

## An Introduction

to **LIPID ANALYSIS**  
in the Cell Biology Laboratory

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Providing students with hands-on experiences is a common goal of many biology laboratory exercises. In this process, we often wish to give students the basic skills, tools and methodology to further explore questions of student origin or to promote student ownership of activities. In this communication I outline a basic thin-layer chromatography (TLC) method that can be used to examine complex mixtures of lipids from most any source in one three-hour laboratory period. I currently use the described TLC procedure in a second-year level undergraduate cell biology course, but the basic methodology presented can be adapted to ask unique questions regarding lipid biology/biochemistry depending upon the needs of the particular course and instructor.

The exploration of biomolecules and their properties is common in the cell biology laboratory. However, current lab manuals tend to emphasize nucleic acids and proteins, while lipids and carbohydrates are somewhat neglected. Part of this neglect, I believe, stems from the lack of classroom methodology for safe, rapid, sensitive and efficient separation and detection of complex mixtures of these biomolecules. Based upon a recent report describing the use of water-soluble dyes for visualization of lipids on thin-

layer chromatography plates (Plekhanov 1999) and the application of a unique sequential chromatography solvent system, I present methodology for the isolation, separation and visualization of polar and nonpolar lipid classes on a single TLC plate that can be carried out within one three-hour laboratory period. The method presented can be used as the basis for student explorations of the lipid composition of biological specimens and for examining the basic properties of lipids.

Procedures for lipid analysis using TLC in the classroom generally follow two approaches (Heidcamp 1995). In the first approach, classes of lipids are separately extracted, and each class of lipid is analyzed via unique TLC methodology. In the second approach, complex mixtures of lipids are separated on TLC plates and then further characterized. As currently practiced, both approaches suffer from one or more of the following drawbacks:

1. Students must work with relatively large amounts of organic solvents that are used in the extraction/separation of lipids.
2. Unique methodology is employed for the separation and visualization of the various classes of lipids – neutral lipids, polar lipids and cholesterol. Additionally, frequently used lipid visualization methods require high temperatures or noxious chemicals, generating safety concerns.

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3. The combination of extraction procedures and/or staining protocols requires more time than is typically available for a single period in the laboratory.

The method that I will describe allows students to study complex mixtures of lipids as in approach number two above, however, the proposed method has the following benefits:

1. The extraction of lipids requires small volumes of organic solvents, generally less than 200  $\mu\text{l}$  per sample, which means that extractions can be carried out in microfuge tubes.
2. Lipids are chromatographed on a single silica gel TLC plate using sequential solvent systems in the same dimension – the relatively nonpolar lipids such as neutral lipids, fatty acids, and cholesterol migrate to unique positions in the upper half of the chromatogram, whereas relatively polar lipids like phospholipids and sphingolipids are separated on the lower half of the chromatogram.
3. All lipids are visualized by staining with a single water-soluble dye, amido black 10B, providing rapid, safe and sensitive staining of lipids.

## Equipment/Supply Requirements

### Thin-Layer Chromatography (TLC) Plates

The author currently uses Analtech GHL Uniplates (scored, inorganic binder; catalog #11511; <http://www.analtech.com>), although any silica gel TLC plate should work. Scored TLC plates allow one to generate variable plate sizes that are appropriate for the number of samples and fit the available chromatography chambers.

### Two Chromatography Chambers

TLC chromatography chambers can be purchased, but are somewhat expensive. One can use any large glass container that can be covered by a flat glass plate. The author has successfully used institutional-size mayonnaise jars as TLC chambers.

### Pipettes for Solvent Transfer in Sample Preparation & Sample Application

Variable volume micropipettes with disposable tips, such as those used in molecular biology labs, work well. If unavailable, one can substitute 1 ml glass serological pipets for solvent transfer or disposable capillary tube micropipettes for solvent transfer (e.g.,

Drummond Microcaps Disposable Micropipets, 100  $\mu\text{l}$  volume, pkg. of 100 = \$9.45; VWR Scientific Products catalog #53440-260) and sample application on the TLC plate (e.g., Drummond Microcaps, 2  $\mu\text{l}$  volume, pkg. of 100 = \$9.45; VWR Scientific Products catalog #53440-023, 1-800-932-5000).

