Mass spectrometry strategies to unveil modified aminophospholipids of biological interest

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Abstract

The biological functions of modified aminophospholipids (APL) have become a topic of interest during the last two decades, and distinct roles have been found for these biomolecules in both physiological and pathological contexts. Modifications of APL include oxidation, glycation, and adduction to electrophilic aldehydes, altogether contributing to a high structural variability of modified APL. An outstanding technique used in this challenging field is mass spectrometry (MS). MS has been widely used to unveil modified APL of biological interest, mainly when associated with soft ionization methods (electrospray and matrix-assisted laser desorption ionization) and coupled with separation techniques as liquid chromatography. This review summarizes the biological roles and the chemical mechanisms underlying APL modifications and comprehensively reviews the current MS-based knowledge that has been gathered until now for their analysis. The interpretation of the MS data obtained by in vitro-identification studies is explained in detail. The perspective of an analytical detection of modified APL in clinical samples is explored, highlighting the fundamental role of MS in unveiling APL modifications and their relevance in pathophysiology.

Keywords: Aminophospholipids, Phosphatidylethanolamine, Phosphatidylserine, Modification, Mass Spectrometry
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I. Introduction

Phosphatidylethanolamine (PE) and phosphatidylserine (PS) are the two classes of glycerophospholipids named aminophospholipids (APL), as they bear a free amine in the polar head group. In mammalian cells, PE and PS are mainly confined to the inner leaflet of the membrane (Schick, Kurica, and Chacko 1976; Higgins and Evans 1978) where they are responsible for both structural and signaling roles. They allow membranes to be fluid as well as impermeable to water and solutes, mediate communication among cells and provide a membrane anchor for signaling macromolecules (Vance and Tasseva 2013). PE is the second most abundant phospholipid class in cell membranes of mammalian organisms, making up 20% of the whole phospholipid profile, while PS is present in a minor amount, constituting 2-10% of all phospholipids (Vance 2008). The biological functions of APL are considerably dependent on their location in the outer or in the inner leaflet on the membrane. Upon physiological conditions, PE and PS are mainly confined to the inner leaflet, maintained by specific flippases, which show a higher selectivity for PS (Daleke 2003), and scramblases (Bevers, Comfurius, and Zwaal 1983; Connor and Schroit 1990; Schroit and Zwaal 1991; Connor et al. 1992; Daleke and Lyles 2000). This specific location is crucial for PS signaling events. Externalized PS is a known activator of the blood-clotting cascade (Higgins and Evans 1978; Bevers, Comfurius, and Zwaal 1983; Connor and Schroit 1990; Connor et al. 1992; Daleke and Lyles 2000; Daleke 2003) and a hallmark of cells facing apoptosis (Fadok et al. 2001; Zwaal, Comfurius, and...
Bevers 2005; Balasubramanian, Mirnikjoo, and Schroit 2007; Segawa et al. 2014). In mammalian cells, PE is involved in the modulation of the cell membrane curvature (Cullis and De Kruijff 1978; Verkleij et al. 1984). The presence of PE in the double layer was also associated with the regulation of contractile ring disassembly during cytokinesis of mammalian cells (Emoto and Umeda 2000) and the arrangement of Golgi membrane fusion in early-divided mitotic cells (Pécheur et al. 2002). Furthermore, PE can modify several proteins in mammalian cells, acting as a donor of ethanolamine moiety (Menon and Stevens 1992; Signorell et al. 2008). Table 1 provides an overview of current knowledge on the functions and roles of PE and PS in mammalian cells.

APL are prone to be modified by oxidation (Domingues et al. 2009; Simões et al. 2010; Maciel et al. 2011; Melo, Santos, et al. 2013), glycation (Simões et al. 2010; Maciel, da Silva, et al. 2013; Melo, Silva, et al. 2013; Annibal et al. 2016), and reactive molecules such as electrophilic aldehydes (Bernoud-Hubac et al. 2004; Bacot et al. 2007). These changes in native APL may occur under physiological and pathological conditions through non-enzymatic reactions with reactive oxygen species (ROS), glucose, and other carbonyl compounds, or through enzyme-catalyzed reactions, generating a vast number of differently modified derivatives which display several biological functions. Oxidized PS (ox-PS) is externalized in cell membranes as an important “eat me” marker of apoptosis, and several studies highlighted it as a more potent signal for activated macrophages when compared to native PS (Kagan et al. 2002; Koty et al. 2002; Fabisiak et al. 2005; Greenberg et al. 2006). In addition to its interaction with macrophages, ox-PS mediates other biological functions that controversially relate it to the modulation of the immune system, from the inhibition of peripheral T-cells proliferation in human blood (Seyerl et al. 2008) to the induction of cytokine production in monocytes and dendritic cells (Silva et al. 2012). Also, PE oxidized by ROS (ox-PE) was found to be an active component of oxidized low-density lipoproteins (ox-LDL), that mediates the binding between platelets and annexin (Zieseniss et al. 2001). Furthermore, several biological roles have been investigated for ox-PE generated by lipoxygenase (LOX) in activated human or murine blood cells (Thomas et al. 2010; Zhao et al. 2011; Hammond et al. 2012). More recently, Kagan et al. (Kagan et
al. 2016) reported the central role of PE oxidation products with modifications in arachidonic acid and adrenic acid chains in the mediation of ferroptosis, a controlled cell death process. Moreover, PE and PS can react with glucose or electrophilic aldehydes and form adducts, which biological functions are far from being understood. The formation of glycated species and advanced glycation end products (AGE) of PE were reported in LDL incubated with glucose (Ravandi, Kuksis, and Shaikh 2000). Glycated PE (gly-PE) was also detected in red blood cells from both healthy and diabetic subjects (Ravandi et al. 1996; Lertsiri, Shiraishi, and Miyazawa 1998; Fountain et al. 1999; Nakagawa 2005). Interestingly, Simões et al. (Simões et al. 2013b; Simões et al. 2013a) reported that glycated PE species are inducers of a pro-inflammatory phenotype in several populations of human peripheral immune cells. Conversely, only one study reported the occurrence of a PS AGE in red blood cells from healthy subjects (Fountain et al. 1999). This study provided evidence that glycation of PS is also likely to occur *in vivo*, despite the authors could not detect an increase in the concentration of the same product in plasma samples from diabetic subjects (Fountain et al. 1999). Aldehyde-modified APL (al-PE and al-PS) were also highlighted as a family of bioactive molecules. Al-PE pyrrole adducts present in LDL from normolipidemic volunteers were found to enhance the viability of macrophages (Riazy et al. 2011). Guo et al. (Guo et al. 2011) treated human umbilical vein endothelial cells (HUVEC) with al-PE and observed an increase in the expression of monocyte adhesion molecules, leading to endothelial dysfunction and inflammation. A comprehensive view of the biological activities modified APL is reported in Table 2, which consists of a summary of the functions that have been so far investigated for each group of derivatives. Moreover, Table 2 merges the studies reporting the occurrence of modified APL in models of inflammatory diseases and clinical samples from inflammation-related pathologies, including diabetes, atherosclerosis, Alzheimer’s disease (AD) and cystic fibrosis.

Even though the biological relevance of modified APL is evident, a link between the structural changes in APL, the biological function of the modified derivatives and their occurrence as biomarkers of diseases has not been identified yet. To this end, it is necessary to be able to predict
and to understand the type of modifications that can occur \textit{in vivo} upon pathophysiological conditions. In parallel, it is of fundamental importance to develop analytical strategies aimed to characterize the structural complexity of modified APL and detect these biomolecules in biological samples. In the following section, the chemistry of APL modifications will be reviewed, sorting the chemical reactions that can induce their modification. The remaining sections of this review will comprehensively examine the information that mass spectrometry (MS-)based approaches have provided for the analysis of modified APL, \textit{in vitro} and biological samples, over the last two decades. The description of the structural modifications that can occur is accompanied by detailed explanations of the MS and MS/MS data that have been acquired for identification, structural characterization, detection and quantification of modified APL.

\textbf{II. Chemistry of APLs Modifications}

APL can undergo several modifications, which overall lead to a variety of products of biological relevance. Figure 1 proposes a schematic representation of the modifications that have been described so far for APL. The chemical reactions leading to modified APL can be categorized as follows:

\textbf{A. Oxidation of the fatty acyl chains} (Khaselev and Murphy 1999; Gugiu et al. 2006; Maskrey et al. 2007; Tyurin et al. 2008; Tyurina et al. 2008; Domingues et al. 2009; Tyurin et al. 2009; Simões et al. 2010; Thomas et al. 2010; Tyurina et al. 2010; Lloyd T. Morgan et al. 2010; Tyurina, Tyurin, et al. 2011; Tyurina, Kisin, et al. 2011; Clark et al. 2011; Hammond et al. 2012; Melo, Santos, et al. 2013; Melo, Silva, et al. 2013; Maciel, Faria, et al. 2013; Simões et al. 2013a);

\textbf{B. Oxidation of the polar head} (Carr, van den Berg, and Winterbourn 1998; Richter et al. 2008; Simões et al. 2010; Üllen et al. 2010; Maciel et al. 2011; Melo, Santos, et al. 2013; Maciel et al. 2014);
C. Adduction of glucose to the polar head with or without further oxidation of the adduct (glycation and glyco-oxidation) (Ravandi, Kuksis, and Myher 1995; Ravandi et al. 1996; Requena et al. 1997; Lertsiri, Shiraishi, and Miyazawa 1998; Pamplona et al. 1998; Fountain et al. 1999; Breitling-Utzmann et al. 2001; Nakagawa 2005; Shoji et al. 2010; Simões et al. 2010; Sookwong et al. 2011; Maciel, Faria, et al. 2013; Maciel, da Silva, et al. 2013; Annibal et al. 2016);

D. Adduction of aldehydes to the polar head (Bhuyan et al. 1986; Guichardant et al. 1998; Bacot 2003; Amarnath et al. 2004; Tsuji et al. 2003; Zamora and Hidalgo 2003; Bernoud-Hubac et al. 2004; Stadelmann-Ingrand, Pontcharraud, and Fauconneau 2004; Hisaka et al. 2010; Bacot et al. 2007).

A. Oxidation of the fatty acyl chains

Oxidation of the fatty acyl chains esterified to APL has been correlated with radical-mediated or enzyme-catalyzed oxidation reactions. Radical-mediated oxidation of fatty acyl chains usually relies on ROS as initial effectors of the modification pathway. ROS are generated in vivo by different sources, such as the mitochondrial electron-transport chain and NAD(P)H oxidases, and participate in the physiological metabolism of mammalian cells (Dröge 2002; Murphy 2009). Under oxidative stress, the equilibrium between cellular detoxification and generation of ROS is lost in favor of an excess of oxidants species. Thus, high concentrations of ROS mediate a randomly extensive oxidative damage to biomolecules, including lipids (reviewed by Dröge) (Dröge 2002). Oxidative stress can also be induced in vitro or ex vivo through a set of chemical, physical and enzymatic systems able to mimic the biological occurrence of the oxidation reaction. These biomimetic systems are used for the characterization of the oxidized APL in vitro and ex vivo, which is necessary for structure-biological activity studies and facilitates the development of targeted methods for the detection and the quantification of these molecules in vivo. A list of biomimetic systems that have been used so far for APL oxidation is reported in Table 3. The most common biomimetic systems are reported in Figure
2 and include Fenton reaction, 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH), photosensitization and lipooxygenases (LOX).

Except for MPO, which catalyzes the formation of a non-radical ROS (HOCl), most of the biomimetic methods grouped in Table 3 lead to the production of partially-reduced oxygen species (*OH, O_2^*, 1O_2) and other free radicals (e.g., R-O-O* from AAPH). Free radicals mediate the hydrogen abstraction and the subsequent generation of a carbon-centered radical which promptly reacts with O_2, leading to the formation of one or more hydroperoxyl derivatives. These radicals are particularly unstable and easily get stabilized by abstracting further (bis-)allylic hydrogen atoms from surrounding lipid molecules, leading to the formation of non-radical fatty acyl hydroperoxides (R-O-O-H). Consequently, metal cations such as Fe^{2+} (in the case of Fenton reaction) or Cu^{2+}, catalyze the decomposition of hydroperoxides into alkoxy radicals (RO*). These radicals may abstract carbon-centered hydrogen from an adjacent lipid molecule and generate hydroxides (R-O-H), or further degrade into other chain-propagation radicals as alkyls (R*), hydroxyl-alkyls (HOR*), epoxy-alkyls (OR*) and epoxy-peroxyls (OROO*) (Reis and Spickett 2012). Esterified APL hydroperoxides can also cyclize and form a 5-membered ring that cleaves, forming a subclass of products named isoketals (isoLGs) (Salomon 2005; Li et al. 2009).

Lipid oxidation derivatives that are named long chain oxidation products contain one or several oxygen atoms inserted on the fatty acyl chains, as in the case of hydroperoxy or hydroxy, keto and epoxy-derivatives. However, radical intermediates as alkoxy radicals can further degrade through a β-cleavage of the C-C bond between the oxygenated carbon atom and the unsaturation in vinylic position. This cleavage leads to the formation of esterified aldehydes, keto-aldehydes, hydroxy-aldehydes, carboxylic acids, keto-carboxylic acids and hydroxy-carboxylic acids, named short chain oxidation products (Figure 1) (Gugiu et al. 2006; Domingues et al. 2009; Maciel, Faria, et al. 2013). When radical-radical reactions occur, in some cases due to antioxidant molecules, the radical-based oxidation process reaches the termination phase (Wefers and Sies 1988; Tadolini et al. 2000).
B. Oxidation of the polar heads

Among the ROS summarized in Table 3, the ones inducing one-electron oxidation on unsaturated fatty acyl chains have also been found to mediate radical-mediated oxidation of the polar head for both PE (Melo, Santos, et al. 2013) and PS (Maciel et al. 2011; Maciel et al. 2014). The serine center in the polar head of PS has hydrogen linked to the α-carbon, which is easily abstracted under radical attack, leading to an α-alkoxyl derivative (Maciel et al. 2011). The oxidation of the α-alkoxyl further proceeds to polar head derivatives modified as terminal acetic acid, terminal acetamide, terminal hydroxy acetaldehyde and terminal hydroperoxy acetaldehyde (Maciel et al. 2011). These modifications were observed for the first time in a study in which oxidation of PS was carried out in vitro through the Fenton reaction (Maciel et al. 2011). Later on, the oxidation of PS polar head to terminal acetic acid was found to occur in human keratinocytes treated with AAPH (Maciel et al. 2014). Similarly, in vitro irradiation of PE standards with white light in the presence of cationic porphyrins as photosensitizers (photo-oxidation) led to oxidative deamination of the ethanolamine group, with the generation of the polar head modified as terminal acetaldehyde (Figure 1) (Melo, Santos, et al. 2013).

