Quantitative assays for esterified oxylipins generated by immune cells

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Principles

The assay is based on the use of LC/MS/MS in multiple reaction monitoring (MRM) mode. Lipids are extracted from samples of tissue culture supernatant or other biological fluids, cells or tissue homogenates, using a hexane/isopropanol/acetic acid liquid extraction procedure. Just prior to extraction, internal standards (IS) are added to all samples. These are phospholipids of the same class, which are not normally present. Typically, dimyristoyl-phosphatidylethanolamine (DMPE, di-14:0-PE) and dimyristoyl-phosphatidylcholine (DMPC, di-14:0-PC) are used for PE and PC, respectively, with both added simultaneously as a mixture to each sample. For studies on immune cells in vitro, at least triplicate samples are analysed, although with human or mouse tissue, more may be required for sufficient power. Lipid extracts are then analysed using reverse-phase LC/MS/MS, monitoring for parent → daughter transitions that are specific for the molecule of interest. We use a Luna (Phenomenex) column at 200 µl min−1 with a methanol/acetonitrile/ammonium acetate gradient. Long separation times (50 min) are needed as these complex mixtures contain many lipids with the same molecular weight (isobaric), and retention time is important for accurate identification. Molecular species are identified based on both retention time and MRM transition. For some molecules, daughter ions specific for positional isomers of eicosanoids can be used (e.g., m/z 115 for the 5-HETE-PE positional isomer). Quantitation is then achieved using standard curves generated using synthetic primary standards. Methods for generation of these are provided in this protocol.

REAGENTS

CAUTION: Use gloves and lab coat when working with biological samples and ensure that work using solvents is carried out in a fume hood.

di-14:0-PE (DMPE; Avanti Polar Lipids, cat. no. 850745)
di-14:0-PC (DMPC; Avanti Polar Lipids, cat. no. 850345)
16:0/20:4-PC (Avanti Polar Lipids, cat. no. 850459)
18:0/20:4-PE (stearoyl-arachidonyl-PE (SAPE); Avanti Polar Lipids, cat. no. 850804)
18:0/22:6-PE (Avanti Polar Lipids, cat. no. 850806)
16:0/18:2-PE (Avanti Polar Lipids, cat. no. 850756)
Soybean lipoxygenase, type V (Sigma-Aldrich, cat. no. L6632)
Hexane, 2-propanol, acetonitrile, methanol, chloroform, water (all HPLC grade; Fisher Scientific)

CAUTION: Organic solvents need to be handled in a fume hood and stored in appropriately vented cabinets.

Free acid eicosanoid standards (all from Cayman Chemical: 15-HETE (cat. no. 34720), 12-HETE (34570), 5-HETE (34230), 11-HETE (34500), 8-HETE (34340), 20-HDOHE (33750), 17-HDOHE (33650), 14-HDOHE (33550), 11-HDOHE (33450), 8-HDOHE (33350), 16-HDOHE (33600), 13-HDOHE (33500), 10-HDOHE (33400), 7-HDOHE (33300), 4-HDOHE (33200), 13-HODE (38600), 9-HODE (38400).

Dess-Martin periodinane, 97% (DMP; Sigma-Aldrich, cat. no. 274623)
CRITICAL: Allow bottle to warm fully to room temperature (20–22 °C) before opening.

Anhydrous dichloromethane (Sigma-Aldrich, cat. no. 270997)

2,2,5,7,8-Pentamethyl-6-chromanol (PMC; Sigma-Aldrich, cat. no. 430676)
CRITICAL: Make up fresh solutions in water on the day of use.

Stannous chloride ($SnCl_2$; Sigma-Aldrich, cat. no. 204722)
CRITICAL: Make up fresh solutions in water on the day of use.

Butylated hydroxytoluene (BHT; Sigma-Aldrich, cat. no. W218405)
CRITICAL: Make up fresh solutions in water on the day of use.

Deoxycholate
Borate buffer
Ammonium acetate
Oxygen source
Nitrogen source (both liquid and gas)
Sodium hydroxide
Hydrochloric acid
Diethylenetriamine penta acetic acid (DTPA)
Phosphate-buffered saline (PBS)

EQUIPMENT

4000 Q TRAP mass spectrometer (Applied Biosystems) or similar model
SIL-HT autosampler (Shimadzu)
Extraction tubes (Chromacol, cat. no. 10-SV T928)
Bottle top dispensers (Fisher Chemical Company)
RapidVap system (Labconco)
Discovery C18 column (25 cm × 4.6 mm, 5 µm; Fisher Scientific, cat. no. 504971)
Spherisorb ODS2 column (15 cm × 4.6 mm, 5 µm; Waters, cat. no. PSS839853)
Luna C18 (2) column (15 cm × 2 mm, 3 µm; Phenomenex, cat. no. 00F-4251-B0)
Analyst 1.4 software
HPLC system (Gilson) or similar model
UV detector, 1100 Series (Agilent)
Uvikon software or similar
Dry block heater (Techne DB.3)
RapidVap vacuum evaporations system (Labconco)
Falcon conical tubes (50 ml)
Pasteur pipettes
Erlenmeyer flasks (250 ml)
Glass tubes, various sizes
Quartz cuvettes
Spectrophotometer
Mortar and pestle

REAGENT SETUP

Biological sample to be analysed: can include cultured cells, human or mouse tissue types including liver, lung, peritoneal lavage fluid, primary peripheral leukocytes or platelets.
CAUTION: Animal and human tissue should be collected in compliance with all relevant governmental and institutional requirements.

Lipid extraction solvent. Combine 1 M acetic acid, 2-propanol and hexane in a ratio of 2:20:30 (vol/vol/vol). Prepare in 500-ml aliquots and store sealed at room temperature (indefinitely).