## Centrifuge

The author uses an Eppendorf 5415 microcentrifuge for this lab, and the noted centrifugation speeds and times in the text refer to this centrifuge. However, other centrifuges give comparable results. For example, red blood cells (RBCs) were also sedimented in a MSE GT-2 clinical centrifuge (often available in anatomy/physiology or hematology labs) at 1200 rpm ( $\sim 250 \times g$ ) for 5 minutes or in a VWR personal Mini Centrifuge (VWR Scientific Products catalog #20668-212, \$290) at 6000 rpm ( $2000 \times g$ ) for 2 minutes. Likewise, chloroform:methanol extracts of membranes were successfully separated in the MSE GT-2 clinical centrifuge (1200 rpm, [ $\sim 250 \times g$ ], 5 minutes) or the VWR personal Mini Centrifuge (6000 rpm, [ $2000 \times g$ ], 2 minutes). Thus, for the sedimentation of RBCs and for chloroform:methanol extraction of membranes, any centrifuge that can generate the above g-forces should be satisfactory. The only place where high g-forces are required is during the sedimentation and washing of RBC membranes in the “scaled-down” version of membrane lipid preparation. Note, however, that an alternative, direct extraction procedure for exploring RBC membrane lipids is presented that does not require the high g-forces used in the isolation and washing of these membranes.

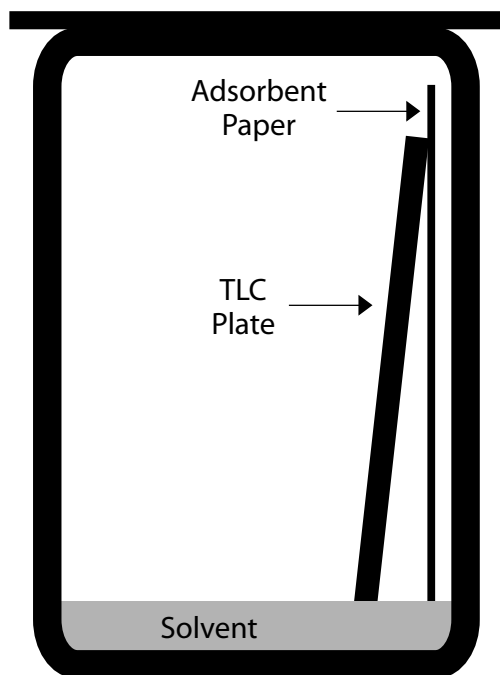
## Chromatography Conditions

### Chromatography Chamber Preparation

Cut a piece of adsorbent paper (e.g., Whatman #1 chromatography paper) with dimensions slightly smaller than those of the back wall of the chromatography chamber. Insert this piece of adsorbent paper into the chromatography chamber, leaning it against the back wall of the chromatography chamber (see Figure 1A). Prepare solvent system #1 (chloroform:methanol:acetic acid:water – 50:30:8:3) (Pai 1988) in a chemical fume hood and pour into chromatography chamber #1 to a depth of 0.5 to 1 cm. (Note: The solvent must be high enough to contact the silica gel on the bottom of the TLC plate when the plate is placed in the chromatography chamber, but not so high as to contact the sample origins. The amount of solvent required depends upon the dimensions of the chromatography chamber; we currently

