XBridge Protein BEH C_4, 300Å Columns

CONTENTS

I. INTRODUCTION

II. GETTING STARTED
- a. Column Installation
- b. Column Equilibration
- c. Initial Column Efficiency Determination
- d. Useful Functional Tests for Benchmarking a New Column

III. COLUMN USE
- a. Sample Preparation
- b. Operating pH Limits
- c. Solvents
- d. Pressure
- c. Temperature

IV. SCALING SEPARATIONS

V. TROUBLESHOOTING

VI. COLUMN CLEANING, REGENERATION, AND STORAGE
- a. Cleaning and Regeneration
- b. Storage

VII. CONNECTING THE COLUMN TO THE HPLC
- a. Column Connectors and System Tubing Considerations
- b. Measuring System Bandspreading Volume

VIII. eCORD INTELLIGENT CHIP TECHNOLOGY
*(Applies only to XBridge **XP** 2.5 µm, <4.6 mm I.D. Columns)*

IX. MEASURING GRADIENT SYSTEM VOLUME (OR DWELL VOLUME)

X. CAUTIONARY NOTE

I. INTRODUCTION

Thank you for choosing a Waters™ reversed-phase Protein Column. The XBridge™ Protein BEH C_4, 300Å packing material was designed to provide excellent peak shape, high efficiency, and good recovery for biological macromolecules that are too large or too hydrophobic for separation on columns with smaller pores or longer chain bonded phases. The base particle and bonding chemistry are chosen to provide exceptional stability at both high and low pH as well as at high temperature. The XBridge Protein BEH C_4, 300Å packing material is manufactured in a cGMP, ISO9002-certified plant using ultra-pure reagents. Each batch of XBridge Protein BEH C_4 packing material has been qualified with a protein test mixture, and the results are held to narrow specification ranges to ensure reproducible performance. Every column is individually tested for efficiency, and a Performance Test Chromatogram, along with a Certification of Acceptance, is provided with each column.

II. GETTING STARTED

Every XBridge Protein BEH C_4, 300Å Column has a Certificate of Acceptance and a Performance Test Chromatogram. The Certificate of Acceptance is specific to each batch of packing material and includes the batch number, analysis of unbonded particles, analysis of bonded particles, and chromatographic results and conditions. The Performance Test Chromatogram is specific to each individual column and contains the information: batch number, column serial number, USP plate count, USP tailing factor, retention factor, and chromatographic conditions. These data should be stored for future reference.

a. Column Installation

Note: The flow rates given in the procedure below are for a typical 3.5 µm packing in a 4.6 mm I.D. column. Scale the flow rate up or down based upon the I.D. of the column being installed. See "Scaling Separations" section for calculating flow rates when changing column I.D. and/or length. See "Connecting the Column to the HPLC" for a more detailed discussion on HPLC connections.

1. Purge the solvent delivery system of any buffer-containing or water-immiscible mobile phases and connect the inlet end of the column to the injector outlet. An arrow on the column identification label indicates the correct direction of solvent flow.
2. Flush column with 100% organic mobile phase (acetonitrile) by setting the pump flow rate to 0.1 mL/min and increase the flow rate to 1 mL/min over 5 minutes.
3. When the mobile phase is flowing freely from the column outlet, stop the flow and attach the column outlet to the detector. This prevents entry of air into the detection system and gives more rapid baseline equilibration.
4. Gradually increase the flow rate as described in step 2.
5. Once a stable backpressure and baseline have been achieved, proceed to the next section.

b. Column Equilibration

XBridge Protein BEH C_4, 300Å Columns are shipped in 100% acetonitrile. 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 mobile phase to be used (refer to Table 1 for column volumes).

To avoid precipitating mobile phase buffers on your column or in your system, flush the column with five column volumes of a water/organic solvent mixture, using the same or lower solvent content as in the desired buffered mobile phase. For example, flush the column and HPLC system with 50% acetonitrile in water prior to introducing 50% acetonitrile/50% buffer mobile phase.

Column equilibration may be judged initially by stable pressure and by a stable detector baseline. For a specific application, it is, however, necessary to test the required duration of equilibration. The criteria for adequate equilibration include reproducibility of retention time for major and minor peaks, resolution for critical pairs, and consistent baseline characteristics.

Note: Low concentration mobile phase additives, particularly those with minimal buffering capacity may require extended equilibration and re-equilibration between gradient analyses.

c. Initial Column Efficiency Determination

1. Perform an efficiency test on the column before using it in the desired application. Waters recommends using the solute mixture and conditions described in the Performance Test Chromatogram to test the column upon receipt.
2. Measure retention of the test compounds and the number of theoretical plates (N).
3. Repeat the test at predetermined intervals to track column performance over time. Slight variations may be obtained on two different HPLC systems due to the quality of the connections, operating environment, system electronics, reagent quality, condition of column, and operator technique.

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

	Column internal diameter (mm)					
Column length (mm)	**2.1**	**3.0**	**4.6**	**10**	**19**	**30**
50	0.17	0.35	0.83	3.9	14	35
100	0.35	0.71	1.7	7.8	28	70
150	0.52	1.06	2.5	12	42	106
250	0.87	–	4.2	20	70	176

d. Useful Functional Tests for Benchmarking a New Column

The Column Efficiency Test described above is a useful measure of the physical state of the packed bed as well as an indicator of the chemical integrity of the bonded phase. It may also be useful to benchmark the column performance with a sample that is more representative of the intended application. Two tests can be suggested as starting points for benchmarking a new column and for monitoring a column during its use.

