Universidade de Aveiro Departamento de Química
2010

Maria Elisabete Alves Maciel

IDENTIFICAÇÃO DOS PRODUTOS DE OXIDAÇÃO DA CARDIOLIPINA POR ESPECTROMETRIA DE MASSA

IDENTIFICATION OF OXIDATION PRODUCTS OF CARDIOLIPIN USING MASS SPECTROMETRY

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Métodos Biomoleculares, realizada sob a orientação científica da Doutora Maria do Rosário Gonçalves Reis Marques Domingues, Professora Auxiliar do Departamento de Química da Universidade de Aveiro

Dedico este trabalho aos meus pais e ao Jorge pelo incansável apoio.

o júri

presidente

Prof. Doutor Armando Jorge Domingues Silvestre
Professor associado com agregação da Universidade de Aveiro

Prof. Doutora Maria Manuel Silva Oliveira
Professora auxiliar da Universidade de Trás-os-Montes e Alto Douro

Prof. Doutora Maria do Rosário Gonçalves Reis Marques Domingues
Professora auxiliar da Universidade de Aveiro

Prof. Doutor Pedro Miguel Dimas Neves Domingues
Professor auxiliar da Universidade de Aveiro

agradecimentos

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Obrigado Família por terem acreditado em mim…

palavras-chave

Cardiolipina, stress oxidativo, radical hidroxilo, mitocondria, espectrometria de massa, LC-MS.

resumo

Cardiolipina (CL) é um fosfolípido de estrutura complexa, que se encontra quase exclusivamente na membrana interna da mitocôndria, onde se encontra associada a diferentes complexos da cadeia respiratória. Pela sua localização, este fosfolípido desempenha um importante papel no metabolismo energético mitocôndrial. Alterações na cardiolipina nomeadamente por modificações oxidativas têm sido relacionadas com a apoptose celular e com várias condições patológicas, particularmente nas doenças neurodegenerativas.

A estrutura da CL apresenta quatro cadeias de ácidos gordos que podem diversificar no comprimento e no grau de insaturação, é muito susceptível a danos oxidativos por espécies reactivas de oxigénio (ROS). A sua localização, na mitocôndria aumenta a probabilidade desta sofrer oxidação, uma vez que existe uma considerável produção de ROS na membrana interna mitocôndrial. ROS estão envolvidas no stress oxidativo em diferentes biomoleculas, nomeadamente nos lípidos, levando a modificações na sua estrutura e consequentemente, à perda da sua função. Apesar da importância da oxidação da CL e das suas possíveis consequências biológicas, existe um conhecimento limitado em relação aos produtos de oxidação formados em condições de stress oxidativo.

Neste estudo, a espectrometria de massa e a espectrometria de massa acoplada à cromatografia liquida (LC-ESI-MS) foram utilizados para identificar modificações oxidativas especificas da tetra-linoleoil CL induzidas pelo radical hidroxilo através da reacção de Fenton (H_2O_2 and Fe^{2+}). Os resultados obtidos neste estudo permitiram identificar pela primeira vez diversos produtos de oxidação de cadeia curta e também outros de cadeia longa durante a oxidação da CL. Os produtos de oxidação de cadeia curta resultam de uma clivagem do ácido gordo oxidado, conduzindo á formação de ácido gordos de cadeia curta com terminal aldeído, e ácidos dicarboxilico, observados em MS na forma de iões $[M-H]^-$ e $[M-2H]^{2-}$. Estes resultados permitiram detectar alguns destes produtos de oxidação na mitocôndria dos rins dos ratos tratados com gentamicina, com nefropatia confirmada.

A identificação detalhada da fragmentação dos produtos de cadeia longa por LC-MS/MS permitiu a diferenciação de isómeros, e a identificação do local de oxidação para CL com 2, 4, 6 e 8 átomos de oxigénios. Este trabalho permitiu identificação de iões produto específicos que permitem a sua atribuição inequívoca, que será fundamental para a identificação destas espécies em amostras biológicas.

keywords

Cardiolipin,oxidative stress, hydroxyl radical, mitochondria, mass spectrometry, LC-MS.

abstract

Cardiolipin (CL) is a phospholipid with a complex structure, found almost exclusively in the inner mitochondrial membrane, where it is found associated with several different complexes of the respiratory chain. Because of its localization, this phospholipid performs an important role in the mitocôndrial energetic metabolism. Alteration of CL namely by oxidative modifications has been related with various pathological conditions, particularly in neurodegenerative diseases.

CL structure bears four chains of fatty acids that can diversify in length and degree of unsaturation; it is susceptible to oxidative damage by reactive oxygen species (ROS). 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. ROS are involved in oxidative stress modifications of different biomolecules, namely lipids, leading to changes in their structure and in consequence, loss of their function. In spite of the importance of CL oxidation and it biological consequence, there is a limited knowledge of the oxidation products of CL formed under oxidative stress.

In this study, mass spectrometry and mass spectrometry coupled with liquid chromatography (LC-MS) was used to identify the specific oxidative modifications of tetra-linoleoyl CL induced by the hydroxyl radical generated under Fenton reaction conditions (H2O2 and Fe2+). The results obtained with this study allowed us to identify, for the first time, several short chain oxidation products along with some long chain products during the oxidation of CL.

Short-chain oxidation products resulted from a cleavage of the oxidized fatty acid, leading to the formation of short chain fatty acids with aldehyde terminal, and dicarboxylic acids, observed in MS in the form of the ions [M-H]- and [M-2H]2-. These results allowed us to detect some of these oxidation products in the mitochondria of kidneys obtained from rats treated with gentamicin, with confirmed nephropathy.

The detailed identification of the fragmentation of the long chain products by LC-MS/MS allowed us to differentiate isomers, and the identification of the oxidation location for the CL with 2, 4, 6 and 8 oxygen atoms. This work allowed the identification of specific product ions which allow their unequivocal assignment that will be paramount for the identification of these species in biological samples.

Index

Chapter I

Introduction

Introduction

Lipidomics is an emerging field that is dedicated to the analysis of lipids from a biological sample, such as tissue, cells, biological fluids, among others. This methodology helps in the study of lipids, their changing in response to different stimulus or situations, as well as their function in biological systems. Phospholipids are important molecules of the biological systems and their study has been growing, namely because the change of their structure induced by oxidative stress conditions that is being associated with several pathological conditions, namely Alzheimer, diabetes, age related diseases and also with inflammatory response.

Oxidative stress causes damage in important biological structures, like proteins, carbohydrates, lipids and nucleic acids. Among phospholipids, cardiolipin oxidation has received particularly special attention since it has been correlated with the cellular apoptosis.

Oxidation reaction of phospholipids can generate numerous oxidation products. The nature of these species may depend on the local and degree of oxidation. It is also known that different oxidation species may have different biological roles in the living species [1].

In spite of the important role of cardiolipin oxidation, there is a limited knowledge about the modifications of this phospholipid that can be generated under oxidative stress conditions. Besides, the possibility of the formation of several oxidation products it turns out that it is important to define exactly which oxidation species are being created, since different cardiolipin oxidation products may exert different biological effects.

Previous studies have lead to speculation that the accumulation of hydroperoxides form cardiolipin may be used has a biomarker of apoptosis *in vivo*. The ability of selective modulation of the cardiolipin oxidation may lead to targeted therapies and in last instance improve results after injury [2].

The identification and characterization of CL oxidations products by mass spectrometry and tandem mass spectrometry will be one important contribute to the fulfillment of the objective mentioned previously and will be executed in this work.

1. Phospholipids

The phospholipids are present in all organisms, from the arcobacter to plants and humans. These hydrophobic molecules are the building blocks of cell membranes, among other functions such as compartmentalization of the cytoplasm, storage of proteins involved in cell signaling, intercellular adhesion and cytoskeleton support, they also serve as a precursor for lipid's biologically active mediators. There are two major groups of phospholipids: glycerophospholipids and sphingolipids.

The glycerophospholipids are the most abundant phospholipids, they have a key role in energy storage and well as in cell structure, they are involved in cell signaling, cell proliferation and may serve as potential biomarkers for several diseases. Glycerophospholipids usually consist of two fatty acids and a phosphate group attached to a glycerol molecule. The phosphate group can bind a polar molecule by an ester bond and this part of the phospholipid molecule is called the headgroup of the phospholipid [3]. These combinations makes the glycerophospholipids, a group that besides abundant is very diverse, since it covers many combinations of lipids that vary according to their length, degree of unsaturation of acyl chains and the composition of the polar head group [4].

The composition of the headgroup is primordial in dividing this phospholipid group to distinct classes, for example if it has a choline, then it belongs to the phosphatidylcholines (PC) class, on the other hand if it has a serine as a headgroup then it is a phosphatidylserine (PS), these classes along with the phosphatidylethanolamines (PE) and phosphatidylinositol (PI) are the main classes of polar phospholipids. It is believed that the temporal and spatial distribution of different classes of lipids has an important biological significance. Each phospholipid class has different combinations of fatty acids in positions *sn*-1 and *sn*-2 on the glycerol [5]. The Figure 1 represents the general structure of the glycerophospholipids.

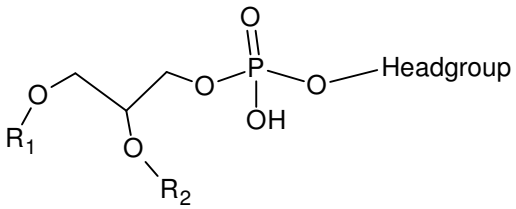
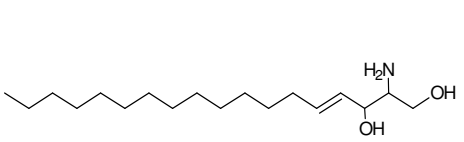


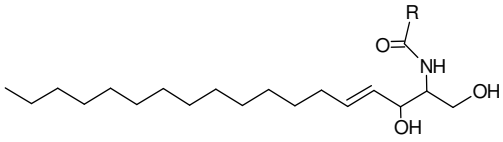
Figure 1: General structure of the glycerophospholipids

The cardiolipins are glycerophospholipids different from those previously presented. They may be considerate a distinct class of glycerophospholipids, since cardiolipin is a dimer that presents two phosphatidic acids linked together through an additional glycerol molecule and also four fatty acyl chains.

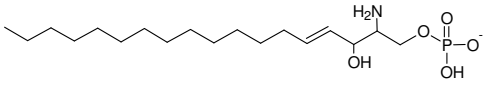
The sphingolipids are amphipathic lipids usually found in cell membranes and contain sphingosine as a backbone (Figure 2). The addition of a fatty acid through an amid bond forms ceramide. Phosphorylation of sphingosine or ceramide results in sphingosine-1-phosphate (S1P) or ceramide-1-phosphate (C1P), respectively [6]. Sphingolipids are emerging as important mediators of cellular signaling, cell growth, and cell death, in addition to their structural contribution to membrane architecture [6-8], per example, sphingolipids are abundant components of the nuclear envelope in mammalian cells, where they modulate cell signaling to profoundly affect the function of the cell [9].



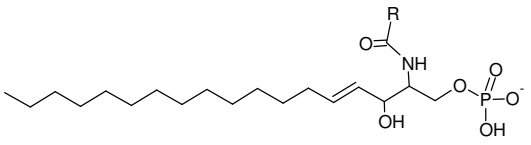
Sphingosine



Ceramide



Sphingosine -1-phosphate



Ceramide-1- phosphate

Figure 2: The molecular structure of sphingosine, sphingosine-1-phosphate, ceramide and ceramide-1-phosphate.

2. Cardiolipin

The cardiolipin (CL) was first isolated in 1942 by Mary Pangborn from bovine heart as 'a substance essential for the reactivity of beef heart antigens in the serologic test for syphilis' [10]. The CL, as described, presents an unusual structure and it can be found in all mammalian tissues, although it is more abundant in mammalian hearts It is found specifically in the mitochondrial inner membrane, where it has a central role in mitochondrial processes and energy metabolism.

In this section I will approach all the features and functions related to the cardiolipin, their association with various proteins, relationship with cytochrome c and mitochondrial apoptosis and also its influence in several pathologies.

2.1. Structure chemical and biosynthetic pathways

CL can be defined as 1,3-bis(sn-3-phosphatidyl)-*sn*-glycerol. It is a dimeric phospholipid in which two phosphatidyl moieties are linked by a central glycerol group and also holds two more molecules of glycerol in its structure. The relationship between the three glycerol molecules creates a unique environment for each ester linkage [11](Figure 3).

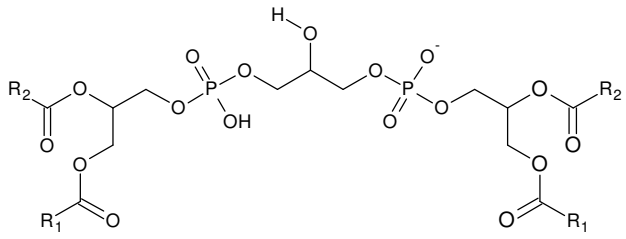
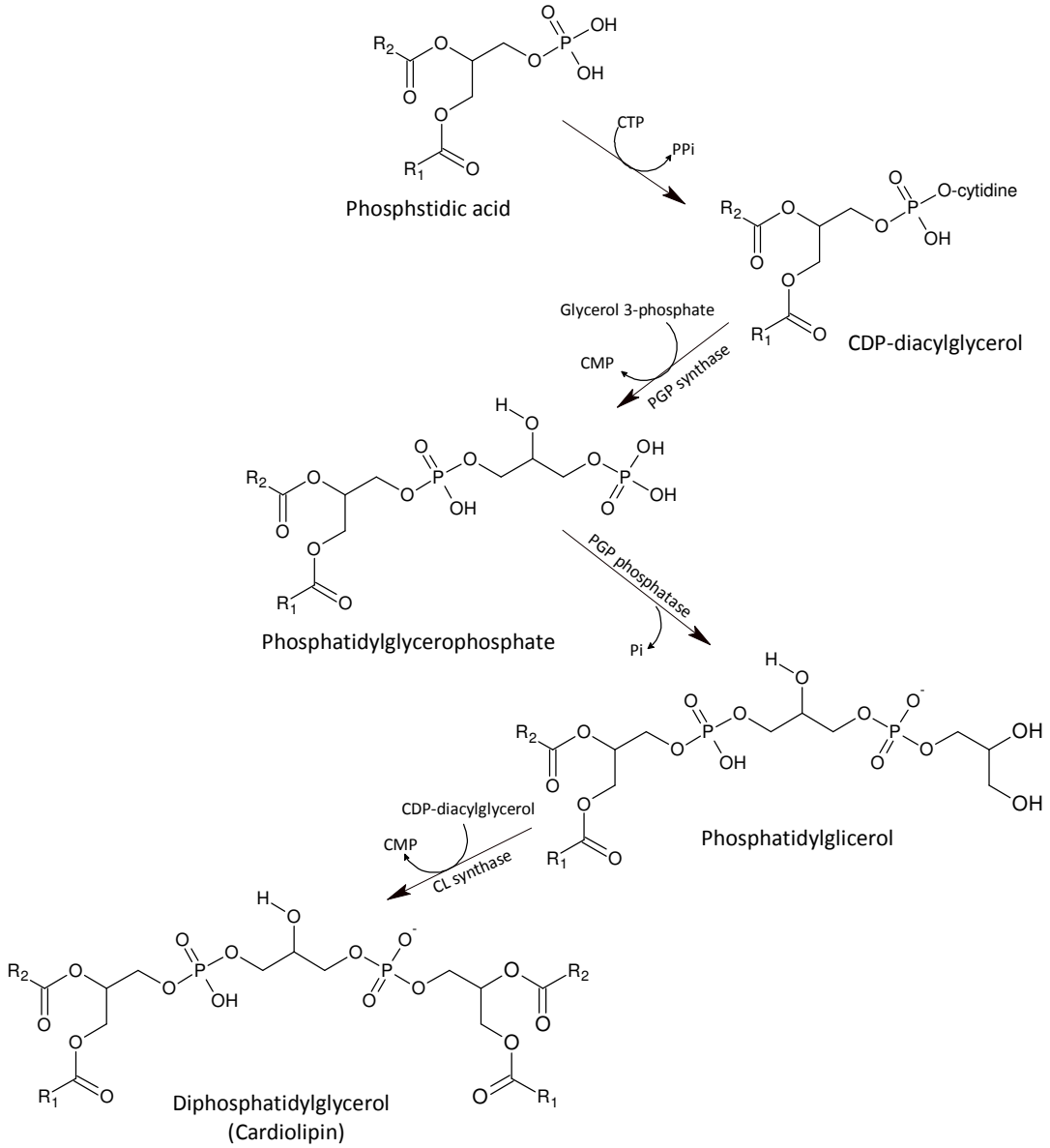


Figure 3: Structure of cardiolipin.

The fact that the headgroup alcohol is shared by two phosphate moieties is a feature with important implications regarding the overall physical properties of CL within the context of a lipid bilayer, namely in their mobility and conformational flexibility [12].

To understand the role of CL in mitochondria, it is necessary to know how it is formed and how it is integrated into mitochondrial membranes. Cardiolipin is synthesized from phosphatidic acid like all phospholipids [13].

However the biosynthesis of the phosphatidic acid occurs in the endoplasmatic reticulum and in the outer mitochondrial membrane [14]. The other steps occur on the matrix side of the inner mitochondrial membrane. The mitochondrial inner membrane contains all the enzymes of the CL pathway, and the final step of this pathway is catalyzed by the cardiolipin synthase [14-15]. The Scheme 1 represented the synthesizing process of the CL from phosphatidic acid via a number of enzymatic steps [11].



Scheme 1: Synthesis of cardiolipin. After synthesis of cytidinediphosphate-diacylglycerol (CDP-DAG) from phosphatidic acid, CL synthesis consist of the three steps, which all take place in the inner mitochondrial membrane. The first step is the formation of phosphatidylglycerophosphate (PGP) from CDP-diacylglycerol 3-phosphate by PGP synthase. In the second step, PGP is dephosphorylated by PGP phosphatase to produce phosphatidylglicerol (PG). In the final step, catalyzed by CL synthase, PG is combined with CDP-diacylglycerol to from CL [13].

