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Research paper

Distribution of antioxidants and phenolic compounds in flour milling fractions from hard red winter wheat

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ABSTRACT

Mature wheat kernels contain three main parts: endosperm, bran, and germ. Flour milling results in multiple streams that are chemically different; however, the distribution of antioxidants and phenolic compounds has not been well documented in terms of conventional milling by-product streams. In this study, multiple analytical methods were used to investigate antioxidant activity and phenolic compound compositions of hard red winter wheat (whole ground wheat), the parts of a wheat kernel (bran, flour, germ), and wheat by-product streams (mill feed, red dog, shorts) for the first time. For each mill stream, phenolic compounds (total, flavonoid, and anthocyanin contents) were determined and antioxidant activities were evaluated with 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity, ferric reducing/antioxidant power (FRAP), and total antioxidant capacity assays. Significant differences ($P < 0.05$) were observed in phenolic concentrations among fractions of bran, flour, and germ milled from the same kernels and noted that germ accounts for the majority of antioxidant properties, whereas bran contains a substantial portion of phenolic compounds and anthocyanins. Mill feed was high in phenolic content (5.29 mg FAE/g), total antioxidant capacity (866 mg/g), and antioxidant activity (up to 75% DPPH inhibition and 20.26 μmol $FeSO_4$/g). The comprehensive information on distribution of antioxidants and phenolic compounds provides insights for future human consumption of commonly produced co-products from flour milling, and for selecting and using different milling fractions to make foods with improved nutritional properties.

1. Introduction

Wheat (*Triticum aestivum* L.) is a staple of human diet and is cultivated worldwide [1] due to its ease of growth, diverse uses, and long-term storage. The annual total for global production of wheat is 762 million tons in 2019–2020 [2]. In the United States, approximately 50% of the domestic wheat crop is exported, 36% is consumed by Americans, while 10% and 4% go to livestock and seeds, respectively [3]. A wheat kernel is composed of approximately 85% endosperm, 13% bran, and 2% germ [4]. Kernel endosperm is used as flour, whereas kernel germ contains high concentrations of lipids that are extracted for oil or processed as higher value feed components [5].

Milling separates bran from endosperm. To optimize yield, millers aim to produce large bran pieces (>2000 μm) and a minimum amount of shorts (broken bran pieces) [4]. Red dog (flour containing high proportions of bran) [6] and mill feed (low quality, highly contaminated bran) [4] are also produced during milling and sold as feed products. Due to the differing chemical compositions and functions of bran, germ, and endosperm from the kernel, milling streams are chemically different [7].

Phenolic compounds are the main antioxidant constituents found in wheat [8,9] and ferulic acid is the dominant phenolic acid in hard red winter wheat [10,11]. The ferulic acid content is approximately 0.8–2.0 g/kg wheat dry weight [12] and is always higher in bran than in flour from the same kernel by 10–20 times [10]. Phenolic compounds are predominantly found in the outer layers of wheat bran as a structural component of the cell wall and provide protection from natural elements, pathogenic organisms, and ultraviolet rays [13,14]. Phytochemical content and availability are affected by environment, farming, mutation, and processing [11,15–17]. In addition, wheat variety is an important factor affecting phenolic acid composition [18].

Many studies have evaluated the variability of nutrients in different

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parts of wheat kernels [19–21]. Studies have investigated bran and germ together as one sample [22,23], bran, flour, and grain [24,25], various compositional differences of select milling streams [26–28], and the distribution of ash and antioxidant capacities for the improvement of flour [29]. Prior investigations have shown that bran and germ serve as possible sources for antioxidants with potential health benefits [26] and even the particle size from milling can change the extractability of these compounds [30]. We should note that different methods have been used to separate different parts of wheat in the literature. In this study, we used a pilot-scale experiment milling system (Fig. 1), which is similar to large-scale commercial dry milling of wheat. Bran, flour, germ, mill feed, red dog, and shorts from the same wheat kernels were obtained and that allowed us to determine the distribution of antioxidants and phenolic compounds in the parts of a wheat kernel (bran, flour, germ), and wheat by-product streams (mill feed, red dog, shorts).

Antioxidants are distinguished as multiple compounds, have the ability to act *in vivo* via multiple mechanisms [31], and no single measurement can express ability, activity, and capacity of antioxidants present in solution [32]. In this study, the detailed distribution of antioxidants and phenolic compounds (total, flavonoid, and anthocyanin contents) in flour milling fractions from hard red winter wheat (HRW) was studied for the first time. HRW wheat is popular in the US and widely used to make breads, hard rolls, croissants, and flat breads. It is also an ideal choice of wheat for some types of Asian noodles, general purpose flour, and as an improver for blending, according to the U.S. Wheat Association. Using a pilot-scale experiment milling system, we separated components of the wheat kernel into different fractions and determined the available antioxidant activity and concentration of phenolic compounds in each wheat kernel fraction and common by-product mill stream produced by dry-milling for enhanced understanding of lesser valued fractions. The bioavailability of these bioactive components could be affected by many factors, including their levels in the original grain and fractions [33–35]. The information gained in this study could provide insights for future human consumption of commonly produced co-products from flour milling, and for selecting and using different milling fractions to make foods with improved nutritional properties.

