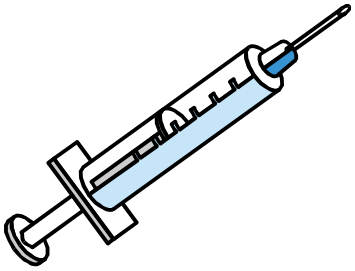
College of the Canyons

COC Biotechnology Program



High Performance Liquid Chromatography

Version 12-12-03

- **Chromatography is used by scientists to separate one substance from another.**
- **In this experiment you will separate the dyes that give grape Kool-aid its color.**
- **You will use a type of chromatography cartridge (called and Sep-Pak) fastened onto a syringe.**
- **Separation is possible because one of the Kool-aid dyes is more strongly attracted to a powdery material in the cartridge than the other dye is.**
- **The kind of cartridge you are using today is used in labs all over the world.**
- **Many everyday products are separated or purified by this method.**
- **This cartridge is used by:**
    - **Food and beverage companies.**
    - **Pharmaceutical companies.**
    - **Oil companies.**
    - **Cosmetic companies.**
    - **Water purification plants.**
    - **Medical laboratories.**
    - **Environmental protection organizations.**
    - **Research laboratories.**

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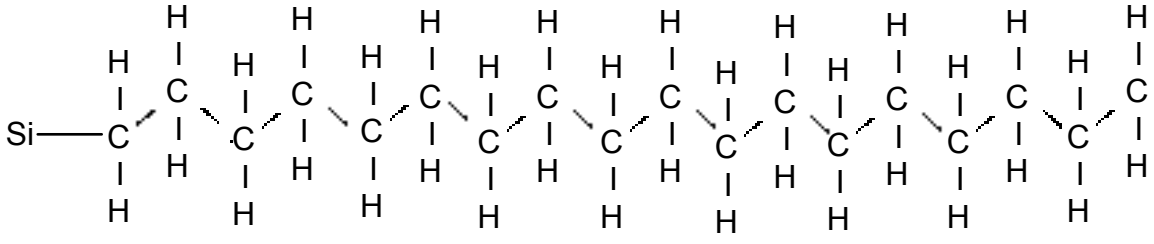
I. Objectives:

1. To understand the usefulness of separating mixtures in order to analyze them.
2. To separate the different dyes that make grape Kool-Aid purple.
3. To compare the use of methyl alcohol and isopropyl alcohol as solvents.
4. To compare some properties of molecules you use for separation based on polarity

II. Background:

A fundamental method of study, whether in science, literature, or any other field, is to isolate the parts that make up the whole, and then to start analyzing each of the parts. In this way, a complicated problem can be solved a little bit at a time. In analytical science, the technique called High Performance Liquid Chromatography (HPLC) uses precision pumps to measure, mix, and propel a liquid through a tube packed full of a powdery solid adsorbent. Separation of a mixture added to the tube occurs because some molecules in the mixture are more attracted to the moving solvent while other molecules in the mixture are more attracted to the solid motionless adsorbing agent. The molecule that is most attracted (most easily dissolved) in the solvent, and least attracted (adsorbed) by the solid, will flow out of the tube first. Electronic and other sensors detect the molecules as they exit the tube.

You will gain experience with HPLC in this laboratory exercise. Your hands, eyes, and brain will perform the work of the expensive pumps and other automated equipment, but you will use the same sorts of solvents and adsorbent used by scientific experts performing sophisticated analyses. It is estimated that over 90% of the scientists using HPLC use the same carbon-18 adsorbent that you will be using in this experiment. The chemical formula of the "C-18" adsorbent is shown below. The powdery substances are C-18 chains attached to a silicon atom.



C 18 SEP-PAK CARTRIDGE

- **Some down-to-earth examples:**
  If two molecules are attracted to each other, they tend to stick to one another and to mix. On a large scale, interactions like this make transparent tape stick to paper and food stains stick to clothing. If molecules repel one another, the substances do not stick and they will not mix.

  Other large scale examples: A ball-point pen fails to write on greasy paper; water rolls off a waxed car; oil and vinegar separate in salad dressing. Molecules attract or repel other molecules depending upon their chemical properties. Chemical properties are the result of which atoms are joined together in each molecule.

