

# Quantitative Profiling Method for Oxylipin Metabolome by Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry

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Cyclooxygenase, lipoxygenase, and epoxygenase derived oxylipins, especially eicosanoids, play important roles in many physiological processes. Assessment of oxidized fatty acid levels is important for understanding their homeostatic and pathophysiological roles. Most reported methods examine these pathways in isolation. The work described here employed a solid phase extraction-liquid chromatography-electrospray ionization MS/MS (SPE-LC-ESI MS/MS) method to monitor these metabolites. In 21 min, 39 oxylipins were quantified along with eight corresponding internal standards. The limits of quantification were between 0.07 and 32 pg (20 pM–10 nM). Finally, the validated method was used to evaluate oxylipin profiles in lipopolysaccharide-exposed mice, an established septic inflammatory model. The method described here offers a useful tool for the evaluation of complex regulatory oxylipin responses in *in vitro* or *in vivo* studies.

Oxylipins are a group of oxidized metabolites of polyunsaturated fatty acids, which perform a variety of functions. Here, the term oxylipin includes the cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP) derived metabolites of polyunsaturated fatty acids (Figure 1). These oxylipins play an important role in regulating cell proliferation, apoptosis, tissue repair, blood clotting, blood vessel permeability, inflammation, immune cell behavior, and other biologies. The best characterized metabolites are prostaglandins and leukotrienes,<sup>1</sup> which are potent eicosanoid lipid mediators derived largely from phospholipase-released arachidonic acid that are involved in numerous homeostatic biological functions and inflammation. Recently, eicosanoid biology has extended beyond the prostaglandins and leukotrienes. The hydroxyl (hydroxy eicosatetraenoic acid; HETE)<sup>2–4</sup> and epoxy (epoxyeicosatetraenoic acid; EET)<sup>5</sup> metabolites of arachidonic acid and lipoxin<sup>6</sup> molecules are also recognized as important biological mediators. EETs demonstrate anti-inflammatory proper-

ties,<sup>7</sup> while lipoxins are considered mediators in the resolution phase of inflammation.<sup>8–11</sup> Similarly, several linoleic acid metabolites in this pathway are also regarded as biologically active compounds. Leukotoxin (9(10)-epoxy-12Z-octadecenoic acid, 9,10 EpOME) and its regioisomer have been associated with multiple organ failure and adult respiratory distress syndrome seen in severe burn patients while the corresponding diols (9,10-dihydroxy-12Z-octadecenoic acid, 9,10 DHOME; 12,13-dihydroxy-octadecenoic acid, 12,13 DHOME) were proven to be even more toxic.<sup>12</sup> In healthy individuals, these metabolites may be endogenous chemical mediators regulating vascular permeability and inflammation. Due to the importance of these mediators, the enzymes responsible for their biosynthesis and metabolism have become therapeutic targets.<sup>13,14</sup> Development of accurate, sensitive, rapid, and specific methods for determining oxylipin levels will facilitate an understanding of the biological functions of these lipid mediators.

Given that these lipid mediators are within the same cascade and are not independent of each other, valuable information can be gained by examining the ratios or patterns of oxylipins or stable analogs. For example, the thromboxane A<sub>2</sub> to prostacyclin ratio (TXA<sub>2</sub>/PGI<sub>2</sub>) is related to the rate of blood clotting<sup>15,16</sup> while 20 HETE and EETs work, in part, in a compensatory fashion.<sup>17</sup> In addition, these lipid mediators fluctuate with physiological or

(7) Node, K.; Huo, Y.; Ruan, X.; Yang, B.; Spiecker, M.; Ley, K.; Zeldin, D. C.; Liao, J. K. *Science* **1999**, *285*, 1276–1279.

(8) Lawrence, T.; Willoughby, D. A.; Gilroy, D. W. *Nat. Rev. Immunol.* **2002**, *2*, 787–795.

(9) Serhan, C. N. *Annu. Rev. Immunol.* **2007**, *25*, 101–137.

(10) Serhan, C. N.; Chiang, N.; Van Dyke, T. E. *Nat. Rev. Immunol.* **2008**, *8*, 349–361.

(11) Serhan, C. N.; Yacoubian, S.; Yang, R. *Annu. Rev. Pathol.* **2008**, *3*, 279–312.

(12) Moghaddam, M. F.; Grant, D. F.; Cheek, J. M.; Greene, J. F.; Williamson, K. C.; Hammock, B. D. *Nat. Med.* **1997**, *3*, 562–566.

(13) Schmelzer, K. R.; Kubala, L.; Newman, J. W.; Kim, I. H.; Eiserich, J. P.; Hammock, B. D. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 9772–9777.

(14) Monti, J.; Fischer, J.; Paskas, S.; Heinig, M.; Schulz, H.; Gosele, C.; Heuser, A.; Fischer, R.; Schmidt, C.; Schirdewan, A.; Gross, V.; Hummel, O.; Maatz, H.; Patone, G.; Saar, K.; Vingron, M.; Weldon, S. M.; Lindpaintner, K.; Hammock, B. D.; Rohde, K.; Dietz, R.; Cook, S. A.; Schunck, W. H.; Luft, F. C.; Hubner, N. *Nat. Genet.* **2008**, *40*, 529–537.

(15) Bouaziz, A.; de Fiequelmont-Loizos, M. M.; Richert, A.; Caprani, A. *Thromb. Res.* **1998**, *90*, 279–289.

(16) Beitz, A.; Taube, C.; Beitz, J.; Goos, H.; Graff, J.; Nohring, J.; Lindenau, K. F.; Mest, H. J. *Biomedica Biochimica Acta* **1988**, *47*, S149–S152.

(17) Luria, A.; Weldon, S. M.; Kabcenell, A. K.; Ingraham, R. H.; Matera, D.; Jiang, H.; Gill, R.; Morisseau, C.; Newman, J. W.; Hammock, B. D. *J. Biol. Chem.* **2007**, *282*, 2891–2898.

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(1) Funk, C. D. *Science* **2001**, *294*, 1871–1875.

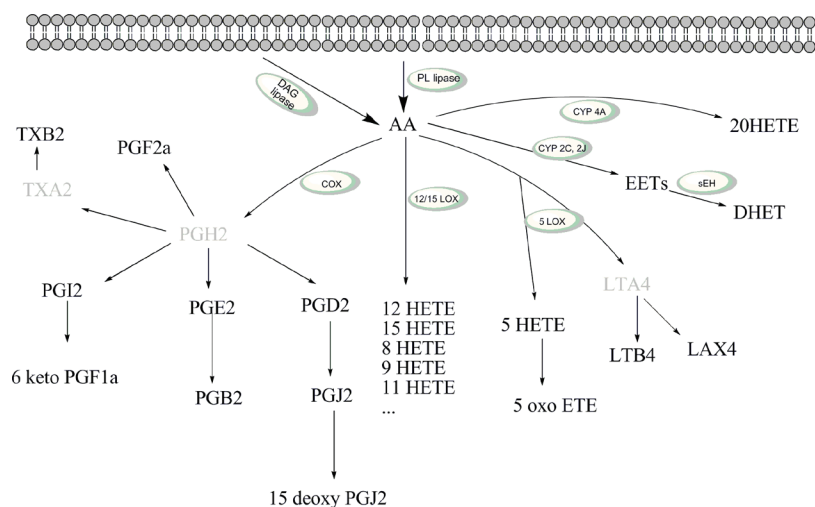
(2) Roman, R. J. *Physiol. Rev.* **2002**, *82*, 131–185.

