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Fractionation of Blue Wheat Anthocyanin Compounds and Their Contribution to Antioxidant Properties

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Demands for anthocyanin-rich foods and supplements are steadily increasing due to their significant roles in human health and food coloration. In the development of blue wheat as a functional food ingredient, dry milling was employed to separate the bran fraction where anthocyanins are concentrated 2.3-fold as compared to whole grain (13.9 mg/100 g). Anthocyanins were then extracted with aqueous ethanol and partially purified into a highly concentrated powder. The total anthocyanin content in the isolated powder as analyzed by high-performance liquid chromatography (HPLC) averaged 3378 mg/100 g and afforded a 243-fold increase in concentration relative to the whole grain product. Four main anthocyanins, delphinidin-3-glucoside (45%), cyanidin-3-glucoside (28%), delphinidin-3-rutinoside (22%), and cyanidin-3-rutinoside (2%), were isolated from the powder using preparative HPLC. Anthocyanin products and compounds were assessed against scavenging of 2,2-diphenyl-1-picryl-hydrazyl and 2,2′-azino-di-(3-ethylbenzthiazoline sulfonate) radicals and inhibition of human low-density lipoprotein cholesterol oxidation. Significant differences in antioxidant capacity were observed with anthocyanin powder and compounds exceeding that of butylated hydroxytoluene, indicating a potential for the development of blue wheat-based natural antioxidants and colorants.

KEYWORDS: Blue wheat; anthocyanins; natural colorants; dietary supplements; LC-UV/vis; LC-MS

INTRODUCTION

Anthocyanins are glycosides or acylglycosides of polyhydroxy 2-phenylbenzopyrylium cations belonging to a larger group of compounds known as flavonoids, a subgroup of polyphenols. They are widespread in fruits and vegetables and found primarily in fresh berries, grape, red cabbage, and red radish with an estimated daily intake 12.5 mg/person in the United States (*1*). Recently, it has become increasingly evident that anthocyanins possess several beneficial health effects including free radical scavenging activity (*2*−*5*), inhibitory capacity against oxidation of low-density lipoprotein (LDL) cholesterol (*6*), anti-inflammatory effects (*7*), anticancer (*8*−*10*), and hypoglycemic responses (*11*). They have also been employed as natural colorants in food and nonfood applications, and there is a great potential for expansion in this area. In addition, they play important roles in plant physiology, for example, as attractants for insect pollinators and seed dispersal (*12*). In this respect, blue wheat and other colored grains would be potential candidates for the development of natural antioxidants and colorants. At present, these grains are underutilized, and their contribution to the human diet is very low due to the limited data available.

Currently, colored cereal grains such as purple or blue corn and blue wheat have drawn the attention of academia and the food industry due to their content of anthocyanin pigments and antioxidant properties. These cereals were found to vary in their anthocyanin composition ranging from a simple profile having a few pigments in black and red rice to a complex profile in blue, pink, purple, and red corn (*13*). Blue wheat was identified among other cereals such as black rice and purple corn as a potential candidate for the development of bioactive food ingredients and natural colorants. This grain was first investigated by our research group in 1999 in a breeding program aimed at developing high pigmented wheat (*14*). The total anthocyanin content distribution in 160 blue wheat lines ranged from 35 to 507 μg/g with a mean of 183 μg/g. Later, it was found that the anthocyanin trait in blue wheat is relatively more stable to environmental changes in a 3 year study as compared

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to purple wheat grown under the same conditions (*15*). This difference between blue and purple wheat cultivars may result from the location of anthocyanin pigments in the wheat kernel. It was reported that the purple pigments are likely to be located in the pericarp outer layers, whereas the blue pigments are mainly found in the aleurone layer (*16*). Composition of anthocyanins was also found to differ between blue and purple wheat (*13, 15*).

Delphinidin was found to be the primary aglycone or anthocyanindin in blue wheat and accounts for about 69% of the total anthocyanins followed by the aglycone cyanidin at 24% with smaller amounts of petunidin, malvidin, and peonidin (*13*). The aglycone delphinidin was estimated to contribute approximately 21% of the total anthocyanin daily intake, and it comes second after cyanidin (45%) (*1*). Four major anthocyanins were found in blue wheat cv. Purendo including delphinidin-3-glucoside, delphinidin-3-rutinoside, cyanidin-3-glucoside, and cyanidin-3-rutionside. However, a study by Hu and others (*17*) found that a dark blue grained wheat cv. Hedong Wumai exhibited a different anthocyanin composition, with cyanidin-3-glucoside being the predominant pigment. Purple wheat cultivars Laval and Konini contained lower amounts of total anthocyanins, but they had a greater variety of anthocyanin compounds as compared to blue wheat, with cyanidin-3-glucoside being the only major anthocyanin (*13*). These data suggest that the anthocyanin composition in wheat varies with genotype.

