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Importance of Insoluble-Bound Phenolics to Antioxidant Properties of Wheat

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Two commercial samples of soft (70% Canadian Eastern soft red spring and 30% Canadian Eastern soft white winter) and hard (90% Canadian western hard red spring and 10% Canadian Eastern hard red winter) wheats were used to obtain different milling fractions. Phenolics extracted belonged to free, soluble esters and insoluble-bound fractions. Soluble esters of phenolics and insoluble-bound phenolics were extracted into diethyl ether after alkaline hydrolysis of samples. The content of phenolics was determined using Folin−Ciocalteu's reagent and expressed as ferulic acid equivalents (FAE). The antioxidant activity of phenolic fractions was evaluated using Trolox equivalent antioxidant capacity, 2,2-diphenyl-1-picrylhydrazyl radical scavenging, reducing power, oxygen radical absorbance capacity, inhibition of oxidation of human low-density lipoprotein cholesterol and DNA, Rancimat, inhibition of photochemilumenescence, and iron(II) chelation activity. The bound phenolic content in the bran fraction was 11.3 ± 0.13 and 12.2 ± 0.15 mg FAE/g defatted material for hard and soft wheats, respectively. The corresponding values for flour were 0.33 ± 0.01 and 0.46 ± 0.02 mg FAE/g defatted sample. The bound phenolic content of hard and soft whole wheats was 2.1 (±0.004 or ±0.005) mg FAE/g defatted material. The free phenolic content ranged from 0.14 ± 0.004 to 0.98 ± 0.05 mg FAE/g defatted milling fractions of hard and soft wheats examined. The contribution of bound phenolics to the total phenolic content was significantly higher than that of free and esterified fractions. In wheat, phenolic compounds were concentrated mainly in the bran tissues. In the numerous in vitro antioxidant assays carried out, the bound phenolic fraction demonstrated a significantly higher antioxidant capacity than free and esterified phenolics. Thus, inclusion of bound phenolics in studies related to quantification and antioxidant activity evaluation of grains and cereals is essential.

KEYWORDS: Alkaline hydrolysis; insoluble-bound phenolics; antioxidant activity; photochemiluminiscence; Rancimat; oxidation of LDL and DNA; iron chelation; phenolic acids; wheat and cereals

INTRODUCTION

Cereals, in general, play an important role in human nutrition. Botanically, cereals are classified in Gramineae, the grass family, and include wheat, rice, barley, oats, rye, maize, sorghum, and millets (*1*). Wheat is an important cereal used as a staple food by the population of temperate areas while rice, sorghum, and millet are mostly used in the tropics (*1*). The importance of wheat has mainly been attributed to its ability to be ground into flour and semolina that form the basic ingredients of bread and other bakery products and pasta, respectively (*1*). The main constituents of wheat kernel are bran, germ, and endosperm (*2*).

Common foods of plant origin contain a variety of flavonoids and phenolic acids in trace amounts to several grams per kilogram of fresh weight (*3*). Cereal grains have also been known to contain phenolic acids, saponins, and phytoestrogens and may contain small quantities of flavonoids (*4*). According to Cassidy (*5*), cereals are a major source of lignan in the human diet. Lignans may function as potent antioxidants by decreasing the production of reactive oxygen species (ROS) thereby exerting anticancer effects. Cereals are known to contain a wide range of phenolic acids that belong to benzoic and cinnamic acid derivatives (*6*). In general, hydroxycinnamic acid (HCA) derivatives are the most common phenolic acids in cereal grains and serve as antioxidants (*7*). Recently, phenolic acids have gained attention due to their antioxidative, antiinflammatory, antimutagenic, and anticarcinogenic properties as well as their ability to modulate some key enzymatic functions in the cells (*8*).

Phenolic acids may form both ester and ether linkages owing to their bifunctional nature through reactions involving their carboxylic and hydroxyl groups, respectively. This allows phenolic acids to form cross-links with cell wall macromolecules (*6*). In one of the early studies, Naczk and Shahidi (*9*) recognized the importance of bound phenolics in the total phenolic content (TPC) of canola meal. Bound phenolics may be released by

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alkali, acid, or enzymatic treatment of samples prior to extraction (*10*−*15*). In barley, most phenolic acids exist in the bound form with other grain components such as starch, cellulose, β-glucan, and pentosans (*6*). A similar observation was made for phenolic acids in ground rye grain that were released upon enzymatic hydrolysis (*16*). With respect to the histological distribution of arabinoxylans, their concentration increases from the center to the periphery of the endosperm. Thus, they are mainly concentrated in the seed coats of cereals (*17*). The increased concentration of ferulates in the outer layers may be implicated in resistance to both insect and fungal pathogens. Thus, cross-linking of phenolic compounds may provide a physical barrier to invasive disease development and consumption by insects (*14*).

Cereal grains are one of the most important food groups, and their fibers are known to render health benefits that may be attributed to the nature of cell wall polymers and chemical architecture (*18*). Epidemiological data have shown that consumption of wheat bran is associated with a reduced risk of colorectal and gastric cancer (*19*). Cereals contain a high amount of hydroxycinnamates (HCA) that may exert potential health benefits (*13*). In cereals, HCA and their dimers exist mainly as esters bound to arabinoxylan. In many studies, phenolic antioxidants of wheat have been tested in their free form only (*20*−*23*). To understand the total antioxidant activity of cereals, it is imperative to consider the contribution from their bound phenolics since under normal conditions phenolics occur mainly in the bound form (*10*−*15*). Ferulic acid is the major phenolic acid in many cereals and exists predominantly in the seed coat (*24*) while traces may be present in the starchy endosperm (*25*).

Oxidative stress arises when the generation of oxidative species exceeds the capacity of the antioxidant system in the body (*26*). The enhancement of naturally occurring endogenous antioxidant defense system through a balanced diet by consuming plant-derived foods may protect the body against oxidative stress. This paper describes the antioxidant capacity of free, soluble esters and insoluble-bound phenolics isolated from soft and hard whole wheats, brans, and flours.

