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## Quantification of fatty acid oxidation products using online high-performance liquid chromatography tandem mass spectrometry

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## ABSTRACT

Oxidized fatty acids formed via lipid peroxidation are implicated in pathological processes such as inflammation and atherosclerosis. A number of methods may be used to detect specific oxidized fatty acids containing a single or multiple combinations of epoxide, hydroxyl, ketone, and hydroperoxide moieties on varying carbon chain lengths from C8 up to C30. Some of these methods are nonspecific and their use in biological systems is fraught with difficulty. Measures of specific oxidized fatty acid derivatives help in identifying oxidation pathways in pathological processes. We used liquid chromatography coupled with electrospray ionization tandem mass spectrometry as an efficient, selective, and sensitive method for identifying and analyzing multiple specific fatty acid peroxidation products in human plasma and other biological matrices. We then distilled the essential components of a number of these analyses to provide an efficient protocol by which fatty acid oxidation products and their parent compounds can be determined. In this protocol, addition of a synthetic internal standard to the sample, followed by base hydrolysis at elevated temperature and liquid–liquid phase sample extraction with lighter-than-water solvents, facilitates isolation of the oxidized fatty acid species. These species can be identified and accurately quantified using stable-isotope dilution and multiple-reaction monitoring. Use of a coupled multiplexed gradient HPLC system on the front end enables high-throughput chromatography and more efficient use of mass spectrometer time.

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## Introduction

Assessment of lipid oxidation levels is essential to the study of biologically relevant reactive oxygen species [1]. Numerous methods exist to ascertain these levels through the systematic analysis of the oxidized fatty acids that originate from the oxidized lipids both in vivo and in vitro [2–25]. In addition to exploring the bulk oxidation, many of these oxidized lipids and their associated oxidized fatty acids have

activities of significant clinical interest [3–6,10,14,18,20,22,26–31]. Numerous GC<sup>1</sup>, LC [32], and hybrid methodologies such as GC–MS [3,5,14,18,20,22,31] and LC–MS [2–4,8–10,12,19,23,27,33] exist to quantify the levels of these compounds. These markers can be used clinically to ascertain the effectiveness of antioxidant therapies; however, there are no universal guidelines regarding the scale of the analyses required for the determination of lipid oxidation (and hence fatty acid oxidation) products in a large clinical study. We have performed and optimized these types of analyses for nearly 2 decades in our laboratory and are presenting our insights into what makes for a good high-throughput oxidized lipid assay.

## Principles

*Liquid/liquid extraction of fatty acids and their oxidation products*

Recovery of free fatty acids and their oxidation products using the classic Folch et al. [34] and Bligh and Dyer [35] extractions is not easily adapted to the small sample volumes required for high-throughput clinical analyses. Hara and Radin's [36] procedure using hexane and isopropanol can be modified and adapted to

**Abbreviations:** AA, arachidonic acid; BHT, butylated hydroxytoluene; DTPA, diethylenetriaminepentaacetic acid; EET, epoxyeicosatrienoic acid; ESI, electrospray ionization; GC, gas chromatography; PGF<sub>2α</sub>, 9α,11α,15S-trihydroxy-5Z,13E-dien-1-oic acid; 15(S)-HETE-d<sub>8</sub>, 15S-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic-5,6,8,9,11,12,14,15-d<sub>8</sub> acid; HETE, hydroxyeicosatetraenoic acid; HPLC, high-performance liquid chromatography; HODE, hydroxyoctadecadienoic acid; LC–MS/MS, liquid chromatography with electrospray ionization online tandem mass spectrometry; LA, linoleic acid; LOD, limit of detection; LOQ, limit of quantitation; MS, mass spectrometry; MRM, multiple-reaction monitoring; oxoETE, oxo-eicosatetraenoic acid; oxoODE, oxo-octadecadienoic acid; PGF<sub>2α</sub>-d<sub>4</sub>, 9α,11α,15S-trihydroxy-5Z,13E-dien-1-oic-3,3,4,4-d<sub>4</sub> acid. See Materials for more specific abbreviations of oxidized fatty acid species.

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these small sample volumes (see Protocol). With this method, in addition to avoiding the toxicity and regulatory issues associated with chlorinated solvents, the organic layer floats on top and is more easily removed. Hexane, being a lighter-than-water solvent, allows one to avoid pushing a pipette or needle through the aqueous layer and risking possible contamination that can occur with heavier-than-water chlorinated solvents. The extraction is efficient because the initial solution is homogeneous, giving a layer that fully permeates and extracts the entirety of the sample solution. In the next step more hexane is added to generate a heterogeneous mixture from which the top layer is easily removed after centrifugation. Even when this liquid–liquid extraction is done manually, its simplicity, low cost, and efficiency allow for rapid processing of multiple samples simultaneously in a short period of time.

#### Use of heavy isotope standards

Internal standards should be incorporated before the extraction step to control for any losses that may occur through poor extraction, inefficient removal of the organic phase, adherence to the container walls, etc. Additionally, one may incorporate a distinct stable-isotope-labeled internal standard of the parent fatty acid to monitor and control for any artificial oxidation products that form during lipid extraction [2,8,9,12,19,23,25,37].

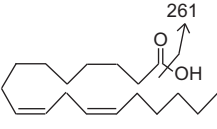
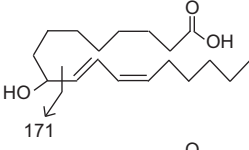
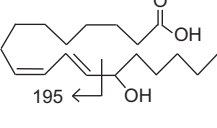
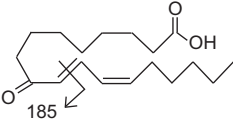
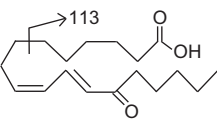
Preferably these are universally  $^{13}\text{C}$ -labeled unsaturated fatty acid derivatives; however, the deuterated analogs are generally less expensive and more readily available from commercial sources.

#### Advantages of column switching

For analyses of biological matrices composed of multiple distinct fatty acid species (both oxidized and unoxidized), a broad gradient elution is often useful to allow for chromatographic separation among the many different compounds present. Once the gradient has been run it is necessary to reequilibrate the column back to its initial state, which takes 10–15 min. This reequilibration period is followed by the injection of the next sample, which with an autosampler could take several minutes followed by 1–2 min (preferred—after the solvent peak) until any peak of interest elutes. During this period the mass spectrometer is sitting idle and not collecting any relevant data from analyte peaks. To make optimal use of the mass spectrometer, the most costly piece in the instrument string, a second column from another HPLC system that has already been injected with another sample immediately at the end of the first column's run can be brought online [28,33].

A				B			
Compound	Precursor (m/z)	Product (m/z)	Structure	Compound	Precursor (m/z)	Product (m/z)	Structure
AA	303	259		5,6-EET	319	191	
5-HETE	319	115		8,9-EET	319	151	
8-HETE	319	155		11,12-EET	319	167	
9-HETE	319	151		14,15-EET	319	175	
11-HETE	319	167		5-oxoETE	317	203	
12-HETE	319	179		12-oxoETE	317	153	
15-HETE	319	175		15-oxoETE	317	113	
PGF<sub>2α</sub>	353	193					

**Fig. 1.** Precursor ion→product ion transitions and suggested structure of the major product ion derived from each eicosanoid. ESI negative-ion full scan was used for identification of precursor ions for each of the analytes and ESI negative-ion product scan was used for identification of the specific product ions from each of the precursors. Suggested structures consistent with the product ions produced are depicted for each analyte.

Compound	Precursor (m/z)	Product (m/z)	Structure
LA	279	261	
9-HODE	295	171	
13-HODE	295	195	
9-oxoODE	293	185	
13-oxoODE	293	113	

**Fig. 2.** Precursor ion  $\rightarrow$  product ion transitions and suggested structure of the major product ion derived from linoleic acid and its oxidation products. ESI negative-ion full scan was used for identification of precursor ions for each of the analytes and ESI negative-ion product scan was used for identification of the specific product ions for each of the precursors. Suggested structures consistent with the product ions produced are depicted for each analyte.

