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Identification of thermal oxidation products of cholesteryl acetate

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Abstract

The polar products separated by solid-phase extraction from the peroxidation mixture of cholesteryl acetate, were investigated. The oxidation products were identified by comparing GC retention times as well as the mass spectra against those of available or synthesized standards. The main oxidation products where 7β-chydroperoxicholesteryl acetate, 7α-hydroperoxicholesteryl acetate, 7-ketocholesteryl acetate, the α and β isomers of 7-hydroxycholesteryl acetate, the α- and β-epoxy isomers in 5,6 position and several derivatives from the loss of groups (especially the acetic and/or hydroxyl groups in the form of acetic acid and water).

1. Introduction

The fact that a number of cholesterol oxidation products are highly toxic [1–4] explains the many detailed studies dealing with their identification [1,5] and formation mechanisms over the last fifteen years. Many analytical problems encountered in determining cholesterol's oxidation mechanisms via the identification of oxidation products are linked to the presence of an hydroxyl group in position 3, that interacts via a polar effect with both the chromatographic materials and substrate components. The present study reports and discusses the identification of the thermal oxidation products of cholesteryl acetate (CA), a molecule in which the importance of the polarity of the –OH group in position 3 is practically nullified, thereby resulting in a polarity similar to that of a fatty acid methyl ester. This approach makes it possible to exploit both the analytical procedure using the experimental conditions adopted in previous studies [6–10] for separations and molecular structure identification.

2. Experimental

2.1. Materials and reagents

Cholesterol, CA (>99%), 5,6α-epoxycholestanol (5,6α-epox), 5,6β-epoxycholestanol (5,6β-epox), 7α-hydroxycholesterol (7α-OH), 7β-hydroxycholesterol (7β-OH), 4β-hydroxycholesterol (4β-OH), 7-ketocholesteryl acetate (7-KA) and 7-keto-3,5-cholestadiene (7-KDA) were supplied by Steraloids (Wilton, NH, USA). The reagents and solvents (analytical or HPLC grade) were supplied by Carlo Erba (Milan, Italy). The solid-phase extraction (SPE) columns (Bond Elut, Analytichem International, Varian, CA, USA) were packed with 500 mg silica.

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2.2. Preparation of 3β-acetyl derivatives of 7α-OH (7α-OHA), 7β-OH (7β-OHA), 5,6α-epox (5,6α-epoxA) and 5,6β-epox (5,6β-epoxA)

Single standard compound was acetylated with pyridine–acetic anhydride (1:1, v/v), for 12 h (epoxides) or 1–2 h (hydroxides) at room temperature. Preparative HPLC and GC–ion-trap detector (ITD) MS were carried out for purification and identification of the 3β-acetyl derivatives.

2.3. CA thermal peroxidation

A 0.5-g amount of CA was oven-heated at 160°C for 90 min, in a 20-ml PTFE-sealed screw cap container.

2.4. SPE collection of cholesteryl acetate oxidation products (CAOPs)

The cholesteryl acetate peroxidized was dissolved in 2 ml of *n*-hexane and loaded onto a

