

Targeted Profiling of Lipid Mediators

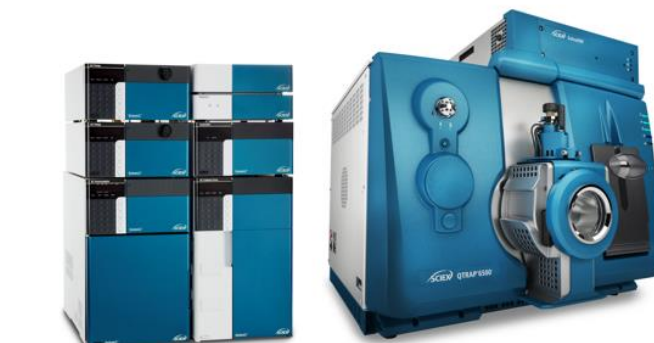
Multiplexed Assay using the SCIEX QTRAP® 6500+ LC-MS/MS System

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Lipid mediators are a class of bioactive molecules that regulate many physiological processes and dysregulation of their biosynthesis and signaling is linked to many inflammatory diseases. They are biosynthesized from arachidonic acid (i.e., eicosanoids) as well as from other fatty acids including EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid). Lipid mediators influence a diverse range of cellular functions that govern both inflammation¹ and its resolution². For example, pro-inflammatory mediators include prostaglandins and leukotrienes, while specialized pro-resolving mediators (SPMs) include lipoxins, resolvins, protectins and maresins. These mediators regulate immune cell motility, migration and cytokine release as well as the presentation of antigens and the generation of antibodies. During acute inflammatory conditions, specific lipid mediators are produced by neutrophils, macrophages and platelets, as well as by endothelial and epithelial cells to accelerate the killing and clearance of infectious agents.^{1,2,3,4}

The ability to identify and differentiate between many structurally related lipid mediators at low concentrations in biological samples is critical to the understanding of human physiology and disease response. Targeted lipidomics strategies using highly sensitive LC-MS/MS has emerged as an important analytical technique that can tackle the challenge of lipid mediator profiling and characterization.^{3,5,6,7}

In this report, a quantitative and qualitative workflow is described for the analysis of different lipid mediator species including



specialized pro-resolving mediators (SPM) leukotrienes, prostaglandins, hydroxy-eicosatetraenoic acids (HETEs) and epoxy-eicosatrienoic acids (EETs) using the QTRAP 6500+ System (Figure 1).

Key Features of the QTRAP 6500+ System for the Analysis of Lipid Mediators

- The QTRAP system offers high sensitivity data acquisition strategies for both quantitative and qualitative experiments
- High-throughput quantitative analysis of lipid mediators is achieved using MRM (Multiple Reaction Monitoring) and the *Scheduled MRM™* Algorithm Pro⁸
- MRM triggered EPI (Enhanced Product Ion) scans enable high sensitivity MS/MS confirmation of low-level analytes
- A single positive ion/negative ion switching method enables the optimum detection of a diverse set of lipid mediator species within one data acquisition

- MultiQuant™ Software enables calculation of the precision, accuracy, sensitivity, and linearity of the quantitative method for all lipid mediators included in the assay
- Complete method provided for easy adoption⁹

