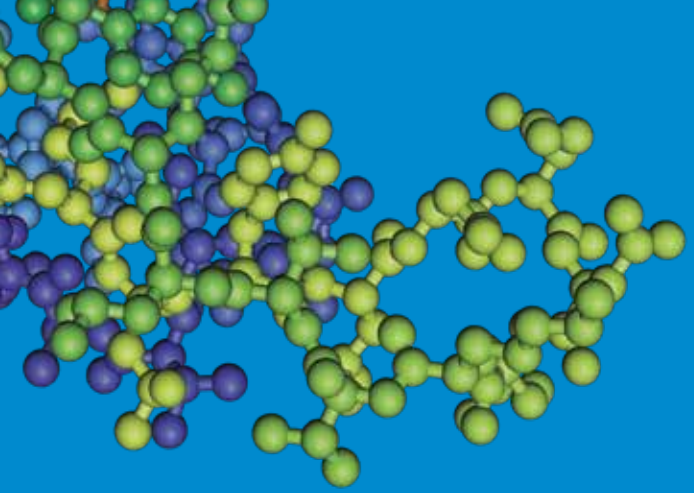
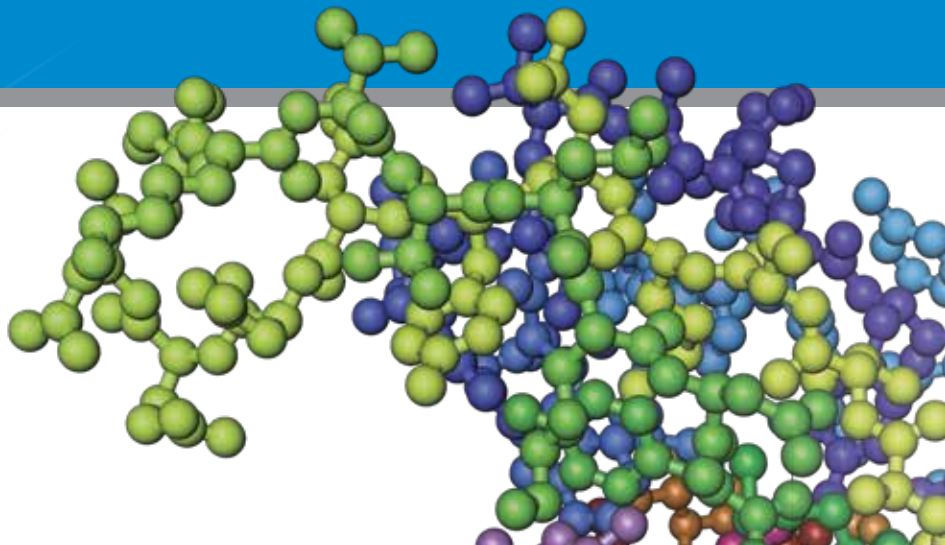


Guide to Size-Exclusion Chromatography (SEC) of mAb Aggregates, Monomers, and Fragments

Waters

THE SCIENCE OF WHAT'S POSSIBLE.™



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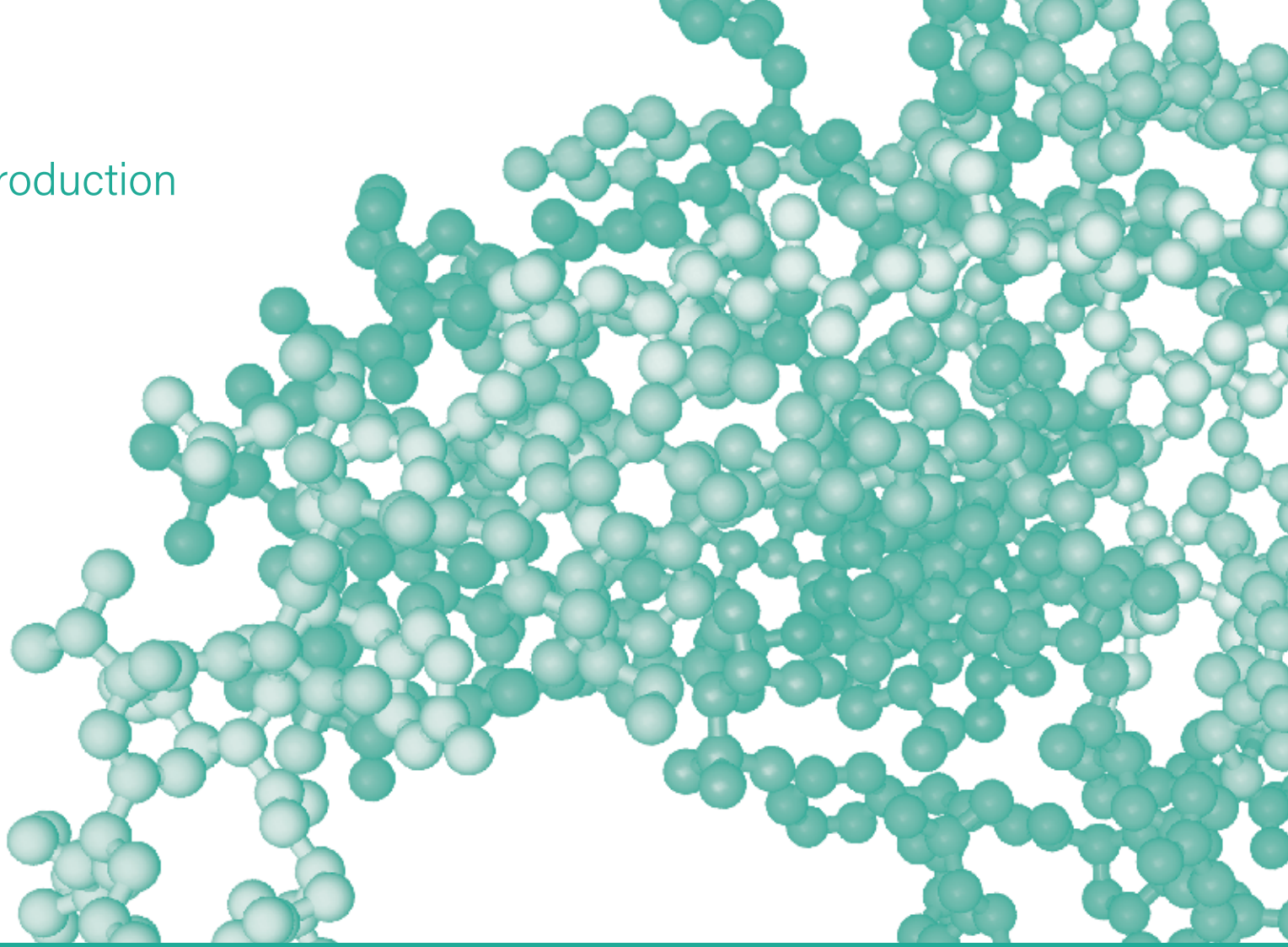
I. Introduction



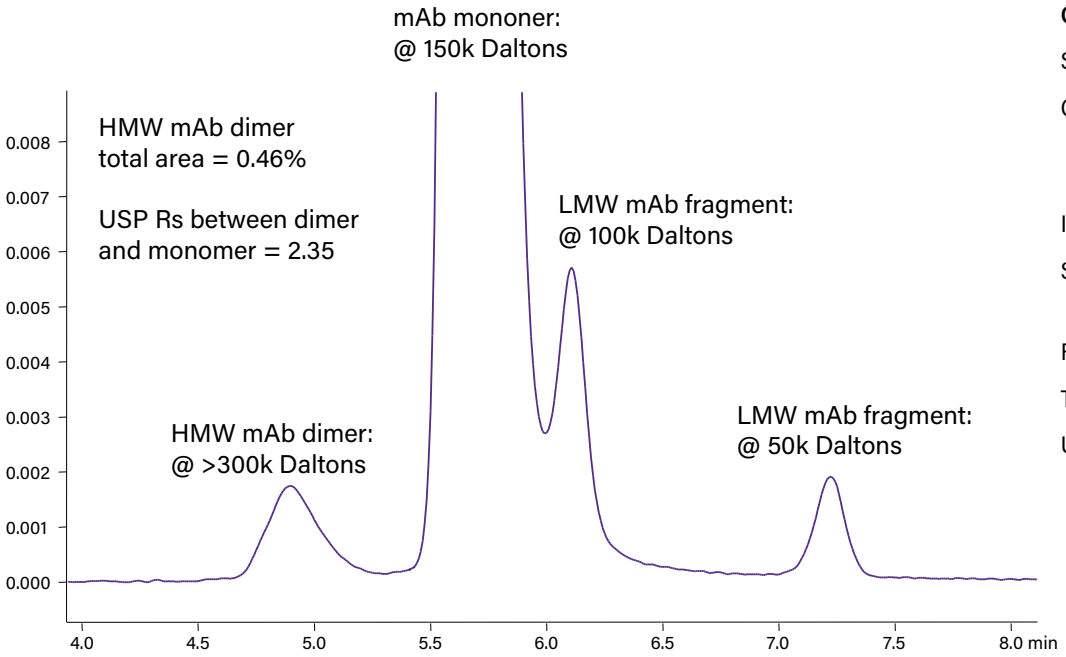
I. Introduction

While numerous analytical techniques have been used for the analysis of biotherapeutic protein aggregates and associated fragments, size-exclusion chromatography (SEC) has been favored in the research, development, and QC testing of these species due to the technique's accuracy, reproducibility, and relative use of use. In the early 1970s, use of LC-based SEC columns containing greater than 20 µm derivatized porous silica particles (e.g., Diol coated) of an appropriate pore size distribution were used for protein separations based ideally on differing hydrodynamic volumes (i.e., relative size in solution or Stokes Radii) of the separated species. To achieve this, there should be no secondary interactions (e.g., hydrophobic or ionic) between the proteins and the SEC chromatographic particles.

