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On-line liquid chromatography–gas chromatography for the analysis of free and esterified sterols in vegetable oil methyl esters used as diesel fuel substitutes

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Abstract

An on-line LC–GC method for the analysis of free and esterified sterols in vegetable oil methyl esters has been set up. Qualitative and quantitative information about this important group of minor components is provided without saponification and off-line pre-separation. Prior to analysis the free sterols are silylated with N-methyl-N-trimethylsilyltrifluoracetamide; betulinol is used as an internal standard. Using concurrent eluent evaporation with the loop-type interface for eluent transfer, transfer temperature and transfer efficiency are carefully optimized. The concentration of the free sterols as well as their qualitative and quantitative composition and the concentration of the sterol esters are determined in five different types of vegetable oil methyl esters. The recovery of the LC–GC procedure and the reproducibility of the quantitative results are evaluated.

1. Introduction

The introduction of quality standards for vegetable oil methyl esters, obtained by alkali-catalyzed transesterification of vegetable oils with methanol, is gaining in importance due to their increased use as diesel fuel substitutes and as technical products. In Austria standards for the quality of rapeseed oil methyl ester used as diesel fuel substitute have been introduced by the standard ÖNORM C 1190 [1] and also several other countries are elaborating standard specifications for these new kinds of oleochemical products. The amounts of the most important organic minor components (mono-, di- and triacylglycerols as well as free glycerol) have been limited by this standardization, since their presence in the fuel causes serious problems through formation of engine deposits and hazardous emissions.

In the production of vegetable oil methyl esters, the sterols as main constituents of the unsaponifiable matter of most vegetable oils remain in the fatty acid methyl ester phase, and are recovered both as free sterols and as esterified sterols in the transesterification product. The influence of the sterols on the technical properties of vegetable oil methyl esters is still not precisely understood, but their presence may influence the combustion characteristics, the storage properties, low-temperature behaviour characteristics and other physical and chemical properties in various ways. Sterol content and composition can also be criteria to distinguish

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between products of different degree of purification or between different types of vegetable oil methyl esters.

Conventional methods for the analysis of the sterol fraction in oils and fats comprise saponification of the triglycerides, extraction of the unsaponifiables, pre-separation by preparative thin-layer chromatography [2–4], chromatography on silica gel columns [5,6], or by off-line HPLC [7], and finally gas chromatographic analysis.

In 1989, Grob et al. described an on-line LC–GC method for the direct determination of minor components in edible oils and fats avoiding saponification [8]. The sterols and related compounds as well as their fatty acid esters were isolated from the rest of the sample, primarily from the triglycerides, by HPLC and transferred on-line to GC. Recently this LC–GC method was modified, replacing the esterification of the free sterols with pivalic acid by trimethylsilylation [9]. A variation of the conventional methods for the analysis of sterols in oils and fats, substituting saponification by transesterification and isolation and pre-separation by on-line LC–GC, was proposed by Biedermann et al. [10].

Recently, we described a method for the direct determination of sterols in vegetable oil methyl esters by capillary GC [11]. In this work, an on-line LC–GC method for the rapid and reliable determination of free and esterified sterols in vegetable oil methyl esters is presented. In contrast to conventional methods for the analysis of the sterol fraction in fats and fat derivatives [4], analysis is carried out without saponification and any off-line pre-separation. Trimethylsilylation of the free sterols prior to analysis allows the determination of the concentration and composition of the free sterols as well as of the concentration of the sterol esters in a single LC–GC run. Transfer temperature and transfer efficiency, the most important parameters for correct solvent transfer and reliable quantitation using concurrent eluent evaporation with the loop-type interface, are optimized. Qualitative and quantitative determination of free and esterified sterols is carried out in five different types of vegetable oil methyl esters. The reproducibility of the quantitative results and the recovery of the LC–GC procedure is evaluated.

2. Experimental

2.1. Chemicals

The reference substances used in this study, stigmasterol, cholesteryl oleate, cholesteryl palmitate, cholesteryl stearate, and betulinol were purchased from Sigma (Deisenhofen, Germany) and were at least 97% pure. MSTFA (N-methyl-N-trimethylsilyltrifluoroacetamide) was obtained from Fluka (Buchs, Switzerland). Analytical grade pyridine and *n*-hexane were supplied by Loba Feinchemie (Fischamend, Austria). *n*-Hexane, dichloromethane and acetonitrile in LiChrosolv quality (Merck, Darmstadt, Germany) were used as LC eluents.

