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Laboratory Experiment

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Phospholipids, Dietary Supplements, and Chicken Eggs: An Inquiry-Based Exercise Using Thin-Layer Chromatography

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Supporting Information

ABSTRACT: This inquiry-based experiment is designed for organic or biochemistry undergraduate students to deduce the identity of phospholipids extracted from chicken eggs and dietary supplements. This is achieved using thin-layer chromatography (TLC) data, a series of guided questions of increasing complexity, and provided relative retention factor (Rf) values for phospohlipids. Colorful visualization agents are used that selectively reveal phospholipids with specific functional groups on TLC plates. The novelty in this experiment lies in the students' ability to use the colorful patterns on the developed plates and compare the spot locations to a list of Rf values without the need for phospholipid standards. Furthermore, the use of krill oil and fish oil provide a unique aspect where an additive purported to be a good source of phospholipids, krill oil, is tested against fish oil (seemingly similar, yet negative for phospholipids). A prelaboratory assignment familiarizes students with concepts of TLC and phospholipids. The experiment takes two, 3 h laboratory periods to complete, but with specific modifications, can be completed in one, 3 h period.

KEYWORDS: *Second-Year Undergraduate, Biochemistry, Organic Chemistry, Laboratory Instruction, Inquiry-Based/Discovery Learning, Bioanalytical Chemistry, Lipids, Membranes, Natural Products, Thin Layer Chromatography*

INTRODUCTION

There are many different types of lipids, all with unique characteristics and functions. Biological membranes are two layers of phospholipids, within which proteins are embedded or associated with varying degrees. Phospholipids each contain a hydrophilic head and a hydrophobic tail; the hydrophilic heads arrange so they are in contact with the intra- and extracellular aqueous environments, which forms a bilayer (Figure 1).

One common phospholipid in membranes is phosphatidylcholine, with a headgroup containing choline and phosphate functional groups. Other phospholipid types vary in their chemical functionality, which can be detected using a variety of techniques. Previously in an undergraduate setting, phospholipids have been extracted and identified using gas chromatography,[1−3] mass spectrometry,[4,5] colorimetric quantitation,[6] and NMR[7] techniques. For undergraduate institutions that lack these resources, phospholipid purification and identification have been done by column chromatography[7] and thin-layer chromatography (TLC).[8] TLC is performed by having a very thin layer of adsorbant, such as silica, on an aluminum, plastic, or glass plate that acts as the stationary phase for chemical separation and a mobile phase that promotes the separation of compounds based on their polarity. Typically, TLC is done using standards that contain known compounds that aid in identification of extracted lipids by comparison of retention factor (Rf) values.

In this guided-inquiry experiment, TLC is used to identify phospholipids extracted from dietary supplements and chicken eggs without the need for known phospholipid standards. Various visualization reagents are used to identify functional groups commonly present in phospholipids as spots on a TLC plate. Iodine is used to detect double bonds, and a yellow or brown color indicates an unsaturated fatty acid.[9] The Dragendorff reagent, a solution of bismuth nitrate and potassium iodide[10] that can be enhanced using aqueous sodium nitrite,[11] tests specifically for choline. Ninhydrin reagent tests for amino acids, amino sugars, and aminophosphatides.[12] Phosphate is detected using a molybdenum visualization agent containing an acidic copper and ammonium molybdate aqueous solution.[13] Additionally, if TLC plates contain an added fluorophore, UV detection can be employed to detect conjugated or aromatic compounds. Students are given a table of common phospholipid structures and "relative Rf" values rather than exact Rf values for all of the major phospholipids. A "relative Rf" value is a ranking of Rf values for seven commonly encountered lipids. Instead of matching an exact Rf value, students are challenged to match the spot to a structure according to the functional group that is present, by choosing which lipid they suspect is present in that spot, and then verify that the relative Rf value makes sense. An overview of the experiment is shown in Figure 2.

EXPERIMENT

An optional prelab handout, to be completed as homework before the experiment, is provided to introduce students to the basics of TLC and phospholipids. Students work in groups of two or three. Greater detail for the experiment and related handouts are provided in the Supporting Information. Week

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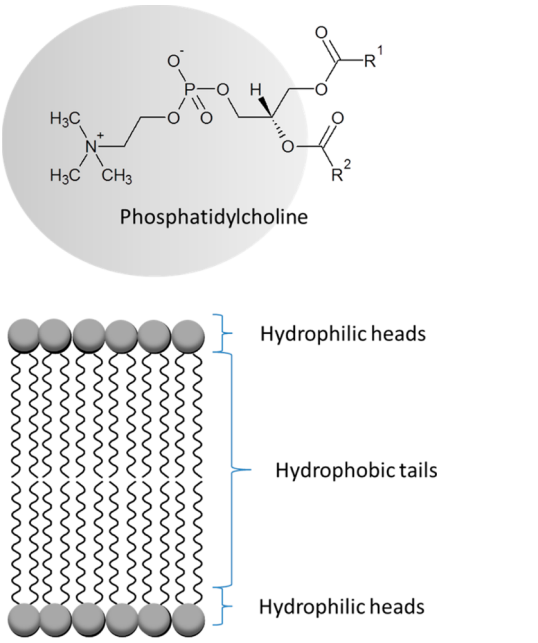

Figure 1. Membrane bilayer with hydrophobic tails of phospholipids facing inward and the hydrophilic heads of the phospholipids facing outward. A general structure of one class of phospholipids, phosphatidylcholine, is shown with the headgroup in gray and the fatty acid tails abbreviated as R^1 and R^2.

