Carbohydrate Research, 147 (1986) 315–324
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

FLAVOLOGLYCAN: A NOVEL GLYCOCONJUGATE FROM LEAVES OF MANGROVE (*Rhizophora stylosa* Griff)

MARTIN J. NEILSON, TERENCE J. PAINTER*, AND GEOFFREY N. RICHARDS†

Department of Chemistry and Biochemistry, James Cook University of North Queensland, Townsville, Queensland 4811 (Australia)

(Received April 29th, 1985; accepted for publication, August 12th, 1985)

ABSTRACT

A novel type of natural, water-soluble copolymer has been obtained from mangrove leaves in ~20% yield. Evidence is presented that the polymers contain covalently bonded flavolan and high-molecular-weight glycan components, probably in a range of proportions, and are therefore designated flavologlycans. Fractionation of the polymers has been conducted by complexing with lead acetate and by sorption on nylon (polyamide) and on Sepharose.

INTRODUCTION

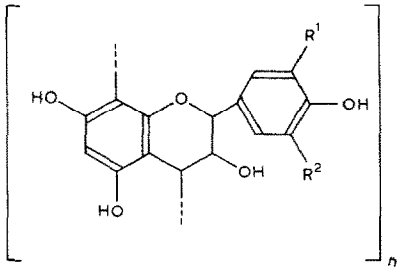
The class of natural polymers variously referred to as *condensed tannins*[1], *flavotannins*[1], *proanthocyanidins*[2,3], or *flavolans*[4,5] have the general formula **1**, with n varying[2,3] from 2 to ~20. Upon heating with alcoholic hydrochloric acid in the presence of oxygen, the inter-unit carbon–carbon bonds are cleaved, with formation of anthocyanidin pigments (**2**) from all but one (terminal) flavanol unit in each chain[2,3]. The polymers are widely distributed, but occur most abundantly in the leaves, fruit, heartwood, and bark of woody plants, from which they are normally extracted, in yields of up to ~3%, by aqueous methanol, ethanol, or acetone[6]. It may be noted that these conditions of extraction are such that, if further quantities of flavolan were covalently linked to polysaccharidic materials, they would remain in the insoluble residue, and we have investigated this possibility with mangrove leaves as part of a wider investigation of the diagenesis of such leaves.

We have found that, after preliminary extraction of pigments and flavonoid materials with hexane and hot acetone, extraction of the fresh mangrove leaves with water or with aqueous (ethylenedinitrilo)tetraacetic acid (EDTA) yields

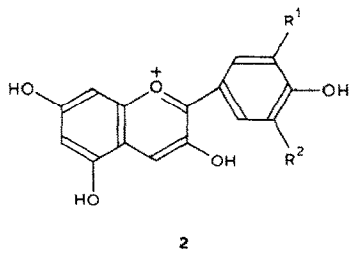
*On leave of absence from the Institute of Marine Biochemistry, University of Trondheim, N-7034 Trondheim-NTH, Norway.

†To whom communications should be addressed. Present address: Wood Chemistry Laboratory, University of Montana, Missoula, Montana 59812, U.S.A.

0008-6215/86/$ 03.50



1



2

~20% of polymeric material containing approximately equal proportions of flavolan and galacturonoglycan. We now report preliminary findings concerning the fractionation of this material and the compositions and properties of the subfractions. We consider that the results detailed herein provide reasonable evidence that the major portion of this extract consists of covalently bonded copolymers of the two species (*viz.*, flavolan and glycan).

The only previous report of a flavonoid–polysaccharide compound appears to be that by Markham[7], who isolated an ~2% yield of what appears to be a related material by water extraction of liverwort. In this case, the molecular weight was ~3200 per flavonoid unit, and the glycoses were shown qualitatively to be the same as those mentioned in Table I, with the exception of arabinose, which was absent from the liverwort product. Markham showed[7] that, in his product, the polysaccharide was bonded glycosidically, *via* galacturonic acid, to O-7 and -4′ of 8-methoxyluteolin (5,7,3′,4′-tetrahydroxy-8-methoxyflavone). Our polymer appears to be present in the *R. stylosa* leaves in much greater proportion, and the gel chromatography and general viscosity behavior suggest that the glycan component is of much higher molecular weight than that in the liverwort product. It is possible that the flavologlycans are of wide occurrence in plants, often in high yield.

