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Spelt (*Triticum spelta* L.) and Winter Wheat (*Triticum aestivum* L.) Wholemeals Have Similar Sterol Profiles, As Determined by Quantitative Liquid Chromatography and Mass Spectrometry Analysis

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From a nutritional point of view, cereal lipids include valuable molecules, such as essential fatty acids, phytosterols, and fat-soluble vitamins. Spelt (*Triticum spelta* L.) is an alternative hulled bread cereal mostly grown in Belgium, where it is mainly intended for animal feed but should increasingly be used for human consumption. The present research focused on phytosterol quantification by LC/APCI-MS^2 in saponified wholemeal extracts of 16 dehulled spelt and 5 winter wheat (*Triticum aestivum* L.) varieties grown in Belgium during 2001−2002 at the same location. Glycosylated sterols and free and formerly esterified sterols could be determined in saponified extracts. Results show that the mean phytosterol content is comparable in both cereals (whereas other lipids, such as oleic and linoleic acids, are increased in spelt wholemeal): spelt extract has, on average, 527.7 μg of free and esterified sterols g^{-1} of wholemeal and 123.8 μg of glycosylated sterols g^{-1} of wholemeal versus 528.5 and 112.6 $\mu g \cdot g^{-1}$ in winter wheat (values not corrected for recoveries). This is the first report on the application and validation of an LC/MS^2 method for the quantification of phytosterols in spelt and winter wheat.

KEYWORDS: Lipid; sterol; spelt; wheat

INTRODUCTION

Cereal phytosterols are present in the free form (free sterols, FS), esterified to fatty or phenolic acids (steryl esters of fatty acids, SE, and steryl esters of hydroxycinnamic acids, HSE), or as steryl glycosides (SG) and acylated steryl glycosides (ASG) (*1*, *2*). Bound forms are known as phytosterol conjugates (*3*) or steryl conjugates.

In cereals, phytosterol determination of saponified and/or acid-hydrolyzed samples is usually performed by gas chromatography/mass spectrometry (GC/MS) after derivatization (*2*, *4*−*9*). Alkaline and acid hydrolyses release the sterol moiety of HSE/SE and SG/ASG, respectively. As a consequence, the sterol moieties from four types of conjugates are combined to FS and determined together. GC/MS is not adapted to the direct study of glycosylated conjugates, as these compounds are not sufficiently volatile without derivatization. In contrast, liquid chromatography/atmospheric pressure chemical ionization mass spectrometry (LC/APCI-MS) does not present this limitation, and samples do not need time-consuming derivatization.

LC/MS has been applied for phytosterol characterization in soybean (*Glycine max* L.) oil, and quantification has been performed by LC with ultraviolet (UV) detection (*10*). With regard to cereals, LC with evaporative light-scattering detection (ELSD) has been useful to determine the five sterol classes in corn fiber oil extracted either with hexane or with supercritical CO_2 (*11*). Liquid chromatography/electrospray ionization mass

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spectrometry (LC/ESI-MS) has allowed the characterization of steryl ferulates in rice (*Oryza sativa*) bran nonsaponified lipid extracts (*12*). We have also analyzed the phytosterol profile of dehulled spelt (*Triticum spelta* L.) and winter wheat (*Triticum aestivum* L.) fine bran (a milling byproduct, also referred to as shorts) by LC/APCI-MS (*13*). We found that both cereals exhibited the same phytosterol profile and contained the following compounds: β-sitosterol, campesterol, Δ^5- and Δ^7-avenasterol, stigmasterol and cholesterol (unsaturated), and sitostanol and campestanol (saturated). Ergosterol and another sterol (both unsaturated), probably a precursor of ergosterol, were also present and could originate from fungal contamination. Some SG were also identified, including glycosylated β-sitosterol, campesterol, Δ^5- and Δ^7-avenasterol, and stigmasterol.

This study is dedicated to the use of our technique for sterol quantification, its validation, and subsequent application to two classes of phytosterols in dehulled spelt and winter wheat wholemeals after direct saponification: FS (free and released by saponification from SE and HSE) and SG (free and released from ASG).

MATERIALS AND METHODS

Plant Material. Five winter wheat and 16 spelt varieties were cultivated in experimental fields at the Agricultural Research Centre of Gembloux, Belgium, during the 2001−2002 growing season. Winter wheat samples comprised European cultivars; spelt material included European cultivars, Belgian landraces, and breeding lines.

After harvest, grains were stored in standard conditions, that is, in Hessian bags at the research station. Spelt grains were dehulled, and grains from both species were graded with a seed grader and then stored in plastic jars at −20 °C. This precaution was taken to protect them from insect contamination. Before grinding, samples were thawed. Ten grams per sample was ground through a 1.0-mm sieve Cyclotec mill (Cyclotec, Tecator, Hoganas, Sweden), and the resulting wholemeal was stored in plastic jars at −20 °C until analyzed. It is pointed out that freezing of ground samples prevents enzymatic degradation; indeed, lipases from both bran and germ are active (*14*), which can negatively influence the study of lipids.

