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ARTICLE

Synthesis of 7-Hydroperoxycholesterol and Its Separation, Identification, and Quantification in Cholesterol Heated Model Systems

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7-Hydroperoxycholesterol is considered to be an intermediate compound of the cholesterol oxidation path as the first product formed when cholesterol is oxidized by triplet oxygen. However, there is a limitation on cholesterol mechanism studies because of the lack of 7-hydroperoxycholesterol analytical standard due to its low stability. To verify the formation of hydroperoxides in cholesterol model systems heated at 140, 180, and 220 °C, 7α-hydroperoxycholesterol was synthesized by cholesterol photooxidation followed by rearrangement at room temperature in chloroform. Its structure was confirmed on the basis of ^{13}C NMR and mass spectra obtained by APCI-LC-MS. The synthesized compound was also used as standard for the quantification of 7-hydroperoxycholesterol as the sum of 7α- and 7β-hydroperoxycholesterol. The results demonstrated that 7-hydroperoxycholesterol is the first compound formed when the temperature is lower (140 °C). However, the concentration of the 7-hydroperoxycholesterol depends on the temperature and time of exposure: the higher the time, the higher the amount of 7-hydroperoxycholesterol at lower temperatures, and the lower the time, the lower the amount of 7-hydroperoxycholesterol at higher temperatures (180 and 220 °C). By the formation of 7-hydroperoxycholesterol, the known cholesterol oxidation mechanism in three phases (initiation, propagation, and termination) could be confirmed; once at lower temperatures, the stage of cholesterol oxidation is at initiation, at which hydroperoxide formation predominates.

KEYWORDS: APCI-LC-MS; cholesterol oxides; thermal degradation; NMR

INTRODUCTION

Cholesterol can undergo oxidation when exposed to high temperatures, light, dehydration, storage, radiation, and combinations of these conditions (*1*−*3*). The resulting cholesterol oxidation products (COP) have been reported to exhibit a wide range of adverse biological effects on animals, such as atherogenesis, cytotoxicity, mutagenesis, and carcinogenesis (*4*).

The mechanism of cholesterol oxidation is similar to that known for unsaturated lipids, which is divided in three phases: initiation, propagation, and termination (*3*). 7-Hydroperoxycholesterol is considered to be the first intermediate compound in the cholesterol oxidation path, as it is formed directly from cholesterol by oxidation promoted by triplet oxygen (*3*, *5*). Thus, the identification of this intermediate plays a key role in quantitative studies of cholesterol and COP by high-performance liquid chromatography (HPLC). However, such studies have been limited because of the lack of a 7-hydroperoxycholesterol analytical standard due to its low stability (*6*−*8*).

The most commonly used HPLC method for COP quantification employs CN column and mixture of *n*-hexane and isopropyl alcohol as mobile phase, allowing the separation of cholesterol and 11 COP (*6*, *9*, *10*); however, the quantification of 7-hydroperoxycholesterol by this method has not been previously reported. Quantification of 7-hydroperoxycholesterol has been currently based on chromophoric formation by the Würster dye method (*6*, *7*) after thin layer chromatography (TLC) or preparative HPLC separation; however, these methods present low sensitivity or accuracy.

In the past decade, liquid chromatography−mass spectrometry (LC-MS) using mostly atmospheric pressure ionization (APCI) has emerged as a powerful analytical technique for the identification and quantification of several COP (*11*−*15*). However, the use of this technique for the identification and quantification of 7-hydroperoxycholesterol has also been limited due to its low stability, so that APCI-MS data for such compounds has not been previously reported.

In this work the synthesis and characterization of 7α-hydroperoxycholesterol by ^{13}C nuclear magnetic resonance (NMR) and atmospheric pressure chemical ionization mass spectrometry (APCI-MS) are described. The synthesized compound was also used as standard for the quantification of 7-hydroperoxycholesterol as the sum of 7α- and 7β-hydroperoxycholesterol in cholesterol heated model systems by HPLC to verify the formation of this compound under high-temperature conditions.

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MATERIALS AND METHODS

Synthesis of 7α-Hydroperoxycholesterol. Cholesterol, 95% pure (Sigma-Aldrich, Steinheim, Germany), and 1% Rose Bengal (Sigma Chemical, St. Louis, MO) were mixed with 10 mL of analytical grade pyridine (Merck, Darmstadt, Germany) and submitted to photooxidation by sodium light exposure (400 W) and oxygen insufflation under continuous stirring and controlled temperature (5 °C), as described by Beckwith and co-workers (*16*).

