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Cardiolipin and oxidative stress: Identification of new short chain oxidation products of cardiolipin in *in vitro* analysis and in nephrotoxic drug-induced disturbances in rat kidney tissue☆

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ABSTRACT

Cardiolipin (CL) is found almost exclusively in the inner mitochondrial membrane. Alteration of CL, namely by oxidative modifications, has been related with various pathological conditions, and with cell apoptosis. Their location, in the mitochondria, makes them even more likely to be oxidized, since that there is a considerable production of ROS in the inner mitochondrial membrane. In spite of the importance of CL oxidation and its biological consequence, there is a limited knowledge of the oxidation products of CL.

In this study, mass spectrometry coupled with liquid chromatography (LC–MS) was used to identify the specific oxidative modifications of tetra-linoleoyl CL induced by the $OH^{\bullet}$ generated under Fenton reaction conditions (H_2O_2 and Fe^{2+}). Short chain products (with fatty acyl shortened) formed during CL oxidation were identified for the first time and were further characterized by LC-ESI-MS/MS. The short chain products identified resulted from β-cleavage of alkoxyl radicals at C9 and C13. These products were identified as $[M-2H]^{2-}$ at *m/z* 691 and 719, with C13 and at *m/z* 685, 693 and 701, with C9 shortened fatty acyl chains. Detailed analysis of the fragmentation pathways of these precursor ions allowed to identify the MS/MS product ions which allow the unequivocal assignment of the oxidized cardiolipin species, very valuable for their detection in biological samples. Some of these products, namely, the CL short chain product at *m/z* 685 and 693 were detected in the mitochondria of kidneys obtained from rats treated with gentamicin. Gentamicin is an aminoglycoside antibiotic that induces nephrotoxicity and that has been associated with mitochondrial dysfunction and lipid peroxidation.

1. Introduction

Cardiolipin (CL) is a phospholipid found mainly in the inner mitochondrial membrane. CL is associated with different proteins of the complexes of the respiratory chain, which are involved in the transduction of electrons and the production of ATP in the mitochondrial inner membrane, conferring to CL a central role in mitochondrial processes and in energetic metabolism [1–3]. Alteration of CL structure, namely by oxidative modifications or change in the fatty acyl profile, and change in mitochondria CL content have been correlated with various pathological conditions, particularly in neurodegenerative diseases, such as Barth syndrome, Parkinson and Alzheimer diseases and in cell apoptosis [3,4].

CL has a more complex structure, when compared with the other phospholipids, bearing four chains of fatty acids that can diversify in length and degree of unsaturation. The structural characteristics of CL and its oxidized species is postulated to be important in different biological functions [5,6]. Like the other phospholipids, and also due to the presence of the double bonds in fatty acyl chains, CL is susceptible to oxidative damage by reactive oxygen species (ROS) [1,7,8]. Their location, in the mitochondria, makes them even more likely to be oxidized, since that there is a considerable production of ROS in the inner mitochondrial membrane [1,4,7]. ROS, and mainly $OH^{\bullet}$, are involved in oxidative stress modification of distinct biomolecules, namely lipids, proteins and nucleic acids, leading to changes in their structure and in consequence, loss or modification of their function. In particular, oxidation of CL is thought to be a key intermediate in cells apoptosis. Oxidation of CL is closely related

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with cellular apoptotic mechanisms which have being associated with nephrotoxicity of several drugs, such as aminoglycosides and immunosupressors [9–11].

Notwithstanding the importance of the consequences of CL oxidation, and the fact that in the last few years there is an increasing interest in the study of CL, there is a limited knowledge of the oxidation products of CL. During ROS oxidation, numerous oxidation products may be formed [6]. It is very important to identify each specific oxidation product in order to understand their specific biological significance and effects [6]. Mass spectrometry has been used for the identification of specific structures of phospholipids oxidation products generated during distinct oxidative processes [12]. ESI-MS and MS/MS in negative mode have been used, by Kagan et al., to identify the CL oxidation induced by different conditions, namely in the presence of cytochrome c/H_2O_2, by γ-irradiation induced intestinal injury [13], after traumatic brain injury [14], during staurosporine-induced apoptosis [15] and by pro-inflamatory stimuli, using LPS [16]. They noticed oxidation of CL by the accumulation of different combinations of hydroxy and hydroperoxy group in CL, although peroxyl derivative seems to be formed preferentially. In a more recent study, they used tandem mass spectrometry to identify and confirm the proposed conjugations and their location along the fatty acyl chain of the CL molecules [16].

Phosphatidic acid and diacylphosphatidyl-hydroxyacetone were also identified as oxidation producst of CL by Yurkova et al. and Shadyro and co-workers, in an oxidative process that conduct to cleavage of the CL molecule [13,17–19]. In these works γ-radiation, Cu^{2+}/H_2O_2/ascorbate, or cytochrome *c* mediate oxidation were used to induce modifications on CL, and the oxidation products were identified by MALDI-MS in positive mode. However, no MS/MS studies were used to confirm and characterize these oxidation products.

In order to bring new knowledge on the oxidative modifications undergone by CL in presence of free-radicals, mass spectrometry coupled with liquid chromatography (LC–MS) was used to identify the specific oxidative modifications of tetra-linoleoyl CL induced by the OH generated under Fenton reaction conditions (H_2O_2 and Fe^{2+}). Tetra-linoleoyl CL has been chosen because it is an abundant form in almost mammalian tissues. Detailed discussion of the fragmentation products will be done in order to pinpoint the specific ions that may allow their unequivocal assignment, which may be useful for their detection in biological samples. Experimental studies have shown that cell apoptosis is induced on *nephrotoxicity* induced by *aminoglycosides* [9–11]. Thus, the nephrotoxicity of aminoglycosides administration could be connected to oxidative modifications on cardiolipin. To evaluate the possible accumulation of CL oxidation products under this apoptotic conditions, comparison of CL profile from kidney mitochondria of rats treated with gentamicin and rats without treatment was done using tandem mass spectrometry, using the data provided by the *in vitro* oxidation of tetra-linoleoyl CL.

2. Experimental

2.1. Materials

Tetra-linoleoyl CL was purchased from Sigma and was used without further purification. Ferrous chloride and hydrogen peroxide (30%, w/v) were used for oxidation reactions and were obtained from Merck (Darmstadt, Germany). Triethylamine (Acros organics), chloroform (Analitycal reagent grade) methanol (HPLC grade), ethanol absolute (Panreac). TLC silica gel 60 with concentrating zone (2.5 cm × 20 cm) was purchased from Merck (Darmstad, Germany).

2.2. Animals

Adult male Wistar rats were maintained at a temperature 23 ± 2 °C, 50–55% humidity, and a lighting cycle of 12 h light and 12 h dark. Commercial rat pellets and water were available ad libitum. Twelve adult male Wistar rats weighting 200–250 g were randomly divided in two groups (6 rats) as follows: (i) control group: treated subcutaneously with isotonic saline solution for 8 days; (ii) gentamicin group (GEN): treated subcutaneously with gentamicin (60 mg/kg) for 8 days. All administrations were made at 9:00 a.m. Rats were anesthetized by inhalation of isofluoran (Abbott Laboratories, Sweden) immediately before being sacrificed. The experiments were performed according to international guidelines concerning the conduct of animal experimentation.

2.3. Isolation of rat kidney mitochondria

Mitochondria were extracted from a homogenate of rat kidney by differential centrifugation according to Cain and Skilleter [20]. Capsule was removed and the remaining organ was cut into pieces and then minced and homogenized with a Potter–Elvehjem in a medium containing 0.25 M sucrose, 20 mM HEPES, 1 mM ethylenediaminetetraacetic (EDTA), 0.2% (w/v) defatted bovine serum albumin, pH 7.4. Mitochondria isolation was performed at 4 °C without delay using differential centrifugation. The homogenate was centrifuged at 800 g for 10 min, and the resulting supernatant was centrifuged at 10,000 g for 10 min. The mitochondrial pellet was washed twice and re-suspended in a medium containing sucrose 0.25 M, 20 mM HEPES, pH 7.4, and suspended at 30 mg protein mL^{-1}. Protein concentration was determined by the biuret method using bovine serum albumin as a standard [21].

2.4. Lipid extraction and TLC analysis

The Bligh and Dyer [22] procedure was used to extract total lipids from each mitochondrial preparation. The lipid extracts were flushed with nitrogen, capped, and stored at −20 °C for ESI–MS analysis. Lipid spots extracts were separated and analyzed by thin-layer chromatography (TLC) plates (silica gel 60 with concentrating zone 2.5 cm × 20 cm, Merck, Darmstadt, Germany). Prior to separation, plates were treated with boric acid 2.3% in ethanol. The plates were developed in solvent mixture chloroform/ethanol/water/triethylamine (30:35:7:35, v/v/v/v). Lipids spots on TLC plates were observed by exposure to iodine vapors and identified by comparison with authentic phospholipid standards. Spots with cardiolipin were scraped from the plates and the CL were extracted using chloroform/methanol (1:1, v/v)

2.5. Oxidation of cardiolipin by Fenton reaction

Ammonium bicarbonate buffer 5 mM (pH 7.4) was added to 0.5 mg of CL, and then the solution was taken to the vortex and the sonicator for the formation of vesicles. The oxidation was performed by adding to the solution 40 μM of $FeCl_2$ and 50 mM of H_2O_2 to a total volume of 250 μL. The mixture was incubated at 37 °C in the dark for several hours. Controls were performed by replacing hydrogen peroxide with water.

