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Ferulic Acid from Aleurone Determines the Antioxidant Potency of Wheat Grain (*Triticum aestivum* L.)

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Grain is an important source of phytochemicals, which have potent antioxidant capacity. They have been implicated in the beneficial health effect of whole grains in reducing cardiovascular disease and type 2 diabetes. The aim of the present study was to identify the most important antioxidant fractions of wheat grain. It was found that the aleurone content of these fractions was highly correlated with the antioxidant capacity of the fractions ($r = 0.96$, $p < 0.0001$). Ferulic acid appeared to be the major contributor to the antioxidant capacity in fractions with higher antioxidant capacity. The contribution of protein was rather limited. It was concluded that the antioxidant potency of wheat grain fractions is predominantly determined by aleurone content, which can be attributed to the presence of relatively large amounts of phenolic compounds, primarily ferulic acid.

KEYWORDS: ***Triticum aestivum* L.; antioxidant capacity; aleurone; ferulic acid; TEAC (Trolox equivalent antioxidant capacity); polyphenols**

INTRODUCTION

Epidemiological studies strongly suggest that whole grain consumption can reduce the incidence of diet related disorders such as cardiovascular disease, type 2 diabetes, and some types of cancers (*1–6*). Grain is an important source of phytochemicals (*7*). Some of them have marked antioxidant activity, as is the case with vitamins (vitamin E, tocotrienols), phenolic compounds (phenolic acids, lignans, and flavanoids), trace minerals (selenium and manganese), and phytic acid (*1, 7–10*). The potential health benefit of antioxidants is associated with protection against oxidative stress (*11, 12*). This is defined as an imbalance in the production of reactive molecules, such as oxygen and nitrogen reactive species, with the capacity for the elimination of these molecules, in favor of the former (*13, 14*). An excess of reactive species will lead to oxidative damage and altered intracellular signaling, for instance, by triggering the activation of serine/threonine kinase cascades such as c-Jun N-terminal kinase, nuclear factor-kB, and others (*15*). In this way, oxidative stress can result in chronic inflammation and possible insulin resistance, leading to the aggravation of type 2 diabetes. Reduction of oxidative stress mediated damage may therefore be implicated in the molecular basis of the reported health benefit of grain (*11, 12, 15*).

Traditionally, the milling process of grain aimed at the refinement of flour and the removal of bran as byproduct (*16*). However, several studies have shown the abundant presence of micronutrients and phytochemicals in bran (*7, 17, 18*). Current pretreatments and new debranning processes preceding milling have been developed in order to obtain innovative wheat fractions. These fractions vary in their composition of micronutrients and phytochemicals (*19*).

The objective of this study was to examine the total antioxidant capacity of different wheat fractions and its distribution within wheat grain. By identifying the most important fractions, the health benefit associated with whole grain can be optimized. Several studies have been performed on the antioxidant capacity of cereals and their milling fractions (*17, 18, 20–22*). Most have focused on the quantification of different antioxidant compounds; however, their relative contribution to the total antioxidant capacity has not been fully evaluated. We determined the total antioxidant capacity of several fractions by the Trolox equivalent antioxidant capacity (TEAC) assay (*23*), which has been designed to quantify the summed activity of all of the antioxidants in a sample. Furthermore, the relative contribution of protein and phenolic compounds to the total antioxidant capacity was also studied.

MATERIALS AND METHODS

Materials. Ferulic acid (4-hydroxy-3-methoxycinnamic acid), ABTS (2,2′ azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)), Bradford reagent, BSA (bovine serum albumin, faction V), gallic acid (97% purity), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were provided by Sigma-Aldrich Canada Ltd. (Oakville, ON). ABAP (2,2-azobis(2-aminopropane) hydrochloride) was obtained from Polyscience

