Phytochemistry, 1963, Vol. 2, pp. 371 to 383. Pergamon Press Ltd. Printed in England

CHANGES IN TANNINS IN RIPENING FRUITS

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(*Received* 15 *April* 1963)

Abstract—Simple methods for separating tannins (flavolans) of different molecular size, and determining the degree of polymerization have been used in a study of the changes in these compounds in several fruits on ripening. It is concluded that loss of astringency, which occurs on ripening, is most probably connected with increased polymerization of tannins.

INTRODUCTION

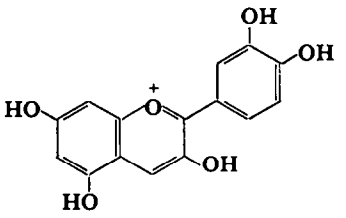
Loss of astringency is one of the major changes which takes place during the ripening of many edible fruits. It is generally agreed[1–3] that this property is due to the presence of tannins, but although some astringent fruits show a reduction in tannins on ripening,[3] others do not.[4] Even in those fruits where both tannins and astringency are reduced on ripening, the biochemistry underlying these changes has not been fully studied, and it is not at all obvious how the two are interrelated.

Part of the difficulty stems from the fact that there appears to be a number of confused ideas about the nature of tannins, mainly due to the fact that many of the chemical properties which are used for their detection and analysis are common to other naturally occurring phenolic compounds which are universally present in plants. Thus Reeve,[5] in an otherwise interesting paper, followed changes in the "tannins" in peaches by a reaction which is given by chlorogenic acid and related compounds which have no tannin-like properties. Barnell and Barnell,[3] on the other hand, drawing attention to the fact that not all "tannins" are astringent,[6] developed a method for following changes in the astringent tannins in ripening bananas which depended on their binding power for protein. A great deal of confusion would obviously be avoided, therefore, if in the context of plant chemistry, the term tannin was reserved for those phenolic compounds of sufficiently high molecular weight (> 500) to form reasonably strong complexes with proteins and other polymers under suitable conditions of concentration and pH.[7] The dry or puckery sensation of astringency[1] undoubtedly arises from the cross-linking of the proteins and glycoproteins in the mouth by tannins with a correspondingly reduced lubricant action. Cross-linking is probably mainly effected by the formation of hydrogen or other bonds which in themselves are relatively weak.[8] The capacity of tannins to form strong cross-links will depend to a large extent on their molecular size,

[1] E. C. BATE-SMITH, *Food* **23**, 124 (1954).
[2] D. G. GUADAGNI and C. C. NIMMO, *Food Technol.* **7**, 59 (1953).
[3] H. R. BARNELL and E. BARNELL, *Ann. Botany, London* **9**, 77 (1945).
[4] C. C. CRAFT, *Am. Soc. Hort. Sci.* **78**, 119 (1961).
[5] R. M. REEVE, *Am. J. Botany* **46**, 645 (1959).
[6] E. CREDE, *J. Am. Leather Chemists Assoc.* **20**, 573 (1925).
[7] T. SWAIN, *Ann. rep. Low Temp. Res. Stn., Cambridge*, p. 34 (1960).
[8] K. H. GUSTAVSON and B. HOLM, *J. Am. Leather Chemists' Assoc.* **47**, 700 (1952).
S. G. SHUTTLEWORTH, *J. Am. Leather Chemists' Assoc.* **47**, 603 (1952).
G. OTTO, *Das Leder* **4**, 193 (1953).

although their overall structure and the number and orientation of the phenolic hydroxyl groups which are responsible for the actual bond formation undoubtedly plays a part.[9] With respect to size, low molecular weight phenolic compounds, including the precursor molecules of the tannins, are apparently too small to form sufficient effective cross-links and are therefore not noticeably astringent; highly polymerized tannins are either too insoluble or too large to fit between suitably oriented protein molecules, and maximum astringency is most probably shown by tannins of intermediate size. In this respect, the astringency of tannins parallels their ability to leather hides[10] and inhibit enzymes.[11]

In ripening fruits, therefore, it may be expected that changes in astringency are a reflection of changes in the molecular size of the tannins. Before discussing such transformations in detail it is useful to consider briefly the chemistry of the tannins that are found in fruits. It is now recognized that in the vast majority of edible fruits these are in fact leuco-anthocyanins.[12–14] Since the only means of detecting leuco-anthocyanins in plants is their ability to yield anthocyanidins (e.g. I) on heating in acid solution, it has often been incorrectly assumed that the term refers specifically to the monomeric flavan-3-4-diols (e.g. II), although it has been stressed[14–16] that it also includes oligomers and polymers (flavolans).[14] Normally



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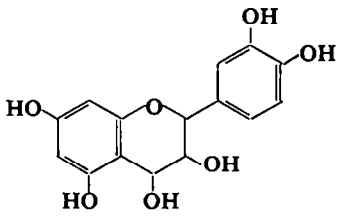


