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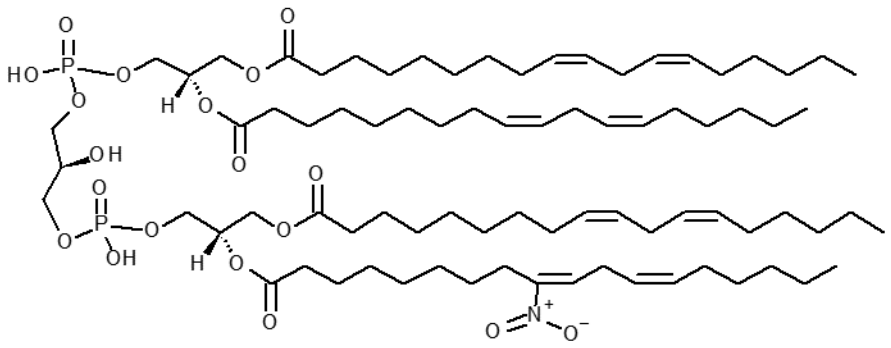

Liquid chromatography/tandem mass spectrometry characterization of nitroso, nitrated and nitroxidized cardiolipin products.

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Running Title: LC/MS of nitrated cardiolipin

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Abstract

Cardiolipins (CL) are anionic dimeric phospholipids bearing four fatty acids, found in inner mitochondrial membrane as structural components and are involved in several processes as oxidative phosphorylation or apoptotic signalling. As other phospholipids, CL can be modified by reactive oxygen species (ROS) and reactive nitrogen species (RNS), which can modulate various cellular functions. Modifications of CL by RNS remain largely unstudied although other nitrated lipids are emerging as bioactive molecules. In this work, we developed a C30-LC-HRMS/MS methodology to identify the nitrated and nitroxidized tetralinoleoyl-cardiolipin (TLCL), using a biomimetic model of nitration, and to disclose specific fragmentation pathways under HCD MS/MS. Using this lipidomics approach, we were able to separate and identify nitro, nitroso, nitronitroso, and nitroxidized TLCL derivatives, comprising 11 different nitrated compounds. These products were identified using accurate mass measurements and the fragmentation pattern acquired in higher-energy collision dissociation (HCD)-tandem MS/MS experiments. These spectra showed classifying fragmentation pathways, yielding phosphatidic acid (PA^-), lysophosphatidic acid (LPA^-), and carboxylate fragment ions with the modifying moiety. Remarkably, the typical neutral losses associated with the added moieties were not observed. In conclusion, this work has developed a new method for the identification of nitroso, nitrated and nitroxidized cardiolipin products by using a C30LC-MS platform method, potentially allowing their detection in biological samples.

Introduction

Cardiolipins (CL) are phospholipids that in mammals are found exclusively in mitochondria, more predominantly in the internal membrane, where they are involved in critical biological processes as energy production through electron transport chain and oxidative phosphorylation [1], or in apoptosis [2]. The average abundance of CLs in the total cellular lipid content is minor, but depending on cell type and tissues, it can achieve a percentage up to 24% of total phospholipids of the mitochondrial membrane [3,4]. In high energy-demand tissues with large numbers of mitochondria, like the brain, liver or muscle, and the cardiac muscle, an increased amount of CL is observed, in the range of nanomolar per mg of protein [5]. Structurally, CLs are dimeric anionic glycerophospholipids with two phosphate groups and three glycerol moieties, allowing the presence of four fatty acids. *In vivo*, the combinations of fatty acids are limited, with a high predominance of chains of 18 carbons with one or two unsaturations (C18:1 and C18:2), and, in less proportion, chains of 16 carbons [5] and, especially in the brain, poly-unsaturated chains of C20:4 and C22:6 [6]. Due to the presence of unsaturated fatty acids, CLs are prone to chemical modification by radicals like reactive oxygen species (ROS) and reactive nitrogen species (RNS), similarly to other phospholipids [7].

Oxidized phospholipids formed by ROS are well-known, showing altered functionality and were linked to pathophysiological conditions [8–10]. Chemical oxidation of CLs is also well-studied [6,11–15]. Long-chain oxidation products of CL have been characterized by LC-MS [12,14,15], as well as short-chain products [16]. Oxidized CLs have been detected *in vivo* and *in vitro* and correlated with important biological processes. They have been detected in endothelial cells and *in vitro* in cultures of neuronal cells under stress and apoptotic stimuli [12], in lymphoblast's treated with rotenone [17], in lung tissue of mice under hyperoxia[12], acute injury [18] or gamma radiation-induced injury [19], in intestinal tissue of mice under gamma radiation [20], in kidney tissue of rats under

nephrotoxic conditions [16], and in brain of rats under chronic stress-induced depression [21]. Nowadays, CL oxidation is considered an important mechanism participating in processes such as apoptosis [6] and with implications in diseases as neurodegeneration, myocardial ischaemia, diabetes, or ageing [13]. Notwithstanding, the modification on CLs by RNS remains unexplored.

It is well-known that RNS can be highly expressed in associated with inflammation, cardiovascular homeostasis, and digestion [22], exerting nitroxidative stress that chemically modifies biomolecules. Nitration of proteins is widely recognized as a marker of cellular dysfunction, usually evaluated by the increase of tyrosine nitration and S-nitrosylation [23,24]. Regarding lipids, the susceptibility of fatty acids and their esterified forms to be modified by RNS has been recently explored. The natural occurrence of nitrated fatty acids is well established, and their biological implications have been addressed [25], evidencing a resolving role in different pathologies as cardiovascular disease [26] and diabetes [27,28]. Indeed, most of the studies in lipid nitroxidation have focused on nitro-fatty acids establishing their role as anti-inflammatory and antioxidant agents [29], antagonizing the effects of oxidation products. The possibility of other nitroxidative changes as the addition of nitroso group or combination with several oxidative moieties has also been observed *in vitro* and *in vivo* [30]. More recently, some of these modifications have also been characterized in phospholipids, namely PEs, PCs and PSs *in vitro* [28,31,32] and *in vivo* [28]. The possible physiological role of nitroxidized phospholipids has just started to be unravelled, indicating that they had antioxidant and anti-inflammatory properties [33]. However, cardiolipins, an important phospholipid class, and their nitroxidation products have not been studied. CLs are a likely target of RNS given that RNS interact with mitochondria and regulate its function [34], and therefore may diffuse in the proximity of mitochondrial membrane promoting CL nitroxidation.

Knowledge of modified fatty acids and phospholipids have been facilitated by previous detailed structural characterization by mass spectrometry (MS). Mass spectrometry is a powerful technique, especially when coupled to liquid chromatography, for molecular characterization of complex

samples containing molecules as oxidized phospholipids [7], nitroxidized fatty acids [35] and phospholipids [28,31]. *In vitro* assays allow the biomimetic generation of these species and their characterization, making possible further detection in complex matrixes, particularly *in vivo*. The aim of this work was to apply LC-MS strategies to identify and characterize *in vitro* products of CLs nitroxidation. We produced nitrated and nitroxidized CL molecular species using a biomimetic protocol of lipid nitration. LC-MS analysis of the products was performed using a C30 reversed-phase column, providing a new method and list of modified CLs that may help to detect and study their functions *in vivo*.

Experimental

Materials

3'-Bis[1,2-Di-(9Z-12Z-octadecadienoyl)-*sn*-glycero-3-phospho]-*sn*-glycerol or tetralinoleoyl cardiolipin (TLCL) was bought from Avanti® Polar Lipids, Inc. (Alabaster, USA) and used without further purification. Nitronium tetrafluoroborate (NO_2BF_4) was purchased from Sigma-Aldrich (Madrid, Spain). Ammonium molybdate ($NaMoO_4 \cdot 4H_2O$) and sodium phosphate monobasic dihydrate ($NaH_2PO_4 \cdot 2H_2O$) were purchased from Riedel-de Haën (Seelze, Germany) and ascorbic acid from VWR International (Leicestershire, UK) HPLC grade chloroform, methanol, isopropanol, formic acid and ammonium acetate were purchased from Fisher Scientific Ltd. (Leicestershire, U.K.). Milli-Q water was used for all experiments, filtered through a 0.22 μm filter and obtained using a Milli-Q Millipore system (Synergy®, Millipore Corporation, Billerica, MA, USA).

In vitro nitroxidation

Cardiolipin (TLCL) nitration (1 mg) was carried out with an excess of nitronium tetrafluoroborate (approximately 5 mg) in chloroform (1 mL) at room temperature for 1 h, under orbital shaking at 750 rpm. Nitration was quenched with water (2 mL), vortexing for 30 s and centrifuged for 5 min at 1000 rpm in a Mixtasel Centrifuge (Selecta, Barcelona, Spain). The water phase containing products from

the hydrolysis of the excess of NO_2BF_4 and salts, in general, was discarded, while the lower organic phase containing the cardiolipin was dried under a nitrogen stream and stored at −20°C to be further quantified using phosphorus assay and analysed by C30-HPLC-ESI-MS and MS/MS.

Phospholipid quantification

The total amount of non-modified and modified CL recovered after reaction and extraction were quantified using colourimetric phosphorus assay as previously described by Bartlett and Lewis [36]. Briefly, 125 microliters of concentrated perchloric acid (70% w/v), $HClO_4$, were added to 10 microliters of 25 µg of dried lipid extract. Samples were then incubated for 1 h at 180 °C in the heating block (Stuart, UK). Afterwards, 0.825 mL of H_2O, 0.125 mL of 2.5% ammonium molybdate (m/v: 2.5 g of $NaMoO_4 \cdot 4H_2O$ in 100 mL of H_2O) and 10% ascorbic acid (m/v; 0.1 g in 1 mL H_2O) were added to all samples. The reaction mixture was homogenized in a vortex mixer and incubated for 10 min at 100 °C in a water bath. Then, the reaction mixture was quickly cooled down to stop the reaction. Standards with 0.1 to 2 µg of phosphorus (in the form of $NaH_2PO_4 \cdot 2H_2O$ salt) were simultaneously treated. Finally, the absorbance of standards and samples was measured at 797 nm in a Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The amount of phosphorus present in each sample was calculated by linear interpolation, based on the calibration curve of the response of absorbance versus phosphorus present in the standards. The mass of phospholipid was calculated by considering the ratio between the molecular mass of the phosphorus detected and molecular mass of tetralinoleoyl cardiolipin.

