Determination by GC-IT/MS of Phytosterols in Herbal Medicinal Products for the Treatment of Lower Urinary Tract Symptoms and Food Products Marketed in Europe

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Key words
- gas chromatography ion trap mass spectrometry
- Δ^7-phytosterols
- pumpkin seed extracts
- saw palmetto extracts
- lower urinary tract symptoms (LUTS)

Abstract

A method for the determination of phytosterols in herbal medicinal products for the treatment of lower urinary tract symptoms and food products is described here. Using a convenient sample preparation protocol and sensitive gas chromatography ion trap mass spectrometry analysis, ten different sterols, among them five Δ^7-phytosterols as typical constituents of pumpkin seed preparations, could be identified and quantified. This protocol was applied to the analysis of 31 marketed products, from which seven were raw materials.

Abbreviations

BPH:	benign prostatic hyperplasia
CS:	calibration standard
DHT:	5α-dihydrotestosterone
dSPE:	dispersive solid phase extraction
HMP:	herbal medicinal products
IPSS:	International Prostate Symptom Score
IS:	internal standard
IT:	ion trap
LUTS:	lower urinary tract symptoms
MSTFA:	*N*-methyl-*N*-trimethylsilyltrifluoroacetamide
M*t*BE:	methyl *tert*-butylether
RRT:	relative retention time
PSA:	primary secondary amine
RSD:	relative standard deviation
S/N:	signal/noise ratio
TMS:	trimethylsilyl
TSIM:	*N*-trimethylsilylimidazole
WS:	working standard

Supporting information available online at http://www.thieme-connect.de/products

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Introduction

For more than 50 years, HMP and food products containing phytosterols have been used in Europe for the treatment of LUTS such as BPH and overactive bladder. Mainly products with preparations out of pumpkin seeds (*Cucurbita* L. species, Cucurbitaceae), saw palmetto fruit [*Serenoa repens* (Bart.) Small, Arecaceae], and nettle root (*Urtica dioica* L., Urticaceae) are used, as well as *β*-sitosterol (**8**, **Fig. 1**) and other poorly defined phytosterol preparations (**Table 1**) [1]. In the United States, saw palmetto ranked third among the herbal dietary supplements sold, with sales of over $18 million in 2011 [2]. Pharmacological and clinical investigations on saw palmetto and stinging nettle extracts have been reviewed by Koch [3]. However, in a recent study, a saw palmetto extract marketed in the US was found to be not more active than a placebo [4].

The Δ^7-phytosterols (**6, 7, 9, 10**, and **12**), typical constituents of pumpkin seeds, are assumed to be effective LUTS therapy due to an inhibition of DHT binding at cellular androgen receptors in the prostate [5]. Urinary obstructive symptoms are improved [5] and a clinical reduction of the IPSS [6], or at least a better quality of life, is achieved [7]. Δ^5-Phytosterols from stinging nettle inhibit the Na^+,K^+-ATPase from BPH cells [8].

Due to the heterogeneity of the phytosterol preparations on the market, an analytical tool is needed for the exact analysis of the sterol compositions as a basis for quality control and estimation of phytoequivalence and comparability of clinical data.

So a differentiation between unique Δ^7-phytosterols and the ubiquitous Δ^5-phytosterol *β*-sitosterol (**8**) and others (**2, 4**, and **5**) was of special interest in our present investigation. Penugonda [9] already analyzed the Δ^5-phytosterols campesterol

