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Identification of free radicals of glycerophosphatidylcholines containing ω-6 fatty acids using spin trapping coupled with tandem mass spectrometry

A. REIS, P. DOMINGUES, A. J. V. FERRER-CORREIA, & M. R. M. DOMINGUES

Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal

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Abstract
Metal-catalysed radical oxidation of diacyl-glycerophosphatidylcholines (GPC) with ω-6 acyl polyunsaturated fatty acids (PAPC, palmitoyl-arachidonoyl-glycerophosphatidylcholine and PLPC, palmitoyl-lineloyl-glycerophosphatidylcholine) was studied. Free radical oxidation products were trapped by spin trapping with 5,5-dimethyl-1-pyrrolidine-N-oxide (DMPO) and identified by electrospray mass spectrometry (ES-MS). The spin adducts of oxidised GPC containing one and two oxygen atoms and one and two DMPO molecules were observed as doubly charged ions. Structural characterisation by tandem mass spectrometry (MS/MS) of these ions revealed product ions corresponding to loss of the acyl chains (*sn*-1-palmitoyl and *sn*-2-oxidised spin adduct of lineloyl or arachidonoyl), loss of the spin trap (DMPO) and product ions attributed to oxidised *sn*-2 fatty acid spin adduct (lineloyl and arachidonoyl). Product ions formed by homolytic cleavages near the spin trap and also from 1,4 hydrogen elimination cleavages involving the hydroxy group in the *sn*-2 fatty acid spin adduct allowed to infer the nature of the radical. Altogether, the presence of GPC hydroxy-alkyl/DMPO and hydroxy-alkoxyl/DMPO spin adducts was proposed.

Keywords: *Phospholipids, carbon-centred radicals, oxygen-centred radicals, spin trapping, tandem mass spectrometry*

Abbreviations: *ESI, electrospray ionisation; ESR, electron spin resonance; HPLC, high performance liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; DMPO, 5,5-dimethyl-1-pyrrolidine-N-oxide; POBN, α-(4-pyridil-1-oxide)-*N-tert-*butylnitrone; PUFA, polyunsaturated fatty acids; ROS, reactive oxygen species; GPC, glycerophosphatidylcholines; POPC, 1-palmitoyl-2-oleoyl-3-glycerophosphatidylcholine; PLPC, 1-palmitoyl-2-lineloyl-3-glycerophosphatidylcholine; PAPC, 1-palmitoyl-2-arachidonoyl-3-glycerophosphatidylcholine*

Introduction

Oxidative damage of biomolecules triggered by free radicals, such as reactive oxygen species (ROS) formed during aerobic metabolism, is associated with the pathogenesis of several age-related diseases and also with atherosclerosis, lung and liver cancer [1] and for this reason much effort has been dedicated to the identification of the structural changes induced by free radicals in lipids, proteins and DNA bases [1]. Free radical species are very reactive species and due to their very short lives are difficult to detect and analyse [2] and so the work published is mainly focused on the identification of more chemically stable non-radical products of biomolecules [3]. On the other hand, the addition of a nitroso or nitrone compound (spin trap), with formation of spin-adducts, stabilises the free radicals making their detection possible [2]. In fact, this approach has been used for the mass spectrometric identification of carbon and/or oxygen centred radicals of amino acids [4], proteins [5] and lipids [6,7]. Phospholipids, found

Correspondence: M. R. M. Domingues, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal. Tel: 351 234 370698. Fax: 351 234 370084. E-mail: mrd@dq.ua.pt

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as major components of cell membranes provide the physical barrier between different organelles and cells and are also susceptible to free radical attack. Although, the structural changes induced by free radicals to phospholipids with formation of radical and non-radical oxidation products [8–10], are reported to affect the physical properties of membranes [11–13], the identification of phospholipid free radicals is scarce [8,14]. The detection of phospholipid free radicals was initially attempted through ESR with the identification of phosphatidylcholine spin adducts [14] and through the hyperfine coupling constants was proposed to correspond to a carbon centred spin adduct. However, the ESR data does not provide detailed structural information about the substitutents nor the location of the spin trap along the carbon chain of the acyl residues present in the phospholipids. Information about the point of radical location can be proposed based on the tandem mass spectrometry (MS/MS), which is already described for lipid spin adducts [15,16] and recently applied in the identification of 5,5-dimethyl-1-pyrrolidine-N-oxide (DMPO) spin adducts of palmitoyl-oleoyl-glycerophosphatidylcholine (POPC), a structurally simple GPC [8]. The results obtained by MS/MS also gave information about the hydroxy groups present (or not) in the fatty acid chain, allowing proposing the presence of both carbon and oxygen centred adducts [8].

In the present work, DMPO spin trapped radicals of glycerophosphatidylcholines (GPC) containing ω-6 lipids at the *sn*-2 residue, namely palmitoyl-lineloyl-glycerophosphatidylcholine (PLPC) and palmitoyl-arachidonoyl-glycerophosphatidylcholine (PAPC), formed by metal-catalysed radical oxidation, were studied by electrospray ionisation mass spectrometry (ES-MS). The ions observed in the mass spectra were further characterised by MS/MS in order to determine the type of radical formed and their location along the unsaturated fatty acid chain.

Experimental

Chemicals

GPC (16:0/18:2 and 16:0/20:4) and DMPO were obtained from Sigma (St Louis, USA) and used without further purification. Iron (II) chloride ($FeCl_2$) and hydrogen peroxide (H_2O_2) used for the peroxidation reaction were purchased from Merck (Darmstadt, Germany).

Preparation of GPC vesicles

Vesicles were prepared from stock solutions, of 1 mg/ml dried under nitrogen stream and ammonium bicarbonate buffer solution (pH 7.4) was added to a final phospholipid concentration of 50 mM and the mixture vortexed.

Oxidation of GPC vesicles by Fenton reaction

Oxidative treatments performed on the GPC were done by addition of 5 mmol $FeCl_2$ solution and 50 mmol H_2O_2 to 50 μl of phospholipid vesicles in 0.5 ml of solution. This mixture was left to react at 37°C for different periods of time with occasional sonication. Spin trapping experiments were performed by adding 1 μl (9 mmol) of DMPO to the reaction mixture 30–60 min after the reaction was initiated. Mass spectra of control reactions (3) performed in the absence of hydrogen peroxide, spin trap (DMPO) and $FeCl_2$, were acquired and none of identified ions attributed to DMPO adducts of phospholipids oxidation products were observed. The phospholipid oxidation products and the spin adducts were extracted using the Folch method with chloroform:methanol (2:1, v/v) [17].

ES mass spectrometry

Positive ion mode ES mass spectra and MS/MS were acquired in a Q-TOF 2 instrument (Micromass, Manchester, UK) using a MassLynx software system (version 4.0, Micromass, Manchester, UK). The samples for electrospray analyses were prepared by diluting 2 μl of the sample in 1000 μl of chloroform: methanol solution (1:1, v/v). Samples were introduced into the mass spectrometer using a flow rate of 10 μl/min, setting the needle voltage at 3000 V with the ion source at 80°C and cone voltage at 30 V. MS/MS of molecular ions were obtained by collision-induced decomposition (CID), using argon as the collision gas (measured pressure in the penning gauge ($\sim 6 \times 10^{-6}$ mbar) and varying collision energy between 15–25 eV. In MS and MS/MS experiments, TOF resolution was set to approximately 9000 (FWHM).

