Lipidomic analysis of polyunsaturated fatty acids and their oxygenated metabolites in plasma by solid-phase extraction followed by LC-MS.

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Abstract

The present work describes the development of a robust and sensitive targeted analysis platform for the simultaneous quantification in blood plasma of lipid oxygenated mediators and fatty acids using Solid-phase Extraction (SPE) and high performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS). The concurrent analysis of these lipid mediators is challenging because of their instability, differences in solubility and the often occurrence of isobaric forms with similar fragmentation patterns. Results demonstrated that the reduction of SPE temperature to 4ºC is a critical parameter for preserving the hydroperoxy derivatives. Polymeric HLB cartridges increased 40-50% ARA, EPA and DHA sensitivity compared to C18 sorbent, and also provided higher global performance for most hydroxides and other oxidation products. The proposed method yields for two tested mass analyzers high sensitivity, good linearity and reproducibility, with detection limits ranging 0.002-7 ng/mL and global recoveries as high as 85-112%. However, it should be noted the additional advantage of the linear ion trap (LIT) mass analyzer acquiring in full scan product ion mode, compared to the triple quadrupole (QqQ) acquiring in Multiple Reaction Monitoring (MRM): the full scan product ion mode provides the full fragmentation spectra of compounds that allowed to discriminate coeluting isomers and false positive identifications without additional chromatography development. The proposed lipidomic procedure demonstrates to be confident, simple and sensitive to profile in plasma a wide range of lipid eicosanoid and docosanoid mediators, including innovatively the analysis of hydroperoxy congeners and non-oxidized PUFA precursors.

Keywords

Oxygenated lipid mediators, solid-phase extraction, liquid chromatography, mass spectrometry, triple quadrupole, ion trap.

1. Introduction

Polyunsaturated fatty acids (PUFAs) exhibit a range of biological effects, many of which are mediated by the formation of lipid derivatives. Such metabolites are produced *in vivo* through the action of cyclooxygenases (COXs), lipooxygenases (LOXs), cytochrome P450 monooxygenases (CYP450) and/or free radical oxidation mechanisms [1]. PUFAs with twenty carbons like arachidonic acid (ARA) and eicosapentaenoic acid (EPA) are precursors of many eicosanoids like prostanoids (prostaglandins and tromboxanes) and leukotrienes, a family of compounds involved in inflammatory processes [2]. From ARA, series-2 prostanoids like PGE_2, PGD_2 or TXB_2 and series-4 leukotrienes like LTB_4 and also hydroxyeicosatetraenoic acids (HETEs) are generated by COXs and LOXs activity [3]; whilst CYP450 and autoxidation reactions result in various hydroxy-, hydroperoxy-, epoxy-fatty acids and F_2-isoprostanes [4]. In a similar

manner, from EPA derive many lipid compounds including series-3 prostanoids (e.g., PGE_3, PGD_3 and TXB_3), series-5 leukotrienes (e.g., LTB_5), hydroxy- and hydroperoxy-eicosapentaenoic acids (HEPEs and HpEPEs) [5][6]. From EPA also derive F_3-isoprostanes (a group of analgesic compounds) [2][7], and series-E resolvins (e.g., RvE_1 and RvE_2) which are a novel discovered family of trihydroxy congeners that result from COX-2 activity and act as anti-inflammatory mediators [3][8].

Enzymatic oxidation or autoxidation of docosahexaenoic acid (DHA) produces hydroxy- docosahexaenoic acids (HDoHEs) and hydroperoxy- docosahexaenoic acids (HpDoHEs); moreover, it has been recently shown that DHA gives rise to a novel family of strong anti-inflammatory compounds termed neuroprostanes with dihydroxy (protectins, PD) and trihydroxy (series-D resolvins) structures [3][8]. (Figure S1 of Electronic Supplementary Material shows the similar formation cascades of ARA, EPA and DHA fatty acids).

Oxygenated metabolites of PUFAs have an important role in a wide range of biological functions. They are part of membrane cells, activate gene transcription, and are also involved in many diseases and inflammatory processes [9][10]. Therefore, these lipid mediators have been investigated in biomarkers discovery and drug development studies [3]. Specific examples include the pro-inflammatory role of ARA eicosanoids and isoprostanes metabolites in many processes related to oxidative stress, such as hypercholesterolemia [11], liver cirrhosis [12], Crest syndrome [13], myocardial reperfusion [14] or type-2 diabetes [15][16]. On the other hand, many hydroxy derived compounds of EPA and DHA are involved in the regulation of vascular tone [17], arteriosclerosis [18] or Alzheimer [19], and are considered markers of lipid peroxidation. Finally, novel studies have related anti-inflammatory and cellular protective activities of RvE_1, RvD_1 and PD_1 [20] to benefits in cardiovascular diseases, dry eyes or even Alzheimer [21][22].

The analytical determination of oxygenated PUFA metabolites is a challenging task, mainly because of their low physiological levels and large number of isomers with very similar physicochemical properties [10]. Therefore, a sensitive and selective detection procedure following an effective separation step is essential to comprehensively study this class of lipids [23]. Methods used for the determination of these lipid mediators currently involve an extraction and/or pre-concentration step followed by the chromatographic separation and mass spectrometry determination. The most common Solid-phase extraction (SPE) sorbents are C18 conventional cartridges [16][24][25], and polymeric sorbents like Oasis-HLB [26][27][28] or Strata-X [29][30]. For many years, gas chromatography coupled to mass spectrometry (GC/MS) was the method of choice for eicosanoids analysis [15][17][31]. Nowadays, the widespread liquid chromatography/mass spectrometry (LC-MS) coupled with high-sensitivity electrospray ionization (ESI) has provided a new approach for quantification, minimizing sample preparation requirements and particularly avoiding derivatization reactions [3][24][25][32][33]. The triple quadrupole (QqQ) mass spectrometer working in the multiple reaction monitoring (MRM) mode [16][26][28][29][30], together with hybrid variants such as quadrupole ion trap (Q-Trap) [27][34] and quadrupole time-of-flight (Q-TOF) [35][36], have become the MS technology of choice for analysis of oxygenated PUFA metabolites.

The aim of this research was to develop an analytical methodology based on SPE and LC-MS, to allow the simultaneous detection and quantification of a wide range of lipid mediators in plasma. PUFA derivatives like prostaglandins, leukotrienes, tromboxanes, hydroxy and hydroperoxy acids, isoprostanes, resolvins and protectins derived from ARA, EPA and DHA and their fatty acid precursors were the target analytes. The proposed method innovatively attempts to analyze PUFA metabolites with a wide range of polarity (octanol-water partition coefficients ranged from 1.1 of PGD_3 to 7.3 of DHA), concentration (up to 4 orders of magnitude), stability diversity (hydroperoxides are highly unstable), and applies polymeric HLB cartridges for the extraction of hydroxy and hydroperoxy PUFA congeners. Two different mass spectrometers were tested in order to discuss and compare both acquisition modes in terms of false positive identifications: a QqQ acquiring in the MRM mode that is commonly applied to lipidomic approaches, and a Linear Ion Trap (LIT) acquiring in full scan product ion mode. Their analytical parameters for optimal sensitivity and selectivity were addressed. Applicability of the analytical platform was validated by analyzing plasma samples of genetically obese spontaneously hypertensive rats.

2. Experimental

2.1. Standards, solvents and sorbents

Thromboxane B_3 (TXB_3, 9α-(±)11,15(S)-trihydroxy-thromba-5Z,13E,17Z-trien-1-oic acid), prostaglandin D_3 (PGD_3, 9α-15(S)-dihydroxy-11-oxo-prosta-5Z,13E,17Z-trien-1-oic acid), prostaglandin E_3 (PGE_3, 11α-15(S)-dihydroxy-9-oxo-prosta-5Z,13E,17Z-trien-1-oic acid), prostaglandin E_2 (PGE_2, 11α-15(S)-dihydroxy-9-oxo-prosta-5Z,13E-dien-1-oic acid), resolvin D_1 (RvD_1, 7(S),8(R),17(S)-trihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid), protectin D_1 (PD_1, 10(S),17(S)-dihydroxy-4Z,7Z,11E,13Z,15E,19Z-docosahexaenoic acid), leukotriene B_4 (LTB_4, 5(S),12(R)-dihydroxy-6Z,8E,10E,14Z-eicosatetraenoic acid), isoprostane $F_{2\alpha}$ ($8isoPGF_{2\alpha}$, 9α,11α,15(S)-trihydroxy-8β-prosta-5Z,13E-dien-1-oic acid), isoprostane $F_{3\alpha}$ ($8isoPGF_{3\alpha}$, 9α,11α,15(S)-trihydroxy-8β-prosta-5Z,13E,17Z-trien-1-oic acid), 15HpEPE (15(S)-hydroperoxy-5Z,8Z,11Z,13E,17Z-eicosapentaenoic acid), 12HpEPE (12(S)-hydroperoxy-5Z,8Z,10E,14Z,17Z-eicosapentaenoic acid), 17HpDoHE (17(S)-hydroperoxy-4Z,7Z,10Z,13Z,15E,19Z-docosahexaenoic acid), 15HEPE (±15-hydroxy-5Z,8Z,11Z,13E,17Z-eicosapentaenoic acid), 12HEPE (±12-hydroxy-5Z,8Z,10E,14Z,17Z-eicosapentaenoic acid), 5HEPE (±5-hydroxy-6E,8Z,11Z,14Z,17Z-eicosapentaenoic acid), 11HETE (±11-hydroxy-5Z,8Z,12E,14Z-eicosatetraenoic acid), 12HETE-d_8 (12(S)-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic-5,6,8,9,11,12,14,15-d_8 acid), 17HDoHE (±17-hydroxy-4Z,7Z,10Z,13Z,15E,19Z-docosahexaenoic acid), 11HDoHE (±11-hydroxy-4Z,7Z,9E,13Z,16Z,19Z-docosahexaenoic acid), 4HDoHE (±4-hydroxy-5E,7Z,10Z,13Z,16Z,19Z-docosahexaenoic acid), eicosapentaenoic acid (EPA, 5Z,8Z,11Z,14Z,17Z-eicosapentaenoic acid), docosahexaenoic acid (DHA, 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoic acid) and arachidonic acid (ARA, 5Z,8Z,11Z,14Z-eicosatetraenoic acid) were purchased from Cayman Chemicals (Ann Arbor, MI, USA). The suppliers stated purities higher than 96% for all standards.

