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Anthocyanin Composition in Black, Blue, Pink, Purple, and Red Cereal Grains

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Anthocyanin pigments from a wide variety of edible and ornamental black, blue, pink, purple, red, and white wheat, barley, corn, rice, and wild rice were identified and quantified to evaluate their potential as natural colorants or functional food ingredients. The total anthocyanin contents varied significantly and exhibited a range of 7−3276 μg/g. Some grains, such as red rice and black rice, contained a limited number of pigments, whereas others, such as blue, pink, purple, and red corns, had complex anthocyanin profiles. Of the 42 anthocyanin compounds observed, 9 were characterized by comparison of the spectroscopic and chromatographic properties with those of authentic standards. The remaining compounds were tentatively identified on the basis of spectroscopic properties and electrospray ionization mass spectra. The most abundant anthocyanins were cyanidin 3-glucoside in black and red rices and in blue, purple, and red corns, pelargonidin 3-glucoside in pink corn, and delphinidin 3-glucoside in blue wheat.

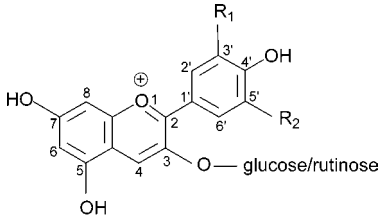
KEYWORDS: Anthocyanins, colored grains; functional grains; natural colorants; LC-UV−vis; LC-MS

INTRODUCTION

Black, blue, and purple grains are currently produced only in small amounts for making specialty foods or for use in ornamentation due to their colorful appearance. Anthocyanins, a group of reddish to purple water-soluble flavonoids, are the primary pigments in these grains (*1*−*4*). The basic structure of common anthocyanins is presented in **Figure 1**. Anthocyanins have been recognized as health-enhancing substances due to their antioxidant activity (*5*−*7*), anti-inflammatory (*8*), anticancer (*9*−*11*), and hypoglycemic effects (*12*). They have been found to be absorbed from the gut into the blood stream and were detected in the urine at 1.0−6.7% of the total amount when six healthy human subjects drank 300 mL of a red wine containing 218 mg of anthocyanins (*13*). The world production of anthocyanins has been estimated at 10000 tonnes from grapes alone, and the average daily intake has been estimated at 215 mg in summer and 180 mg in winter. These numbers reflect the importance of anthocyanins in the human diet and a need to better understand their compositional characteristics and functionality in food and nutrition.

Anthocyanin-pigmented or colored grains hold promise as functional foods (e.g., whole grain products) or functional food colorants (e.g., anthocyanin-rich grain fractions). At present, blue and purple corn grains are used for making blue or pink tortillas. Purple wheat is crushed into large pieces, which are spread over the exterior of multigrain bread (*14*). Red rice has been a functional food in China and is commonly used as a food colorant in bread, ice cream, and liquor (*15*). Purple corn has been identified as a food colorant since 1977 (*16*). Anthocyanin pigments are located in certain layers of the kernel, which could be separated into anthocyanin-rich fractions for use as functional colorants or functional food ingredients. In wheat, the blue pigments are located in the aleurone layer, whereas the purple pigments are concentrated in the pericarp layers (*4*, *17*). The highest concentration of anthocyanin pigments in corn was found in the pericarp, whereas the aleurone layer contained small concentrations (*1*). Currently, grape skin and red cabbage are the main concentrated sources of anthocyanin colorants (*18*).

Although an extensive scientific literature (*2*, *18*) on the composition of anthocyanins in fruits and vegetables exists, little is known about anthocyanin composition in grains. Early studies have shown that cyanidin 3-glucoside and peonidin 3-glucoside



Pelargonidin-3-glucoside: R_1=H, R_2=H, **11**
Cyanidin-3-glucoside: R_1=OH, R_2=H, **6**
Delphinidin-3-glucoside: R_1=OH, R_2=OH, **1**
Peonidin-3-glucooside: R_1=OCH_3, R_2=H, **15**
Petunidin-3-glucoside: R_1=OCH_3, R_2=OH, **9**
Malvidin-3-rutinoside: R_1=OCH_3, R_2=OCH_3, **20**

Figure 1. Structures of common anthocyanins. Each anthocyanin is followed by a number that corresponds to the peak/compound numbers in the tables and figures.

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are the major anthocyanins in purple wheat and purple rye as detected by paper chromatography using rhubarb and plum extracts as standards (*19*, *20*). In a recent LC study, cyanidin 3-glucoside was the most predominant anthocyanin in purple wheat and the second most common in blue wheat, whereas the first major anthocyanin in blue wheat remained unidentified (*3*). In corn, cyanidin 3-glucoside, cyanidin 3-(6″-malonylglucoside), and cyanidin 3-(3″,6″-dimalonylglucoside) were the major anthocyanins, with cyanidin being the main aglycone or anthocyanidin, accounting for 73−87% of the total (*1*). A similar anthocyanin composition was also found in corn flowers (*21*). Black rice contained a wide range of total anthocyanin content, with cyanidin 3-glucoside being the most common anthocyanin (0.0−470 mg/100 g) in most of the 10 varieties studied, whereas peonidin 3-glucoside (0.0−40 mg/100 g) was the second dominant anthocyanin (*22*). Black sorghum possessed the highest total anthocyanin content among selected black, brown, and red sorghum cultivars with luteolinidin and apigeninidin being the major deoxyanthocyanidins in black sorghum, accounting for 50% of the total anthocyanins (*23*, *24*). These studies show substantial differences in anthocyanin content and composition between grains and their potential as natural food colorants.

The present study was aimed at characterizing anthocyanin composition in a diverse array of colored cereals to identify anthocyanin-rich grains and milling fractions for the development of functional foods and/or functional food colorants. The effect of temperature on separation of anthocyanins was investigated to improve separation efficiency and to obtain better identification, particularly for those grains exhibiting a complex anthocyanin profile. The identity and purity of anthocyanin compounds were based on spectroscopic and mass data obtained from liquid chromatography−ultraviolet−visible (LC-UV−vis) and liquid chromatography−mass spectrometry (LC-MS).