MATERIALS AND METHODS

Materials. Whole grains, flours, and brans of commercial soft (70% Canadian Eastern soft red spring and 30% Canadian Eastern soft white winter) and hard (90% Canadian western hard red spring and 10% Canadian Eastern hard red winter) wheat mixtures were obtained from milling suppliers of Robin Hood Multifoods Inc. (Markham, ON) in Saskatchewan. The compounds 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-di[3-ethylbenzthiazolinesulfonate (ABTS), 2,2′-azobis-(2-methylpropionamidine) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), Folin−Ciocalteu phenol reagent, sodium carbonate, monobasic potassium phosphate, dibasic potassium phosphate, human low-density lipoprotein (LDL) cholesterol, hydrogen peroxide, ethylenediaminetetraacetic acid (EDTA), ferric chloride, fluorescein, ferrozine, ferrous chloride, sodium bicarbonate, deoxyribonucleic acid (DNA, pBR 322 Plasmid, *Escherichia coli* strain RRI), Trizma base, boric acid, ethidium bromide, agarose, ferrous sulfate, copper sulfate, bromophenol blue, xylene cyanol, and glycerol were purchased from Sigma-Aldrich Chemical Co. (Oakville, ON). The solvents used were of ACS grade or better quality and were purchased from Fisher Scientific (Nepean, ON).

Preparation of Samples. Whole grains and their milling fractions, when necessary, were ground in a coffee bean grinder (model CBG5 series, Black and Decker Canada Inc., Brockville, ON) and passed through a mesh size 16 sieve (Tylor Test Sieve, Mentor, OH). Wheat flour was used as such for the extraction of crude phenolics. All samples were defatted by blending the ground material with hexane (1:5, w/v, 5 min × 3) in a Waring blender (model 33BL73, Waring Products Dividion, Dynamics Corp. of America, New Hartford, CT) at ambient temperature (21 ± 2 °C). Defatted wheat samples were air-dried for 12 h and stored in vacuum-packaged polyethylene pouches at −20 °C until used for analysis.

Extraction of Free, Esterified, and Bound Phenolics. The free phenolics, soluble esters, and insoluble-bound phenolic acids in wheat extracts were isolated according to the procedure described by Krygier et al. (*11*) and Naczk and Shahidi (*9*) with slight modifications. The defatted samples (2 g) were extracted six times with 40 mL of methanol−acetone−water (7:7:6, v/v/v) at room temperature using a Polytron homogenizer (Brinkman, 15 s, 10000 rpm). The mixtures were then centrifuged (5000*g*, 15 min), and supernatants were collected and combined. The solvent was evaporated at 30 °C under vacuum to approximately 40 mL. Concentrated supernatants were extracted with diethyl ether; phenolic acids so extracted were labeled as free phenolics. The supernatants with esterified phenolic acids were then treated with 30 mL of 4 M NaOH for 4 h at room temperature (21 ± 2 °C). The samples were flushed with nitrogen and packaged in airtight glass sample vials. The resultant hydrolyzate was acidified to pH 2 using 6 M HCl and extracted six times with diethyl ether. The ether extracts were then combined and evaporated to dryness at 30 °C under vacuum. The phenolic acids extracted were those liberated from their esters and labeled as esterified phenolic acids. The leftover meal after extractions was treated with 20 mL of 4 M NaOH for 4 h at room temperature (21 ± 2 °C). The samples were flushed with nitrogen and then acidified to pH 2 with 6 M HCl followed by centrifugation (5000*g*, 15 min). The mixture was extracted six times with diethyl ether. The ether extracts were combined and evaporated to dryness under vacuum at 30 °C. The phenolic acids so extracted were labeled as bound phenolics. Free, esterified, and insoluble-bound phenolics were dissolved separately in 2 mL of methanol and stored at −20 °C until used within 1 week.

Determination of TPC. The content of total phenolics was determined according to a modified version of the procedure described by Singleton and Rossi (*27*). Folin−Ciocalteu's reagent (0.5 mL) was added to a centrifuge tube (50 mL) containing 0.5 mL of the methanolic extract. Contents were mixed, and 1 mL of saturated sodium carbonate solution was added to each tube, followed by adjustment of the volume to 10 mL with distilled water. The contents in the tubes were thoroughly mixed by vortexing. Tubes were allowed to stand at ambient temperature for 45 min until the characteristic blue color developed; centrifugation was then carried out for 5 min at 4000*g* (ICE Centra M5, International Equipment Co., Needham Heights, MA). Absorbance of the clear supernatants was measured at 725 nm using a Hewlett-Packard diode array spectrophotometer, model 8452A (Agilent Technologies Canada Inc., Mississauga, ON). The content of total phenolics in each extract was determined by explaining a standard curve prepared using ferulic acid and expressed as micrograms of ferulic acid equivalents (FAE) per gram of defatted material.

Measurement of Total Antioxidant Capacity (TAC). The total antioxidant activity was determined according to the Trolox equivalent antioxidant capacity (TEAC) assay described by van den Berg et al. (*28*) with slight modifications. The extracts and reagents were prepared in a 0.1 M phosphate buffer (pH 7.4) containing 0.15 M sodium chloride (PBS). A solution of ABTS radical anion ($ABTS^{\bullet-}$) was prepared by mixing 2.5 mM AAPH with a 2.0 mM solution of $ABTS^{2-}$ at a 1:1 (v/v) ratio and heating at 60 °C for 12 min. The absorbance of the freshly prepared radical solution at 734 nm was about 0.4. The radical solution protected from light was stored at room temperature. A standard curve was prepared using different concentrations of Trolox. Thus, the reduction in the absorbance (ΔA) of the $ABTS^{\bullet-}$ solution (1960 μL) at different concentrations of Trolox (40 μL) over a period of 6 min was measured and plotted. The TEAC values of wheat extracts were determined in the same manner and expressed as μmol Trolox equivalents/g of defatted material. A blank was used for each measurement that corresponded to a decrease in the absorbance without any added extract. The TEAC of an unknown compound represents the concentration of a Trolox solution that has the same antioxidant capcity as the compound of concern.