In 2010, Waters™ introduced the use of SEC columns containing porous hybrid organic/inorganic particles for protein-based SEC. Compared to silica-based, diol coated particles, SEC columns containing bridged ethylene hybrid (BEH) particles with surface modified diol groups, provided a significant reduction in silanol activity, thus reducing undesired ionic interactions between SEC particles and proteins. In addition, the high mechanical strength of the BEH particles enables reduction in particle size to 1.7 µm, providing gains in chromatographic efficiency and the ability to effectively reduce SEC analysis times. This technology, as implemented on an appropriately designed UPLC™ System, enabled the separation and accurate quantitation of monoclonal antibody (mAb) aggregate species (>300,000 Da) from the desired mAb monomer (150,000 Da) as well as lower molecular weight fragments (e.g., <100,000 and 50,000 in 8 min [Figure 1]). To address various LC system specifications and configurations (e.g., system operating pressure limitations and dispersion volumes), the same BEH-based, SEC particles are available in 2.5 µm and 3.5 µm sizes to obtain comparable SEC protein separations with proportional increases in analysis times compared to results possible using 1.7 µm particles (see references for more information).

This document is intended to provide important factors to be considered in this application area. While some of the examples detail UPLC-based, SEC separations using 1.7 µm particles, the same principles and "tips and tricks" apply to those using SEC columns containing 2.5 µm and 3.5 µm sizes.

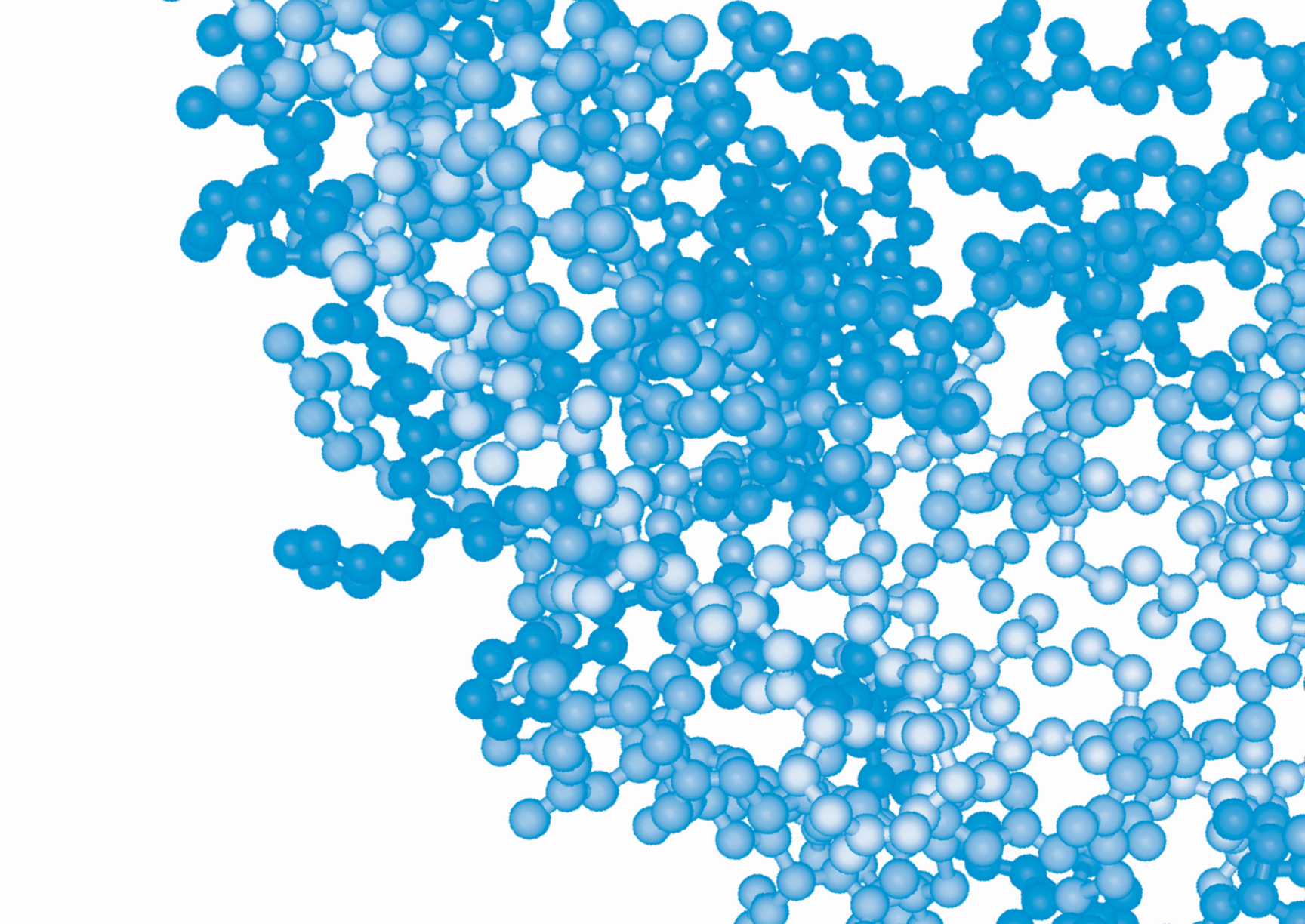
Figure 1



Conditions

System:	ACQUITY UPLC H-Class Bio
Column:	ACQUITY UPLC Protein BEH SEC, 200 Å, 1.7 µm, 4.6 x 300 mm (p/n: 186005226)
Injection:	5 µL
SEC eluent:	25 mM sodium phosphate with 0.15 M sodium chloride, pH 6.8
Flow rate:	0.4 mL/min
Temp.:	30 °C
UV detection:	280 nm

ACQUITY™ UPLC BEH SEC separation of a biotherapeutic monoclonal antibody (mAb).



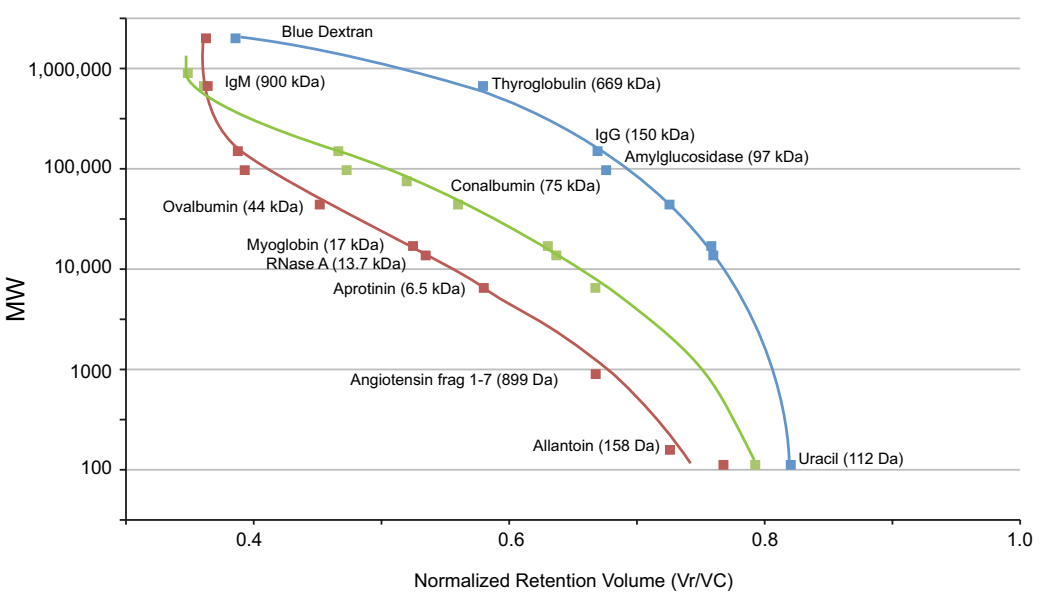
II. Importance of Developing a Robust LC-Based BEH SEC Method

II. Importance of Developing a Robust LC-Based BEH SEC Method

a. Choosing the Appropriate ACQUITY UPLC Protein BEH SEC Column

A number of factors need to be evaluated in the development of a LC-based Protein BEH SEC separation method that can reliably separate and accurately quantitate the various constituents contained in a protein sample. SEC separates compounds primarily based on their relative size in solution. Figure 2 shows calibration curves generated on ACQUITY UPLC Protein BEH SEC Columns of different pore sizes. This data is based on the use of proteins and peptides of known molecular weights. Chromatographers can use this data to select the most appropriate Protein BEH SEC column for a specific application performed on an appropriately configured Waters ACQUITY UPLC System.

