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Measurement of F_2- isoprostanes and isofurans using gas chromatography–mass spectrometry

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ABSTRACT

F_2-Isoprostanes (IsoPs) are isomers of prostaglandin $F_{2\alpha}$ formed from the nonenzymatic free radical-catalyzed peroxidation of arachidonic acid. Since discovery of these molecules by Morrow and Roberts in 1990, F_2-IsoPs have been shown to be excellent biomarkers as well as potent mediators of oxidative stress in vivo in humans. Isofurans (IsoFs) are also oxidation products generated from the nonenzymatic oxidation of arachidonic acid. IsoFs are preferentially formed instead of F_2-IsoPs in settings of increased oxygen tension. The protocol presented herein is the current methodology that our laboratory uses to quantify F_2-IsoPs and IsoFs in biological tissues and fluids using gas chromatography/mass spectrometry (GC/MS). A variety of analytical procedures to measure F_2-IsoPs, including other GC/MS methods and liquid chromatography/MS and immunological approaches, are reported in the literature. This method provides a very low limit of quantitation and is suitable for analysis of both F_2-IsoPs and IsoFs from a variety of biological sources including urine, plasma, tissues, cerebral spinal fluid, exhaled breath condensate, and amniotic fluid, among others.

Introduction

Free radicals have been implicated in a variety of human diseases [1]. Damage to tissue biomolecules, including lipids, proteins, and DNA, by free radicals is postulated to contribute importantly to the pathophysiology of oxidative stress. Polyunsaturated fatty acids (PUFAs) react readily with free radicals resulting in the formation of a number of oxidation products [2]. The isoprostanes (IsoPs) are a unique series of prostaglandin-like compounds formed in vivo via the nonenzymatic free radical-initiated peroxidation of arachidonic acid and other PUFAs. (For comprehensive reviews on IsoP chemistry and biochemistry, please see Refs. [3,4].) Since discovery of these molecules in 1990, one class of IsoPs, the F_2-IsoPs, has become the biomarker of choice for assessing endogenous oxidative stress as these molecules are chemically stable and easily detectable in biological fluids and tissues [5–7]. Isofurans (IsoFs) are another class of compounds generated from the peroxidation of arachidonic acid [8]. The mechanism of formation of F_2-IsoPs and IsoFs is shown in Fig. 1. As shown, IsoPs and IsoFs are generated via similar mechanisms and, in fact, share a carbon-centered radical (**1**) as an intermediate. To generate IsoPs **1** undergoes 5-*exo* cyclization to form a cyclopentane ring while IsoFs are made by the reaction of **1** with molecular oxygen. Consistent with this mechanism, IsoFs represent a better biomarker of oxidative stress under conditions of increased oxygen tension [9–13].

Described herein is the methodology used in our laboratory to simultaneously quantify IsoPs and IsoFs using gas chromatography–mass spectrometry (GC/MS). While no alternative methodologies to quantify IsoFs have been reported, numerous methodologies for the quantification of F_2-IsoPs have been presented in the literature including alternate GC/MS-based assays as well as liquid chromatography (LC)/MS assays and enzyme immunoassays. (A summary of several MS-based assays reported in the recent literature is presented in Table 1.) Quantification of F_2-IsoPs by MS has distinct advantages compared to analysis by immunoassay methodologies such as ELISA. Although ELISA measurement offers high-throughput analysis and does not require costly instrumentation, the polyclonal antibodies used to bind F_2-IsoPs exhibit cross-reactivity with many other molecules similar in structure, including COX-derived $PGF_{2\alpha}$ [14]. This cross-reactivity results in the quantification of inflated concentrations of F_2-IsoPs. Additionally, biological impurities can interfere with antibody binding. MS offers high sensitivity and specificity yielding quantitative results in the picogram per milliliter range.

The GC/MS protocol described herein is a robust and sensitive methodology that has been utilized for the measurement of F_2-IsoPs for more than 20 years. The sample purification procedure is broad-based and thus allows for the quantification of these molecules, as well as more recently IsoFs, in a variety of

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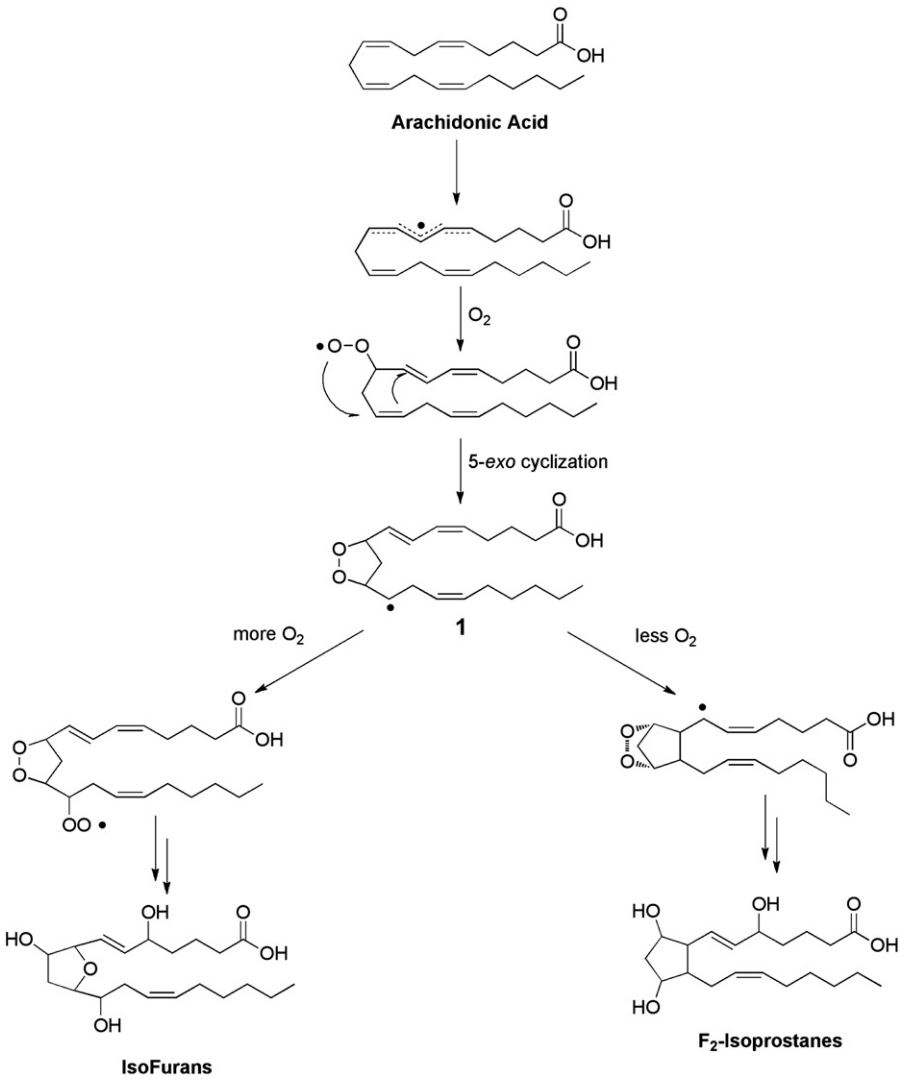

Fig. 1. Mechanism of formation of F_2-IsoPs and IsoFs.