Stock standard solutions of all analytes were prepared in ethanol and stored at -80 ºC under nitrogen. Individual stocks (5 μg/mL) were used to optimize ESI ionization and MS/MS fragmentation conditions by flow injection analysis.

For most species, calibration standard solutions were prepared in the range between 1 and 500 ng/mL. Two additional calibration solutions (1000 and 1500 ng/mL) were

made for 12HEPE. A second series of calibration standards from 100 to 50000 ng/mL was prepared for parent PUFAs (EPA, ARA, DHA) due to their higher concentration in plasma samples. The internal standard (11HETE-d_8) was included in all calibration solutions at a constant level of 500 ng/mL. Calibration lines were calculated by the least-squares linear regression method with the internal standard correction.

Methanol, Optima LC-MS grade, was purchased from Fisher Scientific (New Jersey, USA); ultrapure water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA); methyl formate was purchased from Sigma Aldrich (Poole, UK); n-hexane was provided by Merk (Darmstadt, Germany) and ethanol, formic acid and hydrochloric acid were from AnalR Normapur (Fontenai, France). SPE cartridges (C18-SepPak, 100 mg, 1 mL and Oasis-HLB, 60 mg, 3 mL) were supplied by Waters (Milford, MA, USA).

2.2. Samples and sample preparation

Plasma samples were collected from female SHROB (genetically obese spontaneously hypertensive) rats, an animal model of metabolic syndrome. Animals were kept in an isolated room with a constantly regulated temperature (22±2 ºC) and humidity (50±10%) in a 12 h artificial light cycle, and fed *ad libitum* with water and a standard pelleted chow diet A04 from Harlan Ibérica (Barcelona, Spain), which contained on wet basis 60% carbohydrate, 16% protein and 3% fat. Rats were sacrificed by exsanguination, blood was collected in tubes with ethylenediaminetetraacetic acid (EDTA) to obtain plasma (as described below). All the procedures performed agreed with the national and institutional guidelines of the Animal Care and Use Committee at the CSIC.

Blood was centrifuged at 850 g (4 ºC, 15 min) in the presence of EDTA to remove erythrocytes. Then, plasma was supplemented with 5 mM phenylmethylsulfonyl fluoride (PMSF, protease inhibitor) and erythrocyte free samples were immediately stored at -80 ºC until use. A pre-treatment step for small fluids was applied to plasma samples before the SPE [37]. The defrosting process was carried out slowly in darkness, to prevent possible analytes oxidation and degradation. Samples (90 µL) were diluted with cold methanol 30% (v/v) to a final volume of 1 mL, and spiked with the internal standard (IS) 11HETE-d_8. Samples were incubated on ice for 10 min and then centrifuged at 5000 rpm for 10 min, at 4 ºC, to remove potential proteins that may cause interference. Supernatant and washes of the resultant pellet with 30% methanol were collected in amber glass vials and added to SPE cartridges.

SPE extractions were carried out in a cold room at 4 ºC. Cartridges were conditioned with 5 mL methanol followed by 5 mL Milli-Q water. After sample loading, cartridges were sequentially washed with 5mL 15% methanol (v/v), 5 mL Milli-Q water and 2.5 mL hexane. Then, the sorbent phase was dried under a stream of nitrogen for 30 min and analytes were eluted using 2 mL methyl formate. The extraction procedure was performed using a vacuum manifold; the vacuum was adjusted so that individual drops could be seen from each cartridge. Extracts were evaporated to dryness under a fine stream of nitrogen; the residue was dissolved in 30 µL ethanol and stored at -80 ºC prior to LC-MS/MS analysis.

It is important to notice that during the sample spiking procedure, precursor fatty acids could be degradated rending hydroperoxy derivates. Also, impurities existing in the

commercial standards of EPA, ARA and DHA can become important since their addition level was huge in relation to the rest of compounds. To avoid these potential problems, three sets of spiked samples were prepared in order to separately evaluate the recoveries of precursor fatty acids, hydroperoxy and the rest of analytes. Addition levels used in these sets of spiked samples were 150 ng/mL for PUFAs metabolites, except for 12HEPE (600 ng/mL). Their precursors were added to plasma at much higher levels: EPA (3 μg/mL), DHA (15 μg/mL) and ARA (45μg/mL), which are in the same order of concentration as existing in non-spiked (blank) samples. The added concentration of IS (12HETE-d_8) was 500 ng/mL in all experiments.

Different recovery experiments were performed according to the step procedure where the compounds were spiked: at the beginning of the process (Global recovery, SPE-LC-ESI-MS/MS), after SPE (Matrix effect, ESI-MS/MS) or comparing initial spiked samples with additions done after SPE (SPE recovery). The repeatability of the methodology was expressed as percentage of relative standard deviation (%RSD).

2.3. Apparatus

Analyses were carried out on two LC-MS systems. One system consists of two ProStar 210 high-pressure mixing pumps (Varian, Walnut Creek, CA, USA), a Metachem Technologies vacuum membrane degasser (Bath, UK), and an autosampler and thermostated column compartment ProStar 410 module (Varian). This LC is coupled with a triple quadrupole 320-MS equipped with an electrospray interface (Varian). Nitrogen, used as nebulising and drying gas, is provided by a nitrogen generator (Domnick Hunter, Durham, UK). Argon (99.999%) was used as collision gas. Instrument control and data acquisition were done by Varian MS Workstation software.

The other LC system consists of an Agilent 1260 Series (Agilent, Palo Alto, CA) that includes a binary pump, a degasser system and a thermostated autosampler, coupled to a linear ion trap (LIT) mass spectrometer LTQ Velos Pro equipped with an electrospray interface (Thermo Fisher, Rockford, IL, USA). Nitrogen was used as nebulising and drying gas and helium was the collision gas. Instrument control and data acquisition were done with Xcalibur software.

A Waters C18-Symmetry column, 150×2.1 mm, 3.5 μm (Milford, MA, USA) protected with a 4×2mm C18 guard cartridge provided by Phenomenex (Torrance, CA, USA) was used to perform LC separations in both systems. SPE clean-up and extraction steps were performed using a vacuum manifold system from Supelco (Bellefonte, PA, USA) coupled to a vacuum pump from Millipore (Bedford, MA, USA). Plasma samples were foremost centrifuged in an Avanti J25 refrigerated centrifuge (Beckman Coulter, USA).

2.4. LC-MS/MS conditions

A binary eluent system of water (A) and methanol (B), both with 0.02% (v/v) of formic acid, was used as mobile phase in the LC separation. The gradient was: 0-1 min (60% B), 2-12 min (80% B), 13-23 min (100% B), and 25-30 min (60% B). The flow rate was set to 0.2 mL/min, the column effluent was directly introduced in the ESI interface without splitting, and injection volume was set to 10 μL. The column was maintained at room temperature and extracts were kept at -20 ºC right up the injection moment. Retention times for target compounds are shown in Table 1.

2.4.1. QqQ system

The ESI interface was operated in the negative mode with a needle potential of -4 kV, a source temperature of 50 °C, a desolvation temperature of 200 °C, a nebulising gas pressure of 55 psi (380 kPa) and a drying gas pressure of 20 psi (138 kPa). Argon pressure in the collision cell was kept at 2 mTorr for MS/MS measurements. The mass window of the first and last quadrupoles was adjusted to 2.0 and 1.5 amu, respectively.

Selection of the most intense MS/MS transitions was done by direct infusion of individual standard solutions (5 μg/mL) at a flow rate of 20 μL/min. The intensity of the $[M-H]^-$ ion was optimized by varying the capillary voltage, and subsequently, the $[M-H]^-$ ion was subjected to MS/MS fragmentation experiments and the most intense product ions were obtained by varying the collision energy (0 to 60 eV) with the aid of the automated routines included in the software package. For each analyte, the two most intense or selective transitions were selected (Table 1).

2.4.2. LIT system

Operating conditions of the ESI source were negative ion mode with a sheath gas flow rate of 40 units, spray voltage of 5.5 kV, capillary temperature of 300 ºC and S-lens radio-frequency level of 60%. Mass spectrometer was tuned optimizing voltages on the lenses and trap conditions whilst infusing standard solutions in the same way as QqQ system. The collision induced dissociation (CID) energy was optimized for each compound in order to maximize the intensity of their product ions. The identification of targeted compounds was helped with the full ion product spectra recorded in the range from 90 to 400 *m/z* units. To quantify the analytes, the most intense and selective transition was used. Individual MS/MS parameters for each compound are summarized in Table 1.

