SYSTEM OPTIMIZATION MANUAL

Get the Most out of your Kinetex® Core-Shell Column

Kinetex core-shell media can generate UHPLC-type performance benefits on any HPLC system.

The key to achieving ultra-high performance using Kinetex core-shell columns on an existing legacy HPLC may include optimizing:

All sections may be important for you to achieve maximum benefits from the core-shell media.

1. The HPLC Method

Why This is Important

The following method-related technique improvements will improve performance for all column separations. When using Kinetex® core-shell columns, the following suggestions will dramatically improve performance.

- HPLC methods should first work towards focusing the sample on the head of the column, and then minimizing sample dispersion through the column.
- Incorporating simple injector programming in the method can minimize extra-column volume to improve peak shape and efficiency, especially for fast LC separations.

Trouble: All Peaks are Broad

1. Check: Sample Volume

Errors in sample loading may negate the performance gain the Kinetex core-shell particle can achieve. If you inject the sample in the same organic composition as the mobile phase under isocratic conditions, the initial bandwidth on column will be directly proportional to the injection volume. As such, a large volume sample will load as a broad band on the column (low efficiency).

SOLUTION ▶ Pre-concentrate your sample. To reduce the efficiency loss of large volume injections, it is possible to pre-concentrate the sample on the head of the column by using a weaker diluent than the mobile phase.

2. Check: Sample Diluent

If the organic strength of the diluent is greater than the HPLC mobile phase, then the sample will load as a diffuse band on the head of the HPLC column. This may occur in either isocratic or gradient methods and can negate much of the performance advantages that the Kinetex column delivers.

- Gradient applications where the sample is injected in stronger diluents result in compound surfing effects, which will cause peaks to broaden and split.
- Isocratic methods where the diluent is stronger than the mobile phase will result in excessive sample dispersion.

SOLUTION ▶ Inject a weak solvent. It is best to use a diluent either weaker or equal to the organic strength of the mobile phase so that the sample focuses on the head of the column, resulting in sharper peaks (i.e., higher efficiency).

Trouble: All Peaks are Broad (continued)

3. Check: Injector Program

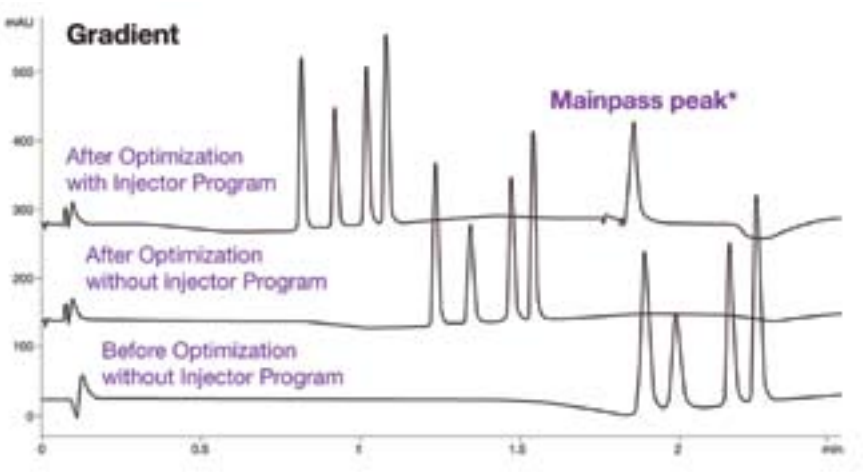
Extra-column volume before the column will cause dispersion of the sample on the way to the column; the injector loop is a major source of extra-column volume.

SOLUTION ▶ Bypass the loop. It is possible to bypass the loop with an injector program during the analysis method (after the sample has left the injector loop).
Table 1 and **Figure 1** show an example where the injection loop is bypassed after injection to reduce system dwell volume.

Table 1:

Steps	Commands	Comments
1	DRAW	Draw volume of sample (injection volume) from vial
2	INJECT	Introduce sample into flow path
3	WAIT 0.06 min (calculated wait time)	Flush sample loop after injection (wait time = 6x (injection volume + 5 µL) / flow rate)
4	VALVE bypass	Direct flow from pump to column, bypassing injection valve to exclude delay volume (~200 – 500 µL from auto-injector path)
5	WAIT 1.5 min	The period of VALVE bypass time (Wait time = Run time - 1 min)
6	VALVE mainpass	Switch valve from bypass to injection position

Figure 1: Dwell Volume Reduction Using an Injector Program



* Mainpass peak is an injector artifact due to switching the injector valve back to include the injector loop in the flow (to flush injector in preparation for the next injection).