Table 1
Summary of several mass spectrometric methods used to quantify F2-isoprostanes.

Citation	Extraction/preconcentration	Internal standard	Matrix	Analytical method	LOQ
Lee et al., 2004 [15]	Oasis mixed anion exchange SPE (Waters Corporation, Milford, MA)	15-F_{2t}-IsoP-d_4	Urine	GC/MS	0.007 ng/mg Cr urine
		$iPF_{2\alpha}$-VI-d_4	Plasma		0.037 ng/mL plasma
Song et al., 2007 [16]	Strata X SPE (Phenomenex, Torrance, CA)	8,12-*iso*-$iPF_{2\alpha}$-VI-d_4	Urine	LC/MS/MS	NR
Mas et al., 2008 [17]	SepPakVac RC C-18 and NH_2 (Waters Corporation, Milford, MA)	4(RS)-F_{4t}-NP	Urine	GC/MS	NR
Taylor and Traber, 2010 [18]	Strata X SPE (Phenomenex, Torrance, CA)	15-F_{2t}-IsoP-d_4	Plasma	LC/MS/MS	0.051 ng/mL
Mas et al., 2010 [19]	SepPak C-18 and Si and HPLC (Waters Corporation, Milford, MA)	8-F_{2t}-IsoP-d_4	Urine	GC/MS	NR
Smith et al., 2011 [20]	IsoP immunoaffinity sorbent (Cayman Chemical, Ann Arbor, MI)	15-F_{2t}-IsoP-d_4	Urine	LC/MS/MS	0.020 ng/mL
Janicka et al., 2012 [21]	Lyophilization	15-F_{2t}-IsoP-d_4	EBC	LC/MS/MS	0.004 ng/mL
Bastani et al., 2012 [22]	Isolute C-18 SPE (Biotage, Uppsala, Sweden)	15-F_{2t}-IsoP-d_4	DBS	LC/MS/MS	0.018 ng/mL
Medina et al., 2012 [23]	Strata X- AW SPE (Phenomenex, Torrance, CA)	15-F_{2t}-IsoP-d_4	Urine	UPLC/MS/MS	0.042 ng/mL
Sterz et al., 2012 [24]	LLE	15-F_{2t}-IsoP-d_4	Urine	UPLC/MS/MS	0.060 ng/mL

DBS, dried blood spot; EBC, exhaled breath condensate; GC, gas chromatography; IsoP, iPF, isoprostane; LC, liquid chromatography; LLE, liquid-liquid extraction; MS, mass spectrometry; NP, neuroprostane; NR, not reported; UPLC, ultrapressure LC; SPE, solid-phase extraction.

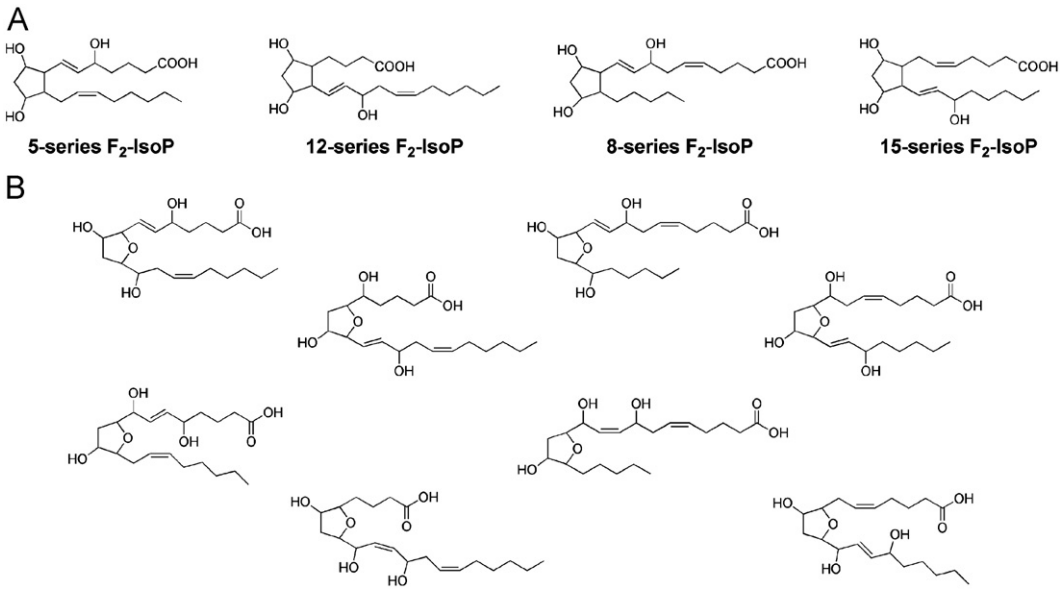

Fig. 2. Structures of F_2-IsoPs and IsoFs.

human and animal tissues and fluids including urine, plasma, tissues, cerebral spinal fluid, exhaled breath condensate, and amniotic fluid, among others.

