Characterization of Side-Chain Oxidation Products of Sitosterol and Campesterol by Chromatographic and Spectroscopic Methods

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ABSTRACT: Based on the cholesterol-lowering effects of phytosterols, known since the 1950s, extra phytosterol amounts have been added to certain food products for almost 10 yr. Literature reports on oxidation of phytosterols in general are limited, and data on side-chain oxidation of these compounds are scarce. The aim of this study was to investigate and characterize autoxidation products from a mixture of the two phytosterols sitosterol and campesterol. A commercial mixture of the two sterols was oxidized for 72 h at 120°C in an air-ventilated oven. The oxidation products were separated by preparative TLC and identified and characterized with GC–MS. The following oxidation products were identified from the two phytosterols: 24-methylcholest-5-en-3β,24-diol, 24-methylcholest-5-en-3β,25-diol, 24-methylcholest-4-en-6α-ol-3-one, 24-methylcholest-4-en-6β-ol-3-one, 24-ethylcholest-5-en-3β,24-diol, 24-ethylcholest-5-en-3β, 25-diol, 24-ethylcholest-4-en-6α-ol-3-one, 24-ethylcholest-4-en-6β-ol-3-one. Full-scan mass spectra of these compounds are reported for the first time as their trimethylsilyl-ether derivatives.

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KEY WORDS: Autoxidation, campesterol, GC–MS, phytosterol, phytosterol oxidation products, side-chain oxidation, sitosterol, TLC.

Phytosterols are 28- and 29-carbon atom steroid alcohols that occur naturally in plants (1,2). Structurally, phytosterols are closely related to cholesterol (cholest-5-en-3β-ol), and the group includes more than 250 different compounds that have been identified from various plant and marine organisms. Phytosterols are essential membrane substances in all eucaryotic organisms where they are involved in the control of membrane fluidity and permeability (3).

Owing to structural similarities to cholesterol, phytosterols are susceptible to oxidation and can produce an array of products similar to the corresponding cholesterol oxidation products (4). Generation of oxidation products by autoxidation of sterols is influenced by factors such as light, temperature, and oxidizing agents (5). However, only a few reports have been published regarding oxidation products formed after autoxidation of phytosterols (6–8) .

Yanishlieva *et al.* (9) studied the products formed after repeated oxidation of sitosterol at 150°C for 1 h. Ten different products including the ring-structure oxidation products were identified, and some ketones and dienes were characterized. In the medium polar region (for an explanation of this term see the Results and Discussion section), two products were identified, stigmasta-5,24-dien-3β-ol and 6-hydroxy-stigmast-4-en-3-one. Daly *et al.* (10) heated sitosterol at 100°C in an air atmosphere for 48 h and examined the formation of oxidation products after purification by TLC and HPLC. The common ring-structure oxidation products and some nonpolar products, mainly ketones, were identified and characterized. Gordon and Magos (11) investigated the formation of oxidation products formed after repeated heating of cholesterol and Δ5-avenasterol at 180°C for 72 h. Despite different conditions, they identified almost the same products as Daly *et al.* However, one oxidation product that originated from the side chain and was characterized for the first time was 5,24(25),28-stigmastatrien-3β-ol.

The pathway for the generation of side-chain autoxidation products from cholesterol, e.g., 20-hydroxy-, 25-hydroxy-, and 26-hydroxycholesterol, has been clearly demonstrated (12). However, similar information for phytosterols is limited. With an additional methyl or ethyl group at C24, sitosterol and campesterol can form a wider range of oxidation products than cholesterol. Despite the demonstrated possibility to generate several side-chain oxidation products of the two phytosterols, no efforts have been made to identify these oxidation products in food products containing plant sterols or in biological samples.

Yanishlieva *et al.* (9) listed the sequence of reactions generating different A,B-ring structure oxidation products from Δ5-sterols. In addition, the authors postulated that, because of the tertiary nature of the carbon at the C24 position of sitosterol, there may be other possible pathways for autoxidation products of the side chain. This postulation may hold also for other phytosterols containing a tertiary carbon at the C24 position. Thus, the formation of 20-hydroxy, 24-hydroxy-, 25-hydroxy-, and 26-hydroxyphytosterols can presumably be generated through hydrogen abstraction from the corresponding hydroperoxides.

The main objective of this study was to isolate possible side-chain oxidation products of phytosterols. In addition, chromatographic characterization and mass spectral analysis of these compounds were undertaken to identify these compounds for their possible presence in food products and in biological samples.

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EXPERIMENTAL PROCEDURES

Reagents. Standard samples of 7α-hydroxycholesterol (cholest-5-en-3β,7α-diol), 7β-hydroxycholesterol (cholest-5-en-3β,7β-diol), 5α,6α-epoxycholesterol (5,6α-epoxy-5α-cholestan-3β-ol), 5β,6β-epoxycholesterol (5,6β-epoxy-5β-cholestan-3β-ol), triol cholesterol (cholestan-3β,5α,6β-triol), 20-hydroxycholesterol (cholest-5-en-3β,20-diol), 25-hydroxycholesterol (cholest-5-en-3β,25-diol), 26-hydroxycholesterol (cholest-5-en-3β,26-diol), and 4-cholesten-6β-ol-3-one were all purchased from Steraloids Inc. (Newport, RI).

Standard samples of 22*R*- and 22*S*-hydroxycholesterol [(22*R*/*S*)-cholest-5-en-3β,22-diol] were purchased from Sigma Chemicals Co. (St Louis, MO). The standard mixture of sitosterol and campesterol (60:40) was purchased from Research Plus Inc. (Bayonne, NJ) and the cholesterol standard from Merck Eurolab AB (Stockholm, Sweden).

All other solutes and chemicals were, unless otherwise stated, of analytical grade and purchased from Merck Eurolab AB.

Autoxidation of phytosterols. Pure samples of sitosterol and campesterol were subjected to thermal oxidation as described by Dutta and Appelqvist (13) but with some modifications. Initially, 1 g of phytosterol standard was dissolved in 20 mL of chloroform in a round-bottomed glass bottle. The solvent was evaporated in a rotary evaporator, and a thin film of sterols was formed on the glass surface. The glass bottle was kept in a ventilated oven for 72 h at 120°C. The oxidation products formed were dissolved in chloroform and separated by preparative TLC as described next.

Preparative and analytical TLC. Two different TLC systems were used to separate the oxidation products. In both, plates with silica gel 60 (20 × 20 cm, 0.25 mm thick; Merck Eurolab) were used.

