JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2003,** *51,* 5861−5866

Quantification of Anthocyanins in Commercial Black Currant Juices by Simple High-Performance Liquid Chromatography. Investigation of Their pH Stability and Antioxidative Potency

INGE LISE F. NIELSEN, GITTE RAVN HAREN, EVA LOFTIN MAGNUSSEN, LARS O. DRAGSTED, AND SALKA E. RASMUSSEN*

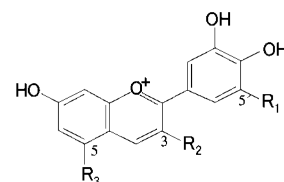
Institute of Food Safety and Nutrition, Danish Veterinary and Food Administration, Mørkhøj Bygade 19, DK-2860 Søborg, Denmark

Quantitative determinations of the four black currant anthocyanins, cyanidin 3-*O*-β-glucoside, cyanidin 3-*O*-β-rutinoside, delphinidin 3-*O*-β-glucoside, and delphinidin 3-*O*-β-rutinoside, were achieved in black currant juices by a rapid and sensitive high-performance liquid chromatographic (HPLC) method. The method was validated, and quantification of anthocyanins in 13 commercially available black currant beverages was demonstrated. To optimize the handling of anthocyanin-containing samples, the pH-dependent stability of the anthocyanins was investigated. Four anthocyanins were incubated for 24 h in aqueous solutions at 13 different pH levels between 0.6 and 5.2, after which the samples were analyzed by HPLC. More than 90% of each anthocyanin remained intact up to pH 3.3. At pH 3.8 a local minimum in stability was detected, and at pH >4.5 the stability rapidly decreased. The antioxidant capacity of all 13 black currant juices was investigated by TEAC and FRAP, and the antioxidant potential of both the anthocyanin and the vitamin C contents in the juices was evaluated. This indicated that <70% of the antioxidant capacity of the juices could be attributed to the anthocyanin content or to vitamin C, signifying that other very potent antioxidants are present in commercial black currant juices.

KEYWORDS: Black currant; *Ribes nigrum* L.; anthocyanins; HPLC; pH stability

INTRODUCTION

Anthocyanins, which belong to the flavonoid class of compounds, are responsible for most red and blue colors in fruits and berries. They have been reported to be very potent antioxidants (*1*) and may therefore contribute to the beneficial effects that red wine is supposed to exert against cardiac diseases. Among rich dietary sources of anthocyanins are red grapes, red wine, strawberries, and black currants. Black currants (*Ribes nigrum* L.) contain ~250 mg of anthocyanins/100 g of fresh fruit (*2*). The four major anthocyanins in the berries are cyanidin 3-*O*-β-glucoside (**1**), cyanidin 3-*O*-β-rutinoside (**2**), delphinidin 3-*O*-β-glucoside (**3**), and delphinidin 3-*O*-β-rutinoside (**4**) (*2*−*4*) (see **Figure 1**). In addition, small quantities of cyanidin and delphinidin (*5*), their respective 3-*O*-β-sophorosides, pelargonidin 3-*O*-β-rutinoside (*6*), and the 3-*O*-β-rutinosides of malvidin and peonidin (*7*) have been found in black currants. Because anthocyanins have a very specific absorption of UV and visible light in the red area, the most widespread technique for the determination and quantification of anthocyanins in black currants is high-performance liquid chromatography (HPLC) with UV detection at wavelengths around 520 nm. Many of the methodologies developed have, however, not been thoroughly validated, and they suffer from long run times between 25 and 75 min (*4*, *7*−*10*) and high limits of detection [0.32 mg/L (*4*)]. In the present study we report on a validated simple and rapid HPLC method with a run time of only 13 min and a limit of detection below 0.018 mg/mL. Furthermore, the present methodology uses purification by solid phase extraction (SPE) of the juice prior to analyses. This removes the sugars in the juice and prolongs the lifespan of the HPLC column. The methodology was successfully applied on 13 different com-



No	Compound	R_1	R_2	R_3
1	Cyanidin-3-*O*-β-glucoside	H	O-glucose	OH
2	Cyanidin-3-*O*-β-rutinoside	H	O-rutinose	OH
3	Delphinidin-3-*O*-β-glucoside	OH	O-glucose	OH
4	Delphinidin-3-*O*-β-rutinoside	OH	O-rutinose	OH
5	Cyanidin-3,5-di-*O*-β-glucoside	H	O-glucose	O-glucose

Figure 1. Structures of the four major anthocyanin aglycons in black currants and the chosen internal standard.

* Corresponding author (telephone +45 33 95 65 48; fax + 45 33 95 60 01; e-mail sen@fdir.dk).

10.1021/jf034004+ CCC: $25.00 © 2003 American Chemical Society
Published on Web 08/12/2003

