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Anthocyanins from Black Currants (*Ribes nigrum* L.)

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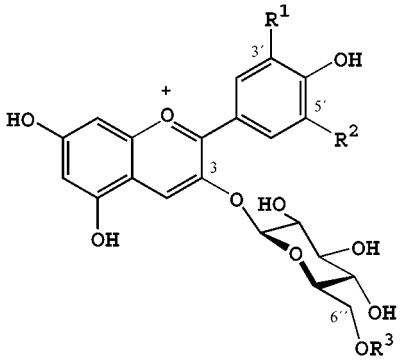
Fifteen anthocyanin structures are reported from an extract of black currant berries (*Ribes nigrum* L.). These are the 3-*O*-glucosides and the 3-*O*-rutinosides of pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin, cyanidin 3-*O*-arabinoside, and the 3-*O*-(6″-*p*-coumaroylglucoside)s of cyanidin and delphinidin. The anthocyanins were characterized by means of size exclusion chromatography, high-performance liquid chromatography, UV−visible spectroscopy, and electrospray mass spectrometry. The four main pigments (the 3-*O*-glucosides and the 3-*O*-rutinosides of delphinidin and cyanidin) made up >97% of the total anthocyanin content. The minor pigments were enriched from the extract by successive partition against ethyl acetate and by gel fractionation. These chromatographic steps were successfully used to isolate the acylated anthocyanins from the ethyl acetate layer and to separate cyanidin 3-*O*-arabinoside from the mixture of anthocyanins. The amounts of anthocyanin rutinosides were found to be higher than the amount of the corresponding glucosides for all detected pigments having the same aglycon moiety.

KEYWORDS: Black currants; *Ribes nigrum* L.; size exclusion chromatography; high-performance liquid chromatography; mass spectrometry; anthocyanins; delphinidin and cyanidin 3-*O*-(6″-*p*-coumaroylglucoside)s

INTRODUCTION

In recent years there has been an increased interest in exploring the health benefits of anthocyanins found in fruits and vegetables. The fruits of black currant (*Ribes nigrum* L.) are known to have a high content of anthocyanins (250 mg/100 g of fresh fruit) (*1*). The fruit is used to prepare extracts enriched in anthocyanins. Such extracts have been reported to have therapeutic benefits on the treatment of cardiovascular disorders and are in general reported to be free radical scavengers (*2*). Recently it has been demonstrated that orally administered anthocyanins from black currants are absorbed by humans and are found as intact anthocyanin glycosides in the blood (*3*, *4*). The focus on the health effects of the anthocyanins from black currants (*5*), together with the introduction of products based on these anthocyanins, has to be followed up by closer analytical inspections of the natural composition of the berries.

The four major anthocyanins in black currants, delphinidin 3-glucoside, delphinidin 3-rutinoside, cyanidin 3-glucoside, and cyanidin 3-rutinoside (**1**−**4**) (**Figure 1**), together with the aglycons cyanidin and delphinidin were first reported by Chandler and Harper (*6*). Pelargonidin 3-rutinoside (**9**), cyanidin 3-sophoroside, and delphinidin 3-sophoroside were reported together with the four main pigments by LeLous et al. (*7*). Upon preparative isolation of the main pigments, the occurrence of two minor compounds was described, peonidin 3-rutinoside (**11**)



	R^1	R^2	R^3
1	OH	OH	H
2	OH	OH	rhamnosyl
3	OH	H	H
4	OH	H	rhamnosyl
5	OMe	OH	H
6	OMe	OH	rhamnosyl
8	H	H	H
9	H	H	rhamnosyl
10	OMe	H	H
11	OMe	H	rhamnosyl
12	OMe	OMe	H
13	OMe	OMe	rhamnosyl
14	OH	OH	coumaroyl
15	OH	H	coumaroyl

Figure 1. Structures of the anthocyanins from black currants. Compound names are listed in **Table 1**.

