XTerra and XTerra Prep Columns

CONTENTS

I. INTRODUCTION

Thank you for ordering an XTerra™ Column. XTerra packing materials are synthesized using Waters Hybrid Particle Technology. XTerra particles contain both inorganic (silica) and organic (organosiloxane) components sharing the advantages of both. Hybrid technology allows for the high efficiency of separation and improved pH stability when compared to Silica based reversed phase packing materials. The manufacture of XTerra Columns starts with ultrapure reagents to control the chemical composition and purity of the Hybrid particle. Every manufacturing step is conducted within narrow tolerances and each column is individually tested. Certificates of Batch Analysis and Column Efficiency are provided with each column.

We recommend the use of VanGuard Column Protection Products to extend the life of your column and protect it from contaminants.

II. COLUMN INSTALLATION

a. Connecting the Column to the HPLC Instrument

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" 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" outer diameter stainless steel tubing. When tightening or loosening the compression screw, place the 5/16" wrench on the compression screw and the other 3/8" wrench on the hex head of the column endfitting.

Note: If one of the wrenches is placed on the column 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.

NOTE: It is important to realize that extra column peak broadening can destroy 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 styles of chromatographic column connectors. The chromatographic performance of your separation can be negatively affected if the style of your column endfittings do not match the existing instrumentation tubing ferrule setting. This page explains the difference between Waters style and Parker style endfittings, which vary in the required length of the tubing protruding from the ferrule. The XTerra Column is equipped with Waters style endfittings which require a 0.130" ferrule depth (see next section for setting ferrule depth). If you are presently using a non-Waters style column, it is critical that you reset the ferrule depth for optimal performance.

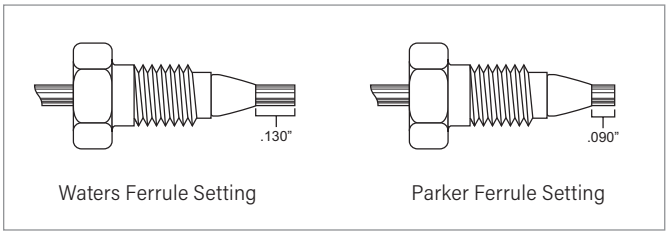

Figure 1: Waters and Parker Ferrule Types

c. The proper tubing/column connection

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

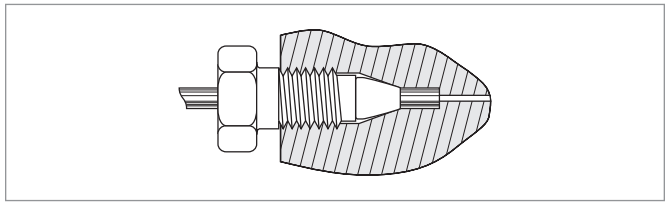

Figure 2. Proper tubing/column connection.

A void appears if a tube with Parker ferrule setting is connected to a Waters style column

The presence of a void in the flow stream down grades the column performance. There is only one way to fix the problem: Cut the end of the tubing with the ferrule, put a new ferrule on the tubing and make the connection. Before tightening the screw, make sure that the tubing bottoms out in the endfitting of the column.

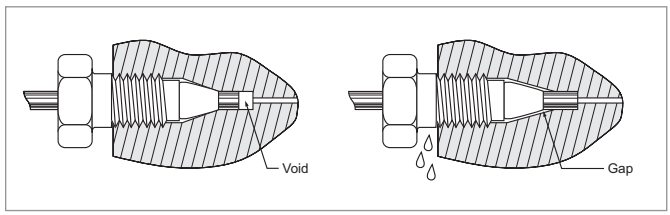

Figure 3: Parker Ferrule in a Waters Style Endfitting (left) & Waters Ferrule in a Parker Style Endfitting (right)

If tubing with a Waters style ferrule setting 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 creating a leak. There are two ways to fix the problem:

1. Just tighten the screw a little bit more. The ferule moves forward, and reaches the sealing surface. Do not overtighten because this may end in breaking the screw.
2. Cut the tubing, put a new ferrule on it and make the connection.

