

## WATERS SPHERISORB COLUMNS

Thank you for choosing a Waters Spherisorb® column. Spherisorb analytical columns are durable, high-efficiency chromatographic columns, featured in thousands of references in the chromatographic literature. The wide range of column lengths and diameters offers you exceptional flexibility in optimizing methods and reducing solvent consumption. Follow the guidelines in this manual to obtain the best performance, reproducibility and durability from your analytical columns and cartridges.

### CONTENTS

#### I. GETTING STARTED

- a. Column Installation
  1. Reversed-Phase Columns
  2. Normal-Phase Columns
- b. Column Equilibration
  1. Reversed-Phase Columns
  2. Normal-Phase Columns
- c. Initial Column Efficiency Determination

#### II. COLUMN USE

- a. Guard Columns
- b. Sample Preparation
- c. pH Range
- d. Solvents
- e. Pressure
- f. Temperature

#### III. SCALING UP/DOWN ISOCRATIC METHODS

#### IV. TROUBLESHOOTING

#### V. COLUMN CLEANING, REGENERATING AND STORAGE

- a. Cleaning and Regenerating
  1. Reversed-Phase Columns
  2. Normal-Phase Columns
- b. Storage
  1. Reversed-Phase Columns
  2. Normal-Phase Columns

#### VI. CONNECTING THE COLUMN TO THE HPLC

- a. Column Connectors and System Tubing Considerations
- b. Band Spreading Minimization
- c. Measuring System Bandspreading Volume & System Variance
- d. Measuring System Volume

#### VII. ADDITIONAL INFORMATION

- a. Use of Narrow-Bore ( $\leq 3.0$  mm i.d.) Columns
- b. Impact of Bandspreading Volume on 2.1 mm i.d. Column Performance
- c. Non-Optimized vs. Optimized LC/MS/MS System:  
System Modification Recommendations



## Spherisorb Column Physical Characteristics

| Chemistry                                      | Particle Shape | Particle Sizes (µm) | Pore Sizes (Å) | Surface Area (m <sup>2</sup> /g) | Pore Volume (cc/g) | % Carbon Load | Endcapped |
|------------------------------------------------|----------------|---------------------|----------------|----------------------------------|--------------------|---------------|-----------|
| Silica                                         | Spherical      | 3, 5 and 10         | 80             | 220                              | 0.50               | n/a           | n/a       |
| ODS2 (C <sub>18</sub> ) - Fully End Capped     | Spherical      | 3, 5 and 10         | 80             | 220                              | 0.50               | 11.5          | yes       |
| ODS1 (C <sub>18</sub> ) - Partially End Capped | Spherical      | 3, 5 and 10         | 80             | 220                              | 0.50               | 6.2           | no        |
| ODSB (C <sub>18</sub> ) - Base De-activated    | Spherical      | 5                   | 80             | 220                              | 0.50               | 11.5          | yes*      |
| C <sub>8</sub>                                 | Spherical      | 3, 5 and 10         | 80             | 220                              | 0.50               | 5.8           | yes       |
| C <sub>6</sub>                                 | Spherical      | 3, 5 and 10         | 80             | 220                              | 0.50               | 4.7           | yes       |
| C <sub>1</sub>                                 | Spherical      | 3, 5 and 10         | 80             | 220                              | 0.50               | 2.2           | no        |
| Nitrile (CN)                                   | Spherical      | 3, 5 and 10         | 80             | 220                              | 0.50               | 3.1           | no        |
| Amino (NH <sub>2</sub> )                       | Spherical      | 3, 5 and 10         | 80             | 220                              | 0.50               | 1.9           | no        |
| Phenyl                                         | Spherical      | 3, 5 and 10         | 80             | 220                              | 0.50               | 2.5           | no        |
| OD/CN (Mixed Mode)                             | Spherical      | 5                   | 80             | 220                              | 0.50               | 5.0           | yes       |
| SAX                                            | Spherical      | 3, 5 and 10         | 80             | 220                              | 0.50               | 4.0           | no        |
| SCX                                            | Spherical      | 3, 5 and 10         | 80             | 220                              | 0.50               | 4.0           | no        |

\*polar endcapping

## I. GETTING STARTED

Each Spherisorb column comes with a Performance Test Chromatogram. This Performance Test Chromatogram is specific to each individual column and contains the following information: gel batch number, column serial number, USP plate count, USP tailing factor, capacity factor, and chromatographic conditions. The performance test chromatogram should be stored for future reference.

