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Color and Antioxidant Properties of Cyanidin-Based Anthocyanin Pigments

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A series of cyanidin-based anthocyanin pigments was investigated to determine the effect of structural variation on a number of chemical and physical properties: CIELAB color coordinates, visual detection thresholds, hydration constants (pK_H), and in vitro antioxidant activities (ORAC). In addition to individual isolated compounds, purified total pigment isolates from blackberry, elderberry, black carrot, red cabbage, and sweet potato were also examined. Acylation with cinnamic acids shifted color tonality (hue angle) to purple, and markedly increased pK_H and antioxidant activity, but lowered the visual detection threshold. Glycosidic substitution at the 5 position moved tonalities toward purple and decreased pK_H, and tended to lower the ORAC value, but raised the visual detection threshold. Increasing the number of sugar substituents at the 3 position also affected all of these parameters, however, the extent was not predictable. Antioxidant levels of purified anthocyanin extracts were much higher than expected from anthocyanin content indicating synergistic effect of anthocyanin mixtures.

KEYWORDS: Colorants; anthocyanins; cyanidin; CIELAB; visual detection threshold; hydration constant; ORAC; antioxidant; blackberry; elderberry; black carrot; red cabbage; sweet potato

INTRODUCTION

In addition to their roles as secondary metabolites in the pigmentation of many flowers, fruits, vegetables, and grains (*1*), anthocyanins have gained increasing interest as functional compounds for coloring food (*2*), and as potent agents against oxidative stress (*3*). Their success as natural alternatives for artificial dyes mainly depends on their economic feasibility, their chemical, biochemical, and physical stability during processing, and their appearance at food pH (*4*). Varying pH levels induces structural transformations (**Figure 1**) which affect both color quality and intensity. The effect of B-ring substitution on color appearance is long established, and there is considerable information on the effect of acylation and glycosidic substitution on pigment stability and a number of color properties (*1*). Furthermore, there is increasing epidemiological evidence that fruit- and vegetable-based diets are likely to improve the antioxidant status of human beings (*5*, *6*). A number of investigators have shown a high correlation between total anthocyanins, total phenolics, and the in vitro antioxidant activities of fruit and vegetable extracts (*7*−*13*). Little has been published regarding the antioxidant potencies of purified anthocyanin pigments and their mixtures. Wang et al. (*14*) measured the oxygen radical absorbing capacities (ORAC) of several purified anthocyanins and demonstrated that variation in B-ring structure and glycosylation can have a marked effect on antioxidant properties.

Our objective was to further examine the effect of structural variation through acylation with cinnamic acids, as well as position and extent of glycosidic substitution on antioxidant activities and several color properties. We limited our investigation to cyanidin-based pigments and selected a range of natural colorants and juice concentrates that could be sources for pigments varying in sugar substitution patterns and acylation. These included blackberry and elderberry juice concentrates, and black carrot, sweet potato, and red cabbage extracts. Individual pigments were purified from these sources by HPLC and solid-phase extraction. Color parameters included lightness (L*), chromatic tonality (hue angle, h°), and metric chroma (C*) measured in the CIELAB scale. The color activity concept developed by Hofmann (*15*) for Maillard reaction products and subsequently used for anthocyanins (*16*) was also applied. Because of the practical significance in food applications of the equilibrium reaction between the flavylium cation and the hemiketal form (**Figure 1**), the pK_H was also determined. The ORAC assay was used for measuring antioxidant properties.

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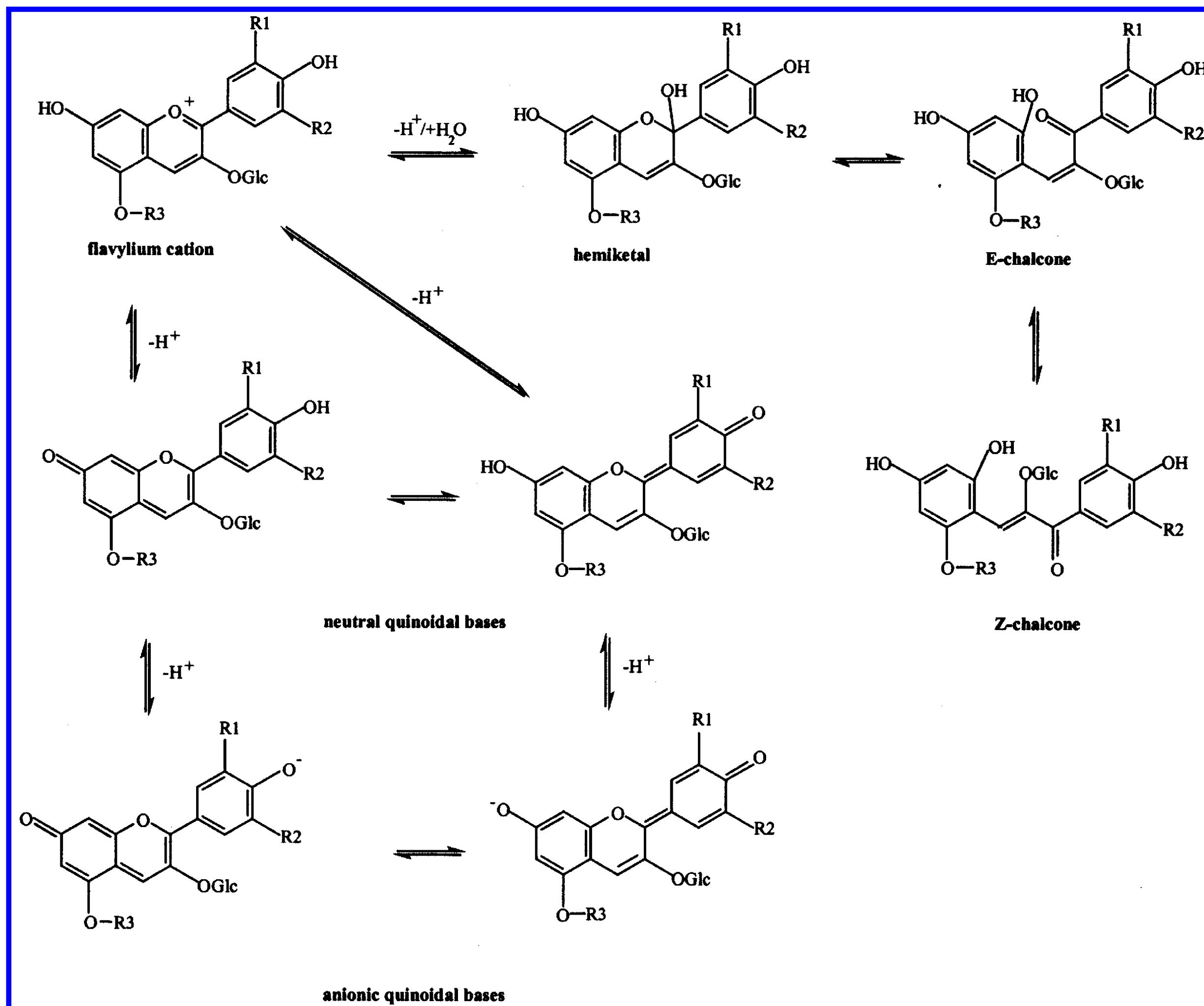

Figure 1. pH-Dependent structural transformations of anthocyanins (modified after (*28*)).

