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EDVO-Kit #

243

Ion Exchange Chromatography

Storage:
Store entire experiment at room temperature.

EXPERIMENT OBJECTIVES:

The objective of this experiment is for students to learn the principles of ion exchange chromatography by separating two charged molecules using a two-step salt gradient.

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Table of Contents

Safety Data Sheets can be found on our website:
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Experiment Components

A Ion Exchanger, CM-Cellulose
B Concentrated Potassium Acetate (KOAc) Buffer, pH 6.0
C Blue/green dye
D Red Dye

- Chromatography Columns
- Plastic Pipets
- 15 ml Conical Tubes
- Microcentrifuge Tubes

This experiment is designed for 10 student groups.

All components may be stored at room temperature.

Requirements

- Test Tubes (8-10 ml capacity)
- Ring Stand with Clamps for Columns
- Glassware (100 or 200 ml beakers or flasks)
- Graduated Cylinder (100, 250, 500 ml)
- Distilled Water
- 5 ml Pipets and Pumps
- Spectrophotometer and Cuvettes (optional)

Background Information

Most biological compounds are positively or negatively charged when exposed to a pH in the range of 2-10. When pH is altered, the net charge on a biomolecule can change from neutral to a net positive or negative charge. Ion exchange chromatography utilizes a solid support (adsorbent) which contains either a permanent positive (cation) or negative (anion) charge. The separation of compounds is based on an equilibrium of the molecules adsorbed to the exchanger versus the elution solvent. This equilibrium can be shifted gradually by changing the ionic strength or pH of the eluting buffer, thereby weakening the electrostatic forces and eluting the molecules from the exchanger. This allows the separation of molecules with small differences in net charges.

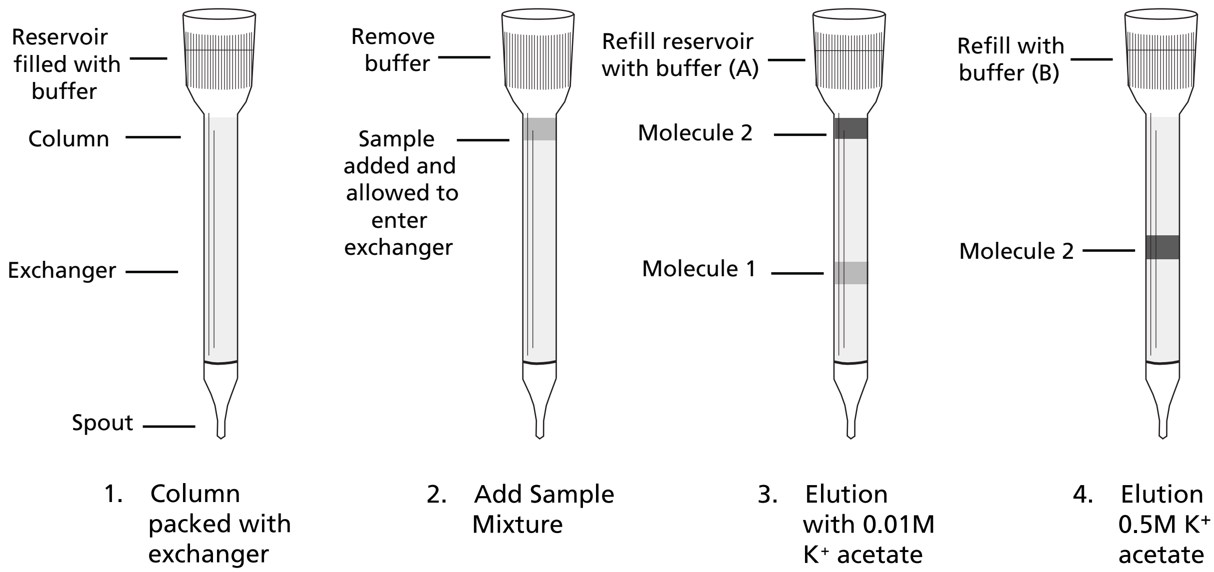
The solid support is usually a synthetic resin (cross-linked polystyrene) or cellulose derivative covalently bonded to the desired functional group to create a weak or strong ion exchanger. For example, weak cation exchanger's functional group is a carboxylic acid, a strong exchanger is sulfonate. Likewise, the anion exchangers are derivatives of either secondary or tertiary amines.