*Multiple-reaction monitoring (MRM) analysis provides an efficient method for quantification of free fatty acids and their oxidized derivatives*

Because of the higher specificity and sensitivity of the MRM mode of operation, it was used for the quantification of oxidized free fatty acids and their precursors. Full ion scan ESI/MS in negative-ion mode was used for identification of the specific mass-to-charge ratio ( $m/z$ ) for each of the fatty acid species monitored. A product ion scan (MS2) was used for identification of the corresponding product ions for each of the analytes. The specific precursor and product ions for each of the oxidized arachidonic acids, including HETEs, EETs, oxoETEs, and  $\text{PGF}_{2\alpha}$  are shown in Fig. 1. The structure and precursor product ion transitions for oxidized linoleic acids including HODEs and oxoODEs are shown in Fig. 2. The product (daughter) ions chosen for the MRM transitions were selected for their specificity for the distinct structural isomers, allowing quantification of the individual isomers even if they coelute during chromatography. The MRM mode of operation shows improved performance over selected-ion mode, particularly in cases of high background noise and those with overlapping peak separation (poor chromatographic resolution) of the isomers.

## Materials

The following compounds were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA):

( $\pm$ )-9-hydroxy-10E,12Z-octadecadienoic acid (9-HODE), Cat. No. 38400;

( $\pm$ )-13-hydroxy-9Z,11E-octadecadienoic acid (13-HODE), Cat. No. 38600;  
 9-oxo-10E,12Z-octadecadienoic acid (9-oxoODE), Cat. No. 38420;  
 13-oxo-9Z,11E-octadecadienoic acid (13-oxoODE), Cat. No. 38620;  
 ( $\pm$ )-5-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid (5-HETE), Cat. No. 34210;  
 ( $\pm$ )-8-hydroxy-5Z,9E,11Z,14Z-eicosatetraenoic acid (8-HETE), Cat. No. 34340;  
 ( $\pm$ )-9-hydroxy-5Z,7E,11Z,14Z-eicosatetraenoic acid (9-HETE), Cat. No. 34400;  
 ( $\pm$ )-11-hydroxy-5Z,8Z,12E,14Z-eicosatetraenoic acid (11-HETE), Cat. No. 34500;  
 ( $\pm$ )-12-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid (12-HETE), Cat. No. 34550;  
 ( $\pm$ )-15-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid (15-HETE), Cat. No. 34700;  
 ( $\pm$ )-5(6)-epoxy-8Z,11Z,14Z-eicosatrienoic acid (5,6-EET), Cat. No. 50211;  
 ( $\pm$ )-8(9)-epoxy-5Z,11Z,14Z-eicosatrienoic acid (8,9-EET), Cat. No. 50351;  
 ( $\pm$ )-11(12)-epoxy-5Z,8Z,14Z-eicosatrienoic acid (11,12-EET), Cat. No. 50511;  
 ( $\pm$ )-14(15)-epoxy-5Z,8Z,11Z-eicosatrienoic acid (14,15-EET), Cat. No. 50651;  
 5-oxo-6E,8Z,11Z,14Z-eicosatetraenoic acid (5-oxoETE), Cat. No. 34250;  
 12-oxo-5Z,8Z,10E,14Z-eicosatetraenoic acid (12-oxoETE), Cat. No. 34580;  
 15-oxo-5Z,8Z,11Z,13E-eicosatetraenoic acid (15-oxoETE), Cat. No. 34730;  
 9 $\alpha$ ,11 $\alpha$ ,15S-trihydroxy-prosta-5Z,13E-dien-1-oic acid (prostaglandin  $\text{F}_{2\alpha}$ ,  $\text{PGF}_{2\alpha}$ ), Cat. No. 16010;  
 9Z,12Z-octadecadienoic acid (linoleic acid, LA), Cat. No. 90150;  
 5Z,8Z,11Z,14Z-eicosatetraenoic acid (arachidonic acid, AA), Cat. No. 90010;  
 15S-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic-5,6,8,9,11,12,14,15-d<sub>8</sub> acid (15(S)-HETE-d<sub>8</sub>), Cat. No. 334720;  
 9 $\alpha$ ,11 $\alpha$ ,15S-trihydroxy-prosta-5Z,13E-dien-1-oic-3,3,4,4-d<sub>4</sub> acid ( $\text{PGF}_{2\alpha}$ -d<sub>4</sub>), Cat. No. 316010;  
 2,3-dinor-8-iso prostaglandin  $\text{F}_{2\alpha}$  (2,3-dinor-8-iso $\text{PGF}_{2\alpha}$ ), Cat. No.16290;  
 8-isoprostaglandin  $\text{F}_{2\alpha}$  (8-iso $\text{PGF}_{2\alpha}$ ), Cat. No.16350;  
 5-isoprostane  $\text{F}_{2\alpha}$  (5-i $\text{PF}_{2\alpha}$ -VI), Cat. No.16300.

The following chemicals and solvents were purchased from Fisher Scientific (Pittsburg, PA, USA):

methanol (Optima grade), Cat. No. A454;  
 water (HPLC grade), Cat. No. W5;  
 acetic acid (glacial, ACS grade), Cat. No. A38;  
 2-propanol (HPLC grade), Cat. No. A451;  
 hexane (HPLC grade), Cat. No. H302;  
 sodium hydroxide (NaOH, ACS grade), Cat. No. S318;  
 hydrochloric acid (HCl, ACS grade), Cat. No.A144.

The following reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA):

tin(II) chloride dihydrate ( $\text{SnCl}_2$ , 98%, ACS grade), Cat. No. 243523;  
 2,6-di-*tert*-butyl-4-methylphenol (BHT, > 99%), Cat. No. B1378;  
 Diethylenetriaminepentaacetic acid (DTPA, >99%) Cat. No. 32319.

Argon (5.0 ultrahigh purity, Part No. AR5.0UH) and nitrogen (5.0 ultrahigh purity, Part No. NI5.0UH) were obtained from Praxair, Inc. (Cleveland, OH, USA).

## Instrumentation

An AB SCIEX API 4000 (Framingham, MA, USA) triple-quadrupole mass spectrometer interfaced to a Shimadzu HPLC system through an electrospray ionization source controlled by a personal computer was used for single-column developmental work. Alternatively, for high-throughput dual high-pressure binary gradient elution (Fig. 8), we used a Shimadzu Prominence HPLC system (Shimadzu Scientific Instruments, Columbia, MD, USA) composed of four Shimadzu LC-20AD pumps, a Shimadzu DGU-20A5 vacuum degasser, and a SIL-HTC autosampler with three two-position, six-port, high-pressure switching valves attached. For higher sensitivity we use an AB SCIEX API 5000 mass spectrometer in the negative ESI mode. We required a centrifuge capable of spinning sample tubes at a relative centrifugal force of 2000g and a vortex mixer (Fisher Scientific MaxiMix, Cat. No. 12-815-50, or equivalent). Organic solutions were measured using a syringe (Gastight; Hamilton Co., Series 1700) or a bottle-top dispenser (Fisher Scientific, Cat. No. 03-692-178). Plasma, urine, and other aqueous solutions were measured using positive displacement pipettors (Fisher Scientific, Cat. No. 21-377-328).

