Plasma Extraction Protocol for CC Diet Samples

Safety: Appropriate PPE such as gloves, goggles and lab coat are to be worn for protection. Waste and tips are disposed of into red bin biohazardous bags. Samples are from mouse plasma and can be worked with on the lab bench.

Samples: Unthaw mouse plasma samples on ice. Use multiple ice buckets as needed.

Standards and Controls: Unthaw on ice and keep cold at all times, stored at -20° C.

Tubes: There are two sets of tubes to label for the samples and controls. Only one set of HPLC vials will be needed for the standards and blanks. Keep volume under 2ml for vials to prevent overflow.

Set 1: Eppendorf Safe Lock Tubes 2.0 mL, natural: Label the tops of the caps.
Set 2: 2.0 mL HPLC vials with blue caps and 150 uL inserts.

Tube preparation, control & standards info:

Controls: For this analysis, three human plasma internal controls with known concentrations of non-deuterated TMAO will be used (C1, C2, and C3). C1 and C3 each contain ~9-10 μM of non-deuterated TMAO while C2 contains ~2-3 μM. Each control will be run in triplicate.

Standards: Standards will be prepared in the HPLC vials with blue caps and 150 uL inserts. There are 13 standards ranging from 0 to 100 uM and 2 blanks (both pure methanol). Use a separate rack for standard tubes. Specifically, the individual standards are as follows:

Standards (μM)
0
0.0475
0.095
0.19
0.39
0.78
1.56
3.125
6.25
12.5
25
50
100

Label sample tubes with mouse ID#.

	# of tubes needed
2	Blanks (MeOH)
13	Standards
3	Control 1 (C1)
3	Control 2 (C2)
3	Control 3 (C3)
x	Samples (incl. duplicates, if any)
x + 24	Total

PRM standards for mass spec:

The PRM standards are used to calculate the standard curve. A two-fold serial dilution with **Optima LC/MS grade** methanol is used to make the standards ranging from 100 uM to 0.0475 uM. A 100 uM stock solution is used to prepare the standards. Use empty 2.0 mL HPLC vials with no inserts labeled with the date made and dilution factor and use red caps for storage. At the end of the entire study, dispose of standards by dumping contents in a properly labeled bottle and toss glass vials into glass waste.

1. Label vials with the date made, PRM, and dilution factor e.g. 100 μM.
2. Add 500 uL of Methanol to each standard HPLC glass vial, check tubes.
3. Add 500 uL of 100uM PRM + 500 uL of Methanol to the 50 uM PRM vial by pipette. Cap the vial with a red cap and mix by vortexing.
4. Transfer 500 uL of the 50 uM PRM to the 25 uM HPLC vial. Cap and mix, and put finished dilution on ice as soon as it is finished being used.
5. Repeat process until finished with 0.0475 PRM. **Keep PRM on ice at all times.**
6. Add 80 uL of 5 uM SSTD to HPLC vials with 150 uL inserts that are labeled with the PRM concentrations. These will be used to generate a standard curve from the mass spec analyses.
7. Add 20 uL of the PRM standards to the respective labeled HPLC vials with the 150 uL inserts and blue caps. Gently vortex to mix blue capped vials.
8. PRM standards are stored in -20 freezer after use. The serial diluted PRM standards will be used for future runs as well.

Preparation of SSTD:

Each sample, standard and control has surrogate standards added. 80 uL of 5 uM SSTD are needed for each. To calculate how much is needed for a run, multiply the total number of samples, standards and controls by 80. For example: If you have 33 samples, 13 PRM standards and 3 controls then one would need 49*80= 3920 uL, so about 4000 uL is needed. To make sure there is enough for all samples, make slightly more; about 4200 ul would be ideal.

The stock SSTD is 100 uM. A 20 fold dilution is required to make a 5 uM SSTD. SSTD can be made in 15 mL eppendorf tube using a 1 ml pipet to add the methanol.

If making 4200 uL of 5 μM SSTD, add the reagents in this order:

3,990 ul Methanol (4200-210 ul)
210 ul 100 μM SSTD stock
4.2 ul CUDA/PHAU stock

CUDA/PHAU are used as controls to make sure run is okay, especially for confirmation of appropriate retention times for the analytes. CUDA is non-polar and comes out later in the run; PHAU is very polar so it shoots out of the column and should be one of the first analytes to appear.

Extraction:

1. Pre-chill centrifuge to 10°C and set acceleration to 9, deceleration to 5 (to avoid breaking the pellet when spinning) for the large centrifuge. Leave lid open when done using the centrifuge to avoid collection of condensation inside.

2. Prepare PRM standards by aliquoting 20 uL of prediluted PRM standards and 80 uL 5 uM SSTD to labeled HPLC vials with 150 ul inserts.

3. Thaw samples on ice or in racks on ice. Keep samples on ice.

4. Mix plasma by pipetting up and down, then pipette 20 uL of plasma directly to the bottom of 2.0 mL Eppendorf tubes avoiding adding bubbles.
   - For samples < 20 uL plasma, add methanol to make up the difference, e.g. if there is only 15 uL of plasma used, add 5 uL methanol.

5. Next vortex briefly and add 80 uL of 5 uM SSTD to each Eppendorf tube containing samples and controls.
   - Make sure to aliquot all plasma samples before adding 5 uM SSTD in order to minimize difference in precipitation times between samples.

6. Vortex tubes for 30 seconds on the highest setting to assure proper precipitation.

7. Centrifuge tubes at 18,000 g for 10 minutes at 10° C. There should be a nice pellet after centrifugation.
   - Make sure centrifuges are set to g (which is rcf) and not RPM; RPM is different between centrifuges – large vs small centrifuges will have different RPMs.
   - ***If pellet breaks:***
     a. Transfer the clear supernatant to a Ultrafree MC Centrifugal filter tubes. Be sure the liquid is pipetted directly on the filter membrane.
     b. Centrifuge filter tubes at 12,000 g for 4 minutes at 10° C and transfer filtrate to the labeled HPLC vials with 150 ul inserts.

8. Carefully transfer 100 uL supernatant to the labeled HPLC vials with 150 ul inserts, avoiding the pellet. If under 100uL supernatant is available, transfer as much as possible.

9. Cap HPLC vials with blue caps; vials are now ready for Mass spectrometry run.
   - Keep tubes in the refrigerator until ready for use.
   - Tubes should be analyzed the day of and not stored for long periods of time if possible.

After Mass spec analysis is completed the next day, take samples out of the auto sampler and cap them with the red caps for long term storage. The red caps do not have slits like the blue caps therefore they are suitable for storage. Store vials in a labeled freezer box at -20 C.