, which he attributed to strong polarization in the coating. Irregular results were obtained when low concentrations, low voltages and short times of deposition were employed.

Hamaker & Verwey[9] have discussed the role of forces between particles in electrodeposition, and they state that a stable suspension is necessary for the formation of a deposit and draw a parallel between deposition obtained electrically and that obtained by sedimentation. They examined deposition under conditions where no energy minimum existed in the charge/distance-of-separation curve for the particles, and showed that deposition was still possible. It was pointed out that deposits could be obtained which had a high mechanical strength whereas others might be very fluid in nature.

Hamaker[10] concluded that deposit-formation would not be greatly influenced by particle size, since the forces exerted on the particle by the electrical field vary similarly to the forces acting between the particles.

Benjamin & Osborn[11] described the electrodeposition of oxide coatings with alkaline-earth carbonates and alumina deposited on nickel or tungsten wire. Deposits of the carbonates were obtained from methanol and acetone, but not from ethanol, propyl alcohol, butyl alcohol or amyl alcohol. Deposition from aqueous suspension was disturbed by gas formation at the electrodes. They reported an apparent polarizing action with the carbonates but not with alumina, only the carbonates giving a drop in current. The addition of small amounts of water, acid or alkali had little or no effect, but larger quantities of acid or alkali inhibited the deposition. Details were given of the use of ethylene glycol to increase the viscosity of the suspending medium, and of nitrocellulose as a binder.

Hill, Lovering & Rees[12] examined, theoretically and experimentally, the deposition of materials from non-aqueous media, taking into account the mutual interaction of the particles. They used a double carbonate of barium and strontium and determined the yield for various concentrations of suspension and at various potential differences. When high polymers were dispersed in the suspending medium a 'critical voltage' appeared. They also found a critical time at which deposition set in, and determined its dependence on applied potential and cathode radius. The critical voltage was thought to be due to a sudden change in electrokinetic properties due to the introduction of the resin into the double layer. These authors stated that on no occasion did they observe any decrease in current during deposition, unless sufficient water was present to cause obvious gas evolution, and they agree, therefore, with Hamaker's conclusions that such a drop is due to polarization, attributing the results of Benjamin & Osborn to the presence of excessive moisture derived either from the suspending medium or from the suspended material.

Experimental

Apparatus

The apparatus used in the present investigation is shown in Fig. 1, the cathode C being a carbon rod 0·5 in. in diameter, and the anode A, a cylinder of stainless-steel gauze 1·5 in. in diameter. The electrodes are positioned and spaced by two Sindanyo rings S S having central holes through which the carbon rod is a push-fit. The rod is held vertically by means of a rubber collar R which rests on the upper insulating ring in such a way that the lower end of the rod is exactly level with the bottom edge of the anode cylinder. In normal operation

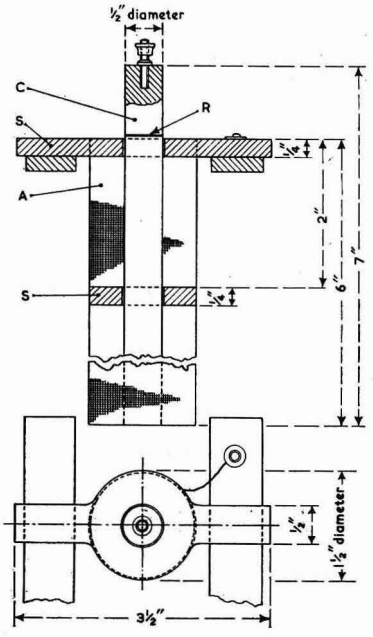

FIG. 1.—*Electrodeposition cell*

about 1 in. of the electrode assembly is immersed in the suspension contained in a suitable vessel, two wooden struts, acting as supports for the electrode system, being attached by screws to two lugs on the upper insulating ring. One of these struts carries the anode terminal.

The electrical circuit is shown in Fig. 3 of the previous paper (p. 185). The potential is controlled (0–250 v) by two rheostats, R_1 and R_2, wired as potential dividers with an overall resistance of 75 Ω and a current rating of 4 A. Voltmeters and ammeters, covering various ranges, are included in the circuit as required, by double-throw switches.

Materials

The materials used were: calcium carbonate (AnalaR); light magnesium carbonate B.P.; heavy magnesium carbonate; magnesium hydroxide (AnalaR); calcium hydroxide (AnalaR); two samples of dolomite from Duror, Argyllshire and from Kishorn, Ross and Cromarty.

Procedure

The method adopted was to make up the suspension in a suitable beaker and immerse the electrode assembly to a fixed depth, supported on the rim of the vessel by the wooden struts. Electrodeposition was then carried out for a given time, readings of the applied potential and current being taken. The electrode assembly was lifted from the suspension, the rubber collar supporting the cathode was removed, and the rod pushed clear of the anode cylinder. The deposit was now removed from the rod, placed in a previously weighed filter-paper, dried in an electric oven and weighed.

The reproducibility of results obtainable by this method was of the order of $\pm$ 2%.

Results

The results obtained are shown in Figs. 2–8, other details being given in Table I.

The standard conditions of experiment adopted were:

(*a*) With calcium carbonate: 50 g. suspended in 500 ml. of ethanol; potential of 150 v applied for 30 seconds at room temperature; length of electrode immersed, 1 in.

(*b*) With magnesium carbonate (light): 20 g. suspended in 500 ml. of ethanol; potential of 150 v applied for 30 seconds at room temperature; length of electrode immersed, $\frac{3}{4}$ in.

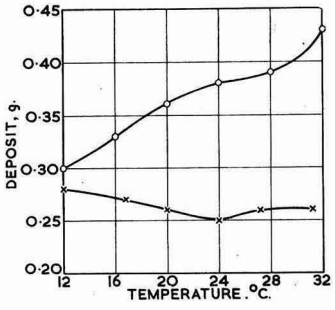

FIG. 2.—*Relation between amount of deposit and temperature*

○ Calcium carbonate
× Light magnesium carbonate

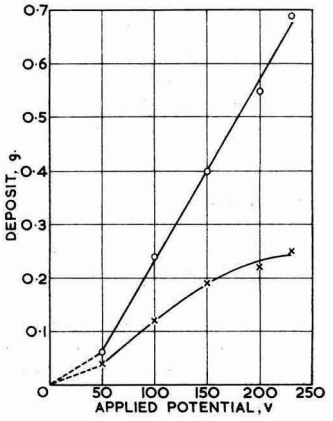

FIG. 3.—*Relation between amount of deposit and applied potential*

○ Calcium carbonate
× Light magnesium carbonate

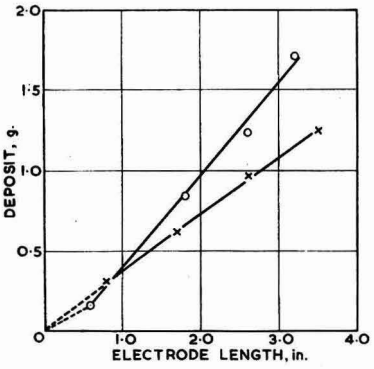

FIG. 4.—*Relation between amount of deposit and electrode surface*

○ Calcium carbonate
× Light magnesium carbonate

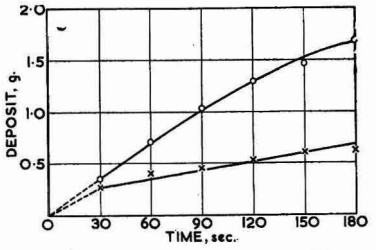

FIG. 5.—*Relation between amount of deposit and time*

○ Calcium carbonate
× Light magnesium carbonate

Table I

Variable	Material	Deposit, g.	Remarks
Temp. (12–32°)	$CaCO_3$	3	Tendency for amount of deposit to increase with increasing temperature
	Light $MgCO_3$	3	Little or no effect
Applied potential (50–230 V)	$CaCO_3$	4	Linear increase in amount of deposit with increasing applied potential
	Light $MgCO_3$	4	Linear increase initially, falling away latterly
Electrode surface (length, 0·6–3·5 in.)	$CaCO_3$	5	Linear increase in amount of deposit with increasing electrode surface
	Light $MgCO_3$	5	Linear increase in amount of deposit with increasing electrode surface
Time (30–180 sec.)	$CaCO_3$	6	Linear increase initially with increase of time, falling away latterly
	Light $MgCO_3$	6	Linear increase initially with increase of time, falling away latterly

Effect of presence of water.—Calcium carbonate only was used. Conditions were standard except that the suspending media used were methanol, methanol containing 2% of water, ethanol, and ethanol containing 2% of water. The results of a series of tests in which the applied potential was varied are given in Fig. 6, from which it may be observed that the presence of water has no apparent effect. When 10–20% of water was present the amount of deposit tended to decrease and was rather wet.

