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CHAPTER THREE

Detection and Quantitation of Eicosanoids via High Performance Liquid Chromatography-Electrospray Ionization-Mass Spectrometry

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Abstract

Eicosanoids constitute a large class of biologically active arachidonic acid (AA) metabolites that play important roles in numerous physiological processes. Eicosanoids are produced by several distinct routes, including the cyclooxygenase, lipoxygenase, and P450 enzymatic pathways, as well as by nonenzymatic processes. In order to completely understand the eicosanoid response of a cell

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or tissue to a given stimulus, measuring the complete profile of eicosanoids produced is important. Since the eicosanoids are products of a single species, AA, and represent, for the most part, the addition of various oxygen species, the hundreds of eicosanoids have very similar structures, chemistries, and physical properties. The identification and quantitation of all eicosanoids in a single biological sample are a challenging task, one that high-performance liquid chromatography-mass spectrometry (LC-MS) is well suited to handle. We have developed a LC-MS/MS procedure for isolating, identifying, and quantitating a broad spectrum of eicosanoids in a single biological sample. We currently can measure over 60 eicosanoids in a 16-min LC-MS/MS analysis. Our method employs stable isotope dilution internal standards to quantitate these specific eicosanoids. In the course of setting up the LC-MS system, we have established a library that includes relative chromatographic retention times and tandem mass spectrometry data for the most common eicosanoids. This library is available to the scientific community on the website www.lipidmaps.org.

1. Introduction

The eicosanoids comprise a broad class of AA metabolites that mediate a wide variety of important physiological functions. Most of the AA found in cells is esterified to the *sn*-2 position of neutral lipids or phospholipids (Schaloske and Dennis, 2006; Six and Dennis, 2000). Upon activation, phospholipase A_2s release the AA from cellular phospholipids. The free AA can then be converted by a score of enzymes in three distinct oxidation pathways (cyclooxygenase [Simmons *et al.*, 2004; Smith *et al.*, 2000], lipoxygenase [Funk, 2001; Peters-Golden and Brock, 2003; Spokas *et al.*, 1999], and cytochrome P450 [Sacerdoti *et al.*, 2003]) into hundreds of bioactive species. These pathways exhibit a fair amount of redundancy, as some eicosanoid species can be produced by more than one enzyme. Likewise, some enzymes are capable of producing more than one eicosanoid product. In addition to these reactions, AA and its metabolites can also undergo nonenzymatic oxidation and dehydration reactions to produce many of these same compounds as well as other metabolic species, including stereoisomers of the enzymatic products. Thus, eicosanoid biosynthesis is a complex network of interacting pathways and interconnected metabolites. It is quite possible that perturbing one arm of this system could produce changes and compensations in the other arms. In order to fully understand how a given cell or tissue responds to a stimulus or how a drug targeted for one eicosanoid might affect the distribution of the other eicosanoids, one must determine the entire eicosanoid spectrum.

Enzyme-linked immunosorbent assays have long been the primary means of quantitating eicosanoids (Reinke, 1992; Shono *et al.*, 1988). This method requires specific antibodies for each eicosanoid to be quantitated; however,

relatively few eicosanoids have commercially available antibodies. This significantly limits the number of eicosanoids that can be detected and quantitated. This technique is also expensive and inefficient in that only a single eicosanoid can be determined with each assay. Thus, it is not amenable to analyzing a large number of different eicosanoids. Gas chromatography-MS (GC-MS) methods were developed that greatly improved upon these limitations (Baranowski and Pacha, 2002) and allowed the simultaneous analysis of multiple eicosanoids. To volatilize the eicosanoids for GC, they must first be chemically derivatized. A wide variety of derivatization methods are available; however, a single derivatization method is not suitable for all eicosanoids. Furthermore, Murphy *et al.* (2005) have found that some eicosanoids are not suited for GC-MS analysis. These volatilization issues were overcome with the development of electrospray ionization (ESI), which allows the eicosanoids to be analyzed by MS directly from an aqueous sample. The eicosanoid carboxylate moiety readily ionizes in the ESI source.

The similarities in eicosanoid structure and chemical characteristics requires that both high performance LC and collision-induced decomposition (CID) be employed, in conjunction with ESI-MS, to isolate and unambiguously identify the individual eicosanoid species. LC isolates the eicosanoids based on their chemical and physical characteristics, while CID produces characteristic precursor/product transitions that can be employed in multi-reaction monitoring (MRM) mode on the MS. ESI-MRM was first employed in this field by Margalit *et al.* (1996) to quantitate 14 eicosanoids directly from a biological sample. In 2002, the resolving power of LC was coupled with the sensitivity of ESI-MRM to study five eicosanoids from LPS (lipopolysaccharide)-stimulated synovial cells (Takabatake *et al.*, 2002). Recently, Kita *et al.* (2005) developed a high-throughput method for the detection of 18 different eicosanoids from biological samples.

We present here a protocol for identifying and quantitating a large number of eicosanoids in a single ESI-based LC-MS/MS run without requiring derivitization. This technique employs a solid-phase extraction procedure to isolate eicosanoids, an LC method to separate species, and a MS-CID technique to unambiguously identify a large number of eicosanoids. To accurately quantitate eicosanoids using these procedures, we have employed the well-established stable isotope dilution method (Hall and Murphy, 1998). We have included deuterated eicosanoids as internal standards to track and measure losses during sample preparation and to account for the various response issues associated with mass spectral analysis.

We currently can identify over 60 discrete chemical species of eicosanoid in a single 16-min run and can quantitate a significant fraction of them (Buczynski *et al.*, 2007; Harkewicz *et al.*, 2007). We present the methods for the extraction and analysis of eicosanoids from media and cells produced during cell culture. We then describe the protocols for these methods and conclude with a discussion of some characteristics of this procedure.

2. Methods

2.1. Sample collection

The following procedure was developed for the isolation of eicosanoids from six-well cell culture plates containing 2.0 ml of media. We have also adapted this method to other sample types and sample volumes by scaling our procedure as needed. The media was removed and 100 μl of a mixture of internal standards (containing 10 ng/100 μl of each standard in EtOH) was added followed by 100 μl of EtOH to bring the total concentration of EtOH to 10% by volume. Samples were centrifuged for 5 min at 3000 rpm to remove cellular debris. The eicosanoids were then isolated via solid-phase extraction.

