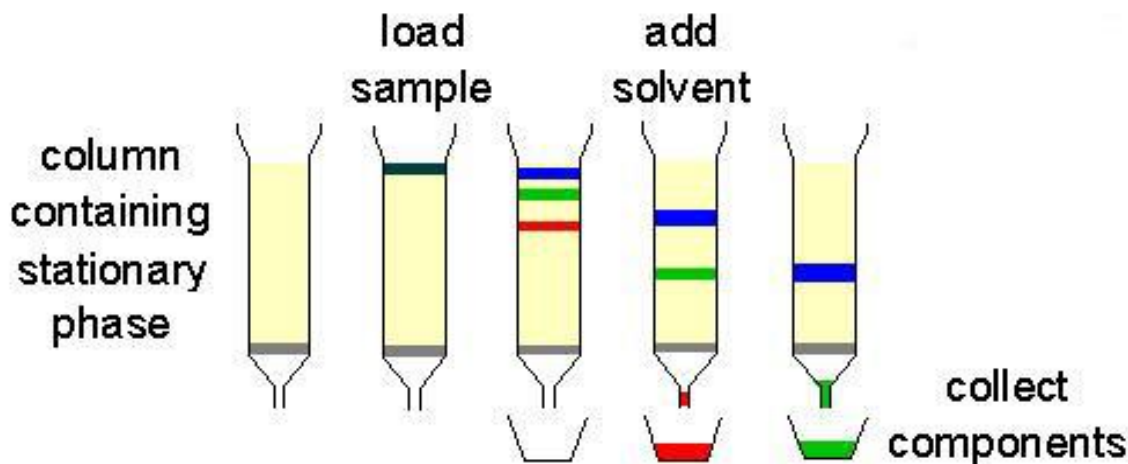


## COC Biotechnology Program



## High Performance Liquid Chromatography (HPLC) Version B

- 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](mailto:jim.wolf@canyons.edu)

**GOT SCIENCE? GET AHEAD!**

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

**OBJECTIVE:** To separate the dyes that make grape Kool-Aid purple.

**I. INTRODUCTION**

What appears to be one substance is often a mixture of several different chemicals. A fundamental method of study, whether in science, literature, or any other field, is to separate the parts that make up the whole. Once the parts are separated, they can be more easily analyzed. In this way, something complicated can be studied and understood a little at a time.

Chromatography allows a scientist to analyze the chemical make-up of a mixture. There are always two vital ingredients in liquid chromatography: a liquid (solvent) and a solid material (adsorbing agent) through which the solvent flows. The solid is ground into a powder and packed in a tube, and the solvent flows through the tube. The chemicals in a mixture entering the tube will separate because some molecules in the mixture are more attracted to the moving solvent while others are more attracted to the 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.

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 washed out with another 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.

In modern analytical science, the technique called High Performance Liquid Chromatography (HPLC) uses precision pumps to measure, mix, and propel solvent through a long tube packed full of adsorbent powder. Sensors detect molecules as they exit the tube.

You will gain experience with HPLC in this laboratory exercise. Your hands, eyes, and brain will replace the expensive pumps, sensors, and other automated equipment, and your tube will be short, but you will use the same sorts of solvents and the same adsorbent (called C-18) used by scientific experts performing sophisticated analyses.

## II. HPLC USING A SEP-PAK C18 CARTRIDGE TO ISOLATE THE FOOD COLORS IN GRAPE DRINK.

A. Check off each item at your lab station.

- Sep-Pak C18 cartridge
- Methanol (methyl alcohol) solutions: 100%, 60%, 20% and 5%.
- Distilled water
- 10 ml syringe with luer tip
- Container for liquid waste, i.e., the fractions you do not want
- Four test tubes to collect the fractions you want to keep
- Grape drink (Kool-Aid) dissolved in water
- 1.0 ml pipette for loading the Kool-Aid onto the cartridge
- Test tube holder

B. Procedure (Wait until your teacher tells you to begin work.)

1. Attach the cartridge to the syringe. Your teacher will demonstrate this, as well as how to fill the syringe with the solvents, how to load the sample and how to pump the solvents through the cartridge.
2. Thoroughly pre-wet the cartridge with 10 ml of 100% methanol. To do this, remove the plunger from the syringe and pour in 10 ml of methanol. Return the plunger to the syringe and push the solution through a drop at a time. Discard the drops in your waste container.
3. Flush wetted cartridge with 10 ml water. Discard the drops in your waste container.
4. Carefully add 1 ml of the Kool-Aid and use the plunger of the syringe to push it into the cartridge. Discard the liquid that comes through in the waste container.

