XBridge and XBridge Premier Columns

CONTENTS

I. INTRODUCTION

Thank you for choosing a Waters™ XBridge™ and/or XBridge Premier BEH™ Column. The XBridge BEH packing materials are designed to provide excellent peak shape, high efficiency, and excellent stability for acidic and basic mobile phases and are manufactured in a cGMP, ISO 9001 certified plant using ultra-pure reagents. Each batch of XBridge BEH packing material is tested chromatographically with acidic, basic, and neutral analytes and the results are held to narrow specification ranges to assure excellent, reproducible performance. Every column is individually tested, and a Performance Test Chromatogram is provided with each column along with the Certificate of Analysis. These documents are available on waters.com/coa or by scanning the QR code on the column label.

XBridge Columns are available in a broad range of sorbent particles and sizes: XBridge e***X***tended ***P***erfomance (***XP***) 2.5 µm Columns for rapid UHPLC analytical separations; 3.5 and 5 µm HPLC columns for reliable analytical method development; and 5 and 10 µm optimum bed density (OBD) preparative columns for purification chromatography.

XBridge Premier Columns utilize MaxPeak™ High Performance Surfaces (HPS) Technology, an innovative technology designed to increase analyte recovery, sensitivity, and reproducibility by minimizing analyte/surface interactions that can lead to sample losses. The XBridge Premier Column is offered with or without a VanGuard™ Fully Integrated Technology [FIT] Cartridge. The VanGuard FIT Cartridge is designed to prevent the unwanted introduction of sample matrix or particulates onto the column without degrading the separation. It can be easily replaced to restore separation performance and extend the analytical column's lifetime.

XBridge™ COLUMNS

XBridge™ PREMIER COLUMNS

II. GETTING STARTED

With each XBridge Column or XBridge Premier Column, a Certificate of Analysis and Performance Test Chromatogram are available in the column box or on the column's eCord™ Intelligent Chip or eConnect™ Column Tag. The Certificate of Analysis is specific to each batch of packing material and includes the batch number, analysis of unbonded and bonded particles, and chromatographic results and conditions. The Performance Test Chromatogram is specific to each individual column and contains the batch number, column serial number, USP tangent efficiency, USP tailing factor, retention factor, and chromatographic conditions. This data should be stored for future reference.

A. ECORD/ECONNECT INSTALLATION

(May not be available for all column configurations)

Depending on the column configuration, your XBridge or XBridge Premier Column may be equipped with either an eCord Intelligent Chip Button or an eConnect electronic column tag. The eCord Button is designed for use on the ACQUITY UPLC™ and ACQUITY™ Arc™ Systems and it should be attached to the side of the instrument's column heater module. The eCord button is magnetized and does not require a specific orientation.

The eConnect Electronic Column Tag is an optional feature for HPLC columns and is designed to be used with the Waters Alliance™ iS HPLC System and the Waters Alliance iS Bio HPLC System. The eConnect Tag is read automatically when the column is installed on the Alliance iS system, and no manual intervention from the user is necessary.

For more information on the eCord Intelligent Chip or eConnect Column Tag functionality, see Section VII in this care and use manual.

B. CONNECTING THE COLUMN TO THE LC SYSTEM

(With or without a VanGuard FIT Cartridge)

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.

i. General Recommendations for Stainless Steel Tubing Connections

Note: If you are using stainless steel tubing with compression screw fittings, you will need a 3/8" wrench and a 5/16" wrench to make proper connections.

1. An arrow on the column identification label indicates the correct direction of solvent flow.
2. Correct connection of 1/16-inch outer diameter stainless steel tubing leading to and/or from the column is essential for high quality chromatographic results. The choice of appropriate column connectors and system tubing is discussed in detail below.
3. 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.

Note: If one of the wrenches is placed on the column tube flat during this process, the endfitting will be loosened and leak.

4. To obtain a void-free connection, the tubing must touch the bottom of the column endfitting. Extra-column peak broadening due to voids can destroy an otherwise successful separation.
5. 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.
6. Alternatively, replace the conventional compression screw fitting with an all-in-one PEEK fitting (P/N 186008714) that allows resetting of the ferrule depth.

ii. Column Connectors and System Tubing Considerations

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 endfitting does not match the existing tubing ferrule settings. This section explains the differences between Waters style and Parker style ferrules and endfittings. Each endfitting style varies in the required length of the tubing protruding from the ferrule. Figure 1 identifies which endfitting style is used on each style of XBridge and XBridge Premier Column.

Figure 1. Standard XBridge Columns with a particle size greater than 3 µm have Waters style endfittings. All other XBridge Columns and XBridge Premier Columns have Parker style endfittings.

Standard XBridge Columns with particle sizes greater than 3 µm are equipped with Waters style endfittings that require a 0.130-inch ferrule depth. XBridge Columns with particle sizes less than 3 µm, as well as all XBridge Premier Columns, are equipped with Parker style endfittings that require a 0.090-inch ferrule depth. It is critical to set the ferrule depth properly prior to installing the XBridge or XBridge Premier Column for optimal performance. In a proper tubing/column connection (Figure 2a), the tubing touches the bottom of the column endfitting, with no void between them.

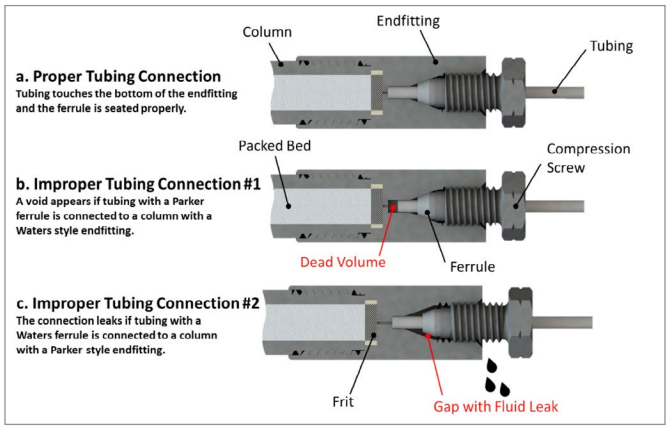

Figure 2. Proper tubing connections require the tubing to touch the bottom of the endfitting and the ferrule to be seated properly.

The presence of a void (dead volume) in the flow stream will reduce column performance. This can occur if a Parker ferrule is connected to a Waters style endfitting, or if proper care is not taken when staking the ferrule onto the tubing (Figure 2b). There is only one way to fix this problem:

1. 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.
    a. Alternatively, replace the conventional compression screw fitting with an all-in-one PEEK® Fitting (P/N: 186008714) that allows resetting of the ferrule depth.

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 2c). There are two ways to fix this problem:

1. Tighten the screw a bit more. The ferrule may move forward and reach 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.
    a. Alternatively, replace the conventional compression screw fitting with an all-in-one PEEK Fitting (P/N: 186008714) that allows resetting of the ferrule depth.

C. COLUMN STARTUP GUIDELINES

Note: Prior to handling XBridge BEH Columns and any chemical, consult with your safety department and/or local regulations on the use of proper protective equipment.