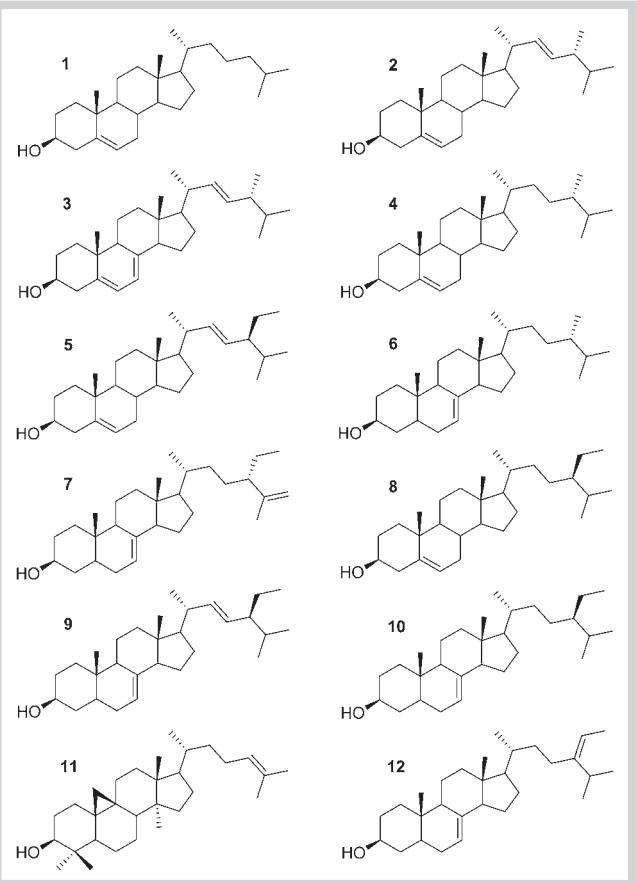

Fig. 1 Overview of the detected sterols. The sterols were analyzed as their corresponding TMS ethers. Cholesterol was used as a calibration standard (CS) and ergosterol was used as an internal standard (IS) (for detailed information about the analyzed sterols, see **Table 3**).

(**4**), stigmasterol, (**5**), and *β*-sitosterol (**8**), but not the Δ^7-phytosterols, in 20 commercially available saw palmetto supplements from the US market.

In our investigation, 31 products, from which seven were raw materials, originating from various medicinal plants (**Table 1**) were surveyed, with an emphasis on the content of both Δ^5- and Δ^7-phytosterols.

The phytosterols of each sample were separated after alkaline hydrolysis by liquid/liquid extraction, and analyzed as their corresponding TMS ethers by GC-IT/MS, because the sensitivity in scan mode is much higher with IT instruments than with quadrupole instruments [10, 11]. Gas chromatography is well established for the analysis of sterols with FID [11–14] and MS detection [9, 11, 15–17].

Results and Discussion

Pumpkin seeds, pumpkin seed oil, and phytosterol-containing preparations (hard and soft gelatin capsules, tablets) from various medicinal plants, among them numerous multicomponent preparations, were analyzed for their phytosterol content. We were able to detect ten different phytosterols, which among them were five Δ^7-phytosterols (**6, 7, 9, 10**, and **12**), in the samples (**Table 1**). The average dosage weight (unprocessed pumpkin seeds and oil excluded; n = 31) was 753 mg (ranging from 250–1400 mg). The recommended daily intake was between one and six dosages per day. The average measured content of phytosterols in mg/dosage was 6.5 mg (ranging from 0.0–59.3 mg). The average content of non-Δ^7-phytosterols per dosage was 2.7 mg (ranging from 0.0–58.4 mg) and the average content of Δ^7-phytosterols per dosage was 1.8 mg (ranging from 0.0–15.4 mg). Detailed information on the composition of each dosage, its origin, and phytosterol content (in mg/dosage) can be found in **Table 1**. The distribution of the detected phytosterols in mg/100 g is shown in **Table 2**.

The ubiquitary phytosterol campesterol (**4**) was detected in 27 samples. *β*-Sitosterol (**8**) was the quantitatively predominating phytosterol among the non-Δ^7-phytosterols and was detected in significant amounts in most (e.g., **S24, S27, S37**, and **S38**), but not all (e.g., **S20** and **S22**) preparations for which this sterol or phytosterol was declared as a main component. It was detected in 23 samples in a range from 2–9016 mg/100 g.

As expected, Δ^7-phytosterols were found as typical components of pumpkin seeds. Surprisingly, a few pumpkin seed preparations (**S13, S24**, and **S29**) contained only non-Δ^7-phytosterols. $\Delta^{7,25}$-Stigmastadienol (**7**) was the main Δ^7-phytosterol in all samples (n = 22) and ranged from 12–993 mg/100 g. The most ubiquitary Δ^7-phytosterol was Δ^7-avenasterol (**12**; n = 25). Cycloartenol (**11**) [18] was detected in five out of ten saw palmetto extract soft capsules and in **S18**, a preparation containing pumpkin seeds, the oil of stinging nettles, and flaxseed oil. No other pumpkin seed preparation contained cycloartenol (**11**).

In conclusion, we have worked out a convenient extraction and isolation method, which in combination with sophisticated GC-IT/MS techniques, is suitable for analyzing phytosterols in complex matrices, like unprocessed material (pumpkins seeds, pumpkin seed oil, and medicinal dosages). Ten phytosterols were rapidly identified and quantified in diverse HMP used for the treatment of LUTS using this protocol (**Fig. 2**). This new, comprehensive analysis of phytosterols (Δ^5 as well as Δ^7) provides reliable data for quality control in phytosterol-containing plant material, food, and pharmaceutical preparations, and for the estimation of phytoequivalence. The analytical data achievable by this protocol should be the basis for future well-grounded pharmacological and clinical investigations.

