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Separation of Δ5- and Δ7-Phytosterols by Adsorption Chromatography and Semipreparative Reversed Phase High-Performance Liquid Chromatography for Quantitative Analysis of Phytosterols in Foods

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A method for the separation, isolation, and identification of phytosterols was developed. A commercial phytosterols mixture, Generol 95S, was fractionated first by adsorption silica gel column chromatography and then separated by means of a semipreparative reverse phase high-performance liquid chromatography fitted with a Polaris C8-A column (250 mm × 10 mm i.d., 5 μm) using isocratic acetonitrile:2-propanol:water (2:1:1, v/v/v) as the mobile phase. Milligram scales of six individual phytosterols, including citrostadienol, campesterol, β-sitosterol, Δ7-avenasterol, Δ7-campesterol, and Δ7-sitosterol, were obtained. Purities of these isolated sterols were 85−98%. Relative response factors (RRF) of these phytosterols were calculated against cholestanol as an authentic commercial standard. These RRF values were used to quantify by gas chromatography−mass spectrometry (GC-MS) the phytosterols content in a reference material, oils, and chocolates.

KEYWORDS: Phytosterol; isolation; column chromatography; semipreparative RP-HPLC; quantitative analysis

INTRODUCTION

Phytosterols are among the most important compounds in plant cells and in the unsaponifiable matter of vegetable foods (*1*−*3*). Research on the biological activities and health-promoting effects of several phytosterols and their conjugates yielded positive results, such as low-density lipoprotein cholesterol reduction (*3*, *4*) and anticancer activity (*5*, *6*).

Sterols may be categorized into three subclasses based on the number of methyl groups on C-4: (I) 4,4′-dimethylsterols, (II) 4-methylsterols, and (III) 4-desmethylsterols (*3*). 4,4′-Dimethyl and 4-methylsterols are metabolic intermediates in the biosynthetic pathway leading to the end product (4-desmethylsterols) and are usually only minor components in most plant sources. Phytosterols contain 28 or 29 carbons and one or two carbon−carbon double bonds, typically one in the sterol nucleus and sometimes a second in the alkyl side chain. According to the different double bond position in the sterol nucleus, phytosterols may also be categorized into Δ5- and Δ7-sterols.

Major phytosterols occurring in vegetable oils are β-sitosterol (40.2−92.3%) and campesterol (2.6−38.6%), accompanied with some other sterols, such as stigmasterol (0.0−31.0%) and Δ5-avenasterol (1.5−18.8%), with a widespread occurrence (*7*−*10*). They differ structurally from cholesterol only with regard to their side chains, which include the addition of a double bond and/or methyl or ethyl group (for structures, see **Figure 1**). A small fraction of cholesterol can also be found in the membranes of some plant cells (*11*). Δ7-Phytosterols are also present in some plant sources, for example, sunflower oil or rice bran oil, but at lower concentrations (*12*, *13*).

Because of the growing interest in the physiological properties of phytosterols and their conjugates with other derivative products, it is important to obtain reliable quantitative data on the content of sterols both in diets and in biological samples. Gas chromatography with mass spectrometric detection (GC-MS) is of great help as it is a rapid method, which allows an unambiguous identification of the sterols in foods by comparison of both the detected mass spectra and the t_R with those of authentic standards. Nevertheless, this detection is linked to the fragmentation pattern of the molecule, which

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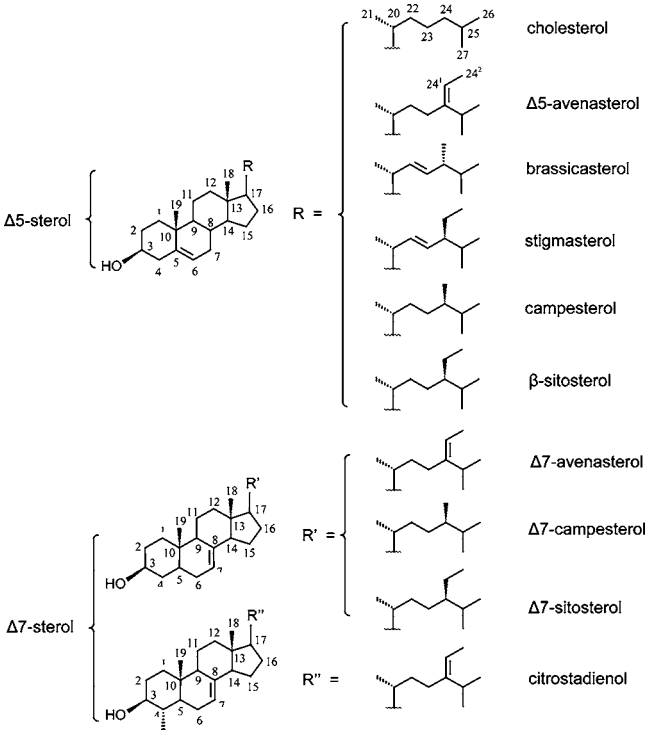

Figure 1. Chemical structures of cholesterol and the studied phytosterols.

depends on its chemical structure and necessitates the determination of GC-MS relative response factors (RRF) and then the availability of commercial highly pure standards. However, with the exception of stigmasterol (Sigma; purity, 95%), β-sitosterol (Acros or Sigma; purity, 97%), and campesterol (Sigma; purity, 60%), no other high-purity individual phytosterols are so far commercially available.

