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POLYPHENOLIC SUBSTANCES OF ARECANUT

II. CHANGES DURING MATURATION AND RIPENING

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Abstract—The polyphenols of Arecanut at all maturity stages are mainly flavonoids and decrease in concentration with maturity on a dry weight basis. The pattern of changes with maturation and ripening is due to insolubilization of higher polymers together with the formation of fresh monomers and intermediate polymers. Reactivities of the fractions of arecanut polyphenols indicate some differences between the areca flavan-3,4-diols (all yielding cyanidin) and others previously reported.

INTRODUCTION

ARECANUT (*Areca Catechu* Linn.) is normally consumed at two different maturities, as a processed mature-green nut, and as a fresh or dried ripe nut. In earlier studies,[1] it was shown that on a dry weight basis high concentration of polyphenols and low alkaloid are found at the young stage. At the ripe stage the polyphenol concentration decreases to a third or quarter of the initial value, while the alkaloid increases. The young nut however is known to be more potent in its physiological action. There is distinct difference in taste between the young nuts and those of higher maturity, although the predominant "taste" at all stages is astringent. Besides the alkaloid, arecoline, the polyphenolic substances of arecanut have also been shown to contribute to physiological activity.[2] In view of the recent interest in plant polyphenols, in relation to its contribution to organoleptic qualities and physiological and pharmacological properties of food and feed materials,[3,4] a closer study of the polyphenols of arecanut at different stages of maturity has been undertaken. In Part I[5] of these studies, the polyphenols of ripe arecanut were shown to contain predominantly polymerized leucocyanidins besides confirming earlier findings of (+)-catechin and monomeric leucocyanidins. Both astringency and physiological action may be expected to vary with the degree of polymerization of the polyphenols.[4] Hillis and Swain[6] studied different parts of *Prunus domestica*, and Craft[7] examined Elberta peaches for qualitative and quantitative changes in the polyphenolics during growth and ripening.

RESULTS

Total extracts from all stages of maturity showed the same absorption pattern with a single maxima at 280 mμ. The freeze-dried aqueous extractives of arecanut of different maturities

[1] A. G. MATHEW, S. D. VENKATARAMU and V. S. GOVINDARAJAN, *Indian J. Technol.* **2**, 90 (1964).
[2] M. SIRSI, A. K. DORLE and V. S. GOVINDARAJAN, *Curr. Sci. (India)*, **32**, 455 (1963).
[3] *The Pharmacology of Plant Phenolics* (Symposium) (Edited by J. W. FAIRBAIRN), Academic Press, London (1959).
[4] *Ann. Rep. Low Temp. Res. Stn, Camb.* **34** (1960).
[5] V. S. GOVINDARAJAN and A. G. MATHEW, *Phytochem.* **2**, 321 (1963).
[6] W. E. HILLIS and T. SWAIN, *J. Sci. Food. Agric.* **10**, 135 (1959).
[7] C. C. CRAFT, *Am. Soc. Hort. Sci.* **78**, 119 (1961).

freed of polysaccharides were analysed for polyphenols by three different methods. These estimations show that there was no significant change with maturity, close agreement being found between all three. The tannin content was considerably lower, amounting to only 70–75 per cent of the other values, and the total leucocyanidins analysed to between 43 and 49 per cent of the extractives in the different stages.

On a dry nut basis, all the estimations of total polyphenolic constituents showed a decrease (Fig. 1) throughout the maturity changes, falling to a third of its initial value, at the final stage. This decrease was greater at the young and at the ripening stage than at the middle stages. Calculated on a wet nut basis, the pattern was similar though the differences were not marked, due to a progressive fall in moisture with growth and maturation. The ripe nut showed a

