

EXERCISE 7

Name _____

How is thin layer chromatography used to separate a mixture of molecules?

Objectives

After completing this exercise, you should be able to:

- ◆ Explain how chromatography is used to separate a mixture of molecules into its components, and describe the role of the mobile and stationary phases in this process.
- ◆ Explain how thin layer chromatography works, and be able to use it to separate a mixture of molecules.
- ◆ Explain what a scanning spectrophotometer is used for, and discuss how the information it provides is useful to scientists.
- ◆ Plot and interpret an absorption spectrum using a scanning spectrophotometer.
- ◆ Explain how absorption spectra can be used to identify molecules.

Prelab

Before you come to lab, read this entire exercise. You must also answer all questions and complete all assignments on the first 5 pages of this exercise. Your instructor will give you directions on when and where to turn in your work.

Cells are composed of thousands of different molecules. To study and learn about these molecules, scientists must be able to isolate them in pure form. To isolate just one type of biomolecule from the thousands of others present in a cell can be a daunting task, like looking for a needle in haystack. Fortunately, powerful separation techniques developed over the past century now make it possible to isolate virtually every type of molecule known to occur in living cells. Two of the most powerful techniques for purifying biomolecules are **chromatography** and **electrophoresis**. In this lab you will use chromatography to separate a mixture of lipid-soluble pigments extracted from spinach leaf cells. In labs 8 and 9 you will have an opportunity to use electrophoresis to separate mixtures of biomolecules.

Chromatography actually includes a range of procedures used to separate mixtures of molecules or ions. During chromatography, a moving substance (called the **mobile phase**) flows past a stationary substance (called the **immobile or stationary phase**). Before the two phases meet, the mixture of molecules to be separated is applied to one of the two phases. Then, when the mobile phase flows past the immobile phase, molecules in the mixture that are most strongly attracted to the mobile phase will move along fastest, while those that are most strongly attracted to the immobile phase will move along slowest. As the molecules in the mixture move along at different speeds, they are gradually separated. In addition, because the migration rate of a molecule is constant when using a given stationary - mobile phase combination, the identity of a particular molecule can often be determined from its migration rate.

In today's lab, you will use a type of chromatography called **thin layer chromatography** (or **TLC**). In TLC, the immobile phase is applied in a very thin layer to the surface of a supporting material such as glass, aluminum, or plastic. For your experiment, you will receive a **chromatography plate** consisting of a thin layer of silica gel (the immobile phase) bound to the surface of a plastic sheet (the supporting material). You will use a capillary tube to apply spinach extract (containing a mixture of lipid-soluble pigments) in a thin line to the silica gel. After applying the spinach extract to the silica gel, one end of the chromatography plate will be immersed in a mixture of organic solvents called the **developing solution**. The developing solution, which acts as the mobile phase, will flow up through the silica gel, much the

way water moves up a dry paper towel if one end of the towel is immersed in the water. As the developing solution moves up through the silica gel, pigments that are most strongly attracted to the developing solution will move up the chromatography plate fastest, while pigments most strongly attracted to the silica gel will move up the plate slowest. The different rates of migration will cause the pigments to separate into different bands.

The pigments that you will be separating have been extracted from spinach leaf cells. Living organisms often use pigments to absorb light energy. The color of a pigment depends on which wavelengths of visible light it reflects and which wavelengths it absorbs. Green pigments, for example, reflect most of the wavelengths in the green region while they absorb light at other wavelengths. A scanning spectrophotometer is an instrument that can measure how much light at each wavelength is absorbed by a given pigment. This is referred to as the pigment's **absorption spectrum**. Because each pigment's absorption spectrum is unique, it can be used to identify the pigment. In this experiment, you will use thin layer chromatography to isolate several pigments found in an extract of spinach leaf cells. You will then use a scanning spectrophotometer to plot the absorption spectra of 3 of these pigments and use this information to identify the pigments.

How can I separate pigments by thin layer chromatography (TLC)?

