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# Cholesterol, Linoleic Acid or/and Tyrosine Yield Different Spectra of Products when Oxidized Alone or in a Mixture: Studies in Various Oxidative Systems

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Identification of reliable biomarkers for oxidative stress for the prediction of the early development of pathological conditions is essential. The detection of biomarkers for oxidative stress such as degradation products of polyunsaturated fatty acid (PUFA), oxysterols, and oxidized proteins, as indicators of oxidative stress are in use, but suffers from insufficient specificity, accuracy and reliability. The overall aim of the present study was to develop new markers which will not only provide information about the presence and level of oxidative stress in biological systems but also on the type of reactive oxygen species (ROS) involved and their metabolic consequences. In the first stage of the study, we compared the level and type of oxidized products formed when different ROS were applied onto three major biomolecules, i.e. cholesterol, linoleic acid (LH) and tyrosine, representing sterols, PUFA and protein, when each compounds was exposed alone or in a mixture to the ROS [copper ions, 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH) and hypochlorous acid (HOCl)]. It was found that different types of oxidants resulted in the formation of different types of oxidation products. Furthermore, oxidation pattern differs when the substrates (cholesterol, PUFA or amino acid) were present alone or in a mixture. As biological systems such as lipoproteins and cell membranes are composed of the above studied molecules, the need for simultaneous detection of the major oxidized products is requires for better characterization of the oxidative stress outcome.

*Keywords*: Oxidative stress; Lipid peroxidation; Cholesterol; Polyunsaturated fatty acids; Amino acids; Free radical generator

## INTRODUCTION

It is generally accepted that in any biological system an important balance must be maintained between the formation and removal of reactive oxygen species (ROS). Otherwise, an increase in the ROS level may result in the development of pathological conditions.

Organs protect themselves from the toxicity of excess ROS by maintaining an oxido/redox balance in different ways, including the use of endogenous and exogenous antioxidants, such as the enzymes superoxide dismutase (SOD), catalase, the glutathione (GSH) system, and other, low molecular weight antioxidants (tocopherols, carotenoids, ascorbic acid and polyphenols).[1,2] An excess of ROS is termed oxidative stress, and is associated with changes in the structure and function of such biomolecules as DNA, proteins and lipids,[3,4] which may lead to the development of cardiovascular diseases, diabetes, neurodegenerative disorders, cataract, cancer and aging.[5,6] Reactions of ROS with DNA modify its bases and lead to the development of mutations. These modifications may cause oxidation and deamination of the DNA bases such as in the formation of 8-oxoguanine, a putative oxidized base.[7]

Proteins can react with peroxyl (ROO·), hydroxyl radicals (HO·) or hypochlorous acid (HOCl), causing

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damage in a number of forms. Examples of such damage are the oxidation of their amino acids, deamination, decarboxylation and modifications in their aromatic rings,[8] which lead to changes in the three-dimensional structure and activity of the protein. Proteins (mainly those containing histidine proline and lysine) can also react with oxidized lipid degradation products, such as ketones and aldehydes, to form Schiff's bases, which change protein activity and membranal functions and cause aggregation.[9]

Cells and tissues contain polyunsaturated fatty acids (PUFA), mainly linoleic acid (LH) and arachidonic acid, as esters with glycerol (phospholipids) and cholesterol. These PUFA are readily auto-oxidized under oxidative stress and, due to their double bonds, react with ROS, thereby initiating other free radical reactions. Auto-oxidation of PUFA affects cell function, and generates changes in membrane density, fluidity, permeability and oxido/redox potential.[10] It has been suggested that such reactions of oxidative stress may participate in an early stage of LDL oxidative modification, instigating changes in LDL-receptor recognition and in the formation of foam cells, the hallmark of early atherosclerosis development.[11,12]

The identification of reliable biomarkers, e.g. modified endogenous compounds formed in biological systems as a result of oxidative stress, is becoming essential for the prediction of the early development of pathological conditions.[13] Several modified endogenous compounds have been proposed as indicators of oxidative stress to proteins (chloro- or nitro-tyrosine) and lipids (oxidized degradation products of PUFA and oxysterols). The detection of these biomarkers as indicators of oxidative stress suffers from limitations, as a result of insufficient specificity, accuracy and reliability. Levels of such biomarkers are determined by a dynamic process, involving their formation, accumulation and removal, which may not be due to the oxidative process within the system, but rather to an alternative processes. Furthermore, their levels may change during their isolation and sample preparation.[14]

In the present study, the effect of different ROS on three major endogenous molecules (amino acids, poly-unsaturated fatty acid and cholesterol) was investigated simultaneously. The molecules analyzed were tyrosine, LH and cholesterol. These compounds were exposed separately, as well as in a mixture, to copper ions, 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH) or HOCl. The level of oxidized products derived from each of the three endogenous molecules was determined qualitatively and quantitatively by means of HPLC, GC-MS and LC-MS-MS. The results of our study may revealed a fingerprint of the damage due to the effect of a specific type of ROS on the endogenous molecules, when all are present in a mixture, competing for the ROS in the various systems under study. As the same way occurs in biological systems when different endogenous compounds are exposed simultaneously to a specific ROS. The present study clearly showed differences in oxidative pattern when each component was exposed separately (as occurs in most *in vitro* experiments) or in combination, as occurs *in vivo*.

## MATERIALS AND METHODS

### Materials

LH, cholesterol (5 cholesten-3-ol), *N-t*-BOC-L-tyrosine (tyrosine-BOC), 1,2-dipalmitoylphosphatidylcholine (DPPC), 3-chlorotyrosine, heptadecanoic acid, *N,O-bis*(trimethylsilyl) acetamide (BSA), ethylendiamine tetraacetic acid (EDTA), butylated hydroxytoluene (BHT), and sodium hypochlorite (NaOCl) were obtained from Sigma Chemical Co. (St Louis, MO). AAPH was purchased from Polysciences, Inc. (Warrington, PA). 7-ketocholesterol (7-keto), 7α and 7β-hydroxycholesterol (7α-OH, 7β-OH), 5α,6α and 5β,6β-poxy-cholesterol (α-epoxy, β-epoxy), 27-hydroxycholesterol (27-OH), 4β-hydroxycholesterol (4β-OH) and 19-hydroxy-cholesterol (19-OH), which were used as internal standards (IS), were purchased from Steraloids Inc., Wilton, NH, USA. All solvents were of either spectrophotometric or HPLC grades.