Peptide Mixture Performance Test

Sample: Waters MassPREP Peptide Standard Mixture (p/n: 186002337)

Reconstitute 1 vial in 100 µL 0.1% TFA: 5% acetronitrile:94.9% water

Injection volume: 2.1 mm – 3.3 µL
3.0 mm – 6.7 µL
4.6 mm – 15.8 µL

Column: XBridge Protein BEH C_4, 300Å, 3.5 µm, 2.1 x 50 mm

Flow rate: 2.1 mm – 0.2 mL/min
3.0 mm – 0.4 mL/min
4.6 mm – 1.0 mL/min

Mobile phase: A: 0.1% TFA in water
B: 0.075% in 71.4% acetonitrile/ 28.6% water

Gradient:

Gradient time for different column lengths						
50 mm	**100 mm**	**150 mm**	**250 mm**	**%A**	**%B**	**Curve**
Initial	Initial	Initial	Initial	100	0	*
30	60	90	150	30	70	6
32	64	96	160	30	70	1
50	100	150	250	100	0	1

Temperature: 40 °C

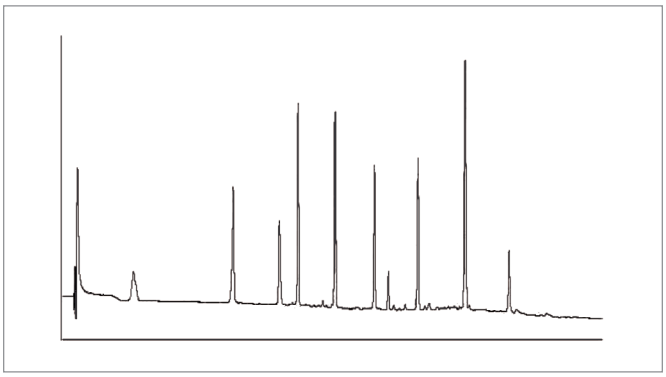


Figure 1. Typical peptide chromatogram using MassPREP Peptide Standard Mixture.

This chromatogram is typical of the results obtained in Waters laboratories with the method described above, using an XBridge Protein BEH C_4, 300Å, 3.5 µm, 2.1 x 50 mm Column. The retention times will double, triple, and be five times greater for the 100 mm, 150 mm, and 250 mm columns, respectively. The exact results observed in any laboratory will depend on the instrument in use. System volume, gradient generation mechanism, mixing, design of temperature control, detector cell dimensions, detector optical properties, and detector electronic properties all have a direct impact on the observed chromatogram. The pattern should be similar, however, on any well-functioning, modern HPLC system. This test is exceptionally valuable for monitoring the life of the column and for troubleshooting separation difficulties that may arise.

Protein Mixture Performance Test

Sample: MassPREP Protein Standard Mixture (p/n: 186004900)

Dissolved in 0.1% TFA:5% acetonitrile:94.9% water

Protein	Sigma p/n	Conc. mg/mL
Bovine ribonuclease A	**R5500**	**0.04**
Horse cytochrome *c*	C7752	0.06
Bovine serum albumin	A8022	0.20
Horse myoglobin	M1882	0.13
Yeast enolase	E6126	0.22
Rabbit phosphorylase b	P6635	0.59

Injection volume: 2.1 mm – 5.0 µL
3.0 mm – 10.2 µL
4.6mm – 24.0 µL

Column: XBridge Protein BEH C_4, 300Å, 3.5 µm, 2.1 x 50 mm

Flow rate: 2.1 mm – 0.2 mL/min
3.0 mm - 0.4 mL/min
4.6 mm – 1.0 mL/min

Mobile phase: A: 0.1% TFA in water
B: 0.075% in 71.4% acetonitrile/ 28.6% water

Gradient:

Time (column length)						
50 mm	100 mm	150 mm	250 mm	%A	%B	Curve
Initial	Initial	Initial	Initial	72	28	*
25	50	75	125	0	100	6
27	54	81	135	0	100	1
45	90	135	225	72	28	1

Temperature: 40 °C

Detection: 220 nm

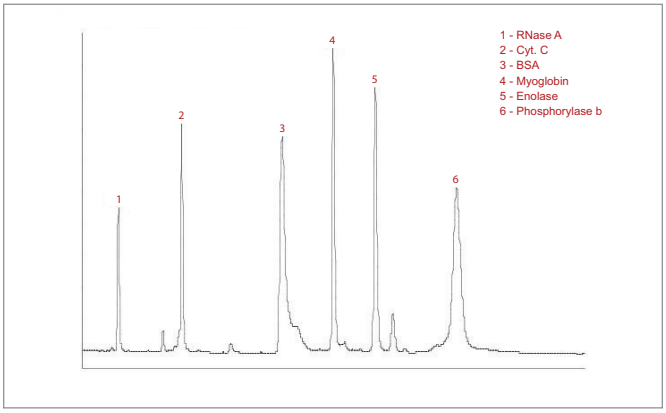


Figure 2. Typical protein test mixture chromatogram using MassPREP Protein Standard Mixture.