MATERIALS AND METHODS

Cereal Grains. A diverse array of edible and ornamental black, blue, pink, purple, red, and white cereal grains were used in the present study. These included blue wheat (cv. Purendo), purple wheat (cvs. Laval and Konini), red wheat (cvs. Ketepwa and Freedom), white wheat (cvs. AC Ron and AC Reed), blue barley (cv. Tankard), black sweet rice, red rice, wild rice, shaman blue corn, cutie blue corn, purple corn, sweet scarlet red corn, cutie pink corn, ruby red corn, crimson red corn, and fiesta Indian multicolored corn. Wheat samples were obtained from the University of Saskatchewan, Saskatoon, SK, Canada, except for cvs. Freedom and AC Ron, which were obtained from the University of Guelph, ON, Canada. Barley samples were provided by the University of Guelph, ON, Canada. Rice and corn samples were purchased or obtained from private grain producers and/or the retail market. The grain samples were ground on a Cyclone sample mill (Udy Co., Fort Collins, CO) equipped with a 500 μm screen except for corn samples, which were ground in an IKA laboratory mill (Janke & Kunkel Co., Staufen, Germany) prior to milling in the Udy mill. Wheat was fractionated into three fractions, namely, flours, shorts (fine bran), and bran (coarse bran) by tempering the grains to 13% moisture, milling on a Quadrumat Jr. flour mill (Branbender Co., South Hackensack, NJ), and sifting the ground materials on a Ro-Tap shaker equipped with 40 mesh (420 μm opening) and 60 mesh (250 μm opening) sieves (Tyler Co., Mentor, OH). Black and red rice kernels were separated into three fractions by a model TM 05C abrasive mill (Satake Co., Hiroshima, Japan). Wheat and rice bran and pearled kernels (intact endosperm) were further ground and passed through a 60 mesh screen. The whole meal flours and milling fractions were mixed to ensure uniformity and kept at 4 °C until analysis.

Anthocyanin Extraction. Anthocyanins in ground grain materials were extracted according to the method described by Abdel-Aal and Hucl with slight modifications (*3*). Three grams of the ground materials was extracted twice by mixing with 24 mL of methanol acidified with 1.0 N HCl (85:15, v/v) and shaking on an IKA Vibrax VXR (Janke & Kunkel Co.) at 1800 rpm for 30 min. The apparent pH of the mixture was adjusted to 1.0 before shaking and was checked and readjusted if necessary after 15 and 30 min of shaking. The crude extracts were centrifuged at 20853*g* and 5 °C for 20 min and then refrigerated for ≈2 days to precipitate large molecules. The extracts were recentrifuged at 20853*g* and 5 °C for 20 min. The partially purified extracts were concentrated ≈10-fold under a stream of nitrogen. The precipitated pellets were separated by centrifugation as described above. The concentrated extracts of ≈1.6 mL were vigorously mixed and filtered through a 0.45 μm Nylon Acrodisc syringe filter (Paill Gelman Laboratory, Ann Arbor, MI) for further cleanup.

Total Anthocyanin Content (TAC). TAC in grain samples was determined using the spectrophotometric method previously described (*4*). The supernatant of the crude extracts was poured into a 50 mL volumetric flask and made up to volume with acidified methanol. Absorbance was measured on a UV−vis spectrophotometer (Varian Inc., Palo Alto, CA) at 535 nm. The TAC (in micrograms per gram) was calculated as follows:

$$\mathrm{TAC} = A \times 288.21$$

where A is the absorbance reading (*4*).

Analysis of Anthocyanins. Anthocyanins in the partially purified extracts were separated and quantified with an 1100 series chromatograph (Agilent, Mississauga, ON, Canada) equipped with a G1311A quaternary pump, G1329A temperature controlled injector, G1316A temperature-controlled column thermostat, G1322A degasser, G1315B photodiode array detector (PDA), and ChemStation v. 8.04 data acquisition system with the capability of conducting isoabsorbance plot and three-dimensional (3D) graphic analyses. A 75 mm × 4.6 mm i.d., 3.5 μm Zorbax SB-C18 rapid resolution column (Agilent) was employed for separation. Separation of anthocyanins was conducted at three temperatures, 25, 38, and 50 °C, to improve separation efficiency, particularly for those grains exhibiting complex anthocyanin composition. The column was eluted with a gradient mobile phase consisting of (A) 6% formic acid and (B) absolute methanol at 1 mL/min. The gradient was programmed as follows: 0−7 min, 82−80% A; 7−10 min, 80−75% A; 10−25 min, 75−40% A; 25−26 min, 40−82% A; and 26−28 min, hold at 82% A. The separated anthocyanins were detected and measured at 525 nm, and the identity of anthocyanins was based on the congruence of retention times and UV−vis spectra with those of pure authentic standards. Seven selected pure anthocyanin compounds including delphinidin 3-glucoside, cyanidin 3-galactoside, cyanidin 3-glucoside, cyanidin 3-rutinoside, peonidin 3-glucoside, petunidin 3-glucoside, and cyanidin chloride were purchased from Polyphenols Laboratories (Sandens, Norway). Later, another two anthocyanin standards, delphinidin 3-rutinoside and pelargonidin 3-glucoside, were also included to identify and quantify pelargonidin- and delphinidin-containing anthocyanins. Additionally, pure anthocyanins were used for recovery experiments using the spiked sample technique. The stock standard solutions were prepared in acidified methanol by weighing a known amount in the range of 0.8−1 mg and dissolving in exactly 1 mL. The working standard solutions were prepared by diluting 1, 5, 20, and 50 μL of the stock solutions into 1000 μL in acidified methanol. The range of the injected standard amounts was from 0 to 1.0 μg (20 μL injection volume). All anthocyanin standard solutions exhibited linear relationships within that range by plotting area response and injected amounts. The coefficient of determinations (R^2) ranged from 0.9930 to 0.9999 for a mixture of pure anthocyanins separated on the C18 column. Anthocyanins that had no standards were quantified on the basis of an anthocyanin standard having the same aglycone.

The purity of each compound in grain samples was verified on the basis of the spectroscopic properties of each peak using isoabsorbance plot or 3D graphic and peak purity analyses provided with the ChemStation software. Peak purity analysis allows the spectrum of the identified compound to be identified and confirmed and to determine whether interference occurs.