TEAC values were determined as follows:

$$\Delta A_{\text{Trolox}} = (A_{t=0\text{minTrolox}} - A_{t=6\text{minTrolox}}) - \Delta A_{\text{radical}(0-6\text{min})}$$

$$\Delta A_{\text{Trolox}} = m \times [\text{Trolox}]$$

$$\text{TEAC}_{\text{extract}} = (\Delta A_{\text{extract}}/m) \times d$$

where ΔA = reduction of absorbance, A = absorbance at a given time, m = slope of the standard curve, [Trolox] = concentration of Trolox, and d = dilution factor.

DPPH Radical Scavenging Assay. The method described by Kitts et al. (*29*) was used with slight modifications in order to assess the DPPH radical scavenging capacity of wheat extracts. A 0.075 mM (final concentration) DPPH solution in ethanol was mixed with wheat extracts and vortexed thoroughly. The absorbance of the mixtures at ambient temperature was recorded for 60 min at 10 min intervals. The absorbance of the remaining DPPH radicals was measured at 519 nm using a Hewlett-Packard diode array spectrophotometer (Agilent Technologies Canada Inc., Mississagua, ON). The scavenging of DPPH was calculated according to the following equation.

$$\% \text{ scavenging} = \{(\text{Abs}_{\text{contol}} - \text{Abs}_{\text{sample}})/\text{Abs}_{\text{control}}\} \times 100$$

where $\text{Abs}_{\text{control}}$ = absorbance of DPPH radical + methanol and $\text{Abs}_{\text{sample}}$ = absorbance of DPPH radical + wheat extract/standard. The scavenging activity was expressed as μmol DPPH radical scavenged/g of defatted material.

Inhibition of Oxidation of Human LDL Cholesterol. The procedure described by Hu and Kitts (*30*, *31*) was employed in this study. LDL was dialyzed in 10 mM PBS (pH 7.4) at 4 °C in the dark for 24 h. LDL (0.2 mg LDL/mL) was mixed with different amounts of wheat extracts dissolved in 10 mM PBS. The reaction was initiated by adding a solution of cuprous sulfate (10 μM), and samples were incubated for 22 h at 37 °C. The formation of conjugated dienes was measured at 234 nm using a diode array spectrophotometer (Agilent Technologies Canada Inc.). The inhibitory effect of wheat extracts on the formation of conjugated dienes (% $\text{inhibition}_{\text{CD}}$) was calculated using the following equation. A separate blank, containing all reagents except LDL, was used for each extract.

$$\% \text{ inhibition}_{\text{CD}} = (\text{Abs}_{\text{oxidative}} - \text{Abs}_{\text{sample}}/\text{Abs}_{\text{oxidative}} - \text{Abs}_{\text{native}}) \times 100$$

where $\text{Abs}_{\text{sample}}$ = absorbance of LDL + $CuSO_4$ + wheat extract or standard, $\text{Abs}_{\text{native}}$ = absorbance of LDL + PBS, and $\text{Abs}_{\text{oxidative}}$ = absorbance of LDL + $CuSO_4$ + PBS. Using percentage values, the amount of protein (μg) that can be protected against copper-mediated oxidation by 1 g of defatted wheat samples was obtained.

Reducing Power of Wheat Extracts. The reducing power of wheat samples was determined following the method of Oyaizu (*32*) with slight modifications. The assay medium contained 2.5 mL of sample in a 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. After incubation at 50 °C for 20 min, 2.5 mL of 10% trichloroacetic acid was added to the mixture followed by centrifugation at 1750*g* for 10 min. One milliliter of the supernatant was mixed with 2.5 mL of high-performance liquid chromatography (HPLC) grade water and 0.5 mL of 0.1% ferric chloride, and the absorbance of the resultant solution was read at 700 nm. A standard curve was prepared using various concentrations of ascorbic acid, and the reducing power was expressed as μmol ascorbic acid equivalents/g of defatted material.

Determination of Oxygen Radical Absorbance Capacity (ORAC) of Wheat Extracts. The ORAC of wheat extracts was determined according to the method of Dávalos et al. (*33*) using 75 mM phosphate buffer (pH 7.4) at 37 °C. A FLUOstar OPTIMA microplate reader (BMG LABTECHNOLOGIES GmbH, Offenberg, Germany) equipped with FLUOstar OPTIMA evaluation software version (1.30-0) and black, polystyrene, nontreated 96 well microplates (Costar Corning Inc., Corning, NY) were used. The outer wells of the microplate were not used in the analysis. The excitation and emission filters were 485-P and 520-P, respectively. The final reaction mixture (200 μL) consisted of extract (20 μL), florescein (120 μL, 70 nM final concentration), and AAPH (60 μL, 12 mM final concentration). The samples were loaded into designated wells in the microplate. A gain adjustment was performed by pipetting 200 μL of florescein into a designated well before starting the program in order to optimize signal amplification. In cycle one, pump 1 was programmed to inject florescein after which the samples and solutions were incubated at 37 °C for 15 min followed by the addition of AAPH using the second pump in cycle 2. The instrument read the florescence of each well after the addition of AAPH over 100 cycles. All of the measurements were expressed relative to the initial reading. The results were calculated using the differences in area under the florescein decay curve between the blank and the sample and expressed as μmol Trolox equivalents/g of defatted material. The standard curve was prepared using a 1−25 μM Trolox solution (final concentration).

Measurement of Iron(II) Chelating Activity of Wheat Extracts. The Fe(II) chelating activity of wheat extracts was measured following the procedure reported by Carter (*34*). The reaction was performed in an aqueous medium. The wheat extracts (2.0 mL) were mixed thoroughly with a 2 mM $FeCl_2$ solution (0.2 mL) and 0.4 mL of 5 mM ferrozine. The mixtures were allowed to stand at room temperature for 10 min. The absorbance of the resultant solution was read at 562 nm. The Fe(II) chelating activity of wheat extracts was calculated as follows.

$$\text{iron(II) chelating activity (\%)} = \{1 - (\text{absorbance of sample at 562 nm}/\text{absorbance of control at 562 nm})\} \times 100$$

The iron(II) chelating capacity of samples was expressed as μg EDTA equivalents/g of defatted material using a standard curve prepared with EDTA.

