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POLYAMIDE COLUMN CHROMATOGRAPHY FOR RESOLUTION OF COMPLEX MIXTURES OF ANTHOCYANINS

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SUMMARY

A method for the separation of anthocyanins on polyamide columns is described. This procedure makes possible the detection and enrichment of minor components and the separation of derivatives which are difficult to separate by paper or thin-layer chromatography. Separation of acylated anthocyanins was effected with minimal degradation. Microcolumns gave excellent resolution of extracts from as little as 15 mg tissue.

INTRODUCTION

Since 1955 it has been well established that polyamide of the polycaprolactam type is suitable for chromatography of phenolic compounds because of its ability to adsorb polar substances through hydrogen bonding[1–4]. Complex mixtures of glycosides and aglycones of isoflavones, flavones, flavonols, dihydroflavonols, and flavanones have been successfully separated with a water–methanol system[5]. From a crude leaf extract of *Cucurbita*, Strack and Reznik[6] purified 10 different flavonolglycosides using water–methanol elution and re-chromatography with a mixture of methanol and chloroform.

Until now, little is known about the application of polyamide column chromatography to anthocyanins. Previous investigations have indicated that polyamide was useful only for preliminary purification of crude anthocyanin extracts[7–9] and offered no advantage over proven classical anthocyanin chromatography methods[10]. Fulek and Francis[11] attempted to develop a quantitative purification method on polyamide columns, but they observed extensive diffusion on the column and some pigment was lost through degradation.

Insoluble polyvinylpyrrolidone (PVP), which has been used mostly for binding phenolic compounds during purification of protein extracts[12], is the first adsorbent which showed both purification and resolution of anthocyanin extracts on columns[13]. Previously PVP was used for preliminary purifications of extracts of anthocyanin

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(*e.g.*, separation from impurities such as sugars, cinnamic acid derivatives or other flavonoid compounds). Other workers have also reported PVP to be preferable for anthocyanin purification (*c.f.*, Fuleki and Francis[11]). Separation of five different anthocyanin-3,5-diglucosides on a PVP column has been reported by Hrazdina[14]. Van Teeling *et al.*[13] partially purified and resolved simple mixtures of anthocyanin derivatives from black raspberry (*Ribes occidentalis*), poinsettia (*Euphorbia pulcherrima*), and cranberry (*Vaccinium macrocarpon*).

Our purpose in this work was to develop a method for anthocyanin chromatography which fulfils the following requests: (1) separation from impurities; (2) resolution of complex natural mixtures; (3) enrichment of minor compounds; (4) purification of fractions containing acylated anthocyanins. Therefore we tested red radish (*Raphanus sativus*) and red cabbage (*Brassica oleracea*), known for containing extremely complex mixtures of different anthocyanins[15–17].

We also tested this procedure on the red genotype (11HHP^{r}P^r) of *Impatiens balsamina*. All the major anthocyanins in the petals have been identified and two acylated derivatives occur[18]. There are also several minor compounds whose identity is putative but through the enrichment procedure described herein, we were able to isolate enough of these compounds to confirm their identity.

EXPERIMENTAL

Pigment extraction

With the exception of *Impatiens* (genotype 11HHP^{r}P^r), which was grown from seed stocks derived from Hagen[18,19], all other plant material was purchased from local merchants. Tissue was extracted overnight at room temperature with 95% ethanol containing 1% (v/v) HCl. The extracts were concentrated in a rotary evaporator at 40° to near dryness and redissolved in a small volume of 0.01 *N* HCl.

Polyamide column chromatography

Polyamide. Polyamide-CC 6 for column chromatography (grain size 0.07 mm) was purchased from Macherey, Nagel & Co., Düren, G.F.R.

Columns. Dimensions of 2 × 25 cm and 2 × 50 cm were used with polyamide bed volumes of 100 and 200 ml, respectively, containing 25 and 50 g polyamide. Pasteur pipets were used for the microcolumns.