2.2. Preparation of samples and standard solutions

Stock solutions of stigmasterol (0.55 mg/ml), cholesteryl stearate (0.72 mg/ml), and betulinol (5.11 mg/ml) in pyridine were used to prepare the standard solutions. The concentrations of the reference substances in the standard solutions varied between 0.3 μg/ml and 6.1 μg/ml for stigmasterol and between 0.4 μg/ml and 8.0 μg/ml for cholesteryl stearate; the concentration of betulinol as internal standard was 5.7 μg/ml in all standard solutions. Appropriate amounts of the stock solutions of stigmasterol and cholesteryl stearate and 10 μl of the betulinol stock solution were transferred to 10 ml screw-cap vials.

For preparation of samples, betulinol stock solution (10 μl containing 51.1 μg betulinol) was added as internal standard to 20.0–30.0 mg of vegetable oil methyl ester in a 10 ml screw-cap vial.

In order to silylate the hydroxyl groups of the free sterols, N-methyl-N-trimethylsilyltrifluoroacetamide (50 μl) was added to the standard and to the sample material. After shaking briefly, the

capped vials were heated at 70°C for 15 min. The silylated mixtures were dissolved in *n*-hexane and diluted to 10 ml.

2.3. Instrumentation

LC–GC analyses were carried out on the Dualchrom 3000 (Carlo Erba, Milan, Italy). The LC system consisted of a Phoenix 30 syringe pump, a pneumatic six-port valve with a 10 μl loop, a pneumatic ten-port valve with the LC separation column, and a variable wavelength detector (MicroUVIS 20). The GC was equipped with a flame ionization detector and with an automated valve system for the interfaces and for the solvent vapor exit. Acquisition of data from both the UV detector and the FID was performed with a Chrom-Card (Fisons Instruments, Milan, Italy) in combination with an IBM compatible personal computer.

2.4. LC pre-separation

In order to simplify peak identification in the subsequent gas chromatogram, the large amounts of fatty acid methyl esters were separated from the sterols by LC pre-separation. A 100 × 2 mm I.D. LC column packed with 5-μm silica (Spherisorb S5W; Phase Separation, Deeside, Clywd, UK) was used with *n*-hexane–dichloromethane–acetonitrile (79.9:20:0.1, v/v/v) as mobile phase at a flow rate of 350 μl/min. Volumes of 10 μl were injected through a Valco six-port valve; detection was carried out at 220 nm. The LC column was backflushed with 1 ml of dichloromethane–acetonitrile (95:5, v/v), followed by mobile phase, 3 min after transfer of the LC fraction to the GC system. The backflush valve returned to stand-by 10 min later.

2.5. LC–GC transfer and GC analysis

Since the elution temperatures of silylated sterols and sterol esters were above 260°C, concurrent eluent evaporation [12,13] with the loop-type interface [14] was the transfer technique of choice. GC separation was carried out on a 12 m × 0.32 mm I.D. fused-silica capillary column coated with a 0.1-μm film of 5% phenyl polydimethylsiloxane (DB-5; J and W Scientific, Folsom, CA, USA), connected in series with a 4 m × 0.53 mm I.D. uncoated, deactivated fused-silica pre-column (Carlo Erba), a 3 m × 0.32 mm I.D. retaining pre-column coated with DB-5 of 0.1 μm film thickness, and an early vapor exit by means of glass press-fit connection.

Carrier gas inlet pressure behind the flow regulator was 250 kPa and the regulated flow-rate was 1.2 ml/min at 40°C (hydrogen). Detector temperature was 370°C.

The transfer of a 1000 μl fraction started 2.67 min after LC injection by switching the sample valve and the carrier gas valve, and by opening the solvent vapor exit. The content of the 1000 μl sample loop containing the silylated sterols and the sterol esters was transferred to the GC column at an oven temperature of 130°C under conditions of concurrent eluent evaporation.

