

High Performance Liquid Chromatographic Identification of Anthocyanins

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Anthocyanins, 3-Deoxyanthocyanins, Partial Hydrolysis, High Performance Liquid Chromatography

A high performance liquid chromatographic procedure for a rapid identification of anthocyanins is described. Besides their characteristic retention times, anthocyanins can easily be identified by an HPLC examination of the kinetics of appearance and disappearance of their partial hydrolysis products. This method is also applicable to 3-deoxyanthocyanins which is demonstrated with an extract from the fern *Blechnum brasiliense*.

The described method allows optimal resolution of complex anthocyanin mixtures in the following sequence of elution: 3-sophoroside-5-glucoside; 3-rutinoside-5-glucoside; 3,5-diglucoside; 3-sophoroside; 3-galactoside; 3-glucoside; 3-rutinoside; 5-glucoside; aglycone, and finally the forms acylated with *p*-coumaric acid. Substitution in the B-ring of the aglycone structure is the key factor for the range of the retention time. Thus cyanidin 3,5-diglucoside elutes earlier (–38 sec) than pelargonidin 3-sophoroside-5-glucoside.

Introduction

Within the last decade high performance liquid chromatography has proven to be almost universally applicable to analyses of secondary phenolic plant constituents [1] and thus the potential exists for applying this technique to the analysis of anthocyanins. Whereas good quality HPLC of anthocyanidins [2–4] will probably lead this application to be well established in plant research, analyses of their glycosides needed further thorough methodological studies. In terms of speed and quality of resolution, only simple mixtures of low glycosylated anthocyanins can, thus far, be satisfactorily analyzed by HPLC [5–7].

In this paper, we present an analytical HPLC technique which enables resolution of complex mixtures of anthocyanins, of high and low substitution. We demonstrate that the described method is applicable to rapid identification of anthocyanins by means of examining the kinetics of appearance and disappearance of their partial hydrolysis products. To date, analyses of intermediate glycosides, formed by controlled hydrolyses of the original glycosides [8–10], has been done by paper and thin-layer methods.

Materials and Methods

Origin of the pigments

3-(*p*-Coumaroylsophoroside)-5-glucoside (raphanusin A) was isolated from roots of *Raphanus sativus* (commercial source). All other anthocyanins came from petals of *Gladiolus* cultivars. Luteolinidin- and apigeninidinglycosides were extracted from young fronds of *Blechnum brasiliense*.

Extraction

Pigments were extracted by placing the plant material into methanol, containing 1% HCl (v/v), for ca. 15 h.

Isolation of individual anthocyanins

Crude extracts were fractionated on polyamide columns [11] and the fractions obtained were resolved into individual pigments by TLC on microcrystalline cellulose (Avicel) using the following solvent systems: 1% HCl and after drying development in the same direction with water-acetic acid-HCl (82:15:3) or *n*-butanol-acetic acid-water (6:1:2). Dry TLC-bands were scraped into 10 ml-syringes and elution was achieved by pressing a few ml of Harborne-mixture (methanol-acetic acid-water, 70:25:5) through the powder. The eluates were taken to dryness in a rotary evaporator and the pigments were redissolved in methanol.

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Sugar identification

Sugars of pigments isolated from *Blechnum* and *Gladiolus* were identified by TLC co-chromatography with standard samples (*n*-butanol-pyridine-water, 3:1:1) on microcrystalline cellulose (Avicel). The upper margin of the plates was connected with chromatographic paper, which hung over the back of the plates and allowed development for 15–20 h. Detection was achieved by spraying with ammoniacal silver nitrate and heating at 100 °C until spots became visible.

Partial hydrolysis

1 ml aliquots of anthocyanin solution were mixed with 1 ml of 4 N HCl and the mixtures were kept in stoppered tubes in a boiling water bath. At suitable time intervals tubes were taken out, cooled in an ice bath and after filtration through a 1 µm Millipore filter (Swinny), the hydrolysates were injected directly onto the column.

