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Capillary column gas–liquid chromatographic separation of Δ5-unsaturated and saturated phytosterols[1]

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Abstract

Diets consisting of food ingredients of both plant and animal origin contain a complex mixture of sterols in the lipid fraction. Analysis of these complex mixtures of sterols is generally accomplished by capillary column GC and GC–MS. A very low polarity liquid phase (methylsilicone) and medium polarity columns of various dimensions are used for the separation and quantitation of sterols. However, these columns may not be able to separate some of the sterols in the mixture. The objective of this study was to accomplish the complete separation of a complex mixture of sterols by GC. In order to achieve this objective, a fused-silica capillary column (14% cyanopropyl-phenyl-methylpolysiloxane) of low/medium polarity was used. Excellent separation of campesterol and campestanol, and sitosterol, sitostanol and Δ5-avenasterol was achieved. In addition to GC resolution, a few critical points for the analysis of sterols are presented.

Keywords: Food analysis; Phytosterols; Sterols; Stanols

1. Introduction

Phytosterols are known inhibitors of cholesterol absorption [1]. Their serum-cholesterol lowering properties have increased the interest in quantifying the plant sterol fraction of food items of vegetable origin. Plant-based foods and food products contain a large number of phytosterols with a backbone structure similar to cholesterol, the main sterol in animal lipids [2]. The major sterols belonging to the group 4-desmethylsterols are cholesterol (cholest-5-en-3β-ol), campesterol (24α-methyl-5-cholesten-3β-ol), sitosterol (24α-ethylcholest-5-en-3β-ol), stigmasterol (5,22-cholestadien-24α-ethyl-3β-ol), and brassicasterol (5,22-cholestadien-24β-methyl-3β-ol) which is generally found in the lipids of the plant family *Cruciferae* [2]. Other sterols of this group are Δ5-avenasterol [24-ethylcholesta-5,24(28)*Z*-dien-3β-ol], Δ7-avenasterol [24-ethylcholesta-7,24(28)*Z*-dien-3β-ol], Δ7-stigmastenol (24α-ethylcholesta-7-en-3β-ol), campestanol (24α-methyl-5α-cholestan-3β-ol) and sitostanol (24α-ethyl-5α-cholestan-3β-ol) [2]. The saturated counterparts of campesterol and sitosterol, campestanol and sitostanol, respectively, commonly known as stanols, are mainly found as a natural constituent in some cereal lipids [3]. Diets consisting of a mixture of food ingredients of both plant and

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animal origin thus contain a complex mixture of sterols in the lipid fraction. Saturated sterols can also be generated in small amounts in hydrogenated vegetable oils [2,3]. The chemical structures of some of the common sterols are presented in Fig. 1.

Analysis of these complex mixtures of sterols is generally accomplished by capillary column gas–liquid chromatography (GC) and a combination of GC with mass spectrometry (MS) [4]. OV-1-type liquid phase (100% methylpolysiloxane) and OV-5 (5% phenyl–95% methylpolysiloxane) capillary columns of various dimensions are used for the quantitative analysis of sterols. These types of columns may not be able to separate a complex mixture of sterols [3–5]. Medium-polarity columns such as Suppelcowax 10 have been shown to separate the common Δ5-unsaturated sterols from their saturated counterparts. However, separation of Δ5-avenasterol and Δ7-stigmastenol was not achieved [6].