(3) Kroetz, D. L.; Zeldin, D. C. *Curr. Opin. Lipidol.* **2002**, *13*, 273–283.

(4) Jacobs, E. R.; Zeldin, D. C. *Am. J. Physiol.* **2001**, *280*, H1–H10.

(5) Spector, A. A.; Fang, X.; Snyder, G. D.; Weintraub, N. L. *Prog. Lipid Res.* **2004**, *43*, 55–90.

(6) Serhan, C. N.; Hamberg, M.; Samuelsson, B. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 5335–5339.



**Figure 1.** Three main branches of the arachidonic and linoleic acid cascades: DAG, diacylglycerol; PL, phospholipid. The compounds in gray font are the unstable metabolites.

pathological status.<sup>18</sup> It is believed that these oxylipins are part of a complex regulatory network. Thus, measuring only one or several oxylipins may not be sufficient to explain a biological phenomenon. By examining the flux of a large number of metabolites across cellular systems, we can begin to investigate not only how levels of the metabolites but also how the profile of the metabolites determine the physiological phenotype.<sup>19–21</sup> This is defined as metabolomics or the identification and quantification of all metabolites in a biological system.<sup>22</sup> Therefore, a comprehensive and robust oxylipin profiling method is vital to advance research in the field of regulatory lipids.

Oxylipins are products of a few fatty acid species and represent the addition of various oxygen species resulting in molecules with similar structures, chemistries, and physical properties. Many of them are isomers, which makes the identification and quantitation of all oxylipins in a single biological sample a challenging task. In addition, most compounds are present at low concentrations, but some of these compounds can vary in concentration by more than 3 orders of magnitude. Some of the methods to measure subsets of these oxylipins include ELISA,<sup>23</sup> GC<sup>24</sup> or GC/MS,<sup>25,26</sup> LC/UV, LC/MS,<sup>27–30</sup> and LC/MS/MS.<sup>31–35</sup> LC/MS/MS currently is the most powerful tool because of its specificity and

sensitivity. Unfortunately, most of the published methods only analyze a small portion of the known oxylipins.<sup>36,37</sup> This is especially true for the eicosanoids<sup>28</sup> where the arachidonate cascade is the target of over 75% of the world pharmaceuticals.

In this paper, we describe a novel sensitive method for the detection and quantification of oxylipins in samples of serum and bronchiolar alveolar lavage fluid (BALF) that employs solid phase extraction (SPE), HPLC separation, and ESI-MS/MS in multiple reaction-monitoring (MRM) mode. This method is quantitative for products of multiple enzymatic pathways, which allows a more complete assessment of localized and systemic pathological changes. To the best of our knowledge, it is the most comprehensive oxylipin profiling method with high sensitivity in a single analysis. The general approach is applicable to monitoring a variety of oxylipins in various matrixes.

## EXPERIMENTAL SECTION

**Chemicals.** Oxylipins were either synthesized or purchased from Cayman Chemical (Ann Arbor, MI), Larodan Fine Lipids (Malmö, Sweden), and Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). Cayman Chemicals provided ( $\pm$ )-12(13)-epoxy-9Z-octadecenoic acid (12, 13 EpOME); ( $\pm$ )-9,10 EpOME; 9, 10 DHOME; ( $\pm$ )-13-hydroxy-9Z,11E-octadecadienoic acid (13 HODE); ( $\pm$ )-9-hydroxy-10E,12Z-octadecadienoic acid (9 HODE); 13-keto-9Z,11E-octadecadienoic acid (13 oxo ODE); 9-oxo-10E,12Z-octadecadienoic acid (9 oxo ODE); 6-oxo-9S,11R,15S-trihydroxy-13E-prostenoic acid (6-keto PGF<sub>10</sub>); thromboxane B<sub>2</sub> (TXB<sub>2</sub>);

- (18) Levy, B. D.; Clish, C. B.; Schmidt, B.; Gronert, K.; Serhan, C. N. *Nat. Immunol.* **2001**, *2*, 612–619.  
 (19) Kell, D. B. *Expert Rev. Mol. Diagn.* **2007**, *7*, 329–333.  
 (20) Oresic, M.; Vidal-Puig, A.; Hanninen, V. *Expert Rev. Mol. Diagn.* **2006**, *6*, 575–585.  
 (21) Morris, M.; Watkins, S. M. *Curr. Opin. Chem. Biol.* **2005**, *9*, 407–412.  
 (22) Dettmer, K.; Aronov, P. A.; Hammock, B. D. *Mass Spectrom. Rev.* **2007**, *26*, 51–78.  
 (23) Quinn, J. V.; Bilgrami, S.; Seidel, G. J.; Slotman, G. J. *Shock* **1996**, *6*, 142–149.  
 (24) Newman, J. W.; Hammock, B. D. *J. Chromatogr., A* **2001**, *925*, 223–240.  
 (25) Leis, H. J.; Malle, E.; Mayer, B.; Kostner, G. M.; Esterbauer, H.; Gleispach, H. *Anal. Biochem.* **1987**, *162*, 337–344.  
 (26) Werner, K.; Schaefer, W. R.; Schweer, H.; Deppert, W. R.; Karck, U.; Zahradnik, H. P. *Prostaglandins, Leukotrienes Essent. Fatty Acids* **2002**, *67*, 397–404.  
 (27) Liminga, M.; Oliw, E. *Lipids* **2000**, *35*, 225–232.  
 (28) Deems, R.; Buczynski, M. W.; Bowers-Gentry, R.; Harkewicz, R.; Dennis, E. A. *Methods Enzymol.* **2007**, *432*, 59–82.  
 (29) Nithipatikom, K.; Grall, A. J.; Holmes, B. B.; Harder, D. R.; Falck, J. R.; Campbell, W. B. *Anal. Biochem.* **2001**, *298*, 327–336.  
 (30) Bylund, J.; Ericsson, J.; Oliw, E. H. *Anal. Biochem.* **1998**, *265*, 55–68.

