Grasas y Aceites
Vol. 50. Fasc. 3 (1999), 193-198

Formation of the 5,6-epoxy derivatives of 7-hydroxy-cholesteryl 3 β-acetates during peroxidation of cholesteryl acetate

By **G. Lercker** [*1], **R. Bortolomeazzi** [2] and **L. Pizzale** [2]

[1] Institute of Agrarian Industries, University of Bologna, via S. Giacomo 7, 40126 Bologna, Italy.
[2] Department of Food Science, University of Udine, via Marangoni 97, 33100 Udine, Italy.

RESUMEN

Formación de derivados 5,6-epoxi de los 7-hidroxi-colesteril 3β-acetatos durante la peroxidación del colesteril acetato.

La peroxidación térmica del colesteril acetato (CA) produce varios compuestos, la mayoría de los cuales fueron ya identificados en estudios precedentes. Los trimetilsilil (TMS) derivados de los productos de la termodegradación de los hidroperóxidos simples del CA (7α- y 7β-) producen espectros GC-MS que son siempre idénticos a aquellos de la peroxidación de CA, a excepción de los cuatro compuestos que son únicamente detectados como derivados TMS. Estas substancias fueron identificadas comparando los espectros de masa y los tiempos de retención GC con los cuatro isómeros de los derivados epoxi-hidroxi del CA. La presencia de una considerable cantidad de derivados epoxi-hidroxi del CA, especialmente a bajas temperaturas de degradación, proveen una explicación de la formación de otras substancias que fueron identificadas precedentemente.

PALABRAS-CLAVE: Colesteril acetato - Derivados epoxi-hidroxi-Peroxidación térmica.

SUMMARY

Formation of the 5,6-epoxy derivatives of 7-hydroxy-cholesteryl 3β-acetates during peroxidation of cholesteryl acetate.

The thermal peroxidation of cholesteryl acetate (CA) generates many compounds, most of which have been identified in previous studies. The trimethylsilyl (TMS) derivatives of the thermodegradation products of the single hydroperoxides of CA (7α- and 7β-) gave GC-MS spectra that were almost identical to those of the thermal peroxidation of CA, except for four compounds that were only detected as TMS derivatives. These substances were identified by comparing their mass spectra and their GC retention time against those of the four synthesized isomers of the epoxy-hydroxy derivatives of CA. The presence of a considerable amount of epoxy-hydroxy derivates of CA, especially at low-temperature degradations, provides an explanation for the formation of other substances that have been previously identified.

KEY-WORDS: Cholesteryl acetate - Epoxy-hydroxy derivatives - Thermal peroxidation.

1. INTRODUCTION

All the oxidation products obtained in the studies with model lipid systems were generated from hydroperoxides (Frankel, 1980; 1987; Lercker, 1987). The formation mechanism of many oxidation products is not completely understood, even though many theories have been suggested in the literature. Cholesterol oxidation has become an interesting issue over the last few years due to the confirmed toxicity of some of the oxidation products (Maerker, 1987; Gallina Toschi and Caboni, 1993; Bösinger *et al.*, 1993). However, there are many analytical problems encountered in determining the oxidative degradation of cholesterol linked to the presence of the hydroxyl group in position 3. By using cholesteryl acetate (CA) instead, these complications can be mostly overcome, thereby allowing a better interpretation of the formation mechanisms.

2. EXPERIMENTAL

2.1 Materials

Cholest-5-en 3β-acetate (cholesteryl acetate, CA) (> 99%), 7α-hydroxy-cholest-5-en 3β-ol (7α-OHC) and 7β-hydroxy-cholest-5-en 3β-ol (7β-OHC) were supplied by Steraloids (Wilton, NH, USA). The acetyl derivatives of standard with hydroxy group in position 3 were synthesized as described in the methodology. 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.