### Preparation of Cholesterol, LH and Tyrosine-BOC Solutions and their Mixture

To prepare cholesterol solution for the reaction with AAPH and $Cu^{2+}$, cholesterol was dissolved in 0.5 ml chloroform, with the addition of 0.6% Tween 20 as emulsifier, and then evaporated under nitrogen. Phosphate buffered saline (PBS — 50 mM phosphate, 110 mM NaCl, pH 7.4) was added to the residue to make the desired concentration. Cholesterol and DPPC in a 1:1 ratio (W/W) in chloroform 0.5 ml were mixed, and then the chloroform was evaporated under nitrogen. The dried residue was dissolved in PBS (50 mM phosphate, 110 mM NaCl, pH 7.4) to a volume with the desired concentration. This was vortexed and bath sonicated, forming liposomes. Micelles of LH were prepared by adding the LH to cold (4°C) PBS and bath sonicated. Nitrogen was bubbled into the solution. Tyrosine-BOC in PBS was prepared by mixing vortexed tyrosine-BOC in PBS, with slight warming. The oxidation of tyrosine was carried out, using tert-butyloxycarbonyl (BOC-tyrosine) to avoid the reaction with the free amino group. The above solutions were freshly prepared before each experiment.

When a mixture of cholesterol, LH and tyrosine-BOC was used, each of the components was first added at the same equivalent to PBS, vortexed, and then used as stock solution for the same day. As ISs for cholesterol and LH, 19-OH and heptadecanoic acid were used, respectively.

### Copper Ions-induced Oxidation of Cholesterol, LH, Tyrosine-BOC and their Mixture

Cholesterol, tyrosine, LH (4 mM) or a mixture of the three (1:1:1 molar ratio) in PBS were treated with $CuSO_4$, at final concentrations of 5, 10, 100 and 200 μM at 37°C and vortex continuously. After 20 h, 10 μl butylated hydroxy toluene (BHT) (5 mg/ml) and 10 μl EDTA (5 mg/ml) were added and the solution was injected to HPLC for the analysis of LH and its oxidized product. To the other part of the reaction mixture, HCl (0.2 N) was added to adjust the pH to 3.0, and then two volumes of diethyl ether (2 ml) was added. The organic layer was evaporated under nitrogen and the residue silylated as described below, prior to GC-MS analysis. The water phase was hydrolyzed with HCl (6 N) at 100°C in sealed glass for 24 h, to obtain the free amino acid, tyrosine and their reaction products by HPLC.

### AAPH — Induced Oxidation of Cholesterol, LH, Tyrosine-BOC and their Mixture

Cholesterol, tyrosine-BOC, LH (15 mM) or a mixture of the three (1:1:1 molar ratio) in PBS were treated with AAPH at a final concentration of 5 or 10 mM AAPH at 37°C, and vortexed continuously. After 2 h, 10 μl BHT (5 mg/ml) was added and the solution was injected to HPLC for the analysis of LH, linoleyl hydroperoxide (LOOH) and hydroxide (LOH). To another portion of the reaction mixture, HCl (0.2 N) was added, the pH was adjusted to 3.0, and two volumes of diethyl ether (2 ml) were then added for the extraction of lipids. The organic layer was evaporated under nitrogen and the residue was silylated, as described below, prior to GC-MS analysis. The water phase was hydrolyzed with HCl (6 N) at 100°C in sealed glass for 24 h, to obtain the free amino acid, tyrosine and their oxidized products, prior to HPLC analysis.

### HOCl-induced Oxidation of Cholesterol, LH, Tyrosine-BOC and their Mixture

For treatment with HOCl, a stock solution of NaOCl was prepared, and the concentration was determined spectrophotometrically ($\varepsilon_{292} = 350\ M^{-1}\ cm^{-1}$).[15] Cholesterol, tyrosine-BOC, LH (2 mM), or a mixture of the three (1:1:1 molar ratio) in PBS were treated with NaOCl, at a final concentration of 0.4 and 4 mM AAPH at 37°C, and vortexed continuously. After 30 min, GSH was added (0.5 or 5 mM) to scavenge any unreacted HOCl.[16] The reaction mixture was treated with HCl (0.2 N), adjusting the pH to 3.0, and then diethyl ether (2 ml) was added to extract the lipids. The organic layer was evaporated by nitrogen to dryness, and the residue was silylated for GC-MS analysis. The aqueous layer was hydrolyzed with 3 N HCl at 100°C in sealed glass for 24 h and then analyzed by HPLC.

### Analysis of Cholesterol Oxidation Products

GC-MS analysis was performed by means of an HP gas chromatograph, Model 5890 Series II (Waldbronn, Germany), fitted with an HP-5 trace analysis capillary column (column 0.32 mm I.D. 0.25 μm film thickness, 5% phenyl methyl silicone), with a mass selective detector, Model 5972, (Waldbronn, Germany) linked to an HP ChemStation data system.

Dried extracts were subjected to a silylating reagent BSA (200 μl), and 1,4-dioxane (dried on 4 Å molecular sieves and passed through aluminum oxide) (200 μl) as solvent and heated to 80°C for 30 min. The GC was operated in splitless mode for 0.8 min and then in split ratio of 1:1. Helium was used as carrier gas, at a flow rate of 0.656 ml/min, pressure 10.4 psi and at a linear velocity of 31 cm/s. The MS transfer line was maintained at 280°C. The injector was set at 300°C, the detector at 330°C and the column heated at a gradient starting at 200°C, increasing to 250°C at 10°/min and then at 5°/min to 300° and maintained for an additional 15 min at 300°C.

### Determination of Oxysterols by GC-MS

Samples were detected in the GC-MS in total ion monitor (TIM), from which the 2–4 most representative ions were selected for re-injection in single ion monitoring mode (SIM). 19-OH was used as ISs. For maximum sensitivity, the oxysterols were injected as their silyl ether derivatives, and the response factor for each oxysterol under the analytical conditions was calculated from the peak area ratio.[2] The selection of the oxysterols 7α-OH, 7β-OH, 4β-OH, β-epoxy, α-epoxy, 3,5,6-trihydroxycholesterol (triol), 25-hydroxycholesterol (25-OH), 7-keto, 26-hydroxycholesterol (26-OH) was based on data from the literature, which presented them as candidates to be formed under cholesterol oxidation.[2,17,18] Under the above conditions, the oxysterols were separated, and the mean quantity of each oxysterol was calculated, using standards calibration curves. A representative GC-MS spectrum of a mixture of oxysterol standards is shown in Fig. 1A.

