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ABSTRACT

Metabolites of non-enzymatic lipid peroxidation of polyunsaturated fatty acids notably omega-3 and omega-6 fatty acids have become important biomarkers of lipid products. Especially the arachidonic acid-derived F_2-isoprostanes are the classic *in vivo* biomarker for oxidative stress in biological systems. In recent years other isoprostanes from eicosapentaenoic, docosahexaenoic, adrenic and α-linolenic acids have been evaluated, namely F_3-isoprostanes, F_4-neuroprostanes, F_2-dihomo-isoprostanes and F_1-phytoprostanes, respectively. These have been gaining interest as complementary specific biomarkers in human diseases. Refined extraction methods, robust analysis and elucidation of chemical structures have improved the sensitivity of detection in biological tissues and fluids. Previously the main reliable instrumentation for measurement was gas chromatography–mass spectrometry (GC–MS), but now the use of liquid chromatography–tandem mass spectrometry (LC–MS/MS) and immunological techniques is gaining much attention. In this review, the types of prostanoids generated from non-enzymatic lipid peroxidation of some important omega-3 and omega-6 fatty acids and biological samples that have been determined by GC–MS and LC–MS/MS are discussed.

Abbreviations: AA, arachidonic acid; AD, Alzheimer disease; AdA, adrenic acid; ALA, α-linolenic acid; AMPP, *N*-(4-aminomethylphenyl)pyridinium; APCI, atmospheric pressure chemical ionization; aSAH, aneurysmal subarachnoid hemorrhage; BHT, butylated hydroxytoluene; BSTFA, *N*,*O*-bis(trimethylsilyl)trifuoroacetamide; CSF, cerebrospinal fluid; DHA, docosahexaenoic acid; DIPEA, *N*,*N*′-diisopropylethylamine; DMF, dimethylformamide; DTPA, diethylene triamine pentaacetic acid; EBC, exhaled breath condensate; EFSA, European Food Safety Authority; EI, electron ionization; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; EPA, eicosapentaenoic acid; ESI, electrospray ionization; GC, gas chromatography; HOTMS, trimethylsilyl hydroxide (trimethysilanol); HPLC, high-pressure liquid chromatography; IAC, immunoaffinity chromatography; IsoPs, isoprostanes; LC, liquid chromatography; *m*/*z*, mass-to-charge ratio; MRM, multiple reaction monitoring; MS, mass spectrometry; NeuroPs, neuroprostanes; NICI, negative-ion chemical ionization; PFB, pentafluorobenzyl; PFB-MO-TMS, pentafluorobenzyl methyloxime trimethylsilyl; PFB-TMS, pentafluorobenzyl trimethylsilyl; PFBBr, pentafluorobenzyl bromide; PGs, prostaglandins; PhytoPs, phytoprostanes; PUFAs, polyunsaturated fatty acids; RIA, radioimmunoassay; ROS, reactive oxygen species; SIL-IS, stable-isotope labeled internal standard; SIM, selected-ion monitoring; SPE, solid-phase extraction; SRM, selected-reaction monitoring; TLC, thin layer chromatography; TMCS, trimethylchlorosilane; TMS, trimethylsilyl; TPP, triphenylphosphine; UPLC, ultra high-pressure liquid chromatography; UV, ultraviolet.

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1. Introduction

Free radicals have been implicated in a number of human diseases such as neurodegenerative, cardiovascular and pulmonary disorders and cancer [1]. Most common free radicals and non-radical species, known as reactive oxygen species (ROS), are able to modify oxidatively lipids, proteins and nucleic acids. Of the lipids in particular the polyunsaturated fatty acids (PUFAs) form a wide variety of oxidized products [2,3].

Among the oxidized lipid products generated, the measurement of isoprostanes (IsoPs) appears to be a promising assay for over two decades due to their specificity and sensitivity for *in vivo* assessment of oxidative stress and lipid peroxidation [4]. The majority of IsoPs are produced *in vivo* by non-enzymatic free-radical-induced peroxidation of PUFAs [5]. These compounds are formed *in situ* on membrane phospholipids and then released in their free form into circulation [6,7]. Depending on the parent PUFAs, different families of IsoPs have been discovered and quantified in several pathological conditions [2]. Among them, F_2-IsoPs are the most represented and extensively studied such that to be the designated "gold" marker by laboratories [8], and to be recently validated by the European Food Safety Authority (EFSA) as biomarkers for oxidative damage in cardiovascular health [9].

Various nomenclature guidelines have been proposed in the literature [10,11]. In this review, the nomenclature reported by Taber and Roberts [10], validated by the International Union of Pure and Applied Chemistry (IUPAC), will be adopted to describe different non-enzymatic oxidized lipid products of arachidonic, adrenic, eicosapentaenoic, docosahexaenoic, and α-linolenic acids. The state-of-the-art analysis of these products, namely F_2-IsoPs, F_3-isoprostanes (F_3-IsoPs), F_4-neuroprostanes (F_4-NeuroPs), F_2-dihomo-isoprostanes (F_2-dihomo-IsoPs) and F_1-phytoprostanes (PhytoPs), in biological samples using GC–MS, GC–MS/MS, LC–MS and LC–MS/MS is reviewed (Fig. 1).

2. Generation of lipid oxidation products

2.1. Metabolites of arachidonic acid

The biosynthesis of the majority of the IsoPs involves a free radical-induced process of arachidonic acid (AA) peroxidation initiated by the presence of radical species mainly centered on the oxygen such as the hydroxyl radical. Generation of prostaglandins (PGs) from free AA is cyclooxygenase dependent, whereas IsoPs made in phospholipid membranes are cyclooxygenase independent. IsoPs are released in their free forms by the platelet-activating factor acetylhydrolase and possibly by other phospholipases [12,13]. It was recently found that they circulate predominantly in high-density lipoproteins (HDL) [14] and then subsequently are metabolized and excreted in urine. It was reported that a significant proportion of F_2-IsoPs in urine are conjugated as glucuronides [15]. Each series of F_2-IsoPs contains eight possible isomers. As each regioisomer includes its racemic counterpart, a total of 64 different F_2-IsoPs can be generated; however, it is unknown and not investigated whether these numerous F_2-IsoPs generated would have similar properties. Among the F_2-IsoPs regioisomers, most human studies have focused on the 15-F_2-IsoP series, in particular 15-F_{2t}-IsoP, which is also known as 8-*iso*-$PGF_{2\alpha}$ and often used as an index for F_2-IsoPs. Nevertheless, Li and coworkers [16] discovered that 5-F_{2t}-IsoPs are present in urine at higher concentration than 15-F_{2t}-IsoPs.

The fundamental difference in the biosynthesis between PGs and IsoPs displays the specificity in the stereostructure of the metabolites. The non-specific initial radical hydrogen atom abstraction at one of the three possible bisallylic positions (7, 10 and 13) in AA is the first difference between the IsoPs synthesis and the PGs synthesis, where H-abstraction is regiospecific and occurs only at the 13 position. The subsequent pentadienyl radicals formed in the IsoPs synthesis react with molecular oxygen generating six different pentadienyl peroxyl radicals. Only four of them can produce the four different series of IsoPs compounds *via* a subsequent irreversible radical cascade (double 5-exo trig cyclization), followed by a final oxygenation leading to G_2-IsoPs. Consecutive hydroperoxide and endoperoxide reductions generate the specific F_2-IsoPs. The endoperoxide reduction can also be perturbed and following a Kornblum–DeLaMare rearrangement can give E- and D-IsoPs. Dehydration of membrane-bound E_2- and D_2-IsoPs is facile under physiological conditions [17] and produces cyclopentenone A_2- and J_2-IsoPs, respectively [18]. The particular *cis*-orientation of the side chains in IsoPs in contrast to *trans*-orientation in PGs reflects another crucial specificity, linked to the localization of the radical process. For IsoPs biosynthesis, the membrane is the place of action, and therefore conventional chemistry rules apply (lower transition state energy during the double 5-exo-trig cyclization) to generate the 1,2-*cis*-orientations of the side chains compared to enzymatically driven three-dimensional *trans*-orientation of the side chains obtained with PGs synthesis. Furthermore, two stereochemistries – the all-*syn* (represented as subscript "c"; see 5-F_{2c}-IsoP) and *syn*-anti-*syn* stereochemistry (represented as subscript "t"; see 15-F_{2t}-IsoP) – are present in IsoPs synthesis, and they are well explained by the Beckwing-Houk model of the lower transition states possible during a radical cyclization process; chair- and boat-like transition states are shown in Scheme 1.

The number of theoretical regioisomers further complicates the complexity of IsoPs biosynthesis; the four F_2-IsoPs regioisomers having each 8 diastereoisomers could generate 64 racemic compounds. It was shown that the 5- and 15-series IsoPs are the most abundant *in vivo*, likely due to the fact that the 8- and 12-series IsoPs are more readily oxidized further [19–22].