Principles

As discussed, F_2-IsoPs and IsoFs can be measured in biological samples from a variety of sources to afford an index of in vivo oxidant stress status. The quantification of these compounds, however, is not trivial as 64 different stereoisomers of F_2-IsoPs and 256 stereoisomers of IsoFs are generated from the free radical-catalyzed peroxidation of arachidonic acid (Fig. 2). Two differing nomenclature systems have been described for F_2-IsoPs and a nomenclature system for IsoFs has been developed as well [25–27]. The nomenclature system for F_2-IsoPs used herein is that described by Morrow and Roberts. In this system, the 64 stereoisomers of F_2-IsoPs are classified into four categories based on the position of the hydroxyl (–OH) group on the side chains of the molecule. The 5- and 15-series F_2-IsoPs are formed more abundantly in vivo than the 8- and 12-series compounds and, thus, are typically the isomers quantified. A major confounding factor, however, in regard to all of the MS-based methodologies reported in the literature to quantify F_2-IsoPs, including the one presented herein, is that the particular F_2-IsoP isomers measured by each report varies. Thus, comparison of F_2-IsoPs quantified in different laboratories is not possible. It should be noted that all of the MS-based techniques use stable isotope dilution methods to quantify F_2-IsoPs. Most commonly, 15-F_{2t}-IsoP (also referred to as 8-iso-$PGF_{2\alpha}$ or $iPF_{2\alpha}$-III), one particular 15-series F_2-IsoP formed in abundance in vivo, is used in the deuterated form as an internal standard for quantification. In this nomenclature system, the subscript '2' in 15-F_{2t}-IsoP refers to the number of double bonds in the molecule while the subscript 't' refers to the fact that the stereochemistry of the hydroxyl group bonds is *trans* to the side-chain bonds (Fig. 3).

F_2-IsoPs and IsoFs can be quantified in a variety of human and animal fluids and tissues. Measurement of these molecules in plasma and urine is least invasive and represents global oxidative stress status in vivo. Quantification of F_2-IsoPs and IsoFs in a tissue sample or in a specific fluid such as exhaled breath condensate (EBC) represents local, organ-specific oxidative stress. In tissues arachidonic acid oxidation products are typically esterified in glycerophospholipids, where they are formed. F_2-IsoPs and IsoFs esterified in glycerophospholipids are also present in plasma. Using the GC/MS methodology presented herein, however, F_2-IsoPs and IsoFs can only be quantified in the free acid form. Thus, the glycerophospholipids must first be extracted from the sample and then subjected to alkaline hydrolysis to release the compounds in the free fatty acid form. Importantly, F_2-IsoP and IsoF free fatty acids must undergo chemical derivatization to the pentafluorobenzyl ester, trimethylsilyl either analogs (Fig. 3) prior to injection on the GC/MS in order to optimize ionization and maintain structural integrity during analysis. Sample collection and preparation procedures as well as the GC/MS methodology are described under Protocol below.

Materials

Reagents

- [$^2H_4$]-15-F_{2t}-IsoP (8-iso-$PGF_{2\alpha}$) internal standard (Cayman Chemical; Cat. No. 316351)
- 15-F_{2t}-IsoP (8-iso-$PGF_{2\alpha}$) (Cayman Chemical; Cat. No. 16350)
- Butylated hydroxytoluene (BHT) (Sigma-Aldrich; Cat. No. B1378)
- Pentafluorobenzyl bromide (PFBB) (Sigma-Aldrich; Cat. No. 10105-2)
- *N*-Ethyldiisopropylamine (DIPE) (Sigma-Aldrich; Cat. No. D3887)
- *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Supelco, Inc.; Cat. No. 33084)
- Dimethylformamide (DMF) (Sigma-Aldrich; Cat. No. 6407) (store over calcium hydride to prevent water accumulation)
- Undecane (Sigma-Aldrich, Cat. No. 94000) (store over calcium hydride to prevent water accumulation)
- Triphenylphosphine (Sigma-Aldrich; Cat. No. T8440-9)
- 10% Phosphomolybdic acid in ethanol (Sigma-Aldrich; Cat. No. P4869)
- Ultrapure water (triply distilled or its equivalent)
- Methanol
- Chloroform (containing ethanol as a preservative)
- Ethyl acetate
- Heptane
- Acetonitrile
- Ethanol
- HCl (American Chemical Society certified or equivalent grade)

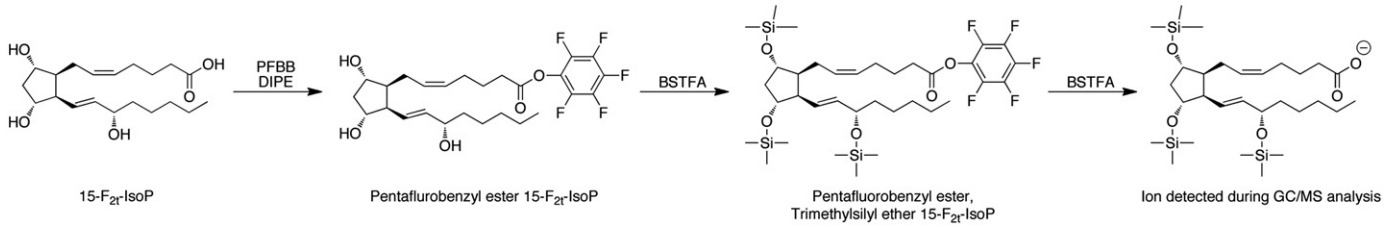

Fig. 3. F_2-IsoPs and IsoFs must undergo chemical transformation to the pentafluorobenzyl ester, trimethylsilyl ether derivatives prior to GC/MS analysis. During the ionization process the pentafluorobenzyl ester is cleaved and the carboxylate anion is the resulting species detected by the mass spectrometer.

- Sodium chloride
- Potassium hydroxide pellets
- Anhydrous sodium sulfate

Equipment

- 15-mL plastic tubes (Denville Scientific; Cat. No. T8173)
- 5-mL plastic vials with cap (Denville Scientific; Cat. No. T8200)
- 50-mL conical centrifuge tubes
- 1.5- to 2-mL microcentrifuge tubes
- Disposable plastic syringes (10 mL) (Laboratory Supply; Cat. No. SMJ512878)
- Nitrogen gas
- SupelcoVisiprep SPE Vacuum Manifold (Sigma-Aldrich; Cat. No. 57265)
- C-18 Plus solid-phase extraction (or Sep-Pak) cartridges, each cartridge contains 500 mg of C-18 (Waters Associates, Milford, MA; Cat. No. WAT036575)
- Silica Plus solid-phase extraction (or Sep-Pak) cartridges, each cartridge contains 500 mg of silica (Waters Associates, Milford, MA; Cat. No. WAT036580)
- TLC paper, cut to be 11″ w × 9″ h (VWR; Cat. No. 28298-020)
- TLC plates: Silica gel HL 250 μm, 5 × 20 cm channeled plates with preadsorbent zone, glass backed (Analtech, Inc., Newark, DE; Cat. No. 43931-2)
- Glass thin-layer chromatography (TLC) tank (11.5″ w × 10″ h × 3.5″ d)
- Analytical evaporation unit with water bath at 37 °C (Organomation Associates, South Berlin, MA; Cat. No. 11634-P))
- Blade homogenizer-PTA 10 S generator (Brinkman Instruments, Westbury, NY)
- Table-top centrifuge (to hold 50-mL conical tubes)
- Microcentrifuge (to hold 1.5- to 2-mL microcentrifuge tubes)
- Aldrich diazomethane generator with System 45 compatible connection (Sigma-Aldrich; Cat. No. Z411736)

Instrumentation

- Capillary gas chromatography column (DB-1701, Agilent; Cat. No. 21512067)
- Gas chromatograph/mass spectrometer (GC/MS, with capabilities for negative-ion chemical ionization (NICI) mass spectrometry)

Protocol

Reagent setup

pH 3 water (0.002 N HCl). Mix 40 mL 1 N hydrochloric acid with 20 L of water.

