Phenolic Acids and Flavonoids of *Theobroma cacao* L. Separation and Identification by Paper Chromatography

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Previous investigations on the phenolic constituents of *Theobroma cacao* L. have been largely limited to studies on the polyphenols of the ripe bean (Forsyth, 1952, 1955; Forsyth & Quesnel, 1957), which have been shown to be of major importance in determining the taste characteristics of processed cacao.

Studies are now being pursued in this Laboratory on the nature of the factors determining the chemical composition of the bean at maturity with particular reference to the phenolic constituents of the cotyledons. The physical and biochemical factors determining the level of polyphenols other than anthocyanins in vegetative and reproductive organs of the green plant are largely unknown, although it has recently been shown by Hillis & Swain (1957) that illumination is an important factor in the formation of the leaf leucocyanidins of *Prunus domestica* var. *victoria*. Other of our current investigations are concerned with the possible role of the phenols in the mechanism of disease resistance of *T. cacao*, evidence having been obtained of an intense metabolic activity involving the host polyphenols of *T. cacao* tissue after invasion by *Marasmius perniciosus* (L. A. Griffiths, unpublished work). For both these projects a systematic investigation of the phenolic constituents of both vegetative and reproductive tissues was a necessary preliminary and the results obtained are presented in this paper. Although there are many reports in the literature describing the sporadic occurrence of phenolic compounds in various plant materials, a study of the distribution and interrelations of this class of compound throughout the vegetative and reproductive structures of a single species of green plant does not appear to have been previously undertaken.

In this survey of the phenolic constituents of *T. cacao*, both methanolic and acid-hydrolysed extracts have been examined by paper chromatography.

MATERIALS AND METHODS

Source of plant material. Unless otherwise stated, all plant material was obtained from trees of *Theobroma cacao*, of the clone I.C.S. 1 (Imperial College Selection 1).

Extraction and separation

Phenolic acids of tissue hydrolysates. Finely chopped fresh tissue (5 g.) was heated with 20 ml. of aq. 2N-HCl for 20 min. on a boiling-water bath. The cooled filtrates were extracted with 5 ml. of diethyl ether and 0.1 ml. of the ethereal extract was applied to the chromatogram by repeated applications in a warm air-stream.

The chromatograms were developed by an ascending method employing benzene-acetic acid-water, 60:70:30 (solvent A) (Griffiths, 1957a). The original procedure was modified only in that the chromatograms were run at 20° instead of 15° as previously used. It was observed that the use of a higher temperature caused an appreciable rise in the R_F values recorded. Although this system was originally developed for the separation of the phenolic acids, it has also proved of utility in the preliminary identification of the flavonols found in these extracts. By extending the development time to 16 hr. and by the use of descending chromatography, flavonols of low R_F , such as quercetin, could be readily separated. Their presence was confirmed with the butanol-acetic acid-water solvent.

Free polyphenols. The polyphenols of *T. cacao* tissue were extracted by homogenizing 5 g. of fresh tissue in 25 ml. of cold absolute methanol in a Micro-Blender for 3 min. A volume (0.05 ml.) of the filtered extract was then applied to the chromatogram.

Both one- and two-dimensional chromatography were employed. With the former the solvent employed was butan-1-ol-acetic acid-water (40:10:50), and, with the latter, water was employed as the first solvent (solvent C), and the butanol-acetic acid-water system (solvent D) as the second. All two-dimensional work was carried out in a Shandon Multi-sheet Chromatank.

Anthocyanidins. A previously described extraction procedure and chromatographic method employing water-acetic acid-HCl, 10:30:3 (solvent B), (Bate-Smith, 1954), was employed. Identification of the anthocyanidin was in all cases confirmed by the use of markers of known identity.

Detection of phenolic compounds

Ultraviolet fluorescence. The hydroxycinnamic acids, their derivatives and the flavonols were detected by examination in ultraviolet light, when they fluoresced in a characteristic manner in the presence and absence of ammonia (Griffiths, 1957a).