3. Results and discussion

3.1. Optimization of LC-ESI-MS/MS conditions

Table 1 summarizes the optimal MS/MS transitions selected for the QqQ and LIT assays. These transitions were recorded in order to facilitate the individual identification of the compounds minimizing further false positive identifications in biological samples. Once transitions were set, compounds were chromatographically separated on a C18 column using a gradient of water/methanol solvents with 0.02% formic acid (v/v). Several compounds coeluted under the same chromatographic peak, but they could be individually quantified using specific transitions except for the particular case of the coeluting isomers PGE_3 and PGD_3 that also showed the same fragmentation pattern (Figure S2 of Electronic Supplementary Materialpresents their individual mass spectrum). MS^3 fragmentations tested for both prostaglandins in the LIT spectrometer did not provide further qualitative information; therefore, they were quantified together.

The LC separation cycle takes 30 min, including a 5 min column conditioning step before the next injection. Overall, this running time was comparable with other reported LC methods of eicosanoids analysis and provides the basis for a rapid assay [4][38]. Some of the selected product ions for QqQ were in agreement with the available literature values as the following: TXB_3 367>169, 8iso-$PGF_{2\alpha}$ 353>192, PGE_2 351>271, 5HEPE 317>115 or 17HDoHE 343>281. However, for these compounds, the found LIT transitions were different than those previously reported. Transitions associated to RvD_1, LTB_4, 12HEPE or 11HETE matched with literature values described for both equipment [3][24][28][29]. Figure 1 shows representative chromatograms of the analysis of these compounds.

3.2. Detection method validation

Table 2 shows the values of linearity (from 1 to 500 ng/mL for oxidized derivates and 100 to 50000 ng/mL for PUFAs), instrumental LODs and LOQs (defined for a signal to noise ratio of 3 and 10, respectively), and repeatability estimated by either of the two mass detectors. The standard calibration functions confirmed that both equipment achieved linear responses for all compounds, R^2 ranged between 0.9860 and 0.9999. Acceptable precision was achieved by both instruments; RDSs % were lower than 12% except for hydroperoxy compounds at 5 ng/mL level.

Finally, instrumental LODs and LOQs for the QqQ system were respectively estimated to be in the range of 0.003-7 and 0.01-23 ng/mL, and that is in agreement with those previously reported in literature [3][16][24][26][28][30][39]. LIT mass spectrometer achieved respectively LODs and LOQs between 0.002-3 and 0.006-10 ng/mL, values similar to those reported in a hybrid system consisting on a quadrupole-ion trap mass analyzer (QTrap) operating in MRM mode [27]. No previous reports were found using a linear ion trap spectrometer operating in full scan product ion mode.

3.3. Optimization of solid-phase extraction (SPE)

3.3.1 Breakthrough and elution solvent volume, washing conditions and extraction sorbent.

In preliminary SPE experiments, aqueous samples (simulating plasma conditions) were used to optimize the SPE process trying two different cartridges, 60 mg Oasis-HLB and 100 mg C18. Methyl formate and ethanol were tested as eluting solvents achieving the same performance; even though, methyl formate was selected due to its higher volatility that facilitates the later stage of concentration. Three consecutive 2 mL fractions of organic solvent were collected from SPE cartridges, previously spiked with 50 μL of a mixture solution of target compounds (200 ng/mL). Responses measured in the first fraction represented more than 98% of the total for all compounds. Thus, 2 mL of methyl formate were the optimal eluting conditions employed in further experiments. This extract was concentrated to dryness and finally re-constituted with 30 μL of ethanol.

The breakthrough volume of both sorbents was investigated using up to 3 mL spiked aqueous samples passed through two cartridges connected in series (C18>C18 and HLB>HLB) and eluted separately. This sample volume, 3 mL, did not achieve the sorbent breakthrough volume due to none of the compounds were detected in the extract from the second cartridge, neither C18 nor HLB.

Considering biological and complex samples, many authors have demonstrated the importance of a washing sample step during the SPE procedure to reduce sample complexity and to prevent matrix effects during LC-ESI-MS determination. Washing conditions were set to 5 mL of water followed by 5 mL of 15% methanol and 2.5 mL of hexane [16][24][26][30] after verifying that none of the compounds were lost in the washing fraction.

Recoveries attained with Oasis-HLB and C18 cartridges were compared for 2 mL of spiked water samples at a final concentration of 150 ng/mL per compound. The comparative recovery results showed that HLB cartridges performed better than C18 ones for precursor fatty acids and hydroxy compounds, for which HLB recoveries

ranged from 70 to 98% while those were reduced to 42 to 82% with the C18 sorbent (Figure 2). Eicosanoids, resolvins and protectins were eluted with similar recoveries (70 to 105%) in both sorbents (Figure 2), and they are in agreement with other published results using HLB [24][25] and C18 [26][27]. Hydroperoxy compounds recoveries were higher using HLB sorbent than C18, but they still ranged from 50 to 70%, so that further optimization of the process was required. On the basis of results depicted in Figure 2, it was decided to select the HLB sorbent (never tested for hydroxy and hydroperoxy metabolites) to continue with optimization of the extraction process.

3.3.2 Effect of sample pH, organic modifiers addition and temperature.

When the SPE conditions described in the above section for aqueous solutions were applied to plasma samples, a decrease in the yield of SPE process was noticed for all compounds. Thus, additional variables were evaluated in order to better understand the behavior of the analytes during the sample preparation process and, obviously, to improve the yield of sample preparation.

Some authors have suggested that acidification of plasma to pH 3 before extraction, improves the affinity of fatty acids and their derivatives by reversed-phase sorbents, and thus the efficiency of SPE extraction [3][40][41]. However, we found similar efficiencies for acidified (pH 3) and non-acidified plasma samples (data not shown); thus, the pH of plasma was not modified.

The three fatty acids involved in this study (ARA, EPA and DHA) and their hydroxy and hydroperoxy derivatives are relatively lipophilic compounds; thus, these compounds are more prone to be lost by lipophilic interactions with the pellet formed after sample centrifugation, and that could partially explain their low recoveries obtained. In order to verify this hypothesis, a series of experiments were performed by diluting spiked plasma samples (90 µL) with aqueous solutions (1 mL) containing different percentages of methanol from 0 to 50%. After centrifugation, the supernatants were submitted to the SPE procedure and absolute recoveries calculated (Figure 3). In general, recoveries obtained for most compounds increased with the percentage of added methanol from 0 to 30% (quantitative recoveries were achieved with 30% of methanol). The exception to this trend corresponded to the free fatty acids (ARA, EPA and DHA). Quantitative recoveries for these species were only achieved using a 50% of methanol, which is in agreement with their high octanol-water partition coefficients ($K_{ow\ (ARA,\ 25\ ºC)}$=6.99, $K_{ow\ (EPA,\ 25\ ºC)}$=6.50, $K_{ow\ (DHA,\ 25\ ºC)}$=7.26). However, methanol proportions above 30%, reduced the recovery for the more water-soluble species (eicosanoids, resolvins and protectins) due to breakthrough problems in the cartridge. Thus, 30% of methanol was the optimized organic modifier used to favor the extraction of PUFAs minor metabolites in plasma. In the specific case of hydroperoxy compounds, the best recoveries were also obtained with 30% of methanol.

The influence of the temperature during the extraction process was then checked comparing spiked plasma processed at room temperature and 4 ºC. Our results demonstrated that temperature had a critical impact on hydroperoxy compounds; whilst it did not affect the rest of studied analytes (Figure 4). Hydroperoxy recoveries were around 50% at room temperature, whereas such recoveries increased to 90-100% at 4 ºC. Therefore, this is a critical parameter to be controlled in the global extraction process of lipid mediators.

To summarize, the optimal extraction conditions were set as follows: 90 µL of internal standard-spiked plasma were diluted with 1 mL 30% methanol. After centrifugation, the supernatant was recovered and loaded into the barrel of an Oasis-HLB 60mg SPE cartridge. The entire process was performed at 4 ºC. A scheme of optimized SPE method is provided as Electronic Supplementary Material in Figure S3.

3.4. Repeatability, SPE-LC-MS recoveries and matrix effects

Table 3 summarizes the repeatability of the entire optimized method, the overall recoveries for the SPE and SPE-LC-MS procedures, and the matrix effects for spiked plasma samples to a final concentration of 150 ng/mL per compound except for 12HEPE (600 ng/mL), EPA (3µg/mL), DHA (15µg/mL) and ARA (45µg/mL), using 60 mg Oasis-HLB cartridges.

Good and similar repeatabilities were obtained for the compounds using the two analyzers, with the exception of hydroperoxy analytes. Repeatabilities were ranged between RSD values of 1-15% (13-26% for hydroperoxy) by SPE-LC-ESI-QqQ and 3-13% (11-25% for hydroperoxy) by SPE-LC-ESI-LIT system. Thus, the precision of the method is controlled by the sample preparation step rather than by the characteristics of the mass analyzer.

Global recoveries for LIT ranged from 80 to 112%, and were generally higher than QqQ global recoveries. In particular, the QqQ system provided global recoveries as low as a 27-38% for the three hydropexides (15HpEPE, 12HpEPE and 17HpDoHE) and the hydroxyl 4HDoHE. Global recoveries for RvD1 and PUFA precursors (ARA, EPA and DHA) were also significantly lower, 50-71%, with the QqQ configuration. SPE recoveries were around 100% in both equipments with the exception of RvD_1 (67-75%). Thus, according to the results compiled in Table 3, the main parameter that affected the global recovery of analytes was the matrix effect.

