XSelect Peptide CSH C_{18}, 130Å, 3.5 µm and 5 µm Columns

CONTENTS

Thank you for choosing a Waters® XSelect® Peptide CSH™ HPLC Column. XSelect Peptide CSH C_{18}, 130Å, 3.5 µm and 5 µm Columns feature Waters Charged Surface Hybrid (CSH) Technology which provides excellent peak shape, high efficiency and loading capacity for basic compounds when using acidic, low ionic strength mobile phases. This same particle technology is used in the ACQUITY UPLC® Peptide CSH C_{18}, 130Å, 1.7 µm and ***XP*** 2.5 µm HPLC columns, thus enabling seamless transferability between HPLC and UPLC® system platforms. The XSelect Peptide CSH C_{18}, 130Å packing materials were designed and are manufactured in a cGMP, ISO 9001 certified manufacturing facility using ultra pure reagents. Each batch of XSelect Peptide CSH C_{18}, 130Å, material is tested chromatographically with acidic, basic and neutral analytes as part of qualification for use in peptide mapping. The results are held to narrow specification ranges to assure excellent, reproducible performance. XSelect Peptide CSH C_{18}, 130Å batches are also QC tested with a gradient separation of a tryptic digest of cytochrome *c* using 0.1% formic acid containing eluents. Finally, every shipped column is individually tested for packed bed efficiency and a Performance Chromatogram and Certificate of Batch Analysis is available upon request.

XSELECT® Columns

I. GETTING STARTED

Each XSelect Peptide CSH C_{18}, 130Å Column comes with a Certificate of Acceptance and a Performance Test Chromatogram. The Certificate of Acceptance is specific to each batch of packing material contained in the Peptide Separation Technology 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 the information: batch number, column serial number, USP plate count, USP tailing factor, retention 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 5 µm packing in a 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 Peptide Separation Technology 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 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

Peptide Separation Technology 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. (e.g., flush the column and HPLC system with 60% methanol in water prior to introducing 60% methanol/40% buffer mobile phase).

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

	Column internal diameter (mm)						
Column Length (mm)	1.0	2.1	4.6	10	19	30	50
50	0.04	0.17	0.83	3.9	14	35	98
100	0.08	0.35	1.7	7.8	28	70	196
150	0.12	0.52	2.5	12	42	106	294

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.

II. COLUMN USE

To ensure the continued high performance of XSelect Peptide CSH C_{18}, 130Å 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 8000 rpm, followed by the transfer of the supernatant liquid to an appropriate vial, could be considered.

c. Operating pH Limits

The recommended operating pH range for XSelect Peptide CSH C_{18}, 130Å, 3.5 µm and 5 µm Columns are 1 to 11. 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.

Important Note:

Waters XSelect Peptide CSH C_{18}, 130Å material is produced from our XBridge Peptide BEH C_{18}, 130Å particles that undergo a surface modification by the addition of a low concentration of weakly basic ionizable silanes, followed by C_{18} bonding and end capping. The optimal surface concentration of the ionizable silane groups is more than an order of magnitude lower than that of the primary C_{18}-bonded phase. The weakly basic silane groups are protonated at a low pH and are neutral at pH greater than 7. Consequently and while the XSelect Peptide CSH C_{18}, 130Å particles can tolerate a pH range from 1 to 11, it will not provide frequently desired positive surface charge benefits when used at a pH greater than 6.

Table 2: Buffer recommendations for using XSelect Peptide CSH C_{18}, 130Å Columns from pH 1 to 11

Additive/Buffer	pKa	Buffer range	Volatility (±1 pH unit)	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_4CH_2COOH)	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_4COOH)	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_4OH)	9.2	8.2 – 10.2	Volatile	Yes	Keep concentration below 10 mM and temperatures below 30 °C.
Ammonium Bicarbonate	10.3 (HCO_3-) 9.2 (NH_4+)	8.2 – 11.3	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_4HCO_3), not ammonium carbonate ($(NH_4)_2CO_3$).
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

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.

e. Pressure

XSelect Peptide CSH C_{18}, 130Å Columns can tolerate pressures of up to 6000 psi (400 bar or 40 Mpa) although pressures greater than 4000 – 5000 psi should be avoided in order to maximize column and system lifetimes.

f. Temperature

Temperatures up to 80 °C are recommended for operating XSelect Peptide CSH C_{18}, 130Å 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.