TABLE I

COMPOSITION (% w/w) OF FLAVOLOGLYCAN FRACTIONS

Fraction	*Rha*	*Ara*	*Xyl*	*Man*	*Gal*	*Glc*	*Uronic acid*[a]	*Total carbohydrate*	*Flavolan*	
									Total[b]	*Insol.*[c]
FG-1	14.4	5.6	tr.	5.5	12.7	4.4	10.4	53.0	45.0	5.0
FG-1c[d]	24.2	10.5	tr.	9.6	23.1	8.2	23.0	98.6	—	—
FG-2A	6.3	5.3	0.6	2.3	6.8	3.3	7.5	32.1	69.0	29.9
FG-2B	4 2	4.2	2.0	2.1	4.2	2 1	1.6	20.4	n.d.[e]	n.d.
FG-2ANF	3.5	6.3	tr.	1.1	4.4	2.2	11.7	29.2	66.0	45.5

[a]Calculated as galacturonic acid. [b]Calculated as loss in weight upon chlorite bleaching. [c]Polymeric fractions precipitated after acid hydrolysis. [d]Galacturonoglycan moiety isolated from FG-1 by chlorite bleaching. [e]N.d., not determined.

Conclusive evidence for covalent, interpolymer linkages in such instances will always be difficult to educe, and contentious, and this situation is reminiscent of the controversy that for many years existed concerning covalent lignin–carbohydrate linkages. The evidence for the latter types of linkages has recently been reviewed[8], and it is apparent that only after several hundred publications on the subject is the accumulated evidence beginning to provide conclusive proof of the presence and nature of the interpolymer linkages.

Our evidences for flavolan–glycan linkages may be summarized as follows. (1) Flavonoid and carbohydrate constituents are both retained during dialysis. (2) Flavonoid and carbohydrate constituents are both sorbed by nylon, the major component is completely resistant to prolonged elution with water, and both constituents are eluted with formamide. (3) Flavonoid and carbohydrate constituents are both sorbed by Sepharose. The major component is completely resistant to elution with water, but both constituents are eluted with increasing concentrations of urea in water. (4) Treatment of the flavologlycan with chlorite, and subsequent dialysis, removes the aromatic components, and leaves a glycan in the anticipated yield. (5) Treatment with acid removes the glycan, and leaves flavolans (now partly soluble in ethyl acetate) in the anticipated yield. (6) Flavolan (nondialyzable) polymer would not be expected to be readily soluble in water, unlike the flavologlycan isolated, whereas the flavologlycan isolated was totally insoluble in all of the solvents normally used to extract flavolans, such as aqueous methanol or aqueous acetone[6].

RESULTS AND DISCUSSION

When boiled and macerated with acetone, the fresh mangrove leaves yielded 16% of soluble material. Most of this material was highly colored and soluble in hexane (pigments); the rest (~3%) consisted of salts and flavonoid compounds, and this fraction was not further investigated. The acetone-extracted leaf-meal was then extracted with aqueous EDTA (water extraction gave yields that were almost as high) and the dialyzed extract yielded 19% of crude flavologlycan (termed FG-1). On hydrolysis, this material gave a total yield of 42.6% of neutral glycoses, with rhamnose and galactose preponderating, plus 10.4% of galacturonic acid. Treatment of FG-1 with chlorite, and subsequent dialysis, gave a good recovery of total carbohydrates, with a total weight-loss of 45%, and this value is therefore assumed to be the approximate content of flavolan. Acid hydrolysis of FG-1 yielded, from the flavolan moiety, products that were partly insoluble in aqueous acid (polymeric flavolans, 5% of FG-1), and the balance, mostly extractable from the hydrolyzate with ethyl acetate, consisted of monomeric 3-flavanols.

