# High-throughput lipidomic analysis of fatty acid derived eicosanoids and N-acylethanolamines

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## MATERIALS AND METHODS

### Sample preparation

The same sample preparation is used when analysing eicosanoids or NAEs. All samples were resuspended in 1.0 ml of 10% methanol water (v/v). Tissue samples were subjected to sonication for 6 s to break up any connective tissue. Samples were spiked with 50 μL of a 50 pg/μL (2.5 ng total) deuterated internal standard solution. Lipid metabolites were extracted using Strata-X 33 u polymerized solid reverse phase extraction columns (Phenomenex, CA; cat # 8B-S100-UBJ) as indicated by manufacturer's directions. Briefly, columns were washed with 3.5 mL of 100% methanol, followed by 3.5 mL of water before samples were extracted. Samples were washed with 3.5 mL of 10% methanol to remove non-specific binding metabolites. Lipids were eluted into 1.0 mL of methanol and stored at − 80C ° before being analysed to prevent metabolite degradation.

### High performance liquid chromatography (HPLC)

Both eicosanoid and NAE samples are subjected to the same treatment for HPLC analysis, although different buffer systems are employed. Extracted samples in 100% methanol are lyophilized to dryness using a Speed-Vac concentrator (Savant, model # SC110-120), and resuspended in 90 μL of their respective solvent A. For the eicosanoids methodology, solvent AEICOS consists of water–acetonitrile–acetic acid (70:30:0.02; v/v/v), while solvent BEICOS consists of acetonitrile–isopropyl alcohol (50:50, v/v). For the NAE methodology, solvent ANAE consists of water–acetonitrile–acetic acid ((70:30:0.1; v/v/v) + 1 g/L ammonium acetate) and solvent BNAE consists of acetonitrile–isopropyl alcohol–acetic acid ((45:45:10; v/v/v) + 1 g/L ammonium acetate). Samples can be subjected to each methodology alone or analysed together in series. After a sample has been analysed for eicosanoids, the presence of NAEs can be determined with the addition of 10 μL of a water–acetonitrile–acetic acid (70:30:0.1; v/v/v) + 5 g/L ammonium acetate solution that makes the sample suitable to be analysed in positive-ion mode.

An aliquot of 40 μL of sample injected on the HPLC system was the standard amount routinely analysed. Eicosanoids were separated on a Synergi reverse-phase C18 column (2.1 × 250 mm; Phenomenex, CA) and NAEs were separated on a Luna reverse-phase C8 column (2.1 mm · 250 mm, Phenomenex, CA). Both sample types use a flow rate of 300 μL/min at 50 °C. The gradient program used to separate the eicosanoids is as follows: 1 min (0% solvent BEICOS), 3 min (25% solvent BEICOS), 11 min (45% solvent BEICOS), 13 min (60% solvent BEICOS), 18 min (75% solvent BEICOS), 18.5 min (90% solvent BEICOS), 20 min (90% solvent BEICOS), 21 min (0% solvent BEICOS). A linear gradient was maintained between each step. The column was re-equilibrated by holding 0% solvent BEICOS between min 21 to 25 before the next sample injection. The gradient program used to separate the NAEs is as follows: 1 min (0% solvent BNAE), 4 min (50% solvent BNAE), 14 min (100% solvent BNAE), 20 min (100% solvent BNAE), and 21 min (0% solvent BNAE). A linear gradient was maintained between each step. The column was re-equilibrated by holding 0% solvent BNAE between min 21 to 25 before the next sample injection.

### Mass spectrometry

An ABI/Sciex (Foster City, CA) 4000 QTRAP hybrid, triple quadrupole, linear ion trap mass spectrometer equipped with a Turbo V ion source was used for all mass spectrometry analysis. Analyst 1.5 software package

was used to operate the mass spectrometer. Nitrogen gas was used as the collision gas for all metabolites. Eicosanoids were detected in negative electrospray ion mode with the following source parameters: CUR = 10 psi, GS1 = 30 psi, GS2 = 30 psi, IS = − 4500 V, CAD = HIGH, TEMP = 525°, ihe = ON, EP = − 10 V, and CXP = − 10 V. NAEs were analysed in positive electrospray ion mode with the following source parameters: CUR = 10 psi, GS1 = 30 psi, GS2 = 30 psi, IS = − 4500 V, CAD = HIGH, TEMP = 525°, ihe = ON, EP = − 10 V, and CXP = − 10 V.

## Quantitation

Eicosanoids and NAEs are quantitated using a stable isotope dilution technique. Primary standard curves are produced separately for eicosanoids and NAEs. Primary standard curves are generated from mixed primary standard stocks at 7 different concentrations (0.1 ng, 0.3 ng, 1 ng, 3 ng, 10 ng, 30 ng, and 100 ng). An aliquot of 50 mL of primary standard stock with the concentration ranging from 1 pg/μL (0.1 ng stock) to 1000 pg/μL (100 ng) are added to an aliquot of 25 μL of internal standard stock. For eicosanoids, 75 μL of 0.2% acetic acid–water were added, while for NAEs 75 μL of solvent ANAE (water–acetonitrile–acetic acid (70:30:0.1; v/v/v) + 1 g/L ammonium acetate). A 40 μL aliquot of each primary standard mix is analysed with mass spectrometric methodologies. Primary standards were run in duplicate and averaged. Primary standard curves are determined by generating a linear regression trend line that is forced through 0. The Multiquant 1.1 Software package (ABI-Sciex) was used to quantitate all metabolites.

# RESULTS

## Sample preparation

These methodologies have been applied to media from cultured primary cells, cultured cell lines (RAW264.7 and HEK293), rat spinal cord tissue, murine papillomas, and murine tibiotarsal ankle joints. The same sample preparation is used when analysing eicosanoids or NAEs. All tissue samples were disrupted with a probe sonicator, while murine papilloma tissue required homogenization prior to sonication. Lipid metabolites were isolated from cultured media and tissue samples using solid phase extraction, except for murine ankle joints where a prior liquid/liquid extraction was employed. A murine ankle joint sample is comprised mostly of bone and required an additional total lipid extraction.

Purification using flash chromatography can significantly enhance lipid detection and quantization limits by removing other chemical species that can diminish the overall sensitivity through processes such as ion suppression. Different extraction methods have been employed in the study of these lipid mediators. The solid phase extraction technique utilizes Strata-X polymerized solid reverse phase extraction columns (Phenomenex, CA; cat # 8B-S100-UBJ). Samples are applied to these columns in 10% methanol, a concentration of organic solvent empirically determined to be sufficient to solubilize the eicosanoids and NAEs in our assay, yet not high enough to prevent them from binding to the column. An additional column volume of 10% methanol is used as a wash, which serves to elute non-specific hydrophobic chemical species and salts from the sample. Subsequently, eicosanoids and NAEs are eluted off the column with 100% of methanol; however, a large fraction of the more lipophilic species that could potentially lead to ion suppression is retained. Lipids are stored in 100% methanol at − 80C ° to minimize non-enzymatic oxidation and degradation until analysis.

A crucial aspect of our methodology is the usage of deuterated internal standards. All samples are spiked with a deuterated internal standard solution prior to lipid extraction. The use of deuterated internal standards is intrinsic to our ability to quantitate these lipids and is covered in greater depth in the Quantitation section. Deuterated internal standards also serve an important role in the extraction and storage process. Since a deuterated internal standard is either an analogous lipid metabolite or a molecule with similar chemical characteristics, both lipid metabolite and internal standard will have similar extraction efficiencies and rates of degradation. Thus, any amount of a lipid metabolite that is lost due to the extraction process or degradation will be accounted for.

## Method design

Our methodology uses a targeted approach to identify and quantitate lipids using mass spectrometry (MS) coupled with high performance liquid chromatography (HPLC). This approach is known as the Comprehensive Lipidomic Analysis by Separation Simplification (CLASS), where lipid metabolites are separated based on their chemical properties prior to mass analysis, then monitored by collision-induced decomposition (CID) in conjunction with electrospray ionization tandem mass spectrometry (ESI-MS/MS). Using CID, whereby each lipid metabolite creates ion fragments unique to its structural components, they can be subsequently measured by monitoring one of these ion fragments. A targeted MS approach allows for a higher sensitivity to detect analytes than an unbiased full scan MS analysis. The trade-off for higher sensitivity is that novel metabolites cannot be detected, and all metabolites of interest must be accounted for prior to analysis.

