

Short Communication

# Characterization of nitrophospholipid-peptide covalent adducts by electrospray tandem mass spectrometry: a first screening analysis using different instrumental platforms

Javier-Fernando Montero-Bullon<sup>1</sup>, Tânia Melo<sup>1</sup>, M Rosário M Domingues<sup>1</sup>, Pedro Domingues<sup>1</sup>

<sup>1</sup>Centro de Espectrometria de Massa, Departamento de Química & QOPNA, Universidade de Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

\* **Corresponding author:** Rosário Domingues, Department of Chemistry, Campus Universitário Santiago, 3810-193 Aveiro, Portugal. **Email:** mrd@ua.pt

**Running Title:** Tandem MS of NO<sub>2</sub>POPC-GSH adducts

**Keywords:** nitration, nitrophospholipid, protein adducts, lipoxidation, post-translational modifications

**Abbreviations:** NO<sub>2</sub>-PL, nitrated phospholipids, NO<sub>2</sub>-FA, nitrated fatty acids.

## Abstract

Lipids are well-known targets of reactive nitrogen species and this reaction leads to the formation of nitrated lipids that have been associated with anti-inflammatory and cytoprotective effects. Nitro-fatty acids (NO<sub>2</sub>-FA) are highly electrophilic compounds that can form covalent adducts with proteins, leading to the formation of lipoxidation adducts,

which modulate the protein structure and function. Nitrated phospholipids (NO<sub>2</sub>-PL) have been detected recently in biological samples, but their biological effects are unknown, although similarly to what it has been described for nitrated lipids, we hypothesize that they may react with peptides and proteins. We used *in vitro* biomimetic assays to synthesize adducts of nitrated POPC (NO<sub>2</sub>POPC), already detected in biological samples, and GSH peptide. The formation of NO<sub>2</sub>POPC-GSH adducts was studied by ESI-MS and MS<sup>2</sup>, using both low and higher energy CID in different MS platforms: a LXQ linear ion trap, a Q-TOF 2, and a Q-Exactive Hybrid Quadrupole-Orbitrap. Typical product ions observed under MS<sup>2</sup> conditions were modified b, y and C ions bearing NO<sub>2</sub>POPC covalently linked, that unequivocally confirmed the presence of the lipid-peptide adduct. Typical loss of HNO<sub>2</sub> was only observed in the MS<sup>2</sup> of the mono charged precursor ions, [M+H]<sup>+</sup>. Product ions at *m/z* 184 or neutral loss of 183 Da were assigned as typical fragmentations that confirm the presence of the phosphatidylcholine. In summary, the characterization of nitro PL-peptide adducts by MS and MS<sup>2</sup> allowed the identification of the structure and specific MS<sup>2</sup> reporter ions to be used to pinpoint these adductions in biological systems.

## **Practical applications**

- The covalent interaction between nitro phospholipids and peptides suggests a new pathway in cellular transduction of nitroxidative stress signal. This adduction can be considered a post-translational modification (PTM) of lipoxidation type, similarly, as it has been described for nitro-fatty acids and with important physiological implications.

The identification and characterization of the nitro phospholipids and peptides adducts is possible by MS and tandem MS. This analytical technique also represents a robust and sensitive approach for detection of nitro-lipids adduction to peptides or proteins in biological samples, allowing to disclose their physiological and clinical implications. Tandem MS fingerprinting is an essential feature for this

purpose, so in this work, we provide the identification of the reporter ions typical for this type of lipoxidation adducts. These reporter ions can be used to design target (NL, PIS or MRM) approaches to detect these type of PTM in biological environments

## Introduction

Phospholipids, main components of lipoproteins and cell membranes, are prone to be modified by reactive oxygen species (ROS) and reactive nitrogen species (RNS), leading to a plethora of oxidized or nitrated and nitroxidized lipids. Among these, oxidized lipids bearing terminal carbonyl group and nitrated lipids, are highly reactive electrophilic compounds, and can form covalent adducts with proteins, leading to the formation of lipoxidation adducts [1, 2]. These reactions are main routes of protein post-translational modifications [3, 4] being responsible for modulation of protein's structure and function. Most of the studies have been focused on lipoxidation adducts formed between peptides and proteins with electrophilic oxidized lipids [5, 6] and, usually, these adducts are reported to have deleterious effects associated with inflammation and several diseases [1].

More recently, peptide and protein modifications by covalent adduction to nitrated fatty acids have been described [7] and have been associated with beneficial and health protective effects [7]. Nitro-fatty acids (NO<sub>2</sub>-FA) have been found both *in vitro* and *in vivo*, namely in red blood cells [8, 9], plasma [8-11], and in different tissues, either in normal or in inflammatory conditions [12]. Several published works described the NO<sub>2</sub>-FA adducts with GSH [13, 14], GAPDH [13, 14], NF-κB [15] and PPARγ [16]. The type of post-translational modification seems to be an important regulatory pathway for the modulation of enzymatic activity, redox homeostasis and in signalling events [7, 13, 17] associated with inhibition of

inflammatory process and pro-survival responses. Also, NO<sub>2</sub>-FA and their protein adducts have been identified in olive and olive oil and were correlated with the beneficial effects of this food and of the Mediterranean diet [18].

