SOLID PHASE EXTRACTION COLUMNS

A Tool for Teaching Biochromatography

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Biochromatography is difficult to teach to undergraduate students because it requires knowledge of molecular interactions not often covered in a typical undergraduate curriculum. But with up to sixty percent of the total protein manufacturing cost directly related to the purification process, a basic knowledge of biochromatography and its inherent efficiencies is an essential part of the biochemical engineer's education. Class lectures on the subject attempt to explain and diagram column dynamics, but hands-on experience will help the students grasp the concepts and remember them far longer. Thus, we have developed two laboratory experiments for protein chromatography.

The experiments were designed to require minimal and inexpensive equipment and to be performed within a two-hour laboratory period. The students were each given three 1 ml Bakerbond spe™ columns (cationic, anionic, and hydrophobic interaction), a size-exclusion column, and an unknown protein or mixture of proteins. Based on the binding and elution properties of the proteins and the known physicochemical properties of several possible protein candidates (pI and relative hydrophobicity), the students were asked to determine which protein(s) were present. Colored proteins were chosen as the unknowns so that no special detection equipment was required.

THEORY

A mixture of proteins can be separated with the use of chromatographic techniques based on the tendency of the various proteins to adsorb to the column packing. In ion-exchange chromatography, adsorption of proteins depends on the proteins' isoelectric point relative to the column pH. Proteins with a high isoelectric point will bind tightly to a cation exchange column in the presence of a low pH and a low salt concentration. Proteins with a low isoelectric point will bind tightly to an anion exchange column in the presence of a high pH and a low salt concentration.

Hydrophobic interaction chromatography uses a high salt concentration to induce an interaction between hydrophobic regions of a protein and a weakly hydrophobic column packing. In all three cases, elution of the bound proteins can be achieved using a salt gradient.

Another type of chromatography, size-exclusion chromatography, separates molecules based on their relative size. The column is filled with a packing with a specific pore size distribution. Smaller molecules enter the pores and linger inside them, while larger molecules, which cannot fit into the pores, pass more rapidly through the column.

Bakerbond spe Wide-Pore columns are small (12 x 65 mm) reusable polypropylene tubes (6 ml total

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In their laboratory reports, the students showed that they recognized that the Gaussian distribution of the pore size for size-exclusion chromatography has an effect on the column resolution, and they noted that protein shape could also have an effect. Several students took their observations one step further and applied them to separation problems that might be encountered in industry.

volume) which contain 500 mg (1.5 ml) of 40-mm silica-based stationary phases. These inexpensive columns are designed to be used for sample preparation prior to conventional open column chromatography or high-performance liquid chromatography (HPLC), and can also be used to determine the physicochemical properties of a particular "target" protein as well as its retention behavior on various chromatographic matrices. The available stationary-phase surface chemistries include Bakerbond Wide-Pore BUTYL (-C_4, reversed-phase matrix), Bakerbond Wide-Pore CBX (-COO^-, weak-cation exchanger), Bakerbond Wide-Pore PEI (-NH_3^+, weak-anion exchanger), Bakerbond Wide-Pore HI-Propyl (-C_3, hydrophobic-interaction matrix), and Sephadex™ G-25 (size-exclusion matrix). Solid phase extraction columns are available from other manufacturers, but have not been tested with these experiments.

TABLE 1
Material Data[4]

Protein	*MW (kDa)*	*pI*	*relative hydrophobicity**
Bovine serum albumin	69.0	5.1	20.5
α-Chymotrypsin	21.6	8.6	16.6
α-Chymotrypsinogen	25.0	9.2	18.1
Conalbumin	77.0	6.3	6.3
Cytochrome C	12.2	9.4	0.6
Ferritin	500.0	4.3	20.8
β-Glucosidase	130.3	7.3	15.6
Hemoglobin	64.0	7.0	1.1**
Lactoperoxidase	85.0	9.5	19.5
Lysozyme	13.9	11.0	8.5
Myoglobin	17.5	7.1	0.8
Ovalbumin	43.5	4.7	6.5
Ribonuclease A	13.5	8.7	1.6
Dyes			
Phenol red	0.35		
Erioglaucine blue	0.78		
Column Packings			
Sephadex G-10	(<0.7 kDa)		
Sephadex G-50	(1.5 to 30 kDa)		
Sephadex G-75	(3 to 70 kDa)		
Sephadex G-100	(4 to 150 kDa)		