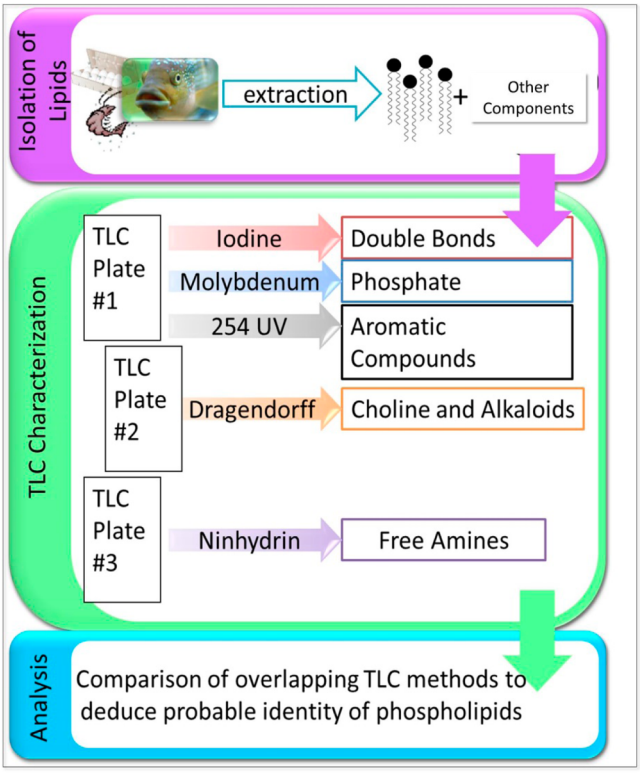

Figure 2. Overview of phospholipid extraction from natural sources and dietary supplements, and characterization by TLC.

one (data collection): dietary supplements in pill form (krill oil, soy lecithin, and fish oil) are punctured or carefully cut; the oil inside is extracted and dissolved in a minimum amount of chloroform. Soy lecithin granules are dissolved in a minimum amount of chloroform. A raw chicken egg is broken, and the yolk is separated from the white. The phospholipids are removed from the yolk using a two-step extraction procedure;[7] acetone removes water and triacylglycerols,[14] which leaves behind phospholipids and proteins, and a chloroform:methanol wash extracts phospholipids.

Samples are then spotted onto three silica TLC plates and eluted using a mixed solvent mobile phase of chloroform/methanol/water. Visualization of the spots on the plate is achieved using the various visualization agents (ninhydrin, Dragendorff, molybdenum, and iodine). Students are instructed to circle the colored spots on the TLC plates using colored pencil and to draw their TLC plate results in the provided handout. The plates are stored by the instructor for the following lab period, and the handouts are collected to preserve the guided-inquiry nature of the lab. Week two (interpretation of results): Students are given back their handouts and TLC plates and are instructed to complete the worksheet with guidance as needed by the instructor to answer questions as they arise.

HAZARDS

Access to a chemical fume hood is required for the use of the solvents and corrosive spray reagents in this experiment. Methanol, chloroform, and *n*-butanol are flammable, toxic, and irritating and should be handled with care. All phospholipid extracts that contain chloroform or methanol should be handled in a fume hood. Caution should be exercised when raw eggs are handled, and all surfaces should be cleaned with hot, soapy water after isolation of the yolk. Students with egg allergies should not be permitted to perform this lab as written; instead, omission of the egg extraction is recommended. Concentrated acids are corrosive to the skin and should be handled with care. Extreme caution should be observed when the corrosive reagents (in particular the Dragendorff and Molybdenum reagents) are sprayed on TLC plates. Bismuth nitrate and sodium nitrite are oxidizers, irritants, and toxic. Appropriate personal protective equipment, including gloves and safety glasses, should be worn when solvents, TLC reagents, and sprayed plates are handled. If TLC plates are heated, they should be handled using tongs in a fume hood. More details on the brand and purity of chemicals are available in the Supporting Information.

STUDENT LEARNING OUTCOMES AND REPRESENTATIVE RESULTS

This experiment was performed by undergraduates in a two-week, 3 h introductory organic chemistry laboratory setting of 16 students in groups of two. Students had previous experience with TLC, but no experience using visualization agents. Students also had no previous knowledge about phospholipids or biochemistry. The majority of students were able to arrive at correct answers using their TLC plates, with only one group unable to visualize spots because of incorrect technique for running the TLC plate. This group in particular included too much solvent in the running chamber and did not realize it until the end of the lab period during week one. The outcomes expected from this experiment are given in Table 1.