Mass spectrometric analysis was performed on an ABI/Sciex 4000 Q-TRAP operating in scheduled multiple-reaction monitoring (sMRM) mode. Multiple-reaction monitoring (MRM) is an MS approach that monitors the transition of a parent ion to a specific daughter ion fragment. PGE2 and anandamide are used as examples to demonstrate the parent to daughter ion transition (Fig. 1). These defined parent and daughter ions are known as an MRM pair. An sMRM pair is an MRM pair with an associated liquid chromatography (LC) retention time. sMRM is an improvement over MRM allowing for better data collection and more analytes to be monitored in a single analysis. The MS operating software (Analyst 1.5) takes into account the total number of sMRM pairs and respective retention times and optimizes how the mass spectrometer scans. We found that a 70 second retention time window for each sMRM pair was sufficient to account for metabolite peaks and slight shifts in their retention times. Although a feature on ABI/Sciex mass spectrometers running Analyst 1.5, scheduling MRM pairs can be replicated on triple quadrupole mass spectrometers from other companies. This method is intended as a starting point for the research community, which can be expanded upon as more metabolite standards become available or scaled down to only include the metabolites of interest.

## Eicosanoid methodology

We have developed a high-throughput CLASS approach to globally monitor a specific lipid class, the eicosanoids. Derived from poly-unsaturated fatty acids (PUFAs), all eicosanoids contain a conserved terminal carboxyl moiety (Fig. 1, PGE2). Eicosanoid are detected in negative electrospray ion mode to take advantage of the terminal carboxyl moiety, which is easily deprotonated during ionization. This feature allows eicosanoids to be detected without any additional derivatization. Unique sMRM pairs have been selected for each eicosanoid species. This was accomplished by analysing product ion scans (MS/MS) from commercial eicosanoid standards (Cayman Chemical & Biomol). Additionally, optimal declustering potential (DP) and the collision energy (CE) parameters have been determined for each sMRM pair. These values were determined by directly infusing commercial standards into the mass spectrometer, while individually ramping these parameters in an MRM experiment. We have compiled the sMRM pair, DP, and CE values for each eicosanoid (Table 1). Table 1 also includes the biosynthetic pathway and PUFA that each eicosanoid is derived from.