Results and discussion

Characterisation of doubly charged ions of PLPC/DMPO spin adducts

The formation of palmitoyl-lineloyl-GPC free radicals by metal catalysed oxidation (Fenton reaction) coupled with DMPO spin trapping was studied by MS. The ES mass spectra of PLPC obtained in the presence of DMPO, (A) under non-oxidative conditions ($PLPC + Fe^{2+}$) and (B) under oxidative conditions ($PLPC + Fe^{2+} + H_2O_2$) are shown in Figure 1. By comparison of the mass spectra in the *m*/*z* range of 450–530, some new ions with *m*/*z* 455.4 and 463.4 are observed and can be assigned as doubly charged $[MH + Na]^{2+}$ ions of PLPC/DMPO adducts containing one and two oxygen atoms, respectively (Scheme 1). The structures shown in Scheme 1 are one of the possible structures that may be formed. Doubly charged ions observed of *m*/*z* 511.9

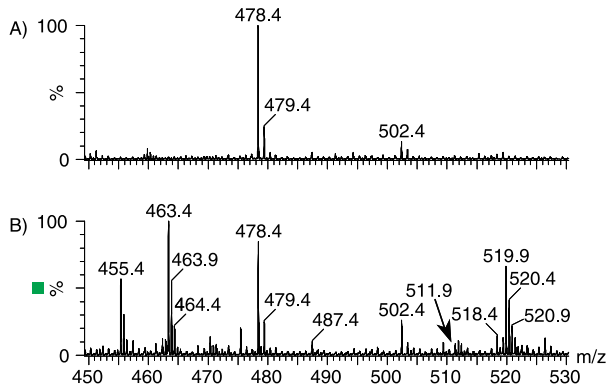
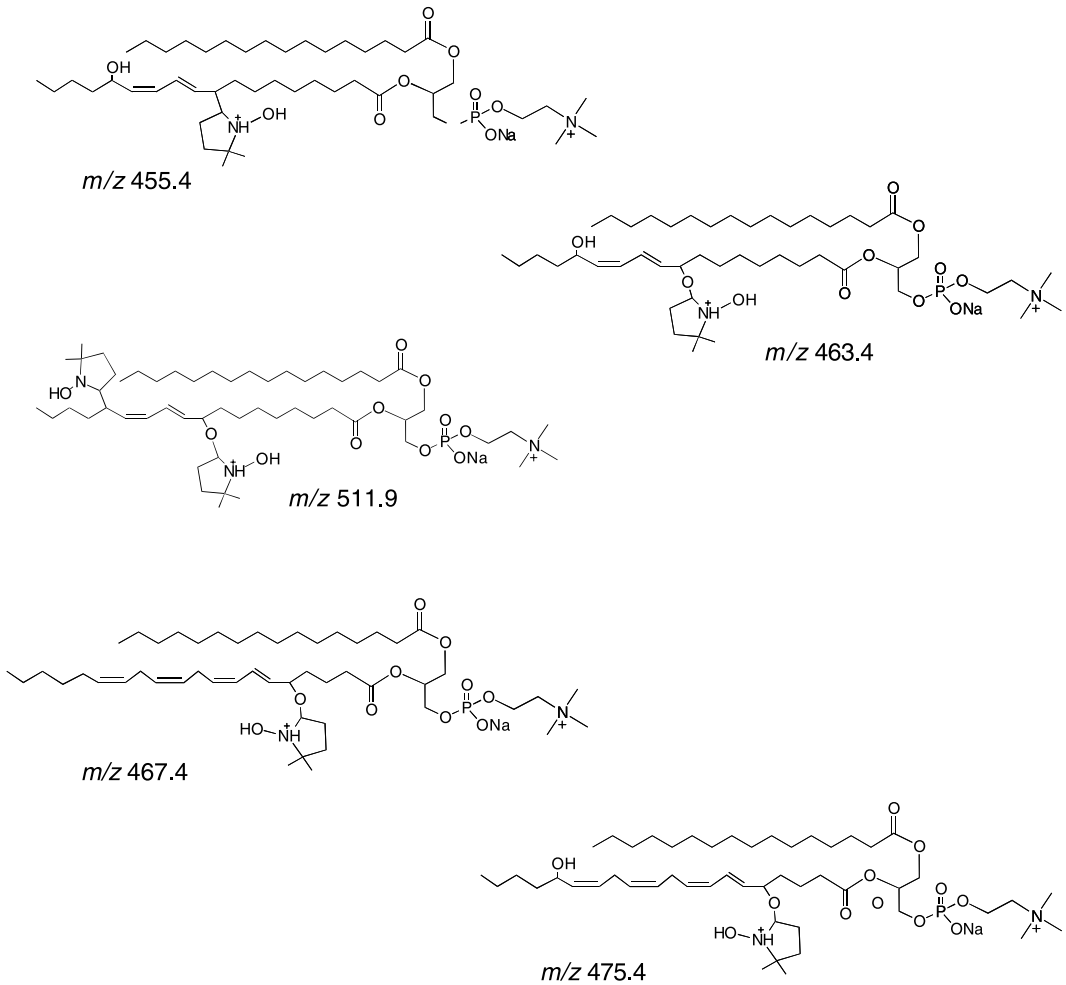


Figure 1. Mass spectra of PLPC vesicles obtained under non-oxidative conditions (A) and in the presence of H_2O_2 + Fe(II) + DMPO (B). The doubly charged ions correspond to PLPC/DMPO spin adducts.

(Scheme 1) and 519.9 were attributed to the ions corresponding to DMPO spin adducts containing two DMPO molecules of intact PLPC with one and two oxygen atoms, respectively, similarly as was reported during the identification of DMPO spin adducts of intact POPC [8]. Other ions observed with very low relative abundance of *m/z* 471.4 and *m/z* 941.8 (data not shown) were attributed, respectively, to sodiated doubly charged and sodiated mono charged ions of DMPO spin adducts of intact PLPC containing three oxygen atoms. However, the low relative abundance of these ions limited the acquisition of MS/MS. The identification of mono and doubly charged ions of oxidised intact GPC is in accordance with previous work [8] performed on the identification of POPC/DMPO spin adducts where doubly charged ions exhibited higher relative abundance over the singly charged ions. In order to identify the free radicals of oxidised PLPC, detailed structural information was obtained by performing MS/MS (product ion scanning) on the most abundant doubly charged ions.

The product ion spectra can either exhibit singly charged product ions, formed by loss of charged molecules and doubly charged ions formed by loss of neutral molecules. The product ion spectra obtained for the doubly charged ions of *m/z* 455.4 and 463.4 and depicted in Figure 2, exhibit abundant singly charged product ions of *m/z* 478.4 and 502.4



Scheme 1. Proposed structures for the doubly charged (A) PLPC/DMPO spin adducts (*m/z* 455.4 and 463.4) and (B) PAPC/DMPO spin adducts (*m/z* 467.4 and 475.4).

