XSELECT HIGH STRENGTH SILICA (HSS) HPLC COLUMNS

CONTENTS

I. INTRODUCTION

Thank you for choosing an XSelect™ High Strength Silica (HSS) HPLC column. The manufacture of XSelect HSS HPLC columns begins with ultrapure reagents to control the chemical composition and purity of the final product. XSelect HSS HPLC columns are manufactured in a cGMP, ISO 9001:2000 certified plant with each step being conducted within narrow tolerances. Every column is individually tested and Certificates of Batch Analysis and a Performance Chromatogram are provided with each column.

XSelect HSS HPLC columns are based on the same particle technology and chemistries as ACQUITY UPLC® HSS columns, thus enabling seamless transferability between HPLC and UPLC® separations.

XSELECT™ Columns

II. CONNECTING THE COLUMN TO THE HPLC SYSTEM

a. Column Connection

Handle the column with care. Do not drop or hit the column on a hard surface as this 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. An arrow on the column identification label indicates correct direction of solvent flow.
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.

Caution: *If one of the wrenches is placed on the column flat during this process, the endfitting will be loosened and leak. Under-tightening compression screws or using worn ferrules can lead to solvent leaking. Care should be taken to check all column connections for leaks to avoid exposure to solvents and the hazards associated with such exposure including risks to health and electrical connections.*

4. 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.

It is important to realize that extra column peak broadening can destroy a successful separation. The choice of appropriate column connectors and system tubing is discussed in detail below.

b. Column Connectors and System Tubing Considerations

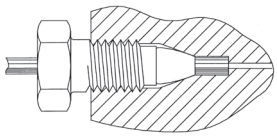
Due to the absence of an industry standard, various column manufacturers have employed different types of chromatographic column connectors. The chromatographic performance of the separation can be negatively affected if the style of the column endfittings does not match the existing tubing ferrule setting. 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. XSelect HSS HPLC columns are equipped with Waters style endfittings, which require a 0.130 inch ferrule. If a non-Waters style column is presently being used, it is critical that ferrule depth be reset for optimal performance prior to installing the XSelect HSS HPLC column. In a proper tubing/column connection (Figure 2), the tubing touches the bottom of the column endfitting, with no void between them.

Figure 1: Waters and Parker Ferrule Types

Waters Ferrule Setting Parker Ferrule Setting

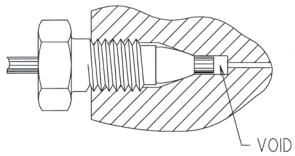
Figure 2: Proper Tubing/Column Connection

Tubing touches the bottom of the column endfitting, with no void between them.



Attention: *A void will occur if a Parker ferrule connection is used with a Waters style endfitting (Figure 3). This will dramatically reduce the efficiency of the column and cause peak shape distortion.*

Figure 3: Parker Ferrule in a Waters Style Endfitting

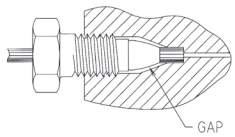


To fix this problem: Cut the end of the tubing with the ferrule, place a new ferrule on the tubing and make a new connection. Before tightening the screw, make sure that the tubing bottoms out in the endfitting of the column.

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).

Caution: *The connection will leak if a Waters ferrule is connected to a column with a Parker style endfitting.*

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 break 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 SLIPFREE® connector to 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

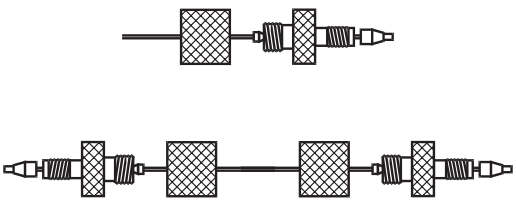


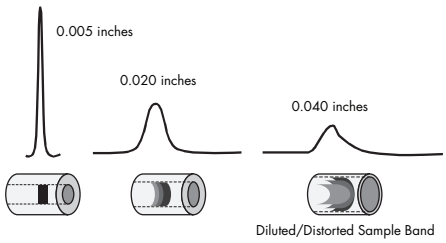
Table 1. Waters Part Numbers for SLIPFREE Connectors

SLIPFREE Type and 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

c. Band Spreading Minimization

Internal tubing diameter influences system band spreading and peak shape. Larger tubing diameters cause excessive peak broadening and lower sensitivity (Figure 6).