### a. Column Installation

*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 Spherisorb column being installed. See "Scaling Up/Down Isocratic Separations" 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. Reversed-Phase Columns

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 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.

#### 2. Normal-Phase Columns

*Note: It is assumed that your system has been used for reversed-phase chromatography. If this is not the case, you can start with step 3.*

1. Purge the pumping system of any buffer containing mobile phases.
2. Flush the system thoroughly with acetonitrile.
3. Switch the system over to the mobile phase that you are planning to use in normal-phase chromatography.
4. Connect the column and equilibrate it with the mobile phase.

*Note: Equilibration with the mobile phase may require a larger amount of solvent than in reversed-phase chromatography.*

## b. Column Equilibration

Spherisorb columns are shipped in test mobile phase. 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).

### 1. Reversed-Phase Columns

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.)

*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.*

### 2. Normal-Phase (Spherisorb Silica, Amino, Cyano) Column

Spherisorb normal-phase (NP) columns are delivered in 96% heptane / 4% isopropyl alcohol. Care should be taken not to pass any mobile phase through the column that might cause a precipitate. Spherisorb normal-phase (NP) columns are compatible with water and all common organic solvents, provided that solvent miscibility is accounted for.

Equilibrate normal-phase silica columns in the mobile phase. Very small quantities of water in the mobile phase can dramatically affect the activity of normal-phase packings. For good reproducibility, ensure that the mobile phase always has the same water content.

It is difficult and usually unnecessary to completely eliminate the water from the mobile phase. Dry mobile phases can take a very long time to equilibrate the column. A water content of 50 percent of saturation is recommended for most applications.

To equilibrate your column:

1. Starting at 0.0 mL/min, increase the flow rate in 0.1 mL/min increments to 1.0 min.
2. Purge the column with the mobile phase until you obtain a stable baseline.
3. Verify that retention times and peak areas for a standard are stable by comparing 2-3 replicate consecutive injections

Before you perform the first analysis on your new column, perform an efficiency test to confirm the performance of the column.

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

|               | Column internal diameter (mm) |      |      |      |    |    |
|---------------|-------------------------------|------|------|------|----|----|
| Column Length | 1.0                           | 2.1  | 3.0  | 4.6  | 10 | 20 |
| 20 mm         | -                             | 0.07 | 0.14 | 0.33 | -  | -  |
| 30 mm         | -                             | 0.1  | 0.2  | 0.5  | -  | -  |
| 50 mm         | 0.1                           | 0.2  | 0.3  | 0.8  | -  | -  |
| 100 mm        | 0.1                           | 0.4  | 0.7  | 1.7  | -  | -  |
| 150 mm        | 0.1                           | 0.5  | 1.0  | 2.5  | 12 | 24 |
| 250 mm        | -                             | 0.9  | 1.8  | 4    | 20 | 40 |

## c. 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 analyze the column upon receipt. However, if the column is used only for a single routine assay, it may be more convenient to test the column under these assay conditions. Keep a record of the initial column performance.
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.

## II. COLUMN USE

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

### a. Guard Columns

Use a Waters Spherisorb guard column of matching chemistry and particle size between the injector and main column. It is important to use a 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 or other changes in chromatographic performance 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 Oasis® solid-phase extraction cartridges/columns or Sep-Pak® cartridges of the appropriate chemistry to clean up the sample before analysis. Link to [www.waters.com/sampleprep](http://www.waters.com/sampleprep)
2. It is preferable to prepare the sample in the operating mobile phase or a mobile phase that is weaker (less organic modifier in the case of reversed-phase chromatography, less polar modifier in the case of normal-phase chromatography or hydrophilic interaction chromatography, less salt in the case of ion exchange) 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. 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 8,000 rpm, followed by the transfer of the supernatant liquid to an appropriate vial, could be considered.