Materials and Methods

Chemicals and reagents

The silylation reagents TSIM and MSTFA were purchased from Macherey Nagel. PSA, particle size 40 µm, for dSPE was from Agilent. Deionized water was self-prepared with an in-house ion exchanger as well as the sodium hydroxide solutions (2 and 4 mol/L). M*t*BE was distilled before use. Methanol, sodium chloride, and anhydrous sodium sulfate were purchased in HPLC grade or in *pro analysis* quality from Sigma-Aldrich. The commercial reference sterols cholesterol (**1**; purity > 99% by an undeclared method), ergosterol (**3**; purity > 95% by HPLC), *β*-sitosterol (**8**; purity > 95% by an undeclared method), and stigmasterol (**5**; purity 90% by an undeclared method) were obtained from Sigma-Aldrich.

Table 1 Overview of analyzed herbal medicinal products and food products **S1–S38** and content of phytosterols in mg/dosage.

	Ingredients [mg]												Content of phytosterols [mg/dosage ± SD] (n = 6)		
Sample	Pumpkin seeds	Pumpkin seed oil	Pumpkin seed extract	Pumpkin seed powder	Saw palmetto extract	Stinging nettle	Others	Dosage form	Country	Status	Daily dose	Average dosage weight [mg] (n = 10)	Total	Not Δ^7	Δ^7
S1	1000							I)	unknown	A	n. q.	n. q.	1.0 ± 0.1	n. d.	1.0 ± 0.1
S2	1000							I)	Austria	A	n. q.	n. q.	0.8 ± 0.2	n. d.	0.8 ± 0.2
S3	1000							I)	Austria	A	n. q.	n. q.	0.7 ± 0.1	n. d.	0.7 ± 0.1
S4	1000							I)	Austria	A	n. q.	n. q.	0.7 ± 0.1	n. d.	0.7 ± 0.1
S5	1000							I)	Germany	A	n. q.	n. q.	3.3 ± 0.7	0.1 ± 0.0	3.3 ± 0.7
S6	1000							I)	Germany	C	n. q.	n. q.	4.4 ± 1.9	0.1 ± 0.0	4.4 ± 2.1
S7	400	340						II)	Germany	C	4	859	5.9 ± 2.4	0.1 ± 0.0	6.0 ± 2.6
S8	400	340						III)	Germany	C	3	1144	3.2 ± 0.7	0.1 ± 0.0	3.1 ± 0.7
S9	400	340			75			II)	Germany	C	3	1049	4.8 ± 2.0	0.1 ± 0.0	4.7 ± 2.2
S10	380				160	170*1)		III)	Nether-lands	B	1	1355	26.6 ± 6.6	26.0 ± 7.2	0.6 ± 0.1
S11	105							II)	Germany	C	3	357	0.1 ± 0.1	n. d.	0.1 ± 0.1
S12	100				160		a)	IV)	Belgium	B	1	1237	3.6 ± 1.0	3.6 ± 1.1	n. d.
S13	100				150	50*2)	b)	II)	Czech/Slovak Republic	B	2	611	3.5 ± 1.0	3.5 ± 1.1	n. d.
S14		1000						V)	Germany	A	n. q.	n. q.	11.1 ± 3.7	0.1 ± 0.0	11.0 ± 4.0
S15		500						III)	France	B	3	707	2.6 ± 0.8	0.1 ± 0.0	2.5 ± 0.9
S16		500					c)	III)	Spain	B	1	931	4.4 ± 1.4	1.5 ± 0.5	2.9 ± 1.0
S17		400					d)	III)	Poland	B	2	1285	2.3 ± 0.7	0.7 ± 0.3	1.5 ± 0.5
S18		400				150	e)	III)	Poland	B	1	1400	2.4 ± 0.5	1.1 ± 0.2	1.4 ± 0.3
S19		350			50		f)	III)	Poland	B	2	1018	1.9 ± 1.1	0.7 ± 0.4	1.2 ± 0.7
S20		350						III)	Poland	B	6	514	2.2 ± 0.5	n. d.	2.1 ± 0.5
S21		300			30	250	g)	III)	Poland	B	1	795	2.3 ± 0.7	0.6 ± 0.2	1.6 ± 0.6
S22		250					h)	III)	Poland	B	4	726	0.8 ± 0.3	n. d.	0.8 ± 0.3
S23		60		50	7.2			III)	Germany	C	4	418	1.1 ± 0.4	0.4 ± 0.2	0.6 ± 0.3
S24		25					i)	III)	France	B	2	818	47.1 ± 16.5	46.7 ± 17.9	0.4 ± 0.1
S25			500*3)					II)	Germany	C	2	611	15.5 ± 2.5	0.1 ± 0.0	15.4 ± 2.7
S26			500					IV)	Austria	B	1	685	< 0.0 ± 0.0	< 0.0 ± 0.0	< 0.0 ± 0.0
S27			262.5		80		j)	IV)	England	B	2	1130	13.7 ± 4.2	13.5 ± 4.5	0.2 ± 0.1
S28			50			75*4)	k)	IV)	Poland	B	1	653	< 0.0 ± 0.0	n. d.	< 0.0 ± 0.0
S29				380				II)	Austria	B	3	515	< 0.0 ± 0.0	< 0.0 ± 0.0	n. d.
S30					320			III)	Austria	C	1	494	1.1 ± 0.2	1.1 ± 0.2	n. d.
S31					320			III)	Germany	C	1	510	1.1 ± 0.2	1.1 ± 0.2	n. d.
S32					320			III)	Germany	C	1	479	1.3 ± 0.5	1.3 ± 0.5	n. d.
S33					320			III)	Poland	C	1	514	1.3 ± 0.4	1.3 ± 0.4	n. d.
S34					320*5)			III)	UK	C	1	492	1.2 ± 0.3	1.2 ± 0.3	n. d.
S35					160			II)	France	C	2	529	1.0 ± 0.5	1.0 ± 0.5	n. d.

