

HPLC/MS/MS based approaches for detection and quantification of eicosanoids

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Summary

Eicosanoids are oxygenated, endogenous, unsaturated fatty acids derived from arachidonic acid. Detection and quantification of these compounds are of great interest because they play important roles in a number of significant diseases, including asthma, chronic obstructive pulmonary disease (COPD), cardiovascular disease and cancer. Because the endogenous levels of eicosanoids are quite low, sensitive and specific analytical methods are required to reliably quantify these compounds. High performance liquid chromatography mass spectrometry (HPLC/MS) has emerged as one of the main techniques used in eicosanoid profiling. Herein, we describe the main LC/MS techniques and principles as well as their application in eicosanoid analysis. In addition, a protocol is given for extracting eicosanoids from biological samples, using bronchoalveolar lavage fluid (BALF) as an example. The method and instrument optimization procedures are presented, followed by the analysis of eicosanoid standards using reverse phase HPLC interfaced with an ion trap mass spectrometer (LC/MS/MS). This protocol is intended to provide a broad description of the field for readers looking for an introduction to the methodologies involved in eicosanoid quantification.

Key Words: eicosanoid, oxylipin, arachidonic acid, mass spectrometry, electrospray, ion trap, LC/MS/MS, bronchoalveolar lavage fluid, BALF

1 Introduction

The biological relevance of arachidonic acid derived mediators (eicosanoids) has been demonstrated in numerous studies.¹⁻⁵ These metabolic products comprise several different classes including leukotrienes (LTs), prostaglandins (PGs), thromboxanes (TXs), hydroxyeicosatetraenoic acids (HETEs), epoxyeicosatrienoic acids (EETs) and oxo-fatty acids. Eicosanoid biosynthesis is accomplished via several distinct enzymatic pathways including cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP). These three different pathways together are responsible for the biosynthesis of the entire suite of compounds collectively known as the arachidonic acid cascade, forming a complex interactive network that is present in almost all tissues.^{1, 2} The most well known eicosanoids are the PGs, TXs, HETEs and LTs, but more recently, the lipoxins (LXs) and EETs have attracted considerable attention. The distribution and synthesis of eicosanoids are key targets for studies involving a range of inflammatory pathologies including asthma and chronic obstructive pulmonary disease (COPD),^{6, 7} nephritis,^{8, 9} cardiovascular diseases^{9, 10} and cancer.^{11, 12}

Historically the detection and quantification of eicosanoids have mainly been performed by high performance liquid chromatography (HPLC) or gas chromatography coupled to mass spectrometry (GC/MS), as well as the more sensitive radio- and enzyme-immunoassay techniques (RIA and EIA).^{13, 14} RIA and EIA approaches have two limitations: 1) generally, only a single product can be detected at a time and 2) cross reactivity can cause variability in sample quantification. HPLC and GC/MS methods are generally less sensitive than RIA and EIA, but offer increased selectivity for the detection of multiple eicosanoids simultaneously.¹⁵⁻¹⁷ However, compounds must be both volatile and thermally stable in order to perform GC/MS-based analyses, which necessitates the use of chemical derivatization for the analysis of most eicosanoids.

An initial limitation in the development of a combined LC/MS method was the interface between the HPLC and the mass spectrometer. However, with the development of MS ionization techniques such as electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), eicosanoids can now be analyzed directly from aqueous samples. A major advantage of this approach is the simplification of sample preparation.¹⁸ As a result, liquid chromatography tandem mass spectrometry (LC/MS/MS or LC/MSⁿ) has become one of the most powerful techniques to quantify large numbers of eicosanoids in a variety of biological matrices.^{3, 7, 19-21} The HPLC separates the compounds based upon physical properties, followed by unambiguous identification based upon the characteristic product ions in the MS. This is generally achieved by scanning in selective reaction monitoring (SRM) mode. The acquisition rate, sensitivity and selectivity provided by SRM enables the acquisition of high quality data for more compounds at faster acquisition rates. The advent of ultra performance liquid chromatography (UPLC) methodologies will further increase this process based on increased chromatographic capacity.²² HPLC/MS/MS has consequently become the only platform capable of performing concurrent quantification of the large numbers of analytes required for studying the biological role of eicosanoids. The choice of chromatography type and mass spectrometer depend on the characteristics of the eicosanoids in the sample and the purpose of the study. Studies have been performed in which a few selected class-specific eicosanoids are analyzed as well as large metabolic profiling studies of a range of eicosanoids.

In this protocol we present some of the main LC/MS/MS techniques and methods used for the detection and quantification of eicosanoids in biological samples. A brief description of MS instrumentation, ionization methodologies, and mass analyzers is discussed followed by the principles and use of SRM in eicosanoid quantification. A method is also described for extracting eicosanoids from biological samples using bronchoalveolar lavage

fluid (BALF) as an example. The steps for preparing eicosanoid standards, gradient solvents and optimization of MS parameters for developing an LC/MS/MS based quantification method for eicosanoid analysis with an ion trap mass spectrometer are then presented. Finally, the instrumentation parameters and preliminary results for method development are shown. It is expected that readers will gain an understanding of the range in technologies available for LC/MS/MS-based analyses of eicosanoids.

1.1 Analysis of eicosanoids using reversed phase high performance liquid chromatography

Endogenous eicosanoids are present in low levels in biological tissues (~pmol/mg to fmol/mg range).^{3, 23} Accordingly, the majority of analytical methods begin with a clean-up and/or separation step to enrich the sample for the compounds of interest. While technically possible,²⁴ direct injection of complex samples into the MS is challenging. Subsequently, the use of an HPLC interfaced with the MS results in improved detection limits and overall quality of the MS data.¹⁸ Separating the analytes reduces background noise and problems associated with ion suppression from coeluting compounds as well as minimizes isobaric interferences. The choice of column, particle size and mobile phase composition depends on the quantity and chemical properties of the target compounds; however there is a limited variety of mobile phases compatible with MS interfaces. Because most eicosanoids are medium to nonpolar, reverse phase (RP) chromatography is most commonly used.^{3, 7, 20, 21, 25-}
³¹ Analytes elute in order of increasing hydrophobicity with a hydrophobic stationary phase (e.g., C₁₈) and a gradient changing gradually from aqueous solvents (with the addition of volatile salts, acids or bases that improve ionization and retention on the HPLC column) to organic solvents (usually methanol and acetonitrile). The gradient is most commonly run

under acidic conditions using a small percentage (~0.01-0.1% v/v) of acetic or formic acid. However there are examples of gradients run under both neutral and basic conditions.^{32, 33}

1.2 Analysis of oxylipin stereoisomers by liquid chromatography mass spectrometry

The use of chiral columns for HPLC-based analysis is a valuable tool for the separation of lipid enantiomers. Chiral liquid chromatography has been extended to both LC/MS and LC/MS/MS for the determination of oxylipin enantiomers. In nature, several oxylipins including hydroperoxyeicosatetraenoic acids (HPETEs), hydroperoxyoctadecadienoic acids (HPODEs), hydroxyoctadecadienoic acids (HODEs), HETEs and EETs are formed as racemic mixtures via nonenzymatic reactions.^{34, 35} On the other hand, when these lipids are oxidized enzymatically, enantiopure products are predominantly produced.³⁶ Thus, chiral phase LC/MS is a valuable tool for diagnosing the mechanism and source of enantiomer formation.