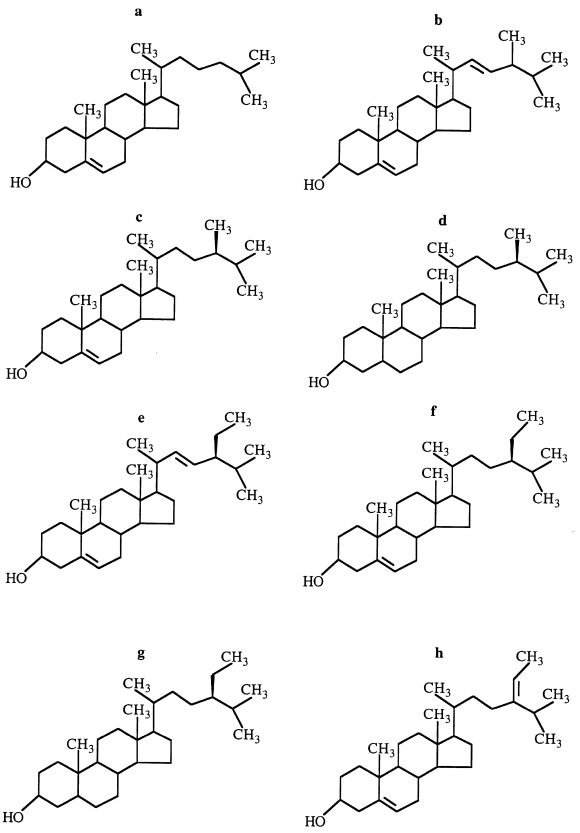

Fig. 1. Structure of some common Δ5-unsaturated and Δ5-saturated sterols showing the differences in the structures of sterols and stanols. (a) Cholesterol, (b) brassicasterol, (c) campesterol, (d) campestanol, (e) stigmasterol, (f) sitosterol, (g) sitistanol, (h) Δ5-avenasterol.

Due to the growing interest in the physiological properties of phytosterols, it is very important to obtain reliable quantitative data on the content of sterols both in diets and in biological samples. The objective of this study was to achieve the complete resolution of complex mixtures of sterols, particularly of campesterol and campestanol, and of sitosterol, sitostanol and Δ5-avenasterol using low/medium polar capillary column GC.

2. Experimental

2.1. Materials

Due to the unavailability of standard samples of Δ5-avenasterol, oat lipids, which contain a considerable amount of this component, were used as the reference sample. The sample of refined oat lipids was a gift from Köttforsknings Institutet (Kävlinge, Sweden), the sample of refined rice bran oil was a gift from Riceland Foods (Stuttgart, AR, USA), and the sample of sunflower oil was purchased from a local market (Uppsala, Sweden). Standard samples of campesterol, sitosterol and brassicasterol were purchased from Larodan Fine Chemicals (Malmö, Sweden), 5α-cholestane and stigmasterol were purchased from Sigma (St. Louis, MO, USA), a mixture of stanols was donated by Raisio Margini (Raisio, Finland), Tri-Sil reagent was procured from Pierce (Rockford, IL, USA), and ethanol from Kemetyl (Hanlinge, Sweden). All other chemicals and solvents were of analytical grade and were purchased from Merck (Darmstadt, Germany) unless otherwise stated.

2.2. Analytical

2.2.1. Saponification of lipids for sterol analyses

Oat lipids, with or without spiking with sitostanol, and other vegetable oils were saponified [3]. In brief, ca. 20 mg total lipids and 20 μg 5α-cholestane as internal standard were well mixed with 2 ml 2 *M* KOH in 95% ethanol in ground-glass stoppered tubes. The tubes were shaken for 45 min at 60°C in a

glycerol bath. The reaction was stopped by cooling the tubes under running cold water and 1 ml water, 2 ml hexane and 0.1 ml absolute ethanol were added. The tubes were shaken vigorously and then centrifuged briefly in a Hettich centrifuge EBA 12 (Hettich, Tuttlingen, Germany). The hexane layer was transferred to small glass tubes, dried under nitrogen and derivatized as described below. The standard samples of sterols and stanols were derivatized without saponification.

2.2.2. Preparation of trimethylsilyl (TMS) ether derivatives of sterols

The sterols were given an addition of 100 μl Tri-Sil reagent in glass tubes, sealed with a ground-glass stopper, and well mixed by submersion in an ultrasonic bath for 2 min and then vortexing briefly. The tubes were then incubated at 60°C for 45 min. Thereafter, the solvent was evaporated under a stream of nitrogen and the TMS ether derivatives were dissolved in 0.2 ml hexane and the tubes sonicated in an ultrasonic bath for 2 min, vortexed and centrifuged for 3 min. The hexane layer was transferred to another tube, avoiding any solid particles [3], and analyzed by GC and GC–MS as described below. After derivatization, the tubes were stored at −20°C for subsequent analyses within 3 days.