Oxysterols obtained by oxidation of cholesterol with AAPH, $CuSO_4$ or HOCl are shown in Fig. 1B–1D

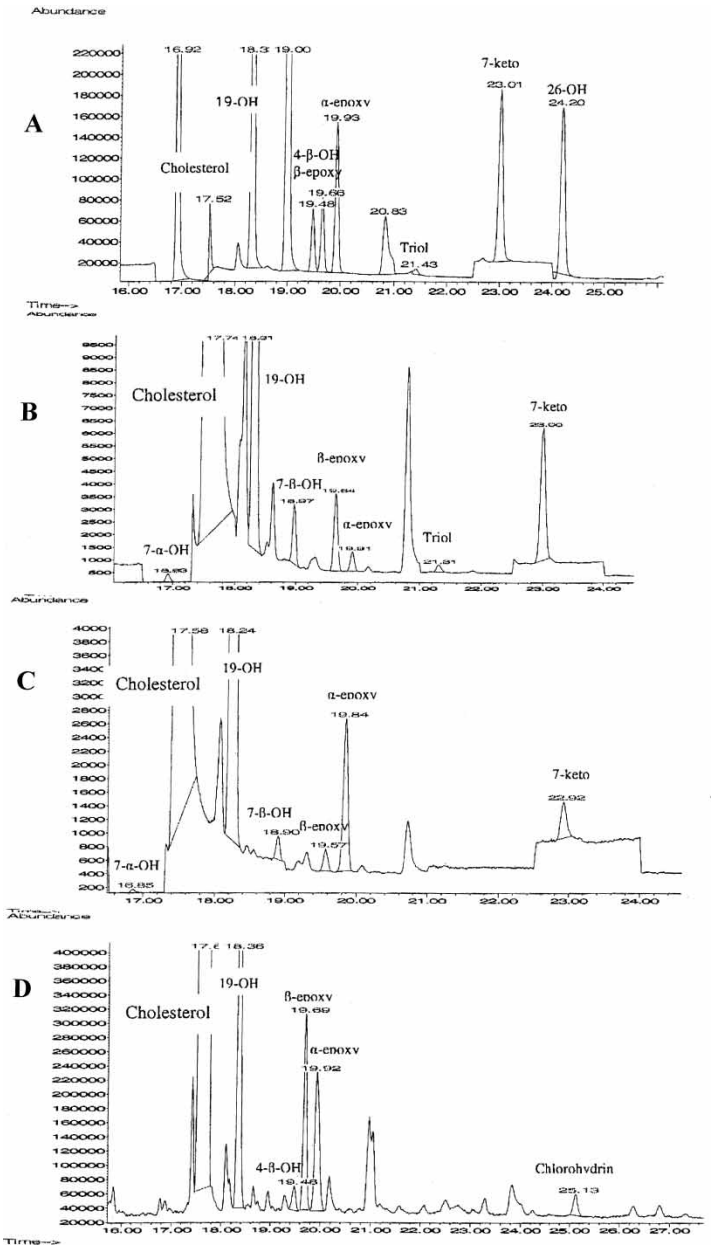


FIGURE 1 GC-MS detection of cholesterol oxidation products in various oxidative systems. Standards of silylether derivatives of oxysterols in concentration of 25 μg/ml were injected (1 μl) in SIM, using 19-OH as internal standard (A). $Me_3Si$-derivatized cholesterol products after oxidation with $Cu^{++}$, 20 μM (B), or with AAPH, 10 mM (C), injected in a SIM and with HOCl, 0.4 mM (D), injected in a TIM.

Treatment of cholesterol with HOCl resulted in the formation of a peak of cholesterol chlorinated product at a retention time of 25 min. This peak was identified as chlorohydrin by GC-MS, presenting characteristic fragmentation at 495, 459, 403 and 366 m/z. It has been mentioned that chlorohydrin can decompose in GC-MS conditions, forming cholesterol epoxides.[19] In order to evaluate, whether such a reactions had taken place in the present GC-MS conditions, and to what extent, cholesterol was treated with HOCl and the products were separated by TLC (ether:petroleumether:acetic acid 70:30:1). The chlorohydrin bands[19] were extracted from the TLC (diethyl ether), silylated as described previously, and injected to the GC-MS. Under the present work conditions, chlorohydrin was converted to α-epoxy and β-epoxy in a ratio of 1:7.9:0.62 (data not shown).

### Analysis of PUFA Oxidation Products

LH, LH hydroxide (L–OH) and hydroperoxide (L–OOH) were analyzed by high-performance liquid chromatography (HPLC), performed with an HP Series 1100, using a 5 μm Lichrospher reverse-phase C-8 column and Photodiode array detector. A mixture of water/acetonitrile/tetrahydrofuran/acetic acid (volume ratio of 40:40:20:0.25) was used as

the isocratic elution at a flow rate of 1.0 ml/min. The detector was monitored at 210 nm for LH and at 234 nm for L–OOH and L–OH. The fractions were identified by their retention times and characteristic UV spectra against synthesized standards.[20] Products were quantified, using the molar-extinction coefficients of $\varepsilon = 23,000$ (l/M/cm) (Cayman Chemical Co. catalog).

### Determination of LH Oxidation Products by GC-MS

LH HOCl-oxidation products were detected by GC-MS, with slight changes from the oxysterols detection conditions. The transfer line was maintained at 200°C, the injector was set at 300°C, the detector at 280°C and the column heated at a gradient starting at 150°C, increasing to 250°C at 5°/min and then maintained for an additional 20 min at 250°C. Heptadecanoic acid was used as IS, and the product was analyzed in extracted ion mode, by a selection of characteristic ions of each product, as described in the "Results" section.

Samples were injected to GC-MS in TIM, from which the most representative ions were selected. Figure 2A shows the GC-MS of the reaction products of LH, with HOCl taken in extract ions mode. The mass spectrum obtained shows the presence of an isomeric mixture of LH reacted with one equivalent HOCl: (9)-chlorohydrin (2B), 10-chlorohydrin (2C), and isomeric mixture of *bis*-chlorination: (10,12)-chlorohydrin (2D) and (10,13)-chlorohydrin (2E). The mass spectra of the monochlrohydrins (peaks at retention times 22:11, 22:22 min) in Fig. 2B and 2C show $(M-CH_3)^+$ ions at 461 m/z, with characteristic fragment ions at 317 m/z (fragmentation between carbons $C_9-C_{10}$), 440 and 365 m/z (fragmentation between carbons $C_{10}-C_{11}$). The high abundance of fragment ions at 317 and 365 m/z indicates a preferential addition reaction of HOCl to the 9,10-double bond of the LH rather than to the 12–13 double bond. The addition of HOCl to the 12,13-double bond gave rise to the formation of compounds 2D–2E (eluted at Rt of 29.74–29.95 min).

The presence of weak $(M-CH_3)^+$ ions at 585 m/z suggests the presence of the molecular ion $M^+$, which is consistent with the addition of two equivalents of HOCl to the LH molecule. Other characteristic ions were detected at 173 m/z (fragmentation between carbons $C_{12}-C_{13}$) and 317 m/z (fragmentation between carbons $C_9-C_{10}$).[21] The isotopic distribution of the 317 and 365 m/z fragment ions is characteristic of the presence of Cl atoms.

### Analysis of Tyrosine Oxidized Products

HPLC analysis was performed by HP series 1100, using a 5 μm Lichrospher reverse-phase C-8 column and photodiode array detector. Tyrosine and its chlorinated products were analyzed by HPLC directly from the reaction mixture, using isocratic eluent solution consisting of 25 mM $KH_2PO_4$ (pH 3.5), at a flow rate of 1.0 ml/min and UV detector set up at 280, 254 and 210 nm. Authentic tyrosine and 3-chlorotyrosine were used as standards.

