Separation and Purification of Anthocyanins by High-Speed Countercurrent Chromatography and Screening for Antioxidant Activity

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The all-liquid chromatographic technique of high-speed countercurrent chromatography (HSCCC) has been applied for separations of anthocyanins. The biphasic mixture of *tert*-butyl methyl ether/*n*-butanol/acetonitrile/water (2:2:1:5) acidified with trifluoroacetic acid was found to be a suitable solvent system for anthocyanin separation. In some cases, enrichment of the pigments on Amberlite XAD-7 resin prior to HSCCC has been carried out. The anthocyanin mixtures from red cabbage, black currant, black chokeberry, and roselle were successfully fractionated using HSCCC. Peak purity control was done by nuclear magnetic resonance spectroscopy as well as electrospray ionization ion trap multiple mass spectrometry. Finally, antioxidant activity of the purified pigments was determined using the Trolox equivalent antioxidant capacity test.

Keywords: *Anthocyanins; red cabbage; black currant; black chokeberry; roselle; high-speed countercurrent chromatography; antioxidants*

INTRODUCTION

Anthocyanins, part of the family of flavonoids, are responsible for red, blue, and purple colors in many fruits and vegetables. Due to the worldwide movement toward the use of "natural" pigments, anthocyanins have received growing importance as food colorants. The color of anthocyanins is pH dependent and will not necessarily obey the Lambert−Beer law, because the color properties also depend on various other factors such as copigmentation with plant flavonoids (Bridle and Timberlake, 1996). The prevalent form of anthocyanins at pH values <2.0 is the red flavylium cation, which undergoes various structural transformations with increasing pH values (Mazza and Miniati, 1993). Although anthocyanin color is very intense, the stability in real food systems is often limited. Sensitivity to bleaching by sulfur dioxide (Bakker and Timberlake, 1997) and chemical conversions at pH values >4.0 (Marcus, 1992) are limiting factors in the use of anthocyanins. Reports of anthocyanins that are stable in food systems over a wide pH range revived the interest in their use as natural colorants (Bakker and Timberlake, 1997; Baublis et al., 1994; Shi et al., 1992a,b). The fact that acylation of the anthocyanin molecule leads to an increased stability (Saito et al., 1995) has resulted in the development of more stable food colorants on the basis of anthocyanins from red cabbage (Mazza and Miniati, 1993).

Moreover, the investigation of the "French paradox" has drawn attention to possible health benefits of the consumption of anthocyanin-rich food. The antioxidative properties of anthocyanins (Frankel et al., 1995; Lapidot et al., 1999; Tamura and Yamagami, 1994; Tsuda et al., 1994; Vinson et al., 1995; Wang et al., 1997) may play a role in preventing or ameliorating some chronic diseases and in reducing the risk of coronary heart disease (Bridle and Timberlake, 1996).

Whereas for the analysis of anthocyanins a variety of chromatographic (Hong and Wrolstad, 1990a,b; Lee and Hong, 1992) and spectroscopic methods (Baldi et al., 1995; Baublis and Berber-Jiménez, 1995; Pedersen et al., 1993; Wang and Sporns, 1999) are available, preparative-scale separations of plant pigments are still a challenge. The polarity and complexity of anthocyanin mixtures often hamper the isolation of pure compounds. Pure reference compounds, however, are required for stability tests, copigmentation studies, and screening of different biological activities. Due to the gentle operation conditions of countercurrent chromatography (CCC) (Conway and Petroski, 1995), we started an investigation into the capability and limits of this technique for anthocyanin separation. As an all-liquid chromatographic technique, CCC uses no solid support; that is, adsorption losses and artifact formation are no longer a problem. The technique allows a complete recovery of the sample and is suitable for separations in the gram range. In the present study, two commercial CCC systems have been applied: a high-speed countercurrent chromatograph (HSCCC) and a multilayer coil countercurrent chromatograph (MLCCC). The application of these techniques for the separation of a number of anthocyanin mixtures is reported in this paper.

