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POLYPHENOLIC SUBSTANCES OF ARECANUT
I. CHROMATOGRAPHIC ANALYSIS OF FRESH MATURE NUT

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Abstract—The polyphenols of arecanut have been examined by use of paper chromatography and specific spray reagents. Besides (+)-catechin and leucocyanidin, a monomeric and some polymeric leucocyanidins have been shown to be present. The data on the high yield of the cyanidin from the polymeric compounds suggest that they contain readily cleavable linkages. Quantitative estimation of the different components shows a preponderance of the polymeric flavan-3:4-diols yielding cyanidin.

INTRODUCTION

ARECANUT, the hard edible endosperm of the palm, *Areca catcehu* Linn. is widely used as masticatory in the Orient.[1,2] The arecanut, a principal component of the chew, contains predominantly polyphenols, carbohydrates (polysaccharides), fat and alkaloids, principally arecoline. While the alkaloid has been very well studied, extremely little is known about the polyphenolic substances.

(+)-Catechin was reported to be present by Yamamoto and Muraoka[3] and has recently been isolated by Seshadri and Nagarajan.[4] The presence of leucocyanidin was indicated by Bate-Smith[5] and by Sastry *et al.*[6] Recently,[7,8] leucocyanidin has been isolated and identified by means of its derivatives and its physical properties. The latter workers[4] indicated that other more complex polyphenolic substances are also present.

Paper chromatographic analysis, combined with the use of specific chromogenic reagents, has in recent years been used with great success in the study of the complex polyphenols of heartwoods[9] and cocoa.[10] The present paper gives the results of similar analysis of the polyphenolic substances in ripe arecanut.

RESULTS

Circular paper chromatography of extracts of fresh ripe nut with three different solvents (water, ethanol and acetone), showed that some seven phenols were present (Fig. 1a). The components were tentatively identified as shown in Table 1. The compound with R_f 0·76 in

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3 R. YAMAMOTO and L. MURAOKA, *Sci. Papers Inst. Phys. Chem. Research* (Tokyo) **19**, 142 (1932).
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5 E. C. BATE-SMITH, *Biochem. J.* **58**, 122 (1954).
6 L. V. L. SASTRY, M. N. SATYANARAYANA, M. SRINIVASAN, N. SUBRAHMANYAN and V. SUBRAHMANYAN, *J. Sci. Ind. Research* (*India*) **15C**, 78 (1956).
7 G. R. NAGARAJAN and T. R. SESHADRI, *J. Sci. Ind. Research* (*India*) **20B**, 165 (1961).
8 S. BANERJEE, S. RAJADURAI and Y. NAYUDAMMA, *Bull. Central Leather Research Inst.* (*India*) **8**, 174 (1961).
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10 W. G. C. FORSYTH and J. B. ROBERTS, *Biochem. J.* **74**, 374 (1960).

BAW was identified as a flavan-3-ol by the colour produced with the specific sprays, by the ultraviolet absorption (max. 280 mμ) and by the fact that no anthocyanidin was produced on treatment with n-butanol–HCl. This compound was identified as (+)-catechin by co-chromatography with authentic material obtained from *Acacia catechu*. The second band probably also contains a flavan-3-ol. The next most prominent band was a flavan-3:4-diol, as seen from the colour reactions to the specific sprays, and from the formation of cyanidin, identified by its absorption maximum at (550–555 mμ) and its R_f in Forestal solvent (0·52) and in formic acid–HCl solvent (0·18). From its position on chromatograms below catechin in BAW and ahead of catechin in 2% acetic acid (R_f 0·54 in both solvents), this substance was identified as