2. Materials and methods

2.1. Wheat and milled wheat fractions

Mixed Kansas hard red winter wheat [36] was tempered to 16% moisture and milled (70% extraction; patent flour contained 0.50% ± 0.00% ash) in the Kansas State University (KSU) Shellenberger Hall industrial–scale milling system (Manhattan, KS). The milling system has been previously described [36,37]. Bran, flour, germ, mill feed, red dog, and shorts were collected from one mill run in the industrial milling system by adjusting mill settings for optimum percentage of the desired fraction. In addition, whole wheat kernels that were collected from the mill run after cleaning were ground on a Burr Mill (Falling Number grinder, Perten Instruments, Springfield, IL) to have less than 2% overs on a 150 μm screen to represent a whole ground wheat sample. Photographs of each fraction were taken for reference, and each wheat sample is described in Fig. 1. Prior to any chemical assay, all samples were ground to pass through a 150 μm screen.

2.2. Chemicals

All chemicals, reagents, and standards were ACS grade. Ascorbic and phenolic acid standards were purchased from Sigma-Aldrich, Inc. (St. Louis, MO).

2.3. Soluble and bound phenolic acids

A two-part extraction for soluble and bound phenolic content was performed for each sample as previously described [22]. Each sample (1.000 g) was extracted for 10 min with 10 mL 80% methanol (*V/V*) at 25 °C under constant stirring. After 10 min, the extract was removed and the extraction procedure was repeated twice on the residual pellet. Extracts were pooled and evaporated under continuous nitrogen gas. Each extracted sample was lyophilized and weights were recorded prior to dissolving in 5 mL methanol. Determination of bound phenolic acids was conducted as previously described [23]. The above pellet was hydrolyzed with 2 M sodium hydroxide at 25 °C for 1 h under continuous nitrogen gas. The extraction was neutralized with 2 M hydrochloric acid

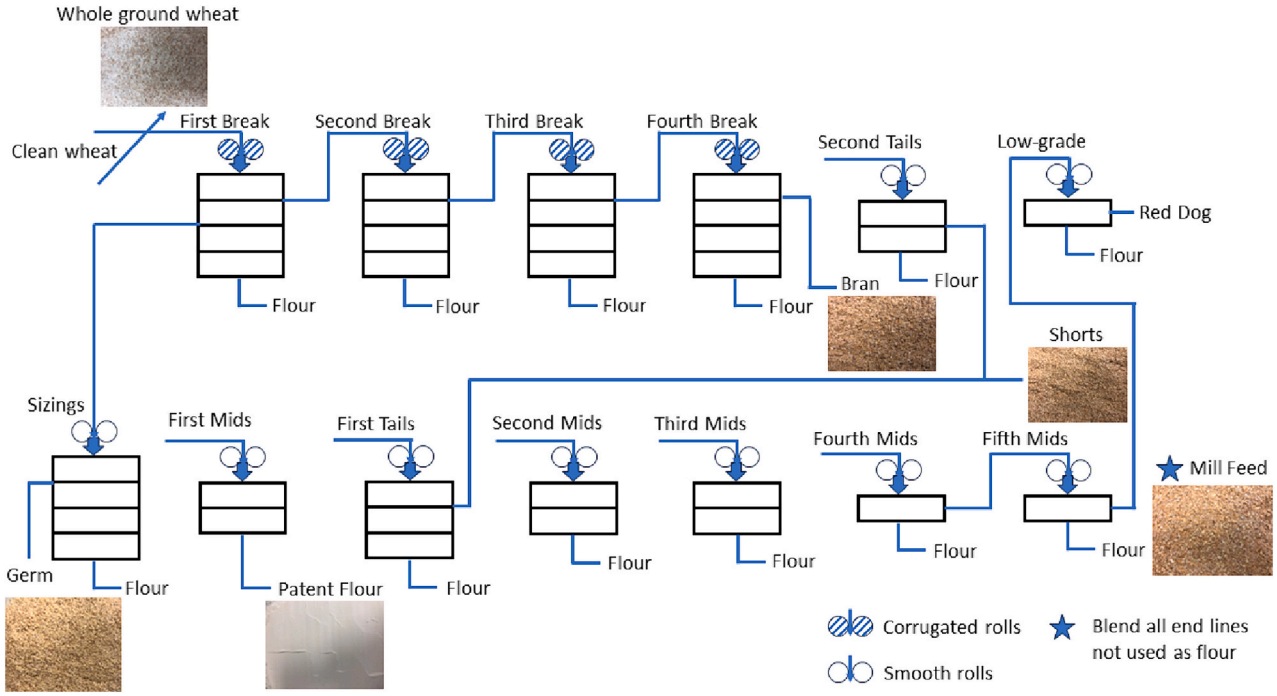

Fig. 1. Sketch of the milling schematic with image located at the fraction output stream. All samples were derived from the same kernels within the mill run.