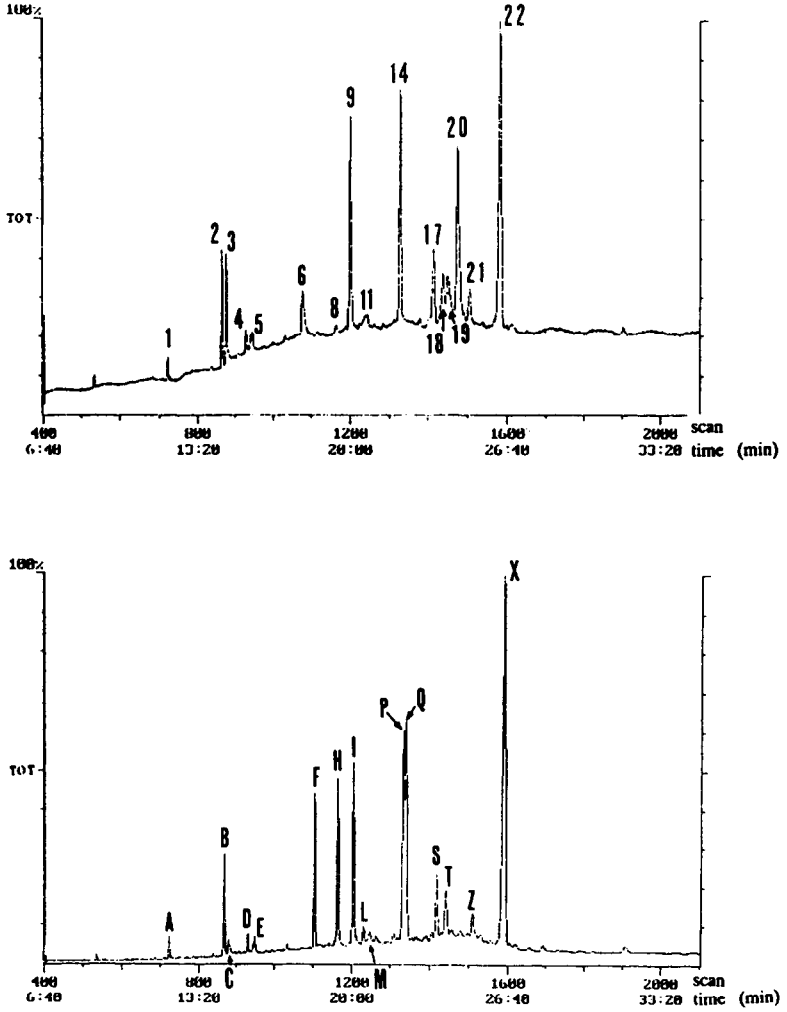

Fig. 1. GC–ITD-MS reconstructed chromatograms of SPE polar fraction: directly injected (top) and injected after TMS derivatization (bottom). Component identification is reported in Table 1.

SPE column preconditioned with 5 ml of *n*-hexane. The column was then eluted with 4 ml of *n*-hexane–diethyl ether (95:5, v/v), 5 ml of chloroform–methanol (1:1, v/v) and 5 ml of methanol.

The second fraction which contained the CA thermal oxidation products was then analyzed by GC–ITD-MS both directly and after silanization.

2.5. Trimethylsilyl ether (TMS) preparation

The samples were silylated with about 0.1 ml of pyridine–hexamethyldisilazane–trimethylchlorosilane (5:2:1, (v/v/v) [11] for 30 min at room temperature, in a desiccator. After drying by evaporation under nitrogen flow, samples were re-dissolved in 30–50 μl of benzene.

2.6. GC–ITD-MS

The capillary gas chromatograph was a Varian 3400 coupled to a Varian Saturn ion-trap detector utilized in the electron impact (EI) mode; the fused-silica column was a DB-5 type (5% phenylmethyl) (J&W, Folsom, CA, USA), with

