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ARTICLE

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

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The effects of environment on the content and composition of phytosterols were examined in 26 wheat genotypes grown at four locations in Europe during a single year and at one location over three consecutive years. Total phytosterol contents varied among the locations, whereas no effect was found for the harvesting year. A significant genetic variation was observed in total sterol contents (700–928 μg/g of dm). The genotype and environment resulted in statistically significant differences in the proportions of the main phytosterols. The high phytosterol contents were characterized by low proportions of sitosterol and high proportions of stanols. Small wheat kernels with proportionally high bran yield and lipid content contained higher levels of phytosterols than large kernels. Knowledge of the level and variability of phytosterols in wheat enables the selection of genotypes with high and stabile phytosterol contents for cultivation or plant breeding purposes.

KEYWORDS: Phytosterol; wheat; year; location; genotype; environment; variation

INTRODUCTION

Phytosterols are plant-derived bioactive compounds with a basic structure similar to that of cholesterol. These compounds are known to interfere with dietary and biliary cholesterol absorption in the small intestine. Numerous studies have shown the serum total and low-density lipoprotein cholesterol-lowering function of phytosterols, and there is also evidence of other health benefits, including cancer-preventing properties (*1−4*). Therefore, these health-promoting compounds are commonly added to functional food products. The excessive consumption of phytosterol-enriched foods may, however, lead to doses above the daily recommended dose of 1.5−2.4 g (*5−7*). Interestingly, the inhibition of cholesterol absorption or the reduction in serum cholesterol levels may be attained with relatively small amounts of naturally occurring phytosterols, as well. Klingberg et al. (*8*) and Andersson et al. (*9*) recently found that habitual diets rich in natural phytosterols resulted in significantly lower serum cholesterol concentrations within British and Swedish populations than diets with low phytosterol contents. The average daily consumption of phytosterols in the preceding populations was under 200 mg in those groups with the lowest intake and over 450 mg in those with the highest intake. Moreover, the cholesterol absorption-restraining effect of phytosterols has been observed with amounts as low as 150 mg (*10, 11*). Thus, there is growing interest in increasing the intake of these compounds in natural diets.

The main natural dietary sources of phytosterols are oils, margarines, cereals, and vegetables. The contribution of cereals is of great importance; cereal-based foods account for up to 42%

of the typical daily intake of phytosterols, which in the average Western diet is approximately 200−300 mg, depending on population and gender (*8, 9, 12−15*). In cereals, the phytosterols are concentrated in the germ and bran layers of the kernel and, thus, wholegrain products are good sources of sterols and also of other phytochemicals, vitamins, minerals, and dietary fiber (*16, 17*). The intake of phytosterols could be enhanced in a natural way by increasing the consumption of cereals, especially wholegrain cereals. Another approach is to enrich the amount of natural phytosterols in cereals and cereal-based foods by selecting the most phytosterol-rich wheat genotypes for cultivation, by plant breeding to develop genotypes with even higher phytochemical contents, or by means of processing. However, background knowledge of the natural variation of these compounds in cereals is needed, because genetic and environmental factors may affect the phytosterol contents and compositions of cereals. An integrated project, HEALTHGRAIN, within the Sixth Framework Program of the European Union, was initiated in 2005 to meet these challenges. One of the aims of the HEALTHGRAIN project is to determine the extent of natural variation in phytochemical and dietary fiber contents of cereals, the main focus being on wheat due to its importance as one of the major cereal grains throughout Europe and the world (*18, 19*).

Prior to the HEALTHGRAIN project, little was known of the natural variation in wheat phytosterols. As part of this project, we previously found that the phytosterol contents of 150 bread wheat genotypes grown in Hungary in 2005 ranged from 670 to 959 μg/g of dry matter (dm), thus showing genetic variation (*20*). More recently, a wide range in variation was also seen in the phytosterol contents of 23 bread wheat genotypes (*21*), whereas less variation was observed in the phytosterol contents in two studies involving five wheat cultivars (*22, 23*). The genetic factors affected the

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Article

J. Agric. Food Chem., Vol. 58, No. 17, 2010   **9315**

Table 1. Phytosterol Contents (Micrograms per Gram of Dry Matter) of 24 Genotypes Grown in Hungary in 2005−2007

genotype	Hungary 2005					Hungary 2006					Hungary 2007				
	sito-sterol	campe-sterol	stanols	others	total sterols	sito-sterol	campe-sterol	stanols	others	total sterols	sito-sterol	campe-sterol	stanols	others	total sterols
Campari	426	132	228	71	857	407	135	248	73	863	424	140	248	74	886
Herzog	382	114	189	54	739	355	109	204	54	722	373	117	194	53	736
Disponent	451	149	217	76	892	423	140	218	75	856	488	168	220	78	954
Tommi	460	125	229	76	890	437	129	244	79	889	449	138	224	69	880
Tremie	468	142	229	75	913	437	132	257	78	904	458	151	224	69	903
CF99105	479	140	237	72	929	468	139	256	92	956	436	137	208	71	852
Valoris	426	138	214	61	839	397	128	246	72	842	451	156	233	70	911
Isengrain	432	140	203	67	842	425	139	233	78	875	417	141	213	65	836
Claire	495	159	228	78	959	467	147	255	91	959	491	162	244	83	980
Maris Huntsman	454	132	248	89	924	437	128	269	97	932	453	140	266	89	948
Lynx	468	146	223	81	918	464	147	244	99	953	490	158	252	95	994
Malacca	449	140	212	65	865	430	137	235	73	876	449	144	228	73	893
Rialto	458	136	222	71	888	438	126	241	86	890	468	146	229	76	920
Riband	461	165	239	91	955	434	151	252	97	934	460	177	244	91	972
Avalon	366	110	200	62	739	427	124	246	87	884	425	138	234	75	872
San Pastore	408	122	164	59	753	409	115	188	68	780	386	103	170	58	717
Estica	440	145	171	75	832	421	142	184	77	824	446	158	164	70	838
Gloria	419	124	168	69	781	439	127	196	96	859	411	119	185	74	790
Spartanka	399	127	184	72	783	439	118	223	83	863	395	126	203	74	799
Obriy	421	134	175	53	783	412	126	201	65	804	406	127	194	54	781
Atlas 66	496	155	215	86	951	479	144	254	100	976	449	143	216	82	890
MV Emese	395	119	171	59	745	409	123	194	72	798	378	113	177	59	728
Chinese Spring[a]	412	122	220	81	836	409	109	251	103	872	411	123	228	91	853
Cadenza[a]	526	153	174	97	949	545	159	220	114	1039	486	143	178	89	896
av[b]	441	136	207	73	857 a	434	132	232	84	881 a	438	140	216	74	868 a
SD	38	14	26	12	74	35	13	25	14	70	35	18	28	12	79
CV (%)	9	10	12	16	9	8	10	11	17	8	8	13	13	16	9
range	366−526	110−165	164−248	53−97	739−959	355−545	109−159	184−269	54−114	722−1039	373−491	103−177	164−266	53−95	717−994