Cation exchangers		Anion exchangers	
$-CH_2COO$	$R-SO_3$	$-CH_2NHR_2$	$-CH_2NR_3$

The cation exchanger carboxymethylcellulose or CM-cellulose has the $-CH_2OH$ groups of cellulose modified to $-CH_2OCH_2COOH$. The corresponding exchanger is substituted with $-CH_2OCH_2CH_2N(CH_2CH_3)_2$ (DEAE-cellulose). The capacity of the exchanger is determined by the number of meq/ml of a standard material that can be adsorbed. In the case of cellulose, there is some limit to the number of substitutions that can be made per unit of cellulose. If it is too highly substituted, the support will become water soluble. Celluloses are the preferred supports for biologically active proteins because they do not denature (deactivate) the protein as readily as synthetic resins.

Background Information

The adsorption and separation are based on the differences between electrostatic interaction of the molecules and support. The following example demonstrates the exchange principle.



Molecule 2 should have a greater attraction for the support than molecule 1. By changing the ionic strength or pH, the elution point for molecule 1 is attained before that of molecule 2.

Ion exchange chromatography can be used to separate both small molecules, such as amino acids and large ones like proteins, RNA and DNA. The molecules to be separated should have either different charges, (positive or negative), or varying degrees of the same charge.

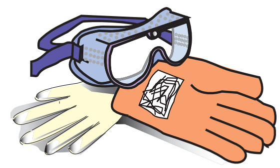
Experiment Overview

EXPERIMENT OBJECTIVE:

The objective of this experiment is for students to learn the principles of ion exchange chromatography by separating two charged molecules using a two-step salt gradient.

SAFETY:

No human materials are used in this experiment. Gloves and safety goggles should be worn as good laboratory practice.



Wear gloves and safety goggles

Student Experimental Procedures

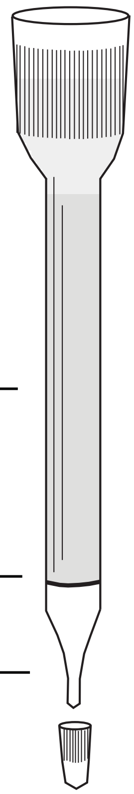


FIGURE 1:
Packing the column.

NOTE:

It is very important to use the correct KoAc buffer as instructed throughout the experimental procedure. Make sure to read the label of the bottle containing the KoAc buffer and not to mix them up.

Read through entire instructions before beginning experiment.

A. PACKING THE COLUMN

1. Vertically mount a column as shown in Figure 1. Slide the cap onto the spout at the bottom of the column.

2. Measure 1 ml water into an empty test tube. This will be used later on as a reference guide.

3. Rinse the column with buffer by filling it with 0.01 M KOAc.

4. Remove the cap to release the buffer. Replace the cap.

5. Thoroughly mix and pipet the entire contents of the slurry to the column by allowing it to stream down the inside walls of the reservoir. If the slurry gets stuck in the reservoir, use a pipet to resuspend the slurry and to continue packing of the column.

   If the flow of slurry 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.

6. Pour additional 0.01 M KOAc buffer into the reservoir. Place an empty beaker under the column and remove the cap to allow the slurry to settle.

7. Add additional 0.01 M KOAc to maintain the level of the buffer above the top bed of the slurry. Do not let the column run dry.

8. After the slurry has settled, replace the cap. Carefully remove any remaining buffer from above the top of the bed by inserting a transfer pipet through the reservoir. Try to minimize disturbance of the bed while removing the buffer.

B. SAMPLE SEPARATION

1. Use a transfer pipet to add the “sample” onto the top of the bed.

2. Remove the cap to allow the sample to slowly enter the bed. After it has completely entered, the top of the bed should be exposed to air.

3. With a transfer pipet, carefully and slowly (2-3 drops at a time) add 0.01M KOAc buffer to the reservoir. Allow the buffer to stream down the inside of the column after the sample has completely entered the gel.