C30-ESI-LC-HRMS and MS/MS analysis

Sample separation was performed using an Accucore C30 column (150mm*2.1mm,2.6um,150 Å, Thermo Fisher Scientific) with an UltiMate U3000RS system, coupled to a high resolution (HR) Orbitrap Q-Exactive mass spectrometer (Thermo Fisher Scientific). The column was run with a flow rate of 300 µL/min and a column temperature set to 40 °C. For each run, 10 µL of a sample containing 5 µg of nitroxidized cardiolipin in methanol was injected. The gradient used was 25% B for

2 min, 25–86% B in 18 min, 86-95% B in 1 min, 95% B for 14 min. The solvents used were acetonitrile/water/formic acid (95/5/0.1; v/v/v), 5mM ammonium acetate (A) and isopropanol/acetonitrile/water/formic acid (85/10/5/0.1;v/v/v/v), 5 mM ammonium acetate (B).

The mass spectrometer was operated in negative ion mode (electrospray voltage 2.7 kV; capillary temperature, 350 °C; sheath gas flow 45 units, auxiliary gas flow 15 units) acquiring a survey mass spectrum with resolving power 70,000 (full width half maximum), *m/z*=1395-1750 using an automatic gain control (AGC) target of $10E^6$ and a maximum injection time of 100 ms. The 5 most intense ions were selected for higher-energy collisional dissociation (HCD) fragmentation (20, 23, and 25% normalized collision energy) and MS/MS spectra were generated with an AGC target of $10E^5$ and a maximum injection time of 200 ms at a resolution of 17,500. The mass spectrometer worked in data-dependent mode, with an inclusion list containing an *m/z* list of possible nitroxidative modifications and dynamic exclusion of 30 seconds.

After analysing this data, a second MS/MS method was run using data-independent acquisition (DIA), for acquiring better quality MS/MS spectra and attempting to identify isomers. For these experiments, a list of the identified nitroxidative products and the respective RT was created. The method surveyed these ranges of *m/z* using a 1 *m/z* quadrupole isolation windows and multiplexing at the maximum 4 of these windows. Maximum injection times were of 300 ms, AGC values were set to 2x10e5 for MS/MS scans. Data acquisition was carried out using the Xcalibur data system (V3.3, Thermo Fisher Scientific, Waltham, MA, USA).

Results

In this study, we analysed the *in vitro* nitration tetralinoleoyl-cardiolipin (TLCL) products (Figure 1) using an C30-LC-HRMS platform. These products, generated by using a biomimetic protocol of lipid nitration using NO_2BF_4, were observed in the negative ion mode, as mono-charged deprotonated molecular ions $[M-H]^-$. Initial structural identification and annotation of the nitration reaction

products were based on accurate mass (< 5ppm) to provide possible products containing nitro and nitroso moieties and enabled the assignment of several products (Table 1). These ions were assigned as nitroso-TLCL derivatives (NO-TLCL, $(NO)_2$-TLCL, $(NO)_3$-TLCL and $(NO)_4$-TLCL) and nitro-TLCL derivatives (NO_2-TLCL and $(NO_2)_2$-TLCL). We have also assigned a nitronitroso product, with both NO with NO_2 groups $(NO_2)(NO)$-TLCL, and few nitroxidized CL derivatives namely hydroxy or hydroperoxy TLCL with a nitroso group (NO+O-TLCL, $(NO)_2$+2O-TLCL) and a nitronitroso derivative ($(NO_2)(NO)$+O-TLCL. Weak signals attributed to the well-known oxidized derivatives, namely keto, hydroxy and hydroperoxy derivatives, were also observed, but not studied because they were out of the scope of this work (data not shown).

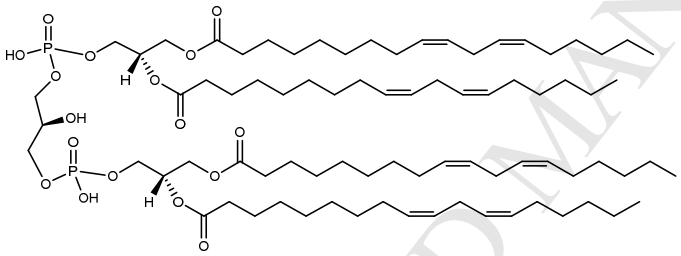


Figure 1- Structure of tetralinoleoyl-cardiolipin (TLCL)

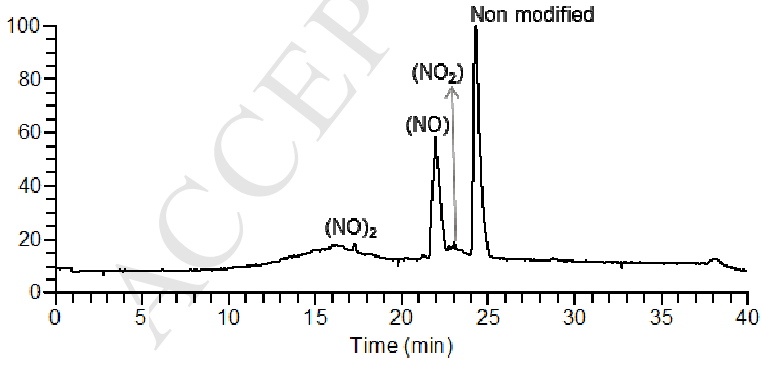
Table 1: Nitrated and nitroso products of tetralinoleoyl-cardiolipin (TLCL) formed by treatment with NO_2BF_4, identified by C30 RP LC-HR-MS as $[M-H]^-$ ions and confirmed by accurate mass measurements and MS/MS data analysis. The relative abundance RA (%) of the various nitrated species was determined by integrating the peak area for each species.

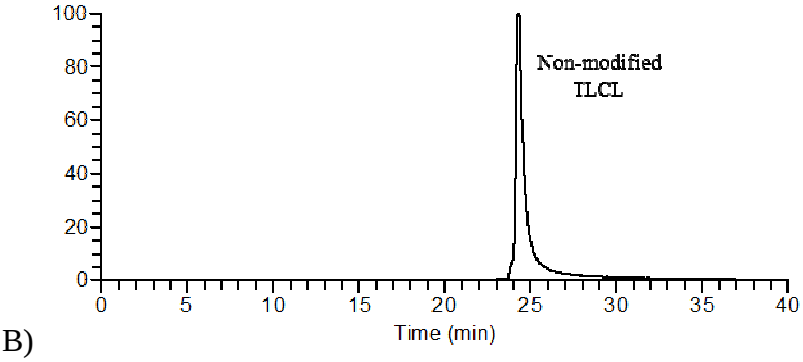
***m/z* theoretical**	***m/z* observed**	**Error (ppm)**	**Shift (Da)**	**Modification**	**RT (min)**	**RA (%)**
1447.9644	1447.9686	2.9	0	-	24.2	-
1476.9546	1476.9595	3.3	29	NO	21.9	60.9
1505.9447	1505.9497	3.3	58	$(NO)_2$	17.5	4.1
1505.9447	1505.9501	3.6	58	$(NO)_2$	18.3	3.8

1534.9349	1534.9396	3.1	87	$(NO)_3$	12.9	1.3
1563.9251	1563.9279	1.8	116	$(NO)_4$	6.2	2.9
1492.9495	1492.9542	3.2	45	NO_2	23.0	11.9
1492.9495	1492.9539	3.0	45	NO_2	21.8	2.6
1492.9495	1492.9518	1.6	45	NO+O	19.6	2.4
1521.9396	1521.9447	3.4	74	$(NO)(NO_2)$	21.9	1.7
1521.9396	1521.9446	3.3	74	$(NO)(NO_2)$	20.2	5.4
1537.9346	1537.9376	2.0	90	$(NO_2)_2$	21.3	1.3
1537.9346	1537.9373	1.8	90	$(NO)(NO_2)$+ O	18.3	1.5

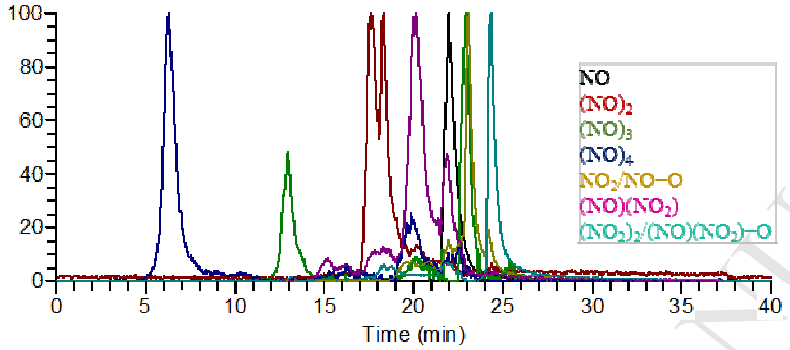
For each of the assigned ions, we plotted a reconstructed ion chromatogram (RIC) and, to confirm its identification (Figure 2), we analysed the HCD MS/MS spectra acquired for each RT of the peaks (also annotated in Table 1), using the conditions previously described. Overall, species with more modifications eluted at shorter retention times, as expected for the LC conditions used. In the following text, we will describe the results obtained for each of the ions presented in Table 1. By using LC-MS/MS data, we will explore the possibility of the presence of positional isomers and of structural isomers. For example, the compounds NO+O-TLCL and NO_2-TLCL are structural isomers and thus indistinguishable by exact mass measurements but can be separated using chromatography.