To improve the limit of detection (LOD), the carboxyl groups of these oxylipins can be derivatized to pentafluorobenzyl esters (PFB)³⁷ and further analyzed by chiral phase liquid chromatography electron capture atmospheric pressure chemical ionization mass spectrometry (chiral LC/EC/APCI/MS). A number of enantiomers of regioisomeric HODEs and HETEs have been analyzed in biological samples by chiral LC/EC/APCI/MS³⁸ and the results have been reviewed elsewhere.^{39, 40} Although PFB-derivatization and chiral LC/EC/APCI/MS have improved the LOD of chiral oxylipins, the method has some disadvantages including extra derivatization steps and sample clean up as well as low yield for conversion to the corresponding PFB-esters. Attempts have been made to analyze oxylipins in their free acid forms. For example, chiral LC/MS/MS was used to analyze underivatized enantiomeric oxylipins and subsequently identified 12(S)-HETE as the major enantiomer in the urine of patients with diabetes mellitus (DM).⁴¹ The effects of

phenobarbital on the synthesis of enantiomers of cytochrome P450 metabolites of arachidonic acid and EETs in rat liver were determined by chiral LC/APCI/MS.⁴²

At present, the main limitation of chiral LC/MS for the analysis of oxylipin enantiomers is the availability of chiral columns. For example, chiral columns are produced predominantly as normal phase, which is not compatible with ESI, but can be used with APCI under well defined experimental conditions. However, even these limitations are being addressed as demonstrated by the recent development of a capillary tandem column chiral phase LC/MS/MS method for the analysis of EET enantiomers.⁴³ This method can simultaneously quantify all 8 EET enantiomers as the free acids with limits of quantification in the low pg range.

1.3 Mass spectrometry techniques used for eicosanoid HPLC/MS/MS analysis

The first step in mass spectrometry analysis is to convert analyte molecules into gas phase ions. Following ion production, the ions are separated by a mass analyzer that measures the mass to charge ratio (m/z). There are a number of different mass analyzers, which vary mainly by their mass range limits (the upper limit of the mass of the ion that can be measured); acquisition rate (the rate at which the mass analyzer measures scans over a particular mass range); transmission range (the ratio of the number of ions reaching the detector to the number of ions leaving the source); mass accuracy (accuracy of the ion mass measurement provided by the mass analyzer); and resolution (ability of a mass analyzer to yield 50% valley separation between distinct signals of two ions). This value is typically defined as the ratio of detected m/z to the full width at half-height maximum (FWHM) of the peak. Essentially all mass analyzers can be used for lipidomics or eicosanoid analysis; however, the field has demonstrated a preference for certain mass analyzers that are particularly suitable for quantifying lipids in complex samples. In this section we will discuss

the main ionization techniques and mass analyzers currently used in eicosanoid HPLC/MS/MS analysis.

1.3.1 Ionization techniques

1. **Electrospray ionization (ESI):** ESI was first proposed as a source of gas phase ions by Dole *et al*⁴⁴ in 1968. However, the success of this technique began when Fenn *et al*,⁴⁵ used ESI in mass spectrometry and demonstrated that multiply charged ions were obtained from proteins. This enabled the determination of the molecular mass of a compound using instruments with limited mass range. In ESI the ionization process occurs by applying a strong electric field (kV), under atmospheric pressure, to a liquid passing through a capillary tube. This field induces a charge accumulation at the liquid surface located at the end of the capillary forming a Taylor cone.^{46, 47} The charge accumulation causes droplets that contain an excess positive or negative charge to detach from the capillary tip and move towards the mass analyzer. An uncharged carrier gas (e.g., nitrogen) is often used to help nebulize the liquid and evaporate the solvent in the droplets. As the solvent evaporates, the molecules are forced closer together which increases the Coulombic electrostatic repulsion (i.e., resulting in high surface charge densities on the droplets). This process continues until the Debye length is reached, after which the repulsion overcomes the surface tension of the droplets, which then break up forming ions in a process that is still not well understood. The main advantage of ESI/MS over other MS techniques is that ESI/MS overcomes the propensity of many biomolecules to fragment following ionization and enables the formation of multiply charged ions. Thus, ESI/MS is critical for the detailed structural analysis of large biomolecules, but it is also useful for the analysis of small polar molecules. Another feature of this technique is its relation with the concentration of the target compound,

and not to the total quantity of sample injected in the source. This fact has led to the development of nanoflow rate ESI (nano-ESI). Consequently, ESI has been widely applied in the analysis of all classes of eicosanoids.^{20, 23, 29, 48, 49} Moreover it is not necessary to chemically modify eicosanoids to enhance ionization efficiently when using this technique. Even when chemical modifications (such as the use of methoxime (MO) derivatization) are used to improve the chromatography it does not affect the ionization efficiency of the eicosanoids. The disadvantages of ESI are its low robustness to matrix interferences and changes of mobile phase and flow rates that can produce ion suppression (caused by ionization competition between compounds) and poor ionization of some compounds.

2. Atmospheric pressure chemical ionization mass spectrometry (APCI/MS):

APCI/MS has proven to be valuable for the analysis of lipids from a variety of classes.^{50, 51} APCI is a soft ionization technique, but unlike ESI, APCI usually produces some degree of fragmentation that can be useful for structural characterization. In contrast to ESI, solvent evaporation and analyte ionization are two separate processes. The latter being a chemical ionization at atmospheric pressure with the mobile phase acting as the reactant gas. As the evaporation of the mobile phase is performed at high temperatures (up to 500°C), APCI is less mild than ESI, thus the thermal stability of compounds is important. APCI normally produces singly charged ions through the addition or abstraction of a proton. This technique is frequently used in the LC/MS/MS analysis of eicosanoids.^{42, 52, 53} Compared to ESI methodologies, LC/MS analysis using an APCI source performs best using normal phase (NP) chromatography methods.^{54, 55} Under these conditions the ion detection capability of APCI methodology is greater due to the low interference of the mobile phase on compound ionization; however, the necessary safety precautions need to be taken.

- 3. Electron capture (EC)/APCI:** EC/APCI is a variation of APCI that can be used to improve ion production and subsequently the LOD. This technique requires sample derivatization with an electron capturing group that will enhance formation of negative ions (see section 1.2). EC/APCI gives essentially equivalent results with either NP- or RP-based systems. The technique has not been used extensively for eicosanoid analysis.^{38,39} However, it has great potential for use in the identification of eicosanoid enantiomers and regioisomers, one of the biggest challenges in eicosanoid analysis.^{34,38} The main disadvantages are that it requires derivatization and the PFB derivatives give relatively weak signals under conventional positive APCI conditions when NP solvents are used.
- 4. Atmospheric pressure photo ionization (APPI):** APPI is an ionization technique that extends the range of ionizable compounds.^{56,57} APPI can ionize compounds that ionize poorly by ESI or APCI, especially nonpolar compounds. The APPI source is a modified APCI source that uses a discharge lamp that emits photons rather than a corona discharge needle that emits electrons. APPI uses photons to ionize gas phase molecules after the sample is vaporized by a heated nebulizer similar to the one used in APCI. The analytes interact with photons, which induces a series of gas phase reactions that lead to the ionization of the sample molecules. APPI gives cleaner spectra and is less susceptible to ion suppression, because it is not based on charge affinity. Cai and Sayge⁵⁸ used this technique to analyze the methyl esters of eicosapentaenoic acid (EPA) as well as other fatty acids and described in detail the potential mobile phase alterations.⁵⁹ One drawback with the technique is that it highly depends on the chromatographic system applied (e.g., RP vs. NP) because the solvents can interfere with ion formation.