MATERIALS AND METHODS

Materials. Calyces from roselle (*Hibiscus sabdariffa* L.) were supplied by Martin Bauer GmbH & Co. KG (Vestenbergsgreuth, Germany). Concentrated liquid extracts of red cabbage (*Brassica oleracea* L.), black currant (*Ribes nigrum* L.), and black chokeberry (*Aronia melanocarpa* Elliott) were donated by Plantextrakt (Vestenbergsgreuth, Germany).

Extraction of Pigments from Roselle. Fifteen grams of calyces from roselle was extracted with 100 mL of 0.1% HCl

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in methanol over 48 h in the dark. The slurry was filtered and the solvent evaporated at 30 °C using a rotary evaporator. The residue was separated by HSCCC without further purification or enrichment.

Cleanup of Pigments from Black Currant and Red Cabbage. Fifteen grams of each of the extracts was acidified with 10% aqueous formic acid (5 mL), diluted with 50 mL of water, and extracted two times with 150 mL of ethyl acetate. The pooled aqueous phase was applied onto an Amberlite XAD-7 column (50 cm × 4 cm, Fluka Chemie, Buchs, Switzerland). The column was washed with 1 L of water, and elution of anthocyanins was carried out with 500 mL of a mixture of methanol/acetic acid (19:1, v/v). The eluate was concentrated in vacuo, water (30 mL) was added, and the aqueous solution was freeze-dried.

The chokeberry fruit extract was used directly without further cleanup or enrichment of anthocyanins.

CCC. *(a)* A high-speed model CCC-1000 (I) manufactured by Pharma-Tech Research Corp. (Baltimore, MD) was equipped with three preparative coils, connected in series (diameter of tubing = 2.6 mm, total volume = 850 mL). The separations were run at a revolution speed of 1000 rpm. A solvent system consisting of *tert*-butyl methyl ether/*n*-butanol/acetonitrile/water (2:2:1:5) acidified with trifluoroacetic acid was used. The elution mode was head to tail with the less dense layer being the stationary phase. The flow rate was set at 5 mL/min and delivered by a Biotronik HPLC pump BT 3020. Freeze-dried XAD-7 extracts and plant extracts were dissolved in a 1:1 mixture of light and heavy phase and injected into the system by loop injection. The amount of sample injected varied from 300 mg to 2 g. Stationary phase retention was in the range of 53−75%. Ten milliliter fractions were collected with a Pharmacia LKB Super Frac fraction collector. Elution was monitored with a Knauer UV−vis detector at 520 nm, and chromatograms were recorded on a Knauer L 250 E plotter.

(b) The second CCC system was a multilayer coil countercurrent chromatograph (II) by P. C. Inc. (Potomac, MD) equipped with a single coil (diameter of tubing = 2.6 mm, volume = 360 mL). Revolution speed was set at 800 rpm, and the flow rate was 2.5 mL/min.

Thin-Layer Chromatography (TLC). TLC was performed on cellulose F plates from Merck (Darmstadt, Germany). As solvent system, the upper phase of the mixture of *n*-butanol/acetic acid/water (4:1:5) was used (Renault et al., 1997).

HPLC with Diode Array Detection (HPLC-DAD). A Jasco ternary gradient unit LG-980-02, with degasser and MD-910 multiwavelength detector driven by Borwin chromatography software, has been used. Peak detection was carried out at 320 and 520 nm. Spectra were also visualized as contour plots in the wavelength region 220−550 nm. The chromatographic separation was performed on a Superspher RP18 column (250 mm × 4 mm) from Merck at ambient temperature. The mobile phase was a linear gradient of 10% aqueous formic acid (solvent A) and acetonitrile/10% aqueous formic acid (9:1, v/v; solvent B). Conditions: initial 95% A, 5% B; in 45 min to 75% A, 25% B; in 15 min to 50% A, 50% B; back to initial conditions; flow rate = 0.8 mL/min.

Proton Magnetic Resonance Spectroscopy (^{1}H NMR). All experiments were performed on a Bruker AMX 300 spectrometer (300 MHz). Spectra were recorded in CD_3OD/CF_3COOD (19:1, v/v). Assignments were made on the basis of spectral data published by Pedersen et al. (1993).