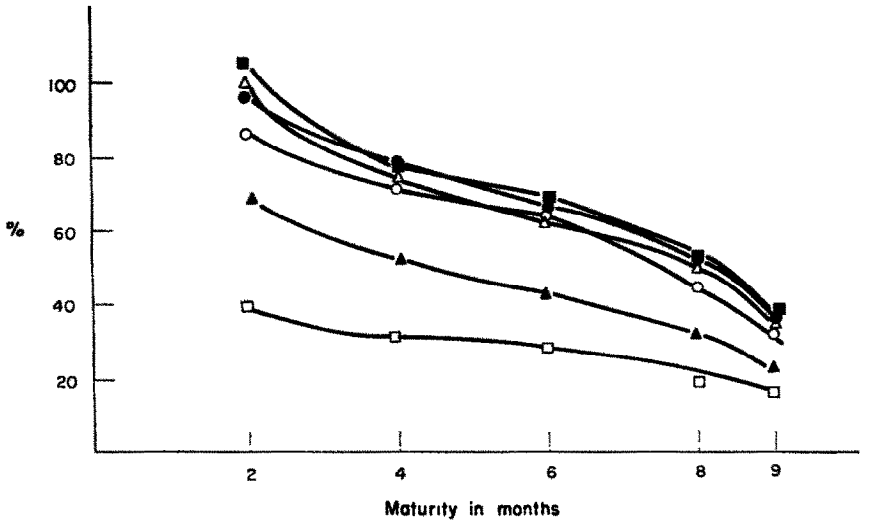

FIG. 1. CHANGES IN POLYPHENOLS WITH MATURITY—ON DRY WEIGHT BASIS.

▲——▲—Total water extractives; ○——○—Tannin content;
●——●—Total phenols; △——△—Total flavnols;
■——■—280 mμ reading; □——□—Total leucocyanidin.

slight increase in values over the middle stages although the value was clearly less than the tender stages. This coincided with a larger drop in moisture content during ripening. The total leucocyanidins on a wet nut basis showed similar variation, though to a much less degree, throughout maturation and ripening. Calculated on a weight per nut basis, total polyphenols, determined by all the methods, and leucocyanidin showed progressive increase with maturity and ripening. The ratio of leucocyanidins to total polyphenols remained nearly the same (40–50 per cent) at the different stages of maturity.

Paper chromatography of extracts of arecanuts of different maturities revealed similar pattern though quantitatively at the ripe stage there was an increased concentration of the spots representing mobile monomeric leucocyanidins and catechin and a decrease at the start and early tailing with lower R_f, representing leucocyanidins of highly polymerized state.

Quantitative fractionation into the ether extractable, ethyl acetate extractable and aqueous residue fractions, and estimations of the last two fractions for total phenols, total flavanols and total leucocyanidins, are expressed as absolute weights in Table 1. With increasing maturity

TABLE 1. YIELD AND REACTIVITIES OF FRACTIONATED ARECA POLYPHENOLS AT DIFFERENT MATURITIES

Maturity in months	Aqueous residue						Ethyl acetate fraction					
	Folin-Denis	Vanillin	Leuco-antho-cyanin	Total solids	V/FD (%)	V/LA (%)	Folin-Denis	Vanillin	Leuco-antho-cyanin	Total solids	V/FD (%)	V/LA (%)
2	35·0	30·1	21·1	32·8	86	143	12·4	10·6	9·7	9·6	85	109
4	33·1	27·6	22·5	31·5	83	123	12·1	10·3	9·2	9·7	85	111
6	33·1	27·2	19·6	30·2	82	139	12·1	10·6	12·7	11·6	88	83
8	33·1	29·1	21·3	29·0	88	137	12·4	10·9	12·3	12·9	88	89
9	33·1	26·6	19·6	26·2	80	136	11·3	11·2	13·1	15·1	99	85

Note: Yield of fractions in mg from 50 mg of total extractives; ether extractable fraction not shown in table.

the total solids increased a little in the ether fraction and markedly in the ethyl acetate fraction while the aqueous residue decreased by a small amount compared to the initial values in the fractions from the very young stage. The chromatographic analysis of the ether fraction revealed mainly catechin with a trace of monomeric leucocyanidin. Measured against catechin as standard, the different methods gave values agreeing closely with the total solids, the values increasing by a small percentage with maturity. The ethyl acetate fraction when chromatographically analysed showed predominantly the monomeric leucocyanidins along with little residual catechin and some polymeric leucocyanidins. The total solids increased with maturity, as did the leucocyanidins but at a slower rate in the later stages. Estimations with Folin-Denis reagent and vanillin–H_2SO_4 on the other hand, gave values agreeing with total solids up to 4 months maturity but did not rise further.

The aqueous residue, after the two solvent extractions, was found by chromatography to contain essentially the polymeric fractions in the lower R_f regions in both directions. Variations between the estimations of the total polyphenols by the different methods were also found with this fraction. The total leucocyanidins and total flavanols, like the total solids, decreased with maturity though to a lesser extent, while the total phenols remained more or less the same with a slight decrease at ripening.

