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Effects of Environment and Genotype on Phenolic Acids in Wheat in the HEALTHGRAIN Diversity Screen[†]

Rebeca Fernandez-Orozco,[§] Li Li, Claudia Harflett,[#] Peter R. Shewry, and Jane L. Ward*

Department of Plant Science, Rothamsted Research, Harpenden, Hertfordshire AL5 2JQ, United Kingdom. [§] Present address: Departamento de Biotecnología de Alimentos, Instituto de la Grasa, CSIC, Avenida Padre García Tejero 4, 41012 Sevilla, Spain. [#] Present address: School of Life Sciences, University of Sussex, Falmer, Brighton BN1 9QG, United Kingdom.

Phenolic acid content and composition have been determined in 26 wheat genotypes grown in Hungary over three consecutive years and at three additional locations (France, United Kingdom, and Poland) during the third year. Fractions comprising free, soluble conjugated, and bound phenolic acids were analyzed using HPLC with measurements being made for individual phenolic acids in each fraction. Statistically significant differences in phenolic acid content occurred across the different growing locations with the average total phenolic acid content being highest in the genotypes grown in Hungary. The growth year in Hungary also had a large impact, especially on the free and conjugated phenolic acid contents. Certain genotypes were more resistant to environmental impacts than others. Of the genotypes with high levels of total phenolic acids, Lynx, Riband, Tommi, and Cadenza were most stable with respect to their total contents, whereas Valoris, Herzog, and Malacca, also high in phenolic acid content, were least stable. Of the three fractions analyzed, the free and conjugated phenolic acids were most variable and were also susceptible to the effect of environment, whereas bound phenolic acids, which comprised the greatest proportion of the total phenolic acids, were the most stable.

KEYWORDS: Phenolic acids; phenolics; wheat; whole grain; wholemeal; flour; cereal; genotype; variation; environment; year; location

INTRODUCTION

Wheat is a globally important crop, grown on the greatest land area compared to any other commercial cereal crop. It is an important component of the human diet, providing energy, due to its high content of carbohydrate, and protein. Regular consumption of whole-grain food products has been shown to be beneficial to humans, reducing the incidence of chronic diseases such as diabetes (*1*), cardiovascular disease (*2*, *3*), and certain types of cancer (*2*, *4*−*7*).

Wheat contains high levels of antioxidants with the content of total phenolics showing strong correlations with total antioxidant activity (*8*−*11*). Phenolics are thus thought to hold great promise for the provision of health benefits (*12*). Phenolic acids represent the most common form of phenolic compounds in whole grains and make up one of the major and most complex groups of phytochemicals in the cereal grain. They exist as soluble free acids, as soluble conjugates that are esterified to sugars and other low molecular mass components, and as insoluble bound forms [reviewed by Piironen et al. (*13*)]. In common with many other wheat phytochemicals they are concentrated in the bran fraction and are typically present in lower levels in white flour (*14*, *15*).

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*Corresponding author [phone +44 (0)1582 763133; fax +44 (0)1582 763010; e-mail jane.ward@bbsrc.ac.uk].

The HEALTHGRAIN Integrated Project (European Union Sixth Framework Programme) aimed to improve the well-being of consumers and to reduce the risk of metabolic diseases by increasing the intake of protective compounds in grains, through the development of health-promoting, safe, and high-quality cereal foods and ingredients. The objective of the present study was to determine the environmental and genetic effects on the phenolic acid contents and compositions of diverse wheat genotypes. In a previous study we showed that the total phenolic acid contents of 150 bread wheat genotypes grown in Martonvásár, Hungary, in 2005 ranged from 326 to 1171 μg/g of dry matter (dm), indicating that genetic variation may exist and be available to plant breeders (*16*). The samples generated in this study were grown in one location, specifically to minimize the effects due to environment. However, the wheat genotype, the environment, and possibly interactions between the genotype and environment are known to strongly influence the levels of grain antioxidants. In a previous study six wheat genotypes grown at four locations in Canada showed large variation in their total phenolic contents and antioxidant activities. Furthermore, environmental effects were greater than genotypic effects, and neither growing temperature nor rainfall from anthesis to maturity was thought to be responsible for the variation (*8*). Bran has also been analyzed from 20 hard winter wheat varieties grown at two locations in Colorado in a single year. The relative contributions of genotype and growing environment to phenolic acid composition were also determined, and, again, greater variation was related to the environment rather than to the genotype (*17*). Whereas a significant negative correlation was observed between total phenolic content and high temperature (hours above 32 °C), this finding was in contrast to previously reported studies (*18*−*20*), which suggested that a range of environmental stresses such as temperature, solar radiation, drought, and excess water can result in the production of antioxidants and increased phenolic content.

To explore the effect of growth year and environment on European wheat cultivars and to further complement our initial study within the HEALTHGRAIN project (*16*), analyses were made of wholemeal flour samples of 26 bread wheat cultivars grown in 6 different environmental conditions. Genotypes were grown in Hungary for three consecutive years, 2005−2007, as well as at three other locations (France, United Kingdom, and Poland) in 2007 under carefully monitored conditions. Total free, soluble conjugated, and bound phenolic acid levels were determined together with individual phenolic acids within each fraction to determine the effects of genotype versus environment and their possible interaction.

MATERIALS AND METHODS

Wheat Lines. The 26 lines selected are listed in **Table 1**. They include 24 winter wheat and 2 spring wheat (*Triticum aestivum* var. *aestivum*) genotypes originating from 11 countries. Twenty-three of these lines, grown in a single year, were analyzed in the HEALTHGRAIN 2005 diversity screen (*21*), and data from these analyses are included in this study for comparative purposes. Genotypes were selected that exhibited either high or low concentrations of a range of phytochemicals and fiber components. Three additional genotypes were included in 2006 and 2007. MV Emese is the standard cultivar used in variety trials at Martonvásár, whereas Tiger and Crousty were selected as standard lines in HEALTHGRAIN module 3 (Technology and Processing) (*22*). Wheat grains were processed at Martonvásár, Hungary, where they were assessed for agronomic and quality parameters including dry matter content, bran yield, and thousand kernel weight (*23*). Grains were milled to yield wholemeal flours of 0.5 mm particle size (*24*), and resulting samples were stored at −20 °C until ready for analysis. Although phenolic acids are concentrated in the outer layers of the grain, which form the bran fraction on milling (*14*, *15*), wholemeal flour samples were utilized in this study to allow comparisons to be made with data for other phytochemical components (e.g., sterols, tocols, folates) collected on the same tissue (see ref *23* and accompanying papers) and also to explore potential correlations with characteristics such as bran yield and grain size. Wholemeal rather than bran samples were also selected for analysis to eliminate differences caused by variation in the efficiency of milling and bran separation between the lines.

Growth Environments. To determine the impact of environment on phenolic acid content, the selected genotypes were grown, with monitoring of soil and weather conditions, in experimental plots at Martonvásár (Hungary) in three years, 2005, 2006, and 2007, as well as in Clermont (France), Saxham (United Kingdom), and Choryn (Poland) in 2007. The choice of growth locations enabled three key comparisons to be made. A comparison of genotypes grown at the same site for three successive years can be made by comparing samples grown in Hungary between 2005 and 2007, whereas analysis of the material grown in 2007 allows a comparison of genotypes grown at four locations in a single year. Taking all growth environments into account, a comparison across six different environments over three years can be made. The heading dates ranged from May 5 to June 14 and harvest dates from July 5 to August 22. During the growth period, temperature and precipitation data were recorded and are described in Shewry et al. (*23*). Precipitation and mean temperatures between heading and harvest ranged from 101.4 to 232.6 mm and between 14.2 and 20.5 °C, respectively (**Table 2**). When the total period from 3 months before heading to harvest was considered, precipitation varied between 205.5 and 360.2 mm. The temperature in Martonvásár varied more widely than at the other three sites, experiencing both the highest maximum temperature and also the lowest minimum temperature. This is in contrast to the U.K. site, which in 2007 was unusually cool (between heading and harvest) and wet. This site also showed the narrowest range in temperature across the whole growing period.