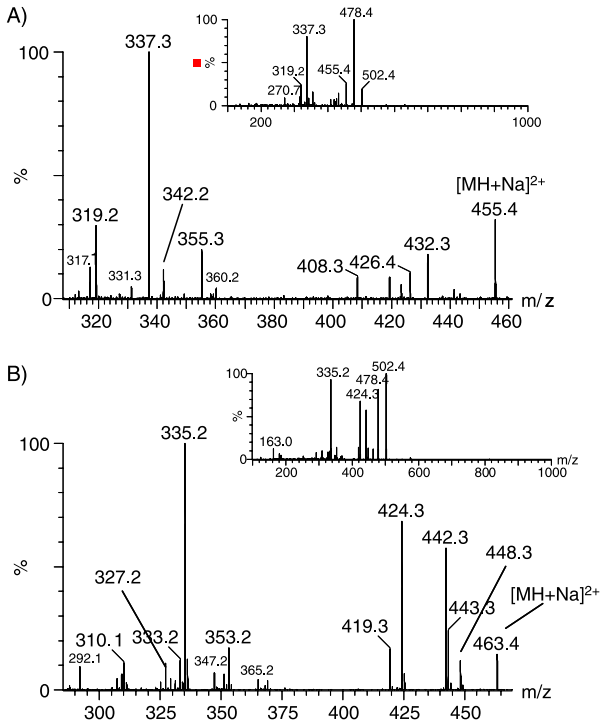


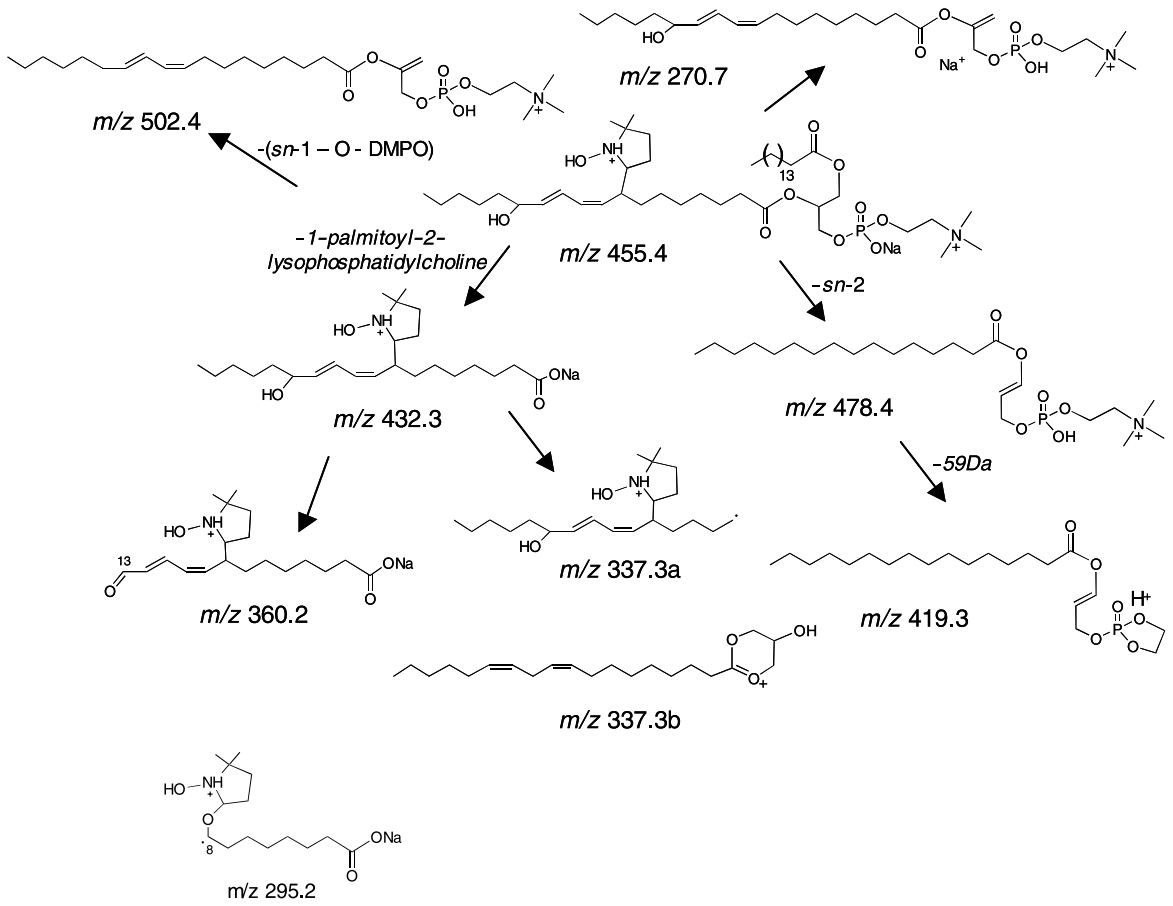
Figure 2. Product ion spectra of doubly charged ions of PLPC/DMPO spin adducts containing (A) one oxygen atom of *m/z* 455.4 (Scheme 2) and (B) two oxygen atoms of *m/z* 463.4 (Scheme 3). Insets were included to visualize the complete product ion spectrum.

(Scheme 2) that correspond to the $[MH]^+$ ion of the dehydrated 1-palmitoyl-2-lyso-phosphatidylcholine and 2-lineloyl-1-lyso-GPC, respectively.

The product ion spectrum of the ion of *m/z* 455.4 (Figure 2(A)), which can be assigned to the alkoxyl/DMPO spin adduct or to the hydroxy-alkyl/DMPO spin adduct of the PLPC, exhibits the doubly charged product ion observed of *m/z* 270.7 that was attributed to the [2-lineloyl-1-lyso-phosphatidylcholine(H) + Na]$^{2+}$ ion (Scheme 2), containing the oxygen atom most likely at the lineloyl moiety. This product ion was also observed, with very low relative abundance, as mono charged product ion with *m/z* 540.4 (data not shown). The presence of the oxygen atom and of the DMPO molecule at the linoleoyl moiety is also evidenced by the singly charged product ion of *m/z* 432.3 $[R_2(O)COONa/DMPO + H]^+$ (Figure 2(A)). This product ion of *m/z* 432.3 (Scheme 2), containing both the DMPO molecule and the oxygen atom, can be assigned to the alkoxyl lineloyl spin adduct ($LO^{\cdot}$/DMPO) or to the hydroxy-alkyl lineloyl spin adduct ($L^{\cdot}(OH)$/DMPO). The product ion of *m/z* 502.4 formed by combined loss of R_1COOH and DMPO—OH may suggest the presence of the alkoxyl spin adduct. The loss of the acyl chains (*sn*-1 and *sn*-2 residues) observed here, lost as free fatty acids (R_1COOH and R_2COOH) and ketene ($R_1{=}C{=}O$ and $R_2{=}C{=}O$) in the case of $[MH]^+$ molecular ions, or lost as salts (R_1COONa and R_2COONa) and free fatty acids (R_1COOH and R_2COOH) in the case of $[MNa]^+$ molecular ions, are also observed in the product ion spectra of GPC [18] and GPC oxidation products [19,20].