The identification *in vitro* or *in vivo* of lipoxidation adducts and of nitrolipid-peptide/protein adducts, is usually done by using mass spectrometry (MS)-based approaches [13]. This approach allows to specify the electrophilic molecule that is covalently linked to peptide or protein, and to localize the addition site [5]. This structural information is obtained by analysis of the fragmentation pattern of peptide-electrophilic adducts obtained using tandem mass spectrometry experiments. [5, 6].

Very recently, nitro-phospholipids (NO<sub>2</sub>-PL) were detected *in vitro* and *in vivo* systems, by our group [19, 20], using MS-based approaches. Nitro derivatives of phosphatidylcholines (NO<sub>2</sub>-PC) and phosphatidylethanolamines (NO<sub>2</sub>-PE), mainly bearing oleic acid (OA) were detected in cardiomyocytes [20] and in cardiac mitochondria from diabetic rats [19]. These results showed that not only free NO<sub>2</sub>-FA, but also esterified NO<sub>2</sub>-FA in PL, can be a target of RNS and can be formed *in vivo*. NO<sub>2</sub>-PL was correlated with beneficial effects in the recovery phase in a cellular model of myocardium infarction in autophagy [20]. Also, NO<sub>2</sub>POPC showed antioxidant properties as scavenging agents and anti-inflammatory properties by inhibiting the expression of iNOS in Raw 264.7 macrophages stimulated with LPS [21].

However, there are no studies on the possible adduction of NO<sub>2</sub>-PLs to peptides. Thus, the aim of our work was to identify for the first time the typical tandem MS fragmentation pattern of the covalent adducts formed between GSH and NO<sub>2</sub>POPC, one of the NO<sub>2</sub>-PL species reported in biological samples [19, 20], and that may have important biological properties. Analysis by tandem MS was performed in three different instruments commonly used in lipidomics and proteomics approaches: an LXQ linear ion trap (LXQ-LIT), a Q-TOF

hybrid quadrupole time-of-flight, and a high-resolution Q Exactive Hybrid Quadrupole-Orbitrap. Herein, we have characterized the covalent Michael adduct formed between GSH and NO<sub>2</sub>-POPC by MS-based approaches, opening a new field of study for nitroxidative stress downstream processes and to unveil the possible role of NO<sub>2</sub>-PL in protein's post-translational modifications.

## Material and methods

Phospholipid nitration was carried out with nitronium tetrafluoroborate (NO<sub>2</sub>BF<sub>4</sub>; Sigma-Aldrich, St Louis, MO, USA), as previously described [19, 20, 22]. A solution of 1-palmitoyl-2-oleoyl-SN-glycero-3-phosphocholine (POPC; 1 mg; Avanti® Polar Lipids, Inc., Alabaster, USA) in chloroform (1 mL; Fisher Scientific Ltd., Leicestershire, UK) was prepared in an amber vial tube. Then, an excess of the solid NO<sub>2</sub>BF<sub>4</sub> (≈ 1 mg) was added. The vial containing the reaction mixture was purged with nitrogen stream, prior to the incubated at room temperature (20 °C) for 1 h, under orbital shaking at 750 rpm. This allow that the reaction occur under nitrogen atmosphere in order to avoid decomposition of the nitro phospholipid formed. After incubation, the reaction was stopped by solvent extraction with Milli-Q water. The organic layer containing the phospholipid products was collected, and was dried under a nitrogen stream. The recovered nitrated PL was quantified using phosphorous assay and analysed by ESI-MS and MS/MS in an LXQ linear ion trap (LIT) mass spectrometer (ThermoFinnigan, San Jose, CA, USA) in positive-ion mode. After analysis of ESI-MS spectrum, it was possible to identify the [NO<sub>2</sub>POPC+H]<sup>+</sup> ion at *m/z* 805.7, confirming the formation of the NO<sub>2</sub>POPC. A smaller ion attributed to the fluorinated derivative [NO<sub>2</sub>POCP+HF+Na]<sup>+</sup> is also observed at *m/z* 847.7, as observed previously for the nitration of fatty acids [22], and may correspond to the addition of fluoride to the double bond of the

monounsaturated fatty acid. Since nitroso or nitroxidized derivatives were not identified in our reaction, in contrast with what was reported before by Melo *et al* [20] and Milic *et al* [22] (because in the previously published work nitration was performed in the presence of air, favouring oxidation and decomposition of nitro POPC) and considering that the hydrofluoride nitro-POPC derivative lose the double bond, thus is not reactive toward protein/ peptides, the NO<sub>2</sub>POPC was used without further purification steps. The adducts between the NO<sub>2</sub>POPC and GSH (reduced form; Sigma-Aldrich, St Louis, MO, USA) were obtained by mixing 0.25 μmol of NO<sub>2</sub>POPC with 1.25 μmol of GSH in 50 μL of NH<sub>4</sub>HCO<sub>3</sub> buffer (5mM, pH 7.4; Sigma-Aldrich, St Louis, MO, USA), under a N<sub>2</sub> atmosphere. The mixture was vortexed for 15 min, sonicated for 5 min and incubated at 37 °C for 6 h in a shaker at 750 rpm. Aliquots of 4 μL of product mixture were diluted to 200 μL of MeOH:H<sub>2</sub>O (3:1, v/v) with 1% formic acid (Sigma-Aldrich, St Louis, MO, USA), and analysed by ESI-MS using different instruments operating in positive-ion mode: a LXQ linear ion trap (LIT) (ThermoFinnigan, San Jose, CA, USA), a Q-TOF 2 hybrid quadrupole time-of-flight (Micromass, Manchester, UK) and a high resolution Q Exactive Hybrid Quadrupole-Orbitrap (Thermo Fisher Scientific, Bremen, Germany) mass spectrometers.

