PREPARATION FOR CSIA

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Tissue prep

1) Rinse wet samples with DI water
    a. For coral samples, after tissue blasting and centrifugation for host/symbiont separation, collect coral tissue and symbiont cell pellet stored in DI water (for lipid, also: with 20 µl / ml BHT and 20 µl / ml indomethacin)
    b. Freeze dry, store in the dry cabinet
2) For dry tissue/plant samples, dry in oven, grind in a mortar and pestle.
3) All samples should be stored in -80 until freeze dried, then stored in dry cabinet.
    a. Do NOT store in ethanol – leaches carbon. Do not store in "cheap" plastic – could leach nitrogen.
4) Weigh samples (>5 mg of dry weight) using a micro balance

FATTY ACID CSIA

Lipid Extraction

1) Prepare **Folch solution** [Chloroform:Methanol in the ratio of 2:1 (v/v)], with 0.01 % (w/v) BHT and Indomethacin
2) Add 5 ml of ice-cold Folch solution into 15 ml conical tube containing sample
3) Homogenize the sample using blade homogenizer or ultrasonication. Keep the conical tube in the icebox.
4) Rinse blade with another 5 ml of Folch solution in the 15 ml conical tube and combine both conical tubes (total volume 10 ml)
    a. ***Add Internal Standard (C19:0, C23:0 etc.)***
5) Seal the conical tube with nitrogen gas and incubate horizontally in the ice box on the shaker for 30 min
6) Add 2 ml aqueous 0.9 % NaCl and vortex vigorously for 30 sec and centrifuge for 10 min at 3000 rpm, 4 °C
7) Transfer the lower phase (Chloroform) into glass vial (if you want to measure total lipid, weigh empty vial) using 230 mm glass Pasteur pipette
8) Dry under the stream of nitrogen gas, on the 37 °C heating block
9) Weigh vial with totally dried lipid to measure total lipid

Hydrolysis & Methylation

1) Redissolve lipid in 3-4 ml of Hexane
2) Add 1 ml of H_2SO_4-Methanol (1:20, v/v) and purge with nitrogen gas and tighten the Teflon liner-cap.
3) Through this step, extracted lipid turns into fatty acid methyl ester (FAME)
4) Incubate in the oven for 2 hrs at 100 °C
5) Add 1 ml of Hexane and 1 ml of DI water
6) Centrifuge for 5 min at 2500 rpm, 4 °C
7) Transfer upper layer (Hexane) which is containing FAME into 4 ml of vial using a Pasteur pipette
8) Dry under the stream of nitrogen gas, on the 37 °C heating block
9) Redissolve in 150-200 µl Hexane and transfer to GC vial
10) Inject to the GC

Amino Acid CSIA

Lipid Extraction (~3 hours – consult FA lipid extraction above as well)

1) Prepare Folch solution - Chloroform : Methanol in the ratio of 2:1 (v/v), with 0.01 % (w/v) BHT to prevent peroxidation. Store at -20 °C.
2) Weigh ~1.0 – 10.0 mg of dry samples into 15 ml falcon tube
    a. ***THIS STEP IS CRITICAL*** – this determines the maximum amino acid/lipid amount that will then be used for the remainder of analysis. Do some research/talk to technician about ideas on how much material should be used, ESPECIALLY for material limited samples. May require testing.
    b. For coral, we use ~3-5mg (host and symbiont) with our current machine and set up BUT check literature or ask for previously run samples and use minimum needed.
3) Add in 5 ml of ice-cold Folch solution to tube containing sample. Also, add 5 ml of additional Folch solution to empty tube to be used as rinse.
4) Place the sample tube in the icebox and Homogenize the sample using blade homogenizer (T25, ULTRA-TURRAX, IKA) at 24,000 rpm in 2 x 20 sec bursts.
5) Rinse the blade for 20 sec with other 5 ml ice-cold Folch solution in 15 ml rinse tube. Combine both portions, totaling 10 ml Folch solution and homogenized sample.
    a. Wash the blade homogenizer with 1 - Milli-Q water and 2 - Methanol in between samples to prevent cross-contamination.
    b. Add Internal Standard (If, quantifying fatty acids)
6) Vortex tube for 30 seconds
7) Incubate horizontally in ice on shaker for 30 min.
8) Vortex tube for 30 seconds
9) Centrifuge at 3000 x *g* for 10 min, 4 °C.
    a. Make sure to wrap tubes in parafilm to eliminate leakage.
10) Pour off supernatant into another 15 ml tube. Keep pellet – this is your lipid extracted sample
11) Dry sample at room temp in hood.
    a. **STOP HERE** if only doing AA analysis. Store dry sample until ready for processing. Then continue to Acid hydrolysis steps.
    b. **IF INCLUDING** lipid analysis as well, go to next steps.
12) Add 2 ml aqueous 0.9 % NaCl to lipid supernatant in order to divide phase separation.
13) Discard upper phase (methanol) and transfer the lower phase (Chloroform) into a glass vial (***measure glass vial weight before transfer***) using 230 mm glass Pasteur pipette.
14) Dry totally under the stream of nitrogen gas, on a 37 °C heating block. (around 90min)
15) ***Measure the glass vial*** and calculate total lipid