It is known that the sensitivity and accuracy of the mass spectrometry determinations can be influenced by the coelution of matrix components with ability to reduce or enhance the intensity of $[M-H]^-$ precursor ions. Matrix effects observed for the QqQ were higher than for the LIT. Seven compounds, i.e. the three hydroperoxides, 4HDoHE and the three PUFA precursors, showed suppression of 28-71% of their signal with the QqQ, whilst with the LIT system only the precursor DHA and 11HDoHE exhibit matrix effect by losing their signal up to 29-20%, respectively. There might be multiple reasons for the different matrix effects observed on the two systems. Ion suppression mainly occurs in the early stages of the ionization process; therefore, the difference in matrix effect contribution may be in part due to the particular design of the ESI source of each system [42].

3.5. Application to plasma analysis

The optimized SPE-LC-MS/MS method was applied to plasma samples of genetically obese spontaneously hypertensive rats. Samples were analyzed by both instrumental systems and results were compared. As it has been assessed above, both equipments achieved similar quantitative performance in the analysis of standard compounds; however, the MRM acquisition mode (QqQ) gave several false identifications when it was applied to rat plasma samples. The QqQ system was not able to distinguish targeted compounds from other coeluting isomers with similar MRM transitions. It should be noted that oxygenated PUFA metabolites comprise an elevated number of isomeric and

isobaric species that may coelute [23]. In contrast, the full ion product mode (LIT) provided full MS/MS spectra which help to minimize the number of false positive identifications. As example, when plasma samples were analyzed by MRM mode, 11HDoHE was tentatively identified based on 343>121 *m/z* quantification and 343>149 *m/z* identification transitions; and on the retention time 18.33 and 18.25 min for the sample and standard solution (Figure 5a,b). However, the LIT MS/MS spectra obtained for the same peak revealed that its fragmentation pattern is different from the 11HDoHE standard (Figure 5c,d). This peak was probably a different coeluting hydroxy derivate of DHA, based on the typical fragments attributed to neutral loses of water (343>325 and 299>281 *m/z*) and CO_2 (343>299) that were detected in both CID spectra. Different fragment ions from C-C cleavages were found (343>233, 255, 161 in the sample and 343>194, 165, 149 in the standard). There are at least other 14 bioactive lipids with the same exact mass than 11HDoHE (MW: 344.235) [23], that in the case of coelution may explain the observed false positive identification in plasma analysis. In a similar way, the full ion product mode confirmed the false positive identification of 15HEPE, 5HEPE and 4HDoHE using the MRM mode.

Quantitative results obtained with the LIT instrument are presented in Table 4. Eight of twenty-three searched compounds were identified. ARA was the metabolite found at highest level (59446 ng/mL) and TXB_3 was that present at the lowest concentration (3 ng/mL). Due to the concentration level of 17HpDoHE was higher than the maximum level of calibration, it was necessary to dilute the samples.

4. Conclusions

The present work describes the development of a robust and sensitive targeted analysis platform for the simultaneous quantification in blood plasma of a larger number of eicosanoids/docosanoids (tromboxanes, prostaglandins, resolvins, protectins, leukotrienes, isoprostanes, hydroxy and hydroperoxy) which are quantified jointly to their PUFA precursors (ARA, EPA and DHA). The developed method overcame issues associated to the wide range of polarity (octanol-water partition coefficients ranged from 1.1 of PGD_3 to 7.3 of DHA), concentration (up to 4 orders of magnitude) and stability diversity (hydroperoxides are highly unstable) of these metabolites. Two mass detectors were tested: the most common used QqQ instrument operating in MRM and LIT spectrometer acquiring in full ion product scan mode. Although the QqQ assays were highly sensitive and selective and chromatographic separation was optimized, the full ion product scan acquisition on the LIT analyzer was more appropriated to minimize false positive identifications for these metabolites. The optimized methodology is simple, sensitive, high-throughput and lends itself to lipidomic applications. The method can be used to biological applications targeted for biomarker discovery or other therapeutic and pharmacological approaches. It can be combined with information on metabolic, proteomic and genomic profiles providing global evidence for evaluating the role of lipids at cellular, tissue and system level.

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7. Captions

Figure captions

Figure 1: Representative LC-MS/MS chromatograms of the 23 lipid mediators (75 ng/mL, per compound) provided by the LIT instrument.

Figure 2: Comparative SPE recovery results between 60 mg HLB and 100 mg C18 cartridges, of 2 mL spiked water samples with a final concentration of 150 ng/mL per compound. The same extraction conditions were applied, at room temperature and neutral pH, n=3 replicates.

Figure 3: Comparative recovery results for the extraction with 60 mg HLB cartridges of spiked plasma samples (final concentration of 150 ng/mL per compound except for 12HEPE (600 ng/mL), EPA (3 μg/mL), DHA (15 μg/mL) and ARA (45 μg/mL)) supplemented with different percentages of methanol, 0 to 50%, n=3 replicates.

Figure 4: Comparative recovery results for the extraction with 60 mg HLB cartridges of spiked plasma samples (final concentration of 150 ng/mL to every compound except 12HEPE (600 ng/mL), EPA (3 μg/mL), DHA (15 μg/mL) and ARA (45 μg/mL)) doing the whole process at room temperature and 4 ºC, n=3 replicates.

Figure 5: Identification of 11HDoHE using QqQ (a, b) and LIT (c, d) mass spectrometers: a) chromatogram obtained in the MRM mode (QqQ) at the transition of 343>121 *m/z* in plasma sample; b) chromatogram obtained in the MRM mode (QqQ) at the transition of 343>121 *m/z* in standard solution of 11HDoHE; c) corresponding MS/MS spectrum of the identified peak in plasma sample with the LIT; d) corresponding MS/MS spectrum of 11HDoHE standard solution with the LIT. Spectra c and d demonstrate the false positive identification of 11HDoHE in plasma by QqQ analysis.

Table captions

Table 1: Retention times, collision energies and MS/MS transitions for QqQ and LIT spectrometers.

Table 2: Linearity (1 to 500 ng/mL for oxidized derivates and 100 to 50000 ng/mL for PUFAs), limit of detection (LOD), limit of quantification (LOQ) and repeatability of LC-ESI/MS/MS assay at three concentration levels (5, 100 and 500 ng/mL) for target compounds for the QqQ and LIT mass spectrometers. *The Repeatability of EPA, ARA and DHA was determined at three different concentration levels: 100, 5000 and 50000 ng/mL (n=5).

Table 3: Repeatability (RSD) of the global process and recovery results including: Global Recovery (SPE-LC-ESI/MS/MS), SPE Recovery and Matrix Effect (LC-ESI/MS/MS); for spiked plasma samples (n=3) to a final concentration of 150 ng/mL per compound except for 12HEPE (600 ng/mL), EPA (3 μg/mL), DHA (15 μg/mL) and ARA (45 μg/mL). Comparison between QqQ and LIT spectrometers.

Table 4: Average concentration and calculated RSD of studied lipid compounds in ten female rat plasma samples by SPE-LC-LIT. A dilution was needed to calculate 17HpDoHE concentration due to his level in samples was higher than the maximum level of calibration.

Electronic Supplementary Material

Figure S1: Cascade of formation to ARA, EPA and DHA oxidized derived compounds from COX, LOX, CYP450 enzyme activities and autoxidation.

Figure S2: Individual mass spectrum and a possible explanation of the fragmentation patterns for PDG_3 and PGE_3 isomers.

Figure S3: Optimized SPE methodology for plasma samples containing lipid mediator derivatives.

8. Tables

Table 1

Compound	Retention Time (min)	QqQ			LIT	
		Collision energy (eV)	Capillary voltage (eV)	MRM quantification[q] and identification[i] transitions (*m/z*)	Collision energy (eV)	Quantification transition (*m/z*)
8iso-PGF$_{3\alpha}$	6.79	18 14.5	-68	351>307 [q] 351>333 [i]	30	351>253
TXB$_3$	6.97	16.0 13.5	-52	367>169 [q] 367>195 [i]	19	367>195
PGD$_3$/PGE$_3$	7.67	12.5 10	-40	349>269 [q] 349>313 [i]	19	349>313
8iso-PGF$_{2\alpha}$	8.28	27.0 24.5	-44	353>192 [q] 353>171 [i]	28	353>299
PGE$_2$	9.09	15.5 10.5	-64	351>271 [q] 351>315 [i]	20	351>315
RvD$_1$	9.80	14.0 18.0	-56	375>141 [q] 375>215 [i]	25	375>141
PD$_1$	12.89	16.0 19.0	-64	359>153 [q] 359>136 [i]	30	359>153
LTB$_4$	13.79	15.0 12.5	-80	335>195 [q] 335>317 [i]	27	335>195
15HpEPE	17.36	9.5 5.0	-30	333>111 [q] 333>315 [i]	20	333>315
12HpEPE	17.80	5.5 9.0	-32	333>271 [q] 333>151 [i]	25	333>315
15HEPE	17.92	11.0 11.5	-56	317>219 [q] 317>255 [i]	27	317>219
12HEPE	18.72	12.5 12.0	-56	317>179 [q] 317>208 [i]	27	317>179
5HEPE	20.47	12.5 10.5	-56	317>115 [q] 317>255 [i]	25	317>255
17HpDoHE	21.90	9.5 5.5	-32	359>111 [q] 359>297 [i]	26	359>341
17HDoHE	21.94	11.5 12.5	-64	343>281 [q] 343>201 [i]	27	343>245
11HETE	22.09	15.0 12.5	-80	319>167 [q] 319>301 [i]	30	319>167
12HETEd$_8$	22.66	13.5 12.0	-68	325>182 [q] 325>307 [i]	30	325>307
11HDoHE	23.20	14.0 12.5	-60	343>121 [q] 343>149 [i]	27	343>149
4HDoHE	23.64	13.5 10.5	-64	343>101 [q] 343>281 [i]	27	343>281
EPA	24.43	10.0 12.5	-64	301>257 [q] 301>203 [i]	27	301>257
DHA	24.93	9.5 11.5	-38	327>283 [q] 327>229 [i]	30	327>283
ARA	25.10	13.5 12.0	-76	303>205 [q] 303>259 [i]	30	303>259

