Supplementary Material

For

Isolevuglandins covalently modify phosphatidylethanolamines in vivo: detection and quantitative analysis of hydroxylactam adducts

by

Wei Li, James M. Laird, Liang Lu, Sanjoy Roychowdhury, Laura E. Nagy, Rong Zhou, John W. Crabb, and Robert G. Salomon

Figure S1

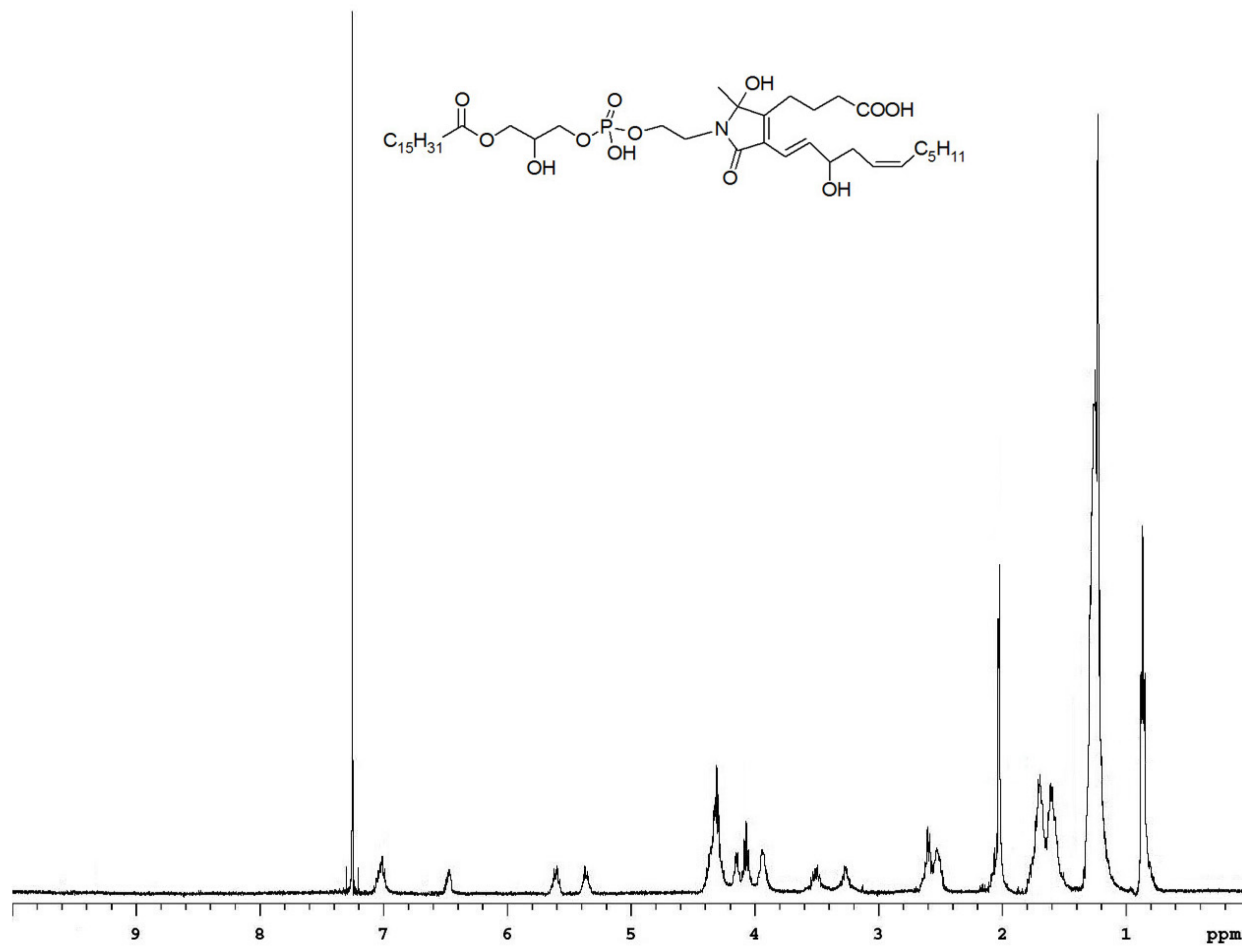


Figure S1 ^{1}H NMR spectrum (400 MHz in $CDCl_3$) of hexadecanoic acid 3-([2-[2-hydroxy-3-(3-carboxy-propyl)-4-((1E,5Z)-3-hydroxyundeca-1,5-dienyl)-2-methyl-5-oxo-2,5-dihydro-1H-pyrrol-1yl]--ethoxy]-hydroxyphosphoryloxy)-2-hydroxy-propyl ester