High performance liquid chromatography

The liquid chromatograph used was Spectra-Physics (Santa Clara, Calif., USA) and included two Model 740 B pumps with 740 pump control units, a 714 pressure monitor, a 744 solvent programmer, and a 755 sample injector (loop and syringe injection mode).

The applied column (250 × 4 mm) was prepacked with LiChrosorb RP-18 (5 µm) (Merck, Darmstadt). Separation was accomplished by gradient elution: in 60 min linear from 25% solvent B in A + B to B (A, 1.5% phosphoric acid in water; B, 1.5% phosphoric acid, 20% acetic acid, and 25% acetonitrile in water). Chromatography of 3-deoxyanthocyanins was achieved with a delay time of 5 min linear from 35% B in A + B in 20 min to B. The flow-rate was 1 ml/min. Anthocyanins were detected at 520 nm and 3-deoxyanthocyanins at 485 nm with a Schoeffel SF 770 UV-VIS detector (Kratos Inc., Trappenkamp). In the case of the anthocyanin acylated with *p*-coumaric acid, a simultaneous detection at 312 nm with a Spectra-Physics SP 8200 dual-beam UV-VIS detector with a SP interference filter kit was monitored.

Retention times and quantitative calculations were obtained with an Autolab System I computing integrator (Spectra-Physics).

Peak identifications of intermediate glycosides from partial hydrolyses were achieved by HPLC co-chromatography with known anthocyanins isolated from various plant sources [12].

Linearity of detection was proven with a set of pigment dilutions.

A very important factor in obtaining the quality of resolution, as to be seen in the figures, is the solvent in which the anthocyanins are dissolved prior to the injection onto the column. Injecting the pigments dissolved in methanol (with 1% HCl), results in severe peak broadening and deficient peak symmetry (leading). The addition of water improves chromatography markedly. The injection solvent should contain at least 50% water or should be water alone (with 1% HCl).

Results and Discussion

Table I lists 14 anthocyanins, 2 anthocyanidins, and the 4 common hydroxycinnamic acids, which were analyzed by reserved-phase HPLC. All compounds eluted as sharp and symmetrical peaks, thus allowing a rapid and high resolution of complex mixtures of anthocyanins.

Table I. Retention times of anthocyanins and hydroxycinnamic acids on LiChrosorb RP-18 using a linear gradient from 25% B in A + B to B within 60 min with a flow-rate of 1 ml/min (A, 1.5% phosphoric acid in water; B, 1.5% phosphoric acid, 20% acetic acid, and 25% acetonitrile in water). The coefficient of variation for early and late peaks was determined to be 1.1% (*n* = 15).

Peak No.	Compound	<i>t_R</i> [sec]
1	Cy* 3,5-diglucoside	716
2	Pg* 3-sophoroside-5-glucoside	754
3	Pg 3-rutinoside-5-glucoside	803
4	Caffeic acid	804
5	Pg 3,5-diglucoside	879
6	Cy 3-glucoside	922
7	Pg 3-sophoroside	957
8	Cy 5-glucoside	982
9	Pg 3-glucoside	1113
10	<i>p</i> -Coumaric acid	1176
11	Pg 3-rutinoside	1210
12	Pg 5-glucoside	1242
13	Ferulic acid	1356
14	Cyanidin	1362
15	Sinapic acid	1404
16	Pelargonidin	1760
17	Pg 3-(<i>p</i> -coumaroylsophoroside)-5-glucoside	1840
18	Pg 3-(<i>p</i> -coumaroylglucoside)-5-glucoside	1918
19	Pg 3-(<i>p</i> -coumaroylsophoroside)	2130
20	Pg 3-(<i>p</i> -coumaroylglucoside)	2246

* Pg = pelargonidin; Cy = cyanidin.