Effect of presence of acid.—Calcium carbonate only was used for this test under standard conditions of concentration in ethanol, varying amounts of sulphuric acid being added to the suspension. A potential of 100 V was applied for 30 seconds. The addition of a small amount of dilute acid showed no effect, but a larger quantity inhibited deposition completely.

Effect of purity of suspending medium.—Calcium carbonate was used for this series of tests in which standard conditions were employed, apart from the suspending media, which

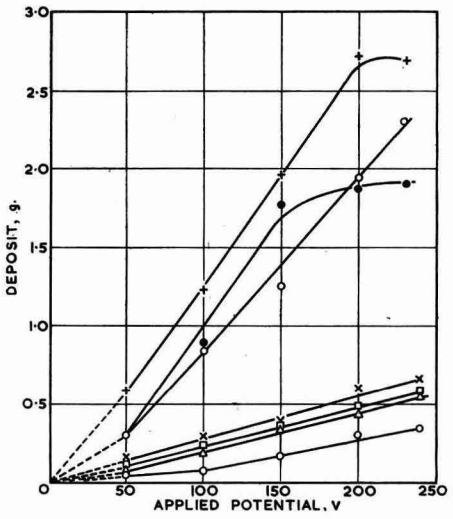

FIG. 6.—*Relation between amount of deposit and applied potential, and the effect of water on it*

+ Absolute methanol
● Methanol/water
○ Methanol
□ Absolute ethanol
× Ethanol/water
△ Ethanol

were absolute methanol, commercial methanol (two samples), commercial methanol with 2% of water added, absolute ethanol, commercial ethanol and commercial ethanol with 2% of water added. In each case, tests were made at different applied potentials. The results are shown in Fig. 6. It will be seen that absolute methanol gave the greatest amount of deposit, one sample of commercial methanol and the same sample with 2% of water giving somewhat less. The other sample of commercial methanol gave completely erratic results. No significant differences were observed in the results obtained with the ethanol.

Effect of different suspending media.—Both carbonates were used in this series of tests which were made in a smaller container. Calcium carbonate (15 g.) and light magnesium carbonate (5 g.) were used, each in 150 ml. of liquid. A potential of 240 v was applied for 30 seconds. The liquids are listed, together with their dielectric constants, in Table II, and were all of AnalaR quality, except *n*-propyl alcohol, which was of B.P. standard, and *cyclo*hexanone, which was redistilled. Fig. 7 shows the amount of deposit plotted against the dielectric constant of the liquid concerned. With two exceptions the amount of deposit increased with increasing dielectric constant, the exceptions being acetone and nitrobenzene.

Effect of concentration.—Standard conditions were used, methanol being employed as suspending medium except with calcium and light magnesium carbonates. Calcium carbonate

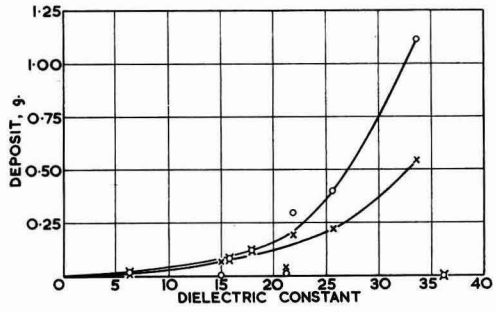

FIG. 7.—*Relation between amount of deposit and dielectric constant*

○ Calcium carbonate
× Light magnesium carbonate

Table II

Liquid	Dielectric constant *
Ethyl acetate	6·4
*cyclo*Hexanone	15·0
n-Amyl alcohol	15·8
n-Butyl alcohol	17·8
Acetone	21·3
n-Propyl alcohol	21·8
Ethanol	25·7
Methanol	33·7
Nitrobenzene	36·1

* These values are taken from the International Critical Tables

(Fig. 8) and two samples of dolomite all gave a linear relationship, but with light and heavy magnesium carbonates, and calcium and magnesium hydroxides, increasing concentration caused a very rapid increase in the amount of deposit.

Additional information about the materials used was obtained from infra-red absorption curves and examination by a Vickers projection-microscope.

The infra-red absorption curves showed, as expected, the presence of the hydroxyl group in calcium and magnesium hydroxides and in light and heavy magnesium carbonates, which are basic in character. No hydroxyl group was found in calcium carbonate nor in either of the two dolomites.

Examination of particle size on the projection microscope yielded the following information :

Calcium carbonate : fairly uniform ; about 10 μ
Light magnesium carbonate : fairly uniform ; about 1 μ or less
Heavy magnesium carbonate : fairly uniform ; about 10–15 μ
Calcium hydroxide : some larger particles but mainly 3–5 μ
Magnesium hydroxide : mainly 1–3 μ
Duror dolomite : mainly 2 μ, though some 4–5 μ
Kishorn dolomite : wide range with main groups 8–10 and 2–4 μ

Discussion

The observed linear relationships between the amount of deposit, on the one hand, and time, concentration (in some cases), applied potential and electrode surface, on the other, are in agreement with the results of Hamaker[8] and of Biguenet & Mano.[13] Biguenet & Mano also reported a decrease in the amount of deposit with increasing temperature, which was not observed in the present investigation.

The initial portions of the graphs, between the origin and the first experimental point are, in some cases, not a smooth continuation of the rest of the curve, and this is attributed to the unique conditions existing at this stage. The initial deposit is formed on the surface of the cathode rod and material deposited later builds up on the previously deposited coating. It follows that, in the lower region of the curve where the weight of deposit is small, the effect of the initial deposit will be relatively large.

The fact that many of the curves depart from a linear relationship is thought to be due to the quantities dealt with. Very large deposits were obtained, and the departures may well have been due to deposited material breaking off under its own weight. The point at which this occurs appears to vary considerably, possibly because the mechanical strength of the deposit is dependent, to some extent, on the experimental conditions. Previous experiments, on the other hand, have been on a very much smaller scale, and deposits were not sufficiently heavy to cause any difficulties.

The results obtained when water and acid were added to the suspending medium agree with those published by Benjamin & Osborn,[11] whereas the very erratic results obtained with a particular sample of commercial methanol (Fig. 6) show the advisability of ensuring the purity of the suspending medium.

The exceptional behaviour of acetone is not in agreement with previously published work, since Hamaker,[8] Benjamin & Osborn[11] and Biguenet & Mano[13] report normal deposit-formation. An obvious difference between acetone and the series of alcohols used is the absence of the hydroxyl group in the acetone, the enol form in acetone being vanishingly small under the experimental conditions. To check this point, *cyclo*hexanone was used as suspending medium, since it is known to have about 12% of the enol form under normal conditions.[14] The results indicate (Fig. 7) that the presence of the hydroxyl group in the suspending medium is necessary for successful deposition, whereas with hydroxyl groups in the suspended solid,

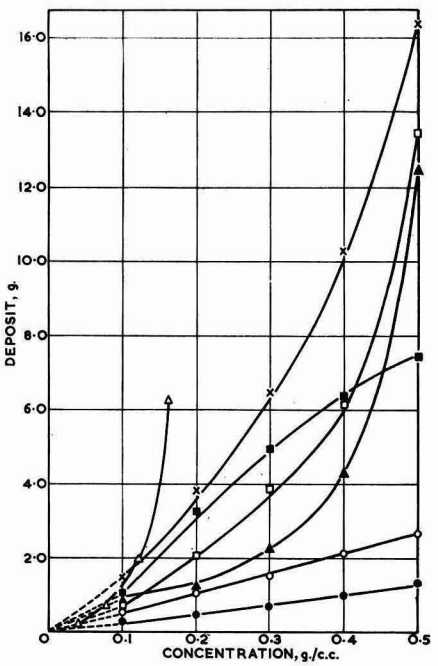

FIG. 8.—*Relation between amount of deposit and concentration*

● Calcium carbonate ■ Kishorn dolomite
○ Duror dolomite × Calcium hydroxide
▲ Heavy magnesium carbonate △ Light magnesium carbonate
□ Magnesium hydroxide

deposition is enhanced and unusual concentration effects are observed (Fig. 8). The possibility of some form of hydrogen bonding involving hydroxyl groups must be considered, a factor which is not possible with acetone suspensions.

