Anthocyanin–Flavanol Condensation Products from Black Currant (*Ribes nigrum* L.)

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Putative flavanol–anthocyanin condensation products were detected in a polyphenol-rich concentrate from black currant (*Ribes nigrum* L.). These compounds had UV–vis spectra similar to those of delphinidin-3-*O*-rutinoside and cyanidin-3-*O*-rutinoside, but eluted before all previously described anthocyanins on reversed phase HPLC. Mass spectrometric data indicated that they were rutinoside derivatives of novel aglycons 304 amu greater than delphinidin and cyanidin, respectively. The compounds were partly purified by semipreparative HPLC and gave MS and MS^2 spectra consistent with anthocyanin rutinosides covalently linked to epigallocatechin or gallocatechin. These compounds are similar in structure to compounds thought to influence color and quality in red wines and strawberry juice products. There was also evidence for the presence of a range of other flavanol–anthocyanin condensation products. The compounds were present at differing levels in juices of 10 black currant varieties, which were roughly correlated to the content of the parent anthocyanins. The flavanol–anthocyanin products were present in polyphenol-enriched concentrates obtained by solid phase extraction, in commercially produced concentrates, and in fresh extracts of black currants. This suggests that the compounds were not artifacts formed during concentration or purification. However, differences in their comparative contents may be related to the lability of the parent anthocyanins during processing. Although present at low levels, the flavanol–anthocyanin products may influence color or quality parameters of black currant juices, and they may confer enhanced stability to the biological activities reported for their anthocyanin parents.

KEYWORDS: Anthocyanins; flavanols; black currant; *Ribes nigrum*; polyphenol; condensation products

INTRODUCTION

Many health experts and government bodies worldwide are promoting the health benefits of increased intake of fruits and vegetables (*1*) to reduce the incidence of cardiovascular disease and other degenerative conditions within the population. A consensus has developed that an increased intake of antioxidants from fruits and vegetables protects against damage to membranes, proteins, and DNA by scavenging free radicals generated through oxidative metabolism (*2*). The promotion of berry fruits through government-funded projects (*3*) has been encouraged following the success of the North Karelian intervention program in Finland (*4*, *5*), where increased dietary intake of berries was associated with reductions of heart disease and strokes by 60%. Berries are rich sources of polyphenol antioxidants, particularly the anthocyanins, which are responsible for the red to purple pigmentation of berries (*6*). For example, a single portion of black currants (*Ribes nigrum* L.) can provide 100–300 mg of anthocyanins (*7*, *8*). The quality, palatability, and acceptability of food products, especially those derived from fruits, is greatly influenced by their color. The rich purple-black color of black

currants is characterized by an anthocyanin profile based on the 3-*O*-glucosides and 3-*O*-rutinosides of cyanidin and delphinidin (*9*); cultivars of black currant show considerable variability in the levels of anthocyanins contained within the berries and subsequent juices, and the selection of higher levels of anthocyanin represents a key objective for many breeders (*10*) to improve the color of berries and berry products. Smaller amounts of the 3-*O*-glucosides and 3-*O*-rutinosides of pelargonidin, petunidin, peonidin, and malvidin have also been reported (*11*–*13*). In addition, cyanidin-3-*O*-arabinoside and cyanidin- and delphinidin-3-*O*-(6′-coumarylglucoside) have been identified (*11*, *13*). Anthocyanins have been reported to produce health benefits through a range of biological activities (*14*). However, claims of efficacy of anthocyanins for some health benefits have been based on ex vivo or in vitro assays, and these claims may be questionable, considering the low bioavailability (*15*) and poor stability (*16*) of anthocyanins at serum or cellular pH. In this paper, we report a new class of black currant pigments analogous to flavanol–anthocyanin products previously identified in red wines (*17*) and strawberries (*18*), which may have altered stability properties that could influence the color and quality of processed black currant products.

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MATERIALS AND METHODS

Plant Material and Extractions. A bulk preparation of polyphenol-rich syrup was produced from 10 kg of ripe fruit of *R. nigrum* L. (cv. Ben Lomond), grown at the Scottish Crop Research Institute and handled after freezing. The berries were extracted in batches using acetonitrile (200 mL per 200 g berries) in a Waring blender (full power; 3×15 s). After filtration through triple muslin sheets to remove pulp, the extracts were dried by rotary evaporation. The dried extracts were dissolved in distilled water (50−100 mL per liter of extract), the same volume of ethanol was added, and the mixture was stirred for 30 min in a cold room and then left overnight to precipitate polysaccharides. The following day, the supernatant was decanted off and ethanol added until the solution was 75% ethanol (v/v). After thorough stirring, the sample was stored on ice for a few hours to precipitate any further polysaccharide material. After centrifugation (5000*g*, 20 min, 4 °C), the supernatant was removed and dried to a syrup in a rotary evaporator at 40 °C. The syrup was stored frozen. A portion of the syrup was redissolved in 0.25% (v/v) aqueous acetic acid and passed through a column (bed volume = 15 mL) of Polyamide 6S (Riedel deHaen A.G., Seelze, Germany) pre-equilibrated in methanol/0.25% acetic acid and then excess aqueous acetic acid. The column was washed with aqueous acetic acid and the bound fraction eluted with methanol/acetic acid and evaporated to dryness (*19*). For the fresh sample, 100 g of black currants (cv. Ben Lomond) was extracted using an equal volume of ice-cold methanol containing 0.25% acetic acid. After filtration, the extract was evaporated to dryness.