Evaluation of Antioxidant Activity of Wheat Extracts Using Photochemiluminescence (PCL). The antioxidant capacity of lipid-soluble (ACL) compounds was assessed using PHOTOCHEM equipment (Analytik Jena USA, Delaware, OH). The extracts were dissolved in methanol followed by centrifugation (4000*g*, 5 min). The supernatants were used in the determination of antioxidant activity with further dilution using respective solvents, if necessary. Analyses were carried out in 0.1 M carbonate buffer (pH 10.5) (*35*−*37*). The photosensitizer luminol (Reagent 3) was procured from Analytik Jena USA. The antioxidant activity of lipid-soluble compounds is expressed as μmol α-tocopherol equivalents/g of defatted material.

Measurement of Antioxidant Activity of Wheat Extracts Using Rancimat. The oxidative stability of fats and oils in the presence or absence of wheat extracts was determined using an automated Metrohm Rancimat apparatus (model 743, Herisen, Switzerland) capable of operating over a temperature range of 50−200 °C. The oxidative stability of stripped corn oil (SCO) was determined at 100 °C. The glassware was thoroughly cleaned and dried prior to each determination. Samples of oil or fat were weighed directly into the reaction vessels. Wheat extracts were added at 0.25 mL/g oil or fat concentration. The air flow rate through the sample was adjusted to 20 L/h. The volatile reaction products released during oxidation of oil or fat sample were collected in 60 mL of distilled water in the collection vessel. The change in the conductivity was plotted automatically until the end point was reached. With each oil or fat, a control test (with no additives) was included and subjected to the same experimental conditions. The oxidative stability was measured in duplicate for each sample, and the induction period (IP; h) was recorded. The relative activity on the antioxidant compounds was expressed as the protection factor (PF) where, PF = IP of oil with additives/IP of the control (no additive) (*38*).

Inhibition of Strand Breaking of Supercoiled DNA by Wheat Extracts. DNA strand breaking by hydroxyl radical was examined according to the method described by Johnson and Grossman (*39*) and Hiramoto et al. (*40*) with slight modifications. The reaction was carried out in 1 M phosphate buffer (pH 7.4). The reaction mixture contained 2 μL of phosphate buffer, 4 μL of a solution of extract, 2 μL of a solution of supercoiled plasmid pBR 322 DNA (4300 base pairs) at 100 μg/mL, 6 μL of 0.33 mM H_2O_2, and 6 μL of 0.33 mM $FeSO_4$ added in the order stated. The reaction was carried out in an Eppendorf tube (1 mL) and incubated at 37 °C for 1 h. Simultaneously, the plasmid DNA was also incubated with the restriction endonuclease Hind III. [Hind III has one restriction site on the pBR 322 plasmid DNA thus producing one fragment having the original number of base pairs. The

Table 1. Free, Esterified, and Bound Phenolics Contents (μg FAE/g Defatted Material) of Whole Grains, Flour, and Bran of Hard and Soft Wheat[a]

milling fraction	free	esterified	bound	total
HWF	137 ± 4 a	234 ± 13 a	328 ± 14 a	699
SWF	161 ± 6 b	278 ± 12 b	464 ± 16 b	903
HWW	353 ± 16 a	954 ± 34 a	2149 ± 43 a	3456
SWW	478 ± 12 b	1196 ± 59 b	2144 ± 52 b	3818
HWB	846 ± 31 a	1365 ± 63 a	11303 ± 126 a	13514
SWB	981 ± 47 b	1432 ± 42 b	12186 ± 149 b	14599

[a] Values are means of three determinations ± standard deviations. Abbreviations: HWF, hard wheat flour; SWF, soft wheat flour; HWW, hard whole wheat; SWW, soft whole wheat; HWB, hard wheat bran; and SWB, soft wheat bran. In each column, pairs carrying different supercripts are significantly ($p < -0.05$) different from one another.

reaction mixture contained 8 μL of DNA (100 μg/mL), 2 μL of Hind III restriction enzyme, 2 μL of restriction buffer (X10), and 8 μL of distilled water]. For identification, the base pair ladder DRIgest III was run along with the extracts. After incubation, 2 μL of the loading dye (0.25% bromophenol blue/0.25% xylene cyanol/50% glycerol) was added and the whole mixture was loaded on to a 0.8% (w/v) agarose gel prepared in Tris/borate/EDTA (TBE) electrophoresis buffer (pH 8.3). Agarose gel electrophoresis was performed using TBE electrophoresis buffer at 116 V for 75 min. The gel was stained with 0.5 μg/mL ethidium bromide, and bands were visualized under ultraviolet (UV) light. The images were analyzed using AlphaEase Stand Alone software (Alpha Innotech Corporation, San Leandro, CA). The protective effects of the crude extracts were measured using the retention percentage of supercoiled DNA. The control used in the experiment contained supercoiled DNA and the phosphate buffer.

Statistical Analysis. All analyses were performed in triplicate, and data were reported as means ± standard deviations, unless otherwise stated. Analyses of variance were performed using General Linear Model of Minitab Release 14 Xtra for Windows (Minitab Inc., State College, PA). Significant differences ($P < 0.05$) among means were determined using the Tukey's multiple range test at a fixed level of $\alpha = 0.05$. The relationship between TPC and other variables within the observed data range was determined as Pearson correlation coefficients in bivariate correlations.