This assay has the advantage of directly assessing the ability of a compound to quench free radicals close to physiological pH.

MATERIALS AND METHODS

Pigment Material. Elderberry and evergreen blackberry juice concentrates were from IFF Flavors North America (Salem, OR), and black carrot concentrate was provided by GNT USA, Inc. (Tarrytown, NY). Red cabbage and purple sweet potato concentrates were supplied by San-Ei Gen F. F. I., Inc. (New York, NY).

Solvents and Reagents. All solvents and reagents were of analytical or HPLC grade. Phosphoric, formic, and acetic acids, methanol, and acetonitrile were from Merck (Darmstadt, Germany). Hydrochloric acid was from Baker Chemical Co. (Phillipsburg, NJ). β-Phycoerythrin was from Sigma Chemical Co. (St. Louis, MO). 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) was obtained from Wako Chemicals USA Inc. (Richmond, VA). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and 2,4,6-tri(2-pyridyl)-*s*-triazine (TPTZ) were purchased from Fluka (Buchs, Switzerland).

Anthocyanin Semi-Purification. Fruit or vegetable concentrates were diluted with purified water at ratios (w/w) of 1/5 for blackberry, 1/20 for elderberry, 1/30 for black carrot, and 1/50 for red cabbage and sweet potato prior to semi-purification on high-load C_{18}-Sep-Pak cartridges (Waters Assoc., Milford, MA), previously activated with methanol followed by aqueous 0.01% HCl (*17*). Using this method, samples (1 mL) were desalted with three volumes of acidified water before removal of nonanthocyanin phenolics by rinsing with 3 vol of ethyl acetate. Elution with methanol containing 0.01% aqueous HCl (v/v) first yielded a colorless and then a red fraction. The red anthocyanin eluate was collected, and it was repeatedly dissolved in methanol and concentrated in vacuo, until complete removal of residual acid. The obtained anthocyanin mixtures were diluted with purified water containing 0.01% HCl for the isolation of different cyanidin pigments by semi-prep HPLC. For color studies, the anthocyanin mixture was repeatedly dissolved in methanol and concentrated in vacuo, until complete removal of residual acid and water.

Pigment Isolation. Individual cyanidin derivatives (**Figure 2**) were isolated from the prepurified samples by semipreparative HPLC applying different gradient systems (see below). Solvents were evaporated in vacuo at 30 °C, and purities were checked using HPLC systems I−IV (see below) and monitoring spectra of the peaks at 280, 320, and 520 nm. The ratio of peak areas of the isolated pigment and total peak area at 280 nm was taken for purity determination (min. 97%). The purified pigments were further concentrated in vacuo at 30 °C. Acid and water were removed by repeated addition of methanol. Pure pigments were dried in a desiccator and stored at −70 °C in a screw-top test tube until further analyses.

High-Performance Liquid Chromatography (HPLC). *Analytical HPLC of Anthocyanins: Apparatus.* A high-performance liquid chromatograph Perkin-Elmer Series 400, equipped with a 50 μL injection loop and a Hewlett-Packard 1040A photodiode array detector was used. Data analysis was performed with Hewlett-Packard HPLC-2D ChemStation software.

Columns, Mobile Phases, and HPLC Conditions. Solvents and samples were filtered through a 0.45-μm Millipore filter type HA (Millipore Corp., Bedford, MA). Chromatographic analysis was carried out using an analytical scale (5-μm, 250 mm × 4.6 mm i.d.) Prodigy

