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# Phenolic Acids in Wheat Varieties in the HEALTHGRAIN Diversity Screen

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The amounts and compositions of free, conjugated, bound, and total phenolic acids were determined in 175 samples of wheat flour grown on a single site in 2005. The highest contents of total phenolic acids were found in flours of winter wheat (1171 μg/g) with average levels of 658 μg/g total phenolics across all of the wheat genotypes. Winter wheats showed a range of >3.5-fold across the concentration range for total phenolic acids. Spelt genotypes displayed the narrowest (1.9-fold) range of total phenolic acid concentration. The concentrations of phenolic acids in the different phenolic acid fractions were in the order bound > conjugated > free, with bound phenolic acids making up around 77% of the total phenolic acid concentration and free phenolic acids constituting between 0.5 and 1%. The results indicate that there is genetic diversity in phenolic acid content and that it should be possible to selectively breed for lines with high contents of phenolic components.

**KEYWORDS: Phenolic; acids; phenolics; wheat; ferulic acid**

## INTRODUCTION

Wheat is a major crop, and its products are an important component of the human diet, mainly because of the energy they provide, due to their high carbohydrate contents. Epidemiological studies show that the consumption of whole grain and wholegrain products reduces the incidence of chronic diseases such as diabetes (*1*), cardiovascular disease (*2, 3*), and cancer (*2, 4*−*7*), and these health benefits have been attributed in part to their unique phytochemical contents. Interest in grain phenolics has increased in recent years because of their activity as antioxidants, with total phenolic content and antioxidant activity being highly correlated (*8*−*10*). Furthermore, it has been suggested that wheats containing high contents of orthophenolic acids have antitumor properties when fed to Min mice [which have a mutant gene that results in spontaneous development of intestinal and colon tumors (*11*)]. In contrast, a high content of bound phenolics may also lead to negative impacts on the solubility of dietary fiber, by cross-linking the arabinoxylan (AX) chains (*12*).

Phenolics are compounds that possess one or more aromatic rings bearing one or more hydroxyl groups. Phenolic acids represent the most common form of phenolic compounds found in whole grains, and they constitute one of the major and most complex groups of phytochemicals in the cereal grain, with a number of types that exist in three forms: as soluble free acids, soluble conjugates that are esterified to sugars and other low molecular mass components, and insoluble bound forms (*13*). The latter are the major form in wheat and are involved in cross-linking polymers, particularly arabinoxylans in the grain cell walls [reviewed by Saulnier et al. (*12*)]. In common with many other phytochemicals they are concentrated in the bran fractions and are present at lower levels in white flour (*14, 15*).

Phenolic acids can be divided into two groups and are derivatives of either hydroxycinnamic acid or hydroxybenzoic acid (**Figure 1**). Hydroxybenzoic acid derivatives include *p*-hydroxybenzoic, protocatechuic, vannilic, syringic, and gallic acids. These acids are commonly present in insoluble bound forms and are typically components of more complex tannins and lignins. Hydroxycinnamic acid derivatives include *p*-coumaric, caffeic, ferulic, and sinapic acids. Again, these acids are present mainly in the bound forms, linked to cell wall structural components such as cellulose, lignin, and proteins through ester bonds. The most abundant phenolics acids in whole grain are ferulic, vanillic, *p*-coumaric, and syringic acids (*16*).

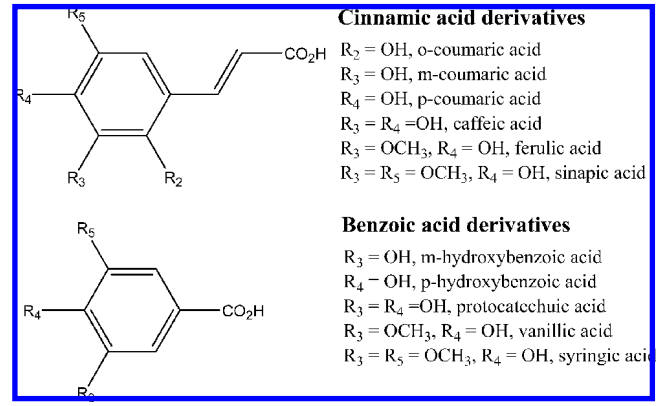


**Figure 1.** Structures of common benzoic and cinnamic acid derivatives.

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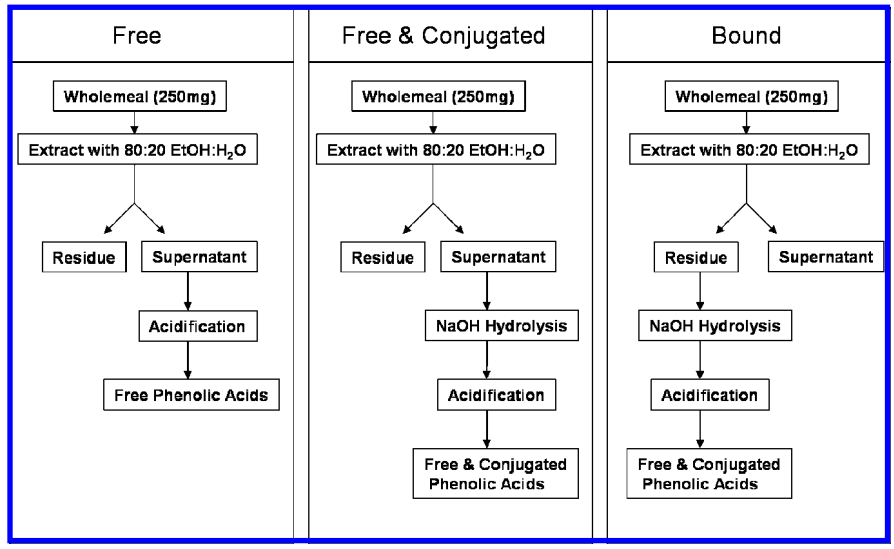


**Figure 2.** Phenolic acid extraction procedure used for analysis of the genotypes in the HEALTHGRAIN diversity screen.

Several previous studies of phenolic acids in Canadian and U.S. wheat genotypes have been reported, but these have compared relatively small numbers of lines (up to 11), although in some cases these were grown on multiple sites (*15, 17*−*20*). This paper therefore presents the first detailed survey of phenolic acids in a wide range of wheat genotypes, different from those used in previous comparative studies, which have been grown on one site to minimize environmental impacts on composition. This study was carried out as part of the EU-funded HEALTHGRAIN project, which aims to exploit the bioactivity of European cereal grains for improved nutrition and health benefits. The analysis of phenolic acids is one part of the diversity screen that was designed to determine the range of variation in phytochemicals and other bioactive components in bread wheat and related *Triticum* species.