Tyrosine and its chlorinated agents were separated by HPLC, as described above, and each of the isolated peaks was then collected and injected into the liquid chromatography mass spectrometer (LC-MS/MS, Micromass Quattro Ultima) in direct injection (ESI) mode. The source temperature of the mass spectrometer was set to 120°C, with a cone gas flow of 101 l/h and a desolvation gas flow of 493 l/h. Peak spectra were monitored between 18 and 408 m/z. Collision-induced dissociation MS was performed, using 19 collision energy and 3.13 capillary voltage

### Determination of Tyrosine Oxidation Products by LC-MS/MS

HPLC analysis of the reaction mixture of *t*-BOC-tyrosine after treatment with HOCl and hydrolysis of the BOC protected group, revealed three major products: tyrosine, 3-chlorotyrosine (both compared with standards) and an additional product at higher retention time, with mass spectra corresponding to 3,5-dichlorotyrosine (data not shown). LCMS/MS analysis of the reaction mixture at full scan in the negative ion mode revealed three ions of 180 m/z for tyrosine $[M-1]^-$, 215 m/z for 3-chlorotyrosine $[M-1]^-$, and of 249 m/z $[M-1]^-$ for 3,5-dichlorotyrosine. Figure 3A shows the fragmentation of the ion at 180 m/z at negative ion resulted in the formation of one major fragment ion at 163 m/z, corresponding to $[M-1-NH_3]^-$. Fragmentation of the ion at 215 m/z, at negative ion (3B), resulted in two major fragment ions at 197 m/z, corresponding to $[M-1-NH_3]^-$, and at 169 m/z, corresponding to $[M-1-HCOOH]^-$. Fragmentation of the ion at 249 m/z at negative ion (3C) resulted in a major fragment ion at 213 m/z, corresponding to $[M-1-HCl]^-$. A peak at 253 m/z (addition of four mass units) was also detected (in a scan mode), at approximately 10% intensity of that at 249 m/z, in agreement with the abundance of the two $Cl^{37}$ isotopes. The fragmentation of ion 249 gave rise to a fragment ion of 213 $[M-1-HCl^{35}]$, while that of ion 253 gave rise to ion 217 $[M-1H-Cl^{37}]$ (data not shown).

### Statistical Analysis

The student *t*-test was used to analyze the significance of the results, which are given as mean ± SD.

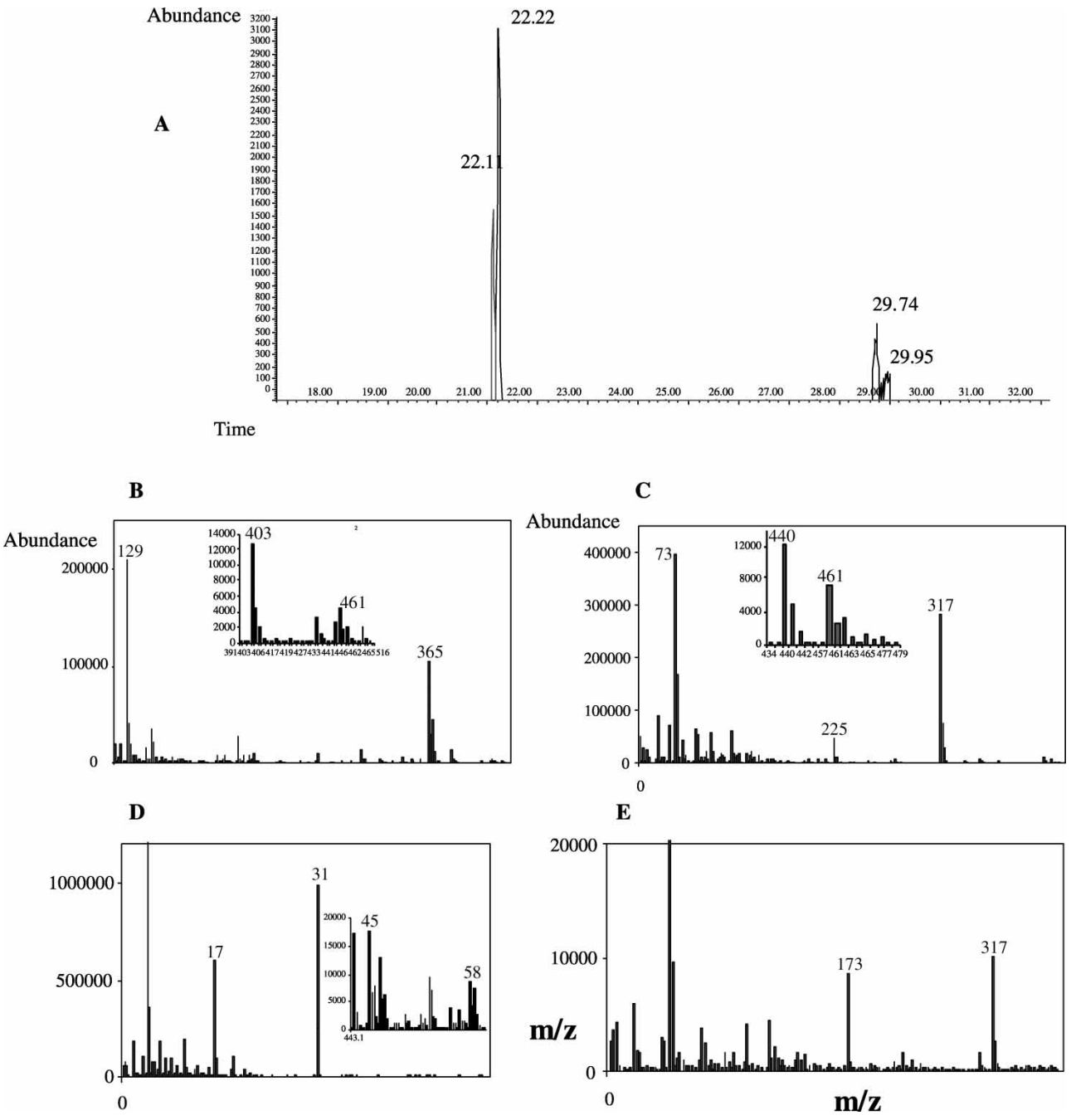


FIGURE 2 GC-MS detection of linoleic acid oxidation products after oxidation with HOCl. $Me_3Si$-derivatized 18:2 products after oxidation (A). Mass spectra of the product at 22:11 min (B) and at 22:22 min (C) were analyzed as two monochlorohydrin isomers. The product at 29:74 min (D) and 29:95 min (E) were identified as bischlorohydrin products. The inset presents a magnification of a specific section of the spectrum.

## RESULTS

The effects of oxidative stress induced by copper ions, AAPH or HOCl were tested on cholesterol, LH and tyrosine. The oxidized products (oxysterols, cholesterol chlorohydrine, hydroxy LH and chlorinated derivatives of LH and tyrosine) were analyzed by HPLC, GC-MS and LC-MS.

