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Online LC−GC Analysis of Free Sterols/Stanols and Intact Steryl/Stanyl Esters in Cereals

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ABSTRACT: The suitability of online liquid chromatography−gas chromatography for the analysis of free sterols/stanols, steryl/stanyl fatty acid esters, and *trans*-steryl/stanyl ferulic acid esters in cereals is demonstrated. The silylated lipid extracts were fractionated via liquid chromatography on a normal phase, and the fractions containing the sterol classes were transferred online to the gas chromatograph for the analysis of their individual compositions. The study provides for the first time data on free sterols/stanols and intact steryl/stanyl esters in sweet corn, popcorn, and proso millet. Sweet corn revealed the highest contents of free sterols/stanols and steryl/stanyl fatty acid esters, and popcorn, in turn, the highest amounts of *trans*-steryl/stanyl ferulic acid esters. The distribution patterns of the proso millet samples revealed pronounced differences from those of sweet corn and popcorn as they particularly exhibited high proportions of free cholesterol and cholesteryl fatty acid esters. Furthermore, no *trans*-steryl/stanyl ferulic acid esters could be detected.

KEYWORDS: *free sterols/stanols, steryl/stanyl esters, sweet corn, popcorn, proso millet, online LC−GC*

INTRODUCTION

Phytosterols and their conjugates, that is, fatty acid esters, phenolic acid esters, glycosides, and acylated glycosides, are minor lipids and well-known for their cholesterol-lowering properties and other health benefits.[1,2] Cereals are important natural sources of these compounds, and depending on population and gender they can contribute up to 42% of the total daily intake of phytosterols.[3] Thus, it is of great interest to establish analytical methods that provide qualitative and quantitative data on the naturally occurring phytosterol classes. However, as some lipid constituents, mainly triglycerides, hamper the direct chromatographic analysis of intact steryl/stanyl esters, a preseparation of cereal lipids is essential. Recently, the suitability of an approach based on solid-phase extraction (SPE) for the fractionation of cereals lipids into free sterols/stanols, steryl/stanyl fatty acid esters, and steryl/stanyl phenolic acid esters has been demonstrated.[4] The individual compositions of the obtained sterol fractions were determined via capillary gas chromatography (GC). The proposed method was successfully applied to the investigation of these compounds in corn, rye, wheat, and spelt kernels; in particular, considerable differences in contents and compositions of steryl/stanyl esters could be detected.[4,5]

A sophisticated and efficient alternative to laborious offline sample preparation steps, for example, SPE, thin layer chromatography, or column chromatography, is the online coupling of liquid chromatography and gas chromatography (online LC−GC). The LC-step is used for cleanup, fractionation, and preconcentration of the sample, and the LC-fraction of interest can be transferred online to the GC for subsequent analysis of the individual composition. By using an online LC−GC system, the fractionation and analysis take place in a closed and automated system, whereby the risk of sample loss and contamination is reduced. Online LC−GC has already been successfully applied to the analysis of sterols and steryl fatty acid esters in oils and fats, of steryl/stanyl ferulates in rice, or of steryl/stanyl fatty acid esters in enriched fat-based foods.[6−13]

The aim of the present study was to establish such a method for the analysis of free sterols/stanols, steryl/stanyl fatty acid esters, and steryl/stanyl phenolic acid esters in cereals, using two corn subspecies, sweet corn (*Zea mays* convar. *saccharata* KOERN.) and popcorn (*Zea mays* convar. *microsperma* KOERN.), as well as seeds of proso millet (*Panicum miliaceum* L.) as examples. Representative structures of the analyzed free sterols/stanols, steryl/stanyl fatty acid esters, and steryl/stanyl phenolic acid esters are shown in Figure 1.

MATERIALS AND METHODS

Sample Material. Fresh sweet corn cobs (distributors: Vitafrisch Gemüse Vertrieb eG, Neckarsulm, Germany (1); Willi Sinn GmbH, Maxdorf, Germany (2); Schweiger's Früchte, Freising, Germany (3); farmer's store, Hallbergmoos, Germany (4)), prepacked popcorn kernels (Seeberger KG, Ulm, Germany (1); Krini GmbH, Weinstadt, Germany (2); Müller's Mühle GmbH, Gelsenkirchen-Schalke, Germany (3); EfeFirat Feinkost GmbH, Achim, Germany (4)), and one hulled proso millet sample (real,-BIO, real,-SB-Warenhaus GmbH, Germany) were purchased in local stores (Freising, Germany). The sweet corn cobs have been harvested in 2011; no other botanical or agronomic information was available for these materials. Additionally, hulled seeds of three proso millet cultivars were provided by PrimaVera Naturkorn GmbH (Mühldorf, Germany): Huangmi, (China, harvested in 2010), Kornberger (Austria, harvested in 2010), and Kornberger (Austria, harvested in 2011).

Chemicals. Cholesteryl palmitate (≥98%), cholesteryl laurate (97%), 5α-cholestan-3β-ol (∼95%), stigmasterol (∼95%), sitostanol (∼95%), *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) + 1%

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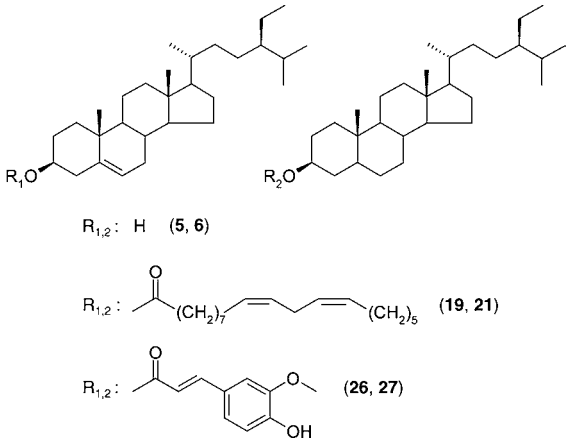