1.3.2 Mass analyzers

- 1. Quadrupole:** The quadrupole analyzer consists of four parallel metal rods that use the stability of the trajectories in oscillating electric fields to separate ions according to their m/z ratios.^{60, 61} This mass analyzer can be used in either a single or triple quadrupole configuration, with the triple quadrupole used to perform MS/MS experiments. In the triple quadrupole configuration, the first and the last quadrupole serve as mass filters and the center quadrupole (or hexapole) is a collision cell consisting of an RF quadrupole (or hexapole) with optimum ion collimating behavior. This geometry enables different types of MS/MS experiments to be conducted such as product, precursor and constant neutral loss (CNL) scanning as well as SRM. Using a triple quadrupole LC/MS system, Kingsley *et al.*⁴⁸ were able to quantify prostaglandin glyceryl esters from cells with a 25 fmol LOQ and Masoodi *et al.*²⁹ analyzed twenty mono- and poly-hydroxy-fatty acid derivatives of linoleic acid, AA, EPA and docosahexaenoic acid (DHA) with a limit of quantitation (LOQ) of 60-177 fmol. Kita *et al.*⁶² used a triple quadrupole system to develop a high throughput method for the detection of 18 different eicosanoids from biological samples. Using a single quadrupole equipment Blewett *et al.* were able to identify 23 eicosanoids in a single HPLC-MS run.²⁰ However, despite these qualities, quadrupoles are fundamentally low resolution instruments and high mass accuracy can only be achieved at the expense of sensitivity. In addition, MSⁿ experiments can only be performed by multi-quadrupole instruments.
- 2. Ion trap:** Paul and Steinwedel first described the ion trap in 1960,^{61, 63} followed by Stafford *et al.*⁶⁴ who modified it for use in a mass spectrometer. An ion trap uses an oscillating electric field to trap ions in either two or three dimensions. Thus, ion traps exist in two types: 2D ion traps (also referred to as linear ion traps), and the 3D ion

traps (also referred to as Paul ion traps). The 2D ion trap is based upon a four rod quadrupole that ends in lenses that repel the ions inside the rods. The 3D ion trap is composed of a ring electrode and two cap electrodes in order to form a closed area where the ions are trapped. 2D and 3D ion traps work similarly; however, 2D ion traps have a >10-fold higher ion trapping capacity compared with 3D traps. The 2D ion trap cannot perform all the scan functions present in 3D traps due to problems with ion ejection from the trap. The main disadvantage of the ion trap is that precursor ion and neutral loss scans cannot be conducted. Ion traps also have a poor dynamic range and quantification capacity, characteristics that limit their use in the quantification of complex samples.

Ion trap mass analyzers exhibit high sensitivity and are most strongly characterized by the ability to perform multiple stages of mass spectrometry (MS^n). Up to 12 stages of tandem mass spectrometry (MS^{12}) have been performed using an ion trap,⁶⁵ which greatly increases the amount of structural information obtainable for a given molecule. Ion traps are normally coupled to ESI or APCI ionization sources for the structural characterization of eicosanoids. Liminga and Oliw⁶⁶ performed quantitative analysis of LOX products in bovine tissues. They demonstrated that 5-, 12-, and 15-HPETE can be identified by scanning the carboxylated ion of the hydroperoxide and the anion of its dehydration product, $[M-H-H_2O]^-$. Using MS^2 and MS^3 scanning mode they elucidated the structures of these compounds, demonstrated the specificity of transition ions and showed how to use transition ions for quantification. Kiss *et al.*⁶⁷ used capillary LC, photodiode array (PDA) and ESI/ MS^2 in negative mode in order to simultaneously identify and quantitate relevant CYP, sEH, LOX, and COX derived eicosanoids (EETs, DHETs, 20-HETE, LTs, LXs, DHETEs, PGs, TXs, 12-HHT, HETEs) and free isoprostanes (iPTs). This method was capable of identifying 44 eicosanoids in a 50 min

run. Kiss *et al.*⁶⁷ also reported a detailed list of transition ions that can be used for SRM analysis of eicosanoids as well as several MS² spectra of eicosanoid standards.

The cysteinyl containing leukotrienes (LTC₄, LTD₄ and LTE₄) and the 5-oxo-7-glutathionyl-8,11,14-eicosatrienoic acid (FOG₇) were analyzed by Hevko and Murphy⁶⁸ using an ESI/MS with a 3D ion trap and a triple quadrupole mass spectrometer. Both singly charged negative and positive ions could be observed. However, in contrast to the triple quadrupole, the collisional activation of the negative ions in the ion trap required high collision energy, and even then few product ions were produced. It is therefore advisable to analyze these compounds in positive ion mode if an ion trap is used. MS² analyses in this mode showed a common series of fragment ions at *m/z* 319, 301, 283, 265 and 189, which can be observed for all cysteinyl leukotrienes (CysLTs). The ion at *m/z* 319 was formed by cleavage of the carbon-sulfur bond to afford a protonated epoxide cation and loss of the neutral cysteine. The other three ions result from the sequential losses of water, and the ion at *m/z* 189 represents the cleavage of the C6-C7 bond of the lipid backbone. For FOG₇ the MS² spectrum is strikingly simple with only one major fragment ion for cleavage of the γ -glutamate amino acid (*m/z* 497). Accordingly, MS³ analysis was necessary to provide unambiguous structural characterization. Hong *et al.*⁶⁹ used electrospray low energy collision induced dissociation (CID) tandem mass spectrometry analysis and reported the correlations between spectra and structure and the fragmentation mechanisms of resolvin D1, protectin D1, mono-hydroxy-DHA and related hydroperoxy-DHA.

- 3. Time of flight (TOF):** This mass analyzer measures the time it takes ions of different masses to move from the ion source to the detector. Although the principle of TOF has been known since Thomson carried out his experiments on ionized particles in 1897,⁷⁰ its use in a mass spectrometer was not proposed until 1946.⁷¹ In principle, a TOF has no

upper mass range limit, which makes it especially suitable for soft ionization techniques. Other advantages of these instruments include their high transmission efficiency that leads to increased sensitivity and easy mass calibration. The disadvantages include the limited precursor ion selectivity for most MS/MS experiments and the inability to work in MSⁿ mode. TOF is one of the most commonly used mass analyzers for protein analysis, but it can also be used for lipids and eicosanoids.^{18, 72} Dickinson and Murphy⁷³ explored the high resolution of a quadrupole time of flight (QTOF) instrument to elucidate the elemental composition of the ions generated by the collisional activation of the carboxylated anion of PGI₂. Harks *et al.*⁷⁴ used a QTOF equipped with an ESI probe to identify the production of PGF_{2α} (identified as the sodium adduct ion [M+Na]⁺) in normal rat kidney fibroblasts.