a. Where does the Kool-Aid settle in the cartridge? \_\_\_\_\_  
\_\_\_\_\_

b. What color is it? Is it spread out in the cartridge? Tell how it looks when viewed from the side, and make a drawing of the cartridge showing the color location to the left. \_\_\_\_\_  
\_\_\_\_\_

5. PUMP 10 ml of water through the cartridge one drop at a time. Collect the drops in a test tube. Label the tube "water" and save it.

a. This sample may contain flavorings. Keeping your nose away from the alcohol beakers, smell the sample in the tube. Can you detect the flavorings? Describe what you smell. \_\_\_\_\_  
\_\_\_\_\_

b. What color is the liquid collected in the tube? \_\_\_\_\_

c. Did the color (or colors) in the cartridge move? If movement took place, tell which color (or colors), and where it moved. Make a sketch of the cartridge to the left.

\_\_\_\_\_

\_\_\_\_\_

d. Is the color more strongly attracted to the moving water or to the stationary C18 Sep-Pak cartridge? \_\_\_\_\_

6. Using the same technique as in step 5, slowly pump 10 ml of 5% methanol through the Sep-Pak. Collect the drops from the bottom of the cartridge in a second test tube. Label the tube "5%" and save it.

- a. What color is the collected liquid? \_\_\_\_\_
- b. Did the color (or colors) in the cartridge move? Describe, and draw to left.

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\_\_\_\_\_

c. Is the color more strongly attracted to the moving solvent or to the stationary C18 absorbent in the cartridge? \_\_\_\_\_

7. Slowly pump 10 ml of 20% methanol through. Collect the drops in the third test tube. Label the tube "20%" and save it.

- a. What color is this liquid? \_\_\_\_\_
- b. What color has remained on the cartridge? Describe color, location, and draw.

\_\_\_\_\_

\_\_\_\_\_

c. Is the color more strongly attracted to the solvent or to the stationary C18 absorbent? \_\_\_\_\_

If some of the first color has remained behind on the cartridge, pump 5 additional ml of 20% methanol through and collect drops in the same tube.

8. Pump 10 ml of 60% methanol through the cartridge and collect the drops in the fourth test tube. Label the tube "60%" and save it.

What color is this liquid? \_\_\_\_\_ Sketch the cartridge to left.  
Is the color more strongly attracted to the solvent or to the C18 absorbent?

\_\_\_\_\_

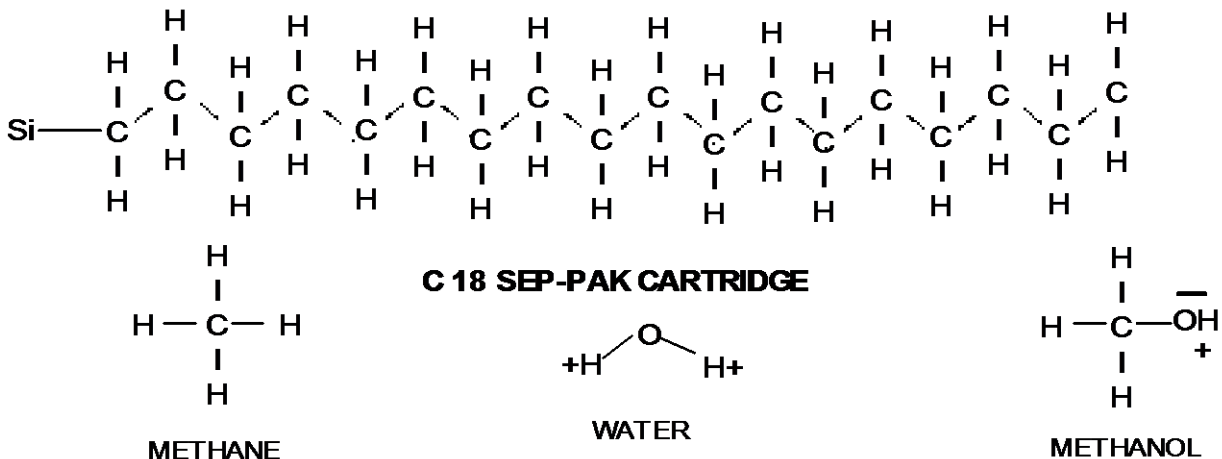
9. Finally, clean the cartridge by pumping 10 ml of 100% methanol through. Discard the drops in the waste container.

