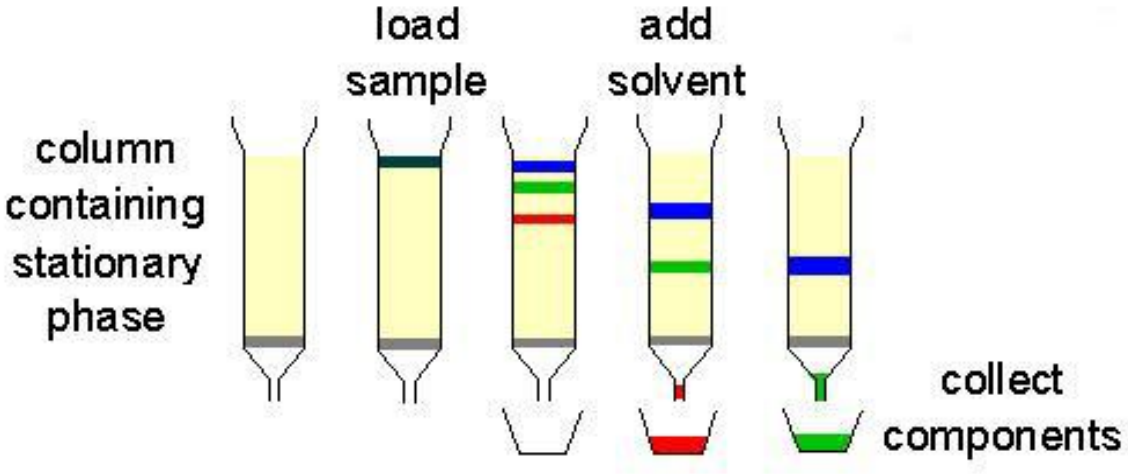
Custom Lab: Introduction to Biotechnology (BioSci 230)



High Performance Liquid Chromatography (HPLC)

Version 7-13-12

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

- **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 Grape 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.**
- **By comparing the number of polar (i.e. C-O) and non polar bonds (i.e. C-H) in a molecule, a general understanding of the ratio of the bonds will give a relative polarity which is especially useful when separating substances (see practice problems).**

- For more information on College of the Canyons' Introduction Biotechnology course, contact Jim Wolf, Professor of Biology/Biotechnology at (661) 362-3092 or email: jim.wolf@canyons.edu. Online versions available @ www.canyons.edu/users/wolfj

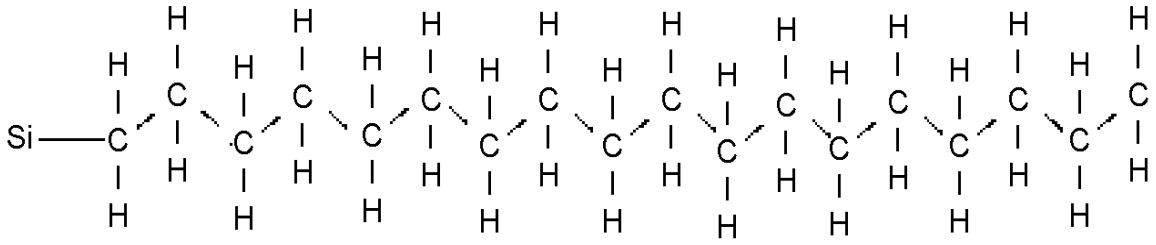
OBJECTIVES:

1. To understand the usefulness of separating mixtures in order to analyze them.
2. To separate the different dyes which make Grape Kool-Aid purple.
3. To compare the use of methanol, ethanol, and isopropyl alcohol as solvents.
4. To compare some properties of molecules you use in the separations.
5. Use predictive analysis to determine an optimum percent mixture of ethanol/ water solutions to achieve separation using a protocol established for methanol and isopropanol solutions.

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.

