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# Fast, reproducible size-exclusion chromatography of biological macromolecules

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**Abstract**

The size-dependent separation of biological macromolecules can be effectively carried out using size-exclusion chromatography (SEC) on silica-based HPLC columns. For this technique to be successful, appropriate methods should be chosen. This paper presents practical guidelines for the development of reproducible SEC methods based upon optimized sample volume, flow-rate, column length and use of mobile phase conditions that reduce non-ideal SEC behavior – parameters often ignored in SEC. Adjustment of these parameters often results in more accurate elution times for proper molecular-mass determination, sharper peaks for improved resolution and shorter run times for increased throughput. In general, sample volume and flow-rate should be kept to a minimum for optimal resolution in SEC. Increasing column length improves resolution and may be achieved by placing columns in tandem. In addition, adjustment of the mobile phase conditions can significantly enhance resolution. However, the results are difficult to predict because the sample plays a major role in this interaction, as does the column packing. When possible, mobile phase ionic strength and pH should be altered until the peak(s) of interest elute at the expected time and with good peak shape. Finally, use of smaller-diameter columns (i.e., 4.6 mm rather than 9.4 mm) and small-diameter packing (4.5 $\mu$m) particles are also briefly discussed. The principles described here are demonstrated, using antibodies and a number of standard proteins under a variety of SEC conditions.

*Keywords:* Size-exclusion chromatography; Mobile phase composition; Flow-rate; Column length; Injection volume; Antibodies; Proteins

## 1. Introduction

Size-exclusion chromatography (SEC) is a powerful tool that is used to separate molecules by size, or hydrodynamic volume. The technique is unique in that it can be used over a broad range of mobile phase conditions, eluting molecules in their native state for use in further experimentation. SEC is commonly used to estimate molecular mass [1]; separate monomer, dimer, trimer and larger aggregates [2]; separate immunoglobulins and antibodies from antibody conjugates or fragments [3]; separate reaction products from the reaction mixture; exchange sample solutions (including desalting); and for a variety of other applications. For a complete review of SEC see [4]. True SEC techniques depend upon the accurate separation of molecules by size, with minimal interaction of sample with the packing surface. However, HPLC columns exhibit non-ideal SEC behavior under certain conditions [5–11]. We have previously characterized sample–surface interactions using a large number of proteins and mobile phase concentrations on a silica-based, zirconia-stabilized, SEC column [12]. At low ionic strength,

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electrostatic interactions can occur between the solute and the surface of the column-packing; at high ionic strength, hydrophobic interactions may occur. The extent of these interactions is specific for each sample molecule, and mobile phase conditions should be optimized to meet the user's separation criteria.

Other operational parameters also affect SEC separations. Short run times result in higher throughput. This saves time and money and decreases the time necessary to scout for mobile phase conditions that promote ideal SEC behavior. Run time can be decreased by reducing column length or increasing flow-rate. Packing materials with good physical stability [13], and pumps capable of sustaining high pressures should be chosen for high-speed separations.

Optimizing column configuration, flow-rate and injection volume, in conjunction with mobile phase conditions, can result in "extra" resolution. This additional resolution insures that the separation will be maintained even with small changes in the chromatographic system. In this paper, we present data exploring the practical uses of column-packing characterization and column configuration to obtain fast, rugged SEC methods.

## 2. Experimental

### 2.1. Instrumentation

HPLC was carried out using an LKB 2150 pump (Pharmacia LKB Biotechnologies, Piscataway, NJ, USA), DuPont 8800 Series multiwavelength detector (DuPont Instruments, Wilmington, DE, USA) and a 9125 injector from Rheodyne Corporation (Cotati, CA, USA). Data was collected with an IBM-compatible 80486 computer running ChromPerfect for Windows chromatography-analysis software (Justice Innovations, Mountain View, CA, USA), ChromPerfect Direct 4I (ver. 6.07) using a DT2804 data-acquisition card, all from Geiser Scientific (Glen Mills, PA, USA). Graphics were exported as HPGL files and enhanced using CorelDraw 5.0 (Corel, Ottawa, Canada).