Figure 2



ACQUITY UPLC Protein BEH SEC, 125 Å, 1.7 µm: 1K–80K Daltons

ACQUITY UPLC Protein BEH SEC, 200 Å, 1.7 µm: 10K–450K Daltons

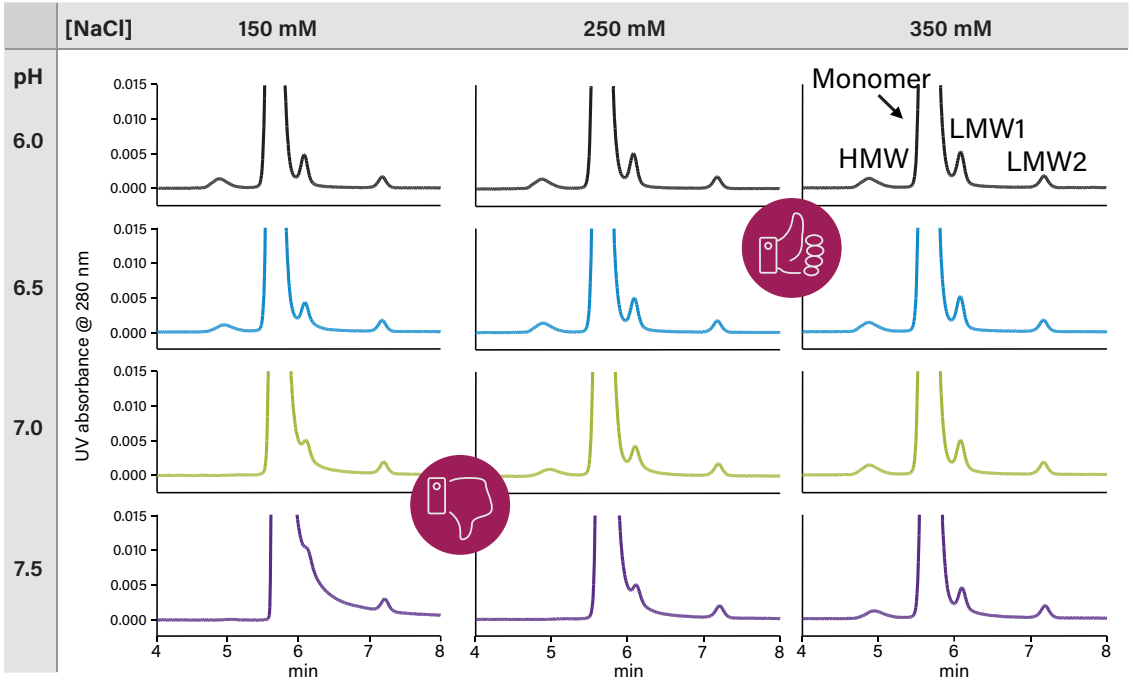
ACQUITY UPLC Protein BEH SEC, 450 Å, 2.5 µm: 100K–1500K Daltons

Calibration curves for ACQUITY UPLC Protein BEH SEC, 125 Å, 200 Å, and 450 Å Columns.

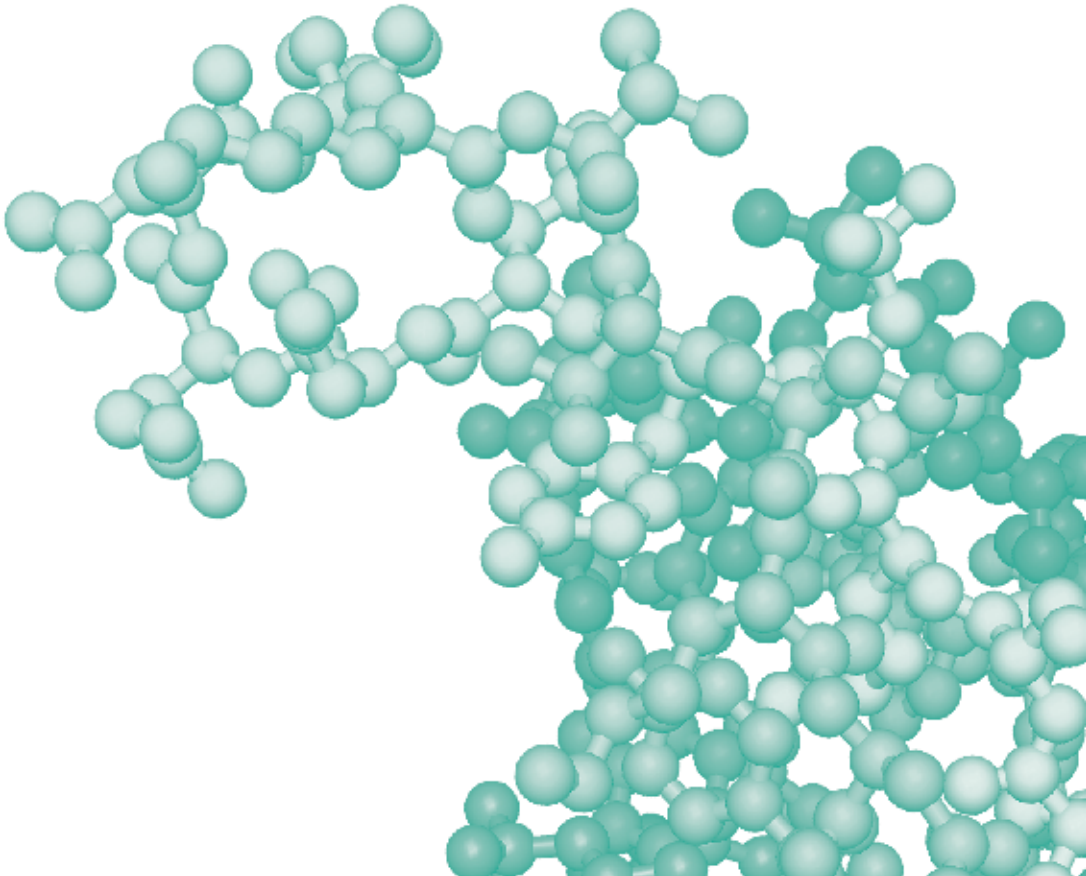
b. Method Development

Many factors need to be evaluated in SEC method development. Ideally, SEC separations are based on differences in the size of native proteins in solution. For this reason, size-exclusion chromatography of biomolecules is usually performed under aqueous, "physiological" conditions. However, the presence of secondary interactions can obscure the desired size-based separation and accurate quantitation of the resolved species. To minimize secondary interactions, the mobile phase and separation conditions need to be evaluated. It should be noted that the conditions used for the SEC separation can alter the protein structure and state. The concentration and type of salt used, as well as, the mobile-phase pH, can affect the three-dimensional structure and the protein-protein interactions. For these reasons, evaluation of a SEC method should be performed with the actual sample to be analyzed. (For more information, reference "Method Development for Size-Exclusion Chromatography of Monoclonal Antibodies and Higher Order Aggregates", p/n: 720004076EN on www.waters.com).

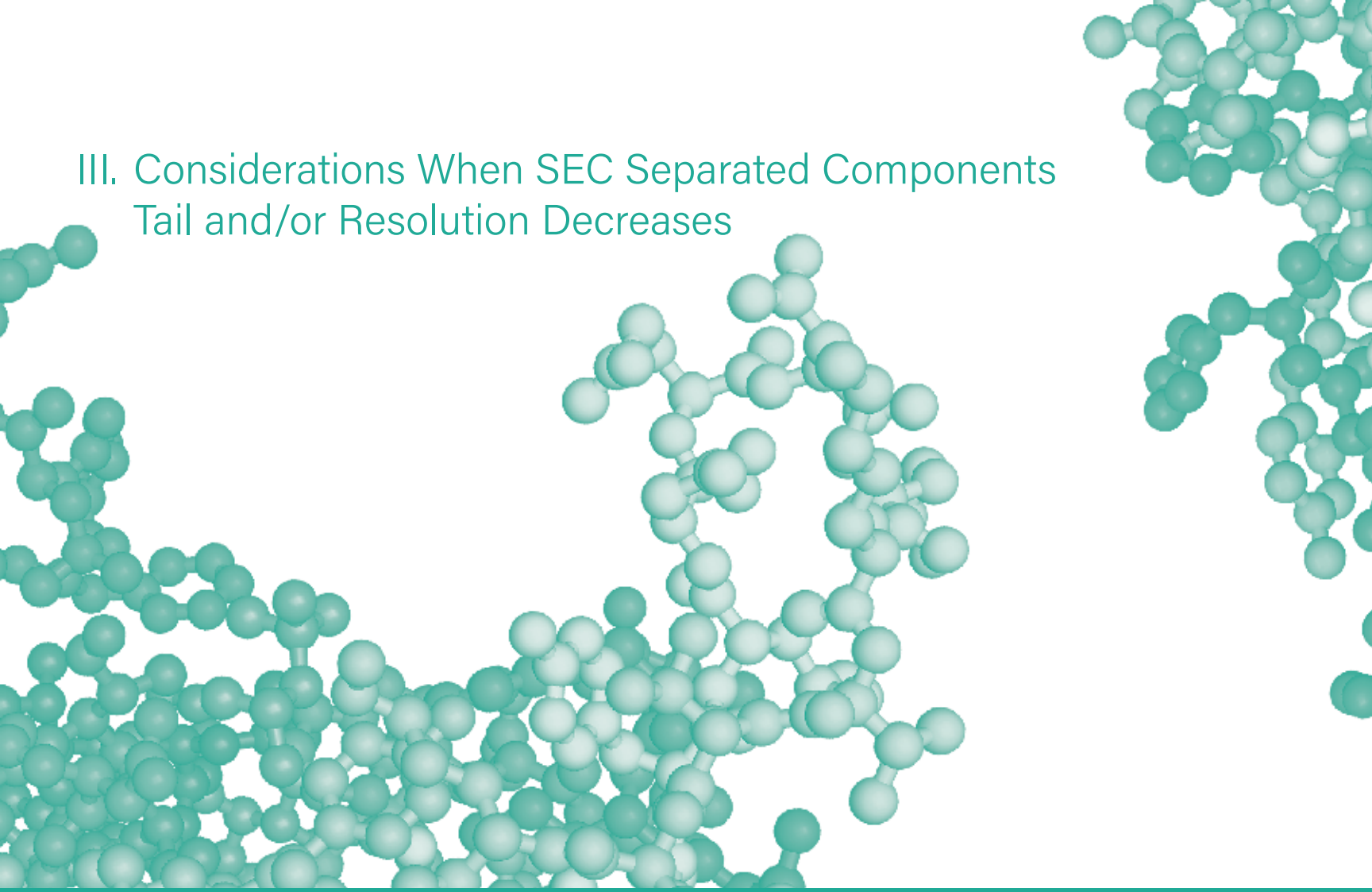
Figure 3



The effect of pH and ionic strength on the separation of a mAb using an ACQUITY UPLC Protein BEH SEC, 200 Å, 1.7 µm, 4.6 x 300 mm Column.



