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XBridge Protein BEH SEC Columns and Standards

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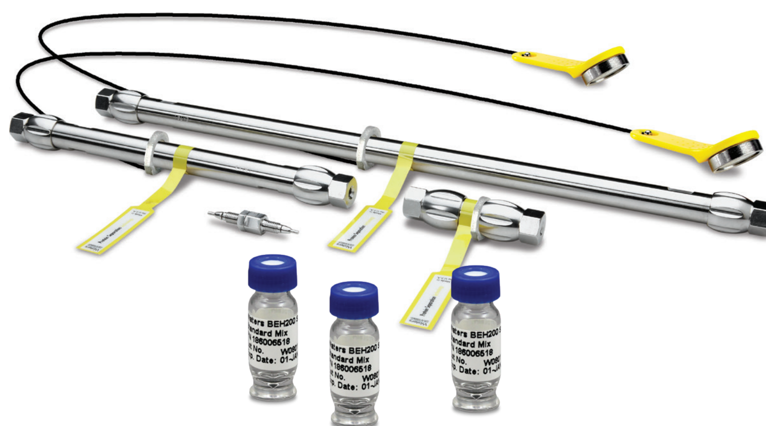
I. INTRODUCTION

Waters™ XBridge™ Protein BEH SEC, 125 Å, 200 Å, and 450 Å, 2.5 and 3.5 µm Columns were developed to complement the existing line of UPLC™-based SEC offerings for use where traditional HPLC-based instrumentation and methods are employed for peptide or protein size-exclusion chromatography (SEC). These HPLC/UHPLC-based, SEC chemistries are based on the same Waters Ethylene Bridged Hybrid (BEH)-based particle technology and diol-bonded surface coating as used in our successful line of UPLC-based SEC columns. This process offers chromatographers the option and ability to easily transfer methods based on lab instrumentation and component resolution or sample throughput needs.

All Waters BEH-based SEC columns are manufactured in a cGMP, ISO 9001 certified plant using stringent manufacturing protocols and ultra pure reagents. Each batch of manufactured material undergoes a series of standard QC measurements (e.g., particle and pore size distribution) followed by an application-specific test using an appropriate peptide and protein test mixture. A packed column efficiency test is then performed on every batch-approved-packed SEC column to further help ensure reproducible batch-to-batch and column-to-column performance for use in research or in a demanding validated method.



