High Performance Liquid Chromatography of Anthocyanidins as a New Approach to Study Flower Pigment Genetics

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The described method allows rapid screening and accurate quantification of anthocyanidins with the aid of HPLC. Using a linear gradient in 40 min from 20 to 40% of solvent B (1.5% phosphoric acid – 20% acetic acid – 25% acetonitrile) in solvent A (1.5% phosphoric acid) + B on LiChrosorb RP-8 the common six anthocyanidins delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin were resolved within 40 min. It is demonstrated that acetonitrile is superior to other solvents in resolving methoxylated phenolic compounds. The application of the described method is shown with *Gladiolus* petals in studies of flower pigment genetics.

Introduction

Applications of HPLC to the analysis of anthocyanidins was reported in 1977 by Adamovics and Stermitz [1] and Wilkinson *et al.* [2] using essentially the same reversed-phase chromatographic system developed by Wulf and Nagel [3] for the separation of flavonols and phenolic acids. Nothing is known about the application of other systems and its usefulness in routine quantitative analyses.

Anthocyanidins occur in plants in the form of glycosides (anthocyanins) and must be set free by acid hydrolysis. During the process of isolation anthocyanidins can undergo structural changes [4] and therefore the preparation for HPLC should be rapid and a prior purification of the hydrolyzed pigment mixture should be avoided.

We report results of the application of reversed-phase HPLC systems to the analysis of anthocyanidins from crude extracts, and we demonstrate efficiency and dependability of the described method.

Materials and Methods

Plant material

Gladiolus cultivars were grown from corm collections of the Department of Floriculture and Ornamental Plants, Volcani Center, Bet Dagan, Israel, and progenies were produced by crossing between the available cultivars. Fully expanded petals were harvested and stored at −25 °C before lyophilization.

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Extraction

Anthocyanins were extracted by placing 200 mg of lyophilized petals for ca. 15 h into 4 ml methanol, containing 1% HCl (v/v).

Acid hydrolysis

1 ml anthocyanin extract was mixed with 1 ml 4 N HCl and the mixture was kept in stoppered tubes for 40 min at 100 °C.

Preparation for HPLC

Acid hydrolysates were filtered through a 1 µm Millipore filter (Swinny) and without further treatment applied to HPLC.

HPL-Chromatograph

The liquid chromatograph used was obtained from Spectra-Physics (Santa Clara, Calif., USA) and is described elsewhere [5].

Chromatographic columns

The following prepacked columns were used: LiChrosorb RP-8/5 µm (250 × 4 mm), E. Merck, Darmstadt, F. R. Germany; µ Bondapack C_{18}/10 µm (300 × 4 mm), Waters Assoc., Milford, Mass., USA; Nucleosil C_{18}/10 µm (250 × 4 mm), Macherey-Nagel + Co., Düren, F. R. Germany; Partisil PXS ODS/10 µm (250 × 4.6 mm), Whatman, Clifton, New Jersey, USA.

Analytical method

Solvents and elution systems are described in "Results and Discussion". The flow-rate was always

2.0 ml/min, detection was at 530 nm (0.2 a. u. f. s.) with a Schoffel SF 770 UV-Vis detector (Kratos Inc., Trappenkamp, F. R. Germany), and the sample size was 25 µl.

Calculations

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

Results and Discussion

Fig. 1 shows a typical chromatogram of the 6 most common anthocyanidins on LiChrosorb RP-8 obtained with a gradient system (see legend). This standard mixture was put together from marker samples of anthocyanidins, which were available in our institute. The separation was accomplished within 40 min and the coefficients of variation in retention times (see Table I, solvent B with acetonitrile) were determined to be 1.2% for delphinidin and 0.5% for malvidin. Table I demonstrates the effect of different solvent systems for B in the gradient elution on retention times and quality of resolution. Only with methanol and acetonitrile baseline separation of all 6 anthocyanidins was obtained. Acetonitrile is superior because the retention time of the last peak malvidin is ca. 40 min, whereas with methanol this peak elutes after 60 min.

Addition of phosphoric acid to both solvent A and B turned out to markedly improve chromatography; peak shape is more symmetrical and retention times are 10 min shorter, compared with those obtained without the addition of phosphoric acid.

The isocratic system used by Wilkinson *et al.* [2] on µ Bondapak C_{18} (Waters Assoc.), which is recommendable for rapid screenings of anthocyanidins, we only could reproduce by using the same column packing. Table II shows that on C_{18}-Nucleosil (Macherey-Nagel + Co.) and on Partisil C_{18} (Whatman) petunidin and pelargonidin could not be sufficiently separated with the solvent mixture used by these authors [2]. However, by the addition of acetronitrile, baseline separation of all 6 anthocyanidins could be achieved on Nucleosil. This phenomenon can be used in optimization of anthocyanidin separations on other reversed-phase column packings.