A)





B)

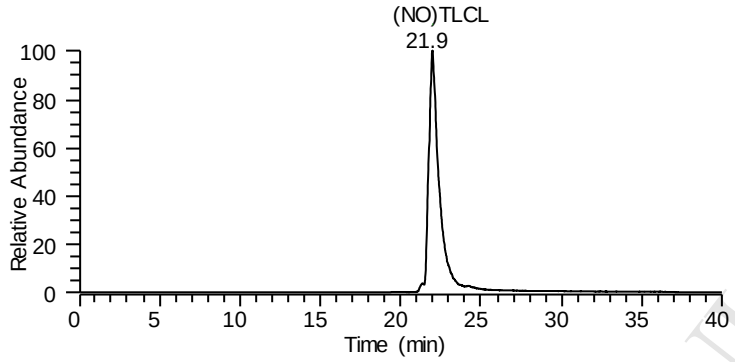


C)

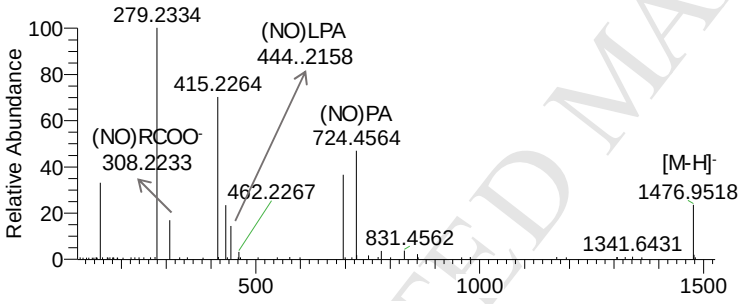
Figure 2- Total ion chromatogram (TIC) obtained after nitroxidation of TLCL (A), reconstructed ion chromatogram (RIC) of non modified TLCL (B) and RIC of the nitroso and nitrated derivatives (C) observed by C30 RP LC-HR-MS as $[M-H]^-$ ions and identified by accurate mass measurement and MS/MS fragmentation pattern, shown in Table 1.

In figure 3A, we show the RIC of the species annotated as NO-TLCL, $[M-H]^-$, at *m/z* 1476.95, corresponding to a mass shift of 29Da (NO) from the native TLCL. The observation of the RIC suggests the presence of only one isomer, although the NO modification could be present in each of the double bonds of the four linoleyl fatty acyl chains. The MS/MS spectra acquired at RT 21.9 min (Figure 3B), validated this identification. In fact, the presence of the diagnostic fragment ions of nitroso phosphatidic acid $(NO)PA^-$ at *m/z* 724.46, the carboxylate anion of the nitroso fatty acid $(NO)RCOO^-$ at *m/z* 308.22, and nitroso lysophosphatidic acid $(NO)LPA^-$ at *m/z* 444.22, allows

identifying this species (figure 3C). However, the neutral loss of HNO was not observed in this analysis, as reported in previous work using an ion trap instrument for nitroso PC and PE. [31] The MS/MS spectra do not give information allowing to assign the position in the fatty acid were NO group in linked. Thus, the structure present in Figure 2C is one possibility since nitration of linoleic FA can occur in C (9,10,12 or 13).



A)



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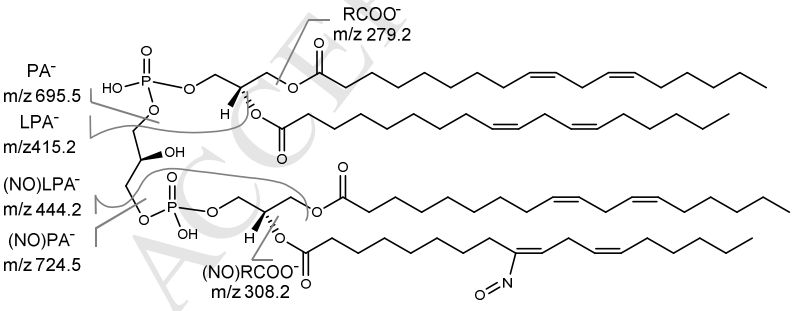
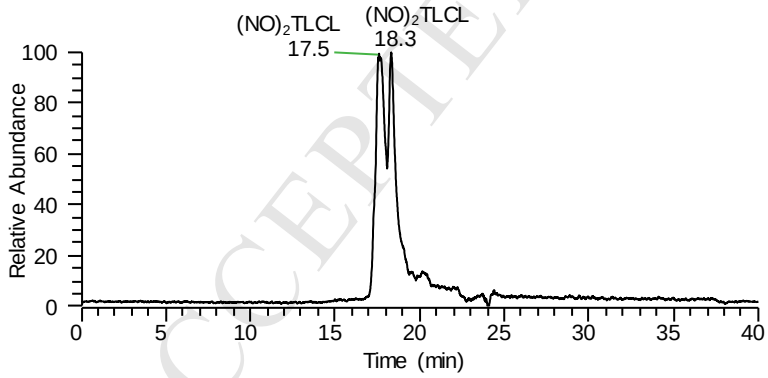


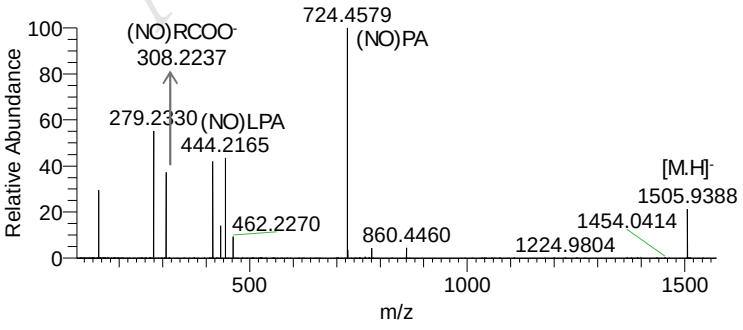
C)

Figure 3: A) Reconstructed ion chromatogram for species with a mass shift of +29Da. B) Correspondent HCD MS/MS spectrum of the species, acquired at RT 21.9 min. C) TLCL with main observed fragmentation pathways.

Figure 4A shows the RIC at *m/z* 1505.94, corresponding to a mass shift of 58Da from the native TLCL. These species were annotated in Table 1 as $(NO)_2$-TLCL. The RIC suggest that we are in the presence of two main isomers with RT of 17.5 min and 18.4 min. The MS/MS spectra acquired at RT 17.5 min show the fragment ions identified as nitroso phosphatidic acid fragments $(NO)PA^-$ at *m/z* 724.46, nitroso fatty acid $(NO)RCOO^-$ at *m/z* 308.22, and the nitroso lysophosphatidic acid $(NO)LPA^-$ at *m/z* 444.22 (figure 4B). These fragment ions suggest the presence of a dinitroso TLCL derivative, with each nitroso group present in different fatty acids and in a different glycerodiacyl monomer of the cardiolipin. The MS/MS of the species eluting latter, at RT 18.3 (figure 4C), show the fragment ion $(NO)_2PA^-$ at *m/z* 753.45 and the fragment ions $(NO)LPA^-$ at *m/z* 444.22 and $(NO)RCOO^-$ at *m/z* 308.22, indicating that, in this isomer, the two nitroso groups were in the same monomer of the cardiolipin, but not in the same linoleyl group. This identification also suggests that the modifications in the same monomer make the nitroso cardiolipin more apolar than when the same modifications occur in different monomers.



A)



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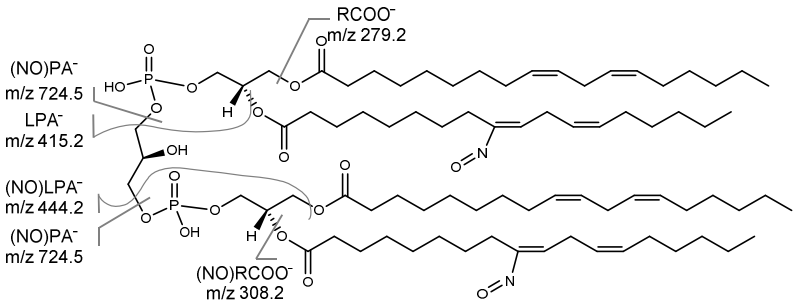

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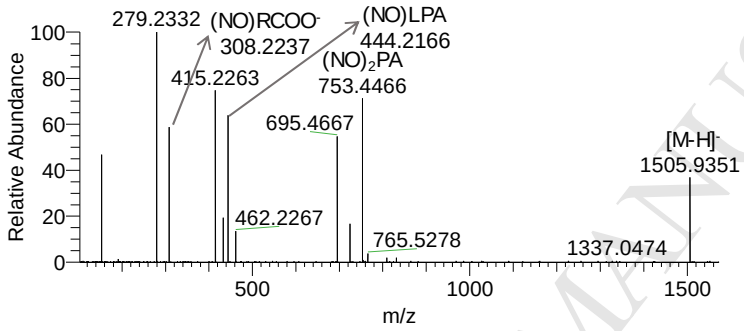

