Supporting Information

A Lipidomic Workflow Capable of Resolving *sn*- and C=C Location Isomers of Phosphatidylcholines

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Experimental Section

Lipid nomenclature

Shorthand notation suggested by LIPID MAPS was used for structural annotation of identified lipids.[1] For example, PC 16:0/18:1(9Z) signifies the fatty acyl chain on *sn*-1 position contains 16 carbons and the fatty acyl chain on *sn*-2 position contains 18 carbons. The "0" and "1" after the carbon number refers to the degree of unsaturation of the fatty acyl chain. The location of C=C is defined by counting from the alpha carbon of the fatty acyl ester, *Z* and *E* is used for annotating C=C bond geometry. "/" means the *sn*-1/*sn*-2 position is determined, while "_" means the position is unknown.

Materials

Lipid standards and the bovine liver polar extract were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Ammonium salts of bicarbonate and formate were purchased from Aladdin (Shanghai, China). HPLC grade acetone and water were purchased from Fisher Scientific Company (Ottawa, ON, Canada). Phospholipase A_2 (PLA_2) from porcine pancreas was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Human breast cancer tissue samples were supplied by the specimen bank of Dongfeng Hospital of Hubei University of Medicine. The procedures were complied with ethical regulations of the Ethical Review Board of Tsinghua University (IRB No. 2017007). Informed consent was obtained from all participants. The lipid extraction procedure from tissue samples was followed Folch method.[2] Tissue sample (70 mg) was added with 1 mL of deionized water in a 10 mL-centrifuge tube. It was homogenized by a handheld homogenizer (Jingxin

Technology, Beijing, China) at 40,000 Hz for 5 min. After homogenization, the lipid was extracted with 1-mL methanol and 2 mL chloroform. After 5 min vortex, the mixture was centrifuged at 11, 269 × g for 8 min (Eppendorf, Shanghai, China). The top layer was used for repeated extraction and the bottom layer was collected and transferred to a 10 mL-glass test tube. The chloroform layers from the two extractions were combined and dried under nitrogen flow. The extract was reconstituted into 1-mL methanol and stored at -20 ℃ before analysis.

PLA_2 Digestion

We followed the PLA_2 digestion protocol from Sigma-Aldrich[3] with some modification. PLA_2 powder was dissolved in 1:1 water-glycerol (v/v) containing 75 mM NaCl and 10 mM Tris-HCl buffer (pH = 8.0) to produce a PLA_2 concentration of 0.89 U μL^{-1}. Five theoretical mole percentages mixtures of *sn*- isomers of PC were mixed in methanol while keeping the total concentration at 10 μM. These mixtures were prepared from the same stock solutions as those used for direct MS analyses. Each sample was reconstituted with 150 μL of the PLA_2 solution and 10 μL of aqueous 100 mM $CaCl_2$ solution. Resulting samples were incubated at 37 ℃ for 7 min, followed by dilution of 50 μL of each sample with 5 mM ammonium acetate in methanol to a final volume of 1 mL.

Mass spectrometry

For method development, experiments were performed on a triple quadrupole/linear ion trap (LIT) hybrid mass spectrometer (4500 QTRAP, Sciex, Toronto, Canada), employing nanoelectrospray ionization (nanoESI). Neutral loss and precursor ion scans were performed

in triple quadrupole mode. Accurate mass measurement data were collected on a quadrupole time-of flight (Q-TOF) mass spectrometer (X500R, Sciex, Toronto, Canada), which was equipped with an ESI source.

LC-PB-MS/MS

The LC-PB-MS/MS system consisted of an ExionLC AC system (Sciex, Toronto, Canada), a QTRAP 4500 mass spectrometer (Sciex, Toronto, Canada) and a home-built flow microreactor. A flow microreactor made from FEP tubing (0.03-in. i.d., 1/16 o.d.) and a low-pressure mercury lamp (emission at 254 nm wavelength) was used for the post-column PB derivation. The LC was equipped with a degasser, two pumps, an automatic sampler, and a column oven. Separation was performed on a HILIC column (150 mm × 2.1mm, silica spheres, 2.7 μm) from Sigma-Aldrich (St. Louis, MO, USA). The column temperature was set at 30 ℃. Mobile phase consisted of A: acetonitrile (ACN) /Acetone (50:50, *v/v*) and B: NH_4HCO_3 aqueous solution (20 mM). A linear gradient was used: 0-8 min: 90% to 85% B; 8-15 min: 85% to 80% B; 15-17 min: 80% to 70% B; 17-23 min: 70% to 70% B; 23-24 min: 70% to 90% B, followed by washing with 90% B at a flow rate of 0.2 mL/min. The injection volume was 2 μL.

