Chemicals needed:

- Chilled 50/50 Methanol/Water
- 0.1% Formic Acid in Water (HPLC-MS grade) as Reconstitution Solution
- 0.1% Formic Acid in Water(HPLC-MS grade) and Acetonitrile (HPLC-MS grade) as Mobile Phases
- Internal Standard Mixes

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)
- Sonicator
- Ice
- Refrigerated Centrifuge
- Nitrogen Dryer
- Labeled LC vials with appropriate caps or 96-well tray
- LC-MS
- ACE PFP 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:

Sonicator,

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.

UHPLC,

Thermo Scientific-Dionex Ultimate 3000: While setting up sequence, ensure that these initial conditions for analysis are as follows: 5 uL injection, 0.300mL/min flow rate, gradient of 100% pump B. Always purge lines if solvent bottles are replaced.

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	120-1,800
Fragmentation	In-Source CID 2 eV
Resolution	35,000
Polarity	Positive
Microscans	1
Lock Masses	Off
AGC target	1e6
Max Inject time	30

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

Set syringe to dispense positive ion calibration solution of a 500uL 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 500 µL of pre-chilled 50/50 Methanol/Water to each pre-weighed sample.
2- Add 20µL Daily Working Internal Standard solution.
3- Vortex sample for 30 seconds to 1 minute.
4- Homogenize in a sonicator for 20 minutes. Add ice to sonicator to reduce temperature rise during sonication. Be sure samples are in water, not ice, to ensure homogenation.
5- Centrifuge samples at 10C and 20,000 RCF for 10 minutes.
6- Pull off 450 uL of supernatant and transfer to a new, labeled microcentrifuge tube.
7- Dry down with clean nitrogen.
8- Reconstitute sample in 50 uL 0.1% FA in water.
9- Vortex
10- Centrifuge to separate any further solid from sample
11- Transfer liquid portion to labeled, glass LC vial.
12- Save the solid portion (if present) left in microcentrifuge tube.
13- Samples are now ready for metabolomic profiling.

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-

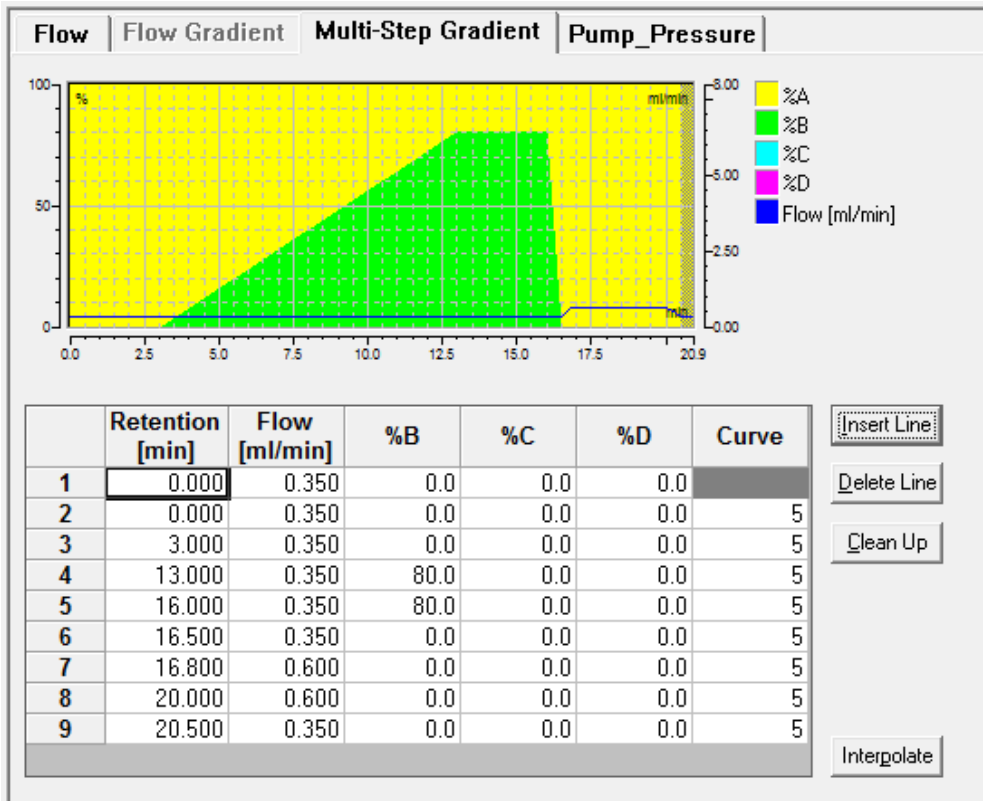
7- Set up sequence starting with 3 blanks, 1 neat QC and 1 Pooled QC followed by unknown samples. After 10 unknown samples run another QC set consisting of one blank, one Neat QC and one Pooled QC.