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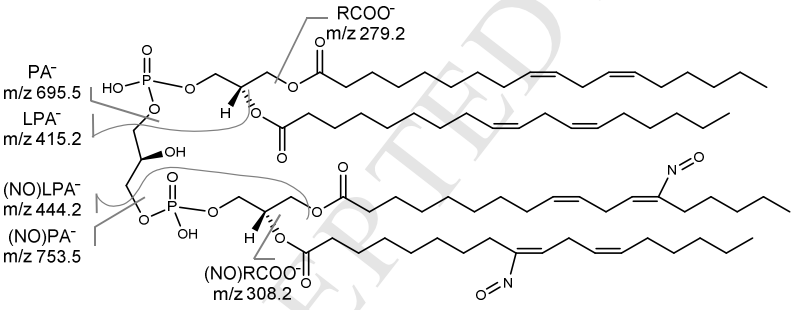
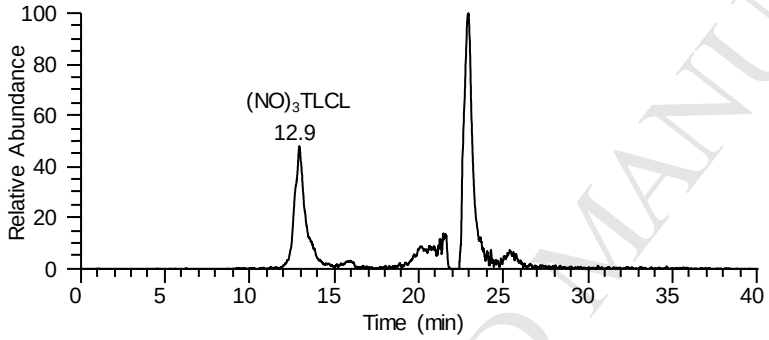


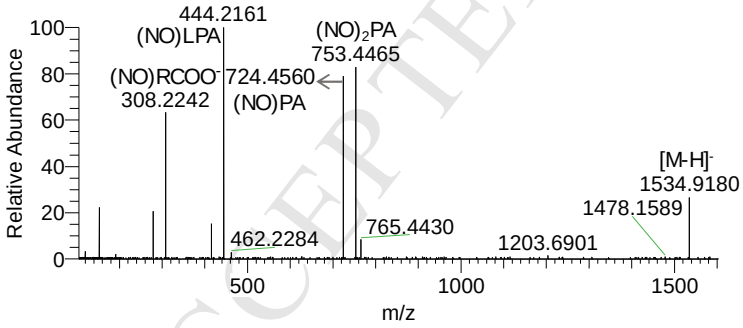
Figure 4: A) Reconstructed ion chromatogram for nitroso species $(NO)_2$-TLCL. MS/MS spectra of the ion at *m/z* 1505.94 acquired at B) RT 17.5 min with C) its fragmentation pathways and D) RT 18.3 min with E) its fragmentation pathways.

In figure 5A, we show the RIC of the species at *m/z* 1534.92, with a mass shift of 87Da from the native TLCL, corresponding to the addition of three nitroso groups, and annotated in Table 1 as $[M-H]^-$ of the $(NO)_3$-TLCL species. The observation of the RIC suggests the presence of, at least, one

abundant isomer that eluted at RT 12.9 min. The MS/MS spectra acquired at RT 12.9 min, shows the presence of the fragment ions $(NO)PA^-$ at *m/z* 724.46 and $(NO)_2PA^-$ at *m/z* 753.45 that validates the interpretation that the isomers bear two NO groups in one monomer and a third one in the other monomer. Each NO group seems to be in different fatty acyl chain as indicated by the presence of $(NO)LPA^-$ at *m/z* 444.22 and $(NO)RCOO^-$ at *m/z* 308.22, and due to the absence of the fragments of $(NO)_2LPA^-$ nor $(NO)_2RCOO^-$. The peak observed at RT 23.2 corresponded to an unrelated contaminant at *m/z* 1534.94.



A)



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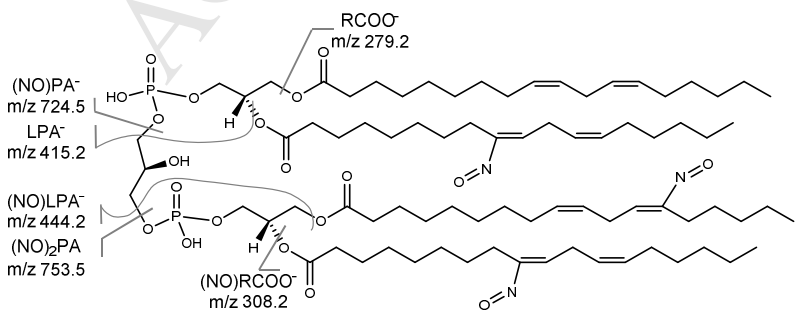
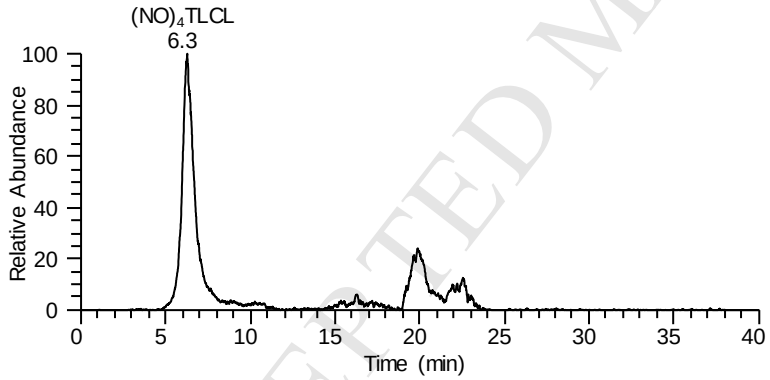


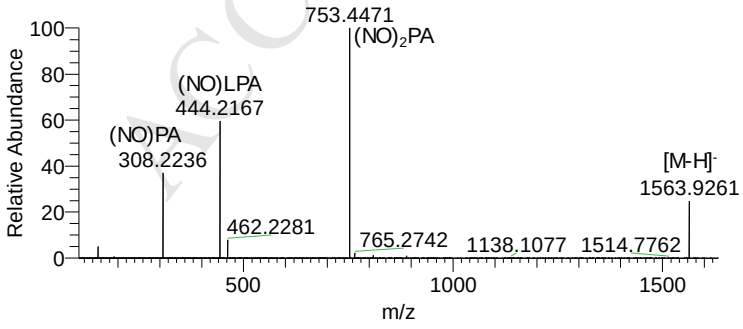
C)

Figure 5: A) Reconstructed ion chromatogram for nitroso species $(NO)_3$-TLCL. B) MS/MS spectrum of the ion at *m/z* 1534.92 acquired at RT 12.9 min with C) its fragmentation pathways.

In figure 6A, we show the RIC of the species at *m/z* 1563.93, with a mass shift of 116Da, corresponding to the addition of four nitroso groups to TLCL, and annotated as the molecular ion $[M\text{-}H]^-$ of $(NO)_4$-TLCL, in Table 1. The observation of the RIC suggests the presence of one isomer. The MS/MS spectra acquired at RT 6.2 min shows the presence of fragment ions identified as $(NO)PA^-$ at *m/z* 308.22 and $(NO)LPA^-$ at *m/z* 444.22 and $(NO)_2PA^-$ at *m/z* 753.45 (figure 6B). This fragmentation pattern is consistent with the presence of one compound with four NO-modified linoleyl fatty acyl chains, thus with each NO group linked to each linoleic acid.



A)



B)

C)

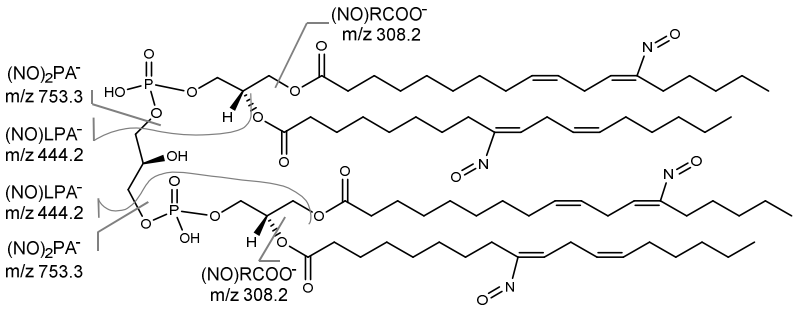
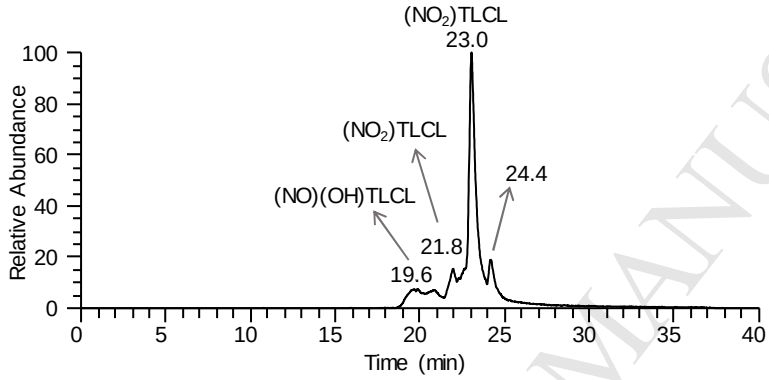


Figure 6- A) Reconstructed ion chromatogram for nitroso species $(NO)_4$-TLCL. B) MS/MS spectrum of the ion at *m/z* 1563.93 acquired at RT 6.2 min with C) its fragmentation pathways.