### Copper Ion-induced Oxidation

#### *The Oxidative Effect of Copper Ion on Cholesterol alone or in Mixture with LH and Tyrosine*

$Cu^{2+}$-induced oxidation products of cholesterol treated alone (4A) or in a mixture (4B) with LH and tyrosine (each at concentration of 4 mM) are shown in Fig. 4. The oxidation of cholesterol alone with

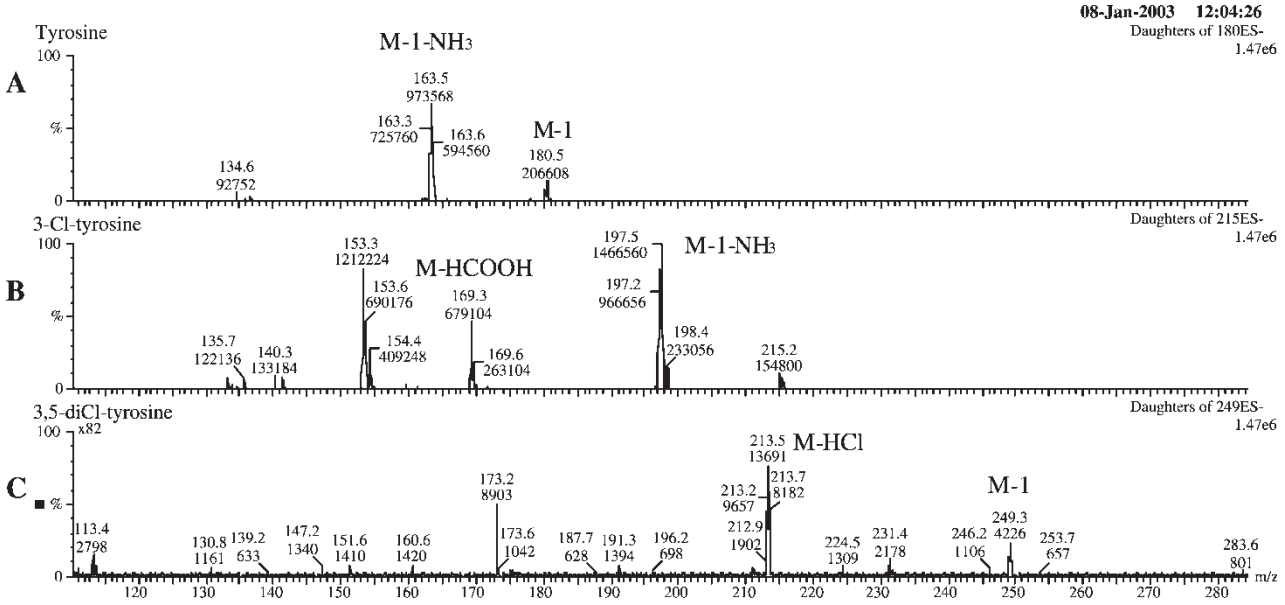


FIGURE 3 LC-MS-MS detection of the tyrosine oxidation products after oxidation with HOCl. Mass spectra are given for: tyrosine (A), 3-chlorotyrosine(B) and 3,5-diCl-tyrosine (C).

copper ions resulted in the formation of three major oxysterols, β-epoxy, α-epoxy and 7-keto, in a dose-dependent manner (from 17.7, 15.3 and 14.0% μM of oxysterol/mM of cholesterol with 10 μM $Cu^{2+}$ to 62.8, 48.6 and 42.2% μM oxysterol/mM cholesterol with 100 μM $Cu^{2+}$, respectively). Increasing copper ion concentration above 100 μM did not further increase the oxysterol level (Fig. 4A).

7β-OH and 7α-OH were also detected, but in much lower amounts (3.7 and 5.3% μM oxysterol/mM cholesterol with 100 μM, respectively). When cholesterol was treated with copper ions together

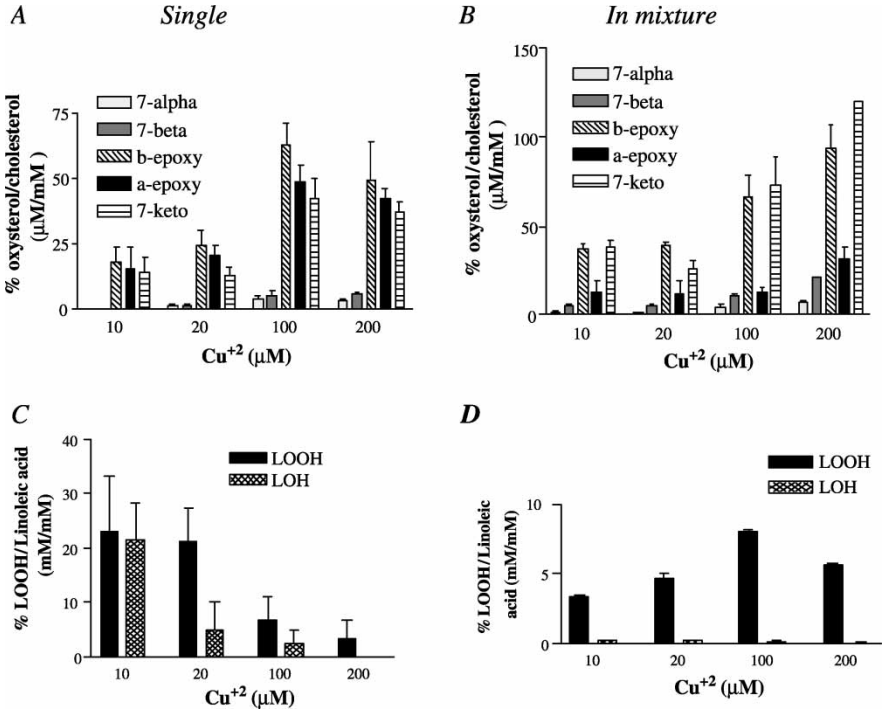


FIGURE 4 Copper ion-induced oxidation products of cholesterol and linoleic acid. The effect of copper ions on oxidation of cholesterol (4 mM) when exposed alone (A) or in the presence of tyrosine and linoleic acid in a molar ratio of 1:1:1 (B). The effect of copper ions on oxidation of linoleic acid (4 mM) when exposed alone (C) or in the presence of 4 mM each of tyrosine and cholesterol in a molar ratio of 1:1:1 (D). Results are presented as mean ± SD of two separate experiments.

with LH and tyrosine in a mixture, the levels of two of the three major oxysterols formed with cholesterol alone increased, i.e. 7β-epoxy and 7-keto (66 and 73% μM oxysterol/mM cholesterol with 100 μM $Cu^{2+}$, respectively), while α-epoxy was significantly lower (12.8% μM oxysterol/mM cholesterol, $p < 0.05$). When cholesterol was oxidized with copper ions in a mixture, the formation of oxysterols continued to increase with increasing copper ion concentration, (up to 200 μM) (Fig. 4B).