Electrospray Ionization Ion Trap Multiple Mass Spectrometry (ESI-MS/MS). Bruker Esquire-LC-MS/MS with electrospray ionization in the positive mode was used. Dry gas was nitrogen with a gas flow of 4 L/min (350 °C); the nebulizer was set at 10 psi. The parameters were as follows: capillary, −2500 V; end plate, −2000 V; capillary exit, 110 V; skim 1, 35 V; skim 2, 8 V. MS/MS experiments were performed with different fragmentation amplitudes.

Trolox Equivalent Antioxidant Capacity (TEAC) Test [According to the Method of Miller et al. (1993)]. Metmyoglobin was prepared by oxidation of commercial myoglobin by potassium ferricyanide and purified prior to use on a Sephadex G-15-120 column. The concentration of metmyoglobin was determined spectrophotometrically, and the solution was diluted to a concentration of 25 μmol/L. For calibration of the assay, Trolox standard was diluted to final concentrations of 0.05, 0.1, 0.15, and 0.2 mmol/L, respectively. One hundred microliters of water for the blank, standard, or sample solutions, respectively, was pipetted into semi-microcuvettes containing 800 μL of phosphate buffer (pH 7.4), 600 μL of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; 300 μmol/L), and 200 μL of metmyoglobin. After mixing, the reaction was initiated by the addition of 300 μL of hydrogen peroxide (0.5 mmol/L). The absorbance at 734 nm was measured exactly after 6 min. Measured values were compared to the results obtained for Trolox standards, which were plotted as a calibration curve.

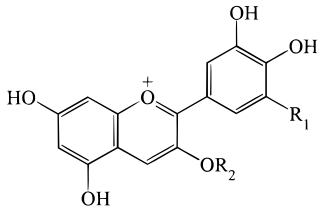

Figure 1. Structure of black currant anthocyanins: cyanidin, R_1 = H; delphinidin, R_1 = OH; R_2 = sugar (glucose/rutinose).

RESULTS AND DISCUSSION

CCC is a support-free all-liquid chromatographic technique that is widely used in natural product analysis due to the gentle operation conditions (Conway and Petroski, 1995). CCC offers several advantages compared to preparative HPLC. As no solid stationary phase is used, no irreversible adsorption on active surfaces can occur. Sample loads in CCC are high; that is, isolation of up to several hundred milligrams of pure compounds can be achieved in a single CCC run. CCC uses inert Teflon tubings, whereas in HPLC the use of very acidic or basic solvent systems often limits the lifetime and performance of HPLC columns. CCC uses large volumes of stationary phase, which allow preparative separations with enhanced peak resolution. This is demonstrated in the case of black currant anthocyanins. When the MLCCC (II) instrument equipped with a single coil (volume of stationary phase = 360 mL) was used, no separation of the mixture of the anthocyanin rutinosides was achieved (cf. Figure 2). The same separation has been repeated with a high-speed CCC (I) device equipped with three preparative coils (total volume of stationary phase = 850 mL). When the volume of stationary phase was increased, almost baseline separation of the two rutinosides was achieved (cf. Figure 3). Consequently, for subsequent separations the preparative HSCCC (I) device has been used.

To check the purity and identity of isolated anthocyanins, HPLC-DAD, TLC, ESI-MS/MS, and ^{1}H NMR have been used. FAB-MS is the technique widely used for molecular weight (MW) determination of anthocyanins (Bakker and Timberlake, 1997; Baublis et al., 1994; Fossen et al., 1996; Kim et al., 1989; Saito et al., 1995). ESI-MS/MS, a fairly recent technique, proved to be very helpful in the characterization of anthocyanins because it reveals characteristic fragments of the molecular peak. Baublis and Berber-Jiménez (1995) showed that molecular weight determinations by ESI-MS are in line with results obtained by FAB-MS measurements.