### c. pH Range

The recommended operating pH range for Spherisorb columns is 2 to 8. A listing of commonly used buffers and additives is given in Table 2. Additionally, the column lifetime will vary depending upon the operating temperature, and 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.

Table 2: Buffer Recommendations for Using Spherisorb Columns from pH 2 to 8

| Additive or Buffer                                     | pK <sub>a</sub> | Buffer range (± pH unit) | Volatility   | Used for Mass Spec? | Comments                                                                                                                                                                      |
|--------------------------------------------------------|-----------------|--------------------------|--------------|---------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 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.                                                                                      |
| Ammonium Formate (NH <sub>4</sub> COOH)                | 3.75            | 2.75-4.75                | Volatile     | Yes                 | Used in the 1-10 mM range for LC/MS. Higher concentrations (typically 20 mM) are recommended for UV applications.<br><i>Note: sodium or potassium salts are not volatile.</i> |
| Trifluoroacetic Acid (TFA)                             | 0.3             |                          | Volatile     | Low conc.           | When used in LC/MS, due at signal suppression, it is generally recommended to use TFA at concentrations <0.1%.                                                                |
| Ammonium Acetate (CH <sub>3</sub> COONH <sub>4</sub> ) | 4.76            | 3.76-5.76                | Volatile     | Yes                 | Used in the 1-10 mM range for LC/MS. Higher concentrations (typically 20 mM) are recommended for UV applications.<br><i>Note: sodium or potassium salts are not volatile.</i> |
| 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 guard column to maximize lifetime.                                                                                       |

#### 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. (Please refer to the filtration section of the Waters Chromatography Columns and Supplies Catalog or the Waters web site ([www.waters.com](http://www.waters.com)) for additional product information.) 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. 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

Spherisorb columns can tolerate pressures of up to 6,000 psi (400 bar or 40 MPa) although long-term, routine operating pressures greater than 4,000 – 5,000 psi should be avoided in order to maximize column and system lifetimes.

#### f. Temperature

Temperatures between 20 – 45 °C are recommended for operating Spherisorb columns in order to enhance selectivity, lower solvent viscosity and increase mass transfer rates. However, any temperature above ambient may have a negative effect on lifetime which will vary depending on the pH and buffer conditions used.

### 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:

$$F_2 = F_1(r_2/r_1)_2$$

or

$$\text{Injection volume}_1 = \text{Injection volume}_2 (r_2/r_1)_2 (L_2/L_1)$$

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

## IV. TROUBLESHOOTING

Changes in retention time, resolution, or backpressure are often due to column contamination. See “Column Cleaning, Regenerating and Storage.” Information on column troubleshooting problems may be found in the current Waters Chromatography Columns and Supplies Catalog. You can also download a copy of the HPLC Troubleshooting Guide at [www.waters.com](http://www.waters.com), click on “Resource Library”, then “Literature Library”, and in the Search Box, enter WA20769.

## V. COLUMN CLEANING, REGENERATING AND STORAGE

### a. Cleaning and Regenerating

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. Changing the guard column being used will often restore column performance. If not (or if no guard column is being used), follow the procedures detailed below. To prevent potential contamination from affecting detector performance, it is recommended that any detector(s) be disconnected from the effluent flow of the column during cleaning. Reversing the direction of the flow through the column (backflushing) may sometimes improve the effectiveness of any cleaning procedure.

#### 1. Reversed-Phase Columns

Flushing with a neat organic solvent, taking care not to precipitate buffers, is usually sufficient to remove most 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 3). Flush columns with 20 column volumes of HPLC-grade solvents (e.g., 80 mL total for 4.6 x 250 mm column). Increasing mobile phase temperature to 35-55 °C increases cleaning efficiency. If the column performance is poor after regenerating and cleaning, call your local Waters office for additional support.

