Phytochemistry, 1969, Vol. 8, pp. 1543 to 1547. Pergamon Press. Printed in England.

THE NATURE OF THE COMPLEX PROANTHOCYANIDINS*

A. G. MATHEW, H. A. B. PARPIA and V. S. GOVINDARAJAN

Central Food Technological Research Institute, Mysore, India

(*Received* 7 *March* 1969)

Abstract—Preliminary separation of the complex proanthocyanidins has been made possible by solvent fractionation using ethyl acetate and ethyl acetate containing ethyl alcohol. Subsequent paper chromatographic separation has yielded good results in the estimation of individual components. Besides leucocyanidin, leucopelargonidin and catechin have been detected in polymeric components.

INTRODUCTION

IN PART I[1] of this study, the polyphenols of arecanut were studied by paper chromatography. The simple polyphenols separate well and have been identified as (+)catechin and (+)leucocyanidin.[1–3] However, during chromatography, there was overcrowding of the areas that represented complex polyphenols, which constituted the major bulk of polyphenols. In recent years, proanthocyanidins from many plant sources were shown to be dimers of catechin and leucocyanidin.[4–6] Later studies also revealed occurrence in plants of dimers of two flavan-3:4-diols, although the nature of linkage was not fully determined.[7] In the light of these developments in the field, the nature of the polymeric polyphenols of arecanut were examined.

RESULTS

It has been noticed that when total polyphenols were extracted with moist ethyl acetate, traces of the complex polyphenols with fair mobility on paper was also extracted along with simple polyphenols. Therefore it was felt that if the polarity of ethyl acetate can be further enhanced by retaining more water, the extraction of this complex polyphenol would be better and thus leave a residue containing fewer components. This was effectively achieved by using small amounts of ethyl alcohol along with ethyl acetate. The solvent fractions—(1) ethyl acetate; (2) ethyl acetate containing ethyl alcohol and (3) aqueous residual fractions—on subsequent two-dimensional chromatography yielded satisfactory separation of all the constituents (Fig. 1). It can be seen that ethyl acetate has extracted most of the monomeric compounds, while ethyl acetate containing alcohol has extracted remaining monomers and the mobile complex (Spot 5). The aqueous residual fraction consisting mostly of other complexes with low mobility was separated into fairly well-defined bands.

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* Part III in the series "Polyphenolic substances of arecanut".

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[6] B. K. NORTJE, *J. Food Sci.* **31**, 733 (1966).
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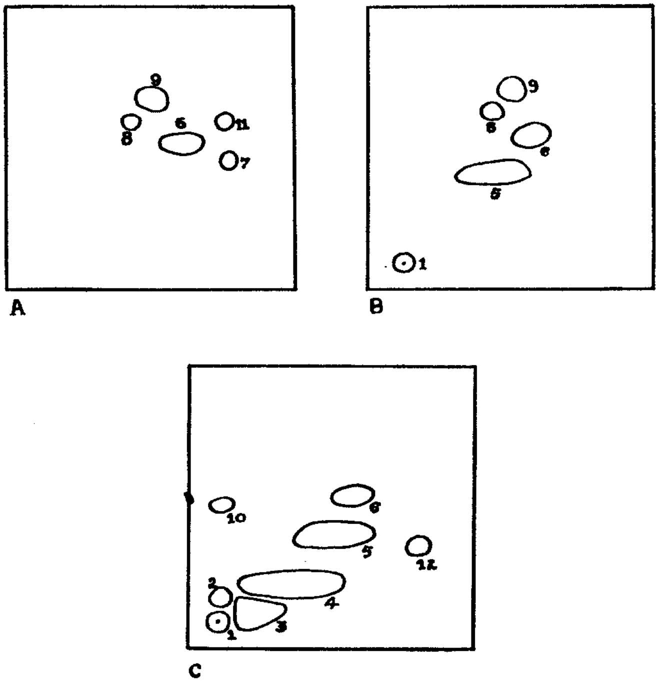

FIG. 1. CHROMATOGRAMS OF THE ARECA POLYPHENOLS—SOLVENT FRACTIONATION.

