

## XSELECT COLUMNS

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Thank you for choosing a Waters XSelect™ column. XSelect HPLC columns feature Waters Charged Surface Hybrid (CSH™) Technology which provides excellent peak shape, high efficiency and loading capacity for basic compounds when using low ionic strength, acidic mobile phases. This same particle technology is used in the ACQUITY UPLC® CSH column family, thus enabling seamless transferability between HPLC and UPLC® system platforms. All XSelect packing materials are manufactured in a cGMP, ISO 9001:2000 certified plant using ultra pure reagents. Each batch of XSelect material is tested chromatographically with acidic, basic and neutral analytes and the results are held to narrow specification ranges to assure excellent, reproducible performance. Every column is individually tested and a Performance Test Chromatogram is provided with each column along with the Certificate of Acceptance.

**XSELECT™**  
Columns



## I. GETTING STARTED

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

### a. Column Installation

*Note: The flow rates given in the procedure below are for a typical 5 µm packing in a 4.6 mm i.d. column. Scale the flow rate up or down accordingly based upon the i.d., length, particle size and backpressure of the XSelect column being installed. See Scaling Up/Down Isocratic Separations section for calculating flow rates when changing column i.d. and/or length. See Connecting the Column to the HPLC section for a more detailed discussion on HPLC connections.*

1. Purge the pumping system of any buffer-containing mobile phases and connect the inlet end of the column to the injector outlet. An arrow on the column identification label indicates the correct direction of solvent flow.
2. Flush column with 100% organic mobile phase (methanol or acetonitrile) by setting the pump flow rate to 0.1 mL/min and increase the flow rate to 1 mL/min over 5 minutes.
3. When the mobile phase is flowing freely from the column outlet, stop the flow and attach the column outlet to the detector. This prevents entry of air into the detection system and gives more rapid baseline equilibration.
4. Gradually increase the flow rate as described in step 2.
5. Once a steady backpressure and baseline have been achieved, proceed to the next section.

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

### b. Column Equilibration

XSelect 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 mobilephase 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).

### c. Initial Column Efficiency Determination

1. Perform an efficiency test on the column before using it in the desired application. Waters recommends using a suitable solute mixture, as found in the "Performance Test Chromatogram", to analyze the column upon receipt.
2. Determine the number of theoretical plates (N) and use this value for periodic comparisons.
3. Repeat the test at predetermined intervals to track column performance over time. Slight variations may be obtained on two different HPLC systems due to the quality of the connections, operating environment, system electronics, reagent quality, column condition and operator technique.

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

Column Length (mm)	Column Internal Diameter (mm)								
	1.0	2.1	3.0	4.6	7.8	10	19	30	50
20	-	0.07	0.14	0.33	-	-	-	-	-
30	-	0.10	0.21	0.50	-	2.4	8.5	-	-
50	0.04	0.17	0.35	0.83	2.4	3.9	14	35	98
100	0.08	0.35	0.71	1.7	4.8	7.8	28	70	-
150	0.12	0.52	1.0	2.5	7.2	12	42	106	294
250	-	0.87	1.8	4.2	-	20	70	176	490

## II. COLUMN USE

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

### a. Guard Columns

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

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

### b. Sample Preparation

1. Sample impurities often contribute to column contamination. One option to avoid this is to use Waters Oasis® solid-phase extraction cartridges/columns or Sep-Pak® cartridges of the appropriate chemistry to clean up the sample before analysis.
2. It is preferable to prepare the sample in the operating mobile phase or a mobile phase that is weaker (less organic modifier) than the mobile phase for the best peak shape and sensitivity.
3. If the sample is not dissolved in the mobile phase, ensure that the sample, solvent and mobile phases are miscible in order to avoid sample and/or buffer precipitation.
4. Filter sample with 0.2 µm filters to remove particulates. If the sample is dissolved in a solvent that contains an organic modifier (e.g., acetonitrile, methanol, etc.) ensure that the filter material does not dissolve in the solvent. Contact the filter manufacturer with solvent compatibility questions. Alternatively, centrifugation for 20 minutes at 8,000 rpm, followed by the transfer of the supernatant liquid to an appropriate vial, could be considered.

