GC-MS Characterization and Quantification of Sterols and Cholesterol Oxidation Products

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Key Words

Gas chromatography-mass spectrometry
Phytosterols
Cholesterol
Cholesterol oxides

Summary

A GC-MS method has been studied for characterization and quantification of phytosterols, cholesterol and cholesterol oxidation products. Baseline separations have been achieved between cholesterol, cholesterol 5α-6α-epoxide, 5-cholestene-3β-ol-7one (7-ketocholesterol), cholestene-3β-5α-6β-triol, 5-cholestene-3β-25-diol (25-hydroxycholesterol), 5-cholestene-3β-20α-diol (20α-hydroxycholesterol), 5-cholestene-3β-7β-diol (7β-hydroxycholesterol) and 5-cholestene-3β-19-diol (19-hydroxycholesterol) as well as between α-cholestane, cholesterol, stigmasterol, campesterol and β-sitosterol. Excellent linearity of response has been obtained permitting reliable quantification. The characterization of each derivatized sterol has been performed by mass-spectrometry. The results confirm the utility of combined gas chromatography-mass spectrometry in the analysis and characterization of sterols and cholesterol oxidation products.

Introduction

Sterols occur widely in plants as well as in animals and are essential components of human and animal diets.

Cholesterol, an important constituent of animal food products, has often been implicated in the etiology of atherosclerosis and coronary heart diseases. On the other hand a hypocholesterolemic property of plant sterols, both in animals and humans, has been demonstrated and certain preparations of plant sterols have been proposed as therapeutic agents for hypercholesterolemia [1]. Moreover recent studies have shown the possible role of cholesterol oxidation products, rather than cholesterol itself, in the initiation of atherosclerotic plaque formation. Some oxysterols have also been reported to be carcinogenic, mutagenic or to produce toxic effects in cell cultures [2].

Given the ubiquitous presence of cholesterol, foods can be susceptible to the formation of oxidation compounds and the difficulty in preventing their occurrence makes this problem really critical [3]. Cholesterol oxidation products have already been found in animal foodstuffs such as egg products, heated tallow, dairy and meat products. Nevertheless, assessing the overall level and the potential intake of oxidized cholesterol in the daily diet is still difficult [4]. In fact a number of methods have been developed for the quantitative determination of the cholesterol oxides in various food products but no standard method yet exists which combines the essential qualities of accuracy, precision and selectivity with that of speed [5].

The aim of the present work was to study a GC-MS method for the characterization and quantification of sterols and oxysterols in food. The method will be used to estimate the potential intake of phytosterols, cholesterol and cholesterol oxides on the basis of the Italian reference diet formulated purposely to monitor food nutrients and contaminants [6].

Experimental

Chemicals

Campesterol, β-Sitosterol, Stigmasterol, Cholest-5-ene-3β-ol (cholesterol), 5α-cholestane, cholesterol 5α-6α-epoxide, 5-cholestene-3β-ol-7one (7-ketocholesterol), cholestene-3β-5α-6β-triol, 5-cholestene-3β-25-diol (25-hydroxycholesterol), 5-cholestene-3β-20α-diol (20α-hydroxycholesterol), 5-cholestene-3β-7β-diol (7β-hydroxycholesterol) and 5-cholestene-3β-19-diol (19-hydroxycholesterol) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

All analytical grade solvents were from Carlo Erba (Milan, I). The silylating agents TMCS (trimethylchlorosilane) and HMDS (hexamethyldisilazane) were from Supelco (Bellofonte PA-USA).

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Food Samples

Fresh eggs were purchased from a local supermarket and freeze-dried egg yolk powder was prepared from fresh egg yolk in the laboratory. Spray-dried whole egg powder was a gift from a local confectionery.

Lipid Extraction and Saponification

Dry samples (0.3 g of egg powder) were diluted with ethanol : dichloromethane (1 : 1) and internal standards (19-hydroxycholesterol and 5α-cholestane) were added. After 1h stirring, the mixture was filtered through defatted cotton wool and rinsed with 10 ml of ethanol : dichloromethane (1 : 1). Lipid extracts were saponified with 20 ml of 1 mol/l KOH in methanol and left overnight at room temperature [7].

