The RP-HPLC Analysis of Anthocyanins

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Key Words

Column liquid chromatography
Acylated anthocyanins
Rudbeckia hirta L.
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Summary

Conditions were determined for the separation of a complex set of anthocyanins (free aglycones, mono- and multiglycosides and esterified forms) by HPLC. The optimised gradient elution method was then used to carry out qualitative and quantitative analysis of anthocyanin compounds present in the callus tissue of *Rudbeckia hirta L.* and the tubular flowers of the soil-based plant. The summary content of anthocyanin pigments and the content of the main pigment was identified in the analysed biomass. The method developed is useful for the purposes of monitoring the process of biosynthesis of anthocyanins in tissues obtained through in vitro cultures. The advantages of the method for anthocyanins and its application to other anthocyanin-rich materials are also discussed.

Introduction

The compounds of the anthocyanin group have a high therapeutic and nutritional value. [1–6]. Anthocyanins are notable for their unstable nature, as well as for the fact, that although they are common in the plant world, relatively few materials are rich in anthocyanins. [7–9]. Although numerous publications include information on separation of anthocyanins by PC, TLC and HPLC techniques, most of them describe research into either isolated standard samples or groups of compounds of fairly similar structure. [10–13]. Attempts to use the chromatographic methods described in the literature to separate anthocyanin compounds from callus tissue *Rudbeckia hirta* did not bring satisfactory results.

The purpose of this research was to develop a new chromatographic system which could be used to analyse quantitatively and qualitatively the content of anthocyanins in *R. hirta* callus.

The method of choice in monitoring the level of biosynthesis of secondary metabolites in in vitro cultures is HPLC, as it is reliable, repeatable and economical, in that it requires only small quantities of extract for analysis [14]. An HPLC system was: specially developed for effective monitoring of the biosynthesis of anthocyanins in biomass grown in vitro under various conditions.

Experimental

Equipment

The HPLC system (Knauer, Berlin) consisted of two Model 64-00 pumps, a solvent dynamic mixing chamber and a Model 87-00 UV detector, equipped with a Model 7125 injection valve (Rheodyne, Cotati, CA, USA) with a 20 µL loop, under computer control (Knauer HPLC, version 211a). The anthocyanins were separated on a LiChrospher RP-18 (5 µm) column (250 × 4 mm I.D.) (Merck, Darmstadt, Germany).

Reagents

The organic solvents were of HPLC grade (acetonitrile, methanol, formic acid-Merck).

Redistilled water was used. After preparation of the mobile phase it was filtered though a 0.48 µm filter (J.T. Baker, Phillipsburg, NY, USA).

Elution

The anthocyanins were separated by gradient elution using solvent A (water:formic acid (90:10) and solvent B (methanol:acetonitrile:water:formic acid 22.5:22.5:45:10) with the following gradient programme:
From 0 min to 1 min–100% A; from 1 min to 30 min–from 0% to 30% B in A (linear gradient); from 30 min to 60 min–30% B in A. A re-equilibration period of 10 min was used between individual runs. Elution was carried out at room temperature with a flow rate of 1.0 mL min^{-1} and detection at 530 nm (sensitivity 0.008AUFS).

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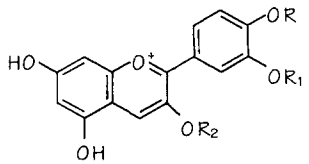

Figure 1
Structure of anthocyanins used as reference compounds.

No.	Compound	R	R_1	R_2
1	cyanidin-3-malonylglucoside	-OH	-H	glucose-malonate
2	delphinidin-3-monoglucoside	-OH	-OH	glucose
3	cyanidin-3-monoglucoside	-OH	-H	glucose
4	petunidin-3-monoglucoside	$-OCH_3$	-OH	glucose
5	paeonidin-3-monoglucoside	$-OCH_3$	-H	glucose
6	pelargonidin-3-monoglucoside	-H	-H	glucose
7	malvidin-3-monoglucoside	$-OCH_3$	$-OCH_3$	glucose
8	delphinidin	-OH	-OH	-H
9	cyanidin	-OH	-H	-H
10	petunidin	$-OCH_3$	-OH	-H
11	paeonidin	$-OCH_3$	-H	-H
12	pelargonidin	-H	-H	-H
13	malvidin	$-OCH_3$	$-OCH_3$	-H

Reference Compounds

Given the considerable difficulties in obtaining good quality standard samples of anthocyanin compounds, even from prominent chemical companies (due to the unstable nature of these compounds) it was necessary to make them as required. This method of obtaining standard samples of anthocyanin compounds for both glycosides and aglycones is commonly applied by many laboratories. [12, 15, 16].

