This protocol is modified from Kevin Wong Metabolomics extraction protocol. Revisions here are to extract metabolites from C-13 labeled larval samples in the Mcap2021 and Pacu2021 projects.

<https://kevinhwong1.github.io/KevinHWong_Notebook/Metabolomics-P-astreoides-sample-prep/>

Modified 4/8/2022 by Ariana Huffmyer for Nov 2022 samples:

Notes from Nov 2022 updates to protocol:

* If needed, use Pacu 2021 samples that are C12 labeled as practice
* For these we are going to separate from all samples but only extract and send the host
* We are only sending for one polarity
* We will only separate into host and symbiont
* On test samples look for symbionts in host fraction and add another centrifuge if necessary
* Set order of sample processing:
  + Clean all equipment
  + First: C12 controls; Second: C13 dark; Last: C13 light
  + Clean in between samples and refresh all washes in between sample type
  + Do separations first; do extractions next
  + Washing/cleaning all equipment and surfaces
* AH ask eric if he wants a backup sample (or keep in freezer as backup)
* Check for symbionts on test samples
* Make new extraction buffers? Make new 15% ammonium bicarbonate? Include recipe and amount we need.
* Add prep list - solutions, labeling, equipment, autoclave the dounces and graduated cylinders
* Shipping to Eric - on dry ice or in dewar? JA ask Kevin
* Hazardous waste disposal?
* C12 (control), C13 (incubated in dark), C13 (incubated in light - came from symbiont)

**—- SEPARATING HOST, HOLOBIONT, SYMBIONT FRACTIONS —-**

Prior to metabolite extraction, separate host, symbiont, and holobiont fractions for further extraction.

Before starting, label the desired tubes. For each biological sample (indicated by #), you will need to label one of each of the following tubes:

1.5mL tubes:

#-Host

#-Sym

#-Holo

1. Thaw sample (2mL tube) on dry ice
2. Add 200uL of ice cold DI water
3. Homogenize with motor for 45 sec-1 min on ice
4. Cap the sample and place on ice.
5. **-CRITICAL-** Clean the motor with 10% bleach, 70% ethanol, and DI water after each sample.
6. Remove 100uL of the homogenized holobiont and place into new tube (#-Holo).
   1. Store on dry ice for further processing or put into -80C depending on samples you need to extract.
7. Remove the remaining homogenate and place into a new tube (#-Sym).
8. Spin the “Sym” tube at 2000g for 90 seconds **at 4C**.
9. Remove the supernatant and place into a new tube (#-Host).
   1. Store on dry ice for further processing or put into -80C depending on samples you need to extract.
10. The “Sym” tube now only contains a symbiont pellet.
    1. Add 100uL of DI water to the tube with the symbiont pellet and pipette up and down to mix
    2. If necessary, move the symbiont homogenate into a new tube.
    3. Store at -80C if not used for extractions.
11. Samples can be stored in the “Pre-Processing” freezer box.

See sections below for extraction with protocol variations for each fraction.

**—- METABOLITE EXTRACTION —-**

Prior to starting, label the following tubes for each sample fraction to intend to extract. Here, # indicates the biological sample ID and “Fraction” indicates the fraction (host, symbiont, or holobiont). Refer to sample list for desired fractions for extraction.

1.5mL tubes:

#-Fraction-A

#-Fraction-B

Autosampler vials:

#-Fraction-A Date Initials

#-Fraction-B Date Initials

Extraction:

1. Thaw chosen sample on dry ice if using frozen homogenized fractions. If proceeding directly after homogenization, immediately proceed with sample of interest in the steps below.
2. Add sample to the ice cold douce with 500uL of ice cold extraction buffer. Sit on dry ice for **5 minutes.**
   1. If extracting the HOST or HOLOBIONT fractions, pipette the liquid fraction obtained from separations into the dounce.
   2. If extracting from the SYM fraction, add 500uL of the ice cold extraction buffer directly to the Sym 1.5mL tube. Use a pipette to gently resuspend the symbiont pellet. Then transfer the extraction buffer and suspended symbiont pellet to the dounce.
3. Homogenize the sample on dry ice for **~1 minute** with the douce.
4. Transfer extract to a new 1.5 mL tube (Tube #-Fraction-A).
5. Centrifuge for **10 minutes** at 15000g at 4C
6. Remove 500uL of the supernatant to a new 1.5 mL tube (Tube #-Fraction-B)
7. Add 44uL of 15% ammonium bicarbonate
8. Vortex and spin down
9. Add 100uL from Tube B into duplicate labeled autosampler vials (#-Fraction-A and #-Fraction-B)
10. Store the two 1.5 mL extract tubes (Tubes A and B) and the two autosampler vials at -80C in the “Post-Extraction” freezer box.