Fourth, how did you come up with the volumes you used for extraction? Often I find it useful to extract in a certain volume that I think will get all the lipids and then extract the pellet again and determine whether there is a substantial quantity of lipids left int he pellet that I am missing. Like we talked about with the weighing after freeze drying, it is important to take a written protocol and validate it in your hands with you own samples.

**Freeze, Freeze Dry under Nitrogen, Vacuum Dry, Extract and Remove Non-Target Lipids**

[Compiled from Hahn lab protocols]

Freeze Dry

*Beforehand prepare samples by* ***weighing*** *larvae* ***then freezing*** *at -80C. Once frozen,* ***slice*** *them in half longitudinally with a razor blade on a blue ice pack so they stay frozen. Put the cut pupa into a medium plastic weighing boat with the cut side up, and place both back at -80 o C until drying and analysis. (Dan recommends freeze-drying to oven drying because you are less likely to have problems with lipid oxidation)*

Freeze dry

*Operating the freeze dryer at the USDA*

*Drain the condenser into a vessel and dispose of melt water immediately before use and hours after turning off condenser.*

*Turn on condenser, allow temp to drop to -80C*

*When ready, quickly place sample inside the drum, close lid and engage the vacuum.*

*The pressure will drop until it reaches approx 400mTor*

*Turn off the vacuum and allow pressure to reduce before opening lid*

*Speak to Chip before and after using instrument as a courtesy.*

Dry

* Place in lyophilizer and apply vacuum
  + Apply vacuum for ~72hrs or until they lose less than 1% of weight in 24hrs
* Open valve, turn off vacuum, remove samples
* Take dry weight of insect sample

Separate

* Turn on centrifuge, set at 4C
* Prepare solvent [ CH2Cl2 + H2O ] and [ H2O + MeOH ]
* Add BHT antioxidant to tissue samples…..Quantity??
* Divide samples into 4 categories of tubes
  + Insect sample + Tri Mix + BHT
  + Insect sample + BHT
  + Tri Mix + BHT
  + BHT only
* Bead beat samples without solvent solution for 20s at “5”
* Add polar solvents (Methanol and H2O) 1.4mL to tube **with** **sample**, 1mL MeOH first then 0.4mL H2O
* Add polar solvents (Methanol and H2O) 1.4mL to tube **without sample**, 1mL MeOH first then 0.4mL H2O
* Add polar solvents (Methanol and H2O) 1.4mL to tube **with TAG spike-in and sample**, 1mL MeOH first then 0.4mL H2O
* Add polar solvents (Methanol and H2O) 1.4mL to tube **with TAG spike-in and without sample**, 1mL MeOH first then 0.4mL H2O
* Bead beat for 20s at “5”
* Switch samples to glass vial before adding solvents
* Add non-polar solvent (Dicholoromethane and H2O) 1.5mL 2:1 [ 1mL CH2Cl2 + .5mL H2O ] to centrifuge tubes **with** **sample** and beads, then homogenize
* Add non-polar solvent (Dicholoromethane and H2O) 1.5mL 2:1 [ 1mL CH2Cl2 + .5mL H2O ] to centrifuge tubes **without sample** and beads, then homogenize
* Add non-polar solvent (Dicholoromethane and H2O) 1.5mL 2:1 [ 1mL CH2Cl2 + .5mL H2O ] to centrifuge tubes **with TAG spike-in and sample** and beads, then homogenize
* Add non-polar solvent (Dicholoromethane and H2O) 1.5mL 2:1 [ 1mL CH2Cl2 + .5mL H2O ] to centrifuge tubes **with TAG spike-in and without sample** and beads, then homogenize
* Bead beat for 20s at “5”
* Centrifuge at max for 5mins, 4C, let contents settle for 5mins
* Draw off supernatant and store in a weighed pooling container
* (2) Re-suspend pellet in solvent
  + Shake tubes at 200rpm for 15mins
  + Centrifuge at max for 4mins, let contents settle for 5mins
  + Draw off supernatant and store in a pooling container
* (3) Re-suspend pellet in solvent
  + Shake tubes at 200rpm for 15mins
  + Centrifuge at max for 4mins, let contents settle for 5mins
  + Draw off supernatant and store in a pooling container
* Add 0.9% NaCl to the pooled fractions, centrifuge at max for 30sec, remove and discard aqueous layer.
* Dry extract under Nitrogen in the hood and evaporate solvent: **Freeze @ -80C to hold until next step**
  + Warm water bath speeds up this process

Remove non target lipids (SPE column)

* Exclude phospholipids using SPE column and a non-polar solvent.
* Re-suspend sample in non-polar solvent (hexane, dichloromethane?), place in column
  + Wash tube with 2x solvent to clear residual lipids
* Use 8mL of non polar solvent at 1mL at a time to wash neutral lipids into collection reservoir.
  + Using TLC test the collected fraction when 6mL of the solvent is eluted. With too much solvent the polar will wash through
* Dry extract under Nitrogen in the hood
* Ready for FAME

**Derivitize, Acidify with H2SO4, Separate Phases, and Store**

 doi:[10.1194/jlr.D001065](http://dx.doi.org/10.1194%2Fjlr.D001065), and Beck Lab protocols

**Make FAME**

* Add in appropriate amount of 10M KOH
  + To make up a 10M KOH soln: 56.11g KOH + 100mL H2O
    - Use a ratio of 0.5 g larvae/ 0.7 mL 10M KOH in water soln
    - Ex : For 0.01g sample, use 14 uL KOH
* Add in appropriate amount of MeOH also
  + Use a ratio of 0.5 g larvae/ 5.3 mL MeOH
  + Ex : For 0.01g sample, use 106 uL MeOH
* Put vial in 55C water bath for 1.5 Hr with vigorous hand-shaking 5 sec every 20 min
  + (weigh vial before and after to note any volume changes)
* After 1.5 Hr, cool vial in cold water bath for ~5 min

**Precipitate Potassium**

* Add in appropriate amount of 12M H2SO4
  + To make up a 12M H2SO4 soln: 31.95mL H2SO4 + 18.05 mL H20
    - Use a ratio of 0.5 g larvae/ 0.58 mL 12M H2SO4 in water soln
    - Ex : For 0.01g sample, use 11.6 uL H2SO4
* Mix tube by inversion and precipitated K2SO4 should form. Incubate again in 55C water bath for 1.5 Hr while shaking tube 5 sec every 20 min
* After 1.5 Hr, cool vial in cold water bath for ~5 min

**Separate Phases**

* Add 1.0 mL hexanes, mix tube and leave at RT for 5 min. Layers should form, and extract the top hexane layer into new vial.
* Add 5-6 granules of Sodium Sulfate to dry hexane layer.
  + This will remove water from the solution.
* Transfer Dried hexane layer to another new GC vial. Cap, Label, and **place vial at -20C until GC analysis**