The unsaponifiable fraction was extracted twice with 25 ml of diethyl ether after addition of 25 ml of doubly distilled water. The pooled organic fractions were rinsed with water and evaporated under vacuum.

Thin Layer Chromatography

The unsaponifiable residue (or the standard mixture) was resuspended into 0.5 ml of chloroform and deposited on a precoated silica gel plate, 20 × 20 cm, 0.25 mm gel thickness.

The plate was developed in diethyl ether : hexane : acetic acid (60 : 40 : 1), dried and sprayed with 2,7-dichlorofluorescein. Sterol and oxidation product areas, identified under UV light by their position on the TLC plate, were scraped off and extracted twice with diethyl ether and chloroform.

Derivatization of Sterols to TMS Ethers

The sterol and cholesterol oxide fractions were dried under a stream of nitrogen and then 100 µl of TMCS and 100 µl of HMDS were added. Derivatization to trimethylsilyl (TMS) ethers was achieved in 1h at 60 °C.

Gas Chromatography-Mass Spectrometry

A Hewlett-Packard 5890 gas chromatograph equipped with a 5970 Mass Selective Detector (MSD) was employed.

Sterols and cholesterol oxides were separated as TMS ethers on an HP-1 crosslinked methyl silicone column, 12 m × 0.2 mm, film thickness 0.33 µm. The carrier gas was helium with an inlet pressure of 83 kPa (12 psi) and a flow rate of 40 cm/sec. The initial column temperature of 70 °C was held for 2 min then programmed at 70 °C/min to 140 °C and at 10 °C/min to 280 °C where it was held for 15 min. Injections (0.5 µl) were made in a splitless mode for 0.2 min, followed by the split mode with a split flow of 50 ml/min and a septum purge of 2 ml/min. Injection temperature was 265 °C.

The total ion mass spectra were recorded in the mass range m/e 100–670 at a scan rate of 1 s/scan. The transfer line temperature was 280 °C and the energy of the electron impact ionization was 70 eV.

Peak identification was based on a relative retention time and total ion mass spectral comparison with an external standard. Sterols and cholesterol oxides were quantified by peak area comparison with the internal standards 5α-cholestane and 19-hydroxycholesterol.

Results

Gas Chromatographic Separation

The GC chromatogram of cholesterol and some plant sterols is shown in Figure 1. Under the conditions employed a baseline separation of all components was achieved.

Separation of cholesterol and cholesterol oxide standards chromatographed as the trimethyl-silylated sterols is shown in Figure 2. Again a complete separation of all relevant cholesterol oxides has been achieved. Cholesterol is also resolved from its oxidation products, but a preliminary TLC separation is advisable because of the high concentration of cholesterol in food samples.

The linearity of the plot for quantification is reported in Table I together with the retention time reproducibility. Regression analysis of the plot of area response ratio versus weight ratio of sterols and oxysterols over internal standard (5α-cholestane and 19 hydroxycholesterol respectively) reveals an excellent linear rela-

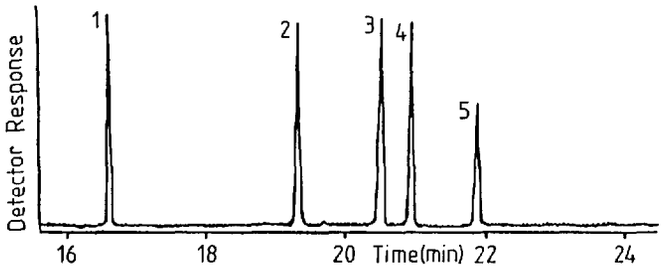

Figure 1

GC-MS separation of α-cholestane (1), Cholesterol (2), Campesterol (3), Stigmasterol (4), β-sitosterol (5). See text for the conditions used.