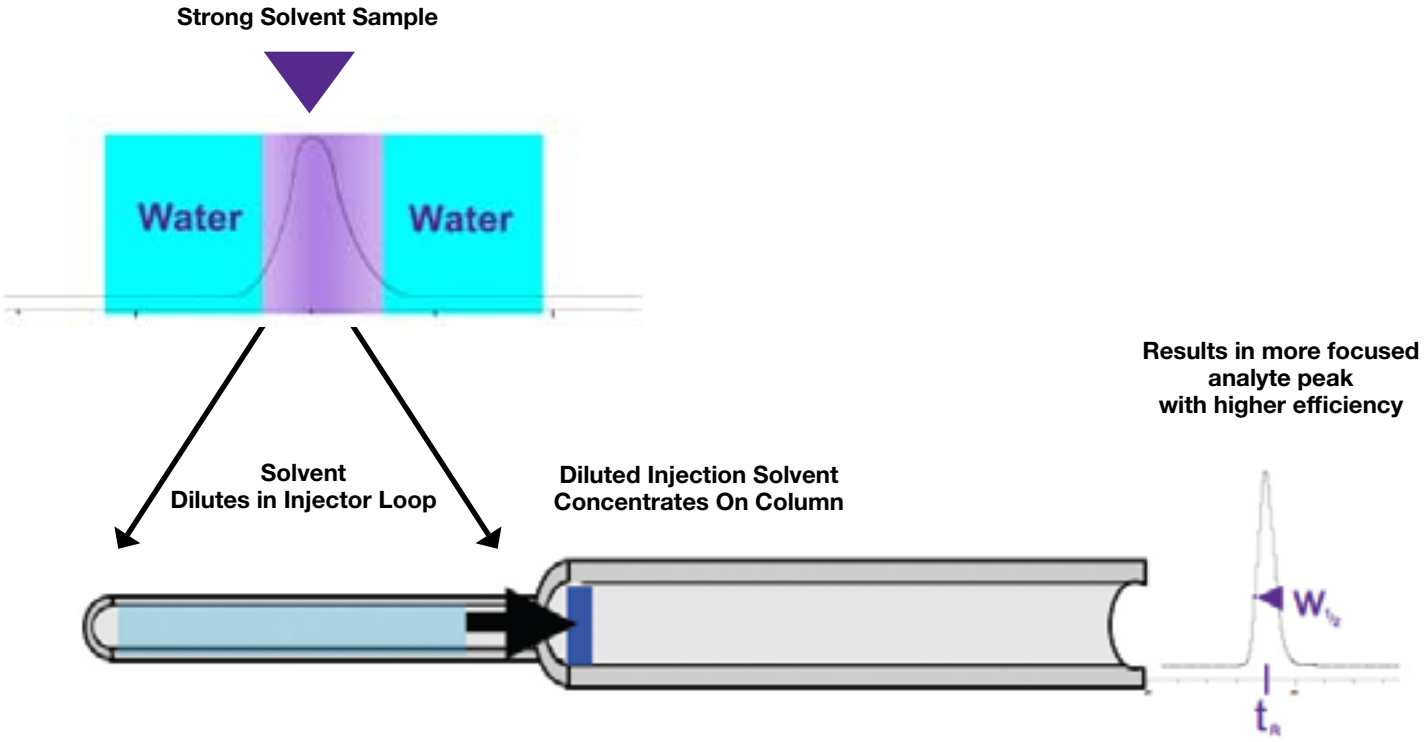
Trouble: The First Peak is Broad

Check: Polar Compound Focusing

In reversed phase HPLC, early eluting compounds typically display the most peak distortion. Under isocratic conditions, the peak volume for early eluters is comparable to the extra-column volume, while under gradient conditions there is little or no refocusing of weakly retained compounds at the column inlet.

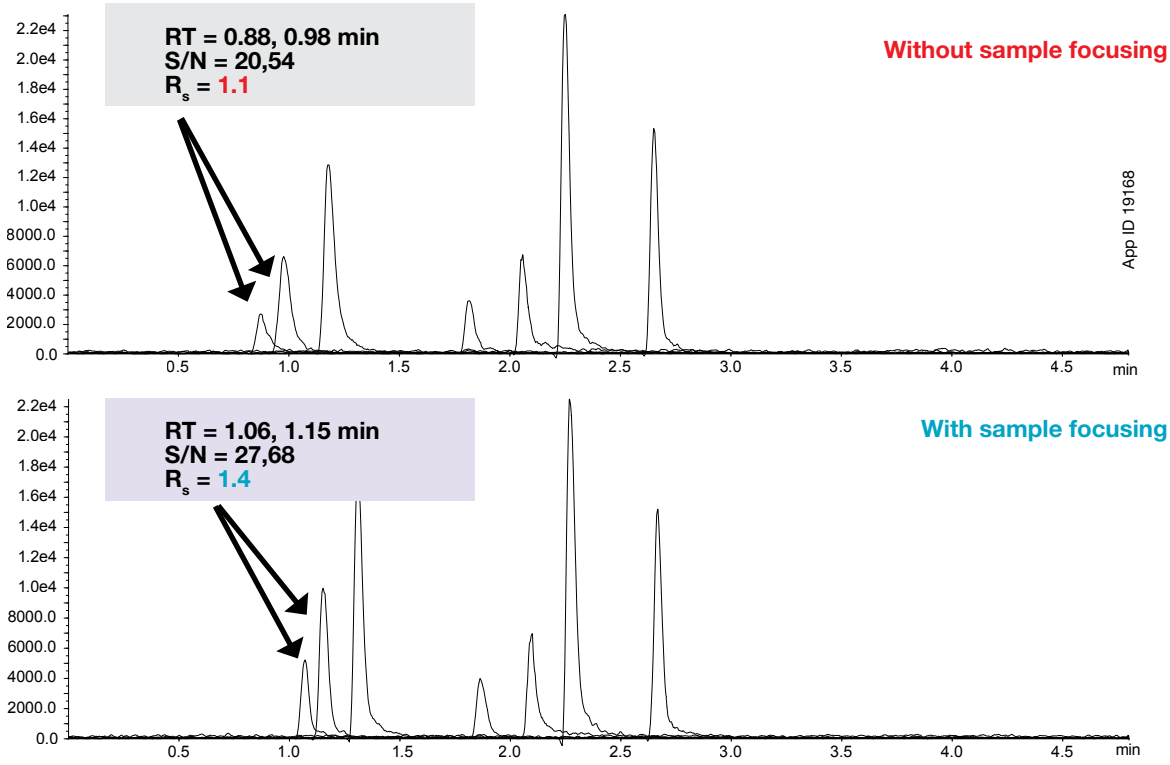
SOLUTION ▶ Focus your sample. Inject a volume of water, approximately 3x the sample injection volume, after the sample using an injector program. This will focus the sample on the head of the column and increase retention, resulting in narrower, more symmetrical peaks. The improvement in resolution is larger with small diameter columns since these provide the smallest peak volumes. An example is shown in **Figures 2** and **3**.

Figure 2: Injector Program For Sample Focusing



Trouble: The First Peak is Broad (continued)

Figure 3: Using Sample Focusing to Improve Early Eluting Peak Shape



HPLC Method Optimization: Pros and Cons

HPLC Method Optimization: Pros and Cons

Benefits

- Reduced run time
- Improved peak efficiency
- Improved resolution of closely eluting peaks
- Improved peak shape of early eluting analytes

Drawbacks

- Increased equilibration time (injector program)
- Limited sample injection volumes
- Possible baseline disruptions in chromatogram (injector program)

2. HPLC System Volume

Why This is Important

The HPLC systems that make up most of the instrument base today are rugged, high performance capable systems optimized by the manufacturer for use with very high volume, fully porous 5 µm media in standard analytical (250 x 4.6 mm) column dimensions. Such a design may significantly mask the separation power of Kinetex® core-shell columns.

To realize the maximum performance of the Kinetex core-shell, the link between extra-column system volume and performance should be considered.