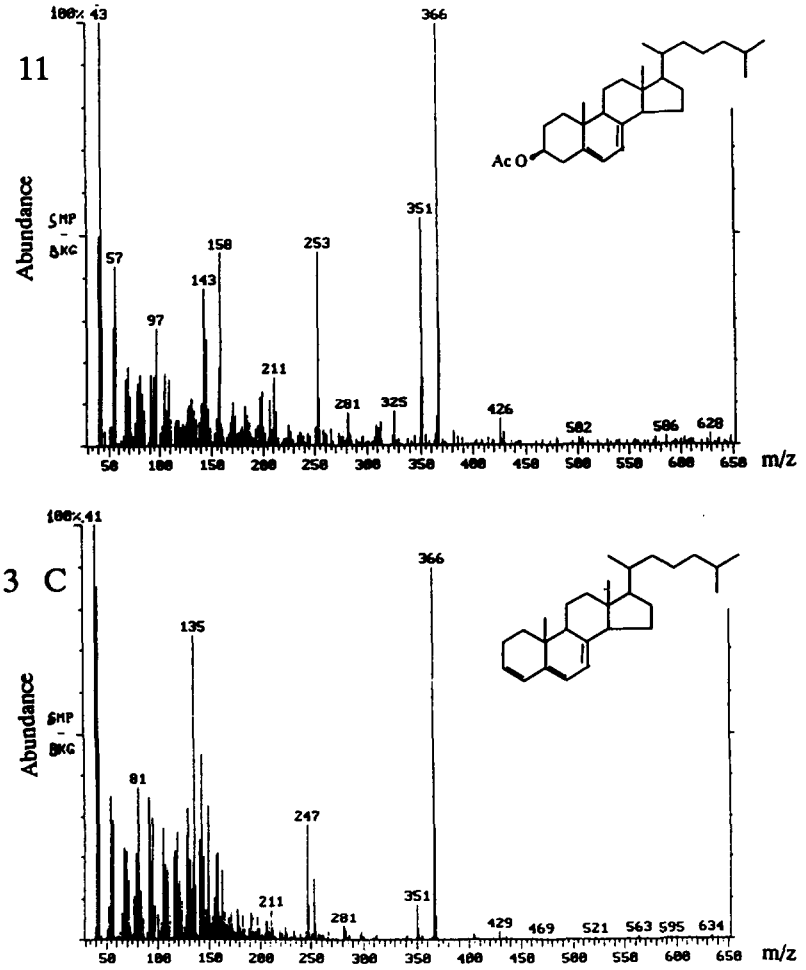

Fig. 2. Mass spectrum of 5,7-cholestadienyl-3β-acetate (11, M) (top): fragments at m/z 426 and 366 correspond to the molecular ion (M^+) and the loss of acetic acid, respectively. Mass spectrum of 3,5,7-cholestatriene (3, C) (tentative) (bottom). Mass spectrum of GC background was substracted (sample – background, SMP – BKG) from mass spectra.

dimensions 30 m × 0.255 mm I.D. and 0.25 μm film thickness. Oven temperature was programmed from 220 to 300°C at a rate of 5°C/min. Injection was in the split mode (1:50 ratio) at a 1 ml/min flow-rate with helium as carrier gas; the injector, transfer line and manifold temperatures were 300, 300 and 220°C, respectively. The filament emission current was 10 μA.

3. Results and discussion

The GC–ITD-MS traces (Fig. 1) were recorded by analyzing the mixture of polar components after SPE collection and purification; these components were examined immediately (Fig. 1, top) and after derivatization by silylation of all –OH groups (Fig. 1, bottom). From the

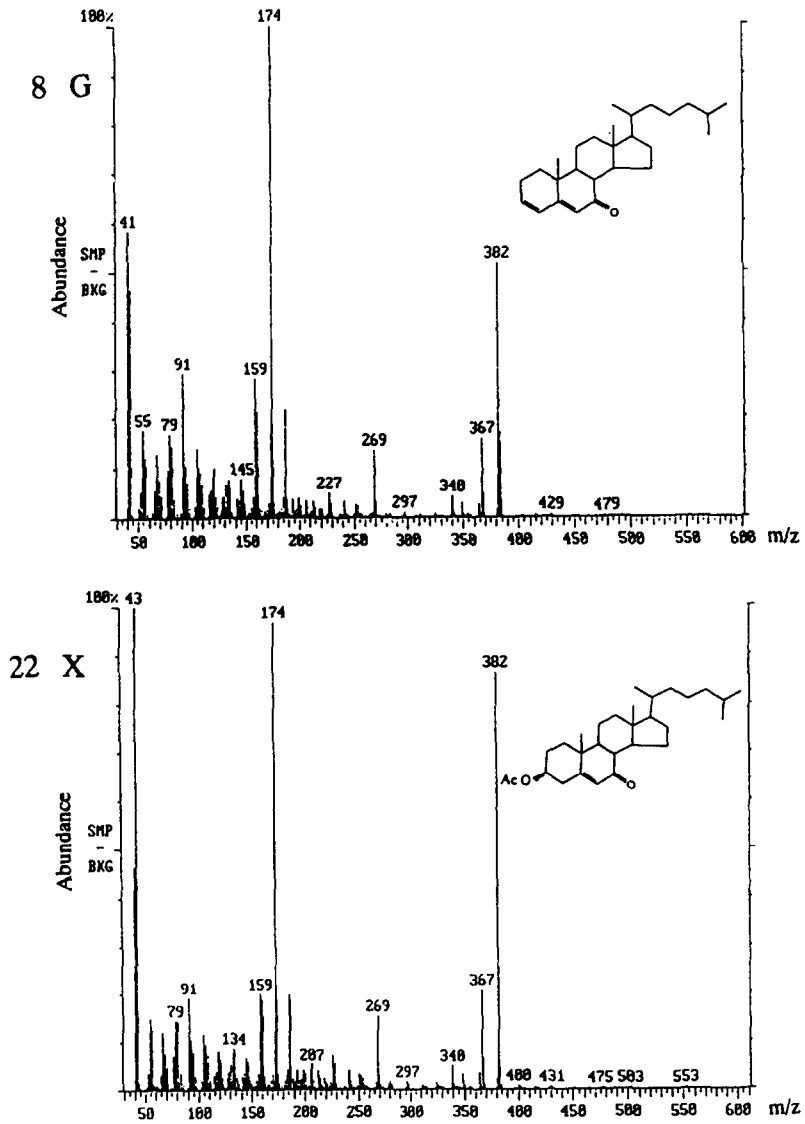

Fig. 3. Mass spectra of 7-keto-3,5-cholestadiene (8, G) (top) and 7-keto-cholesteryl-3β-acetate (22, X) (bottom). Fragment at m/z 382 correspond to the loss of acetic acid (60 u) from molecular ion; fragment at m/z 174 correspond to the elimination of the upper rings (and side chain, except for one methylene group) from the m/z 382 fragment. Mass spectrum of GC background was substracted (sample – background, SMP – BKG) from mass spectra.

found substances, 1-A, 2-B, 3-C, 4-D, 5-E, 11-M, 14-P, 18-T, 17-S and 21-Z are common to both traces.

