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#11 Chromatographic Separation of Methylene Blue and Sodium Fluorescein

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INTRODUCTION

Description

This is a three-part lab. In Part I, the students determine the best solvent system for separating a methylene blue/sodium fluorescein mixture by thin layer chromatography using silica gel as the stationary phase. The students work cooperatively in groups of four. Each student individually conducts an initial TLC separation using ethanol as the mobile phase and silica gel as the stationary phase. Each member of the group then conducts a second TLC separation experiment, with each student in the group using a different solvent mixture. The team members analyze the data of all trials, and determine the best solvent for separating the methylene blue/sodium fluorescein mixture.

In Part II of the lab, the students determine the best solvent system for separating the methylene blue/sodium fluorescein mixture by column chromatography with alumina as the stationary phase.^{1,2} Each member of the team uses a different solvent system to try and separate the mixture. The team members analyze the data of all trials to make their determination. The students are then asked to compare the two (TLC and column) chromatography methods.

Part III is a final project. Based on the previous experiments, the groups determine the best stationary phase and mobile phase to use in separating the components of the methylene blue/sodium fluorescein mixture by column chromatography with the stipulation that the sodium fluorescein must elute first out of the column. The experimental design and subsequent results from each group are compared and discussed in a class setting.

Student Audience

This lab is suitable for students in high school chemistry, beginning college chemistry, general chemistry, beginning organic chemistry, and general organic chemistry.

Goals for the Experiment

In this exercise the students will:

- Learn the techniques associated with thin layer and column chromatography.
- Determine the effects of the stationary phase in separating a mixture by chromatography.
- Determine the effects of the mobile phase in separating a mixture by chromatography.
- Use the acquired knowledge to successfully conduct a specific separation using column chromatography.

Recommended Placement in the Curriculum

This lab would be suited for placement during topics on separation science. It would be appropriate about the third or fourth lab in an organic chemistry course.

Student Handout

Chromatographic Separation of Methylene Blue and Sodium Fluorescein

Purpose

The purpose of this lab is to investigate the role of the stationary phase and mobile phase in chromatography.

Scenario

Sodium fluorescein, the salt of acidic fluorescein, is a fluorescent dye often used in examining subterranean waters, in order to detect water flow patterns and leaks, to determine the approximate volume of water delivered by a natural spring, to detect the source of contamination of drinking water, and to detect infiltration of soil with waste waters of factories.³ Your friend, Tim, is in the business of detecting sources of contamination in drinking water. Unfortunately he has run out of sodium fluorescein. He was able to find a mixture of sodium fluorescein and methylene blue that was being used as a dye. The methylene blue will interfere with his tests. He has no need for the methylene blue, but needs the sodium fluorescein immediately. Your mission, if you decide to accept it, is not only to develop a method to separate the mixture into its pure components, but to also develop a method that will enable you to collect the sodium fluorescein first, so that it can be used ASAP.

Safety, Handling, and Disposal

- Alumina is a body tissue irritant. Avoid inhalation of dust.
- Ethyl alcohol is flammable and should be kept away from all flames.
- Methylene blue is moderately toxic and should be handled with care.
- Chemical splash goggles are required!
- Dispose of all dye solutions in a waste container provided by your instructor.

Materials

Per student

- 2 Pasteur pipettes (5.75 mL)
- 1 Pasteur pipette (9.00 mL)
- 2 latex tubing or aquarium tubing (1/4 in. o.d.)
- 1 spotter or micropipet
- 1 dropper pipette
- 3-6 silica-gel-coated TLC strips (4cm x 10 cm)
- 2 filter paper (11.0 cm)
- 2 250-mL beakers, wide-mouthed bottles, or a developing chamber
- 2 150-mL beakers
- 2 50-mL Erlenmeyer flasks (larger sized flask will also work)
- 2 watch glasses
- alumina (neutral)
- silica gel (35-70 mesh)
- methylene blue
- sodium fluorescein
- ethanol (95%)
- glacial acetic acid

Background

Chromatography is a technique used to separate the components of a mixture by distributing them between two phases, the stationary phase and the mobile phase. The two types of chromatography that will be explored in this lab are thin-layer chromatography (TLC) and column chromatography.

