J. Am. Soc. Mass Spectrom. (2011) 22:1804–1814
DOI: 10.1007/s13361-011-0194-9

RESEARCH ARTICLE

Structural Characterization of Oxidized Glycerophosphatidylserine: Evidence of Polar Head Oxidation

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Abstract

Non-oxidized phosphatidylserine (PS) is known to play a key role in apoptosis but there is considerable research evidence suggesting that oxidized PS also plays a role in this event, leading to the increasing interest in studying PS oxidative modifications. In this work, different PS (1-palmitoyl-2-linoleoyl-sn-glycero-3-phospho-L-serine (PLPS), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS), and 1,2-dipalmitoyl-sn-glycero-3-phospho-L-serine (DPPS) were oxidized in vitro by hydroxyl radical, generated under Fenton reaction conditions, and the reactions were monitored by ESI-MS in negative mode. Oxidation products were then fractionated by thin layer chromatography (TLC) and characterized by tandem mass spectrometry (MS/MS). This approach allowed the identification of hydroxyl, peroxy, and keto derivatives due to oxidation of unsaturated fatty acyl chains. Oxidation products due to oxidation of serine polar head were also identified. These products, with lower molecular weight than the non-modified PS, were identified as $[M - 29 - H]^-$ (terminal acetic acid), $[M - 30 - H]^-$ (terminal acetamide), $[M - 13 - H]^-$ (terminal hydroperoxyacetaldehyde), and $[M - 13 - H]^-$ (terminal hydroxyacetaldehyde plus hydroxy fatty acyl chain). Phosphatidic acid was also formed in these conditions. These findings confirm the oxidation of the serine polar head induced by the hydroxyl radical. The identification of these modifications may be a valuable tool to evaluate phosphatidylserine alteration under physiopathologic conditions and also to help understand the biological role of phosphatidylserine oxidation in the apoptotic process and other biological functions.

Key words: Phosphatidylserine, Oxidation, Hydroxyl radical, Electrospray, Mass spectrometry

Introduction

Phosphatidylserine (PS) is a phospholipid (PL) that is widely distributed among all mammalian cells. It comprises approximately 10% of the total phospholipid of the cell and is found preferentially in the inner leaflet of the plasma membrane and in endocytic membranes. PS plays important roles in many biological processes, with emphasis on blood clotting and apoptosis [1]. In some physiologic processes, the asymmetric distribution of phospholipids of the plasma membrane is lost and PS is translocated to the outer leaf of the plasma membrane [1–3]. The externalization of PS is an early indicator of apoptosis and is essential for the recognition and removal of apoptotic cell. Phagocytes recognize PS as a signal initiator of apoptosis [1, 4–8]. Tyurina et al. reported that inhibition of aminophospholipid translocase, which selectively pumps phosphatidylserine and phosphatidylethanolamine from the outer to the inner plasma membrane monolayer [9], results in externalization of phosphatidylserine, and activation of macrophages [10]. PS asymmetry has also been associated with clinical situations in which apoptosis plays a relevant role, as in cancer, chronic autoimmunity, and infections [3].

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Received: 18 April 2011
Revised: 7 June 2011
Accepted: 8 June 2011
Published online: 19 July 2011

Although non-oxidized PS are major ligands recognized by macrophages on the apoptotic cell, there is growing evidence that oxidized PS (oxPS) also can have an active role [11–14].

When the phospholipids undergo oxidation, a variety of oxidized products can be formed, mainly due to the modification of unsaturated fatty acyl chains [15]. These modifications can be induced in vivo by enzymatic (cytochrome *c*, myeloperoxidase) or non-enzymatic reactions ($^{\bullet}OOH$, $^{\bullet}OH$, Fe^{2+}, Cu^{+}, radiation). Depending on the predominating oxidative process, different PLs oxidation products can be observed [16]. It is thought that oxidized PLs may have new biological properties, participating in various biological processes such as the immune response, inflammation, apoptosis, and age-related diseases [16]. However, little is known about the relationship between specific oxidation products and their biological effects.

Mass spectrometry (MS) has been used to identify non-oxidized and oxidized phospholipids, including native and oxidized PS [17–21]. PS hydroperoxy and hydroxy species were identified by Kagan and co-authors using electrospray mass spectrometry (ESI-MS) and tandem mass spectrometry (ESI-MS/MS) in negative mode. Oxidized PS has been identified in several pathological conditions: Tyurina et al. identified mono-hydroperoxy derivatives of PS after intestinal injury induced by γ-irradiation, [22, 23]; Bayir et al. also proposed the presence of PS hydroperoxides derivatives in traumatic brain injury after controlled cortical impact [24]; hydroperoxyl and hydroxyl PS derivates were also identified by Tyurin et al. during apoptosis induced in neurons by staurosporine [25] and in cells and tissues after pro-apoptotic and pro-inflammatory stimuli [26]; the same group showed that the pattern of phospholipid oxidation during apoptosis is non-random and that PS is one of the preferred peroxidation substrates [8].

Although several studies have produced evidence on the role of oxidation of PS in inflammation and apoptosis, there is still insufficient data on the mechanisms of oxidation and on the nature of the oxidized species formed. The objective of this research is to evaluate the molecular changes induced in phosphatidylserines when subjected to oxidative stress, focusing on the oxidation products resulting from oxidation of the polar head. Different PS species were subjected to oxidation induced by the hydroxyl radical generated under Fenton reaction (H_2O_2/Fe^{2+}) conditions and the oxidation products were subsequently separated by thin layer chromatography (TLC) and analyzed by electrospray (ESI), mass spectrometry (MS), and tandem mass spectrometry (MS/MS).