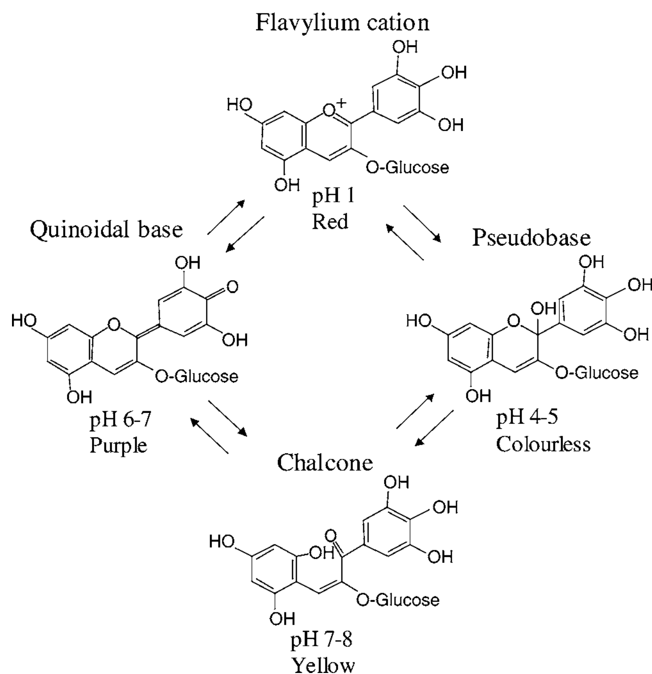


Figure 2. Structural conformations of anthocyanins at different pH levels represented by delphinidin 3-*O*-β-glucoside.

mercially available black currant juices, and their contents of anthocyanins were quantified.

To evaluate the antioxidative potential of anthocyanins in black currant juices, we furthermore investigated the antioxidative capacity of all thirteen juices by measuring the Trolox equivalent antioxidative capacity (TEAC) and the ferric reducing ability of plasma (FRAP). Because black currants also contain high amounts of another very potent antioxidant, vitamin C, we also determined the actual content of vitamin C in each juice to evaluate the contribution of both vitamin C and the anthocyanins to the antioxidant capacity of the juices.

Anthocyanins exist in a pH-dependent equilibrium among at least four different structural forms (**Figure 2**) (*11*). The well-known red color of anthocyanins is present only at low pH in aqueous solution. By raising the pH above 4, yellow, colorless, and blue compounds are produced. Anthocyanins exhibit the highest stability as the red flavylium cation around pH 1−2, whereas the other forms, especially the chalcone, are unstable and eventually lead to the degradation of the anthocyanins. In juices and wines, where the pH typically is between 2.5 and 4, anthocyanins are present as their most stable red flavylium cation, due to copigmentation with various other compounds (*12*). In aqueous solutions, without any copigmentation, such as HPLC standard solutions, purified berry fractions, or biological samples, knowledge about the pH stability of anthocyanins is crucial for sample handling and treatment. We have therefore investigated the stability of pure anthocyanins at different pH levels in aqueous solutions, to identify the pH interval where most of these compounds are preserved to achieve an optimal treatment and storage of anthocyanin-containing samples.

MATERIALS AND METHODS

Standards and Black Currant Beverages. Cyanidin 3-*O*-β-glucoside (**1**), cyanidin 3-*O*-β-rutinoside (**2**), delphinidin 3-*O*-β-glucoside (**3**), delphidin 3-*O*-β-rutinoside (**4**), and cyanidin 3,5-di-*O*-β-glucoside (**5**) of HPLC purity were obtained from Polyphenols (Sandnes, Norway). For structures see **Figure 1**. Thirteen commercially available black currant juices and juice concentrates were purchased from Danish grocery stores: sample A, Fun Light Solbaer from O. Kavli (Hvidovre, Denmark); sample B, Skovgaardens Oekologiske Soed Solbaer Saft, produced by First Brands (Helsingoer, Denmark); sample C, Solmodne Danske Solbaer obtained from Dansk Supermarked Indkoeb (Denmark); sample D, Solbaersaft Oekologisk from Soelyst (Horsens, Denmark); sample E, Sjov Solbaer Light obtained from Pebas Scandinavia (Denmark); sample F, Rynkeby Solbaer Most from Rynkeby Foods (Ringe, Denmark); sample G, Fruiss Tradition from Routin, France; sample H, Clara's Solbaer Drik produced by Routin; sample I, Solbaer Saft den Aegte from Den Gamle Fabrik (Taastrup, Denmark); sample J, Darbo Frugtsirup obtained from Darbo AG (Stans/Tirol, Austria); sample K, Solbaer Sunquick obtained from Cadiso Food (Fredrikssund, Denmark); sample L, Ribena from SB SmithKline Beecham (Ballerup, Denmark); sample M, Rynkeby Soed Saft, produced by Rynkeby Foods (Ringe, Denmark); sample N, a pure black currant juice concentrate obtained from Valloe Saft (Koege, Denmark).

Equipment. HPLC analyses were performed on an HP 1090 system with an 1100 UV−vis detector (Agilent Technologies, Waldbronn, Germany). The system was equipped with a 250 μL injection loop and a 4.6 × 150 mm i.d., 5 μm, Zorbax SB-C18 column with a 4 × 4 mm i.d., 5 μm, C-18 guard cartridge (Agilent Technologies). The columns were maintained at 40 °C during analyses using a thermostatically controlled column compartment.

HPLC/MS analyses were carried out on an 1100 HPLC connected to a G1946 MSD, B-model mass spectrometer using atmospheric pressure chemical ionization (APCI) (Agilent Technology, Waldbronn, Germany) with a UV−vis detector. The system was equipped with a 250 μL injection loop and the same chromatographic columns, maintained at the same temperature as for the HPLC analysis.

Chromatographic Conditions. For the HPLC analysis the flow rate of the system was 1 mL/min using helium-bubbled mobile phases [A, formic acid/H_2O, 1:9 (v/v), pH 1.7; B, acetonitrile (AcCN)]. Elution profile: 0−0.5 min, 1% B (v/v); 0.5−1 min, linear gradient from 1 to 7% B (v/v); 1−4 min, isocratic elution with 7% B (v/v); 4−9.5 min, linear gradient from 7 to 14% B (v/v); 9.5−10.5 min, linear gradient from 14 to 100% B (v/v); 10.5−13 min, column wash with 100% B. Post time: 5 min with 1% B (v/v). The UV−vis spectra were recorded simultaneously during analysis at 520, 350, and 290 nm with a peak scan between 210 and 600 nm (2 nm step) and reference at 650 ± 50 nm.

