JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Composition and Stability of Anthocyanins in Blue-Grained Wheat

EL-SAYED M. ABDEL-AAL*,† AND PIERRE HUCL‡

Food Research Program, Agriculture and Agri-Food Canada, Guelph, Ontario, Canada, and Department of Plant Sciences, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

Wheat grain is recognized as a good source of potentially health-enhancing components such as dietary fiber, phenolics, tocopherols, and carotenoids. Anthocyanins, another group of bioactive compounds, are found in blue and purple wheat grains. In the present study, a blue aleurone spring wheat line "Purendo 38" with relatively high content of total anthocyanins was used to investigate the composition and stability of anthocyanins over three crop years. Commercial cultivars of purple (Konini) and red (Katepwa) wheats were included in the study. Separation of anthocyanins by high-performance liquid chromatography (HPLC) showed that each wheat had a distinct anthocyanin profile. Four major anthocyanins were separated from blue wheat extracts as compared to five anthocyanins in purple wheat. Cyanidin 3-glucoside was the predominant anthocyanin in purple wheat, whereas it was the second major anthocyanin in blue wheat. The predominant anthocyanin in blue wheat, making up approximately 41% of the total anthocyanin content, remains to be structurally unidentified. Blue wheat anthocyanins were thermally most stable at pH 1. Their degradation was slightly lower at pH 3 as compared to pH 5. Increasing the temperature from 65 to 95 °C increased degradation of blue wheat anthocyanins. Addition of SO_2 during heating of blue wheat had a stabilizing effect on anthocyanin pigments. The optimal SO_2 concentrations were 500−1000 ppm for whole meals and 1000−3000 ppm for isolated anthocyanins. Further studies are underway to identify and verify individual anthocyanins in blue wheat and their potential end uses.

KEYWORDS: Blue wheat; purple wheat; anthocyanins composition; stability of anthocyanins

INTRODUCTION

Although pigments exist in wheat grains at very low concentrations, they substantially influence the quality of wheat products such as bread, pasta, and noodles and are used to distinguish the major market classes of wheat, i.e., red vs white wheats. The major pigments in red and amber durum wheats are xanthophylls, carotenoids, and flavones whereas blue and purple wheats primarily contain another group of pigments, the anthocyanins (*1*). These pigments are considered to be physiologically active components and/or health promoters since their role in promoting good health and reducing the risk of chronic diseases has been scientifically documented. For example, diets rich in lutein are associated with a reduced incidence of age-related macular degeneration and cataracts (*2*, *3*), reduced incidence of cancer (*4*), and better health (*5*, *6*). Flavonoids and phenolics were inversely associated with the risk of coronary heart disease (*7*), oxidation of low-density lipoprotein (LDL) and liposomes (*8*, *9*), and cancer (*10*). Anthocyanins were also found to inhibit oxidation of LDL and liposomes (*11*) and to prevent the risk of several chronic diseases (*12*). In this regard, wheat as a unique food ingredient and a staple food for a majority of the world's population can be bred and developed as a source of specific physiologically active and/or health-enhancing components. Recently, einkorn or durum was suggested as a potential candidate for use in the development of high-lutein wheat (*13*).

Anthocyanins are glycosides of polyhydroxy 2-phenylbenzopyrylium or flavylium salts, which are widespread in fruits and vegetables, ranging from 8 to 388 mg/100 g in red grapes (*14*). Anthocyanins were found at relatively high levels in blue wheat whole meals (16 mg/100 g) and brans (46 mg/100 g) (*1*). Barley, corn, rye, rice, sorghum, and millet also are anthocyanin-pigmented cereals (*15*). Grape skin and red cabbage are the only two concentrated sources of anthocyanin colorants (*16*). Because of the structure of grains and their multiple uses in food and nonfood industries, blue cereals may hold promise for the production of naturally colored foods, functional foods, natural colorants, nutraceuticals, and pharmaceuticals. For example, blue and/or purple corn is used for the production of naturally colored blue tortillas and is suggested as food colorants (*17*). Purple wheat is crushed into large pieces, which are spread over the exterior of multigrain breads (*18*). Nevertheless, little is known about anthocyanins in blue and purple cereals. The objectives of the present study were to identify and quantify

* To whom correspondence should be addressed. Tel: 519-829-2400 ext. 3111. Fax: 519-829-2600. E-mail: abdelaale@agr.gc.ca.
† Agriculture and Agri-Food Canada.
‡ University of Saskatchewan.