In TLC, the adsorbent (stationary phase) is thinly spread onto a flat sheet of supportive plastic. The mixture to be separated is applied onto the stationary phase about 1 cm from the bottom of the chromatographic sheet. The sheet is then placed into a developing chamber containing the mobile phase. The mobile phase rises up the chromatography sheet by capillary action. As the mobile phase proceeds up the sheet, the components of the mixture are retained in various degrees by the stationary phase. The chemical composition of the stationary phase and the mobile phase play a significant role in how far the components travel up the chromatographic sheet.

In column chromatography, a glass column is packed with a solid stationary phase. The mixture to be separated is applied at the top of the column. The mobile phase descends by gravity through the column. The components of the mixture to be separated have different properties. The rate at which the components descend through the column depends on several factors. The component is retained by the stationary phase to a certain extent depending on the properties of the stationary phase and the properties of the component. The solvation power of the solvent also affects the rate of elution. The rule of “like dissolves like” applies here. The individual components, with different affinities for the stationary phase and the mobile phase, are continuously absorbed onto the stationary phase, solvated by the mobile phase eluting through the column, reabsorbed onto the stationary phase, etc. The speed at which the components travel through the column is directly related to the number of absorption-elution cycles that occur. Therefore a balance between the solvation power of the mobile phase and the absorption power of the stationary phase determines how fast each individual component travels through the column.^{1,2}

Think of a piece of wood floating down a creek. If there is a lot of grass growing in the stream, the wood will get caught in the grass for awhile, then it will break loose and flow down the creek a short distance, get caught in some more grass or rocks, break free again, and continue this process until it has made its way down the creek. Aluminum cans will travel down the creek at different rates than the wood based on the amount of time they are retained by the grass. If there is no grass in the creek, the piece of wood and aluminum can will both reach the end of the creek at the same time because there will be nothing to restrict their flow.

The careful choice of stationary phase and mobile phase are both critical in order to perform a good separation.

Procedure

Part I: Thin Layer Chromatography (TLC)

Students will work in teams of 3-4. All team members will individually conduct the first experiment. Subsequently, each team member will conduct the experiment with different solvent systems. Team data will be analyzed to aid in solving the problem outlined in the scenario.

A sheet of plastic is often used as a support for a thin layer of adsorbent (silica gel) spread over

the sheet. These silica-coated TLC sheets can be cut into 4-cm wide strips. The length can also be cut so that the strip will fit inside a wide-mouthed bottle, beaker, or other type of developing chamber.

1. For best results, the developing chamber's atmosphere must be saturated with the solvent. This can be accomplished by lining the developing chamber with filter paper. Place a square of filter paper (10-cm x 10-cm) in the jar. Ethanol (95%) will be used as the mobile phase in the first TLC experiment. Transfer enough solvent (ethanol) into the developing chamber or beaker to achieve a level of about 0.5 cm deep. Cover the developing chamber with a glass lid or with a watch glass if a beaker is used.
2. Cut a silica-gel-coated TLC sheet into 4-cm-wide strips with enough length to fit inside the wide-mouthed bottle, beaker, or other type of developing chamber. Measure up 1 cm from the bottom of the TLC strip. Using a #2 pencil, draw a line across the width of the strip. Carefully mark two evenly spaced spots along the line to delineate where to spot the dye solution.
3. The TLC strip can now be spotted. Dip the tip of a micropipet into the dye solution, allowing the pipette to fill by capillary action. The tip of the micropipet is carefully touched to one of the marked spots on the TLC strip. Gently push down on the micropipet until the dye solution transfers to the strip. Push the micropipet down at least three more times to achieve a concentrated spot. Use the spotter to apply a second dot of the dye onto the TLC strip. See Figure 1.