## MATERIALS AND METHODS

**The HEALTHGRAIN Wheat Lines.** Two hundred lines were selected on the basis of the geographical origin, genetic background, and time of origin (i.e., modern varieties, early varieties, and landraces). Grain samples were obtained from seed stocks at Martonvasar (Hungary) and INRA (France) or were donated by partners of the HEALTHGRAIN project. Full details of the selection criteria and the lines are given in an accompanying paper (*21*). The full collection comprised 130 winter cultivars of bread wheat (*Triticum aestivum* var. *aestivum*), 20 spring cultivars, 5 spelts (*T. aestivum* var. *spelta*), 10 durum wheats (*Triticum turgidum* var. *durum*), and 5 lines each of cultivated einkhorn (*Triticum monococcum* var. *monococcum*) and cultivated emmer (*T. turgidum* var. *dicoccum*).

The 150 wheat lines included a number of modern high-yielding commercial varieties. For example, Hereward has been grown in the United Kingdom since 1992 because of its superior breadmaking performance. Although outclassed in terms of yield, it commands a substantial premium from millers. In contrast, Malacca is currently one of the two most widely grown beadmaking wheats in the United Kingdom, yielding about 10% more than Hereward. Claire is currently the most successful biscuit wheat in the United Kingdom and is also widely grown in other countries. The decision to select 120 winter types and 30 spring types is based on the fact that winter wheat is dominant over much of Europe. Winter wheats therefore provide a much more attractive source of material for EU breeders than spring wheats. Seeds from all of the selected genotyes were grown at the Agricultural Research Institute of the Hungarian Academy of Sciences, Martonvásár (MV), Hungary (latitude, 47° 21′ N; longitude, 18° 49′ E; altitude, 150 m) in two plots in 2005 as described by Rakszegi et al. (*21*). Plots were 2 m long, with six rows spaced at a distance of 20 cm. The soil was of the chernozem type with a loam texture and pH 6.8−7.2. The weather was typical arid continental type in 2005. Wholegrain samples (including hulls) from the two plots were combined before milling using a Retch ZM100 mill to produce wholemeal flour. Milled samples were immediately cooled to −20 °C and were kept at this temperature until analysis to protect bioactive components from degradation.

**Standards.** Authentic standards of phenolic acids were obtained from Sigma-Aldrich (Gillingham, U.K.) and included chlorogenic acid, *p*-hydroxybenzoic acid, vanillic acid, syringic acid, *p*-coumaric acid, *o*-coumaric acid, sinapic acid, caffeic acid, and ferulic acid. All standards were prepared as stock solutions at 2 mg/mL in 80:20 ethanol/water. The stock solutions were stored in darkness at −18 °C and remained stable for over 3 months.

**Phenolic Acid Content and Composition.** It is well documented that phenolic acids are concentrated in the aleurone cells and other outer layers of the grain that form the bran on milling (*14, 15, 22*). We analyzed wholemeal flour rather than bran for two reasons: to allow comparisons with studies of other phytochemicals in the same lines (see accompanying papers) and to allow the total phenolics content to be related to characteristics such as grain size and bran yield.

The phenolic acids were extracted from the flour in three separate fractions (soluble free, soluble conjugated, and bound) according to the simplified scheme in **Figure 2**. Separate extractions were carried out for each class of phenolic compound to ensure that the internal standard employed would not be lost during the course of the extraction process. The method employed is based upon previous methods for the extraction of phenolic acids from cereal grains (*17, 23*). Retention times of phenolic acids analyzed in this study are shown in **Table 1**. Phenolic acids were quantified by HPLC by performing a ratio to internal standard and calibration curves generated using authentic standards. Throughout the data collection from samples in the diversity screen a control sample was repeatedly analyzed (MV-Emese) for quality assurance purposes. The quality of this chromatogram was assessed to ensure performance of the HPLC and the extraction procedure. For the majority of the samples two replicates were made. Final calculated concentrations across these samples for the entire data set resulted in a maximum technical error (relative standard deviation) of approximately 15%.

**Internal Standard Preparation.** 3,5-Dichloro-4-hydroxybenzoic acid in 80:20 ethanol/water was used as an internal standard. Two concentrations of internal standard solution were utilized depending on the nature of the phenolic acids being extracted. For the bound phenolic acid fraction the concentration of the internal standard solution was 1.5 mg/mL (S1). For the free and conjugated phenolic acid fractions, 0.075 mg/mL was used (S2). Quantitative measurements of individual phenolic acids were achieved using HPLC.

**Free Phenolic Acids.** Five microliters of the internal standard solution (S2) was added to weighed aliquots of wholemeal flour samples (250 mg) followed by 80:20 ethanol/water (1 mL). Solutions were agitated using a Whirlimixer until all of the flour was suspended before

**Table 1.** Identities and Retention Times of Measured Phenolic Acids by HPLC

| retention time (min) | assignment |
|---|---|
| 25.5 | 4-hydroxybenzoic acid |
| 26.6 | vanillic acid |
| 27.7 | syringic acid |
| 29.9 | syringaldehyde |
| 31.4 | caffeic acid |
| 37.5 | 2,4-dihydroxybenzoic acid |
| 38.6 | sinapic acid |
| 39.5 | ferulic acid |
| 40.1 | *p*-coumaric acid |
| 43.6 | 2-hydroxycinnamic acid |
| 46.9 | 4,4′-dihydroxy-5,5′-dimethoxy-3,3′-bicinammic acid (5-5′-DiFA)[a] |
| 48.4 | ((*Z*)-*β*-[4-[(*E*)-2-carboxyvinyl]-2-methoxyphenoxy}-4-hydroxy-3-methoxycinnamic acid (8-*O*-4′-DiFA)[a] |
| 49.4 | *trans*-5-[(*E*)-2-carboxyvinyl]-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-2,3-dihydrobenzofuran-3-carboxylic acid (8-5′-DiFA benzofuran form)[a] |
| 50.9 | 3,5-dichloro-4-hydroxybenzoic acid (internal standard) |

[a] Putative assignments based on literature reports.

being sonicated for 10 min. Samples were then centrifuged for 15 min at 5000 rpm. The supernatant was removed and evaporated. A second extraction (1 mL) was carried out, and the combined supernatants were evaporated. Two percent (v/v) aqueous acetic acid (500 μL) was added to each sample, followed by the addition of 12 M HCL (2 μL) to acidify the samples to pH 2. The samples were agitated before the addition of ethyl acetate (2 × 500 μL). After centrifugation at 13200 rpm, the upper layer was transferred to a clean Eppendorf tube and evaporated. Two percent (v/v) aqueous acetic acid was added to each sample (100 μL), and after centrifugation, the supernatant was transferred to a clean vial ready for analysis by HPLC.