The operating conditions used in ESI-LXQ-LIT mass spectrometer were as follows: samples were introduced at a flow rate of 8 μL/min; electrospray voltage was +5 kV; capillary temperature was 275 °C; and the sheath gas flow of 25 (arbitrary units). Nitrogen was used as nebulizing and drying gas. Full scan MS spectra were acquired over the *m/z* range 150-1200. For MS/MS experiments, spectra were acquired by CID using helium as the collision gas. The normalized collision energy used was 28 (arbitrary units). Data acquisition and analysis were performed using Xcalibur Data System (version 2.0, ThermoFinnigan, San Jose, CA, USA).

In the ESI-Q-TOF2 mass spectrometer, the flow rate of 10  $\mu\text{L}/\text{min}$ ; cone voltage was set at 30 V; capillary voltage was maintained at +3 kV; source temperature was 80  $^{\circ}\text{C}$ , and desolvation temperature was 150  $^{\circ}\text{C}$ . Full scan MS was acquired scanning the mass range from  $m/z$  150 to 1200. MS/MS spectra were acquired using argon as the collision gas. The normalized collision energy used was 25 (arbitrary units). Data acquisition and analysis were performed using MassLynx 4.0 data system.

In the ESI Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), samples were introduced through direct infusion at flow rate of 10  $\mu\text{L}/\text{min}$  and the operating conditions were as follows: the electrospray voltage was +3 kV; capillary temperature was 250  $^{\circ}\text{C}$ ; sheath gas (nitrogen) flow of 5 (arbitrary units); isolation width of 1 Da; AGC target of 5E06; maximum injection time (IT) was 250 ms; and resolution was set at 140,000 in  $m/z$  range 150-1200. In MS/MS experiments, the resolution was set at 70,000 full width at half maximum (FWHM); AGC target of 1E05; maximum IT 250 ms; isolation window of 1.0  $m/z$ ; and normalized collision energy was 23 for mono charged and 20 for double charged ions, arbitrary units. Collision gas was  $\text{N}_2$ . Data acquisition was carried out using the Xcalibur data system (V3.3, Thermo Fisher Scientific, USA).

## Results and Discussion

The covalent adduct of  $\text{NO}_2\text{POPC}$  and GSH was identified in the ESI-MS spectra by the presence of the  $[\text{M}+2\text{H}]^{2+}$  ions at  $m/z$  556.832 and  $[\text{M}+\text{H}]^+$  ions at  $m/z$  1112.656 (Figure 1), as confirmed by exact mass measurement in the high resolution Q Exactive Orbitrap (not shown), and also by tandem MS. These adducts are formed via Michael addition reaction between reduced cysteine of GSH with the double bond of the  $\text{NO}_2\text{-PL}$ , in a similar way as previously proposed for  $\text{NO}_2\text{-FA-GSH}$  adducts [13, 14] (Scheme 1). [The reaction of  \$\text{NO}\_2\text{POPC}\$](#)

with GSH should occur in aqueous media, as proposed for nitro oleic acid (NO<sub>2</sub>-OA). A recent study evaluated the position of the nitro group of NO<sub>2</sub>-OA, in PC liposomes used as a model of membranes and by fluorescence measurements, vibrational spectroscopy, and coarse-grained computer simulations to demonstrate that NO<sub>2</sub>-OA changes the lipid organization and are mainly accumulated at the membrane–water interface [23]. NO<sub>2</sub>-OA is able to shape the molecular organization of model membranes along the bilayer, and the nitro group is preferentially located near the head group of the phospholipid [23]. This arrangement facilitates the location of the nitro group more closely to the membrane–water interface that favors the electrophilic adduction of the nitro-lipids with the anionic sulfhydryl moiety of the cysteine in peptides and proteins, in an aqueous medium.

Analysis of the MS/MS spectra of both mono and double charged ions, [M+H]<sup>+</sup> and [M+2H]<sup>2+</sup>, allowed to identify the fragmentation pathways and to suggest reporter ions that can be further used to confirm the identity of these adducts. The MS/MS spectra obtained in three different mass spectrometers, an LXQ linear ion trap (CID), a Q-TOF (CID) and a Q Exactive Orbitrap (HCD) are shown in Figures 2 and 3. These MS platforms were selected because they are, nowadays, the most used for the detection of lipid - protein adducts either in biomimetic systems or in searching these compounds in biological samples analysis [13, 24].