Understanding the composition, structure, antioxidant properties, bioactivity, and bioavailability of anthocyanins in blue wheat should help boost the development of this cereal as a functional grain, that is, a potential source of anthocyanins. The objectives of this study were to develop a method for the preparation of anthocyanin powder from blue wheat, to quantify and confirm the identity of anthocyanins in blue wheat products, to fractionate and isolate blue wheat anthocyanins, and to study the antioxidant capacity of the isolated anthocyanins, anthocyanin powder, whole grain, bran, and white flour against free radicals and lipid peroxidation.

MATERIALS AND METHODS

Blue Wheat. Spring type blue wheat (*Triticum aestivum* L. cv. Purendo) was obtained from the University of Saskatchewan (Saskatoon, SK, Canada). The wheat grains were ground on a Cyclone sample mill (Udy Co., Fort Collins, CO) equipped with a 500 μm screen to produce whole grain flour. Wheat kernels were fractionated into white flour and bran fractions 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 a 45 mesh (350 μm opening) sieve (Tyler Co., Mentor, OH). The average yield of the bran fraction was about 26%. The bran fraction was further ground and passed through a 45 mesh screen. The whole grain flour, white flour, and bran fraction were each thoroughly mixed to ensure uniformity and kept at 4 °C until analysis.

Standards, Reagents, and Solvents. The authentic anthocyanin standards delphinidin-3-glucoside, delphinidin-3-rutinoside, cyanidin-3-glucoside, cyanidin-3-galactoside, cyanidin-3-rutionside, peonidin-3-glucoside, petunidin-3-glucoside, pelargonidin-3-glucoside, and cyanidin chloride were purchased from Polyphenols Laboratories (Sandens, Norway). Formic acid, ethanol, methanol, and HCl were obtained from Fisher Scientific (Ottawa, ON, Canada). Butylated hydroxytoluene (BHT), copper sulfate, and 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical were purchased from Sigma (St. Louis, MO). Human LDL cholesterol was purchased from Biomedical Technologies Inc. (Stoughton, MA). Nanopure water was obtained by passing distilled water through a NANO pure Infinity ultra pure water system (Barnstead International, Dubuque, IA).

Preparation of Anthocyanin Powder. A flowchart depicting the preparation of blue wheat milling products, anthocyanin powder, fractionation and isolation of anthocyanins, and characterization of products and compounds is presented in **Figure 1**. Anthocyanin powder was prepared from the bran fraction, from which anthocyanins were extracted with acidified ethanol (ethanol and 1.0 N HCl, 85:15, v/v) and partially purified by passing through a chromatographic column (35 cm × 3 cm i.d.) (Durex Canada, Toronto, ON) packed with 30 g of Amberlite XAD7. Anthocyanins were eluted with 100 mL of ethanol, and the elution was quantitatively transferred into a Petri dish and concentrated to almost dryness in a dark fume hood for about 48 h and then freeze-dried. The partially purified resultant product was called anthocyanin powder.

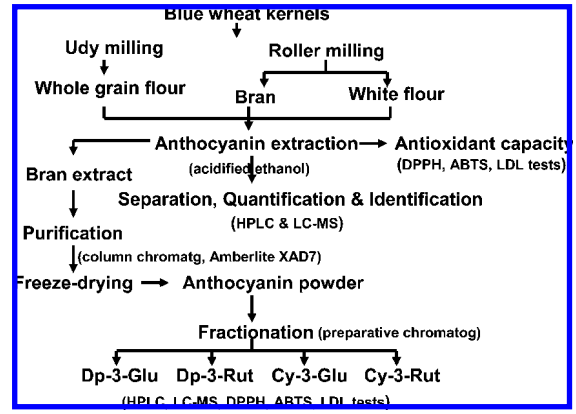

Figure 1. Flowchart showing the preparation of blue wheat anthocyanin powder and anthocyanin compounds as well as their analyses and antioxidant properties.

Extraction of Anthocyanins. Anthocyanins in blue wheat white flour, bran, and whole grain flour and anthocyanin powder were extracted based on the method recently described (*13*). Three grams of blue wheat materials or 0.1 g of anthocyanin powder was used in the extraction process. Following partial purification using column chromatography, the volume of extracts was reduced by nitrogen vaporization to about 1.6 mL, vigorously mixed, and filtered through a 0.45 μm Nylon Acrodisc syringe filter (Pall Gelman Laboratory, Ann Arbor, MI) prior to high-performance liquid chromatography (HPLC) analysis.

Total Anthocyanin Content. The total anthocyanin content in grain and anthocyanin samples was determined using the spectrophotometric method previously described (*14*). The supernatant of the crude extracts was poured into a 50 mL volumetric flask and made up to volume with acidified ethanol. The absorbance was measured on a UV/vis spectrophotometer (Varian Inc., Palo Alto, CA) at 535 nm. The total anthocyanin content in μg/g was calculated as follows: TAC = $A \times 288.21$, where A is absorbance reading (*14*).