The crude FG-1 was fractionated by precipitation with lead acetate, and subsequently by sorption on nylon and on Sepharose CL-4B. All three methods of fractionation rely primarily on interaction with the polyphenol portion of the flavologlycan molecule, and, in the latter cases, the simultaneous sorption and

resistance to desorption of the glycan in such chemically distinct systems (see later) is, perhaps, our strongest evidence for covalent linkage of flavolan and glycan. Admittedly, the flavolans may sometimes form non-covalent complexes with glycans, but the repeated coincidences of sorption and desorption of the two components in three different chemical systems are most simply explained by covalent flavolan–glycan linkage.

The addition of lead acetate to an aqueous solution of FG-1 yielded an insoluble complex (FG-2A), and the supernatant solution yielded a similar amount of complex precipitated by ethanol (FG-2B). Both complexes redissolved in aqueous EDTA, and were recovered after dialysis in 48 and 43% yield, respectively. Analyses of these fractions are shown in Table I, and suggest that a small proportion of carbohydrate or carbohydrate-rich component in FG-1 was not complexed by the lead acetate. The water-insoluble, lead acetate complex (FG-2A) had a higher carbohydrate (especially galacturonic acid) content than the water-soluble complex (FG-2B), and later gel chromatography showed that the former fraction contained some "pectic substances" (see later).

When an aqueous solution of the material (FG-2A) precipitated by lead acetate was passed thrugh a nylon column, 28% of the material was eluted with water. The non-sorbed material gave flavonoid and carbohydrate reactions, and was not further investigated. This fraction presumably contains the "pectic substances" which were coprecipitated with flavologlycan by lead acetate (see later) plus some non-sorbed flavonoids which may or may not be linked to carbohydrate. The sorbed material remaining was completely resistant to elution with water, but was removed by elution with formamide, and was recovered in 72% yield by dialysis and freeze-drying (fraction FG-2ANF); analyses thereof are shown in Table I. About one-third of the fraction consisted of complex galacturonoglycans, and two-thirds was flavolan; the fraction was similar in composition to the compounds precipitated by lead acetate, although containing a higher proportion of galacturonic acid.

Attempted gel chromatography of FG-2A in water on Sepharose CL-4B gave the results shown in Fig. 1. The broadness of the peaks is probably associated with the unusual viscosity behavior of the flavologlycan solutions (see later). Peak I, whose elution commences at V_0, is high-molecular-weight glycan containing little flavolan, and it comprises ~21% of the weight of FG-2A (67% of the total carbohydrates is in this fraction). This preponderantly glycan fraction evidently contains water-soluble glycan, possibly co-extracted by water along with the flavologlycan and subsequently co-precipitated with lead acetate. It presumably includes "pectic substances", as 30% of this material is galacturonic acid. The possibility remains, however, that at least some of peak I was originally bonded to flavolan, extracted into water in this form, and subsequently cleaved from the flavolan by oxidative-reductive depolymerization[9,10] reactions during processing (see later).

About two-thirds of the FG-2A fraction was sorbed to the Sepharose CL-4B

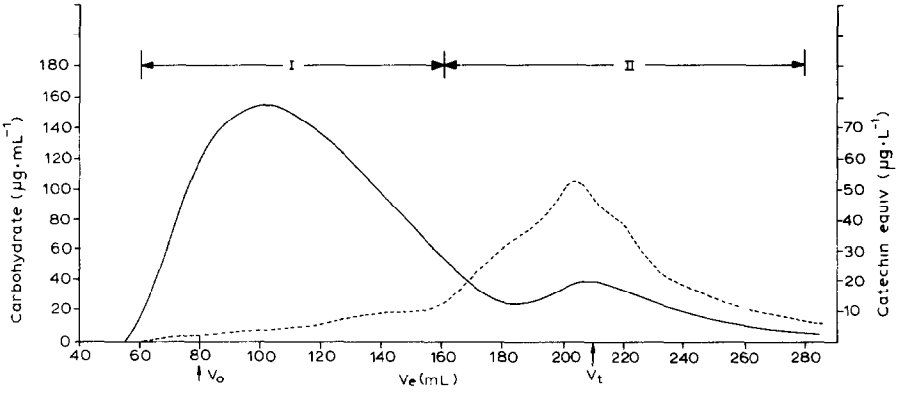


Fig. 1. Gel filtration of FG-2A on Sepharose CL-4B eluted with distilled water. Key: ———, total carbohydrate, by phenol–sulfuric acid method; ....., catechin equivalent, by A_{280}.

