Student Manual

Size Exclusion Chromatography

Lesson 1A Introduction to Chromatography

Chromatography is commonly used in biotechnology for purifying biological molecules, like proteins, for medicine or other uses. Chromatography allows the separation of individual components from complex mixtures. Chromatography consists of a mobile phase (solvent and the molecules to be separated) and a stationary phase either paper (in paper chromatography), or glass beads, called resin (in column chromatography), through which the mobile phase travels. Molecules travel through the stationary phase at different rates because of their chemistry.

Some Common Types of Chromatography

In **gel filtration chromatography**, commonly referred to as **size exclusion chromatography (SEC)**, microscopic beads which contain tiny holes are packed into a column. When a mixture of molecules is dissolved in a liquid and then applied to a chromatography column that contains porous beads, large molecules pass quickly around the beads, whereas smaller molecules enter the tiny holes in the beads and pass through the column more slowly. Depending on the molecules, proteins may be separated, based on their size alone, and fractions containing the isolated proteins can be collected.

In **affinity chromatography**, a biomolecule (often an antibody) that will bind to the protein to be purified is attached to the beads. A mixture of proteins is added to the column and everything passes through except the protein of interest, which binds to the antibody and is retained on the solid support. To get the protein to elute from the column, another buffer is used to disrupt the bond between the protein of interest and the antibody. Often this elution buffer contains high concentrations of salt or acid.

In **ion exchange chromatography**, the glass beads of the column have a charge on them (either + or -). A mixture of protein is added to the column and everything passes through except the protein of interest. This is because the beads are picked to have the opposite charge of the protein of interest. If the charge of the beads is positive, it will bind negatively charged molecules. This technique is called **anion exchange**. If the beads are negatively charged, they bind positively charged molecules (**cation exchange**). Thus, a scientist picks the resin to be used based on the properties of the protein of interest. During the chromatography, the protein binds to the oppositely charged beads. When the contaminant is separated from the protein of interest, a high salt buffer is used to get the desired protein to elute from the column.

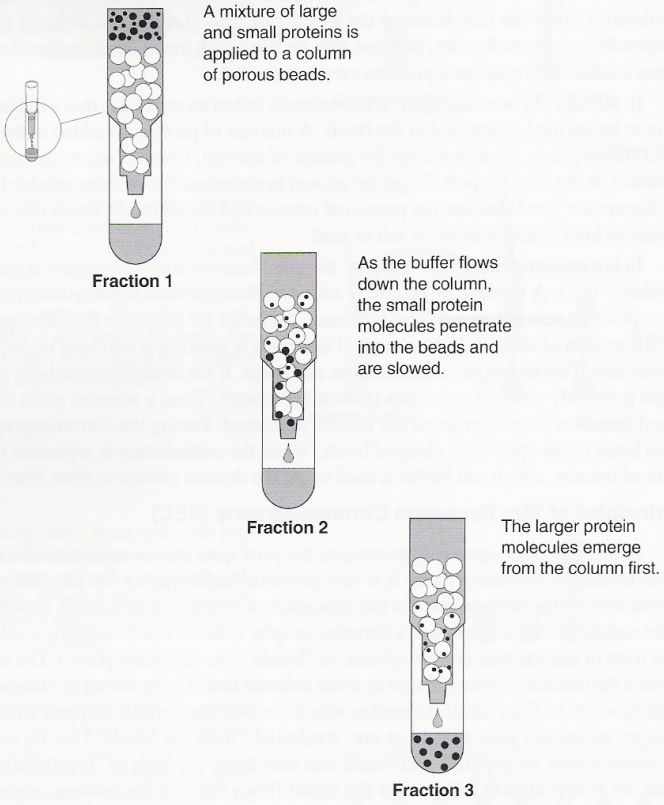
Principles of Size Exclusion Chromatography (SEC)

In this laboratory you will investigate the principles of size exclusion chromatography. Size exclusion chromatography is a very powerful technique for the physical separation of molecules on the basis of size. In this procedure, a mixture of molecules dissolved in liquid (the mobile phase) is applied to a chromatography column which contains a solid support in the form of microscopic porous spheres, or "**beads**" (the stationary phase). The mass of beads within the column is often referred to as the **column bed**. The beads act as "**traps**" or "**sieves**" and function to filter small molecules which become temporarily trapped within the pores. Larger molecules pass around or are "**excluded**" from the beads. This kit contains eight columns which are prefilled with beads that effectively separate or "**fractionate**" molecules that are below 60,000 daltons. As the liquid flows through the column, molecules below 60,000 daltons enter the beads and pass through the column more slowly. The smaller the molecule, the slower they move through the column. Molecules greater than 60,000 pass

around the beads and are excluded from the column—also referred to as the **exclusion limit** of a column.

