XBRIDGE COLUMNS

Thank you for choosing a Waters XBridge™ column. The XBridge™ packing materials were designed to provide excellent peak shape, high efficiency, and excellent stability for acidic and basic mobile phases. The XBridge™ packing materials are manufactured in a cGMP, ISO 9001:2000 certified plant using ultra pure reagents. Each batch of XBridge™ 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 Acceptance.

CONTENTS

I. GETTING STARTED

Each XBridge™ column comes with a Certificate of Analysis and a Performance Test Chromatogram. The Certificate of Analysis, located on the technical information CD, is specific to each batch of packing material contained in the XBridge™ column 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. This data data should be stored for future reference.

a. Column Installation

Note: The flow rates given in the procedure below are for a typical 5 µm packing in a 4.6 mm i.d. column. Scale the flow rate up or down accordingly based upon the column i.d., length, particle size and backpressure of the XBridge™ column being installed. See Scaling Up/Down Isocratic 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 pumping system of any buffer-containing 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 (methanol or 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 steady backpressure and baseline have been achieved, proceed to the next section.

Note: If mobile phase additives are present in low concentrations (e.g., ion-pairing reagents), 100 to 200 column volumes may be required for complete equilibration. In addition, mobile phases that contain formate (e.g., ammonium formate, formic acid, etc.) may also require longer initial column equilibration times.

b. Column Equilibration

XBridge™ 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 a listing of empty column volumes).

To avoid precipitating out 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 60% methanol in water prior to introducing 60% methanol/40% buffer mobile phase).

For XBridge HILIC columns, flush with 50 column volumes of 50:50 acetonitrile:water with 10 mM final buffer concentration. For XBridge HILIC Amide columns, 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 (refer to Table 1 for a list of column volumes). See "Getting Started with XBridge HILIC Columns" or "Getting Started with XBridge HILIC Amide Columns" for additional information.

c. Initial Column Efficiency Determination

1. Perform an efficiency test on the column before using it in the desired application. Waters recommends using a suitable solute mixture, as found in the "Performance Test Chromatogram," to analyze the column upon receipt.
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 obtained on two different HPLC systems due to the quality of the connections, operating environment, system electronics, reagent quality, column condition and operator technique.

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

II. COLUMN USE

To ensure the continued high performance of XBridge™ columns, follow these guidelines:

a. Guard Columns

Use a Waters guard column of matching chemistry and particle size between the injector and main column. It is important to use a high-performance matching guard column to protect the main column while not compromising or changing the analytical resolution.

Guard columns need to be replaced at regular intervals as determined by sample contamination. When system backpressure steadily increases above a set pressure limit, it is usually an indication that the guard column should be replaced. A sudden appearance of split peaks is also indicative of a need to replace the guard column.

b. 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.
2. It is preferable to prepare the sample in the operating mobile phase or a mobile phase that is weaker (less organic modifier) than the mobile phase for the best peak shape and sensitivity.
3. 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.
4. 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 8,000 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 100% organic solvents (e.g., acetonitrile). See "Getting Started with XBridge HILIC Columns" or "Getting Started with XBridge Amide Columns" for additional information.

c. Operating pH Limits

The recommended operating pH limits for XBridge™ columns are listed in Table 2. A listing of commonly used buffers and additives is given in Table 3. Additionally, the column lifetime will vary depending upon the operating temperature, the type and concentration of buffer used.

Table 2: *Recommended pH and temperature Limits for XBridge™ Columns at Ambient Temperatures*

