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GC-Based Analysis of Plant Stanyl Fatty Acid Esters in Enriched Foods

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ABSTRACT: Approaches for the capillary gas chromatographic (GC) based analysis of intact plant stanyl esters in enriched foods were developed. Reference compounds were synthesized by enzyme-catalyzed transesterifications. Their identities were confirmed by means of mass spectrometry. Using a medium polar trifluoropropylmethyl polysiloxane stationary phase, long-chain plant stanyl esters could be separated according to their stanol moieties and their fatty acid chains. Thermal degradation during GC analysis was compensated by determining response factors; calibrations were performed for ten individual plant stanyl esters. For the analysis of low-fat products (skimmed milk drinking yogurts), the GC separation was combined with a "fast extraction" under acidic conditions. For fat-based foods (margarines), online coupled LC−GC offered an elegant and efficient way to avoid time-consuming sample preparation steps. The robust and rapid methods allow conclusions on both, the stanol profiles and the fatty acid moieties, and thus provide a basis for the authentication of this type of functional food ingredients.

KEYWORDS: online LC−GC analysis, plant stanyl fatty acid ester, enriched food

INTRODUCTION

Increased blood cholesterol level is a risk factor for coronary heart disease. Dietary intake of plant sterols and stanols is recommended as a method to reduce low density lipoprotein (LDL) cholesterol. Owing to the inhibition of intestinal cholesterol absorption and other effects on hepatic/intestinal cholesterol metabolism, a reduction in plasma cholesterol concentrations of 10−15% can be achieved by an intake of approximately 2 g of plant sterols/stanols per day.[1,2] Steryl and stanyl esters have also been shown to be effective in reducing total and LDL cholesterol.[3,4] Therefore, for technological reasons in many types of products the fatty acid esters rather than the free sterols/stanols are added.[4]

The first foods placed on the EU market were yellow fat spreads with added phytosteryl esters.[4,5] In the meantime, many types of food categories enriched with plant steryl and stanyl esters have been approved.[6] The concentrations of plant sterols in enriched products vary from 0.3% in milk type products up to 8% in spreads.[6] Vegetable oils as well as tall oil are used as starting materials to obtain sterols and stanols; edible oils are also used as sources to provide the fatty acid moieties employed to produce the respective esters.[7] Accordingly, enriched products are specified regarding their sterol/stanol profiles and the esterified fatty acids, respectively.[5,8,9] There is a need for quantitative and qualitative methods allowing the authentication of these foods. At present, there are official methods for the analysis of plant sterols/stanols[10−12] but not for their intact esters in enriched foods. Approaches allowing the direct quantification of the intact esters are desirable; the chromatographic resolution of the esters should allow conclusions on both the sterol/stanol profile and the fatty acid source. This is of particular importance, as it has been reported that the rate of the intestinal ester hydrolysis, for example by pancreatic cholesterol esterase, depends on the structures of both the sterol and the fatty acid moieties,[13] and, therefore, the composition of the employed ester preparation may have an impact on the cholesterol-lowering effect.[14]

To date, analytical approaches for the determination of plant sterols or fatty acid steryl esters in foods are based on the isolation, for example by thin layer or silica gel chromatography, and the subsequent application of gas chromatography (GC) or high-performance liquid chromatography (HPLC). Regarding sensitivity and precision, gas chromatography is considered as the method of choice for the determination of total sterol content.[15−18] Therefore, steryl esters are commonly subjected to alkaline hydrolysis and the liberated sterols are analyzed by GC either in free or silylated form. However, in this way information regarding the fatty acid composition of the steryl esters is lost.

The analysis of intact steryl esters in foods was achieved by means of GC or reversed-phase HPLC. The HPLC analysis required a mass spectrometry (MS) detector for the identification of incompletely separated as well as coeluted steryl esters and neutral lipids.[19] GC may be a considerably more sensitive analytical procedure for the investigation of intact steryl esters than HPLC,[20,21] but the GC-based analysis of individual steryl esters by GC is still a challenging problem.[22] For example, the degree of unsaturation of C18 fatty acid esters could not be distinguished with nonpolar stationary phases.[20,22,23] The use of a more polar stationary phase (50% phenyl−50% methyl polysiloxane) also resulted in insufficient resolution of a complex mixture of steryl esters regarding their degree of saturation.[21,24] In addition, the high boiling plant steryl esters may be subject to individually differing thermal degradation processes during the GC analysis.[25−27]

Another challenge encountered in particular with foods exhibiting higher fat contents is the need for extensive workup procedures in order to remove di- and triglycerides that may hamper the direct GC analysis of phytosteryl esters. The online coupling

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of LC and GC can offer an efficient alternative to the laborious standard approaches.[23,28]

The objective of the present study was the development of GC-based methods allowing the rapid quantification of intact plant stanyl esters in enriched foods. Using skimmed milk drinking yogurt as example for a low-fat food, a fast method for extraction and separation of phytosteryl esters should be elaborated. Margarines were chosen as examples to demonstrate the usefulness of online LC−GC as an efficient approach for the analysis of plant stanyl esters added to fat-based products.

MATERIALS AND METHODS

Chemicals. Cholesteryl palmitate (≥98%), fatty acid methyl esters (16:0, 16:1, 18:0, 18:1, 18:2, 18:3, 20:0, 22:0 and 22:1; analytical grades), pyridine (puriss), bis(trimethylsilyl)-trifluoroacetamide (BSTFA) + trimethylchlorosilane (TMCS), 99:1, *Candida rugosa* type VII lipase (L1754) and lipase B from *Candida antarctica* (L-4777) were obtained from Sigma-Aldrich (Taufkirchen, Germany). Calcium chloride anhydrous and hydrochloric acid (25%) were purchased from Riedel de Haën (Taufkirchen, Germany). Sodium sulfate anhydrous and sodium methylate (5.4 M in methanol) were obtained from Fluka (Taufkirchen, Germany). *n*-Hexane (SupraSolv) was purchased from VWR International (Darmstadt, Germany). *tert*-Butyl methyl ether (MTBE, DiveronS) was supplied from Oxeno Olefinchemie (Marl, Germany) and was distilled prior to use.

Materials. A mixture of plant stanyl fatty acid esters (plant stanol ester, STAEST-115) was kindly provided by Raisio Group (Raisio, Finland). A mixture of plant stanols (Reducol stanol powder) was a gift from Cognis GmbH (Illertissen, Germany); it consisted of sitostanol (91.2%) and campestanol (8.2%).

Skimmed milk drinking yogurts enriched with plant stanyl esters (Emmi Benecol; produced by Emmi, Switzerland; distributed in Germany by Emmi Deutschland GmbH, Essen) were purchased in local supermarkets (Freising, Germany). Two types of products were selected: taste "orange" (drinking yogurt 1) and "strawberry" (drinking yogurt 2). The labels indicated "added plant stanol esters". In the list of ingredients the following information was included: "plant stanol 3.0 % and fat (without stanol) 2.1 %". The fat (without stanol) was declared as "saturated (0.2 %), monounsaturated (1.4 %) and polyunsaturated fatty acids (0.6 %)".

Margarines enriched in plant stanyl esters were obtained from Finland and The Netherlands. The Benecol margarines from Finland (taste-type "Kevyt kasvirasvalevite 32 %" (margarine 1), "Kevyt kasvirasvalevite Oliivi 32 %" (margarine 2), and "Laktoositon kasvirasvalevite 32 %" (margarine 3), were produced by Ravintoraisio Oy (Raisio, Finland). The plant stanol content was labeled as 8 wt %, total lipids as 32 wt %. As emulsifier soy lecithin was declared. In the case of margarine 2, the olive oil content was declared as 9% of total fat. The Benecol margarines from The Netherlands were produced by McNeill Nutritionals Ltd. (Bracknell, U.K.); the taste-type investigated was "Met Olijfolie" (margarine 4). The list of ingredients included the following information: plant stanol 0.8 g/12 g portion, according to 7 wt %, 55 wt % lipids. The olive oil content was declared as 14% of total fat content. As emulsifiers, soy lecithin and mono- and diglycerides were labeled.

Lipase-Catalyzed Preparation of Stanyl Esters. For plant stanol ester synthesis, fatty acid methyl esters (300 μmol) were transesterified with the Reducol stanol powder (300 μmol) in the presence of 50 mg of *C. rugosa* lipase (VII) and 500 μL of *n*-hexane by shaking a screw-capped tube at 40 °C for 72 h. For the synthesis of the behenates and erucates, *C. antarctica* lipase B was used as biocatalyst.