EQUIPMENT SETUP

LC/MS/MS system

- Mass spectrometry was carried out using an Applied Biosystems 4000 Q TRAP mass spectrometer operating in MRM mode. Ionization was carried out by electrospray, using the Turbo V probe. HPLC separation was performed using a Shimadzu SIL-HT autosampler, online degasser and LC-10ADVPµ

binary pump system. A column oven operating at 22 °C was used, although this is not essential. Data were acquired and analysed using Analyst 1.4. Before analysis of any new compound, it is essential that the instrument is tuned according to the manufacturers' protocols. On our instrument, collision energies of −40 to −50 V are typically required for MS/MS of oxidized phospholipids.

- Because phospholipids are especially 'sticky' molecules, it is important to ensure that extensive washing steps are used between sample injections. On our instrument, we use a 1-min rinse at 35 µl s−1 both before and after aspiration, and our needle is Teflon coated to minimize carryover. It is also essential to run methanol blanks at appropriate defined periods (e.g., between every 10 and 20 samples) to confirm that carryover is not taking place. Methanol blanks are also run at the start and end of all batches. A further system check can be carried out by subjecting cell-free buffers to extraction alongside cell samples and then analysing these for the presence of the lipids.

- When analysing concentrated samples, e.g., standards, it is important to make serial dilutions starting with the lowest concentration; otherwise, contamination of the lines/sources can occur. We find that the best way to remove contamination of HPLC lines with phospholipid standards is with a 20-min wash with chloroform:methanol (50:50) at 1 ml min−1. However, because of the corrosive nature of chloroform, it is important to purge all lines immediately after performing this cleaning procedure.

HPLC system

- HPLC with UV detection was carried out using a Gilson HPLC system comprising 811D dynamic mixer, 306 pumps, 805 manometric module and an Agilent 1100 series UV detector. No column oven was used. Data was acquired and analysed using Uvikon software.

Glassware

- When working with organic solvents, clean glassware is essential. Keep beakers and cylinders aside from general laboratory use, and clean by rinsing with HPLC grade solvents (water, methanol, chloroform, in that order) and leave to dry. Do not use glassware that has been cleaned using detergents. Extraction tubes (Chromacol) and glass Pasteur pipettes are single use only, but tube lids can be recycled by rinsing well with methanol and chloroform. Defined volumes of solvents are aliquotted into tubes using bottle top dispensers (Fisher Chemical Company) that provide an efficient and safe method for working with organic solvents.

Drying techniques

- When evaporating small amounts of solvent, two methods can be used. A dry block heater (e.g., Techne DB.3) set at maximum 30 °C with either nitrogen or argon gas will evaporate multiple samples. Alternatively, we generally use the RapidVap system (Labconco), a shaking vacuum dryer that tends to evaporate faster than blowing with inert gas.

PROCEDURE

1. Synthesis of primary standards for quantitation

Prepare the required standards as described in Option A, B or C. Option A describes generation of purified esterified H(p)ETEs or H(p)ODEs using soybean LOX oxidation of substrates, resulting in single positional isomers, specifically 15-H(p)ETE-PL or 13-H(p)ODE-PL. Option B is for the generation and purification of positional isomer mixtures of H(p)ETE-, H(p)ODE- or H(p)DOHE-PE or -PC standards. Positional isomers do not separate on reverse-phase HPLC sufficiently for purification, thus, they are used as a mixture of known composition. The total amount of these standards is determined using absorbance with $\varepsilon = 28$ mM−1 cm−1, with the proportion of each individual positional isomer determined after saponification and quantitation of relative amounts of free eicosanoid using authentic standards. This then allows calculation of the ng amount of each positional isomer in the mixture. In Option C, KETE-PLs are generated by

oxidation of the corresponding HETE-PLs (either single or mixed positional isomers). Fresh DMP and anhydrous dichloromethane are essential for successful reaction12. The synthesis scheme is summarized in Figure 2, and NMR and MS/MS data are provided in SUPPLEMENTARY DATA section (Supplementary Figures 1, 2, 3, 4 and 5; Supplementary Tables 1 and 2; Supplementary Methods appearing in original paper: https://www.nature.com/articles/nprot.2010.162?message-global=remove)

2. (A) Generation and purification of 15S-H(p)ETE-PL or 13S-H(p)ODE-PL

(i) Dry purified phospholipid (e.g., 18:0a/20:4-PE, 16:0a/20:4-PC or containing 18:2 at sn2, from Avanti Polar Lipids) under inert gas (blow with nitrogen or argon using a glass Pasteur pipette in a 50-ml Falcon tube), then resuspend in 26 ml buffer (10 mM deoxycholate, 0.2 M borate buffer (pH 9.0)) to give a final concentration of 1 mM lipid. Transfer to a large glass (e.g., 250 ml) conical or Erlenmeyer flask to give a large surface area.

(ii) Add soybean 15-LOX type V or IV (5.2 kU ml−1) and incubate for 30 min at room temperature with stirring and bubbling with 95% oxygen. If desired, reduce the hydroperoxides to corresponding alcohols by addition of 1 mM $SnCl_2$ for 10 min at room temperature (261 µl of 19 mg ml−1 in water) after oxidation by LOX. (*CRITICAL STEP: Bubbling with oxygen is essential, as its depletion will cause hydroperoxidase activity of LOX with degradation of the hydroperoxide product).*

(iii) Extract the lipids by adding 65 ml lipid extraction solvent to the sample, then vortex for 1 min. Then add 65 ml hexane and vortex again. Work in a fume hood.

(iv) Decant as much of the upper hexane layer as possible into a conical flask, then pour the rest (aqueous phase) into clean glass tubes and centrifuge at low speed for 5 min. Using a glass Pasteur pipette, you should recover the hexane (upper phase) and add it to what is already decanted. Then pour the aqueous phase back into the conical flask for a second extraction.

(v) Add 65 ml hexane to the aqueous phase and vortex. Repeat the extraction and removal of hexane upper layer as described above. Combine all hexane fractions into the conical flask and dry under a stream of inert gas (nitrogen or argon supplied through a glass Pasteur pipette). Keeping the flask warm by placing in a dry block at a maximum of 30 °C aids evaporation. Periodically swirl to recover lipids that have dried onto the sides of the vessel. Once the sample is less than 4 ml, transfer it to smaller glass vial to finish drying. Alternatively, a separatory funnel may be useful for extraction with large volumes.