Experimental

Materials

1-Palmitoyl-2-linoleoyl-sn-glycero-3-phospho-L-serine (PLPS), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS), 1,2-dipalmitoyl-sn-glycero-3-phospho-L-serine (DPPS), and palmitoyloleoylphosphatidylethanolamine were obtained from Avanti Polar Lipids, Inc. (Alabaster, AI, USA) and used without further purification. $FeCl_2$, EDTA and H_2O_2 (30%, w/w) were acquired from Merck (Darmstadt, Germany). Triethylamine (Acros Organics, Geel, Belgium), chloroform (HPLC grade), methanol (HPLC grade). and ethanol absolute (Panreac) were used without further purification. TLC silica gel 60 plates with concentrating zone (2.5×20 cm) were purchased from Merck.

Oxidation of Phosphatidylserine by Fenton Reaction

Ammonium hydrogen carbonate buffer (5 mM, pH 7.4) was added to 1 mg of phospholipid (2 mg/mL) and the solution was vortex-mixed and sonicated for the formation of vesicles. Oxidative treatments using Fe (II) and H_2O_2 were carried out by adding 40 μM $FeCl_2$/EDTA (1:1) and 10 mM of H_2O_2 to total a volume of 500 μL of solution. The mixture was left to react at 37 °C in the dark for several days with agitation. Controls were performed by replacing H_2O_2 with water and without H_2O_2 and Fe^{2+}.

ESI-MS Conditions (Linear Ion Trap)

The extent of oxidation was monitored by electrospray mass spectrometry in a linear ion trap mass spectrometer LXQ (ThermoFinnigan, San Jose, CA, USA). The LXQ linear ion trap mass spectrometer was operated in negative mode. ESI conditions were as follows: electrospray voltage was 4.7 kV; capillary temperature was 275 °C, and the sheath gas flow was 25 U. An isolation width of 0.5 Da was used with a 30 ms activation time for MS/MS experiments. Full scan MS spectra and MS/MS spectra were acquired with a 50 ms and 200 ms maximum ionization time, respectively. For MS/MS experiments, normalized collision energy (CE) was applied in the range of 17 to 20 (arbitrary units) for MS/MS. Data acquisition was carried out on an Xcalibur data system (ver. 2.0).

Exact Mass Measurement and Elemental Composition

Identification of the oxidation product ions corresponding to the oxidation in the polar head group was confirmed by exact mass measurement and elemental composition determination in a MALDI-TOF/TOF mass spectrometer. Elemental composition of the ion at *m/z* 721.5 (observed for DPPS) was confirmed by exact mass measurement and elemental composition determination using a ESI-Q-TOF2 mass spectrometer due to the presence of a contaminate with the same *m/z* value in the MALDI-MS spectrum.

MALDI-MS Conditions

MALDI mass spectra were acquired using a MALDI-TOF/TOF Applied Biosystems 4800 Proteomics Analyzer

(Applied Biosystems, Framingham, MA, USA) instrument equipped with a nitrogen laser emitting at 337 nm and operating in a reflectron mode. Full scan mass spectra ranging from *m/z* 650 to 4000 were acquired in the negative mode. All spectra were acquired with 2,5-dihydroxybenzoic acid (DHB) matrix. The matrix solution was prepared by dissolving 10 mg of DHB in a 1 mL mixture of methanol: aqueous (1:1, vol/vol). For the accurate mass measurements, the lock mass in each mass spectrum was the calculated monoisotopic mass/charge of the non-modified phosphatidylserine and palmitoyloleoylphosphatidylethanolamine as internal standard.

ESI-MS Conditions (ESI-Q-TOF2)

For the analysis in the ESI-QTOF2 instrument (Micromass, Manchester, UK), the flow rate was 10 μL min^{-1}, the needle voltage was set at 3 kV, the cone voltage at 30 V, the ion source set at 80 °C, and the desolvation temperature at 150 °C. Mass spectra were averaged for 1 min. For the accurate mass measurements, the lock mass in each mass spectrum was the calculated monoisotopic mass/charge of the native phospholipid (non-modified phospholipid).

Separation of Oxidation Products of PS by Thin Layer Chromatography

The oxidation products were separated by thin layer chromatography (TLC) using silica gel 60 plates with concentrating zone 2.5×20 cm (Merck KGaA). Prior to separation, plates were treated with boric acid 2.3% in ethanol. The plates were developed with solvent mixture chloroform/ethanol/water/triethylamine (30:35:7:35, vol/vol/vol/vol). Lipid spots on TLC plates were observed by exposure to primuline. The TLC spots identified as oxidized PS were scraped from the plates and extracted using chloroform/methanol (2:1, vol/vol). The oxidation products separated by thin layer chromatography (TLC) were analyzed by electrospray (ESI), mass spectrometry (MS), and tandem mass spectrometry (MS/MS).

Results and Discussion

This paper focuses on the ESI-MS analysis of PS that was submitted to oxidation induced by the hydroxyl radical generated under Fenton reaction (H_2O_2/Fe^{2+}). Selected PS were dipalmitoyl-phosphatidylserine (DPPS; C16:0/C16:0; *m/z* $[M - H]^-$=734), 1-palmitoyl-2-oleoyl-phosphatidylserine (POPS; C16:0/C18:1; *m/z* $[M - H]^-$ =758), and the 1-palmitoyl-2-linoleoyl phosphatidylserine (PLPS; C16:0/C18:2; *m/z* $[M - H]^-$=756) (Figure 1). Since DPPS contains saturated acyl chains, it is considered as a model for studying oxidation of the phospholipid polar head [15].