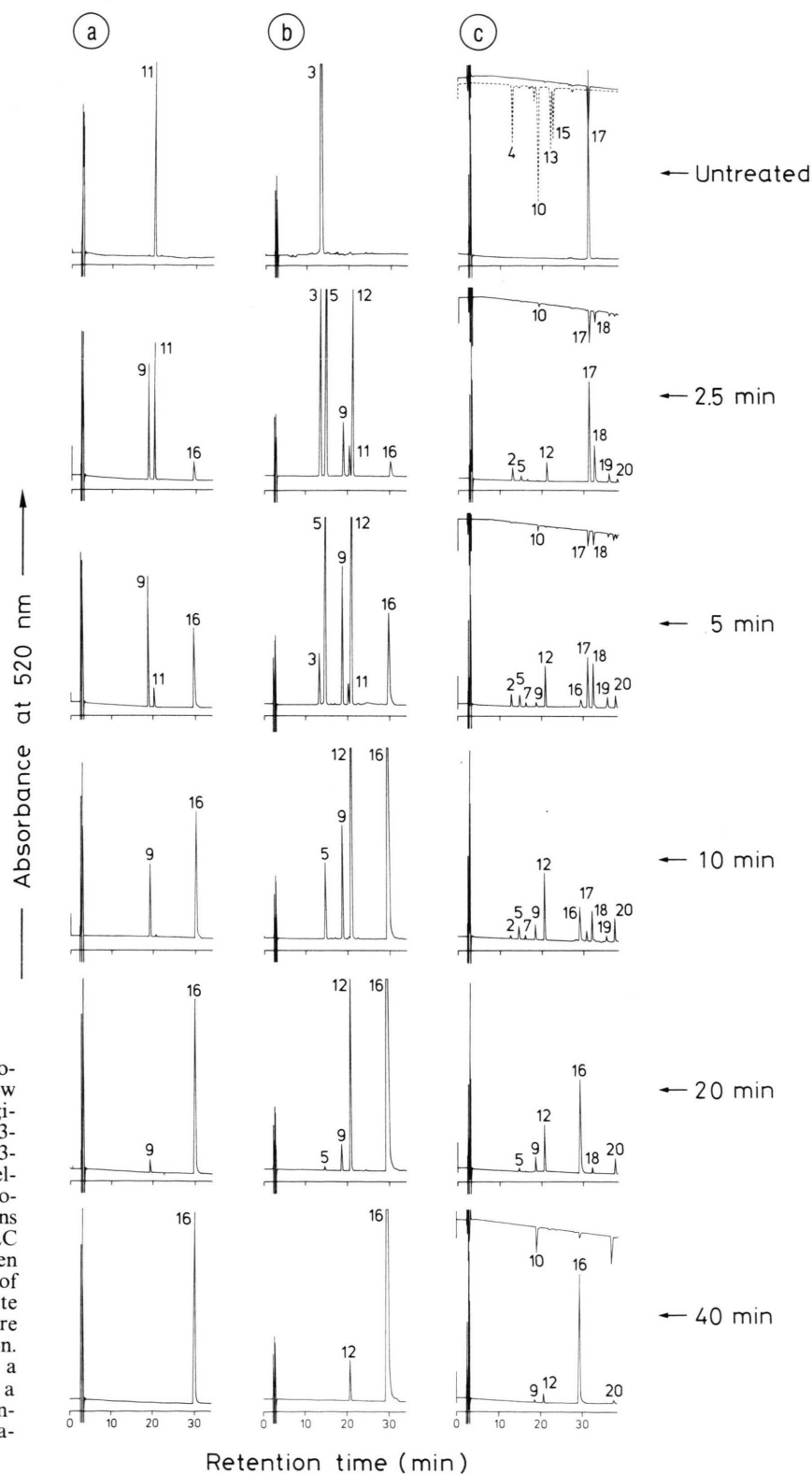


Fig. 1. HPLC analyses of anthocyanin hydrolysates. The first row shows chromatograms of the original pigments: (a), pelargonidin 3-rutinoside; (b), pelargonidin 3-rutinoside-5-glucoside; (c), pelargonidin 3-(*p*-coumaroylsophorose)-5-glucoside. The columns a, b, and c show series of HPLC fingerprints of hydrolysates, taken from 2.5, 5, 10, 20, and 40 min of acid hydrolyses. The opposite chromatograms in column c are derived from a 312 nm detection. The chromatogram drawn with a dashed line was obtained with a standard mixture of hydroxycinnamic acids. For peak identification see Table I.