In the first part of this lab, you will be using thin layer chromatography to separate a mixture of pigments extracted from spinach leaf cells. Before applying the pigments to the silica gel, the silica plate should be thoroughly dried in a desiccator. If this isn't done, the silica gel may contain significant amounts of water, which can interfere with the migration of the pigments through the gel. Similarly, never touch the silica gel with your bare hands because oils from your skin can alter the gel and therefore affect the migration of the pigments.

The mixture of pigments should be applied to the silica gel in a very thin line, so that the pigments separate into distinct bands that do not overlap as they migrate up the plate. The thinner the line, the better the separation will be. At the same time, it is important to apply enough sample so that as the pigment bands separate from each other they don't become too faint. Unfortunately, if a large amount of sample is applied all at once, it would rapidly spread over a large part of the plate, producing a thick line. Therefore, in order to get a thin, yet heavy, sample line at the origin, the sample should be applied in multiple, small applications, allowing the sample to dry thoroughly between each application.

After the pigment sample has been applied to the silica gel, the chromatography plate will be placed in a jar with developing solution. The level of the developing solution should be a couple of mm below the lowest part of the pigment line. If the pigment becomes immersed in the developing solution, it will dissolve in the developing solution at the bottom of the jar, rather than be carried up through the silica gel. Once the chromatography plate is resting at the bottom of the jar, the cover should be tightly closed. It is important to keep your chromatography jar tightly sealed, for two reasons. First, the organic solvents used in the mobile phase are highly volatile and will rapidly evaporate when the chamber is open. The exact ratio of solvents in this mixture is very important to the successful separation of pigments, and if one solvent evaporates faster than the others, the ratio will change quickly. Second, volatile organic solvents tend to be unhealthful, and breathing these vapors should be avoided. For this reason, containers with developing solution should ALWAYS remain inside the fume hood.

As the pigments are carried up through the silica gel by the developing solution, they will also be diffusing into a wider and wider band over time. To minimize this spreading of the pigment bands, the chromatography should be terminated as soon as the solvent front approaches the end of the chromatography plate. When removing the plate from the chamber, however, be aware that the solvents will dry rapidly, so it is important to mark the solvent front as soon as you open the jar. Once the solvents have dried, the solvent front will be invisible.

Your Turn

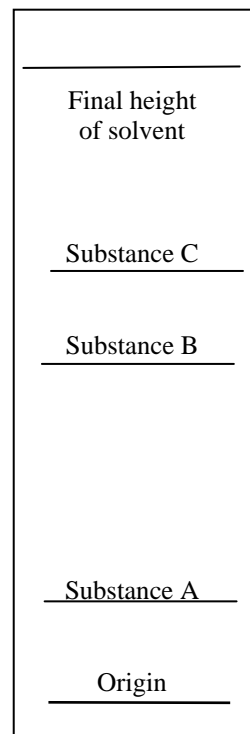
Based on your knowledge of potential problems during TLC, trouble shoot the following results and explain what might have gone wrong in each case:

1. Final chromatogram has fewer bands than expected and the bands appear very faint. Why did this happen?
2. Final chromatogram has bands that are dark enough to see, but the bands are very broad and overlap. What are some reasons that this could have happened?
3. There are no bands in the chromatogram, but the solvent at the bottom of the chromatography chamber has taken on a very bright color. What went wrong here?

How can I compare the migration rate of molecules separated by thin layer chromatography (TLC)?

During TLC, the migration rate of each pigment depends on its relative affinity for the stationary and mobile phases. Pigments that are more strongly attracted to the mobile phase will move up the plate faster, while pigments that are more strongly attracted to the stationary phase will move up the plate slower. This relative affinity for the 2 phases depends only on the molecular structure of the pigment, and, therefore, remains a constant property of the pigment.

