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# Comparison of Antioxidant Activities of Different Colored Wheat Grains and Analysis of Phenolic Compounds

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Extracts from six wheat varieties (three purple, one yellow, two red, and one white) were evaluated and compared for their antioxidant capacities against oxygen radical and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. Phenolic composition in the extracts was examined by high-performance liquid chromatography and mass spectrometry. The results showed that Charcoal purple wheat had remarkable antioxidant activity (up to 6899 $\mu$mol/100 g) followed by Red Fife wheat and yellow Luteus wheat. White AC Vista wheat, due to its lowest phenolic content, exhibited the weakest antioxidant property. The major phenolic composition identified in wheat grains consisted of phenolic acids, flavones, flavonols, and anthocyanins. The former three components were detected in all of the wheat varieties, whereas anthocyanins were identified only in purple wheat. Therefore, anthocyanins could be the major compounds distinguishing purple wheats from other colored wheats with high antioxidant activity.

**KEYWORDS: Colored wheat grain; antioxidant activity; phenolic acid; flavonoid; anthocyanin**

## INTRODUCTION

Wheat is a crop that is cultivated worldwide and consumed as a staple food in many countries. On the market, most wheats are white- or red- grained. Some novel wheat varieties such as purple and blue wheat grains are also commercially available, but in limited amounts. As an important food ingredient, wheat grain is usually ground into flour to make bread, cake, pasta, noodle, or other food products. In recent years, whole grain food products are more favorable than those made from refined grain flour because they have been reported to lower the incidence of chronic diseases such as type II diabetes, cardiovascular diseases, and some cancers (*1*). These health benefits have been partly attributed to the unique phytochemicals present in whole grain (*1*). Therefore, the full characterization of these health-benefiting substances in wheat grain is important for the agricultural industry in breeding and developing value-added wheat containing enhanced nutritional and health-promoting compounds.

Phenolic compounds are among phytochemicals in cereal grains that are actively being investigated as natural antioxidants. The major phenolics reported in wheat are phenolic acids and flavonoids. Although these compounds exist at low concentration, they substantially influence the quality of wheat. The antioxidant activities of different wheat grains and their phenolic profiles have been investigated by several research groups (*2*−*4*). However, most of them are focused on white or red wheat varieties. To our knowledge, there is no report to compare the antioxidant properties of purple, red, yellow, and white wheat grains. Furthermore, the inherent varietal differences in phenolic compounds between different colored wheats have not yet been reported. Thus, the objectives of this study were to compare the antioxidant activities of purple, red, yellow, and white wheat grains and to qualitatively and quantitatively analyze phenolic acids and flavonoids in different colored wheat grains.

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## MATERIALS AND METHODS

**Chemicals and Standards.** Analytical grade acetic acid used in the extraction and Folin−Ciocalteu reagent were purchased from Fisher Scientific (Pittsburgh, PA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH), fluorescein, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2- carboxylic acid (Trolox), catechin, and phenolic acid standards were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). HPLC grade acetone and methanol were used in the extraction. MS grade water, acetonitrile, and acetic acid were used in LC-MS analysis. All of the HPLC grade and MS grade solvents were purchased from Sigma-Aldrich Chemical Co.

**Sample Description and Preparation.** Six different colored bread wheat (*Triticum aestivum*) grains were used in this study including three purple wheats (Charcoal, Indigo, and Konini), one red wheat (Red Fife), one yellow wheat (Luteus), and one white wheat (AC Vista). The wheat varieties were grown under the same organic conditions in 2008. Among them, the three purple wheats, Charcoal, Indigo, and Konini, were grown in the same location at the Danish Agricultural Museum, Gl. Estrup, in Jutland, Denmark (latitute N 56° 26.296′, longitude E 010° 20.522′). The other three wheats were grown at Mørdrupgård, a farm in Lynge north of Copenhagen, Denmark (latitude N 55° 49.530′, longitude E 012° 13.587′). Before analysis, whole grain samples were ground into a fine powder by using an ultracentrifugal mill (Retsch Inc., Haan, Germany) equipped with an 80 mesh sieve (0.5 mm) and stored at −20 °C before extraction. The moisture contents of whole wheat


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flour were determined by AOAC (2003) official method 925.10 (*5*) before extraction.

**Extraction of Soluble Phenolic Compounds.** The soluble phenolic compounds were extracted with two solvent systems. Methanol/1 M HCl (85:15, v/v) was used to extract anthocyanins because anthocyanins are more stable and extractable when the pH $< 2$ (*6*). Aqueous acetone (70%) was used to recover proanthocyanidins and other flavonoids due to its strong ability to break hydrogen bonds, which provides it great extraction efficiency toward polymers (*7*). Whole grain flour (1 g) was extracted with 15 mL of acidic methanol or aqueous acetone on a mechanical shaker (Artisian Scientific, Champaign, IL) for 2 h at room temperature. The mixture was then centrifuged at 5000 rpm and 15 °C (Mandel, Guelph, ON, Canada) for 30 min. The crude methanol and acetone extracts were used for the determination of antioxidant capacity. The remaining supernatant was concentrated under vacuum to dryness and redissolved in 4 mL of 80% MeOH used for the HPLC analysis.

