Introduction:
Egg yolk is a rich source of a variety of biochemically important compounds such as proteins and lipids (glycerides or fats, cholesterol, cholesterol esters, and phospholipids).

Egg lipids may be divided into two classes:
(1) *Non-phosphorylated lipids*: cholesterol, cholesterol esters, and triglycerides (fats):

![Cholesterol and Cholesterol Ester Diagram]

(2) *Phospholipids* (those containing a phosphate entity). These are very similar to triglycerides except that the third fatty acid is replaced with a very polar phosphate ester group. One example is given below:

![Phosphatidylcholine Diagram]

Phospholipids, also known as glycerophosphatides, are widespread and occur in all plant and animal cells as major structural components of cell membranes. They play critical roles in the transport of molecules across membranes, storage and metabolism of fatty acids, and as activators in the blood clotting process.

In this experiment, the lipids will be isolated from egg yolks and separated into the two classes of lipids and these will be characterized through Thin Layer Chromatography.

Prelaboratory Exercise:
Carefully read the laboratory procedure and do a step by step block outline of the experimental protocol.
CAUTIONS:
Chloroform is a suspected carcinogen. Do not allow it to come in contact with your skin or eyes. Work in a hood and avoid breathing vapors. Dispose of it in the proper waste container. H₂SO₄ is extremely corrosive. Do not allow it to come in contact with skin or clothing.

Isolation and Separation:
Note: You will use the glass centrifuge tube from your kit for liquid/liquid extraction only.
Make up 200 mL of CHCl₃:CH₃OH 2:1 (v/v) and place in a labeled bottle. Crack an egg on the side of a beaker; allow the white to slip into the beaker by carefully moving the yolk from one half of the shell to the other. Place the yolk in a clean 250 mL beaker, and extract the lipids by stirring the yolk with 100 mL of the solvent mixture CHCl₃:CH₃OH(2:1) v/v in a fume hood. Allow to stand 10 minutes. Filter the mixture by gravity through 11-cm filter paper.
Take a 5 mL sample (put the unused portion in a 20-mL vial and put it in the refrigerator for later use if necessary). You should extract your 5mL sample in a stoppered 15mL glass centrifuge tube (from the microscale kit) twice with 3 mL of 1% NaCl solution. Do not use a plastic centrifuge tube, it will disintegrate. Be sure to vent the pressure often!
Carefully remove the top aqueous layer with a Pasteur pipette and discard. Dry the lower organic CHCl₃ layer over Na₂SO₄. NOTE: This step is very important, in order to remove residual water. Be sure the Na₂SO₄ is no longer "clumping". Add more if it is.
Pipe filter off the solution into a clean 20-mL vial, and add a crystal of hydroquinone (antioxidant). Evaporate to dryness (a sticky yellow residue) in a beaker of warm water with a stream of nitrogen in the hood. To the residue, add 3 mL of acetone and cool in an ice bath for 15 minutes. (Phosphorylated lipids will precipitate, non-phosphorylated lipids are soluble.)
Carefully decant the acetone solution through a small filter paper, collecting the filtrate in a clean vial (or pipet-filter, if possible).
Wash the precipitated phospholipids with 1 mL of COLD acetone and decant as above. Using a clean microspatula, transfer the precipitate to a clean vial, and dissolve the phospholipids in 1 mL CHCl₃:CH₃OH (2:1). Clearly label the vial, add a crystal of hydroquinone and refrigerate until needed.
Evaporate the acetone solution to dryness with a stream of nitrogen. Dissolve the non-crystalline residue in 1 mL of CHCl₃:CH₃OH (2:1). Transfer to a clean labelled vial and a trace of hydroquinone and refrigerate.
Liebermann-Buchard Test for Cholesterol:
Place 0.5 mL of each lipid solution in a separate small test tube. Add 0.2 mL of acetic anhydride to each and gently swirl. VERY CAREFULLY add 4 drops conc. H₂SO₄ by running it down the side of the tube. Be sure it does not boil. A brown color indicates the absence of cholesterol while a green color is a positive indication of the presence of cholesterol.
Separation of the phospholipids classes by TLC:
Prepare 50 mL of a developing solvent system, chloroform:methanol:hexane/7:2:1 (v/v/v) and 50 mL of hexane:chloroform/10:1 (v/v).
Spot two commercially prepared silica gel TLC plates 5 times with both solutions, the phosphorylated and non-phosphorylated lipid isolates, keeping each spot to approximately 1 mm diameter. Develop one plate in the first solvent system and the other one in the second. When the solvent front almost reaches the top of the plate, remove the plate and dry it in air.
Visualize the plate by placing it in a capped jar with a few iodine crystals. Be sure to mark the starting point and solvent front. How many components are there in the two lanes? Calculate the Rf values for the spots you have identified. The Rf values for the various components are (approximately): 0.9 for phosphatidylethanolamine, 0.5 for phosphatidylcholine, 0.2 for sphingomyelin, and 0.1 for lyso phosphatidylcholine. Sometimes the
lysophosphatidylcholine can not be detected but seems to appear as a slight movement upward of the original spot. Two other components which are not sufficiently well separated from phosphatidylcholine are phosphatidylserine and phosphatidylinositol. This makes the phosphatidylcholine appear as a large streaky spot.

**Cleaning Up:**

The NaCl aqueous layer may be flushed down the drain. All chloroform containing solutions should be disposed of in the Halogenated Organic Waste containers.

**Final Report:**

Include all TLC plates, marked and with $R_f$'s calculated. At the end of the lab report answer these questions:

1. Are the $R_f$'s what you might expect on the basis of the structures given above? Explain.
2. How do phospholipids resemble soaps? How might a cell wall resemble a soap micelle?