**Conjugated Phenolic Acids.** Ten microliters of the internal standard solution (S2) was added to weighed aliquots of wholemeal flour samples (250 mg) followed by 80:20 ethanol/water (1 mL). Solutions were agitated using a Whirlimixer until all of the flour was suspended before being sonicated for 10 min. Samples were then centrifuged for 15 min at 5000 rpm. The supernatant was removed and evaporated. A second extraction (1 mL) was carried out, and the combined supernatants were evaporated. Samples were hydrolyzed with 2 M NaOH (400 μL) for 4 h before acidification with 12 M HCl (80 μL) to pH 2. Free and conjugated phenolic acids were extracted with ethyl acetate (2 × 500 μL). After centrifugation at 13200 rpm, the upper layer was transferred to a clean Eppendorf tube and evaporated. Two percent (v/v) aqueous acetic acid (100 μL) was added to each sample, and after centrifugation, the supernatant was transferred to a clean vial ready for analysis by HPLC.

**Bound Phenolic Acids.** Wholemeal flour samples (250 mg) were extracted with 80:20 ethanol/water (2 × 1 mL). Solutions were agitated using a Whirlimixer until all of the flour was suspended before being sonicated for 10 min. Samples were then centrifuged for 15 min at 5000 rpm. The supernatant was removed and discarded. Internal standard solution (S1, 10 μL) was added to the residue prior to hydrolysis with 2 M NaOH (400 μL) for 4 h. After centrifugation, the supernatant was acidified with 12 M HCl (120 μL) to pH 2. Bound phenolic acids were extracted into ethyl acetate (2 × 800 μL). After evaporation, 2% (v/v) aqueous acetic acid (100 μL) was added to each sample, and after centrifugation, the supernatant was transferred to a clean vial ready for analysis by HPLC.

**HPLC Analysis.** HPLC analysis was carried out using an Agilent 1100 high-performance liquid chromatograph equipped with a photodiode array detector. The wavelength used for quantification of phenolic acids was 280 nm. Separations were carried out using a 250 × 4.6 mm, 5 μm, Discovery Reverse phase-Amide C16 column (Sigma Aldrich) with a 20 × 4.0 mm, 5 μm, Discovery Reverse phase-Amide C16 precolumn. The temperature of the column was set at 30 °C. A gradient elution program was utilized with a mobile phase consisting of acetonitrile (solution A) and 2% (v/v) acetic acid in water (solution B) as follows: isocratic elution, 100% B, 0−30 min; linear gradient from 100% B to 85% B, 30−50 min; linear gradient from 85% B to 50% B, 50−55 min; linear gradient from 50% B to 30% B, 55−65 min; post time, 10 min before the next injection. The flow rate of the mobile phase was 1.0 mL/min, and the injection volume was 20 μL. All phenolic acids were quantified via ratioing to the internal standard (3,5-dichloro-4-hydroxybenzoic acid) added to every sample and using calibration curves of phenolic acid standards having undergone the same extraction procedure. All samples were analyzed in duplicate (unless otherwise stated), and concentrations of individual phenolic acids were expressed in micrograms per gram of dry matter.

**Table 2.** Total Phenolic Acid Contents in Wheat Species (Micrograms per Gram of Dry Matter)

| wheat species | no. of genotypes | mean ± SEM[a] | range |
|---|---|---|---|
| winter wheat *T. aestivum* var. *aestivum* | 130 | 664 ± 15a | 326–1171 |
| spring wheat *T. aestivum* var. *aestivum* | 20 | 612 ± 31a | 456–892 |
| durum wheat *T. turgidum* var. *durum* | 10 | 699 ± 51a | 536–1086 |
| spelt *T. aestivum* var. *spelta* | 5 | 579 ± 57a | 382–726 |
| diploid einkorn *T. monococcum* | 5 | 615 ± 64a | 449–816 |
| tetraploid emmer *T. turgidum* var. *dicoccum* | 5 | 779 ± 109a | 508–1161 |

[a] Values with the same letter are not significantly different ($P < 0.05$) from each other.

**Arabinoxylan and Water-Extractable Arabinoxylan.** Measurement of arabinoxylan and water-extractable arabinoxylan concentrations was carried out, on cereal bran and flour fractions, by partners in the HEALTHGRAIN project using gas chromatography (*24*).

**Statistical Analysis.** Tukey−Kramer tests ($P$ value < 0.05) were carried out using Spotfire Decisionsite software 9.1.1 (Spotfire Tibco) to assess the significance across different cereals with different numbers of members in each group. $r$ and $P$ values for correlation analyses between different measurements were also determined with the same software.

## RESULTS AND DISCUSSION

**Total Phenolic Acids.** The total phenolic acid content was generated by the addition of the total concentrations of the three fractions (free, conjugated, and bound) (**Table 2**). The average total phenolic acid contents of winter, spring, and durum wheat were 664, 612, and 699 μg/g of dry matter (dm), respectively. The average levels of total phenolics in diploid (einkorn) genotypes were comparable at 615 μg/g of dm, whereas those in the tetraploid *T. monococcum* (emmer) genotypes were slightly higher at 779 μg/g of dm. The lowest mean concentration (579 μg/g of dm) of total phenolic acids was found in the spelt lines. Across all of the wheat genotypes, the average total phenolic acid concentration was 658 μg/g of dm. High variation in total phenolic acid content occurred within each cereal type with the greatest range (>3.5-fold) being between the winter wheat genotypes (1171−326 μg/g of dm). However, this may relate, at least in part, to the fact that a much greater number of these lines were analyzed. Disponent, a German genotype, had the highest level of total phenolic acids, whereas Scout 66, an American genotype, had the lowest total phenolic acid concentration. The spelt genotypes displayed the narrowest (1.9-fold) range and also contained the lowest maximum (726 μg/g of dm) when compared to the other cereals in the study. Twenty-eight of the bread wheat genotypes had total phenolic acid contents in excess of 800 μg/g of dm, 58 bread wheat genotypes contents between 600 and 800 μg/g of dm, 58 bread wheat genotypes contents between 400 and 600 μg/g of dm, and only 4 genotypes had total contents below 400 μg/g of dm. In

