LABORATORY 2

Thin Layer Chromatography

Concept goal: polarity, correlation of structure to polarity, understanding inter-molecular interactions, correlate UV activity to presence of chromophoric groups, learn to calculate and interpret R_f values, understand variation of R_f with developing solvent

Operational goal: Preparing a TLC sample, Spotting, developing and visualizing a TLC plate, assess purity of a sample

Introduction

Chromatography is a separation technique based on difference in polarity of molecules. There are different types of chromatography: paper, thin-layer (TLC), column, high performance liquid (HPLC), and gas chromatography (GC). Chromatography can be used as an analytical technique and/or a preparative technique.

When used as an analytical technique, chromatography serves to analyze a sample for relative percentage composition (GC, HPLC) and/or purity (paper chromatography, TLC). When used as a preparative technique, chromatography helps in separating impurities and isolating a chemical in its pure form (flash column chromatography, TLC, HPLC). Some of the chromatographic techniques such as HPLC and TLC can be used as both analytical and preparative methods. Preparative GC, although can result in very pure compounds when carried out carefully, is not entirely non-destructive. It is always accompanied by a material loss of 40-60% due to vaporization of sample (necessary for GC analysis). For this reason, preparative GC is not widely used.

TLC, flash column chromatography and HPLC are in general, widely used for analysis and purification of organic compounds. In particular, TLC is used for a rapid analysis of an organic sample to:
1. Check the purity of a sample
2. Determine the components in a mixture by comparison with pure compounds
3. Monitor the progress of a reaction

In the process, a wide variety of solvents will be tested for their use as the developing solvent, which in turn will aid in choosing a suitable solvent for purification of a large sample using HPLC or flash column chromatography.

Plastic, metal or glass plates are available in general, for TLC. These plates have a coating of silica gel or alumina. This coating is called the "sorbent" and serves as the stationary phase. The sorbent is held tightly to the plate with a binder (plaster, hydrated calcium sulfate). A number of solvents can be used to develop a TLC plate. A mixture of varying ratios of hexanes and ethyl acetate works well in most cases. Other solvents include dichloromethane, ethyl ether, a mixture of methanol with dichloromethane (methanol being 10% or less), etc. The developing solvent used, serves as the mobile phase. In normal phase chromatography (which is more commonly used), the stationary phase is more polar than the mobile phase. There are cases where the polarities are switched or reversed, which is called reverse phase chromatography, which, for example, is used in separation of DNA or RNA molecules.

In this experiment, we will be using a normal phase TLC plate. Therefore, when developing a plate, the more polar components stick more to the silica gel and the relatively less polar components move further along with the developing solvent. This results in a distribution of spots on the TLC plate, which enables one to determine the order of polarities and hence the identities of the samples spotted. A helpful way of determining the polarities is the use of R_f values. R_f value is the ratio of the distance moved by the solute to the distance moved by the solvent. The optimal value for R_f is 0.2-0.4. The choice of a TLC developing solvent is driven by the goal to achieve optimal R_f value, in addition to good separation between spots.

In order to help visualize the spots applied to a TLC plate, usually, fluorescent indicators are added to the plate (there are plates without these indicators, but we will use the plates with UV indicators). This indicator absorbs and emits UV light, which appears green. The presence of a UV active compound on the plate prevents the UV light from reaching those parts of the plate. This results in a darker coloration on the plate. We will be using colored compounds that are visible to the naked eye, as well as those that are visible under UV light.

Reading and Pre-lab Assignments

Read the following sections in *The Organic Chem Lab Survival Manual: A Student's Guide to Techniques* by James W. Zubrick, John Wiley & Sons, Inc.

- Thin Layer Chromatography: TLC

Go through the procedure and make note of all chemicals you will need. Prepare a table of reagents with relevant information on each, as required by your instructor.

Helpful pointers:

Sample spotting:

Plastic backed, UV-active TLC plates are the ones that will be used for this experiment. These plates have a shiny side, and a rough side. The spots need to be placed on the rough, silica-coated side with capillaries meant for this specific use, which will also be available for you. Spotting samples should be liquids. The liquid sample used for TLC should be at the right concentration. Even if you have a solid sample, if you were to prepare a TLC sample, you should dissolve a small amount (2-3 mg) of the solid in a suitable solvent (1 mL). This is the sample that should be applied on the TLC plate. Too dilute a sample will result in inability to visualize the spots whereas too concentrated a sample will result in smearing.