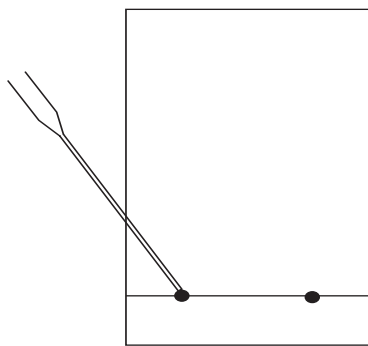


Figure 1: Spotting a TLC plate

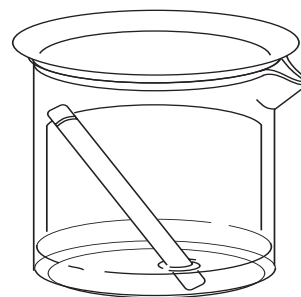
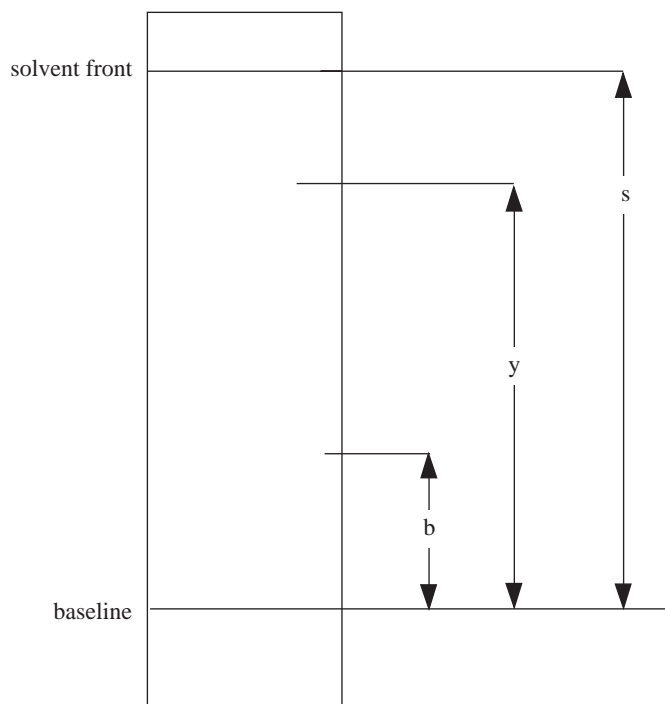


Figure 2: Developing Chamber

4. Place the TLC strip into the developing chamber with the spotted end at the bottom. Cover the developing chamber as shown in Figure 2.
5. Allow the solvent to rise up the sheet until it is within a few centimeters from the top. Remove the strip from the bottle and immediately mark the solvent front with a pencil.

6. Calculate the Retention Factor, R_f , for both the sodium fluorescein and the methylene blue.

Figure 3: Calculation of R_f values



$$R_f = \frac{\text{distance component travels}}{\text{distance solvent travels}}$$

$$\text{For the highest component } R_f = \frac{y}{s}$$

$$\text{For the lowest component } R_f = \frac{b}{s}$$

7. Repeat steps 2-6, replacing the ethanol with one of the mobile phases listed in Table 1. Each member of the team should select a different mobile phase. Additional systems can also be used if the team so desires. Team data will be collected and analyzed.

Table 1: Composition of developing solvent systems					
Volume of each Component in the Mobile Phase					
System	95% Ethanol	Acetone	n-Propanol	Water	Glacial Acetic Acid
A	1	0	0	0	0
B	1	0	0	1	0
C	0	1	1	1	0
D	0	0	1	1	1
E	0	1	1	1	1
F	0	0	0	1	0
G	0	3	2	1	0
H	2	1	0	3	1

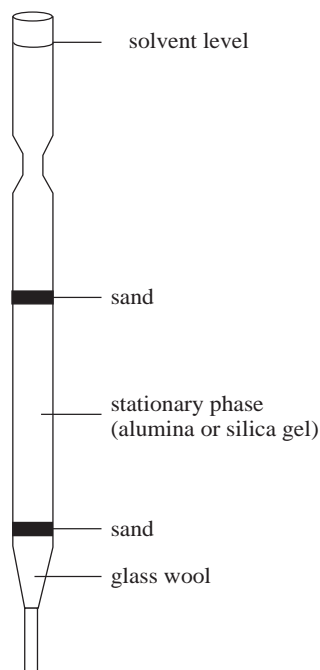
Questions

1. Which component (methylene blue or sodium fluorescein) had the largest R_f value for the various mixtures?
2. Which component (methylene blue or sodium fluorescein) was retained to the greatest extent by the silica gel coating?
3. Which solvent system(s) gave the best separation?
4. Which solvent system(s) gave the poorest separation?
5. Describe any relationship(s) you find between the R_f value and the mobile phase employed.
6. Why are R_f values used instead of just the distance the component traveled?

