XBridge and XBridge Premier Oligonucleotide C_{18} Columns

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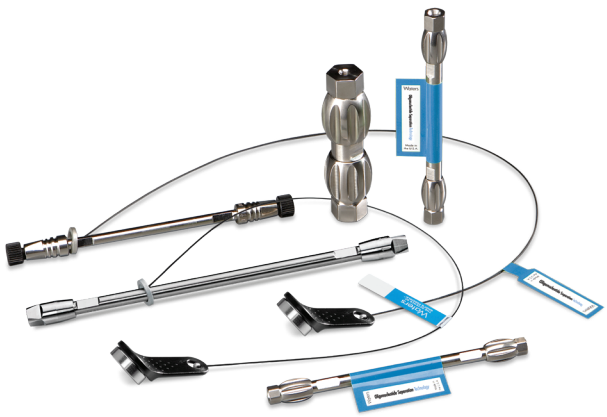
I. INTRODUCTION

Thank you for choosing a Waters™ XBridge™ and/or XBridge Premier Oligonucleotide C_{18} Column designed for lab-scale purifications and analyses of synthetic oligonucleotides. The separation of detritylated oligonucleotides on Waters second generation of hybrid-silica BEH Technology™ particles is based on the well established method on ion-pair, reversed-phase chromatography. The availability of XBridge and XBridge Premier Oligonucleotide C_{18} material contained in several column configurations provides flexibility to meet different application needs.

Your XBridge and XBridge Premier Oligonucleotide C_{18} packing material is manufactured in a cGMP, ISO 9002 certified plant using ultra pure reagents. Each batch of XBridge and XBridge Premier Oligonucleotide C_{18} material is chromatographically tested with acidic, basic, and neutral analytes and the results are held to narrow specification ranges to assure excellent, reproducible performance. In addition, every column is individually tested and the associated Performance Test Chromatogram and Certificate of Acceptance is included with your purchased column.

In addition, the XBridge Premier Oligonucleotide C_{18} Columns utilize MaxPeak™ High Performance Surfaces, an innovative technology designed to increase analyte recovery, sensitivity, and reproducibility by minimizing analyte/surface interactions that can lead to sample losses.



II. GETTING STARTED

Each XBridge and XBridge Premier Oligonucleotide C_{18} Column is shipped with a Certificate of Analysis and a Performance Test Chromatogram document. The Certificate of Analysis is specific to the batch of packing material contained in the XBridge and XBridge Premier Oligonucleotide C_{18} 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 such information as 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.

The XBridge Oligonucleotide C_{18} Column is equipped with Waters-style endfittings that require a 0.130 inch ferrule depth. The XBridge Premier Oligonucleotide C_{18} Column is equipped with a Parker-style endfitting.

a. Connecting the Column

1. Purge the pumping system of any buffer-containing mobile phases and connect the inlet end of the column to the injector outlet.
2. The XBridge and XBridge Premier Oligonucleotide C_{18} Column should be connected to HPLC with 1/16 inch tubing (stainless steel or PEEK™). An arrow on the column identification label indicates the correct direction for solvent flow. Make sure that XBridge and XBridge Premier Oligonucleotide C_{18} Column connection is made with the correct ferrule seating adjustment. Figure 1 shows the differences between Waters and Parker-style of ferules and endfittings. Note the differences in endfitting style and length of tubing protruding from the ferrule end.

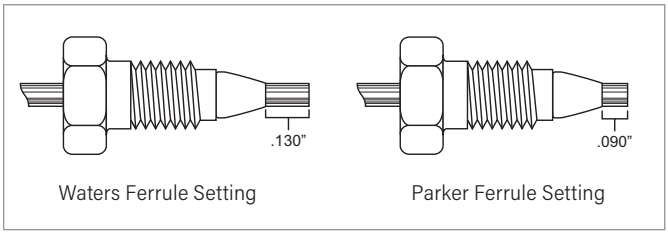


Figure 1. Waters and Parker ferrule types.

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 setting. 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. If a non-Waters-style column is presently being used, 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 XBridge Oligonucleotide C_{18} Column.

