Side-Chain Autoxidation of Stigmasterol and Analysis of a Mixture of Phytosterol Oxidation Products by Chromatographic and Spectroscopic Methods

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ABSTRACT: Although the structure of phytosterols is closely related to cholesterol, there is a gap in knowledge concerning the formation and occurrence of oxidation products from phytosterols. The main objective of this study was to isolate and characterize some side-chain oxidation products formed after autoxidation of stigmasterol. Another objective was to highlight the difficulties in the analysis of phytosterol and a mixture of their oxidation products by GC. Pure stigmasterol was oxidized at 120°C for 72 h in an air-ventilated oven. Preparative TLC separated the oxidation products, and the products were characterized with GC–MS and NMR. In addition to the common ring-structure oxidation compounds, three semipolar oxidation products—24-ethylcholest-5,22-dien-3β,25-diol, 24-ethylcholest- 5,22-dien-3β,24-diol, and 24-ethyl-5,22-choladien-3β-ol-24-one—were characterized for the first time by TLC, GC–MS, and NMR. Moreover, the results of the analysis of a large number of oxidation products from sitosterol, campesterol, and stigmasterol by capillary column GC indicated that further efforts and optimization are required in this area.

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KEY WORDS: Autoxidation, campesterol, GC–MS, NMR, phytosterol oxidation product, sitosterol, stigmasterol, TLC.

The most commonly occurring phytosterols are sitosterol, campesterol, and stigmasterol, which are minor components in plant lipids (1–3). The oxidation of cholesterol, the corresponding steroid in animal lipids, has been studied extensively, and many *in vitro* studies have reported that cholesterol oxidation products can be mutagenic, cytotoxic, or atherogenic (4,5). Because of structural similarities, these conditions may also apply to phytosterols, but limited work has been done in this area (6,7). Among the common phytosterols, most research has focused on campesterol and sitosterol; the latter is the plant sterol for which analyses are most frequently carried out. However, only a few reports have focused on stigmasterol.

The oxidation of sterols is a free radical process (8,9) that is initiated by factors such as light, air, water, heat, and metal contamination (1,2). For cholesterol, more than 70 different oxidation products have been reported, including both ring-structure and side-chain structure products (10,11). The most stable carbon atoms for an oxidative attack in the side-chain are the tertiary carbons, C20 and C25 in cholesterol and C24 in addition in phytosterols (9,12). These carbons have the ability to stabilize the radicals formed. The oxidation in the ring structure for phytosterols has been investigated extensively, but information regarding side-chain oxidation is limited (9).

In a 1989 study, Blekas and Boskou (13) investigated the products obtained after oxidizing a TAG mixture containing 5% stigmasterol at 180°C for 16 h. They identified the polar products 7α-hydroxystigmasterol, 7β-hydroxystigmasterol, and dihydroxystigmasterol and the nonpolar products stigmasta-3,5,22-triene and stigmasta-3,5,22-triene-7-one. The moderately polar product Δ5-pregen-3β-ol-20-one was also separated and characterized.

Dutta (12) investigated the content of phytosterol oxidation products in different oils and french fries prepared in these oils. In addition to this, Dutta reported full-scan mass spectra of several ring-structure oxidation products of stigmasterol. These products were triol-, 7α-hydroxy-, 7β-hydroxy-, 7-keto-, 5α,6α-epoxy-, and 5β,6β-epoxystigmasterol.

Bortolomeazzi *et al.* (14) synthesized 5α-hydroperoxides from sitosterol, campesterol, and stigmasterol by photooxidation of sterols dissolved in pyridine in the presence of hematoporphyrine as sensitizer. Reduction of the 5α-hydroperoxides gave 5α-hydroxysterols, and isomerization of the 5α-hydroperoxides gave 7α-hydroperoxides. Reduction of 7α-hydroperoxide gave 7α-hydroxysterol, and epimerization of this 7α-hydroxy compound gave 7β-hydroxysterol. Some characteristic fragments from GC–MS analysis of the phytosterol oxidation products were reported.

So far, the formation of 25-hydroxystigmasterol after thermal oxidation, reported by Lampi *et al.* (15), and the formation of Δ5-pregen-3β-ol-20-one, reported by Blekas and Boskou (13), are the only side-chain oxidation products from stigmasterol reported in the literature. However, full-scan mass spectra as their trimethylsilyl (TMS)-ether derivatives have not yet been published.

The main aim of this study was to isolate and characterize some stigmasterol side-chain oxidation products that originated from autoxidation initiated by heat. The compounds were characterized with TLC, GC–MS, and NMR. Results of similar studies of campesterol and sitosterol are reported elsewhere (16). Another objective was to illustrate the separation

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of a complex mixture of phytosterols and their oxidation products with capillary column GC.