### *The Oxidative Effect of Copper Ion on LH alone or in Combination with Cholesterol and Tyrosine*

Oxidation of LH with copper ions resulted in the formation of LOOH and LOH. When LH was treated alone (Fig. 4C) the amount of LOOH declined from 2.4 mM (23% mM LOOH/mM LH) with 10 μM $Cu^{2+}$ to 0.5 mM (3.3% mM LOOH/mM LH) with 200 μM $Cu^{2+}$. When the mixture was treated with copper ions, the LOOH concentration rose from 0.28 mM (3.4% mM LOOH/ mM LH) with 10 μM $Cu^{2+}$ to 0.66 mM (8% mM LOOH/mM LH) with 100 μM $Cu^{2+}$, and then declined to 0.47 mM (5.7% mM LOOH/mM LH) with 200 μM $Cu^{2+}$ (Fig. 4D).

It is known that the normal curve of LH oxidation consists of a lag phase, followed by a propagation phase and finally a stationary phase, in which the amount of LOOH formed is equal to the amount of decomposed LOOH. The decline of the LOOH level, which manifested itself later, represents the change in the ratio between LOOH formation and its degradation. In the present study, this decline appeared after 20 h of oxidation, when the LH was oxidized alone. In the contrast of cholesterol and tyrosine, the reaction mixture after 20 h was still in its propagation stage.

### *The Oxidative Effect of Copper Ion on Tyrosine*

Under identical conditions as those above, no tyrosine oxidative products were detected when tyrosine was treated with copper ions alone, or in a mixture together with cholesterol and LH (data not shown).

## AAPH-induced Oxidation

### *The Oxidative Effects of AAPH on Cholesterol*

The AAPH-induced oxidation products of cholesterol treated alone, or in a mixture with LH and tyrosine, each at concentration of 15 mM, are shown in Fig. 5A and 5B. Two major oxysterols, β-epoxy and 7-keto, were observed under both treatments. Treatment of cholesterol alone with AAPH resulted in 0.23 and 0.18% mM oxysterol/mM cholesterol,

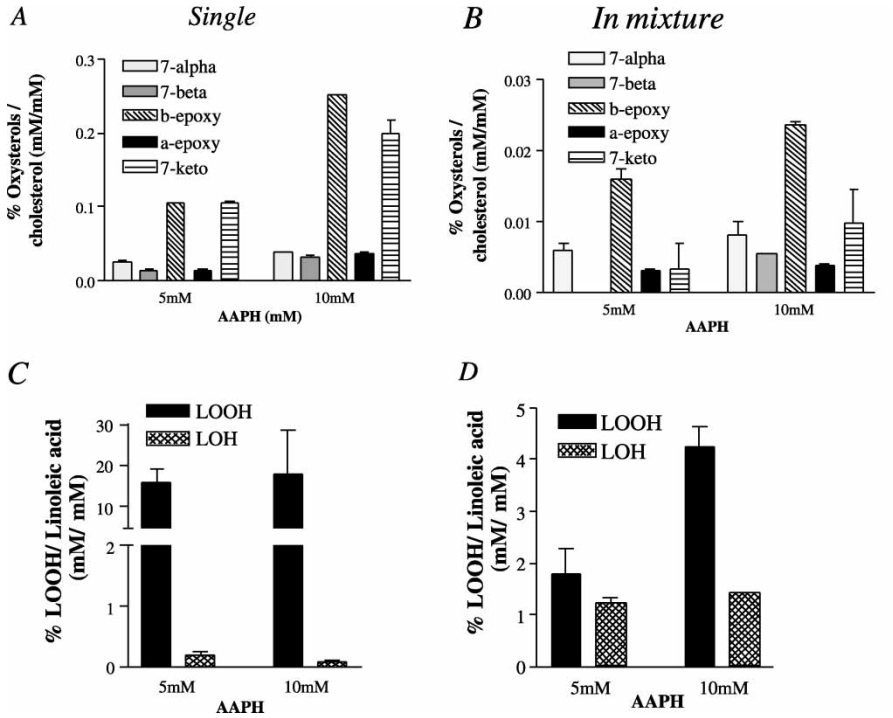


FIGURE 5 AAPH -induced oxidation products of cholesterol and linoleic acid. The effect of AAPH on oxidation of cholesterol (15 mM) when exposed alone (A) or in the presence of tyrosine and linoleic acid in a molar ratio of 1:1:1 (B). The effect of AAPH on oxidation of linoleic acid (15 mM) when exposed alone (C) or in the presence of 15 mM each of tyrosine and cholesterol in a molar ratio of 1:1:1 (D). Results are presented as mean ± SD of two separate experiments.

for β-epoxy and 7-keto with 10 mM AAPH, respectively. When cholesterol was in the mixture, the same oxysterols were obtained with 0.024 and 0.01% mM oxysterol/mM cholesterol, respectively.

### *The Effects of AAPH on LH*

Oxidation of LH with AAPH resulted in the formation of LOOH and LOH. When LH was treated alone (Fig. 5C), the level of LOOH reached to 18.11% mM LOOH/mM LH with 10 mM AAPH, while when the mixture was treated with AAPH, the LOOH concentration rose to 4.25% mM LOOH/mM LH with 10 mM AAPH (Fig. 5D).

### *The Effect of AAPH on Tyrosine*

Treatment with AAPH of tyrosine alone or in a mixture with cholesterol and LH, under the same conditions as above, gave no oxidation products of tyrosine (data not shown).

## HOCl-induced Oxidation

### *The Oxidative Effects of HOCl on Cholesterol*

Oxidation of cholesterol alone with HOCl (Fig. 6A) resulted in the formation of two major oxysterols, β-epoxy and α-epoxy with 23.4, 9.5% oxysterols/mM cholesterol, respectively. Cholesterol chlorohydrin was detected only when cholesterol was treated alone with HOCl (relative amount of 6.6% area/area, with 4 mM HOCl) (Fig. 6A). No oxidation products were detected when cholesterol was treated with 0.4 mM HOCl in the presence of LH and tyrosine (Fig. 6B), while treatment with 4 mM HOCl resulted in the detection of 4β-hydroxy, α-epoxy and β-epoxy (6.0, 18.2 and 8.5%, respectively, Fig. 6B). To further elucidate if the inhibition of cholesterol chlorohydrin