Elution. Water and various mixtures of water–methanol with descending polarity were used, each mixture containing 0.01 *N* HCl. Flow-rates were in the range 1–2 ml/min.

Control of elution. The absorption of the anthocyanins was monitored at 505 or 520 nm in a Beckman spectrophotometer DB-G equipped with a flow cell. The column was monitored continuously and the eluant was collected in 10-ml fractions.

Regeneration of the polyamide. 5 ml of 4 *N* NaOH were washed through the polyamide bed and then the column was washed with 0.01 *N* HCl until the eluent was acidic. In some columns a yellow-brown band near the top remained after recharging. This section was discarded before another pigment extract was applied.

Identification of pigments

Tentative characterization of the isolated pigments was based on comparison

of paper chromatography R_F values in at least four different solvent systems[10,20], absorption maxima, calculations of the extinction ratios from spectroscopic data[21], and color and fluorescence under ultraviolet light (UV). Identification of the acyl substituents of the acylated anthocyanins was done after mild basic hydrolysis (0.01 *N* NaOH, 5 min at 50°). The hydrolysate was re-acidified with HCl and shaken against several aliquots of diethyl ether. The ether fraction was dried, redissolved in methanol and spotted on Sigmacell type 20 thin-layer plates. The plates were developed in toluene–acetic acid (2:1, saturated with water) and 2% formic acid. After drying the spots were located under UV light. Color changes in the presence of ammonia vapor and after spraying with diazotized *p*-nitroaniline oversprayed with 2 *N* NaOH were recorded and compared with known cinnamic acids.

RESULTS AND DISCUSSION

Using polyamide column chromatography we have been able to resolve complex natural mixtures of anthocyanins extracted from red radish roots (*Raphanus sativus*) (Fig. 1), leaves of red cabbage (*Brassica oleracea*) (Fig. 2), and lateral petals of *Impatiens balsamina*, genotype 11HHP^{r}P^{r} (Fig. 3).

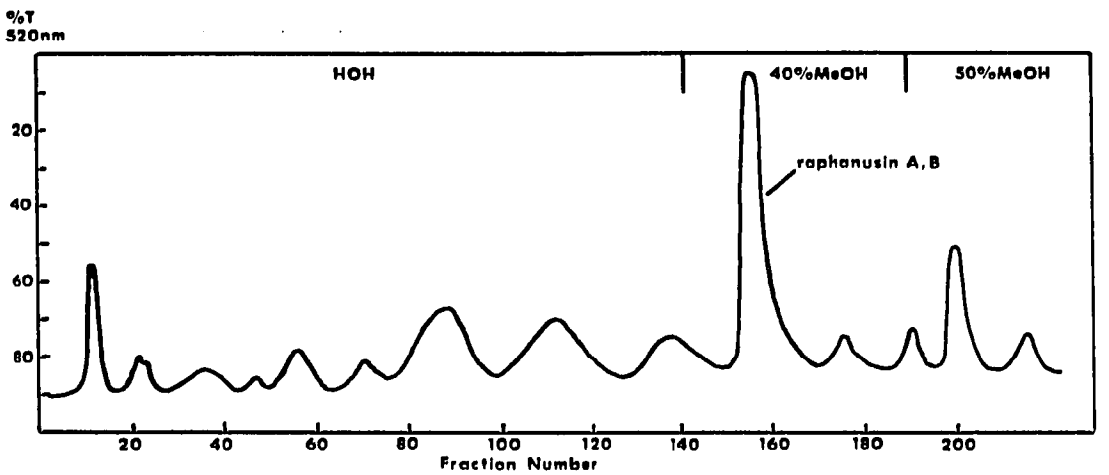

Fig. 1. Elution profile of an alcoholic extract of red radish (*Raphanus sativus*). Column: 25 g polyamide-CC 6 (0.07 mm), 25 × 2 cm. Solvent: water–methanol mixtures with descending polarity; flow-rate: 1–2 ml/min; 10-ml fractions collected.