Alternatively, replace the conventional compression screw fitting with an all-in-one PEEK™ fitting (p/n: PSL613315) that allows resetting of the ferrule depth. Another approach is to use a SLIPFREE® connector to always ensure the correct fit. The finger-tight SLIPFREE connectors automatically adjust to fit all compression screw type fittings without the use of tools (Figure 4).

d. SLIPFREE Connectors

A SLIPFREE Connector guarantees a void-free connection because it pushes the tubing into the endfitting. This design comes installed on the tubing. 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.

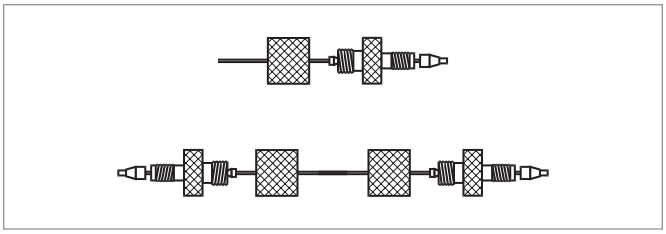

Figure 4. Single and double SLIPFREE connectors.

Table 1: 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

Minimization of band spreading

The following figure 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.

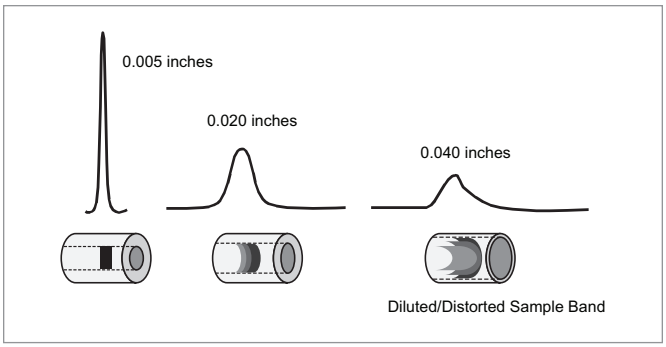

Figure 5. Effect of connecting tubing on system.

f. Measuring System Bandspread Volume

1. Disconnect column from system and replace with a zero dead volume union.
2. Set flow rate to 1 mL/min.
3. Dilute a test mix in mobile phase to give a detector sensitivity of 0.5–1.0 AUFS (system startup test mix can be used which contains uracil, ethyl, and propyl parabens; p/n: WAT034544).
4. Inject 2 to 5 µL of this solution.
5. Using 5 sigma method measure the peak width at 4.4% of peak height:
   - Bandspread (µL) = PW (seconds)/60 (see Figure 6)
   - Typical LC system should be 100 µL ± 30 µL
   - Microbore (2.1 mm i.d. and smaller) system should be no greater than 20-40 µL

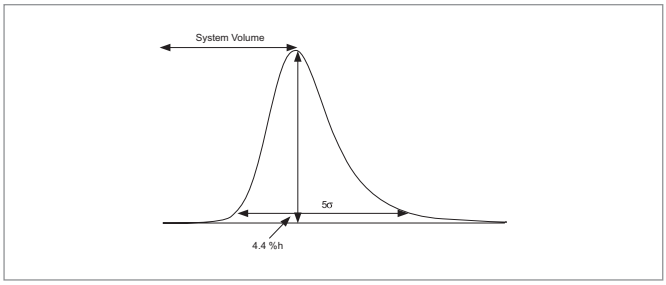

Figure 6: Determination of System Bandspread Volume using the 5-Sigma Method

g. Measuring Gradient Delay Volume

1. Replace the column with a zero dead volume union.
2. Determine the gradient-delay or dwell volume for your system by performing the following test. 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. Switch to 100% eluent B and record the half height of the step. Refer to Figure 7 for an illustration. The dwell volume should be less than 1 mL. If this is not the case, see section on System Modifications to reduce your system volume.

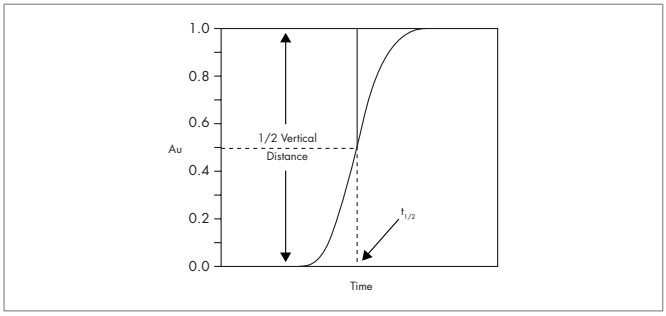

Figure 7: Determination of Dwell Volume

h. Use of Narrow-Bore Columns – (≤3.0 mm i.d.)

