Chemicals needed:

- Precipitate solution, 8:1:1 Acetonitrile: Methanol:Acetone
- 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
- Internal Standard Solution containing Creatine-D3, L-Leucine-D10, L-Tryptophan-D3, and Caffeine-D3.

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)
- 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, 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- 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- Add 100 µL of thawed sample to labeled, clean eppendorf tube using a P200 micropipette.
2- Add 20µL Isotopically-labeled Daily Working Standard Solution as an Internal Standard.*
3- Add 800 µL precipitate solution (8:1:1 Acetonitrile:Methanol:Acetone) with repeater pipette to make a solution of 1:8 (sample:solvent) ratio. Vortex sample to ensure mixing.
4- Cool sample in fridge for 30 minutes to further precipitate proteins.
5- Spin at 20,000 rcf for 10 mins at <10° C to create a pellet of proteins.
6- Transfer 750µL of supernatant to new, labeled tube making sure to leave behind protein pellet.
7- Dry liquid sample using Nitrogen gas in Organomation Associates MultiVap.
8- Reconstitute sample by adding 100 µL H_20 with 0.1% Formic Acid. Vortex.
9- Place on an ice bath for 10-15 minutes. Centrifuge again.
10- Transfer supernatant to labeled, glass LC vial with glass insert. After all samples have been transferred, ensure samples are free of air bubbles
11- 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.

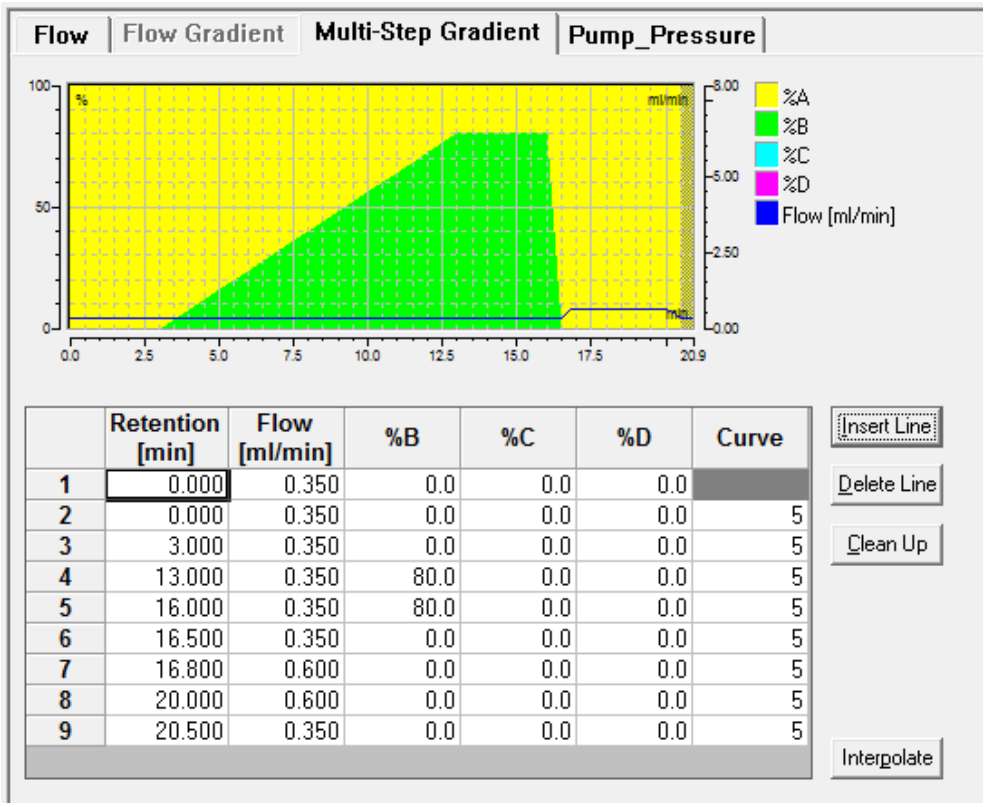
3- Begin equilibration of the system by taking control through chromelean. Set flow rate to 350uL of 100% pump A.
4- Open tunefile "Metabolomics-Pos-Neg-30sLens.mstune" using tuner window. Once this tunefile has been opened set Mass Spectrometer to on.
    a. Steps 2 and 3 combined will allow the system 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-
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