LC-MS Confirmation of Anthocyanin Identity. Confirmation of identity of each peak was carried out by LC-MS (Thermo Finnigan,

Table 1. Total Anthocyanin Contents and Color of Extracts from Selected Edible and Ornamental Colored Grains

cereal	mean[a] ± SD (μg/g)	color of extract: original	color of extract: diluted
rice			
black	3276 ± 93.1 a	dark purple	dark purple
red	93.5 ± 1.3 b	pink-orange	orange-red
wild	27.2 ± 1.2 b	blackish green	yellowish brown
corn			
shaman blue	322.7 ± 1.5 c	dark pink	dark pink
cutie blue	196.7 ± 2.1 d	dark pink	dark pink
cutie pink	163.9 ± 4.7 e	dark pink	dark pink
purple	1277 ± 4.9 a	pink	pink
sweet scarlet red	607.1 ± 21.7 b	dark pink	red
ruby red	69.4 ± 1.9 g	pink-orange	orange
crimson red	50.9 ± 1.7 h	pink-orange	orange
fiesta Indian multicolored	131.7 ± 5.6f	dull dark pink	dark pink
wheat			
blue, cv. Purendo	211.9 ± 3.4 a	dark pink	dark pink
purple, cv. Laval	95.8 ± 5.1 b	light pink	red
purple, cv. Konini	38.0 ± 0.8 c	light pink	light pink
red, cv. Katepwa	7.9 ± 0.4 d	pale yellow	yellow
red, cv. Freedom	6.7 ± 0.2 d	pale yellow	yellow
white, cv. AC Reed	7.3 ± 0.3 d	pale yellow	yellow
white, cv. AC Ron	7.1 ± 0.3 d	pale yellow	yellow
barley			
blue, cv. Tankard	34.6 ± 0.5	light pink	light pink

[a] Means within each cereal category followed by the same letter are not significantly different at $p < 0.05$.

San Jose, CA) equipped with a SpectraSystem UV6000LP ultraviolet detector scanning from 190 to 800 nm and an LCQ Deca ion trap mass spectrometer operated in the ESI positive ion mode scanning from *m*/*z* 50 to 2000. The LC-MS conditions were the same as for the LC-UV−vis analyses.

Machine operating conditions for ESI positive ionization were as follows: sheath gas and auxiliary flow rates were set at 91 and 4 (arbitrary units); voltages on the capillary, tube lens offset, multipole RF amplifier, multipole 1 offset, multipole 2 offset, intermultipole lens, entrance lens, and trap DC offset were set at 35.5, 55.00, 770.00, −4.40, −8.00, −14.00, −58.00, and −10.00 V, respectively; capillary temperature was set at 350 °C; source voltage was 5.00 kV; and source current was 80.0 μA. The mass spectrometer was tuned for maximum response to malvidin 3-glucoside.

Statistical Analysis. The data were subjected to analysis of variance to determine differences between samples and to study the effect of temperature on separation of anthocyanins and to correlation analysis to study the relationship between spectrophotometry and LC method using Minitab software (version 12, Minitab Inc., State College, PA). The data were reported as means of three to six replicates ± standard deviation (SD).

RESULTS AND DISCUSSION

Total Anthocyanin Content. The TAC varied significantly between black, blue, pink, purple, red, and white grains (**Table 1**). Significant differences in the concentration of total anthocyanins were previously found between black, brown, and red sorghum (*23*) as well as among black rice (*22*) cultivars. Black rice had a wide range of total anthocyanins depending upon cultivar (*22*), whereas black sorghum exhibited the highest total anthocyanins in a selection of black, brown, and red sorghum cultivars (*23*). In the present study, black rice, with an average of 3276 μg/g, was found to possess the highest TAC among all of the studied colored grains, which is ≈35 times higher than that of red rice (94 μg/g) (**Table 1**). On the other hand, wild rice had a very small concentration of TAC (27 μg/g), which may belong to one or more other groups of pigments because

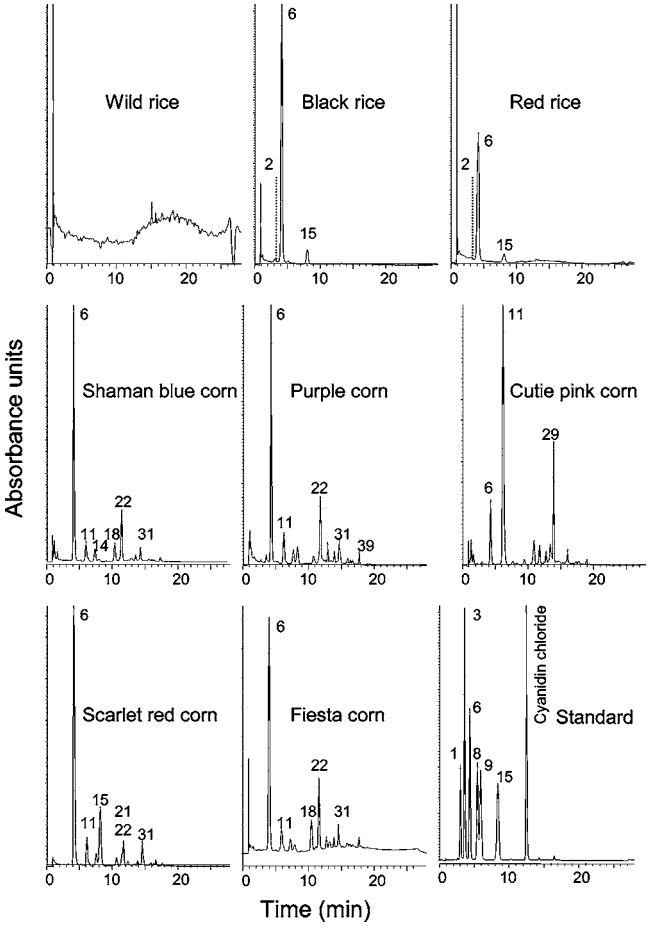

Figure 2. LC-UV–vis chromatograms of anthocyanins in colored rice and corn extracts and anthocyanin standard mixture separated on a C18 column. The peak numbers show the major anthocyanins that are identified in **Table 3**.

no anthocyanin peaks were detected by LC analyses in the wild rice extracts (**Figure 2**).

Eight corn grains exhibiting blue, pink, purple, and red attractive colors were found to contain a wide range of TAC as low as 51 μg/g and as high as 1277 μg/g (**Table 1**). Purple corn had the highest concentration of TAC followed by sweet scarlet red corn and shaman blue corn. At present, most of the colored corn is used in ornamentation due to its colorful appearance; only a small amount is being utilized in making naturally colored tortillas. The TAC results indicate that some of the colored corn grains such as purple and red corn may hold promise for the development of functional foods and/or natural colorants. The availability and agronomic performance of these grains will determine their potential market.