All XBridge Columns are shipped in 100% acetonitrile. The flow rates given in the procedure below are for 2.1 mm ID columns. Scale the flow rate up or down accordingly based upon the column ID, length, particle size, and backpressure limit of the XBridge Column being installed. The following formulas will allow scale up or scale down, while maintaining the same linear velocity, and provide new sample loading values:

If column ID and length are altered:

$F_2 = F_1 (r_2/r_1)^2$

$Load_2 = Load_1 (r_2/r_1)^2 (L_2/L_1)$

$Injection\ volume_2 = Injection\ volume_1 (r_2/r_1)^2 (L_2/L_1)$

Where: r = Radius of the column

F = Flow rate

L = Length of column

1 = Original, or reference column

2 = New column

1. Purge the pumping system of any buffer-containing mobile phases and connect the inlet end of the column to the injector outlet.
2. Flush column with 100% organic mobile phase (methanol or acetonitrile) by setting the pump flow rate to 0.1 mL/min and increase the flow rate to 0.5 mL/min over five 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.
4. Gradually increase the flow rate as described in Step 2.
5. Once a steady backpressure and baseline have been achieved, proceed to the next section.

D. COLUMN EQUILIBRATION

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 (see Table 1 for a list of column volumes). The column may be considered thermally equilibrated once a constant backpressure is achieved.

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

	Column Internal Diameter (mm)								
Column Length (mm)	1.0	2.1	3.0	4.6	7.8	10	19	30	50
20	–	0.07	0.14	0.33	–	–	–	–	–
30	–	0.10	0.21	0.50	–	2.4	8.5	–	–
50	0.04	0.17	0.35	0.83	2.4	3.9	14	35	98
100	0.08	0.35	0.71	1.7	4.8	7.8	28	70	–
150	0.12	0.52	1.0	2.5	7.2	12	42	106	294
250	–	0.87	1.8	4.2	–	20	70	176	490

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 system with 60% methanol in water prior to introducing 60% methanol/40% buffer mobile-phase.)

For columns packed with BEH HILIC material, flush with 50 column volumes of 50:50 acetonitrile: water with 10 mM final buffer concentration. For columns packed with BEH Amide material, flush with 50 column volumes of 60:40 acetonitrile: aqueous. Prior to the first injection, equilibrate with 20 column volumes of initial mobile phase conditions (see Table 1 for a list of column volumes). See Section VIII, "Special Considerations for HILIC and Amide Columns" for additional information.

E. INITIAL COLUMN EFFICIENCY DETERMINATION

1. Perform an efficiency test on the column before using it. This test may consist of:
   a. An analyte test mixture that is commonly used in your laboratory, and/or
   b. The analyte mixture as found on the "Performance Test Chromatogram" that accompanied your column.

Note: If (b) is performed, the isocratic efficiencies measured in your laboratory may be less than those given on the Waters "Performance Test Chromatogram." This is normal. The Waters isocratic column testing systems have been modified to achieve extremely low system volumes. This presents a more challenging test of how well the column was packed and guarantees the highest quality packed column. These special testing systems have been modified to such an extent that they are not commercially viable and have limited method flexibility other than isocratic column testing.

2. Determine the number of theoretical plates (N) and use this value for periodic comparisons.
3. Repeat the test at predetermined intervals to track column performance over time. Slight variations may be observed due to differences in the quality of the connections, operating environment, system electronics, reagent quality, column condition, and operator technique.

F. COLUMN QR CODE

The quick reference (QR) code that is located on the column label provides column-specific information (*e.g.*, the part and serial numbers that are unique identifiers for the column), and its encoding follows a widely adopted industry-standard.

1. Scan QR code using any device that is capable of scanning QR codes (*e.g.*, for smart phones and tablets, use the built-in camera app).
2. Be directed to the column's information hub on waters.com.
3. Access technical and scientific information for the column (*e.g.*, certificate of analysis, application notes).

G. VanGuard/VanGuard FIT COLUMN PROTECTION

For standard XBridge Columns, a separate guard holder and guard cartridge can be easily attached to the inlet of the column (see Figure 3). The guard cartridge holder can be reused multiple times with replacement guard cartridges on the same analytical column. We recommend using a new guard cartridge holder each time you replace the analytical column.

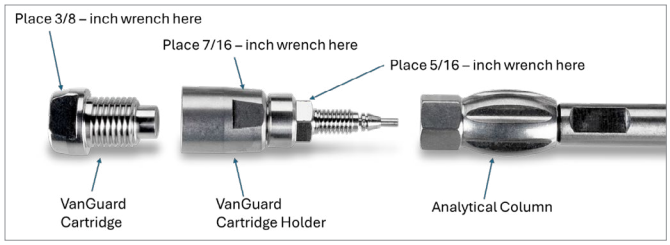

Figure 3. Connection of the VanGuard Cartridge Column, VanGuard Cartridge Holder, and analytical column.

The inlet of the VanGuard Cartridge is designed to be compatible with its intended column configuration and is available in two formats: Parker-style fittings are designated with a crosshair stamped on the VanGuard Cartridge inlet (Figure 4), and can be found in XBridge VanGuard Cartridges with particle sizes less than 3 µm (XBridge ***XP*** Columns). Waters-style fittings are present in XBridge VanGuard Cartridges with particle sizes greater than 3 µm, and do not have the stamped crosshair pattern on the inlet. For more information, visit the VanGuard Column Protection page on waters.com.

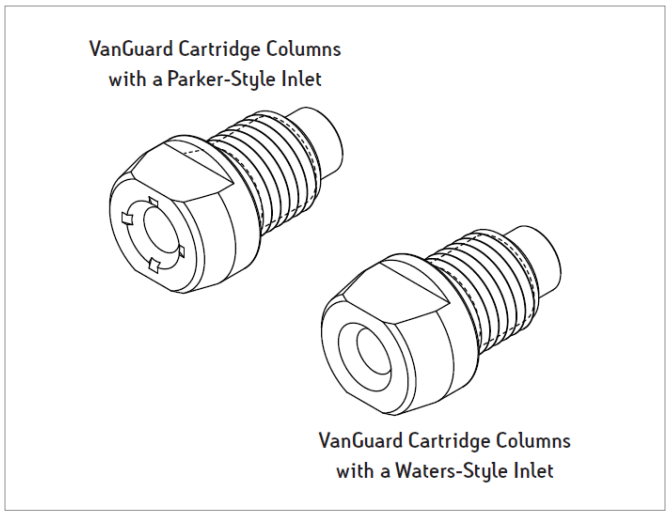

Figure 4. VanGuard Cartridge Columns with Parker-style or Waters-style inlets can be differentiated by the stamped crosshair pattern on the inlet.

The VanGuard FIT Cartridge is designed specifically for XBridge Premier Columns. For the XBridge Premier Columns that have a VanGuard FIT Cartridge, the VanGuard FIT Cartridge may be replaced using two 3/8-inch wrenches. Simply apply the wrenches to the flats on the guard and column end nut and turn in a counterclockwise direction (see Figure 5) to remove the guard cartridge.

A new VanGuard FIT Cartridge can now be used to replace the discarded one. Hand-tighten the new cartridge in a clockwise direction, then tighten using two 3/8-inch wrenches. Proper sealing should not require more than a 1/4 turn past the hand-tightened position. Note that all VanGuard FIT Cartridges feature a Parker style endfitting and require a 0.090-inch ferrule depth.

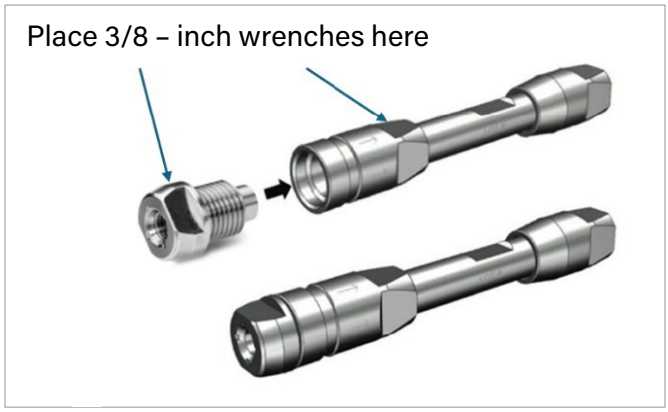

Figure 5. Recommended 3/8" wrench placement to remove the VanGuard FIT Cartridge from the XBridge Premier VanGuard FIT Column.