10.1021/jf021043x CCC: $25.00 © 2003 American Chemical Society
Published on Web 03/13/2003

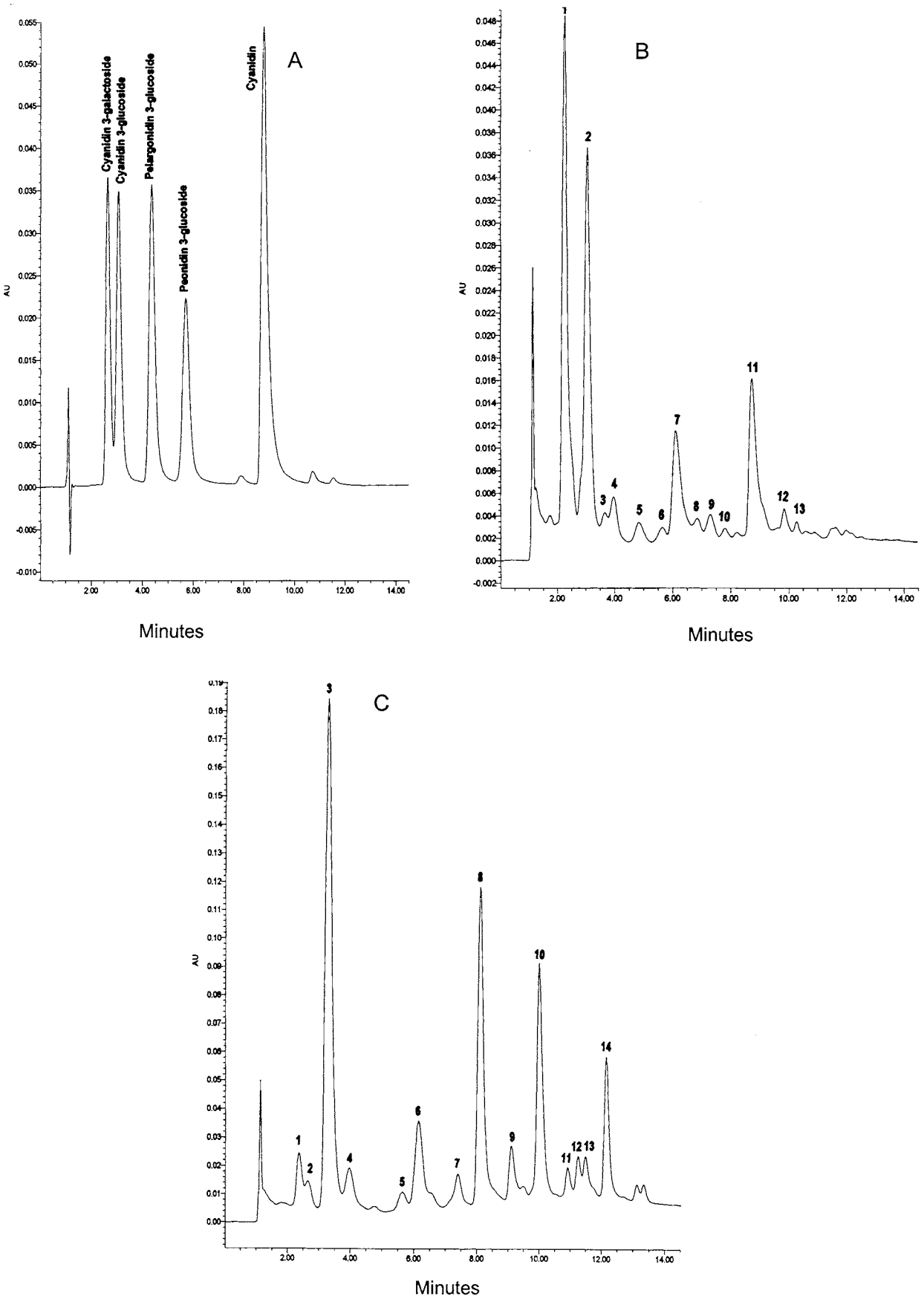

Figure 1. HPLC chromatograms of anthocyanins: (**A**) standard mixture, (**B**) blue wheat, and (**C**) purple wheat.

individual anthocyanins in a blue aleurone wheat cultivar and to investigate the stability of anthocyanins under different processing conditions, such as temperature, time, pH, and SO_2. Because of the stability of anthocyanin pigments at low pH, acidic blue wheat slurries (pH 1−5) were investigated. Characterization of anthocyanins was conducted on grains harvested

from three consecutive year crops. The composition of anthocyanins in blue wheat was compared with that of a commercial purple wheat cultivar.