2.2. Cardiolipin acyl chain composition and subcellular distribution

The composition of the CL is very diverse even within a single organism. The predominant composition in the mammalian tissue is the CL with linoleic acid (18:2) as fatty acid chains. In the hearth the tetralinoleoyl-CL is the more abundant species (the linoleic acid compromises 80-90% of the CL acyl chains). In the brain the most abundant species is the CL with long unsaturated fatty acid chains like arachidonic (20:4), docosatetraenoic (22:4) and docosahexaenoic (22:6). This instauration of the acyl chains of CL make it very susceptible to oxidative damage by reactive oxygen species (ROS) [13].

Some studies have shown that even though the predominant fatty acid species in CL of organisms may differ, there is a high degree of symmetry in the acyl chain distribution of CL in organisms and in tissues within the same organism [16]. The symmetric distribution of acyl chains is being considered to be important in promoting the structured organization of the mitochondrial membrane and to be of importance for the function of CL, as was reviewed elsewhere [17].

The CL if found almost exclusively in the inner mitochondrial membrane. In the bovine heart mitochondria the CL represents about 25% of all phospholipids.

2.3. Cardiolipin and mitochondria

CL is an integral part of the mitochondrial inner membrane where it is associated with many mitochondrial proteins. Therefore it has always been assumed to have an important role in mitochondrial function. CL maintains inner membrane fluidity and osmotic stability [11, 18] and it has a role in mitochondrial bioenergetics; CL electrostatically anchors cytochrome *c* to the inner mitochondrial membrane [19] and may therefore play an important regulatory role in cytochrome *c* release and apoptosis [20-22]. CL also plays an essential role in mitochondrial biogenesis [11], and the assembly of respiratory enzyme supercomplexes [23-24].

2.3.1. Interaction with mitochondrial proteins

Cardiolipin has a unique ability to interact with a large number of mitochondrial proteins (Table 1), most of which reside in the inner membrane. The mitochondrial enzymes have been shown to require CL for optimal activity.

Table 1: Cardiolipin-dependent mitochondrial proteins [11].

Protein	Mitochondrial compartment
ADP-ATP carrier	Inner membrane
Phosphate carrier	
Pyruvate carrier	
Carnitine carrier	
Complex I (NADH: ubiquinone oxidoreductase)	
Complex II	
Complex III (ubiquinol: Cyt *c* oxidoreductase)	
Complex IV (Cytochrome *c* oxidase)	
Complex V (ATP synthase)	
Cytochrome P450SCC	
Cardiolipin synthase	
Cytochrome c	Intermembrane space
Creatine kinase	

CL interacts with all of the major players in oxidative phosphorylation, including respiratory complexes I, III, IV, and V. Restoration of enzymatic activity in phospholipid-depleted complex I and III has been shown to require the presence of cardiolipin [25]. The complex II has been associated with two acyl chains of one CL molecule from *Escherichia coli* [26]. Transfer of electrons between complex III and IV is dependent on cytochrome c which has been shown to interact loosely with CL [21]. Complex IV has been shown to require two associated CL molecules in order to maintain its full enzymatic function [27]. Complex V of the oxidative phosphorylation machinery also displays high binding affinity for CL, binding four molecules of CL per molecule of complex V [28].

The ADP-ATP carrier is a good example of cardiolipin-protein interaction. The carrier belongs to many transport proteins for mitochondrial metabolites, but it has been shown to be optimal only in the presence of the tetralinoleoyl-CL. Other CL species, such as tetraoleoyl-CL and monolyso-CL, and also other phospholipids were not effective in stimulating the ADP-ATP carrier activity. The phosphate carrier [29] and the mitochondrial creatine kinase [30-31] require CL for their activity, however in contrast to cytochrome P-450 (it has been shown to be optimal after CL binding) [32], other phospholipids cannot substitute the CL [30]. Similarly the carnitine acylcarnitine translocase [33] and the pyruvate carrier [34] revealed to be best efficience in the presence of CL. The cardiolipin synthase also require CL for full activity, which is only achieved in combination with PE [35].

2.3.2. The interaction of CL with cytochrome *c* and its role in apoptosis

Recently many studies focused the relationship of cytochrome c and cardiolipin in cellular apoptosis. Several studies indicate that a structural destabilization of this protein, which can be caused by chemical modifications (i.e. oxidation, nitration) or by association with hydrophobic anions including anionic phospholipids [36-38], reveals a new function as a peroxidase [39]. Other studies show the mechanisms through which cyt *c* peroxidase activity propagates the oxidation of cardiolipin, and the essential role of this reaction in the execution of apoptosis [36, 40-41].

The molecular interaction between CL and cytochrome *c* involves electrostatic interactions at the A-site of cyt *c*, whereas hydrophobic interactions and hydrogen bonding take place at its C-site [19]. To allow the release of cyt c to the cytosol it is necessary that the hydrophobic and electrostatic interactions between the CL and the cyt c are degraded [21], this hypothesis is supported by studies demonstrating that the peroxidation of CL virtually eradicate cytochrome c binding [42].

The Figure 4 shows the regulations of cytochrome c release from mitochondria. At the early stages of apoptosis, CL is redistributed to the outer monolayer of the mitochondrial inner membrane and to the outer mitochondrial membrane [40, 43-44]. Redistribution is liable to occur by interactions with proteins associated with apoptosis, such as Bid, tBid

(truncated Bid, the active form of the protein) and Bax, in contact points between the inner membrane and outer membrane [45]. This redistribution will allow a sufficient amount of CL in intermembrane space so that it interacts with cyt *c*. The CL-cyt *c* complex formed can act with a peroxidase potent in the presence of hydrogen peroxides originating hydroperoxides CL. So cyt *c* release occurs after oligomerization and insertion of Bax and Bak promoted by tBid. The CL peroxidation mediated by ROS will stimulate the dissociation of that association, releasing cyt *c* to the cytosol where it activates the pro-caspase-9 via apoptossome mechanism [46].

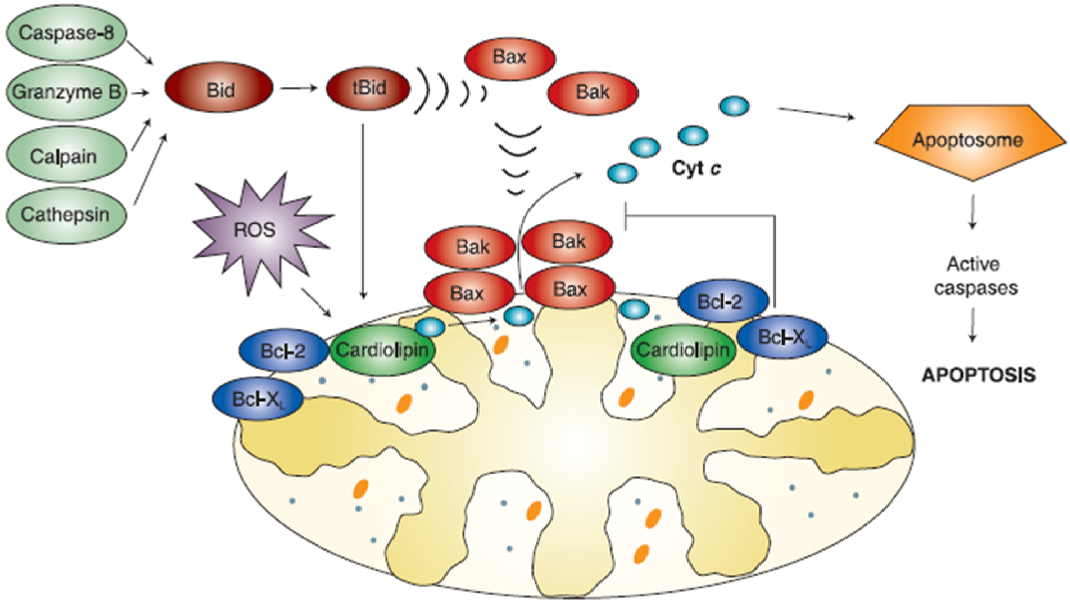


Figure 4: Regulation of cytochrome *c* release from mitochondria. The mitochondrial outer membrane is permeabilized by tBid, which promotes the oligomerization and insertion of Bax and Bak. Cytochrome *c* release is initiated by the dissociation of the hemoprotein from its binding to cardiolipin in the inner mitochondrial membrane, which is stimulated by cardiolipin peroxidation (which is mediated by ROS). Once released into the cytosol, cytochrome *c* triggers pro-caspase-9 activation via the apoptosome mechanism [46].

2.3.3. CL and disease

Alterations of CL have been correlated with various pathological conditions, such as neurodegenerative diseases, Barth syndrome or thyroid dysfunction, among others that will be described below. These associations contribute to the importance of lipidomic study, in order to obtain a deeper knowledge and better understanding of the emergence of some diseases affecting children as well as elderly people.

Barth syndrome

Barth syndrome (BHTS) is an X-linked recessive disorder, clinically characterized by the classical symptoms: cardiomyopathy, neutropenia, muscle weakness and loss of mitochondrial function [13]. BHTS is caused by mutations in the tafazzin (*TAZ*) gene [13, 47]. The *TAZ* gene product, while still unidentified, is believed to be involved in CL remodeling, as the TAZ mutation in BHTS is associated with marked alterations in CL metabolism. Patients with Barth's Syndrome exhibit a loss of functional CL, in particular the tetralinoleoyl (18:2) form, in a variety of tissues. They also exhibit an increase in monolysocardiolipins [13, 48].

Recent studies in yeast expressing the *TAZ* mutation exhibit a decrease in normal CL species containing oleoyl (C18:1) and palmitoleoyl (C16:1) acyl chains, an increase in monolysocardiolipin and destabilization of respiratory chain complexes. Incubation of fibroblasts from patients with BHTS with linoleic acid leads to an increase in tetralinoleoyl CL content, but effects of this treatment in patients await further investigation [13].

Thyroid dysfunction

Thyroid hormone is a regulator of mammalian mitochondrial biogenesis, respiratory function and lipid metabolism, and has been shown to directly modulate CL content by influencing the activity of CL biosynthesis enzymes. An increase in the mitochondrial concentration of CL upon thyroxin treatment was reported in rat heart and rat liver [11, 47], associated with an increase of the activity of multiple CL-dependent mitochondrial proteins and processes (see Table 1). A similar increase in enzyme activity was found for CL synthase and PG phosphate synthase. In contrast, hypothyroidism induced by chronic 6-*n*-propyl-uracil (PTU) treatment decreases levels of CL in heart mitochondria. The reduced level of CL was accompanied by lower activities of pyruvate carrier, the carnitine carrier and cytochrome oxidase [11].

The specific role of CL loss in the PTU-induced decreased in complex IV activity was demonstrated in a study by Paradies *et al*. [49], where enzyme activity was completely restored by reconstituting hypothyroid cardiac mitochondria with exogenous CL liposomes.

Diabetes

Diabetes is a metabolic disease characterized by insulin deficiency (type 1) or insensitivity (type 2), resulting in chronic hyperglycemia and a variety of systemic complications, including neuropathy and cardiomyopathy. Alterations in CL content have been hypothesized to precede the causes of cardiomyopathy, which could be caused by a mitochondrial dysfunction [50].

The influence of the CL changes in the development or progression of diabetic cardiomyophaty is still unclear. There are conflicting results, in streptozotocin-treated rats (a model of type 1 diabetes), mitochondrial CL content has been reported to decrease in the rat brain, increase in the rat liver, or remain unaltered in the rat heart [47].

Tangier disease

Tangier disease is characterized by very low plasma levels of high-density lipoprotein cholesterol, accumulation of cholesteryl esters in tissues and an increased risk for developing cardiovascular disease [50-51]. Tangier disease is mainly caused by abnormal enhanced production of CL. Studies show that there are three to fivefold increase in the levels of CL, monolyso-CL and dilyso-CL in cultured Tangier disease fibroblasts [50, 52]. Because increased CL levels would enhance cholesterol oxidation, and then the formation of oxysterols would consequently increase cholesterol efflux. This process could function as an escape mechanism to remove excess cholesterol from the cell [50, 52].

Aging

An age-related loss of CL was shown in mitochondria from rat heart, rat liver, rat brain mitochondria and in epidermal cells from humans [11].

Oxidative injury of mitochondria is widely believed to play an important role in mitochondrial decay and dysfunction seen in aging [47]. CL comprise in it constitution mainly polyunsaturated acyl chains which are more susceptible to peroxidative damage; age related accumulation of free radicals is responsible for the decline CL [47].

Low levels of CL in aged mitochondria were associated with decreased activities of mitochondrial enzymes such as, a phosphate transporter, pyruvate carrier, carnitine and cytochrome oxidase [11].

Neurodegenerative disease

Alterations in CL have been associated with Alzheimer's disease and Parkison [47]. As reported previously, CL peroxidation is involved in cell apoptosis and mitochondrial dysfunction and is believed to be important contributing factors leading to neuronal loss.

Decreased in CL have been reported to occur in the brain with aging, and probably associated with an increase of lipid peroxidation in rat brain mitochondria exposed to free radical stress. A study by Ellis *et al*. [53] provides intriguing evidence for a potential role of CL alterations in Parkinson's disease.

2.4. Cardiolipin oxidation

Cardiolipin is particularly susceptible to oxidation by reactive oxygen species (ROS) due to the presence of the double bonds in acyl chains, such as linoleic acid or docosahexanoic acid and also due to it association with the electron transport chain (ECT), a major site of ROS production. CL peroxidation has been shown to play a critical role in several physiopathological situations as well as in cell death, as mentioned before.

In order to understand the modification that occur in CL during oxidation some work was done by some research groups in recent years. Oxidation of the CL was promoted by different ways. Kagan and collaborators verified that CL is oxidized by γ–irradiation induced intestinal injury [54]. The irradiation induces radiolysis of water and generation of reactive radicals, which initiated oxidative damage. They hypothesized that CL oxidation may be an important step in the mitochondrial stage of the γ–irradiation-induced apoptotic program in the intestine *in vivo* and, if so, represents an important target for the discovery and development of radioprotectors and radiosensitizers. They also speculated that oxidized molecular species of CL and PS can be used as early biomarkers of γ–irradiation-induced intestinal

apoptosis *in vivo*. They identified using electrospray mass spectrometry (ESI-MS) hydroperoxides derivatives as result of this oxidation process.

Shadyro and co-workers show oxidative modification of cardiolipin induced by γ–irradiation in a model membrane [55-56], using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. They showed the formation of phosphatidic acid in the course of radiolysis of cardiolipin.

The CL oxidation by cyt c/H_2O_2 has been recently studied extensively by Tyurin *et al.* [57-58] as well as, Yourkova *et al.* [59]. They incubate at 37°C the CL liposomes with cytc/H_2O_2 and analyzed their results by ESI-MS and MALDI-MS. Hydroperoxy- and hydroxyl- derivatives of CL, were identified as primary peroxidation products whose subsequent oxidative degradation lead to the formation of a large variety of the other derivatives.

The Yurkova and co-workers used a system under the same conditions where hydrogen peroxide favors a release of the heme iron cyt *c* obtaining phosphatidic acid (PA) and phosphatidylhydroxyacetone (PHA) via an OH-induced fragmentation taking place in the polar moiety of CL.

The Yurkova et al. have been oxidizing the CL in the different ways; apart from oxidation by cyt c / H_2O_2 and γ–irradiation they also tested other methods in order to obtain the PA and PHA in CL liposomes. They induced oxidation by using the hydroxyl radical generated by ascorbate/ Fe^{2+}/H_2O_2 as oxidizing system on CL resulting in the formation of PA [60]. Oxidation by Cu^{2+}/H_2O_2/ascorbate caused the formation of PA and PHA in CL liposomes [61].

The oxidation by hydroxyl radical, usually generated by the Fenton reaction uses iron ions (Fe^{2+}) as reaction catalysts, for the production of ROS, from hydrogen peroxide (H_2O_2).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^-$$

The hydroxyl radical is the most reactive oxygen species generated in living systems, being responsible for the oxidation of biomolecules, including lipids.

The lipid peroxidation induced by the hydroxyl radical is initiated by the abstraction of one hydrogen atom from a reactive methyl group (C-H),

producing alkyl radicals ($R^{\cdot}$). This alteration will promote the reorganization of the adjacent double bonds producing other alkyl radicals. In the presence of oxygen, the alkyl radicals will react to from peroxyl radicals ($ROO^{\cdot}$) that in turn, by acquiring one hydrogen atom, produce one lipid hydroperoxide (ROOH), which will promote various oxidation reactions. The resulting peroxide may be cleaved yielding alcoxil ($RO^{\cdot}$) or epoxiperoxil ($OROO^{\cdot}$) radicals. These species can abstract hydrogen atoms from lipids and propagate the lipid peroxidation reaction.

During oxidation, oxygen atoms are inserted with formation of oxidation products with higher molecular weight. These products are named long-chain oxidation products. But the alkoxyl radical formed during the oxidative reaction may undergo posterior beta cleavage of the fatty acyl chain with formation of oxidation products with shortened fatty acyl chain, and with aldehydic or carboxylic terminal function. These products have lower molecular weight and are called short-chain phospholipid products (Scheme 2). The CL possesses for fatty acid chains that maybe unsaturated, thus being susceptible to oxidation. The tetralinoleoyl-cardiolipin, the most abundant cardiolipin in mammalians, possesses four linoleoyl fatty acyl chains. This fatty acyl chain has two double bonds in the carbon 9 and 12. The bis-allylic hydrogen (found in between the two double bonds) is very unstable; in the presence of oxidant agents this hydrogen is abstracted leading to the dislocation of the double bond for the 10 or 11 carbon, leaving a free radical in the carbon 9 or 13 respectively [62-64].

Scheme 2: Generation of the long chain and short chain oxidation products by radical oxidation.

3. Experimental approaches in Lipidomics research

The typical study of lipids from biological samples (lipidomic) usually involve several steps, beginning with the extraction of lipids from tissues or cells, additional analyses of the lipid profile through mass spectrometry with or without chromatographic separation (Figure 5). This methodology is also applied in the analysis of lipid peroxidation products that are generated in *in vivo* conditions, named as oxidative lipidomic.