Spray reagents. The *p*-nitroaniline-spray reagent previously employed for the detection of phenolic acids (Swain, 1953; Griffiths, 1957a) was found to be of value also in the detection of catechins and leucocyanidins, which gave rise

to yellow-brown coloration on spraying. The toluene-*p*-sulphonic acid reagent of Roux (1957) was employed for the detection of leucocyanidins.

R_F values. Since *R_F* values vary considerably with temperature, it was found essential under tropical conditions to effect direct comparison of the unknown compounds in each case with markers of known identity, although for the two-dimensional chromatograms the individual polyphenols are readily recognized by their position in the overall pattern of spots. The identity of the compounds marked with an asterisk in Table 2 was confirmed in each case by admixture of the extract with an authentic specimen of the compound, when intensification of the corresponding spot was observed.

RESULTS

Phenolic acids and flavonoids of tissue hydrolysates

The general distribution of the phenolic acids in the tissues of *T. cacao* is shown in Table 1. Caffeic acid and a compound of slightly higher *R_F* (0.28 at 20°), exhibiting a green-blue fluorescence in u.v. light, were found to be of widespread occurrence. Other fluorescent phenols detected appeared to be restricted to tissues of a specific morphological type.

Fruit. The tissues of the pod wall show a differentiation in the distribution of the phenolic acids and flavonoids in transverse section. The amounts of individual phenols present, moreover, vary in relation to the degree of maturity of the tissue (Tables 1 and 2). Only trace amounts of the phenolic acids were detected in the hydrolysates of bean tissues from mature pods, and immature beans excised from developing pods were found to contain none of the phenolic acids detected in other cacao tissues.

Leaf. The mature green leaf was shown to contain a high level of compounds of the cinnamic acid group. These acids, with the exception of caffeic acid, are present only in traces in the young leaf, which contains appreciable amounts of quercetin. During the period in which the red pigmentation of the leaf is lost and succeeded by the characteristic green of the mature leaf, a gradual loss of quercetin accompanied by an increase in the amount of caffeic, *p*-coumaric, sinapic and ferulic acid is apparent.

In order to explore the possibility that some of the phenolic acids detected in leaf hydrolysates might occur naturally in the free state, methanolic extracts prepared by the standard procedure were examined in solvent *A*. In all cases, however, it was observed that no mobile fluorescent compounds were present. On submitting the extracts to a hydrolytic treatment with 20% HCl, mobile fluorescent materials made their appearance and were identified as the phenolic acids described

above. It is therefore evident that all of the phenolic acids occurring in the cacao leaf exist in the bound form, possibly as depsides. In several tissue hydrolysates, fluorescent materials of very low *R_F* were detected, but in view of the difficulty of obtaining sufficiently exact measurements of the *R_F* values to characterize these compounds, the substances were not identified.

Anthocyanidins of tissue hydrolysates. All the tissue hydrolysates examined were red in colour, to a varying degree, owing to the liberation of anthocyanidins from anthocyanins and leucocyanidins. Chromatographic examination revealed that cyanidin hydrochloride was the only anthocyanidin present (Table 1).

Methanol-soluble polyphenols of fresh tissue. The distribution of methanol-soluble polyphenols throughout the tissues of *T. cacao* is shown in Table 2. (–)-Epicatechin and leucocyanidins *L*₁ and *L*₂ were found in most tissues examined.

Leaf. A two-dimensional chromatogram of the polyphenols of the leaf shows most of the major polyphenols of *T. cacao* (Fig. 1).

The young leaf of *T. cacao*, I.C.S. 1, is characterized by an intense red coloration, which decreases during development and is succeeded by an olive-green colour and finally by the deep green of the

Fig. 1. Composite diagram showing position of polyphenolic components of leaf tissue. 1, (–)-Epicatechin; 2, leucocyanidin (*L*₁); 3, leucocyanidin (*L*₂); 4, chlorogenic acid; 5, neochlorogenic acid (?); 6, *p*-coumarylquinic acid; 7, compound showing violet fluorescence; 8, compound showing deep-blue fluorescence; 9, 10, 11, 12, unidentified anthoxanthins of leaf; 13, rutin; 14, 3- α -L-arabinosidylecyanidin; 15, 3- β -D-galactosidylecyanidin. Spots shown diagrammatically with a complete outline were detected by u.v. fluorescence; spots shown with a dotted outline were detected either by their colour in visible light (spots 14 and 15) or by the use of spray reagents (spots 1, 2 and 3) as described in the text.

mature leaf. The red coloration was shown to be due to the presence of 3- α -L-arabinosidylecyanidin and 3- β -D-galactosidylecyanidin.