Table 1. Details of the Selected Wheat Lines Utilized in the Study

wheat line	origin	growth habit
Atlas-66	U.S.A.	winter
Avalon	U.K.	winter
Cadenza	U.K.	spring
Campari	Germany	winter
CF99105	France	winter
Chinese Spring	China	spring
Claire	U.K.	winter
Crousty	France	winter
Disponent	Germany	winter
Estica	The Netherlands	winter
Gloria	Romania	winter
Herzog	Germany	winter
Isengrain	France	winter
Lynx	U.K.	winter
Malacca	U.K.	winter
Maris Huntsman	U.K.	winter
MV Emese	Hungary	winter
Obriy	Ukraine	winter
Rialto	U.K.	winter
Riband	U.K.	winter
San Pastore	Italy	winter
Spartanka	Russia	winter
Tiger	Germany	winter
Tommi	Germany	winter
Tremie	France	winter
Valoris	France	winter

Standards. Authentic standards of chlorogenic acid, *p*-hydroxybenzoic acid, vanillic acid, syringic acid, *p*-coumaric acid, *o*-coumaric acid, sinapic acid, caffeic acid, and ferulic acid were obtained from Sigma-Aldrich (Gillingham, U.K.). Standard compounds 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 at least 3 months.

Phenolic Acid Analysis. Separate extractions were carried out, as previously described (*16*), according to modified methods of Adom and Liu (*25*) for free, conjugated, and bound phenolic acids using 3,5-dichloro-4-hydroxybenzoic acid as an internal standard. Free phenolic acids were extracted using an 80:20 ethanol/water solvent mixture. Conjugated phenolic acids were released after alkaline hydrolysis of this initial extract (2 M NaOH, 4 h), and insoluble bound phenolic acids were released via alkaline hydrolysis (2 M NaOH, 4 h) of the residue from the initial ethanol/water extraction. Both conjugated and bound phenolic acid fractions were acidified to pH 2 (12 M HCl) after hydrolysis to enable extraction into organic solvent. Individual phenolic acids were measured using reverse-phase HPLC (Agilent 1100) with detection at 280 nm. Separations were achieved using a 250 × 4.6 mm, 5 μm, Discovery reversed phase Amide C_{16} column with a 20 × 4.0 mm, 5 μm, Discovery reversed phase Amide C_{16} precolumn. Column temperature was set at 30 °C. Injection volume was 20 μL, and the flow rate was set to 1 mL/min. A gradient elution program was utilized, as previously described (*16*), and incorporated a mobile phase of acetonitrile together with a solution of 2% (v/v) acetic acid in water. All phenolic acids were quantified by performing a ratio of phenolic acid peak area to that of the internal standard. Calibration curves of phenolic acid standards were constructed using authentic standards that had undergone the same extraction procedure to ensure that losses due to the extraction were accounted for. All samples were analyzed in duplicate (unless otherwise stated), and concentrations of individual phenolic acids were expressed in micrograms per gram (μg/g) of dry matter (dm).

Statistical Analysis. Calculations of means, standard deviations, and coefficients of variation were carried out using Microsoft Excel. Phenolic acid concentrations across genotypes and environments were compared by analysis of variance (ANOVA) also in Microsoft Excel. To relate phenolic acid values to other phytochemical data and to the physical parameters of the kernels, Pearson correlation coefficients were calculated on a dry weight basis using the Spotfire Decision Site (TIBCO, Somerville, MA).

Table 2. Observed Weather Conditions during 2005−2007 across the Experimental Sites Used in the HEALTHGRAIN Study

	temperature (°C)			precipitation (mm)		
	av min for any 10 day period between heading and harvest	av max for any 10 day period between heading and harvest	mean between heading and harvest	between heading and harvest	3 months before heading	3 months before heading to harvest
Hungary, 2005	−2.53	22.4	19.4	116	201.8	317.8
Hungary, 2006	−4.69	24.91	19.3	128.2	113.3	241.5
Hungary, 2007	0.72	24.5	20.5	126.6	117.1	243.7
France, 2007	−1.43	21.1	18.4	101.4	104.1	205.5
Poland, 2007	−0.43	21.61	17.7	204.2	85.5	289.7
U.K., 2007	2.39	17.18	14.2	232.6	127.6	360.2

Table 3. Free Phenolic Acid Concentrations of Different Genotypes Grown in Hungary (H) in 2005−2007 and in France (F), the United Kingdom (U.K.), and Poland (P) in 2007[a]

	growth location and year						statistics for 3 years at one site (H, 2005−2007)			statistics for 1 year across four locations (H, F, P, and U.K.)			statistics for total data across six environments (H, 2005−2007; P, F, and U.K., 2007)		
	H, 2005	H, 2006	H, 2007	F, 2007	P, 2007	U.K., 2007	av	SD	CV (%)	av	SD	CV (%)	av	SD	CV (%)
Atlas-66	9	11	13	8	7	1	11	2	18	7	5	65	8	4	49
Avalon	10	11	16	8	5	1	12	3	28	8	6	82	8	5	59
Cadenza	11	13	24	7		1	16	7	45	11	12	107	11	8	74
Campari	26	12	23	10	9	2	20	8	38	11	9	78	14	9	66
CF99105	14	11	19	10	8	5	15	4	24	10	6	55	11	5	42
Chinese Spring	6	7	13	8		2	9	4	45	8	6	76	7	4	58
Claire	15	13	24	9	7	8	17	6	32	12	8	67	13	6	50
Crousty	8	11	16	8	6	1	12	4	32	8	6	76	9	5	57
Disponent	26	10	20	7	7	2	19	8	42	9	8	84	12	9	75
Estica	15	24	17	8	8	1	19	5	25	9	6	75	12	8	66
Gloria	6	14	15	10	1	1	12	5	39	11	9	83	8	6	74
Herzog	22	11	23	9	10	2	18	7	36	10	6	60	12	8	65
Isengrain	8	10	19	9	8	5	12	6	49	7	7	96	10	5	49
Lynx	8	20	21	7	11	5	16	7	45	11	7	65	12	7	58
Malacca	9	15	19	11	8	2	14	5	38	10	7	74	11	6	58
Maris Huntsman	8	15	16	8	4	7	13	4	32	9	5	60	10	5	49
MV Emese	7	8	13	7	5	6	9	3	34	8	4	47	8	3	37
Obriy	7	12	16	6	6	4	12	4	36	8	5	65	8	4	51
Rialto	11	20	21	8	8	2	17	5	30	10	8	82	12	7	63
Riband	12	18	24	8	9	4	18	6	32	11	9	79	12	7	59
San Pastore	5	14	15	7	3	1	11	6	50	7	6	93	7	6	76
Spartanka	6	12	12	6	2	1	10	4	36	5	5	95	6	5	73
Tiger	14	17	13	9	6	5	15	2	15	8	3	41	11	5	43
Tommi	7	10	15	9	9	2	11	4	38	9	6	63	9	4	50
Tremie	20	10	23	7	3	6	18	7	39	10	9	90	11	8	70
Valoris	6	11	21	5	3	1	12	8	61	7	9	126	8	7	96
av	11	13	18	8	6	3									
SD	6	4	4	1	3	2									
CV (%)	54	30	22	17	41	72									
min	5	7	12	5	1	1									
max	26	24	24	11	11	8									

[a] Concentrations are given in μg/g of dm and represent a minimum of two independent replicate measurements. Statistical comparisons have been made to show the effects of annual variation, variation due to location, and variation across the six environmental conditions. In each case the average values have been calculated, together with the standard deviations (SD) and the coefficients of variation (CV).

Principal component analysis (PCA) was used to determine the effects of growth year and environment across the data set and to visualize the changes in phenolic acids responsible for these differences. PCA was carried out using SIMCA-P (v.11, Umetrics, Umea, Sweden) on data that had been scaled to unit variance.

RESULTS AND DISCUSSION

Free Phenolic Acids. *Variation in Samples Grown in Three Successive Years*. Free phenolic acids made up only 1−2% of the total (**Table 3**) and thus represent the least abundant class of phenolic acids in wheat. Despite their low concentration, levels of this class of metabolite could be reliably measured with typical relative standard deviations (% RSD) of individual free phenolic acid concentrations ranging from 1 to 18% for replicate extractions of the starting wholemeal flour sample. When grown at Martonvásár between 2005 and 2007, the mean concentrations ranged from 5 μg/g of dm (San Pastore, 2005) to 26 μg/g of dm (Campari, 2005, and Disponent, 2005). Despite the low concentration, the amounts of this class of phenolic acid showed the greatest variation from year to year. Values of the means across the 26 genotypes ranged from 11 to 18 μg/g of dm and showed a high coefficient of variation (CV = 22−54%). The variance of individual genotypes due to year of growth ranged from 15 to 61%, which was higher than for other phenolic acid classes and masked any genotypic effects.

