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Preparative high-performance liquid chromatography for the purification of natural anthocyanins

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Abstract

Preparative HPLC was applied to the purification of anthocyanins from raw extracts of strawberry, elderberry, eggplant and radish and from enocyanin. For each separation, the chromatographic conditions were optimized to achieve an efficient purification in the shortest time. The anthocyanins isolated were compared with reference standards, whenever they were available. In addition, UV–visible characterization was carried out on all the anthocyanins purified. Determination of the complete structure of acylated anthocyanins will require further support from fast atom bombardment MS and NMR analyses, which are in progress.

1. Introduction

Anthocyanins are a widely distributed class of natural pigments, as they are found in many flowers and fruits, where they impart brilliant red and blue colours [1]. For example, the colours of berry fruits, such as strawberry, bilberry and cranberry, are due to many different anthocyanins. Having been eaten for thousands of years without any apparent adverse effects on human health, anthocyanins have attracted the interest of many food scientists and technologists when synthetic food colourants, many of the red type, have been questioned because of their adverse health effects and progressively banned from food composition. Unfortunately, anthocyanins are far less stable than synthetic dyes and undergo structural transformations which end up with loss of colour, especially under pH conditions typically found in many foods and drinks to which anthocyanins would have to be added. For this reason, they have been the subject of many studies addressing the problem of their stability.

We have focused particularly on the study of colour changes undergone by anthocyanins [2] and from the beginning we faced the problem of the limited commercial availability of pure anthocyanins. As analytical HPLC had been proved to be very effective for the identification of anthocyanins in many natural sources [3–8], preparative HPLC seemed to be the right tool to isolate the needed anthocyanins at the high degree of purity desired. In contrast to analytical HPLC, preparative HPLC has received less attention. Teharara and co-workers [9,10] and Sen Lu et al. [11] used preparative HPLC for the purification of acylated anthocyanins from some species of flowers, while Shi and co-workers purified acylated anthocyanins from stems and leaves of Tradescantia [12,13] and from sweet potato [14], in the view of their potential use as food colourants.

We started by studying the simple 3-glucoside

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of the most common anthocyanidins, namely delphinidin (Dp), cyanidin (Cy), pelargonidin (Pg), petunidin (Pt), peonidin (Pn) and malvidin (Mv). Enocyanin, a concentrate from red grape skins, is known to contain the 3-glucosides of Dp, Cy, Pt, Pn and Mv, while strawberry was chosen as the source of Pg-3-glucoside. Elderberry was chosen owing to its higher Cy-3-glucoside content compared with enocyanin, and because anthocyanins with two and three sugar moieties could be isolated [8].

After the first results showing the limited colour stability of simple 3-glucosides [2], the acylated anthocyanins were chosen for further investigation. For this reason, we studied the purification of pigments extracted from eggplant and radish. This paper reports the results of preparative HPLC on the above-mentioned extracts.

2. Experimental

2.1. Materials

Frozen strawberries were purchased at a local supermarket. Eggplant and radish were purchased at a local grocery. Elderberries were collected from bushes in a public garden during September 1991 and stored at −20°C until used. Enocyanin was a gift from Enocanossa (Ciano d'Enza, Italy).

Some anthocyanins were purchased in limited amounts from Extrasynthèse (Génay, France), viz., Pg-3-glucoside, Cy-3-glucoside and 3,5-diglucoside, Pn-3-glucoside and Mv-3-glucoside.

2.2. Pigment extraction

Frozen strawberries (2500 g) were thawed and mixed in a food mixer for 5 min and the pomace obtained was filtered, yielding 1500 ml of clear extract. The residue was extracted three times with 100 ml of methanol (Merck, Darmstadt, Germany) containing 0.01 mol l^{-1} hydrochloric acid (Aldrich, Milwaukee, WI, USA). The methanolic extracts were concentrated under reduced pressure, then mixed with the aqueous extract. As a first purification step, pigments were adsorbed on a column (20 × 2.5 cm I.D.) filled with Amberlite GC-50 ion-exchange resin (Aldrich), then the column was washed with HCl at pH 2 and finally the pigments were eluted with methanol containing 0.01 mol l^{-1} HCl. The eluate was concentrated under reduced pressure and then freeze-dried, yielding 1.2 g of dried extract, to be further purified by preparative HPLC.