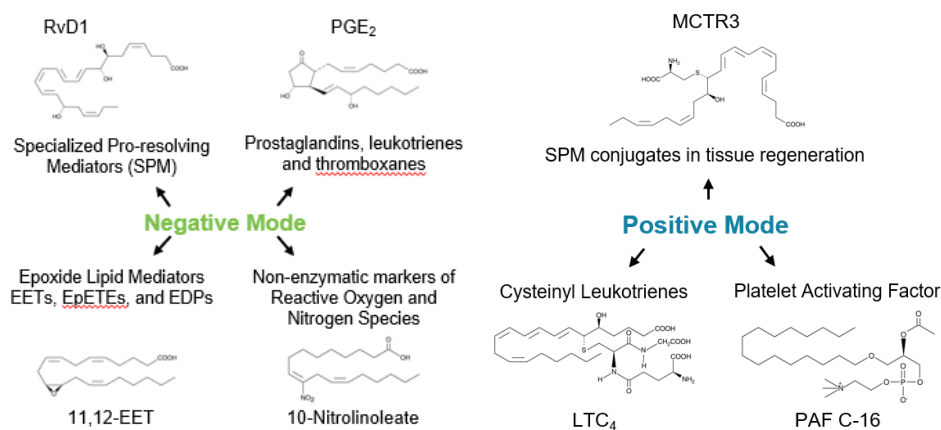


Figure 1. Overview of Lipid Mediator Classes and Their Optimal Modes of Ionization and Detection.

Materials and Methods

Sample Preparation: Standards were obtained from Cayman Chemical and used for optimization of the MRM transitions. Sixteen deuterium labeled internal standards were used in addition to 88 external standards.

Chromatography: Sample separations were performed on an ExionLC™ AD System using a Phenomenex Kinetex 2.6 μm Polar C18 column (100 x 3.0 mm). A gradient elution of methanol in 0.1% formic acid was used to elute the analytes over a 20.5 min run time at 500 μL/min.

Mass Spectrometry: A QTRAP 6500+ System with an IonDrive™ Turbo V Ion Source (electrospray ionization probe) was used for data acquisition in positive and negative mode. Multiple Reaction Monitoring (MRM) was used for quantitation of the various lipid mediators. Details on the LC-MS/MS methods can be found in the supplementary information.⁹

Data Processing: MultiQuant Software was used for computation of calibration curves, percent CVs and standard deviations.

Optimized Separation and Analysis

Lipids, in general, including lipid mediators, are historically difficult to resolve using reversed-phase chromatography. Variations in the positions, geometries, and linkages of fatty acyl chains, modifications to functional groups, and the high number of isomeric and isobaric species, all contribute to the wide diversity of lipid species and the challenge of separation. As shown in Figure 2, the Kinetex Polar C18 column provides both

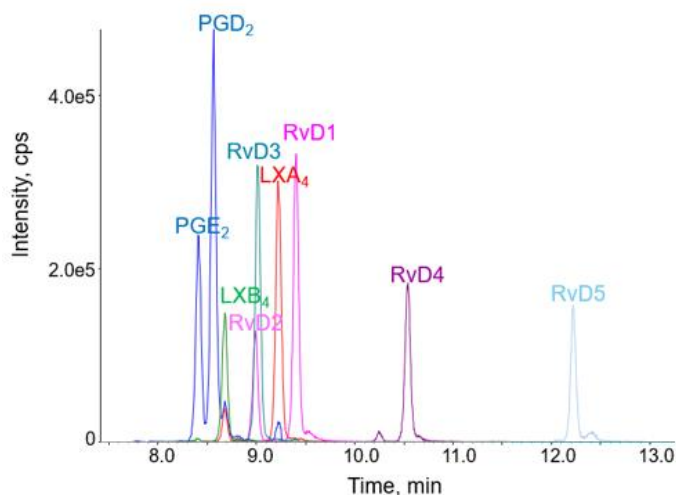


Figure 2. Complete Chromatographic Resolution of Prostaglandins and Specialized Pro-Resolving Mediators (SPM). PGE₂ and PGD₂ as well as 6-keto-PGF_{1α} and TxB₂ (not shown) elute before lipoxins (LX) and D-series resolvins (RvD) with Kinetex Polar C18, unlike other C18 and C8 columns. PGE₂, PGD₂, LXB₄, and LXA₄ are isobaric species that also contain many common fragment ions which are now completely resolved which minimizes false positive identification and gives cleaner fragment spectra for secondary identification with enhanced product ion (EPI) scanning.

polar and non-polar retention that facilitates the separation of lipid mediator epimers and isobaric compounds resulting in improved assay performance statistics and minimization of false positives.