Hypohalous acids as HOCl can also modify the polar head of PE via two-electron oxidation mechanism on the free amino group. This reaction runs by the initial formation of a chloroamine and, upon HOCl excess, continues with the formation of a dichloramine, which eventually loses two moles of HCl and leads to a terminal nitrile derivative (Carr, van den Berg, and Winterbourn 1998; Richter et al. 2008; Üllen et al. 2010).

C. Adduction of glucose to the polar head (glycation and glyco-oxidation)
The amino group present in the polar head of APL is reactive towards electrophilic moieties as carbonyl groups. In fact, several published works mentioned the reactivity of the amino groups of PE and PS towards glucose and the consequent formation of glyated APL (Ravandi, Kuksis, and Myher 1995; Ravandi et al. 1996; Lertsiri, Shiraishi, and Miyazawa 1998; Oak, Nakagawa, and Miyazawa 2000; Nakagawa 2005; Simões et al. 2010; Sookwong et al. 2011; Maciel, da Silva, et al. 2013; Maciel, Faria, et al. 2013; Melo, Silva, et al. 2013; Annibal et al. 2016). APL glycation occurs via reaction between the amino group of the APL polar heads and the carbonyl moiety of glucose, initially leading to a Schiff-base derivative which promptly establishes an equilibrium with the Amadori ketoamine (Simões et al. 2010; Annibal et al. 2016). Glycated APL can be further modified if radical driven oxidation occurs during or after the glycation. Both the Schiff-base and the Amadori derivatives were found to undergo oxidation on the fatty acyl chains and the glycated polar heads when ROS were present, in a process that is identified as glyco-oxidation, and leads to the production of AGE of APL (Simões et al. 2010; Melo, Silva, et al. 2013; Maciel, da Silva, et al. 2013; Maciel, Faria, et al. 2013; Simões et al. 2013a; Annibal et al. 2016). When the radical mechanism involves the polar head glycated as Schiff base, the hydrogen atom on the methylene adjacent to the imine bond is abstracted, leading to peroxyl and alkoxyl radicals. These are often stabilized through reduction to the carbonyl. However, if the radical reaction occurs on the Amadori derivative, the oxidation mainly affects the sugar moiety through sequential backbone cleavages (Simões et al. 2010; Maciel, da Silva, et al. 2013; Annibal et al. 2016) (Figure 1).

D. Adduction of aldehydes to the polar head

The oxidative degradation of lipids generates a variety of low-molecular-weight aldehydes including hydroxy-alkenals, keto-alkenals, γ-keto-aldehydes (Frankel et al. 1977; Benedetti, Comporti, and Esterbauer 1980; Lee and Blair 2000) and isoLGs (Salomon 2005) (reviewed by Reis and Spickett) (Reis and Spickett 2012). These lipid-derived aldehydes contain several electrophilic
centers, which can form covalent adducts with the free amino groups in the polar heads of APL. It has been reported for isoLG a reaction rate with PE that was 4.4-fold faster than the reaction between isoLG and lysine (Amarnath et al. 2004). Bernoud-Hubac et al. studied the mechanism of adduction between isoLG and PE, showing that the reaction runs with an initial formation of a Schiff base, that quickly forms a stable pyrrole through a cyclization (Bernoud-Hubac et al. 2004). On the other hand, the reaction between APL and hydroxy-alkenals was found to occur mainly by Michael addition (Guichardant et al. 1998; Bacot et al. 2007), even though adducts formed by the Schiff base-pyrrole pathway were also reported for the reaction of PE with 4,5(E)-epoxy-2(E)-heptenal (Zamora and Hidalgo 2003).

More recently, Vazdar et al. employed $^1$H nuclear magnetic resonance spectroscopy, Fourier-transform infrared spectroscopy and MS to study the reactivity of PE towards 4-hydroxy-2-nonenal (HNE) and 4-oxo-2-nonenal (ONE) (Vazdar et al. 2017). This study highlighted that PE-HNE adducts, both Michael and Schiff base, preferentially cyclized towards hemiacetal and pyrrole derivatives, in contrast with PE-ONE adducts that did not tend to form cyclic derivatives.

The characterization and the detection of modified APL currently rely on platforms capable of performing specific and sensitive analyzes. Gas chromatography (GC) coupled to MS (GC-MS) has been widely used to analyze oxidized fatty acyl chains of phospholipids in their non-esterified form, as well as modified polar heads from APL, but it is not suitable for direct analysis of intact modified APL (Spickett et al. 2010). Presently, tandem MS using soft ionization methods is the most used platform in the analysis of the complex network of PE and PS modified structures, both in vitro and in biological samples. The next section reviews and describes all the MS strategies that have so far been used in the analysis of modified APL.

III. MS-Based Strategies for the Analysis of APL Modifications
The development of soft ionization methods, such as electrospray ionization (ESI) (Yamashita and Fenn 1984) and matrix-assisted laser desorption ionization (MALDI) (Tanaka et al. 1988; Karas and Hillenkamp 1988), made the MS analysis of intact biomolecules possible, opening a new world of MS-based approaches that has strongly grown through the last 20 years. The introduction of high-resolution MS analyzers in the last decade led to several advantages in the field of lipidomics, including reduced overlap of species in the mass spectrum (due to discrimination at 1-3 ppm or even sub-ppm level) and reduced analysis time (reviewed by Ghaste, Mistrik and Shulaev (Ghaste, Mistrik, and Shulaev 2016). The following sections will review the current state of understanding of the information that MS-based analytical strategies have provided for all the known APL modifications.

A. Applications of GC-MS in the analysis of modified APL

Since APL are non-volatile compounds, their analysis by GC-MS can only be carried out after the first step of hydrolysis of the ester bonds linking the fatty acids and the polar head to the glycerol backbone. In a subsequent step, the fatty acids and the polar head released by the hydrolytic treatment are derivatized with organic acids to produce volatile esters as, for example, trifluoroacetyl, acetyl and methyl esters (van Kuijk et al. 1985; Requena et al. 1997; Fountain et al. 1999). GC-MS typically uses electron ionization (EI) as ionization technique, which is characterized by a high in-source fragmentation that frequently causes the loss of structure of the molecular ion, hindering its observation. However, even though in EI-MS the molecular ion can be absent after the ionization process, information about its structure can be provided by the comparison of its in-source fragmentation pattern with the in-source fragmentation pattern of standards, or by direct interpretation of the fragmentation patterns.

GC-MS has been used to detect AGE of APL bearing glyco-oxidative modifications on the ethanolamine or serine polar heads. The AGE of PE known as carboxymethyl-PE (CM-PE) is a
product of PE glyco-oxidation which has been quantified in vivo through GC-MS analysis of its hydrolysis product, carboxymethyl-ethanolamine (CM-Etn) (Requena et al. 1997; Pamplona et al. 1998). The experimental approach was based on acid hydrolysis of CM-PE that produced the free carboxymethylated polar head (CM-Etn). Subsequently, CM-Etn was derivatized as a trifluoroacetyl-methyl ester (TFAME) and quantified through GC-MS in single ion monitoring (SIM) mode (Requena et al. 1997; Pamplona et al. 1998). This methodology allowed the quantitative assessment of CM-Etn in mitochondrial membranes of several mammals (Pamplona et al. 1998) as well as in lipid extracts of red blood cell membranes of healthy and diabetic subjects (Requena et al. 1997). In another study, two glycation and glyco-oxidation products of APL, namely N-(Glucitol)PE and carboxymethyl-PS (CM-PS), were similarly detected and quantified by GC-MS. N-(Glucitol)PE and CM-PS were hydrolyzed in acid conditions as described above, with the production of the free, modified polar heads N-(Glucitol)ethanolamine (GE) and carboxymethyl-serine (CM-Ser), respectively. The modified polar heads were derivatized as N,O-acetyl methyl esters and finally analyzed by GC-MS in SIM mode (Fountain et al. 1999). In this study, the derivatized polar heads were quantified in the lipid extracts of human red blood cells from healthy and diabetic patients, showing an increased content of GE, but not CM-Ser, in the samples of the diabetic patients when compared to nondiabetic.

GC-MS has also been used to assess the reactivity of PE polar head with fatty aldehydes generated by lipid peroxidation. Bacot (Bacot 2003) studied the in vitro reactivity of the ethanolamine group of PE with 4-hydroxy-2-hexenal (HHE), HNE and 4-hydroxydodeca-(2,6)-dienal (HDDE). Adducted PE were hydrolyzed, and the resulting ethanolamine-hydroxyalkenal adducts were derivatized as pentafluorobenzyloximes trimethylsilyl esters before GC-MS analysis (Bacot 2003). In subsequent work, the same author and others detected PE-hydroxyalkenals Michael adducts in human blood platelets and retinas of streptozocin-induced diabetic rats (Bacot et al. 2007). In this study, Michael-adducted PE were subjected to basic hydrolysis, and the resulting ethanolamine-alkenals moieties were derivatized as trifluoroacetyl esters before the GC-MS analysis. In another
study, aldehyde-carbonylation products of PE with long-chain fatty aldehydes (e.g., pentadecanal, heptadecanal, heptadecenal) were observed through GC-MS analysis (Stadelmann-Ingrand, Pontcharraud, and Fauconneau 2004). These carbonylation products were observed in rat cortex homogenates that were submitted to UV light or Fe$^{2+}$-induced oxidation, after basic methanolysis and derivatization of the modified ethanolamine as trimethylsilyl ether (Stadelmann-Ingrand, Pontcharraud, and Fauconneau 2004). All these works show that GC-MS has undoubtedly been one of the most important analytical techniques in the analysis of lipid modification, and still plays a crucial role in classical lipidomic studies (Li et al. 2011). However, due to several disadvantages such as elaborate sample preparation, fragmentation of the molecular ions in the EI source and risk of degradation for thermolabile molecules, the relevance of this method in the study of APL modification is diminishing. As consequence of these drawbacks, MS analysis of modified APL has started to be performed mainly using soft ionization methodologies (ESI, MALDI) that preserve the structure of the analyte and, in the case of ESI, can be easily coupled with liquid chromatographic separations.

**B. Applications of ESI-MS and MALDI-MS in the analysis of modified APL**

Under ESI-MS and MALDI-MS analysis, APL can ionize in both negative and positive ions modes, with the formation of predominant [M-H]$^-$ ions and [M+H]$^+$ ions, respectively (Pulfer and Murphy 2003). Despite PS can also be analyzed in positive ions mode as [M+H]$^+$ ions, the identification and characterization of PS by MS is preferentially made in negative ions mode, probably due to the presence of the carboxylic acid group in the polar head (Koivusalo et al. 2001; Pulfer and Murphy 2003).

When aiming to unveil APL modifications, the analysis of mass spectra generated by direct infusion MS or by liquid chromatography (LC-)MS runs of mixtures of native and modified APL is technically simple, interpretation of results is straightforward, and the first level of information
regarding the formation of new molecular structures is provided. When the ESI-MS spectra of modified APL are compared with an ESI-MS spectrum acquired in control conditions, the new ions correspondent to modified APL appearing in the former spectrum and the mass shifts can be classified into the following regions (Domingues, Reis, and Domingues 2008):

- Long chain oxidation products region, characterized by ions that result from the insertion of one or more oxygen atoms on the unsaturated fatty acyl chain and therefore show higher m/z values than the native APL. These ions are usually attributed to epoxy-, keto-, hydroxy-, hydroperoxy- and polyhydroxy-derivatives;

- Polar head oxidation products region, characterized by ions that result from deamination or decarboxylation reactions occurring in the polar heads, and therefore show lower m/z values than the native APL.

- Adducts region, characterized by ions that result from the reaction of the free nucleophilic amino group of APL with carbonyl moieties of sugars (e.g. glycation/glyco-oxidation derivatives of PE and PS) and reactive aldehydes/ketones/carboxylic acids generated in the late stages of the fatty acyl chain-shortening oxidative degradations (e.g. HHE, HNE, acrolein, malonaldehyde, hexanoic acid). Ions of these adducts usually show higher m/z values than the native APL;

- Short chain oxidation products region, characterized by ions that result from the oxidative cleavages of the unsaturated fatty acyl chains and therefore can be observed as ions with lower m/z values than the native APL. Shortened APL usually bear carbonyl species on the sn2 position such as aldehydes, keto-aldehydes, hydroxy-aldehydes, as well as carboxylic acids, keto-carboxylic acids, hydroxycarboxylic acids. Oxygen insertions on the fatty acyl chains, as well as products from adduction of sugars and carbonyl species, can also occur on chain-shortened oxidation products leading to other ions with m/z values lower than the native APL, although these types of oxidative modifications have been scarcely reported.
Lyso-APL region, characterized by ions that result from the hydrolysis of the ester linkage between the glycerol backbone and a fatty acyl chain.

I. Identification of long-chain and short-chain oxidation products

The first step when identifying long-chain oxidation products of APL is the observation in the mass spectra of new peaks with different mass shifts. These shifts include ions with +16 Da in the case of hydroxyl derivatives, +32 Da in the case of hydroperoxy (or di-hydroxy) derivatives, and +14 Da in the case of keto or epoxy derivatives (16-2 Da). In Figure 3 we exemplify the presence of these ions for oxidized 1-palmitoyl-2-linoleoyl-sn-3-glycerophosphoethanolamine (ox-PLPE) and in Figure 4 (Panel C) for oxidized 1-palmitoyl-2-linoleoyl-sn-3-glycerophosphoserine (ox-PLPS). Long chain oxidation product with multiple oxidation motifs are detected by identifying mass increments corresponding to combinations of 16 Da and 14 Da. This approach allows proposing several oxidative modifications as hydroxy, di- or poly-hydroxy, hydroperoxy, as well as hydroxy-peroxy, keto and epoxy combined with hydroxy or hydroperoxy (Tyurina et al. 2008; Tyurin et al. 2008; Tyurin et al. 2009; Domingues et al. 2009; Tyurina, Tyurin, et al. 2011; Tyurina, Kisin, et al. 2011; Maciel et al. 2011; Maciel, Faria, et al. 2013; Melo, Silva, et al. 2013; Melo, Santos, et al. 2013; Ni, Milic, and Fedorova 2015). Figure 3B shows a full ESI-MS spectrum ox-PLPE acquired in positive ion mode, which allowed to identify long chain oxidation products bearing 1 oxygen atom, 2, 3 and 4 oxygen atoms (Domingues et al. 2009). Figure 4C shows a full ESI-MS spectrum of ox-PLPS acquired in negative ion mode, reporting long chain oxidation products bearing 1, 2 and 3 oxygen atoms (Maciel et al. 2011).