HSCCC Separation of Anthocyanins from Black Currant (*R. nigrum* L.). The anthocyanin profiles of

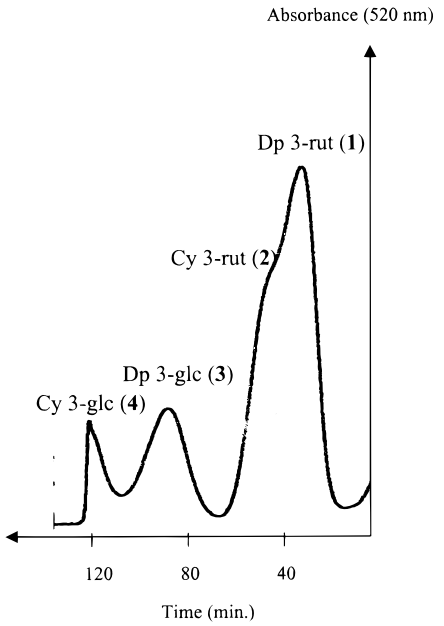

Figure 2. MLCCC separation of anthocyanins from black currant (*R. nigrum* L.) (single-coil system): Cy, cyanidin; Dp, delphinidin; rut, rutinoside; glc, glucoside.

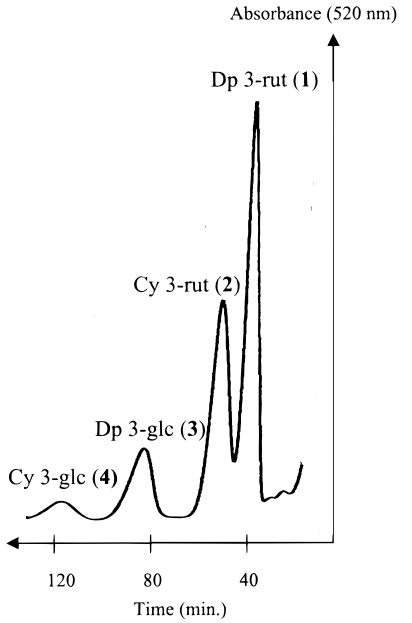

Figure 3. HSCCC separation of anthocyanins from black currant (*R. nigrum* L.) (triple-coil system): see Figure 2 for abbreviations.

different black currant extracts are very similar, whereas red currants can be divided into two subgroups according to their anthocyanin compositions (Roeder, 1997). According to Mazza and Miniati (1993), the four major anthocyanins in black currant are cyanidin 3-rutinoside (35%), delphinidin 3-rutinoside (30%), cyanidin 3-glucoside (17%), and delphinidin 3-glucoside (13%) (cf. Figure 1).

HSCCC separation of an XAD-7 isolate from concentrated black currant juice yielded four fractions (cf. Figure 3 and Table 1). The order of elution is not identical with RP-HPLC. ESI-MS/MS and ^{1}H NMR data revealed that in the case of HSCCC the order of elution is delphinidin 3-rutinoside (**1**), cyanidin 3-rutinoside (**2**), delphinidin 3-glucoside (**3**), and cyanidin 3-glucoside (**4**). In the case of RP-HPLC, the delphinidin-based anthocyanins precede the cyanidin-based ones (Dietrich, 1997). Application of 430 mg of an anthocyanidin-enriched isolate yielded 16, 11, 5, and 3 mg of pure anthocyanins **1**−**4**, respectively. Scaling up of this procedure is possible, and sample loads in the gram range can be applied.

In contrast to separations published by Renault et al. (1997) using gradient elution centrifugal partition chromatography, HSCCC chromatograms show almost baseline separations and resulted in pure compounds.

It should be noted that it is also possible to separate anthocyanins from a concentrated juice without any enrichment or cleanup prior to the separation. The separation characteristics were identical for purified and nonpurified extracts. However, in the NMR spectra some minor impurities were recognized that could not be seen in the HPLC-DAD chromatograms. Therefore, cleanup of the extract by XAD-7 column chromatography prior to HSCCC separation is recommended.

HSCCC Separation of Anthocyanins from Black Chokeberry (*A. melanocarpa* Elliott). Chokeberry is a member of the Rosaceae family and originates from eastern North America. Nowadays, chokeberries are also cultivated in Eastern Europe and Austria (Strigl et al., 1995). Chokeberries are small, dark violet fruits that, due to their high anthocyanin content, are of great interest for food-coloring purposes. In comparison to other dyeing plants, the anthocyanin level in chokeberries is many times higher (Strigl et al., 1995).