2.2 CA thermal peroxidation

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

2.3 Cholesteryl acetate hydroperoxide (CAHP) isolation

After CAHPs preparation by CA thermal peroxidation, SPE fractionation was carried out to recover the more polar products. CAHPs were then collected from this polar fraction by a subsequent HPLC purification.

The peroxidized cholesteryl acetate (about 100 mg) was dissolved in 2 ml of n-hexane and loaded onto an 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 fractionated on a TLC silica plate developed with n-hexane-diethyl ether (1:1, v/v). Two hydroperoxides were formed under these conditions, both in position 7 and with α and β configuration. The TLC band corresponding to the CAHPs, visualized by spraying with 0.2% 2,7-dichlorofluorescein (sodium salt)-ethanol solution and detected by UV light (254 nm), was scraped off and the single CAHP was isolated by HPLC.

The two purified hydroperoxides were then subjected to the following analysis: HPLC of the CAHPs, GC of the corresponding hydroxy derivatives (CAHs) and GC-ITDMS of the trimethylsilyl derivatives (TMS) of the CAHs.

2.4 CAHP reduction

Each CAHP was reduced in methanol with $NaBH_4$ to the corresponding cholesteryl acetate hydroxide (CAH) (Bortolomeazzi *et al.,* 1994b).

2.5 Trimethylsilyl ether (TMS) preparation

Samples were silylated with about 0.1 ml of pyridine:hexamethyldisilazane :trimethylchlorosilane mixture in a 5:2:1 (v/v) ratio (Sweeley *et al.,* 1963), for 30 min at room temperature in a desiccator. After drying in a hot water bath (about 70 °C) by evaporation under nitrogen flow, samples were redissolved in 30-50 μL of benzene.

2.6 High-performance liquid chromatography (HPLC)

The liquid chromatograph was a Knauer 64 pump (Knauer, Berlin, Germany) equipped with a Rheodyne 7125 injector (Cotati, CA, USA), an ACS 750/14 light-scattering detector (Applied Chromatographic System, Macclesfield, UK) and a 3 μm Spherisorb CN column (15 cm x 4.6 mm i.d) (Phase Sep, Deeside, UK). Preparative and analytical HPLC separations were performed under isocratic conditions at 1 mL/min flow rate, using 0.5% anhydrous ethanol in n-hexane as the mobile phase and a splitter device between HPLC column and detector.

2.7 Gas chromatography-ion trap detector mass spectrometry (GC-ITDMS)

The capillary gas chromatograph (GC) was a Varian 3400 with on-line coupling to a Varian Saturn ion-trap detector (ITDMS). The column was a DB-5 fused silica type (5% phenylmethyl) (J&W, Folsom, CA, USA), with 30 m x 0.255 mm i.d. and 0.25 μm film thickness. Oven temperature was programmed from 220 to 300 °C, with a rate of 5 °C/min. Injection was in the split mode (1:50, v/v, ratio) at 1 ml/min flow rate with helium as the carrier gas; the temperatures of the injector, the transfer line and the manifold were 300, 300 and 220 °C, respectively. The filament emission current was 10 mA and an electron beam of 70 eV was used for electron impact ionization (EI). Hydroperoxides were injected after hydroperoxy group reduction and after TMS derivatization.

2.8 Synthesis of the 5,6α- and 5,6β-epoxy-7α-hydroxy-cholest-5-en 3β-acetate (5,6α- and 5,6β-epox-7α-OH CA) and of the 5,6α- and 5,6β-epoxy-7β-hydroxy-cholest-5-en 3β-acetate (5,6α- and 5,6β-epox-7β-OH CA)