** Higher values are more hydrophobic*
*** Estimated*

Scale-up of chromatography columns for protein separation and purification is an important issue for biochemical engineers designing a process. The purest product is obtained with a minimum band width for each protein. Various forces influence the band width, or zone spreading, such as longitudinal diffusion, eddy diffusion, and the lack of a local equilibrium at the front and rear of the band. These forces can be modeled by assuming the molecules move through the column according to the random-walk theory (the molecules move in a series of random stops and starts). A Gaussian distribution of the molecules is also assumed, with $\sigma = 1\sqrt{n}$, where n is the number of random-walk steps and 1 is the step length. The resulting model[1,2] is

$$H = \frac{\sigma^2}{L} = \frac{B}{\bar{u}} + A + C_s\bar{u} + C_m\bar{u} \qquad (1)$$

where

$H = \frac{\sigma^2}{L}$ represents the plate height, used to express the net effect of zone spreading

$\frac{B}{\bar{u}}$ = longitudinal diffusion

A = eddy diffusion

$C_s\bar{u}$ = resistance to mass transfer at the solute-stationary phase interface

$C_m\bar{u}$ = resistance to radial mass transfer caused by particles of the packing material

The magnitude of each of these effects is determined by the velocity of the sample ($\bar{u}$) in the column. If the velocity is too high, the mass transfer resistances predominate and there is more band spreading. If the velocity is too low, the longitudinal diffusion increases the band width. Therefore, there is an optimal sample velocity for each mixture of proteins.

The resolution of two peaks, R, is defined as the distance between the peaks divided by the average width

$$R = \frac{t_{RB} - t_{RA}}{0.5\left(t_{WA} + t_{WB}\right)} \qquad (2)$$

where

t_{RB}, t_{RA} = retention times of components A and B

t_{WA}, t_{WB} = width of peak A and peak B

In column chromatography, peaks become broader proportional to the square root of the column length, but their separation increases in direct proportion to the column length.[3] Thus the resolution is proportional to the square root of the column length. This means that to double the separation between two

bands, a column four times as long is required.

These scale-up concepts, when explained in a class lecture, are not always intuitively obvious to the undergraduate student. Varying these factors in a laboratory exercise, however, allows the students to observe the effects of the column length and sample velocity on the degree of separation.

EXPERIMENT PROCEDURE

Each student is given molecular weight, isoelectric point, and relative hydrophobicity data on a variety of proteins, and size data on two dyes as well as four types of Sephadex that will be used (see Table 1). They are also given the necessary buffers (Table 2), 3 Bakerbond spe columns (WP PEI, WP HI-Propyl, and WP CBX), and a column for size-exclusion chromatography. The students are to process the unknowns through the various columns and determine their identity. The samples can be pulled through the spe columns using a vacuum system or forced through the spe columns using compressed air. In order to use the laboratory time efficiently (to make sure the students have thought about chromatography concepts before lab time) the homework assignment shown in Table 3 is given the week before the experiments.

Chromatography Lab 1

Each student is given a single unknown protein (chosen from the list in Table 4) and a mixture of two or three of these proteins, each dissolved in buffers at both pH 6 and pH 7.5. The Bakerbond spe columns must first be equilibrated to the correct pH by passing 5 to 10 ml of buffer through the column before any protein samples are added. When the column eluant is at the correct pH, about 0.2 ml of protein sample is added to the column. The students slowly process the protein samples through each of the three Bakerbond spe columns and visually observe whether or not binding occurs. By comparing their observations with the table they completed in the homework assignment, the protein(s) can be identified. Proteins can be eluted from the column (and the column cleaned before introducing the next sample) by running a salt solution (200 or 500 mM) through the column.

Chromatography Lab 2

This laboratory exercise has two goals: (1) to determine whether an unknown sample contains ferritin, hemoglobin, cytochrome C and/or a dye, and (2) to determine the effects of the column length and sample velocity on the resolution of the mixture. Each student is given an empty column, a mixture of protein and/or dye, and small quantities of the

TABLE 2
Solutions

20 mM	**KH_2PO_4**	**pH 6 and pH 7.5**
1M	**Na_2SO_4**	
2M	**Na_2SO_4**	
200 mM	**KH_2PO_4**	**pH 7**
500 mM	**KH_2PO_4**	**pH 7**

TABLE 3
Homework Assignment

In order to prepare for next week's laboratory session, you must fill out the table below. Use a "+" sign to indicate binding and a "-" sign to indicate that the protein will flow through the column without binding. This will help you determine which protein(s) you have in the laboratory assignment. Also consider under what conditions the proteins would elute.