The superiority of acetonitrile, resolving mixtures of phenolic compounds containing methoxylated derivatives, was also observed with another class of flavonoids. Isovitexin 2″-arabinoside and 7-methoxy vitexin 2″-rhamnoside are difficult to separate using

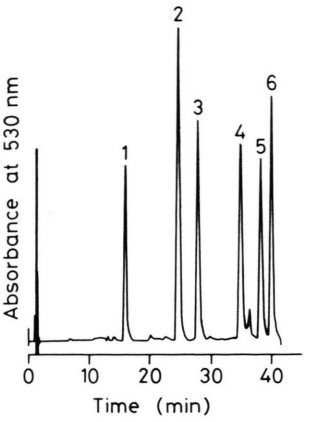

Fig. 1. HPLC resolution of a standard mixture of anthocyanidins on LiChrosorb RP-8 (5 µm). Elution system: Linear gradient in 40 min from 20 to 40% solvent B (1.5% phosphoric acid – 20% acetic acid – 25% acetonitrile in water) in solvent A (1.5% phosphoric acid in water) + B. Peak identification: 1 = delphinidin, 2 = cyanidin, 3 = petunidin, 4 = pelargonidin, 5 = peonidin, 6 = malvidin.

Table I. Retention times (in min) of anthocyanidins on LiChrosorb RP-8 (5 µm). Solvent A, 1.5% phosphoric acid; solvent B, 1.5% phosphoric acid – 20% acetic acid – 25% X*; gradient profile, in 40 min linear from 20 to 40% B in A + B.

No.	Compound	Component X in solvent B				
		* Methanol	Ethanol	i-Propanol	Acetonitrile [a]	Acetone
1	Delphinidin	28.3	19.7	13.8	16.5	16.9
2	Cyanidin	40.4	29.4	21.8	25.1	25.4
3	Petunidin	45.7	32.5	23.4	28.3	27.0
4	Pelargonidin	53.4	40.5	31.5	35.3	34.9
5	Peonidin	57.8	43.0	32.4	38.7	36.4
6	Malvidin	61.1	43.8	32.4	40.5	36.4

[a] Chromatogram in Fig. 1.