Separation and Quantification of Anthocyanins. Anthocyanins in the extracts obtained from blue wheat anthocyanin powder, whole grain, bran, and white flour were separated and quantified with an 1100 Series chromatograph (Agilent, Mississauga, ON) equipped with a G1311A quaternary pump, G1329A temperature-controlled injector, G1316A temperature-controlled column thermostat, G1322A degasser, G1315B photodiode array detector (PDA), and ChemStation Rev. B.0201-SR2[260] data acquisition system with the capability of conducting isoabsorbance plot and 3D graphic analyses. A 75 mm × 4.6 mm i.d., 3.5 μm Zorbax SB-C18 rapid resolution column (Agilent Technologies Canada Inc., Mississauga, ON) was employed for separation. The column was operated at 38 °C and eluted with a gradient mobile phase consisting of (A) 6% formic acid and (B) methanol at 1 mL/min. The gradient was programmed as follows: 0−7 min, 82 to 80% A; 7−10 min, 80 to 75% A; 10−25 min, 75 to 40% A; 25−26 min, 40 to 82% A; and 26−28 min, hold at 82% A. The separated anthocyanins were detected and measured at 525 nm (λ_{max} of delphinidin-3-glucoside, the major anthocyanin pigment in blue wheat), and the identity of anthocyanins was based on the congruence of retention times and UV/vis spectra with those of pure authentic standards. Stock and working standard solutions were prepared and used for the identification and quantification of unknown wheat samples as described in our previous publication (*13*).

The purity of each compound was verified based on the spectroscopic properties of each peak using isoabsorbance plot or 3D graphic and peak purity analyses provided with the ChemStation software. Peak purity analysis allowed the spectrum of the identified compound to be confirmed and to determine whether or not interference occurred.

Anthocyanin compounds in the anthocyanin powder were fractionated using preparative HPLC equipped with an analytical fraction collector. The anthocyanin compounds were collected using a fraction trigger mode timetable with peak-based option in the timetable in which the retention time and duration time of the collected peaks were set up for each peak. The HPLC run time was shortened to 15 min for collecting the four major anthocyanins. The concentration of anthocyanins in the collected fractions was measured by HPLC and spectrophotometric methods.

Confirmation of Anthocyanin Identity. The confirmation of identity of each peak was carried out by liquid chromatography−mass spectrometry (LC-MS) (Thermo Finnigan, San Jose, CA) equipped with a SpectraSystem UV6000LP ultraviolet detector scanning from 190 to 800 nm and a LCQ Deca ion trap mass spectrometer operated in the ESI positive ion (positive) mode scanning from *m/z* 50 to 2000. The LC-MS conditions were the same as for the HPLC analyses. Machine operating conditions for 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; the capillary temperature was set at 350 °C, the source voltage was 5.00 kV, and the source current was 80.0 μA. The mass spectrometer was tuned for maximum response to delphidin-3-glucoside.

Scavenging Capacity of DPPH Radical and 2,2′-Azino-di-(3-ethylbenzthiazoline sulfonate) (ABTS) Radical Cation. The free radical scavenging capacity of wheat extracts was determined using the stable DPPH as outlined by Yu et al. (*18*). The antioxidant reaction was initiated by transferring 1 mL of blue wheat extract or anthocyanin solutions into a test tube containing 4 mL of 80% aqueous methanol and 1 mL (containing 1 mmole) of freshly prepared DPPH solution. The final concentration of DPPH in the reaction mixture was 167 μmol. The reaction was monitored by reading the absorbance at 517 nm over 30 at 1 min intervals. A blank reagent was used to study the stability of DPPH radical over the test time. The scavenging capacity of blue wheat or anthocyanin products was calculated as μmol DPPH/g sample.

The scavenging capacity against radical cation ABTS was measured using the Randox Laboratories assay kit (San Francisco, CA). Trolox (6-hydroxy 2,5,7,8-tetramethylchroman-2-carboxylic acid) provided in the kit was used as an antioxidant standard. The scavenging capacity of blue wheat or anthocyanin products was calculated as μmol ABTS/g sample.

Inhibition of Copper-Induced Human LDL Oxidation. The inhibition capacity against oxidation of copper-induced human LDL cholesterol was based on the method modified by Abdel-Aal and Gamel (*19*). Human LDL cholesterol [5 mg protein/mL in 0.05 M Tris-HCl, 0.15 M NaCl, and 0.3 mM ethylenediaminetetraacetic acid (EDTA) at pH 7.4] was dialyzed against 10 mM phosphate buffer saline or PBS (pH 7.4, 0.15 M NaCl) for 24 h under nitrogen at 4 °C, with one change of buffer once after every 10 h. The EDTA-free LDL was subsequently diluted to obtain a standard protein concentration of 250 μg/mL with PBS. The diluted LDL solution (800 μL) was mixed with 100 μL of diluted sample extract (30 μL of extract and 70 μL of 80% aqueous methanol) in a 4 mL test tube. BHT at a concentration of 75 μg/100 μL was included as a reference antioxidant. The oxidation of LDL was initiated by adding 200 μL of 50 μM cupric sulfate solution. The mixture was incubated at 37 °C, and the formation of conjugated dienes (CDs) was measured spectrophotometrically at 234 nm every 4 h up to 24 h.

