Chemicals needed:

- 2 mL of methanol (with 1mM butylhydroxytoluene) on ice per cell pellet
- 4 mL of chloroform on ice per cell pellet
- 1.5 mL of water on ice per cell pellet
- Reextraction solution (1mL), 2:1 chloroform:methanol on ice
- Reconstitution Solution, 50 µL isopropanol (IPA)
- Mobile phases, 90:10 isopropanol:acetonitrile (IPA:ACN) with 0.1% Formic Acid and 10 mM ammonium formate and 60:40 ACN:Water with 0.1% Formic Acid and 10 mM ammonium formate, LC/MS grade
- Internal standard, Lysophosphatidyl-choline (LPC), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylglycerol (PG) lipid standards were purchased from Avanti Polar Lipids. Triacylglyceride (TG) lipid standards were purchased from Sigma-Aldrich. All lipid standards were diluted prior to analysis in 1:2 (v:v) chloroform/methanol and a working 100 ppm standard mix was then prepared by diluting the stock solution with the same solvent mixture.

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	Triacylglycerol (17:0/17:0/17:0)
Phosphatidylglycerol (17:0/17:0)	Triacylglycerol (15:0/15:0/15:0)

Materials needed:

- Labeled 15 mL conical tubes
- Repeater Pipette
- Calibrated Micropipettes in various volumes* (see table below)
- Appropriate Micropipette tips* (see table below)
- Refrigerator
- Centrifuge
- Vortex
- N_2 Dryer
- Labeled LC vials w/ appropriate caps
- Supelco Titan C18 Column, 75 mm x 2.1 mm, 1.9 µm pore size
- Positive Calibration Solution
- Negative Calibration Solution
- Personal Protective Equipment
- Halo C18 PFP guard Column
- Positive Calibration Solution
- Negative Calibration Solution
- Personal Protective Equipment

Type	Volumes (µL)	Tip color
P200	20 – 200	yellow
P1000	200 – 1000	blue

Precise Micropipette Volume and Transfer capabilities

Instrumentation:

Centrifuge: Set centrifuge to 5°C. Load samples making sure samples and/or weights are evenly distributed among the wheel. Centrifuge cell suspension at 2000 rpm for 5 minutes to pellet cells. Remove supernatant.

N_2 Dryer, Organomation Asscociates, Inc- OA-HEAT: Flip green power switch to "on" (located on bottom right of box). 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. Calibrations should be performed every Monday by a trained staff member and before 24 hour (+) runs. Refer to calibration SOP if needed. The HESI II probe should be installed at position D.

Extraction Procedure:

1- To the cell pellet in a 15 mL conical tube, add 15 µL internal standard mix to samples using repeater pipette.
2- Add 2 mL of ice cold MeOH (1mM BHT).
3- Add 4 mL of ice cold chloroform.
4- Incubate on ice for 20-40 minutes with occasional vortex mixing.
5- Add 1.5 mL of water.
6- Incubate on ice for 10 minutes with occasional vortex mixing.
7- Centrifuge at 2,000 rpm for 5 minutes.

8- Remove the organic layer (bottom layer) to a new 15 mL conical tube and reextract the aqueous phase (top layer) with 1 mL of ice cold $CHCl_3$:MeOH (2:1, v/v).
9- Vortex and centrifuge at 2,000 g for 5 minutes.
10- Combine organic phases and discard the new aqueous phase.
11- Dry organic layer using Nitrogen gas at 30 °C in Organomation Asscociates, Inc- OA-HEAT.
12- Reconstitute sample by adding 50 µL IPA.
13- Transfer to labeled glass LC vial with insert.
14- Load samples into auto sampler.

Data Collection:

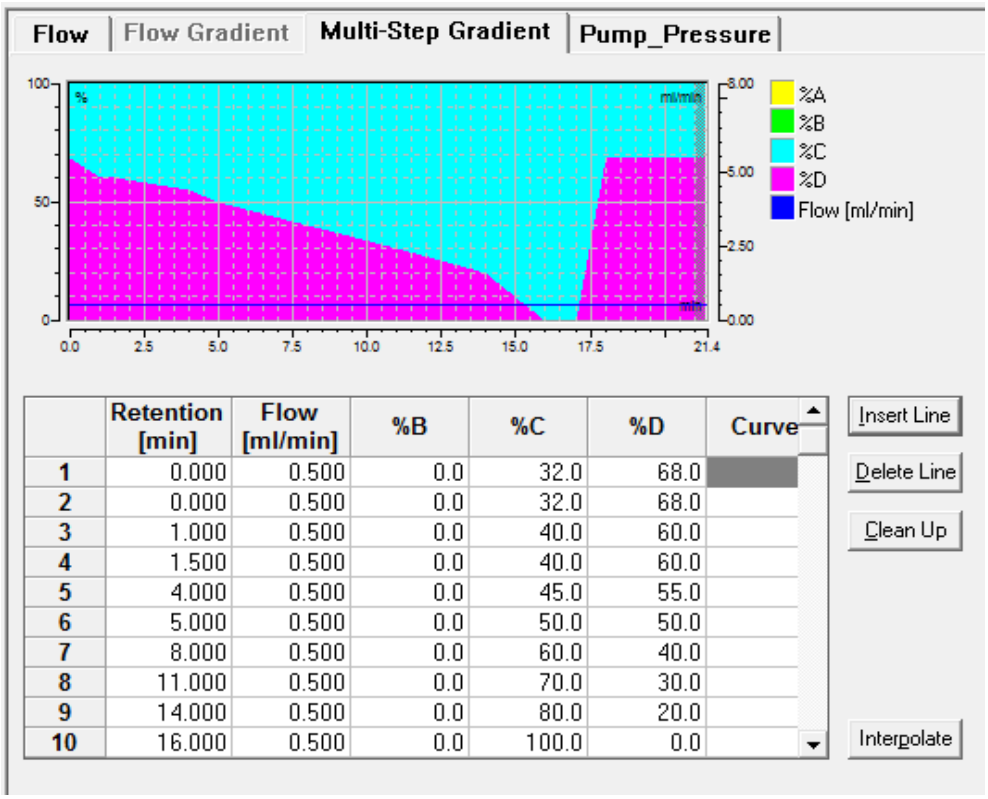
1- Ensure that Column is an Ensure that Column is an Titan C18 with dimensions of 7.5cm x 2.1mm
2- Check total injections on column and make note in read_me file.
3- Begin equilibration of the system by taking control through chromelean. Set flow rate to 500uL of 68% pump C and 32% pump D.
4- Open tunefile "lipidomics_posneg.mstune" using tuner window. Once this tunefile has been opened set Mass Spectrometer to on.
    a. Steps 2 and 3 combined will allow the system to equilibrate before sequence begins. It is recommended to let system equilibrate ~10 minutes before start of run.
5- Create folder where all raw files will be saved and generate folder hierarchy following naming protocol. (see appendix B)
6- Set up sequence starting with 3 blanks, 1 neat QC and 1 Pooled QC followed by unknown samples. After 10 unknown samples run another QC set consisting of one blank, one Neat QC and one Pooled QC.
7- Name samples following protocol, verify location of samples, ensure method is "m_lipid_pos" or "m_lipid_neg" and injection volume is 2uL for injections.

Gradient Information

- Duration of run 21 minutes
- Initial conditions are 32% Pump C (0.1% FA in 90:10 IPA:ACN), 68% Pump D (0.1% FA in 60:40 ACN:Water)
- Beginning at Run Time 1 minute and ending at Run Time 18 minutes, ramp from 68% pump D to 0% pump D.
- 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

Instrument Parameters		
HESI Probe	Positive (+)	Negative (-)
Probe Temperature	350°C	350°C
Spray Voltage	3300 V	3500 V
Capillary Temperature	300°C	350°C
Sheath Gas	35	35
Auxillary Gas	5	5
Mass resolution 70,000 @ m/z 200		



	Retention [min]	Flow [ml/min]	%B	%C	%D	Curve
1	0.000	0.500	0.0	32.0	68.0	
2	0.000	0.500	0.0	32.0	68.0	
3	1.000	0.500	0.0	40.0	60.0	
4	1.500	0.500	0.0	40.0	60.0	
5	4.000	0.500	0.0	45.0	55.0	
6	5.000	0.500	0.0	50.0	50.0	
7	8.000	0.500	0.0	60.0	40.0	
8	11.000	0.500	0.0	70.0	30.0	
9	14.000	0.500	0.0	80.0	20.0	
10	16.000	0.500	0.0	100.0	0.0	

9-Begin Sequence Pre-Processing:

1-Move all .raw files to server and open on processing computer

2- Convert .raw files to .MZxml files

3- process using MZmine

4-Process further with higher level statistics such as Metaboanalyst

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