Figure 1. Structures of representative free sterols/stanols, steryl/stanyl fatty acid esters, and steryl/stanyl ferulic acid esters: (**5**) sitosterol, (**6**) sitostanol, (**19**) sitosteryl-18:2, (**21**) sitostanyl-18:2, (**26**) *trans*-sitosteryl ferulate, and (**27**) *trans*-sitostanyl ferulate. The numbers correspond to those shown in Figures 2 and 3.

trimethylchlorosilane (TMCS), dichloromethane (95%), 2-propanol (CHROMASOLV, for HPLC), and pyridine (99.8%) were obtained from Sigma-Aldrich (Steinheim, Germany). *n*-Hexane (AnalR NORMAPUR and HiPerSolv CHROMANORM) were purchased from VWR International (Darmstadt, Germany, https://de.vwr.com/app/Home). Methyl *tert*-butyl ether (MTBE) was supplied by Evonik Industries AG (Essen, Germany) and was distilled prior to use. A mixture of phytosteryl/phytostanyl fatty acid esters (Vegapure 95E) was provided by Cognis GmbH (Illertissen, Germany). Reference compounds for steryl/stanyl fatty acid esters and steryl/stanyl phenolic acid esters were synthesized according to previously described procedures.[4,14]

Milling and Lipid Extraction. The kernels from 6−8 sweet corn cobs were manually removed and dried in a cabinet dryer at 40 °C for 3 d. About 200 g of the dried sweet corn kernels, popcorn kernels, and seeds of proso millet, respectively, was frozen in liquid nitrogen and ground using a model 1093 cyclone mill equipped with a 500 μm sieve (Cyclotec, Foss, Germany). After freeze-drying of the flours for 48 h, they were stored in plastic bags at −18 °C until analysis.

To 4 g of each flour were added the internal standards (IS) 5α-cholestan-3β-ol (500 μL of 1.0 mg/mL in MTBE) and cholestanyl ferulate (100 μL of 1.0 mg/mL in MTBE). Furthermore, cholesteryl palmitate (500 μL of 1.0 mg/mL in *n*-hexane/MTBE (3:2, v/v)) in case of sweet corn and popcorn or cholesteryl laurate (500 μL of 1.0 mg/mL in *n*-hexane/MTBE (3:2, v/v)) in case of proso millet were applied as IS for the quantitation of steryl/stanyl fatty acid esters. The lipids were extracted with *n*-hexane/dichloromethane (40 mL, 1:1, v/v) under stirring for 1 h at room temperature as previously described.[4] One hundred milligrams of the obtained oil was dissolved in 10 mL of *n*-hexane. An aliquot of that solution (250 μL) was evaporated to dryness by a gentle stream of nitrogen, and the residue was silylated with 75 μL of pyridine and 150 μL of BSTFA:TMCS (99:1) at 80 °C for 20 min. After removal of the silylation reagents by a gentle stream of nitrogen, the residue was dissolved in 250 μL of *n*-hexane/MTBE/2-propanol (96:4:0.1, v/v/v) and used for online LC−GC analysis.

Analysis by Online LC−GC. The online coupling of a 1220 Infinity LC (Agilent Technologies, Waldbronn, Germany) to a 7890A GC (Agilent Technologies) equipped with a flame ionization detector was performed via a 1200 Infinity Series 2-position/6-port switching valve (Agilent Technologies) fitted with a 200 μL sample loop.

LC-separations were carried out on a 250 × 2 mm, 5 μm, Eurospher-100 Si column (Knauer, Berlin, Germany) at 27 °C using *n*-hexane/MTBE/2-propanol (96:4:0.1, v/v/v) as eluent. Ultraviolet detection was performed at 205 nm for free sterols/stanols and steryl/stanyl fatty acid esters and at 325 nm for steryl/stanyl phenolic acid esters. The transfer valve was switched for transfer 1 (free sterols/stanols and steryl/stanyl fatty acid esters; flow rate, 0.25 mL/min; injection volume, 5 μL) 3.7 min after injection and for transfer 2 (*trans*-steryl/stanyl ferulic acid esters; flow rate, 0.20 mL/min; injection volume, 20 μL) 6.75 min after injection. Evaporation of the solvent was performed via the programmable multimode inlet in the solvent vent mode. The injector was set to 50 °C hold for 0.5 min and was then heated with 900 °C/min to 350 °C. Vent flow was adjusted to 1000 mL/min with a vent pressure of 4 psi until 0.5 min. Purge flow to split vent was started at 0.5 min with 2.5 mL/min. The stainless steel transfer line installed between the valve and the inlet was pressure-controlled to avoid a pushing back of solvent vapors into the transfer line. The pressure was set to 5 psi hold for 0.3 min followed by a ramp of 10 psi/min up to 20 psi. GC-separations were carried out on a 30 m × 0.25 mm i.d. fused-silica capillary column coated with a film of 0.1 μm Rtx-200MS trifluoropropylmethyl polysiloxane (Restek GmbH, Bad Homburg, Germany). Hydrogen was used as carrier gas with a constant flow rate of 1.5 mL/min. The oven temperature program was as follows: initial temperature, 40 °C (2 min); programmed at 100 °C/min to 100 °C, then at 15 °C/min to 310 °C (2 min), and at 1.5 °C/min to 340 °C (3 min). The detector temperature was set to 360 °C. Nitrogen was used as makeup gas with a flow rate of 25 mL/min. Data acquisition was performed by ChemStation B.04.03 software.

Quantitation by Online LC−GC. Free sterols/stanols were quantitated as their trimethylsilyl (TMS)-derivatives using a response factor (*Rf*) of 1.0 relative to the IS 5α-cholestan-3β-ol. At first, the responses of individual steryl/stanyl esters were determined within the calibration range of 0.1−0.5 μg/μL using a five-point calibration. The GC-FID detector was also shown to be linear up to an additionally examined concentration of 0.8 μg/μL. On the basis of these results, steryl/stanyl fatty acid esters were subsequently quantitated by generating three-point calibration functions with 0.1, 0.3, and 0.5 μg of total esters (Vegapure 95E) per microliter of injection. Each calibration point was analyzed in triplicate, and linear regression was confirmed in ratio of areas (area steryl or stanyl ester/area IS) and amounts (amount steryl or stanyl ester/amount IS). Steryl/stanyl esters that were not included in the Vegapure 95E mixture were quantitated with an *Rf* of 1.0 in relation to the IS. The TMS-derivatives of *trans*-steryl/stanyl ferulates were determined using the following *Rf* values related to cholestanyl ferulate (IS): 1.01 for stanyl ferulates and 1.15 for steryl ferulates.

