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Isolation and Identification of Novel Pyranoanthocyanins from Black Carrot (*Daucus carota* L.) Juice

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Six novel pyranoanthocyanins were identified by HPLC-ESI-MS^n in black carrot (*Daucus carota* L. ssp. *sativus* var. *atrorubens* Alef.) juice. The two major compounds, namely, the vinylcatechol adducts of cyanidin 3-*O*-(6-*O*-feruloyl-β-D-glucopyranosyl)-(1→6)-[β-D-xylopyranosyl-(1→2)]-β-D-galactopyranoside and cyanidin 3-*O*-[β-D-xylopyranosyl-(1→2)]-β-D-galactopyranoside, respectively, were isolated by a combination of high-speed countercurrent chromatography with semipreparative HPLC. Their structures were fully elucidated by means of one- and two-dimensional NMR spectroscopy and high-resolution mass spectrometry. The four remaining pigments were characterized as the vinylphenol and vinylguaiacol adducts of cyanidin 3-*O*-[β-D-xylopyranosyl-(1→2)]-β-D-galactopyranoside, the vinylguaiacol adduct of cyanidin 3-*O*-(6-*O*-feruloyl-β-D-glucopyranosyl)-(1→6)-[β-D-xylopyranosyl-(1→2)]-β-D-galactopyranoside, and the vinylcatechol adduct of cyanidin 3-*O*-(6-*O*-sinapoyl-β-D-glucopyranosyl)-(1→6)-[β-D-xylopyranosyl-(1→2)]-β-D-galactopyranoside. These compounds are formed during storage of the juice through the direct reaction of either caffeic, ferulic, or coumaric acid with the respective genuine anthocyanins.

KEYWORDS: Anthocyanins; pyranoanthocyanins; black carrot; *Daucus carota* L. ssp. *sativus* var. *atrorubens* Alef.; aging products; cinnamic acids; NMR; HRMS; structure elucidation

INTRODUCTION

The so-called black or purple carrot (*Daucus carota* L. ssp. *sativus* var. *atrorubens* Alef.) originated from Middle Asia, where it has been known for ∼3000 years. It was not cultivated in Europe until the 12th century and is considered to be the archetype of all modern orange carrots, which were bred by Dutch growers around 1750. The identification of the anthocyanins that are responsible for the color of black carrots has been the subject of several studies (*1*−*5*). It was found that all of the major anthocyanins in black carrot plant tissues and cell cultures possess cyanidin as an aglycon. The three major pigments are 3-*O*-(6-*O*-acyl-β-D-glucopyranosyl)-(1→6)-[β-D-xylopyranosyl-(1→2)]-β-D-galactopyranosylcyanidins, with the acyl group being either sinapic **1**, ferulic **2**, or *p*-coumaric acid **3**. The sinapic acid derivative dominated in cell cultures (*1*−*3*), whereas the ferulic acid ester was predominant in black carrot roots, which in addition to the three cinnamic acid derivatives also contained 3-*O*-[β-D-xylopyranosyl-(1→2)-β-D-galactopyranosyl]cyanidin **4** and 3-*O*-(β-D-glucopyranosyl)−(1→6)-[β-D-xylopyranosyl-(1→2)]-β-D-galactopyranosylcyanidin **5** in significant amounts (*3*, *6*). The structures of the five main anthocyanins in black carrot roots are shown in **Figure 1**. Only recently, anthocyanins possessing a peonidin- or pelargonidin-type aglycon were also identified in black carrots, but only in trace amounts (*7*).

The noncolored phenolic compounds of black carrots were found to be mainly cinnamic acid derivatives, whereas flavanols and flavonols were not detected. 5-Caffeoylquinic acid (chlorogenic acid) was present in the highest concentration, followed by caffeic, ferulic, and coumaric acid (*6*, *8*−*11*). Compared to orange, yellow, and white carrot varieties, purple carrots had a much higher (>5 times) content of total phenolics (*11*).

Recently, a novel reaction pathway leading to the formation of pyranoanthocyanins (i.e., anthocyanins that contain an additional pyran ring between C-4 and the hydroxyl group attached to C-5) in red wine was discovered (*12*). It was shown that the concentration of these newly formed pigments increased with storage time through a direct reaction between anthocyanins and free hydroxycinnamic acids. The occurrence of a similar combination of precursors in black carrots led us to explore the likely formation of such pyranoanthocyanin pigments in black carrot juice.

MATERIALS AND METHODS

Chemicals. All solvents were of HPLC quality and all chemicals of analytical grade (>99%). Water was of NANOpure quality.

Black Carrot Juice. A commercially available black carrot juice (pH 3.85; stored for 3 months after production) was obtained from a local health food store.

