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FRACTIONATION AND PROPERTIES OF THE POLYMERIC LEUCOCYANIDIN OF THE SEEDS OF *THEOBROMA CACAO*

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Abstract—The polymeric leucocyanidin of the unfermented cacao seed has been separated into three fractions which differ in their response to phenol reagents and in solubility but do not differ greatly in average molecular size. All the fractions liberate simple dimeric leucocyanidins and epicatechin on hydrolysis with dilute hydrochloric acid and the same sulphur-containing substance on treatment with sulphurous acid. Thus the linkage between the C_{15} units is regarded as being the same as that in leucocyanidin 1. The results suggest that molecular shape may be more important than size in determining the properties of these polymers.

INTRODUCTION

ABOUT 60 per cent of the total polyphenolic matter of the fresh cacao seed is leucocyanidin.[1,2] About half of this consists of the relatively simple compounds (dimers) that have been designated L_1, L_2 and L_3 and the remainder of more complex material.[1,2] A structure has been proposed for L_1 and L_2 is believed to be an isomer.[1,3] The structures of the more complex leucocyanidins are not known but the suggestion has been made that they are either polymers of L_1 or of 5,7,3′,4′-tetrahydroxyflavan-3,4-diol with epicatechin end-groups.[1] This paper gives an account of the fractionation and properties of the polymeric leucocyanidin. It also shows that the linkage between the units is probably the same as that in L_1 and gives evidence to support the view that the polymers arise by condensation of 5,7,3′,4′-tetrahydroxyflavan-3,4-diol units.

RESULTS

Chromatography

On two-way chromatography on paper in 2 per cent aq. acetic acid and *n*-butanol, acetic acid and water (4:1:5), the polymeric leucocyanidin forms a diffuse area on and near the origin (Fig. 1). Fractionation by the methods described later gives three fractions I, II and III which ocupy positions as shown.

Leucocyanidins are labile in acid and, presumably because of this, solvents without acid have been found to be better than those containing acid. No solvent yet tried has separated any fraction into discrete spots but some solvents have shown what appear to be spots embedded in a diffuse background. The R_fs in water-saturated methyl ethyl ketone and in *n*-butanol–ethanol–water (4:1:5) are respectively: I, 0–0·2, 0–0·15; II, 0·1–0·3, 0·1–0·3; and III, 0·2–0·5, 0·2–0·35. As used, the fractions contain traces of pigments, epicatechin and

[1] W. G. C. FORSYTH and V. C. QUESNEL, *Advan. Enzymol.* **25**, 457 (1963).
[2] W. G. C. FORSYTH, *Biochem. J.* **60**, 108 (1955).
[3] W. G. C. FORSYTH and J. B. ROBERTS, *Biochem. J.* **74**, 374 (1960).

the simpler leucocyanidins. There is also a small amount of nitrogen; 2·18 per cent in I, 2·6 per cent in II and 1·99 per cent in III. This probably comes from theobromine which makes up about 14 per cent of the polyphenol storage cells.[1] No caffeine could be detected. Sugars are present in hydrolysates of the fractions which may not be contaminants (see later).

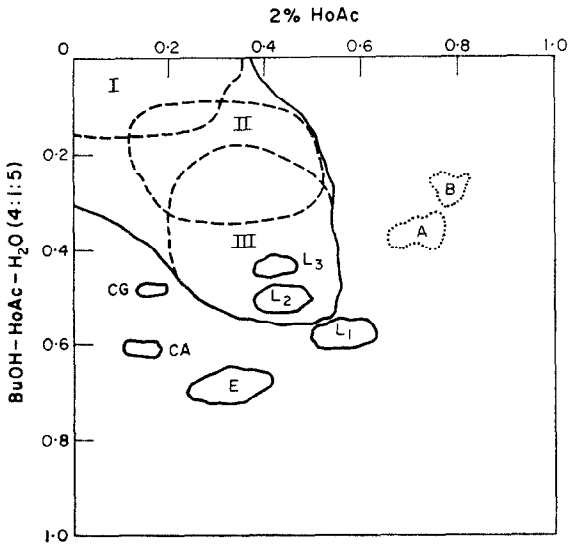


FIG. 1. TWO-WAY CHROMATOGRAM TO SHOW POSITIONS OF THE POLYMERIC LEUCOCYANIDIN FRACTIONS I, II AND III (DASHED LINES) IN RELATION TO THE OTHER MAJOR POLYPHENOLS OF THE FRESH CACAO SEED.