2.6. LC-ESI-MS conditions

The RPLC–MS and RPLC–MS^n studies were conducted on a Waters Alliance (Milford, USA) Model 2690 equipped with a precolumn split (Accurate, LC Packings, USA) and an ACE 3 C18-AR column (150 mm × 1.0 mm, i.d.) kept at room temperature (22 °C). The reaction mixture was diluted 50-fold before injection and a volume of 10 μL was introduced into the column, using a flow rate

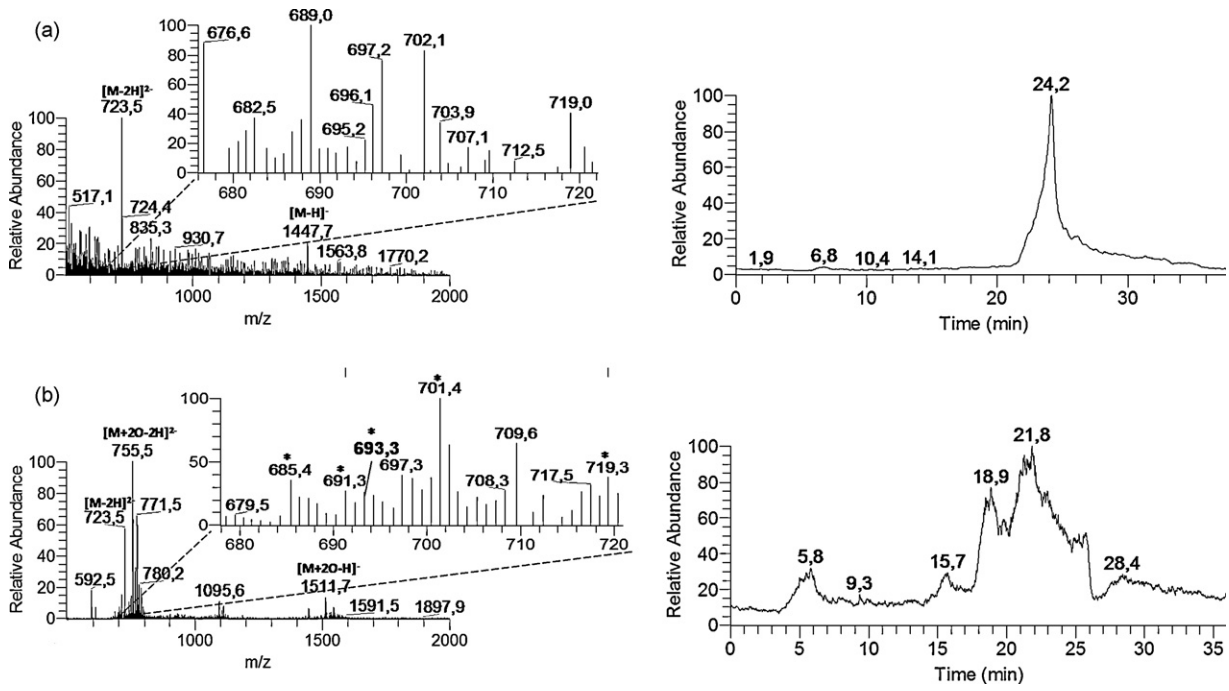


Fig. 1. LC-ESI-MS spectra and total ion chromatogram (TIC) from LC–MS profile obtained for CL under non-oxidative (a) and oxidative conditions (b).

of 25 μL min^{-1}. The phospholipid oxidation products were separated using water–methanol (90:10, v/v, eluent A) and methanol (100%, eluent B) programmed as follows: a linear increase from 90% B to 100% B in 15 min and held isocratically for 20 min. The mobile phase was brought back to the initial elution conditions in 10 min and allowed to equilibrate for 15 min until the next injection.

The LXQ linear ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) was operated in negative mode. Typical ESI conditions were as follows: electrospray voltage was 4.7 kV; capillary temperature was 275 °C and the sheath gas flow was 25 U. An isolation width of 0.5 Da was used with a 30 ms activation time for MS/MS experiments. Full scan MS spectra and MS/MS spectra were acquired with a 50 ms and 200 ms maximum ionization time, respectively. Normalized collision energy^{TM} (CE) was varied between 15 and 20 (arbitrary units) for MS/MS. Data acquisition was carried out on an Xcalibur data system (V2.0).

3. Results and discussion

Oxidation of CL was induced by the hydroxyl radical generated under Fenton reaction and the oxidations products were monitored by ESI-MS in negative mode. Cardiolipin ionizes, under ESI-MS conditions, as mono-charged ions $[M-H]^-$ and di-charged ions $[M-2H]^{2-}$. Comparing the ESI mass spectrum obtained for the cardiolipin after oxidative reaction with the ESI-MS spectrum of cardiolipin in non-oxidative conditions (Fig. 1), new ions were observed as $[M-2H]^{2-}$ species, at *m/z* values lower than the $[M-2H]^{2-}$ ions of native CL and attributed to oxidation products. The same oxidized species were observed as mono-charged ions but with much lower relative abundance, when compared with the double charged ions. These oxidation products (Fig. 1b) are observed at *m/z* 685, 691, 693, 701 and 719 and are proposed to be formed by carbon chain cleavage during fatty acyl oxidation. These species are formed due to an initial abstraction of hydrogen atom from one or more fatty acyl chains, with further cleavage of fatty acyl backbone and formation of carbonylic or carboxylic terminal functions, as reported for lipid peroxidation reaction and reviewed elsewhere [23–25]. In fact, the formation of an additional terminal carboxylic function may justify the most favorable formation of double charged ions, since it corresponds to the removal of one more labile hydrogen that facilitates the formation of $[M-2H]^{2-}$ ion species. Based on the molecular weight of these short chain oxidation products, it is proposed that they correspond to products having the fatty acyl chain shortened either with C13 or C9. To identify the specific structure of these products, LC–MS and LC–MS/MS was performed for the $[M-2H]^{2-}$ of each product.

Interestingly these CL short chain oxidation products were never reported before to be formed during CL oxidation. However, these products formed by cleavage of fatty acyl chains were previously observed during phosphatidylcholine (PC) and phosphatidylethanolamine (PE) oxidation [26,27], being this an usual process during lipid peroxidation, which corroborates the formation of short chain products during CL oxidation. To date, only products formed by insertion of oxygen atoms [16], or formed by cleavage of the inner glycerol backbone with formation of phosphatidic acid and diacylphosphatidyl-hydroxyacetone [28] were mentioned to occur during CL oxidation. Oxidation products formed by insertion of oxygen atoms were also observed in the present work, but since they have been studied, they will not be discussed.

Structural characterization by LC–MS/MS of these new short chain oxidation products were done based in the study of fragmentation pattern of the $[M-2H]^{2-}$ ions, since in the ion trap MS^2 mass spectra the informative fatty acid anions ($RCOO^-$) are observed. For a better analysis of the fragmentation of double charge ions of the oxidation products, fragmentation of double charged ion of tetra-linoleoyl CL was previously analyzed in the same experimental conditions. The MS/MS spectrum obtained is shown in Fig. 2. The structure of the product ions resulting from the fragmentation CL $[M-2H]^{2-}$ are represented in Scheme 1 and includes the product ions $[(M-2H)-RCOO^-]^-$, at *m/z* 1167, formed by the loss of fatty acyl anion and the double charged ion $[M-2H-R{=}C{=}O]^{2-}$, at *m/z* 592, formed by the loss of fatty acyl as ketene. Other abundant product ions are the carboxylate anions, $RCOO^-$, observed at *m/z* 279, the $[M-H]^-$ ions of phosphatidic acid (PA), at *m/z* of 695 (formed due to cleavage between the phosphate group and the inner glycerol) and of phosphatidylhydroxyacetone (PHA) at *m/z* 751, as represented

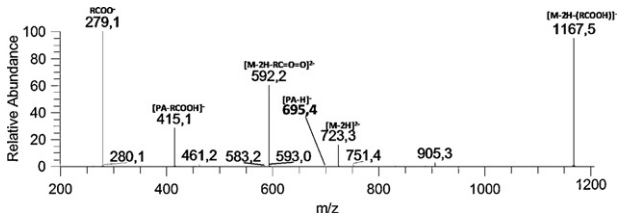
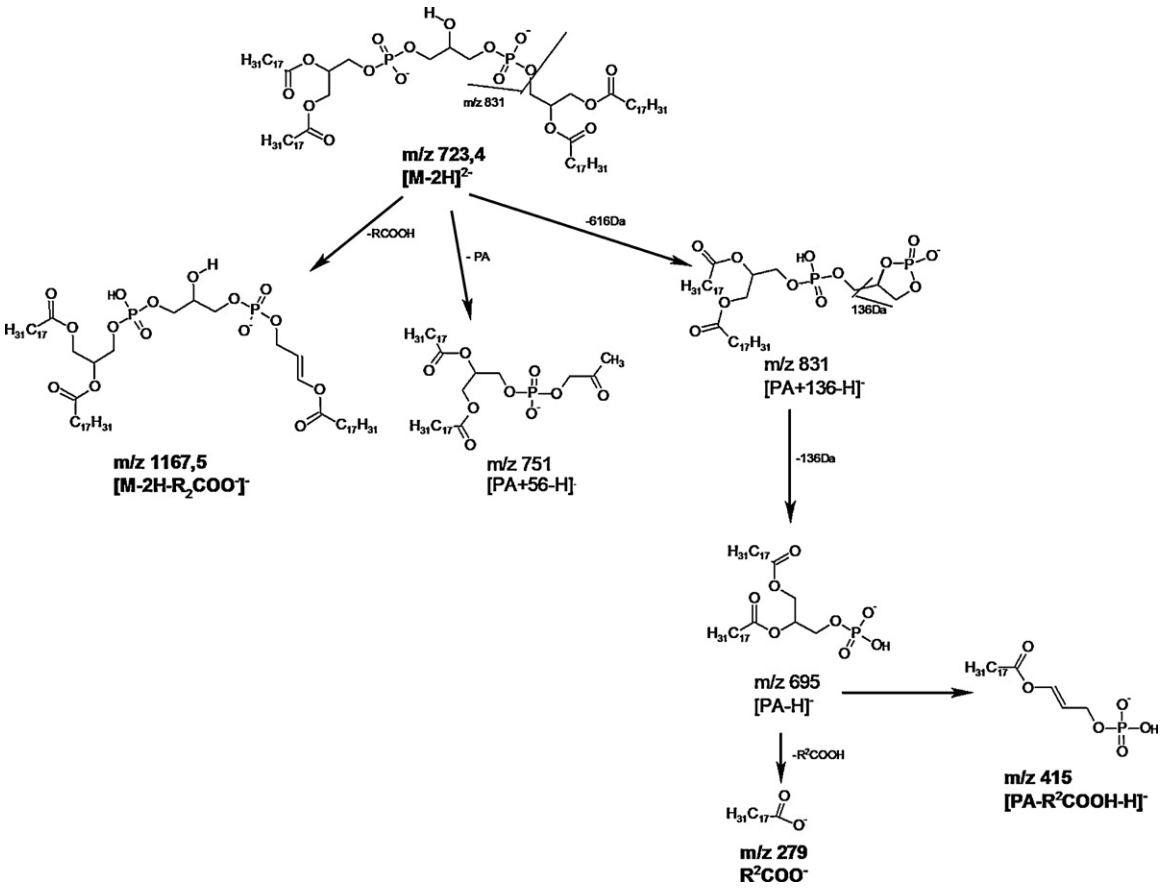


Fig. 2. ESI-MS spectrum of the double charged ion of tetra-linoleoyl cardiolipin ($[M-2H]^{2-}$ at *m/z* 723).