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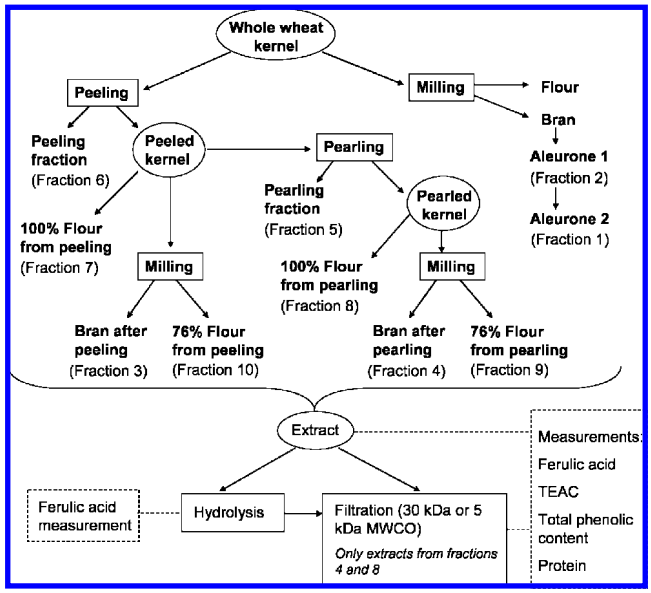

Figure 1. Flowchart of the experimental setup.

(Warrington, PA). Amicon Ultra Millipore Spin columns of 30 and 5 kDa molecular weight cutoff (MWCO) were purchased from Vivascience AG (Hannover, Germany). All chemicals used in the study were of analytic grade quality.

Sample Preparation. Wheat samples (*Triticum aestivum* L.) from the wheat cultivars Tiger and Crousty were provided by Bühler AG Uzwil, Switzerland. The samples were provided as milled fractions.

Aleurone 1 (fraction 2) is a bran fraction enriched in aleurone cells, obtained by grinding and air classification. Aleurone 1 is further purified by electrostatic separation to give aleurone 2 (fraction 1). Details of the preparation of the aleurone fractions can be found in the patent application (*24*).

Bran and flour fractions were obtained by two different debranning processes before milling: peeling and pearling. In the peeling process, bran is removed by friction of the outermost skin of the wheat kernel. This bran fraction is the peeling fraction (fraction 6) that constitutes approximately 4% of the wheat kernel (*25*). The peeled wheat kernels are milled to 76% flour (fraction 10) and bran after peeling (fraction 3). In the pearling process, bran is removed by abrasion from peeled wheat kernels. This bran fraction is the pearling fraction (fraction 5) that constitutes approximately 3% of the wheat kernel (*26, 27*). The pearled wheat kernels are milled to 76% flour (fraction 9) and bran after pearling (fraction 4). Peeled and pearled wheat kernels were used to obtain 100% flour from peeling (fraction 7) and 100% flour from pearling (fraction 8), respectively. **Figure 1** shows an overview of the tested samples. The description and aleurone content of the different wheat fractions is given in **Table 1**. Aleurone content was determined as previously described (*28, 29*).

Sample Extraction. The extraction procedure has been described in a comparative study of different extraction methods of free and bound phenolic compounds (*30*). The selected extraction method showed the highest results in antioxidant capacity. Minor modifications were made in order to optimize the extraction. Briefly, samples were extracted by mixing 0.5 g of sample with 25 mL of diluted hydrochloric acid (pH 1) for 30 min, continuously shaking in a water bath at 80 °C. After centrifugation at 3000*g* for 10 min, the supernatant was removed. The extraction was repeated twice, and the supernatants were pooled. This procedure was designed for the determination of total antioxidant capacity in fractions with a wide range of antioxidant capacity.

A part of the extract (5 mL) was mixed with 1 mL of 2 M NaOH and incubated for 1 h at room temperature (22 ± 3 °C) under a N_2 atmosphere. After this alkaline hydrolysis, extracts were acidified to pH < 2 using 6 M HCl.