The product ion spectra of doubly charged POPC spin adducts previously described [8] exhibited charge remote fragmentations, namely homolytic cleavages that were assigned as the result of cleavages occurring in the vicinity of the spin trap. Hence, the product ion of *m/z* 337.3 (structure 337.3a- Scheme 2) may result from homolytic cleavage of the γ-bond relative to the carboxylic group (Scheme 2). This product ion involves the cleavage of $C_3{-}C_4$ bond, already observed in the product ion spectra of fatty acids [16] and of oxidised fatty acids [21] and is a typical high-energy CID fragmentation [16] although, it has also been observed in low-energy CID spectra [21]. Another mechanism involving charge induced fragmentation can also be proposed for the product ion of *m/z* 337.3 considering the charge placed at the oxygen atom of the carbonyl group (structure 337.3b-Scheme 2). Dehydration of the product ion of *m/z* 337.3 may rationalise the product ion of *m/z* 319.2 (observed in Figure 2(A)). The product ion of *m/z* 319.2 may also be attributed to $[R_2(O)COONa + H]^+$ formed by combined loss of DMPO molecule (113 Da) and loss of 1-palmitoyl-lyso-phosphatidylcholine. The odd numbered singly charged product ion observed of *m/z* 295.2 (not shown in Figure 2(A)) may be identified as resultant from cleavage occurring in the vicinity of the spin trap (Scheme 2) allowing proposing the spin trap to be located at C-8. On the other hand, the even numbered singly charged product ion observed of *m/z* 360.2 (Scheme 2) may result from cleavage involving the hydroxy group at C-13. Thus, the structures of the product ions depicted in Scheme 2, are consistent with the presence of the hydroxy derivative of the carbon centred (alkyl at C-13) spin adduct with the hydroxy group placed at C-8 (Scheme 2).

The product ion spectrum of ion of *m/z* 463.4 (Figure 2(B)) shows the typical fragmentation behaviour described for the doubly charged ions of GPC DMPO spin adducts, with abundant product ions attributed to the lyso-phosphatidylcholines of *m/z* 502.4 and 478.4 formed by loss of the *sn*-1 and the oxidised *sn*-2 fatty acid/DMPO, respectively. Also, the singly charged product ions of *m/z* 419.3 and 443.3 (Figure 2(B)) formed by loss of the oxidised *sn*-2 and *sn*-1 residues, respectively, combined with loss of $N(CH_3)_3$ are observed. The product ion of *m/z* 448.3) gives information regarding the *sn*-2 acyl residue which is attributed to the sodium salt of the oxidised linoleic acid spin adduct $[(OO)R_2COONa/DMPO + H]^+$ (Figure 2(B)). The product ion of *m/z* 448.3 can result from the contribution of the hydroperoxide-alkyl, the hydroxy-alkoxyl or the peroxyl DMPO lineloyl spin adduct. Product ions resulting from loss

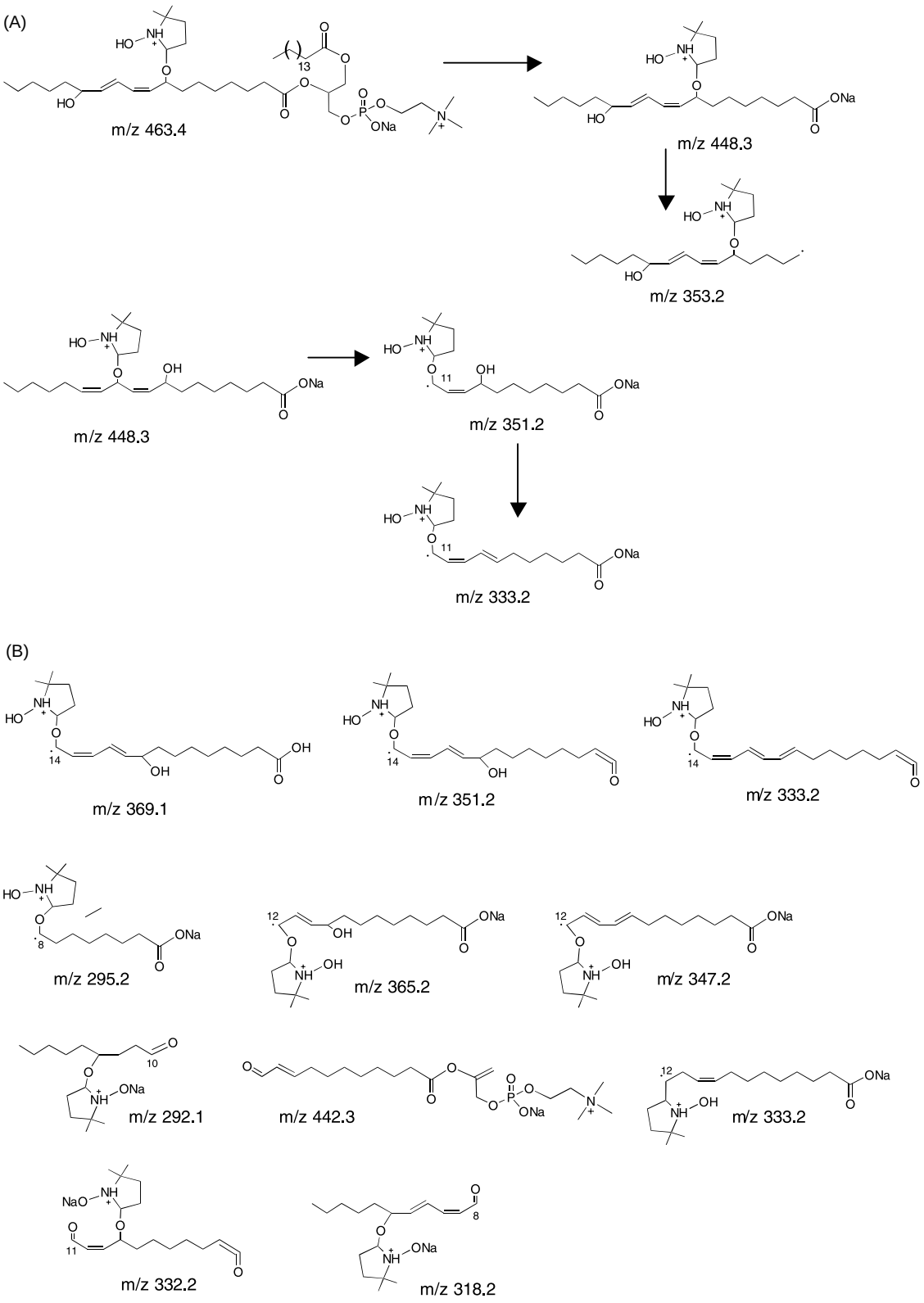


Scheme 2. Proposed structures for the product ions observed in the product ion spectra of ion of *m/z* 455.4.

