Objectives

The objectives of this laboratory are:

a. To use reverse phase liquid chromatography (RPC) to calculate the average retention volume, $V_{Rave}$, for the red and blue dyes in Grape Kool Aid, for each dye calculate a capacity factor, $k'$, calculate selectivity or separation factor, $a$, and then calculate $R$ for this separation.

b. To obtain a paper chromatogram of various gel ink pens, to identify components of ink pens by $R_f$, and then determine which ink colors are mixtures

Background

Although there are several types of chromatography, all types employ a mobile phase or eluent (it can be liquid or gas), which is “forced” through a stationary phase (a solid or semi-solid). Mixtures are separated because some components will be more attracted to the stationary phase (and stick to it) while some components will be more attracted to the mobile phase (and travel with it).

Chromatography is an important analytical tool used to separate the components of a mixture and identifying the components. These components become separated or partitioned between a stationary phase and a moving phase of the chromatography system. The moving phase is either a gas or a liquid and the stationary phase is usually a solid. The mixture to be separated is combined with the mobile phase. As the mobile phase "solution" flows over the stationary phase, the components of the mixture continuously equilibrate between the phases, based on their particular affinity for each phase. A higher attraction for the mobile phase leads to a higher concentration of a component in the mobile phase and a faster transport through the entire system. Components more strongly attracted to the stationary phase take longer to migrate through the system. This results in the components becoming separated into bands that flow through the system at different rates.

The first part of this experiment is a liquid chromatography system. If the separation, or resolution, is sufficient, the bands will exit the system as distinct fractions (see Figure 1 above). The liquid chromatography system consist of six basic components (see Figure 2 above); (1) a separation column, consisting normally of a fine, granular solid packed in a column; (2) a solvent, the mobile phase that washes along the column; (3) an injection system, needed to place the sample mixture on the column; (4) a pump, or solvent delivery system, that forces the solvent through the column; (5) a detector, use to indicate when the components emerge from the column; and (6) a recorder.
Usually, the solid phase is relatively polar and the solvent nonpolar in liquid chromatography. The liquid chromatography part of this experiment utilizes a form of chromatography called reverse phase liquid chromatography (RPC). In RPC, the stationary phase is a nonpolar solid and a polar solvent is used as the mobile phase. When a mixture is injected into the RPC column and washed through it, several processes occur (see Figure 3 below). The more polar components of the mixture are attracted more strongly to the polar solvent, so they will move more quickly through the column with the solvent. The less polar components will move more slowly, as they spend more time adsorbed onto the nonpolar column medium. Ideally, the components should emerge at different times. A measure of the degree of separation that is achieved is called the resolution of the system. As the band of each component moves down the column, the band widens due to diffusion. As bands widen they can overlap each other and may prevent clean separation or resolution of the components.

![Figure 3. Components of Mixture Moving Through Liquid Chromatography Column](image)

The second part of this experiment will be paper chromatography. Paper chromatography uses paper as a stationary phase, but can use a variety of different mobile phases. The trick is to discover which mobile phase is most appropriate – if all the components are attracted to it, there is no separation! The mobile phase takes advantage of differing solubility or polarity of the components of the mixture in order to separate them.

The samples to be separated must be spotted onto the paper (stationary phase). By placing one edge of the paper into a small amount of liquid (mobile phase), the paper will wick up the liquid. The components that are attracted more to the paper will move very little, if at all. The components that are more attracted to the mobile phase will travel with it, at different rates, depending on the level of attraction. This component traveling process is called elution. This experiment relies on gel ink pens which contain dyes. Dyes are colored compounds which are soluble. Some gel ink pens, particularly those which are visible on black paper, contain pigment inks, which are insoluble, and cannot be separated using paper chromatography.
Figure 4 above shows a beaker containing mobile phase and a prepared paper stationary phase. A line is drawn about 1.5 cm above the bottom edge of the paper. This is the starting line. Its height above the bottom edge is chosen to be above the level of the eluting solution. The starting line and ink dots must be above the level of the mobile phase when the paper is placed inside the beaker. If it were below the liquid level, the ink would wash out into the liquid.

Another line is drawn about 10.0 cm above the bottom edge of the paper. This is the finish line. Its location was chosen for this experiment because when the eluting solution reaches that line, any inks that are mixtures should be clearly separated. (Different experiments might require finish lines at a variety of heights above the bottom edge of the paper.) When the solvent front reaches the finish line, the paper should be removed immediately from contact with the mobile phase. If the paper is not removed, the eluting solution will travel to the top edge, and all the eluting inks will travel there eventually and be indistinguishable.