## Protocol

### Standard solutions and plasma and urine sample preparation

Stock solutions of all the standards were prepared in 85% methanol. Glass containers were flushed with argon and kept at  $-20^{\circ}\text{C}$ . Our data indicate that the standard solutions are chemically stable for at least 6 months under these conditions. Working solutions were prepared monthly by diluting the stock solutions into 85% methanol. To prepare the standard curves,  $10\ \mu\text{l}$  of internal standard solution containing  $20\ \text{ng}/\mu\text{l}$   $\text{PGF}_{2\alpha}\text{-d}_4$  and  $4\ \text{ng}/\mu\text{l}$   $15(\text{S})\text{-HETE-d}_8$  was added into  $200\ \mu\text{l}$  of each dilution of the standard solutions. The entire process is detailed below:

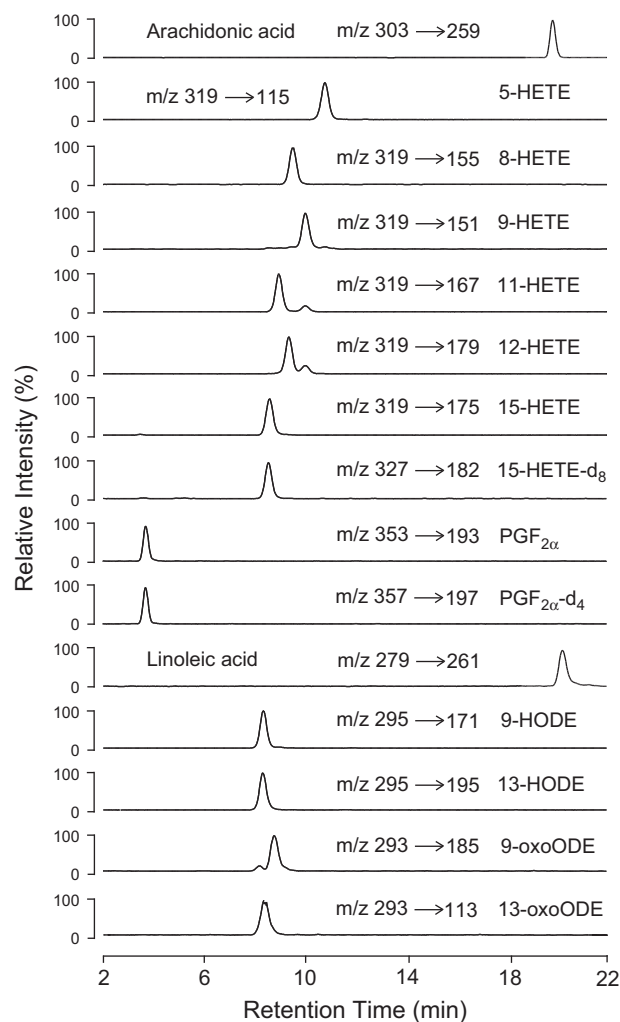
1. Aliquot  $200\ \mu\text{l}$  of plasma (or urine) sample into a clean  $12 \times 75\text{-mm}$  borosilicate glass test tube.
2. Add  $10\ \mu\text{l}$  of internal standard mixture ( $20\ \text{ng}/\mu\text{l}$   $\text{PGF}_{2\alpha}\text{-d}_4$  and  $4\ \text{ng}/\mu\text{l}$   $15(\text{S})\text{-HETE-d}_8$ ).
3. Add  $1.0\ \text{ml}$   $10\%$  v/v acetic acid in water/2-propanol/hexane (2/20/30, v/v/v).
4. Vortex briefly to mix.
5. Add  $2.0\ \text{ml}$  hexane.
6. Cap tube with polypropylene stopper (Fisher Scientific, Cat. No. 14-376-77).
7. Vortex mix for 3 min.
8. Centrifuge sample at room temperature at  $2000\text{g}$  for 5 min.
9. Remove upper hexane layer with a glass Pasteur pipette; place in clean  $12 \times 75\text{-mm}$  test tube.
10. Evaporate hexane extract under  $\text{N}_2$  flow.
11. For unesterified fatty acid analysis, proceed to step 26.  
For total fatty acids profile, suspend pellet in  $1.0\ \text{ml}$  of 2-propanol.
12. Add  $1.0\ \text{ml}$  of aqueous  $2\ \text{M}$  NaOH (saturated with argon).
13. Vortex briefly to mix.
14. Blanket sample with argon gas.
15. Cap tube immediately with polypropylene stopper (Fisher Scientific, Cat. No. 14-376-77).
16. Place in  $60^{\circ}\text{C}$  water bath for 60 min to hydrolyze lipids.
17. Cool to room temperature.  
At this point samples may be stored refrigerated overnight if necessary.
18. Acidify sample by adding  $1.2\ \text{ml}$  of  $2.0\ \text{M}$  HCl in water.
19. Add  $2.0\ \text{ml}$  hexane.
20. Cap tube with polypropylene stopper (Fisher Scientific, Cat. No. 14-376-77).

21. Vortex mix for 3 min.
22. Centrifuge sample at room temperature at  $2000\text{g}$  for 5 min.
23. Remove upper hexane layer with a glass Pasteur pipette; place in clean  $12 \times 75\text{-mm}$  test tube.
24. Repeat steps 19 through 23 and combine both hexane layers.
25. Evaporate hexane extract under  $\text{N}_2$  flow.
26. Vortex pellet into  $100\ \mu\text{l}$  of  $85\%$  v/v methanol in water.
27. Store under argon at  $-20^{\circ}\text{C}$  until analysis by LC-MS/MS.
28. Centrifuge at  $2000\text{g}$  immediately before analysis.

For oxidized fatty acids and their precursor compounds in urine, the same protocol is used as described for plasma above except that a more sensitive mass spectrometer is needed for the analysis.

### LC-MS/MS analysis

A  $40\text{-}\mu\text{l}$  sample (in  $85\%$  methanol) was injected onto a reverse-phase C18 HPLC column (XPERTEX,  $2.1 \times 250\ \text{mm}$ ,  $5\text{-}\mu\text{m}$  particle; P.J. Cobert Associates, St. Louis, MO, USA) at a flow rate of  $0.2\ \text{ml}/\text{min}$ . Solvent A was water (plus  $0.2\%$  v/v acetic acid) and solvent B was methanol (plus  $0.2\%$  v/v acetic acid). The column was equilibrated with  $85\%$  B. The separation was performed starting



**Fig. 3.** Negative-ion LC-MS/MS chromatography of selected unoxidized fatty acids and oxidized fatty acid standards. Chromatographic separation was performed on a reverse-phase C18 column ( $2.1 \times 250\ \text{mm}$ ,  $5\text{-}\mu\text{m}$  particle) using acidified methanol/water as the mobile phase at a flow rate of  $0.2\ \text{ml}/\text{min}$  using a gradient as described in the protocol.

at 85% B for 10 min, then using a gradient to 100% B over 2 min, followed by 100% B for 10 min, and then 100% A for 10 min. HPLC column effluent was introduced into an AB SCIEX API 4000 triple-quadrupole mass spectrometer. The source of the mass spectrometer was configured with the electrospray needle voltage set at  $-4.2$  kV and a turbo ion spray temperature of  $350$  °C. Nitrogen gas was used for the nebulizer, curtain, and collision gas. The gas flow rates, collision energies, declustering potentials, entrance potentials, focusing potentials, and collision cell exit potentials were adjusted to give optimal signal response from the direct infusion of a dilute solution of each standard in aqueous methanol. Analyses were performed using electrospray ionization in negative-ion mode with MRM of precursor and characteristic product ions specific for each analyte monitored for 100 ms per transition. The  $m/z$  transitions monitored are shown in Figs. 1 and 2. The MRM transitions for two internal standards were  $m/z$  327  $\rightarrow$  182 for 15(S)-HETE- $d_8$  and  $m/z$  357  $\rightarrow$  197 for  $PGF_{2\alpha}$ - $d_4$ . The internal standard  $PGF_{2\alpha}$ - $d_4$  was used for quantification of  $PGF_{2\alpha}$  and 15(S)-HETE- $d_8$  was used for quantification of EETs, HETEs, HODEs, oxoETEs, oxoODEs, linoleic acid, and arachidonic acid. In cases in which greater sensitivity was needed for detection, such as in measuring urine  $F_2$ -isoprostanes, an AB SCIEX API 5000 triple-quadrupole mass spectrometer was used.