The synthesized compound was purified by flash column chromatography (silica gel, 4:1 and 1:1 *n*-hexane/ethyl acetate v/v). Twenty fractions of 15 mL each were collected and analyzed by TLC using silica gel G/aluminum precoated plates with 250 μm (Whatman, Freiburg, Germany). The development of TLC was carried out in a glass tank lined with filter paper and equilibrated for 30 min with 5 mL of *n*-hexane/ethyl acetate (4:1 v/v). Approximately 10 μL of each extract was spotted on the plate. The chromatogram was developed over a distance of 3 cm at room temperature, after which the plate was dried and the color development of cholesterol, Rose Bengal, and the reaction product was carried out with an acid solution of anisaldehyde. Reaction product presented three bands, one at the same R_f of cholesterol, another at the same R_f of Rose Bengal, and a third one, which should be 5α-hydroperoxycholesterol. Fractions 11−17 (*n*-hexane/ethyl acetate 1:1 v/v), which presented a single band at the same R_f supposed to be 5α-hydroperoxycholesterol, were grouped and concentrated under a gentle nitrogen stream to afford pure 5α-hydroperoxycholesterol, which was dissolved in chloroform and kept at room temperature for 68 h to rearrange and form 7α-hydroperoxycholesterol. To obtain pure 7α-hydroperoxycholesterol, the solvent was evaporated under a gentle nitrogen stream. The purity of the synthesized compound was determined on the basis of peak area percentages obtained by UV (210 nm) detector in the same chromatographic conditions used to quantify COP. All solvents were of analytical grade.

Structure Confirmation by ^{13}C NMR. The synthetic 7α-hydroperoxycholesterol was solubilized in deuterated chloroform ($CDCl_3$) (Cambridge Isotope Laboratories Inc., Andover, MA) and characterized by ^{13}C nuclear magnetic resonance (NMR) in an INOVA-500 spectrometer (B_0 = 11T) (Varian, Palo Alto, CA) operating at 125.7 MHz for ^{13}C and equipped with a 5 mm probe for direct and indirect detection, selective pulse, and Sun workstation. Spectra were obtained at 24.6 °C with 45° pulses, using broad band decoupling (WALTZ sequence), spectral width of 30 kHz, acquisition time of 1.3 s, and delay time of 1.5 s. The chemical shifts were obtained in parts per million using tetramethylsiloxane (TMS) and deuterated chloroform ($CDCl_3$) as reference standards. The difference between methylic, methylenic, methynic, and not bound carbons was established by DEPT spectra.

Cholesterol and COP Quantification by HPLC-UV-RI. A liquid chromatograph (Shimadzu, Kyoto, Japan), equipped with UV (SPD-10 AVVP) and RI (RID 10A) detectors was used. The analytical column used was a 300 mm × 3.9 mm i.d., 4 μm, Nova Pack CN HP (Waters, Milford, MA); the injection loop was 20 μL, and the oven temperature was 32 °C. A mixture of hexanes (minimum 63% of *n*-hexane) and 2-propanol was used as mobile phase (97:3 v/v) at a flow rate of 1 mL/min (*9*). Cholesterol, 7α- and 7β-hydroxycholesterol, 7-ketocholesterol, and 7-hydroperoxycholesterol (sum of 7α- and 7β-hydroperoxycholesterol) were quantified using the UV detector at 210 nm. α- and β-epoxycholesterols were quantified using an RI detector at 32 °C. Identification of cholesterol and COP was made by comparison of the retention times of peaks in samples with those of reference standards and spiking. The compound identities were further confirmed by LC-APCI-MS.

7-Hydroperoxycholesterol was quantified as the sum of 7α- and 7β-hydroperoxycholesterols using a calibration curve plotted with six points with a concentration range from 0.7 to 182 μg/mL of the synthesized 7α-hydroperoxycholesterol. Cholesterol, 7α- and 7β-hydroxycholesterols, 7-ketocholesterol, and α- and β-epoxycholesterols were quantified by external calibration, with curves ranging from 0.2 to 6 mg/mL for cholesterol and from 0.5 to 100 μg/mL for each COP.