**Table 3.** Ranking of the Winter and Spring Wheat Genotypes According to Their Total Phenolic Content

| | | winter wheat | | spring wheat | |
|---|---|---|---|---|---|
| group | total phenolic content ($\mu$g/g of dm) | no. of genotypes | names of genotypes | no. of genotypes | names of genotypes |
| 1 | 300−400 | 4 | Arthur-71, CF99007, Granbel, Scout66 | 0 | |
| 2 | 400−500 | 16 | Albatros-Odesky, Bankuti-1201, Cf99075, Nomade, Blue/Ag, Sagittario, Kirkpinar-79, Palesio, Karl-92, Nap-Hal, San-Pastore, Magdalena-Fr, Sava, Tam200, Carmen, Ravenna | 6 | Red-River, Sultan95, Chara, Sunstar, Pastor, Chinese-Spring |
| 3 | 500−600 | 31 | Ukrainka, Plainsman-V, Kirac66, Millennium, Cardinal, Korweta, Spartanka, Caphorn, Bilancia, Gloria, Pobeda, Buck-Catriel, Krasnodarskaya-99, Klein-Estrella, Ornicar, Jubilejnaja-50, Alba, Cf99105, Dekan, Fundulea-29,Cf99102, Atay-85, Mieti, Alliance, Arina, Aurora, Seu-Seun-27, Iljicsovka | 5 | Catbird, Saratov-29, Janz, Mexique-50, Red-Fife |
| 4 | 600−700 | 30 | Fertodi-293, Hereward, Agron, Spark, Vona, Thesee, Su321, Galahad, Balkan, Momtchil, Renan, Etoile-De-Choisy, Gerek-79, Manital, Flamura-85, Begra, Libellula, Camp-Remy, Valoris, Ns-Rana-1, Kotuku, Lasta, Mv-Palotas, Isengrain, Key, Martonvasari-17, Sadovo-1, Bezostaja-1, Avalon, Skorospelka-3b | 3 | Manitoba, Kukri, Lona |
| 5 | 700−800 | 21 | Gk-Tiszataj, Mv-Suba, Probstdorfer-Perlo, Qualital, Kanzler, Produttore, Gene, Hana, Maris-Huntsman, Taldor, Malacca, Autonomia, Augusta, Estica, Cubus, Moulin, Soissons, Stephens, Baranjka, Tamaro, Ble-Des-Domes | 4 | Cadenza, Glenlea, Milan, Pan |
| 6 | 800−900 | 12 | Courtot, Lynx, Yumai-34, Sumai-3, Apache, Claire, Ellvis, Fredrick, Rusalka, Rialto, Biscay, Monopol | 2 | Thatcher, Azteca67 |
| 7 | 900−1000 | 10 | Tremie, Recital, Akteur, Geronimo, Riband, Blasco, Guarni, Zvezda, Capo, Tommi | 0 | |
| 8 | >1000 | 4 | Disponent, Amadeus, Herzog, Campari | 0 | |

**Table 4.** Total Free, Conjugated, and Bound Phenolic Acid Contents in Wheat Species (Micrograms per Gram of Dry Matter)[a]

| | | free phenolic acids | | conjugated phenolic acids | | bound phenolic acids | |
|---|---|---|---|---|---|---|---|
| wheat species | no. of genotypes | mean ± SEM | range | mean ± SEM | range | mean ± SEM | range |
| winter wheat *T. aestivum* var. *aestivum* | 130 | 11 ± 0.5a | 3–30 | 162 ± 4ab | 81–276 | 492 ± 13a | 208–878 |
| spring wheat *T. aestivum* var. *aestivum* | 20 | 8 ± 0.5a | 5–13 | 166 ± 12ab | 76–297 | 439 ± 30a | 286–755 |
| durum wheat *T. turgidum* var. durum | 10 | 13 ± 2a | 7–22 | 267 ± 20bc | 184–416 | 418 ± 51a | 288–832 |
| spelt *T. aestivum* var. *spelta* | 5 | 7 ± 1a | 3–12 | 138 ± 17ad | 93–191 | 433 ± 59a | 245–595 |
| diploid einkorn *T. monococcum* | 5 | 10 ± 3a | 3–18 | 229 ± 35bcd | 127–337 | 376 ± 62a | 245–595 |
| tetraploid emmer *T. turgidum* var. dicoccum | 5 | 9 ± 1a | 6–13 | 172 ± 18abd | 103–208 | 599 ± 99a | 398–964 |

[a] Values with the same letter in the mean column are not significantly different ($P < 0.05$) from each other.

comparison between winter and spring wheats, the highest and lowest contents of total phenolic acids were present in the winter wheats (**Table 3**).

**Total Free and Conjugated Phenolic Acids.** Free phenolic acids make the smallest (typically <0.5−1%) contribution to the total phenolic acid content in cereals (*23*). The concentrations of this class of compound varied by 10-fold, from 3 to 30 $\mu$g/g of dm (**Table 4**). The average total free phenolic acid content of the winter wheat genotypes was 11 $\mu$g/g of dm, with similar average levels being present in the durum (13 $\mu$g/g) and diploid einkorn (10 $\mu$g/g) genotypes. The mean concentrations of free phenolic acids in the spring wheat, tetraploid emmer, and spelt genotypes were significantly lower at 8, 9, and 7 $\mu$g/g of dm. Free phenolic acids made up only 1% of the total phenolic acid content across all of the genotypes in the diversity screen. Soluble conjugated phenolic acids make up a greater proportion of the total phenolic acid content in wheat (22%) and range from 76 to 416 $\mu$g/g of dm. The winter and spring wheat genotypes contained similar average levels (162 and 166 $\mu$g/g of dm, respectively) and ranges, whereas the average levels in durum wheat genotypes were much higher (267 $\mu$g/g of dm) and those in spelt were significantly lower (138 $\mu$g/g of dm). Large variation occurred in the levels of total conjugated phenolic acids within each cereal type, with the greatest ranges (>3.5-fold) being displayed across the winter and spring wheat genotypes. Although the highest total level of conjugated phenolic acid contents was present in a durum wheat cultivar (Orjaune), the range within durum wheats was significantly lower, with only a 2.3-fold range across the genotypes. The spelt genotypes displayed the narrowest (2.05-fold) range and also contained the lowest maximum (191 $\mu$g/g of dm) when compared to the other cereals in the study.