III. Considerations When SEC Separated Components Tail and/or Resolution Decreases



III. Considerations When SEC Separated Components Tail and/or Resolution Decreases

a. Connecting an ACQUITY UPLC Protein BEH SEC Column to an LC System

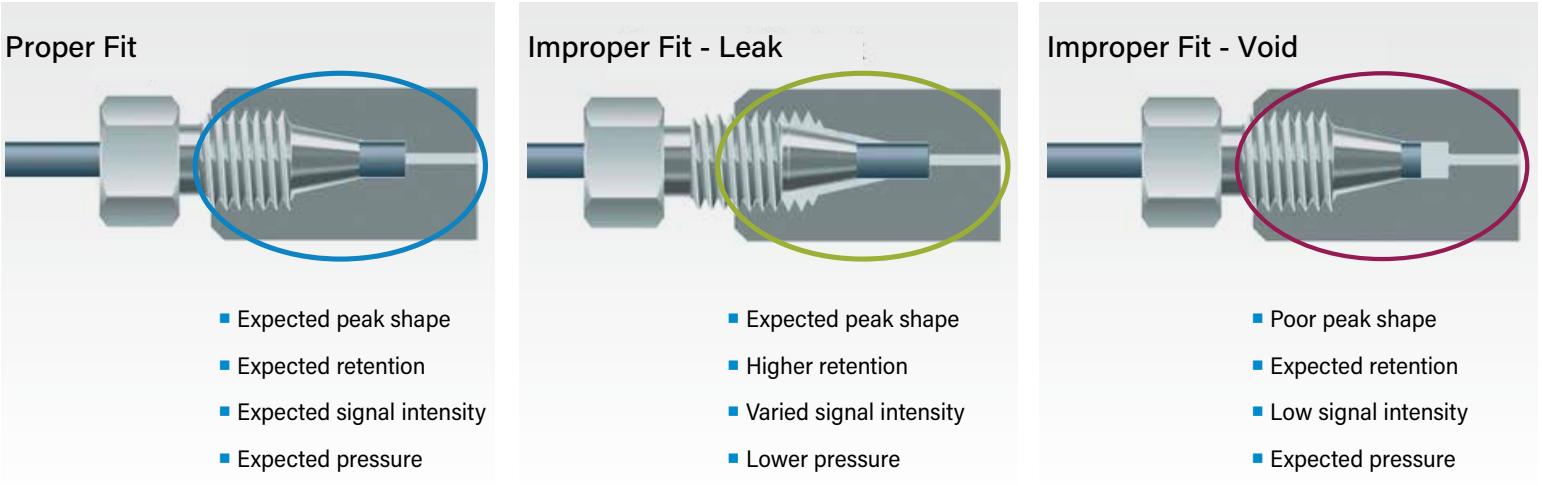
It is critical that the ACQUITY UPLC Protein BEH SEC Column be correctly installed onto the LC system. Figure 4 details the effects of proper vs. improper column connections on chromatographic performance. It is strongly recommended that new column inlet and outlet connections be made whenever a new ACQUITY UPLC Protein BEH SEC Column is installed onto the LC system.

▻► For more information on proper column connections, reference these videos on www.waters.com:

How to Install a UPLC Column onto an ACQUITY UPLC System with an Active Column Preheater

How to Connect an ACQUITY UPLC Column to an ACQUITY UPLC System

Figure 4

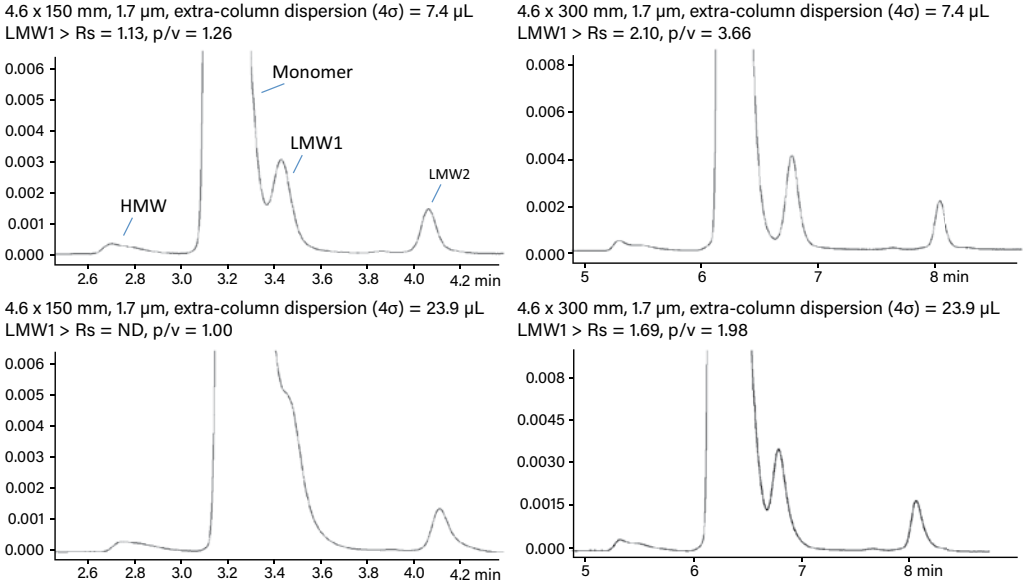


Effects of column-to-LC-system connections on chromatographic performance.

b. LC System Dispersion Effects on a UPLC SEC Separation

In SEC, analytes elute within a single column volume during the isocratic separation. It is therefore critical to appreciate that the total LC system volume, including the injector, tubing, and detector volumes, affect the obtained separation. In general, and as shown in Figures 5 and 6, the lower the total LC system dispersion volume relative to the column volume, the narrower the peaks. The more dramatic effect of LC system dispersion volume on the ability to resolve challenging and closely eluting peaks (e.g., mAb monomer from LMW mAb clip 1) is shown in Figure 5. For ACQUITY UPLC Protein BEH SEC separations, it is recommended that the total UPLC system dispersion volume be less than 15 µL.

Figure 5



Effect of LC system dispersion on an ACQUITY UPLC Protein BEH SEC, 200 Å, 1.7 µm Column separation of infliximab, mAb IgG1.

Conditions

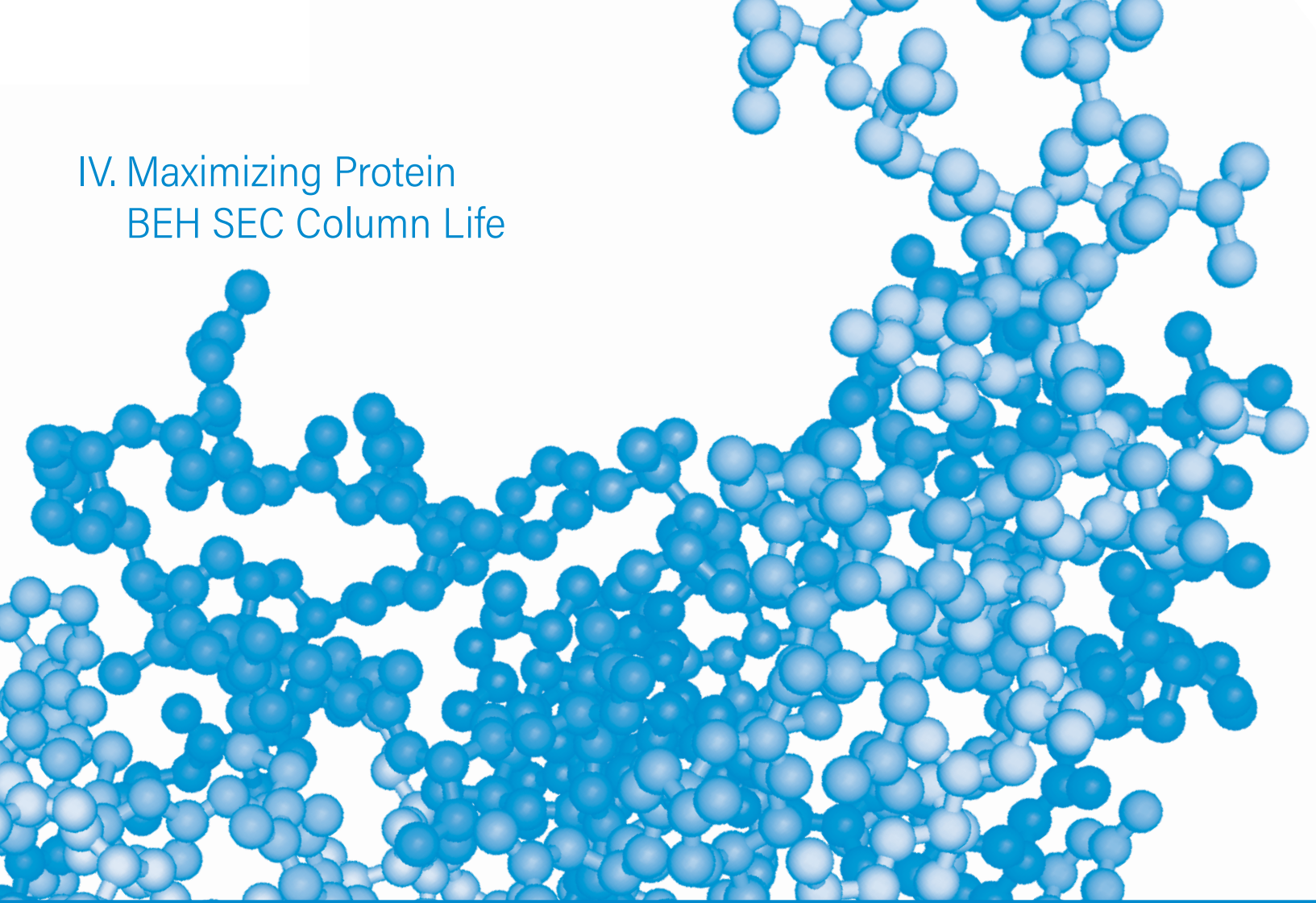
System:	ACQUITY UPLC H-Class Bio with Tunable UV (TUV) detector
Column:	ACQUITY UPLC Protein BEH SEC, 200 Å, 1.7 µm, 4.6 x 150 mm (p/n: 186005225)
Sample:	2 mg/mL mAb IgG1 (infliximab)
Injection:	1 µL for single, 4.6 x 150 mm column; 2 µL for 4.6 x 300 mm column (i.e., two 4.6 x 150 mm columns in series)
SEC eluent:	20 mM sodium phosphate with 350 mM sodium chloride, pH 6.8
Flow rate:	0.35 mL/min
Temp.:	30 °C
UV detection:	280 nm