When the pressure had dropped by 100 kPa after transfer was complete, and a delay of 20 s had passed, sample and carrier gas valves automatically returned to "stand-by". The solvent vapor exit was switched to a high resistance (1 m × 75 μm fused-silica capillary) 55 s after the pressure drop, leaving a small purge flow. After 8 min at an initial temperature of 130°C, the GC oven was heated at 30°/min to 260°C, then at 3°/min to 270°C, and finally at 15°/min to 345°C (held for 7 min). Transfer of 1000 μl took 4.5 min. Total LC–GC run time was 33 min, with 5 min cooling.

3. Results and discussion

3.1. Optimization of transfer temperature

Using fully concurrent eluent evaporation with the loop-type interface for eluent transfer, special attention must be paid to the transfer temperature, i.e. the GC column temperature during transfer. The transfer temperature must be at least equal to the eluent boiling point corrected for carrier gas inlet pressure, to produce an eluent vapor pressure that keeps the liquid out of the GC column. Too low transfer temperatures

result in flooding of the GC column by the LC eluent and consequently, in broad, deformed peaks. Transfer temperatures, exceeding the minimum required column temperature to a great extent, have the drawback that peak broadening and losses through the vapor exit affect solutes of up to unnecessarily high elution temperatures.

For the mobile phase used and an inlet pressure of 250 kPa, a transfer temperature of about 110°C is optimal according to Grob and coworkers [13,15]. Based on the guidelines given in those references, transfer temperature was optimized experimentally by repeated transfer of LC fractions of an rapeseed oil methyl ester (RME) sample at transfer temperatures from 100°C to 160°C. Fig. 1 shows the peak shapes of silylated sterols and betulinol and of the sterol esters observed after transfer at different column temperatures.

A transfer temperature of 100°C creates a vapor pressure in the GC column which is lower than the carrier gas inlet pressure. As a consequence, the eluent flows into the GC column resulting in extremely large initial peak widths, peak broadening and peak deformation. At a GC column temperature of 160°C, the trimethylsilyl derivatives of the sterols exhibit already a considerable vapor pressure and are partially lost through the early vapor exit during the transfer. The resulting peak areas are too small and peaks are broadened due to large initial peak widths.

A transfer temperature of 130°C was found to be optimal for the analysis of free and esterified sterols with the conditions used. The peak areas correspond to the actual amounts of substances and also the peak shapes are satisfactory. The "splitting" of the sterol ester peaks is due to overlapping of esters with different C_{18}-fatty acid residues and not to insufficient transfer.

The time required for the transfer (corresponding to the width of the solvent peak) does not show clear correlation with the transfer temperature. Transfer of 1000 μl at a carrier gas inlet pressure of 250 kPa at GC column temperatures between 90°C and 160°C took from 4.5 min to 6 min.