Impact of Growing Location. Comparison of samples grown at four locations in 2007 showed that free phenolic acids displayed high variation in concentration (**Table 3**). The total amounts of this class of phenolic acid ranged from 24 μg/g of dm (Claire, Riband, and Cadenza, all grown in Hungary) to 1 μg/g of dm (many lines grown in the U.K. and also Gloria when grown in Poland). The mean values for the 26 genotypes across the growing locations ranged from 3 (U.K.) to 18 (Hungary) μg/g of dm and showed a very high coefficient of variation (CV = 17−72%). The variance of individual genotypes due to location was very high, in excess of 41% for all lines, masking any genotypic effects on concentrations. The contents of free phenolic acids were generally highest in the samples grown in Hungary and significantly ($p < 0.001$) lower in those grown in the United Kingdom. The samples grown in France and Poland were more similar in their contents of free phenolic acid but lower than those observed for the samples grown in Hungary.

Genotypic Variation in the Six Site × Year Combinations. With all six environments (i.e., sites and years) taken into account, the genotypes with the highest mean free phenolic acid concentrations were Campari (14 ± 9 μg/g of dm) and Claire (13 ± 6 μg/g of dm), and those showing the lowest mean concentrations across the six environments were Spartanka (6 ± 5 μg/g of dm), San Pastore (7 ± 6 μg/g of dm), and Chinese Spring (7 ± 4 μg/g of dm). Despite the large variation observed between environments, some genotypes appeared to be more "stable" than others. **Figure 1** illustrates the mean phenolic acid contents across the six environmental conditions years for each genotype and each phenolic acid fraction. Plots are ordered by the observed concentration ranges across the 3 year, multienvironment study. For the free phenolic acids (**Figure 1A**), the most stable lines (exhibiting the lowest concentration range across the six conditions) were MV Emese, Chinese Spring, and Spartanka, whereas those showing the highest variation included Campari, Disponent, and Estica. Although some lines were clearly more stable than others, it was nevertheless clear that the free phenolic acid content was extremely variable and that this environmental variation may have masked any true genotypic differences.

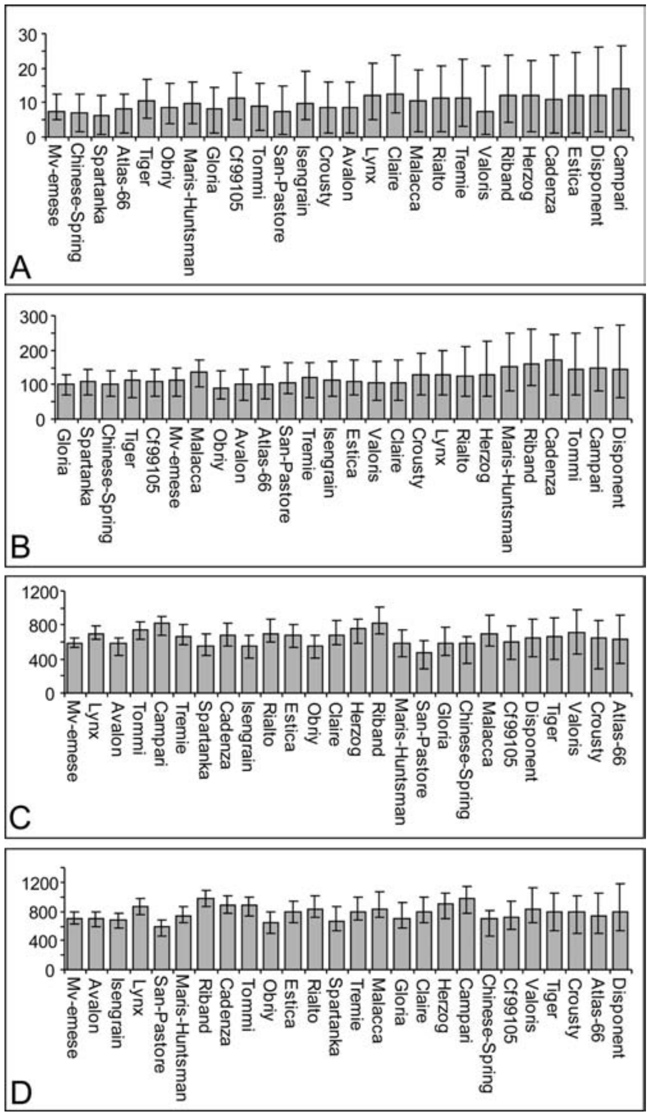

Figure 1. Phenolic acid concentrations across six environmental conditions, four locations (Hungary, France, Poland, U.K.) in 2007 and Hungary in 2005 and 2006: (**A**) free; (**B**) conjugated; (**C**) bound; (**D**) total phenolic acids. Concentrations are expressed in μg/g of dm. Error bars represent measured range. Order represents least to highest variation.

Conjugated Phenolic Acids. *Variation in Samples Grown in Three Successive Years*. Conjugated phenolic acids comprised approximately 20% of the total phenolic acids in wholemeal flour (*16*). Results for this class of phenolic acid are shown in **Table 4**. In contrast to other classes of phenolic acids, the contents of soluble conjugated derivatives were highest in 2005 (180 ± 53 μg/g of dm) and lowest in 2007 (137 ± 19 μg/g of dm). Highest levels in individual genotypes were observed for Disponent (276 μg/g of dm, 2005) and Cadenza (245 μg/g of dm, 2006; and 184 μg/g of dm, 2007), and the lowest levels were for Obriy (87 μg/g of dm, 2005), Estica (97 μg/g of dm, 2006), and San Pastore (110 μg/g of dm, 2007). The coefficient of variation ranged from 2 to 37% for individual genotypes across the 3 years and, unlike for free phenolic acids, some significant differences in the concentrations of conjugated phenolic acids between different genotypes could be identified for a number of the genotypes. In terms of year-to-year variation, genotypes with lower contents of conjugated phenolic acids (e.g., Tiger, Spartanka, Tremie, and CF99105) were more robust (lower CV), whereas lines with high levels of conjugated phenolic acids appeared to be more variable (higher CV) across the three growth years (e.g., Campari, Disponent, Herzog, Tommi, and Riband).

Impact of Growing Location. Conjugated phenolic acids in the material grown at four locations in 2007 showed a similar trend to free phenolic acids, with the U.K. samples having significantly lower contents (mean, 69 ± 10 μg/g of dm) and the samples grown in Hungary the highest contents (137 ± 19 μg/g of dm). The contents in the lines grown in France (103 ± 24 μg/g of dm) were most similar to those grown in Hungary, whereas the contents in the Polish samples (85 ± 15 μg/g of dm) were more similar to those grown in the samples grown in the United Kingdom. The genotypes with the highest contents were Cadenza (184 μg/g of dm, Hungary) and Maris Huntsman (168 μg/g of dm, Hungary), whereas the lowest was Avalon (53 μg/g of dm, U.K.). When data from all growth locations were averaged, as for free phenolic acids, there were few significant differences in mean values between genotypes. Cultivars showing the least variation in soluble conjugated phenolic acids, between locations, included San Pastore, Malacca, and Campari, whereas Cadenza, Disponent, Tremie, Maris Huntsman, and Obriy displayed higher variation across growth locations.

Genotypic Variation in the Six Site × Year Combinations. With all six environmental conditions taken into account, the genotypes with the highest mean conjugated phenolic acid