The epoxy-hydroxy derivatives of the CA were prepared by epoxidation of about 1 mg of 7α-OH CA and 7β-OH CA with m-chloroperoxybenzoic acid in chloroform as suggested in the method of Strocchi and Bonaga (1975) for epoxidation of unsaturated fatty acids. Two products were obtained from each starting 7-OH CA. The interpretation of the mass spectra of these compounds and of their TMS derivatives led to the conclusion that these products had an epoxy-hydroxy structure; as expected, two epoxy derivatives 5,6α- and 5,6β- were formed from the 7α-OH CA and from the 7β-OH CA. The correct assignation of the α or β configuration to the oxyrane ring of the two couples of compounds was based only on chromatographic evaluations. Since the GC retention time of 5,6β-epox CA was lower than that of the 5,6α-isomer, we inferred that also the retention time of 5,6β-epox-7α-OH CA was lower than that of the corresponding 5,6α-isomer. The same considerations were applied to the other couple of 5,6-epox-7β-OH CA.

2.9 Thermal degradation of CAHPs. Degradation in the GC injector

Thermal degradation of the individual hydroperoxides was performed by injection into a piece o glass chromatographic column (15 cm length x 2 mm i.d.), which was inserted in the injection port of a Varian 3700 gas chromatograph. About 7 cm of the column protruded into the oven, which was kept at room temperature, and the last 3 cm were packed with silica gel for trapping. The carrier gas (nitrogen) flow was 20 mL min-1. Four different conditions were tested: two solvents of different polarity (n-hexane and 0.5% methanol) to dissolve each hydroperoxide and injection at two temperatures (308 °C and 200 °C). Injections of 100 μL were made for each test in order to obtain a sufficient quantity of degradation products.

The degradation products were eluted from the column by washing with chloroform-methanol (1:1, v/v; 2 mL) and methanol (1 mL). The solution was then evaporated to dryness under nitrogen flow: 5α-cholestane (internal standard) was added and the extract was redissolved in benzene (40 mL) prior to the GC-ITDMS analysis. The degradation products were also analysed as TMS derivatives.

3. RESULT AND DISCUSSION

The thermal treatment of the hydroperoxides generated a series of compounds almost all of whose structures have been identified (Smith, 1981a; 1996; Bortolomeazzi *et al.*, 1994a; 1994b; Lercker *et al.*, 1996); however, some of these products were present in such small amounts that their mass spectra were difficult to interpret. After silanization of the degradation mixture of the single hydroperoxide, a group of peaks in the GC trace was observed (Figure 1; trace A, peaks 8, 10, 11 and 12) that was not present before the silanization treatment (Figure 1, trace B). The 3β-acetyl derivatives of the 7α- and 7β- hydroxy derivatives of cholesterol were then synthesized and subsequently utilized for the preparation of their corresponding 5,6-epoxy derivatives (Strocchi and Bonaga, 1976). Synthesis of the epoxy derivatives from the single 7α- and 7 β-hydroxy-cholesteryl 3 β-acetates (7α-OH CA and 7 β-OH CA) (Fig. 2), by means of *m*-chloroperoxy-benzoic acid, produces 2 epoxy isomers for each 7-OH CA. It would be useful to distinguish the 5,6α-epoxy isomer from the 5,6 β-epoxy one of each 7-OH CA by relative gas chromatographic retention times, because the only molecular difference is the configuration given by the presence of the epoxy group. The 5,6α-epoxy group shows in CGC a greater retention time than that of the 5,6β-epoxy (Bortolomeazzi, 1994a). These considerations are sufficient to assign the configuration of the two pair of isomers obtained from each 7-OH CAs.