Fig. 2 shows the mass spectra of components 3 (C) and 11 (M) from Fig. 1; Fig. 3 the mass spectra of components 8 (G) and 22 (X); Figs. 4 and 5 the spectra of components 14 (P) and 17 (S) respectively; Fig. 6 the mass spectra of components 18 (T) and 21 (Z); Fig. 7 the mass spectrum of component 19 (H, as TMS) and Fig. 8 the mass spectrum of component 20 (Q, as TMS).

The mass spectra of components 6, F and 9 (I) correspond to those of cholesterol, CA and TMS cholesterol, respectively.

Table 1 shows the identification for the components in Fig. 1. All main components were determined by comparing GC responses as well

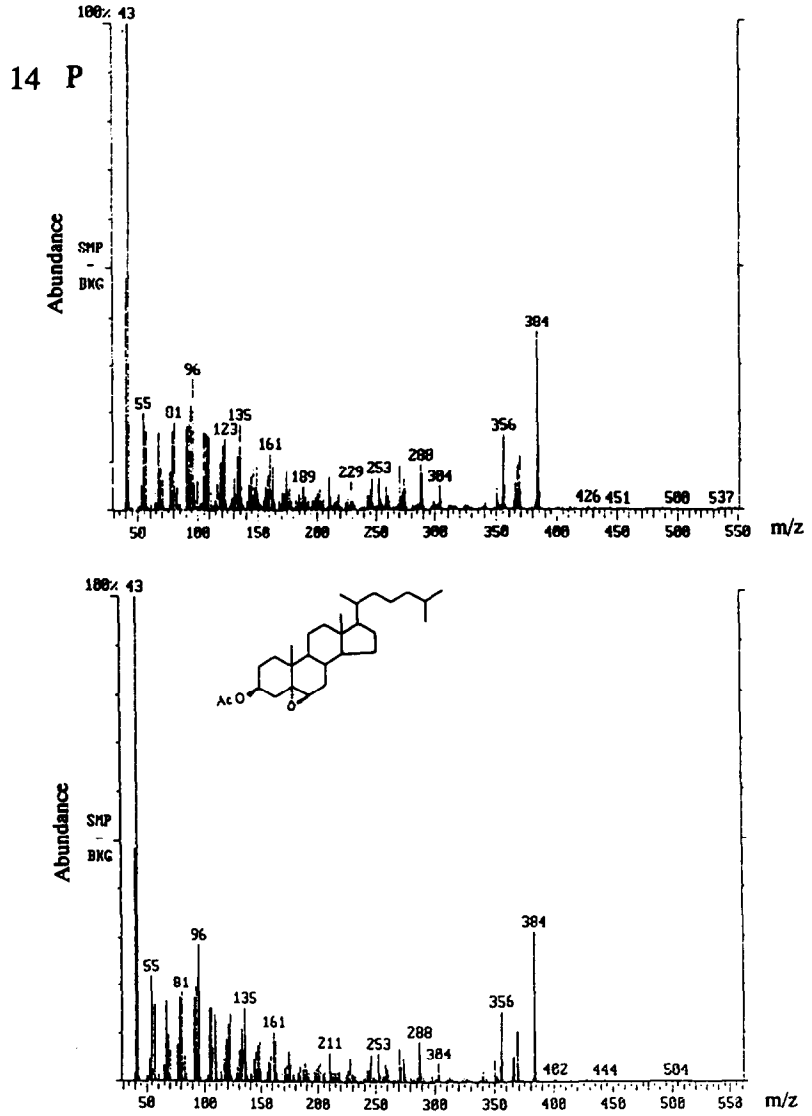

Fig. 4. Mass spectra of 5,6β-epoxy-cholesteryl-3β-acetate (14, P) (top) and corresponding standard (bottom). Fragment at m/z 384 correspond to the loss of acetic acid (60 u) from the molecular ion. Mass spectrum of GC background was substracted (sample − background, SMP − BKG) from mass spectra.