Figure S2

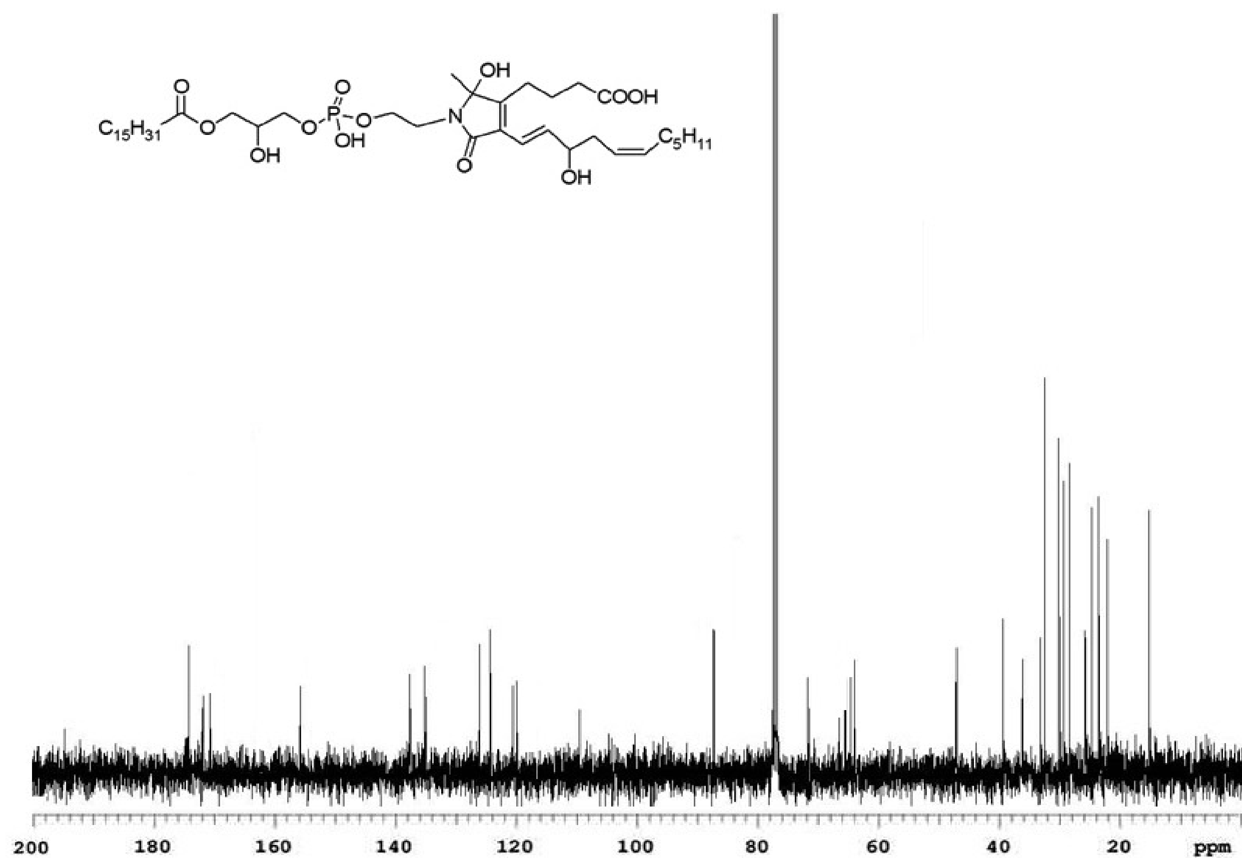


Figure S2 ^{13}C NMR spectrum (100 MHz in $CDCl_3$) of hexadecanoic acid 3-([2-[2-hydroxy-3-(3-carboxy-propyl)-4-((1E,5Z)-3-hydroxyundeca-1,5-dienyl)-2-methyl-5-oxo-2,5-dihydro-1H-pyrrol-1yl]-ethoxy]-hydroxyphosphoryloxy)-2-hydroxy-propyl ester

Figure S3

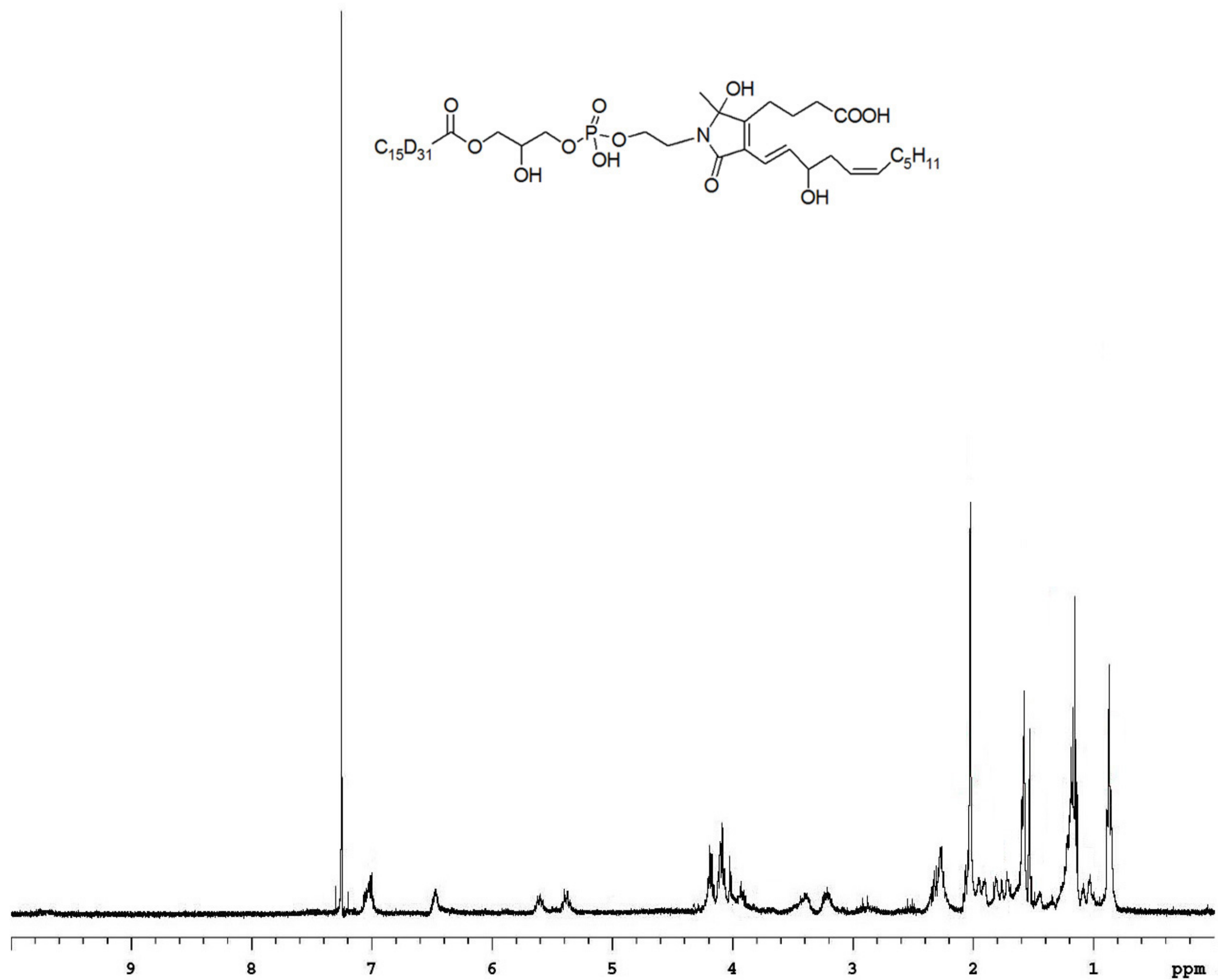


Figure S3 ^{1}H NMR spectrum (400 MHz in $CDCl_3$) of D31-hexadecanoic acid 3-([2-[2-hydroxy-3-(3-carboxy-propyl)-4-((1E,5Z)-3-hydroxyundeca-1,5-dienyl)-2-methyl-5-oxo-2,5-dihydro-1H-pyrrol-1yl]-ethoxy]-hydroxyphosphoryloxy)-2-hydroxy-propyl ester

