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Targeted lipidomics strategies for oxygenated metabolites of polyunsaturated fatty acids

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Abstract

Oxidation of polyunsaturated fatty acids (PUFA) through enzymatic or non-enzymatic free radical-mediated reactions can yield an array of lipid metabolites including eicosanoids, octadecanoids, docosanoids and related species. In mammals, these oxygenated PUFA mediators play prominent roles in the physiological and pathological regulation of many key biological processes in the cardiovascular, renal, reproductive and other systems including their pivotal contribution to inflammation. Mass spectrometry-based technology platforms have revolutionized our ability to analyze the complex mixture of lipid mediators found in biological samples, with increased numbers of metabolites that can be simultaneously quantified from a single sample in few analytical steps. The recent development of high-sensitivity and high-throughput analytical tools for lipid mediators affords a broader view of these oxygenated PUFA species, and facilitates research into their role in health and disease. In this review, we illustrate current analytical approaches for a high-throughput lipidomic analysis of eicosanoids and related mediators in biological samples.

1. INTRODUCTION

Polyunsaturated fatty acids (PUFA) can be oxygenated by means of enzymatic or free radical-mediated reactions into numerous bioactive oxygenated lipid mediators. One of the best-studied classes of these oxygenated mediators are the *eicosanoids* (from the Greek "eikosa," meaning "twenty"), derivatives of the 20-carbon PUFA arachidonic acid (AA;

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20:4n-6), eicosapentaenoic acid (EPA; 20:5n-3) and dihomo- γ -linolenic acid (DGLA; 20:3n-6). For the purpose of this review, the use of the 'eicosanoid' terminology has been extended to include the oxygenated products of other fatty acids including the octadecanoid derivatives of 18-carbon PUFA, such as linoleic acid (LA; 18:2n-6) and alpha-linolenic acid (ALA; 18:3n-3) and the docosanoids produced from 22-carbon PUFA, such as docosapentaenoic acid (DPA; 22:5) and docosahexaenoic acid (DHA; 22:6n-3).

In humans, eicosanoids and related mediators mediate a wide array of critical biological processes such as ovulation, initiation of labor, bone metabolism, nerve growth and development, wound healing, kidney function, blood-vessel tone, blood coagulation, immune responses, and, most notably, inflammation [1–3]. Some of the most successful drug classes, such as the nonsteroidal anti-inflammatory drugs (NSAID) (e.g., acetylsalicylic acid, or aspirin, ibuprofen, and celecoxib, or Celebrex[®]), target prostanoid formation [4].

The enzymatic or non-enzymatic oxygenation of PUFA can result in hundreds of oxygenated species, including prostanoids and iso-prostanoids (isoP), leukotrienes, regio- and stereo-isomers of mono- and poly- hydroxyl-, hydroperoxy-, epoxy- and keto-fatty acids (Figure 1A,B) [1, 5–7]. Because such mediators might have opposite and redundant properties, it would be difficult to explain the molecular mechanisms of biological processes by studying only a limited number of eicosanoid species. Indeed, it is the overall balance between various oxygenated PUFA species that seems to modulate many biological processes. Thus, the ability to simultaneously measure a wide range of metabolites could further our understanding of their roles in health and disease, as well as provide a set of biomarkers for disease diagnosis or prognosis.

The analysis of oxygenated PUFA metabolites faces three major challenges: First, these lipid mediators are present at extremely low concentration in biological tissues. Second, these species are not stored in tissues, but are transiently formed on demand and have limited stability. Third, the same PUFA substrate can be oxidized in different positions of its acyl chain, leading to many isomeric species, each with specific metabolic actions. As a consequence, their measurements require rapid, highly-sensitive, accurate and specific analytical methodologies.

Here we review current analytical approaches for a high-throughput analysis of eicosanoids, octadecanoids, docosanoids and related oxygenated PUFA species in biological samples.

2. Biosynthesis of oxygenated lipid mediators

2.1. Polyunsaturated fatty acid substrates

PUFA are the immediate metabolic precursors for the biosynthesis of oxygenated species. They belong to two main families: omega-3 (n-3) PUFA and omega-6 (n-6) PUFA, so named according to the position of the first double bond in their fatty acyl chain, starting from the methyl end (Figure 1). Mammals cannot insert a double bond at either the n-3 or n-6 position of a fatty acyl chain and must rely entirely on dietary intake for PUFA (hence the characterization of their precursors LA and ALA as essential macronutrients) [8, 9]. Once absorbed in the gut and transported through the lymph in chylomicrons, PUFA can

undergo further elongation and desaturation in the liver, and then circulate through the blood to all tissues, usually via lipoproteins and albumin. Tissue and cellular PUFA can be found as free fatty acids, esterified to complex lipids (e.g., glycerophospholipids, glycerolipids, sphingolipids and cholesteryl esters) or conjugated to amino acids and ethanolamine [10].

Some of the health effects associated with PUFA consumption are mediated through the formation of various oxygenated species [11, 12]. In particular, current nutritional research shows that a diet enriched in n-3 PUFA offers health benefits and anti-inflammatory properties, whereas an excess of n-6 PUFA can contribute to the pathogenesis of many chronic inflammatory diseases, including cardiovascular and autoimmune diseases [13]. Such evidence suggests that, in addition to pharmacological treatments, nutritional interventions can also be used to modulate eicosanoid and related species composition and their biological activities [14–18].

2.2. Reactions mediating the formation and deactivation of eicosanoids and related mediators

Polyunsaturated fatty acids can be oxygenated via enzymatic and/or non-enzymatic reactions. The three major enzymatic pathways involved in the generation of oxygenated species are catalyzed by cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP) isoforms.

Cyclooxygenase Metabolites—COX-1 and COX-2 possess dual cyclooxygenase and peroxidase activities, catalyzing the oxidative cyclization of the central 5 carbons within 20-carbon PUFA to prostaglandin (PG) H₂. PGH₂ is subsequently converted to a variety of prostanoids including PG E, D, and F, prostacyclin (PGI₂) and thromboxanes (TX), by cell-specific terminal synthases and isomerases (Figure 1A,B). 20-Carbon PUFA DGLA, AA and EPA give rise to series-1,-2 and -3 prostanoids, respectively. 18-Carbon PUFA, such as LA, and 22-carbon PUFA, such as DHA, can be partially metabolized to hydroxy-fatty acids such as HODE and HDHA, respectively [19–21]. After acetylation of the active site of COX by aspirin, the enzyme generates (*R*)-hydroxy-derivatives such as 15(*R*)-HETE, 18(*R*)-HEPE and 17(*R*)-HDHA [22, 23].

Lipoxygenase Metabolites—LOX activities catalyze the stereoselective insertion of molecular oxygen into PUFA with the formation of hydroperoxy fatty acids (e.g., HpETE and HpEPE) that are subsequently reduced to the corresponding hydroxy- fatty acids (e.g. HETE and HEPE). LOX isoforms are conventionally named after the stereospecific carbon at which they oxygenate AA, e.g. 5-, 12-15-LOX [24]. In the 5-LOX active site, 5-hydroperoxy-PUFA can also undergo a catalytic rearrangement to form leukotrienes A-series (LTA), which can then be hydrolyzed by the LTA hydrolase to generate LT B-series (LTB), or, alternatively undergo conjugation to glutathione by the LTC synthase to form cysteinyl-LT such as LTC₄ [1]. Monohydroxy fatty acids such as HETE, HEPE and HDHA can participate in transcellular metabolism and, subject to further lipoxygenations, can form polyhydroxy-PUFA such as the AA-derived lipoxins and EPA- and DHA-derived resolvins and protectins [25].