(vi) Resuspend the lipid in 0.5 ml methanol and store at −80 °C (under inert gas) until purification. *PAUSE POINT: The crude mixture can be stored overnight before purification, at −80 °C under inert gas.*

(vii) Purify using reverse-phase HPLC using a Discovery C18 column (25 cm × 4.6 mm, 5 µm) at 1 ml min−1, and a gradient of 50% to 100% mobile phase solvent B in solvent A (solvent A: water, 5 mM ammonium acetate, solvent B: methanol, 5 mM ammonium acetate) over 15 min, and then hold at 100% B for 20 min. Elution is monitored at 205 nm (unoxidized lipid) and 235 nm (HETE-PL).

(viii) Oxidized phospholipids typically elute at 22–24 min, and the 15-HETE- or 13-HODE-PL will elute as a single peak (Fig. 3). For both, the S:R enantiomer ratio will typically be around 9:1, characteristic of the specificity of soybean LOX (not shown). Collect into a glass vial, dry under inert gas or using a RapidVap and resuspend in a small volume of methanol (e.g., 100 µl).

(ix) Quantify the amount of esterified eicosanoid by absorbance (at 235 nm) of the purified lipid mixture using the extinction coefficient $\varepsilon = 28$ mM−1 cm−1. For this, dilute a small amount into methanol and scan in a quartz cuvette from 200 to 400 nm using a scanning spectrophotometer. Store at −80 °C under nitrogen gas in methanol13. *PAUSE POINT: Reduced hydroxy-PL standards are stable for several months, although we find that concentrations gradually decrease because of adsorption to the glass vial. In contrast,*

hydroperoxides are not stable and should be used within 1–2 weeks of generation. Stability may be enhanced by addition of 100 μM BHT to the storage vial.

3. (B) Generation and purification of positional isomer mixtures of H(p)ETE-, H(p)ODE- or H(p)DOHE-PE or –PC standards

(i) Add 64 μl 10 mM PMC in chloroform to 5 mg phospholipid (e.g., 16:0a/20:4-PC, 18:0a/20:4-PE, or with 18:2 or 22:6 at sn2) in 1.5 ml chloroform, then dry under nitrogen in a clean glass tube.

(ii) Incubate the sample at 37 °C for 24 h then resuspend in 200 μl methanol containing 20 mg ml−1 BHT.

(iii) Reduce hydroperoxides using SnCl2 if desired as described in Step 1A(ii).

(iv) Extract lipids by addition of 800 μl methanol, 2 ml chloroform and 0.5 ml water. Vortex and then centrifuge to separate the layers. Remove the bottom layer using a glass Pasteur pipette, then dry and resuspend in 200 μl methanol.

PAUSE POINT: The crude mixture can be stored overnight before purification, at −80 °C under inert gas.

(v) Purify the esterified eicosanoids using reverse-phase HPLC as described in Step 1A(vii), monitoring absorbance at 205 nm (parent) and 235 nm (conjugated diene of esterified eicosanoid). At this stage, H(p)ETE-PLs will appear at 235 nm as six unresolved peaks, each representing a single positional isomer (Fig. 4). HODE-PLs and HDOHE-PLs contain 2 and 10 separate positional isomers, respectively; however, these are not easily resolved using LC-UV.

(vi) Total HETE-PLs in each standard preparation is quantified by absorbance at 235 nm, using ε1mm,1cm = 28 absorbance units (au). For this, dilute a small amount into methanol and scan in a quartz cuvette from 200 to 400 nm using a scanning spectrophotometer. The relative amounts of each isomer are then determined in the analysis of the free acids following saponification.

(vii) Saponification: Remove a small amount of standard (10 μl) and suspend in 1.5 ml of propan-2-ol; add 1.5 ml 0.2 M NaOH and incubate at 60 °C for 30 min under nitrogen.

(viii) Acidify to pH 3.0 with 0.5 M HCl. Add 3 ml hexane, vortex, and centrifuge at 260g for 5 min.

(ix) Remove upper layer and re-extract using 3 ml hexane; combine hexane extracts.

(x) Dry hexane extracts, resuspend in 100 μl methanol and store under nitrogen at −80 °C until analysis by LC/MS/MS for free eicosanoids (below).

PAUSE POINT: Samples can be stored for several weeks if they have been reduced to hydroxylipids, but hydroperoxy standards should be analysed within a few days.

(xi) Analysis of positional isomers as free acids: Separate free eicosanoid positional isomers, using a Spherisorb ODS2 column (5 mm, 150 mm × 4.6 mm; Waters) with a solvent of 50–90% B in solvent A over 15 min (solvent A, water:acetonitrile:acetic acid, 75:25:0.1; solvent B, methanol:acetonitrile:acetic acid, 60:40:0.1) and a flow rate of 1 ml min−1. Elution is monitored using MS/MS transitions listed in Table 1.

(xii) Quantitate using standard curves generated with positional isomer standards for free eicosanoids. These are commercially available from Cayman Chemical, and are listed under REAGENTS.

(xiii) Once relative amounts of each free acid positional isomer have been determined, the absolute amount of each can be calculated based on the total yield of HETE-PLs as determined using absorbance in Step 1B(vi).

PAUSE POINT: Stability information is as for Option A.

4. (C) Generation and purification of KETE-PL standards

(i) Dry down 100 µg purified HETE-PL (either mixed or single isomers) in a glass vial to remove the methanol and reconstitute in 100 µl anhydrous dichloromethane. Add 700 µg DMP and incubate at room temperature for 30 min in the dark, with regular agitation. (*CRITICAL STEP: It is essential that DMP is allowed to warm up fully to room temperature before opening the stock bottle. If this is not done, additional oxidations of the HETE take place, resulting in artifactual product formation)*

(ii) Dry the sample under nitrogen and add 1 ml HPLC-grade water along with 2.5 ml lipid extraction solvent. Vortex vigorously, and then add 2.5 ml hexane. Vortex and centrifuge (260g, 5 min), then recover the lipids using a glass Pasteur pipette by decanting the upper hexane layer.