and extracted with pure hexane. After hexane removal, the hydrolysis was extracted five times with ethyl acetate. Ethyl acetate extracts were pooled together and evaporated to dryness under continuous nitrogen gas. Dried extracts were dissolved in 10 mL methanol and stored at −20 °C until use. Determination of total phenolic content (TPC) in each fraction was conducted as previously described [38]. The reduction of Folin−Ciocalteu reagent in the presence of phenolates was measured spectrometrically on a Perkin-Elmer Lambda 800 UV–Vis spectrophotometer (Perkin-Elmer, Inc., Waltham, MA). Using ferulic acid as the standard, TPC was expressed as ferulic acid equivalents (FAE) per gram of mill fraction. A ferulic acid standard solution or extract sample (125 μL) was added to 0.5 mL deionized water and 125 μL Folin−Ciocalteu reagent in a test tube and vortex-mixed. Samples were allowed to stand for 6 min. Subsequently, 1.25 mL 7% sodium carbonate and deionized water were added to adjust final volume to 3 mL. After 90 min at 25 °C, absorbance was measured at 760 nm against the blank and compared with various concentrations of ferulic acid standards for quantification.

2.4. Phytochemical extraction (flavonoids)

Phytochemicals were extracted from each sample as previously described [23]. Each sample (600 mg) was weighed with 60 mg magnesium carbonate in a loosely closed screw-capped test tube. Solids were blended prior to a rapid extraction with 2 mL 1:1 (*V/V*) methanol/tetrahydrofuran mixture in a water bath at 75 °C for 5 min. Extracts were cooled and centrifuged at 2500 ***g*** for 5 min, and the organic phase was removed. The pellet was extracted two additional times with methanol/tetrahydrofuran (2 mL) in a water bath at 75 °C for 5 min then cooled and centrifuged at 2500 ***g*** for 5 min. Pooled organic phases were dried with anhydrous sodium sulfate and evaporated under continuous nitrogen gas at 35 °C. Residues were dissolved in 1 mL methanol/tetrahydrofuran, stored at −20 °C, and analyzed within 2 weeks.

2.5. Total flavonoid content

Determination of total flavonoid content in each fraction was conducted as previously described [39]. Extracts from the phytochemical extraction (0.25 mL) were mixed with 1.25 mL distilled water in a test tube. Seventy–five microliters of 5% sodium nitrite solution was added, test tubes were held at 25 °C for 6 min, 150 μL 10% aluminum chloride was added, and test tubes were held at 25 °C for 5 min. Subsequently, 0.5 mL 1 M sodium hydroxide was added and solutions were brought up to 2.5 mL with distilled water, then mixed. Samples were immediately measured against a blank at 510 nm on a spectrophotometer. Flavonoid content was calculated as microgram of catechin equivalent (CE) per gram of sample (μg/g CE) against a standard curve of catechin [39].

2.6. Anthocyanin content

Extraction and determination of anthocyanin content in each milling fraction were conducted as previously described [40]. Samples (3.000 g) were extracted twice by turbulent-mixing with 24 mL acidified methanol [1 N hydrochloric acid (85:15, *V/V*)] for 30 min. The pH was adjusted to 1.0 before timing and rechecked after 15 and 30 min of extraction. Extracts were centrifuged at 21,000 ***g*** (4 °C) for 20 min and refrigerated for 2 d to precipitate. Again, extracts were centrifuged 21,000 ***g*** (4 °C) for 20 min and concentrated to 2 mL under continuous nitrogen. For total anthocyanin content determination, the concentrated supernatant was poured into a 50-mL volumetric flask and made up to volume with acidified methanol. Absorbance was measured on a spectrophotometer at 535 nm, and anthocyanin content calculated as micrograms per gram according to the original method [40].

2.7. Total antioxidant capacity

Determination of total antioxidant capacity in each fraction was conducted as previously reported [41]. In one test tube, 0.3 mL from the soluble/bound phenolic extraction and 3 mL of reagent (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) were incubated at 95 °C for 90 min. After the mixtures cooled to 25 °C, absorbance of the solutions were read at 695 nm against a blank on spectrophotometer and calculated against a reference of the total antioxidant capacity of ascorbic acid.

2.8. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) assay

Determination DPPH radical absorbance in each fraction was conducted as previously reported [42]. DPPH reagent (0.004% DPPH in methanol) was prepared the day of analysis. In one test tube, 1.9 mL DPPH reagent and 100 μL extract from the soluble/bound phenolic extraction were mixed. Tubes were kept in a dark room to react. After 30 min, absorbance was tested at 517 nm on a spectrophotometer. IC_{50} value was used to calculate DPPH value and is defined as the concentration of the sample necessary to have 50% inhibition as determined with interpolated linear regression, where a lower IC_{50} value is associated with a higher radical scavenging activity. All DPPH values are reported as '% inhibition'.