The MS/MS spectra of the mono charged [M+H]<sup>+</sup> ions of NO<sub>2</sub>POPC-GSH adduct, obtained in the three instruments (Figure 2) showed some common fragmentation pathways such as the ion arising from the neutral loss of HNO<sub>2</sub> ([M+H-HNO<sub>2</sub>]<sup>+</sup>, at *m/z* 1065.658, confirming the presence of NO<sub>2</sub>-PL species. It was also observed the mono charged *y*<sub>2</sub> fragment ion linked to the nitrated POPC, designed as \**y*<sub>2</sub><sup>+</sup> (at *m/z* 983.615) product ion, (where \* indicates that the product ion has a nitrated POPC moiety) formed by the cleavage of the



peptide backbone in the vicinity of Cys where the NO<sub>2</sub>-POPC is linked (Scheme 2). These MS/MS spectra showed also the loss of HNO<sub>2</sub> from  $*y_2^+$ , inferred by the observation of the product ion at  $m/z$  936.614. A product ion at  $m/z$  663.5, arising from the cleavage of the C9-C10 bond of the modified fatty acyl chain, was only observed in the tandem spectra acquired in the ESI-Linear ion trap and Q-TOF instruments. The presence of product ions attributed to [NO<sub>2</sub>POPC+H]<sup>+</sup> (at  $m/z$  805.6), [POPC+H]<sup>+</sup> (at  $m/z$  760.6) and at phosphatidylcholine head (at  $m/z$  184.1, allowed to confirm the identity of NO<sub>2</sub>-PL. **No loss of HNO was observed in the MS/MS spectra of the [M+H]<sup>+</sup>, and of the [M+2H]<sup>2+</sup>, thus excluding the contribution of possible nitroso derivatives.**

The tandem mass spectra of the double charged [M+2H]<sup>2+</sup> ions showed, in all instruments, (Figure 3), the common product ions  $*y_2^{2+}$  (at  $m/z$  492.311),  $*b_2^{2+}$  (at  $m/z$  519.316), and  $*C_1^{2+}$  (at  $m/z$  440.797), all corresponding to peptide fragments adducted to NO<sub>2</sub>POPC. All these product ions confirmed the presence of the NO<sub>2</sub>POPC covalently linked to the Cys residue of glutathione. Loss of HNO<sub>2</sub> was absent or observed with very low abundance. In all MS/MS spectra was also possible to detect the loss of phosphocholine (-183 Da), observed either from the precursor ion with formation of the product ion at  $m/z$  929.591, or also combined with cleavages of the peptide product ions:  $*y_2^+-183$  (at  $m/z$  800.5471) and  $*b_2^{2+}-183$  (at  $m/z$  854.558). Similarly, as observed in the case of the mono charged ion, the [POPC+H]<sup>+</sup> product ion (at  $m/z$  760.588) and the PC polar head (at  $m/z$  184.074), were observed in all spectra. All these product ions confirmed the presence of a phosphatidylcholine covalently linked to the peptide.

Overall, comparing the MS/MS spectra from the three mass spectrometers, we can highlight as common features that clearly identify the presence of NO<sub>2</sub>POPC-GSH adducts, the product ions  $*y_2$ , and the product ions and combined neutral losses associated with the

phosphocholine polar head ( $m/z$  184 or loss of 183Da). Other important reporter ions were  $^*y_2\text{-HNO}_2$  (observed in the case of fragmentation of mono charged ions), the  $^*b_2$  and  $^*C_1$  (observed in the case of fragmentation of double charged ions). Typical neutral loss (NLs) of  $\text{HNO}_2$  was observed in the case of mono charged ions. The presence of the fragment ions  $^*y_2$ ,  $^*y_2\text{-HNO}_2$  together with the cysteine immonium ions  $^*C_1$  support the hypothesis that the lipid backbone of the  $\text{NO}_2\text{POPC}$  was bonded to the cysteine- moiety of GSH. The main product ions, identified as  $y_2$ ,  $b_2^{2+}$ , and  $C_1$ , now observed for the  $\text{NO}_2\text{POPC-GSH}$  adduct, were also reported in tandem MS of the  $[\text{M}+\text{H}]^+$  ions of the  $\text{NO}_2\text{-FA-GSH}$  adduct [13]. In consequence, these product ions ( $^*y_2$ ,  $^*b_2$ ,  $^*C_1$ ) can be considered as reporter ions for the target analysis of these nitro lipoxidation adducts in biological samples. However, in this case of the  $\text{NO}_2\text{FA}$  adducts, loss of  $\text{HNO}_2$  was not observed in the MS/MS spectrum but was only observed by  $\text{MS}^3$  of  $^*y_2$ , when using the ion trap instrument [13].

The mass spectrometry approach used in this work was able to identify the formation of adducts between nitrated phospholipids and peptides, as exemplified for GSH. POPC is one of the most abundant phospholipids and its nitration *in vivo* has been previously shown [19, 20]. GSH is intracellularly biosynthesized but it is also found in the extracellular medium, as in plasma. In spite of being a hydrophilic peptide, it was chosen for this experiment due to its facility to oxidize and the availability of its cysteine residue. In fact, the susceptibility of being oxidized by the nitro-fatty acyl chain embedded in the bilayer of liposomes shows the accessibility for interaction between  $\text{NO}_2\text{-PL}$  and peptides. This suggests the idea that a great number of peptides, but also proteins can be considered targets of nitrated phospholipids, especially those with a closer relationship with the membrane, which should be further investigated.

## **Conclusions**

In summary, in this work we have characterized a nitro phospholipid-peptide adduct by MS and MS<sup>2</sup>. This approach allows to identify the nature of modified phospholipid that is covalently bound to the peptide/protein and the reported ions identified can be used to pinpoint these adductions in biological systems. The biological effects of these new kind of adducts remains to be studied but it can be considered as promising, based on the importance of nitroxidation events in physiological and pathophysiological circumstances.