A commercial black currant juice concentrate (Blackcurrant 14, Brix 65; Iprona A.G., Lana, Italy) was diluted in distilled water to a phenol concentration similar to that of the fresh sample.

Juices of 10 black currant cultivars were prepared using a standard method (*19*). The cultivars used were from the breeding program at the Scottish Crop Research Institute, namely, Ben Lomond, Ben Connan, Ben Tirran, Ben More, Ben Lair, Ben Vane, Ben Hope, Ben Loyal, and Ben Gairn, and the standard cultivar Baldwin. Briefly, 100 g of fruit was blended in the presence of a pectolytic enzyme preparation (Glaxo Smith Kline Ltd., Uxbridge, U.K.) and then incubated at room temperature overnight. After centrifugation (5000*g*, 20 min, 4 °C), the supernatant was filtered through Whatman no. 1 paper and then frozen.

The total anthocyanin concentration was estimated according to a pH differential method (*7*), and phenol content was measured using a modified Folin−Ciocalteu method (*7*).

Semipreparative Reverse Phase HPLC. The polyamide-concentrated black currant **s**ample was made up to 5% (v/v) acetonitrile in 0.5% formic acid, and 5 mL aliquots were separated using a Gilson 305 liquid chromatography system with a 250 mm × 21.2 mm i.d. octadecyl silica (C-18) column (Phenomenex Ltd., Macclesfield, U.K.). A linear gradient of 5−25% (v/v) acetonitrile in 1% formic acid was applied over 60 min at a flow rate of 5 mL/min followed by a wash at 80% acetonitrile. The eluate was monitored at 280 and 510 nm using a Gilson 155 dual-wavelength detector, and fractions (5 mL) were collected every 1 min. All red fractions that eluted after the breakthrough peak and before the main anthocyanins peaks were pooled, concentrated, and rerun on the same column. Selected fractions were assayed for phenol content and analyzed by liquid chromatography−mass spectrometry (LC-MS).

LC-MS. Samples (containing 40 μg of gallic acid equivalents by Folin assay) were analyzed on an LCQ-DECA system, comprising a Surveyor autosampler, pump, and photodiode array detector (PAD) and a ThermoFinnigan mass spectrometer ion trap (Thermo Electron Corp., London, U.K.). The PAD scanned three discrete channels at 280, 365, and 520 nm. Samples were eluted over a gradient of 10% buffer B (50% acetonitrile, 50% methanol containing 0.5% formic acid) to 50% solvent B in solvent A (0.5% formic acid) on a 150 × 4.6 mm i.d. Synergi Hydro C18 column with polar end capping (Phenomenex Ltd.) over 60 min at a rate of 400 μL/min. The LCQ-Deca LC-MS was fitted with an electrospray ionization (ESI) interface and analyzed the samples in positive ion mode. There were two scan events−full scan analysis followed by data-dependent MS/MS of most intense ions. The data-dependent MS/MS used collision energies (source voltage) of 45% in
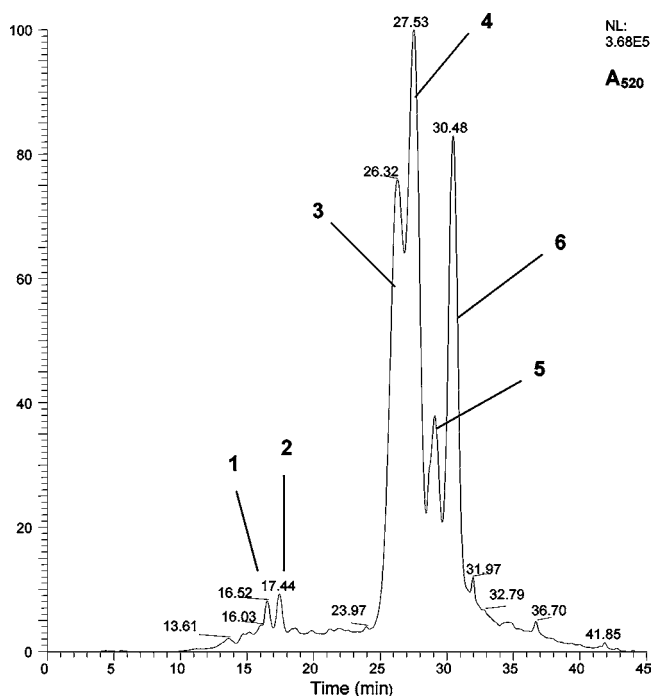

Figure 1. HPLC chromatogram of polyamide-concentrated black currant extract. Arrows denote peaks discussed in the text and referred to in **Table 1**. Peaks are numbered in order of elution. The full scale for the detector response is given.

Table 1. Spectroscopic Properties of Peaks from the Polyamide-Concentrated Black Currant Extract[a]

| peak	t_R (min)	M$^+$ (*m/z*)	MS$_2$	UV−vis[b]	putative identity
1	16.52	915.1 607.1 439.2	607.1, 439.2 439.2, 345.1 303.2	280, 532	flavanol–anthocyanin product
2	17.44	899.1 591.0 423.2	591.0, 423.2 423.1, 329.0 287.3	280, 528	flavanol–anthocyanin product
3	26.34	465.0	303.2	280, 525	delphinidin-3-*O*-glucoside
4	27.62	611.1	303.2 (465.0)	280, 525	delphinidin-3-*O*-rutinoside
5	29.01	449.0	287.2	280, 520	cyanidin-3-*O*-glucoside
6	30.48	595.1	287.2	280, 520	cyanidin-3-*O*-rutinoside

[a] Data relate to peaks shown in **Figure 1**. [b] Only UV−vis maxima are given.

wideband activation mode. The MS detector was tuned against prominent anthocyanins in the black currant extracts.