  In a separation similar to liquid chromatography, a splash of spaghetti sauce on clothing may partly wash out with water. The rest of the stain can be removed with a different solvent, such as soapy water or dry-cleaning fluid, which attracts the oily part of the stain away from the cloth. The stain is separated into two parts, one part dissolved in the water, one part dissolved in the second solvent (i.e. soapy water.)

III. SOP:

Important: Always note any addenda that are posted by the instructor.

A. The needed items are listed below. Collect the following and take to your lab station.

- ☐ Sep-Pak C18 cartridge (1)
- ☐ Methyl alcohol solutions: 100%, 60%, 20% and 5%. (10 mls each solution except for 100 %, 35 mls is needed.)
- ☐ Isopropyl alcohol solutions: 100%, 60%, 20% and 5%. (10 mls each solution except for 100 %, 35 mls is needed.)
- ☐ Distilled water (100 ml)
- ☐ 10 ml syringe with luer tip
- ☐ Container for liquid waste, i.e. the fractions you do not want
- ☐ Test tubes to collect the fractions you want to keep (10 total)
- ☐ Grape drink (Kool-Aid) dissolved in water (10 ml)
- ☐ 1.0 ml pipet for loading the Kool-Aid onto the cartridge
- ☐ Test tube holder

B. Procedure Start the experiment with one alcohol solution and repeat with the other. If you want to (and if there are adequate supplies) one partner can work with one alcohol series and the other partner can work with the other. Enter your data (both partners) in the

spaces for the appropriate alcohol on these sheets. Summary thoughts and data will also appear in your lab notebook.

1. **Preparing the Sep-Pak:** Take either methanol or isopropanol and begin.

a. Attach the cartridge onto the syringe: Your teacher will demonstrate this as well as how to fill the syringe with the solvents and the sample.

b. Precondition the C-18 adsorbent with 10 ml of the 100% solution of the alcohol you are using. To do this, remove the plunger from the syringe and pour in 10 ml of the alcohol. Return the plunger to the syringe and push the solution through a drop at a time. Collect the drops in the waste container.

c. Next, rinse the C-18 adsorbent by pumping through 10 ml of water. Discard the drops into the waste container.

d. Once again remove the plunger from the syringe and, with the pipette, add exactly 1 ml of Kool-Aid. Return the plunger and push the solution into the cartridge. Collect the drops in a test tube. Label the tube and save it.

What color is the liquid collected? (Methanol) _____ (Isopropanol) _____
Does the liquid have any odor? If it smells, describe the smell.
(Methanol) _____
(Isopropanol) _____

Describe the location of the color in the Sep-Pak cartridge (i.e. purple, close to syringe, blue opposite syringe, red in rest of Sep-Pak).
(Methanol) _____
(Isopropanol) _____

2. **SEPARATION STEPS**:

a. Pump 10 ml of water through the cartridge. Collect the drops in another test tube. This fraction may contain Kool-Aid flavorings. Label the tube and save it.

What color is the collected fraction? (Methanol)_____ (Isopropanol)_____

Does the liquid have any odor? If it smells, describe the smell.

(Methanol) _____ (Isopropanol) _____

Did the color in the cartridge move? (Methanol)_____ (Isopropanol)_____

If so, describe the movement. (Methanol) _____ (Isopropanol)_____
Was the color most attracted to the water or to the C-18 adsorbent?
(Methanol) _____ (Isopropanol) _____

b. Pump 10 ml of the 5% solution of your alcohol through the system. Collect the drops in another test tube. Label the tube and save it.
What color is the liquid collected? (Methanol)_____ (Isopropanol)_____
Did the color in the cartridge move? (Methanol)_____ (Isopropanol)_____

c. Pump 10 ml of the 20% alcohol solution through. Collect the drops in another test tube. Label the tube and save it.

What color is the liquid collected? (Methanol)_____ (Isopropanol)_____

What happened to the color in the cartridge? Describe.
(Methanol)_____

(Isopropanol)_____

d. Pump 10 ml of the 60% alcohol solution through the cartridge and collect the drops in another tube. Label the tube and save it.