Offline PB reaction

For determining C=C in *sn*-1 fatty acyls of PCs, PB-MS^4 CID was applied to the bicarbonate adduct of PC. The PB reaction solution was collected from an offline flow microreactor.[4] The flow path was made from fused silica capillaries (363 μm o.d. 100 μm i.d.; Polymicro Technologies/Molex; Phoenix, AZ, USA) with UV-transparent fluoropolymer coating. A

low-pressure mercury (LP Hg) UV lamp (254 nm, BHK, Inc.; Ontario, CA, USA) was utilized to initiate the PB reaction.

OzID on a home-built linear ion trap mass spectrometer

A home-built dual-trap mass spectrometer[5] was employed for performing OzID experiments. First the precursor ion (*m/z* 820) was trapped in linear ion trap 1 (LIT1) and then mass-selectively transferred to LIT2. This fragment ion (*m/z* 419) was formed through beam-type CID by increasing the potential differences between LIT DC float voltages, which was followed by isolation in LIT2. Ozone was produced from dielectric barrier discharge of O_2 using a home-built ozone generator and injected into LIT2 via a pinch valve for 500 ms. Two fragment ions at *m/z* 153 and 169 were observed, proving the existence of C2-C3 double bond on glycerol backbone.

Hypothesis testing for calibration curves generated from NLS 121 Da of $[M+HCO_3]^-$

Hypothesis testing was used to determine whether there were significant differences between the two calibration curves (Fig. 2e) of the *sn*-isomers (PC 16:0/18:1 vs. PC 18:1/16:0). F-test (one-tailed) was first employed to compare the covariances of the two regression lines, S_r^2. T-test (two-tailed) was then employed to compare the slopes (m) of two calibration curves.

F-test for covariance of regression

Covariance of regression, $S_r^2 = \frac{\sum_{i=1}^{N}[y_i-(b+mx_i)]^2}{N-2}$

For calibration curve of PC 16:0/18:1, S_{r1}^2=6.98e-3

For calibration curve of PC 18:1/16:0, S_{r2}^2=1.36e-3

Then, F = $\frac{S_{r1}^2}{S_{r2}^2}$ = 5.12

F_{crit} at 95% confidence level: F_{crit} (0.05, 4, 4) = 6.39

$$F < F_{crit}$$

Therefore, the two calibration curves did not show significant difference in covariance.

T-test for slope

Variance of the slope, $S_m^2 = \frac{S_r^2}{S_{xx}}$ ($S_{xx} = \sum x_i^2 - \frac{(\sum x_i)^2}{N}$)

For calibration curve of PC 16:0/18:1, S_{m1}^2=8.21e-4

For calibration curve of PC 18:1/16:0, S_{m2}^2=1.60 e-4

$S_{pooled}^2 = S_{m1}^2 + S_{m2}^2$ S_{pooled}=0.031

$t = \frac{\Delta m}{S_{pooled}} = 0.40$

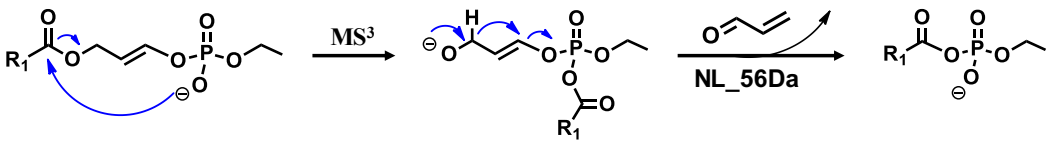
t_{crit} at 95% confidence level (two-tailed): t_{crit} (0.05, 3) = 3.18