**Figure 1A.**

### Chromatography Chamber with Glass Cover

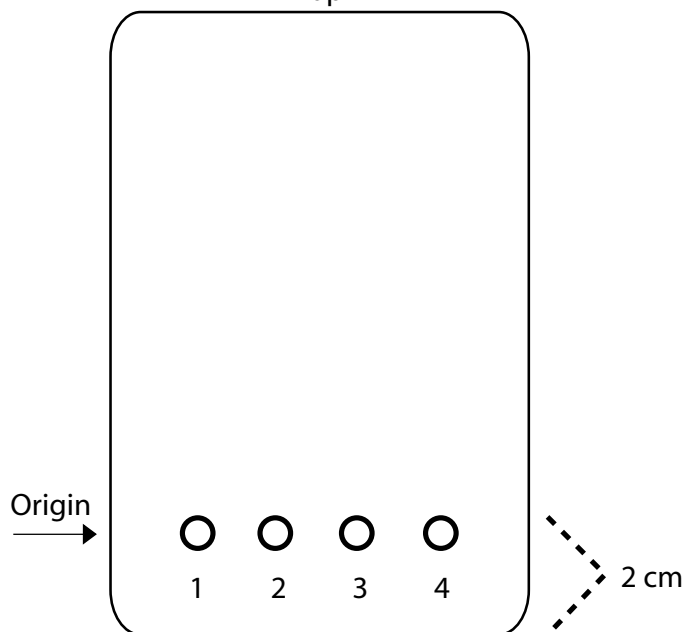


A). Diagram of chromatography chamber in which the positions of the adsorbent paper, TLC plate, and solvent are noted.

**Figure 1B.**

### TLC Plate

Top



B). Diagram of TLC plate showing the placement of origin and lane designations on the silica gel.

use less than 100 ml of each solvent system in our chambers.) Place the lid on the chromatography chamber. Allow the solvent to wick up the adsorbent paper and equilibrate within the chromatography chamber. The presence of the adsorbent paper, though not absolutely necessary, provides uniform chamber saturation by solvent vapors that improve chromatography quality (i.e., reproducibility). Prepare a second chromatography chamber containing adsorbent paper and solvent system #2 (heptane:diethyl ether:acetic acid – 70:30:2) (Plekhanov 1999) as above.

### Preparation of the TLC Plate

The author routinely uses Analtech GHL (inorganic binder) [or HL (organic binder)] Uniplates. One should be able to use any silica gel TLC plate as long as the proper staining protocol from the list below is selected; I would recommend an initial test of TLC plates prior to use in the classroom. Standard TLC plates are 20 cm tall with varying widths. The width required depends upon the number of samples that will be chromatographed. For example, if one spaces the samples 1 cm apart, nine samples can be chromatographed on a TLC plate that is 10 cm in width.

Sites where samples are to be applied (origin) on the TLC plate are lightly marked and numbered directly on the silica gel with a pencil. Sample application points should be approximately 2 cm from the bottom edge of the TLC plate, with at least 1 cm between samples (see Figure 1B). This labeling does not interfere with the chromatography.

### Sample Application

Lipid samples are applied to the TLC plate in a chemical fume hood at the numbered sites of origin as follows. Draw 2.5  $\mu$ l (maximum) of sample into a micropipet or glass capillary tube (see Equipment section). Touch the opening of the micropipet or capillary tube directly to the sample application site and allow the silica gel to absorb the sample, forming a solvent spot on the TLC plate. Allow this solvent spot to dry (turns bright white) prior to application of more sample. Drying can be hastened by gently blowing a stream of air across the TLC plate. (Drying time between sample applications is approximately 5 to 10 seconds.) Repeat sample application 2.5  $\mu$ l at a time until the entire sample has been loaded at the labeled origin for that particular sample, then replicate this process for each sample.