Confirmation of Compound Identities by LC-APCI-MS. A liquid chromatograph (Shimadzu) equipped with quaternary pumps (LC-20AD) and a degasser unit (DGU-20A5) connected in series to a photodiode array detector (PDA) (SPD-M20A) and to an Esquire 4000 mass spectrometer (Bruker Daltonics, Bremen, Germany), fitted with an atmospheric pressure chemical ionization source (APCI) and an ion-trap analyzer was used. The HPLC conditions were the same as described above. The MS parameters were set as follows: positive ion mode of analysis; source temperature, 400 °C; corona, 4000 nA; dry gas (N_2) 300 °C, 5 L/min flow, and 65 psi nebulizer gas; scan range from *m*/*z* 80 to 450. The MS spectrum of each peak was compared with the MS spectrum of the COP standards at the corresponding retention time. The MS/MS spectrum of the synthesized 7α-hydroperoxycholesterol was obtained using the ion *m*/*z* 401 as precursor. The following conditions were used: Collision-induced dissociation was produced with helium 99.999% (White Martins, RJ, Brazil) at a pressure of 30 psi in the ion trap. Capillary voltage was 3500 V; end-plate offset, 500 V; skimmer (I), 10.0 V; and skimmer (II), 6.0 V. The octopole was at 2.5 V, octopole D 2.5, and octopole RF 100.0 (Vpp). The acquisition MS/MS fragmentation amplitude was 1.4 V.

Model Systems. To verify COP formation during heating, pure cholesterol 99% (Sigma-Aldrich, St. Louis, MO) was submitted to a heat process in a heating block (Marconi, Piracicaba, Brazil) under constant oxygen 99.9999% (White Martins) flow (ca. 10 mL/min). The tubes containing 1 mL of a 1 mg/mL cholesterol solution had the solvent (isopropyl alcohol) evaporated under a gentle nitrogen stream and then were placed into the holes of the heating block set at 140, 180, or 220 °C. The tubes were heated until cholesterol concentration reached at least 25% of its initial content. To determine cholesterol, 7-hydroperoxycholesterol, and COP concentrations during the heating process, 12 tubes were sampled at different times. After the heating time, the tubes were immediately chilled to stop any reactions, and their contents were diluted with 1 mL of mobile phase and injected into the HPLC to quantify the remaining cholesterol and COP. The initial amount of cholesterol was measured by the quantification of cholesterol in the test tube without heating.

RESULTS AND DISCUSSION

Synthesis and Identification of 7-Hydroperoxycholesterol. The synthesis produced 7α-hydroperoxycholesterol (39% yield) with 68% of purity. To increase the purity, recrystallization was carried out as described by Beckwith and co-workers (*16*); however, such procedure led to the decomposition of 7α-hydroperoxycholesterol into 7-ketocholesterol and 7α-hydroxycholesterol, which were already identified as part of the impurity of the synthesized 7α-hydroperoxycholesterol. On the other hand, Geiger and co-workers (*17*) reported the formation of 7α- and 7β-hydroperoxycholesterols as the most abundant peroxide species in photooxidized cholesterol liposomes. In the present work, the epimerization of 7α-hydroperoxycholesterol into 7β-hydroperoxycholesterol was not observed.

The identification of 7α-hydroperoxycholesterol was made by comparison of ^{13}C NMR data (**Figure 1**) with previously published data for cholesterol (*18*) and other very closely related structures (*19*). The main difference between data of 7α-hydroperoxycholesterol and 7α-hydroxycholesterol is in the chemical shift of C-7, which is 12.1 ppm deshielded in comparison with the corresponding carbon of 7α-hydroperoxycholesterol. This chemical shift is very similar to that reported for 7-hydroperoxystigmasterol (77.9 ppm) (*19*).

The presence of the hydroperoxy group was easily confirmed by APCI-MS through the loss of 34 mass units directly from the protonated 7α-hydroperoxycholesterol (*m*/*z* 419), which is characteristic for peroxide groups (*20*) (**Figure 2**). The formation of the product ion (*m*/*z* 383) can be considered useful to distinguish 7-hydroperoxycholesterol from other COP, the mass spectra of which are very similar due to water elimination resulting in isobaric ions (*9*).

