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Short communication

Combination of chromatographic techniques for the preparative isolation of anthocyanins — applied on blackcurrant (*Ribes nigrum*) fruits

Cato Frøytlog, Rune Slimestad, Øyvind M. Andersen*

Department of Chemistry, University of Bergen, N-5007 Bergen, Norway

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Abstract

A combination of column chromatography on Toyopearl HW-40F gel and reversed-phase high-performance liquid chromatography enabled us to preparatively separate anthocyanins, without any re-application of overlapping bands of the major (the 3-*O*-glucosides and 3-*O*-rutinosides of delphinidin and cyanidin) and minor (the 3-*O*-rutinosides of peonidin and malvidin) anthocyanins in a 0.81-g sample from blackcurrant (*Ribes nigrum*) fruits. Anthocyanins substituted with methoxyl groups on the aglycone have never been detected previously in the genus *Ribes*. By variation of sample loading, flow-rate and solvent strength, Toyopearl HW-40F gel gave rise to chromatograms with higher resolution between the anthocyanidin 3-rutinoside and anthocyanidin 3-glucoside bands than Sephadex LH-20 gel, in all cases.

Keywords: Blackcurrant; Preparative chromatography; Anthocyanins

1. Introduction

The water-soluble anthocyanins are responsible for most of the red, violet and blue colours in plants [1]. The colour quality of fresh and processed fruits and vegetables, together with documented health benefits, have led to renewed interest in these pigments as part of our diet [2,3]. Anthocyanins consist of an aglycone and one or more sugar units, which may be acylated with aliphatic or aromatic acids.

Blackcurrant (*Ribes nigrum* L) is one of the most examined species with respect to anthocyanins, and the individual anthocyanins have been widely used for reference purposes [4]. Chandler and Harper [5] have identified the 3-glucosides and 3-rutinosides of cyanidin and delphinidin in addition to the aglycones cyanidin and delphinidin in blackcurrant fruits. Other papers [3,6–10] have reported the same anthocyanins and no aglycones in these dark fruits. The same four anthocyanins, together with pelargonidin 3-rutinoside, and the 3-sophoroside of cyanidin and delphinidin, were identified by LeLous et al. [11]. The blackcurrant fruits are a good source of anthocyanins (250 mg/100 g of fresh fruit) [6].

Based on factors like high resolution and speed, high-performance liquid chromatography (HPLC) has, in recent years, been the most promising method for the preparative isolation of anthocyanins [12,13]. However, about 400 different anthocyanins have been reported to occur naturally in plants. They show

*Corresponding author.

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limited structural variation, have similar chromatographic behaviour and, in many cases, preparative chromatographic techniques give rise to band-overlap. In most cases, a single preparative chromatographic technique will not be sufficient for the proper isolation of all of the anthocyanins within an extract containing many compounds, especially if the anthocyanins occur in minor amounts. It would therefore be advantageous to combine preparative HPLC with chromatographic techniques using separation mechanisms other than those previously reported for the preparative isolation of anthocyanins [7,14–16]. In this work, a combination of preparative HPLC and column chromatography was used for the isolation of anthocyanins from blackcurrant fruits of the genus *Ribes*. The performance of the column material Toyopearl HW-40F is compared with that of Sephadex LH-20 material. The paper presents the first application of Toyopearl for the isolation of anthocyanins. This material has previously been used for the separation of flavonoid classes other than anthocyanins in black tea [17], and for the partial fractionation of some isoflavonoids in natto [18].

2. Experimental

2.1. Pigment extraction and purification

Blackcurrant fruits were collected at Foldøy, on the West coast of Norway. Frozen berries (370 g) were extracted for 6 h at 4°C with 1 l of methanol (Fisher Scientific, UK) containing 0.1% trifluoroacetic acid (TFA; Merck, Darmstadt, Germany). The extraction procedure was repeated three times. After concentration under reduced pressure (Büchi R-124 Rotavapor, Switzerland), the extract was diluted with water to a total volume of 250 ml and partitioned against ethyl acetate (Fisons, Loughborough, UK) (3×250 ml). The water layer containing the anthocyanins was then concentrated to 100 ml.

The anthocyanin sample was subjected to an Amberlite XAD-7 (Sigma, USA) column and washed with 1.5–2.0 l of distilled water. At neutral pH, the anthocyanins were eluted using methanol containing 0.05% TFA (1 l). The dried anthocyanin sample (1.62 g) was dissolved in 92.5 ml of 30% aqueous methanol containing 0.05% TFA.