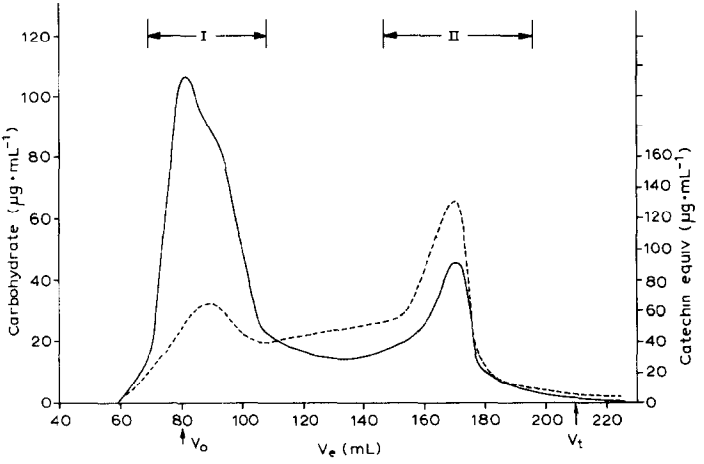


Fig. 2. Gel filtration of FG-2ANF on Sepharose CL-4B eluted with distilled water. Key: ———, total carbohydrate, by phenol–sulfuric acid method; ....., catechin equivalent, by A_{280}.

column and could not be eluted with water. In a follow-up, "short column" experiment, the sorbed fraction was partially eluted with M and 8.0M urea to yield, after dialysis, respectively 7 and 8% of the carbohydrate and 19 and 27% of the flavonoid material from FG-2A. Of the original FG-2A, 20% of the carbohydrate content and 18% of the flavonoid remained bound to the Sepharose. Thus, a large proportion of the carbohydrate material is coincidentally desorbed with the flavonoid material, and this is a major basis for our conclusion that these two components are covalently bonded. The elution of the "pectic substances" at or near to V_0 in Fig. 1 makes it unlikely that the sorbed carbohydrate material was held by non-covalent interaction with sorbed flavonoid material.

Further fractionation on Sepharose CL-4B (see Fig. 2) of the material (FG-2ANF) eluted from nylon with formamide gave two peaks (I and II) which were eluted with water, and contained both carbohydrate and flavolan. Peak I was high in carbohydrate, especially galacturonic acid, arabinose, galactose, and glucose, and low in flavolan. In contrast, peak II was low in carbohydrate and high in flavolan, and hence it interacted to a greater extent with the gel. It seems probable that both peaks may contain a range, or continuum, of molecular types. In this experiment, ~30% of FG-2ANF was sorbed to the column and not removed by elution with water. The sorbed material was mostly removed by elution with 8.0M urea, and it contained both carbohydrate and flavonoid material.

Chlorite treatment, and subsequent dialysis, of FG-2ANF yielded a 60% recovery of the original carbohydrate content (by phenol–sulfuric acid analysis), as non-dialyzable polysaccharide, including 81% of the original galacturonic acid. This result shows unequivocally that most of the carbohydrate that was firmly sorbed to nylon was polymeric in nature. The sorption was obviously *via* the flavonoid, polyphenol compound, and the complete resistance of the polysaccharide to desorption by water is strong evidence of covalent linkage between flavolan and glycan. The molecular weight of the glycan component is evidently very high (or it forms molecular aggregates), because the major portion of the flavologlycan is eluted at the void volume of Sepharose CL-4B (see Fig. 2), and any influence of the aromatic moiety on the Sepharose partition coefficient is likely to be in the direction of increasing the retention volume by interaction with the gel.