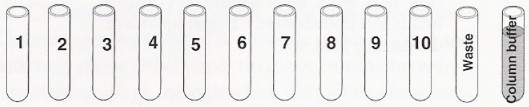
The liquid used to dissolve the biomolecules to make the mobile phase is called a **buffer**. The mixture of biomolecules dissolved in the buffer is called the **sample**. The sample is placed on the column bed and the biomolecules within the buffer enter the top of the column bed, filter through and around the porous beads, and ultimately pass through a small opening at the bottom of the column. For this process to be completed, additional buffer is placed on the column bed after the sample has entered the bed. The mobile phase (liquid) is collected as drops into a series of collection tubes. A set number of drops is collected into each tube. The larger molecules which pass quickly through the column will end up in the early tubes or "**fractions**". The smaller molecules which penetrate the pores of the stationary phase end up in the later fractions.

Hemoglobin and vitamin B12 are the two biomolecules in your sample. Hemoglobin, which is brown, has a molecular weight of 65,000 daltons. Vitamin B12 is pink and has a molecular weight of 1,350 daltons. The schematic below illustrates the differential fractionation of large and small molecules on a size exclusion column.

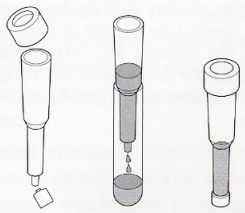


Laboratory Quick Guide
Size Exclusion Chromatography Kit

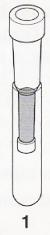
1. Obtain 12 collection tubes and label ten sequentially from 1 to 10. Label the tubes with your name and laboratory period. Label the final two tubes "Waste" and "Column Buffer". Using a clean pipette, transfer 4 ml of column buffer into the tube labeled "Column Buffer".



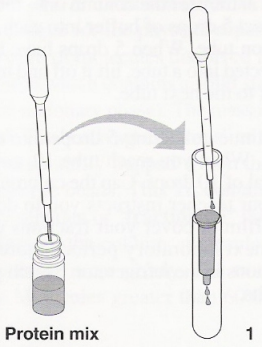
2. Remove the cap and snap off the end of the sizing column. Allow all of the buffer to drain into the waste tube. Observe the upper surface of the matrix and insure that all of the buffer has entered the column. Looking directly over and into the column, you should see the "grainy" appearance of the column matrix. Cap the bottom of the column.



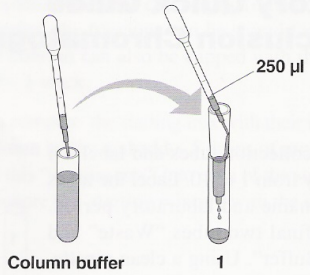
3. Carefully place the column onto tube 1. You are now ready to load (or the teacher may load) the protein sample onto the column.



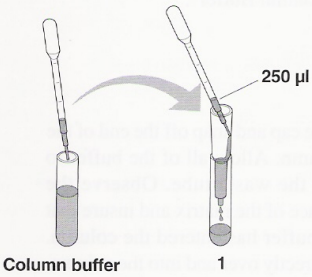
4. When you are ready to load the protein mix, uncap the column. It is important to uncap the column only when you are ready to load your protein—you do not want your column to run dry. Using a pipette, add one drop of protein mix onto the top of the column bed (your teacher may do the loading for you). The pipette should be inserted into the column and the drop should be loaded just above the top of the column so that it minimally disturbs the column bed.



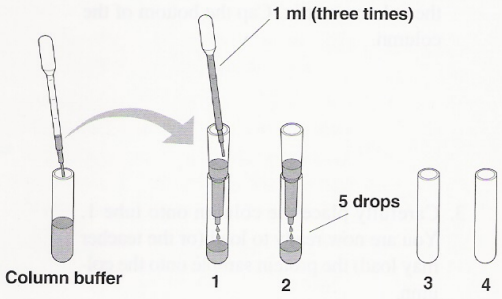
5. As soon as the drop of protein mix enters the column bed, carefully add 250 µl of column buffer to the top of the column. This is best done by inserting the pipette tip into the column so that it rests just above surface of the column matrix. Carefully let the buffer run down the side of the tube and onto the top of the bed. (Note: The size separation will work best when the column bed is left undisturbed). Begin to collect drops into tube 1.



6. Add another 250 µl of column buffer to the top of the column. Add the buffer as before, by placing the pipette just above the top of the column and letting the buffer run down the side of the tube. Continue to collect drops into tube 1.



7. Add 3 ml of column buffer to the top of the column matrix. This can be done by adding 1 ml three times from the pipette. At this time the protein mix has entered the column far enough so that slight disturbances to the column bed will not affect the separation. Transfer the column to tube 2 and begin to count the drops that enter into each tube. Collect 5 drops of buffer into tube 2.



8. When 5 drops have been collected into tube 2, transfer the column onto tube 3. Collect 5 drops of buffer into each collection tube. When 5 drops have been collected into a tube, lift it off and transfer it to the next tube.

9. Continue collecting 5 drops into each tube. When you reach tube 10, collect a total of 10 drops. Cap the column and if your teacher instructs you to do so, parafilm or cover your fractions until the next laboratory period. Store the fractions in the refrigerator. Sketch your results.