Cytochrome P450 Metabolites—The superfamily of CYP monooxygenases catalyzes the hydroxylation and/or epoxygenation of PUFA [26]. In addition to producing LOX-like monohydroxy fatty acids, CYP can add a hydroxyl moiety to the omega-carbons of PUFA to form a unique class of omega-hydroxylated HETE (e.g. 20-HETE) [27]. The epoxidation of PUFA by CYP isoforms results in the formation of epoxyeicosatrienoic acids (EETs) that can be hydrolyzed to the corresponding dihydroxyeicosatrienoic acids (DHETs) by the soluble-enzyme epoxide hydrolase (sEH) [26, 28–30].

Non-Enzymatically Produced Metabolites—Oxidative stress and reactive oxygen species can lead to free-radical catalyzed peroxidation of PUFA, generating a range of isoP and a multitude of hydroxy fatty acids [31–35]. In contrast to enzymatic oxygenation of PUFA, which leads to the formation of pure oxygenated enantiomers and regioisomers, lack of enzymatic control in the free-radical-mediated chemical reactions results in a racemic mixtures of stereo- and regio-isomers. Non-enzymatically derived species have been shown to serve as useful biomarkers of oxidative stress and tissue damage [36–39].

Catabolism of eicosanoids and related species—The biological activities of eicosanoids and other oxygenated lipids are regulated by efficient mechanisms for deactivation and excretion. Human metabolism of eicosanoids involves mainly dehydrogenation of hydroxyl groups and reduction of double bonds [40]. Further catabolism of PUFA oxygenated species might involve hydroxylation (e.g., CYP-mediated omega-hydroxylation), beta-oxidation and glucuronidation, which increase their water solubility and thus urinary excretion from the body [1, 41, 42]. The overall catabolism further contributes to the complexity of the PUFA oxygenated metabolites.

3. Analysis of oxygenated fatty acid metabolites

Historically, measurements of eicosanoids and related species have been performed using radiometric and enzymatic immunoassays, which often lacked specificity and targeted only a few compounds [43–46]. Liquid chromatography with UV or fluorescence detection [47, 48], and gas chromatography coupled to mass spectrometry (GC-MS) have also been used [49]. However, these methodologies require multi-step procedures involving derivatization reactions, increasing the analysis time and the risk of thermal decomposition. Recent advances in biological mass spectrometry of lipids [50] have paved the way to a growing number of liquid chromatography-mass spectrometry (LC-MS) methodologies with much enhanced sensitivity and specificity, and the ability to simultaneously analyze large numbers of oxygenated PUFA species [51–58].

The important biological role of these lipid mediators, and their potential value as biomarkers, has created a growing demand for a comprehensive, high-throughput screening methodology driving the use of the most innovative technologies. A typical analytical platform for eicosanoids and related species includes the optimization of five major steps: 1) sample collection and storage; 2) sample preparation and extraction; 3) liquid chromatography; 4) ionization; and 5) mass spectrometric detection.

3.1. Sample collection and storage

Sample collection is a key step in the workflow for the analysis of PUFA oxygenated metabolites. Tissue degradation and free-radical oxidation might occur within seconds at room temperature, leading to alterations in the composition of eicosanoids and other species. Therefore, to avoid artifacts in the analysis, biological samples should be rapidly collected and processed: tissue samples are quickly snap frozen in liquid nitrogen; biofluids, such as blood, are drawn, stored on ice and rapidly processed for serum or plasma preparation; cells are harvested in cold solvents to immediately quench their metabolism [59–62]. All biological samples should be stored at -80°C until further processed and freeze/thaw cycles should be avoided.

It is worth noting that during the preparation of plasma samples, platelets may become activated and produce large quantities of eicosanoids. This problem can be minimized if blood is collected directly in containers containing anticoagulant, such as EDTA, which can sequester calcium ions and then inhibit phospholipase A_2 's (e.g., sPLA₂, cPLA₂). Similarly, serum samples derived from coagulating blood have much higher concentrations of lipid mediators, compared to plasma [63].

3.2. Sample preparation and extraction

Eicosanoids and similar species are present at very low abundance in both tissues and biofluids. The quality of sample preparation is, therefore, an important factor for a successful analysis. Many PUFA oxygenated species are unstable at room temperature, and the whole sample preparation procedure should be conducted in cold conditions whenever possible and, antioxidants could be used to minimize non-enzymatic oxidation (e.g., butylated hydroxytoluene, BHT) [64]. Normalization of the extraction efficiency and quantitation is achieved by adding stable, isotope-labeled compounds (internal standards) prior to extraction, as described below. Liquid-liquid extraction and/or solid-phase extraction (SPE) procedures are usually used prior to MS analysis, to eliminate non-lipid contaminants and highly abundant species like phospholipids, which may create dynamic-range and ion-suppression problems (Figure 2).

Internal standards—A mixture of deuterated eicosanoids and related species is often used to normalize endogenous metabolite levels for both extraction efficiency and instrument response. With hundreds of oxygenated PUFA species routinely analyzed, using an appropriate internal standard, such as a stable isotope-labeled molecule (e.g., deuterated), for each species is impractical. Many of the deuterated standards are indeed expensive to synthesize, labile, and may contain impurities. We must therefore compromise, normalizing the individual, molecular-ion peak intensity with a limited number of internal standards that would match groups of endogenous species for chemistry, retention time, and ionization efficiencies. Table 1 includes typical deuterated internal standards and the PUFA mediators with which they were paired [40, 52, 54, 56, 65–68].

Solid Phase Extraction (SPE)—In SPE, oxygenated PUFA species are extracted from biological matrix by loading, washing and eluting them through a column using appropriate solvent conditions (Figure 2). SPE involves using commercial columns pre-packed with

various sorbents, which include the reversed-phase C18 (e.g., Sep-Pak, Waters Corp, Milford, MA [64, 69], polymer-based reversed phase materials (e.g., Oasis HLB, Waters Corp, Milford, MA [51, 52, 65, 70] and Strata-X, Phenomenex, Torrance, CA [54, 56]) and anion-exchange polymer-based resins [45, 53, 71, 72] cartridges. A reversed-phase C18 cartridge will allow specificity of retention based on the eicosanoids overall hydrophobicity. Polymeric materials that contain both lipophilic and hydrophilic functional groups will provide a broader retention of more lipid metabolites. Anion-exchange materials with a polymeric backbone in addition to a positively charged functional group will selectively retain eicosanoids based on both hydrophobic interactions with the acyl chains and anion-exchange interactions with the carboxylic groups of the PUFA-derivatives.

Notably, no SPE method gives 100% recovery for all lipid species and the efficiency for each identified lipid must be quantified; hence the importance of including proper internal standards in all studies. Leukotrienes are particularly challenging for certain extraction systems and must be carefully checked. Although SPE cartridges can be expensive in terms of both time and supplies, SPE protocols appear to be indispensable to analyze low abundance of mediators in complex matrices, and can be easily standardized for optimal intra- and inter-lab reproducibility.