MATERIALS AND METHODS

Wheat Grains. A composite sample of blue aleurone spring wheat (*Triticum aestivum* L. cv. Purendo 38) was obtained from selected locations in central Saskatchewan over 3 years (1996−1998). Hard red spring bread wheat (*T. aestivum* L. cv. Katepwa) and purple wheat (*T. aestivum* L. cv. Konini) were grown in the same field trials as checks. The composite sample from each year was ground on a Udy Cyclone sample mill (Udy Co., Fort Collins, CO) equipped with a 500 μm screen to produce whole meals. The grain samples were fractionated into flours and brans by tempering the grains for 18 h to 14.5% moisture and were milled on a Brabender Quadrumat Jr. Flour Mill (Brabender Co., South Hackensack, NJ). For efficient separation of flours, the milled grains were sifted on a 64 mesh sieve using a Ro-Tap sieve shaker (Tyler Co., Mentor, OH) for 3 min. The flour yields were approximately 72%, and the material retained on the sieve was considered bran.

Anthocyanin Analysis. Total anthocyanin content in wheat products was determined using the spectrophotometric method previously described (*1*). Anthocyanins were extracted with acidified methanol (methanol and 1.0 N HCl, 85:15, v/v). Extracts were centrifuged at 27 200*g* for 15 min. The extracts were refrigerated overnight and recentrifuged and then filtered through a 0.45 μm filter. The partially purified extracts were evaporated at 50 °C to dryness and redissolved in acidified methanol. Individual anthocyanins were separated and quantified by high-performance liquid chromatography (HPLC) equipped with a Waters 600 controller, 600 quaternary gradient pump, in-line degasser autosampler, dual wavelength UV/vis detector, and data acquisition system (Millennium Chromatography Manager version 2.15.01) (Waters, Mississauga, ON). A 25 cm × 4.6 mm i.d. reversed-phase Supelcosil LC-18-DB column (Waters) was used and operated at room temperature. The anthocyanins were eluted at 1 mL/min using a gradient system consisting of two solvents: (A) acidified methanol (methanol−0.1% HCl, 85:15, v/v) and (B) 10% formic acid. The gradient was programmed at 20:80 (A:B, v/v) for 1 min, then changed to 85:15 over 19 min, and then returned to the original solvent composition within 1 min. The separated compounds were subsequently detected and identified, at 520 nm, on the basis of chromatographic retention times and by coelution with added standards. Four pure anthocyanins (cyanidin 3-galactoside [ideain], cyanidin 3-glucoside [kuromanin], pelargonidin 3-glucoside [callistephin], and peonidin 3-glucoside) and one anthocyanidin (cyanidin chloride) (Extrasynthese, Genay, France) were used for calibration and quantification. Spiking of wheat extracts with pure anthocyanins was used to assist in identifying individual anthocyanins. Additional identification techniques such as liquid chromatography mass spectrometry (LC-MS) are underway to verify the identity of individual anthocyanins, especially because no data were previously reported on anthocyanins in blue wheat. The stock standard solutions were prepared in acidified methanol (pH 1) by weighing exactly 200−300 μg in 200 μL; 20 μL of stock solution was then diluted to 500 μL to prepare working standard solutions. A typical HPLC chromatogram for standard anthocyanin compounds is presented in **Figure 1A**. The standard anthocyanins exhibited a linear relationship with a HPLC peak area in a concentration range of 0.0−1.0 μg. The coefficient of determination (r^2) ranged from 0.9933 to 0.9998 for a mixture of pure anthocyanins, which were separated by HPLC using 5, 10, and 15 μL of working standard solution.

Stability Tests. The stability of blue wheat anthocyanins was determined on the isolated anthocyanins (20 ppm or 2 mg of anthocyanins in 100 mL) and on whole meals (10% slurries). Anthocyanins were extracted with acidified methanol as described above, and the partially purified extracts were freeze-dried following methanol removal by evaporation at 40 °C. A four-factor full-factorial experiment was designed to study the effects of temperature (65, 80, and 95 °C), time (0, 1, 2, 3, 4, 5, and 6 h), SO_2 (0, 500, 1000, 2000, and 3000 ppm) and pH (1, 3, and 5), giving a total of 315 treatment combinations for the isolated anthocyanin sample. In the case of wheat whole meal, only four concentrations of SO_2 (0, 500, 1000, and 2000 ppm) were studied, giving a total of 252 treatments. The samples were suspended in 0.1 M HCl−phosphate buffer at pH 1, 3, or 5. Each pH mixture was modified to contain 0, 500, 1000, 2000, or 3000 ppm SO_2. The samples were then incubated in a water bath maintained at 65, 80, or 95 °C for the desired period of time. After each treatment, the tubes were cooled to room temperature, readjusted to pH 1 to restore the pinkish color of anthocyanin residues, centrifuged, and filtered. The anthocyanin contents were monitored by reading the absorbance at 535 nm. For treatments heated at 95 °C for long time, i.e., 3 h and longer, the slurries were viscous and hard to filter, and more than one centrifugation was needed to produce clear filtrates.