$t < t_{crit}$

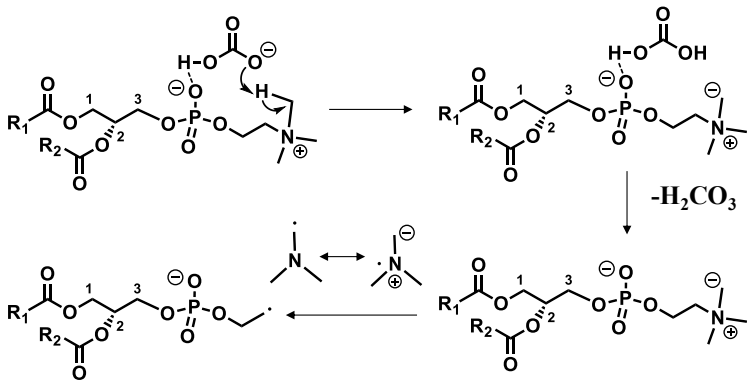
Therefore, there was no significant difference between the two slopes.

The two test results suggest that the two calibration curves of sn-isomers did not show significant difference at 95% confidence level.

Scheme S1. The possible mechanism for neutral loss of acraldehyde (NL_56 Da) from MS^3 CID of the *sn*-1 fragment.



Scheme S2. The "zwitterion intermediate" pathway for the formation of ethyl radical ion



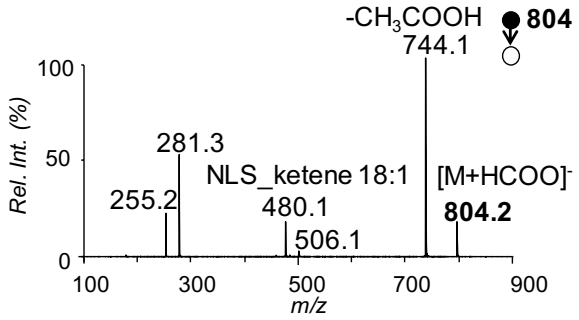


Figure S1. Negative ion mode MS^2 CID of formate adduct of 10 μM PC PC 16:0/18:1 (*m/z* 804.2, CE=30 eV).

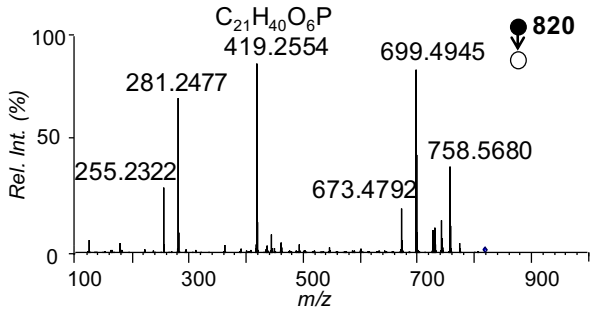


Figure S2. Accurate mass measurement of the *sn*-1 fragment (*m/z* 419). The MS^2 CID spectrum of bicarbonate adduct of PC 16:0/18:1 (*m/z* 820.2, CE=30 eV) was collected on a Q-TOF instrument.