There are two ways to obtain sterols. They may be biosynthesized via a complex time-consuming biosynthetic pathway that includes the reduction of starting material HMG-CoA, the combination of small molecular units, an enzymatic ring closure step, and additional enzymatic reactions (*10*). As phytosterols exist in large amounts in natural plant sources, efforts have been focused on chromatographic techniques, including column chromatography, thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), and GC, for their separation, isolation, and quantification. Abidi (*14*) reviewed the published chromatographic methods for sterol analysis in vegetable oils, seeds, and other plants. Akihisa et al. (*15*, *16*) presented the semipreparative HPLC separation of sterol acetates by Ultrasphere ODS column. Grandgirard et al. (*17*) reported a milligram scale separation of β-sitosterol, campesterol, and brassicasterol by Waters Spherisorb S5 ODS2 column. Zhang et al. (*18*) isolated β-sitosterol at the gram scale by column chromatography, although the method used was not suitable for all phytosterols. On the other hand, there are no reports on the separation of Δ7-phytosterols.

The aim of this study is to present a method to isolate individual Δ5- and Δ7-phytosterols in milligram quantities from a commercial phytosterol mixture (an unsaponifiable fraction of sunflower oil) by adsorption column chromatography followed by a semipreparative reverse phase (RP)-HPLC system. Their chromatographic and spectral data were used for the quantitative analysis of phytosterols in a reference material and in some foods.

MATERIALS AND METHODS

Materials and Reagents. Generol 95S (an unsaponifiable fraction of sunflower oil) was provided by Cognis (Saint-Fargeau-Ponthierry, France) and stored at 4 °C before use. Δ5-Stigmasterol (95%) was purchased from Acros (NJ), and cholesterol (99%) and 3β-hydroxy-5α-cholestane (cholestanol, 95%) were from Sigma (St. Louis, MO). Certified reference material BCR-633 (5 g package) was obtained from the European Commission-Community Bureau of Reference (Geel, Belgium). Sunflower, olive, colza, and soja oils were of edible quality and purchased at a local supermarket. Chocolate Madagascar, chocolate Equateur, and chocolate Venezuela were provided by Valrhona (Tain l'Hermitage, France). Ethyl acetate, isooctane, and 2-propanol of chromatographic grade were purchased from Acros (Geel, Belguim) and Fluka (Steinheim, Germany). Chloroform was obtained from Acros, cyclohexane was from Fluka (Buchs, Switzerland), diethyl ether was from Merck (Darmstadt, Germany), and pyridine was from Prolabo (Fontenay-sous-Bois, France). These solvents were of analytical grade. Heptane of technical grade was purchased from SDS (Peypin, France) and distilled from calcium hydride before use. Water was purified using a Milli-Q system (Millipore Co., MA).

TLC and Column Chromatography. Analytical TLC was performed on silica gel 60 F_{254} precoated aluminum plates (Merck) and developed by heptane:ethyl acetate (2:1, v/v) or ethyl acetate:diethyl ether:petroleum ether (2:1:5, v/v/v) as eluants. After chromatography, the TLC plate was dried briefly in air and the spots were visualized by spraying phosphomolybdic acid reagent in ethanol (20% v/v, Aldrich, Steinheim, Germany) followed by heating at 150 °C.

One hundred milligrams of Generol 95S dissolved in 10 mL of chloroform was applied onto the silica gel column (35 cm × 2 cm i.d., 60 g of silica gel Si 60, particle size 40−63 μm, Geduran, Merck) and eluted with heptane:ethyl acetate (6:1, v/v) at a flow rate of 4 mL/min to be separated into three sterol classes. Elution fractions were monitored by TLC plates. The organic solvents were removed by a rotary evaporator (Rotavapor, Büchi, Switzerland) under reduced pressure at 40 °C.

Analytical HPLC and Semipreparative HPLC. The HPLC system consisted of a Varian ProStar 210 pump (Varian, France), a 7125-038 sample injector (Rheodyne, United States), and a Varian 9050 Variable Wavelength UV−vis detector (Varian).

In the analytical HPLC system, a 20 μL injection loop and a Polaris C8-A column (250 mm × 4.6 mm i.d. stainless steel, 5 μm particles, Varian) were used to separate the phytosterols at ambient temperature with an isocratic mobile phase (acetonitrile:2-propanol:water = 2:1:1, v/v/v) at a flow rate of 0.5 mL/min. The chromatograms were monitored by UV detection at 208 nm, 0.1 AUFS.

For semipreparative separation, a 0.5 mL injection loop and a Polaris C8-A column (250 mm × 10 mm i.d. stainless steel, 5 μm particles, Varian) were used with the same isocratic mobile phase as mentioned above at a flow rate of 2 mL/min. A 0.5 mL amount of saturated acetonitrile solution of sterol classes, which were previously separated by column chromatography, was injected successively into the semipreparative HPLC system. The individual phytosterols were collected during several runs. After removal of organic solvents by rotary evaporation under reduced pressure at 40 °C, the phytosterols were extracted with dichloromethane (3 × 10 mL) from the remaining aqueous phase. The combined organic phases were evaporated to obtain the pure individual sterols as white powder. The purities of isolated phytosterols were determined as the ratio of the area of the chromatographic peak of interest against the sum of the areas of all peaks in the GC chromatogram.

Derivatization. The sterols were converted to trimethylsilyl (TMS) ethers by reaction with 50 μL of pyridine and 40 μL of N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA, Sigma-Aldrich) at 50 °C for 60 min. The solution was then diluted with 410 μL of isooctane and thoroughly mixed prior to GC analysis.