Name of Column	Particle Size	Pore Diameter	Surface Area	pH Limits	Temperature Limits		Surface	Carbon Load
					Low pH	High pH		
XBridge C_{18}	2.5, 3.5, 5 µm	130Å	185 m^2/g	1-12	80 °C	60 °C	3.1 µmol/m^2	18%
XBridge C_8	2.5, 3.5, 5 µm	130Å	185 m^2/g	1-12	60 °C	60 °C	3.1 µmol/m^2	13%
XBridge Phenyl	2.5, 3.5, 5 µm	130Å	185 m^2/g	1-12	80 °C	60 °C	3.0 µmol/m^2	15%
XBridge Shield RP18	2.5, 3.5, 5 µm	130Å	185 m^2/g	2-11	50 °C	45 °C	3.2 µmol/m^2	17%
XBridge HILIC	2.5, 3.5, 5 µm	130Å	185 m^2/g	1-9	45 °C	45 °C	-	-
XBridge Amide	3.5 µm	130Å	185 m^2/g	2-11	90 °C	90 °C	7.5 µmol/m^2	12%

Table 3: *Buffer Recommendations for Using XBridge™ Columns from pH 1 to 12*

Additive/Buffer	pKa	Buffer Range (±1 pH unit)	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_3COOH)	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) 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.

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

d. 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 will generally clog the outside surface of the inlet distribution frit of the column. This will result in higher operating pressure and poor performance.

Degas all solvents thoroughly before use to prevent bubble formation in the pump and detector. The use of an on-line degassing unit is also recommended. This is especially important when running low pressure gradients since bubble formation can occur as a result of aqueous and organic solvent mixing during the gradient.

e. Pressure

XBridge™ columns can tolerate pressures of up to 6,000 psi (400 bar or 40 Mpa) although pressures greater than 4,000 – 5,000 psi should be avoided in order to maximize column and system lifetimes.

f. Temperature

Temperatures between 20 °C – 80 °C (up to 90 °C for XBridge Amide columns) are recommended for operating XBridge 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. Under HILIC conditions XBridge Amide columns can be used at high pH and at high temperatures without issues (see recommended conditions in Getting Started with XBridge Amide section). See Table 2 for recommended pH and temperature limits.

III. SCALING UP/DOWN ISOCRATIC METHODS

The following formulas will allow scale up or scale down, while maintaining the same linear velocity, and provide new sample loading values:

If column i.d. 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

IV. Troubleshooting

Changes in retention time, resolution, or backpressure are often due to column contamination. See the Column Cleaning, Regeneration and Storage section of this Care and Use Manual. Information on column troubleshooting problems may be found in HPLC Columns Theory, Technology and Practice, U.D. Neue, (Wiley-VCH, 1997), the Waters HPLC Troubleshooting Guide (Literature code # 720000181EN) or visit the Waters Corporation website for information on seminars (www.waters.com).

V. 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 4). Flush columns with 20 column volumes each of HPLC-grade solvents (e.g., 80 mL total for 4.6 x 250 mm column) listed in Table 4. Increasing mobile phase temperature to 35-55 °C increases cleaning efficiency. If the column performance is poor after cleaning and regeneration, call your local Waters office for additional support. Flush XBridge 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 XBridge Amide columns, run a 25 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 4: *Cleaning and Regeneration Sequence or Options*

Polar Samples	Non-polar Samples	Proteinaceous Samples
1. water	1. isopropanol (or anappropriate isopropanol/water mixture*)	Option 1: Inject repeated aliquots of dimethyl sulfoxide (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 7M guanidine hydrochloride, or 7M urea
6. mobile phase	6. mobile phase	

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

b. Storage

For periods longer than four days at room temperature, store the reversed-phase XBridge columns and XBridge Amide columns 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. Run a gradient to 100% ACN in order to flush all aqueous solvent from an XBridge 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 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. slightly longer equilibration times may be necessary when the column is re-installed and run again with a formate-containing mobile phase.

VI. 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.

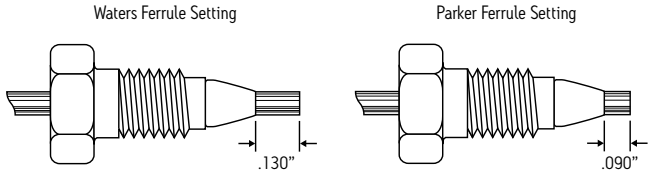
1. Correct connection of 1/16 inch outer diameter stainless steel tubing leading to and from the column is essential for high-quality chromatographic results.
2. When using standard stainless steel compression screw fittings, it is important to ensure proper fit of the 1/16 inch outer diameter stainless 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.