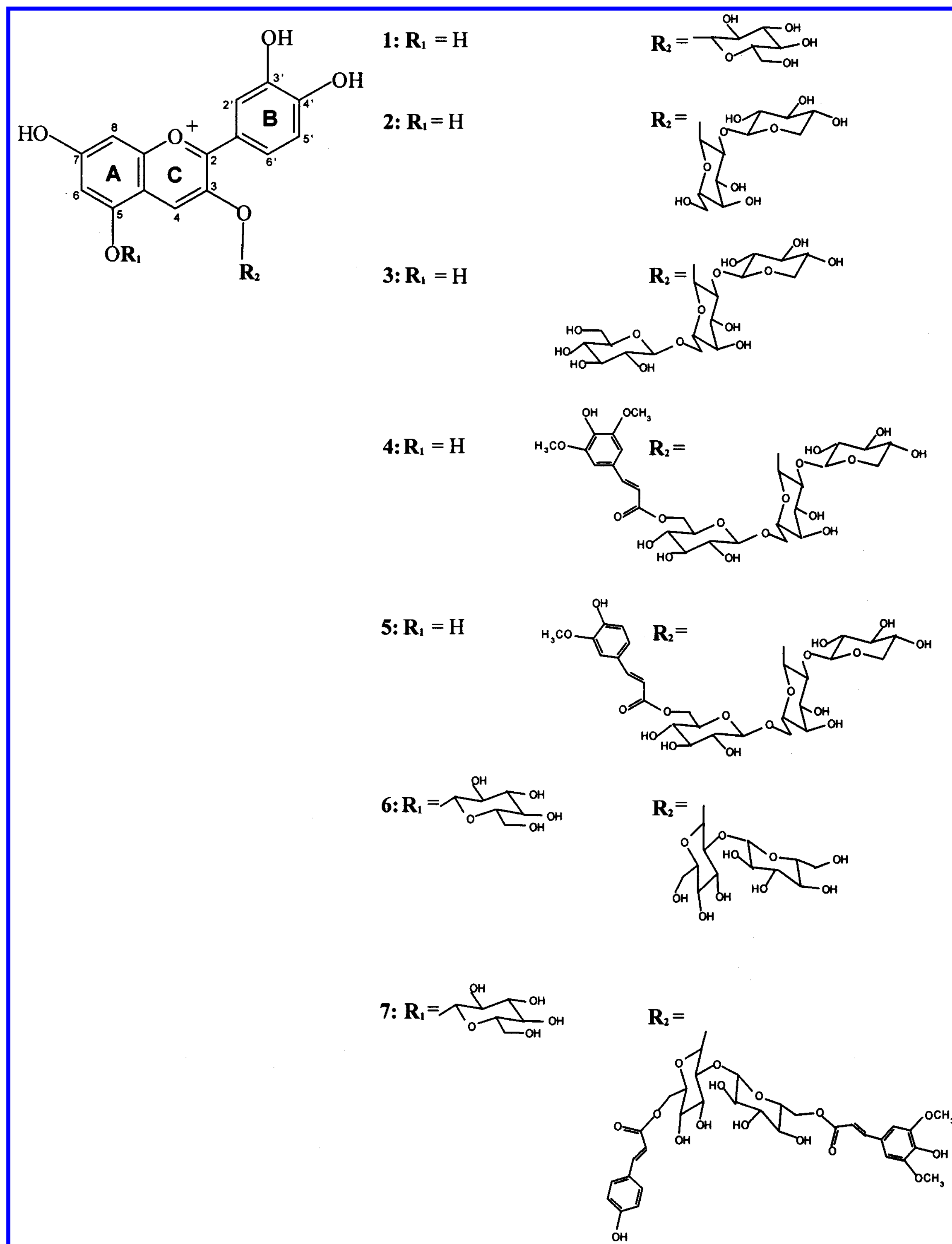

Figure 2. Chemical structures of cyanidin derivatives investigated. Structures for pigments are given as previously reported (*21*, *22*, *26*). Pigment assignments, sources, and mass data are given in **Table 1**.

C_{18} reversed-phase column (Phenomenex, Torrance, CA), fitted with a guard column C_{18} ODS (4 mm × 3.0 mm i.d.). Simultaneous monitoring was performed at 280, 320, and 520 nm at a flow rate of 1 mL/min.

System I (Blackberry and Elderberry Extracts, Cyanidin 3-Glucoside). As described before (*18*), mobile phase B consisted of 100% HPLC-grade acetonitrile, whereas mobile phase A was a mixture of 5% acetonitrile, 10% glacial acetic acid, and 1% phosphoric acid (85%) in water (v/v/v). Separation of anthocyanins was achieved within 25 min. The first 5 min were performed isocratically with 100% A, followed by a linear gradient from 100% A to 80% A in 15 min and then to 60% A in 5 min. The same gradient was used for the purity check of cyanidin 3-glucoside.

System II (Black Carrot Extract and Pigments). Mobile phase B consisted of 90/10 HPLC-grade acetonitrile/purified water (v/v) and mobile phase A was 10% aqueous formic acid. Separation of anthocyanins was achieved within 25 min, starting with 93% A to 85% A in 18 min, and then to 50% A in 7 min. The same system was used for purity control of cyanidin 3-xylosyl-galactoside, cyanidin 3-xylosyl-glucosyl-galactoside, and its sinapoyl and feruloyl derivatives, respectively.

System III (Red Cabbage Extract, Cyanidin 3-Sophoroside-5-Glucoside, and Its Diacylated Derivative). Mobile phase B consisted of 90/10 HPLC-grade acetonitrile/purified water (v/v), and mobile phase A was 10% aqueous formic acid. Separation of anthocyanins was achieved within 50 min. Gradient steps were from 92% A to 89% A, then to 85% A in 15 min, followed by an isocratic elution for 10 min, and finally to 67% A in 15 min. For purity control of cyanidin 3-sophoroside-5-glucoside and its diacylated derivative substituted with coumaric and sinapic acids, the gradient was 92% A to 89% A in 10 min, then to 67% A in 15 min, and finally an isocratic elution for 5 min.

System IV (Sweet Potato Extract). Mobile phase B consisted of 90/10 HPLC-grade acetonitrile/purified water (v/v), and mobile phase A was 10% aqueous formic acid. Separation of anthocyanins was achieved within 60 min. The first 10 min were performed isocratically with 92% A, followed by a gradient to 87% within 18 min, and then to 85% A in 12 min, and finally to 67% A in 20 min.

Semipreparative HPLC of Anthocyanins: Apparatus. A semipreparative Dynamax Rainin model SD-300 high-performance liquid chromatograph was used, and it was equipped with a 1 mL injection loop and a Hewlett-Packard 1040A photodiode array detector. Data were analyzed with Hewlett-Packard HPLC-2D ChemStation software.

Columns, Mobile Phases, and HPLC Conditions. Solvents were filtered through a 0.45 μm Millipore filter type HA (Millipore Corp., Bedford, MA) before use. Semipreparative HPLC was performed using a semipreparative scale (5 μm, 250 mm × 21.4 mm i.d.) Microsorb C_{18} reversed-phase column (Rainin Instrument Co., Inc., Emeryville, CA), fitted with a guard module C_{18} ODS (50 mm × 21.4 mm i.d.). Data were collected at 520 nm at a flow rate of 12 mL/min.

System V (Cyanidin 3-Glucoside). Mobile phase B was 100% HPLC-grade acetonitrile, and phase A consisted of 1% aqueous formic acid. A linear gradient was applied from 80% A to 75% A in 10 min.

System VI (Black Carrot Pigments). Mobile phase B was 100% HPLC-grade acetonitrile, and mobile phase A consisted of 10% aqueous formic acid. A linear gradient was followed from 88% A to 83% A in 13 min.

System VII (Cyanidin 3-Sophoroside-5-Glucoside and Its Diacylated Derivative). Mobile phase B was 100% HPLC-grade acetonitrile, and mobile phase A consisted of 10% aqueous formic acid. A linear gradient was used from 90% A to 77% A in 3 min followed by isocratic elution for 9 min.

Electrospray Mass Spectrometry (MS). Pigment identity was checked using electrospray MS with a Perkin-Elmer SCIEX API III+ mass spectrometer equipped with an ion spray interface (ISV= 4700, orifice voltage of 120 eV) operated in the positive-ion mode. Purified pigments were dissolved in 0.1% aqueous TFA/100% MeOH (1/1, v/v) for direct injection.