## Chromatography

When all samples have been applied to the TLC plate and the sites of application are dry, the TLC plate is ready for chromatography, which is initiated as follows. Remove the lid from pre-equilibrated chromatography chamber #1 (chromatography chamber is located in a chemical fume hood) and rapidly insert the TLC plate into the chamber such that the bottom of the TLC plate is immersed in the solvent and the silica gel is facing the front of the chromatography chamber. Stabilize the TLC plate by leaning it against the adsorbent paper on the back wall of the chamber (see Figure 1A). Quickly replace the lid on the chromatography chamber. (Additional TLC plates can be added to the chamber at any time as long as space permits and it is done rapidly so as to prevent drying of TLC plates already in the chamber.) Solvent should begin to move up the TLC plate, chromatography being allowed to proceed until the solvent front has migrated half way up the plate (approximately 30 minutes). The TLC plate is removed from chamber #1 and air dried in the chemical fume hood. The plate is then placed in pre-equilibrated chromatography chamber #2 in the same orientation as for chamber #1. Chromatography proceeds in the same direction as for

solvent system #1 except that the solvent front is allowed to migrate until it is within 1 cm of the top of the TLC plate (approximately 45 minutes). The TLC plate is removed from chromatography chamber #2 and air dried in the chemical fume hood for 5 to 10 minutes prior to lipid visualization.

## Lipid Visualization

All lipids separated on the chromatogram are visualized with a single stain, the water-soluble dye amido black 10B (Aldrich; Milwaukee, WI; catalog #19,524-3), as originally described by Plekhanov (1999), with modifications noted below if you are using chromatography media that are unstable if immersed in 100% aqueous solutions. The dye preferentially interacts with relatively nonpolar entities and thus when present in a 1 M sodium chloride solution will associate with lipid spots on the chromatogram.

For TLC plates that are stable in 100% aqueous solutions (e.g., Analtech GHL Uniplates with inorganic binder), the developed and dried TLC plate is directly immersed in a tray containing enough staining solution to completely cover the plate. The staining solution is 0.2% (w/v) amido black 10B in 1 M sodium

chloride that has been clarified by filtration through cotton (i.e., pouring the solution through a plug of cotton placed in the bottom of a funnel). Staining at room temperature is rapid and complete within 10 minutes. The stained chromatogram is briefly rinsed (10 to 20 seconds) in a tray containing 1 M sodium chloride, and subsequently dried. An image of the results (see below) can be captured at any time after the chromatogram has been rinsed, although some fading does occur over time; thus I recommend obtaining an image of the chromatogram immediately after staining and rinsing (and drying, if preferred and time permits) for greatest sensitivity.

Some silica gel TLC plates are stable in solutions that are up to 80% (v/v) water (e.g., Analtech HL Uniplates with organic binder). For these TLC plates, lipid visualization is carried out as described above except that the staining solution contains 20% (v/v) ethanol (or methanol). For silica gel TLC plates that are unstable if immersed in 80% (v/v) or greater aqueous solutions, we visualize lipids by spraying (General Glassblowing Company, Inc.; Richmond, VA) the original aqueous amido black 10B solution directly on the chromatogram. Using these staining methods, we are able to detect as little as 25 to 50 ng of phospholipids, 25 to 100 ng of cholesterol, and 50 to 250 ng of neutral lipids and fatty acids, the sensitivity being dependent upon the specific TLC plate used and the staining method (immersion, spraying) employed.

## Image Capture

The image of the blue-purple lipid spots on the chromatogram can be readily captured in a number of ways:

1. Direct photography
2. Use of a CCD camera coupled with appropriate computer software, such as NIH Image, which can be obtained free of charge
3. Use of a computer flatbed scanner and its accompanying software
4. Direct photocopying of the TLC image.

Once captured, the hard-copy image can be analyzed by students as desired. Commonly, students compare the migration ( $R_f$ ) and staining characteristics of unknown lipids and known lipid standards.