GC-MS. GC-MS analyses were performed on a Varian STAR 3400 GC instrument equipped with an on-column SPI injector coupled to a ion trap Varian SATURN 2000 mass sensitive detector (Varian) operating in the electronic impact (EI) ionization mode (accelerating voltage, 70 eV; emission current, 10 μA; axial modulation amplitude,

4.0 V; scan rate, 1 s; trap, manifold, and the transfer line temperature, respectively, 150, 40, and 270 °C; and electronmultiplier voltage, 1800 V) with full scan mode between 40 and 600 *m/z*. The data were acquired using the Auto Gain Control (AGC: 20000 ions) feature of the Saturn 2000 mass spectrometer. Data acquisition and processing and instrumental control were performed by Varian Saturn WS software. The separation of phytosterol mixture was carried on a VF-5ms capillary column (stationary phase: 5% phenyl−95% dimethylpolysiloxane, thickness of 0.1 μm, 60 m × 0.25 mm, Varian). The column temperature was programmed from 80 to 280 °C at 20 °C/min, then to 292 °C at 0.5 °C/min, and to 300 °C at 2 °C/min. The injector temperature was set at 250 °C for 40 min. The detector temperature was 120 °C. Helium (purity 99.9995%, Air Liquide, Paris-La-Défense, France) was used as a carrier gas with a flow rate of 1 mL/min. The injection volume was 1 μL.

Other Apparatus. NMR spectra were recorded on a Bruker AC-300 spectrometer (^{1}H NMR at 300 MHz and ^{13}C NMR at 75 MHz) in $CDCl_3$ as the solvent at ambient temperature. The chemical shifts were reported in units of δ. Melting points (mp) were measured with a SMP3 melting apparatus (Bibby Stuart Scientific, United Kingdom). Optical rotations were measured with a Perkin-Elmer 341 polarimeter (Bodenseewerk, Perkin-Elmer & Co GmbH, Germany).

Citrostadienol. White powder (10 mg, purity 90%); mp 161.6−163.6 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.16 (1H, br d, H-7), 5.15 (1H, br d, H-24^1), 3.59 (1H, m, H-3), 2.04−1.21 [29H, m, including 1d (3H, H-24^2) at 1.55 ppm], 0.91 (3H, d, J = 6.5 Hz, H-26), 0.85 (3H, d, J = 6.8 Hz, H-27), 0.81 (3H, d, J = 6.9 Hz, H-21), 0.79 (3H, s, H-19), 0.77 (3H, d, J = 6.6 Hz, H-28), 0.53 (3H, s, H-18). ^{13}C NMR (75 MHz, $CDCl_3$): δ 145.84, 139.13, 117.46, 116.46, 76.22, 56.00, 54.94, 49.63, 46.64, 43.37, 40.25, 39.53, 36.99, 36.58, 35.90, 34.83, 30.97, 28.60, 27.98, 26.63, 22.92, 21.36, 21.08, 21.00, 18.92, 15.14, 14.14, 12.76, 11.84. $[\alpha]_D^{20}$ +8° (*c* 1, $CHCl_3$).

Campesterol. White powder (20 mg, purity 98%); mp 157−158 °C [literature mp value, 158 °C (*19*)]. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.35 (1H, br d, H-6), 3.53 (1H, m, H-3), 2.27 (2H, m), 2.00 (2H, m), 1.85 (3H, m), 1.56−1.07 (21H, m), 1.01 (3H, s, H-19), 0.91 (3H, d, J = 6.5 Hz, H-21), 0.85 (3H, d, J = 6.5 Hz, H-26), 0.80 (3H, d, J = 6.5 Hz, H-27), 0.77 (3H, d, J = 6.5 Hz, H-24^1), 0.68 (3H, s, H-18). ^{13}C NMR (75 MHz, $CDCl_3$): δ 140.76, 121.72, 71.80, 56.76, 56.09, 50.12, 42.31, 39.77, 38.83, 37.25, 36.50, 35.88, 33.70, 32.42, 31.90, 31.67, 30.26, 28.23, 24.29, 21.08, 20.20, 19.40, 18.70, 18.25, 15.37, 11.86. $[\alpha]_D^{20}$ −34° (*c* 1, $CHCl_3$).

***β*-Sitosterol.** White solid (70 mg, purity 95%); mp 136−138 °C [literature mp value, 138−139 °C (*19*)]. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.34 (1H, br d, H-6), 3.51 (1H, m, H-3), 2.27−1.08 (30H, m), 1.00 (3H, s, H-19), 0.92 (3H, d, J = 6.5 Hz, H-21), 0.84 (3H, t, J = 7.2 Hz, H-24^2), 0.83 (3H, d, J = 6.5 Hz, H-26), 0.80 (3H, d, J = 6.6 Hz, H-27), 0.68 (3H, s, H-18). ^{13}C NMR (75 MHz, $CDCl_3$): δ 140.75, 121.71, 71.79, 56.76, 56.05, 50.13, 45.83, 42.30, 39.77, 37.25, 36.50, 36.14, 33.94, 31.90, 31.65, 29.14, 28.24, 26.07, 24.30, 23.06, 21.08, 19.82, 19.39, 19.03, 18.77, 11.98, 11.85. $[\alpha]_D^{20}$ −34° (*c* 2, $CHCl_3$) [literature value, $[\alpha]_D^{25}$ −38° (*19*)].