## **Acknowledgements**

Thanks are due to University of Aveiro, Fundação para a Ciência e Tecnologia and Ministério da Educação e Ciência (FCT/MEC), European Union, QREN, COMPETE for the financial support to the QOPNA research Unit (FCT UID/QUI/00062/2013) and, through national funds and where applicable co-financed by the FEDER, within the PT2020 Partnership Agreement, and also to the Portuguese Mass Spectrometry Network (LISBOA-01-0145-FEDER-402-022125) and to and to the funding from European Commission's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement number 675132 (MSCA-ITN-ETN MASSTRPLAN). Tânia Melo (BPD/UI51/5388/2017) is grateful to FCT for her post-doctoral grant.

The authors have declared no conflict of interest.

## References

- [1] M. R. Domingues, P. Domingues, T. Melo, D. Pérez-Sala, A. Reis, C. Spickett, Lipoxidation adducts with peptides and proteins: deleterious modifications or signalling mechanisms? *J Proteomics* 2013; 92: 110.
- [2] I. Milic, T. Melo, M. R. Domingues, P. Domingues, M. Fedorova, Heterogeneity of peptide adducts with carbonylated lipid peroxidation products. *J Mass Spectrom.* 2015; 50: 603.
- [3] A. M. Silva, A. C. Borralho, S. A. Pinho, M. R. Domingues, P. Domingues, Cross-oxidation of angiotensin II by glycerophosphatidylcholine oxidation products, *Rapid Commun Mass Spectrom.* 2011; 25: 1413.
- [4] G. Aldini, M. R. Domingues, C. M. Spickett, P. Domingues, A. Altomare, F. J. Sánchez-Gómez, C. L. Oeste, D. Pérez-Sala. Protein lipoxidation: Detection strategies and challenges. *Redox Biol.* 2015; 5: 253.
- [5] M. Fedorova, R. C Bollineni, R. Hoffmann, Protein carbonylation as a major hallmark of oxidative damage: update of analytical strategies, *Mass Spectrom Rev.* 2014; 33: 79.
- [6] Vasil'ev YV, Tzeng SC, Huang L, Maier CS. Protein modifications by electrophilic lipoxidation products: adduct formation, chemical strategies and tandem mass spectrometry for their detection and identification. *Mass Spectrom Rev.* 2014 May-Jun;33(3):157-82.
- [7] A. C. Geisler, T. K. Rudolph, Nitroalkylation--a redox sensitive signaling pathway, *Biochim Biophys Acta* 2012; 1820: 777.

- [8] P. R. Baker, F. J. Schopfer, S. Sweeney, B. A. Freeman, Red cell membrane and plasma linoleic acid nitration products: synthesis, clinical identification, and quantitation, *Proc Natl Acad Sci U S A* 2004; 101: 11577.
- [9] E. S. Lima, P. Di Mascio, D. S. Abdalla, Cholesteryl nitrolinoleate, a nitrated lipid present in human blood plasma and lipoproteins, *J Lipid Res* 2003; 44: 1660.
- [10] E. S. Lima, P. Di Mascio, H. Rubbo, D. S. Abdalla, Characterization of linoleic acid nitration in human blood plasma by mass spectrometry, *Biochemistry* 2002; 41: 10717.
- [11] D. Tsikas, A. A. Zoerner, A. Mitschke, F. M. Gutzki, Nitro-fatty acids occur in human plasma in the picomolar range: a targeted nitro-lipidomics GC-MS/MS study, *Lipids* 2009; 44: 855.
- [12] L. Villacorta, Z. Gao, F. J. Schopfer, B. A. Freeman, Y. E. Chen, Nitro-fatty acids in cardiovascular regulation and diseases: characteristics and molecular mechanisms, *Front Biosci (Landmark Ed)* 2016; 21: 873.
- [13] C. Batthyany, F. J. Schopfer, P. R. Baker, R. Duran, L. M. Baker, Y. Huang, C. Cervenansky, B. P. Branchaud, B. A. Freeman, Reversible post-translational modification of proteins by nitrated fatty acids in vivo, *J Biol Chem* 2006; 281: 20450.
- [14] L. M. Baker, P. R. Baker, F. Golin-Bisello, F. J. Schopfer, M. Fink, S. R. Woodcock, B. P. Branchaud, R. Radi, B. A. Freeman, Nitro-fatty acid reaction with glutathione and cysteine. Kinetic analysis of thiol alkylation by a Michael addition reaction, *J Biol Chem* 2007; 282: 31085.
- [15] T. Cui, F. J. Schopfer, J. Zhang, K. Chen, T. Ichikawa, P. R. Baker, C. Batthyany, B. K. Chacko, X. Feng, R. P. Patel, A. Agarwal, B. A. Freeman, Y. E. Chen, Nitrated fatty