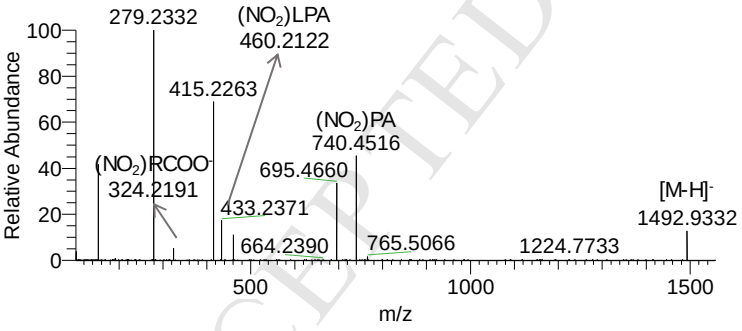
Figure 7A shows the RIC of the *m/z* 1492.93, corresponding to a mass shift of 45Da from the native TLCL. These species were annotated in Table 1 as NO_2-TLCL$^-$ and (NO)+(OH)-TLCL$^-$. The RIC shows an abundant species eluting at RT 23.0 min and several other minor species eluting before (RT 19.6 min; RT 21.8 min) and after (RT 24.4 min). The MS/MS spectrum acquired at RT 23.0 min (figure 7B) shows the fragment ions $(NO_2)PA^-$ at *m/z* 740.45, lysophosphatidic acid $(NO_2)LPA^-$ at *m/z* 460.21, and fatty acid carboxylate anions $(NO_2)RCOO^-$ at *m/z* 324.22. This suggests that this species corresponds to nitrated derivative, NO_2-TLCL. The presence of NO(OH)CL derivative isobaric of NO2CL, could not be excluded, although if it occurs, it is expected to be in lower abundance. The informative neutral loss of HNO_2 or HNO was not observed in this HCD MS/MS spectrum, as it has been previously reported in the CID-MS/MS of other nitrated phospholipids [28,31], thus inducing some uncertainty in the assignments.

Figure 7C shows the MS/MS spectrum of the ion at *m/z* 1492.93, acquired at RT 19.6 min. This spectrum shows the fragment ions identified as $(NO)PA^-$ at *m/z* 724.46 and $(PA+O)^-$ at *m/z* 711.46, $(NO)LPA^-$ at *m/z* 444.22 and $(LPA+O)^-$ at *m/z* 431.22, and $(NO)RCOO^-$ at *m/z* 308.22 and $(RCOO+O)^-$ at *m/z* 295.23. Altogether, this information strongly suggests that this compound is the $(NO+O\text{-}TLCL)^-$ isomer, thus a nitroso hydroxy derivative. Also, these fragment ions suggest that the two groups (nitroso, NO and hydroxy, OH) are present in different fatty acyl chains.

The MS/MS spectra acquired at RT 21.8 min (Figure 7D) is similar to the one described in figure 7B, corresponding to NO_2-TLCL. This most probably can be an indication that we are in the presence of an isomeric species, with the nitro group, NO_2, located in a different position of the fatty acyl chain or in other fatty acid in the TLCL, although the MS/MS spectra provide no evidence of the exact location of the modification. The MS/MS acquired at RT 24.4 min (Figure 7E) does not allow a positive confirmation of the species eluting at this RT.



A)



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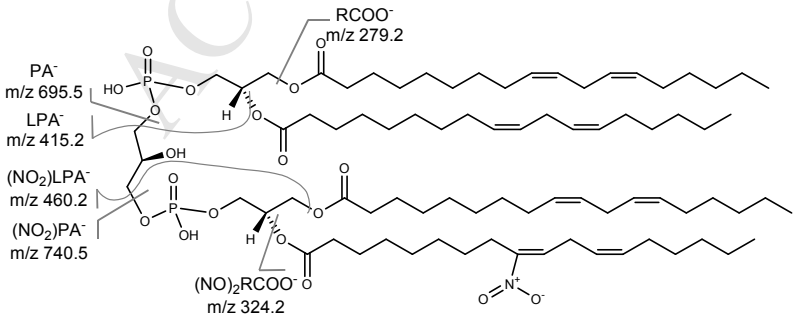
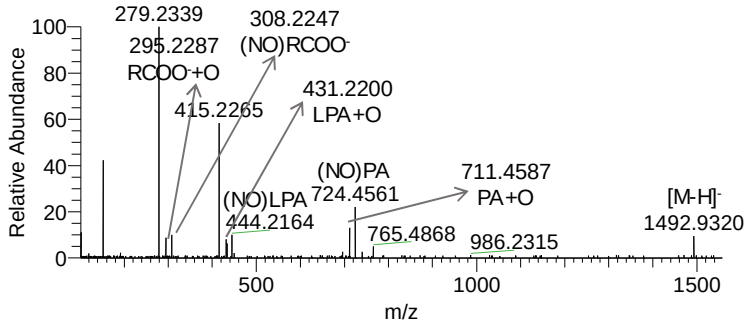
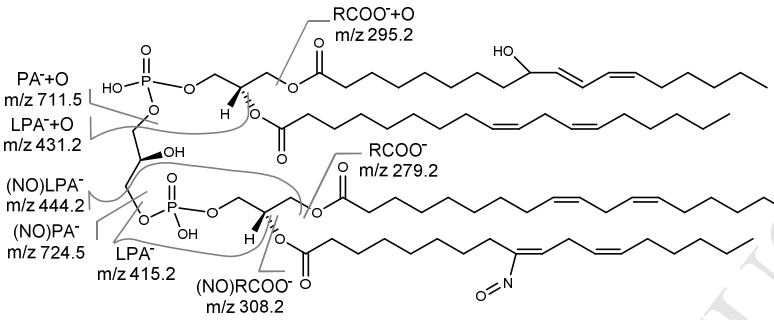


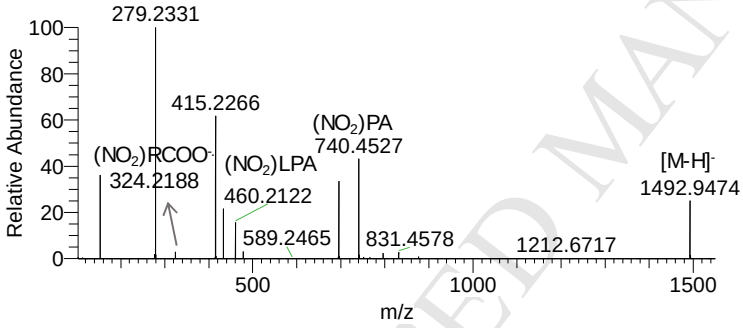
C)



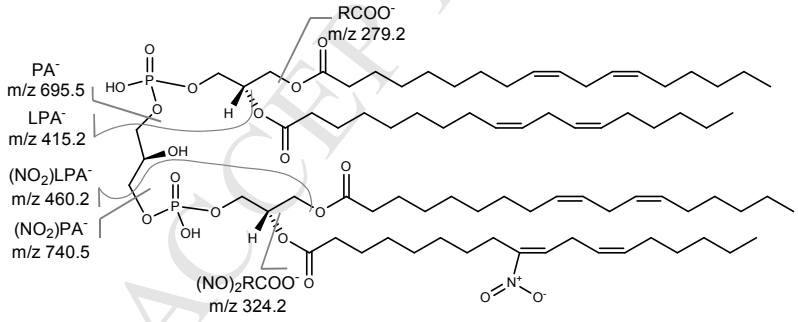
D)



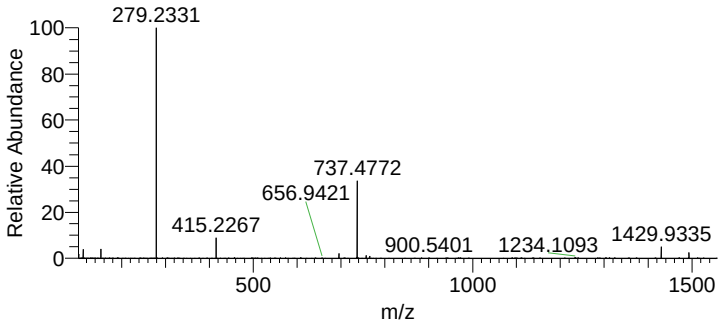
E)



F)



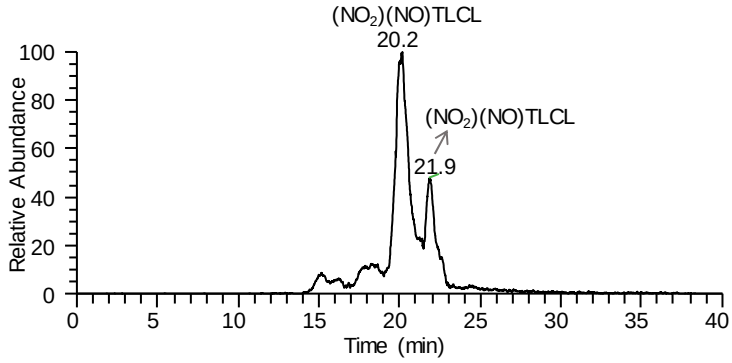
G)



H)

Figure 7: A) Reconstructed ion chromatogram for species *m/z* 1492.93, corresponding to a mass shift of 45Da from the native TLCL. MS/MS spectrum of the ion at *m/z* 1492.93 acquired at B) RT 23.0 min with C) its fragmentation pathways; D) RT 19.6 min with E) its fragmentation pathways; F) RT 21.8 min with G) its fragmentation pathways; and H) RT 24.4 min.

Figure 8A shows the RIC of the *m/z* 1521.93, corresponding to a mass shift of 74Da from the native TLCL. These species were annotated in Table 1 as $(NO+NO_2\text{-TLCL})^-$. The RIC shows two major peaks at RT 20.2 min and 21.9 min. The MS/MS spectrum acquired at RT 20.2min (figure 8B) shows the fragment ions $(NO)PA^-$ at *m/z* 724.45 and $(NO_2)PA^-$ at *m/z* 740.45 indicating that both modifications co-exist and are present in different fatty acyl chains. The MS/MS spectrum acquired at RT 21.9min (figure 7C) does not allow a positive confirmation of the species eluting at this RT. Nevertheless, the presence of the fragment ion at *m/z* 308.22 identified as $(NO)RCOO^-$ also suggest the presence of a $(NO+NO_2\text{-TLCL})^-$, although this fragment ion can also be present due to carryover.