10. Check to be sure that you have recorded results for the four tubes in steps 5-10. Before cleaning up your lab station, slowly pour the blue liquid into the tube containing the red liquid, using the method that was demonstrated to you. Are you able to see a layered effect? Describe what happened.

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**III. SUMMARY:** As you have discovered, Kool-Aid is composed of many different substances including two types of colored molecules. How was separation of the dyes achieved? At first the dyes were strongly attracted to the C-18 powder. The dye molecules continued to stick to the cartridge when water flowed through the system. A dye left the cartridge when the alcohol solution was able to attract its molecules away from the adsorbent. One of the dyes was attracted away by a lower percentage of alcohol than the other. Molecules attract or repel other molecules depending upon their chemical properties. Chemical properties are the result of the atoms joined to make the molecules. Diagrams showing the arrangements of atoms in C-18 adsorbent, water, methanol, and methane are shown below.



Water and methanol contain oxygen (symbol O), an atom that tends to “tug” on the electrons of other atoms. Hydrogen (H) is especially likely to have its electron partly pulled away by an oxygen atom. Since each electron has a negative charge, the oxygen atom of water gets a slight negative charge (from having partly taken another atom’s electron), and the hydrogens end up having a slight positive charge. The same thing happens between the oxygen and one hydrogen atom in methanol. Having areas of slight opposite charge is called “polar,” and the charges cause the molecules to attract and line up with one another, like little magnets. The slight charges on molecules like methanol attract to water, so they are called hydrophilic (“water loving”), and they may feel “watery” to our skin. Polar molecules attract to other polar molecules.

C-18 and methane have no oxygen atom (see diagrams), so the electrons are distributed evenly between the atoms. There is no uneven charge distribution. They are called “non-polar”. Non-polar molecules do not attract to water. They are called hydrophobic (“water fearing”), and they may feel oily or greasy. Fats and oils are non-polar molecules. (Methane is such a small molecule that it is a gas, so it feels like air.) Non-polar molecules stick to one another because of attractions between uncharged atoms that are weaker, and less easy to explain than the attractions between the polar molecules. Non-polar molecules attract to other non-polar molecules.

By looking at the diagram you have noticed that one side of methanol is polar and the other side is non-polar, while water is all polar. Therefore, pure (100%) methanol is less polar than water, but it is more polar than the C-18 molecule, a completely non-polar molecule. By mixing alcohol and water together (5%, 20%, and 60% methanol) this lab used solvents with different polarities. List the solvents you used, in order of polarity, starting with water, the most polar solvent. \_\_\_\_\_, \_\_\_\_\_, \_\_\_\_\_, \_\_\_\_\_, \_\_\_\_\_, \_\_\_\_\_

List the solvents you used, in order of non-polarity. Start with the most non-polar solvent. \_\_\_\_\_, \_\_\_\_\_, \_\_\_\_\_, \_\_\_\_\_, \_\_\_\_\_, \_\_\_\_\_

Since the dyes stuck to the C-18 instead of the water, we know that they are less polar than water molecules. Non-polar attractions were strong enough to draw the dyes away from the water.

Since the dyes were attracted away from the Sep-Pak by the alcohol solutions, we know that the dyes are less non-polar than the C-18 molecules. The combination of polar and non-polar attractions of alcohol solutions was strong enough to draw the dyes away from the Sep-Pak.

**IV. QUESTIONS:**

1. Were you able to separate the dyes completely in steps 5-8 or did your samples contain the original purple color? \_\_\_\_\_

\_\_\_\_\_

2. Which dye (red or blue) was the most polar? Justify your answer. (CLUE: Read the essay on p. 5 and 6.)

\_\_\_\_\_

\_\_\_\_\_

3. What would have happened if you had used the solvents in reverse order? (60% methanol first and 5% last) \_\_\_\_\_

\_\_\_\_\_

4. Was this separation a physical process (pulling different molecules apart) or did chemical reactions (changing colored molecules to new colors) take place? Explain your answer. \_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

5. If you saw a layered effect when you combined the blue and red samples at the end of the experiment, what caused the layered effect? \_\_\_\_\_

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6. Could you use a Sep-Pak C18 cartridge to separate two colorless substances? If you could, how would you detect the separation? (CLUE: Did you detect the flavorings in step 5?)

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The previous lab protocol can be reproduced for educational purposes only. It has been developed by Jim Wolf, and/or those individuals or agencies mentioned in the references.

References:

California Lutheran University's Enriched Science Program: [www.clunet.edu](http://www.clunet.edu)