Marking on the plate:

Any markings on the plate must be done only with pencil (why not use a pen? Think!). Keep in mind that the use of too sharp a pencil may scrap the silica coating.

Preparing the developing chamber:

Before developing the TLC plate, it is important to equilibrate the developing chamber. This is done by placing the developing solvent (4 mL) in the developing chamber and covering it with a lid or a watch glass. A filter paper may also be placed in the developing chamber to uniformly saturate the chamber with solvent vapors thus decreasing the rate of evaporation of solvent from the chamber or the TLC plate.

Developing the TLC plate:

When developing a plate, do not disturb the developing chamber. This may cause the developing solvent in the chamber to rise unevenly on the TLC plate and skew your results.

Interpretation of results:

When visualizing, typically, a pure compound should show only one spot. Multiple spots may indicate that the sample is a mixture or if not, impure and has to be purified. In other words, a mixture of A and B will show the spots corresponding to A as well as B. If the mixture of A and B shows additional spots than those corresponding to A or B, it can be concluded that this mixture has additional unknown contaminants in it.

Things that will be available to you in the lab:

Pre-cut plastic backed or metal backed TLC plates, TLC capillaries, UV lamp

Aim

The goal of your experiment is two fold:
1. To assess the purity of the colored organic compounds provided to you, using TLC.
2. To determine the identities of the analgesic components present in an unknown mixture by comparison with known analgesic samples using TLC.

Experiment 1:

In this first experiment, you will be analyzing samples of colored organic compounds. There are only seven dyes, Red #3, Red #40, Yellow #5, Yellow #6, Green #3, Blue #1, and Blue #2, that together form the base of most of the food coloring used in today's market. The number of dyes on an ingredient list can appear longer as modern food dyes are often formulated as 'lakes', with an inert binder such as a salt attached. Red #40 has largely replaced Red #3 in this country due to consumer concerns, though in some other countries consumers are more concerned with possible safety issues surrounding azo dyes such as Red #40. Green #3 is only rarely used in foods but may be included today as it runs very well under the TLC conditions being used.

Look up the structures of the seven food dyes. Note how all of the structures contain networks of double bonds, known as conjugated systems. This conjugation is what allows for light to absorbed and emitted in the visible range of the spectrum.

Procedure:

Obtain a TLC plate. With a pencil, draw light lines 1 cm from the top and 1 cm from the bottom of the plate. These will serve as your solvent front and baseline respectively. Use the ruler to make 6 evenly placed pencil marks on one of the lines, starting from 0.5 cm from either of the edges. This will be your baseline. Ensure that the marks are spaced no closer than 0.5 cm. Label the markings by writing the codes R3, R40, Y5, **U**, Y6, B1 under the baseline. The lane for **U** indicates the unknown sample. Keep in mind that you need to spot the samples carefully such that the spots are small and round. You can use a scrap TLC plate to practice spotting until you are ready to move on to the next step.