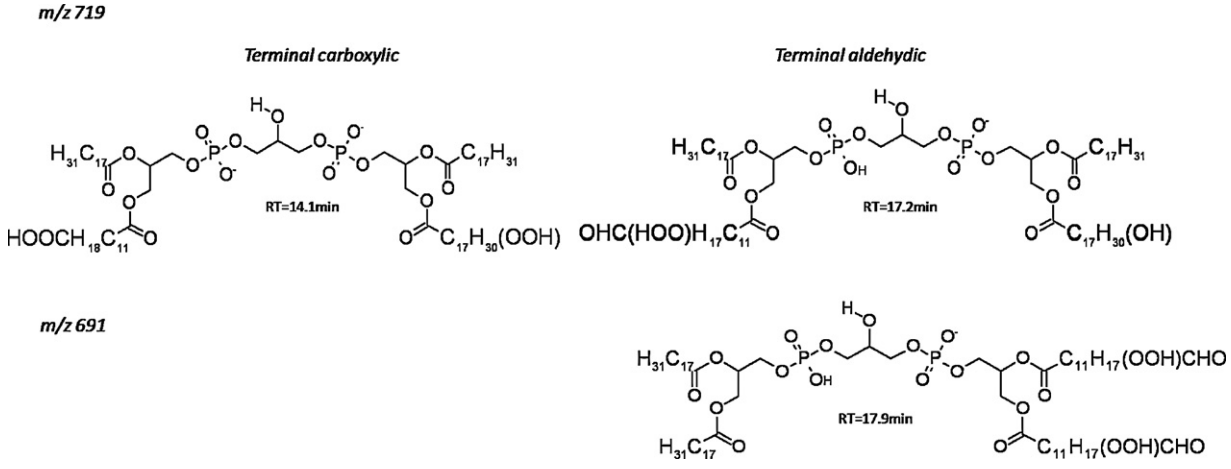
in Scheme 1. The ion at *m/z* 415 is quite abundant and corresponds to the loss of RCOOH from the phosphatidic acid (PA), with formation of the ion $[PA-RCOOH-H]^-$. In this spectrum, we can observe similar fragmentation pathways as previously described by Hsu et al. [29].

In this work, the authors show that if different fatty acyl chains are present in *sn*-1 (or *sn*-1′)and *sn*-2 (or *sn*-2′) of CL, it is expected to see in the MS/MS spectrum of $[M-2H]^-$ ion the product ions $R_2COO^- > R_1COO^-$, $[M-2H-R_2COO^-]^- > [M-2H-R_1COO^-]^-$ and $[M-2H-R_2{=}C{=}O]^{2-} > [M-2H-R_1{=}C{=}O]^{2-}$ [29].

In resume, the $[M-2H]^{2-}$ mass spectrum exhibits three types of products ions that are informative of the structure of CL



Scheme 1. Fragmentation pathways observed in the LC–MS/MS spectrum of the $[M-2H]^{2-}$ ion of tetra-linoleoyl CL (*m/z* 723).



Scheme 2. Short chain products with C13 at *m/z* 719 and 691.

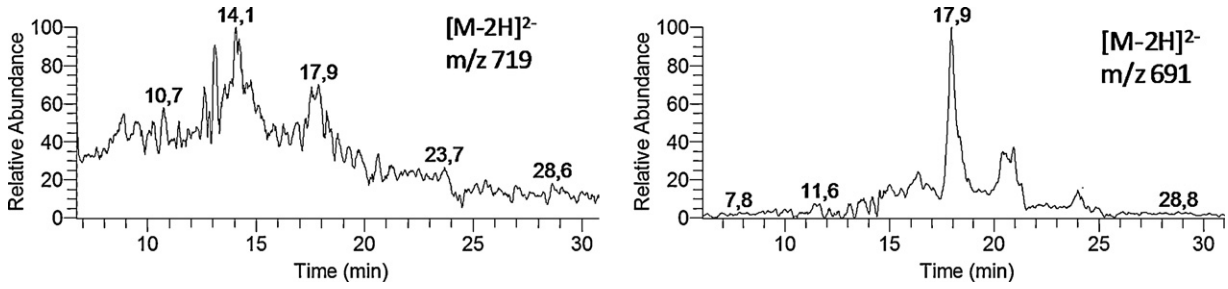


Fig. 3. RIC of the $[M-2H]^{2-}$ ions at *m/z* 691 and 719.

and that that will be used for the assignment of the CL oxidation products. These product ions are: (a) the carboxylate anions ($RCOO^-$); (b) the phosphatidic acid less one fatty acyl chain $[PA-RCOOH-H]^-$ and (c) loss of the $RCOO^-$ yielding the monocharged ion $[(M-2H)-RCOO^-]^-$.

3.1. Short chain oxidation products with fatty acyl chain shortened with C13 carbon length

Based on the molecular weight, the CL oxidation product observed at *m/z* 719, $[M-2H]^{2-}$, correspond to a modified CL molecule with one C13 shortened acyl chain, with an carboxylic acid or aldehyde terminal and also bearing additional 2 or 3 oxygen atoms, probably in other linoleic fatty acyl chains. The oxidative product observed at *m/z* 691, $[M-2H]^{2-}$, may correspond to CL bearing two modified linoleic fatty acyl chain by β-cleavage with formation of two short fatty acyl chain at C13, also with terminal carboxylic acid and plus two oxygen atoms. The reconstructed ion chromatograms (RIC) obtained for these short chain products in C13, (ions at *m/z* 691 and 719) identified as $[M-2H]^{2-}$, are shown in Fig. 3. The ion at *m/z* 691 elutes only in one peak, suggesting that this oxidation product corresponds to only one compound. The ion at *m/z* 719 elutes in two low abundant peaks, meaning that two isomers are present. The LC–MS/MS of these precursor ions are shown in Fig. 4 and are summarized in Table 1. The tandem mass spectra of each of these oxidation prod-

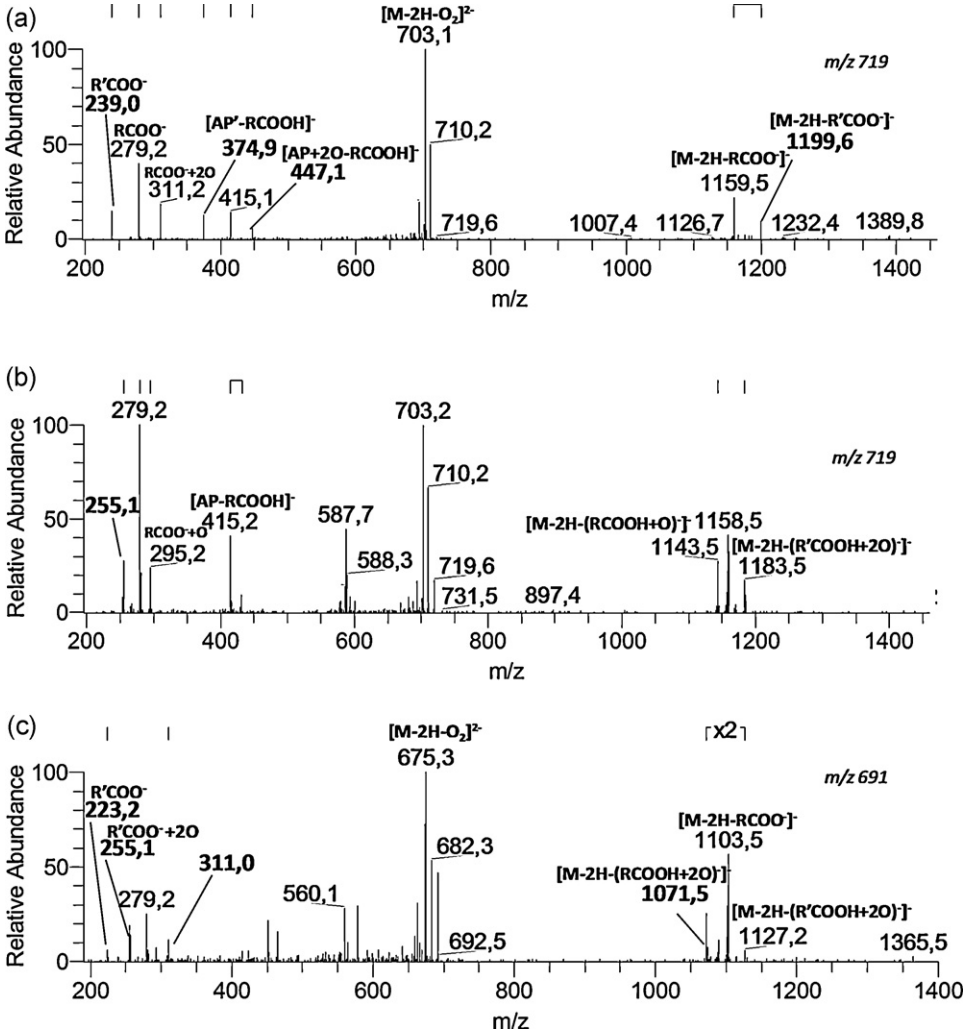


Fig. 4. LC–MS/MS spectra of the ions at *m/z* 719: (a) RT = 14.1 min; (b) 17.2 min and (c) ion at *m/z* 691 with RT = 17.9 min.

