Chromatography

a) Introduction to chromatography

In the fields of cellular biology and biochemistry, research often involves trying to isolate (purify) one molecule away from many other molecules. For example, in the 1960's researchers discovered that the bark of the yew tree could fight many types of cancers. But the yew tree (like any other living organism) contains thousands of different organic molecules. To make an effective medicine from the yew, the researchers had to isolate the one cancer-fighting molecule from the thousands of other molecules. It took them 5 years, but eventually they did isolate the anti-cancer molecule. This molecule, taxol, is now widely used in the fight against breast and ovarian cancer.

To isolate a molecule from a mixture of other molecules, researchers first separate all the molecules from each other. Think of the mixture of molecules as a piggy bank full of different coins. If you were interested in finding one special coin (maybe a 1943 copper penny, worth over $80,000) from the hundreds of others you would first spread all the coins out from each other so you could see which one was the valuable penny.

Researchers use many methods of separating mixtures of molecules, but one of the most common ways is called chromatography, which means separation of a mixture of molecules based on their different migration rates across a surface. The three basic steps in chromatography are:

1) The mixture of molecules is dissolved in a liquid (although sometimes a high pressure gas is used instead).

2) The dissolved molecules flow over a solid substance. The solid substance that the molecules flow across is called the "stationary phase." The liquid that carries the molecules across the stationary phase is called the "mobile phase."

3) The different molecules in the mobile phase flow at different rates across the stationary phase, so the molecules become separated from each other as they travel.

To visualize the process, think of runners in a race. The mixture of molecules in the mobile phase is like the runners bunched up together at the starting line. The race course is like the stationary phase. Because the runners run at different speeds on the race course, they cross the finish line separated from each other.

The molecules in the mixture flow at different rates across the stationary phase because they differ in how much they are attracted to the stationary phase, compared to their attraction to the mobile phase. The molecules that are greatly attracted to the stationary phase (but less attracted to the mobile phase) flow the slowest. The molecules that are greatly attracted to the mobile phase (but less attracted to the stationary phase) flow the fastest.

There are many different stationary phase solids. For example, some are ionic (these separate molecules based on the molecule's attraction to ions), some are hydrophobic (these separate molecules based on the molecule's attraction to hydrophobic substances), and some stationary phases separate molecules based on the size of the molecule.

Many times the stationary phase is placed inside a glass or plastic column (tube). This type of chromatography is called column chromatography.

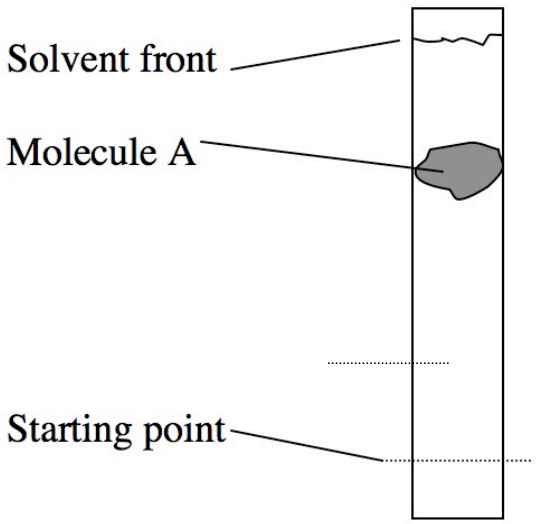
b) Paper chromatography

Paper chromatography is a method that can be used to separate mixtures of molecules. Paper chromatography uses paper as the stationary phase and a liquid solvent as the mobile phase. A glass jar serves as the column to hold the paper.

The mixture of molecules is first "blotted" near the bottom of the paper. The paper is then placed in a jar that has a shallow pool of solvent at the bottom. The solvent creeps upward along the surface of the paper because of paper's "wicking" action (think of the way a paper towel pulls up spilled liquids). Notice that in paper chromatography, the mobile phase does **not** flow downward, but instead upward on the paper.

When the solvent reaches the blotted molecules on the paper, the molecules dissolve into the solvent. The dissolved molecules will travel upward with the solvent, but since each molecule in the mixture has a different attraction to the paper, they will not move at the same speed. Eventually this difference in speed will separate the molecules.

In paper chromatography when the conditions are kept constant, a particular molecule always travels a fixed percentage of the distance traveled by the solvent front (the solvent front is the highest edge of the solvent on the paper). The ratio of the distance the molecule travels to the distance the solvent travels is called the Rf value. The symbol Rf stands for "retardation factor" or "ratio-to-front". It is expressed as a decimal fraction.



In the illustration on the left, the rectangle is the paper. The solvent front has migrated about 45 mm from its starting point, but molecule A has migrated 33 mm. The Rf for the molecule is therefore 0.73

$$\frac{33 \text{ mm}}{45 \text{ mm}} = 0.73$$

The Rf value is a constant for a given molecule. In other words, the same molecule will always have the same Rf value, which will usually (but not always) be different from the Rf values of other molecules. The Rf value is therefore useful in identifying molecules, but other properties should be used in combination with the Rf value to confirm a molecule's identification. Since it is difficult for different laboratories to exactly duplicate conditions for a chromatography experiment, Rf values are more useful for comparisons within one lab than for comparisons of data from different labs.

c) Paper chromatography of food colorings

You will use paper chromatography to test food colorings to see if the color results from a single dye or mixture of dyes. You will be able to see the separated colored dye molecules as different colored spots on the paper. (Substances that are not colored can also be separated by paper chromatography. They are detected using ultraviolet or black light, which makes them appear to glow in the dark.)