**Extraction of Insoluble Phenolic Compounds.** The extraction of insoluble phenolic compounds was based on the published method reported by Holtekjlen et al. (*8*) with minor modifications. Whole wheat flour (0.5 g) was extracted twice with 80% methanol to remove free and soluble conjugated phenolics. The residue was then subjected to alkaline hydrolysis to recover insoluble phenolic compounds. NaOH (2 N, 15 mL) was added to the residue and hydrolyzed for 2 h at room temperature. The suspension was then centrifuged for 45 min at 12000 rpm (Mandel). The supernatant was adjusted to pH 2−3 by using 6 N HCl and extracted twice with 15 mL of hexane to remove the lipids. The phenolic acids were then extracted using ethyl acetate (15 mL × 3). The extracts were combined and dried under vacuum. The residue was redissolved in 4 mL of 50% MeOH and filtered through a 0.22 $\mu$m filter before the LC analysis. The phenolic acids were analyzed by HPLC-Q-TOF-MS.

**DPPH Radical Scavenging Capacity Assay.** The DPPH radical scavenging capacity of crude methanol and acetone extracts was determined according to the method reported by Brand-Williams et al. (*9*) with minor modifications. Briefly, 100 $\mu$L of wheat extract was added to 3.9 mL of freshly prepared DPPH (60 $\mu$M) radical solution. After 60 min of reaction at room temperature, the absorbance of the solution was measured at 515 nm. DPPH radical scavenging capacity was expressed as micromoles of Trolox equivalents per 100 g of wheat grain on a dry weight basis by using Trolox as standard.

**Oxygen Radical Absorbance Capacity (ORAC) Assay.** The ORAC of crude methanol and acetone extracts was determined according to the protocol described by Huang et al. (*10*) with some modifications. A Precision 2000 automated microplate pipetting system (Bio-Tek Instruments Inc., Winooski, VT) was used for plate-to-plate transfer of solutions. An FL×800 microplate fluorescence reader (Bio-Tek Instruments Inc.) controlled by software KC4 3.0 (version 29) was used with fluorescence filters for an excitation wavelength of 485 nm and an emission wavelength of 528 nm. The final assay solution contained 120 $\mu$L of fluorescein, 60 mM AAPH, and 20 $\mu$L of wheat extract or 20 $\mu$L of buffer solution for a reagent blank. The fluorescence of an assay mixture was determined and recorded every minute. Trolox was used as a standard control, and the final results were expressed as micromoles of Trolox equivalents per 100 g of wheat grain on a dry weight basis.

**Determination of Total Phenolic Content.** The Folin−Ciocalteu method was used to determine the total phenolic contents in acidic methanol and aqueous acetone extracts for each sample (*11*). Briefly, 200 $\mu$L of appropriately diluted wheat extract was added to 1.9 mL of 10-fold freshly diluted Folin−Ciocalteu reagent. After 5 min, 1.9 mL of sodium carbonate solution (60 g/L) was added to the mixture and incubated at room temperature for 90 min. The absorbance of the mixture was measured at 725 nm against a blank. Ferulic acid was used as standard to produce the calibration curve. The mean of three readings was used, and the total phenolic content was expressed as milligrams of ferulic acid equivalents per 100 g of wheat grain on a dry weight basis.

**Determination of Total Flavonoid Content.** Total flavonoid content of wheat grain was measured for the aqueous acetone extract by a modified vanillin method (*12*). Vanillin (1%, g/mL) in glacial acetic acid and HCl (8%, g/mL) in glacial acetic acid were prepared and stored at 4 °C. One part of 1% vanillin was freshly mixed with one part of 8% HCl as working reagent just before the analysis. One milliliter of the crude aqueous acetone extract was mixed with 6 mL of working reagent and then incubated at 30 °C for 30 min. The solution absorbance was then measured at 510 nm. Catechin was used as standard, and flavonoid content was expressed as milligrams of catechin equivalents per 100 g of wheat grain on a dry weight basis.

**Determination of Total Anthocyanin Content.** Total anthocyanin content of wheat grain was measured on acidic methanol extracts using the method reported by Abdel-Aal et al. (*6*) with minor modifications. Briefly, acidic methanol (methanol and 1.0 N HCl, 85:15, v/v) was used to extract the anthocyanins at a sample to solvent ratio of 1:15. The absorbance at 535 nm was measured without further dilution of the crude extracts. Cyanidin-3-glucoside was used as standard. Total anthocyanin content of each sample was calculated as

$$(A \times \mathrm{MW} \times 10^3) \times 0.015 \times 100/\varepsilon/\mathrm{Wt}$$

where $A$ is absorbance reading, MW is molecular weight of cyanidin-3-glucoside (449.2 g/mol), $\varepsilon$ is molar absorptivity of cyanidin-3-glucoside (25965/cm/M), and Wt is dry weight of the ground grain sample. The results were expressed as milligrams of cyanidin-3-glucoside equivalents per 100 g of wheat grain on a dry weight basis.