cont.

Table 1 *Continued*

Sample	Ingredients [mg]										Content of phytosterols [mg/dosage ± SD] (n = 6)		
	Pumpkin seeds	Pumpkin seed oil	Pumpkin seed extract	Pumpkin seed powder	Saw palmetto extract	Stinging nettle	Others	Dosage form	Country	Status	Daily dose	Average dosage weight [mg] (n = 10)	Total	Not Δ^7	Δ^7
S36					160	120		III)	Germany	C	2	684	1.5 ± 0.4	1.5 ± 0.4	n. d.
S37							l)	II)	Germany	C	3	250	13.8 ± 8.3	13.7 ± 9.0	0.1 ± 0.1
S38							m)	III)	Germany	C	2	594	59.3 ± 30.9	58.4 ± 33.4	0.9 ± 0.4

a) tea plant leaves [*Camellia sinensis* (L.) Kuntze, Theaceae] 150 mg; b) smallflower hairy willowherb (*Epilobium parviflorum* Schreb., Onagraceae) 50 mg; c) tomato extract (*Solanum lycopersicum* L., Solanaceae) 75 mg, grape extract (*Vitis vinifera* L., Vitaceae) 30 mg; d) ginseng extract (*Panax ginseng* L.; Araliaceae) 50 mg, fish oil 120 mg; e) flaxseed oil (*Linum usitatissimum* L., Linaceae) 300 mg; f) fish oil 100 mg; g) bearberry [*Arctostaphylos uva-ursi* (L.) Spreng., Ericaceae] 50 mg; h) fish oil 250 mg; i) pomegranate fruit (*Punica granatum* L., Lythraceae) 41.25 mg, tomato extract (*Solanum lycopersicum* L., Solanaceae) 10 mg, β-sitosterol 30 mg; j) soya extract [*Glycine max* (L.) Merr., Fabaceae] 50 mg, tomato extract (*Solanum lycopersicum* L., Solanaceae) 50 mg; k) muira puama tree dry extract (*Ptychopetalum olacoides* Benth., Olacaceae) 80 mg; l) phytosterols 10 mg; m) phytosterols 65 mg; dosage form: I) seeds, II) hard gelatin capsules, III) soft gelatin capsules, IV) tablets, V) oil; A: raw material; B: food product; C: medicine; *1) stinging nettle extract; *2) nettle root extract; *3) pumpkin seed soft extract (extraction solvent 92% ethanol); *4) nettle leaves dry extract; *5) saw palmetto fruit extract; < 0.0 content of phytosterols below 0.04 mg/dosage; n. d. not detected; n. q. no quantity (dosage of 1000 mg)

Standard solutions

WSs were prepared according to SANCO guidelines [19] for cholesterol (**1**; 0.5 mg/mL) as a calibration standard and for ergosterol (**3**; 0.5 mg/mL) as an internal standard. The compounds were dissolved in M*t*BE and stored at 5 °C. Before use, the solutions were tempered at room temperature for 1 h and shaken well. Solutions of β-sitosterol (**8**) and stigmasterol (**5**) containing 0.01 mg/mL in M*t*BE were prepared for peak identification.