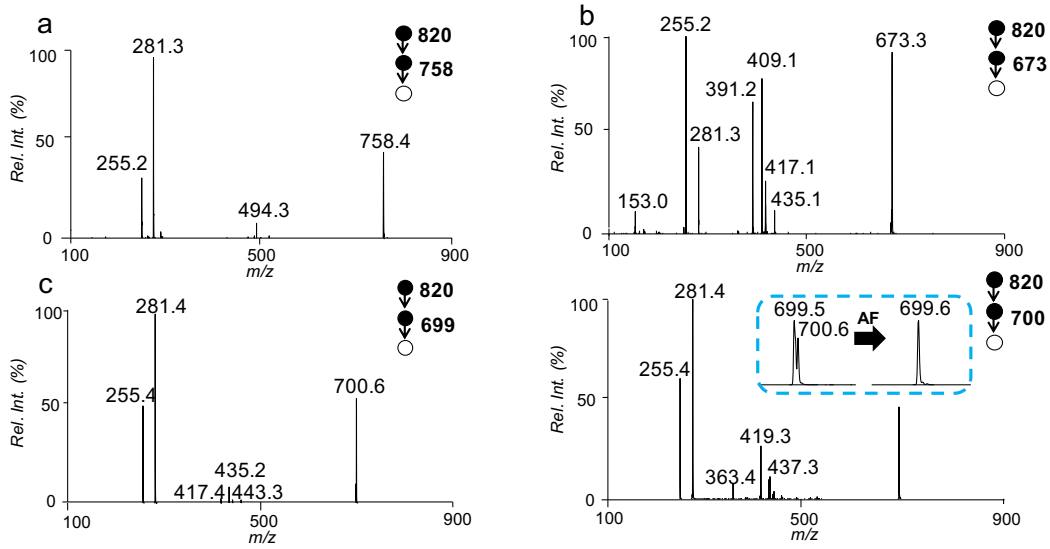


Figure S3. Negative ion mode MS^3 CID of (a) *m/z* 758.4, (b) *m/z* 673.3, (c) *m/z* 699.5, (d) *m/z* 700.6 from the MS^2 CID of the bicarbonate adduct of 10 μM PC 16:0/18:1.

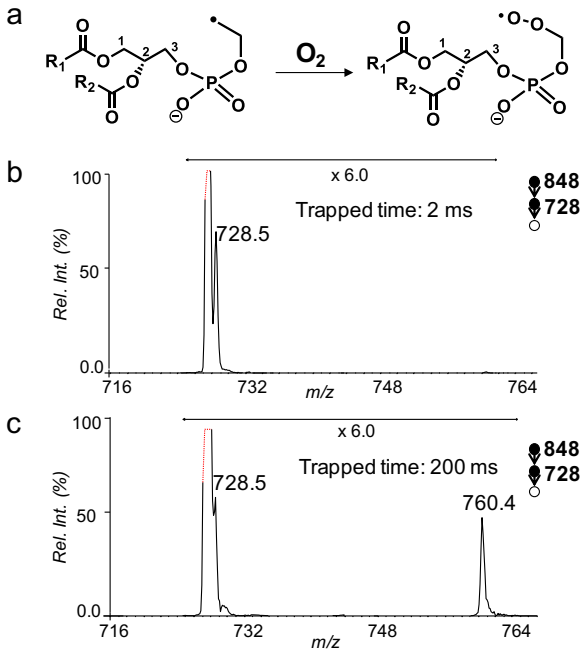


Figure S4. (a) Ion/molecule reactions between the ethyl radical ion and molecular oxygen. Ethyl radical ion at *m/z* 728.5 was generated from MS^2 CID of PC 18:0/18:1 ($[M + HCO_3]^-$, *m/z* 848.5), while oxygen was trace residual in the vacuum system due to atmospheric sampling. Zoomed-in mass spectra after the ethyl radical ion (*m/z* 728.5) being trapped in Q3 LIT for (b) 2 ms and (c) 200 ms. The peak at *m/z* 760.4 suggests O_2 addition to the ethyl radical ion.

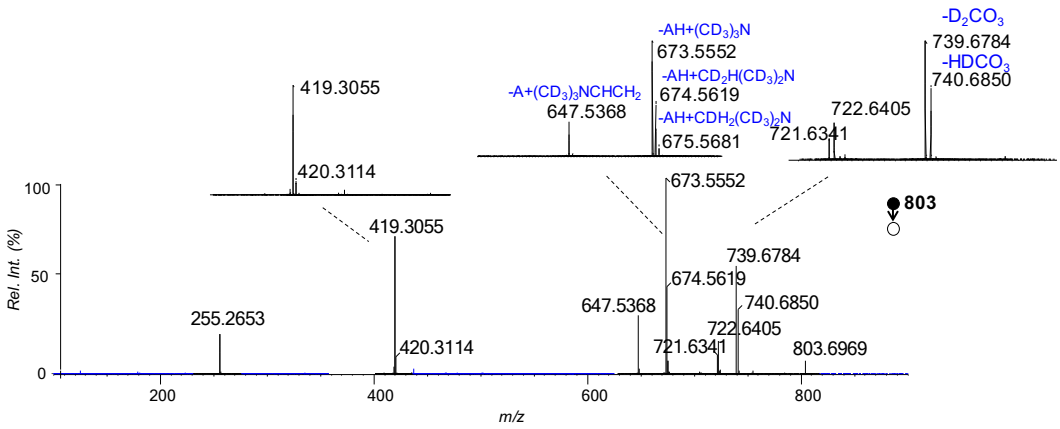


Figure S5. Negative ion mode MS^2 CID of bicarbonate adduct of 10 μM D9-labeled PC 16:0/16:0 (*m/z* 803.7, CE=30 eV), where all nine hydrogen atoms in trimethylamine group were labeled with deuterium atoms.

