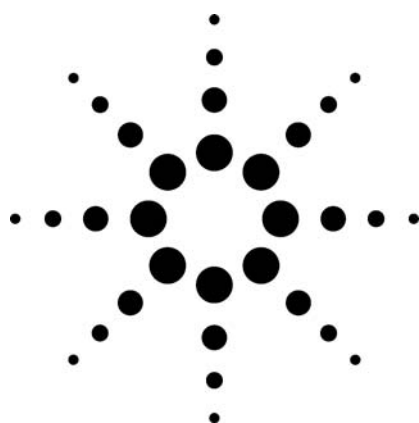


# High-Throughput Gradient Optimization by Easily Minimizing Delay Volume



## Application

### Authors

John W. Henderson, Jr., William Long, and Cliff Woodward  
Agilent Technologies, Inc.  
2850 Centerville Road  
Wilmington, DE 19808  
USA

### Abstract

**The typical approaches to the development of high-throughput gradient analyses involve reduction of column re-equilibration time and reduction of gradient delay volume. Using short ZORBAX Rapid Resolution High Throughput (RRHT) columns significantly shortens re-equilibration time compared to analytical sized columns, and a simple G1312B pump flow path configuration reduces system delay volume. Small delay volumes are needed for gradients using low-volume columns. This is shown by comparing chromatograms from the standard delay volume configuration to the low delay volume configuration. When the pump is optimized for low-volume columns, narrower peaks and increased throughput are achieved. Additionally, substituting an RRHT C18 stationary phase with a C8 stationary phase is also shown to speed up analysis time.**

### Introduction

Short RRHT columns are well suited for high-performance liquid chromatography (HPLC) gradient

methods. The short (30 to 50 mm) columns with 1.8- $\mu\text{m}$  particles can handle higher flow rates than longer conventional 5- $\mu\text{m}$  columns (100 mm to 250 mm), and re-equilibrate much faster. Decreasing re-equilibration time is a key strategy to improve a gradient method to a high-throughput gradient method. Short RRHT columns reduce analysis time, and automatically reduce the re-equilibration time, simply because the column volume is reduced proportionally.

Despite this advantage, there is sometimes a reluctance to modify a gradient method compared to an isocratic method, because the chromatographic result (for example, resolution) may not closely match the original gradient chromatogram. This is especially likely if converting a standard-bore (4.6-mm id) to a narrow-bore (2.1-mm id) gradient. The obstacle is reproducing the time or volume it takes for the gradient to get from its point of formation to the head of the column. This is called the delay time or delay volume. Dissimilar delays cause the mobile phase in the column to be at a different composition at any given time during the gradient. This causes different retention, and possibly different resolution of peaks of interest.

This application describes an easy pump modification that reduces delay volume and improves chromatographic results for low-volume RRHT gradient methods.



## Experimental

The Agilent Rapid Resolution LC (RRLC) system comprises:

- The G1365C multiwavelength detector (MWD) at 254 nm, with a G1315-60024 micro flow cell (3-mm path, 2- $\mu$ L volume), and response time setting of 0.5 s
- A G1312B binary pump SL with mobile phase A: 0.1 % formic acid in water (v/v) B: 0.1% formic acid in acetonitrile (v/v), flow rate: 1.5 mL/min for the 2.1-mm id column or 5 mL/min for the 4.6-mm id column

The column temperature was ambient, as the column compartment heat exchanger was bypassed with a 0.12-mm id capillary.

ZORBAX RRHT columns:

- Eclipse Plus C18, 2.1 x 30 mm, 1.8  $\mu$ , PN 959931-902
- Eclipse XDB-C8, 4.6 x 30 mm, 1.8  $\mu$ , PN 924975-906

The sample contains uracil (20  $\mu$ g/mL), and alkyl phenones (100  $\mu$ g/mL) C3 to C10, C12, and C14, in 50% acetonitrile/water.

## Results and Discussion

### Key Points in High-Throughput Methods

Ideally, short columns provide just enough resolution to handle the particular analysis. For small molecules, separations then can be chiefly influenced by manipulating the mobile phase or gradient. The ZORBAX 1.8- $\mu$ m particles used in RRHT columns maintain the same efficiency of longer columns packed with conventional 5- $\mu$ m particles. For example, a 4.6 x 250 mm 5- $\mu$ m column has about the same number of theoretical plates (N) as a 4.6 x 100 mm 1.8- $\mu$ m column:

$$N_{\text{ideal}} = L/2d_p$$

where L is the column length in microns, and  $d_p$  is the diameter of the particle in microns. The factor 2 in the denominator is sometimes varied (for example, 2.5) to account for less-than-ideal factors that reduce plates, such as extra column volume.

For the 250-mm column:  $N_{\text{ideal}} = 250,000 / 2 (5) = 25,000$ ; for the RRHT 100-mm column  $N_{\text{ideal}} = 100,000 / 2 (1.8) = 27,778$ .