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

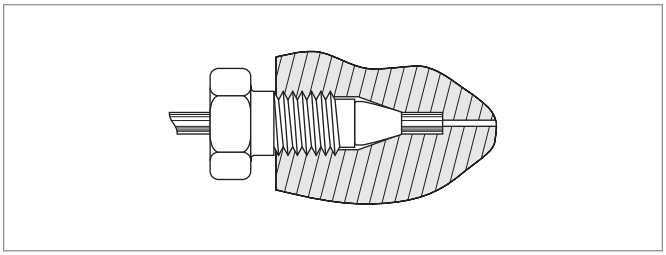


Figure 2. Proper tubing/column connection.

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.

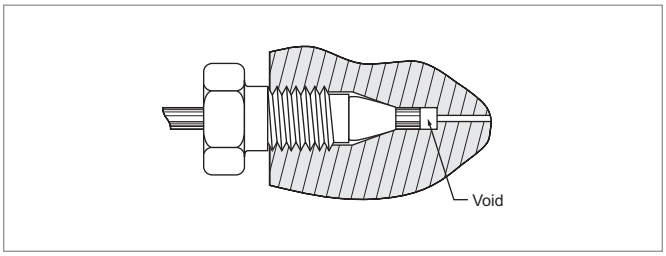


Figure 3. Parker Ferrule in a Waters-style endfitting.

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.

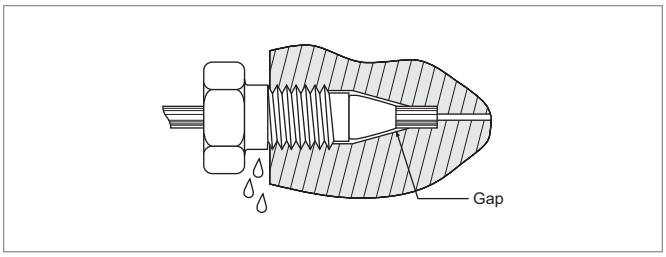


Figure 4. Waters ferrule in a Parker-style endfitting.

The best way to fix the problem is to cut the tubing, replace the ferrule, and make a new connection.

1. Alternatively, replace the conventional compression screw fitting with an all-in-one PEEK fitting (Waters p/n: 186008714) 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).

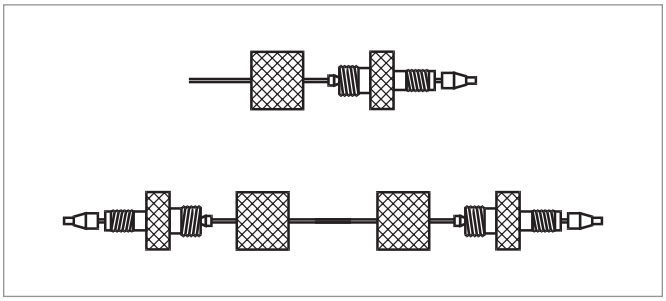


Figure 5. Single and double SLIPFREE connectors.

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 1. 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

2. Flush column with 100% organic mobile phase (acetonitrile with TEAA or methanol for TEA-HFIP ion-pairing method) by setting the pump flow rate to 0.1 mL/min and increase the flow rate to 0.5 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 3.
5. Once a steady backpressure and baseline at 260 nm have been achieved, proceed to the next section.

b. Column Equilibration

XBridge and XBridge Premier Oligonucleotide C_{18} 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 for the oligonucleotide separation.

Note: When mobile phase additives are present in low concentrations (e.g., TEAA or TEA-HFIP ion-pairing reagents), 100 to 200 column volumes may be required for complete XBridge and XBridge Premier Oligonucleotide C_{18} Column equilibration.

Table 2. Empty Column Volumes in mL (multiply by 10 for flush solvent volumes)

Column Length	Internal	Diameter
mm	2.1 mm	4.6
50	0.2	1
100	0.4	2
150	0.5	2.5

c. Initial Column Efficiency Determination (see Section VI below)

It is recommended that an efficiency test be performed before using your XBridge and XBridge Premier Oligonucleotide C_{18} Column for synthetic oligonucleotide applications. Waters recommends using the same solutes and conditions as found in the included Performance Test Chromatogram.

The efficiency test can be repeated to track the column performance over time. Several chromatographic data acquisition software packages (e.g., Waters Empower™ 2 Chromatography Data Software) can determine the number of theoretical plates (N) from the test chromatogram. Slight variations may be observed on two different HPLC systems due to the quality of the connections, operating environment, system electronics, reagent quality, column conditions, and operator technique.

