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Characterization of Anthocyanins and Pyranoanthocyanins from Blood Orange [*Citrus sinensis* (L.) Osbeck] Juice

SILKE HILLEBRAND, MICHAEL SCHWARZ, AND PETER WINTERHALTER*

Institute of Food Chemistry, Technical University of Braunschweig, Schleinitzstrasse 20, 38106 Braunschweig, Germany

Anthocyanins from blood orange [*Citrus sinensis* (L.) Osbeck] juices were isolated and purified by means of high-speed countercurrent chromatography and preparative high-performance liquid chromatography. Structures of the pigments were then elucidated by electrospray ionization multiple mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy. The major anthocyanins of the juice were characterized as cyanidin 3-glucoside and cyanidin 3-(6″-malonylglucoside). Furthermore, six minor anthocyanins were detected and identified as cyanidin 3,5-diglucoside, delphinidin 3-glucoside, cyanidin 3-sophoroside, delphinidin 3-(6″-malonylglucoside), peonidin 3-(6″-malonylglucoside), and cyanidin 3-(6″-dioxalylglucoside). The occurrence of the latter compound in blood oranges is reported here for the first time, together with full NMR spectroscopic data. Further investigations revealed the presence of four anthocyanin-derived pigments, which are formed through a direct reaction between anthocyanins and hydroxycinnamic acids during prolonged storage of the juice. These novel pyranoanthocyanins were identified as the 4-vinylphenol, 4-vinylcatechol, 4-vinylguaiacol, and 4-vinylsyringol adducts of cyanidin 3-glucoside through comparison of their mass spectrometric and chromatographic properties with those of synthesized reference compounds.

KEYWORDS: Blood orange; *Citrus sinensis* (L.) Osbeck; anthocyanins; cyanidin 3-(6″-dioxalylglucoside); oxalic acid; anthocyanin-derived pigments; pyranoanthocyanins; vinylphenol; aging products

INTRODUCTION

Anthocyanins are responsible for the red, purple, and blue colors of many fruits. Besides fresh fruits, fruit juices represent a large amount of the consumer's intake of fruit products. Because of processing and storage of fruit juices, changes in the anthocyanin composition are known to occur. Most notably, the content of polymeric pigments increases, while concomitantly the concentration of the genuine monomeric anthocyanins decreases (*1*−*4*).

Blood orange [*Citrus sinensis* (L.) Osbeck] juices contain anthocyanins in differing concentrations depending on the variety. The two major anthocyanins of blood oranges have previously been identified as cyanidin 3-glucoside and cyanidin 3-(6″-malonylglucoside) (*5*, *6*). So far, only little information concerning the structure of minor pigments has been published. Dugo and co-workers characterized delphinidin 3-glucoside, peonidin 3-glucoside, cyanidin 3-rutinoside, and the 6″-malonylglucose esters of delphinidin, peonidin, and petunidin, respectively, as minor anthocyanins of Sicilian blood orange juice by micro-high-performance liquid chromatography (HPLC)-electrospray ionization mass spectrometry (ESI-MS). The structure of further anthocyanins remained unknown (*7*).

Recently, a novel pathway leading to the formation of pyranoanthocyanins (i.e., anthocyanins that contain an additional pyran ring between the C-4 and the hydroxyl group attached to C-5) was discovered (*8*). The generation of the nongenuine pyranoanthocyanins proceeds through a direct reaction between anthocyanins and free hydroxycinnamic acids, e.g., coumaric, caffeic, ferulic, and sinapic acid. It was found that the concentration of these newly formed pigments increased with the storage time of red wines (*9*). Most recently, six of these pyranoanthocyanins could be identified in black carrot juice (*10*). Blood orange juices contain a similar combination of the reactive precursors, i.e., hydroxycinnamic acids and anthocyanins. Although the structure of the blood orange anthocyanins is different from the pigments commonly encountered in red wines or black carrots, formation of such pyranoanthocyanin pigments in blood orange juice was likely to occur.

Hence, the aim of this work was to characterize the anthocyanin composition as well as possible newly formed anthocyanin derivatives in blood orange juice. Analyses were performed by HPLC with diode array detection (DAD), ESI-MS^n, and nuclear magnetic resonance spectroscopy (NMR).

MATERIALS AND METHODS

Chemicals. All solvents were of HPLC quality, and the chemicals were of p.a. grade. Cyanidin 3-glucoside was isolated from blackberries as described previously (*11*). Cyanidin 3-(6″-malonylglucoside) of ∼90% purity was isolated from blood oranges by high-speed countercurrent chromatography (HSCCC) as described here.

* To whom correspondence should be addressed. Tel: +49-531-391-7200. Fax: +49-531-391-7230. E-mail: P.Winterhalter@tu-bs.de.

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Orange Juices. Besides a commercial blood orange juice obtained from a local health food store, a fresh juice was prepared from blood oranges of the Moro variety. The freshly squeezed juice (pH 3.7) was centrifuged at 4000 rpm, passed through a folded filter, and filled into an amber glass bottle. The air in the headspace was replaced with argon, and the bottle was stored in the dark at 15 °C in a climatized room for 10 months in order to simulate the aging process. The juice was analyzed by HPLC-ESI-MS^n immediately after pressing and after 1, 3, and 10 months of storage.