(iii) Re-extract the lower aqueous layer with 2.5 ml hexane, vortexing and centrifuging as above and decanting the upper hexane layer. Combine this with the first hexane extraction.

(iv) Dry under stream of nitrogen or in vacuum dryer and reconstitute into 100 µl methanol.

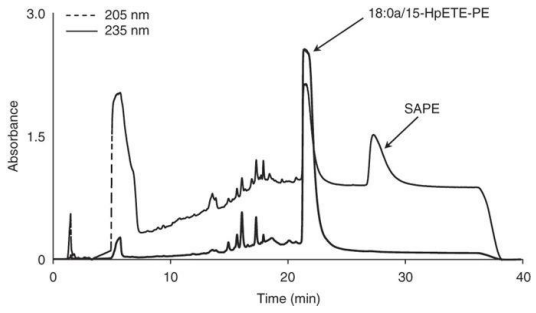
PAUSE POINT: The crude mixture can be stored overnight before purification, at −80 °C under inert gas.

(v) Purify KETE-PL as described for esterified eicosanoids (Step 1A(vii)), using reverse-phase HPLC, monitoring elution at 280 nm (typically around 27 min). Quantify total KETE-PL by measuring absorbance of the purified lipid mixture using ε = 22.96 mM−1 cm−1 (for the conjugated carbonyl, determined using 15-KETE free acid) and store at −80 °C under nitrogen in methanol. For quantification, dilute a small amount into methanol and scan in a quartz cuvette from 200 to 400 nm using a scanning spectrophotometer.

(vi) When isomeric mixtures of HETE-PLs are used to generate KETE-PLs, the relative amount of each KETE-PL will mirror that of the HETE-PL substrate, and thus can then be used to calculate the absolute amount of each positional isomer in the mixture.

PAUSE POINT: Store as for other standards above. Stability information is not yet available for these lipids; however, we anticipate that they may be less stable than hydroxyphospholipids because of the presence of the electrophilic carbonyl group. We recommend, at least at monthly intervals, that the concentration and purity be rechecked by LC/MS/MS analysis (as detailed in Steps 12–13).

Figure 1. Purification of 18:0a/15-HpETE-PE using reverse-phase HPLC-UV.



SAPE was oxidized using soybean LOX as described, then lipids were extracted and 18:0a/15-HpETE-PE was purified using reverse-phase HPLC, with monitoring at 235 nm (product) and 205 nm (substrate). The esterified eicosanoid elutes at about 21–22 min.

Figure 2. Summarized scheme for synthesis of standards

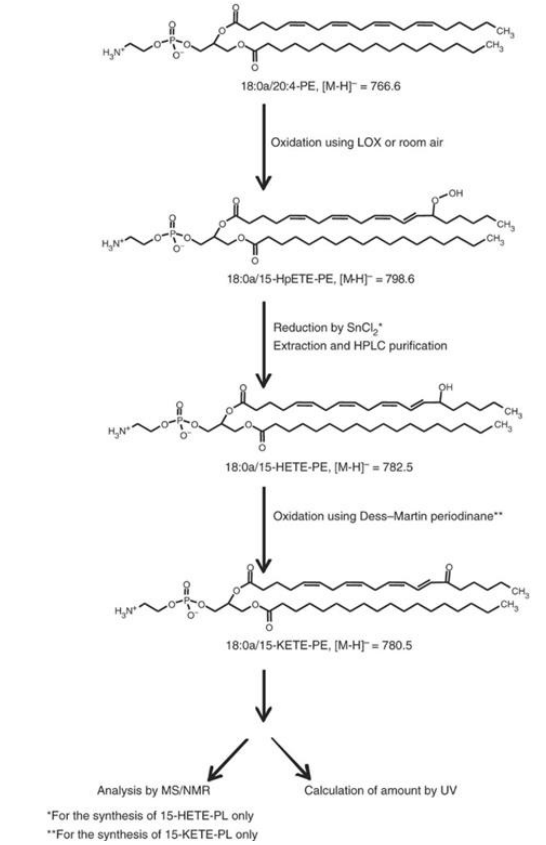
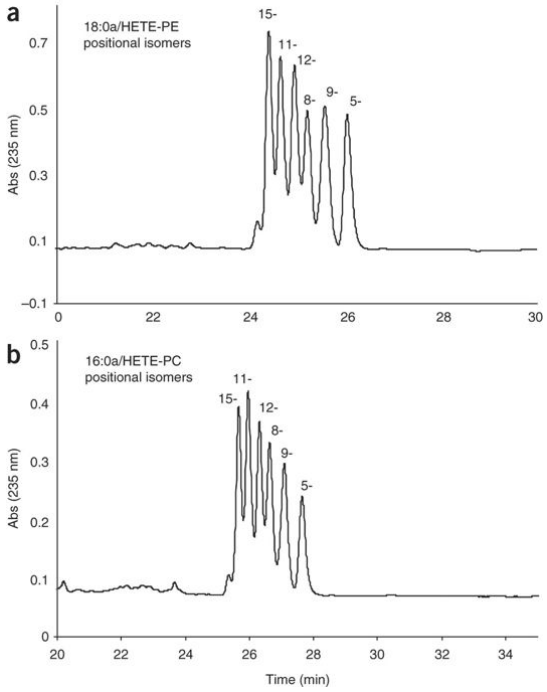


Figure 3. Purification of 18:0a/15-HETE-PE using reverse-phase HPLC-UV.



SAPE was oxidized using air oxidation, then reduced using $SnCl_2$. (a) Chromatogram showing elution of the six 18:0a/15-HETE-PE positional isomers during purification. (b) Chromatogram showing elution of the six 16:0a/15-HETE-PC positional isomers during purification.

