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Edvo-Kit #

113

Edvo-Kit #113

## Principles of Thin Layer Chromatography

### Experiment Objective:

The objective of this experiment is to gain an understanding of the theory and methods of thin layer chromatography.

**Store this experiment at room temperature.**

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## Experiment Components

*Store this experiment at room temperature.*

### Components

- A Blue Dye
- B Violet Dye
- C Yellow Dye
- D Dark Blue Dye
- E Mixture of A-D Dyes
- F Aqueous potassium acetate: ethanol
- G Aqueous sodium citrate: isopropanol
- 10 x 20 cm thin layer cellulose-based plate
- Mini-transfer pipets

### Check (✓)

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Experiment #113 provides enough reagents for 4 experiment sets, for a total of 8 separations.

All experiment components are intended for educational research only. They are not to be used for diagnostic or drug purposes, nor administered to or consumed by humans or animals.

### Requirements *(not included with this kit)*

- 250 mL beakers (6 to 7 cm in diameter)
- Metric rulers
- Pencils
- 5 or 10 mL pipets
- Pipet pumps

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## Background Information

### PRINCIPLES AND PRACTICE OF THIN LAYER CHROMATOGRAPHY

Thin layer chromatography (TLC) is an invaluable method used in chemistry and biochemistry for the separation and analysis of a wide variety of molecular mixtures. TLC methods can be used to separate mixtures of inorganic ions, organic molecules and bioorganic compounds such as pigments, lipids, amino acids, nucleotides and sugars. The TLC plate typically consists of a 0.1 mm thick layer of adsorbent material bonded to a glass or plastic support. The adsorbent consists of many microscopic plates. These surfaces provide a large area for chromatographic separation. After a small volume of sample solution is applied to the adsorbent surface and allowed to dry, the plate is placed in a beaker or tank containing the appropriate solvent. Only the edge of the plate nearest the samples is in contact with the solvent. The solvent is drawn into the dry adsorbent material and travels up the plate through the samples. The migration rate of the sample components over the adsorbent depends on their chemical structure.

An understanding of TLC requires an introduction and the general principles by which they work. Adsorption chromatography was discovered by the botanist Tswett in 1903. He observed that ether solutions of plant pigments, such as the chlorophyll, could be separated into different colored zones by passing them through a column containing calcium carbonate. Significant development of adsorption chromatography occurred in the early 1930s when it was used in the preparative chemistry of pigments. During this period, preparative separation of colorless organic compounds was accomplished with the advent of the appropriate chemical detection methods. Silica gels and paper strips were used in the 1940s for the separation of water soluble substances, such as amino acids and sugars. Other common adsorption chromatography materials include magnesium carbonate, magnesium silicate, alumina and activated charcoal. Most adsorption materials can have surface charges. Substances that are adsorbed to these materials are polar or polarizable molecules. An example of a polar molecule is water.

As shown in Figure 1, the molecule has no net charge. The oxygen atom has slightly more negative charge than the hydrogen atoms, which consequently have slightly more positive charge. This is because the oxygen nucleus attracts the negatively charged electrons in the chemical bonds more strongly than the hydrogen nuclei. Therefore, even though the water molecule is overall electrically neutral, its individual atoms do possess partial negative or positive charge. Molecules that exhibit these properties are called polar. Molecules that contain opposite charges, or partial opposite

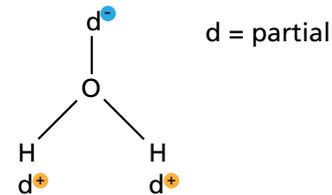
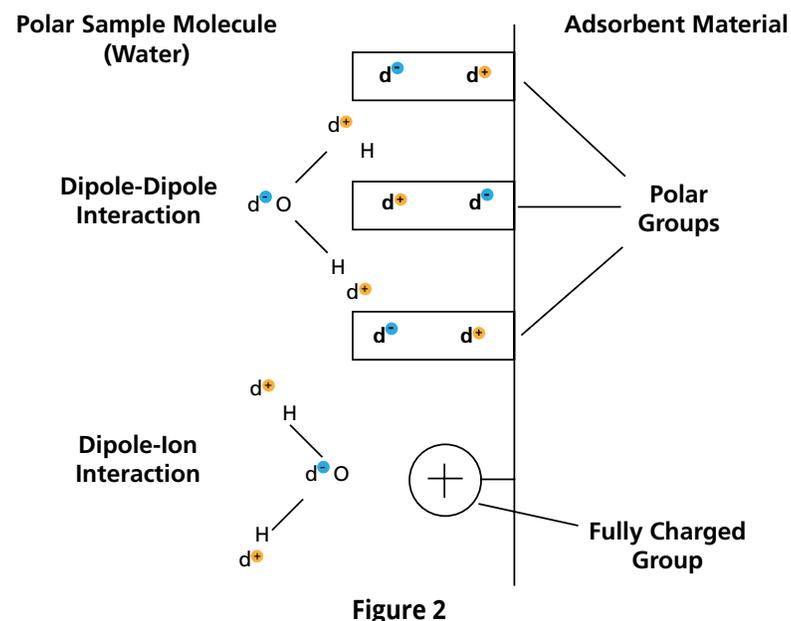