4. Fourier transform ion cyclotron resonance (FTICR/MS): Because biological activity is often regioisomer-specific, it is important to have techniques with the ability to identify complex isomeric oxylipins. FTICR/MS has emerged as a powerful tool for this application. FTICR/MS provides high mass accuracy and high mass resolution to obtain accurate molecular masses and elemental compositions.^{75, 76} MSⁿ analysis of FTICR/MS can be achieved by sustained off resonance irradiation (SORI)/CID, infrared multiphoton dissociation (IRMPD), and electron capture dissociation (ECD).⁷⁷⁻⁸¹ Among these dissociation techniques, ECD provides a softer ionization and gives unique fragments for proteins and peptides. SORI/CID and IRMPD yield similar fragmentation patterns, but each method gives specific spectral characteristics that can be useful for compound identification.⁴⁹

The application of FTICR/MS has been demonstrated for the determination of regioisomers of trihydroxyeicosatrienoic acids (THETAs)⁸²⁻⁸⁴ in biological samples.⁴⁹ With three hydroxyl groups, liquid chromatographic separation of 11,12,15-, 11,14,15-

and 13,14,15-THETA is more difficult and their MS/MS spectra are more complex than HETEs and DHETs. In general, the SORI/CID spectra of these THETAs are similar to IRMPD spectra, suggesting similar major fragmentation pathways for these compounds. The most abundant ions for 11,12,15-, 11,14,15- and 13,14,15-THETA are m/z 197, 167 and 193, respectively. The secondary unique characteristic ions for these THETAs are m/z 157, 85 and 59, respectively. The different positions of the third hydroxyl group and the third double bond in the structures such as in 11,12,15-THETA and 11,14,15-THETA have a remarkable effect on their major fragmentation pathways.⁴⁹ The intensities of the m/z 205 in the IRMPD spectrum for 11,14,15-THETA increased two-fold while the abundance of m/z 85 decreased as compared with SORI/CID. For 13,14,15-THETA, the abundance of the m/z 59 and 173 decreased ten-fold in the IRMPD spectrum as compared with SORI/CID. LC/FTICR using SORI/CID and IRMPD (without a complete LC separation) identified 11,12,15-THETA and 11,14,15-THETA as arachidonic acid metabolites that were synthesized in rabbit aorta.⁴⁹ MS/MS analysis of relatively simple HETEs and DHETs has been successfully achieved by ion trap and triple quadrupole mass spectrometers.^{18, 23, 85-88} In general FTICR/MS generates similar MS/MS spectra for these compounds. However, the high mass accuracy and resolution of FTICR/MS suggest some different molecular fragments for the oxylipins. Characteristic ions indicating the loss of the CO₂ group is unique for FTICR/MS spectra. For example, the m/z 149 and 257 ions of 11-HETE are abundant when a FTICR/MS instrument is used. In contrast they have not previously been detected using a triple quadrupole mass spectrometer.^{18, 49, 86} One example of the powerful ability of FTICR/MS in identifying the fragments using mass accuracy and resolution is the identification of m/z 163 for 12-HETE, 11,12-DHET and 14,15-DHET. This fragment was previously assigned as the same ion formed by the same

fragmentation mechanistic pathway for all compounds.¹⁸ From the FTICR/MS spectra, the m/z 163 for 12-HETE and 11,12-DHET has masses of 163.11239 and 163.11235, respectively. This ion has the elemental composition of $[C_{11}H_{15}O]^+$. However, the m/z 163 for 14,15-DHET has the mass of 163.14947 with the elemental composition of $[C_{12}H_{19}]^+$. These accurate masses indicate that the fragmentation mechanism of the m/z 163 for 11-HETE /11,12-DHET is different from 14,15-DHET and they are in fact different fragments.⁴⁹

- 5. Hybrid analyzers:** A recent trend in mass analyzer development is to combine different analyzers in sequence to form hybrid instruments. This increases the versatility and enables multiple experiments to be performed. Quadrupole TOF instruments, ion trap FT ion cyclotron resonance instruments and quadrupole ion trap mass spectrometers, combine the strengths of each analyzer while often avoiding their weaknesses. Accordingly, improved performance can be obtained with a hybrid instrument relative to individual analyzers. Nowadays, the most popular hybrid MS used for eicosanoid analysis is the quadrupole ion trap mass spectrometer (Quad/trap), which provides excellent quantification, thus it is ideal for complex sample analysis. Using a hybrid quadrupole linear ion trap mass spectrometer, Harkewicz *et al.*⁸⁹ were able to detect 70 diverse eicosanoids and eicosanoid metabolites produced from macrophage-like cells. Following metabolic labeling of the cells with deuterated arachidonic acid (AA- d^8) and stimulation with endotoxin, they were able to identify a "doublet" pattern of eicosanoid products. The method enabled sensitive, comprehensive and quantitative analysis of eicosanoid biology without any previous knowledge or assumptions of the molecular species involved. Using the same method they were able to identify a series of dihomoprostaglandins produced by endotoxin-stimulated RAW cells (dihomo-(PGE₂, PGD₂, PGF_{2 α} , 11(R)-HETE, PGJ₂, and 15d- Δ ^{12,14}-PGD₂)), further

demonstrating the utility of this technique. See Note 1 for more information of hybrid instruments and applications.

1.4 Selective reaction monitoring of eicosanoids

SRM is used in conjunction with LC/ESI/MS to improve the specificity and detection limit for quantification. SRM is a tandem mass spectrometry technique that delivers unique fragment ions that can be monitored and quantified in the midst of a complicated matrix. SRM spectra are highly specific and usually contain only a single peak. This characteristic makes the SRM signal ideal for quantification. In order to use this technique, it is necessary to know the fragmentation patterns of the compounds of interest. A fragment ion is selected from the known mass product spectrum and used for quantification. It is important not to choose a fragment peak ion close to the parent peak, such as dehydrated ions. These type of ions do not have structural significance and can easily be produced equally for isomers (e.g., the range of HETEs), or for unknown contaminants. It is also important to choose a stable peak that consistently appears scan after scan with a constant response.

1.5 Quantitation of eicosanoids using HPLC/MS/MS

One of the most important components in developing methods for the quantification of eicosanoids involves the preparation of standards. While beyond the scope of this chapter, we will provide a brief summary of some of the more important points. Interested readers are directed to the literature.^{19, 90-92}

1. Internal standards (IS) are added prior to sample preparation in order to account for variable losses during the preparation steps. The choices of IS are critical. The ideal standard possesses characteristics similar to those of the eicosanoid during the entire experiment (i.e., extraction, chromatography and mass spectrometry), but does not

interfere with the target analyte(s). Many methods employ isotopically labeled versions of the analytes or odd chain fatty acids. However, since there are cost concerns with the use of multiple labeled standards, usually a single IS is employed for a group of eicosanoids with similar physical properties or structure. For example, the deuterated eicosanoid 5-HETE-d⁸ is an ideal IS for 5-HETE; however, it can also be used for other HETEs.¹⁹ Other examples include 10(11)-epoxyheptadecanoic acid (10(11)-EpHep), which is an odd chain length IS for EETs and 10,11-dihydroxydecanoic acid (10,11-DHN), which is appropriate for DHETEs.^{3,7,27} It is notable that the IS concentration does not need to be accurate, but that it is crucial that the exact same volume is used for both the samples and the primary standards.¹⁹ An important precaution is that sometimes deuterated standards can contain a small percent of nondeuterated analyte. Consequently, it is important to also run all IS separately, in order to obtain information on purity.¹⁹ To account for changes in volume, instrument variability and matrix interferences, an additional technical standard (for example CUDA) can be added prior to analysis.^{7,27} The technical standard should be a stable compound that does not interfere with other standards and analytes.