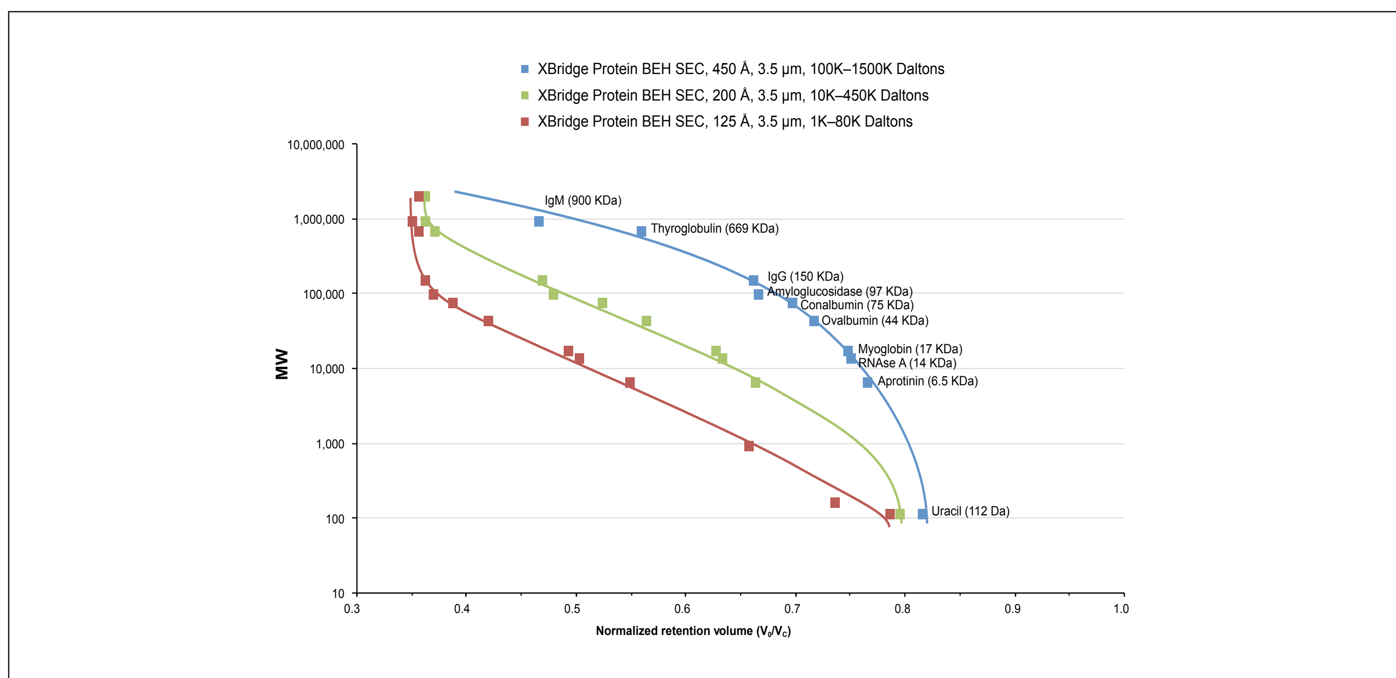


Figure 1. Calibration curves on XBridge Protein BEH SEC, 125 Å, 200 Å, and 450 Å Columns.

II. SYSTEM CONSIDERATIONS FOR SEC SEPARATIONS

a. Getting started

In order to obtain the best performance from your Waters XBridge Protein BEH SEC Column, it is important that your LC system be properly configured. It is recommended that only pre-cut tubing is used, and that the I.D. of all connecting tubing is 0.005" or less for optimal chromatographic performance.

Size-exclusion chromatography may require modifications to an existing LC system. Please refer to “Size-Exclusion and Ion-Exchange Chromatography of Proteins using the ACQUITY UPLC System” (p/n: 715002147A) for examples of LC system components that can affect SEC results.

The sample loop used may affect the performance of your separation. Optimally, select the smallest volume sample loop that is required for the application. Sample loops larger than 20 µL are not recommended.

b. Column installation

1. Prior to placing the column on the system, purge the solvent delivery system of any organic or water-immiscible mobile phases. When connecting the column, orient it in the proper direction as noted by the arrow on the column inlet side which indicates the correct direction of solvent flow.
2. Flush column with 100% aqueous buffer, by pumping at a flow rate of 0.2 mL/min.
3. Ensure that the mobile phase is flowing freely from the column outlet. Attach the column outlet to the detector using 0.004" I.D. tubing (p/n: 430001562). Monitor the system pressure to ensure the column is within its pressure limitations.
4. Gradually increase the flow rate by not more than 0.1 mL/min at a time, as described in Step 2.
5. Once the system pressure has stabilized, ensure that there are no leaks at either the column inlet or outlet.

c. Column equilibration

XBridge Protein BEH SEC Columns are shipped in 20% methanol in water. It is important to ensure mobile-phase compatibility before changing to a different mobile-phase system. Equilibrate the column with a minimum of 10-column volumes of the buffer to be used (refer to Table 1 for column volumes).

Table 1. Empty Column Volumes in mL (multiply by 10 for flush solvent volume)

Column dimension	Approximate volume
4.6 x 150 mm	2.5 mL
4.6 x 300 mm	5.0 mL
7.8 x 150 mm	7.0 mL
7.8 x 300 mm	14.0 mL

d. Useful functional tests for benchmarking LC system and XBridge Protein BEH SEC Column

Waters recommends performing a benchmarking test upon receipt of your column and throughout the lifetime usage. By using a separation of common proteins with an appropriate method, you can:

- Verify the performance of the column upon receipt.
- Monitor the condition of the columns for extended use.
- Troubleshoot separation difficulties that may arise.

Waters BEH125 Protein Standard Mix (p/n: 186006519), BEH200 SEC Protein Standard Mix (p/n: 186006518), and BEH450 SEC Protein Standard Mix (p/n: 186006842) were specifically designed for this purpose with carefully chosen proteins and/or peptides to provide a good representation of the intended application.

The information below details how to successfully prepare and use the included BEH SEC Protein Standard for benchmarking or troubleshooting purposes. Figures 2–4 provide separation conditions and results you should expect to obtain.