Note, however, that the actual distance that a pigment travels during chromatography is not a constant property of the pigment because it can be affected by a number of variables, such as how long the chromatography plate remains immersed in the developing solution. Because the actual migration distance of a pigment can vary from one trial to the next, scientists find it more useful to look at the ratio of the migration distance of the pigment to the migration distance of the solvent front. This ratio is called the **R_f value**, and for any given stationary – mobile phase combination, the R_f value of a pigment should remain constant from one trial to the next. Because the R_f value is a constant property of each pigment, the R_f value of an unknown pigment can be used to help identify it.



$$\mathbf{R_f \text{ value}} = \frac{\mathbf{\text{Distance migrated by the pigment}}}{\mathbf{\text{Distance migrated by the solvent front}}}$$

For example, if the solvent front moves 10 cm from the origin, and one of the pigments in your mixture moves 7cm from the origin, then the R_f value for that pigment would be 0.7.

Your Turn

Practice calculating R_f values by measuring migration distances in the diagram above and then calculating the R_f value for each substance.

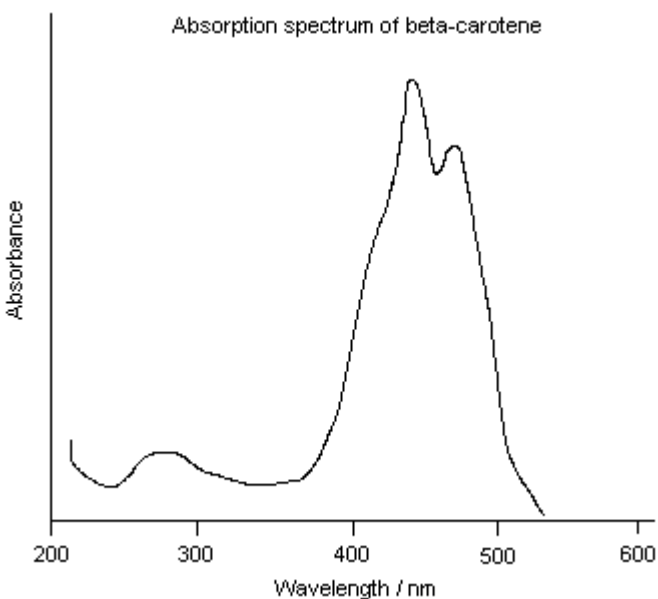
R_f for substance A _____

R_f for substance B _____

R_f for substance C _____

How can a scanning spectrophotometer be used to analyze the purified pigments that were separated using TLC?

In Exercise 2, you learned that a spectrophotometer can be used to shine a specific wavelength (color) of light through a solution and then measure how much of the light is absorbed. This measurement is referred to as the **absorbance** at that wavelength. In today's lab you will use a special type of spectrophotometer, called a **scanning spectrophotometer**, to automatically measure the absorbance of a solution at many different wavelengths. The results are then plotted to form a curve called an **absorption spectrum**, like the one for beta-carotene shown below:



An absorption spectrum shows which wavelengths of light are most strongly absorbed by a solution (peaks on the curve) and which wavelengths are most weakly absorbed (low points on the curve). Wavelengths where peaks occur are called **wavelength maxima** or **absorption maxima**.