- The fraction of time that an analyte spends inside the HPLC column during a run is productive: analytes are being separated with high efficiency.
- The fraction of time that an analyte spends outside of the HPLC column during a run is unproductive: analytes diffuse within connecting tubing, lowering efficiency.
- Lower relative extra-column volume will result in higher overall system performance.
    - Sample dispersion occurs before the column
    - Peak dispersion occurs after the column

? Question: Where should I look to minimize sample dispersion?

1. Check: Volume Before the Column

The part of an HPLC system before the column is typically the most overlooked area to improve overall performance since its effect on overall column performance is less than post-column volume changes. There are many opportunities to reduce pre-column volume and improve system performance.

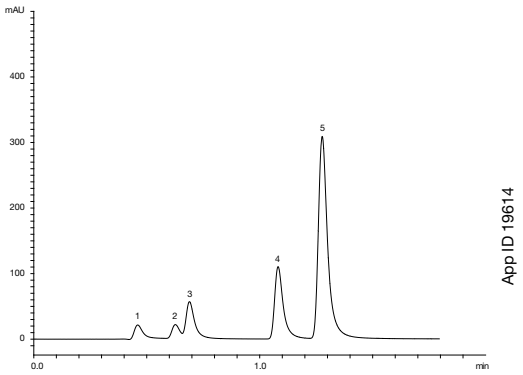
- **Needle Seat.** On many HPLC systems, the injector needle and needle seat are part of the injection loop and represent significant amounts of dead volume.
    - **For both integrated needle and needle seat systems**, the standard needle seat can be easily replaced with a low volume needle seat. The standard needle seat capillary on the Agilent® 1100 (0.17 x 100 mm; green) has a volume of 2.5 µL.

SOLUTION ▶ Use lower volume needle seat. A lower volume needle seat capillary (0.12 x 100 mm; red) reduces the volume by almost one-half to 1.3 µL. **Figure 1** shows an example after an Agilent 1100 has had the needle seat capillary replaced.

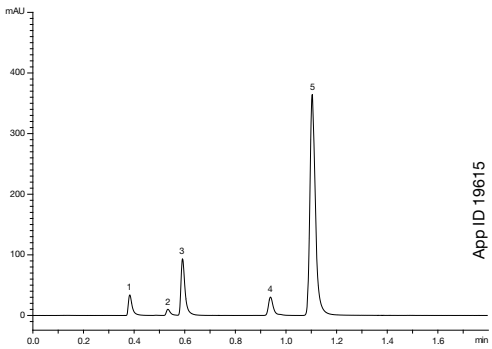
2. HPLC System Volume

Figure 1: Chromatography Improvement by System Optimization

Kinetex® 100 x 2.1 mm Un-optimized HPLC System



Kinetex 100 x 2.1 mm Optimized HPLC System



- **Fixed Volume Loop**. A fixed volume loop is used as part of the injector and oversized sample loops can contribute greatly to extra-column volume of a system.

  SOLUTION ▶ Replace with lower volume loop. In all cases, the injection loop volume can be reduced to volumes more appropriate for a particular application. In some cases where very large diluent volumes are being used, a trap column can be investigated to minimize injection loop volumes.

- **Solvent Heat Exchangers**. In the case of heat exchangers, tubing volume is being added to change the temperature of the mobile phase to more closely match the temperature of the column.

  SOLUTION ▶ Temperature control of solvent reservoir. If mobile phase absolutely must be heated (or chilled), then one should consider controlling the temperature of the solvent reservoir instead and avoid using heat exchangers.

- **Column Switching Valves**. Column switching valves are a great method development tool that adds significant volume to the system.

  SOLUTION ▶ Bypass the switching valve. Alternatively, a lower volume column switching valve can be used.

- **Fittings and Unions**. These can add significant dead volume if fittings are improperly connected. Older tubing unions can also add dead volume to a system (**Figure 2**).

  SOLUTION ▶ Zero volume finger-tight fittings. Tubing unions should be zero dead volume (ZDV) unions. Finger-tight fittings ensure a better connection with parts from multiple vendors.

2. HPLC System Volume

Figure 2: PEEK® Sure-Lok™ Nuts and Zero Dead Volume Union

- **Tubing**. Most legacy HPLC systems are plumbed throughout the system with 0.010 in. ID tubing. The 0.010 in. ID tubing has a solvent volume of 1.3 µL per inch (0.51 µL per cm), adding a significant amount of dead volume to a HPLC system.

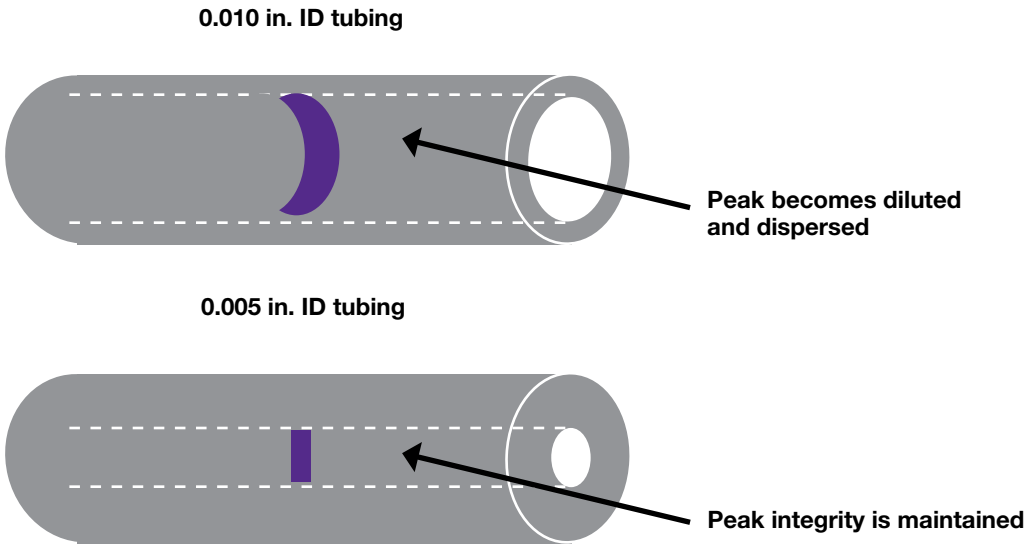
  SOLUTION ▶ Use 0.005 in. tubing. 0.005 in. tubing has 0.3 µL per inch (0.13 µL per cm) internal volume. This will provide an approximate extra-column volume reduction of 1 µL per inch (or 0.38 µL per cm) and should be plumbed from the solvent mixing "T" all the way to the HPLC column inlet. See **Table 1** and **Figure 3**.