Table 1. Total Anthocyanin Contents of Milled Products from Pigmented Wheats (mg/kg)[a]

wheat product	1996 crop	1997 crop	1998 crop	mean ± SD
		blue wheat		
whole meal	163.9a	139.3c	154.6b	152.2 ± 12.3
flour	23.1a	18.5b	21.4b	21.1 ± 2.5
bran	479.7a	415.9c	461.8b	452.9 ± 32.7
		purple wheat		
whole meal	153.3a	61.3b	63.9b	93.1 ± 52.2
flour	14.3a	3.1b	3.2b	6.9 ± 6.2
bran	383.2a	156.7b	166.1b	235.9 ± 127.8
		red wheat		
whole meal	5.3a	4.9a	5.0a	5.1 ± 0.2
flour	1.6a	1.5a	1.7a	1.6 ± 0.1
bran	10.1a	9.9a	10.3a	10.1 ± 0.2

[a] Means within a row followed by the same letter are not significantly different at $p > 0.05$.

Statistical Analysis. Data were subjected to analysis of variance using a general linear model with Minitab Statistical Software Release 12 (Minitab Inc., State College, PA).

RESULTS AND DISCUSSION

Composition of Anthocyanins. The blue aleurone wheat cv. Purendo contained higher concentrations of total anthocyanin as compared to the purple wheat cv. Konini (**Table 1**). This confirms our previous findings (*1*). Both wheats were grown under the same conditions. Significant differences were found in total anthocyanin contents from one year to the next, indicating significant environmental effects. These effects were much stronger in purple wheat than in blue wheat, which may, in part, be due to the different locations of the blue and purple pigments in the wheat grain. The purple pigments are located in the pericarp whereas the blue pigments are found in the aleurone layer (*19*). Blue and purple pigments were concentrated in roller-milled bran fractions. The flour fractions exhibited low levels of anthocyanins, presumably due to contamination with bran. Red wheats contained very low concentrations of total anthocyanin and related pigments, and no significant differences were observed among crop years.

No information is currently available on the types and concentrations of individual anthocyanins in blue wheat or other blue cereal grains. Anthocyanins in blue wheat samples obtained from three year crops were separated and quantified using reversed phase HPLC. Four major anthocyanins were separated from blue wheat in addition to trace concentrations of other anthocyanins (**Figure 1B**). The most common anthocyanin in blue wheat (41.2% of the total anthocyanins) was eluted earlier than the anthocyanin standards having a retention time of 2.3 min (**Table 2**). Cyanidin 3-glucoside was the second most predominant anthocyanin in blue wheat, averaging approximately 28.1% of total anthocyanins. The other two major anthocyanins were separated at retention times of 6.1 and 8.7

Table 2. Concentrations of Individual Anthocyanins in Blue Wheat (mg/kg)

peak no. or anthocyanin	1996 crop	1997 crop	1998 crop	mean ± SD
1	39.96	42.81	40.88	41.21 ± 1.46
2, cyanidin 3-glucoside	29.23	26.95	28.21	28.14 ± 1.15
3	2.64	2.77	2.44	2.61 ± 0.17
4	2.97	3.49	4.75	3.75 ± 0.91
5	1.22	1.27	1.12	1.21 ± 0.08
6, peonidin 3-glucoside	0.76	0.82	0.84	0.81 ± 0.05
7	13.75	14.28	13.96	14.01 ± 0.27
8	0.58	0.55	0.56	0.56 ± 0.02
9	0.92	0.87	0.88	0.89 ± 0.03
10	0.46	0.44	0.44	0.45 ± 0.02
11	11.34	12.34	12.22	11.98 ± 0.56
12	1.36	1.42	1.46	1.41 ± 0.06
13	0.74	0.81	0.79	0.78 ± 0.05
total	105.93	108.81	108.66	107.81

