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Simultaneous Analysis of Free Phytosterols/Phytostanols and Intact Phytosteryl/Phytostanyl Fatty Acid and Phenolic Acid Esters in Cereals

Rebecca Esche, Andreas Barnsteiner, Birgit Scholz, and Karl-Heinz Engel*

Lehrstuhl für Allgemeine Lebensmitteltechnologie, Technische Universität München, Maximus-von-Imhof-Forum 2, D-85350 Freising-Weihenstephan, Germany

ABSTRACT: An approach based on solid-phase extraction for the effective separation of free phytosterols/phytostanols and phytosteryl/phytostanyl fatty acid and phenolic acid esters from cereal lipids was developed. The ester conjugates were analyzed in their intact form by means of capillary gas chromatography. Besides free sterols and stanols, up to 33 different fatty acid and phenolic acid esters were identified in four different cereal grains via gas chromatography−mass spectrometry. The majority (52−57%) of the sterols and stanols were present as fatty acid esters. The highest levels of all three sterol and stanol classes based on dry matter of ground kernels were determined in corn, whereas the oil extract of rye was 1.7 and 1.6 times richer in fatty acid esters and free sterols/stanols than the corn oil. The results showed that there are considerable differences in the sterols/stanols and their ester profiles and contents obtained from corn compared to rye, wheat, and spelt. The proposed method is useful for the quantification of a wide range of free phytosterols/phytostanols and intact phytosteryl/phytostanyl esters to characterize different types of grain.

KEYWORDS: *solid-phase extraction, sterols, steryl fatty acid ester, steryl phenolic acid ester, cereals*

INTRODUCTION

Phytosterols and phytostanols are cholesterol-like molecules that play important roles in the structure and function of cell membranes.[1] The current interest in phytosterols/phytostanols is mainly due to their potency to decrease low-density lipoprotein (LDL) cholesterol levels in plasma, a potential risk factor for cardiovascular diseases.[2−4] This led to the development of functional foods enriched in sterols/stanols and their fatty acid esters (e.g., margarines and drinking yogurts). In addition to the cholesterol-lowering effect, steryl and stanyl ferulates are thought to possess other health-promoting properties, for example, antioxidative and anti-inflammatory effects.[5−7] Thus, there is growing interest in increasing the intake of these compounds in natural diets. The main natural sources of phytosterols are cereals, seeds, and vegetable oils. Cereals and cereal-based foods are of particular importance; depending on the populations and the dietary patterns, they contribute approximately 25−42% to the total daily intake of phytosterols.[8−10] As in many other foods, in cereal grains sterols occur as free sterols, as esters of fatty acids and phenolic acids, as steryl glycosides, and as acylated steryl glycosides.[1]

Currently, the analysis of phytosterols and phytostanols in cereals is mainly based on the determination of total contents after alkaline hydrolysis or after a combination of acidic and alkaline hydrolysis.[11−16] The liberated sterols and stanols can be extracted as part of the unsaponifiable matter, commonly followed by a purification step and final analysis via gas chromatography (GC) or reversed phase high-performance liquid chromatography (RP-HPLC). In whole corn grains, total contents of steryl fatty acid esters were determined by means of normal phase HPLC (NP-HPLC) equipped with an evaporative light scattering detector (ELSD).[17,18] A recently published NP-HPLC-ELSD method was also used to analyze several classes of neutral lipids, for example, steryl esters, free fatty acids, and free sterols, in rye and corn flour as well as in sourdough and bread.[19] Other approaches are based on the isolation of steryl/stanyl fatty acid esters from corn oil by column chromatography (CC) or thin layer chromatography (TLC) and saponification of the ester fraction for the subsequent analysis of liberated sterols by GC and flame ionization detection (FID).[20,21] Thereby, information concerning the distribution and composition of individual steryl/stanyl fatty acid esters gets lost.

Chromatographic methods for the investigation of intact steryl and stanyl fatty acid esters in cereals are rare. Gordon et al. analyzed intact esters in corn oil by means of GC-FID after extraction by preparative NP-HPLC; however, the resolution of individual esters was insufficient, and the esters were only characterized on the basis of relative retention times without identification of individual peaks.[22] In wheat and spelt, the fraction of steryl/stanyl fatty acid esters was extracted from the lipids by TLC, and intact esters were analyzed via GC-FID and RP-HPLC-ELSD.[23] Using the RP-HPLC-ELSD method, several peaks belonging to different steryl series were overlapping. To distinguish between several esters, the analysis had to be done with the use of mass detection by extraction of single ions. In turn, the suggested GC-FID method resulted in a better sensitivity and resolution compared to HPLC-ELSD. However, no resolution between the steryl esters of oleic and linoleic acid could be achieved; moreover, no stanyl esters were included.[23]

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Analytical methods for the investigation of free sterols and stanols in cereals are also lacking. Pelillo et al. determined free sterols and stanols in tetraploid and hexaploid wheat after TLC and GC-FID.[24] In whole corn kernels and corn oil, free sterols/stanols were analyzed in total by NP-HPLC-ELSD or after isolation by TLC or solid-phase extraction (SPE), subsequent saponification, and final analysis via GC-FID.[17,18,20,21,25] A method for analysis of free sterols/stanols besides steryl/stanyl fatty acid esters in several tetraploid and hexaploid wheats was reported by Iafelice et al.[26] The sterol classes were separated by TLC and further saponified for analysis of the sterol moieties by means of GC-FID. Neither qualitative nor quantitative data on the distribution of free sterols and stanols in rye kernels are available.

Investigations on intact steryl and stanyl phenolic acid esters in cereals are commonly based on the isolation of this type of esters by a base−acid cleanup and analysis by means of RP-HPLC. Using this approach, intact phenolic acid esters were determined in wheat and rye grains.[27−29] Nevertheless, drawbacks of this method are an insufficient resolution of main compounds, for example, sitosteryl and campestanyl ferulate, as well as missing information on naturally occurring *cis* isomers of phenolic acid esters. Seitz[30] and Norton[31] analyzed several intact steryl ferulates and coumarates in corn and corn oil via RP-HPLC, but with complex sample preparation steps, for example, liquid−liquid extraction, CC, TLC, and/or base−acid cleanup. Total contents of steryl ferulates in corn grains were determined by means of a NP-HPLC method, which was also used to analyze total amounts of free sterols and steryl fatty acid esters, but without any information on the distribution of individual compounds.[17,18] Data on the contents of total or intact steryl and stanyl phenolic acid esters in spelt grains are not available.

In conclusion, methods for the quantitative analysis of free phytosterols/stanols as well as of intact phytosteryl/stanyl esters in cereals are missing. Therefore, the aim of the present study was the development of an SPE-based method for the separation of free sterols/stanols and steryl/stanyl fatty acid and phenolic acid esters isolated from cereals. GC-FID equipped with a medium polar capillary column should be used for the analysis of the SPE fractions as this chromatographic method has been shown to be suitable for the investigation of individual steryl/stanyl ferulic acid esters in brown rice and of stanyl fatty acid esters in enriched foods.[32,33] The developed methodology should be applied to demonstrate the variability in composition and contents of individual steryl/stanyl conjugates in different types of grain.