Comparative amounts of anthocyanins and condensation products in black currant juice samples were estimated using peak areas calculated by the MS data handling software (Xcalibur QualBrowser software, Thermo Electron Corp.) after peaks had been detected by searching at the appropriate *m/z* value. All peaks were checked for *m/z* value, fragmentation products, and t_R before peak areas were calculated. Areas of double peaks due to isomeric forms of condensation products were summed. The values are expressed as MS detector response units.

RESULTS AND DISCUSSION

The HPLC profile of the bulk black currant sample contained four major peaks detected at 520 nm (**Figure 1**), which gave MS and MS^2 data and UV−vis spectra consistent with delphinidin-3-*O*-glucoside, delphinidin-3-*O*-rutinoside, cyanidin-3-*O*-glucoside, and cyanidin-3-*O*-rutinoside, respectively (peaks 3−6, **Table 1**). The rutinosides of pelargonidin, peonidin, petunidin, and malvidin eluted in the tail of the cyanidin-3-*O*-glucoside peak and were detected by searching the MS data at their respective *m/z* values and confirmed by MS^2 data. Two
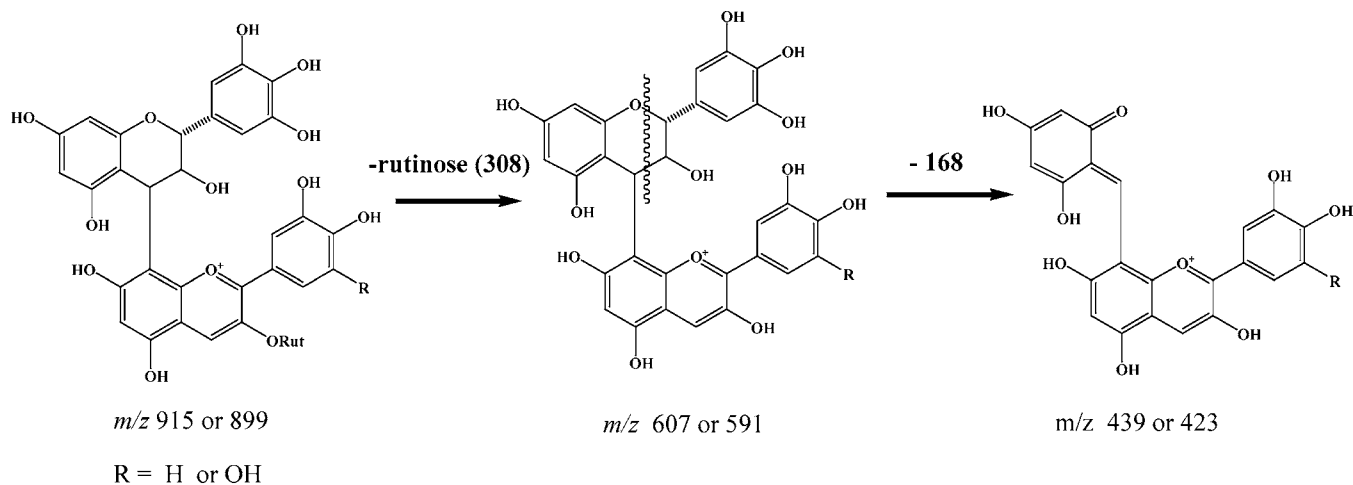
R=H, cyanidin, R=OH, delphinidin
Figure 2. Proposed fragmentation of putative anthocyanin–flavanol condensation product.
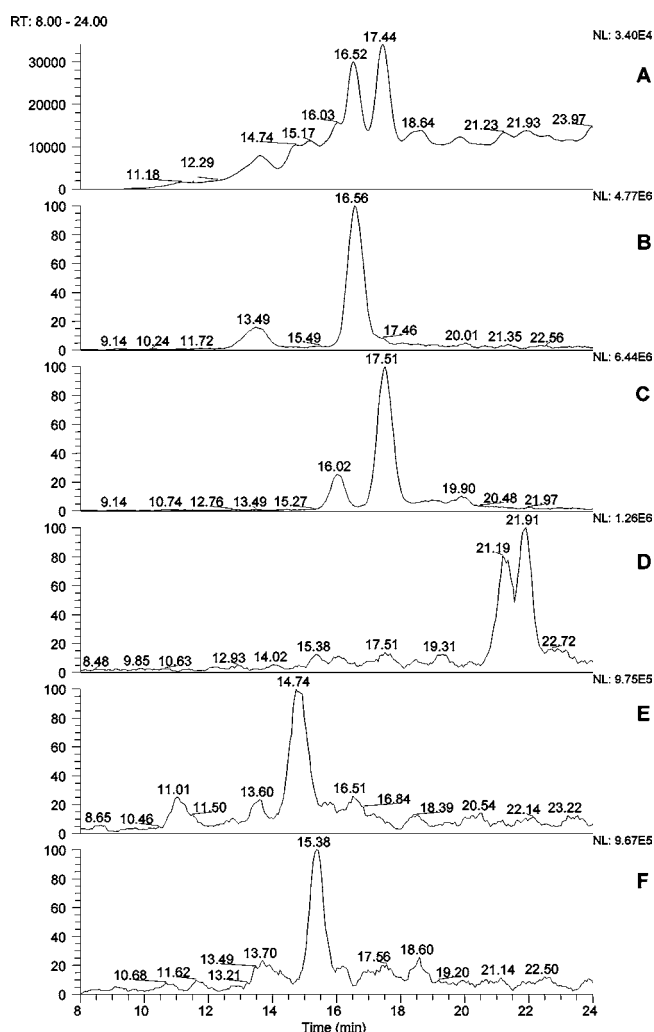