### 2.2. Columns

The columns used were Zorbax BioSeries GF-250 (250×9.4 mm or 250×4.6 mm) obtained from MAC–MOD, Analytical (Chadds Ford, PA, USA). The column packing had a nominal 4.5 μm particle diameter and a 150 Å pore size.

### 2.3. Chemicals and proteins

All antibodies, proteins and low-molecular-mass markers used in this study were of 90% purity or better, purchased from Sigma (St. Louis, MO, USA). Sodium phosphate, monobasic, ultrapure bioreagent and analytical-grade sodium hydroxide were obtained from VWR Scientific (Bridgeport, NJ, USA). Samples and buffers were made using de-ionized water filtered through a 0.22 μm hydrophilic filter (Millipore, Bedford, MA, USA).

### 2.4. Procedures

Protein stock solutions (10 mg/ml) were prepared individually in de-ionized prefiltered water. Samples containing 1–3 proteins with or without sodium azide were prepared for injection by combining the appropriate stock solutions and diluting to the desired volume. This was typically a 10-fold dilution with 0.20 *M* sodium phosphate adjusted to a pH of 7.0 with 10 *M* NaOH. Purified mouse myeloma antibodies were purchased in 20 m*M* Tris-buffered saline, pH 8.0 and were diluted to 1 mg/ml with sterile water. Detection was carried out at a wavelength of 230 nm at ambient temperature, with a flow-rate of 2 ml/min for the 250×9.4 mm column and a flow-rate of 0.96 ml/min for the 250×4.6 mm column.

## 3. Results and discussion

SEC is a well-understood technique; however, specific problems associated with this seemingly simple method are often overlooked. The properties that make it simple are also those that make method

development more difficult. The first property is that the separation is based only on size of the molecules. The second is that peaks elute only between the exclusion volume and the void volume, limiting the number of peaks that can be separated in a single run. The critical factor to obtaining fast methods in any chromatographic technique lies in obtaining high resolution. When extra resolution is available, the chromatographer may increase flow-rate or reduce column size to reduce runtime without losing the desired separation. Also, separations using methods developed with extra resolution will be obtainable over longer periods of time even with slight disturbances in the chromatographic system.

Resolution ($R_S$) is defined by the equation. $R_S = 1/4(\alpha - 1)N^{1/2}\ (k'/1+k')$. An increase in any one of these three parameters, $\alpha$, $N$ and $k'$, will improve resolution. Increasing $k'$ up to a value of about 20 makes a significant contribution to resolution; however, $k'$ cannot be altered in SEC because chromatography occurs without retention of the sample components. Alpha ($\alpha$) is usually improved by increasing differences in the partitioning of two samples between the stationary phase and the mobile phase. However, this partitioning does not occur in SEC separations. In SEC, $\alpha$ can be slightly affected by optimizing the pore size of the packing for the molecular-mass range of the sample and increasing the pore volume of the packing. Efficiency, or theoretical plates ($N$), describes how narrow a peak is relative to the time it takes to elute from the column. Efficiency ($N$) can be improved by decreasing flow-rate, limiting sample volume or increasing effective column length.

### 3.1. Increasing resolution by using longer columns

Increases in effective column length can be obtained by using longer columns or by simply using columns in tandem. The chromatograms in Fig. 1 demonstrate the increased resolution obtained when switching from one 250×4.6 mm column to two in tandem. Notice the increased resolution between peaks 2 and 3 ($R_S$ increased from 0.7 to 1.2). By comparing the theoretical plates ($N$) of peak 4, it can be seen that the increased $R_S$ is the result of