III. COLUMN USE

To ensure the continued high performance of XBridge and XBridge Premier Columns, follow these guidelines:

A. SAMPLE PREPARATION

1. Sample impurities often contribute to column contamination. One option to avoid this is to use Waters Oasis™ Solid-phase Extraction Cartridges/Columns or Sep-Pak™ Cartridges of the appropriate chemistry to clean up the sample before analysis. For more information, visit the Sample Preparation Page on waters.com.
2. It is preferable to prepare the sample in the operating mobile phase or a mobile phase that is weaker than the mobile phase for the best peak shape and sensitivity. Acetone should not be used as a sample solvent/diluent unless a Hexane Tetrahydrofuran Compatibility Kit has been installed.
3. If the sample is not dissolved in the mobile phase, ensure that the sample, solvent, and mobile phases are miscible to avoid sample and/or buffer precipitation.
4. Filter sample with 0.2 µm membranes to remove particulates. If the sample is dissolved in a solvent that contains an organic modifier (*e.g.*, acetonitrile, methanol, *etc.*) ensure that the membrane material does not dissolve in the solvent. Contact the membrane manufacturer with solvent compatibility questions. Alternatively, centrifugation for 20 minutes at 8000 rpm, followed by the transfer of the supernatant liquid to an appropriate vial, could be considered.
5. For Hydrophilic Interaction Chromatography (HILIC) separations, the samples must be prepared in a high percentage of organic solvents (*e.g.*, 95% acetonitrile). See the "Special Considerations for HILIC and Amide Columns" section for additional information.

Table 2. Maximum Tolerated Operating Pressure for Analytical XBridge and XBridge Columns

Particle Size	Column I.D.	Max. Tolerated Operating Pressure
2.5 µm	2.1 mm and 3.0 mm	18,000 psi (1241 bar or 124 MPa)
	4.6 mm	10,000 psi (700 bar or 70 MPa)
3.5µm and 5 µm	2.1 mm, 3.0 mm, and 4.6 mm	6,000 psi (400 bar or 40 MPa)

B. PH RANGE

The recommended operating pH range for XBridge Columns is 1–12 for the BEH C_{18}, BEH C_8, and BEH Phenyl chemistries; 2–11 for the BEH Shield RP18 and BEH Amide chemistries; and 1–9 for the BEH HILIC chemistry. A listing of commonly used buffers and additives is given in Table 3. The column lifetime will vary depending upon the operating temperature, the type, and concentration of buffer used. For example, the use of phosphate buffer at pH 8 in combination with elevated temperatures will lead to shorter column lifetimes.

C. SOLVENTS

To maintain maximum column performance, use high quality chromatography grade solvents. Filter all aqueous buffers prior to use through a 0.2 µm filter. Pall Gelman Laboratory Acrodisc® filters are recommended. Solvents containing suspended particulate materials will generally clog the outside surface of the inlet distribution frit of the column. This will result in higher operating pressure and poorer performance. See Section V (Tips for Maximizing XBridge Column Lifetime) for more information.

D. PRESSURE

Table 2 summarizes the maximum tolerated operating pressure limits for analytical XBridge Columns based on particle size and column internal diameter. XBridge Columns with greater than 3 µm particle size can tolerate operating pressures up to 6,000 psi (400 bar or 40mPa), although greater than 4,000–5,000 psi should be avoided to maximize column and system lifetimes.

Note: Working at the extremes of pressure, pH and/or temperature will result in shorter column lifetimes.

E. TEMPERATURE

Temperatures between 20–90 °C are recommended for operating XBridge Columns to enhance selectivity, lower solvent viscosity, and increase mass transfer rates. Column lifetime at elevated temperature is strongly dependent on mobile phase pH and water content. When operating near the pH limits, lower operating temperatures are recommended for longer column lifetime.

Table 3. Buffer Recommendations for Using XBridge Columns

Additive/Buffer	pKa	Buffer range	Volatility	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 (NH4OH) Ammonium Bicarbonate	9.2 10.3 (HCO_3-) 9.2 (NH_4+)	8.2–10.2 8.2–11.3	Volatile Volatile	Yes 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.
Pyrrolidine	11.3	10.3–12.3	Volatile	Yes	Mild buffer, gives long lifetime.

Table 4. Recommended pH and Temperature Limits for XBridge Columns

Bonded Phase	Particle Size	Pore Diameter	Surface Area	pH Limit	Temp. Guidance* for pH Extremes		Ligand Density	Carbon %
					Low pH	High pH		
XBridge BEH C_{18}	2.5, 3.5, 5 µm	130 Å	185 m^2/g	1–12	80 °C	60 °C	3.1 µmol/m^2	17.7
XBridge BEH C_8	2.5, 3.5, 5 µm	130 Å	185 m^2/g	1–12	60 °C	60 °C	3.1 µmol/m^2	12.8
XBridge BEH Phenyl	2.5, 3.5, 5 µm	130 Å	185 m^2/g	1–12	80 °C	60 °C	3.0 µmol/m^2	14.5
XBridge BEH Biphenyl	2.5 µm	130 Å	185 m^2/g	1.5–10**	60 °C	45 °C	3.3 µmol/m2	16.2
XBridge BEH Shield RP18	2.5, 3.5, 5 µm	130 Å	185 m^2/g	2–11	50 °C	45 °C	3.2 µmol/m^2	16.6
XBridge BEH HILIC	2.5, 3.5, 5 µm	130 Å	185 m^2/g	1–9	60 °C	60 °C	–	–
XBridge BEH Amide	2.5, 3.5, 5 µm	130 Å	185 m^2/g	2–11	90 °C	90 °C	7.5 µmol/m^2	12

**Note: Higher operating temperatures are acceptable when working in the mid-range of the recommended pH limit. When operating near the pH limits, lower operating temperatures are recommended for longer column lifetime.*

***Note: To minimize UV-detected column bleed from BEH Biphenyl columns, the pH should be less than 7. See section IX for additional information.*

IV. 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, or increasing backpressure may indicate contamination of the column. Flushing with a neat organic solvent, taking care not to precipitate buffers, is usually sufficient to remove the contaminant. If the flushing procedure does not solve the problem, purge the column using the following cleaning and regeneration procedures.

Use the cleaning routine that matches the properties of the samples and/or what you believe is contaminating the column (see Table 5). Flush columns with 20 column volumes of solvent.

Increasing column temperature increases cleaning efficiency. If the column performance is poor after regenerating and cleaning, call your local Waters office for additional support.

Flush BEH HILIC columns with 50:50 acetonitrile:water to remove polar contaminants. If this flushing procedure does not solve the problem, purge the column with 5:95 acetonitrile:water.

To clean polar contaminants from BEH Amide columns, run a 10-minute gradient from 0–100% water. Please note that as aqueous concentration increases, backpressure will rapidly increase as well. Reduce flow rate when operating at greater than 60% aqueous. Repeat if necessary.