Table 3. Concentrations of Individual Anthocyanins in Purple Wheat (mg/kg)

peak no. or anthocyanin	1996 crop	1998 crop	mean ± SD
1	3.94	2.56	3.24 ± 0.97
2, cyanidin 3-galactoside	1.12	0.81	0.98 ± 0.21
3, cyanidin 3-glucoside	46.44	19.73	33.11 ± 18.85
4	5.08	1.78	3.43 ± 2.31
5	1.23	0.68	0.97 ± 0.37
6, peonidin 3-glucoside	6.85	2.36	4.61 ± 3.15
7	3.48	1.21	2.35 ± 1.59
8	28.03	9.77	18.93 ± 12.85
9	6.09	2.42	4.27 ± 2.57
10	21.13	7.33	14.23 ± 9.71
11	2.76	0.91	1.85 ± 1.28
12	2.91	1.12	2.01 ± 1.25
13	4.54	2.43	3.49 ± 1.48
14	8.41	3.72	6.09 ± 3.31
total	139.07	56.83	99.56

min, constituting 14.1 and 12.0% of the total anthocyanins, respectively. Peonidin 3-glucoside was found in trace amounts (0.8 mg/kg) in blue wheat. Spiking of samples with pure anthocyanins was used wherever possible to confirm identifications. However, because of the limited number of standard compounds available, most of the major anthocyanins could not be identified. Further research is underway to complete the

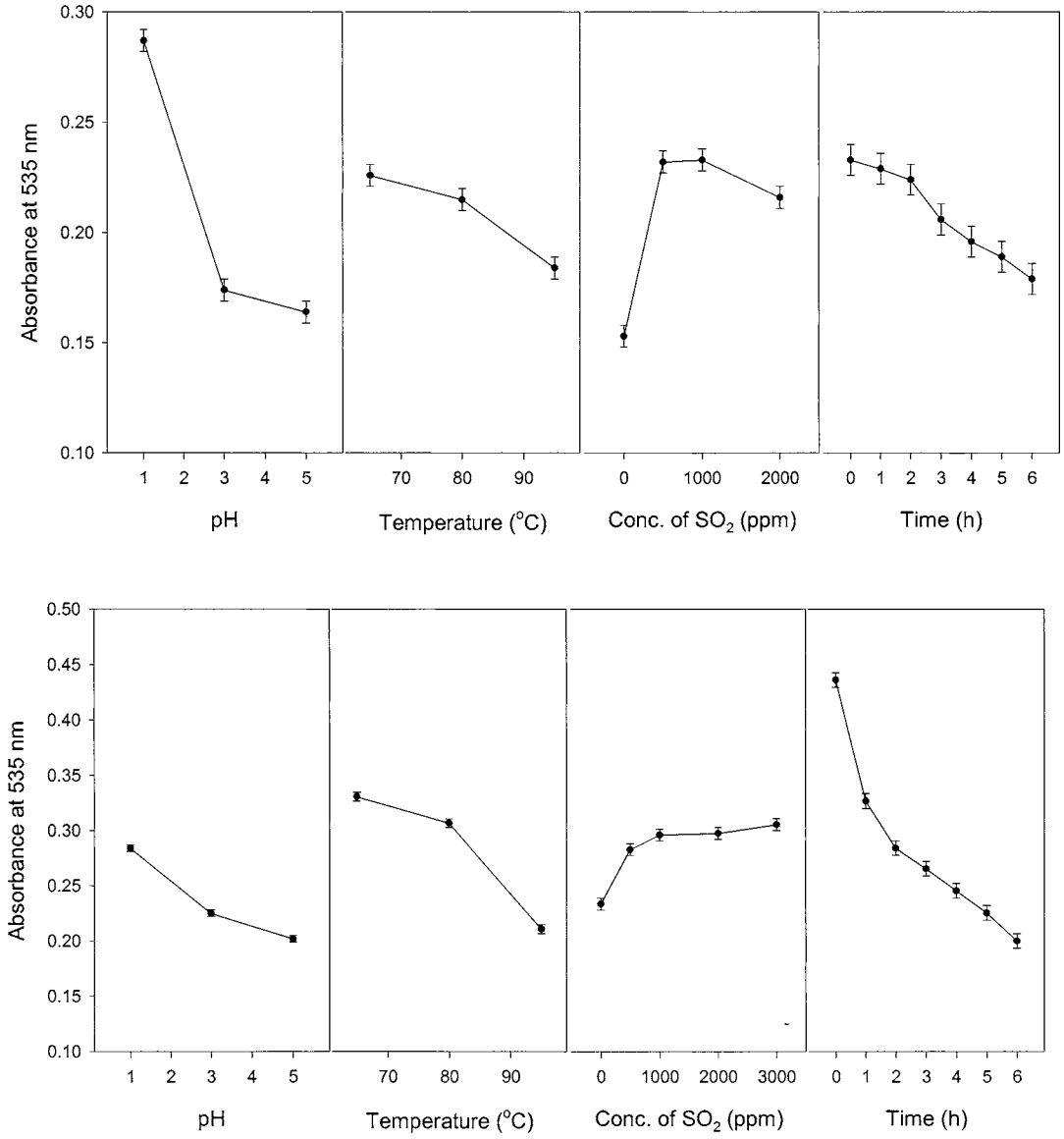

Figure 2. Main effects of pH, temperature, SO_2, and time on stability of blue wheat anthocyanins from whole meals (upper graphs) and isolated pigments (lower graphs).