Liquid-liquid Extraction (LLE)—In LLE, eicosanoids and related species are extracted from biological samples with mixtures of water and organic solvent, leading to phase partitioning of lipids into the organic layer (Figure 2). One of the most commonly used LLE protocols involve the use of chloroform-methanol mixtures according to Bligh and Dyer [73]. Alternative extraction procedures using ether [74], acetone/chloroform [64], hexane–isopropanol [75], and ethyl acetate/ethanol mixtures have been proposed for a wide range of tissues. LLE has proven to be a valid high-throughput alternative to SPE for the extraction of lipid metabolites from biological matrices such as urine samples [53, 76] and brain samples [64, 77]. Additionally, LLE can be used in combination with SPE to maximize the purification of oxygenated PUFA species prior to further MS analysis [66, 78–80].

3.3. Liquid Chromatography

High Performance Liquid Chromatography (HPLC)—Chromatographic separation is necessary for analyzing very low-abundance and isomeric metabolites. Liquid chromatography (LC) has become the chromatographic solution of choice for separating the complex mixture of oxygenated species prior to detection by mass spectrometry. In contrast to GC, in LC no derivatization of the analytes is required, which facilitates the analysis. The high level of chemical similarity and the high number of isomeric species, however, sometimes makes separating the oxygenated PUFA species by conventional high-performance LC (HPLC) quite challenging.

Ultra High Performance Liquid Chromatography (UHPLC)—One approach to increasing chromatographic resolution uses a column packed with reduced particle sizes (<2 μm). The high pressure (10,000–15,000 psi) needed to operate these columns led to the development of ultra-high performance liquid chromatography (UHPLC) [81–83]. Sub-2- μm particles provide narrow chromatographic peaks (often <3 s), which results not only in

better resolution but also in lower detection limits and shorter times for the chromatographic run (<5–10 min), compared with conventional HPLC [81–84]. Thus, in conjunction with mass spectrometry, UHPLC can reduce ion suppression caused by co-eluting lipids, and isobaric and isomeric interferences [53, 58].

Reversed-Phase Liquid Chromatography—Reversed-phase LC columns are often used to separate eicosanoids and related mediators. For underivatized metabolites, protonation of the carboxylic acid using a weak organic acid, such as formic or acetic acid, in the mobile phase allows better retention on the reversed-phase column, ensuring chromatographic separation. Oxygenated PUFA metabolites containing longer acyl chains elute from the reversed phase LC column later than shorter-chain lipids (e.g., C20:3 > C18:3), while the retention time decreases as the number of double bonds increases (e.g., C18:2 > C18:3). N-3 PUFA tend to elute earlier than n-6 PUFA (e.g., docosapentaenoic acid (DPA) n-3 earlier than DPA n-6).

Using an UHPLC with a reversed-phase C18 column, both mediators and their free fatty-acid precursors can be separated in less than 10 minutes in a single chromatographic run, providing key information to help us better understand the intricate regulation of oxygenated PUFA metabolism (Figure 3A,B). Although reversed-phase HPLC and UHPLC applications allow for good separation of most species, the peaks detected might correspond to racemic mixtures; with the exception of diastereo-isomers (e.g., xS,yS and xR,yS), separation of the R and S enantiomers usually requires the use of chiral chromatography (Figure 3C), as discussed below.

Chiral Liquid Chromatography—Most of the high-throughput methods currently used to analyze eicosanoids and related species are based on reversed-phase chromatography, and this approach cannot distinguish between enantiomers. Whilst enzymatic formation of eicosanoids and other PUFA mediators is highly stereoselective, non-enzymatic reactions proceed without stereoselectivity, generating equal amount of enantiomeric species. Thus, the use of either normal phase or reversed-phase chiral chromatography is necessary when attempting to distinguish between enzymatic and non-enzymatic pathways of oxygenated PUFA formation [85–88] (Figure 3C). Chiral chromatography has a number of drawbacks, including the need for long equilibration and elution times (up to 10–60 min) and limited sensitivity. These limitations do not facilitate use in high throughput applications, and chiral chromatography tends to be used for targeted questions aiming to explore specific oxygenated species and not screening assays [89, 90].

3.4. Ionization

Electrospray ionization (ESI) has emerged as the most applied ionization technique for the analysis of all classes of eicosanoids, octadecanoids and docosanoids by LC-MS. ESI generates an abundance of both positive and negative molecular ion species ($[M+H]^+$ and $[M-H]^-$), but the majority of the applications using ESI for non-derivatized metabolites have been in the negative-ion mode [55]. Although mostly detected as deprotonated molecular ion species ($[M-H]^-$), depending on the particular operating conditions of the MS instrument, oxygenated derivatives may be also detected as dehydrated ions ($[M-H-H_2O]^-$) [52].

The presence of the weak acid in most of the mobile phases used for the analysis of oxygenated PUFA species by LC-MS (e.g., 0.1% formic acid and acetic acid), offsets the formation of carboxylate anions in the ESI source. To improve detection limits and method sensitivity, alternative derivatization methodologies have been developed. In charge-reversal derivatization, carboxylic acids are converted into cationic derivatives with quaternary amines, improving the detection by ESI [91–93]. In electron capture atmospheric pressure chemical ionization (APCI), eicosanoids and related species are tagged before analysis with an electron-capturing group such as the pentafluorobenzyl moiety, which enhances detection by APCI [94].

3.5. Mass Spectrometry

Tandem Mass Spectrometry—To maximize the sensitivity and specificity of analysis, tandem mass spectrometry (MS/MS) performed on triple quadrupole instruments is often the preferred technology for the analysis of oxygenated PUFA mediators, offering a very selective and sensitive system appropriate for targeted quantitative analyses. Fragmentation information is required to define chemical structures and distinguish between the numerous isobaric species; an acquisition mode named multiple-reaction monitoring (MRM) is typically used to selectively monitor the transition of a selected precursor ion (M1) to a specific product ion (M2); this can be assisted by LC-separation, as shown in the case of the isobaric species PGE₂ and PGD₂ that have identical fragmentation patterns (Figure 3A) [66]. Furthermore, in the case of co-eluting metabolites, compound-specific precursor ions and their corresponding fragment ions allows selective detection and quantitation of those compounds, a tool important in distinguishing e.g. HETE or HDHA species (Figure 3B; Table 1) [57].

Recent reports have shown that the combination of UHPLC chromatographic separation and MRM transitions performed on triple-quadrupole instruments enables the analysis of over one hundred oxygenated PUFA species in a single acquisition [9–13] including a recent report of 184 metabolites including 26 internal standards separated and accurately quantified including some in picogram/fentomole amounts in a 5 min UHPLC run [68]. To enhance the sensitivity of detection, these MRM transitions are monitored in defined retention-time windows, maximizing dwell times by reducing overlapping transitions. A list of characteristic MRM transitions (compound-specific and selective precursor-to-product ion transitions) for more than 100 common metabolites detected using negative electrospray ionization appears in Table 1.

Quantitation—Quantitation of oxygenated PUFA species is achieved by generating calibration curves using commercially-available standards and class-appropriate deuterated internal standards (see also Internal Standards paragraph under *Sample Preparation and Extraction* section). Although, at an early discovery phase it is often impractical, the use of stable isotope standards for each eicosanoid of interest is ultimately required for the absolute quantitation and validation of the initial discoveries. Usually, eicosanoid concentrations in biological samples are normalized by volume, tissue weight, cell number, or protein or DNA concentration. Despite potential limitations in their measurements (see also the *Sample Preparation and Extraction* Section), the standardization of the operating procedures for

sample collection and preparation can lead to sufficiently accurate results to reveal answers to biochemical questions. For instance, when comparing samples from parallel measurements in which the precision is very high, the lower accuracy in absolute quantity does not impact upon the study outcome.