- acids: Endogenous anti-inflammatory signaling mediators, *J Biol Chem* 2006; 281: 35686.
- [16] P. R. Baker, Y. Lin, F. J. Schopfer, S. R. Woodcock, A. L. Groeger, C. Batthyany, S. Sweeney, M. H. Long, K. E. Iles, L. M. Baker, B. P. Branchaud, Y. E. Chen, B. A. Freeman, Fatty acid transduction of nitric oxide signaling: multiple nitrated unsaturated fatty acid derivatives exist in human blood and urine and serve as endogenous peroxisome proliferator-activated receptor ligands, *J Biol Chem* 2005; 280: 42464.
- [17] G. Bonacci, F. J. Schopfer, C. I. Batthyany, T. K. Rudolph, V. Rudolph, N. K. Khoo, E. E. Kelley, B. A. Freeman, Electrophilic fatty acids regulate matrix metalloproteinase activity and expression, *J Biol Chem* 2011; 286: 16074.
- [18] M. Fazzari, A. Trostchansky, F. J. Schopfer, S. R. Salvatore, B. Sánchez-Calvo, D. Vitturi, R. Valderrama, J. B. Barroso, R. Radi, B. A. Freeman, H. Rubbo, Olives and olive oil are sources of electrophilic fatty acid nitroalkenes. *PLoS One*. 2014; 9:e84884.
- [19] T. Melo, P. Domingues, R. Ferreira, I. Milic, M. Fedorova, S. M. Santos, M. A. Segundo, M. R. M. Domingues, Recent Advances on Mass Spectrometry Analysis of Nitrated Phospholipids, *Anal Chem* 2016; 88: 2622.
- [20] T. Melo, P. Domingues, T. M. Ribeiro-Rodrigues, H. Girao, M. A. Segundo, M. R. M. Domingues, Characterization of phospholipid nitroxidation by LC-MS in biomimetic models and in H9c2 Myoblast using a lipidomic approach, *Free Radical Biol Med* 2017; 106: 219.
- [21] T. Melo, S. S. Marques, I. Ferreira, M. T. Cruz, P. Domingues, M. A. Segundo, M. R. M. Domingues, New Insights in Anti-inflammatory and Antioxidant Properties of Nitrated Phospholipids., *Lipids* 2018, 53,117.

- [22] I. Milic, E. Griesser, V. Vemula, N. Ieda, H. Nakagawa, N. Miyata, J. M. Galano, C. Oger, T. Durand, M. Fedorova, Profiling and relative quantification of multiply nitrated and oxidized fatty acids, *Anal Bioanal Chem* 2015; 407: 5587.
- [23] J. Franz, T. Bereau, S. Pannwitt, V. Anbazhagan, A. Lehr, U. Nubbemeyer, U. Dietz, M. Bonn, T. Weidner, D. Schneider, Nitrated Fatty Acids Modulate the Physical Properties of Model Membranes and the Structure of Transmembrane Proteins, *Chemistry* 2017; 23:9690.
- [24] S. R. Salvatore, D. A. Vitturi, P. R. Baker, G. Bonacci, J. R. Koenitzer, S. R. Woodcock, B. A. Freeman, F. J. Schopfer, Characterization and quantification of endogenous fatty acid nitroalkene metabolites in human urine, *J Lipid Res* 2013; 54: 1998.

**CAPTIONS:**

Figure 1. ESI-MS spectrum obtained after reaction between GSH and NO<sub>2</sub>POPC, showing the NO<sub>2</sub>POPC-GSH indentified by the presence of the [M+2H]<sup>2+</sup> ions at *m/z* 556.832 and [M+H]<sup>+</sup> ions at *m/z* 1112.656.

Figure 2. ESI-MS/MS spectra of [NO<sub>2</sub>POPC-GSH+H]<sup>+</sup> in A) LXQ Linear Ion trap, B) Q-TOF 2, and C) Q-Exactive Orbitrap.

Figure 3. ESI-MS/MS spectra of  $[\text{NO}_2\text{POPC-GSH}+2\text{H}]^{2+}$  in A) LXQ Linear Ion trap, B) Q-TOF 2, and C) Q-Exactive Orbitrap.

Scheme 1. The general mechanism of reaction for adduction of  $\text{NO}_2\text{-PL}$  to GSH with the formation of Michael adducts. [The reaction of  \$\text{NO}\_2\text{POPC}\$  with GSH should occur in aqueous media, as proposed for nitro oleic acid \( \$\text{NO}\_2\text{-OA}\$ \) \[23\].](#)

Scheme 2. Main fragmentation pathways observed in the ESI-MS/MS spectra of the  $\text{NO}_2\text{POPC-GSH}$  adducts detected in all the mass spectrometers, A) mono charged  $[\text{M}+\text{H}]^+$  ion and B) double charged  $[\text{M}+2\text{H}]^{2+}$ .