**HPLC-Q-TOF-MS Analysis.** The chromatographic separation of phenolic compounds was performed on a Waters 2695 HPLC system equipped with a photodiode array detector (PDA) (Waters 996) and autosampler (717 plus, Waters). The analytical column was a 150 mm × 4.6 mm i.d., 5 $\mu$m, Gemini RP-18 column (Phenomenex, Torrance, CA). To separate different phenolic compounds, three gradients were programmed consisting of different mobile phases. The anthocyanin composition was eluted with a gradient mobile consisting of (A) water with 1.0% formic acid and (B) acetonitrile with 1.0% formic acid at 0.5 mL/min. A linear gradient was programmed as follows: 0−5 min, 5−10% B; 5−15 min, 10−15% B; 15−20 min, 15−20% B; 20−30 min, 20−25% B; 30−40 min, 25−40% B; 40−45 min, 40−10% B; 45−50 min, 10% B. The same gradient was used to separate flavonoid compounds, but with a different mobile phase comprising (A) water with 0.1% acetic acid and (B) acetonitrile with 0.1% acetic acid. Phenolic acids were separated by a linear gradient, which was programmed as follows: 0−7 min, 15−20% B; 7−8 min, 20−15% B; 8−20 min, 15% B; 20−21 min, 15−24% B; 21−33 min, 24% B; 33−34 min, 24−13% B; 34−36 min, 13% B; 36−37 min, 13−20% B; 37−45 min, 20% B; 45−46 min, 20−42% B; 46−62 min, 42% B, and finally bringing the mobile phase to 100% B in 1 min followed by 6 min of equilibration. The mobile phase consisted of (A) water with 0.1% acetic acid and (B) methanol with 0.1% acetic acid at a flow rate of 0.7 mL/min. A T-split was used to reduce the flow before the sample was introduced to the mass spectrometer.

Before the MS analysis, the quadrupole time-of-flight (Q-TOF) mass analyzer was calibrated using the NaI standard in negative mode, and a resolution of 5000 was achieved. Full mass spectra were recorded in the range of $m/z$ 100−2000 in negative mode by using a capillary voltage of 2.0 kV and cone voltage of 40 V. The flow rates of desolvation gas ($N_2$) and cone gas ($N_2$) were 900 and 50 L/h, respectively. The desolvation gas temperature and the ion source temperature were set at 350 and 150 °C, respectively.

**Statistical Analysis.** The results were reported as mean ± standard deviation (SD) for triplicate analyses. The statistical calculation employed a one-way analysis of variance (ANOVA) test by using SAS version 9.1 (SAS Institute Inc., Cary, NC). Least significant differences (LSD) at $p < 0.05$ were tested to assess significant differences among the colored wheats.

## RESULTS AND DISCUSSION

**Total Phenolic Contents of Wheat Grains and Their Antioxidant Activities.** Phenolic compounds (phenolics) are the most effective antioxidants in fruits, vegetables, and cereals (*13*). Thereby, measurement of total phenolic contents in wheat grains tends to be important, typically in the assessment of their overall contribution to the total antioxidant capacity of wheat.

The total phenolic contents of crude acidic methanol and acetone extracts from different colored wheat grains are shown in **Figure 1a**, expressed as milligrams of of ferulic acid equivalents per 100 g of grain. Acidic methanol extracts showed significantly higher total phenolic contents than aqueous acetone extracts,

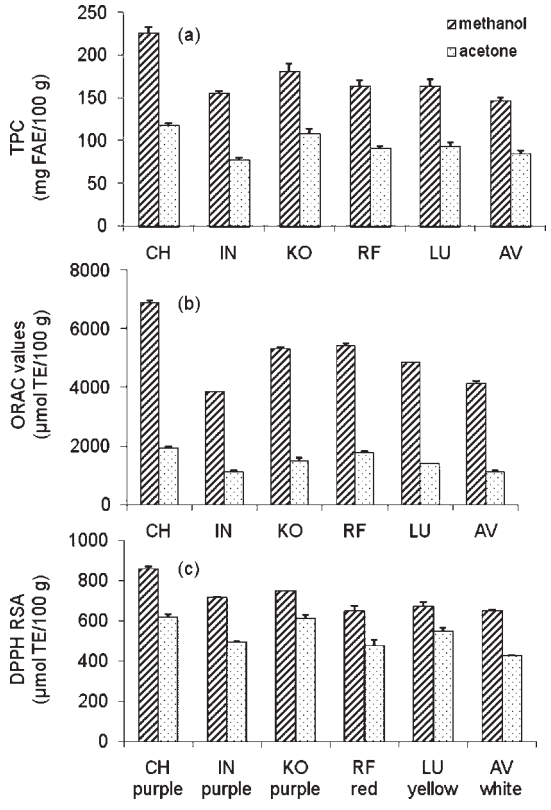


**Figure 1.** (**a**) Total phenolic contents (TPC), (**b**) oxygen radical absorbance capacities (ORAC), and (**c**) DPPH radical scavenging activities (RSA) of different wheat grains: CH, Charcoal; IN, Indigo; KO, Konini; RF, Red Fife; LU, luteus; AV, AC Vista.

indicating that the former solvent may recover more phenolic components from wheat grains. The total phenolic contents of purple, red, yellow, and white wheat grains varied from 146 to 226 mg/100 g in methanol extracts and from 77 to 177 mg/100 g in acetone extracts. Among the samples, purple wheat (Charcoal and Konini) had the highest and second highest phenolic contents, followed by red (Red Fife) and yellow (Luteus) wheat. Indigo, as one of the purple wheat samples, unexpectedly displayed relatively low level of phenolic content. This can be mainly attributed to cultivar because the environmental effects were eliminated by growing all the purple wheats at the same location and under the same environment. The total phenolic content of white wheat (AC Vista) ranked behind the other wheat samples. Significant differences in total phenolic contents were detected between different colored wheat grains, indicating that these wheat grains may exhibit different levels of antioxidant activities.