Folch solution. Combine 2 vol of chloroform with 1 vol of methanol. Dissolve BHT crystals in solution to make a final concentration of 0.005% BHT (w/v). Cool to 4 °C. Solution should be stored in the dark in a brown bottle to prevent light degradation.

PFBB solution. Dilute PFBB to 10% (v/v) in dry acetonitrile. *CAUTION*: *PFBB is a potent lachrymator. Do not use outside of a well-ventilated fume hood.*

DIPE solution. Dilute DIPE to 10% (v/v) in acetonitrile.

Internal standard. The internal standard [$^2H_4$]-15-F_{2t}-IsoP is quantified using a 5-point calibration curve. Each sample contains 0.50 ng of 15-F_{2t}-IsoP, which has been accurately quantified by weighing, and 0.05, 0.25, 0.50, 2.50, or 5.00 ng of [$^2H_4$]-15-F_{2t}-IsoP (amount based on concentration listed on purchased reagent vial). Actual [$^2H_4$]-15-F_{2t}-IsoP/15-F_{2t}-IsoP ratios are compared with the expected ratios in order to quantify the [$^2H_4$]-15-F_{2t}-IsoP.

TLC standard ($PGF_{2\alpha}$ methyl ester). Dissolve 1 mg of $PGF_{2\alpha}$ (fatty acid) in 50 mL of dry methanol (solution will be clear). Methylate by adding dry diazomethane (generated following instructions supplied with the Aldrich diazomethane generator) dropwise into the solution until the solution is a permanent yellow color. Allow to stand for 5 min. Dry solution under a stream of nitrogen. For use, dilute to a concentration 1 mg/mL in methanol. *CAUTION*: *Diazomethane may explosively decompose on shock, friction, or concussion. It may also explode on heating above 100 °C or on contact with rough surfaces or if impurities/solids are present in the undiluted liquid or under high-intensity lighting. Contact with alkali metals and calcium sulfate causes explosions.*

Equipment setup

TLC plate preparation. Prewash all TLC plates with a solution of ethyl acetate/ethanol (90/10, v/v). Air dry completely before sample application.

GC/MS setup. For quantification of F_2-IsoPs by GC/MS, we routinely use an Agilent 5973 mass spectrometer with a computer interface, although other mass spectrometers can be utilized. The F_2-IsoPs are chromatographed on a 15-m DB1701 fused silica capillary column because we have found that this column gives excellent separation of individual regioisomers and separation from confounding PGs and PG metabolites, including cyclooxygenase-derived $PGF_{2\alpha}$ and the PGD_2 metabolite 9α,11β–PGF_2, compared to other columns. The column temperature is programmed from 190 to 300 °C at 20 °C per minute. Methane is used as the reagent gas and helium is used as the carrier gas for NICI. The ion source temperature is 200 °C. The ion monitored for endogenous F_2-IsoPs is the carboxylate anion m/z 569 (M-181, loss of $CH_2C_6F_5$, the pentafluorobenzyl leaving group, Fig. 3). The corresponding carboxylate anion for the deuterated internal standard is m/z 573. Each day the sensitivity of the mass spectrometer is checked by injecting a standard consisting of 40 pg each $PGF_{2\alpha}$ and [$^2H_4$]-15-F_{2t}-IsoP. As noted above, $PGF_{2\alpha}$ elutes at a sufficiently different

retention time from the F_2-IsoPs quantified using this procedure. Therefore, this cyclooxygenase-derived prostaglandin does not interfere with the signal of the non-cyclooxygenase-derived isoprostanes. Injecting both $PGF_{2\alpha}$ and $[^2H_4]$-15-F_{2t}-IsoP in the standard mixture helps the user to predict the retention time of both species during the operation of the mass spectrometer each day. It is important to also note that synthetic standards of the IsoFs are not available; thus IsoFs generated from the oxidation of arachidonic acid are used as retention time standards.

Sample collection and preparation

Plasma

(i) Blood (5–10 mL) should be collected in a tube containing EDTA. (An example of an appropriate collection tube is Cat. No. BD367844 from Laboratory Supply Company.)
(ii) Centrifuge at 4000*g* for 10 min to yield plasma. 0.5–1 mL of plasma is necessary for the quantification of F_2-IsoPs.
(iii) Freeze sample at −80 °C. *As plasma contains a large amount of arachidonic acid, which can oxidize ex vivo to generate F_2-IsoPs, the samples should be frozen at -80 °C immediately. It is imperative that samples are not thawed before analysis.* 0.001% butylated hydroxytoluene (BHT), a potent free radical chain-breaking antioxidant, can be added to the tube to help prevent *ex vivo* autoxidation.
In plasma, F_2-IsoPs and IsoFs are present in both the free fatty acid form and esterified in phospholipids. Most often, only these molecules in the free fatty acid form are measured; however, the total amount of F_2-IsoPs/IsoFs (meaning the sum of both esterified and unesterified compounds) can be measured if the plasma lipids are treated with base prior to analysis. To analyze total F_2-IsoPs/IsoFs begin with step (iv) below. To measure only F_2-IsoPs/IsoFs as free fatty acids begin with step (v) below.
(iv) After thawing, dilute 0.5 mL plasma in an equal volume DI water. Add 1 mL 1 N aqueous KOH. Vortex, purge flask with nitrogen, and cap. Incubate mixture at 37 °C for 30 min. After incubation, dilute to 10 mL with DI water and adjust to pH 3 with 1 N HCl. Proceed to sample purification.
(v) After thawing, dilute 1 mL plasma in 5 mL of purified water and acidify to pH 3 with 1 N HCl. Proceed to sample purification.

