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Assessment of Isoprostanes in Human Plasma: Technical Considerations and the Use of Mass Spectrometry

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Abstract Oxygenated lipid mediators released from non-enzymatic peroxidation of polyunsaturated fatty acids (PUFA) are known to have functional roles in humans. Notably, among these lipid mediators, isoprostanes molecules are robust biomarkers of oxidative stress but those from n-3 PUFA are also bioactive molecules. In order to identify and assess the isoprostanes, the use of mass spectrometry (MS) for analysis is preferable and has been used for over two decades. Gas chromatography (GC) is commonly coupled to the MS to separate the derivatized isoprostanes of interest in biological samples. In order to increase the accuracy of the analytical performance, GC-MS/MS was also applied. Lately, MS or MS/MS has been coupled with high-performance liquid chromatography to assess multiple isoprostane molecules in a single biological sample without derivatization process. However, there are limitations for the use of LC-MS/MS in the measurement of plasma isoprostanes, which will be discussed in this review.

Keywords Lipid peroxidation · Isoprostanes · Neuroprostanes · GC-MS · LC-MS/MS

Abbreviations

15-F _{2t} -IsoP	15-F _{2t} -Isoprostane
4(RS)-4-F _{4t} -NeuroP	4(RS)-4-F _{4t} -Neuroprostane
5-F _{2t} -IsoP	5-F _{2t} -Isoprostane
ARA	Arachidonic acid
AdA	Adrenic acid
ALA	Alpha-linolenic acid
AMPP	<i>N</i> -(4-Aminomethylphenyl)pyridinium
APCI	Atmospheric-pressure chemical ionization
BHT	Butylated hydroxytoluene
DHA	Docosahexaenoic acid
ELISA	Enzyme-linked immunoassay
EPA	Eicosapentaenoic acid
ESI	Electrospray ionization
F ₁ -PhytoP	F ₁ -Phytprostane
F ₂ -IsoP	F ₂ -Isoprostane
F ₂ -dihomo-IsoP	F ₂ -dihomo-Isoprostane
F ₃ -IsoP	F ₃ -Isoprostane
F ₄ -NeuroP	F ₄ -Neuroprostane
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
GC-MS/MS	Gas chromatography-tandem mass spectrometry
HPLC	High performance liquid chromatography
IsoP	Isoprostane
LC	Liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LOD	Limit of detection

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LOQ	Limit of quantification
MECP2	Methyl-CpG-binding protein 2
MRM	Multiple reaction monitoring
MS	Mass spectrometry
<i>m/z</i>	Mass over charge ratio
NeuroP	Neuroprostane
NICI	Negative-ion chemical ionization
PoAF	Postoperative atrial fibrillation
PUFA	Polyunsaturated fatty acid
Q1	Precursor/parent ion
Q3	Fragmented/daughter ion
RIA	Radioimmunoassay
SIM	Selected ion monitoring
S/N	Signal-to-noise ratio
SPE	Solid phase extraction
TOF	Time-of-flight

Introduction

The discovery of isoprostanes (IsoPs) by Morrow and co-workers in 1990 [1] unfolded a whole new area of interest in lipid molecule research. Over the next two and a half decades, enormous amounts of work, including the biosynthesis, chemical total synthesis, and biological relevance of IsoPs, have been extensively published and reviewed [2–4]. The generation of IsoPs is through non-enzymatic oxygenation of polyunsaturated fatty acids (PUFA) initiated by free radical attack, leading to lipid peroxidation [5]. The most classic example of IsoP biomolecules is the family of F₂-isoprostanes (F₂-IsoPs), particularly the isomer 15-F_{2t}-isoprostane (15-F_{2t}-IsoP). The 15-F_{2t}-IsoP was the first and most widely studied IsoP among the family of IsoPs, and the measurement of 15-F_{2t}-IsoP is considered a robust biomarker for accessing oxidative stress status *in vivo* [6]; overproduction of IsoPs is generally regarded as disadvantageous to health. Indeed, numerous metabolic disorders and pathophysiological diseases such as cardiac ischemia–reperfusion [7, 8], atherosclerosis [9], neurodegenerative diseases [10–12], and cancer [13] are frequently associated with elevated plasma F₂-IsoPs.

IsoPs are quantifiable from solid specimens like tissues and cells, or body fluids such as urine [14], plasma/serum [15], or saliva [16] and bronchial lavages [17]. Comparatively, body fluids are much more acquirable than tissues from healthy individuals to access their *in vivo* oxidative status. The choice of specimen sampling, for example plasma versus urine, is important as it might reveal different rate of IsoP metabolism [18]. Although urine is a non-invasive sampling method and readily available, measurement of 15-F_{2t}-IsoP in plasma provides a sensitive index in overall systemic oxidant injury that presumably derived from all tissues in the body. This is unlike the metabolised

F₂-IsoPs found in urine that are in part derived from the kidney, jeopardising the sensitivity and specificity of urinary F₂-IsoPs as a systemic oxidant injury biomarker [18]. As such, the IsoPs measurable in human plasma will be the focus of this article. Plasma sampling, however, possesses a problem of developing artefacts of arachidonic acid autoxidation if samples are not processed or stored properly. To overcome this problem, butylated hydroxytoluene (BHT) and indomethacin [19] is recommended to be added to the plasma before storing them in the deep freezer of $-80\text{ }^{\circ}\text{C}$, but not $-20\text{ }^{\circ}\text{C}$ as storing in such temperature leads to the production of F₂-IsoP artefacts. Addition of BHT and indomethacin in the plasma serve as free radical scavenger and cyclooxygenase inhibitor respectively, preventing *ex vivo* non-enzymatic and enzymatic oxidation to occur [20].