(i) System 1. A solution of the oxidized phytosterols was applied as a 17-cm band over the bottom part of the plate by an automatic sample applicator, CAMAG Linomat IV (Muttenz, Switzerland). The TLC plates were eluted in ether/cyclohexane (9:1, vol/vol), air-dried, and 3.5 cm of the plate was cut and sprayed with a color reagent. The reagent contained equal parts of phosphomolybdic acid and cerium sulfate (1 g) and 5.4 mL concentrated sulfuric acid. Finally, the solution was diluted to 100 mL with distilled water. The colored part of the plate was heated for 10 min at 120°C in a ventilated oven, and the zones that contained oxidized phytosterols were scraped and collected. The scraped zones that contained the oxidation products were extracted with 3 × 5 mL chloroform/methanol (2:1, vo/vol), the solvent was evaporated, and the products were dissolved in 0.5 mL chloroform for subsequent TLC analysis.

(ii) System 2. The same procedure as in system 1 was used, but instead the solvent system was chloroform/ether/methanol (95:5:2) and the chamber was refrigerated (5°C). Standard samples of various oxidation products of cholesterol were used for identification purposes in both TLC systems.

Preparation of trimethylsilyl (TMS)-ether derivatives of sterol oxidation products. The preparation of TMS-ether derivatives of sterol oxidation products involved some modifications of the method previously described by Dutta and Appelqvist (13) . In brief, approximately 20 μg of the sterol oxidation products were mixed with 5 μg of 5α-cholestane in hexane. The solvent was evaporated under a stream of nitrogen gas followed by an addition of 100 μL Tri-Sil reagent (Pierce, Rockford, IL). The reaction mixture was incubated at 60°C for 45 min, then vortexed and dispersed in an ultrasonic bath before the solvent was evaporated under a stream of nitrogen gas. Finally, the products were dissolved in 100 μL hexane, centrifuged at 3000 rpm for 3 min, and stored at –20°C for subsequent analysis by GC–MS.

GC–MS identification of sterol oxidation products. The sterol oxidation products were analyzed on a gas chromatograph (CE Instruments, Milano, Italy) connected to a mass spectrometer (Finnigan, Manchester, England) with 5α-cholestane as an internal standard. The oxidation products were separated on a DB-5ms fused-silica capillary column (30 m × 0.25 mm × 0.5 μm) from J&W Scientific (Folsom, CA). The carrier gas was helium (80 kPa) and the injection mode splitless. The injector temperature was 250°C and the oven was programmed from 60 to 280°C (50°C/min) and from 280 to 300°C (1(C/min). The electron energy was 70 eV, the ion source 200°C, and full-scan mass spectra were recorded.

RESULTS AND DISCUSSION

TLC. The oxidized phytosterols were separated twice with the preparative TLC technique, and the oxidation products were tentatively identified using standard samples of cholesterol oxidation products. All these compounds were further characterized by GC–MS (described shortly). The common ring-structure oxidation products (7α-hydroxy-, 7β-hydroxy-, 7-keto-, 5α,6α-, and 5β,6β-epoxy sito/campesterol) have previously been described, and no further identification was required (5,8,10,13,14). However, the compounds of medium polarity (R_f between epoxides and unoxidized sterols in system 1, just described) have not been characterized before (Table 1, system 1).

The R_f-values for the four medium polarity side-chain oxidation products isolated (24-hydroxy and 25-hydroxy sito/campesterol, where sito is sitosterol) could not be compared with the R_f-value of the existing cholesterol standard. A suggestion is that the extra methyl or ethyl group and the addition of a hydroxy group to the side chain affected the mobility of the molecule, thereby changing the mobility in the TLC analysis. Since the ring structure of cholesterol and sito/campesterol is the same, the influence must derive from changes in the side-chain structure.

Four ring-structure oxidation products of medium polarity were 24-methylcholest-4-en-6α-ol-3-one, 24-methylcholest-4-en-6β-ol-3-one, 24-ethylcholest-4-en-6β-ol-3-one, and 24-ethylcholest-4-en-6β-ol-3-one. The R_f-value for the β-isomers was comparable to the corresponding cholesterol standard, but the α-isomer of cholesterol could not be purchased from any supplier. However, the results for the α-isomers were in

TABLE 1
TLC Mobility for Cholesterol, Sitosterol, and Campesterol Oxidation Products ($R_f = R_{subs}/R_{front}$) and GC–MS Retention Times (relative to internal standard 5α-cholestane) for Sitosterol and Campesterol Oxidation[a]

	TLC				GC–MS		
Compound analyzed	System[b]	R_f[c]	System[d]	R_f[c]	System[e]	RT (min)	RRT[f]
7α-Hydroxycholesterol	1	0.10	2	0.09			
7β-Hydroxycholesterol	1	0.16	2	0.10			
7-Ketocholesterol	1	0.22	2	0.19			
α-Epoxycholesterol	1	0.26	2	0.19			
β-Epoxycholesterol	1	0.26	2	0.19			
20-Hydroxycholesterol	1	0.50	2	0.25			
22-Hydroxycholesterol	1	0.47	2	0.22			
25-Hydroxycholesterol	1	0.30	2	0.17			
26-Hydroxycholesterol	1	0.30	2	0.16			
4-Cholesten-6β-ol-3-one	1	0.39	2	0.23			
Unoxidized cholesterol	1	0.59	2	0.33			
7α-Hydroxy campe/sito	1	0.11	2	0.08	3	32.4/36.3	1.7/1.9
7β-Hydroxy campe/sito	1	0.18	2	0.08	3	39.1/43.1	2.0/2.3
7-Keto campe/sito	1	0.24	2	0.18	3	49.7/55.4	2.6/2.9
α-Epoxy campe/sito	1	0.28	2	0.18	3	41.9/46.4	2.2/2.4
β-Epoxy campe/sito	1	0.28	2	0.18	3	41.2/45.7	2.2/2.4
24-Hydroxy campe/sito	1	0.32	2	0.13	3	40.7/45.2	2.1/2.3
25-Hydroxy campe/sito	1	0.39	2	0.17	3	48.6/53.4	2.5/2.8
24-Methyl/ethyl-cholest-4-en-6α-ol-3-one	1	0.33	2	0.18	3	48.1/53.4	2.5/2.8
24-Methyl/ethyl-cholest-4-en-6β-ol-3-one	1	0.40	2	0.23	3	41.5/46.0	2.2/2.4
Unoxidized campe/sito	1	0.59	2	0.33	3	35.0/39.3	1.8/2.0

[a]Isolated after thermal autoxidation of pure standards. Sito, sitosterol; campe, campesterol.
[b]System 1: TLC, ether/cyclohexane (9:1).
[c]$R_f = R_{substance}/R_{front}$.
[d]System 2: TLC, chloroform/ether/methanol (95:5:2).
[e]System 3: GC–MS, DB5-ms capillary column.
[f]RRT = ($RT_{subs}/RT_{i.s.}$), where RT = retention time; subscript "subs" = substance in question; subscript "i.s." = internal standard.

accordance with data previously reported by Aringer and Nordström (14).