Table 4. Conjugated Phenolic Acid Concentrations of Different Genotypes Grown in Hungary (H) in 2005−2007 and in France (F), the United Kingdom (U.K.), and Poland (P) in 2007[a]

	growth location and year						statistics for 3 years at one site (H, 2005−2007)			statistics for 1 year across four locations (H, F, P, and U.K.)			statistics for total data across six environments (H, 2005−2007; P, F, and U.K., 2007)		
	H, 2005	H, 2006	H, 2007	F, 2007	P, 2007	U.K., 2007	av	SD	CV (%)	av	SD	CV (%)	av	SD	CV (%)
Atlas-66	152	135	122	61	71	64	136	15	11	79	28	36	101	40	40
Avalon	142	121	117	118	65	53	127	13	11	88	34	39	103	35	34
Cadenza	235	245	184	138		70	221	33	15	131	57	44	175	72	41
Campari	268	232	122	92	105	80	207	76	37	100	18	18	150	80	53
CF99105	145	141	131	87	83	67	139	7	5	92	28	30	109	34	31
Chinese Spring	108	125	137	66		66	124	15	12	90	41	46	101	33	33
Claire	172	139	116	78	62	54	143	28	20	78	27	35	104	47	45
Crousty	189	173	143	138	76	72	168	24	14	107	39	36	132	49	37
Disponent	276	206	160	89	78	63	214	58	27	98	43	44	145	84	58
Estica	174	97	119	118	73	71	130	40	31	95	27	28	109	38	35
Gloria	115	98	129	92	93	74	114	15	13	93	26	28	100	19	19
Herzog	230	177	123	105	79	66	177	53	30	93	35	38	130	62	48
Isengrain	168	150	145	81	80	66	154	12	8	97	23	24	115	44	38
Lynx	198	193	115	114	82	72	169	47	28	96	22	23	129	54	42
Malacca	175	168	132	122	119	93	159	23	14	117	17	14	135	31	23
Maris Huntsman	247	186	168	122	109	81	200	42	21	120	36	30	152	60	40
MV Emese	117	124	148	131	96	66	130	16	12	110	36	33	114	29	25
Obriy	87	108	141	77	73	59	112	28	25	87	37	42	91	30	33
Rialto	211	138	147	114	73	69	165	40	24	101	37	37	125	53	42
Riband	264	171	163	140	117	97	199	56	28	129	29	22	159	59	37
San Pastore	166	114	110	84	95	75	130	32	24	91	15	17	107	33	30
Spartanka	134	143	141	86	85	73	139	5	3	96	31	32	110	32	29
Tiger	134	139	136	122	80	62	136	3	2	100	35	35	112	33	29
Tommi	250	183	146	119	91	71	193	52	27	107	33	31	143	66	46
Tremie	165	162	153	118	77	64	160	6	4	103	40	39	123	44	36
Valoris	169	127	117	74	82	56	137	27	20	82	26	31	104	41	40
av	180	154	137	103	85	69									
SD	53	38	19	24	15	10									
CV (%)	29	25	14	23	18	15									
min	87	97	110	61	62	53									
max	276	245	184	140	119	97									

[a] Concentrations are given in μg/g of dm and represent a minimum of two independent replicate measurements. Statistical comparisons have been made to show the effects of annual variation, variation due to location, and variation across the six environmental conditions. In each case the average values have been calculated, together with the standard deviations (SD) and the coefficients of variation (CV).

concentrations were Cadenza (175 ± 72 μg/g of dm), Riband (159 ± 59 μg/g of dm), and Maris Huntsman (152 ± 60 μg/g of dm), and those showing the lowest mean concentration across the six environments were Obriy (91 ± 30 μg/g of dm) and Gloria (100 ± 19 μg/g of dm). As with the free phenolic acid fraction, a large variation in concentration was observed between different environments, but there was again clear evidence that some genotypes were more stable, exhibiting a lower concentration range across the six environments. **Figure 1B** illustrates the concentration ranges observed for this class of phenolic acids. The most stable genotypes (exhibiting the lowest concentration range across the six conditions) were Gloria, Chinese Spring, and Spartanka, whereas those exhibiting the highest variation included Campari, Disponent, and Tommi.

Bound Phenolic Acids. *Variation in Samples Grown in Three Successive Years*. Bound phenolic acids make up the greatest proportion of the total phenolic acids in wheat and typically comprise 75−80% of the total. Mean values for contents of bound phenolic acid ranged from 536 ± 163 μg/g of dm (2005) to 745 ± 119 μg/g of dm (2007) (**Table 5**). The highest levels for individual genotypes were found in Disponent (869 μg/g of dm, 2005), Atlas-66 (921 μg/g of dm, 2006), and Valoris (980 μg/g of dm, 2007), whereas the genotype displaying the lowest level of bound phenolic acids each year was San Pastore (291 μg/g of dm, 2005; 397 μg/g of dm, 2006; and 556 μg/g of dm, 2007). In terms of environmental stability, the lowest variance observed in a single genotype was 2% in MV Emese. Genotypes with higher contents of bound phenolic acids but low variance scores (across 3 years) were Herzog (CV = 5%), Lynx (CV = 10%), Campari (CV = 11%), Riband (CV = 11%), and Tremie (CV = 13%). In contrast, genotypes with high year-to-year variability included Atlas-66 (CV = 44%), Crousty (CV = 47%), Valoris (CV = 36%), and Tiger (CV = 39%).

Impact of Growing Location. The mean contents of bound phenolic acids in the genotypes grown in 2007 ranged from 607 ± 99 μg/g of dm in the material grown in France) to 749 ± 119 μg/g of dm in that grown in Hungary. The highest levels in individual genotypes were Riband (1004 μg/g of dm, grown in the U.K.) and Valoris (980 μg/g of dm, grown in Hungary), whereas the genotypes with the lowest levels were Disponent (434 μg/g of dm) and Spartanka (452 μg/g of dm) (both grown in France). In terms of stability, the lowest variance (due to location) in bound phenolic acid content observed in a single genotype was 7% in Avalon and 8% in Chinese Spring and Rialto. Genotypes showing higher variance related to location included Malacca and Isengrain (CV = 21%), Obriy (CV = 20%), and Valoris, Gloria, Crousty, and Herzog (CV = 17%).

Genotypic Variation in the Six Site × Year Combinations. The contents of bound phenolic acids showed less variation due to

Table 5. Bound Phenolic Acid Concentrations of Different Genotypes Grown in Hungary (H) in 2005−2007 and in France (F), the United Kingdom (U.K.), and Poland (P) in 2007[a]

	growth location and year						statistics for 3 years at one site (H, 2005−2007)			statistics for 1 year across four locations (H, F, P, and U.K.)			statistics for total data across six environments (H, 2005−2007; P, F, and U.K., 2007)		
	H, 2005	H, 2006	H, 2007	F, 2007	P, 2007	U.K., 2007	av	SD	CV (%)	av	SD	CV (%)	av	SD	CV (%)
Atlas-66	344	921	709	510	528	767	658	292	44	629	129	20	630	208	33
Avalon	450	583	658	618	561	659	564	106	19	624	46	7	588	78	13
Cadenza	554	769	614	823		706	646	111	17	714	105	15	693	110	16
Campari	741	905	909	685	794	902	852	96	11	823	106	13	823	97	12
CF99105	399	490	790	630	634	686	560	205	37	685	75	11	605	140	23
Chinese Spring	342	655	673	666		583	557	186	33	640	50	8	584	140	24
Claire	661	656	853	707	572	648	723	113	16	695	119	17	683	94	14
Crousty	293	753	860	559	715	757	635	301	47	723	125	17	656	203	31
Disponent	869	681	689	434	585	621	747	106	14	582	108	19	647	143	22
Estica	530	742	808	709	556	753	694	145	21	707	108	15	683	113	17
Gloria	453	624	779	509	520	661	619	163	26	719	122	17	591	120	20
Herzog	802	820	877	582	693	725	833	39	5	611	77	13	750	106	14
Isengrain	451	416	616	630	507	692	494	107	22	617	128	21	552	110	20
Lynx	681	763	626	702	702	785	690	69	10	704	65	9	710	57	8
Malacca	545	623	919	608	632	874	696	197	28	758	161	21	700	156	22
Maris Huntsman	497	436	678	575	586	745	537	126	23	646	81	12	586	113	19
MV Emese	562	589	572	653	525	593	574	13	2	586	53	9	582	42	7
Obriy	410	628	635	460	472	690	558	128	23	564	116	20	549	115	21
Rialto	594	872	746	631	641	691	737	139	19	677	53	8	696	101	15
Riband	701	813	872	782	737	1004	796	87	11	849	118	14	818	109	13
San Pastore	291	397	556	506	494	610	414	133	32	541	53	10	476	115	24
Spartanka	441	606	699	452	550	507	582	131	22	552	106	19	542	98	18
Tiger	389	896	769	493	755	724	684	264	39	685	129	19	671	190	28
Tommi	646	774	846	650	636	825	756	101	13	739	112	15	729	97	13
Tremie	813	681	634	563	601	746	709	93	13	636	79	12	673	94	14
Valoris	465	695	980	641	781	795	713	258	36	799	139	17	726	173	24
av	536	684	745	607	616	721									
SD	163	147	119	99	94	106									
CV (%)	31	22	16	16	15	15									
min	291	397	556	434	472	507									
max	869	921	980	823	794	1004									

[a] Concentrations are given in μg/g of dm and represent a minimum of two independent replicate measurements. Statistical comparisons have been made to show the effects of annual variation, variation due to location, and variation across the six environmental conditions. In each case the average values have been calculated, together with the standard deviations (SD) and the coefficients of variation (CV).

the environment than other classes of phenolic acids. Among all six growing environments, the genotypes with the highest mean bound phenolic acid concentrations were Campari (823 ± 97 μg/g of dm) and Riband (818 ± 109 μg/g of dm), whereas the genotype with the lowest concentration was San Pastore (476 ± 115 μg/g of dm). The most stable genotypes, in terms of bound phenolic acid contents, were MV Emese (CV = 7%), Lynx (CV = 8%), and Campari (CV = 12%), whereas Atlas-66 (CV = 33%), Crousty (CV = 31%), Tiger (CV = 28%), Valoris (CV = 24%), and Disponent (CV = 22%) showed the highest variance due to environment (**Figure 1C**).

