Simultaneous quantitative profiling of 20 isoprostanoids from omega-3 and omega-6 polyunsaturated fatty acids by LC-MS/MS in various biological samples

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Elsevier Editorial System(tm) for Analytica Chimica Acta

Manuscript Draft

Manuscript Number: ACA-15-2770R1

Title: Simultaneous quantitative profiling of 20 isoprostanoids from omega-3 and omega-6 polyunsaturated fatty acids by LC-MS/MS in various biological samples.

Article Type: Full Length Article

Section/Category: MASS SPECTROMETRY

Keywords: isoprostanes, dihomo-isoprostanes, neuroprostanes, phytoprostanes, MS quantification, oxidative stress

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Manuscript Region of Origin: FRANCE

Cover Letter(including Novelty Statement)

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mars 10, 2016

Revision of a full-length Article

Dear Editor,

Through this letter, we would like to ask you to consider our manuscript entitled "**Simultaneous quantitative profiling of 20 isoprostanoids from omega-3 and omega-6 polyunsaturated fatty acids by LC-MS/MS in various biological samples.**" for revision and publication in Analytica Chimica Acta.

We tried to address all the questions where possible, and clarify in the manuscript. We hope that our modifications coult permit our method to be published in your journal.

Sincerely,

Dr Justine BERTRAND-MICHEL

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Responses to reviewers:

We thank the reviewers for the valuable suggestions, and we have tried to address all the questions where possible, and clarify in the manuscript. Our answers to the referees are in bold in the text, and the text modification are in red in the manuscript.

Reviewer #1: This manuscript describes the identification and quantitative profiling of isoprostanoids by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using either derivatization or two different derivatization protocols of the carboxylic acid function.
The manuscript is interesting and provides an additional answer to investigate the identification of isoprostanoids, which are a group of non-enzymatic oxygenated metabolites of polyunsaturated fatty acids. It is clearly important to improve our understanding of these metabolites and it is arguably equally important the need of selective and sensitive analytical methods.
There are some comments/suggestions that deserve clarification:

1) at page 9, paragraph 3.1.1. It is stated that (i) "IsoPs analyzed in this study have different structures comparatively" (ii) "one specific fragment was selected for each compound and the collision energy was optimized for each SRM transition". Considering that the CID fragmentation of these metabolites has not been discussed and no references are given, how have the Authors choice the SRM transitions?

For each pure molecule: first, the full scan was performed to observe the main ion formed in our MS system, then a product ion spectrum was done to select the most abundant daughter ion to keep for the MRM transition; and finally the collision energy was optimized for this transition. The text of the manuscript has been slightly modified to explain this point (paragraph 3.1.1). As majority of these molecules have never been described previously (except for few of them : references : [25]), it is difficult to make comparisons with some of the new ones in this study.

2) Figure 3. All these plots are not easy to follow and a more explicative view is recommended.

The view has been amended and the name of each molecule has been added, so it should be clearer now.

3) Page 10, line 57. What's the meaning of "using linear regression model with different weight factors." Especially the meaning of different weight factors deserve an explanation. What, where and when have weight factors been applied?

We apologize for the mistake. The linear regression model used the classical 1/X weight factor. It has been modified in the text in the paragraph 3.1.3.

4) Whereas several tabulated data are reported in the manuscript, no one biological sample as an example of LC/MS/MS separation is reported;

As it was difficult to show profile for all different biological samples we measured, we decided to show only human plasma in figure 5, which was the sample used for validated our method. It is also important to display human plasma profile to allow clinical scientists and biochemists to be able to compare their measurements in human plasma with ours. The text (paragraph 3.4) was modified to note this additional figure.

conversely, several redundant Tables are included, namely 2, 3, 4 and 5 which should be moved in the supporting information.

It is true that there are many tables in this paper which present different steps for validation of the method. We think tables 2 and 3 are important information to be conserved close to the text. However, we propose to transfer tables 4 and 5 as supporting materials and indicate them as tables 1 and 2. This has been changed in the text paragraph 3.3.1. Table 6 (in first version) is re-numbered as table 4 in this new version.

5) Table 3. I have serious doubts about the consequence of data like LOD and LOQ. What is the reliability of data such as ".. the LOD values ranged from 0.49 ng/mL to 15.6 ng/mL and the LOQs from 0.98 ng/mL to 31.25 ng/mL." without standard errors and a confidence level (Table 3).

Calibration curves were performed for 14 concentrations : 0.06, 0.12, 0.24, 0.49, 0.98, 1.95, 3.91, 7.81, 15.63, 31.25, 62.5, 125, 250, and 500 ng mL-1 for all primary standards (in fact 500ng/mL division by two). The values considered for LOQ at lower point was s/n>10 and for LOD, the lower point was s/n > 5. This determination is common for analyst to obtain LOD and LOQ values. This information has been added in the text (paragraph 2.2).

6) And data of Table 4; how many samples were examined and what is the meaning of error of matrix effect and extraction yield ?

The experiment for matrix effect and yield extract calculation were performed in triplicate (of three different samples) and these three different preparation were injected in triplicates. We did not describe both matrix effect and extraction yield clearly in the manuscript and it has been corrected in paragraphs 2.8 and 3.3.

7) Another critical points is present in the data presented in Figure 6C and D. Need they really to be displayed or just a comment it is enough?

We agree that this figure doesn't add any information to the manuscript. We have removed the information and replaced in the text with the mention " data not shown".

8) Page 14, Conclusions. Are really conclusions or an abstract is reported? If you look at lines 9-19, it seems a summary of what it is presented in the Abstract!

We have moved the original content of the conclusion to discussion. A new and precise conclusion has been added in the manuscript.

Reviewer #2: This manuscript described a LC-MS/MS methodology for quantification of several isoprostanes in very low concentration. The authors optimized their analytical method in many ways, from LC separation of isomers to derivatization conditions for increasing the signals in SRM analysis, which eventually brought the appropriate method to complex biological samples for application test. In general, the manuscript is well organized and the explanation for each step is very clear. I have only minor suggestions for the author to consider.
1) Figure 1, some annotations of the molecules are on top of the structure, please rearrange it so that it's clear to see.

The format of the figure has been improved, it should be easier to read now.

2) Figure 2, the TIC for PA derivatization and HP derivatization were illustrated using both dotted lines, one is large and one is small. It would easier to compare and distinguish if different colors are used, or with more different lines types.
3) It would be easier for the audiences to follow if the author gives two examples of HP and PA derivatization using any of the IsoPs molecules mentioned. One formed a hydrazide and the other formed an amide bond during derivatization. The structures can be combined into Figure 2 on top of the chromatogram lines, or separate out into a new figure.

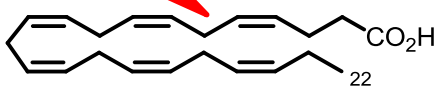
Figure 2 has been improved, the structures of PA and HP derivatized are added on the figure.

4) The yield of extraction of F3t-IsoPs was above 100%, which should be round to 100%, since any numbers beyond 100% is meaningless in terms of yield.

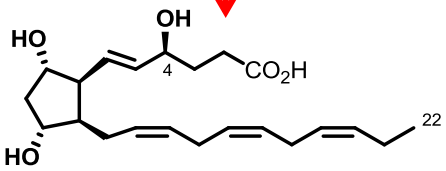
We agree to this. However, as we are determining 20 different molecules simultaneously and using response ratio with deuterated molecules for quantification, the extraction yield should not be the primary confounding factor.

*Graphical Abstract

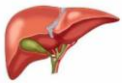
ROS



DHA



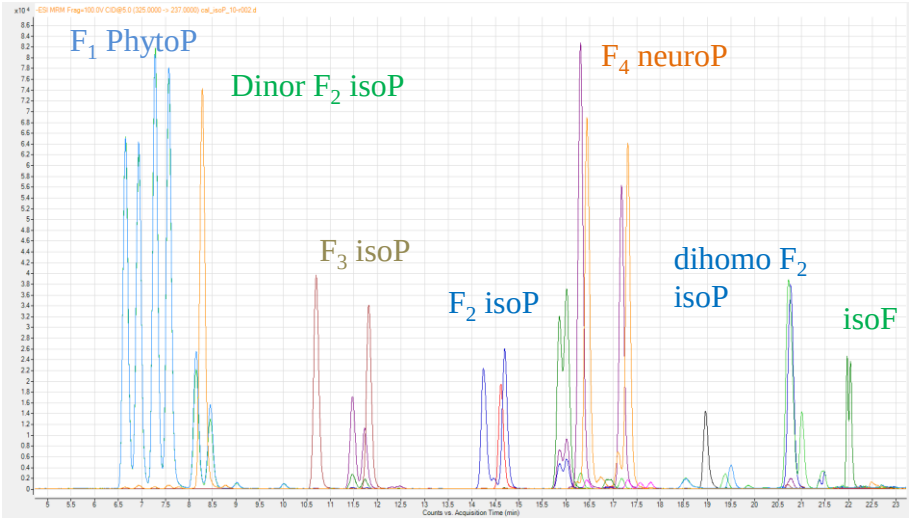
4-F_{4t}-NeuroP



✓ Extraction
✓ Hydolysis
✓ SPE

✓Biomarkers
✓Diagnostic
✓Physiological studies

LC-MS/MS quantification



***Highlights**

Highlights :

Isoprostanoids are a group of non-enzymatic oxygenated metabolites of polyunsaturated fatty acids which are key intermediates in a lot of physiological mechanism. An quantitative LC-MS/MS profiling of these biomarkers was developed, validated and applied it on various biological sample. This method will be highly useful to follow biological process dealing with ROS.

Simultaneous quantitative profiling of 20 isoprostanoids from omega-3 and omega-6 polyunsaturated fatty acids by LC-MS/MS in various biological samples.

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Keywords: isoprostanes, dihomo-isoprostanes, neuroprostanes, phytoprostanes, mass spectrometry, quantification, oxidative stress, ROS

Abbreviations:

AA : Arachidonic acid

AdA : Adrenic acid

ALA : α-Linolenic acid

BHT : Butylated hydroxytoluene

CSF : Cerebrospinal fluid

DHA : Docosahexaenoic acid

DPDS : 2,2’-dipyridyl disulfide

AGTA : Ethylene glycol tetra acetic acid

EPA : Eicosapentaenoic acid

ESI : Electrospray ionization

HP : 2-hydrazinopyridine

HPLC : High-pressure liquid chromatography

IsoPs : Isoprostanes

LOD : limit of detection

LOQ : limit of quantification

m/z : Mass-to-charge ratio

MRM : Multiple reaction monitoring

MS : Mass spectrometry

NeuroPs : Neuroprostanes

OS : Oxidative Stress

PhytoPs : Phytoprostanes

PA : 2-picolylamine

PUFAs : Polyunsaturated fatty acids

ROS : Reactive oxygen species

SPE : Solid-phase extraction

SRM : Selected-reaction monitoring

S/N : signal to noise ratio

TPP : Triphenylphosphine

Abstract

Isoprostanoids are a group of non-enzymatic oxygenated metabolites of polyunsaturated fatty acids. It belongs to oxylipins group, which are important lipid mediators in biological processes, such as tissue repair, blood clotting, blood vessel permeability, inflammation and immunity regulation. Recently, isoprostanoids from eicosapentaenoic, docosahexaenoic, adrenic and ·-linolenic namely F_3-isoprostanes, F_4-neuroprostanes, F_2-dihomo-isoprostanes and F_1-phytoprostanes, respectively have attracted attention because of their putative contribution to health. Since isoprostanoids are derived from different substrate of PUFAs and can have similar or opposing biological consequences, a total isoprostanoids profile is essential to understand the overall effect in the testing model. However, the concentration of most isoprostanoids range from picogram to nanogram, therefore a sensitive method to quantify 20 isoprostanoids simultaneously was formulated and measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The lipid portion from various biological samples was extracted prior to LC-MS/MS evaluation. For all the isoprostanoids LOD and LOQ, and the method was validated on plasma samples for matrix effect, yield of extraction and reproducibility were determined. The methodology was further tested for the isoprostanoids profiles in brain and liver of $LDLR^{-/-}$ mice with and without docosahexaenoic acid (DHA) supplementation. Our analysis showed similar levels of total F_2-isoprostanes and F_4-neuroprostanes in the liver and brain of non-supplemented $LDLR^{-/-}$ mice. The distribution of different F_2-isoprostane isomers varied between tissues but not for F_4-neuroprostanes which were predominated by the 4(*RS*)-4-F_{4t}-neuroprostane isomer. DHA supplementation to $LDLR^{-/-}$ mice concomitantly increased total F_4-neuroprostanes levels compared to F_2-isoprostanes but this effect was more pronounced in the liver than brain.

1. Introduction

Excessive free radicals *in vivo* have been implicated in a number of human diseases such as neurodegenerative, cardiovascular, pulmonary disorder and cancer[1] [2]. The most common free radicals are reactive oxygen species (ROS), which can modify lipids, proteins and nucleic acids. Of the lipids in particular, the polyunsaturated fatty acids (PUFA) form a wide variety of oxygenated metabolites [3] [4]. Among them, the isoprostanes (IsoPs) appears to be a promising group of biomarkers to be assessed for oxidative stress (OS) assessment *in vivo* for over two decades due to its specificity and sensitivity[5] [6]. These compounds are formed *in situ* on membrane phospholipids and then released into their free form via phospholipase A_2 and platelet activating factor hydrolase for circulation. Elevation of IsoPs, in particular those originated from arachidonic acid (AA, 20:4 n-6) also known as F_2-IsoPs in biological fluids (e.g. plasma and urines) are recognized as the reference biomarker for lipid peroxidation and OS in most biological systems. Beyond their capacity of OS as biomarker, IsoPs from n-3 PUFA also demonstrated to be biologically active[7] [8] [9]. Therefore it is crucial to be able to quantify the different isoforms in a large panel of biological samples to integrate this chemical and biological complexity.

Unlike PUFAs, the isoprostanoids are quite complex to assess since the concentration range is very low (from picogram to nanogram) in most biological samples. Moreover, depending on the parent PUFAs, a large diversity of molecule has been discovered as shown in Figure 1. Analysis of these metabolites in biological samples is a challenge and depends on the robustness of the analytical instrumentation. Further, it requires one or several preparation steps, including hydrolysis and extraction from their biological matrix before analysis by radio immunological methods (RIA) or gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-mass spectrometry (LC-MS), which are often coupled to another mass spectrometer (MS/MS) to increase the sensitivity[10]. It is well known to analysts, that RIA is not specific enough to provide efficient quantification of different IsoPs [11] To date, LC-MS/MS is the most common technique to quantify these biomarkers [12], even if the mass spectrometry is not the perfect method to perform absolute quantification compared to GC-MS because of the various ionization efficiency between different molecules. These changes can be very important when comparing compounds with very close structures especially for lipids including the IsoPs. In order to optimize ionization efficacy for each compound, it is essential to have the pure standard to develop a rigorous quantitative method. Although some standards are available commercially, many of the novel ones are unavailable. Through total synthesis,

Durand's group was able to synthesize [13-21] these novel standards, for example dihomo-IsoPs from adrenic acid (C22:4 n-6, AdA) for mass spectrometry analysis.

In this study, we developed a complete quantitative profiling of IsoPs by LC-MS/MS. As IsoPs are present in a very low concentration it was imperative to improve largely the sensitivity of the method therefore we also tried two different derivatization procedures of the carboxylic acid function to improve the ionization of molecules. For both profiles, with and without derivatization, chromatographic separation has been optimized and sensitivity compared. The two methods have been tested on human plasma and the best one was applied in this study. The final methodology was then validated on plasma sample and applied to other biological samples, namely cerebrospinal fluid (CSF), urine, and brain, liver and muscle tissues. Our methodology was finally checked on a mouse model, in which the goal was to determine the isoprostanoids profiling in the brain and liver of $LDLR^{-/-}$ mice and to investigate the effect of docosahexaenoic acid (C22:6 n-3, DHA) supplementation on these profiles.

2. Material and methods

2.1. Chemicals

Commercially available IsoP standards (d_4-15-F_{2t}-IsoP and 2,3-dinor-15-F_{2t}-IsoP) were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Others standards *Ent*-16-*epi*-16-F_{1t}-PhytoP, *Ent*-16-F_{1t}-PhytoP, 9-F_{1t}-PhytoP, 9-e*pi*-9-F_{1t}-PhytoP, *Ent*-15(*RS*)-2,3-dinor-5,6 dihydro-15-F_{2t}-IsoP, 8-F_{3t}-IsoP, 8-*epi*-8-F_{3t}-IsoP, 5-F_{3t}-IsoP, 5-*epi*-5-F_{3t}-IsoP, 15-F_{2t}-IsoP, 15-*epi*-15-F_{2t}-IsoP, 5-F_{2t}-IsoP, 5-*epi*-5-F_{2t}-IsoP, 10-F_{4t}-NeuroP, 10-*epi*-10-F_{4t}-NeuroP, 14(*RS*)-14-F_{4t}-NeuroP, 4(*RS*)-4-F_{4t}-NeuroP, *Ent*-7(*RS*)-7-F_{2t}-dihomo-IsoP, 17(*RS*)-17-F_{2t}-dihomo-IsoP, C21-15-F_{2t}-IsoP, d_4-10-*epi*-10-F_{4t}-NeuroP, d_4-10-F_{4t}-NeuroP, d_4-4(*RS*)-4-F_{4t}-NeuroP were synthesized according to our published procedures[13] .Hexane, ethanol absolute, acetic acid potassium hydroxide (KOH), methanol (MeOH; HPLC gradient Grade), butylated hydroxytoluene (BHT) and formic acid were purchased from Sigma Aldrich (Saint Quentin Fallavier, France). Acetonitrile (ACN; HPLC grade) was obtained from Acros Organics (Illkirch, France). Ammonia solution 30 % (NH_4OH) was purchased from Carlo Erba Reagenti (Cornaredo, Italy). Water used in this study was

purified on a milliQ system (Millipore). The 96 well-plate for solid extraction (SPE) (Oasis Max, 60 mg) was purchased from Waters (Saint-Quentin en Yvelines, France).