2.2. Metabolites of adrenic acid

Adrenic acid (AdA) is the elongated form of arachidonic acid (AA). Being a two-carbon analog of AA, it will also provide four series of dihomo-isoprostanes (dihomo-IsoPs), the 7- and 17-series being the major metabolites [23]. Similarly to IsoPs, 64 racemic F_2-dihomo-IsoPs can theoretically be found *in vivo*, and so far only the F-series was investigated in biological and analytical studies [23–25].

2.3. Metabolites of eicosapentaenoic acid

Eicosapentaenoic acid (EPA) was also found to provide 6 series of F_3-IsoPs, the 5- and 18-isomers being the most abundant *in vivo* [26–28]. Theoretically, 96 racemic isomers of F_3-IsoPs can be observed and quantified as a total *in vivo*. F_3-IsoPs are not the most studied isoprostanoids, probably because EPA is less abundant than AA in human tissues. However, Rokach and coworkers [27,29] have shown that 5-F_3-IsoP can be quantified in urine and may represent a β-oxidized metabolite of 7-F_4-NeuroP from DHA [27,29,30].

2.4. Metabolites of docosahexaenoic acid

The peroxidation of docosahexaenoic acid (DHA) follows a similar process as described for IsoPs. A total of 8 possible regioisomers identified as 4-, 7-, 10-, 11-, 13-, 14-, 17- and 20-series NeuroPs, and a total of 128 theoretical compounds could be generated. Among these 4- and 20-series represent the two most abundantly found NeuroP isomers *in vivo* [31].

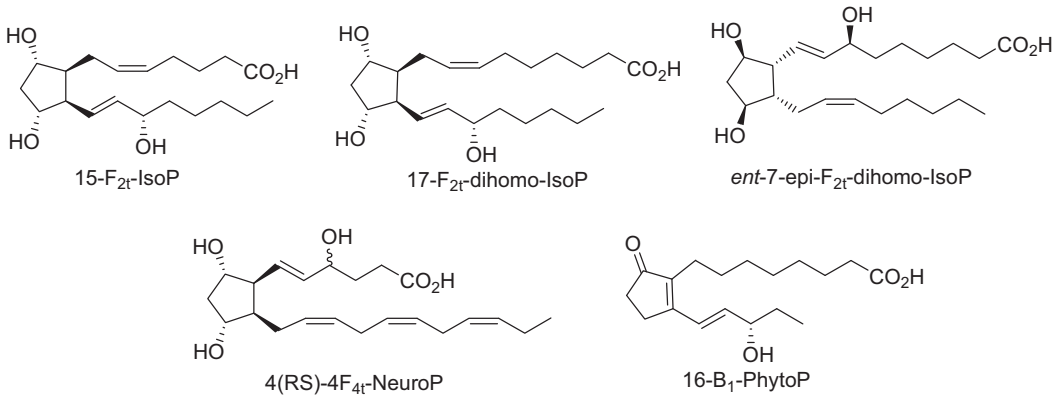


Fig. 1. Representative IsoP, dihomo-IsoP, NeuroP and PhytoP in biological systems.

2.5. Metabolites of α-linolenic acid

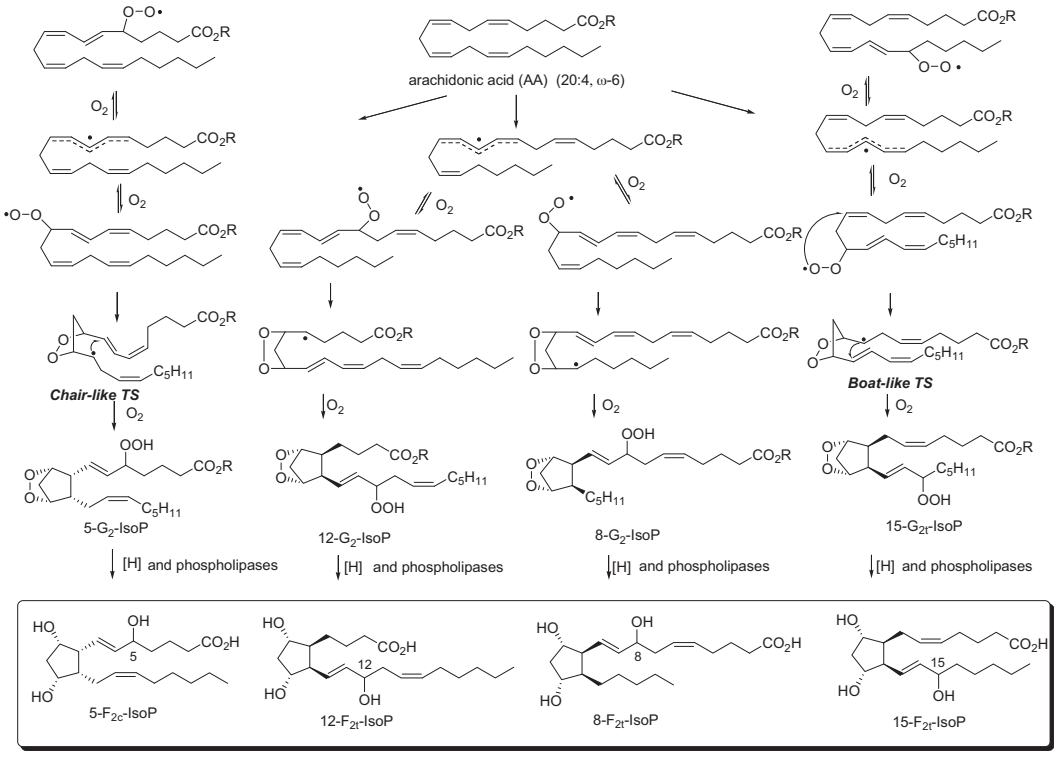
Generation of phytoprostanes (PhytoPs) also involves a free radical-induced reaction of α-linolenic acid (ALA) initiated by the active ROS. These PhytoPs are found in plants in which only two series, *i.e.*, the 9- and 16- isomers, are generated [32]. They have also been encountered in human studies after flaxseed oil supplementation [33] and in vegetable oils [34].

3. Assessment of lipid oxidation products in biological samples

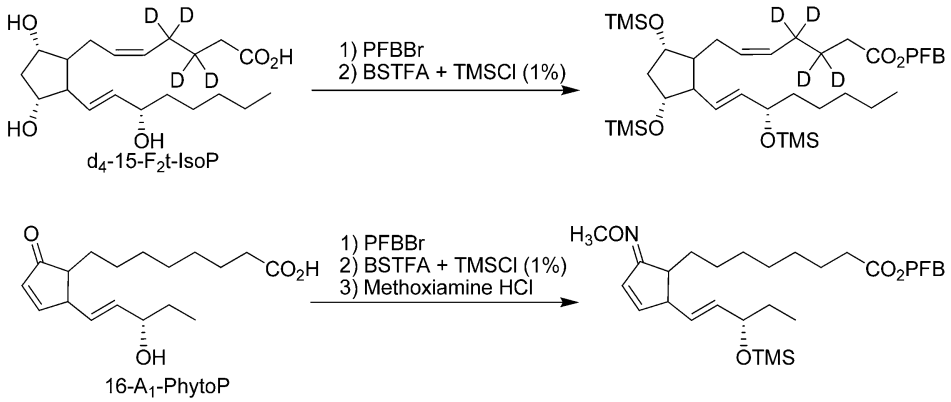
3.1. Sample collection

IsoPs, NeuroPs, PhytoPs and other oxygenated metabolites of n-3 and n-6 PUFAs have been measured in various biological samples. Lipid oxidation products in urine and plasma are indicative of oxidative stress status *in vivo* and tissue samples. In particular fluids such as exhaled breath condensate (EBC) they represent localized, organ-specific oxidative stress status. For accurate analysis, it is essential to minimize any post-drawing oxidation or degradation after sampling that would affect the measurement. PUFAs, particularly n-3 and n-6 and derivatives, are prone to autoxidation or photodegradation. Therefore, numerous cautions must be taken for sample storage as well as in extraction and derivatization steps. To protect samples from direct sunlight, the use of amber glassware or glassware with aluminum foil is recommended. Samples, especially plasma, cerebrospinal fluid (CSF) and tissues, are commonly excluded from oxygen by flushing with nitrogen or argon, after being treated with suitable antioxidant, such as butylated hydroxytoluene (BHT), triphenylphosphine (TPP), diethylene triamine pentaacetic acid (DTPA) and glutathione (GSH), to inhibit free radicals present in matrix that could generate lipid hydroperoxides artificially and consequently oxidized lipid products.