IMPORTANT:

Conditioning a new SEC column is suggested by using the recommended BEH SEC Protein Standard Mix and separation method shown below, if the test chromatograms do not reveal similar results. Several methods can be used to condition a new column however the following has been shown effective:

Using the same (or similar) SEC buffer as used by BEH SEC Protein Standard Mix separations, create a 4.5 mg/mL of BSA or thyroglobulin making sure no particulates contained in sample. If needed, centrifuge, discarding pellet.

Using the same flow rate employed for the BEH SEC Protein Standard Mix, make three repetitive, 10 µL injections of the BSA sample (or thyroglobulin) spaced by approximately 5 minutes between injections.

Make sure all of the injected BSA or thyroglobulin elutes from SEC column before continuing.

Reconstitution, Filtration, and Storage of Waters BEH SEC Protein Standard Mix

The performance and lifetime of an SEC column can deteriorate by the accumulation of sample and/or mobile phase derived >0.2 µm particulates. These include any injected protein containing materials such as microbial or insoluble protein aggregates. As a result, it is recommended to filter the reconstituted Waters BEH SEC Protein Standard Mix with a 0.2 µm filtration device prior to its use in the evaluation of the Waters BEH SEC Column performance. Filtration devices, such as sterile, low-bind, 0.2 µm syringe filters from EMD Millipore (13 mm, catalog no. SLGV013SL or 4 mm, catalog no. SLGV004SL) can be successfully used for this purpose. It is also advised to avoid freeze-thaw cycles of the reconstituted BEH SEC Protein Standard since this can result in the generation of protein aggregates. It is recommended that the reconstituted and filtered BEH SEC Protein Standard be stored at 4–8 °C for no more than a week before considering discarding.

Summary:

- Store the lyophilized BEH SEC Protein Standard in freezer.
- To use, reconstitute in 500 to 1000 µL of buffer (see below).
- Filter the reconstituted protein standard through an appropriate 0.2 µm filter.
- Store reconstituted protein standard at 4–8 °C.
- Discard reconstituted protein standard after 7 days.

Buffer preparation for standards:

Chemicals

- Sodium phosphate monobasic, monohydrate
- Sodium phosphate dibasic, anhydrous
- HPLC-grade water

Preparation of 100 mM sodium phosphate buffer (500 mL):

1. Weigh out 500 ± 0.02 g of water into a 500 mL beaker.
2. Weigh out 3.55 ± 0.02 g of sodium phosphate dibasic and add to the 500 mL beaker.
3. Weigh out 3.45 ± 0.02 g of sodium phosphate monobasic and add to the 500 mL beaker.
4. Stir for a minimum of 30 minutes then filter the solution through a 0.2 µm filter.
5. Take the pH and record the value for reference purposes (approximate pH: 6.8).

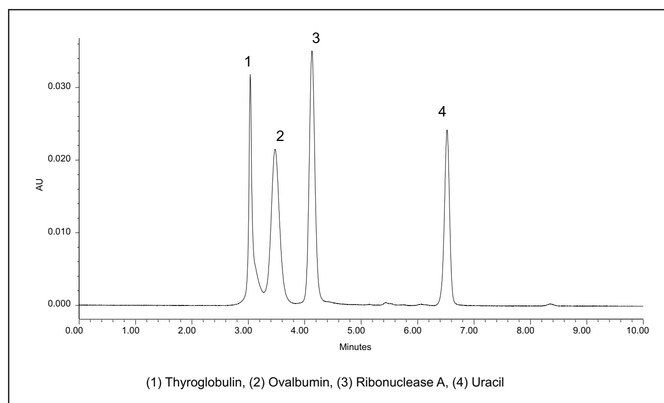


Figure 2. Protein mixture separation on an XBridge Protein BEH SEC, 125 Å, 3.5 µm, 7.8 x 150 mm Column.