Figure 3. MS identification of putative condensation products: (**A**) 520 nm trace of the early eluting peaks; (**B**) presence of molecular ions of *m/z* 915; (**C**) presence of molecular ions of *m/z* 899; (**D**) presence of molecular ions of *m/z* 883; (**E**) presence of molecular ions of *m/z* 769; (**F**) presence of molecular ions of *m/z* 753.

minor peaks absorbing at 520 nm eluted before the main anthocyanins (**Figure 1**; peaks 1 and 2). These gave strong signals in the positive ESI-MS method, which was tuned against the prominent black currant anthocyanins. Peak 1 gave *m/z* values of 915.0, 607.1, and 439.0 (**Table 1**), whereas peak 2
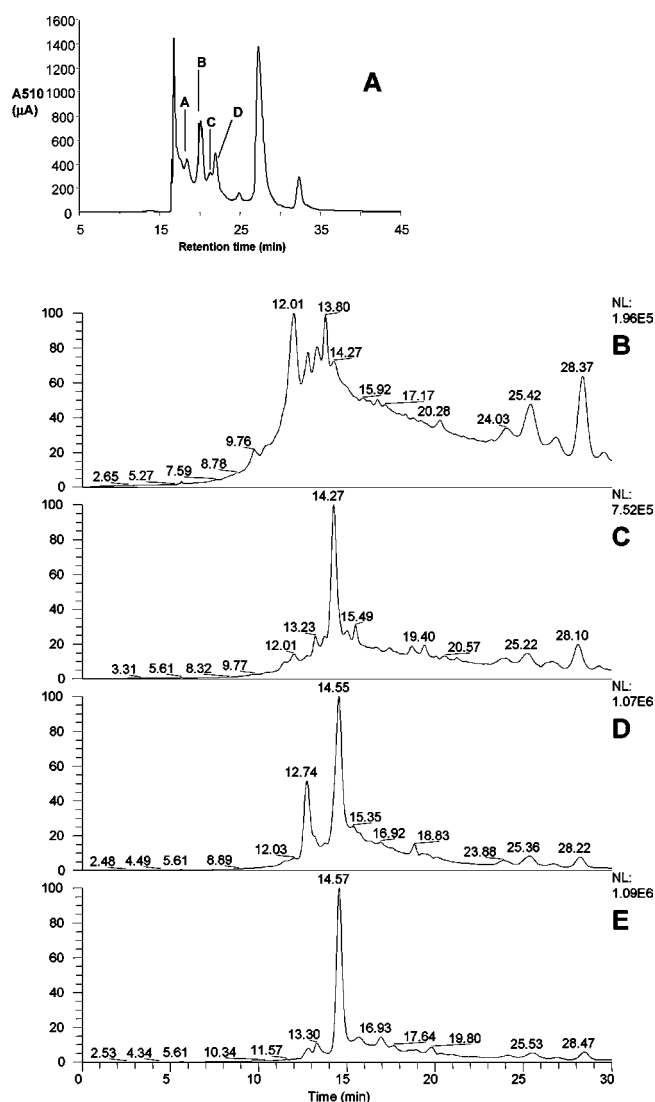

Figure 4. Identification of partially purified flavanol–anthocyanin condensation products: (**A**) A_{510nm} absorbing peaks A–D that elute before the main anthocyanin components; (**B**) LC-MS trace at 520 nm of the material in peak A; (**C**) LC-MS trace at 520 nm of the material present in peak B; (**D**) LC-MS trace at 520 nm of the material present in peak C; (**E**) LC-MS trace at 520 nm of the material present in peak D.

gave *m/z* values of 899.1, 591.0, and 423.1. The less abundant molecular ions in the MS scans were also present as fragments

