

## Biochem Lab

## LIPIDS

**Purpose:** This two-week lab will demonstrate how to extract, separate, and identify nonpolar and polar lipids from various biological tissues and to measure cholesterol.

**Key Concepts and Terms:** Amphipathic, Esterification, Fatty Acids (saturated vs. unsaturated), Phospholipids, Polar vs. Nonpolar, Steroid, Triglycerides, Waxes.

**Background:** Lipids are a heterogeneous group of compounds synthesized by organisms that are present in all biological tissues. These compounds are characterized as being sparingly soluble in water, but highly soluble in nonpolar solvents (e.g., chloroform, ether, hexane, etc.). We can use this solubility difference to extract and separate lipid constituents from the many polar compounds that are contained in biological tissues. Lipids can be classified by a number of schemes, but looking at them according to their biological function is most useful. Triglycerides (or triacylglycerols) are fats and oils that serve as storage and transport forms of metabolic energy. These compounds contain fatty acids (generally 3 molecules of saturated and unsaturated in various combinations) that are esterified to a 3-carbon glycerol molecule. Waxes (long chain fatty acids esterified to a long chain alcohol), hydrocarbons (long chain alkanes) and fatty alcohols form protective surfaces on many plant and animal tissues. Lipids that are membrane components are usually amphipathic and complex (phospholipids, sphingolipids, glycolipids). Finally, there are a large group of low molecular weight, non-polar compounds that are not derived from fatty acids (e.g., steroids, vitamins, carotenoids, quinones, etc.) and function as hormones, vitamins, photopigments and other specialized roles. In the first part of the experiment, you will obtain a crude lipid fraction from either plant and/or animal tissues. You will analyze the lipids contained in your extracts using two dimensional (2-D) thin layer chromatography (TLC) and measure the cholesterol content by spectrophotometry.

**Precautions: Use a hood whenever possible!** Some of the solvents (i.e., ether) used in this experiment are extremely flammable and toxic. **Do not use open flames in the lab!**

**Use a propipette to pipette highly volatile solutions** (e.g., ether) - Draw the solution up into the pipette and let it drain down several times to saturate the space above the liquid. Otherwise, the solution will tend to drip from the tip of the pipette because of the low surface tension. **Be gentle when using either the large propipette or a Pasteur pipette bulb.** When the solvent reaches the bulb, contaminants are introduced and the rubber of the bulb deteriorates. Never invert a pipette with fluid in it!

**Always keep solutions stoppered with glass stoppers or aluminum foil** because many of the solutions used in these experiments are highly volatile. This will prevent accumulation of toxic and/or highly flammable vapors in the lab. Lipids oxidize and polymerize easily in the presence of oxygen and light and should be stored in the dark, under nitrogen (N<sub>2</sub>), and at -20°C.

### Materials:

1. Micropipettes (20 µl, 20 µl, 1,000 µl) one of each per group minimum.
2. Micropipettes tips (two sizes), one box each per group.

3. Capillary tubes 20-40  $\mu$ l.
4. Rulers
5. TLC silica gel plates, 1 per group (SIGMA Z12,272-6)
6. TLC chambers (two plates/groups per chamber)
7. L-a-phosphatidyl choline (SIGMA P-9671)
8. Triolein (C18:1, [cis-9]) (SIGMA T-7190)
9. Cholesteryl Oleate (SIGMA C-9523)
10. Cholesteryl Stearate (SIGMA C-9503)
11. Oleic Acid
12. Nu-Check reference standard
13. Petroleum ether: diethyl ether: acetic acid (70:30:1)
14. Chloroform:methanol:acetic acid:water (85:15:10:3.5)
15. 2',7'-dichlorofluorescein (SIGMA D 5013) in spray bottle
16. UV lamp with viewing chamber
17. Plastic basin for dirty labware. Fill halfway with water and place away from working area.
18. Hexane:isopropanol (3:2 vol/vol) made and kept on ice bath before Lipid Extraction.
19. Cold mortars and pestles.
20. Absolute ethanol.
21. Cholesterol standard: 0.1 mg/ml absolute ethanol.
22. Acid-ferric chloride reagent: 80 ml of 2.5%  $\text{FeCl}_3$  in conc  $\text{H}_3\text{PO}_4$  to which 920 ml of conc  $\text{H}_2\text{SO}_4$  are added. Caution...this reagent contains concentrated acids and must be handled carefully in the hood.