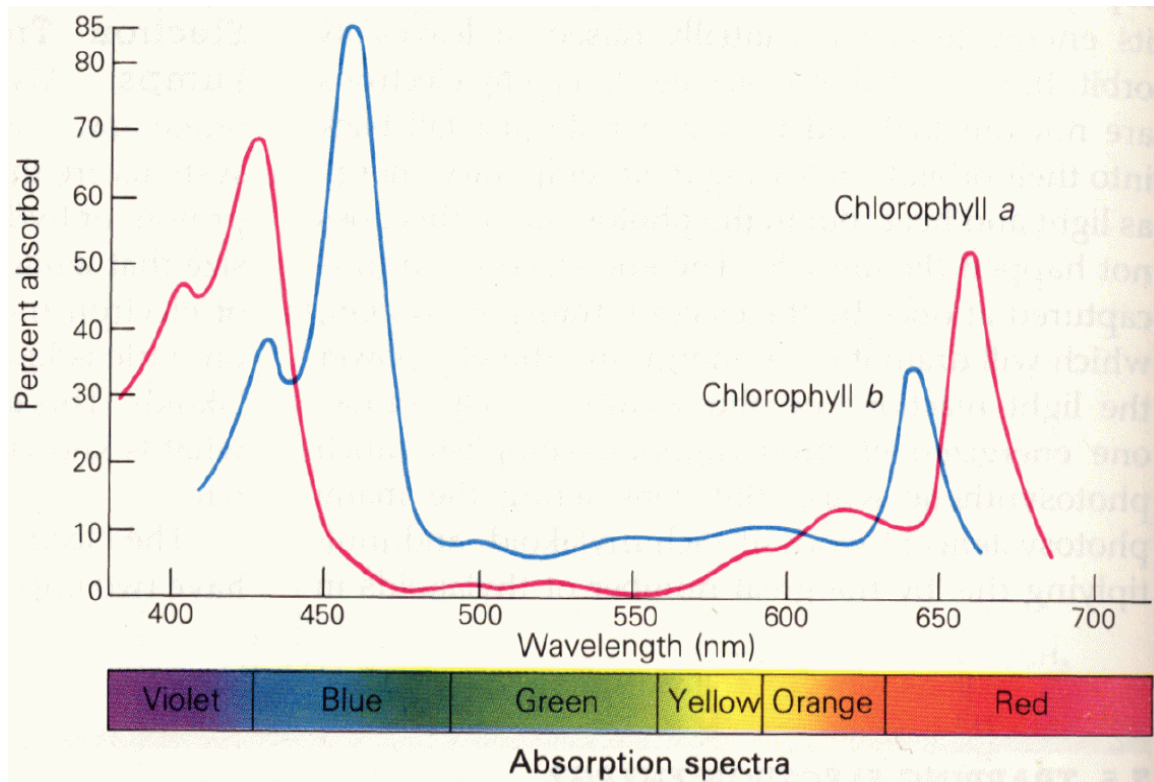
Your Turn

Based on the absorption spectrum shown above, identify the 2 wavelength maximum for beta-carotene:

First wavelength maximum = _____ nm Second wavelength maximum = _____ nm

Because each substance has a characteristic absorption spectrum, examining the absorption spectrum of a solution can help determine what substances are present in the solution and whether any contaminants are present. When examining an absorption spectrum for purposes of identification, it is important to focus on the **location** of any absorption peaks and valleys (i.e. at which wavelengths the peaks and valleys occur) and **NOT** on the **heights** of the peaks. This is because a given substance will always have peaks at the same locations (wavelengths), but the heights of the peaks may vary depending on how concentrated the solution is.

Below you can see the absorption spectra for 2 different pigments, chlorophyll a and chlorophyll b, plotted on the same axis. Note that each type of chlorophyll has its own characteristic absorption spectrum:



Your Turn

Identify the 3 main absorption maxima for chlorophyll a: _____.

Identify the 3 main absorption maxima for chlorophyll b: _____.

A student receives a green solution and is told it contains chlorophyll. The student plots the absorption spectrum of the solution and finds 3 major peaks located at 410 nm, 430 nm, and at 662 nm. Based on his analysis, the solution most likely contains:

- A. pure chlorophyll a B. pure chlorophyll b C. a mixture of chlorophylls a and b

Another student extracts some green pigment from a leaf and then plots the absorption spectrum of the pigment. The spectrum shows major peaks at 410 nm, 430 nm, 463 nm, 642 nm, and 662 nm. Based on her analysis, the solution most likely contains:

- A. pure chlorophyll a B. pure chlorophyll b C. a mixture of chlorophylls a and b

Lab Procedures

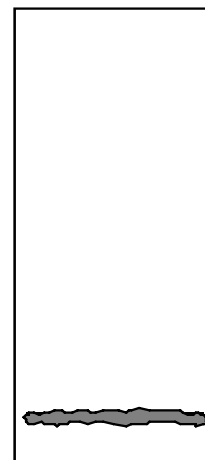
I. Separate photosynthetic pigments by thin layer chromatography (TLC)