Column performance can be tested with the MassPREP Oligonucleotide Standard (p/n: 186004135), a quality controlled synthetic oligonucletoide sample consisting of 15, 20, 25, 30, and 35 mer deoxythymidine. Approximately 0.1 nmol of each oligonucleotide was injected on the XBridge and XBridge Premier Oligonucleotide C_{18} Column. Refer to part number 715001677 for more information on sample preparation for the MassPREP Oligonucleotide Standard. Smaller peaks eluting prior to the main peaks are failure, by-product sequences from the synthesis.

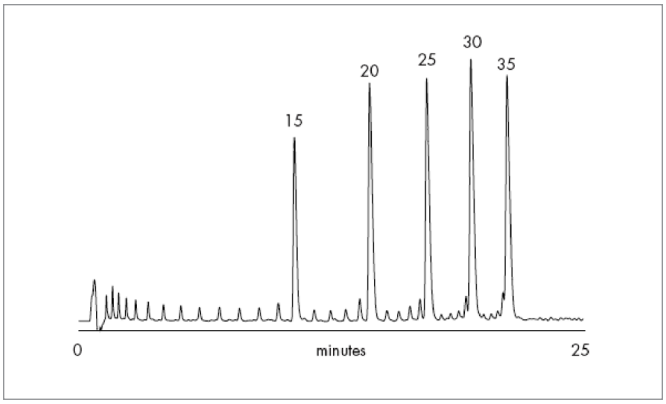


Figure 6. Separation using the MassPREP Oligonucleotide Standard on XBridge Oligonucleotide C_{18} Column.

d. Column QR Code

The quick reference (QR) code that is located on the column label provides column-specific information (i.e., the part and serial numbers that are unique identifiers for the column), and its encoding follows a widely adopted industry-standard.

1. Scan QR code using any device that is capable of scanning QR codes (i.e., for smart phones and tablets, use the built-in camera app).
2. Be directed to the column's information hub on waters.com.
3. Access technical and scientific information for the column (i.e., certificate of analysis, application notes).

HPLC system:	Waters Alliance 2796, PDA Detector with micro UV cell
Sample injected:	Approximately 0.1 nmol of MassPREP Oligonucleotide Standard (p/n: 186004135) diluted in 0.1 M TEAA
Column:	XBridge Oligonucleotide C_{18}, 2.5 µm, 2.1 x 50 mm
Mobile phases:	A: 0.1 M TEAA B: Acetonitrile/0.1 M TEAA, 20/80, v/v
Flow rate:	0.2 mL/min
Column temp.:	60 °C
Gradient delay:	0.45 mL
Gradient:	40 to 59.5% B in 26 minutes (8–11.9% acetonitrile, 0.15% acetonitrile per minute)
Detection:	260 nm, 5 scans per second

III. COLUMN USE

To ensure the continued high performance of your XBridge and XBridge Premier Oligonucleotide C_{18} Columns, follow these guidelines:

a. Sample Preparation

1. Dissolve the detritylated synthetic oligonucleotide sample in mobile phase A (e.g., 0.1 M TEAA). For example, a 0.05–0.2 µmole scale synthesis can be prepared in 0.1 mL of 0.1 M TEAA. Proportionately larger or smaller volumes of 0.1 M TEAA are required when dissolving samples from different scale syntheses. Due to the nature of gradient separations, relatively large volumes of sample (in low organic strength eluent) can be injected and concentrated onto the head of the column before beginning the gradient elution program.
2. Samples must be completely in solution and free of particulates before injection onto the column. Remove all particles from the sample (Controlled Pore Glass Synthesis Support, etc.), which may block the inlet column frit, increase the operating pressure, and shorten the column life time. Sample contamination with high concentration of salts and/or detergents may also interfere with analysis.

3. To remove particulates the sample may be filtered with a 0.2 µm membrane. Be sure that the selected membrane is compatible and does not dissolve with the selected mobile phase diluent. Contact the membrane manufacturer with solvent compatibility questions. An alternative method of particulate removal involves centrifugation for 20 minutes at 8000 rpm, followed by the transfer of the supernatant liquid to an appropriate vial.

b. Recommended Mobile Phases

The most common ion-pair mobile phase for synthetic oligonucleotide separations is based on of triethylammonium acetate (TEAA). This mobile phase can be prepared by titrating glacial acetic acid aqueous solution with triethylamine (TEA).