Table 1. Precursor and product ions of HETEs, HODEs and HDOHE positional isomers analysed using LC/MS/MS.

Eicosanoid	Precursor *m /z*	Product *m /z*
15-HETE	319.2	219.1
12-HETE	319.2	179.1
5-HETE	319.2	115.1
11-HETE	319.2	167.1
8-HETE	319.2	155.1
20-HDOHE	343.2	187.2
17-HDOHE	343.2	273.2
14-HDOHE	343.2	205.2
11-HDOHE	343.2	149.2
8-HDOHE	343.2	109.2
16-HDOHE	343.2	233.2
13-HDOHE	343.2	193.2
10-HDOHE	343.2	153.2
7-HDOHE	343.2	141.2
4-HDOHE	343.2	101.2
13-HODE	295.2	171.1
9-HODE	295.2	195.1

Tissue preparation

5. Homogenization of solid tissue samples (e.g., lung, liver). After tissue collection, snap-freeze the samples in cryovials as quickly as possible, then store at −80 °C until analysis.
   CRITICAL STEP: Care needs to be taken with fresh tissue samples to ensure artifactual oxidation does not take place. This includes freezing samples as quickly as possible after collection.

6. Weigh the frozen tissue (up to 0.5 g), then place in an ice-cold mortar (precooled in freezer) on ice.

7. Add up to 1 ml PBS (pH 7.4), containing 100 μM each DTPA and BHT, followed by some liquid nitrogen (e.g., 10 ml). (*CRITICAL STEP: Addition of antioxidants/chelators during homogenization is essential to prevent artifactual oxidation. Also, liquid nitrogen is added after the buffer so that the solution freezes and making homogenization easier)*

8. Homogenize using the pestle until a smooth homogenate is achieved. Extract as described below.

9. Cells or supernatant samples are extracted without the above steps. (*PAUSE POINT: The homogenate can be stored overnight before extraction, at −80 °C under inert gas. However, in order to minimize freeze-thawing, this is not recommended)*

10. Lipid extraction from samples. Add IS, at 10 ng per sample. For quantitation of PEs or PCs, we use DMPE or DMPC, respectively.
    TROUBLESHOOTING: As mentioned, clean glassware is essential and organic solvents should not be used with regular laboratory plasticware as they will leach compounds from the plastic that can interfere, particularly in the low UV range used for conjugated dienes. This could especially be a problem when purifying newly synthesized standards using LC-UV (Step 1).

    The data shown here used an Applied Biosystems 4000 Q TRAP instrument, considered to be very sensitive when detecting in MRM mode (1–10 pg on column of oxidized phospholipids). When setting up these procedures using a different platform, careful examination of the standard curve will determine the best range for analysis of the lipids, and in some cases, a lower sensitivity may be apparent. If this is the case, adjustment of standard amounts in the curves may be warranted to cover a different range of analyte concentrations than we present here (Step 15).

As internal standards, we typically use di-14:0-PE and PC as we have determined that they are absent in samples (Step 7). If different cells or tissues are to be analyzed, for example, plants, fish or other organisms, then a lipid extract should first be checked using MS to ensure that the internal standard is not present as a constituent lipid. If it is, then a different/absent one should be chosen instead. It is important not to use one that differs substantially, e.g., as in the case of markedly different fatty acid chain lengths, as this may extract with different efficiency to the analyte. If using different internal standards from those listed here, their extraction efficiency should be determined and compared with the analytes of interest to ensure that they are comparable.

11. If preferred, the more unstable hydroperoxides can be reduced to the alcohols, by addition of 10 µl $SnCl_2$ (100 mM in water) per ml sample, for 10 min at room temperature before extraction.

12. Add the sample to a glass extraction tube containing 2.5 ml lipid extraction solvent per 1 ml sample. Cap and vortex vigorously, and then add 2.5 ml hexane. Vortex and centrifuge (260g, 5 min), and then recover the lipids using a glass Pasteur pipette by collecting the upper hexane layer. (*CRITICAL STEP: Extraction is greatly enhanced by vigorous vortexing, for at least 30 s per sample, as well as 30 s manual shaking of the sample before centrifugation*)

13. Re-extract the lower aqueous layer with additional 2.5 ml hexane. Vortex, centrifuge as above and collect the upper hexane layer. Combine this with the first hexane extraction.

14. Dry the combined hexane layers using either nitrogen or a vacuum dryer with warming not greater than 30 °C, then resuspend in 100 µl mobile phase (50:50 solvents A and B as listed in Step 12) and store under nitrogen at −80 °C until analysis by LC/MS/MS, preferably within a week of generating the samples.
    CRITICAL STEP: Resuspend carefully, taking care to recover all lipids from the side of the vial. This is important for recovery. If samples appear cloudy or contain precipitate, this must be removed before LC/MS/MS. Centrifugation or spinning through 0.2-µm filters (e.g., Corning Spin-X filter) will be required. If this is not done, samples may clog the LC column and cause pressure buildup.

    PAUSE POINT: Samples can be stored before analysis, but this time should be kept as short as possible to minimize decay of oxidized lipids. In particular, when analysing hydroperoxy lipids in complex cell extracts, we recommend that samples be analysed immediately after extraction.

