Chemicals needed:

- 2.5 mL per sample methyl tert-butyl ether on ice
- 600 µL per sample of water on ice
- 400 µL per sample of methanol on ice per sample
- Reconstitution Solution, 100 µL isopropanol (IPA)
- Mobile phases, 90:8:2 isopropanol:acetonitrile:water (IPA:ACN:water) with 10 mM ammonium formate and 0.1% Formic Acid and 60:40 ACN:Water with 10 mM ammonium formate and 0.1% Formic Acid LC/MS grade
- Internal standard, Avanti Lipids, Inc. listed in following table. Mixed to 100 ppm solution in 1:2 chloroform:methanol.

• Lysophosphatidylcholine (17:0/0:0)	• Phosphatidylethanolamine (17:0/17:0)
• Lysophosphatidylcholine (19:0/0:0)	• Phosphatidylethanolamine (15:0/15:0)
• Phosphatidylcholine (19:0/19:0)	• Phosphatidylserine (17:0/17:0)
• Phosphatidylcholine (17:0/17:0)	• Phosphatidylserine (14:0/14:0)
• Phosphatidylglycerol (14:0/14:0	• Triglycerol (17:0/17:0/17:0)
• Phosphatidylglycerol (17:0/17:0)	• Triglycerol (15:0/15:0/15:0)

Materials needed:

- Labeled 2 mL Eppendorf tubes
- Repeater Pipette
- Calibrated Micropipettes in various volumes* (see table below)
- Appropriate Micropipette tips* (see table below)
- Refrigerator
- Refrigerated Centrifuge
- Vortex
- Shaker
- N_2 Dryer
- Labeled LC vials with appropriate caps or 96-well tray
- LC-MS
- Waters Acquity C18 BEH (50 x 2.1 mm, 1.7 um) with guard
- Positive Calibration Solution
- Negative Calibration Solution
- Personal Protective Equipment

Type	Volumes (µL)	Tip color
P10	0.5 – 10	white
P20	2 – 20	yellow
P200	20 – 200	yellow
P1000	200 – 1000	blue

Precise Micropipette Volume and Transfer capabilities

Instrumentation:

Centrifuge, Eppendorf- 5417R: Open by pressing blue "open" button on bottom left of display. Check to be sure loading dock is cool. If not cool, close, press fast cool and wait until temperature is <10°C. When temperature is <10°C, press stop, wait for centrifuge to stop spinning, and open. Load samples making sure samples and/or weights are evenly distributed among the wheel.

N_2 Dryer, Organomation Asscociates, Inc- MultiVap 118: Flip green power switch to "on" (located on bottom left of display). Of the three black switches, set the start/reset switch to neutral. Set the heat switch to neutral. Set the gas switch to Manual. To obtain gas flow, turn the gas nozzle on right side of hood. Turn the Harris valve in hood to open position. Adjust LPM air to no more than 15. Place samples in drying tray. Open/close N_2 flow lines depending on where samples are placed. Lower N_2 lines to enable drying.

UHPLC, Thermo Scientific-Dionex Ultimate 3000: While setting up sequence, ensure that these initial conditions for analysis are as follows: 2 µL injection, 0.5000mL/min flow rate, gradient of 68% pump D, 32% pump C. Check the lines for air bubbles and purge line if present.

Mass Spectrometer, Thermo Scientific- Q Exactive: Divert valve set to position 2

To calibrate in positive ion mode: Correctly set up positive ion syringe filled with positive ion calibration solution and attach to MS using positive ion calibration tubing. Open Tuner→ File→ Load Tune File→ Click on StabilityTestMStune. Go to Instrument control tab and ensure conditions are as follows:

For Scan Parameter

Scan Type	Full MS
Scan Range	100-1,500
Fragmentation	In-source CID 20.0 eV
Resolution	70,000
Polarity	Positive
Microscans	1
Lock Masses	Best
AGC target	3e6
Max Inject time	200ms

Go to calibrate tab → source auto → default settings. Ensure settings are as follow

Set syringe to dispense positive ion calibration solution of a 500µL volume at 3µL /min. Begin dispensing. Turn MS on. Begin calibration. Negative calibration completed in similar fashion using negative ion calibration solution. Calibration should only be performed by trained staff.