Procedure

Part II: Column Chromatography

Each member of the group will conduct a column chromatography experiment with one of the solvent systems listed in Table 1. The results from the group will be collected and analyzed to determine a procedure for Part III.



1. Insert a small plug of glass wool into the neck of a Pasteur pipette. The glass wool is packed firmly into the neck using the end of the 9.00 mm Pasteur pipette or an “opened” paperclip. The glass wool is covered with a thin layer of sand.
2. Alumina is added to the column until it reaches about half way up the column. The alumina is packed by gently tapping the outside wall of the column with your finger or a pencil. The eluting solvent is then added to the column and allowed to flow through to “wet” the column. If needed, add more solvent.
3. Allow the solvent to flow through the column until only about 0.5 cm remains above the stationary phase.
4. Add 5 drops of the methylene blue/sodium fluorescein mixture to the top of the column. Allow the mixture to flow just below stationary phase (alumina). Carefully add a thin layer of sand. Slip a 10-cm section of latex or aquarium tubing over the top of the column and fill the tubing with eluting solvent.⁴
5. Allow the solvent to elute through the column. When the solvent gets close to the top sand layer, use a dropper pipette to add more solvent. Never allow the column to become dry.

6. When the first component of the mixture appears ready to elute from the column, place a 50-mL Erlenmeyer flask under the stopcock to collect the eluent. Once the first component has been eluted, remove the flask and label it.
7. Add water or 10% glacial acetic acid to the top of the column. Collect the second dye component.
8. Compare results from all group members.

Questions

1. Which solvent system gave the best separation?
2. Which solvent system gave the poorest separation?
3. Which component (methylene blue or sodium fluorescein) was retained to the greatest extent by the alumina?
4. What is the purpose of the glass wool and the sand?
5. Why do you have to wait for the solvent to go below the sand before you add the dye mixture?
6. What might happen if as you added more solvent the dye mixed with the solvent?

The Problem

Design a method to separate the methylene blue/sodium fluorescein mixture into its pure components, such that the sodium fluorescein is collected first. You have the materials listed below:

glass wool	distilled water
sand	95% ethanol
Pasteur pipettes	n-propanol
alumina	acetone
silica gel	acetic acid

Write a procedure for your proposed method of separation. If time permits, complete this separation.

Results

After performing the separation, turn in the collected container of sodium fluorescein to your instructor.

Instructor Notes

Chromatographic Separation of Methylene Blue and Sodium Fluorescein

Time Required

3 hours

Group Size

3-4 students

Materials

Per class of 24 students

- 48 Pasteur pipettes (5.75 mL)
 - 24 Pasteur pipettes (9.00 mL)
 - 5 m latex tubing or aquarium tubing (1/4 in. o.d.)
 - 6 spotters or micropipets (1 for each group)
 - 24 dropper pipettes
 - 72 silica-gel-coated TLC strips (4cm x 10 cm) (These can be cut from sheets.)
 - 48 filter paper (11.0 cm)
 - 48 250-mL beakers, wide-mouthed bottle, or a developing chamber
 - 48 150-mL beakers
 - 48 50-mL Erlenmeyer flasks (larger sized flask will also work)
 - 48 watch glasses
 - alumina (aluminum oxide, activated, neutral, Brockmann I)
 - silica gel (35-70 mesh)
 - methylene blue (1 mg dissolved in 10 mL 95% ethanol)
 - sodium fluorescein (1 mg dissolved in 10 mL 95% ethanol)
- Combine the methylene blue and sodium fluorescein solutions into a single container.
- ethanol (95%)
 - glacial acetic acid

Safety, Handling, and Disposal

Do not work without chemical splash goggles!