Sample Preparation. The procedure we applied included direct saponification of 3-g wholemeal samples at 80 °C, extraction of unsaponifiable matter with diethyl ether, washing of the organic phase, reversed-phase (C18) solid-phase extraction (SPE) purification, and filtration. This method has been described in detail elsewhere (*13*).

LC/MS. An LC system of Thermo Separation Products (TSP, San Jose, CA) equipped with a P1000XR pump and a TSP AS 3000 autosampler was used. Separation of sterols was performed on an Alltech C18 Prevail column (150 × 2.1 mm i.d., 2 μm) using a linear gradient from 85% methanol−15% water (1% acetonitrile) to 100% methanol in 15 min and maintained at 100% methanol for 20 min or more, depending on the sterol class analyzed. Flow rate was 0.2 mL·min^{-1}, injection volume was 10 μL, and column temperature was kept at 30 °C. Mass spectra were acquired in positive mode using an LCQ mass spectrometer (Finnigan MAT, San Jose, CA) equipped with an APCI source. The following APCI inlet conditions were applied: heated capillary temperature, 175 °C; heated vaporization temperature, 450 °C; sheath gas (N_2), 45 psi; auxiliary gas (N_2), 20 psi. Collision-induced dissociation (CID) was recorded at a relative collision energy of 25%. Data processing was performed with Excalibur software (version 1.1). HPLC grade methanol (Fisher Chemicals, Leicester, U.K.), acetonitrile (Fisher Chemicals), and Milli-Q water (Millipore, Bedford, MA) were used for the LC mobile phase.

Method Validation. The following sterol standards were purchased from Sigma-Aldrich (Steinheim, Germany): campesterol (100 μg·mL^{-1} in chloroform), cholesterol (99% purity), dihydrocholesterol (cholestanol) (95% purity), stigmasterol (95% purity), campestanol, and sitostanol (96% purity). Sitosterol (97% purity) and ergosterol (97% purity) were available from Fluka (Buchs, Switzerland). Absolute ethanol (Labotec, Belgium) was used to prepare standard solutions.

Method validation was performed with saponified spelt samples and included the following parameters, as described by other authors (*10*, *15*−*17*): response function with a linear calibration graph based on an external standard, recovery, limit of detection (LOD), and limit of quantification (LOQ). Selectivity, described in a previous study (*13*), was obtained by detection in the MS^2 mode, which eliminated possible interference with compounds of the same retention time in LC and allowed identification of the quantified molecule.

Given that the most abundant ion in the mass spectra of FS and SG is the same and corresponds to $[M + H - H_2O]^+$ and $[\text{aglycon} + H - H_2O]^+$, respectively (*13*), phytosterol quantification is based on detection in the MS^2 mode using this major ion for both sterol classes. It is noteworthy that SG/ASG content is expressed in phytosterol content; the sugar moiety is not taken into account, as sugars were not identified.

The calibration graph was established with five ergosterol solutions: 0.1, 1, 10, 100, and 400 ng·μL^{-1}. It was revalidated for each quantification sequence by injecting all of the calibration solutions before the samples, to exclude variability due to unexpected changes in the LC/MS system parameters. Accordingly, quantification results were more reproducible. Peak areas of the ergosterol MS^2 major ion (m/z 379) were plotted against corresponding concentrations, and the R^2 was calculated by the Quanbrowser software. Ergosterol was chosen as an external standard for various reasons: under the experimental conditions used, it was the first FS to elute (at 27.8−28 min), it was commercially available in large amounts, whereas other sterols were sold in minute amounts (1−5 mg), and its purity reached 97%, which was adequate for this application.

Although the different sterols found in spelt and wheat extracts have closely related structures, their behaviors might be different in the ionization source. Therefore, we calculated response factors by the ratio of FS over ergosterol peak areas at 100 ng·μL^{-1} for the following seven standard ethanol solutions: stigmasterol, cholesterol, β-sitosterol, campesterol, campestanol, sitostanol, and cholestanol (for recovery studies). Response factors were determined in triplicate, and they allowed us to quantify the different sterol types using a single calibration graph of ergosterol, instead of relying on calibration graphs for all of the FS commercial standards. As pure standard compounds were not available for SG and because their quantification was based on the major ion (i.e., $[\text{aglycon} + H - H_2O]^+$), we applied to this type of derivative the same response factors as those calculated for FS.