- (31) Takabatake, M.; Hishinuma, T.; Suzuki, N.; Chiba, S.; Tsukamoto, H.; Nakamura, H.; Saga, T.; Tomioka, Y.; Kurose, A.; Sawai, T.; Mizugaki, M. *Prostaglandins, Leukotrienes Essent. Fatty Acids* **2002**, *67*, 51–56.  
 (32) Rinne, S.; Ramstad Kleiveland, C.; Kassem, M.; Lea, T.; Lundanes, E.; Greibrokk, T. *J. Sep. Sci.* **2007**, *30*, 1860–9.  
 (33) Hishinuma, T.; Suzuki, K.; Saito, M.; Yamaguchi, H.; Suzuki, N.; Tomioka, Y.; Kaneko, I.; Ono, M.; Goto, J. *Prostaglandins, Leukotrienes Essent. Fatty Acids* **2007**, *76*, 321–329.  
 (34) Newman, J. W.; Watanabe, T.; Hammock, B. D. *J. Lipid Res.* **2002**, *43*, 1563–1578.  
 (35) Cao, H.; Xiao, L.; Park, G.; Wang, X.; Azim, A. C.; Christman, J. W.; van Breemen, R. B. *Anal. Biochem.* **2008**, *372*, 41–51.  
 (36) Masoodi, M.; Mir, A. A.; Petasis, N. A.; Serhan, C. N.; Nicolaou, A. *Rapid Commun. Mass Spectrom.* **2008**, *22*, 75–83.  
 (37) Serhan, C. N. *Prostaglandins Other Lipid Mediators* **2005**, *77*, 4–14.

prostaglandin B<sub>2</sub> (PGB<sub>2</sub>); prostaglandin D<sub>2</sub> (PGD<sub>2</sub>); prostaglandin E<sub>2</sub> (PGE<sub>2</sub>); 9S,11R,15S-trihydroxy-5Z,13E-prostadienoic acid (PGF<sub>2α</sub>); 11-oxo-5Z,9,12E,14E-prostatetraenoic acid (15 deoxy-PGJ<sub>2</sub>); 5-hydroxyeicosatetraenoic acid (5 HETE); 8 HETE; 9 HETE; 11 HETE; 12 HETE; 15 HETE; 20 HETE; 15-oxo-eicosatetraenoic acid (15 oxo-ETE); 5-oxo-ETE; 14,15-epoxy-5Z,8Z,11Z-eicosatrienoic acid (14, 15 EET); 11,12-epoxy-5Z,8Z,14Z-eicosatrienoic acid (11,12 EET); 8,9-epoxy-5Z,11Z,14Z-eicosatrienoic acid (8, 9 EET); 5,6-epoxy-8Z,11Z,14Z-eicosatrienoic acid (5,6 EET); 14,15-dihydroxy-5Z,8Z,11Z-eicosatrienoic acid (14, 15 DHET); 11,12-dihydroxy-5Z,8Z,14Z-eicosatrienoic acid (11, 12 DHET); 8,9-dihydroxy-5Z,11Z,14Z-eicosatrienoic acid (8, 9 DHET); 5,6-dihydroxy-8Z,11Z,14Z-eicosatrienoic acid (5, 6 DHET); leukotriene- B<sub>4</sub> (LTB<sub>4</sub>). Larodan Fine Lipids provided 9,10,13-trihydroxyoctadecenoic acid (9,10,13 TriHOME) and 9,12,13 TriHOME. 5S,6R,15S-trihydroxy-7E,9E,11Z,13E-eicosatetraenoic acid (lipoxin A4) was purchased from Biomol.

The following compounds were synthesized in house: 1-cyclohexyl-dodecanoic acid urea (CUDA); 10,11 dihydroxyheptadecanoic acid (10,11 DHHep); 10,11 dihydroxynonadecanoic acid (10,11-DHN); 11,12, 15 trihydroxy eicosatrienoic (11,12,15 THET); 19 HETE.<sup>34,38,39</sup> Oasis HLB 60 mg SPE cartridges were purchased from Waters Co. (Milford, MA). Acetonitrile, methanol, ethyl acetate, phosphoric acid, and glacial acetic acid of HPLC grade or better were purchased from Fisher Scientific (Pittsburgh, PA). All other chemical reagents were purchase from Sigma (St. Louis, MO).

**Internal Standards.** Two different types of compounds (Table S-6 in the Supporting Information) were used as internal standards. Type I internal standards were added to samples before extraction to mimic the extraction of prostaglandins, diols, epoxides, and other oxylipins. The type I internal standards include 6-keto-PGF<sub>1α</sub>-d4; PGE<sub>2</sub>-d4; 10,11 DHHep; 20 HETE-d6; 9 (S) HODE-d4; 5 HETE-d8; 11,12 EET-d8; and nonendogenous odd chain length monounsaturated fatty acids (10,11 dihydroxynonadecanoic acid, 10,11-DHN). Type II internal standard was added at the last step before analysis to account for changes in volume and instrument variability. A synthetic acid, 1-cyclohexyl-dodecanoic acid urea (CUDA), was selected as type II internal standard.

The analytes were linked to their corresponding type I internal standards for the purpose of quantification.

**Standard Curve Preparation.** Six different batches of standard mixtures were dissolved in methanol with 800nM CUDA to act as the calibration solutions (Table S-1 in the Supporting Information). These calibration solutions were subaliquoted into Wheaton prescored gold-band amber ampules (Fisher Scientific), sealed under nitrogen, and stored at -20 °C. The lowest concentration was diluted further using type II internal standard (IS) solutions for limit of quantification measurement.

**Solid Phase Extraction.** Prior to extraction, Waters Oasis-HLB cartridges were washed with ethyl acetate (2 mL), methanol (2 × 2 mL), and 95:5 v/v water/methanol with 0.1% acetic acid (2 mL). Serum and BALF aliquots (250 μL) were then loaded onto the cartridges and spiked with 10 μL of a 400nM type I internal standard solution. Cartridges were washed with 1.5 mL of 95:5

v/v water/methanol with 0.1% acetic acid; the aqueous plug was pulled from the SPE cartridges with high vacuum, and SPE cartridges were further dried with low vacuum about 20 min. SPE cartridges were eluted into tubes with 0.5 mL of methanol followed by 2 mL of ethyl acetate into 4 mL tubes containing 6 μL of 30% glycerol in MeOH as a trap solution. The volatile solvents were removed using a Speed-Vac until only the trap solution of 2 μL of glycerol remained. The residues were reconstituted in 100 μL of methanol containing 800 nM of internal standard (CUDA). The samples were then mixed on a vortexer for 5 min, transferred to autosampler vials with low volume inserts, and stored at -20 °C until analysis.