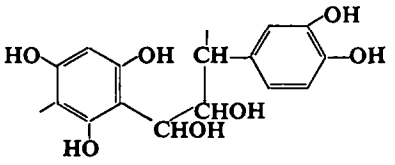
(II)

all three types are present in mature plant tissues,[15,16] and that only molecules of the latter two classes are to be regarded as tannins[16] and therefore to have an effect on astringency. Little work has been done on the mode of polymerization of the flavan-3,4-diols, but by analogy with the related catechins it seems probable that, in part, they link together to give polymers with repeating units of the type (III),[17] (IV),[18] (V)[19] and (VI).[20] The presence of an activated secondary hydroxyl group in the 4-position of flavan-3,4-diols, however, can give rise to other types of linkage.[21,22] The observed ether linkage in cacao leuco-anthocyanin,[23]

[9] W. GRASSMAN, M. ENDRES, M. OPPELT and H. ELI. SISI, *Das Leder* **10**, 149 (1959). S-T TU and R. M. LOLLAR, *J. Am. Leather Chemists' Assoc.* **45**, 324 (1950).
[10] T. WHITE in *The Chemistry of Vegetable Tannins*, p. 1. Soc. Leather Trades Chemists, Croydon (1956).
[11] R. J. BYRDE, A. H. FIELDING and A. H. WILLIAMS in *Phenolic in Plants in Health and Disease* (J. B. PRIDHAM Ed.), p. 95. Pergamon, Oxford (1960).
[12] E. C. BATE-SMITH and T. SWAIN, *Chem. & Ind. London* 377 (1953).
[13] E. C. BATE-SMITH in *The Pharmacology of Plant Phenolics* (J. W. FAIRBAIRN Ed.), p. 133. Academic Press, London (1959).
[14] T. SWAIN in *The Chemistry of Flavonoid Compounds* (T. A. GEISSMAN Ed.), p. 513. Pergamon, Oxford (1962).
[15] W. E. HILLIS and T. SWAIN, *J. Sci. Food Agr.* **10**, 135 (1959).
[16] D. G. ROUX and K. PAULUS, *Biochem. J.* **82**, 320 (1962).
[17] K. FREUDENBERG, *J. Polymer Sci.* **29**, 433 (1958).
[18] D. E. HATHWAY and J. W. T. SEAKINS, *Biochem. J.* **67**, 239 (1957).
[19] D. E. HATHWAY, *Biochem. J.* **70**, 34 (1959).
[20] H. L. HERGERT in *The Chemistry of Flavonoid Compounds* (T. A. GEISSMAN Ed.), p. 553. Pergamon, Oxford (1962).
[21] K. FREUDENBERG and K. WEINGES, *Ibid.*, p. 197.
[22] B. R. BROWN, W. CUMMINGS and G. A. SOMERFIELD, *J. Chem. Soc.* 3757 (1957).
[23] W. G. C. FORSYTH and J. B. ROBERTS, *Biochem. J.* **74**, 374 (1960).

and the link proposed in structure (VI), could explain why polymeric leuco-anthocyanins retain the ability to yield anthocyanidins with hot acid,[15] whereas polymerized catechins are further condensed under the same conditions.

As mentioned earlier, the actual mode of linkage in flavolans (polymeric flavans[14]) probably affects their astringency. However, in view of the difficulty of detecting even gross differences in structure, especially since co-polymerization with several phenolic nuclei is possible,[10] a full investigation of this important aspect of the problem must await further developments.



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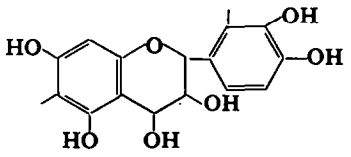

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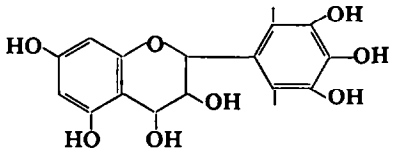

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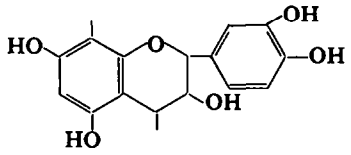


(VI)

However, as stressed above, the overall size of tannins is perhaps of even greater importance than structure in astringency. Several routine methods have been used for roughly determining the molecular size of tannins, the classical hide powder method being one example. Such methods merely divide phenolic compounds into tannins and non-tannins without giving any information about their actual size, and in any case can give incomplete separations.[24] More complex methods, using chromatographic or counter-current separations[16] are not suitable for routine use, and the recovery of the higher polymers may not be quantitative.

It has been known for some time however that flavolans can be separated into groups depending on their solubility in anhydrous and aqueous organic solvents.[25,26] For example it was shown that the flavolans (leuco-anthocyanins) of plum leaves can be divided into three classes, the first two being successively extractable with absolute followed by aqueous methanol, the third, remaining in the residue, being non-extractable by these or other neutral solvents.[15] The first fraction was shown to contain, besides simple phenolic compounds such as chlorogenic acid, leuco-anthocyanins which were mainly mobile on paper chromatograms and therefore likely to be mono- or oligo-meric. On the other hand, the second fraction extracted by aqueous methanol contained mainly non-mobile components which were presumed to have a higher molecular weight (cf.[16]). This latter fraction is not necessarily insoluble in absolute methanol, but is perhaps bound via hydrogen bonds to cell walls or proteins in the plant material, and only released when partial rehydration with the aqueous solvent mixture breaks these bonds. (Even simple phenolic compounds are known to be more easily eluted from paper chromatograms with aqueous rather than absolute solvents.)