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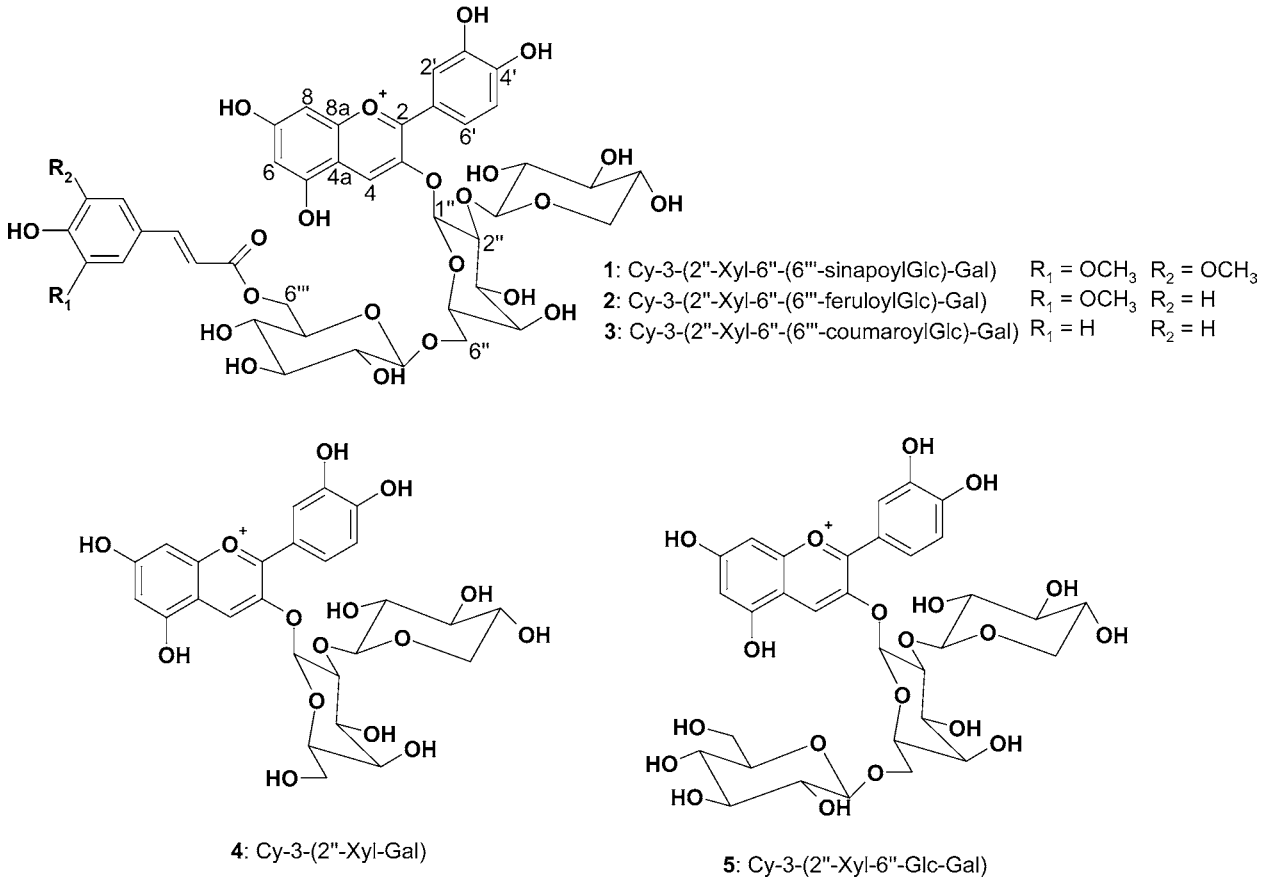


Figure 1. Structures of main anthocyanins in black carrot juice (Cy = cyanidin; Xyl = β-D-xylopyranose; Gal = β-D-galactopyranose; Glc = β-D-glucopyranose).

HPLC with Diode Array Detection (HPLC-DAD). The black carrot juice and countercurrent chromatography (CCC) fractions were analyzed by HPLC with diode array detection. A PU-980 Intelligent HPLC pump equipped with a DG-980-50 three-line degasser, LG-980-02 ternary gradient unit, and MD-1510 multiwavelength detector were used (Jasco, Germany). Samples were injected via a Rheodyne 7175 injection valve (Techlab, Germany) equipped with a 20 μL loop, and separations were carried out on a Synergi MaxRP-12, 4 μm, 250 × 4.6 mm i.d. column (Phenomenex, Germany). Solvents were water/acetonitrile/formic acid (87:3:10, v/v/v, solvent A; 40:50:10, v/v/v, solvent B), and the flow rate was 0.5 mL/min. The linear gradient was from 6 to 20% B for 0−20 min, from 20 to 40% B for 20−35 min, from 40 to 60% B for 35−40 min, from 60 to 90% B for 40−45 min, and held at 90% B for 45−50 min.

For quantification of caffeic acid, ferulic acid, coumaric acid, and chlorogenic acid, calibration curves were obtained in the appropriate concentration ranges. Individual detection wavelengths were 323 nm for cinnamic acid derivatives, 510 nm for pyranoanthocyanins, and 530 nm for anthocyanins.

HPLC with Electrospray Ionization Multiple Mass Spectrometry (HPLC-ESI-MS^n). A Bruker Esquire ion trap LC-MS system was used (Bruker Daltonik, Germany). The HPLC system consisted of a System 1100 binary pump G1312A (Agilent, Germany) and a Rheodyne 7725i injection valve with a 20 μL loop (Techlab, Germany). MS parameters were as follows: positive ion mode, dry gas, N_2, 11 L/min; dry temperature, 325 °C; nebulizer, 60 psi; capillary, −2500 V; capillary exit offset, 70 V; end plate offset, −500 V; skimmer 1, 20 V; skimmer 2, 10 V; scan range, *m/z* 50−1200; chromatographic conditions as above.

Isolation of Anthocyanins by CCC. An anthocyanin-enriched extract from 500 mL of the black carrot juice was prepared by solid-phase extraction (*13*). The solution was poured onto a glass column (100 × 6 cm) filled with Amberlite XAD-7, and the column was washed with water to remove sugars, organic acids, proteins, and salts. Phenolic compounds (such as the anthocyanins) were retained by the resin and eluted using a mixture of methanol/acetic acid (19:1, v/v). Methanol was evaporated in vacuo and the aqueous solution lyophilized.