It is well documented that ultra-low temperatures are necessary for the storage of all biological samples [5] with a restriction



Scheme 1. Biosynthesis of isoprostanes.



Scheme 2. Derivatization process of isoprostanes: (a) derivatization PFB-TMS compounds for GC–MS analysis and (b) derivatization PFB-MO-TMS compounds for GC–MS analysis.

of number of freeze–thaw cycles. Recently, it was reported that blood samples should be collected in EDTA/BHT/GSH tubes [35] and it is crucial the plasma portion be stored at −80 °C to reduce *ex vivo* auto-oxidation of AA that leads to artifact elevation of plasma F_2-IsoPs concentrations [5,35]. Urine does not require special processing since it contains only trace quantities of lipids; thus it is not affected by *ex vivo* formation of n-3 and n-6 PUFA metabolites. However it is suggested to treat it with glucuronidase prior to analysis to release free F_2-IsoPs which are accessible to analysis [36]. In general, plasma and tissue samples can be conserved for up to 6 months at −80 °C but urine samples can be stored for several years at this temperature [37].

3.2. Extraction

Determination of metabolites from lipid peroxidation in biological samples requires one or several preparation steps to extract the compounds from their biological matrix before further analysis by GC–MS, GC–MS/MS, LC–MS, LC–MS/MS or by immunological methods. Sample volumes of biological fluids (plasma, urine, CSF) between 0.2 and 1 mL [38] are required, while tissues samples between 50 and 100 mg [8,39] and 1–5 million cells are needed [40–42]. IsoPs, NeuroPs or PhytoPs are found to be present in conjugated and free forms endogenously. They can be quantified in their free circulating forms or in total, *i.e.*, the sum of free and esterified, in plasma [43] and tissues after extraction. For tissues or cells, lipids are extracted by homogenizing the samples using Folch solution composed of chloroform and methanol (2:1, v/v) with 0.005% BHT, and then continuously agitated at 4 °C for 1 hour. Afterwards, NaCl (0.9%) or phosphated buffered saline (pH 7.4) is added and the organic lipid layer is extracted for analysis [4,44]. For total IsoPs, NeuroPs or PhytoPs measurement, lipid extract is further treated with equal volume of 1 M KOH prepared in methanol and heated at 37 °C. This step is not required for measurement of 'free' IsoPs, NeuroPs or PhytoPs in plasma, lipid extract from tissues, CSF and urine. As for urine, some authors suggest that pretreatment with glucuronidase can reduce matrix effect and provide cleaner sample for analysis [36].

A purification process is needed to avoid matrix effect and to increase sensitivity of the quantification by LC–MS/MS analysis. Thin layer chromatography (TLC) or solid-phase extraction (SPE) using C18 [36] or NH_2 [45] or anionic exchange phase cartridges [46,38] is commonly used in the purification step. Some methods use a combination of hydrophobic (C18) and pure silica, which has high polarity [4] or NH_2 cartridge [45] for the purification. Few authors also combined SPE and TLC in the purification step [4,47]. One method proposes purification with a bimodal SPE non-polar cartridge and strong anion exchange to make it specific for analysis of compound of interest [33]. Others suggested the use of HPLC with extraction column [48] or immunoaffinity chromatography (IAC) using immobilized antibodies directed against specific analytes [49]. However, there is a limitation to IAC as it is exclusively commercialized for 15-F_{2t}-IsoP analysis and not for other metabolites of n-3 and n-6 PUFAs.

3.3. GC–MS and GC–MS/MS analysis

Usually, GC involves a sample being vaporized and injected onto the head of the chromatographic column through a hot injector. The sample is transported through the column by the flow of an inert, gaseous mobile phase such as helium. To increase volatility and thermal stability of the analytes, derivatization steps are often required in the sample preparation process. Over the last three decades, considerable improvements have been achieved in extraction and ionization techniques, in particular the development of GC–MS/MS analysis. The use of SPE for purification and isolation considerably improved the signal-to-noise ratio of the GC–MS and highly increased selectivity for compounds.

3.3.1. Derivatization

Derivatization of oxidized lipid products is generally performed according to Morrow and Roberts [6]. Briefly, after extraction and preliminary purification steps, the carboxylic acid function of compounds is converted into a pentafluorobenzyl (PFB) ester by reacting samples with pentafluorobenzyl bromide (PFBBr) and *N*,*N*′-diisopropylethylamine (DIPEA) acting as the base catalysts. Free hydroxyl groups are further converted into trimethylsilyl (TMS) ether derivatives by incubating with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), trimethylchlorosilane (TMCS; 1%), and dimethylformamide (DMF) leading to the wanted PFB-TMS derivative of the analyte ready for GC–MS analysis. In the specific cases of E-, D-, A- or J-type ring series, carbonyl function is first transformed into *O*-methyloxime derivatives with methoxyamine hydrochloride in pyridine, followed by standard derivatization as shown in Scheme 2 to lead to PFB-MO-TMS compounds.

3.3.2. Ionization and fragmentation

Some assays have been developed to improve GC–MS analysis of oxidized lipid products derived from n-3 and n-6 PUFAs with different ionization modes [50]. However, GC–MS in negative-ion chemical ionization (NICI) mode along with selected-ion monitoring (SIM) of a single ion has been shown to be the most reliable analytical approach to assess these compounds for *in vivo* samples.

Table 1
Oxidized lipid metabolites and internal standards measured by GC-MS and GC-MS/MS in some studies.

Compound name	$[M-PFB]^-$ m/z	Internal standard	$[M-PFB]^-$ m/z	Reference
AA metabolites				
GC-MS				
F_2-IsoPs	569	$[^2H_4]$-15-F_{2t}-IsoP	573	[50]
		$[^2H_4]$-$PGF_{2\alpha}$	573	[64,105,106]
		4-(RS)-4-F_{4t}-NeuroP	593	[107]
		$[^2H_4]$-8-F_{2t}-IsoP	573	[15,108,109]
D_2/E_2-IsoPs	524	$[^2H_4]$-$PGE_{2\alpha}$	528	[17,81]
2,3-Dinor-5,6-dihydro-15-F_{2t}-IsoP	543	$[^{18}O_2]$-15-F_{2t}-IsoP-M	547	[58]
		$[^2H_4]$-15-F_{2t}-IsoP	573	[60,110]
2,3-Dinor-15-F_{2t}-IsoP	541	$[^2H_4]$-15-F_{2t}-IsoP	573	[59]
GC-MS/MS				
F_2-IsoPs	569/299	$[^2H_4]$-15-F_{2t}-IsoP	573/303	[49]
		$[^2H_4]$-$PGF_{2\alpha}$	573/303	[111]
EPA metabolites				
GC-MS				
F_3-IsoPs	567	$[^2H_4]$-5-F_{3t}-IsoP	571	[27]
A_3/J_3-IsoPs	432	$[^2H_4]$-PGA_2	438	[92]
GC-MS/MS				
F_3-IsoPs	567/297	$[^2H_4]$-$PGF_{2\alpha}$	573/303	[25]
ALA metabolites				
GC-MS				
F_1-PhytoPs	545	$[^{18}O_3]$-F_1-PhytoP	551	[34]
	543	$[^2H_4]$-15-F_{2t}-IsoP	573	[33]
DHA metabolites				
GC-MS				
A_4/J_4-NeuroPs	458	$[^2H_4]$-PGA_2	438	[73]
F_4-NeuroPs	593	$[^{18}O_2]$-17-F_{4c}-NeuroP	597.5	[63]
		$[^2H_4]$-15-F_{2t}-IsoP	573	[51,65]
GC-MS/MS				
F_4-NeuroPs	593/323	$[^2H_4]$-$PGF_{2\alpha}$	573/303	[25,62]
AdA metabolites				
GC-MS/MS				
F_2-dihomo-IsoPs	597.5	$[^2H_4]$-15-F_{2t}-IsoP	573	[23]
GC-MS/MS				
F_2-dihomo-IsoPs	597/327	$[^2H_4]$-$PGF_{2\alpha}$	573/303	[24]

The PFB-TMS or PFB-MO-TMS derivatives of several series of IsoPs, NeuroPs and PhytoPs utilize usually methane as reactant gas and produce mass spectra poor in fragmentations. The spectra are dominated by an intense single ion, the carboxylate anion $[M-PFB]^-$ which is formed by cleavage of a highly stabilized PFB radical that follows the capture of an electron by the strong electron capturing PFB moiety of PFB ester. Characteristic $[M-PFB]^-$ fragments of oxygenated metabolites derived from n-3 and n-6 PUFAs such as F_2-IsoPs, F_2-dihomo-IsoPs, F_3-IsoPs, F_4-NeuroPs and F_1-PhytoPs in human fluids and tissues are summarized in Table 1.