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:

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

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

$$Injection\ volume_2 = Injection\ volume_1 (r_2/r_1)^2 (L_2/L_1)$$

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 (p/n: WA20769) or visit the Waters Corporation website for information on seminars (www.waters.com).

VI. 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 3). 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 3. 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 3: Cleaning and regeneration sequence or options

Polar samples	Proteinaceous samples
1. water	Option 1: Inject repeated 100 µL aliquots of dimethyl-sulfoxide (DMSO) using a reduced flow rate delivering 50% Eluent A and 50% Eluent B
2. methanol	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. isopropanol	Option 3: Flush column with 7M guanidine hydrochloride, or 7M urea

Note: To avoid potentially damaging precipitation within your column (e.g., if your separation eluent contains phosphate buffer), be certain to flush column with 5 to 10 column volumes of water BEFORE using suggested organic eluent column wash procedures.

b. Storage

For periods longer than four days at room temperature, store the column 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 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.

V. 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.

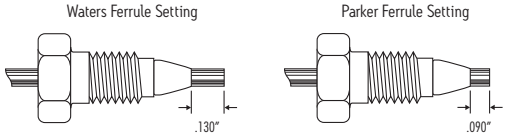
1. Correct connection of 1/16 inch outer diameter stainless steel tubing leading to and from the column is essential for high-quality chromatographic results.
2. When using standard stainless steel compression screw fittings, it is important to ensure proper fit of the 1/16 inch outer diameter stainless 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.

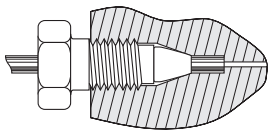
Figure 1. Waters and Parker ferrule types.



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 Peptide CSH C_{18}, 130Å 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 Peptide CSH C_{18}, 130Å Column.

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

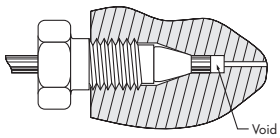
Figure 2. Proper tubing/column connection.



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.

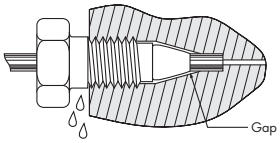


There is only one way to fix this problem: Cut the end of the tubing with the ferrule, place a new ferrule on the tubing and make a new connection. Before tightening the screw, make sure that the tubing bottoms out in the endfitting of the column.

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 Waters ferrule is connected to a column with a Parker style endfitting.

Figure 4. Waters ferrule in a Parker style endfitting.

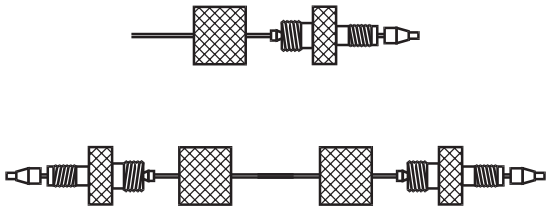


There are two ways to fix the problem:

1. Tighten the screw a bit more. The ferrule moves forward, and reaches the sealing surface. Do not overtighten since this may 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 (p/n: PSL613315) that allows resetting of the ferrule depth. Another approach is to use a Keystone, Inc. 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 I.D.'s 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

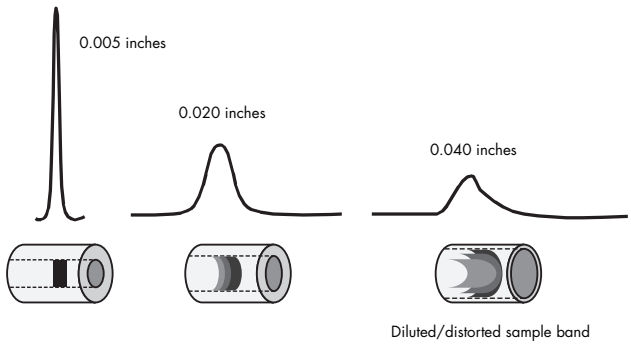
Table 5: Waters part numbers for SLIPFREE Connectors

