Identification of Anthocyanins and Intermediates of Anthocyanin Biosynthesis from *Petunia hybrida* Using High Performance Liquid Chromatography

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A procedure to identify dihydroflavonol (glucosides), cinnamic acids and anthocyanins in flowers of *Petunia hybrida* is presented. Nearly all known intermediates and anthocyanins ocurring in *Petunia* are separated on one column using different elution systems. Hydrolysis of extracts before HPLC and thin layer chromatography is in some cases necessary to make positive identification of flavonoids.

Anthocyanins in flowers of *Petunia hybrida* can occur, depending on genotype, as acylated anthocyanins. Two types of acylated anthocyanins have been identified differing in polarity and thus in retention times on HPLC and TLC. The difference in acylation is genetically determined and gives rise to minor colour differences.

Introduction

The use of High Performance Liquid Chromatography in analysing mixtures of anthocyanins and other flavonoids has been reported frequently the last few years [1–12]. Reversed phase chromatography using organic solvents has shown to be a most useful procedure.

The analysis of flavonoids and intermediates of flavonoid metabolism is of great importance in identifying mutants of flavonoid metabolism [13, 14]. Our collection of mutants of *Petunia hybrida* shows many mutants of flavonoid biosynthesis. These mutants were previously characterized using thin layer chromatography and column chromatography [13, 14]. These procedures are time-consuming and therefore a fast HPLC procedure was developed for identification of flavonoids and cinnamic acids occurring in *Petunia hybrida.* The results are presented in this paper.

Materials and Methods

High Pressure Liquid Chromatography was performed using a Perkin Elmer series 3B liquid chromatograph equipped with a Lichrosorb 10 RP 18 column (Chrompack, dimensions 24 × 0.5 mm). Detection occurred with a LC 75 wavelength detector (Perkin Elmer).

Four solvent systems were used:

I. elution with A = 12.5% methanol, 5% acetic acid and 82.5% water (by vol) at 30 °C with a flow rate of 3 ml/min;

II. elution with B = 30% methanol, 5% acetic acid and 65% water (by vol) at 30 °C with a flow rate of 3 ml/min;

III. steprise lineair gradient elution using C = 95% methanol, 5% acetic acid (by vol) and D = 95% H_2O, 5% acetic acid (by vol), as solvents. Solvents were mixed according to the following gradient: a lineair increase in two minutes of 0.5% to 1.5% methanol (final concentration, by vol) and a lineair increase in five minutes of 1.5% to 4.5% methanol (final concentration, by vol) at a flow rate of 4 ml/min and an elution temperature of 30 °C;

IV. lineair gradient using E = 50% methanol, 10% formic acid and 40% water (by vol) and F = 10% formic acid (by vol) and 90% water. Solvents were mixed according to the following gradient: a lineair increase in 20 min of 10% methanol to 25% methanol (final concentration, by vol) at a flow rate of 4 ml/min and an elution temperature of 45 °C.

Limbs of flowers or flowerbuds were extracted with methanol-HCL (0.5%, by vol, 10 ml/gr. ww). To the extract chloroform and water were added according to Folch *et al.* [15]. The upperphase

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contained the flavonoids and the cinnamic acids whereas the lower phase contained very apolar compounds like chlorophyll. Samples of the upper-phase were injected into the chromatograph.

Intermediates and anthocyanins were isolated from appropriate mutants using Sephadex LH20 chromatography and preparative thin layer chromatography as described elsewhere [13, 14].

p-Coumaric acid, caffeic acid, ferulic acid, sinapic acid and cinnamic acid were purchased from Fluka. Dihydroquercetin and naringenin were obtained from Roth.

Thin layer chromatography of anthocanins was performed on Kieselgel (G 1500, Schleicher and Schüll) and ethylacetate : formic acid : water (60:12:16, by vol, AMW) as eluant.

Paperchromatography of anthocyanins was performed on Whatman I using butanol : acetic acid : water (40:10:50, by vol, upper phase BAW), HCl 1% and acetic acid : conc HCl : water (15:3:82 HOAc-HCl) as eluants.

