**Extraction and concentration of neutral lipids** [Compiled from Hahn lab, Beck lab, and referenced literature]

In vivo and in vitro studies using several insect orders showed DAG is the main lipid in the hemolymph after lipid digestion (10, 22, 90, 128, 155). In *M. sexta* it was demonstrated that dietary lipid is the source of lipophorin DAG because insects raised on a fat-free diet contained a circulating lipophorin essentially depleted of DAG (51). In the same insect, nearly 90% of fatty acid–labeled triolein was absorbed after 4 h, and of that absorbed, more than 70% was found in fat body as TAG (128). In the hemolymph, more than 90% of the label was in DAG and all the DAG was associated with lipophorin. Also, in *M. sexta* larvae, a kinetic model for DAG export showed that its release from the midgut occurs at a rate consistent with the intracellular lipolysis of the TAG pool (22; ER Rubiolo, MA Wells, unpublished data). –Canavoso 2001

Freeze

*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. Or hold them in liquid Nitrogen bath to keep frozen while slicing. Put one cut larva into a 1.5-2mL round bottom polypropylene centrifuge tube and return to the -80˚ C until drying and analysis. (Dan recommends freeze-drying to oven drying because you are less likely to have problems with lipid oxidation)*

*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

*Take wet weight of the samples*

* Place in lyophillizer 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
* Store in polypropylene centrifuge tubes

Solubilize and Extract

DOI:[10.1016/j.jchromb.2009.09.040](http://dx.doi.org/10.1016/j.jchromb.2009.09.040)

Modified from: [doi:10.1016/j.cbpb.2013.05.001](http://dx.doi.org/10.1016/j.cbpb.2013.05.001), Folch 1956, Hahn Lab Methods

* Turn on centrifuge, set at 4C.
* Prepare solvents day of experimentation in Qorpak F217-PTFE vials GLC-01002
  + 5mL MeOH + BHT, 5mL MeOH, 10mL Hexane
    - Add 1% of BHT to MeOH: 1mg/1mL of 2:1 Hex:MeOH
    - BHT: MP biomedicals lot 6029
  + 15mL 0.9% NaCl
    - Use ratio 0.9g NaCl/100mL of H2O = 0.9% solution
  + Using a capillary tube add “spike-in” TAG standards at rate of 20uL per sample directly to sample
* Treatments

|  |  |
| --- | --- |
| * Insect sample + TAG + BHT * Insect sample + BHT * Tri Mix + BHT * BHT only | * Insect sample + TAG * Insect sample * Tri Mix * Blank |

Homogenize

*Take weight of pooling vials.*

* Bead beat samples without solvent for 20s at “5”
  + Grinding media depot beads: 1.1-1.4mm Zirmil.2
* Add 0.3mL of MeOH. Homogenize for 20s at “5”
* Add 0.6mL of Hexane. Homogenize for 20s at “5”
* Centrifuge at max for 5mins, 4C, let contents settle for 5mins
  + Eppendorf Centrifuge 5430R
* Draw off Hexane supernatant and store in a weighed pooling container
* (2) Re-suspend pellet in 0.3mL of 2:1 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 0.3mL of 2:1 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 600uL of 0.9% NaCl to the pooled fractions, centrifuge at max for 30sec, remove and discard the denser aqueous layer.
  + (Salt water density: 1.03g/ml) (hexane density: 0.66g/ml)
* Dry pooled extract under Nitrogen in the hood and evaporate hexane:
  + *If using the “Flexi-Vap”: Turn on Flexi-Vap. Open Nitrogen tank valve, connect instrument hose to hood gas tap, open small valve to hose. Place tubes in holding tray, lower needles and allow time for drying*. Warm water bath speeds up this process
* **Take weight of samples then freeze @ -80C until next step**

Remove non target lipids (SPE column)

doi:[10.1007/s11745-011-3531-7](http://dx.doi.org/10.1007/s11745-011-3531-7)