Comparing the ESI-MS spectra of the three PS obtained before and after oxidation (Figure 2), new ions can be observed. These ions correspond to the $[M - H]^-$ molecular ions of oxidation products. The ESI-MS spectra of PLPS (Figure 2a), acquired after exposure to oxidative conditions, show a larger number of new molecular ions. This is due to the presence in this molecule of two double bonds in the *sn-2* fatty acyl chain, increasing the number of possible oxidation sites. Lower number of ions were observed for POPS (Figure 2c) and even lower for DPPS (Figure 2e). In the spectra of oxPLPS and oxPOPS, some oxidation products can be observed at higher *m/z* values than the non-modified PS, corresponding to the oxidation products with insertion of oxygen atoms as hydroperoxides ($[M - H + 2O]^-$), hydroxide ($[M - H + O]^-$), and keto ($[M - H + O - 2\ Da]^-$) derivatives. These oxidation products will be briefly discussed in this work since they have already been described and fully characterized [13, 23, 25].

In the spectrum obtained after oxidation of the PLPS (Figure 2a) we can observe new ions at *m/z* 666 and 682 (666+16), with lower *m/z* than the non-modified PLPS. These ions correspond to oxidation products with shortened acyl chain at C9 and a carboxylic acid terminal. Short chain oxidation products originated from cleavage of fatty acyl chains have been described for PS [13] and have been studied in phosphatidylcholine, phosphatidylserine, and cardiolipin phospholipids [27–29]. The shortened oxidation products are generated after abstraction of the *bis*-allylic hydrogen atoms by the hydroxyl radical, which through a β-scission mechanism, break down to short-chain phospholipid products with terminal aldehydic or carboxylic acid function. As reviewed elsewhere, these are well known products of lipid peroxidation [30, 31]. No short chain oxidation products were formed during POPS oxidation, similar to previously observed POPC oxidation, using the same oxidative conditions [31].

There are other ions observed at lower *m/z* than the non-modified PS in MS spectra of oxidized PLPS, POPS, and DPPS (Figure 2a, c, and e), which were not assigned as short-chain compounds. These oxidation products, with less 29, 30, and 13 Da compared with the native PS, occur due to oxidative modification in PS polar head, as confirmed by separation using thin layer chromatography (TLC) (Figure 3), and analysis by tandem mass spectrometry will be described latter. Elemental composition determination for the ions with less 29 Da (for DPPS: $C_{37}H_{70}O_{10}$ P, error 18.7 ppm; for POPS $C_{39}H_{72}O_{10}P$, error 27.8 ppm, for PLPS $C_{39}H_{70}O_{10}P$, error −15.4 ppm), with less 30 Da (for DPPS, $C_{37}H_{71}NO_9P$ error 10.3 ppm; for POPS $C_{39}H_{73}NO_9P$, error −1.3 ppm; for PLPS $C_{39}H_{71}NO_9P$ error −32.1 ppm), and with less 13 Da (for DPPS $C_{37}H_{70}O_{11}P$ error −9.9; for POPS $C_{39}H_{72}O_{11}P$ error 20.9 ppm) confirmed the modification of serine polar head.

Mass spectrometry analysis, in positive mode, of these oxidized PS, showed molecular ions that correspond to the same oxidation products, but observed as low abundant $[M + H]^+$, $[M + Na]^+$, and $[M - H + 2Na]^+$ ions (data not shown). However, the oxidation product resulting from loss of 29 Da was not observed. We propose that this oxidation product contains a carboxylic acid group polar head, as will be

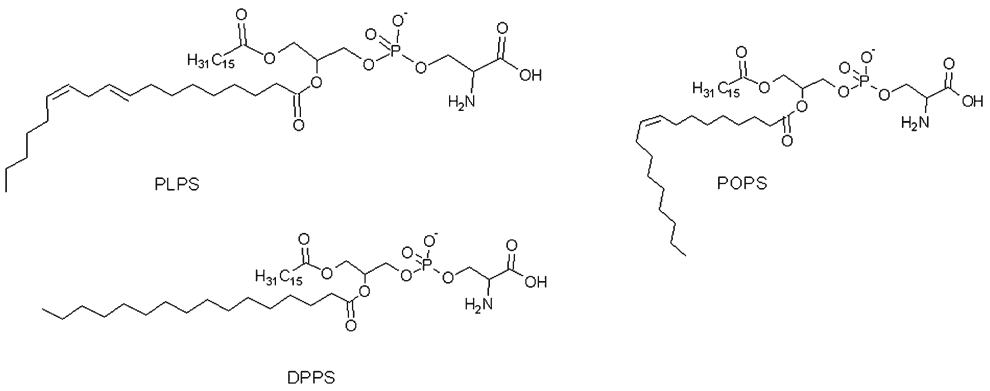


Figure 1. Molecular structures of PLPS, POPS, and DPPS

described latter, forming, preferentially, negative ions. To better characterize these new oxidation products, they were further analyzed by TLC, MS, and MS/MS in negative mode.

Phosphatidylserine Oxidation in Fatty Acyl Chains—Analysis by ESI-MS/MS

Hydroperoxide and hydroxide derivatives are primary oxidation products of lipids and phospholipids, and occur during PLPS and POPS oxidation. DPPS does not yield these oxidation products because it has two saturated fatty acyl chains that do not oxidize [15]. ESI-MS/MS spectra of PLPS and POPS oxidation products show formation of hydroxy [M –H + O]$^-$, keto [M – H + O – 2 Da]$^-$, peroxy [M – H + 2O]$^-$, and hydroxy peroxy [M – H + 3O]$^-$ derivatives, as summarized in Table 1. In all these MS/MS spectra, we can observe a major product ion formed by loss of 87 Da, due to loss of phospholipid polar head. This is an abundant loss, typical of PS [18]. MS/MS analysis (Table 1) shows that insertion of oxygen atoms occur in the linoleic (for PLPS) or