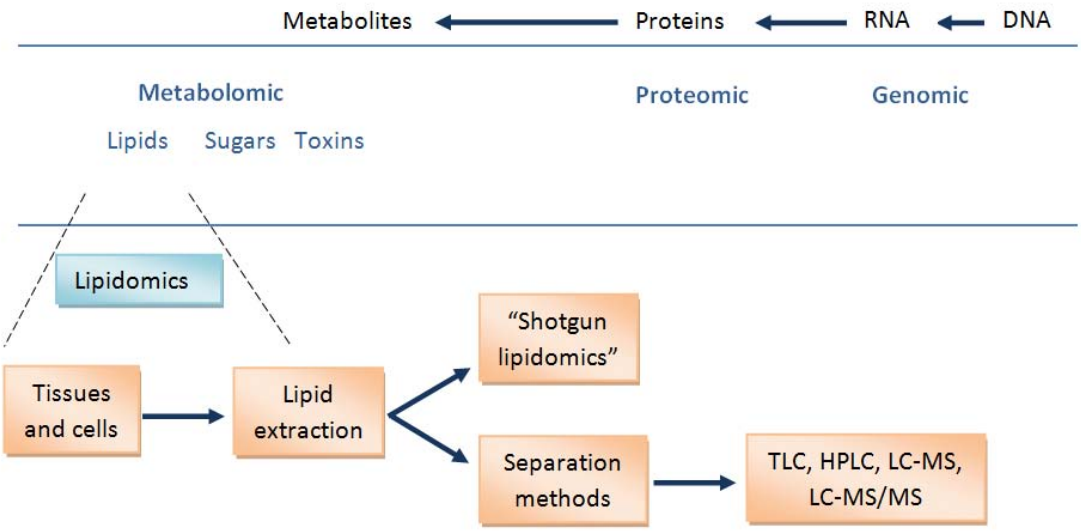


Figure 5: Lipidomics - techniques employed in the analyses of lipids.

Analysis of CL oxidation products has also been carried out using separation techniques and mass spectrometry (MS) and tandem mass spectrometry (MS/MS) [58]. The separation of lipids from biological samples can be conducted using HPLC that has the advantage of being coupled to MS, (LC-MS) or by TLC. TLC is nowadays an important technique used for the separation of different classes of phospholipids in biological samples.

3.1. Separation methods

3.1.1. Thin layer chromatography

Thin layer chromatography (TLC) is a method of separating compounds in the mixture. TLC can also be used for quantify, identify and purify components present, in a mixture. Like all chromatographic methods, TLC takes advantage of the different affinity of analyte with the mobile and

stationary phases to achieve separation of complex mixtures, TLC is then a solid-liquid adsorption technique in which the solvent molecules compete with the molecules of the solute (sample) for binding sites on the stationary phase.

Silica gel, the most commonly used stationary phase, has the empirical formula SiO_2. However, at the surface of the silica gel particles, the dangling oxygen atoms are bound to protons. The presence of these hydroxyl groups renders the surface of silica gel highly polar. Thus, polar functionality in the organic analyte interacts strongly with the surface of the gel particle and nonpolar functionality interacts only weakly. Analyte molecules can bind to the silica gel in two ways: through hydrogen bonds and through dipole-dipole interactions.

For silica gel chromatography, the mobile phase is an organic solvent or mixture of organic solvents. As the mobile phase moves along the surface of the silica gel it transports the analyte particles of the stationary phase. However, the analyte molecules are only free to move with the solvent if they are not bound to the surface of the silica gel. Thus, the fraction of time that the analyte is bound to the surface of the silica gel relative to the time it spends in solution determines the retention factor of the analyte. The ability of an analyte to bind to the surface of the silica gel in the presence of a particular solvent or mixture of solvents can be viewed as a the sum of two competitive interactions. First, polar groups in the solvent can compete with the analyte for binding sites on the surface of the silica gel. Therefore, if a highly polar solvent is used, it will interact strongly with the surface of the silica gel and will leave few sites on the stationary phase free to bind with the analyte. The analyte will, therefore, move quickly past the stationary phase. Similarly, polar groups in the solvent can interact strongly with polar functionality of the analyte and prevent interaction of the analyte with the surface of the silica gel. This effect also leads to rapid movement of the analyte past the stationary phase.

The category in which silica is found as the stationary phase is designed by normal phase TLC in which the more non-polar compounds have more affinity with the mobile phase and so elute in front of the more polar compounds which have higher affinity with the stationary phase. There's another category, TLC of reverse phase, in which the stationary phase is

constituted by linked carbon chains usually with 18 carbons and the mobile phase is constituted by more polar solvents, and therefore the order of solute elution is inverse to the one verified with normal phase TLC.

This technique is used, in lipid analyses for the separation of different lipid classes from total lipid extracts obtained from animal and plant tissues. It is relatively easy and fast to separate, per example, the nonpolar lipids (triglycerides, free fatty acids, cholesterol and diacylglycerols) from more complex lipids like phospholipids, and also achieve separation even within the phospholipids. When using adequate mixture of solvents it is possible to separate and quantify the different lipid classes [65].

3.1.2. High-Performance Liquid Chromatography

The liquid chromatography (LC) is a separation technique based on the distribution of the components of a mixture, which occur in the liquid phase. A sample is separated into its constituent components (or analytes) by distributing between the mobile phase (a flowing liquid) and a stationary phase (sorbents packed inside a column) [66].

The principles of HPLC are similar to TLC. However this is an automated technique and is described and measured in terms of four major concepts: capacity, efficiency, selectivity, and resolution. The capacity and selectivity of the column are variables and depend largely on the column manufacturer, whereas efficiency and resolution can be controlled, to some extent, by the chromatographer. To obtain the best possible separation, the efficiency of the chromatographic system must be optimized in order to minimize band broadening. The column must have the capability to retain the solutes, and it should have the appropriate selectivity to resolve the analytes of interest [67].

A typical HPLC system consists of a pump, an injector, a column, a detector, and a data-handling device (Figure 6).

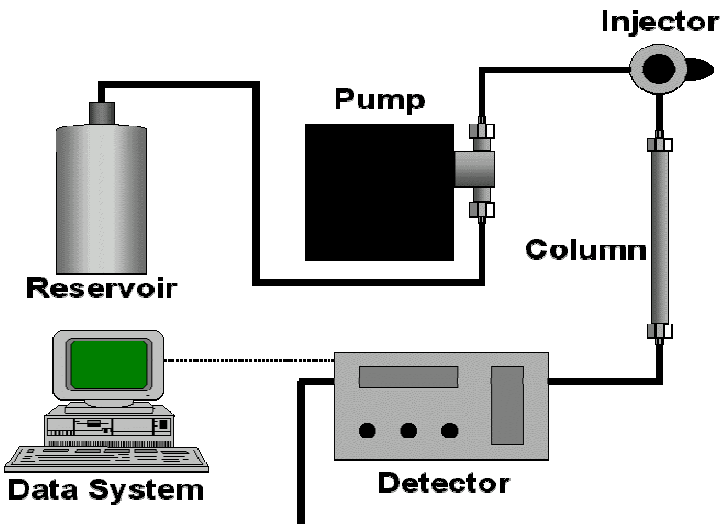


Figure 6: The main components of the HPLC
(http://www.lcresources.com/resources/getstart/)

This technique has been used in the analysis of lipids, either in normal phase and reverse phase, either alone or in association with mass spectrometry, in a lipidomic approach. Nowadays the HPLC is usually coupled with mass spectrometry (LC-MS); this technique has been widely used in the phospholipid study and also in the analysis of phospholipid oxidation products [68-69].

3.2. Mass spectrometry

The mass spectrometry was the main method used for the analysis and monitoring of the CL oxidation. This technique has been widely used in the study of oxidized phospholipids [5, 57-59, 61, 68, 70], since one of its main advantages is its sensibility.

The components of a mass spectrometry instruments are (Figure 7):

- A system to allow the introduction of the sample in the source. This system can be conducted through direct injection or using a coupling of GC-MS, LC-MS;
- Ionization source, where a beam of ions from the sample is produced;
- Analyzers, that allow the separation of the ions in accord with their ratio of mass/charge (*m/z*);
- Detectors where the ions are collected. These are connected to a computer that provides mass spectra recording according to the relative abundance in function of the *m/z* of each ion.

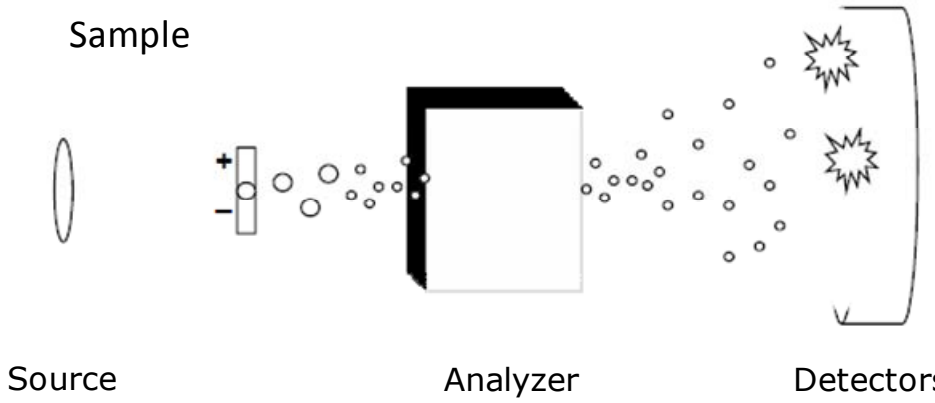


Figure 7: Components of a mass spectrometer.

In the analysis of the phospholipids and phospholipid oxidation products (including CL oxidation products) two ionization methods are used: electrospray (ESI) and matrix assisted laser desorption ionization (MALDI). Several analyzers are available in the mass spectrometers, including quadrupole, ion trap, TOF, among others. They can be alone in a mass spectrometer, or combined such as in the Q-TOF mass spectrometers, triple quadrupole instruments (having two quadrupole analyzers), Q-Trap (combining one quadruple and one ion trap analyzers). In this work we used an ESI-ion trap coupled to LC.

3.2.1. Ionization source

Electrospray (ESI)

In ESI a solution containing the analyte of interest is continuously infused through a metal needle using a syringe pump. A high voltage is applied to the needle. This high voltage will charge the molecules of the solvent, in which the sample is mixed, as well as the sample molecules, producing ions such as $[M+H]^+$ or $[M-H]^-$ depending on the chemical properties of the molecules.

Once they are charged, with the same charge, the molecules will repel one another. This repulsion will force the liquid to exit trough the tip. When the liquid exits it first forms a cone known as Taylor cone; after that, the droplets burst forming the final spray.

After release the droplets, they undergo further division and with the assistance of nitrogen gas the solvent will gradually evaporate. When the

ions become close enough together their electric charges will make them repel each other, because of the Coloumb force. This will force the droplets to divide into smaller droplets until each droplet corresponds to a single charged molecule and the solvent is completely evaporated (Figure 8). This ionization has the advantage that the molecules are not broken apart, instead they remain intact; this is why this ionization is called soft ionization.

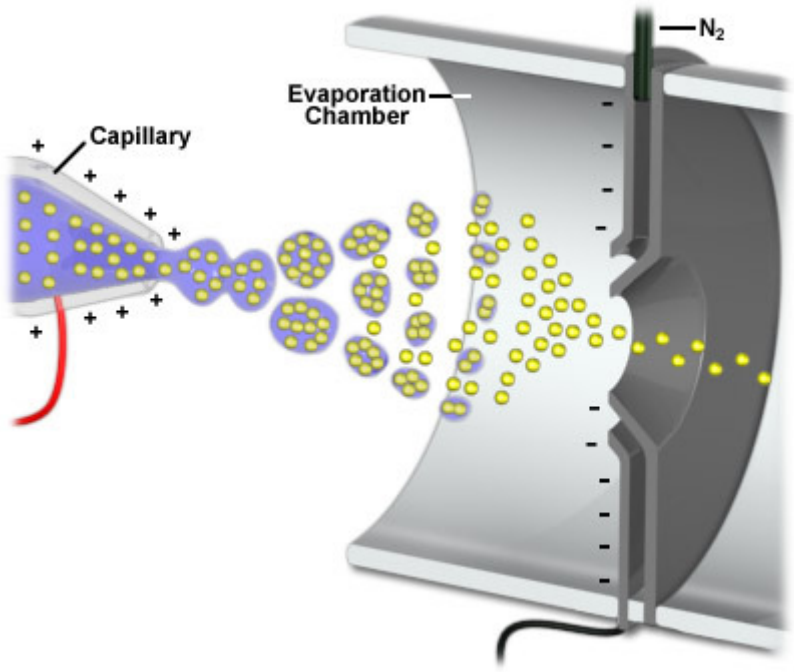


Figure 8: Schematic representation of ionization by ESI (http://www.magnet.fsu.edu/).

The ESI has been widely used in the analysis of biomolecules; its great success in the analysis of proteins is due to its ability of producing multiple charges allowing the analysis of compounds with a higher molecular weight. Electrospray ionization is widely used in lipidomics, and since lipids have a low molecular weight when compared to proteins, they ionize under ESI conditions as single charged ions which facilitate their analysis by ESI-MS.

MALDI

In MALDI, ions are desorbed from the solid phase. A sample is first dissolved in a suitable solvent and mixed with an excessive amount of an appropriate matrix. The most common matrix in lipid analysis is 2,5-dihydroxybenzoic acid (DHB).Than the matrix and analyte mixture are

irradiated with a laser beam. Light energy is absorbed by the crystals and dissipated, with the result that protonated or desprotonated sample molecules and the matrix pass to the gas phase [71] (Figure 9).

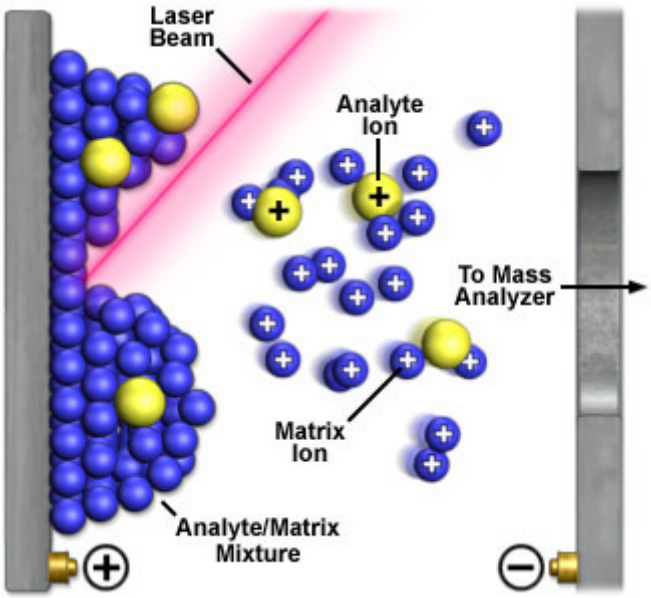


Figure 9: Schematic representation of the ionization of the sample in MALDI (http://www.magnet.fsu.edu/).

This ionization method results predominantly in the generation of mono-charged molecules. Some phospholipids are easily protonated (phosphatidylcholine and phosphatidylethanolamines) and, consequently these are preferably analyzed in the positive ion mode, while others, such cardiolipin and phosphatidylinositol, are more easily desprotonated and analyzed in negative ion mode.

The produced ions are usually analyzed by TOF analyzers, and more recently analyzed by TOF-TOF geometry that allowed tandem mass experiments, which provided additional structural information, with high resolution and accuracy.

3.2.2. Analyzers

Linear Ion trap

The linear ion trap is an analyzer based on the four-rod quadrupole ending in lenses that repel the ions inside the rods. The ions are confined to

a radial dimension by a quadrupole field and to an axial dimension by means of an electric field in the trap extremity.
This analyzer can be combined with other mass analyzers and used to isolate ions of selected mass to charge ratios, to perform tandem mass spectrometry experiments, and to study ion molecule chemistry [72].
Experiments thus far demonstrate the great ion capacity or space charge limits of this device along with a high trapping efficiency of injected ions and low mass discrimination [73].

Quadrupole

The quadrupole is composed by for parallel rolls to which it is applied a direct current and an alternate current that affects the path of the ions. Only the ion with a certain *m/z* value will manage to reach the detector. The rest will collide with rods and will be ejected.

The major advantages of quadrupole analyzers are the low cost, relatively small size, robustness, and ease of maintenance. A quadrupole possesses, however, limited capability in terms of mass range (usually <4000 Da), resolving power, and the ability to perform MS/MS analysis. The final disadvantage can be overcome by attaching a quadrupole to other analyzers such as additional quadrupoles (triple quadrupole instrument) or a quadrupole linked to a TOF (Q-TOF) [74].

Time of flight

The TOF analyzer uses the time that the generated and accelerated ions take to reach the detector. These ions are accelerated by an electric field pulse. All the ions are accelerated with the same energy; their velocities are inversely proportional to the square root of their mass, so the lighter ions gain higher speeds and reach the detector before the ions with higher mass and low velocities.