What color is the liquid collected? (Methanol)_____ (Isopropanol)_____

What colors are still in the cartridge? (Methanol)_____ (Isopropanol)_____

e . Clean the cartridge with 10 ml of the 100% alcohol solution you have been using. Discard the drops in the waste container.

3. Repeat the above procedure exactly as before using the other alcohol, starting over with step 1b. Take care to answer the same questions as you go through every step (unless of course you and you partner did the experiment concurrently!)

Which alcohol gave you more concentrated dye samples? _____

Final step: Using **only** tubes from the methanol experiment, carefully pour the blue fraction into the red fraction VERY SLOWLY (ask for help if not sure) Observe and describe what happens.

_____

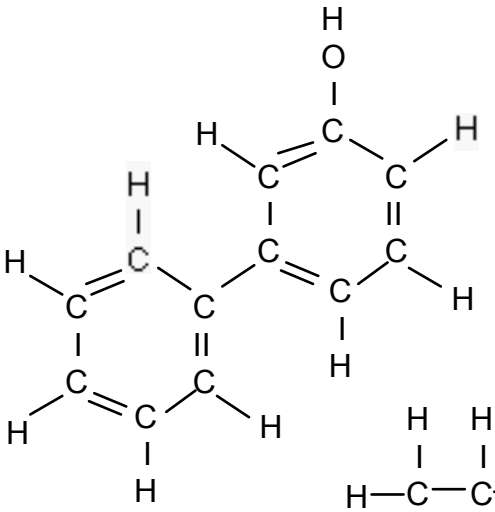
_____

- **SUMMARY:** As you have discovered, Kool-Aid is composed of several substances, including sugar, acids, dyes and flavoring. These substances are differentially soluble in various solvents. Water is a highly polar molecule and alcohol solutions show a range of polarities. The adsorbent in the Sep-Pak cartridge is extremely non-polar, as you would expect with its 18-carbon-long hydrocarbon chains. HPLC takes advantage of these molecular qualities to separate compounds in a mixture.

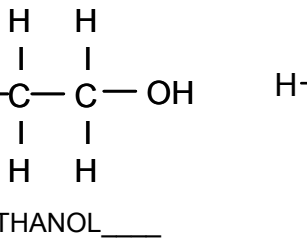
Name _____

a. Write the ratio of hydroxyl group (-OH) to carbon atom (C) in the space beside each molecule. Generally speaking, the higher the number of hydroxyl groups per carbon atom, the more polar the molecule. (Can you find the structural typo?)

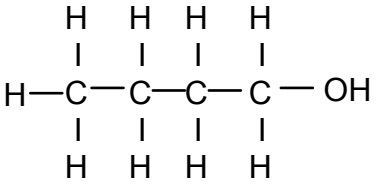
WRITE THE RATIO OF -OH: C IN THE SPACE BELOW EACH MOLECULE



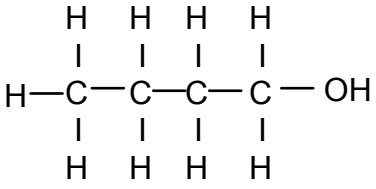
PHENYL PHENOL_____
(LYSOL)



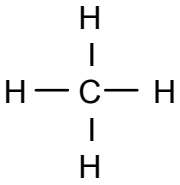
ETHANOL____



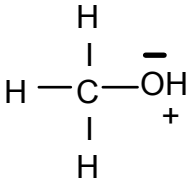
BUTANE____



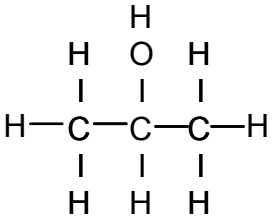
BUTANOL_____



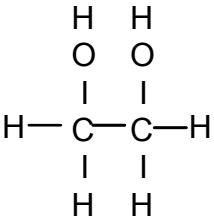
METHANE____



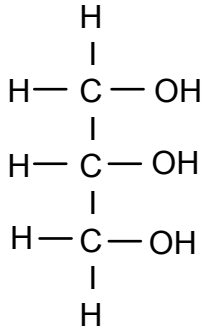
METHANOL____



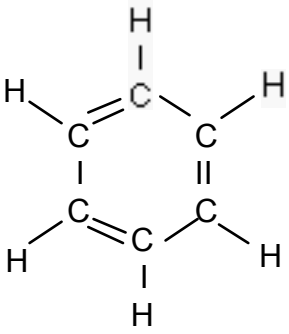
ISOPROPANOL___
(RUBBING ALCOHOL)