MATERIALS AND METHODS

Samples. Corn (*Zea mays*) was provided by Cornexo GmbH & Co. KG (Freimersheim, Germany). Approximately six to eight cobs of the genotype Starsky were harvested in 2010; the kernels (approximately 1 kg) were manually removed and subsequently dried in a cabinet dryer at 40 °C for 3 days. Wheat (*Triticum aestivum* L.) and rye (*Secale cereale*) kernels produced by Alnatura GmbH, Germany, and spelt kernels (*Triticum spelta*) produced by Neuform International, Germany, were purchased in local stores. According to the label, the wheat and rye material had been grown organically; no other botanical or agronomic information was available.

Chemicals and Materials. Cholesteryl palmitate (≥98%), 5-α-cholestan-3β-ol (~95%), stigmasterol (~95%), sitostanol (~95%), *trans*-*p*-coumaric acid (CA, >99%), *trans*-ferulic acid (99%), palmitic acid (98%), linoleic acid (99%), pyridine (99.8%), dichloromethane (DCM, 95%), methanol, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS), thionyl chloride, triethylamine (>99.5%), acetic anhydride (98%), hydrochloric acid (25%), sodium sulfate (anhydrous), magnesium sulfate (anhydrous), piperazine (99%), potassium hydroxide (85%), sodium hydroxide, and 4-dimethylaminopyridine (DMAP, 99%) were obtained from Sigma-Aldrich (Steinheim, Germany). *n*-Hexane (AnalR Normapure), diethyl ether (DEE) (extra pure), and ammonium chloride (Rectapur) were purchased from VWR International (Darmstadt, Germany). Ethyl acetate (EtOAc) and *tert*-butyl methyl ether (MTBE) were supplied by Oxeno Olefinchemie (Marl, Germany) and were distilled prior to use. Tetrahydrofuran (THF) was purchased from BASF (Ludwigshafen, Germany). Sitosterol (75%) was obtained from Acros Organics (Morris Plains, NJ, USA). Mixtures of phytosteryl/phytostanyl fatty acid esters (Vegapure 95E) and of plant stanols (Reducol Stanol Powder) were provided by Cognis GmbH (Illertissen, Germany).

Synthesis of Steryl/Stanyl Fatty Acid Esters. In accordance with the procedure described in a patent (WO2007101580),[34] steryl/stanyl fatty acid esters were synthesized directly without a catalyst under nitrogen atmosphere. Briefly, 0.25 mmol of stigmasterol or sitostanol, respectively, and a 2−3-fold amount of fatty acid were mixed and flushed with nitrogen. The reaction vial was sealed and heated to 180 °C in a heating block for 25 h. Purification was achieved by alkaline liquid−liquid extraction: 2.5 mL of potassium hydroxide solution (1 M) was added to the residue, and the synthesized fatty acid ester was extracted three times with 2.5 mL of *n*-hexane/MTBE (3:2, v/v). After evaporation of the solvent by a gentle stream of nitrogen, the residue was dried at 103 °C for 30 min. The obtained GC purities for stigmasteryl palmitate and sitostanyl linoleate were 90 and 84 area %, respectively.

Synthesis of Steryl/Stanyl Phenolic Acid Esters. On the basis of previously published methods,[35−38] the synthesis of steryl/stanyl phenolic acid esters was performed via (i) acetylation of the phenolic acid, (ii) activation with thionyl chloride, (iii) esterification, (iv) deprotection, and (v) purification. Briefly, 2.5 g of phenolic acid dissolved in 4.5 mL of pyridine and 4.0 mL of acetic anhydride were stirred at room temperature for 4 h. The mixture was transferred into 100 mL of ice−water; the resulting precipitate was isolated by filtration via a Büchner funnel, washed with 20 mL of bidistillated water, and recrystallized from 30 mL of methanol. The acid chloride was prepared by refluxing a mixture of the prepared *O*-acetylphenolic acid (1.2 g) with thionyl chloride at 70−75 °C for 1.5 h. The reagents were removed by rotary evaporation, and the residue was dried by a gentle stream of nitrogen. For esterification, 300 mg of the *O*-acetyl acid chloride and 100 mg of sterol/stanol were dissolved in 5 mL of dry DMAP solution (2.5 mg/mL in DCM) and 50 μL of triethylamine. The mixture was heated to 50 °C for 16 h. The solution was washed three times with 3 mL of hydrochloric acid (0.1 M), twice with 3 mL of a saturated ammonium chloride solution, and finally with 3 mL of bidistillated water. The organic phase was dried with sodium sulfate. After filtration, the solvent was removed by a gentle stream of nitrogen. For deprotection, about 200 mg of the synthesized steryl/stanyl *O*-acetylphenolic acid esters were dissolved in 20 mL of dry THF, and 40 mL of piperazine solution (70 mg/mL in dry THF) was added dropwise at room temperature under argon. The mixture was stirred for 2 h at room temperature, diluted with 50 mL of ethyl acetate, and washed 10 times with 50 mL of saturated ammonium chloride solution. After drying with magnesium sulfate and filtration, the solvent was removed by rotary evaporation. The purification was achieved by an acid−base extraction and subsequent solid-phase extraction. About 100 mg of the residue was dissolved in 20 mL of *n*-hexane, and the solution was mixed with 20 mL of sodium hydroxide (1 M). The aqueous solution was washed twice with 10 mL of *n*-hexane, and the organic layer was discarded. After acidification with hydrochloric acid, steryl/stanyl phenolic acid esters and remaining free phenolic acids were extracted twice with 20 mL of MTBE. The combined extracts were evaporated to dryness by rotary evaporation, and the residue was used for SPE. The cartridge (Chromabond C18ec, 45 μm, 500 mg, Macherey-Nagel GmbH & Co. KG, Germany) was conditioned with 4 mL of methanol. After transfer of the solid onto the cartridge, the first fraction of 6 mL of methanol was discarded.

Elution of steryl/stanyl esters was carried out with 8 mL of MTBE. The solvent was removed by a gentle stream of nitrogen. Fifty micrograms of the synthesized ester was silylated with 100 μL of BSTFA plus 1% TMCS (100 μL) and 20 μL of pyridine at 80 °C for 20 min. The obtained GC purities for cholestanyl-*p*-coumarate, sitostanyl-*p*-coumarate, and sitostanyl ferulate were 95, 84, and 80 area %, respectively.

Milling and Lipid Extraction. About 200 g of the self-dried corn kernels and the commercially obtained wheat, rye, and spelt kernels, respectively, were frozen in liquid nitrogen and subsequently ground using a cyclone mill (1093 Cyclotec, Foss, Germany) equipped with a 500 μm sieve. The resulting flour was freeze-dried (Alpha 1-4 LSC, Christ, Osterode, Germany) for 48 h and stored in plastic bags at −18 °C.

To avoid *cis*−*trans* isomerization of phenolic acid esters, all used devices were wrapped with aluminum foil. Four grams of corn flour and 8 g of rye, wheat, or spelt flour were weighed into an extraction vessel. Five hundred microliters of cholesteryl palmitate (1.0 mg/mL in *n*-hexane/MTBE (3:2, v/v)), 500 μL of 5-α-cholestan-3β-ol (1.0 mg/mL in MTBE), and 80 μL of cholestanyl-*p*-coumarate (1.0 mg/mL in MTBE) were used as internal standards (IS). After the addition of a magnetic stir bar, the oil was extracted with 40 mL of a mixture of *n*-hexane/dichloromethane (1:1, v/v) under stirring for 1 h at room temperature. After filtration, the extraction vessel and the filter were washed, and the combined filtrates were evaporated to dryness by rotary evaporation. The residue was dried at 103 ± 2 °C to constant weight. One hundred milligrams of the obtained oil was dissolved in 10 mL of *n*-hexane and was used for the SPE.