2.2.3. Capillary column gas–liquid chromatography (GC)

In order to achieve separation of TMS ether derivatives of sterols, a fused-silica capillary column DB-1701 (14% cyanopropyl-phenyl-methylpolysiloxane, low/medium polarity), 30 m×0.25 mm, 0.25 μm (J&W Scientific, Folsom, CA, USA), fitted in a Varian GC 3700 gas chromatograph (Varian, Palo Alto, CA, USA) equipped with a falling needle injector and a flame ionization detector was used. The GC conditions were as follows: oven temperature at 250°C for 5 min, and then raised to 268°C at a rate of 1°C/min and maintained at this temperature for a further 27 min. Helium was used as carrier gas at a pressure of 17 p.s.i. and as a make-up gas at a flow rate of 30 ml/min (1 p.s.i.=6894.76 Pa). Detector temperature was 290°C. The peaks were computed using a HP 3396A integrator (Hewlett-Packard, Avondale, PA, USA).

2.2.4. Gas chromatography–mass spectrometry (GC–MS)

GC–MS analyses were performed on a HP5890 series II gas chromatograph (Hewlett-Packard) coupled to a TRIO-1000 mass spectrometer with an Lab-Base data system, version R2.10 (Fisons Instruments, VG Masslab, Manchester, UK). The TMS derivatives of the sterols were separated on the same column as GC analyses using helium as carrier gas at an inlet pressure of 15 p.s.i. The injector temperature was 230°C, the samples were injected in a splitless mode and the purge delay time was 0.8 min. A programmed oven temperature was used at 80°C for 1 min and then raised to 250°C at a rate of 20°C/min and then held at this temperature for 5 min before finally being raised to 265° at 1°/min and kept at this temperature for an additional 25 min. The mass spectra were recorded at an electron energy of 70 eV and the ion source temperature was 200°C.

3. Results

Baseline separation of standard samples of cholesterol, brassicasterol, campesterol, campestanol, stigmasterol, sitosterol and sitostanol was achieved with the capillary column used in this investigation (Fig. 2). Individual standard samples of sterols were tentatively identified first by their GC retention times. Retention times, relative retention times calculated using 5α-cholestane as internal standard, and linearity response of these sterols are presented in Table 1.

A chromatogram of the sterols of oat lipids, spiked with standard samples of stanols, is shown in Fig. 3. Baseline separation of sitosterol, sitostanol and Δ5-avenasterol was achieved under the gas chromatographic conditions used in this study. Separation of Δ7-stimastenol and Δ7-avenasterol was also achieved and was checked using sterol samples from rice bran oil and sunflower oil which contain considerable amounts of these sterols (results not presented).

In order to identify and check the purity of the individual sterol peaks, the sterol fraction from oat lipids, spiked with stanols, was analysed by GC–MS. The total ion GC–MS chromatogram of the TMS

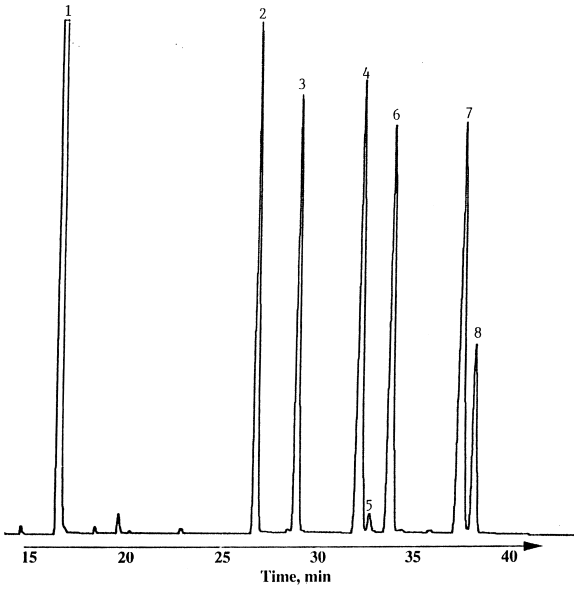

Fig. 2. Capillary column gas–liquid chromatogram showing resolution of standard sample of sterols. Column and GC conditions are given in the Experimental section. Peaks: (1) 5α-cholestane, (2) cholesterol, (3) brassicasterol, (4) campesterol, (5) campestanol, (6) stigmasterol, (7) sitosterol, (8) sitostanol.