Dotted lines show the positions of the sulphur-containing compounds, A and B, derived from the leucocyanidins on hydrolysis with sulphurous acid. E = epicatechin, L_1 L_2 L_3 = simple leucocyanidins, CA = cyanidin arabinoside, CG = cyanidin galactoside.

Molecular Size

Average molecular weights of the acetylated fractions were determined by Rast's method.[4] The melting mixtures were brown and the disappearance of the last crystals difficult to determine accurately. Hence, the molecular weights recorded for the acetylated fractions (I = 2040; II = 3010; III = 1970) must be regarded as minimal ones. I is the least accurate; the true molecular weight could be up to 75 per cent higher. The figures, the means for three determinations, indicate molecules in the size range tetramer to heptamer.

Samples of all fractions were dialysed against 10 per cent aq. methanol for 2 weeks with four changes of medium. The residues remaining within the membrane (I, 27 per cent; II, 16 per cent; III, 26 per cent) indicate that about 20–25 per cent of each sample consisted of molecules above a molecular weight of 5000. The percentage figures are maximal since all the residues were brown indicating oxidation and polymerization. Both of these tests indicate no great differences in average size amongst the molecules of I, II and III.

Hydrolysis with Acid

1 N HCl at 100° rapidly converts all three fractions to "phlobaphene". Although still producing "phlobaphene", 0·1 N HCl at 100° allows intermediate hydrolytic products to be

[4] A. I. VOGEL, *Elementary Practical Organic Chemistry*, p. 366. Longmans Green, London (1959).

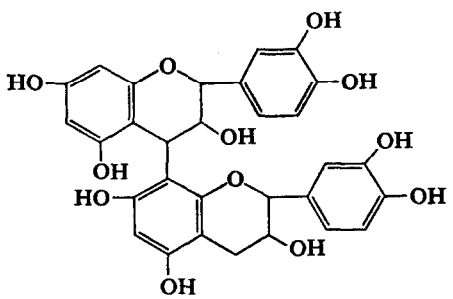
recognized more easily. Chromatography shows that epicatechin, L_1, L_2 and L_3 are present in all samples withdrawn after 3 min of reaction time. Epicatechin and L_1 reach a maximum level between 6 and 9 min and then remain more or less steady or decline slowly. A spot thought to be the L_4 of Griffiths[5] first appears after 6 min, increases during the next few minutes, and then remains unchanged in yield up to 30 min. Catechin appears first at 15 min and thereafter increases in yield.

The response of L_1 to hot 0·1 N HCl is much the same. Epicatechin, L_2 and L_3 are all already present in the earliest sample which has had only a few seconds of hydrolysis. At 3 min half the L_1 is already hydrolysed. L_4 appears only in traces between 9 and 12 min. Catechin appears earlier than 15 min presumably because epicatechin forms a greater part of the molecule of L_1 compared with the fractions and so the concentration of epicatechin in solution is greater.

When a solution of L_1 in 0·1 N HCl is allowed to stand at room temperature (27–30°) for 2 weeks the chromatographic profile of the resulting solution differs from that of the solution obtained from the treatment of L_1 with hot 0·1 N HCl and is indistinguishable from that of an extract of cacao except that there are no anthocyanins. Thus acid promotes condensation as well as hydrolysis and hot acid a rather different condensation from that of cold acid.

