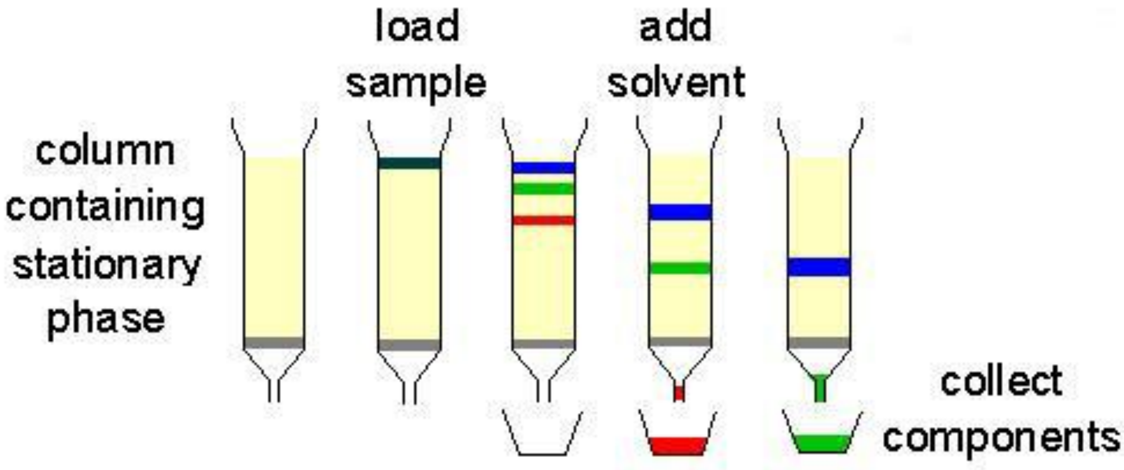
COC Biotechnology Program



High Performance Liquid Chromatography (HPLC)

Version A

- **Chromatography is used by scientists to separate one substance from another in companies such as: food and beverage, pharmaceutical, cosmetic, oil companies and drug testing labs. In fact, this technology is so sensitive that it can detect drug residue in hair up to 5 years after ingestion!**
- **In this experiment you will separate the dyes that give grape Kool-Aid its purple color.**
- **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 dyes in grape Kool-Aid are more non-polar than polar and in fact, Kool-Aid dyes would *dissolve better in gasoline than in water*? Confused? The concept of polarity and like dissolves like will clarify things.**

Did you know that rigorous science training will make you more competitive for ANY type of job? Employers know that students who can tackle hard science will do well with almost ANY challenges presented them. For information on biotechnology and other robust science courses, contact: Jim Wolf, College of the Canyons Biotechnology Director at (661)362-3092 or email: jim.wolf@canyons.edu

GOT SCIENCE? GET AHEAD!

INTRODUCTION TO HIGH PERFORMANCE LIQUID CHROMATOGRAPHY: SEPARATION OF THE DYES IN GRAPE KOOL-AID

OBJECTIVES:

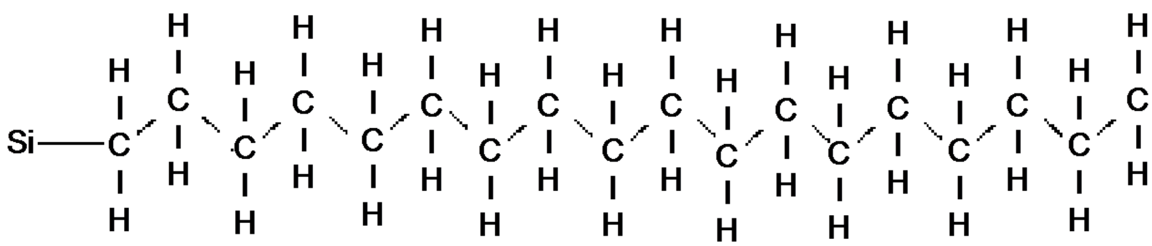
1. To understand the usefulness of separating mixtures in order to analyze them.
2. To separate the different dyes that make grape Kool-Aid purple.
3. To compare the use of methyl alcohol and isopropyl alcohol as solvents.
4. To compare some properties of molecules you use in the separations.

I. 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 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 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 powder is glass, covered with C-18 chains attached at the 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.

II. HPLC

A. Your lab station should be equipped as listed below. You will have either methanol or isopropanol, not both. Before you start, use this list to identify each item at your lab station.

- ☐ Sep-Pak C18 cartridge (1)
- ☐ Methyl alcohol solutions: 100%, 60%, 20% and 5%. (30 ml each solution)
- ☐ Isopropyl alcohol solutions: 100%, 60%, 20% and 5%. (30 ml each solution)
- ☐ 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

For instructions on the lab, go to personal page: "B. Procedure".

III. 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 molecules in a mixture.

IV. Exercises: Use the worksheet on personal page 3 to compare the relative polarity of several alcohols and hydrocarbons .

V. QUESTIONS: Use your previous knowledge about oil and vinegar salad dressing, your observations in this lab, and the information gained in the exercise to answer the following questions: Use a separate piece of paper and attach it to your personal pages.

1. a. Draw a table that compares the dye separations using the two alcohols. Your table should clearly summarize the main results you recorded on your personal pages (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 mixture, to see which alcohol/water mixture is more polar (or more non-polar) than the other. Structures shown on personal page 3.)

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? (60% alcohol solution first and water last)?

