Chemicals needed:

- Cell washing solution, 40 mM Ammonium Formate
- Internal Standard Solution containing Creatine-D3, L-Leucine-D10, L-Tryptophan-D3, and Caffeine-D3.
- Precipitate Solution, Ice-cold 80% methanol
- Reconstitution Solution, H_20 with 0.1% Formic Acid LC/MS grade
- Mobile phases, H_20 with 0.1% Formic Acid LC/MS grade and Acetonitrile LC/MS grade

Materials needed:

- Labeled 1.5 mL or 2 mL Eppendorf tubes
- Repeater Pipette
- Calibrated Micropipettes in various volumes* (see table below)
- Appropriate Micropipette tips* (see table below)
- Ice bucket
- Refrigerator
- Refrigerated Centrifuge
- N_2 Dryer
- Labeled LC vials with appropriate caps or 96-well tray
- LC-HRMS
- ACE Excel 2 C18-PFP Column (100 x 2.1mm) 2.0 µm
- Halo C18 PFP guard Column
- 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, adjust temperature using up and down arrows and 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. Adjust speed and time to fit the needs of the samples.

Vortex, Fisher Scientific, Vortex Genie 2: Flip switch to touch. Adjust turn dial to desired level of vortexing. Gently press sample down on pad to allow vortex to begin.

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 method to be utilized is PFP-metabolomics-neg350-NoBuffer-0-SID and PFP-metabolomics-neg-350-dd-MS2 for negative sequences. For positive sequences utilize PFP-metabolomics-pos-350-0-SID and PFP-metabolomics-pos-350-dd-MS2. Check the lines for air bubbles and purge line if present. Set injection volume to 2uL for positive samples and 4uL for negative samples.

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- Centrifuge cell suspension at 2000 rpm for 5 minutes at 5°C to pellet cells. Discard the supernatant.
2- Wash cell pellet by adding 1 mL of 40mM ammonium formate to cell pellet and centrifuge once again at 2000 rpm for 5 minutes at 5°C. Discard supernatant.
3- Complete step 2 two more times for a total of 3 washings.
4- To cell pellet in Eppendorf tube, add 2 µL isotopically-labeled daily working internal standard solution.
5- Add 1 mL ice-cold 80% methanol with repeater pipette.
6- Gently pipette up and down to disrupt cellular membrane.
7- Vortex sample to ensure mixing
8- Incubate on ice for 10 minutes to further precipitate proteins.
9- Centrifuge at 2000 rpm for 5 minutes at 5°C to separate proteins.
10- Transfer 500 µL of supernatant to new, labeled 2 mL Eppendorf tube making sure to leave behind protein pellet. If needed, place remaining 500 µL in -80 for further testing.
11- Dry liquid sample using clean, nitrogen gas.

12- Reconstitute sample by adding 30 µL 0.1% formic acid in water.
13- Place on an ice bath for 10-15 minutes to further precipitate any proteins that may be left in sample. Centrifuge again.
14- Transfer supernatant to labeled, glass LC vial with insert cap.
15- Load samples into auto sampler.

*See appendix A for Standard Preparation

Data Collection:

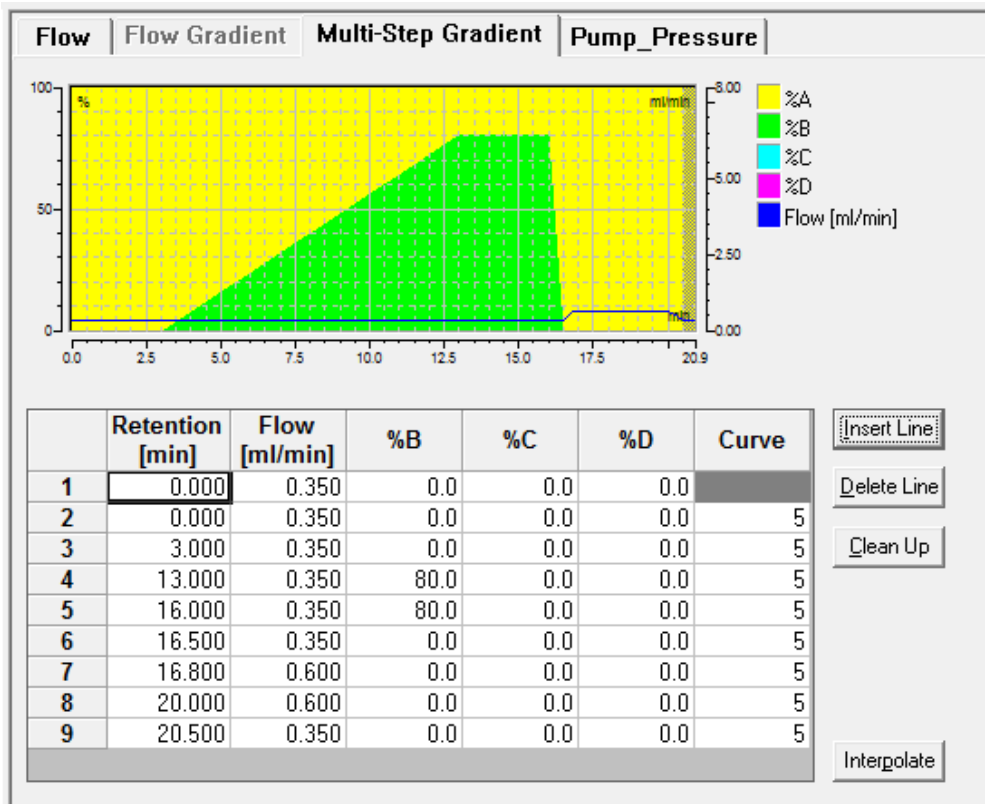
1- Ensure that Column is an ACE Excel 2 C18-PFP with dimensions of 100 x 2.1mm, 2.0 µm with a Halo C18-PFP guard attached
2- Check total injections on column and make note in read_me file.
3- Begin equilibration of the system by taking control through chromelean. Set flow rate to 350uL of 100% pump A.
4- Open tunefile "Metabolomics-Pos-Neg-30sLens.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 "PFP-metabolomics-pos-350-0-SID-17min-new-injector_sycWpump" or "PFP-metabolomics-neg-350-0-SID-17min-new-injector_sycWpump"" and injection volume is 2uL for positive injections and 4uL for negative injections.