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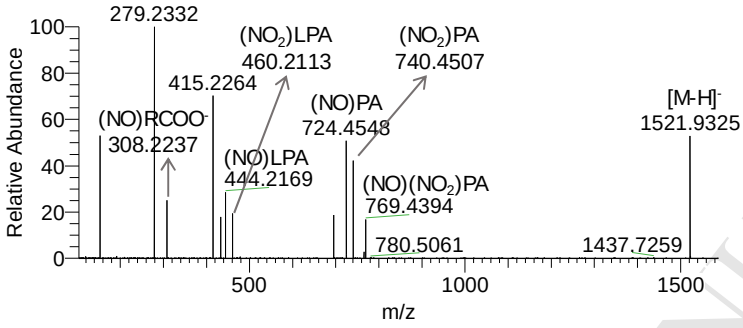

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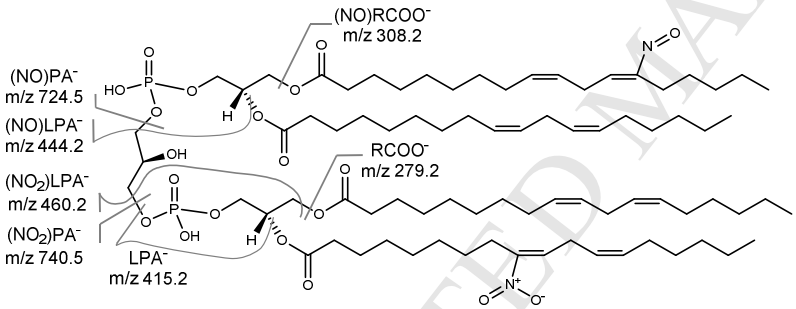

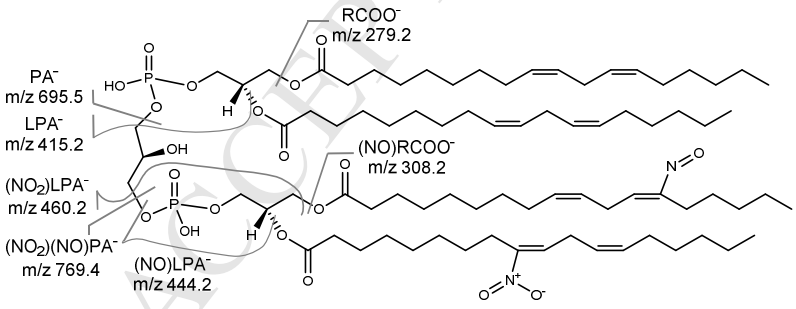

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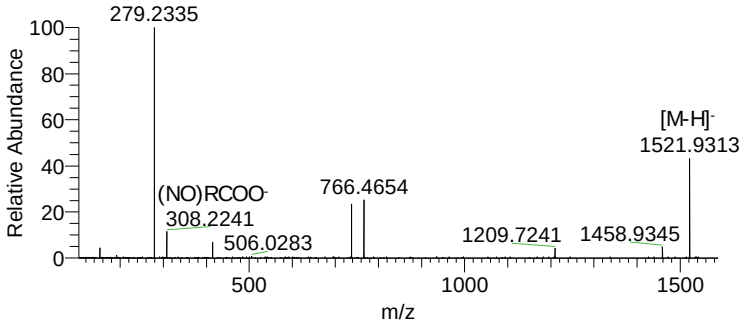
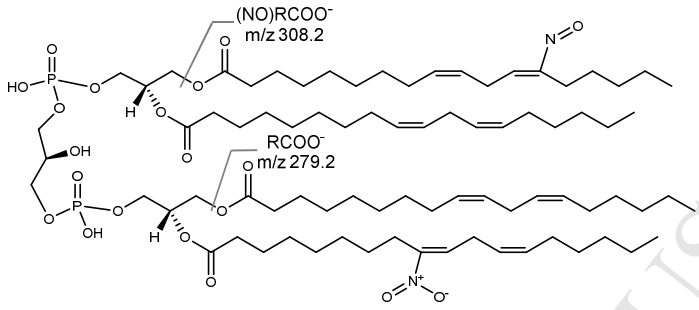


D)

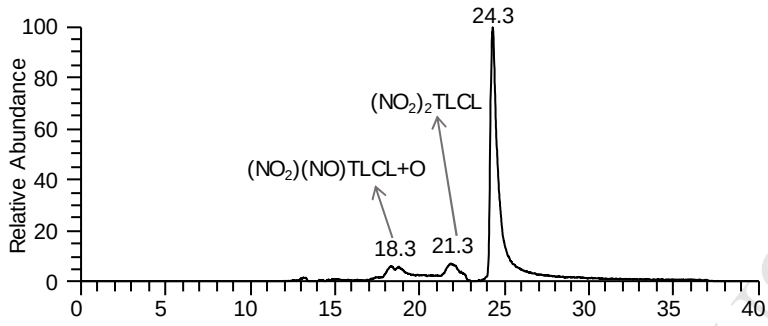


E)

Figure 8: A) Reconstructed ion chromatogram for species *m/z* 1521.93, corresponding to a mass shift of 74Da from the native TLCL. MS/MS spectrum of the ion at *m/z* 1521.93 acquired at B) RT 20.2 min with C) fragmentation pathways for the two possible positional isomers, and D) at 21.9 min with E) its fragmentation pathways.

Figure 9A shows the RIC of the *m/z* 1537.93, corresponding to a mass shift of 90Da from the native TLCL. These species were annotated in Table 1 as $((NO_2)_2$-TLCL$)^-$ and $((NO)(NO_2)$+ O-TLCL$)^-$, eluting at RT 21.3 and 18.3, respectively. The MS/MS spectrum acquired at RT 21.3min (Figure 9B) shows the fragment ion $(NO_2)PA^-$ at *m/z* 740.45. The absence of a fragment ion corresponding to $(NO_2)_2PA^-$ suggest that each of the NO_2 group is located in different monomers. The MS/MS spectrum acquired at RT 18.3min (figure 9C) shows the fragment ions $(NO)PA^-$ at *m/z* 724.46 and $(NO)LPA^-$ at *m/z* 444.21, suggesting that this is a nitroso cardiolipin. Although no fragment ion evidencing the presence of a NO_2 group was observed, the exact mass measurements and the retention time

suggest that this is ((NO)(NO_2)+O-TLCL)$^-$. The MS/MS spectrum acquired at RT 24.3min (figure 9D) does not allow performing a positive identification of this species, corresponding to an unrelated contaminant, probably arising from the nitration reaction.



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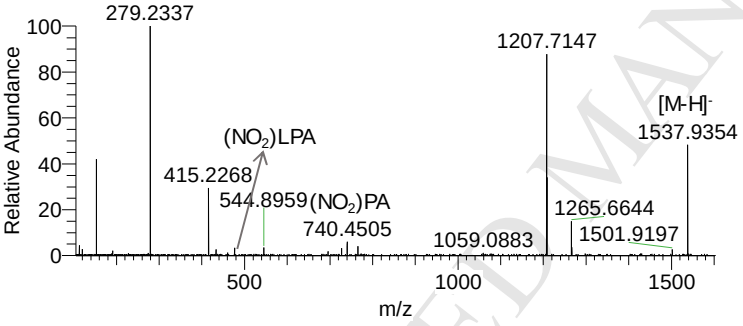

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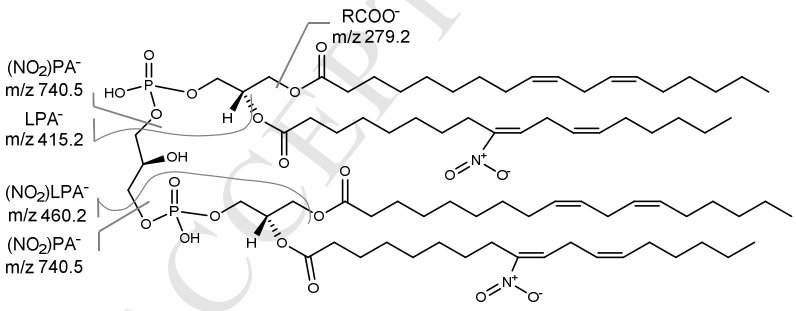

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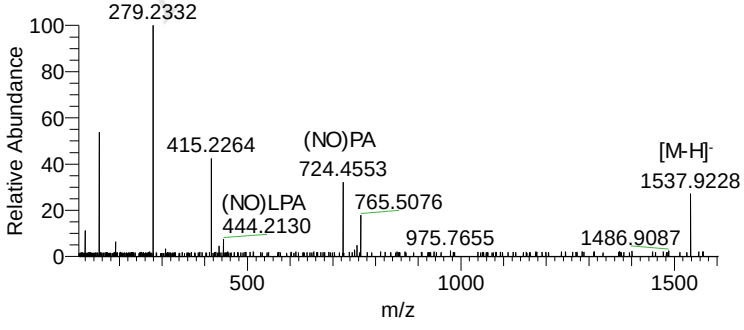

D)

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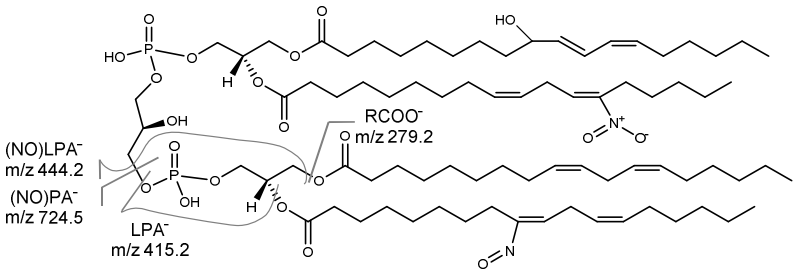