Total Phenolic Acids. *Variation in Samples Grown in Three Successive Years*. The mean amounts of total phenolic acids, across all of the genotypes, ranged from 728 ± 206 to 900 ± 117 μg/g of dm (**Table 6**) and were significantly different [$F(2, 75) = 7.41$, $p = 0.001151$]. The concentrations of total phenolic acids were generally lowest in 2005 and highest in 2007, with the greatest variation (CV = 28%) across the genotypes being evident in the samples grown in 2005. Some variation in total phenolic acid content occurred when the behavior of individual genotypes was considered across different growing years. A clear example of this is Disponent, which had a high total phenolic acid content in 2005 (1171 μg/g of dm) but significantly lower contents in 2006 (897 μg/g of dm) and 2007 (869 μg/g of dm). Conversely, some other genotypes (e.g., Campari and Herzog) had high total phenolic acid contents with low year-to-year variation, suggesting that these genotypes may be better choices for breeders wishing to develop lines with a stable high phenolic acid content. Assessing the genotypes in terms of their total phenolic acid content for each of the three years illustrated such differences in stability of the total phenolic acid content. Only five lines (Campari, Herzog, Riband, and Tommi) showed consistency across the 3 years, whereas most of the genotypes showed variation in total phenolic acid content, which was typically higher in 2006 and 2007.

Impact of Growing Location. The mean contents of total phenolic acids of the 26 genotypes grown at four locations in 2007 ranged from 900 ± 117 to 707 ± 100 μg/g of dm and were significantly different [$F(3,98) = 16.54$, $p < 0.0001$] (**Table 6**). The amounts of total phenolic acids were generally lowest when the lines were grown in Poland and France and highest when grown in Hungary, with more genotypes having > 900 μg/g of dm when grown in Hungary compared with other locations. Genotypes with the highest contents of total phenolic acids included Valoris (1118 μg/g of dm, grown in Hungary), Malacca (1071 μg/g of dm, grown in Hungary), Riband (1105 μg/g of dm, grown in U.K., and 1058 μg/g of dm, grown in Hungary), Campari (1054 μg/g of dm, grown in Hungary), Herzog (1023 μg/g of dm, grown

Table 6. Total Phenolic Acid Concentrations of Different Genotypes Grown in Hungary (H) in 2005−2007 and in France (F), the United Kingdom (U.K.), and Poland (P) in 2007[a]

	growth location and year						statistics for 3 years at one site (H, 2005−2007)			atatistics for 1 year across four locations (H, F, P, and U.K.)			statistics for total data across six environments (H, 2005−2007; P, F, and U.K., 2007)		
	H, 2005	H, 2006	H, 2007	F, 2007	P, 2007	U.K., 2007	av	SD	CV (%)	av	SD	CV (%)	av	SD	CV (%)
Atlas-66	505	1067	843	580	605	832	718	135	19	715	142	20	739	212	29
Avalon	602	715	791	744	631	713	776	41	5	720	68	9	699	71	10
Cadenza	800	1027	822	969		777	961	123	13	856	100	12	879	112	13
Campari	1036	1149	1054	787	909	985	1079	61	6	933	114	12	986	126	13
CF99105	559	643	940	727	725	758	789	258	33	787	103	13	725	128	18
Chinese Spring	456	788	823	740		651	798	282	35	738	86	12	692	147	21
Claire	848	808	993	794	641	710	883	98	11	785	152	19	799	121	15
Crousty	491	937	1019	706	797	830	948	129	14	838	132	16	797	185	23
Disponent	1171	897	869	531	671	686	818	84	10	689	139	20	804	225	28
Estica	720	863	944	835	638	825	975	29	3	811	127	16	804	109	14
Gloria	575	737	923	611	615	736	725	210	29	823	140	17	699	129	18
Herzog	1054	1008	1023	696	781	793	1030	137	13	714	84	12	892	153	17
Isengrain	627	576	780	720	594	763	723	76	10	721	146	20	677	89	13
Lynx	887	977	762	824	795	862	655	96	15	811	42	5	851	76	9
Malacca	729	806	1071	741	760	969	804	248	31	885	161	18	846	141	17
Maris Huntsman	753	637	862	704	700	833	778	121	16	775	85	11	748	86	11
MV Emese	686	721	732	791	626	665	713	24	3	704	73	10	704	58	8
Obriy	504	748	792	543	551	752	593	175	30	660	131	20	648	128	20
Rialto	817	1030	913	753	722	762	921	73	8	788	85	11	833	118	14
Riband	978	1003	1058	930	863	1105	976	78	8	989	112	11	990	87	9
San Pastore	462	525	680	597	593	685	842	176	21	639	51	8	590	87	15
Spartanka	581	761	852	544	637	581	723	125	17	653	138	21	659	121	18
Tiger	537	1052	918	624	841	792	721	238	33	794	124	16	794	189	24
Tommi	903	968	1008	778	735	897	737	239	32	855	123	14	882	106	12
Tremie	998	852	809	688	681	817	891	84	9	749	74	10	807	117	15
Valoris	639	833	1118	719	866	851	902	318	35	888	166	19	838	163	19
av	728	851	900	718	707	793									
SD	206	164	117	111	100	112									
CV (%)	28	19	13	15	14	14									
min	456	525	680	531	551	581									
max	1171	1149	1118	969	909	1105									

[a] Concentrations are given in μg/g of dm and represent a minimum of two independent replicate measurements. Statistical comparisons have been made to show the effects of annual variation, variation due to location, and variation across the six environmental conditions. In each case the average values have been calculated, together with the standard deviations (SD) and the coefficients of variation (CV).

in Hungary), Crousty (1019 μg/g of dm, grown in Hungary), and Tommi (1008 μg/g of dm, grown in Hungary). The lowest contents were in Disponent (531 μg/g of dm, grown in France), Obriy (543 μg/g of dm, grown in France; and 551 μg/g of dm, grown in Poland), and Spartanka (544 μg/g of dm, grown in France; and 581 μg/g of dm, grown in U.K.).

Typical variance due to location across the 26 lines was 15%. For individual genotypes the variance in total phenolic acid content due to growing location ranged from 5 to 21%. Robust lines (exhibiting the lowest variance across four locations) were significantly different from each other and included Lynx (CV = 5%), San Pastore (CV = 8%), Avalon (CV = 9%), and Tremie (CV = 10%). Lines showing large differences in concentration range across the four growing locations included Valoris (CV = 19%), Claire (CV = 19%), Disponent (CV = 20%), Herzog (CV = 17%), and Malacca (CV = 18%). Of these, Valoris, Malacca, and Tremie exhibited the same behavior in the study in which identical cultivars were grown in Hungary across 3 years.

Genotypic Variation in the Six Site × Year Combinations. The mean contents of total phenolic acids of all genotypes across the six environmental conditions ranged from 707 ± 100 μg/g of dm (Poland, 2007) to 900 ± 117 μg/g of dm (Hungary, 2007) and were significantly different [$F(5,148) = 8.13$, $p < 0.0001$]. Cultivars showing the highest contents of total phenolic acids across all environments included Disponent (1171 μg/g of dm, Hungary, 2005), Campari (1149 μg/g of dm, Hungary, 2006), and Valoris (1118 μg/g of dm, Hungary, 2007). The lowest contents were in Chinese Spring (456 μg/g of dm, Hungary, 2005), San Pastore (462 μg/g of dm, Hungary, 2005), and Crousty (491 μg/g of dm, Hungary, 2005).