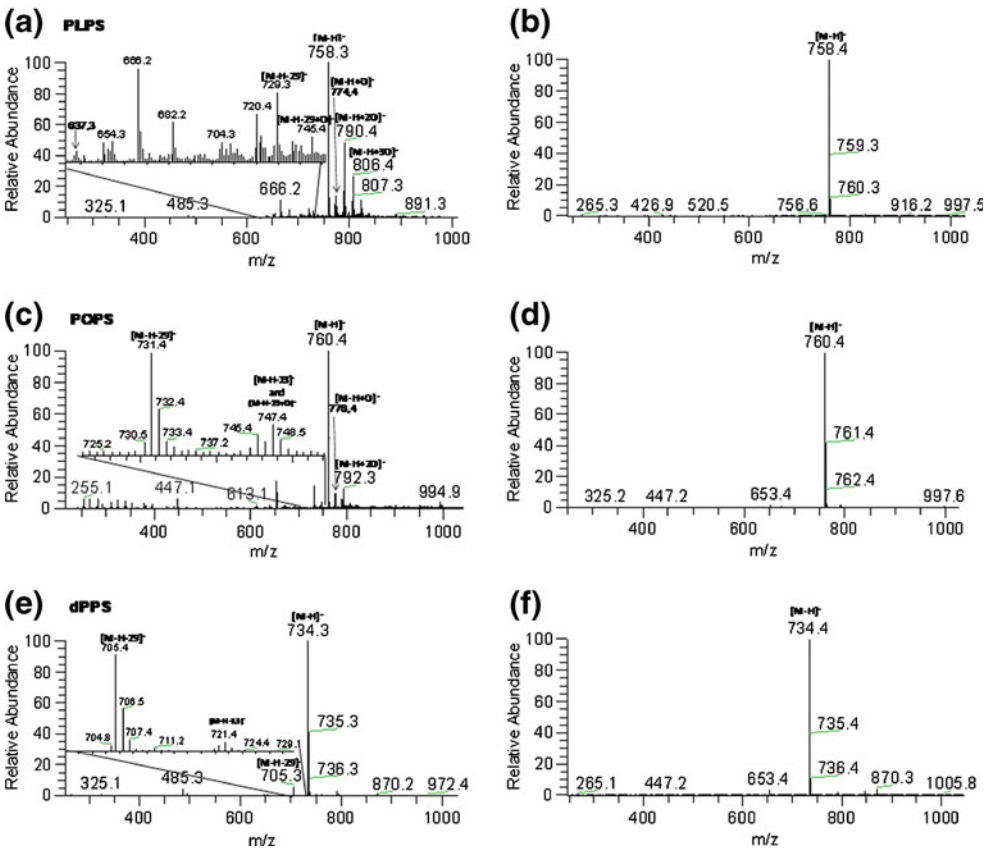


Figure 2. ESI-MS spectra of PLPS (**a**), (**b**), POPS (**c**), (**d**) and DPPS (**e**), (**f**). Spectra a, c, and e were acquired after exposing samples to oxidative stress. Spectra b, d, and f were acquired from control samples

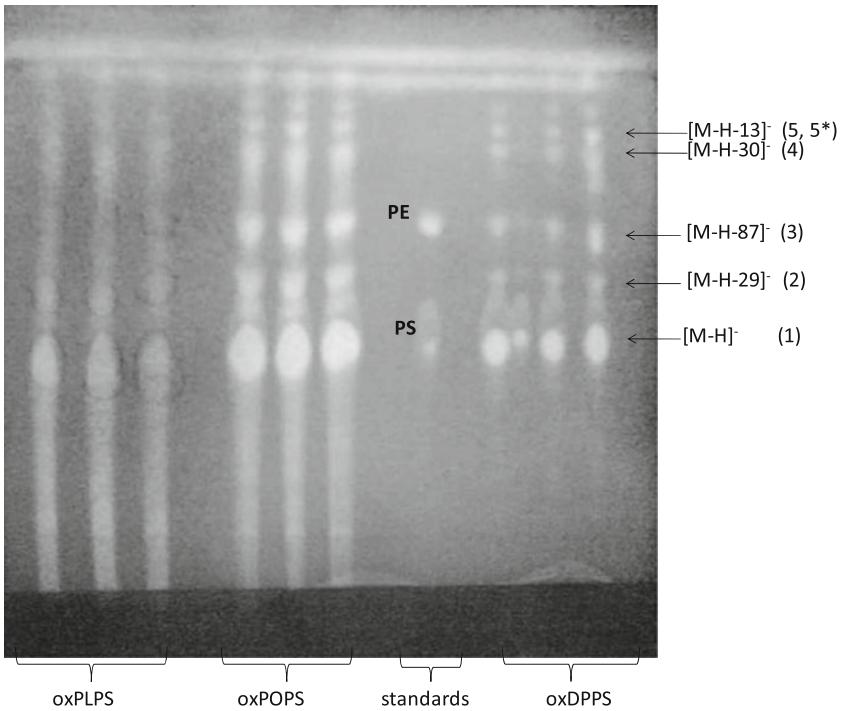


Figure 3. TLC of oxidation products of phosphatidylserines with modifications in the polar head (oxPLPS (lines 1, 2, and 3), oxPOPS (lines 4, 5, and 6), and oxDPPS (lines 8, 9, and 10). PS and PE standards were applied in line 7

oleic acids (for PLPS), as confirmed by the observation of the carboxylate anions of modified fatty acyl chains $R'COO^-$, in agreement with previous published work [26, 28, 31–33].

Phosphatidylserine Oxidation with Modification of in Polar Head—Analysis by TLC and ESI-MS/MS

Oxidative modifications in the PS polar head should change significantly the polarity of these species. Consequently, their separation may be possible through simple techniques such as TLC, which is widely used for separation of different classes of phospholipids. Thus, oxidative mixtures of the three PS under study were fractioned by TLC, as is illustrated in Figure 3.