2.2. Chromatography

The columns (C 10/40, Pharmacia, Sweden) were packed with Sephadex LH-20 (Pharmacia, Sweden) or Toyopearl HW-40F (Tosohaas, Japan) to a height of 36 cm, using 30% methanol with 0.05% TFA. The column (C 16/100, Pharmacia, Sweden) used in combination with preparative HPLC was packed with Toyopearl HW-40F to a height of 80 cm and the sample (0.81 g) was eluted with 30–60% methanol containing 0.05% TFA. Fractions of 16 ml were collected and investigated by thin-layer chromatography (TLC) and analytical HPLC.

The analytical HPLC system (Hewlett Packard, Model 1050) was equipped with a diode-array detector, a 20-μl loop and a 200×4.6 mm ODS Hypersil column, 5 μm (Hewlett Packard). For the preparative separations, a Gilson 305/306 pump system was employed together with a Hewlett Packard 1040A UV detector and a 250×10 mm Econosil C_{18} column, 10 μm (Alltech, USA). Both HPLC systems were operated at room temperature using the same solvents: HCO_2H–H_2O (1:9, v/v) (A) and HCO_2H–H_2O–methanol (1:4:5, v/v/v) (B). The elution profile for analytical HPLC consisted of isocratic elution (90% A, 10% B) in 4 min, a linear gradient from 10% B to 100% B over the next 17 min, isocratic elution (100% B) in 4 min followed by a linear gradient from 100% B to 10% B over 1 min. The flow-rate was 1.0 ml min^{-1}, and aliquots of 15 μl were injected. The elution profile for preparative separations consisted of isocratic elution (90% A, 10% B) in 2 min, a linear gradient from 10% B to 60% B during the next 3 min, a linear gradient from 60% B to 90% B during the next 12 min, a linear gradient from 90% B to 100% B over 1 min, followed by a linear gradient from 100% B to 10% B over 1 min. The flow-rate was 4.0 ml min^{-1}.

TLC was carried out on 0.1 mm cellulose F (Merck) with the solvent FHW (HCO_2H–conc. HCl–H_2O; 25:24:51, v/v).

2.3. Spectroscopy

UV/Vis absorption spectra were recorded on-line during HPLC analysis using a photodiode array detector (HP 1050, Hewlett-Packard). The relative quantitative data were based on the average values of

the absorptions on every second nm between 500 and 540 nm, without taking into account the different molar absorption coefficients of the pigments.

The ^{1}H NMR experiments were obtained at 600.13 MHz on a Bruker DRX-600 instrument at 25°C. The residual ^{1}H signal of the solvent (CF_3CO_2D–CD_3OD; 5:95, v/v) was used as a secondary reference (δ 3.4 from tetramethylsilane, TMS).

The mass spectra were obtained on a Quattro II (Micromass, UK) by flow injection into the electrospray source. The instrument was operated in the positive ion mode and calibrated using NaI. The mobile-phase carrier was a methanol–water (1:1, v/v) mixture containing 0.1% formic acid. The carrier was pumped into the source at a flow-rate of 100 μl/min. Data acquisition was obtained by scanning with the first quadrupole only from 50–1000 Da in 3 s scans. The samples were dissolved in 3% formic acid (in methanol) prior to analysis.

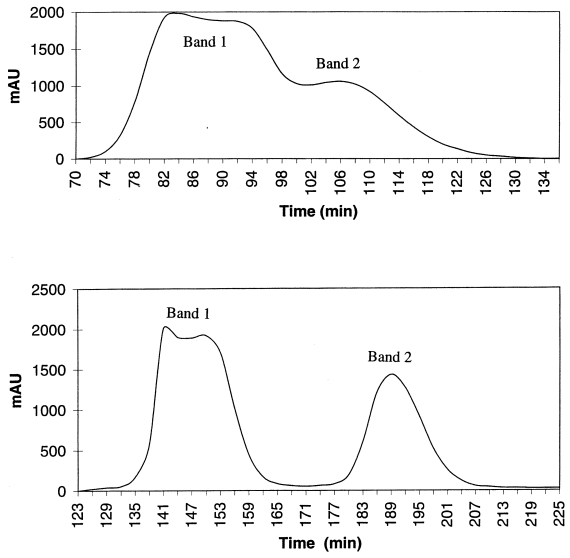


Fig. 1. Separation of anthocyanidin 3-rutinosides (band 1) and 3-glucosides (band 2) from fruits of blackcurrant on Sephadex LH-20 (top) and Toyopearl HW-40F (bottom), detected at 520±20 nm. Loading=1.30 mg, flow-rate=18 ml/h, solvent=60% methanol containing 0.05% TFA.