2.9. Ferric ion reducing antioxidant power (FRAP) assay

Determination of FRAP for each extract was conducted as previously reported [15]. FRAP reagent was prepared the day of analysis and kept in a water bath at 37 °C when not in use. The FRAP reagent contained acetate buffer 300 mM pH 3.6, 2, 4, 6-tripyridyl-*s*-triazine (10 mM in 40 mM hydrochloric acid) and iron (III) chloride hexahydrate (20 mM) in a ratio of 10:1:1 [43]. To determine FRAP of each sample, 1.8 mL FRAP reagent, 300 μL extract from the soluble/bound phenolic extraction, and 180 μL distilled water were combined in one test tube and incubated at 37 °C for 4 min. Absorbance was measured at 593 nm on a spectrophotometer and reported in micromole ferrous sulfate ($FeSO_4$) per gram defatted material.

2.10. Statistical analysis

All tests were performed in triplicate. Means and standard deviations were calculated for all analyses. All values are expressed as mean plus or minus the standard deviation. Significance of differences between groups was compared using two-sample *t*-tests (Excel 2003). *P* values (two-tailed) of less than 0.05 were considered to be a sign of statistical significance. *N* is provided as the number of assays per sample.

3. Results and discussion

3.1. TPC of wheat fractions

Extraction yield of soluble phenolic compounds refers to free and conjugated phenolic acids extracted with 80% methanol, whereas that of bound phenolic compounds refers to those derived by alkaline-hydrolyzed extraction. TPC is expressed as milligram FAE per gram of wheat fraction in Table 1. The highest soluble TPC concentration was observed in germ extract (0.92 mg FAE/g of defatted material), and the lowest was determined in flour extract (0.29 mg FAE/g of defatted material). The order of TPC for soluble extracts was: germ > whole ground wheat > mill feed > bran > red dog > shorts > flour. Similarly, bound extract TPC of wheat fractions was highest in germ (5.43 mg FAE/g of defatted material), and the lowest TPC was found in flour (1.95 mg FAE/g of defatted material). The order of TPC for bound extracts was: germ > whole ground wheat > mill feed > bran > red dog > shorts > flour.

The main components of the wheat kernel were measured in order to correlate the portions of the wheat kernel to that of the wheat milling and by-product streams which contained them. Germ extract contains

Table 1
Total phenolic acid content of wheat fractions milled from the same kernels.

Wheat fraction	Total phenolic compounds[1,2] (mg FAE/g of defatted material)	
	Soluble	Bound
Bran	0.57 ± 0.03[d]	3.81 ± 0.17[c]
Flour	0.29 ± 0.01[e]	1.95 ± 0.01[d]
Germ	0.92 ± 0.11[a]	5.43 ± 0.89[a]
Mill feed	0.69 ± 0.08[c]	4.60 ± 0.56[b]
Red dog	0.56 ± 0.02[d]	3.73 ± 0.13[c]
Shorts	0.34 ± 0.05[e]	2.57 ± 0.04[d]
Whole ground wheat	0.75 ± 0.06[b]	4.87 ± 0.97[a]

Note: [1] Column data with like superscript letters are not significantly different ($P > 0.05$); $n = 3$.
[2] The total phenolic contents are expressed as ferulic acid equivalents (FAE).

the highest portion of oils and antioxidants [44,45], thereby accounting for the high TPC of germ. High TPC values have been reported with the presence of the antioxidant rich pericarp and aleurone layers [46], thereby explaining the TPC value obtained for bran. Subsequently, mill feed and whole ground wheat (which contain relatively high concentrations of bran and germ) also have high TPC values for the same reason [47]. Flour, which contains little bran or germ, had minor detectable phenolic compounds and was found to have low levels of phenolic compounds in previous research [22,41], which complies with the previous theory.

We found whole ground wheat to contain relatively higher [27], and bran relatively lower [24] phenolic compounds than in previous reports. In a previous study, 49 mill streams were investigated for phenolic content, antioxidant activity and iron content to understand the range of said materials within flour streams derived from flour roller milling [29]. Within that study, TPC from flour streams was 0.76–3.69 mg FAE/g (d.w.) which was in range of our reported value. The difference in values between studies may be entirely attributed to the extraction/quantification method. Aprodu and Banu [29] utilized acidified methanol (1% hydrochloric acid)/water (80:10, *V/V*) and 70% ethanol separately to extract antioxidant compound (for TPC and FRAP, respectively). The current study utilized a two-part extraction: a nonacidic, alcohol soluble extraction and an alkaline bound extraction at room temperature for a relatively short extraction time. By this method, we suspect that additional bound phenolic compounds remained within the extracted material [23]. Previous studies have used different extraction solvents on various streams from milling [8,29,48]. There is difficulty in comparing results from the literature because of grave differences in extraction methods [29]; a number of extraction methods have been shown to give various results on the same material [49].