The flavolan components of all fractions were converted into anthocyanidins by oxidation in acid, yielding mainly pelargonidin (**2**, $R_1 = R_2 = H$), cyanidin (**2**, $R_1 = OH$, $R_2 = H$), and delphinidin (**2**, $R_1 = R_2 = OH$), together with another, unidentified product. The molecular size of the flavolan component (*i.e.*, the value of n) in **1** is not yet known, but, by analogy with flavolans from other biological sources[4–6], n probably represents a range of small numbers, and, possibly, a significant proportion of the flavologlycan has $n = 1$.

Although every effort was made to minimize exposure to light and oxygen, including the construction of a special apparatus for dialysis under nitrogen, the flavologlycans were undoubtedly modified somewhat during isolation. The aqueous extracts of the leaf meal were initially colorless, and although very viscous, they did not exhibit marked non-Newtonian ("long flow") characteristics. During the subsequent manipulations, however, the solutions became increasingly yellow and non-Newtonian, to the point of developing a partial gel-like character. This was probably due to cross-linking by free-radical coupling-reactions. Auto-oxidation of flavologlycan solutions would also be expected to result in oxidative-reductive depolymerization (the "ORD" reaction[9,10]) of the galacturonoglycan chains, which may explain, in part, the considerable shifts in composition during fractionation (see Table I). The extraordinary rheological properties of the solutions, and their tendency to undergo irreversible changes on standing, frustrated initial attempts to characterize them by gel-permeation chromatography on Sephadex, Sepharose,

Sephacryl, or Biogel columns, but columns of nylon fabric combined a suitably high flow-rate with separation based upon the highly selective, "tanning" reaction between polyphenols and polyamides.

In conclusion, the flavologlycan of mangrove leaves appears to consist of a family of molecules, in which flavolan chains of various degrees of polymerization are covalently linked to a galacturonoglycan backbone of high molecular weight. Linkages between the two moieties may include the uronic ester type, but the low uronic acid content of one fraction (FG-2B) suggests that other possibilities should also be considered.

EXPERIMENTAL

Sample collection. — Leaves from mangrove (*Rhizophora stylosa* Griff) trees were collected at Hinchinbrook Island (18° 20′ S and 146° 10′ E), and placed in plastic bags containing several pellets of carbon dioxide. These bags were then immediately placed in a black, plastic bag and stored in ice for shipment to the laboratory. Leaves not immediately worked up in experiments were kept at −20° for later use.

Preparation of crude glycoconjugate. — All laboratory operations were carried out with minimal exposure to light and oxygen. Fresh or deep-frozen *R. stylosa* leaves with stalks removed (100 g; 32 g dry-weight) were manually broken into small fragments, dropped into warm acetone (1 L), and the mixture boiled for 10 min, and filtered while hot. The residue was reboiled with acetone (1 L) for 10 min, and the mixture was then cooled, homogenized, and filtered. The resultant leaf meal was washed on the filter with cold acetone, sucked dry, and dried *in vacuo* over silica gel at 25°. The cream-colored meal (yield, 27 g) was stirred under nitrogen with 0.1M aqueous EDTA, pH 6 (1 L) for 24 h at 25° in the dark. The slurry was then cooled, and centrifuged at 4° and 10,000*g* for 30 min. The pellet material was extracted once more with EDTA (500 mL), and the combined centrifugates were exhaustively dialyzed against distilled water under nitrogen, and then freeze-dried, to yield 6 g of orange-tan solid (FG-1). (Note: All dialysis tubing was thoroughly pre-washed in distilled water throughout these experiments.)

Analysis for flavolan. — An estimate of total aromatic content was obtained by warming portions (200–500 mg) of the flavologlycan fractions in water (100 mL) with sodium chlorite (1 g) and glacial acetic acid (1 mL) for 2 h at 80°. The solutions were then cooled, treated with sodium sulfite, dialyzed exhaustively against distilled water, concentrated, and freeze-dried. A minor amount of carbohydrate was lost through the dialysis membrane, and the carbohydrate moiety was recovered intact as a white galacturonoglycan; the aromatic content was accordingly calculated as the loss in weight occurring upon chlorite treatment (see Table I). For FG-2ANF, the retentate yielded 34% of the original material.