### c. Operating pH Limits

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

Table 2: Recommended pH and Temperature Limits for XSelect Columns

Column Name	Particle Size (µm)	Pore Diameter (Å)	Surface Area (m <sup>2</sup> )	pH Limits	Temperature Limits		Ligand Density (µmol/m <sup>2</sup> )	% Carbon
					Low pH	High pH		
XSelect CSH C <sub>18</sub>	3.5, 5	135	185	1-11	60	45	2.3	15
XSelect CSH Phenyl-Hexyl	3.5, 5	135	185	1-11	60	45	2.3	14
XSelect CSH Fluoro-Phenyl	3.5, 5	135	185	1-8	60	45	2.3	10

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

Table 3: Buffer Recommendations for Using XSelect Columns up to pH 11

Additive/Buffer	pKa	Buffer range	Volatility	Used for Mass Spec	Comments
TFA	0.3		Volatile	Yes	Ion pair additive, can suppress MS signal, used in the 0.02-0.1% range.
Acetic Acid	4.76		Volatile	Yes	Maximum buffering obtained when used with ammonium acetate salt. Used in 0.1-1.0% range.
Formic Acid	3.75		Volatile	Yes	Maximum buffering obtained when used with ammonium formate salt. Used in 0.1-1.0% range.
Acetate (NH <sub>4</sub> CH <sub>2</sub> COOH)	4.76	3.76 – 5.76	Volatile	Yes	Used in the 1-10 mM range. Note that sodium or potassium salts are not volatile.
Formate (NH <sub>4</sub> COOH)	3.75	2.75 – 4.75	Volatile	Yes	Used in the 1-10 mM range. Note that sodium or potassium salts are not volatile.
Phosphate 1	2.15	1.15 – 3.15	Non-volatile	No	Traditional low pH buffer, good UV transparency.
Phosphate 2	7.2	6.20 – 8.20	Non-volatile	No	Above pH 7, reduce temperature/concentration and use a guard column to maximize lifetime.
Phosphate 3	12.3	11.3 - 13.3	Non-volatile	No	Above pH 7, reduce temperature/concentration and use a guard column to maximize lifetime.
4-Methylmorpholine	~8.4	7.4 – 9.4	Volatile	Yes	Generally used at 10 mM or less.
Ammonia (NH <sub>4</sub> OH)	9.2	8.2 – 10.2	Volatile	Yes	Used in the 5-10 mM range (for MS work keep source >150 °C). Adjust pH with ammonium hydroxide or acetic acid. Good buffering capacity at pH 10. Note: use ammonium bicarbonate (NH <sub>4</sub> HCO <sub>3</sub> ), not ammonium carbonate ((NH <sub>4</sub> ) <sub>2</sub> CO <sub>3</sub> )
Ammonium Bicarbonate	10.3 (HCO <sub>3</sub> <sup>-</sup> ) 9.2 (NH <sub>4</sub> <sup>+</sup> )	8.2 – 11.3	Volatile	Yes	
Ammonium (Acetate)	9.2	8.2 – 10.2	Volatile	Yes	Used in the 1-10 mM range.
Ammonium (Formate)	9.2	8.2 – 10.2	Volatile	Yes	Used in the 1-10 mM range.
Borate	9.2	8.2 – 10.2	Non-volatile	No	Reduce temperature/concentration and use a guard column to maximize lifetime.
CAPSO	9.7	8.7 – 10.7	Non-volatile	No	Zwitterionic buffer, compatible with acetonitrile, used in the 1-10 mM range. Low odor.
Glycine	2.4, 9.8	8.8 – 10.8	Non-volatile	No	Zwitterionic buffer, can give longer lifetimes than borate buffer.
1-Methylpiperidine	10.2	9.3 – 11.3	Volatile	Yes	Used in the 1-10 mM range.
CAPS	10.4	9.5 – 11.5	Non-volatile	No	Zwitterionic buffer, compatible with acetonitrile, used in the 1-10 mM range. Low odor.
Triethylamine (as acetate salt)	10.7	9.7 – 11.7	Volatile	Yes	Used in the 0.1-1.0% range. Volatile only when titrated with acetic acid (not hydrochloric or phosphoric). Used as ion-pair for DNA analysis at pH 7-9.
Pyrrolidine	11.3	10.3 – 12.3	Volatile	Yes	Mild buffer, gives long lifetime.