[a] A spring wheat type. [b] A statistically significant difference in the average total content among years is denoted with a different letter ($p < 0.05$).

contents of phytosterols, for example, in durum wheat (*20*), rye (*24*, *25*), oat (*26*), and barley (*27*). In addition to genetic variation, environmental factors may also greatly affect the contents and compositions of phytosterols in wheat. Such factors may include weather conditions, soil type, agronomic practices, or other variables related to the growing year and location. Significant effects of both genotype and environment on the phytosterols were observed in three wheat cultivars grown at three locations in the United States (*28*). Variation in phytosterols due to planting location was also reported in soybean seeds (*29*), whereas in oat the effect of location was not significant (*26*). Moreover, the effect of growing year on phytosterol content was demonstrated in rye (*24*). The soybean seeds were considered to be richer in phytosterols when grown in warm areas (*29*), whereas in rye the phytosterol content decreased due to high temperature and dryness (*24*). Thus, although there is some evidence of environmental variation in the phytosterols of cereals, current knowledge concerning wheat phytosterols is still limited.

The aim of the present study was to examine the environmental and genetic variation in phytosterols of wheat as a part of the HEALTHGRAIN project. The effects of environmental and genetic factors were determined by analyzing the phytosterol contents and compositions of 26 bread wheat genotypes, including both winter and spring wheat types, which were systematically grown during three consecutive years at the same location or in a single crop season at four different locations in Europe. Discovery of the extent of natural variation in the bioactive compounds of wheat will provide valuable data for cultivators and plant breeders. The production of wheat-based foods with increased levels of phytosterols would enhance the intake of these compounds and thus promote health and well-being.

MATERIALS AND METHODS

Cereals. The cereal samples included 24 winter wheat and 2 spring wheat (*Triticum aestivum* var. *aestivum*) genotypes. The winter wheat genotypes were Campari, Herzog, Disponent, Tommi, Tremie, CF99105, Valoris, Isengrain, Claire, Maris Huntsman, Lynx, Malacca, Rialto, Riband, Avalon, San Pastore, Estica, Gloria, Spartanka, Obriy, Atlas 66, Crousty, Tiger, and MV Emese. The spring wheat genotypes were Chinese Spring and Cadenza. Detailed descriptions of the genotypes were published by Ward et al. (*18*) and Shewry et al. (*19*).

To determine the variation in the phytosterol content of wheat due to the growing year and location, the wheat genotypes were grown under strictly controlled conditions in experimental fields at Martonvásár (Hungary) over three consecutive years, 2005−2007, and also at three other sites, Enchantillon (France), Woolpit (U.K.), and Choryn (Poland) in 2007 (*19*). Description of the geographic sites is given by Shewry et al. (*19*). Two of the winter wheat genotypes, Crousty and Tiger, were included in the trial only in 2007. Furthermore, only winter wheat genotypes were grown in Poland. The phytosterol contents of the genotypes grown in 2005 in Hungary were reported in our previous publication, but not in detail (*20*). The weather conditions are described elsewhere (*19*, *30*). Briefly, 2005 in Hungary was characterized by high precipitation before the harvest. During the growing season 2007, the average temperature from heading to harvest month was highest in Hungary and lowest in the United Kingdom. During this period the precipitation was very low in Hungary, whereas the highest level of precipitation was observed in the United Kingdom. Moreover, details on the agronomic conditions, soil properties, and heading and harvest dates at various sites are provided in other publications of the HEALTH-GRAIN project partners (*19*, *30*, *31*). The agronomic practices were similar at each location.

After harvesting at crop maturity, the wheat samples of the various growing years and locations were equally milled to 0.5 mm particle size in Hungary (*30*), and wholegrain flours were stored in the dark at −18 °C until analyzed. The winter wheat genotype MV Emese, grown in Hungary,

9316 *J. Agric. Food Chem.,* Vol. 58, No. 17, 2010

Nurmi et al.
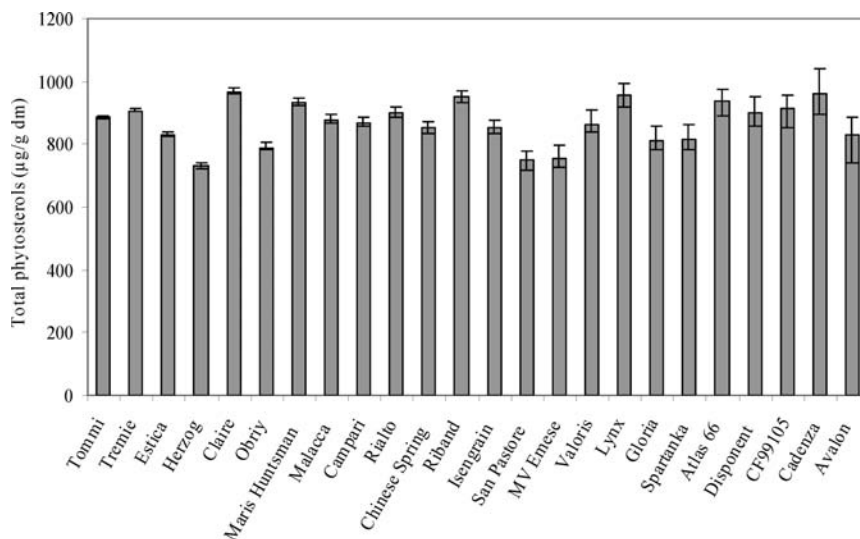
Figure 1. Average total phytosterol contents of various genotypes grown in Hungary in 2005−2007 in order of increasing variation among years. The error bars represent the range in three years.

was applied as an in-house reference. Two batches of the reference were used during the experiment: the first batch with samples grown in 2005 and 2006 and the second with samples grown in 2007. The HEALTH-GRAIN partners kindly provided the moisture contents, total lipid contents, 1000 kernel weights (TKWs), and bran yields of the wheat samples (19).