Solid-Phase Extraction. One milliliter of the oil solution was loaded onto the SPE cartridge (Strata NH_2, 55 μm, 70 Å, 1 g/6 mL, Phenomenex, Germany), previously conditioned with 2 × 5 mL of *n*-hexane. Steryl/stanyl fatty acid esters (fraction A) were eluted with 2 × 5 mL of *n*-hexane/diethyl ether (98:2, v/v). Interfering triglycerides were removed with 4 × 5 mL of *n*-hexane/ethyl acetate (96:4, v/v). Free sterols and stanols (fraction B) were eluted with 2 × 5 mL of *n*-hexane/ethyl acetate (5:95, v/v), and finally steryl/stanyl phenolic acid esters (fraction C) were eluted with 2 × 5 mL *n*-hexane/ethyl acetate (5:95, v/v) followed by 5 mL of MTBE. After evaporation of the solvents by rotary evaporation, the residues of fraction A and B were dissolved in 500 and 1000 μL of *n*-hexane, respectively. Fraction C was silylated with 20 μL of pyridine and 100 μL of BSTFA:TCMS (99:1) at 80 °C for 20 min. The silylation reagents were removed by a gentle stream of nitrogen, and the residue was dissolved in 50 μL of *n*-hexane.

GC-FID Analysis. Analysis was performed using a gas chromatograph equipped with a flame ionization detector (Agilent Technologies Instrument 6890N, Böblingen, Germany). Separations of 1 μL of the sample solutions were carried out on a 30 m × 0.25 mm i.d. fused-silica capillary column coated with a film of 0.1 μm trifluoropropylmethyl polysiloxane (Rtx-200MS, Restek, Bad Homburg, Germany). The temperature of the injector was set to 280 °C, and hydrogen was used as carrier gas with a constant flow rate of 1.5 mL/min. Split flow was set to 11.2 mL/min, resulting in a split ratio of 1:7.5. Nitrogen was used as makeup gas with a flow of 25 mL/min. The oven temperature program was as follows: initial temperature, 100 °C; programmed at 15 °C/min to 310 °C (2 min), then at 1.5 °C/min to 315 °C, and at 15 °C/min to 340 °C (2 min). The detector temperature was set to 360 °C. Data acquisition was performed by ChemStation software.

Quantification by GC-FID. Steryl/stanyl fatty acid esters were quantified by generating three-point calibration functions with 0.1, 0.3, and 0.5 μg of total esters (Vegapure 95E) per microliter of injection.[33] Each calibration point was done in triplicate. Linear regression was confirmed in ratio of areas (area steryl or stanyl ester/area IS) and amounts (amount steryl or stanyl ester/amount IS). Free sterols/stanols were quantified using cholestanol as internal standard with a response factor of 1.00. Steryl/stanyl phenolic acid esters were determined as their trimethylsilyl derivatives with the following response factors related to cholestanyl-*p*-coumarate (IS): stanyl-*p*-coumarates, 1.05; steryl ferulates, 1.05; and stanyl ferulates, 1.15.

Identification by Gas Chromatography−Mass Spectrometry (GC-MS). Identification of the steryl/stanyl esters and the free sterols/stanols was performed on a Finnigan Trace GC ultra (Thermo Electro Corp., Austin, TX, USA) coupled with a Finnigan Trace DSQ mass spectrometer (Thermo Electro Corp.). Mass spectra were obtained by electron impact ionization at 70 eV in the full scan mode at unit resolution from 40 to 750 Da. Helium was used as carrier gas with constant flow rate (1.0 mL/min). The interface was heated to 330 °C and the ion source to 250 °C. Gas chromatographic separations were performed on an Rtx-200MS (30 m × 0.25 mm i.d., 0.1 μm film) fused-silica capillary column (Restek), and conditions were as described for GC-FID analysis. Data acquisition was performed by Xcalibur software.

Validation of the Method. Recoveries were determined by spiking corn flour (4 g) with defined amounts of internal standards and of two selected plant steryl/stanyl derivatives representative for each SPE fraction: 0.5 mg of cholesteryl palmitate, stigmasteryl palmitate, and sitostanyl linoleate, respectively, as reference compounds for steryl/stanyl fatty acid esters; 0.5 mg of cholestanol, stigmastanol, and sitostanol, respectively, as reference compounds for free sterols/stanols; 80 μg of cholestanyl-*p*-coumarate, sitostanyl-*p*-coumarate, and sitostanyl ferulate as reference compounds for steryl/stanyl phenolic acid esters. The repeatability of the method was confirmed by working up a control sample (corn flour) once on each day of analysis (in total, five replicates). The limits of detection (LOD) and the limits of quantification (LOQ) were determined according to standard procedures and criteria.[39]

RESULTS AND DISCUSSION

SPE Separation and GC Analysis. On the basis of SPE, a facile method for the separation of free sterols/stanols, steryl/stanyl fatty acid, and phenolic acid esters from corn oil was developed (Figure 1). Fatty acid esters (fraction A) were eluted

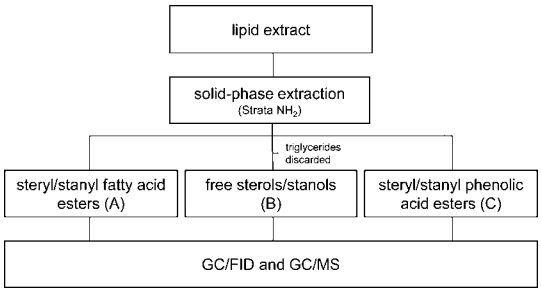

Figure 1. Elution sequence employed to separate sterols/stanols and their esters isolated from cereals via SPE.

with a mixture of *n*-hexane/diethyl ether (98:2, v/v) and were separated from triglycerides, which were subsequently washed out. With aminopropyl-modified silica cartridges a better removal of interfering triglycerides could be achieved compared to normal silica. The elution of free sterols and stanols (fraction B) was performed with a mixture of *n*-hexane/ethyl acetate (5:95, v/v) followed by elution of the steryl/stanyl phenolic acid esters (fraction C). The separation of the lipids from corn into the three classes was very efficient (Figure 2). In fraction C, containing the phenolic acid esters, several other constituents were detected. Preliminary investigations by GC-MS indicated the presence of free fatty acids and monoglycerides. However, a full structural elucidation has not been performed.