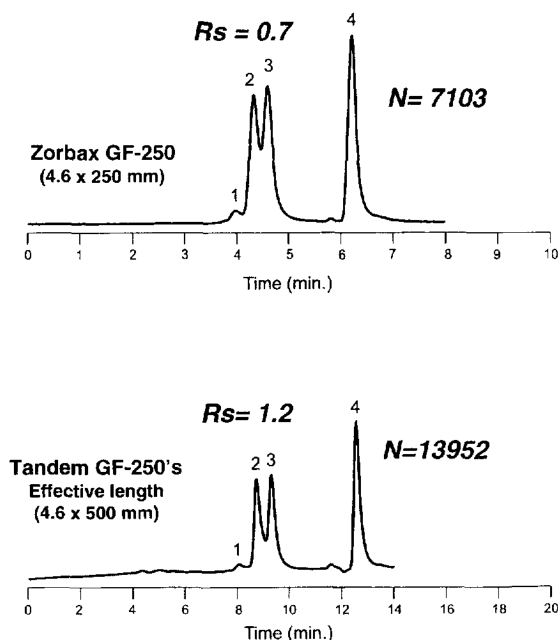


Fig. 1. Effect of column length on efficiency and resolution. A 3-component protein mixture was separated on a single Zorbax GF-250 column (250×4.6 mm) and on two columns in tandem (500×4.6 mm) using a mobile phase of 200 m$M$ sodium phosphate, pH 7.0. The injection volume was 2.5 $\mu$l and the column temperature was ambient. Detection, represented on the y-axis, was carried out at 230 nm. The flow-rate was 0.48 ml/min for both chromatograms. Resolution $R_S$ between BSA and ovalbumin and theoretical plates ($N$) for sodium azide are shown. Peak Identities: 1=BSA-dimer; 2=BSA; 3=ovalbumin and 4=sodium azide.

increased efficiency (change of $N$ from 7100 to 14 000). Increasing resolution in this manner has several possible drawbacks. The first is the increased back-pressure resulting from two columns in tandem. The pressure is double that for a single column. In this experiment, the pressure increased from 80 to 160 bar (1200 to 2400 p.s.i.). The column packing material used must be extremely strong to withstand these back-pressures without collapse of the packed bed. The column packings used in this study are sintered sol beads that will not crush even under very high pressures. Finally, use of two columns in tandem doubles the run time over that for a single column.

### 3.2. Increasing resolution by changing flow-rate

One limitation to high efficiency in size-exclusion chromatography is the rate of diffusion of sample molecules. The larger the sample molecule, the more slowly it diffuses into and out of pores in the packing. This slow diffusion results in peaks that broaden quickly with increased flow-rate. Because the molecules used for size-exclusion chromatography often have high molecular masses, it is important to keep flow-rates to a minimum when performing critical separations. The chromatograms in Fig. 2 show the increased resolution obtained for large molecules by reducing flow-rate. Compare the $R_S$ values of BSA (peak 2) and ovalbumin (peak 3) at 0.25 to 5 ml/min. $R_S$ increases more than 50%, from 1.4 to 2.3. The effect of increased plate values is perhaps best observed for the BSA trimer (the tiny peak eluting just before the BSA dimer, peak 1) which is most easily observed at the lowest flow-rate (0.25 ml/min). Note that in contrast to the proteins, the efficiency of the small molecule sodium azide (peak 5) increases at higher flow-rates. Its rate of diffusion is fast and becomes a major contributor to band broadening at low flow-rates.