Capillary Gas Chromatography (GC/FID). Gas chromatographic analysis of stanyl fatty acid esters was performed on an Agilent Technologies instrument 6890N (Böblingen, Germany) equipped with a flame ionization detector (FID). Separations of 1 μL of the sample were carried out on a 30 m × 0.25 mm i.d. fused-silica capillary coated with a film of 0.1 μm trifluoropropylmethyl polysiloxane (Rtx-200MS, Restek, Bad Homburg, Germany). The temperature of the inlet was set to 280 °C, the detector temperature to 360 °C. Hydrogen was used as carrier gas with the constant flow rate 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 temperature program was 100 °C (2 min), programmed with 15 °C/min up to 310 °C (2 min), then 1.5 °C/min up to 340 °C (3 min). Data acquisition was performed by ChemStation software.

Capillary Gas Chromatography−Mass Spectrometry (GC/MS). Identification of the stanyl esters was performed on a Finnigan Trace GC ultra (Thermo Electron Corp., Austin, TX) equipped with a Finnigan Trace DSQ mass spectrometer (Thermo Electron Corp., Austin, TX). Mass spectra were obtained by electron impact ionization (EI) at 70 eV in the full scan mode at unit resolution from 40 to 750 Da (scan time 0.4 s). Helium was used with the constant flow rate 1 mL/min. The interface was heated to 320 °C, the source to 250 °C. The residual conditions were as described for GC/FID analysis of stanyl esters.

Transesterification of the Stanyl Ester Mixture. The stanyl ester mixture (STEAST-115; 0.5 mg), *n*-hexane (500 μL) and 20 μL of a sodium methylate solution in methanol (2 M) were placed into a 1.5 mL vial. After 60 min at room temperature in the dark, calcium chloride anhydrous was added. The dried *n*-hexane phase containing the fatty acid methyl esters (FAME) was analyzed by GC/FID, performed on a Focus GC (Thermo Electron Corp., Austin, TX) equipped with a flame ionization detector (FID; 320 °C) using a DB-1, 60 m × 0.32 mm i.d. fused silica capillary coated with a 0.25 μm film of polydimethylsiloxane (J&W Scientific, Folsom, CA). Hydrogen was used as the carrier gas (flow = 1.8 mL/min). Split injection (split flow = 27 mL/min) was performed at 250 °C. The column temperature was programmed from 100 to 320 °C (25 min hold) at 4 °C/min. Identification was performed by comparing the retention times to those of a reference FAME mixture.

Extraction of Drinking Yogurts According to Weibull−Stoldt. About 20 g of the shaken yogurt drink sample was weighed into a beaker with an accuracy of ±1 mg. After addition of 3 mL of an internal standard solution [50 mg of cholesteryl palmitate/mL], hydrochloric acid (100 mL; 25%) and 80 mL of aqua dest. were added. The digestion was performed by gentle boiling of the solution for 45 min. Hot distilled water (200 mL) was added, and the solution was filtrated through a moistened folded filter. The filter was washed with hot distilled water until the filtrate exhibited a neutral pH. After drying (2 h at 103 ± 2 °C), the filter was placed into an extraction thimble. The extraction was performed in a Soxhlet apparatus with MTBE (180 mL) for 3 h. Solvent was removed by rotary evaporation, and the residue was dried to constant mass at 103 ± 2 °C. Solutions of the lipid residue, 10 mg/mL in *n*-hexane/MTBE (3:2, v/v), were diluted 1:20 and subjected to GC analysis.

Hydrolysis of Weibull−Stoldt Extracts. In an 11 mL screw capped vial the lipid extracts (0.3 mg) were diluted in 300 μL of *n*-hexane/MTBE (3:2, v/v) and 500 μL of sodium methylate solution (5.4 M). The solutions were flushed with argon, sealed, heated until boiling and then cooled to room temperature. After 30 min at room temperature, the solutions were heated again until boiling and cooled off. MTBE (0.5 mL) and 4 mL of hydrochloric acid (0.7 M) were added. After vigorous shaking, the organic phase was separated. The extraction with 0.5 mL of MTBE was repeated twice. The combined extracts were dried with anhydrous sodium sulfate. The supernatant was transferred into another vial and evaporated to dryness by a gentle stream of nitrogen. The residue was redissolved in 20 μL of dry pyridine and 100 μL of BSTFA + 1% TMCS. The sealed vial was allowed to stand for 20 min at 70 °C in an oil bath. *n*-Hexane/MTBE (3:2, v/v; 380 μL) was added. The solution was analyzed by GC/FID.

"Fast Extraction Method". The internal standard cholesteryl palmitate (750 μg), the yogurt drink sample (100 mg, accuracy of ±0.1 mg), hydrochloric acid (25%; 1000 μL) and bidistilled water (400 μL) were

placed into a reaction vial. After adding a magnetic stir bar, the sealed vials were sonicated until the internal standard was dispersed. The digestion was performed at 130 °C for 45 min. After cooling to room temperature, 1 mL of bidistilled water was added. The solution was filtrated through a 5 mL syringe assembled with a 0.45 μm membrane filter. The vials were washed three times with 5 mL of bidistilled water; the filters were washed until the solution reached a neutral pH value. The lipids were extracted three times with 5 mL of *n*-hexane/MTBE (3:2, v/v); vial and filter were also rinsed with the solvent. The combined extracts were dried with sodium sulfate (anhydrous) and subjected to GC analysis.

GC/FID-Calibration for the Quantification of Stanyl Esters. The five-point calibration functions were generated in the range 0.1−0.5 μg of total stanyl ester per injection (1 μL injection volume). To 150 μL, 300 μL, 450 μL, 600 μL and 750 μL, respectively, of each stock solution (10 mg/mL in *n*-hexane/MTBE (3:2, v/v)) 300 μL of the internal standard (IS) solution (cholesteryl palmitate [2.5 mg/mL] in *n*-hexane/MTBE (3:2, v/v)) was added. Solvents were evaporated by a gentle stream of nitrogen; further workup was as described for the sample preparation of yogurt drinks. Each calibration point was done in triplicate. Linear regression analysis was performed in coordinate ratios of areas (individual stanyl ester/IS) and amounts (individual stanyl ester/IS).

Quantification of Stanols after Hydrolysis by Means of GC/FID. Three-point calibration functions were generated with the plant stanols of the Reducol powder in the range 0.05−0.5 μg of total sterols per 1 μL injection volume. Cholesteryl palmitate (0.1 mg) and 0.1, 0.5, and 1.0 mg of stanol powder were diluted in 300 μL of *n*-hexane/MTBE (3:2, v/v) and prepared as described for the hydrolysis of the Weibull−Stoldt extracts. Each calibration point was done in triplicate. Linear regression analysis was performed in coordinate ratios of areas (plant stanol/cholesterol) and amounts (plant stanol/cholesterol).

Sample Preparation of Margarines. Margarine samples (20−40 mg, accuracy of ±0.1 mg) were weighed into a vessel; internal standard solution (300 μL, 2.5 mg/mL cholesteryl palmitate in *n*-hexane/MTBE (3:2, v/v)), 5 mL of *n*-hexane/MTBE (3:2, v/v) and sodium sulfate (anhydrous) were added and sonicated for 1 min. The solution was filtered through a 0.45 μm membrane filter assembled with a 5 mL syringe. The vessel and the filter were washed twice with 5 mL of hexane/MTBE (3:2, v/v). After dilution (1:40) of the combined extracts, the solution was used for online LC−GC analysis.