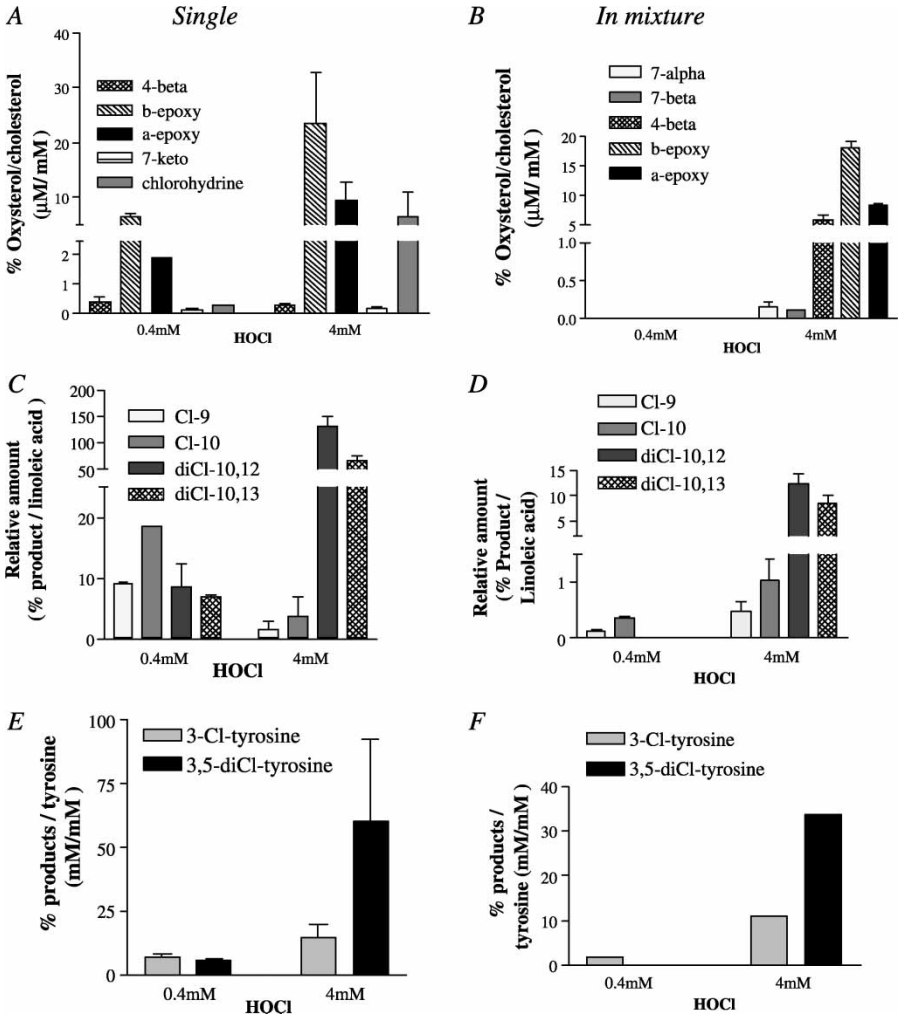


FIGURE 6 HOCl -induced oxidation products of cholesterol, linoleic acid and tyrosine. The effect of HOCl on oxidation of cholesterol (2 mM) when exposed alone (A) or in the presence of tyrosine and linoleic acid in a molar ratio of 1:1:1 (B). The effect of HOCl on the oxidation of linoleic acid (2 mM) when exposed alone (C) or in the presence of 2 mM each of tyrosine and cholesterol in a molar ratio of 1:1:1 (D). The effect of HOCl on oxidation of tyrosine (2 mM) when exposed alone (E) or in the presence of 2 mM each of cholesterol and linoleic acid (F). The cholesterol chlorohydrin and the linoleic acid chlorohydrins results are given as area of chlorohydrin/area of cholesterol (A,C and D). Results are presented as mean ± SD of two separate experiments.

formation when cholesterol was in a mixture, is due to the presence of tyrosine or the LH, we conducted two experiments: cholesterol was induced with HOCl in the presence of (a) tyrosine and heptadecanoic acid (C-18:0) or, (b) LH. The results were that in (a) no cholesterol chlorohydrin was detected while in (b) the amount of chlorohydrin detected was even higher than its amount when cholesterol was in the mixture with LH and tyrosine (relative amount of 8.4% area/area, on using 4 mM HOCl), data not shown.

### *The Effects of HOCl on LH*

Exposure of LH alone in solution to HOCl (Fig. 6C) led to the formation of an isomeric mixture of two monochlorohydrin (9-clorohydrin and 10-chlorohydrin) and two bischlorohydrin derivatives (10,12-bischlorhydrin and 10,13-bischlorohydrin). Changing the LH:HOCl ratio from 5:1 to 1:2 (Fig. 6C) resulted in an increase in the bischlorohydrin mixture from 8.45% mM product/mM LH to 130.95% for 10,12-Cl and from 6.94% mM product/mM LH to 66.04% for 10,13-Cl. As shown for the oxidation of cholesterol (Fig. 6B), the oxidation rate of LH was also lower when it was exposed to HOCl in a mixture with cholesterol and tyrosine (Fig. 6D). In a mixture using 0.4 mM HOCl, monochlorohydrin derivatives were the only products detected (Fig. 6D), but when exposed to 4 mM HOCl, the relative amount of the bischlorohydrins reached 12.43% mM product/mM LH with 10,12-diCl and 8.32% mM product/mM LH with 10,13-diCl.

### *The Effects of HOCl on Tyrosine*

Oxidation of tyrosine alone with HOCl resulted in the formation of two major products, 3-Cl-Tyr and 3,5-dichloro-Tyr (Fig. 6E), which were identified by LC-MS/MS and analyzed by HPLC. When tyrosine alone was exposed to HOCl, the amount of 3-Cl-Tyr increased from 6.87% mM product/mM tyrosine with 0.4 mM HOCl, to 14.35% with 4 mM HOCl, and the amount of 3,5-diCl-Tyr increased from 5.4 to 60%, respectively (Fig. 6E). When tyrosine was treated with 0.4 mM HOCl in a mixture with LH and cholesterol, 3-Cl-Tyr was the only chlorinated product detected, while on using 4 mM HOCl, 3,5-diCl also formed, although less (33.6%) than when tyrosine was alone (60%, Fig. 6F).

These results suggest that the formation of cholesterol chlorohydrin is a relatively slow reaction in comparison to the reaction of tyrosine and/or LH with HOCl. Hence, cholesterol in a mixture with tyrosine and LH, under the present conditions, did not react to form chlorohydrin.