The presence of chlorogenic acid and *p*-coumarylquinic acid was observed in the young leaf (Table 2). Both of these compounds decreased in amount during growth and disappeared at maturity. During these changes two new compounds of higher R_f in solvent *D* appeared on the chromatograms, possessing a blue-green and a violet fluorescence respectively. From the position of the former compound on the chromatogram in relation to the other substances of the chlorogenic acid group, it appears probable that this substance is neochlorogenic acid. It was not, however, possible to confirm this provisional identification, as neochlorogenic acid was not available to us for purposes of chromatographic comparison. Since the rate of increase of the substance resembling neochlorogenic acid paralleled the rate of disappearance of the chlorogenic acid, a possible conversion of the former into the latter during growth is suggested.

(-)-Epicatechin, mobile leucocyanidins (Table 2) and stationary leucocyanidin material were also detected. At least five anthoxanthins, of which one was shown to be identical with rutin, are present in the leaf of cacao. The remaining anthoxanthins were not identified, the work being handicapped by the unavailability of the less-common anthoxanthins as markers.

Fruit. The major polyphenols of the pod wall were (-)-epicatechin leucocyanidins L_1 and L_2 and a fluorescent polyphenol of R_f 0.82. The latter compound, which appeared blue in u.v.-light radiation but became greener on exposure to NH_3 fumes, resembled a member of the chlorogenic acid group, but on chromatographic comparison with authentic specimens of chlorogenic and isochlorogenic acid it was found to possess an R_f value in solvent *D* intermediate between those observed for these two compounds (Table 3). The material was subsequently shown to possess an R_f value closely resembling that of caffeic acid, and the fluorescence also was observed to be very similar to that dis-

played by this compound. That the material is not caffeic acid, however, was shown by comparison in solvent *A*, when the pod-wall compound was found to be immobile whereas the caffeic acid showed an R_f value of 0.22 at 20°.

On submitting the fluorescent pod-wall polyphenol to acid hydrolysis, caffeic acid was detected among the hydrolysis products. Attempts to establish the presence of quinic acid with the sodium metaperiodate, sodium nitroprusside and piperazine reagents of Cartwright & Roberts (1955) were unsuccessful but this may have been due to the small amount of material available.

On spraying with diazotized *p*-nitroaniline the fluorescent compound gave rise to a purple-brown coloration, whereas spraying the chromatogram with an aqueous suspension of polyphenol oxidase, by the technique of Griffiths (1957*b*), gave rise to rapid darkening in the position of this compound.

The young pod wall of the clone, I.C.S. 1, is characterized by an intense-red pigmentation, which decreases as the pod matures. The pigments responsible for this coloration were shown to be 3- α -L-arabinosidylecyanidin and 3- β -D-galactosidylecyanidin.

A violet fluorescent material of R_f 0.93 in solvent *D*, occurring in young-pod-wall tissue, is probably a depside of *p*-coumaric acid, as this acid was detected among the products of acid hydrolysis when the compound was eluted with water and treated with 2*N*-HCl. It is not, however, identical with the *p*-coumarylquinic acid isolated from apple and pear tissue by Cartwright & Roberts (1955), since the *p*-coumaryl derivative of the pod wall possesses a higher R_f value and separates from admixture with an authentic specimen on development with solvent *D*.