Identification by GC−MS. LC-fractions containing the steryl/stanyl conjugates were manually collected after visualization at 205 and 325 nm, respectively, and subjected to GC−MS. GC−MS analyses and identifications were carried out on a Finnigan Trace GC ultra coupled with a Finnigan Trace DSQ mass spectrometer (Thermo Electro Corp., Austin, TX) as previously described.[4] Data acquisition was performed by Xcalibur 1.4 SR1 software.

Identification by Online LC−GC−MS. Online LC−GC−MS analysis was performed by coupling the GC part via a transfer line to a 5975C inert mass spectrometer (Agilent Technologies, Waldbronn, Germany). Mass spectra were obtained by positive electron impact ionization at 70 eV in the scan mode at unit resolution from 40 to 700 Da. The interface was heated to 280 °C, the ion source to 250 °C, and the quadrupole to 150 °C. GC separations were carried out on a 30 m × 0.25 mm i.d., 0.1 μm film Rtx-200MS fused silica capillary column (Restek, Bad Homburg, Germany). The remaining GC conditions were as described for online LC−GC analysis. Data acquisition was performed by MSD Productivity ChemStation E.02.02 software.

Validation of the Online LC−GC-Based Approach. Recoveries were determined by spiking 4 g of flour (corn and proso millet, respectively) with defined amounts of selected steryl/stanyl derivatives: 500 μg of stigmasteryl palmitate and sitostanyl linoleate, respectively, as reference compounds for steryl/stanyl fatty acid esters; 500 μg of stigmasterol and sitostanol, respectively, as reference compounds for free sterols/stanols; and 100 μg of sitostanyl ferulate as reference compound for *trans*-steryl/stanyl ferulic acid esters. The limits of detection (LOD) and the limits of quantitation (LOQ) were determined via the linear regression methodology according to German standard procedures and criteria,[15] and were calculated on

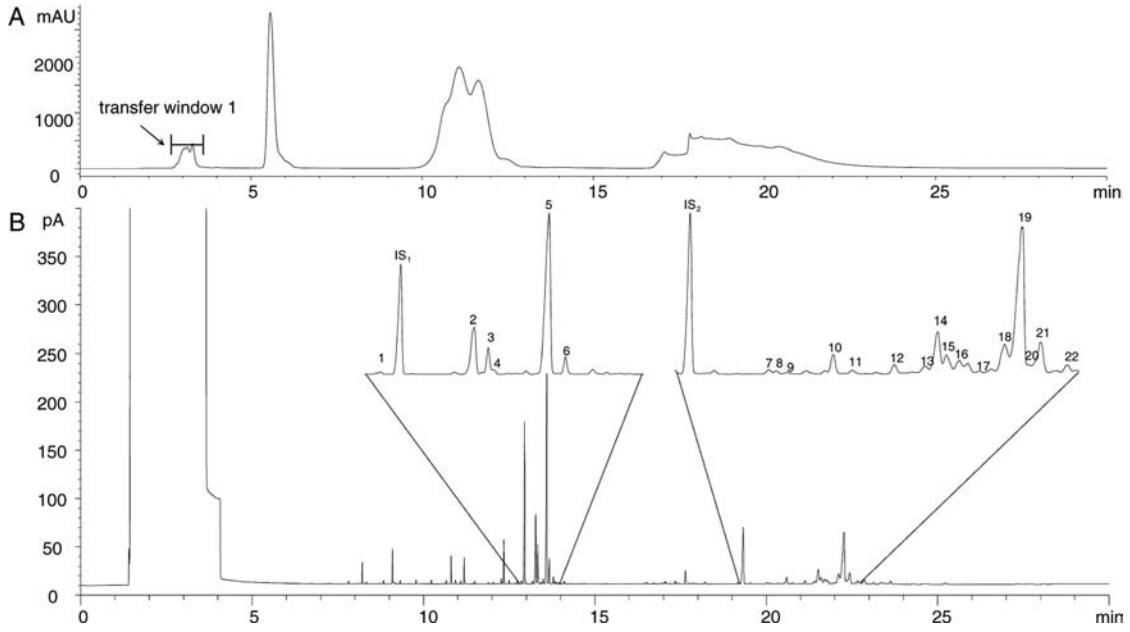

Figure 2. Online LC−GC analysis of free sterols/stanols and steryl/stanyl fatty acid esters in popcorn (sample 1). (A) LC-chromatogram at 205 nm; (B) GC-chromatogram of the transferred LC-fraction. (1) Cholesterol, (2) campesterol, (3) stigmasterol, (4) campestanol, (5) sitosterol, (6) sitostanol, (7) campesteryl-16:0/16:1, (8) stigmasteryl-16:0/16:1, (9) campestanyl-16:0/16:1, (10) sitosteryl-16:0/16:1, (11) sitostanyl-16:0/16:1, (12) Δ^7 sitosteryl-16:0/16:1, (13) campesteryl-18:0/18:1, (14) campesteryl-18:2 + stigmasteryl-18:0/18:1, (15) campestanyl-18:0/18:1 + stigmasteryl-18:2, (16) campestanyl-18:2, (17) Δ^7 campesteryl-18:2, (18) sitosteryl-18:0/18:1, (19) sitosteryl-18:2, (20) sitostanyl-18:0/18:1, (21) sitostanyl-18:2, (22) Δ^7 sitosteryl-18:2, (IS_1) 5α-cholestan-3β-ol, and (IS_2) campesteryl-16:0.