Measurement of plasma IsoPs often start off with lipid extractions commonly employed or modified from established methods, e.g. Folch [21] or Bligh and Dyer [22] methods or protein precipitation with high content of polar solvent. This is followed by further purification, such as solid phase extraction (SPE) [23], thin layer chromatography (TLC) [24] and/or high performance liquid chromatography (HPLC) [25]. Subsequently, measurements of IsoPs and their precursors are widely dependent on the use of mass spectrometry (MS) coupled with either gas chromatography (GC) or liquid chromatography (LC). The choice of gas or liquid chromatographic method to couple with MS however remains as a controversial argument, as both have their respective pros and cons.

This article reviews the technical and biological relevance of quantifying isoprostanooids in human blood plasma. Additionally, we will highlight some important points to take into consideration when choosing which chromatographic separation method to employ in conjunction with MS, i.e. GC–MS(/MS) or LC–MS(/MS).

Biological Relevance of Quantifying Isoprostane Molecules

Isoprostanes are prostaglandin-like molecules that are produced independently and non-enzymatically from various PUFA, most commonly from alpha-linolenic acid (ALA), arachidonic acid (ARA), adrenic acid (AdA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Different classes of IsoPs were discovered with different functional groups on their respective cyclopentane ring structure [26]. For simplicity and coherence of this article, only F-ring IsoPs will be discussed as they are highly abundant *in vivo* and the most well studied. Because of the highly unsaturated nature of PUFAs and to the formation mechanism of IsoP, a substantial amount of racemic regioisomer and diastereoisomer of IsoP molecules can be derived from the same precursors, leading to a vast range of

possible biomolecules being generated [27]. While PUFAs are provided as substrate for peroxidation, they have different rates of oxidation. The most oxidizable PUFA appears to be DHA, due to its great number of double bonds. Interestingly, the biosynthesis of metabolically and chemically stable IsoPs is realised primarily *in situ* in the phospholipid bed. The PUFA esterified in phospholipids are oxidized to IsoPs and released into free form by phospholipase A₂ (PLA₂) hydrolysis [28]. Therefore, IsoP can be quantified in human plasma as free or esterified forms, or even total IsoPs (free plus esterified) [18]. One common approach to differentiate esterified IsoPs from the free ones is via *in vitro* hydrolysis. This is accomplished by the introduction of concentrated alkaline solution, such as potassium hydroxide, to the sample for saponification after total lipid extraction. Alternatively, the addition of exogenous PLA₂ into the sample, prior to total lipid extraction by organic solvents, is able to liberate all the esterified IsoPs [29]. While both methods are commonly employed for the measurement of esterified IsoPs, the use of PLA₂ overcomes the structural alteration on the IsoP as caused by saponification, but may be costly in practice.

F₁-Phytosteranes (F₁-PhytoPs)

F₁-PhytoPs are oxygenated biomolecules from its precursor, α -linolenic acid (ALA) and were first discovered in plants by Parchmann and Mueller [30], which theoretically yield up to two regioisomers (9 and 16 series) and 32 diastereoisomers. ALA is also the precursor for the generation of longer and higher degree of unsaturated PUFAs such as EPA and DHA, which are important for proper foetal development and health maintenance [31]. Mammals do not endogenously produce ALA, but it is found in plants and algae therefore, the mode of acquisition is only possible through diet. Absorbed ALA and PhytoPs are transported in plasma in the conjugated form and excreted as the free form in urine. In addition, F₁-PhytoP is increased in humans with increased consumption of ALA-rich flaxseeds or vegetable oil [32]. Mediterranean diets that are plentiful in ALA-rich vegetables, nuts, and seeds might contribute considerable benefits to health such as preventing cardiovascular disease, anti-inflammation and neuroprotection [33–35]. However, these health benefits may also be due to the PhytoPs found in foods such as rapeseed, olives and almonds, for example [33, 36].

F₂-Isoprostanes (F₂-IsoPs)

Arachidonic acid (ARA), an n-6 PUFA, is one of the most abundant PUFAs in the cell membrane. Large amount of IsoPs molecules are generated from ARA, and the majority of them are from the F-ring group therefore, are termed F₂-IsoPs. A total of 64 compounds from four regioisomers

(5-, 12-, 8-, and 15-series) can be formed theoretically [5]. Because of the high amount of ARA on the cell membrane of most cell types, production of 15-F_{2t}-IsoP would be increased when challenged by free radical attacks, leading to oxidative stress [37]. Therefore, measurement of 15-F_{2t}-IsoP has been generally used as an indicator for overall *in vivo* oxidative stress status in the biological systems [38]. Also, another reason in qualifying it to be suitable candidate for oxidant status biomarker is the stability and robustness of this molecule. Elevation of total F₂-IsoPs in the plasma is often associated with wide range of metabolic disorders, such as in patients with chronic haemodialysis [39], diabetes [40] and atherosclerosis [41]. Apart from being a biomarker, 15-F_{2t}-IsoP also exerts its biological role as vasoconstrictor in most vascular beds, including but not limited to brain, heart, lung, and kidney [42]. Also, it is a ligand for thromboxane receptor and modifies platelet shape and aggregation in human plasma [43, 44].