#### d. Solvents

To maintain maximum column performance, use high quality chromatography grade solvents. Filter all aqueous buffers prior to use. Pall Gelman Laboratory Acrodisc® filters are recommended. Solvents containing suspended particulate materials will generally clog the outside surface of the inlet distribution frit of the column. This will result in higher operating pressure and poor performance.

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

#### e. Pressure

XSelect 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 up to 80 °C are recommended for operating XSelect 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. See Table 2 for recommended pH and temperature operating ranges.

### 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:

$$F_2 = F_1 (r_2/r_1)^2$$

$$\text{Load}_2 = \text{Load}_1 (r_2/r_1)^2 (L_2/L_1)$$

$$\text{Injection volume}_2 = \text{Injection volume}_1 (r_2/r_1)^2 (L_2/L_1)$$

Where:  $r$  = Radius of the column

$F$  = Flow rate

$L$  = Length of column

1 = Original, or reference column

2 = New column

### IV. TROUBLESHOOTING

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

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

#### a. Cleaning and Regeneration

Changes in peak shape, peak splitting, shoulders on the peak, shifts in retention, change in resolution or increasing backpressure may indicate contamination of the column. Flushing with a neat organic solvent, taking care not to precipitate buffers, is usually sufficient to remove the contaminant. If the flushing procedure does not solve the problem, purge the column using the following cleaning and regeneration procedures.

Use the cleaning routine that matches the properties of the samples and/or what you believe is contaminating the column (see Table 4). Flush columns with 20 column volumes each of HPLC-grade solvents (e.g., 80 mL total for 4.6 x 250 mm column) listed in Table 4. Increasing mobile phase temperature to 35-55 °C increases cleaning efficiency. If the column performance is poor after cleaning and regeneration, call your local Waters office for additional support.

**Table 4: Cleaning and Regeneration 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. tetrahydrofuran (THF)	2. methanol	Option 2: gradient of 10% to 90% B where:
3. tetrahydrofuran (THF)	3. dichloromethane	A = 0.1% trifluoroacetic acid (TFA) in water
4. methanol	4. hexane	B = 0.1% trifluoroacetic acid (TFA) in acetonitrile (CH <sub>3</sub> CN)
5. water	5. isopropanol (followed by an appropriate isopropanol/water mixture*)	Option 3: Flush column with 7M guanidine hydrochloride, or 7M urea
6. mobile phase	6. mobile phase	

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

#### b. Storage

For periods longer than four days at room temperature, store XSelect columns in 100% acetonitrile. Immediately after use with elevated temperatures and/or at pH extremes, store in 100% acetonitrile for the best column lifetime. Do not store columns in highly aqueous (<20% organic) mobile phases, as this may promote bacterial growth. If the mobile phase contained a buffer salt, flush the column with 10 column volumes of HPLC grade water (see Table 1 for common column volumes) and replace with 100% acetonitrile for storage. Failure to perform this intermediate step could result in precipitation of the buffer salt in the column or system when 100% acetonitrile is introduced. Completely seal column to avoid evaporation and drying out of the packed bed.