This chromatogram is typical of the results obtained in Waters laboratories with the method described above, using an XBridge Protein BEH C_4, 300Å, 3.5 µm, 2.1 x 50 mm Column. The retention times will double, triple, and be five times greater for the 100 mm, 150 mm, and 250 mm columns, respectively.The exact results observed in any laboratory will depend on the instrument in use. System volume, gradient generation mechanism, mixing, design of temperature control, detector cell dimensions, detector optical properties, and detector electronic properties all have a direct impact on the observed chromatogram. The pattern should be similar, however, on any well-functioning, modern HPLC. This test is exceptionally valuable for monitoring the life of the column and for troubleshooting separation difficulties that may arise

III. COLUMN USE

To ensure the continued high performance of XBridge Protein BEH C_4, 300Å Columns, follow these guidelines:

a. Sample Preparation

Sample impurities often contribute to column contamination. Samples should be free of particles before injection into the system.

It is preferable to prepare the sample in gradient solvent A or in a mobile phase that is weaker (less organic modifier) than the initial strength mobile phase. This ensures the best peak shape.

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.

Filter sample with 0.2 µm filters to remove particulates. If the sample is dissolved in a solvent that contains an organic modifier (e.g., acetonitrile, methanol, etc.) ensure that the filter material does not dissolve in the solvent. Contact the filter manufacturer with solvent compatibility questions. Alternatively, centrifugation for 20 minutes at 12–50,000 g, followed by the transfer of the supernatant liquid to an appropriate vial, could be considered.

b. Operating pH Limits

The recommended operating pH range for XBridge Protein BEH C_4, 300Å Columns is 1 to 12. A listing of commonly used buffers and additives is given in Table 2. Additionally, the column lifetime will vary depending upon the operating temperature as well as the type and concentration of buffer used.

Table 2. Buffer Recommendations for using XBridge Protein BEH C_4, 300Å Columns from pH 1 to 12

Additive/Buffer	pK_a	Buffer range	Volatility (±1 pH unit)	Used for mass spec	Comments
TFA	0.3	–	Volatile	Yes	Ion pair additive, can suppress MS signal, used in the 0.02–0.1% range.
Acetic acid	4.76	–	Volatile	Yes	Maximum buffering obtained when used with ammonium acetate salt. Used in 0.1–1.0% range.
Formic acid	3.75	–	Volatile	Yes	Maximum buffering obtained when used with ammonium formate salt. Used in 0.1–1.0% range.
Acetate (NH_4CH_2COOH)	4.76	3.76–5.76	Volatile	Yes	Used in the 1–10 mM range. Note that sodium or potassium salts are not volatile.
Formate (NH_4COOH)	3.75	2.75–4.75	Volatile	Yes	Used in the 1–10 mM range. Note that sodium or potassium salts are not volatile.
Phosphate 1	2.15	1.15–3.15	Non-volatile	No	Traditional low pH buffer, good UV transparency.
Phosphate 2	7.2	6.20–8.20	Non-volatile	No	Above pH 7, reduce temperature/concentration and use a guard column to maximize lifetime.
Phosphate 3	12.3	11.3–13.3	Non-volatile	No	Above pH 7, reduce temperature/concentration and use a guard column to maximize lifetime.
4-Methylmorpholine	~8.4	7.4–9.4	Volatile	Yes	Generally used at 10 mM or less.
Ammonia (NH_4OH)	9.2	8.2–10.2	Volatile	Yes	Keep concentration below 10 mM and temperatures below 30 °C.
Ammonium bicarbonate	10.3 (HCO_3-) 9.2 (NH_4+)	8.2–11.3	Volatile	Yes	Used in the 5–10 mM range (for MS work keep source >150 °C). Adjust pH with ammonium hydroxide or acetic acid. Good buffering capacity at pH 10. Note: use ammonium bicarbonate (NH_4HCO_3), not ammonium carbonate ($[NH_4]^2CO_3$).
Ammonium (acetate)	9.2	8.2–10.2	Volatile	Yes	Used in the 1–10 mM range.
Ammonium (formate)	9.2	8.2–10.2	Volatile	Yes	Used in the 1–10 mM range.
Borate	9.2	8.2–10.2	Non-volatile	No	Reduce temperature/concentration and use a guard column to maximize lifetime.
CAPSO	9.7	8.7–10.7	Non-volatile	No	Zwitterionic buffer, compatible with acetonitrile, used in the 1–10 mM range. Low odor.
Glycine	2.4, 9.8	8.8–10.8	Non-volatile	No	Zwitterionic buffer, can give longer lifetimes than borate buffer.
1-Methylpiperidine	10.2	9.3–11.3	Volatile	Yes	Used in the 1–10 mM range.
CAPS	10.4	9.5–11.5	Non-volatile	No	Zwitterionic buffer, compatible with acetonitrile, used in the 1–10 mM range. Low odor.
Triethylamine (as acetate salt)	10.7	9.7–11.7	Volatile	Yes	Used in the 0.1–1.0% range. Volatile only when titrated with acetic acid (not hydrochloric or phosphoric). Used as ion pair for DNA analysis at pH 7–9.

c. Solvents

To maintain maximum column performance, use high quality chromatography grade solvents. Filter all aqueous buffers prior to use. Pall Gelman Laboratory Acrodisc® filters are recommended. Solvents containing suspended particulate materials can damage the fluidic components of the HPLC system and will generally clog the inlet distribution frit of the column. This will result in higher operating pressure and poor performance.

d. Pressure

XBridge Protein BEH C_4, 300Å Columns can tolerate pressures of up to 6000 psi (400 bar or 40 Mpa) although pressures greater than 4000–5000 psi should be avoided in order to maximize column and system lifetimes.

e. Temperature

Temperatures between 20–90 °C are recommended for operating XBridge Protein BEH C_4, 300Å Columns in order to enhance selectivity, lower solvent viscosity, and increase mass transfer rates. However, any temperature above ambient will have a negative effect on lifetime which will vary depending on the pH and buffer conditions used.