Online LC−GC/FID. Online LC−GC analysis was performed on a Dualchrom 3000 instrument (Carlo Erba Instruments, Rodano, Italy), equipped with a loop type interface (500 μL sample loop) and a fully automated interface valve system. The HPLC part included two 20 mL syringe pumps (Phoenix 30 CU). One was used as master pump for solvent delivery, the other as slave pump for backflushing the column system. LC detection was performed by a variable wavelength detector (microUVIS 20, Carlo Erba Instruments, Rodano, Italy) at 205 nm. The HPLC preseparation was performed on a Eurospher-100 Si column (5 μm particle size, 250 × 2 mm i.d.) from Knauer (Berlin, Germany). The column was thermostated to 20 °C using a column oven (Jetstream 2 Plus, Advanced Separation Technologies Inc., USA). *n*-Hexane/MTBE (96 + 4, v + v) was used as eluent with a flow rate of 200 μL/min. Backflush of the column was performed directly after the transfer with 200 μL/min MTBE for 7 min. The transfer valve was switched automatically 5.15 min after injection of 5 μL of sample solution. The transfer occurred by concurrent eluent evaporation at 140 °C. The GC separation was performed on a trifluoropropylmethyl polysiloxane column (Rtx-200MS, 27 m × 0.25 mm i.d., 0.1 μm film thickness, Restek, Bad Homburg, Germany) connected in series with an uncoated capillary deactivated with DPTMDS (3 m × 0.53 mm i.d., BGB Analytics Vertrieb, Schlossböckelheim, Germany) and a coated precolumn (3 m × 0.25 mm i.d.) of the same coating as the analytical column. Between the coated precolumn and the separation column, an early solvent vapor exit (SVE) was installed, which was opened during the transfer. The SVE was closed automatically 60 s after a pressure reduction of 80 kPa. Hydrogen was used as the carrier gas with a constant flow rate of 1.7 mL/min and an inlet pressure behind the flow regulator of 250 kPa. After holding the transfer temperature of 140 °C for 5 min, the temperature was programmed with 15 °C/min up to 310 °C (2 min), then 1.5 °C/min up to 340 °C (3 min). The FID temperature was set to 360 °C. To enhance the FID signals, nitrogen was used as make up gas with a pressure of 120 kPa. All components were controlled by the Dualchrom software. The Chromcard software (C.E. Instruments) was used for data collection and processing.

Online LC−GC/MS. For online LC−GC/MS analysis, the Dualchrom 3000 was coupled with a TSQ 700 mass spectrometer (Finnigan-MAT, San Jose, CA). Carrier gas was switched to helium, and the end of the GC separation column was extended with a 1 m uncoated capillary, deactivated with DPTMDS (0.25 mm i.d.), for connection of the GC oven to the MS via a heated transfer line (320 °C). The residual LC−GC conditions were the same as described above. The source and the manifold of the TSQ were heated to 250 and 70 °C, respectively. Emission current was set to 200 μA, the multiplier to 1500 V. The full scan analysis of steryl esters was carried out at unit resolution from 40 to 750 Da at 70 eV and a scan time of 0.4 s.

Calibration of the Online LC−GC/FID. The online LC−GC instrument was calibrated using the plant stanyl ester mixture STAEST-115. Three point calibration functions were generated with varying concentrations of total esters (2.5; 7.5; 12.5 ng/μL) with regard to the internal standard cholesteryl palmitate (1.25 ng/μL). Linear regression analysis was performed in coordinate ratios of areas (individual stanyl ester/IS) and amounts (individual stanyl ester/IS). The performance of the instrument was confirmed daily using the calibration solutions as control samples.

RESULTS AND DISCUSSION

GC/MS Data. Campestanyl palmitate, sitostanyl palmitate and campestanyl linoleate are the only plant stanyl long chain fatty acid esters for which electron impact (EI) mass spectra have been described in the literature.[29,30] In order to build up a reference library, plant stanyl long chain fatty acid esters were prepared by enzyme-catalyzed transesterification of fatty acid methyl esters and stanols, using *C. rugosa* lipase as biocatalyst.[31] Characteristic fragment ions of the enzymatically synthesized reference compounds are summarized in Table 1. As examples, the mass spectra of sitostanyl stearate and sitostanyl oleate are shown in Figure 1. Molecular ions $[M]^+$ of low intensities were obtained for all stanyl esters. The spectra of the saturated stanyl esters exhibited $[M - FA]^+$ as base fragment, esters of unsaturated fatty acid moieties $[M - FA + H]^+$. The characteristic fragment ions reported for stanyl acetates $[M - FA]^+$, $[M - FA - 15]^+$, $[M - FA - SC]^+$, and $[M - FA - SC - D]^+$ [29,32,33] were present in all stanyl fatty acid ester spectra, independent of the fatty acyl moiety (Table 1).

Capillary Gas Chromatographic Separation. The capillary gas chromatographic investigation of plant steryl fatty acid esters using nonpolar stationary phases, e.g. DB-5[22,23] or DB-1,[20] is hampered by the missing resolution of the esters according to the degree of saturation of the fatty acid moiety.[22] The same phenomenon was encountered in the present study when attempting to separate stanyl esters using a DB-5ht as stationary phase (data not shown). The use of the more polar stationary phase TAP (50% phenyl, 50% methyl polysiloxane) also resulted in insufficient separation of a complex mixture of plant steryl esters regarding their degree of saturation.[21] In the present study, the intermediately polar trifluoropropylmethyl polysiloxane (Rtx-200MS) was

Table 1. Characteristic Fragment Ions of Stanyl Fatty Acid Esters (GC/(EI)MS; 70 eV)

				characteristic fragment ions m/z (rel abundance)						
plant stanol ester[a]	RRT[b]	stanyl fatty acid ester	molecular ion M	M − FA + H	M − FA	M − FA − 15	M − FA − 54	M − FA − SC	M − FA − SC − D	others
1	1.057	campestanyl 16:1	638(1)	385(100)	384(47)	369(18)	330(9)	257(20)	215(31)	95(25), 81(28)
2	1.058	campestanyl 16:0	640(3)	385(37)	384(100)	369(36)	330(11)	257(17)	215(80)	471(3), 95(14), 81(18)
3	1.092	sitostanyl 16:0	654(3)	399(35)	398(100)	383(35)	344(10)	257(18)	215(73)	471(3), 95(14), 81(18)
4	1.096	sitostanyl 16:1	652(2)	399(100)	398(45)	383(17)	344(8)	257(17)	215(17)	95(24), 81(29)
5	1.143	campestanyl 18:1	666(1)	385(100)	384(47)	369(17)	330(9)	257(21)	215(25)	95(24), 81(28)
6	1.144	campestanyl 18:0	668(2)	385(34)	384(100)	369(14)	330(10)	257(18)	215(66)	499(2), 95(12), 81(15)
7	1.150	campestanyl 18:2	664(4)	385(100)	384(29)	369(14)	330(7)	257(26)	215(20)	280(67), 95(34), 81(44)
8	1.159	campestanyl 18:3	662(2)	385(100)	384(20)	369(14)	330(5)	257(16)	215(26)	278(48), 277(44), 95(40), 81(43)
9	1.186	sitostanyl 18:0	682(3)	399(36)	398(100)	383(34)	344(10)	257(16)	215(69)	499(2), 95(13), 81(16)
10	1.190	sitostanyl 18:1	680(2)	399(100)	398(51)	383(17)	344(8)	257(19)	215(26)	95(24), 81(29)
11	1.195	sitostanyl 18:2	678(5)	399(100)	398(26)	383(11)	344(5)	257(19)	215(22)	280(82), 95(38), 81(45)
12	1.203	sitostanyl 18:3	676(4)	399(100)	398(24)	383(12)	344(6)	257(16)	215(20)	278(58), 277(62), 95(47), 81(47)
13	1.245	campestanyl 20:0	696(2)	385(37)	384(100)	369(29)	330(11)	257(20)	215(68)	527(2), 95 (12), 81(18)
14	1.293	sitostanyl 20:0	710(2)	399(36)	398(100)	383(32)	344(10)	257(18)	215(71)	527(2), 95(14), 81(18)
15	1.359	campestanyl 22:0	724(3)	385(43)	384(100)	369(36)	330(9)	257(25)	215(73)	555(2), 95(13), 81(20)
16	1.360	campestanyl 22:1	722(2)	385(100)	384(74)	369(32)	330(13)	257(30)	215(28)	95(25), 81(30)
17	1.411	sitostanyl 22:0	738(3)	399(37)	398(100)	383(32)	344(10)	257(21)	215(69)	555 (2), 95(14), 81(18)
18	1.412	sitostanyl 22:1	736(2)	399(100)	398(58)	383(20)	344(9)	257(33)	215(33)	95(27), 81(35)

[a] Peak numbers correspond to Figures 2 and 4. [b] Relative retention time compared to cholesteryl palmitate (GC/FID, Rtx-200MS).

