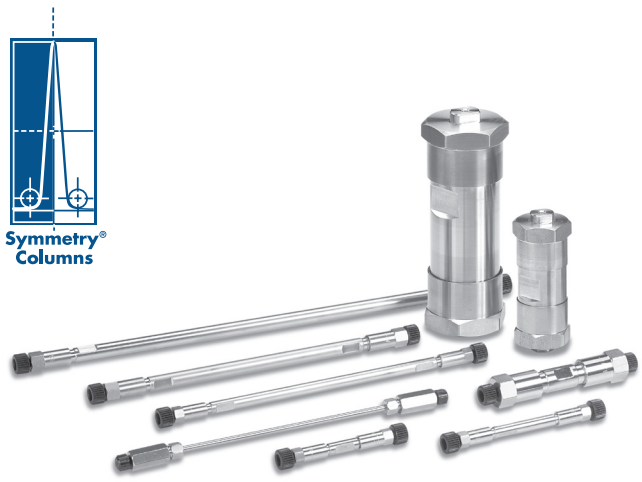
SYMMETRY COLUMNS

I. INTRODUCTION

Thank you for choosing a Waters Symmetry® column. Symmetry columns continue to set the standard of performance for reproducibility. As today's chemists establish new analytical methods for the latest pharmaceutical and biopharmaceutical products, the selection of a reproducible HPLC column is essential. The selected column needs to provide the same chromatographic results over the life of the new drug product. The excellent reproducibility of Symmetry, SymmetryShield™ and Symmetry300™ columns is a result of our commitment to maintaining the tightest specifications in the HPLC column industry.

Physical Characteristics						
Packing	Chemistry	Particle Size	Particle Shape	Pore Size	Carbon Load	End-capped
Symmetry	C_{18}	3.5, 5 µm	Spherical	100Å	19%	Yes
	C_8	3.5, 5 µm	Spherical	100Å	12%	Yes
SymmetryPrep	C_{18}	7 µm	Spherical	100Å	19%	Yes
	C_8	7 µm	Spherical	100Å	12%	Yes
SymmetryShield	RP18	3.5, 5, 7 µm	Spherical	100Å	17%	Yes
	RP8	3.5, 5, 7 µm	Spherical	100Å	15%	Yes
Symmetry300	C_{18}	3.5, 5 µm	Spherical	300Å	8.5%	Yes
	C_4	3.5, 5 µm	Spherical	300Å	2.8%	Yes



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II. GETTING STARTED

Each Symmetry column comes with Certificate of Analysis and a Performance Test Chromatogram. The Certificate of Analysis is specific to each batch of packing material contained in the Symmetry column and includes the gel 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: gel batch number, column serial number, USP plate count, USP tailing factor, capacity factor, and chromatographic conditions. These data 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 Symmetry 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. 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.

b. Column Equilibration

Symmetry 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.)

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.

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.
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	Column internal diameter (mm)						
Length	1.0	2.1	3.0	4.6	7.8	19	30
20 mm	–	0.07	0.14	0.33	–	–	–
30 mm	–	0.1	0.2	0.5	–	8	–
50 mm	0.1	0.2	0.3	0.8	2.4	14	35
100 mm	0.1	0.4	0.7	1.7	5	28	70
150 mm	0.1	0.5	1.0	2.5	7	42	–
250 mm	–	0.9	1.8	4	–	70	–

III. COLUMN USE

To ensure the continued high performance of Symmetry 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 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
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. 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 Symmetry 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, 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 Symmetry Columns from pH 2 to 8*

Additive or Buffer	pK_a	Buffer range (±1 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_4COOH)	3.75	2.75 – 4.75	Volatile	Yes	Used in the 1-10 mM range. *Note: sodium or potassium salts are not volatile.*
Trifluoroacetic Acid (TFA)	0.3		Volatile	Low conc.	When used in LC/MS, due to signal suppression, it is generally recommended to use TFA at concentrations < 0.1%
Ammonium Acetate (NH_4CH_2COOH)	4.76	3.76 – 5.76	Volatile	Yes	Used in the 1-10 mM 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	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. 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

Symmetry 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 – 45 °C are recommended for operating Symmetry 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.

IV. 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

Injection volume$_1$ = 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

V. 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, click on "Literature Library", then in the Information Center Search Box, enter WA20769.

