**Liquid Chromatography**

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**LAB ADV 18**

**INTRODUCTION**

Chromatography is a process used to separate the components of a mixture. A mixture is injected into a chromatography column, where it lands on a substrate, also known as the stationary phase. The stationary phase may be polar, attracting polar substances, or nonpolar, attracting nonpolar substances. When a mixture is injected into a chromatography column, the substances in the mixture cling to the stationary phase. Next, a solvent is injected into the column. The solvent is called the mobile phase. As the solvent moves along the stationary phase, it will carry the components with it. When and how quickly the substances are carried out of the column by the solvent depends on the polarity of the substances and their solubility in the solvent. If the solubilities and/or polarities of the individual parts of the mixture are significantly different, the substances in the mixture will separate from each other as the mixture travels along the substrate. The substance that is the most strongly attracted to the solvent will be the first to leave the chromatography column. The substrate (stationary phase) and the solvent (mobile phase) can be in any phase, depending on the properties and concentrations of the components in a given mixture. Therefore, there is solid, liquid, and gas chromatography.

In this experiment, you will use liquid chromatography to separate the dyes, FD&C Blue and FD&C Red that are found in grape-flavored Kool-Aid®, from the other ingredients in the dry drink product. You will use a special column, called a C18 Sep-Pac® for the experiment. This column contains a silica solid with a C18 hydrocarbon bonded to it, which renders the solid nonpolar.

There are two parts to this experiment. In Part I, you will conduct an isocratic separation, in which one solvent passes through the column at a specified rate. This process allows you to separate the two food dyes from the other ingredients in the mixture. In Part II, you will conduct a step gradient separation. In this process, three solvents are used (each of a different polarity and concentration) to separate most all of the substances in the mixture.

OBJECTIVES

In this experiment, you will

* Conduct an isocratic, liquid-chromatographic separation.
* Conduct a step gradient, liquid-chromatographic separation.
* Complete the necessary measurements and calculations to evaluate the components of a mixture that have been separated by liquid chromatography.

MATERIALS

|  |  |
| --- | --- |
| C18 Sep-Pac® cartridge | 70% isopropanol (2-propanol) |
| 10 mL syringe with male Luer® tip*or* 50 mL dropper bottle with tip*or* 100 mL wash bottle | Grape Kool-Aid® drink mix, unsweeteneddistilled water |
| 1 mL syringe with male Luer® tip | four 50 mL beakers |
| two 10 mL graduated cylinders | three 100 mL beakers |
| two 25 mL graduated cylinders |  |

PROCEDURE

Part I Isocratic Separation

 1. Obtain and wear goggles.

 2. Obtain a 10 mL sample of grape Kool-Aid drink mix that has been prepared according to the label instructions, excluding the sugar.

 3. Prepare the solvent (mobile phase) in a 100 mL beaker by mixing 13 mL of 70% isopropanol with 37 mL of distilled water to make an 18% (v/v) isopropanol solution.

 4. Pretreat the C18 Sep-Pac liquid chromatography cartridge.

1. Obtain a C18 Sep-Pac cartridge and cut off the exit tube (the short end). This will help keep the two food dyes separated.
2. *If you are using a 10-mL syringe*, fill it with 10 mL of undiluted 70% isopropanol. Attach the tip of the syringe to the long end of the Sep-Pac cartridge and inject the isopropanol into the column at a rate of 5-10 mL per minute. Collect the eluate into a 10 mL graduated cylinder to help you monitor the flow rate of the isopropanol.
3. *If you are using a wash bottle or a dropper bottle*, fill the bottle with 70% isopropanol and firmly attach the top of the bottle to the long end of the column. Pump 10 mL of isopropanol slowly through the column.

Wash the Sep-Pac cartridge with 10 mL of distilled water.

 5. Use a 1 mL syringe to draw up 1 mL of your sample of grape Kool-Aid. Slowly inject the 1 mL of Kool-Aid into the Sep-Pac cartridge. Collect and discard the effluent that washes out of the column as you inject the sample.

 6. Elute the components of the grape Kool-Aid sample.

1. Fill a 10 mL syringe or a plastic dropper bottle with 18% isopropanol solution (the solvent).
2. Set up a 10 mL graduated cylinder to collect the dyes as they leave the column.

Slowly pump the 10 mL of 18% isopropanol solution into the column at a steady rate of 5-10 mL per minute.

Repeat c. in order to get all blue dye off the column.

 7. Evaluate the eluate and record volumes in the data table. If there is not a perfect separation of the red and blue bands, record the data for the beginning and end of the intermediate purple band. Use the center of the purple band, if necessary, as the end of the first band and the beginning of the second band.

1. Measure the volume of liquid collected, as the first and last of the colored drops of each dye emerge. Record this value in your data table as *W*, the band width.
2. Determine the volume at the center of the red and blue band width and record it as *V*R, the retention volume.
3. Determine the total volume eluted at the center of the band of the red and blue dye, and record it as *VRavg*.
4. Record the column length, *L*. The C18 Sep-Pac cartridge is 1.25 cm long.

Record the column radius, *r*. The C18 Sep-Pac has a radius of 0.5 cm.

