College of the Canyons

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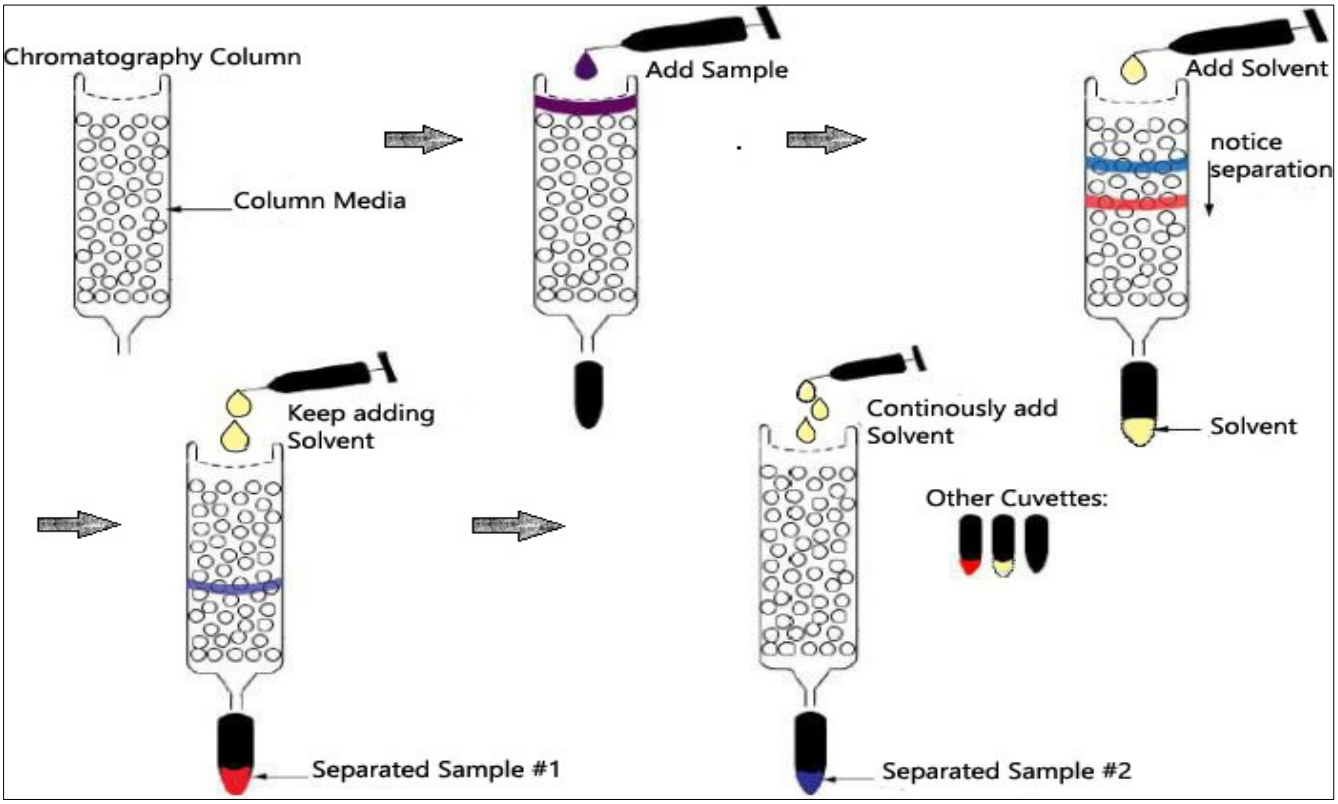

High Performance Liquid Chromatography (HPLC)

Version A

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GOT SCIENCE? GET AHEAD!

CHROMATOGRAPHY: The separation of a mixture/solution based on the polarity of the solutes and the medium (a powdery solid) through which they pass through (and move at different rates).