When intracellular eicosanoids were analyzed, adherent cells were scraped into 500 μl of MeOH, and then 1000 μl of phosphate-buffered saline (PBS) and 100 μl of internal standards were added. Scraping cells in aqueous solutions was shown to activate eicosanoid production, whereas doing so in MeOH effectively stopped the reactions and lysed the cells. These samples were then processed the same as the media.

2.2. Eicosanoid isolation

Eicosanoids were extracted using Strata® X SPE columns (Phenomenex, Torrance, CA). Columns were washed with 2 ml of MeOH followed by 2 ml of H_2O. After applying the sample, the columns were washed with 1 ml of 10% MeOH, and the eicosanoids were then eluted with 1 ml of MeOH. The eluant was dried under vacuum and redissolved in 100 μl of solvent A (water-acetonitrile-formic acid [63:37:0.02; v/v/v]) for LC-MS/MS analysis.

2.3. Reverse-phase liquid chromatography

The analysis of eicosanoids was performed by LC-MS/MS. Eicosanoids were separated by reverse-phase LC on a C18 column (2.1 $\times$ 250 mm; Grace-Vydac, Deerfield, IL) at a flow rate of 300 μl/min at 25°. All samples were loaded via a Pal auto-sampler (Leap Technologies, Carrboro, NC) that maintained the samples at 4° to minimize degradation of eicosanoids while queued for analysis. The column was equilibrated in Solvent A, and samples (dissolved in Solvent A) were injected using a 50-μl injection loop and eluted with a linear gradient from 0 to 20% solvent B (acetonitrile-isopropyl alcohol [50:50; v/v]) between 0 and 6 min; solvent B was increased to 55% from 6 to 6.5 min and held until 10 min; solvent B was increased to 100% from 10 to 12 min and held until 13 min; and then, solvent B was dropped to 0% by 13.5 min and held until 16 min.

2.4. Chiral chromatography

When it was required to isolate isomeric eicosanoids, normal-phase chiral liquid chromatography was carried out using the same pumping system described above for reverse-phase chromatography. Separation was carried out on a 4.6 × 250 mm Chiral Technologies (West Chester, PA) derivatized amylose column (Chiralpak® AD-H) equipped with a guard column (Chiralpak® AD-H guard column) held at 35°. Buffer A was hexane/anhydrous ethanol/water/formic acid: 96/4/0.08/0.02, v/v; buffer B was 100% anhydrous ethanol. This small amount of water in buffer A is miscible in the hexane/anhydrous ethanol mix and was found to be vital for satisfactory chiral separation and peak shape. Gradient elution was achieved using 100/0:A/B at 0 min; linearly ramped to 90/10:A/B by 13 min; linearly ramped to 75/25:A/B by 15 min and held until 25 min; and then linearly ramped back to 100/0:A:B by 27 min and held there until 42 min to achieve column re-equilibration. The chiral chromatography effluent was coupled to a mass spectrometer for further analysis.

2.5. Mass spectrometry

All MS analyses were performed using an Applied Biosystems (Foster City, CA) 4000 QTRAP hybrid, triple-quadrupole, linear ion trap mass spectrometer equipped with a Turbo V ion source and operated in MRM mode. For all experiments, the Turbo V ion source was operated in negative electrospray mode (chiral chromatography utilized the ion source in chemical ionization mode, as shown later) and the QTRAP was set as follows: CUR = 10 psi, GS1 = 30 psi, GS2 = 30 psi, IS = −4500 V, CAD = HIGH, TEM = 525°, ihe = ON, EP = −10 V, and CXP = −10 V. The voltage used for CID (−15 to −35 V) and the declustering potentials (−30 to −100 V) varied according to molecular species and were maximized for each eicosanoid.

The Turbo V ion source was operated in atmospheric pressure chemical ionization (APCI) mode when employing chiral chromatography using the following settings: CUR = 10 psi, GS1 = 45 psi, GS2 = 60 psi, NC = −3.0 μA, CAD = HIGH, TEM = 400°, ihe = ON, DP = −60 V, EP = −15 V, and CXP = −10 V.

2.6. Quantitation

Eicosanoid quantitation was performed by the stable isotope dilution method previously described by Hall and Murphy (Hall and Murphy, 1998). For each eicosanoid to be quantitated, an internal standard was selected that had a different precursor ion mass than the target analyte, but was chemically and structurally as similar to the target analyte as possible. This is ideally achieved by using a deuterated analog of the analyte.

We employed these standards whenever they were commercially available. In other cases, we employed a deuterated analog that was the closest to the desired analog in characteristics. For example, 15d-$\Delta^{12,14}$ PGJ_2 (d4) was employed as the internal standard for PGJ_2, 15d-$\Delta^{12,14}$ PGJ_2, and 15d-$\Delta^{12,14}$ PGD_2. Table 3.1 lists the internal standards that we are currently employing (boxes in gray) and indicates which internal standard is used with which analyte. Presently, eight deuterated internal standards are used to quantitate 16 eicosanoids. An aliquot of the internal standard (10 ng std/100 μl of ethanol) was added to either the media or cell extracts immediately following its isolation. The samples were then processed as previously detailed.

The primary standards contained an accurately known amount of each eicosanoid (non-deuterated) to be quantitated and an accurate aliquot of the internal standards. The concentration of the primary standards must be known with high accuracy. This can be accomplished in one of several ways. In some cases, they are commercially available. Cayman Chemicals, for example, offers a ''Quanta-PAK'' version of many eicosanoids that contains a deuterated internal standard and a vial containing an accurately determined amount of the non-deuterated primary standard. Some of the eicosanoids (e.g., some HETEs and leukotrienes) have significant ultraviolet (UV) absorption that can be employed to determine the concentration of the standard. The amount of standard can also be determined gravimetrically if a microbalance if available.

The set of primary standards was then prepared by adding accurately determined amounts of the given analyte (non-deuterated) to 100 μl of the same internal standard used to spike the samples. (Note: the concentration of the internal standard does not need to be accurate, but it is crucial that an accurately known volume of the exact same internal standard is added to the sample and to the primary standards.) A typical standard curve consisted of 0.3, 1, 3, 10, 30, and 100 ng of primary standard per 100 μl of internal standard containing 10 ng of each internal standard. The internal standard and the primary standard samples were run before and after each set of unknown samples, and 10 μl of each was loaded onto the column.