Table 1
Resume of the main product ions observed in the LC–MS/MS spectra of the CL short chain oxidation products formed from β-cleavage of alkoxy intermediates in C13.

	719 (RT = 14.1) Acid + 2O	719 (RT = 17.2) Aldehyde + 3O	691 (RT = 17.5) 2 Aldehyde + 4O
$[M-2H-RCOO^-]^-$	1159	1159	1103
$[M-2H-(RCOOH+O)^-]^-$	–	1143	–
$[M-2H-(RCOOH+2O)^-]^-$	1127	–	1071
$[M-2H-R'COO^-]^-$	1199	–	–
$[M-2H-(R'COOH+2O)^-]^-$	–	1183	1127
$[PA-RCOOH-H]^-$	415	415	–
$[PA+O-(RCOOH)-H]^-$	–	431	–
$[PA+2O-(RCOOH)-H]^-$	447	–	–
$[PA'-RCOOH-H]^-$	375	–	–
$RCOO^-$	279	279	279
$[RCOO+O]^-$	–	295	–
$[RCOO+2O]^-$	311	–	311
$R'COO^-$	239	–	223
$[R'COO+2O]^-$	–	255	255

ucts will be discussed and presented separately in the following text.

These oxidation products were formed by initial abstraction of one hydrogen atom from the C13, with further uptake of one O_2, with formation of a peroxyl radical intermediate. This intermediate generates the correspondent C13 alkoxyl radical that can undergo cleavage with formation of an aldehyde that further oxidize to terminal carboxylic function. These shortened chain products are typical of lipid peroxidation [25] and their formation was observed under oxidative conditions in PC and PE phospholipids [26,27]. The structure of these CL short chain oxidation products were confirmed by LC–MS/MS of the correspondent $[M-2H]^{2-}$.

3.2. Short chain product at m/z 719

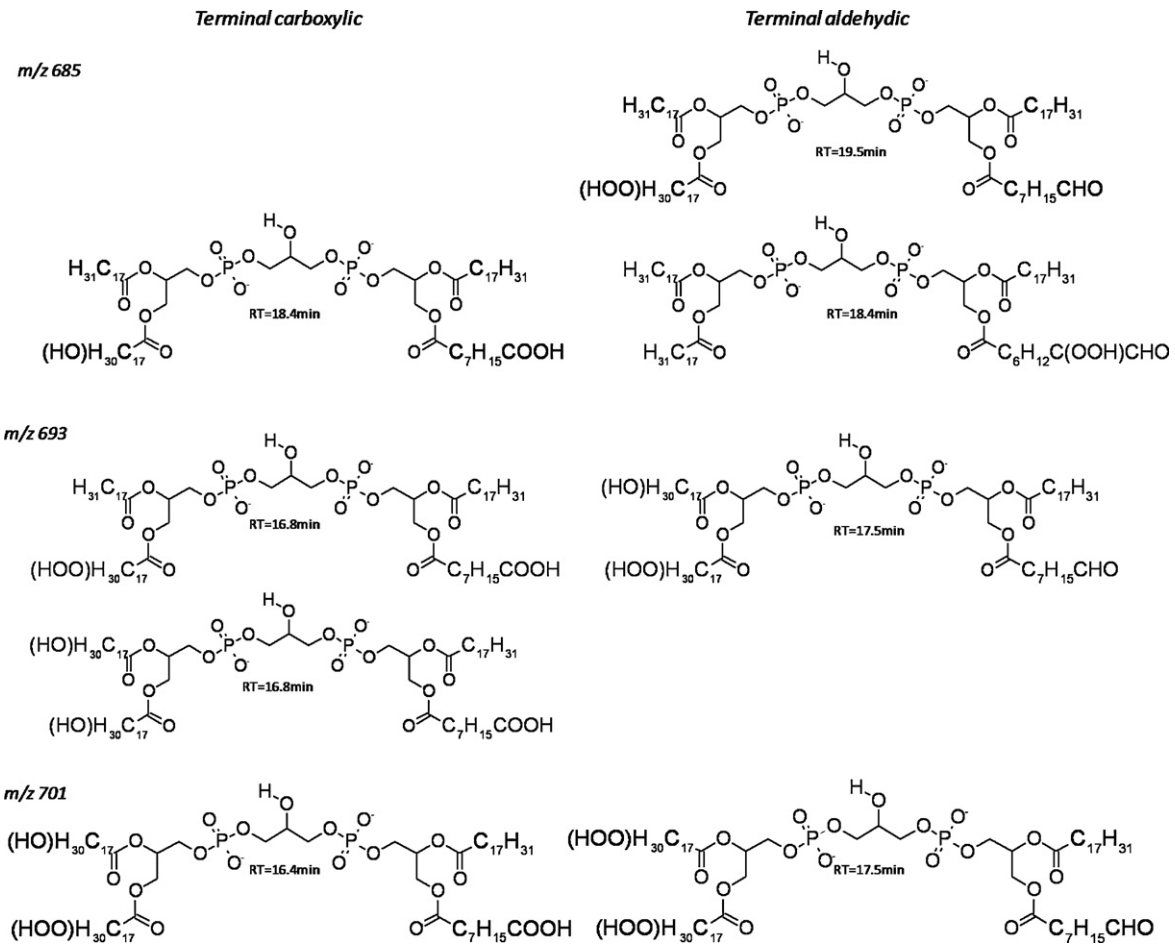
The short chain oxidation product attributed to the ion at *m/z* 719 elutes at 14.1 and 17.2 min. The LC–MS/MS spectra (Fig. 4a) obtained at the two elution times show different product ions confirming that two distinct compounds were separated. The first oxidation product to elute (RT = 14.1 min), corresponding to the most polar structure, shows the presence of the ion with one charge at *m/z* 1199 corresponding to $[M-2H-R'COO^-]^-$ where $R'COO^-$ is the shortened fatty acyl chain with the additional terminal carboxylic acid, with C13. In fact, the product ion at *m/z* 239, attributed to carboxylate anion $R'COO^-$ of $HOOC(CH_2)_{11}COOH$, corroborates the presence of the shortened dicarboxylic acid. The ion at *m/z* 375 attributed to $[PA'-RCOOH-H]^-$ (Scheme 1) confirms also the presence of the modified fatty acyl chain with terminal carboxylic acid in C13 (Scheme 3). Along the manuscript the shortened fatty acyl chains will be generally indicated as R′COOH, independently of the length of the chain. This oxidation product contains two additional oxygen atoms in one of the others fatty acyl chain as a peroxyl group, confirmed by the product ion at *m/z* 311 ($RCOO^-$+2O) and the ion at *m/z* 447, corresponding to the $[PA+2O-RCOOH-H]^-$. The presence of the hydroperoxide was inferred by observing the loss of O_2 from $[M-2H]^{2-}$, leading to $[M-2H-O_2]^{2-}$, at *m/z* 703. Loss of O_2 has already been observed in hydroperoxide derivatives [30]. Formation of hydroperoxides in CL was observed by Kagan et al. [16] during CL oxidation, corroborating the present outcome. In the LC–MS/MS spectrum it was also possible to see the product ions at *m/z* 279 and 415, confirming the presence of unmodified linoleic acid. Altogether, the data allows proposing that this oxidation product is CL modified with one short fatty acyl chain in C13, one linoleic acid with a hydroperoxy and two non-modified linoleic fatty acyl chains ($((C_{18:2})_2/(C_{18:2+OO})/(C_{12}COOH)$) (Scheme 2).

Scheme 3. Fragmentation pathways of the $[M-2H]^{2-}$ ion at *m/z* 719.

The CL oxidation product that elutes at RT = 17.2 min, corresponds to a less polar compound, thus probably with a C13 fatty acyl chain with a terminal aldehyde and the CL modified with plus three oxygen atoms. The presence of the product ion at *m/z* 703, formed by loss of O_2 from the $[M-2H]^-$ ion confirmed the presence of a hydroperoxy group. Thus this oxidation product must have a hydroxy and a hydroperoxy moieties and a shortened C13 aldehyde. The question arises where these hydroxyl and peroxyl moieties are linked. The presence of abundant ions at *m/z* 279 and the ion at *m/z* 415 and *m/z* 1159 $[M-2H-RCOO^-]^-$ show the presence of at least one non-modified linoleic acid. The product ion at *m/z* 295 ($RCOO^-$+O) and the loss of $RCOO^-$+O with formation of the ion $[M-2H-(RCOO+O)^-]^-$ at *m/z* 1143, as well as the ion at *m/z* 431 (415 + 16), confirm the presence of a hydroxyl linoleoyl. The loss of $R'COO^-$+2O (ion at *m/z* 1183), due to the loss of the shortened fatty acyl moiety with a terminal aldehyde plus two oxygen, and the formation of the carboxylate anion $R'COO^-$+2O at *m/z* 255 is indicative of the presence of the hydroperoxy in the shortened C13 aldehyde. This identification is confirmed by the absence of the carboxylate anion of linoleoyl-hydroperoxide at *m/z* 311 ($RCOO^-$+2O) and the absence of the ion due to loss of ($RCOO^-$+2O) (expected at *m/z* 1127). Altogether, the data allows proposing that this oxidation product has two non-modified linoleic acids, one hydroxyl linoleic acid and a short C13 aldehyde with a hydroperoxide ($((C_{18:2})_2/(C_{18:2+O})/(C_{12:2+OO}CHO)$) (Scheme 2).