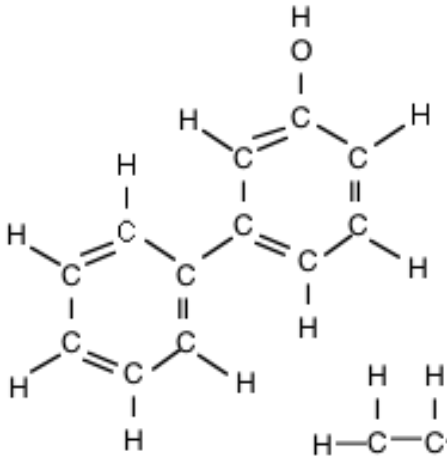
You will gain experience with HPLC in this laboratory exercise. Your hands, eyes, and brain will perform the work of the expensive pumps and other automated equipment, but you will use the same sorts of solvents and adsorbent used by scientific experts performing sophisticated analyses. It is estimated 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.



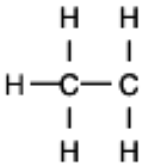
C 18 SEP-PAK CARTRIDGE

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.

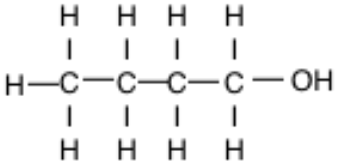
a. Write the ratio of hydroxyl group (-OH) to carbon atom (C) in the space beside each molecule. Generally speaking, the higher the number of hydroxyl groups per carbon atom, the more polar the molecule.



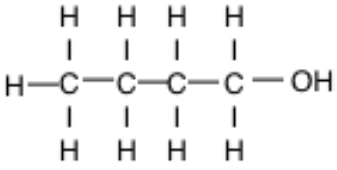
PHENYL PHENOL_____
(LYSOL)



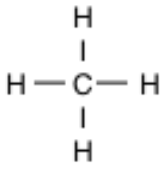
ETHANOL____



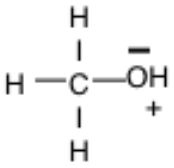
BUTANE____



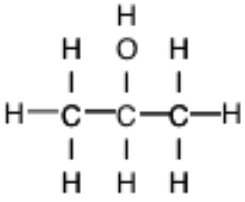
BUTANOL_____



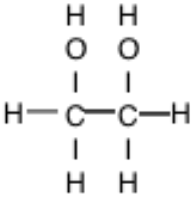
METHANE____



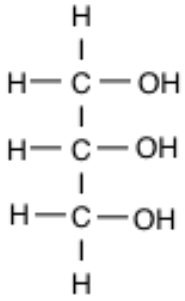
METHANOL____



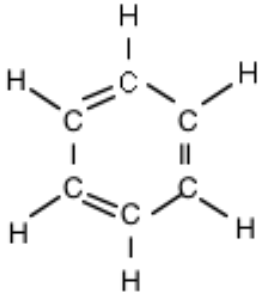
ISOPROPANOL___
(RUBBING ALCOHOL)



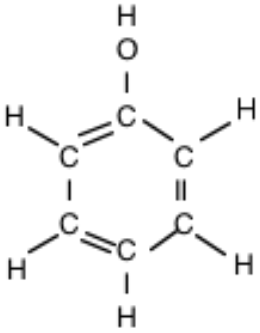
ANTIFREEZE_____
(ETHYLENE GLYCOL)



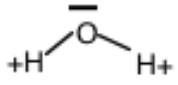
GLYCEROL_____
(USED IN LOTIONS,
COUGH DROPS, AS A
FOOD PRESERVATIVE



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 (in the above worksheet) 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.

II. HPLC

A. Your lab station should be equipped as listed below.

- ☐ Sep-Pak C18 cartridge (1)
- ☐ Methyl alcohol soln's: 100% (30 mls), 60%, (10 mls), 20%, (10 mls) and 5% (10 mls)
- ☐ Isopropyl alcohol solutions (50 ml of 100%)
- ☐ Ethyl alcohol (50 ml 100%, used later, so pick up separately from Prof.)
- ☐ 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 (15 total)
- ☐ Grape drink (Kool-Aid) dissolved in water (10 ml in 15 ml tube).
- ☐ 1.0 ml pipet for loading the Grape Kool-Aid onto the cartridge
- ☐ Test tube holder (Styrofoam rack, hold 15 samples).