Figure 5 on the next page shows a typical paper chromatogram. There are a few difficulties commonly encountered in the elution process. One problem is that spots tend to spread out as they elute, and can bleed into each other as they proceed up the paper. This can be confusing when interpreting the chromatogram. To avoid this problem, space the spots of sample far apart and make repeated, tiny applications of sample to prevent spreading. Another problem is an uneven solvent front. This can happen if the beaker is nudged — if the mobile phase sloshes inside, the elution trails may travel diagonally, which makes interpretation very difficult. This can also happen if the two edges of the chromatogram are allowed to touch when they are stapled or taped together to form a cylinder. Care must be taken to avoid touching edges.
A component with a given solubility travels along with the mobile phase at one rate, regardless of what other components are present in the sample. If the red part of purple ink travels at the same rate as pure red ink, and both stop in the same place, the two should be the same red ink. The two red spots should have the same **Retention Factor**, $R_f$. The $R_f$ is the distance, $D$, traveled by the spot divided by the distance traveled by the eluting solution, or **Solvent Front**, $F$.

$$R_f = \frac{D}{F}$$

Comparing the $R_f$ values allows the confirmation of a component in multiple samples because *unique components have unique $R_f$ values*. The distance traveled by the spots should be taken in the center of the spot, because its beginning position was centered in the starting line.

**Pre-Lab Questions**

1. What is the process of chromatography used for?

2. In chromatography, components of a mixture distribute themselves between the stationary phase and the mobile phase. Explain how the components can be separated with these two phases.

3. In the liquid chromatography column used in this experiment, the solid has a $C_{18}$ hydrocarbon bonded to it. Would a $C_{18}$ hydrocarbon be a polar or a nonpolar substance? Explain.

4. On the next page is typical data for this experiment in Figure 6. 1 mL of a Kool-Aid solution was loaded on a SepPac $C_{18}$ column. The red and blue dyes were eluted from the column with a constant flow of 18% isopropyl alcohol. The eluted solution was collected in a 10-mL graduated cylinder. The volumes of eluant were recorded at the beginning and end of each color band.
This process is represented graphically below in Figure 7. The x-axis represents the milliliters of eluant that emerge from the column, and the y-axis represents the concentration of each dye as it emerges with the eluant.

The first step in calculating the selectivity and resolution of the system is determining the volumes of eluant corresponding to the bandwidths and band centers for each eluted dye.

a. Bandwidth, $W$. This is the volume, in mL, of eluant containing each dye as it emerges from the column. **Calculate** the bandwidth, $W$ for each dye for each of the three runs and then determine the average bandwidth, $W_{ave}$, for each dye.

b. Center of band--called Average Retention Volume, $V_{Rave}$. This volume corresponds to the center of each band. The average retention volume is calculated by taking the average starting volume for each band and adding one half the corresponding average bandwidth. $V_{Rave} = V_{start} + 1/2W_{ave}$ **Calculate** the average retention volume, $V_{Rave}$, for the red and blue dyes.

c. For each dye, a capacity factor, $k'$, can be calculated. This term is a relative measure of the attraction of the dye for the stationary phase as compared to its attraction for the mobile phase. The equation for capacity factor is:

$$k' = \frac{(V_{Rave} - V_M)}{V_M}$$

where $V_{Rave}$ is the average retention volume for each dye and $V_M$ is mobile phase or eluant volume in the cartridge. $V_M$ can be estimated to be one half the cartridge volume, with the stationary phase occupying the other half. For the Sep-Pak cartridges, this $V_M$ value is 0.49 mL. **Calculate** $k'$ for each dye.

d. A selectivity or separation factor, $\alpha$, can now be calculated. This is the ratio of the $k'$ values for each dye, with the larger value in the numerator. For good separation, a mobile phase is usually chosen that gives an $\alpha$ value between 2 and 10. **Calculate** $\alpha$ for this separation using the equation, $\alpha = k'_{Blue} / k'_{Red}$

e. The resolution, $R$, a measure of how well the two dyes are separated by the column and eluant, is given by the equation, $R = \frac{(V_{Rave(Blue)} - V_{Rave(Red)})}{0.5(W_{Blue} + W_{Red})}$ where the numerator is the volume between the band centers and the denominator represents the average bandwidth. The greater the selectivity, the larger the numerator and therefore the greater resolution. The resolution can also increase as the efficiency of the column increases, since this results in a lower average bandwidth. **Calculate** $R$ for this separation.
Safety

Wear safety goggles at all times. Use eluting solution only in the hood. Do not breathe fumes from the eluting solution. Be sure to handle only the dry part or the chromatogram when removing it from the beaker. Wash hands thoroughly if the eluting solution touches your skin.