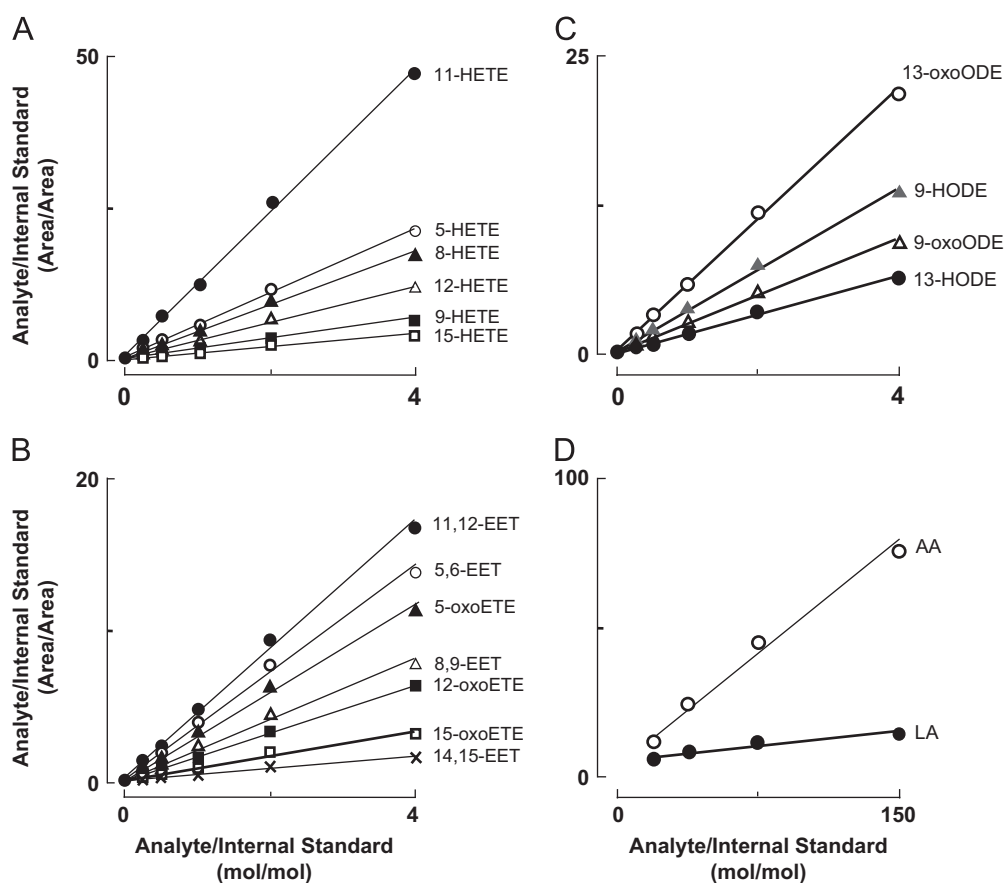
### Calculations and expected results

A typical LC-MS/MS chromatogram for analysis of 15 different fatty acid derivatives including 2 internal standards using MRM mode is shown in Fig. 3. Although the retention time and the

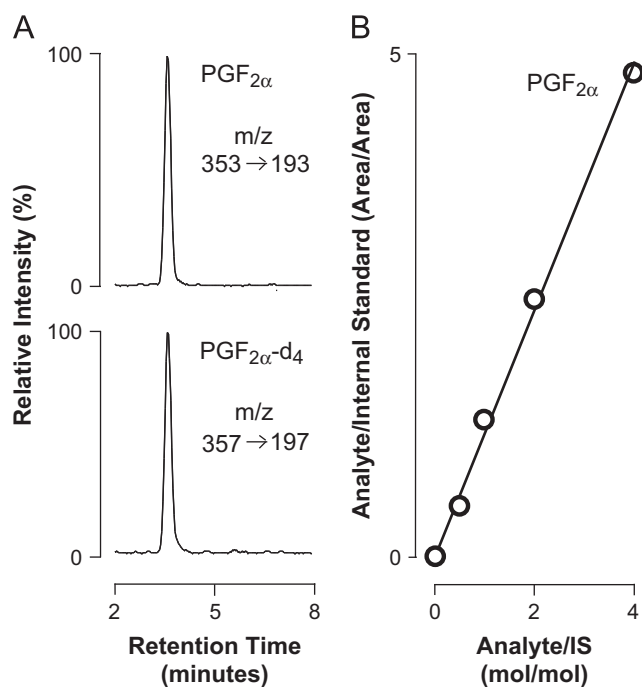
precursor ions for 9-HODE and 13-HODE are nearly the same, they can still be quantified using specific product ions ( $m/z$  171 for 9-HODE and  $m/z$  195 for 13-HODE).

### Calibration curves and limit of detection (LOD)

Calibration curves for all the fatty acid derivatives monitored, relative to their selected internal standard, are shown in Fig. 4. Serial dilutions of authentic fatty acid derivatives including arachidonic acid, linoleic acid, and their oxidation products (HETEs, EETs, oxoETEs, HODEs, and oxoODEs) in an 85% methanol solution demonstrate a linear response over a wide range of the standard concentrations throughout the assay range (Figs. 4A–C). Analyte recovery was quite high, with more than 95% for arachidonic acid and linoleic acid and more than 85% for their oxidation products except for  $PGF_{2\alpha}$ , which had only 10% recovery. Use of the stable isotope synthetic internal standard for  $PGF_{2\alpha}$  allows us to account for the reduced lipid extraction characteristics of this oxidized lipid species. Fig. 5 shows the chromatography and calibration curve of authentic  $PGF_{2\alpha}$  relative to its internal standard  $PGF_{2\alpha}$ - $d_4$ . Because biomarkers of oxidation accumulate only to trace amounts in biological samples, sensitivity is critical. We therefore determined the LOD and limit of quantitation (LOQ) of LC-MS/MS for the species being monitored. The LOD was expressed as the lowest amount of analyte on-column generating a signal-to-noise ratio of at least 3. The LOQ was examined by reducing the concentration of standard solution gradually and is expressed as the lowest concentration yielding a signal-to-noise ratio of at least 10. As shown in Table 1, the most readily detected analyte is 11-HETE, with LOD  $< 2.6$  pg and LOQ  $< 0.09$  ng/ml. The LOD and



**Fig. 4.** Internal standard calibration curves for the unoxidized fatty acids and oxidized fatty acids analyzed using LC-MS/MS. 15(S)-HETE- $d_8$  (12 fmol on column) was used as internal standard for preparing these standard calibration curves. (A–C) Calibration range 0–4 mol/mol, (A) 15(S)-HETE- $d_8$  for HETEs, (B) EETs and oxoEETs, (C) HODEs and oxoODEs. (D) Calibration range 18–150 mol/mol, 15(S)-HETE- $d_8$  for linoleic acid (LA) and arachidonic acid (AA).



**Fig. 5.** Chromatography and internal standard calibration curve for  $\text{PGF}_{2\alpha}$  analyzed using LC–MS/MS.  $\text{PGF}_{2\alpha}\text{-d}_4$  (60 fmol on-column) was used as internal standard for preparing the standard calibration curve for  $\text{PGF}_{2\alpha}$ . (A) Precursor to product ion chromatogram, (B) calibration curve, calibration range 0–4 mol/mol  $\text{PGF}_{2\alpha}\text{-d}_4$ .

**Table 1**

Limit of detection (LOD) and limit of quantitation (LOQ).

Analyte	Transition (m/z)	LOD <sup>a</sup> (pg)	LOQ <sup>b</sup> (ng/ml)
Arachidonic acid	303 → 259	91	3.1
5-HETE	319 → 115	4.8	0.16
8-HETE	319 → 155	4.8	0.1
9-HETE	319 → 151	9.6	0.30
11-HETE	319 → 167	2.6	0.09
12-HETE	319 → 179	3.2	0.11
15-HETE	319 → 175	13	0.45
5,6-EET	319 → 191	16	0.54
8,9-EET	319 → 151	19	0.64
11,12-EET	319 → 167	16	0.54
14,15-EET	319 → 175	26	0.90
5-OxoETE	317 → 203	32	1.1
12-OxoETE	317 → 153	32	1.1
15-OxoETE	317 → 113	64	2.2
$\text{PGF}_{2\alpha}$	353 → 193	7.1	0.24
Linoleic acid	279 → 261	617	21
9-HODE	295 → 171	15	0.50
13-HODE	295 → 195	30	1.0
9-OxoODE	293 → 185	47	1.6
13-OxoODE	293 → 113	29	1.0

<sup>a</sup> LOD was defined as a peak whose signal-to-noise ratio is 3:1.