Blue wheat had an average TAC of 212 μg/g (**Table 1**), which is higher than that reported in our previous study (139−164 μg/g) (*3*). The concentration of anthocyanins in a large population of blue wheat lines was found to range from 35 to 507 μg/g with a mean 183 μg/g (*4*). Additionally, anthocyanin concentrations were significantly influenced by growing conditions and environment in blue and purple wheats and the environmental effect was much stronger in the purple wheat due to the pigment location in the outer pericarp or fruit coat (*3*). Thus, anthocyanins in purple wheat are more prone to environmental effects. Purple wheat used in the present study contained lower TAC compared to blue wheat, and there were significant differences between the two purple wheat cultivars (**Table 1**). Red and white wheat cultivars exhibited small

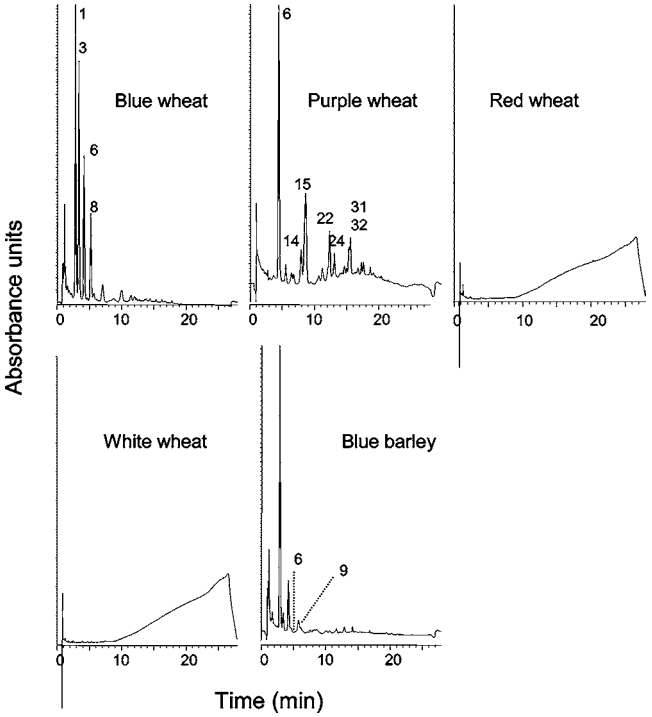

Figure 3. LC-UV–vis chromatograms of anthocyanins in colored wheat and barley extracts separated on a C18 column. The peak numbers show the major anthocyanins that are identified in **Table 3**.

Table 2. Total Anthocyanin Content (TAC), Anthocyanin Recovery, and Milling Yield of Blue Wheat, Red Rice, and Black Rice Milling Fractions

fraction	TAC[a] ± SD (μg/g)	anthocyanin recovery[b] (%)	milling yield ± SD (%)
black rice			
bran, 1 min abrasion	25354 ± 923 a	24.8	3.2 ± 0.1
bran, 1+4 min abrasion	11677 ± 547 b	66.7	18.7 ± 0.9
pearled grain, 1+4 min abrasion	263 ± 8.1 c	6.1	76.6 ± 1.7
red rice			
bran, 1 min abrasion	586.3 ± 18.7 a	27.6	4.4 ± 0.2
bran, 1+4 min abrasion	487.4 ± 7.3 b	59.9	11.5 ± 0.7
pearled grain, 1+4 min abrasion	3.6 ± 0.3 c	3.0	78.2 ± 2.3
blue wheat			
shorts, >250–<420 μm	817.8 ± 15.2 a	27.4	7.1 ± 0.4
bran, >420 μm	495.5 ± 8.7 b	52.8	22.6 ± 1.3
flour, <250 μm	32.4 ± 2.7 c	10.2	66.8 ± 2.1

[a] Means within each cereal category followed by the same letter are not significantly different at $p < 0.05$. [b] [(TAC in fractions × milling yield %)/TAC in whole grain flour] × 100.

concentrations of TAC, and no significant differences were observed between them. These data are in agreement with our previous results (*3*). The LC analysis of red and white wheat extracts showed an absence of anthocyanin compounds in these wheats (**Figure 3**), which indicates that the small amount of TAC may be contributed by one or more other groups of pigment. The blue barley cultivar studied had relatively very small TAC compared to blue wheat (**Table 1**).

Fractionation of rice and wheat kernels into bran and flour fractions by abrasive or roller milling was able to concentrate anthocyanin pigments in the bran fractions (**Table 2**). In fact, the pigments were concentrated by about 8, 6, or 4 times in black rice, red rice, or blue wheat bran fraction, respectively. However, the recovery of pigments in the bran fractions obtained by 1 min of abrasion in the case of rice or in the shorts or fine bran (>250 μm, <420 μm) in the case of wheat was low, ranging from 25 to 28%. The yield of these fractions was also low and ranged from 3 to 7%. In black, brown, and red sorghum, the bran fraction (15% yield) contained 3−4 times higher anthocyanin content than the whole grains, but no data were reported on anthocyanin recovery (*23*, *24*). The addition of 4 min of abrasion in the case of rice or of coarse bran (>420 μm) in the case of wheat increased the pigment recovery to 92, 88, and 80% in the combined bran fractions obtained from black rice, red rice, and blue wheat, respectively (**Table 2**). The average yield of the combined bran fractions was also substantially increased, accounting for approximately 22, 16, and 30% of the total fractions for black rice, red rice, and blue wheat, respectively. The high yield and recovery of anthocyanins in these grain fractions support the use of dry milling and fractionation technologies to produce anthocyanin-rich fractions for use as functional food ingredients or colorants. Further work on the extractability and stability of anthocyanins in these fractions is underway. The flour fractions that represent the bulk of kernel were white, having normal appearance, and can be used as regular ingredients in many food applications.

In general, the colored grains studied showed substantial differences in their TAC, and some of them, such as black rice, purple corn, and blue wheat, had remarkable levels of anthocyanins. In addition, the anthocyanin pigments can be further concentrated by dry milling and fractionation processes to produce fractions that exhibit high levels of anthocyanin even much higher than those found in fruits and vegetables, that is, 25000 μg/g in black rice bran fraction (**Table 2**) versus 200−10000 μg/g in fruits and vegetables (*2*).

Effect of Temperature on the Separation of Anthocyanins. Following the choice of column and mobile phase, the efficiency of separation can be enhanced by increasing the separation temperature. In our previous study, anthocyanins in blue wheat either in whole meal or in isolated form were thermally stable at pH 1 and 65 °C, but as the temperature increased to 95 °C the degradation of anthocyanins increased (*3*). To prevent any thermal degradation during the analysis of anthocyanins and to maintain column quality, the highest temperature employed was 50 °C. Three column temperatures (25, 38, and 50 °C) were investigated to improve the separation efficiency of anthocyanins, particularly for those materials that exhibited complex compositions such as purple corn, pink corn, and purple wheat. When the anthocyanin standard mixture was run at the three temperatures, similar separations and responses were obtained, indicating that no degradation occurred. In the case of sample extracts, poor separation (i.e., several coeluted compounds) was obtained at 25 °C, whereas separation at 38 °C improved the resolution of anthocyanins. The LC chromatograms of anthocyanins separated at 38 °C for 13 grain extracts and a standard mixture are presented in **Figures 2** and **3**. Separation of anthocyanins at 50 °C resulted in slight improvements for only those compounds eluted at longer retention times, that is, separation of cyanidin succinylglucoside and peonidin succinylglucoside in purple corn. Thus, the grain samples were analyzed at 38 °C for the identification and quantification, and corn and purple wheat samples that have complex compositions of anthocyanin were run at 38 and 50 °C for complete separation and better identification.