Using separate capillaries for each of the 5 standard samples, spot them on the appropriate place on the TLC plate. In lane #4, spot your unknown sample. The reason to spot the unknown sample
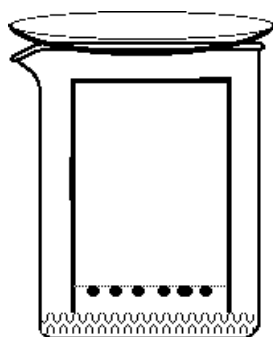
in the middle of the plate as opposed to anywhere else is to make the comparison of the unknown to the standards, easier. The TLC plate is ready to be developed. Get the developing chamber ready for equilibration. The developing solvent (made fresh with in a 25:25:25:10:1 ratio of 1-butanol, ethyl acetate, ethanol, water and glacial acetic acid) should be available in the fume hood, ready for use. Add 5 mL of the developing solvent to the 150 mL beaker, place a filter paper in the beaker and cover it with a watch glass. Let it equilibrate for 2-3 minutes. Place the spotted TLC plate into the developing chamber carefully, cover it with watch glass and observe the solvent rising through the TLC plate by capillary action.
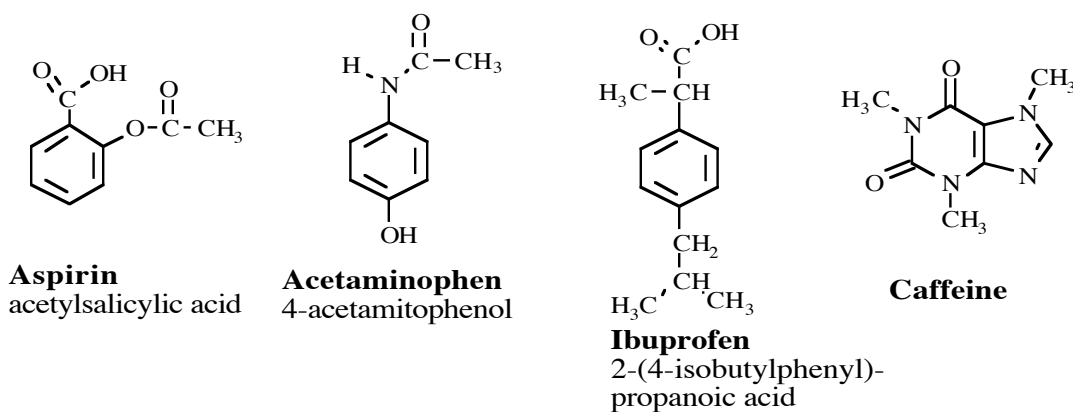
As the solvent rises, note what happens to the spots. It should be possible to see this clearly as the spots are colored and visible to the naked eye. Record the observations. Continue developing the plate until the solvent reaches the line drawn 1 cm from the top. Remove the plate carefully and let it air dry. Visualization is done both visually and under UV lamp. Remember to never look directly at the UV light. Trace each spot with a pencil. Mark the center of each spot. Measure the distance of each spot from the baseline, and the distance between baseline and solvent front. Use the measured distances to calculate the R_f value for each spot. The unknown is a mixture of dyes and should have more than one spot.

$$R_f = \frac{\text{Distance of spot from the baseline}}{\text{Distance between baseline and solvent front}}$$

Trace the TLC plate in your notebook. You may also take a picture of the TLC plate. For later interpretation of the data, it is helpful if a ruler is placed next to the plate before a picture is taken. After recording all the data, discard the used TLC plates, used TLC capillaries and the developing solvent along with any extra TLC samples you might have, in the designated waste containers.

Experiment 2 – Separation Of The Components Of Analgesic Tablets

If you read the label on most over-the-counter headache preparations, you will find that they contain the analgesic (anti-pain) substances aspirin, acetaminophen, and/or ibuprofen. Along with these they may also contain caffeine. Caffeine's role is not only the obvious one of acting as a stimulant. It has also been shown to increase the rate at which the body can absorb many pain relievers, thereby increasing their effectiveness. We will be running a chromatogram spotted with solutions of these four substances, and with an unknown containing one or more of these in a mixture like that found in an analgesic preparation. Our goal will be to identify the substance(s) in the mixture by comparison with the R_f values of the known substances.

Aspirin
acetylsalicylic acid

Acetaminophen
4-acetamitophenol

Ibuprofen
2-(4-isobutylphenyl)-
propanoic acid

Caffeine

4

Preparing the Plate

Following the same procedure you used for the colored food dyes in the first experiment, draw a light pencil line 1 cm from the bottom of a 4 x 6.6 cm chromatographic plate. Since the substances you will be spotting are colorless, it will be helpful to place five light pencil spots along this line starting about 4 mm from each edge. Spot the plate with the smallest possible spots of aspirin, acetaminophen, ibuprofen and caffeine. Save room in the middle of the line for your unknown analgesic.

Place a few mg of the unknown analgesic that your instructor will assign you in a clean scintillation vial. Add 10 drops of ethyl acetate to this unknown and stir to dissolve as much of the substance as you can. The starch binder used in the tablets may not go into solution. Dip a clean capillary pipette into your unknown mixture and place a spot of this on your plate.

After the solvent has dried, check the plate with the ultraviolet light. If you cannot see one of your spots, repeat the spotting so that you have enough compound on your plate. (Beware!! Too much sample is as bad as too little.) The plates we are using contain a fluorescent substance. Your spots will either fluoresce against the plate, or they will absorb the fluorescence of the plate and appear as darkened spots.

Running the Chromatogram

Place 4 mL of the developing solvent (95% ethyl acetate- 5% acetic acid) in your clean, dry 150 mL beaker. *This is a different developing solvent from the one you used in the last experiment!* Place your chromatographic plate in the beaker, cover with the watch glass and develop as before. When the solvent front is near the top of the plate, remove the plate and immediately make a mark on the plate to indicate the distance the solvent front has traveled. Let the solvent evaporate from the plate in the hood.