V/FD (ratio of the reactivities to vanillin–H_2SO_4 and Folin-Denis reagents) showed a slight decrease in the case of aqueous residue and a slight increase in the case of ethyl acetate fractions during maturation (Table 1). V/LA (ratio of V and leuco-anthocyanidins) on the other hand decreased in both cases, especially in the ethyl acetate fraction.

In order to study the differences in the major constituents with maturity, estimation as leucocyanidins of individual spots, after separation by two-dimensional paper chromatography of the total aqueous extract, as shown in Fig. 2, was carried out. The fractions A and B, at the start with little or no movement in either direction, representing highly polymerized leucocyanidins, were definitely greater in young stages and decreased with maturity (from 10 to 5 per cent). Probably because of incomplete separation of very close-lying spots and small variation in movement between the adjacent papers, the remaining zones did not show a clear-cut pattern of change. The other polymerized fractions represented by fractions C and D having lower mobilities than monomers showed slight increases with maturity. While leucocyanidin monomer has not shown any increase, the unidentified leucocyanidin with higher mobility in acetic acid solvent,[5] fraction G, has shown a definite increase with maturity. To reduce the overcrowding of different spots in the beginning and middle portions, a fraction representing moist ethyl acetate extractives of the aqueous extract was used for a similar study. This fraction does not represent the full picture of different polyphenolic compounds, but shows principally the monomeric and some polymeric leucocyanidins. The separation and quantitative analysis of this simpler fraction also gave a similar pattern of changes. The results, expressed as percentages of the total in the original sample, showed that the highly polymerized leucocyanidins located at the starting spot decreased with maturity estimated as total phenols from 4·9–2·4 per cent and as total leucocyanidins from 6·2–3·4 per cent; the monomeric leucocyanidins (fast-moving fraction) increased though less markedly from 22·8–24·2 per cent estimated as total phenols, and from 12·7–18·0 per cent estimated as leucocyanidins. Catechin increased from 8·4–8·7 per cent, estimated as total phenols.

Leucocyanidins extractable in cold 0·3 N HCl showed an increase from 0·22–2·9 per cent from the very young to ripe stage. The insoluble leucocyanidin in the fibrous residue also increased from 2·9–8·6 per cent (Fig. 3). Circular chromatography of the 0·3 N extracts at

equal total phenolic concentration revealed progressively increasing number and concentration of bands of intermediate polymeric stages, while there were strong spots at the start of the highly polymerized fraction for all maturity stages.

Relatively low yields of anthocyanidin were obtained from leucoanthocyanidins in spite of using the improved method of Swain and Hillis.[8] The $E^{1\%}_{cm}$ value for monomer leucocyanidin at 545 mμ on treatment with butan-1-ol-HCl was 150, comparable to the value of 209 obtained for cocao leucocyanidin (T. Swain—private communication). However, a sample of pure leucofisetinidin gave only 10 per cent fisetinidin when subjected to the anhydrous HCl treatment, and a highly polymerized leucocyanidin from pine-bark gave a 5 per cent yield of cyanidin. As against these, the arecanut monomeric leucocyanidin gave 11·0–15·7 per cent yield of cyanidin for different samples, while the combined leucocyanidins of varying degrees of polymerization, represented by aqueous residue, gave a value of 7·7–8·8 per cent.

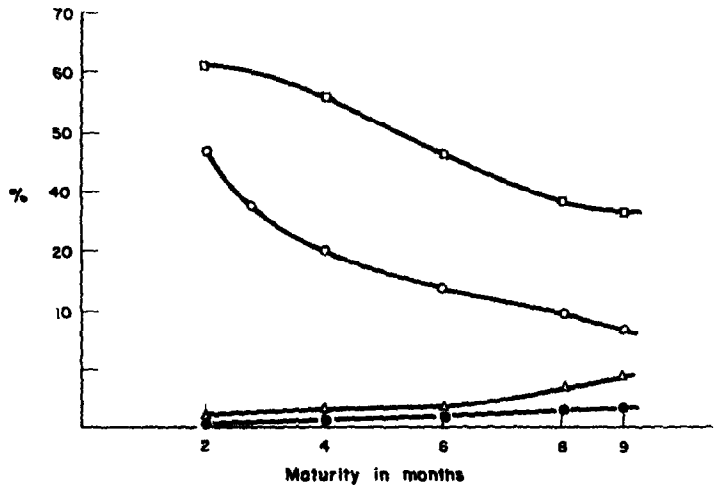

FIG. 2. DISTRIBUTION OF LEUCOCYANIDINS IN THE NUT-CHANGES WITH MATURITY.