of the acyl chains, of the spin trap and of homolytic cleavages occurring at the γ-bond relative to the carboxylic group (*m/z* 353.2, Scheme 3(A)) are fragmentations that do not provide information regarding the location of either the spin trap or the hydroxy groups along the unsaturated acyl chain, as they may be present deriving from either the hydroperoxide-alkyl, the hydroxy-alkoxyl or the peroxyl DMPO lineloyl spin adduct. However, the product ions of *m/z* 369.1, 365.2, 351.2, 347.2 and 333.2 (Figure 2(B)), occurring with minor relative abundance may be assigned to cleavages in the vicinity of the spin trap (Scheme 3(A) and (B)) and are consistent with the presence of the spin adducts at C-8, C-11, C-12 or C-14. Also, the even numbered product ion observed of *m/z* 292.1 (Figure 2(B) evidences the presence of the hydroxy group at C-10 (Scheme 3(B)), although the product ion of *m/z* 442.3 suggests also the hydroxy placed at C-11 (Scheme 3(B)). The product ions described are consistent with the presence of the hydroxy derivative of the oxygen centred spin adduct (hydroxy-alkoxyl/DMPO).

Nonetheless, the product ion of *m/z* 163.0 also observed in the spectrum (Figure 2(B)), which exhibits a 16 Da mass increase relative to the sodiated five-membered cyclophosphane ring (*m/z* 147), suggests that the hydroxy group is also at the phosphocholine head and therefore an additional isomer containing the oxidised polar head must be considered. In this case, the alkyl radical may be considered at C-12, as suggested by the production of *m/z* 333.2 (Scheme 3(B)).

The production spectra obtained for the PLPC/DMPO sodiated doubly charged $[MH + Na]^+$ spin adducts containing two DMPO molecules with *m/z* 511.9 and 519.9 (insets in Figure 3(A) and (B)), exhibited loss of one uncharged DMPO molecule leading to the product ions with *m/z* 455.4 and 463.4, respectively. Both product ions (*m/z* 455.4 and 463.4) are observed in the respective product ion spectrum and seem to be a first step of the fragmentation pattern of these sodiated doubly charged spin adducts, providing no additional structural information on the location of the second DMPO molecule. This behaviour resulted in product-ion spectra very similar to the product-ion spectra of *m/z* 455.4 and 463.4 previously described. In the case of POPC/DMPO spin adducts, the presence of the second DMPO together with the presence of the product ion of *m/z* 130.1 $[DMPO—OH + H]^+$ allowed to propose that the spin adduct containing one oxygen atom and two DMPO molecules (*m/z* 512.9) [8] was simultaneously carbon- and oxygen-centred spin adduct. In the case of ion of *m/z* 511.9 (Figure 3(A)), the doubly charged



Scheme 3. Proposed structures for the product ions observed in the product ion spectra of ions of *m*/*z* 463.4.

product ion of *m*/*z* 398.8, attributed to loss of both DMPO molecules from the precursor ion, does not provide structural information regarding the location of the spin trap nor to the nature of the spin adduct. However, in the case of ion of *m*/*z* 519.9 (Figure 3(B)), the product ions of odd *m*/*z* observed with minor relative abundance of odd *m*/*z* 353.2 may result from γ-bond cleavage (structure in Scheme 3(A)) with

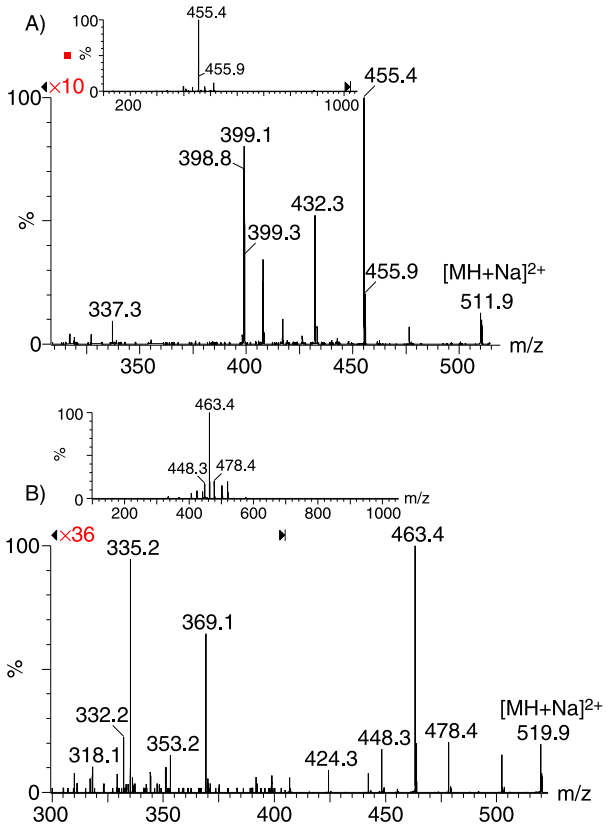


Figure 3. Product ion spectra of doubly charged ions of PLPC/DMPO spin adducts containing two DMPO molecules and (A) one oxygen atom of *m/z* 511.8 and (B) two oxygen atoms of *m/z* 519.9. Insets were included to visualize the complete product ion spectrum.

dehydration (*m/z* 335.2) and the product ions of *m/z* 369.1 and 351.2 (Figure 3(B)) suggest the presence of the spin trap at the C-14 (Scheme 3(B)), while even numbered product ions of *m/z* 318.1 and 332.2 (Figure 3(B)) provided evidence for the presence of the hydroxy group at C-8 and C-11 respectively (Scheme 3(B)).

Characterisation of doubly charged ions of PAPC/DMPO spin adducts

The ES mass spectra obtained for the extracts containing oxidised PAPC in the presence of DMPO (Figure 4(B)) was plotted against the native PAPC in the presence of DMPO (Figure 4(A)). The presence of ions of *m/z* 467.4 and 475.4 were attributed to doubly charged ions of PAPC/DMPO spin adducts containing one and two oxygen atoms, respectively (Scheme 1). The ion with *m/z* 965.7 (data not shown) was also observed although with low relative abundance and may be assigned to the singly charged of the spin adduct PAPC/DMPO containing three oxygen atoms. The ions with *m/z* 523.8 and 531.9 were also observed and may be attributed to doubly charged ions of PAPC spin adducts with one and two

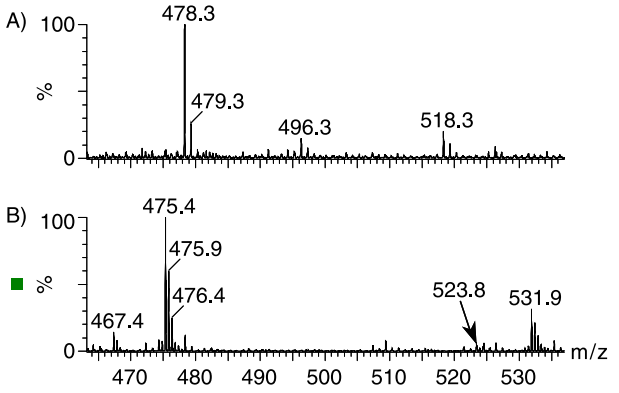


Figure 4. Mass spectra of PAPC vesicles obtained (A) under non-oxidative conditions and (B) in the presence of H_2O_2 + Fe(II) + DMPO. The doubly charged ions correspond to PAPC/DMPO spin adducts.

oxygen atoms containing two DMPO molecules, similar to previous results.