The peak relative to 7α-hydroperoxycholesterol in the model system samples was identified on the basis of comparison of its retention time and mass spectrum with those of the synthesized 7α-hydroperoxycholesterol standard. The epimeric 7β-hydroperoxycholesterol was identified following the elution order of the epimeric 7α-hydroxycholesterol (*9*), as shown in **Figure 3**.

Cholesterol Heating and Formation of Hydroperoxycholesterol. The initial amounts of cholesterol were 1.5, 1.4, and 1.6 mg/mL at

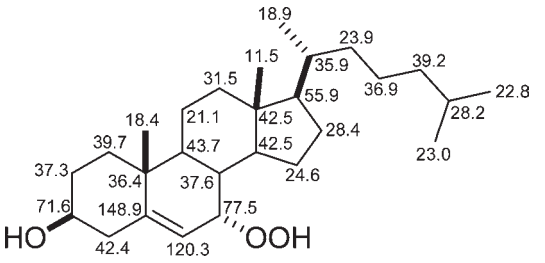

Figure 1. ^{13}C NMR chemical shifts (ppm) of 7α-hydroperoxycholesterol.

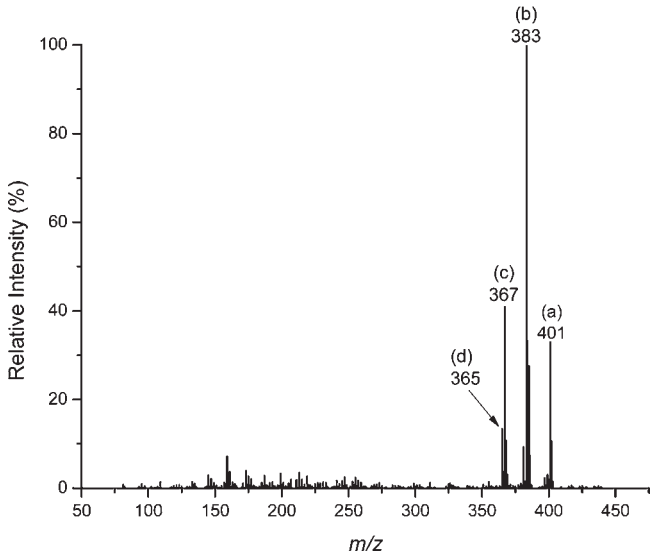

Figure 2. MS spectrum of 7α-hydroperoxycholesterol. Peaks: (a) $[M - H_2O]^+$; (b) $[M - 2H_2O]^+$; (c) $[M - H_2O - H_2O_2]^+$; (d) $[M - 3H_2O]^+$.

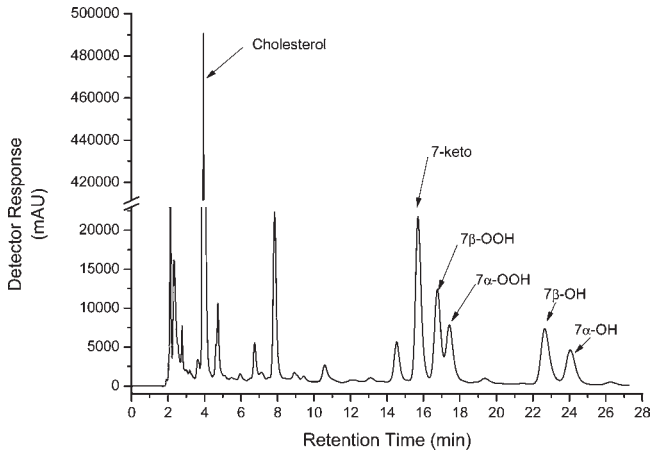

Figure 3. Chromatogram of cholesterol and COP from model system containing pure cholesterol submitted to 220 °C for 45 s obtained by HPLC (UV at 210 nm). Retention times: cholesterol, 3.5 min; 7-ketocholesterol, 14.4 min; 7β-hydroperoxycholesterol, 15.5 min; 7α-hydroperoxycholesterol, 16.1 min; 7β-hydroxycholesterol, 21.3 min; 7α-hydroxycholesterol, 22.7 min.

140, 180, and 220 °C, respectively, which were at least 75% degraded at the end of the heating process.