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

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.

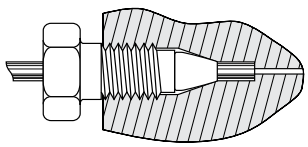
Figure 1: *Waters and Parker Ferrule Types*



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 1). 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™ column.

In a proper tubing/column connection (Figure 2), the tubing touches the bottom of the column endfitting, with no void between them.

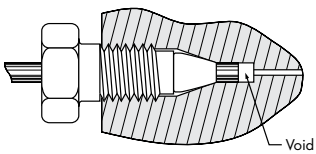
Figure 2: *Proper Tubing/Column Connection*



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 3).

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

Figure 3: *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 4).

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

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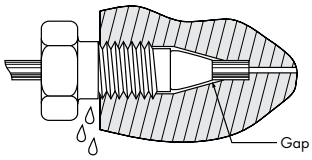
Figure 4: *Waters Ferrule in a Parker Style Endfitting*

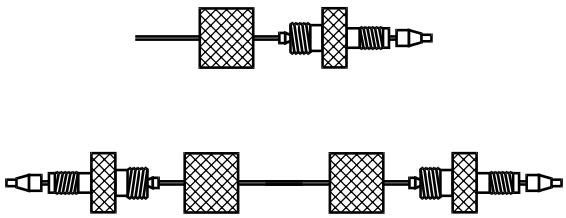


There are two ways to fix the problem:

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 (Waters part number 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 fingertight SLIPFREE® connectors automatically adjust to fit all compression screw type fittings without the use of tools (Figure 5).

Figure 5: *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 IDs 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

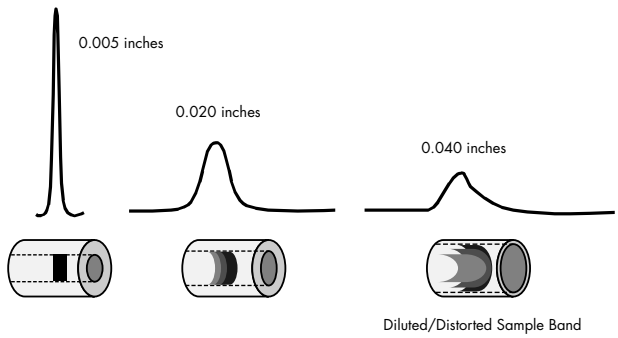
Table 5: *Waters Part Numbers for SLIPFREE® Connectors*

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

Band Spreading Minimization

Figure 6 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 6: *Effect of Connecting Tubing on System*



b. Measuring System Bandspreading Volume and System Variance

This test should be performed on an HPLC system with a single wavelength UV detector (not a Photodiode Array (PDA)).

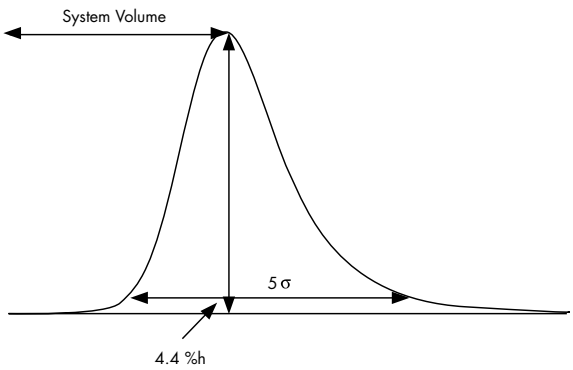
1. Disconnect column from system and replace with a zero dead volume union.
2. Set flow rate to 1 mL/min.
3. Dilute a test mix in mobile phase to give a detector sensitivity of 0.5 - 1.0 AUFS (system start up test mix can be used which contains uracil, ethyl and propyl parabens; Waters part number WAT034544).
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 7: *Determination of System Bandspreading Volume Using 5-Sigma Method*