MS/MS obtained for the ions of *m/z* 467.4 (Figure 5(A)) and 475.4 (Figure 5(B)), exhibit product ions formed by similar fragmentation pathways, namely the product ions with *m/z* 478.3 and 526.3 were attributed to the dehydrated $[MH]^+$ ion of the 1-palmitoyl-lyso-phosphatidylcholine and 2-arachidonoyl-lyso-phosphatidylcholines, respectively. Also, the doubly charged product ions observed with

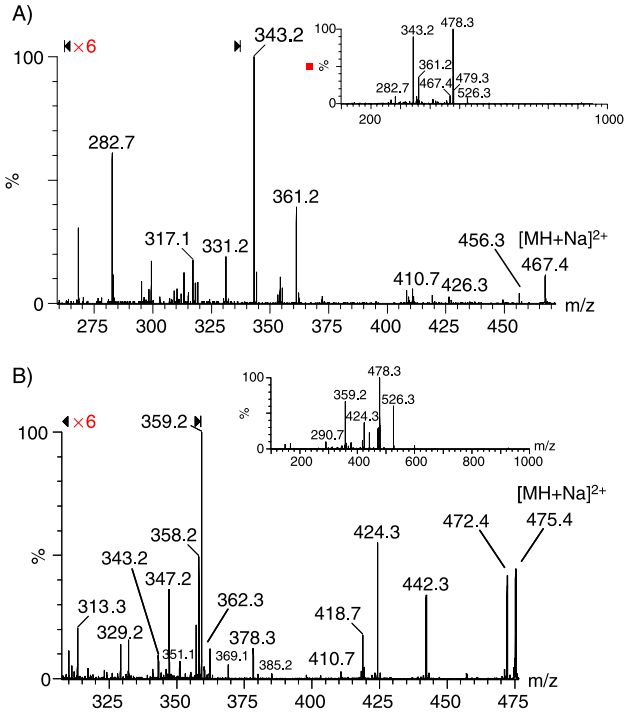
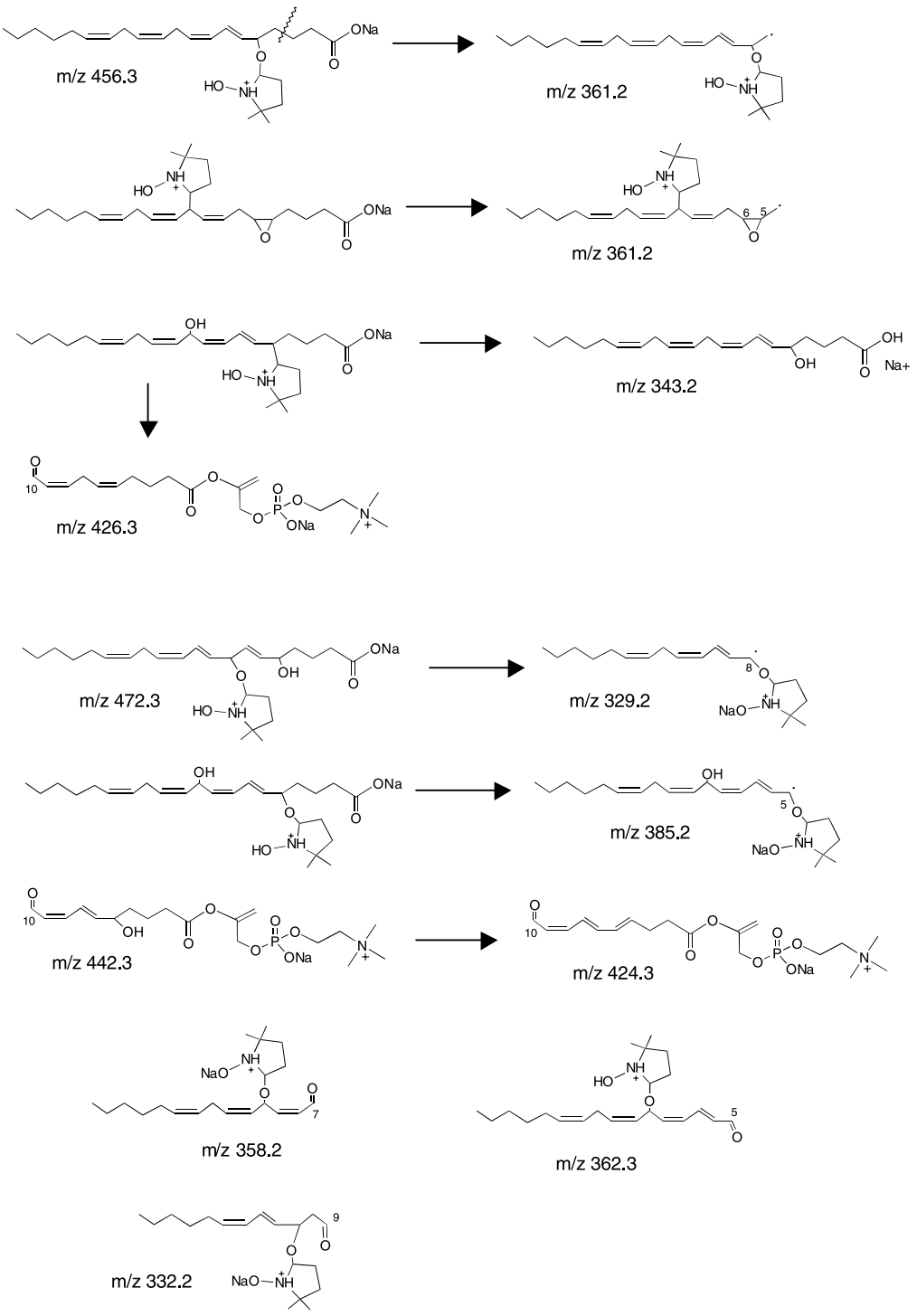


Figure 5. Product ion spectra of doubly charged ions of PAPC/DMPO spin adducts containing (A) one oxygen atom of *m/z* 467.4 (Scheme 4(A)) and (B) two oxygen atoms of *m/z* 475.4 (Scheme 4(B)). Insets were included to visualize the complete product ion spectrum.



Scheme 4. Proposed structures for the product ions observed in the product ion spectra of ion of *m/z* 467.4.

m/z 282.7 (inset Figure 5(A)) and 290.7 (inset Figure 5(B)) may correspond to the dehydrated [2-arachidonoyl-lyso-phosphatidylcholines(H) + Na]$^{2+}$ ions containing one and two oxygen atoms, respectively, confirming the oxidation at the *sn*-2 acyl residue. Also, the product ion attributed to the unsaturated fatty acid chain (arachidonoyl) containing the DMPO molecule and the oxygen atom(s) are observed of *m/z* 456.3 (Figure 5(A)) and 472.3 (Figure 5(B)). The product ions of *m/z* 410.7 (Figure 5(A)) and 418.7 (Figure 5(B)) correspond to the doubly charged product ions formed by loss of DMPO (113 Da) from the correspondent precursor ions.