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Table 1. Chromatographic and Spectroscopic Characteristics of Anthocyanins from Berries of Black Currant, *R. nigrum*

compd	t_R, min	$[M]^+$ *m/z*	fragments, *m/z*	UV–vis, nm	area, % (520 nm)	identity
1	10.33	465	303	278, 526	14.00	delphinidin 3-*O*-glucoside
2	10.80	611	303, 465	278, 528	36.59	delphinidin 3-*O*-rutinoside
3	12.08	449	287	280, 519	7.08	cyanidin 3-*O*-glucoside
4	12.81	595	287, 449	281, 519	40.15	cyanidin 3-*O*-rutinoside
5	13.11	479	317	278, 527	0.05	petunidin 3-*O*-glucoside
6	13.60	625	317, 479	278, 529	0.92	petunidin 3-*O*-rutinoside
7	13.20	419	287	281, 519		cyanidin 3-*O*-arabinoside
8	14.19	433	271	283, 507		pelargonidin 3-*O*-glucoside
9	14.88	579	271	278, 507	0.05	pelargonidin 3-*O*-rutinoside
10	15.09	463	301	280, 519	0.08	peonidin 3-*O*-glucoside
11	15.43	509	301, 463	281, 519	0.91	peonidin 3-*O*-rutinoside
12	15.69	493	331	278, 531	0.03	malvidin 3-*O*-glucoside
13	15.98	639	331, 493	278, 531	0.13	malvidin3-*O*-rutinoside
14	21.41	611	303	282, 315sh, 534		delphinidin 3-*O*-(6″-coumaroylglucoside)
15	22.39	595	287	285, 315sh, 524		cyanidin 3-*O*-(6″-coumaroylglucoside)

and malvidin 3-rutinoside (**13**) (*8*). We here report on the detection and relative amount of 15 anthocyanins from black currants.

MATERIALS AND METHODS

Sample Preparation. Black currant berries (*R. nigrum* cv. Titania) were collected mid-August 2001 at Ogna, Norway, and immediately stored at −10 °C. Voucher specimens are deposited at Polyphenols Laboratories. Frozen berries (500 g) were extracted with 2 L of aqueous methanol [0.1% trifluoroacetic acid (TFA), v/v] for 24 h. The extraction was repeated, and the combined extracts were purified according to established procedures by means of ethyl acetate/water partitioning and adsorption chromatography on a bed of Amberlite XAD-7 (*9*). The ethyl acetate fractions (3 × 250 mL) were combined and concentrated on a rotary evaporator.

Fractionation. Half of the amount of the purified extract was dissolved in 50 mL of 20% methanol (0.1% TFA, v/v), applied to a bed of 500 mL of TSK-gel Toyopearl HW-40F (Toso Corp.,) in a 5 × 30 cm column (XK 50/30, Pharmacia, Uppsala, Sweden), and eluted using a step gradient of methanol in water from 20 to 60%. All solvents contained 0.1% TFA (v/v). The flow rate was adjusted to 3.2 mL/min, and a total of 32 fractions were collected from three chromatographic bands.

Preparative HPLC. Compounds **14** and **15** were isolated as a mixture from the ethyl acetate phase of the crude extract by means of preparative HPLC. The instrument consisted of a Waters 600 pump connected to a Waters 996 photodiode array detector (PAD). Aliquots (1 mL) were manually injected through a Rheodyne injector and loaded onto a preparative 300 mm × 19 mm i.d. Nova-Pak column (Waters). The mobile phase solvents were (A) 5% formic acid and (B) 75% methanol/2.5% formic acid. The elution protocol (B in A) consisted of a linear gradient from 20 to 60% in 8 min, from 60 to 75% for the next 8 min, from 75 to 100% in 1 min, and then isocratic elution for 2 min. The flow rate was 10 mL/min. Fractions containing the two pigments were collected and concentrated under reduced pressure.

Alkaline Hydrolysis. Stepwise and complete hydrolysis of the acyl group of compounds **14** and **15** was performed according to established procedures (*10*).