Acetic Acid

Hazard Alert: Corrosive to skin and tissue, moderate fire risk, toxic by ingestion

Storage: Orange #1 in a dedicated acid cabinet.

Disposal: Dilute slowly with twentyfold excess of water with stirring and rinse down the drain with plenty of water.

Alumina:

Hazard Alert: Body tissue irritant; avoid inhalation of dust

Storage: Inorganic #4

Disposal: Bury in a landfill approved for the disposal of chemical and hazardous waste.

Ethyl Alcohol:

Hazard Alert: Toxic by ingestion

Storage: Organic #2

Disposal: May be disposed of, not to exceed 100 grams per day, by rinsing down the sink

followed by large amounts of water.

Silica Gel:

Hazard Alert: Relatively nonhazardous

Storage: Inorganic #4

Disposal: Bury in a landfill approved for the disposal of chemical and hazardous waste.

Sodium Fluorescein:

Hazard Alert: NONE

Storage: Organic #9

Disposal: Bury in a landfill approved for the disposal of chemical and hazardous waste.

Methylene Blue:

Hazard Alert: Moderately toxic

Storage: Organic #9

Disposal: Bury in a landfill approved for the disposal of chemical and hazardous waste.

The fluorescein and methylene blue can be collected, the solvent evaporated off, and reused.

Points to Cover in the Pre-Lab Discussion

The concept of chromatography should be explained. A background is written at the beginning of the procedure. This background could be presented as an introduction to the scenario. It is very helpful if the students have a basic knowledge of how chromatography separates components of a mixture.

Procedural Tips and Suggestions

- The elution time is much quicker if the Pasteur pipettes are only half filled with the stationary phase.
- If time does not permit the completion of Part III, the students should at least provide a description of how they expect to solve the problem described in the scenario.
- Often in chromatography separation occurs because of polarity. However, in this lab, the separation is also due to acid-base interactions. The students should be aware that all separations are not based on polarity.

Sample Results

Data Table (Merck Index, 12th ed., pp. 705, 1035)			
Substance	M.W. (g/mol)	Color	Solubility
fluorescein, disodium salt	376.28	orange-red powder	water, alcohol (slightly)
methylene blue	319.86	dark green crystals	water, alcohol, chloroform

RESULTS (TLC)

Several different solvent systems were tested to obtain a variety of results.

R_f Values				
System	Methylene Blue	Fluorescein		
EtOH	0.00	0.75	0.68	0.54
H ₂ O	0.02	0.57	0.41	0.88
1:1 Et:W	0.02	0.78	0.80	0.81
1:2 Et:W	0.02	0.58	0.57	0.64
2:1 Et:W	0.02		0.84	0.61
1:1 Ac:W	0.51			0.93

R_f Values		
System	Methylene Blue	Fluorescein
A	0.012	0.72
B	0.014	0.66
C	0.027	0.80
D	0.035	0.70
E	0.370	0.86

Plausible Answers to Student Questions

Part 1

- Which component (methylene blue or sodium fluorescein) had the largest R_f value for the various mixtures?

Sodium fluorescein had the largest R_f value in all cases.

2. Which component (methylene blue or sodium fluorescein) was retained to the greatest extent by the silica gel coating?

Since methylene blue did not travel up the TLC strip very far, methylene blue was most greatly retained.

3. Which solvent system(s) gave the best separation?

Systems A-D gave very good separations.

4. Which solvent system gave the poorest separation?

System E gave the poorest separation. The methylene blue traveled up the TLC strip and exhibited a large amount of band broadening.

5. Describe any relationship(s) you find between the R_f value and the mobile phase employed.

The acidic solvent resulted in the highest R_f value for methylene blue. The methylene blue, being slightly basic is attracted to the acidic solvent.

6. Why are R_f values used instead of just the distance the component traveled?

R_f values are used so that several trials can be compared. This ratio will remain constant no matter when the TLC strip is removed from the solvent. The distance the component travels is dependent on how long the TLC strip is left in the solvent.