For the measurement of conjugated dienes, 100 μL of oxidized LDL mixture was added to 1200 μL of *iso*-propanol in a 1.5 mL Eppendorf tube. After vigorous shaking for 2 min, the sample was centrifuged at 6000*g* for 5 min, and the supernatant was read on a spectrophotometer (Varian Australia Pty Ltd.) at 234 nm. The amount of conjugated dienes formed was calculated using the molar extinction coefficient 29500 M/L/cm (*20*).

Table 1. Average Concentration of Total Anthocyanins (mg/100 g) in Blue Wheat Products

	colorimetry		HPLC	
product	concentration	concentration factor	concentration	concentration factor
white flour	4.1 ± 1.7[a]		2.5 ± 1.1	
whole grain	17.8 ± 3.9		13.9 ± 3.1	
bran fraction	40.5 ± 7.3	2.3	32.1 ± 3.2	2.3
anthocyanin powder	3987.1 ± 112.7	224	3378.1 ± 78.5	243

[a] Mean ± SD, $n = 3$.

Statistical Analysis. All of the analyses were carried out in triplicate, and the data were subjected to statistical analysis using Minitab software (version 12, Minitab Inc., State College, PA). The data were reported as means of three replicates ± standard deviation (SD).

RESULTS AND DISCUSSION

Composition and Confirmation of Anthocyanins. In our previous study, we found that anthocyanins vary significantly in their content and composition among anthocyanin-pigmented cereal grains with blue wheat having an intermediate level of anthocyanins (212 μg/g) as compared to purple wheat (96 μg/g) and purple corn (1277 μg/g) (*13*). Because of the location of blue pigments in the aleurone layer of the blue wheat kernel, anthocyanins can be concentrated in the bran fractions using abrasive or roller milling process. In our previous study (*13*), the fine bran fraction or shorts had 818 μg/g total anthocyanins, whereas the coarse bran fraction contained 496 μg/g. In the current study, wheat kernels were roller milled and separated into white flour and bran fraction at yields of about 74 and 26%, respectively. The bran fraction had 405 and 321 μg/g total anthocyanin content as compared to 178 and 139 μg/g in the whole kernel as determined by colorimetry and HPLC, respectively (**Table 1**). White flour had small amounts of total anthocyanins as compared to the whole kernel, which is close to that obtained in our previous study (*13*). Separation of bran by a small-scale roller mill resulted in an 2.3-fold increase in anthocyanins as compared to the original whole wheat flour based on total anthocyanin content determined by either colorimetry or HPLC. Despite the high content of anthocyanins in the shorts fraction as compared to the coarse bran (e.g., 818 vs 496 mg/kg), in the current study, we combined fine and coarse bran to increase the yield of bran (26%) as compared to only 7% yield for the shorts fraction (*13*). The high yield and anthocyanin concentration in the blue wheat bran would make it a potential candidate for the development of an economically feasible high-anthocyanin functional ingredient due to its low value as a milling byproduct.

Blue wheat grains used in the present study contained lower amounts of anthocyanins as compared to those used in our previous study (e.g., 178 vs 212 μg/g) (**Table 1**). The concentration of anthocyanins in the same wheat variety could vary between years and locations and thus would pose a challenge in the development process. In a large population of blue wheat cultivar/lines, the total anthocyanin content ranged from 35 to 507 μg/g with a mean of 183 μg/g (*14*). The anthocyanin concentration in blue wheat, however, is relatively more stable to growing conditions as compared to purple wheat due to the pigment location, that is, blue pigments in the aleuronic layer and purple pigments in the pericarp outer layers (*15, 16*). Environmental factors such as the quantity of UV-B light received by the plant alter the expression of defense genes, among other factors, and give rise to the synthesis of antho-

Table 2. Average Concentration of Major Anthocyanin Compounds in Blue Wheat Products Determined by HPLC[a]

product	Dp-3-Glu	Cy-3-Glu	Dp-3-Rut	Cy-3-Rut
white flour (μg/g)	11.1 ± 0.43[b]	6.9 ± 0.29	5.2 ± 0.24	0.45 ± 0.03
whole grain (μg/g)	57.1 ± 2.7	37.6 ± 1.5	27.8 ± 0.8	2.3 ± 0.07
bran fraction (μg/g)	135.2 ± 3.1	85.7 ± 3.2	65.1 ± 2.2	5.3 ± 0.17
anthocyanin powder (mg/g)	14.13 ± 0.43	9.03 ± 0.29	6.84 ± 0.27	0.55 ± 0.04

[a] Cy, cyanidin; Dp, delphinidin; Glu, glucoside; Rut, rutinoside. [b] Mean ± SD, $n = 3$.

cyanins (*21*). More research is underway to better understand the stability of the blue pigment trait in wheat during grain development.

