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Edvo-Kit #

**108**

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## **Principles of Gel Filtration Chromatography**

### **Experiment Objective:**

The objective of this experiment is to introduce the principles of gel filtration chromatography as a method that separates molecules according to their size and shape. A mixture of two molecules are separated in this experiment.

**See page 3 for storage instructions.**

# Table of Contents

	Page
Experiment Components	3
Experiment Requirements	3
Background Information	4
Experiment Procedures	6
Study Questions	8
Instructor's Guidelines	
Pre-Lab Preparations	9
Expected Results	10
Study Questions and Answers	10

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## Experiment Components

Store all components below at room temperature.

Component	Check (✓)
A Sample Mixture	<input type="checkbox"/>
B Dry Matrix	<input type="checkbox"/>
C Concentrated Elution Buffer	<input type="checkbox"/>
• Plastic Transfer Pipets	<input type="checkbox"/>
• Microcentrifuge tubes	<input type="checkbox"/>
• Chromatography Columns	<input type="checkbox"/>

Enough reagents and sample are provided for 10 separations.

All components are intended for educational research only. They are not to be used for diagnostic or drug purposes, nor administered to or consumed by humans or animals.

## Requirements *(NOT included in this experiment)*

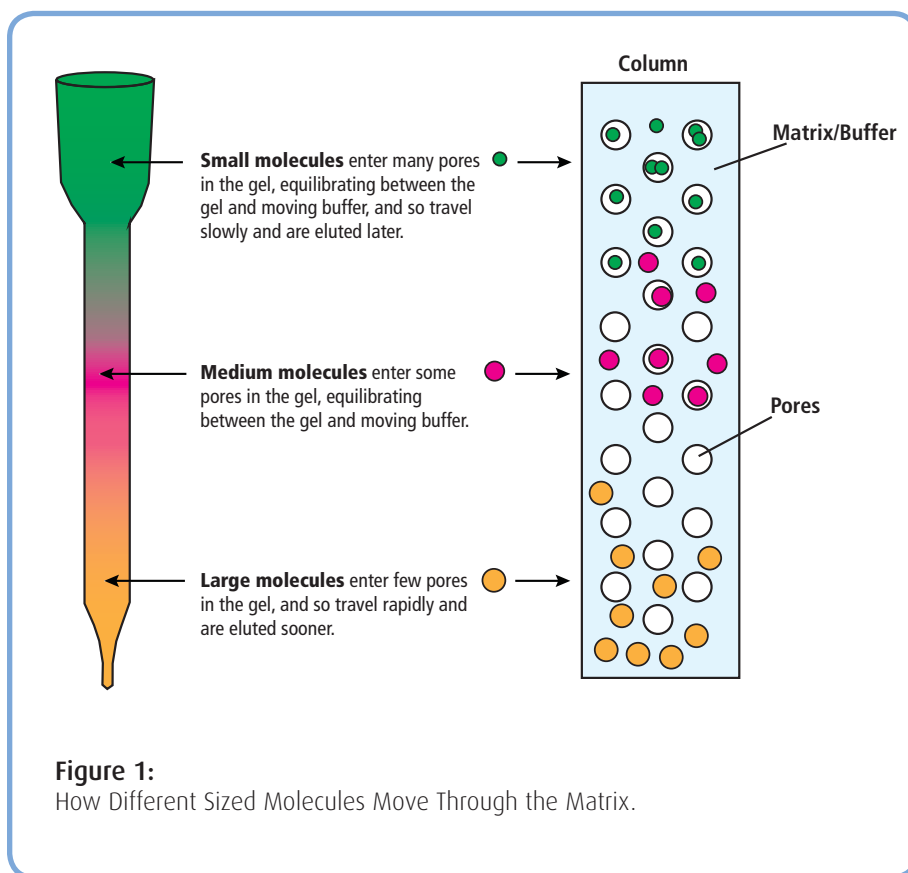
- Ten 50 ml or 100 ml beakers or flasks
- 1 small beaker or flask (10 or 25 ml) or a 10 ml test tube
- 1 ring stand with clamp for each column
- Distilled or deionized water  
(Distilled water available in supermarkets is of suitable quality)
- 5 or 10 ml pipets and pipet pumps (optional)