Because of their fatty acid structure, the majority of lipid mediators are best analyzed using negative ion mode analysis. However, a subset of lipid mediators can be analyzed with higher

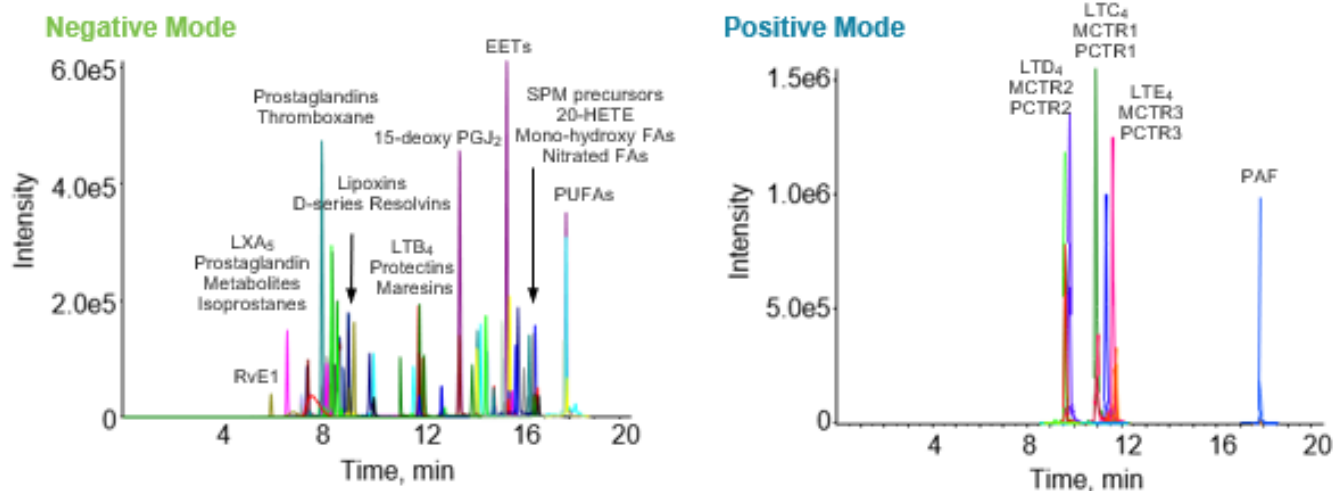


Figure 3. Sensitive and High-Throughput Quantitation Enabled by Rapid Polarity Switching. MRM extracted ion chromatograms (XICs) of the 88 lipid mediators, precursors and metabolites as well as 16 deuterium-labeled internal standards during a 20-minute chromatographic run demonstrate the high-quality separations obtained. The majority of lipid mediators require negative mode ionization due to their fatty acid structure (left panel). Cysteinyl leukotrienes, as well as two new classes of pro-resolving mediators (MCTR and PCTR), are peptide conjugates that are detected with greater sensitivity along with platelet-activating factor in positive mode (right panel).