4. FUTURE DIRECTIONS

Discovery of Novel PUFA-Derived Species

The discovery of novel oxygenated metabolites derivatives of various PUFA present in biological samples could lead to the identification of unexplored biochemical pathways conferring novel biological activities. With the implementation of modern analytical technologies, we can expect that many more novel structures will be discovered and added to current targeted-lipidomics approaches. Three examples are provided below.

1. During the last decade, a growing number of oxygenated PUFA species, including the anti-inflammatory mediators lipoxins, resolvins, protectins and maresins, have been discovered, while their complete biochemical characterization is still underway [95–99]. Furthermore, the existence of very long chain PUFA in humans (C24–C36) [100–103] might suggest the existence of their respective oxygenated metabolites, albeit they have not yet been characterized.
2. Besides existing in their unbound or free form, oxygenated PUFA moieties are found esterified to complex lipids (e.g., glycerophospholipids, glycerolipids and cholesteryl esters) [34, 70, 77, 104–116] or amide-conjugated to ethanolamine (e.g., prostamides) [115, 117] and various amino acids [10, 118], whose mechanism of formation and action has not yet been fully elucidated.
3. Unusual eicosanoids and related molecules derived from gut microbiome metabolism [119, 120] and dietary sources such as plants (e.g., jasmonic acid) [118], animal meat, and dairy (conjugated-linoleic acid) [121], further contribute to the overall complexity of oxygenated PUFA species found in human tissues and biological fluids, possibly mediating yet-to-be explored biological activities.

Biomarkers

Many enzymatically and non-enzymatically formed eicosanoids have been proposed as biomarkers in clinical research [37–39, 122, 123]. As the verification and validation of these markers of health and disease progresses, there will be a need to develop more reproducible and cost-effective means for their measurement; LC-MS is already used in clinical laboratories, and robust and validated assays for oxygenated PUFA mediators could become part of the routine clinical tests.

Systems Biology

Recent evidence indicates that genetic polymorphisms contribute to the absorption, transport, biosynthesis, metabolism and biological effects of PUFA and their derivatives [124–130]. For example, the efficacy of n-3 PUFA supplementation seems to be related to the ApoE polymorphism [125, 127, 128]. Thus, the integration of oxygenated PUFA measurements with various "omics" techniques, including metabolomics, proteomics and

genomics, will allow a more accurate understanding of the role of this system in health and disease, eventually supporting precision and personalized medicine [131, 132].

Novel MS-based approaches

The recent advances in high resolution mass spectrometry and hybrid instruments offer novel analytical capabilities for the analysis of PUFAs and their precursors and metabolites [76, 133, 134]. Although such instruments might suffer from limited dynamic range and sensitivity compared with triple quadrupoles, they can perform additional MS/MS modes of acquisition, such as data-dependent acquisition [133, 135, 136] and data-independent acquisition [76, 134, 137]. Such acquisition modes allow us to acquire extensive fragmentation data from a wider range of lipids in a single analytical run, obviating the need to manually select specific MRM transitions.

In data-dependent acquisition (DDA) mode of operation, the MS/MS experiment performs one scan based on the data acquired in the previous scan. For example, a DDA experiment switches from full-scan MS mode to MS/MS mode when the most intense ions from an inclusion list are detected or after other specific criteria are met. When performing DDA during fast UHPLC analysis, however, the occurrence of narrow, rapidly eluting peaks might result in a loss of data in the MS mode while MS/MS data are being acquired [133, 135, 136].

In data-independent acquisition mode of operation, alternating low and elevated collision energy are utilized independently to the previous scan, without need of inclusion lists and pre-selection of precursor ions by the quadrupole mass analyzer. Low collision-energy acquisition generates information about the precursor ions, and elevated collision energy provides information about product ions. Data-independent acquisitions collect both precursor and product ion information, for virtually all detectable ions, which ultimately constitutes an important repository of knowledge for both qualitative and quantitative applications [76, 134, 137].

Fluxomic approaches

A complete fluxomics analysis of the eicosanoid biosynthetic pathways has recently been developed that allows one to predict and quantitatively determine with LC/MS the temporal changes of each eicosanoid metabolite [135, 138, 139] without using labeled precursors. This is possible because when cells are at rest, levels of free AA and various eicosanoids are minimal, but when cells are activated by various receptor mediated triggers, the cytosolic phospholipase A₂ is activated to release AA instigating changes in AA metabolite levels over time [23].

5. CONCLUSIONS

Oxygenated metabolites of PUFA play an active role in health and disease, conferring a range of beneficial as well as toxic effects. Their production through multiple enzymatic and non-enzymatic pathways results in hundreds of bioactive lipids. The application of MS-based lipidomic methodologies has revealed the challenges presented by increasingly complex lipid profiles, and has precipitated the realization that bioactivities mediated by

eicosanoids and related oxygenated PUFA mediators may largely depend upon their overall balance.

The application of state-of-the-art MS-based analytical platforms permits the rapid identification and quantification of numerous species. These data are creating novel opportunities to enhance our knowledge of oxygenated PUFA functions in the context of health and disease. High-throughput methodology for the measurement of eicosanoids, octadecanoids, docosanoids and other species could lead to the development of panels of biomarkers to assess inflammatory status, disease diagnosis and/or prognosis, assisting pharmacological and/or nutritional interventions.

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Highlights

- Oxidation of polyunsaturated fatty acids can yield an array of eicosanoids
- These lipid mediators play key pathophysiological roles in humans
- Mass spectrometry-based technology allows us to analyze such lipid mediators
- Development of high-throughput approaches facilitates the analysis of eicosanoids