Figure 6: Effect of Connecting Tubing on System

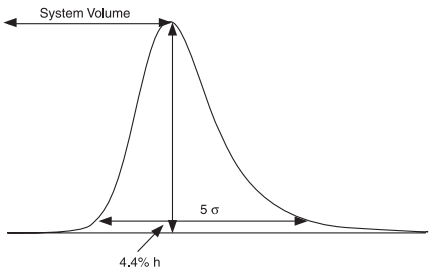


d. Measuring System Band Spread Volume

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 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. Using 5-Sigma Method measure the peak width at 4.4% of peak height:
   Band Spreading (µL) = Peak Width (min) x Flow Rate (µL/min)
   = 0.1 min x 1000 µL/min
   = 100 µL

Figure 7: Determination of System Band Spread Volume Using 5-Sigma Method

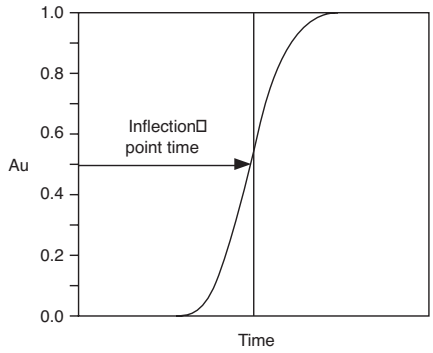


In a typical HPLC system, the Band Spread Volume should be 100 µL ± 30 µL .

e. Measuring Gradient Delay Volume

1. Replace the column with a zero dead volume union.
2. Prepare eluent A (pure solvent, such as methanol) and eluent B (solvent plus sample, such as 5.6 mg/mL propylparaben in methanol).
3. Equilibrate the system with eluent A until a stable baseline is achieved.
4. Switch to 100% eluent B.
5. Record the half height of the step and determine dwell volume (Figure 8).

Figure 8: Determination of Dwell Volume



The dwell volume should be less than 1 mL.

III. WATERS SMALL PARTICLE SIZE (3.5 µm) COLUMNS – FAST CHROMATOGRAPHY

Waters columns with 3.5 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 3.5 µm particles.

***Note**: Columns that contain 3.5 µm particles have smaller pore-size outlet frits to retain packing material than are used at the inlet. These columns should not be backflushed.*

1. Flow Rate — Compared to columns with 5 µm particles, columns with 3.5 µm particles have higher optimum flow rates and 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 3.5 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 XSelect HSS HPLC columns is 20 °C to 45 °C. See "Column Usage" for a discussion on elevated temperature use with XSelect HSS HPLC columns.
4. Sampling Rate — Use a sampling rate of about 10 points per second.
5. Detector Time Constant — Use a time constant of 0.1 seconds for fast analyses.

IV. COLUMN EQUILIBRATION

XSelect HSS HPLC columns are packed and shipped in 100% acetonitrile. It is important to ensure solvent compatibility before changing to a new solvent. Equilibrate with a minimum of 10 column volumes of the mobile phase to be used (refer to Table 2 for a listing of standard column volumes).

Table 2. Standard Column Volumes in mL (Multiply by 10 for Equilibration Mobile Phase Volumes)

Column Volume (mL)				
Column Length	Column Internal Diameter (mm)			
	2.1	3.0	4.6	10
20 mm	.10	.21	.50	2.4
30 mm	.11	2.4	0.5	–
50 mm	.17	.35	.83	4.0
75 mm	.26	.53	1.3	6.0
100 mm	.35	.71	1.7	8.0
150 mm	.52	1.1	2.5	12
250 mm	.87	1.8	4.2	20

V. COLUMN INSTALLATION PROCEDURE

Note: The flow rates given in the procedure below are for a typical 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 XSelect HSS HPLC column being installed. See "Scaling Up/Down" for calculating flow rates when changing column i.d. and/or length.