The GC separation was achieved using a medium polar trifluoropropylmethyl polysiloxane capillary column, which has been shown to be suitable for the separation of steryl/stanyl ferulic acid and stanyl fatty acid esters.[32,33] Under the employed

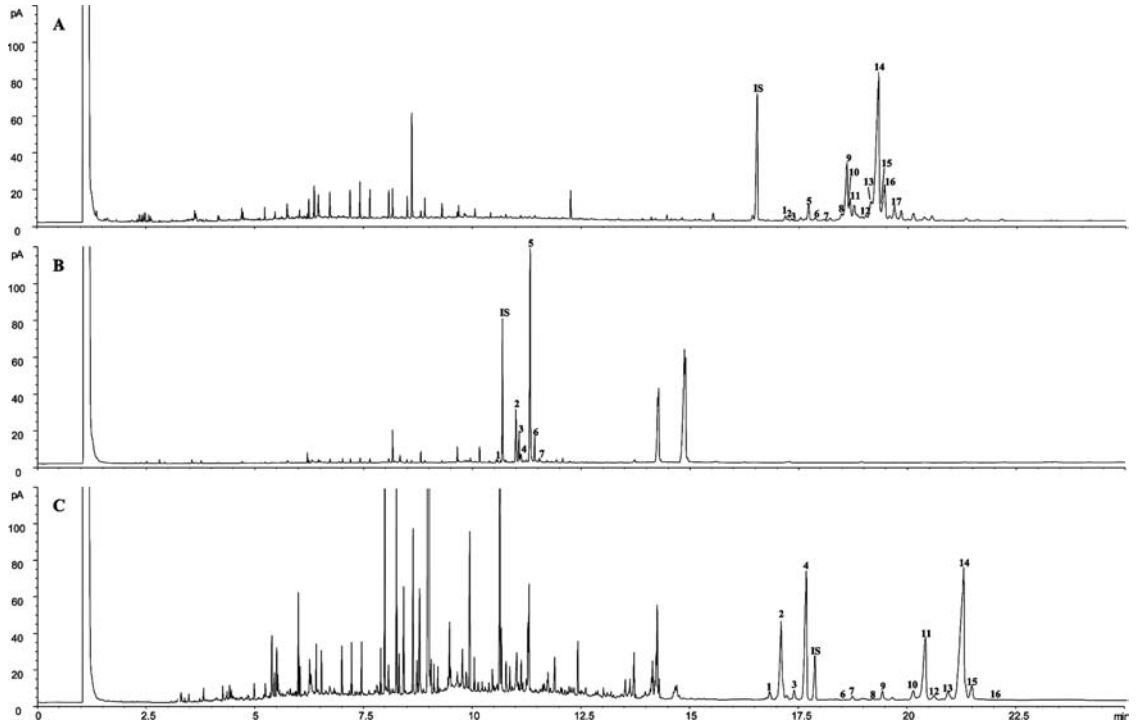

Figure 2. Capillary gas chromatographic analysis of (A) steryl/stanyl fatty acid esters, (B) free sterols and stanols, and (C) steryl/stanyl phenolic acid esters obtained from ground corn kernels. Peak numbering correlates to Tables 3–5. (IS) internal standard: (A) cholesteryl palmitate; (B) cholestanol; (C) cholestanyl-*p*-coumarate.

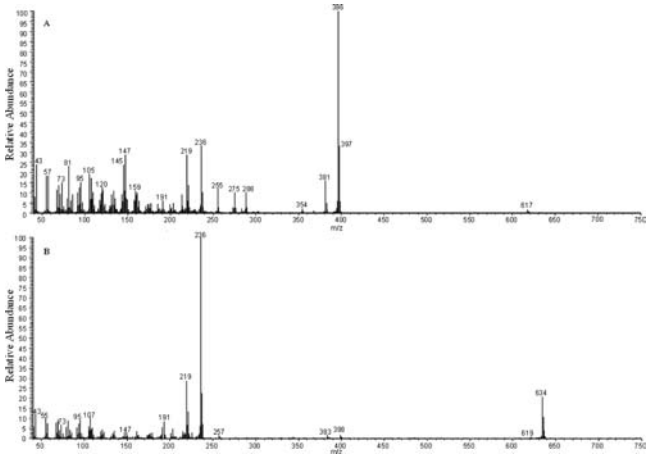

Figure 3. EI mass spectra of TMS ethers of (A) sitosteryl-*p*-coumarate and (B) sitostanyl-*p*-coumarate.

conditions, free sterols and stanols were better resolved than their trimethylsilyl (TMS) derivatives, whereas the response and the resolution of steryl/stanyl phenolic acid esters could be improved by TMS derivatization. Under the applied conditions, besides the steryl and stanyl esters of ferulic acid, also small peaks of coumaric acid esters were detected and quantified (Figure 2C). The stanyl ferulates or coumarates eluted just after the corresponding steryl esters. Additionally, occurring *cis* derivatives of steryl and stanyl ferulic acid esters were completely separated under the applied conditions. Owing to the high temperature stability and the low bleeding, the employed stationary phase is particularly suitable for mass spectrometric investigations.

Identification of individual compounds was performed on the basis of relative retention times and mass spectral data (Table 1). The fragmentation patterns observed for the ferulates were in agreement with previously reported GC-MS data for silylated stanyl ferulates.[40] The steryl/stanyl-*p*-coumarates were resolved and identified in intact and silylated form by GC-MS for the first time. Mass spectra obtained for synthesized sitosteryl and sitostanyl-*p*-coumarate as TMS ethers are shown in Figure 3; characteristic fragments are listed in Table 1. Molecular ions $[M]^+$ were observed only for stanyl esters, whereas the fragment $[M - 15]^+$ was obtained for all steryl/stanyl-*p*-coumarates. The mass spectra of steryl esters exhibited $[M - CA]^+$ and those of stanyl esters the fragment m/z 236 as base ions. The fragments m/z 236, 219, and 191 are characteristic for TMS derivatives of coumaric acid.

The recoveries, LODs, and LOQs of the used internal standards and of representatives of the three substance classes are presented in Table 2. The recoveries of the internal standards and of selected plant sterols/stanols and their esters after lipid extraction, SPE, and GC analysis were >90%. The total contents of fractions A, B, and C from five replicate analyses of the control sample were 643.8 ± 32.5, 351.1 ± 15.5, and 80.3 ± 3.4 μg/g, respectively; the relative standard deviations of <5% indicate good repeatability. The reproducibility in terms of interlaboratory precision of the approach has not been assessed.

Comparison of Different Cereals. The developed methodology was applied to the analysis of steryl/stanyl fatty acid esters (A), free sterols/stanols (B), and steryl/stanyl phenolic acid esters (C) in corn, rye, wheat, and spelt kernels. Lipids were extracted from the ground and freeze-dried cereal grains with a mixture of *n*-hexane/dichloromethane (1:1, v/v), resulting in oil contents of 3.57 ± 0.07, 1.46 ± 0.02, 1.69 ± 0.03, and 2.47 ± 0.03% for corn, rye, wheat, and spelt, respectively. Rye, wheat, and spelt revealed very similar

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Table 1. Mass Spectrometric and Chromatographic Data of Sterols/Stanols and Steryl/Stanyl Esters