HPLC-UV—Vis-MS. For the characterization of the anthocyanins a Waters 2690 instrument connected to a PAD (Waters 996) and a mass spectrometer equipped with an electrospray probe (Platform LCZ, Micromass) was used. Separation was performed on a 125 mm × 3 mm i.d., 3 μm, Hypersil ODS column (Agilent Technologies). The binary solvent system consisted of (A) 0.05% TFA and (B) 0.05% TFA/acetonitrile (1:1). The gradient (B in A) was linear from 20 to 35% for 10 min, from 35 to 55% for 8 min, isocratic for 2 min, from 55 to 80% for 2 min, and finally from 80 to 20% for 2 min. The flow rate was 0.4 mL/min, and aliquots of 10 μL were injected. The column oven temperature was set to 20 °C. PAD spectra were measured over the wavelength range 240−600 nm in steps of 2 nm. Mass spectral measurements were obtained by electrospray ionization in positive mode (ESP+) with the following ion optics: capillary, 3 kV; cone, 30 and 80 V; and extractor, 7 V. The source block temperature was 120 °C, and the desolvation temperature was 150 °C. Inlet flow was adjusted to 100 μL/min. Continuous mass spectra were recorded over the range *m/z* 150−800 with a scan time of 1 s and an interscan delay of 0.1 s.

Cochromatography. Pure analytical standards were prepared by Polyphenols Laboratories AS (Sandnes, Norway). The two aromatic acylated anthocyanins were cochromatographed with an in-house extract of red grapes (*Vitis vinifera*).

RESULTS AND DISCUSSION

The water-soluble anthocyanins from the extract were isolated by use of Toyopearl HW-40F. The pigments were separated according to decreasing molecular weight, and 32 fractions in the range of 50−200 mL were collected. Three chromatographic bands developed on the column: The first band was eluted with 2 L of 20% aqueous methanol and the second with 1 L of 40% aqueous methanol, whereas a last very small band was eluted by means of 0.5 L of 60% aqueous methanol (all solvents contained 0.1% TFA). The fractions were analyzed by HPLC-PAD-MS, which revealed compounds **2**, **4**, **6**, **9**, **11**, and **13** corresponding to the first chromatographic band and compounds **1**, **3**, **5**, **8**, **10**, and **12** to the second, whereas compound **7** was detected from the third band of the size exclusion column.

Compounds **1**−**4** were reported to be the main pigments from this species (*6*). They were assigned as delphinidin 3-glucoside (**1**), delphinidin 3-rutinoside (**2**), cyanidin 3-glucoside (**3**), and cyanidin 3-rutinoside (**4**) due to their PAD spectra and their molecular weights and characteristic fragment ions (**Table 1**). These structures constitute ∼97% of the extractable anthocyanin content and are well-known as constituents of black currants (*11*). The two rutinosides were obtained from the first elution band from the size exclusion column, whereas the glucosides were found in the second band. This confirms the separation mode in which this column material operates (*8*).

Three additional pigments were found in the first fraction of band one from the size exclusion column (**6**, **11**, and **13**), whereas a fourth compound, **9**, was enriched in a later fraction within the same band. Compound **6** gave a maximum absorbance at 529 nm, indicating three oxygen functions on the B-ring system of the molecule (*10*). The molecular ion at 625 together with the fragments at 479 and 317 is consistent with the structure of petunidin 3-*O*-rutinoside. This structure has previously been reported from *Berberis buxifolia* (*12*). The two compounds that coeluted with petunidin 3-*O*-rutinoside were found to have different spectral properties (**Table 1**). Their molecular ions as detected in the MS (*m/z* 509 and 639, respectively) were