## Table 1. Optimized sMRM pairs and parameters for eicosanoids

| Fatty acid | Pathway | Common name | Abbreviation | Parent | Daughter | Retention Time (min)[b] | $LOD_{(pg)}$ | Declustering Potential | Collision energy | Internal standard |
|---|---|---|---|---|---|---|---|---|---|---|
| AA | COX | [a](d4) 6-keto-Prostaglandin $F_{1\alpha}$ | (d4)6k-$PGF1_{\alpha}$ | 373 | 167 | 5.0 | ND | – 65 | – 35 | – |
| AA | COX | (d4) Thromboxane $B_2$ | (d4) $TXB_2$ | 373 | 173 | 6.3 | ND | – 50 | – 25 | – |
| AA | COX | (d4) Prostaglandin $F_{2\alpha}$ | (d4) $PGF_{2\alpha}$ | 357 | 197 | 6.9 | ND | – 50 | – 30 | – |
| AA | COX | (d4) Prostaglandin $E_2$ | (d4) $PGE_2$ | 355 | 275 | 7.1 | ND | – 50 | – 25 | – |
| AA | COX | (d4) Prostaglandin $D_2$ | (d4) $PGD_2$ | 355 | 275 | 7.5 | ND | – 50 | – 25 | – |
| AA | COX | (d4) 15-deoxy-Prostalandin $J_2$ | (d4) 15d-$PGJ_2$ | 319 | 275 | 14.5 | ND | – 60 | – 20 | – |
| AA | COX | (d4) 13,14-dihydro-15-keto Prostaglandin $F_{2\alpha}$ | (d4) dhk $PGF_{2\alpha}$ | 357 | 295 | 8.3 | ND | – 80 | – 30 | – |
| AA | COX | (d4) 13,14-dihydro-15-keto Prostaglandin $E_2$ | (d4) dhk $PGE_2$ | 355 | 211 | 8.0 | ND | – 45 | – 30 | – |
| AA | COX | (d4) 13,14-dihydro-15-keto Prostaglandin $D_2$ | (d4) dhk $PGD_2$ | 355 | 211 | 8.9 | ND | – 45 | – 30 | – |
| AA | non-enz | (d11) Isoprostane $F_{2\alpha}$-IV | (d11) 5-iso $PGF_{2\alpha}$VI | 364 | 115 | 6.6 | ND | – 60 | – 30 | – |
| AA | LOX | (d4) Leukotriene $B_4$ | (d4) $LTB_4$ | 339 | 197 | 11.6 | ND | – 70 | – 22 | – |
| AA | LOX | (d8) 5-hydroxy-eicosatrienoic acid | (d8) 5-HETE | 327 | 116 | 16.7 | ND | – 50 | – 20 | – |
| AA | LOX | (d8) 12-hydroxy-eicosatrienoic acid | (d8) 12-HETE | 327 | 183 | 16.1 | ND | – 60 | – 20 | – |
| AA | LOX | (d8) 15-hydroxy-eicosatrienoic acid | (d8) 15-HETE | 327 | 226 | 15.5 | ND | – 60 | – 20 | – |
| AA | CYP | (d6) 20-hydroxy-eicosatrienoic acid | (d6) 20-HETE | 325 | 295 | 14.7 | ND | – 70 | – 20 | – |
| LA | LOX | (d4) 9-hydroxy-octadecadienoic acid | (d4) 9-HODE | 299 | 172 | 15.6 | ND | – 60 | – 25 | – |
| LA | LOX | (d4) 13-hydroxy-octadecadienoic acid | (d4) 13-HODE | 299 | 198 | 15.4 | ND | – 60 | – 25 | – |
| AA | LOX | (d7) 5-oxo-eicosatetraenoic acid | (d7) 5-oxoETE | 323 | 209 | 17.2 | ND | – 50 | – 25 | – |
| AA | LOX | (d4) Resolvin $E_1$ | (d4) $RvE_1$ | 353 | 197 | 5.2 | ND | – 70 | – 20 | – |
| AA | CYP | (d8) 5(6)-epoxy-eicosatetraenoic acid | (d8) 5, 6 EET | 330 | 202 | 17.6 | ND | – 30 | – 20 | – |
| AA | CYP | (d8) 8(9)-epoxy-eicosatetraenoic acid | (d8) 8, 9 EET | 327 | 158 | 17.5 | ND | – 60 | – 20 | – |
| AA | CYP | (d8) 11(12)-epoxy-eicosatetraenoic acid | (d8) 11, 12 EET | 327 | 171 | 17.5 | ND | – 60 | – 20 | – |
| AA | CYP | (d8) 14(15)-epoxy-eicosatetraenoic acid | (d8) 14, 15 EET | 327 | 226 | 16.9 | ND | – 50 | – 15 | – |
| LA | CYP | (d4) 9,10-dihydroxy-octadecenoic acid | (d4) 9, 10 diHOME | 317 | 203 | 13.0 | ND | – 60 | – 30 | – |
| LA | CYP | (d4) 12,13-dihydroxy-octadecenoic acid | (d4) 12, 13 diHOME | 317 | 185 | 12.4 | ND | – 60 | – 30 | – |
| AA | LOX | (d5) Leukotriene $C_4$ | (d5) $LTC_4$ | 630 | 272 | 9.2 | ND | – 70 | – 35 | – |
| AA | LOX | (d4) Leukotriene $E_4$ | (d4) $LTE_4$ | 441 | 336 | 10.2 | ND | – 60 | – 25 | – |
| – | – | (d8) Arachidonic acid | (d8) AA | 311 | 267 | 20.6 | ND | – 80 | – 20 | – |
| – | – | (d5) Eicosapentaenoic acid | (d5) EPA | 306 | 262 | 19.3 | ND | – 65 | – 15 | – |
| – | – | (d5) Dohexacosaenoic acid | (d5) DHA | 332 | 234 | 20.2 | ND | – 95 | – 20 | – |
| AA | COX | 6-keto-Prostaglandin $F_{1\alpha}$ | 6k-$PGF_{1\alpha}$ | 369 | 163 | 5.1 | 1 | – 65 | – 35 | (d4) 6k-$PGF1_{\alpha}$ |
| AA | COX | Thromboxane $B_2$ | $TXB_2$ | 369 | 169 | 6.3 | 1 | – 50 | – 25 | (d4) $TXB_2$ |
| AA | COX | Prostaglandin $F_{2\alpha}$ | $PGF_{2\alpha}$ | 353 | 193 | 6.9 | 1 | – 50 | – 30 | (d4) $PGF_{2\alpha}$ |
| AA | COX | Prostaglandin $E_2$ | $PGE_2$ | 351 | 271 | 7.1 | 1 | – 50 | – 25 | (d4) $PGE_2$ |
| AA | COX | Prostaglandin $D_2$ | $PGD_2$ | 351 | 271 | 7.5 | 1 | – 50 | – 25 | (d4) $PGD_2$ |
| AA | COX | 11-beta-Prostaglandin $F_{2\alpha}$ | 11βPGF$_{2\alpha}$ | 353 | 193 | 6.2 | ND | – 50 | – 30 | (d4) $PGF_{2\alpha}$ |
| DγLA | COX | Thromboxane $B_1$ | $TXB_1$ | 371 | 171 | 6.1 | ND | – 50 | – 25 | (d4) $TXB_2$ |
| DγLA | COX | Prostaglandin $F_{1\alpha}$ | $PGF_{1\alpha}$ | 355 | 293 | 7.0 | ND | – 75 | – 30 | (d4) $PGF_{2\alpha}$ |
| DγLA | COX | Prostaglandin $E_1$ | $PGE_1$ | 353 | 273 | 7.4 | ND | – 55 | – 25 | (d4) $PGE_2$ |
| DγLA | COX | Prostaglandin $D_1$ | $PGD_1$ | 353 | 273 | 7.5 | ND | – 55 | – 25 | (d4) $PGD_2$ |
| EPA | COX | Δ17-6-keto-Prostaglandin $F_{1\alpha}$ | Δ17 6k-$PGF_{1\alpha}$ | 367 | 163 | 4.4 | ND | – 90 | – 35 | (d4) $PGF_{2\alpha}$ |
| EPA | COX | Thromboxane $B_3$ | $TXB_3$ | 367 | 169 | 5.3 | ND | – 50 | – 25 | (d4) $TXB_2$ |
| EPA | COX | Prostaglandin $F_{3\alpha}$ | $PGF_{3\alpha}$ | 351 | 193 | 5.8 | ND | – 75 | – 30 | (d4) $PGF_{2\alpha}$ |
| EPA | COX | Prostaglandin $E_3$ | $PGE_3$ | 349 | 269 | 6.2 | ND | – 55 | – 25 | (d4) $PGE_2$ |
| EPA | COX | Prostaglandin $D_3$ | $PGD_3$ | 349 | 269 | 6.5 | ND | – 55 | – 25 | (d4) $PGD_2$ |
| ADA | COX | dihomo Prostaglandin $F_{2\alpha}$ | dihomo $PGF_{2\alpha}$ | 381 | 221 | 8.9 | ND | – 75 | – 35 | (d4) $PGF_{2\alpha}$ |
| ADA | COX | dihomo Prostaglandin $E_2$ | dihomo $PGE_2$ | 379 | 299 | 9.1 | ND | – 65 | – 30 | (d4) $PGE_2$ |
| ADA | COX | dihomo Prostaglandin $D_2$ | dihomo $PGD_2$ | 379 | 299 | 9.4 | ND | – 65 | – 30 | (d4) $PGD_2$ |
| ADA | COX | dihomo Prostaglandin $J_2$ | dihomo $PGJ_2$ | 361 | 299 | 12.3 | ND | – 55 | – 25 | (d4) 15d-$PGJ_2$ |
| ADA | COX | dihomo 15-deoxy-Prostaglandin $J_2$ | dihomo 15d $PGJ_2$ | 361 | 299 | 13.9 | ND | – 55 | – 25 | (d4) 15d-$PGJ_2$ |
| DγLA | COX | 6-Prostaglandin $E_1$ | 6k $PGE_1$ | 367 | 143 | 5.3 | ND | – 40 | – 25 | (d4) $PGE_2$ |
| DγLA | COX | 6,15-diketo-, 13, 14-dihydro-Prostaglandin $F_{1\alpha}$ | 6, 15 dk-, dh-$PGF_{1\alpha}$ | 369 | 113 | 6.2 | ND | – 60 | – 40 | (d4) $PGF_{2\alpha}$ |
| DγLA | COX | 15-keto-Prostaglandin $F_{1\alpha}$ | 15k $PGE_{1\alpha}$ | 353 | 113 | 7.4 | ND | – 50 | – 35 | (d4) $PGF_{2\alpha}$ |
| AA | COX | 15-keto-Prostaglandin $F_{2\alpha}$ | 15k $PGF_{2\alpha}$ | 351 | 113 | 7.4 | ND | – 40 | – 35 | (d4) $PGF_{2\alpha}$ |
| AA | COX | 15-keto-Prostaglandin $E_2$ | 15k $PGE_2$ | 349 | 113 | 7.5 | 1 | – 35 | – 30 | (d4) $PGE_2$ |
| AA | COX | 13, 14-dihydro-Prostaglandin $F_{2\alpha}$ | dh $PGF_{2\alpha}$ | 355 | 275 | 7.7 | ND | – 40 | – 25 | (d4) $PGF_{2\alpha}$ |