Short chain oxidation products esterified to an oxidatively truncated carbon chain on the sn-2 position are detected at lower m/z values than the unmodified APL. These species can bear either a terminal aldehydic or carboxylic group, as reported by several authors (Gugiu et al. 2006; Domingues et al. 2009; Simões et al. 2010; Maciel et al. 2011; Maciel, Faria, et al. 2013). These products can be
further oxidized in the shortened carbon chain with additional keto/epoxy, hydroxy and hydroperoxy moieties (Maciel et al. 2011; Maciel, Faria, et al. 2013). As for long-chain derivatives, short chain oxidation products can be analyzed by MS either in positive and negative ion mode. Oxidation products with shortened sn-2 fatty acyl chains (9 carbon atoms) were observed in the MS spectrum of ox-PLPE acquired in positive ion mode, depicted in Figure 3B (Domingues et al. 2009). The MS spectrum of ox-PLPS in negative ion mode, reported in Figure 4C, allows identifying similar short chain oxidation products described above for ox-PE, including esterified aldehydes and carboxylic acids with 9 carbon atoms (Maciel et al. 2011). The mass shifts characteristic for long and short chain oxidation of APL, that can be observed in full MS spectra, are summarized in Table 4.

2. Identification of polar head oxidation products

APL bearing an oxidative modification in the polar heads are easily detected in the MS spectra as peaks with lower m/z values than the unoxidized species (Maciel et al. 2011; Melo, Santos, et al. 2013). Using photosensitized oxidation and direct infusion ESI-MS in negative ion mode, Melo et al. (Melo, Santos, et al. 2013) identified a series of long-chain oxidation products of PE bearing also an oxidative modification in the ethanolamine (Etn) polar head, which are summarized in Table 4. These PE modified in the polar head arose from the oxidative deamination of the Etn moiety of the polar head due to photo-oxidation. Hence they appeared in the MS spectra at odd m/z values with a mass shift of -1 Da when compared to the respective PE. In the case of 1-palmitoyl-2-oleoyl-sn-3-glycerophosphoethanolamine (POPE), the authors identified the [M-H]- ion at m/z 731 as oxidized POPE with one oxygen insertion on the oleyl chain in sn-2 and the Etn polar head modified as terminal acetaldehyde (mass shift from native POPE corresponding to - 1 + 16 Da) (Figure 5).

Maciel et al. (Maciel et al. 2011) identified several modifications in the polar head of PS species, formed during biomimetic oxidation with the Fenton reaction (Figure 4A). The oxidation products of PS with modified polar heads were fractionated by thin layer chromatography (TLC),
exploiting the difference of polarities induced by such modifications (Figure 4B). After scraping and lipid extraction, the modified PS separated in each TLC spots were analyzed by ESI-MS in negative ion mode (Figure 4C). The [M-H]⁻ ions characteristic for PE and PS modified in the polar head are summarized in Table 4 (Maciel et al. 2011).

3. Identification of glycation and glyco-oxidation products

Glycation of APL on the polar heads can be observed in the MS spectra by observing peaks of the native APL with a mass shift of +162 Da (Simões et al. 2010; Maciel, Faria, et al. 2013; Maciel, da Silva, et al. 2013; Melo, Silva, et al. 2013; Annibal et al. 2016). If oxidative conditions are superimposed on glycated APL (glyco-oxidation), the radical-based oxygenation can preferentially affect the fatty acyl chains of the APL and leave the glycated polar head intact. In this case, when the glyco-oxidation mixture is analyzed by ESI-MS the following ions can be observed in the MS spectra (summarized in Table 5):

(I) Short chain oxidation products with a glucose moiety adducted to the polar head: characterized by a negative mass shift due to the chain-shortening β-cleavage, plus a mass shift of +162 Da due to the glycation (Simões et al. 2010; Maciel, Faria, et al. 2013; Annibal et al. 2016);

(II) Long chain oxidation products with a glucose moiety adducted to the polar head: characterized by mass shifts of different combinations of 16 Da and 16-2 Da, due to the oxygen insertions, plus a mass shift of +162 Da due to the glycation (Simões et al. 2010; Melo, Silva, et al. 2013; Maciel, Faria, et al. 2013; Maciel, da Silva, et al. 2013);

Figure 6B shows an ESI-MS spectrum of a gly-ox-PLPE acquired in positive ion mode which shows the several short chains and long chain oxidation products, formed after oxidation of the fatty acyl chain (Simões et al. 2010).
If also the glycated polar head undergoes radical oxidation, the MS spectrum of a glyco-
oxidized mixture can include ions characterized by peculiar mass shifts:

(I) Mass increments higher than $+162\,\text{Da}$ identify glyco-oxidized APL bearing a glucose
 moiety with oxygen insertions (e.g. $162\,\text{Da} + 14\,\text{Da} = 176\,\text{Da}$) (Simões et al. 2010;
 Maciel, da Silva, et al. 2013; Melo, Silva, et al. 2013; Annibal et al. 2016);

(II) Mass increments lower than $+162\,\text{Da}$ identify glyco-oxidized APL bearing an end-
 product of glucose formed through oxidative cleavage (AGE) (e.g. $+28\,\text{Da}$, $+58\,\text{Da}$,
 $+72\,\text{Da}$) (Simões et al. 2010; Maciel, da Silva, et al. 2013; Maciel, Faria, et al. 2013;
 Melo, Silva, et al. 2013; Annibal et al. 2016).

As examples, the characteristic $[\text{M-H}]^-$ ions of POPS AGE identified by ESI-MS are depicted
in Figure 7 (Maciel, da Silva, et al. 2013).

4. Identification of aldehyde-adduction products

ESI-MS has not been extensively used in the analysis of the adducts between APL and
electrophilic aldehydes, except for few studies that focused on the characterization of PE adducts with
iso-LGs (Bernoud-Hubac et al. 2004), HNE (Guichardant et al. 1998), and alkanals (Annibal et al.
2014). There is currently a lack of studies aimed to characterize the potential adduction of PS with
aldehydes and ketones. In what concern PE adducts, Guichardant et al. (Guichardant et al. 1998)
identified the adducts formed by the incubation of 1,2-diheptadecanoyl-sn-3-
glycerophosphoethanolamine (DHPE) and HNE using ESI-MS in negative ion mode. The MS
spectrum of the reaction products are shown in Figure 8, which clearly depicts the characteristic mass
shifts of 120 Da, 138 Da, 156 Da, corresponding to cyclized Schiff base (a), Schiff base (b), and
Michael adduct (c), respectively. More recently, Annibal et al. (Annibal et al. 2014) employed high-
resolution Orbitrap MS analysis to identify modified derivatives of 1,2-dipalmitoyl-sn-3-
glycerophosphoethanolamine (DPPE) arising from its incubation with hexanal and other alkanals.
The MS spectrum of the products of the reaction between DPPE and hexanal acquired in positive ion mode, shown in Figure 9A, shows new ions that have mass shifts corresponding to the sequential adductions of up to three hexanal molecules, resulting in a series of monomeric, dimeric, and trimeric covalent adducts.

Nowadays, accurate mass analysis achievable by mass spectrometers equipped with high-resolution analyzers allows a precise identification of modified APL using data from an MS scan. However, native and modified APL having the same m/z (isobaric species) can be generated within a reaction occurring in vitro or in vivo, and the structural features of the modified products can easily include constitutional isomers that would not be distinguished by using direct infusion MS data, and maybe even using LC separation in the case of isomers of position. Therefore, LC separation and MS/MS structural characterization are highly recommendable to confirm the identity of the modifications. The applications of LC and MS/MS in the structural characterization of modified APL will be addressed in detail in the next paragraphs of the present review.

C. Applications of LC in the MS-based analysis of modified APL

The improvements that LC-ESI-MS methods have brought to the field of lipidomics have already been acknowledged and reviewed (Li et al. 2011; Hyötyläinen and Orešič 2016; Sethi and Brietzke 2017). In fact, LC-MS has also played a key role in the analytical separation of modified APL, mainly for the following reasons:

(I) Oxidation, glycation and aldehyde-adduction reactions generate several modified APL that might have very close m/z, not resolved by low-resolution instruments. Also, in more complex samples, native and modified species can be isobaric. Thus, LC-MS approaches allow the separation of these complex mixtures, based on the differences in the polarities within native and modified species (e.g., separation of hydroperoxy-PE, hydroxy-PE, and native PE);
The ionization efficiency of the modified products can be decreased through ion suppression mediated by the native APL, which are often more abundant. In such case, chromatographic separation of the analyzed mixture would increase the sensitivity towards low-abundant species.

LC protocols allow the separation of constitutional isomers of modified APL.

In most of the LC-MS protocols described in the literature for the analysis of ox-PE, reversed phase high-performance LC (RP-HPLC) was the preferred separation approach, allowing to resolve phospholipids by species through the interaction of the stationary phase with the fatty acyl chains. The insertion of oxygen atoms on the fatty acyl chains increase the polarity of the molecule and, consequently, in RP-HPLC oxidized APL elute earlier than native APL. C\textsubscript{18} microbore columns with an internal diameter of 2 mm or 0.5 mm have been widely used in the chromatographic separation of ox-PE (Gugiu et al. 2006; Maskrey et al. 2007; Domingues et al. 2009; Thomas et al. 2010; Zemski Berry et al. 2010; Alwena H Morgan et al. 2010; Clark et al. 2011; Hammond et al. 2012). Microbore columns operate at low flow rates (e.g., 200 µL/min), increasing the sensitivity of the analysis. However, other LC-MS protocols for ox-PE have relied on C\textsubscript{18} standard analytical columns (Khaselev and Murphy 1999; Hammond et al. 2012). Ox-PS analysis by LC-MS is a less explored field, yet Maciel et al. (Maciel, Faria, et al. 2013) performed a reverse phase separation of a mixture of long chain, short chain and polar head oxidation products of PS using a C\textsubscript{5} microbore column. There are also examples of oxidative phospholipidomics studies in which the analysis of ox-PS species has been performed by normal phase HPLC (NP-HPLC) using silica microbore columns (Tyurina et al. 2010; Tyurina, Tyurin, et al. 2011).

The first LC-MS protocols for gly-PE and glycated PS (gly-PS) were proposed in the nineties and relied on NP analytical columns. Adduction of the polar head to a glucose moiety increases the polarity of the molecule, hence in NP-HPLC glycated APL elute after native APL. This approach succeeded in the separation of native APL from their glycated derivatives, both in vitro (Ravandi, Kuksis, and Myher 1995) and in plasma of diabetic patients (Ravandi et al. 1996). RP-HPLC-MS has
also been carried out for the analysis of gly-PE. A C$_{18}$ medium bore column has been used to separate Schiff bases and Amadori derivatives of PE in human erythrocytes (Breitling-Utzmann et al. 2001). Also, C$_{18}$ microbore columns were used in an LC-MS/MS protocol aimed to quantify PE-AGE in human erythrocytes and blood plasma (CE-PE and CM-PE) (Shoji et al. 2010). More recently, C$_5$ microbore columns have been used in LC-MS/MS protocols aimed to the in vitro characterization and structural elucidation of glyco-oxidized PE (gly-ox-PE) (Simões et al. 2010) and PS (gly-ox-PS) (Maciel, Faria, et al. 2013; Maciel, da Silva, et al. 2013). An overview of the LC columns that have so far been coupled to MS in the analysis of modified APL is reported in Table 6.

D. Applications of MS/MS in the structural characterization of modified APL

Modified APL can be identified in an MS analysis, preferably using high accuracy measurements and interpretation of the new ions appearing at characteristic mass shifts, as described earlier. Besides this, the modifications identified in MS scans should be confirmed by characterizing the new motifs of the modified APL. Such structural characterization can be achieved by MS/MS analysis, as we will describe in the following paragraphs. The characteristic fingerprints and the interpretation of the fragmentation patterns depend on the nature of the modification. Hence, the MS/MS strategies proposed until now for the characterization of modified APL will be addressed, organized by type of modification.

1. Structural characterization of long-chain and short-chain oxidation products

Long and short chain oxidation products can be characterized by the analysis of MS/MS spectra obtained either in positive or negative ion modes. While for native and modified PE both approaches have been used, in the case of PS the most suitable approach is the analysis of the
deprotonated molecular ions [M-H]. MS/MS analysis of long chain and short chain oxidation products performed in the negative ion mode shows two significant families of typical fragment ions:

(I) carboxylate anions of the fatty acids esterified to sn-1 (R1COO-) and sn-2 (R2COO-)

positions;

(II) ions arising the neutral losses of unmodified saturated sn1 acyl chains (R1=C=O, ketene, and R1COOH, carboxylic acid) and modified unsaturated sn2 acyl chains (R2’=C=O, ketene, and R2’COOH, carboxylic acid).

MS/MS spectra of PE acquired in negative ion mode are usually characterized by a higher relative abundance (RA) of R1COO- anions compared to R2COO- anions. The opposite behavior (RA R2COO- > RA R1COO-) is commonly observed for negative ion mode MS/MS spectra of PS (Pulfer and Murphy 2003). However, this behavior depends on the mass spectrometer and should always be confirmed using well-known standards. The MS/MS spectra of the [M-H]- molecular ion of oxidized APL allows the observation of the carboxylate anions of modified unsaturated FA which are usually characterized by the mass increments due to the insertion of n oxygen atoms (n x 16 Da, n x 16-2 Da). Complementarily, the observation of the lyso-APL product ions (due to the neutral loss of the fatty acids as –RCOOH or –R=C=O) can be used to corroborate the oxidative modification (Tyurin et al. 2008; Tyurina et al. 2008; Tyurin et al. 2009; Tyurina et al. 2010; Maciel et al. 2011; Maciel, Faria, et al. 2013; Melo, Silva, et al. 2013; Maciel, da Silva, et al. 2013; Melo, Santos, et al. 2013).

Figure 10 shows the MS/MS spectra of a long chain oxidation product of PAPE (PAPE + 4O), acquired in the negative ion mode using an Orbitrap-higher energy collision-induced dissociation (HCD) (PAPE + 4O, m/z 802.488); the characteristic fragments corresponding to the carboxylate anions can be seen, along with the characteristic neutral loss of modified saturated sn-2 as ketene (R2’=C=O). Figure 11 exemplifies the tandem MS of a short chain oxidation product of PE, namely 1-(palmitoyl)-2-(5-oxovaleroyl)-PE, also acquired upon HCD activation in the negative ion mode; both the native and the shortened fatty acid carboxylate anions, corresponding to the sn-1 and the sn-2 fatty acyl chains, respectively, are shown.
Besides the characteristic product ions described above for all the oxidized APL, the MS/MS analysis of the PS class in negative ion mode always shows a product ion formed due to the neutral loss of aziridine-2-carboxylic acid from the Ser moiety (identified as neutral loss of 87 Da, Figure 12). This abundant neutral loss is typically observed in the negative ion mode MS/MS spectra of native and ox-PS molecular ions (Pulfer and Murphy 2003; Domingues, Reis, and Domingues 2008; Maciel et al. 2011; Maciel, Faria, et al. 2013). Hence, it can be used to further confirm the identity of a long chain or short chain oxidation product of PS, but it is absent in PS modified in the polar head group. PE tandem mass spectra do not display a typical neutral loss in negative ion mode. The identity of long chain and short chain oxidation products of PE can be further verified by MS/MS analysis in positive ion mode through the characteristic neutral loss the polar head (loss of phosphoethanolamine, 141 Da) (Pulfer and Murphy 2003; Domingues, Reis, and Domingues 2008; Domingues et al. 2009; Simões et al. 2010). This neutral loss is a common feature of the tandem mass spectra of native and ox-PE [M+H]+ ions, as it can be observed in Figures 13B and 13C. However, this typical neutral loss is also absent in the case of modifications in the polar head group in PE.