To distinguish the antioxidant properties of different colored wheat grains, several assays were applied. Oxygen radical absorbance capacity (ORAC) assay, due to its biological relevance to in vivo antioxidant efficacy, is one of the most widely used methods to investigate the antioxidant effects of various foods, including cereals. It measures the antioxidant capacity against peroxyl radicals. As seen in **Figure 1b**, the acidic methanol extracts exhibited approximately 3.5 times higher values than aqueous acetone extracts. Although three purple wheat grains displayed significantly different levels of ORAC, on average the values were 5365 $\mu$mol/100 g in their methanol extracts and 1515 $\mu$mol/100 g in acetone extracts. Charcoal purple wheat showed the highest ORAC value, up to 6898 $\mu$mol/100 g in the methanol extract and 1920 $\mu$mol/100 g in the acetone extract. Red wheat (Red Fife) and purple wheats, on average, had ORAC values that were comparable. The ORAC value of yellow wheat (Luteus) was relatively lower than those of purple and red wheats but higher than that of white wheat grain.

Free radical scavenging activity against DPPH radical assay is another method widely used to evaluate the antioxidant activity of plant extracts, although it is focused on hydrogen-donating antioxidants against nitrogen radicals (*9*). Consistent with ORAC values, the acidic methanol extracts exhibited higher DPPH radical scavenging activity than acetone extracts, but with smaller differences (1.2−1.5-fold). Purple wheats still exhibited significantly higher antioxidant capacities than other wheats (**Figure 1c**). In methanol extracts, the DPPH radical scavenging activity of three purple wheats ranged from 715 to 857 $\mu$mol/100 g, with an average value of 773 $\mu$mol/100 g. Yellow wheat (669 $\mu$mol/100 g) showed slightly higher DPPH radical scavenging activity than red wheat (648 $\mu$mol/100 g). This could be due to the higher total flavonoid content (13.44 mg/100 g) than that of Red Fife wheat (10.72 mg/100 g). The lowest DPPH radical scavenging activity was observed in the acetone extract of white wheat, AC Vista (423 $\mu$mol/100 g).

From both assays, significant differences in antioxidant capacity were observed between purple, red, yellow, and white wheat grains. The highest and lowest values were, respectively, found in purple (Charcoal) and white (AC Vista) wheat, indicating that consumption of purple wheat may provide more health benefits than white wheat. To further specify the antioxidants, high-performance liquid chromatography and quadrupole time-of-flight mass spectrometry (HPLC-Q-TOF-MS) was applied in the analysis of phenolic compounds in wheat grains.

**Analysis of Phenolic Compounds in Wheat Grain.** Phenolics are secondary plant metabolites that are widely dispersed throughout the plant in seeds, leaves, roots, and stems. According to their structural complexity, phenolics can be simply divided into two groups, namely, simple phenols and polyphenols (*14*). The simple classes contain only one phenol subunit such as phenolic acids, whereas polyphenols comprise two or more phenol subunits and mainly include flavonoids. Therefore, phenolic acids and flavonoids were the major phenolics analyzed in this study.

**Phenolic Acid Composition.** In cereal grains, the majority of phenolic acids are esterified to the cell wall and present in insoluble bound form. Only a small portion exists as free phenolic acids. Therefore, the insoluble phenolic acids were recovered through alkaline hydrolysis.

The identification of monomeric phenolic acids was accomplished by comparison of UV spectra and retention times with external standards. Further confirmation was made by using $m/z$ values acquired in mass spectra. Five simple phenolic acids were observed in the wheat grains. They were respectively eluted at 21.60, 25.13, 34.73, 44.13, and 50.03 min, representing vanillic acid, caffeic acid, *p*-coumaric acid, ferulic acid, and sinapic acid. To quantify individual phenolic acids, the monitor wavelength was set at 310 nm for caffeic acid, *p*-coumaric acid, ferulic acid, and sinapic acid. Due to the maximum absorption of vanillic acid at 260 and 292 nm, the quantification of this acid was conducted at 292 nm. **Table 1** summarizes the content of each phenolic acid in different colored wheat grains. Ferulic acid as reported before (*15*) was found as the most dominant phenolic acid in all of the wheat samples (74−87 mg/100 g), constituting up to 79% of the total phenolic acids. Vanillic, *p*-coumaric, and sinapic acids were found in moderate levels (about 2 mg/100 g), whereas caffeic acid was present in the least amount (< 1 mg/100 g). Purple wheat, compared with other colored wheat grains, was distinguished by higher contents of vanillic acid (> 2.58 mg/100 g) and ferulic acid (> 81.38 mg/100 g). Red and yellow wheat grains contained significantly higher concentrations of *p*-coumaric acid than other wheats. White wheat, although containing comparable vanillic acid, caffeic acid, and *p*-coumaric acid as red and yellow