2. Primary standards (PS) are prepared for quantification purposes in order to create calibration curves (or standard curves). Consequently the concentration of PS must be known with high accuracy. Calibration curves are generated by the addition of defined amounts of analyte (unlabeled) followed by spiking with an aliquot of the IS.^{3,19,21,90} The calibration curves are based on the ratio of standard peak area to the IS peak area in the primary standards plotted versus the amount of primary standard. The concentrations of the standards are generally selected such that the resultant curve follows a linear regression in the desired detection range. The slope of the curve is a measurement of the sensitivity of the quantification method. The detection range is

restricted on the low end by the detection limit of the MS and the upper limit is restricted by ion self-suppression and detection saturation issues. Standards should be run before and after each set of samples in order to monitor for changes in instrument performance during the run. The ratio of the unknown analyte peak area to IS peak area in each sample is then compared to the respective standard curve in order to calculate the amount of analyte.

3. In addition to using standards for quantification, compound recoveries have to be estimated during sample preparation and instrumental analysis. This can be achieved by preparing a set of samples where a known amount of the analytes is added to the matrix (e.g., phosphate buffered saline (PBS) solution in the protocol below). The samples are then analyzed by LC/MS/MS and the eicosanoid peak intensities are compared to those standards that have not been through the sample preparation procedure. One can also compare the standards with samples in which only the matrix alone has gone through the sample preparation steps. The aliquot of the standard mixture is then added in the last step prior analysis. Recovery values in the range of 75%-120% are preferred.¹⁹
4. Even though the procedures described above limit the effect of systematic errors during quantification, random errors will still occur. Thus, there will always be a difference between the reported and “true” value, and since the possibility of error increases close to the detection limit a formal determination of the LOD is recommended. A common preconception in analytical chemistry is that the signal (S) has to be three times larger than the background noise (N), resulting in the LOD being equivalent to a S/N ratio of 3. However, the formal definition the LOD is the concentration of analyte required to give a signal equal to the background noise (blank) plus three times the standard deviation (STD) of the blank.³ Similarly the LOQ is defined as the concentration of analyte required to give a signal equal to that of the blank plus ten times the standard

deviation of the blank.^{3, 93} Importantly, sample preparations prior to analyses always involve extra factors of error and sample loss. Therefore the overall method detection limit (MDL) can be estimated by determining the STD across seven or more technical replicates of the sample for a concentration within the range or 1-5 times the expected LOD. Subsequently, the MDL can be calculated by multiplying the STD with the Student's *t* value for the appropriate degree of freedom and the 99% confidence limit (2.143 for *n*=7. A selection of *t* values can be found at <http://www.itl.nist.gov/div898/handbook/eda/section3/eda3672.htm>).⁹² The detection limits depend upon the instrument and configuration. Furthermore it depends on the number of eicosanoids and classes of eicosanoids that are being quantified. LOD values between 5-20 fmol and LOQ values between 5-50 fmol have been reported for the quantification of prostaglandin glyceryl esters.⁴⁸ Newman *et al.* simultaneously quantified epoxy and hydroxy fatty acids in urine with estimated MDL values between 0.5-5 fmol and LOQ values between 1.5-15 fmol.³ Recently a method was presented where over 70 diverse eicosanoids were screened with LODs in the range of 2–25,000 fmol.^{19, 89}

2 Materials

2.1 Equipment

1. Surveyor HPLC system (Thermo Finnigan, San Jose, CA, USA) equipped with a Surveyor Autosampler Plus and a Surveyor MS Pump Plus (four solvent channels).
2. LCQ Deca ion trap (Thermo Finnigan) working with an electrospray ionization source equipped with a stainless steel needle kit (Thermo Fisher Scientific, Waltan, MA, USA).

3. Optiplex 755 computer (Dell, Round Rock, TX, USA) equipped with a Windows XP operating system. The system software XcaliburTM 2.0 SR2 (Thermo Finnigan) was used for data processing.

2.2 Reagents

1. LC/MS grade acetonitrile and methanol and HPLC grade glacial acetic acid were obtained from Fischer Scientific (Loughborough, LE, UK). All other solvents were acquired from Rathburn Chemicals Ltd. (Walkerburn, Scotland).
2. 20-hydroxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid (20-HETE) and *N*-cyclohexyl-*N'*-dodecanoic acid urea (CUDA) were obtained from Cayman Chemical (Ann Arbor, MI, USA). All other standards were obtained from Biomol (Plymouth, PA, USA).

2.3 Supplies

1. Luna C₁₈ column (2.0 x 150 mm, 5 μ) (Phenomenex, Allerod, Denmark).
2. C₁₈ security guard cartridges (4 x 2 mm) (Phenomenex).
3. 2 mL amber glass autosampler vials with plastic screw caps (8 mm) and Teflon-faced seals (8 mm) (Chromacol, Thermo Fisher Scientific).
4. 150 μ L polyspring insert for 2 mL autosampler vials (Thermo Fisher scientific).
5. 2 mL amber glass vials with plastic crew caps (8 mm) and Teflon-faced seals (8 mm) (Chromacol, Thermo Fisher Scientific)
6. Glass μ L syringes (10, 100 and 500 μ L) with PTFE-tipped plungers (Hamilton Company, Bonaduz, Switzerland).
7. 2 mL amber volumetric flasks (Volac) (Poulten & Graf Ltda, Barking, UK).
8. FluoroporeTM PTFE membrane filters (47 mm, 0.2 μ m) (Millipore, Billerica, MA, USA).

9. Nylon membrane filters (47 mm, 0.2 μm) (Millipore).

3 Methods

3.1 Protocol

Eicosanoids are frequently measured in a variety of biological matrices including urine, cell culture, plasma and BALF.^{3, 7, 27, 32} Several different methods for sample preparation are available.^{3, 7, 21, 27, 32, 94-96} Generally sample preparation procedures are straight forward, with the exception of biological tissues, which require prior extraction steps.^{21, 23, 96,}⁹⁷ A protocol optimized for the preparation of BALF samples for LC/MS/MS analysis is given below, however the protocol can be readily adapted to other body fluids with slight modifications.^{7, 27} The extraction method is based upon methods developed by Hammock and coworkers^{3, 5, 7, 27, 98} and is described in detail in a recent publication by Yang *et al.*⁹⁹ Subsequently, a set of oxylipin standards was analyzed under various conditions in order to provide an example of the experimental optimizations that need to be performed prior to analysis of biological samples (see Results). For limitations of this method see Note 2.

3.1.1 Off-line solid phase extraction (SPE)

1. Clean Oasis HLB SPE 60 mg columns with one column volume of ethyl acetate followed by two column volumes of methanol. Precondition columns with two column volumes of H₂O:MeOH (95:5) in 0.1% acetic acid (wash solution). Elute the solvents by gravity and avoid letting the sorbents go dry.
2. Thaw BALF aliquots (4 mL) before extraction. Add 200 μL wash solution to columns, 10 μL of IS (400 nM/standard) and 10 μL anti-oxidant and enzyme inhibitor solution (0.2 mg/mL of butylhydroxytoluene (BHT), ethylenediaminetetra acetic acid (EDTA),

thiamine pyrophosphate (TPP) and indomethacin). Apply samples to the columns. In addition to samples, prepare matrix blanks (~ 2 blanks/30 samples) by using 0.1 M PBS instead of BALF.