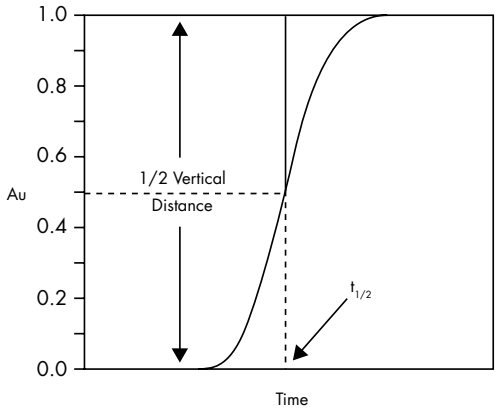
In a typical HPLC system, the Bandspreading Volume should be no greater than 100 µL ± 30 µL (or Variance of 400$µL^2$ ± 36$µL^2$).
In a microbore (2.1mm i.d.) system, the Bandspreading Volume should be no greater than 20 to 40 µL (or Variance no greater than 16$µL^2$ to 64$µL^2$).

c. 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 2 mL/min (the exact conditions are not critical; just make sure the gradient volume is at least 20 mL) with a hold at 100% B.

Figure 8: *Determination of Gradient Delay Volume*



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 8).
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).

$$\text{Dwell Volume } V_D = (t_{1/2} - {}^1/_2\ t_g) \times F$$

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 System Modification Recommendations section on how to reduce system volume.

VII. ADDITIONAL INFORMATION

a. Use of Narrow-Bore (3.0 mm i.d.) Columns

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

b. Impact of Bandspreading Volume on 2.1 mm i.d. Column Performance

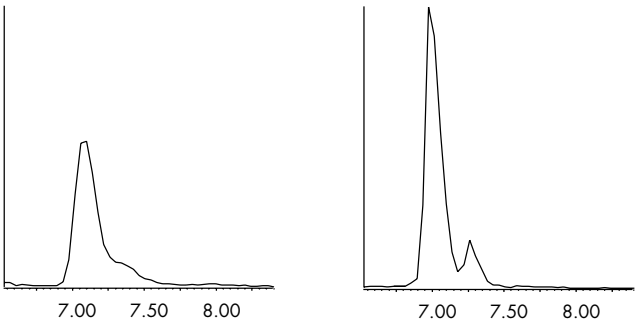
System with 70 µL bandspreading: 10,000 plates

System with 130 µL bandspreading: 8,000 plates (same column)

Note: Flow splitters after the column will introduce additional bandspreading.

System optimization, especially in a system 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 9 where system optimization resulted in a doubling of sensitivity and resolution of the metabolite in an LC/MS/MS system.

Figure 9: *Non-Optimized vs. Optimized LC/MS/MS System*



c. Non-Optimized vs. Optimized LC/MS/MS System: System Modification Recommendations

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

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

2. Minimize injector sample loop volume.
3. Use 0.009 inch (0.25 mm) tubing for rest of connections in standard systems and 0.005 inch (0.12 mm) tubing for narrowbore (2.1 mm i.d.) 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.

d. Waters Small Particle Size (2.5 µm) Columns – Fast Chromatography

Waters columns that contain 2.5 µm particles provide faster and more efficient separations without sacrificing column lifetime. This section describes five parameters to consider when performing separations with columns containing 2.5 µm particles.

Note: Columns that contain 2.5 µm particles have smaller outlet frits to retain packing material. These columns should not be backflushed.

1. Flow Rate—Compared with the 5 µm columns, columns with 2.5 µm particles have higher optimum flow rates. These columns are used when high efficiency and short analysis times are required. These higher flow rates, however, lead to increased backpressure.

   Note: Use a flow rate that is practical for your system.