Whereas the flavologlycans were totally insoluble in all of the solvents normally used to extract flavolans[6], the flavolan moiety was rapidly released on

hydrolysis with 0.2M sulfuric acid for 4 h at 95°; it then showed typical solubility properties. Part of it was insoluble in dilute acid, and was assayed by filtering it off from the hydrolyzate, washing with cold water, drying, and weighing (see Table I). This part appeared to be a polymeric flavolan, as it gave a high yield of anthocyanidins on heating with butanolic hydrogen chloride (see later). The rest was extracted from the acid hydrolyzate with ethyl acetate; from the low yield of anthocyanidins and t.l.c. evidence[3], it appeared to consist mainly of monomeric 3-flavanols (**1**, $n = 1$).

Fractionation with lead acetate. — To a solution of FG-1 (6 g) in water (1 L) was slowly added, with vigorous stirring, lead acetate trihydrate (20 g) in water (50 mL). The flocculent precipitate (complex A) was collected by centrifuging; addition of an equal volume of ethanol to the centrifugate then precipitated another fraction (complex B). After washing with water, or 1:1 (v/v) water–ethanol, respectively, the complexes were separately dispersed in 0.1M EDTA (300 mL), and dialyzed exhaustively against 0.1M EDTA under nitrogen until they had dissolved completely. The solutions were then separately dialyzed against distilled water, and freeze-dried, to yield FG-2A (2.9 g) and FG-2B (2.6 g) as hygroscopic, orange-tan solids.

Analysis for carbohydrate. — *(a) Colorimetric.* Total carbohydrate contents were estimated by the phenol–sulfuric acid method[11]. To correct for background interference produced by the flavonoid components, the height of the inflection at 485 nm in the continuous spectrum of the assay solutions was used, and was related to the total absorbance of a D-glucose standard at the same wavelength. To "spot check" for flavonoids, corresponding blanks were set up wherein the phenol reagent was replaced by water, giving a characteristic, purple color when flavonoids were present.

Uronic acids were determined by the 3-phenylphenol method[12]; to correct for absorbance due to anthocyanidins, samples were also heated with the sulfuric acid–borax reagent. Galacturonic acid was the only hexuronic acid detected in acid hydrolyzates by t.l.c. on cellulose plates with 5:4:1:3 pyridine–ethyl acetate–acetic acid–water, and it was accordingly used as the standard.

(b) G.l.c. Neutral sugars in hydrolyzates were determined, as absolute content of prehydrolysis material, by g.l.c. of alditol acetates, by a procedure adapted for lignin–carbohydrate complexes[13], but using inositol as the internal standard.

Chromatography on nylon. — A glass column (40 × 3.3 cm) was tightly packed, under degassed water, with fine-filament, knitted nylon fabric (total dry wt., ~80 g), and successively washed with methanol, formamide, 0.01M sodium hydroxide, and water. Flavologlycan (FG-2A; 500 mg) in water (50 mL) was then applied to the column. Elution with water (1 L) displaced a component (140 mg) closely similar in composition and properties to fraction FG-2B.

After washing the column with more water (3 L), the main component, FG-2ANF (360 mg), was eluted with formamide, and recovered by exhaustive

dialysis against water, and freeze-drying. No part of a sample of FG-2A from which the flavolan moiety had been removed by chlorite bleaching was retained by the nylon column.

Chromatography on Sepharose CL-4B. — Glass columns (50 × 2.5 cm, and 4 × 3 cm) were packed with Sepharose CL-4B (Pharmacia Fine Chemicals, Sweden), and equilibrated with distilled water. For "long column" experiments, the ascending flow-rate was 30 mL · h^{-1}; void volume (V_0) and V_t were measured with Blue Dextran (Pharmacia) and D-glucose, respectively. Fractions (3 mL) were analyzed for total carbohydrate by the phenol–sulfuric acid method, and for u.v. absorbance (A) at 280 nm. Flavolan content was estimated by A_{280} related to the same wavelength absorption produced by a catechin standard.