3. Wash by gravity with two column volumes of wash solution, vacuum dry for 10 min at 5 psi.
4. Elute eicosanoids with 0.5 mL methanol followed by 1.5 mL ethyl acetate. Collect in 2 mL eppendorf tubes with 6 μ L 30% glycerol in methanol.
5. Use speed vacuum or nitrogen stream to remove solvent until only the glycerol plug remains.
6. Cap and freeze samples for storage at -80 °C or continue to step 7.
7. Prior to LC/MS analysis reconstitute residues to 50 μ L with 200 nM CUDA, centrifuge and transfer supernatant to glass autosampler vials.

3.1.2 Standards, solvents and MS optimization preparations

1. For method development purposes ionization optimization standard solutions were prepared for all analytes as follows: 1-10 μ L of the standard stock solution (0.05-0.5 mg/mL) were transferred to 2 mL amber glass vials with screw caps and Teflon-faced seals and then diluted in methanol to a final volume of 1 mL (0.5 ng/ μ L). Similarly a chromatography optimization solution containing all standards was prepared by adding 2-20 μ L/standard of the stock solutions to a 2 mL amber glass volumetric flask. The standard mixture was then diluted with methanol to a final concentration of 0.5 ng/ μ L. See Note 3 for further comments on sample treatments.
2. LC/MS grade acetonitrile and methanol were vacuum filtered (0.2 μ m PTFE filter) and prepared in a ratio of 88:12. For preparation of the aqueous phase, MilliQ water was vacuum filtered (0.2 μ m Nylon filter). In addition to the neutral aqueous (1) and organic

(2) phases, an acidic phase (3) containing (1) and (2) in the ratio of 85:15 was prepared with an additive of 0.1% glacial HPLC graded acidic acid. See Note 4 for more comments on solvent treatments.

3. In order to optimize the MS parameters for optimal peak detection the ionization optimization standard solutions (0.5 ng/ μ L) were introduced into the MS source directly using a syringe pump. The flow rate was set to 10 μ L/min and T-coupled to the HPLC in order to achieve a flow rate of 350 μ L/min. The LC gradient was set to a similar ratio of solvent (1) and (2) as when the respective compound was known to elute from the column. By recording and comparing intensity changes in the molecular ion to changed parameters of the MS (e.g., heated capillary temperature, capillary voltages, sheath gas flow etc.) optimal conditions could be obtained and saved (as tune files). Compounds were also fragmented in order to determine which fragment ions to select for SRM analysis (based on abundance and specificity). An instrument method was developed based on the compounds retention times, tune files and selected fragment ions. The chromatographic run was divided into different time segments in order to reduce the number of scan events over time, subsequently improving the detection limit (Note 5). The chromatography optimization solution was repeatedly run over time in sequences with up to 15 injections in order to determine the reproducibility of the method. It is vital that the system is reproducible, with little fluctuation in retention times and peak areas (Note 6).

3.1.3 Instrument operation parameters

HPLC:

Autosampler temperature: 10 °C

Column temperature: 40 °C

Mobile phase:

- (1) MilliQ water 18 MΩ (2) Acetonitrile:Methanol (88:12)
(3) Water:Acetonitrile:Methanol (85:13:2), 0.1% Acetic acid

Gradient:

Parameters are described in Table 1 for a set of 24 compounds.

Flow rate: 350 μ L/min

Column temperature: 40 °C

LCQ ion trap:

Capillary temperature: 300 °C

Collision energy (resonance energy, percent of 5 V): 40 – 50 %

Spray voltage: 4 kV

Sheath gas flow rate: 70 arbitrary U

Auxiliary gas flow rate: 20 arbitrary U

Scan mode: Negative

SRM: parameters are described in Table 2.

3.2 Results

1. We compared differences in separation and detection of eicosanoids under acidic (pH 4), neutral and basic (pH 8) conditions (Figure 1A-C, Table 3). Results clearly showed that analytes demonstrated improved performance (i.e., narrower peak shapes, reduced tailing, and improved baseline resolution) under weakly acidic conditions. However, a basic mobile phase can potentially be useful for certain eicosanoid targets. Optimal resolution was achieved after preconditioning of the column at acidic pH followed by a neutral gradient (Figure 1D). These conditions were therefore used for further development of the method.

2. 24 arachidonic and linoleic acid metabolites were analyzed by LC/MS/MS using a 45 min pH gradient (Table 1) and scanning in SRM mode (Table 2). LOD values from 0.5-250 pg/ μ L were observed (Table 2). Figure 2 shows a total ion chromatogram of the analyzed compounds and ion chromatograms obtained for eicosanoids detected 6 to 10 minutes into the run. Compounds with similar properties (i.e., PGs, LTs, HETEs, HODEs, KETEs and EETs) have close retention times but can readily be distinguished by differences in fragmentation pattern. An example of this is shown in Figure 3 in which the MS² spectra of 5-HETE (**A**) and 12-HETE (**B**) are shown. The dehydrated ion at m/z 301 is present in both spectra, as well as the ion at m/z 257. However, scanning in SRM mode for the diagnostic fragment peaks at m/z 115 for 5-HETE and at m/z 179 for 12-HETE enabled the routine detection and quantification of these compounds.

4 Notes

1. The use of new instrumentation such as a quadrupole ion trap time of flight (QITTOF) or FTICR/MS will result in lower LODs in the range of attomol or even zeptomol levels of constituent quantities. The use of these techniques for eicosanoid quantification can greatly increase our ability to detect and quantify a variety of eicosanoids in biological samples that have not been detected previously. This ability is important for clinical applications where the amount of sample available is often a limiting factor. Furthermore, the development of multi-isotope imaging mass spectrometry (MIMS) opens a new field of opportunities, enabling studies of the interaction, detection and quantification of eicosanoids within a single cell.
2. There are specific challenges with the quantification of CysLTs, which are best analyzed directly by on-line SPE coupled to the HPLC/MS/MS run in positive mode.⁷

⁹⁵ This procedure is recommended because CysLTs are sensitive to oxidation and off-line SPE extraction can result in decreased recoveries.

3. Eicosanoids are sensitive to oxidation and consequently standards should always be stored at low temperatures (e.g., ≤ -80 °C) under an inert atmosphere (e.g., argon) in the absence of light, moisture and active surfaces. Samples should be kept in a temperature controlled environment both before as well as during the run (a common feature of many autosamplers).
4. It is important to use HPLC and MS grade solvents for the preparation of chromatographic mobile phases to reduce background noise and keep the instrument in a good working condition. The aqueous phase should be frequently replaced (1/week) in order to reduce the possibility of microorganism growth. In addition, the system should be regularly flushed with organic solvent (e.g. methanol).
5. The limit for the number of compounds that can be identified in a single run depends upon the HPLC separation as well as the ion dwell times (based on the number of analytes in each SRM, the inter-scan delays and the peak width of the analytes). Accordingly, this number will vary greatly with different instruments.
6. System stability is particularly important when operated with large sample sets where the MS conducts multiple scans within a single run.

Acknowledgements

We gratefully acknowledge the assistance of Malin Nording, Katrin Georgi, Jun Yang and Pavel Aronov for helpful discussions and critical reading of the manuscript. This research was supported by The Åke Wibergs Stiftelse, the Fredrik and Ingrid Thuring's Stiftelse, The Royal Swedish Academy of Sciences, The Swedish Council for Strategic Research, The

Swedish Research Council and the European Commission. C.E.W. was supported by a fellowship from the Centre for Allergy Research (Cfa).