The LOD is determined as the minimum concentration of the analyte that the system can detect. In chromatography, it can be defined as the amount of analyte that gives a peak with a signal-to-noise ratio of 2−3 (*15*). The LOQ is the lowest amount of analyte that can be quantitatively determined with a defined accuracy, that is, ±15% for analysis of biological samples (*18*), and corresponds to a signal-to-noise ratio of 10 (*15*, *19*), which is considered to be acceptable in this analytical area. To determine both LOD and LOQ, the following ergosterol solutions were analyzed by LC/MS^2: 0.01, 0.05, 0.1, 0.5, and 1 ng·μL^{-1}.

Cholestanol was chosen for recovery studies because it does not occur naturally in cereal extracts. Consequently, cereal sterols did not influence the recovery percentage. Three-gram cereal samples were spiked with 5-mL cholestanol solutions (25, 100, and 200 ng·μL^{-1}), corresponding to the following amounts of added test analyte: 41.7, 166.7, and 333.3 μg·g^{-1} sample, respectively. The solutions were added before 30 min of saponification at 80 °C or overnight, cold (20 °C), saponification and subsequent steps of sample preparation (unsaponifiable matter extraction, SPE purification, and filtration before LC/MS analysis).

The concentration of cholestanol after sample preparation was calculated in the same way as for the other sterols (see below). Purity of the standard (95%) was taken into account when the recovery percentage was calculated. Data are the means of duplicate determinations for each concentration.

After peak areas were reported in the ergosterol calibration graph, concentrations obtained in ergosterol were recalculated with their corresponding response factors. As ultrapure standards were not available, we used the purest commercial standards we found and corrected our results using the purity value of ergosterol (97%). In the case of sterols for which no standard compound was available (e.g., Δ^5- and Δ^7-avenasterol), their content was expressed in ergosterol. In

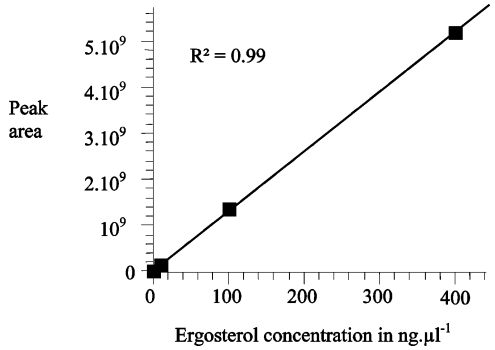

Figure 1. Ergosterol-based calibration graph for sterol quantification, obtained by MS^2.

saponified samples, SG and ASG were quantified together, as well as FS, SE, and HSE (if present). Indeed, ASG, SE, and HSE lost their acid moiety during saponification.

Statistical Analyses. The Mann−Whitney test (nonparametric statistics) was applied to study the variability of sterol content between the two cereals. The software used was SPSS for Windows (*20*).

RESULTS

Method Validation. We have found the following sterol moieties in spelt and winter wheat: stigmasterol (*m*/*z* 395, as SG and FS), Δ^5-avenasterol (*m*/*z* 395, only as FS), Δ^7-avenasterol (*m*/*z* 395, as SG and FS), cholesterol (*m*/*z* 369, only as FS), campesterol (*m*/*z* 383, as SG and FS), β-sitosterol (*m*/*z* 397, as SG and FS), campestanol (*m*/*z* 385, only as FS), and sitostanol (*m*/*z* 399, only as FS). In addition, ergosterol (*m*/*z* 379) was also detected and probably came from fungal contamination, as previously described (*13*). Although both forms of avenasterol and stigmasterol share the same molecular mass (412 g·mol^{-1}) and thus the same major ion (*m*/*z* 395), we were able to distinguish them using their retention time and MS^2 spectra (*13*).

Figure 1 shows the ergosterol calibration graph. In the 0.1−400 ng·μL^{-1} range, the response function is linear, as indicated by a high R^2 value (determination coefficient). Concerning response factors, we obtained the following mean values (standard deviation is indicated in parentheses):

saturated sterols		unsaturated sterols	
sitostanol	0.25 (0.02)	stigmasterol	0.89 (0.02)
campestanol	0.27 (0.01)	cholesterol	1.18 (0.03)
cholestanol	0.31 (0.06)	β-sitosterol	1.41 (0.09)
		campesterol	1.42 (0.05)

It is interesting to note that response factors are related to sterol structures. Indeed, saturated sterols, with no double bond in the ring structure, exhibited low response factors (0.25−0.31), whereas unsaturated sterols with one double bond in the ring structure had high response factors (0.89−1.42). Further differences observed within FS response factors could be explained by differences in their side chains, as described by Kesselmeier et al. (*21*). To quantify cereal sterols, these authors obtained HPLC calibration graphs using stigmasterol and cholesterol and found that their graphs were influenced by the number of double bonds per molecule.