According to Mazza and Miniati (1993) the two major anthocyanins in black chokeberry are cyanidin 3-galactoside (**5**, 64.5%) and cyanidin 3-arabinoside (**6**, 28.9%). Cyanidin 3-galactoside (**5**) eluted first by HSCCC, whereas the second peak was tentatively identified by ESI-MS/MS as cyanidin 3-arabinoside (**6**) (cf. Figure 4 and Table 1).

HSCCC Separation of Anthocyanins from Roselle (*H. sabdariffa* L.). Dried calyces from roselle are consumed as a tea called "karkade" (Marcus, 1992). Anthocyanins originating from this tropical plant are suitable for coloring jams, jellies, and fruit beverages with a brilliant red color (Mazza and Miniati, 1993). Dry calyces contain ~1.5 g of anthocyanins/100 g of dry weight, calculated as delphinidin 3-glucoside (Bridle and Timberlake, 1996). The HSCCC separation of a crude methanolic extract from roselle yielded two major compounds (**7** and **8**). Peak purity was checked by HPLC-DAD and TLC. Through comparison with literature data, compound **7** was tentatively identified on the basis of its ESI-MS/MS spectrum as delphinidin 3-sambubioside; compound **8** was the respective cyanidin derivative (cf. Figure 5 and Table 1).

HSCCC Separation of Anthocyanins from Red Cabbage (*B. oleracea* L.). Red cabbage anthocyanins have gained growing importance as coloring agents. Due to the acylation of anthocyanin molecules (Bridle and Timberlake, 1996), the colorants manufactured from red cabbage possess greater heat and storage stability compared to colorants based on grapes, red beets, or cranberries (Mazza and Miniati, 1993). Furthermore, stabilization of the red cabbage anthocyanins could be achieved by adding copigments such as flavonols or water-soluble antioxidants (Bridle and Timberlake, 1996).

Cleanup of a crude red cabbage extract on an Amberlite XAD-7 column allowed enrichment of anthocya-

Table 1. ESI-MS/MS and HPLC-DAD Data of Anthocyanins from Various Plants[a]

plant	elution time (min) HSCCC (I)	ESI-MS (M^+ of molecule)	ESI-MS/MS (M^+ of aglycon)	λ_{max} (by HPLC-DAD)	name of compd
black currant	32	611	303	527	Dp 3-rut (**1**)
black currant	46	595	287	519	Cy 3-rut (**2**)
black currant	82	465	303	523	Dp 3-glc (**3**)
black currant	114	449	287	515	Cy 3-glc (**4**)
chokeberry	128	449	287	515	Cy 3-gal (**5**)
chokeberry	242	419	287	515	Cy 3-ara (**6**)
roselle	26	597	303	527	Dp 3-sam (**7**)
roselle	46	581	287	519	Cy 3-sam (**8**)

[a] Abbreviations: Cy, cyanidin; Dp, delphinidin; gal, galactoside; ara, arabinoside; rut, rutinoside [α-L-rhamnopyranosyl-(1→6)-D-glucopyranoside]; glc, glucoside; sam, sambubioside [β-D-xylopyranosyl-(1→2)-D-glucopyranoside]. Elution time is measured after equilibration of the HSCCC system.

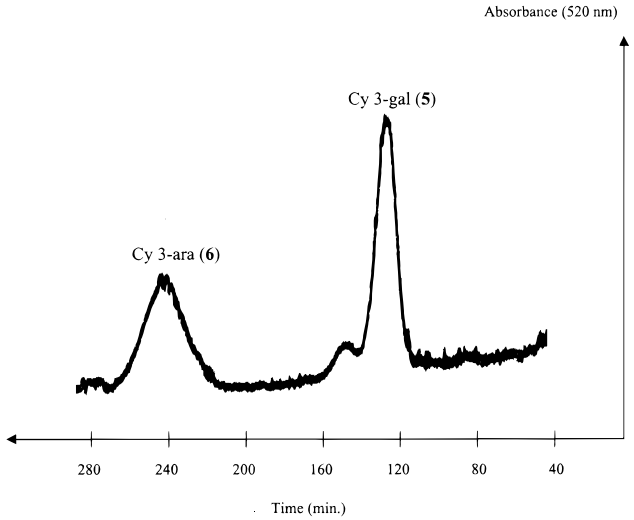

Figure 4. HSCCC separation of anthocyanins from chokeberry (*A. melanocarpa* Elliott): ara, arabinoside; gal, galactoside.