In Figure 5(A), the singly charged product ion of *m/z* 361.2 (Scheme 4) is formed by homolytic cleavage of the γ-bond relative to the carboxylic acid, evidencing the presence of the oxygen and DMPO molecule in the *sn*-2 acyl chain. This product ion can

also be due to homolytic cleavage occurring in the vicinity of the epoxy derivative (Scheme 4), as is described to occur for epoxy derivatives [22]. The product ion of *m/z* 343.2 may be attributed to loss of DMPO (Scheme 4) suggesting the hydroxy-alkyl spin adduct. The product ion of *m/z* 426.3 may suggest the hydroxy group at C-10 (Scheme 4).

In Figure 5(B), the product ion observed of *m/z* 410.7 can be assigned to the doubly charged product ion formed due to loss of $[DMPO{-}OH + H]^+$(−130 Da) from the precursor ion. Also, the loss of the *sn*-1 residue as free fatty acid (R_1COOH) is observed combined with loss of DMPO (−113 Da) leading to the doubly charged product ions of *m/z* 290.7. Product ions observed of odd *m/z* 359.2 and 343.2 (Figure 5(B)) may be attributed to loss of DMPO (113 Da) and DMPO—OH (129 Da) combined with loss of 1-palmitoyl-2-lyso-phosphatidylcholine from the precursor ion, while the product ions of *m/z* 385.2 and 329.2 (Scheme 4) formed by homolytic cleavages in the vicinity of the spin trap at the C-5 and C-8, respectively, suggesting the presence of the hydroxy-alkoxyl spin adduct. The even numbered product ions of *m/z* 442.3 and 424.3 (hydroxy at C-10), 362.3 (hydroxy at C-5) and 358.2 (hydroxy at C-7) (Scheme 4) may result by 1,4-hydrogen elimination mechanism and allow proposing the hydroxy group in different locations. The product ion resulting from homolytic cleavage of the γ-bond relative to the carboxylic group (*m/z* 377.3) was not observed in the product ion spectra, as observed in the previous DMPO spin adducts [8]. Altogether, the product ions point out to the predominance of the hydroxy-alkoxyl-arachidonoyl spin adduct to the relative abundance of the spin adduct of PAPC.

The product ions of *m/z* 408.3 and 426.4 were observed in the product ion spectra of phospholipid spin adducts containing one oxygen atom, namely PAPC (*m/z* 463.4-Figure 5(A)), PLPC (*m/z* 455.4-Figure 2(A)) and POPC (*m/z* 456.4, [8]) and can also be attributed to the palmitoyl-glycerol ion with the DMPO molecule attached most probably at one of the carbon atoms of the glycerol moiety since saturated acids are resistant to radical oxidation. Also, the product ions of *m/z* 442.3 and 424.3 observed in the product ion spectra of phospholipid spin adducts containing two oxygen atoms, namely for PAPC (*m/z* 475.4-Figure 5(B)), PLPC (*m/z* 463.4-Figure 2(B)) and POPC (*m/z* 464.4, [8]) and can also be attributed to the palmitoyl-glycerol ion with one oxygen atom and the DMPO molecule. These structures were never proposed before, although oxidation in other points of the phosphatidylcholine molecule may be susceptible to undergo radical oxidation, as was recently proposed to occur at the phosphocholine polar head [20], which may occur by a mechanism similar to the radical oxidation of amino acids and proteins [23] by hydrogen abstraction from the α-carbon atom of amino acids and proteins and also from aliphatic side chains [23].

The identification in this study of intact oxidised PAPC free radicals centred at C-5, C-8 and C-10 positions of the arachidonoyl chain and considering that the arachidonoyl moiety contains 3 bis-allylic hydrogen atoms each equally susceptible to be removed during non-enzymatic radical oxidation [2], suggests that the C-7 bis-allylic hydrogen atom is most accessible to be abstracted by the hydroxyl radical. The bis-allylic hydrogen atom at C-7 is the one closer to the glycerol moiety. Similar conclusions were drawn through the attack of the thiyl radical ($RS^{\cdot}$) during the study of *cis–trans* isomerisation of PUFA in diacyl-GPC large unilamelar vesicles [24]. The abstraction of the C-7 bis-allylic hydrogen atom generates the alkyl radical centred at C-7 that through isomerisation leads to the alkyl radicals centred at C-5 and C-9. Isomerisation of radical place at C-7 to the C-5 position seems to be favoured due to the predominance of short-chain peroxidation products of PAPC (aldehydes and dicarboxylic terminal groups) with five carbon atoms over others during metal catalysed radical peroxidation [25,26], assuming that no significant differences occur in the ionisation efficiencies of products with various chain lengths.

The product ion spectra of the doubly charged ions with two DMPO molecules containing one (*m/z* 523.8) and two oxygen atoms (*m/z* 531.9) were obtained and particularly the product ion spectrum of ion of *m/z* 531.9 (Figure 6), exhibits the product ion of *m/z* 146.1 $[DMPO{-}OOH + H]^+$ (Figure 6) that suggests the presence of the peroxyl derivative for the PAPC/DMPO spin adduct. In previous studies, performed in the characterisation of linoleic acid DMPO spin adducts, the product ion of *m/z* 146.1 was used together with others to suggest the presence of the peroxyl/DMPO linoleic acid spin adduct [15]. In fact, losses of one and two DMPO molecules from the precursor ion are observed by the doubly charged

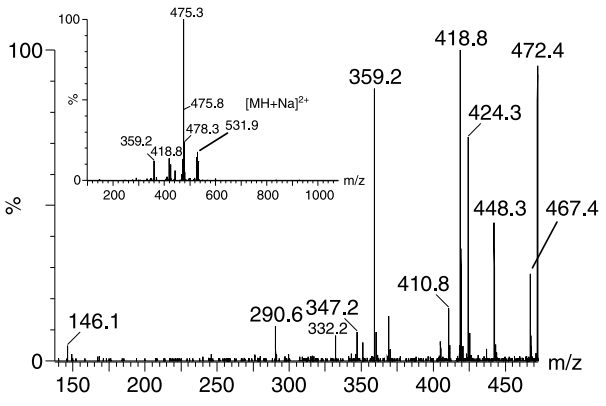


Figure 6. Product ion spectrum of doubly charged ion of PAPC/DMPO spin adducts containing two DMPO molecules and two oxygen atoms of *m/z* 531.9. Inset was included to visualize the complete product ion spectrum.

product ions of *m/z* 475.3 and 418.8 (Figure 6). Also, loss of one DMPO—OH from the precursor ion (*m/z* 467.4) and combined with loss of DMPO molecule at (*m/z* 410.8) are observed as doubly charged ions (Figure 6). Furthermore, product ions observed with low relative abundance of *m/z* 359.2 (loss of DMPO combined with palmitoyl-lyso-phosphatidylcholine) and particularly the product ions of *m/z* 332.2 (hydroxy at C-9) is consistent with the additional contribution of the hydroxy-alkoxyl derivative.