The lyophilisate was separated with a CCC-1000 high-speed countercurrent chromatograph (Pharma-Tech Research Corp., Baltimore, MD) equipped with three coils connected in series (sample loop, 20 mL; total coil volume, 850 mL; speed, 800 rpm; flow rate, 3.5 mL/min). The solvent system consisted of *n*-butanol/TBME/acetonitrile/water (3:1:1:5, v/v/v/v, acidified with 0.1% TFA, solvent system I). The coil residue obtained by this separation was further fractionated using a solvent system composed of *n*-butanol/TBME/acetonitrile/water (1:3:1:5, v/v/v/v, acidified with 0.1% TFA, solvent system II). Prior to injection, the sample was dissolved in 10 mL each of mobile and stationary phase. Solvent systems were delivered by a Biotronik BT 3020 HPLC pump (Jasco, Germany), and the separation was monitored with a variable UV−vis detector (Knauer, Germany) at 520 nm. The CCC system was operated in the head to tail mode; that is, the lower phases of the solvent systems served as the mobile phases. Fractions of 14 mL (4 min) size were collected with a Super Frac fraction collector (Pharmacia LKB, Sweden), combined according to the chromatogram, and lyophilized.

Preparative HPLC. Fractions were purified by semipreparative HPLC. The HPLC system consisted of a Knauer 64 pump (Germany) and a Knauer 87.00 variable UV−vis detector (λ = 510 nm). The sample was injected through a Rheodyne valve 7125 (Techlab, Germany) with a 200 μL loop. Purification was achieved by isocratic elution with water/acetonitrile/formic acid (70:25:5, v/v/v, 6.0 mL/min) on a 250 × 10 mm i.d. Luna RP-18 column (Phenomenex, Germany) equipped with a guard column (50 × 10 mm) of the same material.

Model Experiment on Pyranoanthocyanin Formation. Eleven milligrams of CCC fraction III (separation of the black carrot anthocyanin extract) was dissolved in 30 mL of a saturated aqueous solution of caffeic acid (final pH 3.4) and stored in an amber glass bottle in the dark at 15 °C for 4 months.

Hydrolysis Assay. Chlorogenic acid (17.4 mg) was dissolved in 30 mL of water (final pH 3.4) and stored in an amber glass bottle in the

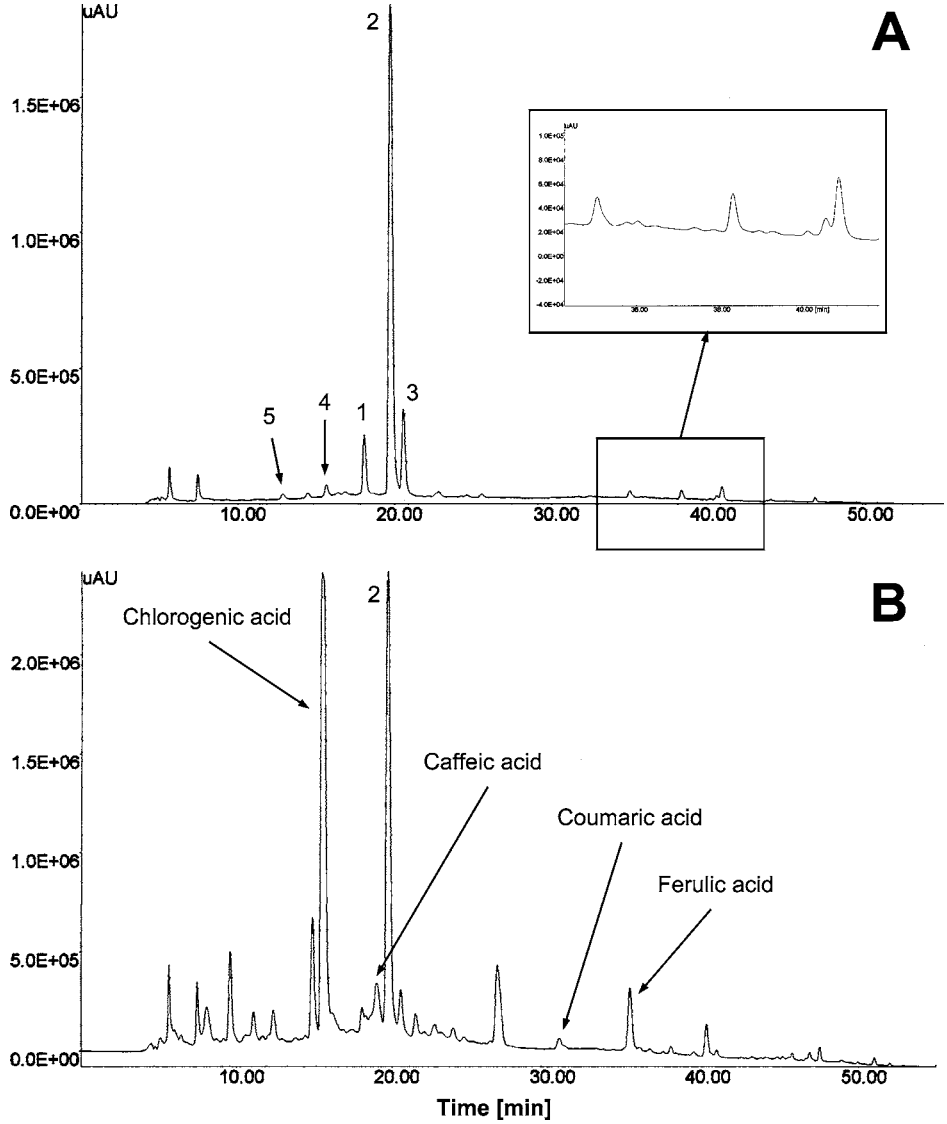


Figure 2. HPLC chromatograms of black carrot juice anthocyanins at 530 nm (**A**) and cinnamic acid derivatives at 323 nm (**B**). For peak assignment cf. **Table 1**.

dark at 15 °C for 4 months. The solution was analyzed at regular intervals by HPLC-DAD for free caffeic acid.

Structure Elucidation. One-dimensional (1D) ^{1}H and two-dimensional (2D) (COSY, TOCSY, HMQC, and HMBC) NMR spectra were recorded on a Bruker AVANCE DMX 600 NMR spectrometer locked to the major deuterium signal of the solvent, CD_3OD. Samples were dissolved in this solvent acidified with a small amount of DCl. Chemical shifts are given in parts per million and coupling constants in hertz. High-resolution electrospray ionization mass spectra (HR-ESI-MS) were recorded on a Micromass Q-Tof 2 mass spectrometer in positive mode.