Whole ground wheat was used to represent the entire kernel and we obtained a TPC value higher than previous studies. The TPC of common wheat has been reported as 1.29 mg FAE/g defatted wheat when extracted by 80% ethanol (*V/V*) [27] and 1.57 mg FAE/ g (d.w.) when extracted with acidified methanol [29]. The wheat cultivar and sample preparation may entirely account for the differences between studies. Additionally, the Folin-Ciocalteu reagent used in the TPC method is not specific to phenolic compounds and can also react with sugars and peptides; many of which are soluble in aqueous solutions. We suspect that these compounds partly account for the soluble extract quantities. As the reagent is nonspecific, it measures all phenolic compounds, not only ferulic acid. The value calculated from TPC is in FAE, yet the method measures reducing capacity, and hence, antioxidant properties of the material [32]; thereby explaining the relative order of values in Table 1 and antioxidant activities discussed in Section 3.3. Phenolic compounds may contribute directly to antioxidant action [50,51]; consequently, it is necessary to investigate TPC when measuring antioxidant properties.

It is well known that there are many factors that have been reported to influence the antioxidant properties of wheat and other cereals. The phytochemical profiles of wheat grains can be substantially influenced by genotypes, environments, management factors, and their interactions, as summarized by a previous study [1]. In addition, extraction and detection methods may also have an impact on bioactive compounds and their biological activities [34]. The extraction, solubility, and separation properties of phenolics are influenced by their complex structure, non-uniform distribution in plants, and high molecular weight [52]. Consequently, the extraction procedure is critical to phenolic extraction [53,54].

3.2. Flavonoid and anthocyanin concentration in wheat fractions

Total flavonoid content for each wheat fraction is reported in Table 2. Mill feed had the highest concentration of flavonoid compounds (360.68 μg/g CE), whereas flour contained the lowest (192.85 μg/g CE). The measured flavonoid content for all wheat fractions was mill feed > shorts > red dog > whole ground wheat > bran > germ > flour. Mixed mill stream samples (whole ground wheat, shorts, mill feed, red dog) were high in flavonoid concentrations compared with separated fractions (bran, flour, germ), although samples did not differ significantly ($P > 0.05$) in many instances. Flour and mill feed were the only samples that were significantly different ($P < 0.05$) from all other samples. The distribution of phenolic compounds in different parts of a wheat kernel is genetically and metabolically determined by secondary metabolism of different tissues in wheat [20,21]. In metabolomic analysis of pealed wheat, Zhu et al. [21] also reported that flavonoid content was higher in the outer layers of the wheat and lower in the kernel.

Anthocyanin content for each wheat fraction is reported in Table 2. Bran had the highest anthocyanin content (72.7 μg/g), followed by red dog (43.7 μg/g). The measured anthocyanin content by wheat fraction was in the following order: bran > red dog > mill feed > shorts > germ > whole ground wheat > flour. Germ and shorts anthocyanin contents did not differ significantly. By using a pilot-scale experimental milling system, anthocyanin content in different milling steams were quantified in this study. Our findings are in general agreement with the metabolomic studies that most bioactive compounds are accumulated in the outer layers of wheat kernels [20].

Extract concentrations, measured with respect to the major plant secondary metabolite catechin, were similar to previous research on the wheat kernel [55]. Although flavonoids have antioxidant capability [35], the assay used does not measure antioxidant properties, and quantified this subset of phenolic compounds. Our study reiterated that flavonoids in germ are highly concentrated and flavonoids in flour are low in concentration and dispersed in red hard winter wheat, most likely as present as phlobaphene or proanthocyanidin [56].

Anthocyanins are flavonoids that are concentrated in the outside layers of the kernel, synthesized through a flavonoid biosynthetic pathway and contain antioxidant ability [35,57,58]. Germ and mill feed

Table 2
Flavonoid and anthocyanin concentrations in wheat fractions milled from the same kernels.

Wheat fraction	Total contents	
	Flavonoid (μg/g CE of extracted material)[1,2]	Anthocyanin (μg/g)[1]
Bran	293.80 ± 4.19[c]	72.7 ± 0.8[a]
Flour	192.85 ± 0.58[d]	1.3 ± 0.1[e]
Germ	283.47 ± 0.64[c]	22.4 ± 1.0[c]
Mill feed	368.68 ± 4.66[a]	24.1 ± 0.5[c]
Red dog	321.71 ± 2.92[bc]	43.7 ± 0.8[b]
Shorts	327.80 ± 1.19[b]	22.6 ± 0.4[c]
Whole ground wheat	314.46 ± 2.21[bc]	4.5 ± 0.3[d]

Note: [1] Column data with like superscript letters are not significantly different ($P > 0.05$); $n = 3$.
[2] Total flavonoid contents are expressed as catechin equivalent (CE).

were high in flavonoids, but not in chemical compounds determined by the total anthocyanin assay. Differentiating the two assays would derive from the 3-phenyl-1,4-benzopyrone structures in these milling fraction extracts that were present to represent phenol groups, but were distinct by degree of unsaturation and oxidation [59]. Anthocyanin concentrations differed from flavonoid concentrations in whole ground wheat and mill feed for the same reason [60]. As anthocyanidin derivatives, these flavonoids are differentiated by R-groups on the isoflavan structure; therefore, total anthocyanin concentration reflects the polyphenol population in a sample as the portion of anthocyanidins with glycosidic compounds [59].