RESULTS AND DISCUSSION

TPC and TAC of Free, Esterified, and Insoluble-Bound Phenolic Fractions. The contents of free, esterified, and insoluble-bound phenolics of whole grain, flour, and bran of hard and soft wheat samples are shown in **Table 1**. The contents of insoluble-bound phenolics were 1.4-, 2.3-, and 8.3-fold higher than those of esterified phenolics for flours, whole grains, and brans of hard wheat, respectively. The corresponding values for soft wheat were 1.7-, 0.63-, and 8.5-fold, respectively. The contents of bound phenolics of flour were 2 and 2.9 times greater than those of free phenolics in hard and soft wheats, respectively. In whole wheat, the contents of bound phenolics were 6.1- and 4.5-fold as compared to those of free phenolics for hard and soft wheats examined, respectively. The corresponding values for bran were 13.4- and 12.4-fold. Thus, the contents of bound phenolics were significantly higher than those of free and esterified fractions, especially for wheat bran.

Andreasen et al. (*13*) determined the TPCs of flour, whole grain, and bran of rye where bound phenolics were released from cell wall materials following treatment with α-amylase and subsequent saponification with NaOH. The phenolic acids were then extracted with ethyl acetate. The TPCs in whole grain and bran of rye were 5.6- and 14.8-fold, respectively, as compared to that of rye flour. Moreover, the antioxidant activity of bran extracts was significantly higher than that of whole grain and flour against copper-induced oxidation of LDL. The amount of phenolic compounds present in cereals was very low, amounting to 0.2−1.3 mg gallic acid equivalents/g of crude phenolic extract (*41*). The low antioxidant activity of cereal extracts even at a 5000 ppm level against oxidation of methyl linoleate was also reported by Kahkonen et al. (*41*). The low TPC in the above study may possibly be due to the inclusion of only free phenolics in the analysis. However, in cereals, phenolic compounds exist primarily in the bound form in association with cell wall materials (*10*−*13*, *42*−*44*). The present study also demonstrated the significant contribution of bound phenolic compounds to the TPCs of whole grains, flours, and brans of soft and hard wheats. Thus, it is apparent why cereals could exhibit low TPCs when tested only for their free phenolic acids. A similar conclusion was reached by Dewanto et al. (*45*) for corn phenolics and dominance of bound phenolics. Sosulski et al. (*10*) reported that 69.2% of TPCs in wheat was due to insoluble-bound phenolics. They also reported that levels of soluble conjugated phenolic acids in cereal flours were 2−5 times higher than those of their free phenolic acids.

In this paper, a nonspecific method was used for quantification of total phenolics. The Folin−Ciocalteu reagent interacts with a number of reducing nonphenolic substances such as certain sugars, amino acids, vitamin C, and other organic acids and thus may lead to overestimation of phenolic content (*46*). In general, identification and quantification of phenolic compounds may be carried out using HPLC/diode array detection or UV detection. Several phenolic compounds such as gallic, protocatechuic, *p*-hydroxybenzoic, gentisic, chlorogenic, vanillic, caffeic, syringic, *p*-coumaric, ferulic, salicylic, and *trans*-cinnamic acids have been reported in wheat (*47*). Flavonoids, which are well-known powerful antioxidants, have also been found in wheat (*44*, *46*). However, determination of phenolics using HPLC was not an objective of this study.

Antioxidant compounds, in general, are located in the bran fraction while endosperm also exhibits considerable antioxidant activity (*48*). It is unlikely that bound or insoluble phenolics are extracted from whole grains under normal extraction conditions (*47*). The TPC of whole grains and bran has been reported in several studies (*20*, *22*, *23*, *47*). In all of these studies, only free and esterified phenolics have been extracted and included in the analysis. Thus, the true TPC may be underestimated in many studies dealing with whole grains. Adom and Liu (*43*) determined the content of free and bound phenolics in the whole grains of wheat, corn, rice, and oat. Bound phenolics contributed 90, 87, 71, and 58% to the TPCs in wheat, corn, rice, and oat, respectively. In our study, bound phenolics contributed approximately 40, 60, and 80% in flours, whole grains, and brans of wheat, respectively. The free phenolics of wheat fractions ranged from 5 to 20% while esterified phenolics contributed 10−30% to TPCs.

The TAC of extracts in μmol TE/g of defatted wheat types is shown in **Table 2**. The TAC of wheat fractions of both hard and soft wheat samples revealed the significance of bound phenolics as >80% of TAC was due to bound phenolics. The contribution of free phenolics to TAC was less than 2% in all wheat fractions examined while the contribution of esterified phenolics was significantly higher as compared to that of free phenolics but significantly less than that of bound phenolics. Bound phenolics in the bran fraction contributed 8.6 and 7.8 times higher to the TAC than that of the total of free and soluble esters for hard and soft wheats, respectively. The corresponding values for flour were 5.1 and 5.2 times, respectively. This

Table 2. TAC (μmol Trolox Equivalents/g Defatted Material) of Free, Esterified, and Bound Phenolic Fractions of Whole Grains, Flour, and Bran of Hard and Soft Wheat[a]

milling fraction	free	esterified	bound	total
HWF	12 ± 0.2 a	90 ± 1.3 a	516 ± 12 a	618
SWF	15 ± 0.4 b	105 ± 2.2 b	628 ± 17 b	748
HWW	75 ± 6 a	412 ± 13 a	10718 ± 134 a	11205
SWW	86 ± 9 b	549 ± 16 b	11582 ± 183 b	12217
HWB	162 ± 11 a	3117 ± 57 a	28270 ± 221 a	31549
SWB	192 ± 9 b	4009 ± 63 b	32765 ± 279 b	36966

[a] Values are means of three determinations ± standard deviations. In each column, pairs carrying different supercripts are significantly ($p < -0.05$) different from one another.