In one experiment, FG-2A (50 mg) in water (10 mL) was loaded onto the column. Despite the viscous nature of the solution, and consequent slight compression of the gel bed, satisfactory resolution occurred with a noticeable broadening of the peaks (see Fig. 1). In a similar experiment, FG-2ANF (20 mg) in water (5 mL) was resolved into two relatively narrow peaks (see Fig. 2). This solution had a much lower viscosity. Neutral-sugar, g.l.c. analysis of peak I showed that rhamnose, arabinose, xylose, glucose, and galactose were present in the weight ratios of 5:46:4:18:27. Galacturonic acid contributed 16% of the material. Peak II contained arabinose and glucose in the ratio of 29:71, traces of rhamnose and galactose, and no galacturonic acid.

In a "short column" experiment, FG-2A (50 mg) in water (10 mL) was placed on the column, and sequentially eluted with water, and 1.0M and 8.0M urea (100 mL of each). The urea solutions were exhaustively dialyzed, and assayed for carbohydrate and A_{280}.

Characterization of polymeric flavolan. — Depolymerization was effected by heating portions (50 mg) of F_1 in a mixture of concentrated hydrochloric acid (10 mL) and 1-butanol (50 mL) in a stream of oxygen for 2 h at 90°. The solutions were evaporated to dryness in a rotary evaporator, and hydrogen chloride was removed by repeated distillation of added ethanol. Two-dimensional t.l.c. was conducted on cellulose plates (Merck DC-Plastikfolien), developed first in 4:1:5 (v/v) 1-butanol–acetic acid–water (organic layer) and then in 30:3:10 (v/v) acetic acid–concentrated hydrochloric acid–water[5]. Pelargonidin, cyanidin, and delphinidin were identified by their R_F and λ_{max} values in methanolic hydrogen chloride, and by the bathochromic shifts on addition of ethanolic aluminum trichloride[5]. In addition, a fourth component, having λ_{max} value similar to that of pelargonidin (520 nm), but with higher R_F values in both solvent-systems, was prominent.

ACKNOWLEDGMENTS

Samples of pelargonidin and delphinidin chlorides were kindly provided by Dr. C. W. Ford. Scientists at the Australian Institute of Marine Science are warmly

thanked for help in identifying mangrove species and in harvesting the leaves, and for their interest. One of us (T.J.P.) is much indebted to Norges Teknisk-og Naturviten-skapelige Forskningsråd for a stipend and travel grant which made his contribution possible. The project was financially supported by grants from the Australian Marine Science and Technology Grants Scheme.

REFERENCES

1 B. C. PLATT, in *Thorpe's Dictionary of Applied Chemistry*, 4th edn., Vol. XI, Longmans, London, 1954, pp. 390–405.
2 E. HASLAM, in J. B. HARBORNE, T. J. MABRY, AND H. MABRY (Eds.), *The Flavonoids*, Chapman and Hall, London, 1975, pp. 505–559.
3 E. HASLAM, in J. B. HARBORNE AND T. J. MABRY (Eds.), *The Flavonoids: Advances in Research*, Chapman and Hall, London, 1982, pp. 417–447.
4 P. RIBEREAU-GAYON, *Plant Polyphenols*, Oliver and Boyd, Edinburg, 1972, pp. 176–197.
5 J. B. HARBORNE, *Phytochemical Methods*, Chapman and Hall, London, 1973, p. 62.
6 L. YEAP FOO AND L. J. PORTER, *Phytochemistry*, 19 (1980) 1747–1754.
7 K. R. MARKHAM, *Phytochemistry*, 11 (1972) 2047–2053.
8 G. N. RICHARDS, in C. MERCIER AND M. RINAUDO (Eds.), *International Workshop on Plant Polysaccharides, Structure and Function*, Nantes, July 9–11, 1984.
9 W. PIGMAN, S. RIZVI, AND H. L. HOLLEY, *Arthritis Rheum.*, 4 (1961) 240–252.
10 O. SMIDSRØD, A. HAUG, AND B. LARSEN, *Acta Chem. Scand.*, 17 (1963) 2628–2637.
11 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350–356.
12 N. BLUMENKRANTZ AND G. ASBOE-HANSEN, *Anal. Biochem.*, 54 (1973) 484–489.
13 M. J. NEILSON AND G. N. RICHARDS, *Carbohydr. Res.*, 104 (1982) 121–138.