Standards and Reagents. All standard compounds, solvents, and reagents used for sample preparation and analysis were reported in our previous publications (20, 32).

Phytosterol Analysis. Phytosterol analysis was based on the method of Piironen et al. (32) and was previously described by Nurmi et al. (20) and Nyström et al. (33). The method included both acid and alkaline hydrolysis prior to phytosterol extraction, purification using silica solid-phase extraction (SiOH-SPE), derivatization to trimethylsilyl (TMS) ethers, and analysis using gas chromatography with flame ionization detection (GC-FID). Each sample was analyzed in duplicate. The identification of phytosterols (15 individual sterol species) was ensured with commercial standards, literature data, and gas chromatography−mass spectrometry (GC-MS). Quantification was performed, using dihydrocholesterol as an internal standard. The limit of determination for GC-FID was 1 μg/g of fresh weight (fw) of flour.

Performance of the Analytical Method. The performance of the analytical method, including method validation and the sample reanalysis procedure, was reported earlier (20). The sterol standard mixture was analyzed daily to confirm the performance of the GC-FID. Moreover, the in-house reference flour was analyzed in each sample set. The two batches of the in-house reference flour contained 652 ± 14 (n = 52) and 657 ± 17 μg/g of fw (n = 39) phytosterols, indicating good repeatability, because the phytosterol contents of the in-house references were within the action limits set at 656 ± 32 and 651 ± 34 μg/g of fw (average ± 2 × standard deviation (SD), n = 10), respectively. The differences between the total phytosterol contents of the duplicate samples did not exceed 5%. All results are presented as means of replicate samples on a dm basis.

Statistical Analysis. Comparison of the phytosterol contents of the wheat genotypes in the various growing years and locations was performed with a randomized block design two-way analysis of variance (ANOVA), using the genotype as a block factor (p < 0.05) and Fisher's least significant difference (LSD) procedure (p < 0.05). In addition, the overall variation was evaluated using one-way ANOVA. Correlations between the total phytosterol contents and TKWs, total lipid contents, bran yields, or contents of individual sterols were analyzed using Pearson correlation coefficients (20). The correlations with p values lower than 0.05 were considered to be statistically significant. Statgraphics Plus 4.0 software (Manugistics, Inc., Rockville, MD) was used for statistical analyses. Moreover, principal component analysis (PCA) was applied to describe the variation and visualize the associations among different variables, using The Unscrambler v 9.0 software (Camo Software AS, Oslo, Norway).

RESULTS AND DISCUSSION

Variation among Years. The yearly variation in phytosterol content of wheat was determined by growing the 24 wheat genotypes over three consecutive years at the same location in Hungary. The lowest phytosterol contents were most frequently found in samples grown in 2005 (**Table 1**). However, the average phytosterol contents did not vary considerably over the growing years, the values being 854 ± 75, 875 ± 64, and 867 ± 82 μg/g of dm in 2005, 2006, and 2007, respectively. For both winter and spring wheats, the respective values remained somewhat similar (857 ± 74, 881 ± 70, and 868 ± 79 μg/g). Thus, the average content of phytosterols was only 3% greater in 2006 and 1% greater in 2007 than in 2005. No statistically significant differences among the average phytosterol contents of the various growing years were observed for the winter wheat genotypes ($F(2,42) = 2.33$, $p = 0.1096$) or all bread wheats ($F(2,46) = 3.08$, $p = 0.0557$). In view of the individual wheat genotypes, however, variation among years was seen in the phytosterol contents of certain genotypes, whereas the contents of some genotypes were very stable during the growing years (**Figure 1**). The narrowest ranges, indicating low variation in total phytosterol contents, were found in the western European varieties Tommi, Tremie, Estica, Herzog, and Claire. The high year-to-year stability may represent a favorable characteristic for cultivators and plant breeders. Conversely, greater variation among years was perceived in genotypes such as Avalon, Cadenza, CF99105, Disponent, and Atlas 66. In those genotypes with wide variation, the highest phytosterol contents were most often found in 2006.

Although no significant year-to-year variation was observed in total content, the phytosterol composition seemed to vary among years. There were statistically significant differences among years in the proportions of the main phytosterols. The most abundant, sitosterol, possessed the highest average value (52 ± 2% of total sterols) in 2005, when the average total sterol content was lower than in other years, and the lowest (49 ± 2%) in 2006, with the higher total contents. Although statistically significant in the bread wheats ($F(2,46) = 117.22$, $p = 0.0000$), this difference was small, because the range among years was no higher than 4 percentage units of the total sterol content in the individual genotypes. Similarly, the lowest proportion of campesterol was perceived in 2006 and the variation among years in the single genotypes was no higher than 2 percentage units. The relative

Article

J. Agric. Food Chem., Vol. 58, No. 17, 2010 **9317**

Table 2. Phytosterol Contents (Micrograms per Gram of Dry Matter) of 26 Genotypes Grown at Four Locations in 2007