	RRT[a]	molecular ion $[M]^+$	characteristic fragments
steryl/stanyl fatty acid esters			
campesteryl-16	1.040	638 (−)	383 (34), 382 (100), 367 (12), 255 (4), 213 (3), 81 (5)
stigmasteryl-16	1.044	650 (−)	395 (27), 394 (100), 379 (13), 255 (13), 213 (3), 81 (7)
campestanyl-16	1.049	640 (7)	385 (37), 384 (100), 369 (31), 257 (11), 215 (57), 81 (10)
Δ^7-campesteryl-16	1.060	638 (100)	383 (−), 382 (19), 367 (3), 255 (23), 213 (1), 81 (8)
sitosteryl-16	1.076	652 (−)	397 (35), 396 (100), 381 (17), 255 (13), 213 (8), 81 (19)
sitostanyl-16	1.083	654 (5)	399 (33), 398 (90), 383 (40), 257 (23), 215 (100), 81 (36)
Δ^7-sitosteryl-16	1.096	652 (88)	397 (4), 396 (20), 381 (15), 255 (100), 213 (19), 81 (1)
campesteryl-18:1	1.118	664 (−)	383 (39), 382 (100), 367 (17), 255 (−), 213 (10), 81 (5)
stigmasteryl-18:1	1.124	676 (−)	395 (34), 394 (100), 379 (7), 255 (28), 213 (7), 81 (18)
campesteryl-18:2	1.125	662 (1)	383 (59), 382 (100), 367 (18), 255 (13), 213 (9), 81 (31)
campestanyl-18:1	1.129	666 (1)	385 (100), 384 (47), 369 (17), 257 (21), 215 (25), 81 (28)
campestanyl-18:2	1.135	664 (4)	385 (100), 384 (48), 369 (15), 257 (−), 215 (13), 81 (45)
stigmasteryl-18:2	1.135	674 (−)	395 (41), 394 (100), 379 (9), 255 (38), 213 (9), 81 (32)
Δ^7-campesteryl-18:2	1.150	662 (25)	383 (100), 382 (28), 367 (5), 255 (14), 213 (1), 81 (8)
sitosteryl-18:1	1.159	678 (−)	397 (38), 396 (100), 381 (14), 255 (12), 213 (10), 81 (22)
sitosteryl-18:2	1.167	676 (3)	397 (53), 396 (100), 381 (20), 255 (11), 213 (11), 81 (45)
sitostanyl-18:1	1.173	380 (2)	399 (100), 398 (51), 383 (17), 257 (19), 215 (26), 81 (29)
sitostanyl-18:2	1.176	678 (5)	399 (100), 398 (26), 383 (11), 257 (19), 215 (22), 81 (45)
sitosteryl-18:3	1.176	674 (4)	397 (70), 396 (100), 381 (12), 255 (16), 213 (11), 81 (25)
Δ^7-sitosteryl-18:2	1.195	676 (22)	397 (100), 396 (51), 381 (1), 255 (20), 213 (50), 81 (77)
free sterols/stanols			
cholesterol	1.011	386 (100)	371 (39), 368 (61), 353 (51), 255 (31), 213 (43), 129 (10)
campesterol	1.041	400 (100)	385 (52), 382 (73), 367 (50), 255 (7), 213 (11), 129 (2)
stigmasterol	1.047	412 (100)	397 (9), 394 (22), 379 (19), 255 (87), 213 (37), 129 (20)
campestanol	1.050	402 (100)	387 (68), 384 (11), 369 (53), 257 (10), 215 (31), 129 (23)
sitosterol	1.072	414 (100)	399 (40), 396 (67), 381 (46), 255 (46), 213 (50), 129 (11)
sitostanol	1.081	416 (100)	401 (36), 398 (17), 383 (15), 257 (16), 215 (81), 129 (3)
steryl/stanyl phenolic acid esters			
cis-campesteryl ferulate	0.944	648 (2)	633 (2), 382 (10), 367 (5), 266 (100), 249 (5), 221 (1), 194 (−)
cis-campestanyl ferulate	0.956	650 (100)	635 (1), 384 (2), 369 (2), 266 (9), 249 (12), 221 (3), 194 (1)
cis-sitosteryl ferulate	0.807	662 (3)	647 (1), 396 (10), 381 (5), 266 (100), 249 (−), 221 (1), 194 (1)
cis-sitostanyl ferulate	0.986	664 (100)	649 (3), 398 (3), 383 (1), 266 (3), 249 (16), 221 (1), 194 (1)
cis-24-methylenecycloartanyl ferulate	1.019	688 (10)	673 (5), 422 (37), 407 (39), 266 (4), 249 (100), 221 (1), 194 (−)
trans-campesteryl-*p*-coumarate	1.029	618 (−)	603 (1), 382 (100), 367 (21), 236 (28), 219 (29), 191 (5), 164 (2)
trans-campestanyl-*p*-coumarate	1.047	620 (42)	605 (1), 384 (13), 369 (4), 236 (100), 219 (35), 191 (21), 164 (2)
trans-sitosteryl-*p*-coumarate	1.056	632 (−)	617 (1), 396 (100), 381 (15), 236 (34), 219 (20), 191 (5), 164 (1)
trans-sitostanyl-*p*-coumarate	1.085	634 (17)	619 (1), 398 (4), 383 (3), 236 (100), 219 (22), 191 (3), 164 (1)
trans-campesteryl ferulate	1.127	648 (−)	633 (3), 382 (14), 367 (4), 266 (100), 249 (4), 221 (1), 194 (−)
trans-campestanyl ferulate	1.144	650 (100)	635 (6), 384 (2), 369 (1), 266 (14), 249 (14), 221 (2), 194 (1)
trans-Δ^7-campesteryl ferulate	1.154	648 (100)	633 (7), 382 (7), 367 (4), 266 (5), 249 (17), 221 (1), 194 (−)
trans-sitosteryl ferulate	1.170	662 (2)	647 (2), 396 (13), 381 (4), 266 (100), 249 (4), 221 (1), 194 (−)
trans-sitostanyl ferulate	1.188	664 (100)	649 (6), 398 (2), 383 (1), 266 (13), 249 (13), 221 (2), 194 (−)
trans-Δ^7-sitosteryl ferulate	1.198	662 (100)	647 (3), 396 (8), 381 (4), 266 (6), 249 (8), 221 (1), 194 (−)
trans-24-methylenecycloartanyl ferulate	1.227	688 (10)	673 (6), 422 (15), 407 (16), 266 (1), 249 (100), 221 (−), 194 (−)

[a]Retention times relative to cholesteryl palmitate for steryl/stanyl fatty acid esters, to cholestanol for free sterols/stanols, and to cholestanyl-*p*-coumarate for steryl/stanyl phenolic acid esters (Rtx-200MS).

patterns; the capillary gas chromatograms of fractions A, B, and C are exemplarily shown for rye in Figure 4.