TABLE 1. QUALITATIVE ANALYSIS BY CIRCULAR PAPER CHROMATOGRAPHY OF ARECA POLYPHENOLS

Solvent	R_f*	Reagent			Probable identity
		Toluene-p-sulphonic acid	Bisdiazotized benzidine	Butanol–HCl 100°C	
BAW	0·00	Brownish pink	Deep maroon	Pink	Flavan-3:4-diol
	0·29	Pink	Faint maroon	Pink	Flavan-3:4-diol
	0·46	Brown–pink	Maroon	Pink	Flavan-3:4-diol
	0·51	Brown–pink	Maroon	Pink	Flavan-3:4-diol
	0·59	Pink	Maroon	Pink	Flavan-3:4-diol (leucocyanidin)
	0·67	—	Faint maroon	Pale brown	Flavan-3-ol
	0·76	Dull yellow	Maroon	Pale brown	Flavan-3-ol (+)-catechin
2% acetic acid	0·00	Dark brownish pink	Deep maroon	Pink	Flavan-3:4-diol
	0·47	Faint yellow–pink	Maroon	No colour	Flavan-3-ol (+)-catechin
	0.56	Brown–pink	Maroon	Pink	Flavan-3:4-diol (leucocyanidin)
	0·71	Pink	Maroon	Pink	Flavan-3:4-diol

* All the bands gave positive reactions with $FeCl_3$–$K_3Fe(CN)_6$ and Vanillin–HCl reagents.

a leucocyanidin. Apart from the leucocyanidin, between two and four other discrete bands with progressively lower R_f in BAW were present which reacted with toluene-*p*-sulphonic acid to give pink colours, and with bisdiazotized benzidine to give claret maroon, indicating that they contained flavan-3:4-diols. All these gave cyanidin on treatment with n-butanol–HCl reagent. A flavan-3:4-diol having an R_f higher than leucocyanidin in 2% acetic acid (R_f 0·71) has been noted in extracts of nuts from some regions.

Better separation of the polyphenolic substances were obtained by a two-dimensional chromatography (Fig. 2). Besides spots corresponding to the positions of catechin and leucocyanidin, other spots with progressively lower R_f values in either directions were found. All the spots except Nos. 9 and 10 gave cyanidin when treated with n-butanol–HCl.

Alkali fusion of the polyphenol mixture and of some of the individual fractions in the high R_f region, gave protocatechuic acid and an unidentified compound. This indicates that all the flavonoids present have the 3′,4′-dihydroxy grouping on their "B" rings.

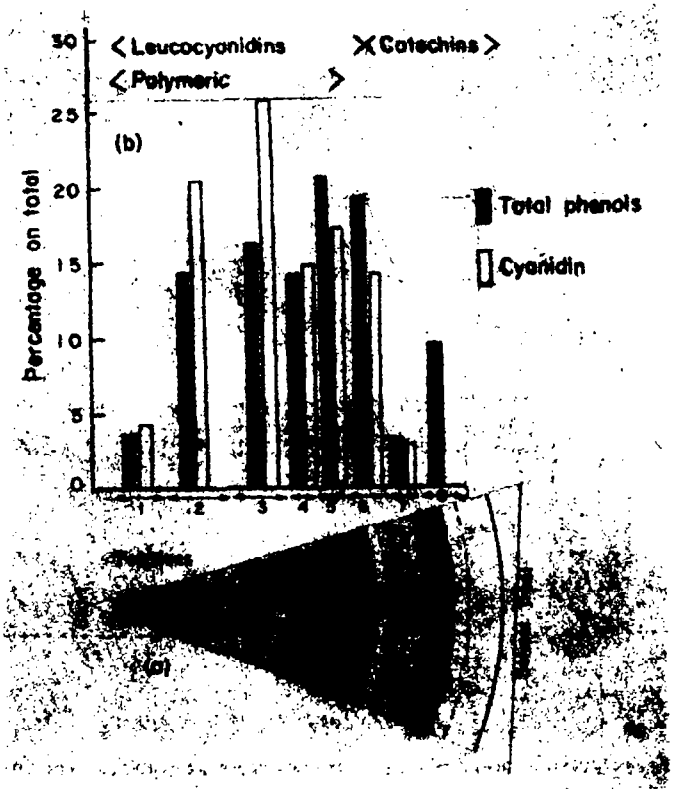

FIG. 1. (a) CIRCULAR PAPER CHROMATOGRAM OF POLYPHENOLS IN AQUEOUS EXTRACT OF ARECANUT. SOLVENT: n-BUTANOL–ACETIC ACID–WATER (4:1:2·2 v/v).