Results and Discussion

Identification of dihydroflavonols and cinnamic acids

In order to analyse dihydroflavonol-glucosides and cinnamic acids occurring in flowers of *Petunia hybrida* we use three solvent systems. The first solvent sytem (I, see materials and methods) separates most of the dihydroflavonol-glucosides. The more polar dihydroflavonol-glucosides (7-glucosides) are eluted within 3 min whereas the 4′-glucosides are eluted between 5 and 7 min after injection (Table I). Dihydromyricetin-4′-glucoside and dihydroquercetin-4′-glucoside could not be separated. The aglucones however are easily separated which indicates that positive identifications can be achieved after hydrolysis of the 4′-glucosidic linkage of the compounds (16). It should be noted however that the 4′- and the 7′-glucosidic linkages of dihdroflavonols show different kinetics on hydrolysis with HCl at 100 °C. Within 5–10 min 4′-glucosides were hydrolysed whereas total hydrolysis of 7-glucosides took 20–25 min. A typical example of an analysis of flavonoids in flowers of the mutants W75 and W39 is shown in Fig. 1. The mutant W39 is homozygous recessive for An3 and is blocked in an early step of biosynthesis of anthocyanins and therefore accumulates flavanones (Fig. 1A). The mutant W75 however is homozygous

Table I. Retention times of flavonoids and intermediates of flavonoid biosynthesis on HPLC. Elutionsystems as described in materials and methods.

Compound	Retention time [min]		
	I	II	III
p-coumaric acid	6.6	2.7	–
cinnamic acid	–	11.1	–
caffeic acid	3.6	1.8	–
caffeic acid derivative	1.5	–	3.4
sinapic acid	10.6	–	–
ferulic acid	9.6	–	–
naringenin	–	12	–
-4′-glucoside	–	5	–
-7-glucoside	–	3.7	–
eriodyctiol	–	6.5	–
-7-glucoside	10.6	2.3	–
dihydrokaempferol	14.6	–	–
-4′-glucoside	6.2	–	–
-7-glucoside	2.7	–	–
dihydroquercetin	7.5	–	–
-4′-glucoside	5.2	–	–
-7-glucoside	1.9	–	4.7
dihydromyricetin	3.2	–	–
-4′-glucoside	5.3	–	–
-7-glucoside	1.2	–	2.2

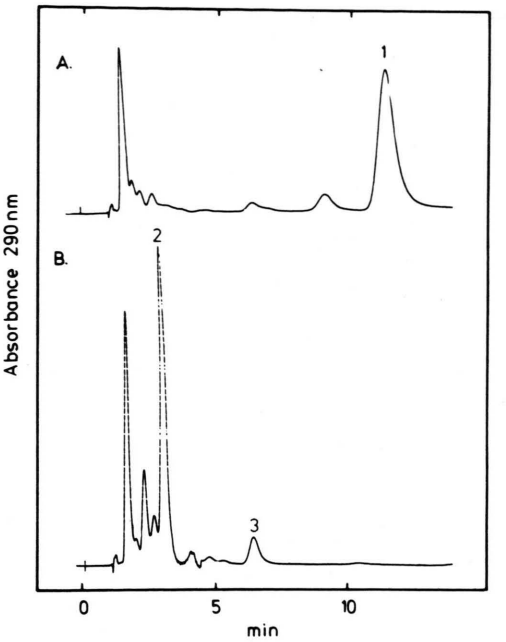

Fig. 1. Liquid chromatography of flavonoids extracted from flowers of the mutant W39 (A) and W75 (B). As eluant system I was used. 1, Eriodyctiol-7-glucoside; 2, dihydrokaempferol-7-glucoside; 3, dihydrokaempferol-4′-glucoside.

recessive for the gene An9 and is able to synthesize dihydroflavonols (Fig. 1 B).

Substituted cinnamic acids can be identified as well using this solvent system. It should be stressed that these compounds occur *in vivo* as far more polar derivatives. These derivatives are converted to the cinnamic acids by hydrolysis in 1 M HCL at 100 °C for 10 min and can be detected as described above.

One derivative of caffeic acid is very abundant in *Petunia hybrida* [17]. The retention time of this compound is almost equal to the retention time of dihydroquercetin-7-glucoside using solvent I. However, caffeic acid can be identified after hydrolysis in HCl using solvent system III. The native compound (and other compounds with a retention time less than 2 min in solvent I) is separated from dihydroquercetin-7-glucoside. Flavanone-glucosides and flavanones can be identified with solvent system II. A higher methanol concentration is needed to elute these less polar compounds. We recently identified a compound which was assumed to be a pentahydroxyflavanone [17]. Recently this was confirmed using the pure pentahydroxyflavanone as referent. This compound is easily separated from the other flavanone-glucosides and flavanones.