Note: To maximize column life, it is ESSENTIAL that all prepared oligonucleotide mobile phases be filtered through a solvent compatible, 0.45 µm membrane and contained in bottles that are clean and particulate free.

TEAA

1 L of 0.1 M TEAA may be prepared as follows:

1. Perform work in a hood.
2. Add 5.6 mL of glacial acetic acid into 950 mL of water and mix well.
3. Slowly add 13.86 mL of TEA.
4. The pH should be adjusted to pH 7.0 +/- 0.5 by careful addition of acetic acid.
5. Adjust final volume to 1 L with water.

Alternatively, premixed TEAA can be used [e.g., Sigma 1 M TEAA (part no. 90357)]. Mix 100 mL with 900 mL of water to prepare 1 L of 0.1 M TEAA mobile phase.

Alternative ion-pairing reagents are recommended for improved separation of phosphorothioates or when performing LC-MS analyses. An ion-pairing mobile phase based on Triethylamine (TEA) and Hexafluoroisopropanol (HFIP) as the buffering acid produces an efficient eluent system for improved separations involving these application types.

As indicated below, two ion-pairing systems are useful. For routine detritylated oligonucleotide applications, aqueous buffer consisting of 8.6 mM TEA and 100 mM HFIP is effective. For applications such as those involving the separation of G-rich oligonucleotides, it is advisable to use aqueous buffer consisting of 15 mM TEA and 400 mM HFIP (pH approximately 7.9).

TEA-HFIP System 1

1 L of 8.6 mM TEA/100 mM HFIP is prepared as follows:

1. Perform work in a hood.
2. Add 10.4 mL of HIFP (16.8 g) into 988.4 g of water and mix well.
3. Slowly add 1.2 mL of TEA.
4. The pH is approximately 8.3 +/- 0.1.

TEA-HFIP System 2

1 L of 15 mM TEA/400 mM HFIP is prepared as follows:

1. Perform work in a hood.
2. Add 41.56 mL (67.17 g) of HFIP into 956.36 g of water and mix well.
3. Slowly add 2.08 mL (1.52 g) of TEA.
4. The pH is approximately 7.9 +/- 0.1.

c. Recommended Injector Wash Solvents

Between analyses, the HPLC System Injector seals should be washed. A 90% water/10% acetonitrile injector wash solvent is recommended.

d. pH Range

The recommended operating pH range for XBridge and XBridge Premier Oligonucleotide C_{18} Columns is 1 to 12.

e. Pressure

XBridge and XBridge Premier Oligonucleotide C_{18} Columns can tolerate pressures of up to 6000 psi (400 bar or 40 Mpa).

f. Temperature

Temperatures between 20 °C–90 °C are recommended for operating XBridge and XBridge Premier Oligonucleotide C_{18} Columns in order to enhance selectivity, lower solvent viscosity and increase mass transfer rates.

Note: Operating at elevated pH, temperature, and/or pressure may potentially result in shortened column life.

g. Flow Rate

The recommended flow rate for oligonucleotide separations performed on a 2.1 x 50 mm XBridge and XBridge Premier Oligonucleotide C_{18} Column is 0.2 mL/minute. 1.0 mL/min is the recommended flow when using a 4.6 x 50 mm column.

IV. COLUMN CLEANING, REGENERATING 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 approximately 20 column volumes of filtered, neat organic solvent (e.g., acetonitrile with the TEAA method of methanol with the TEA/HFIP protocol) is usually sufficient to remove the contaminant. If the neat organic solvent flushing procedure does not solve the problem, purge the column with 20 column volumes of oligonucleotide mobile phase A followed by 20 column volumes of either 7 M guanidine hydrochloride or 7 M urea. Be sure to flush column with an additional 20 column volumes of filtered, LC grade Waters prior to reuse oligonucleotide mobile phases. If the column performance is poor after regenerating and cleaning, call your local Waters office for additional support.

b. Storage

For periods longer than four days at room temperature, store the column in 100% acetonitrile. Immediately after use at elevated temperature and/or pH, store column in 100% acetonitrile for the best column lifetime. Do not store column in highly aqueous (<20% organic) mobile phase, as this may promote bacterial growth. Completely seal column to avoid evaporation and drying out of the packed bed.