This section describes how to minimize extra column effects and gives some guidelines on how to maximize the advantages of your narrow-bore column. The 3.0 mm i.d. narrow-bore column usually requires no system modifications. With the 2.1 mm i.d. column, however, modifications to your HPLC system may be required in order to eliminate excessive system bandspread volume. Without proper system modifications, excessive system bandspread volume causes peak broadening and has a large impact on peak width as peak volume decreases.

i. Impact of bandspreading on column performance (2.1 mm i.d. column)

System with 70 µL bandspread >> 10,000 plates

System with 130 µL bandspread >> ~8,000 plates (same column)

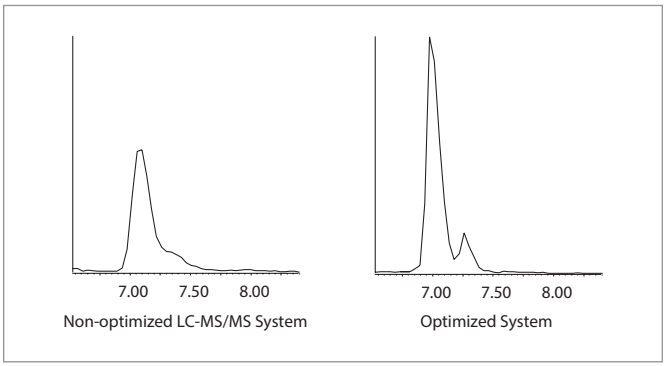

Figure 8: Determination of Gradient Delay Volume

Note flow splitters after the column will introduce additional bandspreading. Optimizing a system, especially one using flow splitters can have a dramatic effect on sensitivity and resolution. Use of correct ferrule depth connectors and minimizing tubing diameter and lengths showed a doubling of sensitivity and enabled resolution of the metabolite on this LC-MS/MS system.

j. Modification Guidelines

1. Use a microbore detector flow cell with the 2.1 mm columns. Recall that due to the shorter pathlength, detector sensitivity is reduced to achieve lower band spread volume.
2. Injector sample loop should be reduced to minimum.
3. Use 0.009" (0.25 mm) tubing between pump and injector.
4. Use 0.009" (0.25 mm) tubing for rest of connections in standard systems and 0.005" (0.12 mm) tubing for narrow-bore (≤2.1 mm i.d.) systems.
5. Use perfect (pre-cut) connections (with a variable depth inlet if using columns from different suppliers).
6. Time constants should be shortened <0.2.

k. Waters Small Particle Size (2.5 µm and 3.5 µm) Columns – Fast Chromatography

The Waters columns with 2.5 µm and 3.5 µm packings provide faster and more efficient separations without sacrificing column lifetime. This section describes five parameters to consider when performing separations on the 2.5 µm and 3.5 µm columns.

Note: All 3.5 µm and 2.5 mm materials have smaller outlet frits to retain packing material. These columns should not be backflushed.

1. Flow Rate — Compared with the 5 µm columns, the 2.5 µm and 3.5 µm columns have a higher optimum flow rate. These columns are used for high efficiency and short analysis times. The higher flow rates, however, lead to increased backpressure. Use a flow rate that is practical for your system.
2. Backpressure — The backpressures on the 2.5 µm and 3.5 µm columns are higher than for the 5 µm columns of the same dimension. Use a shorter column to compensate for increased
   backpressure and obtain a shorter analysis time.
3. Temperature — Use a higher temperature to reduce backpressure caused by smaller particle sizes (see Column Care and Use temperatures recommended in Section III. e.).
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 analysis.

l. Column Performance Validation

Each pre-packed column has an individual quality control report that provides significant information about the column. This report is available as a ready reference and should be kept in your files. It indicates the column specifics: gel lot, column dimensions, bonding chemistry type, particle shape, particle size, porosity, and chromatographic test conditions.

