Konecky Lab – Fatty Acid Purification & Derivatization
Updated 2025-06-04
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Required PPE:

- **Lab Coat**
- **Goggles**
- **Nitrile Gloves**

Overview: Starting with a total lipid extract (TLE) use solid-phase extraction to separate neutral and acidic compounds, the latter of which contains free fatty acids. The acidic fraction is then transesterified using anhydrous methanolic hydrochloric acid, which produces an ester bond on the fatty acid containing the methyl group from the methanol. This yields our desired derivatives: fatty acid methyl esters, or FAMEs. The FAMEs are then liquid-liquid extracted from this solution and further purified by silica gel column chromatography to remove various other acids (particularly FAMEs with hydroxyl functional groups) that could otherwise co-elute with saturated FAMEs during analysis.

This protocol is based on the Brown FAME protocol written by Jessica Tierney. Note that this method has 4 parts.

Materials needed (HPLC+ grade solvents; all aluminum and glassware combusted at 500°C):

- 1:1 Dichloromethane:Methanol
  - **Note:** We originally used 2:1 DCM:Isopropanol, but particularly polar compounds (e.g., monosaccharide anhydrides) are not eluted using this solvent system and have found 1:1 DCM:MeOH as a suitable replacement.
- 4% v/v Acetic acid in ethyl acetate
  - **Note:** We originally used diethyl ether, but found ethyl acetate to be a suitable replacement that has the benefit of never spontaneously exploding.
- Aminopropyl silica gel (SiliCycle #R52030B)
- Methylation methanol (batch with known isotopic composition)
- Acetyl chloride
- Toluene
- 5% w/v NaCl in H2O (25 g / 500 mL is typical batch)
- Hexane
- Dichloromethane
- Silica gel (SiliCycle #R10030B, combusted along with glassware)
- 4 mL screw-cap autosampler vial with PTFE-lined cap
- 4 mL vials for waste collection
- Pasteur pipettes
- Pipette bulbs
- Glass wool

Equipment needed:

- Flexivap N_2 drying station
- Column pushing apparatus
- Heating block with 15 mm insert
- Pipette and vial racks for column chromatography

Part 1: TLE acid/neutral separation by aminopropyl silica gel solid phase extraction

- The aminopropyl functional group of this silica gel forms a covalent bond with the carboxylic acid functional group of compounds loaded onto the gel (similar to a peptide bond in a protein). Once loaded, the gel is rinsed to elute components lacking a carboxylic acid group, which is simply refer to as the 'neutral' fraction. We then elute with acetic acid in ethyl acetate. The acetic acid is a stronger acid than fatty acids, so it preferentially interacts with the aminopropyl groups and releases the fatty acids and most other organic acids, which we refer to as the 'acid' fraction.

1. TLEs should be extracted following the Konecky lab 'ASE Total Lipid Extraction' protocol.
2. Label two 4 mL vials per sample with identification. One vial should also be labeled 'N' for 'neutral' and the other should be labeled 'A' for 'acid.
3. For each sample, place a glass-wool-packed pipette on the pipette rack and load with ~0.5 g (about 1.5 to 2", see image on right) of aminopropyl silica gel using the small funnel. Tap the column a bit to settle the silica gel.

4. Place sample vials in the column chromatography vial rack. The first row should contain empty, clean 4 mL vials for collecting waste, the second row should be your 'neutral' vials, and the third row should be your 'acid' vials.
5. Lift the pipette rack and lower so the pipette ends are centered in your waste vials.
6. Pre-clean each column with 3 reps of 1 mL 1:1 DCM:MeOH using the column pusher to accelerate flow through the columns.
7. Lift the pipette rack and lower into the 'neutral' vials.
8. Dissolve your TLEs in 1 mL (~1 column volume) each of DCM:MeOH and then load the samples onto the columns.
9. Repeat twice for a total of 3 rinses of your sample vials and ~3 column volumes through your columns.
10. Lift the pipette rack and lower into the 'acid' vials.
11. Add 1 mL (~1 column volume) of 4% acetic acid in ethyl acetate to each sample column. Pushing using positive pressure as needed and repeat twice for a total of ~3 column volumes. On the final rinse, the columns should be pushed to dryness into your 'acid' vials.
12. Evaporate both fractions to dryness using the Flexivap. The acid fraction will dry faster and you should remove those vials as they are dried.
13. Cap both fractions and place in the refrigerator. If storing for >1 week, wrap the caps in parafilm.