Table 5. Reversed-Phase Column Cleaning Sequence

Polar Samples	Non-polar samples	Proteinaceous Samples
1. Water	1. Isoproanol (or an appropriate isopropanol/water mixture**)	Option 1: Inject repeated aliquots of dimethylsulfoxide (DMSO)
2. Methanol	2. Tetrahydrofuran (THF)	Option 2: Gradient of 10% to 90% B where: A = 0.1% trifluoroacetic acid (TFA) in water, B = 0.1% trifluoroacetic acid (TFA) in acetonitrile (CH_3CN)
3. Tetrahydrofuran (THF)	3. Dichloromethane	
4. Methanol	4. Hexane	
5. Water	5. Isopropanol (followed by an appropriate isopropanol/water mixture**)	Option 3: Flush column with 7 M guanidine hydrochloride or 7 M urea
6. Mobile phase	6. Mobile phase	

**Prior to using THF or hexane, ensure your system is compatible with these solvents. THF or hexane should only be considered when the column cannot be cleaning by running neat, reversed-phase organic solvents such as acetonitrile. Reduce flow rate, lower operating temperatures, and limit system exposure to THF and/or hexane.*

*** Use low organic solvent content to avoid precipitation buffers.*

B. STORAGE

For periods longer than four days at room temperature, store reversed-phase columns in 100% acetonitrile. For elevated temperature applications, store immediately after use in 100% acetonitrile for the best column lifetime. Do not store columns in buffered eluents. If the mobile phase contained a buffer salt, flush reversed-phase XBridge Columns 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 when 100% acetonitrile is introduced. Run a gradient to 100% ACN

in order to flush all aqueous solvent from an XBridge BEH Amide Column prior to storage in 100% ACN. Completely seal column to avoid evaporation and drying out of the bed.

For periods longer than four days, store XBridge BEH HILIC Columns in 95:5 acetonitrile:water. Do not store in buffered solvent. If the mobile phase contained a buffered salt, flush the column with 10-column volumes of 95:5 acetonitrile:water (see Table 1 for common column volumes).

Note: If a column has been run with a mobile phase that contains formate (e.g., ammonium formate, formic acid, etc.) and is then flushed with 100% acetonitrile, slightly longer equilibration times may be necessary when the column is re-installed and run again with a formate-containing mobile phase.

V. TIPS FOR MAXIMIZING XBRIDGE COLUMN LIFETIMES

1. To maximize XBridge Column lifetime, pay close attention to:
   - Water quality (including water purification system)
   - Solvent quality
   - Mobile-phase preparation, storage, and age
   - Sample, buffer, and mobile-phase solubilities
   - Sample quality and preparation
2. It is recommended to monitor your system's health by incorporating a quality control (QC) reference material into your workflow. (Reference 720004535en)
3. When problems arise, often only one improper practice must be changed.
4. Always remember to:
   - Use an in-line filter unit or, preferably, a VanGuard Cartridge
   - Discourage bacterial growth by minimizing the use of 100% aqueous mobile phases where possible
   - Change aqueous mobile phase every 24–48 hours (if 100% aqueous mobile phase use is required)
   - Discard old 100% aqueous mobile phases every 24–48 hours to discourage bacterial growth
   - Add 5–10% organic modifier to mobile phase A and adjust gradient profile
   - Filter aqueous portions of mobile phase through 0.2 µm filter
   - Maintain your water purification system so that it is in good working order
   - Only use ultra-pure water (18 megohm-cm) water and the highest quality solvents possible. HPLC-grade water is not UPLC-grade water
   - Consider sample preparation (*e.g.*, solid-phase extraction, filtration, *etc.*)
5. Avoid (where possible):
   - 100% aqueous mobile phases (if possible)
   - HPLC-grade bottled water
   - "Topping off" or adding "new" mobile phase to "old" mobile phase
   - Old aqueous mobile phases, remembering to rinse bottles thoroughly and prepare fresh every 24 to 48 hours
   - Using phosphate salt buffer in combination with high ACN concentrations (*e.g.*, >70%) due to precipitation
6. Don't assume a "bad" column is the culprit when high backpressure or split peaks are observed. Investigate cause of column failure by checking:
   - Backpressure
   - Mobile phase(s), bacteria, precipitation, and/or samples
   - Sample quality
   - Injection solvent strength
7. To reduce the chances of mobile-phase contamination or degradation, only prepare what you need for analysis or store excess bulk quantities in a refrigerated environment.
8. Mobile phase-related questions to ask:
   - Am I using 100% aqueous mobile phases? Am I able to add a small amount of organic modifier to my mobile phase A?
   - Do I filter my aqueous mobile phases through 0.2 µm filters?
   - How old is my mobile phase? Do I label the bottle with the preparation date?
   - Do I "top off" or do I prepare fresh mobile phases every 24–48 hours?
   - What is the quality of my water? Has the quality recently changed? How is my water purification system working? When was it last serviced?
   - Am I working with pH 7 phosphate buffer (which is VERY susceptible to bacterial growth)?

9. Sample-related questions to ask:
   - If I inject neat standards prepared in mobile phase, do I observe these problems?
   - If I prepare my standards in water and prepare them like samples (*e.g.*, SPE, filtration, *etc.*) do I still observe these problems?
   - Has the quality of my samples changed over time?

VI. PREPARATIVE XBridge AND MaxPeak XBridge PREMIER COLUMNS

A. OPTIMAL BED DENSITY: THE OBD™ DIFFERENCE

Complex mixtures of sparingly soluble sample components are often dissolved in strong solvents, such as DMSO, THF or DMF, a routine practice in preparative LC. Such solvents cause chromatographic distortion and can introduce issues due to the combination of poor solubility and pressure shocks from large injection volumes. This can compress non-uniformly packed prep column beds, leading to premature column failure, ultimately increasing the potential for lost target compound.

Waters OBD Preparative Columns are built for the harsh demands of target clean-up and purification by HPLC. Their long column lifetimes combined with reliable performance ultimately improve productivity in the purification laboratory, whether purifying singleton targets or libraries batches of target compounds. Waters XBridge OBD Preparative Columns are packed to bed densities that closely match the equivalent analytical column, having uniform bed density throughout the entire length and diameter of the column. OBD Preparative Columns result in predictable scale-up performance, a characteristic that is rare for conventionally packed preparative columns. Matching bed densities are instrumental in providing direct scalability from analytical (UPLC or HPLC) to lab-scale prep.

i. Prep Guards and Holders

Guard columns are essential for protecting both the prep system and column from contaminants or undissolved solids in the sample mixture, which impact chromatographic resolution and column lifetime, or cause LC system issues. Guard columns (10 mm in length) are steel cartridge inserts packed with the same stationary phase as the preparative column and fit in a specialized guard holder. A guard column holder (P/N: 186011548 for XBridge; P/N: 186011737 for MaxPeak Premier XBridge) needs to be plumbed inline ahead of the preparative column. The guard column can be easily replaced when run pressures increase from injection to injection or split peaks emerge.

There are two types of guard holders available, one which can be tightened by hand (Figure 6a), and one that is tightened with the use of a wrench (Figure 6b). Both guard holder types come with a rigid connector (Figure 6c) and transfer tubing and are available in 7.8 mm – 30 mm internal diameters. Select the internal diameter of the guard assembly (column plus required holder) that best matches the prep column diameter for best performance.

Figure 6. Preparative guard column holders and rigid connector assembly for use with 7.8–30 mm internal diameter prep columns.

Sample targets can also precipitate on the head of the column if their solubility is not maintained throughout the preparative run. When this occurs, the system pressure increases and/or a decreasing detector signal of the target peak can become evident. To alleviate the issue and recover the target compound, flush the column with the strong solvent used in the mobile phase (100% or combinations of strong solvents) or perform repeat DMSO injections.