Overall, the experiment was successful with all student groups, except the aforementioned group, able to perform the required extractions, perform TLC, and successfully stain their plates using various visualization agents. The benchmark for success was established as >75% of the students being able to

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Table 1. Student Learning Outcomes

Student Learning Outcomes
At the completion of this exercise, students should be able to
1) Successfully perform thin-layer chromatography to obtain data
2) Successfully stain TLC plates using visualization agents
3) Identify that spots visualized with different stains contain different functional groups
4) Correctly identify specific phospholipids from their data
5) Apply knowledge obtained in the experiment to a new phospholipid (Question #10, student handout)
6) Apply knowledge gained in the experiment to the idea of "purity" via TLC and phospholipid purification (Question #11, student handout)

achieve each outcome in Table 1. For outcomes #1−3 and #6, 88−100% of students achieved the outcome by meeting the benchmark. For outcome #4, 72.9% of the phosphatidylcholine spots were correctly identified. When challenged with applying the knowledge gained in outcome #5, the benchmark was not met, with only 56.3% of students receiving full credit on question #10 of the student handout.

Of the students' calculated Rf data (separate from their interpretation of them), the values that were calculated for each spot were similar to the expected Rf values. For example, the calculated Rf value for PC had a percent error of 19.0% from the accepted Rf value; PE was 7.2%, PI was 0.4%, and PS was 42.6%. Additional information on these results, broken down by student learning outcome, as well as average student calculated Rf values and sample student data is given in the Supporting Information.

The experiment as described is a two-week experiment, but it can be modified to be a one-week experiment by omitting the analysis of two of the phospholipid structures and analyzing only one of the pills and the egg extraction (additional comments are included in the instructor notes in the Supporting Information).

DISCUSSION

The degree of difficulty for this experiment was set for a second-year organic chemistry or biochemistry laboratory course. Students should have previous knowledge of functional groups and experience with best practices for running TLC. This laboratory experiment allowed students to obtain hands-on experience by extracting phospholipids from natural sources, including dietary supplements (fish oil, krill oil, and lecithin) and raw chicken eggs. Students gained additional experience running TLC plates and learned to use them as a tool to characterize extracts from supplements and eggs. The extraction helped students to understand how different solvents affect the solubility. This was seen when the TLC of two extracts obtained from the egg extraction, one from acetone and one from chloroform/methanol, were compared. The extracts showed that little to no phospholipids were extracted in the acetone fraction, whereas phospholipids were selectively extracted in the chloroform/methanol fraction.

All of the information that students collected using visualization reagents helped to guide them to identify the phospholipid at each spot on the TLC plate. This included why some spots were positive for one visualization agent and negative for a different visualization agent. This reinforced the concept that different visualization agents detected different functional groups within the overall structure and that some phospholipid head groups contained more than one functional group. Additionally, students discovered that some spots overlapped and contained multiple phospholipids. For example, phosphatidylcholine and phosphatidylserine had similar Rf values.[15,16] Students used this information to realize the limitations of TLC in separation and identification of phospholipids with similar polarities and that additional information would be needed to conclusively identify phospholipid identity.

Student assessment was achieved by observing the students' resulting TLC plates and grading the completed guided-inquiry handout. Student outcomes #1−6 are given in Table 1. While the benchmark was met for oucomes #1−3 and #6, the benchmark for outcomes #4 and #5 was not met. This may be due to those students not actively engaging in the guided-inquiry process and asking questions as needed, as this experiment was done in a course where the majority of experiments were procedure-based with desired outcomes already specified. It is recommended that instructors remain engaged with students throughout the completion of the exercise to ensure understanding of the content. Additionally, it is hypothesized that deviations in the absolute Rf values determined from the students' data were due to potential errors in mobile-phase solvent preparation, such as using incorrect volumes or inadequate mixing or measuring elongated spots incorrectly, as PC tended to form a long smeared spot depending on its concentration within the extract. However, the relative ranking of phospholipid spot Rf values remained consistent, even when absolute Rfs may have been skewed because of incorrect solvent preparation or spot measurement.

In conclusion, the experiment utilized critical thinking skills, as students were faced with collecting several TLC plates containing various colored spots, and they needed to combine the information from the plates and the relative Rf values to arrive at a conclusion. The experiment handout is designed to guide students by asking questions that require students to think about the data that they are collecting and why they are doing specific steps. The postexperimental step was to compare/contrast the three TLC plates that were developed with the four visualization agents (iodine and molybdenum stains on plate one, Dragendorff on plate two, and Ninhydrin on plate three, with UV detection optional) to determine what functional groups were likely for the specific spots by looking for patterns, with an initial focus on the PC spot that was common among most of the substances studied.

ASSOCIATED CONTENT

Supporting Information

Instructor notes, equipment list, student handouts with answer key, and assessment data are provided. This material is available via the Internet at http://pubs.acs.org.

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Author Contributions

S.E.P. designed and cotaught the laboratory experiment and cowrote the article.

Notes

The authors declare no competing financial interest.

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