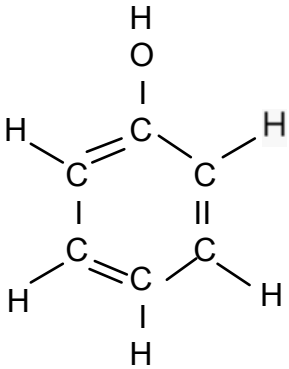
ANTIFREEZE_____
(ETHYLENE GLYCOL)



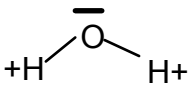
GLYCEROL_____
(USED IN LOTIONS, COUGH DROPS, AS A FOOD PRESERVATIVE



BENZENE_____



PHENOL _____
(USED TO PRODUCE PLASTICS)



WATER____

b. Across the bottom of this page, make a list in which you rank the various alcohols and hydrocarbons according to their relative polarity (from most polar to most non-polar). If two or more molecules are equally polar or non-polar, list them together.

IV. Post-Lab Questions/Activities:

The following post lab questions are for your benefit. The questions will help you to address a range of topics relating to the lab activity. Along with the post lab handouts, these questions will help to ensure that you have both correct information regarding the lab data and crucial lab processes. Complete the post lab questions at the end of the lab and post lab handouts (keys for both of these are available from your instructor) before making any lab-notebook entries.

1. a. Draw a table (percent alcohol and color of fraction collected) that compares the dye separations using the two alcohols. Your table should clearly summarize the main results you recorded on your lab report (steps 2a-d).

b. Explain the differences shown in your table. (Suggestion: include a list of all solvents used in the separation, ranking them according to their relative polarity. Hint: Compare the chemical structures of the two alcohols, and the percentage of water in the mixtures to see which alcohol/water mixture is more polar (or more non-polar) than the other. (Structures are shown on previous page.)

2. Which Kool-Aid dye (red or blue) was the most polar? Justify your answer.

3. What would have happened if you had used the HPLC solvents in reverse order? (100% alcohol solution first and water last)?

4. Was the separation a physical process or did a chemical reaction take place? Review the "final step" idea to aid you in your answer.

5. When you poured the two fractions together ("final steps"), you probably observed a layered effect. What properties of the solutions caused the layering?

6. What do you think would have happened if you had used these mixtures to separate the dyes instead? a. ethyl alcohol (C_2H_5OH) and water? b. butyl alcohol (C_4H_9OH) and water? (See previous page for structures).

7. If two substances have <u>exactly</u> the same polarity, could they be separated using HPLC? Explain your answer.

8. a. If two <u>colorless</u> substances differed in polarity, could they be separated by HPLC?

b. If you answered yes to 8a, describe two different methods you could use to find the colorless substances once they were separated.

V. **Notebook Entries:** Data from the lab should be the focus of this section and if there are any incorrect results, you should discuss this as well as expected results. Section V will contain both your results and discussion. Your data should drive the discussion. An informed discussion is dependent on understanding the post lab questions/activities. **Recall HPLC has a snapshot.**

Your intro should:

- Define HPLC
- State specifically what was done: separation of Kool Aid dyes with varying percentages of solutions of methanol and isopropanol.
- Sep Pak role: Note that accompanying theory is also discussed.

Results should be:

- Simple table of fraction color at different percentages, alcohol types.

Discussion should consider the following:

- Which dye is more polar, reasoning
- Effects of alcohol type (C-H:OH ratio) and percent solution effects.

Your summary (in your lab notebook) for the HPLC lab should be no more than 3 paragraphs long!

References:

California Lutheran University's Enriched Science Program: www.clunet.edu