For the HPLC/MS analysis the flow rate and the eluents were the same as above but a modified elution profile was used to obtain better resolution of the minor black currant anthocyanins: 0−45 min, linear gradient from 1 to 16% B (v/v); 45−50 min, linear gradient from 16 to 100% B (v/v); 50−55 min, column wash with 100% B. Post time: 5 min with 1% B (v/v). The UV−vis spectra were recorded on-line during analysis between 100 and 800 nm with 2 nm steps. Signals were obtained at 520, 350, 290, and 254 nm simultaneously with a reference at 650 ± 50 nm. The following optimal MS conditions were used: positive APCI-MS spectra were recorded in scan mode between m/z 100 and 800; fragmentor voltage, 70 V; APCI capillary voltage, 2500 V; corona current, 10.0 μA; vaporizer temperature, 500 °C; nebulizer pressure, 60 psig; drying gas temperature, 300 °C; and drying gas flow, 4 L/min.

Validation of HPLC Method. Five samples containing 0.2 mg/mL of the anthocyanins **1**, **2**, **3**, **4**, and **5**, respectively, were prepared independently in 10% aqueous formic acid containing 10% AcCN (pH 1.70). Each sample was used to prepare one of five concentration levels in 10% aqueous formic acid for the standard curve. The analyzed samples contained 0 μg/mL ($n = 2$), 0.1 μg/mL ($n = 6$), 4 μg/mL ($n = 2$), 8 μg/mL ($n = 6$), 12 μg/mL ($n = 2$), and 16 μg/mL ($n = 6$), respectively. The rest of one of the samples was diluted to 12 μg/mL in 10% aqueous formic acid for determination of intraday variation ($n = 10$) and interday variation ($n = 4$). The limit of detection (LOD; S/N = 3) and limit of quantification (LOQ; S/N = 10) were determined from analyses of spiked juice samples (see below). Aliquots of 250 μL of each sample were analyzed by HPLC.

Preparation of Samples for pH Stability Studies. Aliquots of 160 μL of a mixture of anthocyanin standards containing 0.125 mg/mL of compound **1**, **2**, **3**, or **5**, respectively, were prepared in 10% aqueous formic acid containing 10% AcCN, and the samples were adjusted to 13 different pH levels in the interval 0.6−5.2 using the appropriate amounts of 2 M HCl, 5 M NaOAc, or 100% formic acid.

After 24 h at 21 °C in darkness, all samples were adjusted to pH 1.7 using 5 M NaOAc, 100% formic acid, or 6 M HCl, and the total volumes were adjusted to 400 μL with 10% aqueous formic acid containing 10% AcCN. Aliquots of 250 μL of each sample were then analyzed by HPLC. All incubations were repeated five times on different days using exactly the same methodology.

Preparation of Juice Samples for HPLC Analyses. The black currant juice concentrates were diluted as described by the manufacturer of each product. Samples A–E were further diluted 4 times in 10% aqueous formic acid, and the other beverages were further diluted 20 times to reduce the sugar concentration. Aliquots of 3 mL of diluted juice sample were added to 1.6 μg of the internal standard, **5**, and were worked up by solid-phase extraction (SPE) on 500 mg Bond Elut-C18 columns (Varian, Middelburg, The Netherlands) to remove the majority of the sugar content prior to HPLC analyses. The columns were preconditioned with 2 × 4 mL AcCN and 2 × 4 mL 10% aqueous formic acid consecutively, and the juice samples were applied onto the columns. The columns were washed with 2 × 4 mL 10% aqueous formic acid before elution with 800 μL of 40% aqueous formic acid containing 40% AcCN (pH 0.9). The eluate was evaporated to dryness, dissolved in 250 μL of 10% aqueous formic acid containing 10% AcCN, and centrifuged for 5 min at 5000*g* prior to analyses by HPLC.

Anthocyanin standard solutions were prepared by dissolving each standard at 1 mg/mL in 10% aqueous formic acid containing 10% AcCN. Spiked juice samples were prepared by the addition of 1.6 μg of each anthocyanin, **1**, **2**, **3**, **4**, and **5**, to the juice samples, which had been worked up by SPE. An external standard mixture of the pure anthocyanins contained likewise 1.6 μg of each anthocyanin per milliliter in 10% aqueous formic acid. Aliquots of 250 μL of each sample were analyzed directly by HPLC.

For positive identification of especially the minor black currant anthocyanins, samples of the pure juice concentrate from Valloe Saft were worked up as described above and analyzed by HPLC using mass spectrometric detection (HPLC-MS).

Analyses of the Vitamin C Content in the Juices. The juices (10 g) were diluted to 50 mL with 2% (w/v) metaphosphoric acid/0.1% (w/v) oxalic acid to stabilize and prevent oxidation to dehydroascorbic acid and stored at −80 °C until analysis. Before analyses, the juices were diluted to a suitable extent in 1% (w/v) metaphosphoric acid/0.05% (w/v) oxalic acid. HPLC analyses were performed on a Waters Alliance 2690 Separations Module (Waters, Milford, MA), equipped with a 250 × 4.6 mm i.d., 5 μm, 300 Å Jupiter C18 column (Phenomenex, Torrance, CA); the column was kept at room temperature, and samples were kept at 5 °C. A Waters 2487 UV detector set at 247 nm was used for direct detection of ascorbic acid, and a Waters 474 scanning fluorescence detector set at an excitation wavelength of 350 nm and an emission wavelength of 430 nm was used for indirect fluorometric detection of dehydroascorbic acid after a postcolumn *O*-phenylenediamine derivatization according to the method described by Kall and Andersen (*13*), with CV% = 1.22.