A linear standard curve was generated where the ratio of analyte standard peak area to internal standard peak area in the primary standards was plotted versus the amount of primary standard (ng). Figure 3.1 shows examples of three typical standard curves. Linear regression analysis was used to calculate the slope and intercept of the standard curves that were then used to calculate the unknowns. R^2 values for these curves of greater than 0.99 were routinely obtained. The ratio of the unknown analyte peak area to internal standard peak area in the sample was then compared to the appropriate standard curve to calculate the amount of analyte in the sample. Since, in some cases, the deuterated standards contained a small percent of non-deuterated analyte, the LC-MS/MS of the internal standard was analyzed to determine the amount of non-deuterated analyte present. In this case, the

Table 3.1 Eicosanoid library

Eicosanoid[a]	Systematic name	[M–H] *(m/z)*	Production *(m/z)*	LC retention time[b] (min)	Internal standard	Recovery[c]	Limit of detection (pg on column)
AA	5Z,8Z,11Z,14Z-eicosatetraenoic acid	303	259	12.4	AA-d_8	E	50
AA-d_8	5Z,8Z,11Z,14Z-eicosatetraenoic acid (5,6,8,9,11,12,14,15-d8)	311	267	12.4		ND	ND
AA-EA	N-(5Z,8Z,11Z,14Z-eicosatetraenoyl)-ethanolamine	346	259	10.6		E	10
5(S)6(R)DiHETE	5S,6R-dihydroxy-7E,9E,11Z,14Z-eicosatetraenoic acid	335	163	9.0		M	5
5(S)6(S)DiHETE	5S,6S-dihydroxy-7E,9E,11E,14Z-eicosatetraenoic acid	335	163	9.0		E	1
5(S)15(S)DiHETE	5S,15S-dihydroxy-6E,8Z,11Z,13E-eicosatetraenoic acid	335	201	7.8		E	5
8(S)15(S)DiHETE	8S,15S-dihydroxy-5Z,9E,11Z,13E-eicosatetraenoic acid	335		ND[d]		ND	ND
±5,6-DiHETrE	5,6-dihydroxy-8Z,11Z,14Z-eicosatrienoic acid	337	145	9.0		E	1
±8,9-DiHETrE	8,9-dihydroxy-5Z,11Z,14Z-eicosatrienoic acid	337	127	8.8		E	1

(*continued*)

Table 3.1 (*continued*)

Eicosanoid[a]	Systematic name	[M–H] (*m/z*)	Production (*m/z*)	LC retention time[b] (min)	Internal standard	Recovery[c]	Limit of detection (pg on column)
±11,12-DiHETrE	11,12-dihydroxy-5Z,8Z,14Z-eicosatrienoic acid	337	167	8.7		E	1
±14,15-DiHETrE	14,15-dihydroxy-5Z,8Z,11Z-eicosatrienoic acid	337	207	8.6		E	1
±5,6-EpETrE	5,6-epoxy-8Z,11Z,14Z-eicosatrienoic acid	319	191	10.1		E	5
±8,9-EpETrE	8,9-epoxy-5Z,11Z,14Z-eicosatrienoic acid	319	127	10.0		E	5
±11,12-EpETrE	11,12-epoxy-5Z,8Z,14Z-eicosatrienoic acid	319	167	9.8		E	10
±14,15-EpETrE	14,15-epoxy-5Z,8Z,11Z-eicosatrienoic acid	319	139	9.7		E	50
5(R)HETE	5R-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid	319	115	9.64	5(S)HETE-d_8	ND	ND
5(S)HETE	5S-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid	319	115	9.64	5(S)HETE-d_8	E	1
5(S)HETE-d_8	5S-hydroxy-6E,8Z,11Z,14Z eicosatetraenoic acid (5,6,8,9,11,12,14,15-d8)	327	116	9.60		ND	ND
8(R)HETE	8R-hydroxy-5Z,9E,11Z,14Z-eicosatetraenoic acid	319	155	9.43		ND	ND
8(S)HETE	8S-hydroxy-5Z,9E,11Z,14Z-eicosatetraenoic acid	319	155	9.43		E	1
9-HETE	9-hydroxy-5Z,7E,11Z,14Z-eicosatetraenoic acid	319	151	9.49		E	1
11(R)HETE	11R-hydroxy-5Z,8Z,12E,14Z-eicosatetraenoic acid	319	167	9.31	5(S)HETE-d_8	ND	ND
11(S)HETE	11S-hydroxy-5Z,8Z,12E,14Z-eicosatetraenoic acid	319	167	9.31	5(S)HETE-d_8	E	1

12(R)HETE	12R-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid	319	179	9.38		ND	ND
12(S)HETE	12S-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid	319	179	9.38		E	1
15(R)HETE	15R-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid	319	175	9.19	5(S)HETE-d_8	ND	ND
15(S)HETE	15S-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid	319	175	9.19	5(S)HETE-d_8	E	1
20-HETE	20-hydroxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid	319	245	8.98		E	1
12(S)HHTrE	12S-hydroxy-5Z,8E,10E-heptadecatrienoic acid	279	163	8.7		E	50
5(S)HpETE	5S-hydroperoxy-6E,8Z,11Z,14Z-eicosatetraenoic acid	335	155	9.7		E	5
12(S)HpETE	12S-hydroperoxy-5Z,8Z,10E,14Z-eicosatetraenoic acid	335	153	9.4		E	1
15(S)HpETE	15S-hydroperoxy-5Z,8Z,11Z,13E-eicosatetraenoic acid	335	113	9.2		E	1
LTB_4	5S,12R-dihydroxy-6Z,8E,10E,14Z-eicosatetraenoic acid	335	195	8.2		M	5
6 trans LTB_4	5S,12R-dihydroxy-6E,8E,10E,14Z-eicosatetraenoic acid	335	195	7.8		E	1
6 trans 12 epi LTB_4	5S,12S-dihydroxy-6E,8E,10E,14Z-eicosatetraenoic acid	335	195	8.0		E	5

(*continued*)

Table 3.1 (*continued*)