3.3. Short chain product at m/z 691

The RIC chromatogram of the $[M-2H]^{2-}$ ion at *m/z* of 691 shows only one peak (RT = 17.9 min), suggesting the elution of only one compound. Based on the molecular weight, it seems that this ion



Scheme 4. Proposed structures for the short chain products with C9 observed at *m/z* 685, 693 and 701, and formed during CL oxidation by the hydroxyl radical generated under Fenton reaction conditions.

has two shortened fatty acyl chains and considering the retention time in comparison with the ion at *m/z* 719, we propose that it contains aldehyde terminal in C13, and also four additional oxygen atoms (Scheme 2). A modified CL with two carboxylic moieties with plus two oxygen should elute earlier, due to higher polarity conferred by the carboxylic acid.

The presence of the ion due to loss of O_2 (*m/z* 675 $[M-2H-O_2]^{2-}$) suggests the presence of a hydroperoxide derivative. The product ions observed in the lower mass region of the spectrum, show the $RCOO^-$ at *m/z* 279, and another ion, at *m/z* 255, with similar relative abundance that is attributable to $R'COO^-$ with C13 shortened with an aldehyde and plus two oxygen atoms. This is consistent with the fatty acyl chain with C13 and a terminal aldehyde bearing also a hydroperoxide. A small ion at *m/z* 223 ($RCOO^-$ of a C13 aldehyde) may be formed by loss of O_2 from the ion at *m/z* 255, since loss of O_2 is a predominant fragmentation pathway from hydroperoxy derivatives, as observed from the previous ion. This confirms the presence of the aldehyde bearing hydroperoxy rather than a shortened fatty acyl with a terminal carboxylic acid bearing a hydroxy group. Analysis of higher mass region of the LC–MS/MS spectrum (Fig. 4c) show the product ions due to loss of a non-modified chain (−280 Da) −RCOOH at *m/z* 1103 and also the ion due to loss of $R'COO^-$+2O, (loss of 256 Da) at *m/z* 1127. These product ions allow proposing the formation of an oxidation product of CL with two shortened aldehyde with C13 each with a hydroperoxide and two non-modified linoleic acids $(C_{18:2})_2/(C_{12+OO}CHO)_2$ (Scheme 2).

The low abundant ions at *m/z* 1071 (−$RCOO^-$+2O) and 311 and 293 (311-H_2O), observed in the LC–MS/MS suggested the co-elution of a positional isomer, with a linoleoyl-hydroperoxide, a non- modified linoleoyl residue, one shortened aldehyde, and a shortened hydroperoxy-aldehyde $(C_{18:2})/(C_{18:2+OO})/(C_{12}CHO)/(C_{12+OO}CHO)$.

3.4. Short chain oxidation products with fatty acyl chain shortened with C9 carbon length

Interestingly, other short chain compounds, with one linoleic acid shortened with C9 length were observed in the LC–MS spectrum, and identified as $[M-2H]^{2-}$ ions at *m/z* 685, 693 and 701. They showed higher relative abundance when compared with the other C13 shortened chains oxidation products. During oxidation of other phospholipids, namely phosphatidylcholine and phosphatidylethanolamine with a linoleic acid, it was observed that the short chain products with C9 were most abundant than the ones in C13 [26,27] and these results are consistent with those findings.

The CL oxidation products with C9 chains were analyzed by LC–MS and MS/MS in order to identify the specific structure and the presence of isomers. The RIC obtained for these ions at *m/z* 685, 693 and 701 are shown in Fig. 5. Observing the RIC for all ions we can verify the separation of two compounds with same *m/z* value, probably one with a carboxylic terminal, eluting earlier, and another with an aldehyde terminal in C9. A more detailed analysis was conducted using LC–MS/MS, and the detailed information for each ion is resumed in Table 2.

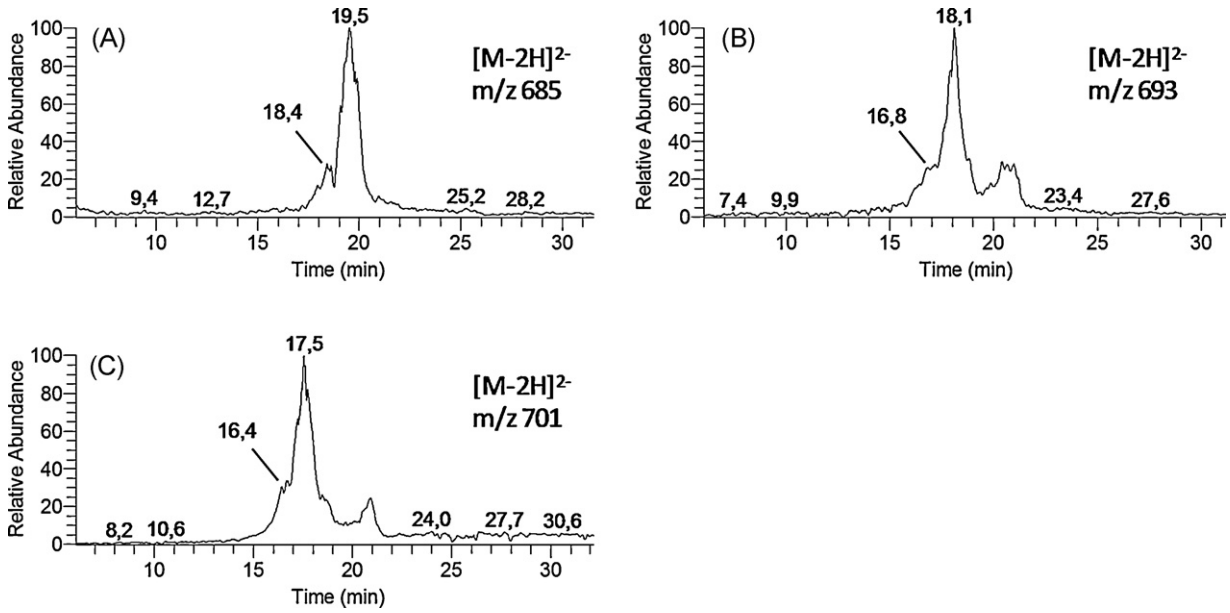


Fig. 5. RIC of CL short chain products with C9: (a) *m/z* 685; (b) *m/z* 693; (c) *m/z* 701.

3.5. Short chain product at m/z of 685

The ion at *m/z* 685 may correspond to CL with a carboxylic acid with C9 plus one oxygen atom in CL or a molecule with a short aldehyde in C9 with plus two oxygen atoms. The presence of the two peaks in the LC profile suggests that both products were present. In this case, the compound that elutes first, at 18.4 min may have a carboxylic acid terminal and the second compound, eluting at RT = 19.5 min has an aldehyde terminal.

LC–MS/MS obtained for the ion that elute in the first peak (RT = 18.4 min) (Fig. 6a) shows, in the higher mass region, the ions formed by the loss of non-modified linoleic acid at *m/z* 1091 ($[M-2H-RCOO^-]^-$), the ion formed by the loss of the shortened acid in C9 at *m/z* 1183 ($[M-2H-R'COO^-]^-$, and the ion $[AP-R'COOH-H]^-$ at *m/z* of 323. Altogether, the data allows proposing that this oxidation product is CL-$(C_{18:2})_2/(C_{18:2+OH})/(C_8COOH)$. Moreover the presence of the ions at *m/z* 1167 (due to loss of short aldehyde plus two oxygen) and at *m/z* of 339 ($[PA'-RCOOH+2O-H]^-$ indicates that another isomer co-elute, identified as CL-$(C_{18:2})_2/(C_{18:2})/(C_{8+OO}CHO)$, with a C9 fatty acyl chain with terminal aldehyde bearing an hydroperoxy group.

The peak at 19.5 shows in the high mass region of the LC–MS/MS spectrum (Fig. 6b), the ions due to loss of non-modified linoleic acid at *m/z* 1091, loss of an oxidized linoleic acid with a peroxyl (RCOO+2O) at *m/z* 1059 and the loss of the short aldehyde at *m/z* 1199. Scheme 5 shows the fragmentation pathways observed for this ion. In carboxylate anions region it is possible to see the ions at *m/z* 279 and 311, corresponding to $RCOO^-$ and $[RCOO+2O]^-$, respectively. Other product ions at *m/z* 307 and 447, assigned as $[PA'-RCOOH-H]^-$ and $[PA+2O-(RCOOH)-H]^-$, confirms the presence of CL with modified fatty acyl chains: $(C_{18:2})_2/(C_{18:2+OO})/(C_8CHO)$.

3.6. Short chain product at m/z of 693

This oxidation product elutes in two peaks at RT = 16.8 and 18.1 min. Based on the molecular weight and considering that the compound that elutes first must be more polar, we propose that the first oxidation product to elute has a carboxylic acid terminal in the C9 short fatty acyl chain and two additional oxygen atoms in CL and the second to elute may have a C9 short fatty acyl chain with a terminal carbonyl group with three additional oxygen atoms. LC–MS/MS data was used to confirm the structure of these CL oxidation products. The LC–MS/MS spectra at those retention times show, as major product ion, the product ion corresponding to the loss of a neutral with 32Da, which confirm the presence of a hydroperoxy group in both molecules.

Table 2
Resume of the main product ions observed in the LC–MS/MS spectra of the CL short chain oxidation products formed from β-cleavage of alkoxy intermediates in C9.