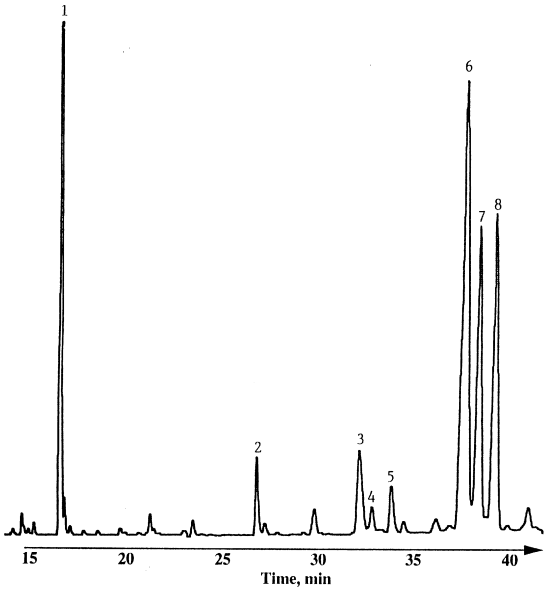

Fig. 3. Capillary column gas–liquid chromatogram showing resolution of the major sterols of oat lipids spiked with a standard sample of stanols. Peaks: (1) 5α-cholestane, (2) cholesterol, (3) campesterol, (4) campestanol, (5) stigmasterol, (6) sitosterol, (7) sitostanol, (8) Δ5-avenasterol.

ether derivatives of this sample is presented in Fig. 4. Mass spectra of some of the components of interest are presented in Fig. 5.

Table 1
Retention times (t_R), relative retention times (RRT) and linearity response (r) of the TMS ether derivatives of different sterols

Sterol	t_R (min)	RRT[a]	r[b]
5α-Cholestane	16.13	1	–
Cholesterol	26.18	1.62	0.999
Brassicasterol	28.25	1.75	0.999
Campesterol	31.70	1.97	0.999
Campestanol	32.05	1.99	0.984
Stigmasterol	33.18	2.06	0.999
Sitosterol	36.82	2.28	0.999
Sitostanol	37.48	2.32	0.998
Δ5-Avenasterol	38.13	2.40	–[c]

[a]Retention time relative to 5α-cholestane.
[b]Linearity response was determined by constructing a calibration curve (mass ratio versus area ratio) using standard solutions containing various concentrations of sterols (0.5–20 μg) except for campestanol (0.004–0.139 μg) and sitostanol (0.555–11.400 μg), and at the same concentration of 5α-cholestane (20 μg).
[c]Not calculated due to the unavailability of a standard sample of Δ5-avenasterol.

4. Discussion

Common vegetable oils and plant-based foods contain a large number of sterols belonging to the

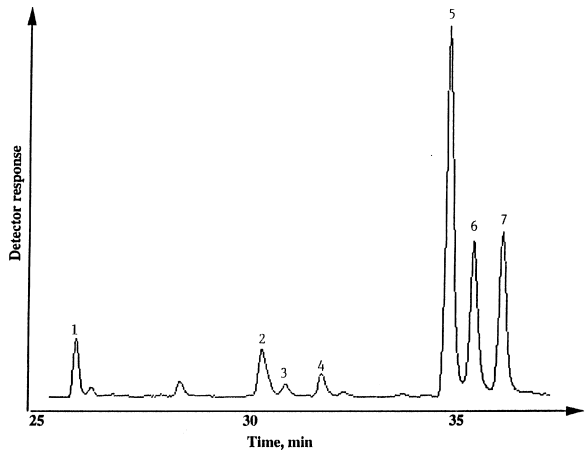

Fig. 4. GC–MS total ion chromatogram of sterols from oat lipids spiked with a standard sample of stanols. (1) Cholesterol, (2) campesterol, (3) campestanol, (4) stigmasterol, (5) sitosterol, (6) sitostanol, (7) Δ5-avenasterol.