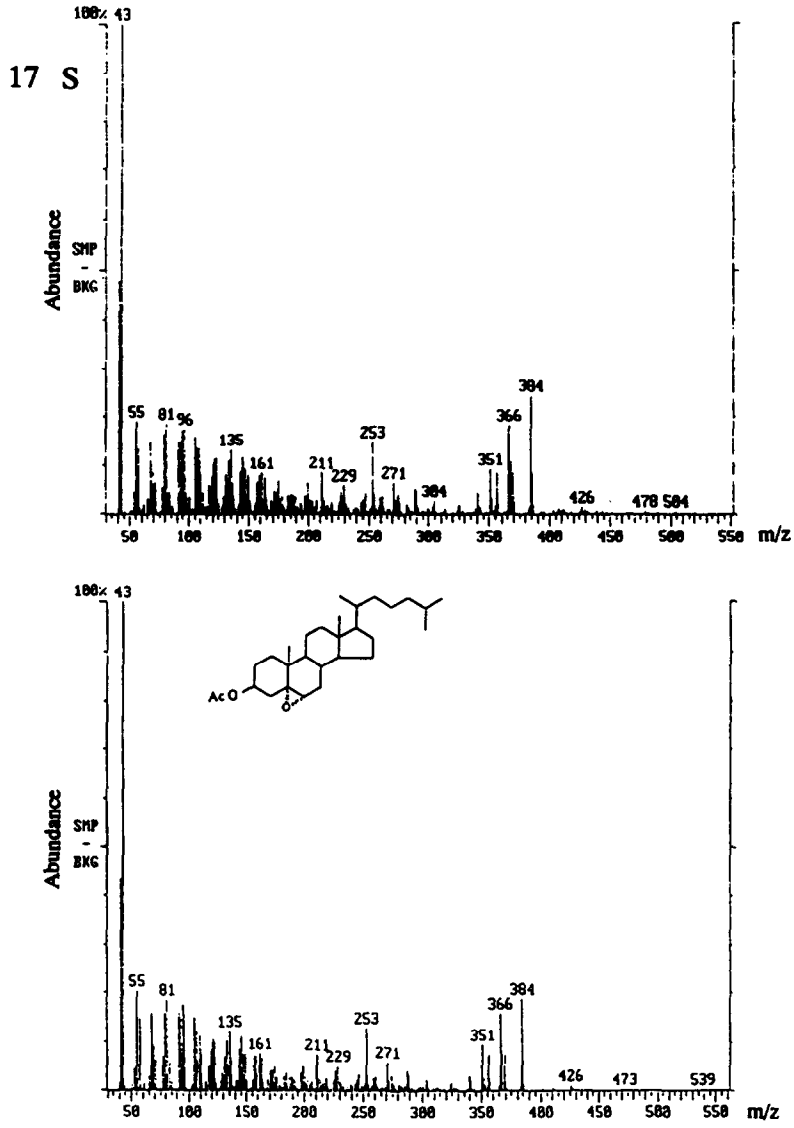

Fig. 5. Mass spectra of 5,6α-epoxy-cholesteryl-3β-acetate (17, S) (top) and corresponding standard (bottom). Fragment at m/z 384 correspond to the loss of acetic acid (60 u) from the molecular ion. Mass spectrum of GC background was substracted (sample – background, SMP – BKG) from mass spectra.

as the mass spectra against those of the standards and of synthesized substances. The identification of some components are tentative, on the basis of GC retention times and mass spectrum interpretation.

The mass spectrum of component 11 (M) (Fig. 2, top) is similar to that of cholesteryl acetate except that it has 2 atomic mass units (u) less, suggesting an additional unsaturation besides the one in position 5,6. The same fragments can also be seen in component 3 (C) (Fig. 2, bottom); the retention time and the quantitative difference in their ratios at m/z 43 and 41 (probably linked to the presence or absence of the acetyl group) suggest for component 3 (C) the structure of a steroid hydrocarbon with three unsaturations (in