EXPERIMENTAL PROCEDURES

Reagents. The standard sample of stigmasterol was purchased from Sigma-Aldrich (Stockholm, Sweden), the cholesterol standard from Merck Eurolab AB, (Stockholm, Sweden), and a mixture of sitosterol/campesterol (60:40) from Research Plus Inc. (Bayonne, NJ). All other solutes and chemicals were, unless otherwise stated, of analytical grade and purchased from Merck Eurolab AB.

Autoxidation of phytosterol. The oxidation products were produced as described by Johnsson and Dutta (16). In brief, 1 g of phytosterol standard was dissolved in 20 mL of chloroform in a round-bottomed glass bottle. The solvent was evaporated, and 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 TLC. Two solvent systems were used to separate the different sterol oxidation products. System 1, which contained ether/cyclohexane (9:1, vol:vol), separated the polar ring-structure products from the semipolar (R_f between epoxides and unoxidized sterols in system 1) ring- and side-chain structure oxidation products (16). The separation was done with preparative silica gel 60 TLC plates (20 × 20 cm, 0.25 mm thickness; Merck Eurolab) and an automatic sample applicator was used (Linomat IV; CAMAG, Muttenz, Switzerland). System 2 was kept at 5°C and contained chloroform/ether/methanol (95:5:1). This system separated the coeluting 24-hydroxystigmasterol and 24-ethyl-5,22-choladien-3β-ol-24-one. Standard samples of cholesterol oxidation products were used whenever necessary in the TLC-systems.

Preparation of TMS-ether derivatives of sterol oxides. The method for preparation of TMS-ether derivatives has been described in detail elsewhere (16). In brief, approximately 20 μg of the sterol oxidation products were mixed with 5 μg of 5α-cholestane in hexane. After incubation and evaporation of the silylated sample, the samples were dissolved in hexane and analyzed with GC–MS.

GC–MS identification of sterol oxidation products. The sterol oxidation products were analyzed on a gas chromatograph (CE Instruments, Milan, 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 was splitless. The temperature on the injector side 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 was at 200°C, and full-scan mass spectra were recorded.

NMR identification of sterol oxidation products. The three stigmasterol side-chain products were analyzed on a 400 MHz NMR instrument (Bruker DRX, Germany). The samples were dissolved in $CDCl_3$ and recorded at 30°C using TMS as a reference. Analyses performed were ^{1}H NMR spectroscopy, COSY (correlation spectroscopy), TOCSY (total correlation spectroscopy), HMBC (heteronuclear multiple bond correlation), and HSQCDEPT (heteronuclear single quantum coherence distortionless enhancement by polarization transfer) using the Bruker standard pulse program.

RESULTS AND DISCUSSION

Autoxidation of stigmasterol produced the common polar ring-structure oxidation products as reported previously (13,14). In addition, three different side-chain structure oxidation products were separated with preparative TLC and further characterized by GC–MS and NMR. The three side-chain compounds were 25-hydroxystigmasterol, 24-hydroxystigmasterol, and 24-ethyl-5,22-choladien-3β-ol-24-one.

TLC. Preparative TLC separated and isolated the three different oxidation products. All three phytosterol oxidation products were of medium polarity (Table 1); these were minor products compared with the common polar products. 25-Hydroxy-stigmasterol was isolated with TLC-system 1 but the two other compounds were separated and isolated with a multiple TLC-system as described in the Experimental Procedures section.

NMR. In general, all three oxidation products gave similar chemical shifts (^{1}H and ^{13}C) for C3, C6, C17, C18, and C19 (Table 2). This evidence, together with the GC–MS fragment at *m/z* 129, indicated that the ring structure was intact and that

TABLE 1
TLC Mobility for Standard Oxidation Products of Cholesterol and Stigmasterol in Two Different TLC Systems

Compound	TLC	
	$R_f^{a,b}$	$R_f^{b,c}$
7α-Hydroxycholesterol	0.1	0.1
7β-Hydroxycholesterol	0.2	0.1
7-Ketocholesterol	0.2	0.1
α-Epoxycholesterol	0.3	0.1
β-Epoxycholesterol	0.3	0.1
20-Hydroxycholesterol	0.5	0.2
22-Hydroxycholesterol	0.5	0.2
25-Hydroxycholesterol	0.3	0.1
26-Hydroxycholesterol	0.3	0.1
Unoxidized cholesterol	0.6	0.3
7α-Hydroxystigmasterol	0.1	0.1
7β-Hydroxystigmasterol	0.2	0.1
7-Ketostigmasterol	0.2	0.1
α-Epoxystigmasterol	0.3	0.1
β-Epoxystigmasterol	0.3	0.1
24-Hydroxystigmasterol	0.5	0.2
24-Ethyl-5,22-choladien-3β-ol-24-one	0.5	0.2
25-Hydroxystigmasterol	0.4	0.1
Unoxidized stigmasterol	0.6	0.2

[a]System 1 TLC eluent, ether/cyclohexane (9:1).
[b]$R_f = R_{substance}/R_{front}$.
[c]System 2 TLC eluent, chloroform/ether/methanol (95:5:1).