The bran fraction obtained from blue wheat was evaluated for the preparation of blue wheat anthocyanin powder by using dry milling and wet extraction processes (**Figure 1**). Following extraction with ethanol, the extracts were purified to remove sugars and proteins and evaporated to remove ethanol prior to freeze drying to obtain anthocyanin powder. Anthocyanin powder had an exceptionally high content of total anthocyanins as determined by either colorimetry or HPLC (**Table 1**). Anthocyanin powder contained significant levels of anthocyanins at 3987 or 3378 mg/100 g as determined by colorimetry and HPLC, respectively. This represents a 243-fold increase over the original whole wheat flour based on total anthocyanins measured by HPLC. The powder is extremely high in anthocyanins as compared to blue wheat milling fractions and would hold potential as a natural colorant or functional ingredient.

The composition of anthocyanins in colored cereal grains was found to vary substantially ranging from a simple profile in black and red rice to a complex profile in blue, pink, purple, and red corn (*13*). In the present study, blue wheat was found to contain four main anthocyanin pigments, namely, delphinidin-3-glucoside, cyanidin-3-glucoside, delphinidin-3-rutinoside, and cyanidin-3-rutinoside (**Table 2**), similar to our previous finding (*13*). In addition to the main anthocyanins, blue wheat extracts contained low concentrations of petunidin-3-rutinoside, petunidin-3-glucoside, malvidin-3-rutinoside, and peonidin-3-rutinoside. Typical HPLC chromatograms showing separation of anthocyanins from blue wheat white flour, whole grain, and bran extracts and anthocyanin powder are presented in **Figure 2**, and the structures of the four main anthocyanin pigments found in blue wheat products are depicted in **Figure 3**. The identity of these pigments was confirmed based on the congruence of UV/vis and MS data as described in our previous study (*13*). Blue wheat extracts showed absorption bands in the blue end of the visible spectrum around 517 and 525 nm in addition to another absorption bands in the UV region around 277−279 nm. Anthocyanin compounds appeared in the MS ESI positive mode as two ions, the molecular ion (true molecular weight or MW) and the fragment ion (MW sugar moiety). For example, in the case of delphidin-3-glucoside, the major anthocyanin pigment in blue wheat, the molecular ion is at m/z 465 and the fragment ion is at m/z 303. Details on UV/vis and MS properties of anthocyanins in blue wheat and other colored grains have been previously reported (*13*).

The concentration of individual anthocyanins in the blue wheat milling products and anthocyanin powder is presented in **Table 2**. As expected, the four main anthocyanins appeared in the four blue wheat products but at different concentrations. The concentrations of the flour anthocyanins were very low in the white flour fraction and extremely high in the powdered product. Delphinidin-3-glucoside constituted the highest proportion at 44−46%, followed by cyanidin-3-glucoside (28−29%), delphinidin-3-rutinoside (21−22%), and cyanidin-3-rutinoside (1.8−1.9%). In another study using the dark blue-grained wheat cv. Hedong Wumai, a different mix of anthocyanin pigments (cyanidin-3-glucoside, cyanidin-3-galactoside, pelargonidin-3-glucoside, and peonidin-3-glucoside) was observed with cyanidin-3-glucoside being the predominant pigment (*17*). This variation in anthocyanin composition in blue wheat may be genotype-dependent.