Part II

1. Which solvent system gave the best separation?

95% ethanol gave the best separation.

2. Which solvent system gave the poorest separation?

The acetic acid/ethanol mixture gave the poorest separation.

3. Which component (methylene blue or sodium fluorescein) was retained to the greatest extent by the alumina?

The sodium fluorescein was retained to greatest extent by the alumina. Sodium fluorescein has an acidic functional group which is attracted to the alumina.

4. What is the purpose of the glass wool and the sand?

The glass wool is used to prevent the stationary phase from falling out of the column. The sand is used so that the stationary phase does not form channels into the glass wool, and so that when the solvent is added the stationary phase remains undisturbed.

5. Why do you have to wait for the solvent to go below the sand before you add the dye mixture?

So that when the dye mixture is added, the stationary phase remains undisturbed.

6. What might happen if as you added more solvent the dye mixed with the solvent?

The dye would make a mixture that would be very spread out in the column (band broadening). It would take a long time for the separation to occur, or possibly everything would elute out as a mixture.

The Problem:

The students should use silica gel as the stationary phase and water as the mobile phase. Ethanol can then be used to elute the methylene blue. When 95% ethanol is used as the mobile phase, the two components tend to come out at approximately the same time with very little separation.

When alumina is used as the stationary phase, sodium fluorescein is more strongly retained. The sodium fluorescein is the salt of acidic fluorescein and is attracted to the alumina.^{6,7} When silica gel is used as the stationary phase, methylene blue is more strongly retained. Methylene blue is basic and is attracted to the acidic silica gel. (See figure 4.) The scenario specifies that sodium fluorescein must be eluted first. Therefore silica gel is the stationary phase of choice. Using the silica gel as the stationary phase and water as the mobile phase, the yellow-green sodium fluorescein elutes first. Methylene blue is then eluted by adding 95% ethanol or an acetic acid/water mixture.

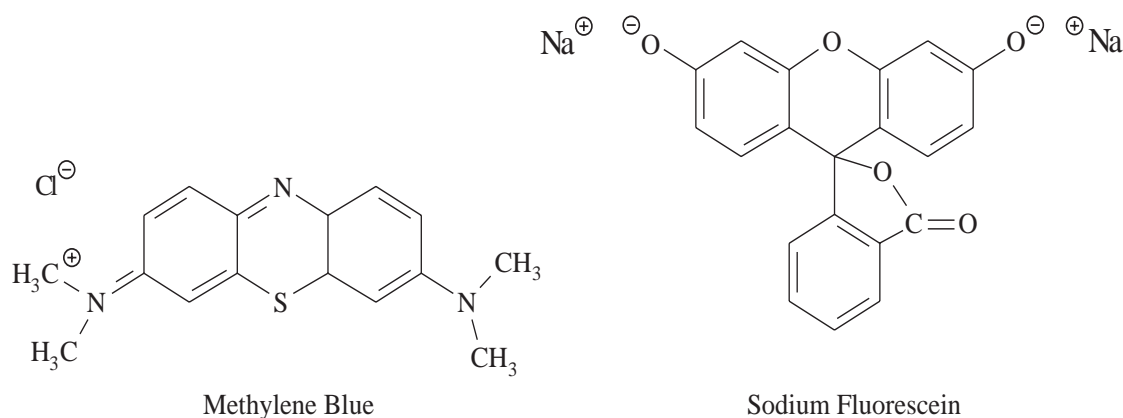


Figure 4: Structures of methylene blue and sodium fluorescein

Extensions and Variations

A combination of Oil Red O, Victoria Blue R, and fluorescein dyes can be easily separated using alumina as the stationary phase and with 95% ethanol as the mobile phase. The fluorescein is retained on the column but can be eluted with ammonia once the other two dyes have been collected.⁵

Fluorescein, Rhodamine B, and methylene blue can be easily separated using a 3:2:1 v/v mixture of acetone, *n*-propanol, and water.⁸

D&C Yellow No. 7 is the free acid fluorescein, and D&C Yellow No. 8 is the disodium salt, uranine.⁹ A scenario can be written to include the separation of one of these dyes and some other impurity.

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