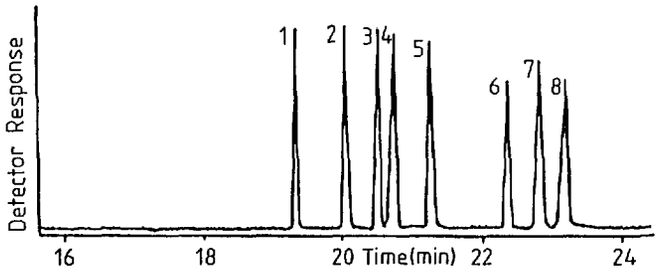

Figure 2

GC-MS separation of Cholesterol (1), 19-hydroxy cholesterol (2), 7β-hydroxy cholesterol (3), 5α-6α-epoxide (4), 20α-hydroxy cholesterol (5), 3β-5α-6β-triol (6), 7-ketocholesterol (7), 25-hydroxycholesterol (8). See text for the conditions used.

tionship. The r^2 values for each compound is nearly unity. These parameters suggest that quantification of sterols and cholesterol oxides by GC-MS is highly reproducible. Under these conditions the lowest detectable level, calculated according to the method of Kaiser [8], is 1.5 ng/µl.

Using the above described method fresh and freeze-dried egg yolk samples have been analyzed. As a reference an old sample of whole egg powder, known to yield high amounts of cholesterol oxides, has been used.

As reported in Table II the cholesterol content is in agreement with similar data obtained using chromatographic methods. Moreover no quantifiable levels of any of the six typical cholesterol oxidation products, which have been identified in the old sample, have been found in fresh egg yolk. The mild freezedrying treatment performed in the laboratory does not induce the formation of cholesterol oxidation products, but the dehydrated egg yolk stored for 6 months developes various oxidation products. The level of cholesterol oxides increases on prolonged storage at 4 °C in air. These results confirm that, as already reported [9], no oxidative reactions occur during lipid extraction and saponification while the storage time seems to be a more critical parameter.

Finally, recoveries close to 100 % have been calculated during a recovery test of cholesterol oxidation products from freeze dried egg powder.

Mass Spectrometric Characterization

The application of mass spectrometry to cholesterol and plant sterol analysis is facilitated since, not whithstanding the common skeleton, the side chain may be regarded as constituting successive members of a homologous series. In fact plant sterols differ from cholesterol in having either one or two extra carbon atoms at C_{24} (Figure 3). Thus ions in a mass spectrum which retain the nucleus will be common to each member of a series, whereas those ions retaining the side chain will show successive increases of 14 mass units [10].

Table I. Linearity of the plot of area response ratio (A_i/A_{is}) versus weight ratio (W_i/W_{is}).*

Compound	RT mean (s.d.)	Slope	Intercept	r^2	RF_i (a) mean (s.d.)
Cholesterol (b)	19.25 (0.06)	1.0152	- 0.0443	0.999	1.009 (0.008)
Campesterol (b)	20.55 (0.06)	0.8790	- 0.0446	0.999	1.163 (0.010)
Stigmasterol (b)	20.91 (0.06)	0.5960	- 0.0200	0.999	1.661 (0.008)
β-sitosterol (b)	21.82 (0.06)	0.6626	- 0.0233	0.998	1.538 (0.018)
7β-hydroxy cholesterol (c)	20.52 (0.04)	1.1507	+ 0.0085	0.999	0.869 (0.010)
5,6α-epoxide (c)	20.75 (0.05)	0.6498	+ 0.0127	0.999	1.505 (0.019)
20α-hydroxide (c)	21.38 (0.05)	0.7580	+ 0.0096	0.999	1.298 (0.010)
3β-5α-6β-triol (c)	22.34 (0.04)	0.6302	- 0.0093	0.998	1.614 (0.013)
7 ketocholesterol (c)	22.89 (0.04)	1.1452	- 0.0028	0.999	0.874 (0.009)
25 hydroxy cholesterol (c)	23.25 (0.04)	0.6352	- 0.0068	0.999	1.598 (0.012)

* Five data points with 3 injections (n = 5, r = 3)
(a) Response factor = (W_i/A_i) (A_{is}/W_{is})
(b) Various amounts of sterols were added to fixed amount of α-cholestane
(c) Various amounts of oxy-sterols were added to fixed amount of 19-hydroxy cholesterol

Table II. Quantification of cholesterol and cholesterol oxides in egg products. Effect of treatments and storage (4 °C in air).