The rapid increase of deposit with increasing concentration is also unexpected. The particle-size examination showed that this was certainly not a controlling factor, and it was observed that these abnormal results were obtained with solids which contained hydroxyl groups and not with those which had no hydroxyl groups. The suspensions in which the effect was noted were observed to be almost paste-like at the higher concentrations, and Hill, Lovering & Rees[12] have pointed out the probability of abnormal results under such conditions due to interaction of the suspended particles. Considering this effect in conjunction with that noted with acetone, it is tentatively suggested that hydrogen bonding may play an important part in determining the interaction of particles in suspension.

The current almost always decreased during deposition by an amount showing a general parallelism with the amount and firmness of the deposit. If the deposit was wet, the drop in current was less than if the deposit was relatively non-porous. Even when every precaution was taken to ensure the absence of water from both the suspended solid and the suspending medium, the current still decreased during deposition. It would seem, therefore, that the effect is due to the insulating effect of the coating of inorganic material on the cathode surface and not to polarization.

Currents were always higher with calcium carbonate than with magnesium carbonate when suspended in methanol, and at higher concentrations were so large that excessive heating was unavoidable, thus preventing any examination of the effect of very high concentrations of this material. It would appear that some reaction must take place between the calcium carbonate and methanol which gives rise to ionization. It proved impossible to work at high concentrations of calcium carbonate in ethanol, owing to the extreme weakness of the structure of the deposit, which broke away from the electrode under its own weight as soon as the applied potential was removed. It was, therefore, not possible to find out whether calcium carbonate could be made to exhibit the abnormal increase with concentration observed with the basic magnesium carbonates.

Acknowledgments

One of the authors (J. C. D.) is indebted to the Scottish Hydroelectric Board for a research scholarship. Grateful acknowledgment is made to the Department of Mining Engineering of the Royal Technical College, Glasgow, for the use of the Vickers projection-microscope. Thanks are also due to Dr. I. A. Brownlie and to Mr. D. Lambie for their assistance in obtaining the infra-red absorption curves.

Technical Chemistry Department
Royal Technical College
Glasgow

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References

[1] Harsanyi, J., Hung. Pat. 102,507 (1928)
[2] Hansgirg, F., Austrian Pat. 124,070 (1930)
[3] Egyesült Izzólámpa és Villamossági R.T., B.P. 381,486 (1932)
[4] Patai, E. & Tomaschek, Z., *Kolloidzschr.*, 1936, **74**, 253
[5] N.V. Philips Gloeilampenfabrieken, B.P. 444,723 (1936)
[6] N.V. Philips Gloeilampenfabrieken, B.P. 478,187 (1938)
[7] de Boer, J. H., Hamaker, H. C. & Verwey, E. J. W., *Rec. Trav. chim. Pays-Bas*, 1939, **58**, 662
[8] Hamaker, H. C., *Trans. Faraday Soc.*, 1940, **36**, 279
[9] Hamaker, H. C. & Verwey, E. J. W., *Trans. Faraday Soc.*, 1940, **36**, 180
[10] Hamaker, H. C., *Trans. Faraday Soc.*, 1940, **36**, 186
[11] Benjamin, M. & Osborn, A. B., *Trans. Faraday Soc.*, 1940, **36**, 287
[12] Hill, C. G. A., Lovering, P. E. & Rees, A. L. G., *Trans. Faraday Soc.*, 1947, **43**, 407
[13] Biguenet, C. & Mano, C., *Vide, Paris*, 1947, **2**, 291
[14] Grignard, V. & Mingasson, G., *Bull. Soc. chim. Fr.*, 1928, **41**, 761

THE WATER-SOLUBLE CARBOHYDRATES OF GRASSES. I.—Changes Occurring during the Normal Life-cycle

By R. WAITE and J. BOYD

The water-soluble carbohydrates [glucose and fructose (reported together as total hexoses), sucrose and fructosan] in the leaf, stem and head portions of four grasses have been determined at regular intervals throughout their growth cycle during two successive years. Hexoses were at all times present in only small amounts. The concentration of sucrose varied with the stage of growth, rising to a peak in May–June, and the effect of the stage of growth on the amount of sucrose was similar in both leaf and stem. Fructosan was present in a much higher concentration in the stem than in the leaf, and stage of growth had a marked effect on the amount present. A relationship is suggested between these fluctuations in fructosan content and morphological changes, first in the development of the growing point from a vegetative to a floral state, and later in seed formation.

It is well known that the stage of growth of grass is the major factor governing its chemical composition. The general trends and order of magnitude of the changes brought about by increasing age in such constituents as crude protein, crude fibre, ether extracts, ash and lignin are well established.[1–3] Considerably less is known about the corresponding changes in the simpler carbohydrates, although, as Archbold[4] mentions in his review, the fructosans of monocotyledons were extensively investigated during the period 1870–1900. Some account of the simpler sugars (glucose, fructose and sucrose) occurring in grasses was given by de Cugnac,[5] but the fructosan content was obviously considered the most important. In a recent paper Laidlaw & Reid[6] have suggested that melibiose, raffinose and stachyose are also present in small amounts. It is reasonable to suppose, moreover, that oligosaccharides intermediate in chain length between sucrose and fructosan may occur.

Much of the earlier work on the carbohydrates of monocotyledons was concerned with cereals, and emphasis was naturally placed on the changes associated with emergence of the flower head and the setting of the seed. This attitude persisted somewhat when the carbohydrates in grasses were first investigated, and changes occurring in the early vegetative stages were neglected. Much of the work of the French school of Colin & de Cugnac[7] and de Cugnac[5] thus deals mainly with the flowering and seed-setting stages of grasses. Norman[8, 9] and Norman & Richardson[10] sampled both rye-grass (*Lolium perenne*) and cocksfoot (*Dactylis glomerata*) from a fairly early stage until after inflorescence and were impressed by the large amounts of fructosan present, particularly in rye-grass. Their method of drying the fresh material, however, was probably insufficient to stop enzyme action completely; for this reason they discounted the free fructose found in many samples and apparently assumed sucrose to be absent, although they pointed out that some carbohydrate was unaccounted for. Thus it can be seen that the fructosan fraction received most attention and provided more purely chemical interest in the elucidation of its structure[11, 12] than a consideration of the relationship between the three major carbohydrate groups, free hexoses, sucrose and fructosan. Recently de Man & de Heus[13] presented an interesting scheme for the determination of glucose, fructose, sucrose and fructosan in grass, but they gave examples of its application only to four samples of rye-grass cut between April and June.

The object of the present series of experiments was therefore to investigate the distribution of these three groups of soluble carbohydrates in four of the more commonly used species of grass. Before examining the carbohydrates in grass grown for some specific agricultural purpose, such as grazing, drying or hay-making, it was considered important first to determine how these constituents changed during the normal growth of the grass from a very young vegetative stage, through inflorescence and seed setting to senescence.

Experimental

Grasses used and samples taken

Perennial rye-grass (strain S23), cocksfoot (strain S143), meadow fescue (*Festuca pratensis* strain S53) and timothy (*Phleum pratense* strain S48) were sown in small plots (36 sq. yd.) in the summer of 1950 on land previously used for a short-term rye-grass ley. No cuts were taken during 1950 other than a trimming cut in November. Superphosphate (18% P_2O_5) and potash (60% K_2O) were applied in February, 1951, followed by 'Nitro-Chalk' at the beginning of April, each at the rate of 2 cwt./acre. The grasses were allowed to grow without check and a sample was cut at weekly intervals from the second week in April until October. To

investigate the effect of weather variations, particularly in the spring, the whole experiment was repeated in 1952, with the same plots, manurial treatment and sampling system as in 1951.

When plant constituents are unequally distributed between leaf and stem the height at which samples are cut is obviously important. This is particularly so in grasses, where the fructosan at certain times in the year is concentrated in the base of the stem.[5, 9] For this reason all samples were cut with scissors or sheep shears at a height of approximately ½ in. above ground level. Usually 1–2 sq. yd. was cut and a rough estimate of the yield of dry matter obtained from the fresh weight of the samples, the area cut and the moisture content of the grass.