The source of standard anthocyanin compounds in this research was plant material described in the literature as containing one quantitatively dominating compound [17]. These basic anthocyanins were isolated by preparative PC and TLC, and their structure was verified by m.p. TLC, PC, hydrolysis, UV, 1H-NMR and 13C-NMR.

The structures of the compounds used are shown on Figure 1.

Calibration

Stock solutions of compound **5**, cyanidin-3-malonylglucoside and **10**, cyanidin-3-monoglucoside, were prepared by dissolving 2 mg of the anthocyanins in 10 mL of methanol. The volumes injected (20 µL) corresponded to amounts of these compounds in the range 1–4 µg. Calibration graphs were obtained by plotting peak area (y) against concentration of standard solutions (x).

Regression equation for compound **5** $y = 6.527\,x + 0.577$ correlation coefficient $r = 0.997$

Regression equation for compound **10** $y = 8.665\,x + 0.098$ correlation coefficient $r = 0.999$

Sample Preparation

The material investigated was the tubular flowers of the plant of *R. hirta*. The plants and seeds to start in vitro cultures were obtained from the Medicinal Plant Garden of the Medical University of Gdańsk (Poland). In addition, chromatographic analysis was performed on callus tissues of *R. hirta* of hypocotyl origin, cultivated on modified Schenk-Hildebrandt medium (SH) and a specially developed two-phase system (SH and Miller media). The plant material was dried (10 g sample), pulverised and extracted at room temperature with a mixture composed of methanol:hydrochloric acid (1000:8.6). The extraction was carried out with 5 portions of the above mixture at pH 1.9. Then the acid methanol extracts were combined and evaporated at reduced pressure at the temperature of 35 °C to form syrup-like residue, and this was in turn diluted in 25 mL methanol (Merck). The solutions were filtered through 0.45 µm filters (J. T. Baker) and injected onto the chromatographic column.

Results and Discussion

The chromatographic system capable of separating anthocyanin compounds from a very complex set of compounds (basic aglycones, monoglucosides and ester compounds) was developed initially on the basis of a mixture of standard compounds. (Figure 2). This led to the optimisation of the chromatographic system with gradient elution. The system allowed for full separation of standard substances from a 13 component mixture, where the structure of the individual substances differed significantly. (Figure 1). Application of these conditions to the separation of the anthocyanins present in callus

tissues of *R. hirta*, resulted in complete separation of the pigments present. (Figures 3 and 4). The HPLC analysis of tubular flowers of the species obtained from the natural plant, showed the presence of only one compound: cyanidin-3-monoglucoside. The anthocyanin compounds, were separated with the use of solvents typical for the RP-HPLC of anthocyanins [7–9]. The mobile phase always included an acid moderator, in this case formic acid. It is widely accepted, that separation of anthocyanin compounds requires an acid component as an essential element in the mobile phase, to ensure a pH of 1.9–2.0. The purpose of such strong acidification is to protect the anthocyanins from the break-down of the permanent red form of flavylium cation into the colourless quinonoidal base [18]. At the same time very strong acidity has the great disadvantage that, it seriously reduces the life of the chromatographic columns. It has to be emphasised therefore, that the use of gradient elution with gradual reduction of formic acid content in the mobile phase made it possible not only to achieve full separation of anthocyanin compounds, but also extended the life of the column (approx. 1000 analyses). Such durability was not observed when anthocyanin compounds were separated by isocratic elution, where the mobile phase showed high acidity (pH = 1.9) throughout the entire process.