FIG. 1. (b) YIELDS OF TOTAL PHENOLS AND CYANIDIN FROM DIFFERENT CUTS OF 1a.

The dotted line on the chromatogram shows the cuts made for estimation. The bands correspond to those referred to in Table 1. The density of shading indicates the intensity of the bands.

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FIG. 2. TWO-DIMENSIONAL CHROMATOGRAM OF POLYPHENOLS IN AQUEOUS-EXTRACT OF ARECANUT. SOLVENTS: 1ST DIRECTION, n-BUTANOL–ACETIC ACID–WATER (4:1:2·2 v/v); 2ND DIRECTION, 2% ACETIC ACID.

Spots 1–5 and 7, polymeric leucocyanidins; 6, 8, monomeric leucocyanidins; 9, possibly a flavan-3-ol; 10, (+)-catechin. The density of shading indicates the intensity of the spots.

The action of potato phenolase on the polyphenols separated by BAW on a one-dimensional chromatogram showed that the highly mobile catechin band turned brown within a few minutes, while the leucocyanidin band and the one next below turned brown after about an hour. The other bands and areas of still lower mobility turned light brown only after keeping the strip overnight in the humid chamber.

The results of quantitative estimation of the polyphenols of arecanut, after separation on paper, are shown in Fig. 1b. The values are given as a percentage of the total given by the complete extract. This procedure has been followed since the nature of the areca leucocyanidins is still under examination and other reference substances were not readily available. It is clear however that the catechins form a minor proportion of the total polyphenols and the leucocyanidin and other flavan-3:4-diols yielding cyanidin on treatment with mineral acid, form the major components.

TABLE 2. QUANTITATIVE ESTIMATION OF PHENOLICS SEPARATED ON A TWO-DIMENSIONAL CHROMATOGRAM

Spot Nos*	Total phenolics: % of total		Leucocyanidins	Anthocyanidin max. at (mμ)†
	Folin–Denis as (+)-catechin	Based on optical density at 280 mμ	% of total	
1	26·2	26·2	35·6	550
2	5·1	5·1	5·2	550–555
3	11·1	10·9	11·1	550–555
4	18·9	19·0	18·4	550
5	1·7	1·7	0·7	550
6	2·0	2·0	2·8	550–555
7	12·6	12·8	11·5	550
8	13·4	13·5	12·9	550–555
9	1·9	1·9	1·7	—
10	6·2	6·2	0·0	—

* The spot Nos. refer to those in Fig. 2.

† Anthocyanidins from all spots, except 5, 6 and 8 showed a minor peak at 450–460 mμ which may be due to degradation products accompanying anthocyanidin formation from the "polymeric" flavan-3:4-diols.

There is however some difference in the percentages of the different leucocyanidin components as determined by the Folin–Denis reagent, and from the cyanidin formed. Some of the slower moving fractions appear to give high yields of cyanidin and react less to the general Folin–Denis reagent. While part of the discrepancy could arise due to overlap of spots and the background spread of some "polymeric" polyphenols, another contributing factor is the incomplete extraction of the polyphenols from paper on the lower R_f regions for reaction with the Folin–Denis reagent.

The quantitative analysis by two-dimensional chromatography confirmed the results of the one-dimensional chromatographic analysis (Table 2). Catechin and a minor phenolic component, spots 10 and 9 (Fig. 2), respectively form about 7 per cent of the total polyphenols while the flavan-3:4-diols make up the rest. Among the flavan-3:4-diols the spot No. 8 corresponding to leucocyanidin forms about 13 per cent; those spots (Nos. 2, 3, 4, 5 and 7) that show definite movement in both the directions, but less than leucocyanidin, form about 50 per cent; spot 6, which is a flavan-3:4-diol, but has greater movement than leucocyanidin in 2% acetic acid, forms about 3 per cent; and the elongated spot which has R_f 0 to 0·5 in BAW,

but does not move in 2% acetic acid, constitutes about 35 per cent. The spots of lower mobilities, except spots 5 and 6, are not formed as discrete round spots but as bands elongated in the direction of the 2% acetic acid run.