Treatment of the grass sample before analysis

In the analysis of plant material, the gain in accuracy of sampling by drying and powdering a relatively large weight of material has always to be balanced against any loss in accuracy through changes in plant constituents during the drying treatment. Raymond[14] has found, when using a forced-draught oven at 95°, that six hours were required to dry 400 g. of fresh grass, and in that time 3–9% of dry matter was lost compared with high-frequency drying; the younger the grass the greater was the loss. This demonstrates the need for rapid drying, and the oven used in the present experiments dried 600 g. of grass from 83 to 2–3% moisture in 45 minutes. This was done in a small vertical oven by passing air at 105–110° through the grass at the rate of 100 cu.ft./minute. Such heat treatment has been shown[15] to cause loss of carotene from young grass of only 9%, and seems well suited to this type of work.

It was to be expected that the simpler carbohydrates would be affected by diurnal variations, and a preliminary experiment showed that this was so. In Table I the results are given

Table I

Diurnal variation of carbohydrate (percentage of dry matter) in the aerial parts of rye-grass

6–7 June, 1951. Total sunshine, 9·8 hr. Temperature, max., 71·8° F.; min., 47·1° F.

Time	Hexoses	Sucrose	Fructosan	Crude protein
9 a.m.	2·9	5·3	16·4	8·7
12 noon	2·5	6·7	17·4	7·9
3 p.m.	2·4	7·0	16·1	8·0
6 p.m.	2·8	7·0	15·8	8·8
9 p.m.	2·7	6·6	15·9	8·3
12 p.m.	2·6	6·6	16·6	8·3
3 a.m.	2·8	5·4	16·9	8·3
6 a.m.	2·9	5·3	16·9	8·9

for rye-grass sampled at intervals of three hours during fairly sunny weather in June. It can be seen that the sucrose content rose to a maximum in the late afternoon, the hexoses fell to their lowest value about the same time, and the fructosan varied irregularly. Evidence will be produced in a later communication to show that fructosan and crude protein are inversely correlated in young grass, and differences in the protein content of the samples may be associated with some of the fructosan variation. The grass was therefore always cut at the same time, between 9 and 10 a.m., to minimize the diurnal effect. The cut sample was placed in the oven within 10–15 minutes and enzyme action would probably have ceased about 30 minutes after cutting. A comparison of this technique with the method of placing the cut grass directly into boiling alcohol in the field showed negligible differences. Typical results of such a comparison are given in Table II. One half of the dried-grass sample containing all the aerial

Table II

Comparison of two methods of sample preparation and the effect on the carbohydrate content (percentage of dry matter) of delay in drying fresh young grass

Treatment	Hexoses	Sucrose	Fructosan
Boiling alcohol in field	2·3	4·7	7·7
Oven drying	2·0	4·8	7·7
Oven drying after 1 hour in laboratory	2·2	4·6	7·4

parts was ground in a small hammer mill to pass through a 50's sieve and the other half was separated into leaf and stem, and head, if present. These fractions were then milled separately. In 1951 the separation of leaf and stem was done on the fresh material, so that about one hour

would have elapsed between cutting and stopping of enzyme action in these fractions. Comparisons of the sugar contents by this method and by separation after drying showed that hexoses and sucrose were unaffected, but that the fructosan content fell by about 9%. This loss is higher than that shown in Table II; the reason for it is probably that the grass which stood for one hour in the laboratory was undisturbed and at a lower temperature than the grass being separated into leaf and stem.

Physical measurements associated with growth

In a series in which the effect of stage of growth is being investigated some measure of the stage reached is obviously required. It has been found in the past[16] that the ratio of the weights of leaf and stem, and the average length of the grass, as given by the measurement of 100 representative tillers, are good indications, and these were again used. In addition, the stage of development of the primary growing-point was noted and its length and position in the stem above ground level were measured. As will be seen below, the information concerning the growing point is of considerable importance to an understanding of carbohydrate changes.

Temperature and rainfall records were obtained by the courtesy of Dr. J. Grainger from the meteorological station at Auchincruive, roughly ½ mile distant from the grass plots.

Chemical analysis of the dried grass

Moisture content and crude protein (total N × 6·25) were determined on all samples.

Sugars were determined by the copper-reduction method of van der Plank,[17] with only minor alterations; the scheme of analysis suggested by de Man & de Heus[13] was used as follows:

Dried grass (0·7 g.) was allowed to stand in 80% ethanol (45 ml.) either for 18 hours in the cold, with shaking during the first two hours and again during the last hour, or refluxed in a boiling-water bath for two hours (both treatments gave the same results). The ethanolic extract was decanted and the grass shaken twice with 20-ml. portions of 80% ethanol, followed by filtration and ethanol washing on the filter to give a total volume of extract of about 100 ml. The ethanol was removed under reduced pressure at 30–37° and the walls of the flask were cleaned with about 10 ml. of warm water. The contents of the distillation flask were then filtered and the residue on the filter was washed with warm water to give a total volume of filtrate, when cool, of 50 ml. This solution (referred to as solution A) was assumed to contain only glucose, fructose and sucrose. The grass residue was quantitatively washed into the original refluxing vessel with 50 ml. of boiling water and refluxed for 30 minutes. It was then filtered and washed with boiling water to give a filtrate volume, when cool, of 100 ml. This solution (referred to as solution B) was assumed to contain only fructosan. Aliquots of both solutions A and B were clarified with dibasic lead acetate and excess lead was removed with disodium phosphate; the precipitates were centrifuged and the clear liquor was finally decanted.

With the copper reagents and conditions given by van der Plank[13] the total reducing power of 5 ml. of each clarified solution A and B was determined, both before and after hydrolysis with 0·02N-sulphuric acid in a boiling-water bath for 30 minutes. Fructose was estimated[18] after oxidation of glucose by hypoiodite at pH 11·2, in solutions A and B before and after hydrolysis. The final titre of thiosulphate was converted into weight of sugar in the 5-ml. aliquot by interpolation on previously prepared standard curves. Two curves, one for glucose and one for fructose, are necessary, since the fructose is determined in presence of considerable quantities of iodide, and the standard value must be obtained similarly.

In solution A, the difference between the value for total reducing-sugars (T.R.S.) and fructose before hydrolysis measures the amount of aldose, and this was assumed to be glucose. Again, in solution A, the increase in the value of the T.R.S. after hydrolysis measures the glucose and fructose produced by the hydrolysis of sucrose. The increase in fructose after hydrolysis should be exactly half the increase in the T.R.S. In solution B, the increase in the value of T.R.S. after hydrolysis should equal the increase in fructose from the hydrolysed fructosan. (The amount of T.R.S. and fructose in the unhydrolysed B solution was always very small, usually 0·1–0·3%.) In practice, the increase in the fructose value fulfilled this condition reasonably well, since it rarely fell outside 96–104% of the increased T.R.S. value. The increase in fructose after hydrolysis of solution A, however, was frequently more than 50% of the increase in T.R.S. This usually occurred when the amount of fructosan in the grass was high, and it is probable that some fructosan was extracted by 80% ethanol. Any

non-sugar reducing-substance would appear in the T.R.S. values both before and after hydrolysis, and would only serve to inflate the value of the free hexoses. As will be seen below, the value of the hexoses was always low (usually about 2–3% of the dry matter), so that interference by such non-sugar compounds could not have been high. Any raffinose present, which would appear as ' sucrose ', is not determined by this system, but it is thought, on the basis of qualitative chromatographic evidence, that the quantity of this sugar present was probably small. Sucrose was calculated by multiplying the increase in the T.R.S. value of solution A after hydrolysis by 0·95, and fructosan by multiplying the increase in T.R.S. of solution B after hydrolysis (which equals the increase in fructose) by 0·90. For the three carbohydrate fractions mentioned, the reproducibility of the method, based on quintuplicate experiments, was $\pm$ 3%.

Thus the values given below for the soluble carbohydrates may suffer from some inaccuracies in analysis. Preliminary experiments had shown, however, that the changes in the soluble-carbohydrate content at various stages of growth were likely to be very much greater than any errors introduced by the inadequacy of the method, and for this reason the system of analysis was justified. It will be seen that relatively small climatic differences caused considerable variation in the rate and type of growth (i.e. vegetative or floral) with a consequent twofold or threefold variation in the carbohydrate values. Hence it is the trend and order of magnitude of the changes, rather than individual values, that are of importance agriculturally.