Extraction of Anthocyanins. An anthocyanin-enriched extract from a commercial blood orange juice was prepared by solid phase extraction (*11*). The juice (0.7 L) was filtered and then poured onto a glass column (100 cm × 6 cm) filled with Amberlite XAD-7 resin. The column was extensively washed with water to remove sugars, organic acids, proteins, and salts. Phenolic compounds (such as the anthocyanins) were retained by the resin. For elution, a mixture of methanol−acetic acid (19:1, v/v) was used (~1 L). Methanol was evaporated in vacuo, and the aqueous phase was lyophilized.

Fractionation of the Anthocyanin-Enriched Extract by HSCCC. Separations were carried out with a CCC-1000 high-speed countercurrent chromatograph (Pharma-Tech Research Corp., Baltimore, MD) equipped with three coils connected in series (total volume, 850 mL; speed, 800 rpm). The solvent system consisted of MTBE/*n*-butanol/acetonitrile/water [1:3:1:5, v/v/v/v, acidified with 0.1% trifluoroacetic acid (TFA)]. The mobile phase (more dense layer) was delivered by a Biotronik BT 3020 HPLC pump (Jasco, Germany) at a flow rate of 4.0 mL/min. Prior to injection, the sample was dissolved in 10 mL of each of the mobile and the stationary phases. Separation was monitored with a UV/vis detector (Knauer, Germany) at $\lambda = 520$ nm. Fractions were collected every 4 min with a Super Frac fraction collector (Pharmacia LKB, Sweden), combined according to the chromatogram, and lyophilized.

HPLC. HPLC-DAD analyses were performed on an MD-910 multiwavelength detector (220−650 nm), equipped with a DG-980-50 three-line degasser, an LG-980−02 ternary gradient unit, a PU-980 Intelligent HPLC pump, and Borwin PDA chromatography software (Jasco, Germany). Anthocyanins were detected at 520 nm. The samples were injected via a Rheodyne 7125 injection valve (Techlab, Germany) equipped with a 20 μL loop, and separations were carried out on a 250 mm × 4.6 mm i.d., 5 μm, Luna RP-18 column (Phenomenex, Germany) at a flow rate of 0.5 mL/min. Two solvent systems were used. Solvent system A consisted of water/acetonitrile/formic acid 87:3:10 (v/v/v), and solvent system B consisted of water/acetonitrile/formic acid 40:50:10 (v/v/v). The linear gradient was as follows: 0 min, 6% B; 20 min, 20% B; 35 min, 40% B; 40 min, 60% B; 45 min, 90% B; 55 min, 6% B.

Preparative HPLC. Purifications were performed on a preparative HPLC system consisting of an HPLC pump 64, a variable wavelength UV/vis detector, and HPLC software 2.0 (Knauer, Germany). The samples (~10 mg) were injected via a Rheodyne 7125 injection valve (Techlab, Germany) equipped with a 200 μL loop, and separations were carried out on a 250 mm × 10.0 mm i.d., 5 μm, Luna RP-18 column (Phenomenex) at a flow rate of 5 mL/min. The solvent system was water/acetonitrile/formic acid 80:15:5 (v/v/v).

HPLC-ESI-MS^n. HPLC-MS analyses of fractions and purified anthocyanins were performed on a Bruker Esquire LC-MS system (Bruker Daltonik, Germany). The HPLC system consisted of a System 1100 binary pump G1312A (Agilent, Germany) and a Lichrograph L-4000 UV/vis detector (Merck Hitachi, Japan). UV chromatograms were recorded with a Chromatopac C-R6A integrator (Shimadzu, Japan). The LC part of the system was controlled by ChemStation version A.06.01, and MS data were processed by Esquire NT 4.0 software (Bruker Daltonik). MS parameters: positive ion mode; capillary, −2500 V; capillary exit offset, 70 V; end plate offset, −500 V; skimmer 1, 20 V; skimmer 2, 10 V; dry gas, N_2, 11 L/min; dry temperature, 325 °C; nebulizer, 60 psi; and scan range, 50−1000 *m*/*z*; chromatographic conditions were as described above.

Alternatively, for MS^n experiments, the sample solution was delivered directly by a syringe pump 74900 (Cole-Parmer, United States) into the ESI source at a flow rate of 240 μL/h. MS parameters: positive ion mode; capillary, −3500 V; capillary exit offset, 60 V; end plate offset, −500 V; skimmer 1, 30 V; skimmer 2, 10 V; dry gas, N_2, 4 L/min; dry temperature, 300 °C; nebulizer, 10 psi; and scan range, 50−1000 *m*/*z*.

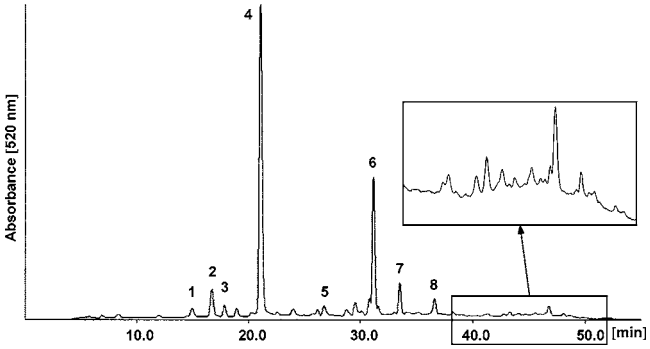


Figure 1. HPLC chromatogram of a commercial blood orange juice at 520 nm.

Table 1. Chromatographic Properties and Relative Amounts of the Anthocyanins in the Blood Orange Juice XAD-7 Extract

peak	retention time (min)	relative composition[a] (%)
1	14.9	1.9
2	16.7	5.1
3	17.8	1.9
4	21.0	57.1
5	26.7	2.1
6	31.2	20.6
7	33.5	4.5
8	36.6	2.9
minor compds	>40	3.9

[a] Relative content of anthocyanins calculated from peak areas at 520 nm. The numbering is according to **Figure 1**.