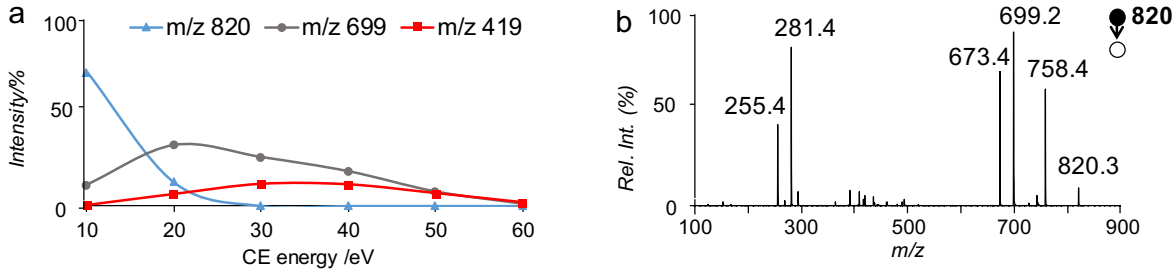


Figure S6. (a) The break down curve derived from MS^2 beam-type CID of bicarbonate adduct of PC 16:0/18:1 (*m/z* 820). (b) MS^2 ion-trap CID of bicarbonate adduct of PC 16:0/18:1 (*m/z* 820, $AF_2 = 0.1$, 25 ms).

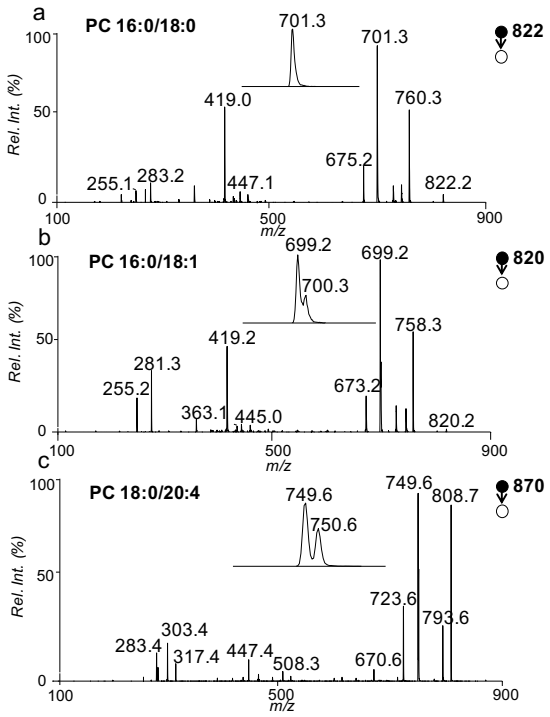


Figure S7. Negative ion mode MS^2 CID of bicarbonate adduct of (a) 10 μM PC 16:0/18:0 (*m/z* 822.2, CE = 30 eV), (b) 10 μM PC 16:0/18:1 (*m/z* 820.2, CE = 30 eV), and (c) 10 μM PC 18:0/20:4 (*m/z* 870.2, CE = 30 eV).

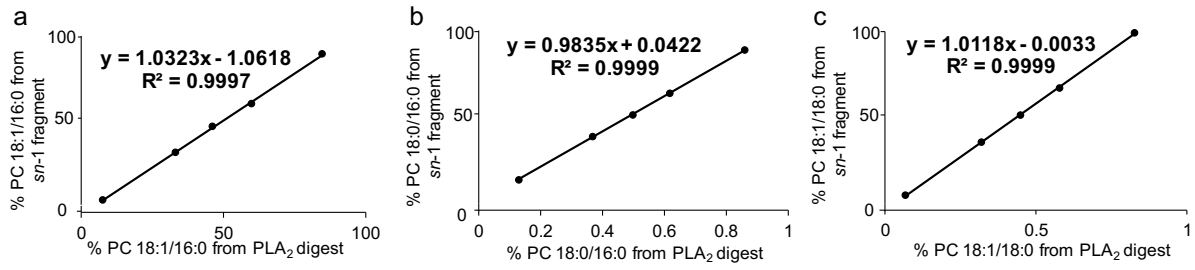


Figure S8. Correlations of *sn*-isomer quantitation by PLA_2 hydrolysis and *sn*-1 fragment method: (a) PC 16:0_18:1, (b) PC 16:0_18:0, and (c) PC 18:0_18:1.