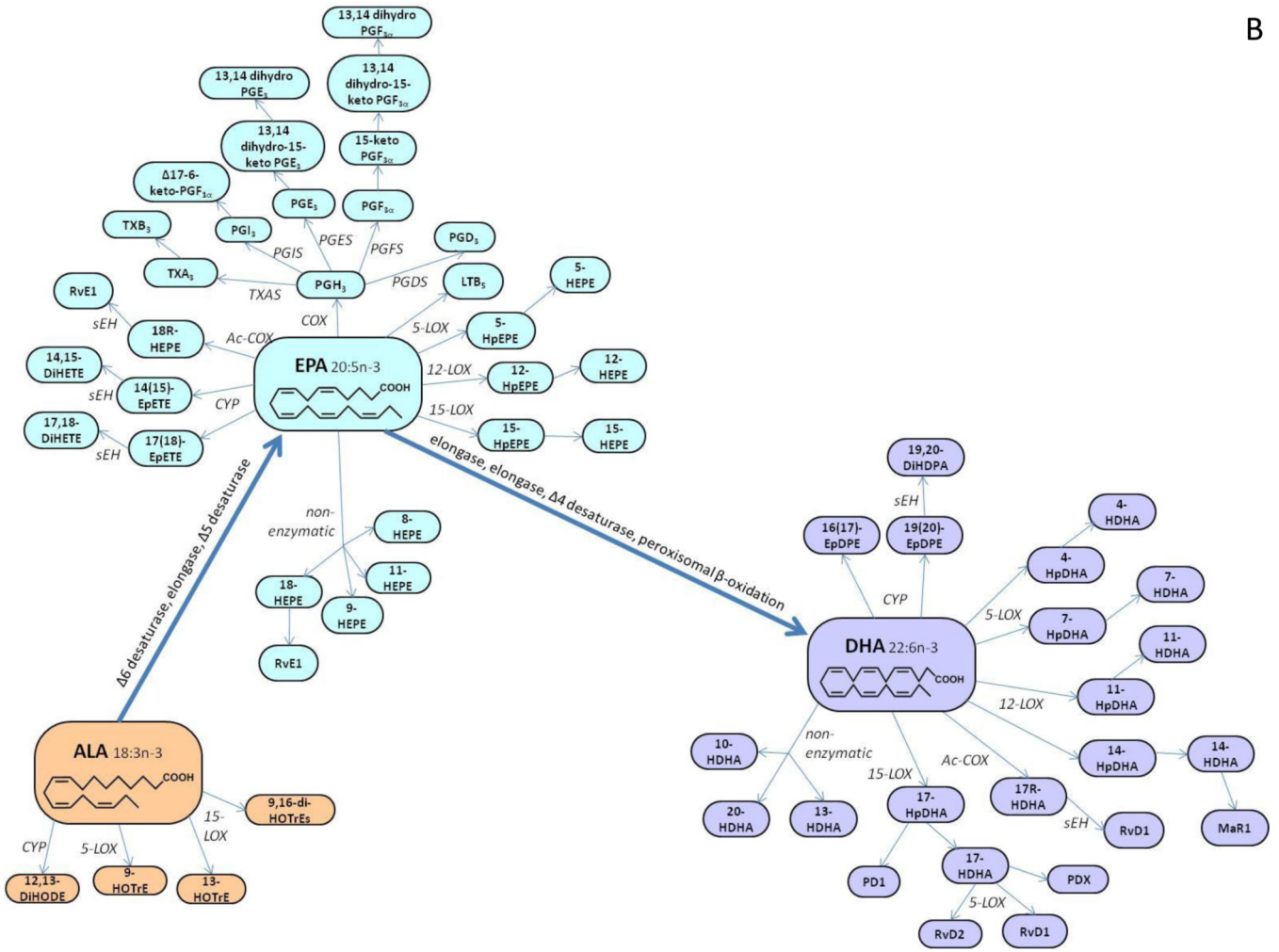


Figure 1.
A. Schematic outline of oxygenated species derivatives of the omega-6 fatty acids linoleic acid C18:2 (LA), dihomo- γ -linolenic acid C20:3 (DGLA) and arachidonic acid C20:4 (AA), via the cyclooxygenase (COX), lipoxygenase (LOX), CYP-450 (CYP) or free radical catalyzed pathways.
B. Schematic outline of oxygenated species derivatives of the omega-3 fatty acids α -linolenic acid C18:3 (ALA), eicosapentaenoic acid C20:5 (EPA) and docosahexaenoic acid C22:6 (DHA), via the COX, LOX, CYP or free radical catalyzed pathways.
 Abbreviations: dihydroxy-eicosatrienoic acid (DHET), dihydroxy-eicosatetraenoic acid (DiHETE), dihydroxy-octadecadienoic acid (DiHODE), dihydroxy-octadecenoic acid (DiHOME), epoxy-keto-octadecenoic acid (EKODE), epoxy-eicosatrienoic acid (EET), epoxy-docosapentaenoic acid (EpDPE), epoxy-eicosatetraenoic acid (EpETE), epoxy-octadecenoic acid (EpOME), hydroxy-docosahexaenoic acid (HDHA), hydroxy-eicosapentaenoic acid (HEPE), hydroxy-eicosatrienoic acid (HETrE), hydroxy-eicosatetraenoic acid (HETE), hydroxy-heptadecatrienoic acid (HHTrE), hydroxy-octadecadienoic acid (HODE), hydroxy-octadecatrienoic acid (HOTrE), hydroperoxy-docosahexaenoic acid (HpDHA), hydroperoxy-eicosapentaenoic acid (HpEPE),

hydroperoxy-eicosatetraenoic acid (HpETE), hydroperoxy-octadecadienoic acid (HpODE), hepxilin (HX), leukotriene (LT), lipoxin (LX), oxo-eicosatetraenoic acid (OxoETE), oxo-octadecadienoic acid (OxoODE), prostaglandin (PG), prostaglandin E metabolite (PGEM), prostaglandin F metabolite (PGFM), resolvin (Rv), soluble epoxide hydrolase (sEH), trihydroxy-octadecenoic acid (TriHOME), thromboxane (TX).