Proton and Carbon NMR. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AMX 300 spectrometer (Bruker Biospin, Germany) at 300.13 and 75.49 MHz, respectively. Anthocyanins were dissolved in a mixture of methanol-d_4/TFA-d_1 (19:1, v/v). Data were processed by WIN NMR software version 6.1.0.0.

Preparation of Pyranoanthocyanins. Cyanidin 3-glucoside and cyanidin 3-(6″-malonylglucoside) were dissolved in aqueous HCl (pH 2.8) at a concentration of 2000 mg/L. To 21 mL of each anthocyanin solution was added 9 mL of an ethanolic solution of one of the following trans-configured hydroxycinnamic acids: *p*-coumaric acid, ferulic acid, caffeic acid, and sinapic acid ($c = 4000$ mg/L). The so-obtained eight model solutions were filled into 30 mL amber glass bottles, and the air in the headspace was replaced with argon. The bottles were stored in the dark in a climatized room at 15 °C, and the solutions were analyzed after 1 month by HPLC-DAD and HPLC-ESI-MS^n.

RESULTS AND DISCUSSION

Identification of Anthocyanins. The HPLC chromatogram of a commercially available blood orange juice is shown in **Figure 1**. Two major pigments (**4** and **6**) and six minor anthocyanins (**1**, **2**, **3**, **5**, **7**, and **8**) were detected at 520 nm; some additional minor pigments eluted at retention times >40 min. The relative composition of the anthocyanin-enriched XAD-7 extract (calculated from the peak areas at 520 nm) is presented in **Table 1** and exactly reflected the original anthocyanin distribution in the entire juice. The XAD-7 isolate was fractionated by HSCCC. Eight fractions as well as the residue that remained on the PTFE column (the so-called coil) were obtained (**Figure 2**). The anthocyanin composition in each fraction was evaluated by HPLC-DAD. Fraction 1 was composed mainly of polymeric pigments that could not be resolved by HPLC, whereas fractions 2−8 contained mixtures of the

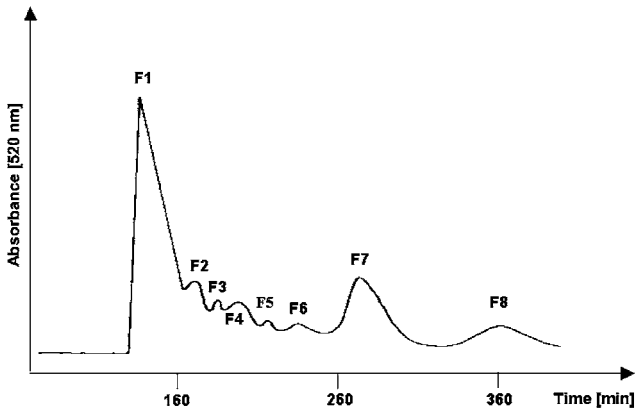


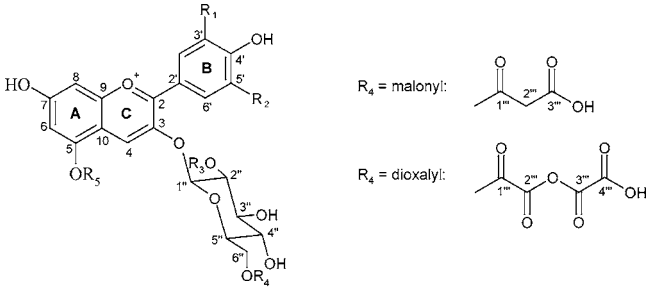
Figure 2. HSCCC separation of anthocyanins from blood orange juice. Solvent system, MTBE/*n*-butanol/acetonitrile/water (1:3:1:5, v/v/v/v, acidified with 0.1% TFA); flow rate, 4.0 mL/min; and detection at 520 nm.

genuine anthocyanins and colorless polyphenols. The majority of anthocyanins **1** and **3** was recovered in fraction 2, pigment **2** was found in fraction 6, and compound **4** was found in fraction 7. Fraction 8 contained anthocyanins **6**−**8** and trace amounts of **5**, but the majority of the latter compound was collected in the coil residue. Prior to further structural characterization by ESI-MS^n and ^{1}H and ^{13}C NMR spectroscopy, the pigments from each HSCCC fraction were purified by preparative HPLC.