Sample preparation equipment

The 10 mL headspace vials and the 1.5 mL autosampler vials were purchased from Macherey Nagel. The 2.0 mL plastic microfuge safe-lock tubes were from Eppendorf. All other consumables were from VWR. Dissolution of capsules and saponification steps at elevated temperatures were performed in a laboratory drying cabinet from WTB Binder Labortechnik. The mixing step was managed on a Vortex Genie 2 from Scientific Apparatus. Separating steps were carried out by using a Megafuge 1.0R from Heraeus/Kendro for 15 mL plastic centrifuge tubes, and an Eppendorf 5415 D centrifuge for the plastic microfuge safe-lock tubes.

Sample acquisition

Herbal medicinal products marketed in Europe and used for LUTS and food products were obtained from internet shops, local supermarkets, and pharmacies (n = 38; ◉ **Table 1**). The preparations were mainly out of pumpkin seeds (n = 28), saw palmetto fruits (n = 17), and nettle roots (n = 6). The dosage forms were raw seeds (n = 6), oil (n = 1), hard capsules (n = 19), soft capsules (n = 8), and tablets (n = 4) (◉ **Table 3**).

Extraction and isolation

The raw seeds and tablets were ground in a common household mill before extraction. For analysis, one dosage for each batch (1 g of raw material; n = 38; six batches were analyzed), respectively, was transferred into a 10 mL headspace vial; 2.5 mL sodium hydroxide solution (4 mol/L) and 2.5 mL methanol were added for dissolution of the capsule and for saponification of the sterol esters. The vial was flooded with nitrogen, closed tightly, and stored at 60 °C for 2 h. After cooling down, 1.0 mL ergosterol solution (internal standard, 0.5 mg/mL) was added and gently shaken. The homogeneous mixture was transferred to into a 15 mL plastic centrifuge tube. The headspace vial was rinsed with 2.5 mL distilled water and 1.5 mL M*t*BE. For phase separation, sodium chloride (1 g) was added to the suspension, shaken vigorously for 1 min, and centrifuged at 2500 × *g* for 3 min. One milliliter of the organic phase was transferred into a second 15 mL plastic centrifuge tube containing 3.0 mL sodium hydroxide solution (2 mol/L). The headspace vial was rinsed a second time with another 2.5 mL M*t*BE, and the mixture was extracted a second time in the same manner. After centrifugation, 2.0 mL of the organic phase were transferred into the second 15 mL plastic centrifuge tube. The combined organic extract was vigorously shaken for 1 min and centrifuged as described above. Then 150 µL of the organic upper layer was transferred into a 2.0 mL plastic microcentrifuge safe-lock tube containing 40 ± 5 mg of a mixture (7 : 1) of anhydrous sodium sulfate and PSA and 1350 µL M*t*BE. The mixture was vigorously shaken for 1 min, followed by a centrifugation step (9000 × *g*, 3 min). Then 1 mL of the purified mixture was transferred into an autosampler vial (1.5 mL) and concentrated to dryness under a gentle stream of nitrogen. The residue was dissolved in 800 µL M*t*BE and 100 µL cholesterol solution (calibration standard, 0.5 mg/mL), and 100 µL of silylation re-

Table 2 Detailed overview of the phytosterol ingredients (in mg/100 g) and percentage of Δ^7-phytosterols in the total sterol fraction.