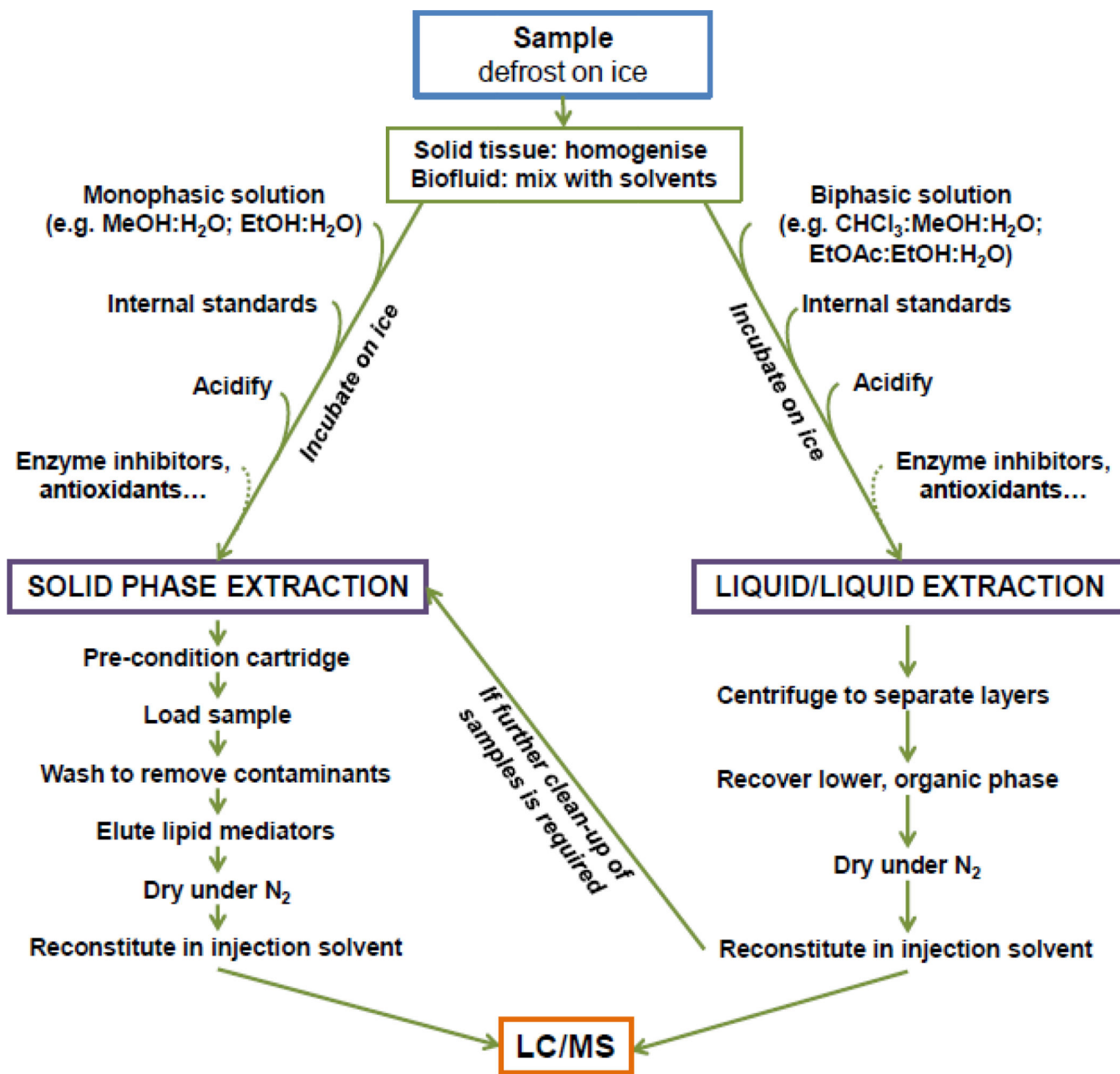
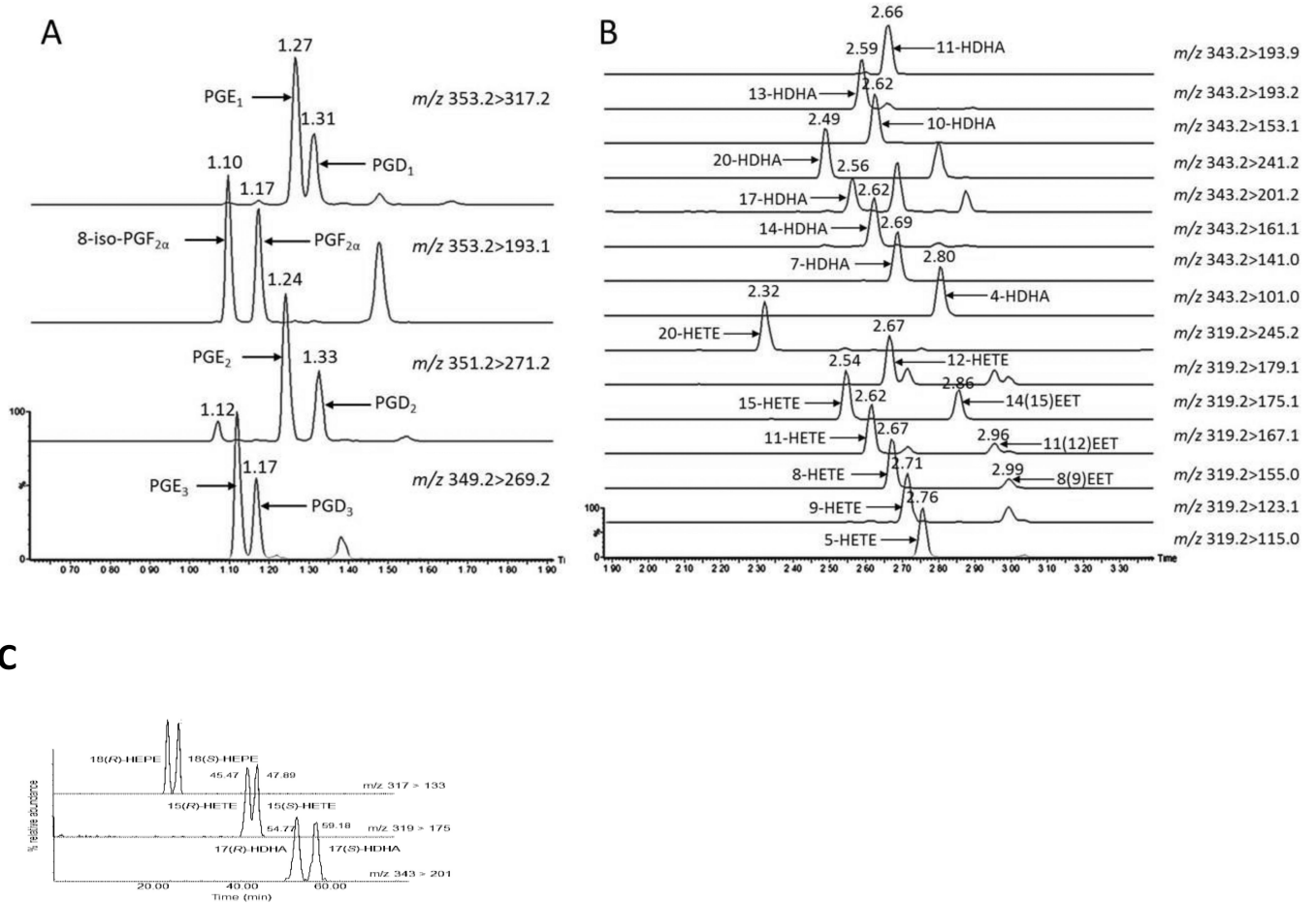


Figure 2.

Workflow for the sample preparation options for the analysis of oxygenated PUFA species found in various biological tissue samples. Samples (liquid and/or solid tissues) can be prepared using solid phase extraction (SPE) or liquid/liquid extraction, or a combination of the two, before analysis by liquid chromatography/mass spectrometry (LC/MS). Acid is used to protonate the analytes prior to reversed phase SPE or liquid-liquid extractions; enzyme inhibitors can include protease or cyclooxygenase inhibitors, depending on the experimental design.

**Figure 3.**

Representative UHPLC-MS/MS chromatograms showing separation of pairs of isobaric species with identical fragmentation patterns by chromatography (A), and of co-eluting metabolites by compound-specific precursor and fragment ions (B). Using a combination of UHPLC with a C18, reversed-phase column, a mixture of commercially available oxygenated PUFA species elutes according to their polarity, number of double bonds and acyl-chain length, allowing the separation of most isomeric and isobaric species (e.g., PGE₂ and PGD₂) in less than 10 minutes according to retention time and identifying fragmentation patterns (Table 1). A representative UHPLC-MS chromatogram for the chiral separation of enantiomers is shown in panel (C) (adapted from [57]).

Table 1

List of MRM transitions (M1 = precursor ion; M2 = production) for most common natural oxygenated PUFA species, and non-natural compounds used as internal standards (ISTD). The precursor fatty acids, metabolite class, and predominant metabolic pathway are also reported for each compound. Compounds can be additionally identified by retention time, which are not listed because they will differ depending on the specific chromatographic methods and experimental conditions employed. An example of selected compounds and their identification by retention time, as well as unique fragmentation pattern, is shown in Figure 3 [52, 54, 56, 65–68].

