

Title: Appendix A

Internal standard preparation for global metabolomics assay

Date Effective: 12/16/14

Compound	Amount (mg)	Product Number (Vendor)
Creatine-D3 H <sub>2</sub> O (methyl-D3)	50 (solid)	D-1972 (CDN isotopes)
L-Leucine-D10	100 (solid)	D-5607 (CDN isotope)
L-Tryptophan-2,3,3-D3	10 (solid)	D-7419 (CDN isotopes)
Caffeine-D3	250 (solid)	D-5912
Salicylic Acid-D4	1mL (liquid)	S-019 (Cerilliant)
Butyric Acid-D7	1 mL (1 g, liquid)	D-171 (CDN isotopes)
Succinic-2,2,3,3-D4 Acid	1 g (solid)	D-197 (CDN isotopes)
2-Acetoxybenzoic-3,4,5,6-D4 Acid	0.01 g (solid)	D6290 (CDN isotopes)

#### Individual stock Preparation

1. Dissolve all 10 mg of L-Tryptophan-2,3,3-D3 in 5 mL of 90/10 water/acetonitrile using a volumetric flask. This yields a solution of 2mg/mL. After complete dissolution, transfer to a clean 5mL microcentrifuge tube or a 15 mL conical tube. Label with compound name, concentration, solvent, date, and initials.
2. Weigh 10mg of Creatine D-3. Transfer to a clean 5 mL volumetric flask and fill to the mark with 90/10 water/acetonitrile. This yields a solution of 2mg/mL. After complete dissolution, transfer to a clean 5mL microcentrifuge tube or a 15 mL conical tube. Label with compound name, concentration, solvent, date, and initials.
3. Weigh 10 mg of L-Leucine-D10. Transfer to a clean 5 mL volumetric flask and fill to the mark with 90/10 water/acetonitrile. This yields a solution of 2mg/mL. After complete dissolution, transfer to a clean 5mL microcentrifuge tube or a 15 mL conical tube. Label with compound name, concentration, solvent, date, and initials.
4. Weigh 10 mg of Caffeine-D3. This yields a solution of 2mg/mL. After complete dissolution, transfer to a clean 5mL microcentrifuge tube or a 15 mL conical tube. Label with compound name, concentration, solvent, date, and initials. Pipette 2.08  $\mu$ L (10 mg) of Butyric acid-D7 into a clean 5 mL volumetric flask. Fill flask to mark with 90/10 water/acetonitrile. This yields a solution of 2mg/mL. Transfer to a clean 5 mL microcentrifuge tube. Label with compound name, concentration, solvent, date, and initials.
5. Dissolve all 10 mg of Acetoxybenzoic acid-D4 in 1 mL methanol and transfer to a clean 5 mL volumetric flask. Fill flask to mark with 90/10 Methanol/water. This yields a solution of 2mg/mL. Transfer to a clean 5 mL microcentrifuge tube. Label with compound name, concentration, solvent, date, and initials.
6. Weigh 10 mg of Succinic acid-D4. Transfer to a clean 5 mL volumetric flask and fill to the mark with 90/10 MeOH/Water. This yields a solution of 2mg/mL. Transfer to a clean 5 mL microcentrifuge tube. Label with compound name, concentration, solvent, date, and initials.

Store all stock solutions in -20 °C freezer

#### Spiking solution preparation

- 1) In a 1.5mL or 2mL microcentrifuge tube add the following:
  - a. 20 $\mu$ L of stock L-Tryptophan-D3 (2mg/mL)
  - b. 2 $\mu$ L of stock L-Leucine-D10 (2mg/mL)
  - c. 2 $\mu$ L of stock Creatine-D3 (2mg/mL)
  - d. 2 $\mu$ L of stock Caffeine-D3 (2mg/mL)

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- e. 4 $\mu$ L of Salicylic Acid-D4 (1mg/mL)
  - f. 4 uL of stock Acetoxybenzoic acid-D4 (2 mg/mL)
  - g. 2 uL of stock Succinic acid-D4 (2 mg/mL)
  - h. 2 uL of stock Butyric acid-D7 (2 mg/mL)
- 2) Add 1mL of 0.1% formic acid in water to each microcentrifuge tube.
  - 3) Close and vortex for 10 seconds
  - 4) This yields the following concentrations
    - a. 40 $\mu$ g/mL L-Tryptophan-D3
    - b. 4 $\mu$ g/mL L-Leucine-D10, Creatine-D3, Caffeine-D3, and Salicylic Acid-D4, Succinic Acid D-4, and Butyric Acid-D7
    - c. 8 $\mu$ g/mL acetoxybenzoic acid- D4
  - 5) 20 $\mu$ L of this spiking solution is used in the extraction method.
  - 6) Store in -20 °C freezer