Assessment of Visual Detection Threshold. Purified anthocyanin extracts or purified pigments were dissolved in 1 mL of pH 3.5 buffer and equilibrated for 30 min prior to stepwise dilution. Minimal pigment concentration at which a difference between two solutions containing purified water and the anthocyanin solution can still be visually discerned is defined as the visual detection threshold (*15*). The dilution factor was determined by diluting purified pigment extracts with buffer until virtually no color was perceived. Visual detection thresholds were obtained in a triangle test by duplicate determination as described earlier (*15*, *16*). The samples (2 mL total volume) were placed in plastic cuvettes (1 cm path length) and inspected in a light booth (Macbeth Color Identification Lamp "Executive" Type B BX 324, Macbeth Corp., Newburgh, NY) against a white background in diffuse daylight mode (Northsky daylight, D_{65}). The color activity value is defined as the ratio of pigment concentration to the detection threshold of the individual pigment (*15*). Concentrations of individual pigments in the extracts were calculated as ratio of total anthocyanin content of the extract determined as cyanidin 3-glucoside (see below) and the relative peak area of the respective anthocyanin at 520 nm. Color contribution was expressed as percentage of the color activity value in relation to the dilution factor of the extract (*15*).

Color Analyses. Color parameters (CIELAB) were recorded with a ColorQuest HunterLab spectrocolorimeter equipped with HunterLab Universal Software Version 3.0 (HunterLab, Hunter Associates Laboratories Inc., Reston, VA). Purified anthocyanin extracts and purified pigments were dissolved in three different buffers (pH 0.45, 3.5, 6.0), and the absorbance was normalized to 0.5 after 30 min. Buffered pigment solutions were filled in a 0.25 cm path length optical glass cell (Hellma, Müllheim, Germany) and CIE L*, a*, and b* values were determined immediately in the total transmission mode in triplicate using Illuminant C and 10° observer angle. Chroma $(a^{*2} + b^{*2})^{1/2}$ and hue angle (arctan b*/a*) were calculated from CIELAB a* and b* coordinates.

Determination of pH. For pH measurements, a Corning pH-meter 340 (Corning Inc., Corning, NY) equipped with a Corning electrode "3-in-1" Combo w/RJ with temperature correction was used under continuous stirring of the solution.

Determination of Hydration Constants. An aqueous hydrochloric acid solution of pH 0.38 was prepared. Purified pigments or extracts were taken up in this solution to reach an initial absorbance of 0.4−0.8 at λ_{max}. Solutions were equilibrated for 30 min at 24 ± 1 °C in the dark. Through addition of 8 N NaOH under constant stirring, the initial pH was set to 0.45 ± 0.01 and the absorbance was measured after 15 min of pH equilibration. Different concentrations of aqueous NaOH (8 N, 4 N, 2 N, 0.5 N, 0.125 N, and 0.03125 N) were used for titration. After each pH change, the absorbance was measured in a 1 cm path-length quartz cell (Hellma, Müllheim, Germany) using a Shimadzu 160 UV spectrophotometer (Kyoto, Japan) with a temperature-controlled cell holder (TCC-240A). Titration curves were obtained by plotting absorbance values versus pH from 0.45 to 6 at 500 nm. A curve fit was performed using Microsoft Excel 97, and the resulting polynomial term of sixth power was processed with Maple V (Release 5, Waterloo Maple Inc.) to calculate the pH of the hydration constant. The pK_H values were obtained from the mean of duplicate measurements.

Total Anthocyanin Content. The monomeric anthocyanin content was determined using a pH-differential method (*19*). Spectral measurements were performed on a Shimadzu 160 UV spectrophotometer (Kyoto, Japan) at 510 and 700 nm with 1 cm path length disposable cuvettes. Anthocyanin contents were calculated using the molar absorptivity of cyanidin 3-glucoside (26 900 L cm^{-1} mg^{-1}) and a molecular weight of 449.2 g mol^{-1}.

Antioxidant Capacity. Purified extracts and isolated pigments (4.7−9.6 mM) were dissolved in methanol. Solutions were subsequently diluted with buffer (1/10, v/v) prior to analysis.

Oxygen Radical Absorbing Capacity (ORAC) Assay. The procedure reported by Cao et al. (*20*) was used and adapted for a Cytofluor 4000 microplate fluorometer (PerSeptive Biosystems, Framingham, MA). In the final reaction mixture, β-phycoerythrin acted as target for the peroxyl radicals generated by AAPH. The decrease of phycoerythrin fluorescence in the absence (control) and presence of antioxidants was monitored for 2 h at 2 min intervals at excitation and emission wavelengths of 485 and 585 nm, respectively. Trolox was used as a standard, and radical absorbing capacities were calculated using the area under the control curve and the quenching curve of β-phycoerythrin in the presence of the respective anthocyanin solution. Results were expressed as μmol Trolox equivalents per μmol pigment. For the extracts, pigment concentrations were calculated on the basis of cyanidin 3-glucoside as described above. The relation between ORAC values and anthocyanin concentration was evaluated by using linear regression analysis (Microsoft Excel 97) with three data points for each of the pigments. Using the least-squares method, a straight line (ORAC [μM Trolox equivalents] = m × anthocyanin concentration [μM] + b) was found as the best fit for the data points with a correlation coefficient above 0.98. Standard errors of intercept (b) and slope (m) were obtained from regression analyses (*14*).

Table 1. Pigment Assignments and Molecular Masses of Isolated Anthocyanins from Different Sources

number	anthocyanin	source	*m*/*z* [M]+
1	cyanidin 3-glucoside	blackberry	449.2
2	cyanidin 3-xylosyl-galactoside	black carrot	581.2
3	cyanidin 3-xylosyl-glucosyl-galactoside	black carrot	743.4
4	cyanidin 3-sinapoyl-xylosyl-glucosyl-galactoside	black carrot	949.4
5	cyanidin 3-feruloyl-xylosyl-glucosyl-galactoside	black carrot	919.6
6	cyanidin 3-sophoroside-5-glucoside	red cabbage	773.6
7	cyanidin 3-coumaroyl-sinapoyl-sophoroside-5-glucoside	red cabbage	1125.8

Table 2. Visual Detection Thresholds and Color Contributions of Individual Anthocyanins in Pigment Extracts

source	DF[a]	anthocyanin	VDT[b] [mg/L]	% peak area at 520 nm	% color contribution
blackberry	1596	cyanidin 3-glucoside	1.3	82.6	52.0
elderberry	2422	cyanidin 3-glucoside	1.3	28.6	18.0
black carrot	19600	cyanidin 3-xylosyl-galactoside	0.9	44.1	36.1
black carrot	19600	cyanidin 3-xylosyl-glucosyl-galactoside	2.4	14.9	4.5
black carrot	19600	cyanidin 3-sinapoyl-xylosyl-glucosyl-galactoside	0.9	27.5	21.2
black carrot	19600	cyanidin 3-feruloyl-xylosyl-glucosyl-galactoside	0.4	13.5	22.6
sweet potato	25003	cyanidin 3-sophoroside-5-glucoside	3.6	1.8	0.6
red cabbage	26147	cyanidin 3-sophoroside-5-glucoside	3.6	17.6	7.0
red cabbage	26147	cyanidin 3-coumaroyl-sinapoyl-sophoroside-5-glucoside	2.0	25.2	18.2

[a] DF: Dilution factor. [b] VDT: Visual detection threshold.