F₂-dihomo-isoprostanes (F₂-dihomo-IsoP)

F₂-dihomo-IsoP is the latest IsoP family to be discovered to date. Free radical attack on adrenic acid (AdA) generate F₂-dihomo-IsoPs molecules, which share many structural similarities to F₂-IsoPs such as the ring structure, hydroxyl moieties and numbers of isomers [45]. The only difference is that F₂-dihomo-IsoPs contains two additional methylene groups than F₂-IsoP, weighting 28 Da more. AdA is highly localized in the myelin (brain white matter) and accounts for the third highest PUFA concentration in the brain after ARA and DHA. White matter is commonly damaged in the event of neuro-oxidative stress and a significant increase of F₂-dihomo-IsoPs is found in the brains of patients with degenerative diseases. The levels of F₂-dihomo-IsoPs in these oxidative injured white matters have greater extent than IsoPs derived from ARA and DHA [46]. Thus, F₂-dihomo-IsoP was proposed as the biomarker for the free radical damage of myelin as observed in the patients with Rett syndrome. Rett syndrome is a congenital condition predominantly found in female patients that is caused by DNA mutation in the gene encoded for methyl-CpG-binding protein 2 (*MECP2*) [47]. The alterations in *MECP2* expression have an impact on the CNS plasticity, leading to learning and memory dysfunction. Although oxidative stress has been shown to be involved in Rett syndrome [48], it remains unclear if it is the cause or the consequence of the pathogenesis [49]. Nonetheless, studies on Rett syndrome patients showed elevated plasma F₂-dihomo-IsoPs, which further affirm its suitability as a reliable biomarker for oxidative damage of brain white matter [50, 51]. To date, little is known about F₂-dihomo-IsoPs molecules and they are generating great interest in the possible role(s) and mechanism of its pathogenesis in neuronal diseases.

*F*₃-isoprostanes (*F*₃-IsoP)

Lipid peroxidation of eicosapentaenoic acid (EPA) converts to the 6-series of *F*₃-IsoPs regioisomers [52]. Physiologically, only a small amount of *F*₃-IsoPs molecules are expected to form *in vivo* since the level of endogenous EPA is relatively low. Dietary supplementation can compensate the EPA level, and DHA can retro-convert to EPA in the metabolism [53]. As EPA is the most abundant PUFA in fish oil, the beneficial effects of fish oil supplementation are generating great interest. Supplementation of fish oil markedly increased the production of *F*₃-IsoPs in mice and showed to be anti-inflammatory [54]. Simultaneously, levels of pro-inflammatory *F*₂-IsoPs were highly reduced due to suppression of ARA by EPA [54]. Furthermore, it was discovered that a specific type of *F*₃-IsoPs had an effect on cardiac cell arrhythmia [55] and 5-*epi*-5-*F*₃-IsoP was reported to reduce K⁺-induced [³H]D-aspartate release in the bovine retina, which was in part associated with the activation of pre-junctional prostanoid EP1-receptors [56]. Only a handful of studies examined *F*₃-IsoPs in plasma. A recent study on the effects of fish oil supplementation in postoperative atrial fibrillation (PoAF) patients showed *F*₃-IsoPs levels in plasma and urine collected before and right after cardiac surgery and 2 days after the surgery. In the report, an association was found with urinary *F*₃-IsoP concentration, but not plasma with higher risk of PoAF development [57]. Further, supplementation of fish oil to Rett syndrome patients lowered plasma *F*₃-IsoP levels and the patients showed strengthened motor related signals [58].

*F*₄-neuroprostanes (*F*₄-NeuroPs)

Neuroprostanes (NeuroPs) molecules are termed based on the initial discovery of IsoP-like molecules from docosahexaenoic acid (DHA), which is highly enriched in the neurons, but not in other tissues. Eight racemic regioisomers of *F*₄-NeuroPs (4-, 7-, 10-, 11-, 13-, 14-, 17-, and 20-series) can be formed from DHA totalling 128 compounds [59]. Comparatively, due to the large number of double bonds, DHA is the most susceptible PUFA to be oxidized. The high abundance of DHA in the brain suggests *F*₄-NeuroPs as the optimal biomarker for neuronal oxidant injury [60]. Indeed, high levels of *F*₄-NeuroPs were detected in brain tissues and cerebrospinal fluids of Alzheimer's disease patients, but were not associated with plasma or urine levels [61]. This might suggest that only neural DHA are oxidised and/or affected in brain-related diseases. The supplementation of fish oil in Rett syndrome patients however, has shown to reduce plasma *F*₄-NeuroP concentration with improvement in clinical severity such as non-verbal communication [58]. Thus, the concentration of plasma *F*₄-NeuroP in Rett syndrome patients might reveal

the severity of their conditions. In addition, plasma *F*₄-NeuroPs also appeared to be a potential predictor for cigarette smokers [62] and pre-eclampsia [63]. These indicate that the measurement of plasma *F*₄-NeuroPs might also serve as an overall health index for an individual. In order to study the biological role of *F*₄-NeuroPs molecules *in vivo*, pure chemical standards are needed. However, *F*₄-NeuroP standards are currently not commercially available. Ironically, despite the synthetic work effort by research groups like Durand, Rokach, Taber, and Cha whose successfully synthesized numerous series of *F*₄-NeuroPs [64–68], the molecular activity of *F*₄-NeuroPs remains largely unexplored. Not until recently, the anti-arrhythmic property of 4(*RS*)-4-*F*₄-NeuroP biomolecule, one of the eight isomers of *F*₄-NeuroPs was discovered [55] in post-myocardial infarcted mice, opening an area of research on the beneficial effects of autoxidized PUFA metabolites.