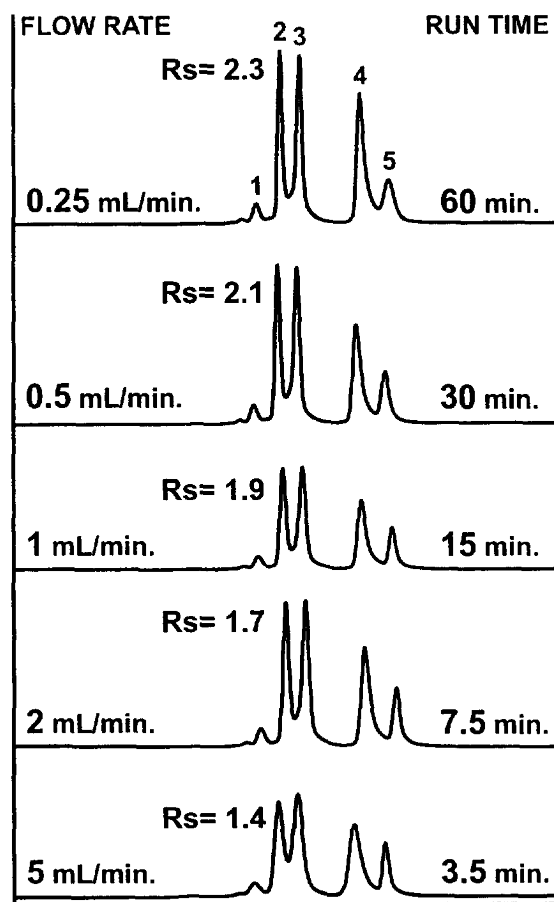


Fig. 2. Effect of flow-rate on resolution and plates in SEC. A 4-component protein mixture was separated on a Zorbax GF-250 column (250×9.4 mm) using a mobile phase of 200 m*M* sodium phosphate, pH 7.0. The injection volume was 10 $\mu$l and ambient temperature was used. Detection, represented on the y-axis, was carried out at 230 nm. The flow-rate was varied from 0.26 ml/min to 6 ml/min and all chromatograms are scaled relative to the flow-rate used. Resolution ($R_S$) between BSA and ovalbumin is shown. Peak Identities: 1=BSA-dimer; 2=BSA; 3=ovalbumin; 4=lysozyme and 5=sodium azide.

### 3.3. Increasing resolution by decreasing sample volume

Under ideal SEC conditions, there is no retention of solute molecules by the column packing. As a result, there is no sharpening of the component peaks as they are applied to the column. As injection volume of the sample is increased, the sample broadens out within the column. If too much sample volume is applied, resolution is lost. The series of chromatograms in Fig. 3 demonstrate the increase in resolution that can be obtained by keeping sample volumes below a certain threshold. This is normally 5–10% of the peak volume. For this system, peak volumes are about 1 ml, 0.1×1 ml=100 $\mu$l injection. The stock sample solution was diluted in proportion to its injection volume so that the same amount of sample could be injected in the indicated volumes. Resolution of peaks 2 and 3 increases as injection volume is decreased. This occurs between 200 and 20 $\mu$l, but levels off so that reducing the injection volume below 20 $\mu$l did not further improve resolution. As with flow-rate, if sufficient resolution exists, sample volume can be increased significantly before resolution diminishes to an extent where the desired separation is lost.

Finally, it should be stressed that sample volume is the limiting factor in SEC not sample concen-