Note: Under certain reversed-phase separation conditions (mobile phase, temperature, etc.) some proteins or peptides may exhibit secondary interactions with the column packing materials or hardware resulting in low recovery or poor peak shape. Repeating several injections of the sample or another protein (for example, bovine serum albumin) until consistent chromatographic performance is achieved can resolve this issue. Additionally, in order to develop a robust separation method the analyst should also optimize the separation conditions being used to minimize any observed secondary interactions.

IV. SCALING SEPARATIONS

Scale-Up Factors

$$\text{Scale-up factor} = \frac{(\text{Diameter prep})^2 \times \text{Length prep}}{(\text{Diameter analytical})^2 \times \text{Length analytical}}$$

Consider scaling up from a **4.6 x 150 mm** column to a **19 x 150 mm** column:

$$\text{Scale-up factor} = \frac{(19)^2 \times 150}{(4.6)^2 \times 150} = 17.1$$

Applying the scale-up factor, we can predict that approximately 17–135 mg of sample could be applied to the larger column (packed with the same material as the analytical column). This range is based on an analytical (4.6 mm I.D.) mass load of 1–8 mg.

Flow Rate

$$\text{Flow rate (prep)} = \text{Flow rate (analytical)} \times \frac{(\text{Diameter prep})^2}{(\text{Diameter analytical})^2} \times \frac{\text{Particle size (analytical)}}{\text{Particle size (prep)}}$$

The calculated flow rate may be used for the larger column to ensure the same linear velocity of mobile phases as used in the analytical run. However, reasonable flow rates are based on column diameters. Systems will be limited by increasing backpressure with increasing column length and decreasing particle size.

Gradient Duration (GD)

$$\text{GD (prep)} = \frac{\text{GD (analytical)} \times \text{Length (prep)}}{\text{Length (analytical)}} \times \frac{\text{Diameter (prep)}^2}{\text{Diameter (analytical)}^2} \times \frac{\text{Flow rate (analytical)}}{\text{Flow rate (prep)}}$$

Mass Loading

Approximate Mass Loading Capacity (mg) for OBD™ Preparative Columns (Gradient Mode)

	Diameter (mm)			
Length (mm)	**4.6**	**10**	**19**	**30**
50	3	15	45	110
75	–	–	–	165
100	5	25	90	225
150	8	40	135	335
250	13	60	225	560
Reasonable flow rate (mL/min)	1.4	6.6	24	60
Reasonable injection volume (µL)	20	100	350	880

The calculated prep gradient duration is entered into the pump's gradient separation over the same number of column volumes as was used in the analytical run.

Reasonable flow rates are based on column diameter. Systems will be limited by increasing backpressure with increasing column length and decreasing particle size.

Reasonable injection volumes are based on column diameter at a length of 50 mm with relatively strong solvents. Increased length is compatible with larger injection, but not proportionately so. Weaker solvents significantly increase injection volume.

Mass loading capacities for peptide purifications depend strongly on the sequence and may be estimated at 5–20% of listed values.

Many factors affect the mass capacity of preparative columns. The listed capacities represent an 'average' estimate.

Capacity is:

- Higher for strongly retained material
- Higher for simple mixtures
- Lower where higher resolution is required
- Very strongly dependent on loading conditions
  - Limited by loading volume
  - Limited by diluent solvent strength

Waters OBD Preparative Calculator

- Convenient scale-up tool provides:
  - Mass load scaling
  - Gradient scaling with appropriate flow rate scale-up and predicting volume consumption
  - Calculations for split flow ratios for those using mass spectrometer driven chromatography
  - Focused gradient UPLC™ to preparative method transfer

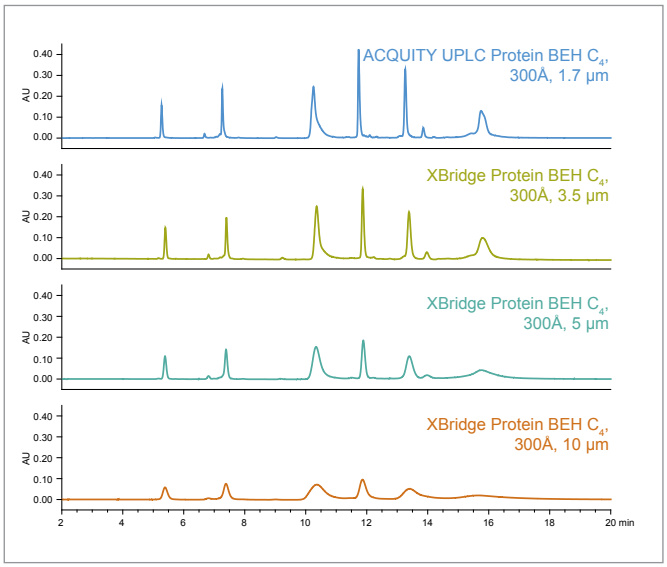


Figure 3. Separation of MassPREP Protein Standard Mixture on ACQUITY™ UPLC Protein BEH C_4, 300Å, 1.7 µm, (top); XBridge Protein BEH C_4, 300Å, 3.5 µm; XBridge Protein BEH C_4, 300Å, 5 µm; XBridge Protein BEH C_4, 300Å, 10 µm Columns using scaling methods obtained from using the Waters OBD Preparative Columns Calculator.