1. Purge the pumping system and connect the inlet end of the column to the injector outlet.
2. Set the pump flow to 0.1 mL/min and increase to 1 mL/min over 5 minutes.
3. When the mobile phase is flowing freely from the column outlet, stop the flow, then attach the column to the detector. This prevents entry of air into the detector and provides more rapid baseline equilibration.

   Caution: Care should be taken to check column connections for leaks to avoid exposure to solvents and the hazards associated with such exposure including risks to health and electrical connections.
4. When the mobile phase is changed, gradually increase the flow rate of the new mobile phase from 0.0 mL/min to 1.0 mL/min in 0.1 mL/min increments.
5. Once a steady backpressure and baseline have been achieved, the column is ready to be used (or equilibrated).

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 slightly longer initial column equilibration times.*

VI. COLUMN PERFORMANCE VALIDATION

Each XSelect HSS HPLC column comes with a Certificate of Batch Analysis and a Performance Test Chromatogram. The Certificate of Analysis is specific to each batch of packing material and includes the batch number, analysis of unbonded particles, analysis of bonded particles and chromatographic results and conditions. The Performance Test Chromatogram is specific to each individual column and contains information such as batch number, column serial number, USP plate count, USP tailing factor, capacity factor and chromatographic results and conditions. These data should be stored for future reference.

VII. INITIAL COLUMN EFFICIENCY DETERMINATION

1. Perform an efficiency test on the column before using it. Waters recommends using a suitable solute mixture, as found in the "Performance Test Chromatogram", to verify the performance of the column upon receipt.
2. Determine the number of theoretical plates (N) and use for periodic comparison.
3. Repeat the test periodically 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.

Note: *If 1) 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 in order to achieve extremely low system volumes. This presents a more challenging test of how well the column was packed. This 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.*

VIII. COLUMN USAGE

Caution: *Accumulation of particulates from solvents, samples, or pump seals may cause the column backpressure to increase over time. This may lead to a system shutdown or leaking of column connections. Accumulation of contaminants from "dirty" samples at the column inlet may lead to a loss of resolution or ion suppression in a mass spectrometer, resulting in erroneous results.*

To ensure the continued high performance of XSelect HSS HPLC columns and cartridges, follow these guidelines:

a. Guard Columns

Use a Waters Sentry guard cartridge of matching i.d., chemistry and particle size between the injector and main column. For best results, the guard column should be replaced prior to the observation of a substantial loss in resolution or increase in system backpressure. It is important to use a high-performance matching guard column to protect

the main column while not compromising or changing analytical resolution.

b. Sample Preparation

1. Sample impurities often contribute to column contamination. Use Waters Oasis® or Sep-Pak® solid-phase extraction cartridges/columns of the appropriate chemistry to cleanup the sample before analysis.
2. It is preferable to prepare the sample in mobile phase or a solvent that is weaker (less organic modifier) than the mobile phase.
3. If the sample is not dissolved in the mobile phase, ensure that the sample and diluent are miscible in the mobile phase(s) in order to avoid sample and/or diluent precipitation.
4. Filter sample through a 0.2 µm membrane 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 careful transfer of the supernatant liquid to an appropriate vial, could be considered.

c. Recommended pH Range

Chemistry	pH Range
XSelect HSS Cyano	2-8
XSelect HSS PFP	2-8
XSelect HSS T3	2-8
XSelect HSS C_{18} SB	2-8
XSelect HSS C_{18}	1-8

Column lifetime will vary depending upon the temperature, type and concentration of buffer used. A listing of recommended and non-recommended buffers is given in Table 3. Please use this as a guideline when developing methods.