c. Determining the LC System Dispersion Volume

1. Replace the column with a Zero-Volume Union (p/n: 700002636). The LC tubing should be 0.005" I.D. or smaller.
2. Purge all LC solvent, wash, and purge lines with water, then 50/50 water/acetonitrile.
3. Set the detector to 273 nm and collect data at >40 points per second.
4. Flow rate: 0.5 mL/min.
5. Run time: 1 min.
6. Sample: 0.16 mg/mL caffeine in 50/50 water/acetonitrile.
7. Injection volume: 0.5 µL.
8. Inject three mobile-phase blanks followed by five caffeine sample injections.
9. To calculate the LC system volume:
   - Measure the caffeine peak width (in minutes) at 4.4% peak height.
   - Multiply the peak width by the flow rate to determine the peak volume width in mL.
   - Multiply the peak volume width in mL by 1000 to determine the peak volume width in µL.

Note: The average ACQUITY UPLC H-Class System volume dispersion, when measured using 4-Sigma Method, should be = 10.0 µL when used with CH-A, = 12 µL for CM-A, and <22.0 µL for 30CH-A column heater. If your value is greater, determine the source(s) of the deleterious extra peak dispersion volume and correct.

IV. Maximizing Protein BEH SEC Column Life

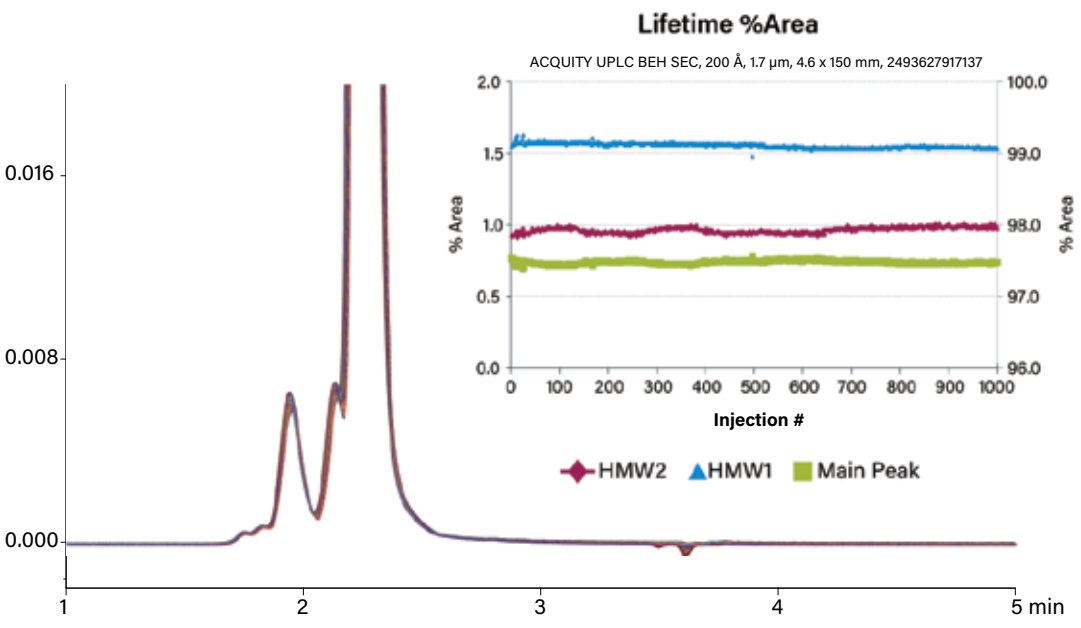


IV. Maximizing Protein BEH SEC Column Life

With proper care in preparing and handling the SEC mobile phase and samples, Protein BEH SEC columns can deliver stable performance for >1000 sample injections (Figure 6). However, the performance and lifetime of a BEH SEC column will deteriorate, if the samples and/or mobile phase introduced into the column contain particulates.

To maximize column lifetime, high quality filtered water (e.g., Milli-Q® Millipak 0.22 µm filtered water) should be used, and mobile-phase buffers should be passed through a ≤0.2 µm filter. It is recommended that mobile-phase buffers be filtered using a sterile disposable filter. It is further recommended that sintered glass filter supports be avoided when filtering mobile phases due to the potential of introducing silicates which could alter column performance. Use of sterile 0.2-µm-filter units is recommended.

Figure 6



One hundred overlays from 1000 repetitive injections of commercial Vectibix® mAb formulation on an ACQUITY UPLC Protein BEH SEC, 200 Å, 1.7 µm, 4.6 x 150 mm Column.

Conditions

System:	ACQUITY UPLC H-Class Bio with Tunable UV (TUV) detector
Column:	ACQUITY UPLC Protein BEH SEC, 200 Å, 1.7 µm, 4.6 x 150 mm (p/n: 186005225)
Sample:	Vectibix, 10 mg/mL diluted 1:1 with water
Injection:	3.5 µL
Mobile phase:	0.1 M sodium phosphate with 0.1 M sodium chloride, pH 7.0
Flow rate:	0.5 mL/min
Temp.:	30 °C
UV detection:	280 nm

a. Mobile Phase Preparation and Use

Mobile phases with pH values in the physiological range that do not contain organic solvent can support microbial growth. Implementation of the following recommendations will help minimize microbial contamination, thus avoiding premature column failure.

- Clean laboratory glassware properly and dry at >170 °C for at least 1 hour to avoid potential microbial growth in remaining water droplets. The use of sterile containers is advised.
- Use only high purity water (18.2 MΩ cm) when preparing SEC mobile phases. If bottled water is used, it should be opened the day of use.
- Always filter prepared mobile phase through compatible 0.22 µm or smaller membrane filters. The use of sterile filters and containers is also recommended.
- Never "top-off" mobile-phase bottles. Always change bottles when replacing the mobile phase.
- Replace low-ionic-strength mobile phases (<150 mM) every 2–3 days.
- High-ionic-strength mobile phases (>150 mM) should be replaced every two weeks. This longer interval is acceptable because high salt concentrations will inhibit microbial growth.
- All mobile-phase bottles should be visually inspected daily for microbial growth and/or particulates. Microbial growth may form a film on the bottle surface and may be observed by swirling the bottle.
- **It is highly recommended to remove and not use the Waters Mobile-Phase Line Sinkers when performing SEC with 100% aqueous mobile phases since they may become contaminated with microbes, thus contaminating freshly prepared solutions of filtered mobile phase.**

Note: Waters Corporation will honor an existing service contract at sites that have removed the Mobile-Phase Line Sinkers provided that the account's SOP states that all SEC mobile phases must be filtered and contained in sterile glassware.

- If microbial growth is observed, immediately discontinue SEC analysis and perform the LC cleaning procedure detailed later in this document.