V. TROUBLESHOOTING

The first step in systematic troubleshooting is a comparison of the column in its current state, to the column when it was functioning properly. The method suggested in Section I for measuring plate count is an essential first step. This technique detects physical changes to the packed bed and chemical changes in the bonded phase surface. The two functional tests with the peptide standard and the protein mixture may reveal more 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 lost 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 measuring pressure with and without the column attached to the instrument. 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 below (Section V). For future stability, it may be useful to incorporate a stronger regeneration step in the method. If a single sample caused the pressure increase, it likely reflects particulates or insoluble components, such as lipids. Cleaning is still an option, but using the more aggressive options. The sudden pressure increase suggests that the user should consider some sample preparation, such as filtration or high speed centrifugation.
2. Loss of retention can reflect a change in the column surface chemistry. Before proceeding with diagnostic or corrective measures, check that the mobile phases have been correctly prepared and the correct method has been selected. Then repeat the efficiency test and the functional peptide or protein test. If both the small and large molecule tests show loss of retention, it is likely that a significant fraction of the bonded phase has been lost, and the column will require replacement. If the changes are small and reflected only for some proteins, one of the cleaning procedures may be effective.
3. For change in peak shape, resolution, or relative retention of peaks, follow the same steps as for loss of retention (Symptom 2).

4. Carryover and memory effects are defined as the appearance of the constituents of one sample in the next gradient analysis. First determine whether the column or the system is the source of carryover. Define a gradient method that includes an "internal gradient". That is, the analytical gradient is repeated within a single method. If the protein peaks appear in both gradients, at the same time after start, the protein came from the column in what is often described as a "memory effect". If the protein peaks only appear when an injection is made, they likely originate from adsorption to some system component. In that case, follow the instrument manufacturer's recommendations.

   Memory effects as a source carryover may be reduced or eliminated in several ways:

   a) Raising the temperature of the separation reduces the possibility of incomplete elution of the protein from the column.

   b) Memory effects may be more pronounced with steep gradients. Keep the gradient slope at 1% per column volume or less.

   c) Memory effects may be exacerbated by high flow rates. Reduce the flow rate by one half while doubling the gradient time to maintain a constant slope.

   d) Memory effects may be reduced by changing the organic solvent to incorporate propanol, typically 70% propanol:30% acetonitrile as a strong solvent.

   e) Carryover may be reduced in routine assays with a regeneration step including a series of fast gradients from 0-100% acetonitrile. The gradients can be as short as two column volumes and three to five repetitions may be effective. This "sawtooth" gradient may be appended to each injection.

   f) Apparent memory effects may actually reflect the solubility of the protein in the mobile phase. Reducing the amount injected may eliminate the effect..

5. Recovery is often improved by elevating the column temperature.

Note: Useful, general information on troubleshooting column problems may be found in "HPLC Columns Theory, Technology and Practice, U.D. Neue, (Wiley VCH, 1997); in Waters' "HPLC Troubleshooting Guide"; or in seminars found on www.waters.com.

VI. COLUMN CLEANING, REGENERATION, AND STORAGE

a. Cleaning and Regeneration

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

1. All cleaning procedures will be more effective at higher temperatures. The XBridge Protein BEH C_4, 300Å Column can be routinely operated at temperatures as high as 90 °C so it is reasonable to conduct cleaning at 70–90 °C.
2. 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.
3. The first and simplest cleaning procedure is to run a series of fast gradients from 0–100% acetonitrile. The gradients can be as short as two column volumes and three to five repetitions may be effective. This "sawtooth" gradient may be appended to each injection to stabilize routine assays.
4. Several different cleaning solutions may be injected to strip strongly adsorbed material or particulates from the column. Make the largest injection possible with the system configuration. With such strong cleaning solutions, it is best to disconnect the detector from the column and to direct the flow to waste.
   a. An injection of 1% formic acid.
   b. An injection of 10% formic acid.
   c. An injection of either 4 M urea or 6 M guanidine-HCl.
   d. If contamination with lipids is suspected, a strong cleaning option is an injection of tetrahydrofuran.
5. Flow reversal or backflushing is often suggested as part of a cleaning procedure. This should be reserved as a last resort. It may further damage the column or provide a short-lived improvement in performance.

b. Storage

For short-term storage, the column should be stored in the mobile phase with 20–50% acetonitrile. For periods longer than four days at room temperature, store the column in 100% acetonitrile. Immediately after use with elevated temperatures and/or at pH extremes, store in 100% acetonitrile for the best column lifetime. Do not store columns in highly aqueous (<20% organic) mobile phases, as this may promote bacterial growth. If the mobile phase contained a buffer salt,

flush the column with 10 column volumes of HPLC grade water (see Table 1 for common column volumes) and replace with 100% acetonitrile for storage. Failure to perform this intermediate step could result in precipitation of the buffer salt in the column or system when 100% acetonitrile is introduced. Completely seal column to avoid evaporation and drying out of the bed.

VII. CONNECTING THE COLUMN TO THE HPLC

a. Column Connectors and System Tubing Considerations

Tools needed:

- 3/8-inch wrench
- 5/16-inch wrench

Handle the column with care. Do not drop or hit the column on a hard surface as it may disturb the bed and affect its performance.