Typical variance across the 26 lines and six environments ranged from 13 to 28%. For individual genotypes the variance in total phenolic acid content ranged from 8 to 29%. As for the different phenolic acid classes, **Figure 1D** shows the different variabilities of genotypes in total phenolic acid content. The genotypes exhibiting the lowest observed range in concentration across the six conditions included MV Emese (626−791 μg/g of dm), Avalon (602−791 μg/g of dm), Isengrain (576−780 μg/g of dm), and Lynx (762−977 μg/g of dm). Genotypes showing large differences in concentration range across the six conditions included Disponent (531−1171 μg/g of dm), Atlas-66 (505−1067 μg/g of dm), Crousty (491−1019 μg/g of dm), and Tiger (537−1052 μg/g of dm).

Variation in Individual Phenolic Acids. *Variation in Samples Grown in Three Successive Years.* Individual phenolic acids were also been determined within each fraction, and the mean concentrations for each environment are given in **Table 7**. The full data set is given in the Supporting Information (Table S1). The most

Table 7. Mean Concentrations with Standard Deviations of Individual Phenolic Acids across 26 Wheat Cultivars[a]

		Hungary, 2005		Hungary, 2006		Hungary, 2007	
class	name	mean ± SD	range	mean ± SD	range	mean ± SD	range
free	4-hydroxybenzoic acid	0.12 ± 0.43	0−2	0.5 ± 0.86	0−3	0.58 ± 0.76	0−2
free	vanillic acid	2.62 ± 0.98	1−5	7.04 ± 1.61	5−11	5.31 ± 1.49	3−8
free	syringic acid	2.20 ± 0.91	0−4	1.00 ± 0.01	1−1	1.27 ± 0.53	1−3
free	dihydroxybenzoic acid[b]	0.27 ± 0.60	0−2	0.88 ± 0.33	0−1	0.08 ± 0.27	0−1
free	sinapic acid	1.77 ± 3.70	0−10	0.27 ± 0.60	0−2	2.65 ± 1.02	0−5
free	ferulic acid	3.50 ± 1.21	1−6	2.38 ± 0.50	2−3	3.00 ± 0.69	2−4
conjugated	4-hydroxybenzoic acid	6.12 ± 2.01	2−10	10.38 ± 3.80	4−21	4.92 ± 1.09	2−7
conjugated	vanillic acid	12.12 ± 3.94	6−20	7.46 ± 4.33	1−17	5.69 ± 2.94	1−12
conjugated	syringic acid	8.23 ± 4.41	2−17	8.19 ± 4.12	4−21	4.42 ± 2.04	1−10
conjugated	dihydroxybenzoic acid[b]	49.50 ± 24.14	9−116	24.27 ± 6.34	15−43	1.12 ± 0.33	1−2
conjugated	sinapic acid	63.54 ± 21.26	30−128	61.85 ± 28.70	18−109	88.46 ± 15.27	69−123
conjugated	ferulic acid	33.77 ± 13.84	8−65	32.08 ± 12.63	15−64	27.04 ± 6.79	18−50
bound	4-hydroxybenzoic acid	2.19 ± 1.20	0−5	5.46 ± 1.92	3−9	0.88 ± 0.43	0−2
bound	vanillic acid	4.23 ± 1.11	3−7	3.35 ± 1.41	2−7	3.85 ± 0.54	3−5
bound	syringic acid	4.12 ± 2.41	1−10	2.19 ± 1.06	1−4	2.15 ± 0.67	1−3
bound	dihydroxybenzoic acid[b]	90.27 ± 44.97	3−198	149.58 ± 61.38	53−281	19.50 ± 2.75	13−25
bound	sinapic acid	25.77 ± 6.36	15−39	69.92 ± 14.66	44−95	94.12 ± 15.88	66−128
bound	ferulic acid	397.77 ± 118.88	225−638	387.65 ± 74.22	248−552	591.00 ± 102.14	431−787

		France 2007		Poland 2007		UK 2007	
class	name	mean ± SD	range	mean ± SD	range	mean ± SD	range
free	4-hydroxybenzoic acid	0.04 ± 0.20	0−1	0.0	0−0	0.0	0−0
free	vanillic acid	4.62 ± 0.90	3−7	2.58 ± 1.77	0−5	1.19 ± 1.55	0−4
free	syringic acid	0.96 ± 0.20	0−1	0.71 ± 0.55	0−2	0.46 ± 0.51	0−1
free	dihydroxybenzoic acid[b]	0.0	0−0	0.0	0−0	0.0	0−1
free	sinapic acid	1.38 ± 0.80	0−3	0.08 ± 0.41	0−2	0.0	0−0
free	ferulic acid	1.58 ± 0.50	1−2	1.83 ± 0.48	1−3	1.23 ± 0.43	1−2
conjugated	4-hydroxybenzoic acid	4.65 ± 0.80	3−6	4.46 ± 0.72	3−6	4.23 ± 0.65	3−6
conjugated	vanillic acid	5.69 ± 1.62	2−9	6.00 ± 1.89	3−10	5.81 ± 2.10	3−10
conjugated	syringic acid	4.88 ± 2.29	2−12	3.25 ± 1.54	1−6	3.27 ± 1.54	1−7
conjugated	dihydroxybenzoic acid[b]	1.35 ± 0.49	1−2	1.04 ± 0.20	1−2	1.08 ± 0.63	0−2
conjugated	sinapic acid	59.00 ± 17.20	31−94	47.96 ± 11.15	26−78	34.04 ± 8.60	21−59
conjugated	ferulic acid	23.85 ± 5.72	11−37	20.79 ± 4.11	16−35	20.50 ± 3.06	14−27
bound	4-hydroxybenzoic acid	5.19 ± 1.77	1−8	1.04 ± 0.36	0−2	1.54 ± 0.51	1−2
bound	vanillic acid	3.77 ± 0.51	3−5	3.83 ± 0.56	3−5	4.19 ± 0.85	3−6
bound	syringic acid	2.08 ± 0.56	1−3	2.04 ± 0.55	1−3	2.38 ± 0.70	1−4
bound	dihydroxybenzoic acid[b]	20.19 ± 2.81	16−26	19.17 ± 2.65	14−26	22.46 ± 3.13	18−28
bound	sinapic acid	100.31 ± 16.17	68−129	77.21 ± 14.98	49−105	91.19 ± 20.66	66−149
bound	ferulic acid	448.27 ± 68.07	335−573	494.71 ± 70.78	386−657	586.81 ± 92.78	405−816

[a] The range represents the maximum and minimum values observed. [b] Predominantly 2,4-dihydroxybenzoic acid with minor amounts of 2,3-dihydroxybenzoic acid, which is unresolved in some cases.

abundant phenolic acids across all fractions were ferulic, sinapic, and dihydroxybenzoic acids. The relative amounts of these varied depending on the fraction being studied. The abundances of many individual free phenolic acids were very low, with vanillic, syringic, and ferulic acids being the most abundant components that could be measured. The most abundant soluble conjugated phenolic acids were ferulic, sinapic, and dihydroxybenzoic acids with sinapic acid being the most abundant in most genotypes. Ferulic acid was, as expected, the dominant component in the bound fraction, and levels of this compound were consistent with those previously reported by Rybka et al. (*26*) at 500 μg/g. These detected phenolic acids are also consistent with the work of Verma et al. (*27*), who also identified ferulic, sinapic, vanillic, and dihydroxybenzoic acids as major components of bran in a study on the effects of hydrolysis conditions in phenolic acid content using six Canadian wheat genotypes.