Sample	Sterol ingredients [mg/100 g ± SD] (n = 6)											Composition of the phytosterol fraction (%)	
	2	4	5	6	7	8	9	10	11	12	Total	Not Δ^7	Δ^7
S1					37 ± 5		31 ± 4			31 ± 4	99 ± 12		100.0
S2					29 ± 7		19 ± 5			21 ± 5	69 ± 17		100.0
S3					25 ± 2		17 ± 1			22 ± 2	64 ± 5		100.0
S4					27 ± 3		17 ± 2			20 ± 5	64 ± 9		100.0
S5		5 ± 1			143 ± 31		103 ± 20			71 ± 15	323 ± 67	1.5	98.5
S6		6 ± 3		3 ± 2	197 ± 95		160 ± 74			76 ± 35	442 ± 209	1.3	98.7
S7		7 ± 5			300 ± 128		245 ± 95			139 ± 56	691 ± 284	1.1	98.9
S8		5 ± 2			96 ± 45		70 ± 32			67 ± 31	238 ± 111	2.0	98.0
S9		9 ± 4			188 ± 82		161 ± 67			96 ± 44	454 ± 196	2.0	98.0
S10		141 ± 34	8 ± 2			1771 ± 445	15 ± 3	8 ± 3		24 ± 6	1968 ± 492	96.6	2.4
S11					15 ± 7						15 ± 7		100.0
S12		90 ± 26	20 ± 5			179 ± 51					288 ± 82	100.0	
S13	7 ± 3	162 ± 47	82 ± 33			323 ± 86					567 ± 165	100.0	
S14		7 ± 2		7 ± 4	432 ± 148		447 ± 149			209 ± 69	1103 ± 372	0.6	99.4
S15				7 ± 2	150 ± 50		90 ± 27	15 ± 5		90 ± 35	353 ± 120		100.0
S16		42 ± 14	43 ± 15	14 ± 5	135 ± 48	72 ± 24	79 ± 21			84 ± 24	470 ± 149	33.3	66.7
S17		14 ± 5	8 ± 3		54 ± 18	34 ± 12	30 ± 8			33 ± 9	172 ± 56	32.6	67.4
S18		18 ± 4	5 ± 1		43 ± 9	43 ± 9	26 ± 6		11 ± 5	27 ± 6	172 ± 39	44.3	55.7
S19		20 ± 11	17 ± 9	5 ± 3	48 ± 25	35 ± 19	30 ± 16			31 ± 17	186 ± 100	38.5	61.5
S20					169 ± 41		118 ± 34			124 ± 34	411 ± 109		100.0
S21		16 ± 5	11 ± 4		86 ± 29	50 ± 17	61 ± 18			54 ± 17	279 ± 90	27.7	72.3
S22					46 ± 15		27 ± 8			35 ± 12	108 ± 35		100.0
S23		17 ± 8			53 ± 22	77 ± 30	52 ± 21			43 ± 17	242 ± 97	39.1	60.9
S24	106 ± 38	993 ± 355	21 ± 8			4608 ± 1615		14 ± 8		35 ± 12	5777 ± 2037	99.2	0.8
S25		18 ± 3		20 ± 4	993 ± 186		938 ± 143			564 ± 73	2533 ± 408	0.7	99.3
S26						2 ± 0.5					2 ± 1		100.0
S27		81 ± 22	8 ± 3			1118 ± 345		8 ± 2		11 ± 3	1225 ± 374	98.4	1.6
S28						4 ± 1					4 ± 1	100.0	
S29						5 ± 2					5 ± 2	100.0	
S30		46 ± 10	11 ± 2			158 ± 32			17 ± 5		232 ± 50	100.0	
S31		42 ± 7	11 ± 2			151 ± 27			13 ± 3		217 ± 39	100.0	
S32		54 ± 20	12 ± 4			198 ± 73			13 ± 4		278 ± 101	100.0	
S33		49 ± 15	13 ± 4			174 ± 56			16 ± 5		251 ± 80	100.0	
S34		49 ± 13	12 ± 3			163 ± 42			13 ± 2		238 ± 60	100.0	
S35		38 ± 19				128 ± 67					166 ± 86	100.0	
S36		37 ± 11				167 ± 48					204 ± 59	100.0	
S37		384 ± 205				5128 ± 3000				39 ± 20	5551 ± 3225	99.3	0.7
S38		801 ± 402	40 ± 19	12 ± 6		9016 ± 4707		50 ± 22		85 ± 36	10 003 ± 5192	98.5	1.5

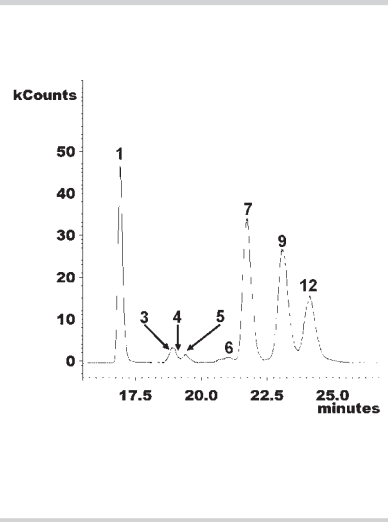

Fig. 2 Representative selected ion chromatogram of an analyzed capsule with high Δ^7-phytosterol content after the workup procedure; the sterols were detected as their TMS ethers: **1**, cholesterol (CS, *m/z* 368); **3**, ergosterol (IS, *m/z* 363); **4**, campesterol (*m/z* 382); **5**, stigmasterol (*m/z* 394); **6**, ergosta-7-enol (*m/z* 472); **7**, $\Delta^{7,25}$-stigmasterol (*m/z* 343); **9**, spinasterol (*m/z* 343); **12**, Δ^7-avenasterol (*m/z* 343).