Procedure:

1- Add 40 µL of thawed plasma sample to clean, 5 µL-size eppendorf tube using a P200 micropipette.
2- Add internal standards (5 µL of 100 ppm)
3- Add 300 µL MeOH (1mM BHT). Vortex.
4- Add 1 mL Methyl-tert-butyl ether (MTBE). Vortex.
5- 1 hour of incubation at room temperature in shaker.
6- Add 250 µL water, Fisher Optima.
7- 10 minutes of incubation at room temperature.
8- Centrifuge at 3000 rcf for 10 minutes.
9- Collect the organic phase (top layer).
10- Re-extract aqueous phase with 1.3 mL MTBE, 387 µL methanol, and 323 µL of water. Vortex and centrifuge.
11- Collect the new organic phase (top layer) and combine the two organic phases.
12- Dry under nitrogen.
13- Reconstitute sample by adding 200 µL IPA.
14- Transfer to labeled glass LC vial with insert.
15- Load samples into auto sampler.

Data Collection:

1- Turn on UHPLC and MS and set to starting conditions by loading method. Calibrate MS if not yet performed.
    a. Ensure that Column is an Titan C18 with dimensions of 7.5cm x 2.1mm
2- Create file and name it following protocol (QE1_Three Initials_Run#).
3- Set up sequence starting with 3 blanks and 1 neat QC. Enter samples with one blank and one QC following every 10 samples.
4- Name samples following protocol, Double check location of samples, Double check Method is correct, and double check injection volume is set to 2 µL for positive mode, 4 µL for negative mode.
5- Check MS settings to make sure they are correct.

For Scan Parameter

Scan Type	Full MS
Scan Range	67-1,500
Fragmentation	None
Resolution	70,000
Polarity	Positive
Microscans	1
Lock Masses	off
AGC target	3e6
Max Inject time	200ms

For HESI Source

Sheath Gas Flow Rate	40
Aux Gas Flow Rate	5
Sweep Gas Flow Rate	**1**
Spray Voltage	3.50
Spray Current	(Blank)
Capillary Temp	300
S-Lens RF Level	35.0
Heater Temp	350

AIF:

Scan Type	All Ion Fragmentation
Scan Range	100-1,500
Resolution	35,000
Polarity	Positive
NCE	25.0

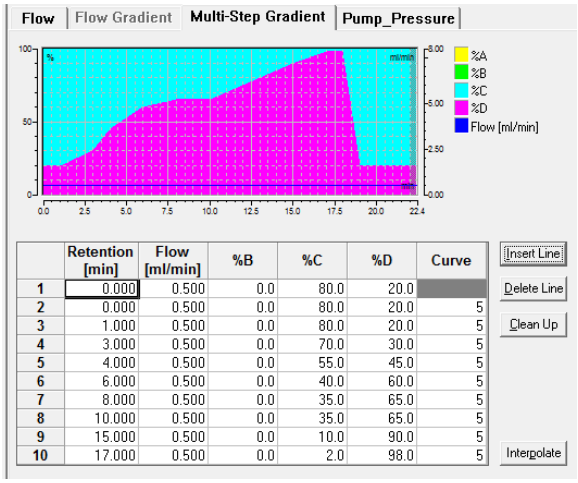
Stepped NCE	20.0%
AGC target	3e6
Max Inject time	175ms

Gradient Information

- Duration of run 21 minutes
- Initial conditions are 80% Pump C (0.1% FA in 60:40 ACN:Water, 10 mM Ammonium Formate), 20% Pump D (0.1% FA in 90:8:2 IPA:ACN:Water, 10 mM Ammonium Formate)
- Gradient begins at Run Time 1.0 minute and ends at Run Time 18 minutes, as shown in the figure below.
- Hold conditions at 0% pump D from Run Time 16 minutes to Run Time 17 minutes
- Return to initial conditions from 17 to 18 Run Time minutes.
- Equilibrate until Run Time 21 minutes

Time (min)	%D
0.00	20
1.00	20
3.00	30
4.00	45
6.00	60
8.00	65
10.00	65
15.00	90
17.00	98
18.00	98
19.00	20
22.00	20

- Flow= 500 µL/min, pump curve=5



	Retention [min]	Flow [ml/min]	%B	%C	%D	Curve
1	0.000	0.500	0.0	80.0	20.0	
2	0.000	0.500	0.0	80.0	20.0	5
3	1.000	0.500	0.0	80.0	20.0	5
4	3.000	0.500	0.0	70.0	30.0	5
5	4.000	0.500	0.0	55.0	45.0	5
6	6.000	0.500	0.0	40.0	60.0	5
7	8.000	0.500	0.0	35.0	65.0	5
8	10.000	0.500	0.0	35.0	65.0	5
9	15.000	0.500	0.0	10.0	90.0	5
10	17.000	0.500	0.0	2.0	98.0	5

6- Begin Sequence

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Approved By:	Tim Garrett	Date:05/28/2014

Revision Number	Name	Reason for Revision	Effective Date
01	Antoine Ducrocq	Creation of SOP	05/29/2014
02	Mike Williams	Changes to Method for Ring Trial	05/07/2015