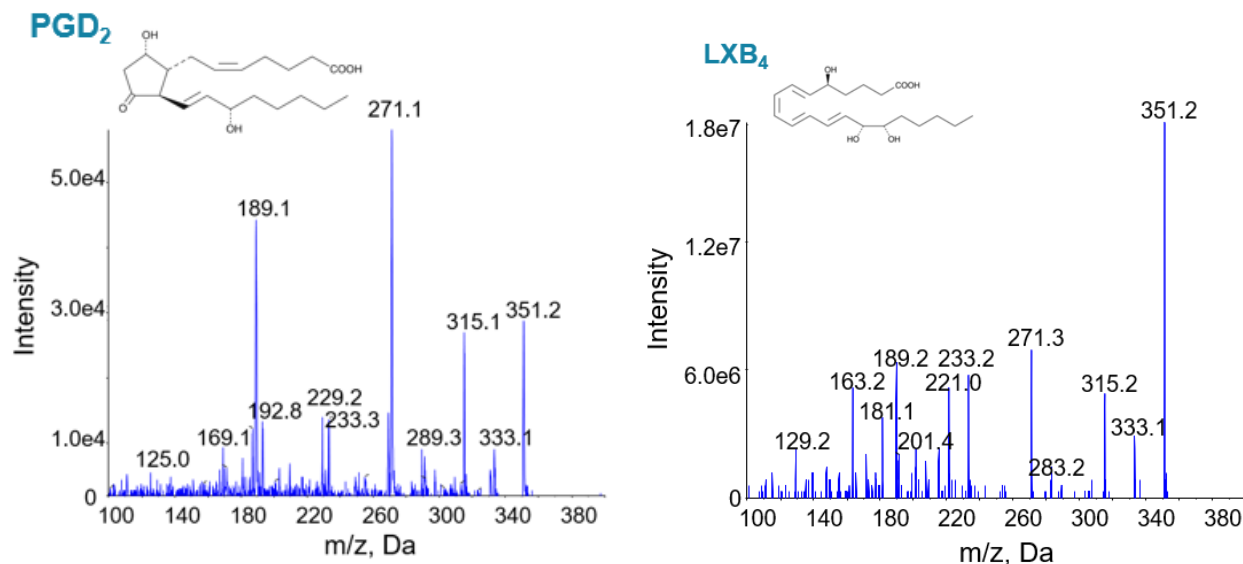


Figure 4. QTRAP 6500+ System Data from MRM Triggered EPI Experiment. MRM signals trigger Enhanced Product Ion (EPI) scans for PGD₂ (left) and LXB₄ (right) using information-dependent criteria to provide qualitative information. The fragmentation pattern observed in the MS/MS data confirms the identity of each lipid mediator.

sensitivity using positive ion mode analysis. Using the fast polarity switching capabilities of the QTRAP 6500+ System, both polarities can be used in a single injection for the optimized detection of all lipid mediators within one assay (Figure 3), greatly reducing the burden of analysis and doubling sample throughput.

Qualitative Confirmation of Lipid Identity

Figure 4 highlights the unique capability of the QTRAP system technology to construct powerful workflows leveraging both the ion trap and triple quadrupole functionality of the system. Here, the MRM triggered EPI workflow was used for the simultaneous collection of both quantitative MRM data as well as qualitative full scan MS/MS data. For isobaric lipid mediator species like PGD₂ and LXB₄ (Figure 4), informative, high-quality MS/MS spectra allow greater confidence in lipid species identification rather than MS alone.

Assay Statistics

Concentration curves were generated by injecting 3 replicate injections of 8 different concentrations of each lipid mediator standard. Excellent linearity is observed with r^2 of 0.999 obtained for the representative concentration curves for selected resolvins, prostaglandins, EETs, and non-enzymatic isoprostanes as shown in Figure 5.

Table 1 displays the data (x3) from the mix of lipid mediators, pathway markers and metabolites. Data for each lipid mediator was processed using MultiQuant Software and the coefficients of variance and accuracy were calculated. Calculated CVs for all compounds were less than 30% and LLOQ were between 0.05 and 1 ng/mL.

Figure 6 shows the complete baseline separation of pro-resolving lipid mediator epimers. Baseline separation of LXA₄ and 15-epi-LXA₄ (top panel) as well as RvD1 and 17-epi-RvD1 (bottom panel) enables the differentiation of enzymatic pathway utilization for pro-resolving lipid mediator production. Specifically, LXA₄ and RvD1 are produced by 5- and 15-lipoxygenase pathways, and 15-epi-LXA₄ and 17-epi-RvD1 are produced by 5-lipoxygenase and acetylated cyclooxygenase-2 pathways. Thus, separation, identification, and quantitation of these species can reveal important biological insights into the mechanisms of inflammatory processes governing various biological observations (Figure 1).