genotype	Hungary 2007					France 2007				
	sitosterol	campesterol	stanols	others	total sterols	sitosterol	campesterol	stanols	others	total sterols
Campari	424	140	248	74	886	380	118	184	61	743
Herzog	373	117	194	53	736	340	100	155	49	645
Disponent	488	168	220	78	954	409	133	165	67	775
Tommi	449	138	224	69	880	396	112	179	58	745
Tremie	458	151	224	69	903	403	128	171	57	759
CF99105	436	137	208	71	852	420	122	181	62	784
Valoris	451	156	233	70	911	405	124	183	55	767
Isengrain	417	141	213	65	836	402	118	182	58	760
Claire	491	162	244	83	980	442	147	187	75	851
Maris Huntsman	453	140	266	89	948	396	120	212	64	792
Lynx	490	158	252	95	994	423	135	180	70	808
Malacca	449	144	228	73	893	391	125	178	56	751
Rialto	468	146	229	76	920	421	122	182	65	791
Riband	460	177	244	91	972	374	136	187	64	760
Avalon	425	138	234	75	872	389	125	190	62	766
San Pastore	386	103	170	58	717	378	101	146	53	678
Estica	446	158	164	70	838	374	133	126	56	690
Gloria	411	119	185	74	790	435	120	152	73	780
Spartanka	395	126	203	74	799	401	122	156	65	745
Obriy	406	127	194	54	781	413	115	161	49	738
Atlas 66	449	143	216	82	890	463	142	163	70	837
Crousty	403	134	198	68	803	383	117	157	66	723
Tiger	370	119	199	54	742	345	105	161	49	660
MV Emese	378	113	177	59	728	391	111	147	54	703
Chinese Spring[a]	411	123	228	91	853	424	112	181	70	788
Cadenza[a]	486	143	178	89	896	452	128	145	75	800
av[b]	434	139	214	73	861 a	402	122	170	62	755 d
SD	37	18	27	12	80	29	12	19	8	50
CV (%)	8	13	13	16	9	7	9	11	13	7
range	370−491	103−177	164−266	53−95	717−994	340−463	100−147	126−212	49−75	645−851

genotype	United Kingdom 2007					Poland 2007				
	sitosterol	campesterol	stanols	others	total sterols	sitosterol	campesterol	stanols	others	total sterols
Campari	414	140	172	57	783	401	125	209	58	794
Herzog	357	114	138	44	653	370	108	181	46	705
Disponent	419	147	140	59	766	444	144	188	63	839
Tommi	433	132	160	55	781	440	127	206	68	840
Tremie	438	140	165	61	804	441	134	200	70	845
CF99105	451	140	173	68	833	477	139	210	80	905
Valoris	422	146	151	56	775	408	134	198	59	798
Isengrain	442	146	176	60	824	427	131	198	68	825
Claire	478	166	162	63	869	495	156	220	77	948
Maris Huntsman	449	152	191	71	864	454	134	229	90	906
Lynx	473	161	179	71	884	439	134	203	74	850
Malacca	451	158	167	64	839	442	140	204	66	851
Rialto	437	137	158	70	801	445	127	202	72	846
Riband	432	169	170	74	846	430	151	221	79	881
Avalon	409	136	170	59	773	428	132	217	74	851
San Pastore	409	124	143	58	734	402	106	174	67	749
Estica	441	168	114	62	785	450	150	157	69	827
Gloria	451	134	154	72	811	420	112	187	86	805
Spartanka	424	141	150	66	781	403	123	202	78	806
Obriy	431	130	152	57	770	405	119	184	56	763
Atlas 66	461	161	151	77	849	458	142	201	84	884
Crousty	408	131	130	58	726	396	123	185	69	773
Tiger	368	123	139	47	677	379	114	183	54	730
MV Emese	399	123	135	52	709	386	112	170	61	730
Chinese Spring[a]	480	135	200	74	889					
Cadenza[a]	531	169	145	85	929					
av[b]	435	143	157	63	798 c	427	130	197	69	823 b
SD	35	16	19	9	66	31	14	17	11	61
CV (%)	8	11	12	15	8	7	11	9	15	7
range	357−531	114−169	114−200	44−85	653−929	370−495	106−156	157−229	46−90	705−948

[a] A spring wheat type. [b] A statistically significant difference in the average total content among locations is denoted with a different letter ($p < 0.05$).
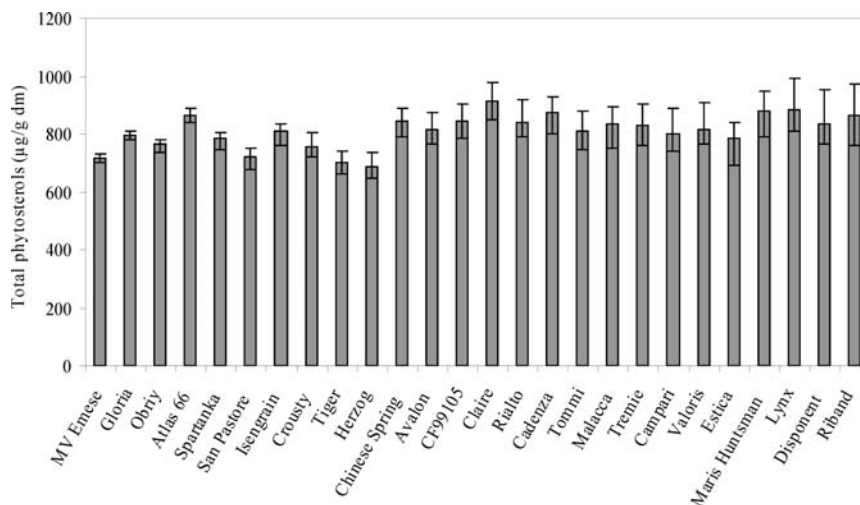
Figure 2. Average total phytosterol contents of various genotypes grown in Hungary, France, the United Kingdom, and Poland in 2007 in order of increasing variation among locations. The error bars represent the range at four locations.

content of total stanols ranged from $24 \pm 2\%$ (2005) to $26 \pm 2\%$ (2006), thus being slightly higher in the year with high total phytosterol contents. The magnitude of variation in individual genotypes was similar to that of sitosterol.

The present study is the first to extensively examine the effect of growing year on wheat phytosterols. The weather conditions varied among crop seasons (*19, 30*). The precipitation was very high during the harvest in 2005, whereas 2006 was more typical and 2007 exceptionally dry during the plant and grain development stages at Martonvásár, Hungary. During this period the average temperature was highest in 2007. The effects of other environmental factors, such as planting location and agronomic practices, were decreased by cultivating the wheat genotypes in strictly controlled experimental fields in the same area every year. The genotypes were, however, grown in different fields with diverse soil properties at Martonvásár over three consecutive years, which may have resulted in some variation (*31*). Despite the differences in weather and soil conditions among growing years, significant interannual effects on average phytosterol contents of wheat were not seen here. In rye phytosterols, however, the effect of growing year was significant, according to Zangenberg et al. (*24*). Within three rye cultivars grown over three consecutive years in 1997−1999 at the same site, the lowest phytosterol contents occurred when the season was warm and dry. This is in contrast with our findings, because in the individual wheat genotypes of the present study the lowest contents were most often found in 2005, a year with exceptionally high precipitation.