TABLE 2
^{1}H NMR and ^{13}C NMR Data in $CDCl_3$ for 24-Hydroxystigmasterol, 25-Hydroxystigmasterol, and 24-Ethyl-5,22-choladien-3β-ol-24-one

	24-Hydroxy		25-Hydroxy		24-Keto compound[b]	
Pos[a]	^{13}C shift	^{1}H shift	^{13}C shift	^{1}H shift	^{13}C shift	^{1}H shift
3	72.0	3.52 (*m*)	72.0	3.52 (*m*)	71.8	3.52 (*m*)
6	121.7	5.35 (*m*)	121.8	5.35 (*m*)	121.8	5.35 (*m*)
17	56.3	1.22 (*m*)	56.1	1.23 (*m*)	55.4	1.25 (*m*)
18	12.5	0.71 (*s*)	12.4	0.71 (*s*)	12.4	0.72 (*s*)
19	19.6	1.01 (*s*)	19.7	1.01 (*s*)	19.7	1.01 (*s*)
20	40.6	2.14 (*overlap*)	40.9	2.12 (*m*)	40.4	2.28 (*m*)
21	21.5	1.05 (*d*)	21.5	1.06 (*d*)	19.6	1.10 (*d*)
22	136.0	5.41 (*dd*)	142.3	5.36 (*dd*)	152.6	6.68 (*dd*)
23	131.3	5.29 (*d*)	127.6	5.07 (*dd*)	127.9	6.01 (*d*)
24	77.0	—	57.7	1.76 (*m*)	201.4	—
25	36.6	1.70 (*m*)	72.3	—	33.6	2.55 (*q*)
26	17.2	0.88 (*d*)	27.1	1.11 (*s*)	8.6	1.09 (*t*)
27	20.0	0.85 (*d*)	27.3	1.17 (*s*)		
28	31.7	1.54 (*overlap*)	27.7	1.60/1.10 (*m*)		
29	8.0	0.85 (*t*)	13.0	0.84 (*t*)		

[a]Numbering of carbon in the molecule.
[b]24-Ethyl-5,22-choladien-3β-ol-24-one.

autoxidation had affected only the side chain. NMR analysis also concluded that the double bond in the side chain was positioned at C22–C23 and was of *trans* conformation ($JH_{22,H23}$ 15 Hz). From the NMR analysis it was also possible to get all the chemical shifts (^{1}H and ^{13}C) for the atoms in the side chain. The data for the carbons were measured with HSQCDEPT.

In the ^{1}H NMR spectrum of 25-hydroxystigmasterol, two singlets corresponding to methyl groups were identified (C26–C27). For unoxidized stigmasterol these singlets would be doublets since C25 would bear an extra hydrogen atom. However, the two singlets had a slightly downfield shift, indicating that C25 was substituted with a hydroxy group (Table 2). In HMBC both methyl groups coupled to C25 at 72.3 ppm (typically hydroxy substituted). COSY and TOCSY, with different delay