RESULTS AND DISCUSSION

Characterization of Anthocyanin Material. The purified anthocyanins examined in this investigation and their sources are listed in **Table 1**. Pigment purities were checked as described above, and their identities were confirmed by chromatographic and mass spectrometric analyses. Cyanidin 3-glucoside (**1**), the most commonly occurring anthocyanin pigment in nature, was isolated from commercial evergreen blackberry (*Rubus laciniatus* Willd.) extract. Additional pigments previously identified in this extract (*18*) included cyanidin 3-rutinoside (2.4%), cyanidin substituted with xylose (7.7%), cyanidin 3-malonyl-glucoside (3.6%), and cyanidin 3-dioxalyl-glucoside (3.0%). Black carrot (*Daucus carota* L. ssp. *sativus* var. *atrorubens* Alef.) extract served as the source for cyanidin 3-xylosyl-galactoside (**2**; 44.1% of total peak area), cyanidin 3-xylosyl-glucosyl-galactoside (**3**; 14.9%), cyanidin 3-xylosyl-glucosyl-galactoside acylated with sinapic acid (**4**; 27.5%), or ferulic acid (**5**; 13.5%). In contrast to the report of Glässgen et al. (*21*), hydroxybenzoyl- and coumaroyl-derivatives were not detected. Red cabbage (*Brassica oleracea* L.) extract was a source for cyanidin 3-sophoroside-5-glucoside (**6**) and a derivative substituted with sinapic and coumaric acids (**7**). Fifteen pigments were separated in this extract, with a predominance of acylated (81.1%) over nonacylated (18.9%) anthocyanins. This pigment profile was in accord with previous reports (*22*−*24*). Sweet potato (*Ipomoea batatas* [L.] Lam.) pigments consisting of peonidin- and cyanidin-3-sophoroside-5-glycosylated structures (*25*, *26*) was considered in this study as an alternative to red cabbage without negative flavor. Its complex pattern comprising more than 15 anthocyanins was characterized by 90.2% acylated and 9.8% nonacylated structures. Consistent with Inami et al. (*27*), elderberry (*Sambucus nigra* L.) extract contained exclusively nonacylated anthocyanins: cyanidin 3-sambubioside (54.7%), cyanidin 3-glucoside (28.6%), cyanidin 3,5-diglucoside (12.9%), and cyanidin 3-sambubioside-5-glucoside (3.8%).

Visual Detection Threshold. The methodology of Degenhardt et al. (*16*) was used to determine visual detection thresholds and the color contribution of individual anthocyanins to the total extract (**Table 2**). Dilution factors for the concentrates allowed assessment of their relative color strengths. Extracts mainly composed of acylated anthocyanins (red cabbage and sweet potato) required 8- to 16-fold higher dilution factors than blackberry and elderberry. This observation could be substantiated by the examination of the purified pigments. Visual detection thresholds of anthocyanins acylated with cinnamic acids were generally lower, indicating a higher tinctorial strength. Acylation of cyanidin 3-xylosyl-glucosyl-galactoside with sinapic (**4**) and ferulic (**5**) acid resulted in a 3-fold and 6-fold increase, respectively. This is consistent with other reports of a hyperchromic effect through cinnamic acid substitution (*28*, *29*). In contrast, Degenhardt et al. (*16*) found that nonacylated wine pigments showed increased tinctorial strength when compared to the respective acylated compounds. These pigments were acylated monoglycosides, however, whereas we investigated acylated diglycosides. Dangles et al. (*28*) reported that two sugars are necessary as spacers to allow folding of the acyl moiety leading to higher chromophore integrity. Diacylation of cyanidin 3-sophoroside-5-glucoside with coumaric and sinapic acids (**7**) did not even reduce the threshold as much as monoacylation of pigment **3**. This was surprising, as diacylation would be expected to be more effective in protecting both faces of the flavylium cation from nucleophilic attack (*30*). Possibly, 5-glycosylation hinders free rotation of the aromatic acyl moiety, thereby reducing the shielding effect on the second face of the flavylium nucleus. In line with another study (*16*), 3,5-diglycosides had lower color strength than 3-glycosides. When comparing cyanidin mono-, di-, and triglycosides, diglycosylation with xylose and galactose reduced the visual detection threshold, whereas additional substitution with glucose increased the threshold. It is evident that the number and position of glycosidic substitution(s), as well as the type of sugar, can affect color strength.

Interestingly, the color contribution of individual anthocyanins is, in almost all cases, less than their respective peak area percentage (**Table 2**). This was particularly true for nonacylated pigments. Although cyanidin 3-glucoside represented the major compound of blackberry extract, its color impact was only about 50%. The same was true for black carrot, for which 100% of

Table 3. Colorimetric and Spectral Characteristics of Isolated Anthocyanins

anthocyanin	L*	C*	h°	λ_{max}
pH 0.45				
cyanidin 3-glucoside	92.9	8.9	11.9	510
cyanidin 3-xylosyl-galactoside	92.7	9.3	5.0	513
cyanidin 3-xylosyl-glucosyl-galactoside	92.6	9.1	5.8	513
cyanidin 3-sinapoyl-xylosyl-glucosyl-galactoside	91.7	10.5	351.1	526
cyanidin 3-feruloyl-xylosyl-glucosyl-galactoside	91.9	10.0	355.2	525
cyanidin 3-sophoroside-5-glucoside	93.2	8.7	0.3	511
cyanidin 3-coumaroyl-sinapoyl-sophoroside-5-glucoside	91.6	11.2	339.7	532
pH 3.50				
cyanidin 3-glucoside	92.5	9.1	8.0	512
cyanidin 3-xylosyl-galactoside	92.3	9.3	2.6	515
cyanidin 3-xylosyl-glucosyl-galactoside	92.1	9.1	3.9	515
cyanidin 3-sinapoyl-xylosyl-glucosyl-galactoside	91.5	10.4	348.8	529
cyanidin 3-feruloyl-xylosyl-glucosyl-galactoside	91.6	10.0	353.7	527
cyanidin 3-sophoroside-5-glucoside	92.6	8.4	355.7	514
cyanidin 3-coumaroyl-sinapoyl-sophoroside-5-glucoside	91.4	10.9	333.4	536
pH 6.00				
cyanidin 3-glucoside[a]	88.9	4.1	304.1	550
cyanidin 3-xylosyl-galactoside[a]	89.6	6.4	341.3	541
cyanidin 3-xylosyl-glucosyl-galactoside[a]	89.8	5.1	340.7	542
cyanidin 3-sinapoyl-xylosyl-glucosyl-galactoside	89.9	8.3	313.3	547
cyanidin 3-feruloyl-xylosyl-glucosyl-galactoside	90.1	7.8	316.1	549
cyanidin 3-sophoroside-5-glucoside[a]	92.2	4.9	321.5	545
cyanidin 3-coumaroyl-sinapoyl-sophoroside-5-glucoside	88.6	7.8	268.3	587