Measurement of Isoprostanes in Human Plasma

Plasma IsoPs are commonly detected via mass spectrometry (MS), coupling to either gas chromatography (GC) or liquid chromatography (LC), and to a lesser extent by immunological methods, such as radioimmunoassay (RIA) and enzyme-linked immunoassay (ELISA). Commercial RIA and ELISA kits are known to provide a high speed, relatively low-cost, high sensitivity, and strong specificity method for IsoPs analysis. Specifically, for 15-*F*₂-IsoP molecule measurements, quantification of ligand concentration is inversely related to radioactivity for RIA, while direct fluorescent intensity is inferred to estimate the antigen amount present for ELISA. Both types of immunological assays rely on the use of specific antibody, but they tend to have positive bias results due to polyclonal antibody reacting with other compounds. A recent study by Klawitter et al. [15] compared some ELISA kits developed by Assay Designs (USA), Cayman Chemicals (USA), and Oxford Biomedical Research (USA) with LC-MS/MS on their 15-*F*₂-IsoP detection performance using human plasma and urine. These immunological assay kits were reported to generate a higher quantity of 15-*F*₂-IsoP than those measured by LC-MS/MS, overestimating the levels of 15-*F*₂-IsoP, particularly in plasma samples. The quantities measured were also disturbingly deviated from one ELISA kit to another, suggesting that concentrations measured from different ELISA kits were not comparable in parallel [15].

On the contrary, the use of MS overcomes such problems and offers fast, genuinely high sensitivity and specificity method for high throughput IsoPs quantification. Although the use of MS instrument is robust, it requires high cost in maintenance and trained personnel to operate the machine. As a consequence, laboratories might be forced to rely on immunological assay kits. Numerous studies have been

published for the detection of IsoPs (mainly on 15-F₂-IsoPs molecule), using either GC or LC coupled to the MS technique (Tables 1, 2). Measurement of human plasma IsoPs in GC–MS was first developed by Morrow and co-workers using negative ion chemical ionization (NICI) mode [5] and was widely adapted and modified by others. Subsequently, with the advances in LC coupled MS techniques, researchers began to investigate the possibility of separating IsoPs using an analytical column, usually C18 reverse phase column. The main difference in using LC to GC is the need to chemically derivatize the purified sample for the latter, which will be discussed later, before subjecting it to GC/MS or GC–MS/MS. Alternatively, some scientists might by pass the chromatographic separation step and perform direct infusion-based MS to acquire cellular lipidome data on a global scale. However, it is generally not usable for the detection and quantification of low abundance lipids, such as IsoPs, because of the interference from complex higher lipids and extracted matrices [69]. This method is usually employed for lipid molecular species analysis and will not be discussed further.

A wide variety of mass spectrometers used in IsoPs quantification are available in the market. They range in different ionization mode like electrospray ionization (ESI) or atmospheric-pressure chemical ionization (APCI) and

mass analysers such as quadrupole, time-of-flight (TOF), and orbitrap. Each has its own merits and demerits depending on the intended purpose, but is not explicitly mentioned here. Rather, the use of single quadrupole or triple quadrupole is what needs to be considered for IsoP measurement. Single MS detection methods such as GC–MS and LC–MS usually report the exact masses of injected analytes over retention time, while MS/MS, as in GC–MS/MS or LC–MS/MS, monitors the precursor (parent) ions (Q1) and fragmented (daughter) ions (Q3) of the molecule over retention time, thus improving the selectivity and precision in detecting targeted IsoPs. The use of MS/MS is particularly useful in detecting interested targets in complex matrices such as plasma at low levels, because the monitoring of Q1 and Q3 transition pair ions further confirms on the target identity among other analytes that might share the same Q1 mass ion. Notably, the selected ion monitoring (SIM) of all F₂-IsoP isomers in LC–MS typically use *m/z* 353 but in order to identify which F₂-IsoP isomers are present in the sample, LC–MS/MS using multiple reaction monitoring (MRM) can further yield for example *m/z* 353→193 for 15-F_{2t}-IsoP and *m/z* 353→115 for 5-F_{2t}-IsoP [14]. Multiple pairs of transition mass ions can be used in MRM. Typically, the transition mass ions were deduced by compound optimization of respective individual commercial

Table 1 Summary of highly cited (>50) articles reporting on human plasma isoprostanes detection using gas chromatography–mass spectrometry (GC–MS)

Study	Sample purification	Derivatization agent	Mass spectrometer (company)	Isoprostanooids detected	<i>m/z</i>	Levels detected (pg/mL)	Limit of detection (pg/mL)	References
Control	SPE: C18 Sep-Pak; and HPLC: LiChrospher 100 RP-18	BSTFA + 1 % TMCS	HP 5989B (Agilent)	Total F ₂ -IsoPs	569	337.5 ± 13.5 ^a	250	[82]
Control	TLC: 60ALK-6Dplates; and SPE: C18 Sep-Pak	BSTFA + DMF	Incos 50B (Finnigan)	Total F ₂ -IsoPs	514	15 ± 4.9	N.A.	[20]
Control	SPE: C18 Sep-Pak + NH ₂ cartridge	BSTFA + 1 % TMCS	VG70SEQ (Fisons)	15-F _{2t} -IsoP	569	112 ± 54	10	[83]
Control renal disease	HPLC: amino column, varian Associates	BSTFA + 1 % TMCS	Trio 1000 (VG Instruments)	Total F ₂ -IsoPs	569	0.3 2.5	50	[25]
Control	SPE: MAX	BSTFA + 1 % TMCS	HP 5973 N (Agilent)	Total F ₂ -IsoPs	569	0.45 ± 0.26	22	[84]
Control	SPE: MAX	BSTFA + 1 % TMCS	HP 5973 (Agilent)	Total F ₂ -IsoPs	569	0.239 ± 0.021	37	[85]

Keywords searched on the Web of Science (Thomson Reuters): isoprostane, plasma, gas chromatography

BSTFA *N,O*-bis(trimethylsilyl)trifluoroacetamide, TMCS trimethylchlorosilane, DMF dimethylformamide, TLC thin layer chromatography, Sep-Pak C18 octadecylsilane cartridge, NH₂ aminopropyl cartridge, MAX mixed anionic exchange, N.A. not reported