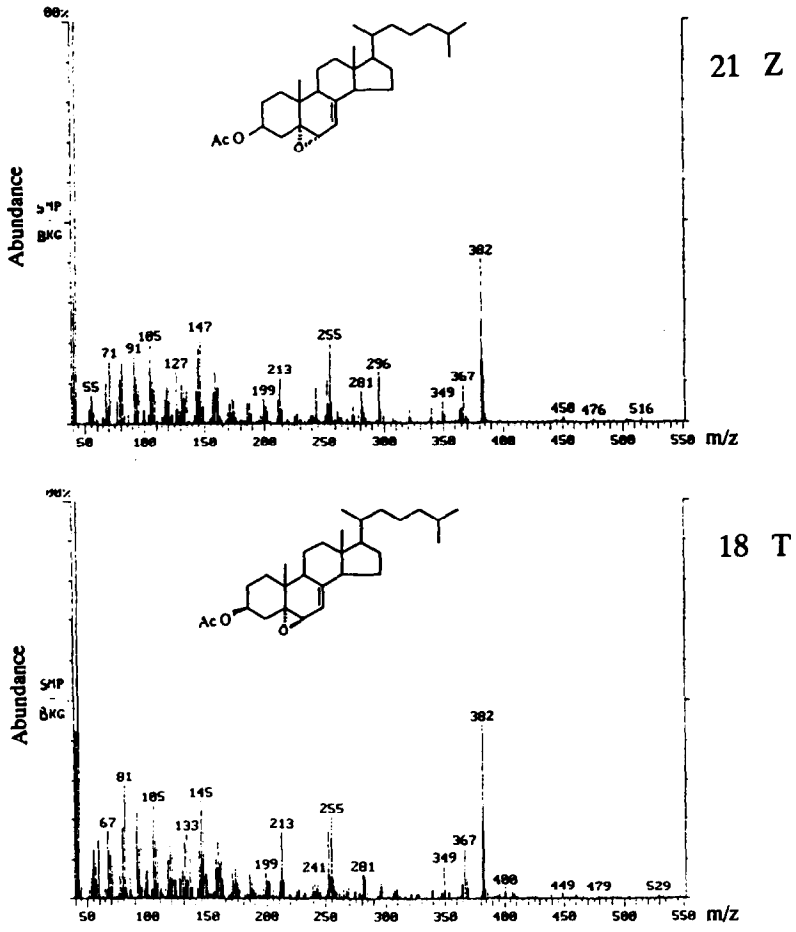

Fig. 6. Mass spectra of 5,6β-epoxy-7-cholestenyl-3β-acetate (18, T) (tentative) (bottom) and 5,6α-epoxy-7-cholestenyl 3β-acetate (21, Z) (tentative) (top). Mass spectrum of GC background was substracted (sample − background, SMP − BKG) from mass spectra.

3,4, in 5,6 and in 7,8), which are likely derived from the CA hydroxide compounds via the loss of H_2O and acetic acid. Component 11 (M) could thus be considered as an intermediate of component 3 (C) since it shows, via its mass spectrum, a structure with a molecular weight of 426, which confirms the presence of the acetyl group (characteristic loss of 60 u). The same holds true for component 8 (G) whose mass spectrum (Fig. 3, top) is very similar to that of 7-KA (Fig. 3, bottom; peaks 22 and X), and which is identical to that of its corresponding standard. The mass spectra of 8 (G) and 22 (X) are quite similar except for the smaller fragment at m/z 43. This indicates the absence of the acetyl group, a fact confirmed by the shorter retention time.

Figs. 4 and 5 show the mass spectra of the two 5,6-epoxyacetate isomers: β-epox (Fig. 4, top; peaks 14 and P) and α-epox (Fig. 5, bottom; peaks 17 and S); their retention times and mass spectra are identical to those of the corresponding standards. Fig. 6 shows the mass spectra of components 18 (T) and 21 (Z) from Fig. 1. The fact that the distribution of fragments with high values of m/z is the same as that of the two identified epoxides (Fig. 4) with 2 u less, suggests that these are epoxy derivatives (position 5,6) which have another, easily eliminated group. These two substances are probably gener-