Identification of Anthocyanins. The acidic methanol extracts of the black, blue, pink, purple, and red grains studied afforded complex mixtures that were characterized on the basis of UV−

Table 3. Anthocyanins Detected in Selected Black, Blue, Pink, Purple, and Red Barley, Corn, Rice, and Wheat and Their Spectroscopic and Mass Characteristics

RT (min)	compd	anthocyanin[a]	λ_{max} (nm)	major ions (*m*/*z*)	observ-ation
2.9	**1**	Dp-3-Glu	525	465, 303[b]	confirmed
3.1	**2**	Cy-diGlu[1]	516	611, 287	presumed
3.4	**3**	Dp-3-Rut	525	611, 303	confirmed
3.5	**4**	Lt-Glu	490	433, 271	presumed
3.8	**5**	Cy-3-Gal[c]	517	449, 287	confirmed
4.1	**6**	Cy-3-Glu	517	449, 287	confirmed
5.0	**7**	Cy-diGlu[1]	515	611, 287	presumed
5.1	**8**	Cy-3-Rut	517	595, 287	confirmed
5.7	**9**	Pt-3-Glu	526	579, 317	confirmed
6.0	**10**	Cy-MalGlu[2]	512	535, 287	presumed
6.2	**11**	Pg-3-Glu	502	433, 271	confirmed
6.3	**12**	Cy-MalGlu[2]	516	535, 287	presumed
7.1	**13**	Pt-3-Rut	529	625, 317	confirmed
7.6	**14**	Cy-MalGlu[2]	516	535, 287	presumed
8.3	**15**	Pn-3-Glu	517	463, 301	confirmed
9.4	**16**	Pg-MalGlu[3]	504	519, 271	presumed
10.0	**17**	Pn-Rut	519	609, 301	presumed
10.6	**18**	Cy-SucGlu[4]	514	549, 287	presumed
10.7	**19**	Pg-MalGlu[3]	506	519, 271	presumed
11.5	**20**	Mv-Rut	530	639, 331	presumed
11.2	**21**	Cy-MalGlu[2]	518	535, 287	presumed
11.6	**22**	Cy-SucGlu[4]	518	549, 287	presumed
12.2	**23**	Cy-SucGlu[4]	520	549, 287	presumed
12.3	**24**	Pn-MalGlu	518	549, 301	presumed
12.7	**25**	Pg-SucGlu[5]	503	533, 271	presumed
12.8	**26**	unknown	498	867, 705, 543, 417, 271	
13.4	**27**	Pg-SucGlu[5]	506	533, 271	presumed
13.5	**28**	Pg-MalGlu[3]	506	519, 271	presumed
13.9	**29**	Pg-SucGlu[5]	502	533, 271	presumed
14.1	**30**	Pn-SucGlu[6]	518	563, 301	presumed
14.6	**31**	Cy-SucGlu[4]	520	549, 287	presumed
14.8	**32**	Pn-SucGlu[6]	520	563, 301	presumed
15.8	**33**	Cy-MalSucGlu[7]	520	635, 287	presumed
15.9	**34**	Pg-SucGlu[5]	506	533, 271	presumed
16.4	**35**	Cy-MalSucGlu[7]	520	635, 287	presumed
16.7	**36**	Pn-SucGlu[6]	520	563, 301	presumed
17.3	**37**	Pg-MalSucGlu[8]	504	619, 271	presumed
17.6	**38**	Pn-MalSucGlu	520	649, 301	presumed
17.7	**39**	Cy-diSucGlu	520	649, 287	presumed
17.8	**40**	Pg-MalSucGlu[8]	520	619, 271	presumed
17.9	**41**	Pg-diSucGlu[9]	522	633, 271	presumed
19.0	**42**	Pg-diSucGlu[9]	506	633, 271	presumed
19.3	**43**	Pn-diSucGlu	520	663, 301	presumed

[a] Cy, cyanidin; Dp, delphinidin; Gal, galactoside, Glu, glucoside; Lt, luteolunidin; Mal, malonyl; Mv, malvidin; Pg, pelargonidin; Pn, peonidin; Pt, petunidin; Rut, rutinoside; Suc, succinyl. [1−9]Compounds with identical molecular mass within each superscript, which suggest they are isomeric. [b] Molecular ion and fragment ion. [c] Only in standard.

vis and MS properties and the retention times of components separated by LC. Wherever possible, confirmation of the identity was achieved by the congruence of these properties with those of authentic anthocyanin standards. Of the 42 anthocyanin compounds observed (**Table 3**), standards of only 9 were available for confirmation. The remaining components were tentatively identified on the basis of spectroscopic, spectrometric, and retention properties.

The assignment of a component to the anthocyanin class was first based upon UV−vis spectra. Anthocyanins show a broad absorption band in the blue end of the visible spectrum with maxima in the 500−535 nm region (*25*). Absorption bands were also observed in the UV region from about 277 to 284 nm, due to the aromatic ring structure, and occasionally another weak UV band was observed in the region from about 328 to 346 nm. The separated anthocyanin peaks from the standard mixture and blue wheat and purple corn extracts and their corresponding absorption spots are shown in the isoabsorbance plot (**Figure 4**).

Anthocyanins could also be characterized by fragmentation patterns arising from MS created by ESI in the positive mode. **Figure 1** illustrates the structures of some typical anthocyanins. They are typified by a central flavonoid ring aglycone structure, which is connected to a saccharide moiety; more than 539 possible combinations are known. The saccharides may be underivatized or have an attached acyl moiety. Different positional and structural isomer possibilities add to the complexity.

A typical ESI positive MS shows two ions: the protonated molecular ion $[M + H]^+$ and a fragment ion $[M + H - X]^+$ arising from loss of the saccharide moiety. However, because the anthocyanins have a natural residual positive charge, one observes a true molecular ion $[M]^+$ and a fragment ion $[M - X]^+$. The fragment ion is that of the underiviatized aglycone. There are six major aglycones generally observed with the associated fragment ions: pelargonidin, *m*/*z* 271; cyanidin, *m*/*z* 287; peonidin, *m*/*z* 301; delphinidin, *m*/*z* 303; petunidin, *m*/*z* 317; and malvidin, *m*/*z* 331. The value for X, based on the difference between the molecular ion and fragment, gives a clue to the nature of the saccharide functionality. The MS of the compound showing ions at *m*/*z* 287 and 449 suggests that the aglycone is cyanidin (*m*/*z* 287), and the difference of *m*/*z* 162 suggests a hexose. Two common hexoses would be glucose or galactose; ESI positive MS cannot distinguish between them. Furthermore, the point of attachment to the aglycone also cannot be distinguished. For this, one could compare retention times and spectra with those of authentic standards. **Table 3** shows that the 3-galactoside and 3-glucoside derivatives of cyanidin are well resolved. Other examples of presumptive assignments in **Table 3** include the loss of *m*/*z* 248 for malonylglucoside, *m*/*z* 262 for succinylglucoside, *m*/*z* 308 for rutinoside, *m*/*z* 324 for diglucoside, *m*/*z* 348 for malonylsuccinylglucoside, and *m*/*z* 362 for disuccinylglucoside.