Urine

(i) Collect voided urine in a sterile container. (Note: Measuring F_2-IsoPs in either a spot urine sample or a 24-h urine collection also represents a reliable probe of the oxidant stress status.)
(ii) Store sample at −80 °C until analysis.
(iii) For analysis, after thawing, dilute 0.200 mL urine in 5 mL purified water and acidify to pH 3 with 1 N HCl.

Tissue samples

(i) Weigh out 100 mg of freshly harvested tissue. Arachidonic acid in tissue samples can oxidize ex vivo to generate F_2-IsoPs. *If tissue is not to be analyzed immediately after collection, it is imperative to flash freeze the sample in liquid nitrogen and store at −80 °C until analysis.*
(ii) Add tissue to 20 mL of ice-cold Folch solution in a 50-mL centrifuge tube with cap. [If sample is < 15 mg then reduce the volume of Folch solution to 10 mL.] *Polypropylene tubes are recommended, as polystyrene is not resistant to chloroform. Keep on ice.*
(iii) Homogenize tissue with blade homogenizer at full speed for 30 s or until fully homogenized.
(iv) Flush centrifuge tube with a stream of nitrogen or argon for 30–60 s to remove air from tube, then cap. Let solution stand at room temperature for 1 h to allow maximal extraction of lipids from ground tissue. Shake tube occasionally for several seconds during this period of time.
(v) Add 4 mL aqueous NaCl (0.9%) prepared in ultrapure water. Vortex or shake vigorously for 1 min at room temperature. [If tissue sample is < 15 mg then reduce volume of NaCl solution to 2 mL.]
(vi) Centrifuge for 10 min in table-top centrifuge at room temperature to separate aqueous and organic layers. Following centrifugation, a semisolid protein layer should have formed between the upper (aqueous) and the lower (organic) layers.
(vii) After centrifugation, carefully pipette off top aqueous layer and discard. Remove the lower organic layer carefully from under the intermediate semisolid protein layer and transfer to a 50-mL conical tube. Evaporate under nitrogen stream in an analytical evaporation unit to dryness.
(viii) Resuspend lipids in 1–2 mL methanol containing 0.005% BHT and vortex. Next add 1 mL aqueous KOH (15%, w/v). [If sample originally weighed < 15 mg, lipid extract can be resuspended in 1 mL methanol, to which 1 mL KOH is added.] Vortex, purge flask with nitrogen, and cap. Incubate mixture at 37 °C for 20 min.
(ix) After incubation, acidify the mixture to pH 3.0 with 1 N HCl. Then dilute the mixture with pH 3 water. *It is important to dilute the methanol in this solution to 5% or less to ensure proper extraction of F_2-IsoPs in the subsequent purification procedure. Also, it is important to continue immediately with the purification steps below. The sample should not be stored in this form due to possible autoxidation.*

Sample purification

1. To sample accurately add 1 ng of the internal standard, $[^2H_4]$-15-F_{2t}-IsoP, and vortex. *To insure accuracy, always add internal standard with an accurate syringe rather than a pipetteman.*
2. Connect one C-18 Sep-Pak cartridge to a 10-mL disposable syringe per sample and place on vacuum manifold. Precondition each cartridge with 5 mL methanol and then 7 mL pH 3 water. *Use a pressure < 5 mm Hg for column conditioning.*
3. Apply acidified lipid mixture to the preconditioned Sep-Pak cartridge. *It is important that samples are corrected to pH 3 or slightly below in order for the carboxylate moiety to be protonated. If not protonated, the compounds will not chromatograph correctly on the Sep-Pak cartridges. During sample loading, the vacuum manifold should be set at < 5 mm Hg. Pressures up to 10 mm Hg are acceptable during the subsequent wash and elution steps, though a steady stream is not recommended.*
4. Wash cartridge first with 10 mL pH 3 water and then 10 mL heptane.
5. Elute F_2-IsoPs from cartridge with 10 mL ethyl acetate/heptane (50/50, v/v) into a 15-mL plastic tube.
6. Add 5 g anhydrous sodium sulfate to the tube to remove residual water from the eluant.
7. Remove used C-18 Sep-Pak cartridges from syringes and discard. Connect one silica Sep-Pak cartridge to each syringe per sample and place on vacuum manifold. Precondition cartridge with 5 mL ethyl acetate.
8. Apply eluant from C-18 Sep-Pak cartridge to silica Sep-Pak cartridge in the same manner as in Step 3. *Care should be taken not to transfer any sodium sulfate to the extraction cartridge.*

9. Wash cartridge with 5 mL ethyl acetate, then elute F_2-IsoPs from silica Sep-Pak cartridge with 5 mL ethyl acetate/methanol (1/1, v/v) into a 5-mL vial.
10. Evaporate eluant under nitrogen in the analytical evaporation unit.
11. Convert F_2-IsoPs to the corresponding pentaflourobenzyl esters. (This derivatization facilitates compound analysis by GC/MS): Add 40 μL of 10% (v/v) PFBB in acetonitrile and 20 μL of 10% (v/v) DIPE in acetonitrile to the sample vial, vortex briefly, and incubate for 20 min at 37 °C. *Caution*: *PFBB is a potent lachrymator. Do not work outside of a well-ventilated fume hood.*
12. Prepare TLC tank by adding 92 mL chloroform, 8 mL ethanol, 1 mL acetic acid, and TLC paper to saturate the tank. Allow tank to equilibrate for 30 min.
13. Dry sample thoroughly under nitrogen in an analytical evaporation unit in a fume hood and resuspend material in 50 μL methanol/chloroform (3/2, v/v). Vortex briefly.
14. Apply mixture from each sample to a prewashed silica TLC plate. Be sure to apply only one sample per lane on each plate. Using a separate TLC plate, apply approximately 2–5 μg of the methyl ester of $PGF_{2\alpha}$to one lane for use as a standard.
15. After ensuring that the application solvent has dried, place TLC plates into the TLC tank, and chromatograph. When the solvent front reaches 13 cm on the TLC plate, remove from tank, and allow solvent to evaporate. Visualize the TLC standard by spraying the standard plate with the phosphomolybdic acid solution and then heating on a hot plate. *Do not spray sample plates.*
16. Scrape silica from the sample TLC plates in the region of the TLC standard (R_f should be approximately 0.18), scraping from 1 cm above the middle of the visualized standard to 1 cm below the standard. *Keep sample plates until GC/MS analysis is complete.*
17. Place scraped silica from each sample into separate microcentrifuge tubes and add 1 mL ethyl acetate. Vortex vigorously for 30 s to extract analytes from the silica, then centrifuge in a bench-top microcentrifuge at 13,000 rpm for 3 min.
18. Carefully remove the ethyl acetate, taking care not to disrupt the silica pellet in the bottom of the tube, and place in a new microcentrifuge tube.
19. Dry under nitrogen. Then add 20 μL BSTFA and 7 μL dry DMF to residue to convert analytes to the trimethylsilyl ether derivatives (Fig. 2).
20. Vortex well and incubate sample at 37 °C for 5 min. Dry reagents under nitrogen.
21. Resuspend sample in 20 μL dry undecane and vortex briefly. Transfer sample to an autosampler vial for GC/MS analysis.