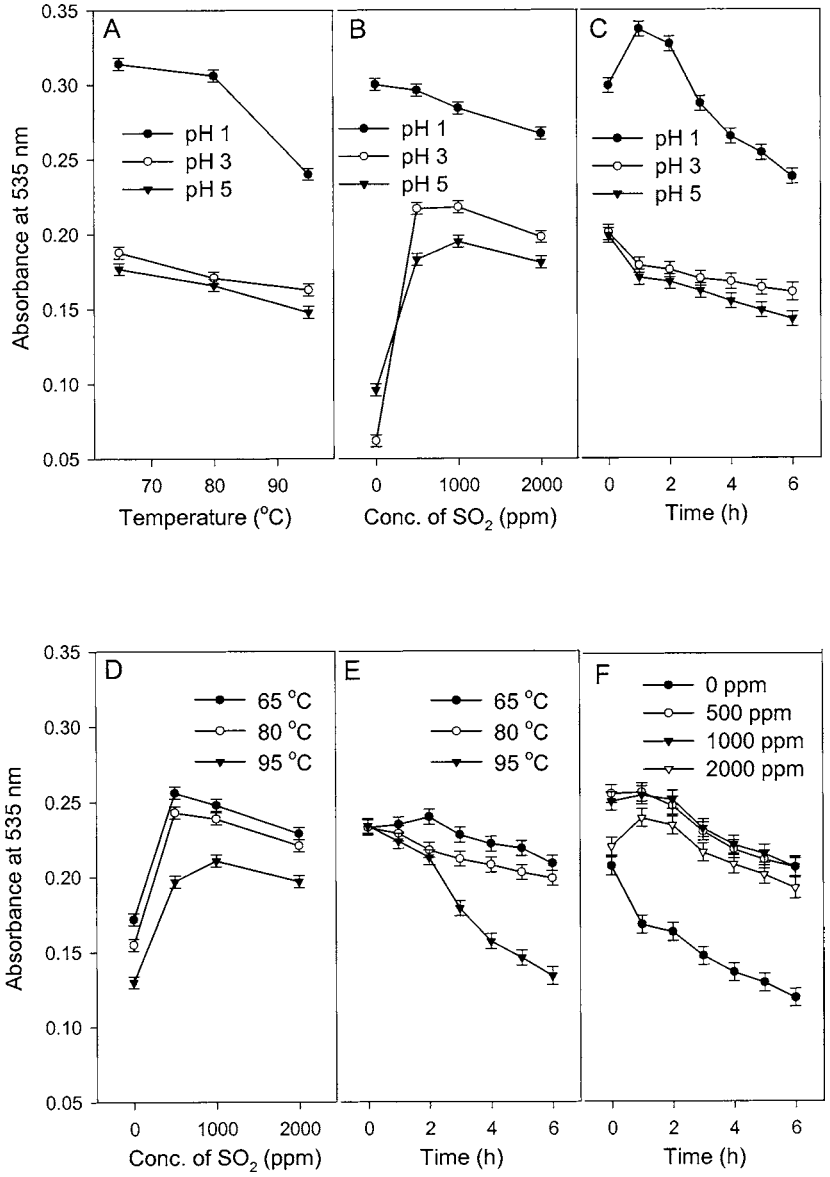

Figure 3. Interaction effects of pH, temperature, SO_2, and time on stability of anthocyanins in blue wheat whole meals.

characterization of anthocyanin pigments in blue wheat and related grains using more reliable identification analytical techniques such as LC-MS and NMR.

Because of severe environmental effects on anthocyanin contents in purple wheat in the 1997 and 1998 years, only the 1998 crop was used along with the 1996 crop for HPLC analysis. There were five major anthocyanins in purple wheat (**Figure 1C**). Cyanidin 3-glucoside was the most common anthocyanin in purple wheat, constituting up to 32.7% of the total anthocyanins in the 1996 crop and 34.7% in the 1998 crop (**Table 3**). Dedio et al. (*20*) reported that the major anthocyanins in the pericarp of the purple wheat line UM 606a were cyanidin 3-glucoside and peonidin 3-glucoside, with trace amounts of the corresponding rutinosides, as detected by paper chromatography using rhubarb and plum anthocyanins as standards. The second, third, and fourth most common anthocyanins were separated at retention times of 8.1, 10.0, and 12.2 min, respectively. The latter anthocyanins constituted 19.7, 14.9, and 5.9% of the total anthocyanins in the 1996 crop and 17.2, 12.9, and 6.5% in the 1998 crop. Peonidin 3-glucoside was the fifth most predominant anthocyanin in purple wheat, accounting for 4.8% of the total anthocyanins in the 1996 crop and 4.2% in the 1998 crop. Trace amounts of other anthocyanins were also observed in purple wheat. The results indicate that the composition of anthocyanins in blue wheat differs from that in purple wheat and may also indicate that the anthocyanin profile might be characteristic of a particular grain tissue, i.e., pericarp or aleurone.