Variation among Growing Locations. Considerable variation was observed in the phytosterol contents of 26 wheat genotypes grown at four locations in 2007 (**Table 2**). The growing location had a statistically significant effect on the total phytosterol content within winter wheat genotypes ($F(3,69) = 55.81$, $p = 0.0000$) and all bread wheats ($F(3,73) = 51.03$, $p = 0.0000$). The level of phytosterols was significantly different in each country as measured with the LSD procedure ($p < 0.05$). The average phytosterol content of winter wheat was highest in those genotypes grown in Hungary ($859 \pm 83 \, \mu g/g$ dm), somewhat lower in those grown in Poland ($823 \pm 61 \, \mu g/g$) and the United Kingdom ($789 \pm 59 \, \mu g/g$), and lowest in France ($752 \pm 51 \, \mu g/g$). All bread wheat genotypes studied contained an average of 861 ± 80, 798 ± 66, and $755 \pm 50 \, \mu g/g$ of phytosterols when grown in Hungary, the United Kingdom, and France, respectively. Only winter wheat types were grown in Poland. Even though statistically significant, the differences among the average phytosterol levels

at the various locations was no more than 12%. All genotypes studied possessed their lowest phytosterol content when grown in France, with the exception of one genotype, Disponent, which was lowest in phytosterols in the United Kingdom. Although nearly all of the winter wheat genotypes contained the highest levels of phytosterols when grown in Hungary, the highest phytosterol contents in both spring wheat genotypes occurred when the growing location was in the United Kingdom. In individual genotypes, the variation was usually wider among growing sites than among years. However, there were six diverging genotypes, namely, Avalon, Gloria, Spartanka, Atlas 66, MV Emese, and Cadenza, with wider variation during growing years than when grown at different sites. The widest variation due to growing site was observed in the western European genotypes Riband, Disponent, and Lynx, whereas the most stable genotypes among the growing locations included the eastern European genotypes MV Emese, Gloria, and Obriy (**Figure 2**). The genotypes with the most stable phytosterol content over the years (Tommi, Tremie, and Estica) were among 10 genotypes possessing the widest range when grown at four locations. Conversely, the genotypes with the widest variation among the harvesting years (CF99105, Cadenza, and Avalon) showed only moderate variation among locations.

The phytosterol composition was affected by the growing location, and there were statistically significant differences among sites in the proportions of major phytosterols in wheat genotypes. The average proportion of sitosterol was lowest ($50 \pm 2\%$) when the genotypes were cultivated in Hungary, that is, in the location with the highest total phytosterol contents, and highest ($55 \pm 1\%$) in the United Kingdom. In contrast, the proportion of stanols was greatest in Hungary and lowest in the United Kingdom. In fact, when the average phytosterol contents of Hungary and the United Kingdom were compared, the contents of sitosterol were similar at both locations, and the differences in total phytosterol content mainly resulted from changes in the stanol content. The greatest relative content of campesterols ($18 \pm 1\%$) was observed in genotypes grown in the United Kingdom. The variation in the proportions of the main phytosterols among locations within individual genotypes was somewhat wider than among years.

Previous knowledge of variation in wheat phytosterols among growing locations is scarce. In agreement with the present findings, Chen et al. (*28*) recently observed significant effects of growing location and genotype on the phytosterol contents of

Table 3. Average Phytosterol Contents (Micrograms per Gram of Dry Matter) of the Wheat Genotypes Grown in Three Years and at Four Locations

genotype	total phytosterol content			
	av[b]	SD	CV (%)	range
Campari	821 c−f	56	7	743−886
Herzog	700 a	42	6	645−739
Disponent	847 e−h	71	8	766−954
Tommi	838 e−g	62	7	745−890
Tremie	855 e−h	63	7	759−913
CF99105	877 f−i	65	7	784−956
Valoris	822 c−f	54	7	767−911
Isengrain	827 d−f	38	5	760−875
Claire	928 i	54	6	851−980
Maris Huntsman	894 g−i	58	6	792−948
Lynx	901 h,l	68	8	808−994
Malacca	846 e−h	50	6	751−893
Rialto	856 e−h	52	6	791−920
Riband	891 g−i	80	9	760−972
Avalon	814 c−e	62	8	739−884
San Pastore	735 a,b	35	5	678−780
Estica	799 c−e	57	7	690−838
Gloria	804 c−e	30	4	780−859
Spartanka	796 b−e	39	5	745−863
Obriy	773 b−d	22	3	738−804
Atlas 66	898 g−i	55	6	837−976
Crousty	756 a−c	39	5	723−803
Tiger	702 a	40	6	660−742
MV Emese	735 a,b	34	5	703−798
Chinese Spring[a]	847 e−h	39	5	788−889
Cadenza[a]	923 i	86	9	800−1039
av	826			
SD	64			
CV (%)	8			
range	700−928			

[a] A spring wheat type. [b] A statistically significant difference in phytosterol content is denoted with a different letter ($p < 0.05$).

three wheat cultivars grown at three different locations in Oklahoma. Little is known of this type of variation in other cereals. The variation among locations was not marked when seven oat cultivars were grown at three locations in Sweden during the same growing season in 1996 (*26*). In contrast, the effect of planting location on the phytosterol contents of two soybean cultivars grown in different parts of Japan was observed, whereas the phytosterol composition was not affected (*29*). Yamaya et al. (*29*) showed that the soybean seeds apparently possessed higher phytosterol contents when the seeds were grown in warm areas. This observation is somewhat consistent with our findings, because the highest average phytosterol contents were found in wheat genotypes grown in Hungary, where the temperature was high and precipitation low. On the other hand, the location with the lowest temperature and highest precipitation detected was in the United Kingdom, but nevertheless the lowest phytosterol levels were found in genotypes grown in France. The climate may to some extent have been responsible for the variation among locations, but other environmental factors may also have a profound effect. This is supported by the finding that the weather did not result in wide variation in phytosterol contents among years in Hungary. The agronomic practices and postharvest conditions were controlled and similar at each location and, thus, no remarkable variation was expected to arise from these factors. It is tempting to speculate that the wheat genotypes may have become adapted to the growing area at Martonvásár, because they were already grown in experimental fields in Hungary during the two seasons before cultivation in the other locations and contained the highest levels of phytosterols there compared to the other locations. However, this is not likely because there were no significant differences in average phytosterol contents among the three growing years in Hungary.