Variations

In certain patient populations, interfering peaks eluting during the GC/MS analysis can confound the quantification of F_2-IsoPs and IsoFs using this methodology. Under these circumstances, we have determined that an additional TLC step is necessary for sample purification. One population that requires this clean-up procedure is surgery patients administered propofol during anesthesia as a metabolite(s) of propofol elute in the same region as F_2-IsoPs. The protocol should be modified as follows:

1. Prepare samples as usual and perform steps 1–10 above.
2. After drying, reconstitute sample in 50 μL methanol.
3. Prepare two TLC tanks (one for TLC standard and one for sample plates) by adding 172 mL chloroform, 28 mL methanol, 2 mL acetic acid, 1.6 mL water, and TLC paper to each. Allow tanks to equilibrate for 30 min.
4. Apply mixture from each sample to a prewashed silica TLC plate. Be sure to apply only one sample per lane on each plate. Using a separate TLC plate, apply approximately 2–5 μg of $PGF_{2\alpha}$ (in the free acid form) to one lane for use as a standard.
5. After ensuring that the application solvent has dried, place sample TLC plates into one TLC tank and the standard TLC plate into another, and chromatograph. When the solvent front reaches 13 cm on the TLC plate, remove from tank, and allow solvent to evaporate. *Note*: *Not all TLC plates may reach* 13 cm *at the same time*. Visualize the TLC standard by spraying the standard plate with the phosphomolybdic acid solution and then heating on a hot plate. *Do not spray sample plates.*
6. Scrape silica from the sample TLC plates in the region of the TLC standard (R_f should be approximately 0.33), scraping from 1.5 cm below the top of the $PGF_{2\alpha}$ free acid band to 0.5 cm above it.
7. Place scraped silica from each sample into separate microcentrifuge tubes and add 1 mL ethyl acetate/ethanol (1/1, v/v) to each. Vortex vigorously for 30 s to extract analytes from the silica, then centrifuge in a bench-top microcentrifuge at 13,000 rpm for 3 min.
8. Carefully remove the ethyl acetate/ethanol mixture, taking care not to disrupt the silica pellet in the bottom of the tube, and place in a new microcentrifuge tube.
9. Evaporate sample to dryness under nitrogen and proceed with analysis steps 11–21 above.

Troubleshooting

If peak signal is extremely low or if no peaks are detected by the mass spectrometer, the sample should be removed from the autosampler vial, dried thoroughly under nitrogen, and steps 19–21 should be repeated. If peaks are still not detected, it is possible that there was a problem with the thin-layer chromatography. Scrape all silica from the bottom half of lane, place in microcentrifuge tube, and extract with ethyl acetate as described in step 17. Continue by finishing the assay as described above. If no result is obtained, it will be necessary to repeat the entire analysis with new sample.

If the internal standard is detected with a strong signal at m/z 573 but there are very low or nonexistent peaks at m/z 569, then the level of F_2-IsoPs is below the limit of detection. The limit of detection for this assay is approximately 5 pg.

Calculations and expected results

Representative chromatograms obtained from the analysis of F_2-IsoPs and IsoFs in plasma are shown in Fig. 4. The ions monitored for endogenous F_2-IsoPs and IsoFs as well as the internal standard are the carboxylate anions obtained following loss of the pentafluorobenzyl leaving group (as shown in Fig. 3). The peaks shown in the middle chromatogram (Fig. 4B) represent different endogenous F_2-IsoPs (m/z 569). This pattern of peaks is virtually identical in all biological fluids and tissues that we have examined to date. To quantify F_2-IsoPs, the height of the peak containing the derivatized 15-F_{2t}-IsoP (labeled with *) is compared with the height of the deuterated internal standard peak (m/z 573, Fig. 4C). For reference, the elution of cyclooxygenase-derived $PGF_{2\alpha}$ is indicated in the chromatogram (labeled with + in Fig. 4).

The peaks in the upper chromatogram (Fig. 4A) represent different endogenous IsoFs (m/z 585). As formation of 256 IsoF isomers from arachidonic acid is possible, the pattern of peaks generated in different biological samples is significantly more variant than that noted for F_2-IsoPs. The peaks eluting between 5.98 and 6.18 min (indicated by dashed line) are most consistently formed and are thus used for quantification of these compounds. To quantify IsoFs, the area of indicated peaks at m/z 585 is compared with the area of the