Eicosanoid[a]	Systematic name	[M–H] (*m/z*)	Production (*m/z*)	LC retention time[b] (min)	Internal standard	Recovery[c]	Limit of detection (pg on column)
LTC_4	5S-hydroxy,6R-(S-glutathionyl), 7E,9E,11Z,14Z-eicosatetraenoic acid	624	272	8.8		P	1
11-trans LTC_4	5S-hydroxy,6R-(S-glutathionyl), 7E,9E,11E,14Z-eicosatetraenoic acid	624	272	9.2		M	1
LTE_4	5S-hydroxy,6R-(S-cysteinyl),7E,9E,11Z,14Z-eicosatetraenoic acid	438	235	10.1		M	5
11-trans LTE_4	5S-hydroxy,6R-(S-cysteinyl),7E,9E,11E,14Z-eicosatetraenoic acid	438	235	10.4		M	1
5(S)6(R)15(S) LXA_4	5S,6R,15S-trihydroxy-7E,9E,11Z,13E-eicosatetraenoic acid	351	115	5.2		M	1
5(S)6(S)15(S) LXA_4	5S,6S,15S-trihydroxy-7E,9E,11Z,13E-eicosatetraenoic acid	351		ND[d]		ND	ND
5(S)14(R)15(S) LXB_4	5S,14R,15S-trihydroxy-6E,8Z,10E,12E-eicosatetraenoic acid	351		ND[d]		ND	ND
5-OxoETE	5-oxo 6E,8Z,11Z,14Z-eicosatetraenoic acid	317	203	9.8		E	5
12-OxoETE	12-oxo-5Z,8Z,10E,14Z-eicosatetraenoic acid	317	153	9.4		E	1
15-OxoETE	15-oxo-5Z,8Z,11Z,13E-eicosatetraenoic acid	317		ND[d]		ND	ND

PGA_2	9-oxo-15S-hydroxy-5Z,10Z,13E-prostatrienoic acid	333		ND[d]		ND	ND
dhk-PGA_2	9,15-dioxo-5Z,10-prostadienoic acid	333		ND[d]		ND	ND
PGB_2	15S-hydroxy-9-oxo-5Z,8(12),13E-prostatrienoic acid	333	175	6.6		E	5
PGD_2	9S,15S-dihydroxy-11-oxo-5Z,13E-prostadienoic acid	351	189	4.6	PGD_2-d_4	M	5
PGD_2-d_4	9S,15S-dihydroxy-11-oxo-5Z,13E-prostadienoic acid (3,3,4,4-d4)	355	193	4.6		ND	ND
PGD_2-EA	N-(9S,15S-dihydroxy-11-oxo-5Z,13E-prostadienoyl)-ethanolamine	394	271	3.3		E	10
15d-$\Delta^{12,14}PGD_2$	9S-hydroxy-11-oxo-5Z,12E,14E-prostatrienoic acid	333	271	8.2	15d-$\Delta^{12,14}PGJ_2$-d4	E	1
dhk-PGD_2	11,15-dioxo-9S-hydroxy-5Z-prostenoic acid	351	207	5.9	dhk-PGD_2-d4	P	1
dhk-PGD_2-d4	11,15-dioxo-9S-hydroxy-5Z-prostenoic acid (3,3,4,4-d4)	355	211	5.9		ND	ND
6-keto PGE_1	6,9-dioxo-11R,15S-dihydroxy-13E-prostenoic acid	367	143	3.1		P	5
PGE_2	9-oxo-11R,15S-dihydroxy-5Z,13E-prostadienoic acid	351	189	4.3	PGE_2-d_4	M	10
PGE_2-d_4	11R,15S-dihydroxy-9-oxo-5Z,13E-prostadienoic acid (3,3,4,4-d4)	355	193	4.3		ND	ND
PGE_2-EA	N-(11R,15S-dihydroxy-9-oxo-5Z,13E-prostadienoyl)-ethanolamine	394	203	3.0		M	10000

(*continued*)

Table 3.1 (*continued*)

Eicosanoid[a]	Systematic name	[M–H] (*m/z*)	Production (*m/z*)	LC retention time[b] (min)	Internal standard	Recovery[c]	Limit of detection (pg on column)
bicyclo-PGE_2	9,15-dioxo-5Z-prostaenoic acid-cyclo[11S,16]	333	175	7.4		E	5
dhk-PGE_2	9,15-dioxo-11R-hydroxy-5Z-prostenoic acid	351	207	5.3	dhk-PGD_2-d4	M	1
19(R)-hydroxy PGE_2	9-oxo-11R,15S,19R-trihydroxy-5Z,13E-prostadienoic acid	367	287	2.4		M	1
20-hydroxy PGE_2	9-oxo-11R,15S,20-trihydroxy-5Z,13E-prostadienoic acid	367	287	2.4		M	5
15-keto PGE_2	9,15-dioxo-11R-hydroxy-5Z,13E-prostadienoic acid	349	161	4.7		M	1
tetranor PGEM	11R-hydroxy-9,15-dioxo-2,3,4,5-tetranor-prostan-1,20 dioic acid	327	291	2.3		P[e]	50
6,15-diketo 13,14-dihydro PGF1α	6,15-dioxo-9S,11R-dihydroxy-13E-prostenoic acid	369	267	3.6		P	50
6-keto $PGF_{1\alpha}$	6-oxo-9S,11R,15S-trihydroxy-13E-prostenoic acid	369	207	2.9	6-keto $PGF_{1\alpha}$-d4	E	10
6-keto $PGF_{1\alpha}$-d4	6-oxo-9S,11R,15S-trihydroxy-13E-prostenoic acid (3,3,4,4-d4)	373	211	2.9		ND	ND
$PGF_{2\alpha}$	9S,11R,15S-trihydroxy-5Z,13E-prostadienoic acid	353	193	4.0	$PGF_{2\alpha}$-d_4	M	10
$PGF_{2\alpha}$-d_4	9S,11R,15S-trihydroxy-5Z,13E-prostadienoic acid (3,3,4,4-d4)	357	197	4.0		ND	ND
$PGF_{2\alpha}$-EA	N-(9S,11R,15S-trihydroxy-5Z,13E-prostadienoyl)-ethanolamine	396	334	3.0		M	10

