

Preparation of Lyophilized Polar extracts for GCMS analysis

Materials (day 1):

- 40% Trichloroacetic Acid (TCA) in water
- GC vials labeled for blank and standard (include the standard series number and the date). Tape these vials to preserve the label.
- The GCMS standards are lyophilized in 4 fractions. They are in 0.5 ml tubes in -80 freezer #5 in boxes labeled "GC/MS standards 5/22/15 J.L.". Tubes are labeled with the series number first and fraction number second (e.g. 20-1, 20-2, 20-3, 20-4). Look carefully and be sure you have the next set in the sequence. Note which set you used for future reference.
- Water with NorLeu Master Mix – 5 nmoles of Norleucine per sample. You will need between 25 and 50 ul for each sample (plus enough for the blank). For example, for 10 samples mix 5 ul 10mM NorLeu and 495 ul water and use 50 ul per sample.
- Liquid Nitrogen for freezing

NOTE: Once the TCA is added, the metabolites can be lost. Samples must be frozen very quickly after adding TCA.

NOTE: One standard contains enough volume to quantify 40-50 samples. Sample sets larger than 50 samples should be split up and prepared in smaller batches.

Procedure (day 1):

1. Defrost lyophilized samples and standards on ice.
2. Prepare LN₂ for freezing the GC vials
3. For the standard, add Master Mix (between 25 and 50 ul; to deliver 5 nmoles of NorLeu) to the fraction #1. Pipet to mix and dissolve then transfer to microfuge tube holding fraction #2. Continue until all 4 fractions of the standard are dissolved and combined, then transfer solution to the labeled GC vial.
 - a. **Add 40% TCA to make final concentration of 10% TCA (if used 50 ul of master mix, add 16.7 ul of 40% TCA for final concentration of 10.1% TCA)**
 - b. FREEZE IMMEDIATELY in LN₂
4. For the samples, add Master Mix (between 25 and 50 ul; to deliver 5 nmoles of NorLeu) to the samples on ice. Vortex to dissolve.
 - a. **Add 40% TCA to make final concentration of 10% TCA (if used 50 ul of master mix, add 16.7 ul of 40% TCA for final concentration of 10.1% TCA)**
 - b. FREEZE IMMEDIATELY in LN₂ This step must be done one sample at a time. Freeze each sample as TCA is added.
5. For the blank, add Master Mix (between 25 and 50 ul; to deliver 5 nmoles of NorLeu) to the labeled GC vial. Freeze in LN₂
6. Lyophilize overnight with a liquid nitrogen trap to trap the TCA.

Materials (day 2):

- Lyophilized, acidified samples, standard, and blank.
- MTBSTFA solution. This solution must be kept sealed and in the dark as much as possible.
- 100 or 200 ul pipet and tips.
- Crimp caps and crimper
- Glass beaker(s) to hold GC vials
- Sonic bath, drain until water level is about 1-2 inches. It should come up the outside of the beaker no higher than the shoulder of the GCMS vial inside the beaker. DO NOT put any trays or racks in the sonic bath.

NOTE: MTBSTFA is highly toxic. It should only be used in the fume hood. All labware contaminated with MTBSTFA must be left in the hood until completely dry.

NOTE: MTBSTFA is also highly hydrostatic and will degrade upon wetting. Care must be taken to keep air OUT as much as possible.

Procedure (day 2):

1. Gather all items (except the sonic bath) in the fume hood. The preference is to use Hood #1 (near the GCMS), unless it is busy.
2. Working with one sample at a time, add 50 ul of the MTBSTFA solution and cap the sample.
 - a. It is advised to have two people for this operation. If working alone, place the cap back on the reagent bottle while capping the sample. It can help stabilize the reagent bottle against tipping over to place it in a small beaker or rack during use.
 - b. Make sure the vial is sealed. If the cap twists on the vial, it is not tight enough. Crimp, turn the vial in the crimper 90-180 degrees, crimp again.
 - c. Make sure the vial labels are taped. You can also label the caps, to be sure. The sonic bath is very effective at removing labels from vials.
3. Place the GC vials in GLASS beaker(s). If the beaker(s) is not full (i.e. vials could fall over), place empty vials in to take up the room. DO NOT PUT ANYTHING PLASTIC IN THE BEAKER – the sonic energy is not effectively transmitted through materials other than glass.
4. Add water to the beaker(s) to a level above the level of the sample in the vials but below the shoulder of the vials. There will be considerable evaporation, so put enough that the water level will still be above the liquid level in the vial after 5 hours of sonication. You do not want your vials to float, however.
5. Place the beakers in the sonic bath and secure with lead weights as needed. Turn on the sonic bath (set dial to “hold”) and set timer to turn it off in 5 hours (the number “5” should be flashing). Leave it overnight.

Materials (day 3):

- Derivatized samples, standard, and blank
- Autosampler vials, labeled to match GCMS submission sample IDs, inserts, and caps.
- 100 or 200 ul pipet and tips.

- Decrimper and dry waste bucket
- GCMS submission file on flash drive

Procedure (day 3):

1. Remove samples from beakers and wipe dry.
2. Collect all materials (except the flash drive) in the fume hood.
3. Decap and transfer samples to inserts in autosampler vials.
 - a. Cap each sample as you transfer – keep air out of the samples as much as possible.
 - b. Discard tips and caps in an empty waste bucket. Leave this and the used GC vials in the hood until the next day. Return the next day and discard the GC vials in glass waste and the tips to an appropriate container.
4. Centrifuge samples in the vacuum centrifuge for 20 minutes.
5. Transfer the GCMS submission file to the instrument computer.