<sup>b</sup> LOQ was defined as a peak whose signal-to-noise ratio is 10:1.

LOQ provided are instrument specific. The instrument selected for these analyses was relatively old and not of the highest sensitivity. Even with the abundance of the molecular species being monitored in plasma or urine this protocol allows for ready detection and quantification even with almost decade-old triple-quadrupole mass spectrometers.

#### Precision and accuracy

Precision and accuracy of the intraday assay were assessed using standards dissolved in phosphate-buffered saline that were

assayed in replicates ( $n=4$ ) for known concentrations of analytes. All samples were analyzed on the same day and their back-calculated concentrations were determined from the internal calibration curves prepared the same day. The interday assay precision and accuracy were assessed by assaying known concentrations of analytes in replicates ( $n=3$ ) over 3 different days. The precision was expressed as the coefficient of variation (%) and the accuracy as the percentage bias (%). For the intraday and interday assay (Table 2) the CV% and percentage bias for almost all of the analytes are substantially less than 10% and typically in the 3–5% range.

#### LC–MS/MS analysis of oxidized fatty acids in plasma

This LC–MS/MS method can be used to identify and quantify fatty acid derivatives in biological matrices such as plasma, serum, or other tissues. Fig. 6 shows an example of quantification of multiple oxidized and unoxidized fatty acid derivatives in 0.2 ml human plasma. This method clearly identified all the peaks of analytes monitored in plasma. The levels of some oxidized and unoxidized fatty acids in human plasma are shown in Table 3.

#### LC–MS/MS analysis of $F_2$ -isoprostanes in urine

Determination of isoprostanes in urine provides a superior noninvasive methodology to assay the index of oxidative stress [2,8,9,12,14,19,23]. Because urine contains markedly less arachidonic acid (and linoleic acid) and other interfering compounds than plasma or serum, these samples are much less prone to the artificial generation of interfering oxidized fatty acids.

#### Caveats

##### Sample collection

For sample collection it is important to minimize any extraneous oxidation that may occur after its initial collection. Storage conditions must be carefully chosen to exclude air, which can be a primary source of contaminating oxidant. It is important to blanket biological tissues and fluids (such as serum or plasma) with an inert atmosphere such as nitrogen or, preferably, with heavier than air argon gas to displace the oxygen around the sample. Ideally, antioxidants are included in the liquid portion of the sample. Commonly used antioxidants are free radical scavenging compounds such as fat-soluble BHT and divalent metal ion scavengers. The choice of divalent metal ion scavengers is critical to avoid extraneous oxidation; pentacoordinated chelators such as diethylenetriaminepentaacetic acid (DTPA), which envelope divalent ions and do not leave open coordination sites available, at which catalysis can occur, are the reagents of choice for this purpose. Tetracoordinated divalent metal ion species in complexes with chelators such as ethylenediaminetetraacetic acid and ethylene glycol tetraacetic acid have open sites that can promote oxidation and even paradoxically enhance the redox potential of the active site on the divalent metal ion [38–40]. In addition, sample storage at low temperatures is crucial. Many laboratories use ultralow (–70 to –80 °C) freezers for this purpose. Experience has shown that lipids in biological samples survive long-term storage better at these lower temperatures. It is also important to limit the number of freeze–thaw cycles a sample will be exposed to during its lifetime. Smaller volume, one-time use aliquots are preferred for storage, because oxidized lipid recovery is degraded in biological samples through multiple freeze–thaw cycles. Indeed, it is wise to aliquot a biological specimen into multiple vials destined for lipid analysis, overlay the samples with argon, and

**Table 2**  
Intraday and interday assay precision and accuracy.

Analyte	Added (ng/ml)	Intraday assay (n=4)				Interday assay (n=9)			
		Mean (ng/ml)	SD	CV% (%)	Bias (%)	Mean (ng/ml)	SD	CV% (%)	Bias (%)
Arachidonic acid	300	313	10	3.3	4.4	280	9.5	3.4	-6.8
5-HETE	15.0	5.5	1.4	9.3	2.8	15.0	1.2	7.7	-0.1
8-HETE	15.0	16.0	1.3	8.3	6.4	15.6	1.2	7.8	4.0
9-HETE	15.0	15.5	2.1	14	3.4	15.0	1.6	10.9	0.1
11-HETE	15.0	15.0	0.6	4.3	0.2	14.7	0.8	5.4	-2.2
12-HETE	15.0	15.4	0.5	3.4	2.7	14.9	0.8	5.3	-3.9
15-HETE	15.0	15.2	1.2	7.7	1.2	15.0	0.8	5.6	-0.1
PGF <sub>2α</sub>	5.0	5.5	0.6	10	9.6	5.4	0.6	10.6	7.8
Linoleic acid	450	473	16	3.3	5.0	431	23	5.3	-4.2
9-HODE	15.0	15.2	1.1	7.2	1.3	14.9	0.8	5.5	-0.5
13-HODE	15.0	15.3	0.6	3.8	1.7	15.1	0.6	4.0	0.8

**Table 3**  
Levels of selected oxidized fatty acids and their precursors in human plasma from healthy volunteers.

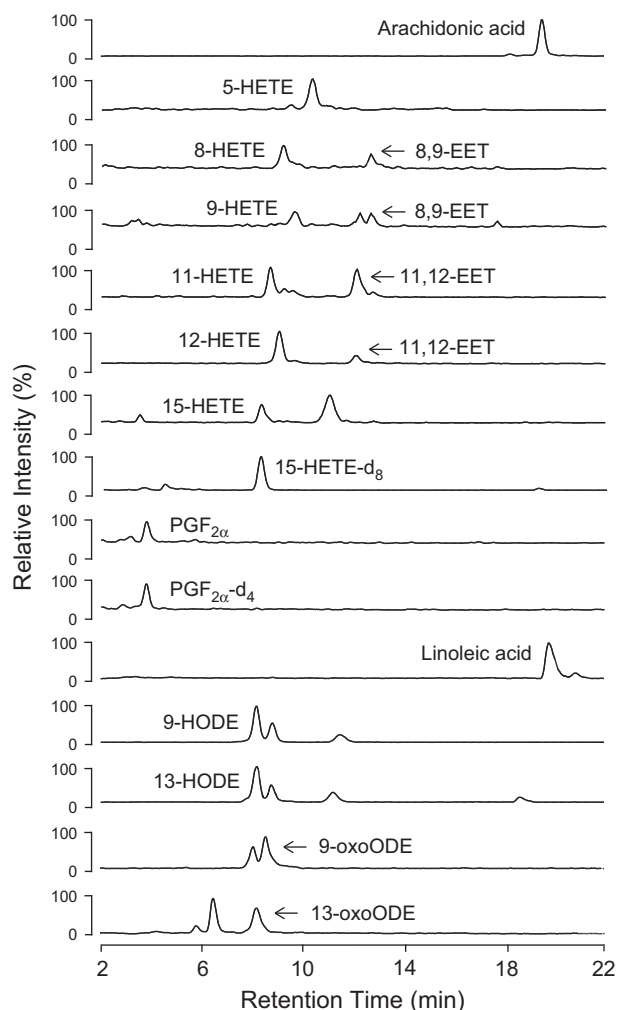
Fatty acid	Mean (ng/ml plasma) (n=15)	SD
Arachidonic acid	638	228
5-HETE	2.9	1.4
8-HETE	2.5	1.1
9-HETE	3.3	1.3
11-HETE	3.2	1.1
12-HETE	12	3.5
15-HETE	4.8	2.0
PGF <sub>2α</sub>	4.1	1.0
Linoleic acid	2124	946
9-HODE	16	5.0
13-HODE	47	20

then “snap freeze” these portions in liquid nitrogen immediately after collection and processing.