Table 2

Compound	R^2		LOD(ng/mL)		LOQ(ng/mL)		Repeatability (RSD % n=5)					
	QqQ	LIT	QqQ	LIT	QqQ	LIT	QqQ			LIT		
							5ng/mL	100ng/mL	500ng/mL	5ng/mL	100ng/mL	500ng/mL
12HpEPE	0.9868	0.9991	2	3	6	10	22	4	5	3	3	2
15HpEPE	0.9868	0.9991	0.4	0.6	1.5	2	16	4	2	3	3	2
17HpDoHE	0.9879	0.9903	1	1	3.5	4	11	7	1	5	3	7
11HDoHE	0.9965	0.9957	0.9	0.1	3	0.3	7	4	4	3	1	2
11HETE	0.9997	0.9931	0.1	0.02	0.4	0.07	8	2	1	4	9	5
12HEPE	0.9974	0.9939	0.7	0.08	2	0.3	7	6	2	4	1	1
12HETEd8			0.003	0.002	0.01	0.006	3	3	4	5	6	4
15HEPE	0.9997	0.9968	0.5	0.07	1.5	0.2	10	3	2	4	1	1
17HDoHE	0.9999	0.9947	0.5	0.2	2	0.7	12	3	6	6	1	1
4HDoHE	0.9921	0.9922	1.5	0.3	5	1	5	1	2	7	5	4
5HEPE	0.9960	0.9938	0.5	0.1	2	0.3	4	3	6	4	1	1
LTB4	0.9994	0.9954	0.3	0.03	1	0.1	6	1	1	3	2	1
PD1	0.9997	0.9971	0.3	0.02	1	0.1	6	2	0.05	5	1	2
PGD3/PGE3	0.9992	0.9985	0.2	0.1	1	0.3	2	1	3	1	1	2
PGE2	0.9993	0.9925	0.1	0.05	0.4	0.2	6	1	1	2	1	1
RvD1	0.9995	0.9958	0.1	0.02	0.4	0.07	6	1	2	1	1	2
TXB3	0.9970	0.9971	0.4	0.1	1	0.5	5	2	1	4	1	1
PGF2α	0.9992	0.9936	1	0.06	3	0.2	4	4	3	8	3	2
PGF3α	0.9997	0.9959	7	0.4	23	1.5	5	3	2	2	1	1
ARA	0.9997	0.9934	0.1	0.04	0.3	0.1	*6	*3	*1	*3	*5	*9
EPA	0.9978	0.9959	0.1	0.01	0.2	0.04	*6	*3	*2	*3	*4	*7
DHA	0.9876	0.9860	0.1	0.01	0.2	0.04	*2	*2	*3	*3	*5	*8

1 **Table 3**

Compound	%RSD (n=3)		%Global Recovery		%SPE Recovery		Matrix Effect	
	QqQ	LIT	QqQ	LIT	QqQ	LIT	QqQ	LIT
12HpEPE	13	11	27	88	91	99	29	88
15HpEPE	19	22	37	88	106	99	35	88
17HpDoHE	26	25	38	97	99	96	39	101
11 HDoHE	5	5	97	94	100	118	97	80
11 HETE	4	8	94	99	101	88	93	113
12 HEPE	4	9	82	87	97	83	85	105
15 HEPE	6	10	100	112	100	103	100	109
17 HDoHE	6	6	111	109	125	123	89	88
4 HDoHE	5	13	37	97	92	116	41	83
5 HEPE	4	7	83	92	100	105	83	88
LTB4	7	11	97	92	101	94	96	98
PD1	5	11	98	86	97	98	100	88
PGD3/PGE3	15	10	80	94	98	94	81	100
PGE2	5	8	112	95	96	99	116	95
RvD1	6	12	61	85	67	75	90	89
TXB3	4	12	81	112	95	84	85	104
8iso-PGF2α	13	8	91	86	92	85	99	102
8iso-PGF3α	12	11	86	80	95	82	91	97
ARA	3	4	71	100	99	111	72	90
EPA	1	6	50	98	101	106	49	93
DHA	6	3	60	88	116	123	51	71

2

Table 4

Compound	Average sample concentration (ng/mL) (n=10)
17HpDoHE	2183 ± 240
11 HETE	3 ± 0.5
12 HEPE	249 ± 69
PGE2	10 ± 1
TXB3	3 ± 0.4
ARA	59446 ± 10105
EPA	8370 ± 1255
DHA	48632 ± 7781