F)

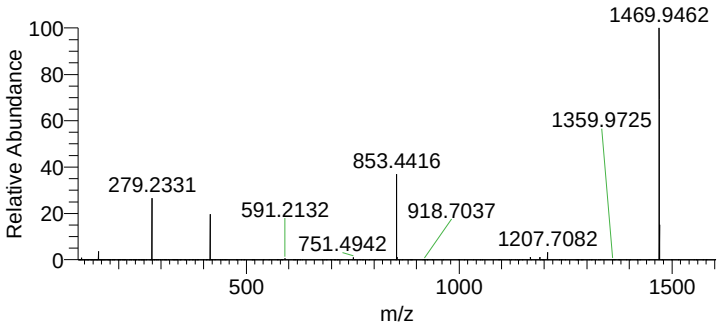

Figure 9: A) Reconstructed ion chromatogram for species at *m/z* 1537.93, corresponding to a mass shift of 90Da from the native TLCL. MS/MS spectra of the ion *m/z* 1537.93 acquired at B) 21.3 min with C) its fragmentation pathways; D) 18.3 min with E) its fragmentation pathways, and F) at 24.3 min.

Discussion

Cardiolipins are structurally a unique phospholipid group, consisting of a dimeric structure, that forms clusters in cell membranes, stabilizing the geometry of curved regions of the membrane [37]. Due to its characteristic structure, cardiolipins form a very large group of diverse molecules, since they include a different combination of fatty acyl chains, in its tetrameric structure, and also with large pools of structural isomers. Recently, using a C8 RP column, G. Oemer *et al.* separated 135 cardiolipins with different masses and referred to the presence of several isobaric CL with limited different retention times [4]. For several years we have been working to characterize biologically-relevant modified phospholipids, mainly by oxidative stress, including the identification of oxidized

cardiolipin (OxCL) that allowed to identify and characterize by MS-based approaches different structural and isomeric OxCL [10,14,16]. Lately, we have published a series of papers identifying phospholipid nitroxidation using LC-MS approaches, through Hydrophilic Interaction Liquid Chromatography-Mass Spectrometry (HILIC-ESI-MS) [28], and reversed phase C5 LC-MS [31]. In these papers, we focused on the identification of nitroalkenes derivatives of phosphatidylcholines, phosphatidylethanolamines, and phosphatidylserines. These LC/MS approaches suffer from a lack of resolution and the ability to separate isomers. The present study extends our previous structural identification platform, by utilizing a C30-ESI-LC-MS and HCD MS/MS analysis to identify nitrated cardiolipins.

The use, for the first time of this platform for the identification of nitrated cardiolipin, has allowed us to separate and identify several isomers of nitroso-TLCL, nitro-TLCL, nitronitroso-TLCL and nitroxidized CL derivatives. In this C30 column TLCL elutes with an RT of 22.3 min, and as expected, nitrated compounds, because they are more polar, elute with a shorter RT. Also, the nitroso compound has shorter retention times than the corresponding nitro derivative. For example, the NO-TLCL has an RT of 21.9 min while the NO_2-TLCL has a retention time of 23.1 min. Remarkably, this platform also allowed to separate positional isomers with good resolution. For example, two different isomers of dinitroso TLCL, $(NO)_2$-TLCL isomers were separated, with an RT difference of 48 seconds and identified (figure 3A). As previously described, the MS/MS data allowed to suggest that one of the isomers had the modification on different monomers of the molecule, while the other had the two nitroso groups in the same monomer of the cardiolipin, but in the different linoleyl group. This data that was further corroborated by other isomers described in this study, suggesting that in linoleyl fatty acids, nitration does not co-occur in the same fatty acid.

By using the platform described in this study, we were also able to resolve and identify several isomeric structures. For example, for the ion at *m/z* 1537.93, corresponding to a mass shift of 90Da from the native TLCL, we were able to identify 3 structures, $((NO_2)_2$-TLCL$)^-$, $((NO)(NO_2)$+O-TLCL$)^-$ and

$((NO)_2$+2O-TLCL)$^-$, with considerable differences in the retention times (RT 21.3, 18.3 and 13.7, respectively) (figure 8A).

The results of this study clearly show that under biomimetic lipid nitration using NO_2BF_4, a wide variety of nitrated structures are formed. Altogether, we have separated and identified in this study 11 different nitrated compounds comprising 7 different mass shifts. Thus, considering the different possible combination of FA in CL in biological samples, the possibility of different nitrated cardiolipins in biological samples increase exponentially, making difficult its detection and identification. To this end, our results show that it is vital to use a high-sensibility, high-resolution instrument, together with good chromatographic selectivity, able to separate isomeric and close related isomeric nitrated cardiolipins.

In this work, we have used higher-energy collision dissociation, HCD-MS/MS spectra to identify the nitrated cardiolipin compounds. These spectra showed the main patterns previously observed in CL [14–16,38]. The main, most informative fragmentation pathways included the cleavage to phosphatidic acid monomers (PA^-) fragment ions, that may lose fatty acids yielding the lysophosphatidic acid monomers (LPA^-), and the carboxylate product ions (RCOO-). PA^- and LPA^- fragment ions were also observed with remaining glycerol (+56) or glycerol-phosphate (+136) moieties from the other monomer of the molecule. Remarkably, the typical neutral losses associated with the modifying moieties, such as neutral loss (NL) of HNO (NL of 31 Da), NO (NL 30) or HNO_2 (NL of 47 Da) from the NO or NO_2 nitrated TLCL precursor ions were not observed, in contrast with the previous works [28,31], which were performed using low-energy collision-induced dissociation (CID). HCD uses higher energy dissociations (<100eV) than those used in resonant-excitation CID (<2 eV), enabling different and more abundant fragmentation pathways [39]. As such, it is important to select the diagnostic ions according to the fragmentation method that it is used.

Conclusions

We have applied C30LC-HRMS and HCD MS/MS to study the nitration products of tetralinoleoyl cardiolipin, after biomimetic lipid nitration using NO_2BF_4. The system performed with high-sensibility, high-resolution and high chromatographic selectivity, allowing to separate and identify a wide variety of nitrated structures. We have separated and identified 11 different nitrated compounds comprising 7 different classes of nitro, nitroso, nitronitroso, and nitroxidized TLCL derivatives. The system also allowed to separate isomers with good chromatographic selectivity. These nitrated cardiolipins were further identified by analysing the LC-HCD-MS/MS spectra. The observed fragmentation pathways included the cleavage to phosphatidic acid monomers (PA^-), lysophosphatidic acid monomers (LPA^-) and carboxylate product ions with the modifying nitro, nitroso, nitronitroso, or nitroxidized moieties, which allowed to identify the nitrated compounds.

Acknowledgements

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References

[1] G. Paradies, V. Paradies, V. De Benedictis, F.M. Ruggiero, G. Petrosillo, Functional role of cardiolipin in mitochondrial bioenergetics, Biochim. Biophys. Acta - Bioenerg. 1837 (2014)

408–17. doi:10.1016/j.bbabio.2013.10.006.

[2] Y.A. Vladimirov, E. V. Proskurnina, A. V. Alekseev, Molecular mechanisms of apoptosis. Structure of cytochrome c-cardiolipin complex, Biochem. 78 (2013) 1086–1097. doi:10.1134/S0006297913100027.

[3] J.J.R. Krebs, H. Hauser, E. Carafoli, Asymmetric Distribution of Phospholipids in the Inner Membrane of Beef Heart Mitochondria*, J. Bio. Chem. 254 (1979) 5308–5316.

[4] G. Oemer, K. Lackner, K. Muigg, G. Krumschnabel, K. Watschinger, S. Sailer, H. Lindner, E. Gnaiger, S.B. Wortmann, E.R. Werner, J. Zschocke, M.A. Keller, Molecular structural diversity of mitochondrial cardiolipins, Proc. Natl. Acad. Sci. 115 (2018) 4158 LP-4163. doi:10.1073/pnas.1719407115.

[5] X. Han, K. Yang, J. Yang, H. Cheng, R.W. Gross, Shotgun lipidomics of cardiolipin molecular species in lipid extracts of biological samples., J. Lipid Res. 47 (2006) 864–79. doi:10.1194/jlr.D500044-JLR200.

[6] V.E. Kagan, Y.Y. Tyurina, V.A. Tyurin, D. Mohammadyani, J.P.F. Angeli, S. V Baranov, J. Klein-Seetharaman, R.M. Friedlander, R.K. Mallampalli, M. Conrad, H. Bayir, Cardiolipin Signaling Mechanisms: Collapse of Asymmetry and Oxidation, Antioxid. Redox Signal. 22 (2015) 1667–1680. doi:10.1089/ars.2014.6219.

[7] A. Reis, C.M. Spickett, Chemistry of phospholipid oxidation, Biochim. Biophys. Acta - Biomembr. 1818 (2012) 2374–2387. doi:10.1016/J.BBAMEM.2012.02.002.

[8] C.M. Spickett, A.R. Pitt, Oxidative lipidomics coming of age: advances in analysis of oxidized phospholipids in physiology and pathology., Antioxid. Redox Signal. 22 (2015) 1646–1666. doi:10.1089/ars.2014.6098.

[9] 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. 5 (2015) 253–266. doi:10.1016/j.redox.2015.05.003.