#### Fatty acid extraction: antioxidants used in sample preservation

To quantify total free oxidized and unoxidized fatty acids, lipids in the sample are hydrolyzed to their free fatty acid components with base at elevated temperature before extraction. Antioxidants need to be included in the hydrolysis medium; these can be BHT and DTPA as described above. Sometimes it is helpful to also include a mild reducing agent such as tin chloride (SnCl<sub>2</sub>), dithionate, or borohydrides (sodium borohydride and sodium cyanoborohydride, for example). If using a mild reducing agent, we prefer tin chloride because all of the others have partial solubility in isopropyl alcohol and may carry through the extraction procedure, and they both interfere with ionization efficiency and, over time, contaminate the mass spectrometer source (i.e., tin chloride does not partition much into the hexane layer of the Hara extraction). With reducing reagents, labile species such as lipid hydroperoxides and cyclic endoperoxides are reduced to their corresponding hydroxyl compounds [32]. Obviously, any assay that requires separate determination of the hydroperoxide or endoperoxide oxidation products must avoid the use of reducing agents such as tin chloride. It should be noted that tin chloride reduction is the classic way to produce prostaglandin D<sub>2</sub> from PGH<sub>2</sub>, raising the possibility of artificial elevation in F<sub>2</sub>-isoprostane levels in plasma with addition of tin chloride [41]. However, in our hands, control studies have shown that addition of tin chloride to plasma does not increase measured F<sub>2</sub>-isoprostane levels, presumably because the level of hydroperoxide present in plasma is exceedingly low relative to the F<sub>2</sub>-isoprostane level. To analyze isoprostanes in plasma it has been shown by others that one should first add a reducing agent (to eliminate unstable hydroperoxides and cyclic endoperoxides) and then hydrolyze for optimal results [42]. In our opinion, analyses of urine F<sub>2</sub>-isoprostane is the superior method for global oxidative stress measures because there is very little precursor arachidonate or hydroperoxide/endoperoxide present, and artifactual formation of the oxidized lipid during prolonged storage does not occur. In plasma matrix, we have found improved recovery of some unoxidized fatty acids (arachidonic and linoleic acid) with shorter hydrolysis times (30 min) at higher temperatures (60 °C), whereas hydrolysis of PGF<sub>2α</sub> is optimal at longer times (60 min) at lower temperatures (45 °C). The choice of base used for saponification and its concentration are also important. Generally sodium hydroxide is used, which is easily neutralized with acid to form a water-soluble salt. Optimization of the conditions of hydrolysis for the analyte of interest needs to be done using authentic standards.

As mentioned above, antioxidants may need to be incorporated into one or both phases of the extraction solvents. Years of



**Fig. 6.** Representative LC-MS/MS chromatograms of unoxidized fatty acids and oxidized fatty acids extracted from human plasma. Extraction was done as described under Protocol; separation was performed on a reverse-phase C18 column (2.1 × 250 mm, 5 μm) using acidified methanol/water as mobile phase at a flow rate of 0.2 ml/min using a gradient as described under Protocol. Note that some of the oxidized fatty acid species have common MRM pairs and unique retention times; 8,9-EET exhibits the same MRM transitions as both 8-HETE and 9-HETE, and 11,12-EET shares the same MRM transitions with both 11-HETE and 12-HETE. The unique retention times of 8,9-EET and 11,12-EET were established by injection of authentic standards (data not shown).

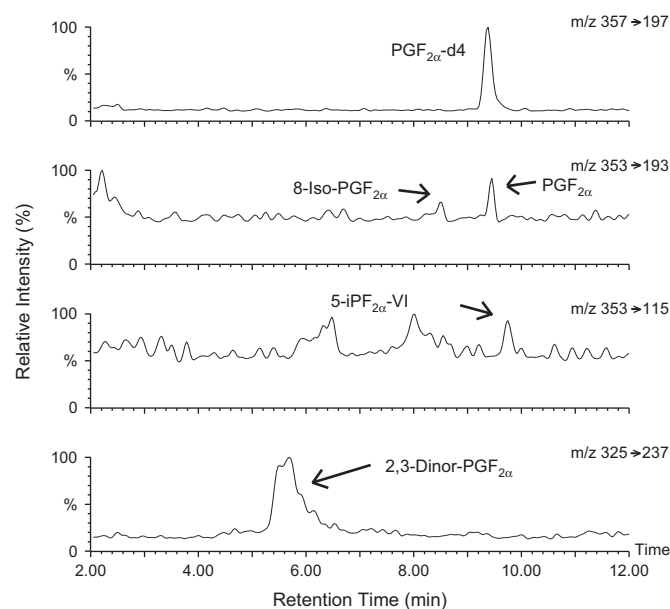
experience have shown us that it is inefficient to include a derivatization step for any compound unless the derivatives are absolutely necessary to provide the sensitivity for the detection of the analytes. Most modern mass spectrometers have the sensitivity to measure compounds down to the femtomole and even attomole range. Careful choice of sample amounts and their dilutions coupled with the resolution and concentrative properties of high-performance liquid chromatography should allow for easy detection of most oxidized fatty acid derivatives and their parent compounds.

### Fatty acid analysis

#### Which fatty acids to monitor?

The last step is the separation and quantitation of the oxidized fatty acid derivatives and their parent compounds from the sample matrix. Which fatty acid and oxidized fatty acid species to monitor is largely dependent on the sample matrix and scientific question. Whereas cyclooxygenase (COX)-derived oxidized species such as  $\text{PGF}_{2\alpha}$  and lipoxygenase products such as HETEs and HODEs predominate in plasma, nonenzymatic free radical-derived oxidized species such as the  $\text{F}_2$ -isoprostanes predominate in urine. As far as biological mechanisms and outcomes are concerned, there may be times when it is desirable to monitor both groups of products in either matrix [3,6]. For a global measure of oxidative stress in a patient it is most desirable to use a noninvasive assay such as urinalysis of oxidized lipid species. The invasive plasma (or serum) matrix determination of oxidized lipid species is useful for the evaluation of COX pathway activity, as in inflammatory disease states such as arthritis and atherosclerosis. In urine the stable end products of the free radical-oxidized fatty acids, such as 2,3-dinor-8-iso $\text{PGF}_{2\alpha}$ , predominate.

Generally speaking, the oxidation products from arachidonic acid can be found in human urine, whereas the linoleate derivatives are typically present at insignificant amounts, often approximating analytical noise levels. The two-step free radical oxidation products from arachidonic acid, the isoprostanes and  $\text{PGF}_{2\alpha}$ , are the most abundant and most persistent (stable). 2,3-Dinor-8-iso $\text{PGF}_{2\alpha}$ , a metabolite of 8-iso $\text{PGF}_{2\alpha}$ , and 5-iso $\text{PGF}_{2\alpha}$  predominate in urine from humans (see Fig. 7). In fact, stability studies with prolonged incubations of urine at 37 °C reveal that addition of an antioxidant cocktail as a sample preservative may not even be necessary. Urine specimens have proven to be stable at room temperature with respect to almost all oxidized fatty acid species examined. The stability has been observed for a number of hours and even many days in specific instances; as expected [2], inclusion of the antioxidant mixtures mentioned above markedly increases this stability.  $\text{F}_2$ -isoprostanes at room temperature are stable for days. What is important is to obtain a clean catch of urine that is free of cell debris or bacteria. Most samples analyzed from males are of this nature; however, those from females can sometimes contain cellular material rich in the precursor arachidonic acid. This interference can mostly be eliminated from all samples by briefly spinning them in a centrifuge before aliquoting for storage. Visible hematuria and urinary tract infections may present samples that yield abnormally high levels of oxidation products. Control studies have found that spot urine collection provides levels of  $\text{F}_2$ -isoprostanes on par with those obtained from a 24-h collection. The dilution levels of these oxidized species from urine are corrected with respect to the urine creatinine level and are expressed as mass of oxidized or unoxidized fatty acid to mass of creatinine. A clean-catch spot urine analysis of oxidized fatty acids such as  $\text{F}_2$ -isoprostanes can provide an immediate noninvasive global systemic index of oxidative stress.