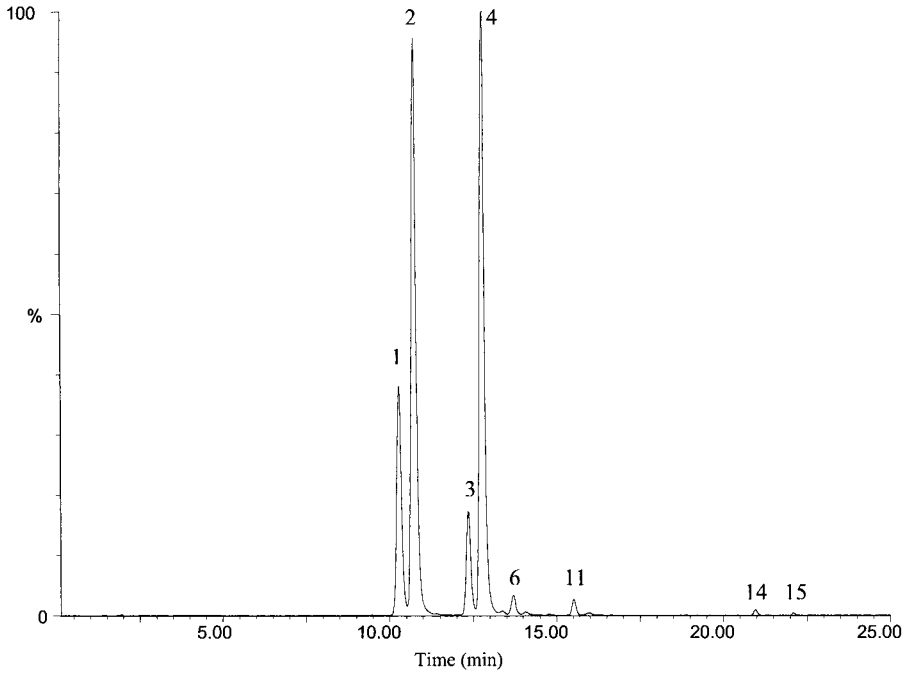

Figure 2. HPLC chromatogram (520 nm) of a crude extract of black currants (*R. nigrum* L.). For peak identity see **Table 1**.

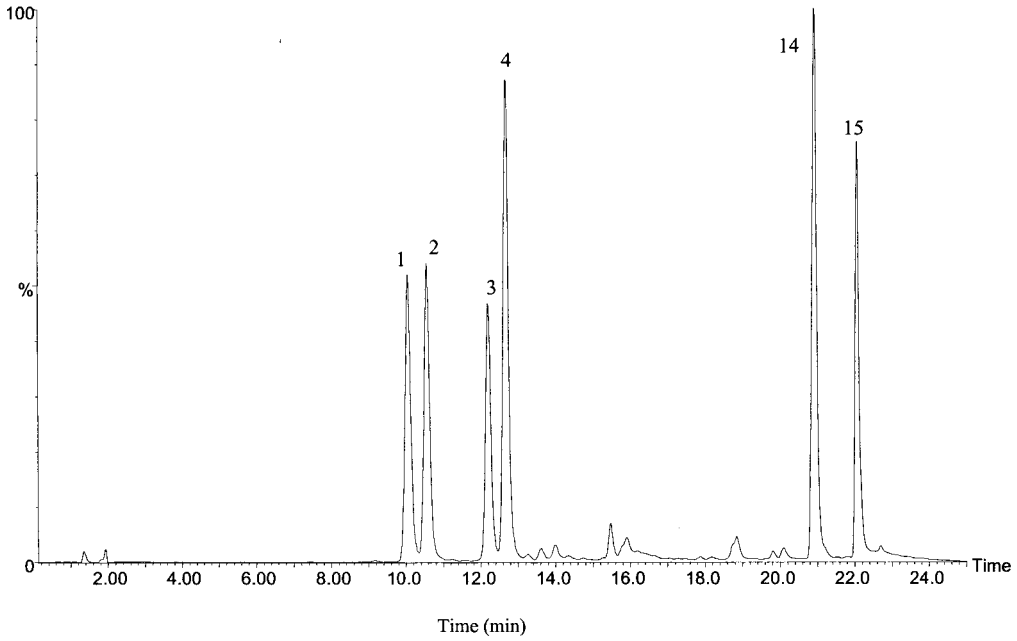

Figure 3. HPLC chromatogram (520 nm) showing the anthocyanin extract enriched in the acylated anthocyanins **14** and **15** after use of a partition against ethyl acetate. For peak identity see **Table 1**.