**Table 1.** Contents of Identified Phenolic Acids in Tested Purple, Red, Yellow, and White Wheat Grains[a]

| | monomeric phenolic acid (mg/100 g) | | | | |
|---|---|---|---|---|---|
| variety | vanillic | caffeic | *p*-coumaric | ferulic | sinapic |
| Charcoal | 3.35 ± 0.74ab | 0.95 ± 0.07bc | 2.87 ± 0.12a | 87.37 ± 1.42a | 3.04 ± 0.08a |
| Indigo | 3.21 ± 0.65ab | 0.84 ± 0.15dc | 2.12 ± 0.20a | 86.59 ± 0.42a | 2.36 ± 0.11b |
| Konini | 2.58 ± 0.64bc | 1.40 ± 0.14a | 2.38 ± 0.26a | 81.38 ± 1.70b | 1.93 ± 0.21c |
| Red Fife | 1.34 ± 0.60d | 0.99 ± 0.22bc | 3.56 ± 0.32a | 69.31 ± 0.04d | 2.40 ± 0.17b |
| Luteus | 1.34 ± 0.62d | 1.07 ± 0.17abc | 3.31 ± 0.51e | 80.98 ± 1.34b | 1.39 ± 0.09d |
| AC Vista | 1.32 ± 0.22d | 0.83 ± 0.17c | 2.73 ± 0.41a | 74.35 ± 0.14c | 0.70 ± 0.23e |

| | diferulic acid (mg/100 g) | | |
|---|---|---|---|
| variety | 5-5′ | 8-*O*-4′ | 8-5′ |
| Charcoal | 1.13 ± 0.15b | 6.62 ± 0.61a | 9.09 ± 0.44b |
| Indigo | 0.89 ± 0.18 cd | 5.31 ± 0.99b | 8.26 ± 0.25c |
| Konini | 1.62 ± 0.26ab | 6.57 ± 0.81a | 11.7 ± 0.31a |
| Red Fife | 0.80 ± 0.15d | 5.08 ± 0.62b | 8.87 ± 1.26c |
| Luteus | 0.92 ± 0.05c | 4.70 ± 0.99c | 12.7 ± 0.96a |
| AC Vista | 0.88 ± 0.10d | 3.26 ± 1.06d | 9.73 ± 0.47b |

[a] The response factors of 5-5′, 8-*O*-4′, and 8-5′ diferulic acids are 0.21, 0.14, and 0.12, respectively. Means in the same column with different letters are significantly different.

**Table 2.** Total Flavonoid Content and Total Anthocyanidin Content in Different Colored Wheat Grains[a]

| grain color | variety | total flavonoid content | total anthocyanidin content (color of extract) |
|---|---|---|---|
| purple | Charcoal | 102.95 ± 1.15a | 23.45 ± 0.06a (purple-red) |
| | Indigo | 35.79 ± 0.25b | 7.24 ± 0.04b (red) |
| | Konini | 21.59 ± 0.58c | 2.54 ± 0.04c (pink) |
| red | Red Fife | 10.72 ± 0.84de | 0.96 ± 0.06d (yellow) |
| yellow | Luteus | 13.44 ± 0.76d | 0.95 ± 0.03d (yellow) |
| white | AC Vista | 9.60 ± 0.43e | 0.77 ± 0.03d (yellow) |

[a] TFC and TAC are, respectively, expressed as mg of catechin equivalents and cyanidin-3-glucoside equivalents/100 g of wheat grain (dry weight basis). Means in the same column with different letters are significantly different.

wheat grains, had a significantly lower level of sinapic acid. Hence, these substantial differences of phenolic acid content between white wheat and other colored wheat grains can mainly be attributed to ferulic acid and sinapic acid.

Besides the above five monomeric phenolic acids, three phenolic acid dehydrodimers were also detected in this study. Ferulate dehydrodimers also referred to as diferulic acids, are the most common dimeric phenolic acids observed in cereal grains. They are abundant in the cell wall and occur only in the insoluble fraction (*16*). By plotting the typical molecular ions at *m*/*z* 385 expected from diferulic acids, three peaks were observed with retention times at 52.15, 53.07, and 63.40 min, respectively, on the reversed-phrase HPLC. By comparing the experimental data with the literature (*8*, *17*, *18*), these peaks were respectively assigned as 5-5′, 8-*O*-4′, and 8-5′ (benzofuran form) diferulic acids. The quantitative analysis of each ferulic acid dehydrodimer was accomplished by using response factors at 280 nm against *trans*-cinnamic acid (*18*). As seen in **Table 1**, 8-5′ (benzofuran form) diferulic acids was found as the most abundant ferulic acid dehydrodimer, ranging from 8.3 to 12.7 mg/100 g. The amounts of 8-*O*-4′ and 5-5′ diferulic acids in wheat grains varied from 3.3 to 6.6 mg/100 g and from 0.8 to 1.6 mg/100 g, respectively. They were more concentrated in purple wheat. White wheat had a significantly lower level of 8-*O*-4′ diferulic acids than other colored wheats. By calculation of the total amount of phenolic acids in each wheat grain, it can be seen that white wheat contained the least phenolic acids and the lowest total phenolic content.

**Flavonoid Composition.** Flavonoids constitute a large group of phenolic compounds in plants, accounting for two-thirds of the dietary polyphenols (*19*). The total flavonoid contents in purple, red, yellow, and white wheat are presented in **Table 2** and expressed as milligrams of catechin equivalents per 100 g of wheat grain. Among all of the colored wheat grains, purple wheat was found to possess significantly highest flavonoid content, ranging from 21.59 to 102.95 mg/100 g. Moderate flavonoid contents were found in yellow and red wheats, with levels of 13.44 and 10.72 mg/100 g, respectively. White wheat had the lowest total flavonoid content (9.6 mg/100 g). These results indicate that dark-colored wheat comprises more flavonoid content than light-colored wheat.