Table 3. Scavenging Capacity (μmol/g Defatted Material) of DPPH Radical of Free, Esterified, and Bound Phenolic Fractions of Whole Grains, Flour, and Bran of Hard and Soft Wheat[a]

milling fraction	free	esterified	bound	total
HWF	1.4 ± 0.02 a	5.4 ± 0.2 a	12.5 ± 0.4 a	19.3
SWF	3.0 ± 0.1 b	6.5 ± 0.3 b	16.0 ± 0.5 b	25.5
HWW	10.4 ± 0.7 a	27.0 ± 0.4 a	249.5 ± 1.2 a	286.9
SWW	12.4 ± 0.9 b	29.1 ± 0.7 b	288.9 ± 1.4 b	330.4
HWB	41.1 ± 0.9 a	107.5 ± 1.3 a	486.0 ± 1.1 a	634.6
SWB	44.3 ± 1.2 b	115.0 ± 1.6 b	502.2 ± 1.7 b	661.5

[a] Values are means of three determinations ± standard deviations. In each column, pairs carrying different supercripts are significantly ($p < -0.05$) different from one another.

Table 4. ORAC (μmol Trolox Equivalents/g Defatted Material) of Free, Esterified, and Bound Phenolic Fractions of Whole Grains, Flour, and Bran of Hard and Soft Wheat[a]

milling fraction	free	esterified	bound	total
HWF	31 ± 2.0 a	71 ± 1.3 a	276 ± 1.9 a	378
SWF	38 ± 0.9 b	79 ± 1.0 b	300 ± 2.2 b	417
HWW	86 ± 1.6 a	342 ± 3.1 a	2978 ± 13 a	3406
SWW	98 ± 1.0 b	392 ± 3.3 b	3180 ± 24 b	3670
HWB	380 ± 2.1 a	3100 ± 23 a	10550 ± 114 a	14030
SWB	440 ± 1.3 b	3500 ± 33 b	11350 ± 174 b	15290

[a] Values are means of three determinations ± standard deviations. In each column, pairs carrying different supercripts are significantly ($p < -0.05$) different from one another.

Table 5. Antioxidant Activity (μmol α-Tocopherol Equivalents/g Defatted Material) of Whole Grains, Flour, and Bran of Hard and Soft Wheat as Evaluated by PCL in an ACL System[a]

milling fraction	free	esterified	bound	total
HWF	3.1 ± 0.2 a	8.4 ± 0.7 a	31.6 ± 1.1 a	43.1
SWF	3.4 ± 0.3 b	9.3 ± 0.9 b	36.9 ± 0.9 b	49.6
HWW	5.8 ± 0.1 a	13.2 ± 1.1 a	89.4 ± 1.4 a	108.4
SWW	6.1 ± 0.4 b	14.6 ± 0.7 b	97.6 ± 1.0 b	118.3
HWB	13.4 ± 0.9 a	29.1 ± 0.8 a	237.8 ± 2.0 a	280.3
SWB	14.2 ± 1.0 b	33.5 ± 0.9 b	269.4 ± 1.7 b	317.1

[a] Values are means of three determinations ± standard deviations. In each column, pairs carrying different supercripts are significantly ($p < -0.05$) different from one another.

difference was remarkable with whole grains, and the contributions of bound phenolics to TACs of hard and soft wheats, respectively, were 22 and 18.5 times higher than those of free and esterfied phenolics (**Table 2**). Thus, this study demonstrated the importance of bound phenolics in the TACs of wheat samples.

In this study, the samples were subjected to alkaline hydrolysis because the conditions employed have been found to be appropriate for the release of bound phenolics from the raw material (*9*). However, hydrolysis may also be carried out under acidic or enzymatic conditions. Nonetheless, the latter conditions do not allow classification of phenolics into free, esterified, and insoluble-bound forms. Further studies may also be required to compare results with those carried out under true physiological conditions, which may not lead to full release of all phenolics present.

DPPH Radical Scavenging Capacity of Wheat Phenolics. The scavenging of the stable DPPH radical was used in the evaluation of antioxidant activity of free, esterified, and bound phenolic fractions of soft and hard wheat. Scavenging of DPPH radical allows evaluation of the hydrogen-donating potency of phenolic compounds (*50*). In the DPPH assay, bound phenolics of flours, whole grains, and brans of hard and soft wheats exhibited 62.7−87% scavenging of total radicals (**Table 3**). The scavenging capacity of DPPH radical was 13−14.9 and 25.9−32.9 times higher in the whole grains and bran, respectively, as compared to that of the flour. Hence, antioxidants that react directly with the DPPH radical reside mainly in the bran fraction. There was dilution of the antioxidants present in the bran by the endosperm; thus, the antioxidant activity of whole grain was significantly lower than that of the bran. The DPPH radical scavenging assay demonstrated the efficiency of wheat phenolics as hydrogen donors.

ORAC of Wheat Phenolics. The ORAC values of wheat extracts were also determined and expressed as μmol Trolox equivalents/g of defatted material (**Table 4**). The ORAC of wheat extracts was in the order of bran > whole grain > flour. The ORACs of bran were approximately 37 and 36.7 times higher than those of the flours of hard and soft wheats, respectively, while the corresponding values for whole grains were 9 and 8.8 times those of the flours. The contribution of bound phenolics in ORAC, especially brans of hard and soft wheats, was also significant. To the best of our knowledge, this is the first report on a determination of ORAC of bound phenolics of wheat.

Free radicals $ABTS^{\bullet-}$ and $DPPH^{\bullet}$ are commonly used to assess antioxidant activity in in vitro assays, although both of these radicals are foreign to biological systems (*51*). ORAC is based on free radical scavenging where different radical generators are used in producing free radicals. Recently, peroxyl radical ($ROO^{\bullet}$) has been adopted as a standard due to its common existence in biological systems (*52*). Thus, it has been stated that ORAC mimics the antioxidant activity of phenolics in biological systems since it uses biologically relevant free radicals and integrates both inhibition time and percentage of ROS by the antioxidant. Thus, ORAC is based on hydrogen atom transfer and in the presence of an antioxidant $ROO^{\bullet}$ can abstract a hydrogen atom from the antioxidant, and the reaction between $ROO^{\bullet}$ and florescein is retarded or inhibited (*53*). The hydrogen-donating properties of wheat phenolics were therefore confirmed using this method (**Table 4**).