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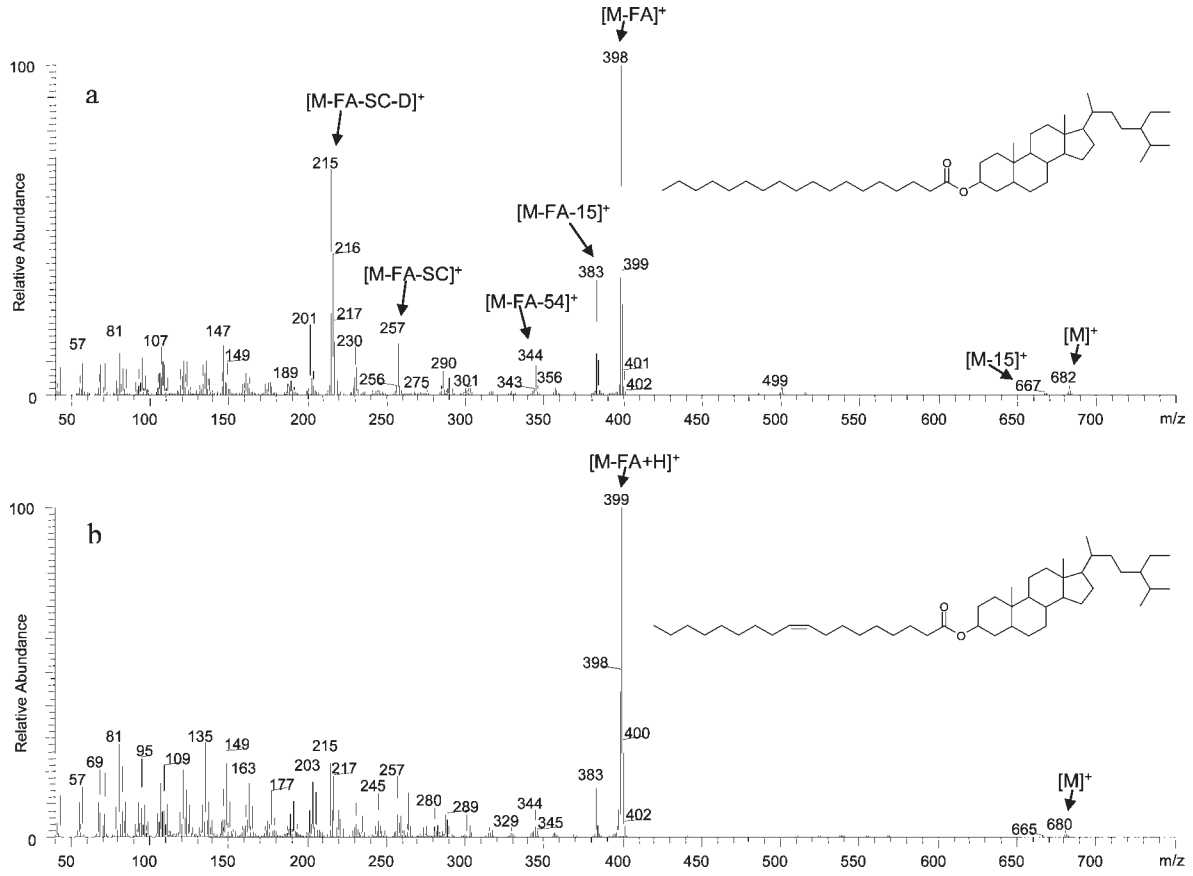

Figure 1. Electron-impact ionization mass spectra of sitostanyl stearate (a) and sitostanyl oleate (b); $[M]^+$, molecular ion; [FA], fatty acid; [SC], side chain; [D], ring D of sterol nucleus; [H], hydrogen.

employed; the achieved separation is shown in Figure 2. The synthesized esters could be distinguished according to their carbon number and, in the case of unsaturated fatty acid moieties, to the number of double bonds. Only the esters of saturated and monounsaturated fatty acids of the same chain length eluted at the same time.

GC/FID Calibration and Quantification. Under the employed gas chromatographic conditions, the plant stanyl esters eluted between 313 and 325 °C from the Rtx-200MS column. The instability of steryl esters, especially of those containing long chain polyunsaturated fatty acid moieties, during high temperature GC analysis is a well-known issue.[25−27] In order to compensate for the thermal degradation of plant stanyl esters, response factors were determined by analysis of an industrially employed plant stanol ester mixture (STAEST-115). The peak areas of the intact esters were compared to those of the respective fatty acid methyl esters obtained after transesterification of the stanyl ester mixture. This allowed the calculation of response factors for individual esters and for stanyl ester groups (Table 2). As expected, the degree of degradation increased with the number of double bonds in the acid moieties and with the time of elution from the column. On the basis of these data, the amounts of individual esters in STAEST-115 were calculated (Table 3). A calibration allowing the selective quantification of the individual esters was performed at concentrations ranging from 0.1 to 0.5 μg of STAEST-115/μL. The limits of detection (LOD) and the limits of quantification (LOQ) determined for the individual stanyl esters are shown in Table 3. The correlation coefficients (R^2) of the calculated calibration functions were in the range of 0.9985−0.9999. The GC/FID detector response was also linear to an additionally examined concentration of 0.8 μg of STAEST-115/μL.

Determination of Plant Stanyl Fatty Acid Esters in Skimmed Milk Drinking Yogurt. Skimmed milk drinking yogurts were selected to demonstrate the applicability of the established capillary gas chromatographic separation of plant stanyl esters. The advantage of this type of product is the lack of an interfering lipid matrix. According to the legal requirements in Germany, yogurt products from skimmed milk may contain up to 0.5% fat.[34] Most commercial skimmed milk yogurts exhibit total fat contents of only 0.1%, an amount that can be considered negligible regarding stanyl ester analysis. On the other hand, the protein contents of these products may hamper the quantitative extraction of lipids. Therefore, sample preparations were carried out by employing the extraction according to Weibull−Stoldt.[35] This classical method involves the "digestion" of the material under acidic conditions as a key step before the extraction of the lipids using organic solvent (Figure 3a). The gravimetrically determined total lipid contents corresponded well to the total amounts of esters determined via GC/FID quantification (Table 4). The capillary gas chromatographic separation of the stanyl esters isolated from the drinking yogurts is presented in Figure 4. The esters in the lipid extracts comprised sitostanyl and campestanyl derivatives, with sitostanyl C18-esters being the predominating fraction. The amounts of esterified stanols (3.02 and 3.16 g/100 g, respectively), calculated from the ester distributions, were in good agreement with the declarations of the products ("plant stanol 3.0 %"). To verify the

