

CHEM105: BIOCHEMISTRY AND SOCIETY

Experiment 2

Separation of Mixtures: Dyes and Vulcan Blood

Introduction

Understanding matter is difficult since most matter exists as complex mixtures. To really understand mixtures, you have to separate the components from each other and study them independently. This could be as simple as separating the salt from salt water by boiling off the water, or as complex as separating one of about 23,000 different proteins present in the human body. That's made even more complicated since proteins are present in cells with lipids, carbohydrates and nucleic acids.

The molecular components of matter can be separated into individual components since the molecules of a given pure substance have different **macroscopic** physical properties (such as melting point, boiling point, solubility, etc) than molecules of a different substance. Pure substances have different macroscopic physical properties since the molecules of given substance have **nanoscopic** properties. Molecules differ in the numbers and connections between atoms, giving rise to different molecular shapes, sizes, and distribution of charges (+, -, $\delta+$, $\delta-$). These nanoscopic differences cause the molecules to dissolve in certain solvents, stick to certain surfaces, evaporate at different temperatures, freeze at different temperatures, etc. In this lab you will perform two different separations. Your first task will be to separate the dye molecules in water soluble transparency pens. The second will be to separate some biological molecules in "Vulcan" blood, which contains:

- blue dextran, a polysaccharide (complex carbohydrate) which has been chemically modified with a blue dye.
- hemoglobin, a red-brown protein molecule from red blood cells that carries molecular oxygen (O_2)
- DNP-alanine, an amino acid which has been chemically modified with a yellow dye.

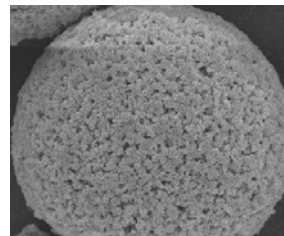
You will use a technique called chromatography to separate the components of a solution (a homogenous mixture). In this technique, the solution is applied to a solid phase through which a solvent (mobile phase) flows. The solid phase can be a paper (2D), or a column of beads (3D). A small amount of a mixture to be separated is spotted on dry paper (paper chromatography) or placed on top of a column (column chromatography) contained beads with solvent flowing through them. In the case of the paper, the spotted paper is placed in a chamber with a small amount of solvent (the mobile phase), which wicks up the paper. When it meets the spot containing the mixture, the individual components, depending on their nanoscopic properties, will either stay bound to the paper or move into the rising solvent, leading to the separation of the components of the mixture. Separation is based on a nanoscopic property of the components, namely the distribution of charges (+, -, $\delta+$, $\delta-$), which

will cause the components to either stick to the paper or “jump” into the mobile solvent and rise with it. In the second experiment, the molecules in the mixture will be separated on the basis of size, as larger molecules flowing through the column are excluded from the microscopic holes and canyons of the beads, while smaller molecules aren't.

Molecular Size and Gel Filtration Chromatography

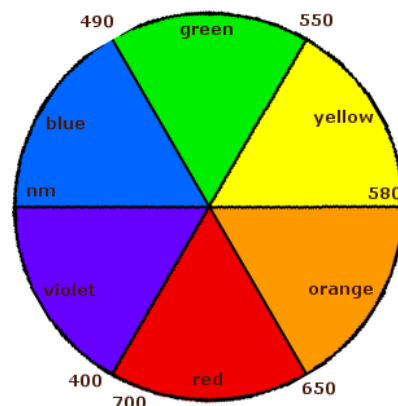
The size of a molecule is closely related to the **molecular weight** of the molecule. The molecular weight for any molecule can be calculated by summing the atomic weight for each atom in the molecule. For instance, H₂O has a molecular weight of 18, 16 from the oxygen and 1 each from each hydrogen. Biomolecules such as proteins and nucleic acids can have very high molecular weights. The Z protein found in skeletal muscle, for example, has a molecular weight in the millions! The three biomolecules used in this lab have very different molecular weights, and hence sizes. These molecules can be separated based on their different sizes using a technique called gel filtration chromatography.

A mixture of the three biomolecules which are all dissolved in a simple salt solution, will be separated, based on their size and hence molecular weight, on a plastic column filled with a slurry of beads that have been swollen in a salt solution. Each bead, as shown to the right, is small (about 150 micrometers in diameter) and has many cavities of similar sizes, likewise filled with the same salt solution. When a mixture of molecules of different molecular weights pass through the column, the size of the molecules will determine whether they enter the solution-filled cavities in the beads. This property of the beads allows the column to separate a mixture of molecules of sufficiently different size. The cavity size in the beads is controlled during the manufacturing process. By answering the pre-lab questions, you should be able to explain how the column separates a mixture of molecules of different molecular weight.