Table 1. Mass Spectrometric Data and Relative Concentration (Determined by HPLC at 530 nm) of the Major Anthocyanins in Black Carrot Juice (for Structures cf. **Figure 1**)

compd	molecular ion [M$^+$] (*m*/*z*)	aglycon (*m*/*z*)	relative concn (%)
1	949	287	9.5
2	919	287	70.1
3	889	287	12.8
4	581	287	2.8
5	743	287	1.4

RESULTS AND DISCUSSION

A chromatogram of the anthocyanin composition of the black carrot juice under investigation is shown in **Figure 2A**. Following mass spectrometric analysis, the five major anthocyanins could be identified by comparison with literature data (*3*). The relative concentration of the individual anthocyanins in the juice was calculated from the 530 nm trace of the HPLC-DAD chromatogram (**Table 1**). The three acylated triglycosides contributed >90% to the total anthocyanin content. Cy-3-(2″-Xyl-6″-feruloylGlc-Gal), **2**, was the most abundant anthocyanin, whereas the sinapic **1** and coumaric acid esters **3** were both present in similar but much lower concentrations. Besides the known anthocyanins with a cyanidin aglycon, several peaks with a retention time of ∼40 min accounting for ∼3% of the total peak area at 530 nm were detected. These unknown compounds had to be of a much less polar nature, and their visible absorbance maxima were hypsochromically shifted to 510 nm (compared to 530 nm of the main compounds). These observations are consistent with the chromatographic behavior and color properties of pyranoanthocyanins isolated from red wine (*14*, *15*).

Figure 2B shows a chromatogram of the cinnamic acid derivatives of the black carrot juice. Chlorogenic acid (243.3 mg/L) was the major phenolic compound. At 30.3 mg/L, caffeic

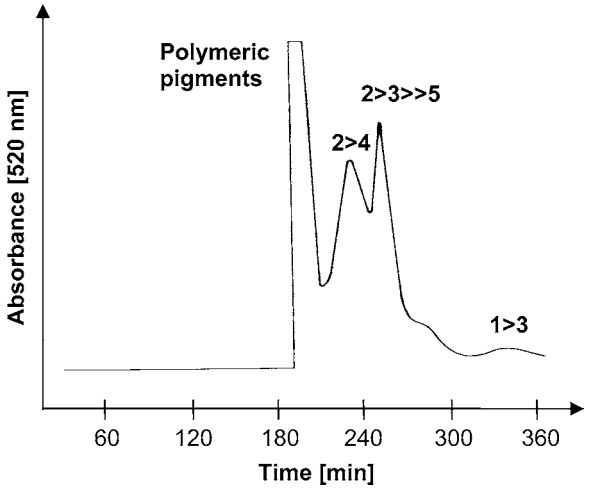


Figure 3. CCC separation of black carrot juice anthocyanin extract and relative distribution of individual anthocyanins **1**–**5** within the fractions. The solvent system was *n*-butanol/TBME/acetonitrile/water (3:1:1:5, v/v/v/v, acidified with 0.1% TFA, flow rate = 3.5 mL/min). For peak assignment cf. **Table 1**.

acid was present in a concentration similar to that recently determined in Pinotage red wines, where the formation of pyranoanthocyanins proceeded rapidly (*16*). The concentration of ferulic acid was one-third lower (20.5 mg/L), whereas only trace amounts of coumaric acid were found (2.3 mg/L). Comparative data for phenolic compounds in black carrot juice are not available. In the only study providing quantitative data on phenolics in fresh black carrots, 541 mg/kg chlorogenic acid and 24 mg/kg caffeic acid were found, whereas in orange carrots only 85 mg/kg chlorogenic acid was determined and free caffeic acid was not detected (*11*). The high levels of free hydroxycinnamic acids provided further evidence for the potential formation of pyranoanthocyanins in black carrot juice. The greater content of cinnamic acids of black carrot juice could also to some extent be responsible for the relatively acidic pH of 3.85, whereas the typical pH of orange carrot juice is between 5.5 and 6.5 (*17*).

The anthocyanin-enriched XAD-7 extract of the juice was separated by CCC by applying solvent system I (*18*). Four fractions were obtained. Polymeric pigments eluted first, followed by three fractions that contained mixtures of the five major anthocyanins. Fraction II was composed of compounds **2** and **4**. Cy-3-(2″-Xyl-6″-feruloylGlc-Gal), **2**, also dominated in fraction III, which in addition contained smaller amounts of **3** and only traces of **5**. The last fraction, IV, was a mix of anthocyanins **1** and **3** (**Figure 3**). The compounds remaining on the coil of the CCC system were recovered and freeze-dried. The HPLC chromatogram of this coil residue (**Figure 4**) showed a strong enrichment of all minor compounds with a retention time of >20 min, which had been hardly visible in the HPLC chromatogram of the integral juice (**Figure 2A**), whereas the major anthocyanins **1**−**5** were almost quantitatively removed.

HPLC-ESI-MS^n analysis of the coil residue revealed the presence of six anthocyanins that upon fragmentation did not produce the typical cyanidin aglycon with *m*/*z* 287. Instead, fragments with *m*/*z* 403, 419, and 433 were observed, which formally correspond to the reaction products of a cyanidin core with coumaric, caffeic, and ferulic acid, respectively. Mass spectrometric properties and tentative identification of these pyranoanthocyanins are provided in **Table 2**; structures are shown in **Figure 5**.