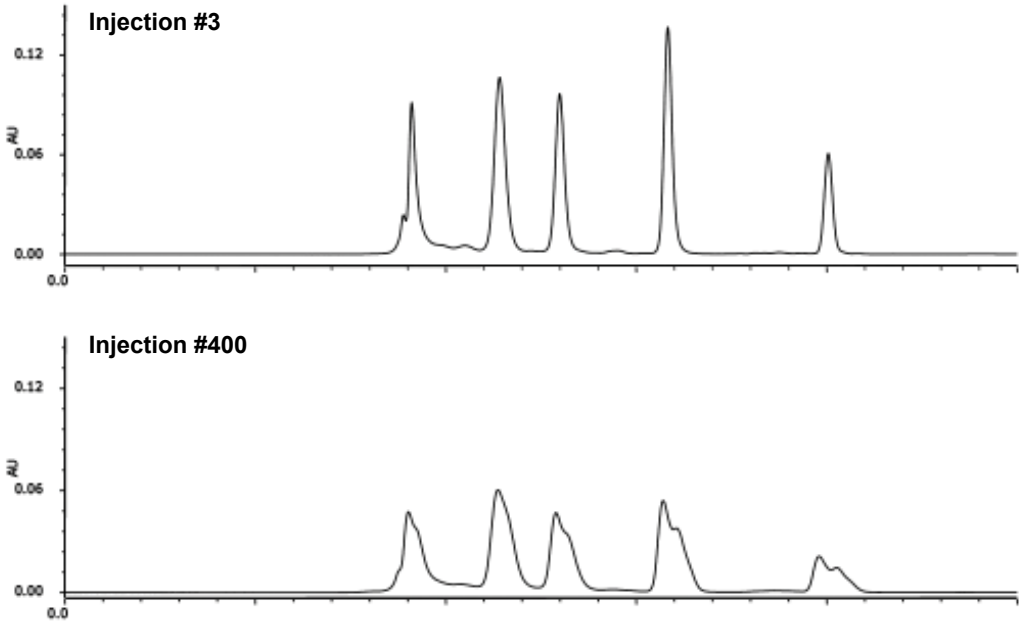
Additional Considerations:

- To prevent potential precipitation of the buffer from the mobile phase in the LC system, good laboratory practice suggests maintaining a low flow rate (e.g., 0.1 mL/min) through the system after the SEC column has been removed and stored as recommended.
- If the LC system will be idle for more than two days, and after the SEC column has been removed and stored as recommended, prime each mobile-phase line for 15 minutes at 1 mL/min with high purity water followed by flushing and storage in 70/30 isopropanol (IPA)/water to prevent microbial growth.

b. Avoiding Microbial Contamination of the Solvent Delivery System

Microbial contamination of the LC system may cause BEH SEC column fouling, resulting in an unacceptable separation (Figure 7). The column pressure may also increase significantly. Note that injection of particulates (e.g., microbes) will result in premature failure of any HPLC or UPLC column. However, failure may occur sooner on columns containing <3 µm particles. If the mobile-phase reservoir becomes contaminated with microbes, the microbes will enter the solvent delivery flow path, and may contaminate fresh mobile phase. The mobile-phase line sinkers are one location that commonly harbors microbes.

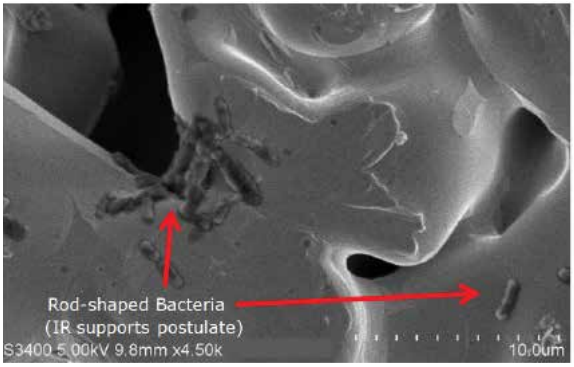
Figure 7



Effect of microbial growth on an ACQUITY UPLC Protein SEC separation of the BEH200 SEC Protein Standard Mix (p/n: 186006519). Contamination was confirmed by analysis of the column inlet frit (Figures 8 and 9).

▻► For sample and separation conditions, reference "Waters ACQUITY UPLC Protein BEH SEC Columns and Standards Care and Use Manual", p/n: 720003385EN on www.waters.com.

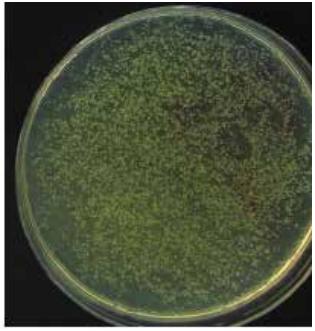
Figures 8 and 9



Scanning electron micrograph of the inlet frit removed from an ACQUITY UPLC Protein BEH SEC, 200 Å, 1.7 µm Column contaminated with bacteria.

a)

b)



Mobile-phase line sinker (Figure a) from a microbe-contaminated SEC mobile-phase bottle. The sinker was aseptically removed, suspended in sterile PBS, then 500 µL was applied to a microbe supporting agar plate. Figure b confirms the presence of microbes contained in the removed SEC mobile-phase line sinker.

c. Cleaning LC Systems

To clean an LC system used in 100% aqueous SEC methods, the use of 100% IPA, as used in the standard UPLC cleaning protocols, should be replaced by the use of 70% isopropanol (IPA) or 70% ethyl alcohol (EtOH) due to their ability to kill bacteria. One hundred percent concentration of either IPA or EtOH does not penetrate the cell wall of bacteria and is therefore not an effective bactericide nor are methanol-containing solutions (Reference 3). Waters has experienced situations where "microbe-contaminated-mobile-phase filters" (a.k.a. sinkers) have resulted in rapid particulate fouling of a new ACQUITY UPLC Protein BEH SEC Column even when freshly prepared and filtered mobile phase is used. This is why Waters recommends that mobile phase sinkers NOT be used for this SEC application as previously detailed.

Note: The suggested LC system cleaning protocols below (e.g., flow rates and times) were developed for the ACQUITY UPLC System. Adjustments in flow rates and flow duration may be necessary for those using different LC systems.

There are three different protocols for cleaning/purging ACQUITY UPLC Systems that can be used under different circumstances.

1. **For a system that is in continuous use with sterile filtered mobile phase:**
   The best practice for the daily use of buffers starts with fresh mobile phase in a new bottle (no topping off). After installing fresh mobile phases, wet prime (a function of Empower™ Software) the buffer lines for 4 minutes if the system was used the day before. If the system was not used the day before, wet prime all lines for 6–10 minutes. This may be accomplished by using the system startup function and changing the prime time as needed. This function also primes the seal wash, purge, and needle wash solvent lines. Keep in mind that when the ACQUITY UPLC System is wet primed, under the control of the ACQUITY UPLC console, the column can remain on the system connected to the pump and detectors.

   The Empower solvent priming function diverts the priming solvents directly to waste. This makes it very easy to purge your lines with mobile phase or the other solvent sequences described below. It does require the movement of your solvent lines, but if you catch a bubble during the process, the wet priming command will eliminate it.

2. **For a system that has been left unused for more than 2–3 days:**
   If your system is left in buffer with or without flow for more than 2–3 days, the lifetime of your column may benefit by wet priming all buffer lines with **water for 4 minutes**, then with **70% IPA for 6–10 minutes** before going **back to water for 4 minutes** and **then your buffer for 4 minutes** before starting another analysis. It is further recommended to leave your system in 70% IPA (after priming the buffer lines with water) when not in use. When restarting, wet prime your system with 70% IPA for 3–4 minutes then water for 4 minutes before wet priming your system with your desired buffer. Before attaching the SEC column to the LC system, allow the freshly prepared SEC buffer to flow through the ACQUITY UPLC System for approximately 5 minutes at a flow of 0.4 mL/min to clear out any previously used mobile phase.

3. **For routine periodic cleaning for preventive maintenance or when the system is contaminated:**
   Table 1 is a summary of the cleaning protocol and reagents required to execute this procedure, which can be used at regular intervals (every 3-6 months) for preventive maintenance.
   a. Disconnect the column and attach a V-Detail Zero Volume Union (p/n: 700002636) to the column inlet and outlet lines.
   b. For each step of the cleaning protocol, monitor the system pressure to keep it <1000 psi to prevent damage to the detector flow cell. If necessary, replace the backpressure device with large bore waste tubing.
   c. If the system has recently been used with mobile-phase buffers or salts, it should be flushed thoroughly with 100% high-purity water prior to the introduction of organic solvents.
   d. Place A, B, C, D, seal wash, sample wash, and purge lines into a clean bottle containing 70% isopropanol as the cleaning solvent.

Note: If there is an incompatibility between the mobile phase or wash solvents with 70% isopropanol, flush first with the appropriate intermediate solutions to ensure compatibility.

e. Prime solvent lines A, B, C, and D for 5 minutes each. The Empower Prime command vigorously forces solvents through the lines and system at 4 mL/min to assist in the cleaning process.

f. Prime the seal wash.

g. Prime the wash solvent for 200 seconds and the purge solvent for 40 cycles.

h. Purge the system at 0.2 mL/min for 10 minutes using 25% A, 25% B, 25% C, and 25% D.

i. Repeat Steps d through h using **100% methanol** as the cleaning solvent, using a flow rate of 1.0 mL/min for Step h.

j. Repeat Steps d through h using **100% high-purity water** as the cleaning solvent, using a flow rate of 1.0 mL/min for Step h.

   Caution: If a nebulization-based detector is connected (MS or ELSD), take it offline before performing Step k. Direct the flow from the union outlet to waste.

k. Repeat Steps d through h using **10% aqueous phosphoric acid** as the cleaning solvent, using a flow rate of 0.2 mL/min for Step h.

   Caution: To avoid damage, do not place the seal wash line in this solution. Place the seal wash line in high purity water.

l. Repeat Steps d through h using **100% high-purity water** as the cleaning solvent, using a flow rate of 1.0 mL/min for Step h.

m. If applicable, reconnect the MS or ELS detector.

n. Repeat Steps d through h using **100% methanol** with a flow rate of 1.0 mL/min for Step h.