B. Procedure: The methanol solutions will be given to you in 100%, 60%, 20% and 5% concentrations. It is your responsibility to make the same concentrations of alcohol using your 50mls of isopropanol or ethanol. Enter your data in the spaces for the appropriate alcohol on these sheets and note that the order of the alcohols for isopropanol and methanol does not mater. Do these before the ethanol (this is the way the lab is laid out, so just follow directions and you will do fine). **CAUTION**: Be very accurate when making your below percent solutions. Since you are making 10 mls of most solutions, use the falcon tubes and appropriate serological pipet to make the solution. For instance, a 5% solution will require 500 ul of alcohol and 9.5 mls of water. Use a micro-pipet for the 500 ul and slowly add water (use squeeze bottle) to bring final volume to 10 mls. The scale on the side of the falcons tube is good, just be careful when reading it. REMEMBER to label all solutions completely (i.e 5% ethanol, not just 5%....). Lastly…when adding solutions to the syringe, take the plunger out, place you finger tip over the end of the Sep-Pak and carefully pour in the 10 mls in. Slowly reinsert the plunger and carefully and very slowly (drop by drop), push on their plunger, making sure the tip of the Sep-Pak n inside the falcon tube with the correct label (the one you just pours the alcohol out of, you will push the fraction into, i.e. 5% alcohol vial gets loaded into the syringe and you will then collect the fraction in the same tube you just poured the alcohol out of *5% alcohol). Please ensure you understand this completely, and do not hesitate to ask!

2. Preparing the solutions for isopropanol:

a. Obtain the 50ml Isopropanol and DI water from your instructor to make your dilutions. (20 mls of 100% and 10 mls each of other dilutions). Use the volume demarcations in the falcon 15 or 50 ml tubes and the transfer pipets to get the volumes you need.0

b.100% solution: separate 20ml of your pure Isopropanol in a suitable container

c. 60% solution:6 mls of Isopropanol + 4 mls of water (10x0.6= 6.0ml)

d. 20% solution: 2 mls of Isopropanol + 8 mls of water (10x0.2= 2ml)

e. 5% solution: 0.5ml of Isopropanol + 9.5ml of water (10x0.05=0.5ml)

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 EXACTLY 1 ml of Grape Kool-Aid solution. 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) _____

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 the other alcohol (so if you just did a isopropanol series, use methanol, or vice versa). Take care to answer the same questions as you go through each step. Which alcohol gave you more ***concentrated***) (intense) dye samples?_____.

Note: For the next two demonstrations, take care to not "flood" the samples. If needed, pour out EQUAL amounts from each tube BEFORE combining, so as to avoid getting too much liquid in the vial when you do the combining exercise as noted.
Final steps:. a. Using **only** tubes from methanol experiment, carefully pour the blue fraction into the red fraction. Observe and describe what happens.

_____

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

_____

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.

QUESTIONS: Note: these questions are designed to help familiarize you with a range of ideas inherent in HPLC. The answer to these questions should NOT be placed in your lab notebook. A KEY for this lab (and the below questions) is available upon request. Feel free to ask the professor, and I will try to help you see what the correct answer is and more importantly, why!! This lab is one of two labs (the other is gel filtration) that will form the basis of your first formal lab write up, so a complete understanding is essential. Other resources for this lab are available and do not hesitate to inquire about ANYTHING regarding this lab that concerns you.

1. a. Draw a table that compares the dye separations using the three alcohols. Your table should succinctly summarize the: percent / type of alcohol that achieved separation of which dye (a similar table should be in your lab notebook FYI..

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 three 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.

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? (i.e 100 % alcohol solution first, 60 %, 20 %, etc..)?

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

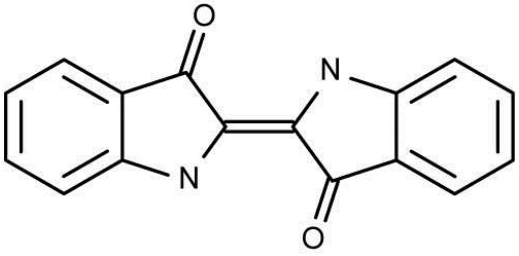
5. When you poured the two fractions together ("final steps on protocol page"), you probably observed a layered effect. What properties of the solutions may cause / contribute to the layering?

6. What do you think would have happened if you had used these mixtures instead:
a. dihyroxymethane $CH_2(OH)_2$ and water?
b. butyl alcohol (C_4H_9OH) and water?