The behaviour of anthocyanins on a polyamide column is similar to other types of flavonoids, *e.g.*, flavonolglycosides[6]. Less polar or highly soluble anthocyanin derivatives (3-sophoro-5-monoglucoside or 3,5-diglucoside) migrate most rapidly and are eluted with water (Figs. 3 and 4). Anthocyanins possessing only one sugar are more strongly retained and can be eluted only with methanol–water mixtures. Most strongly bound are the anthocyanidins, and these can be removed only with methanol concentrations approaching 100% (Fig. 4).

If there are different anthocyanidins with the same type of glycosylation (*e.g.*, pelargonidin, cyanidin and malvidin 3-monoglucoside) in the extract, malvidin-3-

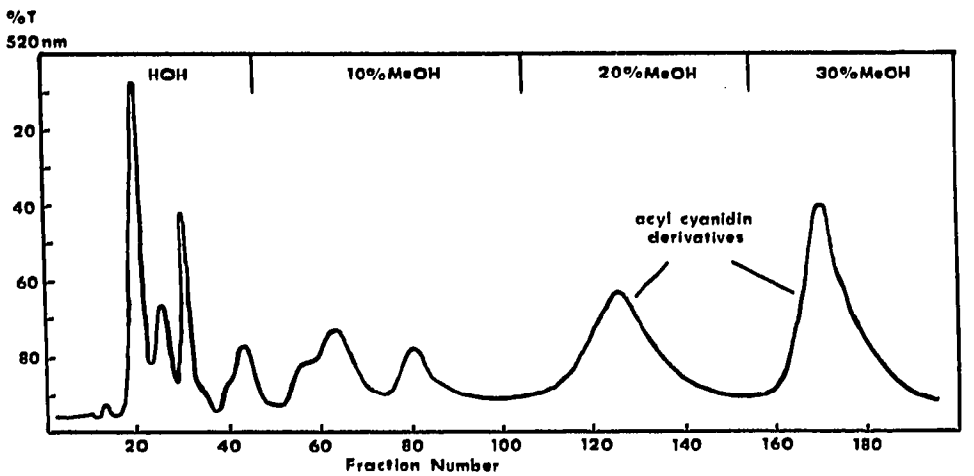

Fig. 2. Elution profile of an alcoholic extract from leaves of red cabbage (*Brassica oleracea*). Column: 50 g polyamide-CC 6 (0.07 mm), 50 × 2 cm. (Other conditions as in Fig. 1).

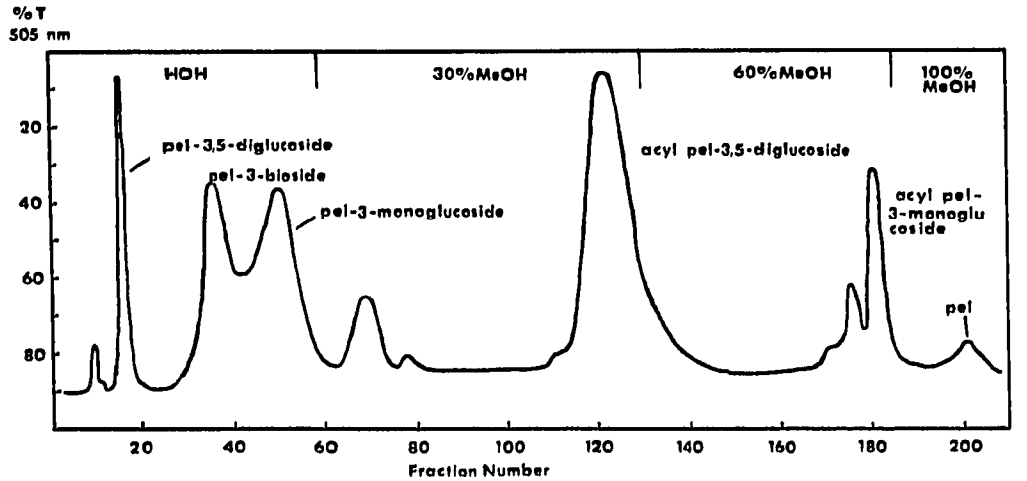

Fig. 3. Elution profile of an alcoholic extract from the lateral petals of *Impatiens balsamina* (genotype 11HHP^{r}P^{r}). For conditions see Fig. 1.

monoglucoside is eluted first, pelargonidin somewhat later followed closely by cyanidin glucoside.