Figure 1



charges, possess a dipole. The adsorbent material has many polar and fully charged (ionic) chemical groups on its surface. Polar sample molecules can interact with these groups by dipole-dipole and dipole-ion interactions. These interactions basically involve the attraction between regions with opposite charges. Such interactions are shown in Figure 2.

Many types of molecules can have a net positive or negative charge. Charged sample molecules interact with the adsorption surface by the same chemical forces discussed. TLC plates can also be prepared to contain a large amount of chemical groups with net positive or negative charges on the surface. Charged sample molecules can be efficiently separated on these plates with solvents having the appropriate pH and salt concentrations. This type of TLC is called ion exchange and involves the interaction of compounds with opposite net charges. Ion-exchange TLC typically involves stronger interactions than adsorption TLC.

TLC is generally very sensitive to small differences in chemical structure. The structure affects the strength and type of interactions between sample and adsorbent. In addition, different sample molecules will have different solubilities in a given solvent. Differences in solubility are also dependent upon chemical structure. The composition of the solvent can easily be varied to provide a virtually unlimited set of conditions for chromatography. For example, aqueous sodium citrate: isopropanol (solvent G) is less polar than aqueous potassium acetate (solvent F) since it contains isopropanol and less water. Isopropanol is much less polar than water. The dyes in this experiment migrate differently on the same kind of TLC plate depending on whether solvent G or F is used.

Solubility differences exhibited by sample molecules between two liquid phases is the basis of partition chromatography. The most obvious example of a two-phase liquid system is oil and water. In partition TLC the solvent is called the mobile phase. The liquid that is associated with the surface of the TLC plate is called the stationary phase. The stationary phase consists of molecular layers of fluid on the surface of the TLC plate.

Sample molecules will have a preferred distribution between the stationary and mobile phases depending on their structure. Samples that have little or no solubility in the mobile phase and high solubility in the stationary phase will migrate slowly. Conversely, samples that have little solubility in the stationary phase but are highly soluble in the mobile phase will have the fastest migration rates.

An example of the partition process is chromatography on paper or on cellulose TLC plates. The stationary phase consists of the water molecules hydrating the cellulose fibers (cellulose is a polymer of glucose). This hydration water does not behave like the "free" liquid and is roughly analogous to a very concentrated aqueous solution of a sugar or polysaccharide, i.e., gel-like. In practice, both partition and adsorption processes operate simultaneously, to different degrees, in the same TLC experiment. For instance, certain sample molecules could interact directly with the cellulose fibers which would involve an adsorption process.

In this experiment, a mixture of dyes will be separated on a cellulose-based TLC plate using two different solvent systems. The solvent should be allowed to develop about half the distance of the plate. The dye molecules contain different types and amounts of charged and polar chemical groups. They also differ from one another with respect to their molecular weights, geometry and the positions and numbers of carbon-carbon double bonds.

After the experiment is completed and the plate is partially dry, a faint wavy line can be observed at the last location of the leading edge of the solvent in the adsorbent. This line is called the solvent front. The distance traveled by the sample from its origin divided by the distance of the solvent front from the sample origin is defined as the  $R_f$ . A substance that does not migrate from the sample origin has an  $R_f = 0$ , while one that is not adsorbed at all (migrated with the front) has a  $R_f = 1$ . The  $R_f$  is a characteristic value for a particular substance chromatographed with a given adsorbent and solvent system. The  $R_f$  cannot be greater than one (1.0).

## Experiment Overview

### EXPERIMENT OBJECTIVE

The objective of this experiment is to gain a basic understanding of chromatographic theory and methods through the use of thin layer chromatography to achieve the separation of a mixture of dyes.

### LABORATORY SAFETY

Be sure to READ and UNDERSTAND the instructions completely BEFORE starting the experiment. If you are unsure of something, ASK YOUR INSTRUCTOR!

- Wear gloves and goggles while working in the laboratory.
- Exercise caution when working in the laboratory – you will be using equipment that can be dangerous if used incorrectly.
- Wear protective gloves when working with hot reagents like boiling water and melted agarose.
- DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS.
- Always wash hands thoroughly with soap and water after working in the laboratory.