9. Figures

Figure 1

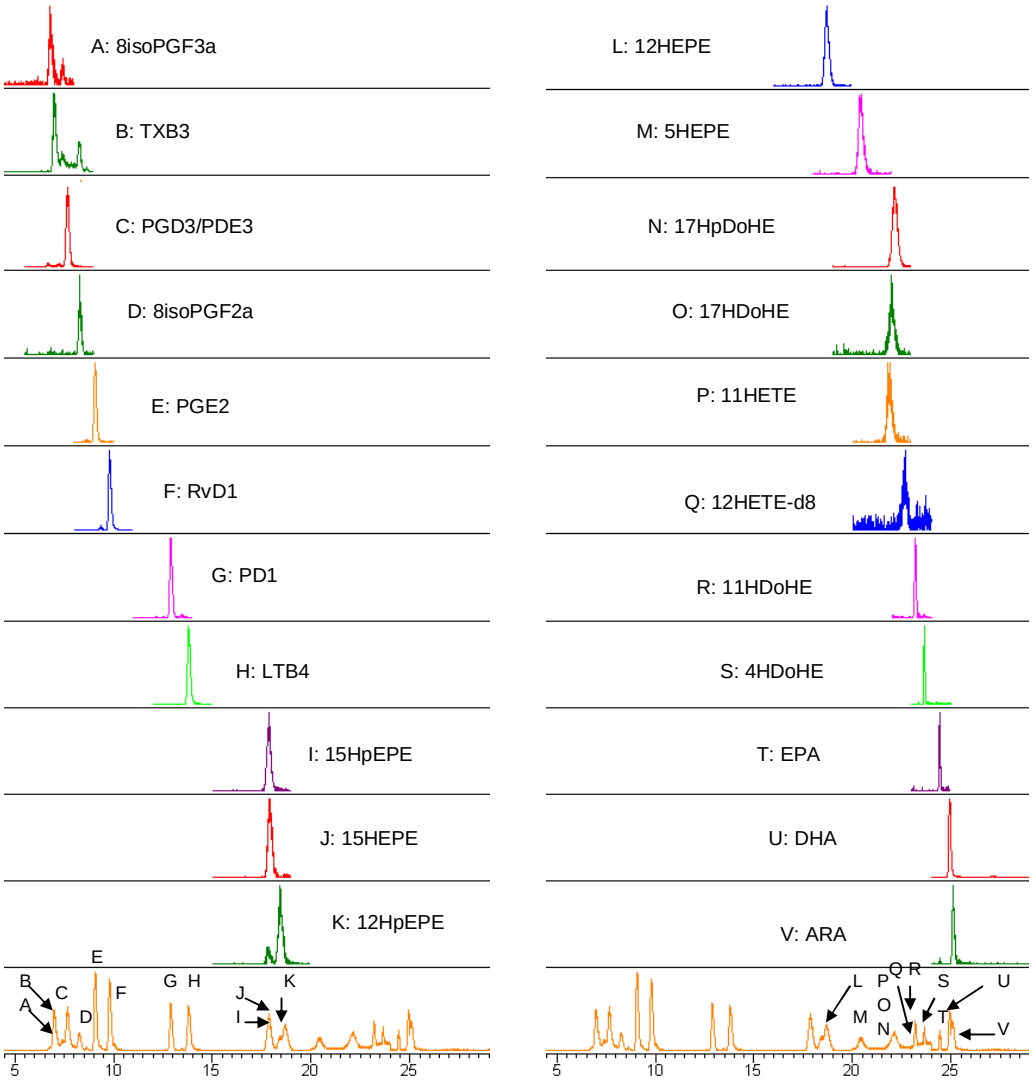


Figure 2

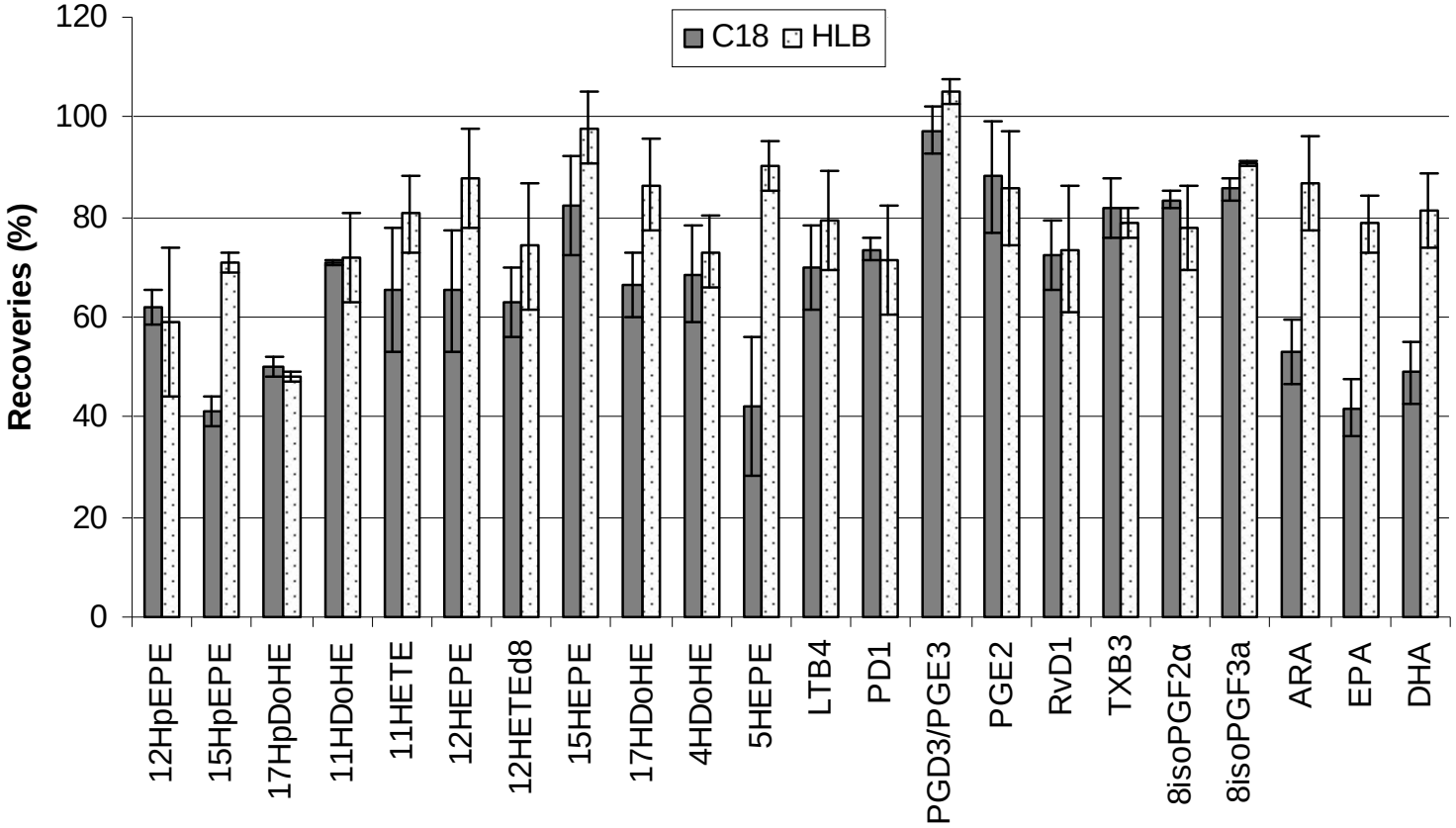

C18
HLB
Recoveries (%)
120
100
80
60
40
20
0
12HpEPE
15HpEPE
17HpDoHE
11HDoHE
11HETE
12HEPE
12HETEd8
15HEPE
17HDoHE
4HDoHE
5HEPE
LTB4
PD1
PGD3/PGE3
PGE2
RvD1
TXB3
8isoPGF2α
8isoPGF3a
ARA
EPA
DHA

Figure 3

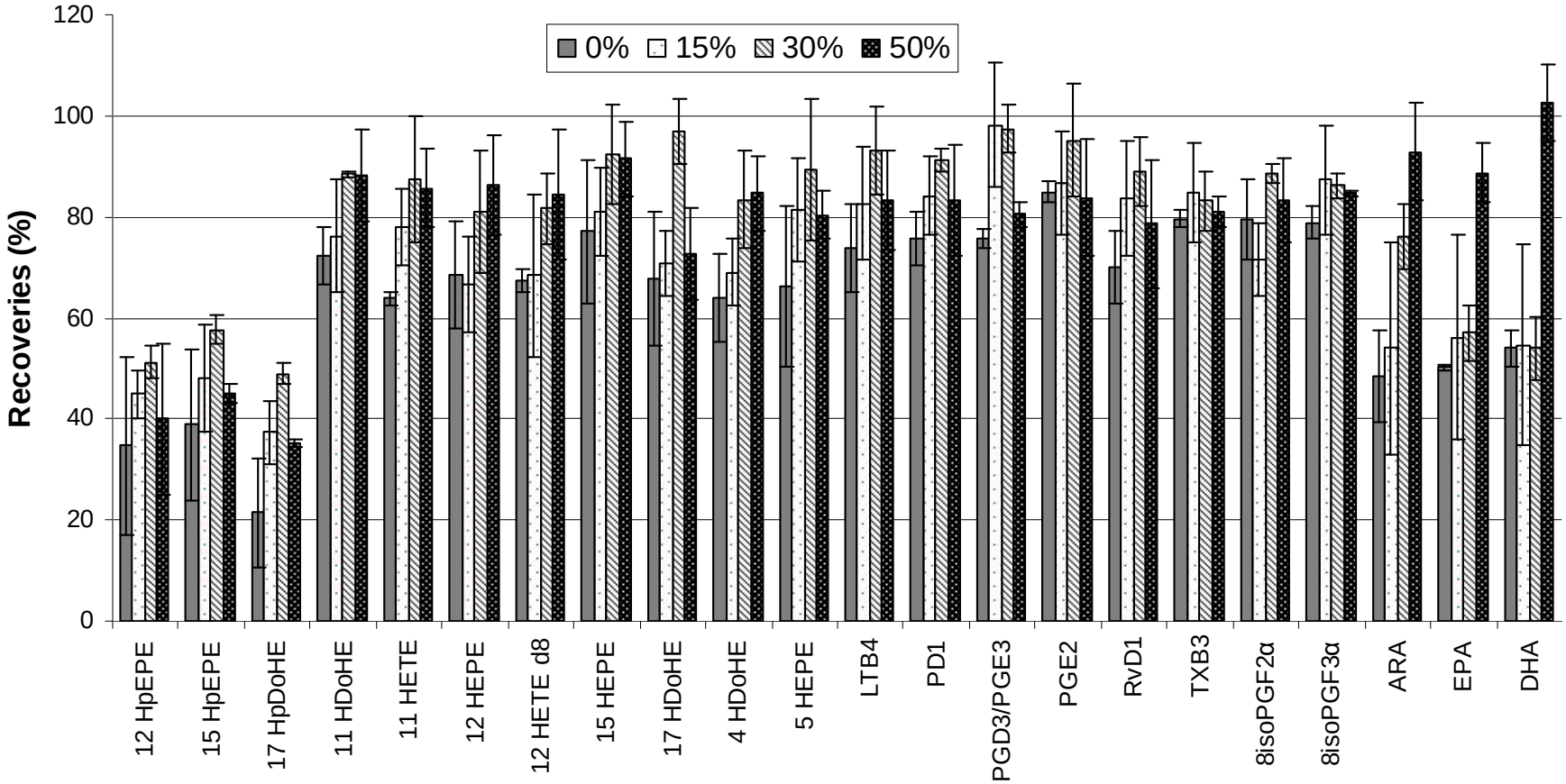

0%
15%
30%
50%
Recoveries (%)
120
100
80
60
40
20
0
12 HpEPE
15 HpEPE
17 HpDoHE
11 HDoHE
11 HETE
12 HEPE
12 HETE d8
15 HEPE
17 HDoHE
4 HDoHE
5 HEPE
LTB4
PD1
PGD3/PGE3
PGE2
RvD1
TXB3
8isoPGF2α
8isoPGF3α
ARA
EPA
DHA