Figure S4

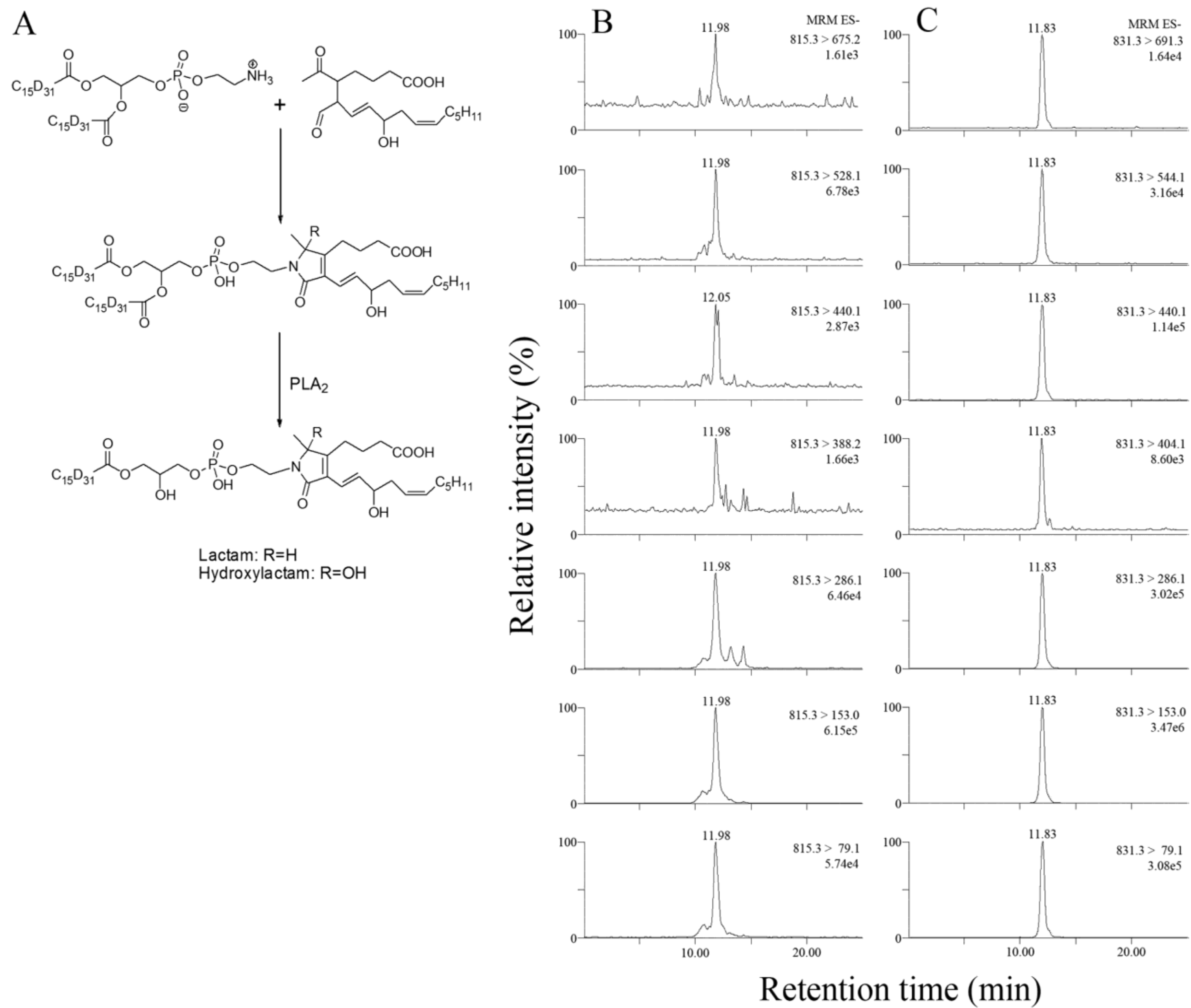
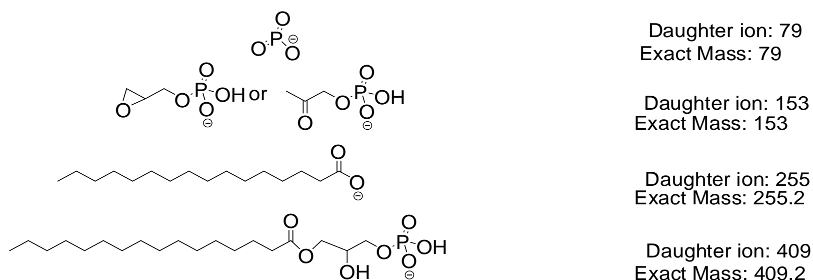


Figure S4 (A) Synthetic rout of isotope labeled lactam and hydroxylactam adducts of iso[4]LGE_2 and lysoPE; (B) Representative MRM spectrum of isotope labeled lactam adducts of iso[4]LGE_2 and lysoPE; (C) Representative MRM spectrum of isotope labeled hydroxylactam adducts of iso[4] LGE_2 and lysoPE

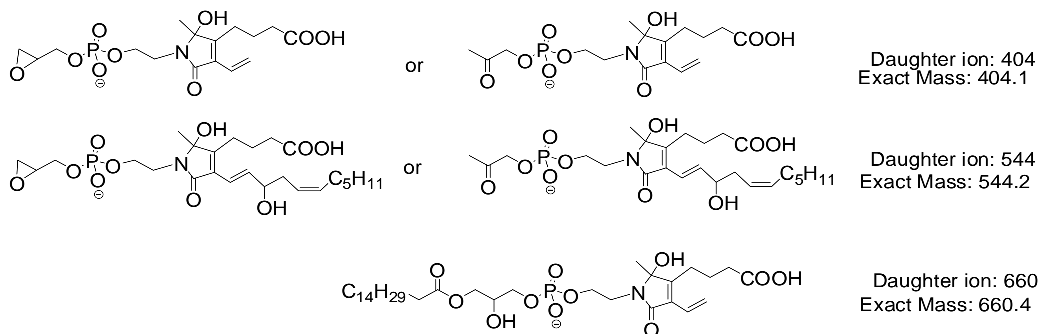
Figure S5

Fragments with PE part (for all adducts)

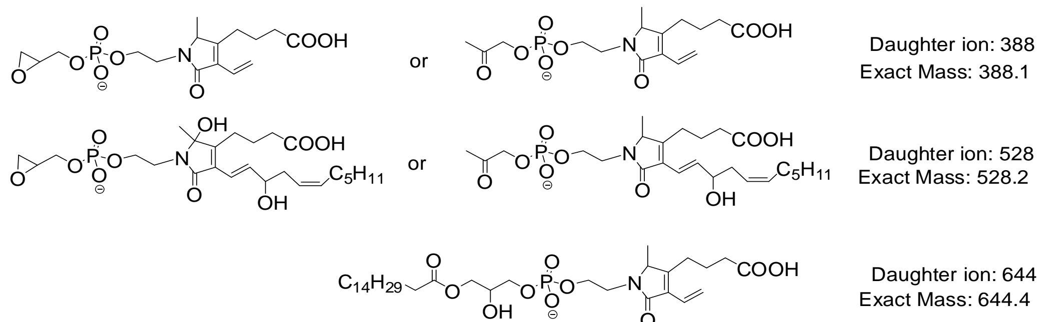


Fragments with iso[4]LGE$_2$ part

Hydroxylactam adduct



Lactam adduct



Pyrrole adduct

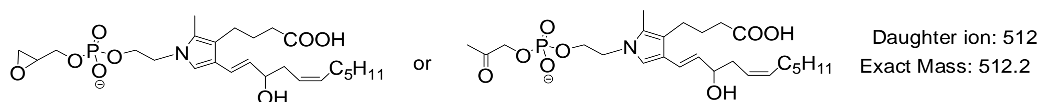
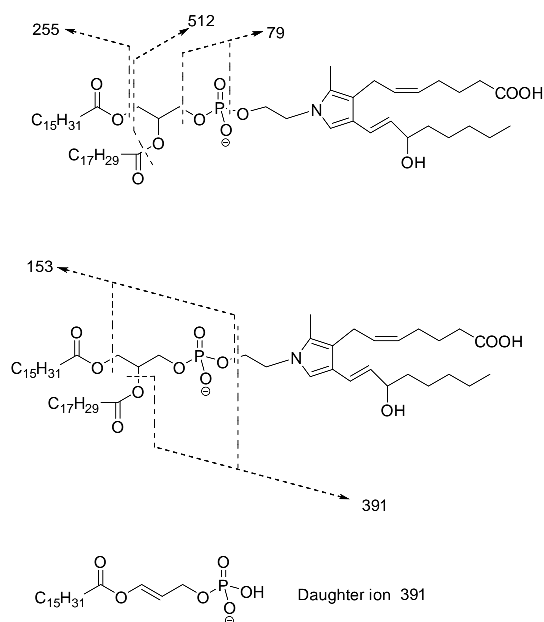


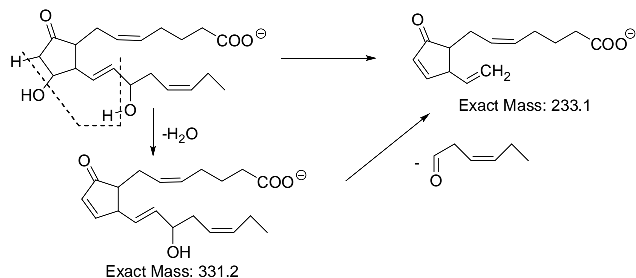
Figure S5 Putative fragmentation assignment of pyrrole, lactam and hydroxylactam adducts of iso[4]LGE$_2$ and 2-lysoPE.