Elderberries (650 g) were blended in a food mixer and the pomace obtained was extracted overnight with methanol containing 0.01 mol l^{-1} HCl (300 ml). After filtration, the solution was concentrated under reduced pressure and then freeze-dried, resulting in 1.8 g of extract.

Enocyanin (50 g) was extracted with 100 ml of 0.01 mol l^{-1} HCl in methanol with magnetic stirring for several hours. The residue was filtered and extracted several times until the solvent was almost colourless. The total volume of acid methanol, 500 ml, was reduced to 100 ml by distillation under reduced pressure and pigments were precipitated with 800 ml of diethyl ether. The precipitation step was then repeated, resulting in 15 g of purified pigment mixture.

Eggplants (1120 g) were peeled, obtaining 87 g of skin, which was cut into small pieces and extracted with 250 ml of formic acid in water (5%, v/v) overnight at 4°C. After filtration, the extract was adsorbed on a Sep-Pak Vac C_{18} cartridge (Waters, Milford, MA, USA), containing 10 g of packing material. The column was washed five times with 20 ml of HCl at pH 1 and dried under vacuum and the pigments were eluted with 25 ml of methanol containing 0.025 g of HCl. After concentration and freeze-drying, 0.48 g of pigment mixture was obtained.

Radishes (900 g) were peeled, yielding 100 g of skin, which was extracted under the conditions used for eggplant; 0.42 g of mixed pigments was obtained.

Tradescantia leaves (4 g) were chopped with scissors, suspended in 50 ml of formic acid solution, treated in a Ultra-Turrax homogenizer (Janke and Kukkal, Staufen Germany) and extracted overnight at 4°C. The filtered extract was adsorbed on a Sep-Pak Vac C_{18} cartridge (2 g),

then the column was washed with 25 ml of HCl at pH 1 and then dried by suction. The pigments were eluted with 3 ml of methanol–water 80:20 (v/v). In this preliminary trial, no quantification was made on the purified extract, owing to the very limited quantity of leaves available.

2.3. Instrumentation and conditions

The chromatographic system was composed of two Model 510 pumps, a model 481 variable-wavelength UV–Vis detector, a Model 730 integrator and a Model 721 system controller (all from Waters). Injections were carried out with a Model 7725i injector (Rheodyne, Cotati, CA, USA) equipped with a 20-μl loop for analytical HPLC and a 1-ml loop for preparative work.

The analytical column was a Spherisorb ODS-2 (250 × 4.6 mm I.D.) (Phase Separations, Deeside, Clwyd, UK), 10 μm, pore size 80 Å; the flow-rate was set at 1 ml min^{-1}. The preparative column (250 × 10 mm I.D.) with the same stationary phase was used at a flow-rate of 3 ml min^{-1}. For both columns, a μ-Bondapak C_{18} guard insert column (Waters) was used. Both columns were operated at room temperature.

The mobile phase was composed of 5% formic acid (pH 2.2 at 20°C) and methanol, in different ratios according to the separation needs. The acid concentration was chosen so that the pH of this solution obtained was a good compromise between the need to avoid too low a pH to hydrolyse silica-ODS bonds while maintaining anthocyanins in the highly coloured flavilium ion form, to enhance detection at 500–530 nm. The organic and aqueous phases were degassed by filtration through 0.45-μm membranes (Millipore, Bedford, MA, USA) under vacuum.

The elution conditions were as follows.

Strawberry: analytical runs, 0–10 min 30% methanol, 10–20 min from 30 to 35% methanol, 20–25 min from 35 to 45%, held constant up to 30 min; preparative runs, isocratic with 35% methanol. A 5-mg amount was injected in each run.

Elderberry: analytical runs, 0–17 min 27% methanol, 17–30 min from 27 to 50% methanol; preparative runs, isocratic with 30% methanol. An 18-mg amount was injected in each run.

Enocyanin: analytical runs, 0–10 min from 30 to 35% methanol, 10–18 min from 35 to 40% methanol, held constant up to 22 min, 22–25 min from 40 to 45% methanol, held constant up to 30 min; preparative runs, 0–15 min 30% methanol, 15–25 min from 30 to 40% methanol, 25–30 min from 40 to 50% methanol. A 10-mg amount was injected in each run.