In order to remove protein, extracts and hydrolyzed extracts were filtrated using spin columns of 30 and 5 kDa molecular weight cutoff (MWCO) centrifuged at 3000*g* for 20 min.

Total antioxidant capacity, total phenolic content, total soluble ferulic acid, and protein were determined in the various extracts.

Determination of the Trolox Equivalent Antioxidant Capacity (TEAC). Total antioxidant capacity was analyzed in the extracts of all of the wheat fractions using the TEAC assay described by van den Berg et al. (*23*) with minor modifications. Briefly, ABTS•- radicals were prepared by mixing 2.5 mM ABAP with 20 mM $ABTS_2^+$ stock solution in phosphate buffered saline (PBS). The solution was heated for 18 min at 60 °C until a maximum absorbance of 0.65 ± 0.05 at 734 nm was reached. A fresh ABTS•- radical solution was prepared each day. Extracts, in a suitable and similar dilution, were added to the ABTS/ABAP solution, and the reduction in absorbance was measured after 6 min. The TEAC of the extract corresponds to the concentration of a Trolox solution that causes an equal decrease in absorbance at 734 nm. The antioxidant capacity of a sample was expressed as μmol Trolox equivalent per gram of sample (μmol TE/g).

Determination of Total Phenolic Content. The total phenolic content of the extract was determined using the method described by Singleton et al. (*31*). Briefly, an appropriate dilution of the extract was mixed with Folin−Ciocalteu reagent, and the mixture was neutralized with sodium carbonate. The absorbance was measured after 1 h at 765 nm. Gallic acid was used as the standard, and total phenolic content was expressed as μmol of gallic acid equivalents per gram of sample (μmol GAE/g).

Determination of Ferulic Acid. Ferulic acid was determined by using HPLC with diode array detection of the UV absorption, on the basis of the method described by Mattila et al. (*32*) with minor

Table 1. Description of the Different Fractions of the Wheat Cultivars Tiger and Crousty

	no.	fraction	description
aleurone fractions	1	aleurone 2	Aleurone fraction high purity. Aleurone content of 75−90%.
	2	aleurone 1	Aleurone fraction. Aleurone content of 55−70%.
bran fractions	3	bran after peeling	Bran fraction of the milling process of peeled wheat kernels. Aleurone content of 40−50%.
	4	bran after pearling	Bran fraction of the milling process of pearled wheat kernels. Aleurone content of 35−40%.
	5	pearling fraction	Bran fraction that comes off during the pearling of peeled wheat kernels. Aleurone content of 30−35%.
	6	peeling fraction	Bran fraction which comes off during the peeling process. Aleurone content of 24−25%.
flour fractions	7	100% flour from peeling	Flour from the whole kernels after peeling. Aleurone content of 7−8%.
	8	100% flour from pearling	Flour from the whole kernels after peeling and pearling. Aleurone content of 5−6%.
	9	76% flour from pearling	White flour from milling of peeled and pearled kernels. Aleurone content of 1−2%.
	10	76% flour from peeling	White flour from milling of peeled kernels. Aleurone content of 1−2%.

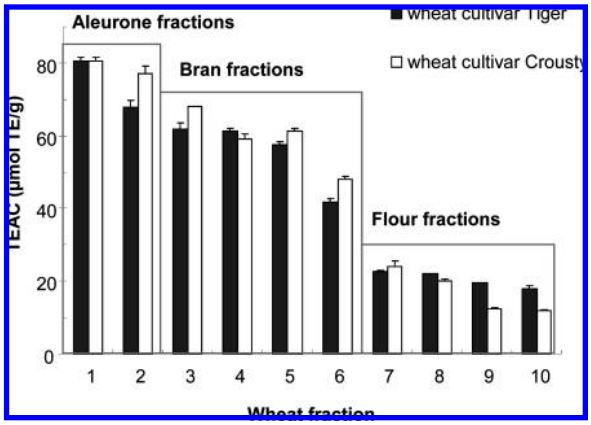

Figure 2. Total antioxidant capacity (TEAC) of different wheat fractions of the wheat cultivars Tiger and Crousty.