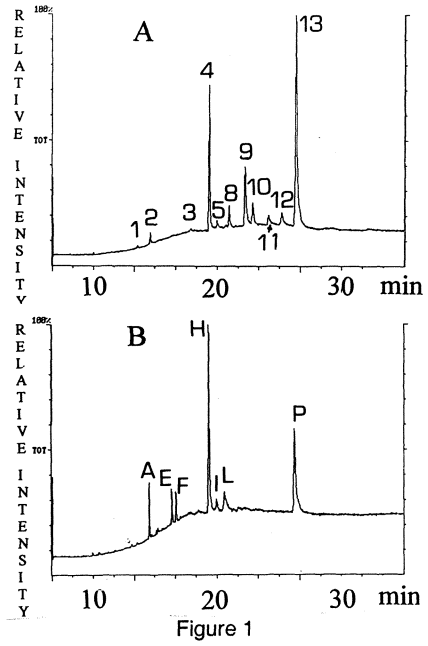

Figure 1

GC-ITDMS trace of TMS derivatives of thermal degradation products of 7α-hydroperoxy-cholesteryl 3β-acetate **(A)** and GC-ITDMS trace of thermal degradation products of 7β-hydroperoxy-cholesteryl 3β-acetate **(B)**. Identification of the main peaks (Bortolomeazzi *et al.*, 1994a; 1994b; Lercker *et. al.*, 1996): *1*, unknown; *2 (A)*, 3,5.7-cholestatriene; *3*, unknown; *4 (H)*, 5-cholesten-7α-trimethylsilyloxy 3β-acetate (7α-OTMS CA); *5 (I)*, 5-cholestenyl 3β-acetate; *(L)*, mixture of 5-cholestenyl 3β-acetate with unknown compound; *8*, cholestan-5,6β-epoxy-7α-trimethylsilyloxy 3β-acetate (5,6β-epox-7α-OTMS CA); *9*, 5-cholesten-7β-trimethylsilyloxy 3β-acetate (7β-OTMS CA); *10*, cholestan-5,6α-epoxy-7α-trimethylsilyloxy 3β-acetate (5,6α-epox-7α-OTMS CA); *11*, cholestan-5,6β-epoxy-7β-trimethylsilyloxy 3β-acetate (5,6β-epox-7β-OTMS CA); *12*, cholestan-5,6α-epoxy-7β-trimethylsilyloxy 3β-acetate (5,6α-epox-7β-OTMS CA); *13 (P)*, 5-cholesten-7-one 3β-acetate.

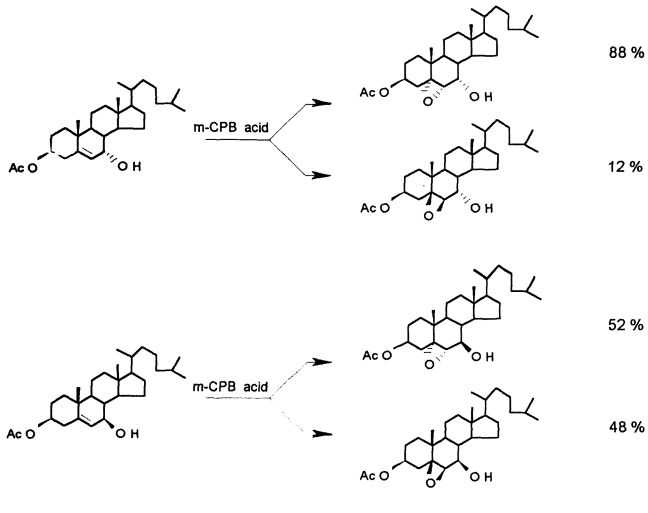

Figure 2

Synthesis of epoxy derivatives of 7-hydroxy cholesteryl 3β-acetates (α and β) (see experimental, 2.8)

Other evidences of these epoxy configurations are described as follows:

— The epoxy synthesis from cholesterol, by means of *m*-chloroperoxybenzoic acid (Smith, 1981b), generates a greater amount of 5,6α-epoxy isomer, which is the same behaviour observed during the epoxy synthesis from 7-OH CAs (Fig. 2);
— The mass spectra of the four epoxy-hydroxy CA isomers show different fragmentations (Fig. 3-6). The 5,6α-epoxy isomers exhibit a greater intensity of the m/z ratio corresponding to H_2O loss (- 18 amu) than that of the CH_3 loss (- 15 amu), which can derive from the molecular ion and the fragment at m/z 382 (Table I); the CH_3 loss is more relevant in the 5,6 β-epoxy isomers, instead.