Table 3: Column Sequence or Options

| Polar Samples      | Non-polar Samples                                                      | Proteinaceous Samples                                                                                                                                               |
|--------------------|------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1. Water           | 1. Isopropanol (or an appropriate isopropanol/water mixture*)          | Option 1: Inject repeated aliquots of dimethyl sulfoxide (DMSO)                                                                                                     |
| 2. Methanol        | 2. Tetrahydrofuran (THF)                                               | Option 2: gradient of 10-90% B where:<br><br>A= 0.1% trifluoroacetic acid (TFA) in water<br>B= 0.1% trifluoroacetic acid (TFA) in acetonitrile (CH <sub>3</sub> CN) |
| 3. Tetrahydrofuran | 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.

#### 2. Normal-Phase Columns

To regenerate, pump 20-30 column volumes each of dichloromethane and isopropanol through the column. Other wash solvents such as tetrahydrofuran (THF) may also be selected based on the suspected contamination.

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. Storage

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

#### 1. Reversed-Phase Columns

For periods longer than four days at room temperature, store the column (with the exception of cyano chemistry columns) in 100% acetonitrile at room temperature. 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 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 when 100% acetonitrile is introduced.

*Note: The exception of the storage recommendations above are cyano (CN) columns used for reversed-phase methods (CN-RP). These columns should never be stored in 100% intermediate polarity solvents (acetonitrile, methanol, IPA). Store CN-RP columns in 0.1 M ammonium acetate/ACN 50/50.*

## 2. Normal-Phase Columns

For rapid equilibration upon start-up, store your normal-phase column in the mobile phase that is commonly used. Completely seal column to avoid evaporation and drying out of the bed.

## VI. CONNECTING THE COLUMN TO THE HPLC

### a. Column Connectors and System Tubing Considerations

All Spherisorb column and cartridges have Parker style endfittings.

Tools needed for Spherisorb analytical column:

1/2 inch wrench

9/16 inch wrench

Tools needed for Spherisorb analytical cartridge column:

1/4 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 1/2 inch wrench on the hex head of the column endfitting.

*Note: If one of the wrenches is improperly placed on the inner column hex head (or the cartridge 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. Tubing touches the bottom of the column endfitting, with no void between them. 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. 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 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 Spherisorb column is equipped with Parker style endfittings that require a 0.090 inch ferrule. If a Waters style column is presently being used, it is critical that the ferrule depth be reset for optimal performance prior to installing a Spherisorb column.

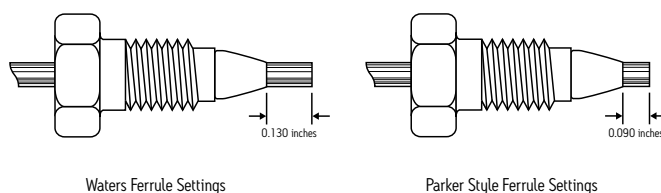


Figure 1: Waters and Parker Style Ferrule Types

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

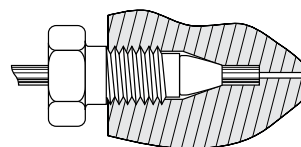


Figure 2: Proper Tubing/Column Connection

The presence of a void in the flow stream reduces column performance. This can occur if a Parker style ferrule is connected to a Waters endfitting (Figure 3).

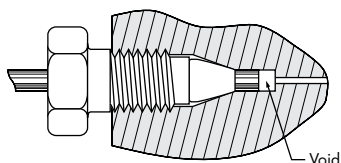


Figure 3: Parker Ferrule in a Waters Style Endfitting

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

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).

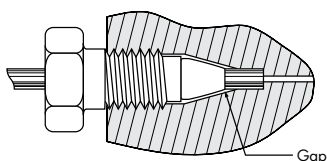


Figure 4: Waters Ferrule in a Parker Style Endfitting

Note: The connection leaks if a Waters ferrule is connected to a column with 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. (Note that PEEK fittings are not recommended for normal-phase applications!) Another approach is to use a Thermo Corporation 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).