Visualizing the Spots

You have probably noticed that, unlike the first experiment, these substances have no pretty colors. Chemists use a variety of techniques for seeing where colorless compounds are on a TLC plate. Today, we will use the UV lamp to help us to visualize where the compounds are. Shine the UV lamp on the plate. With a light pencil mark, circle the shadowy dark spots that appear against the greenish fluorescent background of the plate. Lamps should be turned off after use.

Interpretation

Calculate the R_f values of each of these spots. Compare these values to those of your unknown. Identify the component(s) in your unknown and use these data in your report.

Changing the solvent conditions

In order to see how changing the polarity of the mobile phase influences R_f values, run a second chromatogram of the same compounds. This time, decrease the polarity of the previous mobile phase by making a developing solvent with two components: ethyl acetate and hexanes. The new solvent should be in the range of 80/20 to 60/40 ethyl acetate/hexanes. Visualize the spots and calculate the R_f values as above.

There is a separate waste container for the waste for this experiment in the fume hood. Be certain to place your waste solvent in the proper container.

Results, Discussion and Conclusion

Write your results, discussion of results and your conclusion. Complete any post-lab questions.

Abstract

This part should be filled in after the completion of the experiment and analysis of all data. <u>When submitting the report, the abstract should appear at the beginning of the report.</u>

Report

Adhere to the format required by your instructor and submit the report on time.

Summary

1. Chromatography is a separation technique used to isolate a pure compound from a mixture using the difference in polarities of the various compounds.
2. Thin Layer Chromatography is a separation technique wherein a mixture of compounds can be separated into its individual components based on the difference in polarity of the compounds. The stationary phase used is silica and the mobile phase is in general, an organic solvent or a mixture of organic solvents.
3. Given a mixture of compounds, you should be able to predict how the mixture will appear if spotted and developed on a TLC plate (the separation will be based on the polarity of the compounds and the solvent used as well as the nature of the TLC plate).
4. Given an unknown sample, you should be able to figure out what it is by spotting it with and comparing it to the standard samples.
5. Know how to calculate R_f values.
6. A more polar developing solvent will make the spots move further up on a TLC plate (know why).
7. A more polar compound will stick more to a normal phase TLC plate and move slowly than a relatively less polar compound (know why).
8. You used short-wave UV light to visualize the TLC plates, after developing.

Questions

Your instructor may assign some or all of these questions as pre- or post-Laboratory assignments.

1. Indicate which property of compounds is responsible for the separation in a TLC.
2. How does the separation occur during a TLC?
3. Name 3 adsorbents used in TLC.
4. Define eluent.
5. Indicate 3 ways of visualizing a TLC after elution.
6. Define R_f.
7. Name three other types of chromatography.
8. Indicate 3 uses of Thin Layer Chromatography
9. (a) A student performed a thin layer chromatographic separation experiment. In the first run, four standard substances A – azobenzene, B – benzoin, C –cinnamic acid, and D – dextrose were spotted on the plate. The line indicates the solvent front. In the second TLC run a mixture (X) of two of these same substances was separated. Based on R_f values, what is the identity of the unknown mixture X?
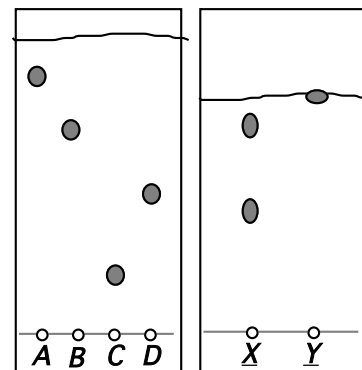
(b) A second mixture, Y, was run at the same time. What is wrong with the solvent system used to try to separate Y?

10. A student was asked to spot the following three compounds on a TLC plate and develop it. Show the diagram of the developed TLC plate appropriately labeled.

A   B   C
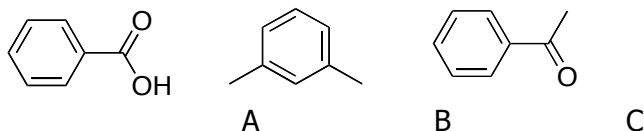
11. Why is it common to mark the TLC plates with pencil rather than ink before developing?
12. What was the visualization technique used to see the developed TLC plates when you used the powdered analgesics? Why did you have to resort to this visualization technique?