2.2. Standards preparation for linearity and reproducibility assessment

Standard solutions with or without derivatization were prepared in MeOH at the following concentrations, 0.06, 0.12, 0.24, 0.49, 0.98, 1.95, 3.91, 7.81, 15.63, 31.25, 62.5, 125, 250, and 500 ng mL-1 for all primary standards. The concentration of the deuterated internal standards (IS) used 5 ng taken from 250 ng mL-1 stock solution. Calibration curves were calculated by the area ratio of the analyte and the internal standard. The linearity and the accuracy of the detection were determined and the limit of detection (LOD : lower point with s/n > 5) and limit of quantification (LOQ: lower point with s/n > 10) were defined for the 20 compounds.

2.3. Biological fluid extraction

Healthy human plasma (1 mL) or CSF (600 µL), LDLR-/- mice plasma (≥200 µL) or urine (≥200 µL) were collected, and supplemented with BHT (1% in ethanol), and stored at -80°C. For the extraction, the samples were thawed and spiked with 5 ng of each internal standard. A volume of 985 µL of hydrolysis solution (KOH 1M in MeOH) was added. The resulting mixture was mixed and incubated at 40°C for 30 minutes (excepted for urine). After cooling in room temperature, 2 mL of 40 mM formic acid (pH 4.5 adjusted with 1 M NaOH) was added. Thereafter, the samples including urine were cleaned and extracted by solid-phase extraction (SPE) on 96-well plate OASIS MAX 60 mg (Waters, USA) modified from Lee et al. method [22]. Briefly, the wells were cleaned with 2 mL of MeOH and conditioned with 2 mL of 40 mM formic acid (pH 4.5). After loading the samples, the wells were washed with 2 mL of 2% NH_4OH followed by 2 mL of MeOH/20 mM formic acid (20:80 v/v) and 2 mL of hexane. The IsoPs were eluted with 2 ml hexane/ethanol/acetic acid (70:29.4:0.6 v/v/v). After drying under nitrogen gas, the samples were re-dissolved with 20 µL of MeOH. A part of the sample (5 µl) was taken for LC-MS/MS analysis and the remaining samples were derivatized prior to LC-MS/MS measurement.

2.4. Biological tissue preparation

The tissue samples were stored at -80°C before preparation. To a total of 200 mg of thawed tissue (brain, liver or muscle) sample, 1 mL of Folch solution ($CHCl_3$:MeOH, 2:1, v/v) containing 10 µL BHT (1 % in ethanol) was added and spiked with 5 ng of each internal standard. The mixture was homogenized with a Fast Prep instrument (MP Biomedicals) for 30 s at 6.5 m/s. Then the homogenized tissue was further extracted with 1.5 mL ice-cold Folch solution ($CHCl_3$: MeOH, 2:1, v/v) and 0.5 mL of ultrapure water. The mix was shaken for 30 s and centrifuge for 10 min at room temperature to separate the aqueous and organic layers. The lower organic layer was carefully removed and transferred to a pyrex tube and then evaporated under nitrogen gas. The extracted lipid was dissolved in 1 mL of hydrolysis solution (KOH 1M in MeOH) and incubated at 40°C for 30 minutes. After cooling in room temperature, 3 mL of 40 mM formic acid was added. The samples were then cleaned and extracted by SPE as described in section 2.3.

2.5. Derivatization of the extracted samples

To a set of extracted samples, 10 µL of freshly prepared solution of 10 mM triphenylphosphine (TPP), 10 mM 2,2'-dipridyl disulfide (DPDS) and 10 µg 2-picolyamine (PA) prepared separately in acetonitrile were added successively. To another set of the extracted samples, freshly prepared 10 µL of TPP and DPDS, and 10 µL of freshly prepared 10 µg 2-hydrazinopyridine (HP) in acetonitrile were added. The sample mixture was incubated at 60°C for 10 min. The mixture was dried under nitrogen and then reconstituted in MeOH for LC-MS/MS analysis.

2.6. LC-MS/MS analysis

High performance liquid chromatography (HPLC) was performed using an Agilent 1290 Infinity equipped with a thermostated autosampler, a binary pump and a column oven. The analytical column was a Zorbax SB-C18 Rapid Resolution HD (2,1 x 100 mm; 1,8 µm) (Agilent Technologies, USA) and maintained at 25°C. The mobile phases consisted of water: formic acid (99.9:0.1;v/v) (A) and acetonitrile : formic acid (99.9:0.1, v/v) (B). The linear gradient was set as follows for the non-derivatized IsoPs analysis: 20% B at 0 min, 30% B at 15 min, 35% B at 20 min, 100% B at 23 min, 100% B at 26 min, and 20% B at 26.5 min for 1.5 min of equilibration. For the derivatized IsoPs, the gradient was set to

18% B at 0 min, 30% B at 22 min, 35% B at 26 min, 100% B at 28 min, 100% B at 29 min, and 18% B at 29.5 min for 1.5 min of equilibration. The flow rate was set at 0.3 mL/min. The autosampler was fixed at 5◦C and the injection volume was 5 µL per analysis. The HPLC system was coupled on-line to an Agilent 6460 triple quadrupole MS (Agilent Technologies, USA) equipped with electrospray ionization (ESI). The ESI was performed in negative ion mode for non-derivatized IsoPs and positive mode for derivatized IsoPs. The MS source parameters were set as follows: source temperature 325ºC, nebulizer gas (nitrogen) flow rate 10 L min-1, sheath gas temperature 350ºC, sheath gas (nitrogen) flow rate 12 L min-1 and the spray voltage adjusted to −3000 V. The dwell time used was 10 ms. The analysis was performed in Selected Reaction Monitoring (SRM) detection mode using nitrogen as the collision gas. The SRM of each compound without (Table 1) or with derivatization (Table 2) were pre-determined by MS/MS analysis. Peak detection, integration and quantitative analysis were performed by Mass Hunter Quantitative analysis software (Agilent Technologies, USA). Concentration of the analytes was calculated by calibration curves obtained in Section 2.2.

2.7. Accuracy and precision

Repeatability and precision were respectively assessed using relative standard deviation (% RSD) and accuracy at 3 concentrations (3.91, 31.25 and 250 ng mL-1) of pure standards in triplicate determination. The concentration was subsequently calculated using the standard curves generated. For inter-day variation, the samples were analyzed on 2 different days, with 15 days in between interval.

2.8. Validation of sample preparation

The preparation of human plasma sample was validated through the yield extraction and the matrix effect. Briefly, three sets in triplicate were prepared : 500 µL of plasma (n = 3) were spiked with 5 ng of IS stock solution and 2 different concentrations of standards (31.25 and 250 ng mL-1) and were extracted as described in Section 2.3: 1) 500 µL of plasma (n = 3) were extracted and then spiked with 5 ng of IS stock solution with 2 different concentrations of standards (31.25 and 250 ng mL-1), and 2) a separate set of pure 5 ng of IS stock solution and standards solutions (31.25 and 250 ng mL-1) in the absence of plasma extract were prepared in MeOH. All sets (of three samples) were analyzed in

triplicate using the LC-MS/MS. The yield extraction was determined as the percent difference between peak areas of standards in pre-spiked and post-spiked samples. The matrix effect was determined as the percentage difference between peak areas of standards added to the extracted samples and pure standard. The plasma matrix effect and yield extraction were calculated for each compound measured in the method described.

2.9. Biological samples

With permission, a sub-group of $LDLR^{-/-}$ mice from a previous study [23] was used to determine the effect of DHA supplementation on the profile of the isoprostanoids in the brain and liver. Briefly, from 8 weeks of age and for 20 weeks, the mice received by daily oral gavages (50 µL, 5 days/week) either oleic acid rich sunflower oil (Lesieur, Asnières-sur-Seine, France; Control group) or a mixture of oleic acid rich sunflower oil and DHA rich tuna oil (OMEGAVIE DHA90TG, Polaris Nutritional Lipids, France containing 90% of DHA as TG) providing 2% (or 35.5 mg/d/mouse) of energy as DHA. At the end of the supplementation, the mice were anaesthetized (40 mg pentobarbital/kg body weight) and the tissue samples were rapidly removed and snap-frozen in liquid nitrogen and stored at -80°C until analysis. Quantification of DHA and AA were performed on brain and liver sample. In brief, after an organic extraction in presence of internal standard, the total fatty acid were methylated, analysed and quantified on a gas chromatography-flame ionization detector (GC-FID) system [24].

3. Results and discussion

3.1 LC-MS/MS method development

In order to achieve the necessary selectivity and sensitivity of the method, the mass detection and chromatographic separation of each standard with or without derivatization were individually optimized.

3.1.1 Mass detection

IsoPs analyzed in this study have different structures comparatively. Nevertheless, due to their common carboxylic acid moiety, they were all detected in the negative ion mode as [M−H]− ions. Firstly, the fragmentor voltage was optimized for each compound in product ion scan mode. This parameter promotes the transmission of the ions between the ionization source and the first quadrupole. Low voltages lead to poor transmission efficiency whereas too high voltage values lead to excessive fragmentation. The optimum value corresponded to a maximum transmission of the [M−H]− ions without fragmentation. The second optimized parameter was the collision energy for each MS/MS transition to monitor; the most abundant one was selected. In this study, one specific fragment was selected for each compound and the collision energy was optimized for each SRM transition (Table 1). SRM transitions observed for this study are divided according to the PUFA type, that includes 15-F_2 series (m/z 353 to m/z 193), 5-F_2 series (m/z 353 to m/z 115), 8-F_3 series (m/z 351 to m/z 127), and 5-F_3 series (m/z 351 to m/z 115) [25]. For the F_1-PhytoP, the carboxylate portion was lost to give SRM m/z 327 to m/z 283, whereas 10-F_4-NeuroP seems to fragment in the same way as 8-F_3 series (m/z 377 to m/z 153). For the remaining compounds, the fragmentations were not as definite.

Since the concentration of the IsoPs is low in biological samples, analysts may opt for a derivatization procedure to increase the volatility and polarity of the compounds. To enhance the detection responses of carboxylic acids in ESI-MS/MS several chemical derivatization procedure can be applied and measured in the positive mode of the LC-MS/MS[26] [27]. However, the derivatization reagents are not always commercially available and the preparation can be long and time consuming. In our study, we tested two simple reagents 2-hydrazinopyridine (HP) and 2-picolylamine (PA), which can be derivatized in one step under mild conditions[28]. When the reagents react with the acidic function of the IsoPs, the HP and PA form a hydrazide and an amide bond, respectively. The sensitivity obtained was preferably for PA derivatives than HP derivatives (Figure 2), therefore MS parameters were optimized only for PA (Table 2). An important drawback of this derivatization method is that for all species a unique PA fragment of m/z = 109 is obtained, which creates poor specificity of the IsoP tested.

3.1.2 Chromatographic separation

The IsoP determined in this study have similar molecular mass and structure. As a result, the liquid chromatographic separation of each metabolite is a crucial step where each isomer of the IsoPs needs to be optimized to be separated in the chromatogram for detection. In this

study, the eluent phase was acidic and a semi-linear gradient elution allows the chromatographic resolution of most compounds on a 10 cm C18 reverse phase column within 30 minutes. The gradient was also designed for the measurement of PA derivatives. As shown in Figure 3, F_{1t}-PhytoPs eluted first, followed by 2,3-dinor-15-F_{2t}-IsoP, F_{3t}-IsoPs, F_{2t}-IsoPs, then F_{4t}-NeuroPs and F_{2t}-dihomo-IsoPs. The isobaric compounds bearing the same transition such as F_{1t}-PhytoPs were successfully separated (Table 1). Furthermore, all the isomers were resolved in the chromatographic separation including 8-F_3t- and 8-*epi*-8-F_3t-IsoPs (Figure 4A and B), while for 5-F_{2t}- and 5-*epi*-5-F_{2t}-IsoPs the separation between the two peaks showed an overlap at the tail of the chromatographic peak (Figure 4C and D). The separation was comparable with PA derivatives indicating derivatization procedure did not improve the separation.

3.1.3 Sensitivity

To quantify our isoprostanoids we first had to choose the appropriate internal internal standard for each metabolite. NeuroPs and 15-F_{2t}-IsoP were quantified through their deuterated equivalent and for other IsoPs, d_4-15-F_{2t}-IsoP and C21-15-F_{2t}-IsoP were used. Based on the internal standard, each calibration curve was obtained with 10 concentrations of the pure IsoPs ranging from 0.9 to 500 ng/mL. The curves were fitted using linear regression model with 1/X factors. The linearity of the method was assessed for each metabolite by evaluating the correlation coefficient (Table 3). The LOD and LOQ of our method were evaluated, and in general LOD corresponding to the lowest concentration had signal to noise ratio above 3 and LOQ corresponding to the lowest concentration had signal to noise ratio above 10. Both of these values however depended on the type of isoprostanoids. For PA derivatives the LOD values ranged from 0.24 ng/mL to 1 ng/mL and the LOQs from 0.12 ng/mL to 2 ng/mL. As shown in Table 3, the LOD values ranged from 0.49 ng/mL to 15.6 ng/mL and the LOQs from 0.98 ng/mL to 31.25 ng/mL. The sensitivity obtained for F_2-IsoP, which is the main IsoP in the literature, is in agreement with LOQ previously reported by others [29] [30]. The sensitivity observed was better than the isoprostanoids with derivatization, in particular for PA. The lack of sensitivity and specificity by PA derivatization indicates that it was not suited for the analysis of IsoPs and more so in biological samples, which often have complicated matrix structure that could further affect the precision of the measurement.

3.2 Plasma sample preparation and analysis

The lipid portion of the tissues or cells was extracted using Folch solution in the presence of antioxidant (0.005% BHT). It is known approximately 70% of the IsoPs are conjugated to phospholipid through ester bond [31], therefore hydrolysis step is required to analyse the total concentration. The lipid extract or fluid (except urine) was treated with 1 M KOH prepared in methanol for 30 minutes at 40 ºC. This step is not required for measurement of non-esterified IsoPs i.e. free form. A purification process is needed to reduce matrix effect and to increase sensitivity of the quantification by LC–MS/MS analysis. Different SPE cartridges were tested in this procedure namely, C18[32], HRX[33] and MAX[22]. It was found MAX cartridge, which is composed of mixed anionic exchange phase provided the cleanest sample and the best LC-MS/MS evaluation. A part of the lipid extracts from SPE were taken for derivatization process. The data obtain from plasma with and without derivatization were compared (data not shown). Despite the peak response and area being bigger for the derivatized samples, the background noise of the chromatogram was much more compared to the non-derivatized samples. Moreover, the derivatization caused the formation of few additional peaks very close to the target ones, making it more difficult to differentiate for peak integration. This observation further support that derivatization procedure is not suitable for IsoP analysis by LC-MS/MS for plasma and likely for other biological samples. Therefore, in order to avoid overestimation in our quantification, we adopted the IsoP measurements without derivatization process in this study.

3.3 Validation of sample processing

3.3.1 Matrix effect and yield of extraction

The efficiency of the sample processing was assessed by measuring the matrix effect and the extraction yield using plasma in triplicate. These two parameters were calculated for two concentrations of IsoPs (250 and 31.25 ng/mL). The peak areas of the chromatogram for each IsoP were compared before and after addition plasma extraction to obtain the matrix effect, as summarized in supporting material Table 1. The matrix effect ranged between 51.4% to 92.7% for low concentration and between 56.7% to 77.5% for high concentration. The values were relatively homogeneous in each group of IsoP. The yield of extraction (supporting material Table 1) was calculated for each IsoP for two concentrations comparing the quantity recovered in presence of plasma to the native one. The yield ranged from 52.0% to 85.3% for low concentration and from

40.5% to 69.2% for high concentration. Surprisingly the yield extraction of F_{3t}-IsoPs was above 100%, and no interference signal was observed for the non-spiked plasma extract; the reason for this observation is unknown.

3.3.2 Performance of the method

Repeatability and precision were then evaluated for intra- and inter-day at 3 concentrations: 3.91, 31.25 and 250 ng/mL (supporting material Table 2) were performed in triplicate. The intra-day accuracy ranged from 81.73% to 114.21% for all IsoP evaluated. The RSD values for 3 injections were ≤ 8%. The inter-day variations were assessed by re-analyzing the samples every 15 days (n=2). The accuracies obtained were between 80.9% and 115.53% with a precision ≤ 15%. These data indicate that the method is highly reproducible for the 20 IsoP compounds analyzed.

3.4 Application on various biological samples

Our extraction and LC-MS/MS methods were applied to quantify non-enzymatic oxidized lipids from PUFAs in different biological samples. The different samples (Table 4) measured include human plasma and CSF, mice plasma, urine, liver, brain and muscle tissues A typical profile for human plasma is displayed in Figure 5. The structural matrix was taken into account for the quantity used for each type of samples and the size is shown in Table 4. Apart from CSF, IsoPs were detected and it appears to vary depending on the type of sample. No IsoPs were found in CSF, and it likely due to small volume used and concentration maybe below our limit of detection.

The method developed was also used to perform the isoprostanoids profiles in the liver and brain of $LDLR^{-/-}$ mice and to investigate if DHA supplementation could affect the profiles. The overall levels i.e. total summation of all the related isomers measured for F_2-IsoPs (19 pg/mg vs 14 pg/mg) and for F_4-NeuroPs (19 pg/mg vs 17 pg/mg) in the liver and brain of control mice (white bars, Figures 6A and 6C) were slightly higher in the liver than the brain (+36% for F_2-IsoP and +11% for F_4-NeuroP). Furthermore, the total levels of F_4-NeuroPs in the control mice liver and brain were similar to the levels of F_2-IsoPs even though the concentration of DHA was 6 times and 34 times higher than AA in the liver and brain respectively (data not shown). Our observation suggest that the presence of high DHA concentration may contribute in protecting the liver and

brain from ROS attack despite it being more prone to peroxidation due to extra double bonds in the structure compared to AA [8]. When looking at the different isomers of the isoprostanoids (Figure 6B and 6D), it is interesting to note that the distribution of the different F_2-IsoP isomers is slightly different in the liver and brain of the control mice with 15-epi-F_{2t}-IsoP and 15-F_{2t}-IsoP being more abundant in the brain; for the liver, the abundance of the different F_2-isomers was as follows: 15-F_{2t}<5-Epi-F_{2t}>15-Epi-F_{2t}>5-F_{2t}.