## Sample Preparation

### Cell Membrane Lipids

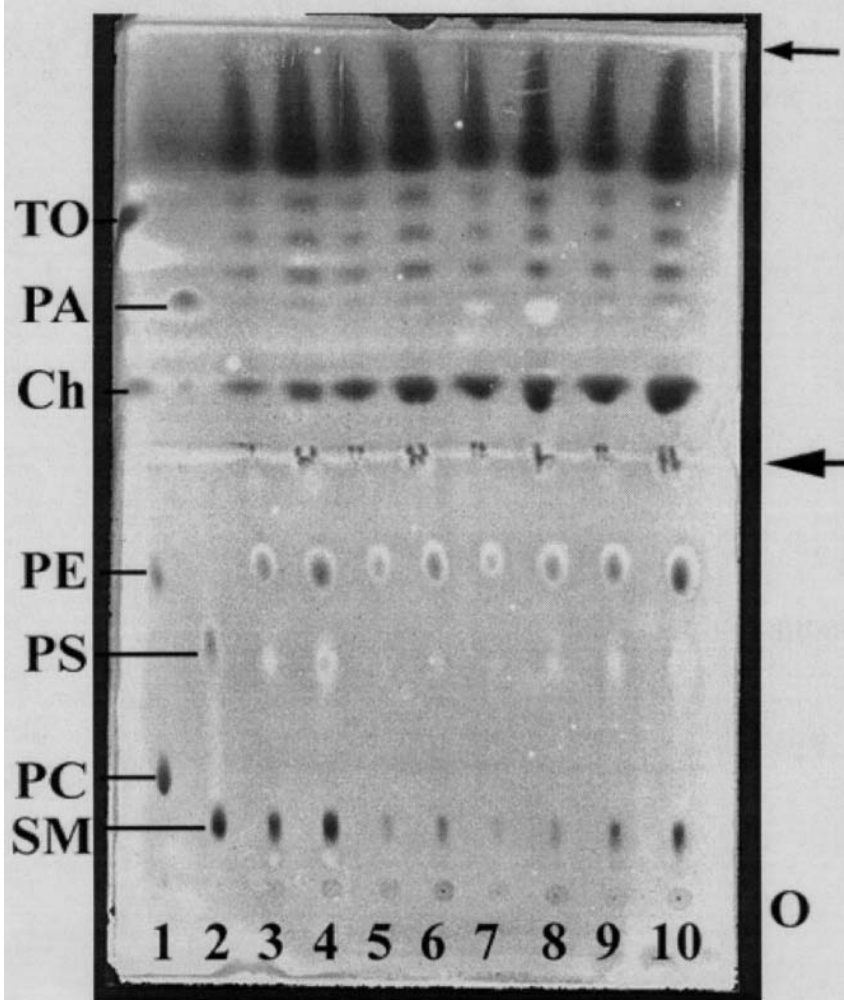
The mammalian red blood cell (RBC) has served as a classical source for explorations of plasma mem-

brane structure and function, as these cells are essentially devoid of internal membranes that could contaminate the plasma membrane lipid preparation. Recently, Gasque (1998) published a lab manual procedure for extracting lipids and proteins from purified sheep RBC membranes. For isolation of membrane lipids only, two time- and reagent-saving modifications of the published procedure were developed. The first modification is a "scaled-down" version of the published method that allows completion of the entire extraction and analysis procedure within one lab period. The second modification is a direct lipid extraction procedure that can be completed within 15 minutes, as compared to 45 to 60 minutes for the "scaled-down" version. The modified procedures provide lipid yields that are adequate for membrane lipid visualization using the methodology noted above.