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Table 1. Gradient parameters for Figure 2.

Time (min)	1(%)^a	2(%)^b	3(%)^c
0.0	85	15	0
0.5	85	15	0
2.0	70	30	0
8.0	45	55	0
28.0	25	75	0
28.5	0	100	0
34.0	0	100	0
37.0	0	0	100
45.0	0	0	100

^a1: Water

^b2: Acetonitrile:Methanol (88:12)

^c3: Water:Acetonitrile:Methanol (85:13:2), 0.1% Acetic acid. Solvent 3 is used to precondition the column prior to the next run.

Table 2. Method parameters for analyzed eicosanoids.

Segment	Analyte	Rt (min)	Transition [<i>m/z</i>]	Collision Energy (%)	LOD (pg/ μ L)
1	6-keto-PGF ₁ α	7.0	369>315	40	0.5
	20-hydroxy-LTB ₄	7.4	351>195	40	0.5
	20-carboxy-LTB ₄	7.4	365>347	40	2.5
	TBX ₂	8.1	369>195	40	0.5
	PGF ₂ α	8.6	353>309	40	0.5
	11-dehydro-TBX ₂	9.3	367>305	40	0.5
2	LXA ₄	10.4	351>115	40	5.0
	LTB ₅	11.5	333>195	40	0.5
	LTD ₄	11.7	495>177	40	0.5
	LTE ₄	12.0	438>333	50	250
3	15-dPGJ ₂	16.9	315>271	40	25
	20-HETE	17.0	319>275	40	5.0
	13-HODE	18.6	295>195	40	0.5
	9-HODE	18.9	295>171	40	0.5
	15-HETE	19.4	319>219	40	2.5
	15-KETE	20.6	317>113	40	0.5
	12-HETE	21.0	319>179	40	0.5
	5-HETE	22.6	319>115	40	0.5
	14,15-EET	23.3	319>219	40	2.5
	11,12-EET	25.0	319>167	40	25
	5-KETE	25.1	317>273	40	25
8,9-EET	25.1	319>155	40	2.5	
4	DHA	30.9	327>283	50	5.0
	AA	31.3	303>259	40	5.0

Table 3. Gradient parameters for Figure 1^a.

Time (min)	1(%)^b	2(%)^c	3(%)^d
0.0	85	15	0
0.5	85	15	0
1.0	70	30	0
5.0	45	55	0
10.0	25	75	0
10.5	0	100	0
15.0	0	100	0
16.0	85 ^e	15 ^e	100 ^f
21.0	85 ^e	15 ^e	100 ^f

^aSolvent (1) and (2) were run under neutral conditions or with an additive of either 0.1% Acetic acid or 0.01% NH₄⁺ (25% aqueous solution) depending on desired pH with a flow rate of 350 μL/min.

^b1: Water

^c2: Acetonitrile:Methanol (88:12).

^d3: Water:Acetonitrile:Methanol (85:13:2), 0.1% Acetic acid.

^eUsed parameters for chromatograms **A-C** in Figure 1.

^fUsed parameters for chromatogram **D** in Figure 1.

Figure 1 . Chromatographic separation of eight eicosanoids (1. 6-keto PFG₁α, 2. LTB₄, 3. 15-HETE, 4. 5-KETE, 5. 12-HETE, 6. 5-HETE, 7. 14,15-EET and 8. 11,12-EET). Gradient parameters are described in Table 3. **(A)** Gradient run with an additive of 0.1 % Acetic acid. **(B)** Gradient run with an additive of 0.01 % NH₄⁺ solution (25%). *This peak contains all eight eicosanoids. **(C)** Gradient run without an additive (neutral conditions). **(D)** Gradient run without additive, but preconditioned under the same acidic conditions as in **A**.

Figure 2. ESI/MS/MS chromatogram obtained for the eicosanoids in Table 2. Details for all SRM parameters are provided in Table 2. **(A)** Total ion chromatogram. **(B-G)** Compounds detected in Segment 1: **(B)** 6-keto PGF₁α, **(C)** 20-hydroxy-LTB₄, **(D)** 20-carboxy-LTB₄, **(E)** TXB₂, **(F)** PGF₂α and **(G)** 11-dehydro-TXB₂.

Figure 3. MS² analysis of 5-HETE (*m/z* 319) **(A)** and of 12-HETE (*m/z* 319) **(B)**. The fragment ions at *m/z* 115 and 179 are specific for 5-HETE and 12-HETE respectively, and can be used in SRM analysis for compound identification and subsequent quantification.