3. Results and discussion

3.1. Toyopearl HW-40F versus Sephadex LH-20 applied on anthocyanins

Using water–methanol mixtures as eluents, both Sephadex LH-20 and Toyopearl HW-40F gels separated the anthocyanins from blackcurrant according to their molecular masses. Thus, the anthocyanidin 3-rutinosides (band 1) were eluted prior to the anthocyanidin 3-glucosides (band 2) on both columns (Fig. 1). When sample loading and the methanol content in solvent was relative low, Sephadex LH-20 partially enabled the separation of each of the two 3-rutinosides and the two 3-glucosides (Table 1). The cyanidin glycosides were eluted prior to their more heavy delphinidin analogues, indicating that the separation mechanism was not based on exclusion alone. Under similar conditions, Toyopearl HW-40F did not separate the anthocyanins according to structural differences on the aglycone level. This property was however advantageous when Toyopearl HW-40F was used in combination with preparative reversed-phase HPLC. A distinct interpretable baseline separation of anthocyanin groups with different numbers of sugar units (Fig. 1, bottom) was favourable above those partially resolved bands obtained by Sephadex LH-20 (Table 1).

Increased flow-rate on Sephadex LH-20 material seemed to reduce the resolution between the anthocyanidin 3-rutinoside and anthocyanidin 3-glucoside bands (Table 1), in agreement with normal observations in liquid chromatography [19]. Surprisingly, on Toyopearl HW-40F under similar conditions, there seems to be a general tendency towards increased resolution with increasing flow-rate. However, there is only one determination for each combination of variables, and it is impossible to judge how much resolution differs under various conditions. For both gels, only small differences in resolution were observed when sample loading was increased from 0.17 to 1.3 mg (Table 1). Even when the same Toyopearl HW-40F column was loaded with a 6.4-mg anthocyanin sample, a resolution of 1.26 was observed.

3.2. Preparative reversed-phase HPLC separation of anthocyanins from blackcurrants

The use of preparative reversed-phase HPLC for the separation of anthocyanins from blackcurrants

Table 1
Anthocyanins in blackcurrants (*Ribes nigrum*), separated on Sephadex LH-20 and Toyopearl HW-40F gels and detected at 520 nm with the backpressure kept below 3 bar

Experiment number	Column material[a]	Loading (mg)	Flow (ml/h)	Methanol (%)	k (band 1)[b]		k (band 2)[b]		R_s		
					Cy	Dp	Cy	Dp	Band 1[b]	Band 2[b]	Main peaks
1	Sephadex LH-20	0.17	6	30	11.2	12.3	16.6	19.0	0.29	0.63	1.11
2	Sephadex LH-20	1.30	6	30	11.2	12.1	17.0	19.2	0.19	0.54	1.11
3	Sephadex LH-20	0.17	18	30	13.4	14.5	19.2	21.7	0.22	0.44	0.89
4	Sephadex LH-20	1.30	18	30	14.4	15.4	21.1	23.8	0.17	0.43	0.91
5	Sephadex LH-20	0.17	6	60	8.2		10.3				1.00
6	Sephadex LH-20	1.30	6	60	6.7		8.7				0.75
7	Sephadex LH-20	0.17	18	60	5.9		7.5				0.61
8	Sephadex LH-20	1.30	18	60	5.2		6.3				0.49
9	Toyopearl HW-40F	0.17	6	30	11.3		17.8				1.96
10	Toyopearl HW-40F	1.30	6	30	4.7		7.8				1.83
11	Toyopearl HW-40F	0.17	18	30	11.4		17.8				2.32
12	Toyopearl HW-40F	1.30	18	30	8.9		15.2				1.84
13	Toyopearl HW-40F	0.17	6	60	3.3		4.1				1.02
14	Toyopearl HW-40F	1.30	6	60	5.1		6.7				1.08
15	Toyopearl HW-40F	0.17	18	60	4.9		6.2				1.34
16	Toyopearl HW-40F	1.30	18	60	5.4		7.0				1.31
17	Toyopearl HW-40F	0.17	6	20	14.2		24.7				2.04
18	Toyopearl HW-40F	0.17	18	75	4.3		5.2				0.91
19	Toyopearl HW-40F	0.17	18	90	4.9		5.7				0.74
20[c]	Toyopearl HW-40F	6.40	18	60	5.8		7.8				1.26