1. Correct connection of 1/16-inch outer diameter stainless steel tubing leading to and from the column is essential for high-quality chromatographic results.
2. When using standard stainless steel compression screw fittings, it is important to ensure proper fit of the 1/16-inch outer diameter stainless steel tubing. When tightening or loosening the compression screw, place a 5/16-inch wrench on the compression screw and a 3/8-inch wrench on the hex head of the column endfitting.
3. If a leak occurs between the stainless steel compression screw fitting and the column endfitting, a new compression screw fitting, tubing, and ferrule must be assembled.
4. An arrow on the column identification label indicates correct direction of solvent flow.

Correct connection of 1/16-inch outer diameter stainless steel tubing leading to and from the column is essential for high-quality chromatographic results. To obtain a void-free connection, the tubing must touch the bottom of the column endfitting. It is important to realize that extra column peak broadening due to voids can destroy an otherwise successful separation. The choice of appropriate column connectors and system tubing is discussed in detail below.

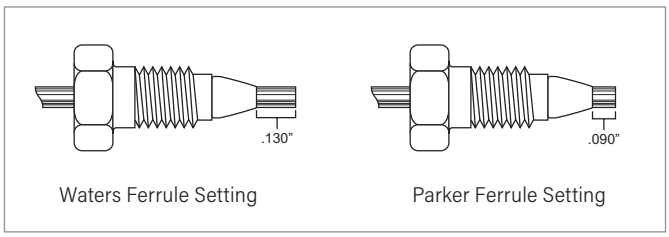


Figure 3. Waters and Parker ferrule types.

Due to the absence of an industry standard, various column manufacturers have employed different types of chromatographic column connectors. The chromatographic separation can be negatively affected if the style of the column endfittings does not match the existing tubing ferrule settings. This section explains the differences between Waters style and Parker style ferrules and endfittings (Figure 3). Each endfitting style varies in the required length of the tubing protruding from the ferrule. The XBridge Column is equipped with Waters style endfittings that require a 0.130-inch ferrule depth. If a non-Waters style column is presently being used, it is critical that ferrule depth be reset for optimal performance prior to installing an XBridge Protein BEH C_4, 300Å Column.

In a proper tubing/column connection (Figure 4), the tubing touches the bottom of the column endfitting, with no void between them.

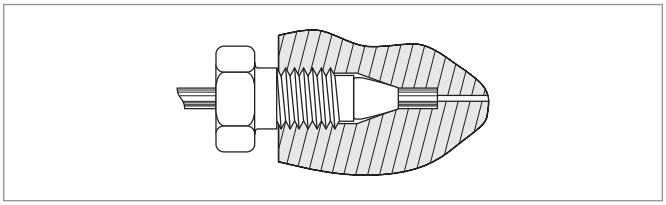


Figure 4. Proper tubing/column connection.

The presence of a void in the flow stream reduces column performance. This can occur if a Parker ferrule is connected to a Waters style endfitting (Figure 5).

Note: A void appears if tubing with a Parker ferrule is connected to a Waters style column.

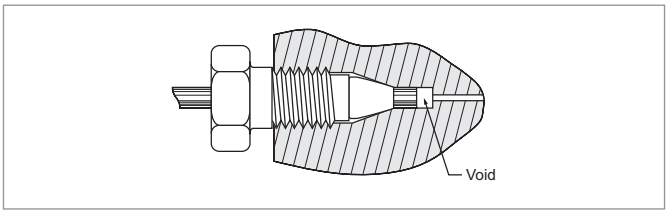


Figure 5. Parker Ferrule in a Waters style endfitting.

There is only one way to fix this problem: cut the end of the tubing with the ferrule, place a new ferrule on the tubing and make a new connection. Before tightening the screw, make sure that the tubing bottoms out in the endfitting of the column.

Conversely, if tubing with a Waters ferrule is connected to a column with Parker style endfitting, the end of the tubing will bottom out before the ferrule reaches its proper sealing position. This will leave a gap and create a leak (Figure 6).

Note: The connection leaks if a Waters ferrule is connected to a column with a Parker style endfitting.

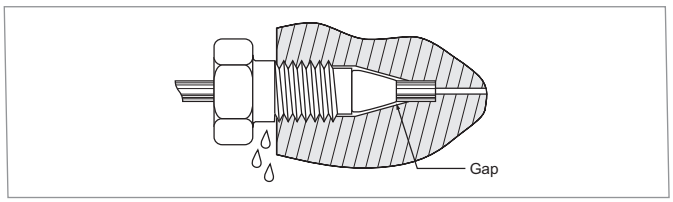


Figure 6. Waters Ferrule in a Parker style endfitting.

There are two ways to fix the problem:

1. Tighten the screw a bit more. The ferrule moves forward, and reaches the sealing surface. Do not overtighten since this may end in breaking the screw.
2. Cut the tubing, replace the ferrule, and make a new connection.

Alternatively, replace the conventional compression screw fitting with an all-in-one PEEK™ fitting (p/n: PSL613315) that allows resetting of the ferrule depth. Another approach is to use a Keystone, Inc. SLIPFREE® connector to always ensure the correct fit. The finger-tight SLIPFREE connectors automatically adjust to fit all compression screw type fittings without the use of tools (Figure 7).