DISCUSSION

The predominant polyphenols of mature arecanut are the flavan-3:4-diols and catechins. The top band on BAW chromatograms is (+)-catechin. The faint band next lower to this is possibly (+)-epicatechin originally present or formed during extraction and concentration. The next lower band is leucocyanidin (5,7,3′,4′-tetrahydroxy-flavan-3:4-diol). Nagarajan and Seshadri[4,7] have reported isolation and identification of both (+)-catechin and leucocyanidin in reasonable yields from ripe arecanuts.

Spot No. 6 (Fig. 2) identified as a leucocyanidin by the cyanidin produced, has a higher mobility, R_f 0·77 in 2% acetic acid compared to the monomeric leucocaynidin (spot 8). This, in view of the observations of Roux[11] on the chromatographic behaviour of stereochemically inter-related flavonoid compounds, is likely to be an enantiomorph of the monomeric leucocyanidin. Roux[12] has reported of an unstable "leucofisetinidin-2" with a higher mobility in water-saturated butanol than (+)-7,3′,4′-trihydroxyflavan-3:4-diol but having similar mobility in 2% acetic acid.

Besides the monomeric catechin and leucocaynidins further discrete bands, with lower mobility in BAW and in 2% acetic acid, with flavan-3:4-diol characteristics have been shown. All these yield cyanidin on treatment with mineral acid. That complex leucocyanidins are part of the normal make-up of the polyphenols of arecanut has further been confirmed by two-dimensional chromatographic analysis. The complex flavan-3:4-diols have been shown to consist of different discrete members showing large differences (R_f 0·0 to 0·5) in their movement in the two developing solvents. Following the studies of Roux[12,13] these different spots with lower mobility would represent the polymeric forms of leucocyanidin where the mode of condensation preserves the flavan-3:4-diol structure intact or potentially available to yield the corresponding anthocyanidin. An interesting observation is that the "polymeric" forms, from the spots having little movement in either directions, give yields of cyanidin comparable to that from monomeric forms.

The cacoa leucocyanidin,[10] a dimeric compound containing a mole of leucocyanidin and a mole of epicatechin, and the dimeric pro-anthocyanidins, studied by Freudenberg and Weinges,[14] both have easily cleavable ether linkages in their structures. Freudenberg and Weinges[15,16] and Hergert[17] also consider that the highly reactive 4-hydroxyl of a flavan-3:4-diol could participate directly in forming a link with the 6, 7 or 8 position of a second molecule and such condensation products could be cleaved by acid to give high yields of the corresponding anthocyanidins.

The action of the potato phenolase on the separated components is also indicative of the nature of the components, the monomeric and simpler polymers being more easily oxidized

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than the complex polymers in the lower regions. The relative higher susceptibility to oxidations by the enzyme of the catechin (compared to the leucocyanidins) in contrast to the easy aerial oxidation of the leucocyanidin (compared to catechin), would also indicate differences in the polymers of biological origin and that from aerial oxidation . The polymeric leucocyanidins of arecanut show absorption maximum at 275–280 mμ but none at 410–430 mμ found for catechin autoxidation products by Hathway and Seakins.[18]

Seshadri and Nagarajan[4] observed that processing of areca nuts, such as sun-drying, and boiling of the aqueous extracts of arecanut reduced the leucocyanidin content while increasing the amount of a red "gummy mass". Seshadri and co-workers[7,19] have also isolated leucocyanidins from a number of other sources which show difference in their physical properties. They have also noted that from tamarind seed testa, an ethyl acetate insoluble fraction was obtained, which resembled leucocaynidin. A similar difference in the easy extractability by ethyl acetate of the catechin and monomeric flavan-3:4-diol fractions of high mobility, in contrast to the other fractions of lower mobility from aqueous solutions of arecanut polyphenols, have been observed by us. It is thus likely that the unidentified fractions isolated earlier by others are similar to the polymeric leucocyanidins with lower mobility shown in this paper. The tanning properties of concentrated extracts of arecanut described by Banerjee *et al.*[8] would be explainable by the condensed forms of leucocyanidin.