We determined LOD and LOQ for ergosterol by MS^2 and found that they were 0.05 ng·μL^{-1} (signal/noise ratio of 3) and 0.1 ng·μL^{-1} (signal/noise ratio of 10), respectively. LOD and LOQ values for the other sterols were calculated using their corresponding response factors (**Table 1**).

Table 1. Limit of Detection (LOD) and Limit of Quantification (LOQ) (in Nanograms per Microliter) of Different Commercial Sterols, Calculated Using LOD and LOQ of Ergosterol and Corresponding Response Factors of Sterol Standards

sterol	LOD	LOQ
cholesterol	0.04	0.08
stigmasterol	0.06	0.11
campesterol	0.04	0.07
β-sitosterol	0.04	0.07
cholestanol	0.16	0.32
campestanol	0.19	0.37
sitostanol	0.20	0.40

Recovery tests (double determinations) were performed with cholestanol, a sterol used as a test analyte with a structure related to the sterols we quantified but not found in cereals, to study the recovery of a sterol when subjected to the whole sample preparation procedure. As several reference compounds were not commercially available in a pure form (some FS and all SG), we did not calculate recoveries for each sterol. As several reference compounds were not commercially available in a pure form (some FS and all SG), we did not calculate recoveries for each sterol. For saponification at 80 °C, we found mean values of 54, 89, and 96% when 41.7, 166.7, and 333.3 μg of cholestanol per gram of sample were added. For cold saponification, recovery values were 60% (41.7 μg·g^{-1}), 90% (166.7 μg·g^{-1}), and 98% (333.3 μg·g^{-1}). Therefore, good recoveries were obtained for the highest amounts (166.7 μg·g^{-1} and 333.3 μg) of added cholestanol. Recovery experiments were repeated twice for the whole process. The error between two determinations reached values as low as 2.5−5.0%, indicating that the repeatability is acceptable for food samples (*15*). As the wholemeals used for recovery went through the whole sample preparation procedure and exhibited low variation [<15% according to FDA (*18*)], we decided to perform a single determination for sterol quantification in each cereal sample.

Determination of SG/ASG and FS/HSE/SE. Tables 2 and **3** present the mean quantitative results for SG/ASG (**Table 2**) and FS/HSE/SE (**Table 3**) for 5 winter wheat and 16 spelt wholemeal saponified samples. All of the free and released sterols identified could be quantified, except Δ^5-avenasterol, because its content lay between LOD and LOQ. It is noteworthy that ergosterol was not taken into account for the calculation of total sterol content in cereal samples, as it is not a cereal sterol in itself.

For glycosylated sterols (**Table 2**), results showed no major difference in SG/ASG contents of spelt and winter wheat samples except for β-sitosterol, the content of which appeared to be significantly higher in spelt ($P = 0.04$). Total SG/ASG mean content was higher in spelt wholemeal than in winter wheat, but mean values were not significantly different ($P = 0.130$). Saturated sterols and cholesterol were either not present in glycosylated forms or their concentration lay below their LODs. In both cereals, β-sitosterol accounted for 74% of total SG/ASG, the campesterol proportion reached 23−25% of glycosylated sterols, and Δ^7-avenasterol and stigmasterol proportions were very low. It also has to be noted that the extraction procedure used was not the most adapted to the extraction of glycosylated sterols, which are generally not freely soluble in diethyl ether. Values given here may account for only a part of the glycosylated sterols present in cereal samples, which should be proportionally the same for both cereals.

For FS/HSE/SE (**Table 3**), mean values indicate that their contents were the same in spelt and winter wheat wholemeals.

Table 2. Combined Steryl Glycoside and Acylated Steryl Glycoside Content (in Micrograms per Gram) of 16 Spelt and 5 Winter Wheat Wholemeal Saponified Samples from 2002 Harvest Year