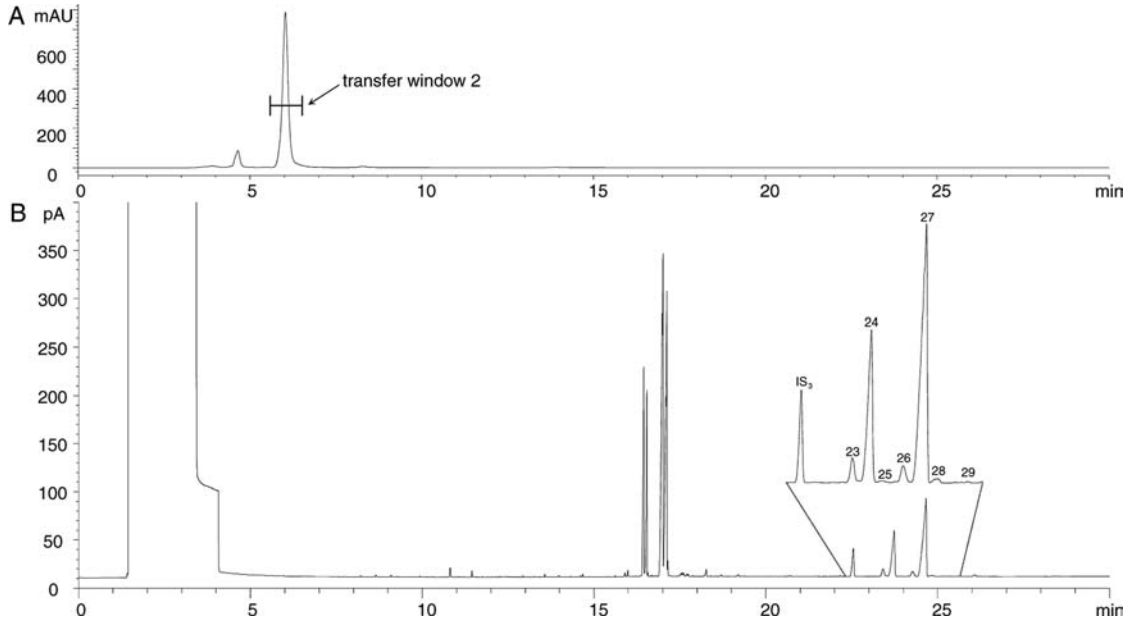

Figure 3. Online LC−GC analysis of *trans*-steryl/stanyl ferulic acid esters in popcorn (sample 1). (A) LC-chromatogram at 325 nm; (B) GC-chromatogram of the transferred LC-fraction. (23) *trans*-Campesteryl ferulate, (24) *trans*-campestanyl ferulate, (25) *trans*-Δ^7 campesteryl ferulate, (26) *trans*-sitosteryl ferulate, (27) *trans*-sitostanyl ferulate, (28) *trans*-Δ^7 sitosteryl ferulate, (29) *trans*-24-methylene cycloartanyl ferulate, and (IS_3) cholestanyl ferulate.

the basis of the respective injection volume (5 or 20 μL). Each regression analysis was performed in triplicate. Additionally, standard solutions were prepared in the range of LOQ−500 μg/mL for selected references of free sterols/stanols (sitosterol and sitostanol) and steryl/stanyl fatty acid esters (stigmasteryl palmitate and sitostanyl linoleate) and in the range of LOQ−250 μg/mL for *trans*-sitostanyl ferulate. The GC-FID responses were linear over the measured concentration ranges (coefficients of determination (R^2) > 0.9993). The repeatability and the stability of the approach were confirmed by working up control samples (corn flour and proso millet flour, respectively) once on each day of analysis (in total, five replicates of each material). The reproducibility in terms of interlaboratory precision has not been assessed.

RESULTS AND DISCUSSION

Online LC−GC Analysis. The lipids extracted from the ground kernels were silylated and subsequently fractionated via

Table 1. Recoveries, LODs, and LOQs of Selected Steryl/Stanyl Derivatives

	recovery [%][a]			
	corn	proso millet	LOD [μg/mL][b]	LOQ [μg/mL][b]
stigmasterol	103.8 ± 1.0	92.7 ± 2.9	0.02	0.03
sitostanol	95.1 ± 2.3	98.5 ± 1.8	0.03	0.09
stigmasteryl palmitate	101.5 ± 0.4	95.4 ± 2.9	0.18	0.38
sitostanyl linoleate	83.3 ± 8.8	86.1 ± 3.1	0.14	0.29
sitostanyl ferulate	94.5 ± 1.2	n.d.[c]	0.13	0.28

[a]Values represent the mean ± standard deviation (n = 3). [b]Limit of detection (LOD) and limit of quantitation (LOQ) expressed as μg/mL of respective injection volume (5 μL for free sterols/stanols and steryl/stanyl fatty acid esters; 20 μL for *trans*-steryl/stanyl ferulic acid esters). [c]Not determined (n.d.).

LC on a silica gel column using *n*-hexane/MTBE/2-propanol (96:4:0.1, v/v/v) as eluent. The transfer times of the different sterol classes were determined using reference compounds, and the respective fractions were transferred online by switching the transfer valve to the GC. Under the employed LC-conditions, the TMS-derivatives of free sterols/stanols and the steryl/stanyl fatty acid esters eluted at the same time and could thus be transferred together to the GC (Figure 2). The applied online LC−GC system was equipped with a PTV interface for the effective evaporation of the solvent, which is transferred from LC to GC. GC separations were carried out on a medium polar capillary column, previously shown to be suitable for the analysis of a wide range of naturally occurring free sterols/stanols and steryl/stanyl esters.[4]

In addition to the steryl/stanyl fatty acid esters, the corresponding phenolic acid esters, also known to be present in certain cereals, should be transferred in a second step.[16−19] However, the LC-separation of the *trans*-steryl/stanyl coumarates and the *cis*-steryl/stanyl ferulates from other matrix constituents was not sufficient. They eluted in a merged peak closely after steryl/stanyl fatty acid esters and free sterols/stanols, so that the online transferred fraction always contained small amounts of cotransferred steryl/stanyl fatty acid esters. Variations of the polarity of the mobile phase did not yield

Table 2. Contents and Compositions of Free Sterols/Stanols and Steryl/Stanyl Esters in Four Commercial Sweet Corn Samples[a]