TEAC and FRAP Analyses of Antioxidant Capacity. The total antioxidant capacity of all 13 juices was determined as the ferric reducing ability of plasma (FRAP) as described by Benzie and Strain (*14*) and as the Trolox equivalent antioxidant capacity (TEAC) using the commercially available total antioxidant status kit Randox NX2332 (Life Science Denmark, Holte, Denmark) on a Cobas Mira S (Triolab, Brøndby, Denmark), both with CV% < 2.

RESULTS

pH Stability of Anthocyanins in Aqueous Solution. The stability of anthocyanins in aqueous solution was explored at 13 different pH levels and is shown in **Figure 3**. After incubation at room temperature for 24 h at pH levels up to 3.3, >90% of the anthocyanins remained intact. A local minimum in stability was detected around pH 3.8, above which the stability again increased up to pH 4.5, and thereafter the degradation of the anthocyanins accelerated. The phenomenon of a local minimum in stability between pH 3.3 and 4.5 was confirmed by five repetitions of the study and has not previously been reported.

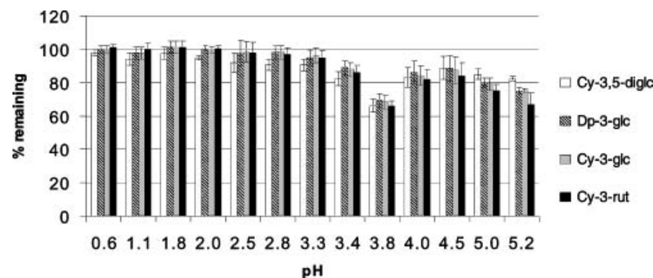


Figure 3. Stability of four anthocyanins: cyanidin 3,5-di-*O*-β-glucoside (Cy-3,5-diglc), delphinidin 3-*O*-β-glucoside (Dp-3-glc), cyanidin 3-*O*-β-glucoside (Cy-3-glc), and cyanidin 3-*O*-β-rutinoside (Cy-3-rut). The compounds were incubated at different pH levels for 24 h, after which the pH was adjusted to 1.7 and the samples were analyzed by HPLC.

On the basis of the result from this study, all standard solutions were prepared at pH 1.7, and highly acidic conditions were chosen for the HPLC analyses using 10% aqueous formic acid as mobile phase A, to maximize the stability of all analytes throughout the study.

Validation of the HPLC Methodology, Identification, and Quantification of Anthocyanins in Black Currant Juices. The chromatograms resulting from the developed HPLC method showed a fast and selective separation of the four major black currant anthocyanins, **1**–**4**, and the internal standard, **5** (see **Figure 4**). The validation of the HPLC method demonstrated linearity within the dynamic range 0–16 mg/L, with *r* values > 0.999, LOD < 0.018 mg/L, and LOQ < 0.06 mg/L. The intra- and interday variations were below 0.044 and 1.23%, respectively. Recovery of the internal standard, **5**, added to the juices prior to SPE was >95% ($n = 6$).

The developed HPLC method was applied to 13 different commercially available black currant beverages to evaluate their content of anthocyanins (see **Figure 5**). As shown, the analyses were highly reproducible and had a coefficient of variation of 5.3%. The total content of anthocyanins ranged from 1.4 mg/L in sample A to 492 mg/L in sample M, and also the relative content of individual anthocyanins varied among the different juices. However, the two rutinosides (**2** and **4**) were in general the major anthocyanins present in all of the investigated juices, which is also the case in black currant berries.

The pure black currant juice concentrate from Valloe Saft (sample N) (**Figure 4**, chromatogram C) contained five additional minor peaks with anthocyanin-like UV–vis absorption (peaks 1 and 7–10) apart from the four major black currant anthocyanins. Peaks 7–10 were also detectable as minor impurities in the aqueous solution of the commercial standards (**Figure 4**, chromatogram A), because these standards all were isolated from black currants by the manufacturer. HPLC-MS analyses confirmed the identity of the four major black currant anthocyanins, **1**–**4**, as peaks 3, 4, 5, and 6 respectively (see **Figure 4** and **Table 1**). Preparative HPLC of peak 1 eluting with the solvent front, followed by HPLC-MS analyses of the collected fraction, showed that peak 1 contained several coeluting compounds. The major part of this fraction showed UV absorption at 280 nm and multiple masses below *m/z* 200. A minor part of this fraction contained four minor anthocyanins all in too small amounts for further identification. Thus, peak 1 possibly consists of small sugars, acids, or salts coeluting with small amounts of anthocyanins resulting in the absorption at 520 nm.