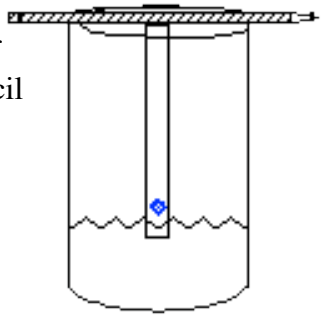
1) Obtain 5 chromatography paper strips. When you pick up a strip, try to hold it at only one end to avoid getting your skin oils on it. Cut each strip to a length of 10 cm.

2) On one strip, use a pencil to mark a line about 2 cm from the bottom. This will be the spot where you apply the food coloring blot.

3) Add a drop of green food color to a spot plate. Dip the blunt end of a cut toothpick into the food color. Use the toothpick to place a dot of green food color on the pencil mark start line and allow it to dry. Repeat the dotting in the same spot 3 more times. The dot should be about a few millimeter across.

4) Attach a piece of tape to the top end of the strip of paper. Tape the paper to the pencil and lower the paper into the glass chromatography jar. The paper bottom should just touch the bottom of the jar. Adjust the height of the paper until the bottom of the paper just touches the jar bottom.

5) Gently and slowly add water to container until the water level touches the bottom of the strip of paper; the water level needs to be well below the pencil mark start line on your strip of paper and at least 1 cm below the green spot. The water must **NOT** touch the green spot of food color.



6) Let the water wick (climb) up the paper. The top of the wet area is the "solvent front". The solvent front will climb the first few centimeters quickly. The food color will probably trail behind the solvent front.

7) When the front edge of the water reaches four fifths of the way up the paper remove the paper from the glass jar. Use a pencil (not a pen) to mark the front edge of the solvent. Allow the paper to dry. Use the pencil to mark the center of all dye spots.

8) Note if more than one color appears on the paper. If so, find the center of each dye.

9) Measure the distance from the start line (where the green spot was placed) to the mark for the upper edge of the solvent front. Record this distance. Measure the distance from the start line to the center of each dye spot. Record this distance. Using these distances, calculate the Rf of each dye. Record your observations and Rf's on the Report Sheet.

10) Repeat steps 1 through 9 using the blue food coloring, and then again with the red food coloring, then again with the purple food color. Record your observations and Rf's on the Report Sheet. **Show your results to your instructor before proceeding.**

d) Re-analysis of red food color using a hydrophobic mobile phase

1) Repeat steps 1 through 9 in the above procedure using red food color but this time use isopropyl alcohol (a hydrophobic solvent) instead of water (a hydrophilic solvent) as the mobile phase. By comparing the Rf of each dye in the red food color in the two solvents, determine which dye is more hydrophobic and which is more hydrophilic. Record your observations and Rf's in the results table.

e) Results sheet

Each colored spot on the chromatography paper is a dye molecule in the food coloring. Some food colorings are mixtures of several dyes and some are only one dye.

Food color	Is the food color a mixture of dyes (Yes/No)	List the color and Rf of **each** Dye in the food color*
Green		
Blue		
Red		
Purple		

Red in isopropyl alcohol

* The entries in this column should be the name of the dye (you can invent any name that best describes the dye color) and it's Rf: For example, "Aqua-blue (Rf = 0.65)"

f) Review questions

1) In chromatography, what is the definition of "mobile phase"? What substance was the mobile phase in today's experiment?

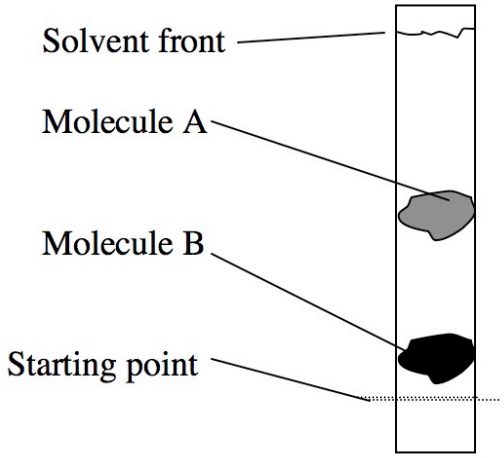
2) In chromatography, what is the definition of "stationary phase"? What substance was the stationary phase in today's experiment?

3) If you had let each chromatography experiment go only until the solvent front was half way up the paper (instead of 4/5 up the paper), do you think the Rf's would have been different? Why or why not?

4) Calculate the Rf's for the two molecules shown on the right.

Molecule A Rf: _____

Molecule B Rf: _____



5) Do you think it is possible for two different molecules to have the same Rf? Why or why not?

6) What does "Rf" stand for?

7) If the stationary phase was a hydrophobic substance, the mobile phase was water, and all the molecules dissolved in the mobile phase were hydrophilic, do you think the molecules would have high or low Rfs? Justify your answer.

8) Which dye molecule in the red food color was hydrophobic? Which was hydrophilic? Justify your answer using your experimental data.

9) If the stationary phase was a substance with positive ions, the mobile phase was water, and all the molecules dissolved in the mobile phase were negative ions, do you think the molecules would have high or low Rfs? Justify your answer.

10) If you had a mixture of molecules that you wished to separate by chromatography, but all the molecules in your mixture were hydrophobic (will not dissolve in water) could you use chromatography to separate them? Why or why not?