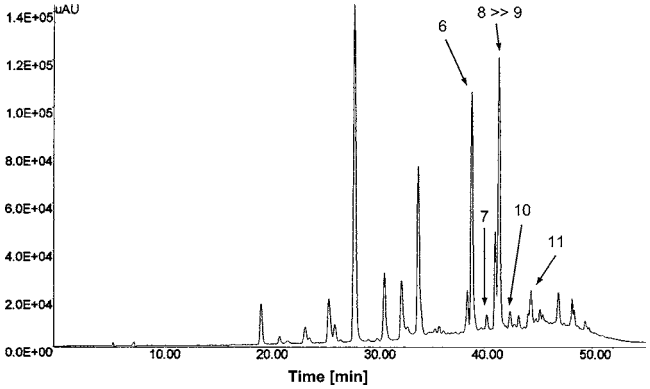
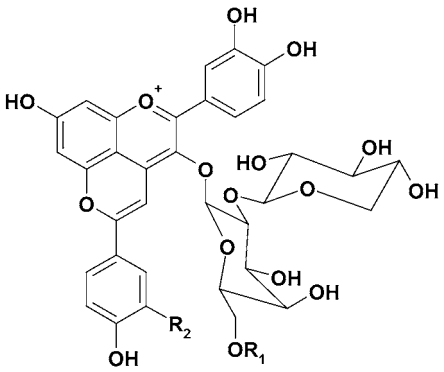


Figure 4. HPLC chromatogram (510 nm) of the coil residue obtained after CCC separation of black carrot juice anthocyanin extract. For peak assignment cf. **Table 2**.

Table 2. Mass Spectrometric Properties and Tentative Identification of Pyranoanthocyanins in Black Carrot Juice (for Structures cf. **Figure 5**)

compd	molecular ion [M$^+$] (*m*/*z*)	aglycon (*m*/*z*)	identified as
6	713	419	vinylcatechol adduct of **4**
7	697	403	vinylphenol adduct of **4**
8	1051	419	vinylcatechol adduct of **2**
9	1081	419	vinylcatechol adduct of **1**
10	727	433	vinylguaiacol adduct of **4**
11	1065	433	vinylguaiacol adduct of **2**



6: R_1 = H R_2 = OH
7: R_1 = H R_2 = H
8: R_1 = feruloylGlc R_2 = OH
9: R_1 = sinapoylGlc R_2 = OH
10: R_1 = H R_2 = OCH_3
11: R_1 = feruloylGlc R_2 = OCH_3

Figure 5. Structures of pyranoanthocyanins identified in black carrot juice (Glc = β-D-glucopyranose).

The coil residue was further fractionated by CCC. Use of the less polar solvent system II led to a reduced elution time of the pyranoanthocyanins. Their distribution within the four fractions collected is shown in **Figure 6**. A comparison with the HPLC chromatogram of the coil residue (**Figure 4**) clearly shows the difference in selectivity of CCC versus HPLC. The combination of both techniques results in an advantageous increase in separation power (e.g., pyranoanthocyanins **8** and **9** could not be separated by HPLC but are well resolved by CCC) and thus simplifies the isolation of pure compounds from complex natural sources. The major compounds **6** and **8** coeluted with other pyranoanthocyanins, and the CCC fractions also contained minor amounts of noncolored phenolic impurities.

Table 3. ^{1}H NMR Spectroscopic Data for Pyranoanthocyanins **6** and **8**

Aglycon

	δ ^{1}H, ppm (*J*, Hz)								
compd	H-6	H-8	H-9	H-2′	H-5′	H-6′	H-2″	H-5″	H-6″
6	7.15 (1.8)	7.18 (1.8)	7.99	7.97 (2.2)	6.98 (8.5)	7.97 (2.2, 8.5)	7.74 (2.2)	7.06 (8.5)	7.81 (2.2, 8.5)
8	7.15 (1.6)	7.17 (1.6)	7.99	8.05 (2.0)	6.99 (8.4)	8.05 (2.0, 8.4)	7.74 (2.0)	7.01 (8.4)	7.79 (2.0, 8.4)

β-D-Galactopyranose

	δ ^{1}H, ppm (*J*, Hz)						
compd	H-1	H-2	H-3	H-4	H-5	H-6A	H-6B
6	4.88 (7.5)	4.23 (7.5, 9.0)	3.80 (3.0, 9.0)	3.89 (3.0, <1)	3.30–3.45 (no[a])	3.30–3.45 (no)	3.30–3.45 (no)
8	4.85 (7.7)	4.23 (7.7, 9.2)	3.79 (3.2, 9.2)	3.85 (3.2, <1)	3.69 (no)	3.74 (2.3, 11.3, no)	3.65 (2.3, 11.3, no)

β-D-Xylopyranose

	δ ^{1}H, ppm (*J*, Hz)					
compd	H-1	H-2	H-3	H-4	H-5A	H-5B
6	4.83 (7.3)	3.56 (7.3, 9.5)	3.48 (9.3, 9.5)	3.64 (5.5, 9.5, 11.5)	3.92 (5.5, 11.5)	3.34 (no)
8	4.81 (7.7)	3.55 (7.7, 9.0)	3.48 (9.0, 9.0)	3.64 (~5, 9, 11)	3.92 (no)	3.32 (no)

β-D-Glucopyranose

	δ ^{1}H, ppm (*J*, Hz)						
compd	H-1	H-2	H-3	H-4	H-5	H-6A	H-6B
8	3.90 (7.8)	2.98 (7.8, 9.0)	3.13 (~9)	3.16 (~9)	3.02 (~2, 6.3, ~ 9)	4.30 (~2, 11.9)	3.97 (6.3, 11.9)

E-Feruloyl Moiety

	δ ^{1}H, ppm (*J*, Hz)					
compd	H-2‴	H-5‴	H-6‴	H-7‴	H-8‴	OMe
8	7.17 (1.6)	6.85 (8.2)	7.06 (1.6, 8.2)	7.52 (15.9)	6.31 (15.9)	3.94

[a] no = not observable due to signal overlap.