## Background Information

Gel filtration chromatography (sometimes referred to as molecular sieve chromatography) is a method that separates molecules according to their size and shape. The separation of the components in the sample mixture, with some exceptions, correlates with their molecular weights. In these cases, gel filtration can be used as an analytical method to determine the molecular weight of an uncharacterized molecule. Gel filtration is also an important preparative technique since it is often a chromatographic step in the purification of proteins, polysaccharides and nucleic acids.

The basic components of the gel filtration experiment are the matrix, chromatography column and the elution buffer. The matrix is the material in the column that is actually the separation medium. It is the stationary phase of the chromatography.

The column is a tube with a frit and elution spout fitted at the bottom. The frit is a membrane or porous disk that supports and retains the matrix in the column but allows water and dissolved solutes to pass. The elution buffer is the mobile phase of the chromatography and flows through the matrix and out of the column. The column, with the matrix and applied sample, is "developed" by the elution buffer. This means that the molecules in the sample are carried by the flow of buffer into the matrix where they are gradually separated. The separated zones of molecules then flow out of the column where they are collected for analysis.



Filling the chromatography column with matrix is referred to as "packing". The packed matrix is called the "bed" and the volume it occupies is termed the "bed volume". It is very important not to allow the bed to run dry. Otherwise, cracks and fissures develop and the matrix has to be removed and repacked.

The gel filtration matrix consists of microscopic beads that contain pores and internal channels. The larger the molecule, the more difficult it is for it to pass through the pores and penetrate the beads. Larger molecules tend to flow around and in between the beads. The total volume of buffer between the beads is the "void volume". Smaller molecules tend to spend more time in the maze of channels and pores in the bed. Consequently, the larger, higher molecular weight molecules are eluted from the column before smaller molecules. Larger molecules take the faster, more direct

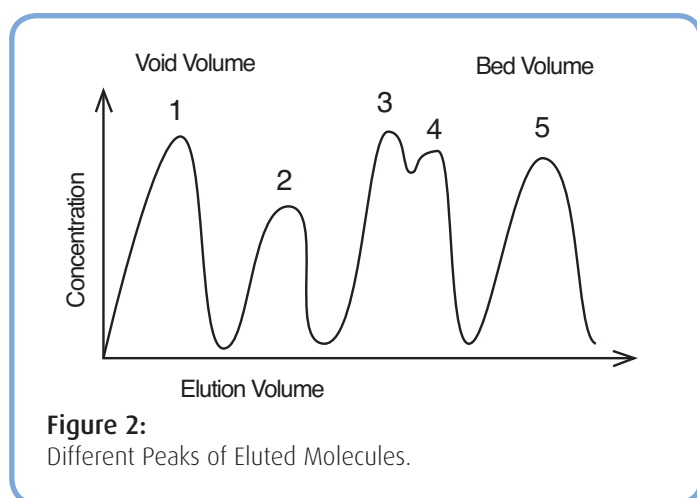
## Background Information, continued

path that involves less time in the beads (Figure 1). This is somewhat analogous to finding your way out of a complicated maze or simply walking around the outside of the maze and avoiding the whole situation entirely.

Molecules can have the same molecular weight but radically different shapes. Molecules with a more compact shape, such as a sphere, will penetrate the beads more easily than those having an elongated shape, like a rod. Therefore, a rod-like molecule will elute before a spherical one of the same molecular weight.

There are many different types of gel filtration matrices. The spectrum of molecular weights the matrix is capable of separating is called the fractionation range. For example, consider a matrix that has a fractionation range (in molecular weight) of 1000 to 100,000 daltons. Molecules with an average molecular weight of 1000 or less will not be separated from each other since they all penetrate the beads completely and with equal efficiency. These molecules take the maximum volume of buffer for elution, which is equal to one bed volume.