**LC/MS/MS Analysis.** The liquid chromatography system used for analysis was an Agilent 1200 SL liquid chromatography series (Agilent Corporation, Palo Alto, CA). The autosampler was kept at 4 °C. Liquid chromatography was performed on a Pursuit Plus C18 2.0 × 150 mm, 5 μm column (Varian Inc. Palo Alto, CA). Mobile phase A was water with 0.1% glacial acetic acid. Mobile phase B consisted of acetonitrile/methanol (84:16) with 0.1% glacial acetic acid. Gradient elution was performed at a flow rate of 400 μL/min. Chromatography was optimized to separate all analytes in 21 min. The gradient is given in Table S-2 in the Supporting Information.

The column was connected to a 4000 QTrap tandem mass spectrometer (Applied Biosystems Instrument Corporation, Foster City, CA) equipped with an electrospray source (Turbo V). The instrument was operated in negative MRM mode. Individual analyte standards were infused into the mass spectrometer and MRM transitions and source parameters optimized for that analyte. Source parameters, were then reoptimized under flow injection acquisition (FIA) mode (infusion of analytes into the column eluent flow). The LC gradient was selected to distribute analytes among various periods allowing for an increase in dwell time and lowering the limits of detection. In the whole optimization process, the two most abundant transitions were kept for each standard. While at the last step, the more specific or sensitive transitions were selected to avoid interference with the close isomers and to obtain better detection limits. The optimized mass spectrometric parameters are given in Table S-3 in the Supporting Information.

**Method Validation.** Limit of quantification (LOQ), linearity, inter- and intraday accuracy, recovery, and precision were determined for the profiling method.<sup>40</sup>

**LOQ and Linearity Range.** The six batches of standard mixtures and three further dilutions were used to determine the LOQ and linearity range. The calibration curves were calculated by least-squares linear regression using an 1/x weighting factor.<sup>41</sup> The standard concentrations were back-calculated from constructed calibrations curves for each analyte.

**Accuracy and Precision.** The accuracy and precision of the method were established by analyzing quality control (QC) samples of each analyte. These QC samples consisted of 10 μL of S2, S3, S5, and S6 and 190 μL of 100 mM phosphate buffer saline (PBS) solution. Three replicates of each sample were analyzed together with a complete set of calibration standards in three

(38) Kim, I. H.; Morisseau, C.; Watanabe, T.; Hammock, B. D. *J. Med. Chem.* **2004**, *47*, 2110-2122.

(39) Watanabe, T.; Morisseau, C.; Newman, J. W.; Hammock, B. D. *Drug Metab. Dispos.* **2003**, *31*, 846-853.

(40) *Guidance for Industry-Bioanalytical Method Validation*; Food and Drug Administration (FDA); 2001, <http://www.fda.gov/cder/guidance/4252fnl.pdf>.

(41) Almeida, A. M.; Castel-Branco, M. M.; Falcao, A. C. *J. Chromatogr.* **2002**, *774*, 215-222.

analytical runs. The intraday accuracy was determined as the percent difference between the mean concentration per analytical run and the expected concentration. The interday accuracy was determined as the percent difference between different days. The coefficient of variation provided the measure of intra- and interday precision.

**Recovery.** Method recovery determines the amount of analyte spiked in the matrix that can be recovered and quantified. The phosphate buffer saline (PBS) solutions spiked with different analyte concentrations were extracted by SPE and analyzed by LC/MS/MS to determine recovery for each analyte. This was repeated for different sample matrixes.

**Sample Collection and Analysis.** BL57/6 mice (8-week old) were obtained from Charles River Laboratories (Wilmington, MA) and housed in metabolic chambers with ad libitum food and water for four days, until they were given either saline or lipopolysaccharide (LPS). A 10 mg/kg dose of LPS or the equivalent volume of saline was administered either intranasally (i.n.) or intraperitoneally (i.p.). The intranasal injections were administered while a slightly anesthetized mouse was inhaling, to ensure that the LPS was inhaled into the lungs. After this time, the animals were given water but restricted from food for 24 h and then euthanized by overdose of pentobarbital. Each group had three replicate animals. Animals were handled in accordance with standards established by the University of California, Davis, Animal Care and Use Committee.

Blood was collected via cardiac puncture 24 h after LPS administration. The BALF was collected using 2 mL of a 5% dextrose solution. Both the blood and BALF were centrifuged, and the supernatants were transferred to polypropylene tubes and stored at  $-20\text{ }^{\circ}\text{C}$  until analysis. Triphenylphosphine (TPP) and butylated hydroxytoluene (BHT) (0.2% w/w) were added to the matrixes at the time of sample collection. TPP was used to reduce peroxides to their monohydroxy equivalents, and BHT was used to quench radical catalyzed reactions. Both reagents prevent peroxy radical propagated transformations of polyunsaturated fatty acids.<sup>42,43</sup> Serum and BALF aliquots (250  $\mu\text{L}$ ) were extracted and analyzed as described above.

## RESULTS AND DISCUSSION

**Method Development.** As mentioned above, there are three major challenges in development of an optimal oxylipin profiling method. First, the structural similarity between members of the oxylipin metabolome, particularly the isomers, requires excellent chromatographic separation especially for compounds that have identical transitions. Second, the endogenous concentrations of these oxylipins are very low and require careful optimization at every step to achieve the best detection limit. When only including more oxylipins in a single method without providing enough sensitivity, the measurement of the oxylipins in the real samples will fail. Lastly, in order to develop a high throughput method, the gradient and ion transitions must be optimized to accomplish a short run time. Additional problems arise due to the instability of some analytes and the complexity of some matrixes.

**Critical Separation Pairs.** Compounds which have identical molecular compositions, similar fragmentation, and close retention

times were considered to be “critical separation pairs”. In this method, the following pairs were regarded as critical separation pairs: 9,12,13 TriHOME/9,10,13 TriHOME; PGE2/PGD2; 14,15-DHET/11,12DHET; 19 HETE/20HETE; 13 HODE/9-HODE; 13 oxo-ODE/9 oxo-ODE; 12,13 EpOME/9,10 EpOME; 11,12 EET/8,9 EET; 8,9 EET/5,6 EET. In the LC optimization, column temperature and gradients were adjusted to accomplish complete separation of these critical separation pairs. The final gradient is given in Table S-2 in the Supporting Information. The typical chromatogram and PGE2/PGD2 and 19HETE/20HETE extraction ion chromatograms are shown in Figure 2.

**Mass Spectrometry Optimization.** After LC parameters were optimized, MS parameters were optimized to attain the lowest detection limit. Tables S-3 and S-4 in the Supporting Information provide the optimized mass transitions and mass spectrometric parameters.

Most of these fragments are produced from cleavages adjacent to double bonds and/or hydroxyl moieties. In general, these transitions were selected to yield the greatest sensitivity and selectivity for MRM quantification. In certain cases, more characteristic fragmentation ions were chosen to gain selectivity at the expense of sensitivity. For example, although 8,9 EET gets the higher response when transition 319.2/167 is used, the more specific transition 319.2/123 was selected to avoid the interference from 11,12 EET in transition 319.2/167.