Column Conditions:
- CATION EXCHANGER: Equilibrate column with pH 6 buffer
- ANION EXCHANGER: Equilibrate column with pH 7.5 buffer
- HIC: Equilibrate column with 1 M salt solution (assume 6.1 relative hydrophobicity)

	COLUMN TYPE						
				Sephadex			
Protein	Cation Exchange	Anion Exchange	HIC	G-10	G-50	G-75	G-100
Bovine serum albumin							
α-Chymotrypsin							
α-Chymotrypsinogen							
Conalbumin							
Cytochrome C							
Ferritin							
β-Glucosidase							
Hemoglobin							
Lactoperoxidase							
Lysozyme							
Myoglobin							
Ovalbumin							
Ribonuclease A							
Phenol red							
Erioglaucine blue							

What mixtures of proteins could not be separated by any combination of the columns and conditions above?

four types of Sephadex gels. Before starting the experiment, the Sephadex beads must be swollen in 20 mM salt solution. The beads should be poured carefully into the column, so that cracks and holes in the packing are avoided. In addition, the column should not be allowed to become dry during the course of the experiment.

To accomplish the first goal, the sample is processed through similar columns filled with different sizes of Sephadex. The students choose which sizes to use in order to determine what protein/dye combination is in their sample. For at least two different sizes of beads, the students observe whether the compounds pass quickly through the columns or stay near the top of the column. From their observations they can identity the unknowns in the mixture. To observe scale-up effects, only one size of Sephadex is used (one that separated the student's unknown into two bands). Samples are processed under four conditions: (1) short column (2 cm) and gravity flow; (2) short column and vacuum-induced flow; (3) long column (8 cm) and gravity flow; and (4) long column and vacuum-induced flow. The students can easily observe the effects of column length and sample velocity on the degree of separation.

The results of this experiment can be quantified by collecting samples of the eluant and measuring the absorbance with a spectrophotometer. The students can then produce a chromatogram and calculate the resolution under the different conditions.

CONCLUSIONS

The students found the labs relatively uncomplicated and possible to complete within the two-hour laboratory period. The use of colored proteins allowed them to actually observe the "binding" rather than simply studying a chromatogram that comes from a spectrophotometer. The students did request that proteins of different colors be used in the exercise (all of the proteins used are brown or reddish-brown) so that they could confirm their deductions based on color observation. By limiting the proteins to one color, however, the students were forced to determine the protein identity based on adsorption properties, not on color. By the end of the first laboratory exercise the students had a clearer idea of how proteins could be separated based on differing isoelectric points. Properties of size-exclusion chromatography were understood after the first half of the second experiment. Several students commented on the unexpected property exhibited by the smaller dye molecules which moved more slowly than the larger protein molecules through the size-exclusion column—a concept not easily grasped in class lectures.

TABLE 4
Proteins Used as Unknowns

Conalbumin	**50 mg/ml**
Cytochrome C	**20 mg/ml**
Hemoglobin	**20 mg/ml**
Ferritin	**20 mg/ml**

Consequences of scale-up, such as the effects of velocity and column length on zone spreading (band width) were similarly difficult to comprehend during the lecture, but were very clear when observed firsthand. In their laboratory reports, the students showed that they recognized that the Gaussian distribution of the pore size for size-exclusion chromatography has an effect on the column resolution, and they noted that protein shape could also have an effect. Several students took their observations one step further and applied them to separation problems that might be encountered in industry, suggesting changes that would have to be made.

It is important that the theoretical concepts be explained in class before the students attempt the laboratory exercises. Operational problems also became clear while the students were performing the experiments. For example, the importance of equilibrating the column before introducing samples was discovered by several students who found that none of the proteins would bind to their column if it had not been equilibrated. The problems that occur when the column is allowed to become dry (cracks or holes in the packing) were noted by several students performing size-exclusion chromatography.

The students rated the laboratory experiment highly and as "very worthwhile." It allowed them to perform and validate what they had learned in class. They were able to use chromatography techniques and to more clearly understand the interaction of protein properties and column phases.

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