Product	Storage months	Cholesterol*	Cholesterol oxides**					
			7β hydroxy-	5α, 6α epoxide	20α hydroxy	3β, 5α, 6β triol	7keto cholesterol	25-hydroxy-cholesterol
Fresh egg yolk	0	18.70 (0.06)	ND	ND	ND	ND	ND	ND
Freeze-dried egg yolk	0	18.70 (0.06)	ND	ND	ND	ND	ND	ND
	6		4.79 (0.20)	7.19 (0.02)	6.96 (0.09)	TR	TR	TR
	12		8.69 (0.04)	15.12 (0.33)	9.96 (0.13)	1.03 (0.05)	10.94 (0.06)	TR
Spray-dried whole egg	?? years	15.00 (0.06)	36.85 (0.09)	13.34 (0.14)	20.67 (0.12)	2.83 (0.06)	17.87 (0.17)	6.00 (0.12)

ND: not detectable, detection limit ca 1 ppm
TR: traces (< 1 ppm)
* milligrams per gram (dry weight): means (s.d.), n = 3
** micrograms per gram (dry weight): means (s.d.), n = 3

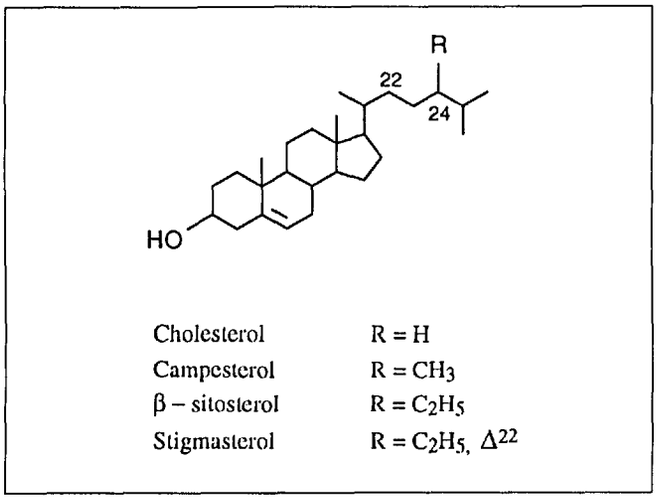

Figure 3
Cholesterol and plant sterols: structures

In Table III are listed details of the TMS-sterols studied in the present work together with the observed base peak and the principal ions above m/e 129.

The ion (M-129) is specific Δ^5 sterol trimethylsilyl ether derivatives and permits the distinction between mono-unsaturated di-unsaturated sterols. A corresponding abundant ion is also observed at m/e 129 and is thought to contain C_1 to C_3 with the oxygen function [10].

Ions involving loss of the side chain may be divided into those which have retained the C_3 oxygen and those which have lost this group as TMSOH or TMSO. The presence of a double bond in the side chain (e.g. stigmasterol) gives two characteristic ions due to elimination of the side chain with two additional hydrogen atoms and to further elimination of TMSOH.

In addition stigmasterol exhibits loss of 43 mass units from the M-TMSOH ion probably due to loss of the isopropyl group at the end of the side chain (C_{25}, C_{27}).

Cholesterol oxides examined in this work are shown in Figure 4 and Figure 5. The relative principal ions for the TMS derivatives are listed in Table IV.

The TMS ether of 7-keto cholesterol shows two abundant peaks relative to the molecular ion, stabilized by the conjugated ketone group, and to m/e 129 fragment. Nevertheless, under our conditions, a fragment at m/e 174 is the base peak.

α-epoxide TMS ether gives fairly intense peaks at m/e 474 (molecular ion) and m/e 384 (loss of trimethylsilanol). A fragment at m/e 143, the origin of which was not identified, is the base peak.

Based on the molecular ion peaks of the TMS derivatives, oxysterols having one or more hydroxyl groups can be identified [12]. The presence of characteristic peaks corresponding to M-90, M-180 or M-270 in TMS sterols is additional information to identify mono or more TMS ethers.

Loss of trimethylsilanol (M-90), due to easy elimination of the allylic trimethylsilyloxy group at C_7, forms the base peak of the bis-TMS ether of 7β-hydroxycholesterol. In the mass spectrum there are no other fragments amounting to more than 6 % of the intensity of the base peak.