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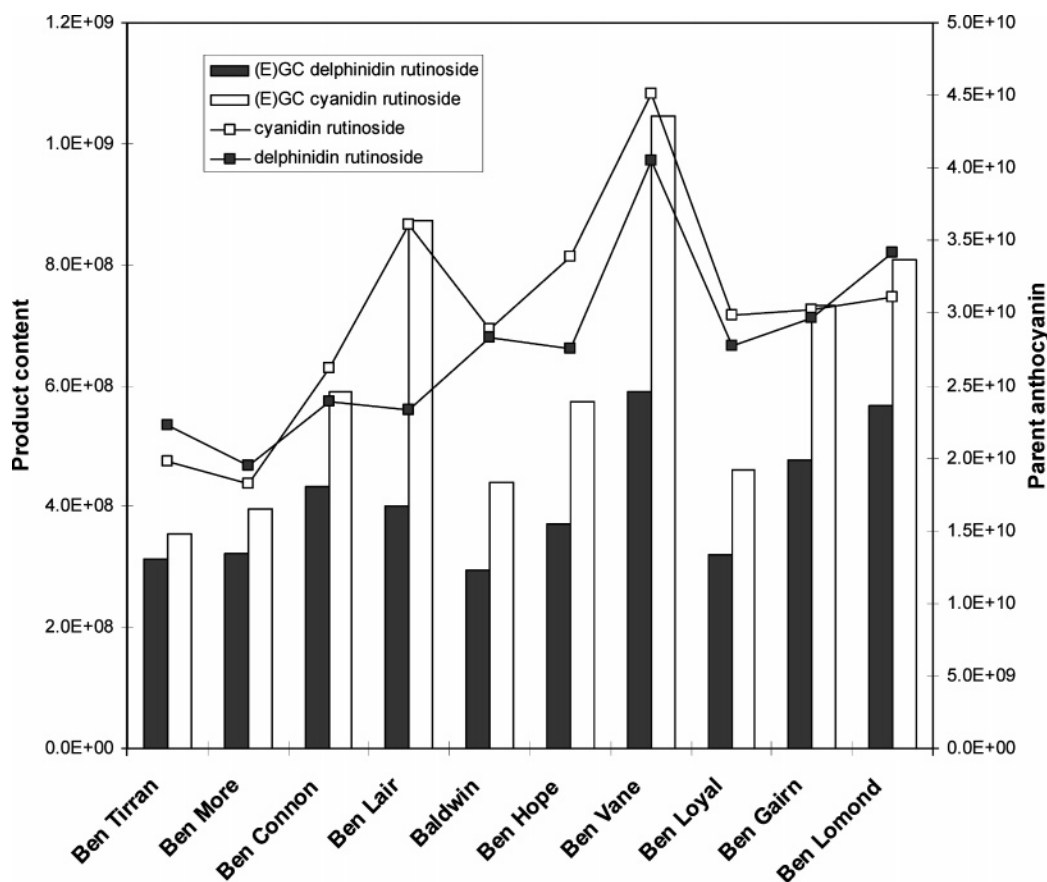
Figure 5. Product content in 10 black currant varieties. Condensation product and anthocyanin content are expressed as MS detector response units.

(MS^2) of the larger molecular ions, which suggested that the original ions fragment in source to some extent. The major compounds in peaks 1 and 2 lost fragments of 308 amu, suggesting the loss of a rutinoside moiety (*11, 21*). Considering that these compounds differ by 16 amu and that this difference is maintained throughout their fragmentation, they may be products of delphinidin-3-*O*-rutinoside and cyanidin-3-*O*-rutinoside, respectively, with an additional mass of 304 amu. The putative delphinidin product (*m/z* 915) elutes before the putative cyanidin product (*m/z* 899), which is commonly the case for delphinidin and cyanidin glucosides and rutinosides on reversed phase chromatography (*11*).

The putative condensation products had properties (i.e., reduced retention on C18, increased aglycon mass and altered UV−vis spectra with a bathochromic shift) similar to those noted for anthocyanin−flavan-3-ol products in red wine (*17*) and strawberries (*18*) and compounds synthesized in vitro (*22*). Black currants contain low levels of the flavan-3-ols, (+)-catechin (C), (−)-epicatechin (EC), (+)-gallocatechin (GC), (−)-epigallocatechin (EGC), (+)-catechin, (−)-epicatechin, and (−)-epigallocatechin (*9*), and all of these have been identified by LC-MS^n (*21*).

The loss of 168 amu from the aglycon (**Table 1**; see **Figure 2**) of the products (*m/z* 915 and 899) may be analogous to the loss of 152 amu noted in the MS/MS fragmentation of the aglycon of the epicatechin product (*22*) with malvidin-3-*O*-glucoside. This fragmentation is proposed to arise from a characteristic retro-Diels-Alder decomposition of the flavanol (*22, 23*). If all of these attributes are into account, it seems to be reasonable to assume that these compounds are condensation products of epigallocatechin or gallocatechin with delphinidin- and cyanidin-3-*O*-rutinosides (611 and 595 plus 304 amu, respectively).

All of the A_{520}-absorbing peaks (**Figure 3A**) that eluted before the main anthocyanin peaks can be assigned putative flavanol−anthocyanin structures. Searching the MS data at *m/z* 915 (**Figure 3B**) and 899 (**Figure 3C**) uncovered two distinct peaks for each *m/z* value with identical MS^2 fragmentation, which may arise from condensation products of delphinidin- and cyanidin-3-*O*-rutinosides with epigallocatechin and gallocatechin, respectively. This phenomenon has been noted before for flavanol−anthocyanin products (*17, 22*) and was attributed to condensation with catechin and epicatechin (*22*). Two further peaks with *m/z* 899 could be discerned at t_R = 19.91 and 18.98, which both yielded MS^2 products (591.1 and 439.1) that were different from the other peaks (591.1 and 423.1). These compounds could be epicatechin or catechin condensation products of delphinidin-3-*O*-rutinoside (288 + 308 + 303 amu) as retention of the products appears to be inversely related to the number of hydroxyl groups on the flavanol (**Figure 3C**). Searching the MS data at *m/z* 883 (**Figure 3D**) also uncovered two peaks (t_R = 21.25 and 21.86), which yielded a main MS^2 fragment at *m/z* 575, consistent with condensation products of epicatechin and catechin with cyanidin-3-*O*-rutinoside. Searching the MS data at *m/z* values of 769 (**Figure 3E**) and 753 (**Figure 3F**) uncovered two further peaks at t_R = 14.74 and 15.38, which gave MS^2 fragmentation consistent with epigallocatechin or gallocatechin condensation products with delphinidin-3-*O*-glucoside and cyanidin-3-*O*-glucoside, respectively. However, further work is required to define the structure of these less abundant products.