Molecular Structure and the Interaction with Light

Each of the biological molecules used in the “Vulcan blood” appear colored when dissolved in a salt solution. It is not that the molecules themselves have color, but rather they each have a unique structure which absorbs certain wavelengths of visible light from the visible spectrum, which imparts a color to the solution. Not every solution of a molecule dissolved in water has a color. Sunlight and light from incandescent bulbs are examples of white light, a mixture of all of the colors of the spectrum. Each color is a range of light wavelengths as indicated by the color wheel depicted to the right.



Consider a liquid which appears green. The color on the opposite side of the color wheel, namely red, is absorbed by the liquid and the green wavelengths pass through the liquid. Thus red and green are known as complementary colors. The green solution absorbs wavelengths over the range of approximately 650-750 nanometers. Many biological molecules, such as those used in this lab, as well as commercial products like foodstuffs, clothes, ink and paints contain dyes which selectively absorb light of a particular wavelength or range of wavelengths to cause the materials to appear the complementary color. Many of these commercial products contain mixtures of dyes to produce the desired color. Ultraviolet-visible or UV-Vis spectrophotometry is used to analyze the light absorption characteristics of colored solution.

The UV-Vis spectrophotometer will produce a graph known as a spectrum showing the absorption intensity of the y-axis versus the wavelength on the x axis. A hill or peak in the graph is a wavelength region of greater absorption. Don't worry about the humps or valleys below 400 nm as they are in the ultraviolet region of the electromagnetic spectrum.

Procedures

A. Separation of Water Soluble Dyes in Transparency Pens: Paper Chromatography

Spot a red, yellow, and orange water soluble transparency pen about one inch from the bottom of the filter paper and inch from each other. Let it dry. Next place the paper into a container with about a ¼ inch of water in the bottom. Let the water rise by capillary action, carrying the dyes with it. Let it run until the water nears the top of the paper. Remove the paper and dry it.

B. Gel Filtration Chromatography

1. Each group in the lab will be given a gel filtration column clamped to a column and fitted with a stopcock and a piece of tubing. The column has been washed in a solution of sodium chloride
2. Remove the top liquid from the column with a plastic pipette. Open the stopcock as described in lab and elute the column just until the top level of liquid in the column reaches the top bed of the gel. Shut the stopcock off to prevent further flow.
3. With the plastic pipette, carefully add the mixture of the three substances, to the column. Take care to disrupt the top bed of the gel beads as little as possible.
4. Open the column and start eluting the mixture through the column. Just as soon as the mixture completely enters the column, shut off the column. **Carefully**, add a small amount of the sodium chloride solution to the column, open the column, and wash the mixture into the column. This step is very important if you wish the mixture to be separated. Keep adding small volumes of sodium chloride solution until the mixture is clearly into the column.
5. Carefully fill the column with sodium chloride solution and elute the mixture through the column.

6. Collect in three different clean tubes the “purist” sample of each of the three substances eluting from the column.
7. Make sure to draw a sketch of the column in your notebook at various times during the gel filtration experiment, documenting the progress of the separation.

C. UV-Vis Spectrophotometry

Blank Measurement

1. Place the cuvette (a sample holder for the spectrophotometer) containing pure water in the cuvette holder of the spectrophotometer.
2. Pull down the **Measure** menu and select **Blank**. Wait until the spectrum of the blank appears.

Sample Measurements

3. Replace the cuvette containing water with one contain blue dextran. Pull down the **Measure** menu and select **Sample**. Wait until the spectrum of the sample appears.
4. Repeat step 3 with the cuvette containing hemoglobin. The spectrum for this sample will be overlain onto the spectrum for blue dextran.
5. Repeat step 3 with one of the purified samples from your gel filtration column.
6. Pull down the **File** menu and select **Print Reports**. Select **Results**. Be sure to label the plot after it prints out if the necessary information is lacking on the output.
7. When you are finished and all of the spectra are printed, pull down the **Edit** menu and select **Clear**. All of your data will disappear.

MATERIAL SAFETY DATA FOR EXPERIMENT 1

The solutions of DNP-alanine and blue dextran are considered irritants and should not be allowed to contact your skin for prolonged times.

The hemoglobin solution is from a bovine sources, and is biologically safe.

The sodium chloride solution is non-toxic.