Figure 4

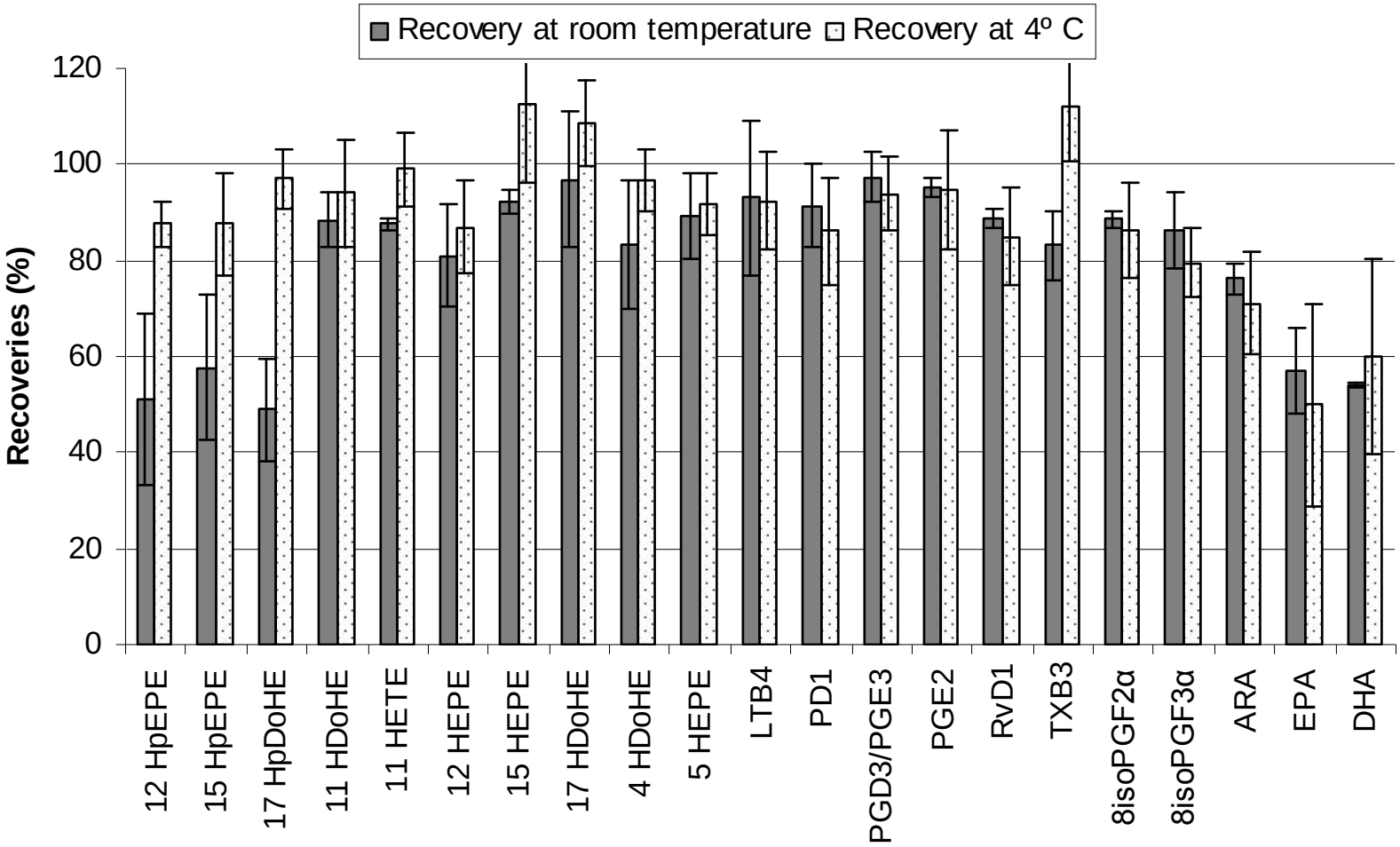
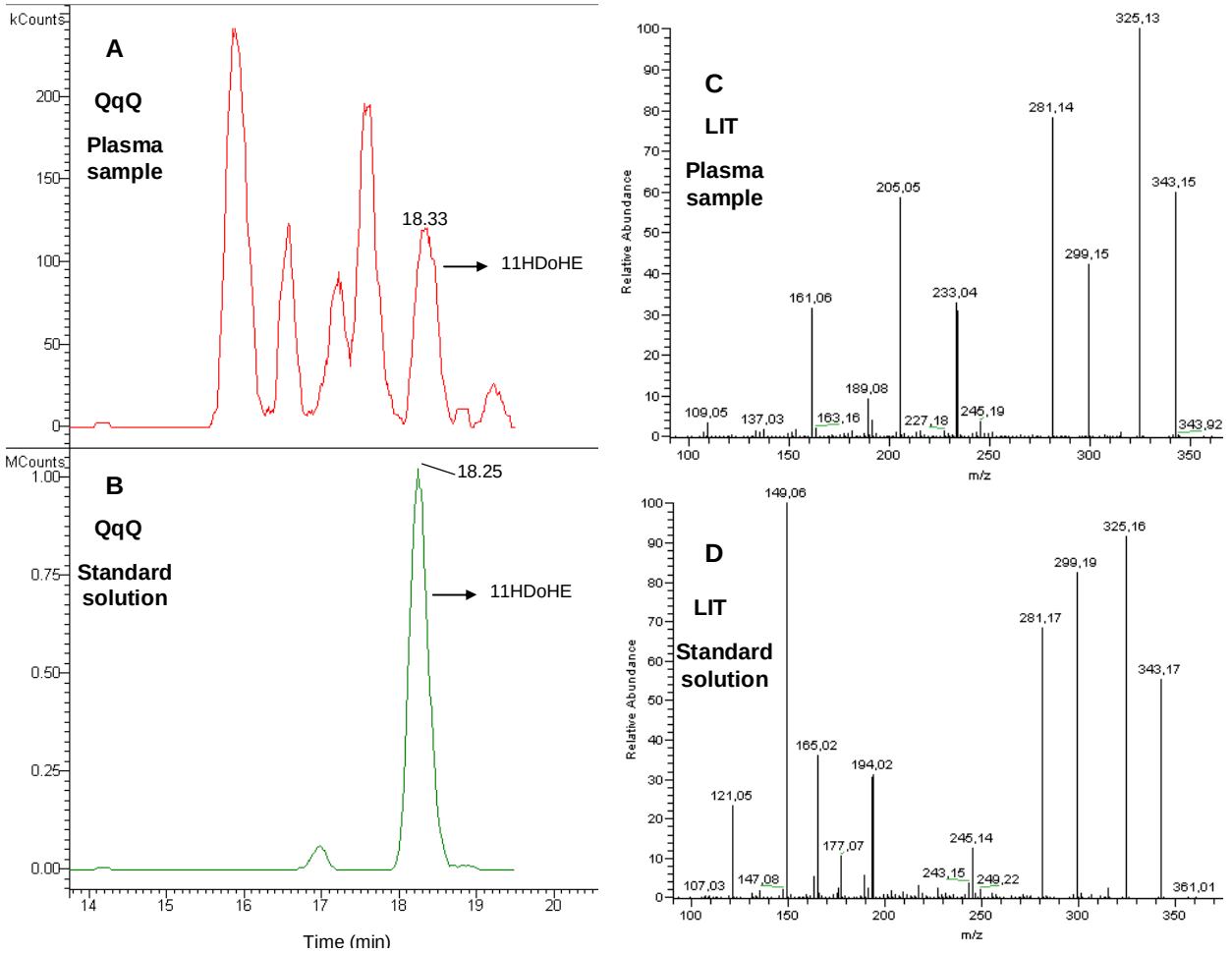

Recovery at room temperature
Recovery at 4º C
Recoveries (%)
120
100
80
60
40
20
0
12 HpEPE
15 HpEPE
17 HpDoHE
11 HDoHE
11 HETE
12 HEPE
15 HEPE
17 HDoHE
4 HDoHE
5 HEPE
LTB4
PD1
PGD3/PGE3
PGE2
RvD1
TXB3
8isoPGF2α
8isoPGF3α
ARA
EPA
DHA

Figure 5


A
QqQ
Plasma sample
kCounts
18.33
11HDoHE
B
QqQ
Standard solution
MCounts
18.25
11HDoHE
Time (min)
C
LIT
Plasma sample
Relative Abundance
m/z
325,13
281,14
343,15
205,05
299,15
233,04
161,06
189,08
109,05
137,03
163,16
227,18
245,19
343,92
D
LIT
Standard solution
149,06
325,16
299,19
281,17
343,17
165,02
194,02
121,05
177,07
245,14
107,03
147,08
243,15
249,22
361,01