Cholestane-triol has one hydroxyl group sterically hindered, but a small peak observed at m/e 636 and some other fragments due to a loss of 270 mass units strongly suggest a tris-TMS ether structure. This hypothesis is also in agreement with the presence of a single peak in the gas chromatogram. The base peak at m/e 403 arises from the loss of ring A, according to the mechanism already proposed [13].

Table III. Mass spectrometric data for sterol trimethylsilyl ethers. Relative abundances are reported in square brackets.

Fragmentation	Cholesterol TMS	Campesterol TMS	β-Sitosterol TMS	Stigmasterol TMS
M (molecular ion)	458 [29]	472 [36]	486 [31]	484 [66]
M-CH_3	443 [9]	457 [10]	471 [4]	469 [6]
M-TMSOH	368 [77]	382 [86]	396 [78]	394 [84]
M-(CH_3 + TMSOH)	353 [41]	367 [40]	381 [35]	379 [33]
M-(TMSOH + C_5H_7)	301 [5]	315 [4]	329 [-]	327 [-]
M-(TMSOH + C_7H_9)	275 [3]	289 [4]	303 [2]	301 [-]
M-(TMSOH + C_9H_{13})	247 [16]	261 [14]	275 [11]	273 [-]
M-(C_1, C_3 + TMSO)	329 [100]	343 [100]	357 [100]	355 [38]
M-(C_{25}, C_{27})	415 [-]	429 [-]	443 [-]	441 [-]
M-(C_{25}, C_{27} + TMSOH)	325 [1]	339 [-]	353 [-]	351 [42]
M-(TMSOH + C_8H_{12})	260 [3]	274 [-]	288 [-]	286 [-]
M-side chain	345 [-]	345 [3]	345 [-]	345 [-]
M-(side chain + TMSOH)	255 [16]	255 [18]	255 [16]	255 [100]
M-(side chain + 2H)	343 [-]	343 [-]	343 [-]	343 [14]
M-(side chain + 2H + TMSOH)	253 [-]	253 [-]	253 [-]	253 [19]
M-(side chain + C_3H_6)	303 [-]	303 [-]	303 [2]	303 [-]
M-(side chain + C_3H_6 + TMSOH)	213 [10]	213 [11]	213 [7]	213 [29]
M-(side chain + C_{16}, C_{17})	318 [-]	318 [-]	318 [-]	318 [-]
M-(side chain + C_{16}, C_{17} + TMSO)	229 [3]	229 [2]	229 [-]	228 [5]
C_1, C_3 + TMSO	129 [68]	129 [78]	129 [67]	129 [94]