Plant Steryl/Stanyl Fatty Acid Esters (A). The majority (51.5−56.5%) of the sterols and stanols in the investigated cereal samples occurred as fatty acid esters. Amounts of individual esters are shown in Table 3. The total contents of fatty acid esters (A) ranged from 370 μg/g in wheat to 651 μg/g in corn. The steryl and stanyl ester profile of corn was clearly distinguishable from those obtained from rye, wheat, and spelt. In corn, the steryl/stanyl esters of unsaturated fatty acids, mainly campesteryl and sitosteryl linoleate, made up 95.6% of total esters. In contrast, in rye, wheat, and spelt approximately 50% of the sterols/stanols in fraction A were esterified to palmitic acid. It is interesting to note that the level of fatty acid esters based on micrograms per 100 mg of extracted oil in rye was 1.7-fold above the level in corn oil (Table 3); however, taking into account the low fat content of rye, the amounts based on dry matter of ground kernels were highest in corn. The determined total contents of steryl/stanyl fatty acid esters in wheat and spelt were in line with reported results.[23] To the

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Table 2. Recoveries, Limits of Detection (LOD), and Limits of Quantification (LOQ) for Steryl/Stanyl Fatty Acid Esters (A),[a] Free Sterols/Stanols (B), and Stanyl Phenolic Acid Esters (C)

	recovery[b] (%)	LOD[c] (μg/mL)	LOQ[c] (μg/mL)
internal standards			
(A) cholesteryl palmitate	97.9 ± 1.9	0.04	0.09
(B) cholestanol	98.0 ± 2.0	0.03	0.07
(C) cholestanyl-*p*-coumarate	93.6 ± 5.3	1.48	3.36
plant sterols/stanols and esters			
(A) stigmasteryl palmitate	96.7 ± 1.9	0.13	0.34
(A) sitostanyl linoleate	102.9 ± 1.4	0.16	0.52
(B) stigmasterol	99.1 ± 0.6	0.03	0.07
(B) sitostanol	95.2 ± 0.3	0.03	0.07
(C) sitostanyl-*p*-coumarate	96.9 ± 4.5	1.10	3.30
(C) sitostanyl ferulate	92.8 ± 3.7	3.60	7.01

[a]Designations (A–C) according to the SPE fractions. [b]Average of three values ± standard deviation. [c]Determined according to ref 39 and expressed on μg/mL of injection volume of fraction A, B, or C.

authors' knowledge, there are presently no data available for fatty acid esters in rye. In corn kernels the fatty acid esters have been analyzed by means of NP-HPLC.[17] Thereby, the ranges of total contents reported for ground kernels (0.76–3.09 wt % of oil; n = 49) were in good agreement with the data determined for the corn sample in this study (1.82 ± 0.04 wt % of oil).

Free Plant Sterols/Stanols (B). In all cereal grains, sitosterol was the dominating free sterol (55.4–61.5%, Table 4). Sitostanol and campestanol represented 9.5% of total free sterols/stanols in corn, 10.6% in rye, 16.3% in wheat, and 15.5% in spelt. The amounts of stigmasterol in corn and rye were higher compared to campestanol; in wheat and spelt the amounts of campestanol were higher. Cholesterol was present in all cereals and made up 0.7–1.2% of free sterols/stanols. Comparable to the fatty acid esters, rye contained the highest amounts of free sterols/stanols in the lipid extract and corn the highest contents based on ground kernels. The levels of free sterols/stanols in corn, wheat, and spelt were comparable to those previously reported.[17,24]

Plant Steryl/Stanyl Phenolic Acid Esters. Up to 16 intact phenolic acid esters were identified in the investigated cereal samples. The contents of individual steryl and stanyl phenolic acid esters are presented in Table 5. The highest total amounts were determined in corn grains and were mainly composed of steryl and stanyl ferulic acid esters, representing 97.6% of total phenolic acid esters. Additionally, small amounts of coumarates occurred in all samples. Steryl and stanyl coumarates have been described in corn and corn bran as minor components, but to the authors' knowledge not in other cereals.[30,31,41] In this study, campestanyl and sitostanyl-*p*-coumarate could also be identified in rye, wheat, and spelt grains. The contents of coumarates were very low; however, this is the first report on the occurrence of these particular esters in cereals other than corn.

In accordance with data from previous studies, the contents of sitosteryl/sitostanyl ferulate in corn were higher compared to those of campesteryl/campestanyl ferulate; in the samples of rye, wheat, and spelt the esters of campesterol and campestanol are dominating.[27,29,30] Wheat contained the highest total amounts of phenolic acid esters in the oil extract and corn the highest level based on ground kernels. The phenolic acids occurred predominantly as esters of stanols (74.6–90.8%), whereas in fraction A 79.0–85.5% of the fatty acids were esterified to sterols. The employed GC analysis also allowed the determination of *cis* derivatives of steryl and stanyl ferulates. The *cis*/*trans* ratios were 0.64, 0.17, 0.05, and 0.13 for corn, rye,

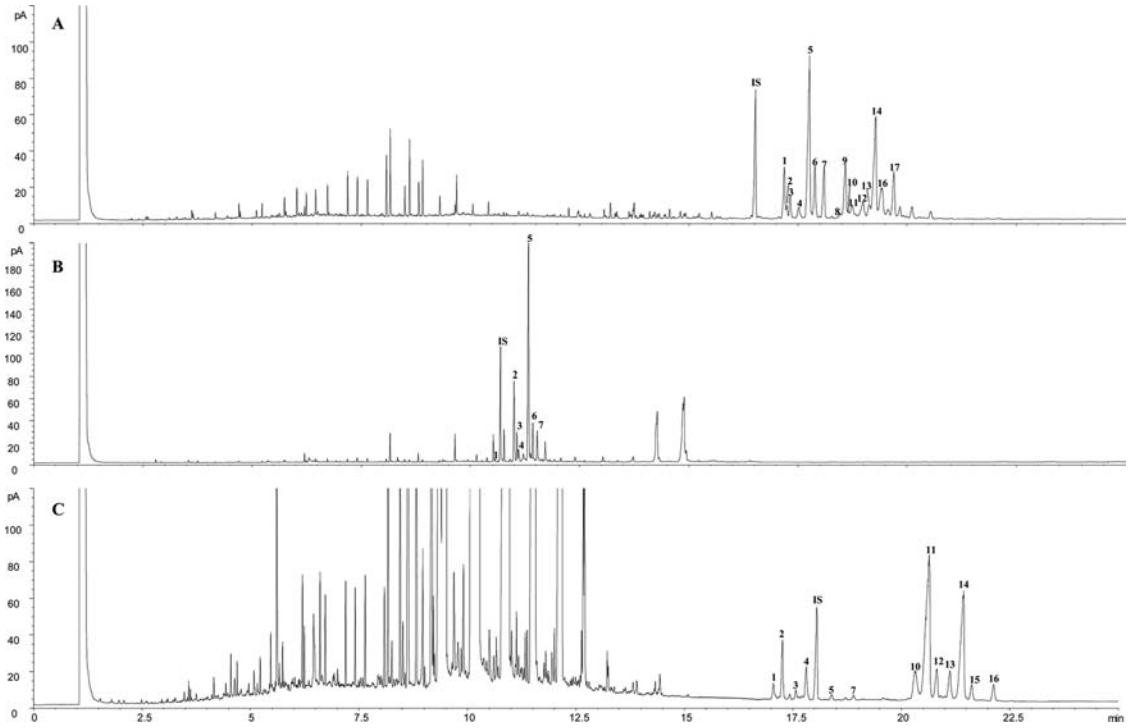

Figure 4. Capillary gas chromatographic analysis of (A) steryl/stanyl fatty acid esters, (B) free sterols and stanols, and (C) steryl/stanyl phenolic acid esters obtained from ground rye kernels. Peak numbers correlate to Tables 3–5. (IS) internal standard: (A) cholesteryl palmitate; (B) cholestanol; (C) cholestanyl-*p*-coumarate.