	1	2	3	4
oil content [%]	8.83 ± 0.21	10.77 ± 0.24	8.97 ± 0.12	10.50 ± 0.20
total free sterols/stanols [μg/g dm[b]]	993.1 ± 15.7	1049.1 ± 10.2	870.7 ± 11.6	946.5 ± 20.9
total free sterols/stanols [μg/100 mg oil]	1125.5 ± 17.5	974.3 ± 31.1	971.2 ± 4.7	901.6 ± 27.7
sitosterol [%]	59.7 ± 0.5	62.6 ± 0.8	61.7 ± 0.6	59.3 ± 0.5
campesterol [%]	22.4 ± 0.2	19.7 ± 0.3	20.8 ± 0.2	22.6 ± 0.2
stigmasterol [%]	7.8 ± 0.1	6.6 ± 0.3	7.4 ± 0.3	6.8 ± 0.1
sitostanol [%]	2.3 ± 0.0	3.7 ± 0.1	3.7 ± 0.1	6.1 ± 0.3
campestanol [%]	1.1 ± 0.1	1.5 ± 0.3	1.5 ± 0.0	2.6 ± 0.1
cholesterol [%]	1.1 ± 0.2	0.9 ± 0.1	0.7 ± 0.1	0.6 ± 0.1
others [%]	5.6 ± 0.1	5.1 ± 0.1	4.3 ± 0.6	1.9 ± 0.3
total steryl/stanyl fatty acid esters [μg/g dm[b]]	822.6 ± 35.3	1067.8 ± 20.0	905.1 ± 27.8	932.4 ± 52.4
total steryl/stanyl fatty acid esters [μg/100 mg oil]	932.1 ± 40.2	991.3 ± 20.7	1009.5 ± 25.7	887.6 ± 39.2
sitosteryl-18:2 [%]	47.0 ± 0.2	53.7 ± 1.4	53.6 ± 0.6	54.3 ± 0.9
campesteryl-18:2 + stigmasteryl-18:0/18:1 [%]	16.7 ± 0.2	14.1 ± 0.2	16.6 ± 0.6	15.6 ± 0.2
sitosteryl-18:0/18:1 [%]	13.0 ± 0.2	11.8 ± 0.1	10.5 ± 0.1	10.6 ± 0.2
Δ^7 sitosteryl-18:2 [%][c]	5.5 ± 0.5	4.7 ± 0.3	5.3 ± 0.3	3.8 ± 0.5
sitostanyl-18:2 [%]	4.7 ± 0.1	4.6 ± 0.4	4.3 ± 0.4	4.6 ± 0.6
campesteryl-18:0/18:1 [%]	4.6 ± 0.3	3.2 ± 0.5	3.1 ± 0.3	2.8 ± 0.1
campestanyl-18:0/18:1+ stigmasteryl-18:2 [%]	3.7 ± 0.2	2.9 ± 0.4	2.7 ± 0.2	3.0 ± 0.2
sitosteryl-16:0/16:1 [%]	3.0 ± 0.2	3.0 ± 0.0	2.7 ± 0.0	3.2 ± 0.1
campesteryl-16:0/16:1 [%]	1.0 ± 0.1	1.1 ± 0.1	0.8 ± 0.1	1.0 ± 0.1
sitostanyl-16:0/16:1 [%]	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.4 ± 0.0
stigmasteryl-16:0/16:1 [%]	0.3 ± 0.0	0.4 ± 0.1	0.2 ± 0.0	0.4 ± 0.0
campestanyl-16:0/16:1 [%][c]	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0
campestanyl-18:2 [%]	<LOQ[d]	<LOQ	<LOQ	<LOQ
sitostanyl-18:0/18:1 [%]	<LOQ	<LOQ	<LOQ	<LOQ
total *trans*-steryl/stanyl ferulic acid esters [μg/g dm[b]]	33.5 ± 2.8	111.7 ± 7.0	47.6 ± 1.3	47.6 ± 0.8
total *trans*-steryl/stanyl ferulic acid esters [μg/100 mg oil]	37.9 ± 2.4	103.6 ± 4.4	53.0 ± 0.8	45.3 ± 1.4
trans-sitostanyl ferulate [%]	43.2 ± 1.4	42.2 ± 0.9	41.9 ± 0.5	42.1 ± 0.4
trans-campestanyl ferulate [%]	31.2 ± 1.5	36.9 ± 0.5	36.1 ± 0.3	36.4 ± 0.5
trans-sitosteryl ferulate [%]	13.0 ± 0.6	9.5 ± 0.6	9.9 ± 0.2	10.0 ± 0.2
trans-campesteryl ferulate [%]	7.4 ± 0.6	9.8 ± 0.1	6.9 ± 0.5	6.6 ± 0.9
trans-24-methylene cycloartanyl ferulate [%]	5.3 ± 1.1	<LOQ	2.7 ± 0.4	2.5 ± 0.3
trans-Δ^7 sitosteryl ferulate [%]	<LOQ	1.6 ± 0.2	2.4 ± 0.1	2.5 ± 0.0

[a]Values represent the means ± standard deviations (n = 3). [b]Based on dry matter (dm) of ground kernels. [c]Compound calculated with Rf = 1. [d]Content below LOQ (Table 1).