ESI-MS^n analysis provided information about the molecular mass of the anthocyanins, their characteristic fragmentation pattern, and the nature of the respective aglycon moiety. Anthocyanins **1**, **3**, **4**, **6**, and **7** were identified as pigments based on cyanidin as aglycon with *m*/*z* 287. Compounds **1** and **3** possessed identical molecular masses (*m*/*z* 611), but a different fragmentation pattern was observed. Two individual hexose moieties with *m*/*z* 162 were cleaved from **1**, resulting in an intermediate fragment of *m*/*z* 449 in addition to the fragment of the aglycon with *m*/*z* 287. In contrast, **3** showed the loss of only one unit composed of two hexoses (324 u = 2 × 162 u). Structure elucidation by ^{1}H and ^{13}C NMR spectroscopy of both pigments confirmed the presence of a cyanidin core and identified all sugar residues as β-D-glucose. The β-D-glucosidic linkage points in **1** were—in compliance with the observed fragmentation pattern—determined at positions C-3 and C-5. In **3**, the sugar moiety was established to be a β-D-glucosyl-(1→2)-β-D-glucoside (sophoroside) unit bound to position C-3 of the aglycon. The two pigments were thus identified as cyanidin 3,5-O-β-D-diglucoside (**1**) and cyanidin 3-O-sophoroside (**3**). Compound **4** produced a mass spectrum with $[M]^+$ at *m*/*z* 449 and a loss of 162 upon fragmentation. It was identified as cyanidin 3-O-β-D-glucoside after NMR analysis. Upon fragmentation of **6** with a molecular mass of 535, an ion at *m*/*z* 449 $[M - 86]^+$ was observed, corresponding to the cleavage of a malonic acid ester. Further fragmentation resulted in the loss of a hexose moiety. After NMR spectroscopic evaluation, this pigment was identified as cyanidin 3-O-β-D-(6″-malonylglucoside). The detected ions with *m*/*z* 303 following fragmentation of compounds **2** and **5** were indicative of the presence of a delphinidin aglycon, whereas pigment **8** was determined to contain a peonidin core (*m*/*z* 301). The molecular ion of **2** was found at *m*/*z* 465, and cleavage of one hexose was observed. NMR spectroscopy established the identity of this pigment as delphinidin 3-O-β-D-glucoside. The isolated amounts of compounds **5** and **8** were not sufficient for analysis by NMR. Fragment ions at $[M - 86]^+$ and $[M - 86 - 162]^+$ suggested for both compounds the presence of one malonyl group and one hexose. Thus, these pigments were tentatively identified based on their mass spectrometric properties and comparison with literature

Table 2. ^{1}H and ^{13}C NMR Spectroscopic Data of Cyanidin 3-Glucoside (**4**) and Cyanidin 3-(6″-Dioxalylglucoside) (**7**) in CD_3OD/TFA-d_1 (19:1, v/v)

	cyanidin 3-glucoside (**4**)		cyanidin 3-(6″-dioxalylglucoside) (**7**)	
position	^{1}H NMR [ppm] ($J_{H,H}$ in Hz)	^{13}C NMR (ppm)	^{1}H NMR [ppm] ($J_{H,H}$ in Hz)	^{13}C NMR (ppm)
aglycon				
2		164.5		164.4
3		145.7		145.3
4	8.96 s	137.1	8.96 s	137.2
5		159.3		159.9
6	6.90 dd (0.5, 2.0)	103.4	6.91 s	110.3
7		170.5		170.5
8	6.66 d (2.0)	95.2	6.59 d (1.5)	103.6
9		157.8		157.6
10		113.4		113.2
1′		121.3		121.1
2′	8.05 d (2.0)	118.5	8.04 d (2.5)	117.8
3′		147.4		147.3
4′		155.8		155.8
5′	7.02 d (9.0)	117.4	7.04 d (9.0)	118.4
6′	8.26 dd (2.0, 9.0)	128.3	8.26 dd (2.5, 9.0)	128.3
3-glucosyl				
1″	5.29 d (7.5)	103.9	5.29 d (7.0)	110.3
2″	4.67 dd (7.5, 9.0)	74.8	4.27 dd (7.0, 9.0)	74.6
3″	3.55 dd (9.0, 9.0)	78.2	3.89 dd (9.0, 9.0)	76.0
4″	3.43 dd (9.0, 9.5)	71.2	3.51 dd (9.0, 9.5)	71.2
5″	3.55 ddd (2.0, 6.0, 9.5)	78.8	3.94 ddd (2.0, 5.5, 9.5)	77.8
6″-A	3.71 dd (6.0, 12.0)	62.4	4.27 dd (5.5, 12.0)	64.5
6″-B	3.91 dd (2.0, 12.0)		4.52 dd (2.0, 12.0)	
6″-dioxalyl				
1‴				158.1
2‴				172.4
3‴				172.4
4‴				174.8



compd	R_1	R_2	R_3	R_4	R_5
1	OH	H	H	H	glucosyl
2	OH	OH	H	H	H
3	OH	H	glucosyl	H	H
4	OH	H	H	H	H
5	OH	OH	H	malonyl	H
6	OH	H	H	malonyl	H
7	OH	H	H	dioxalyl	H
8	OCH_3	H	H	malonyl	H

For compound labeling cf. Figure 1

Figure 3. Structures of the anthocyanins from blood orange juice: cyanidin 3,5-diglucoside (**1**), delphinidin 3-glucoside (**2**), cyanidin 3-sophoroside (**3**), cyanidin 3-glucoside (**4**), delphinidin 3-(6″-malonylglucoside) (**5**), cyanidin 3-(6″-malonylglucoside) (**6**), cyanidin 3-(6″-dioxalylglucoside) (**7**), and peonidin 3-(6″-malonylglucoside) (**8**).

data (*7*) as delphinidin 3-(6″-malonylglucoside) (**5**) and peonidin 3-(6″-malonylglucoside) (**8**).