## VI. CONNECTING THE COLUMN TO THE HPLC

### a. Column Connectors and System Tubing Considerations

Tools needed:

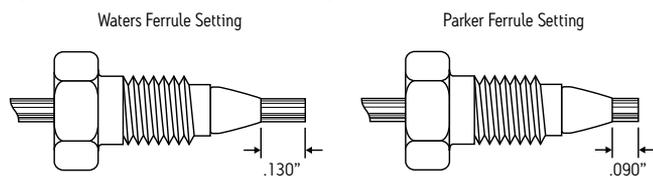
- 3/8 inch wrench
- 5/16 inch wrench

Handle the column with care. Do not drop or hit the column on a hard surface as it may disturb the bed and affect its performance.

1. Correct connection of 1/16 inch outer diameter stainless steel tubing leading to and from the column is essential for high-quality chromatographic results.
  2. When using standard stainless steel compression screw fittings, it is important to ensure proper fit of the 1/16 inch outer diameter stainless steel tubing. When tightening or loosening the compression screw, place a 5/16 inch wrench on the compression screw and a 3/8 inch wrench on the hex head of the column endfitting.
- Note: If one of the wrenches is placed on the column tube flat during this process, the endfitting will be loosened and leak.*
3. If a leak occurs between the stainless steel compression screw fitting and the column endfitting, a new compression screw fitting, tubing and ferrule must be assembled.
  4. An arrow on the column identification label indicates correct direction of solvent flow.

Correct connection of 1/16 inch outer diameter stainless steel tubing leading to and from the column is essential for high-quality chromatographic results. To obtain a void-free connection, the tubing must touch the bottom of the column endfitting. It is important to realize that extra column peak broadening due to voids can destroy an otherwise successful separation. The choice of appropriate column connectors and system tubing is discussed in detail below.

**Figure 1: Waters and Parker Ferrule Types**

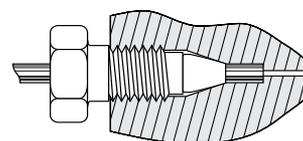


Due to the absence of an industry standard, various column manufacturers have employed different types of chromatographic column connectors. The chromatographic separation can be

negatively affected if the style of the column endfittings does not match the existing tubing ferrule settings. This section explains the differences between Waters style and Parker style ferrules and endfittings (Figure 1). Each endfitting style varies in the required length of the tubing protruding from the ferrule. The XSelect column is equipped with Waters style endfittings that require a 0.130 inch ferrule depth. If a non-Waters style column is presently being used, it is critical that ferrule depth be reset for optimal performance prior to installing an XSelect column.

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

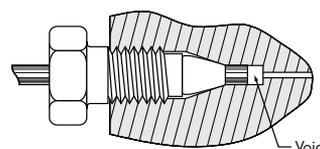
**Figure 2: Proper Tubing/Column Connection**



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

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

**Figure 3: Parker Ferrule in a Waters Style Endfitting**

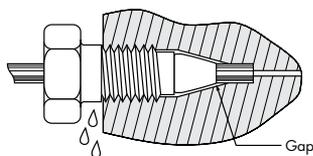


There is only one way to fix this problem: Cut the end of the tubing with the ferrule, place a new ferrule on the tubing and make a new connection. Before tightening the screw, make sure that the tubing bottoms out in the endfitting of the column.

Conversely, if tubing with a Waters ferrule is connected to a column with Parker style endfitting, the end of the tubing will bottom out before the ferrule reaches its proper sealing position. This will leave a gap and create a leak (Figure 4).

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

Figure 4: Waters Ferrule in a Parker Style Endfitting

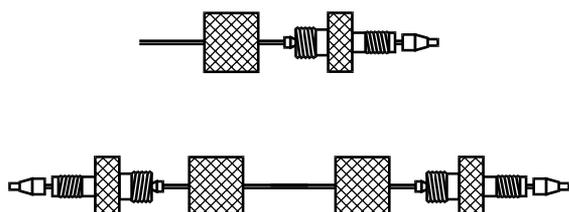


There are two ways to fix the problem:

1. Tighten the screw a bit more. The ferrule moves forward, and reaches the sealing surface. Do not over tighten 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 SLIPFREE® connector to always ensure the correct fit. The fingertight SLIPFREE® connectors automatically adjust to fit all compression screw type fittings without the use of tools (Figure 5).