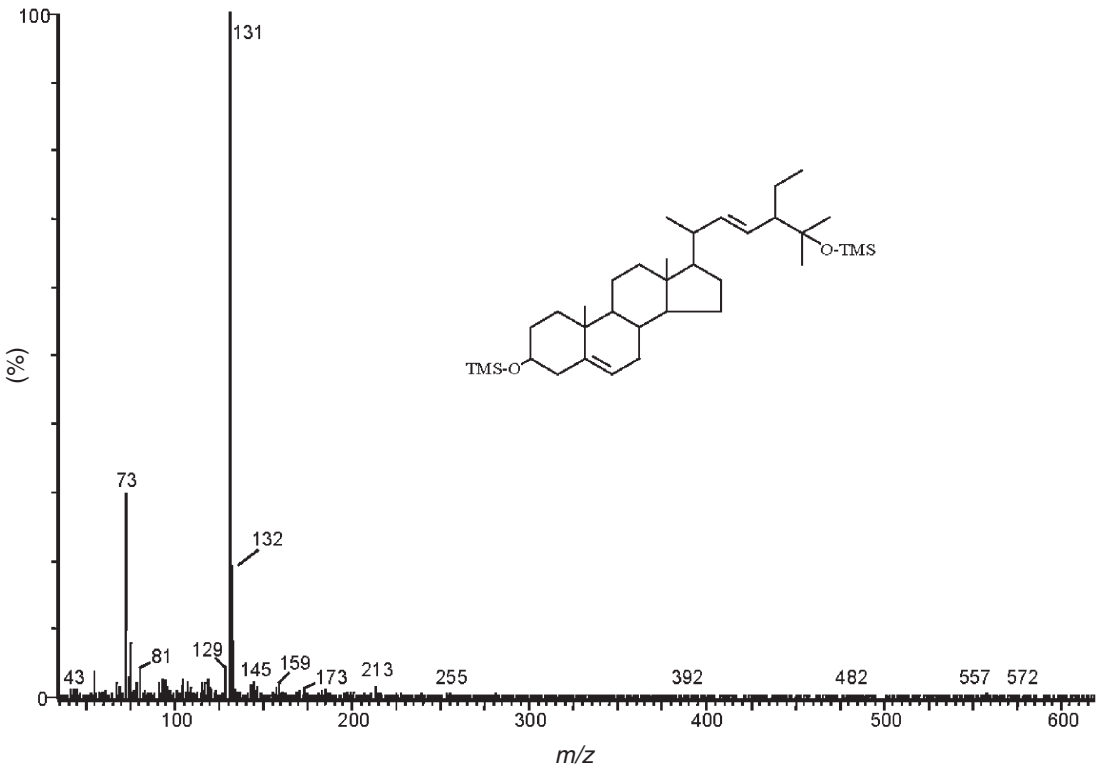

FIG. 1. GC–MS spectrum of 25-hydroxystigmasterol as trimethylsilyl (TMS)-ether derivative. The molecular ion at *m/z* 572 and the base peak at *m/z* 131 are both present in the figure.

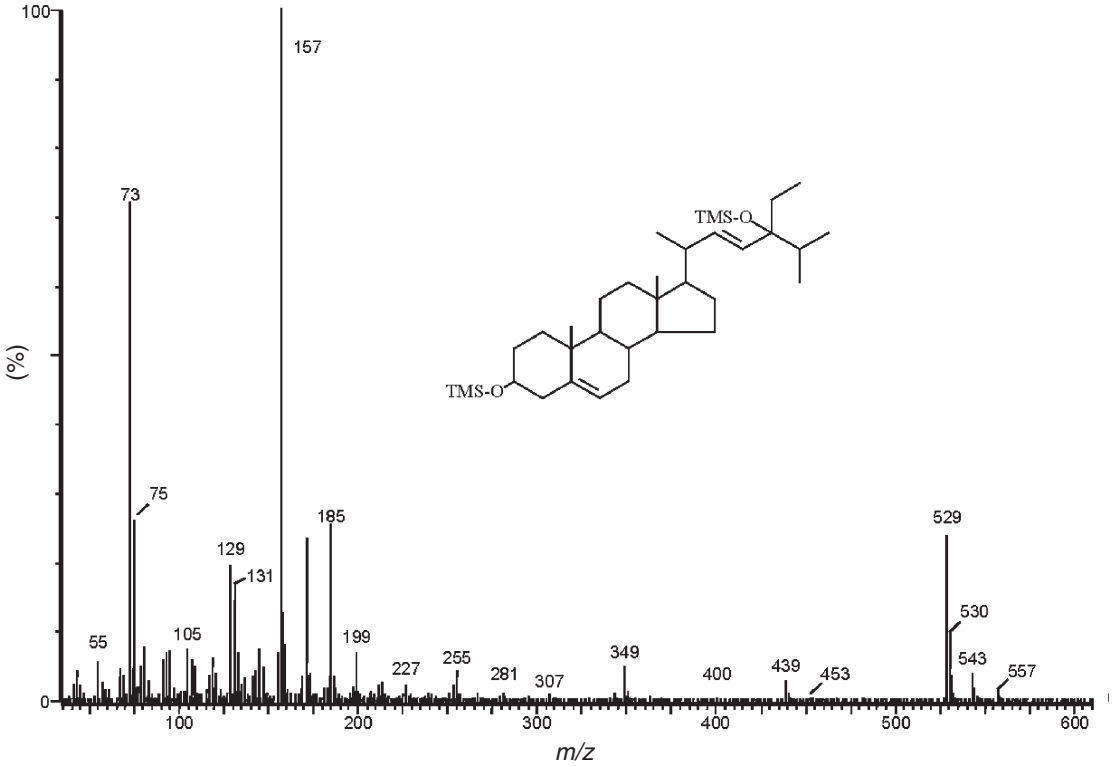

FIG. 2. GC–MS spectrum of 24-hydroxystigmasterol as TMS-ether derivative. The base peak at *m/z* 157, tentatively identified as C22–C29 (without C25–C27), and the peak at *m/z* 529, identified as the molecular ion after the loss of C25–C27, were both important fragments. For abbreviation see Figure 1.