| AA | COX | 13, 14-dihydro-15-keto Prostaglandin $F_{2\alpha}$ | dhk $PGF_{2\alpha}$ | 353 | 291 | 8.1 | 1 | – 60 | – 25 | (d4) dhk $PGF_{2\alpha}$ |
| AA | COX | 13, 14-dihydro-15-keto Prostaglandin $E_2$ | dhk $PGE_2$ | 351 | 207 | 8.2 | 1 | – 40 | – 25 | (d4) dhk $PGE_{2\alpha}$ |
| AA | COX | 13, 14-dihydro-15-keto Prostaglandin $D_2$ | dhk $PGD_2$ | 351 | 207 | 8.9 | 1 | – 40 | – 25 | (d4) dhk $PGD_2$ |
| AA | COX | bicyclo Prostaglandin $E_2$ | bicyclo $PGE_2$ | 333 | 113 | 10.6 | ND | – 60 | – 35 | (d4) $PGE_2$ |
| AA | COX | 11beta-13, 14-dihydro-15-keto-Prostaglandin $F_{2\alpha}$ | 11β dhk $PGF_{2\alpha}$ | 353 | 113 | 8.0 | ND | – 50 | – 35 | (d4) $PGF_{2\alpha}$ |
| AA | COX | 19-hydroxy-$PGF_{2\alpha}$ | 19oh $PGF_{2\alpha}$ | 369 | 193 | 3.3 | ND | – 50 | – 35 | (d4) $PGF_{2\alpha}$ |
| AA | COX | 20-hydroxy-$PGF_{2\alpha}$ | 20oh $PGF_{2\alpha}$ | 369 | 193 | 3.2 | ND | – 50 | – 35 | (d4) $PGF_{2\alpha}$ |
| AA | COX | 19-hydroxy-$PGE_2$ | 19oh $PGE_2$ | 367 | 189 | 3.6 | ND | – 40 | – 25 | (d4) $PGE_2$ |
| AA | COX | 20-hydroxy-$PGE_2$ | 20oh $PGE_2$ | 367 | 189 | 3.5 | ND | – 40 | – 25 | (d4) $PGE_2$ |
| AA | COX | 2, 3-dinor-11-beta-Prostaglandin $F_{2\alpha}$ | 2, 3 dinor 11β $PGF_{2\alpha}$ | 325 | 145 | 5.2 | ND | – 40 | – 25 | (d4) $PGF_{2\alpha}$ |
| AA | COX | tetranor-Prostanglin F Metabolite | tetranor-PGFM | 329 | 293 | 2.5 | ND | – 40 | – 25 | (d4) $PGF_{2\alpha}$ |
| AA | COX | tetranor-Prostanglin E Metabolite | tetranor-PGEM | 327 | 291 | 2.5 | ND | – 40 | – 25 | (d4) $PGE_2$ |
| AA | LOX | tetranor 12-hydroxy-eicosatetraenoic acid | tetranor 12-HETE | 265 | 109 | 13.0 | 0.1 | – 75 | – 14 | (d8) 15-HETE |
| AA | COX | 11-beta-Prostaglandin $E_2$ | 11β $PGE_2$ | 351 | 271 | 7.1 | ND | – 40 | – 25 | (d4) $PGE_2$ |
| AA | COX | Prostaglandin $K_2$ | $PGK_2$ | 349 | 205 | 7.1 | ND | – 50 | – 30 | (d4) $PGE_2$ |
| AA | COX | 12S-hydroxy-heptadecatrienoic acid | 12-HHT | 279 | 163 | 13.4 | 1 | – 30 | – 30 | (d8) 5-HETE |
| AA | COX | 11-hydroxy-eicosatetraenoic acid | 11-HETE | 319 | 167 | 16.0 | 0.1 | – 60 | – 20 | (d8) 5-HETE |
| AA | COX | 11-hydroxy-eicosapentaenoic acid | 11-HEPE | 317 | 121 | 14.8 | 1 | – 70 | – 24 | (d8) 15-HETE |
| AA | COX | 13-hydroxy-docosahexaenoic acid | 13-HDoHE | 343 | 221 | 15.8 | 0.1 | – 60 | – 17 | (d8) 15-HETE |
| AA | COX | Prostaglandin $A_2$ | $PGA_2$ | 333 | 271 | 9.7 | ND | – 30 | – 20 | (d4) 15d-$PGJ_2$ |
| AA | COX | Prostaglandin $B_2$ | $PGB_2$ | 333 | 271 | 9.5 | ND | – 30 | – 20 | (d4) 15d-$PGJ_2$ |
| AA | COX | 15-deoxy-Prostaglandin $A_2$ | 15d-$PGA_2$ | 315 | 271 | 15.0 | ND | – 50 | – 15 | (d4) 15d-$PGJ_2$ |
| AA | COX | Prostaglandin $J_2$ | $PGJ_2$ | 333 | 271 | 9.7 | 1 | – 30 | – 20 | (d4) 15d-$PGJ_2$ |
| AA | COX | 15-deoxy-Δ12, 14-$PGD_2$ | 15d-$PGD_2$ | 333 | 271 | 11.7 | 1 | – 30 | – 20 | (d4) 15d-$PGJ_2$ |
| AA | COX | 15-deoxy-Δ12, 14-$PGJ_2$ | 15d-$PGJ_2$ | 315 | 271 | 14.5 | 0.1 | – 50 | – 15 | (d4) 15d-$PGJ_2$ |
| AA | non-enz | Isoprostane $F_{2\alpha}$-IV | 5-iso $PGF_{2\alpha}$VI | 353 | 115 | 6.7 | 1 | – 60 | – 30 | (d11) 5-iso $PGF_{2\alpha}$VI |
| AA | non-enz | Isoprostane $F_{2\alpha}$-III | 8-iso $PGF_{2\alpha}$III | 353 | 193 | 6.2 | 1 | – 50 | – 30 | (d11) 5-iso $PGF_{2\alpha}$VI |
| AA | non-enz | 9-hydroxy-eicosatetraenoic acid | 9-HETE | 319 | 151 | 16.5 | 0.1 | – 60 | – 20 | (d8) 5-HETE |
| EPA | non-enz | 9-hydroxy-eicosapentaenoic acid | 9-HEPE | 317 | 149 | 15.0 | 0.1 | – 75 | – 20 | (d8) 5-HETE |
| DHA | non-enz | 8-hydroxy-docosahexaenoic acid | 8-HDoHE | 343 | 109 | 16.2 | 0.1 | – 70 | – 20 | (d8) 5-HETE |
| DHA | non-enz | 16-hydroxy-docosahexaenoic acid | 16-HDoHE | 343 | 233 | 15.5 | 0.1 | – 75 | – 19 | (d8) 15-HETE |
| DHA | non-enz | 20-hydroxy-docosahexaenoic acid | 20-HDoHE | 343 | 241 | 15.3 | 0.1 | – 60 | – 20 | (d8) 15-HETE |
| AA | LOX | Leukotriene $B_4$ | $LTB_4$ | 335 | 195 | 11.5 | 1 | – 70 | – 22 | (d4) $LTB_4$ |
| AA | LOX | 20-hydroxy-Leukotriene $B_4$ | 20oh $LTB_4$ | 351 | 195 | 5.1 | ND | – 60 | – 25 | (d4) $LTB_4$ |
| AA | LOX | 20-carboxy-Leukotriene $B_4$ | 20cooh $LTB_4$ | 365 | 195 | 5.1 | ND | – 60 | – 25 | (d4) $LTB_4$ |
| AA | LOX | 5, 6 dihydroxy-eicosatetraenoic acid | 5, 6 diHETE | 335 | 163 | 15.0 | 1 | – 60 | – 25 | (d4) $LTB_4$ |
| AA | LOX | 20-trans-Leukotriene $B_4$ | 6t $LTB_4$ | 335 | 195 | 11.1 | ND | – 70 | – 22 | (d4) $LTB_4$ |
| AA | LOX | 12-epi-Leukotriene $B_4$ | 12epi $LTB_4$ | 335 | 195 | 11.1 | ND | – 70 | – 22 | (d4) $LTB_4$ |
| AA | LOX | 6-trans-, 12-epi-Leukotriene $B_4$ | 6t, 12epi $LTB_4$ | 335 | 195 | 11.1 | ND | – 70 | – 22 | (d4) $LTB_4$ |
| AA | LOX | 12-oxo-Leukotriene $B_4$ | 12-oxoLT$B_4$ | 333 | 179 | 12.4 | ND | – 60 | – 17 | (d4) $LTB_4$ |
| AA | LOX | Leukotriene $C_4$ | $LTC_4$ | 625 | 272 | 9.2 | ND | – 70 | – 28 | (d5) $LTC_4$ |
| AA | LOX | Leukotriene $D_4$ | $LTD_4$ | 495 | 177 | 10.6 | ND | – 60 | – 25 | (d4) $LTE_4$ |
| AA | LOX | Leukotriene $E_4$ | $LTE_4$ | 438 | 333 | 10.2 | 1 | – 60 | – 25 | (d4) $LTE_4$ |
| AA | LOX | 11-trans-Leukotriene $C_4$ | 11t $LTC_4$ | 625 | 272 | 9.8 | ND | – 70 | – 35 | (d5) $LTC_4$ |
| AA | LOX | 11-trans-Leukotriene $D_4$ | 11t $LTD_4$ | 495 | 177 | 11 .5 | ND | – 60 | – 25 | (d4) $LTE_4$ |
| AA | LOX | 11-trans-Leukotriene $E_4$ | 11t $LTE_4$ | 438 | 333 | 10.9 | ND | – 60 | – 25 | (d4) $LTE_4$ |
| AA | LOX | 5-hydroxy-eicosatetraenoic acid | 5-HETE | 319 | 115 | 16.6 | 0.1 | – 60 | – 20 | (d8) 5-HETE |
| EPA | LOX | 5-hydroxy-eicosapentaenoic acid | 5-HEPE | 317 | 115 | 15.3 | 0.1 | – 40 | – 17 | (d8) 5-HETE |
| DHA | LOX | 7 -hydroxy-docosahexaenoic acid | 7-HDoHE | 343 | 141 | 16.2 | 0.1 | – 60 | – 18 | (d8) 5-HETE |
| DHA | LOX | 4-hydroxy-docosahexaenoic acid | 4-HDoHE | 343 | 101 | 16.8 | 0.1 | – 70 | – 17 | (d8) 5-HETE |
| LA | LOX | 9-hydroxy-octadecatrienoic acid | 9-HOTrE | 293 | 171 | 14.1 | 0.1 | – 70 | – 20 | (d8) 5-HETE |
| MA | LOX | 5-hydroxy-eicosatrienoic acid | 5-HETrE | 321 | 115 | 18.3 | 0.1 | – 70 | – 19 | (d8) 5-HETE |
| AA | LOX | 5S,15S-dihydroxy-eicosatetraenoic acid | 5,15 diHETE | 335 | 201 | 11.1 | 1 | – 50 | – 30 | (d4) $LTB_4$ |
| AA | LOX | 5(S),6(R)-lipoxin $A_4$ | 6R-$LXA_4$ | 351 | 115 | 8.0 | 1 | – 30 | – 20 | (d4) $LTB_4$ |
| AA | LOX | 5(S),6(S)-lipoxin $A_4$ | 6S-$LXA_4$ | 351 | 115 | 8.3 | 1 | – 30 | – 20 | (d4) $LTB_4$ |
| AA | LOX | 5(S),14(R)-Lipoxin $A_4$ | 14R-$LXA_4$ | 351 | 115 | 8.1 | ND | – 70 | – 25 | (d4) $LTB_4$ |
| EPA | LOX | Lipoxin $A_5$ | $LXA_5$ | 349 | 113 | 6.7 | ND | – 60 | – 25 | (d4) $LTB_4$ |