Among the F_4-NeuroPs, the most abundant isomer was 4(RS)-4-F_{4t}-NeuroP regardless of the tissue type. This finding is consistent with previous analysis performed in rat brain and heart tissues[34] .When the mice were supplemented with DHA (Figure 6, dark bars), the total level of F_2-IsoPs decreased in the brain (-40% for the sum of F_2-IsoP) and the liver (-57% for the sum of F_2-IsoP) whereas the total level of F_4-NeuroPs increased by 51% in the brain and 247% in the liver. The concomitant decrease of F_2-IsoPs and increase of F_4-NeuroPs could be attributed at least in part by the replacement of AA by DHA in the membrane phospholipids. It should nevertheless be noted that the modulation of isoprostanoids profiles is much more pronounced in the liver than in the brain emphasizing the high "plasticity" of liver towards DHA supplementation. Consistently, correlations between levels of AA and F_2-IsoP as well as DHA and F_4-NeuroP were strong in the liver (R^2=0.71 and 0.96 respectively, Figure 7A and B) whereas they were much weaker in the brain (R^2=0.02 and 0.16 respectively, data not shown).

It should be noted in this report that we profiled the isoprostanoids in the liver and brain of an atherosclerotic mice and not normal mice. It is also anticipated that the profile of a normal mice may be different from our observation. Regardless, the objective of this study is to understand the differential changes between tissues. Our observation particularly displayed the importance of performing an integrated analysis of isoprostanoids levels since biological interpretation regarding tissue distribution and dietary modulation of lipid peroxidation is complex, and may lead to incorrect interpretation of experimental findings[10]. Moreover, the distribution of different types of isomers depends on the tissue type, which indicates the importance of tissue selection for studies evaluating bioactivity and organ crosstalk.

In summary, we have described a LC-MS/MS methodology allowing simultaneous quantification of several isoprostanes derived from n-3 and n-6 PUFA which are potential biomarkers in biological systems. Using the LC-MS/MS, we first characterized the analytical and quantification parameters of the 20 studied IsoPs and the 4 internal standards including LOD and LOQ concentration range. We optimized the sample preparation and the extraction process of these isoprostanoids which include Folch extraction, basic hydrolysis and SPE purification to obtain

low matrix effect and good yield of extraction. The method was validated on human plasma (repeatability and accuracy). The optimized chromatographic separation permits to separate nearly all the isomers in 30 minutes with a good sensitivity. We then applied this method to human samples (plasma and CSF) and mice samples (plasma, urine, liver, brain, muscle) to optimize the quantity required to be able to profile the isoprostanoids. Finally, the method was tested on brain and liver samples of $LDLR^{-/-}$ mice with and without DHA supplementation to observe the change on the isoprostanoids profiles. We found a variation in the distribution of different F_2-IsoP isomers between tissues but not for F_4-NeuroP. DHA supplementation concomitantly increased F_4-NeuroP levels. OS is a key feature in a number of human diseases, since ROS are likely to be involved in all disease stages. Many of these diseases are associated to PUFA therefore it is important to identify and evaluate the IsoP compounds simultaneously, not only as reliable biomarkers but also for its functional roles in the PUFA metabolism; we believe that the information we obtained from such profiling will allow us to understand the interaction of the compounds in diet and disease studies.

4. Conclusion

In this report, we developed a quick and robust method to determine multiple numbers of non-enzymatic oxidized lipid products of PUFAs, namely isoprostanoids in various biological sample in particular human plasma by LC-MS/MS. Unlike other reports, we were able to measure and incorporate new isoprostanoids from AdA and ALA as well as some isomers of AA, DHA and EPA into our method. However, it should be noted that not all 20 of the products determined were found in all biological samples therefore care must be taken when selecting them in metabolism studies.

Acknowledgment and Grant support

This work was supported by the Fondation pour la Recherche Médicale (grant ING20121226373), the Agence Nationale de la Recherche, the Institut National de la Santé et de la Recherche Medical and the French National Infrastructure MetaboHUB ANR-11-INBS-010.

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Figure legends

Figure 1. Chemical structure of isoprostanes derived from non-enzymatic oxidation of n-6 PUFA, adrenic acid (AdA) and arachidonic acid (AA), and n-3 PUFA, α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) measured in this study.

Figure 2. Total ion current chromatogram of IsoP: with PA derivatization (A), with HP derivatization (B), and without derivatization (C) of the same sample mix.

Figure 3. Chromatogram of selected reaction monitoring (SRM) of metabolites from each PUFA A: adrenic acid, B: arachidonic acid, C: α-linolenic acid, D: eicosapentaenoic acid and E: docosahexaenoic acid. ~~The numbers indicated for each molecule refers to the compounds annotated in Table 1.~~

Figure 4. Optimum chromatographic separation of diastereoisomers for 8-F_{3t}-IsoP (a) and 8-*epi*-8-F_{3t}-IsoP (b). The diastereoisomers of 5-F_{2t}-IsoP (c) and 5-*epi*-5-F_{2t}-IsoP (d) were unable to resolve as well in the chromatographic analysis.

Figure 5. Chromatogram of selected reaction monitoring (SRM) of metabolites detected in human plasma : 15-F_2t-IsoP, 15-*epi*-15-F_2t-IsoP (A) ; 5-F_2t-IsoP, 5-*epi*-5-F_2t-IsoP (B) ; 10-F_4t-NeuroP, 10-*epi*-10-F_4t-NeuroP (C) ; 4(*RS*)-F_4t-NeuroP (D) ; internal standard d_4-15-F_{2t}-IsoP (E).

Figure 6. Isoprostanoids levels in the liver and brain of $LDLR^{-/-}$ mice given either oleic acid rich sunflower oil (Ctrl, n=3) or a mixture of oleic acid rich sunflower oil and DHA rich tuna oil providing 2% of energy as DHA (DHA, n=3). The sum of F_2-IsoPs and sum of F_4-NeuroPs represents the 'total' sum of the isomers measured for the respective group.

Figure 7. Correlations between the levels of AA or DHA and the corresponding isoprostanoids (i.e. sum of F_2-IsoPs and sum of F_4-NeuroPs) in the liver (A and B).

Table 1. Selected reaction monitoring (SRM) of the isoprostanes derived from polyunsaturated fatty acids.

Compounds	RT (min)	Precursor ion (*m/z*)	Product ion (*m/z*)	F (V)	CE (V)
Adrenic acid					
Ent-7(*RS*)-7-F_{2t}-dihomo-IsoP	20.76	381	143	120	18
17(*RS*)-F_{2t}-dihomo-IsoP	20.90	381	337	120	12
Arachidonic acid					
15-*epi*-15-F_{2t}-IsoP	14.24	353	193	120	20
15-F_{2t}-IsoP	14.69	353	193	120	20
5-F_{2t}-IsoP	15.85	353	115	120	12
5-*epi*-5-F_{2t}-IsoP	16.01	353	115	120	12
2,3-dinor-15-F_{2t}-IsoP	8.29	325	237	100	5
Ent-15(*RS*)-2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP	8.30	327	283	120	20
d_4-15-F_{2t}-IsoP	14.61	357	197	120	20
C21-15-F_{2t}-IsoP	18.97	368	193	120	22
alpha-Linolenic acid					
Ent-16-*epi*-16-F_{1t}-PhytoP	6.66	327	283	120	15
Ent-16-F_{1t}-PhytoP	6.94	327	283	120	15
9-F_{1t}-PhytoP	7.30	327	283	120	15
9-e*pi*-9-F_{1t}-PhytoP	7.58	327	283	120	15
Eicosapentaenoic acid					
8-F_{3t}-IsoP	10.70	351	127	120	18
8-*epi*-8-F_{3t}-IsoP	11.82	351	127	120	18

5-F_{3t}-IsoP	11.48	351	115	120	15
5-*epi*-5-F_{3t}-IsoP	11.72	351	115	120	15
Docosahexaenoic acid					
10-F_{4t}-NeuroP	16.43	377	153	120	15
10-*epi*-10-F_{4t}-NeuroP	17.31	377	153	120	15
4(*RS*)-F_{4t}-NeuroP	19.50	377	101	120	15
d_4-10-*epi*-10-F_{4t}-NeuroP	17.19	381	157	120	15
d_4-10-F_{4t}-NeuroP	16.31	381	157	120	15
d_4-4(*RS*)-4-F_{4t}-NeuroP	20.90	382	239	120	15

The deuterated form of IsoP and NeuroP, and C21-15-F_2t isoP were used as internal standards for quantification of samples in this study. IsoP: isoprostane; NeuroP: neuroprostane; F: Fragmentor; CE: collision energy; N : number.

Table 2. Selected reaction monitoring (SRM) of the derivatized isoprostanes derived from polyunsaturated fatty acids.

Compound	RT (min)	Precursor ion (*m/z*)	Product ion (*m/z*)	F (V)	CE (V)
Adrenic acid					
Ent-7(*RS*)-7-F_{2t}-dihomo-IsoP	24.17	455	109	130	30
17(*RS*)-F_{2t}-dihomo-IsoP	23.41	455	109	130	30
Arachidonic acid					
15-*epi*-15-F_{2t}-IsoP	14.73	427	109	120	25
15-F_{2t}-IsoP	15.34	427	109	120	25
5-F_{2t}-IsoP	18.32	427	109	120	30
5-*epi*-5-F_{2t}-IsoP	18.50	427	109	120	30
Ent-15(*RS*)-2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP	8.89	401	109	120	30
d_4-15-F_{2t}-IsoP	15.26	431	109	120	25
C21-15-F_{2t}-IsoP	20.83	442	109	120	20
alpha-Linolenic acid					
Ent-16-*epi*-16-F_{1t}-PhytoP	5.97	401	109	120	30
Ent-16-F_{1t}-PhytoP	6.21	401	109	120	30
9-F_{1t}-PhytoP	6.62	401	109	120	30
9-e*pi*-9-F_{1t}-PhytoP	6.86	401	109	120	30
Eicosapentaenoic acid					
8-F_{3t}-IsoP	10.58	425	109	120	35
8-*epi*-8-F_{3t}-IsoP	11.91	425	109	120	35
5-F_{3t}-IsoP	12.77	425	109	130	30
5-*epi*-5-F_{3t}-IsoP	13.03	425	109	130	30
Docosahexaenoic acid					

10-F_{4t}-NeuroP	16.80	451	109	120	20
10-*epi*-10-F_{4t}-NeuroP	18.17	451	109	120	20
14(*RS*)-14-F_{4t}-NeuroP	18.53	451	109	130	25
4(*RS*)-F_{4t}-NeuroP	23.81	451	109	130	25
d_4-10-*epi*-10-F_{4t}-NeuroP	16.64	455	109	120	20
d_4-10-F_{4t}-NeuroP	18.04	455	109	120	20
d_4-4(*RS*)-4-F_{4t}-NeuroP	23.90	455	109	140	30

The deuterated form of IsoP and NeuroP, and C21-15-F_2t isoP were used as internal standards for quantification of samples in this study. IsoP: isoprostane; NeuroP: neuroprostane; isofurane ; F: Fragmentor; CE: collision energy.

Table 3. Limit of detection (LOD) and limit of quantification (LOQ) of the pure compounds analyzed.

Compound	Linear regression	R^2	LOD (ng/mL)	LOQ (ng/mL)	IS
Adrenic acid					
Ent-7(*RS*)-7-F_{2t}-dihomo-IsoP	y = 0.0044 x - 0.0339	0.998	1.95	3.91	d_4-15-F_2t isoP
17(*RS*)-F_{2t}-dihomo-IsoP	y = 0.0065 x - 0.0639	0.998	7.81	15.63	d_4-15-F_2t isoP
Arachidonic acid					
2,3-dinor-15-F_{2t}-IsoP	y = 0.0079 x - 0.0558	0.998	0.98	1.95	d_4-15-F_2t isoP
Ent-15(*RS*)-2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP	y = 0.0079 x - 0.0747	0.998	3.91	7.81	d_4-15-F_2t isoP
15-*epi*-15-F_{2t}-IsoP	y = 0.0024 x - 0.0183	0.998	1.95	3.91	d_4-15-F_2t isoP
15-F_{2t}-IsoP	y = 0.0027 x - 0.0208	0.998	1.95	3.91	d_4-15-F_2t isoP
5-F_{2t}-IsoP	y = 0.0032 x - 0.0240	0.998	1.95	3.91	d_4-15-F_2t isoP
5-*epi*-5-F_{2t}-IsoP	y = 0.0042 x - 0.0348	0.998	1.95	3.91	d_4-15-F_2t isoP
Alpha-Linolenic acid					
Ent-16-*epi*-16-F_{1t}-PhytoP	y = 0.0136 x - 0.1393	0.998	1.95	3.91	d_4-15-F_2t isoP
Ent-16-F_{1t}-PhytoP	y = 0.0133 x - 0.1109	0.998	1.95	3.91	d_4-15-F_2t isoP

9-F_{1t}-PhytoP	y = 0.0166 x - 0.1316	0.998	1.95	3.91	d_4-15-F_2t isoP
9-e*pi*-9-F_{1t}-PhytoP	y = 0.0160 x – 0.1293	0.998	1.95	3.91	d_4-15-F_2t isoP
Eicosapentaenoic acid					
8-F_{3t}-IsoP	y = 0.0042 x - 0.0314	0.998	0.98	1.95	d_4-15-F_2t isoP
8-*epi*-8-F_{3t}-IsoP	y = 0.0035 x - 0.0299	0.998	0.98	1.95	d_4-15-F_2t isoP
5-F_{3t}-IsoP	y = 0.0018 x - 0.0186	0.998	3.91	7.81	d_4-15-F_2t isoP
5-*epi*-5-F_{3t}-IsoP	y = 0.0012 x - 0.0111	0.998	3.91	7.81	d_4-15-F_2t isoP
Docosahexaenoic acid					
10-F_{4t}-NeuroP	y = 0.0017 x - 0.0117	0.998	0.49	0.98	d_4-10-F_4t-NeuroP
10-*epi*-10-F_{4t}-NeuroP	y = 0.0023 x - 0.0162	0.998	0.98	1.95	d_4-10-*epi*-10-F_4t-NeuroP
14(*RS*)-14-F_{4t}-NeuroP	$y = 6.2450.10^{-5}$ x - $6.7300.10^{-4}$	0.998	15.63	31.25	d_4-10-*epi*-10-F_4t-NeuroP
4(*RS*)-F_{4t}-NeuroP	$y = 1.8386.10^{-4}$ x - 0.0019	0.998	7.81	15.63	d_4-4(RS)-F_4t-NeuroP

The deuterated form of IsoP and NeuroP were used as internal standards to determine the LOD and LOQ of the analytes. IsoP: isoprostane; NeuroP: neuroprostane.

Table 4. Types of isoprostanes quantified in human and mice biological samples using the method described in Section 3.

Biological samples	Sample size	IsoPs detected
Human plasma	1 mL	15-F_2t-IsoP, 15-*epi*-15-F_2t-IsoP, 5-F_2t-IsoP, 5-*epi*-5-F_2t-IsoP, 10-F_4t-NeuroP, 10-*epi*-10-F_4t-NeuroP, 4(*RS*)-F_4t-NeuroP
Human CSF	600 µL	n.d
Mouse Plasma	≥ 200 µL	15-F_2t-IsoP, 15-*epi*-15-F_2t-IsoP, 5-F_2t-IsoP, 5-*epi*-5-F_2t-IsoP
Mouse Urine	≥ 200 µL	*Ent* 15 (*RS*) 2,3 dinor 5,6 dihydro 15-F_2t-isoP, 15-F_2t-IsoP, 15-*epi*-15-F_2t-IsoP, 5-F_2t-IsoP, 5-*epi*-5-F_2t-IsoP
Mouse Liver	200 mg	15-F_2t-IsoP, 15-*epi*-15-F_2t-IsoP, 5-F_2t-IsoP, 5-*epi*-5-F_2t-IsoP, 10-F_4t-NeuroP, 10-*epi*-10-F_4t-NeuroP, 4(*RS*)-F_4t-NeuroP
Mouse Brain	200 mg	15-F_2t-IsoP, 15-*epi*-15-F_2t-IsoP, 5-F_2t-IsoP, 5-*epi*-5-F_2t-IsoP, 10-F_4t-NeuroP, 10-*epi*-10-F_4t-NeuroP, 4(*RS*)-F_4t-NeuroP, Ent-7(*RS*)-F_2t-Dihomo IsoP
Mouse Muscle	200 mg	5-F_2t-IsoP, 5-*epi*-5-F_2t-IsoP

n.d. not detected; IsoP: isoprostane; NeuroP: neuroprostane

Supporting material Table 1.

Plasma matrix effect and extraction efficiency of healthy human plasma.