Both L_1 and polymeric leucocyanidin are known to be split by sulphurous acid at 100°.[3, 6] L_1 is split in 2·5 hr to epicatechin and substance A, a sulphur-containing, acidic compound that can be converted to cyanidin.[3] Chromatography of samples removed during the course of the treatment has shown that a second compound, substance B (Fig. 1), appears for a while before disappearing. The polymeric fractions I, II and III behave similarly but treatment must be continued for much longer periods to obtain complete degradation and the transient compounds are much more numerous. Both A and B are easily recognized. In the early stages of treatment epicatechin, L_1 and L_2 are all present.

The polymeric fractions thus behave towards dilute hydrochloric acid and sulphurous acid in the same way as L_1 which is itself recognizable in the products of their hydrolysis. Therefore it follows that the mode of linkage in the polymers is the same as in the dimer. Forsyth and Roberts[3] had proposed two structures for L_1 in both of which epicatechin was joined to 5,7,3′,4′-tetrahydroxyflavan-3,4-diol by acetal-type links. Mass spectra and NMR spectra recently obtained on methylated L_1 (unpublished data) show that neither structure can be correct and support a structure (IV) identical with the one proposed by Geissman and



(IV) Leucocyanidin 1

[5] L. A. GRIFFITHS, *Biochem. J.* **74**, 362 (1960).
[6] V. C. QUESNEL, *Tetrahedron Letters* **48**, 3699 (1964).

Dittmar[7] for a similar leucocyanidin from avocado. Therefore all three fractions I, II and III are considered to be linear chains of 5,7,3′,4′-tetrahydroxyflavan-3,4-diol units joined by C_4–C_8(C_6) linkages, each chain being terminated by an epicatechin unit. Since epicatechin has two asymmetric carbon atoms and the diol three, possibilities for molecules differing in shape are immense. L_1 itself differs from the avocado dimer by having epicatechin instead of catechin.

Portions of the fractions were dialysed in a Wood electrodialyser[8] to remove the sugar-containing anthocyanins. Hydrolysis with 1 N HCl and chromatography then revealed the presence of arabinose, glucose and galactose in all the fractions. Hydrolysis and chromatography of the residues from the dialysis against 10 per cent methanol revealed arabinose, galactose and an unidentified sugar in all. Glucose may have been present in traces.

The sugars could have come only from the leucocyanidin fractions themselves or from sugar-containing contaminants that were too big to pass through dialysis membrane. The second possibility cannot be rejected out of hand but polysaccharides other than starch are not known to occur in the polyphenol storage cells. Also the first possibility seems more likely since arabinose and galactose are the sugars occurring in the anthocyanins.[9]

If sugars do form part of the leucocyanidin polymers it is likely that they are attached at C_3 of the C_{15} unit at one end of the chain. Although the evidence is slight the following facts are relevant. The sugars occur in very small amounts. They are the same sugars that occur in the anthocyanins where they are attached at C_3. The polyphenols of the young cotyledon at the stage *before* the appearance of anthocyanin are chromatographically different from those of the mature cotyledon and resistant to attack by the polyphenol oxidase that is already present in the cotyledon suggesting the blocking of the reaction by some group (sugar?) attached to the polyphenols. It is conceivable therefore that the polyphenols of the mature seed are derived from those of the young seed by processes that include a removal of sugar. If the process is incomplete at maturity some sugar-containing leucocyanidins may remain.

Phenol Determinations

Several standard methods are available for the determination of polyphenols. Five of these, together with one developed in this laboratory, were applied to the fractions, with the results shown in Table 1. All colorimetric determinations were made in the same instrument