Anthocyanins are water-soluble pigments contributing to the blue, purple, and red color of cereal grains. Because of their antioxidant properties, anthocyanins are among the major flavonoids studied in cereal grains. The total anthocyanin contents in different colored wheat grains were determined in their acidic methanol extracts and expressed as milligrams of cyanidin-3-glucoside equivalents per 100 g of wheat grain. As seen in **Table 2**, significant differences in anthocyanin contents were detected between purple and other light-colored wheats. Three purple wheats (Charcoal, Indigo, and Konini) were found to contain remarkable anthocyanin contents ranging from 2.5 to 23.5 mg/100 g. Their extracts, respectively, exhibited purple-red, red, and pink colors. However, the extracts from red, yellow, and white wheat grains appeared in a light yellow color. The total anthocyanin contents of these light-colored wheats were found to be <1 mg/100 g. Therefore, it is reasonable that they had lower total anthocyanin content than purple wheat. These results suggest that anthocyanins likely exist only in purple wheat, whereas red, yellow, and white wheat grains may not comprise significant amounts of anthocyanins or their concentrations are too low to detect.

To further confirm the presence of anthocyanins, the LC chromatogram of each wheat grain was recorded at 520 nm. As seen in **Figure 2**, six anthocyanin pigments (peaks 1−6) were detected in Charcoal purple wheat. Under collision-induced dissociation (CID), each peak gave rise to a deprotonated molecular ion $[M-H]^-$ (namely, parent ion) and a fragment ion. This typical fragment ion can be used to illustrate the aglycone structure of anthocyanins. For instance, the fragment ions of peaks 1 and 2 had the same *m*/*z* 285 value, indicating that both consisted of cyanidin as the aglycone. Despite the same aglycone, the sugar substituent of these two peaks was different based on the mass

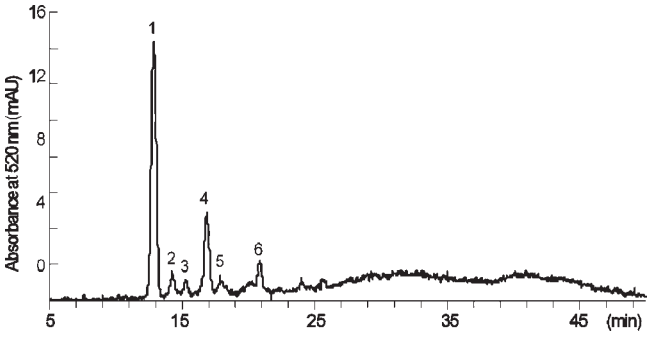


**Figure 2.** Identification of six anthocyanins in acidic methanol extract of Charcoal purple wheat by HPLC-MS.

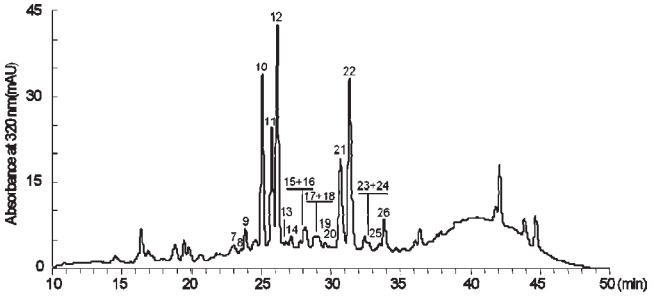


**Figure 3.** HPLC chromatogram of acidic methanol extract from Charcoal purple wheat recorded at 320 nm.

losses observed from the parent ion. For peak 1, the mass difference of 162 (447 − 285) Da was observed between the parent ion and the fragment ion, indicating that a glucose residue was cleaved from the precursor ion. However, a mass loss of 308 (593−285) Da was observed for peak 2, suggesting a rutinose moiety conjugated to the cyanidin aglycone. Therefore, peak 1 was designated cyanidin-3-glucoside, and peak 2 was assigned as cyanidin-3-rutinoside. Analogously, the other four peaks were respectively designated pelargonidin-3-glucoside, delphinidin-3-glucoside, peonidin-3-glucoside, and petunidin-3-rutinoside. Among the detected anthocyanins, peak 1 was the largest peak observed in the LC chromatogram (**Figure 2**), indicating that cyanidin-3-glucoside was the most abundant anthocyanin in Charcoal purple wheat. According to Abdel-Aal et al. (*20*), cyanidin-3-glucoside also appeared in variable amounts in black and red rice and in blue, purple, and red corn. In the other two purple wheats, Indigo and Konini, although comprising relatively higher TAC than red, yellow, and white wheats, only cyanidin-3-glucoside was detected without the presence of the other five anthocyanins. Therefore, the remarkable anthocyanin content of purple wheat is mainly due to the cyanidin-3-glucoside constituent. In our study, no anthocyanin was observed in red, yellow, and white wheat grains.

In addition to anthocyanins, 20 other peaks were detected at 320 nm in the HPLC chromatogram of acidic methanol extract from Charcoal purple wheat (**Figure 3**). As seen in **Table 3**, most of them were recognized as flavones bearing apigenin, luteolin, or chrysoeriol as the aglycone, and a few of them were identified as flavonols comprising kaempferol or quercetin as the backbone.