Some 510 nm absorbing fractions eluted before the main anthocyanin peaks upon separation of the polyamide-concentrated black currant sample by semipreparative HPLC (**Figure 4A**) and were found to be enriched in particular condensation products when analyzed by LC-MS. Peak A (**Figure 4B**)
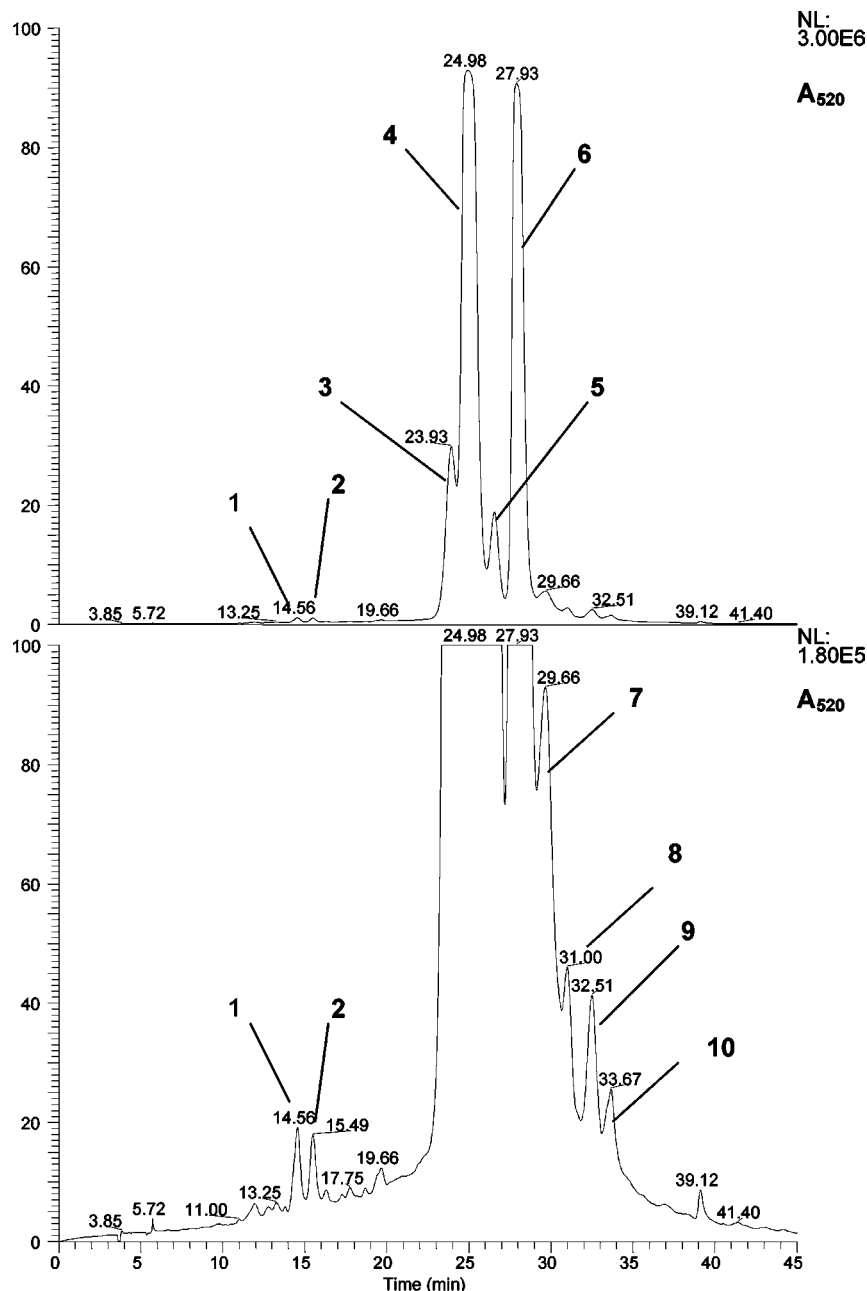
Figure 6. HPLC chromatogram of fresh extract. Arrows denote peaks discussed in the text and referred to in **Table 2**. Peaks are numbered in order of elution. The full scale for the detector response is given for each graph.

contained a number of peaks absorbing at 520 nm, but only two gave MS information suggesting that they may be flavanol−anthocyanin products. The peak at $t_R = 13.80$ gave one clear molecular ion at m/z 1219.1 and MS^2 fragments of 911.1 and 607.1 consistent with a putative structure of (E)GC−(E)GC−delphinidin-3-*O*-rutinoside [where (E)GC stands for epigallocatechin or gallocatechin]. The peak at $t_R = 12.01$ gave a molecular ion of m/z 915.1 with MS^2 fragments of 607.2 and 439.2 consistent with a putative structure of (E)GC−delphinidin-3-*O*-rutinoside.

Peak B (**Figure 4C**) contained one main peak that absorbed at 520 nm ($t_R = 14.27$), which was enriched with a molecular ion of m/z 899 with MS^2 fragments of 591.0 and 423.0 consistent with (E)GC−cyanidin-3-*O*-rutinoside. A smaller peak ($t_R = 12.01$) had a molecular ion of m/z of 753.1 and MS^2 fragments of 591.1 and 423.0, which is consistent with a structure of (E)-GC−cyanidin-3-*O*-glucoside.