There is one determination for each combination of variables.
Cy=cyanidin; Dp=delphinidin.
[a] Bed size: Toyopearl (8.0 g), Sephadex (6.8 g).
[b] Anthocyanidin 3-rutinosides (band 1), anthocyanidin 3-glucosides (band 2).
[c] UV detection at 440 nm.

showed some of the same limitations as found for the Sephadex LH-20 material, namely band overlapping at high loading (Fig. 2). However, when used after the removal of either the two 3-rutinosides (Toyopearl Band 1) or the two 3-glucosides (Toyopearl Band 2), we achieved baseline separation between the peaks in the HPLC chromatograms of each of the two Toyopearl bands. Toyopearl band 1 contained, in addition to the anthocyanidin 3-rutinosides (**2** and **4**), two anthocyanidin 3-rutinosides (**5** and **6**) that had not been reported previously from this species.

Table 2
Chromatographic and spectroscopic data on the anthocyanins in fruits of the blackcurrant (*Ribes nigrum*)

Pigment[a]	Aglycone	Sugar	Online HPLC				TLC (FHW) hR_F
			t_R (min)	Area (%)	Vis. max (nm)	A_{440}/A_{max} (%)	
1	Dp	Glc	11.09	12.9	525	28	15
2	Dp	Rut	11.76	30.6	529	26	35
3	Cy	Glc	12.21	9.3	518	30	25
4	Cy	Rut	12.88	43.6	520	30	48
5	Pn	Rut	13.57	1.5	522	26	60
6	Mv	Rut	14.58	2.1	528	32	57

[a] See Fig. 4.

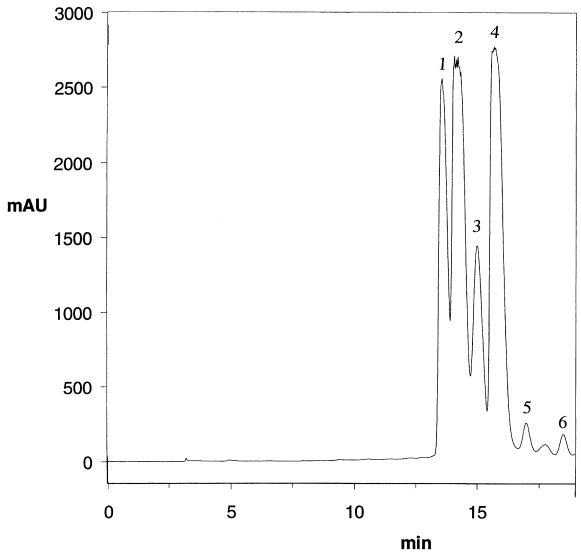


Fig. 2. Preparative HPLC separation of anthocyanins in the fruits of blackcurrant using an ODS 250×10 mm C_{18} (10 μm) Econosil column, detected at 520±20 nm. The amount of sample applied on the column was 4.3 mg. For peak identification, see Fig. 4.

3.3. Structural determination of anthocyanins in blackcurrant

Based on chromatographic and spectroscopic data (Table 2) and co-chromatography with authentic pigments, the identities of the major pigments (**1–4**) were confirmed to be delphinidin 3-glucoside (**1**), delphinidin 3-rutinoside (**2**), cyanidin 3-glucoside (**3**) and cyanidin 3-rutinoside (**4**).

The UV/Vis spectra of **5** showed a λ_{max} at 522 nm with an $A_{440}/A_{vis—max}$ of 26% (Table 2), indicating a cyanidin or peonidin nucleus with a sugar unit in the 3-position [20]. In the UV-region, 290–340 nm, there was no indication of acylation with an aromatic acid. The downfield part of the one-dimensional proton NMR spectrum of **5** showed a 3H ABX system at δ8.37 (*dd, J*=8.7, 2.4 Hz, H−6′), δ8.33 (*d, J*=2.4 Hz, H−2′) and δ7.16 (*d, J*=8.7 Hz, H−5′); a 2H AX-system at δ6.78 (*d, J*=2.0 Hz, H−6) and δ6.93 (broad *d,* H−8); a 1H singlet at δ9.08 (H−4), and a 3H singlet at δ4.06, in accord-