Table 3. Contents and Compositions of Free Sterols/Stanols and Steryl/Stanyl Esters in Four Commercial Popcorn Samples[a]

	1	2	3	4
oil content [%]	4.33 ± 0.04	4.00 ± 0.04	4.04 ± 0.01	3.75 ± 0.03
total free sterols/stanols [μg/g dm[b]]	331.0 ± 5.5	273.7 ± 9.1	276.4 ± 0.7	290.2 ± 1.5
total free sterols/stanols [μg/100 mg oil]	804.0 ± 19.0	683.3 ± 16.8	683.8 ± 5.5	773.4 ± 8.3
sitosterol [%]	64.9 ± 0.1	63.5 ± 0.7	62.4 ± 0.1	64.2 ± 0.2
campesterol [%]	18.9 ± 0.2	19.7 ± 0.1	19.7 ± 0.1	18.3 ± 0.0
stigmasterol [%]	6.4 ± 0.1	6.5 ± 0.3	6.6 ± 0.1	6.2 ± 0.1
sitostanol [%]	6.1 ± 0.2	5.7 ± 0.1	5.7 ± 0.0	6.4 ± 0.0
campestanol [%]	1.7 ± 0.2	1.5 ± 0.0	1.5 ± 0.1	1.7 ± 0.1
cholesterol [%]	0.2 ± 0.0	1.5 ± 0.0	2.4 ± 0.1	1.3 ± 0.1
others [%]	1.8 ± 0.1	1.5 ± 0.0	1.6 ± 0.0	1.8 ± 0.0
total steryl/stanyl fatty acid esters [μg/g dm[b]]	343.3 ± 5.3	240.0 ± 2.0	246.2 ± 1.5	276.6 ± 6.4
total steryl/stanyl fatty acid esters [μg/100 mg oil]	792.8 ± 5.3	599.5 ± 9.0	609.2 ± 4.3	736.9 ± 12.0
sitosteryl-18:2 [%]	43.2 ± 0.4	33.8 ± 1.1	41.1 ± 0.2	44.2 ± 1.3
sitosteryl-18:0/18:1 [%]	14.9 ± 0.3	22.3 ± 0.0	12.4 ± 0.8	7.7 ± 1.1
campesteryl-18:2 + stigmasteryl-18:0/18:1 [%]	10.9 ± 0.1	10.7 ± 0.2	11.0 ± 0.0	11.1 ± 0.1
sitostanyl-18:2 [%]	9.8 ± 0.2	7.3 ± 0.2	8.1 ± 0.3	10.8 ± 0.1
campestanyl-18:0/18:1+ stigmasteryl-18:2 [%]	5.5 ± 0.1	6.4 ± 0.1	5.4 ± 0.1	6.1 ± 0.1
sitosteryl-16:0/16:1 [%]	2.4 ± 0.1	3.6 ± 0.0	5.0 ± 0.0	4.3 ± 0.1
Δ^7 sitosteryl-18:2 [%][c]	2.5 ± 0.1	2.4 ± 0.0	2.8 ± 0.1	3.4 ± 0.1
sitostanyl-18:0/18:1 [%]	2.2 ± 0.4	1.9 ± 0.3	3.3 ± 0.2	3.2 ± 0.5
campestanyl-18:2 [%]	3.1 ± 0.1	2.0 ± 0.1	2.0 ± 0.1	2.5 ± 0.0
campesteryl-18:0/18:1 [%]	1.9 ± 0.1	2.5 ± 0.2	3.0 ± 0.1	1.6 ± 0.1
Δ^7 campesteryl-18:2 [%][c]	0.7 ± 0.0	0.6 ± 0.0	0.7 ± 0.1	0.7 ± 0.1
Δ^7 sitosteryl-16:0/16:1 [%][c]	0.9 ± 0.1	2.1 ± 0.0	2.3 ± 0.1	2.0 ± 0.0
campesteryl-16:0/16:1 [%]	0.7 ± 0.0	0.8 ± 0.1	0.8 ± 0.0	0.7 ± 0.0
sitostanyl-16:0/16:1 [%]	0.3 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
stigmasteryl-16:0/16:1 [%]	0.5 ± 0.1	2.7 ± 0.4	1.3 ± 0.0	0.7 ± 0.0
campestanyl-16:0/16:1 [%][c]	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.1	0.5 ± 0.0
total *trans*-steryl/stanyl ferulic acid esters [μg/g dm[b]]	165.4 ± 3.4	185.4 ± 5.5	173.0 ± 6.0	186.5 ± 4.3
total *trans*-steryl/stanyl ferulic acid esters [μg/100 mg oil]	401.8 ± 6.2	466.6 ± 7.9	433.8 ± 7.8	521.0 ± 18.0
trans-sitostanyl ferulate [%]	60.7 ± 0.0	60.3 ± 0.1	59.7 ± 0.2	63.0 ± 0.6
trans-campestanyl ferulate [%]	28.7 ± 0.1	30.0 ± 0.4	30.3 ± 0.0	25.0 ± 0.3
trans-campesteryl ferulate [%]	4.1 ± 0.1	4.1 ± 0.3	4.1 ± 0.0	4.6 ± 0.5
trans-sitosteryl ferulate [%]	3.1 ± 0.1	3.1 ± 0.3	3.0 ± 0.0	4.3 ± 0.2
trans-Δ^7 sitosteryl ferulate [%]	1.8 ± 0.2	1.4 ± 0.1	1.5 ± 0.1	1.5 ± 0.3
trans-Δ^7 campesteryl ferulate [%]	0.7 ± 0.0	0.7 ± 0.1	0.9 ± 0.1	0.9 ± 0.2
trans-24-methylene cycloartanyl ferulate [%]	0.9 ± 0.1	0.3 ± 0.0	0.5 ± 0.1	0.6 ± 0.0

[a]Values represent means ± standard deviations (n = 3). [b]Based on dry matter (dm) of ground kernels. [c]Compound calculated with Rf = 1.

sufficient improvements as it either resulted in a broadening of the LC-peak accompanied by a loss in resolution or in a coelution with triglycerides. Thus, only the *trans*-steryl/stanyl ferulates could be analyzed as their TMS-derivatives in a second transfer (Figure 3).

Identification of individual compounds was performed by GC−MS after multiple manual collections of the LC-fractions of interest. In the case of proso millet, identification was additionally carried out by means of online LC−GC−MS, which enabled a much faster analysis. The identities were confirmed by comparison of relative retention times and mass spectrometric data to commercially obtained or synthesized references substances or by comparison to literature data.[4,10,14,20−22]

The recoveries determined for corn and proso millet as well as the LODs and LOQs of selected steryl/stanyl derivatives are summarized in Table 1. The repeatability of the approach, comprising lipid extraction and online LC−GC analysis, was confirmed by the analysis of five replicates of two control samples (corn flour and proso millet flour); for both materials, low relative standard deviations were determined for free sterols/stanols (1.2%), steryl/stanyl fatty acid esters (6.7% for corn and 3.3% for proso millet), and *trans*-steryl/stanyl ferulic acid esters (8.5% for corn). The stability of the approach was supported by the results obtained upon the repeated analyses of the routinely employed control samples and by the fact that the most critical step of the analysis, the high temperature-GC, resulted in consistent responses for the employed reference substances (relative standard deviations <6%). These data demonstrated the stability of the analytical procedure; nevertheless, lipid extraction, silylation, and analysis of samples were normally performed within 1 d.