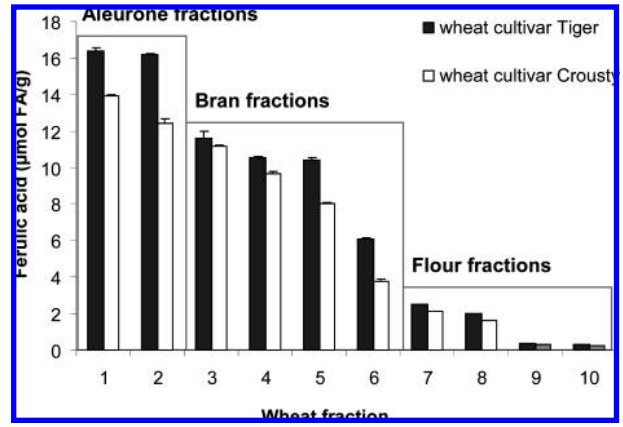

Figure 3. Total soluble ferulic acid content (μmol FA/g) of different wheat fractions of the wheat cultivars Tiger and Crousty.

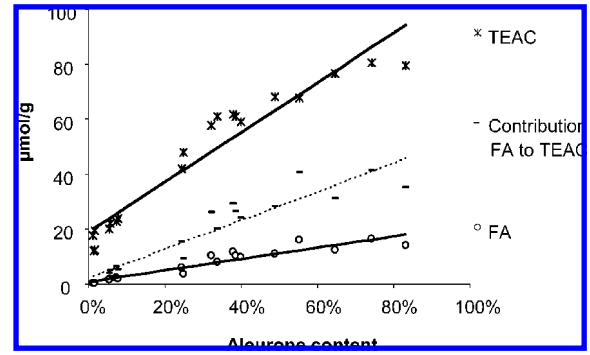

Figure 4. Correlation of aleurone content in the fraction with the content in total soluble ferulic acid (FA) and the antioxidant capacity (TEAC). The dotted line shows the contribution of ferulic acid to the TEAC of the fraction.

modifications. The separation was achieved using a Hypersil BDS 5 C18 column. The solvents consisted of 0.085% (w/v) of H_3PO_4 in water (solvent A) and acetonitrile (solvent B). The linear gradient used was 0 min 90% A, 13 min 78% A, 14.0 min 60% A, and 15 min 90% A. The flow rate was 1.0 mL/min, volume of injection was 10 μL, and temperature of the column was 30 °C. Detection was performed at 330 nm. Ferulic acid concentrations of the extracts were extrapolated from a pure *trans*-ferulic acid standard curve.

Protein Determination. Protein was determined in the extracts according to Bradford (*33*). Absorbance was measured at 595 nm and related to calibration standards of bovine serum albumin (BSA).

Calculation of the Contribution of Ferulic Acid and Protein to the Total Antioxidant Capacity. Standard solutions containing different concentrations of ferulic acid were added to the $ABTS^{\bullet-}$ radical solution (prepared as described above), and the resulting reduction in absorbance was measured at 734 nm and related to that of Trolox. The TEAC of ferulic acid shows the antioxidant potency of ferulic acid relative to Trolox on a molar basis. Knowing the concentration of ferulic acid of the extract and the TEAC of ferulic acid, the antioxidant capacity due to ferulic acid in the mixture can be calculated by multiplying the concentration of ferulic acid by the TEAC of ferulic acid.

The contribution of protein to the total antioxidant capacity was estimated from the decrease in TEAC after protein removal. Protein was removed by filtration as previously described in the sample extraction.

Statistical Analysis. Data are reported as the mean and relative percentage difference (RPD) of duplicate analyses. Pearson's correlation test was performed using SPSS 14.0 windows software. Statistical significance was declared at $p < 0.05$.