### LABORATORY NOTEBOOKS:

Scientists document everything that happens during an experiment, including experimental conditions, thoughts and observations while conducting the experiment, and, of course, any data collected. Today, you'll be documenting your experiment in a laboratory notebook or on a separate worksheet.

#### Before starting the Experiment:

- Carefully read the introduction and the protocol. Use this information to form a hypothesis for this experiment.
- Predict the results of your experiment.

#### During the Experiment:

- Record your observations.

#### After the Experiment:

- Interpret the results – does your data support or contradict your hypothesis?
- If you repeated this experiment, what would you change? Revise your hypothesis to reflect this change.

## Applying Dye to the Thin Layer Plate

1. Use a fresh mini-transfer pipet for each dye stock.
2. Tap each tube of dye to dislodge any fluid from inside the cap.
3. Place the end of the mini-transfer pipet just below the surface of the dye in the microtest tube. Gently squeeze the bulb and allow the liquid to fill just the very tip of the small stem at the base of the pipet and then withdraw the pipet from the tube. This is approximately 1 microliter ( $\mu\text{L}$ ).
4. To apply the dye to the thin layer plate:
  - Hold the pipet vertically and touch the end to the sample origin line on the adsorbent thin layer plate and very gently squeeze the pipet to slowly release the dye.
  - Quickly lift the pipet away from the adsorbent plate.



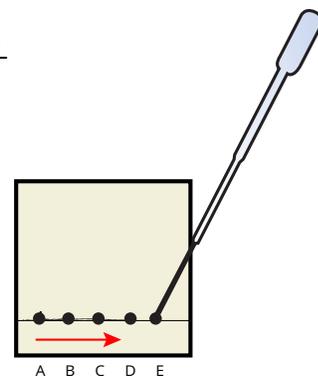
**NOTE:**  
The exact volume withdrawn with the pipet is not critical.

### APPLICATION OF DYES TO PLATES F AND G

1. Obtain two thin layer plates. At the top (edge opposite the sample origin line), label one plate F and the second plate G.

#### For each plate:

2. Start the application of dyes with Light Blue (A) towards the left side of the plate. Apply 1  $\mu\text{L}$  to each plate.
3. Using a fresh or rinsed pipet, apply 1  $\mu\text{L}$  of Violet (B) to the right of dye A on both plates. Leave a small space between the samples.
4. Apply 1  $\mu\text{L}$  of Yellow (C) to the right of dye B on both plates.
5. Apply 1  $\mu\text{L}$  of Dark Blue (D) to the right of dye C on both plates.
6. Apply 1  $\mu\text{L}$  of the mixture (E) to the right of dye D on both plates.
7. Let the sample spots dry for 5 minutes.



*continued*

## Applying Dye to the Thin Layer Plate, continued

### CHROMATOGRAPHY

- Place the bottom edge of plate F (edge nearest sample origin line) into a beaker containing aqueous potassium acetate (solvent F).

It is important that the level of the solvent is lower than the sample origin. Stand the plate straight up using the wall of the beaker as a support.



- In the same manner, place plate G into a beaker containing aqueous sodium citrate: isopropanol (solvent G).
- Let the plates stand in solvent for approximately 12 minutes.

The solvent front should not reach the top of the plates in this time.

- Before the solvent front reaches the top edge, remove the plates and lay them flat (adsorbent side up) on a paper towel to dry.



- Calculate the  $R_f$  of samples A - D on both plates:
  - In millimeters, measure the distances from the sample origin to the approximate center of each spot. This may be more difficult with dye D.
  - The  $R_f$  is the distance traveled by the sample from its origin, divided by the distance of the solvent front from the origin. The  $R_f$  cannot be greater than 1.

$$R_f = \frac{\text{Distance traveled by sample}}{\text{Distance traveled by front}}$$