Our group has published results from several ESI-MS/MS structural characterizations of oxidized APL, either in negative ion mode (Maciel et al. 2011; Maciel, Faria, et al. 2013) or in the positive ion mode (Domingues et al. 2009; Simões et al. 2010; Simões et al. 2013a). Long chain and short chain oxidation products of PE can be characterized by positive ion mode MS/MS. The complete interpretation of the fragmentation patterns should include the observation and interpretation of the following product ions (Domingues et al. 2009; Simões et al. 2010):

(I) Ions arising from the neutral losses of the polar head (141 Da);

(II) Ions arising from the neutral losses of unmodified saturated sn1 acyl chains (R1=−C=O, ketene, and R1COOH, carboxylic acid) and modified unsaturated sn2 acyl chains (R2′=−C=O, ketene, and R2′COOH, carboxylic acid);

(III) Ions arising from the neutral losses of the fatty acyl chains plus the polar head;
(IV) Ions arising from the neutral loss of the polar head, plus the fragmentation of the C-C bond between a carbon atom bearing an oxygen insertion and the unsaturation in vinylic position. These typical product ions observed in MS/MS spectra in positive ion mode of ox-PE are exemplified in Figures 13B and 13C. These figures compare the positive LC-MS/MS spectra of two ox-PE isomers (PLPE + 3O) (Domingues et al. 2009); ions labelled as A, B and C were formed by the fragmentation occurring between the carbon atom bearing the hydroxyl or hydroperoxyl functional group and the adjacent double bond, thus were used to indicate which carbon atom was modified by the oxygenated moieties (Figures 13B and 13C). Domingues et al. (Domingues et al. 2009) also correlated the neutral loss of H$_2$O$_2$ (34 Da), that was observed for the ox-PLPE isomer in Figure 13C through the fragment ion at m/z 730.6, with the presence of a hydroperoxy moiety on the sn-2 fatty acyl chain. The neutral loss of H$_2$O$_2$ was also reported for PCs oxidized as hydroperoxy derivatives (Adachi et al. 2004; Adachi et al. 2005; Spickett et al. 2001; Reis et al. 2007). Such a neutral loss would not appear in the MS/MS spectra of hydroxylated species. Differently, multiple losses of H$_2$O molecules (losses of n x 18 Da) are observed for poly-hydroxy derivatives, as exemplified in Figures 13B and 10 (Tyurin et al. 2009).

2. Structural characterization of polar head oxidation products

The structural characterization of APL bearing an oxidized polar head group is a poorly explored topic that should deserve more attention. These molecules were found to be pro-inflammatory factors in peripheral blood (Silva et al. 2012) and were detected in keratinocytes treated with the oxidant AAPH (Maciel et al. 2014). APL oxidative modifications of the polar head were characterized by negative ion mode MS/MS for ox-PS. The MS/MS spectra are characterized by the following features (Maciel et al. 2011):

(I) Absence of the ions arising from the neutral loss of aziridine-2-carboxylic acid (-87 Da), typical of native PS;
Carboxylate anions of the fatty acids esterified to sn-1 and sn-2 positions;

Ions arising from specific neutral losses due to the modification of Ser in the oxidized polar head.

Figures 14A and 14 B show two MS/MS spectra of ox-PS bearing a modification in the polar head, acquired in negative ion mode upon collision-induced dissociation (CID) and HCD activation, respectively. In both the MS/MS spectra, the neutral loss of 87 Da from the precursor is not observed, yet it is possible to detect a neutral loss of 58 Da. This fragmentation pattern is characteristic for ox-PS bearing a glycerophosphoacetic acid derivative in the polar head. The structures of this ion are depicted in Figure 14 and Figure 5A (Maciel et al. 2011).

A summary of the main product ions that have been characterized by the MS/MS analysis of ox-PE and ox-PS in both positive and negative ion modes is reported in Table 4.

3. Structural characterization of glycation and glyco-oxidation products

The MS/MS-based fingerprinting of glycated and glyco-oxidized APL relies on the interpretation of specific neutral losses, which allow confirming the adduction of a glucose moiety to the polar head. A correct reading of such neutral losses also allows the elucidation of the oxidative modifications that have eventually been introduced on the glycated polar head. A summary of the mass shifts and the characteristic neutral losses that have been observed in the MS/MS spectra of glycated and glyco-oxidized APL acquired in both positive and negative ion modes is reported in Table 5. Importantly, the neutral loss of phosphoethanolamine polar head from PE occurs upon MS/MS in positive ion mode, whereas the neutral loss of Ser from the polar head of PS takes place upon MS/MS in negative ion mode (Pulfer and Murphy 2003). Hence, the fragmentation in positive ion mode is preferred for the analysis of gly-PE and gly-ox-PE, since it allows to observe the neutral loss of the glycated polar head (Simões et al. 2010; Melo, Silva, et al. 2013; Annibal et al. 2016). Conversely, the analysis in negative ion mode is more suitable for gly-PS and gly-ox-PS, because it
allows the observation of the neutral loss of the glycated Ser moiety from the polar head (Maciel, da Silva, et al. 2013). However, MS/MS analysis in negative ion mode is also suitable for gly-PE and gly-ox-PE (Simões et al. 2010; Melo, Silva, et al. 2013). The MS/MS fragmentation of gly-PE in positive ion mode results in typical fragmentation patterns that include the following ions:

(I) Ions arising from the neutral loss of 303 Da, due to the elimination of the phosphoethanolamine polar head (-141 Da) adducted to one glucose molecule (-162 Da) (Simões et al. 2010; Melo, Silva, et al. 2013; Annibal et al. 2016). Figure 15 shows an MS/MS spectrum of gly-PLPE acquired in positive ion mode, in which it is possible to observe a diagnostic neutral loss of polar head adducted to glucose (-303 Da). (Simões et al. 2010);

(II) Ions arising from multiple neutral losses of H$_2$O and H$_2$CO, characteristic for the insertion of a glucose moiety (Wang et al. 2008; Simões et al. 2010).

When the glycated polar head of gly-ox-PE is oxidized, different ions arising from neutral losses can be observed in the MS/MS spectra acquired in positive ion mode. These explanatory neutral losses from the parent molecular ion can be classified in two main groups (Table 5) (Simões et al. 2010; Shoji et al. 2010; Melo, Silva, et al. 2013; Annibal et al. 2016):

(I) Ions arising from neutral losses greater than 303 Da, due to the elimination of the phosphoethanolamine polar head adducted to glucose, with additional oxygen insertions (e.g., glucuronic acid, glucose adducted to α-keto-ethn) (Simões et al. 2010; Melo, Silva, et al. 2013; Annibal et al. 2016) (Simões, Simões, et al.; Melo, Silva, et al.; Annibal, Riemer, et al.);

(II) Ions arising from neutral losses smaller than 303 Da, due to the elimination of the phosphoethanolamine polar head adducted to end-products of glucose oxidative cleavage (e.g., carboxymethyl, carboxyethyl, formamide, carbamino); Figure 16 shows an MS/MS spectrum of gly-ox-PLPE acquired in positive ion mode. In this
case, the new structural feature is characterized by the neutral loss of glyco-oxidized polar head.

The common fragmentation pathways observed in the negative ion mode MS/MS spectra of gly-PS and gly-PE are also characterized by specific product ions that are different from those observed in the positive ion mode. The following fragment ions are common to gly-PS and gly-PE, when analyzed in negative ion mode (Table 5) (Simões et al. 2010; Maciel, da Silva, et al. 2013; Maciel, Faria, et al. 2013):

(I) Ions arising from the neutral loss of 162 Da, due to the elimination of the glucose moiety;

(II) Carboxylate anions of the fatty acids, R\text{1}COO^{-} and R\text{2}COO^{-}. Nevertheless, it is still possible to observe oxidative modifications that can occur on the fatty acyl chains;

(III) Ions formed by neutral losses of C\text{3}H\text{6}O\text{3}, C\text{4}H\text{8}H\text{4}, and C\text{3}H\text{10}O\text{5} (-90, -120 Da and -150 Da, respectively) due to the cleavage of the glucose moiety that occurs along the glycosidic linkages upon MS/MS (Asam and Glish 1997; Simões et al. 2007).

As an illustration of these fragmentation patterns, Figure 17 shows the MS/MS fragmentation of gly-PLPE in negative ion mode, showing the characteristic neutral losses of glucose from the polar head (Simões et al. 2010). The fragmentation of gly-POPS in negative ion mode is shown in Figure 18 and is characterized by the typical neutral losses of glycated Ser (Maciel, da Silva, et al. 2013).

The MS/MS spectra acquired in negative ion mode of gly-ox-PS also show characteristic neutral losses. If the oxidation occurs on the glycated Ser moiety, the following diagnostic neutral losses can be observed (Table 5) (Maciel, da Silva, et al. 2013; Maciel, Faria, et al. 2013):

(I) Ions arising from neutral losses greater than 249 Da, due to the elimination of the Ser moiety adducted to glucose with additional oxygen insertions; Figure 19B shows the MS/MS spectrum of gly-ox-POPS acquired in negative ion mode, which highlights the oxidation of the glucose moiety adducted to the polar head (Maciel, da Silva, et al. 2013);
(II) Ions arising from neutral losses smaller than 249 Da, due to the elimination of the Ser moiety adducted to end-products from glucose oxidative cleavage (AGE of glycated Ser).

The MS/MS spectrum of gly-ox-POPS bearing an AGE of glycated Ser acquired in negative ion mode is depicted in Figure 20. This spectrum shows the characteristic fragmentation pattern of a derivative which is oxidatively shortened at C2 of the glucose moiety (Maciel, da Silva, et al. 2013).

4. Structural characterization of aldehyde-adduction products

Adducts of APL with aldehydes have been highlighted as bioactive molecules, able to promote macrophage viability (Riazy et al. 2011) and monocyte adhesion (Guo et al. 2011). MS/MS allowed the structural elucidation of several adducts of PE produced in-vitro using reactive products from lipid peroxidation as hexanoic acid (Tsuji et al. 2003) and iso-LGs (Bernoud-Hubac et al. 2004). Bernoud-Hubac and co-authors (Bernoud-Hubac et al. 2004) performed the structural characterization of the adducts formed by the reaction of PLPE and a mixture of synthetic iso-LGs. MS/MS in negative ion mode elucidated the structures of one AL-PE Schiff-base adduct and one AL-PE pyrrole adduct. Figures 21 and 22 show two MS/MS spectra of AL-PLPE adducts, acquired in negative ion mode. The spectrum in Figure 21 shows the fragmentation pattern of the AL-PLPE pyrrole adduct (m/z 1031). The spectrum in Figure 22 shows the fragmentation pattern acquired in negative ion mode MS/MS of the second AL-PLPE (m/z 1035), that allowed its characterization as Schiff base adduct and the differentiation from the pyrrole adduct (Bernoud-Hubac et al. 2004). Annibal et al. (Annibal et al. 2014) performed an elegant structural characterization of a Schiff adduct formed by the reaction of DPPE and hexanal, based on multistage tandem MS analysis. The MS², MS³ and MS⁴ spectra acquired in positive ion mode for the characterization of Schiff base DPPE-hexanal are shown in Figure 23A, 23B, and 23C, respectively.
Upon the whole, unbiased LC-MS/MS analyzes of modified PE and PS allow the identification of the modified species by characteristic mass shifts in the MS spectra, and the structural characterization of the modifications by the acquisition of the MS/MS spectra. This knowledge can be further applied to the detection and the quantification of biologically relevant species in vivo, for example, by developing targeted MS-based analytical methods. The next section reviews the MS approaches that have so far been employed for the targeted analysis of modified APL.

E. Targeted MS approaches for the detection of modified APL in biological samples

The detailed knowledge acquired, based on the analysis of the MS/MS spectra of modified APL, allowed the development of MS-based targeted approaches, aimed at their detection and quantification ex-vivo or in-vivo. The targeted detection in cells or biofluids is fundamental, as it can relate the occurrence of modified APL with the biological mechanisms underpinning the onset of a disease, and validate these molecules as biomarkers of a pathological condition. The applications of the targeted MS approaches such as precursor ion scanning (PIS), neutral loss scanning (NLS) and single/multiple reaction monitoring (SRM/MRM) to detect or quantify modified APL in biological samples will be discussed in detail below.

1. Precursor ion scanning (PIS)

PIS identifies all the precursor ions that fragment in the collision cell to generate a selected reporter ion. The carboxylate anions of the fatty acyl chains esterified to the glycerol backbone (R₁COO⁻ and R₂COO⁻) are typical reporter ions formed during the fragmentation of APL upon MS/MS in negative ion mode (Pulfer and Murphy 2003). In the case of long-chain oxidation products, the observation of characteristic mass shifts (n x 16 Da, n x 16-2 Da) on the carboxylate reporter ions confirms the insertion of oxygen atoms on the unsaturated acyl chains, as reported above. Several
studies used the carboxylate anion of hydroxy-arachidonic acid (hydroxy-eicosatetraenoic acid, HETE) as selected reporter ion for PIS analysis. LC-MS methods based on RP separation and PIS of the precursors of HETE (m/z 319) were employed to detect HETE-PE generated by LOXs in activated human platelets (Maskrey et al. 2007; Thomas et al. 2010), neutrophils (Clark et al. 2011) and monocytes/macrophages (Maskrey et al. 2007; Morgan et al. 2009). A similar PIS approach reported by Hammond et al. (Hammond et al. 2012) used the selection of the carboxylate anion of arachidonic acid bearing one keto insertion (keto-eicosatetraenoic acid, KETE). The authors employed an LC-MS method based on RP separation and PIS with the selection of KETE as reporter ion (m/z 317.2), which detected KETE-containing PE generated by LOX in monocytes and macrophages of patients affected by cystic fibrosis. Morgan et al. (Lloyd T. Morgan et al. 2010) used a similar PIS approach in negative ion mode, by selecting the carboxylate anion of docosahexaenoic acid with the insertion one hydroxy group (hydroxy-docosahexaenoic acid, HDOHE) as fragment ion. The LC-MS method was based on the PIS of the HDOHE reporter ion at m/z 343 and detected four HDOHE-PE generated by 12-LOX in activated human platelets.