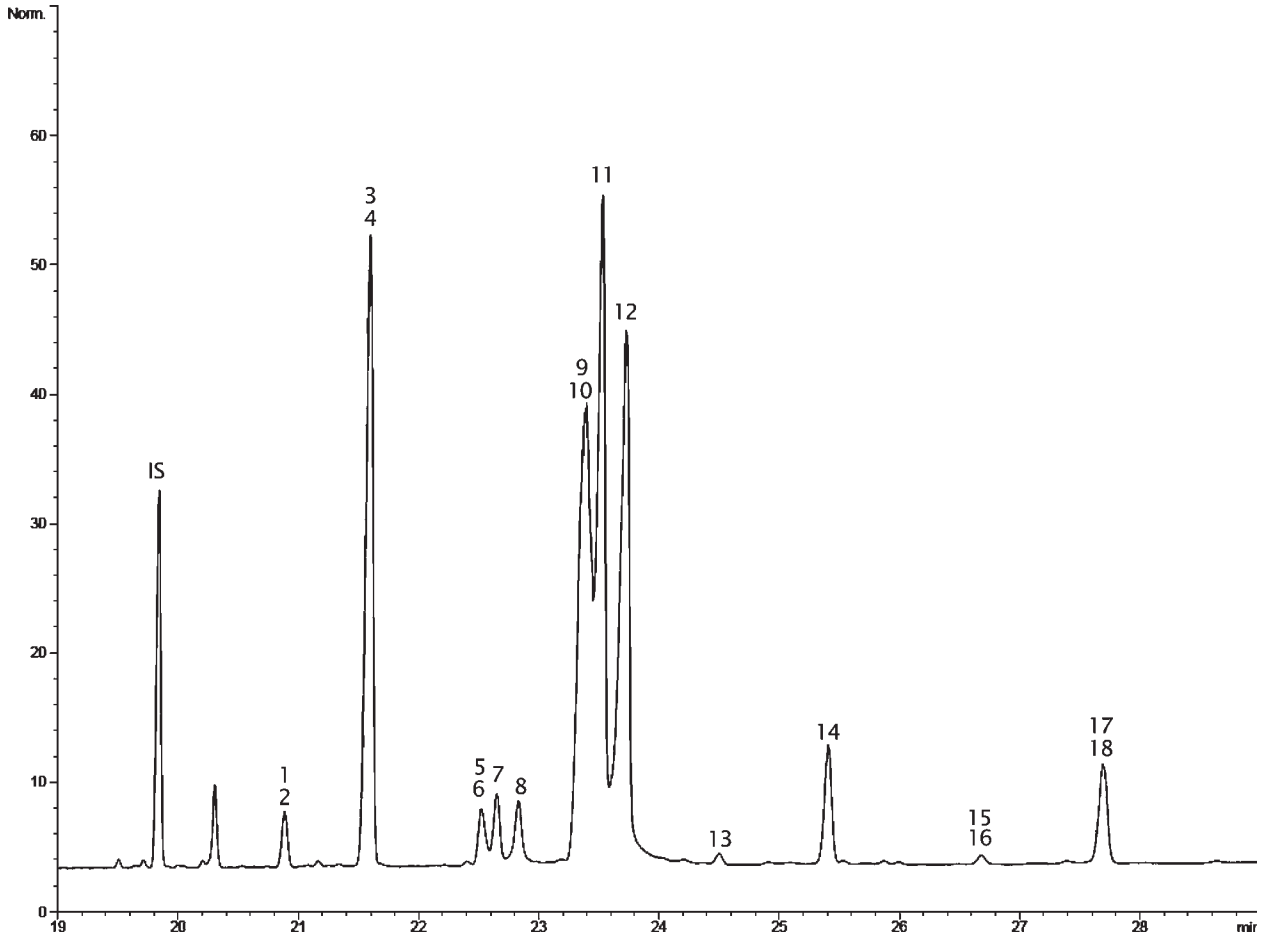

Figure 2. Capillary gas chromatographic separation of enzymatically synthesized stanyl fatty acid esters on an Rtx-200MS stationary phase; peak numbering according to Table 1; IS (internal standard), cholesteryl palmitate.

Table 2. GC/FID Response Factors (RF) for Plant Stanyl Fatty Acid Esters Determined by Analysis of an Industrially Employed Plant Stanyl Ester Mixture (STAEST-115)

fatty acid moieties	FAME[a] [area %]	stanyl esters[b] [wt %]	RF[c]
16:0/16:1	4.70	4.84	0.94
18:0/18:1/18:2	83.50	69.35	1.16
18:3	8.90	5.14	1.68
20:0/20:1	2.03	1.26	1.55
22:0/22:1	0.82	0.28	2.86

[a] Distribution of fatty acid methyl esters obtained after transesterification of the plant stanyl ester mixture STAEST-115. [b] Distribution of stanyl esters determined by GC/FID quantification of the mixture STAEST-115; stanyl esters represent 96.7% of total peak area. [c] Response factors calculated as RF = FAME [area %]/stanyl esters [wt %] × 0.967.

Table 3. Limits of Detection (LOD) and Limits of Quantification (LOQ) Determined by Analysis of an Industrially Employed Plant Stanyl Ester Mixture (STAEST-115)

plant stanyl ester	content[a] [g/100 g]	LOD[b] [μg/mL]	LOQ[b] [μg/mL]
campestanyl 16:0/16:1	0.5	0.05	0.15
sitostanyl 16:0/16:1	4.1	0.05	0.16
campestanyl 18:0/18:1	5.7	0.11	0.32
campestanyl 18:2	2.1	0.10	0.30
campestanyl 18:3	1.1	0.10	0.30
sitostanyl 18:0/18:1	54.2	0.14	0.41
sitostanyl 18:2	18.8	0.18	0.53
sitostanyl 18:3	7.5	0.43	1.28
sitostanyl 20:0/20:1	2.0	0.18	0.54
sitostanyl 22:0/22:1	0.8	0.27	0.80

[a] Determined by GC/FID quantification using the calculated response factors. [b] Determined according to ref 41.

developed calibration and quantification approach, the amounts of esterified stanols were also analyzed after alkaline hydrolysis of the lipid extracts (Table 4). The calculated values for campestanol and sitostanol as well as those obtained after alkaline hydrolysis were comparable to those reported in a yogurt from the Portuguese market.[36] Finally, the proportions of the fatty acid moieties were calculated; they indicated rapeseed oil as a fatty acid source of the esters.[37] The reproducibility of the Weibull—Stoldt extraction method is given in Table 6. Spiking of skimmed milk yogurt with the STAEST-115-mixture and subsequent analysis by GC/FID resulted in a recovery of 96.3 ± 1.7%.

Development of a "Fast Extraction Method". The sample preparation according to the Weibull—Stoldt method requires

about 12 h (Figure 3a). To accelerate the procedure and to render the approach more appropriate for routine analysis, the method was miniaturized. This involved, for example, reduction of the sample size and the volume of acid employed for digestion, filtration via syringe filter and extraction in vials with smaller amounts of solvent rather than in a Soxhlet apparatus (Figure 3b). These modifications allowed a triplicate analysis of drinking yogurts within two hours. This "fast extraction method" in combination with the GC/FID analysis gave excellent results regarding the recovery (100.1 ± 1.0%) of a stanyl ester mixture (STAEST-115) added to skimmed milk yogurt. No hydrolysis of esters during the workup procedure was observed. The repeatability of the method was demonstrated by triplicate analyses of all six bottles contained in commercially available drinking yogurt sales box. As shown for two drinking yogurt samples, the standard deviations of the analytical results obtained for the individual esters were very low (Table 5). The reproducibility of the results obtained after analysis of the same sample by different operators is shown in Table 6.

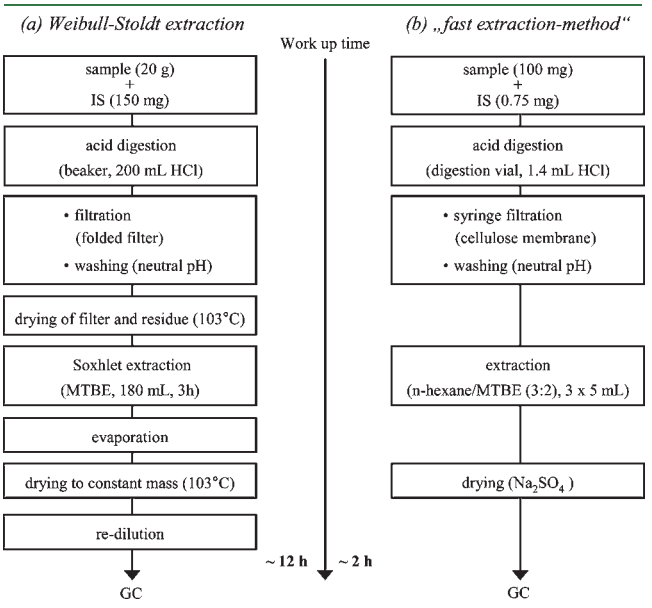

Figure 3. Comparison of the workup procedures according to the Weibull−Stoldt extraction (a) and the "fast extraction method" (b).

Determination of Plant Stanyl Fatty Acid Esters in Enriched Margarines. The investigated commercial margarines contained 32 and 55% fat, respectively, without the added phytostanols. Some lipids may interfere with the plant stanyl esters during the capillary gas chromatographic separation. Hence, a fractionation step before the GC analysis is crucial. Standard approaches are off-line techniques such as TLC, SPE, or HPLC.[17,27] An elegant alternative to perform the preseparation and the GC investigation in a single run is provided by an online coupled LC−GC system.[38]

The LC preseparation step was achieved on a silica gel column, as these are known to be very suitable for retaining triglycerides,[39] with *n*-hexane/MTBE (96 + 4, v + v) as eluent. The transfer

Table 4. Results Obtained after the Isolation of Lipids from Enriched Skimmed Milk Drinking Yogurts by Weibull−Stoldt Extraction and GC/FID Analysis

	drinking yogurt 1		drinking yogurt 2	
total lipids[a] [g/100 g]	5.1 ± 0.0		5.1 ± 0.0	
stanyl ester[b] [g/100 g]				
campestanyl 16:0/16:1	0.07 ± 0.00		0.07 ± 0.00	
sitostanyl 16:0/16:1	0.21 ± 0.00		0.21 ± 0.00	
campestanyl 18:0/18:1	0.69 ± 0.00		0.72 ± 0.02	
campestanyl 18:2	0.23 ± 0.00		0.25 ± 0.01	
campestanyl 18:3	0.09 ± 0.02		0.11 ± 0.01	
sitostanyl 18:0/18:1	2.31 ± 0.03		2.41 ± 0.04	
sitostanyl 18:2	0.87 ± 0.01		0.91 ± 0.02	
sitostanyl 18:3	0.27 ± 0.00		0.31 ± 0.01	
sitostanyl 20:0/20:1	0.08 ± 0.00		0.08 ± 0.00	
sitostanyl 22:0/22:1	0.03 ± 0.00		0.03 ± 0.00	
others[c]	0.07 ± 0.00		0.07 ± 0.00	
total esters	4.93 ± 0.02		5.16 ± 0.10	
esterified stanols [g/100 g]	3.02 ± 0.01[d]	3.03 ± 0.01[e]	3.16 ± 0.06[d]	3.05 ± 0.01[e]
campestanol[f] (%)	22.1 ± 0.4	21.4 ± 0.1	22.1 ± 0.4	21.7 ± 0.0
sitostanol[f] (%)	76.4 ± 0.5	76.5 ± 0.1	76.4 ± 0.3	76.7 ± 0.2
esterified fatty acids[g] (%)				
16:0/16:1	5.7 ± 0.1		5.4 ± 0.1	
18:0/18:1	60.8 ± 0.4		60.7 ± 0.1	
18:2	22.3 ± 0.3		22.4 ± 0.1	
18:3	7.3 ± 0.5		7.8 ± 0.2	
20:0/20:1	1.7 ± 0.0		1.6 ± 0.0	
22:0/22:1	0.7 ± 0.0		0.7 ± 0.0	