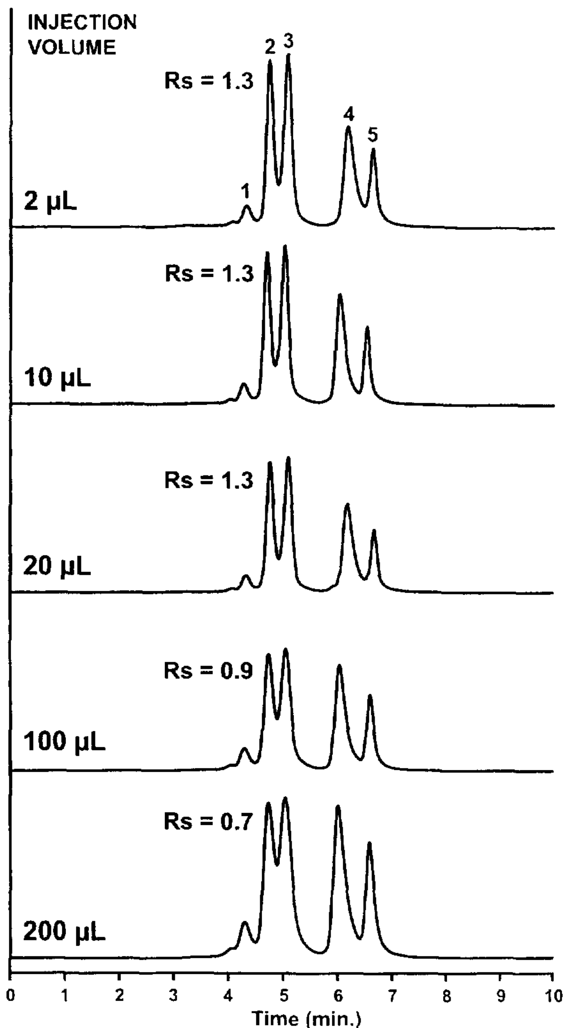


Fig. 3. Effect of injection volume on separation efficiency in SEC. A 4-component protein mixture was separated on a Zorbax GF-250 column (250×9.4 mm) using a mobile phase of 200 m*M* sodium phosphate, pH 7.0. The injection volume was varied from 2 to 200 $\mu$l and ambient temperature was used. Detection, represented on the y-axis, was carried out at 230 nm. The flow-rate was 2 ml/min. Resolution ($R_S$) between BSA and ovalbumin are shown. Peak Identities: 1=BSA-dimer; 2=BSA; 3=ovalbumin; 4=lysozyme and 5=sodium azide.

tration. One does not see concentration-dependent loss of resolution until samples become very viscous. As a rough estimate, this usually begins to occur at sample concentrations of 5–10 mg/ml.

### *3.4. Effect of mobile phase conditions on resolution and elution volume*

As mentioned earlier in this paper, SEC is meant to be non-interactive; sample should pass through the column without being retained by the packing surface. The sample molecules should be separated purely on the basis of size, from largest to smallest, and all sample components should elute before the column void volume ($V_0$). However, as described in our earlier paper [12], all SEC columns exhibit electrostatic and hydrophobic effects under certain mobile phase conditions and with certain sample components. At low ionic strength, it is common for electrostatic interactions to occur, and at high ionic strengths, hydrophobic effects are dominant. In either case, the sample components are retained by interaction with the packing surface and elute with broadened peaks. This interaction distorts the chromatography, making it impossible to estimate molecular mass (since retention time is not solely due to molecule size) and often making it difficult to obtain the desired separation.

Antibodies and their related reaction components make up a large portion of samples run by SEC. Examples of these molecule types are used in Fig. 4 and Fig. 5 to demonstrate mobile phase effects. In the experiment shown in Fig. 4, the effect of mobile phase ionic strength on chromatography is demonstrated using three mouse myeloma antibodies of similar molecular mass but of differing overall charge. The mobile phase contained sodium phosphate (pH 7) ranging in concentration from 20 to 1000 m*M*, as indicated. A number of sample–packing interactions can be observed for the three example antibodies. For the most weakly basic antibody, Fig. 4A, notice that the antibody elutes properly and with good peak shape at concentrations of sodium phosphate as low as 50 m*M*. Only at 20 m*M* phosphate does the peak begin to broaden and to be retained. Because there is no predominant positive charge on the antibody surface it is not attracted to the column packing. At high ionic strength (i.e., 600 and 1000 m*M*) the peak begins to broaden and to be retained due to hydrophobic effects, overlapping the peak observed at the void volume. For the somewhat more basic antibody shown in Fig. 4B, the pattern is