Zemski Berry et al. (Zemski Berry et al. 2010) proposed a PIS approach based on the derivatization of PE with 4-(dimethylamino)benzoic acid (DMABA) for detecting ox-PE in RAW 264.7 cells. In this study, the treatment of lipids extracted from RAW 264.7 cells with AAPH led to a complex mixture of native and ox-PE, which were separated by RP-HPLC, and detected by PIS using a peculiar ion formed during the fragmentation of ox-PE derivatized with D6-DMABA (m/z 197.1) (Figure 24). The structures of the ox-PE precursor ions identified by PIS were elucidated by MS/MS, allowing the characterization of long chain and short chain oxidation products of PE (Zemski Berry et al. 2010).

Maciel et al. (Maciel et al. 2014) reported a PIS approach for the detection of ox-PS derivatives in keratinocytes subjected to in vitro radical oxidation with AAPH. The authors studied the fragmentation of ox-PS with polar head modified as GPAA (Figures 4A and 14) by MS/MS in negative ion mode, observing the formation of the reporter ion at m/z 137.1 (HOPO₃CH₂COO⁻).
Subsequently, the PIS scan detected several oxidation derivatives of PS with polar head oxidized as GPAA in the oxidatively stressed cells (Maciel et al. 2014).

2. Neutral loss scanning (NLS)

NLS identifies all the precursor ions that fragment in the collision cell by the loss of a reporter neutral fragment. Both AGE and early glycation products of PE have been detected using this tandem MS approach. Amadori gly-PE in plasma from diabetic patients were detected by scanning of the neutral loss of 303 Da, corresponding to the loss of glycated polar head and formation of a diacylglyceride ion (see also Figure 15 and Table 5) (Nakagawa 2005). In another study, profiling of the PE AGE CM-PE and CE-PE in human diabetic plasma was carried out by NLS of parent ions, yielding neutral losses of 199 Da and 213 Da, respectively, which again correspond to the loss of modified polar heads (Shoji et al. 2010). Also, Maciel et al. (Maciel et al. 2014) profiled PS oxidation products with polar head modified as GPAA in keratinocytes subjected to in vitro radical oxidation with AAPH, using an NLS of the reporter neutral fragment corresponding to the acetic acid moiety of GPAA (58 Da) as shown in Figure 14A.

3. Single and multiple reaction monitoring (SRM/MRM)

Reaction monitoring routines are more suitable when the presence of an analyte has been confirmed, and a quantitative assessment is required. In single reaction monitoring (SRM) a single fragmentation step is monitored, and the reporter ion that is selected is usually the most diagnostic and abundant among the ions characterizing the MS/MS pattern. If the fragmentation of the parent ion leads to several reporter ions which are diagnostic and intense, multiple transitions, from one precursor ion to one or more reporter ions, can be monitored. This approach is known as multiple reaction monitoring (MRM).

Positive mode reaction monitoring has been used for detecting gly-PE in biological samples,
through the formation of the diacylglyceride derivative fragment ions arising from the loss of glucose-
adducted polar head (303 Da) (Nakagawa 2005; Sookwong et al. 2011). In these reports, the transition
906.5 -> 603.7 was used to quantify Amadori-dioleoyl-PE in plasma from healthy and diabetic
humans (Nakagawa 2005). Also, the transition 908.8 -> 605.7 was used to quantify Amadori-1-
stearoyl-2-oleoyl-PE in several tissues from healthy and diabetic rats (Sookwong et al. 2011).
Similarly, CE-PE and CM-PE were quantified by reaction monitoring in human erythrocytes and
blood plasma, using the transition leading to the elimination of modified polar head and formation of
diglyceride product ion (Shoji et al. 2010). SRM and MRM were also used in the quantification of
different ox-PE from rat tissues. Positive mode MRM was used to quantify 14 different short chain
oxidation derivatives of PE in rat retina, by setting one specific parent-product transition for each
oxidatively truncated analyte (Gugiu et al. 2006).

Different isomers of long-chain oxidation products of PE are generated by LOXs in activated
blood cells, including platelets, neutrophils, and monocytes/macrophages. In these cells, negative ion
mode MRM based on the modified fatty acid carboxylate product ions as KETE (m/z 317) and HETE
(m/z 319) has been used for detection and quantification of HETE-PE (Thomas et al. 2010; Clark et
al. 2011; Morgan et al. 2009) and KETE-PE (Hammond et al. 2012) derivatives, respectively.

IV. Conclusive remarks

PE and PS, also named APL, are essential phospholipids that are found in the plasma
membrane of mammalian cells, displaying structural and signaling roles. APL can be modified to
oxidized, glycated, glyco-oxidized and aldehyde-adducted derivatives. Over the last 20 years, several
studies have reported the bioactivities of modified APL and their occurrences in several
inflammation-related pathologies, highlighting potential roles as signaling molecules and biomarkers
of disease. However, structural complexity and low in vivo-concentration have represented the two
Main challenges for the analysis of modified APL. MS is a sensible and selective analytical platform that has been fundamental in the identification, structural characterization, detection and quantification of modified APL. Some MS-based strategies have already provided brilliant insights on modified PE, with several studies focusing on its characterization in vitro and, to a lesser extent, on its detection in biological samples. Nevertheless, the literature focused on the MS analysis of APL modifications is still scarce, particularly in the case of PS. As a challenge for the future, a more comprehensive knowledge of the modifications that can occur in PE and PS is needed, relying on systematic experimental approaches that merge in vitro biomimetic methods to modern MS platforms. Databases of fragment ions of modified APL can be generated from an extensive in vitro characterization, and finally translated to MS-based targeted methods, which represent a reliable tool for the detection of these molecules in clinical samples. This bioanalytical knowledge, accompanied by a more detailed investigation of the biological functions of modified PE and PS, will contribute to unveil the implications of modified APL in, for example, inflammatory diseases, and will provide new biomarkers of highly-debilitating pathologies like cancer, diabetes, and atherosclerosis.

V. Abbreviations

- AAPH: 2,2'-Azobis(2-amidinopropane) dihydrochloride
- AD: Alzheimer’s disease
- AGE: advanced glycation end product(s)
- Al-PE: aldehyde-PE
- Al-PS: aldehyde-PS
- APL: aminophospholipid
- CE: carboxyethyl
- CID: collision-induced dissociation
- CM: carboxymethyl
889  DHPE   1,2-diheptadecanoyl-sn-3-glycerophosphoethanolamine
890  DPPE   1,2-dipalmitoyl-sn-3-glycerophosphoethanolamine
891  DPPS   1,2-dipalmitoyl-sn-3-glycerophosphoserine
892  EI     electronic impact
893  ER     endoplasmic reticulum
894  ESI    electrospray ionization
895  Etn    ethanolamine
896  GC     gas chromatography
897  Gly-ox-PE  glyco-oxidized PE
898  Gly-ox-PS  glyco-oxidized PS
899  Gly-PE    glycated PE
900  Gly-PS    glycated PS
901  GPAA   glycerophosphoacetic acid
902  H₂O₂  hydrogen peroxide
903  HCD    higher energy collision-induced dissociation
904  HDDE   4-hydroxydodeca-(2,6)-dienal
905  HDOHE  hydroxydocosahexaenoic acid
906  HETE  hydroxyeicosatetraenoic acid
907  HHE    4-hydroxy-2-hexenal
908  HNE    4-hydroxy-2-nonenal
909  HUVEC human umbilical vein endothelial cells
910  Iso-LG iso-ketal
911  KETE   keto-eicosatetraenoic acid
912  LC     liquid chromatography
913  LDL    low-density lipoprotein
914  LOX    lipoxygenase
| Page | Term | Definition |
|------|------|------------|
| 915  | MALDI | matrix-assisted laser desorption/ionization |
| 916  | MDA  | malondialdehyde |
| 917  | MPO  | myeloperoxidase |
| 918  | MRM  | multiple reaction monitoring |
| 919  | MS   | mass spectrometry |
| 920  | NL   | neutral loss |
| 921  | NLS  | neutral loss scanning |
| 922  | NP-HPLC | normal phase high performance liquid chromatography |
| 923  | O₂   | molecular oxygen |
| 924  | O₂⁻* | superoxide radical |
| 925  | ¹O₂  | singlet oxygen |
| 926  | *OH  | hydroxyl radical |
| 927  | ONE  | 4-oxo-2-nonenal |
| 928  | Ox-PE | oxidized PE |
| 929  | Ox-PS | oxidized PS |
| 930  | PE   | phosphatidylethanolamine |
| 931  | PLPE | 1-palmitoyl-2-linoleoyl-<i>sn</i>-3-glycerophosphoethanolamine |
| 932  | PLPS | 1-palmitoyl-2-linoleoyl-<i>sn</i>-3-glycerophosphoserine |
| 933  | POPE | 1-palmitoyl-2-oleoyl-<i>sn</i>-3-glycerophosphoethanolamine |
| 934  | PS   | phosphatidylserine |
| 935  | RA   | relative abundance |
| 936  | ROS  | reactive oxygen species |
| 937  | RP-HPLC | reversed phase high performance liquid chromatography |
| 938  | Ser  | serine |
| 939  | SIM  | single ion monitoring |
| 940  | SRM  | single reaction monitoring |
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Table 1. Main biological activities of phosphatidylserine (PS) and phosphatidylethanolamine (PE) in mammalian cells.

| BIOLOGICAL ACTIVITY                                                                 | REFERENCES                                                                 |
|------------------------------------------------------------------------------------|---------------------------------------------------------------------------|
| **Phosphatidylserine**                                                              |                                                                           |
| Interaction with PS receptor of macrophages after externalization: apoptosis         | (Fadok et al. 2001; Zwaal, Comfurius, and Bevers 2005; Balasubramanian, Mirnikjoo, and Schroit 2007; Segawa et al. 2014; Fadok et al. 1992) |
| Inhibition of inflammatory response                                                 | (Fadok et al. 1998; Kimani et al. 2014)                                   |
| Inhibition of proinflammatory cytokine production, alleviation of arthritis         | (Yeom et al. 2013)                                                        |
| Immune system modulation                                                            | (Kimani et al. 2014; Tietjen et al. 2014; Elliott et al. 2005; Fischer et al. 2006; Hoffmann et al. 2005) |
| Promotion of the blood-clotting cascade in injury-activated platelets               | (Schick, Kurica, and Chacko 1976; Schroit and Zwaal 1991; Connor and Schroit 1990; Connor et al. 1992; Bevers, Comfurius, and Zwaal 1983) |
| Modulation of the activity of membrane-bound proteins (PKC, Annexin V, Rac 1) at the intracellular leaflet | (Powell et al. 2000; Swairjo et al. 1995; Finkielstein, Overduin, and Capelluto 2006; Lemmon 2008) |
| Intracellular signaling pathway in neuronal cells: neurite growth, neuronal cell survival, and synaptogenesis | (Verdaguer et al. 1999; Huang et al. 2011; Improta-Brears, Ghosh, and Bell 1999; Kim, Huang, and Spector 2014; Kim 2007; Suzuki et al. 2001) |

| **Phosphatidylethanolamine**                                                        |                                                                           |
| Increase in the percentage of cytokine-producing cells (monocytes and mDC)         | (Simões et al. 2013b)                                                    |
| Modulation of mammalian cell membrane curvature                                    | (Cullis and De Kruijff 1978; Verkleij et al. 1984)                       |
| Biosynthesis of glycosylphosphatidylinositol-ethanolamine anchors                  | (Menon and Stevens 1992)                                                 |
| Topic                                                                 | Reference                      |
|----------------------------------------------------------------------|--------------------------------|
| Post-translational modification of Eukaryotic Elongation Factor 1A   | (Signorell et al. 2008)        |
| Regulation of contractile ring disassembly during cytokinesis of mammalian cells | (Emoto and Umeda 2000; Emoto et al. 1996) |
| Regulation of Golgi membrane fusion in early-divided mitotic cells   | (Pécheur et al. 2002)          |
| Cofactor activity in propagation and infectivity of mammalian brain prions | (Deleault et al. 2012)         |
| Covalent modification and recruitment of common autophagosome markers | (Hailey et al. 2010)           |
| Implication with sarcolemmal damage after ischemia and reperfusion   | (Post, Bijvelt, and Verkleij 1995) |
| Exposure to the outer leaflet in mammalian tumors                    | (Stafford and Thorpe 2011)     |
| Table 2. Occurrences of modified PS and PE in biological samples and their main biological activities in mammalian cells. |
|---|
| **OXIDIZED PHOSPHATIDYLSTERINE** |
| **Occurrence** | **References** |
| Formation and externalization during apoptosis | (Kagan et al. 2002; Fabisiak et al. 1998; Arroyo et al. 2002; Shvedova et al. 2002; Koty et al. 2002; Matsura et al. 2002; Matsura et al. 2004; Kagan et al. 2004) |
| Formation during oxidative lung injury | (Tyurina, Kisin, et al. 2011; Tyurina et al. 2010; Tyurina, Tyurin, et al. 2011) |
| Formation in a mouse model of the AD and post-mortem brain samples from AD patients | (Maki et al. 2009) |
| Formation in rat apoptotic cortical neurons | (Tyurin et al. 2008) |
| Formation in plasma membranes of apoptotic cells from alcoholic liver disease | (Vay et al. 2006) |
| **Biological activity** | **References** |
| “Eat-me” signal for phagocytes during clearance of apoptotic cells | (Greenberg et al. 2006; Hochreiter-Hufford and Ravichandran 2013; Kagan et al. 2002) |
| Non-enzymatic scramblase activity | (Tyurina et al. 2004) |
| Inhibition of peripheral blood T-cells proliferation | (Seyerl et al. 2008) |
| Upregulation of cytokines production in monocytes and dendritic cells | (Silva et al. 2012) |
| Inhibition of respiratory burst | (Bluml et al. 2008) |
| Protection of pulmonary endothelium | (Birukova et al. 2006) |
| Multi-inhibition of the Toll-like receptor 4 pathway | (von Schlieffen et al. 2009) |
| Stimulation of the expression of pro-atherogenic genes | (Afonyushkin, Oskolkova, and Bochkov 2012) |
| Pro-coagulant activity via regulation of PCI | (Malleier et al. 2007) |
| **GLYCATED AND GLYCO-OXIDIZED PHOSPHATIDYLSTERINE** |
| **Occurrence** | **References** |
| CMS detected in human red blood cells membranes | (Fountain et al. 1999) |
| **ALDEHYDE-ADDUCTED PHOSPHATIDYLSTERINE** |
| **Occurrence** | **References** |
| PS-malondialdehyde (PS-MDA) adducts detected in human senile cataractous tissue | (Bhuyan et al. 1986) |
| N-hexanoyl-PS detected in red blood cells of carbon tetrachloride-treated rats | (Hisaka et al. 2010) |
| **OXIDIZED PHOSPHATIDYLETHANOLAMINE** |
| **Occurrence** | **References** |
| Direct enzymatic oxidation of membrane PE to 15-hydroxy-eicosatetraenoic acid (15-HETE-PE) in ionophore-activated human platelets and peripheral monocytes | (Maskrey et al. 2007) |
| Direct enzymatic oxidation of membrane PE to 12-hydroxy-eicosatetraenoic acid (12-HETE-PE) in ovalbumin-treated murine peritoneal macrophages | (Morgan et al. 2009) |
| Biological activity | References |
|---------------------|------------|
| Enhanced thrombin generation | (Zieseniss et al. 2001; Thomas et al. 2010) |
| Shift in the mobility of soluble CD14 | (von Schlieffen et al. 2009) |
| Inhibition of cytokines generation in human monocytes | (Morgan et al. 2009) |
| Increase in the frequency of cytokine-producing cells (monocytes and mDC) | (Simões et al. 2013a) |
| Activation of PPARγ in mouse macrophages | (Hammond et al. 2012) |
| Inhibition of extracellular traps and enhanced \( \text{O}_2^- \) generation in human neutrophils | (Clark et al. 2011) |
| Pro-coagulant activity via regulation of PCI | (Malleier et al. 2007) |
| Regulation of MAPK signaling in human airway epithelial cells | (Uderhardt et al. 2012) |
| Regulation in the clearance of apoptotic cells and maintenance of immunologic tolerance | (Sankhagowit et al. 2016) |
| Modification in cellular membrane topology | (Kagan et al. 2016) |
| Mediation of ferroptotic cell death | (Kagan et al. 2016) |