| Fatty acid | Pathway | Common name | Abbreviation | Parent | Daughter | Retention time (min)[b] | $LOD_{(pg)}$ | Declustering potential | Collision energy | Internal standard |
|---|---|---|---|---|---|---|---|---|---|---|
| AA | LOX | Lipoxin $B_4$ | $LXB_4$ | 351 | 221 | 7.3 | ND | −80 | −25 | (d4) $LTB_4$ |
| EPA | LOX | Resolvin $E_1$ | $RvE_1$ | 349 | 195 | 5.2 | ND | −70 | −20 | (d4) $RvE_1$ |
| EPA | LOX | Resolvin $D_1$ | $RvD_1$ | 375 | 141 | 7.8 | ND | −45 | −20 | (d4) $LTB_4$ |
| DHA | LOX | Neuroprotectin $D_1$ | $PD_1$ | 359 | 153 | 10.5 | ND | −40 | −20 | (d4) $LTB_4$ |
| DHA | LOX | 15-trans Neuroprolectin $D_1$ | 15t $PD_1$ | 359 | 153 | 10.8 | ND | −40 | −20 | (d4) $LTB_4$ |
| DHA | LOX | 10S, 17S-dihydroxy Docosahexaenoic acid | 10S,17S-DiHoHE | 359 | 153 | 11.0 | ND | −40 | −20 | (d4) $LTB_4$ |
| AA | LOX | 8S,15S-dihydroxy-eicosatetraenoic acid | 8,15 diHETE | 335 | 127 | 10.7 | 1 | −50 | −25 | (d4) $LTB_4$ |
| AA | LOX | 15-hydroxy-eicosatetraenoic acid | 15-HETE | 319 | 219 | 15.6 | 0.1 | −50 | −15 | (d8) 15-HETE |
| EPA | LOX | 15-hydroxy-eicosapenlaenoic acid | 15-HEPE | 317 | 219 | 14.6 | 0.1 | −60 | −20 | (d8) 5-HETE |
| DHA | LOX | 17-hydroxy Docosahexaenoic acid | 17-HDoHE | 343 | 245 | 15.6 | 0.1 | −60 | −20 | (d8) 15-HETE |
| LA | LOX | 13-hydroxy-octadecadienoic acid | 13-HODE | 295 | 195 | 15.4 | 1 | −60 | −25 | (d4) 13-HODE |
| αLA | LOX | 13-hydroxy-ocladecatrienoic acid | 13-HOTrE | 293 | 195 | 14.3 | 1 | −80 | −25 | (d4) 13-HODE |
| γLA | LOX | 13-hydroxy-γ-octadecatrienoic acid | 13-HOTre-γ | 293 | 193 | 14.5 | 0.1 | −70 | −20 | (d4) 13-HODE |
| $D_7$LA | LOX | 15-hydroxy-eicosatrienoic acid | 15-HETrE | 321 | 221 | 16.3 | 0.1 | −70 | −21 | (d8) 15-HETE |
| AA | LOX | 8-hydroxy-eicosatetraenoic acid | 8-HETE | 319 | 155 | 16.3 | 0.1 | −60 | −20 | (d8) 5-HETE |
| EPA | LOX | 8-hydroxy-eicosapenlaenoic acid | 8-HEPE | 317 | 127 | 14.9 | 1 | −70 | −25 | (d8) 5-HETE |
| DHA | LOX | 10-hydroxy Docosahexaenoic acid | 10-HDoHE | 343 | 181 | 15.8 | 1 | −60 | −17 | (d8) 5-HETE |
| $D_7$LA | LOX | 8-hydroxy-eicosatrienoic acid | 8-HETrE | 321 | 157 | 16.8 | 1 | −70 | −23 | (d8) 5-HETE |
| AA | LOX | Eoxin $C_4$ | $EXC_4$ | 625 | 272 | 7.2 | ND | −70 | −35 | (d5) $LTC_4$ |
| AA | LOX | Eoxin $D_4$ | $EXD_4$ | 495 | 177 | 10.7 | ND | −60 | −25 | (d4) $LTE_4$ |
| AA | LOX | Eoxin $E_4$ | $EXE_4$ | 438 | 333 | 8.7 | ND | −60 | −25 | (d4) $LTE_4$ |
| AA | LOX | 12-hydroxy-eicosatetraenoic acid | 12-HETE | 319 | 179 | 16.2 | 1 | −60 | −20 | (d8) 15-HETE |
| EPA | LOX | 12-hydroxy-eicosapentaenoic acid | 12-HEPE | 317 | 179 | 14.9 | 0.1 | −70 | −20 | (d8) 15-HETE |
| DHA | LOX | 14-hydroxy-eicosapentaenoic acid | 14-HDoHE | 343 | 205 | 15.8 | 1 | −60 | −18 | (d8) 15-HETE |
| DHA | LOX | 11-hydroxy-eicosapentaenoic acid | 11-HDoHE | 343 | 149 | 16.0 | 1 | −60 | −19 | (d8) 5-HETE |
| LA | LOX | 9-hydroxy-octadecadienoic acid | 9-HODE | 295 | 171 | 15.6 | 1 | −60 | −25 | (d4) 9-HODE |
| AA | LOX | Hepoxilin $A_3$ | $HXA_3$ | 335 | 127 | 14.3 | 1 | −50 | −25 | (d8) 14,15 EET |
| AA | LOX | Hepoxilin $B_3$ | $HXB_3$ | 335 | 183 | 14.4 | 1 | −40 | −20 | (d8) 14,15 EET |
| AA | LOX | 5-oxo-eicosatelraenoic acid | 5-oxoETE | 317 | 203 | 17.2 | 1 | −40 | −25 | (d7) 5-oxoETE |
| AA | LOX | 12-oxo-eicosatelraenoic acid | 12-oxoETE | 317 | 153 | 16.1 | 1 | −40 | −25 | (d7) 5-oxoETE |
| AA | LOX | 15-oxo-eicosatetraenoic acid | 15-oxoETE | 317 | 113 | 15.9 | 1 | −40 | −25 | (d7) 5-oxoETE |
| LA | LOX | 9-oxo-octadecadienoic acid | 9-oxoODE | 293 | 185 | 16.1 | 1 | −60 | −25 | (d7) 5-oxoETE |
| LA | LOX | 13-oxo-octadecadienoic acid | 13-oxoODE | 293 | 113 | 15.7 | 1 | −70 | −30 | (d7) 5-oxoETE |
| EPA | LOX | 15-oxo-eicosadienoic acid | 15-oxoEDE | 321 | 113 | 17.7 | 1 | −100 | −32 | (d7) 5-oxoETE |
| AA | CYP | 20-hydroxy-eicosatelraenoic acid | 20-HETE | 319 | 289 | 14.8 | 1 | −75 | −25 | (d6) 20-HETE |
| AA | CYP | 19-hydroxy-eicosatelraenoic acid | 19-HETE | 319 | 231 | 14.6 | 1 | −80 | −25 | (d6) 20-HETE |
| AA | CYP | 18-hydroxy-eicosatetraenoic acid | 18-HETE | 319 | 261 | 15.0 | 0.1 | −80 | −25 | (d6) 20-HETE |
| AA | CYP | 17 -hydroxy-eicosaletraenoic acid | 17-HETE | 319 | 247 | 15.1 | 0.1 | −80 | −25 | (d6) 20-HETE |
| AA | CYP | 16-hydroxy-eicosaletraenoic acid | 16-HETE | 319 | 189 | 15.1 | 1 | −80 | −25 | (d6) 20-HETE |
| EPA | CYP | 18-hydroxy-eicosapenlaenoic acid | 18-HEPE | 317 | 215 | 14.2 | 0.1 | −60 | −20 | (d6) 20-HETE |
| AA | CYP | 5(6)-epoxy-eicosatrienoic acid | 5,6 EET | 319 | 191 | 17.8 | 1 | −30 | −20 | (d8) 5,6 EET |
| AA | CYP | 8(9)-epoxy-eicosatrienoic acid | 8,9 EET | 319 | 155 | 17.5 | 1 | −60 | −20 | (d8) 8,9 EET |
| AA | CYP | 11(12)-epoxy-eicosatrienoic acid | 11,12 EET | 319 | 167 | 17.3 | 1 | −60 | −20 | (d8) 11,12 EET |
| AA | CYP | 14(15)-epoxy-eicosatrienoic acid | 14,15 EET | 319 | 219 | 16.9 | 1 | −50 | −15 | (d8) 14,15 EET |
| EPA | CYP | 14,15-epoxy Eicosaletraenoic acid | 14,15 EpETE | 317 | 208 | 15.8 | 1 | −65 | −18 | (d8) 14.15 EET |
| EPA | CYP | 17,18-epoxy Eicosatetraenoic acid | 17,18 EpETE | 317 | 215 | 15.6 | 1 | −65 | −16 | (d8) 14,15 EET |
| DHA | CYP | 16,17-epoxy Docosapentaenoic acid | 16,17 EpDPE | 343 | 193 | 17.0 | 1 | −70 | −16 | (d8) 14.15EET |
| DHA | CYP | 19,20-epoxy Docosapentaenoic acid | 19,20 EpDPE | 343 | 241 | 16.5 | 1 | −70 | −17 | (d8) 14.15 EET |
| DHA | CYP | 19,20-dihydroxy-docosapenlaenoic acid | 19,20 DiHDPA | 361 | 229 | 13.1 | 1 | −70 | −25 | (d8) 14,15 EET |
| LA | CYP | 9(10)-epoxy-octadecenoic acid | 9,10 EpOME | 295 | 171 | 17.1 | 1 | −60 | −25 | (d4) 9,10 diHOME |
| LA | CYP | 12(13)-epoxy-octadecenoic acid | 12,13 EpOME | 295 | 195 | 17.1 | 1 | −60 | −25 | (d4) 12,13 diHOME |
| AA | CYP | 5,6-dihydroxy-eicosatrienoic acid | 5,6 DHET | 337 | 145 | 15.0 | 0.1 | −75 | −25 | (d8) 5-HETE |
| AA | CYP | 8,9-dihydroxy-eicosatrienoic acid | 8,9 DHET | 337 | 127 | 14.4 | 1 | −60 | −30 | (d8) 5-HETE |
| AA | CYP | 11, 12-dihydroxy-eicosatrienoic acid | 11,12 DHET | 337 | 167 | 14.0 | 0.1 | −60 | −25 | (d8) 5-HETE |
| AA | CYP | 14,15-dihydroxy-eicosatrienoic acid | 14,15 DHET | 337 | 207 | 13.3 | 0.1 | −60 | −25 | (d8) 5-HETE |
| LA | CYP | 9, 10-dihydroxy-octadecenoic acid | 9,10 diHOME | 313 | 201 | 12.9 | 0.1 | −60 | −30 | (d4) 9,10 diHOME |
| LA | CYP | 12, 13-dihydroxy-octadecenoic acid | 12,13 diHOME | 313 | 183 | 12.4 | 1 | −60 | −30 | (d4) 12,13 diHOME |
| - | - | Arachidonic acid | AA | 303 | 259 | 20.6 | ND | −80 | −20 | (d8)AA |
| - | - | Adrenic acid | ADA | 331 | 287 | 21.3 | ND | −80 | −20 | (d8)AA |
| - | - | Eicosapentaenoic acid | EPA | 301 | 257 | 19.4 | ND | −65 | −15 | (d5) EPA |
| - | - | Docohexaenoic acid | DHA | 327 | 229 | 20.2 | ND | −95 | −20 | (d5)DHA |