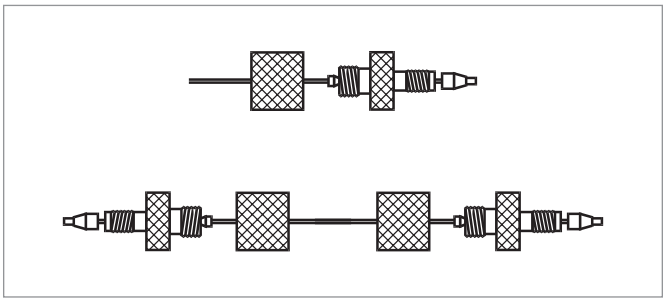


Figure 7. Single and double SLIPFREE connectors.

SLIPFREE Connector Features:

- Tubing pushed into endfitting, thereby guaranteeing a void-free connection
- Connector(s) come(s) installed on tubing
- Various tubing I.D.s and lengths available
- Fingertight to 10,000 psi – never needs wrenches
- Readjusts to all column endfittings
- Compatible with all commercially available endfittings
- Unique design separates tube-holding function from sealing function

Table 3. Waters Part Numbers for SLIPFREE Connectors

SLIPFREE type	Tubing Internal Diameter		
Tubing length	**0.005"**	**0.010"**	**0.020"**
Single 6 cm	PSL 618000	PSL 618006	PSL 618012
Single 10 cm	PSL 618002	PSL 618008	PSL 618014
Single 20 cm	PSL 618004	PSL 618010	PSL 618016
Double 6 cm	PSL 618001	PSL 618007	PSL 618013
Double 10 cm	PSL 618003	PSL 618009	PSL 618015
Double 20 cm	PSL 618005	PSL 618001	PSL 618017

Minimizing Band Spreading

Figure 8 shows the influence of tubing internal diameter on system band spreading and peak shape. As can be seen, the larger tubing diameter causes excessive peak broadening and lower sensitivity.

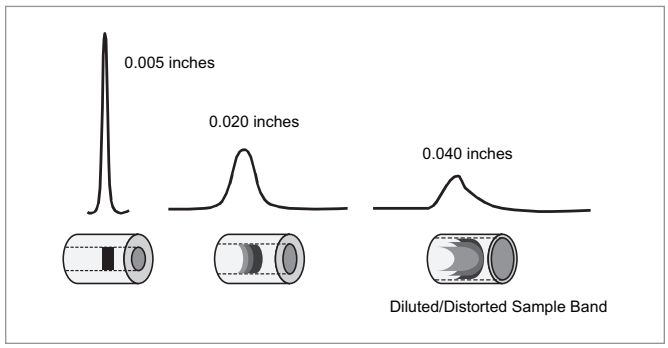


Figure 8. Effect of connecting tubing on system.

b. Measuring System Bandspreading Volume

This test should be performed on an HPLC system with a UV detector.

1. Disconnect column from system and replace with a zero dead volume union.
2. Set flow rate to 1 mL/min.
3. Dilute a test mix in mobile phase to give a detector sensitivity of 0.5–1.0 AUFS (system startup test mix can be used which contains uracil, ethyl, and propyl parabens; p/n: WAT034544).
4. Inject 2 to 5 µL of this solution.
5. Measure the peak width at 4.4% of peak height (5-sigma method):
   - 5-sigma Bandspreading (µL) = Peak Width (min) x Flow Rate (mL/min) x (1000 µL/1 mL)
   - System Variance (μL^2) = (5-sigma bandspreading)^2/25

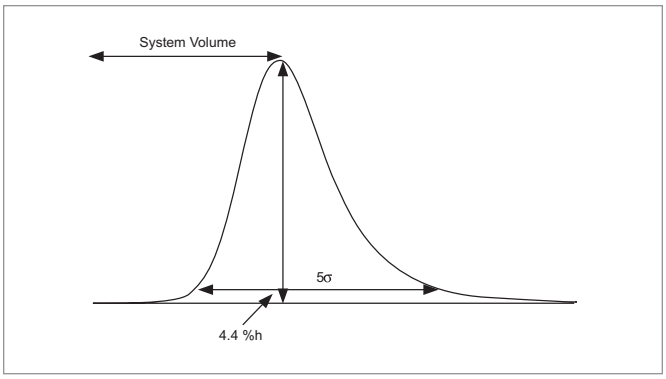


Figure 9. Determination of system bandspreading volume using 5-sigma method.

In a typical HPLC system, the bandspreading volume should be no greater than 100 µL ± 30 µL (or variance of 400µL^2 ± 36µL^2).

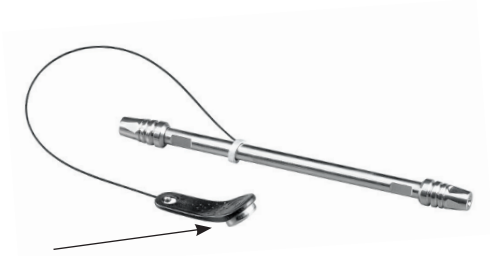
In a microbore (2.1 mm I.D.) system, the bandspreading volume should be no greater than 20 to 40 µL (or variance no greater than 16 µL^2 to 64 µL^2).

VIII. eCORD INTELLIGENT CHIP TECHNOLOGY

*(Applies only to XBridge **XP** 2.5 µm, <4.6 mm I.D. Columns)*

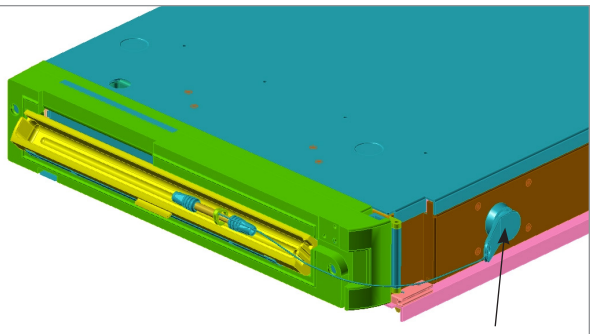
a. Introduction

The eCord™ Intelligent Chip technology provides the history of a column's performance throughout its lifetime. The eCord is permanently attached to the column to assure that the column's performance history is maintained in the event that the column is moved from one instrument to another.