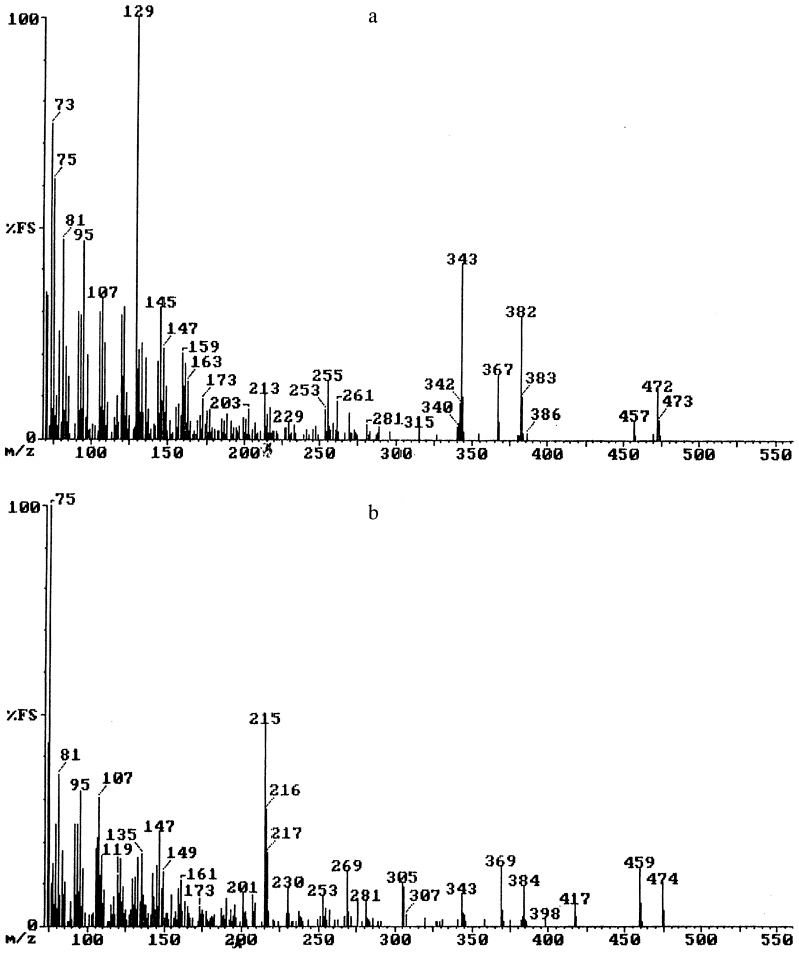

Fig. 5. Full scan mass spectra of some common sterols and stanols from the unsaponifiable fraction of oat lipids spiked with standard stanols. (a) A full scan mass spectrum of the TMS ether derivative of campesterol showing the molecular ion (M^+=472) and other prominent ion fragments; (b) a full scan mass spectrum of the TMS ether derivative of campestanol showing the molecular ion (M^+=474) and the most typical ion fragment at *m/e* 215 [M^+-side chain – 42 –$(CH_3)_3SiOH$]; (c) a full scan mass spectrum of the TMS ether derivative of sitosterol showing the molecular ion (M^+=486) and other prominent ion fragments; (d) a full scan mass spectrum of the TMS ether derivative of sitostanol showing the molecular ion (M^+=488) and the most typical ion fragment at *m/e* 215 [M^+-side chain – 42 –$(CH_3)_3SiOH$]; (e) a full scan mass spectrum of the TMS ether derivative of Δ5-avenasterol showing the molecular ion (M^+=484) and other major ion fragments.