Results

Before considering the carbohydrate values it is necessary to note the differences in the weather conditions between the two years and their effect on the growth of the four species of grass. The total monthly rainfall and average monthly temperature are shown in Fig. 1 and the leaf–stem ratio and the height of the grasses in Fig. 2. The spring of 1952 was warmer and drier than 1951, and this was more effective in promoting growth than the higher rainfall of 1951. None of the four grasses used is an ' early ' grass, but, of the four, the fescue and cocksfoot are earlier than the timothy and the rye-grass. In 1951 the growth of the cocksfoot was abnormal in that it never flowered ; this is reflected in its lower height and considerably higher leaf–stem ratio throughout the year. The slower general growth in the spring of 1951 also resulted in a higher leaf–stem ratio for the other three grasses. The autumn of 1951 was warmer than that of 1952, but weather at this time of year has little influence on the growth of primary tillers. Table III lists the dates of the major morphological changes during the two growing seasons. The development of the primary growing-point from a vegetative

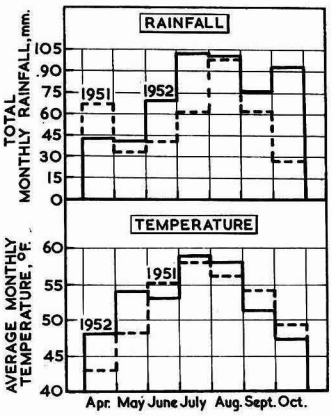

FIG. 1.—*Rainfall and temperature during the growing seasons of* 1951 *and* 1952

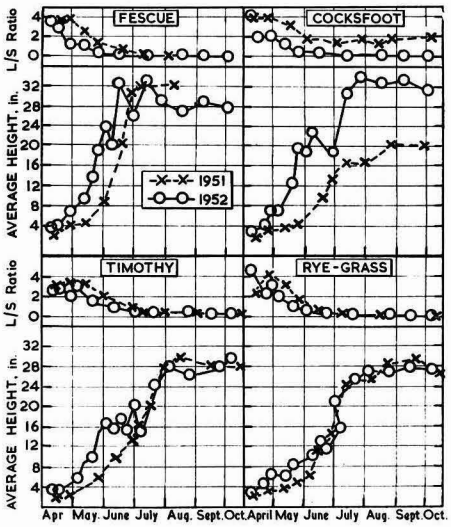

FIG. 2.—*Leaf–stem ratio and height of the grasses (L/S ratio = ratio of dry weights of leaf and stem)*

to a floral form occurs early in the year and is most easily recognized under low-power magnification. This change is immediately followed by greatly increased growth of the growing point and a corresponding elongation of the true stem behind it. (The growing point may enlarge from 0·3 to 12 mm. and move a distance of 3–4 cm. up the stem in 10 days.) These changes will be considered in more detail in a later communication; it is sufficient to note here that they represent an extremely sudden demand by the plant for energy.

Table III

Dates of occurrence of the major morphological changes in the four grasses in 1951 and 1952

Morphological change	Year	Fescue	Cocksfoot	Timothy	Rye-grass
Growing-point change	1951	14 May	—	31 May	25 May
	1952	30 April	1 May	12 May	10 May
Head emergence (50% tillers) ..	1951	9 June	—	4 July	28 June
	1952	26 May	26 May	23 June	23 June
Anthesis	1951	29 June	—	31 July	24 July
	1952	10 June	10 June	23 July	6 July
Starch in head	1951	8 Aug.	—	18 Aug.	24 Aug.
	1952	14 July	14 July	11 Aug.	4 Aug.

The changes in the three carbohydrate fractions, hexoses, sucrose and fructosan, are shown separately for leaf in Figs. 3 and 4 and for stem in Figs. 5 and 6; all values are given as a percentage of the dry matter. In all the samples examined glucose and fructose occurred in roughly equal proportions, with fructose slightly in preponderance. In view of the general low level at which they occurred, only the sum of these two sugars has been reported. The carbohydrate contents of the whole aerial parts have not been given since they can be calculated from the present values and those of the leaf–stem ratios (e.g. whole aerial parts by analysis; hexoses 2·9%, sucrose 5·0%, fructosan 9·0%; by calculation 2·9%, 4·7%, 9·2%). Moreover, they mask to some extent the change in fructosan content, which is associated chiefly with the stem.

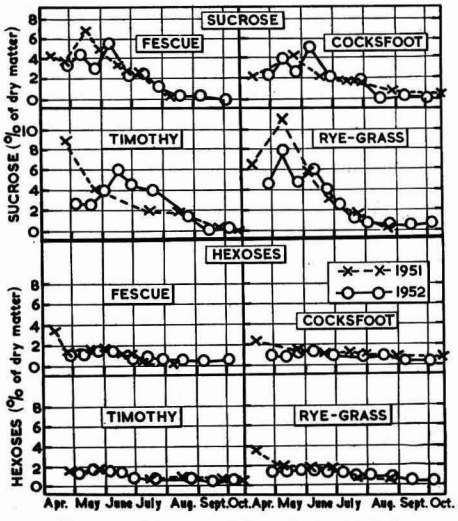

FIG. 3.—*Sucrose and hexoses in the leaf*

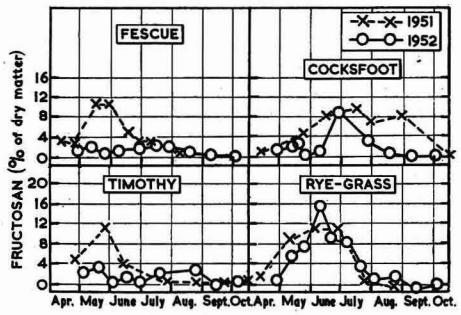

FIG. 4.—*Fructosan in the leaf*

The separated 'head' samples contained not only the flower and, later, the fertilized seed, but also at all times the rachilla, the glumes and the pales. The hexose, sucrose and fructosan contents were measured in these samples from head emergence onwards. It is unnecessary to give these values in full since it is uncertain how much the individual parts of the head contributed, and also the general pattern for all species was similar. For example, at the first sampling (May–June) sucrose and fructosan were present in the head in about equal quantity, each amounting to 2–3% of the dry matter, with hexoses only half that value. By late July the sucrose content had risen to a peak (5–7%) from which it fell steadily to a low value (1%) at the time when starch was first observed. The fructosan and hexose contents in the head barely rose from their initial values, and when starch appeared were less than 1%. The appearance of starch was judged qualitatively by the colour reaction of the extracts with iodine, which indicated that dextrins were present a week or so before the starch.

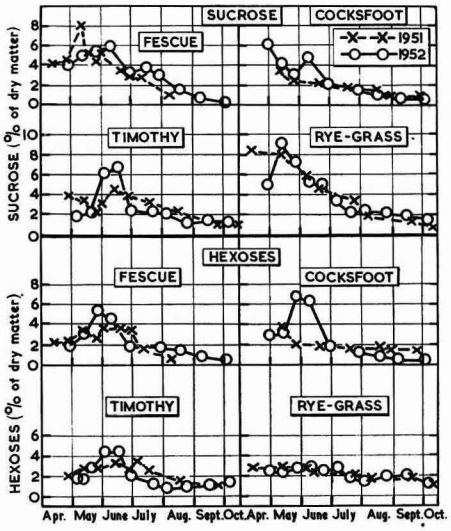

FIG. 5.—*Sucrose and hexoses in the stem*

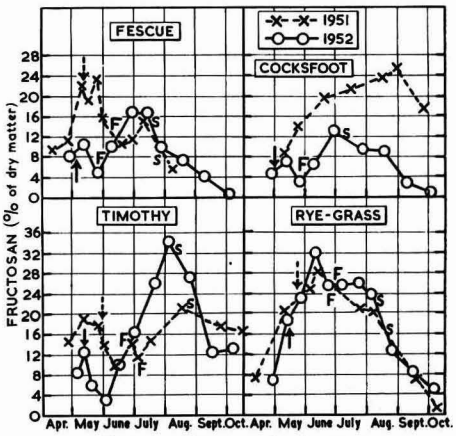

FIG. 6.—*Fructosan in the stem*

↑↑ indicates change of growing point from vegetative to floral
F indicates flower-head emergence
S indicates starch in the seed

In Fig. 6 the appearance of starch in the head is indicated by the letter S, and it can be seen that soon after its appearance the fructosan content of the stem fell rapidly.