**GLYCATED AND GLYCO-OXIDIZED PHOSPHATIDYLETHANOLAMINE**

| Occurrence | References |
|------------|------------|
| Formation in red blood cells and plasma of healthy and diabetic subjects | (Ravandi et al. 1996; Requena et al. 1997; Fountain et al. 1999; Breitling-Utzman et al. 2001; Nakagawa 2005; Shoja et al. 2010; Lertsiri, Shiraishi, and Miyazawa 1998) |
| Formation in glucose-treated LDL | (Ravandi, Kuksis, and Shaikh 2000) |
| Formation in diabetic rats | (Sookwong et al. 2011) |
| CME formation in mitochondrial membranes of different mammalian species | (Pamplona et al. 1998) |

| Biological activity | References |
|---------------------|------------|
| Promotion of lipid peroxidation | (Oak, Nakagawa, and Miyazawa 2000; Nakagawa 2005; Annibal et al. 2016) |
| Different modulation of the frequency of cytokine-producing cells | (Simões et al. 2013b; Simões et al. 2013a) |
| Modulation of the expression levels of proteins involved in several metabolic cell pathways | (Annibal et al. 2016) |
| Occurrence                                                                 | References                           |
|----------------------------------------------------------------------------|--------------------------------------|
| PE-malondialdehyde (PE-MDA) adducts detected in human senile cataractous tissue | (Bhuyan et al. 1986)                 |
| N-hexanoyl-PE detected in red blood cells of carbon tetrachloride-treated rats | (Hisaka et al. 2010)                 |
| PE-HNE adducts detected in rats affected by diabetic retinopathy            | (Bacot et al. 2007)                  |
| IsoLG-PE adducts detected in patients with macular degeneration            | (Li et al. 2009)                     |
| Para-hydroxyphenylacetaldehyde (pHA-PE) adducts detected in the human atherosclerotic intima | (Heller et al. 2000)                 |

| Biological activity                                                                 | References                           |
|-------------------------------------------------------------------------------------|--------------------------------------|
| Increase of the macrophages viability in humans                                      | (Riazy et al. 2011)                  |
| Endothelial dysfunction, ER curvature and expression of monocyte adhesion molecules in HUVEC | (Guo et al. 2011)                    |
| Increase in platelet aggregation and prothrombinase activity                       | (Zieseniss et al. 2001)              |
| Inhibition of macrophage phagocytosis                                               | (Shiratsuchi et al. 2008)            |
| Modification in cellular membrane topology                                          | (Annibal et al. 2014)                |
Table 3. Chemical, physical and enzymatic biomimetic methods that have been used for studying APL oxidation and that were coupled with mass spectrometry analysis for the identification and structural characterization of oxidized APL.

| System          | Species        | Matrix                                                                 | Experimental conditions                                      | References                          |
|-----------------|----------------|------------------------------------------------------------------------|-------------------------------------------------------------|-------------------------------------|
| Fe²⁺/H₂O₂       | *OH            | Ammonium bicarbonate buffer (5 mM, pH 7.4)                            | 40 µM FeCl₂, 50 mM H₂O₂, 72 h and 192 h, 37 °C, dark         | (Simões et al. 2010)               |
|                 |                |                                                                       | Final volume 50 µL, 144 h, and 192 h, 37 °C dark             | (Domingues et al. 2009)            |
|                 |                |                                                                       | 40 µM FeCl₂/EDTA (1:1), 10 mM H₂O₂, 37 °C, dark              | (Maciel et al. 2011)               |
|                 |                |                                                                       | 80 mM FeSO₄, 50 mM H₂O₂, 2 h, 37 °C                          | (Annibal et al. 2016)              |
|                 |                |                                                                       | 40 µM FeCl₂, 50 mM H₂O₂, 48 h, 37 °C, dark                   | (Maciel, da Silva, et al. 2013; Maciel, Faria, et al. 2013) |
|                 |                |                                                                       | 500 µM FeCl₂, 50 mM H₂O₂, 120 h, 37 °C, dark                 | (Simões et al. 2013b; Simões et al. 2013a) |
| Fe²⁺/ascorbate   | *OH            | Rat cerebral cortex homogenates                                       | 20 µM FeCl₂, 250 µM ascorbate, 6 h, 37 °C                   | (Stadelmann-Ingrand, Pontcharraud, and Fauconneau 2004) |
| Cu²⁺/H₂O₂       | *OH            | Phosphate buffer saline (50 mM, pH 7.4)                               | 100 µM CuCl₂, 70 mM H₂O₂, up to 3 h, 37 °C                  | (Khaselev and Murphy 1999; Gugiu et al. 2006)  |
| AAPH            | ROO*           | Hank's balanced salt solution                                         | 10 mM AAPH, 5 h, 37 °C                                      | (Zemski Berry et al. 2010)         |
|                 |                | HaCaT Keratinocytes (15 x 10⁶)                                        | 30 mM and 50 mM AAPH, 24 h, 37 °C                           | (Maciel et al. 2014)               |
| Gly-PE          | O₂*            | Tris-HCl buffer (10 mM, pH 7.4)                                       | 15 mol %, 0.3 mol %, 0.05 mol % Amadori PE, up to 120 h, 37 °C | (Breitling-Utzmann et al. 2001)    |
| Method       | Species | Buffer/Reagents                        | Conditions                                                                 | References                                    |
|-------------|---------|----------------------------------------|-----------------------------------------------------------------------------|-----------------------------------------------|
| **PHYSICAL**|         |                                        |                                                                             |                                               |
| UVA         | Primary rat cardiomyocytes             | 1 mM gly-PE or 1 mM gly-ox-PE, 30 min and 16 h                              | (Annibal et al. 2016)                         |
|             | Ammonium bicarbonate buffer (5 mM, pH 7.4) | 20 min, 75.4 J/cm²                                                          | (Melo, Silva, et al. 2013)                    |
|             | Phosphate buffer                        | 28 °C                                                                        | (Gugiu et al. 2006)                           |
|             | Rat cerebral cortex homogenates         | 0-90 min, 0-10.8 J/cm²                                                       | (Stadelmann-Ingrand, Pontcharraud, and Fauconneau 2004) |
| White light | 1 O₂    | Ammonium bicarbonate buffer (5 mM, pH 7.4) | Artificial white light irradiation, 30 min, 90 min, 270 min, 7.2 J/cm², 21.6 J/cm², 64.8 J/cm² | (Melo, Santos, et al. 2013)                    |
| Electrochemistry | *OH | 1:1 MeOH:20 mM ammonium formate         | 20µL/min, 37 °C, 2.5 V or 3 V (depending on the APL species)                 | (Colombo et al. 2018)                         |
| LOX         | -      | 10 mM deoxycholate, 0.2 M borate buffer | 5.2 KU/mL LOX, 30 min, 95% O₂                                                | (Alwena H Morgan et al. 2010)                |
| MPO         | HOX    | Sodium phosphate buffer (50 mM)         | 5 nM MPO, 100 ng/mL glucose oxidase, 500 µM NaNO₃, 100 µg/mL glucose, 200 µM DTPA | (Gugiu et al. 2006)                           |
Table 4. Oxidative modifications of APL identified by mass spectrometry either in biomimetic systems and in vivo: mass shifts observed in the MS spectra when compared with the respective non modified APL. Characteristic fragmentations (fatty acid carboxylate anions and neutral losses) noticeable in the MS/MS spectra that allow confirming the structural features of the oxidized APL.

| Modification                  | Modified fatty acid carboxylate anion (m/z) [M-H]⁻ | Observed neutral loss [M-H]⁻ and [M+H]⁺ | References                                                                 |
|-------------------------------|--------------------------------------------------|------------------------------------------|-----------------------------------------------------------------|
| sn-2 keto (+14 Da)            | 295 (oleic acid) 293 (linoleic acid) 317 (arachidonic acid) 341 (docosahexaenoic acid) | --                                       | (Melo, Santos, et al. 2013; Hammond et al. 2012; Alwena H Morgan et al. 2010) |
| sn-2 hydroxy (+16 Da)         | 297 (oleic acid) 295 (linoleic acid) 319 (arachidonic acid) 343 (docosahexaenoic acid) | [M+H-18]⁺ [M-H-18]⁻                           | (Domingues et al. 2009; Melo, Santos, et al. 2013; Clark et al. 2011; Maskrey et al. 2007; Tyurin et al. 2008; Tyurina et al. 2010; Tyurina et al. 2008; Tyurin et al. 2009; Alwena H Morgan et al. 2010; Lloyd T. Morgan et al. 2010) |
| sn-2 dihydroxy (+32 Da)       | 313 (oleic acid) 311 (linoleic acid) 335 (arachidonic acid) 359 (docosahexaenoic acid) | [M-H-36]⁻ (18 + 18)                         | (Tyurin et al. 2009)                                           |
| sn-2 hydroperoxy (+32 Da)     | 313 (oleic acid) 311 (LA linoleic acid) 335 (arachidonic acid) 359 (docosahexaenoic acid) | [M+H-34]⁺                                    | (Melo, Santos, et al. 2013; Tyurin et al. 2008; Tyurin et al. 2009; Tyurina et al. 2008; Tyurina et al. 2010; Domingues et al. 2009) |
| sn-2 hydroxy-hydroperoxy (+48 Da) | 329 (oleic acid) 327 (linoleic acid) 351 (arachidonic acid) 375 (docosahexaenoic acid) | [M+H-34]⁺ [M+H-18]⁺                           | (Domingues et al. 2009; Melo, Santos, et al. 2013; Tyurin et al. 2008) |
| sn-2 carboxylic acid terminal C9 | 187 (linoleic acid) | [M+H-379]⁺ (141+R₁=C=O)                   | (Maciel et al. 2011; Domingues et al. 2009; Simões et al. 2010) |
| sn-2 carboxylic acid terminal C5 | 131 (arachidonic acid) | [M-H-190]⁻ (58+R₂'COOH) [M+H-141]⁺             | (Maciel, Faria, et al. 2013; Gugiu et al. 2006) |
| Polar head deamination + decarboxylation + oxidation (-29 Da) | -- | [M-H-58]⁻                                      | (Maciel et al. 2011)                                           |
| Polar head decarboxylation + oxidation (-30 Da) | -- | [M-H-57]⁻                                      | (Maciel et al. 2011)                                           |
| Polar head deamination (-1 Da) | -- | --                                           | (Melo, Santos, et al. 2013)                                   |
Table 5. Glycative and glyco-oxidative modifications of APL: Characteristic product ions observed in the MS spectra and corresponding neutral losses. *This neutral loss was used for the targeted MS detection of modified APL in biological samples.

| Modification | Structure (PE is chosen as an example) | Observed neutral loss | References |
|--------------|----------------------------------------|-----------------------|------------|
| Glucose (+162 Da) | ![Glucose Structure](image) | [M+H-84]+ (18+18+18+30) | (Simões et al. 2010) |
| | | [M-H-90] | (Maciel, da Silva, et al. 2013; Maciel, Faria, et al. 2013) |
| | | [M+H-120]+ | (Simões et al. 2010; Annibal et al. 2016) |
| | | [M-H-120]- (*) | (Breitling-Utzmann et al. 2001) |
| | | [M+H-150]+ | (Simões et al. 2010) |
| | | [M+H-162]+ | (Simões et al. 2010) |
| | | [M-H-162]- | (Ravandi, Kuksis, and Shaikh 2000; Maciel, da Silva, et al. 2013; Maciel, Faria, et al. 2013) |
| | | [M-H-249]- (87+162) | (Maciel, da Silva, et al. 2013; Maciel, Faria, et al. 2013) |
| | | [M+H-303]+ (141+162) (*) | (Simões et al. 2010; Melo, Silva, et al. 2013; Ravandi, Kuksis, and Shaikh 2000; Sookwong et al. 2011; Nakagawa 2005) |
| Adduct                        | Formula         | References                                      |
|-------------------------------|-----------------|-------------------------------------------------|
| Glucose adducted to α-keto-ctn(+176 Da) | ![Glucose adducted to α-keto-ctn(+176 Da)](image1.png) | [M+H-317]$^+$ (141+162+14) (Simões et al. 2010) |
| Glucuronic acid (+176 Da)     | ![Glucuronic acid (+176 Da)](image2.png) | [M-H-263]$^-$ (87+162+14) (Maciel, da Silva, et al. 2013) |
|                               |                 | [M+H-317]$^+$ (141+162+14) (Melo, Silva, et al. 2013; Annibal et al. 2016) |
| Carboxymethyl (+58 Da)        | ![Carboxymethyl (+58 Da)](image3.png) | [M+H-199]$^+$ (141+58) (*) (Shoji et al. 2010) |
|                               |                 | [M-H-58]$^-$ (Maciel, da Silva, et al. 2013; Melo, Silva, et al. 2013) |
|                               |                 | -- (Annibal et al. 2016)                        |
| Carboxyethyl (+72 Da)         | ![Carboxyethyl (+72 Da)](image4.png) | [M+H-213]$^+$ (141+72) (*) (Shoji et al. 2010) |
|                               |                 | -- (Annibal et al. 2016)                        |
| Formamide (+28 Da)            | ![Formamide (+28 Da)](image5.png) | [M-H-115]$^-$ (87+28) (Maciel, Faria, et al. 2013; Maciel, da Silva, et al. 2013) |
| Carbamino acid (+44 Da) | ![Chemical Structure](image) | -- | (Simões et al. 2010; Melo, Silva, et al. 2013) |
|-------------------------|-------------------------------|-----|-----------------------------------------------|
|                         |                               | --  | (Melo, Silva, et al. 2013)                    |
Table 6. Chromatographic Columns used for LC-MS separation and analysis of modified APL.