[a] Data given do not represent a stable appearance of the solution because degradation proceeded quickly.

the peak area contributed to only about 85% of the overall coloring strength. Because coelution of anthocyanins was excluded, this discrepancy can be explained only by interactions between the individual anthocyanins requiring higher dilution factors for the extracts. While the visual detection thresholds were determined for isolated pigments, extract dilution factors were assessed with intermolecular arrangements between different anthocyanin structures being possible (*31*). For red cabbage, the color contribution of two pigments accounting for about 43% of the peak area was only 25%. From this it can be deduced that other pigments, in particular the acylated anthocyanins, constituted the major color fraction. It is worth noting that cyanidin 3-feruloyl-xylosyl-glucosyl-galactoside (**5**) from black carrot accounted for only half of the peak area compared to the sinapoyl-derivative (**4**), but equally contributed to color appearance (**Table 2**). When applying the color activity concept to anthocyanins, it should be remembered that quantification of the flavylium cation is carried out at low pH, whereas thresholds are determined at the pH of the food application. Transition of the flavylium ions to colorless structures will occur to a varying extent at higher pH values depending on the nature of the pigment investigated (*32*, **Figure 1**).

Color Attributes of Anthocyanin Solutions. In determining CIELAB coordinates, pigments and extracts were diluted to a common absorbance value of 0.5, which accounted for the similarity in lightness (L*) for the different samples (**Tables 3** and **4**). Because chroma was only slightly affected by different substitution patterns, it was of particular interest how anthocyanin structure affected hue angle. The latter is expressed on a 360° color wheel where 0°/360° = purplish-red, 90° = yellow, 180° = bluish-green, and 270° = blue. Although it is well established that methoxylation and hydroxylation of the B-ring has a predictable effect on color and wavelength of maximum absorption (*29*), this investigation examined the effect of acylation with cinnamic acids and glycosidic substitution on these parameters. At pH 0.45, color quality of the pigments can be completely ascribed to the flavylium ion (*33*) allowing assessment of the impact of structural modifications (**Table 3**). Acylation with cinnamic acids had a pronounced effect on hue angle, shifting the color from red to purplish-red and purple. Substitution of cyanidin 3-xylosyl-glucosyl-galactoside with sinapic acid had a greater effect than with ferulic acid. The diacylated pigment from red cabbage exhibited a pronounced shift in hue angle compared to the nonacylated parent compound of 21° at pH 0.45 and of 22° at pH 3.5. At pH 6, the diacylated cyanidin derivative even displayed a blue appearance. Glycosidic substitution at 3 or 5 position decreased hue angle. Glycosylation at the 3 position with a di- or trisaccharide reduced the hue angle slightly by 4 to 7°. The color indices for nonacylated pigments at pH 6.0 need to be regarded as transient because these pigments rapidly degraded at that pH.

Table 4. Colorimetric and Spectral Characteristics of Purified Anthocyanin Extracts

anthocyanin extract	L*	C*	h°	λ_{max}
pH 0.45				
blackberry	92.1	9.0	10.6	510
elderberry	92.0	9.2	4.7	510
black carrot	91.8	10.3	358.3	520
red cabbage	92.0	10.7	348.9	520
sweet potato	91.8	11.2	348.2	520
pH 3.50				
blackberry	90.9	8.5	17.4	510
elderberry	90.9	8.5	10.9	520
black carrot	91.2	10.3	356.2	520
red cabbage	91.1	11.0	340.4	530
sweet potato	91.1	11.3	342.3	530
pH 6.00				
blackberry[a]	88.78	5.74	30.9	530
elderberry[a]	88.33	4.63	17.4	540
black carrot	88.84	7.01	325.1	550
red cabbage	89.20	8.36	304.4	550
sweet potato	89.32	9.23	321.4	550

[a] Data given do not represent a stable appearance of the solution because degradation proceeded quickly.

The color properties of the extracts (**Table 4**) are in accord with the values determined for the purified pigments. For extracts predominantly consisting of nonacylated anthocyanins

Table 5. Hydration Constants (pK_H) of Individual Anthocyanins and Pigment Extracts

	pK_H	literature data	ref
anthocyanin			
cyanidin 3-glucoside	3.01 ± 0.04	3.02 ± 0.06	(*38*)
cyanidin 3-xylosyl-galactoside	3.13 ± 0.02	3.41 ± 0.01	(*34*)
cyanidin 3-xylosyl-glucosyl-galactoside	3.26 ± 0.06	3.37 ± 0.05	(*34*)
cyanidin 3-sinapoyl-xylosyl-glucosyl-galactoside	3.79 ± 0.08	4.41 ± 0.05	(*34*)
cyanidin 3-feruloyl-xylosyl-glucosyl-galactoside	4.42 ± 0.20		
cyanidin 3-sophoroside-5-glucoside	2.32 ± 0.01		
cyanidin 3-coumaroyl-sinapoyl-sophoroside-5-glucoside	3.12 ± 0.01		-
anthocyanin source			
blackberry	3.07 ± 0.02		
elderberry	2.96 ± 0.02	2.99 ± 0.06	(*35*)
black carrot	3.67 ± 0.06		
red cabbage	2.69 ± 0.00		
sweet potato	3.15 ± 0.02		