	Δ^7-avenasterol[a,b]	stigmasterol	campesterol	β-sitosterol	total
		Spelt			
Oberkulmer	1.0	0.3	31.7	97.4	130.4
Ebners Rotkorn	1.8	0.6	29.0	97.5	128.8
Béryl	1.3	0.4	25.9	90.0	117.6
Alkor	0.9	0.3	27.2	90.8	119.3
Ostar	0.9	0.2	31.0	92.3	124.5
Redouté	1.9	0.3	27.5	90.7	120.4
Rouquin	1.2	0.2	26.4	84.0	111.9
Line 115.11	1.0	0.3	24.2	84.7	110.2
Line 115.6.2	0.7	0.3	27.9	86.3	115.2
LR 134[c]	1.5	0.2	32.8	98.5	133.0
LR 135	2.1	0.4	32.8	99.9	135.2
LR 140	1.9	0.5	25.5	92.5	120.4
LR 229	1.3	0.3	26.5	77.9	106.0
LR 260.1	1.5	0.5	27.7	99.4	129.1
LR 268	1.6	0.4	31.6	103.4	137.1
LR 286.1	2.0	0.5	35.7	103.6	141.9
av	1.4a	0.4a	28.9a	93.1a	123.8a
SD[d]	0.4	0.1	3.2	7.4	10.4
		Winter Wheat			
Corbeil	0.5	0.4	27.7	77.0	105.5
Rialto	1.1	0.6	26.3	87.8	115.8
Pajero	1.6	0.4	22.4	68.8	93.2
Estica	1.0	0.5	33.5	91.8	126.7
Eléphant	0.6	0.5	31.4	89.4	121.8
av	1.0a	0.5b	28.3a	82.9b	112.6a
SD	0.4	0.1	4.3	9.7	13.4
signif, $P =$	0.091	0.050	0.780	0.040	0.130

[a] Values for Δ^7-avenasterol are given in ergosterol, as no commercial standard is available. [b] Within the same column, mean values with the same letters are not significantly different ($P > 0.05$), whereas mean values with different letters are significantly different ($P \leq 0.05$). [c] LR are Belgian landraces. [d] Standard deviation.

However, more variability was observed among spelt samples ($n = 16$) than among winter wheat samples ($n = 5$), which could be due to the higher number of spelt samples analyzed. For individual sterols, the most important difference concerned Δ^7-avenasterol (calculated in ergosterol): on average, spelt contained 45% more Δ^7-avenasterol than winter wheat, which was statistically significant ($P = 0.019$). For the other sterols, differences were smaller (2−17%) and were not statistically significant (P values in **Table 3**). Regarding the proportion of each sterol, it was the same in spelt and winter wheat: the major sterol was β-sitosterol and accounted for 70% of total sterols, campesterol represented 20%, and all other sterols accounted for the remaining 10%. Hence, sterol proportions were slightly different in FS/HSE/SE as compared to SG/ASG, given that the former included more sterols than the latter. We also noted that unsaturated sterols were by far more abundant than saturated sterols (sitostanol and campestanol), which together represented <5% of total sterols. Finally, the contents of both stigmasterol and cholesterol were negligible.

We calculated the total sterol content as the sum of SG/ASG and FS/HSE/SE (not corrected for recoveries as explained before) and found that spelt contained an average of 651.5 $\mu g \cdot g^{-1}$, which was higher, but not significantly, than winter wheat average (641.1 $\mu g \cdot g^{-1}$) total sterol content. Results also showed that the FS/HSE/SE proportion was the same in spelt and winter wheat, reaching 80% of total sterols; the proportion of glycosylated sterols was consequently 20% of total sterols.

DISCUSSION

Sample Preparation. Phytosterol quantification in cereal products is usually performed with saponified and even acid-hydrolyzed samples (see Introduction). Sample preparation is a critical step for the analysis of phytosterols because the category of phytosterols that can be analyzed depends on the procedure applied. For instance, in saponified samples, part of SG and FS may come, respectively, from ASG and SE/HSE, and they are quantified together, as mentioned in the Introduction and Results. For analytical purposes, the determination of total sterol content, regardless of the origin of the sterol moiety (from SG, FS, HSE, ASG, or SE) can be interesting. Toivo et al. (*8*) stated that the ideal sample preparation procedure for total sterol determination would include sterols from all possible conjugates. In this case, only the sterol moiety is considered. However, from a nutritional perspective, we believe that it would be better to quantify each sterol class separately. Indeed, the bioavailabilities of different steryl conjugates may be different, as well as their physiological effects (*22*, *23*). Besides, even individual sterols have been described as presenting specific physiological effects, such as Δ^7-sterols found particularly in pumpkin seed oil (*24*).

Although ether is not the most adapted solvent to extract glycosylated sterols, these conjugates are partially extracted, because their sterol moiety is lipophilic. Besides, the LC/MS method applied for sterol determination in cereal extracts was also suitable for SG/ASG quantification; therefore, we took advantage of this possibility to analyze SG/ASG. To improve extraction, and thus recovery, of glycosylated sterols, the use of solvent mixtures such as chloroform/methanol (2:1) would be better, as described by other authors (*21*, *25*, *26*). However, the goal of our study was not to optimize SG extraction.