Radish: analytical runs, 0–20 min from 35 to 45% methanol, 20–25 min from 45 to 55% methanol, held constant up to 30 min; preparative runs, 0–10 min 40% methanol, 10–25 min from 40 to 50% methanol, held constant up to 30 min. A 5-mg amount was injected in each run.

Eggplant: analytical runs, 0–4 min 25% methanol, 4–25 min from 25 to 35% methanol, 25–30 min from 35 to 50% methanol, held constant up to 35 min; preparative runs, isocratic with 25% methanol. A 5-mg amount was injected in each run.

All gradient steps were linear.

UV–visible spectra were recorded on a Lambda 15 spectrophotometer (Perkin-Elmer, Norwalk, CT, USA) in 1-cm quartz cells, using 0.01% (v/v) HCL in methanol as solvent.

3. Results and discussion

Results obtained from the purification of the different raw extracts are reported in separate tables, along with the UV–visible data. Retention times reported in Tables 1–5 were obtained on the analytical column.

3.1. Strawberry

The three more abundant anthocyanins found in strawberry extract were isolated and characterized as reported in Table 1. F1 and F2 were compared with authentic samples and were identified without uncertainty as Cy-3-glucoside and Pg-3-glucoside, respectively, while the identification of F3 as Pg-3-arabinoside was based on UV–Vis data, which show that the aglycone is

Table 1
Characterization of the three more abundant anthocyanins isolated from strawberry

Peak No.	Anthocyanin	t_R (min)[a]	Concentration in raw extract (%)[b]	Purity %[b]	UV–visible characterization				
					λ_{max} (nm)	E_{UV}/E_{vis} (%)	E_{acyl}/E_{vis} (%)	E_{440}/E_{vis} (%)	Shift with $AlCl_3$ (nm)
F1	Cy-3-glucoside	14.15	2.2	95.0	281, 524	75	–	22	19
F2	Pg-3-glucoside	19.02	90.0	99.0	270, 508	63	–	38	None
F3	Pg-3-arabinoside[c]	23.18	4.2	95.0	270, 511	81	–	40	None

[a] Retention times refer to the separation on the analytical column.
[b] Calculated from the ratio of the peak area to the total area of peaks corresponding to anthocyanins.
[c] Tentative identification based on spectral characterization and chromatographic behaviour.

pelargonidin, and on the later elution of 3-arabinoside with respect to 3-glucoside [4].

The maximum amount of extract that could be purified in a single run on the preparative column was 5 mg. Injections of larger amounts resulted in strong distortion of Pg-3-glucoside peak, which started to overlap with the Pg-3-arabinoside peak with a consequent loss of separation.

3.2. Enocyanin

Table 2 summarizes the characterization data obtained for the fractions isolated from enocyanin. The UV–Vis data are in good agreement with those reported in the literature [15,16]. The enocyanin sample used in this study contained very limited amounts of diglucosides and acylated pigments which would have preceded Dp-3-glucoside (the former) and followed Mv-3-glucoside (the latter). This observation suggests the use of *Vitis vinifera* berries as the starting material for preparing the pigment concentrate.

3.3. Elderberry

Table 3 reports the results of the purification and characterization of pigments from elderberry. The purity of Cy-3-glucoside and Cy-3-sambubioside was not as high as expected. The separation of these two anthocyanins with methanol as organic solvent is then difficult; the same problem has been reported by other workers using acetonitrile [4]. Brønnum-Hansen and Hansen [8] reported on this separation carried out with tetrahydrofuran as a substitute for methanol. It should be noted that the comparison between the composition reported by Brønnum-Hansen and Hansen [8] and that reported

Table 2
Characterization of anthocyanins isolated from enocyanin

Peak No.	Anthocyanin	t_R (min)[a]	Concentration in raw extract (%)[b]	Purity %[b]	UV–visible characterization				
					λ_{max} (nm)	E_{UV}/E_{vis} (%)	E_{acyl}/E_{vis} (%)	E_{440}/E_{vis} (%)	Shift with $AlCl_3$ (nm)
E1	Dp-3-glucoside	10.46	17.4	99.5	279, 540	62	–	20	45
E2	Cy-3-glucoside	13.45	3.7	96.0	281, 530	66	–	22	35
E3	Pt-3-glucoside	15.64	20.9	98.5	279, 539	63	–	20	46
E4	Pn-3-glucoside	19.15	5.8	93.5	280, 529	64	–	26	None
E5	Mv-3-glucoside	20.63	45.8	99.6	278, 540	58	–	20	None

[a,b] See Table 1.