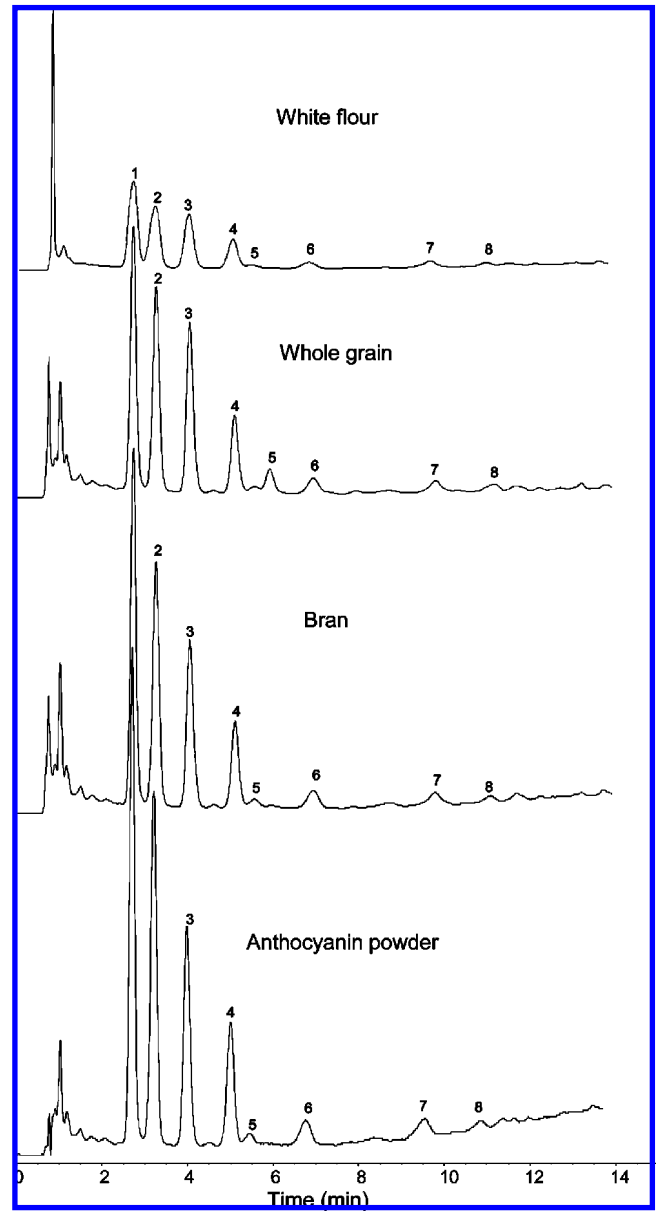

Figure 2. HPLC chromatograms showing separation of anthocyanins from blue wheat white flour, blue wheat whole grain flour, blue wheat bran, and anthocyanin powder extracts. Peaks: 1, delphinidin-3-glucoside; 2, delphinidin-3-rutinoside; 3, cyanidin-3-glucoside; 4, cyanidin-3-rutinoside; 5, petunidin-3-glucoside; 6, petunidin 3-rutinoside; 7, peonidin-3-rutinoside; and 8, malvidin-3-rutinoside.

Scavenging Capacity of Free Radicals. It is generally accepted that reactive oxygen species and free radicals generated during cellular metabolism or peroxidation of lipids and other biological molecules play important roles in the pathogenesis of chronic diseases such as coronary heart disease and certain cancers. Dietary antioxidants could combat such reactive oxygen species and free radicals and help reduce the risk of chronic diseases. Wheat contains a variety of bioactive compounds that

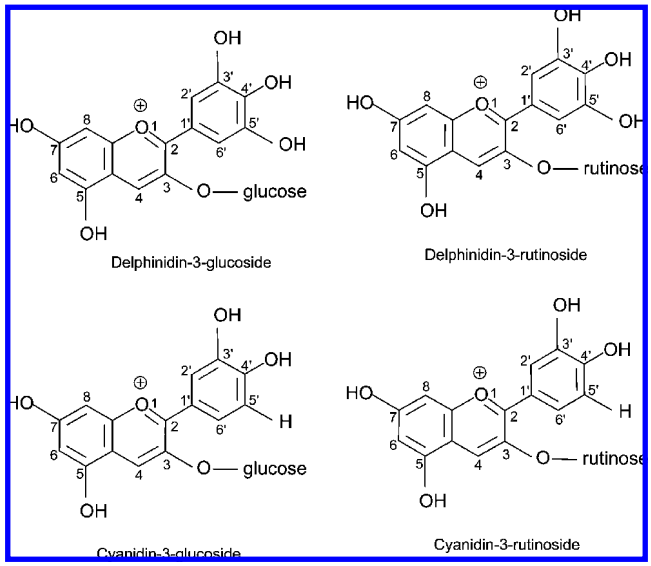

Figure 3. Structures of the main anthocyanin pigments in blue wheat.

Table 3. Scavenging Capacity of Blue Wheat Milling Products, Anthocyanin Powder, and Anthocyanin Compounds against DPPH Radical and ABTS Radical Cation

	DPPH (μmol/g)		ABTS (μmol/g)	
blue wheat	10 min	30 min	3 min	10 min
	products			
white flour	30.2 ± 0.02[a]	36.0 ± 0.33	14.8 ± 0.92	30.7 ± 0.70
whole grain	33.1 ± 0.06	40.4 ± 0.28	22.5 ± 0.45	45.0 ± 0.53
bran fraction	40.0 ± 0.48	45.8 ± 0.24	29.9 ± 0.66	57.2 ± 0.25
anthocyanin powder	1685.8 ± 81.3	2525.9 ± 125.2	1609.9 ± 35.3	2925.1 ± 48.1
	anthocyanin fractions			
delphinidin-3-glucoside	792 ± 21.3	831 ± 26.2	192 ± 22.7	382 ± 15.1
cyanidin-3-glucoside	1113 ± 15.7	1232 ± 21.3	533 ± 4.7	1015 ± 7.2
delphinidin-3-rutinoside	567 ± 17.1	613 ± 15.9	232 ± 13.8	430 ± 16.8
cyanidin-3-rutinoside	3967 ± 45.9	4346 ± 61.7	1673 ± 19.9	3261 ± 34.6

[a] Mean ± SD, $n = 3$.