1. Perform an efficiency test on your column before you use it. Waters recommends using a suitable solute mixture, such as found in the "Column Test Report", to immediately analyze the column once you receive it. Determine the number of theoretical plates (N) and use for periodic comparison. 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. Please report any column problems observed upon receipt of the column.

m. Sample Preparation

1. It is preferable to prepare sample in the mobile phase or a weaker solvent than the mobile phase.
2. If the sample is not dissolved in the mobile phase, ensure sample, solvent and mobile phases are miscible to avoid sample or buffer precipitation.
3. Filter sample with 0.2 µm membrane to remove particulates.

n. Column Equilibration

Waters delivers the column in 100% acetonitrile. It is important to ensure solvent compatibility before changing to a new solvent. Equilibrate your column with a minimum of 10 times its internal volume with the mobile phase to be used (refer to Table 2 for some standard column volumes).

1. Purge your pumping system and then connect the inlet end of the column to the injector outlet. Turn on the pump flow at 0.1 mL/min. and increase to 1 mL/min over 5 minutes.
2. When the solvent is flowing freely from the column outlet, attach the column to the detector. This procedure prevents entry of air into the detection system and gives more rapid equilibration.
3. When the mobile phase is changed, gradually increase the flow rate of the new mobile phase from zero mL/min to 1.0 mL/min in 0.1 mL/min increments.
4. Once a steady backpressure and baseline have been achieved, the column is ready to be used.

Note: If mobile phase additives are present in low concentrations (such as ion-pairing reagents, at 5 to 10 mmol/L) 100 to 200 column volumes may be required for complete equilibration.

Table 2. Volume of standard columns (mL), multiply by 10 for flush solvent volume)

Column Length	Column internal diameter (mm)									
	1.0	2.1	3.0	3.9	4.6	7.8	10	19	30	50
30 mm	-	0.1	0.2	-	0.5	-	2.4	8	-	-
50 mm	0.1	0.2	0.3	-	0.8	2.4	4	14	35	98
100 mm	0.1	0.4	0.7	1.2	1.7	5	8	28	70	-
150 mm	0.1	0.5	1.0	1.8	2.5	7	12	42	106	294
250 mm	-	0.9	1.8	-	4	-	20	70	176	490
300 mm	-	-	-	-	-	14	24	85	212	589

III. COLUMN USAGE

To ensure the continued high performance of your columns and cartridges, follow these guidelines:

a. Guard columns

Samples: Sample impurities very often contribute to column contamination. Two ways to avoid this are:

1. Use of Waters Oasis™ solid-phase extraction sample clean-up cartridges or columns or Sep-Pak cartridges of the appropriate chemistry to clean up your sample before analysis.
2. Use of a Waters guard cartridge 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 analytical resolution.

b. pH Range

XTerra Columns have a widened useable pH range over silica based columns. The pH range is pH 1-12 for the MS columns and 2-12 for the RP columns. Lifetime although greater is still finite and will vary depending upon what buffers are used, the concentration of those buffers and the temperature at which they are used. Here is a table of recommended and non-recommended buffers to be used as a guideline when developing methods. Note that high pH use of Phosphate is not recommended even though it will generally give longer lifetimes than silica based columns. For a table on appropriate buffers to use, please see Tables 3, 4 and 5.

Table 3: XTerra buffers for use from pH 1-7

Additive or Buffer	pka	Buffer Range (± 1 pH unit)	Volatile or Non-Volatile	Recommended Use with XTerra Packagings
TFA	<1.0		Volatile	Yes (0.02-0.1%)
Acetic Acid	4.76		Volatile	Yes (0.1-1.0%)
Formic Acid	3.75		Volatile	Yes (0.1-1.0%)
Acetate (Ammonium)	4.76	3.76-5.76	Volatile	Yes (1-10mM) note Na+, K+ salts are not volatile
Formate (Ammonium)	3.75	2.75-4.75	Volatile	Yes (1-10mM) note Na+, K+ salts are not volatile
Phosphate 1	2.15	1.15-3.15	Non-Volatile	Yes
Phosphate 2	7.2	6.20-8.20	Non-Volatile	pH's >7.0 lifetime decreases significantly with this buffer. See also note on Phosphate 3 below. The lower the temperature and buffer molarity, the longer the column lifetime achievable.