11β-$PGF_{2\alpha}$	9S,11S,15S-trihydroxy-5Z,13E-prostadienoic acid	353	193	3.7		M	1
dhk-$PGF_{2\alpha}$	9S,11S-dihydroxy-15-oxo-5Z-prostenoic acid	353	209	5.2	dhk-$PGF_{2\alpha}$-d4	M	5
dhk-$PGF_{2\alpha}$-d4	9S,11S-dihydroxy-15-oxo-5Z-prostenoic acid (3,3,4,4-d4)	357	213	5.2		ND	ND
2,3 dinor-11β PGF2α	9S,11S,13S-trihydroxy-2,3-dinor-5Z,13E-prostadienoic acid	325	145	3.0		M	1
20-hydroxy $PGF_{2\alpha}$	9S,11S,15S,20-tetrahydroxy-5Z,13E-prostadienoic acid	369	193	2.3		M	50
15-keto $PGF_{2\alpha}$	9S,11R-dihydroxy-15-oxo-5Z,13E-prostadienoic acid	351	217	4.5		M	1
$PGF_{2\beta}$	9R,11R,15S-trihydroxy-5Z,13E-prostadienoic acid	353		ND[d]		ND	ND
tetranor PGFM	9S,11R-dihydroxy-15-oxo-2,3,4,5-tetranor-prostan-1,20-dioic acid	329	293	2.1		P[e]	10
PGG_2	9S,11R-epidioxy-15S-hydroperoxy-5Z,13E-prostadienoic acid	367		ND[d]		ND	ND
PGH_2	9S,11R-epidioxy-15S-hydroxy-5Z,13E-prostadienoic acid	351		ND[d]		ND	ND
PGJ_2	11-oxo-15S-hydroxy-5Z,9Z,13E-prostatrienoic acid	333	189	6.5	15d-$\Delta^{12,14}PGJ_2$-d4	E	1
$\Delta^{12}PGJ_2$	11-oxo-15S-hydroxy-5Z,9Z,12E-prostatrienoic acid	333		ND[d]		ND	ND
15d-$\Delta^{12,14}PGJ_2$	11-oxo-5Z,9Z,12E,14Z-prostatetraenoic acid	315	271	8.8	15d-$\Delta^{12,14}PGJ_2$-d4	E	5

(*continued*)

Table 3.1 (*continued*)

Eicosanoid[a]	Systematic name	[M–H] (*m/z*)	Production (*m/z*)	LC retention time[b] (min)	Internal standard	Recovery[c]	Limit of detection (pg on column)
15d-$\Delta^{12,14}PGJ_2$-d4	11-oxo-5Z,9Z,12E,14Z-prostatetraenoic acid (3,3,4,4-d4)	319	275	8.8		ND	ND
PGK_2	9,11-dioxo-15S-hydroxy-5Z,13E-prostadienoic acid	349	205	4.4		ND	ND
TXB_2	9S,11,15S-trihydroxy-thromboxa-5Z,13E-dien-1-oic acid	369	169	3.6	TXB_2-d4	M	10
TXB_2-d4	9S,11,15S-trihydroxy-thromboxa-5Z,13E-dien-1-oic acid (3,3,4,4-d4)	373	173	3.6		ND	ND
11-dehydro TXB_2	9S,15S-dihydroxy-11-oxo-thromboxa-5Z,13E-dien-1-oic acid	367		ND[d]		ND	ND
2,3-dinor TXB_2	9S,11,15S-trihydroxy-2,3-dinor-thromboxa-5Z,13E-dien-1-oic acid	341		ND[d]		ND	ND

[a] Gray boxes indicate internal standards.
[b] The retention times are given to indicate the relative elution position of the compounds with the understanding that the absolute values are not significant.
[c] Recoveries were grouped as P for poor (<25%), M for moderate (25–75%), E for excellent (<76%), and ND for not determined.
[d] These compounds were not analyzed quantitatively for this table, but the MS/MS spectra and LC retention times are available in the LIPID MAPS Eicosanoid Library at www.lipidmaps.org or in Harkewicz *et al.*, 2007.
[e] Tetranor PGFM and tetranor PGEM did not bind to the Strata-X SPE column and thus could not be detected by this method.

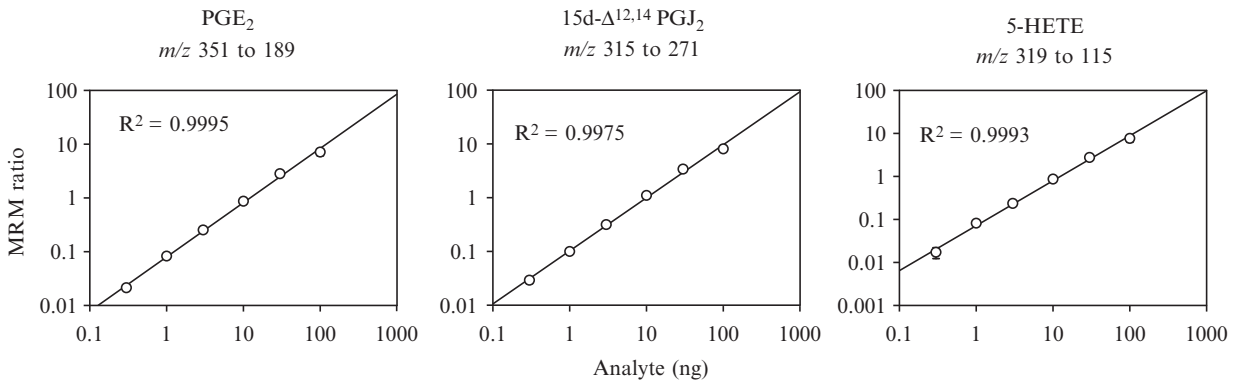


Figure 3.1 Eicosanoid standard curves. Three typical standard curves are shown. The standard solutions are prepared as described in the "Methods" section. The solutions contained 10 ng of an internal standard for each analyte and 0.3 to 100 ng of the analyte in 140 μl of which 10 μl were analyzed. The multi-reaction monitoring (MRM) transitions employed to monitor the analytes are listed in the figure. The internal standards and transitions were: PGE_2 (d4) *m/z* 355–193, 15d-$\Delta^{12,14}$ PGJ_2 (d4) *m/z* 319–275, and 5-HETEs (d8) *m/z* 327–116. The data were presented in log scale, but the linear regression analysis to determine parameters was done on the original non-log data.

non-deuterated contaminant was subtracted from each analysis. The dynamic range that can be covered is limited on the low end by the amount of non-deuterated analyte in the internal standards and the sensitivity of the mass spectrometer. The upper limit is restricted by ion suppression and detector saturation issues.