Beans. The polyphenols of the ripe bean have previously been described by Forsyth (1952, 1955). In addition to those reported by him, evidence was obtained by two-dimensional chromatography of the presence in trace quantity of *p*-coumarylquinic acid, which was detected by its violet fluorescence in u.v.-light radiation. A fluorescent compound possessing a similar R_f value to the chlorogenic

Table 3. Comparison of fluorescent polyphenols of pod wall and phenolic compounds showing similar fluorescent or chromatographic properties

	R_f in solvent <i>D</i> (29°)	R_f in solvent <i>A</i> (20°)	Fluorescence in the presence of NH_3
Major fluorescent polyphenol of pod wall	0.82	0	Blue-green
Minor green fluorescent polyphenol	0.90	.	Blue-green
Minor violet fluorescent polyphenol	0.93	.	Violet
Chlorogenic acid	0.73	.	Green
Isochlorogenic acid	0.85	.	Green
Caffeic acid	0.83	0.22	Blue-green
<i>p</i> -Coumarylquinic acid	0.79	.	Violet

acid was detected but it differs from the latter in not showing the same degree of greening with NH_3 fumes.

During maturation of the fruit the white ovules enlarge and give rise to the heavily pigmented beans, and the tissue surrounding the ovules gives rise to the semi-fluid pulp. Samples were taken from the composite tissues internal to the pod wall, and the development of the polyphenols was followed chromatographically. Examination of the chromatograms revealed that large amounts of the leucocyanidin material, stationary in solvent *D*, were present throughout development but that the mobile leucocyanidins and (-)-epicatechin first appeared at the stage at which the anthocyanins make their appearance. Of the two anthocyanins, the 3- α -L-arabinosidylecyanidin was the first formed. The fluorescent material resembling chlorogenic acid appeared at a relatively late stage in the ripening of the bean.

Flower. The pigments present were identified as 3- α -L-arabinosidylecyanidin and 3- β -D-galactosidylecyanidin.

A large amount of (-)-epicatechin and leucocyanidins L_1 and L_2 were present.

Root tissue. Among the compounds detected were small amounts of the major fluorescent polyphenol observed in pod wall, the R_f values, fluorescence and colour reaction with *p*-nitroaniline being identical.

DISCUSSION

From the observations recorded, it is evident that in *T. cacao*, I.C.S. 1, the phenolic compounds vary not only with the morphology of the tissue but also with the physiological age of the material.

Of the phenolic acids detected in hydrolysates, caffeic and *p*-coumaric acid are known to arise at least in part from chlorogenic acid and *p*-coumarylquinic acid respectively, whose presence has been established. Evidence was also obtained for the presence of bound forms of ferulic and sinapic acid in the leaf but the nature of the parent substances remains unknown.

Young-leaf tissue is characterized by the presence of large amounts of the leucocyanidins, which show a progressive decrease during growth. A similar relationship between the age of the leaf and the leucocyanidin content of *Eucalyptus sieberiana* has been noted by Hillis (1956). The metabolic fate of the leucocyanidin in the developing leaf of *T. cacao* is uncertain but the parallel increase in the phenolic acid content is suggestive. It has recently been shown by the use of radioactive tracers that certain of the aromatic acids, namely cinnamic and *p*-hydroxycinnamic acid, were very good precursors of quercetin in buckwheat (Underhill, Watkin & Neish, 1957), and it

appears not unlikely that the biosynthetic route is reversible. Whether or not the more complex polyphenols of young cacao-leaf tissues are degraded to the phenolic acids detected in mature-leaf tissue is a question which could be settled conclusively only by the use of labelled compounds.