I. BACKGROUND

Chromatography's main job in Biotechnology and Forensics is to separate. One type of chromatography technique is called High Performance Liquid Chromatography (HPLC). It is sometimes called High Pressure Liquid Chromatography, but as for this lab, the pressure will come from the syringe. How does HPLC's ability to separate compounds useful for science? It is so powerful that it can take strands of hair and tell what, when, and how much of a drug someone has used. Cocaine can be detected in urine for 2-3 days after use, but with chromatography, scientists can detect cocaine 2-6 MONTHS after use **[1]**. It can also isolate other drugs such as heroine, methadone, and THC from marijuana all at the same time (Table 1)!

HPLC begins with a long tube, or column full of media. This packing material will do the separation, acting as one complicated coffee filter (but made of Silica and C-18 hydrocarbon chains) [diagram 1]. The sample (in this case, the chemically dissolved hair strand), is added to the top of the column media like Olympic swimmers lining up for a race. Now automatic pumps will push a liquid through the Chromatography tube, so the sample is pushed through the tube and the race is off! Now some drugs will be faster and finish before the slower drugs.

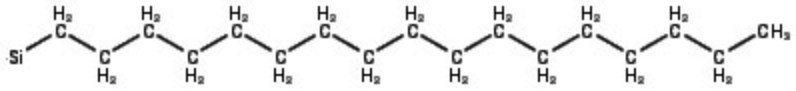
Table 1: The Race Results **[2]**:

1st place – Methadone (7.4 minutes)
2nd place- Cocaine (8.2 minutes)
3rd place- THC (9 minutes)

Why do some drugs move at different speeds through the chromatography column? . It has to do with the column media and how different media interact with samples moving through the column. In this case, the column media had gaps that let small molecules go through, while getting in the way of bigger molecules. Smaller molecules will have faster run times, and bigger molecules will travel slower through the column. You use this column when you want to separate things based on size, but what if every drug in a sample is the close to the same size? What do you have to change?

Your lab will differ from the drug analysis above in two relevant ways. Number one, you will be separating Kool-Aid because school district does not have the purchasing power to perform the experiment above. Number two, your column shown below will separate your sample based on polarity, NOT size.

Diagram 1:



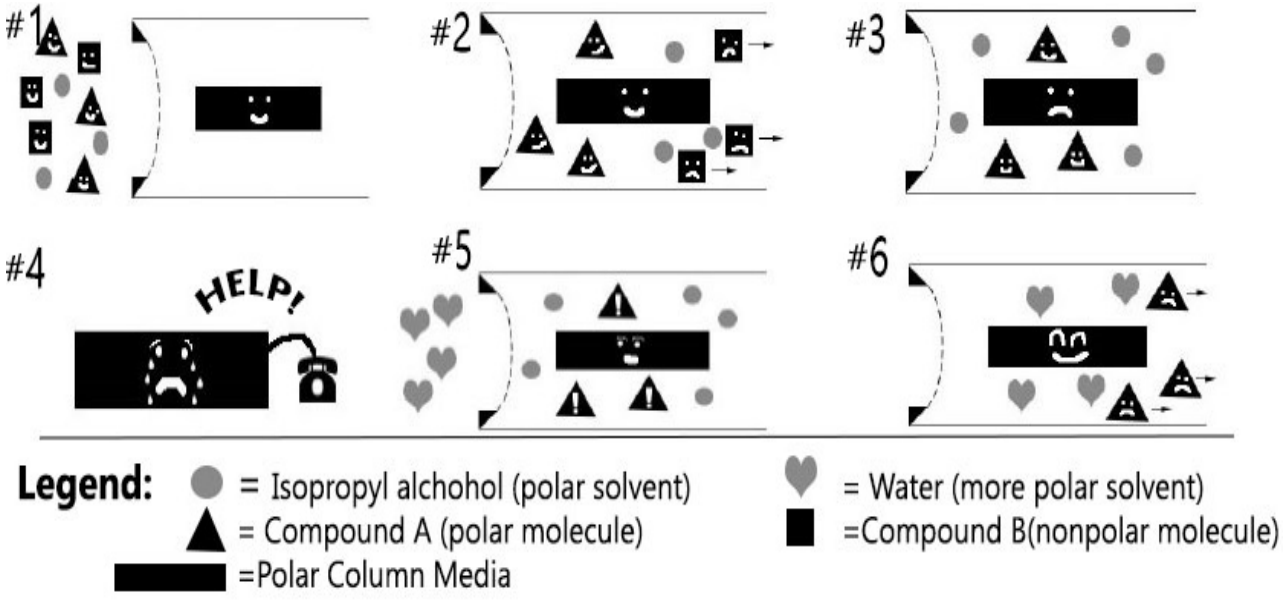
C 18 SEP-PAK CARTRIDGE

If you have a polar column media, then polar molecules will move slower because they are attracted to the media in the column. “Like attracts like” here in Biochemistry. The more polar a molecule is the greater the attraction and the slower the speed. Now if you have a non-polar packing material the polar molecules are not attracted and will now move quickly. Look at your C-18 column media, is is polar or nonpolar? What kind of molecules will move quickly through your column, and what kind of molecules will move slowly?

Here's a metaphor that will hopefully clear any confusion up. Imagine the column media (neither polar or nonpolar) is like a house, and your sample is a brother (non-polar) and sister (polar). You're the parent and want to separate these two and get them out of the house. You know if you play loud Country music (polar) the brother will leave because it all sounds the same to him, and the sister will stay. If you play loud Dubstep (non-polar) the sister will leave because she thinks real songs should have lyrics, and the brother will stay. This is how you separate samples based on their attraction or lack of attraction.

But what if you play one kind of music to separate the brother and sister, how do you then get the second sibling out of the house? You can't change the column media because your sample is still inside your column! What you need is something to change the attraction between the molecules and the media. Now this is where the solvent comes in, which is the liquid pushing the sample through the chromatography column. It is the equivalent of badly singing and dancing to music in order to change the siblings' attraction to the music. Suddenly, it isn't enjoyable to them anymore and the second sibling will also leave the house. Now both polar and non-polar substances are separated and out of your column (Diagram 2)!

Diagram 2: *The hearts and circles represent that two solventare needed to separate the samples (squares and triangles) from the packing column as seen in steps #2 and #6.*



How does the solvent change the attraction between the packing material and the samples? If you start with a polar column media you can change to a new, more polar solvent. This way the solvent will have a stronger attraction than the polar component. It competes with the polar sample and wins. Now that the polar sample is moving quickly, why didn't you just start with the more polar solvent to begin with? Why might that be a bad idea? How would it affect how well you separate polar and nonpolar components?

By now you understand the fundamentals of Reverse Phase and Size Exclusion HPLC (RPHPLC & SEHPLC), how they are used in science, and how to change the packing material AND solvents to separate different molecules.

Sources (Always Cite your Sources!!**)-**

[1] Musshof, F. "Results of Hair Analysis for Drugs of Abuse and Comparison with Self Reports and Urine Tests." National Center for Biotechnology Information. U.S. National Library of Medicine, 2004. Web. 08 Oct. 2015.
[2] Strano-Rossi, S., and M. Chiarotti. "Solid-Phase Microextraction for Cannabinoids Analysis in Hair and Its Possible Application to Other Drugs." Journal of Analytical Toxicology 23.1 (1999): 7-10. Web.

35. 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.

☐ -*Column or Chromatography Column*- Sep-Pak C18 cartridge (1)

☐ -*Solvent B*- Methyl alcohol solutions: 30 ml of100% (10 µl of 60%, 20% and 5%).
-*Solvent A*- Isopropyl alcohol solutions: 30 ml of 100% (10 µl of 60%, 20% and 5%).
-Grape drink (Kool-Aid) dissolved in water (10 ml)
-Distilled water (100 ml)

☐ -1.0 ml transfer pipet for loading the Kool-Aid onto the syringe cartridge
-Test tubes to collect the fractions you want to keep (10 total)
-10 ml syringe
-Test tube holder

☐ -Container for liquid waste, i.e. leftover liquids.

For instructions on the lab, go to personal page: "B. Procedure" (*last 3 pages of the lab*).

III. SUMMARY: As you have discovered, Kool-Aid is composed of several substances, including acids, dyes and flavoring. These substances will be soluble depending on their own polarity and the polarity of the solvent. Water is the most common polar solvent and will be more polar than all alcohol solutions. This weaker polarity is useful in dissolving things that water cannot, such as non-polar molecules. 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 different polarities to separate molecules in a mixture.