A, Ethyl acetate; B, ethyl acetate + ethyl alcohol, and C, aqueous residue fractions. ↑ 1st direction BAW; → 2nd direction 2% HAc

Analysis of the Fractions

The amount of polyphenols in each fraction was estimated by Folin–Denis method[8] after taking them into aqueous phase. The individual components of each fraction was then separately estimated by quantitative paper chromatography. Finally, estimated values

TABLE 1. CHROMATOGRAPHIC CHARACTERISTICS AND ANALYSIS OF ARECA POLYPHENOLS

Spot No., Fig. 2	R_f in BAW	R_f in 2% acetic acid	Leucoanthocyanidin test	Chemical nature	% of the total Folin–Denis
1	0·00	0·00	+ve	Very highly polymerized proanthocyanidin	14·70
2	0·10–0·23	0·00	+ve	Highly polymerized proanthocyanidins	3·46
3	0·00–0·10	0·05–0·38	+ve		27·98
4	0·11–0·22	0·07–0·58	+ve		17·68
5	0·25–0·35	0·20–0·62	+ve	Low polymer proanthocyanidin	8·13
6	0·50	0·56	+ve	Monomer (+)leucocyanidin	12·06
7	0·45	0·68	+ve	Isomer of 6	1·26
8	0·59	0·34	−ve	(−)Epicatechin	2·57
9	0·72	0·34	−ve	+Catechin	10·27
10, 11, 12	—	—	—	Minor spots	2·00

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

of identical spots in different fractions were pooled together and expressed as percentage of the total (Table 1).

Proanthocyanidin Nature of Complex Polyphenols

When anthocyanidin mixture obtained from the aqueous residual fraction on treatment with acid was spotted in high concentration in the form of long bands, generally two faint bands were seen apart from cyanidin, which has been the only anthocyanidin noticed by earlier workers.[9, 10] Both the faint bands have higher R_f in Forestal and formic acid solvents.

The anthocyanidin with the highest R_f was identified as the *n*-butyl derivative of cyanidin formed during the conversion.[11] The middle band, which was obtained from only the proanthocyanidin complexes, was identified as pelargonidin, after purification, chemical study[12] (Table 2) and co-chromatography.[13]

TABLE 2. COMPARISON OF THE CHARACTERISTICS OF PELARGONIDIN OBTAINED FROM ARECANUT WITH LITERATURE VALUES

Characteristics	Obtained	Literature[9, 12]
R_f in Forestal solvent	0·70	0·71
R_f in formic acid solvent	0·36	0·33
R_f in BuOH–HCl	0·92*	0·80
E_{max}. mμ	530	530
Shift with $AlCl_3$[17]	nil	nil
Phenolic acid on alkali fusion[18]	*p*-hydroxy benzoic acid	*p*-hydroxy benzoic acid

* Cyanidin gave a value of 0·85 instead of the reported value of 0·69.[9]

The ratio of pelargonidin to cyanidin in the anthocyanidin mixture obtained from arecanut is about 1:20. Pelargonidin was obtained from both the mobile complex extracted by ethyl acetate containing alcohol and the higher polymers remaining in the aqueous phase, demonstrating the presence of leucopelargonidin in the different complex polyphenols along with leucocyanidin. No monomer leucopelargonidin has been noticed.

Catechin in Complex Polyphenols

The two fractions containing complexes were then hydrolysed by dilute hydrochloric acid, after purification by repeated washing with dry ethyl acetate. Both the fractions gave (+)catechin as identified by extraction with ethyl acetate from the reaction mixture, separation by paper chromatography and reaction with specific spray reagents. Hydrolysis with sulphurous acid also confirmed the release of (+)catechin from the complex polyphenols.

When an extract of areca polyphenols, from which monomeric components have been removed by repeated extraction with ethyl acetate, is allowed to stand for a few days, solids

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[9] E. C. BATE-SMITH, *Biochem. J.* **58**, 122 (1954).

[10] L. V. L. SASTRY, M. N. SATYANARAYANA, M. SRIVINASAN, N. SUBRAMANYAN and V. SUBRAHMANYAN, *J. Sci. Ind. Res.* **15C**, 78 (1956).

[11] A. G. MATHEW, *Phytochem.* **8**, 677 (1969).

[12] D. G. ROUX, *Nature* **179**, 305 (1957).

[13] S. LAKSHMINARAYANA and A. G. MATHEW, *J. Food Sci.* **38**, 451 (1967).

separate out. These solids on mild acid hydrolysis gave (+)catechin and, on longer heating, cyanidin. The fractions remaining in solution after the precipitation showed on paper chromatography a pattern similar to the original starting material.

DISCUSSION

From the chromatographic pattern of the fractions (Fig. 1) and the analysis (Table 1) a total chromatogram of the polyphenols can be reconstructed. Examination of the present improved analysis shows that the highly complex polyphenols represent a lower proportion than shown in the earlier study[1] done without fractionation. Similarly (+)catechin (Spot 9) and monomer (+)leucocyanidin (Spot 6), which represent the major monomeric components, are found to be present in amounts over 10 per cent of the total polyphenols. The improvements in this two-stage estimation have been (1) avoiding of overcrowding in paper chromatographic separation, by using simpler fractions, and (2) grouping of components of more or less equal adsorption in each chromatographic estimation. The other spots that are clearly separated are (—)epicatechin (Spot 8), the isomer of monomer (+)leucocyanidin (Spot 7) and some unidentified minor components.