10. Electronic Supplementary material
Figure S1

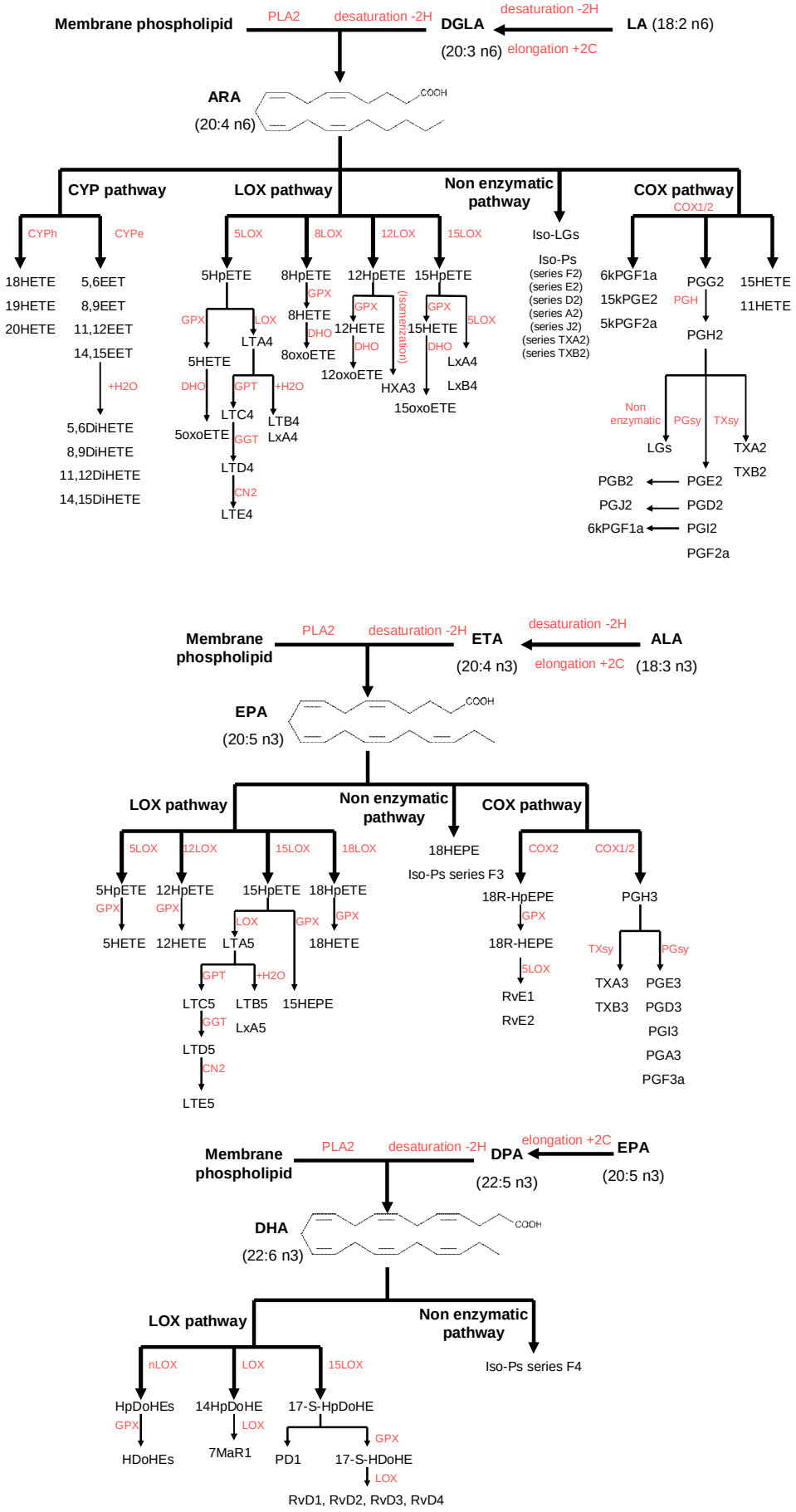


Figure S2

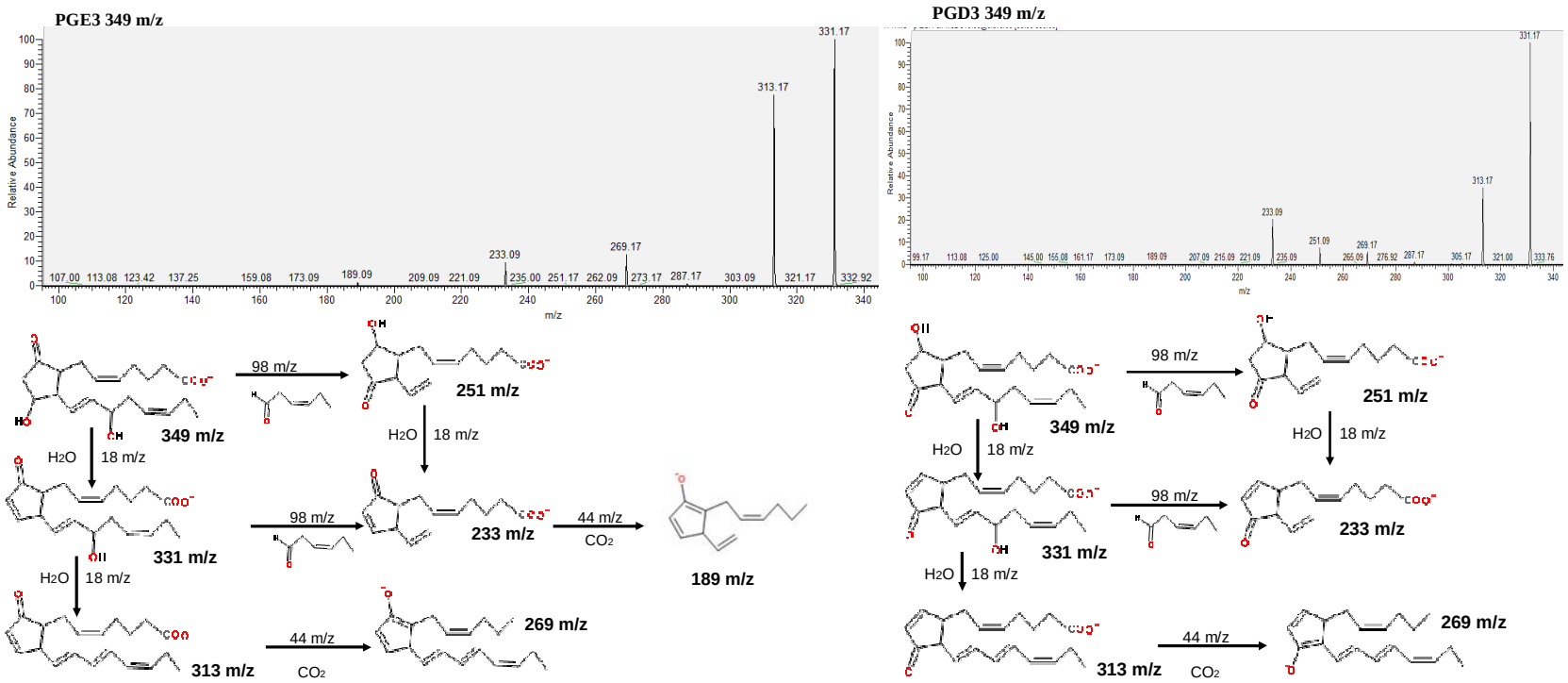
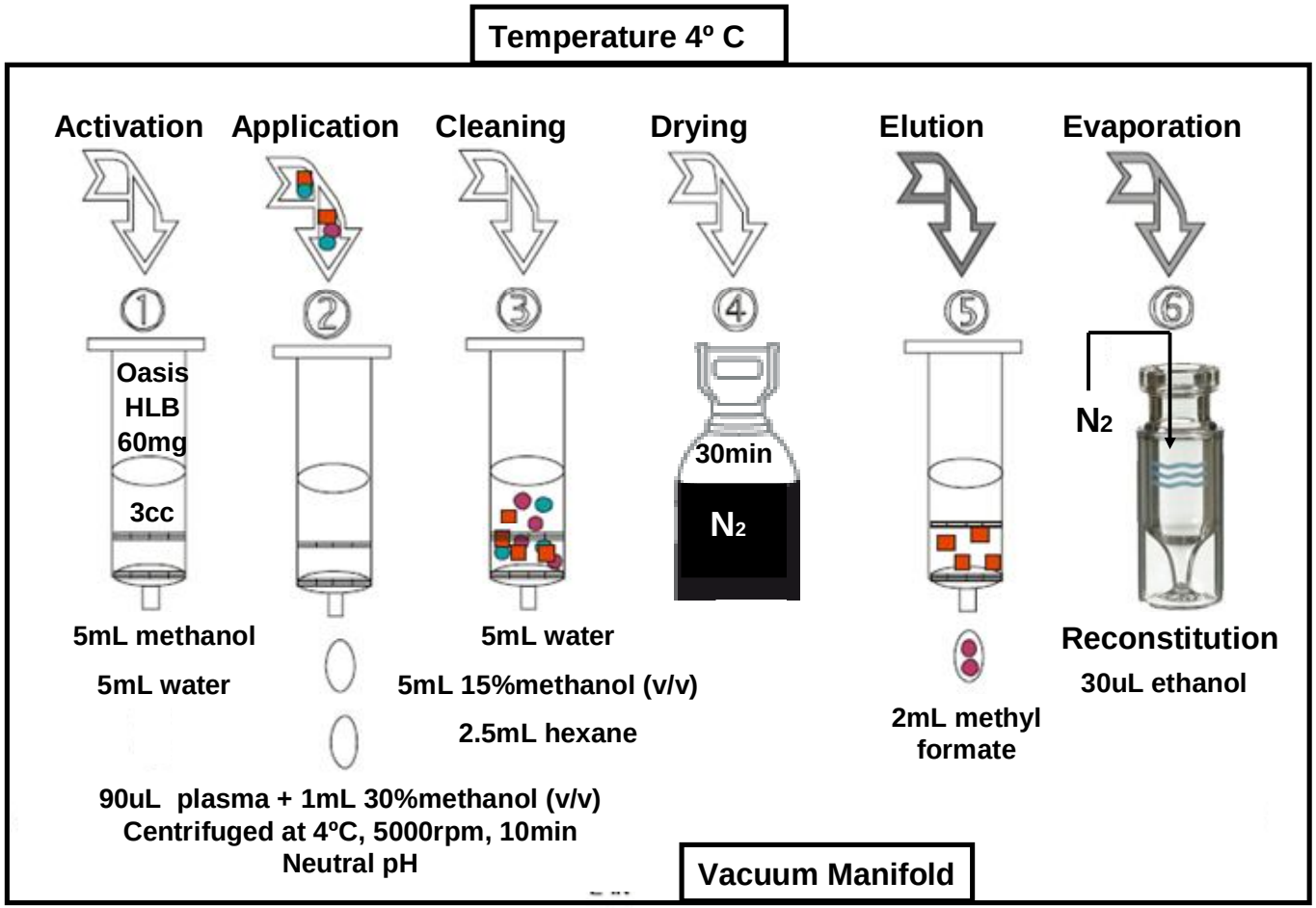

PGE3 349 m/z
Relative Abundance
m/z
331.17
313.17
233.09
269.17
PGD3 349 m/z
349 m/z
331 m/z
313 m/z
251 m/z
233 m/z
269 m/z
189 m/z
98 m/z
44 m/z
CO2
H2O 18 m/z

Figure S3


Temperature 4º C
Activation
Application
Cleaning
Drying
Elution
Evaporation
Oasis
HLB
60mg
3cc
30min
N2
N2
5mL methanol
5mL water
5mL water
5mL 15%methanol (v/v)
2.5mL hexane
2mL methyl formate
Reconstitution
30uL ethanol
90uL plasma + 1mL 30%methanol (v/v)
Centrifuged at 4ºC, 5000rpm, 10min
Neutral pH
Vacuum Manifold