□——□—Total in nut; ○——○—Water soluble;
●——●—0·3 N HCl extract of residue; △——△—Insoluble residue.

DISCUSSION

Almost similar values obtained for total phenols, total flavanols, and the absorption at 280 mμ, indicate that the arecanut polyphenols are mostly of the flavonoid type and that there is no significant change in the nature of the polyphenols at the different stages of maturity. The Lowenthal method is known to give lower estimates of the flavonoid polyphenols[9] which explains the low values obtained by this estimation. The fairly close agreement between the other three methods indicates that we are dealing with fairly pure isolates of total polyphenols.

The considerable decrease in the concentration of polyphenols on dry basis with maturity is consistent with the observed change in the pattern of the other chemical constituents which

[8] T. SWAIN and W. E. HILLIS, *J. Sci. Food Agric.* **10**, 63 (1959).
[9] C. J. B. SMIT, M. A. JOSLYN and A. LUKTON, *Analyt. Chem.* **27**, 1159 (1955).

are synthesized at increased rates with maturation and ripening.[1] Calculated on weight per nut basis, however, all the estimations show an increase with maturity of polyphenolics and leucoanthocyanidins. The ratio of leucoanthocyanidin to total phenols did not show any profound change at any stage. Similar observations have been made by Craft[7] for Elberta peaches and by Hillis and Swain[6] for plums. It has earlier been shown[5] that 80–85 per cent of the total polyphenol in ripe nut is accounted for by monomeric and polymeric leucocyanidin and that most of the leucocyanidins in ripe arecanut would appear to be in the polymerized state. The yield of cyanidins in the case of arecanut tends to remain nearly constant. However, the relative amounts of monomeric and polymeric leucoanthocyanidins will have an influence on the total yield of cyanidin and this aspect is considered later.

Two-way chromatography of total aqueous extracts of ripe nut shows a polyphenolic pattern with catechin and monomeric leucocyanidin as prominent, clearly separated spots along with highly polymerized leucocyanidins at the start and leucocyanidins of varying degrees of polymerization represented by elongated spots in the direction of 2% acetic acid from R_f 0 to 0·6. In addition to these, there are minor spots provisionally identified as (−)-epicatechin and an enantiomorph of monomeric leucocyanidin.[5] Comparative chromatography of extracts of arecanuts of different maturities reveals that the number of bands and spots are similar for all the stages. However, as the maturity proceeds there is an increase in concentration of catechin and monomeric leucocyanidins and a decrease of polymerized leucocyanidin represented by the bands at the start and some of the early portion of the tailing while the major portion of intermediate stages of polymerization remains the same. Chromatograms of extracts of very young nuts made under non-oxidizing conditions showed essentially the same pattern, suggesting that little change is due to the short-term storage of the polyphenols under the conditions of the study.

Ether extraction of total aqueous extractives of different stages of maturity gives mainly catechin. The subsequent extraction of the extracts with ethyl acetate gives fractions rich in leucocyanidin monomers. The aqueous residues represent mainly the polymerized constituents. It is clear from the analysis of these fractions that with maturity there is an increase of monomeric compounds and a decrease of the highly polymerized leucocyanidins and this confirms the qualitative results of the chromatographic separation. Quantitative estimation of the individual spots of total aqueous extract (Fig. 2) and moist ethyl acetate extractable fraction also prove the same pattern of quantitative changes.

Continued slow synthesis of polyphenols as the nut matures is shown by the steady increase in the amount of polyphenols on a nut basis. This phenomenon has been reported in other plant materials as well. Increased recovery of anthocyanidin from cold 0·3 N HCl extract and insoluble residue suggests progressive insolubilization of the polymeric leucocyanidin as the maturity proceeds. Hillis and Swain[6] have shown a possible trend for an increase in the tightly bound leucoanthocyanins in leaf marc after extraction with solvents. Due to continued formation of monomeric polyphenols and insolubilization of some of the highly polymerized leucocyanidins with maturity, it is possible for polyphenolic estimations in total extract not to show any quantitative change.