3. Results and Discussion

3.1. MRM transition selection

To date, we have compiled a library of over 60 eicosanoids that we can detect and quantitate with these methods. These compounds are listed in Table 3.1 with the precursor and product ions used in the MRM analysis. We have also included in this table several compounds for which we have MS/MS spectra but for which we have not selected MRM transitions. We have not included any recovery or limit of detection data for these compounds.

We have compiled the MS/MS spectra of each of these compounds. We have published these data (Harkewicz *et al.*, 2007). These data also can be accessed on the LIPID MAPS web page at http://www.lipidmaps.org. In addition to MS/MS spectra, the web visitor can obtain chemical structures for standards in both GIF and ChemDraw® formats, specific details regarding LC and MS parameters employed in our analysis, structures of dominant fragment ions (including literature references, when available, for fragment assignments), and retention times for a stated set of chromatographic conditions. Lastly, a web link to Cayman Chemical provides useful information and references on specific eicosanoids.

The product ions employed here for the MRM detection were selected to yield the best discrimination from other eicosanoids that co-elute in the vicinity of the analyte and to yield the highest signal. By balancing LC retention time and product ion selection, we were able to successfully distinguish the large majority of the eicosanoids listed. Various product ions can be selected to obtain greater sensitivity if conflicting eicosanoids are not present in a given set of samples.

The MS/MS spectra of most eicosanoids show numerous product ions. While a similar eicosanoid may have the same product ions, their relative intensities usually vary. The ratio of intensities of these product ions can be used to distinguish these species. In this case, multiple MRM transitions can then be analyzed, and the ratio of product ions found in the unknown can be compared with either an MS/MS library spectra or a pure standard run under the same conditions. This would aid in confirming the identity of a chromatographic peak.

Figure 3.2 shows the chromatograms of a few selected eicosanoids to illustrate several points about this procedure. All of the panels in Fig. 3.2 were generated by overlaying the individual MRM chromatograms for the various MRM pairs onto a single plot.

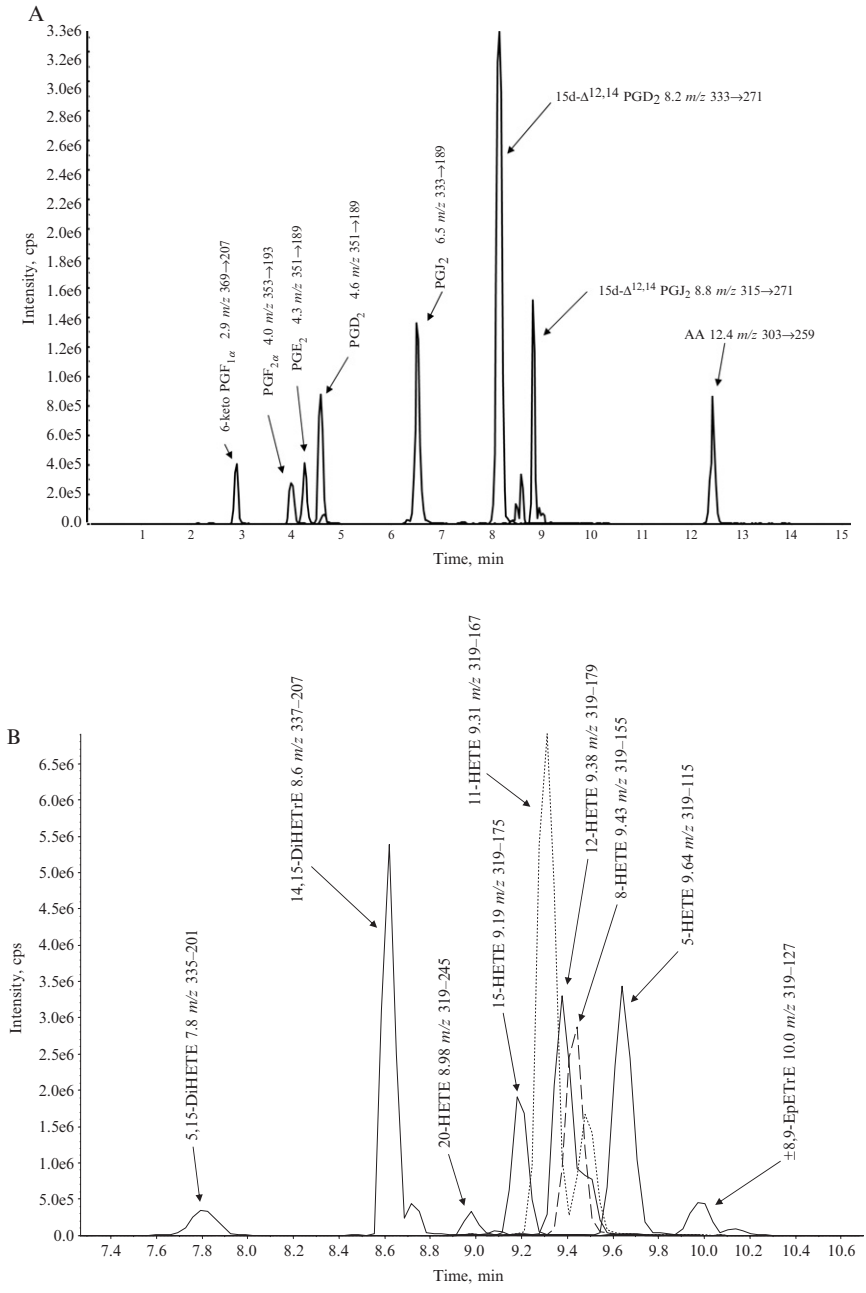
The sharpest peaks have a width at half height of 6 s and the baseline peak width on the order of 18 s. The Applied Biosystems 4000 QTrap can handle over 100 MRM pairs in a single scan. The dwell time employed when we are scanning 60 to 70 analytes is 25 ms. This produces a cycle time of 2 s per scan. This translates to at least nine data points per peak for the narrowest peaks, which is sufficient to accurately define the peak shape for quantitation. The Applied Biosystems 4000 QTrap software allows the user to break the MRM pairs into sets, and these sets can be run in series during the course of a single analysis so that only a fraction of the MRM pairs are being scanned during any time period. Running fewer MRM pairs in each scan allows the dwell time to be increased. Although this would not increase the absolute intensity of the peaks, it would increase the time averaging for each data point, thus decreasing the noise levels and increasing the signal-to-noise (S/N) ratio.