  SOLUTION ▶ Proper Tubing Cuts. Rough cut tubing can potentially increase dead volume if such fittings compromise a smooth coupling. Precut tubing of fixed lengths can be purchased from numerous sources.

Table 1: Estimating Capillary Tubing Volume

ID (inches)	ID (mm)	µL/inch	µL/cm
0.010	0.254	1.287	0.51
0.007	0.178	0.633	0.25
0.005	0.127	0.322	0.13

Figure 3: Large ID Tubing Increases System Volume and Sample Dispersion



2. HPLC System Volume

Pre-Column System Optimization: Pros and Cons

Benefits

- Reduced run time
- Increased efficiency
- Better resolution of closely eluting peaks

Drawbacks

- Higher system backpressure
- Potential method reproducibility concerns (column heater and static mixer)
- Limited sample injection volumes

? Question: Where should I look to minimize peak dispersion?

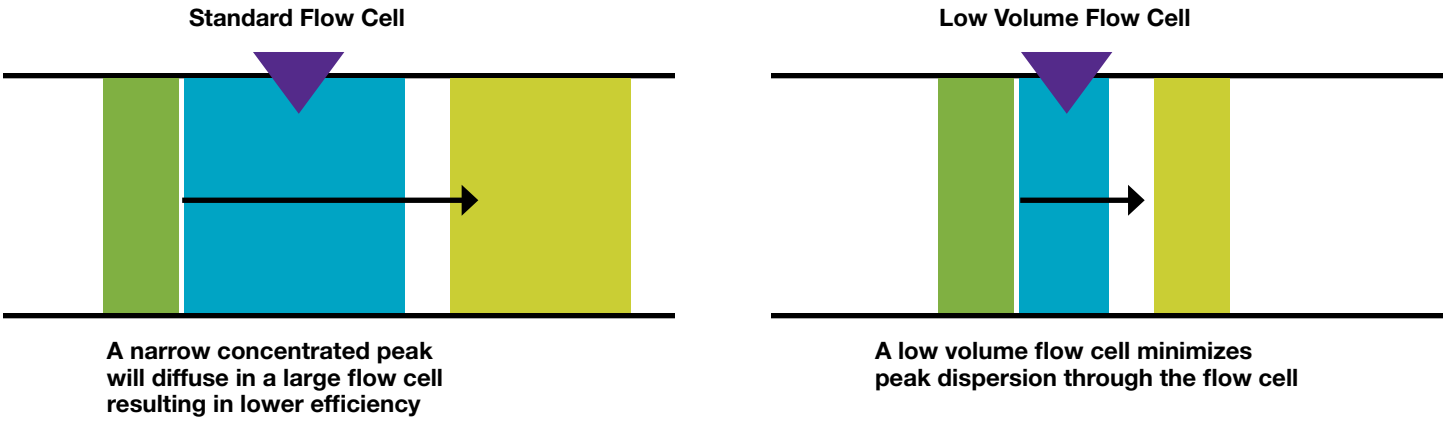
1. Check: Volume After the Column

Minimizing post-column volumes significantly decreases peak dispersion and has the greatest effect on the apparent performance of a column. The areas of post-column volume where system optimization can influence column performance will depend on the type of detection used.

- UV Detection. The UV flow cell is an important place where a minor change like using a lower volume flow cell can make a big difference in column performance. Larger flow cells tend to disperse narrow peaks and reduce the apparent efficiency of the column; this can also result in loss of resolution for closely eluting peaks.

  SOLUTION ▶ Install a lower volume flow cell. Moving to a lower volume flow cell (≤3 µL) can produce sharper peak shapes and better resolution for most closely eluting peaks (**Figure 4**).

Figure 4: Peak Dispersion Due to Flow Cell Incompatibilities



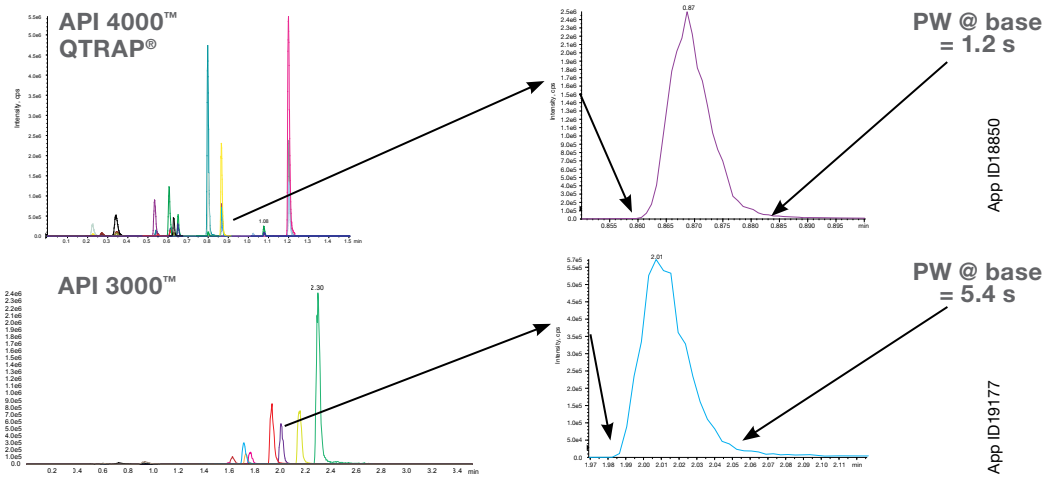
- **MS Interface**. Extra volume in the electrospray interface can reduce apparent performance similar to what is seen with a UV detector flow cell. You must also consider that when an MS detector is being used, the distance between the column outlet and the MS detector can be quite large.

  SOLUTION ▶ Use a lower volume ESI interface. Using capillary lines in the interface can reduce volume as can using a sheath-flow for low volume separations. **Figure 5** shows an example of the differences between the API 3000™ and API 4000™ interfaces; the API 4000™ shows a four-fold improvement in peak width due in part to a lower volume ESI interface.