Table 1. ACQUITY UPLC System Cleaning Protocol Summary. *Check (✓) when step is completed.*

Eluent	Flow Rate	Prime Lines A, B, C, D (5 min each)	Prime Wash Solvent (200 sec)	Prime Purge Solvent (40 cycles)	Prime Seal Wash	Purge A/B/C/D 25/25/25/25 (10 min)
IPA	0.2 mL/min					
Methanol	1 mL/min					
High Purity Water	1 mL/min					
Leave seal wash line in high purity water. If used, remove nebulization-based detectors (MS or ELSD) from flow path.						
10% Aqueous Phosphoric Acid	0.2 mL/min					
High Purity Water	1 mL/min					
If used, return nebulization-based detectors to flow path.						
Methanol	1 mL/min					

Required Cleaning Solvents	
100% high purity water	Milli-Q water or equivalent
100% methanol	Fisher Methanol, Optima™ #A454-4 or equivalent
70% isopropanol (IPA) in high purity water (v,v)	Fisher 2-Propanol, Optima #A464-4 or equivalent
10% phosphoric acid (aqueous)	Prepare 10% (v/v) using HPLC-grade 85% phosphoric acid and high-purity water

d. Minimizing Sample Particulate Contamination of Protein BEH SEC Columns

The performance and lifetime of an ACQUITY UPLC Protein BEH SEC Column can deteriorate due to the accumulation of particulates. These include insoluble protein aggregates contained in the injected sample. As a result, it is recommended to filter all protein-containing samples using sterile, low protein binding, 0.2 µm syringe filters. Alternatively, ACQUITY UPLC Sample Vials containing sample can be centrifuged so that any insoluble particulates form a solid pellet at the bottom of the vial.

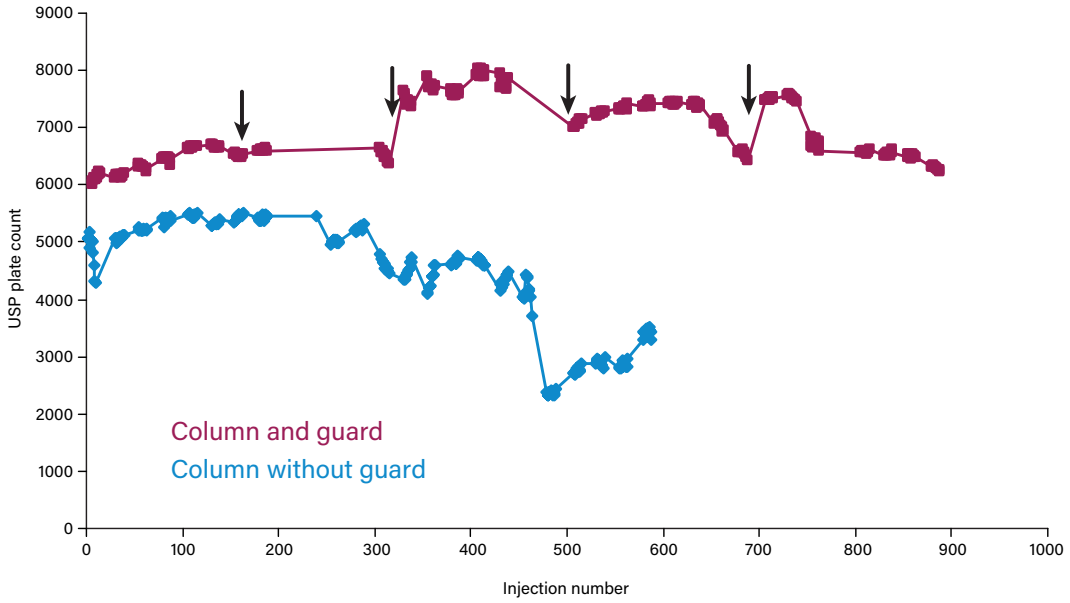
Note: Be sure to adjust the ACQUITY UPLC needle depth to prevent sampling any particulates located at the bottom of the centrifuged vial. For more information on setting the needle depth and minimum volume in vials, reference "Sample Vials and Accessories", p/n: 720001818EN on www.waters.com.

e. Preventing Sample Formulation Constituent Contamination of Protein BEH SEC Columns

Studies have found a variety of chemical and physical conditions that can cause the creation of biotherapeutic protein aggregates. To minimize this occurrence, excipients are typically added to therapeutic protein formulations. However, these excipients can cause inaccurate SEC quantitation due to changes in retention, peak shape, or resolution. While a variety of SEC column cleaning protocols may be used to restore a column's performance, this can be time consuming and ultimately ineffective. As a last resort, column replacement is often required, resulting in higher costs and delays. As indicated in Figure 10, the use of guard columns may protect Protein BEH SEC columns from deterioration due to chemical contamination.

▻► For more information, reference “Improving the Lifetime of UPLC Size-Exclusion Chromatography Columns Using Short Guard Columns”, p/n: 720004034EN on www.waters.com.

Figure 10



Effect of using an ACQUITY UPLC Protein BEH SEC Guard Column (4.6 x 30 mm) on column efficiency. The arrows indicate where the guard column was changed, noting how the column life was extended (top/red) vs. a control that did not use a guard column (bottom/blue).

f. Column Storage

To prevent the potential of microbial growth in SEC columns that will be removed from your system and stored, we recommend the following:

1. Start by using a solvent line that is already in 100% water. Then using this line, flush the existing mobile phase from the column with approximately 10-column volumes of HPLC grade and filtered water (e.g., 25 mL for 4.6 x 150 mm columns) to remove any buffer and salts from the SEC column.
2. After preparing the UPLC system as needed, flush the SEC column with approximately 10-column volumes of filtered 20% methanol/80% water to prevent bacterial growth. The column can then be safely stored at room temperature.
3. For short-term storage (e.g., <3 days), removing the SEC column from the LC system and storing at 4 °C can also be employed. Care must be taken to avoid freezing the mobile phase in the SEC column.

Note: It is important that all salt and/or protein be flushed from the BEH SEC column BEFORE storage in 20% MeOH. Failure to remove salt and/or protein may cause precipitation in the column when it is exposed to the 20% organic storage solvent.

V. Summary and References

V. Summary and References

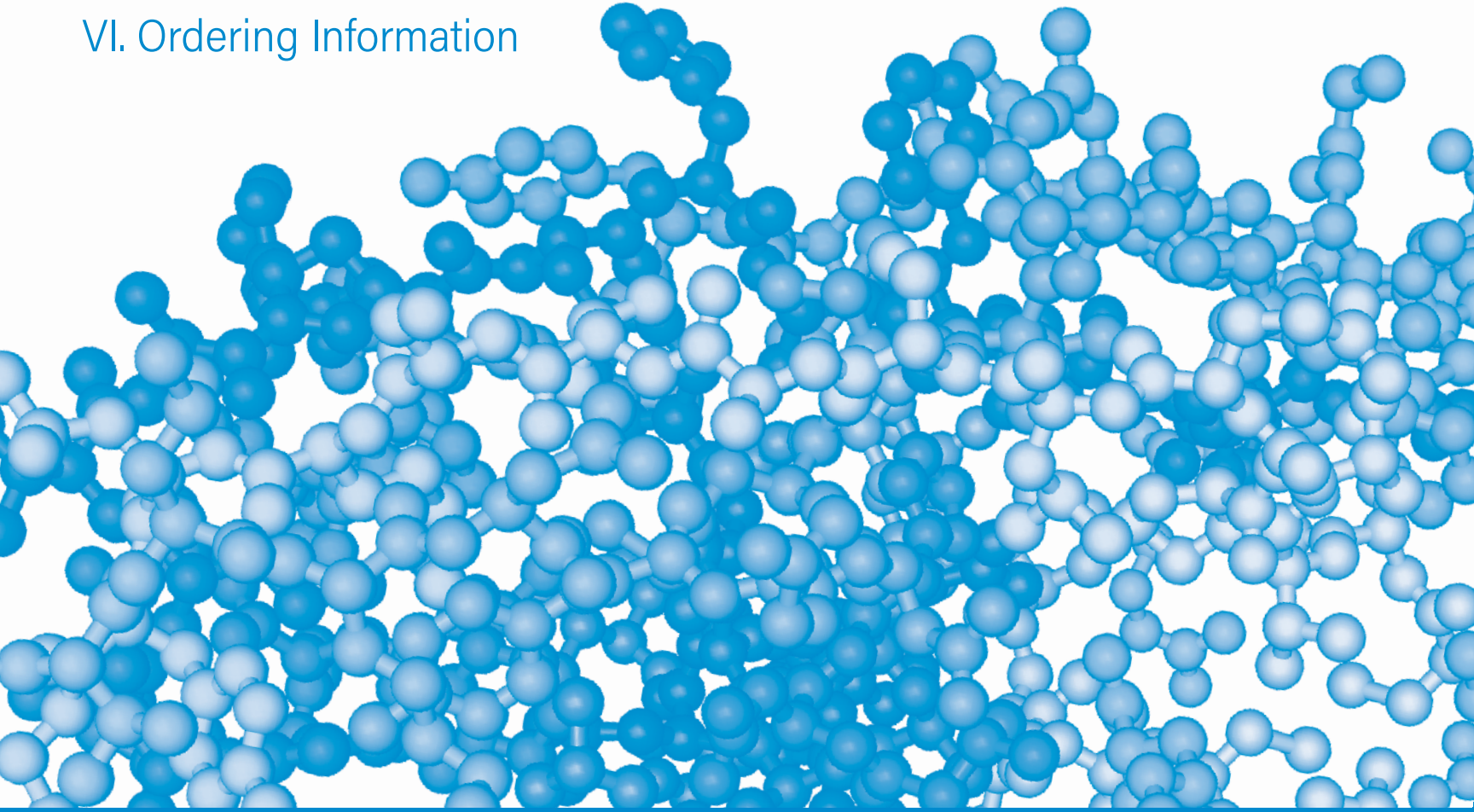
Summary

With the practices described, robust ACQUITY UPLC Protein BEH SEC analyses can be achieved, offering faster and higher resolution separations than those obtained using traditional HPLC-based SEC methods. To maximize the lifetime of an ACQUITY UPLC Protein BEH SEC Column, it is important to avoid microbial growth in the mobile phase as well as to ensure that injected samples are free of particulates. The use of BEH SEC guard columns can be an effective means to protecting your analytical SEC column from deterioration due to these various types of failure mechanisms.