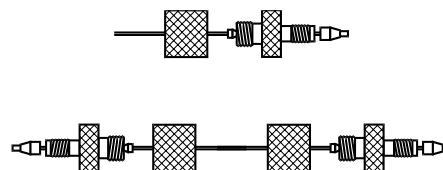


Figure 5: Single and Double SLIPFREE Connectors

#### SLIPFREE Connectors Features

- Tubing pushed into endfitting, thereby guaranteeing a void-free connection
- Connector(s) come(s) installed on tubing
- Various tubing i.d. 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 4. Waters Part Numbers for SLIPFREE Connectors

| SLIPFREE Type and Tubing Length | Tubing Internal Diameter |            |            |
|---------------------------------|--------------------------|------------|------------|
|                                 | 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 |

### b. 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.

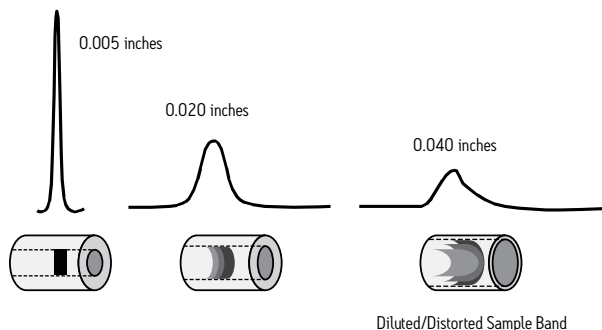


Figure 6: Effect of Connecting Tubing on System

### c. 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 P/N WAT034544).
4. Inject 2 to 5  $\mu\text{L}$  of this solution.
5. Measure the peak width at 4.4% of peak height (5-sigma method):

$$\text{5-sigma Bandspreading } (\mu\text{L}) = \text{Peak Width (min)} \times \text{Flow Rate (mL/min)} \times (1000 \mu\text{L}/1 \text{ mL})$$

$$\text{System Variance } (\mu\text{L}^2) = (5\text{-sigma bandspreading})^2 / 25$$

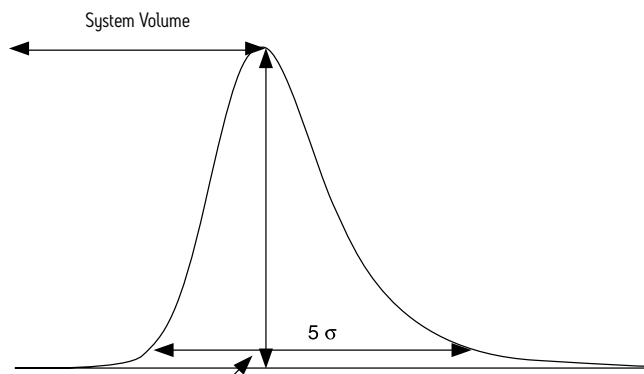


Figure 8: Determination of System Bandspreading Volume Using 5-Sigma Method

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

### d. Measuring System Volume

System volume is important in scaling separations because it creates an isocratic hold at the start of every run. This hold is often several column volumes on a small scale, but a fraction of the volume of a prep column. Compensation for this volume must be included in planning a scaling experiment to avoid distorting the chromatography (Figure 9).