Figure S6

Bernoud-Hubac, N. et al. Free Radical Biology & Medicine (2004)37:1604-1611



Lu, Y., Hong, S., Yang, R., Uddin, J., Gotlinger, K. H., Petasis, N. A., and Serhan, C. N. (2007) *Rapid Commun Mass Spectrom* **21**, 7-22



Murphy, R. C., Fiedler, J., and Hevko, J. (2001) *Chemical Reviews* **101**, 479-526

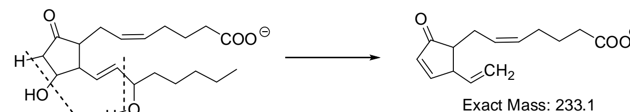


Figure S6 Proposed fragmentation mechanisms of the literatures cited for the explanation of the precedent ions at m/z 79, 153, 255, 512 and those unprecedented daughter ions at m/z 409, 528, 544, 628, 660 which have analogous fragmentation mechanism to the literatures.

Figure S7

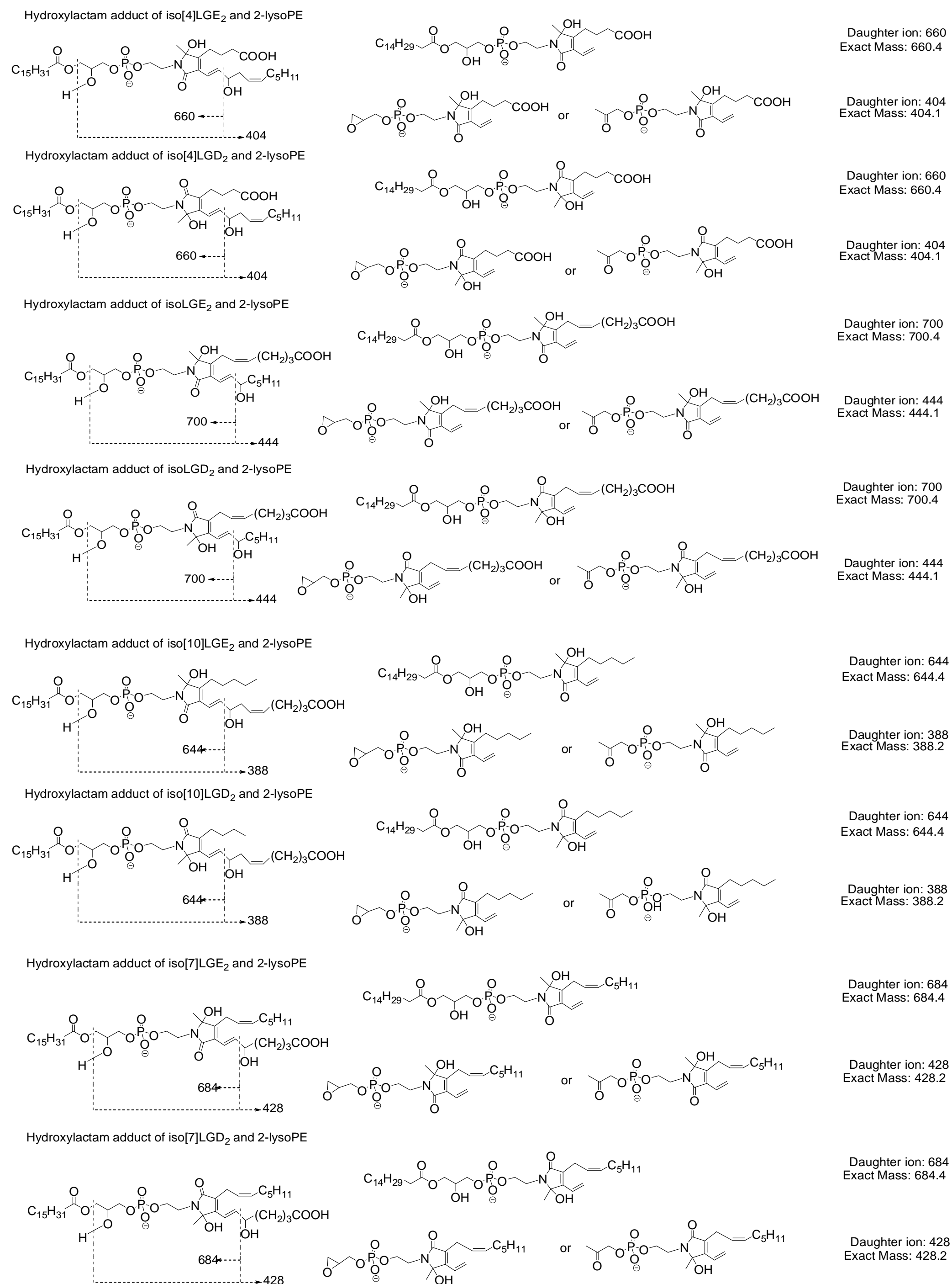


Figure S7 Proposed structurally specific fragmentation of different hydroxylactam adducts of 8 isoLG regioisomers and 2-lysoPE.