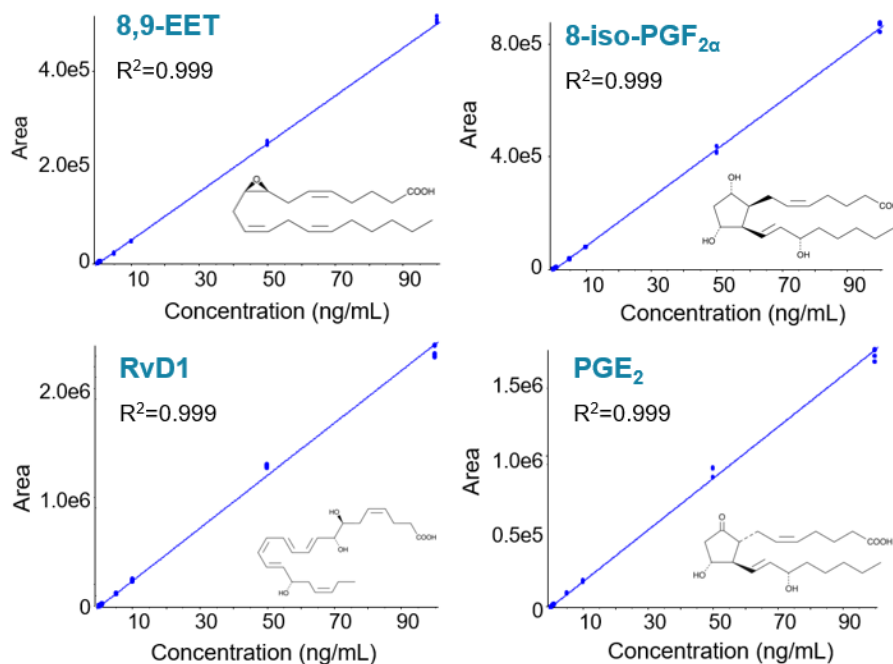


Figure 5. Representative Concentration Curves for Selected Resolvins, Prostaglandins, EETs, and Non-Enzymatic Isoprostanes. 3 replicate injections of each lipid species at 8 different concentrations were performed, and concentration curves were generated. Linearity and reproducibility were determined for each compound.

Conclusions

A high-throughput targeted quantitative LC-MS/MS assay was designed to monitor lipid mediators. By taking advantage of the fast positive-ion/negative-ion polarity switching of the QTRAP 6500+ System, individualized and optimized conditions could be used to monitor a wide diversity of lipid mediator species within one 20 minute MRM assay. Additionally, the HPLC method enabled resolution of isobaric and isomeric species that are historically challenging to separate using reversed-phase chromatography. Excellent sensitivity was obtained for all lipid mediator species with LLOQ between 0.05 and 1 ng/mL and CV < 30%. The unique qualitative/quantitative QTRAP platform also enabled MRM triggered EPI experiments which provided high sensitivity MS/MS data for confirmation of low-level analytes.