[24] A. H. Williams, *Ann. Rep. Agr. and Hort. Res. Stn., Long Ashton*, p. 219 (1952).
[25] F. Stather, R. Lauffmann and T. Bau Miau, *Collegium* 66 (1936).
D. W. Duthie, *Analyst* 63, 27 (1938).
[26] W. R. C. Handley, *Plant and Soil* 15, 37 (1961).

In a similar way, the "insoluble" third fraction probably contains some flavolans which are again not truly insoluble, but are more firmly bound to cell-wall polysaccharides or other polymers, or perhaps are present as salts.[14] In any case they are probably of higher molecular weight than compounds in the first two classes.

From an examination of the changes in the three fractions of flavolans present in plum leaves during the growing season[15] it would appear that the proportion of polymeric compounds increases as the leaves aged. Such changes probably account for the increasing indigestibility to animals of protein in the leaves of certain tannin-containing plant species as the season progresses, since the older leaves, containing more polymeric tannins, would be expected to have a higher proportion of indigestible tanned protein.[26] The polymerization of flavans in leaves may well be an enzymically controlled process, at least in the first stages, but it can take place in dead tissue,[15,27] and also during the course of oven-drying.[15,26,28]

It may be expected by analogy, therefore, that polymerization of flavans also occurs during the maturation and ripening of fruits. As plum fruits mature, the leuco-anthocyanin content per fruit increases[15] (although decreasing on a fresh weight basis) probably up to the point of maximum cell elongation. Net synthesis probably ceases during the ripening process, but there may well be a continuation of the polymerization process leading to an increased proportion of higher molecular weight flavolans in the fully ripe fruit. Changes of this type certainly appear to take place in the banana, the tannin cells of which are reported to appear "caked" in the ripe fruit.[3] Similarly in *Malus tavingo* and *M. flovibunda*, the tannin cells of the riper fruit are reported to contain solidified material, whereas those in the unripe contain soluble diffusable tannins in their vacuoles.[29] It is, of course, possible that some catabolism of tannins also takes place in ripening fruits, but it should be remembered that these compounds are more resistant to degradation by enzymes than most other cellular constituents.[27]

If the hypothesis outlined above is accepted, any reduction observed in the amount of flavolan during the ripening of fruits could be accounted for either by a reduction in their reactivity with the reagents used for analysis, or by their reduced extractability, both due to increased polymerization. Providing the increase in polymerization is such that most of the oligomeric forms in the unripe fruit which are responsible for astringency are further transformed, there will also be a reduction in this property. In cases where no reduction is observed to take place in flavolans on ripening the situation is more complex. In most fruits, however, other changes which occur on ripening may affect the extractability of the flavolans, especially increases in the water content of the cells, and in the polysaccharide constituents. Changes in the degree of methylation of the pectins,[30] for example, might reduce their binding power for these phenolic polymers. It is notable that in the peach, which undergoes large changes in texture on ripening,[30] no observed reduction in flavolans takes place.[4] This is in contrast to the banana, where changes in texture on ripening are less drastic, and where flavolans are markedly reduced.[3] It has also been reported that tannins and pectin "disappear" at the same time in artificially ripened persimmons, which suggests that here there may be an increased adsorption of the tannins onto the cell wall,[31] thus reducing the extractability of both polymers. One further important difference is the distribution of the flavolans within

[27] C. B. COULSON, R. I. DAVIES and D. A. LEWIS, *J. Soil Sci.* **11**, 20 (1960).
[28] A. T. MARKH and YU G. SKORIKOVA, *Isvest. Vysshikh. Uchet. Zavedenii Pishchevaya Tekhnol.* **1**, 37 (1959).
[29] D. MILICIC, *Protoplasm* **41**, 327 (1952).
[30] C. STERLING and A. J. KALB, *Botan. gaz.* **922**, **121**, 111 (1959).
[31] S. KIMURA, T. SHIBATA and S. SUDO, *Rep. Food Research Inst. (Tokyo)* **6**, 7 (1952).
F. E. LLOYD, *Trans. Roy. Soc. Canad.* **16V**, 1 (1912).

the fruit: in the banana and the persimmon they are mainly localized in special tannin cells, whereas in the peach they are distributed throughout the tissue.[32]

Polymerization, besides affecting extractability, will also affect chemical reactivity due to the fact that the majority of the polymerization processes outlined earlier involve the formation of C–C links between the most reactive positions of monomers. The resulting polymers will thus be less reactive than their precursors to analytical reagents which form C–C substitution compounds.[33–37] On the other hand analytical reagents which react with the phenolic hydroxyl groups[33, 38, 39] of tannins should be little affected by polymerization, except in so far as the number of phenolic groups may be reduced by quinone (e.g. IV) or ether formation. In general therefore, it may be expected that the ratio of reactivity of the tannins to the two types of reagent will change on polymerization. This method for following polymerization processes was suggested by Swain,[40] who reported that the vanillin reagent[33] reacts stoichiometrically with the phloroglucinol nucleus of 5,7-dihydroxy substituted flavans (e.g. II), and this reaction was reduced on polymerization. Hillis and Urbach,[34] demonstrated the utility of this approach using formaldehyde as the substitution reagent, and showed that, as expected, catechin (VII) lost one reactive position per mole on autoxidative polymerization[18] to give (IV).