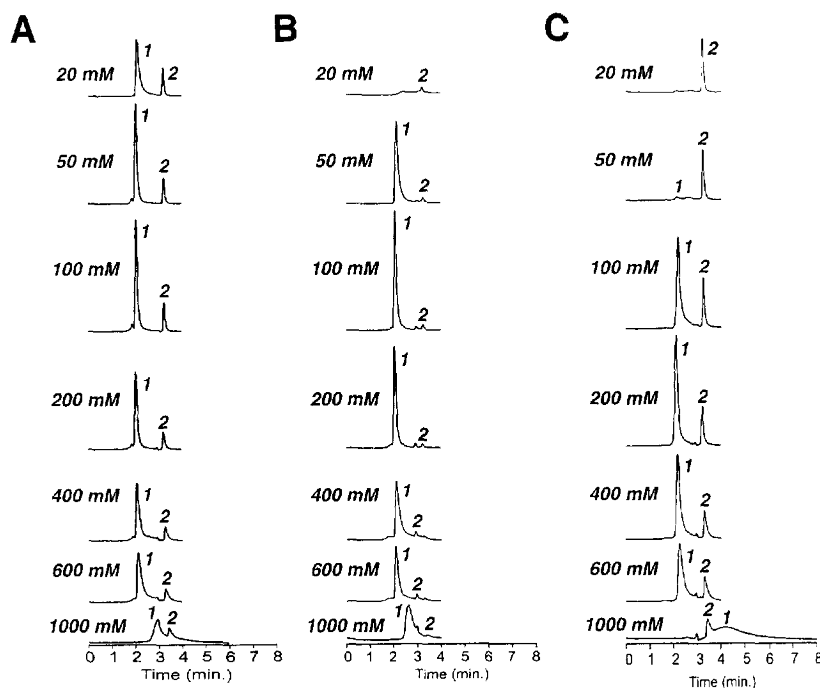


Fig. 4. Effect of mobile phase ionic strength on elution characteristics of mouse myeloma antibodies at pH 7.0. Three different antibodies were separated on a Zorbax GF-250 column (250×4.6 mm) using a mobile phase of sodium phosphate, pH 7.0 at the concentrations indicated. Flow was 0.96 ml/min to achieve run times of less than 3.5 min. The injection volume was 2.5 $\mu$l and the column temperature was ambient. Detection, represented on the y-axis, was carried out at 230 nm. (A); peak 1=Mouse IgG1,k (MOPC 31C); (B); peak 1=IgG2a,k (UPC 10); (C); peak 1=IgG1,k (MOPC 31C). Peak 2 is Tris salt (permeation volume).

quite similar except that the antibody is permanently retained at the 20 m*M* phosphate concentration. The extent of positive charges available on the surface of this antibody appears greater than the antibody in Fig. 4A, attracting it more strongly to the column packing at low ionic strengths. For the strongly basic antibody shown in Fig. 4C, elution does not occur at 20 or 50 m*M* phosphate. The peaks are broadened at all but the 200 m*M* phosphate concentration. Also, the peak is extremely broad and strongly retained at 1000 m*M* phosphate.

It is not often that the chromatographer would operate under the extreme mobile phase conditions used here. These examples were used to demonstrate elution characteristics that one might observe for certain antibodies (and other proteins) when using the wide variety of mobile phase conditions often used for SEC chromatography. As mentioned in our previous paper, the elution characteristics in SEC are highly dependent upon the protein molecule used [12].

In SEC separations, the pH of the mobile phase has a significant effect on the peak shape and elution time of molecules. This is ultimately due to the change in charge on sample molecules being separated. In addition, the column packing can be charged, having a significant effect on chromatography. The column used in these experiments is silica based and its surface contains silanols that are ionized and negatively charged except at very low pH. The pH of the mobile phase affects equilibrium between the charged and uncharged forms of functional groups on both the sample and the column packing. This charge-effect changes the affinity of the molecules for each other and for the column packing, having an effect on peak shape and retention through electrostatic, hydrophobic and solubility effects. As in Figs. 4 and 5 shows the effect of

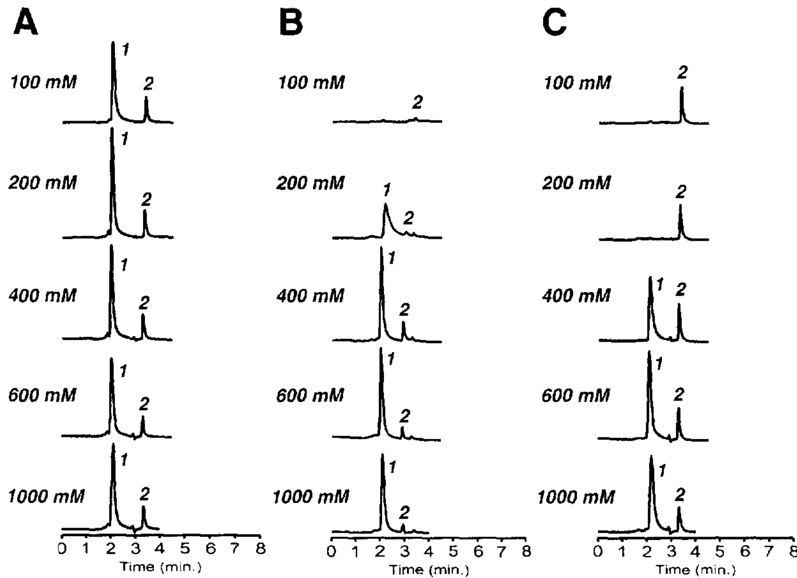


Fig. 5. Effect of mobile phase ionic strength on elution characteristics of mouse myeloma antibodies at pH 5.5. Three different antibodies were separated on a Zorbax GF-250 column (250×4.6 mm) using a mobile phase of sodium phosphate, pH 5.5 at the concentrations indicated. Other conditions and sample identities as in Fig. 4.