3.3.3. *Metabolites assessed in biological samples*

3.3.3.1. F_2-isoprostanes. Several GC–MS methods have been applied to a variety of biological sources including urine, plasma, tissue (brain, liver, kidney, colon), CSF, sputum, skin, erythrocytes and fungi [4,39,51–53] (Tables 2a and 2b). GC–NICI-MS analysis of F_2-IsoPs was first developed by Morrow et al. [5] in human plasma and urine, and rat plasma and kidney. In this method, F_2-IsoPs are determined by SIM of m/z 569 for endogenous F_2-IsoPs and m/z 573 for the internal standard d_4-F_2-IsoP. The GC–MS method by Morrow et al. [5] is one of the most widely used methods for F_2-IsoPs to measure lipid peroxidation and oxidative stress in various clinical studies, mainly vascular related diseases such as atherosclerosis and stroke, and neurodegenerative diseases such as Parkinson and Alzheimer diseases [51,54,55]. In addition, the use of GC–MS for the measurement of F_2-IsoPs and other E_2/D_2-IsoPs has also helped to elucidate the biological activities of these products in humans [50].

In 1996, Roberts et al. [56] showed that the major urinary metabolite of 15-F_{2t}-IsoP in human is 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP. Subsequently, Chiabrando et al. [57] found one more metabolite in human urine, 2,3-dinor-15-F_{2t}-IsoP. The sensitive and accurate GC–NICI-MS method developed for IsoPs further allowed the identification and quantitation of these compounds in urine, which are present in higher amounts than the parent F_2-IsoPs [57,58]. A rapid approach to quantify simultaneously urinary 15-F_{2t}-IsoP and its metabolites 2,3-dinor-15-F_{2t}-IsoP and 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP prior to final quantitation by GC–NICI-MS was also shown by Nourooz-Zadeh et al. [59]. Measurement of free F_2-IsoPs in urine can be confounded by the potential contribution of local F_2-IsoPs production in the kidney [51]. Therefore, urinary measurement of the systemic oxidation status in mammals could be best represented by the analysis of these F_2-IsoP metabolites as demonstrated by Dorjgochoo et al. [60]. Their results further suggest that 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP may be a more sensitive marker of endogenous oxidative stress status than F_2-IsoPs in the assessment of antioxidants' effects on age-related diseases.

Measurements of F_2-IsoPs by GC–NICI-MS are not limited to biological fluids such as plasma and urine. It has been determined in sputum to investigate oxidative stress and pulmonary disorders, in amniotic fluids in pre-eclampsia pregnancy and in CSF in

Table 2a
Metabolites measured in biological fluids by GC-MS and GC-MS/MS in some studies.

Sample	Study	Organism	Sample preparation	Precursor fatty acid	Precursor/product ion *m/z*	Metabolite	Baseline level[a]	Reference
Plasma Urine	Healthy	Human	SPE: MAX	AA	569	F_2-IsoPs	239 ± 21 pg/mL 502 ± 404 pg/mg Cr	[38]
Plasma Urine	Healthy	Human	LLE and SPE: Si	ALA	545	F_1-PhytoPs *Plasma* *Urine*	 <LOD <LOD	[112]
Plasma Urine	Flaxseed oil supplementation to healthy subjects	Human	SPE: Certify II	AA	569	F_2-IsoPs *Plasma* *Urine*	 1.99 ± 0.17 nmol/L 10.30 ± 1.54 nmol/24 h	[33]
				ALA	543	F_1-PhytoPs *Plasma* *Urine*	 4 nmol/L[b] 275 nmol/24 h[b]	
Plasma Urine	Overweight Dislipidemia Type 2 diabetes	Human	SPE: Certify II	AA	569	F_2-IsoPs *Plasma* *Urine*	 207.1 ± 7.8 pg/mL 2100 pg/mg Cr	[108,109]
Plasma Urine	Age-matched control and Parkinson's disease (stage 1)	Human	SPE: MAX	AA	569	F_2-IsoPs Control *Plasma* *Urine* PD *Plasma* *Urine*	 0.36 ± 12 ng/mL 0.54 ± 0.27 ng/mg Cr 0.76 ± 0.30 ng/mL 0.57 ± 0.26 ng/mg Cr	[113]
				DHA	593	F_4-NeuroPs Control *Plasma* PD *Plasma*	 0.45 ± 0.25 ng/mL 0.67 ± 0.24 ng/mL	
Plasma Urine	Healthy	Human	SPE: C-18, Si TLC	AA	569	F_2-IsoPs *Plasma* *Urine*	 35 ± 6 pg/mL 1600 ± 600 pg/mg Cr	[4]
Plasma	CCl_4 treatment	Rat	SPE: C-18 TLC	AA	569	F_2-IsoPs	430 ± 70 pg/mL	[8]
Plasma	Healthy Dengue fever Parkinson's disease Ischemic stroke	Human	SPE: MAX	AA	569	F_2-IsoPs *Healthy* *Dengue fever* *PD* *Ischemic stroke*	 0.58 ± 0.29 ng/mL 0.59 ± 0.22 ng/mL 0.72 ± 0.28 ng/mL 0.94 ± 0.36 ng/mL	[55]
Plasma	Rett syndrome	Human	SPE: C-18, NH_2	AA AdA	567/297 569/299	F_2-IsoPs F_2-dihomo-IsoPs	144 pg/mL 1.0 ± 0.11 pg/mL	[24,25,62,114]
				EPA DHA	593/323 597/327	F_3-IsoPs F_4-NeuroPs	15.0 ± 10 pg/ml[b] 1.0 pg/mL	
Urine	Polytraumatized patients	Human	SPE: C-18, NH_2	AA	569	F_2-IsoPs	811 ± 359 pg/mg Cr	[107]
Plasma Cord blood	Pregnant women Pre-eclampsia	Human	SPE: Certify II	AA DHA	569 593	F_2-IsoPs F_4-NeuroPs	727 pg/mL 731 pg/mL	[67]
Urine	Healthy	Human	SPE: Certify II	AA	569	F_2-IsoPs	1904 ± 94 pg/mg Cr	[36]
Platelets	Human platelets aggregation	Human	SPE: Certify II	AA	569	F_2-IsoPs 8-F_{2t}-IsoP	50 fmol[b] 250 fmol[b]	[115]
CSF	Aneurismal subarachnoid hemorrhage	Human	SPE: C-18, Si TLC	DHA	593	F_4-NeuroPs	77.8 ± 19.5 pg/mL	[51]
CSF	Inspired oxygen during limb surgery	Human	SPE: Certify II	AA DHA	585 593	F_2-IsoPs F_4-NeuroPs	400 pmol/L[b] 300 pmol/L[b]	[65]
Sputum	Inflammatory airway diseases	Human	C18 Sep-Pak Cartridge	AA	569	F_2-IsoP	Not reported	[116]

AA: arachidonic acid; AdA: adrenic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; PD: Parkinson's disease; SPE: solid phase extraction; LLE: liquid-to-liquid extraction; MAX: mixed anionic exchange; Certify II: mixed mode/strong anionic exchange; TLC: thin layer chromatography; Cr: creatinine.

[a] Baseline values indicate healthy group, age-matched group, pre-treatment prior intervention or initial stage of disease development.

[b] Approximate reading from graphical display reported in the literature.

Table 2b
Metabolites measured in biological cells and tissues by GC-MS and GC-MS/MS in some studies.