The changes in flavolans during the ripening of several fruits have now been examined using both solvent separation and chemical methods for the determination of polymerization processes. In general the results obtained support the hypothesis that loss of astringency is connected with polymerization, but further experiments are required before this hypothesis can be confirmed.

RESULTS AND DISCUSSION

Using a number of model substances, an examination was first made of the methods used by Swain and Hillis[33] for the analysis of phenolic compounds. The results obtained confirmed that the vanillin reagent reacts in an approximate stoichiometric manner with compounds containing the phloroglucinol group.[40] Thus both phloroglucinol and theaflavin gallate (VIII) which differ widely in molecular weight have nearly the same molecular absorptivity ($3{\cdot}5 \times 10^4$ and $3{\cdot}0 \times 10^4$ respectively, Table 1). Compounds of this type (e.g. II and VII) all gave an adduct[34] of the same colour (λ_{max} 500 mμ). It may be noted that the catechins give consistently higher ϵ values than their epimers (including cocoa leuco-anthocyanin). The reagent also reacts with compounds containing the resorcinol group (e.g. IX) but in this case both the λ_{max} (520 mμ) and the molecular absorptivity (average $4{\cdot}14 \times 10^4$) are higher. Pyrogallol behaves like resorcinol in this respect but (−) epigallocatechin (X) shows no augmentation of molecular absorptivity over that of epicatechin (VII) and the pyrogallol group present in (X) obviously does not react with the reagent, perhaps due to steric factors. Gallic acid also shows no reaction due to deactivation by the carboxyl group, and in this respect behaves like other compounds containing deactivating substituents (cf.[32]. No

[32] J. L. Goldstein and M. A. Joslyn (unpublished).
[33] T. Swain and W. E. Hillis, *J. Sci. Food Agr.* **10**, 63 (1959).
[34] W. E. Hillis and G. Urbach, *J. Appl. Chem.* **9**, 474 (1959).
[35] H. G. Bray, B. G. Humphris, W. V. Thorpe, K. White and P. B. Wood, *Biochem. J.* **52**, 416 (1952).
[36] P. Baruah and T. Swain, *Biochem. J.* **66**, 321 (1957).
[37] F. E. King, T. J. King and L. C. Manning, *J. Chem. Soc.* 563 (1957).
[38] C. J. B. Smit, M. A. Joslyn and A. Lukton, *Analyst. Chem.* **27**, 1159 (1955).
[39] A. L. Kursenov and M. N. Zeprometov, *Biokhimiya* **14**, 467 (1949).
[40] T. Swain, *Ann. Rep. Food Investigation Board*, London, 40 (1956).

reaction was obtained with numerous phenolic compounds not containing *meta* oriented hydroxyl groups, except for homocatechol which perhaps reacts through the methyl group.

It should be noted that in both the phloroglucinol and resorcinol series of compounds C-substitution reduces the ϵ value. In the case of flavolans, therefore, a similar if not greater reduction might be expected, due to the formation of new C–C bonds. This hypothesis was confirmed by the results obtained for a series of tannins and catechin polymers (Table 2) which were all found to have lower $E^{1\%}_{1cm}$ values than (+) catechin itself (ϵ could not be

TABLE 1. MOLECULAR ABSORPTIVITIES OF THE COLOURED PRODUCTS FORMED FROM VARIOUS MODEL COMPOUNDS BY THE FOLIN–DENIS REACTION WITH VANILLIN, 4-AMINO PHENAZONE AND MOLYBDATE REAGENTS

Compound	$\epsilon \times 10^{-3}$			
	Folin–Denis*	Vanillin*	4 Amino-phenazone†	Molybdate‡
Catechol	20·2	0·04	6.1	1·8
Homocatechol	17·6	3·6	4·0	1·7
Tertiary butyl catechol	9·7	0·05	2·0	1·3
Chlorogenic acid	21·8	0	—	—
Resorcinol	15·8	45·7	6·8	0
4-Ethylresorcinol	10·4	38·4	2·5	0
2,4-Dimethyl resorcinol	9·3	38·4	1·6	0
Pyrogallol	10·9	44·6	0·50	5·3
2-Methylpyrogallol	13·2	21·6	0·20	—
Gallic acid	18·5	0	—	—
Phloroglucinol	7·7	35·0	2·6	0
2-Methylphloroglucinol	5·2	28·0	0·18	0
7-Hydroxyflavan	8·7	42·1	—	0
5,7-Dihydroxyflavan	13·3	30·5	—	0
(+)-Catechin	30·7	32·9	3·3	1·8
(+)-Epicatechin	26·7	24·7	3·2	1·8
(+)-Gallocatechin	30·1	28·0	—	—
(−)-Epigallocatechin	16·7	24·0	2·5	2·4
(−)-Epigallocatechin gallate	43·5	32·1	4·0	10·7
Theaflavin gallate	49·8	30·0§	—	—
Brazilin	36·8	39·1	—	—