Goldstein and Swain[10] have suggested that the decreasing values of V/FD in a general way indicate the increasing degree of polymerization. The results with the polyphenols of arecanut of increasing maturity show that the ratios do not vary very much from 2–9 months maturity (Table 1). The ratio (V/FD) decreases to a small extent in the polymerized aqueous fraction while it increases a little nearing unity with the monomeric forms in the ethyl acetate

[10] J. L. GOLDSTEIN and T. SWAIN, *Nature* **198**, 587 (1963).

fraction. More recently the same authors[11] have suggested that an increase in the V/LA ratio may indicate early stages of polymerization more conclusively. These ratios obtained with the polyphenols of arecanut show interesting differences. The decreasing ratio of V/LA with maturity in the ethyl acetate extractives, due to slightly increased yield of cyanidin from leucocyanidins at mature stages and nearly the same ratio at all maturities in the aqueous residue, are at variance with the above generalizations of Goldstein and Swain,[11] who have also observed some significant exception in the case of persimon and peaches.

Yields of anthocyanidin for the polymerized leucocyanidins of arecanut are comparable to the yield for monomeric fraction unlike the large differences in yields reported by Roux and Paulus[12] from monomeric and polymeric leucoanthocyanidins. This would suggest that the linkages in the polymers of areca leucocyanidin polymers are comparatively more labile. Hergert[13] has also observed differences in yields of cyanidin from polymeric leucoanthocyanidins of different conifer wood and bark.

The lower reactivity based on total solids of the ethyl acetate soluble monomeric fractions at higher maturities and the slightly higher reactivity of the polymeric water-soluble fractions taken together with comparable yields of cyanidin from the monomeric and different polymeric forms of leucocyanidins would indicate structural differences of the areca leucocyanidins from other sources. Nagarajan and Seshadri[14] have recently shown considerable variation in the optical rotation and other characteristics of the methyl ethers of leucocyanidins isolated from different sources including arecanut. The existence of 4-epimers of leucoanthocyanidins in nature and the low reactivity of melacacidin due to the conformation of the 4-OH has been shown by Clark-Lewis and Mortimer.[15]

The increased astringency of the young arecanut over the ripe one is mostly due to differences in the concentration of polyphenols on a dry basis (Fig. 1) and the absence of constituents such as fat, polysaccharides and fibre which tone down the astringency. A taste evaluation of the freeze-dried extractives from nuts of different maturity still showed a slightly increased astringency for the very young sample, although there was not much of a conclusive change in the rest of the maturities. Probably astringency may require a certain degree of polymerization which, however, should not increase to the extent of making it difficultly soluble and thus inactive.[4] The slightly increased astringent taste of the tender nut may reflect a slightly higher amount of polymeric forms (occupying the starting point in chromatograms) in tender stage and disappearance of a part of these by insolubilization at the mature stage. The possibilities of complexing of the polyphenols with other constituents such as pectin, alkaloids and polysaccharides[16] which increase with maturity, may also reduce astringency and reactivity with the different reagents.

EXPERIMENTAL

Nut samples. Arecanuts were procured from a local commercial garden from marked trees. The fruits of specified maturities[1]—very young (2 months); young (4 months); mature-green (6 months); semi-ripe (8 months) and ripe (9 months) were hand-picked, brought to the laboratory immediately and processed for extraction.

Compounds. Catechin and epicatechin were prepared by ether extraction of a commercial

[11] J. L. GOLDSTEIN and T. SWAIN, *Phytochem.* **2**, 371 (1963).
[12] D. G. ROUX and K. PAULUS, *Biochem. J.* **82**, 320 (1962).
[13] H. L. HERGERT, *Forest Prods J.* **10**, 610 (1960).
[14] G. R. NAGARAJAN and T. R. SESHADRI, *J. Sci. Ind. Res. (India)* **20B**, 165 (1961).
[15] J. W. CLARK-LEWIS and P. I. MORTIMER, *J. Chem. Soc.* 4106 (1960).
[16] T. O. M. NAKAYAMA and C. O. CHICHESTER, *Nature* **199**, 72 (1963).

total extract of *Acacia catechu*. Catechin was also prepared by ether extraction of freeze-dried areca extracts. Areca leucocyanidin was obtained from freeze-dried aqueous extract of arecanut, freed from catechin, by repeated extraction with anhydrous ethyl acetate. Chromatographic analysis showed this fraction to be a pure monomeric leucocyanidin with a trace of catechin. Crystalline leucofisetinidin was obtained from Dr. D. G. Roux, and a sample of polymerized leucocyanidin from pine bark from Dr. H. L. Hergert.