3.2.3. Tandem Mass Spectrometry (MS/MS)

The Tandem mass spectrometry is based in the isolation of a specific *m/z* value (precursor ion) that may be submitted to dissociation and subsequent production of ion fragments. To achieve this goal, several analyzers are

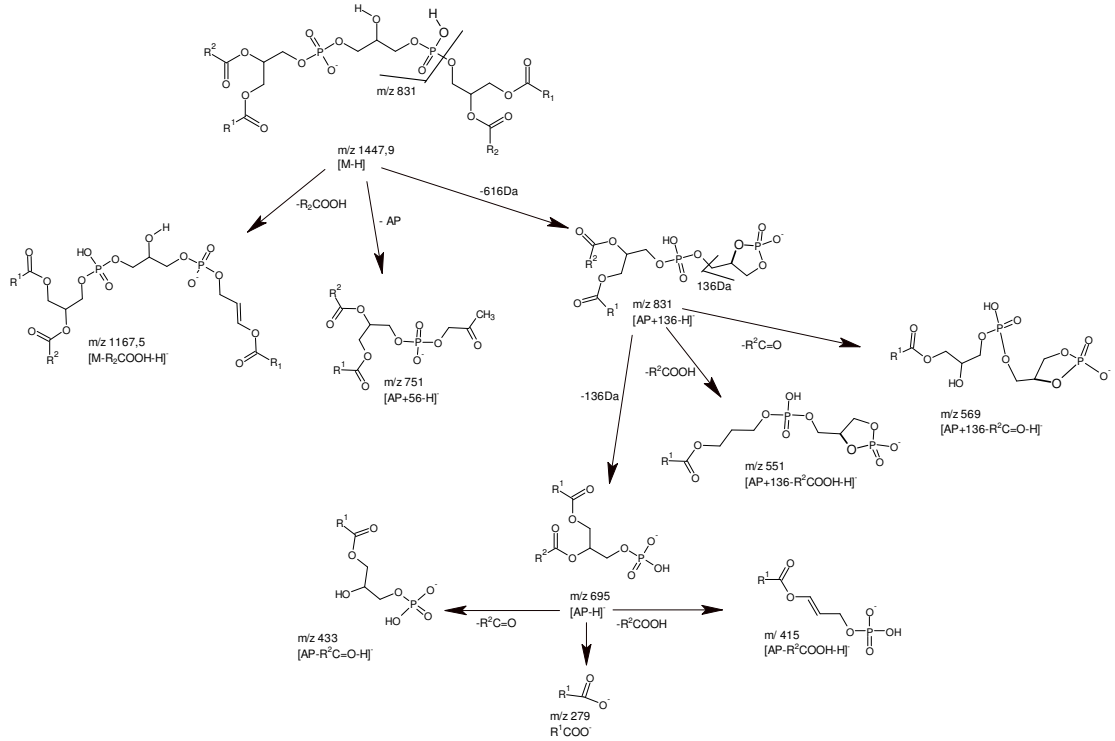
coupled in series in a way that the isolation is performed by the first analyzer, followed by fragmentation in the collision cell; the fragmented ions are separated based on their *m/z* value by the last analyzer. However some instruments (linear ion trap and FT-ICR) with just one analyzer are able to perform multiple MS (MS^n), becoming efficient instruments in the structural identification of several molecules including the phospholipids. [75-77].

This technology is necessary since the soft ionization methods used nowadays produce mass spectra where fragmentation is absent, as so, they do not provide much information about the molecular structure. For that purpose we need to proceed to the fragmentation of the desired ion inducing the dissociation.

Using this technology we can draw several conclusions about the structure of the analyzed molecules and the spectra obtained give us not only the most frequent molecular fragmentation but also as the most favorable.

4. Mass spectrometry in the analysis of cardiolipin

Mass spectrometry has been used for the structural characterization of cardiolipin. Hsu and Turk described the cardiolipin fragmentation by electrospray tandem mass spectrometry using either a quadrupole or multiple-stage quadrupole Ion-trap, in negative mode. Cardiolipin ionizes under ESI conditions as double- and mono-charged ions ($[M-2H]^{2-}$ and $[M-H]^{-}$) [76] and also as sodium adducts ($[M-2H+Na]^{-}$) [78]. The analysis of sodium adducts was also performed in positive mode ($[M+Na]^{+}$ or $[M-2H+3Na]^{+}$) [75]. This group proposed specific fragmentation pathways of the $[M-H]^{-}$ ion of CL, as resumed in Scheme 3.



Scheme 3: Typical Fragmentation of CL as $[M-H]^{-}$ ion.

Studies using mass spectrometry to analyze CL included one published by, Beckedorf and co-works [79] that use in positive and negative mode obtained by nanoESI-QTOF-CID-MS, and MS/MS for detailed structural characterization of the D-glucopyranosylcardiolipin. Valianpour *et al*. [80] also used electrospray ionization mass spectrometry for CLs analysis in *in vivo* samples from patients Barth syndrome.

Cardiolipin oxidation was studied, as reported before, using ESI-MS and MS/MS by Kagan group. They analyzed oxidation products of CL in several biological samples in negative mode $[M-H]^-$and $[M-2H]^{2-}$ [2, 54, 57-58, 81]. MS/MS of the $[M-H]^-$ ions, yielded the oxidized R'_2COO^-, confirming the occurrence of the oxidative modifications in *sn*-2 fatty acyl chain. MS^3 was used for the analysis of R_2COO^- of the CL-OOH and CL-OH. The authors suggested, for the CL-OOH, the formation of the 9-s-OOH-$C_{18:2}$ and 13-s-OOH-$C_{18:2}$; and for CL-OH, the formation 9-s-OH-$C_{18:2}$ and 13-s-OH-$C_{18:2}$ in a model system during cyt *c* driven CL peroxidation *in vitro* [54].

MALDI-MS have been used by Shadyro and collaborates [55-56, 59-61] for analysis of cardiolipin oxidation and identify the phosphatidic acid and phosphatidylhydroxyacetone as oxidation products products of CL generated from cleavage on central glycerol molecule.

However, there is still need of a deeper knowledge about oxidation product that can be generated during CL induces from different conditions.

5. Aims

As mentioned previously, the oxidation of CL may generate numerous oxidation products and the nature of these species may depend of the local and degree of instauration, so it is important to define exactly which oxidative species are being created, since different oxidation products may potentially exert distinct biologic effects.

Despite the importance of CL oxidation there are few studies in this topic; only the products that are formed initially were identified and their characterization lacked detail.

So the aim of this work is to identify and characterize with great detail, using mass spectrometry coupled with separation techniques, all the oxidation products resulting from the oxidation of cardiolipin: long-chain and short-chain.

Mass spectrometry is a very useful technique in the identification and characterization of the products formed due to oxidative stress. It is used in study of cardiolipin oxidation, as mentioned before, as well as other phospholipids oxidation like the PC and the PE [62-63, 68, 70]. In this work the oxidation products of cardiolipin will be studied in detail using ESI-MS, ESI-MS/MS and LC-MS/MS.

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CHAPTER II

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

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

Elisabete Maciel[a], Pedro Domingues[a], Diane Marques[a], Cláudia Simões[a], Ana Reis[a], Maria Manuel Oliveira[b], Romeu A. Videira[c], Francisco Peixoto [c], M. Rosário M. Domingues[a]*

[a]Mass Spectrometry Centre, QOPNA, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal

[b]CQ-VR, Department of Chemistry, University of Trás-os-Montes e Alto Douro, 5001-801 Vila Real, Portugal

[c]CECAV, Department of Chemistry, University of Trás-os-Montes e Alto Douro, 5001-801 Vila Real, Portugal

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* Author to whom correspondence should be addressed:

M.Rosário M. Domingues

e-mail: mrd@ua.pt

Phone:+351 234 370 698

Fax:+ 351 234 370 084

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.

Keywords (6): cardiolipin, oxidation, hydroxyl radical, mass spectrometry, LC-MS, mitochondria

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 oxidize 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 with cellular apoptotic mechanisms which have being associated with nephrotoxicity of several drugs, such as aminoglycosides and immunosupressors [9-11]. In spite of the importance of CL oxidation and its biological consequence, there is a limited knowledge of the oxidation products of CL.

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 products 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, 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 on *nephrotoxicity* of *aminoglycosides* have shown that cell apoptosis is induced [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.

Experimental

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 (2.5×20cm) was purchased from Merck (Darmstad, Germany).

Animals

Adult male Wistar rats were maintained at a temperature 23 ± 2°C, 50-55% humidity, and a lighting cycle of 12 hours light and 12 hours 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.

Isolation of rat kidney mitochondria

Mitochondria were extracted from a homogenate of rat kidney by differential centrifugation according to Cain and Skilleter (1987) [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 ethylene-diaminetetraacetic (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].

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 -20ºC for ESI/MS analysis. Lipid extracts were separated and analyzed by thin-layer chromatography (TLC) plates (silica gel 60 with concentrating zone 2.5×20cm, Merck, Darmstadt, Germany). Prior to separation, plates were treated with boric acid 2.3% in ethanol. The plates were developed in solvent mixture chloroform/methanol/water/triethylamine (30:35:7:35, v/v/v/v). Lipids spots on TLC plates were observed by exposure to iodine vapors and indentified 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)

Oxidation of cardiolipin by Fenton reaction

Ammonium bicarbonate buffer 5mM (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 40μM of $FeCl_2$ and 50mM 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.

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 pre-column split (Accurate, LC Packings, USA) and an ACE 3 C18-AR column (150×1.0mm 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 of 25μLmin^{-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 minutes 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 units. 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 200ms 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).

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 (Figure 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 monocharged ions but with much lower relative abundance, when compared with the double charged ions. These oxidation products [Figure 1(b)] 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.

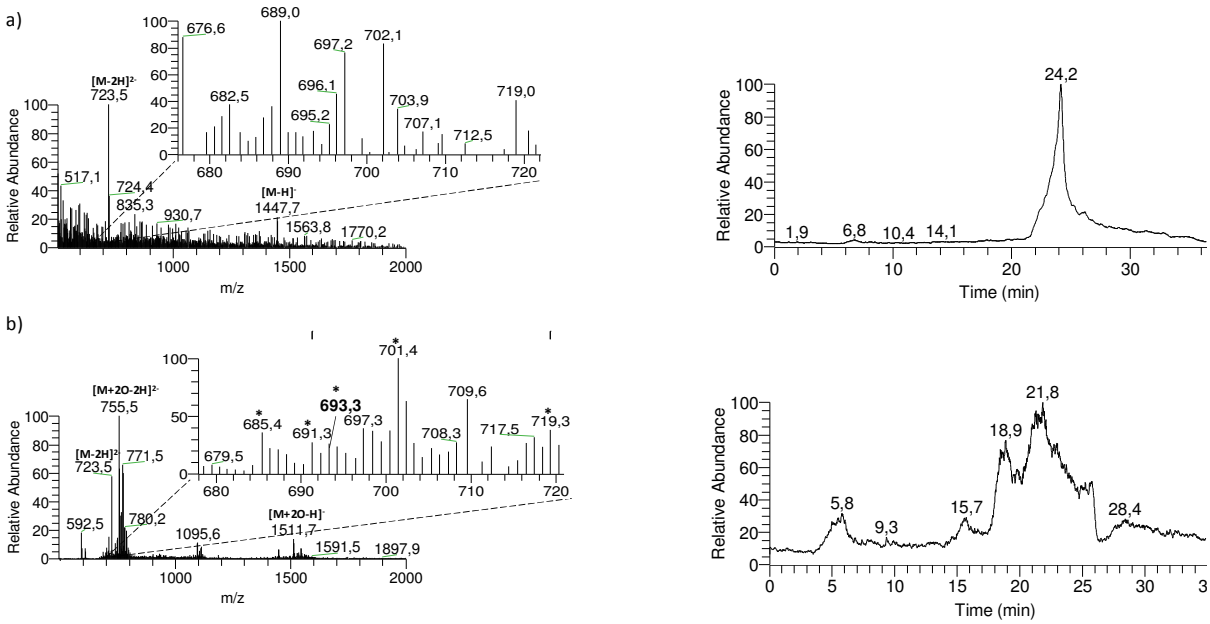


Figure 1: LC-ESI-MS spectra and total ion chromatogram (TIC) from LC-MS profile obtained for CL under non-oxidative (a) and oxidative conditions (b).

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 Figure 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* of 751, as represented 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 and Turk [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].

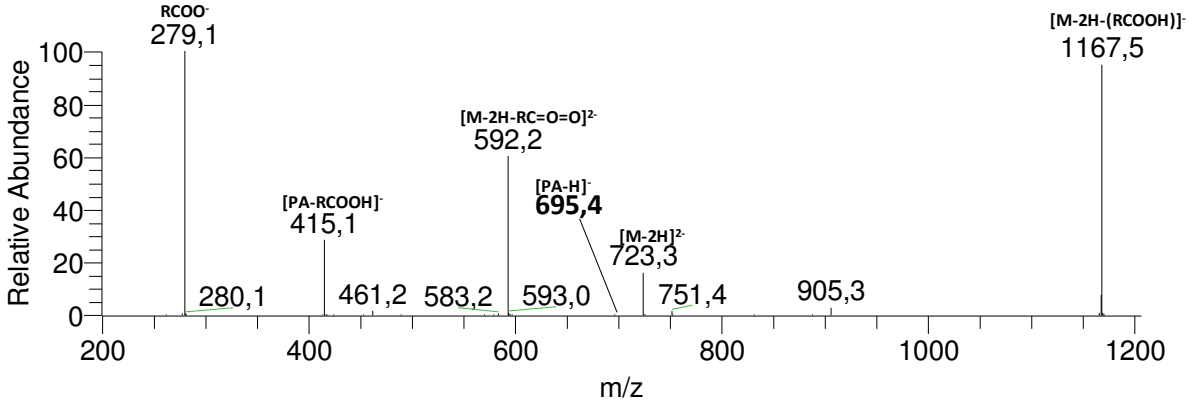
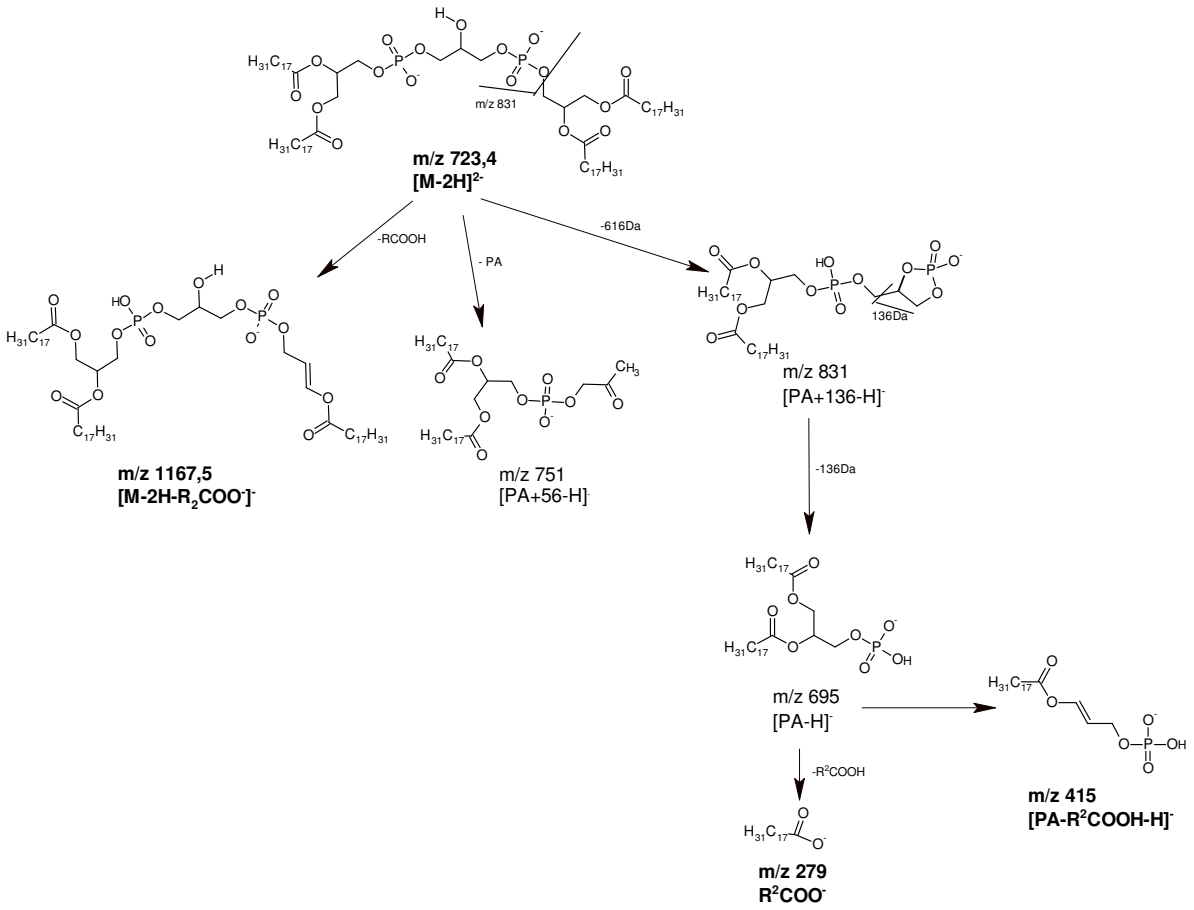


Figure 2: ESI-MS spectrum of the double charged ion of tetra-linoleoyl cardiolipin ($[M-2H]^{2-}$ at *m/z* 723).



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

In resume, the $[M-2H]^{2-}$ mass spectrum exhibits three types of products ions that are informative of the structure of CL and 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 mono-charged ion $[(M-2H)-RCOO^-]^-$.

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 Figure 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 Figure 4 and are summarized in Table 1. The tandem mass spectra of each of these oxidation products 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 $[M-2H]^{2-}$.

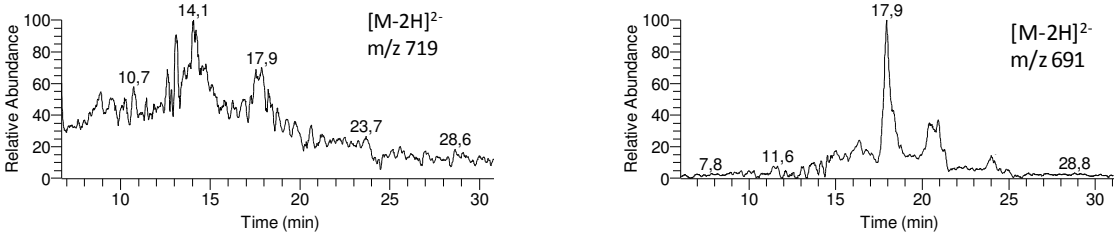


Figure 3: RIC of the $[M-2H]^{2-}$ ions at *m/z* 691 and 719

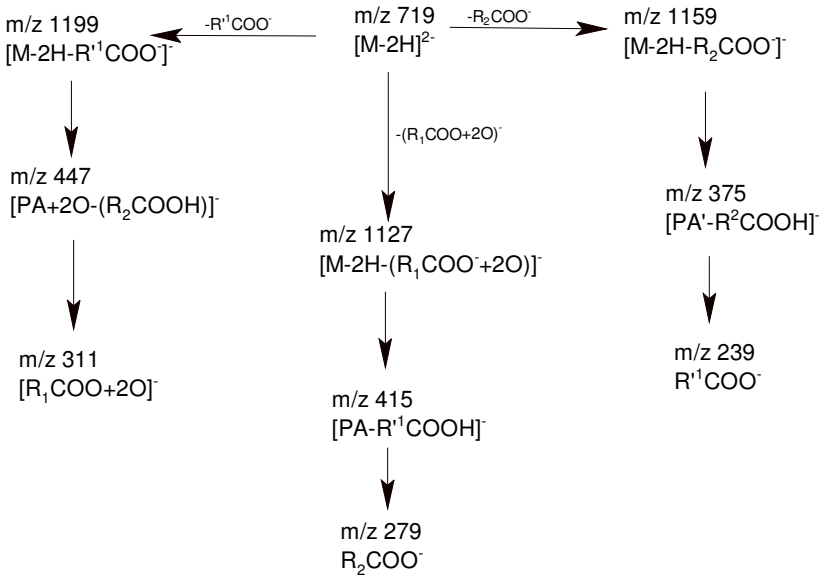
Short chain product at m/z 719

The short chain oxidation product attributed to the ion at *m/z* 719 elutes at 14.1 and 17.2 minutes. The LC-MS/MS spectra (Figure 4 a) obtained at the two elution times show different product ions confirming that two distinct compounds were separated. The first oxidation product to elute (RT 14.1min), 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 2). 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 3).