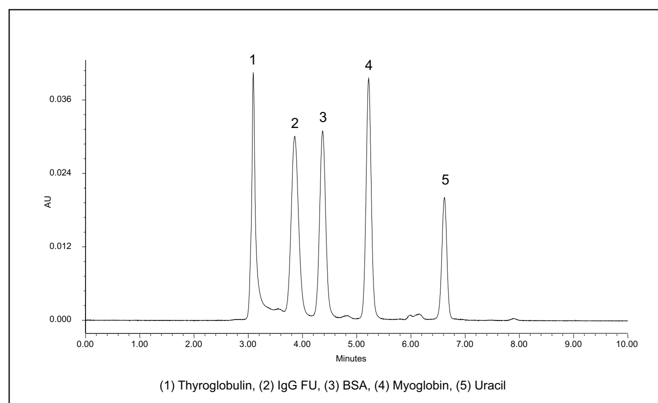


Figure 3. Protein mixture separation on an XBridge Protein BEH SEC, 200 Å, 3.5 µm, 7.8 x 150 mm Column.

Table 2. BEH125 SEC Test Mix

Analyte		pI	MW
1	Thyroglobulin, 0.1 mg/mL	4.6	669,000
2	Ovalbumin, 0.3 mg/mL	4.5	44,200
3	Ribonuclease A, 0.3 mg/mL	9.6	13,700
4	Uracil, 0.05 mg/mL	N/A	112

System: ACQUITY™ TUV with Tunable UV detector

Column: XBridge Protein BEH SEC, 125 Å, 3.5 µm, 7.8 x 150 mm that includes BEH125 SEC Protein Standard Mix (p/n: 176003592)

Sample: BEH125 SEC Protein Standard Mix (p/n: 186006519)

Standard: Dissolve the BEH125 SEC Protein Standard preparation in 500 µL of 100 mM sodium phosphate buffer, pH 6.8. Once solubilized, do not freeze sample, store at 2–8 °C for no more than a week.

Mobile phase: 100 mM sodium phosphate, pH 6.8

Weak needle wash: 100% Milli-Q® water

Strong needle wash: 100% Milli-Q water

Seal wash: 90/10 water/methanol

Injection type: Full loop

Injection volume: 2 µL

Flow rate: 0.86 mL/min

Column temp.: Ambient

UV detection: 220 nm

Table 3. BEH200 SEC Test Mix

Analyte		pI	MW
1	Thyroglobulin, 3 mg/mL	4.6	669,000
2	IgG, 2 mg/mL	6.7	150,000
3	BSA, 5 mg/mL	4.6	66,400
4	Myoglobin, 2 mg/mL	6.8, 7.2	17,000
5	Uracil, 0.1 mg/mL	N/A	112

System: ACQUITY TUV with Tunable UV detector

Column: XBridge Protein BEH SEC, 200 Å, 3.5 µm, 7.8 x 150 mm that includes BEH200 SEC Protein Standard Mix (p/n: 176003595)

Sample: BEH200 SEC Protein Standard Mix (p/n: 186006518)

Standard: Dissolve the BEH200 SEC Protein Standard preparation in 500 µL of 100 mM sodium phosphate buffer, pH 6.8. Once solubilized, do not freeze sample, store at 2–8 °C for no more than a week.

Mobile phase: 100 mM sodium phosphate, pH 6.8

Weak needle wash: 100% Milli-Q water

Strong needle wash: 100% Milli-Q water

Seal wash: 90/10 water/methanol

Injection type: Full loop

Injection volume: 2 µL

Flow rate: 0.86 mL/min

Column temp.: Ambient

UV detection: 280 nm

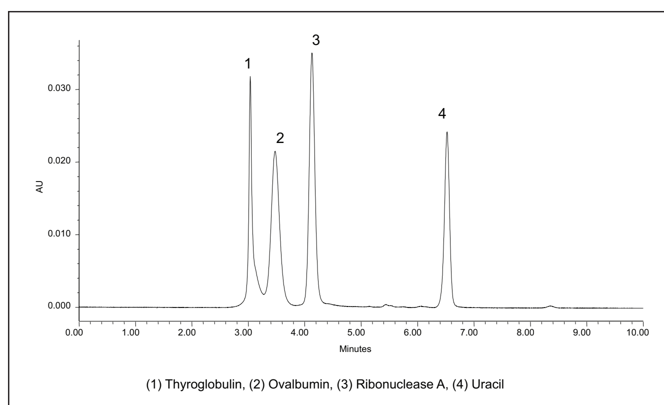


Figure 4. Protein mixture separation on an XBridge Protein BEH SEC, 450 Å, 3.5 μm, 7.8 x 150 mm Column.