a Deuterated internal standards are shaded in grey. b Retention times are representative values

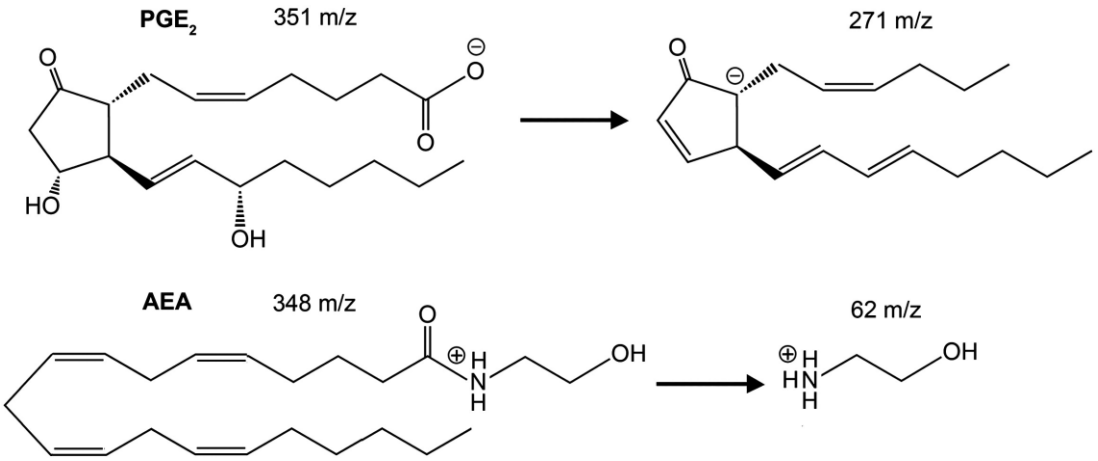


**Fig. 1**. The structures of the parent and daughter ions for PGE2 and AEA used in this method.

Representative retention time values for each eicosanoid are reported in Table 1. Representative retention times are reported because retention fluctuations occur between experiments which can be due to column usage, buffer preparation, and instrument variation. A chromatograph from a single analysis of a 100 ng primary standard solution containing a total of 102 eicosanoid species demonstrates the robustness of our methodology (Fig. 2A). Specific eicosanoid ion chromatographs have been extracted to illustrate different features of our methodology (Fig. 2B). Chromatographic separation is paramount when monitoring a large number of structurally similar metabolites. The COX pathway derived metabolites, prostaglandin E2 (PGE2) and prostaglandin D2 (PGD2) have the same molecular formula, differing only by the placement of a keto- and hydroxyl-group at positions 9 and 11. Subsequent full MS/MS scan analyses of these two molecules produce the exact same ion fragmentation pattern. The use of mass spectrometry alone is not sufficient to distinguish between metabolites that share the same sMRM pair. Adequate chromatographic separation of PGE2 and PGD2 allows us to differentiate between these two metabolites (Fig. 2B). There are 39 occurrences in our eicosanoid methodology where a metabolite shares an sMRM pair with another metabolite.

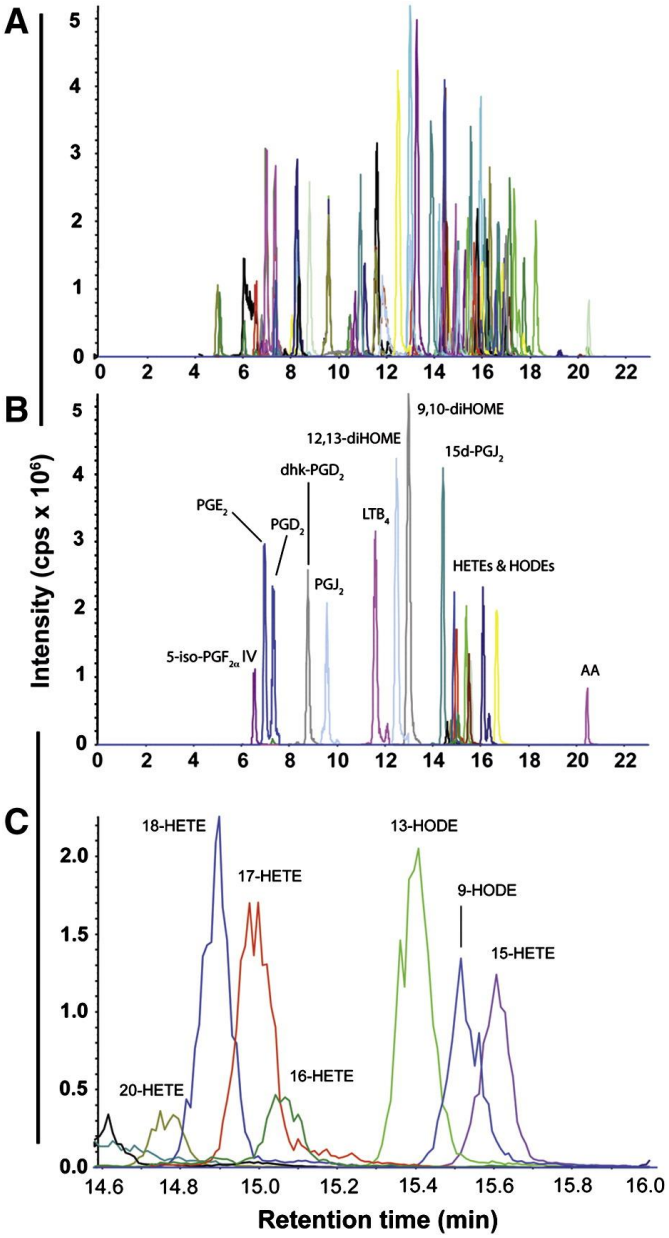


**Fig. 2.** Chromatograph from a single 100 ng standard solution subjected to our eicosanoid methodology. (A) 102 sMRM pairs were extracted from a single run. (B) Selected eicosanoid sMRM pairs from the major biosynthetic pathways were extracted from a single analysis. (C) A magnified view of the chromatograph of extracted HETE and HODE metabolites.