Figure 1

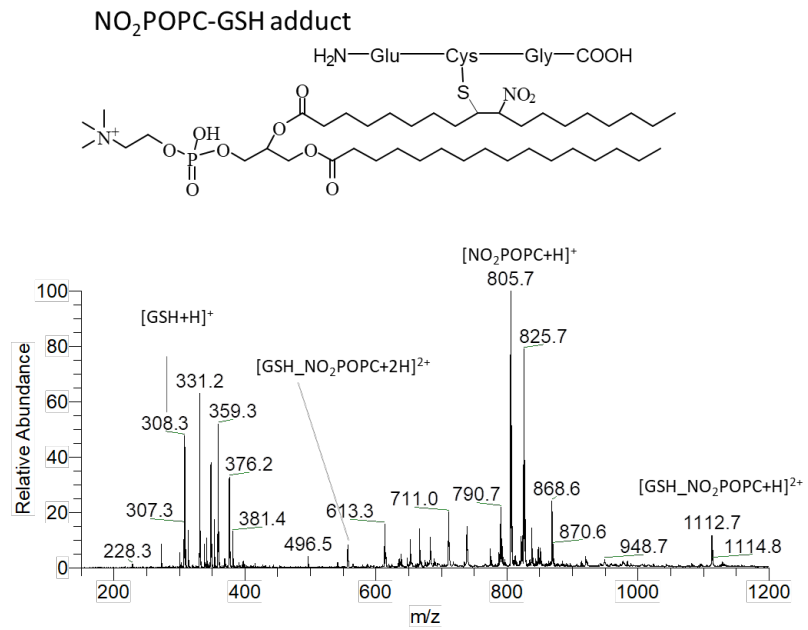


Figure 2

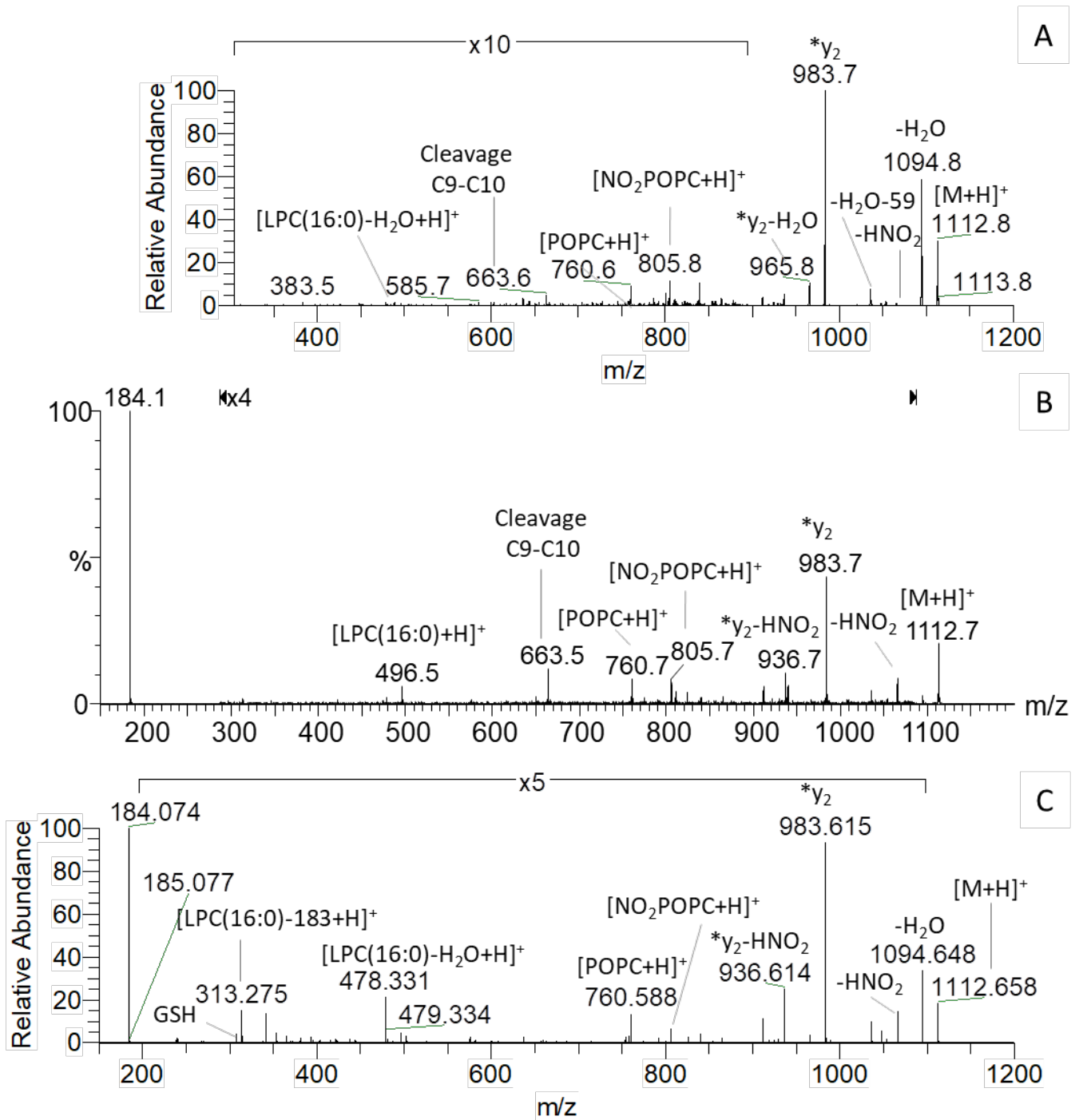
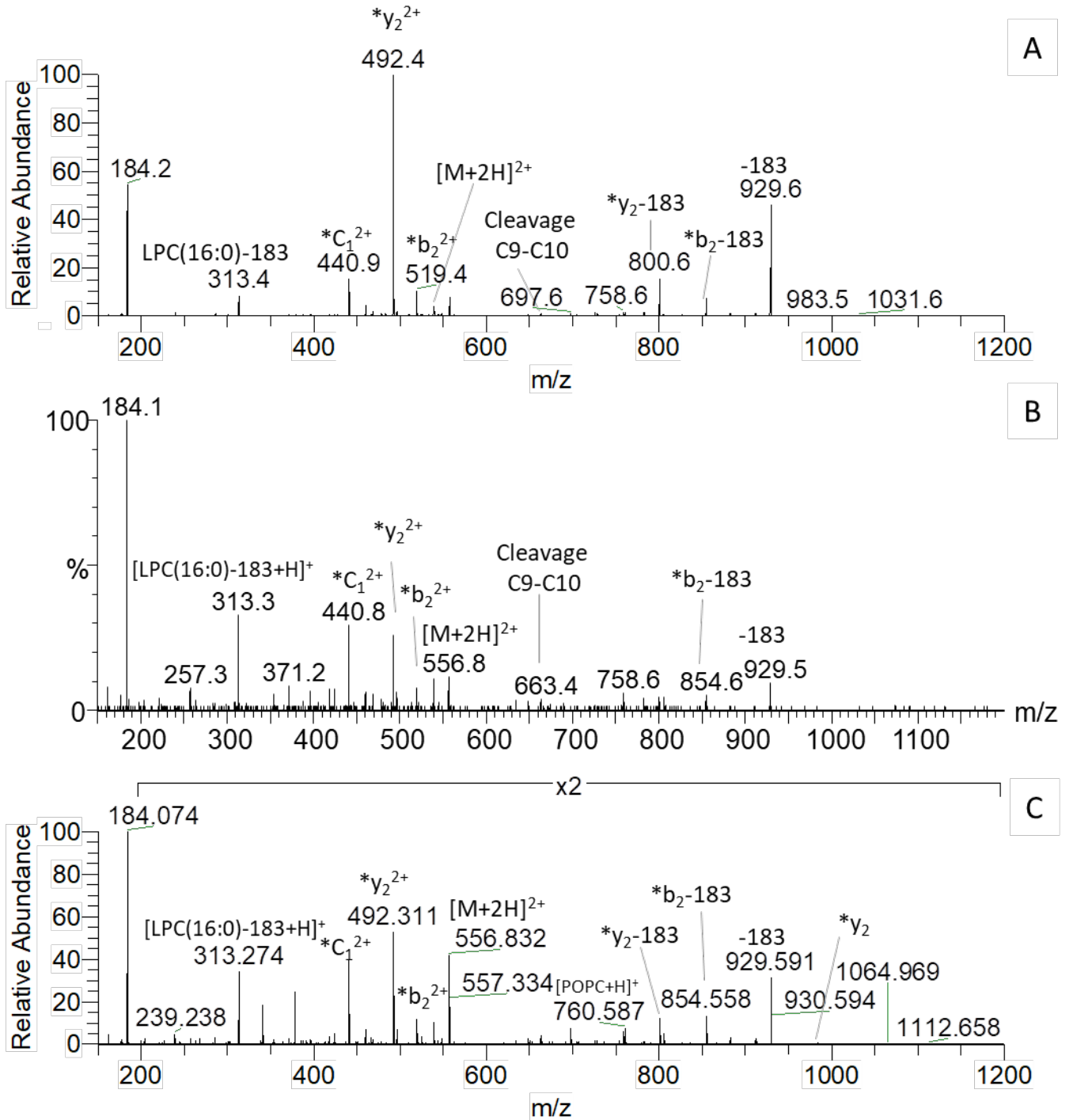
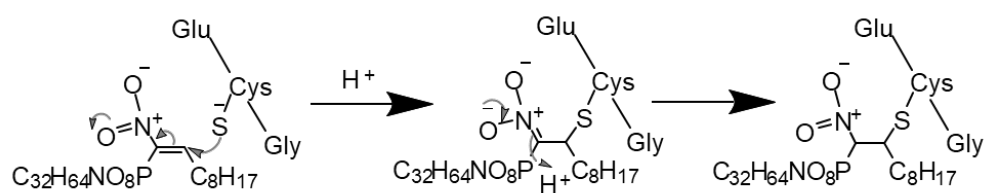


Figure 3

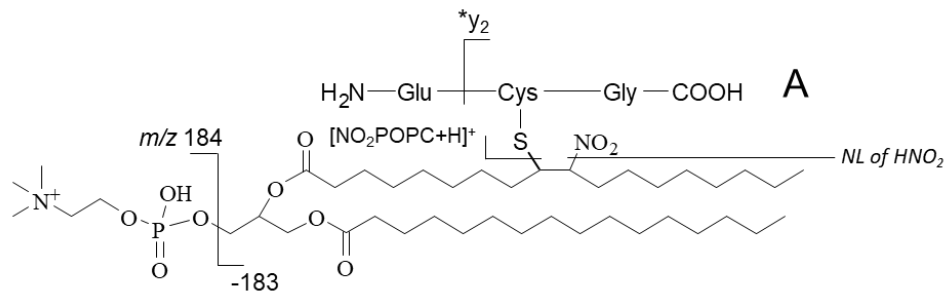


### Scheme 1



## Scheme 2

Common fragmentation pathways of the mono charged ions



Common fragmentation pathways of the double charged ions