**Total Bound Phenolic Acids.** Bound phenolic acids contribute the highest proportion of the total phenolic acids in cereals (*23*) and accounted for 77% of the total phenolic content across all of the wheat genotypes in the HEALTHGRAIN study. The average total phenolic acid contents of winter, spring, spelt, and durum wheat were similar, with mean concentrations of 492, 439, 433, and 418 $\mu$g/g of dm, respectively (**Table 4**). The average level of bound phenolics in diploid einkorn genotypes was significantly lower at 376 $\mu$g/g of dm, whereas the levels in the tetraploid emmer genotypes were slightly higher at 599 $\mu$g/g of dm. There was large variation in the total bound phenolic acid content within each cereal type, with the greatest range (>4-fold) being present in the winter wheat genotypes (208−878 $\mu$g/g of dm). The 10 lines with the highest levels of bound phenolic acids (MVGB349, Amadeus, Disponent, MV-Makaroni, Recital, Geronimo, Tremie, Zvezda, Herzog, and Blasco) were all of European origin, with bound phenolic acid contents between 771 and 964 $\mu$g/g of dm. Similar concentration ranges of bound phenolics acids were present in the other genotypes (2.4−2.9-fold).

**Phenolic Acid Composition.** Phenolic acids commonly found in whole grains include ferulic acid, vanillic acid, caffeic acid,

**Table 5.** Compositions of Individual Phenolic Acid Contents in Wheat Species (Micrograms per Gram of Dry Matter)[a]

| | winter wheat ($n = 130$) *T. aestivum* var. *aestivum* | | spring wheat ($n = 20$) *T. aestivum* var. *aestivum* | | durum wheat ($n = 10$) *T. turgidum* var. *durum* | | spelt ($n = 5$) *T. aestivum* var. *spelta* | | diploid einkorn ($n = 5$) *T. monococcum* | | tetraploid emmer ($n = 5$) *T. turgidum* var. *dicoccum* | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | mean | range | mean | range | mean | range | mean | range | mean | range | mean | range |
| **4-hydroxybenzoic acid** | | | | | | | | | | | | |
| free | 0.1 ± 0.024 | 0–1.8 | | | | | | | | | | |
| conjugated | 5.4 ± 0.14ab | 2.3–11.1 | 6.3 ± 0.34abd | 3.6–9.6 | 10.9 ± 0.85c | 6.0–16.2 | 6.9 ± 0.42abd | 5.3–7.6 | 4.6 ± 0.38ab | 3.7–5.4 | 7.8 ± 1.19bd | 5.5–12.3 |
| bound | 2.1 ± 0.12abc | 0.2–8.3 | 2.8 ± 0.35ab | 0.8–6.5 | 2.0 ± 0.76abc | 0.4–8.6 | 1.9 ± 0.41abc | 0.6–2.8 | 0.7 ± 0.24ac | 0–1.5 | 3.9 ± 0.75ab | 2.0–6.1 |
| **vanillic acid** | | | | | | | | | | | | |
| free | 2.3 ± 0.059ad | 0–4.4 | 1.7 ± 0.076bcd | 1.2–2.5 | 1.9 ± 0.13abcd | 1.1–2.5 | 1.3 ± 0.42bcd | 0–2.4 | 0.4 ± 0.37c | 0–1.8 | 1.7 ± 0.11abcd | 1.3–2.0 |
| conjugated | 14.1 ± 0.29ab | 8.8–24.5 | 14.0 ± 0.69abc | 8.2–22.7 | 15.2 ± 0.77ab | 11.6–18.3 | 11.4 ± 0.23abc | 10.5–11.9 | 9.6 ± 1.16ac | 7.0–12.5 | 11.3 ± 1.40abc | 7.6–16.3 |
| bound | 4.5 ± 0.10ab | 2.1–9.0 | 3.8 ± 0.25abc | 2.4–5.9 | 3.3 ± 0.56ac | 1.8–8.1 | 2.7 ± 0.34ac | 1.8–4.0 | 2.2 ± 0.24ac | 1.7–3.1 | 2.7 ± 0.46ac | 1.9–4.2 |
| **syringic acid** | | | | | | | | | | | | |
| free | 2.0 ± 0.072ac | 0–4.0 | 2.4 ± 0.22ac | 0.8–5.1 | 2.3 ± 0.32ac | 1.0–4.1 | 0.7 ± 0.47ab | 0–2.8 | 0.2 ± 0.21ab | 0–1.0 | 1.2 ± 0.35abc | 0–2.0 |
| conjugated | 10.9 ± 0.35ab | 3.9–22.0 | 11.2 ± 0.71ab | 6.7–18.8 | 5.8 ± 0.42ac | 3.7–7.9 | 8.2 ± 1.05abc | 4.6–10.7 | 3.8 ± 0.41ac | 2.8–5.1 | 5.7 ± 2.81ac | 1.2–16.8 |
| bound | 4.7 ± 0.20abc | 1–13.4 | 3.8 ± 0.35abcd | 1.5–6.7 | 1.2 ± 0.20ade | 0.6–2.8 | 2.3 ± 0.3abcde | 1.3–3.1 | 1.1 ± 0.2abde | 0.7–1.7 | 1.0 ± 0.3abde | 0.2–1.7 |
| **syringaldehyde** | | | | | | | | | | | | |
| free | 0.1 ± 0.027 | 0–1.6 | | | | | 0.3 ± 0.26 | 0–1.6 | 0.8 ± 0.48 | 0–2.0 | | |
| conjugated | 0.4 ± 0.07 | 0–2.9 | | | | | | | | | | |
| bound | 0.4 ± 0.05 | 0–2.0 | | | | | | | | | 0.1 ± 0.08 | 0–0.4 |
| **caffeic acid** | | | | | | | | | | | | |
| free | 0.4 ± 0.080 | 0–3.3 | | | | | 0.9 ± 0.64 | 0–4.3 | 0.8 ± 0.76 | 0–3.8 | 0.6 ± 0.62 | 0–3.1 |
| conjugated | nq | nq | nq | nq | nq | nq | nq | nq | nq | nq | nq | nq |
| bound | nq | nq | nq | nq | nq | nq | nq | nq | nq | nq | nq | nq |
| **2,4-dihydroxybenzoic acid** | | | | | | | | | | | | |
| free | 0.3 ± 0.055ab | 0–4.6 | 0.2 ± 0.09ab | 0–1.6 | 1.1 ± 0.33bc | 0–2.5 | 0.6 ± 0.23abc | 0–1.7 | 1.2 ± 0.61bc | 0–3.5 | 0.8 ± 0.55abc | 0–2.9 |
| conjugated | 50.6 ± 1.51ab | 7.6–116 | 57.1 ± 6.59abc | 5.2–119 | 108.4 ± 6.82c | 80.5–153.0 | 38.6 ± 8.10ab | 11.3–57.9 | 86.8 ± 16bcd | 63.7–147 | 58.8 ± 12.6abc | 11.0–79.5 |
| bound | 76.4 ± 3.64ac | 0–197.9 | 24.8 ± 4.52ab | 3.1–74.6 | 23.9 ± 6.47ab | 9.0–65.6 | 47.8 ± 7.32abc | 20.5–71.7 | 55.8 ± 9abc | 36.8–88 | 109.1 ± 30.0ac | 40.5–215.1 |
| **sinapic acid** | | | | | | | | | | | | |
| free | 2.2 ± 0.34a | 0–12.3 | | | 3.6 ± 1.48a | 0–9.2 | | | 3.6 ± 2.18a | 0–9.1 | | |
| conjugated | 55.5 ± 1.54ab | 27.8–128 | 47.4 ± 3.5ab | 21.5–83 | 86.6 ± 7.86ac | 55.9–136.6 | 53.0 ± 7.39ab | 39.6–77.0 | 92.8 ± 9.0ac | 76.1–121 | 63.4 ± 3.45abc | 54.3–73.5 |
| bound | 23.2 ± 0.44a | 12.9–40 | 20.3 ± 0.87a | 15.1–30 | 23.8 ± 1.45a | 18.4–34.7 | 17.8 ± 0.89a | 13.6–21.3 | 25.3 ± 1.33a | 22.1–29 | 25.1 ± 3.20a | 19.1–36.6 |
| **ferulic acid** | | | | | | | | | | | | |
| free | 3.3 ± 0.08ab | 1.2–6.2 | 2.7 ± 0.132ac | 1.9–4.1 | 2.9 ± 0.18abc | 2.1–3.8 | 3.7 ± 0.24abc | 2.6–4.7 | 4.0 ± 0.38ab | 3.2–5.2 | 3.2 ± 0.35abc | 2.6–4.5 |
| conjugated | 29.3 ± 1.02ab | 9.4–62.3 | 28.5 ± 2.14ab | 11.6–51 | 44.9 ± 5.65bcd | 26.2–87.8 | 31.0 ± 3.04abd | 22.0–38.9 | 56.6 ± 5.3cd | 44.0–70 | 26.3 ± 8.73abd | 15.9–61.1 |
| bound | 366.2 ± 10.60a | 162–721 | 375.0 ± 25.2a | 252–628 | 355.5 ± 43.1a | 241.0–701 | 333.7 ± 31.56a | 200.6–475 | 241.4 ± 34.9a | 163–372 | 449.3 ± 73.50a | 304.2–693 |
| ***p*-coumaric acid** | | | | | | | | | | | | |
| free | 0.6 ± 0.04ab | 0–2.2 | 0.6 ± 0.14abc | 0–2.2 | 1.1 ± 0.22ac | 0–2.3 | | | 0.3 ± 0.2abc | 0–0.9 | 0.8 ± 0.38abc | 0–2.0 |
| conjugated | 5.2 ± 0.14a,bc | 3.0–12.1 | 6.3 ± 0.59ac | 3.8–14.6 | 6.1 ± 0.52abc | 4.9–10.5 | 3.5 ± 0.38ac | 2.5–4.9 | 5.0 ± 0.6abc | 3.2–6.7 | 4.6 ± 1.11abc | 1.7–8.2 |
| bound | 10.0 ± 0.30a | 3.0–19.1 | 4.1 ± 0.49b | 2.8–13.3 | 3.4 ± 0.074b | 3.1–4.0 | 5.1 ± 1.31b | 2.7–13.5 | 2.9 ± 0.012b | 2.8–2.9 | 3.1 ± 0.22b | 2.4–3.6 |
| **2-hydroxycinnamic acid** | | | | | | | | | | | | |
| free | 0.03 ± 0.01 | 0–0.8 | | | 0.2 ± 0.11 | 0–1.1 | 0.1 ± 0.04 | 0–0.3 | 0.1 ± 0.11 | 0–0.5 | 0.4 ± 0.09 | 0.2–0.6 |
| conjugated | 1.9 ± 0.03ab | 1.3–3.6 | 2.2 ± 0.09ac | 1.8–3.0 | 2.5 ± 0.10ac | 2.0–3.1 | 2.4 ± 0.22abc | 1.9–3.0 | 1.8 ± 0.3abc | 1.3–2.8 | 2.5 ± 0.16ac | 2.0–2.8 |
| bound | 4.3 ± 0.09a | 2.4–7.3 | 4.6 ± 0.22a | 3.3–6.8 | 5.1 ± 0.36a | 3.9–7.4 | 4.0 ± 0.18ab | 3.4–4.9 | 2.7 ± 0.14b | 2.5–3.2 | 4.7 ± 0.56a | 3.6–6.4 |