The CL oxidation product that elutes at RT 17.2 minutes, corresponds to a less polar compound, thus probably with a C13 fatty acyl chain with a terminal aldehyde and the CL modified with plus 3 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 an 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 3).



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

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 minutes), suggesting the elution of only one compound. Based on the molecular weight, it seems that this ion 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 3). A modified CL with two carboxylic moieties with plus two oxygens should elute earlier, due to higher polarity conferred by the carboxylic acid.

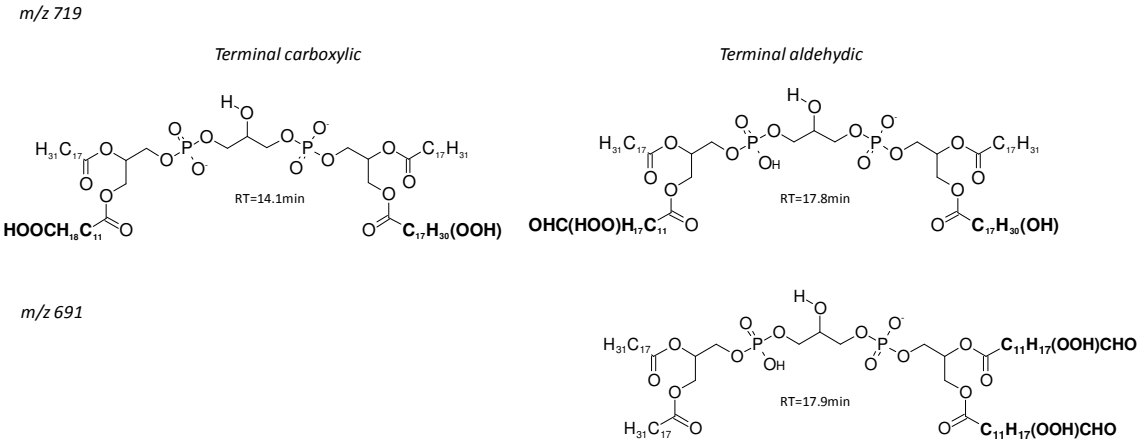
The presence of the ion due to loss of O_2 (*m/z* 675 $[M-2H-O_2]^{2-}$) suggests the presence of a hydroperoxide derivative. The product ions observed in the

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 (Figure 4 c) show the product ions due to loss of a non-modified chain (-280Da) -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 $\mathbf{(C_{18:2})_2}$ **/** $\mathbf{(C_{12+OO}CHO)_2}$ (Scheme 3).

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 $\mathbf{(C_{18:2})}$ **/** $\mathbf{(C_{18:2+OO})}$ **/** $\mathbf{(C_{12}CHO)}$ **/** $\mathbf{(C_{12+OO}CHO)}$.

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

	719(RT=14.1) Acid+2O	**719(RT=17.2) Aldehyde+3O**	**691(RT=17.9) 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



Scheme 3: Short chain products with C13 at *m/z* 719 and 691

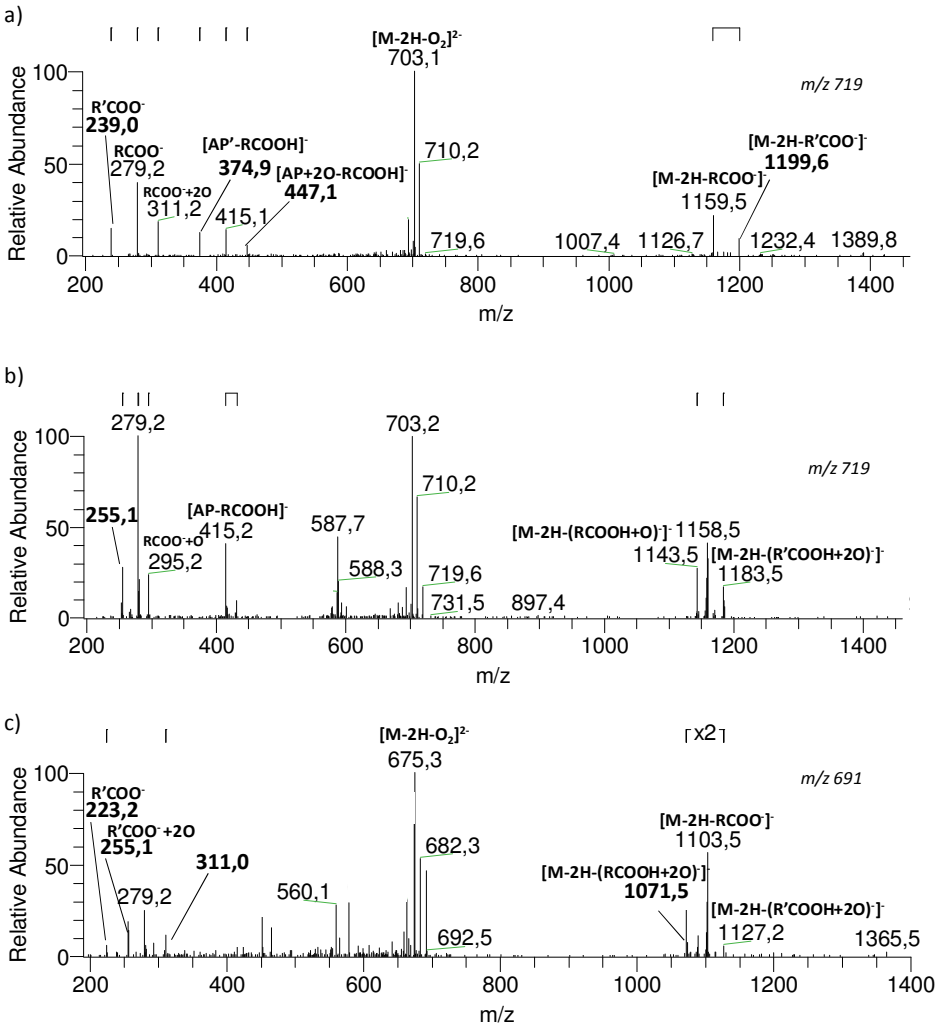


Figure 4: LC-MS/MS spectra of the ions at *m/z* 719. a) RT= 14.1 minutes; b) 17.2 minutes and c) ion at *m/z* 691 with TR=17.9 minutes

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 Figure 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.

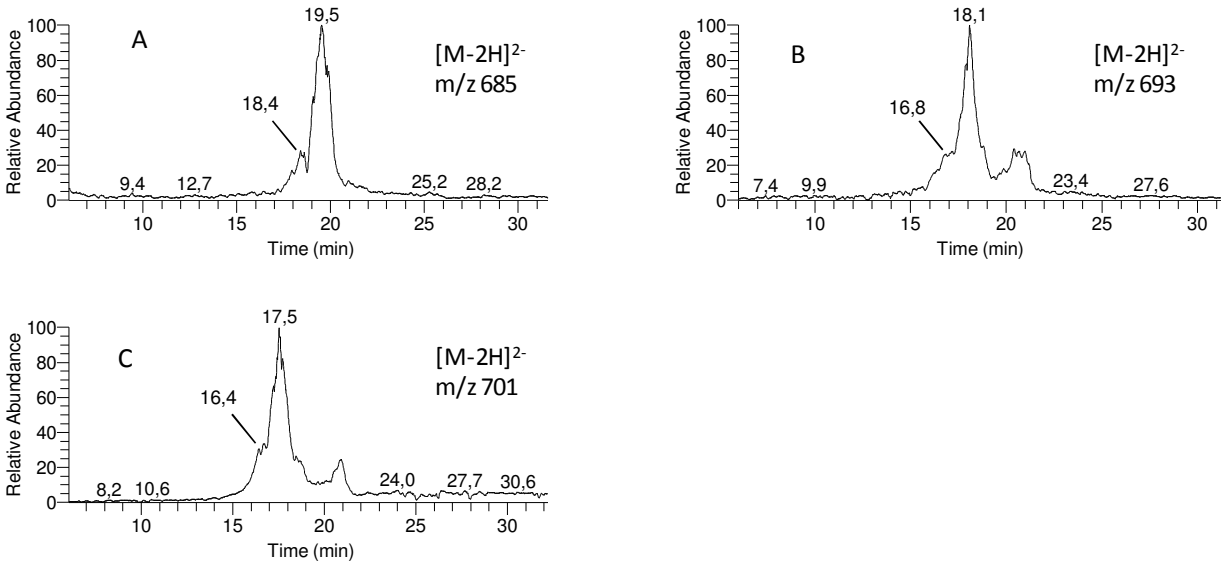


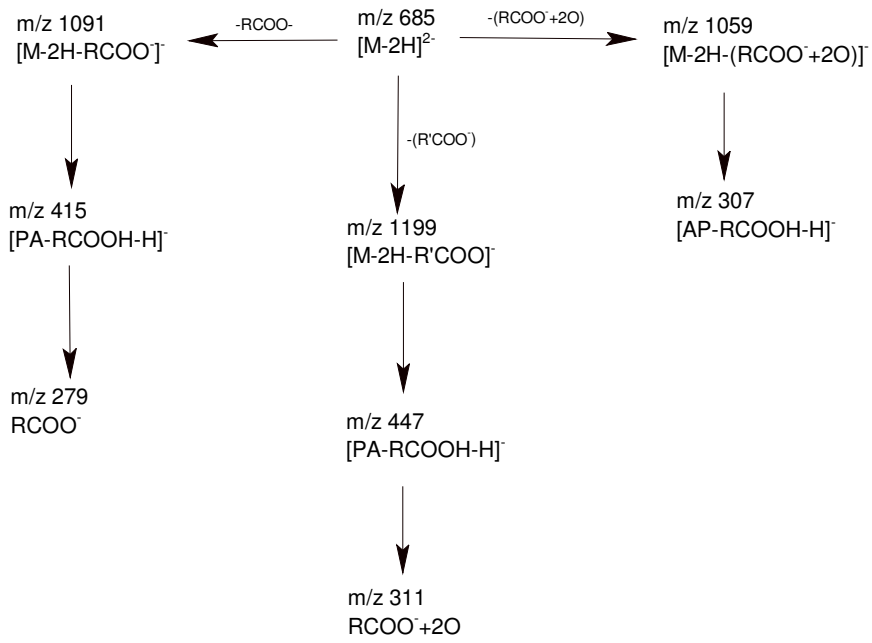
Figure 5: RIC of CL short-chain products with C9; A –*m/z* 685; B – *m/z* 693; C – *m/z* 701

***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 minutes 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) (Figure 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 (Figure 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 4 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 $[AP+2O-(RCOOH)-H]^-$, confirms the presence of CL with modified fatty acyl chains: **($C_{18:2}$)$_2$/($C_{18:2+OO}$)/(C_8CHO).**



Scheme 4: Fragmentation pathways of the $[M-2H]^{2-}$ ion at *m/z* 685 with aldehyde terminal in C9 (TR=19.5min)

Short chain product at m/z of 693

This oxidation product elutes in two peaks at RT 16.8 and RT 18.1min. 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.

In the LC-MS/MS (Figure 6 c) of the compound that elutes first (RT=16.8min) 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 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 5).

The compound that elutes later (RT=18.1 minutes), 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 (Figure 6 d) 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 [AP'-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 5).

***Short chain product at m/z* of 701**

The RIC of the ion [M-2H]$^{2-}$ at *m/z* of 701 (Figure 5 C) 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

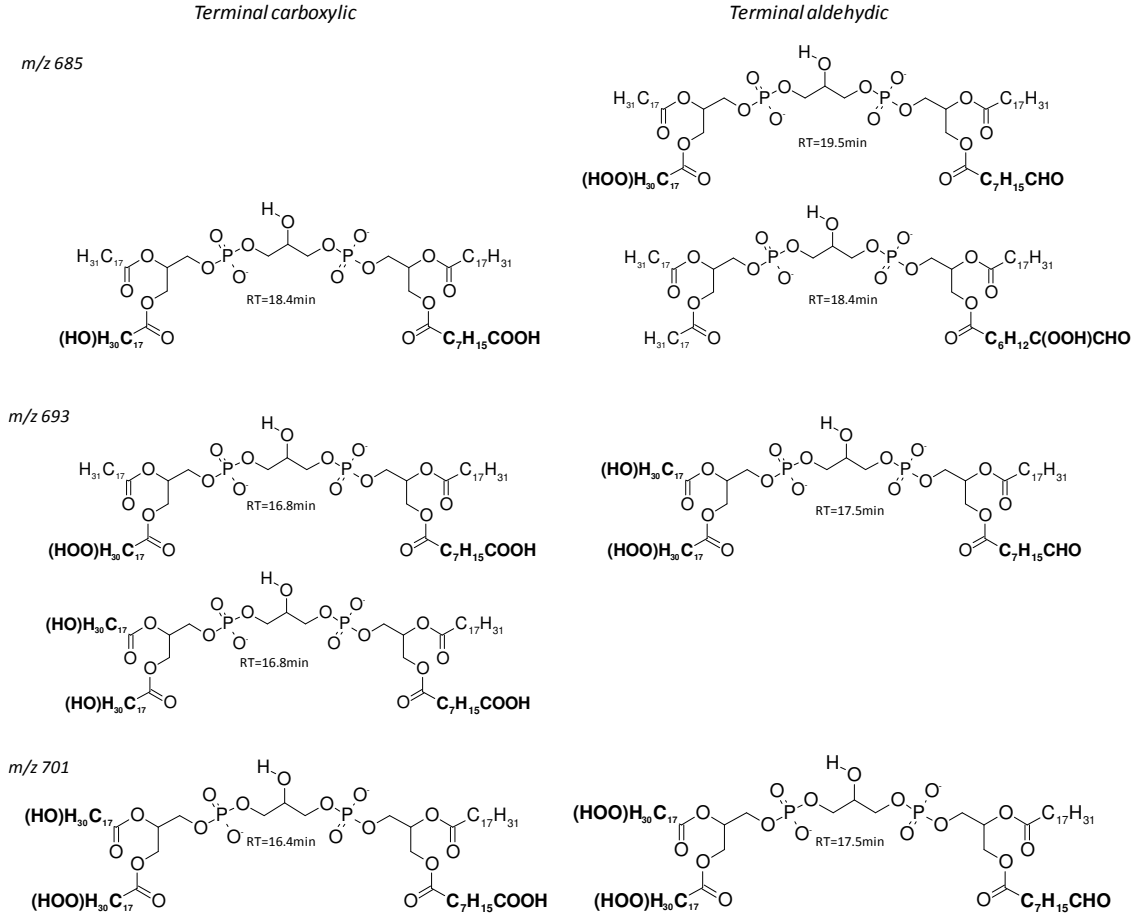
additional oxygen in CL and the second should has a C9 short chain aldehyde and four additional oxygen atoms in CL.

For the compound that eluted at RT=16.4 minutes the LC-MS/MS spectrum (Figure 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 with one additional oxygen. The ions at *m/z* 311 ($RCOO^-$+2O) and 447 ($[AP+2O-(RCOOH)-H]^-$) 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 5).

The analysis of the LC-MS/MS spectrum of the compound eluting at 17.5 minutes (Figure 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 confirms 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 5).

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

	685(RT=18.4) Acid+O	685(RT=19.5) Aldehyde +2O	693(RT=16.8) Acid+2O	693(RT=18.1) Aldehyde+3O	701 (RT=16.4) Acid+3O	701(RT=17.5) 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



Scheme 5: 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.