GC–MS. The most favorable carbon atoms for an oxidative attack in the side chain of a steroid molecule are the tertiary carbons (5,6). These carbons can stabilize the radicals formed, making them more susceptible to oxidation. Consequently, one of the reported side-chain oxidation products from cholesterol is 25-hydroxycholesterol (cholest-5-en-3β,25-diol). Similar products were isolated by preparative TLC from campesterol and sitosterol, but the two compounds co-eluted.

The GC–MS retention time for 25-hydroxycampesterol (24-methylcholest-5-en-3β,25-diol) on the DB-5ms capillary column used in this study was 48.6 min (Table 1). The characteristic mass fragments were *m/z* 560 (M^+, <1%), with 545 ($M^+ - CH_3$), 470 (M^+ – TMSO), and 455 (M^+ – TMSO – CH_3) also in low abundance (<1%) (Fig. 1). The base peak was identified at *m/z* 131 and derived from the α-fragmentation of C24–C25 (14–17). A similar fragmentation pattern was observed for 25-hydroxycholesterol (Fig. 2), and previously published papers have reported that *m/z* 131 is an exclusive fragment for the 25-hydroxy product (14,16). Another characteristic fragment for 25-hydroxycampesterol was *m/z* 215. This fragment derived from the α-cleavage of the C17–C20 bond and contained the complete side chain.

The corresponding 25-hydroxysitosterol (24-ethylcholest-5-en-3β,25-diol) had a GC–MS retention time at 53.4 min (Table 1), and mass-spectral data indicated that the molecular ion was *m/z* 574 (M^+, <1%) (Fig. 3). Similar to 25-hydroxycampesterol and 25-hydroxycholesterol, the characteristic fragments were 559 ($M^+ - CH_3$) and 484 (M^+ – TMSO), both fragments being low in abundance (<1%). As for 25-hydroxycampesterol and 25-hydroxycholesterol, the most important fragment for the identification was the characteristic base peak, *m/z* 131.

The 24-hydroxy oxidation product from both sitosterol and campesterol was tentatively identified from the thermal oxidation products. These have not been reported from cholesterol, probably because C24 is a secondary carbon atom in cholesterol. However, in the two phytosterols discussed in this paper, an additional methyl or ethyl group is attached to C24, making it a tertiary carbon and therefore more susceptible to an oxidative attack.

24-Hydroxycampesterol (24-methylcholest-5-en-3β,24-diol) was of medium polarity in TLC analysis (R_f = 0.3 in system 1), and the retention time on the GC–MS system was 40.7 min (Table 1). The molecular ion at *m/z* 560 (M^+, 6.5%) was followed by the characteristic fragmentation pattern of a dihydroxy steroid molecule (Fig. 4). The loss of a methyl group generated *m/z* 545 ($M^+ - CH^3$, 3.5%), and the loss of TMSO

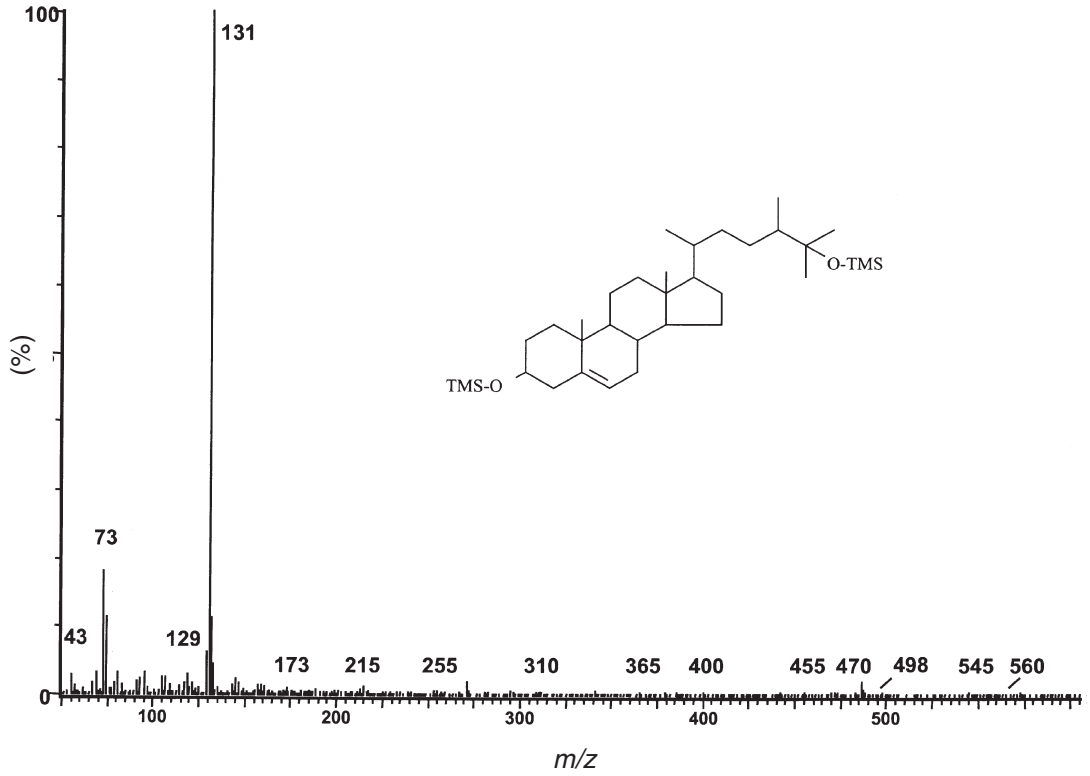

FIG. 1. GC–MS spectrum of 25-hydroxycampesterol as trimethylsilyl (TMS)-ether derivative. The characteristic base peak was identified at *m/z* 131 and the molecular ion at *m/z* 560.