TABLE 1. REACTIVITY OF THE LEUCOCYANIDIN FRACTIONS I, II AND III WITH VARIOUS PHENOL REAGENTS

Polyphenol	Stiasny reaction (mg recovered)	$KMnO_4$ oxidn. (μ eq./0·01 mg)	Periodate oxidn. (E/10 μg/ml)	Leucocyanidin reaction (E/10 μg/ml)	Folin–Denis reagent (E/10 μg/ml)	Vanillin reagent (E/10 μg/ml)
Fraction I	71·4 (65)*	0·650 (70)	0·042 (45)	0·137 (92)	0·577 (66)	0·214 (42)
Fraction II	79·9 (73)	0·773 (84)	0·058 (62)	0·198 (132)	0·784 (90)	0·344 (67)
Fraction III	100 (90)	0·864 (94)	0·066 (70)	0·230 (155)	1·01 (115)	0·469 (92)
Dimer L_1	111	0·923	0·094	0·149	0·875	0·510
Epicatechin	123	1·02	0·216	—	1·42	—

* Result as percentage of the reaction given by the dimer L_1.

[7] T. A. GEISSMAN and H. F. K. DITTMAR, *Phytochem.* **4**, 359 (1965).
[8] T. WOOD, *Biochem. J.* **62**, 611 (1956).
[9] W. G. C. FORSYTH and V. C. QUESNEL, *Biochem. J.* **65**, 177 (1957).

and are directly comparable, although no ϵ values were calculated because of the inaccurately known molecular weights of the polymers. Extinction has been calculated per 10 μg per ml. Leucocyanidin 1 and epicatechin are included for comparison.

The main features of the results are as follows. Among the fractions reactivity decreases in the order III, II, I; the decrease is least (25 per cent) in the permanganate oxidation test and most (54 per cent) in the vanillin reaction; L_1 is not always more reactive than III though it is in four of the six tests.

Permanganate is an unspecific reagent and may oxidize the compounds completely. The complete oxidation of 10 μg epicatechin would require 1·07 μg eq of $KMnO_4$ and the complete oxidation of L_1 and polymers of like structure would require 1·05 μg eq. For epicatechin the observed result is only 5 per cent below theoretical, for L_1 12 per cent and for fractions III, II and I 18 per cent, 26 per cent and 38 per cent respectively. Thus, oxidation of the fractions is not complete under the conditions of the test. Both the Folin–Denis reaction[10] and the periodate reaction depend on oxidations and since in both tests epicatechin reacts more strongly than I, II and III it may be assumed that the fractions are incompletely oxidized here too.

TABLE 2. REACTIVITY RATIOS OF THE LEUCOCYANIDIN FRACTIONS FROM CACAO

Polyphenol	V/LA × 100*	V/FD × 100	V/F × 100
Fraction I	156	37·1	510
Fraction II	173	43·9	594
Fraction III	203	46·4	711
Dimer L_1	342	58·4	544

* V = Vanillin reaction, LA = leucocyanidin reaction, FD = Folin–Denis reaction, P = periodate reaction.

There is no obvious reason for the decreased reactivity of L_1 compared with III in the Folin–Denis reaction. On the other hand, there is no difficulty in suggesting a probable explanation for the results observed in the leucocyanidin reaction. L_1 is a dimer of epecatechin and 5,7,3′,4′-tetrahydroxyflaven-3,4-diol; thus only one-half the molecule can give rise to cyanidin.[3] If the fractions consist of linear polymers of the diol with epicatechin end-groups, then in each fraction more than half of each molecule will give rise to cyanidin and all fractions should give more cyanidin than L_1. Two of the fractions, II and III, give more cyanidin than L_1; the third, I, gives slightly less presumably because decreased reactivity counteracts the effect of the increased proportion of cyanidin precursor.

Table 2 gives some ratios derived from the figures in Table 1. The values always decrease in the order III, II, I. Goldstein and Swain[11] had used the first two ratios as a measure of polymerization and, as shown, the ratios behave in the expected way if average molecular weight increases in the order III, II, I. However, Goldstein and Swain arrived at these ratios by arguing that in the flavans they studied polymerization occurred through the A ring which would block the site of the vanillin reaction while leaving unchanged the site of the Folin–Denis reaction. According to present knowledge, linkage in the cacao polymers is also

[10] T. SWAIN and J. GOLDSTEIN, in *Methods in Polyphenol Chemistry* (edited by J. B. PRIDHAM), p. 131. Pergamon Press, Oxford (1964).