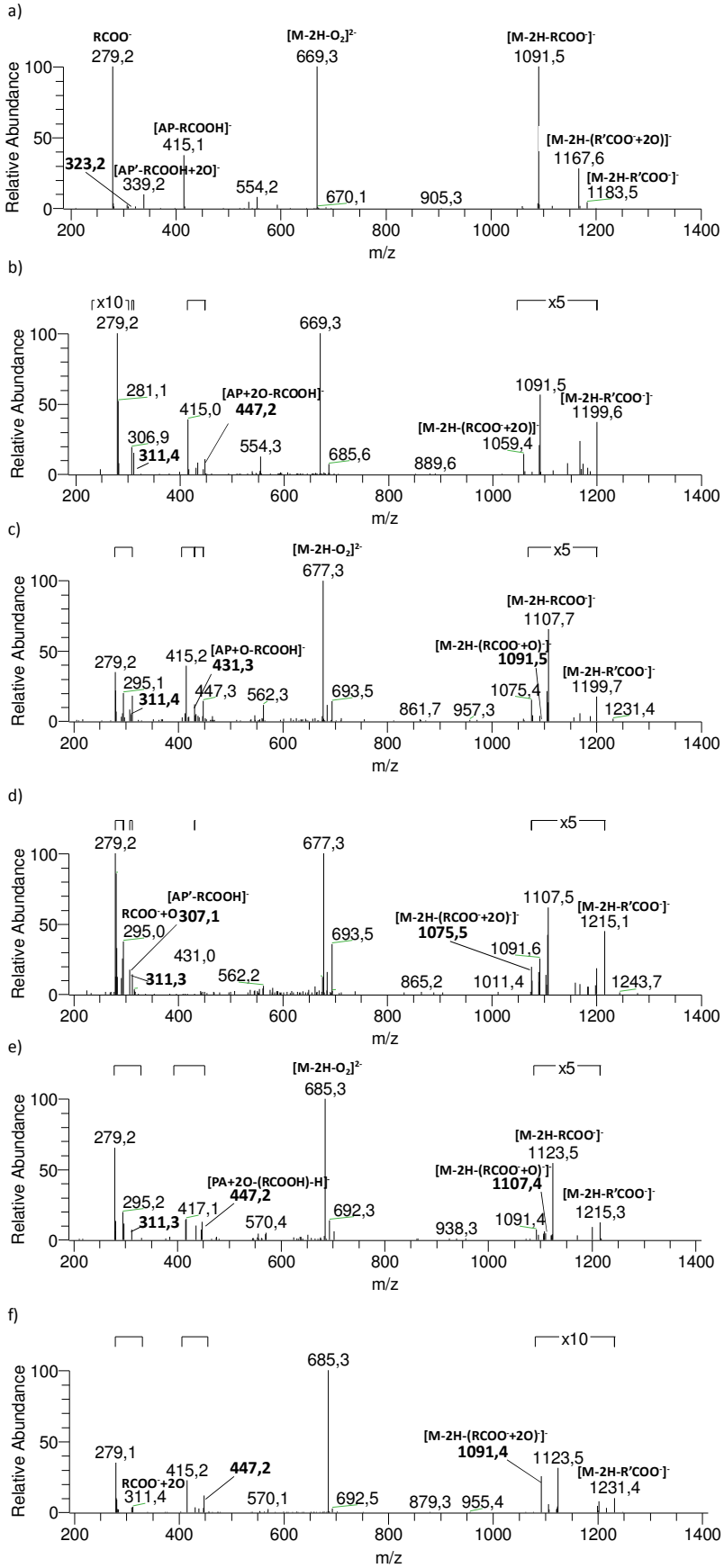


Figure 6: LC-MS/MS spectra of the ions at *m/z* 685. a) RT= 18.4 min; b) 19.5 min, of the ion at *m/z* 693. c) RT=16.8 min d) RT=18.1 min and of the ion at *m/z* 701. e) RT=16.4min f) RT=17.5 min

Identification of short chain oxidation products of CL in mitochondria

It is know 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 Figure 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 most abundant oxidation products identified as $[M-2H]^{2-}$ ions correspond to the tetralineoylCL (the most abundant CL observed in the extract from rat control) hydroxyl and peroxyl 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 (Figure 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 Figure 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 (Figure 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.

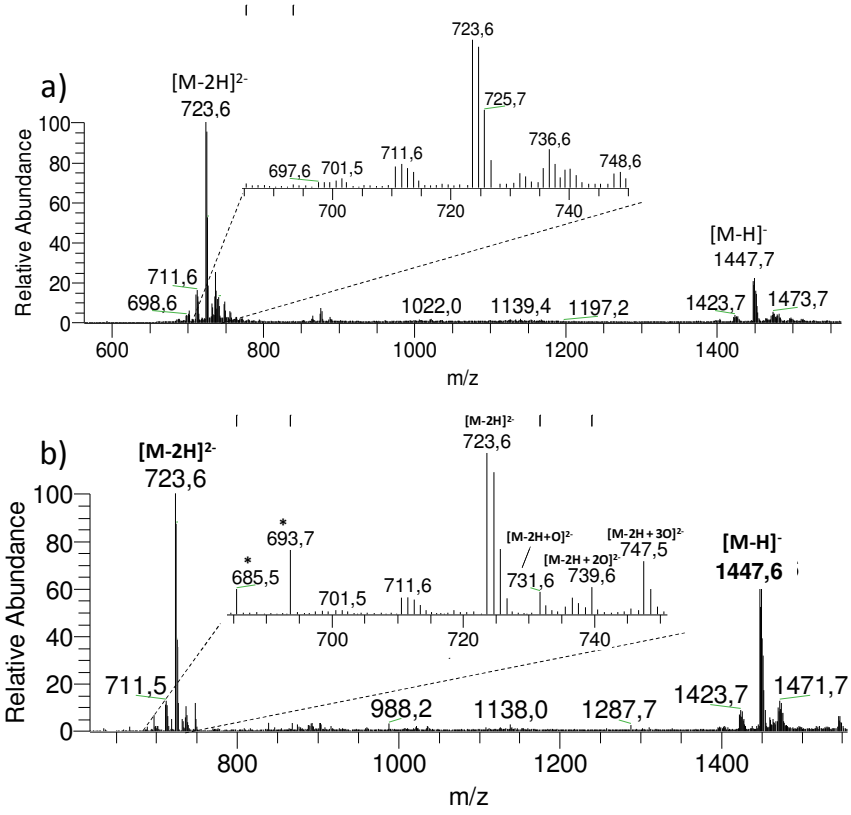


Figure 7: ESI-MS spectra obtain from cardiolipin extracted from mitochondria of rats control (a) and with treatment with gentamicin (b)

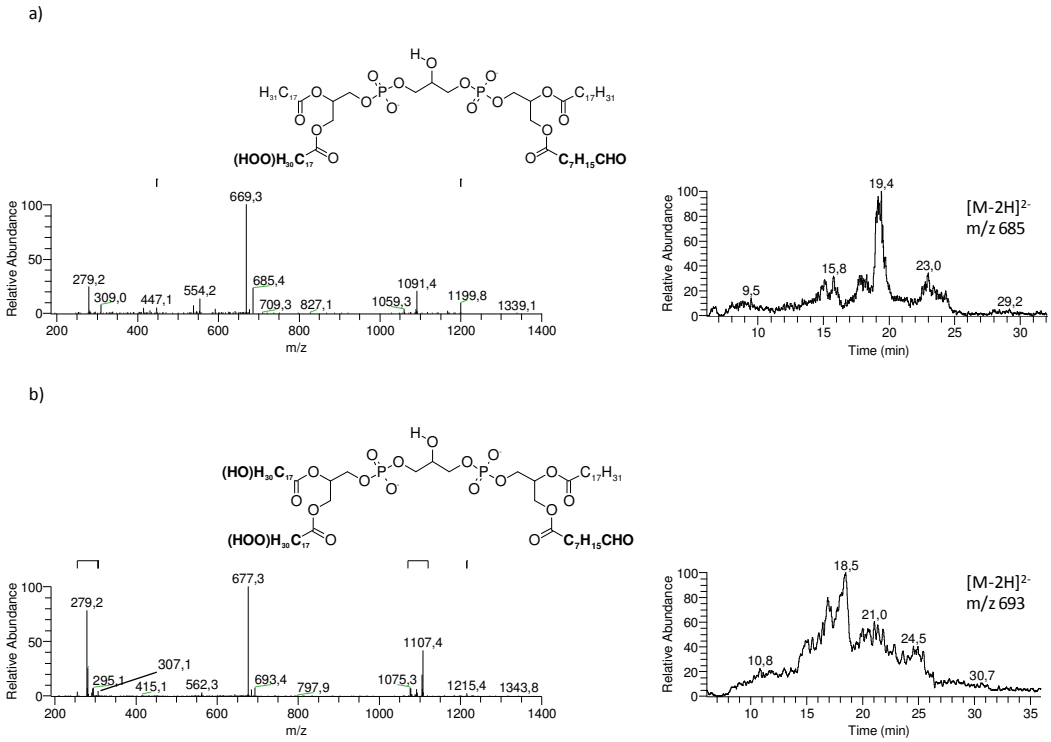


Figure 8: LC-MS/MS spectra and RIC chromatograms of the ions at *m/z* 685 (a) and 693 (b) observed in the ESI-MS spectra of cardiolipin from rats treated with gentamicin and attributed to the short chain oxidation product of cardiolipin: (C18:2)2/(C18:2+OO)/(C8CHO) and (C18:2) / (C18:2+O) / (C18:2+OO) / (C8CHO), respectively.

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.

Acknowledgments

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CHAPTER III

Liquid chromatography tandem mass spectrometry analysis of long chain oxidation products of cardiolipin induced by the hydroxyl radical

Liquid chromatography tandem mass spectrometry analysis of long chain oxidation products of cardiolipin induced by the hydroxyl radical

Elisabete Maciel, Pedro Domingues, Rosário M. Domingues*

Mass Spectrometry Centre, QOPNA, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal

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Running title: Cardiolipin long chain oxidation products

* Author to whom correspondence should be addressed:
M.Rosário M. Domingues
e-mail: mrd@ua.pt
Phone:+351 234 370 698
Fax:+ 351 234 370 084

Abstract

The anionic phospholipid cardiolipin is found almost exclusively in the inner membrane of mitochondria, playing an important role in energy metabolism. Oxidation of CL has been associated with apoptotic events and various pathologies. In this study, electrospray mass spectrometry coupled with liquid chromatography (LC-ESI-MS) was used to identify tetralinoleoyl-cardiolipin (TLCL) modifications induced by the $OH^{\cdot}$ radical generated under Fenton reaction conditions (H_2O_2 and Fe^{2+}). The identified oxidation products of TLCL contained 2, 4, 6 and 8 additional oxygen atoms. These long chain oxidation products were characterized by LC-ESI-MS/MS as double $[M-2H]^{2-}$ and mono charged $[M-H]^{-}$ ions. A detailed analysis of the fragmentation pathways of these precursor ions allowed the identification of hydroperoxyde derivatives of CL. MS/MS analysis indicate that CL oxidation products with 4, 6 and 8 oxygen atoms have one fatty acyl chain bearing 4 oxygen atoms ($[RCOO+4O]^{-}$). Even when the TLCL molecule was oxidized by the addition of eight oxygen atoms, one of the acyl chains remains non-modified and one fatty acyl chain contains three or four oxygen atoms. This led us to conclude that under oxidative conditions by the $OH^{\cdot}$ radical, the distribution of oxygens/peroxy groups in the CL molecule is not random, even when CL has the same fatty acyl chains in all the positions. Using mass spectrometry, the oxidation products have been unequivocal assigned, which may be useful for their detection in biological samples.

Keywords - cardiolipin, LC-MS, oxidation, hydroxyl radical, eletrospray, mass spectrometry

Introduction

Cardiolipin (CL) is a dimeric phospholipid with two phosphatidyl moieties and four fatty acyl chains. The fatty acyl groups of CL in mammalian tissues are almost exclusively 18-carbon fatty acids, the majority of which are linoleic acids (tetralinoleoyl-cardiolipin (TLCL)). In the cells, this phospholipid is located most exclusively in the mitochondrial inner membrane where it is associated with several mitochondrial proteins. In the intermembrane mitochondrial space cardiolipin interacts with cytochrome *c* (cyt *c*), forming a complex CL-cyt *c*. It was observed that an increase of production of hydrogen peroxide in mitochondria, induces the oxidation of CL, with formation of hydroperoxy derivatives. These hydroperoxides have been associated with the release of cyt *c* into the cytosol, a key step in the cell apoptosis [1-3]. TLCL is very susceptible to oxidative damage by reactive oxygen species (ROS), due to the presence of polyunsaturated fatty acyl chains [4-6]. Oxidation of CL has been associated with Barth syndrome, thyroid dysfunction, aging, diabetes and also in the pathogenesis of several neurodegenerative diseases namely, Parkinson and Alzheimer [7-9].

Currently there's a limited knowledge on the nature of the oxidation products of CL, even though its relevant biological consequences. In the recent years there is an increasing interest in the observation of CL modification under oxidative conditions. However this is a challenge, since lipid peroxidation, occurring during ROS oxidation, is a chain reaction and thus numerous oxidation products may be formed[3]. These different oxidation products may be responsible for distinct biological effects thus increasing the relevance of identifying each specific oxidation product in order to understand their specific biological significance and effects [3].

Mass spectrometry (MS) has been previously used to identify phospholipids oxidation products, including cardiolipin's oxidative products, generated during oxidative processes (review on [10]). However, few studies have used MS to identify the oxidation products of CL. Yurkova, Shadyro and co-workers using MALDI-MS in positive mode have identified phosphatidic acid and diacylphosphatidyl-hydroxyacetone resulting from cleavage of the CL molecule in consequence of the oxidative process. In these studies, γ-radiation, Cu^{2+}/H_2O_2/ascorbate, or cytochrome *c* was used to induce oxidative modifications on CL[11-14].

Kagan *et al*, using electrospray mass spectrometry (ESI-MS) and tandem mass spectrometry (MS/MS) in negative mode, have identified oxidative modifications of CL induced by the presence of cytochrome *c*/H_2O_2[15], by γ–irradiation[16], after traumatic brain injury[6], during staurosporine induced apoptosis[17] and by pro-inflammatory stimuli using LPS[18]. In these studies, it was noticed the oxidation of CL by accumulation of hydroxy and hydroperoxy groups in CL, with the insertion of one to eight oxygen atoms. Among these, oxidation products with peroxyl groups were preferentially formed. In a more recent study these authors have used tandem mass spectrometry to identify and confirm the location of peroxy groups along the fatty acyl chain of the CL molecules[18]. In this important study, the MS/MS spectra of oxidation products as $[M-H]^-$ of CL plus one and two oxygen atoms, and tandem mass spectra of $[M-2H]^{2-}$ for the CL plus one, two, three and four oxygens namely hydroxy and hydroperoxy derivatives, were analyzed. However the distribution of the oxygen atoms along the CL molecule and fatty acyl chains for the other oxidation products with higher number of oxygen atoms was not confirmed by tandem mass spectrometry and no previous separation using chromatography techniques were used to identify possible isomers.

In this paper we discuss the identification and structural characterization by LC-MS/MS of TLCL long-chain oxidation products, correspondent to the addition of two to eight oxygen atoms formed during oxidation using the Fenton system. A LC-MS approach was developed and applied to verify the presence of isomers. A detailed interpretation of MS/MS spectra will be done to identify ions that allow the unequivocal assignment of distribution of oxygen atoms in oxidized TLCL. This should provide significant information for the detection of these oxidative products in biological samples.

Experimental

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). Chloroform and methanol used were HPLC grade.

Oxidation of cardiolipin by Fenton reaction

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

LC-ESI-MS conditions

The LC-MS and LC-MS^n studies were conducted on a Waters Alliance (Milford, USA) Model 2690 equipped with a pre-column split (Accurate, LC Packings, USA) and an 150×1.0mm i.d. ACE 3 C18-AR column (Advanced Chromatography Technologies, Scotland, United Kingdom), 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 of 25$\mu L min^{-1}$. 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 minutes 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 units. 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 200ms maximum ionization time, respectively. Normalized collision energy™ (CE) was varied between 15 and 20 (arbitrary units) for tandem mass spectrometry. Data acquisition was carried out on an Xcalibur data system (V2.0).

Results and Discussion

Oxidation of CL was induced using Fenton reaction conditions (H_2O_2/Fe^{2+}), as described in the experimental section, and the formation of the oxidation products was monitored by direct injection and LC-ESI-MS, in negative mode. Both $[M-2H]^{2-}$ and $[M-H]^-$ ions of CL and CL oxidation products were observed in the ESI-MS spectra. When comparing the ESI-MS spectra obtained for the CL after the oxidative reaction (Figure 1b) with the CL in the absence of oxidation conditions (Figure 1a) we can observe new $[M-2H]^{2-}$ and $[M-H]^-$ ions correspondent to oxidation products, with higher *m/z* than TLCL (*m/z* $[M-H]^-$=1447 and *m/z* $[M-2H]^{2-}$ =723). These products are designated as long chain oxidation products in analogy with the oxidation of other phospholipids[10]. The long-chain oxidation products correspond to the insertion of 2, 4, 6 and 8 oxygen atoms are observed as $[M-H]^-$ at *m/z* 1479, 1511, 1543, 1575 and as $[M-2H]^{2-}$ at *m/z* 739, 755, 771 and 787, respectively. Long chain oxidation products were already identified as oxidation products in other phospholipids such as phosphatidylcholines[19] and in phosphatidylethanolamines[20]. CL long-chain products were identified by Kagan et al[18]. It was found that main oxidation products comprised hydroperoxy derivatives even after addition of eight oxygen atoms (meaning four hydroperoxy groups linked to CL), although only species with 2 ($[M-H]^-$) and 4 ($[M-2H]^{2-}$) additional oxygen atoms were studied, as described in the introduction section.

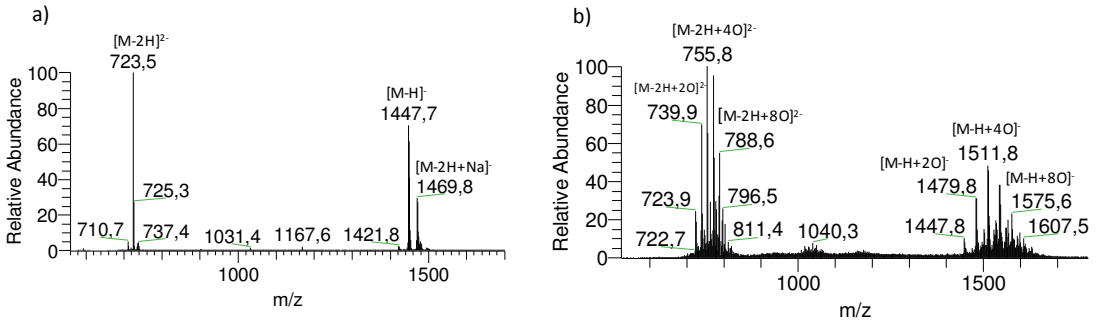

Figure 1: ESI-MS spectra obtained for CL under non-oxidative (a) and oxidative conditions (b).

In order to understand the distribution of oxygen atoms along the TLCL oxidized molecule, we analyzed in detail the fragmentation pathways of

both of $[M-H]^-$ and $[M-2H]^{2-}$ oxidation products observed in the LC-ESI-MS/MS spectra.

In developing the separation method, we tried to obtain the best possible resolution in the shortest period of time. Thus, it was possible to resolve the oxidation products containing a different number of atoms of oxygen. Nevertheless, the simultaneous separation of the isomers of these oxidation products proved to be an impossible task. In fact, the chromatographic peaks corresponding to different isomeric forms of a particular oxidation product appear as broad bands, containing several peaks not completely resolved. However, it was possible to obtain different LC-MS/MS spectra in different areas of these chromatographic peaks. As we shall see, these MS / MS spectra correspond to different isomers of the oxidation products of CL and the information contained therein can be used to uniquely identify these products.