Identification of anthocyanins

Anthocyanins were separated using the same column as used for other flavonoids and C_9 intermediates. However, instead of acetic acid, formic acid was added to the solvents. Besides, the elution temperature was increased to 45 °C. These modifications improved separation by diminishing peak broadening. The retention times of anthocyanins are summarized in Table II. Solvent system IV separates most of the naturally occurring anthocyanins. In some cases however, hydrolysis to the corresponding aglucone is necessary (compare R_f peonidin-3RG ac5G and malvidin-3RG ac5G) which are easily identified on TLC. In most mutants of *Petunia hybrida* anthocyanins are acylated with *p*-coumaric acid. Some mutants however, show a different acylation pattern. This could be due to the nature of the acyl-group or the linkage between *p*-coumaric acid and rhamnose. Preliminary experiments have indicated that in both acylation types *p*-coumaric acid is present. This difference in acylation causes an increase in polarity. The retention times are lowered as is indicated in Table II. Furthermore there is a minor change in colour of the flower. To obtain definite identification of these compounds it is necessary to perform thin layer chromatography. The data are shown in Tables II and III. Treatment of these compounds with NaOH gives malvidin-3RG5G clearly indicating that the difference in structure is restricted to the acyl moiety of the molecule. The difference in acylation is genetically determined (Results not shown). Fig. 2 shows a typical example of the identification of cyanidin-3G and cyanidin-3GG in flowers of the mutant R27. It should be noted that the gradient system is changed from 10% to 25% methanol (final concentration, by vol) in 20 min (method IV) to 10% to 25% methanol (final concentration, by vol) in 7 min. This has no influence on the separation of the low substituted anthocyanins in this particular experiment but

Table II. Retention times on HPLC and R_f values on TLC of anthocyanins present in mutants of *Petunia hybrida*. As eluants solvent system IV (HPLC) and AMW on kieselgel (TLC) were used.

Compound	Retention time [min]	R_f value
delphinidin-3G	3.8	–
cyanidin-3GG	4.3	–
malvidin-3RG5G	4.5	–
delphinidin-3RG	4.9	–
cyanidin-3G	5.4	–
cyanidin-3RG	6.8	–
petunidin-3RG ac5G [a]	12.2	0.24
delphinidin-3RG ac5G	12.7	–
cyanidin-3RG ac5G	15.1	–
petunidin-3RG ac5G	15.5	0.36
malvidin-3RG ac5G [a]	17.3	0.26
peonidin-3RG ac5G	17.8	–
malvidin-3RG ac5G	18.0	0.41

[a] Different acylation (see text).

Table III. R_f values of acylated anthocyanins on paper chromatography using different solvent systems.

Compound	R_f value		
	BAW	1% HCL	HOAc-HCl
malvidin 3RG ac5G	0.35	0.30	0.56
malvidin 3RG ac[a] 5G	0.19	0.09	0.23
treatment with NAOH of both anthocyanins, malvidin 3RG5G	0.26	0.48	0.69

[a] Different acylation.

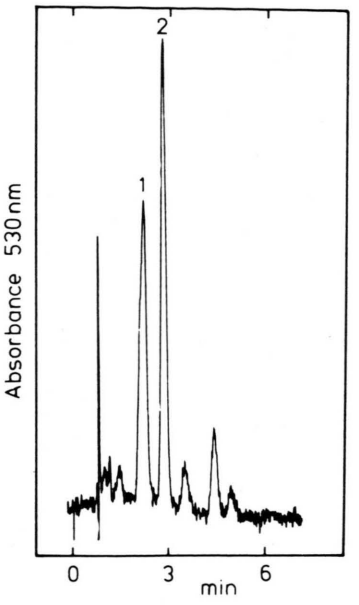

Fig. 2. Liquid chromatography of anthocyanins extracted from a flower of the mutant R27 (9 mg wet weight) 25 µl of a total Folch upperphase was injected. 1, Cyanidin-3-diglucoside; 2, Cyanidin-3-glucoside.

decreases separation of the higher substituted anthocyanins.

These results clearly indicate that, using one column and four solvent systems, information can be obtained on the flavonoid composition and cinnamic acid composition of flowers of *Petunia hybrida.* In some cases TLC is needed to obtain definite identification. Limited amounts of material are needed (less than one petal of a flower), because of the sensitivity of the method and the possibility to use one extract for detection of intermediates and anthocyanins.

The HPLC analysis of anthocyanins and other flavonoids described above is also of particular use in enzymological experiments. It provides a rapid and accurate determination of products formed upon incubation of anthocyanins with anthocyanin modifying enzymes [10].

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