[11] J. GOLDSTEIN and T. SWAIN, *Phytochem.* **2**, 371 (1963).

through the A ring. If, therefore, the observed decrease in the ratios results from an increase in polymer size from III to I the argument behind the derivation of the ratios is supported. If, on the other hand, there is no great difference in molecular size of the fractions (as is indicated above) then the conclusion must be either that small differences in size exert a great influence on the ratios or that the values of the ratios are determined by features of the polymers other than size, e.g. shape. If the second of these possibilities is correct then the ratios are no sure guide to degree of polymerization in these polymers or in others derived from sources other than cacao.

Solubility

The usual method of determining solubility is inappropriate to mixtures. The method here used (Table 3) is better but not entirely free from objection. Values must necessarily be somewhat inaccurate inasmuch as the time for solution of the sample is not always constant. Generally, tannins are insoluble in anhydrous organic solvents but soluble in water and aqueous organic solvents. The figures for the cacao leucocyanidins do not entirely support this. All fractions are relatively insoluble in water and dilute acid but soluble in dilute

TABLE 3. SOLUBILITIES OF LEUCOCYANIDIN FRACTIONS

	Solubility (in g/100 g solvent) in						
Leucocyanidin	Water	0·1 N NaOH	0·1 N HCl	0·1 N Borax	MeOH	EtOH	EtOH–H_2O (1:1)
Fraction I	0·46	4·7	0.030	0·26	0·22	⩽0·019	2·5
Fraction II	0·40	12	0·027	4·5	35	0·061	20
Fraction III	<0·037	9·9	0·018	6·5	47	21	13
Dimer L_1	4·76	23	32	12	39	30	21

alkali and aqueous ethanol. In borax, methanol and ethanol solubilities differ widely. I is rather insoluble in all; II is soluble in borax and methanol but not in ethanol; III is outstanding for its solubility in ethanol and it is also soluble in borax and methanol. L_1 is soluble in all solvents.

Forsyth[2] has stated that all the polyphenols of cacao are soluble in 0·1 N HCl but the figures of Table 3 show that this is far from true for the polymers. With Forsyth's extraction technique, only a part of the polymeric material could have been extracted. This would have introduced errors into the calculations of the proportions of the polyphenols in the storage cells. Most, if not all, of the 14 per cent of the polyphenol storage cells labelled as unknown[1] must be polymeric material insoluble in 0·1 N HCl. If this is so, recalculation shows the polymeric material to be the biggest fraction of the total phenol (39 per cent) with catechin next (31 per cent) and simple leucocyanidin next (26 per cent).

Condensed tannins are known to be salted out of solution. This is true too of all the polymeric leucocyanidin fractions, the percentage of each fraction precipitated by salt being as follows: I, 91 per cent; II, 82 per cent; III, 82 per cent.

Reaction with Gelatin

50-mg quantities of each fraction and of L_1 were dissolved in 50 ml water (about 5 per cent remained undissolved in the case of III) and the solutions were tasted. L_1 and III were both

strongly astringent and bitter, the bitterness being experienced on the tongue as with epicatechin not with the palate as with caffeine; I was a little less astringent and not bitter; II was barely astringent and not bitter. Addition of 1 ml of 1 per cent gelatin solution caused no precipitation of I or II, produced an opalescence in the solution of L_1 and threw down a flocculent precipitate from the solution of III. The close association between astringency and ease of precipitation with gelatin is evident.

This is a more exacting test for tannins than the usual one employing a saline solution of gelatin and was used because of the known tendency of the fractions to precipitate from saline solutions.