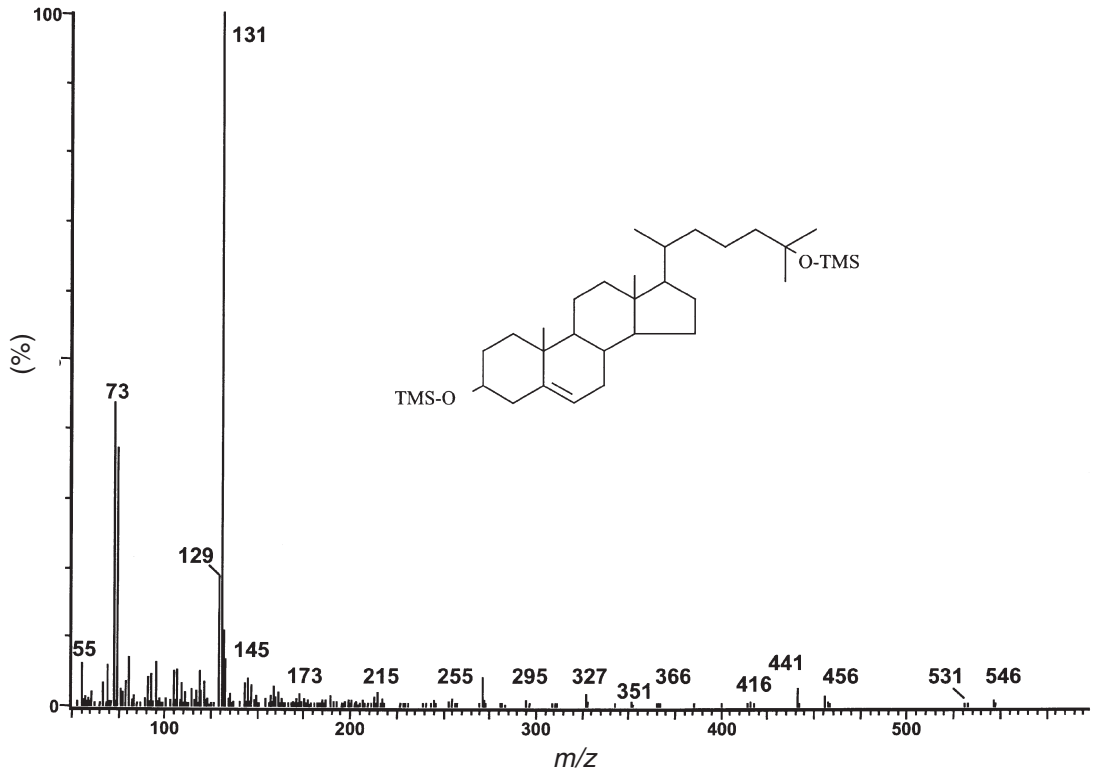

FIG. 2. GC–MS spectrum of 25-hydroxycholesterol as TMS-ether derivative. The characteristic base peak was identified at *m/z* 131 and the molecular ion at *m/z* 546. For abbreviation see Figure 1.

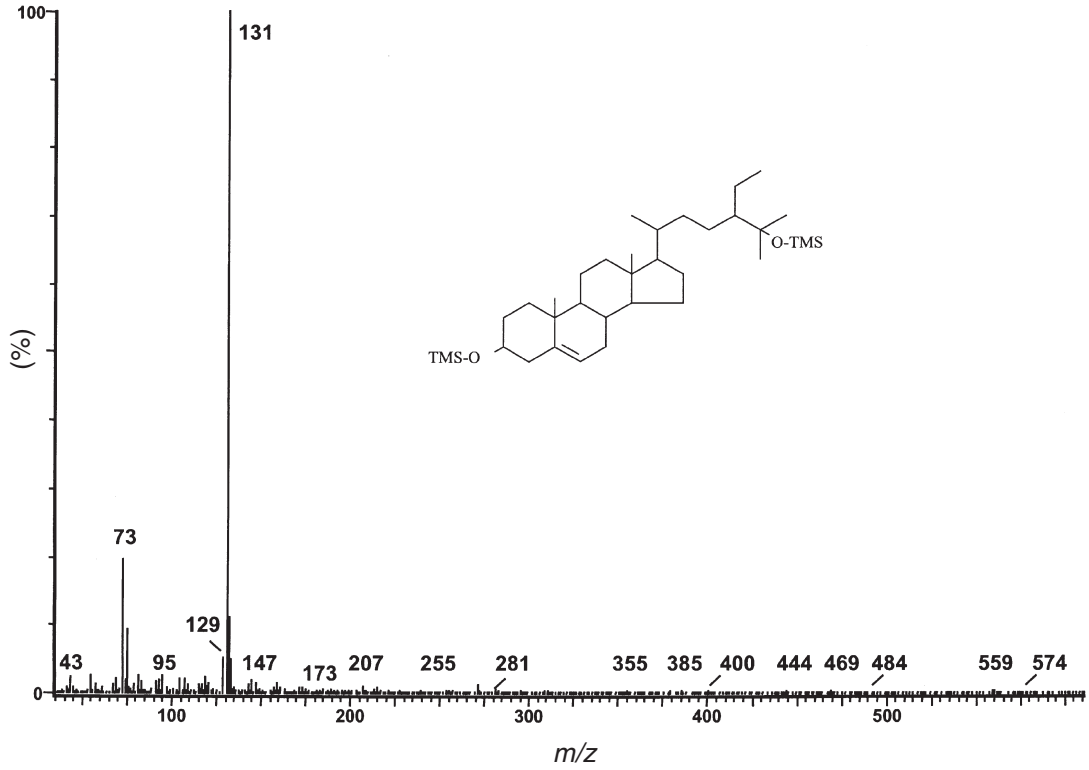

FIG. 3. GC–MS spectrum of 25-hydroxysitosterol as TMS-ether derivative. The characteristic base peak was identified at *m/z* 131 and the molecular ion at *m/z* 574. For abbreviation see Figure 1.