Δ7-Avenasterol. White powder (27 mg, purity 98%); mp 143.8−144.8 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.15 (1H, br d, H-7), 5.10 (1H, d, J = 6.8 Hz, H-24^1), 3.59 (1H, m, H-3), 2.83 (1H, m, H-25), 2.42−1.24 [27H, m, including 1d (3H, J = 6.6 Hz, H-24^2) at 1.59 ppm], 0.98 (3H, d, J = 7.0 Hz, H-26), 0.96 (3H, d, J = 7.0 Hz, H-27), 0.94 (3H, d, J = 7.0 Hz, H-21), 0.79 (3H, s, H-19), 0.54 (3H, s, H-18). ^{13}C NMR (75 MHz, $CDCl_3$): δ 145.83, 139.59, 117.44, 116.46, 71.05, 56.01, 55.03, 49.44, 43.40, 40.25, 39.55, 37.99, 37.14, 36.58, 35.91, 34.14, 31.48, 29.64, 28.60, 28.00, 27.95, 22.96, 21.55, 21.08, 21.00, 18.92, 13.04, 12.76, 11.84. $[\alpha]_D^{20}$ +9° (*c* 1, $CHCl_3$).

Δ7-Campesterol. White powder (3 mg, purity 85%); mp 129.6−131.8 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.16 (1H, br d, H-7), 3.59 (1H, m, H-3), 2.09−1.20 (28H, m), 0.91 (3H, d, J = 6.5 Hz, H-21), 0.85 (3H, d, J = 6.8 Hz, H-26), 0.81 (3H, d, J = 6.6 Hz, H-27), 0.79 (3H, s, H-19), 0.76 (3H, d, J = 6.6 Hz, H-24^1), 0.53 (3H, s, H-18). ^{13}C NMR (75 MHz, $CDCl_3$): δ 139.63, 117.41, 71.06, 56.12, 55.04, 49.44, 43.38, 40.25, 39.56, 38.83, 37.99, 37.13, 36.31, 34.20, 33.65, 32.41, 31.48, 30.37, 29.64, 27.95, 22.95, 21.55, 20.20, 18.82, 18.26, 15.37, 13.04, 11.84. $[\alpha]_D^{20}$ +9° (*c* 1, $CHCl_3$).

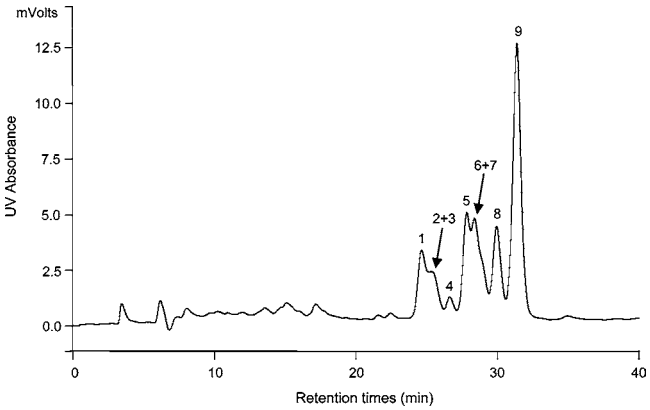

Figure 2. Total analytical HPLC chromatogram of Generol 95S on a Polaris C8-A column (250 mm × 4.6 mm i.d., 5 μm particles); mobile phase, acetonitrile:2-propanol:water (2:1:1, v/v/v); flow rate, 0.5 mL/min; UV detection, 208 nm; 0.1 AUFS. Peaks are identified as in **Table 1**: 1, Δ7-avenasterol; 2, brassicasterol; 3, Δ5-avenasterol; 4, Δ7-campesterol; 5, campesterol; 6, citrostadienol; 7, stigmasterol; 8, Δ7-sitosterol; and 9, *β*-sitosterol.

Δ7-Sitosterol. White powder (23 mg, purity 95%); mp 144−145 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.16 (1H, br d, H-7), 3.59 (1H, m, H-3), 2.04−1.19 (30H, m), 0.92 (3H, d, J = 6.4 Hz, H-21), 0.84 (3H, d, J = 7.2 Hz, H-24^2), 0.83 (3H, d, J = 6.5 Hz, H-26), 0.81 (3H, d, J = 6.5 Hz, H-27), 0.79 (3H, s, H-19), 0.53 (3H, s, H-18). ^{13}C NMR (75 MHz, $CDCl_3$): δ 139.62, 117.42, 71.06, 56.07, 55.04, 49.44, 45.83, 43.38, 40.25, 39.55, 37.99, 37.14, 36.58, 34.20, 33.89, 31.48, 29.64, 29.14, 27.97, 26.17, 23.06, 22.96, 21.55, 19.83, 19.03, 18.90, 13.04, 11.97, 11.84. $[\alpha]_D^{20}$ +7° (*c* 1, $CHCl_3$).

Soxhlet Extraction of Phytosterols from Chocolates. Chocolate samples (5 g) were frozen under liquid nitrogen, finely ground, and placed in extraction thimbles (22 mm × 80 mm, Schleicher&Schuell MicroScience GmbH, Dassel, Germany) with 250 mL of chloroform in a Soxhlet apparatus (2 cycles h^{-1}) (Verrerie Striegel, Strasbourg, France). Extractions lasted 16 h (further cycles did not lead to more phytosterol extractions). The extracts (about 2 g) were dried with a rotary evaporator (35 °C, 200 mbar) and stored at 4 °C until further analysis.

Isolation and Purification of Phytosterols from Food Samples. The procedure for phytosterol preparation used here was adapted from the analytical method described in NF EN ISO 12228 (*20*) and the one used for BCR-633 (*21*).