4. Was the separation a physical process or did chemical reactions take place?

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

6. What do you think would have happened if you had used these mixtures instead:
a. ethyl alcohol (C_2H_5OH) and water?
b. butyl alcohol (C_4H_9OH) and water? (See personal page 3 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.

Lab partner(s) name(s): ____

B. Procedure: The alcohol solutions will be shared by two lab stations. One lab station will use the methyl alcohol solutions first and then repeat the procedure using isopropyl alcohol solutions. The neighboring lab station will start with isopropyl alcohol and then use methyl alcohol. Enter your data in the spaces for the appropriate alcohol on these sheets.

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 pipet, add 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.
(methanol) ____
(isopropanol) ____

Sketch the Sep-Pak to left and show the location of the color.

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) ___________________________
Sketch the Sep-Pak to left and show the location of the color.

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)_________

Sketch the Sep-Pak to left and show the location of the color.

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)__ _____________________

Sketch the Sep-Pak to left and show the location of the color.
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)_________

Sketch the Sep-Pak to left and show the location of the color.

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 the other alcohol

Step 1b. Take care to answer the same questions as you go through every step.

Which alcohol gave you more concentrated dye samples?_______________________
Final steps:. a. Using **only** tubes from methanol experiment, carefully pour the blue fraction onto the red as was demonstrated. Observe and describe what happens.

____

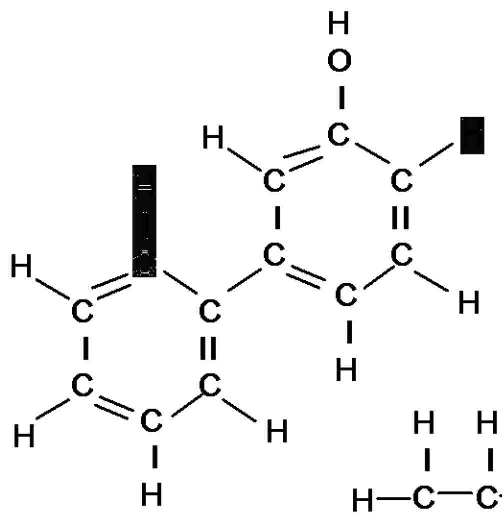
b. Using **only** tubes from isopropanol experiment, carefully pour the blue fraction onto the red as was demonstrated. Observe and describe what happens.

____

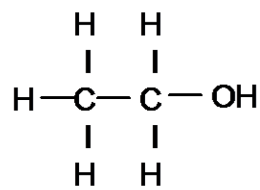
At this point, return to the Summary, Exercise, and Questions on HPLC page 2 and 3.

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.

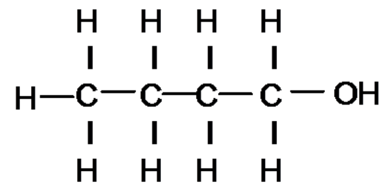
WRITE THE RATIO OF -OH: C IN THE SPACE BELOW EACH MOLECULE



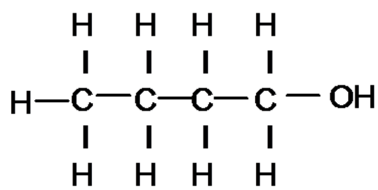
PHENYL PHENOL_____
(LYSOL)



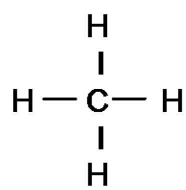
ETHANOL____



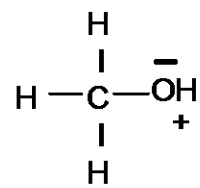
BUTANE____



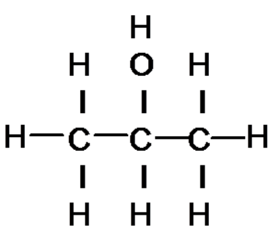
BUTANOL______



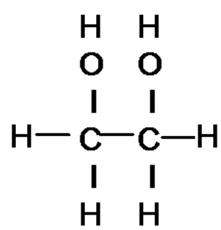
METHANE____



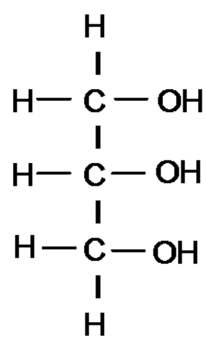
METHANOL____



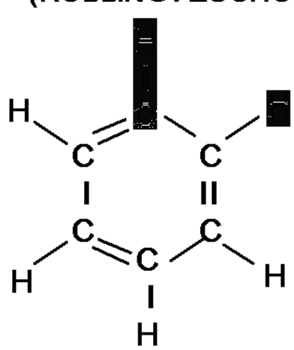
ISOPROPANOL___
(RUBBING ALCOHOL)



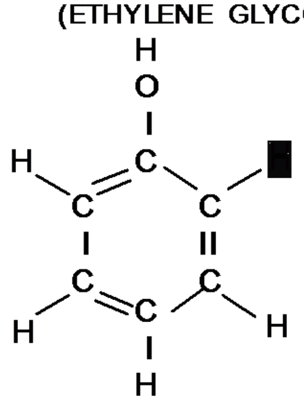
ANTIFREEZE______
(ETHYLENE GLYCOL)



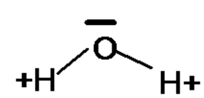
GLYCEROL______
(USED IN LOTIONS, COUGH DROPS, AS A FOOD PRESERVATIVE



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.