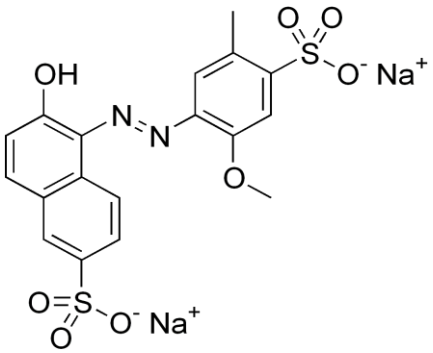
7. If two substances have <u>exactly</u> the same polarity, could they be separated using HPLC? Explain your answer.

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.



Indigo (Dark Blue, blue dye # 1)
$C_{12}H_{10}N_2O_2$
Molecular Weight 262.27g/mol



Allura (Red dye # 40)
$C_{18}H_{14}N_2Na_2O_8S_2$
Molecular Weight 496.42 g/mol

B. Calculate the charge to mass ratio of the dyes above. These are the dyes in in Grape Kool-Aid FYI. Predict their behavior in a HPLC column (which one would emerge first, last?) Using a similar approach to the just completed worksheet, calculate the number of polar bonds (C---O or C----N) to non-polar bonds (C----C, C----H). Please note…there is some ambiguity as determining bond number from the simple line diagrams can be a little tricky. Despite this issue, you should be able to generate a reasonable guess and get some results that make sense. After creating your guess, take a minute to review the lab key to see how accurate your results were!

	# of C---C bonds	**# of C----H bonds**	**# of C----N bonds**	**# of C-----O bonds**
Allura:	_____	_____	_____	_____
Indigo:	_____	_____	_____	_____

DO THIS EXERCISE AFTER YOU HAVE COMPLETED ALL THE EXERCISES IN THE LAB DEALING WITH ISOPORPANOL AND METHANOL.

a. Obtain 50ml of ethanol from the instructor, and looking back at the lab results, predict how ethanol will act as eluent in this lab. Predict what concentrations of ethanol will work in the HPLC column.

Start by drawing the complete chemical structure of all three alcohols below. Next to each drawing, state the ratio of polar to non-polar bonds.

Methanol Isopropanol Ethanol

Now arrange methanol, isopropanol and ethanol in order from most polar to least polar.

What percent concentrations did the other alcohols elute the dyes at?
Isopropanol eluted the red dye @ _____ % The blue dyes @ _____ %

Methanol eluted the red dye @ _____% The blue dye eluted @ _____ %

Now predict what percent solutions will be effective at removing the dyes. Show you predictions to the instructor. Please note..there are many ways to determine the optimum concentration, so be prepared to explain you rational for your numbers….

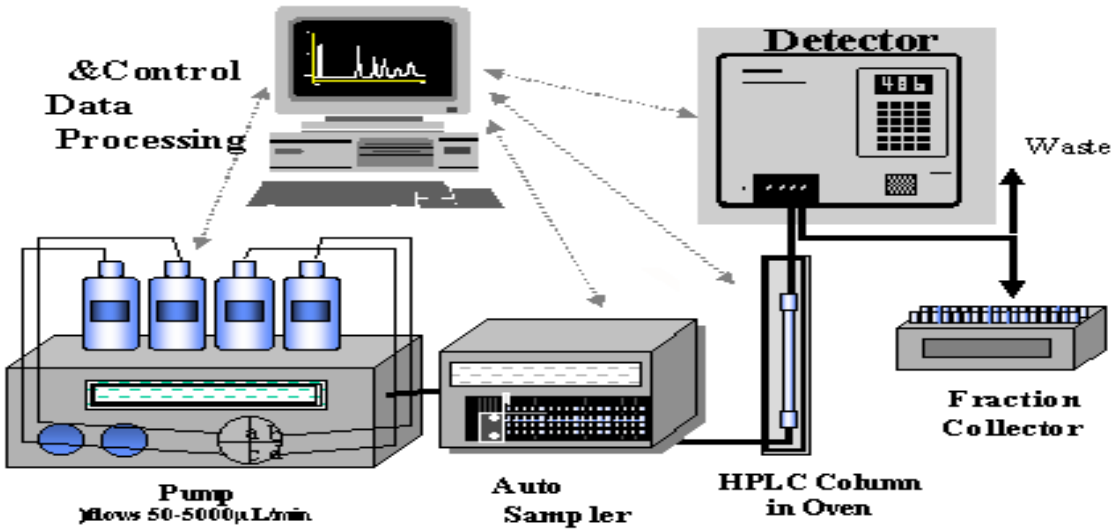
_____ % ethanol should elute the red dye _____ % ethanol should elute the blue dye.