Column Length (mm)	1.0	2.1	4.6
SLIPFREE Type	Tubing Internal Diameter		
Tubing length	0.005"	0.010"	0.020"
Single 6 cm	PSL 618000	PSL 618006	PSL 618012
Single 10 cm	PSL 618002	PSL 618008	PSL 618014
Single 20 cm	PSL 618004	PSL 618010	PSL 618016
Double 6 cm	PSL 618001	PSL 618007	PSL 618013
Double 10 cm	PSL 618003	PSL 618009	PSL 618015
Double 20 cm	PSL 618005	PSL 618001	PSL 618017

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 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; (p/n: WAT034544).
4. Inject 2 to 5 µL of this solution

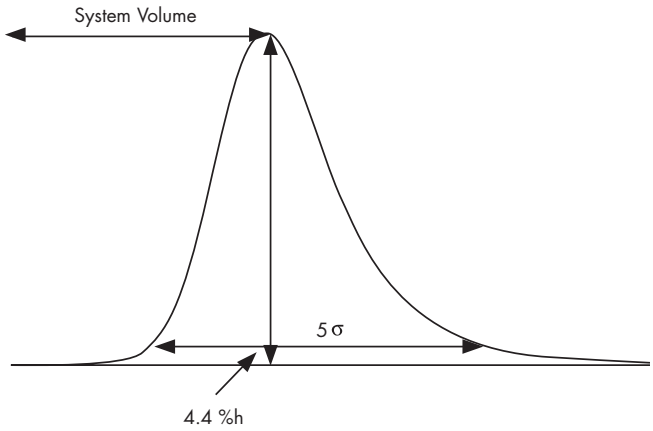
5. Measure the peak width at 4.4% of peak height (5-sigma method):

5-sigma Bandspreading (µL) = Peak Width (min) x

Flow Rate (mL/min) x (1000 µL/1 mL)

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

Figure 7. Determination of system bandspreading volume using 5-Sigma method.



In a typical HPLC system, the Bandspreading Volume should be no greater than 100 µL ± 30 µL (or Variance of 400 µL ± 36 µL).

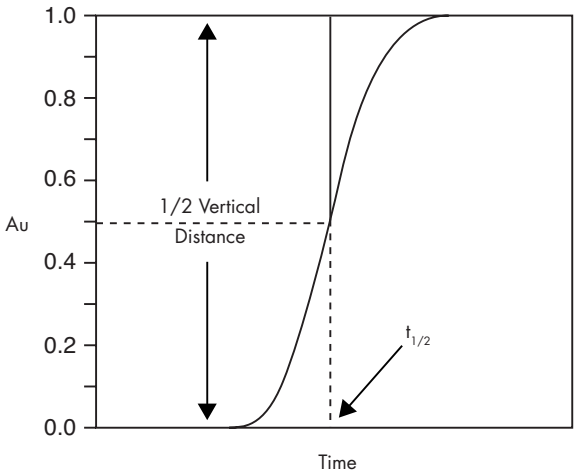
In a microbore (2.1 mm I.D.) system, the Bandspreading Volume should be no greater than 20 to 40 µL (or Variance no greater than 16 µL to 64 µL).

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).

Figure 8. Determination of gradient delay volume.



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.
6. Determine the dwell time by first locating the time at the midpoint of the formed gradient (t1/2) (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_g) (10 min/2 = 5 min in this example) from the gradient midpoint (t1/2) to obtain the dwell time (tD).
8. Convert the dwell time (tD) to the dwell volume (VD) by multiplying by the flow rate (F).

$$\text{Dwell Volume } V_D = (t_{1/2} — 1/2\ t_g) \times F$$

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 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 2.1 mm I.D. column 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

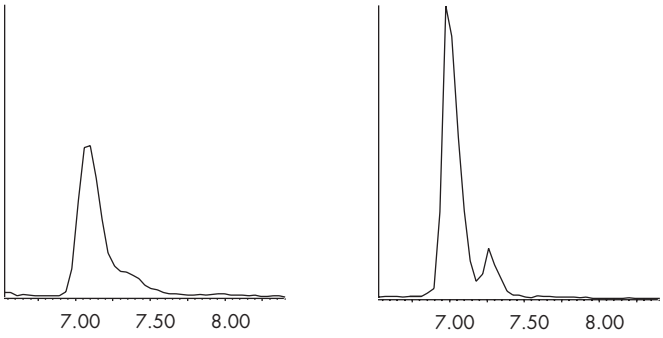
System with 70 µL bandspreading: 10,000 plates

System with 130 µL bandspreading: 8000 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.