Sample	Study	Organism	Sample preparation	Precursor fatty acid	Precursor/product ion *m/z*	Metabolite	Baseline level[a]	Reference
Liver Kidney	Effects of age and caloric restriction	Rat	SPE: C-18, Si TLC	AA	569	F_2-IsoPs *Liver* *Kidney*	 10.34 ± 0.85 ng/g 5.04 ± 0.49 ng/g	[117]
Brain Liver, erythrocytes	Antioxidants	Mouse	SPE: C-18, NH_2	AA	481	F_2-IsoPs *Brain* *Liver* *Erythrocytes*	 29.78 ng/g 22.08 ng/g 4.68 ng/mL	[39]
Liver Liver	Hypercholesterolemia PGs formation *via* IsoPs pathway	Rabbit Rat	SPE: ion exchange SPE: C-18, Si, HPLC	AA AA	569 524	F_2-IsoPs PGE_2/D_2	1.3 ± 0.3 ng/mg 1240 ± 450 ng/mg arachidonate	[118] [119]
Brain	Oxidative stress induction with oxygen	Pig	SPE: C-18, Si	AA DHA	569 593	F_2-IsoPs F_4-NeuroPs	6.05 ± 2.0 ng/g 23.27 ± 6.6 ng/g	[120]
Brain	Alzheimer's disease	Mouse Human	SPE: C-18, Si TLC	DHA	593	F_4-NeuroPs *Mouse brain* *Human brain*	 8.7 ± 2.0 ng/g 4.9 ±0.6 ng/g	[68]
Brain	Alzheimer's disease	Human	SPE TLC	AA	569	F_2-IsoPs *Frontal* *Temporal* *Occipital* *Cerebellum*	 159 ± 36 pg/mg 154 ± 27 pg/mg 110 ± 15 pg/mg 118 ± 20 pg/mg	[54]
Brain	Healthy	Human	SPE: C-18, Si TLC	AA DHA AdA	569 593 597	F_2-IsoPs F_4-NeuroPs F_2-dihomo-IsoPs	1.5 ± 0.1 ng/g 11.3 ± 0.6 ng/g 14.5 ± 0.5 ng/g	[23]
Skin microdialysis	Healthy female	Human	SPE: C-18	AA	569 569	F_2-IsoPs 9α-11α-$PGF_{2\alpha}$	0.28 ± 0.06 pmol/mL 0.65 ± 0.08 pmol/mL	[52]
Catfish epidermal secretion	Healthy	Fish	LLE TLC	AA	569	F_2-IsoPs *Male* *Female*	 4.17–5.63 ng/mL 3.60–3.81 ng/mL	[121]
Aspergillus fumigatus	Production of eicosanoids	Fungus	IAC	AA	569/299 524/268 569/299 524/268 524/268	15-F_{2t}-IsoP 15-E_{2t}-IsoP $PGF_{2\alpha}$ PGE_2 PGD_2	<1–5.6 pg/mL <1 pg/mL <1 pg to 17.7 pg/mL <1 pg/mL <1 pg/mL	[53]

AA: arachidonic acid; AdA: adrenic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; SPE: solid phase extraction; LLE: liquid-to-liquid extraction; TLC: thin layer chromatography; IAC: immunoaffinity column.

[a] Baseline values indicate healthy group, age-matched group, pre-treatment prior intervention or initial stage of disease development.

aneurysmal subarachnoid hemorrhage (Table 2a). Various biological tissues have been analyzed for F_2-IsoPs, mainly animal liver and brain are investigated in toxicological studies and neurodegenerative diseases respectively. Both liver and kidney tissues of rats were also analyzed to understand the effect of age and calorie restriction and its relationship to DNA oxidation. Analysis of F_2-IsoPs in biological samples in recent years further expanded in human skin after UV light exposure, in fish epidermis and even in fungi (Table 2b).

3.3.3.2. F_2-dihomo-isoprostanes. In 2008, VanRollins et al. [23] described the formation of F-ring IsoPs from adrenic acid (AdA, C22:4, n-6). These compounds were named F_2-dihomo-IsoPs. AdA, like DHA, is highly enriched in the brain but is primarily found in white matter and is associated with oxidative stress in the myelin portion. From the results provided by GC–MS analysis, it was deduced that these compounds could be considered as indicators of oxidative stress-driven white matter damages in human brain. For example, phospholipids derived from human cerebral gray matter, white matter, and myelin were similarly oxidized *ex vivo* and showed the ratio of esterified F_2-dihomo-IsoPs to F_4-NeuroPs approximately 10-fold greater in myelin-derived than in gray matter-derived phospholipids. The study suggests that quantification of dihomo-IsoPs may be a selective marker of white matter injury *in vivo*. De Felice et al. [24] systematically investigated the impact of AdA and non-enzymatic peroxidation in plasma of Rett patients (Table 2a). For this study two dihomo-IsoPs, 7- and 17-F_2-dihomo-IsoPs, were synthesized by the Durand team [61], and accurately measured by GC–NICI-MS(MS) based on their previous method [62] after SPE (C18 and NH_2 cartridge) and standard derivatization steps. It was found that average plasma F_2-dihomo-IsoP levels in Rett syndrome were approximately two orders of magnitude higher at early stage of the disease followed by a steady decrease during the natural clinical progression [24] (Table 2a).

3.3.3.3. F_3-isoprostanes. The development of F_3-IsoPs *in vivo* from EPA was identified and reported by Nourooz-Zadeh et al. [26] and Gao et al. [28]. Quantitation of total F_3-IsoPs level is performed by determining merged peaks of *m/z* 567 signal and using d_4-F_2-IsoP as SIL-IS in GC–NICI-MS analysis. Since physiological EPA concentration is very low, endogenous level of F_3-IsoPs in tissues of animals and humans is reported to be approximately <30 pg/g of tissue only. Apart from the above two reports very little information [25] is available concerining assessing F_3-IsoPs levels by using GC–NICI-MS.

3.3.3.4. Neuroprostanes. Docosahexaenoic acid (DHA, C22:6, n-3) is concentrated in the brain and enriched in neurons; therefore,

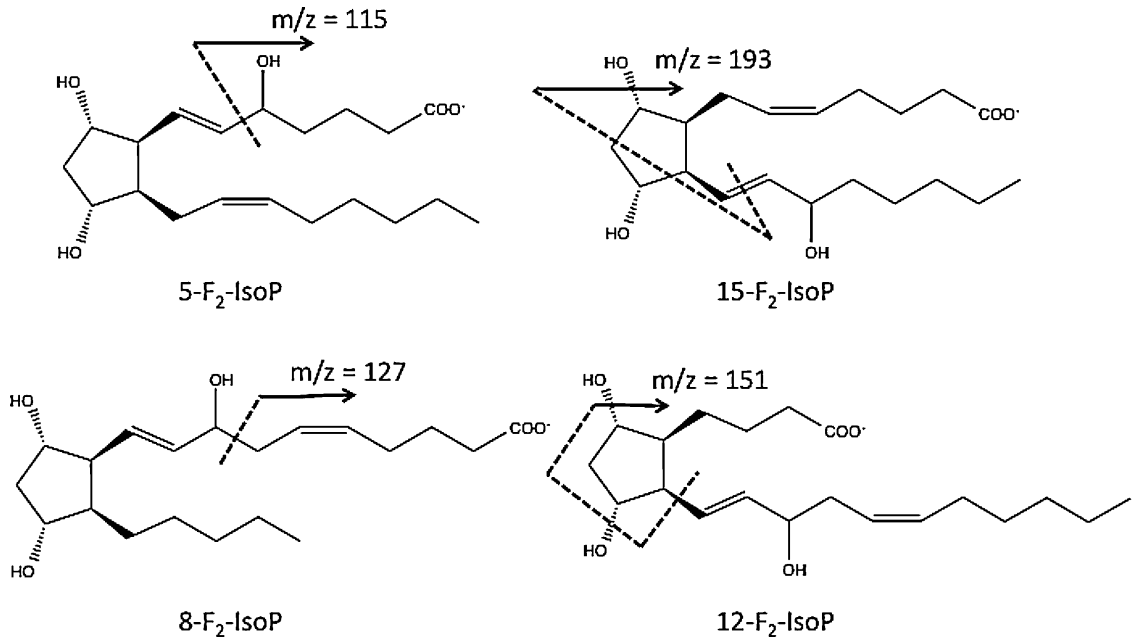


Fig. 2. Characteristic fragmentation of four series of F_2-IsoPs.

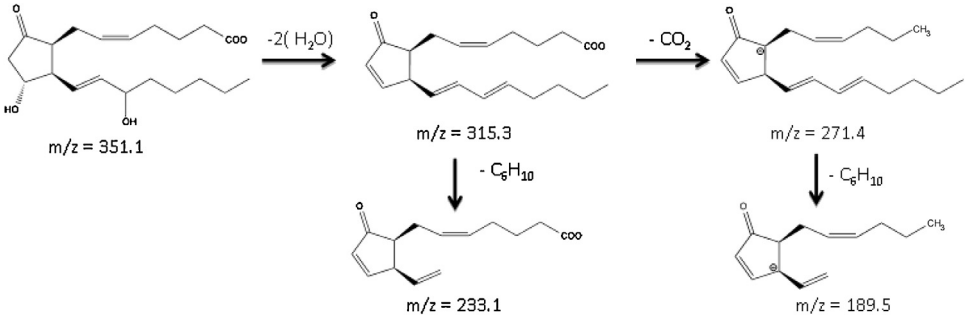
F_4-NeuroPs are considered as the most selective indicator for oxidative stress and neurodegenerative diseases [55]. In 1998, Roberts et al. [63] showed that the presence of F_4-NeuroPs has no counterpart in the PG family (in contrary to AA, EPA and AdA). They showed that the metabolite is purely from free radical-mediated lipid peroxidation of DHA, both *in vitro* and *in vivo*, and measurable with the same GC–NICI-MS method as F_2-IsoPs [63]. F_4-NeuroPs is determined by merged peaks of *m/z* 593 signal and using [$^{18}O_2$]-17-F_{4C}-NeuroP, [$^2H_4$]-15-F_{2t}-IsoP or [$^2H_4$]-PGF$_2\alpha$ as SIL-IS in GC–NICI-MS analysis.