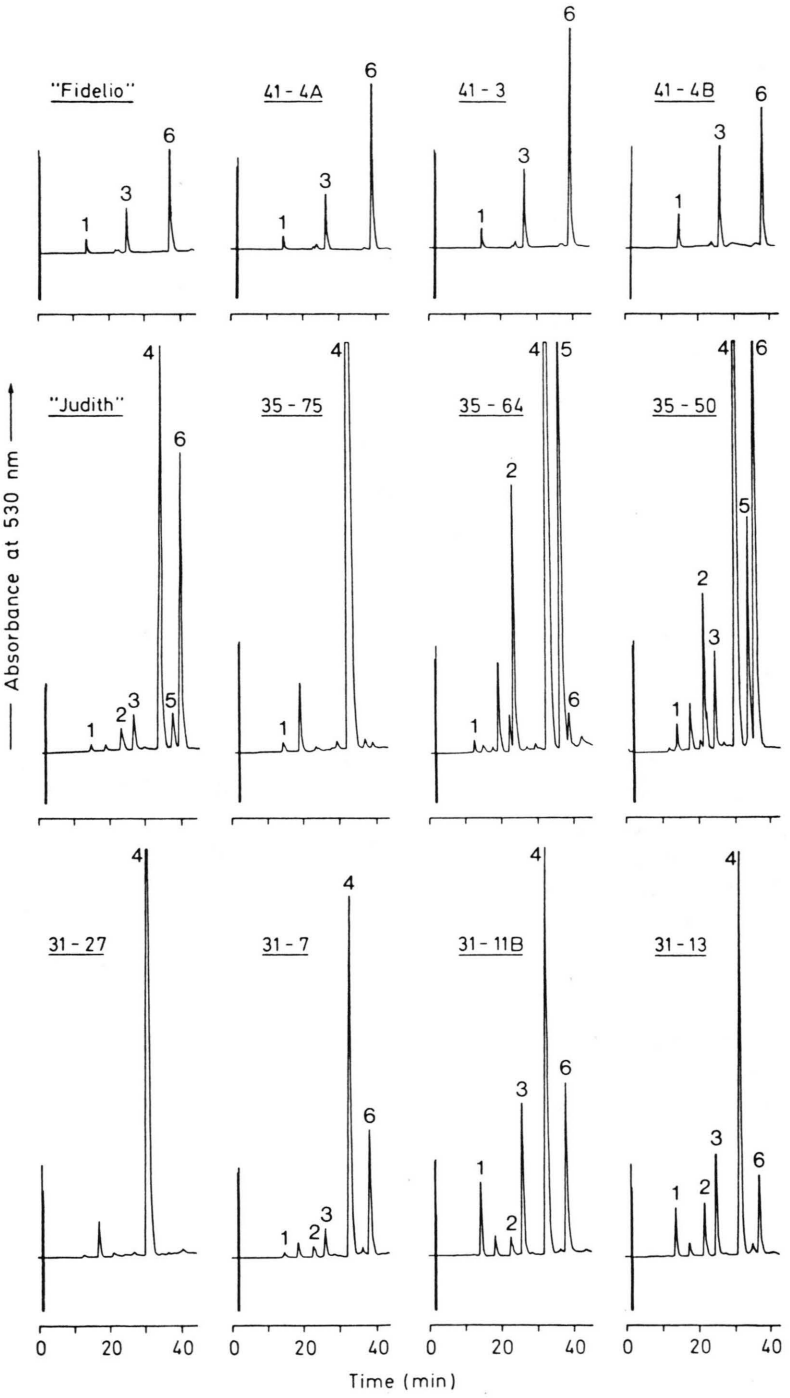

Fig. 2. Application of HPLC on LiChrosorb RP-8 (5 µm) to crude hydrolysates of anthocyanin extracts from petals of *Gladiolus* cultivars and some progenies (elution system as described in Fig. 1). Peak identification as in Fig. 1. *Row 1:* 3 F_1-progenies (41-) derived from self-pollination of "Fidelio" (purple); *Row 2:* 3 F_1-progenies (35-) from a crossing of "Judith" (deep red) and "Oscar" (dark red); *Row 3:* F_1-progenies from a crossing of "Judith" and "Gold field" (yellow).

a water-methanol gradient ($\alpha = 1.02$) [6], whereas with a water-acetonitrile gradient α was determined to be 1.19.

Another advantage in using acetonitrile instead of methanol concerns the stability of the elution system. Water-acetic acid-methanol mixtures as used by Wilkinson *et al.* [2] are unstable because of ester formation, which results in changes of retention times. Using the gradient system with methanol as described in Table I, the retention times of delphinidin

Table II. Retention times (in min) of anthocyanidins with isocratic systems on 10 μm C_{18}-column packings.

No.	Compound	Water-acetic acid-methanol (71 : 10 : 19) according to Wilkinson *et al.* [2]			Water-acetic acid-methanol-acetonitrile (75 : 10 : 7 : 8)		
		μ Bondapak	Nucleosil	Partisil	μ Bondapak	Nucleosil	Partisil
1	Delphinidin	5.1	4.6	4.8	3.8	3.7	4.0
2	Cyanidin	7.8	6.8	6.5	5.8	5.6	5.7
3	Petunidin	9.7	8.7	8.6	7.1	7.2	7.4
4	Pelargonidin	11.5	9.5	8.6	8.9	8.5	7.8
5	Peonidin	14.5	12.5	11.6	11.0	11.1	10.2
6	Malvidin	17.6	15.9	15.0	13.3	14.0	13.1

and malvidin decreased within 24 h by 4.2 and 7.8 min, respectively.

Fig. 2 illustrates the application of the described method, showing selected anthocyanidin-chromatograms obtained from *Gladiolus* petals of a few progenies and their parents. This method is a new approach for a rapid characterization of petal phenotypes as required for studies in the genetic control of colour formation in flowers. The tetraploid *Gladiolus* petals can produce the 6 common anthocyanidins [7] and we expect a complex pigment pattern in the progenies. In row 1 of Fig. 2 there are chromatograms of 3 F_1-progenies derived from self-pollination of the cultivar "Fidelio", showing 2 different results: progenies 41-3 and 41-4A depict different total amounts of anthocyanidins, yet the per cent ratio of the individual compounds is very similar; comparison of the progenies 41-4B with 41-4A shows, that there is the same total level, but the per cent composition is different. The other progenies in Fig. 2 exhibit a more complex picture, illustrating possible differences in gene combinations and activities.

By collecting such data on a large number of progenies from these crossings and others it could be possible to gather enough information, to draw a conclusion about the genetic control of anthocyanidin production in *Gladiolus* petals.

The coefficient of variation ($n = 9$) for the quantitative estimations of anthocyanidins, including extraction and hydrolysis, was determined to be 4.9% in this study. After hydrolysis anthocyanidins undergo decomposition [4]. In the hydrolysis mixture (2 N HCl in water-methanol, 1 : 1), at room temperature in light half-life was determined to be 34 h, at room temperature in darkness 70 h, and at 4 °C in darkness ca. 200 h. At −20 °C in darkness no decomposition was observed over a period of several weeks.

HPLC systems for the separation of anthocyanins were published by Wulf and Nagel [8], Williams *et al.* [9], and Asen [10]. Such techniques could be employed to gain an insight into the regulation of glycosides biogenesis. Work along these lines is underway with selected *Gladiolus* pedigries, and it will be published elsewhere.

Acknowledgements

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