Experiment Procedures

Student Experimental Procedures

4. Collect fractions containing red dye:
   - Label 2 tubes "R".
   - Hold each of the empty test tubes under the column and collect approximately 1 ml fractions (use the tube from step A-2 as a reference).
5. Monitor the level of buffer in the reservoir, refill with 0.01M KOAc if needed.
6. After the red has completely eluted the column, place the beaker under the column and allow the remaining 0.01M KOAc to empty from the reservoir.
7. With a transfer pipet, carefully and slowly (a few drops at a time) add 0.5 M KOAc buffer to fill the reservoir.
8. Collect fractions containing Blue/green dye:
   - Label 6 tubes "G".
   - Hold each of the empty test tubes under the column and collect approximately 1 ml fractions (use the tube from step A-2 as a reference).
9. Monitor the level of buffer in the reservoir, refill with 0.5 M KOAc if needed.
10. After most of the green has completely eluted from the column, replace the cap.
11. Measure the volume of buffer required to elute each dye. Compare your results to other groups.

C. SAMPLE QUANTIFICATION (OPTIONAL)

1. Prepare standard curve.

   a. Stock is 1 mg/ml. Prepare serial dilutions as follows:

1 mg/ml	=	stock		
0.5 mg/ml	=	3 ml of 1 mg/ml	+	3 ml distilled water
0.25 mg/ml	=	3 ml of 0.5 mg/ml	+	3 ml distilled water
0.125 mg/ ml	=	3 ml of 0.25 mg/ml	+	3 ml distilled water
0.0625 mg/ml	=	3 ml of 0.125 mg/ml	+	3 ml distilled water

Student Experimental Procedures

NOTE:

If your spectrophotometer cannot read 1 ml samples, you may want to consider making dilutions of the fractions in order to read the A550. Don't forget to include the dilution factor in your calculation.

b. Blank spectrophotometer at 550 nm with distilled water.

c. Record A_{550} and plot absorbance on Y axis and Blue/green dye concentration on X axis.

2. Samples

a. Transfer green chromatograph fractions to cuvettes.

b. Read and record A_{550} for each fraction.

c. Pool green fractions in a beaker or large test tube. Mix and measure total volume with a pipet or graduated cylinder.

d. Transfer a portion of mixed fractions to a cuvette. Read and record A_{550}.

e. From standard curve, determine concentration of Blue/green dye in mixed fractions.

f. To determine yield, multiply concentration in mg/ml by the total volume (in ml).

g. Sample loaded on column contained 0.4 mg (0.4 ml of 1 mg/ml) of Blue/green dye. Calculate percentage recovery from column.

Study Questions

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

1. What is the basis for the separation of different compounds by ion exchange?
2. How can molecules with the same charge at varying amounts be separated by chromatography?
3. Why are celluloses often used as supports to separate large biologically active proteins?
4. What do you think would happen if 0.5M K^+ acetate were used first to elute the sample? Why?
5. Why is it important to prepare a standard curve for each spectrophotometer?

Notes to the Instructor

This experiment module was designed for 10 lab groups.

APPROXIMATE TIME REQUIREMENTS

The pre-lab materials can be prepared the day before the lab. The preparation should take about one or two hours. Cover buffers and exchanger with foil or plastic wrap.

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PreLab Preparations

A. PREPARATION OF BUFFERS

1. Label 10 small beakers or flasks “0.5 M KOAc (potassium acetate)”.
2. Dilute the concentrated Potassium Acetate Buffer, pH 6.0 (Component B) by adding 25 ml of buffer to 100 ml of distilled water. This is now 0.5 M KOAc buffer.
3. Aliquot 10 ml of 0.5 M K^+ acetate to each of the beakers or flasks labeled “ 0.5 M KOAc”.
4. Label 10 small beakers or flasks “ 0.01 M KOAc”.
5. Add 15 ml of 0.5 M KOAc buffer from step 2 to 735 ml distilled water. This is now 0.01 M KOAc.
6. Aliquot 30 ml of 0.01 M KOAc to each of the 10 beakers or flasks labeled “ 0.01 M KOAc”.