 8. Repeat Steps 4-6 twice, starting with 4b, using two new samples of grape Kool-Aid. Record the results of all three trials in your data table.

Part II Step Gradient Separation

 9. Prepare the solvents (mobile phase).

1. Mix 7.0 mL of 70% isopropanol with 46.5 mL of distilled water into a 100 mL beaker to make a 10% isopropanol solution*.*
2. Mix 20 mL of 70% isopropanol with 30 mL of distilled water into a 100 mL beaker to make a 28% isopropanol solution.

Obtain a dropper bottle of distilled water as the third solvent for the step gradient separation.

 10. Repeat Parts b-d of Step 4 to pre-treat the Sep-Pac cartridge.

 11. Repeat Step 5 to inject a Kool-Aid sample into the column.

 12. Elute the components of the grape Kool-Aid sample and separate by the step gradient process.

1. Obtain four 50 mL beakers. Label the beakers, 1-4.
2. Set up Beaker 1 to collect the first eluate. Pass 5 mL of distilled water through the column to elute the polar components of the mixture and collect them in the first of four 50 mL beakers.
3. Set up Beaker 2 to collect eluate from the column. Pass 5 to 10 mL of 5% isopropanol solution through the column to elute the red dye.
4. Set up Beaker 3 to collect eluate. Pass 5 to 10 mL of 28% isopropanol solution through the column to elute the blue dye.

Set up Beaker 4 to collect eluate. Pass 8 mL of 70% isopropanol solution through the column to elute flavor oils and other nonpolar ingredients.

 13. Place the four beakers of eluate in a hood, or well-ventilated area away from open flames, and allow the solvents to evaporate. When the beakers are dry, observe the contents of each beaker and record your observations. Use a hotplate set on low to speed up the process.

 14. All of the solutions may be discarded down the sink. The Sep-Pac cartridge is reusable.

DATA TABLE

|  |  |  |  |
| --- | --- | --- | --- |
|  | Trial 1 | Trial 2 | Trial 3 |
| *VR* (start) |  |  |  |
| *VR* (end) |  |  |  |
| *W* = *VR* (end) – *VR* (start) |  |  |  |
| *VRavg* = *VR* (start) + 0.5 W |  |  |  |
| *L* |  |  |  |
| *r* |  |  |  |
| *VM* (mobile phase volume) |  |  |  |
| *k*′ (capacity factor) |  |  |  |
| α (selectivity factor) |  |  |  |
| *N* (theoretical plates) |  |  |  |
| *R* (resolution) |  |  |  |

CALCULATIONS

The following calculation guides will help you complete the data table.

 1. *VM* is the mobile phase volume, determined by the following equation: *VM* = 0.5 πr2*L*. This factor represents about half of the total empty column volume. The unit for *VM* will be cm3 (or mL) if the values of r and L are measured in cm.

 2. *k* ′ is the capacity factor, which is a unitless measure of the retention for each of the dyes and is determined by solving the following equation: *k* ′ = (VRavg – *VM*)/ *VM*. In this experiment, you will calculate two *k*′ values. Optimum values of *k* ′ are commonly between 1 and 10.

 3. α is the selectivity, or separation, factor and it is the ratio of the separation of the *k*′ values. In this experiment, you will calculate one selectivity factor, because you separated only two substances (the two food dyes). The equation for the selectivity factor is: α = *k* ′2 / *k* ′1. The value of α is always larger than 1, therefore you will use the larger of your k′ values as *k* ′2.

 4. *N* represents the number of theoretical plates in the column. Think of *N* as the number of times a dye molecule is exchanged back and forth between the stationary phase (the silica in the column) and the mobile phase (the isopropanol solution). The equation for *N* is: *N* = 16 (*VR*/*W*)2. The value of *N* is generally based on the dye which is eluted last. A large value of *N* means that the column is more efficient. The range of N values is normally between 20 and 200.

 5. *R* is the resolution, which is the major objective of a chromatographic separation. *R* measures how well the two dyes were separated by the Sep-Pac cartridge. The equation for *R* is:
*R* = (*VR1* – *VR2*) / 0.5 (*W1* + *W2*). The numerator is the volume between the bands made by the two dyes when they were in the column, which is related to the selectivity factor (α). The denominator is the average band width, which is proportional to the efficiency of the column. As the value of *R* increases above a value of 1, there is much greater total separation of the dyes.

DATA ANALYSIS

 1. Evaluate your testing based on the calculated values of *k*′, *α*, *N*, and *R*. Do your values for these factors fall within the normal range? Explain how your values for these factors describe how well the two food dyes were separated in this process.

 2. Describe the contents of the four 50 mL beakers in which you collected the various ingredients of the grape Kool-Aid mix. Estimate the relative amounts of the substances.

 3. Describe how the solvents worked as the mobile phase of the liquid chromatography experiment. Why was it necessary to use different concentrations of aqueous isopropanol in the step-gradient separation?

 4. The most important factor, *R*, should be greater than 1. Was this the case in your experiment? If so, what observations would support a high value of *R*? If not, how could the experiment be modified to increase the value of *R*, other than using a longer column?