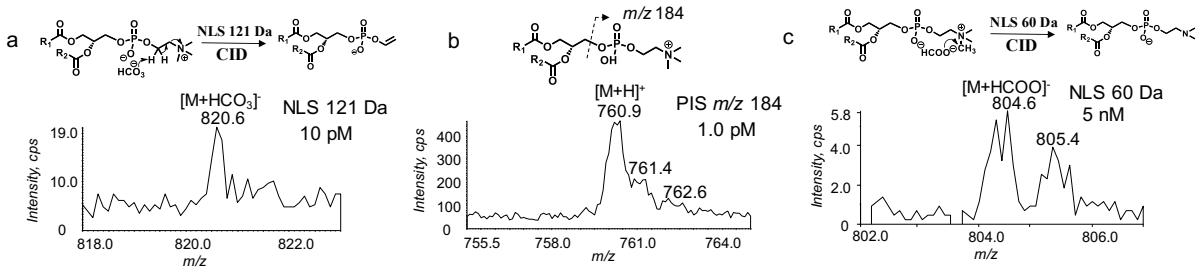


Figure S9. Limit of detection (LOD) for PC 16:0/18:1 based on signal to noise ratio higher than 3 using different MS/MS methods. (a) LOD =10 pM from NLS 121 Da of $[M+HCO_3]^-$, (b) LOD =1.0 pM from PIS *m/z* 184 of $[M+H]^+$, and (c) LOD = 5 nM from NLS 60 Da of $[M+HCO_2]^-$.

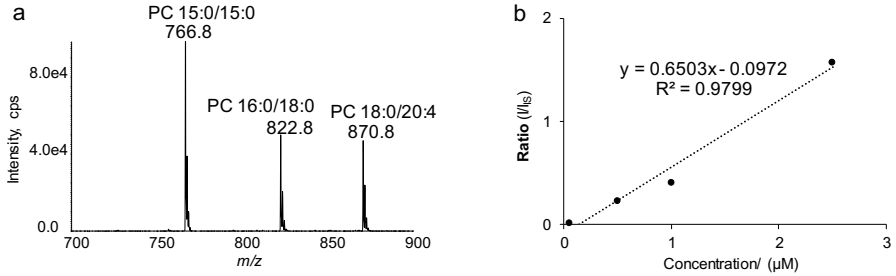


Figure S10. (a) NLS 121 Da of $[M+HCO_3]^-$ derived from an equal molar mixture of PC 15:0/15:0, PC 16:0/18:0, and PC 18:0/20:4 (5 μM each, CE=30 eV). (b) Calibration curve for PC 18:0/20:4 using NLS 121 Da with IS (PC 15:0/15:0) kept at 0.5 μM.

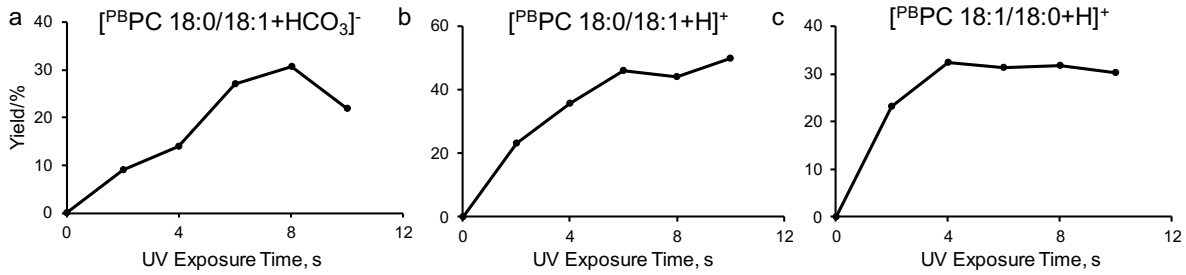


Figure S11. Comparisons of the PB reaction kinetic curves of PC 18:0/18:1 (5 μM) prepared in 1:1 acetone: H_2O with (a) 5 mM NH_4HCO_3 added, and (b) without NH_4HCO_3 added. (c) The PB reaction kinetic curve of PC 18:1/18:0 (5 μM) prepared in 1:1 acetone: H_2O.