2. HPLC System Volume

Figure 5: ESI Interface Volume Influences Peak Dispersion

Meperidine peak width comparison using two different MS sources



- **Fittings and Unions**. These can add significant dead volume if fittings are improperly cut and connected. Older tubing unions can also add dead volume to a system.

  SOLUTION ▶ Zero volume finger-tight fittings. Tubing unions should be zero dead volume (ZDV) unions. Finger-tight fittings ensure a better connection with parts from multiple vendors.

- **Tubing**. Most legacy HPLC systems are plumbed throughout the system with 0.010 in. ID tubing. The 0.010 in. ID tubing has a solvent volume of 1.3 µL per inch (0.51 µL per cm), adding a significant amount of dead volume to a HPLC system.

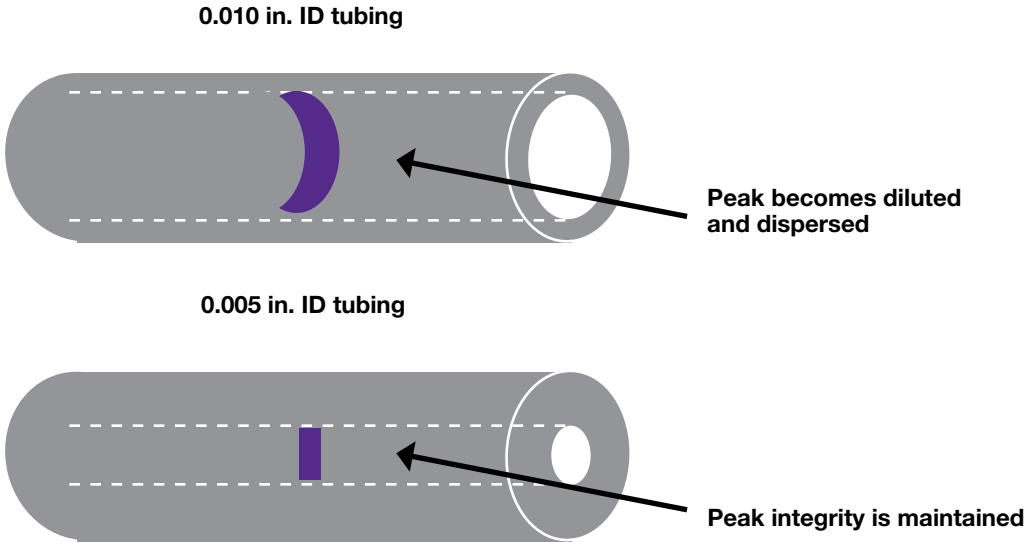
  SOLUTION ▶ Use 0.005 in. tubing. 0.005 in. tubing has 0.3 µL per inch (0.13 µL per cm) internal volume. This will provide an approximate extra-column volume reduction of 1 µL per inch (or 0.38 µL per cm) and should be plumbed from the HPLC column outlet to the inlet of the UV flow cell or electrospray interface (**Table 2** and **Figure 6**).

  SOLUTION ▶ Proper Tubing Cuts. Rough cut tubing can potentially increase dead volume if such fittings compromise a smooth coupling. Precut tubing of fixed lengths can be purchased from numerous sources.

Table 2: Estimating Capillary Tubing Volume

ID (inches)	ID (mm)	µL/inch	µL/cm
0.010	0.254	1.287	0.51
0.007	0.178	0.633	0.25
0.005	0.127	0.322	0.13

Figure 6: Large ID Tubing Post-Column Increases Peak Dispersion and Reduces Peak Efficiency



Post-Column System Optimization: Pros and Cons

Benefits

- Increased peak efficiency
- Improved resolution of closely eluting peaks

Drawbacks

- Potential reduction in sensitivity (flow cell)

3. Detector Settings

Why This is Important

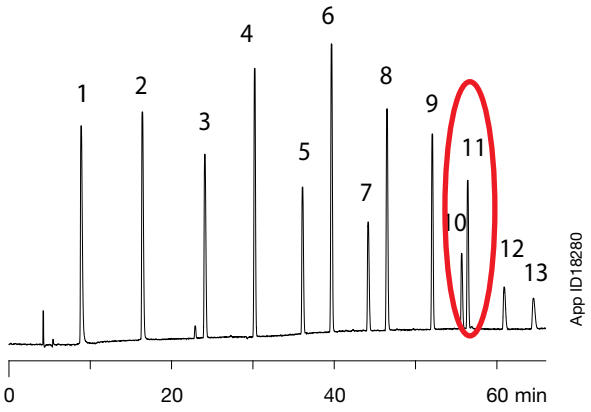
Most HPLC detectors have their data acquisition optimized for high volume peak widths typically seen with 5 µm fully porous media.

- The ultra high performance chromatography of Kinetex® core-shell columns produce narrow analyte peaks that are highly concentrated, elute quickly and are low volume (**Figure 1**). The detector sampling rate may require adjustment.
- On a variety of different systems this can be found as sampling rate, peak width, or detector time constant.

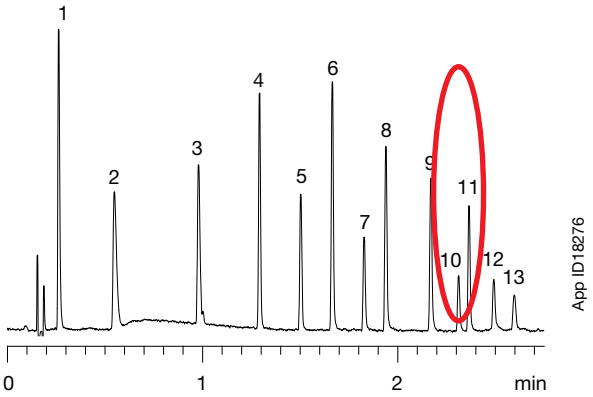
Figure 1: Kinetex fast, low volume peaks