Variation among Genotypes. As in our previous study (*20*), the genetic factors greatly affected the phytosterol contents and compositions of wheat. The impact of genotype was clearly seen both during the years 2005−2007 and at the different growing locations, as demonstrated in **Figures 1** and **2**. When the data of all environments were considered, there were statistically significant differences in the total sterol contents among the genotypes within both winter wheat types ($F(23,111) = 24.69, p = 0.0000$) and all bread wheats ($F(25,119) = 21.91, p = 0.0000$), due to a pure genotype effect. The average phytosterol contents of 26 genotypes varied from 700 to 928 μg/g of dm, indicating a 1.3-fold difference (**Table 3**) and, on the basis of the average contents of the genotypes, 12 homogeneous groups could be identified with the LSD procedure ($p < 0.05$) when only the genetic factors were taken into account (data not shown). The most phytosterol-rich wheat genotypes throughout the growing years and locations were Claire, Cadenza, Lynx, Atlas 66, and Maris-Huntsman, most of these being of British origin. The genotypes with relatively low total phytosterol contents included Herzog, Tiger, San Pastore, and MV Emese. The phytosterol levels were somewhat higher when the genotypes were grown over three years in Hungary (range of 732−966 μg/g) than during 2007 at four sites (range of 685−912 μg/g).

High phytosterol contents combined with low interannual variation were found in genotypes such as Claire, Maris Huntsman, and Tremie. However, many of the genotypes with high phytosterol contents tended to vary widely in total phytosterols among locations. The old American variety Atlas 66 was characterized by both substantially high phytosterol contents and high stability throughout the growing locations. Among the two spring wheat genotypes, Cadenza contained more phytosterols, but showed wider variation among the growing years and locations than Chinese Spring.

Genetic variation was also observed in the composition of phytosterols. The genetic factors resulted in statistically significant differences in the proportions of wheat phytosterols, over both the three years and the locations. The major phytosterol in all genotypes was sitosterol, followed by campesterol, sitostanol, and campestanol. Sitosterol accounted for 49−55% of the total sterols of the wheat genotypes over the years and growing sites. The lowest proportions of sitosterol were found in the British cultivars Riband and Maris Huntsman and the highest in the British spring wheat genotype Cadenza. The stanols, including sitostanol and campestanol, contributed 19−26% of total sterols over the various environments, with Cadenza and Estica possessing the lowest proportions and Campari, Maris Huntsman, and Avalon the highest proportions of stanols. The relative content of campesterol varied from 15% (e.g., Chinese Spring) to 19% (Estica) of total phytosterols. Sitosterol, campesterol, and the corresponding stanols together comprised the majority ($92 \pm 1\%$) of all phytosterols. The other 4-desmethyl sterols, namely, brassicasterol, stigmasterol, Δ^5-avenasterol, Δ^7-avenasterol, stigmasta-5,24(25)-dienol, and Δ^7-stigmastenol, accounted for an average of $7 \pm 1\%$ of total sterols. Only 1% of the phytosterols were 4-monomethyl and 4,4′-dimethyl sterols (gramisterol, α-amyrin, cycloartenol, 24-methylenecycloartanol, and citrostadienol).

The genetic variability was first seen in our previous study, in which the total phytosterol contents of 130 winter wheat genotypes grown at the same location in Hungary in 2005 varied from 670 to 959 μg/g and those of 20 spring wheat genotypes varied from 797 to 949 μg/g of dm (*20*). The present findings are in accordance with these values. More recently, the effect of genetic variation on wheat phytosterols was also demonstrated in other
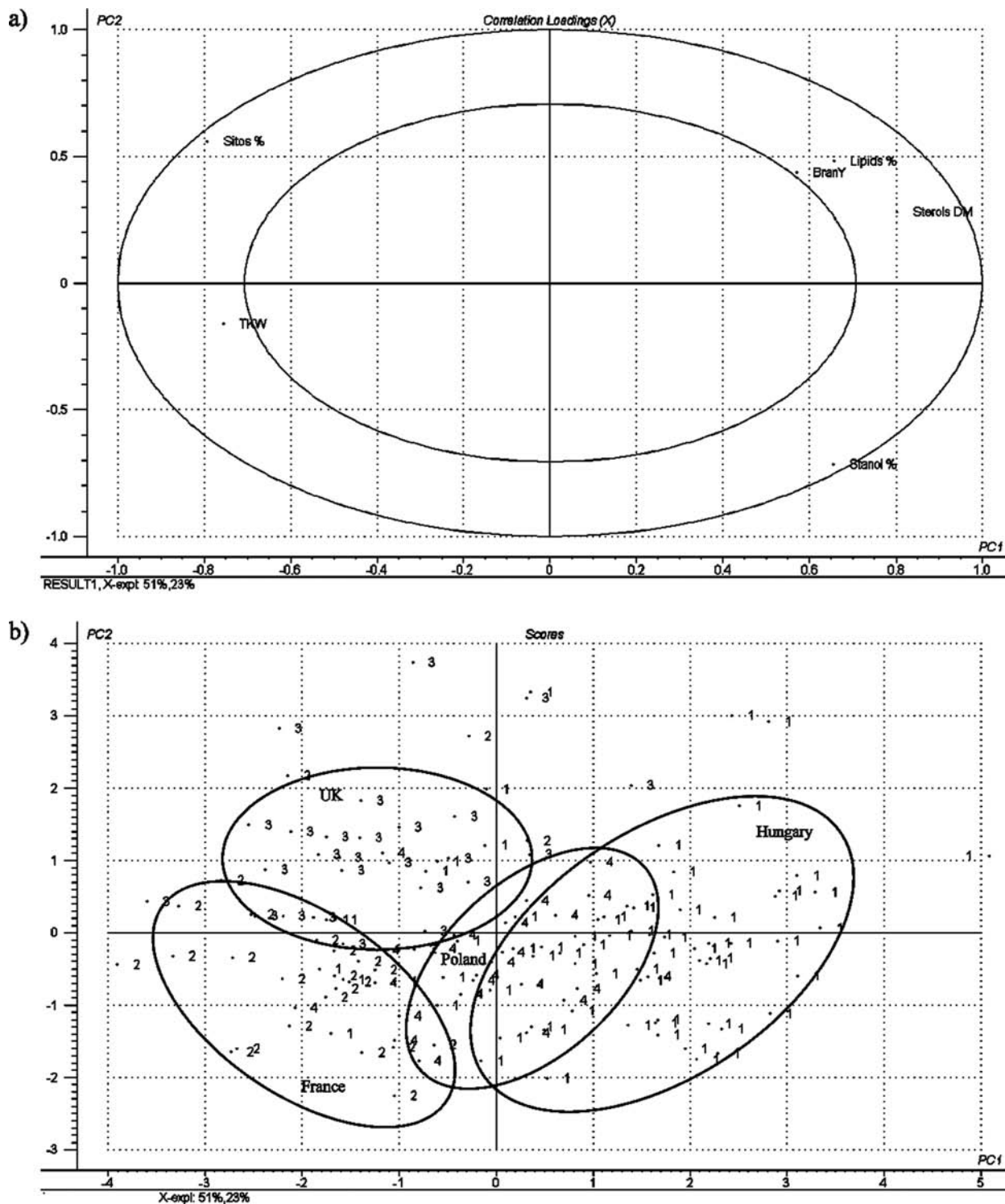
Figure 3. Principal component analysis of phytosterol contents and compositions and other characteristics of the wheat grains grown in different environments (*N* = 150): (**a**) loadings plot of the variables (sterols DM, total phytosterol content; sitos %, proportion of sitosterol; stanol %, proportion of stanols; TKW, 1000 kernel weight; BranY, bran yield; lipids %, lipid content); (**b**) scores plot of samples displayed by the growing location (1, Hungary; 2, France; 3, United Kingdom; 4, Poland).