References

1. Hong, P.; Koza, S.M.; Bouvier, E.S.P. A Review: Size-exclusion chromatography for the analysis of protein biotherapeutics and their aggregates. *J. Liq. Chrom. Rel. Tech.* **2012**, *35*(20), 2923-2950.
2. Bouvier, E.S.P.; Koza, S.M. Advances in size-exclusion separations of proteins and polymers by UHPLC. *Trends Analyt. Chem.* **2014**, *63*, 85-94.
3. Guideline for Disinfection and Sterilization in Healthcare Facilities, 2008, William A. Rutala, David J. Weber, M.D., and the Healthcare Infection Control Practices Advisory Committee. US CDC, Atlanta, Georgia. (https://www.cdc.gov/hicpac/pdf/guidelines/Disinfection_Nov_2008.pdf)
4. Koza, S. M.; Reed, C. E.; Chen, W. Evaluating the impact of LC system dispersion on the size-exclusion chromatography analysis of proteins. Waters Application Note, 720006337EN (2019).
5. Koza, S. M.; Reed, C.; Chen, W. Impact of LC system dispersion on the size-exclusion chromatography analysis of monoclonal IgG antibody aggregates and fragments: selecting the optimal column configuration for your method. Waters Application Note, 720006336EN (2019).
6. Hong, P.; Fountain, K. Method development for size-exclusion chromatography of monoclonal antibodies and higher order aggregates. Waters Application Note, 720004076EN (2011).

VI. Ordering Information



a. BEH SEC Column Selection Guide

Important Considerations in SEC Column Selection			
What is the molecular weight of your sample?			
	MW 1–80K Da	MW 10–450K Da	MW 100–1500K Da
Pore Size Recommendation	125 Å	200 Å	450 Å
What is the dispersion of your LC system?*			
	<20 µL (UPLC™)	>20–≤35 µL (UHPLC)	>35 µL (HPLC)
Particle Size Recommendation	1.7 µm	2.5 µm	2.5 µm
Are you separating components <2-fold difference in MW?**			
	1.7 µm	2.5 µm	2.5 µm
Column Dimension Recommendation	4.6 x 300 mm	7.8 x 300 mm	7.8 x 300 mm
Are you separating components ≥2-fold difference in MW?			
	1.7 µm	2.5 µm	2.5 µm
Column Dimension Recommendation	4.6 x 150 mm	7.8 x 150 mm	7.8 x 150 mm

**System dispersion measures at 5 σ peak width. For guidance on measuring system dispersion, reference page 18.*

***Larger column length provides more resolution. To understand the "why" behind these recommendations, read the Application Note (p/n: 720006336EN) on waters.com.*

b. BEH SEC (UPLC, UHPLC, HPLC) Column Offerings

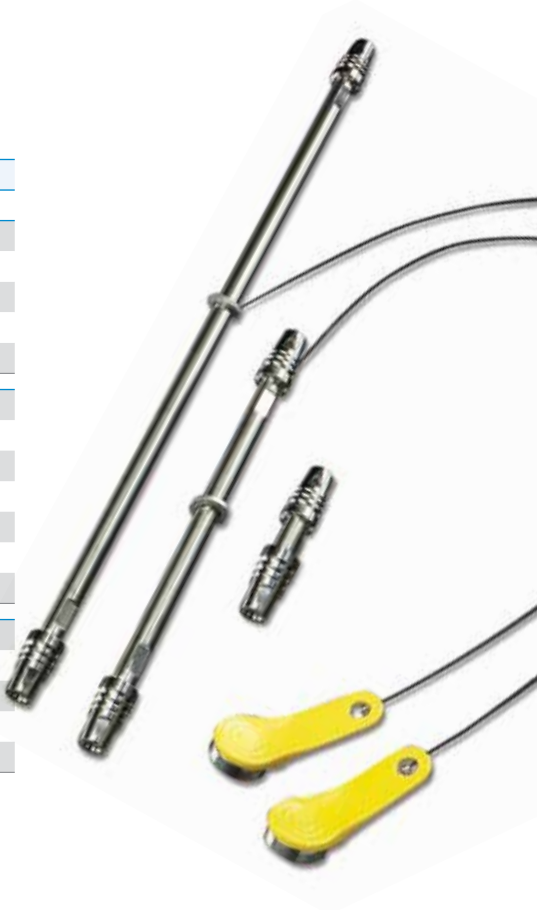
ACQUITY UPLC Protein BEH SEC Columns and Guard Kits

			Particle Size: 1.7 µm	Particle Size: 2.5 µm
BEH SEC, 125 Å	**Dimension**	**Configuration**	**P/N**	**P/N**
	4.6 × 150 mm	Column and Standard	176003906	–
	4.6 × 150 mm	Column	186006505	–
	4.6 × 300 mm	Column and Standard	176003907	–
	4.6 × 300 mm	Column	186006506	–
	4.6 × 30 mm	Guard Kit[1]	186006504	–
BEH SEC, 200 Å	2.1 × 150 mm	Column	186008471	–
	4.6 × 50 mm	Column	186009082	–
	4.6 × 150 mm	Column and Standard	176003904	–
	4.6 × 150 mm	Column	186005225	–
	4.6 × 300 mm	Column and Standard	176003905	–
	4.6 × 300 mm	Column	186005226	–
	4.6 × 30 mm	Guard Kit[1]	186005793	–
BEH SEC, 450 Å	4.6 × 150 mm	Column and Standard	–	176002996
	4.6 × 150 mm	Column	–	186006851
	4.6 × 300 mm	Column and Standard	–	176002997
	4.6 × 300 mm	Column	–	186006852
	4.6 × 30 mm	Guard Kit[1]	–	186006850

1) Size-exclusion chromatography may require modifications to an existing ACQUITY UPLC System. Please reference "Size-Exclusion and Ion-Exchange Chromatography of Proteins using the ACQUITY UPLC System" (p/n: 715002147) or "Size Exclusion and Ion-Exchange Chromatography of Proteins using the ACQUITY UPLC H-Class System" (p/n: 715002909) for specific recommendations.

2) To connect two UPLC SEC Columns together in series, we recommend using a Waters Sample Loop (p/n: 430001516).

[1] All Guard Kits contain a straight piece of 0.005" × 1.75" tubing and end fittings (p/n: WAT022681) to connect the guard column to the analytical SEC column.



XBridge™ Protein BEH SEC HPLC and UHPLC Columns

Description	Dimension	Configuration	P/N	Dimension	Configuration	P/N
	Particle Size: 2.5 µm			Particle Size: 3.5 µm		
BEH SEC, 125 Å Column with BEH125 SEC Protein Standard Mix	4.6 × 30 mm	Guard Kit[1]	176004331	7.8 × 30 mm	Guard Kit[1]	176003591
	4.6 × 150 mm	Column	176004332	7.8 × 150 mm	Column	176003592
	4.6 × 300 mm	Column	176004333	7.8 × 300 mm	Column	176003593
	7.8 × 30 mm	Guard Kit[1]	176004322			
	7.8 × 150 mm	Column	176004323			
	7.8 × 300 mm	Column	176004324			
BEH SEC, 200 Å Column with BEH200 SEC Protein Standard Mix	4.6 × 30 mm	Guard Kit[1]	176004334	7.8 × 30 mm	Guard Kit[1]	176003594
	4.6 × 150 mm	Column	176004335	7.8 × 150 mm	Column	176003595
	4.6 × 300 mm	Column	176004336	7.8 × 300 mm	Column	176003596
	7.8 × 30 mm	Guard Kit[1]	176004325			
	7.8 × 150 mm	Column	176004326			
	7.8 × 300 mm	Column	176004327			
BEH SEC, 450 Å Column with BEH450 SEC Protein Standard Mix	4.6 × 30 mm	Guard Kit[1]	176004337	7.8 × 30 mm	Guard Kit[1]	176003597
	4.6 × 150 mm	Column	176004338	7.8 × 150 mm	Column	176003598
	4.6 × 300 mm	Column	176004339	7.8 × 300 mm	Column	176003599

Note: To connect two HPLC/UHPLC SEC columns together in series, we recommend using a Waters Sample Loop, p/n: 430001516.
[1]All Guard Kits contain a straight piece of 0.005" × 1.75" tubing and end fittings (p/n: WAT022681) to connect the guard column to the analytical SEC column.

ACQUITY UPLC BEH SEC Protein Standards

Description	P/N
BEH125 SEC Protein Standard Mix	186006519
BEH200 SEC Protein Standard Mix	186006518
BEH450 SEC Protein Standard Mix	186006842

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