[a] Values with the same letter in a row are not significantly different ($P < 0.05$) from each other.

**Table 6.** Total Ferulic Acid Content in Wheat Species (Micrograms per Gram of Dry Matter)[a]

| wheat species | no. of genotypes | total ferulic acid | | total diferulate | | |
|---|---|---|---|---|---|---|
| | | mean ± SEM | range | mean ± SEM | range | % of total ferulate |
| winter wheat *T. aestivum* var. *aestivum* | 130 | 395 ± 11a | 181–742 | 19 ± 1ac | 51–8 | 7–3 |
| spring wheat *T. aestivum* var. *aestivum* | 20 | 401 ± 25a | 284–651 | 25 ± 2bc | 45–17 | 8–5 |
| durum wheat *T. turgidum* var. *durum* | 10 | 400 ± 43a | 290–737 | 30 ± 3be | 45–17 | 9–6 |
| spelt *T. aestivum* var. *spelta* | 5 | 365 ± 50a | 223–502 | 19 ± 2abcd | 23–14 | 6–4 |
| diploid einkorn *T. monococcum* | 5 | 298 ± 39a | 207–442 | 9 ± 1cd | 13–7 | 4–3 |
| tetraploid emmer *T. turgidum* var. *dicoccum* | 5 | 476 ± 71a | 323–711 | 24 ± 5abce | 43–16 | 6–4 |

[a] Values with the same letter in the mean column are not significantly different ($P < 0.05$) from each other.