UV−vis data also provide some means of confirming some of the aglycones. **Table 3** shows that UV−vis maxima for pelargonidin-based compounds were observed at 502−506 nm, for cyanidin at 512−520 nm, for peonidin at 517−520 nm, for delphinidin at 525 nm, for petunidin at 526−529 nm, and for malvidin at 530 nm. For a given saccharide combined with the various possible aglycones, the relative LC elution of the compounds follows the order delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin.

In **Table 3**, the anthocyanins for which structures are noted as confirmed on the basis of congruence of properties with authentic standards are completely named. The remainder are listed as presumptive on the basis of analogy of the chromatographic, UV, and MS properties. Low levels of anthocyanins precluded the obtaining of quantities sufficient to enable use of other analytical techniques, such as nuclear magnetic resonance, to identify unambiguously the nature and position of the glycoside and acyl moieties. Because glucose has been reported as the most common hexose in grain anthocyanins (*1*, *3*, *22*), it was presumed as the hexose in our study.

Several compounds with similar mass spectra but different retention times and spectroscopic properties were detected (**Table 3**). Two isomers of cyanidin diglucoside were found in black rice and red rice at different retention times; four isomers of cyanidin malonylglucoside, three isomers of pelargonidin malonylglucoside, four isomers of cyanidin succinylglucoside, four isomers of pelargonidin succinylglucoside, three isomers of peonidin succinylglucoside, two isomers of cyanidin malonylsuccinylglucoside, two isomers of pelargonidin malonylsuc-

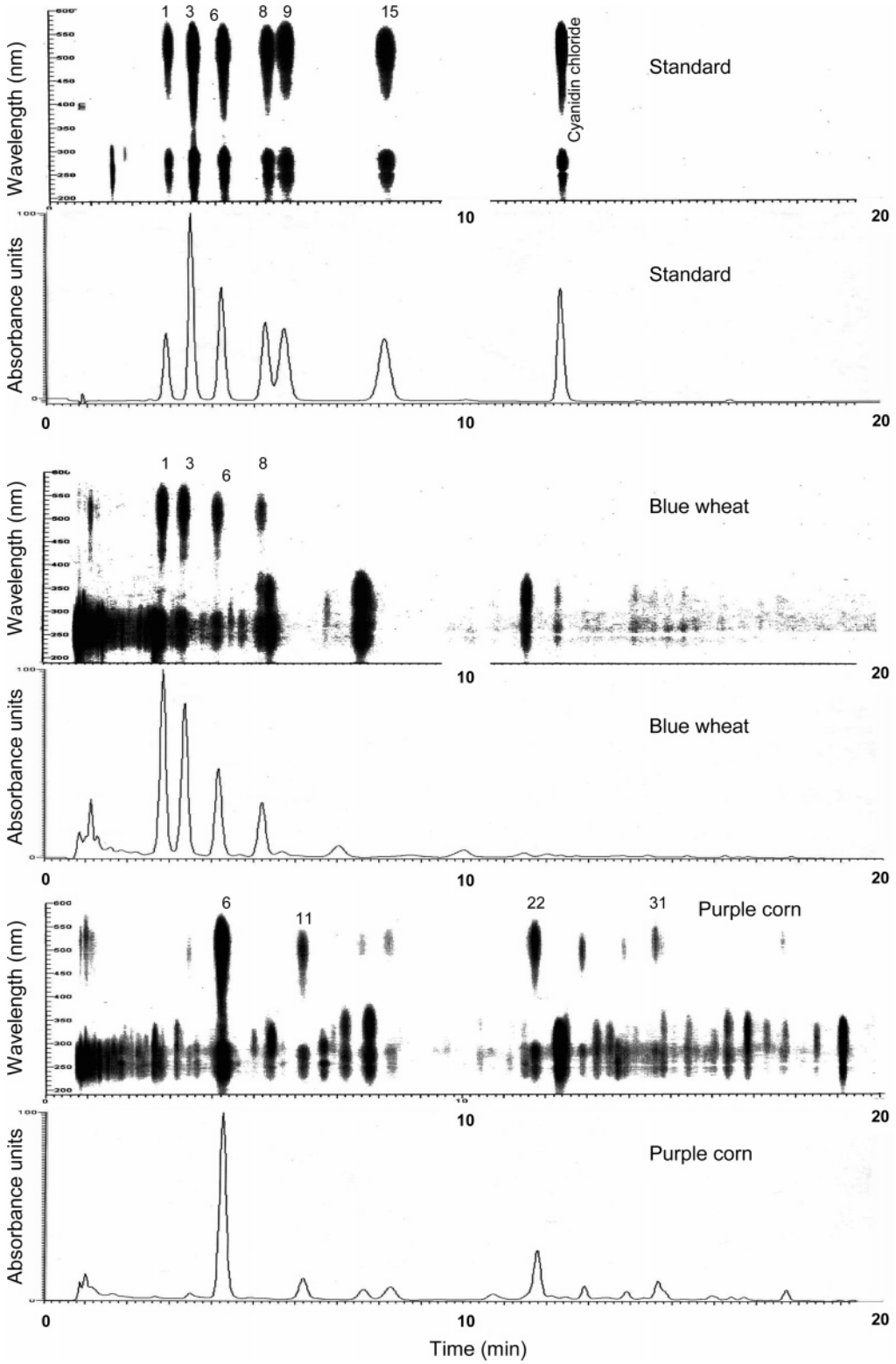

Figure 4. Typical illustration for isoabsorbance plots of anthocyanins separated from standard mixture, blue wheat, and purple corn. The peak numbers show the major anthocyanins that are identified in **Table 3**.

cinylglucoside, and two isomers of pelargonidin disuccinylglucoside were observed. Of the 43 anthocyanin compounds, only one unknown compound, presumably proanthocyanindin or a dimer of anthocyanin, had four major ions (i.e., *m*/*z* 867, 705, 417, and 271).