It was found by Nourooz-Zadeh et al. [64] that the levels of F_4-NeuroPs were 4-fold higher than F_2-IsoP levels in plasma of Alzheimer disease (AD) patients. Hsieh et al. [51] and others [65] detected F_2-IsoPs and F_4-NeuroPs in CSF using [$^2H_4$]-15-F_{2t}-IsoP as IS and analyzed by GC–NICI-MS in aneurysmal subarachnoid hemorrhage (aSAH) patients. Their results suggest that F_4-NeuroPs in CSF could be a better predictor for outcome of aSAH than F_2-IsoPs at early stage. They also indicated that the analysis of F_4-NeuroPs that use [$^{18}O_2$]-17-F_{4c}-NeuroP as SIL-IS could potentially interfere with F_2-dihomo-IsoPs chromatographic peak in CSF samples [51,66]. Nevertheless, the same GC–NICI-MS method was also applied to elucidate the role of F_2-IsoPs, IsoFs and F_4-NeuroPs in pre-eclampsia and normal pregnancy maternal plasma and cord blood plasma [67] (Table 2a). Again, measurement of F_4-NeuroPs is not restricted to biological fluids. Results by Musiek et al. [68] showed an increase of 2.1-fold of F_4-NeuroPs in the temporal lobe of AD patients' brain compared to that in control samples. They developed an assay using synthesized [$^{18}O_2$]-17-F_{4c}-NeuroP as SIL-IS and showed low limit of quantification at approximately 10 pg by GC–NICI-MS analysis.

Signorini et al. [69] also developed a GC–MS/MS method to quantify F_4-NeuroPs; extraction, purification and derivatization procedures of F_2-IsoPs and F_4-NeuroPs from brain tissue were adapted from Nourooz-Zadeh et al. method [64], then compounds were analyzed by GC–NICI-MS(MS) using [$^2H_4$]-PGF$_{2\alpha}$ as SIL-IS. The MS/MS spectrum of the brain sample was dominated by ions produced by loss of trimethylsilyl hydroxide (HOTMS). Evidently, ions at *m/z* 503, 413 and 323 were attributable to elimination of one, two or three HOTMS groups, respectively, from the ions [M−PFB]$^-$ of F_4-NeuroPs. Similar fragmentation and neutral loss of HOTMS were reported for the SIL-IS (*m/z* 303) and F_2-IsoPs (*m/z* 299). With this GC–NICI-MS(MS) method, the detection limit was approximately 10 pg/mL for plasma [62].

Reports also showed that E_4-NeuroPs and D_4-NeuroPs can be detected by GC–NICI-MS in normal rat and human brains [70], and interestingly the F-ring to D/E-ring ratio for NeuroPs, but not IsoPs was 40–70% lower in all brain regions of AD patients compared to controls [71]. Indeed, the levels of E_4/D_4-NeuroPs in normal brain are found to be one-third compared to levels of F_4-NeuroPs [72]. J_4/A_4-NeuroPs were also analyzed by GC–NICI-MS with [$^2H_4$]-PGA$_2$ as SIL-IS after SPE and derivatization to PFB-MO-TMS compounds [73]. The major ions present in the NICI mass spectra of A_4/J_4-NeuroPs, and the [$^2H_4$]-PGA$_2$ derivatives are the carboxylate anions at *m/z* 458 and *m/z* 438, respectively.

3.3.3.5. Phytoprostanes. In 1998, Parchmann and Mueller [74] were the first ones to discover the formation of dinor-E_1-IsoP from ALA in plants. These compounds named E_1-phytoprostanes (E_1-PhytoPs) were initially analyzed by GC–EI-MS after derivatization to the PFB ester and TMS ether derivatives. Thereafter, the use of GC–NICI-MS and [$^{18}O_1$]-B_1-PhytoP as SIL-IS enabled the separation of the two regioisomers of B_1-PhytoPs chromatographically. Following this discovery, Imbusch and Mueller [32] showed F_1-PhytoPs could be quantified by separating F_1-PhytoPs to the acyclic triols (derived from both enzymatic and non-enzymatic oxidation of linolenate in plants) since these compounds had identical chromatographic and mass spectrometric properties with same molecular weight. The only difference between the two types is that F_1-PhytoPs have one double bond compared to two for acyclic compounds. Thus, during the sample preparation step, and after SPE extraction, the eluate was hydrogenated before derivatization. In this report, the most intense ion identified is the [M−PFB]$^-$ without double bond at *m/z* 545. Nevertheless, A_1-PhytoPs and B_1-PhytoPs quantification include two main challenges: (1) the A-ring-to-B-ring isomerization at higher temperature (GC injector port) and (2) the low derivatization ability in combination with isotope SIL-IS becomes unstable during analysis. Consequently, Thoma et al. [75] proposed this measurement in two steps using GC–NICI-MS. The first analysis conduces to the determination of the A_1-PhytoPs and B_1-PhytoPs sum by using [$^{18}O_1$]-B_1-PhytoP as SIL-IS. The second



Scheme 3. Characteristic product ions of E_2-IsoP fragmentations.

analysis, performed after methyloximation of the keto function, is to prevent isomerization of A_1-PhytoP into B_1-PhytoP. From the combined information of the two experiments, A_1-PhytoP and B_1-PhytoP respective quantitation could be calculated.

PhytoP levels were also measured in human blood and urine before and after plant oil consumption [34]. Interestingly, it was found that total F_1-PhytoPs circulating in plasma levels were below the limit of detection of GC–MS analysis but urinary F_1-PhytoP was elevated. In 2009, Barden et al. [33] analyzed simultaneously levels of F_2-IsoPs and F_1-PhytoPs in plasma and urine of male subjects to investigate the effect of flaxseed oil supplementation (Table 2a). They developed a facile extraction/separation method with SPE before derivatization for GC–NICI-MS analysis. The F_1-PhytoPs are measured by monitoring *m/z* 543 signal and comparing to the SIL-IS [$^{18}O_1$]-F_1-PhytoP. The results showed that after four weeks supplementation with flaxseed oil, plasma ALA phospholipids were significantly increased and highly correlated with increased F_1-PhytoPs in plasma and urine. Although PhytoPs have been reported to possess a broad spectrum of biological activities in different plant species [76], further studies are required to determine physiological activities in human diseases.

3.4. LC–MS and LC–MS/MS analysis

LC is able to separate different kinds of oxygenated metabolites of PUFAs and allows multiple compound evaluation in one analysis. To date, there is no report on the levels of PhytoPs measured by LC. In the following part, we will not distinguish performances between high-pressure liquid Chromatography (HPLC), ultra high-pressure liquid chromatography (UHPLC), micro- or nano-flow HPLC, but we will concentrate on their respective use.

Sample preparation procedures, such as extraction, derivatization or purification, are often critical and time-consuming and may lead to a substantial loss of target compounds, or transformation of them by *ex vivo* autoxidation. Compared to GC, LC presents the advantage to omit derivatization steps and also no compound vaporization for elution is necessary, thus limiting the risk of deterioration for thermosensitive compounds. With LC methodology, sample preparation usually consists of a single extraction step, so analyte recovery may be higher by one order of magnitude [36,37]. However, derivatization of eicosanoids in LC–MS/MS may improve sensitivity as outlined below. Because the sample preparation protocols are relatively generic, it is easy to analyze metabolites across multiple classes resulting in even greater timesaving as shown by Massey [77] or Shinde [78]. However, strong matrix effect and signal suppression can be observed. Unfortunately, these parameters are not systematically studied and reported in the literature. A report by Prasain et al. [46] indicated a total recovery yield of 86% for urinary eicosanoids, after accounting for extraction, matrix and suppression ion effects. Also, Sterz et al. [79] reported that liquid/liquid extraction procedure could replace SPE in UPLC–MS analysis of urinary eicosanoids.