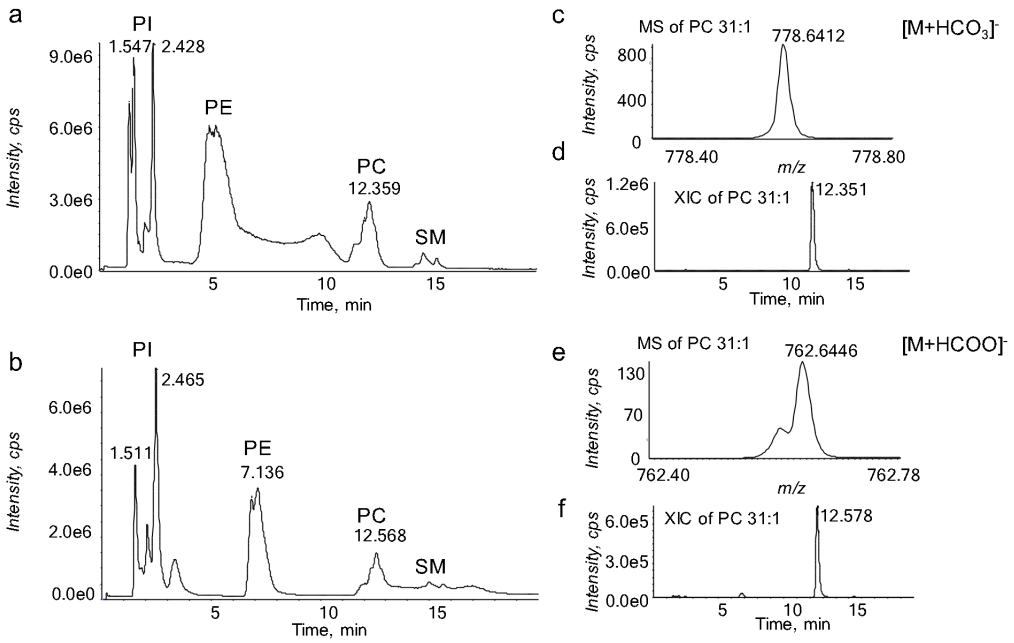


Figure S12. Comparisons of negative ion mode LC-MS from polar lipid extract of bovine liver with (a) 20 mM NH_4HCO_3 and (b) 20 mM NH_4HCO_2 added in the mobile phase as buffer, while all other parameters were kept the same. Retention of different classes of lipids was comparable between the two buffer conditions. (c) MS spectrum of a low abundance PC species, PC 31:1, from condition (a) and (d) corresponding extracted ion chromatogram (XIC). (e) MS spectrum of PC 31:1 from condition (b) and (f) corresponding XIC. Detection of PC 31:1 in the form of $[M+HCO_3]^-$ (*m/z* 778) is about 8 times more abundant than in the form of $[M+HCO_2]^-$ (*m/z* 762).

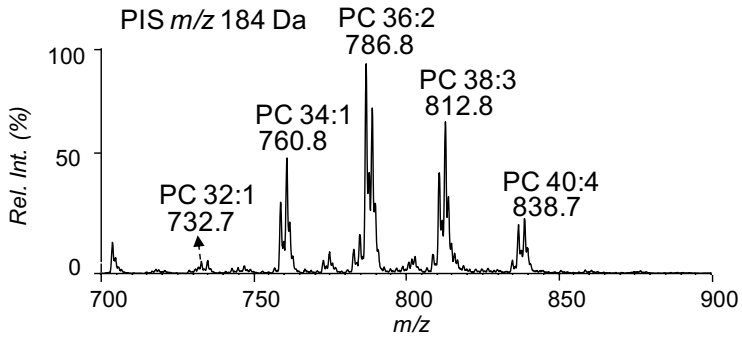


Figure S13. PIS *m/z* 184 of $[M+H]^+$ (CE=35 eV) of PCs in bovine liver extract (200 ppm.)