Table 4. BEH450 SEC Test Mix

	Analyte	pI	MW
1	Thyroglobulin, 0.1 mg/mL	4.6	669,000
2	Thyroglobulin, approx. 3 mg/mL	4.6	669,000
3	IgG, 2 mg/mL	6.7	150,000
4	BSA, 5 mg/mL	4.6	66,400
5	Myoglobin, 2 mg/mL	6.8, 7.2	17,000
6	Uracil, 0.1 mg/mL	N/A	112

System: ACQUITY TUV with Tunable UV detector

Column: XBridge Protein BEH SEC, 450 Å, 3.5 μm, 7.8 x 150 mm that includes BEH450 SEC Protein Standard Mix (p/n: 176003598)

Sample: BEH450 SEC Protein Standard Mix (p/n: 186006842)

Standard: Dissolve the BEH450 SEC Protein Standard preparation in 500 μL of 100 mM sodium phosphate buffer, pH 6.8. Once solubilized, do not freeze sample, store at 2–8 °C for no more than a week.

Mobile phase: 100 mM sodium phosphate, pH 6.8

Weak needle wash: 100% Milli-Q water

Strong needle wash: 100% Milli-Q water

Seal wash: 90/10 water/methanol

Injection type: Full loop

Injection volume: 2 μL

Flow rate: 0.86 mL/min

Column temp.: Ambient

UV detection: 280 nm

III. COLUMN SPECIFICATIONS AND USE

Note: Particulates impact SEC column lifetime.

The performance and lifetime of any HPLC or UPLC-based SEC column can be significantly compromised by the injection and accumulation of sample and/or mobile-phase-derived particulates onto bed of the packed column. Particulate can include protein aggregates in the sample or microbial contaminants contained in prepared SEC eluent. For more information, reference the “Size-Exclusion Chromatography (SEC) Optimization Guide” (p/n: 720006067EN).

Consequently, it is highly recommended that one use high quality, filtered water (i.e., Milli-Q Millipak 0.22 μm filtered water) when preparing the SEC mobile phase and that it be filtered with a <0.2 μm filter (e.g., through a single use, sterile disposable filter).

It is also recommended to avoid use of silica-based filter supports when filtering mobile phases of pH >6.8 due to the potential of introducing soluble and/or insoluble silicates into the SEC eluent which could alter column performance. Sterile 0.2 μm nylon, nalgene units have been used for this purpose (i.e., Fisher Scientific catalog no. 09-740-46).

Protein samples should be centrifuged or passed through a sterile 0.2 μm low-bind filter to ensure they do not contain significant amounts of particulates. Prior to routine use, a filter should be evaluated to ensure it is able to quantitatively yield analytes of interest. If not filtered, samples can be centrifuged at 16,000 x g for 5 minutes in a temperature-controlled microcentrifuge. Where it is not possible to filter or centrifuge samples, it should be accepted that the lifetime of SEC columns will decrease as a result of their exposure to >0.2 μm particulates.

To check the peak shape performance of an SEC column, and to monitor its lifetime, it is recommended to perform intermittent injections of 0.2 μm filtered Waters BEH SEC Protein Standard Mixes and/or 0.05 mg/mL buffered solutions of uracil.

To help ensure the continued high performance of your XBridge Protein BEH SEC Columns, follow these guidelines:

a. SEC eluent and needle wash preparation

- Use HPLC-grade buffers, water, and organic solvents when possible.
- Filter solutions through a compatible 0.2 µm or smaller pore size filter. The use of a sterile filtration apparatus is recommended for buffers capable of supporting microbial growth.
- Solutions that are susceptible to microbial growth should be replaced at regular intervals to avoid column contamination. Do NOT refill partially-full SEC eluent bottles with new eluent. Rather, when required, use new bottle containing freshly prepared SEC eluent.
- Select solvent inlet filters that are compatible with solutions used, and clean or replace filters regularly when using solutions that are susceptible to microbial growth.

b. Sample preparation

- Ensure that samples are free of particulates before injecting onto the SEC column. If samples appear cloudy or turbid, they should not be injected, as this could lead to column pressure increases. Sample preparation such as filtration or centrifugation may be used, if appropriate.
- If the sample is not dissolved in the mobile phase, ensure that the sample, solvent, and mobile phases are miscible in order to avoid sample and/or buffer precipitation.