Compound **7** had an apparent molecular mass $[M]^+$ of 593 u, and two fragment ions at *m*/*z* 449 $[M - 144]^+$ and *m*/*z* 287 $[M - 144 - 162]^+$, the latter corresponding to the loss of one hexose. A pigment with the same mass spectrometric properties was earlier reported to occur in blood orange juice by Dugo et al. (*7*), but its structure was not elucidated. The ^{1}H NMR spectrum of **7** was almost identical with the spectroscopic data of cyanidin 3-glucoside (**4**), the only exception being a strong downfield shift of the glucose protons H-6″A and H-6″B. This result is indicative for the linkage of an acidic ester group in this position; however, no signals of additional protons were observed. The ^{13}C NMR spectrum of **7** is displayed in comparison to cyanidin 3-glucoside (**4**) four additional carbon resonances in the region of quaternary carbonyl signals between 158.1 and 174.8 ppm, two of them showing a symmetry within

Table 3. Identity and Mass Spectrometric Properties of the Anthocyanins from Blood Orange Juice

compd	$[M^+]$ (*m*/*z*)	MS/MS (*m*/*z*)	identified as
1	611	287, 449	cyanidin 3,5-O-β-D-diglucoside[a]
2	465	303	delphinidin 3-O-β-D-glucoside[a]
3	611	287	cyanidin 3-O-sophoroside[a]
4	449	287	cyanidin 3-O-β-D-glucoside[a]
5	551	303, 465	delphinidin 3-(6″-malonylglucoside)[b]
6	535	287, 449	cyanidin 3-O-β-D-(6″-malonylglucoside)[a]
7	593	287, 449	cyanidin 3-O-β-D-(6″-dioxalylglucoside)[a]
8	549	301, 463	peonidin 3-(6″-malonylglucoside)[b]

[a] Structure identification based on ESI-MS^n analysis as well as ^{1}H and ^{13}C NMR data. [b] Structure identification based on ESI-MS^n analysis. The numbering is according to **Figure 1**, for structures cf. **Figure 3**.

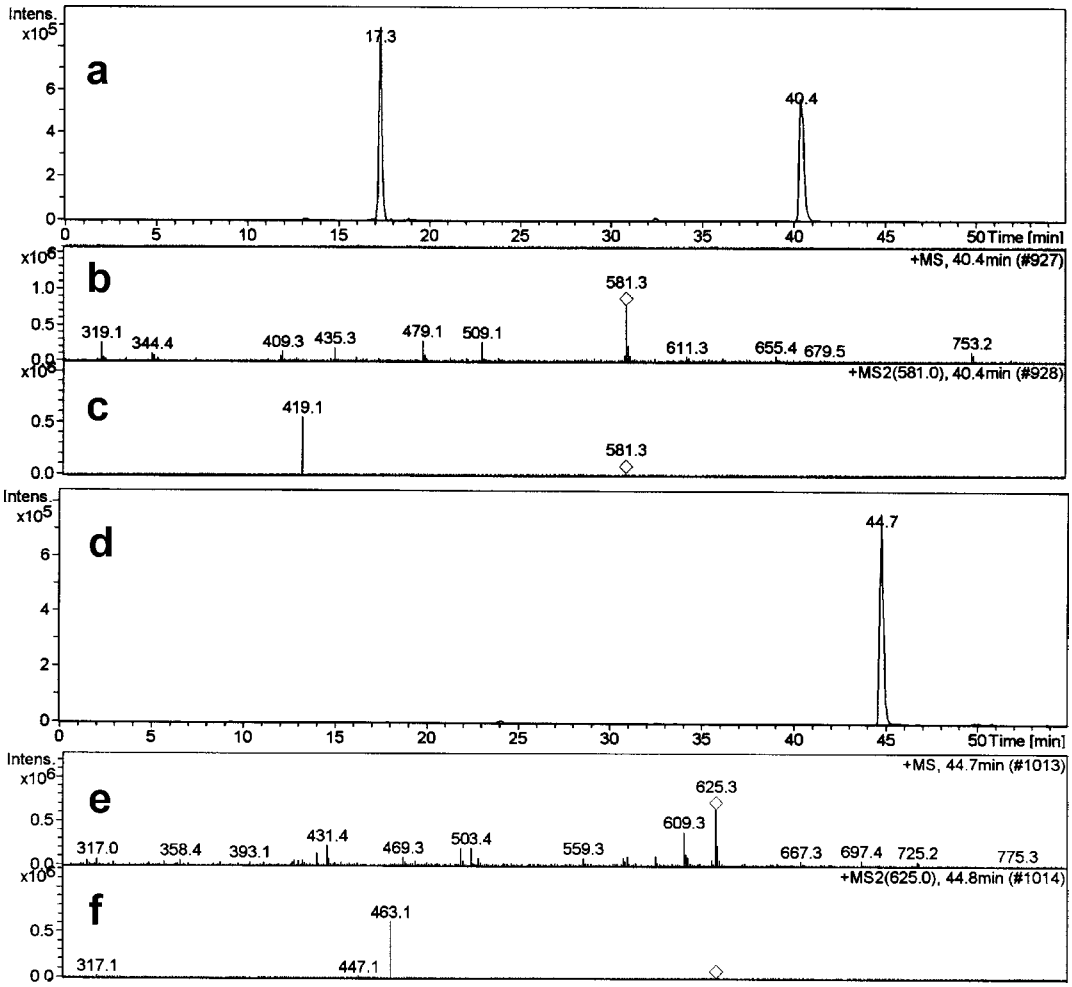


Figure 4. HPLC-ESI-MS ion chromatogram of the coil residue at *m*/*z* 581 (**a**) and *m*/*z* 625 (**d**), and mass spectra and fragmentation pattern of the peaks at 40.4 (**b**,**c**) and 44.7 (**e**,**f**) min.