During heating, the amount of 7-hydroperoxycholesterol (sum of 7α- and 7β-hydroperoxycholesterols) reached a maximum concentration, after which time degradation occurred (**Figure 4**). The time to reach the maximum concentration of 7-hydroperoxycholesterol varied at each temperature as well as the amount of

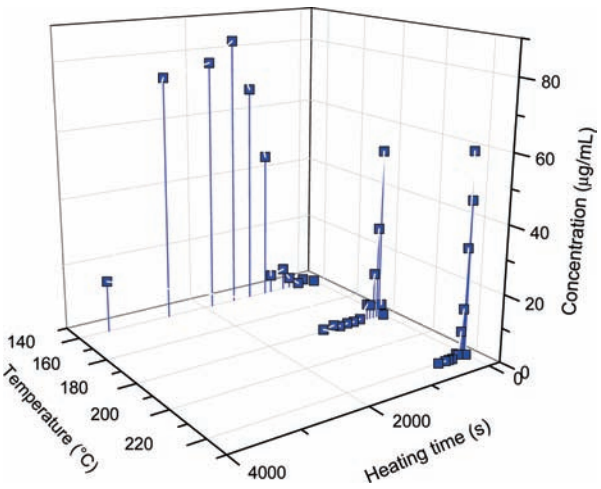

Figure 4. Concentration of 7-hydroperoxycholesterol during heating at 140, 180, and 220 °C.

Table 1. Cholesterol Degradation and Formation of 7-Hydroperoxycholesterol and COP at the Heating Time at Which Maximum 7-Hydroperoxycholesterol Formation Occurred

	140 °C	180 °C	220 °C
cholesterol degradation (%)	42	21	31
7-hydroperoxycholesterol (μg/mL)	83	52	59
time[a] (s)	1494	87	45
total COP (μg/mL)	79	92	110

[a] Time at which 7-hydroperoxycholesterol presented the maximum formation.

7-hydroperoxycholesterol formed, possibly because of differences in the cholesterol oxidation rates (*21*).

A direct relationship was verified between 7-hydroperoxycholesterol formation and cholesterol degradation, where the lowest temperature (140 °C) presented higher values than the other temperatures (**Table 1**). At 180 °C smaller amounts of 7-hydroperoxycholesterol and cholesterol degradation percentages were found, whereas at higher temperatures the amount of 7-hydroperoxycholesterol increased again, as well as the cholesterol degradation, suggesting that cholesterol degradation can be correlated to 7-hydroperoxycholesterol formation, independently of temperature. At 140 °C the formation of 7-hydroperoxycholesterol was slower than at the higher temperatures, and probably its degradation was slower, too, explaining the higher concentration of 7-hydroperoxycholesterol when compared to the higher temperatures.

The maximum amounts of 7-hydroperoxycholesterol detected represented 5, 5, and 10% of the remaining cholesterol and 6, 4, and 4% of the initial content cholesterol at 140, 180, and 220 °C, respectively. Nawar and co-workers (*12*) reported the amount of COP as representing 22% of initial cholesterol; however, the authors heated the cholesterol at 180 °C for 1 h.

Chien and co-workers (*6*) used much higher quantities of cholesterol (100 mg) in a heated model system and detected ca. 0.35 μg of 7-hydroperoxycholesterol at 150 °C for 90 min and ca. 8 μg of 7-hydroperoxycholesterol for each gram of cholesterol at 140 °C for 120 min (*7*). However, the authors quantified the 7-hydroperoxycholesterol by using the Würster dye method, which uses preparative HPLC analysis followed by color reaction and UV detection.

Cholesterol Oxidation and 7-Hydroperoxycholesterol Formation. Cholesterol oxidation is known to occur by two different paths: C-7 oxidation and epoxidation (*6*−*8*). The C-7 oxidation path is observed by the formation of 7-ketocholesterol and

Table 2. Content (Micrograms per Milliliter) of Cholesterol Oxides Formed at the Heating Time at Which Maximum Formation of 7-Hydroperoxycholesterol Occurred

COP	140 °C	180 °C	220 °C
7-ketocholesterol	13	28	21
7α-hydroxycholesterol	13	17	20
7β-hydroxycholesterol	14	20	25
α-epoxycholesterol	14	11	17
β-epoxycholesterol	27	17	27