3.4.1. Ionization and fragmentation

For lipid analysis, electrospray ionization (ESI) is widely used and usually operated in negative ionization mode which generates carboxylate ions $[M-H]^-$ from any "free" IsoPs. An alternative to ESI is the atmospheric pressure chemical ionization (APCI) which minimizes matrix effect such as ion suppression [48,80]. From an analytical point of view, also shown by others, the four classes of F_2-IsoPs produce the same intermediate ion (carboxylate anion) at *m/z* 353, under ESI conditions in the negative-ion mode [50]. After collision-induced dissociation of the molecular ion, product ions below *m/z* 200 differ between various isomers: *m/z* 115 for 5-F_{2t}-IsoP, *m/z* 127 for 8-F_{2t}-IsoP, *m/z* 151 for 12-F_{2t}-IsoP and *m/z* 193 for 15-F_{2t}-IsoP [50] (Fig. 2). These intermediate transitions allow selected-reaction monitoring (SRM) of *m/z* 353 (parent ion) to *m/z* 193 (product ion). A characteristic SRM for 15-series F_{2t}-IsoPs is shown in Fig. 2. Thus, SRM can be employed to differentiate between the four regioisomers. Although studies of IsoPs formation have mainly focused on F_2-IsoPs, other IsoPs such as E- and D-IsoPs have been investigated as well [81]. However, only few groups have developed LC–MS or LC–MS/MS methods for the study of these metabolites which share two main specific multiple reaction monitoring (MRM) transitions *m/z* 351 → 271 and *m/z* 351 → 189, but also the additional MRM transitions *m/z* 351 → 233, *m/z* 351 → 315, and *m/z* 351 → 333 (Scheme 3) [37,82,83]. One limitation of LC–ESI-MS(MS) is the missing sensitivity which is in the 10–20 pg range. Charge reversal derivatization was tested [84–86] by which the carboxylic acid is converted to an amide bearing a permanent positive charge [84–86]. Recent improvement by Bollinger et al. [87] highlighted the use of commercially available AMPP (*N*-(4-aminomethylphenyl)pyridinium). In combination with LC–ESI-MS(MS) in positive ion mode, this derivatization method improved the sensitivity for eicosanoids by one order of magnitude compared to standard protocols.

3.4.2. Analytical application in biological samples

3.4.2.1. F_2-isoprostanes. In the firstly reported LC–MS/MS study on the separation and identification of F_2-IsoP regioisomers and diastereoisomers, Yin et al. [88] showed that the 15-series and the 5-series of IsoPs diastereoisomers with *cis*-alkyl chains on the prostane ring are formed in greater amount than those with *trans*-configuration. Precisely, these diastereoisomers are also formed to a greater extent than the 8- and 12-compounds probably because only the transient peroxyl radical intermediates of the 8- and 12-series of F_2-IsoPs can form a novel class of IsoPs termed dioxolane IsoPs [21]. For the past decade, interest in F_2-IsoPs measurement by LC–MS/MS and more recently by UHPLC–MS/MS has grown, especially in 15-F_{2t}-IsoP determinations. While it is not the most abundant isoprostanes in human urine [89], it is the most

Table 3a
Metabolites measured in biological fluids by LC-MS and LC-MS/MS in some studies.

Sample	Study	Organism	Sample preparation	Precursor fatty acid	$[M-H]^-$ m/z	Metabolite	Baseline level[a]	Reference
Plasma Urine Full blood	Healthy	Human	SPE: C-18	AA	353	15(RS)-15-F_{2t}-IsoP *Plasma* *Urine* *Full blood*	 40–170 pg/mL 30–303 ng/g Cr 1–2 ng/mL	[122]
Plasma	Normotensive pregnant women	Human	LLE	AA	353 353 353 353 353 353 353	15-F_{2t}-IsoP 15(R)-15-F_{2t}-IsoP 15(R)-$PGF_{2\alpha}$ 8-F_{2t}-IsoP 5-F_{2t}-IsoP 5(R)-5-F_{2t}-IsoP 5(RS)-5-F_{2c}-IsoP	195 pg/mL 177 pg/mL 338 pg/mL 137 pg/mL 416 pg/mL 393 pg/mL 879 pg/mL	[123]
Plasma	Aspirin administration in male healthy subjects	Human	SPE: C-8	AA	353	15-F_{2t}-IsoP (32 arachidonic acid related metabolites)	52 ± 26 pg/mL	[78]
Urine	Smokers	Human	SPE: C-18/hydro	AA	353 325 327	15-F_{2t}-IsoP 2,3-Dinor-15-F_{2t}-IsoP 2,3-Dinor-5,6-dihydro-15-F_{2t}-IsoP	200–1500 pg/mg Cr 3000–5000 pg/mg Cr 1000–3000 pg/mg Cr	[124]
Urine	Cardiac surgery	Human	SPE: polymeric weak anion exchange	AA	353 353	15-F_{2t}-IsoP 15(R)-15-F_{2t}-IsoP	395 ± 51 pg/mg Cr 477 ± 81 ng/mg Cr	[46]
Urine	Smokers	Human	LLE SPE: C-18/polymeric/weak/strong anion exchange	AA	353 325	15-F_{2t}-IsoP 2,3-Dinor-15-F_{2t}-IsoP	178 pg/mg Cr 1080 pg/mg Cr	[79]
CSF	Neuro-degenerative disorders	Human	SPE: C-18	AA	353 353	15-F_{2t}-IsoP 5-F_{2t}-IsoP	5–80 pg/mL 12.5–250 pg/mL	[91]
Exhaled breath condensate	Asbestosis	Human	SPE: IAC	AA	353	15-F_{2t}-IsoP	36 pg/mL	[125]
Exhaled breath condensate	Smokers	Human	No preparation	AA	353	15-F_{2t}-IsoP 15(R)-15-F_{2t}-IsoP PGs	<LOD <LOD <LOD	[126]
Amniotic fluid	Preeclampsia	Human	HPLC C-18 partition	AA	353	15-F_{2t}-IsoP	73.8 ± 36.6 pg/mL	[97]

AA: arachidonic acid; AdA: adrenic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; SPE: solid phase extraction; LLE: liquid-to-liquid extraction; TLC: thin layer chromatography; IAC: immunoaffinity column; Cr: creatinine.

[a] Baseline values indicate healthy group, age-matched group, pre-treatment prior intervention or initial stage of disease development.

thoroughly investigated F_2-IsoPs [90] and it is assessed in other biological fluids such as urine, plasma, CSF and EBC (Table 3a). Analogous to GC–MS, tissue samples obtained from the brain, liver, kidney, *Caenorhabditis elegans*, fish muscle and carcinoma cells have been analyzed by LC–MS/MS in various studies (Table 3b). Human brain and liver samples were also analyzed by LC–MS/MS in different studies [91].

3.4.2.2. F_3-isoprostanes. Only a few papers describe the LC–MS/MS measurement of F_3-IsoPs formed from the oxidation of EPA *in vivo*. In 2006, Lawson et al. [29] proposed LC–MS/MS quantification of 5-F_{3t}-IsoP in human urine, and they discovered the β-oxidation of 7-F_{4t}-NeuroP to 5-F_{3t}-IsoP. The same year, other groups have identified F_3-IsoPs in heart tissues of EPA-supplemented mice [28] and showed that the 5- and 18-series F_3-IsoPs are the most abundant type *in vivo*. More recently the same group has demonstrated the generation of A_3- and J_3-IsoPs from the oxidation of EPA using an almost identical approach [92]. Furthermore, the Rokach group [27,30] showed that the *all-syn* stereoisomer, 5-F_{3c}-IsoP and its 5-epimer are the major F_3-metabolites in human urine. In other biological samples, Chung et al. [93] quantified F_3-IsoP in toxicological study of marine fish, and Leung et al. [42] assessed it in sonoporated Jurkat cells. A recent study using UHPLC–MS/MS methodology for quantification of endogenous oxidative damage in the nematode *C. elegans* found F_3-IsoPs as the best biomarker for lipid peroxidation by ROS [40,45] (Table 3b).

3.4.2.3. F_4-neuroprostanes. Initially, LC–MS/MS studies on F_4-NeuroPs mainly focused on structural information, specifically the regiochemistry of F_4-NeuroPs formation after autoxidation of DHA [31]. In addition to F_4-NeuroPs, the highly unstable E_4- and D_4-NeuroPs which lead to the highly reactive A_4- and J_4-NeuroPs were also investigated using a variety of complementary chemical (GSH adducts formation) and MS approaches using LC–ESI-MS(MS) but mainly for identification rather than quantification [71,73]. Neuroprostanes have been measured in mice and rat brains in studies related to neurodegenerative disorders [82,83]. Chung et al. [93] showed F_4-NeuroPs can be quantified by LC–MS/MS in marine fish muscle and in Jurkat cells by Leung et al. [42] (Table 3b).

Table 3b
Metabolites measured in biological cells and tissues by LC-MS and LC-MS/MS in some studies.