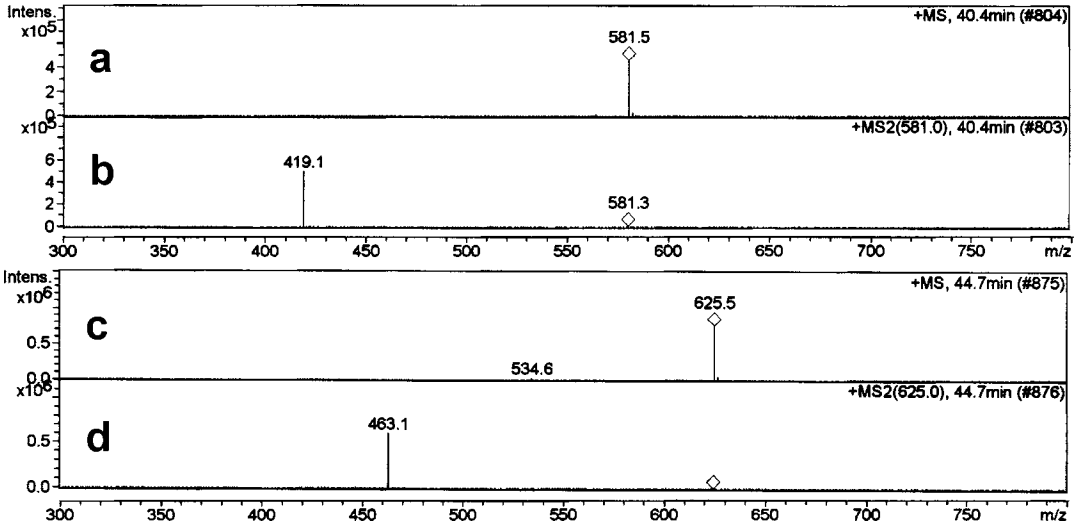


Figure 5. Mass spectra and fragmentation pattern of the synthesized reaction products of cyanidin 3-glucoside with caffeic acid (**a**,**b**) and sinapic acid (**c**,**d**).

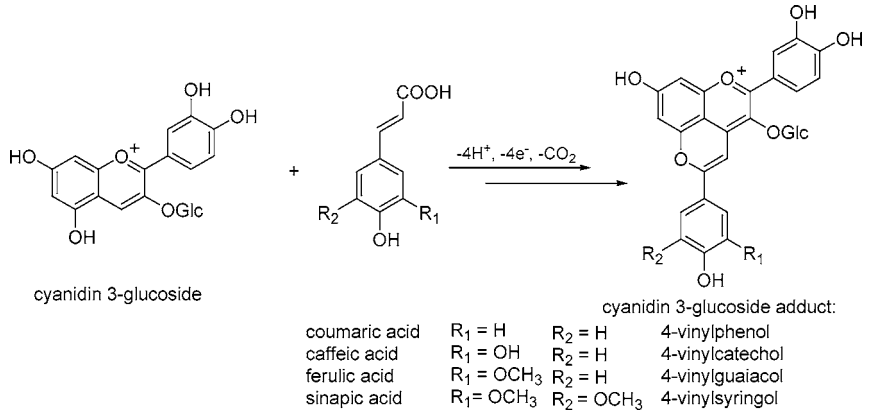


Figure 6. General reaction scheme for the formation of cyanidin 3-glucoside-derived pyranoanthocyanins. For details on the reaction pathway, please refer to ref *8*.

the acidic ester residue (**Table 2**). The four carbonyl groups together account for a weight of 112 u. The remaining difference of 32 u to the observed fragment of *m*/*z* 144 can be explained by the presence of two additional oxygen atoms; hence, the composition of the acidic ester moiety must be C_4O_6. Thus, pigment **7** was in compliance with its mass spectrometric properties and NMR spectroscopic data identified as cyanidin 3-(6″-dioxalylglucoside). On the basis of MS data and information obtained from the hydrolysis of the pigment, cyanidin 3-(6″-dioxalylglucoside) has already been tentatively identified by Stintzing et al. in blackberries, but further investigation of the structure and binding site of the oxalic acid residue by NMR techniques was not possible due to the low quantity available (*12*). In our earlier studies on anthocyanins from blackberries, we obtained an HSCCC fraction that contained the described pigment (*11*). By HPLC coinjection with the cyanidin 3-(6″-dioxalylglucoside) now isolated from blood orange juice, we were able to confirm that both anthocyanins are fully identical. This is the first report on anthocyanins with an oxalic acid residue in the family of the Rutaceae. So far, apart from blackberries (Rosaceae), oxalylated anthocyanins were only described to occur in flowers of the Orchidaceae family (*13*, *14*). The mass spectrometric properties and identities of anthocyanins **1**−**8** are summarized in **Table 3**, while the structures are shown in **Figure 3**.

Identification of Pyranoanthocyanins. The CCC separation of the anthocyanin-enriched XAD-7 extract from the commercial blood orange juice was terminated after the elution of the eight major anthocyanins. The solvent mixture remaining on the coil was recovered, the organic solvents were evaporated in vacuo, and the solution was freeze-dried.

Table 4. Mass Spectrometric Properties of the Synthesized Cyanidin 3-(6″-Malonylglucoside)-Derived Pyranoanthocyanins

cyanidin 3-(6″-malonylglucoside) adduct	retention time (min)	molecular ion $[M]^+$ (*m*/*z*)	aglycon (*m*/*z*)
4-vinylphenol	44.5	651	403
4-vinylcatechol	41.7	667	419
4-vinylguaiacol	45.0	681	433
4-vinylsyringol	45.2	711	463

The HPLC-DAD chromatogram of this fraction revealed a strong enrichment of less polar components with a retention time >30 min (chromatogram not shown), which had been hardly visible in the chromatogram of the entire juice (**Figure 1**). The visible absorbance maximum of some of the pigments in this fraction was hypsochromically shifted to ∼510 nm as compared to a maximum of ∼530 nm of the major anthocyanins. These observations were consistent with the chromatographic behavior and color properties of pyranoanthocyanins isolated from red wine (*15*, *16*).