Compound name	Matrix effect		Yield extraction	
	250 ng/mL	31.25 ng/mL	250 ng/mL	31.25 ng/mL
Adrenic acid				
Ent-7(*RS*)-F_{2t}-dihomo IsoP	66.8 ± 9.6	63.1 ± 8.2	63.2 ± 2.3	77.6 ± 1.2
17(*RS*)-F_{2t}-dihomo IsoP	68.2 ± 9.2	69.1 ± 8.4	62.9 ± 2.8	83.9 ± 2.5
Arachidonic acid				
15-*epi*-15-F_{2t}-IsoP	70.5 ± 9.2	74.1 ± 7.7	58.9 ± 2.5	73.2 ± 2.0
15-F_{2t}-IsoP	68.6 ± 9.7	73.2 ± 9.0	59.6 ± 2.0	69.9 ± 2.3
5-F_{2t}-IsoP	64.7 ±10.7	82.1 ± 7.6	61.3 ± 2.6	62.4 ± 10.5
5-*epi*-5-F_{2t}-IsoP	69.2 ± 9.5	64.9 ± 10.3	58.0 ± 3.0	72.3 ± 8.0
2,3-dinor-15-F_{2t}-IsoP	59.6 ± 8.2	57.0 ± 6.9	51.6 ± 0.9	71.1 ± 11.6
Ent-15(*RS*)-2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP	62.5 ± 9.3	73.6 ± 2.4	57.9 ± 0.9	79.1 ± 5.5
d_4-15-F_{2t}-IsoP	67.1 ± 8.8		69.8 ± 8.6	
C21-15-F_{2t}-IsoP	61.2 ± 8.6		79.2 ± 9.9	
Alpha-Linolenic acid				
Ent-16-epi-F_{1t}-PhytoP	58.0 ± 8.7	53.5 ± 6.1	40.5 ± 2.1	58.3 ± 5.7
Ent-16-F_{1t}-PhytoP	58.7 ± 8.6	53.8 ± 6.4	42.7 ± 1.6	60.4 ± 3.2
9-F_{1t}-PhytoP	56.7 ± 8.5	53.7 ± 5.5	46.7 ± 1.7	65.2 ± 7.3
9-e*pi*-9-F_{1t}-PhytoP	59.1 ± 8.9	51.8 ± 6.4	45.0 ± 1.2	67.1 ± 7.6
Eicosapentaenoic acid				
8-F_{3t}-IsoP	65.5 ± 8.8	57.8 ± 8.2	54.0 ± 2.1	74.2 ± 4.9
8-*epi*-8-F_{3t}-IsoP	64.5 ± 8.9	62.1 ± 6.2	55.5 ± 2.4	65.5 ± 3.4
5-F_{3t}-IsoP	59.9 ± 8.0	65.1 ± 6.4	104.9 ± 4.3	117.9 ± 5.8
5-*epi*-5-F_{3t}-IsoP	64.7 ± 7.7	68.2 ± 8.9	112.0 ± 6.4	131.8 ± 0.6

Docosahexaenoic acid				
10-F_{4t}-NeuroP	75.3 ± 9.7	68.2 ± 9.8	54.9 ± 2.5	74.0 ± 5.4
10-*epi*-10-F_{4t}-NeuroP	72.5 ± 9.1	64.6 ± 8.4	55.7 ± 2.5	77.4 ± 0.1
14(*RS*)-14-F_{4t}-NeuroP	77.3 ± 8.1	82.6 ± 7.1	58.9 ± 4.8	52.0 ± 10.6
4(*RS*)-F_{4t}-NeuroP	73.1 ± 11.1	92.7 ± 4.0	61.0 ± 3.9	76.6 ± 0.1
d_4-10-*epi*-10-F_{4t}-NeuroP	66.2 ± 4.1		72.5 ± 7.2	
d_4-10-F_{4t}-NeuroP	73.5 ± 4.7		68.1 ± 7.6	
d_4-4(*RS*)-F_{4t}-NeuroP	64.8 ± 12.1		63.8 ± 7.4	

The matrix and extraction yield were tested by the addition of low and high concentration of the respective compounds as described in Section 2. IsoP: isoprostane; NeuroP: neuroprostane.

Supporting material Table 2

Repeatability and accuracy of the method for isoprostanes evaluation in human plasma.

Compound	Nominal conc.	Intraday			Interday		
	(ng/mL)	Measured	RSD %	Accuracy %	Measured	RSD %	Accuracy %
Adrenic acid							
Ent-7(*RS*)-F_{2t}-dihomo-IsoP	3.91	3.91 ± 0.28	0.32	102.28	4.48 ± 0.81	18.04	114.61
	31.25	31.31 ± 0.37	0.24	101.53	33.79 ± 0.67	1.97	82.35
	250	256.91 ±1.65	0.17	102.77	273.58 ± 4.50	1.65	111.12
17(*RS*)-F_{2t}-dihomo-IsoP	3.91	n.d	n.d	n.d	n.d	n.d	n.d
	31.25	31.24 ± 1.77	0.16	96.37	26.41 ± 0.83	3.13	84.43
	250	253.60 ±0.53	0.11	101.44	262.76 ± 7.84	2.98	105.10
Arachidonic acid							
15-*epi*-15-F_{2t}-IsoP	3.91	3.96 ± 0.54	0.52	108.20	4.50 ± 0.18	4.02	115.29
	31.25	31.57 ± 1.04	0.42	97.39	33.44 ± 0.50	1.50	86.94
	250	254.41 ± 1.72	0.30	101.77	269.80 ± 10.52	3.90	104.69
15-F_{2t}-IsoP	3.91	4.10 ± 0.27	0.47	108.82	4.21 ± 0.59	13.98	107.85
	31.25	30.94 ± 1.44	0.38	100.42	32.60 ± 0.45	1.39	84.51
	250	257.20 ± 4.92	0.27	102.88	275.25 ± 5.98	2.17	106.05
5-F_{2t}-IsoP	3.91	3.64 ± 0.02	0.49	93.26	4.14 ± 0.37	8.91	105.95
	31.25	31.48 ± 0.60	0.33	99.68	30.02 ± 0.97	3.22	88.39

	250	260.76 ±1.08	0.23	104.30	268.06 ± 3.60	1.34	107.19
5-*epi*-5-F_{2t}-IsoP	3.91	3.71 ± 0.13	0.35	93.84	3.79 ± 0.41	0.71	91.19
	31.25	31.41 ± 0.86	0.26	102.67	32.24 ± 0.30	0.92	80.90
	250	254.45 ±5.61	0.17	101.78	271.10 ± 4.77	1.76	108.36
2,3-dinor-15-F_{2t}-IsoP	3.91	3.50 ± 0.11	3.09	87.01	4.14 ± 0.52	12.56	105.99
	31.25	31.39 ± 0.20	0.62	100.34	30.06 ± 0.49	1.64	84.98
	250	253.89 ± 1.57	0.62	101.56	245.48 ± 8.11	3.30	98.61
Ent-15 -(*RS*)-2,3-dinor 5,6 dihydro-15-F_{2t}-IsoP	3.91	3.97 ± 0.32	8.05	110.80	4.80 ± 0.41	8.44	115.53
	31.25	31.27 ± 0.71	2.27	99.21	33.17 ± 1.22	3.68	92.75
	250	254.19 ± 1.94	0.09	101.67	237.99 ± 9.28	3.90	96.48
Alpha-Linolenic acid							
Ent-16-*epi*-16-F_{1t}-PhytoP	3.91	3.37 ± 0.25	7.49	81.73	4.04 ± 0.19	4.63	103.49
	31.25	31.39 ± 0.27	0.86	100.26	32.84 ± 0.55	1.68	87.89
	250	252.93 ± 2.70	1.07	101.17	251.57 ± 7.61	3.02	100.06
Ent-16-F_{1t}-PhytoP	3.91	3.66 ± 0.18	4.93	95.35	3.94 ± 0.10	2.56	100.96
	31.25	31.43 ± 0.22	0.70	100.12	31.82 ± 0.92	2.88	87.51
	250	254.35 ± 2.88	1.13	101.74	246.90 ± 9.44	3.82	98.89
9-F_{1t}-PhytoP	3.91	3.64 ± 0.09	2.38	90.53	4.24 ± 0.02	0.47	108.65
	31.25	31.31 ± 0.04	0.13	100.18	32.04 ± 0.87	2.71	85.74
	250	257.24 ± 3.60	1.40	102.90	256.15 ± 8.56	3.34	101.28

9-e*pi*-9-F_{1t}-PhytoP	3.91	3.57 ± 0.02	0.61	91.37	4.44 ± 0.28	6.22	113.55
	31.25	31.31 ± 0.58	1.87	99.36	31.25 ± 0.84	2.70	85.27
	250	256.35 ± 2.21	0.86	102.54	253.11 ± 7.69	3.04	100.54
Eicosapentaenoic acid							
8-F_{3t}-IsoP	3.91	3.48 ± 0.14	3.92	89.71	4.06 ± 0.77	18.90	103.87
	31.25	31.15 ± 0.61	1.96	100.59	32.05 ± 0.69	2.16	88.59
	250	253.18 ± 1.67	0.17	101.27	243.29 ± 8.38	3.45	97.93
8-*epi*-8-F_{3t}-IsoP	3.91	3.57 ± 0.13	0.41	94.08	4.42 ± 0.29	6.53	113.12
	31.25	31.48 ± 0.85	0.31	97.61	32.55 ± 1.19	3.66	89.77
	250	252.98 ± 3.52	0.21	101.19	243.60 ± 4.45	1.83	97.99
5-F_{3t}-IsoP	3.91	3.85 ± 0.61	0.86	86.07	3.60 ± 0.26	7.17	92.08
	31.25	31.02 ± 0.59	0.59	99.26	30.36 ± 1.21	3.97	89.52
	250	254.47 ± 4.08	0.40	101.79	226.63 ± 5.18	2.29	93.51
5-*epi*-5-F_{3t}-IsoP	3.91	3.75 ± 0.54	1.48	85.89	3.95 ± 0.26	6.57	101.03
	31.25	31.19 ± 1.11	0.86	102.31	30.20 ± 1.82	6.01	88.80
	250	254.04 ± 4.84	0.61	101.62	232.31 ± 8.13	3.50	95.10
Docosahexaenoic acid							
10-F_{4t}-NeuroP	3.91	3.47 ± 0.20	0.19	93.71	3.76 ± 0.11	2.81	96.17
	31.25	31.27 ± 0.96	0.16	103.17	32.18 ± 0.37	1.16	85.61
	250	256.74 ± 1.01	0.10	102.70	259.93 ± 3.98	1.53	102.25

10-*epi*-10-F_{4t}-NeuroP	3.91	3.75 ± 0.16	0.22	95.14	3.94 ± 0.47	11.83	100.92
	31.25	31.29 ± 0.16	0.17	99.53	31.72 ± 0.70	2.22	84.31
	250	256.15 ± 3.60	0.12	102.46	262.56 ± 1.97	0.75	102.93
14(*RS*)-14-F_{4t}-NeuroP	3.91	n.d	n.d	n.d	n.d	n.d	n.d
	31.25	33.66 ± 0.83	5.49	109.19	29.42 ± 2.84	9.66	94.15
	250	255.05 ± 9.44	4.32	102.02	258.99 ± 16.26	6.25	97.67
4(*RS*)-F_{4t}-NeuroP	3.91	n.d	n.d	n.d	n.d	n.d	n.d
	31.25	31.41 ± 2.40	2.03	101.15	35.16 ± 3.18	9.04	93.60
	250	256.93 ± 3.24	1.46	102.77	261.07 ± 3.00	1.15	102.47

Three nominal concentrations of the compounds were used to assess the stability of the compounds within the day (intraday) and between 15 days intervals (interday). IsoP: isoprostane; NeuroP: neuroprostane; IsoF: isofuran.

Figure

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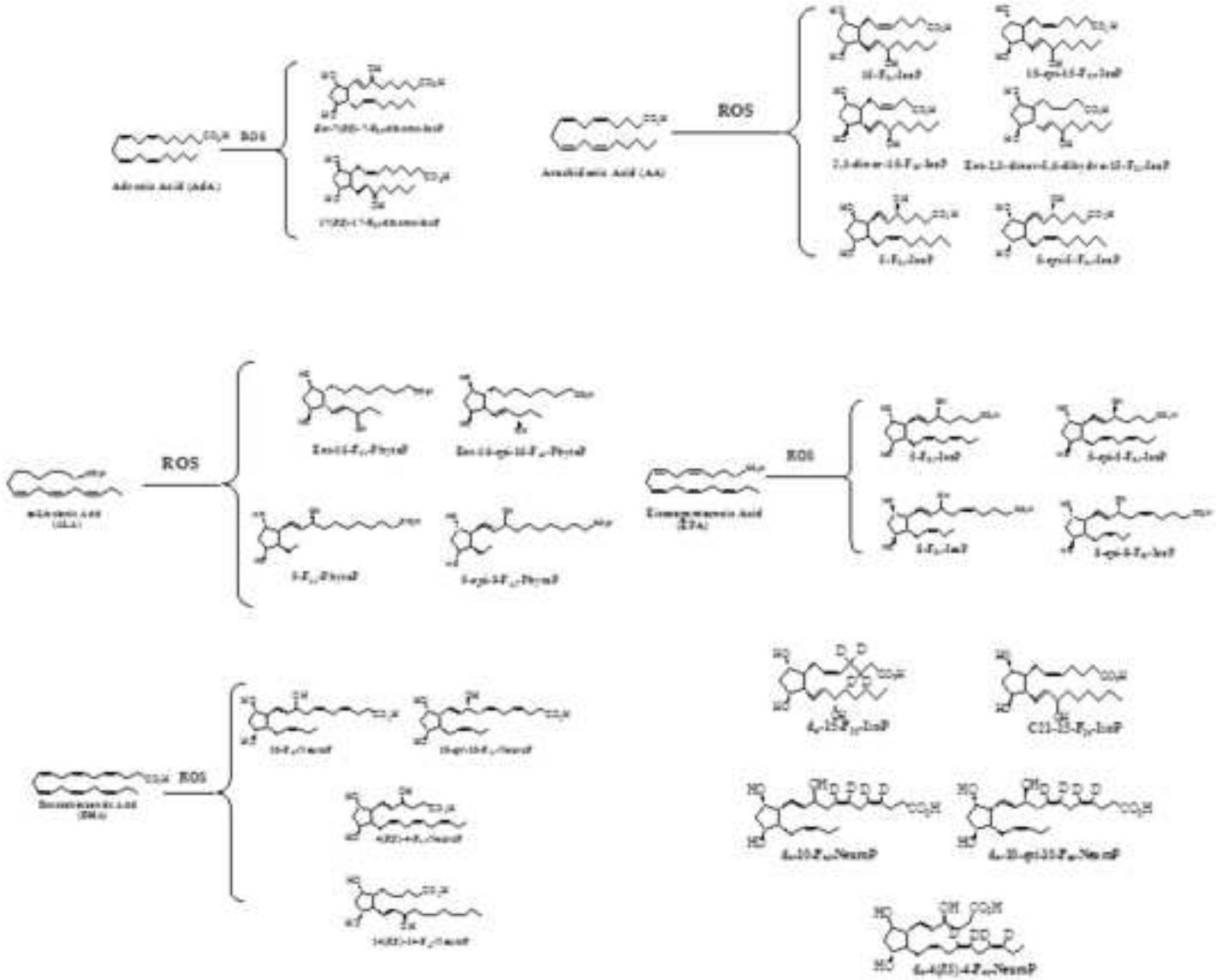
Figure 1



Figure

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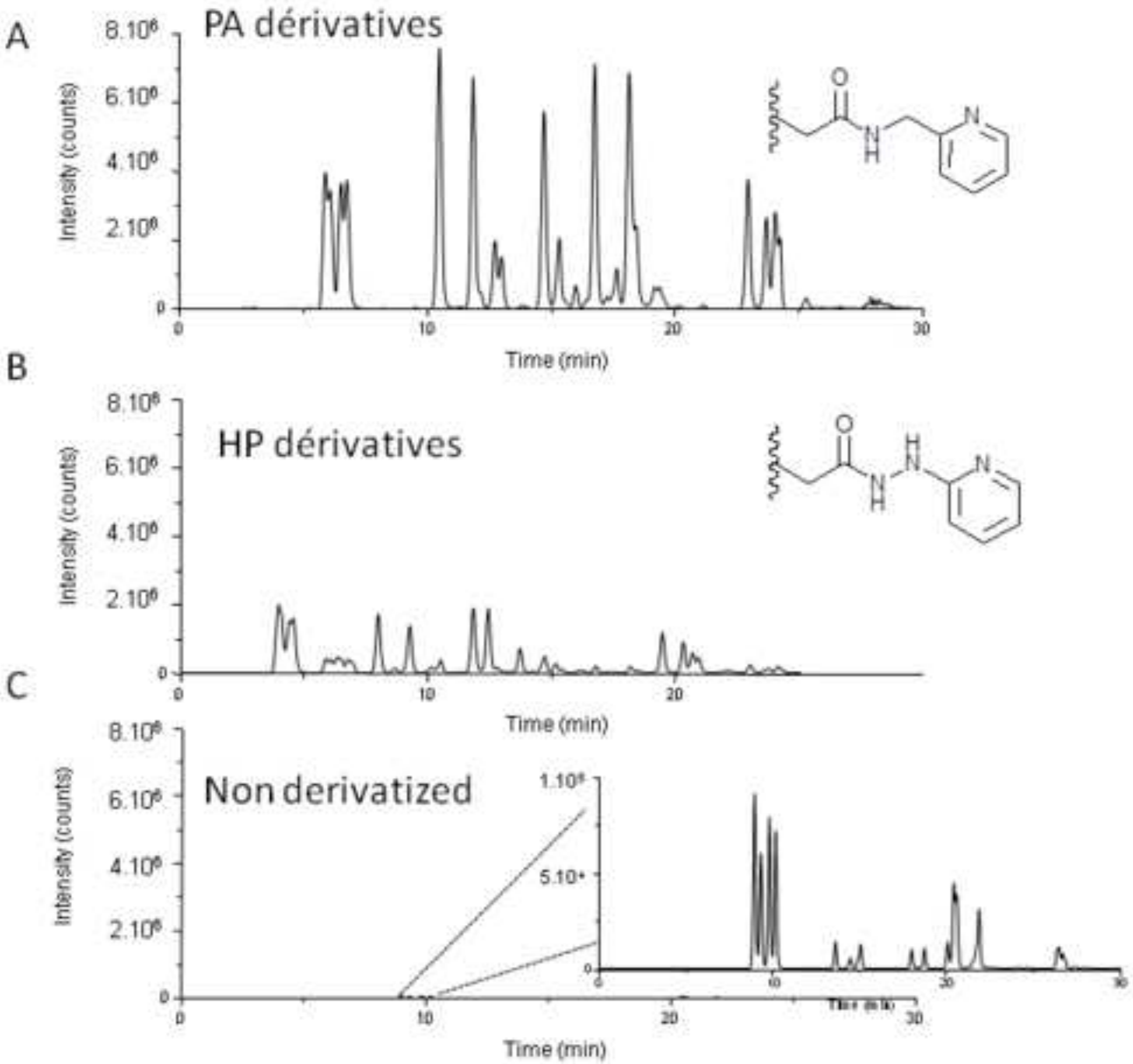
Figure 2