DISCUSSION

The cacao fractions are undoubtedly polyphenolic in that they react with phenol reagents in the expected way and they give recognizable polyphenols on hydrolysis. They are polymeric, although molecular weight determinations and dialysis indicate polymers of fairly low average molecular weight (1200–1800) and at least one is a tannin since it precipitates gelatin from solution. From the way in which they were prepared and their behaviour on chromatography it was thought at first that I is the most highly polymerized fraction and III the least. However, the results of molecular weight determinations and dialysis show the fractions to be of much the same average molecular size; the solubility results show the anomaly of supposedly smaller molecules of similar chemical substances being less soluble than larger molecules. Specifically, it is difficult to see why III is so much less soluble in water and dilute acid than I on this theory.

If the properties cannot be explained satisfactorily as the result of differences in molecular size a possible explanation may be that they result from differences in molecular shape. For instance, maleic acid (*cis*-butenedioic acid) is over 100 times more soluble in water and ten times more soluble in ethanol than is fumaric acid (*trans*-butenedioic acid).[12] If this is the explanation for the differences in solubility between III and I then it follows that the original fractionation may have been achieved as much on the basis of molecular shape as of molecular size. If this is admitted, the results of chromatography, molecular weight determination and dialysis are immediately interpretable and the results of solubility determination and reaction with phenol reagents present fewer difficulties.

In this interpretation, III must be considered to be held by intramolecular hydrogen bonding in such a shape that relatively few hydrophilic groups are available for bonding with solvent molecules in aqueous solvents so that the fraction is insoluble in water but very soluble in organic solvents compared with I. The hydrogen bonding must be assumed to remain intact in acid solution but to be disrupted in alkali so as to allow hydrogen bonding with solvent molecules. This would account for the very greatly increased solubility of III in 0·1 N sodium hydroxide and 0·1 N borax compared with water and 0·1 N hydrochloric acid. I, which is relatively insoluble in all solvents except 0·1 N sodium hydroxide and ethanol–water (1 : 1), must consist of molecules in which intermolecular hydrogen bonding is facilitated so that the fraction is relatively insoluble in all solvents. Fraction II seems to be intermediate between I and III in nearly every respect.

In reactions with phenol reagents, steric hindrance caused by differences of shape is at least as good a reason for decreased reactivity as is the blocking of an active site by polymerization. Therefore, *a priori*, the results recorded in Table 1 are as easily explained by one

[12] *Handbook of Chemistry and Physics* (edited by G. D. HODGEMAN), pp. 823, 900. Chemical Rubber Publishing Co. Cleveland (1947).

as by the other. Steric hindrance is favoured by the fact that the decrease in reactivity from III to I is much the same for all reactions regardless of the mechanism or the site of the reaction. Thus, for instance, the difference in reactivity between L_1 and I in the periodate reaction is only slightly less than that in the vanillin reaction. Yet vanillin reacts by substitution in the A-ring,[10] the site of polymerization, whereas periodate reacts by oxidation of the *o*-dihydroxy group of the B-ring far removed from the site of polymerization. Goldstein and Swain[11] favoured polymerization as the explanation for such decreases despite their mention of the possibility of steric hindrance and despite their results which show the operation of this factor. They record in the Folin–Denis reaction ϵ values for epigallocatechin (16×10^3) and gallocatechin ($30{\cdot}7 \times 10^3$) that differ by a factor of 2 though the two compounds differ only in configuration.[11]

If shape plays an important part in determining the properties of the polymeric leucocyanidin then astringency may result as much from peculiarities of shape as of size. Goldstein and Swain [11] acknowledge this possibility but prefer to interpret their results as the effect of size. Another factor possibly affecting the results is contamination, but all the fractions are contaminated to about the same extent and it is difficult to believe that the contaminants can have influenced the behaviour of the fractions sufficiently to have produced the observed results. Caffeine can form a loose complex with epicatechin[13] but no caffeine has been detected in the fractions. The contaminating nitrogen probably comes from theobromine but this is not known to form complexes with phenolic compounds.