IV. **Summary:** The ability to organize information onto a page from months of experiments and notes is is vastly underrated. It is a valuable skill for every scientists but that does not mean that every scientist can do it well. Don't be like those kinds of scientists. Practice organizing your **<u>own lab notes</u>** according to the instructions below.

a. Draw a table that compares the dye separations using the two alcohol series. 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 others. Relevant structures shown on personal page 3.)

V. **QUESTIONS:** Use what you have learned from your observations/notes in this lab, and your background knowledge of HPLC to answer the following questions: Use a separate piece of paper and attach it to your personal pages(or follow instructions from your instructor.

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

2. What would have happened if you had used the HPLC solvents in reverse order? (60% alcohol solution first and water last)?

3. Write a better story/metaphors/etc. for how to separate polar and nonpolar solvents using HPLC. (like how the country music and the brother and sister were used as metaphors to represent separation processes in the *<u>Background section</u>*).

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

5. 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 ideas.)

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. Prepare 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.

_____

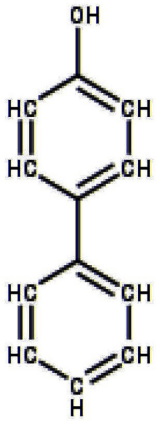
At this point, return to the Summary, Exercise, and Questions on HPLC page 2 and 3.

Name _____ Unit 1/ Module 1 /Version A Personal page 3

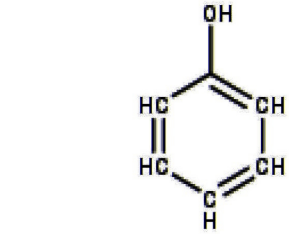
a. Write the ratio of hydroxyl group (-OH) to carbon atom (C) in the space beside each molecule. Generally speaking, the higher the number of hydroxyl groups per carbon atom, the more polar the molecule.

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 side by side.

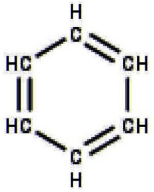
Write the ratio of -OH : C in the space below each molecule



Phenyl Phenol ____
(Lysol)



Phenol____
(Used to produce plastics)

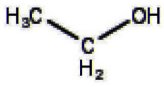


Benzene____

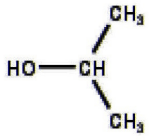
Water_____

Methane___

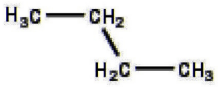
Methanol___



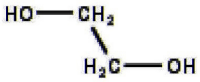
Ethanol____



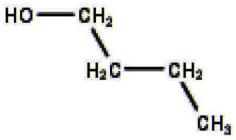
Isopropanol____
(Rubbing Alcohol)



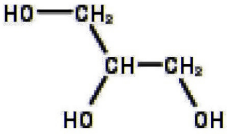
Butane___



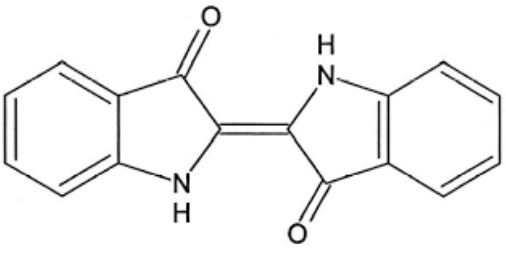
Ethylene Gycol___



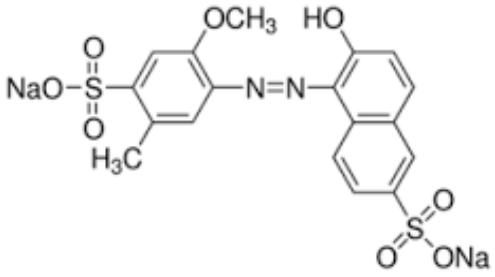
Butanol____



Glycerol____
(Used in lotions,
cough drops, and
as a food preservative)



Indigo *(blue)* dye_____



Allura *(red)* dye_____