In addition, several compounds with MRM transitions that are different from other published data were compared (Table 1).<sup>28,44–47</sup> The result shows that the careful selection of transitions could improve sensitivity several fold by optimizing for sensitivity or for selectivity.

At first, DP, declustering potentials, were regarded as compound dependent parameters, which were optimized under direct infusion of the single compounds. However, they also proved to be affected by the solvent composition. Therefore, all the transitions were compared with different DPs (range from  $-30$  to  $-100$  V) across the LC gradient. Nearly all of these compounds achieved the best sensitivities when DPs were set at  $-60$  V.

Dwell time was found to play a very important role in increasing the signal-to-noise ratio with a shorter dwell time resulting in a much higher noise. On the other hand, the longer dwell time used made it necessary to break the whole acquisition into several periods to keep an adequate number of measurements across a peak ( $>10$  points per peak). The dwell times and the periods needed to be balanced in the optimization process. In this method, the dwell times were set above 25 ms with three acquisition periods.

**Method Validation.** This method was designed to be applicable to multiple biological matrixes; therefore, extraction and cleanup procedures were developed in 100 mM phosphate-buffered

(42) Nourooz-Zadeh, J.; Tajaddini-Sarmadi, J.; Wolff, S. P. *Anal. Biochem.* **1994**, *220*, 403–409.

(43) Carlin, G. J. *Free Radical Biol. Med.* **1985**, *1*, 255–261.

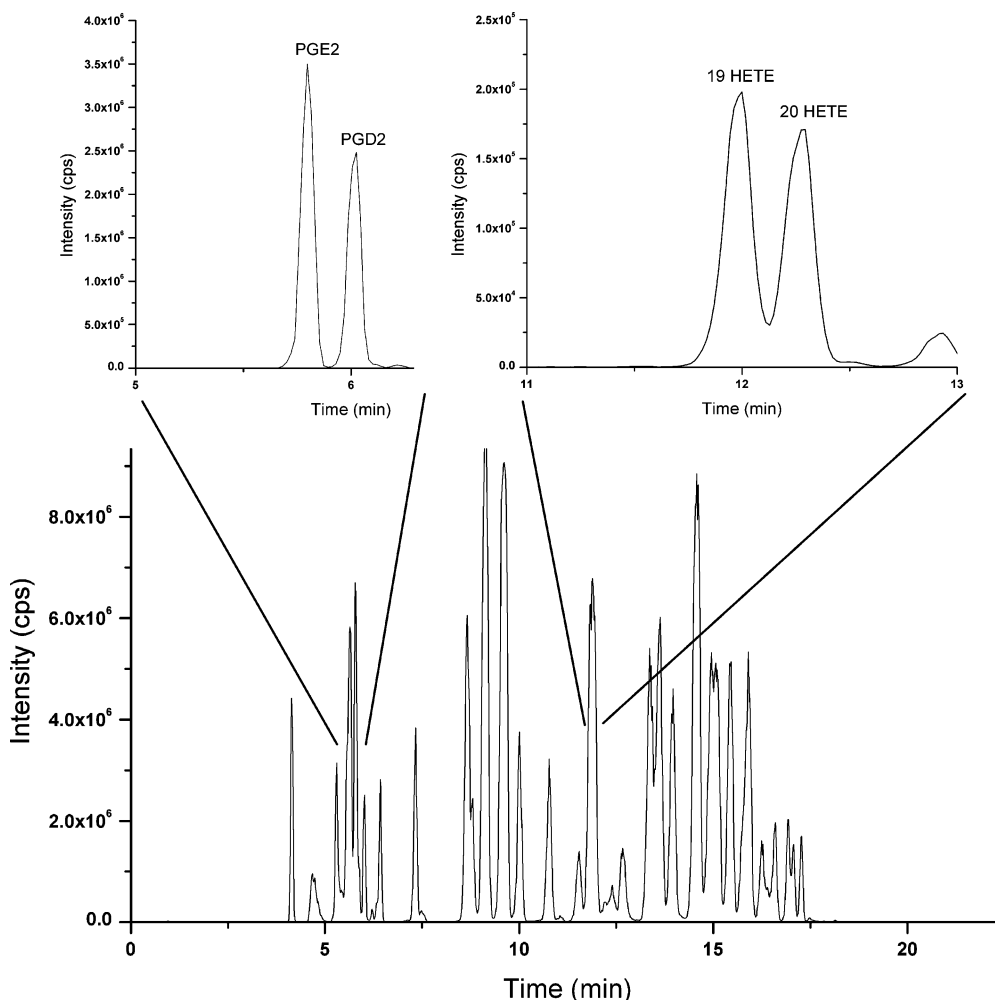
(44) Blewett, A. J.; Varma, D.; Gilles, T.; Libonati, J. R.; Jansen, S. A. *J. Pharm. Biomed. Anal.* **2008**, *46*, 653–662.

(45) Yue, H.; Jansen, S. A.; Strauss, K. I.; Borenstein, M. R.; Barbe, M. F.; Rossi, L. J.; Murphy, E. *J. Pharm. Biomed. Anal.* **2007**, *43*, 1122–1134.

(46) Kita, Y.; Takahashi, T.; Uozumi, N.; Shimizu, T. *Anal. Biochem.* **2005**, *342*, 134–143.

(47) Zhang, J. H.; Pearson, T.; Matharoo-Ball, B.; Ortori, C. A.; Warren, A. Y.; Khan, R.; Barrett, D. A. *Anal. Biochem.* **2007**, *365*, 40–51.

(48) Dieter, P.; Kolada, A.; Kamionka, S.; Schadow, A.; Kaszkin, M. *Cell. Signalling* **2002**, *14*, 199–204.



**Figure 2.** Total ion chromatogram (TIC) of 49 oxylipins standards in the oxylipin metabolome. In 21 min, all 49 compounds could be measured accurately. The extracted ion chromatograms (XIC) in the insets illustrate that two critical separation pairs, PGE2/PGD2 and 19 HETE/20 HETE, could be separated well. cps: counts per second.

**Table 1. Comparison of Selected MRM Transitions with the Ones from Literature<sup>a</sup>**

compound	transitions	signal-to-noise (S3)	signal-to-noise (QC4)
PGD2 <sup>b</sup>	351/271	322	452
PGD2 <sup>c</sup>	351/189	247	307
6 keto PGF1 $\alpha$ <sup>b</sup>	369/163	28.0	66.3
6 keto PGF1 $\alpha$ <sup>c</sup>	369/207	16.0	33.7
TXB2 <sup>b</sup>	369/169	282	739
TXB2 <sup>c</sup>	369/195	73.9	203
15 HETE <sup>b</sup>	319/219	585	979
15 HETE <sup>c</sup>	319/175	221	788

<sup>a</sup> The MS settings for both MRM transitions were optimized representatively before comparison using direct infusion of the standards. <sup>b</sup> MRM transitions used in this study. <sup>c</sup> MRM transitions used in previous literature.

saline (pH 7.4) that contained no endogenous analytes. It was subsequently tested with multiple matrixes.