	685 (RT = 18.0) Acid + O	685 (RT = 20.2) Aldehyde + 2O	693 (RT = 15.9 Acid + 2O	693 (RT = 17.9) Aldehyde + 3O	701 (RT = 14.7) Acid + 3O	701 (RT = 16.4) Aldehyde + 4O
$[M-2H-RCOO^-]^-$	1091	1091	1107	1107	1123	1123
$[M-2H-(RCOO^-+O)^-]^-$	–	–	1091	1091	1107	1107
$[M-2H-(RCOO^-+2O)^-]^-$	–	1059	1075	1075	1091	1091
$[M-2H-R'COO^-]^-$	1183	1199	1199	1215	1215	1231
$[M-2H-(R'COO+O)^-]^-$	1167	–	–	–	–	–
$[PA-R{=}C{=}O-H]^-$	–	433	433	–	–	–
$[PA-RCOOH-H]^-$	415	415	415	–	415	415
$[PA+O-(RCOOH)-H]^-$	–	–	431	431	–	–
$[PA+2O-(RCOOH)-H]^-$	–	447	447	–	447	447
$[PA'-RCOOH-H]^-$	323	307	323	307	–	–
$[AP'+O-(RCOOH)-H]^-$	339	–	–	–	–	–
$RCOO^-$	279	279	279	279	279	279
$RCOO^-+O$	–	–	295	295	295	–
$RCOO^-+2O$	–	311	311	311	311	311

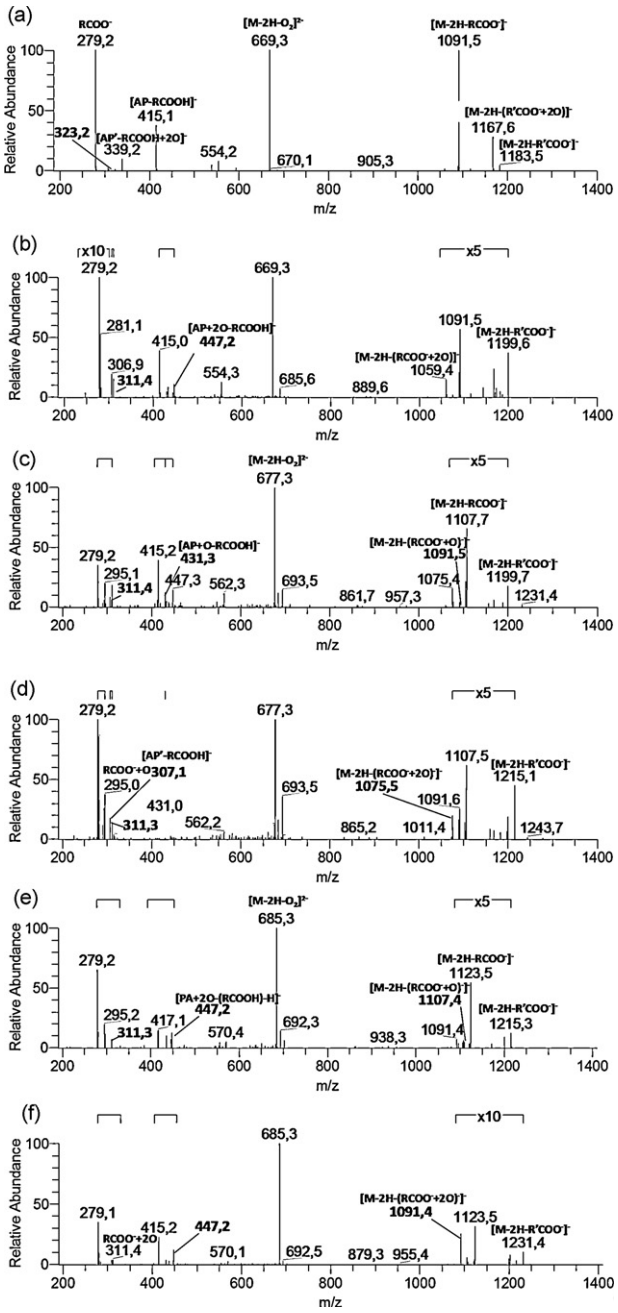
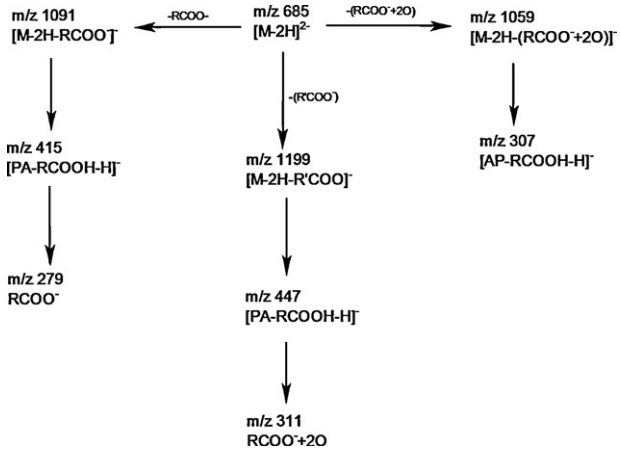


Fig. 6. LC–MS/MS spectra of the ions at *m/z* 685: (a) RT = 18.4 min; (b) 19.5 min, of the ion at *m/z* 693; (c) RT = 16.8 min; (d) RT = 18.1 min and of the ion at *m/z* 701; (e) RT = 16.4 min; (f) RT = 17.5 min.



Scheme 5. Fragmentation pathways of the $[M-2H]^{2-}$ ion at *m/z* 685 with aldehyde terminal in C9 (RT = 19.5 min).

In the LC–MS/MS (Fig. 6c) of the compound that elutes first (RT = 16.8 min) we can observe in the high mass region: the product ion corresponding to the losses a non-modified fatty acyl chain $[M-2H-RCOO^-]^-$ at *m/z* 1107, a product ion formed by loss of a fatty acyl chain with an additional oxygen $[M-2H-(RCOO+O)^-]^-$ at *m/z* 1091, a product ion formed by loss of a fatty acyl chain with two additional oxygens $[M-2H-(RCOO+2O)^-]^-$ at *m/z* 1075 and other ion due to the loss of the short chain fatty acyl chain with a carboxylic acid terminal $[M-2H-R'COO^-]^-$ at *m/z* 1199. A low abundant ion at *m/z* of 323, $[AP'-RCOOH-H]^-$, confirms the presence of a short chain with carboxylic acid terminal. Knowing that the compound only has two additional oxygen atoms the presence of all these ions indicates that two isomers co-elute, regarding the oxygen atoms distribution along the linoleic acyl chains, thus having an isomer with two hydroxy linoleoyl acyl (due to loss of $RCOO^-+O$) and the other one, one peroxy linoleoyl acyl chain (due to loss of $RCOO^-+2O$). The anions $[RCOO+O]^-$ and $[RCOO+2O]^-$ ions at *m/z* 295 and 311 respectively, confirms the presence of two oxidation products of the CL $(C_{18:2})/(C_{18:2+O})_2/(C_8COOH)$ and $(C_{18:2})_2/(C_{18:2+OO})/(C_8COOH)$ (Scheme 4).

The compound that elute later (RT = 18.1 min), is a less polar compound, suggesting the presence of the short chain fatty acyl bearing a terminal aldehyde terminal, with three additional oxygen atoms in the CL molecule. The LC–MS/MS spectrum (Fig. 6d) shows the product ions corresponding to the loss of four fatty acyl moieties: $[M-2H-(RCOO)^-]^-$ at *m/z* 1107, $[M-2H-(RCOO+O)^-]^-$ at *m/z* 1091, $[M-2H-(RCOO+2O)^-]^-$ at *m/z* 1075 and an ion formed by loss of the shortened fatty acyl chain with C9 and a terminal aldehyde $[M-2H-R'COO^-]^-$ at *m/z* 1215. Altogether, the data allows proposing that this oxidation product is $(C_{18:2})/(C_{18:2+O})/(C_{18:2+OO})/(C_8CHO)$. In the lower mass region of this LC–MS/MS spectrum, several carboxylate anions corresponding to the non-modified and oxidized fatty acyl chain were assigned: $RCOO^-$, $RCOO^-+O$ and $RCOO^-+2O$ at *m/z* 279, 295 and 311 respectively. The ion $[PA-RCOOH-H]^-$ at *m/z* 307 is also present, confirming the presence of the C9 short chain with terminal aldehyde, providing further evidence to the presence of this oxidation product of CL (Scheme 4).

3.7. Short chain product at m/z of 701

The RIC of the ion $[M-2H]^{2-}$ at *m/z* of 701 (Fig. 5c) shows two distinct peaks. Considering the molecular weight and the fact that these ions correspond to the presence of an additional oxygen when compared with the oxidation products discussed above, it is suggested that the compound that elutes first has a C9 short chain with a carboxylic terminal and three additional oxygen in CL and the second should has a C9 short chain aldehyde and four additional oxygen atoms in CL.

For the compound that eluted at RT = 16.4 min the LC–MS/MS spectrum (Fig. 6e) shows on the higher mass region the product ions formed by the loss of the fatty acyl moieties, namely $[M-2H-RCOO^-]^-$ at *m/z* of 1123, $[M-2H-(RCOO+O)^-]^-$ at 1107, $[M-2H-(RCOO+2O)^-]^-$ at *m/z* 1091 and loss of short fatty acyl chain as $[M-2H-(R'COO^-]^-$ at *m/z* 1215. This means that this oxidation product has one fatty acyl non-modified chain, one fatty acyl chain with an additional oxygen, one chain with two oxygens and one C9 short chain with carboxylic terminal. The carboxylate anion observed at *m/z* 279 $(RCOO^-)$ confirms the presence of a non-modified chain while the presence of the product ion at *m/z* 295 $(RCOO^-+O)$ confirms the presence of a chain

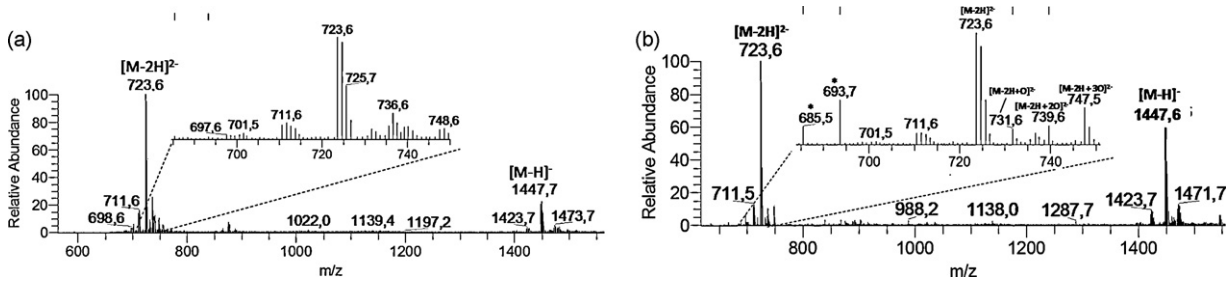


Fig. 7. ESI-MS spectra obtain from cardiolipin extracted from mitochondria of rats control (a) and with treatment with gentamicin (b).

with one additional oxygen. The ions at *m/z* 311 ($RCOO^-$+2O) and 447 ([PA+2O–(RCOOH)–H]$^-$) corroborate the presence of linoleoyl chain with two additional oxygen atoms. Altogether, the data allows proposing that this oxidation product is a CL with $(C_{18:2})/(C_{18:2+O})/(C_{18:2+OO})/(C_8COOH)$ (Scheme 4).