8- 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

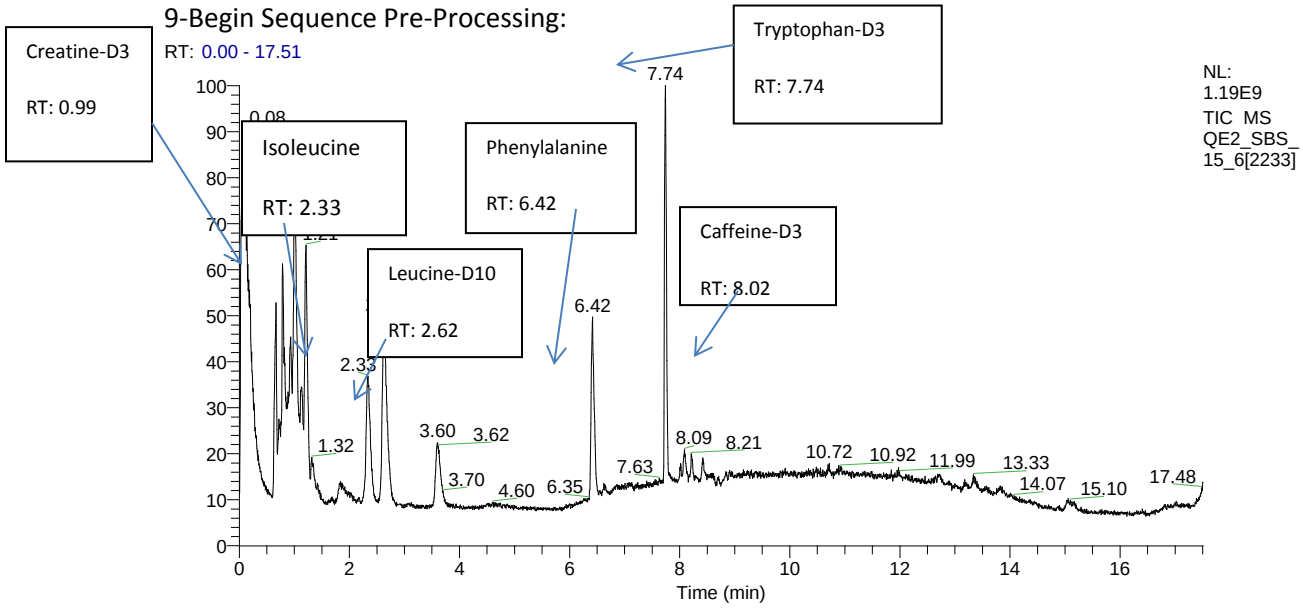
- Duration of run is 20.5 minutes
- Initial conditions are 100% Pump A (0.1% FA in Water)
- Flow rate is .350mL/min until run time 16.8
- Beginning at Run Time 3 minutes and ending at Run Time 13 minutes, begin a ramp gradient up to 80% pump B (Acetonitrile)
- Hold conditions at 80% pump B from Run Time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



Created By:	Sandi Batson	Date: 12/16/14
Reviewed By:	Tim Garrett	Date: 12/16/14
Approved By:		

Revision Number	Name	Reason for Revision	Effective Date
01	Sandi Batson	Creation of SOP	12/16/14
02	Sandi B. Sternberg	• Changed method and gradient information to reflect new pump. • Updated the retention times in pre-processing	03/17/15