**LOQ and Linearity.** For this procedure, we defined the limit of quantifications (LOQ) as the amount of sample required to produce a signal-to-noise ratio (S/N) of 10 or greater. Analysis of calibration standards established the LOQs between 0.07 and 32 pg. This is equivalent to 8 pM to 4 nM in a 250  $\mu$ L plasma

sample analyzed at a final volume of 100  $\mu$ L. Table 2 gives the method's LOQ. To the best of our knowledge, it is the most sensitive comprehensive oxylipin profiling method in a single analysis. A recent paper reported the development of an eicosanoid profiling method for 104 unique lipid species.<sup>49</sup> This is a less sensitive method although it covers more analytes. In other words, the LOD of the reported method is much worse than the LOQ of our method, as illustrated by an LOD of PGE2 for their method of 1 pg compared to the LOQ of our method of 0.07. The lower sensitivity may result from insufficient dwell time in their method. This comparison brings up the point that one must often balance the number of analytes in a method against sensitivity for individual metabolites. This brings up the broader challenge to the field of balancing the number of analytes, sensitivity, speed, accuracy, sample size, and other parameters against the biological goals of the analysis.

The linearity of the method was determined by the calibration curves constructed for each analyte. Curves plotted the ratio of the analyte area to its internal standard area against concentration using 1/x weighting factors in the regression.

(49) Balho, V. a.; Buczynski, M. W.; Brown, C. R.; Dennis, E. A. *J. Biol. Chem.* **2009**, In press.

(50) Buczynski, M. W.; Dumlao, S. D.; Dennis, E. A. *J. Biol. Chem.* **2009**, *50*, 1015-1038.

**Table 2. LOQ and Linearity Range**

analytes	limit of quantitation (nM)	limit of quantitation (pg)	linear range <sup>a</sup> (nM)
6k PGF1a d4	2.00	7.47	2–1000
6k PGF1a	0.60	2.22	0.6–1000
TXB2	0.20	0.74	0.2–100
9–12–13 TriHOME	0.02	0.07	2–100
9–10–13 TriHOME	0.02	0.07	0.6–100
PGF2a	0.10	0.35	0.3–500
PGE2-d4	0.02	0.07	0.2–100
PGE2	0.02	0.07	0.2–100
PGD2	0.20	0.70	0.2–1000
11 12 15 THET	1.00	3.53	1–500
Lipoxin A4	0.06	0.21	0.2–100
PGB2/PGJ2	0.07	0.23	3–5000
THF Diols	1.00	3.53	1–500
LTB4	0.06	0.20	0.06–1000
12 13 DHOME	0.20	0.63	2–1000
10 11 DHHep	0.20	0.60	0.2–100
9 10 DHOME	0.20	0.63	0.6–1000
14 15 DHET	0.02	0.07	0.02–100
11 12 DHET	0.06	0.20	0.06–100
8 9 DHET	0.20	0.67	0.2–100
15 deoxy PGJ2	0.04	0.13	0.04–200
19 HETE	5.00	15.96	5–500
20 HETE d6	10.00	32.52	10–1000
20 HETE	5.00	15.96	5–500
5 6 DHET	0.20	0.67	0.2–1000
13 HODE	0.10	0.30	1–500
9 HODE d4	0.60	1.80	0.6–100
9 HODE	0.10	0.30	1–500
10 11 DHN	0.06	0.20	0.06–1000
15 HETE	0.10	0.32	1–500
13 oxo ODE	5.00	14.66	5–500
11 HETE	0.30	0.96	1–500
15 oxo ETE	0.01	0.03	0.1–50
9 oxo ODE	0.30	0.88	0.3–50
12 HETE	0.10	0.32	1–500
8 HETE	1.00	3.19	1–500
9 HETE	0.10	0.32	1–500
5 HETE d8	0.06	0.20	0.2–100
5 HETE	0.10	0.32	1–500
12 13 EpOME	0.20	0.59	0.2–100
14 15 EET	0.30	0.96	0.3–500
9 10 EpOME	0.02	0.06	0.2–100
11 12 EET d8	0.02	0.07	0.2–100
11 12 EET	0.10	0.32	0.1–50
5 oxo ETE	5.00	15.86	5–500
8 9 EET	0.30	0.96	0.3–500
5 6 EET	1.00	3.19	5–500

<sup>a</sup>  $R^2 > 0.998$ .

Regression analysis determined *R* values of 0.999 or greater for each analyte.

**Recovery.** Recoveries range from 88% to 127% (Table S-5 in the Supporting Information). The precision was good (most of the RSDs < 15%), and the extraction efficiencies were stable across low and high concentrations of analytes.

**Accuracy and Precision.** Table 3 lists the accuracy and precision data acquired for intra- and interday analyses. The accuracy and precision were determined using QC samples prepared at four concentrations spanning the entire concentration range measured by the method. Triplicate injections were made daily to determine the method's intraday variability. Accuracy was calculated as the percent difference between the daily mean QC value and the expected value. Precision was calculated as the relative standard deviation of these three daily

values. To determine the interday variability of the method, QC samples were run on three different days to determine the accuracy and precision (Table 3). New calibration curves were run each day in conjunction with the QC samples. The QC samples (QC1–QC4) for each component had greater than 80% accuracy, indicating that the method can be considered accurate and precise across the range of concentrations used.

**Analysis of Biological Samples.** The data summarized in Table 4 (statistically significant changes in each analyte are shown) illustrate the power of this method to define quantitative changes in oxylipin profiles in mice following administration of LPS intranasally (i.n.) or intraperitoneally (i.p.). In these models of murine sepsis, the oxylipin method illustrates the large difference in route of administration changes in serum vs bronchiolar lavage samples. The data also illustrate the power of the method of oxylipin in very small samples. There are numerous conclusions which can be drawn.

The mice that were administered LPS via both intraperitoneal (i.p.) injection and intranasal (i.n.) injection had increased levels of almost all of the detected oxylipins in serum when compared to saline treated animals. The pro-inflammatory mediator PGE2 had the greatest change (~30 fold). These data imply that the common substrate-arachidonic acid is released from lipid layers under inflammatory stimuli. This observation is consistent with research results<sup>48</sup> that the LPS-induced release of arachidonic acid and prostaglandins are regulated in part by phospholipase A2 (PLA2). The biggest change for PGE2 indicated that COX-2, the enzyme largely responsible for PGE2 production in inflammation was induced after LPS administration. These data support our previous study.<sup>13</sup>

Similar fold increases are seen when comparing the serum from the LPS (i.p.) injection and LPS (i.n.) administration. There are six oxylipins that have statistically different concentrations when comparing the i.p. to i.n. injections of LPS (Table 4), which reflect the slight difference between different administration methods.