| Analyte(s)                                | Stationary phase | Column     | ID    | Comment                                                                 | References                                                                 |
|-------------------------------------------|------------------|------------|-------|-------------------------------------------------------------------------|----------------------------------------------------------------------------|
| 4-(dimethylamino)benzoic acid (DMABA)-PE  | C<sub>18</sub>   | Gemini®    | 2.0 mm|                                                                         | (Zemski Berry et al. 2010)                                                 |
| HETE-PE KETE-PE                           | C<sub>18</sub>   | Luna®      | 2.0 mm|                                                                         | (Lloyd T. Morgan et al. 2010; Maskrey et al. 2007; Clark et al. 2011; Hammond et al. 2012; Morgan et al. 2009) |
| HETE-PE                                   | C<sub>18</sub>   | Luna®      | 2.0 mm| Separation of the following isobaric species: 18:0a/12-HETE-PE and 16:0a/12-HETE-PC | (Thomas et al. 2010)                                                       |
| HPETE-PE HETE-PE KETE-PE                  | C<sub>18</sub>   | Luna®      | 2.0 mm| Separation of positional isomers of 18:0a/HETE-PE                      | (Alwena H Morgan et al. 2010)                                              |
| Short chain oxidation products of PE       | C<sub>18</sub>   | Prodigy™   | 2.0 mm|                                                                         | (Gugiu et al. 2006)                                                       |
| Oxidized plasmeryl-PE                     | C<sub>18</sub>   | Ultramex   | 4.6 mm|                                                                         | (Khaselev and Murphy 1999)                                                 |
| Short chain and long chain oxidation products of PE | C<sub>5</sub> | Discovery® BIO Wide Pore | 0.5 mm| Separation of the following couples of long-chain oxidation products: (i) PLPE-OH-OH and PLPE-OOH (ii) PLPE-OH-OH-OH and PLPE-OH-OH-OH | (Domingues et al. 2009)                                                   |
| Long chain oxidation products of PS        | Silica           | Luna®      | 2.0 mm|                                                                         | (Tyurina et al. 2008; Tyurina et al. 2010)                                 |
| Gly-PE Gly-PS                             | Silica           | Spherisorb®| 4.6 mm|                                                                         | (Ravandi, Kuksis, and Myher 1995; Ravandi et al. 1996)                     |
| Gly-PE                                    | C<sub>18</sub>   | XTerra™    | 3 mm  |                                                                         | (Breitling-Utzmann et al. 2001)                                            |
| Gly-PE Gly-ox PE                          | C<sub>5</sub>    | Discovery® BIO Wide Pore | 0.5 mm| Separation of two positional isomers for glyco-oxidized PLPE: (i) Gly-PLPE with ketone modification on the polar head; (ii) Gly-PLPE with ketone modification on the sn-2 fatty acyl chain | (Simões et al. 2010)                                                     |
| Gly-PS | C<sub>5</sub> | Discovery® BIO Wide Pore | 0.5 mm | Separation of two positional isomers for glyco-oxidized POPS:
(i) Gly-POPS with ketone modification on the polar head;
(ii) Gly-POPS with ketone modification on the sn-2 fatty acyl chain | (Maciel, da Silva, et al. 2013) |
|--------|--------|-----------------|-------|---------------------------------------------------------------|--------------------------------|
| Gly-PS | C<sub>5</sub> | Discovery® BIO Wide Pore | 0.5 mm | Separation of two isobaric short chain oxidation products of PAPS | (Maciel, Faria, et al. 2013) |
| CM-PE, CE-PE | C<sub>18</sub> | XBridge™ | 2.1 mm | - | (Shoji et al. 2010) |
| PE adducted to 4,5(E)-Epoxy-2(E)-heptenal | HILIC | LiChrospher® | 4 mm | - | (Zamora and Hidalgo 2003) |
| PE adducted to isoketals | C<sub>18</sub> | Nucleosil 100-5® | 2.1 mm | - | (Bernoud-Hubac et al. 2004) |
Figure 1

Schematic representation of the production of glycated APLs and the subsequent formation of ROS and Michael adducts. Glycated APLs can undergo further oxidation to form Schiff bases, which can be converted back to glycated APLs. ROS can also promote the oxidation of APLs to form hydroperoxides, which can be further oxidized to form aldehydes. These aldehydes can react with amino groups to form Schiff bases, which can undergo further oxidation to form glycated APLs. The diagram also shows the involvement of APLOH in the metabolism of APLs and the production of short chain aldehydes.
**Figure 2**

- **Fenton reaction:** production of hydroxyl radical
  \[ \text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \cdot\text{OH} + \cdot\text{OH} \]

- **Electrochemical oxidation:** production of hydroxyl radical
  \[ \text{H}_2\text{O} \rightarrow \cdot\text{OH} + \text{H}^+ + \text{e}^- \]

- **AAPH reaction:** production of hydroperoxyl radical
  \[ \text{R} \cdot\text{N}=\text{N} \cdot \text{R} \xrightarrow{\Delta} \text{N}_2 + \cdot 2\text{R} + \text{O}_2 \rightarrow \text{ROO}^* \]

- **Photosensitization:** production of singlet oxygen
  \[ \text{P}(\text{S}_0) \xrightarrow{\text{hv}} \text{P}(\text{S}_1) \rightarrow \text{P}(\text{T}_1) \]
  \[ \text{P}(\text{T}_1) + \cdot\text{O}_2 \rightarrow \text{P}(\text{S}_0) + \cdot\text{O}_2 \]

- **Lipooxygenases-catalyzed reaction**
  \[ \text{Fe}^{3+} \rightarrow \text{LOX} \rightarrow \text{Fe}^{2+} \]

- **APL oxidation products**
  \[ \text{APLH} \rightarrow \text{APL}^* \rightarrow \text{APL} \]
  \[ \text{ROO}^* \rightarrow \text{ROOH} \]
  \[ \text{APL} \rightarrow \text{APLOOH} \]
  \[ \cdot\text{O}_2 \rightarrow \text{APLOOH}^* \]
Figure 3

A

B

Short chain oxidation products

Long chain oxidation products

[M+H]^+  

[M+H + 1O]^+

[M+H + 2O]^+

[M+H + 3O]^+

[M+H + 4O]^+
Figure 4

A

B

C

[Chemical structures and diagrams with labels and annotations]
Figure 5

- Oxidative deamination
  Mass shift: -1 Da

- Non-modified POPE (m/z 716)

- POPE with deaminated polar head (m/z 715)

- Insertion of hydroxyl function
  Mass shift: +16 Da

- Hydroxy-POPE (m/z 732)

- Hydroxy-POPE with deaminated polar head (m/z 731)
Figure 6

A

PE $[M+H]^+$

Gly-PE $[M+H]^+$

B

PE long chain oxidation products

Gly-PE long chain oxidation products
Figure 7
Figure 8

The diagram shows a mass spectrometry profile with peaks labeled at m/z values of 718.6, 815.7, 838.9, 856.8, and 874.5. The structures A, B, and C are annotated with corresponding chemical formulas and annotations.
Figure 9

A

[Graph showing m/z data with peaks labeled 692.52, 714.50, 774.60, 856.68, 906.72, 936.74, 954.75]
Figure 10
Figure 11

![Diagram of molecular structure with m/z values and relative abundance]
Figure 13
Figure 14

A

Relative Abundance

-58 Da
695.4

R_1COO^- 255.2

R_2COO^- 303.3

439.2

m/z 303

m/z 255

NL 58 Da

[M-H]^-
753.5

B

Relative Abundance

-58 Da
671.469

R_1COO^- 255.233

R_2COO^- 279.232

136.963

204.684

391.225

m/z 279.232

m/z 255.233

NL 58 Da

[M-H]^-
729.472
Figure 16
Figure 18
Figure 19

A. Glyco-oxidized POPS isomers

B. RT 11.0 min

C. Structure with m/z 673 and m/z 760

D. RT 19.7 min

E. Structure with m/z 687 and m/z 774
Figure 20
Figure 21
Figure 23
Figure 24

Identification of oxidized phosphatidylethanolamines in the mixture of non-oxidized and oxidized lipids

LC-MS/MS (Scan: Precursors of m/z 197.1)
Figures captions.

**Figure 1.** Schematic diagram of the chemistry involved in the oxidative and glyco-oxidative modifications of APL. The shaded green boxes depict the main modified products.

**Figure 2.** Diagram reporting the main biomimetic methods used in the study of APL oxidation (Fenton reaction, electrochemical oxidation, AAPH reaction, photosensitization, LOX) and the radical chemical reactions induced or catalyzed by each method.

**Figure 3.** MS spectrum of a PLPE before and after the oxidation induced by *OH radical formed during the Fenton reaction (A and B, respectively). The [M+H]^+ molecular ion of PLPE is depicted at m/z 716.5; the [M+H]^+ molecular ion at m/z 748.5 (Panel B) corresponds to the long-chain oxidation product of PLPE +2O (PLPE+ 32 Da). PLPE +2O can be assigned as dihydroxy-PLPE and as hydroperoxy-PLPE. The [M+H]^+ ion at m/z 764.5 corresponds to the long-chain oxidation product of PLPE +3O (PLPE+48Da) and can be identified both as hydroxy-hydroperoxy-PLPE and trihydroxy-PLPE. The [M+H]^+ ion at m/z 608.4 is attributed to the short chain product 1-(palmitoyl)-2-(9-oxo-nonanoyl)-PE, in which sn-2 position is esterified to a nonanoic acid with a terminal aldehyde in C9. The [M+H]^+ ion at m/z 624.4 corresponds to 1-(palmitoyl)-2-(9-carboxy-nonanoyl)-PE, which is formed by the oxidation of the terminal aldehydic function of 1-(palmitoyl)-2-(9-oxo-nonanoyl)-PE. Reprinted with permission from Domingues et al. (2009), copyright 2009 [John Wiley & Sons].

**Figure 4.** (A) Structures of oxidized PS derivatives with modifications in the polar head group obtained after the oxidation induced by *OH radical generated by Fenton reaction: terminal hydroperoxyacetaldehyde (−13 Da), terminal acetic acid (−29 Da), and terminal acetamide (−30 Da). (B) Thin Layer Chromatography (TLC) plate under UV light of PS oxidation products with modifications in the polar head: lines 1, 2, and 3: oxPLPS; lines 4, 5 and 6: oxPOPS; lines 8, 9 and 10: oxidized 1,2-dipalmitoyl-sn-3-glycerophosphoserine (DPPS); line 7: PS and PE standards. (C)
ESI-MS spectra of PLPS acquired after and before oxidation induced by the *OH radical (right and left panels, respectively). In Panel C, the ion at m/z 758.4 corresponds to the [M-H] molecular ion of unoxidized PLPS; The oxidized molecular [M-H] ions were observed at: m/z 774, PS+O (hydroxy-PLPS) m/z 790.4, PLPS +2O, hydroperoxy-PS and/or di-hydroxy-PS (mass shift from native PLPS: M+32 Da); m/z 806.4, PLPS +3O hydroxy-hydroperoxy-PS and tri-hydroxy-PS (mass shift from native PLPS: M+48 Da); m/z 666.2, 1-(palmitoyl)-2-(9-oxo-nonanoyl)-PS; m/z 682.2, 1-(palmitoyl)-2-(9-carboxy-nonanoyl)-PS; m/z 728.4, ox-PLPS with Ser polar head modified to terminal acetamide (mass shift from native PLPS: M - 30 Da), m/z 745.4, ox-PLPS with Ser polar head modified to terminal hydperoxy-acetaldehyde (mass shift from native PLPS: M-13 Da); m/z 729.4, ox-PLPS with Ser polar head modified to terminal acetic acid (mass shift from native PLPS: M - 29 Da). Reprinted with permission from Maciel et al. (2011), copyright 2011 [Springer].

**Figure 5.** Schematic representation of the oxidative modifications reported for POPE on the unsaturated fatty acyl chains and on the polar head group, along with their characteristic mass shifts.

**Figure 6.** ESI-MS spectrum of gly-PLPE acquired after and before oxidation induced by the *OH radical (Panels A and B, respectively). In Panel A, the ion at m/z 878.5 corresponds to the [M+H]+ molecular ion of glycated PE. In Panel B, the glyco-oxidized [M+H]+ molecular ions were observed at: m/z 894.6, gly-PLPE bearing 1 oxygen insertion on the sn-2 linoleoyl chain (mass shift from gly-PLPE: M+16 Da); m/z 908.6, gly-PLPE bearing 2 oxygen insertions on the sn-2 linoleoyl chain (mass shift from gly-PLPE: M+32Da); m/z 926.6, gly-PLPE bearing 3 oxygen insertions on the sn-2 linoleoyl chain (mass shift from gly-PLPE: M+48 Da); m/z 786.4, glycated 1-(palmitoyl)-2-(9-carboxy-nonanoyl)-PE, a short chain product of glycated PE. Reprinted with permission from Simões et al. (2010), copyright 2010 [Springer].

**Figure 7.** AGEs formed during oxidation of glycated POPS induced by the *OH radical (Fenton reaction). The ion at m/z 788.4 (POPS + 28 Da) corresponds to the product with a polar head group
bearing a terminal formamide, arising from the oxidative cleavage between C1 and C2 of the oxidized glucose moiety. The ion at \( m/z \) 818.4 (POPS + 58 Da) corresponds to the modified POPS with terminal carboxymethyl, arising from the oxidative cleavage between C2 and C3 of the glucose moiety, while the ion at \( m/z \) 832.4 (POPS+ 72 Da) corresponds to the modified PS with terminal carboxyethyl, arising from the oxidative cleavage between C3 and C4 of the glucose moiety. The ion at \( m/z \) 934.4 was formed by the oxidation of the glucose moiety to glucuronic acid, that resulted in a mass increment of 176 Da from native POPS \((162 \text{ Da} + 14 \text{ Da} = 176 \text{ Da})\).

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**Figure 8.** Full ESI-MS of PE after 2 h of treatment with 2 equivalents of HNE. The schematic representation of native DHPE and its different adducts with HNE is also illustrated. In this spectra, the ion at \( m/z \) 718.6 corresponds to the \([M-H]\) molecular ion of the native DHPE, the ion at \( m/z \) 874.5 corresponds to the Michael adduct formed by DHPE and HNE (mass shift of 156 Da, structure C), the ion at \( m/z \) 856.8 corresponds to the Schiff base adduct (mass shift of 138 Da, structure B), the ion at \( m/z \) 838.9 was assigned as 2-pentylpyrrole-PE (mass shift of 120 Da, structure A) arising from the cyclization and dehydration of the Schiff base. Reprinted with permission from Guichardant et al. (1998), copyright 1998 [Elsevier].