VI. 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. 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 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% 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.*

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

For periods longer than four days at room temperature, store the column in 100% acetonitrile. For elevated temperature applications, store immediately after use in 100% acetonitrile for the best column lifetime. Do not store columns in buffered eluents. If the mobile phase contained a buffer salt, flush 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. Completely seal column to avoid evaporation and drying out of the bed.

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.

VII. 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.

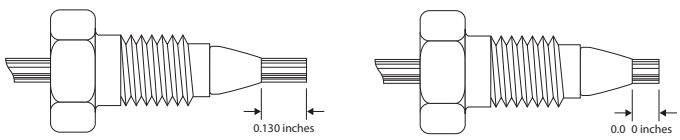
Note: If one of the wrenches is placed on the column tube flat during this process, the endfitting will be loosened and leak.

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. 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 Symmetry column is equipped with Waters style endfittings that 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 a Symmetry column.

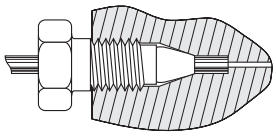
Figure 1: *Waters and Parker Style Ferrule Types*



Waters Ferrule Settings | Parker Style Ferrule Settings

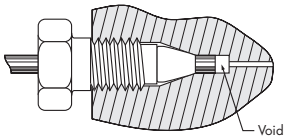
In a proper tubing/column connection (Figure 2), the tubing touches the bottom of the column endfitting, with no void between them.

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*

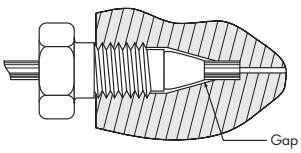


Note: A void appears if tubing with a Parker style ferrule is connected to a Waters style column.

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*



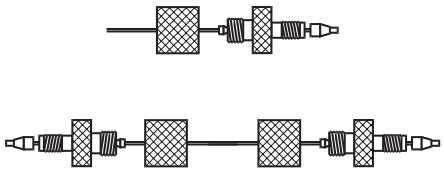
Note: The connection leaks if a Waters ferrule is connected to a column with a Parker style endfitting.

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. 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

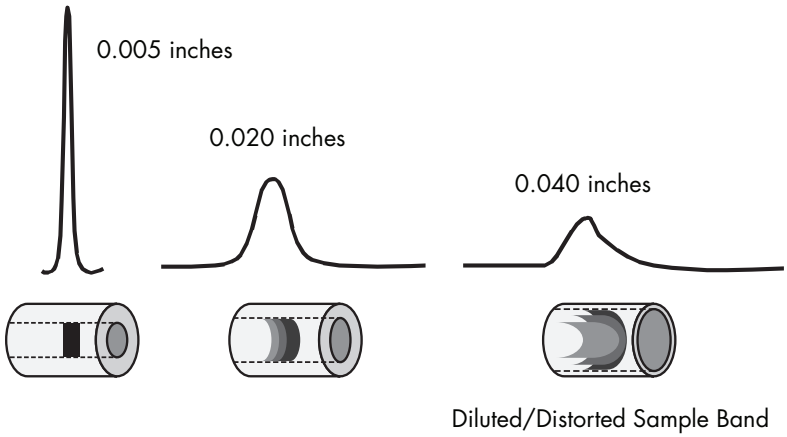
Table 4. *Waters Part Numbers for SLIPFREE Connectors*

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.

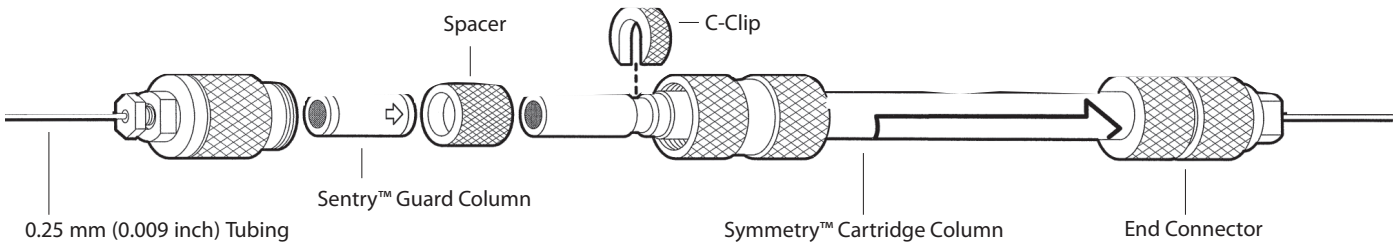
Figure 6: *Effect of Connecting Tubing on System*



c. Cartridge Installation

Refer to the installation illustration in Figure 7. Unscrew the end connectors from the old cartridge column, leaving them connected to the inlet and outlet lines of the instrument.