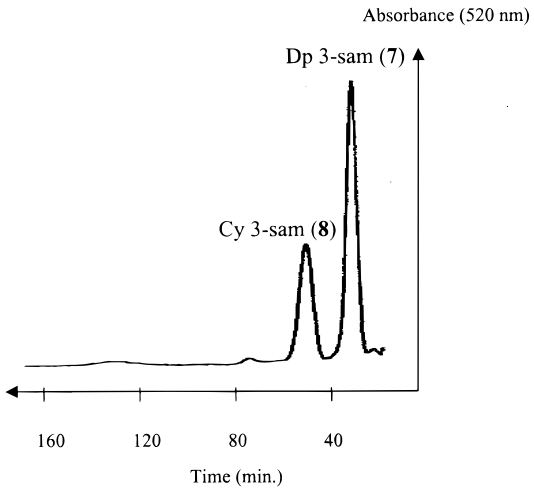

Figure 5. HSCCC separation of anthocyanins from roselle (*H. sabdariffa* L.): sam, sambubioside.

nins and removed unpleasant odorants that were present in the extract. It is known that red cabbage can contain up to 15 different anthocyanins (Mazza and Miniati, 1993). Their common basic structural feature is a cyanidin 3-sophorosido-5-glucoside backbone. Red cabbage anthocyanins differ by varying degrees of acylation with substituted cinnamic acids.

HSCCC allowed the purification of four major pigments in red cabbage (cf. Figure 6). According to ESI-MS, ^{1}H NMR, and HPLC-DAD data, the fractions are of good purity except for the first fraction, which was a

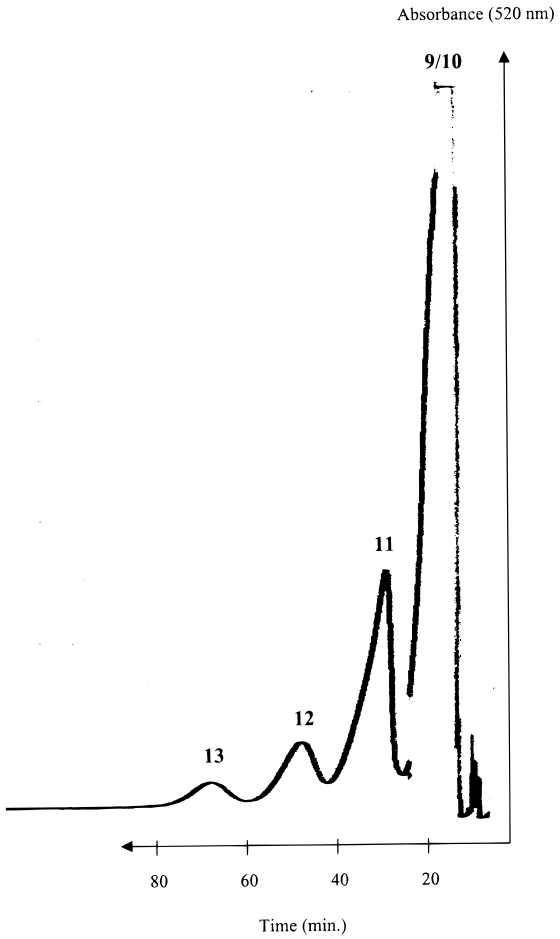

Figure 6. HSCCC separation of anthocyanins from red cabbage (*B. oleracea* L.).

mixture of anthocyanins **9** and **10**. After comparison with literature data (Mazza and Miniati, 1993; Shimizu et al., 1996), the isolated anthocyanins were tentatively identified as outlined in Figure 7 and Table 2.

On the basis of the spectral data available, the position of attachment of the sugar groups and cinnamic acids could not be determined. Further 2D-NMR as well as NOE experiments are necessary to elucidate the exact structures of the pigments. Separation of 300 mg of the XAD-7 extract yielded 30 mg of compounds **9/10**, 32 mg of **11**, 5 mg of **12**, and 4 mg of pure compound **13**, respectively.