confirmed by the presence of adduct ions ($[M + Na]^+$ and $[M + K]^+$). The spectroscopic data, together with the chromatographic behavior of the compounds (**Figure 2**; **Table 1**), are in accordance with the structures of peonidin 3-*O*-rutinoside (**11**) and malvidin 3-*O*-rutinoside (**13**). These compounds have previously been characterized as part of a chromatographic work on anthocyanins from black currants (*8*). Compound **9** gave an absorbance spectrum typical of those of pelargonidin derivatives (*10*). The fragment loss $[M - 308]^+$ revealed the structure to be pelargonidin 3-*O*-rutinoside, which previously has been reported from this source (*7*).

The second band from the size exclusion chromatography column contained six compounds with spectral properties analogous to those of the first band. The systematic difference in molecular weights by *m*/*z* 146 between the anthocyanins from the first band compared to the corresponding structures from the second indicated the absence of the rhamnosyl unit as compared to the corresponding anthocyanidin rutinosides. This is in accordance with the chromatographic behavior of the 3-*O*-rutinosides and 3-*O*-glucosides of delphinidin and cyanidin. In addition, the pigments gave the same order of retention values on HPLC as did the rutinosides, but with a slightly lower factor compared to the corresponding rutinosides; $\Delta t_R = 0.2-0.7$. Thus, the structures were assigned to be petunidin 3-*O*-glucoside (**5**), pelargonidin 3-*O*-glucoside (**8**), peonidin 3-*O*-glucoside (**10**), and malvidin 3-*O*-glucoside (**12**).

The amounts of anthocyanin 3-*O*-rutinosides were found to be higher than those of anthocyanidin 3-*O*-glucosides (**Figure**

2; **Table 1**). For the delphinidin derivatives the level of the rutinoside was 2.6 times higher than that of the glucoside. Similar relations were found for the other pairs: cyanidin (5.7), petunidin (18.4), peonidin (11.4), and malvidin (4.3).

Compound **7** was found within the last fractionated band from the size exclusion column. Its absorbance spectrum and the fragment weight from the MS corresponding to the aglycon fragment were in accordance with cyanidin. The fragment loss $[M - 132]^+$ indicated an arabinosyl or a xylosyl moiety. Cochromatography with an authentic compound showed **7** to be cyanidin 3-*O*-arabinoside. To our knowledge this is the first report of flavonoids connected to arabinose from this plant species.

Two compounds detected from the ethyl acetate phase of the extract were strongly retained on the RP-HPLC column: t_R = 21.41 (**14**) and t_R = 22.39 min (**15**) (**Figure 3**). The aglycon units were determined to be delphinidin and cyanidin on the basis of their typical fragment masses (**Table 1**). Their absorbance maxima were similar to the derivatives of delphinidin and cyanidin with the exception of an additional band occurring at 315 nm. This indicates a cinnamic acid-type moiety with one oxygen function in a para position of the ring system. The MS fragmentation gave a fragment loss of *m*/*z* 308 in both cases. This is consistent with coumaroyl glucoside. Partial alkaline hydrolysis of **14** and **15** gave delphinidin 3-*O*-glucoside and cyanidin 3-*O*-glucoside along with *p*-coumaric acid. The acylated pigments coeluted with the 3-*O*-(6″-coumaroylglucoside)s of delphinidin and cyanidin obtained from an in-house standard extract of *Vitis vinifera*. Thus, the compounds were assigned to be delphinidin 3-*O*-(6″-*p*-coumaroylglucoside) (**14**) and cyandin 3-*O*-(6″-*p*-coumaroylglucoside) (**15**), which has been given the trivial name hyacinthin. Besides being present in several grapes and grape products (*13*), these compounds have been isolated from *V. vinifera* (*14*) (compound **14**) and *Camellia* species (*15*) (compound **15**).

This is the first report of acylated anthocyanins from black currant berries. Although the amount of these compounds in the berries is low, this study shows that these structures are easily transferred into a lipophilic solvent (ethyl acetate). The difference in distribution may be of importance for the absorption and metabolic route for such structures in the human body.

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