Figures 3, 4, 5 and 6 show the mass spectra of the unknown peaks from Figure 1, each of them next to the mass spectrum of the corresponding synthesized compound. Although the mass spectra are quite similar but not identical because of variations in a series of contributing factors (purity of the GC peak, concentration, temperature, etc.), the GC response together with the spectra allows a precise identification of the products. The presence of the epoxy-hydroxy compounds in the thermal degradation mixture of the CA was not surprising because these types of substance have been already found in methyl oleate (Lercker *et al.,* 1978; Lercker *et. al.,* 1984a; 1984b; Capella *et al.,* 1988). Nevertheless, this is an important fact with regard to cholesterol and cholesteryl acetate, since these compounds have never been reported among the oxidation products of cholesterol.

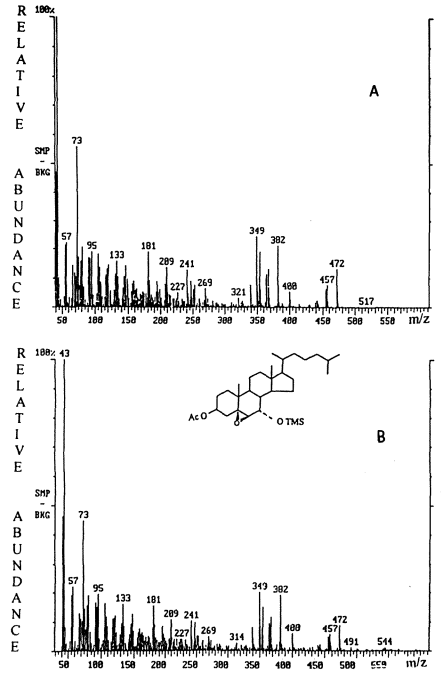

Figure 3
Mass spectra of the components that correspond to peak *8* (Figure 1) and of the TMS derivative of the 7α-hydroxy-5,6β-epoxy-cholesteryl 3β-acetate

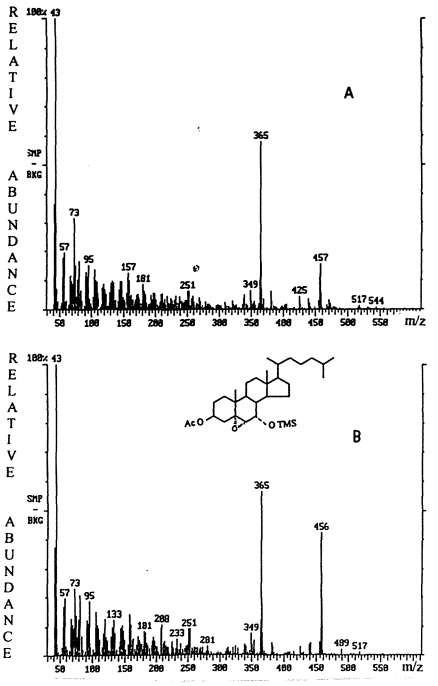

Figure 4
Mass spectra of the components that correspond to peak *10* (Figure 1) and of the TMS derivative of the 7α-hydroxy-5,6α-epoxy-cholesteryl 3β-acetate

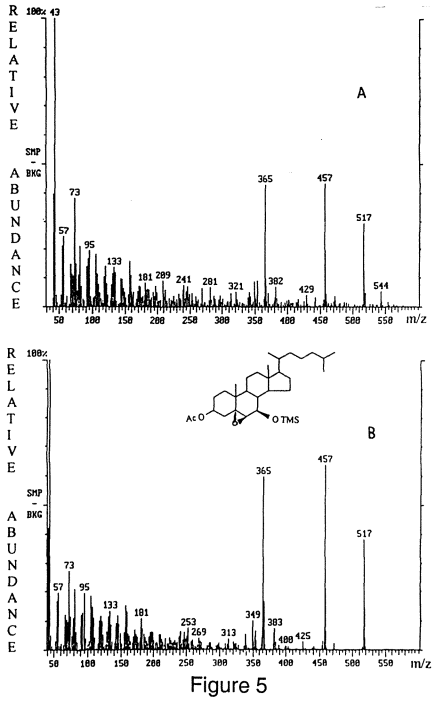

Figure 5
Mass spectra of the components that correspond to peak *11* (Figure 1) and of the TMS derivative of the 7β-hydroxy-5,6β-epoxy-cholesteryl 3β-acetate