Evaluation of Antioxidant Potential of Wheat Phenolics Using PCL. The antioxidative potential of free, esterified, and bound phenolics of whole grains, flours, and brans of hard and soft wheats as measured by PCL method is shown in **Table 5**. The highest inhibition of PCL was exhibited by phenolic fractions derived from the bran while flour exhibited the weakest

Table 6. Oxidative Stability (Expressed as a PF) of SCO in the Presence of Free, Esterified, and Bound Phenolic Extracts of Whole Grains, Flour, and Bran of Hard and Soft Wheat as Evaluated by the Rancimat[a]

milling fraction	free	esterified	bound
HWF	1.12	1.31	1.82
SWF	1.16	1.33	1.89
HWW	1.19	1.61	2.19
SWW	1.21	1.73	2.36
HWB	1.9	2.26	3.93
SWB	2.1	2.4	4.43

[a] Values are averages of two determinations, which were always within a maximum of 5% of each value.

antioxidant effect. The inhibition of PCL varied from 13.4 to 237.8 and 14.2 to 269.4 μmol of α-tocopherol equivalents/g defatted material, respectively, for brans of hard and soft wheats. The contribution from bound phenolics of hard and soft wheat brans was >84%. The PCL assay is based on temporary inhibition of a photoinduced, superoxide radical anion-mediated chemiluminescence originating from the oxidation of luminol (*35*). In the presence of an antioxidant, chemiluminescence may be delayed and the resulting integral in the ACL system is indicative of the radical scavenging potential of the compound. The method is quite sensitive and hence allows detection of low concentrations of antioxidants relative to a reference compound (*54*). In PCL, both hydrophilic and hydrophobic antioxidants present in the extracts can be evaluated. This study reports for the first time the antioxidant activity of whole grains, flours, and brans of wheat using the PCL method.

Evaluation of Antioxidant Potential of Wheat Phenolics Using the Rancimat Method. The influence of free, esterified, and bound phenolics of wheat on the oxidation of SCO was determined at 100 °C using the Rancimat method. The bound phenolics were more effective in extending the induction period and reducing autoxidation of virtually antioxidant-free corn oil at elevated temperatures than free and esterified phenolics (**Table 6**). The PF obtained for the bran fraction of both hard and soft wheats was remarkable and indicated the concentration of bioactive components in the external layers of the grain. Oxidation of lipids, which is the main cause of quality deterioration in many food systems, may lead to off-flavor development and formation of toxic compounds that compromise the quality and nutritional value of foods. Moreover, lipid oxidation products have been known to be associated with aging, membrane damage, heart disease, and cancer (*55*). Although synthetic antioxidants such as butylated hydroxytoluene, butylated hydroxyanisole, tertiary-butylhydroquinone, and propyl gallate have widely been used in retarding lipid oxidation, their safety has recently been questioned (*56*). Thus, there is much interest in the development of safer antioxidants using natural extracts from oilseeds, spices, and other plant materials (*57*). In general, phenolic acids and flavonoids, among others, have been recognized to confer stability to vegetable oils against autoxidation (*57*).

Inhibition of Oxidation of LDL by Wheat Phenolics. An increased level of LDL is associated with an increased risk of atherosclerosis and cardiovascular disease. LDL is the major cholesterol carrier in the blood, and although LDL may not form atherosclerotic plaques in its native form, its oxidative modification leads to the formation of key factors in pathogenesis of atherosclerosis (*58*). Hence, dietary antioxidants that inhibit LDL oxidation may prevent atherosclerosis as well as cardiovascular diseases (*59*). The bound phenolics of whole grain and bran and esterified phenolics of bran of hard and soft wheats completely inhibited (100%) copper-induced oxidation of LDL at concentrations tested. Among various fractions of phenolics of flours of hard and soft wheats, only the bound phenolics exhibited at least 50% inhibition in the LDL assay (data not shown). A low concentration of bioactive compounds in the flour fraction is responsible for its low activity.

Table 7. Inhibition of Oxidation of LDL (μg Protein/g Defatted Material) by Free, Esterified, and Bound Phenolic Extracts of Whole Grains, Flour, and Bran of Hard and Soft Wheat[a]

milling fraction	free	esterified	bound
HWF	1472	3584	6502
SWF	1997	4634	7373
HWW	3315	4551	25600
SWW	3795	4691	25600
HWB	4845	12,800	>25600
SWB	4978	12,800	>25600

[a] Values are averages of two determinations, which were always within a maximum of 5% of each value.

Table 8. Retention (μg DNA/g Defatted Material) of pBR 322 Supercoiled DNA against Hydroxyl Radical-Mediated Single Strand Scission by Free, Esterified, and Bound Phenolic Fractions of Whole Grains, Flour, and Bran of Hard and Soft Wheat[a]

milling fraction	free	esterified	bound
HWF	110	240	920
SWF	150	290	880
HWW	270	450	1300
SWW	290	470	1380
HWB	430	590	1660
SWB	470	560	1740

[a] Values are averages of two determinations, which were always within a maximum of 5% of each value.

The inhibition of oxidation of LDL, expressed as μg protein retained/g of defatted soft or hard wheat, is shown in **Table 7**. Brans of both hard and soft wheats exhibited the highest retention capacities followed by the whole grains and flour. The bound phenolics of brans, whole grains, and flours contributed significantly to inhibition of LDL oxidation as compared to that of free and esterified phenolics. The free phenolic fraction possessed the lowest ability to inhibit oxidation of LDL among the three types of phenolics present. The inhibition of LDL oxidation by bound phenolics was 5.3 and 5.1 times higher than that of free phenolics for hard and soft wheat brans, respectively. The corresponding values for whole wheat and flour were 7.2 and 6.7 times and 4.4 and 3.7 times, respectively. Moreover, the retention capacity of bound phenolics of soft and hard wheat brans was 3.9 and 3.5 times higher than those of bound phenolics of the flours, respectively. Results indicated the significance of bound phenolics of wheat against LDL oxidation.