B. SAMPLE PREP GUIDANCE

When direct scaling to prep scale is intended (*i.e.* mixture component elutes from the prep column in the same order and column volumes as observed during on the analysis or screening run), the identical column packing should be employed. Consistency should be kept between both the base particle type (*i.e.* XBridge BEH 130 Å, BEH 300 Å, *etc.*) and the ligand (C_{18}, C_8, C_4, *etc.*) bound to it to maintain the column chemistry when scaling. The particle size can vary during scale-up to prep as long as the column length to particle size (L/dp ratio) is maintained and linear velocity is scaled appropriately.

The sample mixture should be filtered using an Acrodisc 13 or 25-mm syringe filter (P/N: WAT200500 or WAT200558) or a bed of Celite to remove residual solids (reaction catalysts, *etc.*). This ensures that that sample mixtures are fully solubilized before injecting onto the prep LC system for purification. The mobile phase (pH adjusted) is an ideal solvent, but strong solvents such as acetonitrile, methanol or DMSO are common. DMF should be avoided due to the extent of chromatographic distortion (*e.g.*, peak smearing) which can occur with its use.

C. COLUMN CAPACITY

Injection volumes should not exceed more than about 5–10% of the prep column's volume (and up to about 75% of the system's loop volume). Sample diluent volume can be increased when sample solubility is low which may be necessary for certain molecular types (*e.g.*, peptides).

Keep in mind that adjusting the pH is a well-known option to optimize crude sample mixture loads. Acidic and basic components chromatograph well in their neutral forms, when the pH is not near the pKa value. The XBridge Column enables pH flexibility from 1–12. Hence, the amount of a crude sample load of a basic compound mix (*e.g.*, small molecule drugs, *etc.*) can be maximized on a preparative column when the diluent pH is also basic. For example, LC purifications employing a base (*e.g.*, 0.1% ammonium hydroxide), in both the diluent and the mobile phase, can significantly increase sample loads on prep columns.

The total mass capacity of a preparative column is greatly dependent on the sample complexity, molecular hydrophobicity, separation method, sample diluent, target compound retention, and column chemistry. To determine the amount of crude sample mixture that can be loaded onto a specific XBridge Preparative Column, perform a loading study on the analysis or scouting column. Consult the Prep Ordering Guide (720007209EN) for more details.

D. SCALE-UP GUIDANCE

When directly scaling to prep, the identical column packing should be employed for desired results (*e.g.*, components eluting in the same order and column volumes). Consistency should be kept between both the base particle type (XBridge BEH 130 Å, BEH 300 Å, *etc.*) and the ligand (C_{18}, C_8, C_4, *etc.*) The particle size can vary during scale-up to prep as long as the column length to particle size (L/dp ratio) is maintained and linear velocity is scaled appropriately. For more information on scale-up and loading, visit waters.com/prep, or reference the Preparative OBD Columns Wall Chart for loading and mobile-phase charts, scaling tools, and more.

VII. ADDITIONAL INFORMATION

A. ECORD AND ECONNECT ELECTRONIC COLUMN TAGS

"Smart columns" are those enabled with electronic devices (tags) permanently affixed to enable identification and/or tracking of column history and use information. These tags contain unique product identifying information, such as part numbers, serial numbers, and product descriptions. Data residing on these devices is automatically imported by complementary Waters' systems, whether LC instrumentation or the Empower Chromatographic Data System. This type of product data is used to aid in tracking and decision-making and troubleshooting, especially valuable for GLP/GMP compliant laboratories.

Tags come in two types: the tethered eCord Button and the untethered eConnect Tag. The eCord is a standard feature on ACQUITY UPLC™ Columns and an optional feature on some XBridge/XBridge Premier Columns for use with Waters ACQUITY and Arc UHPLC Systems. The eConnect column tag is an optional feature for some XBridge Columns and is designed to be used with the Waters Alliance iS and Alliance iS Bio HPLC system. Both the eCord chip and the eConnect tag are permanently attached to the column to assure that the column performance history is maintained if the column is moved from one instrument to another.

Both the eCord chip and the eConnect tag are designed to be robust and durable. When in operation, the eCord enabled column is connected via a cable tether to the system, allowing the electronic tag to be physically located outside the column manager. This placement reduces exposure to variable operating temperatures and solvents. In contrast, the eConnect tag is designed to be within the column manager. Because it is exposed to the full range of operating temperatures and may be exposed to solvents, extensive testing has been conducted to ensure the performance of the eConnect tag in this environment.

i. eCord Intelligent Chip Technology

(may not be available for all column configurations)

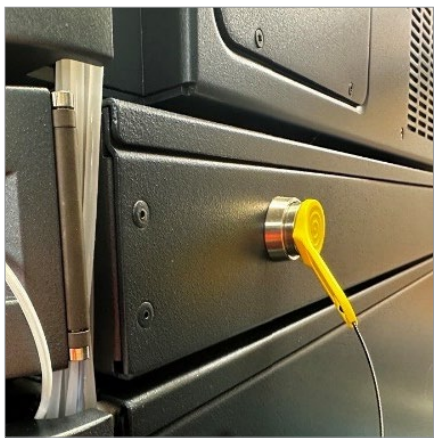

Figure 7. eCord Intelligent Chip attached to the side of column heater.

The eCord chip provides the customer with a history of column use data and performance throughout its lifetime. Once the column is installed into the column heater, attach the eCord to the side of the column heater (Figure 7) and the Empower or MassLynx™ Software will automatically download key parameters into a column history file stored on the chip, allowing access to the column information. The information will include the column 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, username, 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. Up to 50 sample sets can be stored on the eCord chip.

ii. eConnect Electronic Column Tag

(may not be available for all column configurations)

The eConnect electronic column tag is a digital device that enables fully automated identification, verification and tracking of column use. eConnect tags operate with Empower 3.8 with extended capabilities. When a column equipped with an eConnect tag is installed into the Alliance iS column heater (Figure 8), the column information is automatically imported into Empower.

Figure 8. An eConnect enabled column installed in an Alliance iS column heater.

In addition, eConnect tags also offer a convenient, easy-to-use connection to the Waters website. Using an NFC enabled internet connected device to scan the eConnect tag will provide the user with a web address which will take them directly to the Waters product page for that consumable. This page provides access to product information, like Care and Use or Certificate of Analysis, as well as reorder and related product support.

B. TIPS FOR OPERATING ON HIGH DISPERSION SYSTEMS

i. Measuring System Bandspreading Volume and System Variance

This test should be performed on an LC system with a single wavelength UV detector (not a Photodiode Array (PDA)). Suggested operating parameters are appropriate for typical HPLC systems, but these general guidelines apply to any LC system regardless of system dispersion. Adjust the flow rate and injection volume as needed according to your specific LC system parameters.

1. Disconnect column from system and replace with a zero dead volume union.
2. Set flow rate to 1 mL/min.
3. Dilute a caffeine standard (Waters P/N: 70003233) in mobile phase to give a detector sensitivity of 0.5–1.0 AUFS.
4. Inject 2 to 5 µL of this solution.
5. Measure the peak width at 4.4% of peak height (5-sigma method, Figure 9):

   5-sigma Bandspreading (µL) = Peak Width (min) x Flow Rate (µL/min)

   System Variance (μL^2) = (5-sigma Bandspreading)^2 / 25

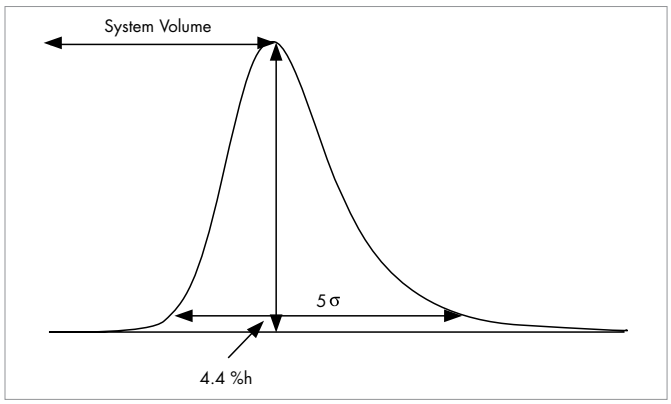

Figure 9. Determination of System Bandspreading Volume Using 5-sigma Method

In a typical HPLC system, the Bandspreading Volume should be no greater than 70 to 130 µL (or Variance of 364 to 436 µL^2). In a microbore system, the Bandspreading Volume should be no greater than 20 to 40 µL (or Variance no greater than 16 to 64µL^2). Figure 10 shows the influence of tubing internal diameter on system band spreading and peak shape. Larger tubing diameters can cause excessive peak broadening and lower sensitivity.