The MS/MS spectrum of the $[M-H]^-$ ion of non modified TLCL (*m/z* 1447) can be divided in three distinct zones, as described by Hsu and Turk[21-23]: the phosphatidic acid ($[PA-H]^-$, *m/z* 695), the $[(PA-H)+136]^-$ (*m/z* 831) and the mono phosphatidic acid ($[PA-RCOOH]^-$, *m/z* 415) zone. The structures of each ion are found in Figure 2.

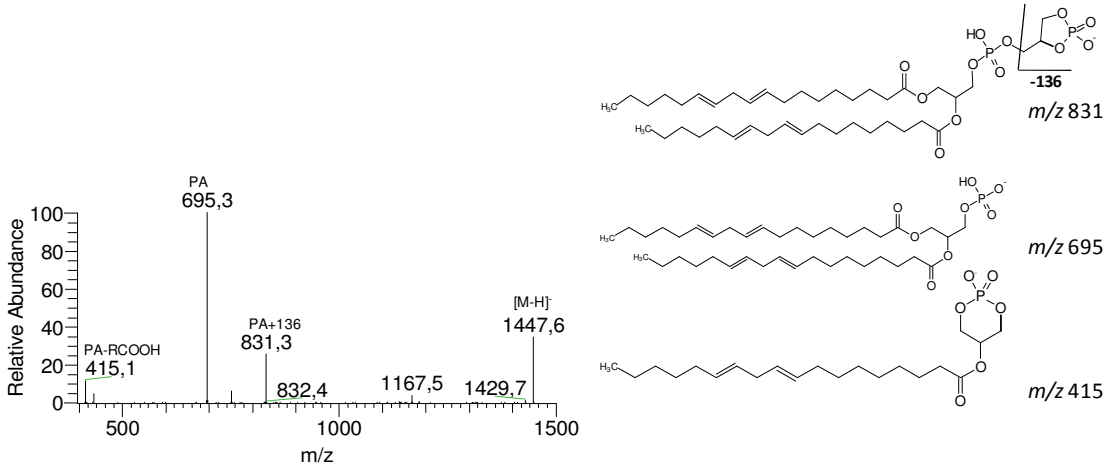

Figure 2: LC-MS/MS spectrum of the $[M-H]^-$ ion of TLCL at *m/z* 1447, showing the most diagnostic product ions, their *m/z* values and structures.

The MS/MS spectrum of the $[M-2H]^{2-}$ ion of non modified CL (*m/z* 723) show two distinct zones: the carboxylate ion ($RCOO^-$, *m/z* 279); and the $[PA-RCOOH]^-$ ion(*m/z* 415)[24].

Long-chain oxidation products of CL analyzed by LC-ESI-MS

The oxidation products formed by the insertion of two oxygen atoms, elute in one peak (RT 23.1min), indicating that, most probably, this is a non-isomeric oxidation product. In fact, this is in agreement with the previous published work by Kagan group that identify this oxidation product as hydroperoxy–CL[18]. The LC-MS/MS spectrum of the ion $[M-H]^-$ at *m/z* 1479 (Figure 3a), corresponding to the $[CL+2O]^-$ molecular ion, shows , in the $[(PA-H)+136]^-$ zone, the ions at *m/z* 831 ($[(PA-H)+136]^-$) and 863 ($[(PA-H)+136+2O]^-$). The $[PA-H]^-$ zone shows the same information with the presence of the ions at *m/z* 695 ($[PA-H]^-$) and 727 ($[(PA-H)+2O]^-$). In this zone, a fragment ion at *m/z* 709 is also observed, corresponding to the loss of H_2O from the hydroperoxy-PA (*m/z* 727). In the PA-RCOOH zone, both the ions at *m/z* 415 $[PA-RCOOH]^-$ and 447 $[(PA+2O)-RCOOH]^-$ are present; indicating that the two additional oxygen atoms are in the same acyl chain. Based on the LC-MS/MS data, the oxidation product of TLCL with two oxygen atoms is TLCL-$(C_{18:2})_2$/$(C_{18:2})(C_{18:2+OO})$ (Figure 4).

In order to confirm this information the LC-MS/MS spectrum of the molecular ion at *m/z* 739 ($[M-2H]^{2-}$, (CL+2O)) was analyzed (figure 3c). Carboxylate anions are observed at *m/z* 279 ($RCOO^-$) and 311 ($[RCOO+2O]^-$), and also the ions *m/z* 415 and 447, also observed in the MS/MS of $[M-H]^-$, confirming the presence of a hydroperoxy CL.

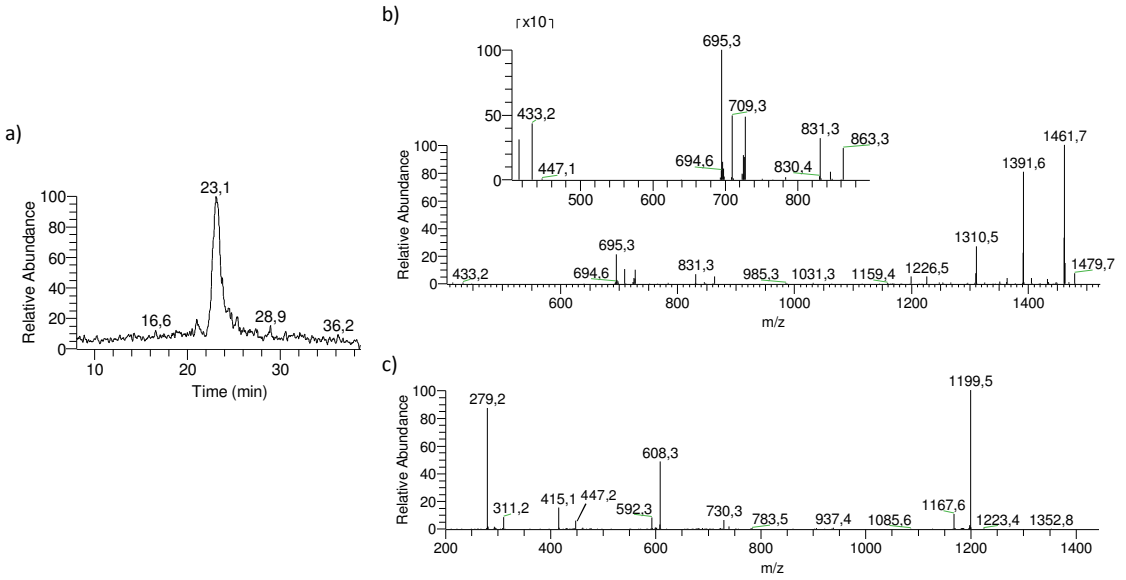

Figure 3: (a) RIC of the $[M-H]^-$ ion at *m/z* 1479 and $[M-2H]^{2-}$ ion at *m/z* 739 of the TLCL plus two oxygen atoms. (b) LC-MS/MS of the $[M-H]^-$ ion. (c) LC-MS/MS of the $[M-2H]^{2-}$.

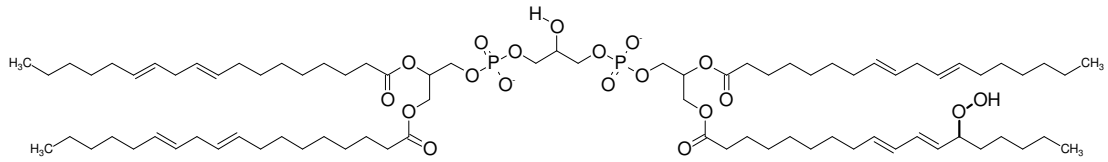

Figure 4: Proposed structure of the ion at *m/z* 739 $(CL+2O)^-$

The oxidation product formed by the insertion of four oxygen atoms to TLCL, correspondent to the molecular ion at *m/z* 1511 ($[M-H]^-$) and at *m/z* 755 ($[M-2H]^{2-}$) elutes in two overlapping peaks, at 18.3min and at 19.3min (Figure 5a). This suggests the presence of two isomeric structures, confirmed by the LC-MS/MS spectra obtained for each retention time.

The LC-MS/MS of the molecular ion at *m/z* 1511 ($[M-H]^-$), obtained at RT 18.3min (Figure 5b) shows the ions at *m/z* 831 ($[(PA-H)+136]^-$) and 863 ($[(PA-H)+136+2O]^-$). It also shows the ions at *m/z* 695 ($[PA-H]^-$), 727 ($[(PA-H)+2O]^-$). These product ions indicate that in this oxidation product each hydroperoxy group is located in different PA moieties of the TLCL molecule. The LC-MS/MS spectrum of the $[M-2H]^{2-}$ (Figure 5c) of the isomer that elutes at 18.3min shows the $RCOO^-$ fragment ion at *m/z* 279 and the $[RCOO+2O]^-$ fragment ion at *m/z* 311, confirming the presence of non modified fatty acyl chains and a fatty acyl chain with two additional oxygen atoms (hydroperoxy linoleoy fatty acyl chain). The fragment ions

PA-RCOOH, observed at *m/z* 415 and the PA-RCOOH+2O ion, observed at *m/z* 447 confirm this isomer is **TLCL-($C_{18:2}$)($C_{18:2+OO}$)/($C_{18:2}$)($C_{18:2+OO}$)**.

The LC-MS/MS of the molecular ion at *m/z* 1511 ($[M-H]^-$), obtained at RT 19.3 (Figure 5b) shows the fragment ions at *m/z* 831 ($([PA-H)+136]^-$), 863 ($[(PA-H)+136+2O]^-$), and 895 ($[(PA-H)+136+4O]^-$). Also, in the other region of this spectrum, we can see the fragment ions at *m/z* 695 ($[PA-H]^-$), 727 ($[(PA-H)+2O]^-$) and 759 ($[(PA-H)+4O]^-$). The ions with *m/z* 759 and 895, identified as $[PA-H]^-$ and $[PA+136]^-$ ions with 4 additional oxygen atoms, only appear in the LC-MS/MS spectrum obtained at 19.3min, and are absent from the MS/MS spectrum of the isomer that elutes at RT 18.3. This indicates that this TLCL oxidation product has the 4 oxygen atoms in the same PA moiety. Due to the poor chromatographic resolution of these two isomers, the ion at *m/z* 727 (corresponding to PA plus two oxygen atoms) is present in both spectra but its presence at oxygen atoms 19.3min we due to the tailing of the isomer eluting at 18.3 min. Despite our efforts, we were unable to obtain better chromatographic separation. Furthermore, the fragment ions at *m/z* 279 ($RCOO^-$), 311 ($[RCOO+2O]^-$) and 343 ($[RCOO+4O]^-$), corresponding to the non-modified acyl chain, the acyl chain with two and with four additional oxygen atoms, are observed in the LC-MS/MS spectra of the $[M-2H]^{2-}$ molecular ion of the isomer eluting at 19.3min. In the $[PA-RCOOH]^-$ zone it is also possible to observe the fragment ion at *m/z* 479, corresponding to the $[PA-RCOOH]^-$ plus four oxygen atoms. This allows us to conclude that the isomer bears the 4 oxygen atoms in only one fatty acyl chain. As previously noted, the observation of the fragment ion at *m/z* 311 can be due to some co-elution of the isomer with RT 18.3.

Altogether, these results allow us to propose the following structures for these compounds: **CL-($C_{18:2}$)($C_{18:2+OO}$)/($C_{18:2}$)($C_{18:2+OO}$)** (RT 18.3min) and **CL-($C_{18:2)}$)$_2$/($C_{18:2}$) ($C_{18:2+(OO)_2}$)** (RT 19.3min) (Figure 6).

Interestingly, in a previous study that identified short chain oxidation products formed during TLCL oxidation, it was found that multiple oxidation occurs in only one fatty acyl chain, while the other three fatty acyl chains remain unmodified [25]. Also, oxidation in more than one place of linoleoyl fatty acyl chain was observed during oxidation of PLPC[19].

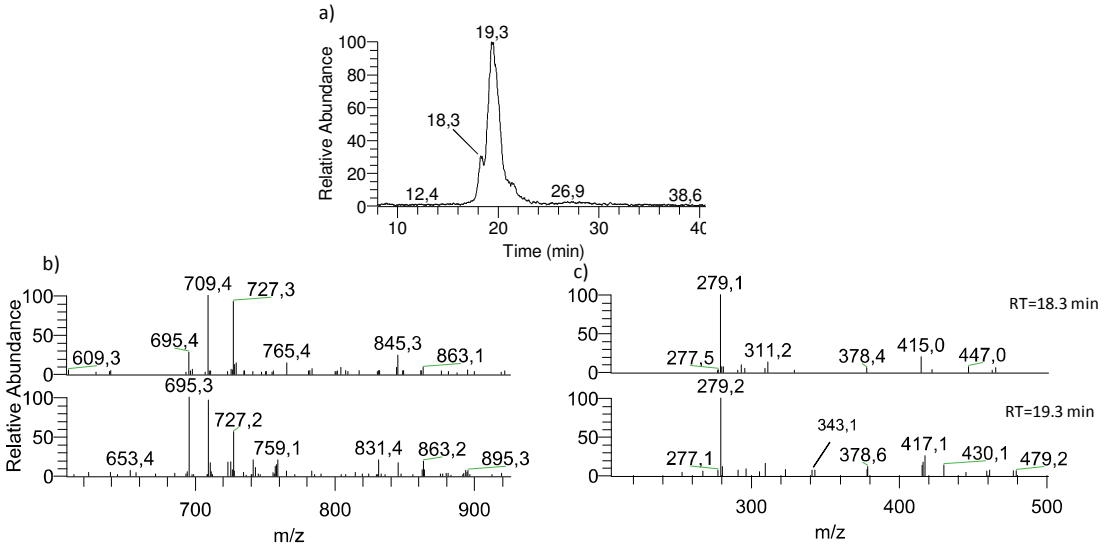

Figure 5: (a)RIC of the $[M-H]^-$ ion at *m/z* 1511 and $[M-2H]^{2-}$ ion at *m/z* 755 of the CL plus four oxygen atoms. (b)LC-MS/MS of the $[M-H]^-$ ion. (c) LC-MS/MS of the $[M-2H]^{2-}$

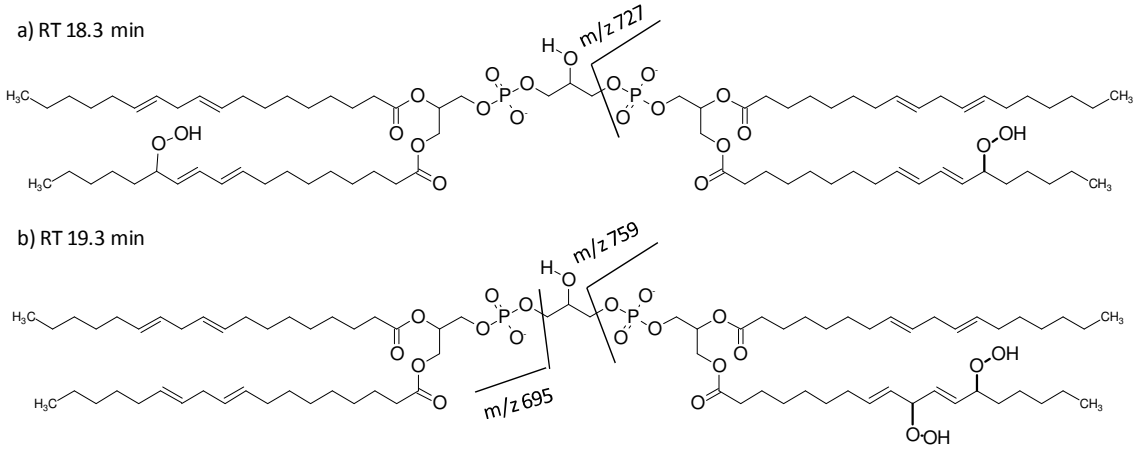

Figure 6: Proposed structures for the ion with *m/z* 1511: a) RT 18.3 min; b) 19.3 min

Long chain CL oxidation products with 6 additional oxygen atoms were also analyzed by LC-MS/MS. The RIC obtained for this CL plus six oxygen atoms, shows the elution of, at least four isomers, since four overlapping peaks are identified in Figure 7a.

The LC-MS/MS spectrum of the molecular ion at *m/z* 1543 ($[M-H]^-$) acquired at 15.0 and 16.1min are similar (Figure 7b), the most abundant product ion is $[PA-H+2O]^-$, at *m/z* 727. We can also observe the fragment ion at *m/z* 863, corresponding to $[(PA-H)+136+2O]^-$. Besides, diagnostic ion of the presence of 4 additional oxygen atoms is observed at *m/z* 895 ($[(PA-H)+136+4O]^-$). It is important to point out that the ions at *m/z* 695 and

831, corresponding to the non-modified $[PA-H]^-$ and $[(PA-H)+136]^-$ respectively, are of very low abundance. Also, the carboxylate anions observed in the LC-MS/MS spectrum of $[M-2H]^{2-}$ acquired at the same RT are observed at *m/z* 279 and 311 ($[RCOO+2O]^-$) and the ions at *m/z* 295 ($[RCOO+O]^-$), 327 ($[RCOO+3O]^-$) and 343 ($[RCOO+4O]^-$) are absent. With these results we propose the following structure for the ion eluting at 16.3min: **CL-($C_{18:2}$)($C_{18:2+OO}$)/($C_{18:2+OO}$)$_2$**.

The LC-MS/MS spectrum of the molecular ion at *m/z* 1543 ($[M-H]^-$) acquired at 17.5min shows the product ions at *m/z* 727, 743 and 759, corresponding respectively to $[PA-H]^-$ with 2, 3 and 4 additional oxygen atoms. Fragment ions of the zone $[(PA-H)+136]^-$ with 2, 3, 4 and 5 additional oxygen atoms are observed at *m/z* 863, 879, 895 and 911. These ions suggest the presence of a mixture of positional isomers, as shown in the Figure 8.