## N-acylethanolamine (NAE) methodology

A similar CLASS approach used to monitor eicosanoids is employed in the detection of NAEs. The NAE methodology can be employed as a lone analysis or performed in tandem after samples have subjected to the eicosanoid methodology. All NAEs contain an acyl linkage to the nitrogen of an ethanolamine molecule (Fig. 1, AEA). NAEs are detected in positive electrospray ion mode. Unlike the eicosanoids, NAEs ionize more efficiently in positive ion mode due to their conserved amide nitrogen. Since the carboxyl-amide bond is labile, fragmentation of an N-acylethanolamine [M + H]+ species produces a 62 m/z ion (C2H8N1O1+). The presence of salts such as Na+ and K+, which can remain associated with these lipids even after sample preparation, can facilitate the formation of other NAE ion species such as [M + Na]+ and [M + K]+, diminishing the overall sensitivity of these lipids. Ammonium acetate, when used in concentrations above 1 mM, can shift the equilibrium toward [M + NH4]+ species, which degrade to [M + H]+ + NH3 during ionization. Similarly to the eicosanoids, no additional chemical derivatization is required to monitor NAEs. The current NAE methodology monitors 36 different metabolites, derived from saturated fatty acids, mono- to poly-unsaturated fatty acids, and eicosanoids. Some of these metabolites can be purchased (Cayman Chemical), while others were synthesized in our laboratory by standard methods. The sMRM pair and representative retention time for each NAE have been determined in the same manner as the eicosanoids (Table 2). Fragmentation of the carboxyl-amide bond required the same declustering potential (50) and collision energy (40) values for all NAEs.

### Table 2. Optimized sMRM pairs and parameters for N-acylethanolamines.

| Common name | Abbreviation | Parent | Daughter | Retention time (min)[b] | LOD (pg) | Internal standard |
|---|---|---|---|---|---|---|
| (d4) Prostaglandin $F_{2\alpha}$ Ethanolamide[a] | (d4) $PGF_{2\alpha}$-EA | 384 | 62 | 5.1 | ND | – |
| (d4) Palmitoyl Ethanolamide | (d4) 16:0-EA | 312 | 62 | 12.1 | ND | – |
| (d4) Oleoyl Ethanolamide | (d4) 18:1-EA | 328 | 62 | 12.5 | ND | – |
| (d8) Arachidonyl Ethanolamide | (d8 20:4-EA | 356 | 62 | 11.4 | ND | – |
| Prostaglandin $F_{2\alpha}$ Ethanolamide | $PGF_{2\alpha}$-EA | 380 | 62 | 5.1 | 1,000 | (d4) $PGF_{2\alpha}$ EA |
| 11-beta-Prostaglandin $F_{2\alpha}$ Ethanolamide | 11β- $PGF_{2\alpha}$-EA | 380 | 62 | 4.8 | ND | (d4) $PGF_{2\alpha}$-EA |
| 8-iso-Prostaglandin $F_{2\alpha}$ III Ethanolamide | 8-iso-$PGF_{2\alpha}$ III-EA | 380 | 62 | 4.7 | 100 | (d4) $PGF_{2\alpha}$-EA |
| Prostaglandin $E_2$ Ethanolamide | $PGE_2$-EA | 378 | 62 | 5.2 | 100 | (d4) $PGF_{2\alpha}$-EA |
| Prostaglandin $D_2$ Ethanolamide | $PGD_2$-EA | 378 | 62 | 5.4 | 10 | (d4) $PGF_{2\alpha}$-EA |
| 5(6)-Epoxy-Eicosatrienoic acid Ethanolamide | 5,6-EET-EA | 346 | 62 | 9.8 | 1 | (d4) $PGF_{2\alpha}$-EA |
| 8(9)-Epoxy-Eicosatrienoic acid Ethanolamide | 8,9-EET-EA | 346 | 62 | 9.4 | 10 | (d4) $PGF_{2\alpha}$-EA |
| 11 (12)-Epoxy-Eicosatrienoic acid Ethanolamide | 11 ,12-EET-EA | 346 | 62 | 9.2 | 1 | (d4) $PGF_{2\alpha}$-EA |
| 14(15)-Epoxy-Eicosatrienoic acid Ethanolamide | 14,15-EET-EA | 346 | 62 | 8.9 | 1 | (d4) $PGF_{2\alpha}$-EA |
| 15-Hydroxy-Eicosatetraenoic acid Ethanolamide | 15-HETE-EA | 346 | 62 | 8.4 | 0.1 | (d4) $PGF_{2\alpha}$-EA |
| 20-Hydroxy-Eicosatetraenoic acid Ethanolamide | 20-HETE-EA | 346 | 62 | 7.6 | 0.1 | (d4) $PGF_{2\alpha}$-EA |
| Lauroyl Ethanolamide | 12:0-EA | 244 | 62 | 8.9 | 10 | (d4) 16:0-EA |
| Myristoyl Ethanolamide | 14:0-EA | 272 | 62 | 10.6 | 10 | (d4) 16:0-EA |
| Pentadecanoyl Ethanolamide | 15:0-EA | 286 | 62 | 11.4 | 0.1 | (d4) 16:0-EA |
| Palmitoyl Ethanolamide | 16:0-EA | 300 | 62 | 12.2 | 0.1 | (d4) 16:0-EA |
| Heptadecanoyl Ethanolamide | 17:0-EA | 314 | 62 | 12.9 | 0.1 | (d4) 16:0-EA |
| Stearoyl Ethanolamide | 18:0-EA | 328 | 62 | 13.7 | 0.1 | (d4) 16:0-EA |
| Arachidoyl Ethanolamide | 20:0-EA | 356 | 62 | 14.8 | 0.1 | (d4) 16:0-EA |
| Tricosanoyl Ethanolamide | 23:0-EA | 398 | 62 | 16.2 | 0.1 | (d4) 16:0-EA |
| Lignoceroyl Ethanolamide | 24:0-EA | 412 | 62 | 16.6 | 0.1 | (d4) 16:0-EA |
| Palmitoleoyl Ethanolamide | 16:1-EA | 298 | 62 | 11.1 | 0.1 | (d4) 18:1-EA |
| Oleoyl Ethanolamide | 18:1-EA | 326 | 62 | 12.5 | 0.1 | (d4) 18:1-EA |
| Docosaenoyl Ethanolamide | 22:1-EA | 382 | 62 | 14.9 | 0.1 | (d4) 18:1-EA |
| Nervonoyll Ethanolamide | 24:1-EA | 410 | 62 | 15.8 | 0.1 | (d4) 18:1-EA |
| Linoleoyl Ethanolamide | 18:2-EA | 324 | 62 | 11.5 | 0.1 | (d8) 20:4-EA |
| α-Linolenoyl Ethanolamide | 18:3(α)-EA | 322 | 62 | 10.8 | 10 | (d8) 20:4-EA |
| γ-Linolenoyl Ethanolamide | 18:3(γ)-EA | 322 | 62 | 10.4 | 100 | (d8) 20:4-EA |
| Meadoyl Ethanolamide | 20:3-EA | 350 | 62 | 12.4 | 0.1 | (d8) 20:4-EA |
| Dihomo-γ-Linolenoyl Ethanolamide | 20:3-EA (DγLA) | 350 | 62 | 12 | 0.1 | (d8) 20:4-EA |
| Arachidonoyl Ethanolamide | 20:4-EA | 348 | 62 | 11.4 | 0.1 | (d8) 20:4-EA |
| Docosatetraenoyl Ethanolamide | 22:4-EA | 376 | 62 | 12.5 | 0.1 | (d8) 20:4-EA |
| Eicosapenaenoyl Ethanolamide | 20:5-EA | 346 | 62 | 10.5 | 0.1 | (d8) 20:4-EA |
| Docohexaenoyl Ethanolamide | 22:6-EA | 372 | 62 | 11.2 | 0.1 | (d8) 20:4-EA |

[a] Deuterated internal standards are shaded in gray. [b] Retention times are representative values.

The NAE metabolites are identified using a 25 min LC/MS/MS analysis. The identity of detected NAE metabolites are confirmed against pure NAE standards subjected to the same LC/MS/MS analysis. A chromatograph from a 100 ng standard stock solution containing 33 NAEs subjected to this methodology is depicted in Fig. 3A. Selected metabolites have been extracted to simplify the data produced by this method (Fig. 3B). Since every NAE generates the daughter ion fragment (62 m/z)+, many of metabolites share the same MRM transition. This can be

seen when examining the different HETE-EA and EET-EA species which all have a 346.0/62.0 sMRM transition (Fig. 3C). Having adequate chromatographic separation is the key in distinguishing these metabolites from each other. There are 15 NAEs that have a shared sMRM pair.