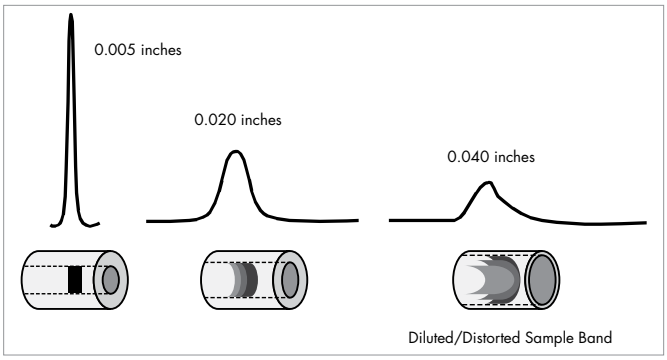

Figure 10. Influence of tubing internal diameter on system bandspreading and peak shape.

ii. Measuring Gradient Delay Volume (or Dwell Volume)

For successful gradient-method transfers the gradient delay volumes should be measured using the same method on both HPLC systems. The procedure below describes a method for determining the gradient delay volumes.

1. Replace the column with a zero dead volume union.
2. Prepare mobile phase A (pure solvent, such as methanol) and mobile phase B (mobile phase A with a UV absorbing sample, such as (v/v) 0.1% acetone in methanol).
3. Equilibrate the system with mobile phase A until a stable baseline is achieved.
4. Set the detector wavelength to the absorbance maximum of the probe (265 nm for acetone).
5. Program a 0–100% B linear gradient in 10 min at 0.5 to 2 mL/min (the exact conditions are not critical; choose a flow rate that will provide a sufficient gradient volume for your LC system type) with a hold at 100% B.
6. Determine the dwell time by first locating the time at the midpoint of the formed gradient ($t_{1/2}$) (half the vertical distance between the initial and final isocratic segments as shown in Figure 11).
7. Subtract half the gradient time (1/2 t_g) (10 min/2 = 5 min in this example) from the gradient midpoint ($t_{1/2}$) to obtain the dwell time (t_D).
8. Convert the dwell time (t_D) to the dwell volume (V_D) by multiplying by the flow rate (F).

$$\text{Dwell Volume } V_D = (t_{1/2} - 1/2\ t_g) \times F$$

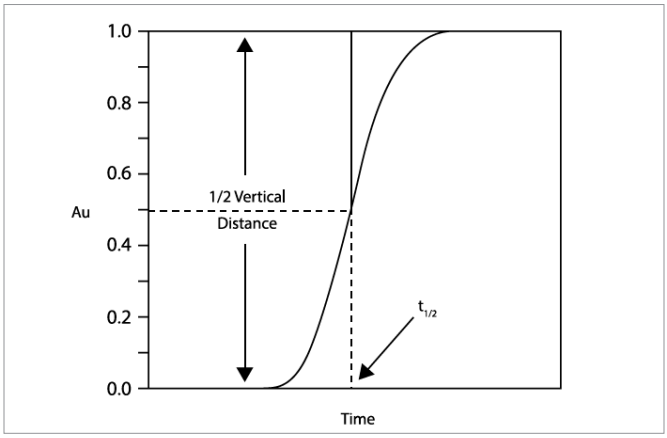

Figure 11. Determination of Gradient Delay Volume.

For fast gradient methods, the gradient delay volume (or dwell volume) should be less than 1 mL. If the gradient delay volume is greater than 1 mL, see the Use of Narrow-Bore Columns on High Dispersion Systems section for some system modification recommendations to reduce system volume.

iii. Impact of Bandspreading Volume on Column Performance

The system bandspreading volume will influence column performance. When comparing the same column across three different systems, there is a decrease in observed column efficiency as the system bandspreading volume increases (Figure 12).

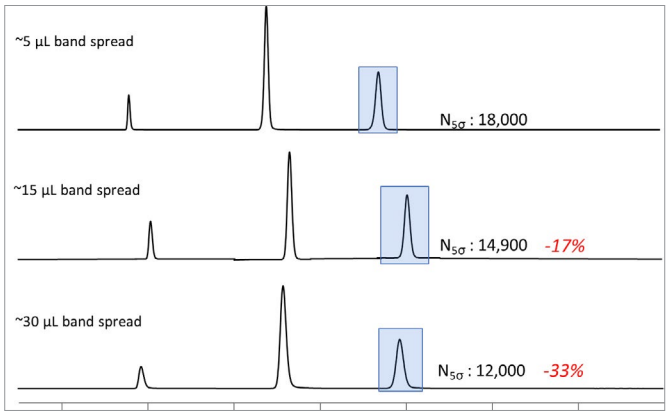

Figure 12. Efficiency decreases as the same column is run on three different LC systems with increasing bandspreading volume.

System optimization, especially in a system with high dispersion volume or one that contains a flow splitter, can have dramatic effects on sensitivity and resolution. Optimization includes using correct ferrule depths and minimizing tubing inner diameters and lengths. An example

is given in Figure 13 where system optimization resulted in a doubling of sensitivity and resolution of the metabolite in an LC-MS/MS system.

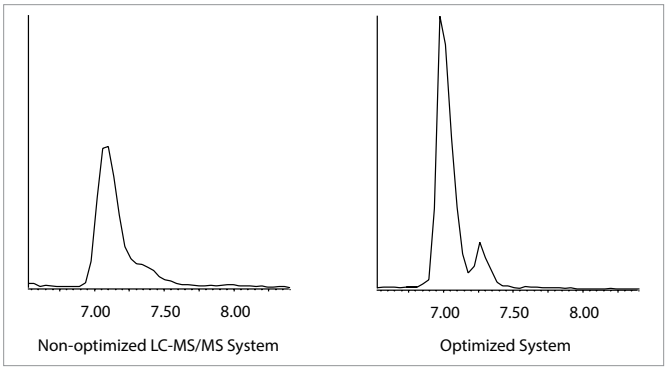

Figure 13: Non-Optimized vs. Optimized LC-MS/MS System.

iv. System Modification Recommendations and Use of Narrow-Bore Columns on High Dispersion Systems

This section describes how to minimize extra column effects and provides guidelines on maximizing the performance of a narrow-bore column on an HPLC system with high dispersion. A 3.0 mm ID narrow-bore column usually requires no system modifications. A 2.1 mm ID column, however, requires modifications to the HPLC system to eliminate excessive system bandspreading volume (Figure 14). Without proper system modifications, excessive system bandspreading volume causes peak broadening and has a large impact on peak width as peak volume decreases.