### "Scaled-down" Version of Membrane Lipid Preparation

Two hundred fifty microliters of defibrinated sheep blood (Remel; Lenexa, KS) are placed in a microfuge tube and red blood cells (RBCs) are sedimented by centrifugation at 5000 rpm (~2,000 X g) for two minutes in a microfuge (Eppendorf). The liquid above the RBC pellet is discarded in biohazardous waste, and the RBC pellet is resuspended in 0.5 ml of 10 mM phosphate buffer (pH 7) which causes the RBCs to lyse. Membrane storage buffer (MSB; MSB = 10 mM HEPES [pH 7.4], 130 mM KCl, 0.5 mM MgCl<sub>2</sub>, 0.05 mM CaCl<sub>2</sub>) (0.8 ml) is added to the lysed RBC prep; this isotonic buffer allows the membranes to pack more tightly upon centrifugation (Gasque 1998). The sample is centrifuged for 10 minutes at 14,000 rpm (16,000 X g) in a microfuge located in the cold room. (Note: I have also had success carrying out the centrifugation at room temperature.) Discard the supernatant in biohazardous waste and resuspend the cloudy sediment containing the RBC membranes in 1.3 ml of MSB. Centrifuge again at 14,000 rpm (16,000 X g) as above; remove the supernatant and resuspend in 1.3 ml of MSB, repeating the washing procedure one or two more times until the supernatant is clear with relatively little hemoglobin. After the final wash, remove all of the supernatant. Add 0.1 ml of chloroform:methanol (9:1) to the membrane pellet and mix vigorously to dissolve the membrane lipids. (NOTE: Carry out work with chloroform and methanol in a chemical fume hood.) Centrifuge the membrane extract at 14,000 rpm (16,000 X g) for two minutes in the microfuge. Using a micropipet, transfer the lower, clear chloroform:methanol phase containing the membrane lipids to a clean microfuge tube. Spot 10 to 30  $\mu$ l (2.5  $\mu$ l at a time) of this membrane lipid extract on

**Figure 2.**



Separation and visualization of sheep red blood cell (RBC) lipids via thin-layer chromatography. Lanes 1, 2: lipid standards (SM = sphingomyelin, PC = phosphatidylcholine, PS = phosphatidylserine, PE = phosphatidylethanolamine, Ch = cholesterol, PA = palmitic acid, TO = triolein; all standards were purchased from Sigma, dissolved in chloroform:methanol [9:1], and stored at  $-80^{\circ}\text{C}$ . Five micrograms of each standard were applied to the TLC plate.). Lanes 3, 4: 15  $\mu\text{l}$  and 30  $\mu\text{l}$ , respectively, of sheep RBC lipids extracted as per the procedure described by Gasque; Lanes 7, 8: 15  $\mu\text{l}$  and 30  $\mu\text{l}$ , respectively, of sheep RBC lipids using the direct extraction method; Lanes 9, 10: 15  $\mu\text{l}$  and 30  $\mu\text{l}$ , respectively, of sheep RBC lipids extracted using the "scaled-down" version of the Gasque protocol; Lanes 5, 6: a duplicate sample of sheep RBC lipids prepared as for Lanes 9 and 10. The large arrow indicates the solvent front for solvent system #1; the small arrow indicates the solvent front for solvent system #2. O = origin.

your TLC plate, and develop and visualize as described above. Figure 2 compares the lipids extracted from RBC membranes using the large-scale published procedure (Lanes 3,4) and the scaled-down version proposed here (Lanes 9,10). The composition and amounts of lipids extracted using the two procedures

are very nearly identical.

### Direct Extraction of Sheep RBC Lipids

Since sheep (mammalian) RBCs contain only one membrane system, the plasma membrane, I tested them to see if direct extraction of RBCs would provide membrane lipid preparations suitable for TLC analysis. The procedure outlined below proved satisfactory.

Two hundred fifty microliters of defibrinated sheep blood is placed in a microfuge tube and RBCs are sedimented for two minutes at 5,000 rpm ( $\sim 2,000 \times g$ ) in a microfuge (Eppendorf). The supernatant is discarded in biohazardous waste, and the RBC pellet is resuspended in 0.1 ml of chloroform:methanol (9:1) and mixed vigorously for 10 to 20 seconds using a vortex mixer. (NOTE: Work with chloroform and methanol is carried out in a chemical fume hood.) The mixture is centrifuged for two minutes at 14,000 rpm ( $16,000 \times g$ ) in a microfuge, and the clear, lower chloroform:methanol layer is transferred to a clean microfuge tube. Ten to thirty microliters (2.5  $\mu\text{l}$  at a time) of this lipid extract are spotted on a TLC plate and developed as above. The direct extraction method (Figure 2, Lanes 7,8) gives results that are similar to the previous methods, but the yields are slightly reduced. However, the savings in time and reagents, and the reduced time of exposure of students to the sheep blood, coupled with adequate yields for visualization of lipids, make this a viable alternative. [Note: While this manuscript was in preparation, Keys (2000) described a procedure for direct extraction of membrane lipids from sheep erythrocytes that is similar to the method described here.]