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Table 3. Contents of Steryl/Stanyl Fatty Acid Esters (A) Determined in the Investigated Cereals

		corn		rye		wheat		spelt	
steryl/stanyl fatty acid esters		μg/g of dm[a]	μg/100 mg of oil	μg/g of dm	μg/100 mg of oil	μg/g of dm	μg/100 mg of oil	μg/g of dm	μg/100 mg of oil
1[b]	campesteryl-16:0/16:1[c]	4.2 ± 0.2[f]	11.7 ± 0.5	25.0 ± 0.5	171.0 ± 0.9	27.3 ± 0.5	161.5 ± 3.9	37.0 ± 0.5	149.6 ± 4.0
2	stigmasteryl-16:0/16:1[c]	1.7 ± 0.1	4.8 ± 0.2	6.5 ± 0.2	44.1 ± 1.4	1.4 ± 0.1	8.2 ± 0.4	2.9 ± 0.3	11.9 ± 1.2
3	campestanyl-16:0/16:1[c,d]	1.4 ± 0.1	3.8 ± 0.3	13.1 ± 0.2	90.0 ± 2.5	13.9 ± 0.3	82.3 ± 2.2	20.5 ± 0.4	83.0 ± 0.6
4	Δ^7-campesteryl-16:0/16:1[c,d]	–[g]	–	8.9 ± 0.7	60.8 ± 4.2	3.4 ± 0.2	19.9 ± 1.4	4.2 ± 0.4[i]	16.8 ± 1.3[i]
5	sitosteryl-16:0/16:1[c]	13.0 ± 0.8	36.5 ± 2.3	106.3 ± 0.3	727.4 ± 9.1	111.1 ± 1.3	657.0 ± 7.0	175.1 ± 1.0	707.9 ± 12.8
6	sitostanyl-16:0/16:1[c]	5.2 ± 0.2	14.5 ± 0.5	26.3 ± 0.4	179.9 ± 1.4	22.1 ± 0.3	130.9 ± 1.4	33.7 ± 1.8	136.4 ± 8.9
7	Δ^7-sitosteryl-16:0[c,d]	3.2 ± 0.1	9.1 ± 0.2	26.6 ± 0.6	181.9 ± 3.1	3.3 ± 0.1	19.3 ± 0.3	4.7 ± 0.2	19.1 ± 0.5
8	campesteryl-18:0/18:1[e]	12.8 ± 2.0	35.7 ± 4.9	2.6 ± 0.8	17.9 ± 5.2	1.7 ± 0.5	10.2 ± 2.9	6.2 ± 0.1	25.1 ± 0.7
9	stigmasteryl-18:0/18:1[e] + campesteryl-18:2	96.2 ± 1.6	269.4 ± 4.2	39.6 ± 0.7	270.8 ± 3.4	27.6 ± 0.2	163.2 ± 1.5	37.2 ± 0.1	150.3 ± 1.7
10	stigmasteryl-18:2 + campestanyl-18:0/18:1[e]	35.5 ± 0.3	99.5 ± 2.7	3.6 ± 0.3	24.6 ± 1.8	3.4 ± 0.2	20.4 ± 1.6	6.7 ± 0.4	27.2 ± 1.3
11	campestanyl-18:2	17.7 ± 0.3	49.6 ± 0.8	9.6 ± 0.5	65.8 ± 3.1	9.0 ± 0.3	53.3 ± 0.9	12.0 ± 0.1	48.4 ± 0.8
12	Δ^7-campesteryl-18:2[d]	4.8 ± 0.2	13.3 ± 0.8	16.4 ± 0.4	112.5 ± 2.5	6.2 ± 0.2	36.8 ± 1.0	8.7 ± 0.8	35.0 ± 3.1
13	sitosteryl-18:0/18:1[e]	22.8 ± 0.7	63.8 ± 1.9	20.6 ± 0.7	140.7 ± 5.7	18.1 ± 0.1	107.0 ± 1.3	31.1 ± 0.4	125.7 ± 3.4
14	sitosteryl-18:2	341.0 ± 4.8	954.9 ± 24.9	86.5 ± 0.8	591.9 ± 3.6	80.9 ± 1.7	478.2 ± 5.7	114.5 ± 0.8	462.7 ± 3.0
15	sitostanyl-18:0/18:1[e]	9.7 ± 1.9	27.0 ± 5.2	–	–	5.2 ± 0.3	30.9 ± 2.2	13.8 ± 0.9	55.9 ± 3.3
16	sitostanyl-18:2	60.5 ± 0.9	169.4 ± 2.8	36.9 ± 0.9[h]	252.4 ± 5.2[h]	26.2 ± 0.2	154.9 ± 1.5	37.1 ± 0.8	150.1 ± 4.1
17	Δ^7-sitosteryl-18:2[d]	20.8 ± 0.4	58.3 ± 1.0	30.9 ± 0.1	211.7 ± 2.7	8.9 ± 0.1	52.6 ± 0.1	12.1 ± 0.3	48.7 ± 1.4
total fatty acid esters		650.6 ± 9.0	1821.5 ± 35.2	459.3 ± 6.2	3143.3 ± 36.0	369.9 ± 5.2	2186.8 ± 23.4	557.6 ± 2.6	2253.9 ± 36.9

[a]Based on dry matter (dm) of ground kernels. [b]Peak numbering according to Figures 2A and 4A. [c]Ratio of palmitic acid to palmitoleic acid was determined as fatty acid methyl esters after methanolysis of the respective steryl ester fraction: corn, 30:1; rye, 55:1; wheat, 37:1; and spelt, 30:1. [d]Compound calculated with Rf = 1. [e]Ratio of stearic acid to oleic acid was determined as fatty acid methyl esters after methanolysis of the respective steryl ester fraction: corn, 1:4; rye, 1:6; wheat, 1:4; and spelt 1:5. [f]All contents are the average of three values ± standard deviation. [g](–) content below LOD (Table 2). [h]Sitostanyl-18:2 and coeluting sitosteryl-18:3. [i]Δ^7-Campesteryl-16:0 and unidentified steryl ester.

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Table 4. Contents of Free Sterols and Stanols (B) Determined in the Investigated Cereals

		corn		rye		wheat		spelt	
free sterols/stanols		μg/g of dm[a]	μg/100 mg of oil	μg/g of dm	μg/100 mg of oil	μg/g of dm	μg/100 mg of oil	μg/g of dm	μg/100 mg of oil
1[b]	cholesterol	2.7 ± 0.7[c]	7.5 ± 1.9	1.8 ± 0.2	12.3 ± 1.8	2.7 ± 0.4	16.1 ± 2.2	2.3 ± 0.2	9.1 ± 0.9
2	campesterol	69.8 ± 2.9	195.3 ± 4.3	54.1 ± 0.4	370.5 ± 7.3	38.1 ± 0.7	225.2 ± 3.9	37.0 ± 0.2	149.4 ± 2.3
3	stigmasterol	25.2 ± 1.1	70.4 ± 1.8	14.4 ± 0.3	98.3 ± 2.3	6.8 ± 0.1	40.5 ± 0.1	6.9 ± 0.0	28.0 ± 0.5
4	campestanol	11.5 ± 0.5	32.3 ± 0.7	8.5 ± 0.2	57.8 ± 0.5	13.5 ± 0.4	80.0 ± 3.6	11.6 ± 0.3	46.9 ± 1.1
5	sitosterol	238.7 ± 9.5	668.1 ± 13.8	145.1 ± 1.8	992.7 ± 12.9	136.8 ± 3.7	808.7 ± 16.4	131.7 ± 1.5	532.4 ± 12.0
6	sitostanol	25.5 ± 0.6	71.3 ± 1.1	19.4 ± 0.9	132.7 ± 6.0	23.1 ± 0.8	136.8 ± 3.5	21.1 ± 0.8	85.4 ± 4.1
7	others[d]	15.0 ± 1.7	41.8 ± 4.1	18.4 ± 0.3	126.0 ± 2.9	3.7 ± 0.2	21.6 ± 1.0	3.7 ± 0.5	15.1 ± 2.2
total free sterols/stanols		388.3 ± 15.8	1086.7 ± 23.9	261.6 ± 3.7	1790.4 ± 12.9	224.8 ± 4.7	1328.8 ± 18.7	214.3 ± 2.4	866.3 ± 20.9