Stability of Anthocyanins. The tested variables and their interactions showed highly significant effects on the stability of anthocyanins, either in whole meals or as isolated pigments, except for the interaction of pH and time in the case of isolated anthocyanins. The main effect of pH indicates that blue wheat anthocyanins, either in whole meal or in isolated form, were thermally most stable at pH 1, and their degradation was insignificantly lower at pH 3 as compared to pH 5 (**Figure 2**). Color and stability of anthocyanins were mainly dependent on the pH of the anthocyanin-containing products (*21*). As the temperature was increased from 65 to 95 °C, degradation of blue wheat anthocyanins increased, as indicated by the reduction in absorbance readings. Mok and Hettiarachchy (*22*) reported that thermal degradation of sunflower hull anthocyanins followed first-order kinetics with an average activation energy of 23.1 kcal/mol. Addition of sulfur dioxide during heating of blue

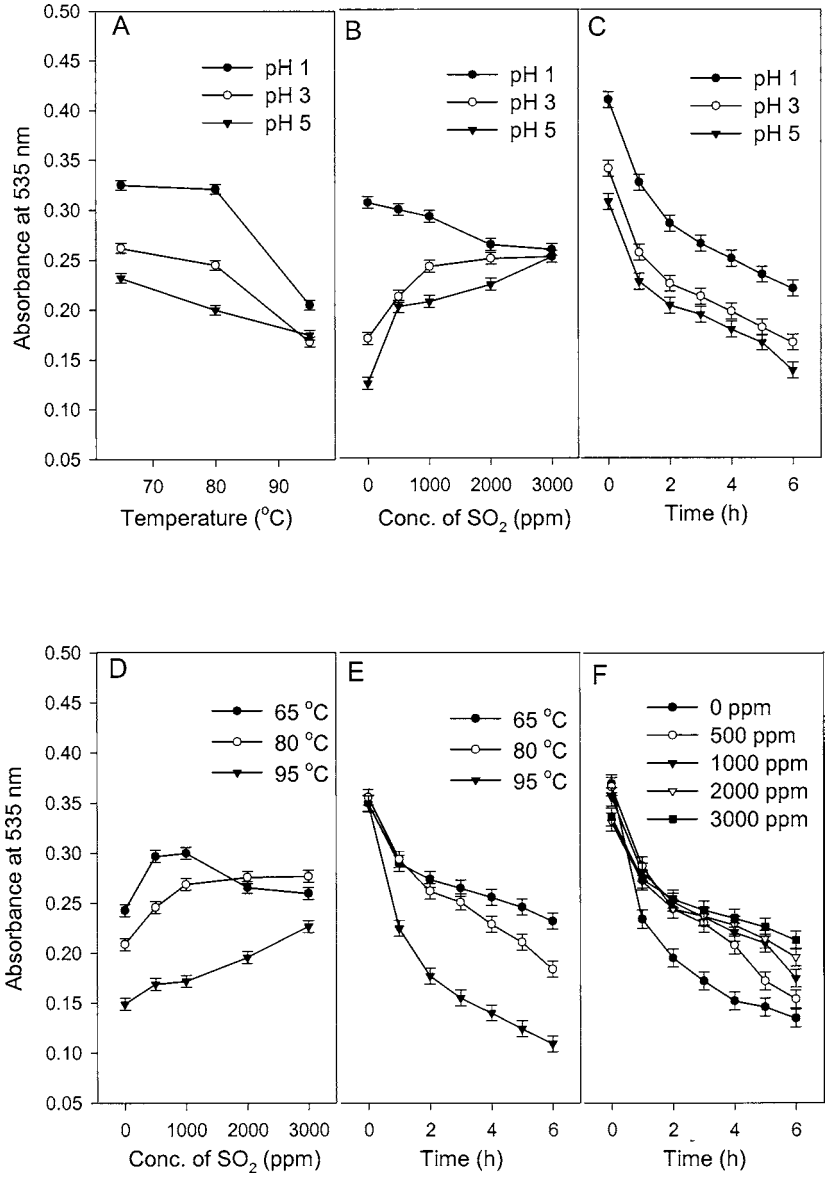

Figure 4. Interaction effects of pH, temperature, SO_2, and time on stability of the isolated anthocyanins from blue wheat.