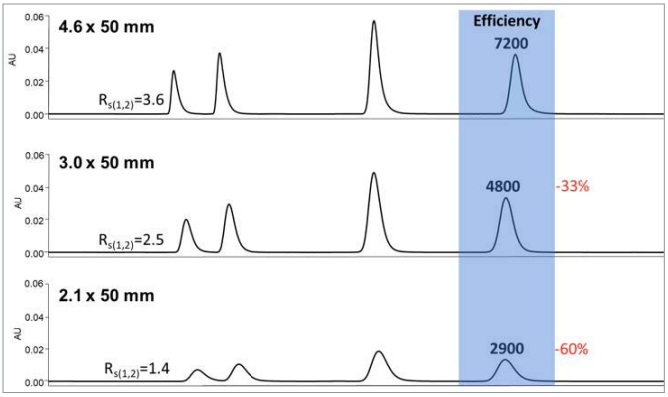

Figure 14. The system bandspreading on this LC system is approximately 50 µL. The observed efficiency decreases as column inner diameter decreases.

Consider the following system modification/optimization recommendations when operating on a high dispersion LC system:

1. Use a microbore detector flow cell with 2.1 mm ID columns.

Note: Detector sensitivity is reduced with the shorter flow cell path length to achieve lower bandspreading volume.

2. Minimize injector sample loop volume.
3. Use 0.009-inch (0.25 mm) tubing for connections in standard systems and 0.005-inch (0.12 mm) tubing for connections in narrow-bore (2.1 mm ID) systems.
4. Use perfect (pre-cut) connections (with a variable depth inlet if using columns from different suppliers).
5. Detector time constants should be shortened to less than 0.2 seconds.

C. MAXPEAK PREMIER COLUMN HARDWARE

Most LC column hardware is stainless steel, which has excellent pressure tolerances and a highly reproducible machining process for a wide range of dimensions/formats. Non-Specific Adsorption (NSA) can be a problem with stainless steel column hardware, as certain analytes (such as acidic, phosphorylated or carboxylated compounds) can bind to the metal oxide layer, resulting in poor peak shape, poor analyte recovery and poor reproducibility. This problem is often noticed as a loss in signal intensity or inconsistent results injection to injection and/or column to column.

MaxPeak Premier Columns feature the MaxPeak High Performance Surfaces (HPS) Technology, which is a hybrid organic/inorganic silica surface comprised of a resilient, highly crosslinked layer containing ethylene bridged siloxane groups that is covalently bonded to the metal surface. MaxPeak Premier Columns are designed to increase analyte recovery, sensitivity, and reproducibility by minimizing analyte/surface interactions that can lead to sample losses.

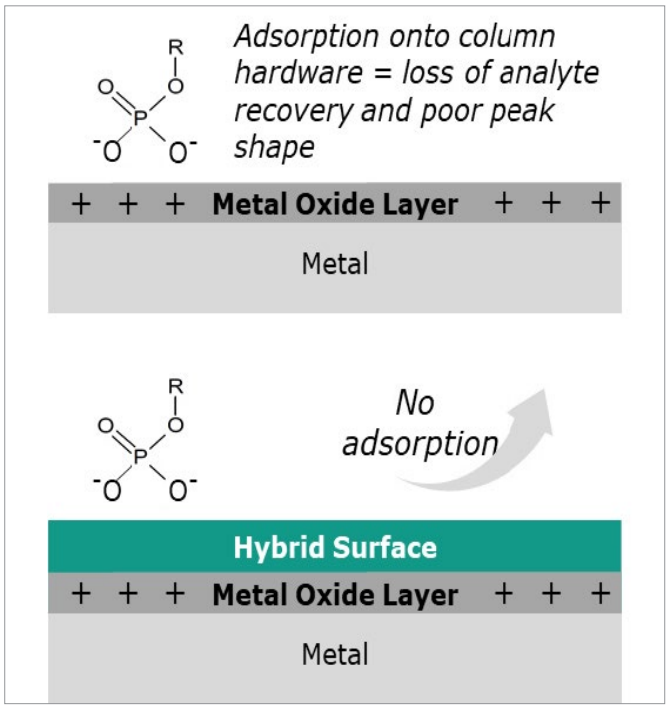

Figure 15. MaxPeak High Performance Surfaces Technology prevents adsorption of analytes to metal surfaces.

VIII. SPECIAL CONSIDERATIONS FOR BEH HILIC AND BEH AMIDE COLUMNS

A. RECOMMENDATIONS FOR BEH HILIC COLUMN USE

Because BEH HILIC columns do not possess a bonded phase, the pH operating range is 1–9, and they can operate at temperatures up to 60 °C when working near the upper and lower pH limits. As with any LC column, operating at the extremes of pH, pressures, and temperatures will result in decreased column lifetime.

i. Column Equilibration

1. When the column is first received, flush in 50% acetonitrile: 50% water with 10 mM final buffer concentration for 50 column volumes.
2. Equilibrate with 20-column volumes of initial mobile-phase conditions before making first injection.
3. If gradient conditions are used, equilibrate with 8–10 column volumes between injections.
4. Failure to appropriately equilibrate the column could result in drifting retention times.

ii. Mobile-phase Considerations

1. Always maintain at least 3% polar solvent in the mobile phase or gradient (*e.g.*, 3% aqueous/3% methanol or 2% aqueous/1% methanol, *etc.*). This ensures that the XBridge BEH particle is always hydrated.
2. Maintain at least 40% organic solvent (*e.g.*, acetonitrile) in your mobile phase or gradient.
3. Avoid phosphate salt buffers to avoid precipitation in HILIC mobile phases. Phosphoric acid is okay.
4. Buffers such as ammonium formate or ammonium acetate will produce more reproducible results than additives such as formic acid or acetic acid. If an additive (*e.g.*, formic acid, *etc.*) must be used instead of a buffer, use 0.2% (v:v) instead of 0.1%.
5. For the best peak shape, always maintain a buffer concentration of 10 mM in your mobile phase/gradient.

iii. Injection Solvents

1. If possible, injection solvents should be 95% acetonitrile. The polar solvent (*i.e.*, water, methanol, isopropanol) should be minimized to 25% of the total volume.
2. A generic injection solvent is 75:25 acetonitrile:methanol. This is a good compromise between analyte solubility and peak shape.
3. Avoid water and dimethylsulfoxide (DMSO) as injection solvents. These solvents will produce very poor peak shapes.
4. Exchange water or DMSO with acetonitrile by using reversed-phase solid-phase extraction (SPE). If this is not possible, dilute the water or DMSO with organic solvent.

iv. Miscellaneous Tips

1. BEH HILIC columns are designed to retain very polar bases. Acidic, neutral, and/or non-polar compounds will have limited retention.
2. Optimal flow rates for small (<200 Daltons) very polar bases are in the 0.4 to 0.8 mL/min range.
3. As compared to Atlantis™ HILIC Silica HPLC Columns, the BEH HILIC columns are approximately 20% less retentive for gradient analysis and 35–65% less retentive for isocratic analysis. This is due to the lower residual surface silanol concentration of the BEH particle.
4. In HILIC, it is important to remember that water is the strongest solvent. Therefore, it must be eliminated or minimized in the injection solvent.
5. For initial scouting conditions, run a gradient from 95% acetonitrile to 50% acetonitrile. If no retention occurs, run isocratically with 95:3:2 acetonitrile:methanol:aqueous buffer.
6. Alternate polar solvents such as methanol, acetone, or isopropanol can also be used in place of water to increase retention.
7. If using an ACQUITY UPLC System, the weak needle wash should closely match the % organic present in the initial mobile-phase conditions, otherwise, analyte peak shape or retention may suffer.
8. It is recommended to monitor your system's health by incorporating the HILIC Quality Control (QC) Reference Material into your workflow. (Reference 720004535EN).