^a Mathematical conversion to match the unit

Table 2 Summary of highly cited method development articles reporting on plasma isoprostanes detection using liquid chromatography–mass spectrometry (LC–MS/MS)

Study	Sample purification	Column	Mass spectrometer (company)	Isoprostanooids detected	SIM/MRM	Levels detected (pg/mL)	Limit of detection (pg/mL)	References
Control	HPLC: XDB-C8	C18	QTRAP 4000 (ABSciex) QTRAP 5000 (ABSciex)	Free 15-F _{2t} -IsoP Free 15-F _{2t} -IsoP	353/193 353/193	3–25	10 2.5	[70]
Non-smokers Smokers	SPE: Empore disk cartridge; 0.45 μm filters	C8	MStation 700 (JEOL)	Total F ₂ -IsoPs	353.24 353.24	42 ± 2 46 ± 2	20	[86]
Non-smokers Smokers	LLE: ethyl acetate:hexane (3:1)	C18	QTRAP 3000 (ABSciex)	15-F _{2t} -IsoP 15- <i>epi</i> -15-F _{2t} -IsoP 15-F _{2t} -IsoP 15- <i>epi</i> -15-F _{2t} -IsoP	353/193	82 ± 23 108 ± 32 78 ± 27 121 ± 35	N.A.	[87]
Healthy control	IAC: Isoprostane affinity column	C18	Surveyor MSQ (Finnigan)	Total F ₂ -IsoPs	353.2	37.6	0.5	[88]
Control	HPLC: XDB-C8	C18	QTRAP 5000 (ABSciex)	Free 15-F _{2t} -isoP	353/193	21.1 ± 27.9	2.5	[15]
Control	7-mm cellulose filter; protein precipitated	C18	QTRAP 4000 (ABSciex)	15-F _{2t} -IsoP	353.1/193.2	N.A.	1.3 ^a	[89]
Control	SPE: 100 mg C18 EC	ABZ + Plus	QTRAP 4000 (ABSciex)	15-F _{2t} -IsoP	353.4/197.1	40–170	4	[90]

Keywords searched on the Web of Science (Thomson Reuters): isoprostane, plasma, liquid chromatography

HPLC high performance liquid chromatography, *SPE* solid phase extraction, *LLE* liquid–liquid extraction, *IAC* immune-affinity column, *N.A.* not reported

^a Mathematical conversion to match the unit

standards performed by the spectrometer using direct infusion through a syringe pump. Therefore, one is required to optimize all the target standards of interests for MS/MS settings, individually, before sample analysis.

In addition, to quantify accurately the absolute amount of target molecules, deuterated internal standards are commonly added in sample preparation or extraction but before purification steps. This is because deuterated internal standards are not biologically available and adding known amount of internal standards to sample aids compound quantification by comparing the peak areas of same analyte in deuterated and non-deuterated forms. The use of deuterated IsoPs is ideally of similar chemical structure as to the target molecule, or as close as possible if commercially unavailable, so as to mimic the ionization efficiency of analyte in the matrix. Of note, losses of deuterated internal standards during sample purification are taken into account for the calculation of final concentrations.

A large number of known IsoPs are present physiologically and increasing types of IsoP species were successfully detected through IsoP research. Especially F₂-dihomo-IsoPs and F₄-NeuroPs molecules that arise from AdA and DHA, respectively, which are highly present in the brain

[46], and F₃-IsoPs from EPA, were all recently reported to be quantifiable in human plasma (Table 3). Compared to F₂-IsoPs from ARA, the concentrations of the IsoPs from ALA, EPA or DHA are very low but measurable by the GC–MS, GC–MS/MS, or LC–MS/MS.1

Parameters to Consider on Using GC or LC Coupled to MS for Human Plasma IsoPs Measurement

Detection and Quantitation Limits

The limit of detection (LOD) and limit of quantitation (LOQ) are two parameters most widely discussed in MS analytical methods. LOD and LOQ are closely related, but have different definitions and confusion must be avoided. LOD refers to the lowest analyte concentration detectable, but not necessarily quantifiable under the test condition, while LOQ refers to the lowest concentration of an analyte that can be determined with satisfactory precision and accuracy under the test condition. To find out the LOD and LOQ of an analyte, sufficient analytical signal must be instantly spotted out from the analytical noise, which is produced in the absence of analyte. More often than not,

Table 3 Newly found isoprostanes in human plasma

Isoprostanoid	Study	Purification	MS-method	Mass spectrometer (company)	Levels detected (pg/mL)	References
F ₂ -Dihomo-IsoP	Control RTT	SPE: Sep-Pak C18 & NH ₂	GC-MS/MS	Polaris Q (ThermoFinnigan)	1.0 ± 0.11 (free) 3.3 ± 0.31 (free)	[50]
F ₃ -IsoPs ^a	Control RTT	SPE: Sep-Pak C18 & NH ₂	GC-MS/MS	Polaris Q (ThermoFinnigan)	13 (free) 40 (free)	[58]
	No PoAF PoAF	TLC: 60ALK6D plates & SPE: C18 Sep-Pak	GC-MS/MS	HP 5973 N (Agilent)	5 (total) 6 (total)	[57]
F ₄ -NeuroP	Control	SPE: MAX	GC-MS	HP 5973 N (Agilent)	250 ± 110 (total)	[62]
	Smokers				460 ± 200 (total)	

RTT Rett syndrome, PoAF post-operative atrial fibrillation, TLC thin layer chromatography, SPE solid phase extraction, Sep-Pak C18 octadecylsilane cartridge, NH₂ aminopropyl cartridge, MAX mixed anionic exchange

^a Estimated values from graphical depiction in the report

the signal-to-noise ratio (S/N) is used in calculating their respective LOD and LOQ. A S/N greater than three is generally acceptable as LOD and S/N of at least ten for LOQ is commonly practised.

