

Chromatographic Techniques: Ion Exchange Chromatography and Gel Filtration

Introduction

Chromatography of both small molecules and macromolecules is widespread in biochemistry. Two of the most common techniques used for the separation of macromolecules are size exclusion chromatography (SEC, also called gel filtration) and ion exchange chromatography (IEC).

SEC separates molecules on the basis of their size. Formally, SEC is a separation based on hydrodynamic radius; however, for similarly shaped molecules hydrodynamic radius is proportional to molecular weight. Therefore we generally talk about SEC as a mass based separation, even though this is not strictly true. Many different chromatographic materials for SEC are available. Each material is characterized by an “exclusion limit” which specifies an approximate upper limit for the size of molecules able to be separated using the material. Separation in SEC is based on the partitioning of molecules between the bulk fluid between the beads of chromatographic media and the aqueous space with the pore of these beads. The smaller the molecule the larger fraction of the total volume it “sees” and the slower it moves through the column. Thus, the largest molecules elute first while the smallest elute last.

SEC separations are performed by placing the appropriate chromatographic medium in a long thin column. The sample is introduced at the top of the column and material eluted by flowing an appropriate buffer through the column. SEC columns are characterized by two parameters, the void volume (V_0) and the total volume (V_T). V_0 is essentially the volume of the space between the “beads” of chromatographic medium; molecules larger than the exclusion limit elute in the V_0 . V_T is the volume of all of the liquid with in column (i.e. both within the porous beads, as well as between them). The smallest molecules appear in the V_T .

IEC separations are based on molecular charge. Chromatographic materials for the separation of both cations (cation exchange media) and anions (anion exchange media) are available. A cation exchange medium contains anionic functional groups while an anion exchange medium is cationic. Thus these materials bind molecules with a charge opposite to their own, while allowing similarly charges molecules to pass through. Changing the composition of the buffer elutes materials bound to the column. In some cases the ionic strength of the buffer is increased, in others, the pH is changed in order to elute material from the column.

In this experiment you will be given a mixture of five colored compounds that vary in their size and charge, a number of different chromatographic media and an assortment of solutions for eluting columns. Your task is to devise a method for the separation of all five components by performing a number of trial separations using test mixtures of the compounds.

Materials

Samples

Note: Use 0.3 mL of these samples for each column you run.

Trail Mixture #1: blue dextran (3 mg/mL), cytochrome C (3 mg/mL), DNP-glycine (1 mg/mL)

Trail Mixture #2: blue dextran (3mg/mL), yellow dextran (3mg/mL), vitamin B₁₂ (0.2 mg/mL)

The “real” sample: blue dextran (3 mg/mL), yellow dextran (3mg/mL), cytochrome C (3 mg/mL), vitamin B₁₂ (0.2 mg/mL), DNP-glycine (1 mg/mL)

Chromatographic Media

Sephadex G25, equilibrated in 0.1 M KAc, pH 6

This SEC medium has an exclusion limit of about 25,000 Da. Use this medium in a column with a bed height of about 12 cm.

CM-Sephadex, equilibrated in 0.1 M KAc, pH 6

This ion exchange medium contains carboxymethyl [-CH₂COO⁻] groups for binding cations. It can be eluted by using KAc, pH 6 buffers of increasing concentration.

DEAE-Cellulose, equilibrated in 0.1 M KAc, pH 6

This ion exchange medium contains diethylaminoethyl [-CH₂CH₂N⁺H(CH₂CH₃)₂] groups for the binding of anions. It can be eluted by using KAc, pH 6 buffers of increasing concentration. Solutions of HCl (1.0 and 6.0 M) can also be used to elute compounds that remain bound at pH 6.

Solutions

0.1 M potassium acetate (KAc), pH 6

Use for elution of the SEC column and as the initial buffer for IEC columns.

The following solutions are for elution of IEX columns. They are listed in order of increasing elution power. Use HCl only on DEAE-cellulose.

0.5 M KAc, pH 6

1.0 M KAc, pH 6

1.0 M HCl

6.0 M HCl

Methods

General Procedure

- 1) Use the two test mixtures to determine the chromatographic properties of the various compounds on the various media.
- 2) Use this information to devise a separation procedure involving the use of two different columns that will result in the separation of all five compounds.
- 3) Obtain the “real” sample from the instructor and perform the procedure you devised.
- 4) Hand in (before you leave the lab) 5 test tubes, each containing (hopefully!) a single component of the original sample.

Helpful Hints:

- 1) Collect fractions of equal volume (1 mL is recommended) while eluting columns during the trial phase. During the separation of the “real” sample, act as a “smart” fraction collector and try to collect each component of the mixture in a single test tube.
- 2) Remember how colors combine (e.g. red + blue = purple) and the effect of dilution on color (e.g. red diluted out = pink) and make careful observations of the colors of the various fractions.
- 3) After finishing an IEC experiment, remember to re-equilibrate the column back to its original state before running another sample on it. Re-equilibration is accomplished by running 3 to 5 column volumes of “starting” buffer (0.1 M KAc, pH 6 in this case) through the column.
- 4) Properties of the compounds to be separated:

Compound	Color	MW (Da.)	Ionic Character at pH 6
Blue dextran	Blue	> 500,000	Very strong anion
Yellow dextran	Yellow	~ 40,000	Strong anion
Cytochrome c	Red-orange	12,400	Strong cation
Vitamin B12	Cherry red	1,357	Weak cation
DNP*-glycine	Yellow	241	Weak anion

*DNP = dinitrophenyl