Considering first the leaf hexoses (Fig. 3) it can be seen that the level was low throughout the season and very similar for all four grasses. Leaf sucrose, on the other hand, usually rose to a maximum in May or June, after which it declined steadily as an increasing proportion of the leaves became aged and brown. The slower-growing rye-grass had the highest sucrose values of the four grasses. In both hexoses and sucrose content the difference between the values for the two years was not great. The leaf fructosan (Fig. 4) showed marked differences both between the grasses and between the two years, but, since fructosan is stored mainly in the stem, the actual values, with perhaps the exception of those for rye-grass, were not high. Discussion of the pattern of fructosan change in the leaf will be deferred until after consideration of the corresponding changes in the fructosan stored in the stem.

The corresponding figures for the stem carbohydrates (Figs. 5 and 6) show that the hexose components were again present in only small quantity (although greater than in the leaf) and, with the exception of the rye-grass and the non-flowering cocksfoot in 1951, they showed a marked rise in early June. Stem sucrose followed a similar pattern to leaf sucrose and at much the same level. Again, the difference between the four grasses and between the values of both hexoses and sucrose in 1951 and 1952 was not large. It is only when the stem-fructosan values are considered that divergences appear. In fescue, cocksfoot (1952 only)

and timothy the fructosan curve showed two peaks, whereas in rye-grass and cocksfoot (1951) there was only a single peak. The values for the two years, particularly in the spring, showed marked differences, except in the rye-grass, for which the two curves were remarkably similar. In 1952, the cocksfoot flowered normally at almost the same time as the fescue, and both grasses had similar stem-fructosan values at all times of the year.

In contrast with the fluctuations in the values of the carbohydrate fractions, the crude protein content decreased smoothly from the high values of the youngest grasses. Typical crude protein figures for the leaf and stem in April, June and October respectively were: 23 and 14%, 8 and 3% and 4 and 2% (dry-matter basis). The values for all four grasses fell within a narrow range.

The yield of dry matter, starting at 500–600 lb./acre in April, rose sharply in May and reached a fairly constant value by mid or late July of 6000–7000 lb./acre, after which there was little change except for a slight decline in autumn. The yields in 1952 were about 10–15% higher than in 1951.

Discussion

It would appear that in the production of sugars by photosynthesis fructose and glucose are formed before sucrose and raffinose.[19, 20] The present results suggest that there must be a rapid synthesis of the more complex sugars, leading to the formation of a fructosan as the carbohydrate reserve. It is not clear what governs the equilibrium between the various sugars, but a limiting osmotic pressure may be one such factor. Formation of the higher sugars would be a simple way of preventing the osmotic pressure from rising beyond a certain limit while allowing the formation of a reserve. The magnitude of this reserve and the part of the plant in which it is stored are important, since they will affect the development of the grass and its power of recovery after defoliation. It appears from the results that fructosan can be formed in the leaf but that it does not accumulate there to any great extent. Most of the carbohydrate produced in excess of the plant's immediate needs is presumably passed to the stem and stored there as fructosan.

The first peak in the stem-fructosan curves for fescue, cocksfoot (1952) and timothy occurred within a few days of the change of the growing point from a vegetative to a floral development (this approximate date is indicated in Fig. 6 by an arrow). As a working hypothesis it is suggested that the continued rise in fructosan storage is interrupted by the heavy demands made by the rapidly-elongating growing point. When this period of rapid growth and development draws to an end, as shown by the visible emergence of the flower head (denoted by F in Fig. 6), the amount of carbohydrate synthesized by the leaves is again in excess and the fructosan curve again rises. The second peak resulted similarly from a new demand for carbohydrate, this time to form the reserve in the developing seed. Many of the leaves of the grass are brown at this stage, and, as shown by the low values for sucrose and hexoses, probably little carbohydrate is being synthesized. The rye-grass-fructosan curve did not follow this general pattern, and it would appear that in both years this grass was capable of synthesizing sufficient carbohydrate at the time of development of the growing point to prevent a fall in the fructosan value. The reason for this is not clear, but the greater efficiency of the rye-grass leaf in producing sucrose and a slower growth in spring may partly account for the single peak.

It is now possible to interpret some of the differences between the fructosan values for the two years. In 1952, active growth started earlier and was at a greater rate than in the cold spring of 1951. This is shown in Fig. 2 by the greater heights and lower leaf–stem ratios of all grasses in 1952, and by the early dates at which the growing points became floral (Table III). This early growth resulted in less fructosan being accumulated than in the slower-growing period of 1951, and the development of the growing point then reduced the fructosan reserve still further. At about the time of flower emergence in 1952, growth was slowed for about two weeks by a sharp drop in temperature (Fig. 1), and this may partly account for the higher values of the second fructosan peak, although it is unlikely to be entirely responsible for the extremely high value reached by the timothy stem. The assumption that, as growth and development proceed, all except late-flowering grasses will normally show a double peak in their stem-fructosan curve, because of flower initiation and seed development, goes far to explain the fructosan values of the cocksfoot in 1951. It has already been stated that in that year, for no immediately apparent reason, this grass never progressed beyond the vegetative stage and few flower heads were present in the whole plot. This reduced growth and development would make less demand on the carbohydrate reserve, and consequently the stem fructosan increased steadily throughout the season. In 1952, however, the cocksfoot

flowered normally and as early as the fescue, resulting in very similar double-peaked curves for the stem-fructosan content of both grasses.

It is interesting to note that the values for fructosan given by Norman[8–10] for rye-grass and cocksfoot, which were considered to rise to a single peak, do in fact fit the present hypothesis. In the early part of the year there was a fall in the fructosan value of both grasses before the main rise, and it is probable that this represented the decrease associated with flower development.

From the present results it would therefore appear that, in perennial grasses growing to maturity, fructosan accounts for the greater part of the water-soluble carbohydrates and is the constituent most likely to fluctuate in concentration at different seasons. These fluctuations would appear to be associated with flower initiation and development and later with seed formation. Earliness of flowering habit, or a warm 'early' spring, may be expected to keep the fructosan values before flowering at a low level. The fructosan content of the later-flowering grasses, on the other hand, does not appear to be so much affected by spring conditions and tends to follow a more regular pattern.

Summary

1. Four perennial grasses—meadow fescue, cocksfoot, timothy and rye-grass—were sampled weekly from earliest spring growth to autumnal senescence in 1951 and again in 1952. The average height of the grass, the ratio of the weight of leaf to stem and the yield of dry matter were recorded at each sampling. Examinations were also made of the stage of development of the primary growing-point to establish the date of flower initiation.

2. The aerial parts were separated into leaf and stem, and, when available, head. These fractions were analysed for glucose, fructose, sucrose and fructosan. Crude protein was also determined on all samples.

3. The results show that the total free-hexose content in all plant fractions was low at all times of the year. The sucrose content in leaf and stem was similar and rose to a maximum value during May–June. Fructosan was present in all grasses and was the major carbohydrate constituent. The curves for the fructosan in fescue, cocksfoot and timothy showed two peaks, the first in May and the second in July–August; rye-grass exhibited a single peak in June. Starch was present only in the mature seed.

4. The fluctuations in the fructosan content are explained as the result, first, of flower initiation and development in the primary growing-point, and secondly, of carbohydrate formation in the seed.

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The Hannah Dairy Research Institute
Kirkhill, Ayr

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References

[1] Wilson, D. J., *Trans. Highl. agric. Soc. Scot.* (*Ser.* 5), 1889, **1**, 1
[2] Fagan, T. W. & Milton, W. E. J., *Welsh J. Agric.*, 1931, **7**, 246
[3] Pazur, J. H. & DeLong, W. A., *Sci. Agric.*, 1948, **28**, 39
[4] Archbold, H. K., *New Phytology*, 1939, **2**, 185
[5] Cugnac, A. de, *Ann. Sci. nat.*, 1931, **13**, 1
[6] Laidlaw, R. A. & Reid, S. G., *J. Sci. Fd Agric.*, 1952, **3**, 19
[7] Colin, H. & Cugnac, A. de, *Bull. Soc. Chim. biol., Paris*, 1926, **8**, 621
[8] Norman, A. G., *Biochem. J.*, 1936, **30**, 1354
[9] Norman, A. G., *Biochem. J.*, 1939, **33**, 1201
[10] Norman, A. G. & Richardson, H. L., *Biochem. J.*, 1937, **31**, 1556
[11] Challinor, S. W., Haworth, W. N. & Hirst, E. L., *J. chem. Soc.*, 1934, p. 676
[12] Laidlaw, R. A. & Reid, S. G., *J. chem. Soc.*, 1951, p. 1830
[13] Man, T. J. de & Heus, J. G. de, *Rec. Trav. chim. Pays-Bas*, 1949, **68**, 43
[14] Raymond, W. F., 'Experiments in Progress', 1951, p. 57 (Stratford-on-Avon: Grassland Research Station)
[15] Waite, R. & Sastry, K. N. S., *J. agric. Sci.*, 1949, **39**, 174
[16] Waite, R. & Sastry, K. N. S., *Emp. J. exp. Agric.*, 1949, **17**, 179
[17] van der Plank, J. E., *Biochem. J.*, 1936, **30**, 457
[18] Ingles, O. G. & Israel, G. C., *J. chem. Soc.*, 1948, p. 810
[19] Aronoff, S. & Vernon, L., *Arch. Biochem.*, 1950, **28**, 424
[20] Benson, A. A., *Arch. Biochem.*, 1951, **32**, 223

THE DETERMINATION OF CAFFEINE

By F. J. T. HARRIS

Very many modifications of the basic method for the determination of caffeine in coffee products have been proposed from time to time. In a search for a rapid and reliable method all the steps and many alternative procedures have been considered. As a result, a method is now described which, although presenting few novel features, meets all the requirements and gives results of better-than-average accuracy. The accuracy of the method has been proved by checking actual manufactures.