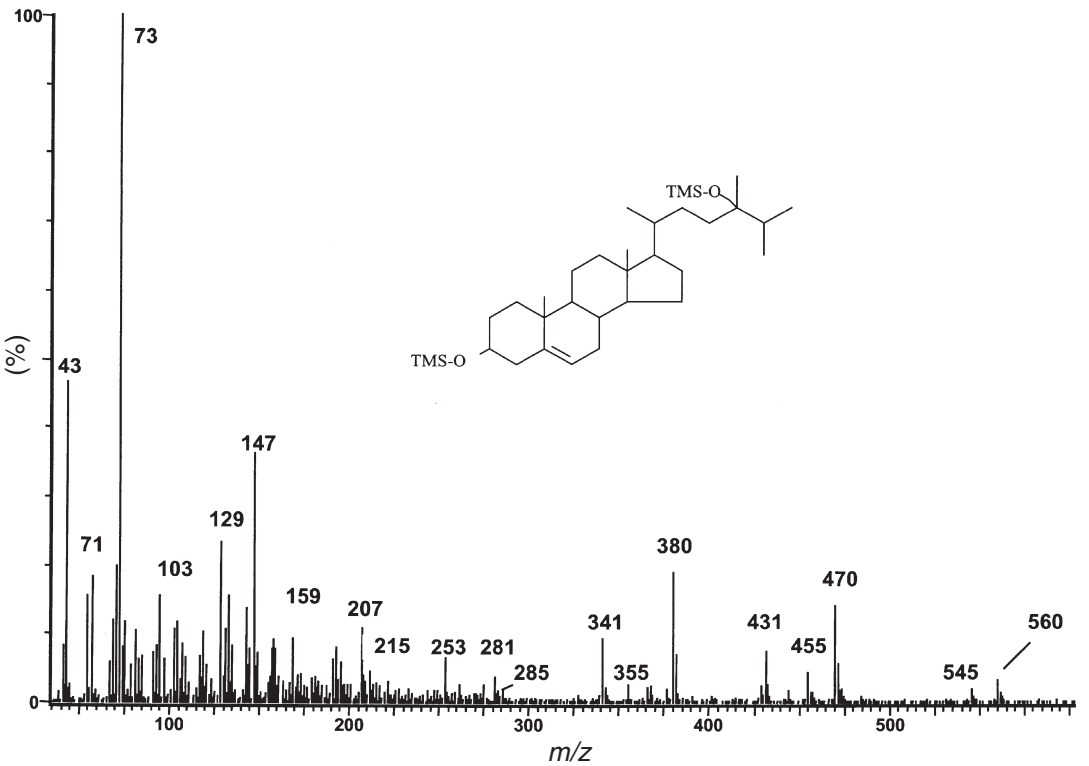

FIG. 4. GC–MS spectrum of the tentatively identified 24-hydroxycampesterol. The molecular ion was identified at *m/z* 560, and the characteristic losses of two TMSO groups for a dioxygenated steroid compound were identified ($M^+ - 90$ and $M^+ - 180$). For abbreviation see Figure 1.

groups produced *m/z* 470 (M^+ – TMSO, 25.9%) and 380 (M^+ – 2TMSO, 31.1%). Other characteristic fragments identified were *m/z* 455 (M^+ – TMSO – CH_3, 7.4%), 431 (M^+ – 129, 12.9%), 345 (M^+ – C_9H_{18}TMSO, <1%), 159 (M^+ – $C_{23}H_{36}$TMSO, 9.3%), and the common Δ5-steroid fragment *m/z* 129. The side-chain fragment *m/z* 215 (M^+ – $C_{19}H_{28}$, 3.1%) was also important for the identification and characterization of this compound. Further characterization of this compound would include NMR. However, the NMR technique was used only for further identification of the corresponding oxidation products of stigmasterol (18).

From the oxidation of sito- and campesterol, 24-hydroxysitosterol (24-ethylcholest-5-en-3β,24-diol) was also tentatively identified. The separation on the TLC plate indicated a compound of medium polarity (R_f = 2.3 in system 1), and the retention time on GC–MS for 24-hydroxysitosterol was 45.2 min (Table 1). The molecular ion was identified at *m/z* 574 (M^+, 6.4%), and the loss of TMSO groups generated *m/z* 484 (M^+ – TMSO, 27.3%) and 394 (M^+ – 2TMSO, 32.9%) (Fig. 5). Three other important fragments were *m/z* 559 (M^+ – CH_3, 3.75%), 469 (M^+ – CH_3 – TMSO, 6.7%) and 445 (M^+ – 129, 13.1%). Some characteristic side-chain fragments were *m/z* 229 (M^+ – $C_{19}H_{28}$TMSO, 1.2%) and 173 (M^+ – $C_{23}H_{36}$TMSO, 5.2%). Fragment *m/z* 129 confirmed that the molecule had a Δ5-steroid structure.

Two medium polar ring-structure oxidation products that were isolated by TLC were 24-methylcholest-4-en-6α-ol-3-one and its isomer 24-methylcholest-4-en-6β-ol-3-one. Chromatographic data for these two substances are listed in Table 1. Compared with the standard sample of 4-cholesten-6β-ol-3-one, the β-isomer of campesterol was similar in mobility on the TLC plate (Table 1). The retention time on GC–MS for 24-methylcholest-4-en-6α-ol-3-one was longer than for its β-isomer, 48.1 and 41.5 min, respectively (Table 1), the same results as previously reported by Aringer and Nordström (14). The MS spectra of these two isomers (Figs. 6 and 7) were similar to the corresponding cholesterol spectra of 4-cholesten-6β-ol-3-one (Fig. 8), and these compounds have previously been reported as products after oxidation of phytosterols (9). However, the full-scan mass spectra as their TMS-ether derivatives have not been reported.

The mass spectra of the two isomers were almost identical, but the relative abundance of the fragments was slightly different, which concurred with published results on the corresponding oxidation products from cholesterol (14). The molecular ion of the campesterol oxidation product was *m/z* 486 (for the α-isomer 47.2% and for the β-isomer 40.2%), followed by a fragment at 471 (M^+ – CH_3, 80.4 and 70.6%, respectively) and 430 (M^+ – C_3H_4O, 83.6% and 100%, respectively). This fragmentation pattern was characteristic of these isomers and derived from the loss of one methyl group and a fragment from ring A (19-21). The base peak in the mass spectrum was identified at *m/z* 43 for the β-isomer and *m/z* 430 for the β-isomer. Some other characteristic fragments were the side-chain fragments *m/z* 127 (M^+ – $C_{19}H_{42}$TMSO, 3.3 and 2.1%, respectively) and 359 (M^+ – C_9H_{19}, 1.6 and 0.7%, respectively). Both derived from the