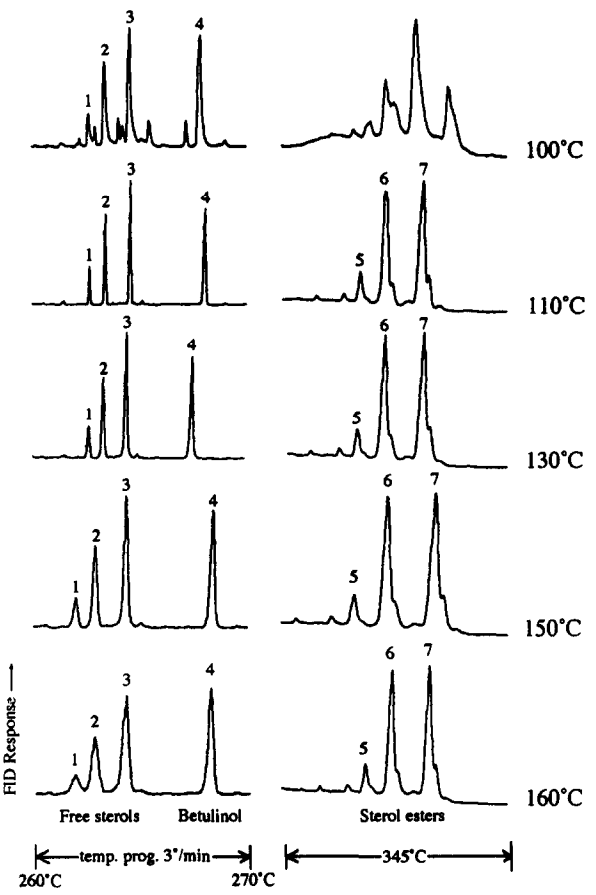

Fig. 1. Peak shapes observed after transfer to the GC system at varying transfer temperatures. 1000-μl fractions were introduced into the GC system via a loop-type interface at a maximum applied inlet pressure of 250 kPa. Eluent: *n*-hexane–dichloromethane–acetonitrile (79.9:20:0.1, v/v/v). GC column: 12 m × 0.32 mm I.D. fused-silica capillary column coated with DB-5 (0.1 μm film thickness), equipped with a 4 m × 0.53 mm I.D. uncoated pre-column, a 3 m × 0.32 mm I.D. retaining pre-column and an early vapor exit. Peak assignment: 1 = brassicasterol; 2 = campesterol; 3 = β-sitosterol; 4 = betulinol, I.S.; 5,6,7 = sterol esters.

3.2. Optimization of transfer efficiency

For the approximate determination of the fraction window, silylated stigmasterol and cholesteryl stearate were injected to LC and detected at 220 nm. With the phase system used, silylated sterols and sterol esters show lower retention than fatty acid methyl esters. Sterol

trimethylsilyl ethers as well as fatty acid steryl esters exhibit poor UV absorption and are not eluted exactly at the same time. At the transfer time, when the sample loop is decoupled from the LC flow path by switching the sample valve from the "stand-by" to the "transfer" position, the major part of the fatty acid methyl esters should not have reached the sample loop, whereas the sterols and sterol esters remain there completely. Transfer efficiency, i.e. the proportion of solute material injected to LC that is recovered by GC, was optimized to come close to 100% by adjustment of the transfer time. Losses by incomplete transfer, by degradation in the LC column or by leaks were carefully avoided to ensure that the transferred fraction contained all material of interest.

In order to determine the fraction window exactly, LC fractions of an RME sample were transferred to the GC with slight variations of the transfer time. As a reference, the same sample volume as injected into the LC was directly introduced into the GC by loop injection without pre-separation (10 μl loop mounted in the sample valve; Fig. 2). Completeness of transfer was checked by comparison of the peak areas obtained by LC–GC transfer and by loop injection. In Table 1, the transfer efficiencies for β-sitosterol and for the sum of the sterol esters are given for different transfer times. With the conditions used, 2.67 min after LC injection turned out to be the optimum transfer time.

Table 1
Efficiency of LC–GC transfer at different transfer times

Transfer time (min)	Transfer efficiency (%)[a]	
	β-Sitosterol	Sterol esters
2.33	94	96
2.50	98	107
2.67	100	104

[a] Percentages refer to peak areas obtained by loop injection as 100%.

An LC chromatogram of silylated rapeseed oil methyl ester is shown in Fig. 3. The fraction window of interest (indicated by the hatched area) lasted from the dead time of the LC column to the beginning of the fatty acid methyl ester peaks and had a volume of 650 μl. Since high accuracy of the analysis was essential and no interfering signals were observed, the fraction