While total phenolic acid, flavonoid and anthocyanin contents in different milling fractions were determined in this study, individual bioactive compounds such as specific phenolic acids (e.g., ferulic acid, vanillic acid, caffeic acid, syringic acid, and coumaric acid) and specific flavonoids (e.g., rutin, quercetin, apigenin, kaempferol, luteolin, and naringenin) were not analyzed. Nevertheless, Zhu et al. [21] conducted an extensive analysis, quantifying 1202 metabolites in the metabolome of dried mature kernels from six wheat varieties. Their research also included an examination of the contributions of these metabolites through Principal Component Analysis (PCA). The identified compounds in wheat primarily consisted of flavonoids, sugars, polyphenols, phenolamides, lipids, and vitamins. The data strongly indicate that bioactive compounds accumulate more prominently in the outer layer (bran) compared to other parts of the wheat grain. The variations in the distribution of these antioxidants and/or metabolites across milling fractions likely have implications for the flour and bakery industries, and suggest health benefits of consuming whole grain products and foods containing wheat bran.

3.3. Total antioxidant capacity of wheat fractions

Total antioxidant capacity (TAC) of each wheat fraction is reported in Table 3. For the soluble extracts, the highest total antioxidant capacity was observed in the whole ground wheat extract (193.59 mg/g of defatted material). The order of total antioxidant capacity was: whole ground wheat > mill feed > red dog > shorts > bran > flour > germ. For the bound extracts, the highest total antioxidant capacity was observed in the mill feed extract (710.16 mg/g of defatted material). The order of total antioxidant capacity was: mill feed > germ > bran > whole ground wheat > red dog > shorts > flour.

Table 3
Antioxidant activities of wheat fractions milled from the same kernels.

Wheat fraction	Total antioxidant capacity (equivalent to ascorbic acid [mg/g])		Antioxidant activities[1]			
			DPPH (% Inhibition)		FRAP (μmol $FeSO_4$/g of defatted material)	
	Soluble	Bound	Soluble	Bound	Soluble	Bound
Bran	78.63 ± 0.05^{e}	448.36 ± 0.1^{c}	19.94 ± 0.02^{c}	52.83 ± 0.15^{c}	2.73 ± 0.02^{d}	15.75 ± 0.37^{b}
Flour	70.34 ± 0.12^{f}	111.23 ± 0.1^{g}	6.03 ± 0.06^{e}	13.23 ± 0.08^{f}	0.81 ± 0.01^{e}	1.23 ± 0.01^{f}
Germ	35.84 ± 0.10^{g}	509.98 ± 1.9^{b}	18.89 ± 0.14^{d}	87.12 ± 0.29^{a}	6.57 ± 0.11^{a}	25.13 ± 0.03^{a}
Mill feed	156.38 ± 0.21^{b}	710.16 ± 0.20^{a}	38.36 ± 0.14^{a}	75.27 ± 0.22^{b}	4.47 ± 0.02^{b}	15.79 ± 0.01^{b}
Red dog	115.48 ± 0.24^{c}	409.80 ± 0.29^{e}	31.11 ± 0.04^{b}	73.60 ± 0.29^{b}	3.57 ± 0.17^{c}	13.36 ± 0.01^{d}
Shorts	97.37 ± 0.03^{d}	360.16 ± 0.20^{f}	18.60 ± 0.10^{cd}	44.23 ± 0.17^{d}	2.62 ± 0.02^{d}	14.57 ± 0.02^{c}
Whole ground wheat	193.59 ± 0.02^{a}	444.94 ± 0.07^{d}	18.17 ± 0.00^{cd}	30.86 ± 0.44^{e}	4.35 ± 0.06^{b}	11.42 ± 0.09^{e}

Note: [1] Column data with like letters are not significantly different ($P > 0.05$); $n = 3$.

Mill feed and whole ground wheat had approximately the same TPC but different soluble extract contributions to total antioxidant capacity. As observed in the TPC analysis, total antioxidant capacity in soluble extracts were at least two times higher than in bound extracts from the same material (Table 3), confirming the increased presence of reducing compounds in the soluble fraction. Previous studies have also noted the correlation between TPC and total antioxidant capacity values [46]. Total antioxidant capacity quantifies by cumulative capacity to scavenge free radicals [61] and the reagent in the TPC assay also allows for continued reduction of all reducible material.

Aprodu and Banu [29] noted the highest antioxidant activity of the flour streams analyzed derived from the streams at the last two break rolls. From our milling schematic (Fig. 1), the last break roll is where the bran fraction was obtained and flour obtained at that point would be heterogeneous in size and lower quality than patent flour. A previous study also found that bran and bran containing outlet streams have significantly higher TPC and total antioxidant capacity over those samples containing high amounts of endosperm [27]. Knowing the composition of mill streams is important for producing the type of flour desired [29] and knowing the composition of by-product streams is important for enhanced utilization of by-products.