### Student Exploration

Provided with the above basic methodology, students can explore the lipid content of any sample of

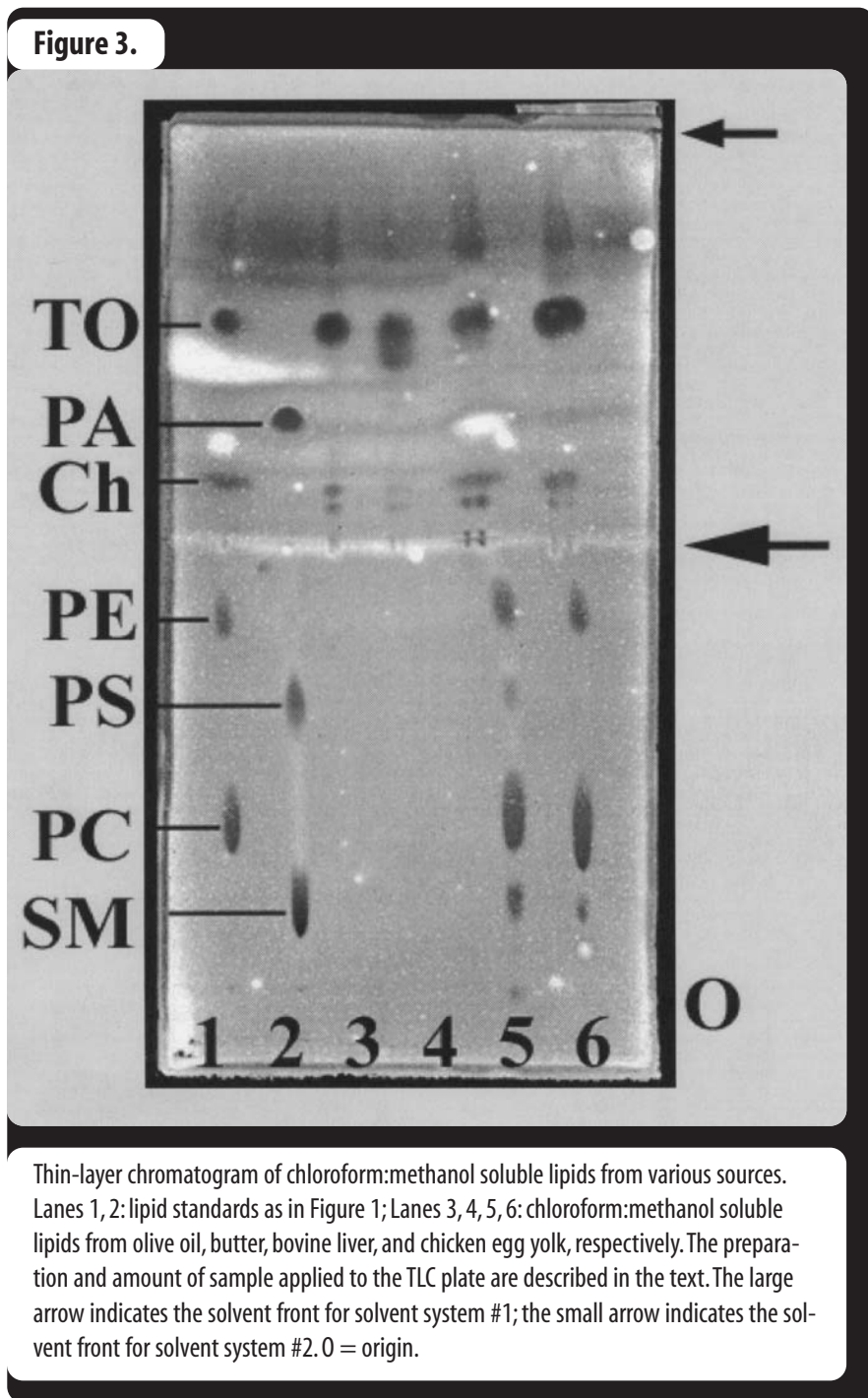
interest. Samples of chloroform:methanol (9:1) soluble lipids from various sources are shown in Figure 3 and are provided as starting points for students to explore lipids from other sources. For example, Lanes 3 and 4 depict chloroform:methanol soluble lipids present in olive oil and melted butter, respectively. In each case 1  $\mu$ l of sample (olive oil or butter) was added to 99  $\mu$ l of chloroform:methanol, and 2  $\mu$ l of this mixture was spotted at the origin of the TLC plate. Both of these sources show a preponderance of nonpolar lipids, as might be anticipated. The lipids from 0.15 g of minced bovine