Peak C (**Figure 4D**) contained two main peaks absorbing at 520 nm, the larger of which ($t_R = 14.56$) was enriched in the m/z 915 product, (E)GC−delphinidin-3-*O*-rutinoside, whereas the smaller peak (at $t_R = 12.74$) yielded one major molecular ion at m/z 769 and MS^2 fragments of 607.0 and 439.0, which is consistent with a putative structure of (E)GC−delphinidin-3-*O*-glucoside. The small peak at $t_R = 15.35$ yielded a molecular ion with m/z 1203.1 and MS^2 fragments of 895.0 and 591.0, which is consistent with a structure of (E)GC−(E)GC−cyanidin-3-*O*-rutinoside.

Peak D (**Figure 4E**) contained one main peak absorbing at 520 nm ($t_R = 14.57$), which gave a molecular ion of m/z 915.1 and MS^2 products of 607.1 and 439.0 consistent with (E)GC−delphinidin-3-*O*-rutinoside. Derivatives of malvidin-*3-O*-glucoside linked to two epicatechin units have been reported (*21*). Therefore, black currants may contain a family of flavanol−anthocyanin condensation products.The four major condensation

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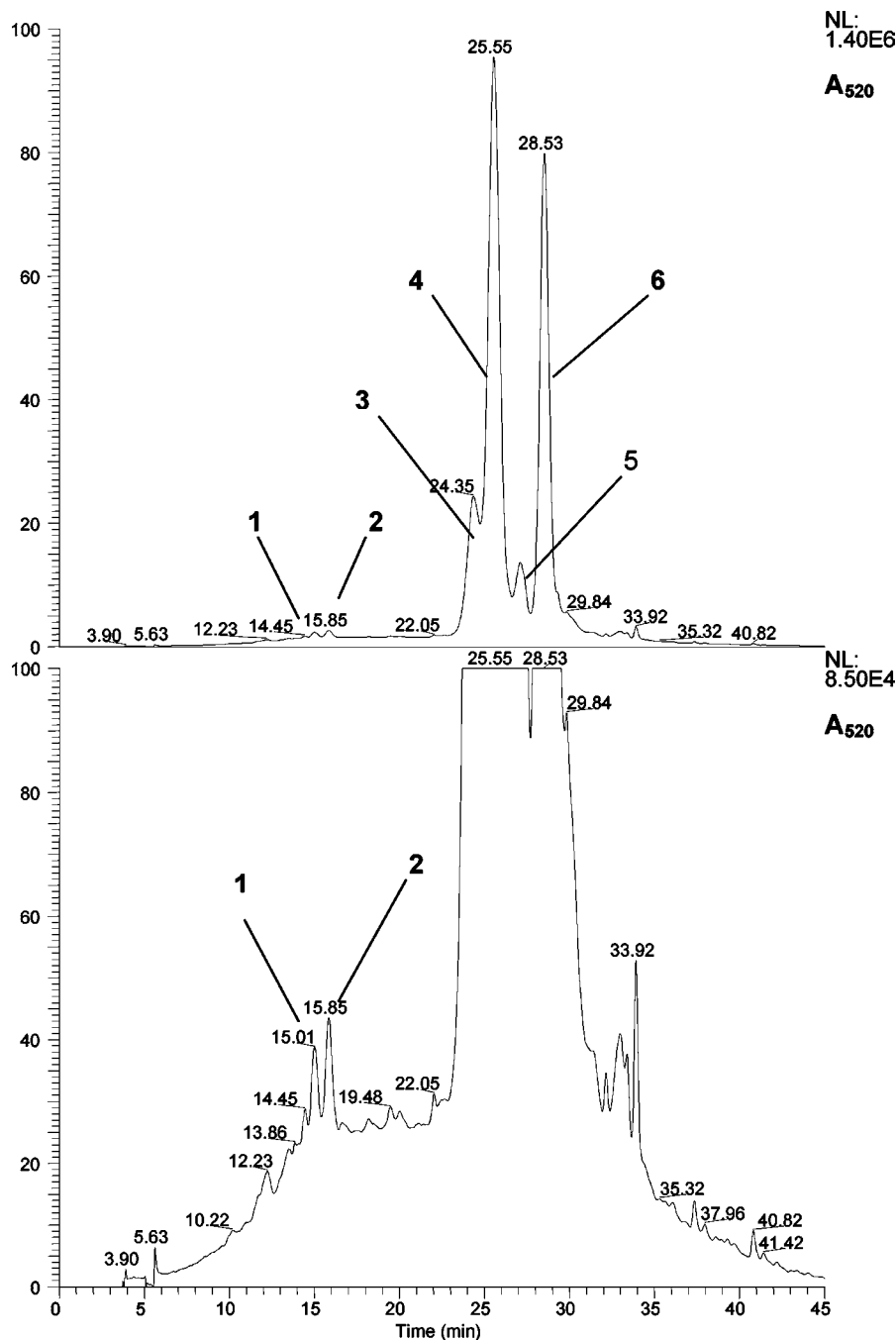

Figure 7. HPLC chromatogram of commercial juice concentrate Arrows denote peaks discussed in the text and referred to in **Table 1**. Peaks are numbered in order of elution. The full scale for the detector response is given for each graph.