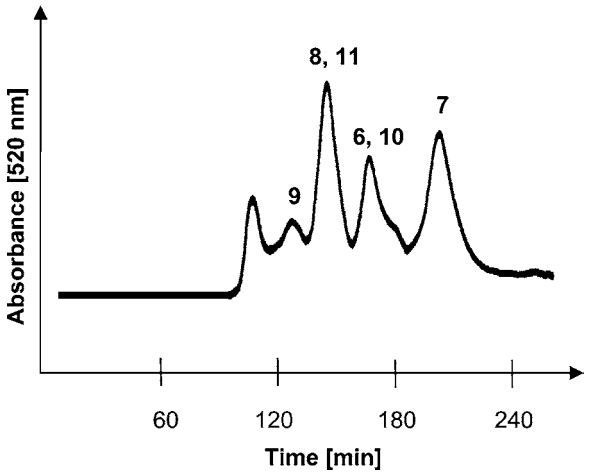


Figure 6. Further CCC fractionation of the coil residue. The solvent system was *n*-butanol/TBME/acetonitrile/water (1:3:1:5, v/v/v/v, acidified with 0.1% TFA, flow rate = 3.5 mL/min). For peak assignment cf. **Table 2**.

Semipreparative HPLC was applied for final purification prior to full structure elucidation by NMR spectroscopy.

The proposed structures of the pyranoanthocyanins **6** and **8** (**Figure 5**) were unambiguously verified by means of NMR spectroscopy and high-resolution mass spectrometry. The ^{1}H NMR spectra confirmed the presence of a cyanidin-type aglycon for both compounds, in which the signals for H-4 were absent and additional signals belonging to a catechol ring system were observed. In the case of **6**, two anomeric protons were present, and three were detected in the spectra of compound **8**. All of these protons exhibited coupling constants in the range of 7−8 Hz, which are indicative of the β-configuration of each glycosidic linkage. Individual spin systems of the sugars and aromatic rings were identified by means of 2D TOCSY experiments. 2D COSY correlations in combination with the

Table 4. ^{13}C NMR Spectroscopic Data for Pyranoanthocyanin **8**

position	δ ^{13}C	position	δ ^{13}C	position	δ ^{13}C
Aglycon		β-D-Galactopyranose		*E*-Feruloyl Moiety	
2	162.6	1	104.0	1‴	127.2
3	134.5	2	82.2	2‴	111.5
4	108.2	3	74.7	3‴	148.8
4a	107.7	4	69.9	4‴	150.1
5	153.1	5	76.2	5‴	116.1
6	100.5	6	70.3	6‴	123.7
7	167.3	β-D-Xylopyranose		7‴	146.8
8	100.6	1	107.3	8‴	114.6
8a	153.8	2	75.8	9‴	168.4
9	98.5	3	77.7	OCH_3	56.2
10	168.6	4	70.3		
1′	121.6	5	67.2		
2′	118.3	β-D-Glucopyranose			
3′	145.8	1	104.7		
4′	152.4	2	74.5		
5′	116.4	3	77.3		
6′	125.7	4	71.3		
1″	122.6	5	74.7		
2″	115.2	6	64.2		
3″	146.9				
4″	153.3				
5″	116.7				
6″	122.9				

[a] Shifts of protonated carbons were taken from the HMQC spectrum and those of quaternary carbons from the HMBC spectrum. Cross-peaks were calibrated relative to the methanol signal at 49.0 ppm.

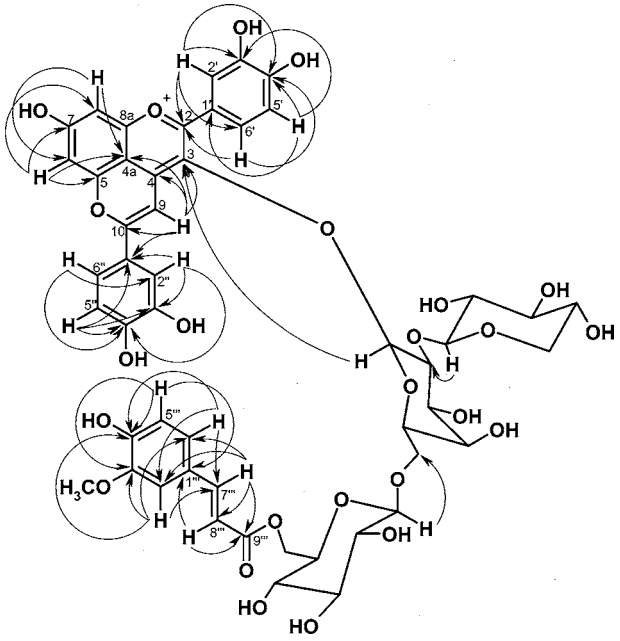


Figure 7. Illustration of the observed two- and three-bond H–C correlations (HMBC experiment) in pyranoanthocyanin **8**.

1D ^{1}H spectra allowed full assignment of all protons and hence unambiguous identification of the nature of the sugar system present (**Table 3**). ^{13}C shifts of all proton-bearing carbon atoms of pyranoanthocyanin **8** were available from the 2D HMQC correlation, whereas long-range correlations in the HMBC spectrum allowed the assignment of the ^{13}C shifts of all quaternary carbon atoms as well as identification of the linkage points of the individual sugar moieties (**Table 4**). The observed two- and three-bond H−C connectivities confirmed the proposed structure (**Figure 7**). By HR-ESI-MS the molecular ions [M^+] of **6** and **8** were detected at *m*/*z* 713.171 (calculated, *m*/*z* 713.1718) and *m*/*z* 1051.272 (calculated, *m*/*z* 1051.2719), respectively. The associated molecular formulas, $C_{34}H_{33}O_{17}$ and $C_{50}H_{51}O_{25}$, are in exact accordance with the proposed structures (**Figure 5**).