mobile phase concentration on the peak shape and elution time of the three mouse myeloma antibodies. In this experiment, the concentration of sodium phosphate was again varied between 20 and 1000 m*M*; the pH used was 5.5. With the change of mobile phase pH from 7 to 5.5, two significant differences in the chromatography are observed. First, all three antibodies are retained more strongly on the column (i.e., higher sodium phosphate concentration was required for proper elution). Second, even at very high ionic strengths, none of the antibody peaks are badly broadened or retained. These observations are consistent with the explanation that little change in ionization of surface silanols occurs when mobile phase pH is changed from 7 to 5.5; while accessible charge on the antibody becomes increasingly positive. This increased positive charge would cause increased attraction of the molecule to the packing, causing an increase in the concentration of phosphate buffer needed to shield these charges and restore expected elution. Using this same explanation, the increased positive charge on the antibody surface would make the molecule appear more polar and would function to reduce its hydrophobic interactions with the column. At first, this seems counterintuitive; ionization of silanols to $-Si-O^-$ should occur in the pH range 3 to 8 [14] and ionization of amine groups on proteins in the range of 9 to 12 [15]. The rate of increase in un-ionized silanols should exceed the rate of $NH_3^+$ formation. This is most likely the case; however, we are looking at the overall ability of sample molecules to interact with the column packing. It is not difficult to envision pH-dependent structural changes in the protein molecule that expose more positively charged domains. At the same time, this structural shift could mask hydrophobic domains of the molecule, as they become internalized.

## 4. Conclusions

SEC continues to be an important technique in bioseparations because it can be performed using non-denaturing mobile phase conditions and because it can separate molecules on the basis of molecular mass. However, while these mobile phase conditions may be best for the sample molecule, they can

promote non-ideal SEC interactions and the development of methods that are not strictly based on molecular size and therefore, are not rugged. This non-ideal behavior is due to electrostatic and hydrophobic interaction of the sample with the column packing. The resulting change in elution time and peak shape is an inconvenience when trying to base a separation solely on size in order to estimate molecular mass. Most importantly, SEC column packings are not manufactured for operation under interactive conditions and as a result, may not operate reproducibly. Since electrostatic and hydrophobic interactions have large effects on retention, any differences in the column packing or system conditions will be dramatically observed. For this reason, the following guidelines for rugged SEC method development are suggested.

1. Determine the separation goal. Enough is sufficient!
2. Minimize non-ideal SEC effects. Start with mobile phase conditions suggested by the manufacturer. Determine adequacy of the separation at 50 and 200% of the mobile phase ionic strength. Remember that retention due to electrostatic and hydrophobic interaction changes logarithmically with ionic strength.
3. Depending on mobile phase pH, a certain range of ionic strength is required to minimize interactions between the sample and the packing. For sodium phosphate, pH 7 (200 to 400 m*M*) and pH 5.5 (600 m*M*).
4. Separation efficiency may be increased by increasing the column length. Decreasing particle size of the column packing also increases efficiency.
5. Separation efficiency in SEC is not limited by sample concentration but is reduced by high sample viscosity large and injection volumes. To obtain optimal efficiency, reduce the injection volume to ≤10% of the peak volume (ca. ≤100 μl).
6. When reduced separation time is required, use increased flow-rate. There is only a small loss in efficiency when using these (250×9.4 mm) silica-based columns at 2 ml/min. Tolerable separation can be obtained even at 5 ml/min.
7. When working under sample-limiting conditions, use a narrower column diameter. A 250×4.6 mm column was used in some of these experiments.
8. Use simple mobile phases. Complicated mobile phases lead to complicated separations.

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