* Method essentially the same as used by Swain and Hillis.[33]

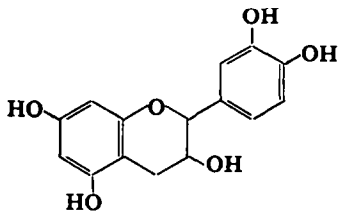
† Method essentially the same as used by Baruah and Swain.[36]

‡ To 1 ml of phenol, 3 ml of 1·3 % (w/v) sodium molybdate in phosphate buffer (0·066 M pH 6·5) was added. The adsorption at 350 mμ was determined after 15 min, see Experimental.

§ Per "phloroglucinol" ring.

calculated as the molecular weights were not known). It is apparent therefore that as the molecular weight increases the reactivity to vanillin decreases in a similar way as found for formaldehyde.[34]

Unlike vanillin, the Folin–Denis reagent[33] did not react in a stoichiometric manner with phenolic compounds, even when the number of hydroxyl groups was taken into account (cf. catechol and pyrogallol in Table 1). It is thus similar to other oxidizing agents[24] and the differences are presumably due to the relative oxidation–reduction potentials of the different phenolic groupings. However, the molecular absorptivities for the more complex molecules is approximately the same as that obtained by addition of the coefficients of the separate phenolic nuclei. For example, this applies to epigallocatechin gallate (II) which contains resorcinol, pyrogallol and gallic acid nuclei. One may assume therefore that polymeric



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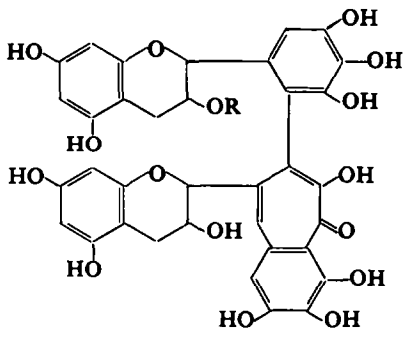

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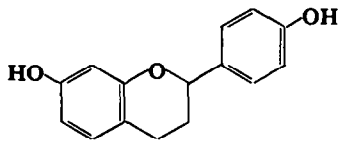

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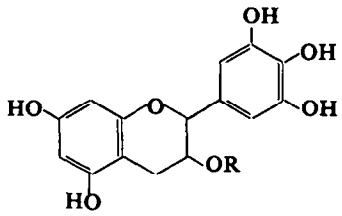

(X) R = H

(XI) R = Galloyl

tannins will give approximately the same amount of colour with this reagent as the monomers unless hydroxyl groups are involved in linkage or are transformed into stabilized quinonoid groups during polymerization (e.g. IV). The results on the tannins (Table 2) showed that in each case the colour formed with this reagent was less than that of (+) catechin but the reduction in $E^{1\%}_{1cm}$ was not as great as that obtained with vanillin. Thus the ratio of the values

TABLE 2. $E^{1\%}_{1cm}$ VALUES FOR VARIOUS TANNINS AND CATECHIN POLYMERS*

Tannin	$E^{1\%}_{1cm} \times 10^{-2}$ Folin–Denis	$E^{1\%}_{1cm} \times 10^{-2}$ Vanillin	% V/FD
Catechin	10·6	11·4	108
Acid catalysed polymer†	11·3	10·3	91
Autoxidation polymer	4·9	1·6	33
Gallocatechin	9·9	9·1	92
Acid catalysed polymer†	9·9	8·2	83
Autoxidation polymer	2·2	0·9	42
Grape fruit	4·1	1·2	29
Grape leaf	4·6	1·8	39
Persimmon	5·6	3·4	61
Cacao	4·6	2·2	48
Quebracho Bark	6·5	2·5	38
Wattle Bark	6·1	3·1	51

* Polymers prepared by heating catechin or gallocatechin (1 μM) in 0·1 M H_2SO_4 or 0·1 M sodium acetate solution for 5 hr at 100°.

† Plus unreacted monomer.

obtained with the two reagents (V/FD) is lower for polymers. During the course of autoxidation of (+) catechin and (+) gallocatechin, this ratio was found to fall in a linear fashion and to parallel the formation of chromatographically non-mobile polymeric material. The ratio can be taken, therefore, as a measure of polymerization.

No study could be made of the effect of increasing molecular size on the conversion of leuco-anthocyanins into anthocyanins with butanol–HCl,[33] as model compounds were not available. Roux and Paulus,[16] however, have shown that although monomeric leucofisetinidin (3,4,7,3′,4′-pentahydroxyflavan) gives three and a half times the yield of fisetinidin than does the trimer, little further change in yield occurs up to the decamer. It can be presumed, therefore, that a reduction in the leuco-anthocyanin content of a fruit on ripening, may represent either the conversion of a monomer into a dimer or trimer, or may be due to the reduced extractability of higher polymers.