EXPERIMENTAL

Fractionation Procedure

Polyphenol storage cells were isolated from dried seeds of the clone I.C.S. 95 by methods previously described.[2,14] 10-g samples were extracted twice with 50 ml methanol and the supernatants, recovered by centrifugation, were pooled. Ether (100 ml) was added to the supernatant with stirring. After 15 min at room temperature (*ca.* 28°) precipitate 1 was collected by centrifugation. In like manner, precipitate 2 was collected by adding 400 ml of ether to the supernatant from the first precipitate. The original cell residue, which still contained much polyphenol, was extracted with 100 ml methanol and centrifuged. The supernatant was mixed with precipitate 1 and 100 ml ether was added. Fraction I precipitated and 400 ml ether was added to the supernatant. The precipitate that formed was collected by centrifugation, mixed with precipitate 2 and dissolved in 50 ml methanol. Ether (250 ml) was added and fraction II which precipitated was removed by centrifugation. Fractions I and II were separately suspended in chloroform, recovered by centrifugation and dried. All the supernatants were pooled and solvent was removed on a flash evaporator at 45°. Recoveries from a total of 30 g of polyphenol storage cells were as follows: I, 3·9 g (13 per cent); II, 3·5 g (12 per cent); supernatant, 18·5 g (62 per cent); cell residue, 3·3 g (11 per cent).

The supernatant fraction was mixed with 1 l. water and a putty-like material was removed with a glass rod. The solution was saturated with salt and ethyl acetate, and the sticky material that separated was collected and added to the putty-like material. The combined material was triturated with water and then with several changes of ethyl acetate until it became hard. After drying, the material (4·2 g) was dissolved in methanol (40 ml). A small amount of undissolved matter was removed by centrifugation and ether (400 ml) was added to the supernatant. The resulting precipitate, fraction III, was collected by centrifugation, washed with chloroform and dried. The saturated salt solution was then extracted with ethyl acetate several times and the extract used for the isolation of L_1 by Forsyth's procedure.[2]

Chromatography

Two-dimensional chromatograms of the fractions and the products of mild acid hydrolysis were obtained by downward development on Whatman No. 1 paper first in 2 per cent aqueous acetic acid and then in *n*-butanol, acetic acid and water (4:1:5). One-dimensional chromatograms of the fractions were obtained by ascent in ethyl methyl ketone saturated with water and in *n*-butanol, ethanol and water (4:1:5). The polyphenols were detected with the reagent of Barton *et al.*[15]

[13] W. G. C. FORSYTH, *Nature* **169**, 33 (1952).
[14] H. B. BROWN, *Nature* **173**, 492 (1954).
[15] G. M. BARTON, R. S. EVANS and J. A. F. GARDNER, *Nature* **170**, 249 (1952).

Hydrolysis with Dilute Hydrochloric Acid

15 ml 0·2 N HCl was heated under reflux and a hot solution of the polymeric fraction (100 mg) in water (15 ml) was added at zero time. Samples were withdrawn after 10 sec, 3, 6, 9, 12, 30 and 60 min. They were cooled rapidly and 0·005 ml used for two-dimensional chromatography in solvents 1 and 2.

Hydrolysis with Sulphurous Acid

Water (5 ml) was heated under reflux with a gentle stream of sulphur dioxide bubbling through. At zero time a solution of the fraction (100 mg) in water (5 ml) was added and samples removed after 10 sec, 10, 20, 40, 90 and 150 min. Chromatography was carried out as for the HCl hydrolysates.

Dialysis and Sugar Determination

Sugars were determined only on hydrolysates of the fractions after recovery from dialysis. Dialysis through cellophane was carried out in the normal way. For electrodialysis in the Wood dialyser, which was supplied by Shandon, 25 mg of the fraction was dissolved in 10 per cent aq. acetic acid (5 ml) to which drops of 2 N HCl were added as required. The dialyser was set up with ion-exchange membranes and dialysis was carried out for 2 hr with a current of about 0·2 A. After dialysis the solution was straw-coloured and did not turn red on the addition of more acid (i.e. the anthocyanins had been removed completely).