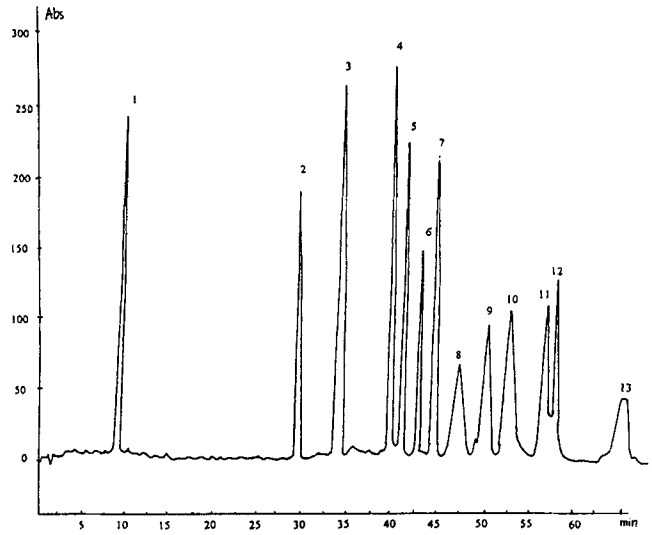

Figure 2

HPLC of anthocyanins used as reference compounds (gradient elution).
Peaks: **1** = cyanidin-3-malonylogluoside; **2** = delphinidin-3-monoglucoside; **3** = cyanidin-3-monoglucoside; **4** = petunidin-3-monoglucoside; **5** = paeonidin-3-monoglucoside; **6** = pelargonidin-3-monoglucoside; **7** = malvidin-3-monoglucoside; **8** = delpinidin; **9** = cyanidin; **10** = petunidin; **11** = paeonidin; **12** = pelargonidin; **13** = malvidin.

The idea of the chromatographic programme developed here was to apply interchangeably isocratic elution and a linear gradient. The system made it possible to separate the 13 component group of anthocyanin compounds in callus tissues of *R. hirta*. The compounds were washed away from the column in the sequence: first the most polar, i.e. esterified anthocyanin glycosides (comp. **1, 2, 3, 4, 5**), then multiglycosides (compounds **6, 7, 8, 9**), monoglycosides (comp.**10, 11, 12**) and finally free aglycone (comp. **13**).(Figures 3 and 4).

As shown on Figures 3 and 4 and Table I the separated anthocyanin group which was present in the material examined was only partially identified. One reason was the absence of standards, the other was low concentration of individual compounds in the group which made full identification impossible.

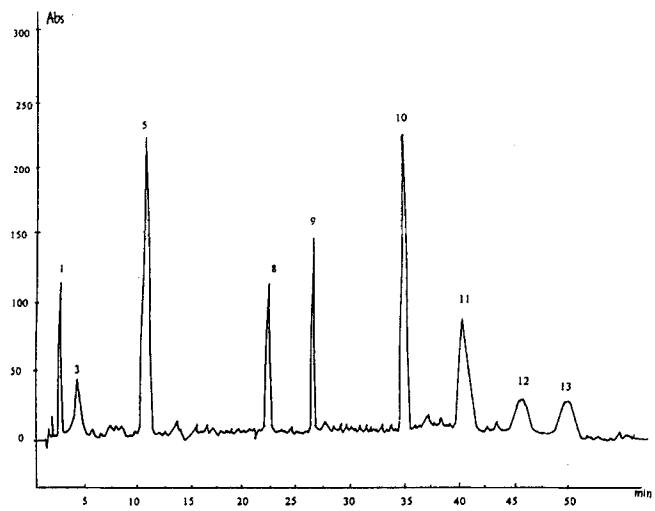

Figure 3

HPLC of anthocyanins from callus cultures of *R. hirta L.* maintained on Schenk–Hildebrandt medium (gradient elution).
Peaks: **5** = cyanidin-3-malonylogluoside; **10** = cyanidin-3-monoglucoside; **11** = petunidin-3-monoglucoside; **12** = malvidin-3-monogluoside; **13** = cyanidin.

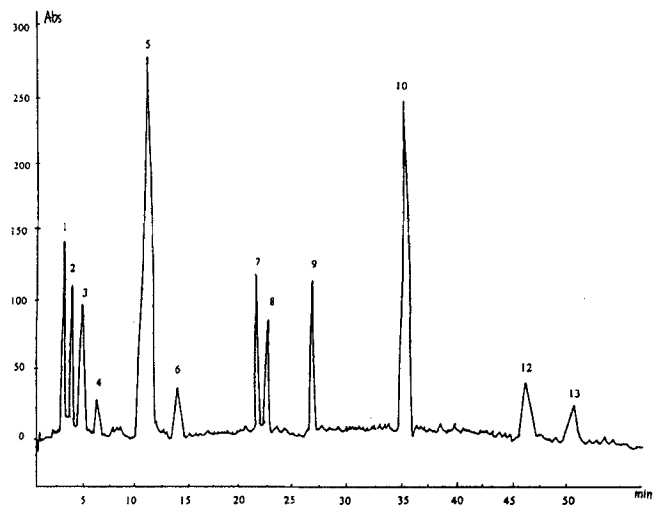

Figure 4

HPLC of anthocyanins from callus cultures of *R. hirta L.* maintained on two- phase growth system.
Peaks: **5** = cyanidin-3-malonylogluoside; **10** = cyanidin-3-monoglucoside; **12** = malvidin-3-monogluoside; **13** = cyanidin.