Intranasal LPS exposure also increased oxylipins locally as indicated by analysis of the BALF. There was more variability in these samples, so fewer metabolites show statistically significant differences (Table 4). The variability is probably due to mucosal ciliary clearance of the LPS, variable distribution following administration, and variable LPS transport within proximal and distal airways. Lower airways can be effectively sealed off in inflamed lungs. There was an increasing trend in LTB4 levels in BALF after LPS exposure although the data were not significant (Table 4). In contrast, LTB4 could not be detected in serum and PGE2 showed a different response between serum and BALF. This reflects that oxylipin profiles may differ dramatically not only temporally but also with location. LTB4 may be a good indicator of inflammation in the airway system.

## CONCLUSIONS

In this paper, we describe the development, validation, and application of a quantitative assay to profile the levels of 39 members of the oxylipin metabolome by solid phase extraction-liquid chromatography-electrospray ionization MS/MS (SPE-LC-ESI MS/MS). Oxylipins are an important group of biologically active compounds. Many of them are important lipid

**Table 3. Accuracy and Precision<sup>a</sup>**

analytes	QC1			QC2			QC3			QC4		
	accuracy	precision	interday	accuracy	precision	interday	accuracy	precision	interday	accuracy	precision	interday
6k PGF1a d4	N.D. <sup>b</sup>	N.D.	N.D.	2.16%	1.48%	2.74%	6.76%	3.64%	0.06%	7.25%	1.63%	8%
6k PGF1a	N.D.	N.D.	N.D.	15.94%	7.84%	7.94%	0.21%	3.31%	4.94%	2.50%	3.38%	7%
TXB2	18.33%	9.78%	13%	2.69%	4.32%	8.54%	1.41%	3.52%	4.46%	6.54%	4.28%	12%
9-10-13 TriHOME	<i>o.r.</i> <sup>c</sup>	<i>o.r.</i>	<i>o.r.</i>	15.00%	16.7%	7.56%	12.75%	2.35%	11.65%	10.08%	4.47%	14%
9-10-13 TriHOME	<i>o.r.</i>	<i>o.r.</i>	<i>o.r.</i>	14.44%	4.6%	19.33%	4.78%	0.75%	6.93%	1.33%	2.30%	4%
PGF2a	10.71%	7.97%	9.5%	16.67%	4.43%	18.00%	13.94%	2.30%	10.91%	2.42%	0.54%	6%
PEE2-d4	7.12%	14.98%	7%	1.33%	2.64%	3.53%	4.00%	2.43%	12.27%	10.00%	5.15%	6%
PEE2	10.67%	8.18%	6.2%	7.66%	8.45%	11.27%	9.56%	2.10%	1.73%	11.11%	4.89%	7%
PGD2	17.7%	11.71%	11.5%	13.6%	9.54%	18.36%	17.14%	5.50%	6.43%	11.31%	3.07%	5%
11 12 15 THET	N.D.	N.D.	N.D.	15.90%	8.18%	8.82%	6.41%	5.83%	10.46%	15.90%	3.46%	2%
Lipoxin A4	16.72%	10.33%	15.5%	7.22%	11.35%	11.33%	6.85%	0.34%	5.33%	11.85%	2.55%	17%
PGB2/PGJ2	<i>o.r.</i>	<i>o.r.</i>	<i>o.r.</i>	12.04%	4.35%	11.35%	5.31%	4.38%	8.37%	2.93%	3.27%	4%
THF Diols	N.D.	N.D.	N.D.	18.33%	11.95%	18.36%	1.85%	8.89%	2.55%	3.03%	1.08%	9%
LTB4	N.D.	N.D.	N.D.	12.78%	12.61%	11.44%	2.96%	2.01%	1.33%	0.19%	1.70%	8%
12 13 DHOME	15.00%	3.64%	9.61%	15.00%	3.11%	11.44%	14.44%	2.91%	13.33%	3.33%	2.34%	6%
10 11 DHHEP	12.97%	10.95%	7.2%	5.24%	12.15%	10.71%	4.40%	1.20%	13.14%	3.93%	2.44%	9%
9 10 DHOME	17.04%	4.76%	11.78%	13.33%	2.92%	16.89%	17.04%	0.91%	15.67%	1.30%	6.02%	10%
14 15 DHET	17.78%	8.11%	16.09%	8.61%	7.53%	0.58%	13.89%	2.96%	0.67%	17.78%	4.60%	5%
11 12 DHET	15.83%	7.76%	13.83%	12.78%	5.64%	1.17%	8.33%	3.28%	8.50%	6.39%	4.48%	10%
8 9 DHET	15.94%	6.79%	10.73%	14.17%	6.40%	6.83%	6.39%	5.93%	8.17%	4.72%	3.42%	9%
15 deoxy PGJ2	18.75%	7.20%	10.08%	10.08%	0.67%	12.30%	16.42%	2.30%	11.05%	3.42%	1.01%	11%
19 HETE	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	4.80%	9.48%	10.56%	8.80%	4.67%	12%
20 HETE d6	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	5.83%	10.41%	2.06%	7.46%	4.96%	12%
20 HETE	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	17.47%	7.28%	4.52%	5.52%	4.38%	5%
5 6 DHET	10.00%	5.12%	8.7%	4.72%	6.43%	6.83%	1.67%	5.15%	9.58%	3.61%	2.78%	10%
13 HODE	13.52%	5.34%	8.67%	1.85%	0.56%	6.89%	1.22%	1.66%	0.18%	9.37%	2.47%	9%
9 HODE d4	N.D.	N.D.	N.D.	0.19%	8.50%	9.18%	6.11%	3.13%	4.67%	1.33%	2.79%	11%
9 HODE	2.48%	5.89%	4.2%	3.44%	1.63%	6.22%	1.96%	0.92%	3.60%	10.63%	5.00%	9%
10 11 DHN	13.42%	3.91%	19.54%	0.73%	6.69%	2.25%	10.10%	3.43%	1.88%	7.81%	5.60%	10%
15 HETE	18.89%	10.18%	15.67%	9.11%	2.50%	2.80%	2.11%	1.86%	2.10%	8.72%	4.66%	10%
13 oxo ODE	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	9.29%	7.23%	11.57%	11.43%	6.09%	11%
11 HETE	10.56%	9.81%	12.52%	17.78%	4.65%	12.50%	12.56%	4.36%	15.30%	8.89%	10.92%	5%
9 oxo ODE	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	1.67%	4.26%	3.29%	14.76%	4.22%	10%
12 HETE	16.94%	10.44%	10.67%	8.94%	6.63%	12.73%	3.50%	1.48%	0.13%	3.11%	3.68%	11%
8 HETE	13.89%	8.80%	10.08%	11.17%	10.57%	9.20%	1.56%	3.77%	0.57%	7.00%	2.48%	12%
9 HETE	9.17%	4.07%	11.97%	9.94%	2.46%	11.37%	4.44%	3.34%	9.53%	12.33%	3.31%	11%
5 HETE d8	7.75%	2.42%	9.00%	8.89%	4.67%	13.42%	11.67%	1.89%	15.00%	8.42%	3.72%	5%
5 HETE	10.83%	4.49%	8.83%	9.83%	7.75%	11.00%	3.50%	2.25%	10.43%	14.72%	2.22%	10%
12 13 EpOME	11.67%	3.68%	9.45%	8.50%	5.69%	10.50%	6.17%	1.63%	12.50%	5.67%	2.92%	9%
14 15 EET	N.D.	N.D.	N.D.	12.00%	17.24%	6.75%	10.38%	1.81%	8.10%	2.54%	4.77%	12%
9 10 EpOME	10.83%	5.96%	9.2%	9.33%	2.62%	9.60%	3.00%	1.86%	8.90%	5.33%	4.27%	7%
11 12 EET d8	11.04%	10.25%	10.13%	11.88%	4.87%	2.75%	5.42%	3.17%	6.13%	11.88%	4.45%	6%
11 12 EET	6.63%	3.47%	7.38%	10.44%	1.78%	6.25%	11.00%	1.83%	7.35%	12.71%	2.41%	12%
8 9 EET	10.63%	7.50%	8.00%	11.42%	8.18%	12.58%	17.08%	9.41%	12.05%	13.21%	2.10%	13%
5 6 EET	N.D.	N.D.	N.D.	<i>o.r.</i>	<i>o.r.</i>	<i>o.r.</i>	6.17%	10.22%	6.60%	9.38%	3.09%	11%
15 oxo ETE	10.37%	14.26%	8.61%	14.00%	9.26%	15.33%	9.44%	4.26%	12.49%	6.37%	9.46%	9%
5 oxo ETE	N.D.	N.D.	N.D.	16.52%	6.91%	14.22%	18.78%	6.45%	2.60%	2.67%	3.58%	7%