Lipid Storage

1) Redissolve lipids in 1 ml Methanol containing 0.005 % BHT
2) Purge with nitrogen gas and store in the freezer.

Acid Hydrolysis (~1 hour)

1) Add lipid extracted sample into a hydrolysis tube
2) *FUTURE STEP: Add in "internal standard" (norleucine?) to sample – amount to be decided.*
3) Add 1ml of ultra-clean 6M HCl, from purified glass vials.
4) Flush with nitrogen before sealing with caps (about 10 seconds, start at bottom of vial and move up).
5) Place in oven at 110C for 20 hours (check caps after 5 minutes, tighten if needed).
    a. See Figure 1 for hydrolysate.
6) Transfer to small muffled vials with Teflon caps

a. If dirty, do this by filtering through small filter using "broken" glass pipette setup, Figure 2. These filters do not work great, looking at better options for future.

Dowex ion-exchange chromatography purification (2-day set-up, ~6 hour process)

1) Dowex 50W8-400, Sigma-Aldrich needs to be activated in order for ion-exchange to work. So, **TWO DAYS before you want to derivatize**:
    a. Mix Dowex (about 10mg for ~6 samples) with 3 M NaOH. Let sit overnight. Stir a few times.
    b. Pour off NaOH (let Dowex settle), then rinse 3x with MQ water. (each time stir, let settle, pour off).
    c. Add 6M HCL to Dowex. Let sit overnight.
    d. Repeat rinse step. Then keep Dowex suspended in clean MQ water (Dowex slurry) – it is ready for use.
2) Set up Pasteur glass pipettes for column chemistry (see Figure 3). Make sure you have added in glass wool to taper of pipette. Make sure you have some stopper at tip to stop eluting liquids.
3) Stir Dowex slurry to suspend settled particles. Then, in 1-ml aliquots, pipette (can use standard plastic tips) into Pasteur glass pipettes. Let settle (MQ water will settle on top, creating a water layer). Continue until you have added ~5cm of Dowex to the column, eluting some water if needed for space.
4) Drain the settled water down until it is just ABOVE the layer of Dowex (it is important to never let the Dowex be exposed to air).
5) Add ~500 uL of filtered hydrolysate (sample). Run down into the column by eluting water until sample is right at Dowex level.
6) Elute salts
    a. In 0.5-1 mL increments, run 6 mL of MQ water through the column, and elute out. Collect, and discard.
7) Elute AA
    a. In 0.5-1 mL increments, run 6 mL of 2M NH_4OH through the column, and elute out. Collect in small glass muffled vials, and KEEP.
        i. Some literature only uses 4mL to elute. But this has not been tested yet at HKU.
    b. Because the small muffled vials are only ~4ml, after half the process stop and dry down a bit, and then finish elution OR collect in two vials then combine.
8) Dry down collected NH_4OH+AA elute under N2 at 80C.
    a. Because of the MQ water, this will take ~5 hours.
9) After fully dried, add in 0.5 mL of 0.1 M HCL to dried glass vial. Store in freezer until derivatization.

Derivatization (~4-6 hours)

AMINO ACID DERIVITIZATION:

1) Add ~100 uL of stock standard solution OR sample to small muffled glass vial
    a. If Dowex ion-exchange purified step was used, add half of the sample (~250 uL) to glass vial, and archive the other half.
2) Dry down at 110C under N2

Carboxyl Derivatization (acetyl chloride & isopropanol):

1) On ice, add 1ml of acetyl chloride to 4ml of isopropanol (2-proponol)
    a. Mix solution in glass tube. ALWAYS add acetyl chloride to the isopropanol.
    b. The ratio, 1:4 acetyl chloride to isopropanol is what's important. If you have more than 5 samples, you will need to make more solution. It is smart to make one EXTRA aliquot then number of samples, in case of imprecise pipetting.
2) Add 1ml of isopropanol /acetyl chloride solution to sample vials and cap vials
3) Heat at 110C for 1 hour (check caps after 5 minutes, tighten if needed)
4) COOL samples in FREEZER for ~5-10 minutes before opening