Difficulties are often observed when there are different derivatives of anthocyanidins differing in polarity; this might be the limiting feature of both polyamide and PVP column chromatography.

Van Teeling *et al.*[13], using PVP columns, obtained incomplete separation and overlapping bands with an extract from poinsettia bracts which contain the 3-monoglucoside and 3-rutinoside of both pelargonidin and cyanidin. We also attempted to resolve the poinsettia pigments and obtained results similar to those of Van Teeling *et al.* We were able to separate partially cyanidin-3-rutinoside and pelargonidin-3-

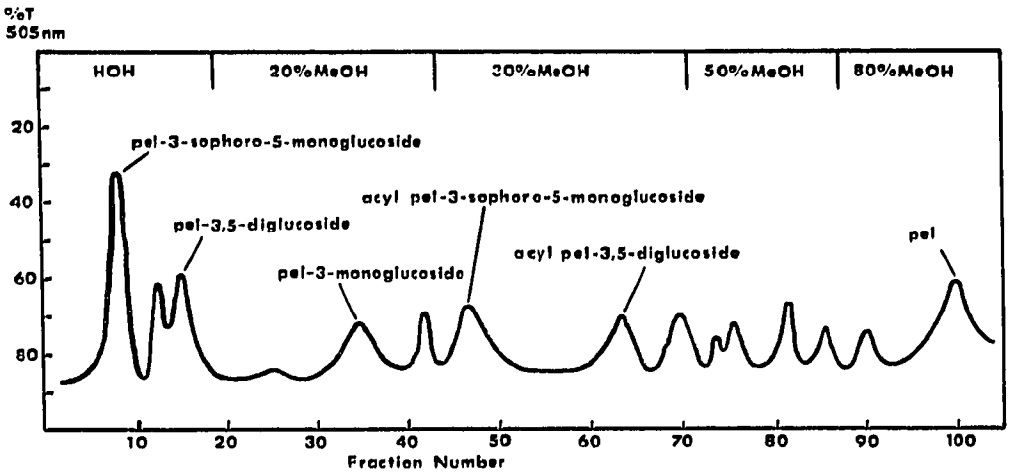

Fig. 4. Elution profile of products from a mild acid hydrolysis of the main fraction of red radish (raphanusin A and B). For conditions see Fig. 1.

monoglucoside but with a large overlapping volume. Pelargonidin-3-monoglucoside and cyanidin-3-monoglucoside from strawberry extracts were eluted in bands which overlapped in the middle 8 of 40 fractions. Thus the intersection of two characteristics of this type of chromatography can be seen: the solubility of the anthocyanin in the solvent, and the adsorption strength of polyamide.

As with PVP chromatography, polyamide columns demonstrate that the nature of the sugar moiety has a greater effect on the rate of movement of the pigment than the hydroxylation pattern of the B ring (*c.f.*, Van Teeling *et al.*[13]). The best resolution is obtained from mixtures of anthocyanins containing different derivatives of the same anthocyanidin (Figs. 3 and 4).

The retention time for a complex mixture of anthocyanins increases in the following sequence: high glycosylation, low glycosylation, acyl-high glycosylation, acyl-low glycosylation, aglycone. The acyl anthocyanins also show a very similar chromatographic mobility to the corresponding flavonol glycosides. Table I shows that the acyl pelargonidin-3-monoglucoside moves together with kaempferol-3-monoglucoside.