Table 1. Product Ions Observed in ESI-MS/MS Spectra of Oxidized PLPS and POPS, *which* were Determined by Tandem Mass Spectrometry to Contain Modified Fatty Acyl Chains

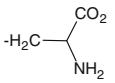
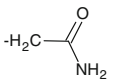
	Modifications		$[M–H]^-$	$R_2'COO^-$	$[M–R_1COOH–87–H]^-$
PLPS	+1 O	–OH	774	295	431
		– =O	772	293	429
	+2 O		790	311	447
	+3 O		806	327	463
	Short (C9)*		666	187	323
	Short (C9) + O		682	203	339
POPS	+1 O		776	297	433
			774	295	431
	+2 O		792	313	449
DPPS	-		-	-	-

*Short chain oxidation product in C9 with carboxylic acid terminal.

In the TLC of the oxidized PS, five new spots were resolved using the same conditions used to separate phospholipid classes, demonstrating that significant changes of polarity have occurred, presumably with formation of new compounds with different polar head structures. The new spots were scraped from the plate, the OxPL extracted, and further analyzed by ESI-MS and MS/MS. Table 2 summarizes are all molecular ions observed in MS spectra for each spot of TLC and the proposed structure.

In spots #1 (Figure 3, Table 2) we have identified the non-modified PS together with the hydroperoxides, keto, and hydroxides oxidation products (Table 1), in agreement with previous results [26]. In the spots #2 (Figure 3, Table 2) we identified, for all PS, oxidation products with polar head modifications $[M – H – 29]^-$. Spots #3 (Figure 3, Table 2) shows a Rf similar to the phosphatidic acid (PA) standard. The MS spectra acquired from samples of these spots show $[M – H – 87]^-$ ions, thus indicating that PA is generated during PS oxidation, with complete loss of polar head. PA was also observed during cardiolipin oxidation by γ-irradiation [34, 35]. The oxidation products from spots #4 (Figure 3, Table 2) contained a head modification, with a mass difference of less 30 Da. It is also possible to observe spots #5 (Figure 3, Table 2) in the DPPS and POPS. Analysis of the MS spectrum of these samples shows the presence of ions at *m/z* 747 and 763 for POPS, and an ion at *m/z* 721 for DPPS. These ions have a difference of 13 Da compared with the non-modified PS. In the case of PLPS, these oxidation products were not observed, probably because they might decompose to fatty acyl short chain

Table 2. Molecular Ions Observed in the ESI-MS Spectra from the Different Spots Identified in the TLC Plate for Each PS. The Table Shows the *m/z* Value of the $[M - H]^-$ Ions, their Most Probable Identification, Including the Modified Polar Headgroup, and the Typical Neutral Loss, Observed in the MS/MS Spectra

Spots #	Modification	[M-H]⁻			Polar head	Neutral loss
		PLPS	POPS	DPPS		
1	–	758	760	734		87
	+14	772	774	–		
	+16	774	776	–		
	+30	788	790	–		
	+32	790	792	–		
	+46	804	–	–		
	+48	806	–	–		
2	−29	729	731	705		58
	−29+14	743	745	–		
	−29+16	745	747	–		
	−29+30	759	761	–		
	−29+32	761	763	–		
3	−87	671	673	647	absent	–
	−87+14	685	687	–		
	−87+16	687	689	–		
	−87+30	701	703	–		
	−87+32	703	705	–		
4	−30	728	730	704		57
	−30+14	742	744	–		
	−30+16	744	746	–		
	−30+30	758	760	–		
	−30+32	760	762	–		
5	−13	–	747	721		74
	−13+16	–	763	–		
5*	−13		747			58

derivatives, which are not observed in TLC plate. The MS spectra of POPS samples from spots #5 show ions at the same *m/z* of ions found in spots #2. These are necessarily different compounds, since they have different Rf. The nature of these ions was determined by MS/MS analysis and will be discussed in another section of this manuscript. In each spot of PLPS and POPS oxidized samples, additional ions with plus 14 Da (+O – 2 Da), 16 Da (+O), 30 Da (+2O – 2 Da), 32 Da (+2O) 46 Da (+3O – 2 Da), and 48 Da (+3O) were observed. These ions resulted from additional oxidation in unsaturated fatty acyl chain, as is shown in Table 2.

In order to confirm the structure of the oxidation products found in spots #2 to #5, MS/MS of all the identified molecular ions (Table 2) were obtained and analyzed. These spots corresponded to oxidation products resulting from modification of PS polar head.

The oxidation products with less 29 Da than the unoxidized PS, found in spots #2, show a neutral loss of 58 Da, instead of the typical 87 Da of PS (Figure 4). The MS/MS of these ions $[M - H - 29]^-$ show the non-modified carboxylate anions $RCOO^-$, thus confirming that the structural changes occurred at the polar head and not at the fatty acyl chains. This main fragmentation pathway (loss of 58 Da) is also observed for the oxidation products with additional oxidation (+nO) in the unsaturated fatty acyl chains observed for PLPS and POPS. Tandem mass spectra of these ions also showed the modified $RCOO^-$. Oxidation products with less 29 Da were observed during the oxidation of amino acids and were identified as the result of oxidative reactions that lead to loss of an amine group and CO_2, with formation of a terminal carboxylic acid group [36]. We propose that oxidation products observed in spots #2 are glycerophosphoaceticacid derivatives (Scheme 1).

The new oxidation products identified in spots #3 are due to oxidation in polar head group of the PS. These oxidative modifications are not observable when a neutral loss scan of aziridine-2-carboxylic acid (neutral loss of 87 Da) is performed to detect oxidized PS from in vivo samples [13].