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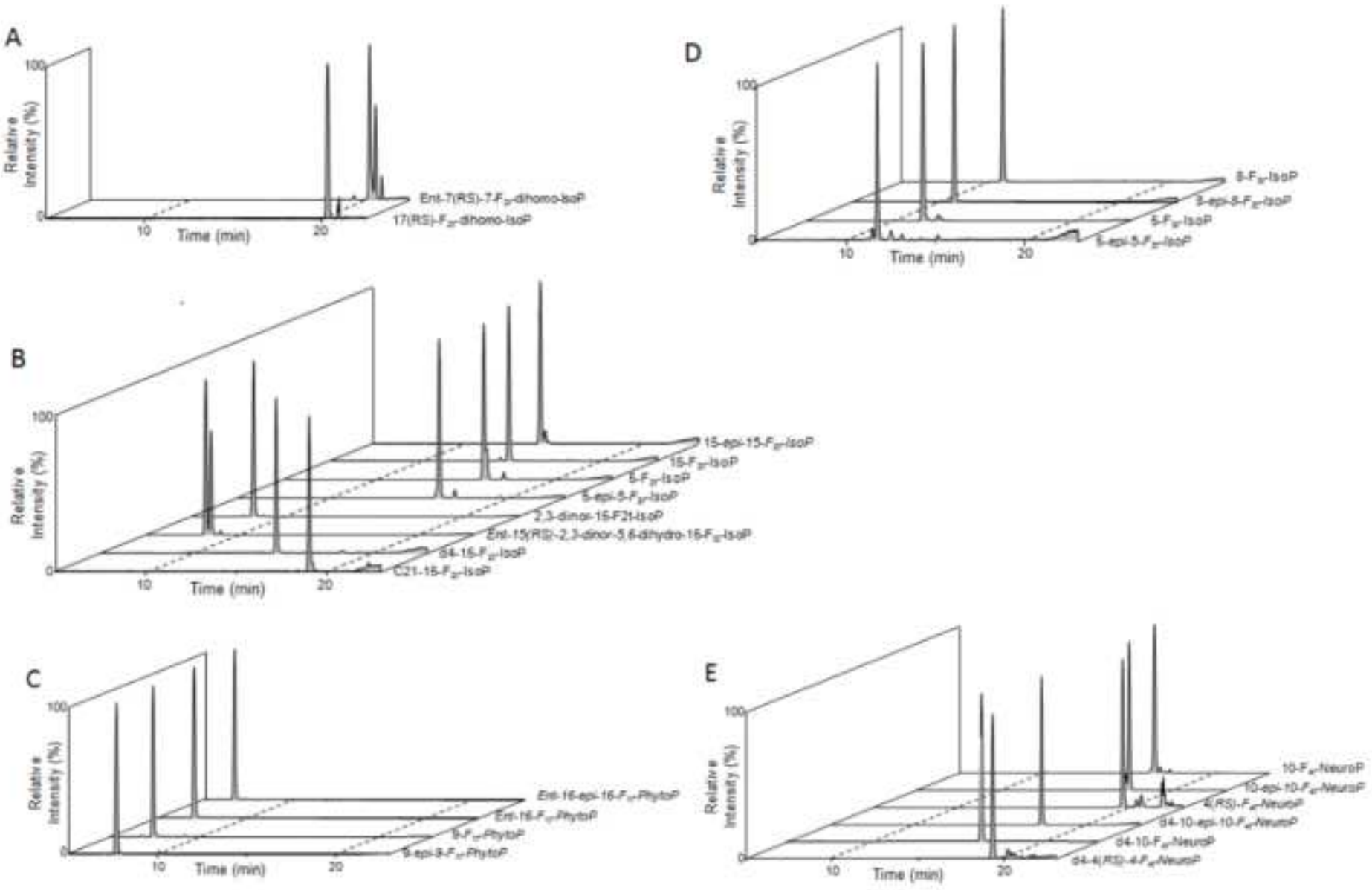

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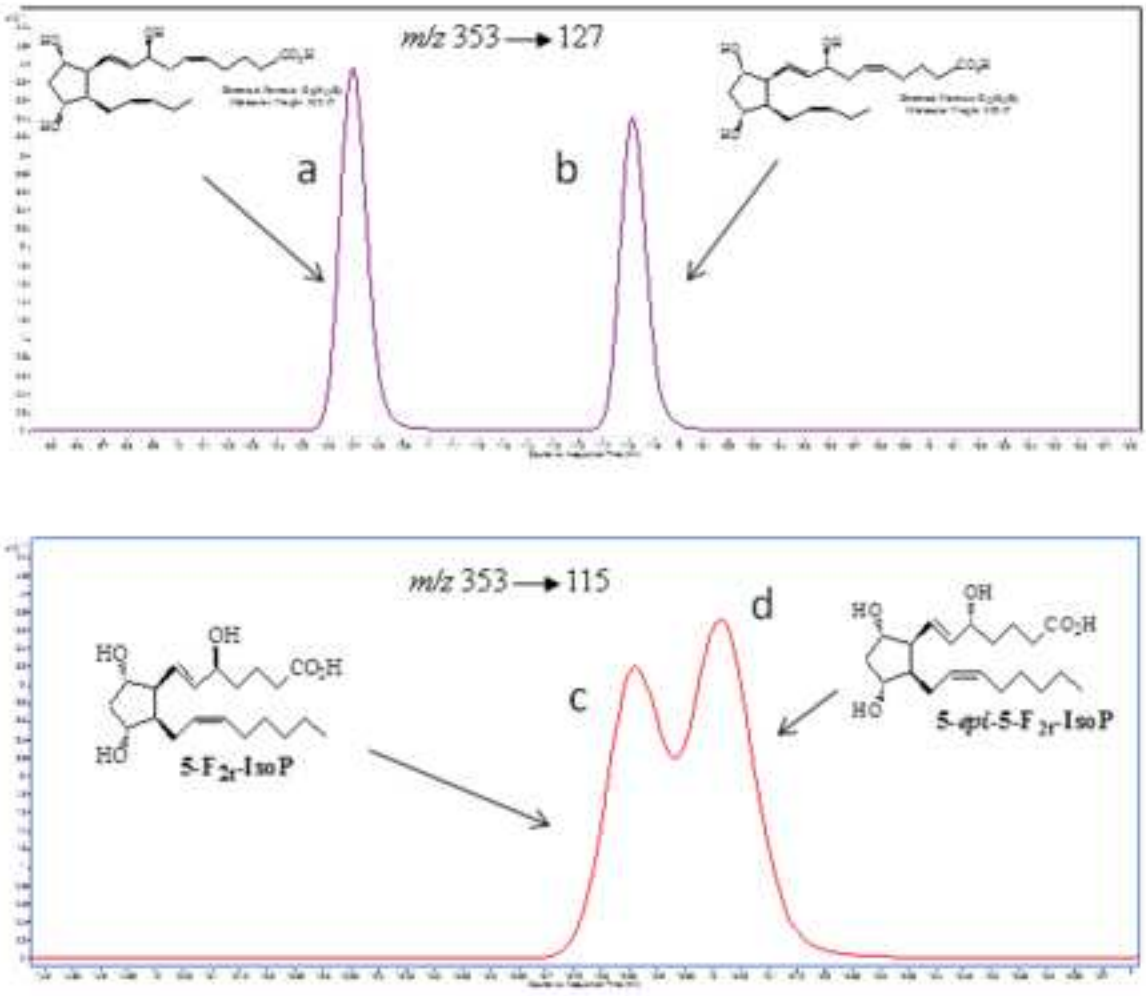

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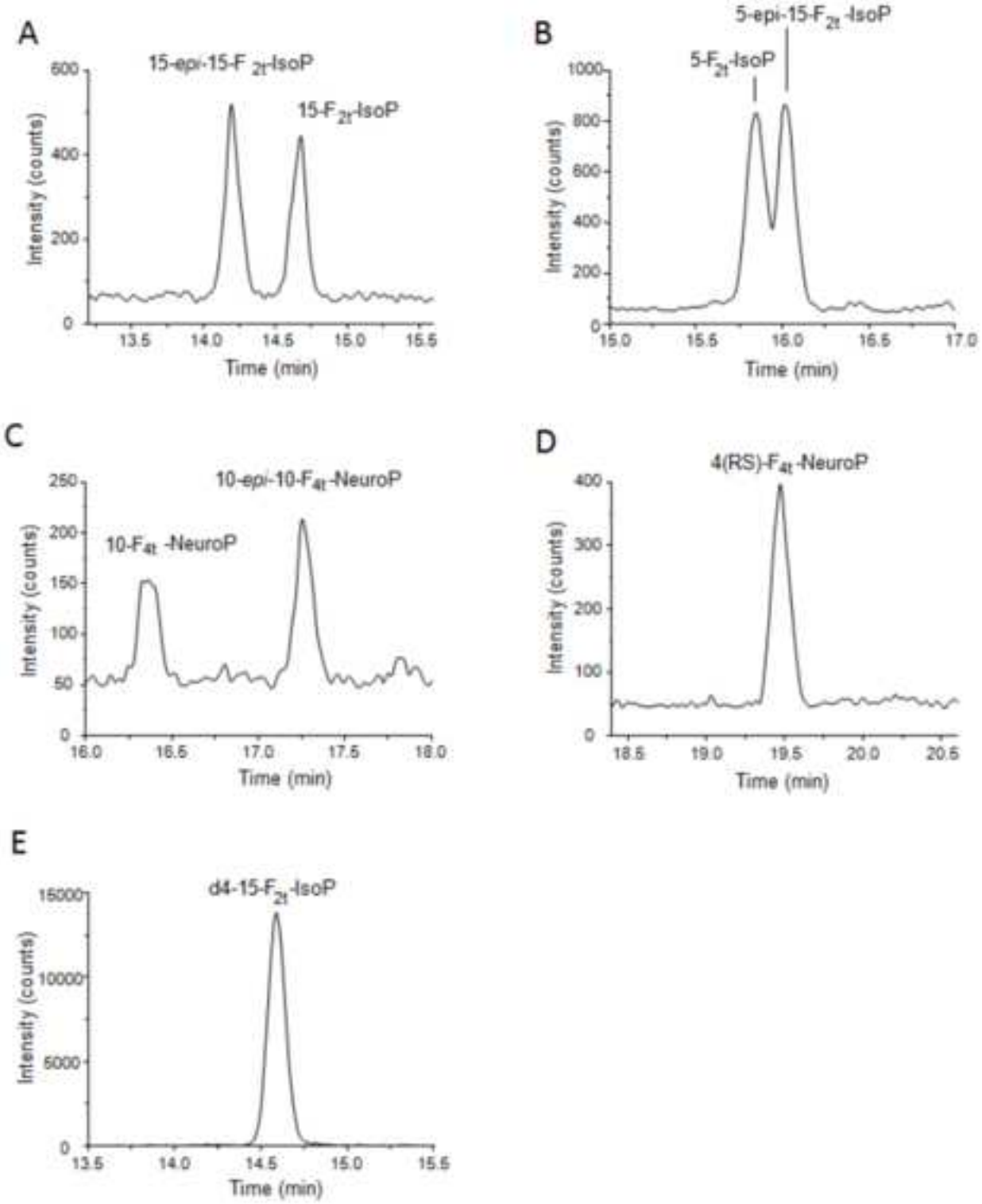

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Figure 6

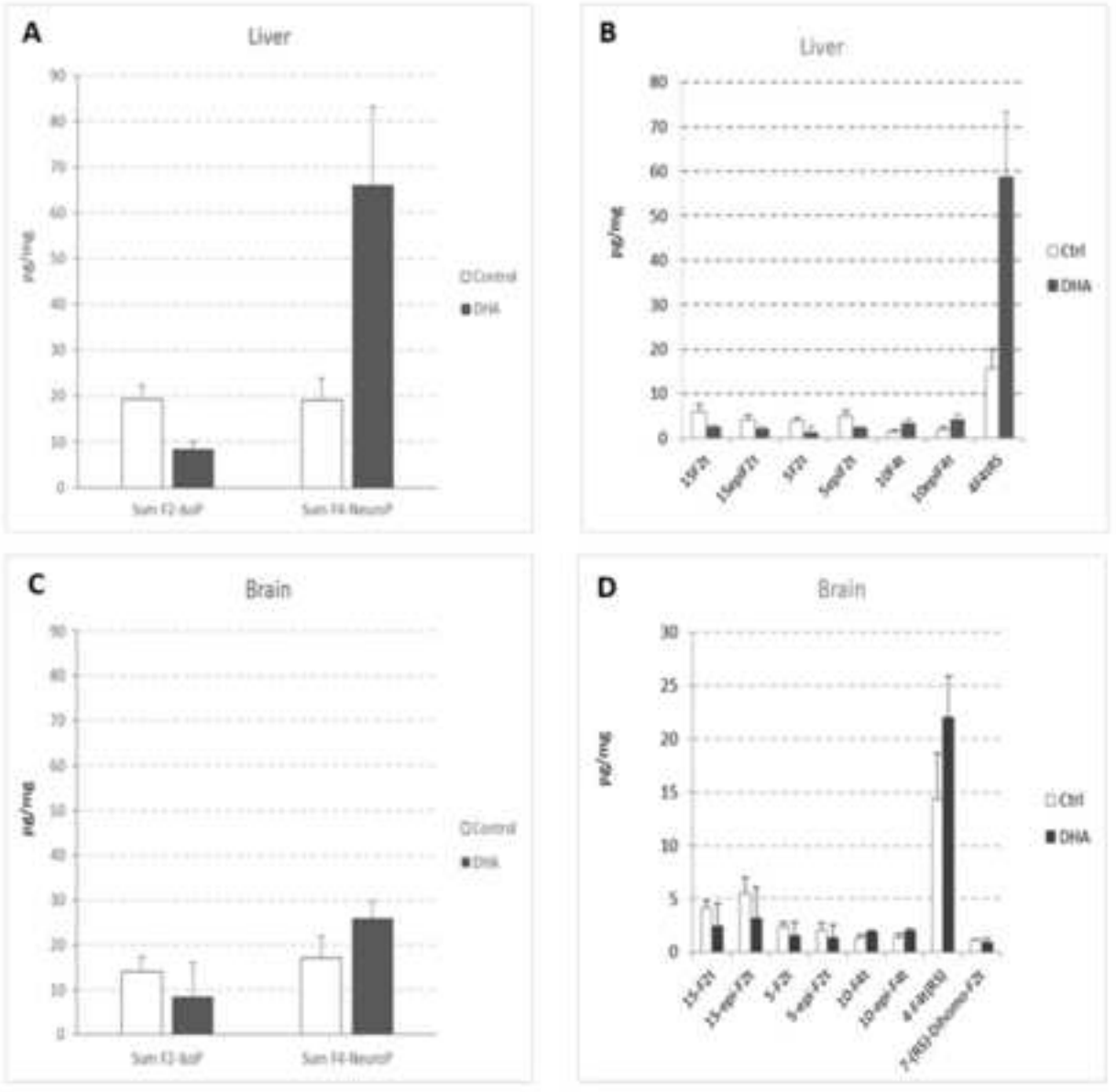

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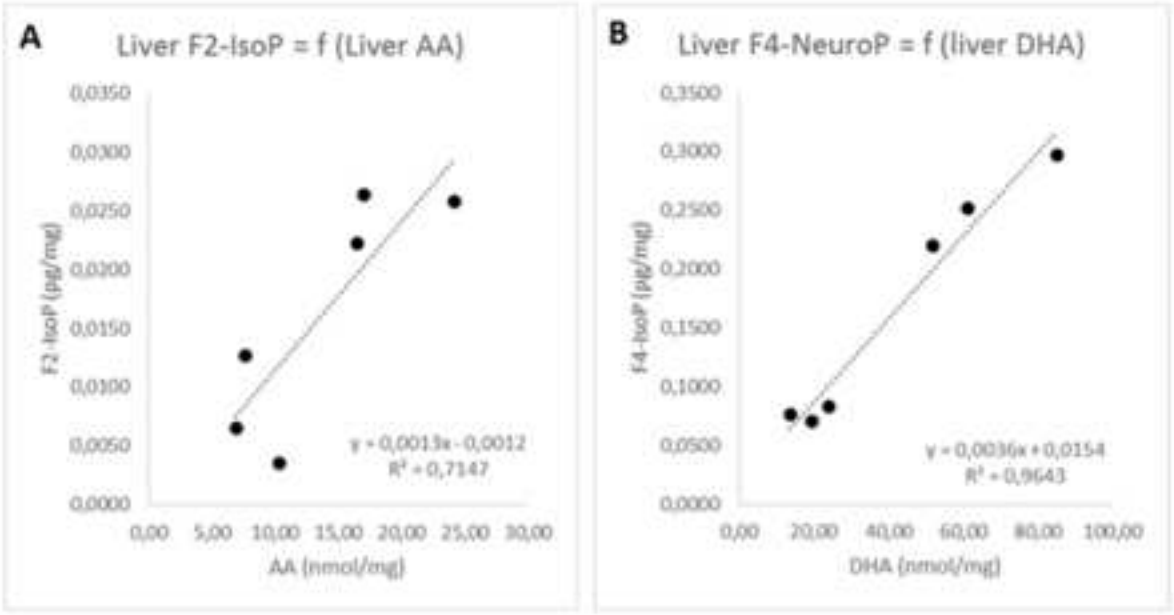
Figure 7



Simultaneous quantitative profiling of 20 isoprostanoids from omega-3 and omega-6 polyunsaturated fatty acids by LC-MS/MS in various biological samples.