Table 3
Characterization of anthocyanins isolated from elderberry

Peak No.	Anthocyanin	t_R (min)[a]	Concentration in raw extract (%)[b]	Purity %[b]	UV–visible characterization				
					λ_{max} (nm)	E_{UV}/E_{vis} (%)	E_{acyl}/E_{vis} (%)	E_{440}/E_{vis} (%)	Shift with $AlCl_3$ (nm)
S1	Cy-3,5-diglucoside	6.70	15.7	99.7	279, 526	56	–	14	37
S2	Cy-3-sambubioside	16.62	59.8	94.0	282, 529	58	–	22	34
S3	Cy-3-glucoside	17.83	20.4	90.7	282, 529	60	–	23	36

[a,b] See Table 1.

here reveals a higher content of Cy-3,5-diglucoside in our sample (15.7% instead of 0.8%).

3.4. Radish

Radish extract was purified to obtain acylated anthocyanin. Fig. 1 shows the chromatograms of radish extract before and after a mild alkaline treatment by which acyl acids are selectively removed from sugars [17]. It appears clearly that the three main anthocyanin in radish extract are acylated, along with at least other four minor pigments. The preparative purification was then directed to the isolation of the three main anthocyanins and the more abundant of the four minor anthocyanins.

Table 4 gives the characterization data for the purified radish anthocyanins. The high values of E_{acyl}/E_{vis} ratios and the comparison between the chromatograms obtained before and after the mild alkaline hydrolysis of each pigment after purification confirmed the presence of acyl acids. The UV–vis data also suggest that Pg is the aglycone in all the pigments and this was further confirmed by comparing the chromatogram of an authentic Pg sample (obtained from the acid hydrolysis of Pg-3-glucoside) with the chromatograms of purified pigments R1–R4 after the same treatment [18]. The values of the E_{440}/E_{vis} ratios compare well with those published for Pg pigments substituted at the 3- and 5-positions [19].

The identification of the different anthocyanins is made more complex by the absence of reference standards. The determination of sugar/aglycone ratios and of sugars and acyl acids is in progress but the complete structures will have to be confirmed by fast atom bombardment (FAB) MS and NMR studies, in order to determine the exact position of sugar–aglycone and acyl acid–

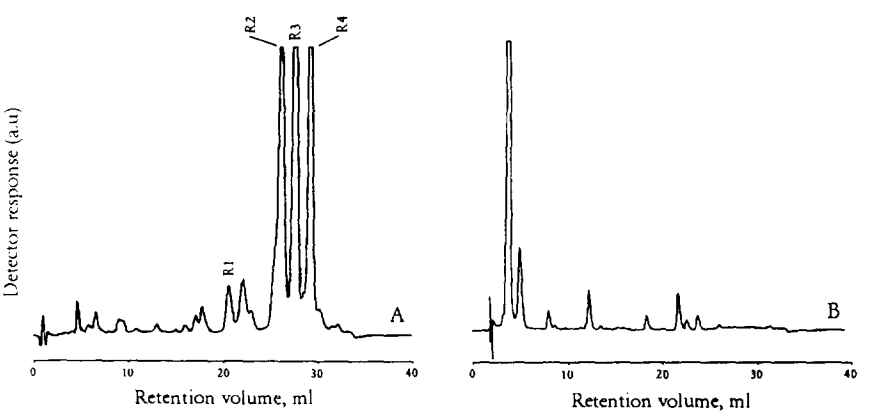

Fig. 1. Chromatograms obtained with the analytical column for radish extract. (A) Raw extract as such; (B) extract after mild alkaline hydrolysis. Peaks are labelled according to Table 4.

Table 4
Characterization of anthocyanins isolated from radish

Peak No.	Anthocyanin	t_R (min)[a]	Concentration in raw extract (%)[b]	Purity %[b]	UV–visible characterization				
					λ_{max} (nm)	E_{UV}/E_{vis} (%)	E_{acyl}/E_{vis} (%)	E_{440}/E_{vis} (%)	Shift with $AlCl_3$ (nm)
R1	Pg-3,5-diglycoside, acylated[c]	20.71	3.1	92.1	279,328,509	78	68	20	None
R2	Pg-3,5-diglycoside, acylated[c]	26.30	25.3	91.4	286,317,507	77	64	20	None
R3	Pg-3,5-diglycoside, acylated[c]	27.75	38.0	94.1	286,318,508	72	58	19	None
R4	Pg-3,5-diglycoside, acylated[c]	29.36	26.0	92.0	287,318,508	72	59	19	None

[a–c] See Table 1.

sugar linkages. At present a tentative identification based on the few literature data available [20] suggests that R2, R3 and R4 are Pg-5-glucoside-3-diglucosides acylated with caffeic, ferulic and coumaric acid, respectively.