The mass spectrum of peak 7 revealed a small molecular ion at *m/z* 625 $[M]^+$ and a major fragment ion at *m/z* 317 (**Table 1**). This corresponded to petunidin 3-*O*-β-rutinoside (M_w 625),

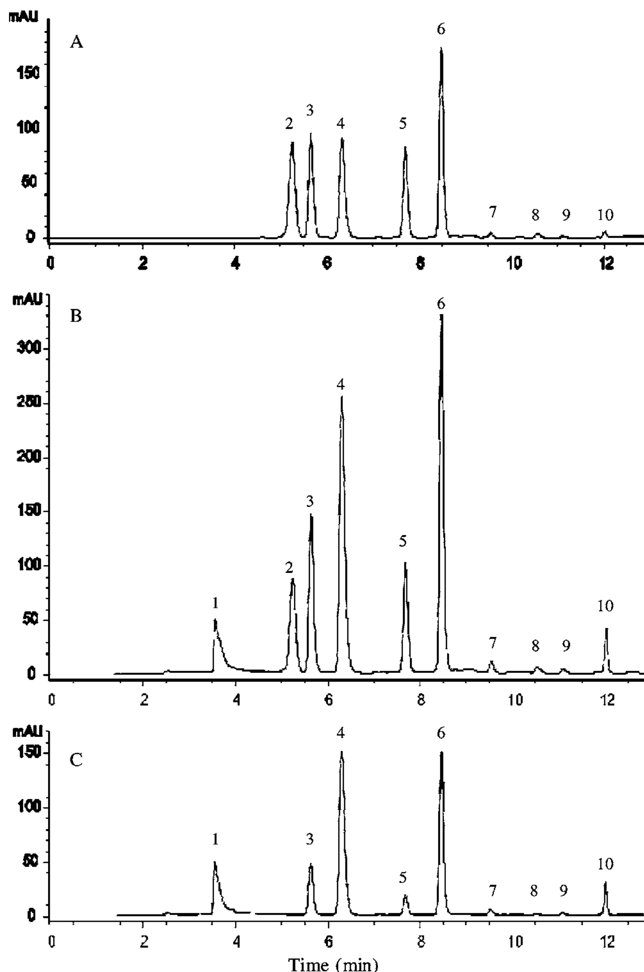


Figure 4. HPLC chromatograms detected at 520 nm of (A) an aqueous solution of the four major black currant anthocyanins and the internal standard [peak 2, **5** (cyanidin 3,5-di-*O*-β-glucoside); peak 3, **3** (delphinidin 3-*O*-β-glucoside); peak 4, **4** (delphidin 3-*O*-β-rutinoside); peak 5, **1** (cyanidin 3-*O*-β-glucoside); peak 6, **2** (cyanidin 3-*O*-β-rutinoside)]; (B) black currant juice spiked with compounds **1**–**5**, and (C) black currant juice. Areas of peaks 3–6 (compounds **3**, **4**, **1**, and **2**, respectively) correspond to 0.80, 3.20, 0.30, and 1.71 mg/L, respectively. Peak 1 represents low molecular mass compounds coeluting with small amounts of anthocyanins. Peak 7 was tentatively identified as petunidin 3-*O*-β-rutinoside. Peak 8 represents peonidin 3-*O*-β-rutinoside, corresponding to ~0.05 mg/L. Peak 9, represents delphinidin 3-*O*-(6″-coumaroylglucoside). Peak 10 is a major anthocyanin component identified as cyanidin 3-*O*-(6″-coumaroylglucoside).

and the fragment ion $[M - 308]^+$ originated from the loss of the rutinoside moiety, resulting in petunidin (M_w 317). Petunidin 3-*O*-β-rutinoside has recently been identified in black currants by Slimestad and Solheim (*8*). The mass spectrum of peak 8 showed two significant signals corresponding to peonidin 3-*O*-β-rutinoside and the fragment ion peonidin (see **Table 1**), which was confirmed by comparison with a commercially available standard of peonidin 3-*O*-β-rutinoside. This anthocyanin has also previously been found in black currants (*8*).

Peak 9 revealed m/z 611 and m/z 303. These masses correspond to delphinidin 3-*O*-(6″-*p*-coumaroylglucoside) and its characteristic loss of m/z 308, the coumaroyl glucoside moiety, forming delphinidin. Delphinidin 3-*O*-(6″-coumaroylglucoside) was not commercially available as a standard but has recently been identified in black currants as one of the more apolar anthocyanins identified from black currants (*8*).

Peak 10, eluting with 100% AcCN, contained a mixture of more apolar components in the juice, including some anthocyanins. The major anthocyanin component could be identified as cyanidin 3-*O*-(6″-*p*-coumaroylglucoside) having m/z 595 and 287 corresponding to the loss of the coumaroyl glucoside moiety. This has also recently been identified in black currants by Slimestad and Solheim (*8*). The identifications of the two anthocyanins with coumaroyl glucoside moieties in peaks 9 and 10 were further confirmed by their UV spectra having a characteristic additional band at 310−315 nm indicating a cinnamic acid-type moiety (*8*).

Antioxidant Capacity of the Black Currant Juices. The antioxidant capacities of all 13 black currant juices were determined by the TEAC and FRAP assays as shown in **Figure 5**. Furthermore, to evaluate the antioxidant potential of the anthocyanins in comparison with the vitamin C content, the vitamin C concentration was additionally determined in all 13 juices (**Figure 5**). The correlation between the total content of anthocyanins, vitamin C, and the sum of these with TEAC or FRAP is seen in **Figure 6**. It can be seen that the combination of the total content of vitamin C and anthocyanins results in the best correlation with the antioxidative measurements ($R^2 =$ 0.65 and 0.66 for FRAP and TEAC, respectively) and that the vitamin C content is the major contributor to the antioxidative potential of the juices. The data for all 13 juices using the two different methodologies for measurement of the antioxidative capacities of the juices, TEAC and FRAP, correlated well ($R^2 = 0.98$, data not shown).