Table 6. Polynomial Fits for pK_H-Determination of Isolated Anthocyanins and Pigment Extracts

	coefficients[a]							
	a[b]	*b*[b]	*c*[b]	*d*[b]	*e*[b]	*f*[b]	*g*[b]	*r*[c]
anthocyanin								
cyanidin 3-glucoside	−0.0022	0.0285	−0.1154	0.1357	0.0565	−0.2507	1.1516	0.99
	−0.0015	0.0176	−0.0519	−0.0304	0.2398	−0.3277	1.2018	0.99
cyanidin 3-xylosyl-galactoside	−0.0047	0.0662	−0.3426	0.7972	−0.8751	0.3573	0.9446	0.99
	0.002	−0.0233	0.1122	−0.3163	0.5013	−0.4724	1.0519	0.99
cyanidin 3-xylosyl-glucosyl-galactoside	−0.00007	−0.0006	0.0247	−0.1643	0.3695	−0.4092	1.1186	0.99
	−0.0017	0.0250	−0.1323	0.2966	−0.2921	0.0492	0.8703	0.99
cyanidin 3-sinapoyl-xylosyl-glucosyl-galactoside	0.00004	−0.0013	0.0148	−0.0744	0.1599	−0.1671	0.3981	0.99
	−0.00009	0.0009	0.0007	−0.0309	0.0934	−0.1107	0.3366	0.99
cyanidin 3-feruloyl-xylosyl-glucosyl-galactoside	−0.00003	0.0005	−0.0008	−0.0146	0.0572	−0.0875	0.3807	0.99
	0.00005	−0.0013	0.0138	−0.0684	0.1531	−0.1594	0.3056	0.99
cyanidin 3-sophoroside-5-glucoside	0.0011	−0.0211	0.1541	−0.5110	0.7534	−0.5633	0.6902	0.99
	0.0022	−0.0373	0.2372	−0.6930	0.8969	−0.5498	0.6653	0.99
cyanidin 3-coumaroyl-sinapoyl-sophoroside-5-glucoside	0.0002	−0.0046	0.0370	−0.1349	0.2129	−0.1656	0.3817	0.99
	0.0003	−0.0067	0.0528	−0.1874	0.2835	−0.2096	0.5152	0.99
anthocyanin extract								
blackberry	−0.0028	0.0388	−0.1953	0.4272	−0.4224	0.0699	0.9915	0.99
	−0.0012	0.0173	−0.0839	0.1575	−0.1122	−0.0831	0.9545	0.99
elderberry	−0.0007	0.0077	−0.0168	−0.0547	0.2044	−0.3047	0.9952	0.99
	−0.0012	0.0147	−0.0563	0.0427	0.0724	−0.2485	1.2166	0.99
black carrot	−0.0004	0.0058	−0.0223	0.0006	0.0999	−0.1917	0.9386	0.99
	−0.0005	0.0071	−0.0286	0.0163	0.0747	−0.1818	0.9902	0.99
red cabbage	0.0007	−0.0143	0.1062	−0.3498	0.4694	−0.3242	0.8758	0.99
	0.0009	−0.0192	0.1458	−0.5072	0.7783	−0.5877	0.9561	0.99
sweet potato	0.0008	−0.0147	0.1082	−0.3788	0.6096	−0.4985	0.8461	0.99
	−0.0002	0.0005	0.0223	−0.1510	0.3141	−0.3202	0.8118	0.99

[a] Coefficients for the polynomial term $y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$, with *y* for the absorbance and *x* for pH. [b] The two coefficients given are for the duplicates. [c] Regression coefficient.

such as blackberry and elderberry, hue angles ranged between 5 and 17°, corresponding to red color and increased (i.e., tonality moved yellower) with higher pH. Red cabbage and sweet potato extracts were composed primarily of highly acylated anthocyanins and were characterized by h° values indicative of purple tonalities. Black carrot extract consisted of nearly equivalent proportions of nonacylated and acylated anthocyanins and displayed intermediate h° values. Although red cabbage and sweet potato extracts had similar tonalities at pH 0.45 and pH 3.5, there was a difference of 17° at pH 6.0. It is worth noting that hue angle and values of maximum absorbance did not necessarily correspond. This phenomenon is due to the considerable variation in absorption curve shape throughout the extent of the visible spectrum.

Hydration Equilibrium Constants. pK_H values were determined by titration starting at pH 0.45 where 100% of the pigment would be present as the flavylium cation (*33*). The pK_H was reached when 50% of the red flavylium form had been transformed into the colorless hemiketal corresponding to a 50% decline in absorbance. Because absorbance was measured at 500 nm, interference with the blue quinoidal base could be excluded (*34*). This method permitted determination of hydration constants of both purified extracts and isolated pigments (**Table 5**). The best fit for the titration curves was obtained with a polynomial term of sixth power (**Table 6**) from which the pHs of the hydration constants were calculated using Maple V. Assuming an equilibrium between the flavylium cation and the hemiketal structures, the pK_H can be described by the following equation (*35*, *36*):

$$pK_H = -\log \frac{c_{hemiketal}}{c_{flavyliumcation}} + pH$$

By comparing the values for nonacylated 3-glycosides with cyanidin-3-sophoroside-5-glucoside (**Table 5**), it is clear that substitution at the 5 position markedly lowered the hydration

Table 7. Peroxyl Radical Absorbing Capacities for Pigment Extracts and Isolated Anthocyanins

	coefficients[a]		
	m (slope)[b]	*b* (intercept)[b]	*r*[c]
anthocyanin extract			
blackberry	13.868 ± 0.093	−0.232 ± 0.172	0.999
elderberry	9.220 ± 0.225	0.977 ± 0.895	0.999
black carrot	9.154 ± 0.033	0.745 ± 0.099	0.999
red cabbage	9.781 ± 0.425	−0.581 ± 1.422	0.999
sweet potato	10.011 ± 0.398	2.704 ± 1.082	0.999
anthocyanin			
cyanidin 3-glucoside	2.257 ± 0.135	2.732 ± 1.220	0.996
cyanidin 3-xylosyl-galactoside	2.255 ± 0.049	1.119 ± 0.536	0.999
cyanidin 3-xylosyl-glucosyl-galactoside	1.479 ± 0.123	2.321 ± 3.159	0.996
cyanidin 3-sinapoyl-xylosyl-glucosyl-galactoside	2.020 ± 0.160	−0.057 ± 1.599	0.997
cyanidin 3-feruloyl-xylosyl-glucosyl-galactoside	3.035 ± 0.035	0.556 ± 0.272	0.999
cyanidin 3-sophoroside-5-glucoside	1.581 ± 0.061	2.167 ± 1.012	0.999
cyanidin 3-coumaroyl-sinapoyl-sophoroside-5-glucoside	2.852 ± 0.162	1.279 ± 1.857	0.998

[a] The coefficients are given for the term ORAC [μM Trolox equivalent] = $m \times$ anthocyanin concentration [μM] + b. [b] The slopes (m) are significantly different from zero ($p < 0.05$) whereas the intercepts (b) do not significantly differ from zero ($p > 0.05$). Therefore, the antioxidant capacity is directly expressed by the slope (m). [c] Multiple correlation coefficient.

constant. Others have reported similar decreases when comparing 3-glucosides with their corresponding 3,5-diglucosides (*37*−*40*). As described previously, glycosylation at the 5 position results in decreased electron-density of the pyrilium ring which favors nucleophilic attack by water, thereby enhancing hemiketal formation (*41*, *42*). A higher number of sugar residues at the 3 position increased the hydration constant to a small degree. Acylation with cinnamic acids substantially raised the hydration constant. The ferulic acid moiety of cyanidin 3-xylosyl-glucosyl-galactoside increased the hydration constant more than the sinapoyl residue. Acylation of cyanidin 3-sophoroside-5-glucoside with sinapic and coumaric acids only partially compensated for the destabilizing effect of 5-substitution.