may contribute to its antioxidant capacity. Polyphenol compounds such as anthocyanins are among the major contributors to the antioxidant properties of wheat products. The scavenging capacity of blue wheat milling products, anthocyanin powder, and anthocyanin compounds were evaluated against DPPH free radicals (**Table 3**). The DPPH test is based on an electron transfer reaction (*22*) in which the colored stable DPPH free radical is reduced in the presence of an antioxidant into a nonradical DPPH-H. The DPPH free radical shows a strong absorption at 517 nm in the visible spectrum because of the odd electron. As this electron becomes paired off in the presence of a free radical scavenger, the absorption diminishes, and the resulting decolorization is proportional to the number of electrons taken up (*23*). Among the blue wheat milling products, the bran extract showed the highest capacity to scavenge DPPH free radicals, followed by whole grain and white flour. This could be attributed to a higher concentration of anthocyanins in the bran fraction as compared to whole grain and white flour (**Table 1**). Hu et al. (*17*) reported that 69% of the overall free radical scavenging capacity of dark blue grained wheat is attributed to the anthocyanin content, as compared to 19% for the extractable phenolic acids. The DPPH scavenging capacity of the bran extracts was about 2-fold higher than that of whole grain extracts prepared from four wheat genotypes (black, blue, purple, and white) (*24*). Blue wheat white flour showed a reasonable level of scavenging capacity toward DPPH radicals (**Table 3**) despite its low content of anthocyanins (**Table 1**). This may indicate that other kernel constituents contribute to the total antioxidant activity in the white flour.

The DPPH test was monitored over a 30 min time course to measure the reaction rate. The scavenging capacity after 10 and 30 min is reported here. The reaction rate was relatively rapid during the first 10 min, and afterward, it became progressively slower. The DPPH scavenging capacity was positively correlated with anthocyanin content under the experimental conditions ($r = 0.92$). These results are in agreement with those reported by Zhou et al. (*25*).

The DPPH scavenging capacity of aqueous plant extracts was found to be concentration-dependent, that is, as the amount of phenolic compounds increased, the reducing power increased (*26*). This was obvious in the case of anthocyanin powder and anthocyanin compounds, which showed exceptionally high scavenging capacities (**Table 3**). Anthocyanin powder had a DPPH scavenging capacity 42-fold higher than blue wheat bran. There were significant differences between anthocyanin compounds in their DPPH scavenging capacity. Cyanidin-containing anthocyanins had higher DPPH scavenging capacities as compared to delphinidin-based anthocyanins. This finding emphasizes the importance of the molecular structure of anthocyanins in antioxidant properties and perhaps bioactivity.

The antioxidant capacities of blue wheat milling products, anthocyanin powder, and anthocyanin compounds were also assessed based on their ability to quench ABTS radical cation (**Table 3**). This test is based on the formation of ABTS radical cation by reacting ABTS with metmyoglobin and H_2O_2 at 37 °C. The ABTS radical cation has a relatively stable blue-green color, which is measured at 600 nm. In the presence of antioxidants, the color production will be suppressed in proportion to the concentration of antioxidants. ABTS scavenging capacity after 3 and 10 min exhibited a similar increasing trend as observed for the DPPH test (**Table 3**). Again, among the milling products, blue wheat bran had the highest scavenging capacity followed by whole grain and white flour. When a range of diverse grains was examined based on their capacity to quench ABTS radical cation, normal wheat cv. Henika came third after buckwheat and barley and was followed by oat, rye, and wheat cv. Almari (*27*). The ABTS scavenging capacity was found to be related to phenolic compounds and their concentration (*28*).

Anthocyanin powder and anthocyanin compounds were exceptionally high in ABTS scavenging capacity as compared to the blue wheat milling products (**Table 3**). Anthocyanin powder had an ABTS scavenging capacity 54-fold higher than blue wheat bran. In addition, there were significant differences between anthocyanin compounds in their ABTS scavenging capacity. Again, cyanidin-containing anthocyanins exhibited higher ABTS scavenging capacity as compared to delphinidin-based anthocyanins. This trend is similar to that obtained with DPPH scavenging capacity. The sugar moiety was also found to influence ABTS scavenging capacity of anthocyanin compounds. In this respect, rutinose-containing anthocyanins showed higher scavenging capacity as compared to glucose-containing anthocyanins. The effect of aglycone and sugar moiety on antioxidant capacity requires further research to better understand their impact on scavenging capacity.

Inhibition of LDL Cholesterol Oxidation. High levels of plasma LDL cholesterol and its oxidation products are important factors in the development of atherosclerosis and heart disease (*29, 30*). LDL oxidation has been studied extensively in vitro using a variety of agents to initiate the oxidation process, in which cupric ion is the most frequently used (*31*). In this