Ketone mixture

R_s (10,11) = 3.11



R_s (10,11) = 3.12



Fully Porous 5 µm C18

- 250 x 4.6 mm
- 0.7 mL/min
- Agilent® 1100

60 min

Average Peak Width: 0.37 min (22 seconds)
Average Peak Volume: 300 µL

Kinetex 2.6 µm C18

- 50 x 4.6 mm
- 3.4 mL/min
- Agilent 1100

2.5 min

Average Peak Width: 0.043 min (2.5 seconds)
Average Peak Volume: 146 µL

3. Detector Settings

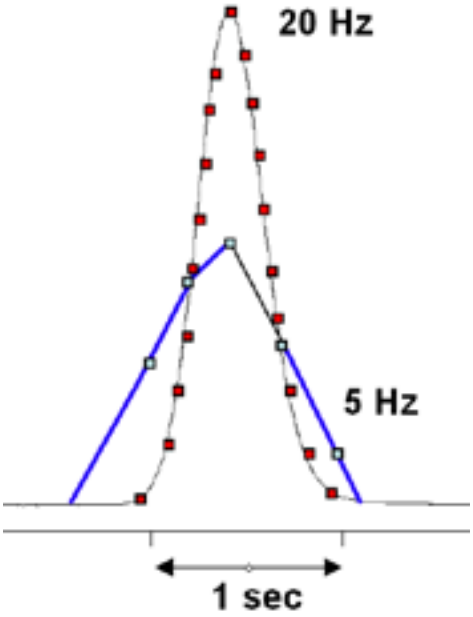
Trouble: Broad and Low Intensity Peaks

1. Check: UV Detector Scan Rate

Many chromatographers using fully porous 10, 5, and even 3 µm analytical columns are not in the habit of changing the detector scan rate.

SOLUTION ▶ Collect 20 points across the peak. For proper integration of a peak, one ideally needs at least 20 data points. Increasing the sampling rate to 20 Hz results in an accurate determination of 1 second wide peaks. An example of the limitations of low sampling rate is shown in **Figure 2**.

Figure 2: Detector Setting Influence on Peak Efficiency and Peak Shape



2. Check: UV Detector Time Constant

A slow detector time constant is normally set to filter out high frequency noise. Unfortunately, this noise filtering can also filter out the sharp peaks and high resolution that Kinetex® core-shell columns deliver.

SOLUTION ▶ Increase Detector time constant. **Figure 3** and **Table 1** show the efficiency improvement when the detector time constant is optimized on an Agilent® 1100 using ChemStation. **Figure 4** and **Figure 5** show where the settings for the detector sampling rate can be modified in the software for the Agilent® and Waters® instruments, respectively.

3. Detector Settings

Trouble: Broad and Low Intensity Peaks (continued)

2. Check: UV Detector Time Constant (continued)

Figure 3: Effect on Peak Efficiency by Changing Time Constant

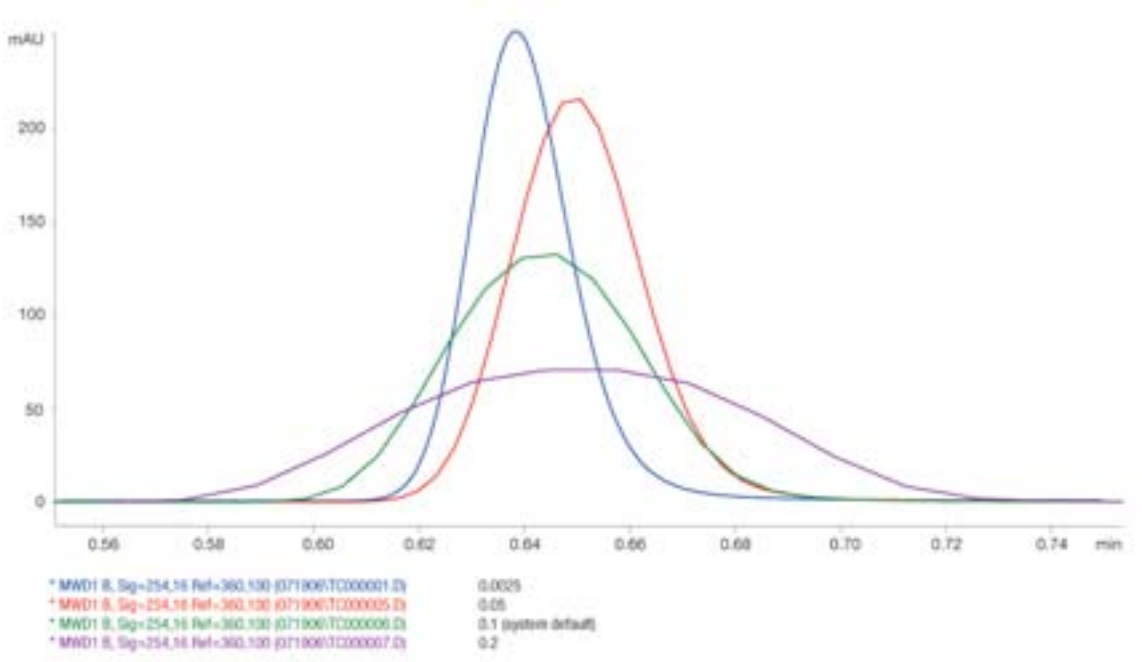


Table 1: Lists of Time Constants

Time Constant (min)	Efficiency (plates/column)
0.0025	***8073***
0.005	8027
0.01	7955
0.03	7598
0.05	***6612***
0.1*	***3978***
0.2	***1459***

*The system default for Agilent® HPLC instruments

Trouble: Broad and Low Intensity Peaks (continued)