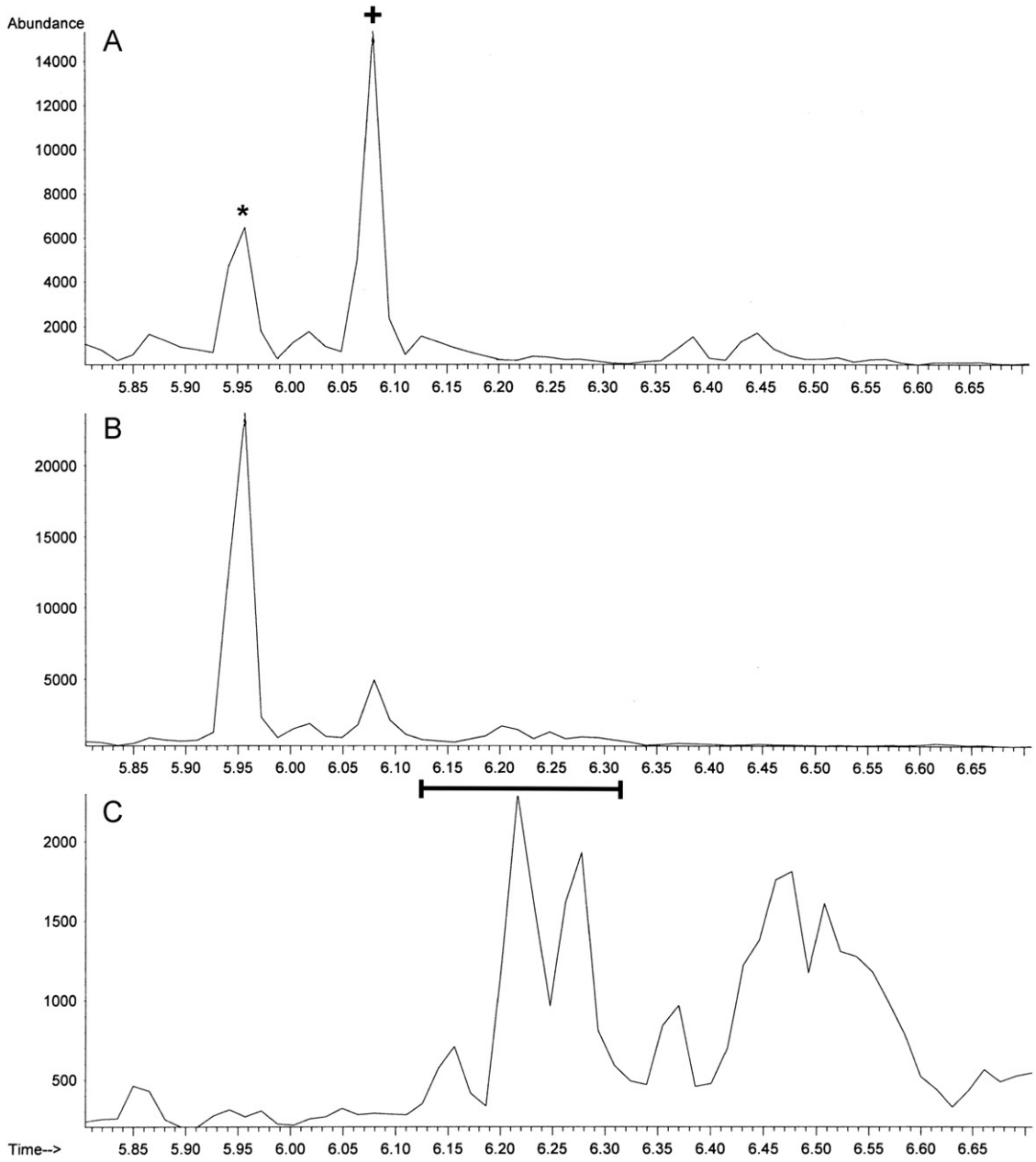

Fig. 4. Representative chromatogram showing the elution of endogenous F_2-IsoPs and IsoFs in human plasma. (A) Endogenous F_2-IsoPs. F_2-IsoP isomers, including 15-F_{2t}-IsoP, eluting in the peak indicated by the '*' are quantified. Cyclooxygenase-derived $PGF_{2\alpha}$, indicated by the '+', does not interfere with this analysis. (B) [$^2H_4$]-15-F_{2t}-IsoP internal standard. (C) Endogenous IsoFs. IsoF isomers eluting in the peaks indicated by the bracket are quantified.

[$^2H_4$]-15-F_{2t}-IsoP internal standard. Note: Area rather than height is used in the quantification of IsoFs as multiple peaks, rather than a single peak, are used in the quantification.

The following equation is used in the calculation of both F_2-IsoPs and IsoFs:

$$\frac{\text{Area/Height of Endogenous Compounds}}{\text{Area/Height of Internal Standard}} \times \frac{\text{Amount of Internal Standard}}{\text{Sample size (tissue/plasma/urine)}} \times \frac{1}{\text{Creatinune (urine)}}$$

Levels of F_2-IsoPs and IsoFs in plasma are reported in nanograms per milliliter while levels in tissues are reported in nanograms per gram of tissue. Levels of F_2-IsoPs in urine are normalized to creatinine clearance and reported as nanograms per milligrams of creatinine.

Normal levels of F_2-IsoPs in human plasma quantified using this methodology are 0.035 ± 0.006 ng/mL while normal levels in human urine are 1.6 ± 0.6 ng/mg Cr. Normal levels of IsoFs in human plasma are 0.071 ± 0.010 ng/mL and in human urine are 5.8 ± 1.0 ng/mg Cr. Quantification of F_2-IsoPs and IsoFs using this method is highly precise and accurate. The precision is $\pm 6\%$ and the accuracy is 96%. The lower limit of quantitation is 0.002 ng/mL.

Caveats

The GC/MS protocol described herein is a robust and specific methodology for the quantification of F_2-IsoPs and IsoFs and has been

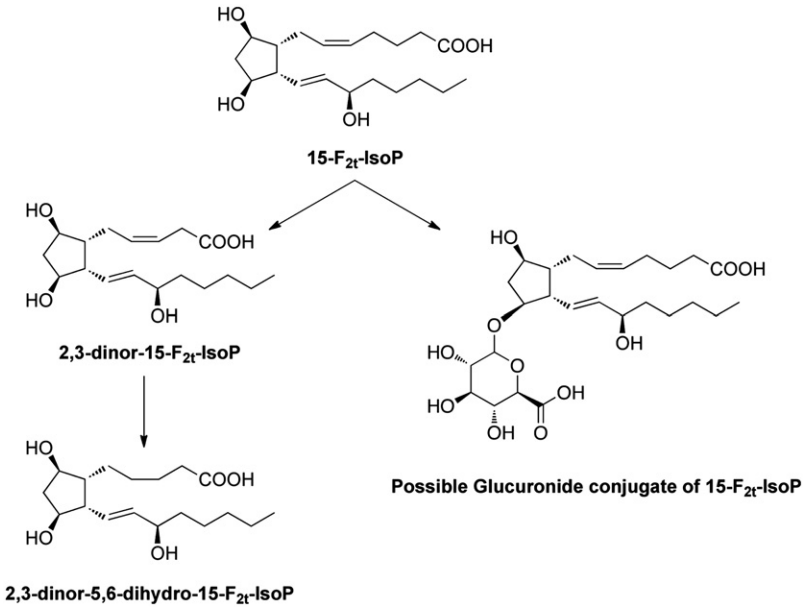

Fig. 5. Urinary metabolites of 15-F_{2t}-IsoP.

utilized for this purpose for more than 20 years. Importantly, this method offers the lowest limit of quantitation of any reported mass spectrometric methodology for F_2-IsoPs. This is particularly important in the quantification of these molecules in plasma, CSF, EBC, and other biological fluids in which low levels of F_2-IsoPs are found. Further, according to our knowledge, this is the only reported methodology for the quantification of IsoFs. However, the labor-intensive purification and derivatization steps limit the throughput of the assay—a maximum of 20 samples can be analyzed in one day by experienced personnel.