Compound Name	M1	M2	ISTD	Precursor	Pathway	Class
1 12-HHTe	279.2	179.2	(d8) 12(S)-HETE	AA	COX	Alcohol
	279.2	163.2				
2 (d4) 15-deoxy- 12,14-PGJ ₂	319.2	275.3	ISTD	AA	COX	Prostanoid
3 2,3-dinor-11b PGF _{2α}	325.2	145.1	(d4) PGF _{2α}	AA	COX	Prostanoid
4 Tetranor-PGEM	327.1	309.2	(d4) PGE ₂	AA	COX	Prostanoid
	327.1	291.1				
5 Tetranor-PGFM	329.2	311.2	(d4) PGF _{2α}	AA	COX	Prostanoid
	329.2	293.2				
6 PGJ ₂	333.2	233.1	(d4) PGD ₂	AA	COX	Prostanoid
	333.2	271.2				
7 ¹² -PGJ ₂	333.2	233.1	(d4) 15-deoxy- ^{12,14} -PGJ ₂	AA	COX	Prostanoid
	333.2	271.2				
8 15-deoxy- 12,14-PGJ ₂	315.2	271.2	(d4) 15-deoxy- 12-PGJ ₂	AA	COX	Prostanoid
9 PGA ₂	333.2	271.2	(d4) PGE ₂	AA	COX	Prostanoid
10 PGK ₂	349.2	205.1	(d4) PGE ₂	AA	COX	Prostanoid
11 13,14-dihydro-15-keto PGD ₂	351.2	175.2	(d4) PGD ₂	AA	COX	Prostanoid
12 13,14-dihydro-15-keto PGE ₂	351.2	175.2	(d4) PGE ₂	AA	COX	Prostanoid
	351.2	333.2				
	351.2	207.2				
13 15-keto PGF _{2α}	351.2	219.1	(d4) PGF _{2α}	AA	COX	Prostanoid
	351.2	113.1				
14 PGD ₂	351.2	271.2	(d4) PGD ₂	AA	COX	Prostanoid
15 PGE ₂	351.2	271.2	(d4) PGE ₂	AA	COX	Prostanoid
16 11β-PGE ₂	351.2	271.2	(d4) PGE ₂	AA	COX	Prostanoid
17 13,14-dihydro-15-keto PGF _{2α}	353.2	113.1	(d4) PGF _{2α}	AA	COX	Prostanoid
	353.2	183.1				
	353.2	291.1				

Compound Name	M1	M2	ISTD	Precursor	Pathway	Class
18 11 β -13,14-dihydro-15-keto PGF _{2α}	353.2	113.2	(d4) PGF _{2α}	AA	COX	Prostanoid
19 PGF _{2α}	353.2	193.2	(d4) PGF _{2α}	AA	COX	Prostanoid
20 11 β -PGF _{2α}	353.2	193.2	(d4) PGF _{2α}	AA	COX	Prostanoid
21 (d4) PGD ₂	355.2	275.2	ISTD	AA	COX	Prostanoid
22 (d4) PGE ₂	355.2	275.2	ISTD	AA	COX	Prostanoid
23 13,14-dihydro PGF _{2α}	355.2 355.2	275.2 311.2	(d4) PGF _{2α}	AA	COX	Prostanoid
24 (d4) PGF _{2α}	357.3	197.2	ISTD	AA	COX	Prostanoid
25 6-keto PGE ₁	367.2	143.1	(d4) PGE ₂	AA	COX	Prostanoid
26 20-hydroxy PGE ₂	367.2	287.2	(d4) PGE ₂	AA	COX	Prostanoid
27 6-keto PGF _{1α}	369.2	163.1	(d4) 6-keto PGF _{1α}	AA	COX	Prostanoid
28 TXB ₂	369.2	169.1	(d4) TXB ₂	AA	COX	Thromboxane
29 (d4) 6-keto PGF _{1α}	373.2	167.2	ISTD	AA	COX	Thromboxane
30 (d4) TXB ₂	373.2	173.1	ISTD	AA	COX	Thromboxane
31 15-KETE (15-OxoETE)	317.2	113.2	(d8) 5(S)-HETE	AA	LOX	Ketone
32 5-KETE (5-OxoETE)	317.2	203.2	(d8) 5(S)-HETE	AA	LOX	Ketone
33 12-KETE (12-OxoETE)	317.2	273.3	(d8) 12(S)-HETE	AA	LOX	Alcohol
34 5-HETE	319.2	115.1	(d8) 5(S)-HETE	AA	LOX	Alcohol
35 8-HETE	319.2	155.1	(d8) 5(S)-HETE	AA	LOX	Alcohol
36 11-HETE	319.2	167.1	(d8) 12(S)-HETE	AA	LOX	Alcohol
37 15-HETE	319.2 319.2	175.1 219.2	(d8) 5(S)-HETE	AA	LOX	Alcohol
38 12-HETE	319.2	179.2	(d8) 12(S)-HETE	AA	LOX	Alcohol
39 (d8) 5(S)-HETE	327.3	116.1	ISTD	AA	LOX	Alcohol
40 (d8) 12(S)-HETE	327.3	184.2	ISTD	AA	LOX	Alcohol
41 15-HpETE	335.2 317.2	113.1 113.1	(d8) 5(S)-HETE	AA	LOX	Hydroperoxide
42 LTB ₄	335.2	195.1	(d4) LTB ₄	AA	LOX	Leukotriene

Compound Name	M1	M2	ISTD	Precursor	Pathway	Class
43 6-trans-LTB ₄	335.2	195.1	(d4) LTB ₄	AA	LOX	Leukotriene
44 5-HpETE	335.2 317.2	203.2 203.2	(d8) 5(S)-HETE	AA	LOX	Hydroxyperoxide
45 Hepoxilin A3	335.2 335.2	273.2 127.2	(d8) 12(S)-HETE	AA	LOX	Hepoxilin
46 12-HpETE	335.2 317.2	273.3 153.3	(d8) 12(S)-HETE	AA	LOX	Hydroxyperoxide
47 (d4) LTB ₄	339.2	197.1	ISTD	AA	LOX	Leukotriene
48 5(S),6(R)-Lipoxin A4	351.2	115.1	(d4) LTB ₄	AA	LOX	Lipoxin
49 5(S),6(S)-Lipoxin A4	351.2	115.1	(d4) LTB ₄	AA	LOX	Lipoxin
50 20-hydroxy LTB ₄	351.2	195.1	(d4) LTB ₄	AA	LOX	Leukotriene
51 5(S),14(R)-Lipoxin B ₄	351.2	221.2	(d4) LTB ₄	AA	LOX	Lipoxin
52 20-carboxy LTB ₄	365.2	347.2	(d4) LTB ₄	AA	LOX	Leukotriene
53 LTE ₄	438.2	333.2	(d3) LTE ₄	AA	LOX	Leukotriene
54 11-trans LTE ₄	438.2	333.2	(d3) LTE ₄	AA	LOX	Leukotriene
55 14,15-LTE ₄	438.2	333.2	(d3) LTE ₄	AA	LOX	Leukotriene
56 (d3) LTE ₄	441.2	336.2	ISTD	AA	LOX	Leukotriene
57 LTD ₄	495.2	177.1	(d3) LTE ₄	AA	LOX	Leukotriene
58 11-trans LTD ₄	495.2	177.1	(d3) LTE ₄	AA	LOX	Leukotriene
59 20-HETE	319.2 319.2	275.2 245.2	d6-20-HETE	AA	CYP	Alcohol
60 8(9)-EpETE (8(9)-EET)	319.2	155.1	(d11) 14,15-DiHETE	AA	CYP	Epoxide
61 11(12)-EpETE (11(12)-EET)	319.2	167.1	(d11) 14,15-DiHETE	AA	CYP	Epoxide
62 14(15)-EpETE (14(15)-EET)	319.2 319.2	175.1 219.2	(d11) 14,15-DiHETE	AA	CYP	Epoxide
63 5(6)-EpETE (5(6)-EET)	319.2	191.2	(d11) 14,15-DiHETE	AA	CYP	Epoxide
64 (d6) 20-HETE	325.3	279.2	ISTD	AA	CYP	Alcohol
65 bicyclo-PGE ₂	333.2	113.2	(d4) PGE ₂	AA	CYP	Prostanoid
66 5,6-DiHETE	335.2	115.1	(d4) LTB ₄	AA	CYP	Diol