Part 2: Transesterification by methanolic hydrochloric acid

- This section replaces the hydrogen (H) in the hydroxyl (-OH) of the carboxylic acid (O=C-OH) of the fatty acid with a methyl ($-CH_3$), resulting in the methyl-ester derivative form of the carboxylic acid ($O=C-O-CH_3$). This creates your fatty acid methyl ester, or FAME, which is both less polar and much more volatile than the original free fatty acid. The increased volatility enables the analysis of FAMEs by gas chromatography.

1. Make enough 5% v/v acetyl chloride in methanol (i.e., acidified methanol) using the following protocol:
   1.1. Locate the 'Methylation Methanol'. This is a specific batch whose isotopic composition has been measured. This is critical because we are placing a methyl group from the methanol onto our compounds and its composition must be accounted for!
   1.2. Measure out an appropriate volume of methylation methanol using a graduate cylinder and transfer to an appropriately sized volumetric flask. If making 10 mL of solution, you need 9.5 mL methanol. If making 20 mL of solution, you need 19 mL of methanol. In both of those cases, a 25 mL volumetric flask is sufficient. You should make a few milliliters excess of this solution to make transfers easy.
   1.3. Using a glass pipette, transfer an appropriate amount of acetyl chloride into the methanol bottle **drop by drop**, swirling between drops. This reaction creates HCl (and by-product methyl acetate) and is very exothermic. Swirling the jar is critical to dissipating the heat generated by this reaction.
   1.4. If you hear any popping sounds, then you are pipetting too quickly. If these occur every drop, you should cap the flask and allow to cool.
2. Add 0.3 mL of toluene into each sample vial to re-dissolve.
3. Add 1 mL of the acidified methanol to your sample vials and roll the sides of the vial using the 1.3 mL of liquid in each vial.
4. Open four adjacent (in a square) ports on the Flexivap. For each sample, displace the headspace with N_2 and then quickly cap while centered under the four ports.
5. Place your samples on a heating block at 60 °C for 12 hours. This should be done overnight using the heating block's timer. The samples should be cool by the next morning.

Part 3: Liquid-liquid purification of FAMEs

- This section is meant to remove your derivatized FAMEs (and other, co-extracted components) from the acidifed MeOH. The added saltwater helps improve the extraction efficiency of the liquid-liquid extraction.

1. Add 2 mL of the 5% NaCl in H_2O solution to each sample.
2. Add 1 mL of hexanes to each sample.
3. Working in sets of two, cap tightly and vortex each sample for 20 seconds. Let stand to separate your phases.
4. Once the phases have separated, carefully pipette the hexane phase (upper phase) into clean, labeled 4 mL vials. Take care to avoid water on the walls of the tube. Leave a small disc of hexane to avoid in the tube to help avoid drawing any water.
5. Repeat twice for a total of three liquid-liquid extractions. You may, if concerned, perform a fourth liquid-liquid extraction in the event you are not confident about your transfer volumes.

6. After finishing all the samples, dry them down using the Flexivap, cap, and store in the refrigerator.

Part 4: Final clean-up

- This section is meant to elute minor contaminants in the initial hexane rinse and elute saturated FAMEs during the DCM rinse. The majority of the contaminants will remain on the column and could, if desired, be eluted using a strongly polar solvent such as MeOH.
- Veterans of this method may prefer to transfer their upper hexane phase of the liquid-liquid extraction directly onto silica gel columns. This saves time and glassware but requires additional skill and attention and should not be done until sufficiently skillful.

1. Prepare a set of silica gel columns for your samples. Add enough silica gel to occupy ~1.5" of a glass-wool-packed pipette. Tap lightly to settle the gel. **Note**: this step uses 'normal' silica gel that is kept in the drying oven. Don't use aminopropyl silica gel here!
2. Mount the columns and place waste 4 mL vials in the first row and clean, labeled 4 mL vials in the second row.
3. Pre-wet your columns with 1-2 column volumes of hexane.
4. Redissolve your samples in hexane (~1 mL) and load them onto the columns. Repeat twice for a total of three column volumes.
5. Move your columns over the second row containing your clean, labeled 4 mL vials. Add ~1 mL of DCM to your sample vials and then transfer to the columns. Repeat twice for a total of three column volumes, pushing the columns to dryness on the last rinse.
6. Blow down your purified FAMEs using the Flexivap, cap, and store in the refrigerator until analysis.