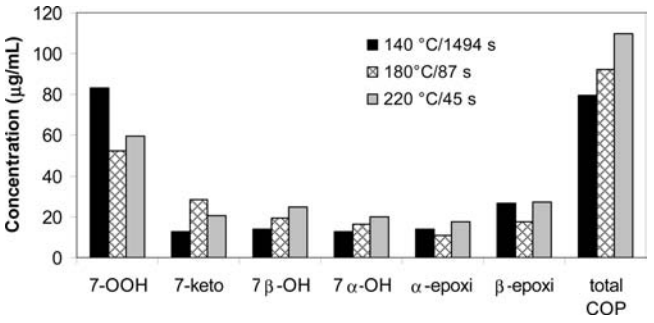

Figure 5. Formation of 7-hydroperoxycholesterol (maximum) and COP observed in pure cholesterol model system heated at 140, 180, and 220 °C for 1494, 87, and 45 s, respectively.

7α- and 7β-hydroxycholesterols, which are formed by the decomposition of 7α- and 7β-hydroperoxycholesterols, respectively. The epoxidation path is observed by the formation of α- and β-epoxycholesterols due to the presence of 7-hydroperoxycholesterol by a bimolecular reaction, at which 7-hydroperoxycholesterol probably attacks another cholesterol molecule to form epoxycholesterol (*3*).

The COP detected in the present study were those known to result from cholesterol oxidation such as 7-ketocholesterol, 7α- and 7β-hydroxycholesterols, and α- and β-epoxides. Side-chain derivatives or 3β-,5α-,6β-cholestanetriol were not detected. At 140 °C, only 7-hydroperoxycholesterol was formed until 213 s; between 213 and 593 s, 7-ketocholesterol and 7α- and 7β-hydroxycholesterols were formed; and after that, the formation of α- and β-epoxycholesterols began. However, this behavior was not observed at higher temperatures, at either 180 and 220 °C, when at the first sampling time, all five of these cholesterol oxides were detected and quantified. COP formation presented a direct correlation with temperature: the higher the temperature, the higher the COP formation, which is consistent with previously published data (*21*).

Table 2 shows the content of COP at each temperature when the 7-hydroperoxycholesterol reached its maximum concentration. At 140 °C the relationship between epoxides and C-7 derivatives is higher than at 180 or 220 °C, indicating that, at 140 °C, the epoxidation path is preferred.

The differences between 7-hydroperoxycholesterol and COP contents and the sum of COP (total COP) are shown at **Figure 5**. At 140 °C the amount of 7-hydroperoxycholesterol was quite similar to the total COP formed; however, at higher temperatures the quantity of 7-hydroperoxycholesterol formed was lower than the amount of COP. Possibly, the amount of 7-hydroperoxycholesterol was only partially transformed into COP, meaning that temperature must interfere in this balance.

The difference among heating times is due to the different reaction rates; as can be observed in the COP formation results, cholesterol degradation occurred more rapidly at higher temperatures. Similar results were found by Kim and Nawar (*21*). Moreover, degradation of cholesterol at 140 °C occurs slowly, as this temperature is lower than its melting point. These data indicate that a greater part of degraded cholesterol did not form 7-hydroperoxycholesterol or other COP, but other polymers or volatile compounds, which were not identified in this work. Heating cholesterol at too high temperatures (> 200 °C) may have caused thermal degradation rather than cholesterol oxidation (*22*). Moreover, the amount of COP detected could be reduced by thermal degradation, because the quantity observed after a given time interval represents the net balance between formation and degradation of each COP (*21*).

In summary, the method used takes advantage of mass spectrometry for the identification of 7-hydroperoxycholesterol. The potential of this technique for the analysis of such compounds has not been previously investigated in the literature. By applying this method in cholesterol heated model systems it was possible to verify that 7-hydroperoxycholesterol is the first compound formed when the temperature is lower (140 °C). However, the concentration of the 7-hydroperoxycholesterol is dependent on the temperature and time exposure: the higher the time, the higher the amount of 7-hydroperoxycholesterol at lower temperature, and the lower the time, the lower the amount of 7-hydroperoxycholesterol at higher temperature (180 and 220 °C).

These results corroborate the three phases of cholesterol oxidation, being that at 140 °C the oxidation is still at initiation phase at which the formation of hydroperoxides predominates, whereas at higher temperatures the oxidation is already at propagation phase at which the formation of other COP predominates.

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