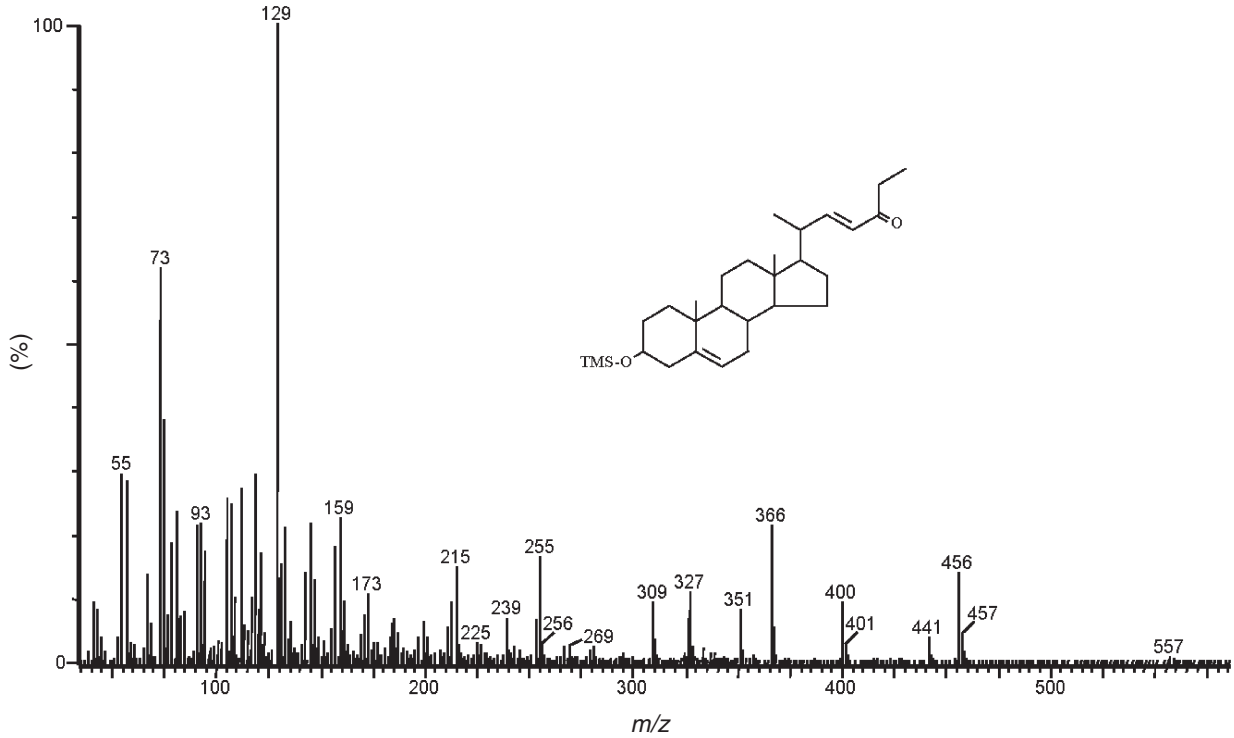

FIG. 3. GC–MS spectrum of 24-ethyl-5,22-choladien-3β-ol-24-one as TMS-ether derivative. The base peak at *m/z* 129, the molecular ion at *m/z* 456, and the loss of TMSO at *m/z* 366 were all characteristic fragments for this compound. For abbreviation see Figure 1.