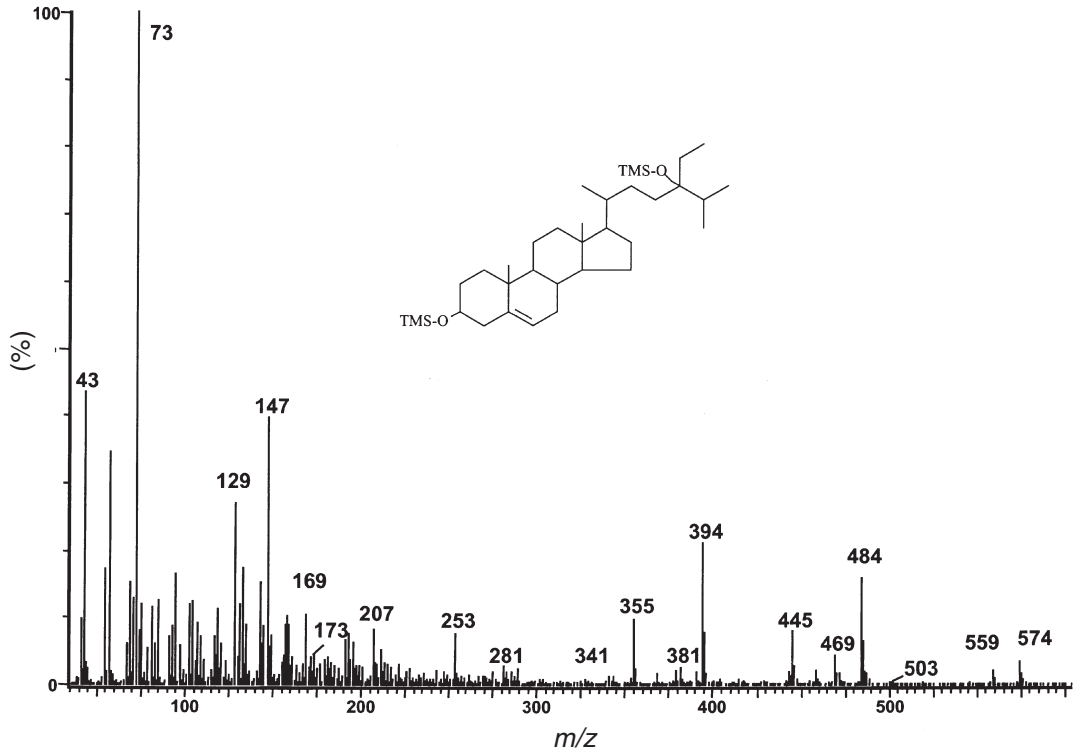

FIG. 5. GC–MS spectrum of the tentatively identified 24-hydroxysitosterol. The molecular ion was identified at *m/z* 574, and the characteristic losses of two TMSO groups for a dioxygenated steroid compound were identified (M^+ – 90 and M^+ – 180). For abbreviation see Figure 1.

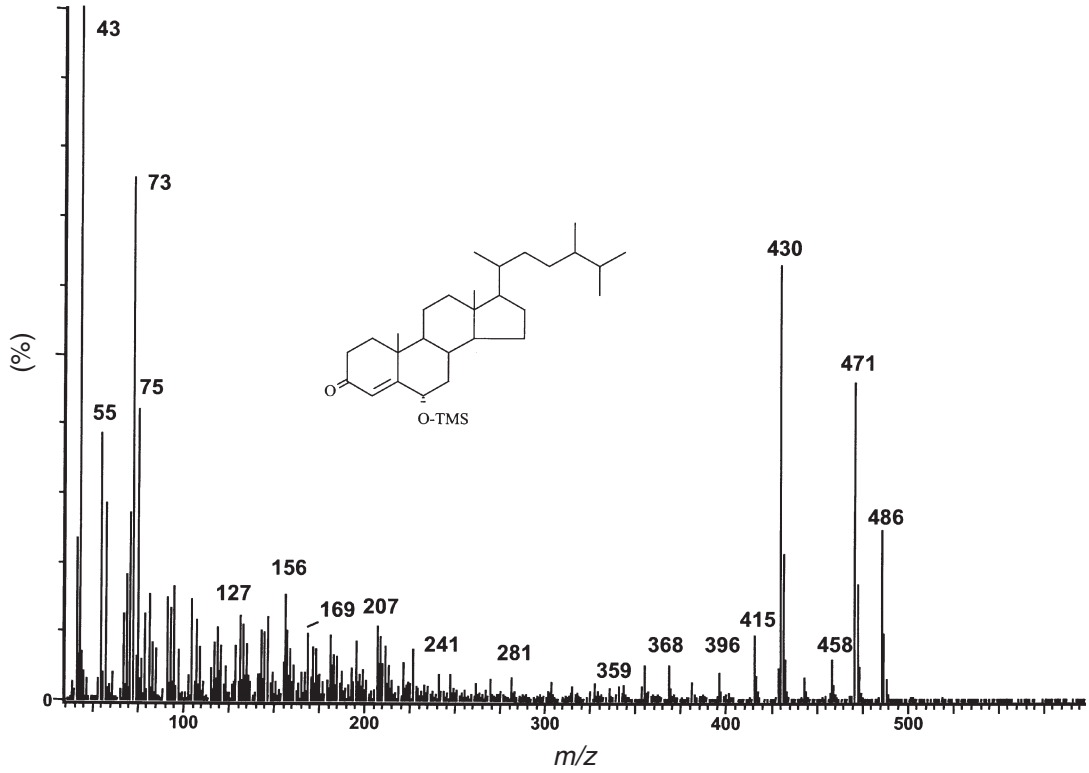

FIG. 6. GC–MS spectrum of 24-methylcholest-4-ene-6β-ol-3-one as TMS-ether derivative. The molecular ion was identified at *m/z* 486, and characteristic fragmentation patterns of $M^+ - 15$ and $M^+ - 56$ were reported. For abbreviation see Figure 1.