$$\text{mg/dosage} = \frac{PA_s \times m_{Chol_{CS}} \times PA_{Erg_{WSS}} \times 100 \times DF}{PA_{Chol_{CS}} \times PA_{Erg_{\varnothing WS}} \times m_S}$$

Fig. 3 Determination of phytosterol content in mg/dosage; PA_S = sterol TMS ether peak area; m_{CholCS} = weight cholesterol (calibration standard) (mg); PA_{ErgWSS} = ergosterol TMS ether peak area (WS) sample; DF = dissolution factor (= 50); PA_{CholCS} = cholesterol TMS ether peak area (calibration standard); $PA_{Erg\varnothing WS}$ = average ergosterol TMS ether peak area (WS) of blank sample analysis (= 24); m_S = mass of the sample [g].

agent MSTFA/TSIM (9 : 1) was added. The sample was gently shaken and stored at room temperature for at least 30 min. Silylation was complete after this period, as no free sterols (identified by RRT and MS data) were detectable in the samples. For removal of precipitates, the mixture was transferred into a 2.0 mL plastic microcentrifuge safe-lock tube and centrifuged at 9000 × *g* for 3 min, then transferred back into the autosampler vial before being subjected to GC-IT/MS analysis.

GC-IT/MS analysis

A Varian 3800 gas chromatograph was coupled with a Saturn 2200 IT from Varian. The autosampler was a CombiPal from CTC Analytics and the used injector was a Varian 1177 with split/splitless option. Data analysis and instrument control was made with Varian Workstation 6.9 SP 1 software. The instrument was equipped with an Agilent VF-5MS capillary column (30 m × 0.25 mm; inner diameter 0.25 µm and 10 m EZ-Guard column). The carrier gas was helium 5.0 (purity 99.99%) at a constant flow rate of 1.4 mL/min. The inlet injector temperature was maintained at 280 °C and the injection volume was 1 µL (split 15). The GC oven started at 55 °C (1.0 min hold), was ramped up to 265 °C (heating rate 50 °C/min) followed by a gradient of 1 °C/min up to 287 °C, and then was ramped up to 310 °C (heating rate 50 °C/min) (hold time 0.84 min). The total run time was 28.5 min. Transfer line temperature was 270 °C and the IT temperature was 200 °C. The IT/MS was switched on after 15.5 min (solvent delay) and scanned at a mass range from 100 to 600 *m/z* (EI, 70 eV) (**Fig. 1S**, Supporting Information).

The phytosterol TMS ethers were identified by mass spectral analysis in comparison with commercial references, the NIST™ database, or data from literature (**Table 3**) [15–17, 20–24].

Determination of phytosterol content

Each dosage was weighted for determining the phytosterol content in mg/100 g and for the average dosage weight (n = 10) (**Table 3**). The phytosterol TMS ether peaks were referred to the TMS ether peak area of the base peak of cholesterol (**1**) TMS ether. Beveridge et al. [26] and Mandl et al. [27] also used such approximation because no commercial references of Δ^7-phytosterols were available. The base peaks of each sterol TMS ether were taken as a quantifier ion for calculating the peak area (**Fig. 3**, **Table 3**). The content for each phytosterol (mg/100 g) was calculated according to **Fig. 3** [14, 25]. The total phytosterol content was calculated by the addition of all detected phytosterol TMS ethers, the content of non-Δ^7-phytosterols by summation of the phytosterol TMS ethers of **2, 4, 5, 8**, and **11**, and for the Δ^7-phytosterols by summation of **6, 7, 9, 10**, and **12** (**Tables 1** and **2**).