The protocol was as follows: 20 μg of cholestanol (40 μL of 0.5 mg/mL in ethyl acetate) used as an internal standard was spiked into 100 mg of BCR-633, oils, or chocolate extracts in a 100 mL flask. After removal of ethyl acetate under nitrogen, 5 mL of 0.5 M KOH ethanolic solution was added, and the mixture was stirred and refluxed at 90 °C for 60 min. After the reaction system was cooled to room temperature, the solution was extracted with diethyl ether (3 × 20 mL). The combined organic extracts were washed with 20 mL of 0.5 M aqueous KOH solution and 0.2 M aqueous Na_2SO_4 solution (3 × 15 mL). The organic solvents were removed with a rotary vacuum evaporator (30 °C, 400 mbar), and the residue was dissolved in 1 mL of a solution of cyclohexane/diethyl ether (9:1, v/v). The sterol fraction was purified by SiOH solid phase extraction (SPE) cartridge (3 mL/500 mg, Chromabond, Macherey-Nagel, Düren, Germany). The cartridges were first conditioned with 5 mL of cyclohexane before the samples were loaded. Low-polarity lipids were eluted with 5 mL of cyclohexane/diethyl ether (9:1, v/v) and discarded, and the sterol fractions were then eluted with 4.5 mL of cyclohexane/diethyl ether (1:1, v/v) in test tubes and dried under gentle nitrogen flow. The purified sterols were converted to TMS ethers as mentioned above before GC analysis.

Quantitative Analysis. Quantitation was performed by GC-MS with total ion count (TIC) mode against cholestanol as the internal standard. The MS RRF values of each sterol TMS ether were determined toward

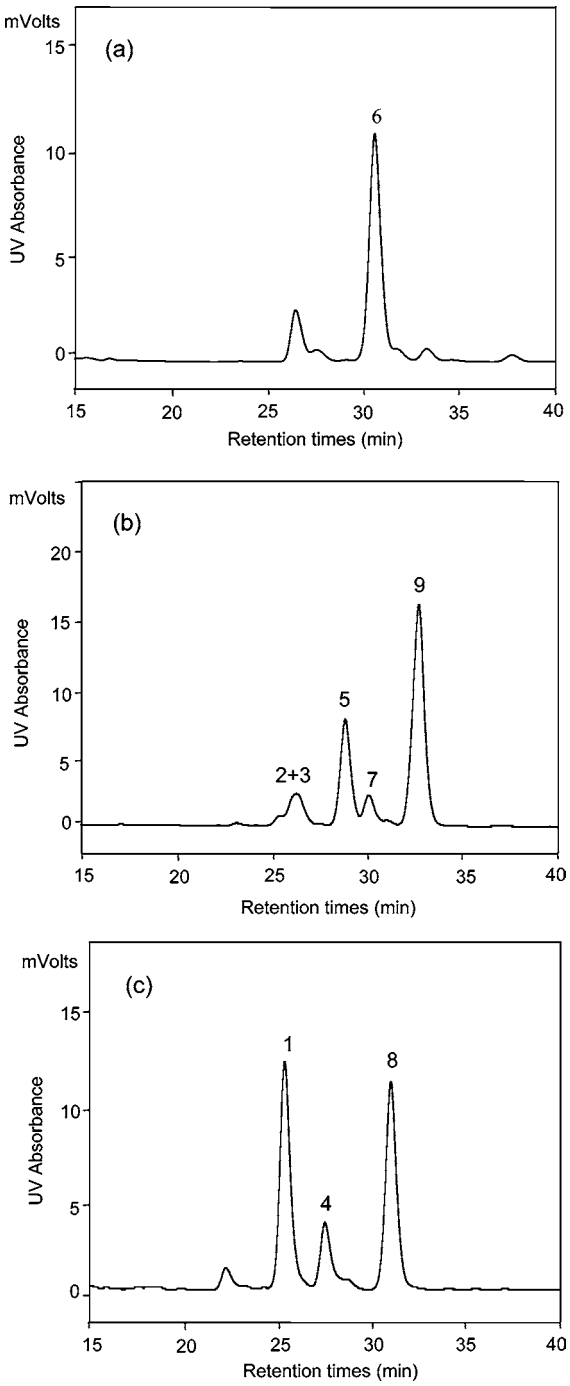

Figure 3. HPLC chromatograms of three sterol classes obtained from silica gel column chromatography: (**a**) 4-methylsterols, (**b**) Δ5-4-desmethylsterols, and (**c**) Δ7-4-desmethylsterols. Analytical HPLC conditions are as **Figure 2**. Peaks are identified as in **Table 1** and **Figure 2**.

cholestanol TMS ether by the formula:

$$\mathrm{RRF_c} = \frac{A_{is}}{A_c} \times \frac{n_c}{n_{is}} \times \mathrm{RRF_{is}}$$

where A_c and A_{is} were the peak areas of studied sterol and of the internal standard, respectively; n_c and n_{is} were the moles of studied sterol and of the internal standard, respectively; and RRF_{is} was the response factor of internal standard, which was set to 1.

Statistical Analysis. The statistical analysis of RRF values of each sterol (performed in eight replicates) was done with a two-sided Student test within a 95% confidence interval, after having verified the equality of the standard deviations by a two-sided Fischer−Snedecor test within a 95% confidence interval.