## DISCUSSION

The present study demonstrates, for the first time, the different pattern of oxidation products of cholesterol, LH and tyrosine when present, alone or together in a mixture, following exposure to different types of ROS produced by copper ions, AAPH or HOCl. The major findings were as follows:

(a) In the copper ion system, oxidation of cholesterol alone led to the formation of three major products; β-epoxy- > α-epoxy- > 7-keto- cholesterol, which reached maximum level with 100 μM of copper ion. In contrast, when cholesterol was oxidized in a mixture (together with LH and tyrosine), the major oxysterols formed were 7-keto- > β-epoxy- > α-epoxy-cholesterol, and their levels continue to increase in a dose-dependent manner, up to 200 μM of copper ion. The oxidative effect of copper ions on LH alone showed that the linoleate oxidized product (L–OOH) reached a steady state at as low as 10 μM copper ions, whereas when LH was oxidized in the mixture system, the levels of L–OOH continued to increase up to 100 μM of copper ions.
(b) In the AAPH system, the oxidation products had the same pattern when each component was oxidized alone or in the mixture. Under both copper ions or AAPH, tyrosine alone or in a mixture was not oxidized at all.
(c) In HOCl system, oxidation of cholesterol alone resulted in the formation of α-epoxy-, β-epoxy-cholesterol and chlorohydrin, while in the mixture chlorohydrin was not formed. Testing the effects of tyrosine and LH on the oxidation of cholesterol demonstrated that tyrosine inhibited the formation of chlorohydrin, while LH significantly enhanced the formation of chlorohydrin, 4β-OH, β-epoxy- and α-epoxy- cholesterol. Tyrosine was the preferred reactant in the mixture when oxidized with HOCl, in comparison to LH and cholesterol.

The mechanism of oxidative damage to biomolecules by ROS is under extensive investigation, but an understanding of the complex interactions of different biomolecules expose simultaneously to various ROS is still ambiguous. The present study demonstrates some of this complexity, showing that exposure of cholesterol, unsaturated fatty acid and amino acid together to various types of oxidative stress resulted in the formation of an oxidized product fingerprint, which differed from that obtained when each molecule was exposed separately to the same ROS under identical conditions. It is suggested that, as biological system consist of a mixture of biomolecules, the analysis of many

oxidized products from different classes of biomolecules is essential to a better understanding of the type and kinetics of ROS involved.

The oxidation of cholesterol, alone or in a mixture with copper ions, yielded different fingerprint analyses of the oxidized products. These may have resulted from the appearance of new ROS formed from the oxidation of LH in the mixture, such as ROO·, RO· and/or ROOH. 7-Keto cholesterol was also demonstrated as the major oxidized product of LDL that was treated with copper ions.[2,17,18,22] Indeed, LDL is an example of biological system that contains both protein (apolipoprotein B-100) and lipid components, including LH, in the LDL phopholipids and cholesteryl–ester moieties, as well as cholesterol in free and in esterified forms. Under the above experimental conditions, tyrosine alone or in a mixture with LH and cholesterol was not oxidized by copper ions.

The oxidative effect of copper ions on LH alone shows that, at a low concentration of copper ion, the oxidized product, L–OOH, reached a steady state (a stage where the formation of L–OOH is equal to its degradation). However, when LH was treated with copper ions in a mixture (with cholesterol and tyrosine), the level of L–OOH continued to increase (the propagation phase). This pattern is probably not due only to the competition of LH with cholesterol, since similar effect was observed when copper ion concentration was increased by 10-fold (up to 100 μM). It may thus, be due to the formation of a new ROS, such as cholesteryl hydroperoxide.

Oxidation with AAPH had the same effect on the mixture as on the individual components (cholesterol, LH or tyrosine). Copper ions and AAPH are two *in vitro* inducers of oxidation that are frequently used[9,23,24] with different modes of action. AAPH generates free carbon radicals, leading to the formation of ROO·,[25] the main radical involved in AAPH induced oxidation. On the other hand, copper ion-induced oxidation required the presence of preformed lipid hydroperoxide.[26] In the presence of cholesterol and/or LH and their oxidized products, copper ion may be reduced to $Cu^{1+}$, which reacts with lipid hydroperoxides (such as LOOH or cholesteryl hydroperoxide) to form other types of peroxyl radicals oxidative products (RO·).

HOCl is a potently toxic oxidant formed by the enzyme myeloperoxidase (MPO), which is present in neutrophils and monocytes, during the reaction of $H_2O_2$ in the presence of chloride ions ($Cl^-$). It reacts with protein free amino groups to form unstable chloramines (which can further form other reactive species). HOCl also reacts with unsaturated lipid double bonds, such as LH and cholesterol, to yield lipid chlorohydrins which have been detected in atherosclerotic lesions. HOCl reacts with LDL,[27] and the preferential reacting components of the LDL are protein amino acids, such as tryptophan, lysine, methionine, cysteine and tyrosine, while unsaturated fatty acid and cholesterol demand higher concentration of HOCl and longer incubation time.[28] In the present study, the oxidation of cholesterol alone, but not in a mixture with HOCl, resulted in the formation of α-epoxy-, β-epoxy-cholesterol and chlorohydrin. The possibility was explored that the absence of chlorohydrin in the treated mixture was due its transformation to α-and β-epoxy cholesterol (which may occur under GC-MS conditions, by intramolecular nucleophilic substitution, subsequent to elimination of HCl), chlorohydrin was synthesized, purified, and injected to the GC-MS.[19] It was found indeed that such a transformation does occur, and about 80% of the chlorohydrin were transformed to epoxy cholesterol.

In order to test, whether one of the component in the mixture inhibits chlorohydrin formation, tyrosine and LH effects were tested separately: the results showed that tyrosine is the inhibitor. This inhibitory effect remained valid even with 4 mM HOCl, while when cholesterol was treated alone, 0.4 mM HOCl, (within the physiological concentration,[29]) was sufficient to form significant amount of cholesterol chlorohydrin. These results may be supported by a previous study which demonstrated that the amount of HOCl needed to obtain chlorohydrin from reaction of HOCl in cells and membranes is 5–15 times higher than that required with cholesterol alone in liposomes[19] and that a molar ratio of 1000:1 of HOCl:LDL was not sufficient to permit the detection of cholesterol chlorohydrin in LDL.[30] These results were explained by the existence of competition with other components. The present study showed that an inhibitory effect of the tyrosine may also be a possible explanation.

The effects of HOCl on tyrosine was carried out, using BOC-tyrosine to avoid the reaction of HOCl with the free amino group and formation of chloroamine. The reaction of HOCl with tyrosine alone or in a mixture yielded 3-Cl-tyrosine and 3,5-diCl-tyrosine, suggesting that tyrosine is a preferred reactant in the HOCl system, in comparison to LH and cholesterol. These findings are in agreement with previous findings that, in LDL, the lipid components do not compete effectively for HOCl with the LDL protein.[31] In the present study, a mixture of cholesterol, LH and tyrosine showed that both oxidized products were formed, i.e. chlorohydrins of LH and oxysterols, in addition to tyrosine chlorinated products.

We may conclude that different ROS affect endogenous bio-molecules differently when they are exposed to oxidative stress alone or when they are present in a mixture with other molecules. As biological system are composed of a mixture of components, this finding may emphasize the need

for simultaneous detection of the major oxidized products from divers types of body building components, for a better characterization of different oxidative stress related pathological conditions. Thus, the design of a new biomarker, which contains together as one compound the main endogenous oxidative stress susceptible molecules, is requested. Such a biomarker may provide a fingerprint of the oxidative stress outcome in biological systems.

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