The bed volume is equal to the volume of the beads plus the void volume. Molecules in the range of 1000 to 100,000 daltons will enter the beads with varying efficiencies and be partially or completely separated from one another. Molecules greater than 100,000 daltons will not enter the beads and be eluted in the void volume. Note that in this example, any number of different molecules having molecular weights of 100,000 daltons or greater will all elute at the same time since they are not sieved by the matrix. The partially or completely separated zones of molecules that are eluted from the column are called peaks. A peak consists of an increasing and decreasing concentration gradient of molecules (Figure 2).

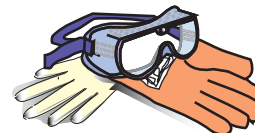


This experiment includes columns which will be packed with the appropriate matrix for separation of the sample mixture. The sample in this experiment contains a mixture of orange and blue molecules. The orange dye has a molecular weight of 452. The blue is a polymer of glucose with an average molecular weight of 2,000,000 daltons, and has a rod-like shape. The fractionation range of the matrix is 4,000 to 150,000 daltons. The smaller orange dye enters the pores of the beads and slowly works through them while the very large blue glucose polymer cannot enter the bead pores and must travel around them, moving much faster.

## Experiment Procedures

### EXPERIMENT OBJECTIVE:

The objective of this experiment is to introduce the principles of gel filtration chromatography as a method that separates molecules according to their size and shape. A mixture of two different molecules will be separated in this experiment.



### LABORATORY SAFETY

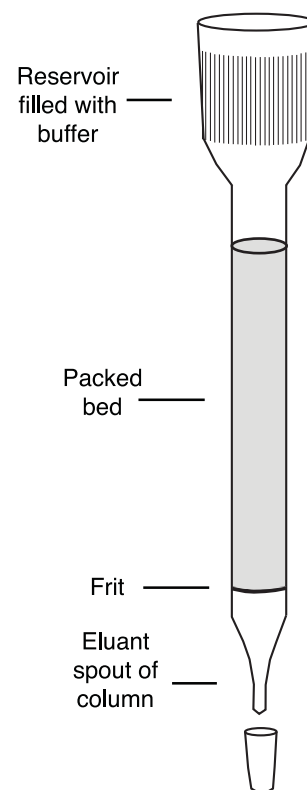
Gloves and safety goggles should be worn as good laboratory practice.

### CHROMATOGRAPHY: PACKING THE COLUMN

1. Mix the matrix thoroughly by swirling or gently stirring.
2. With a 5 or 10 ml pipet, carefully pipet all of the mixed slurry into the column by letting it stream down the inside walls of the reservoir or pour the slurry into the column.

If the flow of matrix is stopped by an air pocket, stop pouring and firmly tap the column until the air is removed and the slurry flows down. Continue pouring the rest of the slurry.

3. With a transfer pipet, add elution buffer to fill the reservoir.
4. Place an empty beaker under the column.
5. Remove the cap from the spout of the column.
6. Let the buffer flow through the column for approximately 10 minutes. The matrix will pack down into the column.
7. Place the cap onto the spout of the column.
8. The matrix is packed when it stops compressing.



### OPTIONAL STOPPING POINT

If time is limited, the experiment can be temporarily stopped here. **The columns must be securely closed so they do not run dry.** If you are stopping the experiment here, make sure there is buffer above the bed and the reservoir is covered with plastic wrap or parafilm.



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## Experimental Procedures, continued

### CHROMATOGRAPHY: FRACTION COLLECTION

1. Label 8 test tubes 1-8. Put your initials or lab group number on all the tubes.
2. Carefully remove all the buffer from above the bed with a transfer pipet. The top of the bed should be exposed to air.  
  