In the course of this study consideration was given to the possible origin of the phenolic substances of the bean. It would appear equally possible that the bean polyphenols are synthesized completely in the cotyledon or that the aromatic ring or flavonoid skeleton is elaborated in the leaf and translocated to the developing ovule. In support of the latter view Lindner, Fitzpatrick & Weeks (1950) found that any type of phloem blockage produced accumulation of polyphenol, and from their description it appears that these polyphenols could have been leucocyanidins. The view that the bean polyphenols may arise from pre-formed leucocyanidin is also supported by the observation that the simpler hydroxycinnamic acids, which have been recognized as early precursors of the flavonoid skeleton (Underhill *et al.* 1957), were absent from the developing bean although present as depsides in the mature leaf. The possibility must be recognized also of formation of the flavonoids of the ripe bean from the immobile leucocyanidin material, which is present before and throughout the period during which the anthocyanins, catechins and mobile leucocyanidins make their appearance in the developing bean. That the leucocyanidins of certain plants may be the precursors of anthocyanin pigments has previously been suggested by Robinson & Robinson (1939), Simmonds (1954) and Alston & Hagan (1955). That the leucocyanidins are present in large amounts in rapidly growing leaf tissue and low in the metabolically inert tissues, e.g. bark and heart wood, suggests that in *T. cacao* the leucocyanidins play an active role in the intermediary metabolism of the plant and are not to be regarded as inert end-products.

It has also been noted that large amounts of leucocyanidin are always found in tissues containing appreciable quantities of the anthocyanins, the young leaf, the bean and the flower being particularly rich in both types of compound. The converse, that colourless tissues are necessarily low in leucocyanidins, does not, however, hold since the young unpigmented inner-pod-wall tissue contains appreciable amounts of leucocyanidin material.

SUMMARY

1. The distribution of phenolic compounds in the tissues of *Theobroma cacao* is described.
2. (-)-Epicatechin and leucocyanidins L_1 and L_2 were present to a varying degree in all tissues.

3. Caffeic acid, *p*-coumaric acid and an unidentified fluorescent compound of R_F 0.28 are present in many cacao tissues, but ferulic and sinapic acids are of limited occurrence.

4. The major anthocyanin of the young leaf and young pod wall was shown to be 3- α -L-arabinosidylecyanidin, and in the flower 3- α -L-arabinosidylecyanidin and 3- β -D-galactosidylecyanidin were present in approximately equal amounts.

5. Cyanidin hydrochloride was the only anthocyanidin detected in tissue hydrolysates of *T. cacao*.

6. *p*-Coumarylquinic acid was detected in the cotyledons of the bean and in young-leaf tissue. Chlorogenic acid was also shown to occur in young-leaf tissue.

7. A fluorescent phenolic compound releasing caffeic acid on hydrolysis, but not identical with chlorogenic or isochlorogenic acid, was detected in pod-wall tissue.

8. Leucocyanidin material is present in large amounts in young-leaf tissue but the content falls to a very low level at maturity. Similar developmental changes were observed in pod-wall tissue.

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Purification of Free Staphylococcal Coagulase

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Two types of material capable of interacting with plasma or fibrinogen are formed by pathogenic staphylococci. One of these, termed bound coagulase, is apparently part of the cell wall and is released only by disintegration or autolysis, whereas the second, known as free coagulase, is liberated into the medium during growth (Duthie, 1954*a*). Free coagulase has been shown by numerous workers to interact with a so-called coagulase activator found in the plasma of certain animals to give a thrombin-like material capable of clotting fibrinogen (Smith & Hale, 1944). The nature of the coagulase activator is disputed, but recently the work of Tager (1956) suggests that both prothrombin and another factor, possibly a prothrombin-breakdown product, can function in this respect. The nature of their interaction is unknown.

Partial purification of free coagulase from cultures in digest broth was described by Duthie & Lorenz (1952). They found that coagulase was

easily precipitated by cadmium salts, which could be removed by dialysis at an acid pH, giving a coagulase which was relatively stable in solution at pH 2.0. Jacherts (1956) has recently submitted material prepared in this way to further purification by column chromatography on aluminium hydroxide, followed by paper electrophoresis. A single active component staining with ninhydrin was obtained, but no details regarding its specific activity are given.

In the present purification studies, use was made of the finding by Duthie (1954*b*) that if 1 volume of a fully grown shaken culture of staphylococci in digest broth is inoculated into 9 vol. of fresh warm broth, i.e. to give a concentration of about 40 μ g. of bacterial nitrogen/ml., and is then shaken at 37°, there is a maximal release of free coagulase in less than 2 hr. By inoculating into a casein-hydrolysate medium instead of digest broth and growing for exactly 1 hr. 20 min. a maximal ratio of free