**Figure 9.** MS spectrum of DPPE incubated with 400 mM hexanal for 1 h at 37 °C (A). The ion at \( m/z \) 692.52 corresponds to \([M+H]^+\) molecular ion of DPPE; the ion at \( m/z \) 774.60 corresponds to the Schiff base adduct (mass shift of 82 Da); the ion at \( m/z \) 856.68 corresponds to the dimeric adduct due, arising from the covalent adduction of a second molecule of hexanal (mass shift of 164 Da), the ion at \( m/z \) 874.69 was identified as the hydrated form of this dimer; the ion at \( m/z \) 936.74 corresponds to the pyridinium ring adduct formed by covalent adduction of a third hexanal molecule, and followed by cyclization and loss of one H₂O molecule; the ion at \( m/z \) 954.75 corresponds to the hydrated precursor of the pyridinium adduct; the ion at \( m/z \) 938.76 corresponds to the trimeric adduct. The mechanism of the consecutive covalent adductions of hexanal to DPPE
Figure 10. MS/MS spectrum (Orbitrap HCD activation) acquired in negative ion mode of the [M-H'] molecular ion of the long chain oxidation product of PAPE bearing 4 inserted oxygen atoms (m/z 802.486). The ion at m/z 255.231 corresponds to the R₁COO⁻ of palmitic acid; the ion at m/z 367.210 corresponds to the R₂'COO⁻ of oxidized arachidonic acid (arachidonic acid +4O, 303+64 Da); the ion at m/z 452.277 corresponds to the anion of lyso-PAPE, arising from the neutral loss of oxidized arachidonic acid from sn-2 as ketene (-R₂’=C=O). The ions at m/z 784.475 and m/z 766.470 were formed by the neutral losses of 1 and 2 water molecules, respectively (-18 Da and -36 Da). The schematic representation of two possible isomers, along with their fragmentation mechanisms, is also illustrated.

Figure 11. MS/MS spectrum (Orbitrap HCD activation) acquired in negative ion mode showing the fragmentation of a short chain oxidation product of PAPE (m/z 550.343, 1-palmitoyl-2-(5-oxovaleroyl)-sn-3-glycerophosphoethanolamine). The MS/MS spectrum of the [M-H'] molecular ion shows the fragment ions corresponding to the carboxylate anion of palmitic acid (m/z 255.231), and the carboxylate anion of the shortened arachidonic acid derivative with terminal aldehydic function (5-oxovalerate) esterified at sn-2 (m/z 115.038). The schematic representation of the fragmentation is also illustrated.

Figure 12. MS/MS spectrum acquired in the negative mode (Ion Trap CID activation) of the PAPS bearing 2 inserted oxygens (m/z 814). The fragment ion at m/z 727.4 arises from the neutral loss of the Ser moiety from the precursor molecular ion (-87 Da). The schematic representation of the fragmentation is also illustrated.

Figure 13. (A) Reconstructed ion current (RIC) chromatogram of the [M+H]⁺ molecular ion of PLPE long chain oxidation product, at m/z 764.5 (3 inserted oxygen atoms), showing the separation
of two constitutional isomers. (B, C) MS/MS spectra acquired in the positive mode of the isomers eluted at 18.32 min (identified as the poly-hydroxy derivative) (B) and 21.88 min (identified as the hydroperoxy derivative) (C). The following ions were observed in both spectra: ion at \( m/z \) 623.6 ([M+H-141]^+) which arises from the typical neutral loss of the PE polar head; ion at \( m/z \) 313.3, is due to the combined loss of 141 Da and \( R_2'=\text{C}=\text{O} \); ion at \( m/z \) 467.4, arising from the fragmentation of the C9-C10 bond (C9 with a hydroxyl group and C10 with a double bond), combined with the loss of polar head (“A1” and “A2” in B and C, respectively); ion at \( m/z \) 523.5, arising from the fragmentation of the C12-C13 bond (C12 with a hydroxyl group and C13 with a double bond), combined with the loss of polar head (fragments “A1” and “A2” in B and C, respectively); ion at \( m/z \) 646.4, arising from the fragmentation of the C12-C13 bond (C13 with a hydroxyl or hydroperoxy group and C12 with a double bond) (fragments “B1” and “B2” in B and C, respectively); ion at \( m/z \) 535.4, arising from the fragmentation of the C13-C14 bond (C13 with a hydroperoxy group) combined with the neutral loss of polar head (fragment “C2” in B). In the MS/MS spectrum at Panel B, it is possible to see the product ions at \( m/z \) 746.6 and \( m/z \) 728.6, arising from the loss of one (18 Da) and two H\(_2\)O molecules (36 Da), respectively, pinpointing the presence of a polyhydroxy-derivative. In the MS/MS spectrum at Panel C, it is possible to observe the product ions at \( m/z \) 730.6 and \( m/z \) 712.6, formed by the neutral loss of H\(_2\)O\(_2\) (34 Da) and the combined losses of H\(_2\)O\(_2\) plus H\(_2\)O (52 Da), respectively, pinpointing the presence of a hydroxy-hydroperoxy derivative. Reprinted with permission from Domingues et al. (2009), copyright 2009 [John Wiley & Sons].

**Figure 14.** (A) MS/MS spectrum acquired in the negative mode (Ion Trap CID activation) of the PAPS oxidized in the polar head (PAPS -29 Da) and (B) MS/MS spectrum acquired in the negative mode (Orbitrap HCD activation) of PLPS oxidized in the polar head (PLPS -29 Da). Both derivatives were formed upon oxidation by the *OH radical (Fenton reaction). The MS/MS spectrum of PAPS -29 Da (A) shows a base peak at \( m/z \) 695.4 corresponding to the fragment ion
formed by the neutral loss of acetic acid (-58 Da) from the precursor molecular ion. The fragment ion at m/z 255.2 corresponds to palmitic acid (R\textsubscript{1}COO\textsuperscript{-}) and the fragment ion at m/z 303.3 corresponds to arachidonic acid (R\textsubscript{2}COO\textsuperscript{-}). In the MS/MS spectrum of PLPS -29 Da (B) the fragment ion at m/z 671.469 corresponds to the neutral loss of acetic acid (-58 Da) from the precursor molecular ion. The fragment ion at m/z 255.233 corresponds to palmitic acid (R\textsubscript{1}COO\textsuperscript{-}) and the fragment ion at m/z 303.232 corresponds to linoleic acid (R\textsubscript{2}COO\textsuperscript{-}). The schematic representations of the fragmentations are also reported.

**Figure 15.** MS/MS spectrum acquired in the positive mode of gly-PLPE. The neutral loss of glycated polar head (-303 Da) is evidenced by the base peak at m/z 575.6; the fragment ion at m/z 794.6 arises from the neutral loss of 84 Da, due to the elimination of three H\textsubscript{2}O molecules and one H\textsubscript{2}CO molecule from the precursor molecular ion. The schematic representation of the fragmentation leading to the neutral loss of the glycated polar head is also illustrated. Reprinted with permission from Simões et al. (2010), copyright 2010 [Springer].

**Figure 16.** MS/MS spectrum acquired in the positive mode of gly-ox-PLPE. The fragment ion at m/z 575.5 (base peak) arises from the neutral loss of the oxidized polar head (303+14 Da); the fragment ion at m/z 318.1 corresponds to the protonated oxidized polar head group. The schematic representation of the fragmentation leading to the neutral loss of the oxidized glycated polar head is also illustrated. Reprinted with permission from Simões et al. (2010), copyright 2010 [Springer].

**Figure 17.** MS/MS spectrum acquired in the negative mode (Orbitrap HCD activation) of gly-PLPE. The fragment ion at m/z 714.508 arises from the neutral loss of glucose (-162 Da). The fragment ions at m/z 255.233 and m/z 279.233 correspond to the unmodified R\textsubscript{1}COO\textsuperscript{-} and R\textsubscript{2}COO\textsuperscript{-}, respectively. The fragment ions at m/z 756.519 and m/z 786.529 arise from the neutral losses of 90 Da (-C\textsubscript{3}H\textsubscript{6}O\textsubscript{3}), and 120 Da (-C\textsubscript{4}H\textsubscript{8}O\textsubscript{4}) from the precursor ion, respectively, and arise from characteristic fragmentation patterns of the glucose moiety upon negative ion mode MS/MS. The schematic representation of the fragmentation is also reported.
Figure 18. MS/MS spectrum acquired in the negative mode (Orbitrap HCD activation) of gly-PLPS. The fragment ion at \( m/z \) 758.497 arises from the neutral loss of glucose (-162 Da). The base peak at \( m/z \) 671.466 arises from the combined neutral losses of glucose and aziridine-2-carboxylic acid from the precursor ion (162+87 = 249 Da). The fragment ions at \( m/z \) 255.233 and \( m/z \) 279.233 correspond to the unmodified \( R_1\text{COO}^- \) and \( R_2\text{COO}^- \), respectively. The ion at \( m/z \) 830.519 arises from the neutral loss of 90 Da (-C\(_3\)H\(_6\)O\(_3\)). The schematic representation of the fragmentation is also reported.

Figure 19. (A) Reconstructed ion current (RIC) chromatogram of the [M-H]\(^-\) molecular ion of gly-POPS long chain oxidation product, at \( m/z \) 936.4 (mono-keto-derivative) showing the separation of two constitutional isomers. (B) MS/MS spectrum and (C) scheme of fragmentation of the isomer that eluted at 11.0 min; in the MS/MS spectrum at Panel B, the fragment ion at \( m/z \) 673.3 (base peak) arises from the neutral loss of 263 Da, which is due to the loss of aziridine-2-carboxylic acid (87 Da) adducted to the glucuronic acid moiety (162+14 = 176 Da), confirming the insertion of one oxygen atom on the glycated polar head; the fragment ion at \( m/z \) 760.4 can be attributed to the neutral loss of glucuronic acid (176 Da) and further confirms the oxidation of the glucose moiety. (C) MS/MS spectrum and (D) scheme of fragmentation of the isomer eluted at 19.7 min; in the MS/MS spectrum at Panel B, the fragment ion at \( m/z \) 687.4 (base peak) arises from the neutral loss of 249 Da, which corresponds to the aziridine-2-carboxylic acid (87 Da) adducted to the glucose moiety (162 Da), confirming that the keto insertion (+14 Da) is located on the oleoyl chain of gly-POPS; the fragment ion at \( m/z \) 774.3 (neutral loss of glucose, 162 Da) also shows that the glucose moiety does not bear any oxidative modification. Reprinted with permission from Maciel et al. (2013), copyright 2013 [Elsevier].

Figure 20. MS/MS spectrum acquired in the negative mode of the advanced gly-ox-POPS end product at \( m/z \) 818.4 (cleavage in the C\(_2\)-C\(_3\) bond of the glucose moiety). In this spectrum, the fragment ion at \( m/z \) 673.4 (base peak) arises from the neutral loss of 145 Da, that corresponds to the
Ser moiety adducted to a carboxymethyl group, formed by the oxidative cleavage of glucose (87 + 58 Da). This fragmentation pathway confirms that the precursor ion at m/z 818.4 is a PS AGE in which the glucose moiety has been shortened by an oxidative cleavage between C2 and C3. The minor fragment ion at m/z 760.5 arises from the neutral loss of a carboxymethyl moiety from the precursor ion (-58 Da) and further confirms that an oxidative cleavage occurred between C2 and C3 of glucose. Reprinted with permission from Maciel et al. (2013), copyright 2013 [Elsevier].

**Figure 21.** MS/MS spectrum acquired in the negative mode of the IsoLG–PE pyrrole adduct at m/z 1031; the fragment ion at m/z 768 arises from the neutral loss of R2=C=O; the fragment ion at m/z 512 is due to the combined losses of R2COOH and R1COOH; the fragment ion at m/z 415 arises from the neutral losses of the polar head, modified as pyrrole adduct (361 Da), and R1COOH; the fragment ion at m/z 391 arises from the neutral losses of the polar head modified as pyrrole adduct (361 Da) and R2COOH.; the fragment ions at m/z 279 and m/z 255 are R2COO⁻ and R1COO⁻, respectively; the fragment ion at m/z 153 is due to the combined neutral losses of the polar head modified as pyrrole adduct (361 Da) and of R1COOH and R2COOH. The schematic representations of the fragmentations are also illustrated. Reprinted with permission from Bernoud-Hubac et al. (2004), copyright 2004 [Elsevier].

**Figure 22.** MS/MS spectrum acquired in negative ion mode of IsoK–PE Schiff base adduct at m/z 1035; the fragment ion at m/z 772 arises from the neutral loss of R2=C=O; the combined neutral losses of R2COOH and R1COOH led to fragment ion at m/z 516; the fragment ion at m/z 415 is formed by the combined neutral losses of the polar head modified as Schiff base adduct (-365 Da) and R1COOH; the fragment ion at m/z 391 arises from the combined neutral losses of the polar head modified as Schiff base adduct (-365 Da) and R2COO⁻; the fragment ions at m/z 279 and m/z 255 are R2COO⁻ and R1COO⁻, respectively; the fragment ion at m/z 153 arises from the neutral losses of the polar head modified as Schiff base adduct (-365 Da) and of R1COOH and R2COOH. The
schematic representations of the fragmentations are also illustrated. Reprinted with permission from Bernoud-Hubac et al. (2004), copyright 2004 [Elsevier].

**Figure 23.** Positive ion mode multistage tandem MS spectra of a DPPE-hexanal Schiff base adduct. (A) MS/MS spectrum of the DPPE-hexanal Schiff base adduct; the fragment ion at $m/z$ 551.51 corresponds to the diacylglyceride fragment ion, arising from the neutral loss of the phosphoethanolamine moiety covalently linked to hexanal (-224 Da); the fragment ion corresponding to the modified polar head group can be seen at $m/z$ 224.15 and was selected for further fragmentation. (B) The MS$^3$ spectrum of phosphoethanolamine-hexanal Schiff base product ion; the fragment ion at $m/z$ 126.10 corresponds to the of the vinylamine-hexanal Schiff base adduct due to the neutral loss of phosphoric acid from the precursor ion (-98 Da); this fragment ion at $m/z$ 126.10 was further isolated and fragmented. (C) The MS$^4$ spectrum of the vinylamine-hexanal Schiff base product ion; several peaks ($m/z$ 112.34, 98.15, 83.97, 69.98, 55.99) arise from the sequential mass losses of 14 Da, which confirm the presence of a hexanal alkyl chain adducted to the polar head. The schematic representations of the fragmentations are also illustrated. Reprinted with permission from Annibal et al. (2014), copyright 2014 [John Wiley & Sons].

**Figure 24.** Schematic representation of the PIS approach based on D6-DMABA derivatization proposed for the identification of oxidized PE in lipid extracts from RAW 264.7 cells.