Each group requires:

- 1 column attached to a ring stand
- 1 beaker of 0.01 M KOAc, 30 ml
- 1 beaker of 0.5 M KOAc, 10 ml
- 1 tube “sample”, 0.5 ml
- 1 test tube of "standard", 6 ml
- 1 tube of ion exchange matrix (slurry), 6 ml
- 3 transfer pipets
- 1 small empty beaker
- 5 ml pipet and pump
- 6 microcentrifuge tubes
- Test tube (8-10 ml capacity) and rack for Sample Quantification (optional)

B. PREPARATION OF ION EXCHANGE MATRIX (SLURRY)

1. Add the entire contents of component A, the CM-cellulose ion exchanger to a medium-sized beaker (250 ml size).
2. Add 150 ml 0.01 M KOAc to the beaker containing the ion exchanger. Stir occasionally for 5 min. Use a spoon or spatula to break apart any hard clumps of cellulose. Allow the exchanger to settle for 10 minutes.
3. After most of the exchanger has settled, carefully decant and discard the liquid plus fines (be extremely careful to avoid dumping the exchanger that has settled at the bottom).
4. Add 150 ml of fresh 0.01 M KOAc to the previously hydrated ion exchanger. Stir briefly to mix and allow the exchanger to settle for 10 minutes. Decant as in step 3.
5. After the second settling and decanting, add 50 ml 0.01 M KOAc to the exchanger and stir to mix well.
6. Mix the exchanger in between pouring each tube and aliquot approximately 6 ml of the resuspended exchanger into the 15 ml conical tubes provided with the kit. Cap the tubes and distribute one tube per group.

C. PREPARATION OF THE SAMPLE MIXTURE AND STANDARD

1. Label 10 microcentrifuge tubes “sample”.
2. Add 2 ml of the Blue/green dye (C) to the bottle of red dye (D). Cap & mix well. Aliquot 0.5 ml into the tubes labelled "sample".
3. Label 10 test tubes “standard”.
4. Aliquot 6 ml of the Blue/green dye (C) into test tubes labeled "standard".

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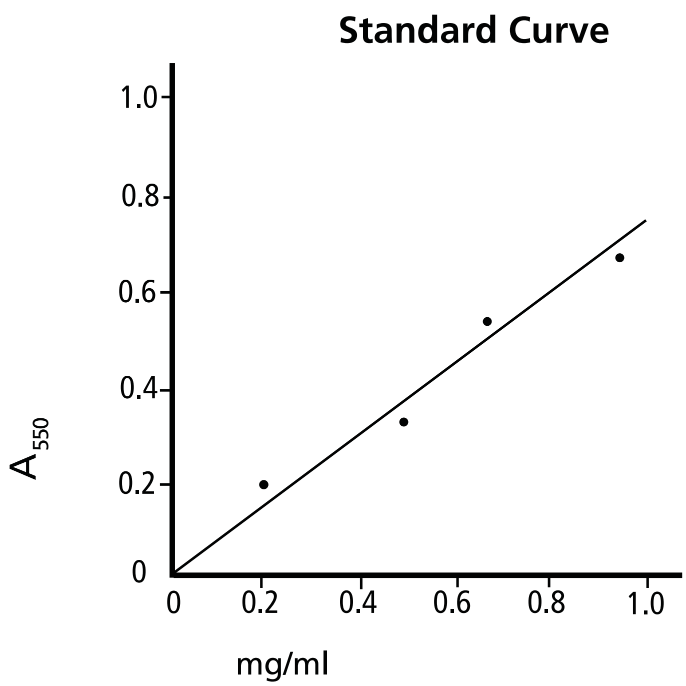
Avoiding Common Pitfalls

1. Dilute buffers properly.
2. Avoid discarding the slurry when decanting the water from the mixture.
3. When packing the columns, avoid bubbles and air pockets that will interrupt the flow of the sample.

Expected Results

Red dye will be eluted first after addition of 0.01M KOAc. Blue/green dye will follow after adding 0.5 M KOAc.



A standard curve must be made for each spectrophotometer used. Do not use the standard curve to the right. It is for illustration purposes only.

Please refer to the kit insert for the Answers to Study Questions