<sup>a</sup> Accuracy represents the difference between the measured and expected value (*n* = 3). Precision represents the relative standard deviation of the measurements (*n* = 6). <sup>b</sup> N.D. the concentration is below the LOQ. <sup>c</sup> *o.r.* out of the linear range.

**Table 4. Statistical Significance of Increases in Oxylipins in a Sepsis Murine Model<sup>a</sup>**

compound	serum +/- LPS ( <i>i.p.</i> )	Serum +/- LPS ( <i>i.n.</i> )	serum +LPS <i>i.p./i.n.</i>	BALF +/- LPS ( <i>i.n.</i> )
6 keto PGF1 $\alpha$	2.83	2.61	1.10	1.71
TXB2	2.37	2.18	0.92	2.47
9, 10, 13 TriHOME	3.05	0.89	0.29	9.07
PGE2	29.63	33.94	1.15	0.22
PGD2	0.63	0.25	0.39	N.D.
LTB4	N.D. <sup>b</sup>	N.D.	N.D.	82.50
12, 13 DHOME	4.85	7.64	1.58	21.26
9, 10 DHOME	6.42	11.58	1.80	5.08
14, 15 DHET	4.46	8.66	1.94	6.44
11, 12 DHET	5.41	19.85	3.67	2.92
5,6 DHET	1.71	3.83	2.24	N.D.
13 HODE	11.18	10.34	0.92	1.83
9 HODE	5.09	3.98	0.78	2.66
15 HETE	2.52	1.93	0.77	2.54
11 HETE	6.28	3.21	0.51	4.18
12 HETE	3.67	2.04	0.56	3.63
5 HETE	2.20	2.30	1.05	N.D.
12, 13 EpOME	1.69	3.39	2.01	N.D.
9, 10 EpOME	2.71	3.58	1.32	N.D.

<sup>a</sup> Three biological replicates were used for each group, the samples were acquired after 24 h after LPS challenge. Statistical significance was set at  $p = 0.1$ . Grey = statistically significant change and white = nonsignificant changes. The numbers depict fold increases in individual analytes. <sup>b</sup> N.D. = non detect.

mediators in initiation and resolution of inflammation. The thorough evaluation of the oxylipin metabolome is useful to understand the physiology and pathology behind these processes. In addition, oxylipin metabolomics has already been shown to be useful in elucidation of a compensatory mechanism for homeostatic blood pressure regulation.<sup>17</sup>

To improve the quantitative accuracy, an internal standard (type II internal standard) was chosen to monitor the injection variation and eight type I internal standards were selected to compensate the differences in the extraction and ionization efficiency due to differences in chemical structure and chromatographic elution. When commercially available, we used deuterated internal standards. As more heavy atom standards become available for oxylipins, we can anticipate improved accuracy and precision in the measurement. The validation shows this method meets the criteria for several important parameters. Linearity of each compound was greater than 0.999 over the calibration range of 0.07 to 32 pg (20 pM–10 nM) injected on the column. The accuracy and precision of the method were established by examining the reproducibility of several QC samples over extended times and conditions. Finally, the murine sera and BALF oxylipin profiles and the variations in oxylipins levels treated by LPS were successfully quantified. The results show this method provides a powerful platform to monitor changes in the oxylipin metabolome in systemic (serum) and local systems (BALF) after stimuli. In

addition, the oxylipin profiles also reflected the different response between different administration methods.

The oxylipins are of great importance to a wide range of physiological functions, including inflammatory response and resolution. The ability to relate a physiological state to a specific oxylipin profile increases the understanding of a disease process and should lead to more efficacious therapeutics to treat and prevent inflammatory diseases. This robust method can provide this profiling.

New methods for oxylipin analysis will continually be needed. Currently, well over 100 biologically active oxylipins have been identified,<sup>50</sup> and the number continues to grow indicating that broader coverage of structures is needed. For example multiple oxylipins in the  $\omega$ -3 series appear biologically important. On the other hand, the basal concentrations of several oxylipins are below the detection limit of even the most sensitive methods. Thus, even with careful optimization, there will be a trade off between the number of analytes monitored and the sensitivity. In addition, the chirality of many oxylipins is critical to biological activity, and this method does not address the chirality problem. Coverage, sensitivity, accuracy, precision, and speed must of course be weighed against cost of analysis. For any biological problem, there will be trade offs among these drivers, requiring analytical chemists to adjust their procedures for the goals of the project, develop new techniques to obtain more information from existing



instrumentation, and of course adapt their methods to improved instrumentation.

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#### **SUPPORTING INFORMATION AVAILABLE**

Additional information as noted in text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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