3.4. DPPH radical scavenging activity of wheat fractions

All wheat fraction extracts showed DPPH scavenging activities in concentration measured by IC_{50} value (Table 3). As the standard, ascorbic acid was measured at 96% DPPH inhibition with this sample set. Soluble and bound germ extracts displayed significantly higher ($P < 0.0001$ and $P < 0.0012$, respectively) DPPH values than other fractions. The order of ability to scavenge DPPH radicals by wheat fractions was: mill feed > red dog > bran > germ > shorts > whole ground wheat > flour for soluble extracts, and germ > mill feed > red dog > bran > shorts > whole ground wheat > flour for bound extracts. Mill feed and red dog demonstrated the highest abilities to scavenge DPPH radicals.

The ability to act as donors of hydrogen atoms in DPPH radical transformation to a reduced form proved to be distinctive when measuring antioxidants within mill streams. DPPH assay measures single electron transfer to determine antioxidant reducing capacity by IC_{50} [29]. Higher inhibition demonstrates stronger antioxidant activity [62], as measured in the flouring milling waste streams. High DPPH activity in mill feed and red dog is thought to derive from those mill streams containing large amounts of germ and bran compared with other by-products in milling streams. Differences between by-product mill streams may be due to the DPPH assay reaction depending on the structural conformation of the antioxidants being examined [63,64]. Although mill feed showed high concentrations of DPPH in our experiment, note that milling products vary in composition when produced from different mills, kernels, and operational conditions; therefore, analysis from multiple mills and wheat cultivars should be compared in future studies.

A previous study has investigated DPPH of milling fractions [46]. Common fractions used in our study and the previous publication showed differences and commonalities. In the current study, shorts had an insignificantly lower DPPH value than bran (Table 3), while a previous study reported the difference between the DPPH values of shorts and bran to be significant [46] another by the same group reported the difference as insignificant [46]. These results reiterate the diversity of certain phytochemical compounds between varieties [65,66]. Liyana-Pathirana and Shahidi [46] reported a trend with TPC and DPPH, which noted a decrease in activity with decrease of bran contamination. This trend was consistent in the current study when significant differences were observed.

3.5. FRAP of wheat fractions

Antioxidant activity of wheat fraction extracts was evaluated for

power by FRAP assay (Table 3). Mill feed and red dog had similar ($P = 0.41$) soluble portions; however, the remaining samples had significantly different abilities to reduce iron. Germ-soluble extracts (6.57 μmol of $FeSO_4$/g defatted material) had significantly higher ($P < 0.0001$) FRAP values than other fractions, whereas flour extracts had significantly lower FRAP values (0.81 μmol of $FeSO_4$/g defatted material). For wheat fraction soluble extracts, ability to reduce Fe^{3+} to Fe^{2+} was in the order of germ > mill feed > whole ground wheat > red dog > bran > shorts > flour. For bound extracts from different fractions of wheat, germ extracts had significantly higher ($P < 0.0001$) FRAP values (25.13 μmol of $FeSO_4$/g defatted material) than other fractions, and extracts from flour were the lowest (1.23 μmol of $FeSO_4$/g defatted material). For all samples, ability of bound extracts to reduce Fe^{3+} to Fe^{2+} was in the order of germ > mill feed > bran > shorts > red dog > whole ground wheat > flour.

In a previous study, FRAP activity correlated with TPC of brewers' spent grain extracts [67], but in the current study only germ, whole ground wheat, and mill feed had consistently higher TPC and FRAP values. In a previous study utilizing the FRAP assay to investigate mill streams, Aprodu and Banu [29] did not analyze any stream in the milling process designated as germ (which had our highest FRAP value). The range for the FRAP value of flour was vast (0.2 nmol Fe^{2+}/mg to 7.1 nmol Fe^{2+}/mg), as multiple flour streams were analyzed. Common milling terms were not assigned to the streams reported in the study by Aprodu and Banu; therefore, it is only assumed that patent flour in their milling scheme would derive from reduction flour fractions. Although various extraction methods and data interpretations (use of various standards, etc.) were compared, this study and several previous studies have confirmed the increase of antioxidant activity with increase in bran concentration within the fraction [27,29,35]. Bran containing material is high in nutritive value and may be accessible in human digestion [27,35,66].

We should note that DPPH (2,2-diphenyl-1-picrylhydrazyl) and FRAP (Ferric Reducing Antioxidant Power) are both methods used to measure the antioxidant capacity of compounds, particularly in the context of food and biological samples. DPPH method is based on the reduction of a stable free radical (DPPH) by antioxidants [68], while FRAP measures the ability of an antioxidant to reduce a ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex to its ferrous form (Fe^{2+}) [69]. DPPH analysis is commonly used for assessing radical scavenging activity in various samples. FRAP is widely employed to measure the overall reducing power and antioxidant capacity of samples. It is noteworthy that both methods provide valuable information about the antioxidant potential of compounds. However, the selection between them depends on the specific needs of the study and the characteristics of the samples under examination. Researchers frequently employ a combination of assays to acquire a thorough comprehension of the antioxidant activity.