Waters eCord Intelligent Chip.

At the time of manufacture, tracking and quality control information will be downloaded to the eCord. Storing this information on the chip will eliminate the need for a paper Certificate of Analysis. Once the user installs the column, the software will automatically download key parameters into a column history file stored on the chip. The eCord provides a solution to easily track the history of column usage.

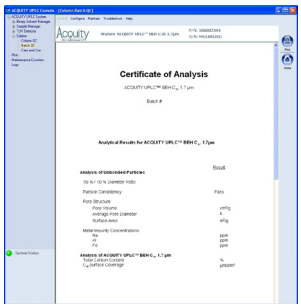


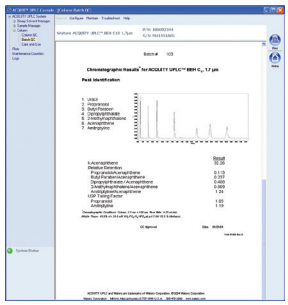
eCord inserted into side of column heater.

b. Installation

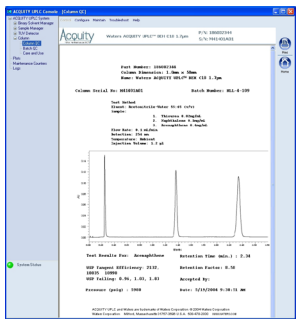
Install the column into the column heater. Plug the eCord into the side of the column heater. Once the eCord is inserted into the column heater the identification and overall column usage information will be available in Empower™ and MassLynx Software allowing the user to access column information on their desktop.

c. Manufacturing Information



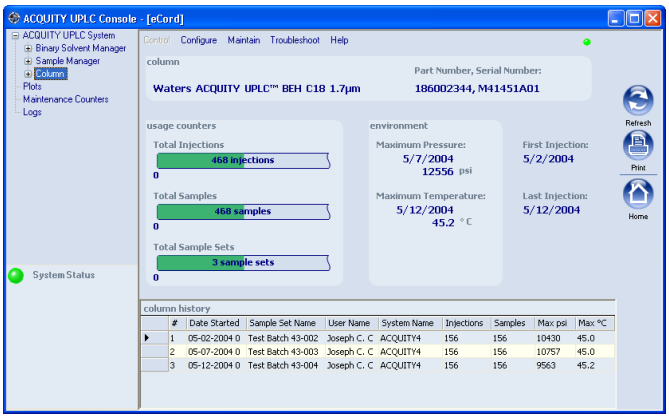


The eCord Chip provides the user with an overview of the bulk material QC test results.



The eCord Chip provides the user with QC test conditions and results on the column run by the manufacturer. The information includes mobile phases, running conditions, and analytes used to test the columns. In addition, the QC results and acceptance is placed onto the column.

d. Customer Use Information



The eCord Chip will automatically capture column use data. The top of the screen identifies the column including chemistry type, column dimensions, and serial number. The overall column usage information includes the total number of samples, total number of injections, total sample sets, date of first injection, date of last injection, maximum pressure, and temperature. The information also details the column history by sample set including date started, sample set name, user name, system name, number of injections in the sample set, number of samples in the sample set, maximum pressure, and temperature in the sample set; and if the column met basic system suitability requirements.

IX. MEASURING GRADIENT SYSTEM VOLUME (OR DWELL VOLUME)

1. Remove column.
2. Use acetonitrile as A, and acetonitrile with 0.05 mg/mL uracil as B (eliminates non-additive mixing and viscosity problems).
3. Monitor 254 nm.
4. Use the flow rate in the original method and the intended flow rate on the target instrument.
5. Collect 100% A baseline for 5 minutes.
6. At 5.00 min, program a step to 100% B, and collect data for an additional 5 minutes.
7. Measure absorbance difference between 100% A and 100% B.
8. Measure time at 50% of that absorbance difference.
9. Calculate time difference between start of step and 50% point.
10. Multiply time difference by flow rate.

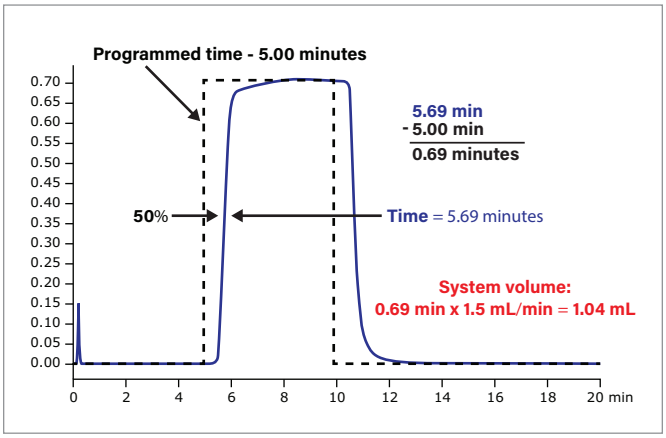


Figure 10. Determination of system volume.

X. 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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