Figure S8

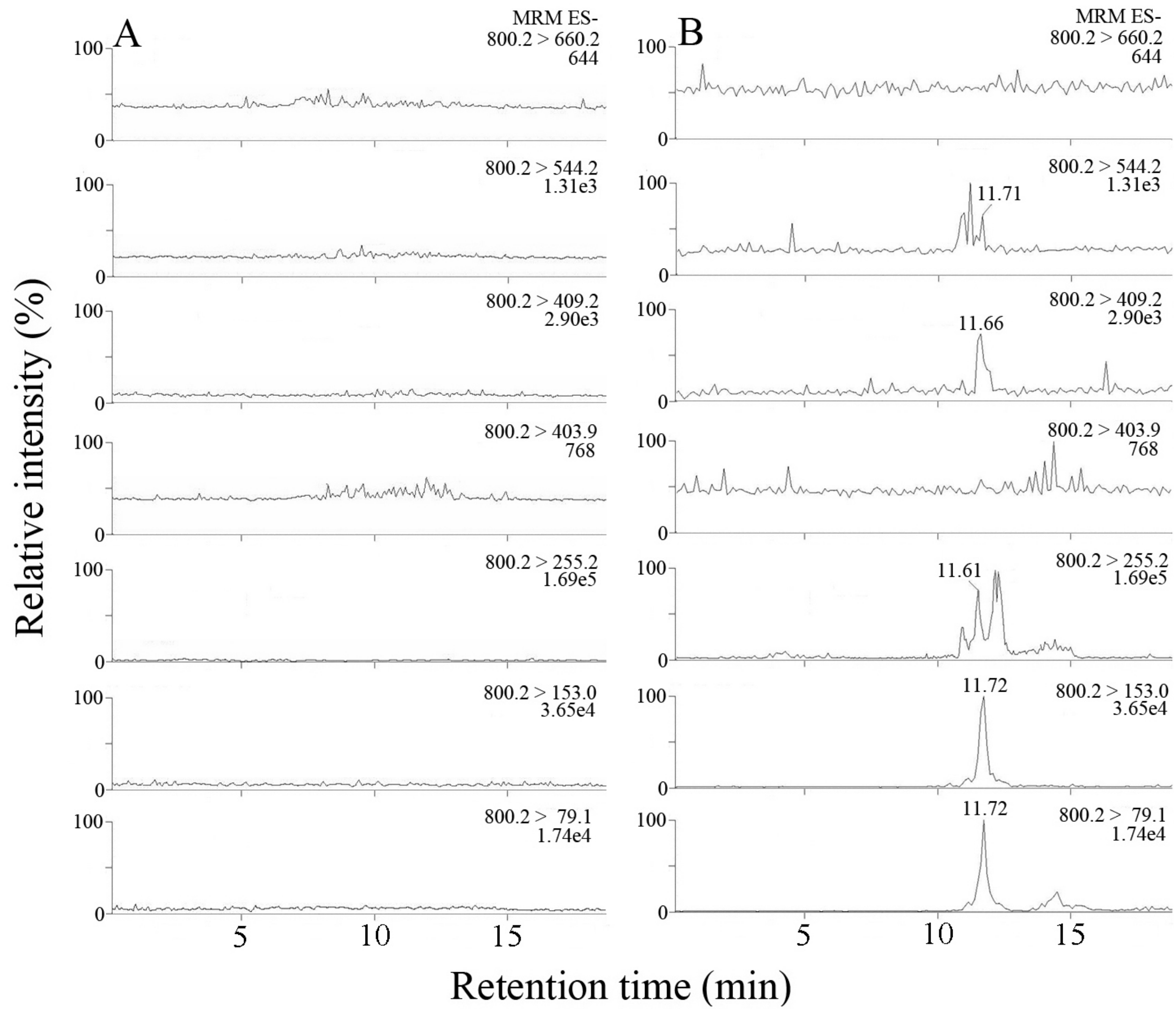


Figure S8 LC/ESI/MS/MS analysis of putative isoLG-lysoPE-hydroxylactam adducts in lipid extract from human plasma samples: (A) before PLA_2 treatment; (B) after PLA_2 treatment.

Figure S9

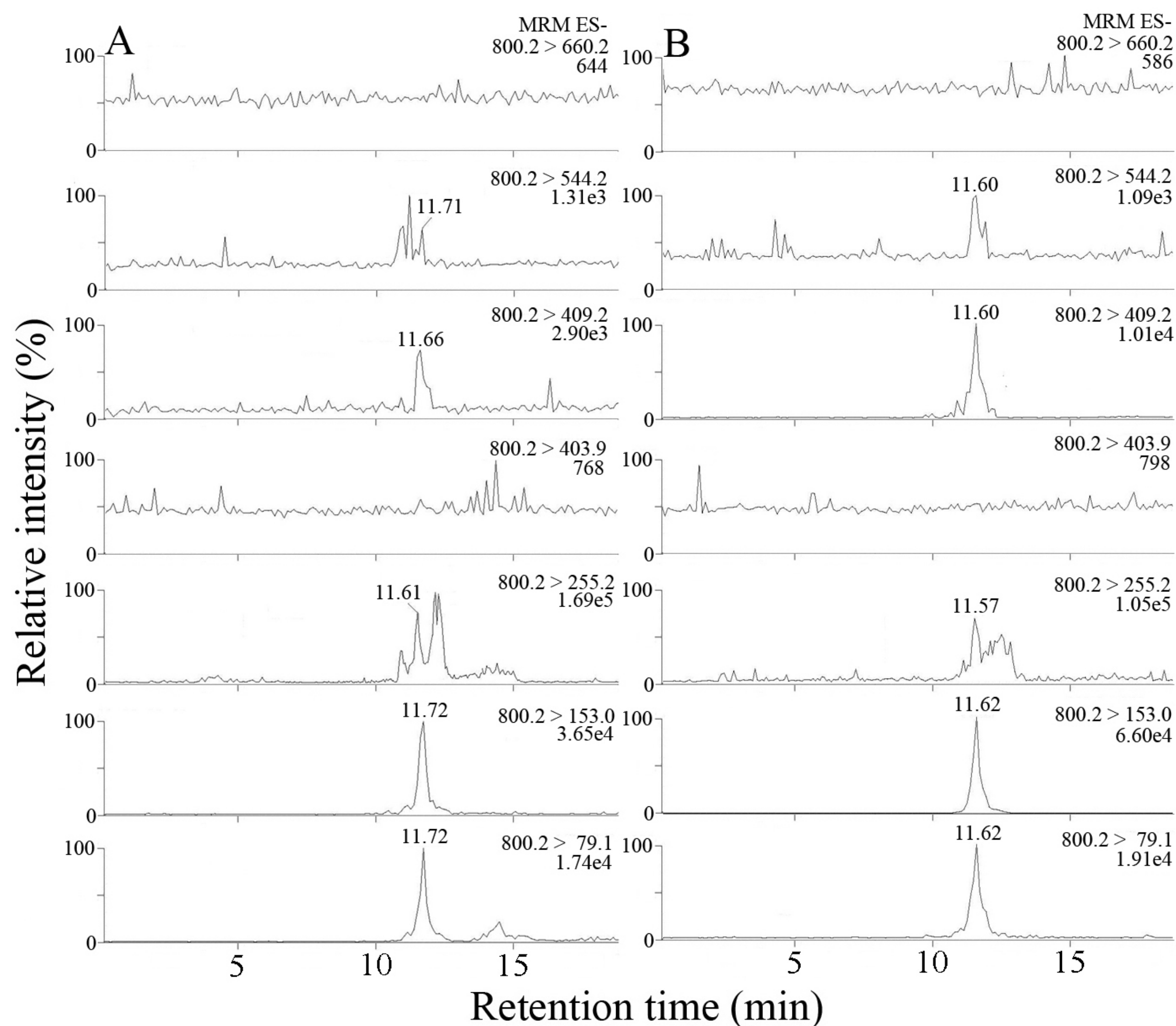


Figure S9 LC/ESI/MS/MS analysis of isoLG-lysoPE-HL adducts in human plasma samples: (A) putative isoLG-lysoPE-HL adducts in lipid extract from human plasma with PLA_2 treatment; (B) synthetic standard of isoLG-lysoPE-HL adducts was spiked into blank human plasma (without PLA_2 treatment).