Figure 7: *Installing a Symmetry Cartridge Column with a Sentry Guard Column*



Attach the new cartridge column between the connectors so that the direction of the flow arrow on the label is pointing towards the detector. Finger-tighten all fittings.

d. Measuring System Bandspreading Volume and System Variance

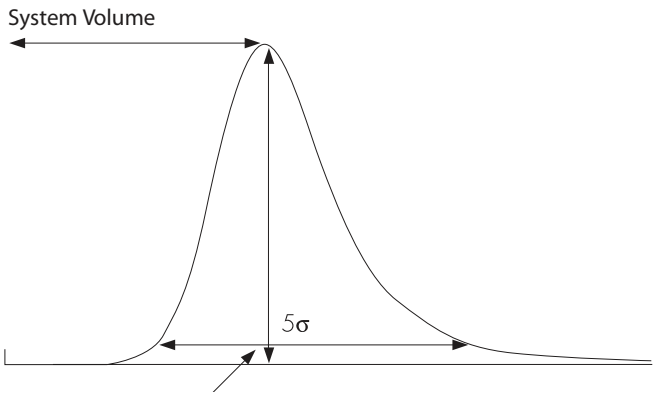
This test should be performed on an HPLC system with a single wavelength UV detector (not a Photodiode Array [PDA]).

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 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) Flow Rate (mL/min x (1000 µL/1 mL)

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

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



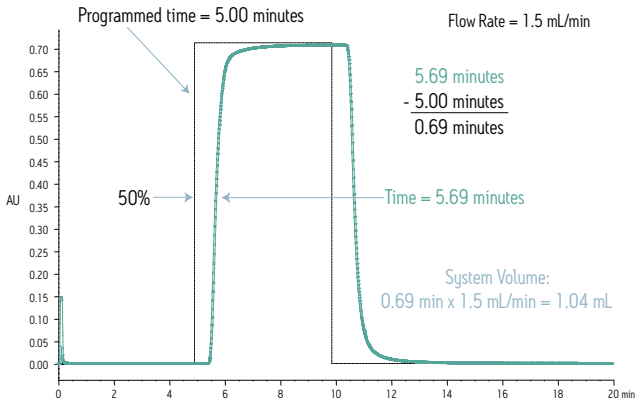
In a typical HPLC system, the Bandspreading Volume should be 100 µL ± 30 µL (or Variance of 400 μL^2 +/- 36 μL^2).

In a microbore (2.1 mm 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).

e. 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.

VIII. ADDITIONAL INFORMATION

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

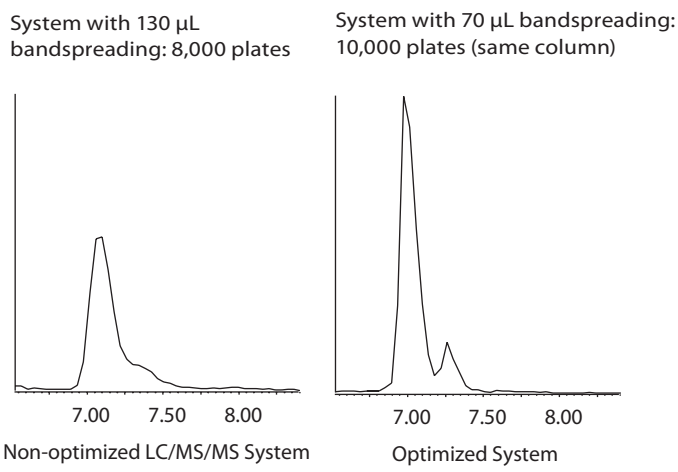
This section describes how to minimize extra column effects and provides guidelines on maximizing the performance of a narrowbore column in an HPLC system. A 3.0 mm i.d. narrow-bore column usually requires no system modifications. A 2.1 mm i.d. column, however, requires modifications to the HPLC system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

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 ≤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.

d. Waters Small Particle Size (3.5 µm) Columns – Fast Chromatography

Waters columns that contain 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 outlet frits to retain packing material. These columns should not be backflushed.

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