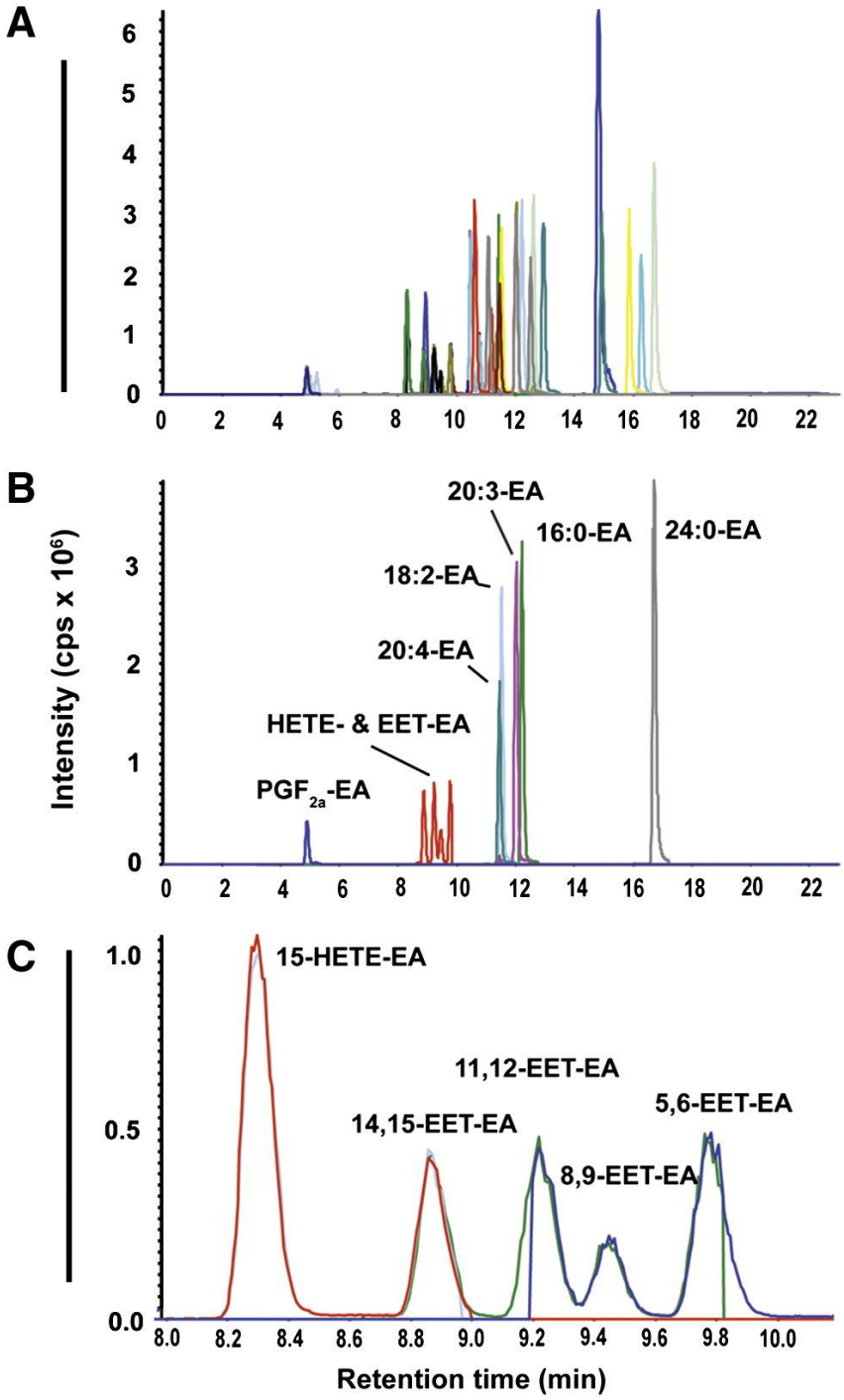


Fig. 3. Chromatograph from a single 100 ng standard solution subjected to our ethanolamine methodology. (A) 40 sMRM pairs were extracted from a single run. (B) A diverse group of selected ethanolamides sMRM pairs were extracted from a single analysis. (C) A magnified view of the chromatograph of extracted HETE-EA and EET-EA metabolites.

## Quantitation

Our current methodology allows for both relative and exact quantitation using a stable isotope dilution technique. This technique applies for the quantitation of eicosanoids and NAEs. First, we employ measures to filter out aberrant signals from the dataset, whereby a detected peak is considered valid if the peak's height is 3-fold greater than the background noise. Peaks that do not meet this requirement are disregarded. Also, the addition of deuterated internal standards can add contaminating lipid metabolites to the sample, which arise from impurities in the standard. The deuterated internal standard is mass analysed in every experiment, and contaminating peaks are subtracted from the samples.

Both relative and exact quantitation heavily rely upon making comparisons between the integrated areas of a given lipid metabolite and its corresponding internal standard (AreaMETABOLITE/AreaINSTD). The internal standard solution for the eicosanoids is comprised of 30 deuterated eicosanoids, while the NAE's internal standard solution contains 4 deuterated NAEs. The criteria used to pair a metabolite with a particular internal standard include: 1) matching the metabolite with the analogous deuterated species (PGD2 and d4-PGD2), or 2) matching the metabolite with an internal standard that has a similar chemical structure and retention time (PGJ2 and d4-15d-PGJ2). Table 1, Table 2 list the deuterated internal standards (highlighted in grey) and lipid metabolite they were paired with. This ratio, which is established prior to lipid extraction, is maintained throughout the entire sampling process. This technique allows for accurate quantitation because lipid metabolites and corresponding internal standards have similar ionization efficiencies and will be equally affected by factors such as ion suppression. An important caveat is that the internal standard must be detectable above the background for quantitation.

Relative quantitation is determined by comparing the ratio (AreaMETABOLITE/AreaINSTD) between two different samples. This can be useful in monitoring how a single or a group of lipid metabolites change during different stages of a disease, or how these metabolic pathways are globally affected by pharmacological intervention. To quantitate the exact amount of a given metabolite in a sample, a primary standard curve generated from commercially bought standards is required. Primary standard curves for eicosanoids and NAE are generated separately. A primary standard curve is produced from 7 different concentrated eicosanoid or NAE standard solutions (0.1 ng, 0.3 ng, 1 ng, 3 ng, 10 ng, 30 ng, and 100 ng) that have been spiked with the internal standard solution. Since the primary standard and internal standard solutions are maintained in 100% ethanol, the addition of aqueous buffer is required to reproduce a metabolite's chromatographic retention time. For eicosanoids, an equal volume of 0.2% acetic acid–water is added to the primary/internal standard mixture, while an equal volume of water–acetonitrile–acetic acid (70:30:0.1; v/v/v) + 1 g/L ammonium acetate is added to the NAE primary/internal standard mixture. Each primary standard concentration is analysed in duplicate and averaged. The primary standard curve is determined by generating a linear regression trend line that is forced through 0.

Representative primary standard curves from eicosanoid and NAE metabolites are shown to illustrate this technique (Fig. 4). These primary standard curves were generated from 3 separate experiments performed in duplicate spanning a 2-month period. Each standard curve displays a correlation value (R2 value) above 0.999 indicating the high reproducibility of this methodology. Exact quantitation of a metabolite in a given sample is determined by extrapolating the amount (X-axis value) from where the ratio ((AreaMETABOLITE/AreaINSTD); (Y-axis value)) intercepts the primary standard curve (Fig. 4). Alternately, the exact amount of a given lipid metabolite in a sample can be determined by dividing the ratio (AreaMETABOLITE/AreaINSTD) by the slope of the standard curve. We routinely quantitate about 100 eicosanoids out of the total 141 monitored in a single analysis. Our NAE methodology contains 33 quantifiable metabolites out of a total of 36 monitored in a single analysis. Some metabolites are not quantitated because either a pure standard is not available (such as the dihomo-prostaglandins) or are routinely observed in very low abundance in experimental models that we have examined.

Monitoring sMRM pairs instead of full MS/MS scans greatly increases the sensitivity of detection. A 100 ng mixture of standards containing eicosanoids or NAE was serially diluted and analyzed by our methodology to determine the lower limit of detection (LOD) for a portion of the routinely quantitated eicosanoids and NAEs. These values are detailed in Table 1, Table 2. The LOD for the eicosanoids analyzed ranged between 0.1 pg and 1 pg. This is a definite improvement over our previous method with reported LOD values ranging from 1 pg to 10 pg on average [30]. The LOD values for a majority of NAEs were detected at 0.1 pg, while in a few instances a higher LOD value was observed ranging from 10 pg to 1000 pg. The difference in sensitivity is due to the efficiency and stability of the parent to daughter ion transition.