Reverse-phase LC/MS/MS of esterified eicosanoids

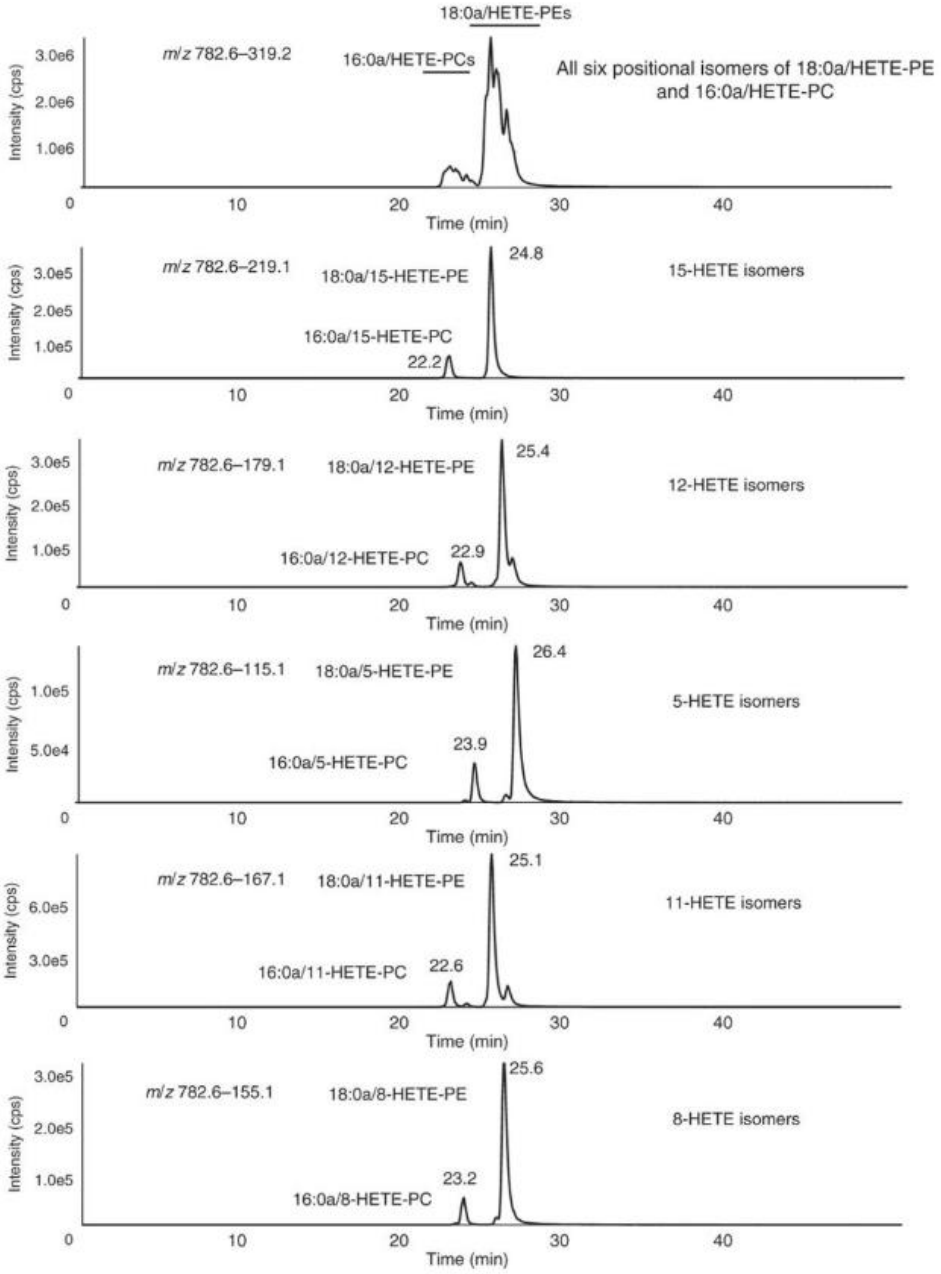
15. Analyse samples using reverse-phase chromatography. We use a Luna 3 µm C18 (2) 150 mm × 2 mm column (Phenomenex) with a gradient of 50–100% solvent B in solvent A over 10 min followed by 30 min at 100% solvent B (solvent A: methanol:acetonitrile:water, 1 mM ammonium acetate, 60:20:20; solvent B: methanol, 1 mM ammonium acetate) with a flow rate of 200 µl min^{-1}.
16. On our LC/MS/MS instrument (Applied Biosystems 4000 Q TRAP), the typical optimum system settings to determine esterified HETEs are: declustering potential −140 V, collision energy −45 V. However, this will differ on other models and other manufacturers' instruments and needs to be determined by investigators according to the standard protocols for their instrument. It is important that the user tunes the instrument separately for each analyte and standard (including collision energy) to be measured, to obtain the optimum settings for analysis.
17. MRM transitions to monitor and retention times these lipids on our system are shown in Table 2. Figures 5,6,7,8 show the LC/MS/MS elution of positional isomer standards for HETE, HDOHE, HODE and KETE-PE lipids analysed using this method.

    CRITICAL STEP: It is essential to include a number of blanks (typically methanol), especially at the start and end of each batch, to monitor for carryover. If carryover occurs, extensive wash steps between samples may need to be introduced. See EQUIPMENT SETUP for further information on our washing procedure. Timing: 50 min per sample

Table 2: Precursor and product ions, and retention time of some esterified HETEs, HODEs, HDOHEs and KETE standards.