Diverse levels of LOD and LOQ were reported for the same analyte measured by different GC-MS or LC-MS/MS models of different companies. For example, Haschke et al. [70] reported the measurement of 15-F_{2t}-IsoP in human plasma via QTRAP 4000 (ABSciex, USA) and QTRAP 5000 MS (ABSciex, USA) machines and found four-fold improvement for LOD in QTRAP 5000, suggesting that the latter model of the MS machine might be a better tool in compound analysis. We investigated highly cited articles in the Web of Science (Thomson Reuters) reporting on the detection of IsoPs (mainly 15-F_{2t}-IsoPs) in plasma using GC- and LC-coupled MS, respectively (Tables 1, 2). It suggests that the LOD and LOQ levels are of far superior for GC- than LC-coupled MS and the difference is the enhancement by GC derivatization, which will be discussed below. Comparatively, from Tables 1 and 2, LOD for LC-MS(/MS) seemed to perform better than GC-MS(/MS). However, the S/N used by different groups for the stated LOD were not in coherence, implying that the LOD reported might not be comparable with others.

Derivatization

A distinct difference in employing GC or LC coupled MS is the need to derivatize the sample. Derivatization is needed in GC-MS(/MS), but not necessarily so for LC-MS(/MS). The need to derivatize compound is to improve volatility for GC separation by adding chemical functions to its native structure, as well as increase thermal stability. However, the downside of performing derivatization is more labour intensive as well as exposure to potentially toxic chemicals. Different derivatization agents are

available in the market and its suitability is based on which targeted compounds are being analysed.

Commonly, the three types of derivatization reactions for GC are silylation, acylation, and alkylation. Silylation is typically used for the detection of IsoPs in GC-MS. As illustrated in Fig. 1 the typical steps in derivatizing 15-F_{2t}-IsoP for GC-MS analysis is shown [71]. Briefly, 15-F_{2t}-IsoP is converted to pentafluorobenzyl (PFB) esters by the means of pentafluorobenzyl bromide (PFBBR), followed by the use of derivatization agents *N,O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS). BSFTA readily silylate alcohol groups and TMCS act as a silylation catalyst to increase reactivity of BSTFA. It should be noted that the use of BSFTA + TMCS is extremely sensitive to moisture and, therefore, should be handled under dry conditions.

The use of derivatization reagents was also reported for LC-MS/MS platform. Simultaneous detection of multiple IsoPs is often performed under negative ionization mode, which has low ionization efficiency because free carboxylic acids are difficult to be detected, especially in large number of carboxylic acid-containing analytes. The derivatization of IsoP compatible for positive ionization is designed to overcome such inefficiencies. For example, Bollinger et al. [72] introduced a derivatization strategy that converts the carboxy moiety of oxylipins to an amide with positively charged *N*-(4-aminomethylphenyl) pyridinium (AMPP). They reported the improvement of ionization efficiency and approximately 10–20-fold enhancement in detection sensitivity of derivatized eicosanoids [72, 73] and fourfold for 4(*RS*)-4-F_{4t}-NeuroP in human plasma (*unpublished data*) (Fig. 2a). The AMPP derivatizing agent is now commercialized by Cayman Chemical (USA) as AMP⁺ Mass Spectrometry kit. Nevertheless, as mentioned by Bollinger and co-workers [73] and others [74], the product mass ion of AMPP-derivatized eicosanoids was consistently reported

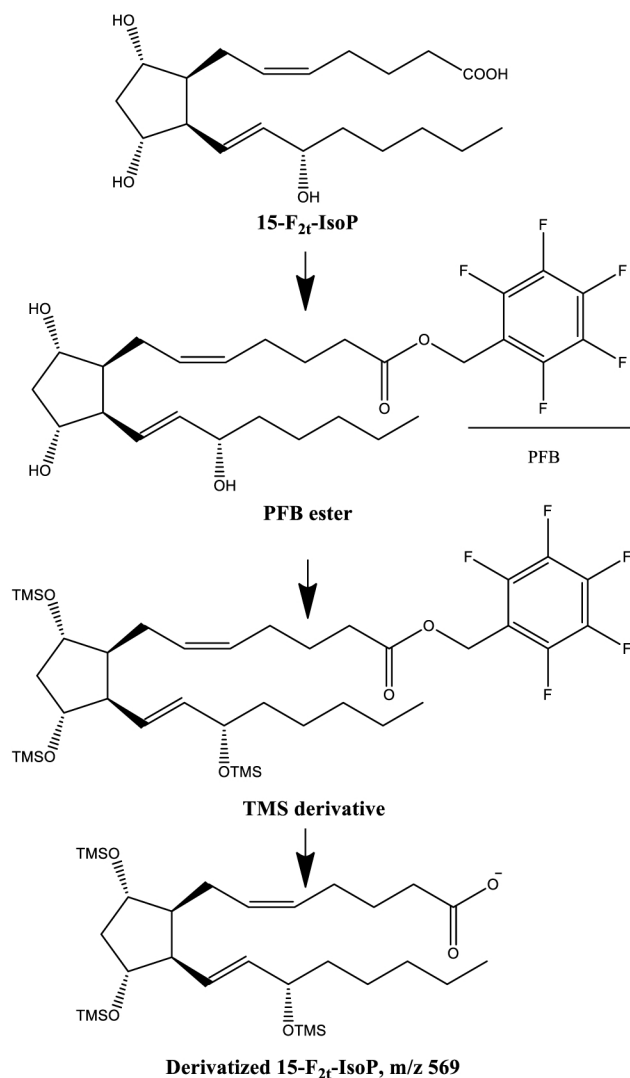


Fig. 1 Two derivatization steps for 15-F_{2t}-IsoP analyses by GC-MS. First derivatization is with pentafluorobenzyl bromide for the formation of pentafluorobenzyl (PFB) ester with 15-F_{2t}-IsoP and second derivatization is with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) to form pentafluorobenzyl ester-trimethylsilyl ether