DISCUSSION

Stability of Anthocyanins. The stability of anthocyanins in aqueous solution was explored at 13 different pH levels as shown in **Figure 3**. It was observed that all four anthocyanins remained intact up to pH 2, because ~100% was recoverable in the red flavylium cation form after 24 h of incubation in the dark, whereas a small reduction in stability was observed between pH 2 and 3.3. However, around pH 3.8, a local minimum in the stability of the anthocyanins was observed at which a decrease in stability of anthocyanins was seen in aqueous solution. According to Mazza and Brouillard, all four anthocyanin forms shown in **Figure 2** are present in aqueous solution between pH 1 and 6, and at pH 3.8 the maximal concentration of the pseudobase is almost reached (*15*). The observation of a local minimum in stability around pH 3.8 could thus indicate the presence of an unstable intermediate formed when there is a maximal transition from the red flavylium cation present at pH 1 to the colorless pseudobase present at pH 4−5 (**Figure 2**), but this has to be investigated further by LC-MS and NMR. Attempts were made to isolate the degradation products of anthocyanins. However, no new compounds were detected by HPLC at any of the employed wavelengths after incubation at pH 3.8 or higher as compared to pH 0.6, indicating rapid degradation of the compounds. It can thus be concluded that it is crucial to control the pH when anthocyanin-containing samples are handled, and a pH <2 is recommended.

All 13 anthocyanin-containing beverages employed had a pH between 2.5 and 3.0 at purchase (data not shown). This indicates that the beverages are produced and stored at a lower pH than the local minimum in stability observed between pH 3.2 and 4.5 but still above pH 2, where the anthocyanins exhibited the highest stability. However, the content of other phenolic compounds in the juices very likely results in copigmentation (*16*) increasing the stability during storage of the anthocyanins as recently demonstrated by Eiro and Heinonen (*17*). Brouillard et al. (*18*) state that copigmentation in aqueous solution is maximal in the range from pH 3 to 5. Even though copigmentation may stabilize the anthocyanins, avoiding the observed

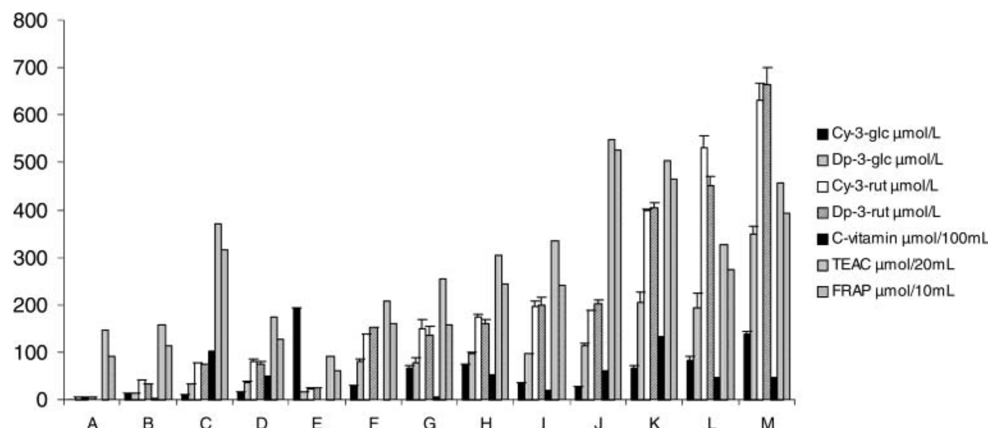


Figure 5. Content of anthocyanins and vitamin C in 13 different commercially available black currant juices and the antioxidant capacity determined as TEAC and FRAP. The anthocyanin content is given as mean ± SD (n = 3). Juices are designated A–M as under Materials and Methods. The vitamin C content was determined in duplicate with a maximum deviation of 1.84 μmol/100 mL. TEAC and FRAP analyses were performed as single determinations (CV% < 2). To ease comparison between the individual juices, different units are chosen for each dataset. All analyses were performed on "ready to drink juices", so that each beverage is diluted as described by the manufacturer.

Table 1. Chromatographic and Spectrophotometric Characteristics of Anthocyanins from Black Currant

	peak	t_R, min, HPLC	t_R, min, LC-MS	$[M]^+$, m/z[a]	fragment ions, m/z[a]
delphinidin 3-*O*-glucoside (**3**)	3	5.7	12.3	465 (55)	303 (100)
delphinidin 3-*O*-rutinoside (**4**)	4	6.4	14.3	611 (18)	465 (10), 303 (100)
cyanidin 3-*O*-glucoside (**1**)	5	7.7	16.2	449 (45)	287 (100)
cyanidin 3-*O*-rutinoside (**2**)	6	8.5	19.1	595 (25)	449 (7), 287 (100)
petunidin 3-*O*-rutinoside	7	9.6	22.3	625 (100)	479 (80), 317 (83)
peonidin-3-*o*-rutinoside	8	10.6	26.7	609 (57)	301 (100)
delphinidin 3-*O*-(6′′-coumaroylglucoside)	9	11.1	29.3	611 (100)	303 (95)
cyanidin 3-*O*-(6′′-coumaroylglucoside)	10	12.0	41.0	595 (100)	287 (65)

[a] Numbers in parentheses are the percentage relative intensity observed.

minimum in stability between pH 3.2 and 4.5, the results from the present study on the stability in aqueous solution at different pH values are still crucial for samples such as purified anthocyanin fractions, standard solutions, and biological samples.