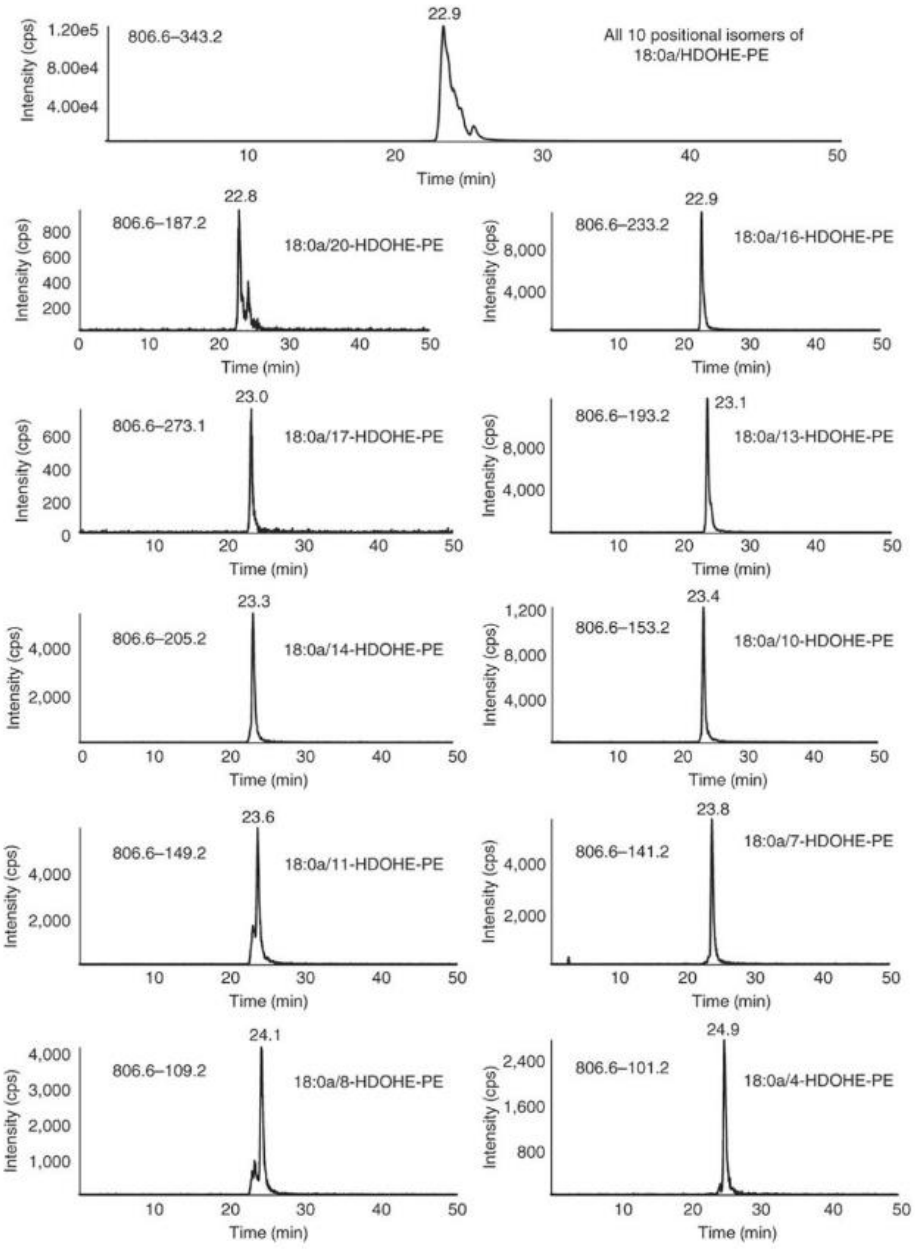
Lipid name	Precursor *m* /*z*	Product *m* /*z*	Retention time (min)
18:0a/HETE-PE	782.6	319.2	24.5-26.5
18:0a/15-HETE-PE	782.6	219.1	24.8
18:0a/12-HETE-PE	782.6	179.1	25.4
18:0a/5-HETE-PE	782.6	115.1	26.4
18:0a/11-HETE-PE	782.6	167.1	25.1
18:0a/8-HETE-PE	782.6	155.1	25.6
16:0a/HETE-PC	782.6	319.2	22–24
16:0a/15-HETE-PC	782.6	219.1	22.2
16:0a/12-HETE-PC	782.6	179.1	22.9
16:0a/5-HETE-PC	782.6	115.1	23.9
16:0a/11-HETE-PC	782.6	167.1	22.6
16:0a/8-HETE-PC	782.6	155.1	23.2
18:0a/HDOHE-PE	806.6	343.2	22–25
18:0a/20-HDOHE-PE	806.6	187.2	22.8
18:0a/17-HDOHE-PE	806.6	273.2	23
18:0a/14-HDOHE-PE	806.6	205.2	23.3
18:0a/11-HDOHE-PE	806.6	149.2	23.6
18:0a/8-HDOHE-PE	806.6	109.2	24.1
18:0a/16-HDOHE-PE	806.6	233.2	22.9
18:0a/13-HDOHE-PE	806.6	193.2	23.1
18:0a/10-HDOHE-PE	806.6	153.2	23.4
18:0a/7-HDOHE-PE	806.6	141.2	23.8
18:0a/4-HDOHE-PE	806.6	101.2	24.9
16:0a/HODE-PE	730.6	295.2	20.6
16:0a/13-HODE-PE	730.6	171.1	20.6
16:0a/9-HODE-PE	730.6	195.1	20.6
18:0a/15-KETE-PE	780.6	317.1	23.8

Figure 4: LC/MS/MS of a mixture of standards comprising six positional isomers of 16:0a/HETE-PC and 18:0a/HETE-PE.



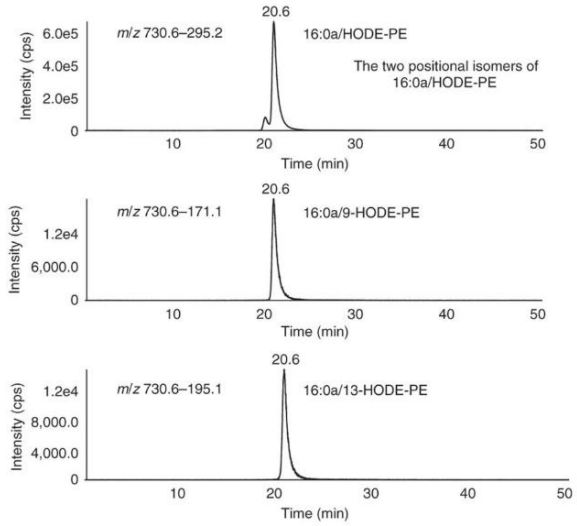
All isomers have m/z 782.6. HETE-PE and -PC lipids were generated by air oxidation, as described in Step 1B, and purified by reverse-phase HPLC. A sample containing both HETE-PEs and -PCs was separated using LC/MS/MS as described in Steps 12–14. Elution was monitored by either m/z 782 → 319 (all positional isomers) or product ions specific for each positional isomer.