**Fig. 7.** A representative LC-MS/MS chromatogram of  $\text{PGF}_{2\alpha}$ 's extracted from human urine. The top shows heavy-isotope-labeled internal standard  $\text{PGF}_{2\alpha}$ -d<sub>4</sub> from urine, the second graph shows the corresponding transition for  $\text{PGF}_{2\alpha}$ 's that are chromatographically resolved from their free radical-derived isomers, the 8-iso $\text{PGF}_{2\alpha}$ 's. The bottom two graphs show other free radical-derived  $\text{PGF}_{2\alpha}$  isomers with different MRM transitions.

#### Standards

The standards themselves are carefully chosen, based on their chemical structure, to avoid deuterium exchange with the solvents. When possible, the concentrations of the stable-isotope-labeled internal standards are verified against primary standards, typically from the linearity plots of their unlabeled isotopologues. Care must be taken to include an amount of stable-isotope-labeled unsaturated parent fatty acid (arachidonic or linoleic acid) in the general concentration range of endogenous fatty acid derivative levels. If oxidation is observed in this internal standard channel for a particular sample, it could be remade and rerun. At worst, if all samples show some degree of artificial oxidation, the extraction method itself may need to be modified by incorporating either better or higher concentrations of antioxidants. Use of distinct stable-isotope-labeled precursors to monitor for potential artificial oxidation (by monitoring of potential MRM of heavy-isotope-labeled isotopologues) is critical for assay development to ensure the assay is not artificially generating the oxidation products monitored in the endogenous sample. Absolute quantitation of the contribution from artifactual oxidation products to samples should be less than 10% (maximum) of the amount measured.

#### Quality control standards for fatty acid analysis performance

It is helpful to first run a solution containing both the analytes and the internal standards in the same solvent mixture used for the sample dissolution but not in the sample matrix (i.e., in a simple solvent system). This standard is used primarily to assess the suitability of the chromatographic system to perform the assay. If peaks are poorly resolved, out of sequence, or absent, the run can be stopped immediately and the chromatographic issues addressed without losing a precious sample. The sample matrix itself presents a formidable challenge to the chromatography system as well as the mass spectrometer. It is wise to prepare a significant volume of control standard consisting of analytes spiked into the matrix of interest at physiologically relevant concentrations (typically at the midpoint of the compound's



standard curve). Several of these control standards are processed in parallel with the samples (at least 1 for every 5 to 10 samples). Minimally, this check standard is analyzed first and a blank is run immediately thereafter. These high-throughput analyses demand a robust HPLC system with an autosampler allowing for the storage of a run-full of samples (up to 100 per day) under refrigeration.

#### *HPLC system hardware*

There are a plethora of HPLC systems available for this task from numerous manufacturers. Herein, we explore just a few of the options and alternatives. Nano-flow HPLC (flow less than 1  $\mu\text{l}/\text{min}$ ) offers the best chromatographic effects and sensitivity, but suffers from its expense, fragility, and ease of contamination. One stray dust particle is enough to clog a flow path and shut the system down for a major prolonged and expensive repair. It is our opinion that it is best to reserve nano-flow for stringent analyses requiring the utmost in sensitivity, not routine high-throughput analyses. Next is micro or narrow bore chromatography (flow rates from 1 to 200  $\mu\text{l}/\text{min}$ ). This is the optimal operational region for a high-throughput chromatography system even though 2.1-mm-diameter columns are starting to be considered old technology. Most HPLC hardware developed over the past 20 years is able to operate reliably in this flow window, and most importantly, most mass spectrometers have heated sources that can handle these flow rates without splitting. At a 200  $\mu\text{l}/\text{min}$  flow rate, solvents are still used slowly, with amounts of less than 300 ml of solvent consumption and waste per system per day. A note on ultrahigh-pressure liquid chromatography (UPLC): although smaller particle sizes in stationary phases offer enhanced rapid resolution of components, the chromatographic resolution can make peaks of interest so thin that they cannot be properly digitized by the mass spectrometry system. This can be especially true for cases such as these analyses, for which many different MRM channels of data must be simultaneously collected during the chromatographic run. Large-scale studies and the desire for robust reproducible performance with few difficulties (e.g., 300–600 sample injections before needing to replace the column) make the high-pressure mixing pump configuration at a 200  $\mu\text{l}/\text{min}$  flow rate our preferred system because it provides adequate sensitivity and resolution for the analytes being quantified. Normal bore chromatography with flow rates greater than 200  $\mu\text{l}/\text{min}$  and up to 1 ml/min is now old technology, with solvent usage and waste amounts of up to a liter and a half per day. Chromatographic dilution at these higher flow rates and inefficient vaporization and sampling of the resulting spray in the mass spectrometer source attenuate signals, making these high-flow methods less desirable.

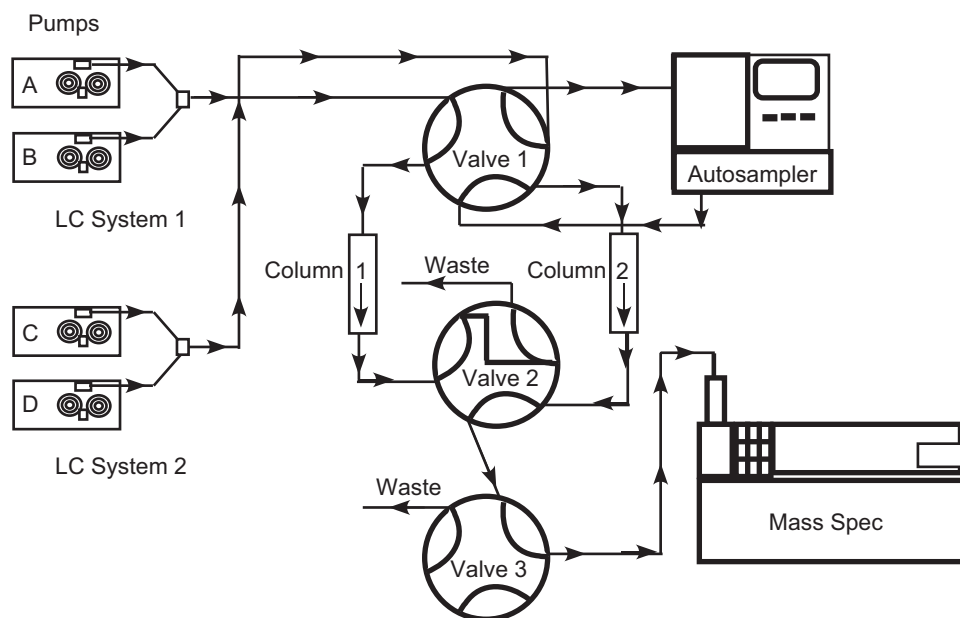
#### *Multiplexing considerations*

Efficient use of mass spectrometer time may be further optimized in this multiplexing approach when three HPLC systems and columns are used. The actual advantage of a three-column versus a two-column system amounts to an approximate 10% time savings. This becomes important only when thousands of samples must be analyzed under the same conditions on the same data acquisition system. There are multiplexed HPLCs consisting of four (or more) chromatographic systems run in parallel in a “gatling gun” fashion to maintain 100% usable time on the mass spectrometer. Although such systems sound ideal, it is our experience that a fourfold multiplex system is not typically necessary. The system complexity, cost, and maintenance become increasingly prohibitive as each chromatographic system is added. Typically, chromatography conditions can be fine-tuned to allow for faster overall runs, and a two- or three-column multiplex system can provide near optimal instrument efficiency. Additionally, we have found

that column manufacturers often do not supply separation columns that demonstrate the same chromatographic performance that column switching requires. Even consecutive columns from the same lot will not provide the same performance (same peak retention times, peak resolution, and tailing factor). Consequently, we purchase one dozen or more of the same column type at a time and screen all the columns with standards to identify pairs of columns that work best with each other in terms of retention characteristics (times and peak shapes). This process greatly facilitates the programming of the multiplex HPLC system. When some columns in each purchase seem to have no matching partner, these unusable columns can usually be returned for credit, provided only a single clean standard solution has been injected and the column has been well maintained.