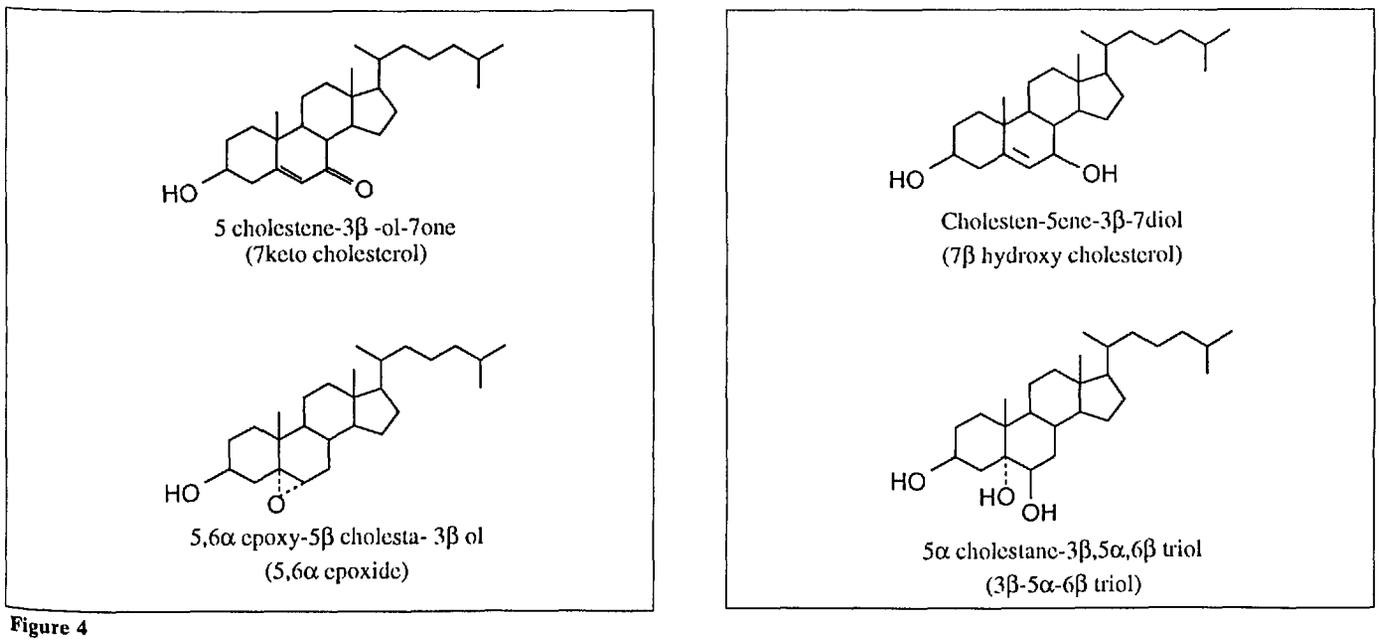

Figure 4
Cholesterol oxidation products: structures

Table IV. Mass spectrometric data for cholesterol oxide trimethylsilyl ethers. Relative abundances are reported in square brackets.

Fragmentation	7keto cholesterol TMS	5α, 6α epoxide TMS	7β-hydroxy cholesterol TMS	3β, 5α, 6β triol TMS	20α-hydroxide cholesterol TMS	25-hydroxy cholesterol TMS
M (molecular ion)	472 [97]	474 [20]	546 [1]	636 [3]	546 [4]	546 [4]
M-CH_3	457 [16]	459 [19]	531 [-]	621 [-]	531 [2]	531 [2]
M-TMSOH	382 [42]	384 [54]	456 [100]	546 [18]	456 [83]	456 [4]
M-2TMSOH			366 [3]	456 [73]	366 [58]	366 [4]
M-3TMSOH				366 [19]		
M-(CH_3 + TMSOH)	367 [72]	369 [34]	441 [2]	531 [9]	441 [22]	441 [2]
M-(CH_3 + 2TMSOH)			351 [4]	441 [25]	351 [78]	351 [3]
M-(CH_3 + 3TMSOH)				351 [17]		
M-(TMSOH + C_5H_7)	315 [-]	317 [-]	389 [-]	479 [-]	389 [23]	389 [-]
M-(2TMSOH + C_5H_7)			299 [0.5]	389 [-]	299 [18]	299 [-]
M-(3TMSOH + C_5H_7)				299 [-]		
M-(TMSOH + C_7H_9)	289 [-]	291 [6]	363 [-]	453 [-]	363 [-]	363 [-]
M-(2TMSOH + C_7H_9)			273 [-]	273 [3]	273 [-]	273 [-]
M-(3TMSOH + C_7H_9)				183 [16]		
M-(TMSOH + C_9H_{13})	261 [-]	263 [-]	335 [-]	425 [-]	335 [-]	355 [-]
M-(2TMSOH + C_9H_{13})			245 [0.5]	335 [-]	245 [-]	245 [3]
M-(3TMSOH + C_9H_{13})				245 [6]		
M-(C_1, C_3 + TMSO)	343 [-]	345 [-]	417 [-]	507 [-]	417 [-]	417 [-]
M-(C_1, C_3 + 2TMSO)			327 [0.5]	417 [-]	327 [20]	327 [8]
M-(C_1, C_3 + 3TMSO)				327 [5]		
M-(C_{24}, C_{25} + TMSO)						415 [-]
M-(TMSOH + C_8H_{12})	274 [-]	276 [-]	438 [-]	438 [-]	348 [-]	348 [-]
M-(2TMSOH + C_8H_{12})			348 [-]	348 [-]	258 [-]	258 [-]
M-(3TMSOH + C_8H_{12})				258 [-]		
M-side chain	359 [-]	361 [-]	433 [-]	523 [-]	417 [-]	417 [-]
M-(side chain + TMSOH)	269 [31]	271 [21]	343 [-]	433 [-]	32 [20]	327 [8]
M-(side chain + 2TMSOH)			253 [2]	343 [-]	237 [-]	237 [-]
M-(side chain + 3TMSOH)				253 [12]		
M-(side chain + C_3H_6)	317 [-]	319 [-]	391 [-]	481 [-]	375 [-]	375 [-]
M-(side chain + C_3H_6 + TMSOH)	227 [17]	229 [19]	301 [-]	391 [-]	285 [-]	285 [-]
M-(side chain + C_3H_6 + 2TMSOH)			211 [2]	301 [-]	195 [-]	195 [-]
M-(side chain + C_3H_6 + 3TMSOH)				211 [11]		
M-(side chain + C_{16}, C_{17})	332 [-]	334 [-]	406 [-]	496 [-]	390 [10]	390 [-]
M-(side chain + C_{16}, C_{17} + TMSO)	242 [-]	244 [-]	316 [-]	406 [-]	300 [-]	300 [-]
M-(side chain + C_{16}, C_{17} + 2TMSO)			226 [-]	316 [-]	210 [-]	210 [-]
M-(side chain + C_{16}, C_{17} + 3TMSO)				226 [-]		
C_1, C_3 + TMSO	129 [99]	129 [78]	129 [6]	129 [39]	129 [100]	129 [23]
C_{24}, C_{25} + TMSO	131 [35]	131 [42]	131 [4]	131 [18]	131 [43]	131 [100]
M-Ring A	403 [-]	403 [-]	403 [-]	403 [100]	403 [-]	403 [-]
?	143 [29]	143 [100]	143 [6]	143 [33]	143 [33]	143 [4]
?	174 [100]	174 [9]	174 [0.5]	174 [-]	174 [7]	174 [-]