studies (*21, 22, 28*). Chen et al. (*28*) found statistically significant genotype effects within different locations when they examined the phytosterol contents and compositions of three wheat cultivars grown at three locations in Oklahoma in 2005. Nevertheless, they observed total phytosterol contents of only up to 355 μg/g of fw, which is considerably lower than the values in the present study. The phytosterol contents of five Italian wheat cultivars harvested in 2002 in an experimental field in Italy and five

European winter wheat cultivars grown at the same location in Belgium in 2001−2002 were more comparable to our findings, 600−677 μg/g of dm and 622−655 μg/g of fw, respectively, as reported by Iafelice et al. (*22*) and Ruibal-Mendieta et al. (*23*). The variation, however, was not as wide as in the present study, probably due to the small number of genotypes analyzed. Alignan et al. (*21*) measured wide genetic variation in the phytosterol contents of 23 European bread wheat genotypes grown under

organic conditions in France; the total contents ranged from 494 to 796 μg/g of dm. To some extent the low phytosterol contents in contrast to the present results may be explained by the different environmental conditions and genotypes. In addition, these dissimilarities may have resulted from analytical differences, for example, in sample preparation steps, chromatographic conditions, quantification methods, or the number of sterols identified. In the present method, acid hydrolysis prior to saponification was used to release the phytosterols from their glycoside conjugates. In wholegrain wheat, approximately 10% of the sterols were reported to exist in the glycosylated form and, thus, excluding the acid hydrolysis step will result in underestimation of the total amount of phytosterols (*16*). Sample preparation in the methods of Chen et al. (*28*) and Alignan et al. (*21*) included only the saponification step, and all of the conjugated forms of phytosterols were not analyzed. Furthermore, we applied SPE purification instead of the thin-layer chromatography (TLC) method, during which losses are more likely to occur. The use of internal standard also compensated for the losses during sample preparation. The two Finnish wheat cultivars grown in Finland in 1997 were analyzed by Piironen et al. (*32*) with a procedure similar to the method used in this study, and the total phytosterol contents, 763 and 818 μg/g of dm, were similar to the present findings. In our previous study, somewhat wider variation was observed in the proportions of sitosterol and the other main sterols in 150 winter and spring wheat genotypes grown in 2005 (*20*). However, the number of wheat genotypes was considerably higher than in the present study.

Overall Variation. The growing location tended to have the greatest effect on wheat phytosterols, followed by the impact of genetic factors, whereas the year of growth caused less variation in phytosterols than the other factors. Among all of the different environments studied, the highest average phytosterol content was found in bread wheats grown in Hungary in 2006 and the lowest in those grown in France in 2007. However, the difference between these values was only 1.2-fold. The extent of environmental variation differed among the various genotypes. The overall variation was greatest in the modern cultivar Cadenza and lowest in the old genotype Obriy. The highest individual phytosterol content in the present study was detected in the spring wheat Cadenza grown in Hungary in 2006 (1039 μg/g of dm), which was 1.6-fold higher than the lowest value in the winter wheat Herzog grown in France in 2007 (645 μg/g).

When the average phytosterol contents over all of the environments were considered, there were 8 wheat genotypes containing 700−799 μg/g, 15 genotypes containing 800−899 μg/g, and only 3 genotypes containing > 900 μg/g of dm of phytosterols. The one-way ANOVA reflecting the overall variation showed statistically significant differences in average total phytosterol contents among the genotypes grown in the studied environments ($F(25,124) = 7.67$, $p = 0.0000$). The LSD procedure ($p < 0.05$) allowed the distribution of the 26 genotypes into nine homogeneous groups, based on the average total sterol contents over the years and locations (**Table 3**). The genotype Claire possessed the highest average content and also showed considerably less overall variation in sterol content than the other genotypes with high contents (Cadenza and Lynx). The spring wheat Chinese Spring and winter wheats Gloria, Isengrain, and Rialto were genotypes with moderately high and stable phytosterol contents over the years and sites. The genotypes with the narrowest overall ranges in sterol content tended to be the poorest sources of these compounds.