Figure 5: Single and Double SLIPFREE® Connectors



SLIPFREE® Connector Features:

- Tubing pushed into endfitting, thereby guaranteeing a void-free connection
- Connector(s) come(s) installed on tubing
- Various tubing IDs and lengths available
- Fingertight to 10,000 psi – never needs wrenches
- Readjusts to all column endfittings
- Compatible with all commercially available endfittings
- Unique design separates tube-holding function from sealing function

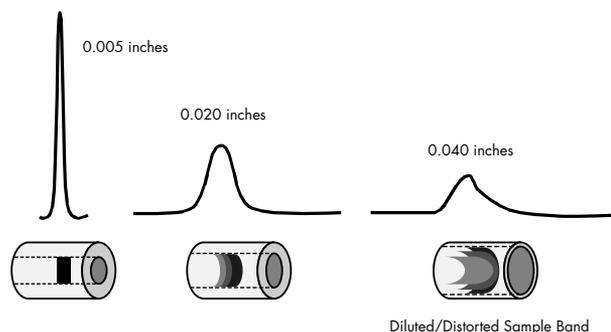
Table 5: Waters Part Numbers for SLIPFREE® Connectors

SLIPFREE® Type	Tubing Internal Diameter		
	0.005"	0.010"	0.020"
Single 6 cm	PSL 618000	PSL 618006	PSL 618012
Single 10 cm	PSL 618002	PSL 618008	PSL 618014
Single 20 cm	PSL 618004	PSL 618010	PSL 618016
Double 6 cm	PSL 618001	PSL 618007	PSL 618013
Double 10 cm	PSL 618003	PSL 618009	PSL 618015
Double 20 cm	PSL 618005	PSL 618001	PSL 618017

### Band Spreading Minimization

Figure 6 shows the influence of tubing internal diameter on system band spreading and peak shape. As can be seen, the larger tubing diameter causes excessive peak broadening and lower sensitivity.

Figure 6: Effect of Connecting Tubing on System



### b. Measuring System Band-spreading Volume and System Variance

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

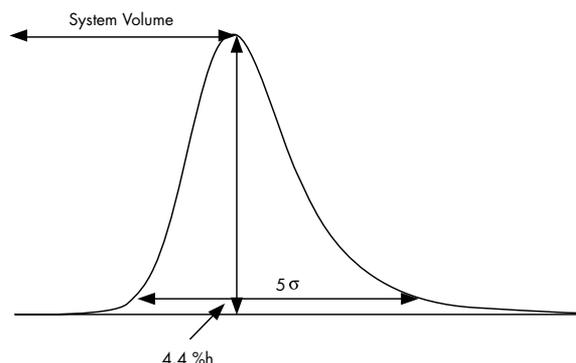
1. Disconnect column from system and replace with a zero dead volume union.
2. Set flow rate to 1 mL/min.
3. Dilute a test mix in mobile phase to give a detector sensitivity of 0.5 - 1.0 AUFS (system start up test mix can be used which contains uracil, ethyl and propyl parabens; Waters part number WAT034544).
4. Inject 2 to 5 µL of this solution.

5. Measure the peak width at 4.4% of peak height (5-sigma method):

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

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

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



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

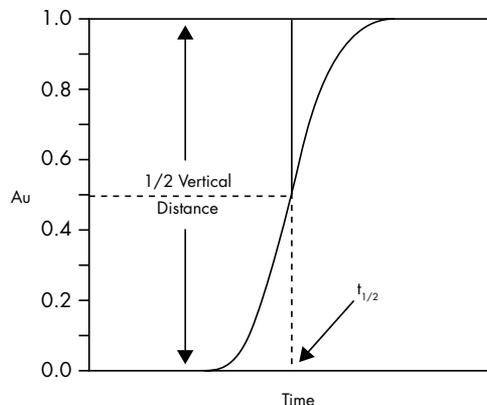
### c. Measuring Gradient Delay Volume (or Dwell Volume)

For successful gradient-method transfers the gradient delay volumes should be measured using the same method on both HPLC systems. The procedure below describes a method for determining the gradient delay volumes.