The analysis of the LC–MS/MS spectrum of the compound eluting at 17.5 min (Fig. 6f) shows abundant product ions at *m/z* 1123 and 1091, [M–RCOOH–H]$^-$ and [M–(RCOOH+2O)–H]$^-$ respectively, indicative of the presence of a non-modified chain and a linoleoyl chain with two additional oxygens. The ions [M–2H–R′COO$^-$]$^-$ at *m/z* 1231 confirm the presence of the short chain C9 with aldehyde terminal. It is also observed the ion [PA+2O–(RCOOH)–H]$^-$ at *m/z* 447 and the carboxylate anions at *m/z* 279 ($RCOO^-$), at *m/z* 311 ($RCOO^-$+2O). Therefore the ion [M–2H]$^{2-}$ at *m/z* of 701 is a CL oxidation product with $(C_{18:2})/(C_{18:2+OO})_2/(C_8CHO)$ (Scheme 4).

3.8. Identification of short chain oxidation products of CL in mitochondria

It is known that administration of the antibiotic gentamicin induces nephrotoxicity and that this adverse effect of the aminoglycosides is mediated by activation of mitochondria apoptosis pathways, namely by the release of cyt *c* to the cytosol [9,11,31–33]. Cyt *c* release has an essential role in apoptosis and it is considered to be initiated by CL oxidation [3,34,35]. However, gentamicin induced nephrotoxicity has also been co-related with lipid peroxidation [10]. Based on these findings we studied changes in CL found mitochondria's from rat kidney subjected to a treatment with gentamicin. Information was compared with the ones from mitochondria's of rat controls (without treatment). The presence of nephropathy in the sample group, after gentamicin administration, was confirmed by the urea and creatinine increase in plasma (data not shown). Phospholipids of mitochondria were isolated and fractionated by thin-layer chromatography. The cardiolipin pools were extracted and analyzed by ESI-MS and the phospholipids identified were analyzed by ESI-MS/MS.

In Fig. 7, it is shown the ESI-MS spectra of the CL from mitochondria from control rats and from rat treated with gentamicin. It is possible to see as major ions the ions at *m/z* 1447 [M–H]$^-$ and 723 [M–2H]$^{2-}$ (tetra-linoleoyl CL). Other cardiolipin molecules were identified but with lower abundance. Further analysis of the mass spectra allowed us to confirm the presence of CL oxidative species, in the extracts obtained from rats treated with gentamicin. The

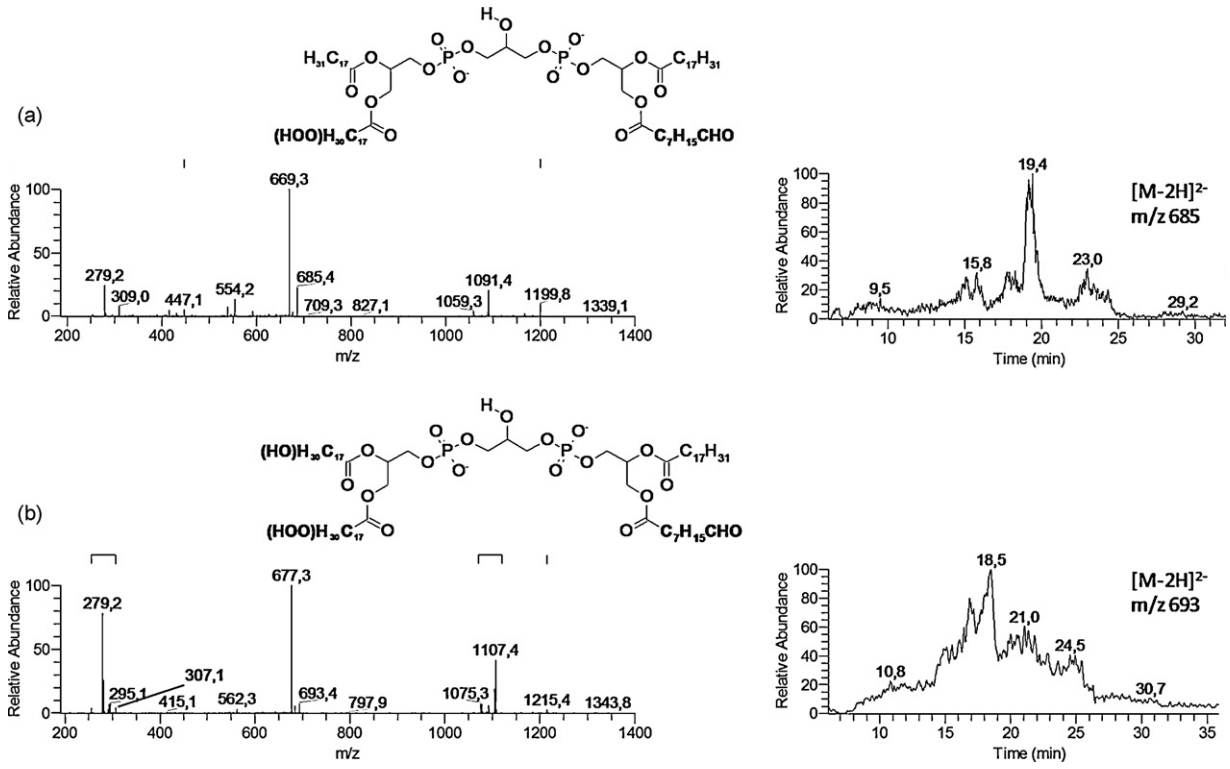


Fig. 8. LC–MS/MS spectra and RIC chromatograms of the ions at *m/z* 685 (a) and 693 (b) observed in the ESI-MS spectra of cardiolipin from rats treated with gentamicin and attributed to the short chain oxidation product of cardiolipin: $(C_{18:2})2/(C_{18:2+OO})/(C_8CHO)$ and $(C_{18:2})/(C_{18:2+O})/(C_{18:2+OO})/(C_8CHO)$, respectively.

most abundant oxidation products identified as $[M-2H]^{2-}$ ions correspond to the tetra-linoleoyl CL (the most abundant CL observed in the extract from rat control) hydroxy and peroxy derivatives at *m/z* 731 (723 + 8) and 739 (723 + 16), derived from the oxidation of the most abundant CL. Hydroxy and peroxy CL are typical CL oxidation products, as reported by Kagan and co-workers [16]. This result evidences the occurrence of lipid peroxidation and specifically cardiolipin oxidation, during gentamicin induced nephrotoxicity.

Moreover, in the lower mass region of the spectrum of the CL extract of mitochondria from rat treated with gentamicin, we can see new ions at *m/z* 685 and 693, which are absent in the spectrum of the control rats. These products have the same *m/z* values and by LC–MS analysis showed a similar retention time (Fig. 8) of some of the new short chain products of CL that were identified in our model for oxidation of CL molecule and reported above. They were identified as being short chain products with C9 shortened fatty acyl chains. In order to confirm their structure, LC–MS/MS was obtained and the LC–MS/MS spectrum of the ion at *m/z* 685 in Fig. 8a shows the ions at *m/z* 311, 447, 1059 and 1199 which confirm the formation of C9 aldehyde plus a peroxy linoleoyl chain: $(C_{18:2})_2/(C_{18:2+OO})/(C_8CHO)$, as previously identified. Also analysis of the LC–MS/MS spectrum of the ion at *m/z* 693 observed in mitochondria CL extract (Fig. 8b) allow to identify this oxidation product as $(C_{18:2})/(C_{18:2+O})/(C_{18:2+OO})/(C_8CHO)$, based on ions at *m/z* 295, 307, 1075, 1107 and 1215 observed in the LC–MS/MS spectrum.

Since the *in vivo* analysis show that CL oxidation products are formed in mitochondria during nephrotoxicity induced by gentamicin, our results corroborate that gentamicin induced nephrotoxicity and CL oxidation are two correlated events.

4. Conclusions

The short chain products formed by reaction with the hydroxyl radical with cardiolipin were identified by liquid chromatography Electrospray Tandem Mass Spectrometry. These products comprised CL with one short fatty acyl chain containing terminal aldehyde and carboxylic groups, some of them were substituted with hydroperoxide groups. Although CL has four linoleic fatty acyl chains, major oxidations products have only one chain shortened. However, almost all products have an additional hydroperoxide in another linoleoyl chain. Based in the previous results, we were able to find short chain oxidation products (with C9) in mitochondria isolated from kidney rats treated with gentamicin, which could explain the previous mitochondrial dysfunctionality observed in these rats. This finding may open new perspectives in the identification of specific molecular targets that may be helpful in the understanding of the biological process and the discovery of new biomarkers of diseases mediated by CL oxidation.