Application of the Online LC−GC-Based Approach. The established methodology was applied to the qualitative and quantitative analysis of free sterols/stanols, steryl/stanyl fatty acid esters, and *trans*-steryl/stanyl ferulic acid esters in kernels of sweet corn, popcorn, and proso millet.

Sweet Corn and Popcorn. The oil contents and quantitative data of individual steryl/stanyl conjugates determined in sweet corn and popcorn are given in Tables 2 and 3. The oil contents of the sweet corn kernels averaged 9.77 ± 1.01% and were more than twice as high as those determined in popcorn.

Table 4. Contents and Compositions of Free Sterols/Stanols and Steryl/Stanyl Esters in Four Proso Millet Cultivars[a]

	1	2	3	4
	Huangmi (2010)	Kornberger (2010)	Kornberger (2011)	–[g]
oil content [%]	3.05 ± 0.08	3.15 ± 0.02	3.51 ± 0.04	4.01 ± 0.05
total free sterols/stanols [μg/g dm[b]]	188.3 ± 3.5	211.5 ± 3.0	259.8 ± 3.8	208.5 ± 2.1
total free sterols/stanols [μg/100 mg oil]	613.3 ± 3.4	671.6 ± 13.1	741.8 ± 5.2	518.4 ± 6.1
sitosterol [%]	64.2 ± 0.6	65.7 ± 0.7	61.0 ± 0.3	58.4 ± 0.2
campesterol [%]	11.6 ± 0.1	12.1 ± 0.1	12.1 ± 0.1	12.2 ± 0.0
cholesterol [%]	6.0 ± 0.2	6.7 ± 0.2	7.4 ± 0.1	9.1 ± 0.2
stigmasterol [%]	6.9 ± 0.6	5.9 ± 1.4	5.4 ± 0.1	5.7 ± 0.0
cycloartanol [%]	3.1 ± 0.0	2.7 ± 0.2	4.4 ± 0.2	3.2 ± 0.1
cycloartenol [%]	2.6 ± 0.2	3.3 ± 0.3	2.9 ± 0.1	4.0 ± 0.1
Δ^5 avenasterol [%]	2.7 ± 0.1	2.4 ± 0.1	3.6 ± 0.1	3.8 ± 0.1
sitostanol [%]	1.4 ± 0.2	1.0 ± 0.1	1.2 ± 0.1	1.2 ± 0.2
24-methylene cycloartanol [%]	<LOQ[f]	<LOQ	1.2 ± 0.1	1.1 ± 0.0
clerosterol [%]	0.9 ± 0.1	0.8 ± 0.0	0.8 ± 0.0	0.8 ± 0.0
campestanol [%]	0.6 ± 0.1	<LOQ	<LOQ	0.6 ± 0.1
total steryl/stanyl fatty acid esters [μg/g dm[b]]	524.4 ± 11.9	345.4 ± 1.1	419.1 ± 16.2	751.0 ± 4.6
total steryl/stanyl fatty acid esters [μg/100 mg oil]	1708.9 ± 57.5	1096.5 ± 8.0	1196.3 ± 35.2	1867.3 ± 14.4
sitosteryl-18:2 [%]	42.9 ± 0.4	32.5 ± 2.9	35.0 ± 0.2	38.0 ± 0.1
cholesteryl-18:2 + sitosteryl-16:0/16:1 [%][c]	15.7 ± 0.2	19.8 ± 2.7	18.1 ± 0.3	18.3 ± 0.2
sitosteryl-18:0/18:1 [%]	13.1 ± 0.7	12.3 ± 0.4	11.2 ± 0.1	9.4 ± 0.1
cycloartenyl-18:2 [%][d]	8.8 ± 0.4	10.4 ± 0.8	11.4 ± 0.3	9.1 ± 0.4
campesteryl-18:2 + stigmasteryl-18:0/18:1 [%]	8.0 ± 0.2	8.1 ± 0.8	7.4 ± 0.2	7.8 ± 0.2
campesteryl-18:0/18:1 [%]	5.7 ± 0.8	7.6 ± 0.6	8.5 ± 0.2	8.0 ± 0.2
cycloartanyl-18:2 [%][d]	2.6 ± 0.1	4.6 ± 0.0	4.6 ± 0.0	3.4 ± 0.2
stigmasteryl-18:2 [%]	2.0 ± 0.2	2.0 ± 0.2	1.7 ± 0.2	2.2 ± 0.1
cholesteryl-18:0/18:1 [%][e]	0.9 ± 0.2	1.4 ± 0.2	1.3 ± 0.2	2.3 ± 0.1
cholesteryl-16:0/16:1 [%][d]	0.7 ± 0.1	0.7 ± 0.2	0.5 ± 0.0	0.7 ± 0.0
campesteryl-16:0/16:1 [%]	0.6 ± 0.1	0.5 ± 0.1	0.3 ± 0.0	0.6 ± 0.1
stigmasteryl-16:0/16:1 [%][d]	0.3 ± 0.1	0.2 ± 0.0	<LOQ	0.1 ± 0.0

[a]Values represent means ± standard deviations (n = 3). [b]Based on dry matter (dm) of ground kernels. [c]Quantitated via the calibration curve of campesteryl-18:2. [d]Compound calculated with Rf = 1. [e]Quantitated via the calibration curve of campesteryl-18:0/18:1. [f]Content below LOQ (Table 1). [g]No information available.