The identification of the epoxy-hydroxy compounds and that of the other oxysterols could be helpful in explaining the presence of some oxysterols that are generated by dehydration or loss of acetic acid from these epoxy-hydroxy 3 β-acetoxy compounds (Figure 7).

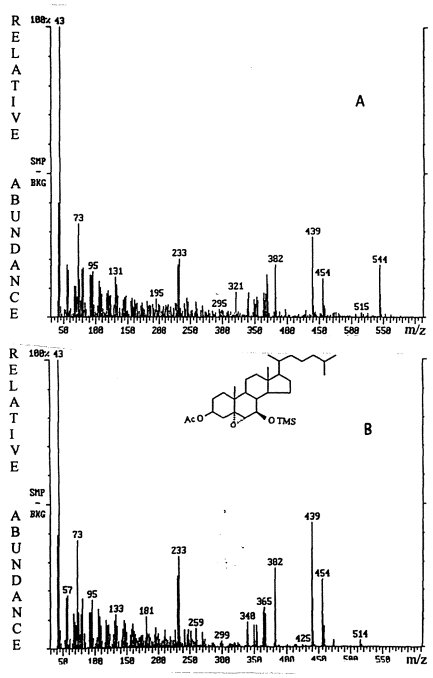

Figure 6
Mass spectra of the components that correspond to peak *12* (Figure 1) and of the TMS derivative of the 7β-hydroxy-5,6α-epoxy-cholesteryl 3β-acetate

Table I
Main significative fragments of mass spectra obtained from epoxy-hydroxy cholesteryl 3β-acetate isomers (as TMS derivatives)

m/z	Neutral fragment loss	amu
517	– CH_3	15
472	– CH_3COOH	60
457	– CH_3 - CH_3COOH	15 + 60
454	– CH_3COOH - H_2O	60 + 18
439	– CH_3COOH - H_2O - CH_3	60 + 18 + 15
425	– H_2O - TMS O	18 + 89
383	– CH_3COOH - TMS O	60 + 89
382	– CH_3COOH - TMS OH	60 + 90
365	– CH_3COOH - H_2O - TMS O	60 + 18 + 89
349	– CH_3COOH - H_2O - CH_3 - TMS OH	60 + 18 + 15 + 90
340	– CH_3COOH - H_2O - CH_3 - 99[a]	

[a] Fragment m/z = 99 corresponds to the ion CH_3 CO O = CHCH = $CH_2]^+$

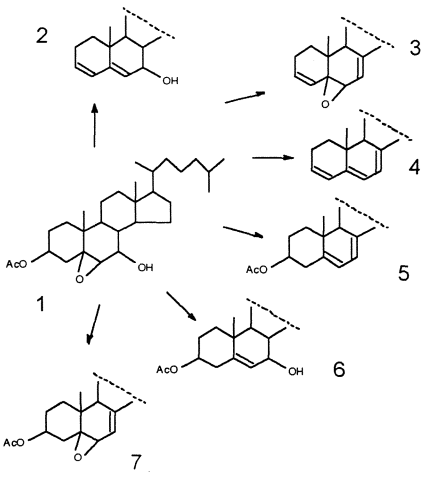

Figure 7
Formation scheme of some cholesterol oxides from the epoxy-hydroxy-cholestan 3β-acetate derivatives: **1)** 5,6-epoxy-7-hydroxy-cholestan 3β-acetate (four isomers); **2)** 5,6-epoxy-cholest-3-en (two isomers); **3)** 5,6-epoxy-cholest-3,7-dien (two isomers); **4)** 3,5,7-cholestatriene; **5)** cholest-5,7-dien 3β-acetate; **6)** 7-hydroxy-cholest-3-en 3β-acetate (two isomers); **7)** 5,6-epoxy cholest-7-en 3β-acetate (two isomers)

ACKNOWLEDGEMENTS

This study was supported by the National Research Council of Italy.