5) Carefully dry down at room temperature (under N2); do no over/under dry
6) At room temperature, rinse 2X with 250ul of DCM to remove acetyl chloride
7) If break is necessary, add 200ul of DCM and put in freezer overnight

Amine Derivatization (TFA anhydride & DCM):

1) Add 0.5ml DCM and 0.5ml of TFA Anhydride to each sample vial
2) Heat at 110C for 10 minutes (check caps after 5 minutes, tighten if needed)
3) COOL samples in FREEZER for ~5-10 minutes before opening
4) Carefully dry down at room temperature (under N2)

Concentration/dilution

1) Add anywhere from 200ul or 500ul DCM to samples for machine run
    a. This is dependent on whether you are running Carbon (more dilute) or nitrogen (more concentrated). Also, dependent on the amino acid concertation of sample.
    b. As an example, standards are usually diluted with 500 uL as they have a strong signal. Samples typically diluted 200 uL for carbon analysis. But check literature/test out first.
    c. If completely unknown, test with 200uL DCM and then watch initial run of sample. If too strong a signal, remove and dilute before next run. If too weak, take off and dry down. Then dilute with less DCM quantity. Work with lab tech during process.
2) Place in freezer until ready for GC-IRMS
3) Before run, add to small Thermo vials, and cap.
    a. Use a glass insert if quantity if less then 500uL.

Chemicals

- Folch solution - Chloroform : Methanol in the ratio of 2:1 (v/v)
- HCL (6M purified)
- Ammonium hydroxide, 2M
- Dowex 50W8-400, Sigma-Aldrich (100-200 mesh, though literature uses 200-400)
- DCM (dichloromethane)
- Acetyl Chloride: Fluka (00990)
- Isoproponol (2-proponol): Fisher Scientific, HPLC Grade (A451-1)
- TFA (trifluoroacetic) anhydride: Pierce (67363)

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Pictures

Figure 1 - Hydrolysate after 20 hours. Note, some samples may be clean (left two) or "dirty" (right two) – remnant material. These will need to be filtered and (likely) Dowex purified.

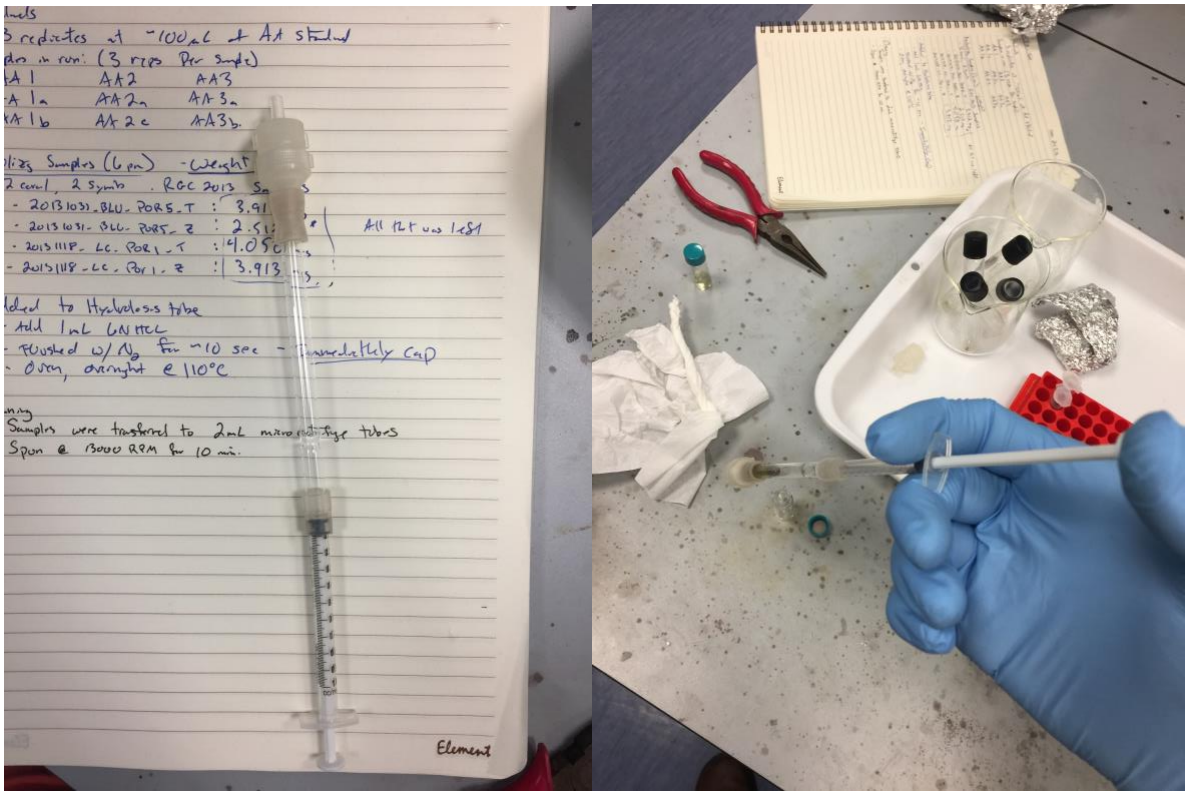

Figure 2 – Filter setup. After hydrolyzation, suck up sample with just broken glass pipette, then attach filter tip, then pass through filter into vial.