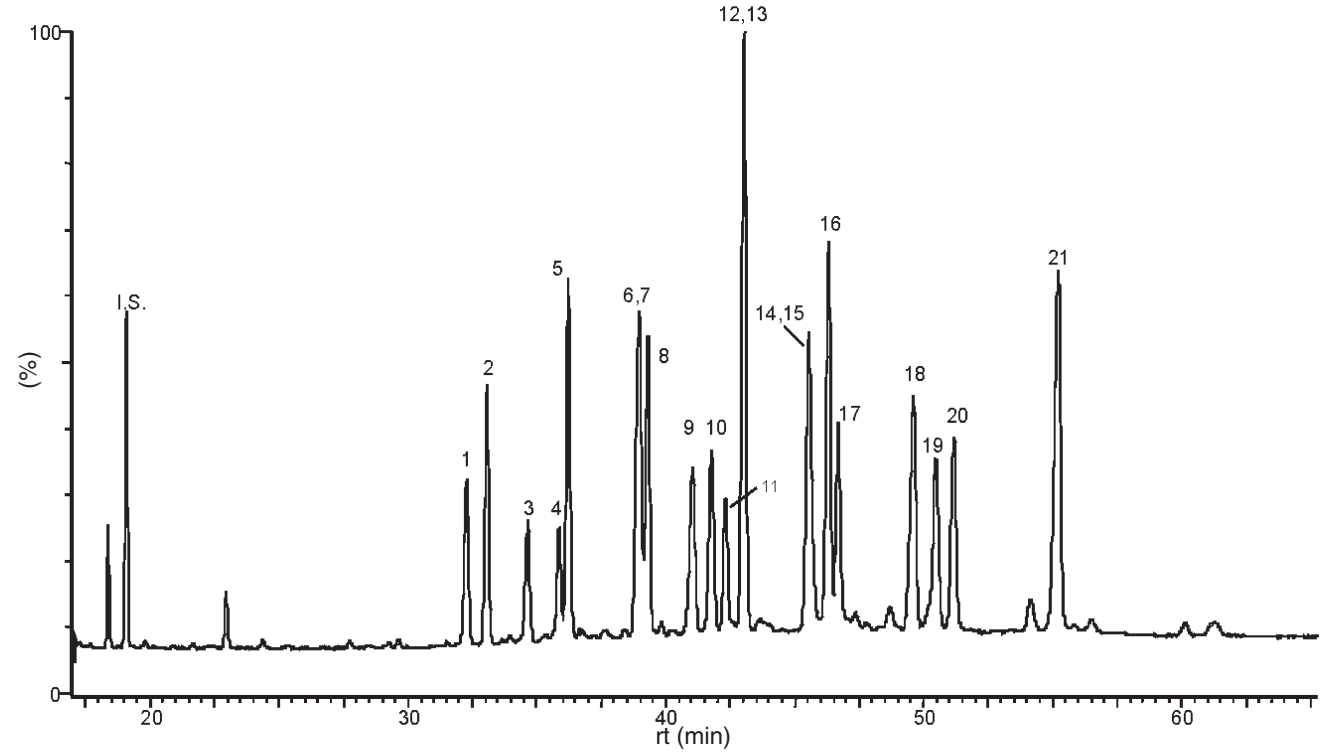

FIG. 4. GC separation of a mixture of oxidation products from sitosterol, campesterol, and stigmasterol. Seven of the most common oxidation products from each phytosterol were analyzed (see Table 3 for identification of the peaks). I.S., internal standard; rt, retention time.