The MS/MS spectra of molecular ions $[M - H - 30]^-$ found in spots #4 of the three PS show loss of the head group as loss of 57 Da, but typical loss of 87 Da is not observed. We propose that these oxidation products resulted from decarboxylation and generation of a keto moiety, as observed during amino acid and peptides oxidation (Scheme 1) [36]. In these species, loss of modified polar head group (−57 Da) is not the base peak of the spectra, in

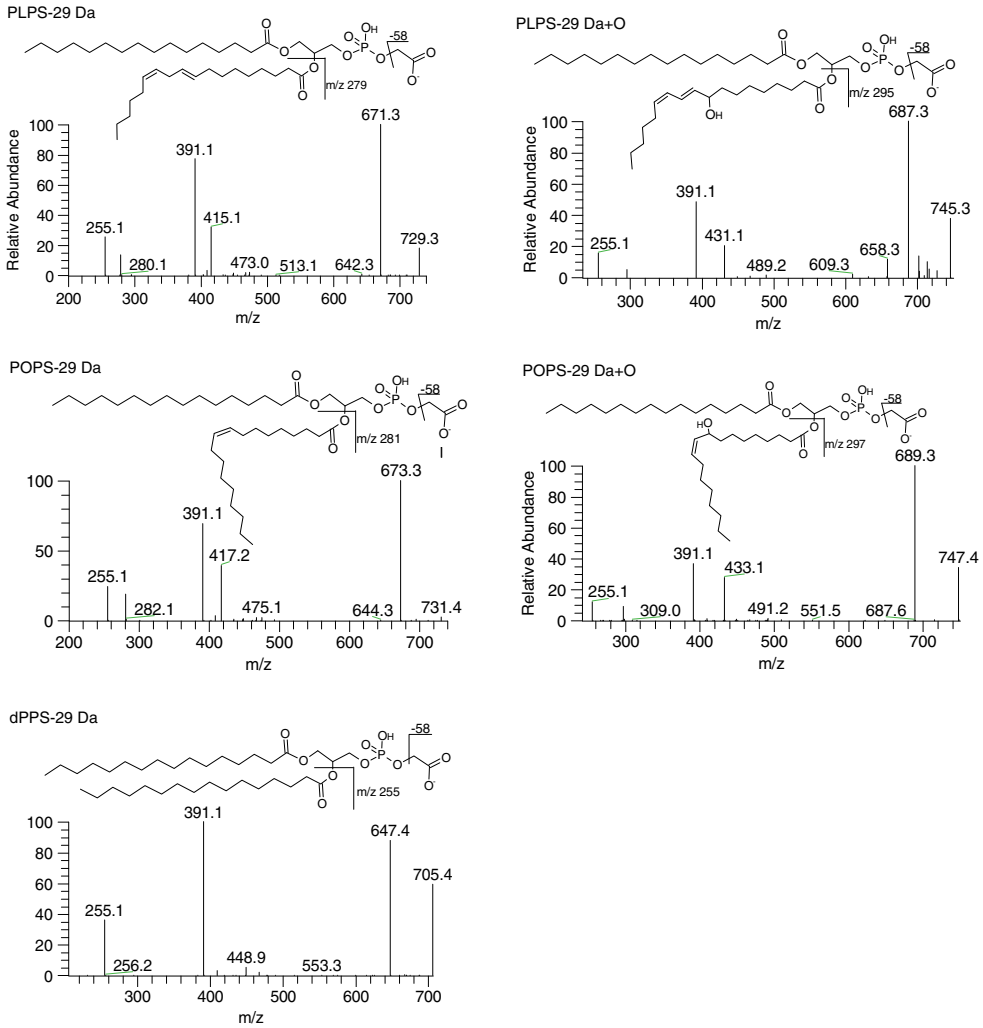
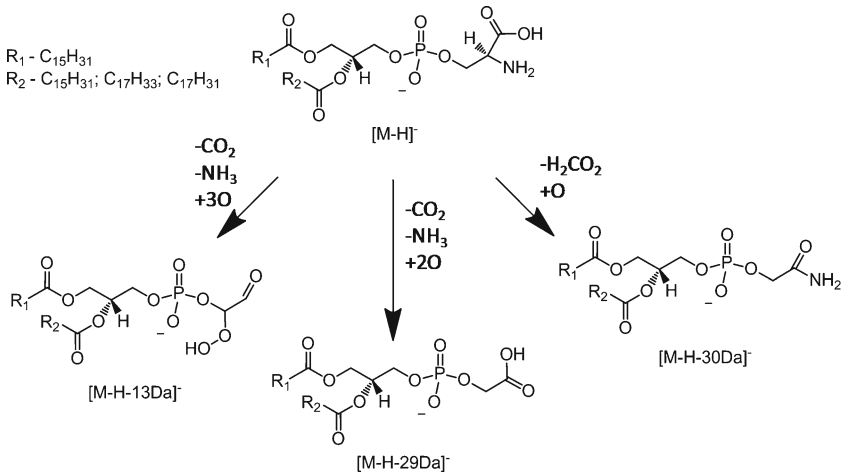


Figure 4. ESI-M/MS spectra of $[M - H - 29]^-$ molecular ions that resulted from oxidation of PLPS (*m/z* 729 and 745), POPS (*m/z* 731 and 747), and DPPS (*m/z* 705), found in spots #2. The proposed structures of each molecular ion are also shown. The structures of the oxidation products PLPS – 29 Da + O and POPS – 29 Da + O illustrate one possible location of the hydroxyl group in the unsaturated fatty acyl chains

contrast with the typical behavior of PS and the other identified PS oxidation products. A minor loss of polar head group resembles the behavior observed in negative mode tandem mass spectra of PE. Also, as observed in PE tandem mass spectra, R_1COO^- ions were found to be more abundant than R_2COO^- ions. This is in contrast with the typical behavior of PS and other oxidation products with a terminal carboxylic acid function, which show in their MS/MS spectra that RA of $R_2COO^- > R_1COO^-$ [37]. These findings also contributed to proposing the presence of a free amine moiety in these oxidation products (M-30 Da). We propose that oxidation products observed in spots #3 are glycerophosphoacetamide derivatives (Scheme 1) (Figure 5).