A number of other reagents for the estimation of phenolic compounds were examined to see whether they could be used in determining the degree of polymerization. Since autoxidative polymerization involves the formation of quinones, and hence a reduction of vicinal hydroxyl groups, an attempt was made to measure changes in such groups. King and White[41] described a modification of the Mitchell ferrous tartrate method[42] for the estimation of vicinal phenolic groups but as this involved aqueous buffers, the method was not very suitable for methanolic extracts. Instead, a method based on the use of sodium molybdate[43] was modified for use with alcoholic solutions. The results obtained (Table 1) show a reasonable degree of stoichiometry with a number of model compounds containing catechol-like groups, but it was found that there was much variation on polymerization which made the method difficult to apply.

As mentioned above the vanillin reagent reacts only with the *meta*-substituted benzene ring and it was felt that other non-selective substitution-type reagents might be more advantageous. Several substituted diazotized amines were examined[35] including a number of commercially available stabilized salts, but all were found to suffer from the disadvantage of high and variable blanks in the presence of organic solvents, and the fact that Beer's law was only obeyed over a small concentration range. 4-Aminophenazone,[36] was also examined, but although it showed a marked decrease in reactivity with substituted phenols (Table 1) it gave very variable results with tannins and polymerized catechins probably due to the fact that it requires an alkaline medium for reaction. Similar disadvantages apply to the use of Gibb's reagent.[37]

It was decided therefore to investigate the changes in flavolans in the absolute methanol extracts and aqueous methanol extracts of ripening fruits using only the first three analytical methods described above.[15] However, although the V/FD ratios can be used to show the degree of polymerization of flavanols in model systems it cannot be properly measured in respect of flavanols and their polymers in fruit extracts. As mentioned earlier such extracts contain, besides leuco-anthocyanins and other flavanols, a number of simple phenolic compounds all of which react with the Folin–Denis reagent, and this gives rise to meaningless V/FD ratios. Instead, therefore, vanillin/leuco-anthocyanin ratios (V/LA) have been used as a measure of polymerization, since in this case both reagents react only with flavanols and their congeners. That this ratio is more useful than V/FD in determining the degree of

[41] H. G. C. KING and T. WHITE in *The Chemistry of Vegetable Tannins*, p. 31. Society Leather Trades Chemists, Croydon, England (1956).
[42] C. A. MITCHELL, *Analyst* **61**, 295 (1936).
[43] L. P. KENDAL, *Biochem. J.* **44**, 433 (1949).

polymerization of flavanols in fruit extracts is shown by the results given in Table 3. It can be seen that the V/FD ratios are in most cases lower in the absolute methanolic extracts of the fruits than in the aqueous methanolic extracts, in spite of the fact that the latter extracts, judged chromatographically, contain only polymerized material. The V/LA ratios, on the

TABLE 3. V/LA AND V/FD RATIOS FOR ABSOLUTE AND AQUEOUS METHANOL EXTRACTS OF FRUITS

	Extract % MeOH*			
	100		50	
Fruit	% V/LA	% V/FD	% V/LA	% V/FD
Apple	67	45	32	55
Pear	86	15	41	24
Banana	74	15	48	53
Grape	49	53	47	57
Persimmon	71	35	46	40
Cacao†	112	59	57	55

* Outer flesh was exhaustively extracted successively with absolute methanol followed by aqueous methanol. The Folin–Denis and vanillin reacting compounds in each extract were expressed as catechin, and the leuco-anthocyanin as cacao leuco-anthocyanin all on a dry weight basis.
† Defatted fermented seed cotyledons.

other hand, show the expected behaviour being higher in the absolute methanolic extracts (cf. Table 2). When considering changes in the tannins in fruits on ripening, however, it should be recalled that polymerization of monomeric flavan-3,4-diols causes an initial reduction in reactivity with both reagents. If in the polymer the reduction in leuco-anthocyanins reactivity (conversion to anthocyanidin) is greater than the reduction in the reactivity

TABLE 4. $E^{1\%}_{1cm}$ VALUES FOR CACAO LEUCO-ANTHOCYANIN AND ITS POLYMERS*

	Folin–Denis	Vanillin	Leuco-anthocyanin	Ratio % V/LA
Cacao leuco-anthocyanin	10·2	8·5	2·1	405
Acid-catalysed polymer†	11·7	5·8	0·9	603
Autoxidation polymer†	8·8	2·2	0·7	315

* Produced as described in Table 2, but heated for 1 hr only.
† Plus some unreacted monomer.

to vanillin, as might be expected from the results of Roux and Paulus,[16] then the ratio V/LA will be higher after polymerization; such changes will be expected to occur only in the fraction soluble in absolute methanol. The aqueous methanolic extracts, which contain polymeric leuco-anthocyanins, should show a greater reduction in vanillin reactivity on further polymerization than in their yield of anthocyanidin, and so the V/LA ratios should fall. That this hypothesis may be true is shown by the results for the acid-catalysed and

autoxidative polymerization products of cacao–leuco-anthocyanin (Table 4). Both show the expectable reduction in vanillin reactivity (cf. Table 2), but the V/LA ratio of the acid-catalysed polymer (which is perhaps a mixture of dimer or trimer) is higher, and that of the autoxidative polymer lower, than the monomer itself.