Attention: *Operating at the upper or lower end of the pH range in combination with elevated temperatures will lead to shorter column lifetime and/or may result in the column generating high backpressure.*

Table 3: Buffer recommendations for using HSS HPLC columns from pH 1 to 7

Additive or 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 0.01-0.1% range.
Formic Acid	3.75	–	Volatile	Yes	Maximum buffering obtained when used with Ammonium Formate salt. Used in 0.1-1.0% range.
Acetic Acid	4.76	–	Volatile	Yes	Maximum buffering obtained when used with Ammonium Acetate salt. Used in 0.1-1.0% range.
Formate (NH_4COOH)	3.75	2.75 – 4.75	Volatile	Yes	Used in the 1-10mM range. *Note*: sodium or potassium salts are not volatile.
Acetate (NH_4CH_3COOH)	4.76	3.76 – 5.76	Volatile	Yes	Used in the 1-10mM range. Note: 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	Much shorter colum lifetimes will be realized using phosphate at pH 7.

d. Solvents

To maintain maximum column performance, use high quality chromatography grade solvents. Filter all aqueous buffers prior to use. The addition of at least 5% organic to buffers is recommended to discourage bacterial growth. Acrodisc® filters are recommended. Solvents containing suspended particulate materials will generally clog the outside surface of the inlet frit of the column. This will result in higher operating pressure and poorer 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

XSelect HSS HPLC 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, and the risk of system shutdowns and leaking.

f. Temperature

Temperatures between 20 °C - 45 °C are recommended for operating XSelect HSS HPLC columns in order to enhance selectivity, lower solvent viscosity and increase mass transfer rates. However, any temperature rise above ambient will have a negative effect on lifetime which will vary depending on the pH and buffer conditions used. The combination of operating at elevated temperatures and at pH extremes should be avoided.

g. Scaling Up/Down Isocratic Methods

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

If column i.d. and length are altered: $F_2 = F_1(r_2/r_1)^2$

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

or: $Inj\ vol_1 = Inj\ vol_2\ (r_2/r_1)^2\ (L_2/L_1)$

Where: r = Radius of the column, in mm
F = Flow rate, in mL/min
L = Length of column, in mm
1 = Original, or reference column
2 = New column

XIII. COLUMN CLEANING, REGENERATING AND STORAGE

a. Cleaning and Regenerating

A sudden increase in pressure or shift in retention or resolution may indicate contamination of the column.

Flush with a neat organic solvent to remove the non-polar contaminant(s). If this flushing procedure does not solve the problem, purge the column with a sequence of progressively more non-polar solvents. For example, switch from water to tetrahydrofuran to methylene chloride. Return to the standard mobile-phase conditions by reversing the sequence.

For Normal-Phase Conditions:

The XSelect HSS Cyano column can be used for both reversed-phase separations as well as normal-phase separations. The column is originally shipped in acetonitrile and is ready to use for reversed-phase conditions. If you intend to use the column for normal-phase applications you will need to condition the column with the following procedure:

1. Flush the column with a minimum of 20 column volumes of 100% methanol using a low flow rate to avoid overpressuring the LC system. Refer to Table 2 for minimum solvent volume.
2. Flush the column with a minimum of 20 column volumes of 100% isopropanol using a low flow rate to avoid overpressuring the LC system. Refer to Table 2 for the minimum solvent volume.
3. Flush the column with a minimum of 20 column volumes of 100% dichloromethane using a low flow rate to avoid overpressuring the LC system. Refer to Table 2 for the minimum solvent volume.
4. Flush the column with the intended mobile-phase conditions until a stable baseline is achieved.

b. Storage

For periods longer than four days, store the column in 100% acetonitrile. Do not store columns in buffered eluents. If the mobile phase contained a buffer salt, flush the column with 10 column volumes of HPLC grade water (see Table 2 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.

Completely seal column to avoid evaporation and drying out of the bed.

Note: *If a column has been run with a formate-containing mobile phase (e.g., ammonium formate, formic acid, etc.) and is flushed to remove the buffer, slightly longer equilibration times may be required after the column is re-installed and run again with a formate-containing mobile phase.*

For Normal-Phase Use:

For rapid equilibration upon start-up, it is recommended that you store the XSelect HSS Cyano column in the mobile phase that is commonly used for your normal-phase separation. Completely seal the column to avoid evaporation and drying out of the bed.

X. TROUBLESHOOTING

Changes in retention time, resolution, or backpressure are often due to column contamination (refer to "Column Cleaning, Regenerating and Storage"). Information on column troubleshooting problems may be found in HPLC Columns Theory, Technology and Practice, U.D. Neue, (Wiley-VCH, 1997) or the Waters HPLC Troubleshooting Guide (Literature Code 720000181EN).

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