2. Backpressure—Backpressures for columns with 2.5 µm particles are higher than for 5 µm columns with the same dimensions. Waters suggests using a shorter column to compensate for increased backpressure and to obtain a shorter analysis time.
3. Temperature—Use a higher temperature to reduce backpressure caused by smaller particle sizes. The recommended temperature range for XBridge™ columns is 20 °C to 60 °C. See Column Use section for a discussion of elevated temperature use with XBridge™ columns.
4. Sampling Rate—Use a sampling rate of about 10 points per second or higher. A minimum of 20 points across the earliest eluting peak of interest is needed for optimum reproducibility.
5. Detector Time Constant—Use a time constant of 0.1 seconds or lower for fast analyses.

e. Getting Started With XBridge HILIC Columns

1. Because XBridge HILIC columns do not posses a bonded phase, the pH operating range is 1 to 9, and they can be operated at temperatures up to 45 °C.
2. As with any LC column, operating at the extremes of pH, pressures and temperatures will result in decreased column lifetime.

Column Equilibration

1. When 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.

Mobile-Phase Considerations

1. Always maintain at least 5% polar solvent in the mobile phase or gradient (e.g., 5% aqueous/5% methanol or 2% aqueous/3% methanol, etc.). This ensures that the XBridge 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 best peak shape, maintain a buffer concentration of at least 10 mM in your mobile phase/gradient at all times.

Injection Solvents

1. If possible, injection solvents should be 100% organic solvent of the initial mobile phase conditions. Water must be eliminated or minimized. Choose weak HILIC solvents such as acetonitrile, isopropanol, methanol, etc.
2. A generic injection solvent is 75:25 acetonitrile:methanol. This is a good compromise between analyte solubility and peak shape. If solubility is still poor, 0.2% formic acid can be added.
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.

Miscellaneous Tips

1. As compared to Atlantis® HILIC Silica HPLC columns, the XBridge HILIC columns are approximately 20% less retentive for gradient analysis and 35 to 65% less retentive for isocratic analysis. This is due to the lower residual surface silanol concentration of the BEH particle.
2. In HILIC, it is important to remember that water is the strongest solvent. Therefore, it must be eliminated or minimized in the injection solvent.
3. For initial scouting conditions, run a gradient from 95% acetonitrile to 50% acetonitrile. If no retention occurs, run isocratically with 95:3:2 acetonitrile:isopropanol:aqueous buffer.
4. Alternate polar solvents such as methanol, ethanol or isopropanol can also be used in place of water to increase retention.

f. Getting Started with XBridge Amide Columns

Operating Ranges

1. XBridge 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.
2. As with any LC column, operating at the extremes of pH, pressures and temperatures will result in decreased column lifetime.

Column Equilibration

1. When column is first received, flush in 60% acetonitrile: 40% aqueous (or initial starting conditons) 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.

Mobile Phase Considerations

1. Always maintain at least 5% polar solvent in the mobile phase or gradient (e.g., 5% aqueous, 5% methanol or 2% aqueous/3% methanol, etc.).
2. Maintain at least 40% organic solvent (e.g., acetonitrile) in your mobile phase or gradient.
3. At aqueous concentrations greater than 60%, lower flow rates should be used due to high backpressure. This includes all aqueous wash procedures.
4. Avoid phosphate salt buffers to avoid precipitation in HILIC mobile phases. Phosphoric acid is OK.

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.
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 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.

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.

Tips for Separating Sugars/Saccharides/Carbohydrates

1. If separating sugars or sugar-containing compounds that do not include reducing sugars (see below) follow generic 'Getting Started with XBridge Amide Columns' recommendations described above.
2. If separating reducing sugars, please review the following information.
3. Reducing sugars can undergo mutarotation which produces the undesired separation of the α and β ring forms (anomers).
4. Collapsing anomers into one peak is accomplished through the use of 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 (NH_4OH)) and/or
   b. Use of >80 °C with 0.05% TEA high temperature (>80 °C)
5. 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 to facilitate anomer collapse.
   b. With longer columns, increased flow rates 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 (e.g., 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

6. More complex sample mixtures may require the use of gradient conditions and/or longer column lengths.

8. 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 minutes

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 guard columns for column protection.

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