Effects of Wheat Phenolics on HO$^\bullet$-Mediated DNA Scission. Table 8 shows the effects of free, esterified, and bound phenolic extracts of whole grains, flours, and brans of hard and soft wheats in inhibiting DNA strand cleavage by the Fenton reaction-mediated hydroxyl radical (HO$^\bullet$). The results are expressed as the amount of DNA retained by wheat (μg DNA/g defatted wheat). The free, esterified, and bound phenolic extracts of whole grains, flours, and brans of hard and soft wheats differed considerably in their ability to protect DNA from nicking by HO$^\bullet$. The HO$^\bullet$ cleaved supercoiled plasmid pBR

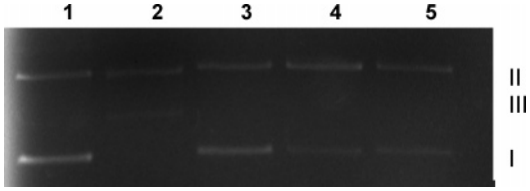

Figure 1. Agarose gel electrophoresis of supercoiled DNA treated with hydroxyl radical in the presence of free, esterified, and bound phenolics of flour of hard wheat (lane 1, supercoiled DNA, control; lane 2, supercoiled DNA + $OH^{\bullet}$; lane 3, supercoiled DNA + $OH^{\bullet}$ + bound phenolics; lane 4, supercoiled DNA + $OH^{\bullet}$ + esterified phenolics; lane 5, supercoiled DNA + $OH^{\bullet}$ + free phenolics; form I, supercoiled DNA; form II, nicked open circular DNA; and form III, linear DNA).

Table 9. Iron(II) Chelation Capacity (μg EDTA Equivalents/g Defatted Material) of Free, Esterified, and Bound Phenolic Fractions of Whole Grains, Flour, and Bran of Soft and Hard Wheat[a]

milling fraction	free	esterified	bound
HWF	304 ± 15 a	914 ± 41 a	4270 ± 101 a
SWF	398 ± 21 b	1074 ± 56 b	4802 ± 122 b
HWW	639 ± 43 a	1829 ± 99 a	7064 ± 135 a
SWW	794 ± 39 b	2158 ± 81 b	8310 ± 111 b
HWB	1238 ± 76 a	1945 ± 76 a	9459 ± 205 a
SWB	1563 ± 64 b	2487 ± 33 b	10572 ± 221 b

[a] Values are means of three determinations ± standard deviations. In each column, pairs carrying different supercripts are significantly ($p < -0.05$) different from one another.

DNA completely into nicked circular and linear DNA in the absence of antioxidants (**Figure 1**, lane 2). The bound phenolics of bran of hard and soft wheats were most effective in inhibiting nicking and retained 1660 and 1740 μg DNA/g of defatted wheat bran, respectively. Moreover, the esterified phenolics of bran and bound phenolics of whole grain of both wheat types inhibited DNA scission by retaining 560−590 μg DNA/g. The free phenolics of flour demonstrated weak activity against protecting DNA from oxidation and the retention capacities varied from 110 to 150 μg DNA/g for hard and soft wheats, respectively.

Antioxidant properties of a compound may be evaluated by monitoring $HO^{\bullet}$-induced single strand breaks in DNA in the presence of that compound (*59*). Ascorbate and Fe(III) together form a H_2O_2 and $HO^{\bullet}$ generating system that readily induces DNA strand cleavage. The bound phenolics of bran effectively prevented the cleavage of supercoiled DNA induced by Fenton reactants, demonstrating their scavenging of $HO^{\bullet}$. In general, $HO^{\bullet}$ is a highly reactive ROS formed in biological systems. It can react with biological molecules such as lipids, sugars, phospholipids, proteins, and nucleic acids, among others (*60*).

Iron(II) Chelation Capacity of Wheat Phenolics. The iron-(II) chelating capacity of free, esterified, and bound phenolics of whole grains, flours, and brans of hard and soft wheat samples was determined by measuring the iron−ferrozine complex (**Table 9**). Despite its significant role in oxygen transport, respiration, and as a cofactor in the activity of several enzymes, iron is an extremely reactive metal that catalyzes oxidative changes in lipids, proteins, and nucleic acids, among others (*61*). Any compound that can chelate ferrous ions may render them inactive or poorly active in the Fenton reaction as in the DNA scission assay.

The bound phenolics of bran of hard and soft wheats demonstrated considerable chelating properties and were 9459 and 10572 μg EDTA equivalents/g of defatted material, respectively (**Table 9**). The free phenolics of soft and hard wheats did not chelate Fe(II) efficiently. The Fe(II) chelation capacity of free, esterified, and bound phenolics may also be explained by considering the TPCs of these extracts. Thus, there was a positive association between the TPC and the Fe (II) chelation property. When wheat samples were subjected to alkaline hydrolysis, the phenolic aglycons are released and they may participate in Fe(II) chelation. When phenolic acids exist in esterified or bound form, their Fe(II) chelating properties may be hindered. In general, glycosylation of phenolic compounds is known to diminish their antioxidant activity as measured by the aqueous radical trapping capacity (*62*) as the OH group is not available for reaction. The same phenomenon may also be observed for Fe(II) chelation.

Conclusions. The content of bound phenolics in the hard and soft wheat samples examined in the current study was significantly higher than that of free and esterified phenolics, and this corresponded to their higher contribution to the TAC and antioxidant potential in the latter groups. The correlation analysis between phenolic contents and results of other antioxidant assays demonstrated a strong positive association ($r > 0.9$, $p < 0.01$, or $p < 0.05$). Despite the existing differences in the mechanism of actions involved in different assays, similar trends were observed with respect to the antioxidant potential of wheat samples tested. Therefore, inclusion of bound phenolics in the estimation of phenolic content and evaluation of their antioxidant effects are important. In addition, as antioxidants exert their effects via different mechanisms, such as free radical scavenging, electron donation, and chelation of metal ions, use of a range of tests representing different action mechanisms is essential for evaluation of antioxidant efficacy of food phenolics. Finally, retention of bran layers in wheat products is essential for taking advantage of these benefits.

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