to be m/z 183 and m/z 169 with high intensity, which are the AMPP derivative fragments (Fig. 2b). However, the group also indicated for this reason, certain compounds e.g. PGF_{2 α} and arachidonic acid were measured by using a fragment in the analyte portion rather than the AMPP tag, i.e. non-tag fragment ion derived by cross-ring cleavage of the cyclopentanone ring. The application of AMPP eliminates the advantages of using the two-stage tandem MS as one is unable to identify the unique Q3 mass ion, in particular for IsoPs, that is derived from the analyte portion instead of the AMPP tag; especially when the MS machine loses its sensitivity when used extensively. Also, derivatization might affect the hydrophobicity of analyte causing retention time shift when passed through a HPLC column.

Fig. 2 Derivatization of carboxylic acid of isoprostanoids with *N*-(4-aminomethylphenyl) pyridinium (AMPP) for LC-MS/MS analysis. The effect of AMPP is shown by chromatographic display (a) of human plasma for the measurement of 4(*RS*)-4-F_{4t}-NeuroP with (m/z 545→183) and without (m/z 377→101) derivatization process; the response amplified approximately fourfold after derivatization (unpublished data). The formation of m/z 183 (benzenemethanimine pyridinium ion) and 169 (tolylpyridinium distonic radical ion) products (b) in LC-MS/MS analysis serves as derivative fragments of carboxylic acid and does not alter for compounds with carboxylic acid group attached

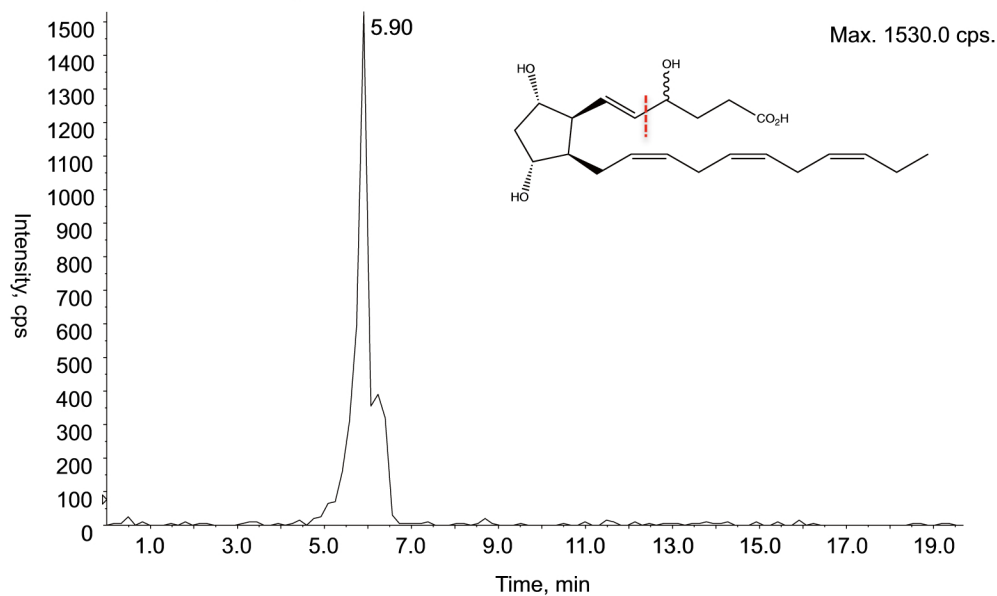
Similarly, other derivatization compounds were reported for the use of lipid and their mediator detections in LC-MS/MS system. For example, 2-(4-aminophenoxy)ethyl (4-bromophenethyl)-dimethylammonium bromide hydrobromide (4-APEBA) was used to couple the carboxylic group of prostanoid molecules and measured at positive ionization mode. This method was found to be simple and able to enhance ESI efficiency in human urine analysis [75]. In a more recent study, *N*-hydroxysuccinimide (NHS) and 1-ethyl-3-[3-dimethylaminopropyl]-carbodiimide hydrochloride (EDC) were used to derivatize the carboxyl group of fatty acids in rat plasma and showed an increase in the sensitivity for LC-(ESI)MS/MS analysis as low as femto-gram level [76]. Therefore, the use of derivatization may be a benefit in LC-MS/MS analysis using lower models (e.g. QTrap 3200) that are less sensitive for plasma IsoPs measurement (Fig. 2a), in which the levels normally range from picogram to nanogram per millilitre in healthy subjects; this allows better chromatographic peak detection for integration.