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References

[1] A.J. Chicco, G.C. Sparagna, Role of cardiolipin alterations in mitochondrial dysfunction and disease, American Journal of Physiology – Cell Physiology 292 (1) (2007) C33–C44.
[2] S. Orrenius, A. Gogvadze, B. Zhivotovsky, Mitochondrial oxidative stress: implications for cell death, Annual Review of Pharmacology and Toxicology 47 (2007) 143–183.
[3] R.H. Houtkooper, F.M. Vaz, Cardiolipin, the heart of mitochondrial metabolism, Cellular and Molecular Life Sciences 65 (16) (2008) 2493–2506.
[4] K. Sas, H. Robotka, J. Toldi, L. Vecsei, Mitochondria, metabolic disturbances, oxidative stress and the kynurenine system, with focus on neurodegenerative disorders, Journal of the Neurological Sciences 257 (1–2) (2007) 221–239.
[5] M Schlame, M.D. Ren, Y. Xu, M.L. Greenberg, I. Haller, Molecular symmetry in mitochondrial cardiolipins, Chemistry and Physics of Lipids 138 (1–2) (2005) 38–49.
[6] S. Pope, J.M. Land, S.J.R. Heales, Oxidative stress and mitochondrial dysfunction in neurodegeneration; cardiolipin a critical target? in: 15th European Bioenergetic Conference, Elsevier Science BV, Dublin, Ireland, 2008.
[7] G.M. Petrosillo, M. Casanova, G. Ruggiero, F.M. Paradies, Mitochondrial dysfunction in rat brain with aging Involvement of complex I, reactive oxygen species and cardiolipin, Neurochemistry International 53 (5) (2008) 126–131.
[8] H. Bayir, V.A. Tyurin, Y.Y. Tyurina, R. Viner, V. Ritov, A.A. Amoscato, Q. Zhao, X.J.J. Zhang, K.L. Janesko-Feldman, H. Alexander, L.V. Basova, R.S.B. Clark, P.M. Kochanek, V.E. Kagan, Selective early cardiolipin peroxidation after traumatic brain injury: an oxidative lipidomics analysis, Annals of Neurology 62 (2) (2007) 154–169.
[9] H. Servais, P. Van Der Smissen, G. Thirion, G. Van der Essen, F. Van Bambeke, P.M. Tulkens, M.P. Mingeot-Leclercq, Gentamicin-induced apoptosis in LLC-PK1 cells: involvement of lysosomes and mitochondria, Toxicology and Applied Pharmacology 206 (3) (2005) 321–333.
[10] C. Yanagida, K. Ito, I. Komiya, T. Horie, Protective effect of fosfomycin on gentamicin-induced lipid peroxidation of rat renal tissue, Chemico-Biological Interactions 148 (3) (2004) 139–147.
[11] H. Servais, A. Ortiz, O. Devuyst, S. Denamur, P.M. Tulkens, M.P. Mingeot-Leclercq, Renal cell apoptosis induced by nephrotoxic drugs: cellular and molecular mechanisms and potential approaches to modulation, Apoptosis 13 (1) (2008) 11–32.
[12] M.R.M. Domingues, A. Reis, P. Domingues, Mass spectrometry analysis of oxidized phospholipids, Chemistry and Physics of Lipids 156 (1–2) (2008) 1–12.
[13] Y.Y. Tyurina, V.A. Tyurin, M.W. Epperly, J.S. Greenberger, V.E. Kagan, Oxidative lipidomics of gamma-irradiation-induced intestinal injury, Free Radical Biology and Medicine 44 (3) (2008) 299–314.
[14] H. Bayir, V.A. Tyurin, Y.Y. Tyurina, R. Viner, V. Ritov, A.A. Amoscato, Q. Zhao, X.J. Zhang, K.L. Janesko-Feldman, H. Alexander, L.V. Basova, R.S.B. Clark, P.M. Kochanek, V.E. Kagan, Selective early cardiolipin peroxidation after traumatic brain injury: an oxidative lipidomics analysis, Annals of Neurology 62 (2) (2007) 154–169.
[15] V.A. Tyurin, Y.Y. Tyurina, W. Feng, A. Mnuskin, J.F. Jiang, M.K. Tang, X.J. Zhang, Q. Zhao, P.M. Kochanek, R.S.B. Clark, H. Bayir, V.E. Kagan, Mass-spectrometric characterization of phospholipids and their primary peroxidation products in rat cortical neurons during staurosporine-induced apoptosis, Journal of Neurochemistry 107 (6) (2008) 1614–1633.
[16] V.A. Tyurin, Y. Tyurina, M.Y. Jung, M.A. Tungekar, K.J. Wasserloos, H. Bayir, J.S. Greenberger, P.M. Kochanek, A.A. Shvedova, B. Pitt, V.E. Kagan, Mass-spectrometric analysis of hydroperoxy- and hydroxy-derivatives of cardiolipin and phosphatidylserine in cells and tissues induced by pro-apoptotic and pro-inflammatory stimuli, Journal of Chromatography B – Analytical Technologies in the Biomedical and Life Sciences 877 (26) (2009) 2863–2872.
[17] I. Yurkova, M. Kisel, J. Arnhold, O. Shadyro, Iron-mediated free-radical formation of signaling lipids in a model system, Chemistry and Physics of Lipids 137 (1–2) (2005) 29–37.
[18] O. Shadyro, I. Yurkova, M. Kisel, O. Brede, J. Arnhold, Formation of phosphatidic acid, ceramide, and diglyceride on radiolysis of lipids: Identification by MALDI-TOF mass spectrometry, Free Radical Biology and Medicine 36 (12) (2004) 1612–1624.
[19] O.I. Shadyro, I.L. Yurkova, M.A. Kisel, O. Brede, J. Arnhold, Radiation-induced fragmentation of cardiolipin in a model membrane, International Journal of Radiation Biology 80 (3) (2004) 239–245.
[20] K. Cain, D.N. Skilleter, Preparation and use of mitochondria in toxicological research, in: K. Snell, B. Mullock (Eds.), Toxicology: A Molecular Approach, IRL Press, Oxford, 1987, pp. 217–253.
[21] A.G. Gornall, C.J. Bardawill, M.M. David, Determination of serum proteins by means of the buiret reaction, The Journal of Biolgical Chemistry 177 (1949) 751–766.
[22] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Canadian Journal of Biochemistry and Physiology 37 (1959) 911–917.
[23] E. Niki, Lipid peroxidation: Physiological levels and dual biological effects, Free Radical Biology and Medicine 47 (5) (2009) 469–484.
[24] P. Spiteller, W. Kern, J. Reiner, G. Spiteller, Aldehydic lipid peroxidation products derived from linoleic acid, Biochimica Et Biophysica Acta – Molecular and Cell Biology of Lipids 1531 (3) (2001) 188–208.
[25] G. Spiteller, Peroxyl radicals: inductors of neurodegenerative and other inflammatory diseases. Their origin and how they transform cholesterol, phospholipids, plasmalogens, polyunsaturated fatty acids, sugars, and proteins into deleterious products, Free Radical Biology and Medicine 41 (3) (2006) 362–387.
[26] A. Reis, P. Domingues, A.J.V. Ferrer-Correia, M.R.M. Domingues, Fragmentation study of short-chain products derived from oxidation of diacylphosphatidylcholines by electrospray tandem mass spectrometry: identification of novel short-chain products, Rapid Communications in Mass Spectrometry 18 (23) (2004) 2849–2858.
[27] M.R.M. Domingues, C. Simoes, J.P. da Costa, A. Reis, P Domingues, Identification of 1-palmitoyl-2-linoleoyl-phosphatidylethanolamine modifications under oxidative stress conditions by LC–MS/MS, Biomedical Chromatography 23 (6) (2009) 588–601.

[28] I.L. Yurkova, F. Stuckert, M.A. Kisel, O.I. Shadyro, J. Arnhold, D. Huster, Formation of phosphatidic acid in stressed mitochondria, Archives of Biochemistry and Biophysics 480 (1) (2008) 17–26.
[29] F.F. Hsu, J. Turk, E.R. Rhoades, D.G. Russell, Y.X. Shi, E.A. Groisman, Structural characterization of cardiolipin by tandem quadrupole and multiple-stage quadrupole ion-trap mass spectrometry with electrospray ionization, Journal of the American Society for Mass Spectrometry 16 (4) (2005) 491–504.
[30] A. Reis, P. Domingues, A.J.V. Ferrer-Correia, M.R.M. Domingues, Tandem mass spectrometry of intact oxidation products of diacylphosphatidylcholines: evidence for the occurrence of the oxidation of the phosphocholine head and differentiation of isomers, Journal of Mass Spectrometry 39 (12) (2004) 1513–1522.
[31] Y.Y. Tyurina, V. Kini, V.A. Tyurin, I.I. Vlasova, J.F. Jiang, A.A. Kapralov, N.A. Belikova, J.C. Yalowich, I.V. Kurnikov, V.E. Kagan, Mechanisms of cardiolipin oxidation by cytochrome *c*: relevance to pro- and antiapoptotic functions of etoposide, Molecular Pharmacology 70 (2) (2006) 706–717.
[32] N.A. Belikova, J. Jiang, Y.Y. Tyurina, Q. Zhao, M.W. Epperly, J. Greenberger, V.E. Kagan, Cardiolipin-specific peroxidase reactions of cytochrome *C* in mitochondria during irradiation-induced apoptosis, International Journal of Radiation Oncology Biology Physics 69 (1) (2007) 176–186.
[33] V.E. Kagan, Y.Y. Tyurina, H. Bayir, C.T. Chu, A.A. Kapralov, I.I. Vlasova, N.A. Belikova, V.A. Tyurin, A. Amoscato, M. Epperly, J. Greenberger, S. DeKosky, A.A. Shvedova, J. Jiang, The "pro-apoptotic genies" get out of mitochondria: oxidative lipidomics and redox activity of cytochrome *c*/cardiolipin complexes, Chemico-Biological Interactions 163 (1–2) (2006) 15–28.
[34] V.E. Kagan, V.A. Tyurin, J.F. Jiang, Y.Y. Tyurina, V.B. Ritov, A.A. Amoscato, A.N. Osipov, N.A. Belikova, A.A. Kapralov, V. Kini, I.I. Vlasova, Q. Zhao, M.M. Zou, P. Di, D.A. Svistunenko, I.V. Kurnikov, G.G. Borisenko, Cytochrome *c* acts as a cardiolipin oxygenase required for release of proapoptotic factors, Nature Chemical Biology 1 (4) (2005) 223–232.
[35] M. Ott, J.D. Robertson, V. Gogvadze, B. Zhivotovsky, S. Orrenius, Cytochrome *c* release from mitochondria proceeds by a two-step process, Proceedings of the National Academy of Sciences of the United States of America 99 (3) (2002) 1259–1263.