RESULTS AND DISCUSSION

Total Antioxidant Capacity. The total antioxidant capacity, determined by TEAC, was unevenly distributed over the various fractions of wheat grain. Similar fractions from the wheat cultivars Crousty and Tiger showed no substantial differences in antioxidant capacity (**Figure 2**). The highest TEAC values were found in the aleurone fractions, followed by the bran fractions; the flour fractions had the lowest TEAC values.

The bran fractions were obtained using different debranning processes before milling, i.e., peeling or pearling. The peeling fraction displayed the lowest antioxidant capacity of the bran fractions (**Figure 2**). The peeling fraction contains the outermost layers of the bran and less of the aleurone layer than the other bran fractions (**Table 1**). Thus, within the bran fractions, the antioxidant capacity was in line with the aleurone content of the fraction. In general, there was a very strong correlation between the aleurone content (**Table 1**) and the antioxidant capacity of all fractions ($r = 0.962$, $p < 0.0001$) (**Figure 4**).

Aleurone is a monolayer of cubic cells that form the tissue overlaying the endosperm. Because of its high adherence to the pericarp, aleurone is mainly found in bran fractions after the milling (*34, 35*). From a nutritional point of view, aleurone is an important source of dietary fiber, minerals, B-vitamins, proteins, phytate, and phenolic compounds (*35–38*). In fractions with little or no aleurone content, some antioxidant capacity was also found. This demonstrates the presence of antioxidants in other tissues of wheat grain. Nevertheless, the results indicate that the content of aleurone obtained in the fractionation of grain is the major determinant of the antioxidant capacity of the fraction.

Ferulic Acid and Contribution to the Total Antioxidant Capacity. There was a large variation in ferulic acid content, determined as total soluble ferulic acid, among the various wheat fractions (**Figure 3**). A strong correlation was found between the antioxidant capacity (TEAC) and ferulic acid ($r = 0.960$, $p < 0.000001$). The TEAC of ferulic acid was found to be 2.5 ± 0.1. This means that 2.5 μmol of Trolox is needed to scavenge the same amount of $ABTS^{\bullet-}$ radicals than 1 μmol of ferulic acid. In other words, ferulic acid has 2.5 times higher antioxidant capacity than Trolox, for which the TEAC value is 1. Using the TEAC value of ferulic acid and its concentration, the contribution of ferulic acid to the antioxidant capacity was calculated for each fraction. The results show that the contribution of ferulic acid to the antioxidant capacity was larger in the aleurone fractions (41−60%) than in the bran fractions (20−47%). In the flour fractions, the antioxidant capacity was low, and the contribution of ferulic acid to the total antioxidant capacity was limited (4−28%).

The highest ferulic acid concentrations were found in the extracts of the aleurone fractions, and a strong correlation was