## Study Questions

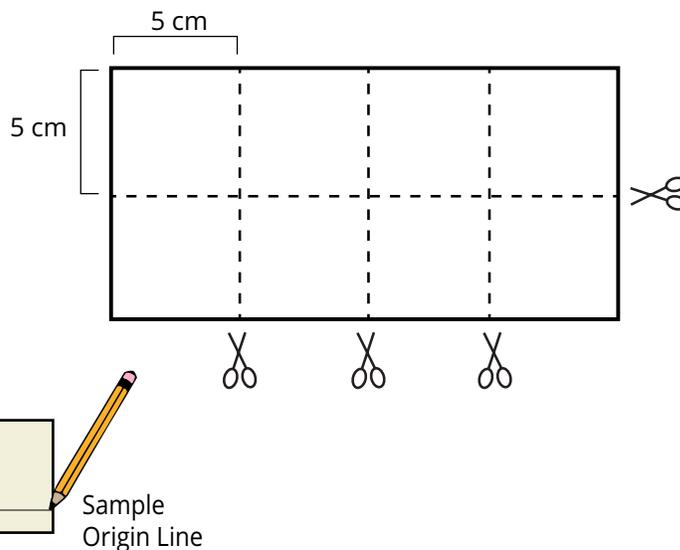
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1. Are the  $R_f$  values of the dyes the same in solvent F and solvent G?
2. Assume that partition chromatography was only operating during the experiment. Which dyes were more soluble in solvent F? Which dyes were more soluble in solvent G?
3. Assume that only adsorption chromatography was operating during the experiment. Which dye was adsorbed most strongly in solvent F? Which dye was least adsorbed in solvent F?
4. Assume that a solvent having more isopropanol than solvent G was used in the experiment. Predict whether sample B would migrate a smaller or larger distance in this solvent than in solvent F or G. Why?
5. TLC can be one of several methods used to help identify an unknown compound. Explain how this might be accomplished using the dyes as an example.

# Instructor's Guide

## PREPARATION OF THIN LAYER PLATES

1. Handle the plate by its edges. Divide the plate into eight (8) 5 x 5 cm squares by lightly drawing a line using a blunt pencil and the straight edge of a ruler on the cellulose adsorbent.
2. Carefully cut the plate into 8 pieces with a sharp pair of scissors. Do not use a paper cutter. You will need two pieces for each experiment set.
3. Measure 1 cm up from an edge on each piece and lightly draw a straight line across using a pencil. Do not press hard, otherwise you will scrape the cellulose off of its support. This line will be the sample origin.



## PREPARATION OF SOLVENTS

*(Prepare just before the lab.)*

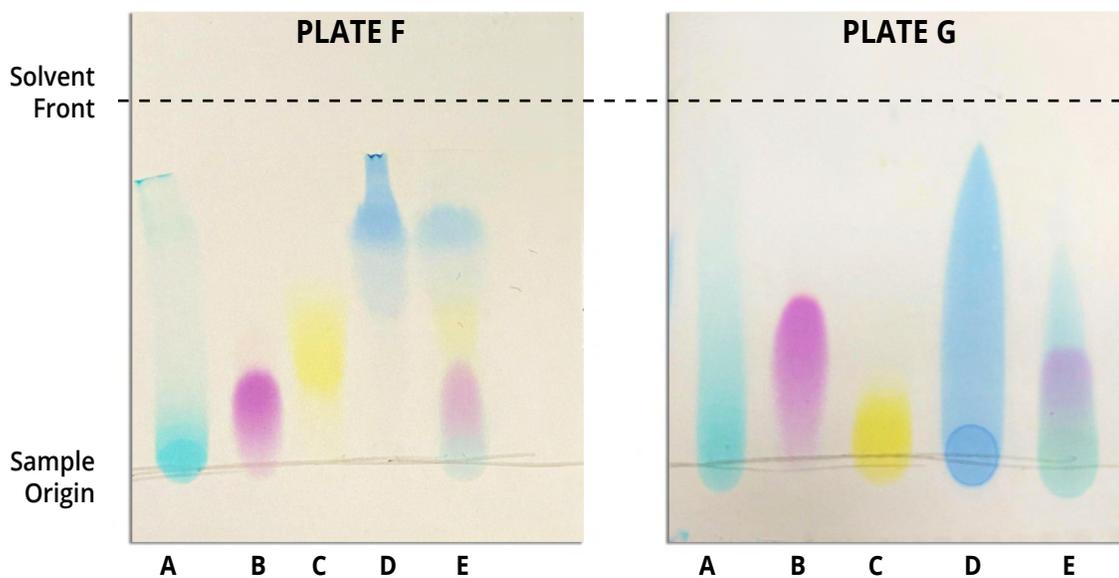
1. Aqueous potassium acetate: ethanol (Solvent F) is used undiluted.
2. Aqueous sodium citrate: isopropanol (Solvent G) is used undiluted.
3. For each group performing the experiment, label two beakers - one 250 mL beaker "F" and another "G".
4. Add approximately 4 mL of aqueous potassium acetate: ethanol (F) to each beaker F (or just enough fluid to cover the bottom).
5. Add approximately 4 mL of aqueous sodium citrate: isopropanol (G) to each beaker G.

## PREPARATION OF DYES

*(Prepare just before the lab.)*

Add 150  $\mu$ L distilled water to each tube of dye (Components A-E). Allow sample to hydrate for 5 minutes. Use a vortex to mix the samples or tap them with your finger to mix until the color of each dye has been evenly distributed.

## Expected Results



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