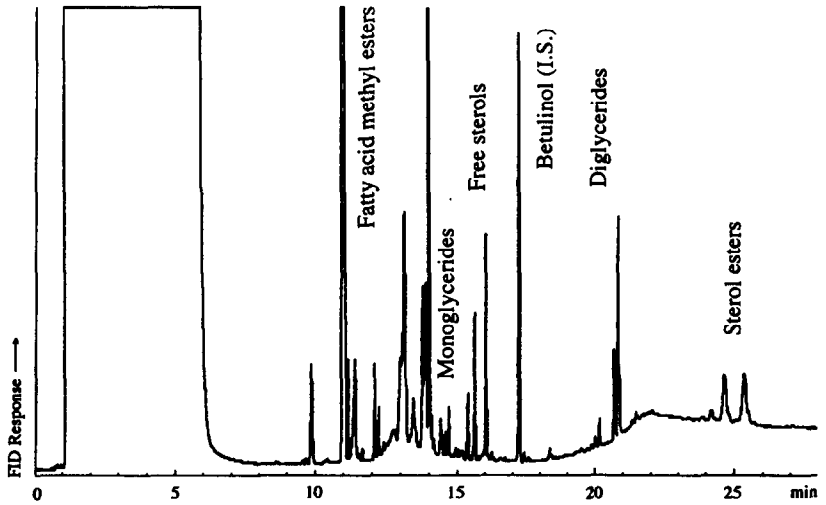

Fig. 2. Gas chromatogram of silylated rapeseed oil methyl ester obtained by large-volume loop injection without LC preseparation. Betulinol was added as internal standard. GC column: as in Fig. 1. GC temperature programme: 130°C (8 min), at 30°C/min to 260°C, at 3°C/min to 270°C, at 15°C/min to 345°C (7 min).

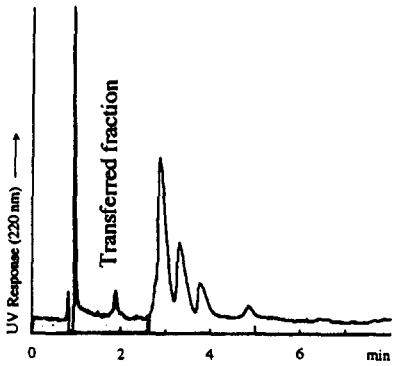

Fig. 3. Liquid chromatogram of silylated rapeseed oil methyl ester. The fraction indicated was transferred to the GC system and contained the TMS-derivatives of the free sterols and betulinol, as well as the sterol esters. LC column: 100 × 2 mm I.D. Spherisorb S5W silica. Mobile phase: as in Fig. 1. Flow-rate: 350 μl/min. Injected volume: 10 μl. Detection: 220 nm.

volume was extended to 1000 μl. As a consequence, LC retention times had not to be precisely controlled.

3.3. Qualitative analysis

An LC–GC–FID chromatogram of a rapeseed oil methyl ester fraction containing free sterols, the internal standard betulinol (as TMS derivatives) and the sterols esters is shown in Fig. 4. The group of the originally free sterols is eluted first, with the peaks of β-sitosterol, campesterol and brassicasterol clearly dominating. In addition, cholesterol, stigmasterol, and Δ^5-avenasterol could be identified by analyses of samples spiked with reference substances and by comparison with literature data [4,16,17]. Then follows the peak of betulinol, which appeared to be a suitable internal standard eluting between the groups of the originally free sterols and the sterol esters. Finally, the esterified sterols form a multiplet of peaks. Due to insufficient resolution and the lack of commercially available standard material, the individual sterol ester peaks could not be reliably identified. Tentative identifications were, however, made, as the pattern of the free sterols was repeated with some changes in the quantity ratios.

3.4. Calibration

For the quantitative determination of the free and esterified sterols in vegetable oil methyl esters, a calibration with stigmasterol and cholesteryl stearate as reference substances was carried out. As already noted [11] for a reliable gas chromatographic determination of free and es-

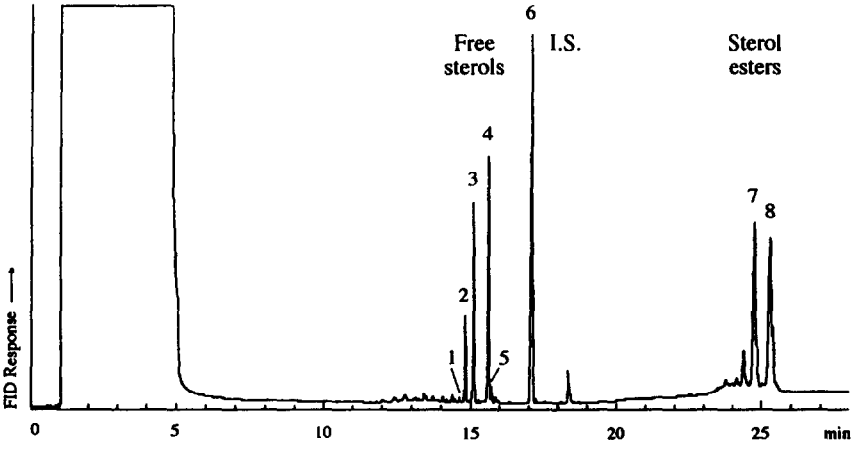

Fig. 4. Gas chromatogram of the rapeseed oil methyl ester fraction shown in Fig. 2. Conditions as in Fig. 1, 2, 3. Peak assignment: 1 = cholesterol; 2 = brassicasterol; 3 = campesterol; 4 = β-sitosterol; 5 = Δ^5-avenasterol; 6 = betulinol I.S.; 7 = campesteryl-C_{18}-esters; 8 = β-sitosteryl-C_{18}-esters.