Table 4: XTerra buffers for use from pH 7-12

Additive or Buffer	pka	Buffer Range (± 1 pH unit)	Volatile or Non-Volatile	Recommended Use with XTerra Packagings
4-Methyl-Morpholine	~8.4		Volatile	Yes (10mM)
Ammonia	9.2		Volatile	<10 mM and <30 °C
Ammonium Bicarbonate	10.3 (HCO_3)	9.3-11.3	Volatile	Yes 5-10 mM (keep source >150 °C) **(do not use carbonate)** (total pH range 6.-11.3, natural pH=8.4) adjust pH with either ammonium hydroxide or acetic acid
	9.2 (NH_4)	8.2-10.2		
	7.8 (H_2CO_3)	6.8-8.8		
Ammonium (Acetate) or (Formate)	9.2	8.2-10.2	Volatile	Yes (1-10mM)
Borate	9.2	8.2-10.2	Non- Volatile	
1-Methyl- Piperidine (Acetate or Formate)	10.3	9.3-11.3	Volatile	Yes
Triethylamine (Acetate or Formate)	10.7	9.7-11.7	Volatile	Yes (0.1-1%)
Pyrrolidine	11.3	10.3-12.3	Volatile	
Phosphate 3	12.3	11.3-13.3	Non- Volatile	

Table 5: XTerra buffers for use from pH 9-12 with alternative buffers

Additive or Buffer	pka	Buffer Range (± 1 pH unit)	Volatile or Non-Volatile	Recommended Use with XTerra Packagings
Glycine	9.8	8.8-10.8		Yes
CAPSO	9.7	8.7-10.7		Yes (0.1-1.0mM)
CAPS	10.5	9.5-11.5		Yes (0.1-1.0mM)

c. Solvents

To maintain maximum column performance, use high quality chromatography grade solvents. Filter all buffers before 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 poorer performance. Degas all solvents thoroughly before use to prevent bubble formation in the pump and detector.

d. Pressure

All XTerra Columns, regardless of dimension, can be operated at pressures up to 6000 psi, 400 bar or 40 Mpa.

e. Temperature

Temperatures between 20 – 60 ˚C are recommended for operating XTerra Columns 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.

IV. SCALING UP/DOWN

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 only column i.d. is changed:

$$X = (r_2/r_1)^2$$

If both column i.d. and length are altered:

$$F_2 = F1(r_2/r_1)^2$$

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

Where: X = Factor by which original flow must be modified (also adjusts sample load)

L = Length of column, in mm

r = Radius of the column, in mm

F = Flow rate, in mL/min.

1 designates the original, or reference column

2 designates the new dimension column.

V. COLUMN CLEANING, REGENERATING AND STORAGE

a. Cleaning and Regeneration

A shift in retention or resolution may indicate contamination of the column. Flushing with a neat organic solvent is usually sufficient to remove the contaminant. If the flushing procedure does not solve the problem, purge the column with a sequence of progressively more nonpolar or hydrophobic solvents. For example, switch from water to tetrahydrofuran (THF) to methylene chloride. Return to the standard mobile phase conditions by reversing the sequence.

Guard columns need to be replaced at regular intervals as determined by sample contamination. When system back-pressure steadily increases above a set pressure limit, it is usually an indication that the guard column should be replaced.

b. Storage

For maximum column lifetime and performance, store the column in 100% acetonitrile after use. Do not store the column in aqueous-based mobile phases containing buffers or salts. If the mobile phase eluent contained acidic or alkaline additives, buffers or salts, flush the column with at least 10 column volumes of HPLC grade water (see Table 2 for common column volumes) followed at least 15 column volumes of 100% HPLC grade acetonitrile prior to storage. Failure to perform these steps could result in precipitation of the buffer salt in the column or degrade column performance. Completely seal the column using the supplied end plugs to avoid evaporation and drying out of the bed.

VI. TROUBLESHOOTING

Changes in retention time, resolution, or backpressure are often due to column contamination. See the Column Cleaning, Regeneration and Storage section of this instruction sheet. 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).

Waters

THE SCIENCE OF WHAT'S POSSIBLE.™

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com