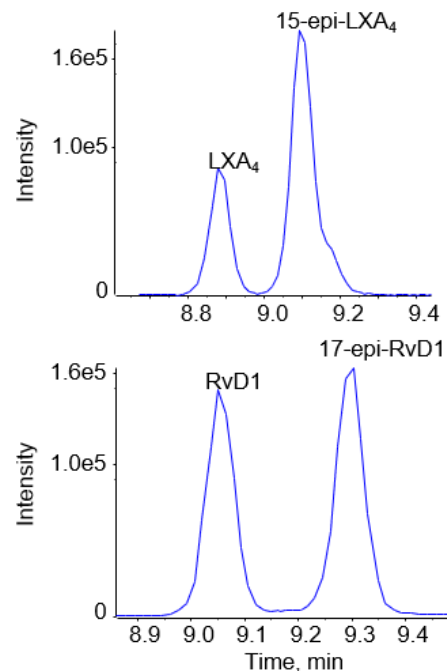


Figure 6. Chromatographic Resolution of Pro-Resolving Lipid Mediator Epimers. Baseline separation of LXA₄ and 15-epi-LXA₄ (top panel), as well as RvD1 and 17-epi-RvD1 (bottom panel), enables the differentiation of enzymatic pathway utilization for pro-resolving lipid mediator production. Specifically, LXA₄ and RvD1 are biosynthesized via 5- and 15-lipoxygenase pathways, and 15-epi-LXA₄ and 17-epi-RvD1 are biosynthesized via 5-lipoxygenase and acetylated cyclooxygenase-2 pathways (Figure 1).

Table 2. Replicate Injections and Limits of Quantitation Data for Lipid Mediator Species.

Compound	%CV	% Accuracy	LLOQ (ng/mL)	R ²	Compound	%CV	% Accuracy	LLOQ (ng/mL)	R ²
6k-PGF _{1α}	10	91	1	0.998	RvD1	24	116	0.05	0.999
PGE ₂	19	120	0.05	0.999	RvD2	7	105	0.5	0.998
15-keto-PGE ₂	5	94	0.5	0.999	RvD3	9	91	0.05	0.999
13,14-dihydro-15-keto PGE ₂	5	102	0.5	0.999	RvD4	2	107	0.1	0.999
PGD ₂	13	112	0.05	0.999	RvD5	3	106	0.1	0.999
11-beta-PGF _{2α}	9	95	0.5	0.999	PD1	16	106	0.05	0.998
13,14-dihydro-15-keto PGD ₂	4	85	0.1	0.999	PDX	14	105	0.05	0.999
PGJ ₂	27	109	0.1	0.999	Maresin 1	12	93	0.1	0.999
15-deoxy-PGJ ₂	11	116	0.1	0.999	17-HDHA	14	111	0.5	0.999
LTB ₄	12	112	0.05	0.999	7,8-EpDPA	16	99	1	0.999
LXA ₄	10	102	0.05	0.999	10,11-EpDPA	14	114	0.1	0.999
LXB ₄	7	116	0.05	0.999	13,14-EpDPA	12	85	0.1	0.999
5,6 EET	20	111	0.5	0.997	16,17-EpDPA	11	88	0.5	0.999
8,9 EET	19	118	0.5	0.999	19,20-EpDPA	27	102	1	0.999
11,12 EET	20	113	0.1	0.998	DHA	16	106	0.1	0.999
14,15 EET	15	112	0.5	0.999	DPA	22	83	1	0.997
15-HETE	13	103	0.1	0.999	Adrenic Acid	6	118	1	0.999
8-iso-PGF _{2α}	23	115	0.1	0.999	9-HODE	28	110	0.1	0.999
RvE1	18	118	0.05	0.999	13-HODE	6	83	0.5	0.999
18-HEPE	3	99	0.5	0.999	10-Nitrolinoleate	14	110	0.1	0.999
8,9-EpETE	10	113	1	0.999	LTC ₄	19	106	0.1	0.999
11,12-EpETE	22	120	0.1	0.999	LTD ₄	26	115	0.05	0.998
14,15-EpETE	19	111	0.1	0.999	MCTR1	0.7	99	0.1	0.996
17,18-EpETE	19	98	0.5	0.999	MCTR2	22	110	0.05	0.999
EPA	15	106	0.5	0.999	MCTR3	21	94	0.1	0.999
PAF	24	93	0.1	0.999	PCTR1	9	120	0.1	0.997
AA	14	96	1	0.998	PCTR2	8	116	0.1	0.998
LTE ₄	8	112	0.5	0.999	PCTR3	19	99	0.5	0.999

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