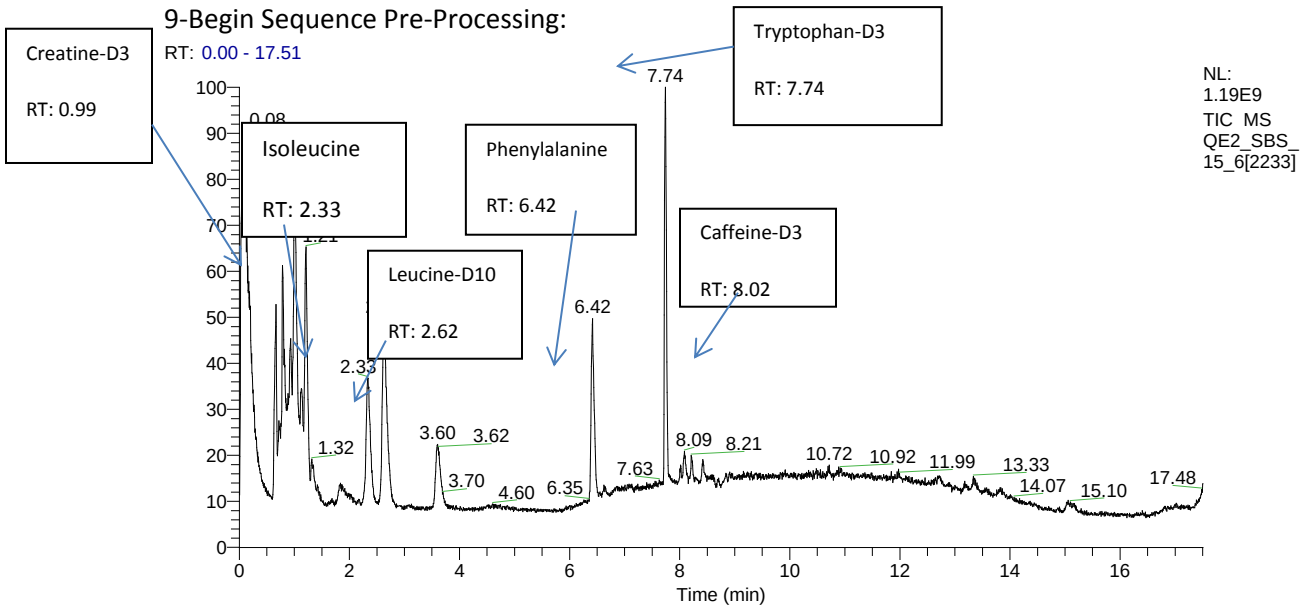
- Duration of run is 20.5 minutes
- Initial conditions are 100% Pump A (0.1% FA in Water)
- Flow rate is .350mL/min until run time 16.8
- Beginning at Run Time 3 minutes and ending at Run Time 13 minutes, begin a ramp gradient up to 80% pump B (Acetonitrile)
- Hold conditions at 80% pump B from Run Time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



1-Check to see presence of amino acids and standards within individual runs

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: 04/22/14
Reviewed By:	Tim Garrett	Date: 04/22/14
Approved By:	Rick Yost	Date: 04/22/14

Revision Number	Name	Reason for Revision	Effective Date
01	Sandi Batson	Creation of SOP	04/22/14
02	Sandi B. Sternberg	Changed Precipitate solution to 8:1:1 Acetonitrile:MeOH:Acetone. Added new step (9) in procedure. Both changes will give a cleaner sample.	08/01/14
03	Sandi B. Sternberg	Changed Reconstitution amount from 150uL to 100uL	09/18/14
04	Sandi B. Sternberg	Changed amount of Internal Standard to be added from 10uL to 20uL due to change in Internal Standard mix.	10/04/14
05	Sandi B. Sternberg	• Update in Instrumentation to reflect purchase and use of new nitrogen dryer. • Took out calibration info and added reference to calibration SOP. • Added the exact method(s) used with Dionex.	10/29/14
06	Sandi B. Sternberg	• Updated Data collection section to name exact method and tunefile to be used. • Removed Instrument parameters since they are saved in tunefile and	10/31/14

		method. • Changed gradient information to show increased use of pump B. • Added section for Pre-processing	
07	Sandi B. Sternberg	• Added step in Pre-processing to accommodate XReport.	12/5/14
08	Sandi B. Sternberg	• Changed Step 6 in Extraction Procedure from 250µL to 750µL. • Added instrument parameters	12/15/14
09	Sandi B. Sternberg	• Changed method and gradient information to reflect new pump. • Updated the retention times in pre-processing	03/17/15