DISPOSAL

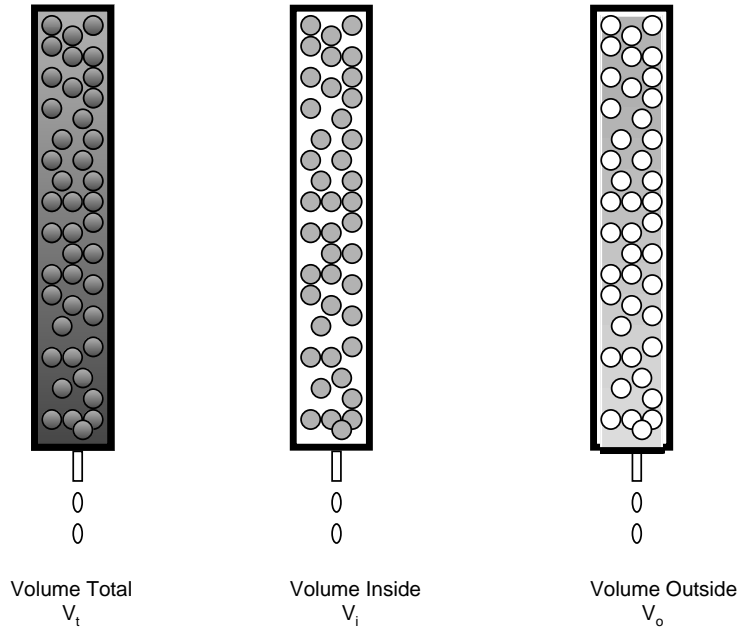
Place waste containing blue dextran and DNP-alanine in the labeled waste container in the hood.

PRE-LABORATORY QUESTIONS

1. A gel filtration column separates molecules by size. Imagine a column filled with these beads, as shown below. Three different volumes, shown in the shaded regions below, can be defined for the column.

In reality, much of the volume of the beads consists of the solution-filled pores. V_t is the total volume of the packed beads in the column, V_i is the internal volume of the beads, and V_o is the volume of solution surrounding the beads. In general V_o is about 35-40% of V_t . Answer the following questions before you come to the lab. In the lab, you will review your answers in small groups before you turn in your responses to the T.A..

GEL FILTRATION CHROMATOGRAPHY: DEFINITION OF VOLUMES



a. Derive a mathematical formula which describes the relationship among V_t , V_i , and V_o .

b. A gel filtration column is packed from a slurry of the beads in a solution. The column is continually washed with the solution which is added on top of the packed beads, and eluted at the bottom of the column. A mixture of three different kinds of molecules, X, Y, and Z, where the molecular weight of X is much greater than Y and the molecular weight of Y is much greater than Z, is placed on top of the packed column. X is of sufficient size that it can not enter the pores in the beads. Z is sufficiently small that it can fit into all of the volume within the beads. Y is intermediate in its ability to fit into the pores. The mixture is allowed to flow into the column, and then washed through and eluted with more solution. Pretend you are molecules X, Y, and Z. Use the diagram of the columns above to visualize the paths of molecules X and molecules Z as they flow through the column. Using these diagrams, predict the order of elution of X, Y, and Z from the column. Explain how you arrived at your prediction.

2. UV-Vis Spectrophotometry

Molecule	Color in Solution	Expect wavelength absorbed
DNP-alanine	yellow	
hemoglobin	red-orange	
blue dextran	blue	

Names _____

LABORATORY REPORT

Experiment 2: Separation of Mixtures: Dyes and Vulcan Blood

A. Hand in your dried paper chromatography paper showing the separation of the red, orange, and yellow dyes. Which of the dye components was least soluble in water and most attracted to the solid paper phase?

B. Gel Filtration Chromatography

1. Draw a sketch in the right margin of the gel filtration column showing the separation of the biomolecules just before the first started to elute from the column.
2. From the experiment, what conclusions can you draw about the relative molecular weights of each of the three biomolecules that you were given?
3. What if you were given a sample of hemoglobin dissolved in a solution of sodium chloride, or common table salt. You wish to remove the sodium chloride from the hemoglobin and replace it with potassium chloride. Both salts have molecular weights much less than hemoglobin. Explain how you could use gel filtration chromatography to accomplish this.
4. You have a mixture of two different proteins, cytochrome C (present in mitochondria of all cells) and lactalbumin, (a protein found in milk). The proteins have approximately the same molecular weight, but different charges. One is positively charged, the other negatively charged. Obviously, gel filtration chromatography could not be used to separate these protein, but another column chromatography method can. Suggest how chromatography beads could be made which when used in a column could be used to separate these molecules.

C. UV-Vis Spectrophotometry

1. Hand in the overlapping spectrum of the three separate biomolecules, and one of the samples you purified on your gel filtration column. Identify each on the graph.
2. From the overlapping spectra, identify the sample you purified on the column. From the spectra, speculate as to how pure the sample is. How could you have increased its purity?
3. Chlorophyll is a plant pigment which imparts a green color to leaves. In what range of wavelengths would chlorophyll absorb visible light? _____ nm
4. The spectra of various photosynthetic pigments are shown below. What color would an aqueous (water) solution of phycocyanin appear?
_____nm