The present work opened the possibility of purifying and enriching derivatives of anthocyanins which occur in extremely low amounts. From red radish, red cabbage, and *Impatiens* we were able to purify and enrich the very minor anthocyanins. Fuleki found thirteen different pelargonidin derivatives in red radish roots[17]. In leaves of red cabbage 5 different anthocyanins have been described[15,16]. From a red radish extract we obtained 14 separate bands showing absorption at 520 nm (Fig. 1). Using a longer polyamide column we found eight 520-nm absorbing fractions from red cabbage (Fig. 2). In the lateral petals of *Impatiens balsamina* (red genotype 11HHP^{r}P^r) Hagen[18,19] has described ten different anthocyanins, derivatives of pelargonidin. Our investigations of this tissue has confirmed these previous findings. They were resolved clearly and we obtained those anthocyanins which had been given a putative identification.

TABLE I

RESOLUTION OF ANTHOCYANIN AND FLAVONOL DERIVATIVES OF A STAGE 3* FLOWER PETAL OF THE RED GENOTYPE 11HHP^{r}P^r OF *IMPATIENS BALSAMINA* ON A MICRO-SCALE

Conditions: Polyamide-CC 6 (0.07 mm), 6.5 × 0.5 cm; flow-rate, 0.3 ml/min; alcoholic extract from 15 mg of tissue. Compounds: pelargonidin: 4 = 3,5-diglucoside; 5 = 3-monoglucoside; 6 = acyl 3,5-diglucoside; 37 = 3-bioside; 41 = aglycone; 52 = acyl 3-monoglucoside. Kaempferol: 2 = aglycone; 7 = 3-monoglucoside; 8 = 3-bioside.

Solvent	*Fraction (ml)*	*Compound*								
		4	*37*	*5*	*6*	*8*	*7*	*52*	*41*	*2*
water	5	+								
	25		+	+						
40% methanol	15				+	+				
50% methanol	12						+	+		
80% methanol	10								+	
100% methanol	10									+

* Flower stage and compound number after Hagen[18,19].

This technique makes it thus possible to determine more accurately the total number of anthocyanins present in a plant organ. It is also likely that the qualitative anthocyanin content of many well known plants will be found to be greater than previously thought, since this procedure permits the isolation of most minor compounds in amounts sufficient for further investigations.

Mabry *et al.* observed polyamide columns separating flavone and flavonol derivatives which could not be separated by paper chromatography[5]. Our results confirm the same to be true for anthocyanins. In petals of *Impatiens* there are two pelargonidin derivatives (putative 3-diglycoside and acyl-3,5-diglucoside) which have nearly identical R_F values in four different solvent systems. These compounds can only be distinguished by their fluorescent properties under UV light. The polyamide elution profile (Fig. 3) shows that these derivatives have very different retention times and are easily separated.

During the identification of the major compounds from radish we carried out a mild acid hydrolysis (15 min at 100° in 1 *N* HCl) of the raphanusin A and B fraction (Fig. 1). This hydrolysate was re-chromatographed on polyamide and numerous anthocyanin derivatives were found (Fig. 4). Each of the pigments labelled in Fig. 4 were identified by paper chromatography.

In recent investigations of the kinetics and turnover of the anthocyanins during development, we obtained a rapid and quantitative resolution of the alcoholic extract of *Impatiens balsamina*, genotype 11HHP^{r}P^r (Table I). All compounds listed are clearly resolved and excellent reproducibility can be obtained if definite volumes of solvents are used. Even kaempferol-3-monoside and 3-bioside, which show very similar rates of migration on polyamide columns[2,6], are quantitatively separated.

An important parameter for obtaining good resolution is the grain size of the polyamide powder. In earlier experiments with the regular grain size of 0.16-mm broadening of the bands was observed which resulted in great overlapping and contamination of the individual fractions.

Since anthocyanins are weakly retained on polyamide compared with other

types of flavonoids[7] and this behaviour might be strengthened by using HCl in the solvent (*c.f.*, Mabry *et al.*[5]) for stabilization of the pigments, the water–methanol gradient must be as low as possible.

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