The generation of pyranoanthocyanin **8** in black carrot juice can be explained by the reaction of **2** with caffeic acid, following the recently elucidated reaction pathway (*12*). The presence of large amounts of **6**, however, is surprising, as its apparent precursor **4** is only a very minor component of the juice. The most likely explanation for the formation of the high concentrations of **6** is that pyranoanthocyanin **8** is susceptible to hydrolysis and slow cleavage of the feruloylglucose group occurs under the acidic conditions in the juice. A reversed reaction order, hydrolytic cleavage of the acylglucose moiety prior to pyranoanthocyanin formation, is less likely, as this should also result in a higher concentration of **4** in the juice, which was not observed. The reaction between anthocyanins and hydroxycin-

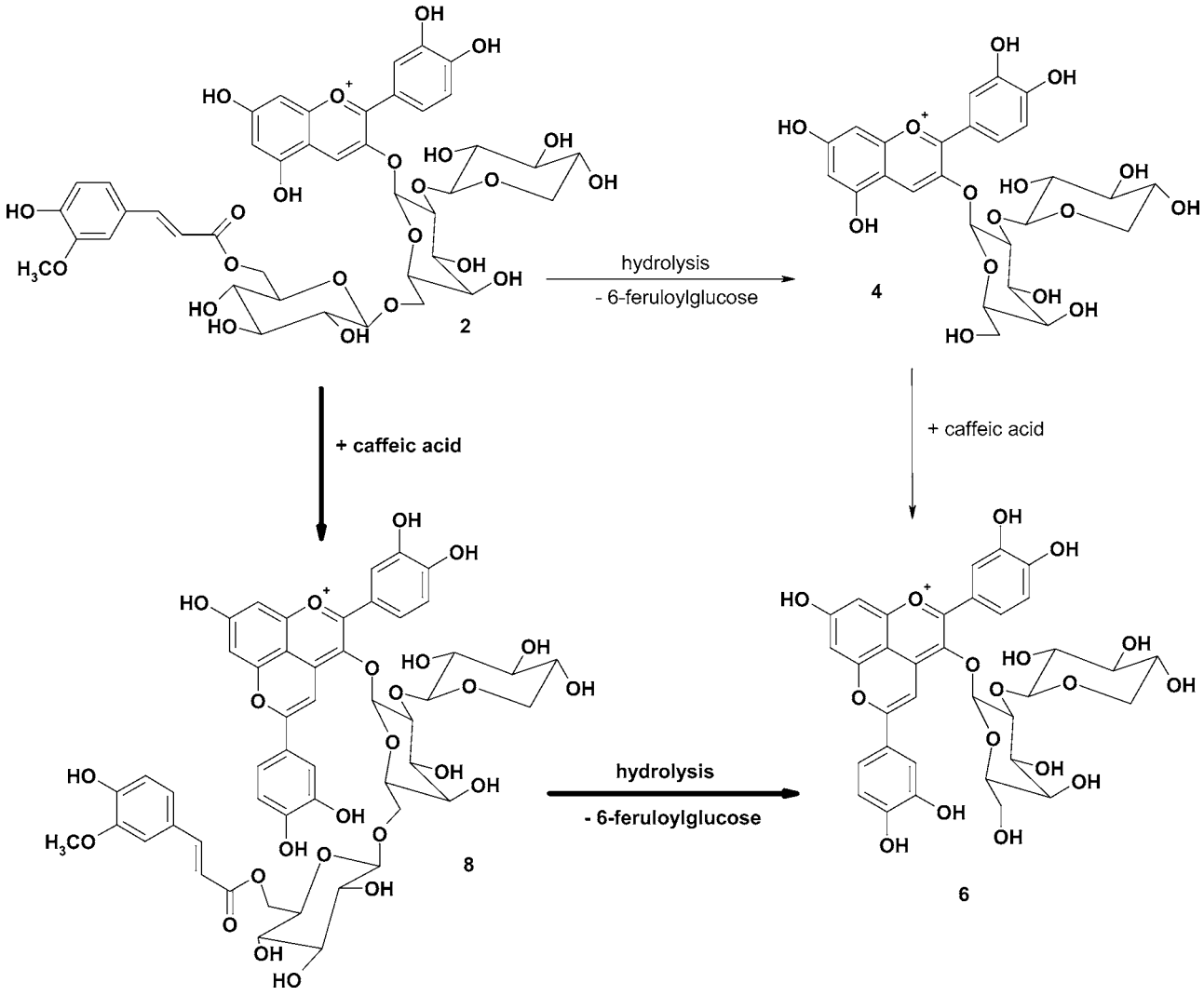


Figure 8. Routes leading to formation of pyranoanthocyanins **6** and **8**.

namic acids proceeds rather slowly (*12*); therefore, hydrolytically generated anthocyanin **4** would accumulate in the juice instead of being transformed into pyranoanthocyanin **6** immediately. The possible reaction pathways are shown in **Figure 8**. Anthocyanins **1**, **3**, and **5** could also play a role, as hydrolysis of the corresponding caffeic acid reaction products (of which only the vinylcatechol adduct **9** of **1** was detected) would in each case lead to the formation of **6**. However, because of its high concentration, it is more likely that the major route starts from Cy-3-(2″-Xyl-6″-feruloylGlc-Gal) **2**.

In a model experiment we could prove the facile formation of pyranoanthocyanin **6** through reaction of caffeic acid with compound **2**. For this experiment, the third fraction of the CCC separation of the black carrot isolate juice XAD-7 extract containing anthocyanins **2** (76.1%), **3** (23.7%), and **5** (0.2%), but not **4** (**Figure 3**), was dissolved in a saturated aqueous solution of caffeic acid. With pH 3.4 this medium was only slightly more acidic than the original black carrot juice. After 3 months, the presence of pyranoanthocyanins **6** and **8** as well as of Cy-3-(2″-Xyl-Gal), **4**, was confirmed by HPLC-DAD and HPLC-ESI-MS^n. Detection of the latter compound in the reaction mixtures supports our assumption that formation of **4** proceeds under acidic conditions via a cleavage of the feruloylglucose moiety from compound **2**.