### Procedure Week One:

**Extraction of Total Lipids.** Lipids can be extracted from intact cells by grinding in relatively, nonpolar, organic solvents (hexane-isopropanol). The tissue is re-extracted several times and tissue debris is removed by centrifugation. The lipid and non-lipid contaminants are separated by partitioning in a separatory funnel using an aqueous salt solution.

1. Each group will be given a sample to extract (liver, egg yolks, peanuts, avocado, etc.). Weigh 5.0 g of the tissue, record the weight to the nearest 0.1 g, and transfer into a mortar.
2. Add about an equal amount of acid-washed sand and grind with a pestle.
3. Add 5 ml of hexane-isopropanol (3:2) and grind to a smooth paste; add an additional 5 ml of solvent and continue grinding.
4. Transfer to a Sorvall centrifuge tube and prepare a balance tube by weight (your instructor will demonstrate how to do this).
5. Centrifuge at 10,000 x g for 5 min. Decant the supernatant fluid into a Sorvall centrifuge tube.
6. Re-extract the pellet from the original extract with 10 ml of hexane-isopropanol (3:2). Centrifuge and combine the supernatant fluids.

7. Add 5 ml of 15% Na<sub>2</sub>SO<sub>4</sub> to the combined supernatants and vortex for 1 min. Centrifuge at 10,000 x g for 5 min to separate the phases.
8. Remove the organic (top) layer using a pipette and transfer to a 50 ml centrifuge tube. Evaporate to dryness under a gentle stream of air IN THE HOOD.
9. Resuspend the lipid residue in 1 ml of CHCl<sub>3</sub>. Transfer the total lipid extract to a screw cap glass tube, label it with what was extracted (fish, egg, etc.), group number and section. Give it to the TA to be stored in the freezer for analysis by 2-D TLC during week 2 of this lab.

### **Procedure Week Two:**

**Thin Layer Chromatography of Lipids.** Separate the hexane:isopropanol extract of your tissue (dissolved in CHCl<sub>3</sub>) into its component lipid classes using the following thin-layer chromatography method:

1. Apply your extract and lipid standards onto two Silica Gel G TLC plates – apply the spots approximately 2-3 cm from the bottom of each plate (remember not to touch the TLC plates with your bare hands or fingers).
2. The neutral lipid components are separated by developing one plate with a mixture of petroleum ether (a combination of hexanes): diethyl ether: acetic acid (70:30:1). Development should take about 45 min – 1 h.
3. The polar lipids are separated by developing the second plate in a mixture of chloroform: methanol: acetic acid: water (CHCl<sub>3</sub>:CH<sub>3</sub>OH:CH<sub>3</sub>COOH:H<sub>2</sub>O) 85:15:10:3.5. Development should take about 1 h.
4. After development, the plates are removed from the tank, air dried, sprayed with fluorescein to label the lipid spots with the fluorescent dye, and viewed under UV light. Spots are identified by comparison with the lipid standards. Compare the different extracts qualitatively for presence of particular lipid classes and variation in amounts of the classes.

**Determination of Cholesterol.** Cholesterol concentration can be determined spectrophotometrically. The procedure does use concentrated reagents, particularly acids so be careful and do the pipetting in a hood with some sodium-bicarbonate near by to neutralize any spills. You will prepare blanks and standard curve and samples in 13 x 100 mm tubes that fit in to the spectrophotometers, be careful to select optically clear tubes.

1. The blank contains 2 ml of ethanol and 2 ml of **acid-ferric chloride** reagent. Mix carefully at the low setting making sure no drops escape from the tube.
2. Standard Curve tubes: Cholesterol standard contains 0.1 mg cholesterol /ml. Pipet the

correct volumes to tubes so that you have 20, 40, 80, 120, 160 and 200  $\mu\text{g}$ /tube in a final volume of 2 ml.

3. Three sample tubes: add 20  $\mu\text{l}$  to one tube, 0.1 ml to another and 0.5 ml to the third. Evaporate these to dryness with a gentle stream of air in a hood. Add 2 ml ethanol to each tube.
4. Add 2 ml of **acid-ferric chloride** reagent carefully to each tube and vortex CAREFULLY.
5. Incubate for 30 minutes at room temperature and then determine the absorbency at 550nm in the spectrophotometer using the blank tube to zero the instrument.
6. Construct a standard curve and calculate the cholesterol content of your sample.