The overall variation in the phytosterol content and composition of wheat grains grown in different environments was also illustrated, using PCA. The first principal component (PC1)

explained 51% and the second (PC2) 23% of the variation (**Figure 3**). The first two principal components thus accounted for 74% of the total variance. The loadings plot in **Figure 3a** shows the associations between the measured continuous variables, including the total sterol content, proportions of sitosterol and stanols, TKW, bran yield, and lipid content. The figure clearly shows the distribution of sterol species; the total phytosterol content and the proportion of stanols were positively correlated with each other and negatively correlated with the proportion of sitosterol. An increased proportion of stanols and a decreased proportion of sitosterol were thus found in the genotypes with high total phytosterol contents. The relationship between total phytosterol content and the proportion of sitosterol ($r = -0.476$, df = 148, $p < 0.01$) or stanols ($r = 0.274$, df = 148, $p < 0.01$) was relatively weak but statistically significant. In addition, the high kernel weight was associated with low proportions of bran and lipids and low total sterol content. All of the continuous variables were important in explaining the variance, because they were situated within the two ellipses representing explanation rates of 50 and 100%. The standardized data were also classified on the basis of categorical variables, which included the genotype, geographic origin of the genotypes, and country and year of growth. The scores plot in **Figure 3b** demonstrates the relationship among 150 wheat samples and clearly allows discrimination among the four different growing locations. The genotypes contained high levels of total phytosterols when grown in Hungary and low levels when grown in France in 2007, as already demonstrated with ANOVA. The figure also supported the finding that the samples grown in the United Kingdom were characterized by high proportions of sitosterol and those grown in Hungary with high proportions of stanols. Moreover, the largest kernels with low proportions of bran and lipids were developed in France.

As already described in the PCA loadings plot, there were relationships between total phytosterol contents and the other kernel characteristics, that is, the lipid and bran contents and the weight of the wheat kernels. The high proportions of bran indicated high phytosterol contents; a positive and statistically significant correlation was found between the total sterol contents and the bran yields of the wheat genotypes ($r = 0.425$, df = 148, $p < 0.01$). The high lipid content of the kernel was also related to the high level of phytosterols, because there were significant and moderately strong positive correlations between these variables in all bread wheats ($r = 0.574$, df = 148, $p < 0.01$). The situation contrasted with that of the TKW. Small kernels tended to contain more phytosterols than large kernels, because the moderately strong correlation observed between the TKW and total sterol content was negative within all wheat genotypes ($r = -0.608$, df = 148, $p < 0.01$). The relationships between the variables were also similar when only the winter wheat types were considered. Hence, the genotypes with small kernel size, characterized by low TKW and high bran and lipid content, contained high levels of phytosterols. This is quite expected, in view of the fact that phytosterols are concentrated in the bran and germ, and the proportions of these fractions are greater in small kernels than in large kernels (*16*). This is in agreement with our previous findings, even though the correlations found in the present study were somewhat stronger (*20*). The differences in total phytosterol contents may partly be explained by the differences in kernel sizes, because in 2007 the smallest kernels developed under dry and warm conditions in Hungary, where the highest phytosterol contents were observed, and the largest kernels and the lowest sterol contents developed in France, which was more rainy and cold. Therefore, genetic and environmental factors may have influenced the kernel size and thereby also the phytosterol content.

Conclusions. The HEALTHGRAIN project enabled us to examine the natural variation in wheat phytosterols caused by genetic and environmental factors. In the present study the effects of environment on the phytosterol content and composition of wheat genotypes were investigated more extensively than in former studies and with a highly controlled experimental design. The variation among growing locations was wider than that among growing seasons, and the magnitude of environmental variation was different among the various genotypes. As expected, the genetic factors also strongly affected the phytosterols of wheat. The differences in phytosterol contents were in general relatively small, only up to 1.6-fold. The beneficial health effects of phytosterols can, however, be achieved with even modest increases in the phytosterol contents of cereals due to the high consumption of the cereal products. On the basis of the knowledge of natural variation obtained here, genotypes with high stable phytosterol contents can be selected for cultivation and for plant breeding to develop wheat genotypes with even better qualities, thus increasing the concentration of phytosterols in cereal foods and the intake of these health-promoting compounds in the natural diet.

ABBREVIATIONS AND NOMENCLATURE USED

α-Amyrin, urs-12-en-3β-ol; ANOVA, analysis of variance; av, average; Δ^5-avenasterol, stigmasta-5,24(28)-dien-3β-ol; Δ^7-avenasterol, (24Z)-5α-stigmasta-7,24(28)-dien-3β-ol; branY, bran yield; brassicasterol, (22E)-ergosta-5,22-dien-3β-ol; campestanol, (24R)-5α-ergostan-3β-ol; campesterol, (24R)-ergost-5-en-3β-ol; cholesterol, cholest-5-en-3β-ol; citrostadienol, (24Z)-4α-methyl-5α-stigmasta-7,24(28)-dien-3β-ol; CV, coefficient of variation; cycloartenol, 9,19-cyclolanost-24-en-3β-ol; dihydrocholesterol, 5α-cholestan-3β-ol; dm, dry matter; FID, flame ionization detector; fw, fresh weight; GC, gas chromatography; gramisterol, 4α-methyl-5α-ergosta-7,24(28)-dien-3β-ol; LSD, least significant difference; 24-methylenecycloartanol, 24-methylene-9,19-cyclolanostan-3β-ol; MS, mass spectrometry; PC1, first principal component; PC2, second principal component; PCA, principal component analysis; SD, standard deviation; SiOH, silica; sitostanol, 5α-stigmastan-3β-ol; sitosterol, stigmast-5-en-3β-ol; SPE, solid-phase extraction; stigmasta-5,24(25)-dienol, stigmasta-5,24(25)-dien-3β-ol; Δ^7-stigmastenol, stigmast-7-en-3β-ol; stigmasterol, (22E)-stigmasta-5,22-dien-3β-ol; TKW, 1000 kernel weight; TLC, thin-layer chromatography; TMS, trimethylsilyl.

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