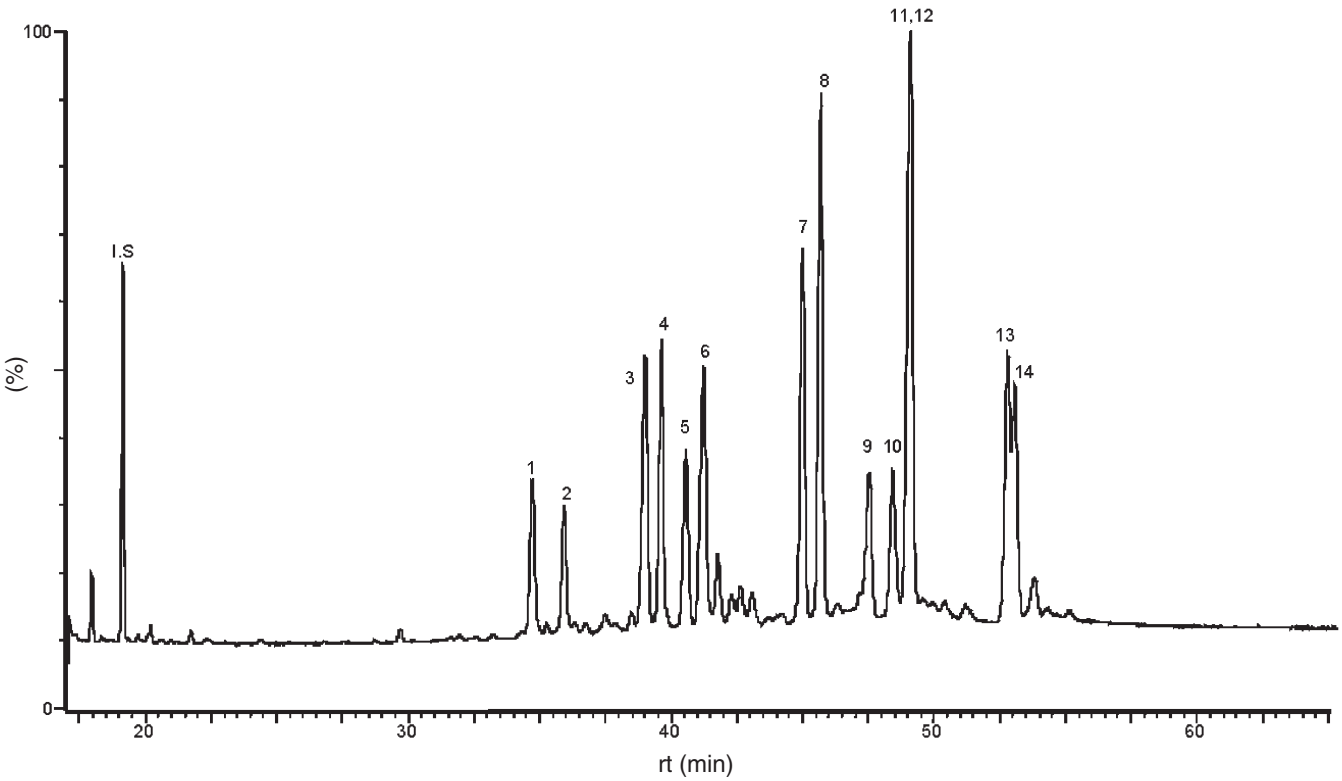

FIG. 5. GC separation of a mixture of medium polarity side-chain and ring-structure oxidation products from sitosterol, campesterol, and stigmasterol. The autoxidation products were isolated with TLC and analyzed on GC–MS (see Table 3 for identification of the peaks). For abbreviations see Figure 4.

times, gave the complete spin-system system (H20–H24 → H28–H29).

In the ^{1}H NMR spectrum of 24-hydroxystigmasterol, two olefinic protons [5.29 (*d*) and 5.41 ppm (*dd*)] that coupled to each other were identified (C22–C23) (Table 2). The doublet at 5.29 ppm (C23) indicated a quaternary carbon adjacent to C23. COSY and TOCSY gave the intact spin system (H20–H24), and in HMBC cross-peaks from methyl protons at 0.88 and 0.85 ppm to a carbon atom at 77.0 ppm also provided strong evidence of a hydroxylated C24.

In the ^{1}H NMR spectrum for 24-ethyl-5,22-choladien-3β-ol-24-one two methyl signals were missing. A pair of olefinic protons was identified further downfield [6.68 ppm (*dd*) and 6.01 ppm (*d*)], indicating a carbonyl function close to the olefinic protons (Table 2). COSY and TOCSY analyses indicated the intact spin system (H20–H24), and it was concluded that C25–C27 had been removed from this molecule. In HMBC, cross-peaks from H22, H23 and H25, H26 clearly indicated that the keto function (201.4 ppm) was in the C24 position.

GC–MS. GC–MS analysis of 25-hydroxystigmasterol gave the very characteristic fragmentation pattern that also has been reported for 25-hydroxysitosterol, 25-hydroxycampesterol, and 25-hydroxycholesterol (10,15) as well as by Johnsson and Dutta (16). The characteristic base peak (17–20) that corresponded to α-cleavage between C24–C25 was identified at *m/z* 131 (Fig. 1). Other important fragments for the characterization of this molecule were the molecular ion *m/z* 572 (M^+), the loss of one and two silyl groups ($M^+ - 90$, $M^+ - 180$), and the loss of a methyl group ($M^+ - 15$). However, the abundance of all these fragments was low (<1%).

The molecular ion for 24-hydroxystigmasterol was identified at *m/z* 572 (<1%) (Fig. 2). Some other important fragments were *m/z* 529 [M^+ – (C25–C27), 21.1%], *m/z* 439 (529 – TMSO, 2.5%), and *m/z* 349 (529 – 2TMSO, 4.6%). The base peak *m/z* 157 (tentatively identified as C22–C29, without C25–C27) and the loss of a methyl group at *m/z* 557 ($M^+ - CH_3$, 1.0%) were also fragments that were important for characterization of 24-hydroxystigmasterol.

The last compound to be characterized was 24-ethyl-5,22-choladien-3β-ol-24-one. The mass spectrum indicated the molecular ion at *m/z* 456 (13.7%), the loss of TMSO at 366 (21.4%), and the loss of the side chain at *m/z* 345 (<1%) (Fig. 3). The loss of a methyl group was reported at *m/z* 441 (3.5%), and the characteristic Δ5-steroid fragment at *m/z* 129 was identified as the base peak.