TABLE 5. CHANGES IN THE PHENOLIC COMPOUNDS IN THE BANANA ON RIPENING

Stage of ripeness	Extract* % MeoH	% Of total† in each extract	mg/gm Dry weight‡ Folin–Denis	Vanillin	Leuco-anthocyanin	% V/LA
Unripe	100	86	9·3	6·3	9·0	70
	50	14	0·7	0·4	1·5	27
Ripe	100	11	3·2	1·4	0·6	230
	50	89	2·1	1·2	4·7	25

* Outer mesocarp was exhaustively extracted successively with methanol followed by aqueous methanol (1:1 v/v). The residue still contained leuco-anthocyanins.
† Based on leuco-anthocyanin value.
‡ On the same basis as the fruit in Table 3.

Bearing in mind these considerations, the results obtained for banana, persimmons, peach and plum can now be discussed (Tables 5–8). It can be seen that in each case on ripening there is a decrease in the proportion of phenolic compounds which are extracted by absolute methanol regardless of the analytical method used. This is consistent with the fact

TABLE 6. CHANGES IN THE PHENOLIC COMPOUNDS IN THE PERSIMMON ON RIPENING

Stage of ripeness	Extract % MeoH*	% Of total† in extract	mg/gm Dry weight† Folin–Denis	Vanillin	Leuco-anthocyanin	% V/LA	% V/FD
Unripe	100	91	142	51·0	171	30	
	50	8·6	12·6	6·6	16·1	41	52
	0	0·3	2·3	0·9	0·6	150	41
Ripe	100	37·5	38·0	13·9	25·9	54	
	50	50·5	36·2	14·9	34·8	43	41
	0	12·0	10·7	3·4	8·3	41	31

* As in Table 5, but water used for final extract.
† As in Table 5.

that, on ripening, there is a polymerization of flavolans thus rendering them less easily extractable by absolute methanol. It will be noted that in banana (Table 5) and persimmon (Table 6) this change is accompanied by an overall reduction in the total extractable phenolic compounds, whereas in the peach (Table 7) and plum (Table 8) there is a small increase probably due to the reasons mentioned earlier. In each fruit also there is an increase in the V/LA ratio in the absolute methanolic extracts, and a small decrease of this ratio in the

aqueous methanolic extract which, on the basis of the considerations put forward above, is again consistent with polymerization.

It appears reasonable to assume, therefore, that in the fruits examined, reduction in astringency on ripening is due to polymerization of flavans. However, there are a number of discrepancies which bear further examination. For example, an examination of the

TABLE 7. CHANGES IN THE PHENOLIC COMPOUNDS IN PEACHES ON RIPENING

Stage of ripeness	Extract* % MeoH	% Of total* in extract	mg/gm Dry weight* Folin–Denis	Vanillin	Leuco-anthocyanin	% V/LA
Unripe	100	90	18·0	7·8	13·1	60
	50	10	0·6	0·3	1·4	22
Ripe	100	81	21·0	10·4	15·3	69
	50	19	1·3	0·5	3·6	14

* As in Table 5.

changes in the water extracts of persimmon (Table 6) shows that although on ripening there is an expected increase in the proportion of total extractable tannins which are present in this fraction (which judged chromatographically contains high molecular weight flavolans only) and a fall in the V/LA ratio, the value of the latter ratio in the unripe fruit is higher than in the methanol extractable fractions. At first sight this would appear to indicate that the water-extractable fraction contains less polymerized material than the absolute or aqueous

TABLE 8. CHANGES IN THE PHENOLIC COMPOUNDS IN PLUMS ON RIPENING

Stage of ripeness	Extract* % MeoH	% Of total* in extract	mg/gm Dry weight* Folin–Denis	Vanillin	Leuco-anthocyanin	% V/LA
Unripe	100	75	12·0	6·0	9·0	67
	50	25	1·0	1·0	3·0	33
Ripe	100	50	16·0	10·0	7·0	143
	50	50	4·0	2·0	7·0	29

* As in Table 5.

methanol extracted fractions. However, when the V/FD ratios of the aqueous methanol and water-soluble fractions are compared (which is allowable as all simple phenolic compounds which would add to the Folin–Denis reactivity would have been extracted with absolute methanol) it can be seen that the latter fraction contains, on this basis, more highly polymerized material. It is probable, therefore, that the water-extractable fraction contains a higher proportion of non-leuco-anthocyanin flavolans. Another discrepancy was found when

ripe astringent and non-astringent peaches were compared (Table 9). Here again there is a smaller proportion of absolute methanol extractable tannins in the astringent fruit, but the V/LA ratios (and in this case also the V/FD ratios of the aqueous methanol extracts) are not in accord with expectation. It could be argued that in the peach, lower polymers are more astringent than higher ones, or that the yield of anthocyanidins from different polymeric leuco-anthocyanidins is not in accordance with the assumptions which have been made on the basis of the results of Roux and Paulus.[16] Obviously, then, much more work is required before the relationship between the changes in flavolans and in astringency in ripening fruits can be completely described; it is hoped, however, that the use of the analytical techniques described here, together with the development of further methods for the determination of

TABLE 9. PHENOLIC COMPOUNDS IN ASTRINGENT AND NON-ASTRINGENT PEACHES

	Extract* % MeoH	% Of total* in extract	mg/gm Dry weight* Folin–Denis	Vanillin	Leuco-anthocyan	% V/LA
Non-Astringent	100	85	20·0	10·0	15·2	67
	50	15	1·4	0·7	2·6	27
Astringent	100	71	20·0	10·1	8·9	114
	50	29	1·5	1·1	3·7	30

* As in Table 5.

the molecular size of tannins now in progress, and an examination of the relation between these parameters and astringency, will enable the problem to be solved in the not too distant future.