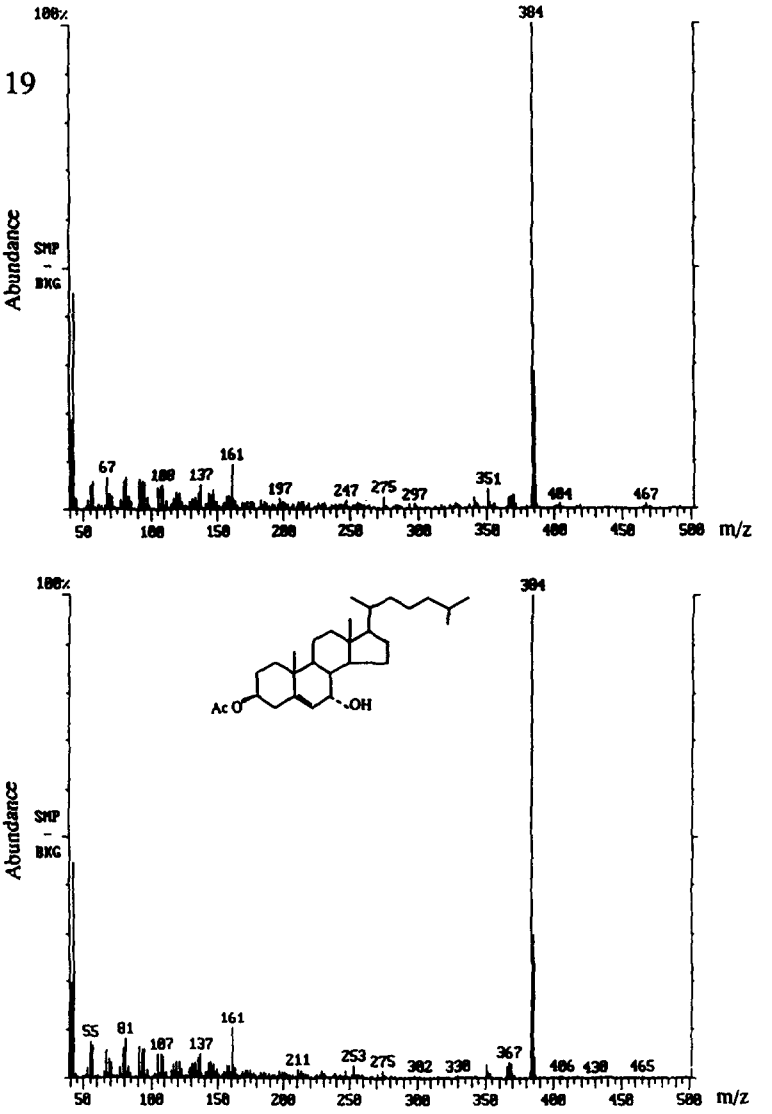

Fig. 7. Mass spectra of 7α-hydroxycholesteryl-3β-acetate (19; H, as TMS) (top) and corresponding standard (bottom). Fragment at m/z 384 correspond to the loss of acetic acid (60 u) from the molecular ion. Mass spectrum of GC background was substracted (sample – background, SMP – BKG) from mass spectra.

ated from the 5,6-epoxy-7-hydroxy-cholesteryl 3β-acetates, which were found in other research [12], by dehydration in 7,8 position of 7β-OHA-epoxide and 7α-OHA-epoxide, respectively.

The mass spectra of components 19 (H) and 20 (Q) are shown in Figs. 7 and 8. They exhibit the same characteristics (retention time and mass spectra) as 7α-OHA (peak 19), 7β-OHA (peak 20), [7α-OTMSA (H) and 7β-OTMSA (Q)].

The component 3 (C) is found in small amounts after silanization of peroxidation mixture (Fig. 1, bottom), since it is probably produced by the direct degradation of hydroxides which are, in this case, mostly stable in the form