HPLC and HPLC-MS Analyses of Black Currant Juices. The HPLC and HPLC-MS analyses of the black currant juice concentrate and the 13 different beverages demonstrate that this rapid HPLC method separates all of the major anthocyanins present in black currants including the internal standard cyanidin 3,5-di-*O*-β-glucoside (**5**). This good separation combined with the fact that all of the anthocyanins investigated in the stability study follow the same pattern of stability supports the choice of **5** as an internal standard during the workup procedure and subsequent analyses of black currant beverages. At all times during the workup procedure, the pH of the samples did not exceed 1.7. During the HPLC analyses, the pH starts at 1.7 and the last major peak elutes at pH 1.8, which shows that there is no significant risk of degeneration of the anthocyanins during the analyses. The developed HPLC method proved to be robust and highly reproducible within the chosen linear range, with low inter- and intraday variation and r values >0.999. The LOQs below 0.06 mg/L and the linear range of the methodology cover sufficiently the level of anthocyanins detected in juice samples (**Figure 5**).

Anthocyanin Content in Commercial Black Currant Juices. The HPLC analyses of the 13 different commercially available black currant beverages for their content of anthocyanins were highly reproducible as seen in **Figure 5**. The analyses of the content of anthocyanins in the investigated beverages revealed a high variation of the total anthocyanin content ranging from 1.4 mg/L in sample A to 492 mg/L in sample M. The content was surprisingly low (around or below 200 μmol/L) for the products having names indicating careful processing of the berries or organic production methods (samples B−D). However, sample A, which was based mainly on artificial aromas with declared low content of black currants, was also the one with the lowest content of black currant anthocyanins. This large variation in the anthocyanin content indicates that habitual dietary intakes of black currant anthocyanins cannot merely be calculated from the intake of black currant juices in general, but must be determined by intake data of specific beverages or by the use of a biomarker estimating the total intake of anthocyanins. In all of the beverages except sample E, **4** was the major anthocyanin present. The different pattern for sample E is due to the fact that this beverage contains only 6% black currant juice but has added elderberry to obtain the desired color. This suggests that the present methodology can also be used for the determination of the authenticity of black currant products.

Antioxidant Capacity of Black Currant Juices. The antioxidant potential of the black currant juices determined as TEAC and FRAP showed only a modest correlation with the anthocyanin content (**Figure 6**), whereas the content of vitamin C proved to be a more important determinant of the antioxidative capacity of the juices. As shown, the combination of these two antioxidants probably accounts for about two-thirds of the antioxidant capacity of the juices, suggesting that other juice components, presumably other phenolics in black currant, such as hydroxycinnamic acids (*19*), are also important antioxidants with a potency equal to that of the anthocyanins. It must, however, be taken into consideration that a content of additives, including stabilizing agents such as sodium benzoate and malic acid, which may add to or increase the antioxidative properties of the juice, was declared on several of the juices.

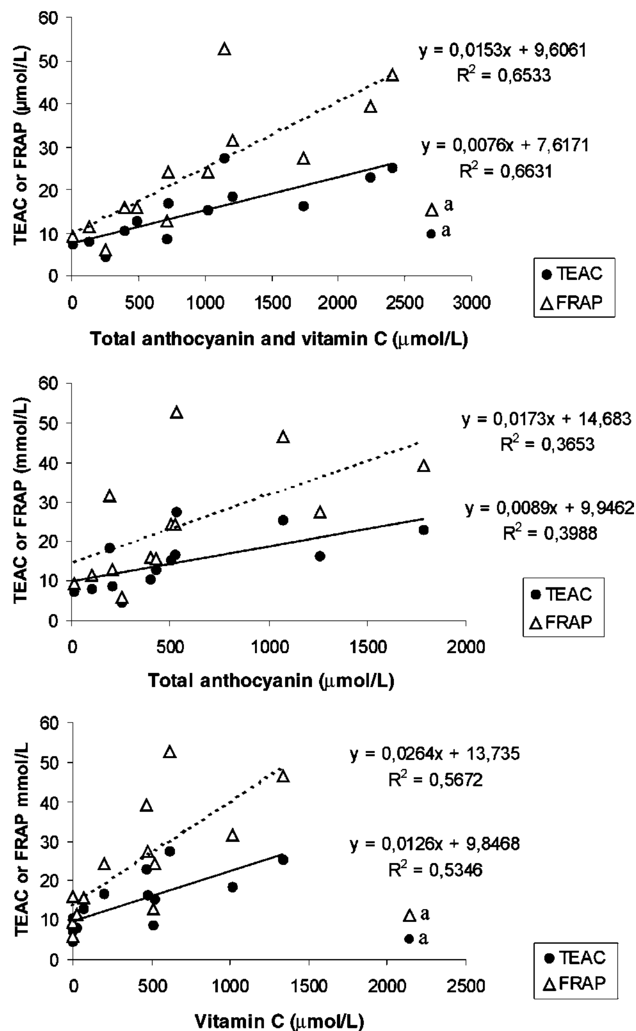


Figure 6. Correlations between the anthocyanin content, the vitamin C content, and the combinations of both with the antioxidant capacities of the juices determined as TEAC or FRAP. Equations and regression coefficients are given for all correlations, *p* value for R^2 was <0.005 in upper diagram and <0.05 in the two lower diagrams. [a] These data were considered as outliers because they fall outside the 95% confidence bands.