Table I. Anthocyanins from *Rudbeckia hirta L.* in soil grown material and in vitro cultures.

		Plant material		
		soil grown material	in vitro cultures	
No.	Compound	tubular flowers	callus from SH medium	callus from 2-phase growth medium
1*	Ester of delphinidin-3-0-monoglucoside	–	+	+
2*	Ester of cyanidin-3-0-monoglucoside	–	–	+
3*	Ester of cyanidin-3-0-monoglucoside	–	+	+
4*	Ester of cyanidin-3-0-monoglucoside	–	–	+
5	Cyanidin-3-malonylglucoside	–	+	+
6*	Multiglucoside of cyanidin	–	–	+
7*	Multiglucoside of cyanidin	–	–	+
8*	Multiglucoside of cyanidin	–	+	+
9*	Multiglucoside of cyanidin	–	+	+
10	Cyanidin-3-0-monoglucoside	+	+	+
11	Petunidin-3-0-monoglucoside	–	+	–
12	Malwidin-3-0-monoglucoside	–	+	+
13	Cyanidin	–	+	+

* - sup. Structure
SH- Schenk-Hildebrandt medium

Table II. Quantitative analysis of anthocyanins in plant material of *Rudbeckia hirta L.* (Amount in % dry material, n = 5).

Plant material	Amount of comp. **5** % dry material (n=5)	Amount of comp. **10**	Amount of all anthocy-anins	Stand-ard de-viation
tubular flowers (soil based plant)	–	0.28	0.28	0.00024
callus cultures from Schenk-Hildebrandt medium	0.40	0.44	1.60	0.0285
callus cultures from two-phase growth system	2.03	0.85	4.47	0.014

Detection at wavelength 530 nm in our chromatographic system also proved beneficial. It is known, that anthocyanins absorb at this wavelength and also at 280 nm. In HPLC analysis of anthocyanins both wavelengths are used interchangeably for various tasks [6, 8, 9]. There is, however, a degree of risk in using 280 nm, as this wavelength is also absorbed by other polyphenolic compounds which are often present in plant extracts (i.e. flavonoids, phenolic acids). Using 530 nm is useful, as in the case of primary plant material extracts which are a mixture of polyphenolic compounds including anthocyanins, separation produces a chromatogram which is a picture of the anthocyanin pigments only. This makes it possible to identify the total of anthocyanin compounds in the material under investigation even though the individual compounds are not available. This method of assessing the content of anthocyanins materials is commonly used when only one standard substance is available [6, 8]. That is why also in our case, the general amount of anthocyanins was calculated by summing the areas of all the peaks in the chromatogram. The total for anthocyanin compounds was determined by recalculating the results into 3-gl-cyanidin, which was used to create the regression equation.

It has to be emphasised, that detection at 530 nm meant that the extracts did not have to be purified by removing other, non-anthocyanin polyphenolic substances, which are normally an undesirable presence. This is particularly important, as any laboratory operations on anthocyanin extracts may result in decomposition of these compounds. In addition, the analysis time was shortened. The chromatography conditions developed here made it possible to determine qualitative differences between plant materials (Table I). The richest source of anthocyanins was callus tissue from the 2-phase growth system (12 compounds). The biomass grown on SH medium contained 9 compounds and the tubular flowers of the natural plant only contained a minimal quantity of one compound, cyanidin-3-monoglucoside (0.28 %). (Tables I, II). Moreover, the quantitative analysis showed, that the callus tissue from the 2-phase growth system contained 4.47 % of anthocyanins, so it is a rich source of this class of pigments. The dominant component of the anthocyanin group in this tissue was cyanidin-3-malonylglucoside (2.03 %). Similarly, the callus grown on SH medium produces a significant amount of anthocyanins (1.60 %) and within this pigment group, cyanidin-3-monoglucoside (comp. **10**)

(0.44 %) dominated slightly over compound **5**, cyanidin-3-malonylglucoside (0.40 %) (Table II).

Summing up, we have developed an effective chromatographic system for the separation of anthocyanis in *R. hirta* material from natural plants and in–vitro cultures. This in turn made it possible to monitor the biosynthesis of anthocyanins in the tissues micropropagated in-vitro, resulting finally in an increase of their contents in the callus to 4.47 %, which is a significant increase from that in the natural plant (0.28 %). The separation of standard compounds led to the conclusion, that the chromatographic system developed here may be used to separate anthocyanins from other plant materials.

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