- **Mass load:**
  4.6 x 150 mm column: <100 µg
  7.8 x 150 mm column: <300 µg
- **Volume load:**
  4.6 x 150 mm column: <21 µL
  7.8 x 150 mm column: <60 µL
- **Recommended pH range:** 2.5–8. The column lifetime will vary depending upon the operating temperature as well as the type and concentration of buffer used.
- **Recommended salt conc.:** ≤0.5 M
- **Recommended organic conc.:** <20% acetonitrile (Caution: Many proteins are insoluble at elevated organic concentrations. Prior to chromatography, test to ensure the sample does not precipitate at the organic concentration to be used for the chromatography. Also, if column is run under denaturing conditions (greater than 10% organic), subsequent column performance under 100% aqueous conditions may be affected.)
- **Recommended temperature:** 4–60 °C. Reduce flow rate when operating at low temperatures (e.g., 10 °C) to avoid excessive column pressure.
- **Recommended storage:** See Page 8 (Section V, Part b. Storage).

c. Column specifications

- **Shipping solvent:** 20% methanol in water
- **Maximum recommended pressure drop across column:**

Column Name	Maximum Suggested Back Pressure			
	4.6 x 150 mm	4.6 x 300 mm	7.8 x 150 mm	7.8 x 300 mm
XBridge Protein BEH SEC, 125 Å, 2.5 µm	5500 PSI (379 Bar)	7500 PSI (517 Bar)	5500 PSI (379 Bar)	7500 PSI (517 Bar)
XBridge Protein BEH SEC, 125 Å, 3.5 µm	Not Available	Not Available	3200 PSI (221 Bar)	3200 PSI (221 Bar)
XBridge Protein BEH SEC, 200 Å, 2.5 µm	4500 PSI (310 Bar)	6500 PSI (448 Bar)	4500 PSI (310 Bar)	6500 PSI (448 Bar)
XBridge Protein BEH SEC, 200 Å, 3.5 µm	Not Available	Not Available	3200 PSI (221 Bar)	3200 PSI (221 Bar)
XBridge Protein BEH SEC, 450 Å, 2.5 µm	3000 PSI (207 Bar)	5000 PSI (345 Bar)	Not Available	Not Available
XBridge Protein BEH SEC, 450 Å, 3.5 µm	Not Available	Not Available	3200 PSI (221 Bar)	3200 PSI (221 Bar)

The maximum recommended pressure drop across the column is provided as recommendations to ensure longest possible column lifetimes. This guidance should not be interpreted as an absolute upper pressure limit. For example, some methods may exceed the suggested maximum pressure yet deliver desired SEC separation performance, although shorter column lifetimes may be expected.

Additionally, the stated maximum recommended pressure drop across the column refers only to the column itself and NOT from the added pressure contribution from the LC System. To determine pressure drop across column: Install a zero dead volume union in place of the column and determine the system pressure at operating conditions. Subtract that value from the system back pressure observed with the column installed.

IV. TROUBLESHOOTING

The first step in systematic troubleshooting is comparing the column performance in its current state to the performance when it was functioning properly. The functional tests with the protein mixture may reveal subtle changes in surface chemistry that affect the application.

There are several common symptoms of change in the column.

1. **An increase in pressure is often associated with decreased performance in the application.** The first step in diagnosis is to ensure that the elevated pressure resides in the column rather than somewhere else in the system. This is determined by monitoring pressure of the system as each connection is broken from the outlet end to the inlet. If the system is occluded, the blockage should be identified and removed. If the pressure increase resides in the column, it is helpful to know whether the problem was associated with a single injection or whether it occurred over a series of injections. If the pressure gradually built up, it is likely that the column can be cleaned as described in Section V. If a single sample caused the pressure increase, it likely reflects particulates or insoluble components, such as lipids or higher order aggregates. Cleaning is still an option, but using the more aggressive options. If samples appear cloudy or turbid, they should not be injected, as this will lead to pressure increases. Sample preparation such as filtration or centrifugation may be used, but one should first check whether this impacts the results.
2. **Loss of resolution and increased peak tailing can be caused by microbial contamination.** It is important to follow good standard laboratory practices to prevent microbial contamination. This includes changing buffer bottles frequently, using high purity water, using a sterile filtration apparatus, and storing system and column under recommended conditions. If microbial contamination has occurred, cleaning the column will have no effect on performance. When changing the flow rate, ramp it at a rate of 0.1 mL/min and avoid immediate flow-rate increases greater than 0.1 mL/min.
3. **Increased peak tailing can be caused by failure of a tubing connector or a buildup of material on the column inlet frit.** Before proceeding with diagnostic or corrective measures, check all connections that the mobile phases have been correctly prepared and the correct method has been selected. Then repeat the protein standard test. If the proteins show increased peak tailing, it is likely that there is significant buildup of material on the column inlet and the column will require replacement.
4. **Carryover is defined as the appearance of the constituents of one sample in the next analysis. In size-exclusion chromatography, carryover is typically due to system components or improper wash solvents.** Run a blank injection. If the protein peaks only appear when an injection is made, they likely originate from system component or inadequate wash solvents. Adsoprtion in the system components most likely occurs in the loop or needle. In these instances the component may need to be changed.