Isopropyl alcohol is a flammable liquid and a fire hazard. Do not use near flames or other ignition sources. It is slightly toxic by ingestion and inhalation. Wear chemical splash goggles and a chemical-resistant apron. Wash hands thoroughly with soap and water before leaving the laboratory.

Materials and Equipment

Chromatography paper, Pilot Precise® color pen set, and eluting solution. 600-mL beaker, pencil, ruler, plastic wrap, tape and paper towels, Isopropyl alcohol, C\textsubscript{3}H\textsubscript{7}OH, 70%, 50 mL isopropyl alcohol, C\textsubscript{3}H\textsubscript{7}OH, 28%, 10 mL isopropyl alcohol, C\textsubscript{3}H\textsubscript{7}OH, 18%, 50 mL isopropyl alcohol, C\textsubscript{3}H\textsubscript{7}OH, 5%, 10 mL Water, distilled or deionized, 300 mL Grape Kool-Aid® solution, 20 mL Sep-Pak® C\textsubscript{18} cartridge Syringe, 10-mL with male Luer® tip syringe, 100-mL Graduated cylinders, 10-mL and 25-mL Beaker, 10-mL or 50-mL Syringe, 3-mL with male Luer® tip (optional)

Procedure

Part A: Liquid Chromatography

Part 1. Isocratic Separation (Flow rate and solvent concentration are held constant.)

1. Pretreat the Sep-Pak C\textsubscript{18} cartridge.
   a. To help eliminate remixing of closely eluting bands in the cartridge, cut off the exit tube of the cartridge (the shorter end) at the point where it meets the body of the cartridge. This may already be done from previous years!
   b. Fill the syringe with 10 mL of the 70% isopropyl alcohol.
   c. Attach the tip of the syringe cartridge to the long end of the Sep-Pak cartridge, and pump the isopropyl alcohol through the syringe cartridge at a rate of 5-10 mL per minute.
   d. Collect the eluted alcohol in a 10-mL graduated cylinder to monitor the flow rate.
   e. Repeat steps 2-4 using distilled or deionized water.

2. Inject the sample.
   a. Use the 10-mL syringe to slowly inject 1 mL of the Kool-Aid sample onto the column. Optional: Use a clean 3 mL syringe to inject the Kool-Aid sample.
   b. Discard the column effluent (the portion that washed out as the sample was injected).
   c. Remove the cartridge from the syringe.
   d. If the 10-mL syringe was used in step 1, rinse the syringe with 10 mL of distilled water three times to remove any traces of Kool-Aid sample.

3. Elute the sample.
   a. Use the 10-mL syringe to slowly elute the dyes. Fill the syringe with the 18% isopropyl alcohol eluant and attach the syringe to the Sep-Pak cartridge.
   b. Pump the 18% isopropyl alcohol through the cartridge at a steady rate of 5-10 mL per minute.
   c. Collect the column effluent in a 10-mL graduated cylinder.
   d. Record, in the Part 1 data table, the volume of effluent collected as the first and last of the colored drops of each of the dyes emerge. If there is not a perfect separation between the blue and red-colored bands, record data for the beginning and end of the intermediate purple band. The center of the purple band will serve as the end of the first band and beginning of the last.
e. Regenerate the column and repeat the measurements.
f. Repeat the measurements two more times. Between injections, wash the column with 10 mL of distilled water at the same flow rate of 5-10 mL per minute. If colored material builds up on the column, repeat the pretreatment procedure (step 2).

Part 2. Step Gradient Separation

4. In this procedure, the composition of the eluting liquid is changed. Since the column is nonpolar, first a very polar solvent, water, is used. Then its composition is changed to less polar by adding more isopropyl alcohol. This procedure allows the separation of the citric acid and flavoring oils as well as the dyes.
   a. Pretreat the cartridge.
   b. Follow the same pretreatment as in Part 1. Inject the sample and elute the components.
   c. Slowly inject 1 mL of the Kool-Aid sample onto the column.
   d. Elute the polar components of the mixture (citric acid and any sugar present) by passing 5 mL of water through the column.
   e. Collect the effluent in a small beaker.
   f. Elute the red dye by passing 10 mL of 5% isopropyl alcohol through the column. Note that large amounts of the 5% isopropyl alcohol can be used without eluting the blue dye. Collect this effluent in a second beaker.
   g. Use 10 mL of the 28% isopropyl alcohol to elute the blue dye. Collect it in a third beaker.
   h. Use 10 mL of 70% isopropyl alcohol to elute the nonpolar flavor oils and other nonpolar additives. Collect this fraction in a fourth beaker. Record the color of each eluted fraction in the Part 2 data table.