[a]Based on dry matter (dm) of ground kernels. [b]Peak numbering according to Figures 2B and 4B. [c]All contents are the average of three values ± standard deviation. [d]Tentatively identified as Δ^7-sitosterol and unidentified sterol.

Table 5. Contents of Steryl/Stanyl Phenolic Acid Esters (C) Determined in the Investigated Cereals

		corn		rye		wheat		spelt	
steryl/stanyl phenolic acid esters		μg/g of dm[a]	μg/100 mg of oil	μg/g of dm	μg/100 mg of oil	μg/g of dm	μg/100 mg of oil	μg/g of dm	μg/100 mg of oil
6[b]	*trans*-campesteryl-*p*-coumarate	<LOQ[c]		–[d]	–	–	–	–	–
7	*trans*-campestanyl-*p*-coumarate	1.0 ± 0.1[e]	2.9 ± 0.3	0.5 ± 0.0	3.5 ± 0.2	1.7 ± 0.2	10.3 ± 1.3	<LOQ	
8	*trans*-sitosteryl-*p*-coumarate	<LOQ		–	–	–	–	–	–
9	*trans*-sitostanyl-*p*-coumarate	2.7 ± 0.2	7.7 ± 0.8	0.3 ± 0.0	1.8 ± 0.2	0.3 ± 0.0	2.0 ± 0.2	<LOQ	
1	*cis*-campesteryl ferulate	<LOQ		1.9 ± 0.1	13.2 ± 0.9	0.9 ± 0.0	5.2 ± 0.3	1.5 ± 0.1	6.0 ± 0.3
10	*trans*-campesteryl ferulate	2.8 ± 0.1	7.7 ± 0.8	6.5 ± 0.6	44.6 ± 3.5	11.6 ± 0.2	68.6 ± 2.5	6.8 ± 0.8	27.6 ± 2.9
2	*cis*-campestanyl ferulate	23.4 ± 3.1	65.5 ± 8.6	5.7 ± 0.2	39.0 ± 1.7	3.0 ± 0.3	18.0 ± 1.7	4.7 ± 0.2	19.0 ± 1.2
11	*trans*-campestanyl ferulate	21.7 ± 0.6	60.8 ± 1.4	34.5 ± 1.8	236.2 ± 9.4	53.0 ± 2.0	313.5 ± 14.5	35.7 ± 1.3	144.4 ± 3.4
12	*trans*-Δ^7-campesteryl ferulate	1.5 ± 0.4	4.2 ± 1.2	4.3 ± 0.3	29.3 ± 1.6	2.1 ± 0.2	12.3 ± 1.2	1.5 ± 0.1	6.1 ± 0.5
3	*cis*-sitosteryl ferulate	2.7 ± 0.3	7.5 ± 0.9	0.9 ± 0.0	6.0 ± 0.3	<LOQ		<LOQ	
13	*trans*-sitosteryl ferulate	3.1 ± 0.5	8.5 ± 1.5	4.7 ± 0.5	32.4 ± 2.8	6.4 ± 0.1	37.6 ± 1.0	4.4 ± 0.6	18.0 ± 2.2
4	*cis*-sitostanyl ferulate	35.4 ± 5.8	99.1 ± 15.5	4.0 ± 0.2	27.4 ± 1.3	2.2 ± 0.1	13.2 ± 0.7	4.6 ± 0.2	18.7 ± 0.6
14	*trans*-sitostanyl ferulate	58.5 ± 1.0	164.5 ± 2.2	23.6 ± 1.5	161.4 ± 9.3	39.9 ± 1.4	236.0 ± 10.7	31.8 ± 3.2	128.3 ± 12.2
15	*trans*-Δ^7-sitosteryl ferulate	4.4 ± 1.1	12.3 ± 3.2	2.1 ± 0.1	14.4 ± 0.6	1.5 ± 0.1	8.9 ± 0.5	1.3 ± 0.0	5.1 ± 0.1
5	*cis*-24-methylene-cycloartanyl ferulate	–	–	0.7 ± 0.1	4.7 ± 0.6	<LOQ		–	–
16	*trans*-24-methylene-cycloartanyl ferulate	<LOQ		2.2 ± 0.2	15.4 ± 1.8	1.5 ± 0.1	8.6 ± 0.8	<LOQ	
total phenolic acid esters		157.5 ± 13.3	441.0 ± 35.8	92.0 ± 5.1	629.2 ± 29.0	124.2 ± 4.1	734.3 ± 31.0	92.4 ± 5.9	373.2 ± 21.5

[a]Based on dry matter (dm) of ground kernels. [b]Peak numbering according to Figures 2C and 4C. [c]Content below LOQ (Table 2). [d](–) content below LOD (Table 2). [e]All contents are the average of three values ± standard deviation.

wheat, and spelt, respectively. Comparably high *cis*/*trans* ratios in cereals have been reported earlier.[42] It has been shown that the *trans* forms of phenolic acids can be partially converted into their *cis* forms by ultraviolet light and daylight.[43,44] Therefore, the sample preparation was performed protected from daylight, and during the analysis no isomerization of the internal standard cholestanyl-*p*-coumarate was observed. Steryl and stanyl ferulates were determined, among others, by Nurmi et al. via RP-HPLC.[29] Thereby, the total amounts of steryl/stanyl ferulates were 65–74 μg/g in rye and 74–114 μg/g in wheat and were a little lower compared to the investigated samples. The determined amounts of phenolic acid esters in corn were in agreement with previous data.[17]

In conclusion, the described solid-phase extraction method enables the effective isolation of free sterols/stanols, steryl/stanyl fatty acid esters, and steryl/stanyl phenolic acid esters from corn, rye, wheat, and spelt grain oils. The analysis of intact steryl/stanyl esters by means of capillary gas chromatography provides detailed information on the profiles and contents of individual esters. The methodology revealed quantitative differences between the patterns of fatty acid and phenolic acid esters in corn compared to rye, wheat, and spelt.

The approach should be a suitable tool for future investigations of the natural variability of individual phytosterol/phytostanol conjugates in various types of grain and could, for example, be employed to investigate the phenotypic plasticity of sterol patterns as a response to environmental factors.

AUTHOR INFORMATION

Corresponding Author

*Phone: +49 8161 714250. Fax: +49 8161 714259. E-mail: k.h.engel@wzw.tum.de.

Notes

The authors declare no competing financial interest.

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