Figure 3.2A displays the retention times of several typical prostaglandins. Most of the prostaglandins show baseline separation from all other prostaglandins, and the analysis and identification of the peaks is fairly clear cut.

Figure 3.2B displays the region of the chromatogram that contains the HETEs, DiHETEs, EpETrEs, and DiHETrEs. Some prostaglandins and the leukotrienes also elute in this region; however, their molecular ions m/z are different from the hydroxy eicosanoids so that they do not appear in these scans. Even so, Fig. 3.2B clearly indicates that significant care must be taken when assigning the peaks in this region to the correct eicosanoid. While the differences in the retention times of the HETEs are reproducible, for the HETEs that elute between 8- and 12-HETE, they are not very large. The absolute value of the retention time for a given eicosanoid fluctuates due to batch variability in the LC solvents, and these shifts can be larger than the differences between the retention times of two neighboring eicosanoids. For this reason, a complete set of standards should be run both before and after each set of unknowns to accurately determine eicosanoid retention times, especially when assigning peak identities. If these HETEs are present in the samples, the gradient in this region of the chromatogram can be altered to spread out this region and improve the resolution.

Figure 3.2C shows the chromatograms of LTB_4, LTC_4, 11-trans LTC_4, LTE_4, and 11-trans LTE_4. This panel also demonstrates one of the significant powers of the MRM method. Examination of Fig. 3.2A and B shows that the region from 8 to 11 min is very crowded, and that a significant number of eicosanoids elute in this region. Yet, when the MRM transitions are different, the chromatograms are very clean, and the assignments are easily made. This panel also highlights an important precaution that must be considered with the MRM method. While the chromatogram of a

particular analyte may appear to be very clean because no other compounds exhibit the same MRM transition, a tremendous amount of material may be eluting in the same place, but with different MRM transitions.



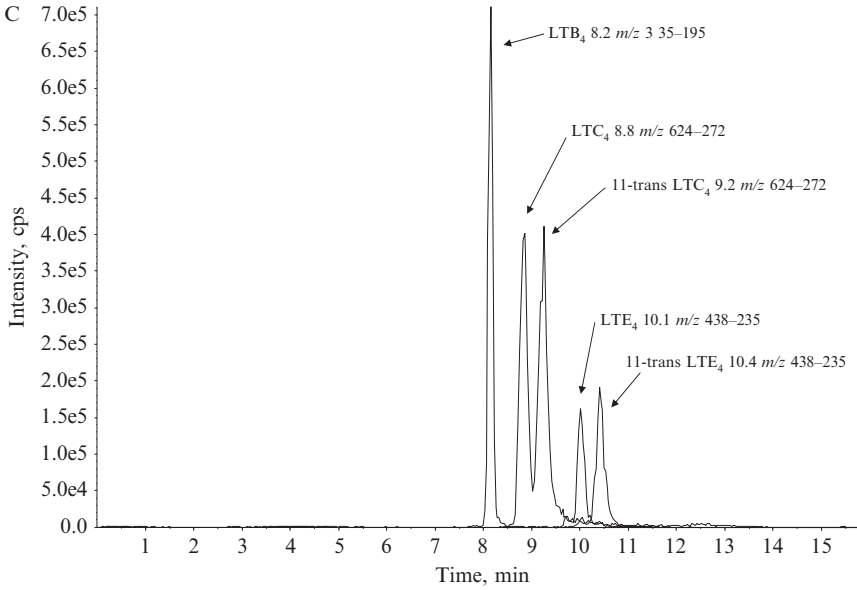


Figure 3.2 High performance liquid chromatography (HPLC) eicosanoid chromatography on reverse-phase C18. The chromatography profiles of selected eicosanoid standards run on reverse-phase C18 HPLC (see "Methods" section). In each panel, the individual chromatograms produced by a given multi-reaction monitoring (MRM) pair have been overlaid. Each label lists the eicosanoid, retention time, and multi-reaction monitoring transition that produced a given chromatogram. (A) Representative sample of prostaglandins and arachidonic acid (AA). (B) Representative sample of the hydroxy-eicosanoids, including examples of HETEs, diHETEs, diHETrEs, and EpETrEs. (C) Representative sample of leukotrienes.

This material can still cause significant ion suppression of the analyte being examined. For example, we have found that PGE_2 and PGD_2 appear to co-elute with material co-extracted from Dulbecco's Modification of Eagle's Medium (DMEM) cell culture media, significantly diminishing the MS sensitivity of these eicosanoids (see "Recoveries" section). The proper use of internal standards can compensate for some of these issues during quantitation.

3.2. Stereoisomer detection

Table 3.1 contains eight pairs of isomers that have identical retention time and MS/MS spectra, and thus cannot be distinguished with the C18 column. To identify and quantitate these isomers normal phase chiral chromatography is required.

3.3. Lower limit of detection

Table 3.1 also contains an estimate of the lower limit of detection (LOD) for the eicosanoid when employing our standard procedures. A set of standards was made and then serially diluted to produce a set of standards such that 1 to 5000 pg would be loaded onto the column. A signal was judged to be significant if the signal area was three times the noise at the three-standard-deviation level. Most limits were between 1 and 50 pg loaded onto the column. Only PGE_2-EA had an LOD greater than 50 ng. The detection limits that we obtained were achieved with a 2.1 × 250 mm Grace-Vydac reverse-phase C18 column with 5 μm particle size. Decreasing the column diameter, the particle size, and the flow rate can increase sensitivity, as can changing the column packing. We have analyzed only one isomer of any isomeric analytes and only non-deuterated analytes. Therefore, we have listed the LOD and recovery values for the other isomers and for the deuterated standards as not determined (ND) in Table 3.1. However, their recoveries and LODs should be very similar to the corresponding isomer that was analyzed or the equivalent non-deuterated analyte.