b. Repeat the lab using Ethanol but use the concentrations you predicted would give the best separation. Don't forget to prime the HPLC column with ethanol and then water before starting the experiment. GO in order of the preceding solutions (5, 20, 60 and 100 %).

c. Save and label ALL fractions collected from experiment. Label the % ethanol and bring your vials to the instructor.

d. Your results will be compared to the rest of the class to clarify the scope ideas and approaches to the solution concentration determination, and which numbers gave the best separation.

High Performance Liquid Chromatography (HPLC): Theory verse technique:
Some consider HPLC the use of a column and solvents to achieve separation based on a molecule's polarity. Other associate HPLC with the technology that makes it happen. Who is correct? Well, the truth is, both groups are partially. HPLC involves a very basic theoretical idea, but like many principles in science, has been taken to the extreme through the use of technology. To be an effective scientist, the theory should come first. With this theory comes a deeper understanding and you can then apply your newly acquired theory to a novel experiment and or technology. The experiment you conducted conveys most of the theory and ideas behind HPLC. This said, a modern HPLC like device can run into the millions of dollars and requires months to fully train and or understand. Sadly there is often a real disconnect between theory and applied. Some folks are very comfortable with the underlying theory, but stop short at mastering the complexities of the device. Others know their device, but do not truly understand the underlying theory. To give you a better appreciation of the idea, take a look at the following example of a modern HPLC. **Complete the exercise as best you can and on the next page are some answers for you to ponder.**



A. Compare the HPLC setup above to the equipment in your lab. Which are similar, and what is different? Predict the function of each equipment depicted above.
1) Describe the function and relate the these pieces of HPLC equipment to the ones used in lab:
The pump:

The HPLC column:

The auto sampler:

Fraction collector:

Detector:

Data Processor:

ANSWERS to question on preceding page.

1) Describe the function and relate the these pieces of HPLC equipment to the ones used in lab:
The pump: The pressure you applied with your hand is the pump. Often very expensive systoltic pumps are used. These pumps very gently apply constant pressure by squeezing tubing. This type for pump helps avoid damage to the reagents (i.e. a propeller based pump, called an impellor, can damage cells, lager proteins, etc.) and does not require hooking up of tubes to the pump, vial, etc
The HPLC column: The 2 cm long "Sep Pak can be replaced with short 10-30 cm columns (designed for "gross separation" of things that are quite different. If some of the chemical component are very similar, the process may involve a 50 or 75 meter long column to achieve separation!
The auto sampler: This device allows for sampling of samples that have been placed in small vials or jars. As you might imagine, numerous samples are often run with once machine and automating the placement of these samples is a good thing. You acted as an auto-sampler when you collected and added 1 ml of Grape Kool-Aid to the column.
Fraction Collector: The fraction collector was vou, placing vials in front of the Sep Pak and collect the vials of solvents as they came out of the device. In a real HPLC set up, the fractions collected may be a few milliliters and number of the 10,000's.
Detector: Your eyes were adequate to see the fractions collected. Many reagents can be detected using colorimetric tests (adding Biuret's reagent to a protein for example) or more elaborate tests like mass spectroscopy or nuclear magnetic resonance (NMR). Sometime the HPLC device is a separate gizmo, other times, it part of a "all in one" device that separate, detects and identifies novel substances within a few minutes of starting testing.
Data Processor: Crunching the numbers, etc…was your responsibility in lab. When you calculated the % concentration for the ethanol series, this calculations is the start of what could be a very analytic and statistically based assessment of the data.
Take a minute to reflect on this idea. Many aspects of the technology in science are noting more that putting a bunch of "bells and whistles" on a very simple process. These added technology simply allow for more precise control of what is often at its heart, a relatively simple process.

References: This lab has been prepared by Dave Bowlus as part of California Lutheran Universities' Science Outreach Program and further modified and expanded by Jim Wolf at College of the Canyons.