[10] Y.Y. Tyurina, R.M. Domingues, V.A. Tyurin, E. Maciel, P. Domingues, A.A. Amoscato, H. Bayir, V.E. Kagan, Characterization of cardiolipins and their oxidation products by LC–MS analysis, Chem. Phys. Lipids. 179 (2014) 3–10. doi:10.1016/j.chemphyslip.2013.12.003.

[11] S.L. Iverson, S. Orrenius, The cardiolipin-cytochrome c interaction and the mitochondrial regulation of apoptosis., Arch. Biochem. Biophys. 423 (2004) 37–46.

[12] V. A. Vladimir Tyurin, Y.Y. Tyurina, M.-Y. Jung, M.A. Tungekar, K.J. Wasserloos, H. Bayır, J.S. Greenberger, P.M. Kochanek, A.A. Shvedova, B. Pitt, V.E. Kagan, Mass-spectrometric analysis of hydroperoxy-and hydroxy-derivatives of cardiolipin and phosphatidylserine in cells and tissues induc1, J. Chromatogr. B. 877 (2009) 2863–2872. doi:10.1016/j.jchromb.2009.03.007.

[13] H. Yin, M. Zhu, Free radical oxidation of cardiolipin: chemical mechanisms, detection and implication in apoptosis, mitochondrial dysfunction and human diseases, Free Radic. Res. 46 (2012) 959–974. doi:10.3109/10715762.2012.676642.

[14] E. Maciel, P. Domingues, M.R.M. Domingues, Liquid chromatography/tandem mass spectrometry analysis of long-chain oxidation products of cardiolipin induced by the hydroxyl radical, Rapid Commun. Mass Spectrom. 25 (2011) 316–326. doi:10.1002/rcm.4866.

[15] J. Kim, P.E. Minkler, R.G. Salomon, V.E. Anderson, C.L. Hoppel, Cardiolipin: characterization of distinct oxidized molecular species., J. Lipid Res. 52 (2011) 125–35. doi:10.1194/jlr.M010520.

[16] E. Maciel, P. Domingues, D. Marques, C. Simões, A. Reis, M.M. Oliveira, R.A. Videira, F. Peixoto, M.R.M. Domingues, Cardiolipin and oxidative stress: Identification of new short chain oxidation products of cardiolipin in in vitro analysis and in nephrotoxic drug-induced disturbances in rat kidney tissue, Int. J. Mass Spectrom. 301 (2011) 62–73. doi:10.1016/J.IJMS.2010.06.036.

[17] Y.Y. Tyurina, D.E. Winnica, V.I. Kapralova, A.A. Kapralov, V.A. Tyurin, V.E. Kagan, LC/MS

characterization of rotenone induced cardiolipin oxidation in human lymphocytes: Implications for mitochondrial dysfunction associated with Parkinson's disease, Mol. Nutr. Food Res. 57 (2013) 1410–1422. doi:10.1002/mnfr.201200801.

[18] Y.Y. Tyurina, V.A. Tyurin, A.M. Kaynar, V.I. Kapralova, K. Wasserloos, J. Li, M. Mosher, L. Wright, P. Wipf, S. Watkins, B.R. Pitt, V.E. Kagan, Oxidative lipidomics of hyperoxic acute lung injury: mass spectrometric characterization of cardiolipin and phosphatidylserine peroxidation, Am. J. Physiol. Cell. Mol. Physiol. 299 (2010) L73–L85. doi:10.1152/ajplung.00035.2010.

[19] Y.Y. Tyurina, V.A. Tyurin, V.I. Kapralova, K. Wasserloos, M. Mosher, M.W. Epperly, J.S. Greenberger, B.R. Pitt, V.E. Kagan, Oxidative Lipidomics of γ-Radiation-Induced Lung Injury: Mass Spectrometric Characterization of Cardiolipin and Phosphatidylserine Peroxidation, Radiat. Res. 175 (2011) 610–621. doi:10.1667/RR2297.1.

[20] Y.Y. Tyurina, V.A. Tyurin, M.W. Epperly, J.S. Greenberger, V.E. Kagan, Oxidative lipidomics of γ-irradiation-induced intestinal injury, Free Radic. Biol. Med. 44 (2008) 299–314. doi:10.1016/J.FREERADBIOMED.2007.08.021.

[21] R. Faria, M.M. Santana, C.A. Aveleira, C. Simões, E. Maciel, T. Melo, D. Santinha, M.M. Oliveira, F. Peixoto, P. Domingues, C. Cavadas, M.R.M. Domingues, Alterations in phospholipidomic profile in the brain of mouse model of depression induced by chronic unpredictable stress, Neuroscience. 273 (2014) 1–11. doi:10.1016/J.NEUROSCIENCE.2014.04.042.

[22] M.C. Martínez, R. Andriantsitohaina, Reactive Nitrogen Species: Molecular Mechanisms and Potential Significance in Health and Disease, Antioxid. Redox Signal. 11 (2009) 669–702. doi:10.1089/ars.2007.1993.

[23] D.A. Butterfield, T. Reed, R. Sultana, Roles of 3-nitrotyrosine- and 4-hydroxynonenal-modified

brain proteins in the progression and pathogenesis of Alzheimer's disease., Free Radic. Res. 45 (2011) 59–72. doi:10.3109/10715762.2010.520014.

[24] M.W. Foster, D.T. Hess, J.S. Stamler, Protein S-nitrosylation in health and disease: a current perspective, Trends Mol. Med. 15 (2009) 391–404. doi:10.1016/j.molmed.2009.06.007.

[25] A.J. Deen, V. Sihvola, J. Härkönen, T. Patinen, S. Adinolfi, A.-L. Levonen, Regulation of stress signaling pathways by nitro-fatty acids, Nitric Oxide. (2018) S1089-8603(17)30323–3. doi:10.1016/J.NIOX.2018.03.012.

[26] 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. 21 (2016) 873–89.

[27] F.J. Schopfer, Y. Lin, P.R.S. Baker, T. Cui, M. Garcia-Barrio, J. Zhang, K. Chen, Y.E. Chen, B.A. Freeman, Nitrolinoleic acid: an endogenous peroxisome proliferator-activated receptor gamma ligand, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 2340–2345. doi:10.1073/pnas.0408384102.

[28] 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. 88 (2016) 2622–2629. doi:10.1021/acs.analchem.5b03407.

[29] A. Trostchansky, L. Bonilla, L. González-Perilli, H. Rubbo, Nitro-Fatty Acids: Formation, Redox Signaling, and Therapeutic Potential, Antioxid. Redox Signal. 19 (2013) 1257–1265. doi:10.1089/ars.2012.5023.

[30] 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. 407 (2015) 5587–5602. doi:10.1007/s00216-015-8766-3.

[31] T. Melo, P. Domingues, T.M. Ribeiro-Rodrigues, H. Girão, 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 Radic. Biol. Med. 106 (2017) 219–227. doi:10.1016/j.freeradbiomed.2017.02.033.

[32] B. Neves, P. Domingues, M. Oliveira, M. Domingues, T. Melo, B. Neves, P. Domingues, M.M. Oliveira, M. do R. Domingues, T. Melo, Profile of Phosphatidylserine Modifications under Nitroxidative Stress Conditions Using a Liquid Chromatography-Mass Spectrometry Based Approach, Molecules. 24 (2018) 107. doi:10.3390/molecules24010107.

[33] T. Melo, S.S. Marques, I. Ferreira, M.T. Cruz, P. Domingues, M.A. Segundo, M.R.M. Domingues, New Insights into the Anti-Inflammatory and Antioxidant Properties of Nitrated Phospholipids, Lipids. 53 (2018) 117–131. doi:10.1002/lipd.12007.

[34] F.J. Larsen, T.A. Schiffer, E. Weitzberg, J.O. Lundberg, Regulation of mitochondrial function and energetics by reactive nitrogen oxides, Free Radic. Biol. Med. 53 (2012) 1919–1928. doi:10.1016/j.freeradbiomed.2012.08.580.

[35] S.R. Woodcock, G. Bonacci, S.L. Gelhaus, F.J. Schopfer, Nitrated fatty acids: Synthesis and measurement, Free Radic. Biol. Med. 59 (2013) 14–26. doi:10.1016/j.freeradbiomed.2012.11.015.

[36] E.M. Bartlett, D.H. Lewis, Spectrophotometric determination of phosphate esters in the presence and absence of orthophosphate, Anal. Biochem. 36 (1970) 159–167. doi:10.1016/0003-2697(70)90343-X.

[37] K.C. Huang, R. Mukhopadhyay, N.S. Wingreen, A curvature-mediated mechanism for localization of lipids to bacterial poles, PLoS Comput. Biol. 2 (2006) e151. doi:10.1371/journal.pcbi.0020151.

[38] F.F. Hsu, J. Turk, E.R. Rhoades, D.G. Russell, Y. Shi, E.A. Groisman, Structural characterization of cardiolipin by tandem quadrupole and multiple-stage quadrupole ion-trap mass

spectrometry with electrospray ionization, J. Am. Soc. Mass Spectrom. 16 (2005) 491–504. doi:10.1016/j.jasms.2004.12.015.

[39] J. V. Olsen, B. Macek, O. Lange, A. Makarov, S. Horning, M. Mann, Higher-energy C-trap dissociation for peptide modification analysis, Nat. Methods. 4 (2007) 709–1. doi:10.1038/nmeth1060.

Liquid chromatography/tandem mass spectrometry characterization of nitroso, nitrated and nitroxidized cardiolipin products.

Highlights

- C30 LC-MS allows to separate nitro-oxidation products of nitroxidized TLCL.
- C30 LC-MS separated and identified 11 different nitrated compounds.
- HCD-MS/MS showed classifying fragmentation pathways
- HCD-MS/MS fragmentation confirmed the identity of each isomer after LC separation.