Caffeine can be extracted from a solution buffered with trisodium phosphate, and determined by a Kjeldahl digestion. Zinc ferrocyanide is a satisfactory clarifying agent, provided that it is removed by filtration within, say, 15 minutes.

The accuracy of the proposed method is approximately 1% from the recovery experiments. A number of experimental batches of coffee and chicory essence were examined, and satisfactory agreement was found between calculated and determined caffeine contents.

Both the Power–Chesnut[1] and the Bailey–Andrew[2] methods for the determination of caffeine are time-consuming. The Bailey–Andrew method, now an official method for caffeine in coffee, though more rapid than the Power–Chesnut method, still requires at least seven hours per sample. Modifications[3] have been made in the Power–Chesnut method, but the method is still very lengthy. In a search for a speedy, accurate method, suitable for routine use, a number of published methods have been examined, and a satisfactory method has been produced.

A procedure for the determination of caffeine usually includes the following five steps: (*a*) preparation of the sample, (*b*) clarification of the solution, (*c*) extraction of the caffeine, (*d*) purification of the extracted caffeine and (*e*) quantitative measurement.

Proposed method

(*a*) *Preparation of the sample*

Prepare a solution or extract of the material that will contain 25–200 mg. of caffeine/100 ml. of solution. The method employed should be the simplest consistent with the material, and any chemical treatment should be avoided as far as possible at this stage. Transfer 100 ml. of solution to a 200-ml. standard flask.

(*b*) *Clarification*

To the solution in the standard flask add 10 ml. of zinc acetate solution,[4] mix, and then immediately add, with constant swirling, 10 ml. of potassium ferrocyanide solution.[4] Dilute to 200 ml. and shake well. After 4 minutes filter through a rapid filter-paper (e.g. Postlip BL or C), discard the first 2 ml. of filtrate, and then collect for not more than 15 minutes. Pipette 100 ml. of the filtrate into a 200-ml. standard flask, add about 50 ml. of distilled water, 20 ml. of the trisodium phosphate buffer solution, dilute to 200 ml., mix, and filter through a dry filter-paper.

(*c*) *and* (*d*) *Extraction and purification*

Transfer 150 ml. of the filtrate to a 250-ml. separating funnel; the pH should now be about 11. Shake the clarified extract with 30 ml. of chloroform and, after setting, run off the chloroform into another separating funnel containing 5 ml. of N-sulphuric acid. Repeat the operation 4 times. After the chloroform extraction is complete, shake the combined chloroform extracts with the sulphuric acid. Allow to settle for 10 minutes, then draw off the chloroform, passing it through a small dry filter-paper into a round-bottomed flask. Wash the sulphuric acid with one 30-ml. portion of chloroform, run this off and add it to the bulk.

(*e*) *Determination*

Distil off the bulk of the chloroform and transfer the remaining 10–15 ml. quantitatively to a 100-ml. digestion flask. Evaporate the chloroform extract to dryness in a boiling-water bath. Add 25 ml. of the potassium sulphate–cupric sulphate solution and a few glass chips, then heat until the solution boils briskly. Digest for 45 minutes, then allow to cool.

Transfer the digest quantitatively to an ammonia-distillation apparatus; dilute as necessary, according to the size of the apparatus. Add zinc and an excess of sodium hydroxide solution (about 75 ml.). Distil the ammonia into 25 ml. of the boric acid solution containing 2 drops of the mixed indicator. When sufficient distillate has collected to give a quantitative recovery of the ammonia, the amount depending on the size of the apparatus, titrate the distillate with 0·1N-hydrochloric acid to the colourless stage of the indicator.

Blanks

Carry through the complete procedure on a sample of water. The blank titration is usually less than 0·1 ml. of 0·1N-hydrochloric acid.

1 ml. of 0·1N-acid = 4·85(5) mg. of anhydrous caffeine.

Reagents

1. *Chloroform.*—AnalaR.
2. *Sulphuric acid.*—Approximately N.
3. *Zinc acetate solution.*—Dissolve 219 g. of A.R. zinc acetate dihydrate and 30 ml. of A.R. acetic acid in water and dilute to 1 l.
4. *Potassium ferrocyanide solution.*—Dissolve 106 g. of A.R. potassium ferrocyanide trihydrate in water and dilute to 1 l. Store in a brown bottle.
5. *Trisodium phosphate buffer solution.*—Dissolve 190 g. of pure trisodium phosphate duodecahydrate in water and dilute to 1 l.
6. *Potassium sulphate and cupric sulphate solution.*—Dissolve 40 g. of M.A.R. potassium sulphate, 4 g. of M.A.R. cupric sulphate and 100 ml. of M.A.R. sulphuric acid in water and dilute to 500 ml.
7. *Boric acid solution.*—Dissolve 30 g. of A.R. boric acid in water and dilute to 1 l.
8. *Indicator.*—Dissolve 0·06 g. of bromocresol green and 0·04 g. of methyl red in 100 ml. of 95% alcohol.
9. *Hydrochloric acid.*—Either prepare an approximately 0·1N-solution and standardize, or preferably prepare a standard solution by dilution of the constant-boiling acid.[5]
10. *Sodium hydroxide solution.*—About 5N.

Discussion

(*a*) *Preparation of samples*

With ground roasted coffee it is well known that extraction of caffeine is higher in acid or alkaline than in neutral solutions. It has been shown[6] that an acid extraction is rather more efficient than an alkaline extraction.

With the essences and extracts, it is sufficient to dilute and heat the solution. The potassium caffeine chlorogenates or tannates are soluble in warm water. Frequently a sludge is found in the bottom of bottles of coffee essence or extract, and this sludge is relatively rich in caffeine. It is therefore necessary to see that the contents of any bottle are well mixed before sampling.

(*b*), (*c*) *and* (*d*) *Clarification and extraction of the solution and purification of the crude caffeine*

The determination of caffeine in coffee infusions by the repeated extraction of the alkaline solution with chloroform[7] is tedious because the chloroform separates slowly, and fatty materials are extracted as well as the caffeine. The use of a clarifying agent prevents the formation of emulsions and also increases the purity of the extracted caffeine.

The use of lead acetate to clarify the coffee infusion has been suggested,[8] as has zinc ferrocyanide.[9] Both Bower *et al.*,[10] and Ishler *et al.*[6] have used magnesium oxide as a clarifying agent.

Silvestri[11] has proposed treatment of coffee with neutral potassium permanganate in two stages, followed by the addition of cupric sulphate and sodium hydroxide. Maes[12] purifies the crude caffeine by dissolving in water, oxidizing the impurities with permanganate and re-extracting with chloroform. However, the use of permanganate has been shown to cause loss of caffeine.[13]

Bührer[14] makes the solution alkaline with ammonia and extracts with chloroform, whereas Washburn & Krueger,[15] working with pharmaceuticals, extract the caffeine with chloroform from a solution made acid with hydrochloric acid.

Hadorn & Jungjunz[16, 17] used a column of activated alumina to remove impurities after extraction of ammoniacal coffee infusions, which made the method long and not suitable for routine use.