**Table 3.** LC-MS/MS Identification of *C*-/*O*-Glycosyl Flavonoids in Charcoal Purple Wheat Including Their Retention Times, Deprotonated Molecular Ions, and Main Fragments (Ion Intensity) Obtained in Negative Mode under Collision-Induced Dissociation

| | | | | product ion (*m*/*z*) and its relative intensity (%) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| peak | assignment | $t_R$ (min) | $[M-H]^-$ *m*/*z* | −18 | −60 | −90 | −120 | −150 | A + 113[a] | A + 83 |
| | **C-glycosides** | | | | | | | | | |
| 7 | apigenin-6,8-di-*C*-hexoside isomer | 23.09 | 593 | 575 (8) | | 503 (20) | 473 (42) | 443 (15) | 383 (59) | 353 (100) |
| 8 | apigenin-6,8-di-*C*-hexoside isomer | 23.64 | 593 | 575 (18) | | 503 (12) | 473 (30) | 443 (18) | 383 (51) | 353 (100) |
| 9 | chrysoeriol-6,8-di-*C*-pentoside | 23.91 | 579 | 561 (10) | 519 (10) | 489 (31) | 459 (15) | 429 (30) | 399 (100) | 369 (99) |
| 10 | apigenin-6-*C*-pentoside-8-*C*-hexoside isomer | 25.15 | 563 | 545 (5) | 503 (7) | 473 (30) | 443 (38) | 413 (18) | 383 (80) | 353 (100) |
| 11 | apigenin-6-*C*-pentoside-8-*C*-hexoside isomer | 25.84 | 563 | 545 (5) | 503 (7) | 473 (28) | 443 (38) | 413 (15) | 383 (72) | 353 (100) |
| 12 | apigenin-6-*C*-pentoside-8-*C*-hexoside isomer | 26.25 | 563 | 545 (5) | 503 (7) | 473 (28) | 443 (38) | 413 (15) | 383 (72) | 353 (100) |
| 13 | chrysoeriol-6-*C*-pentoside-8-*C*-hexoside isomer | 26.39 | 593 | 575 (10) | 533 (8) | 503 (40) | 473 (60) | 443 (10) | 413 (62) | 383 (100) |
| 14 | chrysoeriol-6-*C*-pentoside-8-*C*-hexoside isomer | 27.21 | 593 | 575 (12) | 533 (8) | 503 (40) | 473 (60) | 443 (10) | 413 (62) | 383 (100) |
| 16 | chrysoeriol-6-*C*-pentoside-8-*C*-hexoside isomer | 28.28 | 593 | 575 (10) | 533 (10) | 503 (40) | 473 (32) | 443 (20) | 413 (51) | 383 (100) |
| 17 | apigenin-6,8-di-*C*-pentoside isomer | 28.82 | 533 | 515 (7) | 473 (17) | 443 (21) | 413 (19) | | 383 (90) | 353 (100) |
| 18 | apigenin-6,8-di-*C*-pentoside isomer | 29.58 | 533 | 515 (7) | 473 (15) | 443 (25) | 413 (18) | | 383 (90) | 353 (100) |

| | | | | product ion (*m*/*z*) and its relative intensity (%) | | | |
|---|---|---|---|---|---|---|---|
| peak | assignment | $t_R$ (min) | $[M-H]^-$ *m*/*z* | −206 | −224 | −224 − 90 | −224 − 120 |
| | **acylated C-glycosides** | | | | | | |
| 21 | apigenin-6-*C*-sinapoylpentoside-8-*C*-hexoside | 30.77 | 769 | 563 (5) | 545 (100) | 455 (5) | 425 (84) |
| 22 | apigenin-8-*C*-sinapoylpentoside-6-*C*-hexoside | 31.14 | 769 | 563 (5) | 545 (100) | 455 (5) | 425 (72) |

| | | | | product ion (*m*/*z*) and its relative intensity (%) | | | |
|---|---|---|---|---|---|---|---|
| peak | assignment | $t_R$ (min) | $[M-H]^-$ *m*/*z* | −162 | −146 | −162 − 146 | −162 − 146 − 18 |
| | **O-glycosides** | | | | | | |
| 15 | kaempferol-7-*O*-sopheroside | 27.92 | 609 | 447 (18) | | | 285 (100) |
| 19 | kaempferol-7-*O*-neohesperidoside | 29.71 | 593 | | | 285 (100) | |
| 20 | quercetin-3-*O*-rutinose | 29.94 | 609 | 447 (60) | | 301 (100) | |
| 23 | luteolin-7-*O*-hexoside | 32.14 | 447 | 285 (100) | | | |
| 24 | luteolin-7-*O*-neohesperidoside | 32.51 | 593 | | | 285 (100) | |
| 25 | apigenin-7-*O*-neohesperidoside | 33.52 | 577 | | | 269 (100) | |
| 26 | chrysoeriol-7-*O*-neosperidoside | 34.00 | 607 | | | 299 (100) | |

[a] A = aglycone.

These flavones and flavonols were not present as free aglycone, but existed as glycoconjugates (**Table 3**).