1. An extract of lipids from spinach leaves can be found at your workstation. This dark green liquid contains a mixture of several pigments that spinach plants use to collect light energy for photosynthesis. During this exercise you will use TLC to separate this mixture of pigments into its components.
2. Before you begin the experiment, you will practice applying the extract to a **sample** chromatography plate in order to develop your technique for making thin applications of extract. The chromatography plate consists of a thin layer of silica gel bonded to a plastic sheet. These sheets should only be handled by the edges; never touch the silica gel with your fingers.

Obtain some **practice** extract and a small **sample piece** of the chromatography plate. Dip a capillary tube into the sample extract, and then make a thin line of extract along the chromatography plate. Do this by touching the tip of the tube to the surface of the silica gel *briefly*—just long enough to make a very small dot of pigment on the plate. Then make another small dot next to it and then another, and so on.

Note: rather than making individual dots, you can also try dragging the capillary tip very lightly on the surface of the silica gel to make a thin, smooth line of pigment. Use whatever technique works best for you. The goal is to produce a line of pigment that is as thin as possible. **However, make sure you do not scratch the silica gel off the plate with the tip of the tube!** Once you have developed a “feel” for how to apply the extract so as to make a thin, straight line of pigment, you are ready to streak the actual plate.

3. Obtain the chromatography plate for the experiment from your instructor. Make a thin line of pigment, approximately 1.5 cm above the bottom of the plate. This is called “streaking the plate.” The streak should remain about 2 mm away from either side edge of the plate. (See the diagram on the right.)
4. When you have made one thin line of pigment extract, allow the streak to dry thoroughly, then apply another line on top of the first—making your line darker and more concentrated. Repeat this process until you have transferred ALL 0.3 mL of the extract, in several layers, onto the plate.
5. Allow the solvent in the extract to evaporate completely after you are finished streaking the plate. This may take several minutes. While you are waiting, examine the glass jar, called a chromatography tank, located in the fume hood.



⇒ **CAUTION:** The developing solution inside the chromatography tank is highly volatile and will rapidly evaporate when the jar is open. Volatile organic solvents tend to be unhealthful, and breathing these vapors should be avoided. For this reason, containers with developing solution should ALWAYS remain inside the fume hood and should never be kept open any longer than absolutely necessary.

The solution in the chromatography tank should rise to a height of about 1 cm above the bottom of the jar. If it does not, add enough of the “chromatography developing solution” so that it rises to the required height. The developing solution consists of a mixture of organic solvents. Your developing solution may be 58% petroleum ether, 30% ethyl acetate, and 12% diethylamine, or it may be a 7:3 mixture of petroleum ether and acetone. With the cover firmly screwed on, swirl the jar so that the developing solution completely saturates the air inside the chamber.

6. When your streak is completely dry, carefully place the chromatography plate vertically into the developing tank with the streak near the bottom of the jar. Make sure the streak itself does not dip into the solution. If it looks like it might, pour some of the developing solution out of the jar and into the container labeled “chromatography developing solution” to prevent this from happening. The lid should remain on the jar, except for the second or so needed to place the plate inside.
7. As the developing solvent moves up the plate, it will pull the pigments along with it. Pigments that are more strongly attracted to the developing solvent (the **mobile phase**) will move up the plate faster than those more strongly attracted to the silica gel (the **immobile or stationary phase**). The leading edge of the solvent is called the **solvent front** and the original site of the streak is called the **origin**. Allow the chromatogram to develop until the solvent front is about 1 cm below the top edge of the sheet, then remove the plate from the developing tank.
8. Place your plate on a paper towel in the fume hood. Quickly (while you can still see the level of the solvent) mark the level of the solvent front by placing a ruler across the top of the plate at the level of the solvent front and scratching a straight line through the silica gel. This should create a thin line that clearly marks how far the solvent traveled from the origin.
9. Leave the developed plate in the fume hood until all of the solvent evaporates. This may take 5 minutes or longer. Then make a diagram of the chromatogram, showing the location and color of all the pigment bands.
10. Measure and record the final height of the solvent front above the origin (the distance from the middle of the origin to the scored line at the top of the plate.) Also measure and record the final height of each pigment band above the origin. Heights of the bands should be measured from the middle of the origin to the middle of each band.