GC separation. A large number of phytosterol oxidation products may be present in food systems with vegetable origin. To complete the data regarding the content of phytosterol oxidation products in food products, an optimized method for the separation and quantification of all the compounds should be developed. A poor analytical method may account for the overestimation of the content of different oxidation products in food products, thereby constituting a hindrance to the development of knowledge.

Figure 4 illustrates the separation of some ring-structure oxidation products from sitosterol, campesterol, and stigmasterol together with the corresponding unoxidized substances (see Table 3 for identification of the peaks). Peaks that coeluted were unoxidized stigmasterol/7α-hydroxysitosterol (peaks 4 and 5 in Fig. 4), unoxidized sitosterol/7β-hydroxycampesterol/7β-hydroxystigmasterol (peaks 6, 7, and 8 in Fig. 4), α-epoxystigmasterol/7β-hydroxysitosterol (peaks 12 and 13 in Fig. 4), β-epoxysitosterol/triol campesterol (peaks 14 and 15 in Fig. 4), and α-epoxysitosterol/triol stigmasterol (peaks 16 and 17 in Fig. 4). The coelution of these peaks clearly illustrates that system optimization is crucial for future studies. The side-chain oxidation products from sitosterol and campesterol were identified and characterized elsewhere (16).

Figure 5 illustrates the separation of oxidation products characterized in this article (16). (See Table 3 for identification of the peaks.) The products, 25-hydroxystigmasterol/

TABLE 3
Oxidation Products of Sitosterol, Campesterol, and Stigmasterol with Corresponding Peak Number in Figures 4 and 5. Retention Time from GC–MS and Relative Retention Time Compared to 5α-Cholestane[a]

Peak no.	Figure	Compound	rt (min)	rrt
1	4	7α-Hydroxycampesterol	32.4	1.7
2	4	7α-Hydroxystigmasterol	33.3	1.7
3	4	Unoxidized campesterol	35.0	1.8
4	4	Unoxidized stigmasterol	36.3	1.9
5	4	7α-Hydroxysitosterol	36.3	1.9
6	4	Unoxidized sitosterol	39.3	2.0
7	4	7β-Hydroxycampesterol	39.1	2.0
8	4	7β-Hydroxystigmasterol	39.5	2.1
9	4	β-Epoxycampesterol	41.2	2.1
10	4	α-Epoxycampesterol	41.9	2.2
11	4	β-Epoxystigmasterol	42.5	2.2
12	4	α-Epoxystigmasterol	43.3	2.2
13	4	7β-Hydroxysitosterol	43.1	2.3
14	4	β-Epoxysitosterol	45.7	2.4
15	4	Triol campesterol	45.7	2.4
16	4	α-Epoxysitosterol	46.4	2.4
17	4	Triol stigmasterol	46.9	2.4
18	4	7-Ketocampesterol	49.7	2.6
19	4	Triol sitosterol	50.6	2.6
20	4	7-Ketostigmasterol	51.4	2.7
21	4	7-Ketositosterol	55.4	2.9
1	5	Unoxidized campesterol	35.0	1.8
2	5	Unoxidized stigmasterol	36.3	1.9
3	5	Unoxidized sitosterol	39.3	2.0
4	5	24-Ethyl-5,22-choladien-3β-ol-24-one	40.1	2.1
5	5	24-Hydroxycampesterol	40.7	2.1
6	5	24-Methylcholest-4-en-6β-ol-3-one	41.5	2.2
7	5	24-Hydroxysitosterol	45.2	2.3
8	5	24-Ethylcholest-4-en-6β-ol-3-one	46.0	2.4
9	5	24-Methylcholest-4-en-6α-ol-3-one	48.1	2.5
10	5	25-Hydroxycampesterol	48.6	2.5
11	5	25-Hydroxystigmasterol	50.1	2.6
12	5	24-Hydroxystigmasterol	49.6	2.6
13	5	24-Ethylcholest-4-6α-3-one	53.4	2.8
14	5	25-Hydroxysitosterol	53.4	2.8

[a]rt, retention time; rrt, relative retention time.

24-hydroxysitosterol (peaks 11 and 12 on Fig. 5), and 24-ethylcholest-4-en-6α-ol-3-one/25-hydroxysitosterol (peaks 13 and 14 in Fig. 5), were not separated. However, the remaining oxidation products and the unoxidized substances were separated satisfactorily. If the common polar oxidation products and the newly characterized semipolar products were analyzed simultaneously, the difficulties with coeluting peaks would increase dramatically.

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