three major groups: 4-desmethylsterols, 4-monomethylsterols and 4,4′-dimethylsterols, of which the first group dominates [2]. Analysis of the minor components requires further analytical steps. The one generally employed is preparative thin-layer chromatography of the unsaponifiable fraction [7,8]. The sterols may also be found in foods and in vegetable oil as free sterols, steryl esters, steryl glycoside and acylated steryl glycosides. Analysis of the composition of these individual forms of sterols also requires further preparative steps [9]. For refined vegetable oils, the common saponification procedure described in this paper may be adequate, since the steryl glycosides are generally removed during refining processess [2]. For this reason, total sterols can be analysed by alkali hydrolysis and subsequent analysis of the sterols by GC or other methods. On the other hand, quantification of total sterols in the lipids

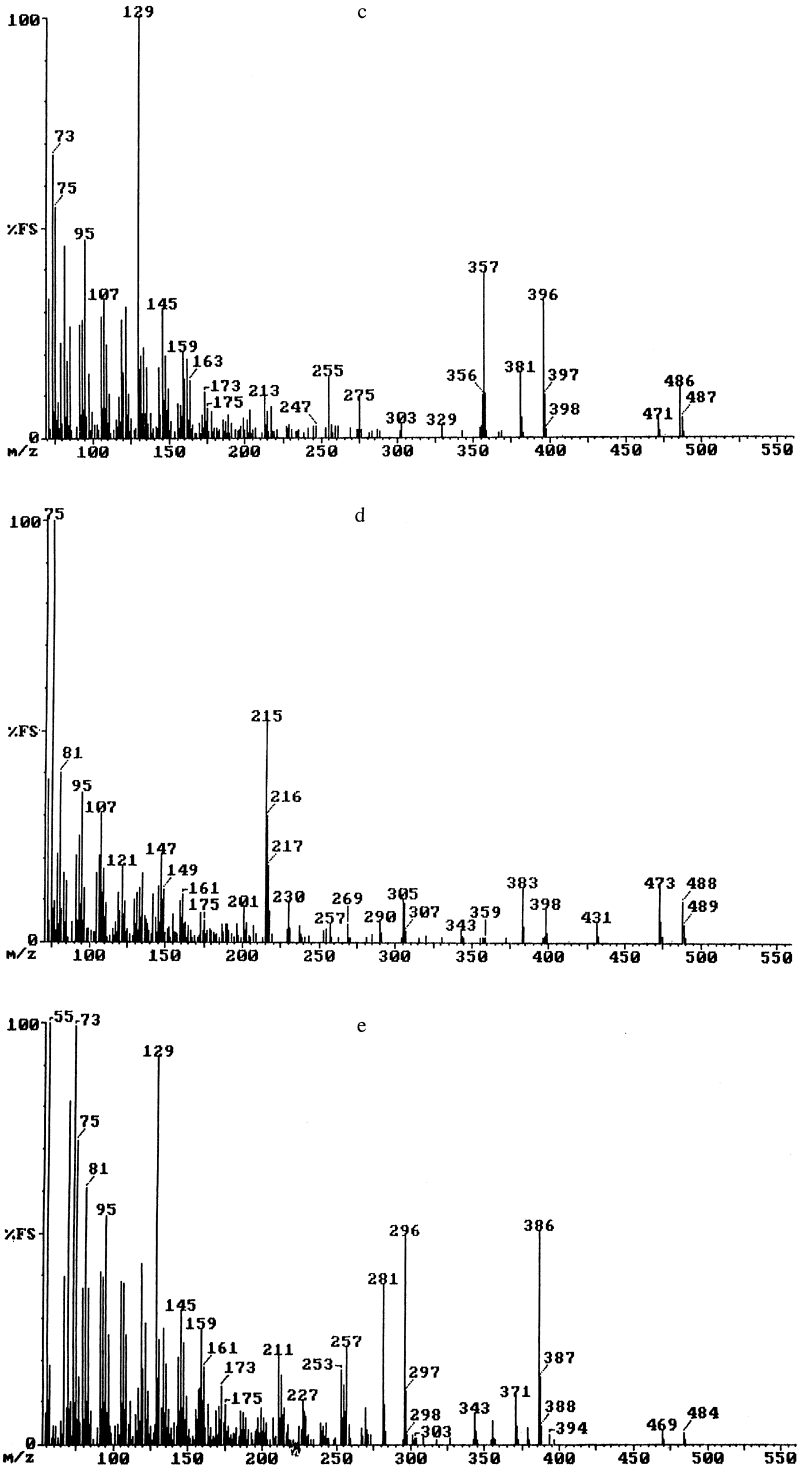

Fig. 5. (*continued*)