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Keywords: isoprostanes, dihomo-isoprostanes, neuroprostanes, phytoprostanes, mass spectrometry, quantification, oxidative stress, ROS

Abbreviations:

AA : Arachidonic acid

AdA : Adrenic acid

ALA : α-Linolenic acid

BHT : Butylated hydroxytoluene

CSF : Cerebrospinal fluid

DHA : Docosahexaenoic acid

DPDS : 2,2’-dipyridyl disulfide

AGTA : Ethylene glycol tetra acetic acid

EPA : Eicosapentaenoic acid

ESI : Electrospray ionization

HP : 2-hydrazinopyridine

HPLC : High-pressure liquid chromatography

IsoPs : Isoprostanes

LOD : limit of detection

LOQ : limit of quantification

m/z : Mass-to-charge ratio

MRM : Multiple reaction monitoring

MS : Mass spectrometry

NeuroPs : Neuroprostanes

OS : Oxidative Stress

PhytoPs : Phytoprostanes

PA : 2-picolylamine

PUFAs : Polyunsaturated fatty acids

ROS : Reactive oxygen species

SPE : Solid-phase extraction

SRM : Selected-reaction monitoring

S/N : signal to noise ratio

TPP : Triphenylphosphine

Abstract

Isoprostanoids are a group of non-enzymatic oxygenated metabolites of polyunsaturated fatty acids. It belongs to oxylipins group, which are important lipid mediators in biological processes, such as tissue repair, blood clotting, blood vessel permeability, inflammation and immunity regulation. Recently, isoprostanoids from eicosapentaenoic, docosahexaenoic, adrenic and ·-linolenic namely F_3-isoprostanes, F_4-neuroprostanes, F_2-dihomo-isoprostanes and F_1-phytoprostanes, respectively have attracted attention because of their putative contribution to health. Since isoprostanoids are derived from different substrate of PUFAs and can have similar or opposing biological consequences, a total isoprostanoids profile is essential to understand the overall effect in the testing model. However, the concentration of most isoprostanoids range from picogram to nanogram, therefore a sensitive method to quantify 20 isoprostanoids simultaneously was formulated and measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The lipid portion from various biological samples was extracted prior to LC-MS/MS evaluation. For all the isoprostanoids LOD and LOQ, and the method was validated on plasma samples for matrix effect, yield of extraction and reproducibility were determined. The methodology was further tested for the isoprostanoids profiles in brain and liver of $LDLR^{-/-}$ mice with and without docosahexaenoic acid (DHA) supplementation. Our analysis showed similar levels of total F_2-isoprostanes and F_4-neuroprostanes in the liver and brain of non-supplemented $LDLR^{-/-}$ mice. The distribution of different F_2-isoprostane isomers varied between tissues but not for F_4-neuroprostanes which were predominated by the 4(*RS*)-4-F_{4t}-neuroprostane isomer. DHA supplementation to $LDLR^{-/-}$ mice concomitantly increased total F_4-neuroprostanes levels compared to F_2-isoprostanes but this effect was more pronounced in the liver than brain.

1. Introduction

Excessive free radicals *in vivo* have been implicated in a number of human diseases such as neurodegenerative, cardiovascular, pulmonary disorder and cancer[1] [2]. The most common free radicals are reactive oxygen species (ROS), which can modify lipids, proteins and nucleic acids. Of the lipids in particular, the polyunsaturated fatty acids (PUFA) form a wide variety of oxygenated metabolites [3] [4]. Among them, the isoprostanes (IsoPs) appears to be a promising group of biomarkers to be assessed for oxidative stress (OS) assessment *in vivo* for over two decades due to its specificity and sensitivity[5] [6]. These compounds are formed *in situ* on membrane phospholipids and then released into their free form via phospholipase A_2 and platelet activating factor hydrolase for circulation. Elevation of IsoPs, in particular those originated from arachidonic acid (AA, 20:4 n-6) also known as F_2-IsoPs in biological fluids (e.g. plasma and urines) are recognized as the reference biomarker for lipid peroxidation and OS in most biological systems. Beyond their capacity of OS as biomarker, IsoPs from n-3 PUFA also demonstrated to be biologically active[7] [8] [9]. Therefore it is crucial to be able to quantify the different isoforms in a large panel of biological samples to integrate this chemical and biological complexity.

Unlike PUFAs, the isoprostanoids are quite complex to assess since the concentration range is very low (from picogram to nanogram) in most biological samples. Moreover, depending on the parent PUFAs, a large diversity of molecule has been discovered as shown in Figure 1. Analysis of these metabolites in biological samples is a challenge and depends on the robustness of the analytical instrumentation. Further, it requires one or several preparation steps, including hydrolysis and extraction from their biological matrix before analysis by radio immunological methods (RIA) or gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-mass spectrometry (LC-MS), which are often coupled to another mass spectrometer (MS/MS) to increase the sensitivity[10]. It is well known to analysts, that RIA is not specific enough to provide efficient quantification of different IsoPs [11] To date, LC-MS/MS is the most common technique to quantify these biomarkers [12], even if the mass spectrometry is not the perfect method to perform absolute quantification compared to GC-MS because of the various ionization efficiency between different molecules. These changes can be very important when comparing compounds with very close structures especially for lipids including the IsoPs. In order to optimize ionization efficacy for each compound, it is essential to have the pure standard to develop a rigorous quantitative method. Although some standards are available commercially, many of the novel ones are unavailable. Through total synthesis,

Durand's group was able to synthesize [13-21] these novel standards, for example dihomo-IsoPs from adrenic acid (C22:4 n-6, AdA) for mass spectrometry analysis.

In this study, we developed a complete quantitative profiling of IsoPs by LC-MS/MS. As IsoPs are present in a very low concentration it was imperative to improve largely the sensitivity of the method therefore we also tried two different derivatization procedures of the carboxylic acid function to improve the ionization of molecules. For both profiles, with and without derivatization, chromatographic separation has been optimized and sensitivity compared. The two methods have been tested on human plasma and the best one was applied in this study. The final methodology was then validated on plasma sample and applied to other biological samples, namely cerebrospinal fluid (CSF), urine, and brain, liver and muscle tissues. Our methodology was finally checked on a mouse model, in which the goal was to determine the isoprostanoids profiling in the brain and liver of $LDLR^{-/-}$ mice and to investigate the effect of docosahexaenoic acid (C22:6 n-3, DHA) supplementation on these profiles.

2. Material and methods

2.1. Chemicals

Commercially available IsoP standards (d_4-15-F_{2t}-IsoP and 2,3-dinor-15-F_{2t}-IsoP) were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Others standards *Ent*-16-*epi*-16-F_{1t}-PhytoP, *Ent*-16-F_{1t}-PhytoP, 9-F_{1t}-PhytoP, 9-e*pi*-9-F_{1t}-PhytoP, *Ent*-15(*RS*)-2,3-dinor-5,6 dihydro-15-F_{2t}-IsoP, 8-F_{3t}-IsoP, 8-*epi*-8-F_{3t}-IsoP, 5-F_{3t}-IsoP, 5-*epi*-5-F_{3t}-IsoP, 15-F_{2t}-IsoP, 15-*epi*-15-F_{2t}-IsoP, 5-F_{2t}-IsoP, 5-*epi*-5-F_{2t}-IsoP, 10-F_{4t}-NeuroP, 10-*epi*-10-F_{4t}-NeuroP, 14(*RS*)-14-F_{4t}-NeuroP, 4(*RS*)-4-F_{4t}-NeuroP, *Ent*-7(*RS*)-7-F_{2t}-dihomo-IsoP, 17(*RS*)-17-F_{2t}-dihomo-IsoP, C21-15-F_{2t}-IsoP, d_4-10-*epi*-10-F_{4t}-NeuroP, d_4-10-F_{4t}-NeuroP, d_4-4(*RS*)-4-F_{4t}-NeuroP were synthesized according to our published procedures[13] .Hexane, ethanol absolute, acetic acid potassium hydroxide (KOH), methanol (MeOH; HPLC gradient Grade), butylated hydroxytoluene (BHT) and formic acid were purchased from Sigma Aldrich (Saint Quentin Fallavier, France). Acetonitrile (ACN; HPLC grade) was obtained from Acros Organics (Illkirch, France). Ammonia solution 30 % (NH_4OH) was purchased from Carlo Erba Reagenti (Cornaredo, Italy). Water used in this study was

purified on a milliQ system (Millipore). The 96 well-plate for solid extraction (SPE) (Oasis Max, 60 mg) was purchased from Waters (Saint-Quentin en Yvelines, France).

2.2. Standards preparation for linearity and reproducibility assessment

Standard solutions with or without derivatization were prepared in MeOH at the following concentrations, 0.06, 0.12, 0.24, 0.49, 0.98, 1.95, 3.91, 7.81, 15.63, 31.25, 62.5, 125, 250, and 500 ng mL-1 for all primary standards. The concentration of the deuterated internal standards (IS) used 5 ng taken from 250 ng mL-1 stock solution. Calibration curves were calculated by the area ratio of the analyte and the internal standard. The linearity and the accuracy of the detection were determined and the limit of detection (LOD : lower point with s/n > 5) and limit of quantification (LOQ: lower point with s/n > 10) were defined for the 20 compounds.

2.3. Biological fluid extraction

Healthy human plasma (1 mL) or CSF (600 µL), LDLR-/- mice plasma (≥200 µL) or urine (≥200 µL) were collected, and supplemented with BHT (1% in ethanol), and stored at -80°C. For the extraction, the samples were thawed and spiked with 5 ng of each internal standard. A volume of 985 µL of hydrolysis solution (KOH 1M in MeOH) was added. The resulting mixture was mixed and incubated at 40°C for 30 minutes (excepted for urine). After cooling in room temperature, 2 mL of 40 mM formic acid (pH 4.5 adjusted with 1 M NaOH) was added. Thereafter, the samples including urine were cleaned and extracted by solid-phase extraction (SPE) on 96-well plate OASIS MAX 60 mg (Waters, USA) modified from Lee et al. method [22]. Briefly, the wells were cleaned with 2 mL of MeOH and conditioned with 2 mL of 40 mM formic acid (pH 4.5). After loading the samples, the wells were washed with 2 mL of 2% NH_4OH followed by 2 mL of MeOH/20 mM formic acid (20:80 v/v) and 2 mL of hexane. The IsoPs were eluted with 2 ml hexane/ethanol/acetic acid (70:29.4:0.6 v/v/v). After drying under nitrogen gas, the samples were re-dissolved with 20 µL of MeOH. A part of the sample (5 µl) was taken for LC-MS/MS analysis and the remaining samples were derivatized prior to LC-MS/MS measurement.

2.4. Biological tissue preparation

The tissue samples were stored at -80°C before preparation. To a total of 200 mg of thawed tissue (brain, liver or muscle) sample, 1 mL of Folch solution ($CHCl_3$:MeOH, 2:1, v/v) containing 10 µL BHT (1 % in ethanol) was added and spiked with 5 ng of each internal standard. The mixture was homogenized with a Fast Prep instrument (MP Biomedicals) for 30 s at 6.5 m/s. Then the homogenized tissue was further extracted with 1.5 mL ice-cold Folch solution ($CHCl_3$: MeOH, 2:1, v/v) and 0.5 mL of ultrapure water. The mix was shaken for 30 s and centrifuge for 10 min at room temperature to separate the aqueous and organic layers. The lower organic layer was carefully removed and transferred to a pyrex tube and then evaporated under nitrogen gas. The extracted lipid was dissolved in 1 mL of hydrolysis solution (KOH 1M in MeOH) and incubated at 40°C for 30 minutes. After cooling in room temperature, 3 mL of 40 mM formic acid was added. The samples were then cleaned and extracted by SPE as described in section 2.3.

2.5. Derivatization of the extracted samples

To a set of extracted samples, 10 µL of freshly prepared solution of 10 mM triphenylphosphine (TPP), 10 mM 2,2'-dipridyl disulfide (DPDS) and 10 µg 2-picolyamine (PA) prepared separately in acetonitrile were added successively. To another set of the extracted samples, freshly prepared 10 µL of TPP and DPDS, and 10 µL of freshly prepared 10 µg 2-hydrazinopyridine (HP) in acetonitrile were added. The sample mixture was incubated at 60°C for 10 min. The mixture was dried under nitrogen and then reconstituted in MeOH for LC-MS/MS analysis.

2.6. LC-MS/MS analysis

High performance liquid chromatography (HPLC) was performed using an Agilent 1290 Infinity equipped with a thermostated autosampler, a binary pump and a column oven. The analytical column was a Zorbax SB-C18 Rapid Resolution HD (2,1 x 100 mm; 1,8 µm) (Agilent Technologies, USA) and maintained at 25°C. The mobile phases consisted of water: formic acid (99.9:0.1;v/v) (A) and acetonitrile : formic acid (99.9:0.1, v/v) (B). The linear gradient was set as follows for the non-derivatized IsoPs analysis: 20% B at 0 min, 30% B at 15 min, 35% B at 20 min, 100% B at 23 min, 100% B at 26 min, and 20% B at 26.5 min for 1.5 min of equilibration. For the derivatized IsoPs, the gradient was set to

18% B at 0 min, 30% B at 22 min, 35% B at 26 min, 100% B at 28 min, 100% B at 29 min, and 18% B at 29.5 min for 1.5 min of equilibration. The flow rate was set at 0.3 mL/min. The autosampler was fixed at 5◦C and the injection volume was 5 µL per analysis. The HPLC system was coupled on-line to an Agilent 6460 triple quadrupole MS (Agilent Technologies, USA) equipped with electrospray ionization (ESI). The ESI was performed in negative ion mode for non-derivatized IsoPs and positive mode for derivatized IsoPs. The MS source parameters were set as follows: source temperature 325ºC, nebulizer gas (nitrogen) flow rate 10 L min-1, sheath gas temperature 350ºC, sheath gas (nitrogen) flow rate 12 L min-1 and the spray voltage adjusted to −3000 V. The dwell time used was 10 ms. The analysis was performed in Selected Reaction Monitoring (SRM) detection mode using nitrogen as the collision gas. The SRM of each compound without (Table 1) or with derivatization (Table 2) were pre-determined by MS/MS analysis. Peak detection, integration and quantitative analysis were performed by Mass Hunter Quantitative analysis software (Agilent Technologies, USA). Concentration of the analytes was calculated by calibration curves obtained in Section 2.2.

2.7. Accuracy and precision

Repeatability and precision were respectively assessed using relative standard deviation (% RSD) and accuracy at 3 concentrations (3.91, 31.25 and 250 ng mL-1) of pure standards in triplicate determination. The concentration was subsequently calculated using the standard curves generated. For inter-day variation, the samples were analyzed on 2 different days, with 15 days in between interval.

2.8. Validation of sample preparation

The preparation of human plasma sample was validated through the yield extraction and the matrix effect. Briefly, three sets in triplicate were prepared : 500 µL of plasma (n = 3) were spiked with 5 ng of IS stock solution and 2 different concentrations of standards (31.25 and 250 ng mL-1) and were extracted as described in Section 2.3: 1) 500 µL of plasma (n = 3) were extracted and then spiked with 5 ng of IS stock solution with 2 different concentrations of standards (31.25 and 250 ng mL-1), and 2) a separate set of pure 5 ng of IS stock solution and standards solutions (31.25 and 250 ng mL-1) in the absence of plasma extract were prepared in MeOH. All sets (of three samples) were analyzed in

triplicate using the LC-MS/MS. The yield extraction was determined as the percent difference between peak areas of standards in pre-spiked and post-spiked samples. The matrix effect was determined as the percentage difference between peak areas of standards added to the extracted samples and pure standard. The plasma matrix effect and yield extraction were calculated for each compound measured in the method described.

2.9. Biological samples

With permission, a sub-group of $LDLR^{-/-}$ mice from a previous study [23] was used to determine the effect of DHA supplementation on the profile of the isoprostanoids in the brain and liver. Briefly, from 8 weeks of age and for 20 weeks, the mice received by daily oral gavages (50 µL, 5 days/week) either oleic acid rich sunflower oil (Lesieur, Asnières-sur-Seine, France; Control group) or a mixture of oleic acid rich sunflower oil and DHA rich tuna oil (OMEGAVIE DHA90TG, Polaris Nutritional Lipids, France containing 90% of DHA as TG) providing 2% (or 35.5 mg/d/mouse) of energy as DHA. At the end of the supplementation, the mice were anaesthetized (40 mg pentobarbital/kg body weight) and the tissue samples were rapidly removed and snap-frozen in liquid nitrogen and stored at -80°C until analysis. Quantification of DHA and AA were performed on brain and liver sample. In brief, after an organic extraction in presence of internal standard, the total fatty acid were methylated, analysed and quantified on a gas chromatography-flame ionization detector (GC-FID) system [24].

3. Results and discussion

3.1 LC-MS/MS method development

In order to achieve the necessary selectivity and sensitivity of the method, the mass detection and chromatographic separation of each standard with or without derivatization were individually optimized.

3.1.1 Mass detection

IsoPs analyzed in this study have different structures comparatively. Nevertheless, due to their common carboxylic acid moiety, they were all detected in the negative ion mode as [M−H]− ions. Firstly, the fragmentor voltage was optimized for each compound in product ion scan mode. This parameter promotes the transmission of the ions between the ionization source and the first quadrupole. Low voltages lead to poor transmission efficiency whereas too high voltage values lead to excessive fragmentation. The optimum value corresponded to a maximum transmission of the [M−H]− ions without fragmentation. The second optimized parameter was the collision energy for each MS/MS transition to monitor; the most abundant one was selected. In this study, one specific fragment was selected for each compound and the collision energy was optimized for each SRM transition (Table 1). SRM transitions observed for this study are divided according to the PUFA type, that includes 15-F_2 series (m/z 353 to m/z 193), 5-F_2 series (m/z 353 to m/z 115), 8-F_3 series (m/z 351 to m/z 127), and 5-F_3 series (m/z 351 to m/z 115) [25]. For the F_1-PhytoP, the carboxylate portion was lost to give SRM m/z 327 to m/z 283, whereas 10-F_4-NeuroP seems to fragment in the same way as 8-F_3 series (m/z 377 to m/z 153). For the remaining compounds, the fragmentations were not as definite.

Since the concentration of the IsoPs is low in biological samples, analysts may opt for a derivatization procedure to increase the volatility and polarity of the compounds. To enhance the detection responses of carboxylic acids in ESI-MS/MS several chemical derivatization procedure can be applied and measured in the positive mode of the LC-MS/MS[26] [27]. However, the derivatization reagents are not always commercially available and the preparation can be long and time consuming. In our study, we tested two simple reagents 2-hydrazinopyridine (HP) and 2-picolylamine (PA), which can be derivatized in one step under mild conditions[28]. When the reagents react with the acidic function of the IsoPs, the HP and PA form a hydrazide and an amide bond, respectively. The sensitivity obtained was preferably for PA derivatives than HP derivatives (Figure 2), therefore MS parameters were optimized only for PA (Table 2). An important drawback of this derivatization method is that for all species a unique PA fragment of m/z = 109 is obtained, which creates poor specificity of the IsoP tested.