Table 4. Inhibition of Human LDL Cholesterol Oxidation by Blue Wheat Milling Products, Anthocyanin Powder, and Anthocyanin Compounds Determined by the Amounts of Conjugated Dienes (CDs) Formed

samples	4 h	8 h	12 h	24 h
control and reference samples (μmol CDs/mg sample/g LDL)				
control (no antioxidants)	381.3 ± 13.3[a]	528.1 ± 14.7	530.6 ± 13.9	584.7 ± 17.1
BHT (75 μg)	19.4 ± 0.57	23.2 ± 0.93	30.5 ± 0.85	36.6 ± 1.12
blue wheat products (μmol CDs/mg sample/g LDL)				
white flour	9.6 ± 0.39	48.0 ± 1.52	49.9 ± 1.44	51.8 ± 1.31
whole grain	2.7 ± 0.07	7.3 ± 0.31	32.1 ± 0.91	46.1 ± 1.45
bran fraction	1.4 ± 0.08	16.8 ± 0.72	31.9 ± 1.07	42.7 ± 1.37
anthocyanin powder	0.055 ± 0.002	0.062 ± 0.005	0.292 ± 0.015	1.445 ± 0.058
anthocyanin fractions (μmol CDs/μg anthocyanin/g LDL)				
delphinidin-3-glucoside	42 ± 1.71	119 ± 3.55	241 ± 6.0	244 ± 5.8
cyanidin-3-glucoside	308 ± 7.2	695 ± 11.7	720 ± 14.6	928 ± 17.4
delphinidin-3-rutinoside	47 ± 1.63	92 ± 1.82	261 ± 5.1	266 ± 5.1
cyanidin-3-rutinoside	312 ± 6.32	721 ± 13.9	799 ± 15.3	1004 ± 19.9

[a] Mean ± SD, $n = 3$.

study, effects of blue wheat milling products, anthocyanin powder, and anthocyanin compounds on copper-induced human LDL oxidation were investigated and are reported in **Table 4**. The LDL oxidation was found to occur in three consecutive phases; a lag phase, in which the formation of CD increases slightly, followed by a propagation phase with a rapid increase of the diene absorption, and finally a decomposition phase (*32*). In the current study, the in vitro human LDL oxidation reaction was monitored by measuring the amount of conjugated dienes at 234 nm after 4, 8, 12, and 24 h with and without anthocyanin products. In the control sample (e.g., no antioxidants added), there was a rapid increase in the concentration of CD until 8 h of incubation; afterward, a slight increase occurred (**Table 4**). In the presence of blue wheat milling product, anthocyanin powder and anthocyanin compound extracts or BHT solution, the concentration of CD dropped significantly to varying extents depending upon the type of antioxidant. This demonstrates the ability of blue wheat products and anthocyanin compounds to inhibit the oxidation of LDL cholesterol in vitro. Barley and barley milling fractions were also found to possess good inhibition effects against copper-induced human LDL cholesterol oxidation (*19, 32*).

Among blue wheat milling products, the bran fraction exhibited a greater inhibition capacity against oxidation of LDL as compared to the whole grain and white flour (**Table 4**). These differences were obvious during the first 4 and 8 h, where the rate of oxidation reaction was considerably high. With a longer incubation time, differences in inhibition capacity between bran and whole grain were insignificant. Anthocyanin powder and anthocyanin compounds were remarkably high in their ability to inhibit copper-induced human LDL cholesterol oxidation as compared to the blue wheat milling products (**Table 4**). For instance, anthocyanin powder exhibited inhibition capacity 25-fold higher than blue wheat bran and was comparable to that of the isolated anthocyanin compounds. Significant differences between anthocyanin compounds in inhibition capacity of LDL oxidation were also observed. Contrary to the free radical scavenging capacity, cyanidin-containing anthocyanins possessed lower inhibition capacity against LDL oxidation as compared to delphinidin-based anthocyanins. The inhibition of LDL oxidation reaction is based on hydrogen atom transfer that is different from DPPH or ABTS reaction (*22*). The aglycone delphinidin has a structure similar to cyanidin except that it contains one more hydroxyl group at the C5-position (**Figure 3**). This additional hydroxyl group would change the release of hydrogen ions and thus the hydration constant (pK_H). Hydration constants, color, and antioxidant properties of cyanidin-based anthocyanins were influenced by their structure, that is, site of glucosylation as well as type and degree of acylation with cinnamic acid (*33*). The present study demonstrated the presence of significant differences between the isolated blue wheat anthocyanin pigments in terms of free radicals scavenging capacity and inhibitory effects of oxidation of human LDL cholesterol in vitro.

DPPH and ABTS scavenging reaction and LDL oxidation reaction data clearly demonstrate that blue wheat anthocyanins are potent antioxidants. In addition, the composition of anthocyanins such as type of aglycone and sugar had a significant impact on antioxidant properties. Anthocyanins having different aglycones and sugar moieties were also found to have different responses in term of bioavailability and health effects (*34*−*36*). In addition, acylated anthocyanins also showed different stability and bioavailability as compared to nonacylated anthocyanins (*37*−*39*). Those studies demonstrate differences in anthocyanin characteristics in terms of antioxidant properties and bioavailability and thus their health effects. The current study has shown that blue wheat bran offers an anthocyanin-rich functional ingredient, which could be used as a functional natural colorant/antioxidant or could be processed further to produce anthocyanin powder and anthocyanin compounds as dietary supplements. The anthocyanin powder exhibited extremely high levels of anthocyanins and antioxidant capacity holding a promise for the development of natural colorants and antioxidants. However, more research on the stability of blue wheat anthocyanin powder and compounds is required.

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