Table S1 Calculation of factor of isotope labeled internal standard correction

Parent ion/Daughter ion	Peak intensity of isotope labeled hydroxylactam adducts (internal standard) in the LC/MS/MS run of the experiment Fig. 3C	Peak intensity of isotope labeled hydroxylactam adducts (internal standard) in the LC/MS/MS run of the experiment Fig. 5B	Factor of isotope internal standard correction for the peak intensity of various transition in Figure 5B
800>79	1.34e05	3.85e05	2.87
800>153	9.65e05	5.71e05	0.59
800>255	4.85e05	2.25e05	0.46
800>404	3.42e04	1.85e04	0.54
800>409	8.64e04	6.98e04	0.81
800>544	2.46e04	2.76e04	1.12
800>660	1.44e04	6.04e03	0.42

Table S2 Comparison between the peak intensity of hydroxylactam adduct in Figure 3C and peak intensity of hydroxylactam adduct in Figure 5B with internal standard correction

Parent ion/Daughter ion	Peak intensity of hydroxylactam adduct in Figure 3C	Peak intensity of hydroxylactam adduct in Figure 5B without internal standard correction	Peak intensity of hydroxylactam adduct in Figure 5B with internal standard correction
800>79	1.46e05	5.73e05	1.99e05
800>153	9.96e05	8.68e05	1.47e06
800>255	5.02e05	3.25e05	7.01e05
800>404	3.61e04	2.65e04	4.90e04
800>409	9.24e04	1.04e05	1.29e05
800>544	2.66e04	3.74e04	3.33e04
800>660	1.56e04	8.45e03	2.01e04

Table S3 Comparison between ratios of peak intensities of various transition to those of 800>153 in Figure 3C, ratio of peak intensities of various transition (without internal standard correction) to those of 800>153 in figure 5B and ratio of peak intensities of various transition (with internal standard correction) to those of 800>153 in figure 5B.

Parent ion/Daughter ion	Ratio of peak intensities of various transition to those of 800>153 in Figure 3C	Ratio of peak intensities of various transition (without internal standard correction) to those of 800>153 in figure 5B	Ratio of peak intensities of various transition (with internal standard correction) to those of 800>153 in figure 5B
800>79	0.15	0.66	0.14
800>153	1.00	1.00	1.00
800>255	0.50	0.37	0.48
800>404	0.04	0.03	0.03
800>409	0.09	0.12	0.09
800>544	0.03	0.04	0.02
800>660	0.02	0.01	0.01

Table S4 Calculation of factor of isotope labeled internal standard correction

Parent ion/Daughter ion	Peak intensity of isotope labeled hydroxylactam adducts (internal standard) in the LC/MS/MS run of the experiment Fig. 3C	Peak intensity of isotope labeled hydroxylactam adducts (internal standard) in the LC/MS/MS run of the experiment Fig. 6A	Factor of isotope internal standard correction for the peak intensity of various transition in Figure 6A
800>79	1.34e05	8.72e04	0.65
800>153	9.65e05	1.74e05	0.18
800>409	8.64e04	1.53e04	0.18

Table S5 Comparison between the peak intensity of hydroxylactam adduct in Figure 3C and peak intensity of hydroxylactam adduct in Figure 6A with internal standard correction

Parent ion/Daughter ion	Peak intensity of hydroxylactam adduct in Figure 3C	Peak intensity of hydroxylactam adduct in Figure 6A without internal standard correction	Peak intensity of hydroxylactam adduct in Figure 6A with internal standard correction
800>79	1.46e05	1.74e04	2.67e04
800>153	9.96e05	3.65e04	2.02e05
800>409	9.24e04	2.90e03	1.64e04

Table S6 Comparison between ratios of peak intensities of various transition to those of 800>153 in Figure 3C, ratio of peak intensities of various transition (without internal standard correction) to those of 800>153 in figure 6A and ratio of peak intensities of various transition (with internal standard correction) to those of 800>153 in figure 6A.

Parent ion/Daughter ion	Ratio of peak intensities of various transition to those of 800>153 in Figure 3C	Ratio of peak intensities of various transition (without internal standard correction) to those of 800>153 in figure 6A	Ratio of peak intensities of various transition (with internal standard correction) to those of 800>153 in figure 6A
800>79	0.15	0.48	0.13
800>153	1.00	1.00	1.00
800>409	0.09	0.08	0.08

PROTOCOL

MATERIALS

REAGENTS

- 1,2-Dipalmitoyl-D62-*sn*-glycero-3-phosphoethanolamine (Avanti Polar Lipids, Inc., cat. no. 860372)
- 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE, Avanti Polar Lipids, Inc., cat. no. 850705)
- Palmitoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (Avanti Polar Lipids, Inc., cat. no. 856705)
- Phospholipase A_2: from porcine pancreas. min.10,000 unit/mL (Sigma-Aldrich, cat. no. P0861)
- Acetic acid, glacial (Sigma-Aldrich, 99.99+%, cat. no. 338826)
- Ammonium acetate (Sigma-Aldrich, 99.999%, cat. no. 372331)
- Butylated hydroxytoluene (Sigma-Aldrich, 99+%, cat. no.W218405)
- Calcium chloride (Fisher Scientific, cat. no.C79-500)
- Ethylenediamine tetraacetic acid (EDTA, Fisher Scientific, cat. no.E478-500)
- Triethylamine (Sigma-Aldrich, 99.5%, cat. no. 47128-3)
- 1.5% sodium chloride: 1.5 g NaCl in 100 mL of distilled water
- 50 mM of calcium chloride: 367.5 mg $CaCl_2.2H_2O$ (FW 147.02 g/mol) in 50 mL of water.
- Phosphate-buffered saline (PBS) from phosphate buffered saline tablet: 1 (Sigma, cat. no. 79382), dissolve 1 tablet in 200 mL of water to obtain 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4 at 25 °C (store solution at 0-5 °C).
- Water (distilled and 10 mΩ filtered, Milli-Q™ water system)
- Methanol HPLC grade (Fisher Scientic, cat. no. A452SK-4)
- Chloroform HPLC grade (Fisher Scientic, cat. no. C606SK-4)
- Solvent M: chloroform:methanol:water (60:35:5, v/v/v) 100 mL (60 mL chloroform, 35 mL methanol and 5 mL distilled water)
- Solvent S: chloroform:methanol (50:50, v/v)
- Bligh & Dyer solution with BHT (chloroform-methanol 1:2, v/v with 1 mM butylated hydroxytoluene): dissolve 26 mg of butylated hydroxytoluene in 40 mL of chloroform and 80 mL methanol
- Folch solution with BHT (chloroform-methanol (2:1, v/v) with 1 mM butylated hydroxytoluene): dissolve 26 mg of butylated hydroxytoluene in 80 mL of chloroform and 40 mL of methanol.
- HPLC Solvent 1: water/methanol (50:50 v/v), 1mM ammonium acetate
- HPLC solvent 2: water/methanol (5:95 v/v)
- HPLC Solvent 3: methanol, 2 mM ammonium acetate
- HPLC Solvent 4: water, 2 mM ammonium acetate