Quantitative Analysis of Phytosterols. Our method was developed by taking into account the usual performances tested for quantitative analysis. Concerning method sensitivity, the LOD and LOQ for β-sitosterol and stigmasterol were, respectively, 0.04 and 0.07 ng·μL^{-1} and 0.06 and 0.11 ng·μL^{-1} (**Table 1**). Careri et al. (*10*) found LOD and LOQ values of 0.42 and 0.52 ng·μL^{-1}, respectively, for β-sitosterol and 0.42 and 0.54 ng·μL^{-1}, respectively, for stigmasterol by LC/UV analysis. Hence, the method we developed is 5−10 times more sensitive. In addition, our LC/MS^2 method is 100 times more sensitive than GC/MS without derivatization, as we found with commercial standards (unpublished results).

Recovery was highest with the highest addition of cholestanol per gram of sample, and it did not seem to be influenced by saponification temperature. We can note from our results that the amount of sterol lost in each experiment was in the same range (~15 $\mu g \cdot g^{-1}$ of wholemeal) but the relative losses, depending on the amount added, were different. Except for the low-concentration solution, recovery percentages were high, indicating that, although the sample preparation procedure included many steps, it did not automatically result in high analyte losses. Nevertheless, as we did not have the possibility to calculate recoveries for each sterol quantified (several were not commercially available and particularly SG), and also because recoveries were lower for low concentrations, we did not use this recovery value to correct the quantification results.

Spelt and winter wheat wholemeal extracts have the same sterol contents. Data reported in the literature show that the sterol content determined in wheat wholemeal depends on the sample preparation procedure: Toivo et al. (*7*) found a lowest value of 24.5 mg·100 g^{-1} (245 $\mu g \cdot g^{-1}$) and a highest value reaching 66 mg·100 g^{-1} (660 $\mu g \cdot g^{-1}$), whereas we found values ranging

Table 3. Combined Free and Esterified Sterol Content (Micrograms per Gram) of 16 Dehulled Spelt and 5 Winter Wheat Wholemeal Saponified Samples, Cultivated in Belgium during 2001–2002

	ergosterol[a]	Δ^7-avenasterol[b]	stigmasterol	cholesterol	campesterol	β-sitosterol	campestanol	sitostanol	total[c]
				Spelt					
Oberkulmer	7.7	9.5	2.4	1.4	124.8	394.2	11.4	12.1	555.7
Ebners Rotkorn	9.3	12.7	3.5	2.4	119.0	403.8	11.6	9.8	562.8
Béryl	7.6	7.1	2.4	1.8	101.6	329.4	10.1	10.0	462.3
Alkor	9.2	7.5	2.3	2.1	108.1	345.5	10.2	12.6	488.3
Ostar	9.8	9.0	3.1	1.8	117.5	343.2	8.3	8.5	491.4
Redouté	7.1	13.1	1.7	2.0	104.8	359.5	7.3	9.0	497.3
Rouquin	7.4	8.3	2.2	1.5	100.0	328.6	8.9	11.9	461.4
Line 115.11	7.9	9.6	2.7	1.5	111.2	351.7	9.7	11.2	497.6
Line 115.6.2	10.1	6.2	2.2	1.7	106.1	377.4	10.6	14.5	518.7
LR 134[d]	8.9	9.7	2.1	1.8	123.7	409.0	11.0	10.6	567.8
LR 135	10.1	12.1	2.7	2.1	119.4	353.5	12.0	13.5	515.3
LR 140	8.2	12.2	2.4	1.8	96.2	378.1	12.0	19.5	522.3
LR 229	6.7	9.0	2.9	1.8	102.9	307.1	8.3	5.7	437.6
LR 260.1	11.0	12.4	3.6	2.4	127.6	383.7	11.5	12.4	553.7
LR 268	13.6	8.7	2.3	1.9	136.9	463.3	15.0	17.6	645.8
LR 286.1	12.1	15.3	3.3	1.8	155.5	450.7	19.6	19.1	665.4
av	9.2a	10.1a	2.6a	1.9a	116.0a	373.7a	11.1a	12.4a	527.7a
SD[e]	1.9	2.5	0.5	0.3	15.6	43.1	2.9	3.8	62.7
				Winter Wheat					
Corbeil	6.8	5.8	2.3	1.4	145.3	354.3	16.8	11.4	537.2
Rialto	9.3	7.2	2.8	2.1	113.3	349.6	14.6	16.4	506.1
Pajero	6.4	11.2	2.6	1.5	125.0	394.0	11.6	11.8	557.7
Estica	8.8	5.6	2.3	2.4	140.9	359.1	10.7	7.2	528.2
Eléphant	8.8	5.3	2.0	1.4	114.2	376.8	7.3	6.2	513.2
av	8.0a	7.0b	2.4a	1.8a	127.8a	366.8a	12.2a	10.6a	528.5a
SD	1.3	2.5	0.3	0.5	14.8	18.4	3.7	4.1	20.4
signif, $P =$	0.208	0.019	0.495	0.495	0.130	0.905	0.495	0.398	0.660

[a] Within the same column, mean values with the same letters are not significantly different ($P > 0.05$), whereas mean values with different letters are significantly different ($P \leq 0.05$). [b] Values for Δ^7-avenasterol are given in ergosterol, owing to the lack of a commercial standard. [c] Total values exclude ergosterol, as it is not a cereal sterol. [d] LR are Belgian landraces. [e] Standard deviation.

from 543.5 to 807.2 $\mu g \cdot g^{-1}$. These data correspond to the sum of SG/ASG and FS/HSE/SE, which is the main limitation of this method.