A rapid, simple, and highly sensitive HPLC method for quantification of the four major anthocyanins in black currant juice has been developed successfully. The pH-dependent stability of anthocyanins was investigated in aqueous solution, and it was concluded that the pH should preferentially be kept below 2 when aqueous solutions of anthocyanins are being used. At pH 3.8 a local minimum in stability was detected, suggesting the presence of an unstable intermediate, but this phenomenon needs to be further investigated, for example, by NMR. The analyses of commercially available black currant juices revealed a large variation in the anthocyanin content. Small adjustments of the present methodology should allow the development of similarly rapid analyses for a range of other food products containing anthocyanins and anthocyanidins. The evaluation of the antioxidative capacity of the juices showed that the total content of vitamin C and anthocyanins in the investigated juices probably accounted for about two-thirds of the antioxidative potency of the juices.

LITERATURE CITED

(1) Tsuda, T.; Watanabe, M.; Ohshima, K.; Norinobu, S.; Sang, W. C.; Kawakishi, S.; Osawa, T. Antioxidative activity of the anthocyanin pigments cyanidin 3-*O*-β-D-glucoside and cyanidin. *J. Agric. Food Chem.* **1994**, *42*, 2407−2410.

(2) Koeppen, B. H.; Herrmann, K. Flavonoid glycosides and hydroxycinnamic acid esters of blackcurrants (*Ribes nigrum*). Phenolics of fruits 9. *Z. Lebensm. Unters. Forsch.* **1977**, *164*, 263−268.

(3) Goiffon, J.-P.; Mouly, P. P.; Gaydou, E. M. Anthocyanic pigment determination in red fruit juices, concentrated juices and syrups using liquid chromatography. *Anal. Chim. Acta* **1999**, *382*, 39−50.

(4) Nyman, N. A.; Kumpulainen, J. T. Determination of anthocyanidins in berries and red wine by high-performance liquid chromatography. *J. Agric. Food Chem.* **2001**, *49*, 4183−4187.

(5) Chandler, B. V.; Harper, K. A. A procedure for the absolute identification of anthocyanins: the pigments of blackcurrant fruit. *Aust. J. Chem.* **1961**, *15*, 114−120.

(6) Le Lous, J.; Majoie, B.; Moriniere, J. L.; Wulfert, E. Etude des flavonoides de *Ribes nigrum* [The flavonoids of *Ribes nigrum*]. *Ann. Pharm. Fr.* **1975**, *33*, 393−399.

(7) Froytlog, C.; Slimestad, R.; Andersen, O. M. Combination of chromatographic techniques for the preparative isolation of anthocyanins—applied to blackcurrant (*Ribes nigrum*) fruits. *J. Chromatogr. A* **1998**, *825*, 89−95.

(8) Slimestad, R.; Solheim, H. Anthocyanins from Black Currants (*Ribes nigrum* L.). *J. Agric. Food Chem.* **2002**, *50*, 3228−3231.

(9) Bub, A.; Watzl, B.; Heeb, D.; Rechkemmer, G.; Briviba, K. Malvidin-3-glucoside bioavailability in humans after ingestion of red wine, dealcoholized red wine and red grape juice. *Eur. J. Nutr.* **2001**, *40*, 113−120.

(10) Lamuela-Raventós, R. M.; Waterhouse, A. L. A direct HPLC separation of with polyphenolics. *Am. J. Enol. Vitic.* **1994**, *45*, 2−6.

(11) Lapidot, T.; Harel, S.; Akiri, B.; Granit, R.; Kanner, J. pH-dependent forms of red wine anthocyanins as antioxidans. *J. Agric. Food Chem.* **1999**, *47*, 67−70.

(12) Davies, A. J.; Mazza, G. Copigmentation of simple and acylated anthocyanin with colorless phenolic compounds. *J. Agric. Food Chem.* **1993**, *41*, 716−720.

(13) Kall, M. A.; Andersen, C. Improved method for simultaneous determination of ascorbic acid and dehydroascorbic acid, isoascorbic acid and dehydroisoascorbic acid in food and biological samples. *J. Chromatogr. B* **1999**, *730*, 101−111.

(14) Benzie, I. F.; Strain, J. J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxident power": the FRAP assay. *Anal. Biochem.* **1996**, *239*, 70−76.

(15) Mazza, G.; Brouillard, R.; Miller, N. J.; Rice-Evans, C.; Davies, M. J.; Gopinathan, V.; Milner, A. Color stability and structural transformations of cyanidin 3,5-diglucoside and four 3-deoxyanthocyanins in aqueous solutions. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *J. Agric. Food Chem.* **1987**, *35*, 422−426.

(16) Mazza, G.; Brouillard, R. Recent Developments in the Stabilization of Anthocyanins in Food Products. *Food Chem.* **1987**, 207−225.

(17) Eiro, M. J.; Heinonen, M. Anthocyanin Color Behavior and Stability during Storage: Effect of Intermolecular Copigmentation. *J. Agric. Food Chem.* **2002**, *50*, 7461−7466.

(18) Brouillard, R.; Wingand, M.-C.; Dangles, O.; Cheminal, A. pH and solvent effects on the copigmentation reaction of malvin with polyphenols, purine and pyrimidine derivatives. *J. Chem. Soc., Perkin Trans. 2* **1991**, 1235−1241.

(19) Stöhr, H.; Herrmann, K. Die phenolischen inhaltsstoffe des Obstes. *Z. Lebensm. Unters. Forsch.* **1975**, *159*, 31−37.

Received for review January 3, 2003. Revised manuscript received July 17, 2003. Accepted July 21, 2003. This research was part of the project "Anthocyanin Bioactivities" QLRT-1999-00124, supported by the European Commission under the Framework V Program.

JF034004+