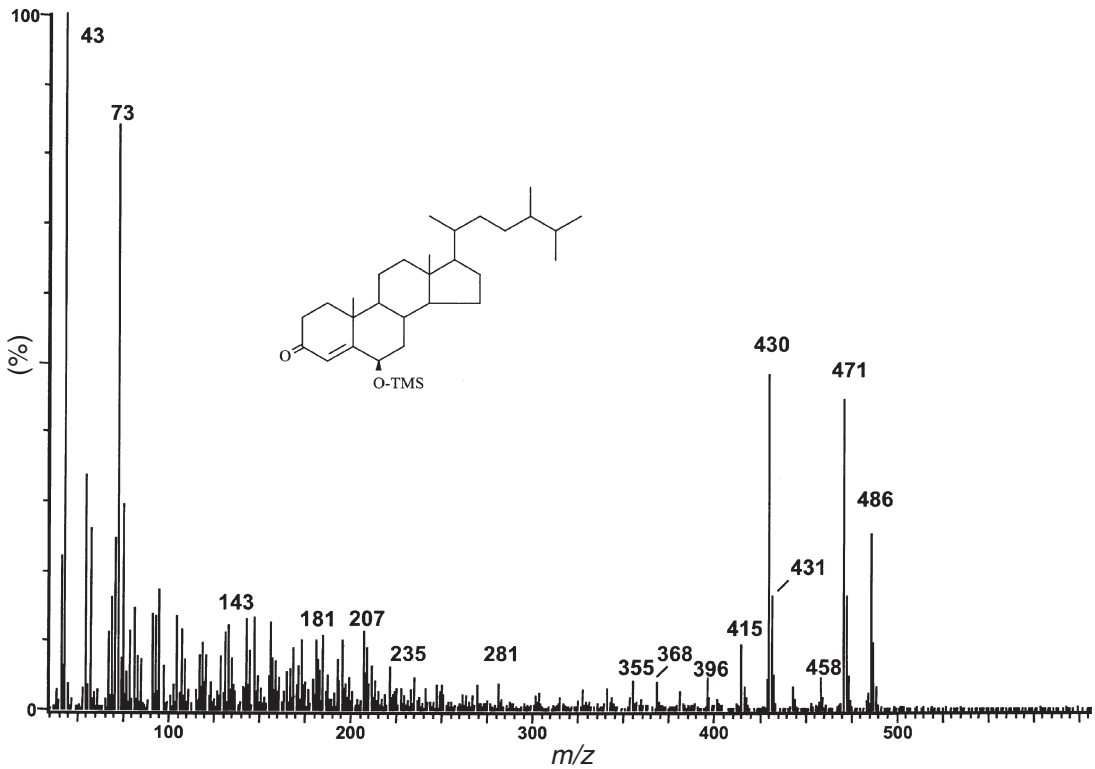

FIG. 7. GC–MS spectrum of 24-methylcholest-4-ene-6α-ol-3-one as TMS-ether derivative. The molecular ion was identified at *m/z* 486, and characteristic fragmentation patterns of $M^+ - 15$ and $M^+ - 56$ were reported. For abbreviation see Figure 1.

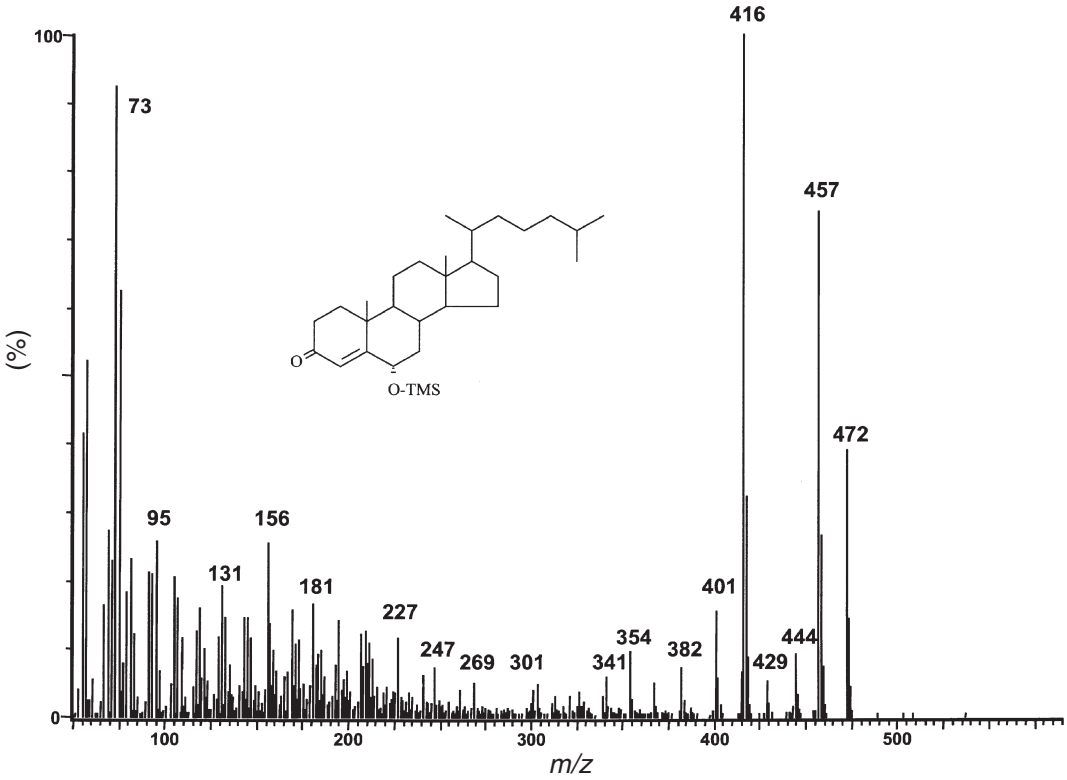

FIG. 8. GC–MS spectrum of an authentic standard sample of the TMS-ether derivative of 4-cholest-6β-ol-3-one. The molecular ion was found at *m/z* 472, and the base peak at *m/z* 416. For abbreviation see Figure 1.