[a] Gravimetric determination; values represent average ± standard deviation. [b] GC/FID analysis; values represent average ± standard deviation. [c] Others: compounds calculated with RF = 1 (campestanyl-C20 and -C22 esters contained in STAEST-115 in amounts not allowing a calibration; minor unknown peaks). [d] Calculated on the basis of stanyl esters. [e] Determined by GC analysis after alkaline hydrolysis of the lipid extract. [f] Relative to total esterified stanols. [g] Relative to total esterified fatty acids.

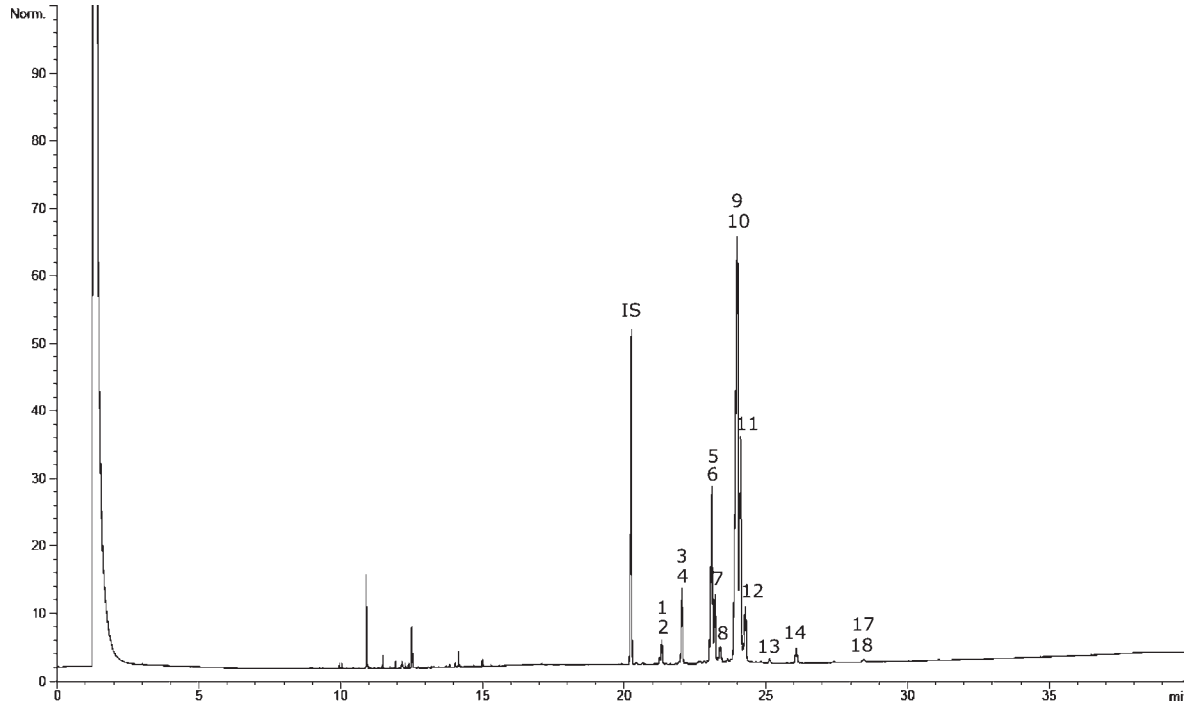

Figure 4. Analysis of stanyl esters extracted from an enriched skimmed milk drinking yogurt by means of GC/FID; peak numbering according to Table 1.

Table 5. Repeatability of the "Fast Extraction Method" by Means of GC/FID

	drinking yogurt 1[a]	drinking yogurt 2[a]
stanyl ester [g/100 g]		
campestanyl 16:0/16:1	0.07 ± 0.00	0.07 ± 0.00
sitostanyl 16:0/16:1	0.19 ± 0.00	0.17 ± 0.00
campestanyl 18:0/18:1	0.78 ± 0.01	0.86 ± 0.02
campestanyl 18:2	0.26 ± 0.00	0.29 ± 0.01
campestanyl 18:3	0.11 ± 0.00	0.15 ± 0.01
sitostanyl 18:0/18:1	2.41 ± 0.04	2.10 ± 0.06
sitostanyl 18:2	0.81 ± 0.04	0.84 ± 0.07
sitostanyl 18:3	0.28 ± 0.01	0.29 ± 0.01
sitostanyl 20:0/20:1	0.07 ± 0.00	0.08 ± 0.00
sitostanyl 22:0/22:1	0.02 ± 0.00	0.03 ± 0.00
others[b]	0.07 ± 0.01	0.10 ± 0.02
total esters	5.07 ± 0.07	4.98 ± 0.12
esterified stanols [g/100 g]	3.10 ± 0.05	3.05 ± 0.07

[a] Each of the six bottles contained in a commercial sales box was analyzed in triplicate; values represent average ± standard deviation of 18 analyses. [b] Calculated with RF = 1.

Table 6. Reproducibility Data on the Stanyl Ester Analysis in Skimmed Milk Drinking Yogurts

	operator 1	operator 2	operator 3
Weibull−Stoldt extraction[a]			
total lipids[b] [g/100 g]	5.10 ± 0.07	5.03 ± 0.01	5.05 ± 0.01
total esters[c] [g/100 g]	4.98 ± 0.11	4.92 ± 0.04	4.89 ± 0.06
esterified stanols[d] [g/100 g]	3.05 ± 0.07	3.01 ± 0.02	2.99 ± 0.04
"fast extraction method"[e]			
total esters[c] [g/100 g]	5.01 ± 0.19	5.11 ± 0.08	5.12 ± 0.04
esterified stanols[d] [g/100 g]	3.07 ± 0.11	3.12 ± 0.05	3.13 ± 0.02

[a] The combined skimmed milk drinking yogurts from six bottles contained in a commercial sales box were worked up in triplicate by three operators; values represent average ± standard deviation. [b] Gravimetric determination. [c] GC/FID analysis. [d] Calculated on the basis of stanyl esters. [e] One bottle of enriched skimmed milk drinking yogurt was worked up in triplicate by three operators; values represent average ± standard deviation.

window for stanyl esters was determined with synthesized references, which eluted about 4 min after injection. The loop type technique with fully concurrent solvent evaporation was used as interface. The GC separation (Figure 5) was comparable to that obtained by a single GC (Figure 4), only the resolution was slightly reduced: Long chain fatty acid esters of campestanol and sitostanol were well separated and the esters of unsaturated fatty acid moieties could be distinguished. Only the saturated and monounsaturated fatty acid esters eluted at the same time. The use of a silver ion LC column as recently described might be useful to solve this issue.[40] The identities of the esters were confirmed by means of online LC−GC/(EI)MS (data not shown).