To 1·5 ml of each fraction after dialysis 0·15 ml of conc. HCl was added and the fraction hydrolysed in a sealed tube at 100° for 2 hr. Much anthocyanidin and "phlobaphene" was extracted from the hydrolysates with isoamyl alcohol and the aqueous layers were evaporated to dryness. Water was added to the residues and evaporated again. The residues were then taken up in pyridine for chromatography in the top phase of a mixture of ethyl acetate, pyridine and water (3:1:3) against standards of arabinose, galactose and glucose. Sugars were detected with the aniline hydrogen oxalate spray.

Phenol Determination

The leucocyanidin and vanillin reactions were carried out as recommended by Swain and Hillis.[16] For the Folin–Denis reaction the procedure followed was that of Swain and Hillis[16] but the quantities of reagents used were those given in the AOAC official methods of analysis.[17] The Stiasny reaction was carried out as described by Forsyth[2] on 100-mg samples. Recoveries on simple phenols by this method are well above 100 per cent, e.g. 137 per cent for catechol and 150 per cent for resorcinol, but are not consistent and may be influenced by the age of the formalin solution used for preparing the reagent. All the determinations reported here were done within 2 days and with the same batch of reagent.

The periodate reaction was developed as an analytical method by K. Jugmohunsingh of this laboratory. A portion of the solution (1 ml) is mixed with 1 per cent aq. sodium periodate (2 ml) and diluted with distilled water to a convenient volume. After 15 min the absorption is measured in a spectrophotometer at 370 nm. The brown colour fades slowly (20 per cent loss over 24 hr) but not fast enough to produce any detectable difference after 1 hr. The colour obeys Beer's law. The method is specific, reproducible and extremely simple. Titration with 0·01 N potassium permanganate was done in the usual way. When the end point was approached the solution was heated to temperatures of 50° to 60° and the titration completed at this temperature. The end point was taken to be the point at which there was no noticeable fading of the added permanganate within 15 sec.

For the above reactions (with the exception of the Stiasny reaction) fractions I and II were dissolved in water at a concentration of 1 mg/ml. Fraction III, which is less soluble, was used at 0·5 mg/ml but even at this concentration about 1 per cent of the material remained undissolved. Determinations were done in duplicate except for the Stiasny and vanillin reactions where only single determinations were done. All colorimetric determinations were done with a Bausch and Lomb, Spectronic 20 spectrophotometer in test tubes of 11·67 mm i.d. which with test solutions gave the same results as 1 cm cuvettes.

Solubility Determinations

Portions of the substances (20–30 mg) were accurately weighed into flasks and solvent was added to the flasks dropwise with shaking. The flasks were stoppered between addition of solvent. When the substance had dissolved the flasks were weighed again to determine the weight of solvent added. All determinations were done at room temperature (27°–30°).

To determine precipitation by salt, just enough 0·1 N sodium hydroxide solution was added to known weights of each fraction to bring them into solution. Water (25 ml) was then added by pipette to each. The solutions were saturated with salt and filtered through Whatman No. 1 paper that had been wetted with a saturated salt solution. The volume of the filtrates were determined in measuring cylinders and portions were

[16] T. SWAIN and W. E. HILLIS, *J. Sci. Food Agri.* **10**, 63 (1959).

[17] *Official Methods of Analysis* (edited by W. HOROWITZ), p. 111. Association of Official Agricultural Chemists, Washington (1960).

taken for determination of the phenol by the Folin–Denis method. By comparison of these results with the results recorded in Table 1 the phenol precipitated was calculated as a per cent.

Acetylation

Portions (100 mg) of the fractions were acetylated by the method of Forsyth and Roberts.[3] The acetylated products were recovered from the ice mixtures, washed with water, dried and used directly.

Nitrogen Determination

The method was that of Miller and Miller.[18]

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[18] G. L. MILLER and E. E. MILLER, *Anal. Chem.* **20**, 481 (1948).