B. RECOMMENDATIONS FOR BEH AMIDE COLUMN USE

BEH Amide columns can be used routinely under HILIC conditions between pH 2 to 11, and they can be operated at temperatures up to 90 °C. As with any LC column, operating at the extremes of pH, pressures and temperatures will result in decreased column lifetime.

i. Column Equilibration

1. When column is first received, flush in 60% acetonitrile:40% aqueous (or initial starting conditions) for 50 column volumes.
2. Equilibrate with 20-column volumes of initial mobile phase conditions before making first injection.
3. If gradient conditions are used, equilibrate with 8-10 column volumes between injections.
4. Failure to appropriately equilibrate the column could result in drifting retention times.

ii. Mobile Phase Considerations

1. Always maintain at least 3% polar solvent in the mobile phase or gradient (*e.g.*, 3% aqueous, 3% methanol, or 2% aqueous/1% methanol, *etc.*).
2. Buffered mobile phases are recommended over additives. For best results, always maintain at least a 10 mM buffer concentration on column.
3. If additives must be used instead of a buffer, it is recommended to always maintain at least a 0.2% additive concentration on column.
4. Maintain at least 40% organic solvent (*e.g.*, acetonitrile) in your mobile phase or gradient.
5. At aqueous concentrations greater than 60%, lower flow rates should be used due to high backpressure. This includes all aqueous wash procedures.
6. Avoid phosphate salt buffers to avoid precipitation in HILIC mobile phases. Phosphoric acid is okay.

iii. Injection Solvents

1. If possible, injection solvents should be as close to the mobile phase composition as possible (if isocratic) or the starting gradient conditions. Acetone should not be used as a sample solvent/diluent unless a Hexane Tetrahydrofuran Compatibility Kit has been installed.
2. A generic injection solvent is 75:25 acetonitrile:methanol. This is a good compromise between analyte solubility and peak shape. When separating saccharides with limited solubility in organic solvents, higher concentrations of aqueous solvent in the sample are acceptable. 50:50 acetonitrile:water can provide satisfactory results.
3. The injection solvent's influence on peak shape should be determined experimentally. In some cases, injections of water (or highly aqueous solutions) may not adversely affect peak shape.

iv. Miscellaneous Tips

1. For initial scouting conditions, run a gradient from 95% acetonitrile to 50% acetonitrile. If no retention occurs, run isocratically with 95:3:2 acetonitrile:methanol: aqueous buffer.
2. Alternate polar solvents such as methanol, acetone, or isopropanol can also be used in place of water to increase retention.
3. Ensure that your weak needle wash solvent/purge solvent is your starting mobile phase (*i.e.*, high organic), or your peak shapes will suffer. Typical needle wash conditions: 800 µL strong wash in 20:80 ACN/H_2O, 500 µL weak wash in 75:25 ACN/H_2O.
4. Acetone should not be used as a sample solvent/diluent unless a Hexane Tetrahydrofuran Compatibility Kit has been installed.

v. Tips for Separating Sugars/Saccharides/ Carbohydrates

If separating sugars or sugar-containing compounds that do not include reducing sugars, follow the generic recommendations described above. If separating reducing sugars, please review the following information:

1. Reducing sugars can undergo mutarotation which produces the undesired separation of the α and β ring forms (anomers).
2. Collapsing anomers into one peak is accomplished with a combination of elevated temperature and high pH:
   a. Use of 35 °C with high pH (0.2% triethylamine (TEA) or 0.1% ammonium hydroxide (NH4OH)) and/or
   b. Use of high temperature >80 °C with 0.05% TEA.
3. When separating reducing sugars (*e.g.*, fructose, glucose, maltose, lactose, arabinose, glyceraldehyde, *etc.*) please pay attention to the following suggestions. Failure to do so will result in the appearance of split peaks (anomer separation) for these analytes:
   a. Operate at a slow flow rate (*e.g.*, 0.10–0.13 mL/min on 2.1 x 50 mm column) to facilitate anomer collapse.
   b. With longer columns, increased flow rates (*e.g.*, up to 0.3 mL/min) can be used. As with all LC separations, optimal flow rates should be determined experimentally.
   c. Add triethylamine (TEA) or ammonium hydroxide (NH_4OH) modifiers to aqueous and organic mobile phase reservoirs at equal concentrations.

d. For HPLC separations of mono- and/or disaccharides using XBridge Amide columns typical isocratic conditions include:
   i. 75% acetonitrile (ACN) with 0.2% TEA, 35 °C
   ii. 77% acetone with 0.05% TEA, 85 °C
e. For HPLC separations of more complex sugar mixtures (*e.g.*, polysaccharides) using XBridge Amide Columns typical gradient conditions include (add TEA modifier to both mobile phases A and B):
   i. Gradient going from 80% to 50% ACN with 0.2% TEA, 35 °C,
   ii. 80%–55% acetone with 0.05% TEA, 85 °C
f. For HPLC/MS separations of mono- and disaccharides using XBridge Amide columns typical isocratic conditions include:
   i. 75% ACN with 0.1% NH_4OH, 35 °C
g. For HPLC/MS separations of more complex sugar mixtures (tt., polysaccharides), using XBridge Amide Columns typical gradient conditions include (add NH_4OH modifier to aqueous and organic mobile phase reservoirs):
   i. Gradient going from 75% to 45% ACN with 0.1% NH_4OH, 35 °C

4. More complex sample mixtures may require the use of gradient conditions and/or longer column lengths.
5. If acetone is used in one or more mobile phases, do not use acetone as a sample diluent or needle wash solvent. Refer to injection solvents section for sample diluent recommendations and miscellaneous tip (#3) for needle wash solvent/purge solvent recommendations.
6. Typical sample preparation suggestions for samples that contain sugars/saccharides/carbohydrates:
   a. Liquid Samples
      i. Dilute with 50:50 ACN/H_2O.
      ii. Filter using 0.45 µm or 0.22 µm syringe filter (if necessary).
   b. Solid Samples
      i. Weigh out sample (~3 g) into 50 mL centrifuge tube.
      ii. Add 25 mL of 50:50 ACN/H_2O and homogenize (mechanically).
      iii. Centrifuge at 3200 rpm for 30 min.
      iv. Collect supernatant and filter using 0.45 µm or 0.22 µm syringe filter (if necessary).
   c. Depending on sample and/or analyte concentrations, additional sample dilutions may be necessary.
   d. More complex samples and/or lower analyte concentrations may require additional sample preparation steps and/or procedures such as solid-phase extraction (SPE).
   e. Consider VanGuard column protection.
   f. It is recommended to monitor your system's health by incorporating the HILIC Quality Control (QC) Reference Material into your workflow. (Reference 720004535en).

IX. SPECIAL CONSIDERATIONS FOR MINIMIZING UV-DETECTED BIPHENYL COLUMN BLEED

Due to the high molar absorptivity of the biphenyl group at 250 nm, the elution of bonded phase hydrolysis products ("column bleed") from BEH Biphenyl columns may cause a rising baseline when using gradient elution with high pH mobile phases and UV detection at wavelengths near 250 nm. The severity of the baseline rise depends on the gradient span, the mobile phase pH, the column temperature and the use history of the column. To minimize this issue, it is recommended that the mobile phase pH be no greater than 7. Higher pH mobile phases (up to 10) may be used with mass spectrometry detection. As discussed in section IVb, BEH Biphenyl columns should be stored in 100% acetonitrile to prevent hydrolysis, ensuring the lowest column bleed.

X. CAUTIONARY NOTE

Depending on the 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 waters.com/sds.

For your local sales office, please visit waters.com/contact

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