MATERIALS AND METHODS

Areca fruits of eight to nine months' maturity with the bright orange-yellow skin were hand-plucked and brought from a local commercial garden. Fruits which were similar in size, colour and firmness were used in the study. They were dehusked with a stainless-steel knife and the hard endosperm cut into thin slices with a stainless-steel cutter. The thin slices, further crushed in a heavy pestle-and-mortar and fragmented in a Waring blender, were extracted with water, ethanol, acetone and ethyl acetate in the cold repeatedly. The combined extracts were filtered to get fairly clear solutions and stored in the refrigerator to settle some fine particles. The supernatants were either examined straight by chromatography or concentrated for fractionation by extraction and precipitation.

Qualitative surveys of polyphenolic compounds in the extracts were done by one- and two-dimensional paper chromatography on Whatman No. 1 and No. 3 papers. Both ascending and circular techniques were used for one-dimensional runs. BAW, i.e. n-butanol–acetic acid–water (v/v 4:1:2·2) and 2% acetic acid were generally used for developing polyphenols, while the Forestal solvent and the formic acid–HCl solvent according to Roux[20] were used for developing anthocyanidins. Simpler phenols and phenolic acids were developed with the organic layers of the mixtures, butanol–acetic acid–water (4:1:5) and *m*-cresol–acetic acid–water (50:2:48). Two-dimensional chromatograms were made on Whatman No. 1 and No. 3 papers of 20 cm^2 in a Dent and Datta frame, using the developing solvent BAW for the first direction and 2% acetic acid for the second direction.

The polyphenolic areas on the papers were located by examining under ultraviolet, before and after exposure to ammonia vapour, for fluorescent areas, by spraying with a mixture of equal parts of aqueous 0·3% $FeCl_3$ and 0·3% $K_3Fe(CN)_6$[21] and ammoniacal $AgNO_3$.[22]

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Catechins and leucoanthocyanins were located on the sheet with specific reagents such as vanillin–HCl,[23] toluene-*p*-sulphonic acid in ethanol[9] and bisdiazotized benzidine.[24] The vanillin reacting areas were further characterized as leucoanthocyanins by boiling the corresponding chromatogram areas in n-butanol–HCl[25] and characterizing the developed anthocyanidin by their absorption maxima and chromatography. Absorption measurements were made on eluates of cut areas of the chromatograms.

Degradation of the flavonoids by fusion with caustic potash was also done on a few extracts and fractions and the resulting phenols and phenolic acids identified by paper chromatography.

The susceptibility of the polyphenolic areas to enzymic oxidation was determined by spraying areas with partially purified preparation of potato phenolase, hanging the paper in a humid chamber and noting the development of brown colour.[26] Quantitative estimation of total polyphenolic components was done with the Folin–Denis reagent. This method is not specific for polyphenols, but gives results of the same order as other available methods and is simple for routine estimation. The total phenols and fractions have also been examined by their absorption characteristics. The results were calculated with a preparation of catechins from *Acacia catechu* as the reference substance.

Total flavans were estimated by the vanillin–H_2SO_4 reaction, and the flavan-3:4-diols by the conversion to the anthocyanidins, both according to the standardized conditions described by Swain and Hillis.[25] The results are expressed as percentages of the total.

One-dimensional or two-dimensional separations of known quantities of the extracts were carried out in quadruplicate in the same chamber and one of the papers developed with a polyphenolic reagent, usually $FeCl_3$–$K_3Fe(CN)_6$ mixture. With this as reference pattern different areas from the other chromatograms were cut out. The total polyphenol and leucocyanidin content were then estimated by direct treatment of the cut bits of the areas in test tube with specific reagents. The vanillin–H_2SO_4 reagent could not be used in this way.

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