Matrix Effects

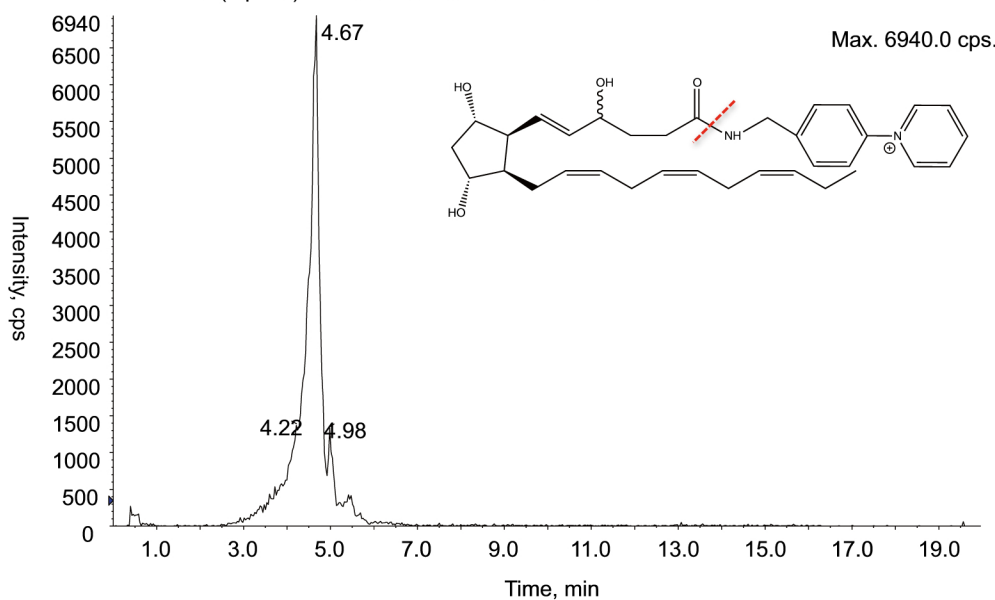
Matrix effect occurs when the compound of interest co-elutes with other molecules from the column, almost exclusively in LC resulting in alteration of ionization efficiency. The possible phenomenon of matrix effects is signal suppression, reduction or even amplification. The exact mechanism of how matrix effects develops is unknown as the possible interacting molecules with the analyte in a matrix sample like plasma is affected in many ways. The current understanding of matrix effects is caused by the competition of non-volatile matrix components with analyte ions to retain at the surface of liquid droplet for transfer to the gas phase [77]. Analysis of multiple analytes under the same MS conditions might cause some, but not all analytes signals to be suppressed due to matrix effects [78].

The solution to reduce, but not remove matrix effects is switching the ionization source from ESI to APCI as some researchers found that under the APCI ionization mode, it appeared to be less severe [70, 79]. However, APCI mode

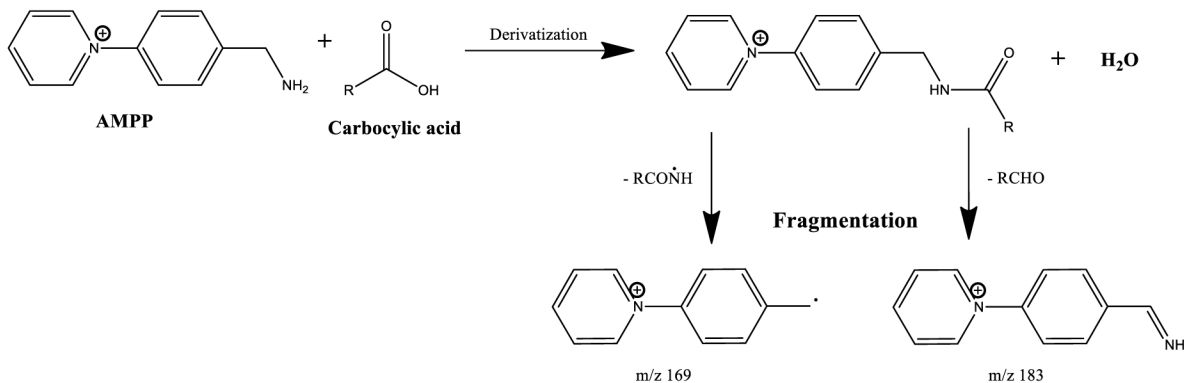
A ■ XIC of -MRM (48 pairs): 377.000/101.000 Da ID: 4(RS)-4-F4t-NeuroP



■ XIC of +MRM (9 pairs): 545.000/183.000 Da ID: F4-NP



B



is not ideal to ionize IsoPs due to sample loss therefore, to reduce this effect, the interfering matrix components are often removed. Alternatively, protein precipitation by organic solvents is used to remove most of the dirtiest contaminants from the plasma matrix in the analysis for IsoPs. This is often followed by SPE or liquid–liquid extraction (LLE) purification. Some researchers also performed purification by on-line HPLC extraction prior to LC–MS/MS analysis using a column-switching device, which is advantageous over others for its automation in the analysis procedures [80, 81].

Conclusions

Given the relevance and emergence of lipid mediators in the field of lipidomics, the measurement of IsoPs molecules is essential in lipid research especially when investigating fatty acid metabolism and oxidative stress. The choice of whether GC- or LC-coupled MS technique for IsoPs measurement is one of the concerns by many, especially when the laboratory has access to both platforms. The design and development of GC is originally for the analysis of less polar and LC for more polar analytes. The measurement of human plasma IsoPs is possible in both platforms and the key is to ensure its sensitivity for measurement at very low concentrations. LC–MS/MS is gaining popularity in recent years for IsoPs detections and quantifications, offering a fast and high-throughput analysis with the help of MRM. Although MRM mode is also possible in the GC–MS/MS platform, the fragmented ions of most, if not all, analytes are the derivatives and therefore, reduce the advantages of using two-stage MS. Nonetheless, reducing matrix effects from plasma samples are often an obstacle when analysed by LC–MS/MS. Appropriate clean-up procedures are necessary and derivatization for the analysis of less concentrated compounds are perhaps advantageous when subjecting the samples to LC-based MS analysis. However, due to some limitations in the use of GC- and LC-coupled MS platforms, as discussed in this article, one has to carefully consider the principle aim of the research project and decide on either platform depending on detectability, particularly when quantifying IsoPs that some are known to be low in abundance.

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