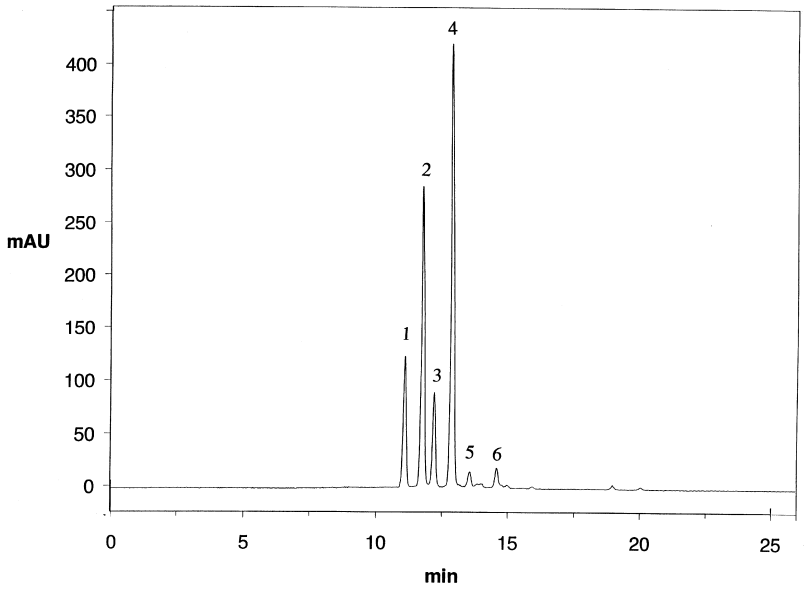
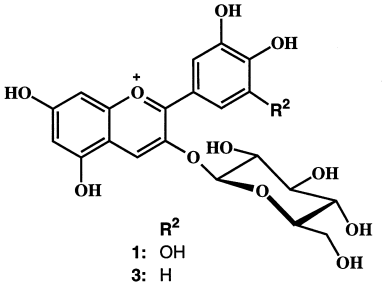


Fig. 3. Analytical HPLC separation of anthocyanins in fruits of the blackcurrant (*Ribes nigrum*) using an ODS Hypersil 5 μm, 200×4.6 mm column, detected at 520±20 nm. For peak identification, see Fig. 4.

ance with a peonidin nucleus [21]. The anomeric proton signals in the ^{1}H NMR spectrum of **5** appear considerably downfield of the other sugar resonances. Thus, the two doublets at δ5.38 (*d, J*=7.6 Hz, H−1 glucose) and δ4.74 (*d, J*=1.5 Hz, H−1 rhamnose), the 3H doublet at δ5.38 (*d, J*=6.2 Hz, H−6 rhamnose), together with the integration data and a molecular ion $[M+H]^+$ of *m/z* 609.2 found in the electrospray MS spectrum, confirmed the identity of **5** as peonidin 3-*O*-rutinoside.

The UV/Vis spectrum of **6**, taken during on-line HPLC, showed a visible maximum at 528 nm with an A_{440}/A_{528} value of 32% (Table 2), indicating a 3-glycoside with a delphinidin, petunidin or malvidin nucleus [20]. A molecular ion $[M+H]^+$ at *m/z* 639.2 identified **6** to be malvidin 3-*O*-rutinoside.



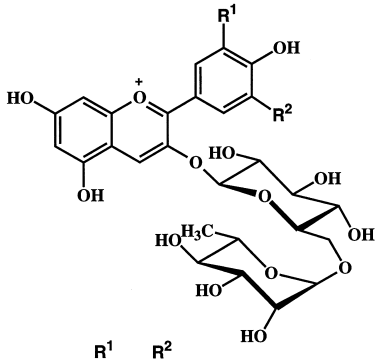


Fig. 4. Structures of the anthocyanins identified in fruits of the blackcurrant (*Ribes nigrum*); **1** and **3**: the 3-glucosides of delphinidin and cyanidin, respectively, **2** and **4–6**: the 3-rutinosides of delphinidin, cyanidin, peonidin and malvidin, respectively.

4. Conclusion

The analytical HPLC chromatogram of a crude extract of blackcurrant fruits showed four major anthocyanins (**1–4**) in addition to two minor pigments (**5** and **6**) (Figs. 3 and 4). Difficulties were experienced during the preparative HPLC isolation of both the major and minor compounds. The strategy, including the use of exclusion chromatography on Toyopearl HW-40F and ODS-HPLC, enabled us to preparatively isolate even the minor pigments **5** and **6**, which were elucidated to be peonidin 3-*O*-rutinoside and malvidin 3-*O*-rutinoside, respectively. Anthocyanins substituted with methoxyl groups on the aglycone, like **5** and **6**, have previously never been detected in the genus *Ribes*.

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