Quantification of Anthocyanins. The colored grains investigated exhibited diverse anthocyanin compositions. Anthocyanin composition ranged from only a few pigments or a simple profile such as in black rice, red rice, and blue barley (**Table 4**; **Figure 2**) to an intermediate profile such as blue and purple wheat (**Table 4**; **Figure 3**) to a complex profile such as blue, pink, purple, and red corn (**Table 5**; **Figure 2**). Cyanidin 3-glucoside was the most abundant anthocyanin in black rice and red rice, accounting for 88 and 67% of the total anthocyanins, respectively. Peonidin 3-glucoside came second in black and red rice, whereas cyanidin diglucoside was the third major anthocyanin in both rice grains. Two isomers of cyanidin diglucoside were observed in black and red rice, which could be positional or

Table 4. Average Concentration of Anthocyanins (Micrograms per Gram) in Black and Red Rice, Blue and Purple Wheat, and Blue Barley

anthocyanin[a]	compd	black rice	red rice	blue wheat	purple wheat	blue barley
Dp-3-Glu	**1**			56.5 ± 4.6		
Cy-diGlu[1]	**2**	71.8 ± 2.0[b]	1.7 ± 0.1			
Dp-3-Rut	**3**			49.6 ± 2.4		
Cy-3-Glu	**6**	2013 ± 57.1	14.0 ± 0.3	20.3 ± 1.5	4.0 ± 0.1	1.2 ± 0.0.02
Cy-diGlu[1]	**7**	16.7 ± 0.2	tr[c]			
Cy-3-Rut	**8**	19.9 ± 0.4	1.3 ± 0.1	16.8 ± 0.8		
Pt-3-Glu	**9**			2.2 ± 0.1		2.9 ± 0.1
Pt-3-Rut	**13**			4.5 ± 0.2		
Cy-MalGlu	**14**				1.2 ± 0.1	
Pn-3-Glu	**15**	162.1 ± 4.6	2.5 ± 0.1		2.1 ± 0.1	
Pn-Rut	**17**			1.2 ± 0.1		
Cy-SucGlu[2]	**18**				0.6 ± 0.04	
Mv-Rut	**20**			2.0 ± 0.1		
Cy-SucGlu[2]	**22**				1.1 ± 0.2	
Pn-MalGlu	**24**				0.6 ± 0.2	
Cy-SucGlu[2]	**31**				1.2 ± 0.1	
Pn-SucGlu[3]	**32**				0.9 ± 0.1	
Pn-SucGlu[3]	**36**				0.6 ± 0.1	
Pn-MalSucGlu	**38**				0.5 ± 0.1	
total		2283.5	21.8	153.1	12.8	4.1

[a] Cy, cyanidin; Dp, delphinidin; Glu, glucoside; Mal, malonyl; Mv, malvidin; Pn, peonidin; Pt, petunidin; Rut, rutinoside; Suc, succinyl. [1−3]Compounds with identical molecular mass within each superscript, which suggest they are isomeric. [b] Mean ± SD. [c] Trace, <0.1 μg/g.

Table 5. Average Concentration of Anthocyanins (Micrograms per Gram) in Blue, Pink, Purple, Red, and Fiesta Multicolored Corn

anthocyanin[a]	compd	shaman blue	cutie pink	purple	scarlet red	fiesta corn
Lt-Glu	**4**			31.0 ± 1.4		1.2 ± 0.1
Cy-3-Glu	**6**	110.2 ± 5.1[a]	7.8 ± 0.4	298.9 ± 14.4	284.5 ± 11.0	47.1 ± 2.2
Cy-3-Rut	**8**	1.1 ± 0.03				
Cy-MalGlu[1]	**10**	1.5 ± 0.03				
Pg-3-Glu	**11**	12.1 ± 0.3	39.8 ± 2.1	55.3 ± 2.6	27.7 ± 1.9	6.8 ± 0.3
Cy-MalGlu[1]	**12**	2.9 ± 0.1	0.6 ± 0.03	3.6 ± 0.3	4.2 ± 0.4	1.2 ± 0.1
Cy-MalGlu[1]	**14**	13.2 ± 0.7	1.2 ± 0.1	32.0 ± 0.5	16.8 ± 0.9	3.9 ± 0.2
Pn-3-Glu	**15**	2.3 ± 0.1	0.7 ± 0.1	27.3 ± 1.2	58.7 ± 2.5	2.2 ± 0.1
Pg-MalGlu[2]	**16**	0.8 ± 0.1	1.3 ± 0.1		0.7 ± 0.1	0.3 ± 0.04
Cy-SucGlu[3]	**18**	12.2 ± 0.7	1.1 ± 0.1	14.7 ± 0.4	11.3 ± 0.5	8.9 ± 0.2
Pg-MalGlu[2]	**19**	1.2 ± 0.1	4.9 ± 0.3	5.5 ± 0.3	2.1 ± 0.1	0.5 ± 0.02
Cy-MalGlu[1]	**21**	6.9 ± 0.4	0.4 ± 0.01	34.8 ± 1.7	20.2 ± 1.1	4.7 ± 0.1
Cy-SucGlu[3]	**22**	30.9 ± 1.6	3.3 ± 0.2	101.6 ± 7.1	30.1 ± 1.3	12.6 ± 0.3
Cy-SucGlu[3]	**23**			11.2 ± 1.1	4.8 ± 0.2	
Pg-SucGlu[4]	**25**	0.3 ± 0.03	2.4 ± 0.2		0.6 ± 0.1	0.2 ± 0.02
Pg-SucGlu[4]	**27**	1.4 ± 0.1	3.5 ± 0.1		1.8 ± 0.2	1.6 ± 0.1
Pg-MalGlu[2]	**28**		0.5 ± 0.02	21.2 ± 1.1	1.6 ± 0.1	
Pg-SucGlu[4]	**29**	2.6 ± 0.2	15.9 ± 0.7	22.1 ± 1.2	2.1 ± 0.2	1.9 ± 0.2
Pn-SucGlu[5]	**30**			12.3 ± 0.5	3.7 ± 0.1	
Cy-SucGlu[3]	**31**	12.9 ± 0.7	1.4 ± 0.2	97.1 ± 5.1	43.5 ± 2.1	3.9 ± 0.3
Pn-SucGlu[5]	**32**	1.1 ± 0.1		14.5 ± 0.7	7.1 ± 0.4	0.8 ± 0.1
Cy-MalSucGlu[6]	**33**	2.1 ± 0.2		25.3 ± 1.3	4.8 ± 0.3	
Pg-SucGlu[4]	**34**	2.3 ± 0.2	3.6 ± 0.2	23.9 ± 1.4	4.3 ± 0.3	
Cy-MalSucGlu[6]	**35**	2.6 ± 0.2		35.1 ± 2.1	5.1 ± 0.2	
Pn-SucGlu[5]	**36**			13.5 ± 0.7	9.5 ± 0.5	
Pg-MalSucGlu[7]	**37**		0.9 ± 0.1	3.7 ± 0.2	0.6 ± 0.1	
Cy-diSucGlu	**39**	4.6 ± ± 0.2	1.5 ± 0.1	57.6 ± 3.1	8.7 ± 0.4	2.1 ± 0.1
Pg-MalSucGlu[7]	**40**			3.1 ± 0.1	0.9 ± 0.1	
Pg-diSucGlu[8]	**41**		0.4 ± 0.03			
Pg-diSucGlu[8]	**42**		2.1 ± 0.2	14.3 ± 0.1	0.8 ± 0.1	
Pn-diSucGlu	**43**			5.6 ± 0.3	2.3 ± 0.1	
total		225.2	93.3	965.2	558.5	100.0