wheat had a stabilizing effect on anthocyanin pigments as compared to the control sample or heating without sulfur dioxide. In the case of blue wheat whole meal, there were no significant differences in stabilizing effect between 500 and 1000 ppm SO_2, whereas addition of 2000 ppm SO_2 resulted in reduced stability. The reason that anthocyanin at 2000 ppm SO_2 was less stable might be attributed to an irreversible bleaching effect of SO_2 at high concentrations. However, in the case of the isolated anthocyanins, the optimal SO_2 concentration was 1000−3000 ppm. Anthocyanin degradation occurred at different rates during the course of experiment time (6 h). For whole meal, a slow rate occurred during the first few hours of heating followed by sharp degradation during the last 3 h. In the case of the isolated anthocyanins, a more rapid degradation (sharper slope) was observed (**Figure 2**).

The interaction effects indicate that the influence of temperature on color degradation of blue wheat slurries was dependent upon the pH of the slurry being greater at pH 1 as compared with pH 3 or 5, particularly for the samples heated at 95 °C (**Figure 3A**). Addition of sulfur dioxide also showed different effects depending upon slurry pH (**Figure 3B**). At pH 3 and 5, a noticeable increase in color occurred, whereas at pH 1 there was a slight decrease in pigment content. This increase in pigment content suggests that anthocyanin extraction continued during the first hour of heating. The presence of SO_2 in the extracting medium improved extractability and stability of anthocyanins (*16*). The effect of time was also dependent on the pH of slurry, with pH 1 having a distinct effect as compared with pH 3 or 5 (**Figure 3C**). The interaction effects of SO_2 and temperature indicate that degradation of blue wheat pigments in whole meals heated at 65, 80, or 95 °C was diminished by addition of 500−1000 ppm SO_2, whereas at 2000 ppm the stabilizing effect was reduced but stabilizing was still much greater than in the control sample (0.0 ppm SO_2) (**Figure 3D**). The effect of time on degradation of anthocyanins in blue wheat slurries heated at 95 °C was much greater than in those heated at 65 or 80 °C (**Figure 3E**), and the rate of color degradation was significantly reduced over time with the addition of SO_2, particularly at 500 and 1000 ppm (**Figure 3F**).

In the case of isolated anthocyanins, the interaction effects of temperature and SO_2 with pH were also dependent on pH (**Figure 4A**,**B**), whereas time had similar effects at different pH values (**Figure 4C**). Addition of SO_2 improved the stability of anthocyanin pigments at the three temperatures tested (**Figure**

4D). Furthermore, slight differences in pigment stability were observed between heating at 65 and 80 °C, especially at high concentrations of SO_2 (2000 and 3000 ppm). Adding 3000 ppm of SO_2 had a stabilizing effect on the pigments at the three temperatures. On the other hand, degradation of anthocyanins over the course of the experiment (up to 6 h) occurred at a higher rate at 95 °C as compared to 80 or 65 °C (**Figure 4E**). The interaction effect between time and SO_2 indicates that addition of SO_2 reduced the rate of anthocyanin degradation as compared with the control sample (no SO_2 added) (**Figure 4F**). This effect was much higher at the higher concentrations of SO_2 (2000 and 3000 ppm).

In general, blue wheat could provide a naturally colored and/or functional food ingredient for the cereal industry based on its anthocyanin content. The anthocyanin profile in blue wheat was distinct from that of purple wheat, which may reflect diverse stabilities and characteristics. Addition of sulfur dioxide and maintaining a given pH during processing of blue wheat whole meals or of the isolated anthocyanins is crucial for stabilization and color expression of the pigments. Blue wheat exhibited a reduced effect of growing environment on anthocyanin content as compared to purple wheat, perhaps due to the location of the anthocyanins in the aleurone as opposed to the pericarp. Further quantification and identification of individual anthocyanins in blue wheat and other blue cereals would facilitate the use of these grains as natural colorants and functional food ingredients.

ACKNOWLEDGMENT

We are grateful for the technical assistance provided by N.-Y. Jai, H. Braitenbach, and M. Grieman.

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Received for review October 14, 2002. Revised manuscript received January 24, 2003. Accepted January 31, 2003. The financial support of the Saskatchewan Agriculture Development Fund is greatly appreciated.

JF021043X