3.5. Eggplant

Fig. 2 shows the chromatograms of a raw eggplant extract before and after mild alkaline treatment. Eggplant extract purification was also carried out to obtain acylated anthocyanins, but it turned out that they were just a minor fraction in the pigment mixture. It was known from the literature that eggplant varieties harvested in Japan contain mainly highly acylated anthocyanins [21,22], whereas varieties from other countries contain no acylated pigments at all [23]. The variety used in this study is intermediate, but in any case it is not a good source of acylated anthocyanins.

Table 5 reports the characterization data for the two fractions isolated. The aglycone is Dp for both fractions, as suggested by UV–vis data and confirmed by comparison with a chromatogram of an authentic Dp sample obtained from Dp-3-glucoside.

The E_{440}/E_{vis} ratio for M1 is in good agree-

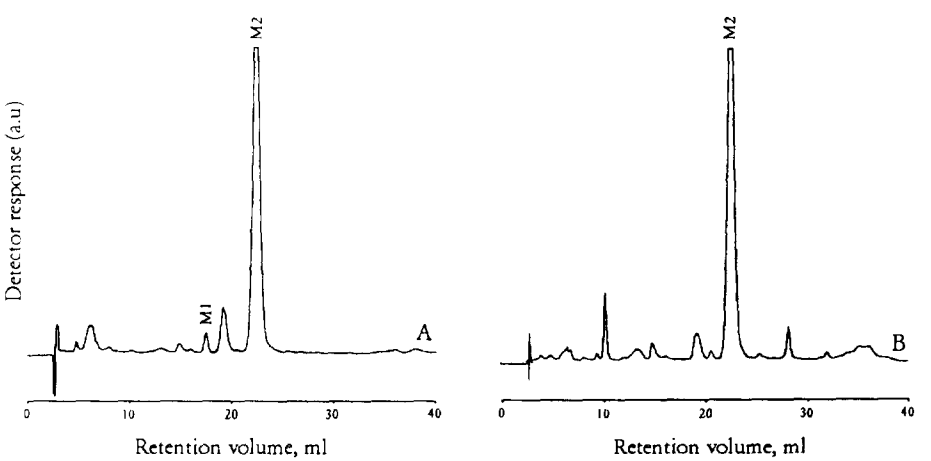

Fig. 2. Chromatograms obtained with the analytical column for eggplant extract. (A) Raw extract as such; (B) extract after mild alkaline hydrolysis. Peaks are labelled according to Table 5.

Table 5
Characterization of anthocyanins isolated from eggplant

Peak No.	Anthocyanin	t_R (min)[a]	Concentration in raw extract (%)[b]	Purity %[b]	UV–visible characterization				
					λ_{max} (nm)	E_{UV}/E_{vis} (%)	E_{acyl}/E_{vis} (%)	E_{440}/E_{vis} (%)	Shift with $AlCl_3$ (nm)
M1	Dp-3,5-diglucoside, acylated[c]	17.53	7.5	97.1	281,323,540	80	80	11	48
M2	Dp-3-arabinoside[c]	22.47	87.0	95.0	283,542	75	–	17	46

[a–c] See Table 1.

ment with the value reported for Dp-3,5-diglucoside [18], so that M1 can be an acylated derivative of such an anthocyanin, whereas the value for M2 is the same as that reported for a Dp-3-glycoside. As M2 is eluted after Dp-3-glucoside, the sugar in M2 could be arabinose, but further evidence is needed to support this suggestion.

In conclusion, it has been shown that preparative HPLC is an efficient method to isolate pure anthocyanins, either simple monoglucosides or more complex acylated di- and triglycosides, from natural sources. For these more complex types of pigments, the lack of reference standards makes identification difficult and further evidence from FAB-MS and NMR spectroscopy is needed. Such experiments are in progress, along with the identification of sugars and acyl acids.

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