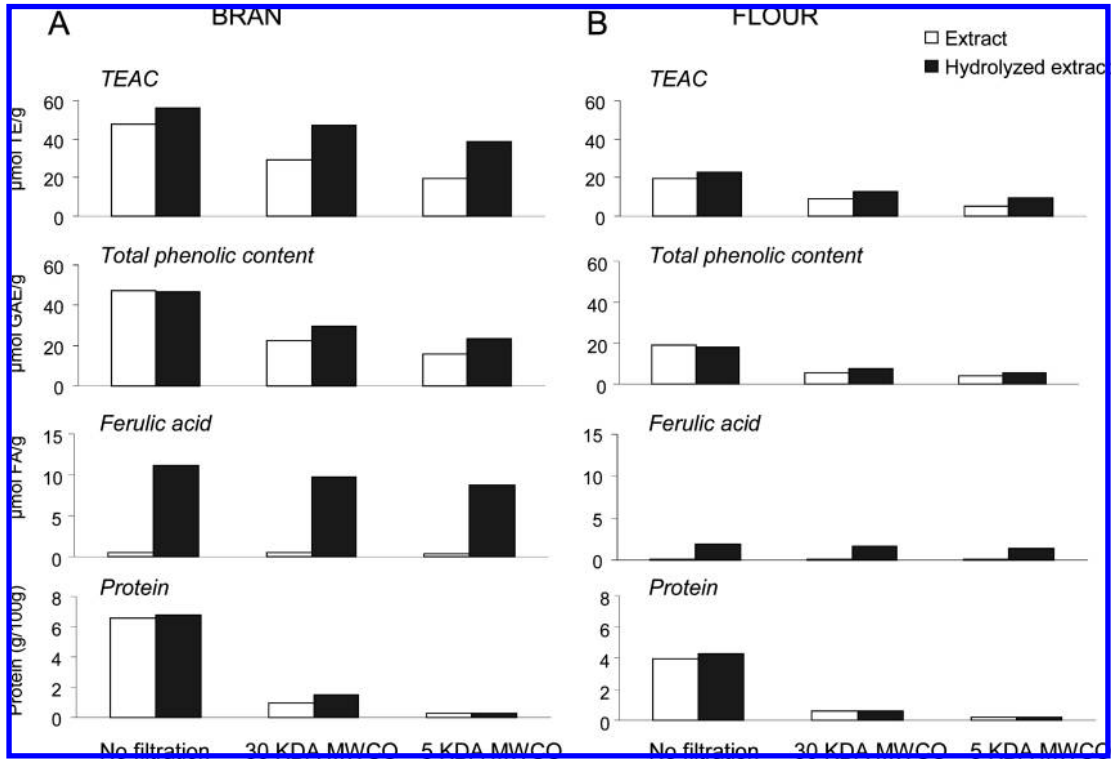

Figure 5. Antioxidant capacity (TEAC) (μmol TE/g), total phenolic content (μmol GAE/g), ferulic acid (μmol FA/g), and protein (g/100 g) in extracts and hydrolyzed extracts of bran fraction 4 (bran after pearling) (**A**) and flour fraction 8 (100% flour from pearling) (**B**) before and after filtration with 30 or 5 kDa of molecular weight cutoff (MWCO). Results are expressed as the mean of duplicate determinations with RPD < 10%.

found between ferulic acid and aleurone content ($r = 0.936$, $p < 0.0001$) (**Figure 4**). This can be explained by the localization of ferulic acid as a structural component in the cell walls of aleurone cells (*39*). Among the wide variety of phenolic compounds, ferulic acid is the most abundant in wheat. In wheat grain, ferulic acid is found free or bound by ester and ether linkages to complex carbohydrates or proteins (*40*). Several studies have reported the occurrence of bound ferulic acid in cereals (*7, 40*). This observation was confirmed in the present study. Bound ferulic acid was released after hydrolysis, resulting in a large increase in free ferulic acid in the hydrolyzed extracts, e.g., from 0.5 to 11.1 μmol/g (bran fraction 4) and from 0.1 to 1.9 μmol/g (flour fraction 8).

In the hydrolyzed extracts, the antioxidant capacity (TEAC) was also increased. This can be explained by the increase in free ferulic acid and possibly other phenolic compounds that are released from their bound forms by hydrolysis. Binding of antioxidants has been reported to reduce their antioxidant capacity (*41–43*); esterification and dimerization of ferulic acid affect the antioxidant capacity of the resulting structure (*44*). Merely from the increase in ferulic acid ($\sim$10 μmol ferulic acid/g bran), an increase in antioxidant capacity of at least $\sim$25 μmol TE/g bran (i.e., the increase in ferulic acid multiplied by the TEAC value of ferulic acid) was expected. However, only an increase of $\sim$10 μmol TE/g bran was detected (**Figure 5A**). Release of bound ferulic acid did not result in the expected increase of antioxidant capacity from the rise in free ferulic acid. From these results, we can conclude that ferulic acid, still bound to other molecules, has a substantial antioxidant capacity. However, it should be noticed that binding of ferulic acid to other molecules might limit its bioavailability and compromise the systemic health effects (*45*).