II. Remove your pigments from the silica gel and suspend each in ethanol

1. With label tape, label three centrifuge tubes with the colors of the three major bands found on your chromatography plate (the 2 green bands and the orange-yellow band nearest the solvent front.) Ask your instructor if you are not sure which are the major bands.
2. With a spatula, carefully scratch off the silica gel containing the orange-yellow band nearest the solvent front. Be careful to scratch off **ONLY** this band. Transfer the loosened silica gel containing the pigment onto a piece of waxed paper, and then pour it into the correspondingly labeled centrifuge tube.
3. With a 5 mL pipette, transfer 3.0 mL of ethanol into the centrifuge tube containing the silica gel. Mix the contents with a glass rod to dissolve the pigment in the ethanol.
4. Remove each of the two major green bands from the chromatogram and place them in the other labeled centrifuge tubes using the same procedure you followed for the orange-yellow band. Add 3.0 mL of ethanol to each of these tubes and mix as was done with the orange-yellow band.
5. Place a 150 mL beaker on each of two separate electronic balances and tare the balances. Place one of the conical centrifuge tubes in each beaker and balance the two tubes by adding ethanol to the lighter tube. Place the balanced tubes directly opposite each other in the rotor of a clinical centrifuge, and then tare the balances again with the empty beakers on them.
6. Add 3.0 mL dH₂O to a clean centrifuge tube and place the tube in one of the two beakers. Place the third tube of pigment in the other beaker and balance the two tubes by adding ethanol to the pigment tube or more dH₂O to the other tube. Place the balanced tubes directly opposite each other in the clinical centrifuge.
7. Centrifuge for 3 minutes. Once the centrifuge has come to a complete stop, remove the tubes and pour each supernatant into a separate, labeled test tube without disturbing the pellet.
8. You should now have 3 test tubes of pigment, each containing approximately 3 mL of solution. Discard the pellets of silica gel as directed by your instructor.

III. Plot an absorption spectrum for each pigment using the scanning spectrophotometer

1. Pour each of the 3 tubes of pigment into a separate plastic cuvette. (NOTE: when handling the cuvettes, touch the frosted or ridged sides only, not the smooth sides!) Prepare a reagent blank by filling a fourth cuvette with ethanol.
2. Take the 4 cuvettes to the **Genesys 2 Scanning Spectrophotometer**. Familiarize yourself with the parts and operation of the spectrophotometer before you begin. Check with your instructor to see if any supplemental instructions are available.
3. From the Main Menu, select “4” (Advanced Scanning) by pressing “4” on the keypad.
4. Press “Setup Tests” on the keypad.
5. Select “1”, Test Name
6. Enter a test name of your group’s choosing, and press “Exit” on the keypad.
7. Select “4”, Start Wavelength, and set to 400nm. Press “Enter”.
8. Select “5”, Stop Wavelength, and set to 720 nm. Press “Enter”.
9. Select “6”, Scan Speed, and set to Medium (1pt/1.0nm) by repeatedly pressing “6” on the keypad until display reads “Medium”.
10. Select “7”, Measurement Mode, and set to ABS (absorbance) by repeatedly pressing “7” on the keypad until display reads “ABS”.
11. Select “8”, Number of Samples. Set to desired number of samples (NOT counting the blank) by entering the number “3” on the keypad. Press “Enter”.
12. Select “Next Page”. This is done by pressing the yellow arrow on the keypad under the “Next Page” icon on the display.
13. Select “1”, Overlay Scans, by pressing, “1” on the keypad until display reads “ON”.
14. Press “Exit”
15. Open the sample compartment on the spectrophotometer, and examine the sample holder. It has 7 cells (cell # 1 is closest to the front of the spectrophotometer and cell # 7 is closest to the back). The cuvettes are placed in the cells with the smooth sides facing left and right, and the ridged or frosted sides facing front and back. Wipe off the smooth sides of each cuvette with a Kimwipe, and place them in the sample holder as follows:

Place the reagent blank (ethanol) in cell # 1
Place the orange-yellow pigment in cell # 2
Place the first green pigment band in cell # 3
Place the second green pigment band in cell # 4
16. Select “Collect Baseline” by pressing the yellow arrow on the keypad under the “Collect Baseline” icon on the display. Wait until machine is finished scanning.
17. Select “Scan” by pressing the yellow arrow on the keypad under the “Scan” icon.
18. Machine will scan all of the samples, and a graphic representation will be made for each sample. The scale of the graph will automatically be corrected at the end of all scans.

19. Print out the graph of the scans by pressing the “Printed Icon” on the bottom left of the keypad. If a “Printer Error” message is displayed, select “Retry” by pressing the yellow arrow on the keypad below the “Retry” icon on the display.
20. Ask your instructor for help if you have any difficulties. When you are finished, remove the graph from the printer and label the three curves that were plotted so you know which one corresponds to each pigment band.
21. Remove the cuvettes from the machine, and press “Exit” to return to the Main Menu.

Clean up

Remove all labels and clean your glassware.

All disposable glassware goes into the special glass disposal receptacle.

All instruments should be turned off and unplugged.

Wipe off your workspace with a damp paper towel.

Make sure everything that you have used is clean, put away, or discarded. Leave your work area in the same order that you found it in.

Ask your instructor to check your work area before you leave.

Postlab

1. What is the function of the pigments present in spinach leaves?
2. During chromatography, a mixture of molecules is separated when a mobile phase flows past a stationary phase.
 - a. Identify the mobile phase in your experiment.
 - b. Identify the stationary phase in your experiment
 - c. Explain **how** the different pigments were separated as the mobile phase flowed past the immobile phase.
3. Using the data you collected during lab, determine the R_f value of every pigment separated on your TLC plate. (The R_f value for each pigment is defined as the migration distance of the pigment divided by the migration distance of the solvent front.)
4. Some pigments found in plants are listed in the table below. Make a list to show which of these pigments you were able to identify in your chromatogram.

<u>Pigment</u>	<u>Color</u>
Anthocyanins	red
Carotenes	orange
Chlorophyll a	bluish-green
Chlorophyll b	yellow-green
Pheophytin	gray
Xanthophyll	yellow

5. In this experiment you plotted the absorption spectra for three different pigments isolated from spinach leaves: beta-carotene, chlorophyll a, and chlorophyll b. Compare your 3 absorption spectra with the labeled absorption spectra for beta-carotene, chlorophyll a, and chlorophyll b that are shown in the Prelab. Then label each absorption spectrum on your graph with the name of the pigment that it corresponds to.
6. One way to characterize the absorption spectrum of a particular substance is to identify the wavelengths where peaks in absorbance occur. The wavelengths where these peaks occur are called “wavelength maxima” or “absorbance maxima.”
 - a. Identify the wavelength of each absorbance maximum for chlorophyll a.
 - b. Identify the wavelength of each absorbance maximum for chlorophyll b.
 - c. Identify the wavelength of each absorbance maximum for beta-carotene.
7. Of the 3 pigments you used for plotting absorbance spectra:
 - a. What is the name and R_f value of the one **most** strongly attracted to the mobile phase?
 - b. What is the name and R_f value of the one **least** strongly attracted to the mobile phase?
 - c. What is the name and R_f value of the one with **intermediate** attraction to the mobile phase?
8. Goldfish skin and chicken egg yolks typically have a yellow, orange, or reddish-orange color. However, these tissues are practically colorless if the respective animal is not permitted to eat photosynthetic plant tissue. Propose a hypothesis to explain these observations, and then explain how you would test your hypothesis.