The fairly discrete band (Spot 5) has been identified as a low polymeric flavan from the following observations: solubility in moist ethyl acetate and ethyl acetate containing alcohol; partial elution from cellulose column with BAW along with monomeric leucocyanidin and fairly good mobility on paper and in countercurrent distribution between ethyl acetate and water. From the study of anthocyanidin mixture and hydrolysis products, this oligomeric fraction can be defined as a complex formed of predominently leucocyanidin, some amount of catechin and traces of leucopelargonidin. Similar types of polymers have been detected in apple,[14] while several cases of dimers of catechin and leucocyanidin[4–6] and of two flavan 3:4 diols[7] have also been reported.

It can be seen that because of the extraction of Spot 5 into ethyl acetate, containing alcohol, the aqueous residual fractions are resolved into separate areas. The areas represented by Spots 4, 3 and 2 are high polymers, as progressively lower mobility would normally represent higher stages of polymerization. The components which have become very highly polymerized remain at the start. Collectively, these higher polymeric polyphenols yield (+)catechin on mild acid hydrolysis and cyanidin and traces of pelargonidin on treatment with hot *n*-butanol–HCl.

The relative ratio of cyanidin to pelargonidin in the complexes was found to be 20:1. Thus, if at least 1 molecule of leucopelargonidin is present in the simplest of all polymers, there must be at least 20 molecules of leucocyanidin, on the assumption that the yield of the two anthocyanidins are roughly equal from the corresponding 3:4-diols. Moreover there are some molecules of catechin also in the polymer. Thus, according to these observations, even the simplest of polymer has to be made up of over 20 flavan units, a possibility which has to be considered very unlikely in view of the mobility on paper and solubility in water. On the other hand the alternate possibility of each area representing a mixture of different complexes of similar mobility and extractability cannot be ruled out.

The formation of higher insoluble polymers involving catechin and leucoanthocyanidin has been demonstrated by the analysis of insoluble precipitate formed from lower polymers in areca polyphenolic extract. It would therefore appear that in the plant a similar insolubilization is also likely resulting in polymers of increasing complexity without the involvement of

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[14] S. ITO and M. A. JOSLYN, *J. Food Sci.* **30**, 44 (1965).

enzymes. Recovery of cyanidin from the unextractable portions of the nut lends support to this assumption. Study of the changes with maturity also points out that there are progressive polymerization and insolubilization as the nut matures.[15]

EXPERIMENTAL

Material

Freeze-dried polyphenolic extract of the locally available ripe arecanuts was used in different studies.[1]

Solvent Fractionation of Areca Polyphenols

250 mg of the above freeze-dried material was dissolved in 50 ml of water. The aqueous solution was extracted three times in 20-ml portions of ethyl acetate in a separating funnel. The remaining aqueous phase was further extracted with three 20-ml portions of ethyl acetate containing ethyl alcohol (8 ml of ethyl aclohol per 100 ml of ethyl acetate). The two solvent fractions were evaporated off and taken in water for freeze-drying together with aqueous residual fraction.

Chromatographic Procedure

Two-dimensional chromatography was run on No. 3 Whatman paper using *n*-butanol–acetic acid–water, 4:1:2·2 v/v (BAW), in the first direction and 2% aqueous acetic acid in the second direction. The chromatograms were sprayed with a mixture of 0·3% aqueous solution $K_3Fe(FCN)_6$ and $FeCl_3$ and with other specific spray reagents.[16] Quantitative paper chromatographic procedure has been as described earlier.[15]

The anthocyanidins obtained by treatment of proanthocyanidins with acid were tested as per another communication from this laboratory.[13]

Hydrolysis of Proanthocyanidins

0·05 g of each fraction was dissolved in 5 ml of 0·1 N hydrochloric acid and boiled for 3–5 min. For hydrolysis with sulphurous acid, 0·05 g of the material was dissolved in 5 ml of water and sulphur dioxide was bubbled at 100° for 45 min.[6]

Acknowledgement—The authors wish to thank Shri C. P. Natarajan, Chairman, Spices & Flavour Technology Department of this Institute, for his interest in this work.

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[17] J. B. Harborne, *Biochem. J.* **70**, 22 (1958).

[18] C. L. Hsia, B. S. Luh and C. O. Chichester, *J. Food Sci.* **30**, 5 (1965).