Contribution of Other Compounds to the Antioxidant Capacity. The strong correlation found between the TEAC and protein content of the various fractions ($r = 0.687$, $p < 0.001$) prompted us to investigate other potential contributors than ferulic acid to the antioxidant capacity, such as protein itself and other polyphenols. Two fractions were selected to study these possible contributors: a bran fraction (fraction 4: Bran after pearling), with high antioxidant capacity, and a flour fraction (fraction 8: 100% flour from pearling), with low antioxidant capacity.

In order to study the contribution of protein to the total antioxidant capacity, protein was removed from the extracts by filtration. Filters of two molecular weight cut offs (MWCO), 30 kDa and 5 kDa, were used for the removal of protein. The filter of 5 kDa MWCO was more efficient in removing protein, namely, 95% of the protein was removed, compared to the filter of 30 kDa MWCO that removed 85% (**Figure 5**). Filtration with the lowest MWCO had also a more pronounced effect on the antioxidant capacity and total polyphenols. The antioxidant capacity was decreased by 59% in the bran extract and by 74% in the flour extract. Also, total polyphenols were reduced by 57% in the bran extract and by 80% in the flour extract. This indicates the presence of polyphenols of high molecular weight or a strong interaction of low molecular weight phenolic compounds with protein or other large molecules that are removed in the filtration. Cereal cell walls are composed of complex polysaccharides of large molecular weight (20−300 kDa), such as β-glucans and arabinoxylans (*39*). Some studies have reported the binding of polyphenols with proteins and cell wall polysaccharides (*46, 47*). This may explain the reduction in phenolic content observed after filtration. In the hydrolyzed extracts, the effect of filtration on the antioxidant capacity and total polyphenols was relatively small, probably due to the release of polyphenols from these large molecular weight complexes.

The concentration of free ferulic acid in the extracts was not strongly affected by filtration (**Figure 5**). Filtration with 5 kDa produced a small decrease in ferulic acid of 0.5 μmol/g flour

fraction and 2.4 μmol/g bran fraction. However, because of the high TEAC value of ferulic acid, a loss of 2.4 μmol/g implicates a reduction of the antioxidant capacity of 6 μmol TE/g. In total, the antioxidant capacity decreased by 18 μmol TE/g in the bran fraction due to filtration and consequently protein removal of the hydrolyzed extract (**Figure 5A**). As 6 μmol TE/g of the 18 μmol TE/g is due to the loss in ferulic acid, apparently only 12 μmol TE/g can be attributed to the removal of protein. This represents approximately 20% of the antioxidant capacity of the not filtrated extract. From these experiments, we can conclude that the contribution of protein to the antioxidant capacity of bran is low, certainly much lower than 20% since some polyphenols were also removed along with protein. Protein is, therefore, not considered as a major contributor to the antioxidant capacity of the wheat fractions. The correlation found between protein and antioxidant capacity ($r = 0.687$, $p < 0.001$) is likely to have resulted from the high protein content of aleurone (*35–37*) together with the strong correlation of the aleurone content with the antioxidant capacity of the fraction ($r = 0.962$, $p < 0.0001$).

The results of the present study show that the wheat fractions with high aleurone content have the highest antioxidant capacity, which can be attributed to the high phenolic content and primarily to ferulic acid. Other factors besides the high antioxidant content of the wheat grain fraction are important to consider in the ultimate health effect as the effect of processing and bioavailability. The fact that ferulic acid and other phenolics occur in grain mostly bound to indigestible cell wall material may limit their bioavailability (*45, 48*). Nevertheless, it has been recently reported that wheat bran consumption increased total phenols and antioxidant capacity in plasma to a comparable extent to some other phenol-rich foods (*49*). Regarding the antioxidant capacity within the wheat kernel, aleurone is the fraction with the highest potential. Wheat fractions with the highest aleurone content might be used in cereal products to optimize the beneficial health effect associated with whole grain.

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