products, (E)GC−cyanidin-3-*O*-rutinoside (*m/z* 899), (E)GC−delphinidin-3-*O*-rutinoside (*m/z* 915), (E)C−cyanidin-3-*O*-rutinoside (*m/z* 883), and (E)C−delphinidin-3-*O*-rutinoside (also *m/z* 899), could be detected in the juices of 10 varieties of black currant and compared to the contents of the parent anthocyanins cyanidin-3-*O*-rutinoside and delphinidin-3-*O*-rutinoside. The order of abundance, assuming equivalent ionization of products, was always (E)GC−cyanidin-3-*O*-rutinoside > (E)GC−delphinidin-3-*O*-rutinoside > (E)C−cyanidin-3-*O*-rutinoside > (E)C−delphinidin-3-*O*-rutinoside. For clarity, only the levels of (E)GC−cyanidin-3-*O*-rutinoside (*m/z* 899) and (E)GC−delphinidin-3-*O*-rutinoside (*m/z* 915) are shown (**Figure 5**). The content of each product differed between varieties, but it was roughly correlated with the content of the parent anthocyanins. This is consistent with formation by nonenzymatic means. On average, (E)GC−cyanidin-3-*O*-rutinoside was present at 2.1%

of cyanidin-3-*O*-rutinoside content, (E)GC−delphinidin-3-*O*-rutinoside at 1.5% of delphinidin-3-*O*-rutinoside content, (E)C−cyanidin-3-*O*-rutinoside at 0.6% of cyanidin-3-*O*-rutinoside content, and (E)C−delphinidin-3-*O*-rutinoside at 0.3% of delphinidin-3-*O*-rutinoside content.

The main flavanol−anthocyanin condensation products [peaks 1 (*m/z* 899) and 2 (*m/z* 915)] could also be identified in the fresh black currant extract (**Figure 6**) and the commercial black currant juice concentrate (**Figure 7**). Their presence in the fresh extract suggests that they are not artifacts formed during extraction and/or concentration of the berries or that they are formed very readily after disruption and processing of the berries. However, the commercial juice concentrate contained >3 times as much of the condensation products (related to the amount of the parent anthocyanin) compared with the freshly prepared black currant extract and the polyamide-purified

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Table 2. Spectroscopic Properties of Peaks from Fresh Black Currant Extract[a]

peak	t_R (min)	M^+ (m/z)	MS$_2$	UV–vis[b]	putative identity
1	14.56	915.1 607.1 439.2	607.1, 439.2 439.2, 345.1 303.2	280, 532	flavanol–anthocyanin product
2	15.49	899.1 591.0 423.2	591.0, 423.2 423.1, 329.0 287.3	280, 528	flavanol–anthocyanin product
3	23.93	465.0	303.2	280, 525	delphinidin-3-O-glucoside
4	24.98	611.1	303.2 (465.0)	280, 525	delphinidin-3-O-rutinoside
5	26.51	449.0	287.3	280, 520	cyanidin-3-O-glucoside
6	27.93	595.1	287.2 (449.0)	520, 280	cyanidin-3-O-rutinoside
7	29.66	625.1	317.2 (479.0)	ND	petunidin-3-O-rutinoside
8	31.00	579.1	271.2 (443.0)	ND	pelargonidin-3-O-rutinoside
9	32.46	609.1	301.2 (462.9)	ND	peonidin-3-O-rutinoside
10	33.64	639.1	331.1 (492.9)	ND	malvidin-3-O-rutinoside

[a] Data relate to peaks shown in **Figure 6**. [b] Only UV–vis maxima are given. ND, compounds **7**–**10** were not adequately separated for their UV–vis spectra to be measured.

sample. The commercial and polyamide-purified concentrates had substantially lower anthocyanin-to-phenol ratios than the fresh sample (0.325 and 0.735 polyamide versus 1.273 fresh, respectively). The thermal evaporation and rotary evaporation procedures used to produce the commercial and laboratory juice concentrates, respectively, may favor the production of condensation products and/or accelerate the breakdown of the parent anthocyanins. Indeed, it was notable that the rutinosides of pelargonidin, peonidin, petunidin, and malvidin were apparent as minor but distinct peaks in the fresh black currant extract (**Figure 6**, peaks 7–10, respectively; **Table 2**) but could only be discerned by searching the MS data in the laboratory (**Figure 1**) and commercial black currant extracts (**Figure 7**). This may have been caused by enhanced anthocyanin degradation in the more processed samples, leading to comparably higher levels of the more stable condensation products. In addition, small amounts of cyanidin-*3-O*-arabinoside, cyanidin-*3-O*-(6′-coumarylglucoside), and delphinidin-*3-O*-(6′-coumarylglucoside) could be detected (by searching at their respective *m/z* values) in the polyamide-concentrate and fresh extracts but not in the commercial concentrate (results not shown). The analogous flavanol–anthocyanin condensation products in red wine were thought to be more stable than their parent anthocyanins, a property that ensured their survival during fermentation and aging (*24*). Considering the variation in product content among the 10 varieties examined (**Figure 5**), it is also possible the use of mixtures of different cultivars in the large-scale production of the commercial juice concentrate affects the content of condensation products.

In conclusion, naturally occurring anthocyanin–flavanol compounds with enhanced color stability could be useful food coloring agents (*25*). Although only present at ∼1% of the total anthocyanin content, the persistence of flavanol–anthocyanin compounds may affect the color of processed black currant products and therefore their quality and palatability. In addition, the flavanol–anthocyanin products may share the bioactivities associated with positive health benefits reported for anthocyanins (*14*) but be more stable under physiological conditions.

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