Owing to the possibility to subject the lipid extract directly to online LC−GC without prior purification steps, the workup time for a triplicate analysis of a margarine was less than 0.5 h. This online LC−GC approach gave excellent results regarding the recovery (99.0 ± 2.1%) of a stanyl ester mixture (STAEST-115) added to a commercial margarine. The results obtained after investigation of four commercial margarines with added stanyl esters are shown in Table 7. The stanol contents calculated on the basis of the analyzed esters were in accordance with the package labeling. Comparable to the analyzed yogurt drink samples, the fatty acid moieties determined in the stanyl esters contained in the enriched margarines indicated rape seed oil as a fatty acid

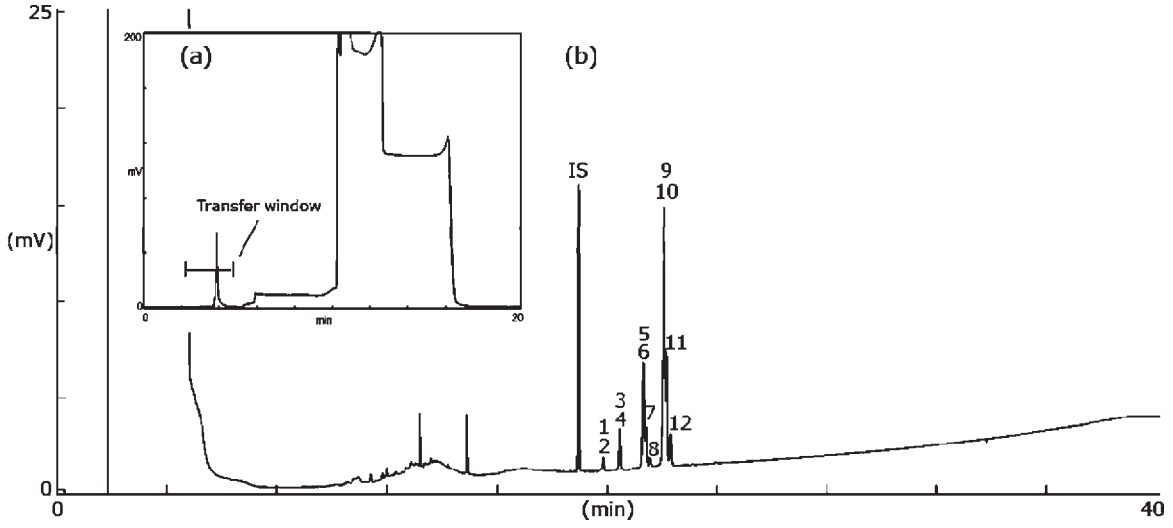

Figure 5. Analysis of stanyl esters in an enriched margarine by means of online LC−GC/FID; the transfer window is indicated in the LC/UV chromatogram (a), the GC/FID chromatogram (b) after online transfer of the stanyl ester fraction; peak numbering according to Table 1.

Table 7. Analysis of Plant Stanyl Esters in Enriched Margarines by Means of Online LC−GC/FID

	margarine 1[a]	margarine 2[a]	margarine 3[a]	margarine 4[a]	margarine 4[b]
stanyl ester [g/100 g]					
campestanyl 16:0/16:1	0.19 ± 0.00	0.22 ± 0.01	0.19 ± 0.01	0.18 ± 0.02	0.17 ± 0.02
sitostanyl 16:0/16:1	0.55 ± 0.01	0.60 ± 0.03	0.52 ± 0.02	0.40 ± 0.01	0.41 ± 0.06
campestanyl 18:0/18:1	2.04 ± 0.07	2.33 ± 0.11	2.05 ± 0.07	1.58 ± 0.04	1.60 ± 0.09
campestanyl 18:2	0.92 ± 0.03	1.11 ± 0.08	0.93 ± 0.02	0.57 ± 0.02	0.60 ± 0.05
campestanyl 18:3	0.35 ± 0.02	0.45 ± 0.03	0.37 ± 0.02	0.24 ± 0.00	0.24 ± 0.02
sitostanyl 18:0/18:1	6.71 ± 0.23	7.14 ± 0.29	6.46 ± 0.15	5.09 ± 0.07	5.12 ± 0.44
sitostanyl 18:2	2.41 ± 0.21	2.74 ± 0.04	2.27 ± 0.13	1.91 ± 0.03	1.94 ± 0.31
sitostanyl 18:3	0.86 ± 0.04	0.98 ± 0.01	0.85 ± 0.03	0.71 ± 0.01	0.70 ± 0.05
campestanyl 20:0/20:1	–[c]	–	–	0.06 ± 0.01	0.05 ± 0.01
sitostanyl 20:0/20:1	0.05 ± 0.01	–	0.05 ± 0.03	0.15 ± 0.02	0.16 ± 0.02
total esters	14.1 ± 0.5	15.6 ± 0.6	13.7 ± 0.3	10.9 ± 0.1	11.0 ± 0.8
esterified stanols[d] [g/100 g]	8.6 ± 0.3	9.5 ± 0.4	8.4 ± 0.2	6.7 ± 0.1	6.7 ± 0.5
campestanol[c] (%)	24.9 ± 0.4	26.3 ± 0.4	25.8 ± 0.2	24.1 ± 0.3	24.3 ± 1.5
sitostanol[c] (%)	75.1 ± 0.4	73.7 ± 0.4	74.2 ± 0.2	75.9 ± 0.3	75.7 ± 1.5
esterified fatty acids[d] (%)					
16:0/16:1	5.2 ± 0.2	5.3 ± 0.1	5.2 ± 0.3	5.3 ± 0.2	5.2 ± 0.3
18:0/18:1	62.2 ± 1.4	60.8 ± 0.5	62.1 ± 0.4	61.3 ± 0.7	61.3 ± 1.7
18:02	23.7 ± 1.2	24.7 ± 0.3	23.4 ± 0.6	22.7 ± 0.6	23.1 ± 1.9
18:03	8.6 ± 0.2	9.2 ± 0.2	8.9 ± 0.1	8.7 ± 0.1	8.6 ± 0.4
20:0/20:1	0.4 ± 0.0	–	0.4 ± 0.2	1.9 ± 0.2	2.0 ± 0.2

[a] Values represent average ± standard deviation; each package was worked up in triplicate. [b] Repeatability determined by the triplicate analysis of each of the 8 packages contained in the commercial sales box, values represent average ± standard deviation of 24 analyses. [c] Not determined. [d] Calculated on the basis of stanyl esters.

source. The repeatability of the method was verified by triplicate analyses of all eight packages of margarine 4 contained in the commercially used sales box (Table 7). The reproducibility of the results obtained after the extraction of the same sample by different operators is shown in Table 8.

In conclusion, the combination of a "fast extraction" under acidic conditions with high temperature GC using a medium polar stationary phase proved to be suitable for the analysis of intact plant stanyl esters in low-fat products. A remaining limitation of the approach is the coelution of saturated and monounsaturated fatty acid esters of the same chain length rendering them undistinguishable under the employed GC conditions. For fat-based foods, online coupled LC−GC offers an elegant and efficient way to avoid time-consuming sample preparation steps. Using commercial skimmed milk drinking yogurts and margarines with added plant stanyl esters as examples, the developed workup procedures were shown to be easily performed, robust and fast. For analysis of semiskimmed products containing proteins, e.g.

Table 8. Reproducibility of the Online LC−GC Approach

	operator 1	operator 2	operator 3
total esters [g/100 g]	12.5 ± 0.7	13.4 ± 0.7	12.7 ± 0.4
esterified stanols [g/100 g]	7.6 ± 0.5	8.2 ± 0.4	7.7 ± 0.3

a

b

One package of margarine 1 was worked up in triplicate by three operators; values represent average ± standard deviation. Calculated on the basis of stanyl esters.

enriched milk, a combination of both the fast extraction step and the online LC−GC analysis of the intact esters would be suitable. The methods result in information on the identities and contents of individual plant stanyl esters. Thus, a basis for the authenticity assessment of this type of functional food ingredients by means of capillary gas chromatography is provided. Preliminary studies demonstrated that the approaches are also applicable to the analysis of plant steryl esters. Therefore, further work is in progress to extend the methods to other types of enriched foods containing mixtures of plant steryl and stanyl esters.

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REFERENCES

(1) Brufau, G.; Canela, M. A.; Rafecas, M. Phytosterols: physiologic and metabolic aspects related to cholesterol-lowering properties. *Nutr. Res. (New York, NY, United States)* **2008**, *28* (4), 217–225.

(2) Katan, M. B.; Grundy, S. M.; Jones, P.; Law, M.; Miettinen, T.; Paoletti, R. Efficacy and safety of plant stanols and sterols in the management of blood cholesterol levels. *Mayo Clin. Proc.* **2003**, *78* (8), 965–978.

(3) Plat, J.; Kerckhoffs, D. A. J. M.; Mensink, R. P. Therapeutic potential of plant sterols and stanols. *Curr. Opin. Lipidol.* **2000**, *11* (6), 571–576.

(4) Thompson, G. R.; Grundy, S. M. History and Development of Plant Sterol and Stanol Esters for Cholesterol-Lowering Purposes. *Am. J. Cardiol.* **2005**, *96* (1A), 3D–9D.