The LC-MS/MS spectrum of the $[M-2H]^{2-}$ obtained at 17.5min, shows the fragment ions at *m/z* 295 ($[RCOO+O]^-$) and 327 ($[RCOO+3O]^-$), as well as the ions at *m/z* 279 ($RCOO^-$) and 311 ($[RCOO+2O]^-$). The fragment ion at *m/z* 295 shows the presence of a hydroxyl fatty acyl chain. The fragment ion at *m/z* 327 shows the presence of an acyl chain with three additional oxygen atoms, corresponding to a hydroxyl-hydroperoxy fatty acyl chain. Altogether, we propose that this compound should have one non modified acyl chain, one chain with an hydroxyl group, one chain with two additional oxygen atoms and another chain with three additional oxygen atoms. The two isomers that co-elute considering the different distribution of the oxidized fatty acyl chains (**CL-($C_{18:2}$)($C_{18:2+OO}$)/ ($C_{18:2+O}$)($C_{18:2+3O}$), CL-($C_{18:2}$)($C_{18:2+O}$)/($C_{18:2+OO}$)($C_{18:2+3O}$)**). And are shows in Figure 8.

The compound(s) with the LC-MS/MS spectrum shown in Figure 7a elutes at 18.9min; It yields the product ions at *m/z* 695 ($[PA-H]^-$), 727 ($[(PA-H)+2O]^-$), 759 ($[(PA-H)+4O]^-$), and 791 ($[(PA-H)+6O]^-$). In the $[(PA-H)+136]^-$ zone, the $[(PA-H)+136]^-$ with 2 and 4 additional oxygen atoms are observable, due to the presence of the ions at *m/z* 863 and 895. The presence of these ions, as described previously, suggests that mixture of positional isomers is co-eluting (Figure 8). The LC-MS/MS spectrum of the ion $[M-2H]^{2-}$ shows in carboxylate anions zone the ions at *m/z* 279 ($RCOO^-$), 311 ($[RCOO+2O]^-$) and 343 ($[RCOO+4O]^-$) and the spectrum shows in the $[(PA-H)-RCOOH]^-$ zone, the ions at *m/z* 415 ($[(PA-H)-RCOOH]^-$), 447 ([(PA-

H+2O)-RCOOH]$^-$) and 479 ([(PA-H+4O)-RCOOH]$^-$). This data shows that the derivatives that elute at 18.9 min possesses two non modified acyl chains (*m/z* 279), one chain with two oxygen atoms (*m/z* 311) and another with four additional oxygen atoms (*m/z* 343). In conclusion, based in the LC-MS/MS spectrum of the ions [M-H]$^-$ and [M-2H]$^{2-}$ we can affirm that, at least, two isomers co-elute: **CL-($C_{18:2}$)($C_{18:2+(OO)2}$)/ ($C_{18:2}$)($C_{18:2+OO}$)** and **CL-($C_{18:2}$)$_2$/($C_{18:2+(OO)2}$)($C_{18:2+OO}$)**

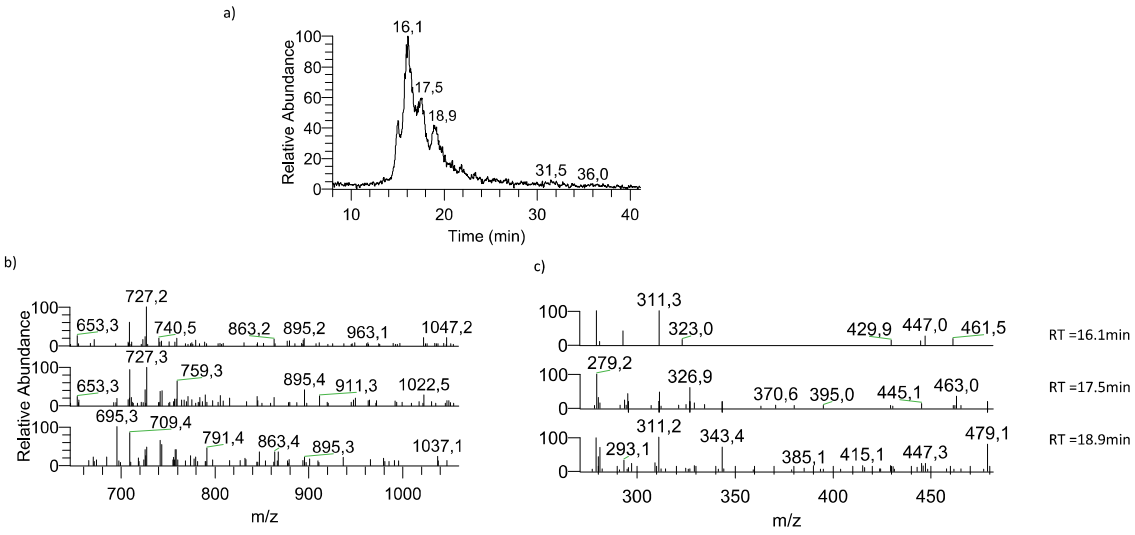

Figure 7: (a)RIC of the [M-H]$^-$ ion at *m/z* 1543 and [M-2H]$^{2-}$ at *m/z* 771 attributed to CL plus 6 oxygen atoms. (b) LC-MS/MS of the [M-H]$^-$ ion. (c) LC-MS/MS of the [M-2H]$^{2-}$ ion.

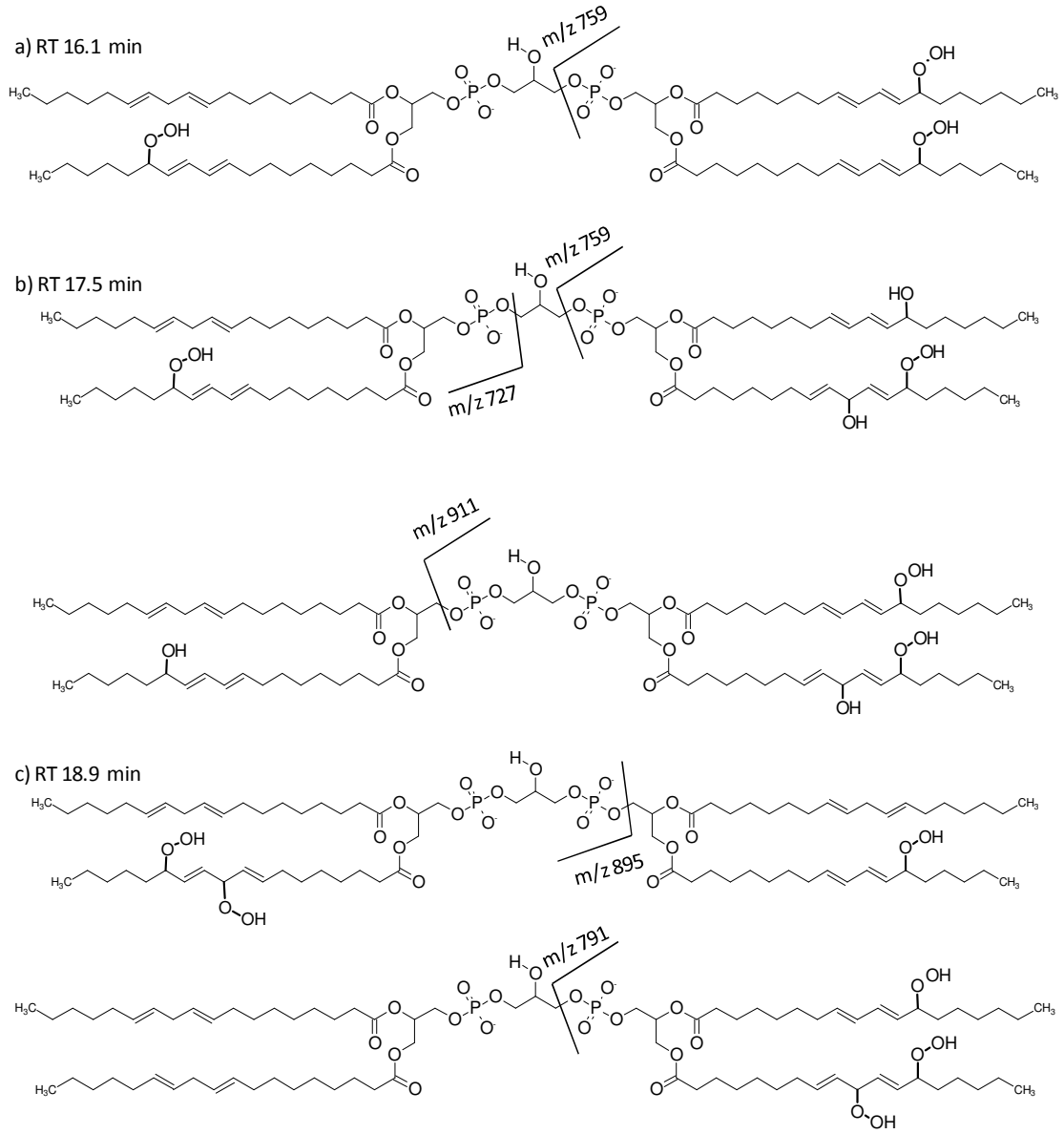

Figure 8: proposed structures for the ion with *m/z* 1543 (TLCL+6O): a) RT 16.1min;b) RT 17.5min; c) RT 18.9min.

The RIC obtained for the CL oxidation product plus 8 oxygen atoms (Figure 9a), is a broad peak, probably due to the presence of several isomers. However we can observe two distinct LC-MS/MS spectra, one at 13.8min and another at 15.6min.

The LC-MS/MS spectrum of the $[M\text{-}H]^-$ ion obtained at 13.8min shows, with high relative abundance, the fragment ion at *m/z* 759, corresponding to $[PA\text{-}H]^-$ with four additional oxygen atoms. The LC-MS/MS spectrum of the ion $[M\text{-}2H]^{2-}$ shows the ion $[RCOO+4O]^-$ at *m/z* 343, indicating that one acyl chain possesses two additional oxygen atoms and another possesses

four additional oxygen atoms The absence in the tandem mass spectra of other informative ions lead us proposing the structure in which each peroxy group is quantitatively distributed in each linoleic acid chain(**CL-($C_{18:2+(OO)2}$)/ $C_{18:2+(OO)2}$**) as shown in Figure 10.

The LC-MS/MS spectrum of the ion $[M-H]^-$ obtained at 15.6min (Figure 9a) shows ions at *m/z* 743 ($[(PA-H)+3O]^-$), 759 ($[(PA-H)+4O]^-$), and 775 ($[(PA-H)+5O]^-$) and $[PA+136+5O]^-$ at *m/z* 911. In the LC-MS/MS the ion $[M-2H]^{2-}$ spectrum obtained at 15.6min we can observe the ions at *m/z* 295, 311 and 343, correspondent to the $RCOO^-$ plus, respectively, 1, 2 and 4 additional oxygen atoms in the acyl chains . The presence of the all these product ions lead us to conclude that several isomers co-elute in this chromatographic peak (RT=15.6min, Figure 9a). However, the identified ions lead us to propose the following structures: **CL-($C_{18:2+O}$)($C_{18:2+(OO)2}$)/ ($C_{18:2+O}$)($C_{18:2+OO}$)** and **CL-($C_{18:2+OO}$)$_2$/($C_{18:2}$)($C_{18:2+(OO)2}$)** as represented in Figure 10.

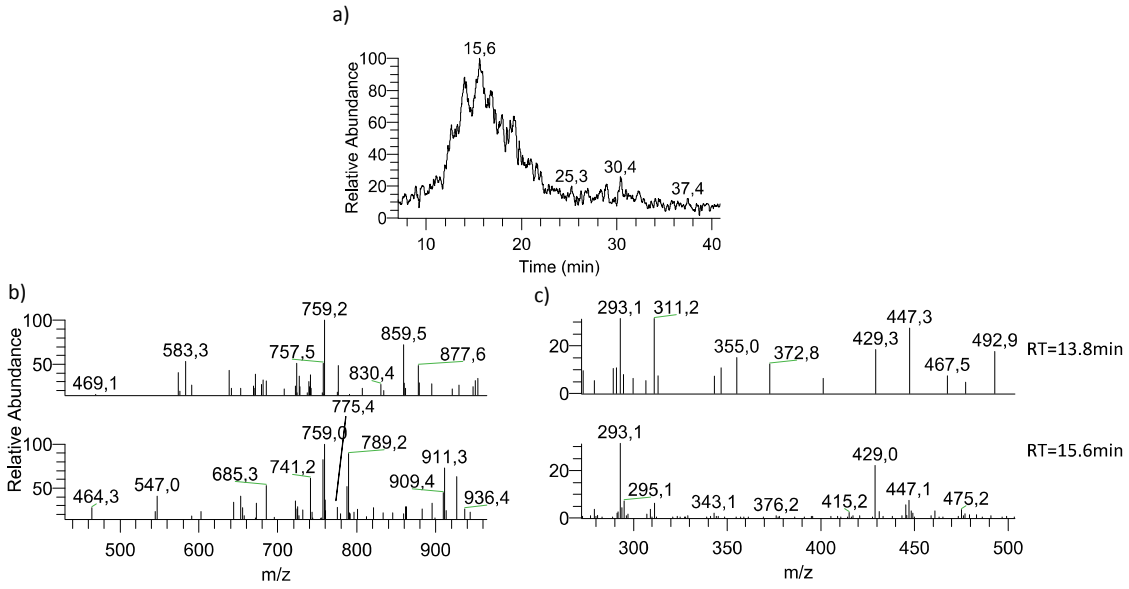

Figure 9: (a) RIC of the $[M-H]^-$ ion at *m/z* 1575 and $[M-2H]^{2-}$ ion at *m/z* 787 of the CL plus eight oxygen atoms. (b) LC-MS/MS of the $[M-H]^-$ ion. (c) LC-MS/MS of the $[M-2H]^{2-}$.

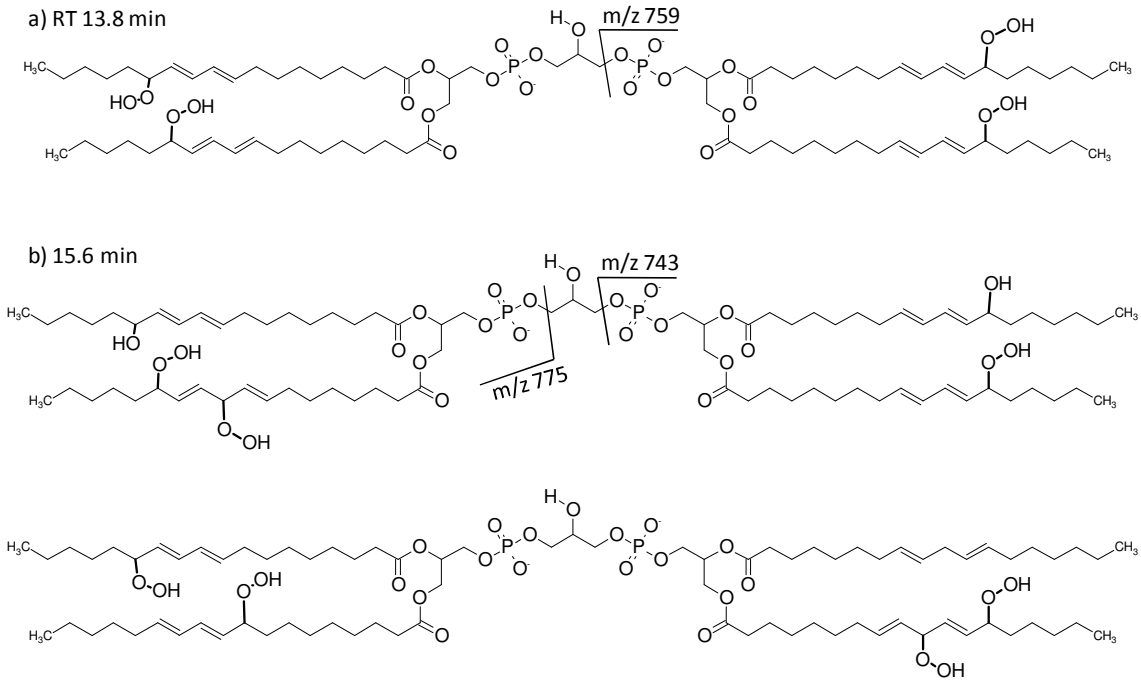

Figure 10: Proposed structures for the ion with *m/z* 1575: a) RT 13.8min; b) 15.6min.

Conclusions

The long-chain oxidation products, formed by reaction with the hydroxyl radical with cardiolipin were identified by liquid-chromatography electrospray tandem mass spectrometry. Long chain oxidation products formed by the addition of 2, 4, 6 and 8 oxygen atom to CL molecule were identified and characterized by reverse phase LC-MS/MS. This approach allowed determining the distribution of the oxygen atoms along the CL molecule and to separate several isomers of the long chain products with higher number of oxygen atoms. It was found that the linoleoyl fatty acyl chains TLCL molecule doesn't oxidize equally. Even when TLCL molecule oxidized by the addition of eight oxygen atoms, there is still one non-modified acyl chain and thus another fatty acyl chain with three or four oxygen atoms. This means that, under oxidative conditions by the $OH^{\cdot}$, the distribution of oxygen atoms/peroxy groups is random in the CL molecule, even when CL have equal fatty acyl chains in all the positions. Specific product ions of each isomer observed in the MS/MS spectra were identified which will be a valuable tool for their detection in biological samples.

These findings may open up 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.

Acknowledgments

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Chapter IV

Conclusion

Conclusions

The oxidation products, formed during the *in vitro* reaction of the hydroxyl radical with cardiolipin, were identified by Liquid- Chromatography Electrospray Tandem Mass Spectrometry (LC-MS and MS/MS). The CL oxidation products identified comprise long chain products, which are products that preserve the phospholipid skeleton and short chain products, formed by cleavage of the unsaturated fatty acyl chains. The shortened fatty acyl chain contains either a terminal aldehyde or carboxylic groups. Although the CL studied 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.

Analysis of biological samples using the same methodology allow the identification of CL short chain oxidation products (with C9) in mitochondria isolated from kidney rats with nephropathy induce by gentamicin, which could explain the mitochondrial dysfunctionality observed in these rats.

Long chain oxidation products formed by the addition of 2, 4, 6 and 8 oxygen atom to CL molecule were also characterized by reverse phase LC-MS/MS. This approach allows to separate several isomers of the long chain products with higher number of oxygens and to identify their distribution along the CL fatty acyl chain. The TLCL molecule doesn't oxidize the same way in all acyl chains. Even a TLCL molecule with eight additional oxygen atoms still possesses one non-modified acyl chain. Specific product ions of each isomer observed in the MS/MS spectra were identified which will be a valuable tool for their detection in biological samples.

These findings may open up 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.