To understand more fully the contributions of the individual phenolic acids to the variation observed, principal component analysis (PCA) was carried out using the individual phenolic acid data for each genotype. This method allows us to highlight similarities and differences between groupings of samples. **Figure 2A** shows a PCA of data obtained from samples grown over three successive years in Hungary. Individual statistical models were constructed for each class of phenolic acid, and data were scaled to unit variance to remove any bias caused by comparing more abundant metabolites with those that are less abundant. Samples grown in different years could be separated by the first two components of the model. Separation between the three years was very clear for the free and bound phenolic acid fractions. Although the separation of the soluble conjugated phenolic acids was less clear-cut, the trends were the same as observed for the other fractions. **Figure 2B** is a heatmap showing the individual phenolic acids responsible for the separation of the different clusters. The components responsible for the separation of the free and conjugated phenolic acid fractions tended to be derived from benzoic acid (e.g., 4-hydroxybenzoic, vanillic, and syringic acids), whereas for the bound fraction, components derived from cinnamic acid (ferulic and sinapic acid), as well as 4-hydroxybenzoic acid, were the major components responsible for the separation of samples from different years. The 2005 samples were therefore separated from those grown in other years on the basis of higher concentrations of free syringic acid and conjugated

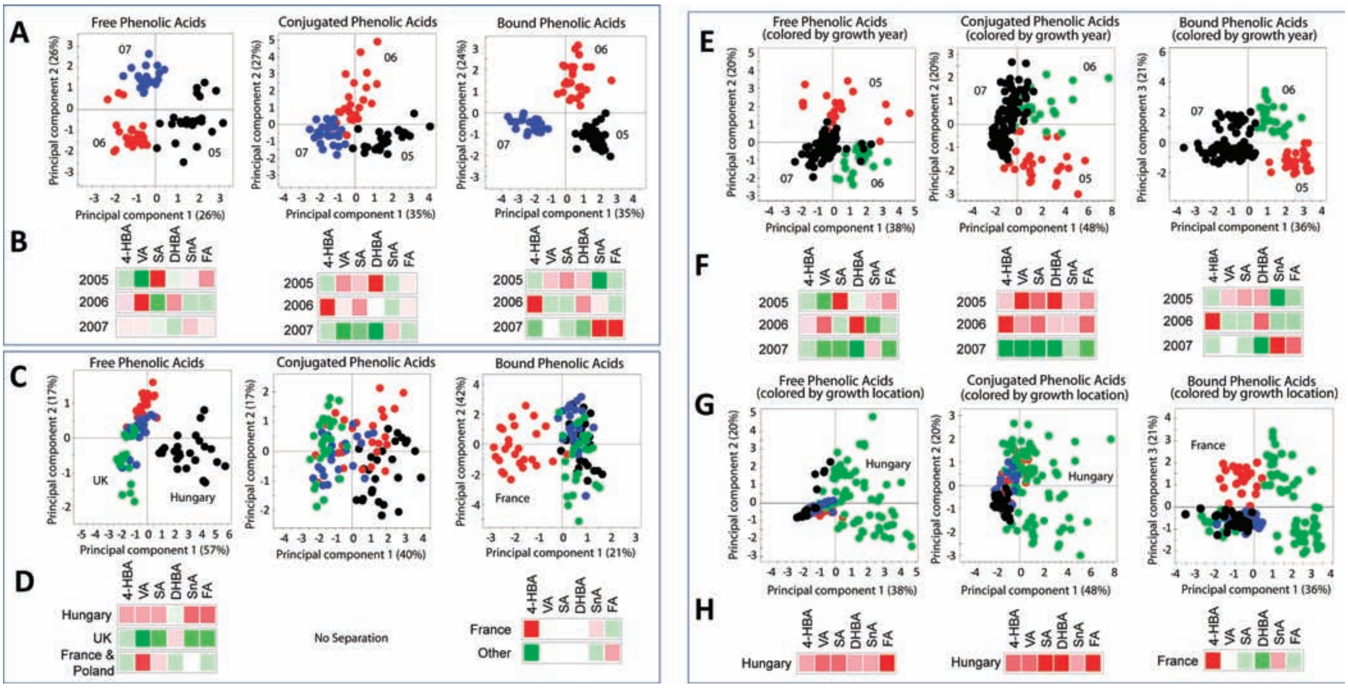

Figure 2. Principal component analysis (PCA) scores plots and associated heatmap representations describing differences constructed from individual phenolic acid data (free, conjugated, and bound fractions). 4-HBA, 4-hydroxybenzoic acid; VA, vanillic acid; SA, syringic acid; S-ald, syringealdehyde; DHBA, dihydroxybenzoic acid; SnA, sinapic acid; FA, ferulic acid. Red squares in the heatmaps represent metabolites that are increased and green squares represent those that are reduced in concentration. (**A**) PCA scores plots of separate data models from samples grown in Hungary (2005−2007): black, 2005; red, 2006; blue, 2007. (**B**) Heatmap representations illustrating which changes in phenolic acids are responsible for year-to-year separations. (**C**) PCA scores plots from separate data models of samples grown in 2007 (at four locations; red, France; green, U.K.; black, Hungary; blue, Poland). (**D**) Heatmap representations illustrating which changes in phenolic acids are responsible for separations due to growth location in 2007. (**E**) PCA scores plots from individual phenolic acid data of samples grown between 2005 and 2007 (across four locations; red, 2005; green, 2006; black, 2007). (**F**) Heatmap representations illustrating which changes in phenolic acids are responsible for separation by growth year across the six environments. (**G**) PCA scores plots of individual phenolic acid data from samples grown between 2005 and 2007 (across four locations; green, Hungary; red, France; black, U.K.; blue, Poland). (**H**) Heatmap representations illustrating which changes in phenolic acids are responsible for separation by growth location across the six environments.

dihydroxybenzoic acid and lower levels of bound sinapic acid. The 2006 samples are characterized by higher levels of free vanillic acid, together with higher levels of 4-hydroxybenzoic acid in the soluble conjugated and bound forms. Finally, the 2007 samples were characterized by higher levels of bound sinapic and ferulic acids compared to samples grown in other years.

Impact of Growth Location. **Figure 2C** shows a PCA of data from samples grown at different locations in 2007. Unlike the PCA of growth years, where samples grown in different years were clearly grouped on the basis of the compositions of each phenolic acid fraction, data from samples grown at four locations could not be similarly completely separated. However, the separations that were observed tended to be greater than the differences related to growth year. The samples grown in Hungary were clearly separated from those grown at other locations on the basis of free phenolic acids, and analysis of the metabolites responsible for the separation (**Figure 2D**) showed higher levels of the majority of free phenolic acids, with the exception of dihydroxybenzoic acid, which remained unchanged. This separation occurred in the direction of PC1 and explained 57% of the total variance. PC2, explaining 9% of the variation, separated the U.K. samples from those grown in France. The U.K. samples were characterized by a general reduction in the levels of most free phenolic acids. The French samples, although lower in most free phenolic acids, were unusually high in free vanillic acid, which was very low in the samples grown in the United Kingdom. Unlike for free phenolic acids, PCA modeling of the soluble conjugated phenolic acids did not separate the data set by growth location. Modeling of bound phenolic acid data separated the samples grown in France, on the basis of an increase in 4-hydroxybenzoic acid.

Genotypic Variation in the Six Site × Year Combinations. PCA of data from samples grown at the six environments is shown in **Figure 2E−H**. The data for free phenolic acids and conjugated phenolic acids showed separations due to year and also due to growth location. This contrasts with the data for bound phenolic acids, for which a separation by year only was observed. Considering all of the data together, the samples grown in Hungary were generally separated from the other growth locations, due to increases in most of the individual phenolic acids (**Figure 2H**). No discernible differences in the compositions of phenolic acids were observed between growth locations. When the data points are colored by growth year, separations can be seen for each class of phenolic acid, including the bound phenolic acid fraction. For the free phenolic acid fraction, these year-to-year separations are mainly due to syringic and dihydroxybenzoic acids. In addition, the samples grown in 2007 generally contained lower total contents of free phenolic acids. Similarly, lower contents of individual conjugated phenolic acids were observed in samples grown in 2007. The samples grown in 2005 and 2006 were separated on the basis of different contents of components derived from benzoic acid (vanillic, 4-hydroxybenzoic, and dihydroxybenzoic acids). Variation in bound phenolic acids was mainly due to different contents of 4-hydroxybenzoic acid in 2006 and increased contents of sinapic and ferulic acids in 2007.