Method validation

For verification of selectivity, ten different dosages were analyzed in the presence or absence of cholesterol (**1**) and ergosterol (**3**). Both WSs were not detectable in the unspiked samples. All compounds (**Fig. 1**, **Table 1**) were identified on the basis of RRT, detection of the molecular ion, and the specific fragmentation pattern of each sterol TMS ether including the relative intensities of ion fragments. The detector response was linear for sterol TMS ethers, as demonstrated in previous measurement data [23, 27]. The European Pharmacopoeia uses betulin (lup-20(29)-en-3*β*-28-diol) for single-level calibration in phytosterol analysis [14], but this compound has physicochemical properties strongly different from our analytes. Hence, we decided to use cholesterol as a calibration standard [25, 26]. The RSD of cholesterol TMS ether (**1**; 0.05 mg/mL) and ergosterol TMS ether (**3**; 0.01 mg/mL) were accomplished by blank sample analysis (n = 6). The peak area of the cholesterol (**1**) TMS ether showed an RSD of 11.8%, and the peak area of the ergosterol (**3**) TMS ether showed an RSD of 13.1%. The ergosterol content was calculated by the rule of three with 0.0112 mg/mL (RSD = 2.1%, n = 6). Using the peak area of the quantification ion of each phytosterol TMS ether (**Table 1**), LOD and LOQ were determined for an S/N of 3 and 10, respectively. As a factor of robustness, the same sample was analyzed in the same manner after one batch analysis (n = 38). The RSD of **1** was 17.8%, for **3** 20.0%, and the calculated ergosterol content was 0.0116 mg/mL (RSD = 3.4%). Four blank samples spiked with the cholesterol (**1**) TMS ether (0.05 mg/mL) and ergosterol (**3**) TMS ether (0.01 mg/mL) were analyzed for determining the intraday precision. The interday precision was calculated by analyzing the four blank samples in all six batches. The average intraday precision for **1** was ± 17.9% (ranging from 10.4–24.6%) and for **3** ± 17.6% (ranging from 10.4–22.5%). The interday precision was 22.0% for **1** and 24.3% for **3**. Recovery analysis was performed with all samples (n = 38) during each batch (n = 6). The recoveries were calculated by comparing the measured area of the TMS ether of ergosterol (**3**, IS) of each sample and the average measured area (n = 4) of the blank sample analysis of the ergosterol TMS ether from the intraday determination. The average recovery (n = 38) was 88.9% (median 84.9%) and ranged from 43.0% (**S7**) to 138.1% (**S28**). The average standard deviation of the determined recoveries in one sample (n = 6) was 15.4% (median 13.6%) and ranged from 4.6% (**S25**) to 36.2% (**S24**). With regard to the complex sample matrix, multistep sample workup

Table 3 Identification of sterols.

Substance	Trivial name IUPAC name	M_R (OH) M_R (TMS) [g/mol]	RT TMS ether [min]	RRT TMS ether (cholesterol)	RRT TMS ether (ergosterol)	RRT TMS ether (sitosterol)	Quantifier ion (*m/z*)	Reference
1	Cholesterol (CS)	386	16.77	1.00	0.89	0.77	368	RS
	Cholest-5-en-3β-ol	458						
2	Brassicasterol	398	17.62	1.05	0.94	0.81	380	[20, 21]
	Ergosta-5,22-dien-3β-ol	470						
3	Ergosterol (IS)	396	18.83	1.12	1.00	0.87	363	RS
	Ergosta-5,7,22-trien-3β-ol	468						
4	Campesterol	400	19.27	1.15	1.02	0.89	382	[22, 25]
	Ergost-5-en-3β-ol	472						
5	Stigmasterol	412	19.93	1.19	1.06	0.92	394	RS
	Stigmasta-5,22-dien-3β-ol	484						
6	Ergosta-7-enol	400	20.83	1.24	1.11	0.96	472	[23, 24]
	Ergost-7-en-3β-ol	472						
7	$\Delta^{7,25}$-Stigmastadienol	412	21.56	1.29	1.14	1.00	343	[16, 26]
	Stigmasta-7,25-dien-3β-ol	484						
8	β-Sitosterol	414	21.67	1.29	1.15	1.00	396	RS
	Stigmast-5-en-3β-ol	486						
9	Spinasterol	412	23.02	1.37	1.22	1.06	343	[17]
	Stigmasta-7,22-dien-3β-ol	484						
10	Δ^7-Sitosterol	414	23.31	1.39	1.24	1.08	486	[17, 25]
	Stigmast-7-en-3β-ol	486						
11	Cycloartenol	426	23.46	1.40	1.25	1.08	365	[21, 24]
	Cycloart-24-en-3β-ol	498						
12	Δ^7-Avenasterol	414	24.05	1.43	1.28	1.11	343	[17, 25]
	Stigmasta-7,24(28)-dien-3β-ol	486						

Trivial and IUPAC name, molecular mass of free sterol [M_r(OH)] and TMS ether derivative [M_r(TMS)], retention time (RT) and relative retention time (RRT) of TMS ether relative to cholesterol (CS), ergosterol (IS), and β-sitosterol; CS = calibration standard, IS = internal standard, RS = authentic reference material

procedure, and the determined precisions, the recoveries were in an expected and adequate range.

Supporting information

Full mass spectra of all detected sterol TMS ethers are available as Supporting Information.

Conflict of Interest

▼

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