To better understand the relationship between measured antioxidant capacity and certain types of antioxidants, statistical correlations between bioactive compounds and antioxidant activities in various wheat milling fractions are analyzed (Table 4). Remarkably, a strong correlation ($P < 0.001$) emerged between soluble and bound TPC in the present investigation. TPC (both soluble and bound) had the strongest association with the soluble FRAP assay ($P < 0.01$). These findings align with the outcomes reported by Li et al. [70], underscoring the influence of milling methods and distinct milling fractions on antioxidant properties of wheat. Our finding corresponds with the observations of Benzie and Strain [43], who identified a substantial positive correlation between total phenolic content and the FRAP assay. However, our study did not identify any significant correlation between total phenol content and flavonoids, anthocyanin, soluble TAC, and soluble DPPH. Our analysis also revealed that flavonoids exhibited correlations with bound TAC and soluble DPPH. Conversely, no significant correlation was observed between anthocyanins and the other parameters examined. TAC bound demonstrated a significant correlation ($P < 0.05$) with bound TPC and flavonoids. These outcomes suggest that not only do milling fractions exhibit variations in bioactive compounds, but each compound category contributes differentially to biological activities through distinct mechanisms or chemical reactions. Additional investigation is warranted to enhance our understanding of the correlations among these compounds and their associated biological activities, taking into consideration various potential influencing factors.

4. Conclusion

Antioxidant activity, total phenolic content, flavonoid and anthocyanin concentrations varied among bran, germ, flour, and various mill streams from hard red winter wheat. In addition, antioxidants did not have the same distribution pattern throughout the flour milling fractions. Germ extract was high in antioxidant ability and phenolic acids, bran-containing fractions were high in anthocyanins, and flour extracts had the lowest concentrations in all phenolic compounds and antioxidant responses. The use of the same wheat kernels was implemented for optimum fraction comparison in this study. This is the first comprehensive study on distribution of antioxidants and phenolic compounds in flour milling fractions from US hard red winter wheat using an industrial-scale milling system. The knowledge gained is useful when selecting and using different mill fractions to enhance antioxidant activity or other nutritional properties in foods. Antioxidant capacity, total phenolic acid, flavonoid and anthocyanin contents in wheat fractions milled for the same kernels were determined in this study and the information would be useful for making food products using those different milling fractions. There are many phytochemicals in wheat; specific phenolic acids such as ferulic, vanillic, caffeic, syringic, and coumaric acids were not quantified in this study and merit further studies. Additional work should also be conducted to study more hard wheat cultivars and other types of wheat. Prospective research includes

Table 4
Correlations between bioactive compounds and antioxidant activities of different wheat milling fractions.

Items	TPC soluble	TPC bound	Flavonoids	Anthocyanin	TAC soluble	TAC bound	DPPH soluble	DPPH bound	FRAP soluble	FRAP bound
TPC soluble	1									
TPC bound	0.99***	1								
Flavonoids	0.39	0.50	1							
Anthocyanin	0.06	0.10	0.28	1						
TAC soluble	0.13	0.24	0.56	−0.25	1					
TAC bound	0.71	0.78*	0.85*	0.29	0.37	1				
DPPH soluble	0.39	0.48	0.87*	0.35	0.43	0.84*	1			
DPPH bound	0.66	0.67	0.59	0.43	−0.18	0.75	0.73	1		
FRAP soluble	0.94**	0.94**	0.50	0.01	0.06	0.74	0.46	0.77*	1	
FRAP bound	0.75	0.75	0.54	0.38	−0.23	0.71	0.45	0.86*	0.86*	1

Note: *** P-value <0.001; ** P-value <0.01; * P-value <0.05.
TPC = Total phenolic compounds; TAC = Total antioxidant capacity; DPPH = 1,1-diphenyl-2-picrylhydrazyl radical-scavenging activity; FRAP = ferric reducing/antioxidant power.

utilization of by-product streams for higher profit uses, effects of different milling fractions on quality and sensory properties of foods, and investigation of antioxidant activity deterioration post-processing in products for human consumption.

CRediT authorship contribution statement

Lauren Renee Brewer: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Jittawan Kubola:** Writing – original draft, Investigation, Formal analysis. **Sirithon Siriamornpun:** Writing – review & editing, Supervision, Methodology, Formal analysis. **Yong-Cheng Shi:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Ethical statement

This study does not involve human or animal testing.

Declaration of competing interest

All authors consented to the publication of this work. The authors declare that they have no competing interests. The author Yong-Cheng Shi is an Editorial Board Members for Grain & Oil Science and Technology and was not involved in the editorial review or the decision to publish this article.

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