(5) SCF. Opinion of the Scientific Committee on Food on a request for the safety assessment of the use of phytosterol esters in yellow fat spreads. 2000.

(6) EFSA. Consumption of food and beverages with added plant sterols in the European Union. *EFSA J.* **2008**, (*133*), 1−21.

(7) Weber, N.; Mukherjee, K. D. Plant sterols and steryl esters in functional foods and nutraceuticals. *Nutraceutical Sci. Technol.* **2006**, *5* (Nutraceutical and Specialty Lipids and Their Co-Products), 483−508.

(8) SCF. Opinion of the Scientic Committee on Food on an application from ADM for approval of plant sterol-enriched foods. 2003.

(9) SCF. Opinion of the Scientific Committee on Food on Applications for Appproval of a Variety of Plant Sterol-Enriched Foods. 2003.

(10) AOCS. Determination of the Composition of the Sterol Fraction of Animal and Vegetable Oils and Fats by TLC and Capillary GLC (Method Ch. 6-91). *Off. Methods and Recomm. Pract. Am. Oil Chem. Soc.* **1991**.

(11) Naudet, M.; Hautfenne, A. Standard method for the determination of total sterols in fats and oils. *Pure Appl. Chem.* **1985**, *57* (6), 899–904.

(12) ISO 6799: Determination of composition of the sterol fraction of fats and oils by gas-liquid chromatography; International Organization for Standardization: 1991.

(13) Brown, A. W.; Hang, J.; Dussault, P. H.; Carr, T. P. Plant sterol and stanol substrate specificity of pancreatic cholesterol esterase. *J. Nutr. Biochem.* **2010**, *21* (8), 736–740.

(14) Rasmussen, H. E.; Guderian, D. M., Jr.; Wray, C. A.; Dussault, P. H.; Schlegel, V. L.; Carr, T. P. Reduction in cholesterol absorption is enhanced by stearate-enriched plant sterol esters in hamsters. *J. Nutr.* **2006**, *136* (11), 2722–2727.

(15) Santos, R.; Limas, E.; Sousa, M.; Castilho, M. d. C.; Ramos, F.; Noronha da Silveira, M. I. Optimization of analytical procedures for GC-MS determination of phytosterols and phytostanols in enriched milk and yoghurt. *Food Chem.* **2007**, *102* (1), 113–117.

(16) Laakso, P. Analysis of sterols from various food matrices. *Eur. J. Lipid Sci. Technol.* **2005**, *107* (6), 402–410.

(17) Lagarda, M. J.; Garcia-Llatas, G.; Farre, R. Analysis of phytosterols in foods. *J. Pharm. Biomed. Anal.* **2006**, *41* (5), 1486–1496.

(18) Clement, L. M.; Hansen, S. L.; Costin, C. D.; Perri, G. L. Quantitation of Sterols and Steryl Esters in Fortified Foods and Beverages by GC/FID. *J. Am. Oil Chem. Soc.* **2010**, *87* (9), 973–980.

(19) Mezine, I.; Zhang, H.; Macku, C.; Lijana, R. Analysis of Plant Sterol and Stanol Esters in Cholesterol-Lowering Spreads and Beverages Using High-Performance Liquid Chromatography-Atmospheric Pressure Chemical Ionization-Mass Spectroscopy. *J. Agric. Food Chem.* **2003**, *51* (19), 5639–5646.

(20) Evershed, R. P.; Male, V. L.; Goad, L. J. Strategy for the analysis of steryl esters from plant and animal tissues. *J. Chromatogr.* **1987**, *400*, 187–205.

(21) Gordon, M. H.; Griffith, R. E. Steryl ester analysis as an aid to the identification of oils in blends. *Food Chem.* **1991**, *43* (1), 71–8.

(22) Gunawan, S.; Melwita, E.; Ju, Y.-H. Analysis of trans-cis fatty acids in fatty acid steryl esters isolated from soybean oil deodoriser distillate. *Food Chem.* **2010**, *121* (3), 752–757.

(23) Kamm, W.; Dionisi, F.; Fay, L. B.; Hischenhuber, C.; Schmarr, H. G.; Engel, K. H. Analysis of steryl esters in cocoa butter by on-line liquid chromatography-gas chromatography. *J. Chromatogr., A* **2001**, *918* (2), 341–349.

(24) Caboni, M. F.; Iafelice, G.; Pelillo, M.; Marconi, E. Analysis of Fatty Acid Steryl Esters in Tetraploid and Hexaploid Wheats: Identification and Comparison between Chromatographic Methods. *J. Agric. Food Chem.* **2005**, *53* (19), 7465–7472.

(25) Evershed, R. P.; Prescott, M. C.; Goad, L. J. Deuteration as an aid to the high-temperature gas chromatography-mass spectrometry of steryl fatty acyl esters. *J. Chromatogr.* **1992**, *590* (2), 305–12.

(26) Lechner, M.; Reiter, B.; Lorbeer, E. Determination of free and esterified sterols in potentials new oil seed crops by coupled online liquid chromatography-gas chromatography. *Fett/Lipid* **1999**, *101* (5), 171–177.

(27) Duchateau, G. S. M. J. E.; Janssen, H.-G. M.; Louter, A. J. H., Plant sterol analysis in relation to functional foods. *Nutraceutical Sci. Technol.* **2004**, *1*, (Phytosterols as Functional Food Components and Nutraceuticals), 75−131.

(28) Grob, K.; Lanfranchi, M.; Mariani, C., Evaluation of Olive oils through the fatty alcohols, the sterols and their esters by coupled LC-GC. *JAOCS* **1990**, *67* (10).

(29) Lusby, W. R.; Thompson, M. J.; Kochansky, J. Analysis of sterol esters by capillary gas chromatography-electron impact and chemical ionization-mass spectrometry. *Lipids* **1984**, *19* (11), 888–901.

(30) Evershed, R. P.; Prescott, M. C.; Spooner, N.; Goad, L. J. Negative ion ammonia chemical ionization and electron impact ionization mass spectrometric analysis of steryl fatty acyl esters. *Steroids* **1989**, *53* (3−5), 285–309.

(31) Vu, P.-L.; Shin, J.-A.; Lim, C.-H.; Lee, K.-T. Lipase-catalyzed production of phytosteryl esters and their crystallization behavior in corn oil. *Food Res. Int.* **2004**, *37* (2), 175–180.

(32) Budzikiewicz, H.; Djerassi, C.; Williams, D. H. *Steroids, terpenoids, sugars, and miscellaneous classes*; Holden-Day: San Francisco, 1964; Vol. ll.

(33) Rahier, A.; Benveniste, P., Mass spectral identification of phytosterols. In *Analysis of Sterols and Other Biologically Significant Steroids*; Nes, W. D., Parish, E. J., Eds.; Academic Press, Inc.: San Diego, 1989; pp 223−250.

(34) Verordnung über Milcherzeugnisse *(Milcherzeugnisverordnung - MilchErzV) vom 21. Dezember 2007 (BGBl. I S. 3282)*

(35) Bestimmung des Fettgehaltes von Milchprodukten - Verfahren nach Weibull. *Amtliche Sammlung von Untersuchungsverfahren nach* § *64 LFGB: Untersuchung von Lebensmitteln L-02.00−11* 2005.

(36) Saraiva, D.; Castilho, M. d. C.; Martins, M. d. R.; da Silveira, M. I. N.; Ramos, F. Evaluation of Phytosterols in Milk and Yogurts Used as Functional Foods in Portugal. *Food Anal. Methods* **2011**, *4*, 28–34.

(37) Standard for Named Vegetable Oils. *Codex-Stan 210*, 1999.

(38) Hyotylainen, T.; Riekkola, M.-L. On-line coupled liquid chromatography-gas chromatography. *J. Chromatogr., A* **2003**, *1000* (1−2), 357–384.

(39) Grob, K.; Kaelin, I.; Artho, A. Coupled LC-GC: the capacity of silica gel (HP)LC columns for retaining fat. *J. High Resolut. Chromatogr.* **1991**, *14* (6), 373–6.

(40) Momchilova, S. M.; Nikolova-Damyanova, B. M. Separation of isomeric octadecenoic fatty acids in partially hydrogenated vegetable oils as p-methoxyphenacyl esters using a single-column silver ion high-performance liquid chromatography (Ag-HPLC). *Nat. Protoc.* **2010**, *5*, 473–478.

(41) Chemische Analytik - Nachweis-, Erfassungs- und Bestimmungsgrenze unter Wiederholbedingungen - Begriffe, Verfahren, Auswertung; *DIN 32645*. 2008−11.