3.1.2 Chromatographic separation

The IsoP determined in this study have similar molecular mass and structure. As a result, the liquid chromatographic separation of each metabolite is a crucial step where each isomer of the IsoPs needs to be optimized to be separated in the chromatogram for detection. In this

study, the eluent phase was acidic and a semi-linear gradient elution allows the chromatographic resolution of most compounds on a 10 cm C18 reverse phase column within 30 minutes. The gradient was also designed for the measurement of PA derivatives. As shown in Figure 3, F_{1t}-PhytoPs eluted first, followed by 2,3-dinor-15-F_{2t}-IsoP, F_{3t}-IsoPs, F_{2t}-IsoPs, then F_{4t}-NeuroPs and F_{2t}-dihomo-IsoPs. The isobaric compounds bearing the same transition such as F_{1t}-PhytoPs were successfully separated (Table 1). Furthermore, all the isomers were resolved in the chromatographic separation including 8-F_3t- and 8-*epi*-8-F_3t-IsoPs (Figure 4A and B), while for 5-F_{2t}- and 5-*epi*-5-F_{2t}-IsoPs the separation between the two peaks showed an overlap at the tail of the chromatographic peak (Figure 4C and D). The separation was comparable with PA derivatives indicating derivatization procedure did not improve the separation.

3.1.3 Sensitivity

To quantify our isoprostanoids we first had to choose the appropriate internal internal standard for each metabolite. NeuroPs and 15-F_{2t}-IsoP were quantified through their deuterated equivalent and for other IsoPs, d_4-15-F_{2t}-IsoP and C21-15-F_{2t}-IsoP were used. Based on the internal standard, each calibration curve was obtained with 10 concentrations of the pure IsoPs ranging from 0.9 to 500 ng/mL. The curves were fitted using linear regression model with 1/X factors. The linearity of the method was assessed for each metabolite by evaluating the correlation coefficient (Table 3). The LOD and LOQ of our method were evaluated, and in general LOD corresponding to the lowest concentration had signal to noise ratio above 3 and LOQ corresponding to the lowest concentration had signal to noise ratio above 10. Both of these values however depended on the type of isoprostanoids. For PA derivatives the LOD values ranged from 0.24 ng/mL to 1 ng/mL and the LOQs from 0.12 ng/mL to 2 ng/mL. As shown in Table 3, the LOD values ranged from 0.49 ng/mL to 15.6 ng/mL and the LOQs from 0.98 ng/mL to 31.25 ng/mL. The sensitivity obtained for F_2-IsoP, which is the main IsoP in the literature, is in agreement with LOQ previously reported by others [29] [30]. The sensitivity observed was better than the isoprostanoids with derivatization, in particular for PA. The lack of sensitivity and specificity by PA derivatization indicates that it was not suited for the analysis of IsoPs and more so in biological samples, which often have complicated matrix structure that could further affect the precision of the measurement.

3.2 Plasma sample preparation and analysis

The lipid portion of the tissues or cells was extracted using Folch solution in the presence of antioxidant (0.005% BHT). It is known approximately 70% of the IsoPs are conjugated to phospholipid through ester bond [31], therefore hydrolysis step is required to analyse the total concentration. The lipid extract or fluid (except urine) was treated with 1 M KOH prepared in methanol for 30 minutes at 40 ºC. This step is not required for measurement of non-esterified IsoPs i.e. free form. A purification process is needed to reduce matrix effect and to increase sensitivity of the quantification by LC–MS/MS analysis. Different SPE cartridges were tested in this procedure namely, C18[32], HRX[33] and MAX[22]. It was found MAX cartridge, which is composed of mixed anionic exchange phase provided the cleanest sample and the best LC-MS/MS evaluation. A part of the lipid extracts from SPE were taken for derivatization process. The data obtain from plasma with and without derivatization were compared (data not shown). Despite the peak response and area being bigger for the derivatized samples, the background noise of the chromatogram was much more compared to the non-derivatized samples. Moreover, the derivatization caused the formation of few additional peaks very close to the target ones, making it more difficult to differentiate for peak integration. This observation further support that derivatization procedure is not suitable for IsoP analysis by LC-MS/MS for plasma and likely for other biological samples. Therefore, in order to avoid overestimation in our quantification, we adopted the IsoP measurements without derivatization process in this study.

3.3 Validation of sample processing

3.3.1 Matrix effect and yield of extraction

The efficiency of the sample processing was assessed by measuring the matrix effect and the extraction yield using plasma in triplicate. These two parameters were calculated for two concentrations of IsoPs (250 and 31.25 ng/mL). The peak areas of the chromatogram for each IsoP were compared before and after addition plasma extraction to obtain the matrix effect, as summarized in supporting material Table 1. The matrix effect ranged between 51.4% to 92.7% for low concentration and between 56.7% to 77.5% for high concentration. The values were relatively homogeneous in each group of IsoP. The yield of extraction (supporting material Table 1) was calculated for each IsoP for two concentrations comparing the quantity recovered in presence of plasma to the native one. The yield ranged from 52.0% to 85.3% for low concentration and from

40.5% to 69.2% for high concentration. Surprisingly the yield extraction of F_{3t}-IsoPs was above 100%, and no interference signal was observed for the non-spiked plasma extract; the reason for this observation is unknown.

3.3.2 Performance of the method

Repeatability and precision were then evaluated for intra- and inter-day at 3 concentrations: 3.91, 31.25 and 250 ng/mL (supporting material Table 2) were performed in triplicate. The intra-day accuracy ranged from 81.73% to 114.21% for all IsoP evaluated. The RSD values for 3 injections were ≤ 8%. The inter-day variations were assessed by re-analyzing the samples every 15 days (n=2). The accuracies obtained were between 80.9% and 115.53% with a precision ≤ 15%. These data indicate that the method is highly reproducible for the 20 IsoP compounds analyzed.

3.4 Application on various biological samples

Our extraction and LC-MS/MS methods were applied to quantify non-enzymatic oxidized lipids from PUFAs in different biological samples. The different samples (Table 4) measured include human plasma and CSF, mice plasma, urine, liver, brain and muscle tissues A typical profile for human plasma is displayed in Figure 5. The structural matrix was taken into account for the quantity used for each type of samples and the size is shown in Table 4. Apart from CSF, IsoPs were detected and it appears to vary depending on the type of sample. No IsoPs were found in CSF, and it likely due to small volume used and concentration maybe below our limit of detection.

The method developed was also used to perform the isoprostanoids profiles in the liver and brain of $LDLR^{-/-}$ mice and to investigate if DHA supplementation could affect the profiles. The overall levels i.e. total summation of all the related isomers measured for F_2-IsoPs (19 pg/mg vs 14 pg/mg) and for F_4-NeuroPs (19 pg/mg vs 17 pg/mg) in the liver and brain of control mice (white bars, Figures 6A and 6C) were slightly higher in the liver than the brain (+36% for F_2-IsoP and +11% for F_4-NeuroP). Furthermore, the total levels of F_4-NeuroPs in the control mice liver and brain were similar to the levels of F_2-IsoPs even though the concentration of DHA was 6 times and 34 times higher than AA in the liver and brain respectively (data not shown). Our observation suggest that the presence of high DHA concentration may contribute in protecting the liver and

brain from ROS attack despite it being more prone to peroxidation due to extra double bonds in the structure compared to AA [8]. When looking at the different isomers of the isoprostanoids (Figure 6B and 6D), it is interesting to note that the distribution of the different F_2-IsoP isomers is slightly different in the liver and brain of the control mice with 15-epi-F_{2t}-IsoP and 15-F_{2t}-IsoP being more abundant in the brain; for the liver, the abundance of the different F_2-isomers was as follows: 15-F_{2t}<5-Epi-F_{2t}>15-Epi-F_{2t}>5-F_{2t}.

Among the F_4-NeuroPs, the most abundant isomer was 4(RS)-4-F_{4t}-NeuroP regardless of the tissue type. This finding is consistent with previous analysis performed in rat brain and heart tissues[34] .When the mice were supplemented with DHA (Figure 6, dark bars), the total level of F_2-IsoPs decreased in the brain (-40% for the sum of F_2-IsoP) and the liver (-57% for the sum of F_2-IsoP) whereas the total level of F_4-NeuroPs increased by 51% in the brain and 247% in the liver. The concomitant decrease of F_2-IsoPs and increase of F_4-NeuroPs could be attributed at least in part by the replacement of AA by DHA in the membrane phospholipids. It should nevertheless be noted that the modulation of isoprostanoids profiles is much more pronounced in the liver than in the brain emphasizing the high "plasticity" of liver towards DHA supplementation. Consistently, correlations between levels of AA and F_2-IsoP as well as DHA and F_4-NeuroP were strong in the liver (R^2=0.71 and 0.96 respectively, Figure 7A and B) whereas they were much weaker in the brain (R^2=0.02 and 0.16 respectively, data not shown).

It should be noted in this report that we profiled the isoprostanoids in the liver and brain of an atherosclerotic mice and not normal mice. It is also anticipated that the profile of a normal mice may be different from our observation. Regardless, the objective of this study is to understand the differential changes between tissues. Our observation particularly displayed the importance of performing an integrated analysis of isoprostanoids levels since biological interpretation regarding tissue distribution and dietary modulation of lipid peroxidation is complex, and may lead to incorrect interpretation of experimental findings[10]. Moreover, the distribution of different types of isomers depends on the tissue type, which indicates the importance of tissue selection for studies evaluating bioactivity and organ crosstalk.

In summary, we have described a LC-MS/MS methodology allowing simultaneous quantification of several isoprostanes derived from n-3 and n-6 PUFA which are potential biomarkers in biological systems. Using the LC-MS/MS, we first characterized the analytical and quantification parameters of the 20 studied IsoPs and the 4 internal standards including LOD and LOQ concentration range. We optimized the sample preparation and the extraction process of these isoprostanoids which include Folch extraction, basic hydrolysis and SPE purification to obtain

low matrix effect and good yield of extraction. The method was validated on human plasma (repeatability and accuracy). The optimized chromatographic separation permits to separate nearly all the isomers in 30 minutes with a good sensitivity. We then applied this method to human samples (plasma and CSF) and mice samples (plasma, urine, liver, brain, muscle) to optimize the quantity required to be able to profile the isoprostanoids. Finally, the method was tested on brain and liver samples of $LDLR^{-/-}$ mice with and without DHA supplementation to observe the change on the isoprostanoids profiles. We found a variation in the distribution of different F_2-IsoP isomers between tissues but not for F_4-NeuroP. DHA supplementation concomitantly increased F_4-NeuroP levels. OS is a key feature in a number of human diseases, since ROS are likely to be involved in all disease stages. Many of these diseases are associated to PUFA therefore it is important to identify and evaluate the IsoP compounds simultaneously, not only as reliable biomarkers but also for its functional roles in the PUFA metabolism; we believe that the information we obtained from such profiling will allow us to understand the interaction of the compounds in diet and disease studies.

4. Conclusion

In this report, we developed a quick and robust method to determine multiple numbers of non-enzymatic oxidized lipid products of PUFAs, namely isoprostanoids in various biological sample in particular human plasma by LC-MS/MS. Unlike other reports, we were able to measure and incorporate new isoprostanoids from AdA and ALA as well as some isomers of AA, DHA and EPA into our method. However, it should be noted that not all 20 of the products determined were found in all biological samples therefore care must be taken when selecting them in metabolism studies.

Acknowledgment and Grant support

This work was supported by the Fondation pour la Recherche Médicale (grant ING20121226373), the Agence Nationale de la Recherche, the Institut National de la Santé et de la Recherche Medical and the French National Infrastructure MetaboHUB ANR-11-INBS-010.

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Figure legends

Figure 1. Chemical structure of isoprostanes derived from non-enzymatic oxidation of n-6 PUFA, adrenic acid (AdA) and arachidonic acid (AA), and n-3 PUFA, α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) measured in this study.

Figure 2. Total ion current chromatogram of IsoP: with PA derivatization (A), with HP derivatization (B), and without derivatization (C) of the same sample mix.

Figure 3. Chromatogram of selected reaction monitoring (SRM) of metabolites from each PUFA A: adrenic acid, B: arachidonic acid, C: α-linolenic acid, D: eicosapentaenoic acid and E: docosahexaenoic acid. ~~The numbers indicated for each molecule refers to the compounds annotated in Table 1.~~

Figure 4. Optimum chromatographic separation of diastereoisomers for 8-F_{3t}-IsoP (a) and 8-*epi*-8-F_{3t}-IsoP (b). The diastereoisomers of 5-F_{2t}-IsoP (c) and 5-*epi*-5-F_{2t}-IsoP (d) were unable to resolve as well in the chromatographic analysis.

Figure 5. Chromatogram of selected reaction monitoring (SRM) of metabolites detected in human plasma : 15-F_2t-IsoP, 15-*epi*-15-F_2t-IsoP (A) ; 5-F_2t-IsoP, 5-*epi*-5-F_2t-IsoP (B) ; 10-F_4t-NeuroP, 10-*epi*-10-F_4t-NeuroP (C) ; 4(*RS*)-F_4t-NeuroP (D) ; internal standard d_4-15-F_{2t}-IsoP (E).

Figure 6. Isoprostanoids levels in the liver and brain of $LDLR^{-/-}$ mice given either oleic acid rich sunflower oil (Ctrl, n=3) or a mixture of oleic acid rich sunflower oil and DHA rich tuna oil providing 2% of energy as DHA (DHA, n=3). The sum of F_2-IsoPs and sum of F_4-NeuroPs represents the 'total' sum of the isomers measured for the respective group.

Figure 7. Correlations between the levels of AA or DHA and the corresponding isoprostanoids (i.e. sum of F_2-IsoPs and sum of F_4-NeuroPs) in the liver (A and B).

Table 1. Selected reaction monitoring (SRM) of the isoprostanes derived from polyunsaturated fatty acids.

Compounds	RT (min)	Precursor ion (*m/z*)	Product ion (*m/z*)	F (V)	CE (V)
Adrenic acid					
Ent-7(*RS*)-7-F_{2t}-dihomo-IsoP	20.76	381	143	120	18
17(*RS*)-F_{2t}-dihomo-IsoP	20.90	381	337	120	12
Arachidonic acid					
15-*epi*-15-F_{2t}-IsoP	14.24	353	193	120	20
15-F_{2t}-IsoP	14.69	353	193	120	20
5-F_{2t}-IsoP	15.85	353	115	120	12
5-*epi*-5-F_{2t}-IsoP	16.01	353	115	120	12
2,3-dinor-15-F_{2t}-IsoP	8.29	325	237	100	5
Ent-15(*RS*)-2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP	8.30	327	283	120	20
d_4-15-F_{2t}-IsoP	14.61	357	197	120	20
C21-15-F_{2t}-IsoP	18.97	368	193	120	22
alpha-Linolenic acid					
Ent-16-*epi*-16-F_{1t}-PhytoP	6.66	327	283	120	15
Ent-16-F_{1t}-PhytoP	6.94	327	283	120	15
9-F_{1t}-PhytoP	7.30	327	283	120	15
9-e*pi*-9-F_{1t}-PhytoP	7.58	327	283	120	15
Eicosapentaenoic acid					
8-F_{3t}-IsoP	10.70	351	127	120	18
8-*epi*-8-F_{3t}-IsoP	11.82	351	127	120	18

5-F_{3t}-IsoP	11.48	351	115	120	15
5-*epi*-5-F_{3t}-IsoP	11.72	351	115	120	15
Docosahexaenoic acid					
10-F_{4t}-NeuroP	16.43	377	153	120	15
10-*epi*-10-F_{4t}-NeuroP	17.31	377	153	120	15
4(*RS*)-F_{4t}-NeuroP	19.50	377	101	120	15
d_4-10-*epi*-10-F_{4t}-NeuroP	17.19	381	157	120	15
d_4-10-F_{4t}-NeuroP	16.31	381	157	120	15
d_4-4(*RS*)-4-F_{4t}-NeuroP	20.90	382	239	120	15

The deuterated form of IsoP and NeuroP, and C21-15-F_2t isoP were used as internal standards for quantification of samples in this study. IsoP: isoprostane; NeuroP: neuroprostane; F: Fragmentor; CE: collision energy; N : number.

Table 2. Selected reaction monitoring (SRM) of the derivatized isoprostanes derived from polyunsaturated fatty acids.

Compound	RT (min)	Precursor ion (*m/z*)	Product ion (*m/z*)	F (V)	CE (V)
Adrenic acid					
Ent-7(*RS*)-7-F_{2t}-dihomo-IsoP	24.17	455	109	130	30
17(*RS*)-F_{2t}-dihomo-IsoP	23.41	455	109	130	30
Arachidonic acid					
15-*epi*-15-F_{2t}-IsoP	14.73	427	109	120	25
15-F_{2t}-IsoP	15.34	427	109	120	25
5-F_{2t}-IsoP	18.32	427	109	120	30
5-*epi*-5-F_{2t}-IsoP	18.50	427	109	120	30
Ent-15(*RS*)-2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP	8.89	401	109	120	30
d_4-15-F_{2t}-IsoP	15.26	431	109	120	25
C21-15-F_{2t}-IsoP	20.83	442	109	120	20
alpha-Linolenic acid					
Ent-16-*epi*-16-F_{1t}-PhytoP	5.97	401	109	120	30
Ent-16-F_{1t}-PhytoP	6.21	401	109	120	30
9-F_{1t}-PhytoP	6.62	401	109	120	30
9-e*pi*-9-F_{1t}-PhytoP	6.86	401	109	120	30
Eicosapentaenoic acid					
8-F_{3t}-IsoP	10.58	425	109	120	35
8-*epi*-8-F_{3t}-IsoP	11.91	425	109	120	35
5-F_{3t}-IsoP	12.77	425	109	130	30
5-*epi*-5-F_{3t}-IsoP	13.03	425	109	130	30
Docosahexaenoic acid					

10-F_{4t}-NeuroP	16.80	451	109	120	20
10-*epi*-10-F_{4t}-NeuroP	18.17	451	109	120	20
14(*RS*)-14-F_{4t}-NeuroP	18.53	451	109	130	25
4(*RS*)-F_{4t}-NeuroP	23.81	451	109	130	25
d_4-10-*epi*-10-F_{4t}-NeuroP	16.64	455	109	120	20
d_4-10-F_{4t}-NeuroP	18.04	455	109	120	20
d_4-4(*RS*)-4-F_{4t}-NeuroP	23.90	455	109	140	30

The deuterated form of IsoP and NeuroP, and C21-15-F_2t isoP were used as internal standards for quantification of samples in this study. IsoP: isoprostane; NeuroP: neuroprostane; isofurane ; F: Fragmentor; CE: collision energy.