Gradient Information

- Duration of run is 20.5 minutes
- Initial conditions are 100% Pump A (0.1% FA in Water)
- Flow rate is .350mL/min until run time 16.8
- Beginning at Run Time 3 minutes and ending at Run Time 13 minutes, begin a ramp gradient up to 80% pump B (Acetonitrile)
- Hold conditions at 80% pump B from Run Time 13 minutes to Run Time 16 minutes

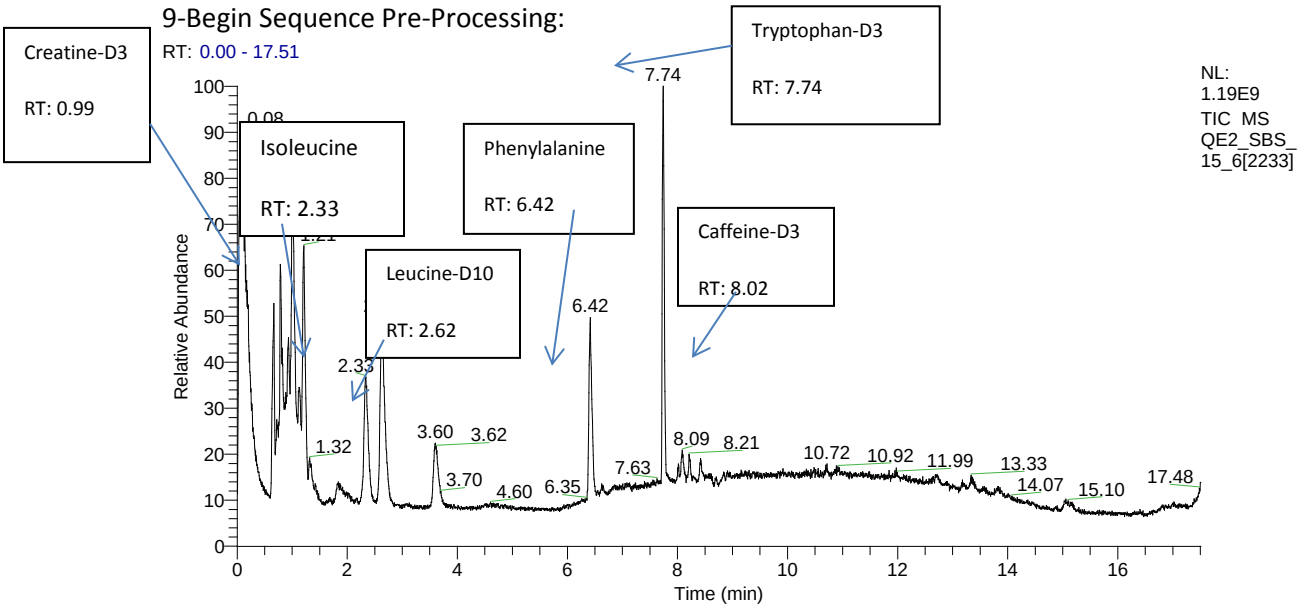
- Beginning at Run Time 16 minutes, return to initial conditions at ending at Run Time 16.5 minutes
- At run time 16.8 increase flow rate to .600mL/min
- Continue until run time 20 and decrease flow rate back to 350 mL/min until Run Time 20.5 minutes
- pump curve=5

Instrument Parameters		
HESI Probe	Positive (+)	Negative (-)
Probe Temperature	350°C	350°C
Spray Voltage	3500 V	3500 V
Capillary Temperature	320°C	320°C
Sheath Gas	40	45
Auxillary Gas	10	10
Spare gas	1	1
Mass resolution 70,000 @ m/z 200		



	Retention [min]	Flow [ml/min]	%B	%C	%D	Curve
1	0.000	0.350	0.0	0.0	0.0	
2	0.000	0.350	0.0	0.0	0.0	5
3	3.000	0.350	0.0	0.0	0.0	5
4	13.000	0.350	80.0	0.0	0.0	5
5	16.000	0.350	80.0	0.0	0.0	5
6	16.500	0.350	0.0	0.0	0.0	5
7	16.800	0.600	0.0	0.0	0.0	5
8	20.000	0.600	0.0	0.0	0.0	5
9	20.500	0.350	0.0	0.0	0.0	5

Insert Line
Delete Line
Clean Up
Interpolate



2-Generate summary report and sample report.

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

4- Convert .raw files to .MZxml files

5- process using MZmine

6-Process further with higher level statistics such as Metaboanalyst

Created By:	Sandi Batson	Date: 02/05/15
Reviewed By:	Tim Garrett	Date: 02/05/15
Approved By:	Rick Yost	Date: 02/05/15

Revision Number	Name	Reason for Revision	Effective Date
01	Sandi Batson	Creation of SOP following C. Ulmer's protocol for Metabolomics/Lipidomics.	02/05/15
02	Sandi B. Sternberg	• Changed method and gradient information to reflect new pump. • Updated the retention times in pre-processing	03/17/15