EQUIPMENT

- Flat top microcentrifuge tube-1.5 mL (Fisher Scientific, cat. no. 05-408-129)
- Polyethylene transfer pipette (Fisher Scientific, cat. no. 13-711-31)
- Polypropylene centrifuge tube-15 mL (Fisher Scientific, cat. no. 14-959-70C)
- Sonicator (Fisher Scientific, FS20, Ultrasonic Cleaners)
- Nitrogen evaporator (Organomation Associates, Inc., N-EVAP™ III)
- Tabletop centrifuge (Beckman, Accuspin FR™)
- HPLC column: (analytical column, Phenomenex, Luna 5µ C18 (2), 250 x 6 mm, 00G-4252-E0)
- HPLC column: (analytical column, Phenomenex, Luna 5µ C18 (2), 150 x 2.0 mm, 00F-3300-B0)
- HPLC column: (semi-preparative column, Phenomenex, Luna 5µ C18 (2), 250 x 10 mm, 00G-4252-E0)
- HPLC instrument: model 600 Controller, 717 Plus Autosampler, 2790 HPLC (Waters, Milford, MA)
- Detector: Evaporative Light Scattering (ELS), SEDERE Inc., SEDEX75
- HPLC coupled to a triple-quadrupole mass spectrometer (Micromass Quattro Ultima)
- Retsch Qiagen TissueLyser Universal Laboratory Mixer-Mill Disrupter (Qiagen)

EQUIPMENT SET-UP

HPLC set up

- Inlet line for Solvent A is placed into Solvent 1 and inlet line for Solvent B is placed in Solvent 2. The instrument is wet-primed with solvent A at 4 mL/min for 5 min.
- The HPLC column is connected to the outlet line from the HPLC and then connected to the inlet line of the ELS detector.
- The column is equilibrated for 15 minutes running at 2 mL/min at 100% Solvent A.
- The gradient is programmed to run at 2 mL/min as follows:

  0-10 min A/B 100:0

  10-20 min a linear gradient from 100:0 to 50:50

  20-45 min A/B 50:50

  45-46 min a linear gradient from 50:50 to 0:100

  47- 77 min A/B 0:100

  77-78 min a linear gradient from 0:100 to 100:0

  78-90 min A/B 100:0
- Fractions containing hydroxylactam adducts are collected from 59 to 61 min.

Mass spectrometer tuning

To optimize the MS parameters, direct infusion was performed on a Quattro Ultima triple-quadrupole mass spectrometer interfaced HPLC system. The total ion current was measured in the mass range of m/z 50 - 900 at 30 V of cone energy in the negative ion mode.

- MS pump Solvent A inlet line is placed into Solvent 3 and inlet line for Solvent B placed in Solvent 4.
- The instrument is wet-primed for 5 minutes each at 4 mL/min with mixture of 50% solvent A and 50% Solvent B.
- A syringe (500μl, Hamilton) is washed with methanol 3 times, and filled with methanol as solvent control, or with sample.
- The syringe is connected with tubing from the outlet line of the HPLC and then connected to the inlet of the electrospray ionization source, at 20 μl/min
- Operate the mass spectrometer in the negative ion mode
- Optimize MS parameters to maximize the intensity of the parent ion peak, m/z 800.2, in the full scan mode, with the mass range of m/z 50-900.
- Activate collision gas, and vary the collision energy to maximize the intensity of daughter ion, in the daughter ion scan mode.

LC/MS/MS set-up

- MS pump Solvent A inlet line is placed into Solvent 3 and inlet line for Solvent B placed in Solvent 4.

  The instrument is wet-primed for 5 minutes each at 4 mL/min with mixture of 50% solvent A and 50% Solvent B.

- The column is equilibrated for 15 minutes running at 200 µl/min with the mixture of 75% solvent A and 25% Solvent B.
- The Phenomenex Luna C18 column (150 x 2.0 mm i.d., 5-µm particle size) is connected with tubing from the outlet line of the HPLC and then connected to the inlet of the electrospray ionization source.
- The gradient is programmed to run at 200 µl /min as follows:

  0-10 min a linear gradient from A/B 75:25 to 100:0

  10-17 min A/B 100:0

  17-17.5 min a linear gradient from A/B 100:0 to 75:25

  17.5-25 min A/B 75:25
- Operate the mass spectrometer in the negative ion mode.
- Set the capillary voltage to 3.5 kV, the cone voltage to 90 v, source temperature to 120 °C, the desolvation temperature to 250 °C, the cone gas flow to 65 L/h and desolvation gas flow to 658 L/h.
- Set the multiple reaction monitoring (MRM) parameters as follows:

  iso[4]LGE_2-lyso-PE-hydroxylactam, m/z 800.2-153@ 45eV

  iso[4]LGE_2-D31-lyso-PE-hydroxylactam, m/z 831.2-153@ 45eV

REAGENT SET-UP

Preparation of internal standard (iso[4]LGE_2-D31-lyso-PE-hydroxylactam)

TIMING 1 h workup + 12h incubation+ PLA_2 enzyme hydrolysis overnight + 1.5 h HPLC run time

1. Synthesis of iso[4]LGE_2-1,2-pamitoyl-D62-PE-hydroxylactam
   a. Prepare a 10 mM solution of 1,2-dipalmitoyl-D62-*sn*-glycero-3-phosphoethanolamine by adding 3.77 mg (5 µmol) of deuterated reagent to 500 µL of solvent M. Prepare a 10 mM solution of synthetic iso[4]LGE_2 by adding 1.76 mg (5 µmol) iso[4]LGE_2 to 500 µL of solvent M. Combine the two reagents in a vial, and the total volume is 1 mL.
   b. Add 5 µL triethylamine (0.05 mmol) to the mixture solution and vortex for 1 min.
   c. Place a magnetic stirring bar in the vial, and stir the mixture solution for 12 h at room temperature
   d. Extract the reaction mixture as follow by 3 times: add 1.5 mL binary solvent (chloroform: methanol 2:1v/v). After vortexing 1 min and centrifugation at 3000 rpm for 10 min, the lower phase is collected with a polyethylene transfer pipette.
   e. Evaporate the organic solvent by gently blowing argon gas over the sample.
2. Preparation of iso[4]LGE_2-D31-lyso-PE-hydroxylactam by PLA_2-catalyzed hydrolysis
   a. Put 900 µL of PBS (10mM, pH 7.4) in a 3.7 mL glass vial.
   b. Add 100 µL of 50 mM $CaCl_2$ solution.
   c. Add 100 µL of iso[4]LGE_2-1,2-pamitoyl-D62-PE-hydroxylactam (50 mg/mL in methanol, estimated concentration).
   d. Sonicate for 10 min to dissolve the hydroxylactam adducts in the PBS solution.
   e. Add 100 µL of PLA_2 (1000 units).
   f. Incubate the mixture solution under argon at 37 °C overnight.

g. Extract the reaction mixture as follow: add 1.5 mL binary solvent (chloroform: methanol 2:1 v/v). After 1 min of vortex and centrifugation at 3000 rpm for 10 min, the lower phase is collected with a polyethylene transfer pipette.

h. Evaporate the organic solvent by gently blowing argon gas over the sample.

3. Separate the iso[4]LGE$_2$-D31-lyso-PE-hydroxylactam from iso[4]LGE$_2$-D31-lyso-PE-lactam using HPLC.
4. Make aliquots of the purified samples (20 ng/vial, for analysis of 10 samples) and store them at –80 °C until use for LC/MS analysis.