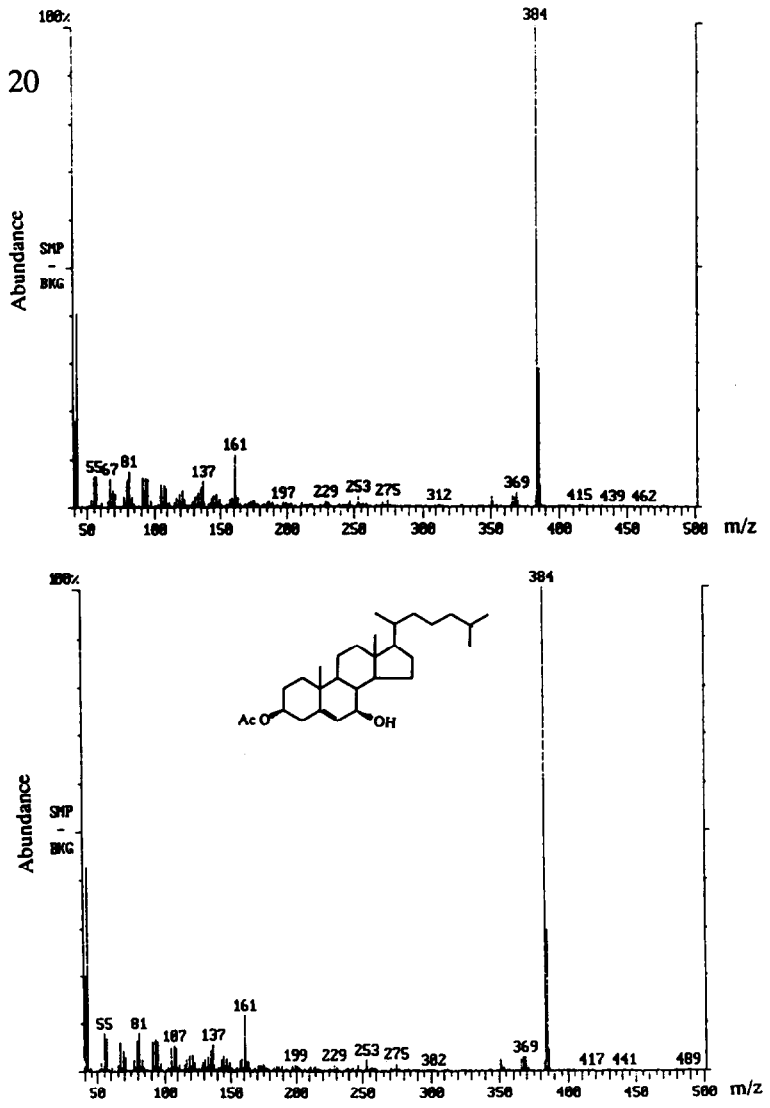

Fig. 8. Mass spectra of 7β-hydroxycholesteryl-3β-acetate (20; Q, as TMS) (top) and corresponding standard (bottom). Fragment at *m/z* 384 correspond to the loss of acetic acid (60 u) from the molecular ion. Mass spectrum of GC background was substracted (sample – background, SMP – BKG) from mass spectra.

of TMS derivatives. Therefore, previous identifications, as well as other data, could be confirmed from the resulting products of silanization.

Fig. 9 shows the identified products and their structures. Note the main components of the CA oxidation products, except for 7β-OOHA and 7α-OOHA, are 7-KA, 7β-OHA, 7α-OHA, 5,6β-epoxA and 5,6α-epoxA. Although SPE fractionation distinguishes well between polar and non-polar products, it cannot be ruled out that CA (peak 9) and cholesterol (peak 6) derive from the starting substrate; the former as residue and the latter as polar impurity. When CA is peroxidized, like methyl oleate, isolable hydro-

Table 1
Main thermo-oxidation products of cholesteryl acetate

GC peak (Fig. 1)	Name	Identification
1	Unknown	
2 (A)	Unknown	
3 (C)	3,5,7-Cholestatriene	Tentative
4 (D)	Unknown	
5 (E)	Unknown	
6	5-Cholesten-3β-ol (cholesterol)	t_R, MS
8 (G)	3,5-Cholestadien-7-one	t_R, MS
9 (I)	5-Cholesten-3β-acetate (CA)	t_R, MS
11 (M)	5,7-Cholestadien-3β-acetate	Tentative
14 (P)	5,6β-Epoxycholestan-3β-acetate	t_R, MS
17 (S)	5,6α-Epoxycholestan-3β-acetate	t_R, MS
18 (T)	5,6β-Epoxy-7-cholesten-3β-acetate	Tentative
19	5-Cholesten-7α-ol-3β-acetate	t_R, MS
20	5-Cholesten-7β-ol-3β-acetate	t_R, MS
21 (Z)	5,6α-Epoxy-7-cholesten-3β-acetate	Tentative
22 (X)	5-Cholesten-7-oxo-3β-acetate	t_R, MS
F	3β-Trimethylsilyloxy-5-cholestene	t_R, MS
H	7α-Trimethylsilyloxy-5-cholesten-3β-acetate	t_R, MS
L	Unknown	
Q	7β-Trimethylsilyloxy-5-cholesten-3β-acetate	t_R, MS

Identification carried out by mass spectrum interpretation (tentative) or by retention time (t_R) and mass spectrum (MS) comparison with those of standard availables or synthesized.

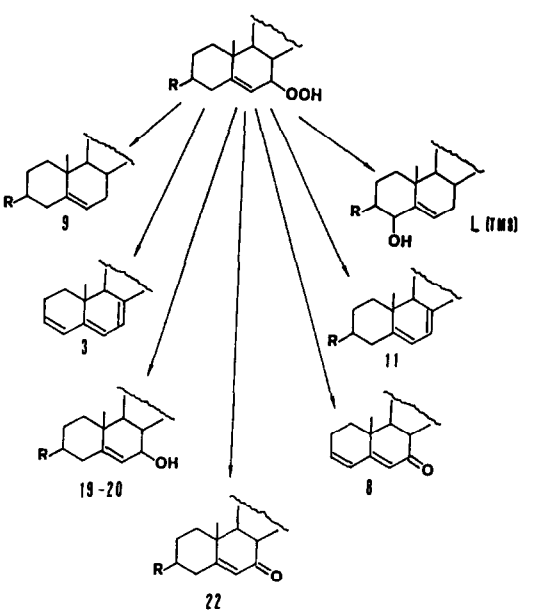

Fig. 9. Structures of the identified products

peroxides are formed [13]; these, in turn, give rise both directly or not to all the identified products [12].

Acknowledgements

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