Note: Useful, general information on column troubleshooting problems may be found in "HPLC Columns Theory, Technology and Practice", U.D. Neue (Wiley-VCH, 1997), the Waters "HPLC Troubleshooting Guide" (p/n: 720000181EN), and the Waters website, www.waters.com.

V. COLUMN CLEANING, REGENERATION, AND STORAGE

a. Extending SEC Column Life

The inadvertent injection of sample or SEC eluent particles have shown to be a primary cause of premature loss for desired SEC column performance. In addition, the injection of some excipients contained in therapeutic protein formulations can also result in shortened column life as noted by changes in retention, peak shape, and/or component 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. In such situations, it is recommended to use an XBridge Protein BEH SEC Guard Column.

For more information, please reference: "Improving the Lifetime of UPLC Size-Exclusion Chromatography Columns Using Short Guard Columns" (720004034) and the "Guide to Size-Exclusion Chromatography of mAb Aggregates, Monomers, and Fragments" (720006067EN).

b. Cleaning and regeneration

Changes in peak shape, such as increased tailing, shoulders on the peak, shifts in retention, change in resolution, ghost peaks, or increased backpressure may indicate contamination of the column. Choose a cleaning option that may be expected to dissolve the suspected contaminant.

It may be useful to conduct cleaning procedures at one-half the flow rate typically used with that column. In this way, the possibility of high pressure events is reduced.

Recommended cleaning solvents:

- A concentrated salt solution at low pH (e.g., 0.5 M Na SO, pH 2.7).
- A low concentration of methanol (e.g., 20%) in HPLC-grade water.
- Use of ionic detergents and other surfactants should be avoided if the SEC column is to subsequently be used to analyze native proteins.

Note: Choose a cleaning solvent based on sample properties, for example, use (a) to remove basic protein and (b) to remove hydrophobic proteins. Chaotrophic agents can solvate strongly adsorbed proteins via hydrogen bond disruption.

As a last resort, flow reversal or back flushing can be tried at a low flow rate (e.g., 0.1 mL/min). However, this approach may further damage the column or only provide short-lived improvement in performance.

c. Storage

For overnight storage, with the SEC column on the LC system:

Continuously flush the column with the used SEC mobile phase at 10-20% of the flow rate used for separations.

For short-term storage (<2 weeks), when the SEC column is removed from the LC system:

Tightly cap column and store at 4 °C to prevent microbial growth. DO NOT FREEZE.

For long-term storage, when the SEC column is removed from the LC system:

Flush column (approx. 10-column volumes) and store in a mobile phase containing 5–25 mM phosphate buffer, pH 6.5–7.0, and 20–100 mM (KCl or NaCl) with the addition of 10% acetonitrile, 20% methanol, or 0.05% sodium azide to prevent microbial growth. This storage solution can be a dilute version of the mobile phase with an antimicrobial added to it. To further mitigate the risk of microbial growth, tightly cap column and store at 4 °C. DO NOT FREEZE.

Note: Working at extremes of pressure, pH, and/or temperature may result in shorter column lifetime.

VI. CAUTIONARY NOTE

Depending on user's application, these products may be classified as hazardous following their use, and as such are intended to be used by professional laboratory personnel trained in the competent handling of such materials. Responsibility for the safe use and disposal of products rests entirely with the purchaser and user. The Safety Data Sheet (SDS) for this product is available at www.waters.com.

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