Due to the softer mode of ionization utilized, LC/MS methodologies for the measurement of F_2-IsoPs offer a more rapid alternative to GC/MS as only extraction from the biological matrix is required; the extensive chemical derivatization steps necessary for GC/MS can be eliminated. For reference, a summary of several MS-based assays reported in the recent literature is presented in Table 1. The existence of these multiple methodologies, however, is a major challenge to the field as the specific F2-IsoP isomers quantified differ from assay to assay and, consequently, results cannot be directly compared between laboratories. Further, more and more new techniques are being published each year. Within the past year alone, at least five new methodologies have been reported in the literature and each paper reports a unique way to extract and analyze samples depending on the sample matrix and available equipment. This avalanche of technical papers can be overwhelming to a newcomer in the field; thus, great care should be exercised when choosing a methodology for the analysis of these molecules.

When analyzing samples for F_2-IsoPs, it is important to consider that these molecules are not only excreted intact in the urine but are also metabolized in the liver. Two major metabolites of 15-F_{2t}-IsoP found in human urine are 2,3-dinor-15-F_{2t}-IsoP (2,3-dinor-8-IsoP-$F_{2\alpha}$) and 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP (2,3-dinor-5,6-dihydro-8-IsoP-$F_{2\alpha}$) (Fig. 5) [28]. Further, Yan and colleagues recently established that F_2-IsoPs are excreted in the urine as glucuronide conjugates [29]. These authors noted that levels of urinary F_2-IsoPs were significantly increased after treatment of the urine with β-glucuronidase (0.43 ± 0.02 vs. 0.61 ± 0.03 nmol/mmol Cr) and that this increase was, in fact, dependent on the activity of the enzyme. (The exact structure of F_2-IsoP glucuronide conjugates has not been determined.) Multiple mass spectrometric methodologies have been developed to quantify F_2-IsoP metabolites, but most clinical reports in the literature have focused on the measurement of unmetabolized F_2-IsoPs in urine. Very few studies have measured both F_2-IsoPs and urinary IsoP metabolites. In a recent commentary on the utility of F_2-IsoPs as biomarkers of oxidative stress, Halliwell and Lee rightly suggest that further study is needed to determine factors that affect F_2-IsoP metabolism in humans and that measurement of either intact F_2-IsoPs or metabolized F_2-IsoPs alone might not fully represent endogenous oxidant stress [30].

Finally, it is important to note that F_2-IsoPs are only one class of an entire series of IsoPs that can be generated from the oxidation of arachidonic acid [3]. Several different classes of IsoPs have been identified in humans and are shown in Fig. 6. Despite the fact that F_2-IsoPs are the most well-studied IsoPs, under certain biological conditions, the quantification of these other species may be more relevant. For example, E_2/D_2-IsoPs, which are formed competitively with F_2-IsoPs by isomerization rather than reduction of the endoperoxide in Fig. 6, are favored to form in vitro when cellular reducing agents, such as glutathione (GSH) or α-tocopherol, are depleted [31]. Thus, the ratio of E_2/D_2-IsoPs to F_2-IsoPs provides information not only about lipid peroxidation in a given organ, but also about the redox environment in that tissue. E_2/D_2-IsoPs levels are increased, and have been shown to be the favored products of the IsoP pathway, in affected brain regions of patients with Alzheimer's disease [31], in settings of cerebral ischemia [32,33], and after traumatic brain injury [34].

Taking into account the complex mechanisms that regulate arachidonic acid autoxidation and the resulting array of possible oxidation products including F_2-IsoPs, E_2/D_2-IsoPs, IsoFs, and their metabolites as well as many other compounds, care should be taken when determining the appropriate species to quantify as the most suitable biomarker of endogenous oxidant stress.

Acknowledgments

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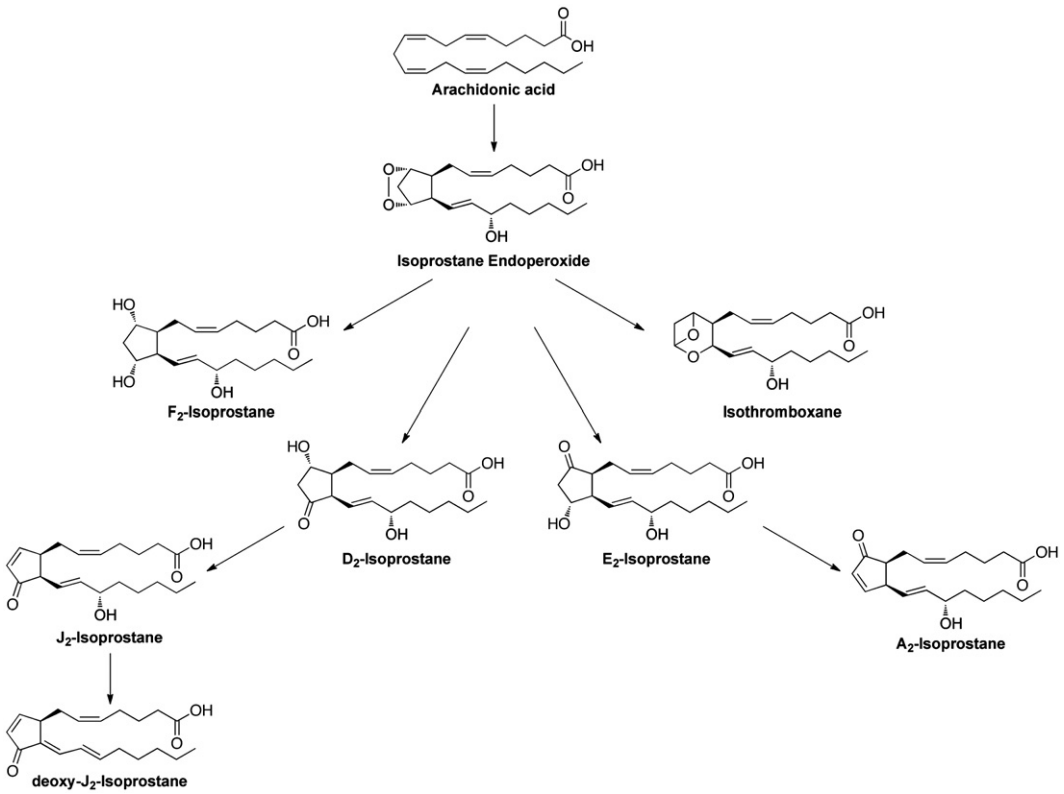

Fig. 6. Structures of IsoPs with alternate ring configurations.

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