**Table 7.** Correlation Matrix of Different Phenolic Acid Fractions with Other Bioactive Component Data[a]

| | free phenolic acids | soluble conjugated phenolic acids | bound phenolic acids | total phenolic acids |
|---|---|---|---|---|
| bran yield | $r = 0.091$<br>$P < 0.001$ | $r = -0.282$<br>$P = 0.530$ | $r = 0.091$<br>$P = 0.0014$ | $r = 0.177$<br>$P = 0.035$ |
| 1000 kernal weight | $r = -0.04$<br>$P < 0.001$ | $r = -0.01$<br>$P = 0.189$ | $r = -0.025$<br>$P = 0.997$ | $r = -0.027$<br>$P = 0.606$ |
| total sterols | $r = 0.256$<br>$P = 0.113$ | **$r = 0.552$**<br>**$P < 0.001$** | $r = -0.051$<br>$P = 0.291$ | $r = 0.139$<br>$P = 0.591$ |
| total folates | $r = 0.135$<br>$P = 0.314$ | **$r = 0.437$**<br>**$P < 0.001$** | $r = -0.071$<br>$P = 0.701$ | $r = 0.211$<br>$P = 0.0262$ |
| total alkylresorcinols | $r = 0.048$<br>$P = 0.018$ | **$r = 0.305$**<br>**$P < 0.001$** | $r = -0.150$<br>$P = 0.0206$ | $r = 0.237$<br>$P < 0.001$ |
| total tocols | $r = 0.166$<br>$P < 0.001$ | **$r = 0.390$**<br>**$P < 0.001$** | $r = 0.079$<br>$P = 0.203$ | $r = 0.204$<br>$P = 0.0035$ |
| total phenolic acids | $r = 0.270$<br>$P = 0.693$ | **$r = 0.431$**<br>**$P < 0.001$** | **$r = 0.943$**<br>**$P < 0.001$** | |
| total free phenolic acids | | $r = 0.176$<br>$P = 0.251$ | $r = 0.199$<br>$P = 0.040$ | $r = 0.27$<br>$P = 0.693$ |
| total soluble conjugated phenolic acids | $r = 0.176$<br>$P = 0.251$ | | $r = 0.109$<br>$P = 0.0297$ | **$r = 0.431$**<br>**$P < 0.001$** |
| total bound phenolic acids | $r = 0.199$<br>$P = 0.040$ | $r = 0.109$<br>$P = 0.0297$ | | **$r = 0.943$**<br>**$P < 0.001$** |
| total arabinoxylan in flour | $r = 0.053$<br>$P = 0.305$ | $r = 0.081$<br>$P = 0.001$ | $r = 0.208$<br>$P = 0.069$ | $r = 0.288$<br>$P = 0.0882$ |
| total arabinoxylan in bran | $r = -0.011$<br>$P = 0.00101$ | **$r = -0.506$**<br>**$P < 0.001$** | $r = 0.129$<br>$P < 0.001$ | $r = -0.063$<br>$P = 0.073$ |
| water extractable arabinoxylan in flour | $r = -0.078$<br>$P = 0.279$ | **$r = -0.308$**<br>**$P = 0.346$** | $r = -0.086$<br>$P = 0.147$ | $r = -0.026$<br>$P = 0.125$ |
| water extractable arabinoxylan in bran | $r = 0.120$<br>$P = 0.602$ | $r = -0.059$<br>$P = 0.0012$ | $r = 0.139$<br>$P = 0.478$ | $r = 0.110$<br>$P = 0.083$ |

[a] Data generated from Pearson correlation analysis. Figures in bold represent those correlations with $r > 0.3$ and thus representing a weak correlation.

syringic acid, and *p*-coumaric acid (*16*). In this study, the concentrations of individual phenolics acids were measured for each phenolic acid fraction (**Table 5**). These included 4-hydroxybenzoic acid, vanillic acid, syringic acid, syringealdehyde, caffeic acid, 2,4-dihydroxybenzoic acid, sinapic acid, ferulic acid, *p*-coumaric acid, and 2-hydroxycinnamic acid. The proportions of these compounds vary from fraction to fraction and from cereal to cereal. The composition of free phenolic acids varied most across the cereals (**Figure 3**), the predominant components in the winter and spring wheats being ferulic, vanillic, syringic, and sinapic acids. The durum wheat genotypes showed less variation in free phenolic acid composition, whereas the spelt genotypes contained the highest proportions of free ferulic acid. There was less variation in the composition of the conjugated phenolic acid fractions of the different cereals, the major phenolic acids being ferulic, sinapic, and 2,4-dihydroxybenzoic acids. The latter component is present in small amounts in the free and bound fractions but is among the highest components in the conjugated phenolic acid fraction (25−39%). The profiles of the bound phenolic acids were similar across all of the different wheat genotypes, with ferulic acid predominating (72−85%). The highest mean concentrations of ferulic acid were in spring wheat and durum wheat genotypes (84 and 85%, respectively). The concentrations of ferulic, vanillic,

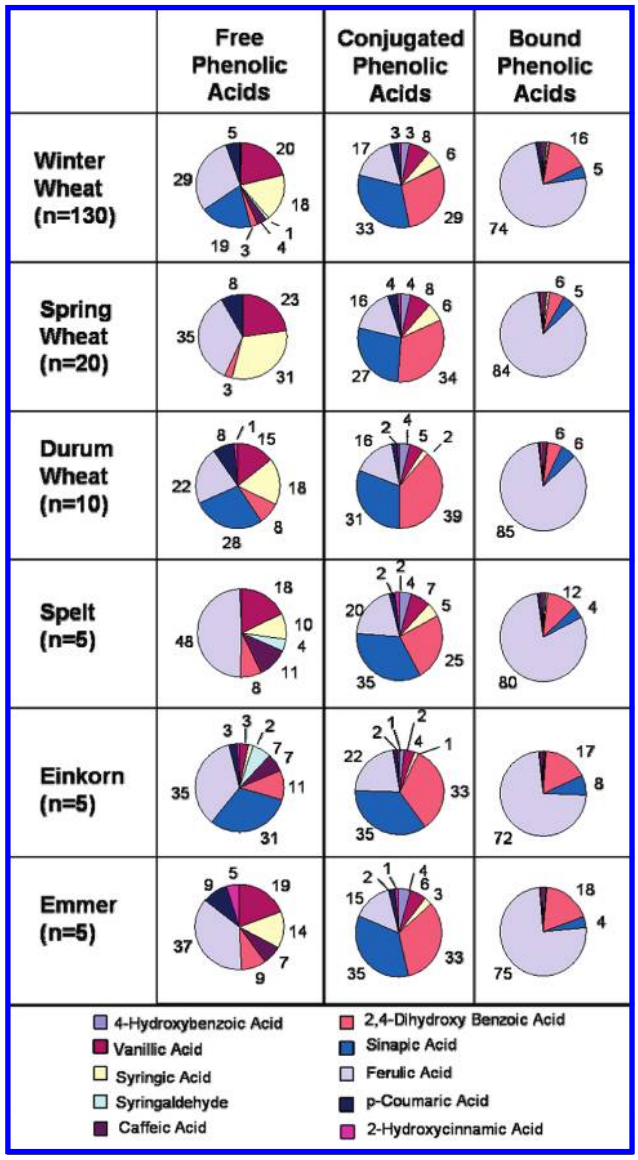


**Figure 3.** Distribution of individual phenolic acids across free, conjugated, and bound phenolic acid fractions for different cereals in the HEALTHGRAIN diversity screen.