Figure 1

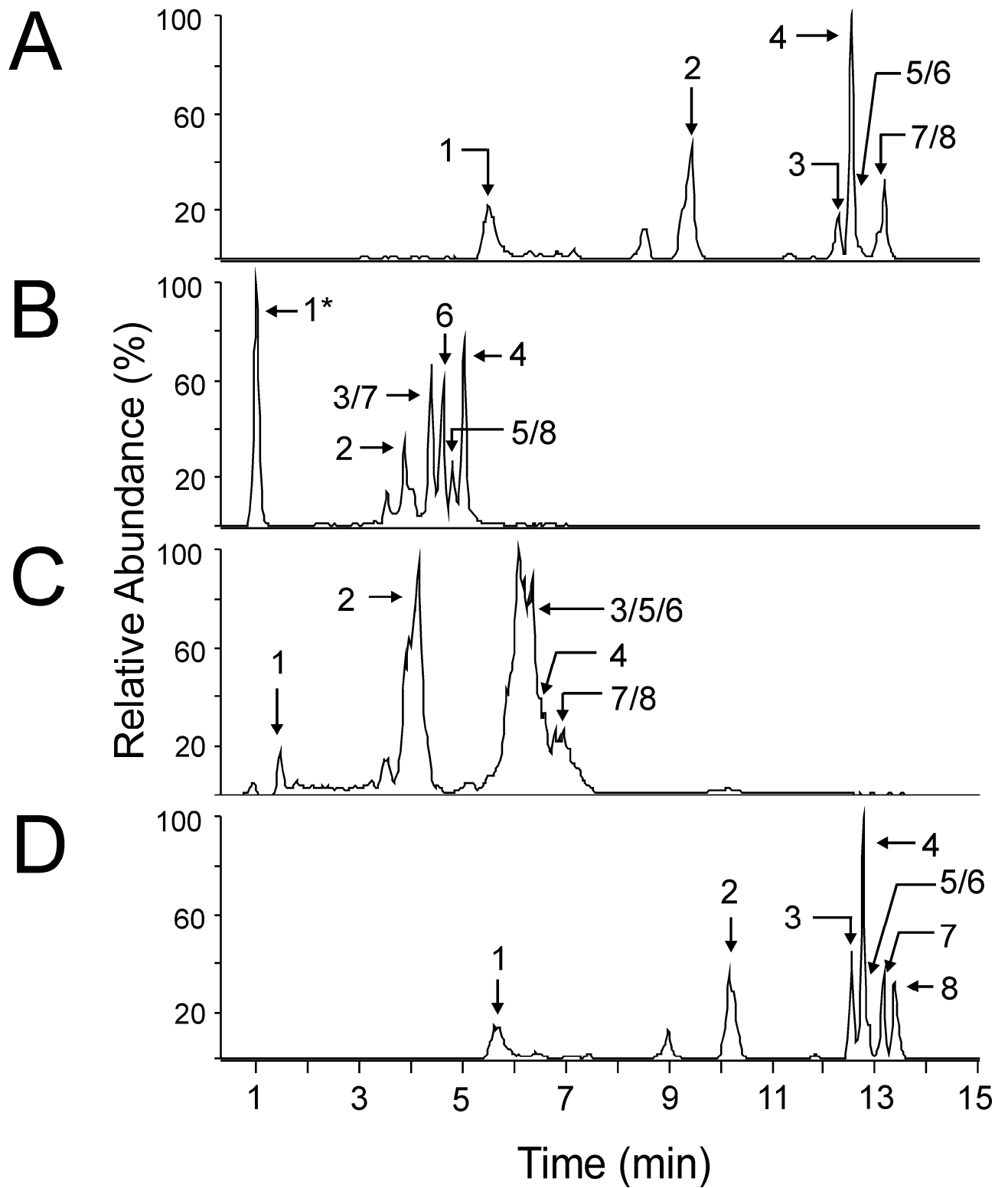


Figure 2

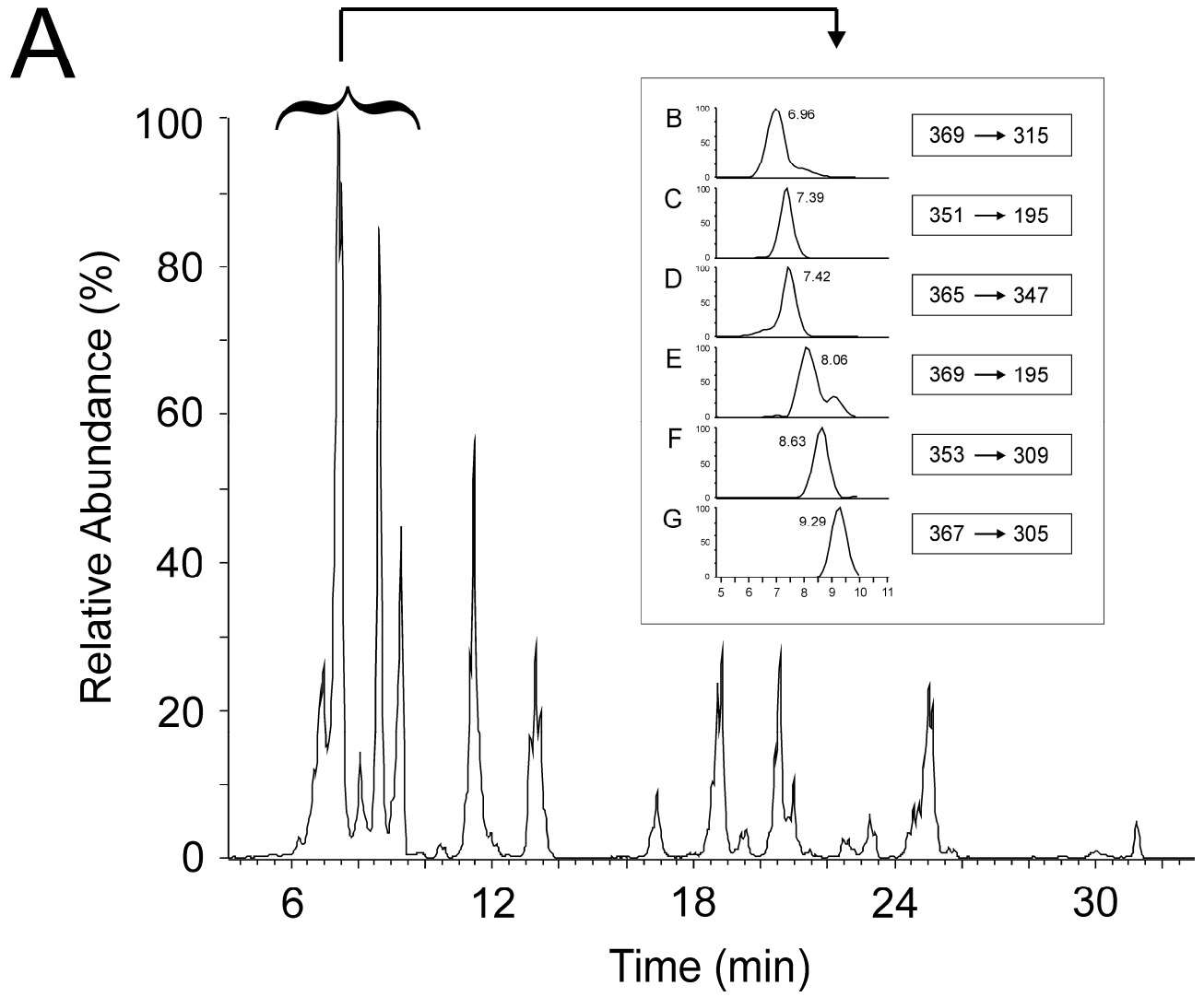
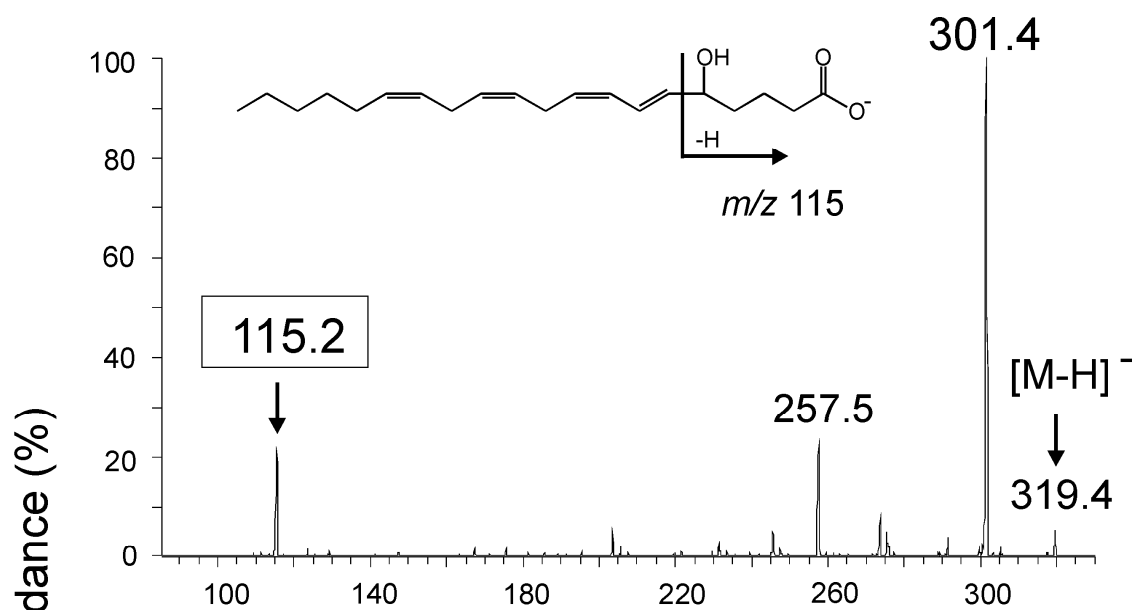


Figure 3

A



B