Influence of Weather Conditions. The average temperature (heading to harvest) across the six environments varied from 14.2 to 20.5 °C, whereas precipitation in the 3 months before

Table 8. Correlations between Contents of Phenolic Acids and Other Phytochemical Data in the HEALTHGRAIN Project across Grain of 26 Wheat Lines Grown in Six Site × Year Combinations[a]

	free phenolics		conjugated phenolics		bound phenolics		total phenolics	
	R	*p* value	*R*	*p* value	*R*	*p* value	*R*	*p* value
alkylresorcinols	0.382	**<0.0001**	0.186	**0.019**	**0.594**	**<0.0001**	**0.575**	**<0.0001**
folates	**0.723**	**<0.0001**	**0.621**	**<0.0001**	0.314	**0.001**	**0.462**	**<0.0001**
tocols	**0.465**	**<0.0001**	0.257	**0.009**	**0.543**	**<0.0001**	**0.573**	**<0.0001**
sterols	**0.462**	**<0.0001**	**0.515**	**<0.0001**	0.272	**0.001**	**0.412**	**<0.0001**
stanols	0.279	**<0.0001**	0.256	**0.001**	−0.104	0.201	0.000	0.996
total free phenolic acids			**0.634**	**<0.0001**	**0.419**	**<0.0001**	**0.572**	**<0.0001**
total conjugated phenolic acids	**0.634**	**<0.0001**			0.302	**<0.0001**	**0.563**	**<0.0001**
bound phenolic acids	**0.419**	**<0.0001**	0.302	**<0.0001**			**0.957**	**<0.0001**
total phenolic acids	**0.572**	**<0.0001**	**−0.563**	**<0.0001**	**0.957**	**<0.0001**		
bran total arabinoxylan	−0.235	**0.003**	0.306	**<0.0001**	−0.266	**0.001**	−0.334	**<0.0001**
bran water extractable arabinoxylan	0.104	0.197	0.182	**0.023**	**0.417**	**<0.0001**	**0.421**	**<0.0001**
flour total arabinoxylan	0.204	**0.011**	0.250	**0.002**	0.348	**<0.0001**	0.386	**<0.0001**
flour water extractable arabinoxylan	0.060	0.457	0.140	0.080	0.330	**<0.0001**	0.331	**<0.0001**
β-glucan	0.305	**<0.0001**	0.357	**<0.0001**	0.213	**0.008**	0.306	**<0.0001**
lignin (% DM)	**0.592**	**<0.0001**	**0.571**	**<0.0001**	0.393	**0.001**	**0.510**	**<0.0001**
digestible starch (% DM)	**−0.591**	**<0.0001**	**−0.485**	**<0.0001**	−0.227	**<0.0001**	**-0.339**	**<0.0001**
amylose in grain (% DM)	**−0.491**	**<0.0001**	−0.321	**<0.0001**	−0.202	**0.0066**	−0.274	**<0.0001**
bran yield	0.015	0.855	0.219	**<0.0001**	0.113	0.168	0.171	**0.0355**
thousand kernel weight	0.324	**<0.0001**	0.091	0.268	−0.183	**0.0243**	−0.200	**0.0140**

[a] Values in bold represent significant ($p < 0.05$) correlations with a correlation coefficient (R) > 0.4. R, correlation coefficient; p value, significance.

heading varied from 85.5 to 201.8 mm and that between heading and harvest from 101 to 233 mm (**Table 2**). The samples grown in Hungary over three successive years showed a significant correlation ($R^2 = 0.81$, $p = 0.014$) of temperature with the contents of free phenolic acids and a weaker correlation ($R^2 = 0.57$, $p = 0.084$) with conjugated phenolic acids. Correlations of total and bound phenolic acids with precipitation before heading were also observed but were not statistically significant ($p > 0.05$) when data from 2005 were included. When 2005 data were excluded, the correlations were highly significant (e.g., $R^2 = 0.88$ and $p = 0.019$ for bound phenolic acids).

Relatively few studies have been reported describing variation in phenolic acids when wheat is grown at the same location in different years. However, several studies describing variation due to growing location are available. For example, Yu et al. (*28*) determined total phenolic acids in bran from cv. Akron grown at five locations, with and without irrigation. Measurements were also made of solar radiation and the number of hours exceeding 32 °C. The nonirrigated test locations were characterized by below-normal precipitation and drought stress during vegetative development (prior to heading stage), and clear differences were observed between the irrigated and nonirrigated samples, with the former having higher phenolic acid contents in bran. Correlations with total solar radiation, daily average solar radiation, or the number of hours exceeding 32 °C were not significant. In our study the average precipitation levels, both between the heading to harvest dates and also including 3 months before heading, were similar in 2006 and 2007 but differed in 2005 (**Table 2**). This was in contrast to average temperature between heading and harvest dates, which was more similar in 2005 and 2006. No significant correlations were found between total phenolic acid content and precipitation or temperature.

In 2007, samples were grown on four European sites, with the average temperatures from heading date to harvest being highest in the Hungarian site and lowest in the United Kingdom, with the French and Polish sites experiencing similar mean temperatures across the grain development period. The precipitation from heading to harvest was lower in the sites in Hungary and France and higher in those in Poland and the United Kingdom, whereas the precipitation 3 months before heading to heading was more similar in the sites in Hungary and the United Kingdom and generally higher than that experienced in the sites in France and Poland. The lines grown in Hungary had the highest contents of total phenolic acids, and this site also had a hot dry summer. In addition, the Hungarian site experienced the greatest extremes of temperature. The samples grown in the United Kingdom generally showed the second highest levels of total phenolic acids, and although the total precipitation in the 3 months before heading was similar to that in Hungary, the total precipitation to harvest date was greater. In addition, the mean temperatures at the sites in the United Kingdom and in Hungary were the most extreme of any of the growing locations. Thus, it appears that rainfall in the period before heading may be a more relevant factor influencing phenolic acid content than total precipitation. This is consistent with the study of Yu et al. (*28*), who demonstrated that irrigated plants had higher contents of phenolic acids in bran.

Correlations with Other Phytochemicals and Physical Properties. Strong correlations were observed between the total amounts of the different classes of phenolic acid (**Table 8**). The highest observed correlation was between bound phenolic acids and total phenolic acids ($R = 0.957$, $p < 0.001$), but this was to be expected as bound phenolic acids make up a significant proportion of the total. Similar correlations have previously been reported (*29*) in a study of 51 Canadian wheats grown at a single site in Saskatoon, Canada, where the total amounts of both free and bound phenolic acids correlated well with the total phenolic acid content.

In addition to correlations between the classes of phenolic acids, there were strong correlations between the contents of phenolic acids and those of other phytochemicals analyzed in the HEALTHGRAIN project [described in Shewry et al. (*23*) and the accompanying papers]. Significant positive correlations of free phenolic acids occurred with folates ($R = 0.723$, $p < 0.0001$), tocols ($R = 0.465$, $p < 0.001$), and sterols ($R = 0.462$, $p < 0.001$). However, strong correlations were not observed with the two major types of dietary fiber component in wheat grain (arabinoxylans and β-glucan). Total conjugated phenolic acids showed strong correlations with folates ($R = 0.621$, $p < 0.001$) and sterols ($R = 0.515$, $p < 0.001$) and again no clear correlations with fiber components. Bound phenolic acids, unlike other forms, correlated with total alkylresorcinols ($R = 0.594$, $p < 0.001$), tocols ($R = 0.543, p < 0.001$), and water-extractable arabinoxylan in bran ($R = 0.417$, $p < 0.001$). Although total starch was not measured within the HEALTHGRAIN project, the content of digestible starch (% dm) was measured (Boros et al., unpublished data) and was negatively correlated with the contents of all phenolic acid fractions. Whereas free and conjugated phenolic acid fractions showed the strongest correlations ($R = -0.591$ and -0.485, respectively), the correlation with bound phenolic acids was lower and not significant. Conversely, all fractions showed positive correlations with the content of lignin, an integral part of the cell wall of the outer pericarp of the grain, which again was not surprising given that lignin is made up of a series of polymeric phenolic acid linkages and that both classes of metabolite share common biosynthetic ancestry (*30*). Although phenolic acids are known to be concentrated in the bran, no significant correlations were found between phenolic acid content (for any class) and grain properties such as bran yield, thousand kernel weight, or protein content.

We have determined the contents of phenolic acids in 26 wheat genotypes grown on the same site for three successive years and on four sites in different countries for a single year, giving six site × year combinations. Variation related to growing location was greater than that observed between growing seasons. These environmental effects were larger than genotypic differences, especially for the low abundance free and conjugated phenolic acid fractions. Bound phenolic acids, which form the largest proportion of the total phenolic acids, were less influenced by environment, and more genotypic differences were discernible. Furthermore, the extent of variation related to environmental conditions varied from genotype to genotype with some genotypes being more stable in composition. This suggests that genotypes with higher and more stable contents of phenolic acids could be selected for cultivation and for use in plant breeding. This was also suggested by Menga et al. (*31*), who found similar variation in phenolic acid content due to environment across a range of cereals when grown at different locations across Italy. Alternatively, grain producers may exploit environmental impact by selecting growing locations that result in higher contents of phenolic acids.

ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; PCA, principal component analysis; dm, dry matter; CV, coefficient of variation; SD, standard deviation; 4-HBA, 4-hydroxybenzoic acid; VA, vanillic acid; SA, syringic acid; S-ald, syringealdehyde; DHBA, dihydroxybenzoic acid; SnA, sinapic acid; FA, ferulic acid.

Supporting Information Available: Supplementary Table 1. This material is available free of charge via the Internet at http://pubs.acs.org.

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