HPLC-ESI-MS^n analysis of the coil residue revealed the presence of two pigments that did not contain the previously

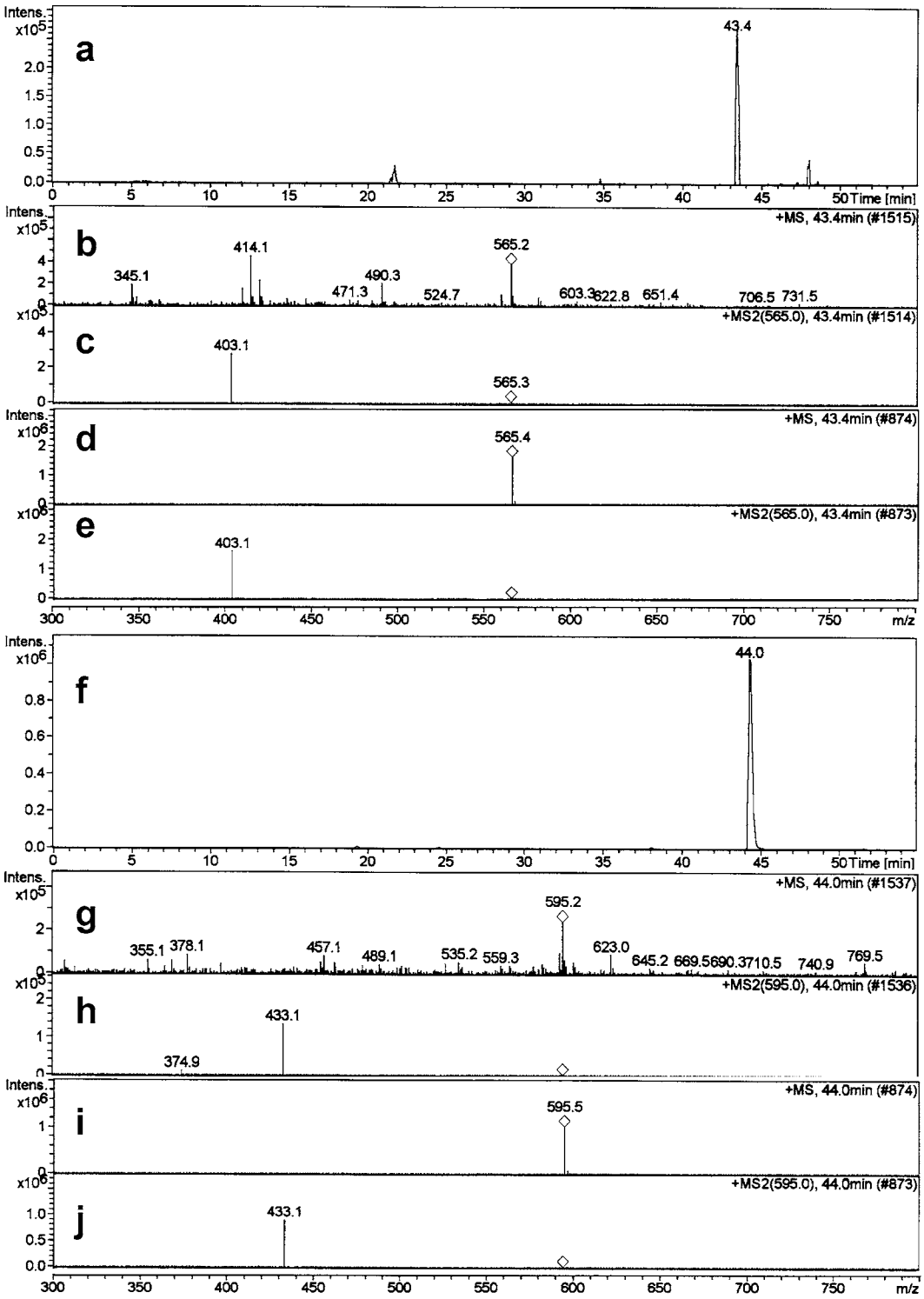


Figure 7. HPLC-ESI-MS chromatogram of the freshly squeezed blood orange juice at *m*/*z* 565 (**a**) after 3 months of storage and at *m*/*z* 595 after 10 months (**f**). Mass spectra and fragmentation pattern of the peaks at 43.3 (**b**,**c**) and 44.0 (**g**,**h**) min are compared to the properties of the reaction products of cyanidin 3-glucoside with coumaric acid (**d**,**e**) and ferulic acid (**i**,**j**).

encountered cyanidin or delphinidin aglycons. The molecular masses $[M]^+$ of the two pigments were determined to be at 581 and 625 mass units, respectively. Upon fragmentation, we observed for both compounds the cleavage of a hexose moiety (162 u), resulting in fragments with $m/z = 419$ and $m/z = 463$, respectively (**Figure 4**). Formally, these aglycons correspond to the adducts of either vinylcatechol or vinylsyringol to a cyanidin core. For red wines, it was recently shown that this type of pyranoanthocyanins can be formed through a direct reaction between anthocyanins and hydroxycinnamic acids (*8*). We therefore concluded that the two pyranoanthocyanins in question are the reaction products of cyanidin 3-glucoside, one of the most abundant anthocyanins of blood orange juices, with caffeic acid and sinapic acid, respectively.