terified sterols a calibration of the response factors is necessary. Especially for the high-boiling sterol esters, thermal degradation on the GC column results in substantial deviations of the response factors from unity. In LC–GC analysis, LC pre-separation and the LC–GC transfer can show additional influence on the peak sizes of the solutes of interest. For these reasons, external calibration and the addition of an internal standard are inevitable.

Standard solutions containing known amounts of the reference substances (stigmasterol and cholesteryl stearate) and the internal standard (betulinol) were analyzed three times each by LC–GC, in order to determine the ratio A_c:A_{st} (peak area of component (A_c):peak area of internal standard (A_{st})). Using the phase systems and the conditions mentioned above, the following calibration functions have been obtained: W_c:$W_{st} = (1.13 \pm 0.02).A_c$:$A_{st}$ for stigmasterol; W_c:$W_{st} = (1.65 \pm 0.03).A_c$:$A_{st}$ for cholesteryl stearate (W_c, weight of component; W_{st}, weight of internal standard). Good linearity of the calibration functions was observed (stigmasterol: standard error = 0.025, $n = 6$, $r^2 = 0.996$; cholesteryl stearate: standard error = 0.032, $n = 6$, $r^2 = 0.996$) within the concentration range of interest (10–2500 ppm for free sterols; 15–3200 ppm for sterol esters).

The concentrations of all free sterols were calculated based on the internal standard betulinol, using the response factor determined for stigmasterol. The sterol esters were quantified analogously using the response factor obtained for cholesteryl stearate.

3.5. Quantitative analysis

The concentration of the free sterols, as well as their qualitative and quantitative composition, and the total concentration of the sterol esters were determined by on-line LC–GC in five different vegetable oil methyl esters. Due to insufficient resolution, quantitation of the individual sterol ester species was not possible. The total concentrations of free sterols and sterol esters in rapeseed oil methyl ester (RME), sunflower oil methyl ester (SFME 1), high oleic sunflower oil methyl ester (SFME 2), soybean oil methyl ester (SBME) and used frying oil methyl ester (UFME) are given in Table 2. Rapeseed oil methyl ester shows the highest content of free and esterified sterols followed by used frying oil methyl ester, which contains a great proportion of transesterified rapeseed oil, also indicated by the relatively high content of brassicasterol.

The compositions of the free sterols in the analyzed vegetable oil methyl ester samples were calculated based on the concentrations of the individual free sterols related to the total amount of free sterols and are given in Table 3. Like in vegetable oils, the sterol composition is more characteristic of a certain type of vegetable oil methyl ester than the fatty acid composition. β-Sitosterol represents the main component of the free sterols in all vegetable oil methyl esters considered. Brassicasterol was only present in rapeseed oil methyl ester and used frying oil methyl ester. A relative content of Δ^7-stigmastenol of more than 10% is typical of sunflower oil methyl esters. Only in used frying oil methyl

Table 2
Total concentration of free sterols and sterol esters in different vegetable oil methyl esters ($n = 4$)

	Concentration (weight%)				
	RME	SFME1	SFME2	SBME	UFME
Free sterols	0.33	0.25	0.25	0.20	0.25
Sterol esters	0.73	0.18	0.15	0.15	0.50

Abbreviations: RME, rapeseed oil methyl ester; SFME 1, sunflower oil methyl ester; SFME 2, high oleic sunflower oil methyl ester; SBME, soybean oil methyl ester; UFME, used frying oil methyl ester.