Analyses. "Tannin" content was determined by the Lowenthal method of permanganate oxidation and expressed as gallotannic acid.[17] The Folin-Denis method for total phenols, vanillin–H_2SO_4 method for total flavanols, and butanol–HCl method for total leucocyanidins were carried out as described by Swain and Hillis.[8] Photometric method[18] of 280 mμ absorption reading was carried out using the Beckman spectrophotometer. The straight permanganate titration method,[19] without the use of indigo carmine indicator, had interference from coloured oxidation products and therefore could not be used.

The mixture of catechin and epicatechin from *Acacia catechu* was used as the standard for total phenols, whereas chromatographically pure catechin was used as a standard for total flavanols and the 280 mμ absorption readings. Areca monomeric leucocyanidin served as the standard for total leucocyanidin estimations.

Two-dimensional chromatograms were run on Whatman No. 1 paper using butan-1-ol: acetic acid: water (4:1:2·2 v/v) as the first solvent in the machine direction and 2% aqueous acetic acid as the second solvent.[20] The polyphenolic areas were located by examining under u.v. light before and after exposure to ammonia and by spraying with various reagents.[21] Quantitative chromatographic separation and estimation of components was carried out using a Dent and Dutta frame with Whatman No. 3 paper as described in the earlier communication.[5] Spots were marked from an adjacent paper sprayed with a mixture of 0·3% $FeCl_3$ and 0·3% $K_3Fe(CN)_6$, and cut out for estimation of polyphenols and leucocyanidin.

Absolute yields of anthocyanidins were calculated using the standard molar extinction values of fisetinidin[22] as no similar values for cyanidin were available.

Extraction. The fresh fruits of different maturities were analysed for the average weight, moisture content and total water extractives of the nut. Eighty grams of fresh nut in each maturity were macerated immediately on opening with 150 ml of water and 0·1% of potassium metabisulphite to avoid oxidation.[23] The contents, after transferring into a beaker, were boiled gently for 15 min, keeping the mouth of the beaker covered with a watch-glass. The extract was separated by passing through a filter cloth. The residue was boiled gently with two more portions of 150 ml water for 10 min and 5 min respectively. The combined extracts were centrifuged to remove fine particles and then freeze-dried to yield an almost white material. The residue from the exhaustive aqueous extract was shaken with three portions of 15 ml, 5 ml and 5 ml of cold 0·3 N hydrochloric acid,[24] and the insoluble residue dried.

Analysis of the freeze-dried material was carried out on an aqueous solution from which the polysaccharides and other non-tan materials had been precipitated by addition of an equal volume of alcohol.

[17] W. HORWITZ (Editor) *Official Methods of Analysis*, p. 185. Association of Official Agricultural Chemists, Washington (1960).
[18] D. G. ROUX, *J. Soc. Leather Trades' Chemists* **35**, 322 (1951).
[19] M. HOLDEN, *J. Sci. Food Agric.* **8**, 553 (1957).
[20] R. A. CARTWRIGHT and E. A. H. ROBERTS, *Chem. & Ind. (London)* 1389, (1954).
[21] D. G. ROUX and A. E. MAIHS, *J. Chromatog.* **4**, 65 (1960).
[22] S. G. ROUX and S. R. EVELYN, *Biochem. J.* **69**, 530 (1958).
[23] L. VUATAZ, H. BRANDENBERGER and R. H. EGLI, *J. Chromatog.* **2**, 173 (1959).
[24] W. G. C. FORSYTH, *Biochem. J.* **51**, 511 (1952).

Seventy per cent alcohol-soluble portion of the freeze-dried extractives was freed of alcohol and was taken in water for further fractionation. The aqueous solution (5 ml) so obtained, representing total polyphenols, was extracted six times (24 ml total) with ether, followed by extraction of the residue six times by ethyl acetate (24 ml total). The solvent was removed from each combined fraction under reduced pressure and the residues dissolved in water for further analysis.