from diets of plant origin requires acidic conditions for hydrolysis of steryl glycosides [10]. The composition of sterols in the steryl esters and steryl glycosides, however, is mainly 4-desmethylsterols [9,11]. A detailed discussion of this aspect is presented elsewhere [12].

In cereal lipids and certain vegetable oils, some of the above 4-desmethylsterols may be present together. These are campesterol, campestanol, sitosterol, sitostanol and Δ5-avenasterol [3,5,13]. The separation of sitosterol and Δ5-avenasterol can be achieved using very non-polar and non-polar columns. The separation of campesterol and campestanol, of sitosterol and sitostanol, and of sitostanol and Δ5-avenasterol is, however, rather difficult to achieve using this type of column [3–6]. Baseline separation of these components has been achieved using the DB-1701 capillary column in this study (Figs. 2 and 3). Here, it should be mentioned that not only is the polarity of the capillary column materials important for good resolution of closely eluting peaks, but other parameters such as length, internal diameter, and film thickness should also be considered [14]. To accomplish baseline separation, particularly of sitosterol, sitostanol and Δ5-avenasterol, in addition to the DB-1701 column, a few other columns were also tested in this investigation (results not shown). These columns were of DB-1, 30 and 60 m long, and both having 0.25 mm I.D. and 0.25 μm film thickness, and a column of DB-5MS, 12 m×0.2 mm I.D. and 0.33 μm film thickness. However, none of these columns could provide a good separation of the above-mentioned sterols. Non-polar columns of different dimensions, particularly with larger film thickness, may also be able to separate these components at the expense of analysis time. Some oils, for example sunflower oil and rice bran oil, contain considerable amounts of Δ7-avenasterol and Δ7-stigmastenol [2], which also require separation for accurate quantitative results. No overlap between Δ7-stigmastenol and Δ7-avenasterol was observed, which was checked in this study by GC and GC–MS using unsaponifiables from oat lipids, rice bran oil and sunflower oil (results not shown).

The total analytical time needed to elute the common sterols, including Δ7-avenasterol and Δ7-stigmastenol, was accomplished in less than 45 min under the analytical conditions used in this investigation. The linear response of all the standard sterols is almost 1, except that of campestanol, which shows a slightly lower value. This may be explained by the very low concentration of this compound (0.004–0.139 μg) compared with other compounds (0.5–20 μg). The reason for the very low concentration of campestanol is that it was a co-product present in a sample of pure stanols where sitostanol was the major component (>90%).

Sterols can be analyzed by GC as free form, as 3β-acetoxy, or as TMS ether derivatives. However, analysis of free sterols does not produce good quantitative results [6]. The analysis of sterols as TMS derivatives by GC is generally preferred as this analysis gives good reproducible results, and allows positive identification by GC–MS because of the abundance of molecular ions compared with 3β-acetoxy sterols [6,15]. For these reasons, TMS ether derivatives of sterols were analysed by GC in this study. The mass spectra of some of the sterols of interest (Fig. 5) show the abundances of different characteristic ion fragments concurring with published results [3,8,15]. The purity of the peaks of cholesterol, campesterol, campestanol, stigmasterol, sitosterol, sitostanol, Δ5-avenasterol, Δ7-avenasterol and Δ7-stigmastenol was checked by analyzing the mass spectrum of these components from different regions of the peaks. No overlapping with other components was observed.

In conclusion, this study has shown that the capillary column used in this investigation gives excellent resolution of some common sterols and stanols, enabling accurate quantification of these interesting compounds.

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