Sample	Study	Organism	Sample preparation	Precursor fatty acid	$[M-H]^-$ m/z	Metabolite	Baseline level[a]	Reference
Kidney Liver	CCl_4 treatment	Rat	LLE	AA	353	15-F_{2t}-IsoP *Kidney* *Liver* 5-F_{2c}-IsoP *Kidney* *Liver*	 6.5 ng/g 8.4 ng/g 20.6 ng/g 43.9 ng/g	[127]
Liver	Hepatitis C or B virus infected patient	Human	LLE	AA	353	15-F_{2t}-IsoP	24.78 ng/g	[13]
Brain	Ischemia	Rat	SPE: HLB	AA	351	E_2/D_2-IsoPs	1300 ± 317 pg/g	[82]
Brain	Neuro-degenerative disorders	Human	SPE: C-18	AA	353 353	15-F_{2t}-IsoP 5-F_{2t}-IsoP	30–70 pg/0.1 g 60–150 pg/0.1 g	[91]
Brain	Ischemia	Mouse	LLE	AA	351	15-E_{2t}-IsoP E_2/D_2-PGs	1400 pg/gww	[83]
Whole animal	Wild or mutant type	Nematode *C. elegans*	LLE SPE: NH_2	EPA	351	F_3-IsoPs	10 pg/μg protein[b]	[40]
Muscle tissue	Hydrogen peroxide treatment	Medaka fish	SPE: MAX	AA	353	15-F_{2t}-IsoP *Male* *Female*	 1.88 ± 0.11 ng/g 3.18 ± 0.21 ng/g	[93]
					325	2,3-Dinor-15-F_{2t}-IsoP *Male* *Female*	 2.40 ± 0.37 ng/g 9.70 ± 0.19 ng/g	
					327	2,3-Dinor-5,6-dihydro-15-F_{2t}-IsoP *Male* *Female*	 119.80 ± 9.47 ng/g 50.0 ± 6.22 ng/g	
				EPA	351	F_3-IsoPs *Male* *Female*	 0.86 ± 0.07 ng/g 2.46 ± 0.26 ng/g	
				DHA	377	F_4-NeuroPs *Male* *Female*	 0.84 ± 0.18 ng/g 0.55 ± 0.06 ng/g	
Carcinoma cell line	Pro- or antioxidant treatment	Human	LLE SPE: NH_2	AA	353 353	15-F_{2t}-IsoP 5-F_{2t}-IsoP	8 pg[b] 50 pg[b]	[128]
Jurkat cell line	Sonoporation	Human	SPE: MAX	AA EPA DHA	353 351 377	15-F_{2t}-IsoP F_3-IsoPs F_4-NeuroPs	2.26 ng/ng arachidonate 0.35 ng/ng eicosa-pentaenoate 0.29 ng/ng docosa-hexaenoate	[42]
Lung epithelial cell line	Nanoparticle	Human	SPE: HLB	AA	353 353	15-F_{2t}-IsoP 15(R)-15-F_{2t}-IsoP	0.0294 ± 0.0007 ng/million cells 0.036 ± 0.0023 ng/million cells	[41]
Erythrocytes	Healthy	Human	SPE: C-18	AA	353	15(RS)-15-F_{2t}-IsoP	0.4–1.1 ng/mL	[122]

ALA: α-linolenic acid; AA: arachidonic acid; AdA: adrenic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; SPE: solid phase extraction; LLE: liquid-to-liquid extraction; MAX: mixed anionic exchange; HLB: hydrophilic–lipophilic-balanced; TLC: thin layer chromatography; Cr: creatinine.

[a] Baseline values indicate healthy group, age-matched group, pre-treatment prior intervention or initial stage of disease development.

[b] Estimated reading from graphical display reported in the literature.

3.5. Immunological methods

Enzyme immunoassay (EIA) and radioimmunoassay (RIA) techniques are based on the same principle, *i.e.*, on the immunological reaction between an antibody that recognizes and binds a specific antigen (analyte) in the sample being tested. The underlying basic principle of immunoassay is competitive binding, where a "tracer" competes with the "analyte" for a fixed number of antibodies. Eicosanoid concentrations are calculated by means of calibration curves which are prepared with the respective synthetic eicosanoid. The type of detection, *i.e.*, the choice of the tracer, makes the only difference between EIA and RIA with the use of an antigen linked to an enzyme able to induce a colorimetric reaction or the use of a radioactive antigen, respectively.

Theoretically, because each antibody is prepared against one specific antigen, only certain metabolites can be measured at a time. In practice, however, because of the large number of different oxidation products formed *in vivo* from lipids, and because of their very slight discrepancies in their structures and properties, cross-reactivity is common. Cross-reactivity can vary from "negligible" (less than 1%) to 10% or even exceptionally high to 20% for some eicosanoids. This issue was investigated in detail by Basu [94] and Wang et al. [95], who determined cross-reactivity for a number of synthetic F_2-IsoPs using EIA and RIA. Klawitter et al. [80] compared the quantification of 15-F_{2t}-IsoPs with enzyme-linked immunosorbent assay (ELISA) with chromatographic methods in human plasma and urine. They noticed that commercially available ELISA kit grossly overestimated the concentration of 15-F_{2t}-IsoP especially in plasma, even after dilution.

EIA, ELISA and RIA are commercially available as kits for many eicosanoids, are easy to use, sensitive (about 23 pM), and allow rapid analysis of a large number of samples within a short time.

In addition, EIA and RIA do not require expensive instrumentation such as GC–MS, GC–MS/MS, LC–MS and LC–MS/MS. EIA and RIA are therefore very often used in large experimental and clinical studies. Immunoassays are widely employed for 15-F_{2t}-IsoP determination, in urine, plasma, CSF or EBC in correlation studies between lipid peroxidation level and oxidative stress due to numerous environmental or pathological conditions including, smoking, air pollution, diabetes, preeclampsia, hypercholesterolemia, atherosclerosis or neurodegenerative disease [96–104]. However, unspecificity due to cross-reactivity and for other poorly recognized reasons represents, besides limitation to particular eicosanoids, the most important disadvantage of EIA and RIA compared to mass spectrometry-based approaches for eicosanoids [129] and other biomarkers of oxidative stress such as 3-nitrotyrosine [130].

4. Future perspectives

GC–MS(/MS) coupled first with EI and later with NICI was essential in discovering the PG-like compounds IsoPs or NeuroPs, cyclic metabolites of n-6 and n-3 PUFAs, respectively, in biological samples, and in studying their *in vitro* and *in vivo* formation, and evaluating the role of this important class of oxidized lipids in human physiology and pathology. Measurement of these compounds in biological samples by GC–MS and GC–MS/MS has allowed defining them as specific biomarkers of lipid peroxidation, to evaluate the extent of oxidative stress related to human diseases, and to define reference values and intervals for IsoPs and NeuroPs. Combined measurement of multiple metabolites increased information in biological systems related to vital organs and oxidative stress status [131,132].

In general, LC–MS/MS is regarded as a more versatile analytical technique compared to GC–MS and GC–MS/MS methodologies. LC–MS/MS will surely be very valuable in future platform technologies for the analysis of oxidative stress biomarkers including IsoPs and NeuroPs. At present, LC–MS/MS seems to take the liberty of dispensing with time-consuming steps in sample preparation including eicosanoids extraction and their subsequent derivatization. However, despite recent great advances in LC–MS/MS instrumentation including ionization and ions transfer, LC–MS/MS has not yet achieved the sensitivity of GC–MS/MS when performed in the NICI mode. To date, many different GC–MS/MS or LC–MS/MS methods are available for PGs, IsoPs and NeuroPs. However, reliable comparison of these techniques with respect to accuracy, one of the most important analytical features in quantitative analysis, has not been performed thus far, but it is warranted. For the time being, the use of LC–MS/MS and GC–MS/MS methods in the analysis of eicosanoids and their oxidatively modified metabolites will continue in parallel. As the older and more experienced approach in eicosanoids analysis, GC–MS/MS may serve as a landmark in the further improvement of the LC–MS/MS methodology.

Immunoassays are less specific than LC–MS/MS and GC–MS/MS assays with regard to current native and oxidatively modified eicosanoids analysis. It is expected that immunoassays will constitute important analytical tools in the analysis of a large number of samples. It is also expected that immunoassays will further improve by generating antibodies of high specificity and affinity for a wider spectrum of eicosanoids. In this context, the chemical synthesis of oxidized lipids in the highest available chemical and optical purity is indispensable.

Oxidative stress is a key feature in a number of human diseases, because ROS are likely to be involved in all disease stages. It is therefore important to identify and evaluate reliably biomarkers that allow us to monitor and quantify oxidative stress status in healthy and diseased individuals. F_2-IsoPs are widely used in biological samples and have been recognized as “gold markers” of non-enzymatic lipid peroxidation. In the future, other non-enzymatic metabolites such as isofuranoids will be important to complete the panel of oxidative stress biomarkers. In this article, we reviewed and discussed the analysis of oxidized lipid metabolites in different biological samples by GC–MS(MS) and LC–MS/MS. To our understanding, LC–MS/MS methods are currently more specific and versatile methods with respect to the quantification of all series of these metabolites, whereas especially GC–MS/MS methods are more sensitive.

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