Non-optimized LC-MS/MS System

Optimized System

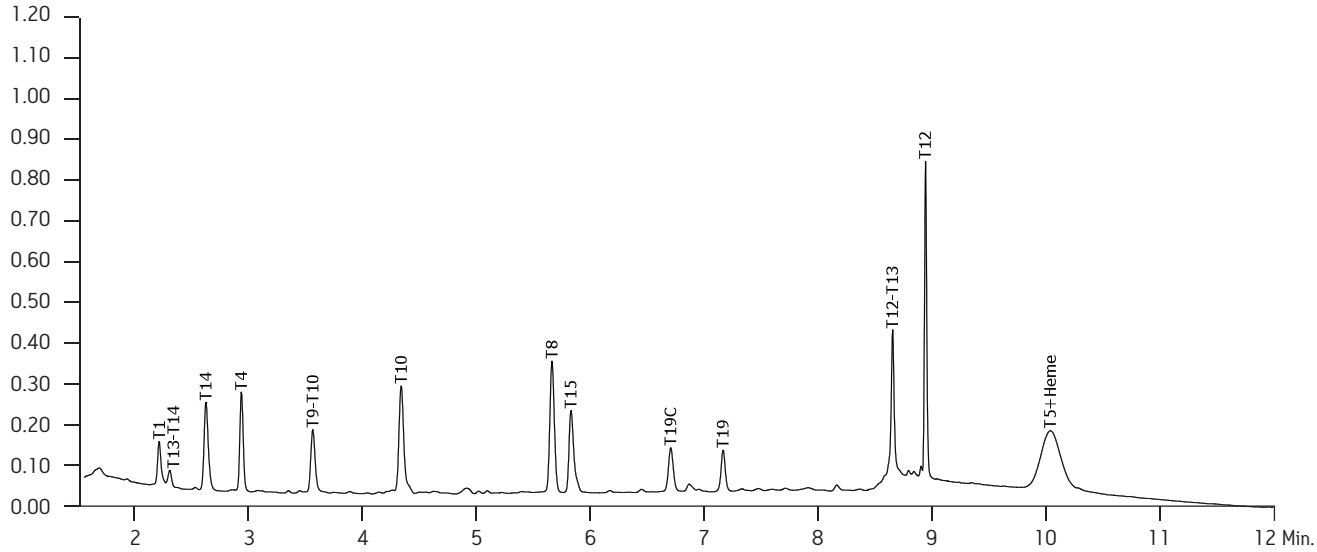
c. Non-Optimized vs. Optimized LC-MS/MS System: System Modification Recommendations

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 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.

Figure 10. Representative test chromatography and conditions for separation of a cytochrome c tryptic digest.



Chromatographic conditions

Column:	XSelect Peptide CSH C_{18}, 130Å, 3.5 µm, 2.1 mm x 50 mm (p/n: 186006950)
Sample:	Tryptic digest of bovine cytochrome *c* (p/n: 186006371)
	Reconstituted w/ 200 µL of MPA, 5.0 µL injection
Flow rate:	0.2 mL/min
Temperature:	40 °C
Mobile phase A:	0.1% formic acid in 100% water MPA
Mobile phase B:	0.085% formic acid in 75% acetonitrile MPB
Gradient:	1 to 21% B in 5 min; 21 to 31.4% B in 2.5 min; 31.4 to 95% B in 2.5 min; 95% B for 1.5 min; 1% B for 2.5 min
UV detection:	214 nm

Peak identification

T1	N-AcGDVEK
T13-T14	KYIPGTK
T14	YIPGTK
T4	IFVQK
T9-T10	KTGQAPGFSYTDANK
T10	TGQAPGFSYTDANK
T8	TGPNLHGLFGR
T15	MIFAGIK
T19C	EDLIAY
T19	EDLIAYLK
T12-T13	GITWGEETLMEYLENPKK
T12	GITWGEETLMEYLENPK
T5	CAQCHTVEK (heme attached)

Retention time (min)	**Result**
T19C	6.71

Retention time difference (min)	**Result**
T4 and T14	0.312
T10 and T9-T10	0.780
T19 and T19C	0.461
T12 and T12-T13	0.290

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