1. Replace the column with a zero dead volume union.
2. Prepare mobile phase A (pure solvent, such as methanol) and mobile phase B (mobile phase A with a UV absorbing sample, such as (v/v) 0.1% acetone in methanol).
3. Equilibrate the system with mobile phase A until a stable baseline is achieved.
4. Set the detector wavelength to the absorbance maximum of the probe (265 nm for acetone).

5. Program a 0-100% B linear gradient in 10 min at 2 mL/min (the exact conditions are not critical; just make sure the gradient volume is at least 20 mL) with a hold at 100% B.

Figure 8: Determination of Gradient Delay Volume



6. Determine the dwell time by first locating the time at the midpoint of the formed gradient ( $t_{1/2}$ ) (half the vertical distance between the initial and final isocratic segments as shown in Figure 8).
7. Subtract half the gradient time ( $1/2 t_{1/2}^g$ ) (10 min/2 = 5 min in this example) from the gradient midpoint ( $t_{1/2}$ ) to obtain the dwell time ( $t_D$ ).
8. Convert the dwell time ( $t_D$ ) to the dwell volume ( $V_D$ ) by multiplying by the flow rate (F).

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

For fast gradient methods, the gradient delay volume (or dwell volume) should be less than 1 mL. If the gradient delay volume is greater than 1 mL, see System Modification Recommendations section on how to reduce system volume.

## VII. ADDITIONAL INFORMATION

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

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

peak broadening and has a large impact on peak width as peak volume decreases.

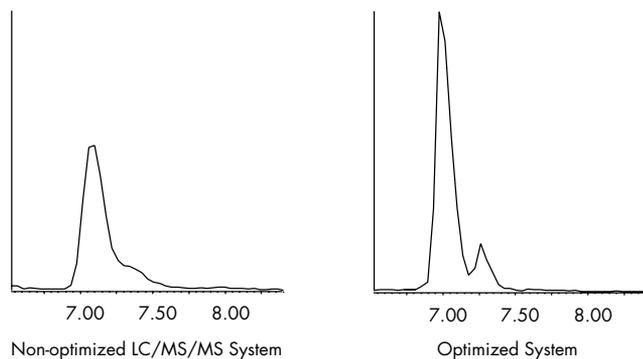
### b. Impact of Band-spreading Volume on 2.1 mm i.d. Column Performance

System with 70  $\mu$ L band-spreading: 10,000 plates  
 System with 130  $\mu$ L band-spreading: 8,000 plates (same column)

*Note: Flow splitters after the column will introduce additional band-spreading.*

System optimization, especially in a system that contains a flow splitter, can have dramatic effects on sensitivity and resolution. Optimization includes using correct ferrule depths and minimizing tubing inner diameters and lengths. An example is given in Figure 9 where system optimization resulted in a doubling of sensitivity and resolution of the metabolite in an LC/MS/MS system.

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



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

1. Use a microbore detector flow cell with 2.1 mm i.d. columns.  
*Note: Detector sensitivity is reduced with the shorter flow cell path length in order to achieve lower band-spreading volume.*
2. Minimize injector sample loop volume.
3. Use 0.009 inch (0.25 mm) tubing for rest of connections in standard systems and 0.005 inch (0.12 mm) tubing for narrowbore (2.1 mm i.d.) systems.
4. Use perfect (pre-cut) connections (with a variable depth inlet if using columns from different suppliers).
5. Detector time constants should be shortened to less than 0.2 seconds.

### 购买联系方式：

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010-67113925/67136152/67100708

Fax : 010-67114016

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