Substitution in the B-ring is the key factor for the range of elution time. Thus cyanidin 3,5-diglucoside elutes earlier (– 38 sec) than the higher glycosylated pelargonidin 3-sophoroside-5-glucoside. The nature of an attached sugar displays a marked influence on retention time. This is evident when comparing the retention times of pelargonidin 3-sophoroside-5-glucoside (754 sec) with pelargonidin 3-rutinoside-5-glucoside (803 sec) or alternatively the 3-sophoroside (957 sec) with 3-rutinoside (1210 sec). The latter is even more retained than the 3-monoglucoside (1113 sec). Attached galactose instead of glucose decreases

retention time: cyanidin 3-galactoside (888 sec) elutes earlier than cyanidin 3-glucoside (922 sec). Pigments acylated with *p*-coumaric acid are the most retained and elute later than the aglycone.

The described method is applicable to rapid identification of anthocyanins by means of examining the kinetics of appearance and disappearance of intermediate glycosides, formed by controlled hydrolyses of the original glycoside. This is demonstrated in Figs. 1, 2, and 3. It can be seen that all intermediate derivatives, which would be expected, appeared. Provided that the sugars attached to the

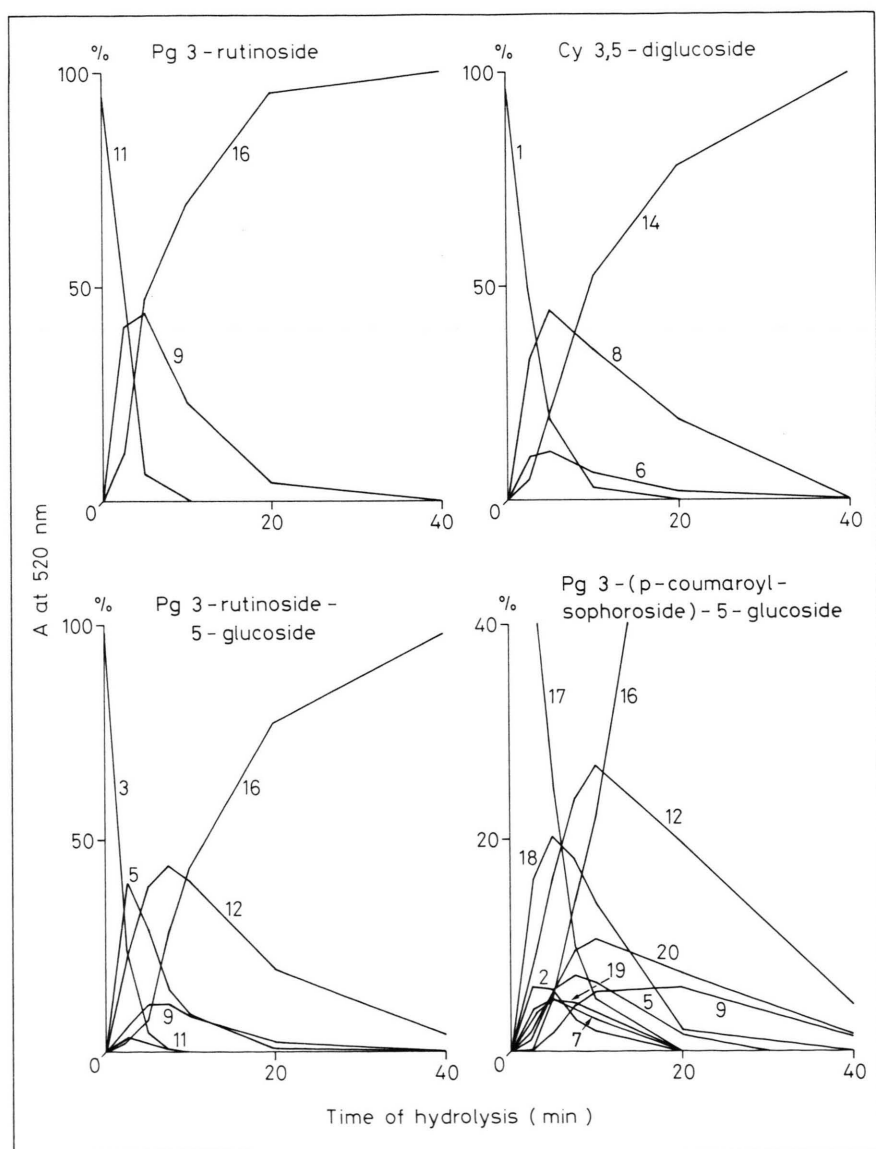


Fig. 2. Kinetics of appearance and disappearance of partial hydrolysis products, formed by controlled acid hydrolyses. Values are expressed as per cent of total absorption of all pigments in one chromatogram at 520 nm. For identification see Table I.

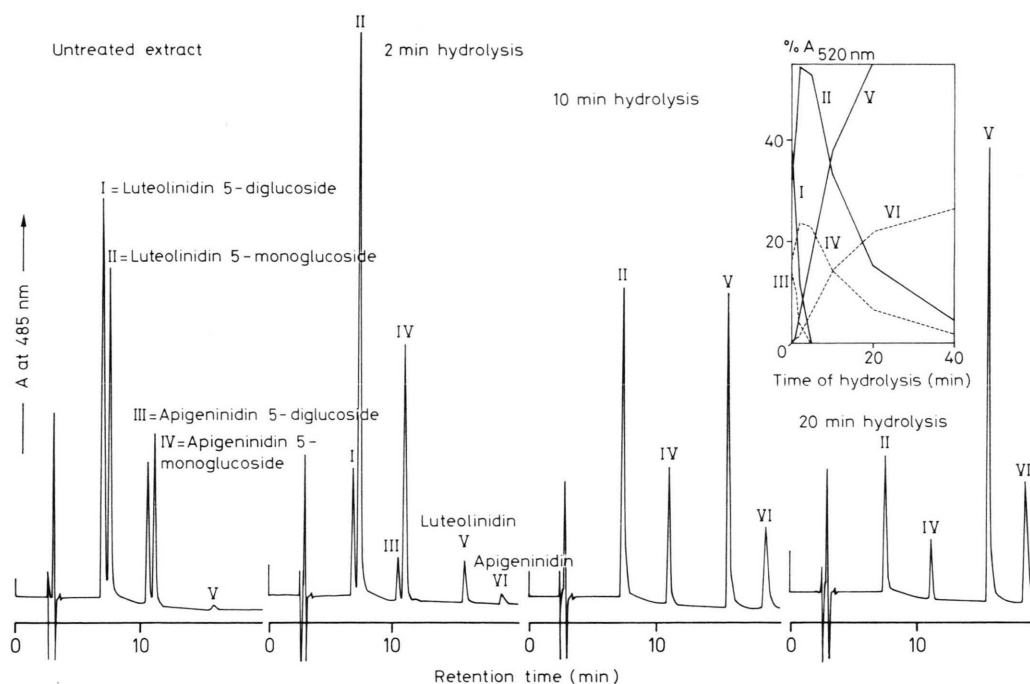


Fig. 3. HPLC chromatograms of 3-deoxyanthocyanins from an alcoholic extract of *Blechnum brasiliense* and their hydrolysate fingerprints, taken at 2, 10, and 20 min of acid hydrolysis. Inserted are the plotted quantitative values as per cent of total absorption of all pigments in one chromatogram at 485 nm.

anthocyanidin are known, the described procedure enables a rapid structural elucidation of an unknown anthocyanin. Even without knowing the sugars tentative identifications are possible because this HPLC selects between anthocyanins differing only in the nature of the attached sugars (see Table I and Fig. 4).