Another issue with multiplexed HPLC systems is high-pressure versus low-pressure mixing with the HPLC pumps. Low-pressure mixing, in which the solvents are mixed by a proportioning valve located ahead (upstream) of the pumps, is much simpler to maintain and operate because only one pump per analytical column is required to deliver the gradient. However, low-pressure pumps have larger delay volumes with a resulting longer gradient delay. They also cannot generate as reproducible a gradient as a high-pressure mixing system. High-pressure mixing requires two pumps, one for the aqueous phase and one for the organic phase, with their flow outputs connected together on the high-pressure side of the pumps. Differential flow rates through the two pumps generate the gradient and because pump flows can be tightly controlled, the gradients supplied by binary high-pressure systems are very accurate.

We have settled on a dual high-pressure binary gradient elution system for all multiplexed high-throughput applications, such as oxidized fatty acid analyses [23,27,28,33]. Maintaining four HPLC pumps and one autosampler with two or three switching valves is a tenable goal (Fig. 8 illustrates the plumbing for a two-HPLC multiplex system). The heart of the dual-column switching system are two six-port two-position valves that simultaneously switch one HPLC system or the other in-line with the autosampler and mass spectrometer (Fig. 8). The dual-column advantage is that data can immediately be collected from a second column after gradient elution and concurrent data collection from the first column. The chromatographic run on the second column takes place while the first column is being washed, reequilibrated, and reinjected. In a single-column system the mass spectrometer would not be collecting any useful data during the column washout reequilibration and sample injection steps. Even in the most rapid chromatographic gradient separations, the dual column approach will offer this time savings in the dwell time of column washout, reequilibration, and subsequent sample injection. One has to decide if the added complexity is worth the increase in speed. In our experience, column switching increases sample throughput because it takes significantly more time to wash, reequilibrate, reinject, and wait for the salt front to elute from a reverse-phase chromatography column compared to the time it takes to run the analytical part of the gradient in which peaks of interest elute and data are collected. Specifically, in the analysis of the oxidized and unoxidized fatty acids, the mass spectrometer idle time (during the 10 min of column equilibration back into the aqueous phase and the 2 min of the sample injection and salt front washout) is utilized for data collection from the second column. Because the actual data collection occurs over 22 min (of a total run time of 34 min) this translates into an increase in used mass spectrometer time of 12 min per sample. When thousands of clinical samples must be analyzed, 12 min equates to 23 more samples that can be run per day or a difference of between 24 (no multiplexing) and 15 days (with multiplexing) per 1000 samples.



**Fig. 8.** Dual high-pressure mixed binary gradient elution HPLC system. The plumbing schematic for the HPLC system used for high-throughput chromatography is shown. The valves are configured so that column 2 is in-line with the mass spectrometer, and valve 3 serves as a bypass valve to waste, preventing effluent from reaching the mass spectrometer during any period of non-data collection.

More complicated front-end chromatography systems with more than two binary pump HPLC systems require a more complicated system of valves including a multiposition selector valve. We have chosen to incorporate a bypass valve into our chromatographic systems that directs the HPLC stream to waste during any time when there is no data collection occurring [28], which helps keep the mass spectrometer and its source clean. This setup also requires that the autosampler or system controller have suitable relay-type outputs for controlling the valves and starting data collection on the mass spectrometer. The dual high-pressure binary systems we use have four contact closure outputs used for this purpose: one to start data collection on the mass spectrometer, two to control the valves that switch between the HPLC systems and columns, and one to control the additional bypass valve. Valves such as these that can accommodate lower flow rates (in the nano-flow range) and higher pressures (UPLC) are also more expensive to purchase and maintain.

#### Mass spectrometer considerations

We purchase mass spectrometers based on their sensitivity to the compounds in our assay. It is necessary to be able to detect a number of MRM pairs to accurately quantify the compounds of interest. With an average peak width of 30 s in our chromatographic runs, a data sampling rate of 1.5 s (the time it takes for the mass spectrometer to cycle through every MRM pair) provides adequate digitization (more than 15 data points over each peak). For a typical Gaussian-shaped peak, we recommend collecting at least 10–12 data points over the duration of the peak for accurate digitization [43]. More sensitive high-end instruments allow for faster sampling rates, permitting the use of thinner (low volume) chromatographic peaks, such as those from UPLC. The trade-off for the thinner UPLC peaks is a faster, more sensitive mass spectrometry instrument with the possibility of some uncertainty in the quantitation if an adequate data sampling rate can barely be maintained. Faster scan rates at higher resolutions on newer instrumentation increase the amount of MRM data that can be collected over time. Higher order scanning (MS3, MS4, etc.) aids in structural confirmation, though sometimes at the expense of time

needed to collect enough data for accurate MRM quantitation. Widely available triple-quadrupole instruments, because of their specificity and sensitivity, are a must. We envision each MRM pair as a specific detector for each individual compound. Thus, if we require confirmation of the identity of a compound we typically collect more than one MRM transition for that compound. Matching chromatographic retention time and the ratio of the signals between two (or more) distinct MRM channels for the same analyte is a powerful argument to confirm identity. This also brings to light the issues with the sensitivity range that can be addressed by the careful selection of MRM pairs. Some fatty acids, such as the precursor (unoxidized) species (e.g., linoleic and arachidonic acid), are present at much higher concentrations than their oxidized products. Selection of an alternate MRM pair in which the precursor or product ion is not the base peak (at 100% intensity) allows for the quantitation of compounds when one or another MRM pair reaches saturation levels in the mass spectrometer detector. This lower abundance channel is then used for quantitation. Linearity is determined as the ratio of the concentration to a stable-isotope-labeled fatty acid of similar structure and the peak area ratio of the MRM pair from the native fatty acid derivative to the corresponding MRM pair from the isotopologue standard.

#### Summary and conclusion

Rapid and efficient high-throughput LC-MS/MS analysis of oxidized fatty acid derivatives is feasible. The sample preparation entails addition of stable isotope synthetic standards, basic hydrolysis, and liquid-liquid phase extraction. Specific oxidized free fatty acids and their precursors in the extract are separated through a reverse-phase C18 column using acidified methanol/water as the mobile phase and detected by multiple-reaction monitoring using negative ion ESI-MS/MS. In the examples provided, more than 20 different oxidized fatty acids and their precursors were analyzed, including hydroxyoctadecadienoic acids, oxo-octadecadienoic acids, hydroxyeicosatetraenoic acids, epoxyeicosatrienoic acids, oxo-eicosatetraenoic acids, 9 $\alpha$ ,11 $\alpha$ ,

15S-trihydroxy-prosta-5Z,13E-dien-1-oic acid, and their precursors (linoleic acid and arachidonic acid). The internal standard PGF<sub>2α</sub>-d<sub>4</sub> was used for the calibration of PGF<sub>2α</sub>, and 15-HETE-d<sub>8</sub> was used for the calibration of the other lipids monitored. This LC-MS/MS method enables high sample throughput (more than 20 specific lipid peroxidation products may be analyzed from a single injection) and is optimal for obtaining a complex lipid oxidation profile. The recovery of oxidized fatty acid derivatives extracted from plasma under the methods employed is typically over 85%, except for PGF<sub>2α</sub>. All the aforementioned specific lipid peroxidation products have been detected in human plasma from healthy subjects and found to be above the limit of quantitation. For intraday precision, CVs ranged from 3.3 to 10% and accuracy ranged from 99 to 109%. For the interday precision, CVs ranged from 3.4 to 11% and accuracy ranged from 93 to 108%. This LC-MS/MS method is specific, sensitive, reproducible, and practical for analyses of lipid peroxidation products in human plasma and F<sub>2</sub>-isoprostanes in urine.

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