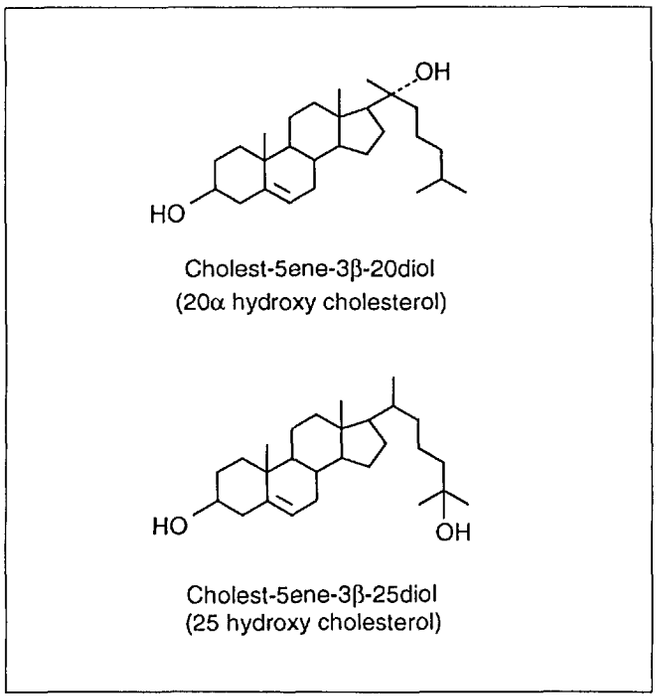

Figure 5
Cholesterol side-chain oxidation products: structures

Location of side-chain hydroxyl groups is facilitated by the α-cleavages typical of the TMS ethers. The terminal alcohol of 25-hydroxycholesterol, in the TMS form, gives the base peak at m/e 131, while 20-hydroxycholesterol gives the base peak at m/e 129 even if it includes the expected fragment at m/e 131.

Conclusion

The results surveyed above confirm that mass spectrometric data, when interpreted in conjunction with gas chromatographic data, provide powerful evidence for characterization and quantification of sterols and oxidation products.

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