Comparable high fat contents based on dry matter of matured sweet corn kernels have already been described.[23] The oil contents of popcorn are also in agreement with earlier reported data.[24]

The distribution patterns of free sterols/stanols revealed almost no variation between the samples of sweet corn and popcorn. Sitosterol was dominating, accounting for 59.3–64.9% of total free sterols/stanols, followed by campesterol and stigmasterol. Free stanols, that is, campestanol and sitostanol, represented 3.4–8.7%. The detected profiles generally agree with already published data concerning total or free sterols/stanols in corn kernels or corn oils.[5,25−27] Total contents of free sterols/stanols based on dry matter of sweet corn flour were approximately 3 times higher than those determined in the flours of popcorn. The amounts calculated for the extracted oils averaged 0.99 ± 0.09% in sweet corn and 0.74 ± 0.06% in popcorn.

Overall, esters of C18-fatty acids were more abundant than esters of C16-fatty acids, with sitosterol and campesterol as main esterified sterols. Thereof, sitosteryl-18:2, campesteryl-18:2 (coeluted with stigmasteryl-18:0/18:1), and sitosteryl-18:0/18:1 were the main steryl/stanyl fatty acid esters, accounting together for 63.0–80.7% of total esters. The total contents of steryl/stanyl fatty acid esters determined in sweet corn as well as in popcorn were in the same order of magnitude as the respective levels of free sterols/stanols. This is in contrast to the distribution of other corn subspecies, where steryl/stanyl fatty acid esters were reported to be more abundant than free sterols/stanols.[5,18]

Within *trans*-steryl/stanyl ferulic acid esters, the esters of sitostanol and campestanol were dominating, together accounting for 74.4–90.3%, followed by esters of sitosterol, campesterol, and 24-methylene cycloartanol. However, all investigated popcorn samples revealed a higher degree of esterified stanols than the samples of sweet corn. The total contents of *trans*-steryl/stanyl ferulic acid esters in the flours and oils of sweet corn were on average about 3 and 8 times lower, respectively, than those determined in popcorn. Additionally, a 3-fold margin was observed between the lowest and highest content of total *trans*-steryl/stanyl ferulic acid esters in sweet corn (samples 1 and 2, respectively), whereas the total contents analyzed in the four popcorn samples were of the same order of magnitude.

Proso Millet. The oil contents of the four investigated millet samples averaged 3.43 ± 0.43% (Table 4), which is in agreement with earlier results, where oil contents of various hulled proso millet cultivars were reported to be between 2.9% and 4.5%.[28,29]

The total contents as well as the percentage distributions of free sterols/stanols are presented in Table 4. In accordance with other cereal grains, sitosterol was predominant and made up >58.4% of total free sterols/stanols, followed by campesterol and stigmasterol. The levels of cholesterol were relatively high (6.0–9.1% of total free sterols/stanols) as compared to sweet

corn, popcorn (Tables 2 and 3), or other cereal grains.[4] A previous study also reported high amounts of cholesterol in seeds of proso millet as cholesterol represented 3−16% of total sterols.[30] The sum of free sterols/stanols made up on average 217.0 ± 30.3 μg/g dry matter flour, being lowest in cultivar Huangmi (China, 2010) and highest in cultivar Kornberger (Austria, 2011). The free sterol/stanol levels determined in the flours as well as in the extracted oils were lower as compared to those determined in sweet corn and popcorn (Tables 2 and 3) and were also lower than those reported for other cereal grains.[4,18,21]

Cholesterol was not only detected in free form, but also esterified to C16 and C18 fatty acids (Table 4), which represented together approximately 20% of total steryl/stanyl fatty acid esters. Because of the natural presence of cholesteryl palmitate, which has been used as IS for the quantitation of steryl/stanyl fatty acid esters in the corn samples, cholesteryl laurate was applied as IS in case of the seeds of proso millet. Despite the comparably high amounts of cholesteryl esters in proso millet, sitosteryl esters were dominating with sitosteryl-18:2 as the most abundant ester, accounting for 37.1 ± 4.5%. To the authors' knowledge, it is not only the first time that individual steryl/stanyl fatty acid esters were analyzed in millet, but also that intact cholesteryl esters were detected in cereal grains. Distinct differences were observed in total contents of steryl/stanyl fatty acid esters between the samples; amounts ranged from 345.4 to 751.0 μg/g dry matter flour and from 1.1−1.9% in extracted oil, being highest in the commercially obtained proso millet seeds. The cultivar Huangmi (China, 2010) contained, in contrast to the total contents of free sterols/stanols, higher levels of steryl/stanyl fatty acid esters than both Kornberger samples. All four investigated cultivars exhibited higher total amounts of steryl/stanyl fatty acid esters than of free sterols/stanols. This is contradictory to the results reported by Sridhar and Lakshminarayana,[31] who determined more free sterols than steryl esters in the nonpolar lipid fraction of proso millet seeds.

Steryl/stanyl ferulic acid esters, which are unique compounds in a wide range of cereals, for example, rice, corn, wheat, rye, or spelt,[4,17,32] could not be detected in the seeds of proso millet. To the authors' knowledge, no quantitative data on steryl ferulates in small millets have been reported to date. Only the sum of steryl ferulates in two sorghum hybrids has been determined via normal phase high-performance LC.[33] However, the amounts reported were low (0.03% of extracted sorghum oil) as compared to other cereals.[4,17,32]

In conclusion, the established online LC−GC-based approach enables the simultaneous analysis of free sterols/stanols and intact steryl/stanyl fatty acid esters in cereal lipids; *trans*-derivatives of steryl/stanyl ferulic acid esters could further be analyzed in a second run. Whereas the previously reported SPE-based approach provided more information on the spectrum of steryl/stanyl phenolic acid esters,[4] the advantage of the online LC−GC technique is in particular the far less complex sample preparation. Consequently, the workup time is strikingly decreased, and less solvent amount is needed as compared to the previously reported SPE-based approach.[4] Furthermore, due to the automation of the instrumentation and the performance in a closed system, the risk of sample loss and contamination is reduced, thus enabling a robust and fast analysis. The results elaborated regarding the contents and the distribution patterns of free sterols/stanols and intact steryl/stanyl esters in sweet corn, popcorn, and proso millet demonstrate the potential of the technique.

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Notes

The authors declare no competing financial interest.

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