Table 1. Retention Times (t_R) and Relative Retention Times (t_{RR}) of Phytosterols in an Analytical RP-HPLC System

		HPLC[a]	
peak no.	compound	t_R (min)	t_{RR}[b]
	cholesterol	25.4	1.00
	cholestanol	25.4	1.00
1	Δ7-avenasterol	24.6	0.97
2	brassicasterol	25.4	1.00
3	Δ5-avenasterol	25.4	1.00
4	Δ7-campesterol	26.7	1.05
5	campesterol	27.9	1.10
6	citrostadienol	28.4	1.13
7	stigmasterol	29.0	1.14
8	Δ7-sitosterol	30.0	1.18
9	β-sitosterol	31.8	1.25

[a] Analytical HPLC was carried on a reversed phase Polaris C8-A column (250 mm × 4.6 mm i.d.) with an isocratic mobile phase (acetonitrile:2-propanol:water = 2:1:1, v/v/v) at a flow rate of 0.5 mL/min. UV detection was at 208 nm; 0.1 AUFS. [b] $t_{RR} = t_R$ of a phytosterol/t_R of internal standard (cholesterol).

Table 2. Retention Times (t_R) and Relative Retention Times (t_{RR}) of Phytosterols in GC Analysis

	GC[a]			GC[a]	
compound	t_R (min)	t_{RR}[b]	compound	t_R (min)	t_{RR}[b]
cholesterol	30.22	0.99	β-sitosterol	34.23	1.12
cholestanol	30.51	1.00	Δ5-avenasterol	34.65	1.14
brassicasterol	31.05	1.02	Δ7-sitosterol	35.84	1.18
campesterol	32.35	1.06	Δ7-avenasterol	36.28	1.19
stigmasterol	32.85	1.08	citrostadienol	38.71	1.27
Δ7-campesterol	33.77	1.11			

[a] GC chromatography was achieved on a VF-5ms capillary column. [b] $t_{RR} = t_R$ of a phytosterol/t_R of internal standard (cholestanol) as TMS ether.

The Student coefficient t was computed as follows:

$$t = \frac{|m_1 - m_2|}{\sqrt{\left(\frac{\nu_1 {s_1}^2 + \nu_2 {s_2}^2}{\nu_1 + \nu_2}\right)\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}}$$

where m_1 and m_2 were the means of the RRF values of each sterol, respectively; s_1 and s_2 were the standard deviations, respectively; ν_1 and ν_2 were the degrees of freedom; and n_1 and n_2 were the numbers of measures, respectively. This coefficient was then compared to the Student coefficient $t_{(\nu,0.975)}$ found in the tables (*22*).

RESULTS AND DISCUSSION

Chromatographic Behavior of Phytosterols on TLC and Column Chromatography. In a crude plant lipid exact, plant sterols occur as free sterols, steryl esters, steryl glycosides, and acylated steryl glycosides (*3*, *23*). They can be preliminarily separated by preparative TLC or column chromatography with suitable mobile phases, such as chloroform:ethanol, petroleum ether:diethyl ether, or hexane:diethyl ether, to get enriched sterol fractions for GC or HPLC analysis (*14*, *24*, *25*).

In the present work, TLC was used to find a suitable eluant for the column chromatography. The three sterol classes (Δ7-4-methylsterols, Δ5-4-desmethylsterols, and Δ7-4-desmethylsterols) present in Generol 95S were separated on TLC plates using heptane:ethyl acetate (2:1, v/v) or ethyl acetate:diethyl ether:petroleum ether (2:1:5, v/v/v) as mobile phase. With both eluants, the TLC mobilities were as follows: $R_{f\Delta 7-4-\mathrm{methylsterols}} > R_{f\Delta 5-4-\mathrm{desmethylsterols}} > R_{f\Delta 7-4-\mathrm{desmethylsterols}}$. This result showed that the sterols separation achieved on the

Table 3. Fragmentation Ions of the Phytosterols as TMS Ethers

	main fragmentation ions, *m/z* (relative abundance)[a]					
	M^+	$M^+ - 15$	$M^+ - 90$	$M^+ - 105$	$M^+ - 129$	others
brassicasterol	470 (48)	455 (16)	380 (65)	365 (45)	341 (26)	283 (18), 255 (55), 213 (26), 159 (31), 129 (76), 69 (100)
campesterol	472 (28)	457 (12)	382 (100)	367 (59)	343 (63)	315 (9), 255 (35), 213 (12), 129 (76), 73 (71), 43 (57)
stigmasterol	484 (21)	469 (9)	394 (28)	379 (11)	355 (6)	281 (8), 255 (12), 213 (15), 129 (39), 117 (65), 73 (100), 55 (49)
Δ7-campesterol	472 (100)	457 (45)	382 (32)	367 (83)		345 (20), 318 (11), 255 (91), 213 (82), 147 (32), 105 (39), 75 (51), 43 (44)
β-sitosterol	486 (25)	471 (14)	396 (100)	381 (52)	357 (60)	255 (37), 213 (12), 129 (85), 73 (79), 43 (55)
Δ5-avenasterol	484 (4)	469 (7)	394 (2)	379 (4)	355 (8)	379 (19), 344 (14), 281 (94), 255 (35), 213 (21), 129 (46), 73 (100), 55 (65)
Δ7-sitosterol	486 (67)	471 (36)	396 (24)	381 (65)	357 (12)	355 (22), 281 (31), 255 (90), 213 (100), 147 (65), 105 (73), 75 (95), 43 (52)
Δ7-avenasterol	484 (1)	469 (5)	394 (1)	379 (4)		385 (19), 343 (100), 281 (9), 255 (33), 213 (9), 145 (8), 105 (14), 75 (21)
citrostadienol	498 (1)	483 (3)	408 (2)	393 (6)		400 (6), 357 (100), 267 (35), 145 (9), 105 (8), 75 (13), 55 (20)

[a] Intensity relative to base peak (percent).