Comparison with previous studies (*2*, *8*) shows that the values we found for β-sitosterol and campesterol contents are comparable to those already published for wheat wholemeal, which also contains trace amounts of cholesterol, in agreement with our own results. On the other hand, these authors [and also Pelillo et al. (*9*)] found stanol contents 5−10 times higher than in our samples. Discordant results might be explained by the fact that they did not rely on response factors to correct their values. Furthermore, they determined total sterol content after acid and alkaline hydrolysis. Therefore, they cannot indicate the proportion of FS/HSE/SE versus glycosylated sterols. In this sense, our results contribute to the determination of two sterol classes (after saponification and extraction with diethyl ether) found in spelt and winter wheat. A last remark we can make concerning sterol content is that, although spelt wholemeal has a significantly higher lipid content than winter wheat wholemeal (*27*), this observation does not apply to phytosterol content.

Conclusions and Perspectives. Spelt and winter wheat exhibit not only similar sterol profiles but also similar sterol contents, as studied in saponified samples. The LC/MS^2 method we applied was suitable to determine both FS and sterols released from their esters, as well as glycosylated sterols (SG and released ASG). For the latter compounds, the extraction step could be improved by using more polar solvents, such as methanol. Also, the quantification procedure could be further elaborated by studying recovery of the various sterols present in spelt and wheat samples, using commercial standards, when available, but many of them are not. The next step is the qualitative and quantitative study of other conjugated phytosterols in nonsaponified cereal samples, to spare conjugated structures. It would also be of particular interest to quantify sterols and their conjugates in lipid-rich milling byproducts, such as bran and shorts, because they could also be used in the human food chain, provided that they present a nutritional advantage in comparison with wholemeal.

ABBREVIATIONS USED

APCI, atmospheric pressure chemical ionization; ASG, acylated steryl glycoside; CID, collision-induced dissociation; ELSD, evaporative light-scattering detection; ESI, electrospray; FS, free sterol; GC, gas chromatography; HSE, hydroxycinnamate steryl ester; LC, liquid chromatography; LOD, limit of detection; LOQ, limit of quantification; LR, landrace; MS, mass spectrometry; SD, standard deviation; SE, steryl fatty acid ester; SG, steryl glycoside; SPE, solid-phase extraction; UV, ultraviolet.

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LITERATURE CITED

(1) Moreau, R. A.; Whitaker, B. D.; Hicks, K. B. Phytosterols, phytostanols, and their conjugates in foods: structural diversity, quantitative analysis and health-promoting uses. *Prog. Lipid Res.* **2002**, *41*, 457−500.

(2) Piironen, V.; Toivo, J.; Lampi, A. M. Plant sterols in cereals and cereal products. *Cereal Chem.* **2002**, *79*, 148−154.