Preparation of authentic standard (iso[4]LGE$_2$-lyso-PE-hydroxylactam)

TIMING 1 h workup + 12h incubation+ 1.5 h HPLC run time

1. Synthesis of iso[4]LGE$_2$-1,2-pamitoyl-D62-PE-hydroxylactam
   a. Prepare a 10 mM solution of 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (lyso-PE) by adding 2.27 mg (5 µmol) of deuterated reagent to 500 µL of solvent M. Prepare a 10 mM solution of synthetic Iso[4]LGE$_2$ by adding 1.76 mg (5 µmol) iso[4]LGE$_2$ to 500 µL of solvent M. Combine the two reagents in a vial, and the total volume is 1mL.
   b. Add 5 µL triethylamine (0.05 mmol) to the mixture solution and vortex for 1 min.
   c. Place a magnetic stirring bar in the vial, and stir the mixture solution for 12 h at room temperature
   d. Extract the reaction mixture as follow by 3 times: add 1.5 mL binary solvent (chloroform: methanol 2:1v/v). After 1 min of vortex and centrifugation at 3000 rpm for 10 min, the lower phase is collected with a polyethylene transfer pipette.
   e. Evaporate the organic solvent by gently blowing argon gas over the sample.
2. Separate the iso[4]LGE$_2$-lyso-PE-hydroxylactam from iso[4]LGE$_2$-lyso-PE-lactam by HPLC.
3. Make aliquots of the purified standard and store them at –80 °C until use for LC/MS analysis.

Standard curve for iso[4]LGE$_2$-lysoPE-HL

1. Prepare serial dilutions of iso[4]LGE$_2$-lysoPE-HL – 0.2, 0.8, 1.6, 2.5, 3.2, 5 6.4, 12.8, 25.6, 51.2 ng/mL – in 190 µL of methanol. Add 10 µL containing 2 ng of internal standard to each vial.
2. Transfer each 200 µL of solution to an autosample vial.
3. Inject 20 µL from each vial into the HPLC system with the autosampler.
4. Run the LC/MS/MS with the program described above.
5. Peak areas of iso[4]LGE$_2$-lyso-PE-hydroxylactam, m/z 800.2-153 and iso[4]LGE$_2$-D31-lyso-PE-hydroxylactam, m/z 831.2-153 are integrated with Masslynx.
6. Plot peak area ratio vs analyte concentration, $Y = 0.1131X - 0.0164$, where Y = peak area ratio, X = analyte concentration.

PROCEDURE

Sample preparation

1. Lipid extraction

- **Plasma samples (Bligh & Dyer method)**

  TIMING 1.5 h workup

  a. Transfer 200 µL of plasma into a 1.5 mL centrifuge tube.
  b. Add 750 µL of Bligh & Dyer solution with BHT. For LC/MS analysis, this solution should include 2 ng of internal standard
  c. Vortex vigorously for 2 min to mix and then keep at 4 °C for 15 min.
  d. Add 250 µL of chloroform.
  e. Vortex vigorously for 1 min to mix.
  f. Add 250 µL of 1.5% sodium chloride solution.
  g. Vortex vigorously for 1 min to mix.
  h. Centrifuge at 3000 RPM for 10 min to give a three-phase system (aqueous top, protein disk, organic bottom).
  i. Recover the bottom phase as follows: insert a polyethylene transfer pipette through the upper phase with gentle positive-pressure (i.e., gentle bubbling) so that the upper layer does not get into the pipette tip. When the pipette tip is at the bottom of the tube, carefully withdraw bottom phase through the pipette, making sure to avoid the interface or upper layer (WARNING: only try to recover ~90% of the bottom layer, not all of it).
  j. Evaporate the solvent by blowing argon gas over the sample.
  k. Store the sample at –80 °C until subsequent PLA_2 treatment.

- **Tissue samples (Folch method)**

  TIMING 2 h workup

  a. Chill a 15 mL polyethylene tube with Folch solution with BHT antioxidant on ice for 15 min. (i.e., for 100 mg tissue use 5 mL of Folch solution)
  b. Transfer frozen tissue sample to a new tube which has been weighted, then cap the tube very quickly.
  c. Weight out frozen tissue exactly and record. Keep tissue on dry ice before measurement.
  d. Transfer tissue to a tube with Folch solution with BHT antioxidant.
  e. Add internal standard (200 µL of solution with 2 ng of iso[4]LGE_2-D31-lyso-PE-hydroxylactam).
  f. Homogenize manually (tissue weight ≥100 mg) using a stainless steel pestle coated with Teflon, or using a TissueLyser (tissue weight ≤100mg), 30 Hz for 10 min.
  g. After homogenization, place the tubes in a 0-4 °C freezer for 30 min to maximize lipid extraction efficiency.
  h. Add an aqueous solution of sodium chloride (1.5% (w/w), ? volume of organic extract solution).
  i. Vortex vigorously 1 min to mix.
  j. Centrifuge at 3000 rpm for 15 min to give a three-phase system (aqueous top, protein disk, organic bottom).
  k. Transfer the lower layer containing the lipids to a clean brown vial with a polyethylene transfer pipette.

l. Dry the collected organic fraction under a stream of argon.
m. Store the sample at –80 °C until subsequent PLA_2 treatment.

2. PLA_2-catalyzed hydrolysis of isolevuglandin-diacyl-PE adducts from biological extracts

TIMING 0.5 h workup + incubation overnight

a. Add 50 µL of methanol into the extract from 200 µL of plasma.
b. Add 450 µL of PBS (10 mM, pH 7.4).
c. Add 50 µL 50 mM $CaCl_2$ solution.
d. Place the mixture in the sonicator for 5 min.
e. Add 50 µL PLA_2 (500 units).
f. Seal the vial under argon.
g. Incubate the mixture solution under argon at 37 °C overnight.
h. Transfer the solution (600µL) to a 15mL polyethylene tube.
i. Add 2.25 mL of Bligh & Dyer solution with BHT.
j. Vortex vigorously for 15 min to mix.
k. Add 750 µL of chloroform.
l. Vortex vigorously for 1 min to mix.
m. Add 750 µL of 1.5% sodium chloride solution.
n. Vortex vigorously for 1 min to mix.
o. Centrifuge at 3000 RPM for 10 min to give a three-phase system (aqueous top, protein disk, organic bottom).
p. Transfer the lower layer containing the lipids to a clean brown vial with a polyethylene transfer pipette
q. Evaporate the solvent by blowing argon gas over the sample.
r. Store the sample at –80 °C until LC/MS analysis.

Quantitive analysis by LC/MS/MS

TIMING 1 h workup + 25 min HPLC run time for each sample

a. Resuspend samples in 200 µL Solvent S and transfer to autosample vial.
b. Inject 20 µL of each sample into LC/MS/MS instrument with the same gradient as described above.
c. MRM for isoLG-lysoPE-HL and internal standard are performed.
d. The amount of isoLG-lysoPE-HL and internal standard in the sample are determine by peak area in the LC/MS/MS traces.
e. Calculate isoLG-lysoPE-HL as follows: the concentration of isoLG-lysoPE-HL = (peak area ratio of isoLG-lysoPE-HL to internal standard +0.00164)/0.1131