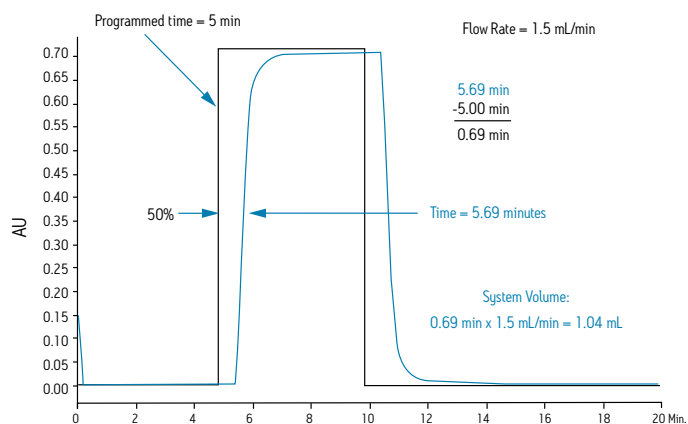


Figure 9: Determination of Gradient Delay Volume

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

## VII. ADDITIONAL INFORMATION

### a. Use of Narrow-bore (<3.0 mm i.d.) Columns

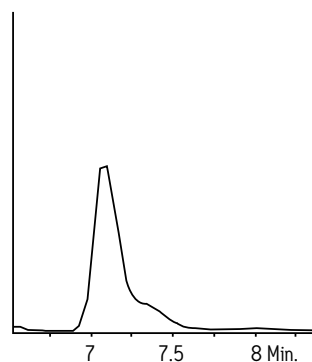
This section describes how to minimize extra column effects and provides guidelines on maximizing the performance of a narrowbore column in an HPLC system. A 3.0 mm i.d. narrow-bore column usually requires no system modifications. A 2.1 mm i.d. column, however, requires modifications to the HPLC system 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

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

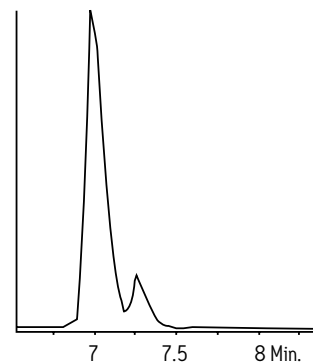
System optimization, especially in a system that contains a flow splitter, can have dramatic effects on sensitivity and resolution. Optimization includes using correct-depth ferrules and minimizing tubing diameter and lengths. An example is given in Figure 10 where system optimization resulted in a doubling of sensitivity and resolution of the metabolite in an LC/MS/MS system.

System with 130  $\mu$ L  
bandspreading: 8,000 plates



Non-optimized LC/MS/MS System

System with 70  $\mu$ L bandspreading:  
10,000 plates (same column)



Optimized System

Figure 10: 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  $\leq 2.1$  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 between pump and injector.
4. 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.
5. Use perfect (pre-cut) connections (with a variable depth inlet if using columns from different suppliers).
6. Detector time constants should be shortened to less than 0.2 seconds.

Sales Offices

**Austria and European Export  
(Central South Eastern Europe, CIS  
and Middle East)** 43 1 877 18 07

**Australia** 61 2 9933 1777

**Belgium** 32 2 726 1000

**Brazil** 55 11 5094-3788

**Canada** 1 800 252 4752 x2205

**China** 86 21 6879 5888

**CIS/Russia** +497 727 4490/290 9737

**Czech Republic** 420 2 617 1 1384

**Denmark** 45 46 59 8080

**Finland** 09 5659 6288

**France** 33 1 30 48 72 00

**Germany** 49 6196 400600

**Hong Kong** 852 29 64 1800

**Hungary** 36 1 350 5086

**India and India Subcontinent**  
91 80 2837 1900

**Ireland** 353 1 448 1500

**Italy** 39 02 265 0983

**Japan** 81 3 3471 7191

**Korea** 82 2 820 2700

**Mexico** 52 55 5524 7636

**The Netherlands** 31 76 508 7200

**Norway** 47 6 384 60 50

**Poland** 48 22 833 4400

**Puerto Rico** 1 787 747 8445

**Singapore** 65 6273 7997

**Spain** 34 93 600 9300

**Sweden** 46 8 555 11 500

**Switzerland** 41 56 676 70 00

**Taiwan** 886 2 2543 1898

**United Kingdom** 44 208 238 6100



©2008 Waters Corporation. Waters, The Science of What's Possible, Spherisorb, Oasis and SepPak are trademarks of Waters Corporation. Acrodisc is a trademark of Pall Corporation. PEEK is a trademark of Agilent Technologies. SLIPFREE is a trademark of Thermo Corporation.

December 2008 WAT094178 Rev A VW-PDF

**Waters Corporation**  
34 Maple Street  
Milford, MA 01757 U.S.A.  
T: 1 508 478 2000  
F: 1 508 872 1990  
[www.waters.com](http://www.waters.com)