The use of lead acetate leads to high and variable results. The extracted caffeine is still contaminated with fatty materials, an observation in agreement with those of Ishler.[6] Zinc ferrocyanide has been used by Moores & Campbell[18] followed by sodium phosphate to render the solution alkaline. Zinc ferrocyanide has one disadvantage, namely that the precipitate slowly absorbs caffeine, but provided the time of contact is short, the recovery is quantitative, as will be seen from Table I. The time of contact of the zinc ferrocyanide and the caffeine solution has therefore been standardized in this method.

After removal of the zinc ferrocyanide by filtration, the solution may be kept overnight without loss of caffeine.

It is generally considered necessary to extract the caffeine from an alkaline solution. The use of ammonia may cause contamination of the extract and high results, if the caffeine is finally determined by a Kjeldahl digestion. Sodium hydroxide, although free from this objection, may cause loss of caffeine at room temperature (see Table II). Trisodium phosphate acts as a buffering agent and the loss of caffeine is slow. It has the further advantage that it helps to clarify the solution.

Table I

Absorption of caffeine by zinc ferrocyanide

Time of contact of zinc ferrocyanide and caffeine sol.	Caffeine found, * mg.		
Normal	27·20	17·98	28·89
30 min.	27·18	—	28·86
2 hr.	26·98	—	—
Overnight	25·49	16·99	27·67

* Each figure is the mean of a number of results

The extracting agent is normally chloroform (partition coefficient between solvent and water, 22·2), though benzene can also be used (partition coefficient, 0·74). With benzene, theobromine is not extracted at any pH; with chloroform it is extracted if the pH is below about 10.[18] Trisodium phosphate in the quantity suggested above raises the pH to 10·5–11·5 and theobromine is not extracted from tea or cacao materials.

The distillation of the chloroform without the acid wash leads to a loss of caffeine, as may be seen in Table II.

Table II

Loss of caffeine when kept under alkaline conditions

Treatment	Caffeine found, mg.		
Normal	35·25	27·19	16·75
Made alkaline with sodium hydroxide, held 30 min. at 20° before extraction	9·71	—	—
Trisodium phosphate, held 30 min.	35·20	—	16·75
,, ,, ,, overnight	—	26·75	—
Chloroform evaporated without the acid wash	34·76	—	—

(*e*) *Determination*

The caffeine after extraction may be determined by weighing, by spectrophotometric methods, by conversion into ammonia, or titration with perchloric acid. A bromometric[19] and an iodometric method[20] have also been described. Weighing can be used for routine purposes provided the extracted caffeine is sufficiently pure. This method has been found to yield caffeine 97–98% pure. The direct titration of caffeine in dioxan[21] or other organic liquid with perchloric acid has been considered, but rejected on the grounds of safety in routine use. The Kjeldahl digestion is easily made and lessens the possibility of interference.

The use of cupric sulphate as a catalyst in the digestion is simpler than the use of mercuric oxide, and more reliable than selenium[22] or tellurium.[23] The time of digestion needed is relatively short and there is little to be gained by adding hydrogen peroxide as in the method of G. L. Miller & E. E. Miller.[24] The ammonia may be determined by the method of Casas & Dominguez[25] or by distillation and titration. Casas & Dominguez oxidize the ammonia in the presence of phenol to give a blue colour. We have investigated the method using hypochlorite, chloramine-T and bromine water as oxidizing reagents; the colour produced is capable of measurement by an absorptiometer and the method is sensitive, but seems to need very careful control of conditions to give reproducible results. Determination of ammonia without distillation is possible[26, 27] but requires so much care that it is not practicable for routine use. The distillation of the ammonia into 3% boric acid[28–31] and its titration with standard acid require very little attention.

The method described above yields reproducible results (see Table III).

Table III

Reproducibility of proposed method

Sample	Caffeine, % w/w	Sample	Caffeine, % w/w
A	0·260, 0·261	H	0·262, 0·261
B	0·222, 0·219	I	0·151, 0·152
C	0·305, 0·306	J	0·173, 0·174
D	0·403, 0·400	K	0·336, 0·340
E	0·257, 0·263	L	0·192, 0·190, 0·192
F	0·204, 0·207	M	1·31, 1·33
G	0·400, 0·412		

Table IV

Recovery of caffeine added to coffee and chicory essence

	Caffeine in sample, mg.	Added caffeine, mg.	Recovery of added caffeine Mg.	%
Sample I	28·02	0		
	,,	8·62	8·68	100·69
	,,	17·25	17·36	100·63
Sample II	29·93	0		
	,,	15·00	15·01	100·07
	,,	15·00	15·11	100·73
	,,	15·00	15·40	102·67
Sample III	28·02	0		
	,,	50 (7 tests)	49·55 to 51·05 Average, 49·85	99·10 to 102·10 Average, 99·70
Grand average				100·22

The recovery of caffeine when added to coffee and chicory essence is demonstrated in Table IV.

A number of experimental batches of coffee and chicory essence of about 160 gallons each have been prepared, and from the weight of the coffee used and the yield the caffeine content has been calculated. These figures are compared in Table V with the caffeine content found by the method described above.

Table V

Comparison of calculated and determined caffeine contents

Batch	Calc., % (w/w)	Found
1	0·204	0·204
2	0·204	0·191
3	0·204	0·207
4	0·226	0·218
5	0·267	0·260

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References

1 'Official and Tentative Methods of Analysis', 6th ed. (Washington: Association of Official Agricultural Chemists)
2 *J. Ass. off. agric. Chem., Wash.*, 1947, **30**, 70
3 Crowell, G. K., *J. Ass. off. agric. Chem., Wash.*, 1946, **29**, 38
4 *Analyst*, 1930, **55**, 111
5 Vogel, A. I., 'Textbook of Quantitative Inorganic Analysis', 1944, p. 279 (London: Longmans Green & Co. Ltd.)
6 Ishler, N. H., Fincane, T. P. & Barker, E., *Analyt. Chem.*, 1948, **20**, 1162
7 Edwards, F. W. & Nanji, H. R., *Analyst*, 1937, **62**, 849
8 Taylor, D. J. & Taylor, A., *Analyst*, 1949, **74**, 463
9 Kidd, J. D., Nanji, H. R. & Edwards, F. W., *Analyst*, 1941, **66**, 240
10 Bower, R. S., Anderson, A. D. & Titus, R. W., *Analyt. Chem.*, 1950, **22**, 1056
11 Silvestri, N., *Boll. chim.-farm*, 1948, **87**, 236
12 Maes, E., *Meded. vlaamsche chem. Ver.*, 1947, **9**, 152
13 Lepper, H. A., *J. Ass. off. agric. Chem., Wash.*, 1921, **4**, 526
14 Bührer, N. E., *Rev. Quím. industr., Rio de J.*, 1949, **18**, 23
15 Washburn, W. H. & Krueger, E. O., *J. Amer. pharm. Ass.*, 1950, **39**, 473
16 Hadorn, H. & Jungjunz, R., *Mitt. Lebensm. Hyg., Bern.*, 1949, **40**, 190
17 Hadorn, H. & Jungjunz, R., *Mitt. Lebensm. Hyg., Bern.*, 1950, **41**, 155
18 Moores, R. G. & Campbell, H. A., *Analyt. Chem.*, 1948, **20**, 40
19 Vallanua, L., *An. Acad. Farm., Madr.*, 1948, **14**, 707
20 Wade, P. & Hannen, J., *J. Sci. Fd Agric.*, 1950, **1**, 177
21 Fritz, J. S., *Analyt. Chem.*, 1950, **22**, 578
22 Patel, S. M. & Sreenivasan, A., *Analyt. Chem.*, 1948, **20**, 63
23 Bradstreet, R. B., *Analyt. Chem.*, 1949, **21**, 1012
24 Miller, G. L. & Miller, E. E., *Analyt. Chem.*, 1948, **20**, 481
25 Casas, J. R. & Dominguez, R., *An. Soc. esp. Fis. Quím.*, 1949, [B] **45**, 1607
26 Marcali, K. & Rieman, W., *Industr. Engng Chem. (Anal.)*, 1946, **18**, 709
27 Marcali, K. & Rieman, W., *Analyt. Chem.*, 1948, **20**, 381
28 Machemer, P. E. & McNabb, W. M., *Analyt. chim. acta*, 1949, **3**, 428
29 Klein, L., *J. Inst. Sew. Purif.*, 1947, **1**, 223
30 Silverstein, R. M. & Perthel, R., *Analyt. Chem.*, 1950, **22**, 949
31 Vignau, M., *Bull. Soc. chim. Fr.*, 1947, **14**, 1032

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