syringic, and *p*-coumaric acids for each phenolic class have been reported for eight varieties of Maryland soft wheat (*25*). The total ferulic acid levels ranged from 455 to 621 μg/g, which compares well with the range reported here. Similarly, reported ranges for total vanillic acid (8.4−12.7 μg/g), syringic acid (8.9−17.8 μg/g), and *p*-coumaric acid (10.4−14.10 μg/g) were also consistent with our determinations of the average levels of these metabolites (20, 17, and 16 μg/g of dm, respectively).

**Ferulic Acid.** Ferulic acid, a derivative of *trans*-cinnamic acid and a component of lignin, is largely concentrated in the cereal cell wall, where it is esterified to arabinose components of arabinoxylans and can form cross-links by ether bonds [reviewed by Saulnier et al. (*12*)]. In addition, ferulic acid is a precursor for the synthesis of other aromatic compounds in the plant. Ferulic acid comprised a significant proportion of each of the phenolic acid fractions, with concentrations ranging from 1.2−6.2 μg/g of dm for free ferulic acid to 163−721 μg/g of dm for the bound form (**Table 5**). The average concentrations of bound ferulic acid (the major form) were similar for spelt and the winter, spring, and durum wheats, being approximately 350 μg/g of dm. However, the average concentrations were slightly lower in the diploid einkorn samples (240 μg/g of dm) and higher in the tetraploid emmer samples (450 μg/g of dm). Similarly, the average total concentrations of ferulic acid (**Table 6**) were similar for spelt, durum, winter, and spring wheats (about 400 μg/g of dm) but were lower in einkorn samples (298 μg/g of dm) and higher in emmer samples (476 μg/g of dm). Previous reports of the concentration of ferulic acid in wheat grain vary greatly. Our findings were consistent with the studies of Pussnawin and Wetzel (*26*), who reported total ferulic acid contents of 500 μg/g. Rybka et al. (*27*) reported that ferulic acid was the major free phenolic acid, whereas Lempereur et al. (*28*) showed high genetic (variety-dependent) variation in the content of ferulic acid content in durum wheat grains (from 0.693 to 2.443 mg/g of dm).

Determination of phenolic acids in five quality classes of Canadian wheat (*29*) showed that about 80% of the total was present in insoluble complexes. In this study ferulic acid ranged from 274.8 to 337.6 μg/g and was proposed to affect the quality of the wheat. Six of the varieties in our study originated from Canada (Augusta, Frederick, Glenlea, Manitoba, Red Fife, and Thatcher). The ferulic acid content of these genotypes ranged from 284 to 651 μg/g of dm, with an average level of 487 μg/g of dm. These levels are higher than those reported in ref *29*, which could relate to differences in genotype or growth site (Hungary compared to Canada).

**Diferulic Acid.** Diferulic acids are potent antioxidants and are dimers of ferulic acid, which have been found in the cell walls of several plant species (*30*). These phenolic compounds are ester-linked to cell wall polysaccharides and cannot be absorbed by humans in this form. They can, however, be released from cereal brans by intestinal enzymes (*31*), and thus these components are now regarded as bioavailable. In addition to the free phenolic acids measured in this study, peaks consistent with 5-8-, 5-5-, 8-*O*-4-, and 5-8-dehydrodiferulate esters have also been measured. Semiquantification was carried out using the ferulic acid calibration curves (no authentic standards could be purchased), allowing a comparison of these data across the HEALTHGRAIN diversity screen.

Total measured diferulate content varied from 51 to 7 μg/g of dm (**Table 6**), with the highest concentration range being present in winter wheat genotypes. The highest mean concentrations of diferulate components were found in spring and durum wheats (25 and 30 μg/g of dm, respectively). Interestingly, diploid einkorn and tetraploid emmer genotypes showed very large differences in diferulate content, with the diploid einkorn genotypes showing the smallest range (13−7 μg/g of dm) and the lowest mean concentration (9 μg/g of dm). Conversely, the tetraploid emmer genotypes possessed much higher levels of diferulate (mean = 24 μg/g of dm) with a concentration range of 43−16 μg/g of dm. The proportion of total ferulate that was present as dimeric forms ranged from 3 to 9% across all of the wheat genotypes in the HEALTHGRAIN diversity screen.

**Correlations with Other Bioactive Components in Winter Wheats. Table 7** shows Pearson correlation coefficients between the contents of phenolics acids and other bioactive components. Prior to generation of the correlation plots, all data were logged to allow for the differing scales across the variety of measurements. The clearest correlations were between the classes of phenolic acids themselves. It was not surprising that the clearest correlation was between total phenolic acids and bound phenolic acids ($r = 0.94$) as the bound phenolic acids contriubte a high proportion of the total phenic acid content. The contents of bound and total phenolic acids also showed very weak positive correlations ($r = 0.2$ and 0.29) with total arabinoxylan content in flour but not with the content of water-extractable arabi-

noxylan. Conjugated phenolic acids negatively correlated with the total arabinoxylan content in bran ($r = -0.51$) as well as with other bioactive components (sterols, tocols, folate, and alkylresorcinols). In contrast, free phenolic acids showed no significant correlations with either arabinoxylan content or phytochemical content. Given that phenolic acids are concentrated in the bran, similar correlation plots were generated for bran yield. Only a very weak correlation ($r = 0.18$) of total phenolic acids with bran yield was observed across the winter wheats, indicating that variation in phenolic acid content is not due simply to variation in bran yield but also to genotypic differences.

In summary, results from this study have demonstrated a wide range in total phenolic content across a large number of wheat genotypes. Although more research is required to understand the effect of growing location and conditions, the observed multifold differences between varieties with the highest and lowest concentration of phenolic acids suggest that it may be possible to develop varieties which are enriched in phenolics acids with benefits to the health of consumers.

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