Testing of Red Cabbage and Black Currant Anthocyanins for Antioxidative Activity. The antioxidative activity of pure anthocyanins was determined using the TEAC test (Miller et al., 1993). This test is based on quenching of the absorbance of a radical

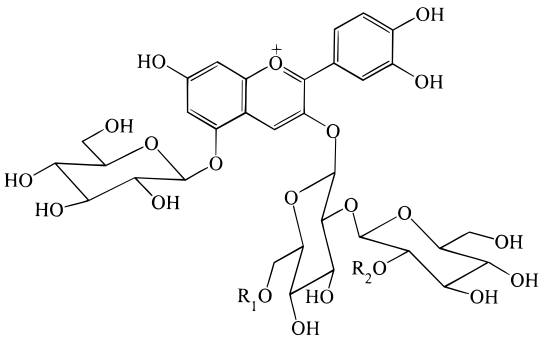

Figure 7. Structure of red cabbage anthocyanins [adapted from Shimizu et al. (1996)]: compound **9**, R_1 = sinapoyl, R_2 = H; compound **10**, R_1 = H, R_2 = sinapoyl; compound **11**, R_1, R_2 = sinapoyl; compound **12**, R_1 = feruloyl, R_2 = sinapoyl; compound **13**, R_1 = *p*-coumaroyl, R_2 = sinapoyl.

Table 2. ESI-MS Data for Red Cabbage Anthocyanins[a]

compd	elution time (min) HSCCC (I)	ESI-MS/MS prominent ions
9/10	18	**979** (M^+), daughter ions of M^+: 817 (Cy + 2Glc + sinapoyl)$^+$, 449 (Cy + Glc)$^+$, 287 (Cy)$^+$
11	30	**1185** (M^+), daughter ions of M^+: 1023 (Cy + 2Glc + 2sinapoyl)$^+$, 449 (Cy + Glc)$^+$, 287 (Cy)$^+$
12	50	**1155** (M^+), daughter ions of M^+: 993 (Cy + 2Glc + sinapoyl + feruloyl)$^+$, 449 (Cy + Glc)$^+$, 287 (Cy)$^+$
13	72	**1125** (M^+), daughter ions of M^+: 963 (Cy + 2Glc + coumaroyl + sinapoyl)$^+$, 449 (Cy + Glc)$^+$, 287 (Cy)$^+$

[a] Elution time is measured after equilibration of the HSCCC system.

Table 3. TEAC Values for Extracts, Isolated Anthocyanins, and Other Known Antioxidants

compd	MW	TEAC value: μmol of Trolox/mg	TEAC value: mmol of Trolox/mmol
XAD-7 isolate black currant		3.0	
delphinidin 3-rutinoside (**1**)	611	3.7	2.3
XAD-7 isolate red cabbage		2.1	
9/10	979	2.6	2.6
11	1185	3.0	3.6
rutin	610	4.7	2.8
catechin	290	9.8	2.8
ascorbic acid	176	7.8	1.4

cation ($ABTS^{\bullet+}$) in the presence of antioxidants. The XAD-7 isolates of red cabbage and black currant as well as several of the purified compounds have been screened. Results are calculated on a milligram basis and are given as Trolox (a water soluble vitamin E analogue) equivalents. The pH of the test system was 7.4. The values determined for anthocyanin extracts are, on a molar basis, comparable to those of known antioxidants such as flavanols and flavonol glycosides (cf. Table 3). The TEAC values for the reference compounds (rutin and catechin) were in the same activity range and showed the same ranking order as published by Rice-Evans et al. (1996). Results published by Tsuda (1994) indicated antioxidative activity of anthocyanins such as delphinidin 3-glucoside in a linoleic acid test system at pH values of 7. With the TEAC system, delphinidin 3-rutinoside gave a good response at pH 7.4. The red cabbage anthocyanins (**9**/**10** and **11**), which differ only in their substitution with cinnamic acid derivatives, showed high antioxidative activities. Calculated on a molar basis, the acylated anthocyanins from red cabbage seem to be more active in the TEAC system than nonacylated species from black currant (Tamura and Yamagami, 1994). These results indicate that anthocyanins may exhibit an antioxidative effect at physiological conditions (pH 7.4) and that a different substitution pattern obviously modulates the antioxidative properties.

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