By comparing the hydration constants of individual anthocyanins with those of their respective sources, it is evident that cyanidin 3-glucoside was largely responsible for the value of blackberry extract (**Table 5**). Although blackberry contains minor acylated pigments (*18*), their concentration was probably too low to have a measurable impact. Black carrot and blackberry extracts contain exclusively 3-substituted cyanidin pigments. The markedly higher hydration constant for black carrot can be attributed to the substantial degree of cinnamic acid acylation (41.0%). Whereas red cabbage has a high proportion of acylated anthocyanins (81.1%), its hydration constant is much lower than those for black carrot, blackberry, and elderberry. This can most likely be attributed to glycosidic substitution at the 5 position. Although sweet potato anthocyanins are also characterized by 3,5-substitution, the higher degree of acylation (90.2%) compensates for 5-glucosylation giving it an intermediate position between red cabbage and black carrot. The hydration constants obtained in this work are in good agreement with those found by others (*35*, *38*). The pK_H values reported by Redus et al. (*34*) showed the same trend, but differed in absolute numbers (**Table 5**). Hydration constants have very practical ramifications because higher values will translate into substantially greater color intensity at the pH of most food systems.

Peroxyl-Radical Absorbing Capacity. The ORAC assay has received widespread acceptance as a measure of antioxidant capacity, as it is believed to mimic the ability of biological compounds to trap free radicals (*43*). Another advantage of the ORAC assay is that it is conducted close to physiological pH at 7.4, which is appropriate when evaluating pharmacological properties. The matter of pH is even more important, because anthocyanin structure is dependent on the acidity of the medium (**Figure 1**) with the pseudobase, *cis*- and *trans*-chalcones, and quinoidal bases predominating at pH 7 (*32*). The chalcones and quinoidal bases display double bonds in conjugation with a keto group, a structural element defined by Bors et al. (*44*) to be essential for efficient antioxidant action. Anthocyanin pigments are known to be effective quenchers of free radicals, and a number of investigators have reported high linear correlations between total anthocyanin pigment content and ORAC values (*10*−*13*, *45*). Wang et al. (*14*) studied the effect of glycosylation and variation in anthocyanin B-ring structure on ORAC values. B-ring structure had a marked effect on antioxidant activity with *ortho*-hydroxylation and methoxylation substantially increasing antioxidant activity. Anthocyanidins had higher ORAC values than their glycosides, which is to be expected because the aglycons are very unstable and highly reactive. We took a very similar experimental approach, focusing on the effect of cinnamic acid acylation and glycosidic substitution patterns. Considering this good correlation ($r > 0.99$) between ORAC and anthocyanin content in this study (**Table 7**), higher pigment concentrations went along with higher antioxidant capacities. As assessed by regression analysis, this dose-dependent relationship could be adequately described by a straight line ($R^2 > 0.99$, data not shown). Our results (**Table 7**) also show that the intercepts (b) were not significantly different from zero ($p > 0.05$), thus antioxidant activity could be directly expressed by the slope (m). Whereas cyanidin 3-glucoside and cyanidin 3-xylosyl-galactoside had equivalent values, addition of a third sugar to form cyanidin 3-xylosyl-glucosyl-galactoside reduced the antioxidant level. Cyanidin 3-sophoroside-5-glucoside and cyanidin 3-xylosyl-glucosyl-galactoside had similar values, therefore, one cannot conclude whether the reduction is due to 5-glycosylation or the presence of a third sugar residue. However, Terahara et al. (*46*) found that cyanidin 3-sophoroside-5-glucoside had lower antioxidant activity than the corresponding 3-monoglucoside using the β-carotene bleaching assay. The more striking finding is the impact of cinnamic acid acylation: esterification of cyanidin 3-xylosyl-glucosyl-galactoside with sinapic acid distinctly increased the ORAC value, whereas ferulic acylation doubled the antioxidant activity. Diacylation of cyanidin 3-sophoroside-5-glucoside with coumaric and sinapic acids also markedly increased the ORAC value. Tamura and Yamagami (*47*) reported that acylation of malvidin-3-glucoside with *p*-coumaric acid increased antioxidant activity

as measured by linoleic acid oxidation. The only pigment studied by Wang et al. (*14*) in common with those of the present study was cyanidin 3-glucoside. We obtained a value of 2.26 μmol Trolox equivalents per μmol anthocyanin, whereas Wang and co-workers (*14*), dissolving anthocyanins in dimethyl sulfoxide and performing the assay at pH 7.0, found 3.49. This might indicate that different solvent systems (solvent type and pH) yield varying ORAC values for the same commodity investigated.

Surprisingly, the pigment extracts had ORAC values 3- to 6-fold higher than those of the individual pigments (**Table 7**). One possible explanation is a synergistic effect arising from anthocyanin mixtures. Furthermore, principles found for the purified pigments were not reflected in the antioxidant potencies of the extracts. In part, this can be explained by the fact that calculation of ORAC values for the extracts was based on cyanidin 3-glucoside concentration.

This study was undertaken to relate color quality and antioxidant properties to structural functions of cyanidin derivatives. Tonality was influenced by the site of glucosylation as well as type and degree of hydroxycinnamic acid substitution. Increasing the number of sugar substituents at the 3 position can also affect all of these parameters, however, the extent was not predictable. Although we could confirm an increase in color strength through acylation, it was also proved that slightly different substitution of the acyl moiety affected chromophore protection and color appearance. Diacylation does not necessarily result in improved color strength, as shown in the case of 5-glycosylation. Besides their coloring function, anthocyanins are also believed to display an array of pharmacological properties (*3*). As was demonstrated in this study, not only concentration, but also distinct structural elements of anthocyanins may influence the antioxidant properties in vitro. Whereas 5-glycosylation led to a decrease, acylation raised antioxidant potency. Because anthocyanin mixtures exhibited stronger capacities than expected by their qualitative and quantitative pigment compositions, synergistic effects were suspected. These assumptions need to be verified in vivo. However, learning more about their chemical properties may help in understanding principles influencing anthocyanin bioavailability, tissue retention, and mechanisms of antioxidant action.

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