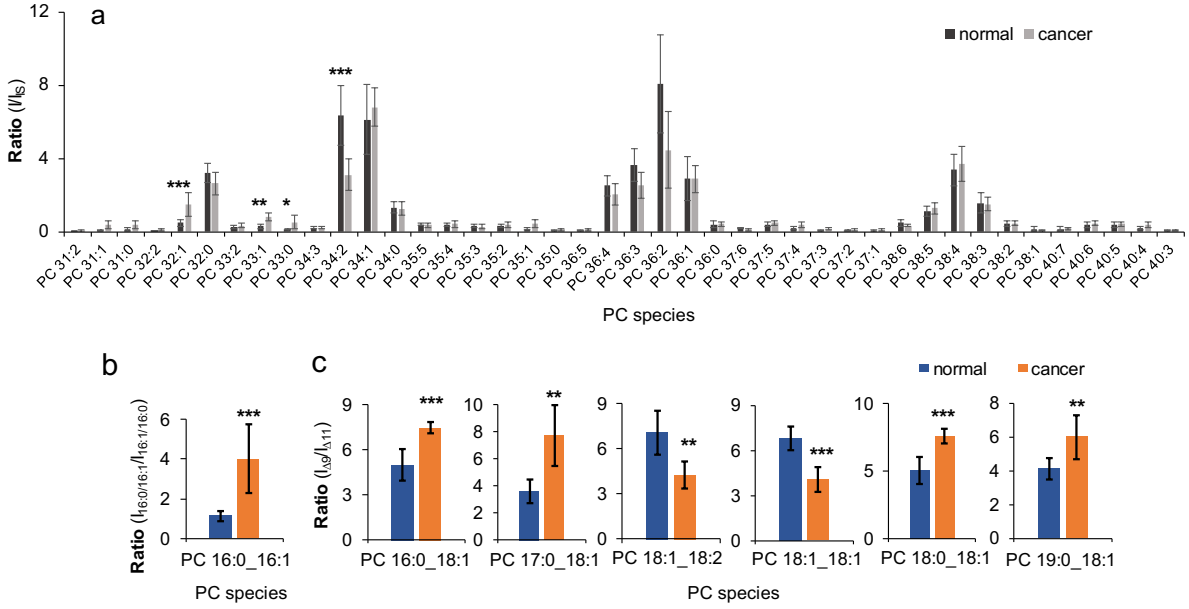


Figure S14. (a) Relative quanitation of PCs at subclass level from normal (N=3) and cancerous (N=3) human breast tissue. (b) Changes of *sn*-isomeric ratio of PC 16:0_16:1 from normal (N=3) and cancerous (N=3) human breast tissue. (c) Relative changes of Δ9/Δ11 ratios of C18:1 in PCs from normal (N=3) and cancerous (N=3) human breast tissue. Differences between normal and cancerous samples were evaluated for statistical significance

using the two-tailed student's t-test (* $P<0.05$, ** $P<0.01$, *** $P<0.001$). Error bar represents ± s.d. (N=3).

Table S1. Comparison of *sn*- purities for commercial PC standards by PLA_2 digestion and *sn*-1 fragment derived from MS^2 CID of $[M+HCO_3]^-$

Lipids	PLA_2 (mol%)	*sn*-1 fragment (mol%)	%Dev
PC 16:0/18:1	92.5±0.4	93.2±1.4	+1%
PC 18:1/16:0	85.3±0.4	87.2±1.4	+2%
PC 16:0/18:0	87±3	83±5	-5%
PC 18:0/16:0	86±4	89±4	+3%
PC 18:0/18:1	93±4	93±3	0%
PC 18:1/18:0	83±4	84±3	+1%

Table S2. Comparison of t*sn*-purities for commercial PC standards by PLA_2 digestion and MS^3 CID of $[M+HCOO]^-$ (all data were taken from publication by Ekroos et al.) [6]

Lipids	PLA_2 (mol%)	Ion trap MS^3 CID	%Dev
PC 16:0/18:1	88	83	-5%
PC 18:1/16:0	83	79	-5%
PC 16:0/18:0	88	83	-6%
PC 18:0/16:0	94	93	-1%
PC 18:0/18:1	96	89	-7%
PC 18:1/18:0	81	75	-7%

Reference

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