Insert a pipet through the reservoir. Try to minimize disturbance of the bed while removing buffer.
3. Load the contents of the "Sample" tube (x) onto the top of the bed with a transfer pipet. Let the sample drip down the inside walls of the column.
4. Place a beaker under the column.
5. Remove the cap from the spout. The sample will slowly enter the bed. When it has **completely** entered the bed (the top of the bed will be exposed to air), replace the cap.
6. **Carefully** add several drops of buffer over the bed with a transfer pipet. Open the cap and allow the buffer to enter the column.
7. Continue adding buffer, several drops at a time, pausing to allow the buffer to enter the bed.
8. When the blue dye reaches near the bottom of the column, begin to collect 0.5 ml fractions. Hold tube #1 directly under the column. (The tubes are graduated to help you measure 0.5 ml.)
9. As the dyes gradually separate in the column, periodically add fresh buffer to the reservoir to keep it full.
10. Continue collecting 0.5 ml fractions in each of the 2-8 tubes.
11. After all tubes of column effluent (column fractions) have been collected, replace the cap onto the spout.
12. Identify the tube with the greatest amount of blue dextran that eluted from the column.
13. Identify the tube that has the greatest amount of orange dye that eluted from the column.

## Study Questions

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Answer the following study questions in your laboratory notebook or on a separate worksheet.

1. Compare gel filtration chromatography to other techniques of separation.
2. Draw and label the parts of a column. Include the amounts of buffer which is placed above/below the bed. Define: frit, matrix, and eluant.
3. What is the purpose of an elution buffer.
4. Which would elute from a column first: a spherical or a linear molecule? Why?
5. What is the stationary phase of the chromatography? The mobile phase?





# Instructor's Guide

## Pre-Lab Preparations

### A. PREPARATION OF 10 COLUMNS

- Vertically mount each column on a ring stand, making sure they are straight. Keep both the top and bottom caps on the column.

### B. PREPARATION OF ELUTION BUFFER

1. Dilute the Concentrated Elution Buffer by adding the following:
  - Concentrated Elution Buffer (entire contents of Component C)
  - 540 ml of distilled water
  - Mix well.
2. Dispense approximately 50 ml of the diluted elution buffer for each separation in a beaker. Keep the extra buffer on hand in case of spills for additional sample application.
3. Pipet 0.2 ml of the Sample Mixture (Component A) into a microtest tube. Label this tube "X". (There is enough sample to divide into ten tubes, each containing 0.2 ml).

Assemble the following:

- 8 Microcentrifuge tubes
- 2 Plastic transfer pipets
- An empty beaker

### C. PREPARATION OF DRY MATRIX

1. Add the Dry Matrix (Component B) to a flask or beaker. Add 52 ml diluted elution buffer.
2. Cover the tube or beaker and swirl to mix the matrix.
3. Allow the matrix to swell for at least 2 hours at room temperature before dispensing (can also be done the day before the lab).
4. After the matrix has swollen, stir or swirl to make a uniform suspension and quickly dispense 5 ml into a small beaker, flask, or tube for each group.

## Expected Results

The tubes containing the greatest amounts of each of the separated components of the sample mixture represent the chromatographic peaks. Blue Dextran, the larger molecule, elutes first. Since the Orange G is a small molecule, it will tend to diffuse more than the dextran during the chromatography. Diffusion causes broadening of the Orange G zone in the column. This causes the Orange G peak to be wider, and consequently, the dye elutes in a larger volume (more fractions).

## Study Questions and Answers

1. Three separation techniques are summarized in the table below.

	GEL FILTRATION CHROMATOGRAPHY	GEL ELECTROPHORESIS	PAPER/THIN LAYER CHROMATOGRAPHY
BASIS OF SEPARATION	Size and Shape	Size, Shape, Net Charge	Polarity, Charge, Solubility
STATIONARY PHASE	Matrix	Gel	Adsorbent
MOLECULAR MOBILITY BY:	Buffer flow	Electrical current	Solvent Diffusion

2. Refer to the figure in the Experimental Procedure instructions.

frit: porous disk which supports and retains the matrix in the column.

matrix: separation medium

eluant: the liquid that flows out of the column

- As the mobile phase, the elution buffer transports the molecules to be separated through the matrix.
- A linear molecule would elute from the column first. The molecule can not enter the beads as readily and passes around the matrix beads.
- The stationary phase is the matrix and the mobile phase is the elution buffer.