2. Check: UV Detector Time Constant (Continued)

Figure 4: Agilent® System Detector Rate Settings

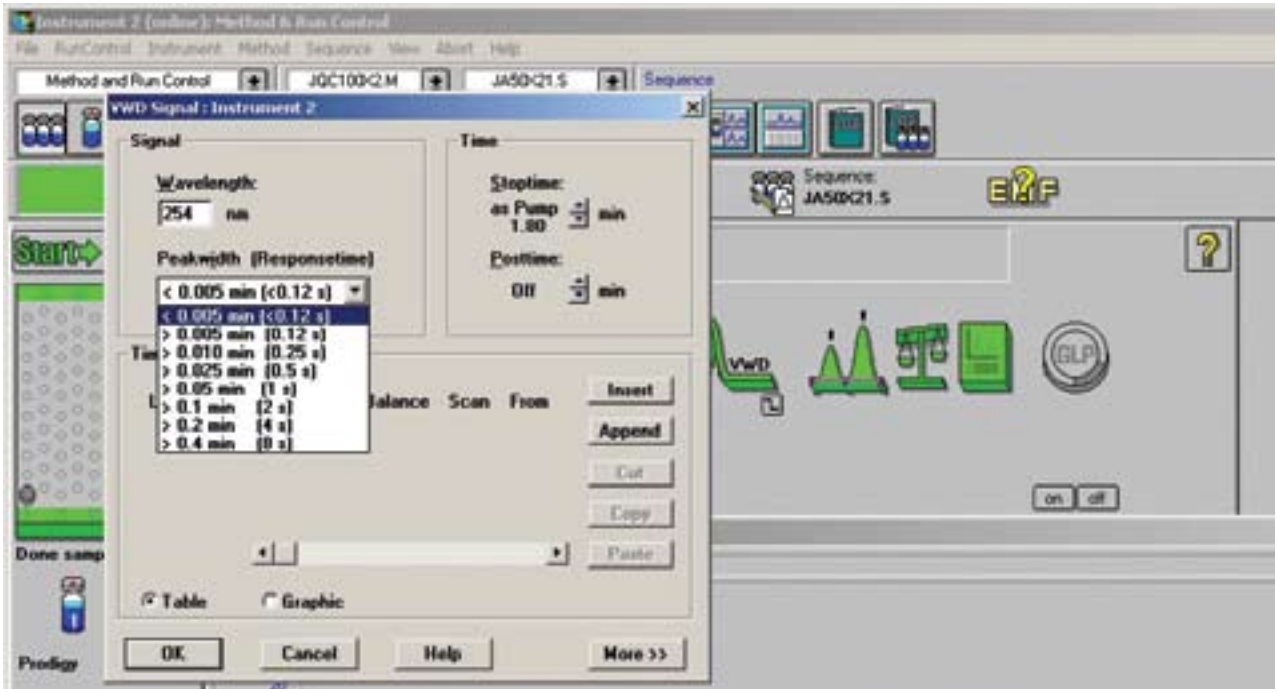
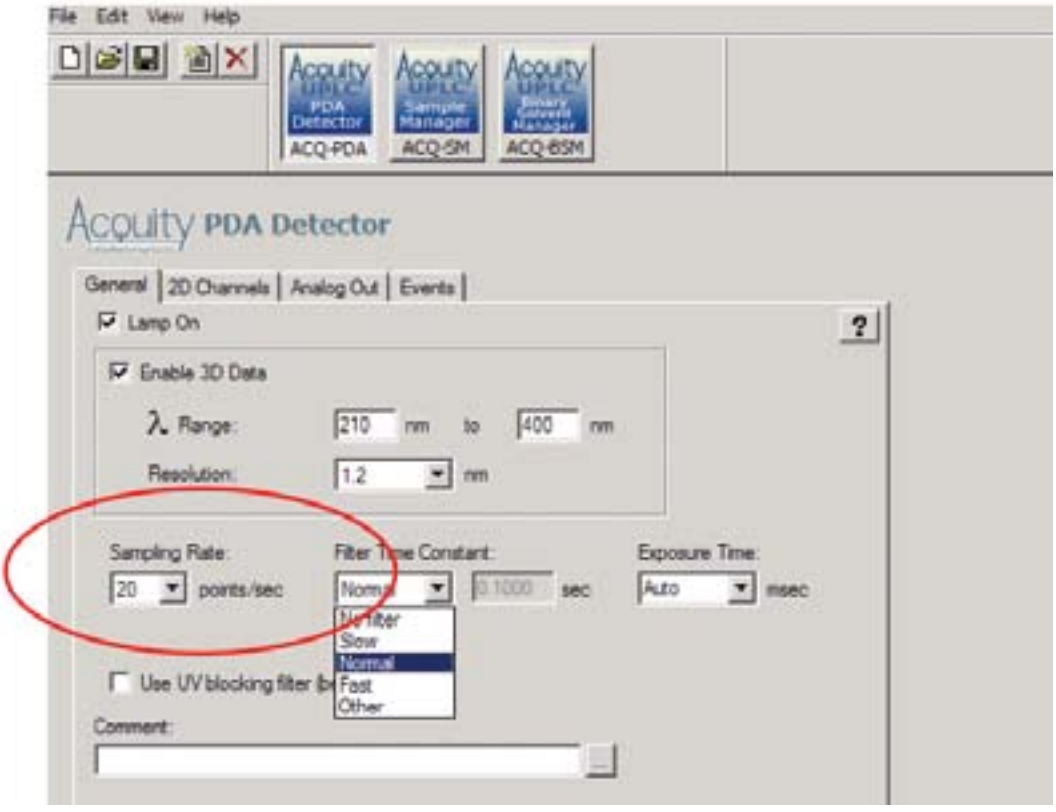


Figure 5: Waters® Empower Acquisition Rate Settings



3. Detector Settings

Trouble: Broad and Low Intensity Peaks (continued)

Changing UV Detection Rate: Pros and Cons

Benefits

- Increased efficiency of all analyte peaks
- Improved resolution of closely eluting peaks
- More accurate quantitation of peak height and area

Drawbacks

- Increased size of chromatography data file
- Increased baseline noise
- Poor quantitation of very low level peaks

3. Check: MS Sampling Rate

The total number of sample points recorded per second (pts/s). Sampling rate is dictated by the dwell time, settling time, and pause between mass ranges.

- Dwell time: the scan time required for one sample point. An MRM will be monitored for the period specified by the dwell time giving one sample point.
- Pause between mass ranges: the length (millisecond) of pause between scanning separate mass ranges.
- Settling time: the amount of time (millisecond) allowed for the system to pause at a m/z setting before data acquisition begins.
    - **Table 2** shows an example calculation for the sampling rate used in an application (5 MRM transitions monitored) (Phenomenex HPLC 2010 Poster)

Table 2: Relationship Between Dwell Time and Sampling Rate

Dwell time for each MRM transition (ms)	Settling Time (ms)	Pause between mass ranges (ms)	Total sampling time for 5 transitions (ms)	Sampling Rate (pts/s)
10	*0.1*	*3*	*(10x5) + (0.1) + (3x4) = 62.1 ms/pt*	*16*

3. Detector Settings

Trouble: Broad and Low Intensity Peaks (continued)

Fixed Mass Methods (MRM, SIM, etc.)

- Fixed mass methods optimization is often straightforward for maximizing performance of Kinetex® core-shell columns.

  SOLUTION ▶ Optimize the dwell time to obtain 20 or greater points per second for methods with only a few analytes (**Table 3**).