Table 3
Composition of the free sterols in different vegetable oil methyl esters ($n = 4$)

	Composition (% of the free sterols)				
	RME	SFME1	SFME2	SBME	UFME
Cholesterol	1.7	1.0	<0.1	0.7	12.6
Brassicasterol	12.7	<0.1	<0.1	<0.1	6.7
Campesterol	34.2	9.5	8.6	22.8	22.7
Stigmasterol	0.5	7.5	8.6	18.6	3.4
β-Sitosterol	49.2	61.6	66.6	53.1	47.9
Δ^5-Avenasterol	1.7	4.3	3.9	1.8	1.5
Δ^7-Stigmastenol	<0.1	14.7	11.5	2.7	5.0
Δ^7-Avenasterol	<0.1	1.3	0.8	0.3	0.2

Abbreviations as in Table 2.

ester was cholesterol found to any considerable extent.

3.6. Recovery and reproducibility of results

For the evaluation of the recovery of the LC–GC procedure, a reference sample and a spiked RME sample containing known amounts of standard substances were analyzed four times. The reference sample contained a mixture of silylated sterols, cholesteryl palmitate and cholesteryl oleate at concentrations typical of authentic vegetable oil methyl ester samples. Distilled rapeseed oil methyl ester (not containing any sterols) was spiked with the standard substances mentioned. By comparison of the quantitative results obtained by LC–GC analysis of the reference sample and the spiked RME sample with the actual concentrations of the standard substances, the percentage recoveries were calculated. As shown in Table 4, the recovery of the LC–GC analysis is excellent for all solutes of interest.

In order to check the precision of the method, an RME sample was prepared and injected five times into the LC–GC. The quantitative results obtained for β-sitosterol as major component of the free sterols, for the total free sterols, and for the total sterol esters are given in Table 5.

As an additional test for reproducibility, five samples of an RME specimen were prepared and analyzed by LC–GC following the procedure outlined above. The results are summarized in Table 6.

The data in Tables 5 and 6 indicate the excellent reproducibility of the outlined method. Besides the errors resulting from repeated injection, further inaccuracies might be caused by sample weighing, the addition of the internal standard, and by possible insufficiency of the

Table 4
Average recovery of the LC–GC procedure for free sterols and sterol esters ($n = 4$)

	Concentration (μg/ml)	Recovery (%)	
		Reference sample	Spiked RME sample
Free sterols	0.15	100	96
Cholesteryl palmitate	0.15	110	109
Cholesteryl oleate	0.25	105	102

Table 5
Concentrations of free β-sitosterol, of total free sterols, and of total sterol esters in RME, obtained by consecutive injections into LC—GC ($n = 5$)

Analysis	Concentration (weight%) in RME		
	β-Sitosterol	Free sterols	Sterol esters
1	0.120	0.253	0.568
2	0.119	0.254	0.577
3	0.120	0.256	0.581
4	0.120	0.255	0.588
5	0.117	0.250	0.593
Mean	0.119	0.254	0.582
Standard deviation	0.001	0.002	0.009
Standard deviation (%)	0.92	0.82	1.52

derivatization. Comparison of the data in Tables 5 and 6 shows that the contribution of these errors to the total standard deviation is small.

4. Conclusion

The proposed LC–GC method provides qualitative and quantitative information about free and esterified sterols in vegetable oil methyl esters. Advantageous in comparison to conventional techniques involving saponification and off-line pre-separation are the simple preparation of samples, the extremely short analysis time and the possibility of complete automation. Peak identification is easy due to the absence of interfering components. The excellent recovery and reproducibility of quantitative results allow reliable qualitative and quantitative determination of free and esterified sterols in these new kinds of oleochemical products.

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Table 6
Concentrations of free β-sitosterol, of total free sterols, and of total sterol esters, obtained by repeated complete analyses of the same RME sample ($n = 5$)

Analysis	Concentration (weight%) in RME		
	β-Sitosterol	Free sterols	Sterol esters
1	0.118	0.254	0.571
2	0.122	0.260	0.576
3	0.122	0.257	0.570
4	0.124	0.261	0.594
5	0.120	0.256	0.588
Mean	0.121	0.258	0.580
Standard deviation	0.002	0.003	0.010
Standard deviation (%)	1.57	1.00	1.66

Vienna Chamber of Commerce is gratefully acknowledged.

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