Because both pigments were only present at very low concentrations, isolation and complete structure elucidation by NMR were not possible. To obtain authentic reference compounds for comparison of retention times and mass spectrometric

properties, we performed small-scale model experiments. Pure cyanidin 3-glucoside, recently isolated from blackberries (*11*), was reacted separately with *p*-coumaric acid, caffeic acid, ferulic acid, and sinapic acid under previously optimized conditions (*16*). After 1 month, the newly formed pyranoanthocyanins were analyzed by HPLC-DAD and HPLC-ESI-MS^n. Retention time, molecular mass, and fragmentation pattern of the reaction products of cyanidin 3-glucoside with caffeic acid and sinapic acid were in agreement with the properties of the two pigments detected in commercial blood orange juice (**Figure 5**). Structures and pathways of formation for the two novel pyranoanthocyanins are shown in **Figure 6**. Neither the coumaric acid nor the ferulic acid adduct was found in the juice.

Accordingly, we synthesized the 4-vinylphenol, 4-vinylcatechol, 4-vinylguaiacol, and 4-vinylsyringol adducts of cyanidin 3-(6″-malonylglucoside). We expected them to be present in the juice, as cyanidin 3-(6″-malonylglucoside) was initially one of the most abundant anthocyanins. Again, the mass spectrometric data of the synthesized compounds complied with the expected structures (**Table 4**). However, even with knowledge of the exact retention time, none of these pyranoanthocyanins could be detected in the juice. This unexpected result can be explained by an observation made during the synthesis of the malonylated pyranoanthocyanins: the isolated cyanidin 3-(6″-malonylglucoside) that was used for the synthesis contained only 4.6% cyanidin 3-glucoside as an impurity (as determined by the relative peak areas at 530 nm). However, after 1 month of reaction time, the malonylated as well as the nonesterified pyranoanthocyanins were present in almost equal concentrations. This confirms that the malonyl esters are susceptible to rapid hydrolysis under acidic conditions (*6*, *17*). Steric hindrance could also be a possible reason for the preferential reaction between the hydroxycinnamic acids and the cyanidin 3-glucoside, whose content in the juice was also higher than that of cyanidin 3-(6″-malonylglucoside).

The formation of pyranoanthocyanins through reaction with hydroxycinnamic acids is not only taking place under optimized model reaction conditions but also in blood orange juice during storage. Both, anthocyanins and free cinnamic acids, are readily available, although blood oranges do not contain free cinnamic acids; instead, the better water soluble esters with glucose or glycaric acids are present (*18*). Free cinnamic acids in the juice form quickly through hydrolysis of the esters under acidic conditions, favored by thermal treatment during juice processing (*19*, *20*), and their concentration also increases with storage time. Contents up to ~10 mg/L of free cinnamic acids (coumaric, caffeic, ferulic, and sinapic acid) in blood orange juices have been described (*19*, *21*, *22*).

As an alternative reaction pathway leading to the formation of pyranoanthocyanins, the reaction with free vinylphenols, as originally postulated by Fulcrand et al. (*23*), can be considered. The occurrence of 4-vinylphenol and 4-vinylguaiacol in blood orange juices is well-documented. The latter compound was identified as being one of the most detrimental off-flavors developing in orange juices during storage at elevated temperatures (*19*, *24*). The vinylphenols were shown to arise from the decarboxylation of free hydroxycinnamic acids under acidic conditions (*25*, *26*). A strong increase in the amount of 4-vinylguiacol was observed after addition of ferulic acid when the juice was stored at 35 and 50 °C (*20*). The temperature dependency of the decarboxylation reaction was evaluated in more detail by Lee and Nagy (*27*). They reported a strong accumulation of 4-vinylguaiacol at temperatures of 30 and 40 °C (13 and 45 times higher, respectively), while the concentration at 20 °C only doubled as compared to storage at −10 °C.

Because no details were known about any thermal treatments of the commercial blood orange juice under investigation, we prepared a freshly squeezed juice and stored it for several months at 15 °C. In this way, any thermally induced formation of pyranoanthocyanins could be excluded. After 1 month of storage, HPLC-ESI-MS^n analysis allowed the detection of the 4-vinylcatechol and 4-vinylsyringol adducts of cyanidin 3-glucoside, i.e., the same pigments as in the commercial juice. After 3 months, we also detected the 4-vinylphenol adduct, and after 10 months, the presence of the 4-vinylguaiacol adduct was confirmed by comparison with the synthesized reference compounds (**Figure 7**). Once again, none of the malonylated pyranoanthocyanins could be detected.

It is interesting to note that the reaction between hydroxycinnamic acids and anthocyanins is likely to explain the increased color stability of blood orange juice after fortification with caffeic acid (*28*). Maccarone et al. reported that by adding caffeic acid at a concentration of 500 mg/L, the half-life of the color intensity determined by photometric measurement at 535 nm could be improved to 90 days, as compared to only 40 days of the untreated reference juice. The authors explained this phenomenon by copigmentation effects and formation of anthocyanin−caffeic acid complexes as a result of the structural similarity with two vicinal OH groups in both compounds. On the basis of our experiments, it is more likely that such a high content of free caffeic acid will in fact lead to the rapid formation of the respective 4-vinylcatechol-substituted pyranoanthocyanins, as it was observed in red wines (*9*). Their stronger resistance against degradation as compared to the genuine anthocyanins (*29*) in combination with their greater stability toward decolorization (*16*) at a pH typical for blood orange juices is able to explain the observed increased color stability during prolonged storage.

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