*Take weight of pooling test tube*

* Exclude phospholipids using SPE column and a non-polar organic solvent.
* Re-suspend sample in 500uL of hexane (vortex for 5 sec) and place on silica gel column (C18), then repeat.
* Use 8mL of hexane (1mL increments) to wash neutral lipids into collection reservoir.
  + Too much hexane will wash polar compounds off column
* Verify collected sample at 5mL using TLC
  + Setup TLC plate with detector and visualize using UV
    - Mark TLC silica plates using pencil (plate with an indicator preferred)
    - Place a filter paper into the solvent to help permeate the solvent throughout the chamber.
    - Apply test 1-10uL of sample solution to TLC plate
* Dry collected fraction under Nitrogen in the hood.
* **Take weight of samples and freeze @ -80C until next step (up to 5 days).**

Alkylate, Acidify, Separate Phases, and Store

doi:[10.1194/jlr.D001065](http://dx.doi.org/10.1194%2Fjlr.D001065), and Beck Lab protocols (SLF-B27-74)

Alkylate

* Turn on hot plate to 55C
* Prepare consumable volumes of 10M KOH, 12M H2SO4 MeOH, & Hexane
  + 10M KOH soln: 56.11g KOH + 100mL H2O

|  |  |  |  |
| --- | --- | --- | --- |
| 10M KOH | 56.11g KOH | .100L 10M KOH | =56.11g KOH |
| 1L KOH | 1M KOH | 1 KOH soln |  |

* + 12M H2SO4 soln: 31.95mL H2SO4 + 18.05 mL H20
    - C1 = [(% x density) / MW] x 10
    - C1 V1=C2V2
* Add 10M KOH
  + Use a ratio of 0.5 g larvae/ 0.7 mL 10M KOH in water soln
  + C1V1= C2V2 : 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
  + C1V1= C2V2 : For 0.01g sample, use 106 uL MeOH
* Warm tubes for 1.5hr at 55C, vigorous hand-shaking for 5 sec every 20 min
* Cool tubes in cold water bath for ~5 min

Acidify to form precipitate

* 12M H2SO4
  + Use a ratio of 0.5 g larvae/ 0.58 mL 12M H2SO4 in water soln
  + C1V1= C2V2 : 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

*Take weight of transfer vials*

* 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.
* **Take weight of samples**
* Add 5-6 granules of Sodium Sulfate to dry hexane layer.
  + This will remove water from the solution.
* Transfer Dried hexane layer to GC vial. **Store at -20C until GC analysis**

GC Methodology

Sequence

* Warm up runs high concentration of real matrix
  + this step equilibrates the column and coats any possible hotspots of the column to allow for a consistent signal recovery
* Calibration Curve
  + this step is batched into the sequence at the beginning and the end and batches are flanked by blanks.
  + calibration curve is run from low concentration to high concentration
* Samples
  + QC’s are one point calibration curve data, comprised of a random data point from the calibration curve data, these runs flank a batch of samples.
    - this packages data into packets and allows for each packet to be compared to a known standard and that is compared to the calibration curve data
    - QC’s are given a range of signal detection varibility (5-10% of the calibration curve signal).
    - If the QC works then the samples can be said to be true, if the QC fails then that packet of data can be removed from the final analysis and rerun
  + The experimental samples
    - Run within the batched set, flanked by QC’s and blanks
  + Calibration curve is run at the end of all the batches
    - this allows insight into instrument or sample decline
  + Clean up run
    - this is to remove any goop left on the column, usually a high temperature run

SPME-HS GC analysis

* Prepare samples and run GC-MS in triplicate
  + Program GC-MS (Arwen) to Run HS-SPME
  + Aliquot 10uL onto a filter paper dot and allow the paper to dry for a set time, then follow PEST standards.
    - **P**ermeate: place dot into a vial with septa lid for *X-*mins. This allows the methyl esters to volatilize and adsorb to the SPME fiber
    - **E**xposure: after permeation is complete, inject the SPME needle through the septa on the vial and expose the fiber to the volatilized methyl esters in the vial for *X-*mins
    - **S**torage: after exposure is complete, store the fiber, retract the needle from the vial, move towards the GC all within 2 mins
    - **T**emperature: The temperature of the chamber where in the fiber will be exposed. After the storage time is complete, inject the SPME needle into the heated chamber, select RUN on the face of the instrument.
    - After 6 mins retract the fiber into the GC, and remove the SPME from the GC.
  + After the GC completes its run cycle, compare the peaks to the library to determine the compound.