Peaks 10−12 were among the most dominant components eluted from Charcoal purple wheat extract. In the negative mode, they produced the same deprotonated molecular ion with an *m*/*z* 563 value, indicating they are isomers. The major fragments obtained from these three peaks were as follows: *m*/*z* 545, 503, 473, 443, 413, 383, and 353 ions. The fragments at *m*/*z* 545 denoted the mass loss of 18 Da from the precursor ion $[M - H]^-$, representing the elimination of one water molecule. The ions at *m*/*z* 503, 473, and 443 corresponding to the mass loss of 60, 90, and 120 Da were obtained by cross-ring cleavages in hexose and pentose residues. These typical mass losses indicated that peaks 10−12 were *C*-glycosyl flavonoids. Unlike *O*-glycosides, the complete aglycone in *C*-glycosides is hardly cleaved from the parent ion. Thus, the ions A + 83 and A + 113 are used to illustrate the aglycone present in flavonoid *C*-glycosides. In peaks 10−12, the fragments at *m*/*z* 353 and 383 are, respectively, corresponding to A + 83 and A + 113, indicating that the molecular weight of the aglycone was 270. Thus, the only possibility of such an aglycone was apigenin. To date, the sugar substitution of *C*-glycosides is observed at only the 6- and 8-positions (*21*). Therefore, peaks 10−12 are presumed as apigenin-6/8-*C*-pentoside-8/6-*C*-hexoside isomers (MW 564 = apigenin 270 + hexose 162 + pentose 132). They are also found in durum wheat (*15*) and other cereal grains such as wild rice (*22*). The other *C*-conjugated glycosides identified in Charcoal purple wheat were apigenin-6,8-di-*C*-hexoside, chrysoeriol-6,8-di-*C*-pentoside, apigenin-6,8-di-*C*-pentoside, chrysoeriol-6-*C*-pentoside-8-*C*-hexoside, and their isomers.

Peaks 21 and 22 were also found as the major components in Charcoal purple wheat. According to their mass spectra, the same deprotonated molecular ions at *m*/*z* 769 were observed with a series of product ions at *m*/*z* 563, 545, 455, and 425. Among the fragments, the one at *m*/*z* 545 showed the highest intensity. It was formed by the loss of 224 Da from the parent ion, indicating a neutral loss of a sinapic acid. The loss of sinapoyl (206 Da) was revealed by the ions at *m*/*z* 563. These two fragments indicated that peaks 21 and 22 were sinapoyl acylated flavonoids. The other fragments at *m*/*z* 445 and 425 corresponding to the subsequent losss of 90 and 120 u from the fragment at *m*/*z* 545, suggested a *C*-hexosyl linkage. According to Cao et al. (*23*), the corresponding compounds could be apigenin-6/8-*C*-sinapoylpentoside-8/6-*C*-hexoside or apigenin-6/8-*C*-sinapoylhexoside-8/6-*C*-pentoside (MW 770 = apigenin 270 + hexose 162 + pentose 132 + sinpoyl 206). However, a specific mass loss of 60 Da for the cross-ring cleavage of pentose was absent, suggesting that sinapoyl was acylated with pentose instead of hexose. Therefore, under CID the cleavages happened between sinapoyl and pentose and within the ring of hexose. Peaks 21 and 22 were identified as apigenin-6/8-*C*-sinapoylpentoside-8/6-*C*-hexoside isomers.

Although most flavones identified were *C*-conjugated glycosides, a few of them were recognized as *O*-glycosidic derivatives (**Table 3**). In the case of *O*-glycosides, the cleavage usually happens at the glycosidic *O*-linkage, resulting in the direct loss of saccharide residues. Thus, the characteristic mass losses are 162 Da (hexose), 146 Da (deoxyhexose), and 132 Da (pentose). Sometimes the aglycones of *O*-glycosides can be directly obtained by applying high collision energy, such as peaks 23−26. As seen in **Table 3**, under CID these four peaks gave rise to only one fragment ion. They were respectively observed at *m*/*z* 285, 285, 269, and 299, indicating that luteolin, apigenin, and chrysoeriol are the aglycones. The sugar substituent of each peak can be illustrated by the observed mass losses. According to Cuyckens and Claeys (*21*), the 3- and 7-positions are the most common glycosylation sites observed in *O*-glycosyl flavones and flavanols. Thereby, these four peaks are respectively assigned as luteolin-7-*O*-hexoside, luteolin-7-*O*-neohesperidoside, apigenin-7-*O*-neohesperidoside, and chrysoeriol-7-*O*-neohesperidoside.

Among the identified 20 flavones and flavonols, there are 11 *C*-conjugated glycosides, 7 *O*-glycosidic derivatives, and 2 acylated *C*-glycosides. Thus, *C*-glycosidic linkage is the most common conjugating form in wheat grain. *C*-Glycosyl flavonoids are not only predominant in wheat grain but also found as the major flavonoid glycosides in the leaves and stems of wheat plants (*24*,*25*). Unlike anthocyanins, flavones and flavonols were detected in all of the tested wheat grains, indicating they are the common metabolites in wheat grain.

No proanthocyanidins were detected in the selected wheat grains, indicating that proanthocyanidins were not common phenolic constituents in wheat. Overall, in terms of phenolic acid and flavonoid composition in the tested wheat varieties, purple, red, yellow, and white grains exhibited similar phenolic profiles, except for anthocyanin contents. Purple wheat, due to significantly high levels of anthocyanins, displayed outstanding antioxidant activity. When compared with other cereal grains, the antioxidant activity of Charcoal purple wheat was higher than that of white rice, black barley, purple barley, and yellow barley and comparable to that of wild rice (*16*, *26*), which demonstrates the great potential of purple wheat as a functional food ingredient.

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