As shown with raphanusin A (Fig. 1), it is also possible to identify rapidly a hydroxycinnamic acid, commonly found in acylated anthocyanins, when monitoring simultaneously in the UV region (*e. g.* 312 nm). However, the yield of the free hydroxycinnamic acid is low [12]. Peaks which appear simultaneously at 520 and 312 nm in column c of Fig. 1, indicate the *p*-coumaroyl derivatives of intermediate glycosides of the original raphanusin A.

The quantitative values obtained can be taken as an additional important criterion in anthocyanin identification. In the described procedure, the ratio between the 5- and 3-monoglucosides from the original cyanidin 3,5-diglucoside (Fig. 2) was calculated to be ca. 4:1. The identity of the 5-glucoside was obtained by TLC and

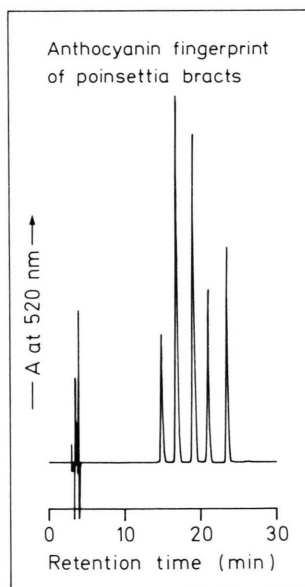


Fig. 4. Anthocyanins from poinsettia bracts resolved by HPLC. The pigments elute in the following sequence: cyanidin 3-galactoside (888 sec); cyanidin 3-glucoside; cyanidin 3-rutinoside (1027 sec); pelargonidin 3-glucoside; pelargonidin 3-rutinoside.

by the characteristic yellow fluorescence of 5-substituted anthocyanins [8, 12, 13]. Pelargonidin 3-rutinoside-5-glucoside yields, after 5 min hydrolysis, quantities of the intermediates 3,5-diglucoside and the 5-glucoside in the ratio of ca. 1:1.

Fig. 3 demonstrates that the described HPLC system is also applicable to analyses of deoxyanthocyanins. From an alcoholic extract from fronds of *Blechnum brasiliense* luteolinidin and apigeninidin 5-di- and monoglucosides (Reznik, unpublished) were enriched on a polyamide column with 50% methanol. Simultaneous hydrolyses of the 4 naturally occurring constituents lead to the conclusions that the two major compounds are the 5-diglucoside and 5-monoglucoside of luteolinidin and the two minor ones those of apigeninidin. The nature of the 3-deoxyanthocyanidins can easily be determined by HPLC. Using the system described in ref. [4] and in this paper, luteolinidin elutes between delphinidin and cyanidin, apigeninidin between petunidin and pelargonidin, both baseline separated from the anthocyanidins.

The application of the described analytical method to characterize cultivars of *Gladiolus* by fingerprinting the anthocyanin-pattern of petals showed excellent resolution of mixtures containing low and

highly glycosylated derivatives of the 6 common anthocyanidins delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin. This will be published elsewhere [14]. Some difficulties concerning overlapping peaks in extremely complex mixtures can be eliminated by varying the solvent flow-rate and by slight changes of the gradient profile. However, in a broad application, mostly concerned with simple mixtures of anthocyanins, the presented method does not need any variations. For example Fig. 4 shows an anthocyanin fingerprint of poinsettia bracts. The peaks were identified by their retention time (Table I) and/or by co-chromatography with anthocyanins isolated from other plants [12]. The anthocyanin pattern in Fig. 4 is in agreement with results published by Asen [7], who resolved the poinsettia anthocyanins isocratically on μ Bondapak C₁₈.

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