(3) Wojciechowski, Z. A. Biochemistry of phytosterol conjugates. In *Physiology and Biochemistry of Sterols*; Patterson, G. W., Nes, D., Eds.; American Oil Chemists' Society: Champaign, IL, 1991; pp 361−395.
(4) Nourooz-Zadeh, J.; Appelqvist, L. A. Isolation and quantitative determination of sterol oxides in plant-based foods: soybean oil and wheat flour. *J. Am. Oil Chem. Soc.* **1992**, *69*, 288−293.
(5) Dutta, P. C.; Appelqvist, L. A. Saturated sterols (stanols) in unhydrogenated and hydrogenated edible vegetable oils and in cereal lipids. *J. Sci. Food Agric.* **1996**, *71*, 383−391.
(6) Määttä, K.; Lampi, A. M.; Petterson, J.; Fogelfors, B. M.; Piironen, V.; Kamal-Eldin, A. Phytosterol content in seven oat cultivars grown at three locations in Sweden. *J. Sci. Food Agric.* **1999**, *79*, 1021−1027.
(7) Toivo, J.; Lampi, A. M.; Aalto, S.; Piironen, V. Factors affecting sample preparation in the gas chromatographic determination of plant sterols in whole wheat flour. *Food Chem.* **2000**, *68*, 239−245.
(8) Toivo, J.; Phillips, K.; Lampi, A. M.; Piironen, V. Determination of sterols in foods: Recovery of free, esterified, and glycosidic sterols. *J. Food Compos. Anal.* **2001**, *14*, 631−643.
(9) Pelillo, M.; Iafelice, G.; Marconi, E.; Caboni, M. F. Identification of plant sterols in hexaploid and tetraploid wheats using gas chromatography with mass spectrometry. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 2245−2252.
(10) Careri, M.; Elviri, L.; Magia, A. Liquid chromatography-UV determination and liquid chromatography-atmospheric pressure chemical ionization mass spectrometric characterization of sitosterol and stigmasterol in soybean oil. *J. Chromatogr. A* **2001**, *935*, 249−257.
(11) Moreau, R. A.; Powell, M. J.; Hicks, K. B. Extraction and quantitative analysis of oil from commercial corn fiber. *J. Agric. Food Chem.* **1996**, *44*, 2149−2154.
(12) Fang, N.; Shanggong, Y.; Badger, T. M. Characterization of triterpene alcohol and sterol ferulates in rice bran using LC-MS/MS. *J. Agric. Food Chem.* **2003**, *51*, 3260−3267.
(13) Rozenberg, R.; Ruibal-Mendieta, N. L.; Petitjean, G.; Cani, P.; Delacroix, D. L.; Delzenne, N. M.; Meurens, M.; Quetin-Leclercq, J.; Habib-Jiwan, J. L. Phytosterol analysis and characterisation in spelt (*Triticum aestivum* ssp. *spelta* L.) and wheat (*T. aestivum* L.) lipids by LC/APCI-MS. *J. Cereal Sci.* **2003**, *38*, 189−197.
(14) O'Connor, J.; Perry, H. J.; Harwood, J. L. A comparison of lipase activity in various cereal grains. *J. Cereal Sci.* **1992**, *16*, 153−163.
(15) Huber, L. Validation of analytical methods: Review and strategy. *LC−GC Int.* **1998**, *Feb*, 96−105.
(16) Breinhölder, P.; Mosca, L.; Lindner, W. Concept of sequential analysis of free and conjugated phytosterols in different plant matrices. *J. Chromatogr. B* **2002**, *777*, 67−82.
(17) De Baere, S.; Cherlet, M.; Baert, K.; De Backer, P. Quantitative analysis of amoxycillin and its major metabolites in animal tissues by liquid chromatography combined with electrospray ionization tandem mass spectrometry. *Anal. Chem.* **2002**, *74*, 1393−1401.
(18) FDA. *Guidance for Industry: Bioanalytical Method Validation*; U.S. Department of Health and Human Services, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER); FDA: Rockville, MD, 2001.
(19) Conseil de l'Europe. Techniques de séparation chromatographique. Méthode 2.2.46. In *Pharmacopée Européenne*; Direction de la Qualité du Médicament du Conseil de l'Europe, Ed.; Conseil de l'Europe: Strasbourg, France, 2002; p 68.
(20) SPSS for Windows package, release 10.0.5; Bristol Technology Inc., 1999.
(21) Kesselmeier, J.; Eichenberger, W.; Urban, B. High performance liquid chromatography of molecular species from free sterols and sterylglycosides isolated from oat leaves and seeds. *Plant Cell Physiol.* **1985**, *26*, 463−471.
(22) Awad, A. B.; Fink, C. S. Phytosterols as anticancer dietary components: evidence and mechanism of action. *J. Nutr.* **2000**, *130*, 2127−2130.
(23) Bouic, P. J. D. The role of phytosterols and phytosterolins in immune modulation: a review of the past 10 years. *Curr. Opin. Clin. Nutr.* **2001**, *4*, 471−475.
(24) Mandl, A.; Reich, G.; Lindner, W. Detection of adulteration of pumpkin seed oil by analysis of content and composition of specific Δ^7-phytosterols. *Eur. Food Res. Technol.* **1999**, *209*, 400−406.
(25) Yamauchi, R.; Aizawa, K.; Inakuma, T.; Kato, K. Analysis of molecular species of glycolipids in fruit pastes of red bell pepper (*Capsicum annuum* L.) by high-performance liquid chromatography−mass spectrometry. *J. Agric. Food Chem.* **2001**, *49*, 622−627.
(26) Ramadan, M. F.; Mörsel, J. T. Analysis of glycolipids from black cumin (*Nigella sativa* L.), coriander (*Coriandrum sativum* L.) and niger (*Guizotia abyssinica* Cass.) oilseeds. *Food Chem.* **2003**, *80*, 197−204.
(27) Ruibal-Mendieta, N. L.; Delacroix, D. L.; Meurens, M. A comparative analysis of free, bound and total lipid content on spelt and winter wheat wholemeal. *J. Cereal Sci.* **2002**, *35*, 337−342.

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