[a] Cy, cyanidin; Glu, glucoside; Lt, luteolunidin; Mal, malonyl; Pg, pelargonidin; Pn, peonidin; Pt, petunidin; Rut, rutinoside; Suc, succinyl. [1−8]Compounds with identical molecular mass within each superscript, which suggest they are isomeric. [b] Mean ± SD.

structural isomers due to differences in hexose type and/or position. Ryu et al. (*22*) found two main anthocyanins in 10 black rice varieties in which cyanidin 3-glucoside is the most common (0.0−470 mg/100 g), whereas peonidin 3-glucoside (0.0−40 mg/100 g) is the second.

The anthocyanin composition of blue wheat differed from that of purple wheat. This study is the first to identify the main anthocyanin in blue wheat as delphinidin 3-glucoside, being ≈37% of the total anthocyanins (**Table 4**). In our previous study on blue wheat, the main anthocyanin was not identified but accounted for 38% of the total anthocyanins (*3*). Delphinidin 3-rutinoside was the second dominant anthocyanin at 32% of the total anthocyanins. Delphinidin is the main aglycone in blue wheat, being ≈69% of the total anthocyanidins. In purple wheat, 10 anthocyanin compounds were observed at small concentration with cyanidin 3-glucoside and peonidin malonylglucoside being

the main ones (**Table 4**). Cyanidin 3-glucoside was the first predominant anthocyanin in purple wheat (*3*). Only two anthocyanins were detected in blue barley, not including the major peak (**Table 4**; **Figure 3**). This indicates that one or more other pigments may contribute to the color of the grain giving higher TAC.

Blue, pink, purple, red, and multicolored corn exhibited complex anthocyanin composition having from 18 to 27 compounds (**Table 5**). The highest number of anthocyanins (27 compounds) was found in scarlet red corn, whereas the highest amount was observed in purple corn (965 μg/g), with 25 anthocyanins. Cyanidin 3-glucoside was the most common anthocyanin in colored corn, except for pink corn, accounting for 51, 49, 47, and 31% in red, blue, multicolored, and purple corn, respectively. In pink corn, the major anthocyanin was pelargonidin 3-glucoside at 43% of the total anthocyanins. The second most abundant anthocyanin was different in corn, being cyanidin 3-succinylglucoside in blue, purple, and multicolored corn, cyanidin 3-glucoside in pink corn, and peonidin 3-glucoside in red corn. Moreno and others (*1*) reported that cyanidin 3-glucoside, cyanidin 3-(6″-malonylglucoside), and cyanidin 3-(3″,6″-dimalonylglucoside) are the major anthocyanins, with cyanidin being the main aglycone accounting for 73−87% of the total anthocyanins in purplish-red corn.

The majority of the anthocyanins found in colored corn were in acylated form having malonyl or succinyl moieties and several isomers. For example, isomers of cyanidin malonylglucoside, pelargonidin malonylglucoside, cyanidin succinylglucoside, and pelargonidin succinylglucoside were found in blue, pink, purple, and red corn, whereas isomers of pelargonidin succinylglucoside and cyanidin malonylsuccinylglucoside were observed in blue, purple, and red corn. Pelargonidin malonylsuccinylglucoside and pelargonidin disuccinylglucoside isomers were found only in pink, purple, and red corn but not in blue corn. Because glucose is the most common sugar in corn anthocyanins, it was presumed to be the hexose in those pigments. However, it is possible that other hexoses such as galactose may also be present in colored corn. In addition, sugar is commonly attached at position 3 (**Figure 1**), which may suggest that most of the identified anthocyanins in **Tables 4** and **5** may have the hexose at position 3. A deoxyanthocyanin, luteolunidin glucoside, was also detected in purple and fiesta multicolored corn. This deoxyanthocyanin and apigeninidin or apigeninidin glucoside were the major deoxyanthocyanins in black sorghum, accounting for 50% of total anthocyanins (*24*).

When the relationship between the total anthocyanin content determined by colorimetry (**Table 1**) versus that determined by LC (**Tables 4** and **5**) was examined, a significant positive correlation was obtained with a correlation coefficient (r) of 0.9973 and a slope of 1.410. This shows that the colorimetric method overestimated the LC values by ≈41%. The difference may be due to the contribution of other pigments present in the grains that have an absorbance at 535 nm. This overestimation was consistent from one grain to another.

Anthocyanins have been recognized as health-enhancing substances and have been found in many types of grains. The present study showed a diversity of anthocyanins in a selection of black, blue, pink, purple, and red cereal grains. It also shows substantial differences in anthocyanin content and composition among the grains studied. Some of the grains exhibited a few pigments or a simple anthocyanin profile such as black rice, red rice, and blue barley; others had an intermediate profile such as blue and purple wheat, whereas blue, pink, purple, and red corn possessed a complex profile. Such diversity in anthocyanin composition would help in the selection process for the development of anthocyanin-rich grain products. In addition, the anthocyanin pigments in grains can be concentrated by dry milling and fractionation processes to produce fractions that are high in anthocyanin contents, even much higher than those found in fruits and vegetables, that is, 25000 μg/g in the black rice fraction versus 200−10000 μg/g in fruits and vegetables. The data suggest that some of the colored grains such as black rice, purple corn, and blue wheat may hold promise for the development of grain-based functional foods or natural colorants on the basis of their anthocyanin content and composition. These grains could also be used as genetic resources of anthocyanin pigments in breeding programs.

ABBREVIATIONS USED

cv., cultivar; ESI, electrospray ionization; LC, liquid chromatography; MS, mass spectrometry; PDA, photodiode array detector; SD, standard deviation; TAC, total anthocyanin content; UV; ultraviolet; vis, visible.

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