BIBLIOGRAPHY

Bortolomeazzi, R., Pizzale, L., Conte, L.S. and Lercker, G. (1994a).—«Identification of thermal oxidation products of cholesteryl acetate».—J. Chromatogr. **683**, 75-85.

Bortolomeazzi, R., Pizzale, L., Vichi, S. and Lercker, G. (1994b).—«Analysis of isomers of hydroperoxides of cholesteryl acetate».— Chromatographia **39**, 577-580.

Bösinger, S., Luf, W. and Brandl, E. (1993).—«Oxysterols: their occurrence and biological effect».—Int. Dairy Journal **3**, 1-33.

Capella, P., Caboni, M.F., Bonaga, G. and Lercker, G. (1988).—«Meccanismi di formazione dei prodotti di evoluzione degli idroperossidi monoinsaturi: idrossi- e cheto-esteri».—Riv. Ital. Sostanze Grasse **65**, 629-631.

Frankel, E.N. (1980).—«Lipid oxidation».—Progr. Lipid Res. **19**, 1-22.

Frankel, E.N. (1987).—«Secondary products of lipid oxidation».—Chem. Phys. Lipids **44**, 73-85.

Gallina Toschi, T. and Caboni, M.F. (1993).—«Cholesterol oxides: biological behaviour and analytical determination».—Ital. J. Food Sci. **4**, 223-228.

Lercker, G., Capella, P., Conte, L.S. and Pallotta, U. (1978).—«Sur certains produits de transformation thermique des hydro-peroxydes de l'oleate de methyle».—Rev. Fr. Corps Gras **25**, 227-237.

Lercker, G., Capella, P. and Conte, L.S. (1984a).—«Thermo-oxidative degradation products of methyl oleate».—Riv. Ital. Sostanze Grasse **61**, 337-344.

Lercker, G., Capella, P. and Conte, L.S. (1984b).—«Thermal decomposition of methyl oleate hydroperoxides».—Riv. Ital. Sostanze Grasse **61**, 623-627.

Lercker, G. (1987).—«Autoxidation process».—in «Fat Production and Consumption», 269-280, C. Galli and E. Fedeli (Eds.), Plenum Publ. Co., London.

Lercker, G., Bortolomeazzi, R., Pizzale, L. and Vichi, S. (1996).—«Thermal degradation of single cholesteryl acetate hydroperoxide».—Chromatographia **42**, 29-33.

Maerker, G. (1987).—«Cholesterol autoxidation: current status».—J. Am. Oil Chem. Soc. **64**, 388-392.

Smith, L.L. (1981a).—«Cholesterol Autoxidation», 359-458, Plenum Press, New York and London.

Smith, L.L. (1981b).—«Cholesterol Autoxidation», 505, Plenum Press, New York and London.

Smith, L.L. (1996).—«Review of progress in sterol oxidations: 1987-1995».—Lipids **31**, 453-487.

Strocchi, A. and Bonaga, G. (1975).—«Epoxydation reaction to determine the structure of unsaturated fatty acids».—Riv. Ital. Sostanze Grasse **52**, 84-87.

Sweeley, C.C., Bentley, R., Makita, M. and Wells, W.W. (1963).—«Gas-liquid chromatography of trimethylsilyl derivatives of sugars and related substances».—J. Am. Chem. Soc. **85**, 2497-2507.

Recibido: Abril 1998
Aceptado: Septiembre 1998