The GPC used in this study contained one saturated fatty acid (palmitic acid), which is resistant to radical oxidation and also linoleic (18:2) and arachidonic acid (20:4), which are ω-6 PUFA. The mass spectra obtained for both GPC (PLPC and PAPC) showed the presence of ions that were assigned to DMPO spin adducts of oxidised intact GPC formed by insertion of up to three oxygen atoms. In the literature, it is acknowledged that the extent of oxidation reaction is related to the number of double bonds (i.e. the number of bis-allylic hydrogen atoms). The presence of 3 bis-allylic hydrogen atoms in the arachidonoyl moiety of PAPC vesicles, would be expected to induce oxidation to a greater extent, when compared to the PLPC vesicles. Thus, the insertion of three oxygen atoms may be rationalized considering that the oxidative damage of PUFA by free radicals may not be strictly related to the number of bis-allylic hydrogen atoms or to the surface area [27], but also to the acyl chain conformation in PC bilayers [28]. However, the identification of radical peroxidation products of PAPC containing up to six oxygen atoms, in previous published results obtained during radical oxidation of PAPC vesicles by the Fenton reaction [20], rules out the hypothesis that acyl chain packing might have influence on the accessibility of ROS into the liposomes, as was proposed earlier to explain the higher oxidative stability shown by PDPC vesicles relative to PAPC vesicles [28]. Another explanation is to consider that the DMPO may have a retarding effect on the lipid peroxidation reaction by trapping the GPC radicals and blocking subsequent reactions with the oxygen, as suggested elsewhere [5,29]. On the other hand, some authors have reported that larger alkyl radicals, as is the case of GPC free radicals, are trapped more slowly by DMPO than the smaller ones, such as $^{\cdot}CH_3$ or $^{\cdot}CH_2CH_3$ [30], due either to spin–spin reactions of larger alkyl radicals [30] or to increasing steric hindrance effects [2]. The rate constants of alkyl radicals with DMPO ($k \sim 10^6$–$10^7\,M^{-1}\,s^{-1}$), although with little variations, are chain length-dependent [30], in detriment of the formation of higher carbon chain lengths alkyl DMPO spin adducts. However, in the present study, MS/MS data supported the presence of both carbon and oxygen centred radicals of GPC, namely of alkoxyl PLPC/DMPO spin adducts placed at the C-9 and C-11 and the presence of alkoxyl PAPC/DMPO spin adducts that can be placed at the C-5 and C-10, the latter formed by abstraction of the C-7 bis-allylic hydrogen atom.

Evidence for the presence of the alkoxyl radical of the dilinoleoyl-glycerophosphatidylcholine (DLPC), was recently proposed during enzymatic and non-enzymatic oxidation, based on the identification of the $^{\cdot}C_5H_{11}$/POBN spin adduct [31], which was interpreted as indicative of the alkoxyl radical located at the C-13 in one of the linoleoyl residues. However, the authors do not mention the presence of the carbon centred spin adduct resultant from the alkoxyl radical placed at C-9. This would also be expected since both 9-alkoxyl and 13-alkoxyl lipid radicals have previously been described during linoleic acid enzymatic oxidation [6,7,32], although the ratios of POBN/$^{\cdot}C_5H_{11}$ vs. POBN/$^{\cdot}C_8H_{15}O_2$ observed during lipoxygenase oxidation were consistent with preferential formation of 13-LO$^{\cdot}$ over 9-LO$^{\cdot}$ [7], which is in accordance with the specificity of the enzyme [33]. Both 9-alkoxyl and 13-alkoxyl lipid radicals were identified as oxidation products of linoleic acid during non-enzymatic metal catalysed oxidation [15,16]. It is assumed that the LO$^{\cdot}$ radicals play a minor role in the propagation steps during lipid peroxidation and instead undergo cyclisation leading to the carbon centred epoxy-alkyl radical (OL$^{\cdot}$) [34]. Thus, cyclisation of the 13-alkoxyl linoleic radical leads to the epoxy carbon centred lipid radical placed at C-9 and after β-scission the epoxy-alkyl radical may lead to the heptanoic radical ($^{\cdot}C_7H_{13}O_2$). Similarly, the 9-alkoxyl linoleic radical would, through the same route, lead to the butyl radical ($^{\cdot}C_4H_9$). Interestingly, the heptanoic acid DMPO spin adduct (*m/z* 244), but not the butyl DMPO spin adduct (*m/z* 172), was identified during the study of linoleic acid radicals by tandem mass spectrometry coupled with liquid chromatography (LC-MS/MS) [16], thus suggesting the presence of the carbon centred radical (epoxy-alkyl) at the C-9. These findings suggest that in the case of linoleic acid, after C-11 bis-allylic hydrogen abstraction, the isomerisation would be preferably towards the formation of the radicals at C-9 over the C-13 position, leading to the more favourable or more stable formation of the C_9 short-chain products. Other studies performed on the identification of peroxidation products of GPC in model liposomes [19] and in oxLDL [35,36] revealed the 1-palmitoyl-2-(9-oxo-nonanoyl)-3-GPC to be one of the major aldehyde products, among the short-chain products with terminal aldehyde at *sn*-2 acyl residues esterified to the 1-palmitoyl-GPC moiety. On the other hand, the predominance of C_9 aldehydes (9-oxo-nonanoyl) over C_{12} aldehydes (12-oxo-dodecenoyl) may be related to the higher ionisation efficiency, which is also dependent on the acyl chain length [37].

In the present study, the mass spectrum of ion of *m/z* 531.9 exhibited the product ion of *m/z* 146.1

identified as $[DMPO{-}OOH + H]^+$, which was earlier used to proposed the presence of the peroxyl linoleic acid DMPO adduct [15] may be corroborated by the product ion of *m/z* 526.4 (data not shown) formed by combined loss of R1COOH and DMPO-—OOH. Although the lipid peroxyl radicals are reported to give unstable spin adducts [38] these have shown to be stable enough to allow DMPO trapping [15]. Lipid alkoxyl radicals and particularly the hydroperoxide derivatives, are intermediate lipid peroxidation products known to be the precursors of cytotoxic α,β-unsaturated aldehydes, such as acrolein, crotonaldehyde and 4-hydroxy-nonenal (HNE), among others [39,40]. The cytotoxicity of these secondary products is reported to be related to the chain length [41] and also to the electrophilicity at C-3 given by electron-withdrawing groups [42,43]. The chemical stability of unsaturated aldehydes, when compared to the lipid radicals, the ability to permeate across the lipid bilayer membrane and the nucleophilic reaction that they undergo with primary amino groups, found in proteins and DNA bases [23], is though to be responsible for the initiation of apoptotic events that different cells exhibited [41–43].

In conclusion, carbon and oxygen centred free radicals of intact diacyl-GPC were identified using the DMPO as spin adducts and based on the MS/MS data, the free radicals were identified to occur in carbon atoms closer to the glycerol moiety. The product ions observed with minor relative abundance were the most informative regarding the location of the spin trap and of the hydroxy groups.

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