V. ADDITIONAL INFORMATION

a. Bandspreading Minimization

Detritylated synthetic oligonucleotide separations are almost exclusively performed using gradient elution techniques. As such, the effect of pre-column sample band broadening can be minimized by allowing the sample to bind to the column before beginning the actual separation gradient. However, proper connection from the XBridge Oligonucleotide C_{18} Column outlet to the detector is critical in order to minimize the deleterious effect of post-column sample bandspreading. Use of appropriate internal diameter tubing (e.g., 0.005 inch PEEK tubing for UV detector applications) is recommended for reasons illustrated in Figure 7.

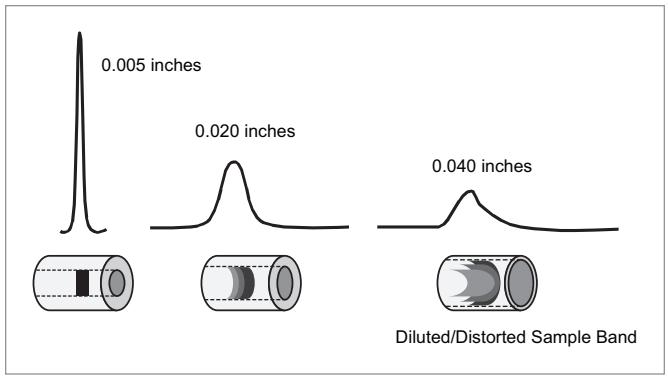


Figure 7. Effect of connecting tubing on system.

b. Measuring System Bandspreading Volume and System Variance

1. Disconnect column from system and replace with a zero dead volume union.
2. Set flow rate to 0.5 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

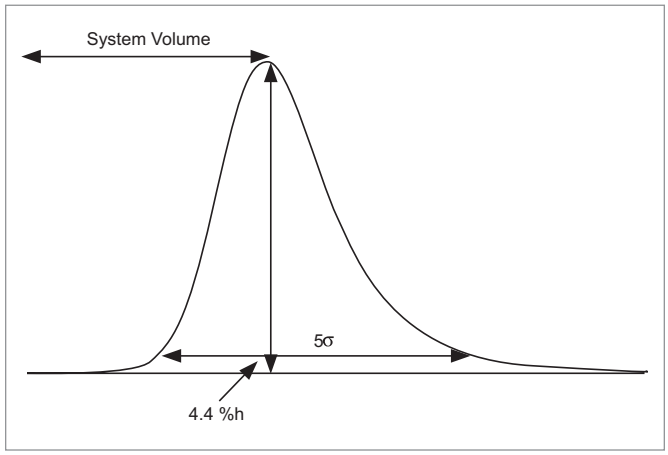


Figure 8. Determination of system band spreading volume using 5-Sigma Method.

c. Impact of Bandspreading Volume on 2.1 mm I.D. Column Performance

Use of a 2.1 mm I.D. XBridge and XBridge Premier Oligonucleotide C_{18} Column may require modification to the HPLC system tubing in order to eliminate excessive system bandspreading volume. Without proper system modification, excessive system bandspreading volume will cause peak broadening and will have a detrimental impact on peak resolution. For example, an HPLC System with 70 µL bandspreading might yield 10,000 plates while the same column tested on a LC System with 130 µL bandspreading would yield only 8000 plates.

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 ferrule depths and minimizing tubing inner diameters and lengths. An example is given in Figure 9 where system optimization resulted in a doubling of component sensitivity and resolution in an LC-MS/MS system.

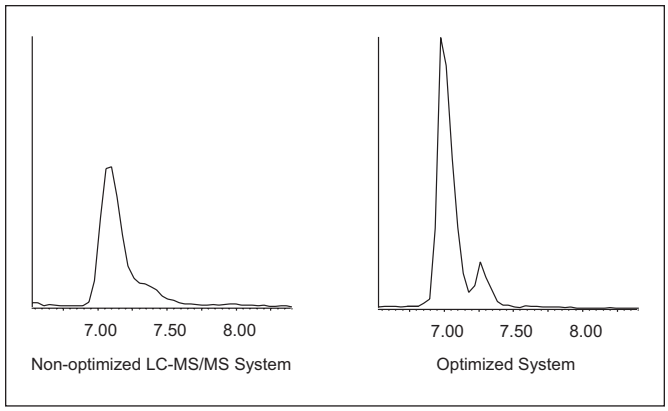


Figure 9. Non-optimized vs. optimized LC-MS/MS system.

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