Table 3. Limit of detection (LOD) and limit of quantification (LOQ) of the pure compounds analyzed.

Compound	Linear regression	R^2	LOD (ng/mL)	LOQ (ng/mL)	IS
Adrenic acid					
Ent-7(*RS*)-7-F_{2t}-dihomo-IsoP	y = 0.0044 x - 0.0339	0.998	1.95	3.91	d_4-15-F_2t isoP
17(*RS*)-F_{2t}-dihomo-IsoP	y = 0.0065 x - 0.0639	0.998	7.81	15.63	d_4-15-F_2t isoP
Arachidonic acid					
2,3-dinor-15-F_{2t}-IsoP	y = 0.0079 x - 0.0558	0.998	0.98	1.95	d_4-15-F_2t isoP
Ent-15(*RS*)-2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP	y = 0.0079 x - 0.0747	0.998	3.91	7.81	d_4-15-F_2t isoP
15-*epi*-15-F_{2t}-IsoP	y = 0.0024 x - 0.0183	0.998	1.95	3.91	d_4-15-F_2t isoP
15-F_{2t}-IsoP	y = 0.0027 x - 0.0208	0.998	1.95	3.91	d_4-15-F_2t isoP
5-F_{2t}-IsoP	y = 0.0032 x - 0.0240	0.998	1.95	3.91	d_4-15-F_2t isoP
5-*epi*-5-F_{2t}-IsoP	y = 0.0042 x - 0.0348	0.998	1.95	3.91	d_4-15-F_2t isoP
Alpha-Linolenic acid					
Ent-16-*epi*-16-F_{1t}-PhytoP	y = 0.0136 x - 0.1393	0.998	1.95	3.91	d_4-15-F_2t isoP
Ent-16-F_{1t}-PhytoP	y = 0.0133 x - 0.1109	0.998	1.95	3.91	d_4-15-F_2t isoP

9-F_{1t}-PhytoP	y = 0.0166 x - 0.1316	0.998	1.95	3.91	d_4-15-F_2t isoP
9-e*pi*-9-F_{1t}-PhytoP	y = 0.0160 x – 0.1293	0.998	1.95	3.91	d_4-15-F_2t isoP
Eicosapentaenoic acid					
8-F_{3t}-IsoP	y = 0.0042 x - 0.0314	0.998	0.98	1.95	d_4-15-F_2t isoP
8-*epi*-8-F_{3t}-IsoP	y = 0.0035 x - 0.0299	0.998	0.98	1.95	d_4-15-F_2t isoP
5-F_{3t}-IsoP	y = 0.0018 x - 0.0186	0.998	3.91	7.81	d_4-15-F_2t isoP
5-*epi*-5-F_{3t}-IsoP	y = 0.0012 x - 0.0111	0.998	3.91	7.81	d_4-15-F_2t isoP
Docosahexaenoic acid					
10-F_{4t}-NeuroP	y = 0.0017 x - 0.0117	0.998	0.49	0.98	d_4-10-F_4t-NeuroP
10-*epi*-10-F_{4t}-NeuroP	y = 0.0023 x - 0.0162	0.998	0.98	1.95	d_4-10-*epi*-10-F_4t-NeuroP
14(*RS*)-14-F_{4t}-NeuroP	$y = 6.2450.10^{-5}$ x - $6.7300.10^{-4}$	0.998	15.63	31.25	d_4-10-*epi*-10-F_4t-NeuroP
4(*RS*)-F_{4t}-NeuroP	$y = 1.8386.10^{-4}$ x - 0.0019	0.998	7.81	15.63	d_4-4(RS)-F_4t-NeuroP

The deuterated form of IsoP and NeuroP were used as internal standards to determine the LOD and LOQ of the analytes. IsoP: isoprostane; NeuroP: neuroprostane.

Table 4. Types of isoprostanes quantified in human and mice biological samples using the method described in Section 3.

Biological samples	Sample size	IsoPs detected
Human plasma	1 mL	15-F_2t-IsoP, 15-*epi*-15-F_2t-IsoP, 5-F_2t-IsoP, 5-*epi*-5-F_2t-IsoP, 10-F_4t-NeuroP, 10-*epi*-10-F_4t-NeuroP, 4(*RS*)-F_4t-NeuroP
Human CSF	600 µL	n.d
Mouse Plasma	≥ 200 µL	15-F_2t-IsoP, 15-*epi*-15-F_2t-IsoP, 5-F_2t-IsoP, 5-*epi*-5-F_2t-IsoP
Mouse Urine	≥ 200 µL	*Ent* 15 (*RS*) 2,3 dinor 5,6 dihydro 15-F_2t-isoP, 15-F_2t-IsoP, 15-*epi*-15-F_2t-IsoP, 5-F_2t-IsoP, 5-*epi*-5-F_2t-IsoP
Mouse Liver	200 mg	15-F_2t-IsoP, 15-*epi*-15-F_2t-IsoP, 5-F_2t-IsoP, 5-*epi*-5-F_2t-IsoP, 10-F_4t-NeuroP, 10-*epi*-10-F_4t-NeuroP, 4(*RS*)-F_4t-NeuroP
Mouse Brain	200 mg	15-F_2t-IsoP, 15-*epi*-15-F_2t-IsoP, 5-F_2t-IsoP, 5-*epi*-5-F_2t-IsoP, 10-F_4t-NeuroP, 10-*epi*-10-F_4t-NeuroP, 4(*RS*)-F_4t-NeuroP, Ent-7(*RS*)-F_2t-Dihomo IsoP
Mouse Muscle	200 mg	5-F_2t-IsoP, 5-*epi*-5-F_2t-IsoP

n.d. not detected; IsoP: isoprostane; NeuroP: neuroprostane

Supporting material Table 1.

Plasma matrix effect and extraction efficiency of healthy human plasma.

Compound name	Matrix effect		Yield extraction	
	250 ng/mL	31.25 ng/mL	250 ng/mL	31.25 ng/mL
Adrenic acid				
Ent-7(*RS*)-F_{2t}-dihomo IsoP	66.8 ± 9.6	63.1 ± 8.2	63.2 ± 2.3	77.6 ± 1.2
17(*RS*)-F_{2t}-dihomo IsoP	68.2 ± 9.2	69.1 ± 8.4	62.9 ± 2.8	83.9 ± 2.5
Arachidonic acid				
15-*epi*-15-F_{2t}-IsoP	70.5 ± 9.2	74.1 ± 7.7	58.9 ± 2.5	73.2 ± 2.0
15-F_{2t}-IsoP	68.6 ± 9.7	73.2 ± 9.0	59.6 ± 2.0	69.9 ± 2.3
5-F_{2t}-IsoP	64.7 ±10.7	82.1 ± 7.6	61.3 ± 2.6	62.4 ± 10.5
5-*epi*-5-F_{2t}-IsoP	69.2 ± 9.5	64.9 ± 10.3	58.0 ± 3.0	72.3 ± 8.0
2,3-dinor-15-F_{2t}-IsoP	59.6 ± 8.2	57.0 ± 6.9	51.6 ± 0.9	71.1 ± 11.6
Ent-15(*RS*)-2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP	62.5 ± 9.3	73.6 ± 2.4	57.9 ± 0.9	79.1 ± 5.5
d_4-15-F_{2t}-IsoP	67.1 ± 8.8		69.8 ± 8.6	
C21-15-F_{2t}-IsoP	61.2 ± 8.6		79.2 ± 9.9	
Alpha-Linolenic acid				
Ent-16-epi-F_{1t}-PhytoP	58.0 ± 8.7	53.5 ± 6.1	40.5 ± 2.1	58.3 ± 5.7
Ent-16-F_{1t}-PhytoP	58.7 ± 8.6	53.8 ± 6.4	42.7 ± 1.6	60.4 ± 3.2
9-F_{1t}-PhytoP	56.7 ± 8.5	53.7 ± 5.5	46.7 ± 1.7	65.2 ± 7.3
9-e*pi*-9-F_{1t}-PhytoP	59.1 ± 8.9	51.8 ± 6.4	45.0 ± 1.2	67.1 ± 7.6
Eicosapentaenoic acid				
8-F_{3t}-IsoP	65.5 ± 8.8	57.8 ± 8.2	54.0 ± 2.1	74.2 ± 4.9
8-*epi*-8-F_{3t}-IsoP	64.5 ± 8.9	62.1 ± 6.2	55.5 ± 2.4	65.5 ± 3.4
5-F_{3t}-IsoP	59.9 ± 8.0	65.1 ± 6.4	104.9 ± 4.3	117.9 ± 5.8
5-*epi*-5-F_{3t}-IsoP	64.7 ± 7.7	68.2 ± 8.9	112.0 ± 6.4	131.8 ± 0.6

Docosahexaenoic acid				
10-F_{4t}-NeuroP	75.3 ± 9.7	68.2 ± 9.8	54.9 ± 2.5	74.0 ± 5.4
10-*epi*-10-F_{4t}-NeuroP	72.5 ± 9.1	64.6 ± 8.4	55.7 ± 2.5	77.4 ± 0.1
14(*RS*)-14-F_{4t}-NeuroP	77.3 ± 8.1	82.6 ± 7.1	58.9 ± 4.8	52.0 ± 10.6
4(*RS*)-F_{4t}-NeuroP	73.1 ± 11.1	92.7 ± 4.0	61.0 ± 3.9	76.6 ± 0.1
d_4-10-*epi*-10-F_{4t}-NeuroP	66.2 ± 4.1		72.5 ± 7.2	
d_4-10-F_{4t}-NeuroP	73.5 ± 4.7		68.1 ± 7.6	
d_4-4(*RS*)-F_{4t}-NeuroP	64.8 ± 12.1		63.8 ± 7.4	

The matrix and extraction yield were tested by the addition of low and high concentration of the respective compounds as described in Section 2. IsoP: isoprostane; NeuroP: neuroprostane.

Supporting material Table 2

Repeatability and accuracy of the method for isoprostanes evaluation in human plasma.

Compound	Nominal conc.	Intraday			Interday		
	(ng/mL)	Measured	RSD %	Accuracy %	Measured	RSD %	Accuracy %
Adrenic acid							
Ent-7(*RS*)-F_{2t}-dihomo-IsoP	3.91	3.91 ± 0.28	0.32	102.28	4.48 ± 0.81	18.04	114.61
	31.25	31.31 ± 0.37	0.24	101.53	33.79 ± 0.67	1.97	82.35
	250	256.91 ±1.65	0.17	102.77	273.58 ± 4.50	1.65	111.12
17(*RS*)-F_{2t}-dihomo-IsoP	3.91	n.d	n.d	n.d	n.d	n.d	n.d
	31.25	31.24 ± 1.77	0.16	96.37	26.41 ± 0.83	3.13	84.43
	250	253.60 ±0.53	0.11	101.44	262.76 ± 7.84	2.98	105.10
Arachidonic acid							
15-*epi*-15-F_{2t}-IsoP	3.91	3.96 ± 0.54	0.52	108.20	4.50 ± 0.18	4.02	115.29
	31.25	31.57 ± 1.04	0.42	97.39	33.44 ± 0.50	1.50	86.94
	250	254.41 ± 1.72	0.30	101.77	269.80 ± 10.52	3.90	104.69
15-F_{2t}-IsoP	3.91	4.10 ± 0.27	0.47	108.82	4.21 ± 0.59	13.98	107.85
	31.25	30.94 ± 1.44	0.38	100.42	32.60 ± 0.45	1.39	84.51
	250	257.20 ± 4.92	0.27	102.88	275.25 ± 5.98	2.17	106.05
5-F_{2t}-IsoP	3.91	3.64 ± 0.02	0.49	93.26	4.14 ± 0.37	8.91	105.95
	31.25	31.48 ± 0.60	0.33	99.68	30.02 ± 0.97	3.22	88.39

	250	260.76 ±1.08	0.23	104.30	268.06 ± 3.60	1.34	107.19
5-*epi*-5-F_{2t}-IsoP	3.91	3.71 ± 0.13	0.35	93.84	3.79 ± 0.41	0.71	91.19
	31.25	31.41 ± 0.86	0.26	102.67	32.24 ± 0.30	0.92	80.90
	250	254.45 ±5.61	0.17	101.78	271.10 ± 4.77	1.76	108.36
2,3-dinor-15-F_{2t}-IsoP	3.91	3.50 ± 0.11	3.09	87.01	4.14 ± 0.52	12.56	105.99
	31.25	31.39 ± 0.20	0.62	100.34	30.06 ± 0.49	1.64	84.98
	250	253.89 ± 1.57	0.62	101.56	245.48 ± 8.11	3.30	98.61
Ent-15 -(*RS*)-2,3-dinor 5,6 dihydro-15-F_{2t}-IsoP	3.91	3.97 ± 0.32	8.05	110.80	4.80 ± 0.41	8.44	115.53
	31.25	31.27 ± 0.71	2.27	99.21	33.17 ± 1.22	3.68	92.75
	250	254.19 ± 1.94	0.09	101.67	237.99 ± 9.28	3.90	96.48
Alpha-Linolenic acid							
Ent-16-*epi*-16-F_{1t}-PhytoP	3.91	3.37 ± 0.25	7.49	81.73	4.04 ± 0.19	4.63	103.49
	31.25	31.39 ± 0.27	0.86	100.26	32.84 ± 0.55	1.68	87.89
	250	252.93 ± 2.70	1.07	101.17	251.57 ± 7.61	3.02	100.06
Ent-16-F_{1t}-PhytoP	3.91	3.66 ± 0.18	4.93	95.35	3.94 ± 0.10	2.56	100.96
	31.25	31.43 ± 0.22	0.70	100.12	31.82 ± 0.92	2.88	87.51
	250	254.35 ± 2.88	1.13	101.74	246.90 ± 9.44	3.82	98.89
9-F_{1t}-PhytoP	3.91	3.64 ± 0.09	2.38	90.53	4.24 ± 0.02	0.47	108.65
	31.25	31.31 ± 0.04	0.13	100.18	32.04 ± 0.87	2.71	85.74
	250	257.24 ± 3.60	1.40	102.90	256.15 ± 8.56	3.34	101.28

9-e*pi*-9-F_{1t}-PhytoP	3.91	3.57 ± 0.02	0.61	91.37	4.44 ± 0.28	6.22	113.55
	31.25	31.31 ± 0.58	1.87	99.36	31.25 ± 0.84	2.70	85.27
	250	256.35 ± 2.21	0.86	102.54	253.11 ± 7.69	3.04	100.54
Eicosapentaenoic acid							
8-F_{3t}-IsoP	3.91	3.48 ± 0.14	3.92	89.71	4.06 ± 0.77	18.90	103.87
	31.25	31.15 ± 0.61	1.96	100.59	32.05 ± 0.69	2.16	88.59
	250	253.18 ± 1.67	0.17	101.27	243.29 ± 8.38	3.45	97.93
8-*epi*-8-F_{3t}-IsoP	3.91	3.57 ± 0.13	0.41	94.08	4.42 ± 0.29	6.53	113.12
	31.25	31.48 ± 0.85	0.31	97.61	32.55 ± 1.19	3.66	89.77
	250	252.98 ± 3.52	0.21	101.19	243.60 ± 4.45	1.83	97.99
5-F_{3t}-IsoP	3.91	3.85 ± 0.61	0.86	86.07	3.60 ± 0.26	7.17	92.08
	31.25	31.02 ± 0.59	0.59	99.26	30.36 ± 1.21	3.97	89.52
	250	254.47 ± 4.08	0.40	101.79	226.63 ± 5.18	2.29	93.51
5-*epi*-5-F_{3t}-IsoP	3.91	3.75 ± 0.54	1.48	85.89	3.95 ± 0.26	6.57	101.03
	31.25	31.19 ± 1.11	0.86	102.31	30.20 ± 1.82	6.01	88.80
	250	254.04 ± 4.84	0.61	101.62	232.31 ± 8.13	3.50	95.10
Docosahexaenoic acid							
10-F_{4t}-NeuroP	3.91	3.47 ± 0.20	0.19	93.71	3.76 ± 0.11	2.81	96.17
	31.25	31.27 ± 0.96	0.16	103.17	32.18 ± 0.37	1.16	85.61
	250	256.74 ± 1.01	0.10	102.70	259.93 ± 3.98	1.53	102.25

10-*epi*-10-F_{4t}-NeuroP	3.91	3.75 ± 0.16	0.22	95.14	3.94 ± 0.47	11.83	100.92
	31.25	31.29 ± 0.16	0.17	99.53	31.72 ± 0.70	2.22	84.31
	250	256.15 ± 3.60	0.12	102.46	262.56 ± 1.97	0.75	102.93
14(*RS*)-14-F_{4t}-NeuroP	3.91	n.d	n.d	n.d	n.d	n.d	n.d
	31.25	33.66 ± 0.83	5.49	109.19	29.42 ± 2.84	9.66	94.15
	250	255.05 ± 9.44	4.32	102.02	258.99 ± 16.26	6.25	97.67
4(*RS*)-F_{4t}-NeuroP	3.91	n.d	n.d	n.d	n.d	n.d	n.d
	31.25	31.41 ± 2.40	2.03	101.15	35.16 ± 3.18	9.04	93.60
	250	256.93 ± 3.24	1.46	102.77	261.07 ± 3.00	1.15	102.47

Three nominal concentrations of the compounds were used to assess the stability of the compounds within the day (intraday) and between 15 days intervals (interday). IsoP: isoprostane; NeuroP: neuroprostane; IsoF: isofuran.