The MS/MS spectra of $[M - H - 13\ Da]^-$ molecular ions that resulted from oxidation of DPPS and POPS (Figure 6) and were found in spots #5 (Table 2), are very similar. In these spectra, we observe a major fragment ion formed by loss of 32 Da ($-O_2$), indicating the presence of a hydroperoxide [28, 38]. Due to the fact that DPPS does not oxidize in the fatty acyl chain, the hydroperoxide moiety should be located in polar head group. We propose that these oxidation products resulted from oxidation in the polar head group, with formation of terminal hydroperoxydeacetaldehyde, as shown in Table 2. The presence of a modified polar head group is also evidenced by the presence of carboxylate anions of the non-modified fatty acyl chains in the MS/MS spectra and the observation of a neutral loss of 74 Da (hydroperoxydeacetaldehyde). The MS/MS spectra of POPS (*m/z* 747) found in spots #5* also shows oleoyl carboxylate anion + 16 (*m/z* 297) and loss of 58 Da ($CH_2(OH)HC{=}O$). This suggests the presence of an isobar originated by oxidation of the polar head group, with formation of a



Scheme **1.** Oxidation products of PS formed by Fenton reaction with observed modifications on the polar head group: terminal hydroperoxyacetaldehyde (–13 Da), terminal acetic acid (–29 Da), and terminal acetamide (–30 Da)

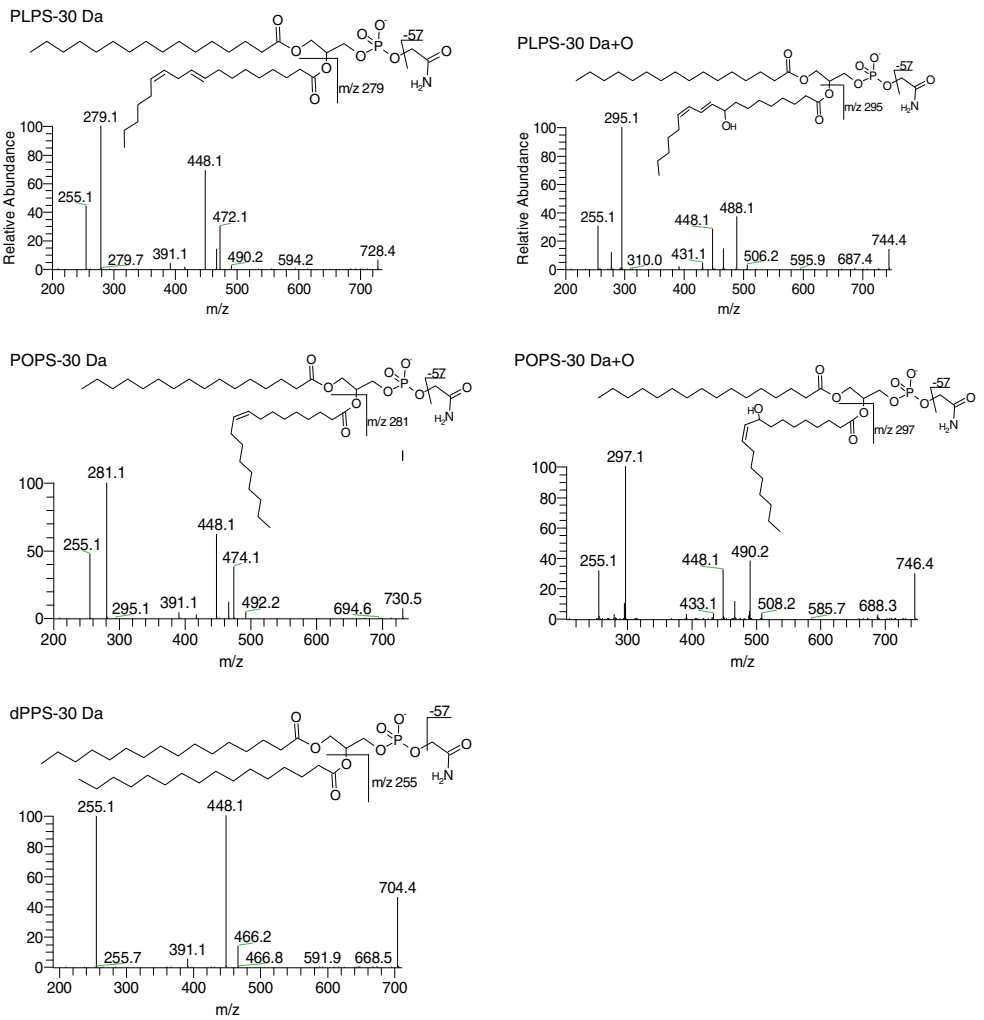


Figure 5. ESI – MS/MS spectra of $[M - H - 30]^-$ ions that resulted from oxidation of PLPS, POPS and DPPS, found in spots #4. The proposed structures of each molecular ion are also shown