Compound Name	M1	M2	ISTD	Precursor	Pathway	Class
67 5,15-DiHETE	335.2	115.2	(d4) LTB ₄	AA	CYP	Diol
68 8,15-DiHETE	335.2 335.3 353.2	127.2 155.1 235.2	(d4) LTB ₄	AA	CYP	Diol
69 8,9-DiHETE (8,9-DHET)	337.2	127.1	(d11) 14,15-DiHETE	AA	CYP	Diol
70 5,6-DiHETE (5,6-DHET)	337.2	145.1	(d11) 14,15-DiHETE	AA	CYP	Diol
71 11,12-DiHETE (11,12-DHET)	337.2	167.2	(d11) 14,15-DiHETE	AA	CYP	Diol
72 14,15-DiHETE (14,15-DHET)	337.2	207.2	(d11) 14,15-DiHETE	AA	CYP	Diol
73 (d11) 14,15-DiHETE	348.3	207.1	ISTD	AA	CYP	Diol
74 9-HETE	319.2 319.2	123.1 167.1	(d8) 12(S)-HETE	AA	non-enzymatic	Alcohol
75 (+/-) 5-IPF _{2α} -VI	353.2	115.1	(d4) PGF _{2α}	AA	non-enzymatic	IsoP
76 8-iso PGF _{2α}	353.2	193.2	(d4) PGF _{2α}	AA	non-enzymatic	IsoP
77 1α,1β-dihomo PGF _{2α}	381.3	337.2	(d4) PGF _{2α}	ADA	COX	Prostanoid
78 9-HOTe	293.2	171.1	(d4) 9(S)-HODE	ALA	LOX	Alcohol
79 12,13-DiHODE	311.2	293.2	(d4) 9,10-DiHOME	ALA	CYP	Diol
80 15-keto PGF _{1α}	353.2 353.2	113.1 221.1	(d4) 6-keto PGF _{1α}	DGLA	COX	Prostanoid
81 13,14-dihydro-15-keto PGD ₁	353.2	209.1	(d4) PGD ₂	DGLA	COX	Prostanoid
82 PGD ₁	353.2 353.2	273.2 317.2	(d4) PGD ₂	DGLA	COX	Prostanoid
83 PGE ₁	353.2 353.2	273.2 317.2	(d4) PGE ₂	DGLA	COX	Prostanoid
84 13,14-dihydro-15-keto PGF _{1α}	355.2	193.2	(d4) PGF _{2α}	DGLA	COX	Prostanoid
85 PGF _{1α}	355.2 355.2	293.2 311.2	(d4) PGF _{2α}	DGLA	COX	Prostanoid
86 TXB ₁	371.2	171.1	(d4) TXB ₂	DGLA	COX	Thromboxane
87 15-HETE	321.2 321.1	221.2 303.2	(d8) 5(S)-HETE	DGLA	LOX	Alcohol
88 17-HDoHE (17-HDHA)	343.2 343.2	201.2 281.3	(d8) 5(S)-HETE	DHA	LOX	Alcohol

Compound Name	M1	M2	ISTD	Precursor	Pathway	Class
89 Neuroprotectin D1 (PDI)	359.2	153.1	(d8) 12(S)-HETE	DHA	LOX	Protectin
	359.2	206.1				
90 10,17-DiHDoHE (PDX)	359.2	153.2	(d8) 12(S)-HETE	DHA	LOX	Protectin
	359.2	206.1				
91 Resolvin D1	375.2	141.0	(d11) 14,15-DiHETrE	DHA	LOX	Resolving
92 16(17)-EpDPE	343.2	233.2	(d11) 14,15-DiHETrE	DHA	CYP	Epoxide
93 19(20)-EpDPE	343.2	281.3	(d11) 14,15-DiHETrE	DHA	CYP	Epoxide
	343.3	285.2				
94 19,20-DiHDPa	361.2	229.3	(d11) 14,15-DiHETrE	DHA	CYP	Diol
	349.2	269.2				
95 PGD ₃	349.2	269.2	(d4) PGD ₂	EPA	COX	Prostanoid
96 PGE ₃	349.2	269.2	(d4) PGE ₂	EPA	COX	Prostanoid
97 PGF _{3α}	351.2	193.2	(d4) PGF _{2α}	EPA	COX	Prostanoid
98 17-6-keto PGF _{1α}	367.2	163.1	(d4) 6-keto PGF _{1α}	EPA	COX	Prostanoid
99 TXB ₃	367.2	169.1	(d4) TXB ₂	EPA	COX	Thromboxane
100 5-HEPE	317.2	115.1	(d8) 5(S)-HETE	EPA	LOX	Alcohol
101 12-HEPE	317.2	179.1	(d8) 12(S)-HETE	EPA	LOX	Alcohol
102 15-HEPE	317.2	175.1	(d8) 5(S)-HETE	EPA	LOX	Alcohol
	317.2	219.2				
103 LTB ₅	333.2	195.1	(d4) LTB ₄	EPA	LOX	Leukotriene
104 Resolvin E1	349.2	195.1	(d11) 14,15-DiHETrE	EPA	LOX	Resolving
105 14(15)-EpETE	317.2	207.1	(d11) 14,15-DiHETrE	EPA	CYP	Epoxide
106 17(18)-EpETE	317.2	259.2	(d11) 14,15-DiHETrE	EPA	CYP	Epoxide
107 14,15-DiHETE	335.2	207.1	(d11) 14,15-DiHETrE	EPA	CYP	Diol
108 17,18-DiHETE	335.2	247.2	(d11) 14,15-DiHETrE	EPA	CYP	Diol
109 13-KODE (13-OxoODE)	293.2	113.1	(d4) 9(S)-HODE	LA	LOX	Ketone
110 9-KODE (9-OxoODE)	293.2	185.2	(d4) 9(S)-HODE	LA	LOX	Ketone
111 9-HODE	295.2	171.1	(d4) 9(S)-HODE	LA	LOX	Alcohol
112 13-HODE	295.2	195.2	(d4) 9(S)-HODE	LA	LOX	Alcohol
113 (d4) 9(S)-HODE	299.2	172.1	ISTD	LA	LOX	Alcohol

Compound Name	M1	M2	ISTD	Precursor	Pathway	Class
114 13-HpODE	311.2	113.2	(d4) 9(S)-HODE	L.A	LOX	Hydroxyperoxide
	293.2	113.2				
115 9-HpODE	311.2	185.2	(d4) 9(S)-HODE	L.A	LOX	Hydroxyperoxide
	293.2	185.2				
116 9,10,13-TriHOME	329.2	171.1	(d4) 9(S)-HODE	L.A	LOX	Triol
117 9,12,13-TriHOME	329.2	211.2	(d4) 9(S)-HODE	L.A	LOX	Triol
118 9(10)-EpOME	295.2	171.2	(d4) 9,10-DiHOME	L.A	CYP	Epoxide
119 12(13)-EpOME	295.2	195.2	(d4) 12,13-DiHOME	L.A	CYP	Epoxide
120 12,13-DiHOME	313.2	183.2	(d4) 12,13-DiHOME	L.A	CYP	Diol
121 9,10-DiHOME	313.2	201.1	(d4) 9,10-DiHOME	L.A	CYP	Diol
122 (d4) 12,13-DiHOME	317.3	185.2	ISTD	L.A	CYP	Diol
123 (d4) 9,10-DiHOME	317.3	203.2	ISTD	L.A	CYP	Diol