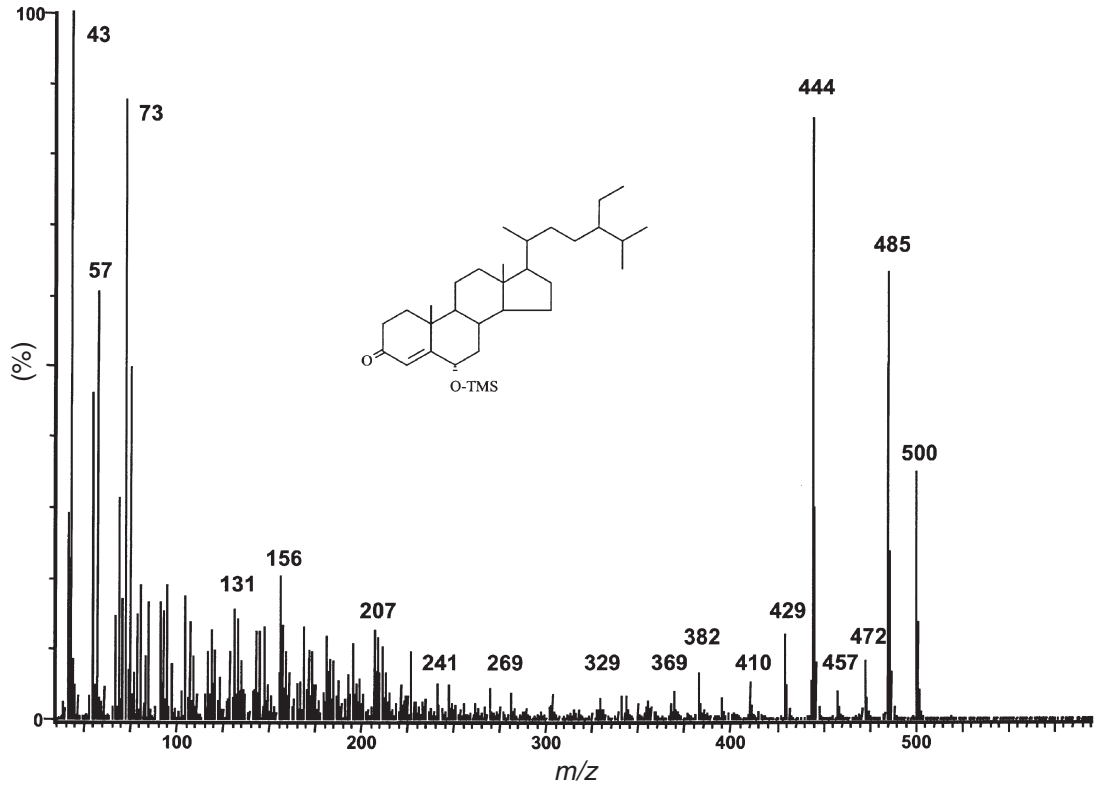

FIG. 9. GC–MS spectrum of 24-ethylcholest-4-ene-6β-ol-3-one as TMS-ether derivative. The molecular ion was identified at *m/z* 500, and characteristic fragmentation patterns of $M^+ - 15$ and $M^+ - 56$ were reported. For abbreviation see Figure 1.

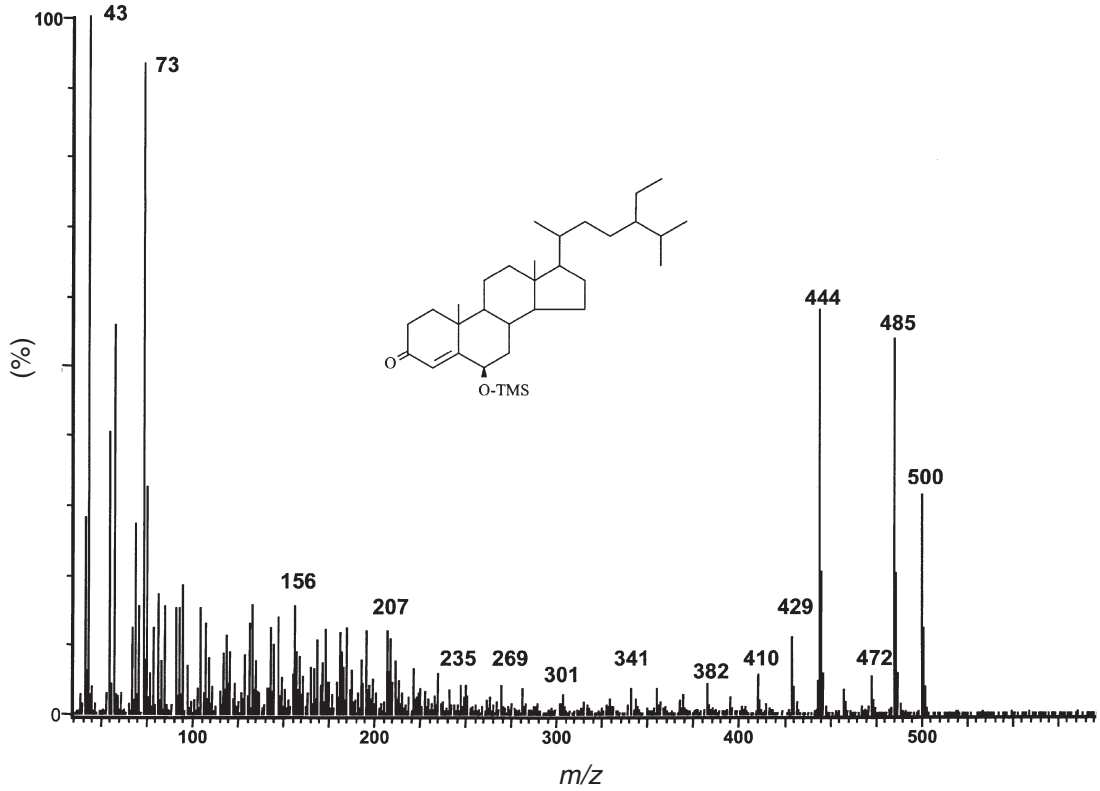

FIG. 10. GC–MS spectrum of 24-ethylcholest-4-ene-6α-ol-3-one as TMS-ether derivative. The molecular ion was identified at *m/z* 500, and characteristic fragmentation patterns of $M^+ - 15$ and $M^+ - 56$ were reported.

cleavage between C17 and C20, and these fragments confirmed the proposed structure.

Since the starting material was a mixture of sitosterol and campesterol, the corresponding oxidation products from sitosterol were also isolated after the autoxidation. These products, 24-ethylcholest-4-en-6α-ol-3-one and 24-ethylcholest-4-en-6β-ol-3-one, had retention times of 53.4 and 46.0 min, respectively, on the GC–MS system used in this study (Table 1). The molecular ion of the oxidation product was *m/z* 500 (relative intensity 55.0% for the α-isomer and 42.3% for the β-isomer), and the fragmentation pattern was similar for the two phytosterol isomers (Figs. 9 and 10). As for the campesterol isomers, the most characteristic fragments were $M^+ - CH_3$ (71.7 and 91.8%, respectively) and $M^+ - C_3H_4O$ (99 and 100%, respectively). The side-chain fragments *m/z* 131 (C_9H_{21}, 16.8 and 14.7%, respectively) and 169 ($M^+ - C_9H_{21}$, 13.9 and 12.1%, respectively) were also identified in the two spectra.

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