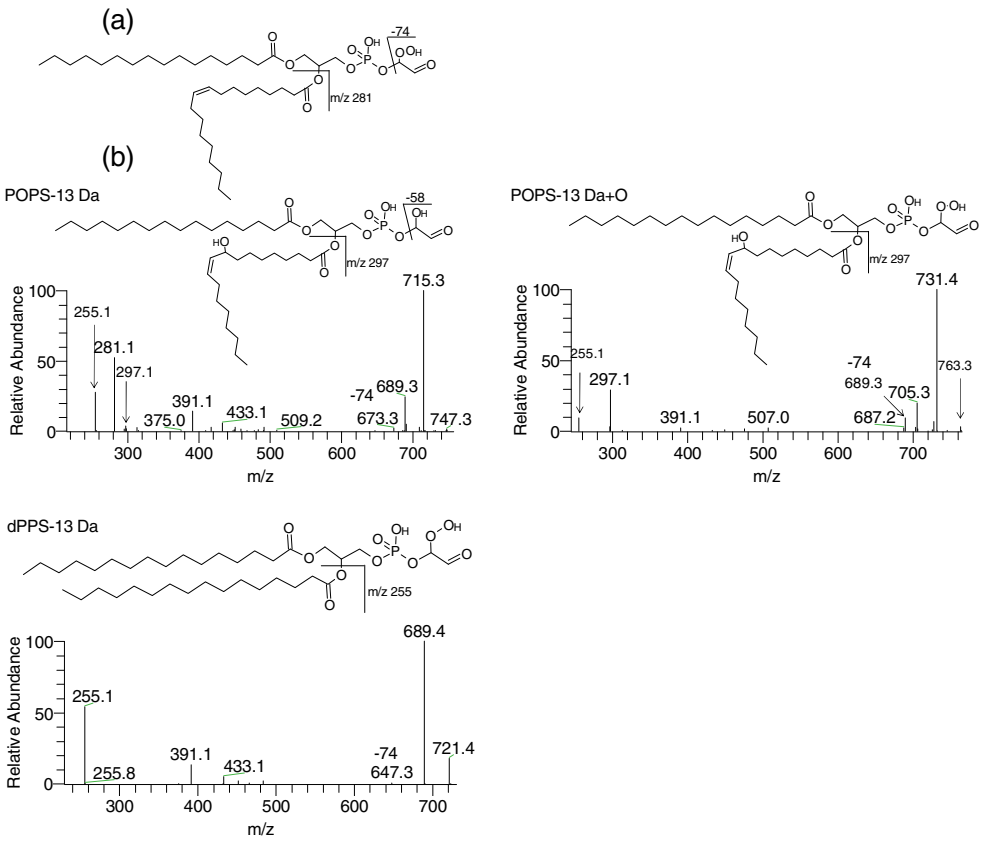


Figure 6. ESI-M/MS spectra of the $[M - H - 13]^-$ ions that resulted from oxidation of POPS (*m/z* 747 and 763) and DPPS (*m/z* 721), found in spots #5. The proposed structures of each molecular ion are also shown

species containing a terminal hydroxyacetaldehyde, and a hydroxylated acyl chain (Figure 6a). The ion at *m/z* 763, observed in POPS spots #5, corresponds to a species with a hydroperoxydeacetaldehyde polar and a hydroxylated acyl chain, as suggested by the fragment ion at *m/z* 297 $[RCOO + O]^-$ and a neutral loss 74 Da (Figure 6b).

Phosphatidylserine polar head group has a serine amino acid moiety linked to the phosphate group It is well known that amino acids are prone to oxidation, not only in the side chains but also in the alfa carbon [36, 39, 40]. Oxidative modification of the PS polar head group has been reported to occur during oxidation induced by HClO and catalyzed by myeloperoxidase [41, 42], confirming the reactivity of serine under oxidative conditions. The oxidation products reported to be formed in those conditions were not the same as the ones reported in this work. In those works, the authors reported the formation of 1,2dipalmitoyl-*sn*-glycero-3-phosphoacetaldehyde and 1,2dipalmitoyl-*sn*-glycerol-3-phosphonitrile derivatives.

The changes of the serine polar head group induced by the hydroxyl radical resemble the oxidative modifications of the amino acid structure, such as the oxidative decarboxylation with formation of an additional keto group (oxidation products with −30 Da). Decarboxylation of amino acids is a well-known reaction occurring during amino acid and peptide oxidation. Loss of CO_2 from the C terminal is due to β-scission of an alkoxyl radical at the C terminal alpha carbon. This reaction is proposed to be initiated from the abstraction of hydrogen linked to an alfa carbon, generating a tertiary radical that is stabilized by the amine nitrogen and carbonyl group [40, 43].

The modification in the PS polar head group leading to the formation of the oxidation products with less 29 Da should occur by decarboxylation plus deamination with formation of a terminal carboxylic acid moiety (oxidation products with −29 Da). Loss of amine and CO_2 resembles typical modification already observed during amino acid oxidation, as reviewed elsewhere [36, 39].

Conclusions

Oxidative modifications induced to phosphatidylserine were identified and characterized by thin layer chromatography combined with tandem mass spectrometry. Oxidation products were fractionated by TLC and further characterized by MS/MS. This approach allowed the identification of hydroxyl, peroxy, and keto derivatives due to oxidation of unsaturated fatty acyl chains. Additionally, we have identified for the first time five families of oxidation products, with lower molecular weight than the non-modified PS, which resulted from oxidative modifications on the serine polar head group. They were identified as $[M - 29 - H]^-$

(terminal acetic acid), $[M - 30 - H]^-$ (terminal acetamide), $[M - 13 - H]^-$ (terminal hydroperoxideacetaldehyde), and $[M - 29 - H]^-$ (terminal hydroxyacetaldehyde). Phosphatidic acid derivatives were also formed as result of PE oxidation. Hydroxy, keto, peroxy, and short chain acyl derivatives of these new molecules were also identified for PS phospholipids with unsaturated fatty acyl chains.

The results presented here allowed the identification, for the first time, of modification of PS polar head group induced by the hydroxyl radical mediated oxidation in vitro conditions. The identification of oxidation products of PS is far from being completely explored, although it is essential to understanding the relation between the PS oxidation products formed and the specific biological activity that they mediate.

Acknowledgments

The authors acknowledge Fundação para a Ciência e a Tecnologia and COMPTE for funding of projects PTDC/QUI- BIQ/104968/2008, REDE/1504/REM/2005 (that concerns the Portuguese Mass Spectrometry Network) and Ph.D. grants to E.M. (SFRH/BD/73203/2010) and to C. S. (SFRH/BD/46293/2008).

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