liver are shown in Lane 5. The lipids were extracted with 100  $\mu$ l of chloroform:methanol, using a toothpick to disrupt the tissue; the disrupted tissue mixture was briefly centrifuged (30 to 60 seconds at 14,000 rpm [16,000 X g] in a microfuge), and 20  $\mu$ l of the liquid phase was applied at the origin of the TLC plate. The chloroform:methanol soluble lipids from chicken egg yolk (3  $\mu$ l yolk plus 57  $\mu$ l of chloroform:methanol; 2.5  $\mu$ l of the mixture applied to the TLC plate) are shown in Lane 6. In the case of liver and egg yolk extracts, both polar and nonpolar lipids appear to be present.

## Discussion

In this article I have outlined a thin-layer chromatography development and visualization system that allows students to: (1) separate polar and nonpolar lipids, plus cholesterol, on one TLC plate and (2) rapidly visualize polar and nonpolar lipids, plus cholesterol, on their chromatograms using a common, water-soluble dye, amido black 10B, and capture these images via readily accessible means. In addition, I have provided examples of sample preparation that allow students to: (1) perform the classic examination of lipids in mammalian RBC membranes within a single three-hour laboratory period and (2) explore the lipid composition of other biological materials of interest.

Using the basic methodology presented here, the course instructor can design lab exercises and pose questions to meet student/curriculum needs. For example, one could introduce the method by performing the sheep RBC lipid extraction and analysis in one lab period, and in a subsequent lab period compare RBC membrane lipids from various vertebrate species such as sheep (a ruminant), another mammal (dog, cat, rat), and nucleated RBCs of amphibians. Alternatively, the instructor might allow students to bring samples of materials for lipid extraction and analysis in the subsequent laboratory period. Students can explore the lipid composition of several foodstuffs, for



example, or explore the ability of different solvents to extract lipids from a single source. They could compare lipids extracted from a single tissue of an animal fed various diets or bacteria grown on different media. Students could also examine the effects of enzymatic or chemical manipulations on lipids extracted from a source either pre- or post-chromatography (the staining is reversible) as an aid to characterizing a specific lipid. With some guidance, students can formulate their own questions and explore topics of interest to them in the area of lipid biology/biochemistry.

The reduced levels of organic solvents used, and the safe, sensitive and rapid staining procedure decrease the safety concerns usually associated with such lab exercises. The use of a single TLC plate, and a single development and staining system for visualization of both polar and nonpolar lipids, reduces equipment and supply needs. Lastly, the staining provides for readily accessible means of obtaining hard-copy images of student results for documentation and analysis.

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