TLC was based not only on the degree of methyl substitution at C-4 as described in the literature (*14*, *25*) but also on the position of double bonds at C-5 or C-7 on the ring structure.

Consequently, a chromatographic separation of the Generol 95S phytosterol mixture was performed on a silica gel glass column. The sample size applied onto the column should not exceed 100 mg to avoid any overload, which may result in poor resolution. Despite the very close polarities of Δ5- and Δ7-4-desmethylsterols, chromatographic separation of the three sterol classes could be achieved using the mobile phase heptane:ethyl acetate (6:1, v/v). This chromatographic step is necessary to reduce the number of compounds present in each sterol class and to increase their concentration, then allowing better separations of individual sterols on the following semipreparative HPLC.

Analytical HPLC and Semipreparative HPLC for the Separation of Phytosterols on Polaris C8-A Column. Analytical HPLC was used to analyze the content of each separated three sterol fractions and of the Generol 95S mixture. **Figures 2** and **3** show analytical HPLC chromatograms of, respectively, the total sterols content of the mixture Generol 95S and its three sterol classes separated with an analytical Polaris C8-A column using acetonitrile:2-propanol:water (2:1:1, v/v/v) as the mobile phase. Isocratic conditions were chosen since, while providing a good separation of sterols, they did not require equilibration between runs and allowed minimal variations in individual sterol retention times and resolution.

The retention times (t_R) and relative retention times (t_{RR}) of the phytosterols in relation to cholesterol as internal standard in analytical HPLC are listed in **Table 1**. An interesting result was that Δ5- and the corresponding Δ7-sterols could be resolved as pairs, with the Δ7-sterols eluting earlier. HPLC retention times of these nine phytosterols yielded the following elution order: $t_{R\Delta7-avenasterol} < t_{R(\Delta5-avenasterol+brassicasterol)} < t_{R\Delta7-campesterol} < t_{Rcampesterol} < t_{R(citrostadienol+stigmasterol)} < t_{R\Delta7-sitosterol} < t_{R\beta-sitosterol}$. Using this separation system, Δ5-avenasterol coeluted with brassicasterol, and citrostadienol coeluted with stigmasterol (**Figure 2**). However, as mentioned above, 4-desmethylsterols could be separated from 4-methylsterols by silica gel chromatography. Therefore, in the subsequent HPLC separation, stigmasterol (in **Figure 3b**) would not interfere with the isolation of citrostadienol (in **Figure 3a**), since each sterol class could be chromatographied separately.

Table 4. RRFs of Phytosterols as TMS Ethers in Relation to that of Cholestanol[a]

sterols	RRF[a]
cholestanol (internal standard)	
cholesterol	1.20 ± 0.03
campesterol	1.15 ± 0.04
stigmasterol	1.23 ± 0.05
β-sitosterol	1.20 ± 0.05
Δ7-campesterol	1.58 ± 0.08
Δ7-sitosterol	1.50 ± 0.06
Δ7-avenasterol	1.56 ± 0.10
citrostadienol	1.70 ± 0.09

[a] Mean ± standard deviation ($n = 8$). [b] In relation to cholestanol (internal standard).

In the Δ7-4-methylsterol class (**Figure 3a**), the major component was citrostadienol. In the Δ5-4-desmethylsterol class (**Figure 3b**), five compounds were detected including campesterol, stigmasterol, β-sitosterol, and brassicasterol coeluting with Δ5-avenasterol whatever the mobile phase used. As these two compounds were minor constituents of food phytosterols, their separation was ignored. In the Δ7-4-desmethylsterol class (**Figure 3c**), Δ7-avenasterol, Δ7-campesterol, and Δ7-sitosterol were recovered.

In a semipreparative HPLC system, scale-up generally leads to increase overlapping of neighboring peaks and loss of resolution. Therefore, the final sample size of sterol classes, which was previously separated by column chromatography, was limited to 0.5 mL of saturated acetonitrile solutions of sterols and injected into semipreparative HPLC system. The phytosterol peaks were monitored by UV detector and collected manually except for stigmasterol because of the poor resolution of the semipreparative HPLC between campesterol and stigmasterol. As stigmasterol was commercially available with high purity and at an affordable price, efforts were focused on the collection of pure campesterol.

At last, six individual phytosterols, including one Δ7-4-methylsterol (citrostadienol), two Δ5-4-desmethylsterols (Δ5-campesterol and β-sitosterol), and three Δ7-4-desmethylsterols (Δ7-avenasterol, Δ7-campesterol, and Δ7-sitosterol), were isolated at the milligram scale after several semipreparative HPLC injections. Their ^1H and ^{13}C NMR profiles determined