Figure 5: LC/MS/MS of a mixture of standards comprising 10 positional isomers of 18:0a/HDOHE-PE.



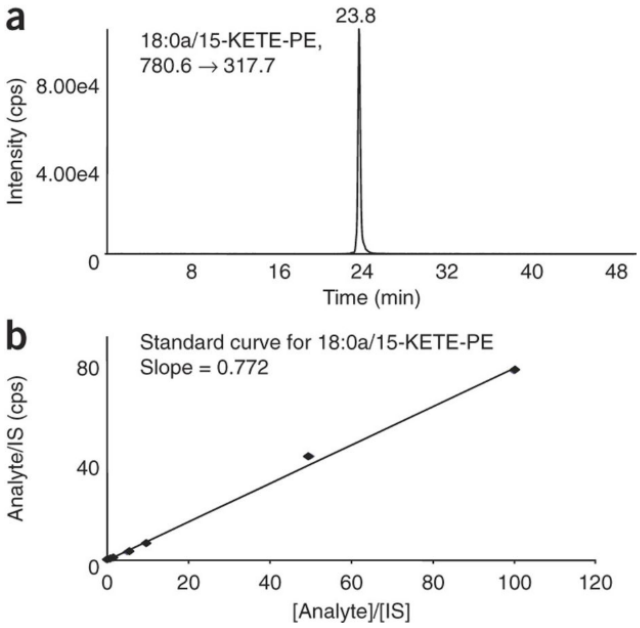
All isomers have m/z 806.6. HDOHE-PE lipids were generated by air oxidation, as described in Step 1B, and purified by reverse-phase HPLC. A sample was separated using LC/MS/MS as described in Steps 12–14. Elution was monitored by either m/z 806 → 343 (all positional isomers) or product ions specific for each positional isomer.

Figure 6: LC/MS/MS of a mixture of standards comprising two positional isomers of 16:0a/HODE-PE.



Isomers have m/z 730.6. HODE-PE lipids were generated by air oxidation, as described in Step 1B, and purified by reverse-phase HPLC. A sample was separated using LC/MS/MS as described in Steps 12–14. Elution was monitored by either m/z 730 → 295 (all positional isomers) or product ions specific for each positional isomer.

Figure 7: LC/MS/MS and standard curve for 18:0a/15-KETE-PE.



(a) LC/MS/MS elution profile of KETE-PE standard, showing single peak at 23.8 min. (b) A standard curve was created by LC/MS/MS analysis of a fixed amount of DMPE with varying amounts of 15-KETE-PE.

Standard curves and quantitation

18. Generate a standard curve by serial dilution of primary esterified eicosanoid analytes (A) (e.g., HETE-PE, KETE-PE, HETE-PC) in methanol that contains an equal amount of internal standard (IS) per vial (e.g., DMPE, DMPC). We typically work with a range that gives the following amounts: 10 ng, 5 ng, 1 ng, 500 pg, 100 pg, 50 pg, 10 pg, 5 pg, 1 pg primary standard per 10 µl injection, with 100 pg internal standard.

19. *When using mixtures of positional isomers, amounts of each will be slightly different and need to be accounted for during quantitation.*

20. Analyse 10 µl of each standard using reverse-phase LC/MS/MS as described above for esterified eicosanoids.

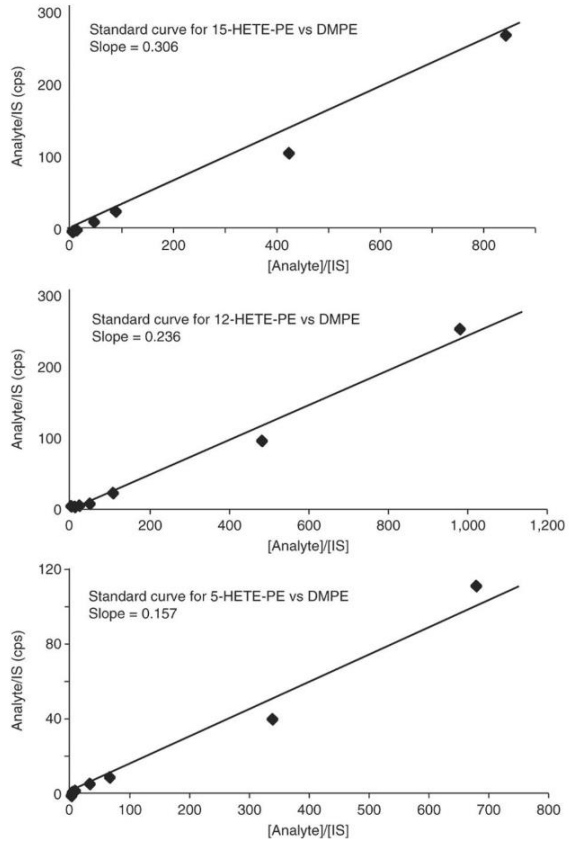
21. Integrate peaks for all lipids. Calculate the ratio of A:IS for ng amounts of lipid in each standard vial and for A:IS in counts per second (c.p.s.) for each standard run. Plot A:IS (c.p.s.) versus A:IS (ng) as shown by examples in Figures 8 and 9. Calculate slope for each analyte.

22. For all samples, analyse 10–20 µl (depending on sensitivity), then integrate the area and use the following equation to determine amounts of each analyte in all samples:

23. $$A_{ng} = (A_{cps} / IS_{cps})(I_{ng} / \text{slope})$$

24. Data are given as ng per sample extracted and should then be normalized per cell number or tissue wet weight.

Figure 8: Generation of standard curves for 5-, 12- and 15-HETE-PE isomers.



Standard curves were created by adding a fixed amount of DMPE to varying amounts of the standard HETE-PE positional isomer mixture, generated by air oxidation, and analysed using LC/MS/MS as described in Steps 12–14.