EXPERIMENTAL

Compounds

Except where mentioned below, model compounds were commercial samples which were purified where necessary by crystallization. (−)-Epicatechin was isolated from unfermented dried cocoa beans, and (+)-catechin from cutch extract. Cocoa tannin was prepared from fermented cocoa beans by extraction and precipitation with ammonium sulphate. The samples of quebracho and wattle tannin were obtained from commercial tannin extracts and purified by alcohol precipitation. Samples of tannins from persimmon, grape leaf, and grape fruit were kindly given by Drs. H. W. Siegleman, W. L. Porter and P. Ribereau-Gayon respectively. Cocoa leuco-anthocyanin was kindly donated by Dr. W. G. C. Forsyth, 7-hydroxy- and 5,7-dihydroxy-flavan by Dr. D. E. Hathway and the other catechins and theaflavin gallate by the late Drs. E. A. H. Roberts and A. E. Bradfield.

Analytical Methods

(a) Folin–Denis: The method was scaled down from Swain and Hillis.[33] To 1·0 ml of sample solution and 1·0 ml 0·25 N Folin–Denis reagent (complex phosphotungstomolybdate) followed 3 min later by 1·0 ml 1 N Na_2CO_3. The absorbance was read 1 hr later against a suitable blank at 725 mμ. (b) Vanillin: carried out using half the quantities given by Swain and Hillis.[33] (c) Leuco-anthocyanins: as given by Swain and Hillis[33] but heating carried out

in 25-ml wide-mouthed bijou bottles fitted with a screw cap and rubber gasket. These gave more reproducible results than glass-stoppered test tubes. (d) Molybdate: to 1·0 ml of solution was added 3·0 ml of a 2:1 mixture of 0·1 M phosphate buffer (pH 6·5) and 5% $Na_2MoO_42H_2O$; the absorbance was measured after 15 min at 350 mμ against a suitable blank. (e) 4-Aminophenazone: to 1·0 ml of sample solution was added 1·0 ml of reagent (0·2% in 0·5 N ethanolic ammonium hydroxide solution) followed by 1·0 ml 0·2% aqueous solution of $K_3Fe(CN)_6$. The absorbance was determined after 1 min at 500 mμ against a suitable blank. (f) Diazotized amines: to 1·0 ml of solution was added 1·0 ml of diazotized amine or stabilized diazonium salt (0·1% w/v) followed by 2·0 ml 2·5 N sodium acetate. The absorbance was read at a suitable wavelength against the appropriate blank. The stabilized salt included, those from 4-amino-4'-methoxydiphenylamine (Fast blue V.B.), 1-amino-anthraquinone (Fast red A.L.), 2-amino-4-nitroanisole (Fast red B), and 5-nitroaninsidine (Fast red R).

Source of fruit. Unripe bananas (*Musa sapientum* L. var. Gros Michel) grown in W. Africa were obtained through commercial channels and kept at 11·8° before ripening at 20°. Unripe plums (*Prunus domestica* L. var. Gaviota) and peaches (*P. persica* stokes var. Elberta) were specially picked and transported by the Western Provinces Fruit Research Station, Department of Agriculture, Union of South Africa, through the courtesy of Dr. L. Ginsberg. Depending on condition they were ripened between 10 and 15°. Unripe persimmons (*Diospyrus Kaki* L. var. Hachiya) were obtained from the Department of Agriculture, Israel, through the courtesy of Prof. M. A. Joslyn and ripened at 15°.

Extraction. Samples of 3–5 fruits were sectioned and several discs of the outer mesocarp taken with an 8 mm stainless steel cork-borer. A portion was taken for dry weight determination and the rest (2–3 gm) weighed under methanol (10–15 ml/gm). The discs were crushed with a glass rod and extracted by boiling for 10 min. The extract was decanted and the residue extracted four times more, the combined extract being concentrated and made up to 50 ml. The residue was then extracted with three portions of aqueous methanol (10–15 ml/g) at boiling point, and these extracts combined, concentrated and made up to 25 ml. Analyses on the extracts, diluted where necessary, were carried out in triplicate.

Acknowledgements—Besides all those mentioned in the Experimental section, we wish to thank the Forestal Land Timber and Railway Co. for a supply of commercial tannins and Messrs. Cadbury's Ltd for a supply of cacao beans. We are also grateful to Dr. R. Gane of this laboratory for helping with the ripening of the fruits. Part of the experimental work was carried out with the assistance of Mr. A. S. Mead.