Table 5. Concentration of Phytosterols in Oils and Chocolates (mg/kg)[a]

					chocolate		
	sunflower oil	olive oil	colza oil	soja oil	Madagascar	Equateur	Venezuela
cholesterol			31.4 ± 2.8		28.2 ± 0.5	34.6 ± 0.8	35.2 ± 0.2
brassicasterol			790.1 ± 28.3	16.5 ± 1.8			
campesterol	359.4 ± 1.0	46.4 ± 6.7	2810.1 ± 24.0	577.6 ± 15.0	288.4 ± 13.2	226.3 ± 5.8	268.0 ± 6.9
stigmasterol	301.6 ± 1.8	13.8 ± 0.3	30.4 ± 11.9	580.2 ± 20.5	662.2 ± 15.1	619.3 ± 11.7	705.0 ± 11.1
Δ7-campesterol	116.3 ± 0.5						
β-sitosterol	2337.1 ± 14.1	1320.1 ± 8.3	3541.5 ± 19.3	1690.7 ± 17.5	1446.3 ± 25.6	1491.1 ± 5.2	1561.8 ± 16.2
Δ5-avenasterol	175.6 ± 12.5	117.2 ± 5.0	122.0 ± 1.9	42.5 ± 8.6	114.7 ± 2.0	76.9 ± 5.5	85.4 ± 0.0
Δ7-sitosterol	375.3 ± 9.0			93.0 ± 8.3			
Δ7-avenasterol	249.2 ± 1.3			48.8 ± 2.3			
citrostadienol	809.3 ± 11.7			270.5 ± 10.6			
total phytosterols (mg/kg)	4723.8 ± 51.9	1483.7 ± 20.0	7325.5 ± 88.2	3319.8 ± 84.6	2539.8 ± 56.4	2448.2 ± 29.0	2655.4 ± 34.4

[a] Mean ± standard deviation ($n = 3$).

as described in Materials and Methods section were consistent with the data available in the literature (*26*, *27*).

GC-MS Analysis. GC is the most common method for the analysis of sterols (*14*, *28*, *29*). To reduce the boiling points of the hydroxy-containing GC detectants, to enhance their chromatographic resolution, and to stabilize the thermolabile unsaturated sterols, the title compounds are commonly analyzed as their TMS or acetate derivatives. It is well-known that the sterol structures play a pivotal role on GC elution characteristics (*14*). In the present study, the chromatographic separation of Generol 95S as TMS ether was achieved on a VF-5ms capillary column and Δ7-phytosterols tended to have longer retention times as compared to their Δ5-analogues (**Table 2**). This result is consistent with data available in the literature (*9*, *30*). As expected, the addition of methyl groups resulted in an increase of retention times such as $t_{R\text{citrostadienol}} > t_{R4-\text{desmethylsterols}}$. The mass spectra of the different sterols are presented in **Table 3**.

Generally, methods for the determination of sterols in food consist of a GC-FID detection. Nevertheless, such a poor selective detection constrained the analyst to use only the retention times for the identification of the detected sterols. Mass spectrometric detection is of great help as it allows an unambiguous identification of the sterols in foods by comparison of both the detected mass spectra and t_R with those of authentic standards. Nevertheless, this detection is linked to the fragmentation pattern of the molecule, which depends on its chemical structure and necessitates the determination of GC-MS RRFs. The amounts of isolated phytosterols obtained by semipreparative HPLC procedure were sufficient to allow the determination of their RRF in relation to cholestanol as internal standard. Eight replicates were performed. The Student test showed that the RRF results (**Table 4**) were not significantly different within all of the sterols belonging to each class: 1.20 ± 0.04 for the Δ5-desmethylsterols, 1.55 ± 0.08 for the Δ7-4-desmethylsterols, and 1.70 ± 0.09 for the Δ7-4-methylsterol. Brassicasterol and Δ5-avenasterol, which could not be separated by semipreparative HPLC, were affected of the same RRF value (1.20) than the other sterols of the Δ5-desmethylsterols class.

Validation of the RRF Value. BCR-633 is a European certified reference material (anhydrous butter fat). Some tracers, including β-apo-8′-carotenic acid ethyl ester, *n*-heptanoic acid triglyceride, β-sitosterol, and stigmasterol, were present in certified amounts (*21*). To validate the obtained RRF values, 100 mg of BCR-633 spiked with 20 μg of cholestanol as internal standard was assayed in triplicate with the NF-EN-ISO 12228 analytical method proposed by BCR (saponification of the nonsterol compounds with KOH in ethanolic solution, extraction of the sterols with diethyl ether, and trimethylsilylation of the analytes prior to GC separation) modified by a chromatographic detection by mass spectrometry. The results obtained with the mass spectrometric detection (β-sitosterol, 521 ± 6 mg/kg; stigmasterol, 155 ± 3 mg/kg) are consistent with the standard values provided by the BCR (β-sitosterol, 530 ± 29 mg/kg; stigmasterol, 147 ± 11 mg/kg); the proposed RRF values can be then considered as validated and may be applied for GC-MS analysis of foods.

Phytosterol Contents in Oils and Chocolates. The phytosterols in food were identified by comparison of their t_R and mass spectra with those of standards phytosterols. Quantitative estimation of the levels of phytosterols in oils and chocolates is listed in **Table 5**.

β-Sitosterol was the major component followed by campesterol and stigmasterol in oils; other sterols existed in minor quantities. In colza oil, the concentration of β-sitosterol (∼3500 mg/kg of oil) was considerably higher than in other oils (1300−2300 mg/kg of oil). A trace amount of cholesterol (about 30 mg/kg of oil) was found in the colza oil. Considerable amounts of Δ7-phytosterols (∼700 mg/kg of sunflower oil and 140 mg/kg of soja oil) were present in sunflower and soja oils, while they were not found in olive and colza oils.

Only Δ5-phytosterols could be found in the chocolates, with β-sitosterol being the major component (∼1500 mg/kg of chocolate). The concentration of stigmasterol was higher as compared with campesterol, followed by Δ5-avenasterol. Cholesterol (about 30 mg/kg of foods) was also present in chocolates.

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