- For methods with 5 or more analytes being monitored, it is often difficult for the mass spectrometer to analyze all the analytes in parallel with a high sampling rate.

  SOLUTION ▶ Time programmed acquisition method. Such methods can maximize the data acquisition rate for the limited time during analyte elution and make high performance possible for methods with large numbers of analytes.

- Signal Sensitivity versus Analyte Quantitation

  SOLUTION ▶ Sometimes one must balance between maximizing analyte sensitivity with increasing dwell time, and improving peak shape and quantitation with higher sampling rate. An example of this balance between sensitivity and resolution is shown in **Figure 6**.

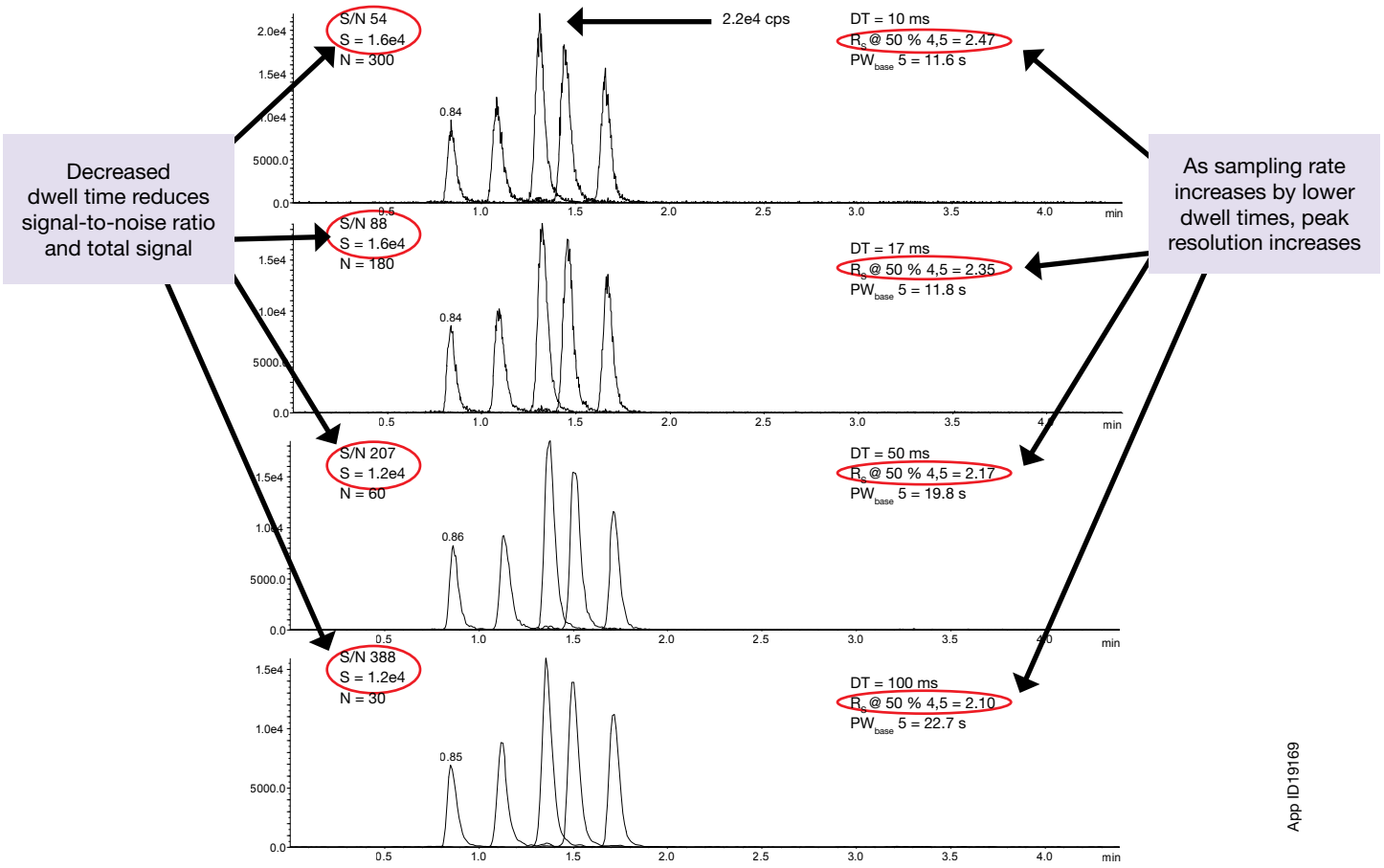
Table 3: Relationship Between Dwell Time and Sampling Rate for an API 3000™ and API 4000™ MS

	Dwell Time (ms)	Sampling Rate (SR = pts/s)	Peak Width (PW) in sec (5th Peak @ Base)	# Points across the peak (PW x SR)
API 3000	10	16	11.6 s	186
	17	10	11.8 s	118
	50	4	19.8 s	79
	100	2	22.7 s	45

	Dwell Time (ms)	Sampling Rate (SR = pts/s)	Peak Width (PW) in sec (5th Peak @ Base)	# Points across the peak (PW x SR)
API 4000	10	16	7.6 s	122
	17	10	8.8 s	88
	50	4	15.1 s	60
	100	2	19.1 s	38

Trouble: Broad and Low Intensity Peaks (continued)

Figure 6: Balancing Between Sensitivity and Resolution by Adjusting MS Dwell Time



3. Detector Settings

Trouble: Broad and Low Intensity Peaks (continued)

Full scan MS or MS/MS methods

- Depending on the mass spectrometer being used, scan rates are based on several factors: dwell time (time acquiring a specific m/z), mass resolution (mass difference between neighboring m/z), and scan window (mass range of m/z from low to high mass).

SOLUTION ▶ Depending on the analytes being scanned, all three parameters can be modified to increase the sampling rate. Dwell time can be reduced if sensitivity is not a factor in the method, mass resolution can be decreased if isotopic resolution is not necessary, and the scan range can be narrowed if some information is known about the analytes being analyzed.

Reducing Dwell Time and Increasing MS Sampling Rate: Pros and Cons

Benefits

- Improved resolution of closely eluting peaks
- Improved quantitation of analytes

Drawbacks

- Reduced MS sensitivity
- Reduced signal-to-noise ratio
- Narrower MS scan range
- Reduced isotopic resolution

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