Protocol MSU_MSMC_004, version 1.2
Two-phase extraction of metabolites from animal tissues

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Purpose

This protocol describes procedures for extraction of polar- and lipid-based metabolites from animal (including human) tissue for subsequent metabolomic analysis via GC/MS and LC/MS respectively. Before tissues are collected, all appropriate ethical oversight approvals and safety training (e.g. for handling of biohazardous materials) must be in place.

Equipment needed

Lab shaker or vortexer
Ultrasound water bath
Centrifuge with rotor for 2-mL polypropylene tubes
Vacuum centrifuge with rotor for 2-mL tubes (e.g. SpeedVac) and cold trap
Explosion-proof or flammable storage freezer (-20˚C)
Analytical balance (to ± 0.0001 g)
Pipetters (1000 µL and 100 µL) and pipet tips (positive displacement pipetters preferred)

Supplies needed

Nitrile gloves
Eye protection (e.g. safety glasses/goggles)
3 mm Chrome Stainless steel ball bearings (#Kit12064, VXB.com) or suitable substitute (may be available from Mass Spec Core
2-mL Safe-Lock polypropylene microcentrifuge tubes
Labels for microcentrifuge tubes and vials (Core lab may provide bar-coded labels)
Vial storage boxes (plastic or cardboard) with labels
Methanol, HPLC grade
Chloroform, HPLC grade
Formic acid, 98%, Millipore-Sigma (Fluka) #94318
Butylated hydroxytoluene (BHT), solid

GENERAL REQUIREMENTS

- Gloves must be worn at all times when handling tissue specimens, removing lids from microcentrifuge tubes, centrifugation, pipetting, disposal of contaminated tubes, and cleanup of spills. All plasticware must be properly disposed of in biohazard containers in accordance with institutional requirements.
- All other institutional requirements and local health and safety procedures for working with tissue samples should be followed, including gloves, eye protection or working in a biosafety cabinet for subsequent processing.
- All equipment and samples (during storage and shipping) must be labeled as biohazard.
- Steps should be taken to prevent thawing of samples during transportation (i.e. keeping on dry-ice during transportation).

Preparation of Reagents and Supplies

1. 3-mm stainless steel ball bearings should be cleaned by sonication in methanol in a clean glass beaker followed by drying on clean paper towels.
2. Prepare a mixture of 1:2 methanol/chloroform containing 0.01% BHT and 1% formic acid (by volume), with enough total volume to provide at least 1000 µL for each sample including process blanks. Store in a glass media bottle with PTFE-lined cap in an explosion-proof or flammable-storage freezer (-20˚C).

Metabolite Extraction

1. If specimen is frozen, initially defrost in refrigerator (4˚C) or on ice prior to exposure to room temperature. The frozen tissue may be cut with a razor blade or scalpel to suitably-sized portions.
2. Transfer 50 mg of tissue to a labeled 2-mL polypropylene microcentrifuge tube (Safe-Lock)
3. Add internal standard cocktails (both polar cocktail and lipid cocktail) to each tube (composition of internal standard to be defined as appropriate for metabolites of interest). Initial suggestion: add ***25 µL of polar internal standard cocktail*** (containing 10 µg of [$^{13}C_6$]D-glucose; 12.5 µg of phenylalanine-d_7; 12.5 µg of [$^{13}C_3$]pyruvic acid, sodium salt; 2.5 µg of inosine-1',2',3',4',5'-$^{13}C_5$, and ***25 µL of nonpolar internal standard cocktail*** (containing 12.5 µg of palmitic acid-d_{31}). See **Protocol MSU_MSMC_005** for details regarding preparation of internal standard cocktails.
4. Add 600 µL of methanol/chloroform (1:2 v/v) + 1% formic acid and 0.01% BHT and one 3-mm ball bearing per tube; seal the tube
5. Homogenize using a lab shaker or vortexer for 3 minutes.
6. Sonicate in water bath for 15 minutes
7. Add 500 µL of Milli-Q water (> 18 MΩ) and vortex for 15 seconds
8. Centrifuge at 13500 x *g* for 15 minutes at ambient temperature
9. Transfer the upper (aqueous layer) to an additional 2- mL polypropylene microcentrifuge tube
10. Repeat steps 7-10, pooling the two aqueous volumes into the same receiving tube (~ 1000 µL).
11. Remove 200 µL of the combined aqueous extracts and transfer to a labeled microcentrifuge tube for storage (at -80 ˚C). This aliquot may be used for analysis of volatile metabolites that would otherwise be lost during solvent evaporation.
12. Evaporate the remaining aqueous fraction (~800 µL) to dryness in the SpeedVac without application of heat.
13. The lower (chloroform) layer may be evaporated to dryness under a stream of nitrogen.
14. After solvent evaporation, seal the microcentrifuge tubes from both the aqueous and chloroform phases, and store them at -20˚C for up to 30 days (store at -80˚C) if storage for longer is required.

Derivatization procedures for methoximation and trimethylsilylation are described in **Protocol MSU_MSMC_007**.