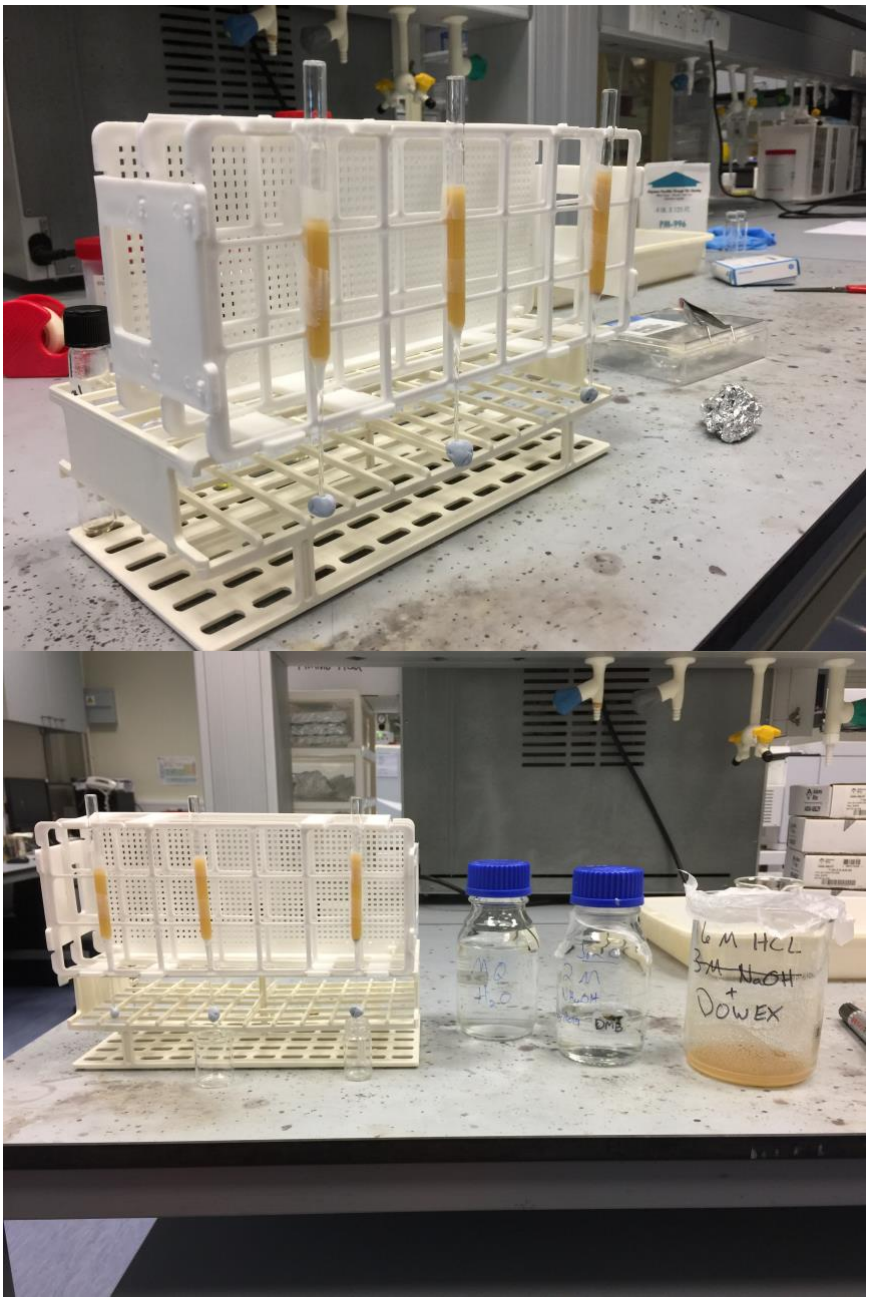

Figure 3 – Dowex ion-exchange column set up.

Figure 4 – Drying in and out of the block / heating in the block. Note, for all steps BUT the first dry down, the vials are dried at room temp (second picture, not in the heated block).