5. Evaporate the solvents and examine the components.
   a. Allow the solutions to evaporate by leaving them in the fume hood until the next laboratory period. Be sure to label those solutions containing isopropyl alcohol as the solvent.
   b. Observe and describe the contents of each of the beakers. Look for color, odor, and appearance. Enter these observations in the Part 2 data table.
Calculations

1. Determine the following values and show calculations. Refer to question four in the Pre-Lab Questions. Enter results in the Part 1 data table.
   a. Bandwidth, \( W \), for each dye.
   b. Average Retention Volume, \( V_{\text{R ave}} \), for each dye.
   c. Capacity Factor, \( k' \), for each dye.
   d. Selectivity, \( \alpha \), for the two dyes with this isocratic separation.
   e. Resolution, \( R \), for the two dyes with this isocratic separation.

2. For good separation of the dyes, the resolution should be greater than one. What was the value you calculated? Did the two dyes overlap as they emerged from the column, or was the separation a good one?

3. In the step gradient separation, four separate fractions were collected. How were these related to the polarities of the column and of the eluting solvent?
Part B: Preparation of Chromatography Paper

1. Wash your hands thoroughly to remove excess oils from your skin. Obtain a ruler and a piece of chromatography paper from your instructor. Handle the paper only on the edges to avoid leaving fingerprints, as these may hinder the elution process.

2. Place the chromatography paper on a sheet of clean notebook paper or paper towel to avoid picking up dirt or contaminants from the bench top. Orient the paper into a “landscape” position and write your name on the top edge of the paper in one corner. Using a pencil and the ruler to measure accurately, lightly draw a straight line across the paper, about 1.5 cm above the bottom edge. This is the starting line. Lightly draw another line about 10 cm above the bottom edge. This is the finish line.

3. On the starting line, measure in from one side about 2.5 cm and lightly draw a small “X” centered on the starting line. Draw seven more, 1.5 cm apart.

4. In the center of each X, make a small spot of ink of a different color in this order: blue, black, red, pink, violet, turquoise, green. When you have finished, you should have something that looks like Figure 3.

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\begin{center}
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\draw[->] (0,0) -- (0,5); \draw[->] (0,0) -- (5,0);
\draw[thick] (0,0) -- (0,1.5) node[above] {1.5 cm} -- (2.5,2.5) node[above] {10 cm} -- (5,0) -- (0,0);
\fill (0.5,1.5) circle (0.1); \fill (1.0,1.5) circle (0.1); \fill (1.5,1.5) circle (0.1); \fill (2.0,1.5) circle (0.1); \fill (2.5,1.5) circle (0.1); \fill (3.0,1.5) circle (0.1); \fill (3.5,1.5) circle (0.1); \fill (4.0,1.5) circle (0.1); \fill (4.5,1.5) circle (0.1); \fill (5.0,1.5) circle (0.1);
\end{tikzpicture}
\end{center}
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5. Go back over each ink spot one time to make sure there is enough ink in the spot.

6. Obtain a small piece of tape and gently curl the paper into a cylinder. Tape or staple the ends together near the top, taking care that the two edges of the paper do not touch! (If they do touch, the eluent will creep on a diagonal and the spots will run together and ruin the chromatogram.)

7. Take a 600-mL beaker and place about 25-mL of eluting solution into the beaker. Obtain a piece of plastic wrap from your instructor of a size adequate to cover the top of the beaker to prevent evaporation of the eluting solution.

8. Gently place the paper cylinder into the beaker and cover the top with the plastic wrap. Remember that the spots must be above the liquid level for the experiment to work. Watch the eluent creep up the paper until it begins to move some of the ink. Your instructor may have another assignment for you to work on while the chromatogram is developing. It will take about 30 minutes for the solvent front to reach the finish line.

9. When the solvent front reaches the finish line, remove the paper from the beaker, being careful to touch only the top. Let excess eluent drip into the beaker. Gently remove the tape and lay the chromatogram on a piece of paper towel in the hood. Immediately mark the line of the solvent front with a pencil. Leave the paper in the hood and allow it to dry completely.

10. When the chromatogram is completely dry, take back to your bench. Take the ruler and draw a plus sign centered in each of the spots that have eluted. Measure the distance between the center of each plus sign and the starting line. Record this distance in a data table.

11. Measure the distance between the starting line and the line you marked for the solvent front. It may be slightly different than the finish line. Record this distance in a data table.

12. Calculate the $R_f$ for each spot and record the values in a data table. Staple your chromatogram to your lab report to be handed in.
### Lab 4

**Chromatography of Gel Ink Pens and Grape Kool Aid®**

**Grading Protocol**

Name: ________________________

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