Figure 3.3 shows an example of the analysis of a sample that was close to our lower limit of detection. It is the chromatogram showing the analysis of PGE_2 and PGD_2 in a sample of spinal fluid from a rat that had been

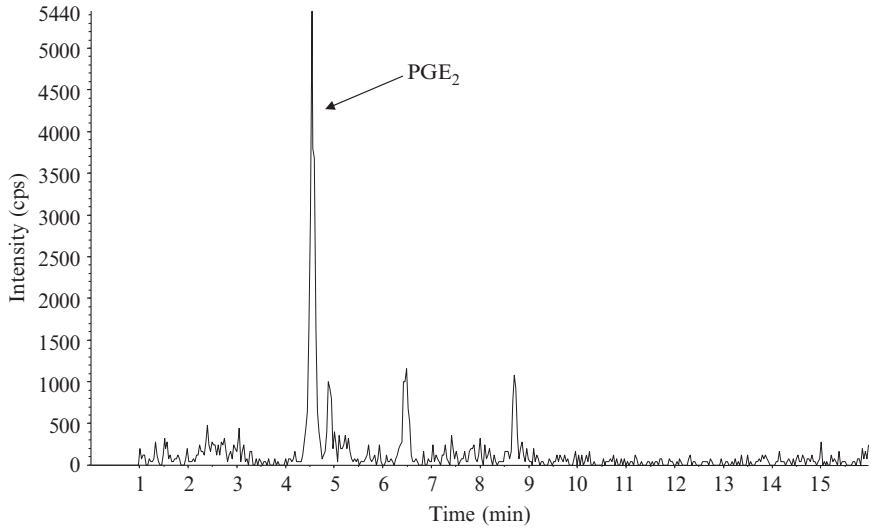


Figure 3.3 PGE_2 analysis of rat spinal fluid. Spinal fluid was collected from a rat whose paw had been injected with carrageenan to induce an inflammatory pain state. A sample of spinal fluid was removed (42 μl), processed as described in the "Methods" section and analyzed by liquid chromatography–mass spectrometry–mass spectrometry. Eighty percent of the sample was analyzed. The PGE_2 peak corresponds to 52 pg of PGE_2 being loaded onto the column. The signal-to-noise (S/N) ratio was 16-fold over a three-standard-deviation noise level.

subjected to the carrageenan model of inflammatory pain. This represents 52 pg of PGE_2 load onto the column and correlates with 150 pg of PGE_2 present in the original 42-μl spinal fluid sample.

3.4. Recoveries

Our primary goal was to analyze as many eicosanoids in a single run as possible. We chose to focus on maximizing the recovery of AA, prostaglandins, and HETEs. Table 3.1 shows the recoveries that this system achieved for most of the analytes that we detect. These recoveries were determined by adding a known amount of each analyte, contained in a standard mix, to 2.0 ml of water or DMEM, and then isolating the eicosanoids via the standard sample preparation method previously outlined. We also did an "add back" experiment where DMEM alone was extracted by the same method; however, instead of adding an aliquot of the standard eicosanoid mixture to the DMEM sample before extraction, the eicosanoids were added to the post-extraction methanol column effluent. Losses in the "add back" samples would indicate that materials in the media are being co-extracted with the eicosanoids, and that these contaminants affect the eicosanoid MS response. All three sets of samples were analyzed with our LC-MS/MS procedure. The eicosanoid peak intensities of these samples were compared to those of standards that had not been through the isolation procedure, but instead were directly analyzed by LC-MS/MS.

Table 3.1 reports the relative recoveries of standards that were extracted from DMEM. The mono- and di-hydroxy eicosanoids had excellent recoveries at between 75 and 100%. The leukotrienes and prostaglandins had only moderate recoveries in the range of 50%. In most cases, the recoveries of these compounds from water were in the 80 to 100% range. The "add back" experiment showed that, for the prostaglandins, most of the losses occurred in the "add back" experiment, implying that some component from the media is being extracted that decreases the MS response to the prostaglandins. However, the "add back" levels for the cysteinyl leukotrienes were 100% within experimental error, suggesting that the losses occurred during the extraction process, and that media components are not affecting leukotriene detection. Tetranor PGEM and PGFM do not bind to the Strata-X SPE columns under these conditions and could not be detected by this protocol. The PGK_2 had very large errors, and extraction could not be measured. Again, the yield of a given class of compounds could be improved by altering the conditions or column type, but often at the expense of other analytes.

3.5. Miscellany

The process of culturing cells can affect the eicosanoid levels in other ways as well. For example, PGD_2 can undergo dehydration to form PGJ_2, 15d-$\Delta^{12,14}$ PGJ_2, and 15d-$\Delta^{12,14}$ PGD_2. This dehydration has been reported to

be accelerated in serum albumin (Fitzpatrick and Wynalda, 1983; Maxey *et al.*, 2000). This decomposition will not be compensated for by the internal standards since they are not added until after the cell incubations. We have also found that when 10% serum is present during the cell culture, no LTC_4 could be detected; however, LTE_4, which is a breakdown product of LTC_4, was detected. When the same experiments are run in serum-free media, significant levels of the LTC_4 were detected, but very little LTE_4. Presumably, the serum is catalyzing the conversion of LTC_4 to LTE_4. Clearly, the types of recovery experiments outlined above must be conducted whenever applying this system to a new type of sample (e.g. media, serum, or tissues) or when changing the conditions of an already-tested sample. Care must also be taken to determine the effects that the addition of any agents (e.g., inhibitors, activators, or drugs) have on the extractions and on the quantitation of any other analytes. LC-MS/MS should also be done on the agents being added to determine if they have any MRM transitions that could be mistaken for one of the standard analytes.

Traditionally, lipids solutions were routinely acidified before subjecting them to liquid extractions. We compared our recoveries with and without acidifying the media before application to the SPE columns. We found that there were no significant differences; therefore, we do not routinely acidify the media. It is possible that eicosanoids other than those we employed in our testing could benefit by acidification. In addition, some eicosanoid species are susceptible to air oxidation and/or adhere to vessels and should not be taken to dryness. To guard against these losses, 20 μl of a 50/50 solution of glycerol/ethanol can be added to the methanol column elution just before drying down the samples on the Speedvac. The glycerol remains, and the eicosanoids are concentrated into the glycerol, which can then be taken up in the LC equilibration buffer for LC-MS/MS analysis.

3.6. Summary

The method outlined above is a sensitive, accurate one for identifying and quantitating a large number of eicosanoids in a single LC-MS/MS run. This method can detect 1 pg (on column) of many eicosanoids, which is similar to the sensitivity of EIA analysis. Its primary advantage is that a large number of different species can be measured in a single analysis, while EIA analysis requires specific antibodies that are often not commercially available.

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