Chlorogenic acid does not actively take part in pyranoanthocyanin formation as it was shown recently that hydroxycinnamic acid esters are unable to undergo this reaction (*12*). However, chlorogenic acid hydrolyzes slowly at a typical pH of fruit juices as demonstrated in a second model experiment and thus constantly increases or at least maintains the level of reactive free caffeic acid.

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LITERATURE CITED

(1) Harborne, J. B.; Mayer, A. M.; Bar-Nun, N. Identification of the major anthocyanin of carrot cells in tissue culture as cyanidin 3-(sinapoylxylosylglucosylgalactoside). *Z. Naturforsch., C: Biosci.* **1983**, *38*, 1055−1056.

(2) Glässgen, W. E.; Wray, V.; Strack, D.; Metzger, J. W.; Seitz, H. U. Anthocyanins from cell suspension cultures of *Daucus carota*. *Phytochemistry* **1992**, *31*, 1593−1601.

(3) Glässgen, W. E.; Seitz, H. U.; Metzger, J. W. High-performance liquid chromatography/electrospray mass spectrometry and tandem mass spectrometry of anthocyanins from plant tissues and cell cultures of *Daucus carota* L. *Biol. Mass Spectrom.* **1992**, *21*, 271−277.

(4) Baker, D. C.; Dougall, D. K.; Glässgen, W. E.; Johnson, S. C.; Metzger, J. W.; Rose, A.; Seitz, H. U. Effects of supplied cinnamic acids and biosynthetic intermediates on the anthocyanins accumulated by wild carrot suspension cultures. *Plant Cell Tissue Organ Cult.* **1994**, *39*, 79−91.

(5) Dougall, D. K.; Baker, D. C.; Gakh, E. G.; Redus, M. A.; Whittemore, N. A. Studies on the stability and conformation of monoacylated anthocyanins. Part 2—Anthocyanins from wild carrot suspension cultures acylated with supplied carboxylic acids. *Carbohydr. Res.* **1998**, *310*, 177−189.

(6) Kammerer, D.; Berardini, N.; Schieber, A.; Carle, R. Charakterisierung von Anthocyanen und phenolischen Säuren aus Schwarzen Karotten mittels HPLC-MS. *Lebensmittelchemie* **2003**, *57*, 28−29.

(7) Kammerer, D.; Carle, R.; Schieber, A. Detection of peonidin and pelargonidin glycosides in black carrots (*Daucus carota* ssp. *sativus* var. *atrorubens* Alef.) by high-performance liquid chromatography/electrospray ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 2407−2412.

(8) Stöhr, H.; Herrmann, K. Über die Phenolsäuren des Gemüses. III. Hydroxyzimtsäuren und Hydroxybenzoesäuren des Wurzelgemüses. *Z. Lebensm. -Unters. -Forsch.* **1975**, *159*, 219−224.

(9) Malien-Aubert, C.; Dangles, O.; Amiot, M. J. Color stability of commercial anthocyanin-based extracts in relation to the phenolic composition. Protective effects by intra- and intermolecular copigmentation. *J. Agric. Food Chem.* **2001**, *49*, 170−176.

(10) Braun, G.; Seitz, U. Verlauf der Akkumulation von Kaffee-, Ferula- und Chlorogensäure in Beziehung zur Cyanidinakkumulation bei 2 Zellinien von *Daucus carota*. *Biochem. Physiol. Pflanz.* **1975**, *168*, 93−100.

(11) Alasalvar, C.; Grigor, J. M.; Zhang, D. L.; Quantick, P. C.; Shahidi, F. Comparison of volatiles, phenolics, sugars, antioxidant vitamins, and sensory quality of different colored carrot varieties. *J. Agric. Food Chem.* **2001**, *49*, 1410−1416.

(12) Schwarz, M.; Wabnitz, T. C.; Winterhalter, P. Pathway leading to the formation of anthocyanin-vinylphenol adducts and related pigments in red wines. *J. Agric. Food Chem.* **2003**, *51*, 3682−3687.

(13) Schwarz, M.; Hillebrand, S.; Habben, S.; Degenhardt, A.; Winterhalter, P. Application of high-speed countercurrent chromatography to the large-scale isolation of anthocyanins. *Biochem. Eng. J.* **2003**, *14*, 179−189.

(14) Schwarz, M.; Jerz, G.; Winterhalter, P. Isolation and structure of pinotin A, a new anthocyanin derivative from Pinotage wine. *Vitis* **2003**, *42*, 105−106.

(15) Schwarz, M.; Winterhalter, P. A novel synthetic route to substituted pyranoanthocyanins with unique colour properties. *Tetrahedron Lett.* **2003**, *44*, 7583−7587.

(16) Schwarz, M.; Hofmann, G.; Winterhalter, P. Investigations on anthocyanins in wines from *Vitis vinifera* cv. Pinotage: Factors influencing the formation of pinotin A and its correlation with wine age. *J. Agric. Food Chem.* **2004**, *52*, 498−504.

(17) Schobinger, U. *Frucht- und Gemüsesäfte*, 3rd ed.; Ulmer: Stuttgart, Germany, 2001.

(18) Degenhardt, A.; Habben, S.; Winterhalter, P. Isolation of physiologically active compounds from nutritional beverages and vegetables by countercurrent chromatography. In *Neutraceutical Beverages. Chemistry, Nutrition, and Health Effects*; Shahidi, F., Weerasinghe, D. K., Eds.; ACS Symposium Series 871; American Chemical Society: Washington, DC, 2004; pp 443−456.

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