The 100-mm RRHT column's smaller length simply speeds throughput 2.5 times. Additionally, lower back pressures are found compared to other sub-2-micron columns because of RRHT's proprietary engineered particle size distribution. This allows for higher flow rates and throughput.

Typical approaches to develop high-throughput gradient analyses are to reduce column re-equilibration time and to reduce gradient delay volume.

### Short RRHT Columns Automatically Reduce Column Re-Equilibration

Short columns packed with 1.8- $\mu$  particles, known as RRHT columns, have much reduced column volume compared to larger analytical-sized columns. For example, a 4.6 x 150 mm column has a volume of about 2.49 mL (without packing material); a 50-mm length column of the same diameter would be two-thirds less, or 0.83 mL. A 2.1 x 30 mm column has a volume only 0.10 mL, or approximately 25 times less than a standard analytical-sized column; therefore, column re-equilibration can be 25 times less mobile phase or 25 times less time. Factoring in the volume displacement of the column packing material reduces the actual volumes roughly 60% for ZORBAX 80- $\text{\AA}$  particles. A well-packed 4.6 x 150 mm column then would have a volume of about 2.49 mL x 0.60 = 1.5 mL, and a 2.1 x 30 mm packed column would be about 0.1 mL x 0.60 = 0.06 mL, still a 25-fold smaller volume.

Assume five column volumes are needed to re-equilibrate a column to starting mobile phase conditions. For a ZORBAX 4.6 x 150 mm column operating at 1 mL/min, this would be 5 column volumes x 1.5 mL/col. vol. = 7.5 mL, 7.5 mL / 1 mL/min = 7.5 min. re-equilibration time.

Inherently, the RRHT columns re-equilibrate much more quickly:

- 2.1 x 30 mm: 5 col. vol. x 0.06 mL/col. vol. = 0.3 mL; 0.3 mL / 0.2 mL/min = 1.5 min re-equilibration time
- 4.6 x 30 mm: 5 col. vol. x 0.3 mL/col. vol. = 1.5 mL; 1.5 mL / 1 mL/min = 1.5 min re-equilibration time

In practice, RRHT equilibration times could likely be even shorter because the 1.8- $\mu$ m particles found in short RRHT columns are designed to operate at faster flow rates without loss of efficiency.

## Gradients Using Low-Volume RRHT Columns Need Small Delay Volume

In order to achieve identical column flow velocities, typically flow rates for 2.1-mm id methods are about five times less (4.8 X) than 4.6-mm id methods:

$$F_2 = F_1 (id_2/id_1)^2$$

$F_2$  and  $F_1$  are the new and existing flow rates, and  $id_2$  and  $id_1$  are the column internal diameters, respectively. Because the flow rates are proportional to the squares of the internal diameter, scaling down from a 4.6 to a 2.1 mm is about a five-fold decrease in flow rate.

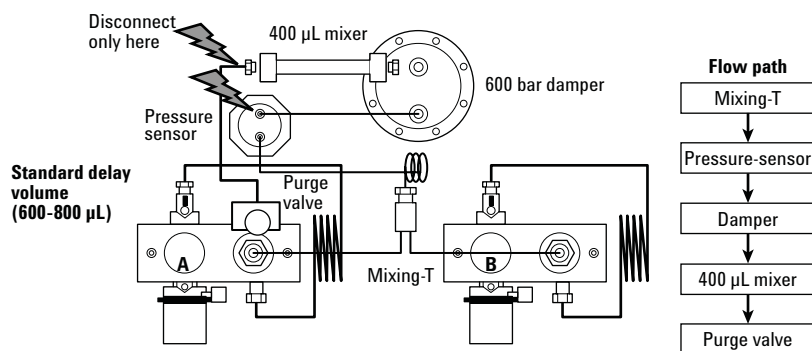
In a gradient method, the delay time (the time it takes the gradient to get from its point of formation to the head of the column) would then be five times longer. Since the sample is injected at time zero minutes for both 4.6- and 2.1-id columns, the sample travels isocratically through the column five times longer when a 2.1-id column is used, until the gradient reaches the head of the column. Because the delay time is out of synchronization by a factor of five, the chromatographic result can be quite different.

There are two ways to reduce the delay time: operate at a higher flow rate or reduce the precolumn volume so the gradient reaches the column faster.

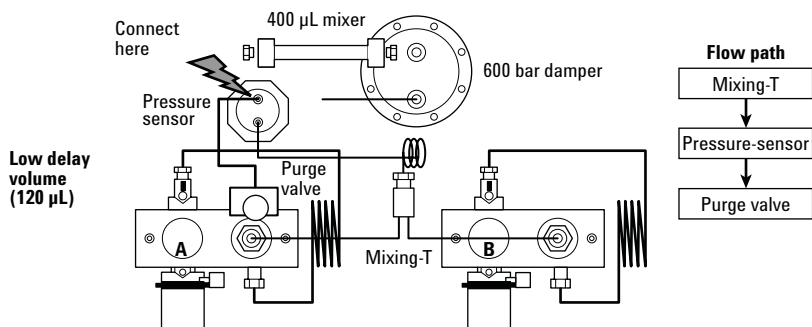
There are a variety of options to reduce the delay volume of the 1200 HPLC system, including:

- Using 0.12-mm id tubing in place of 0.17-mm id tubing
- Bypassing the standard heat exchanger in the column compartment with a short capillary, or using the low-dispersion heat exchanger kit (G1316-80003 with G1316-83200)
- Programming the autosampler for bypassing the sample loop and metering device (Automatic Delay Volume Reduction ON)
- Replumbing the binary pump from standard delay volume to low delay volume

Converting a G1312B binary pump between standard delay volume and low delay volume is straightforward [1], requiring only 1/4" and 9/16" wrenches and the diagram in Figures 1a and 1b. A slotted 1/4" nut-driver (PN 05023-0240) is available to facilitate connecting the capillary to the pressure transducer.



**Figure 1a. Standard delay volume configuration of G1312B LC pump indicating the two connections in the flow path for easily reducing delay volume.**



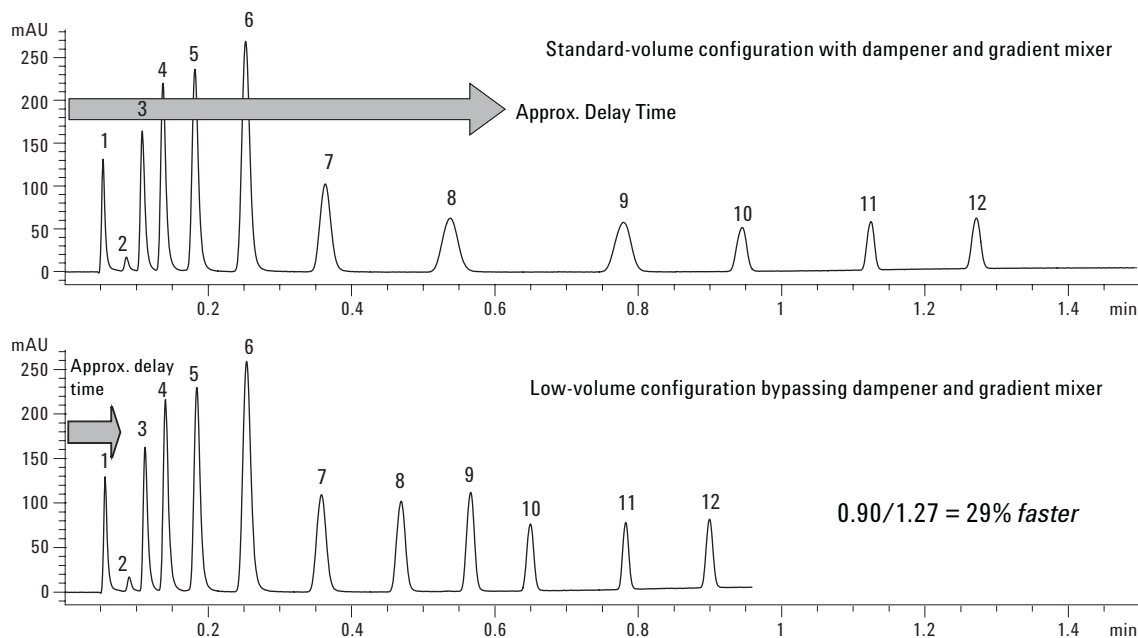
**Figure 1b** Low delay volume configuration of G1312B LC pump. The capillary connected to the purge valve is now connected to the pressure sensor.

The results of the minimized delay volume can be readily seen in Figures 2 and 3.

Figure 2 is a RRHT gradient method for alkyl phenones developed on a 2.1 x 30 mm RRHT Eclipse Plus C18 with the binary pump configured in its standard delay volume, compared to the low-volume configuration. Although, by convention, it is stated that the gradient starts at time zero and ramps up to 100% in 0.5 minutes, in actuality the precolumn volume causes a noticeable delay time before the gradient reaches the column. This is seen as broadening peaks (peaks 1 to 10) eluting off the column. It is not until approximately 0.5 minute that the gradient reaches the injected sample and sharpens peaks 10 to 12.

In the low-volume configuration, the earliest eluting peaks are still eluted in the isocratic delay, but the later eluting peaks are narrower, indicating the gradient reached the head of the column more quickly. Overall the analysis time decreased about 30% by configuring the pump for low delay volume.

Figure 3 is an example using a 4.6 x 30 mm RRHT Eclipse XDB-C8, instead of the 2.1 x 30 mm Eclipse Plus C18. The flow rate is 5 mL/min, so the difference in delay time between the standard delay volume and low delay volume is small. Band broadening is not noticeable like the 2.1 id method. However, there is still a 14% difference in analysis time when the pump is configured with low delay volume.



**Column:** ZORBAX Rapid Resolution HT Eclipse Plus C18, 2.1 mm × 30 mm

**Mobile phase:** A: 0.1% formic acid, B: 0.1% formic acid in ACN

**Gradient:** 70 to 100% B/0.5 min

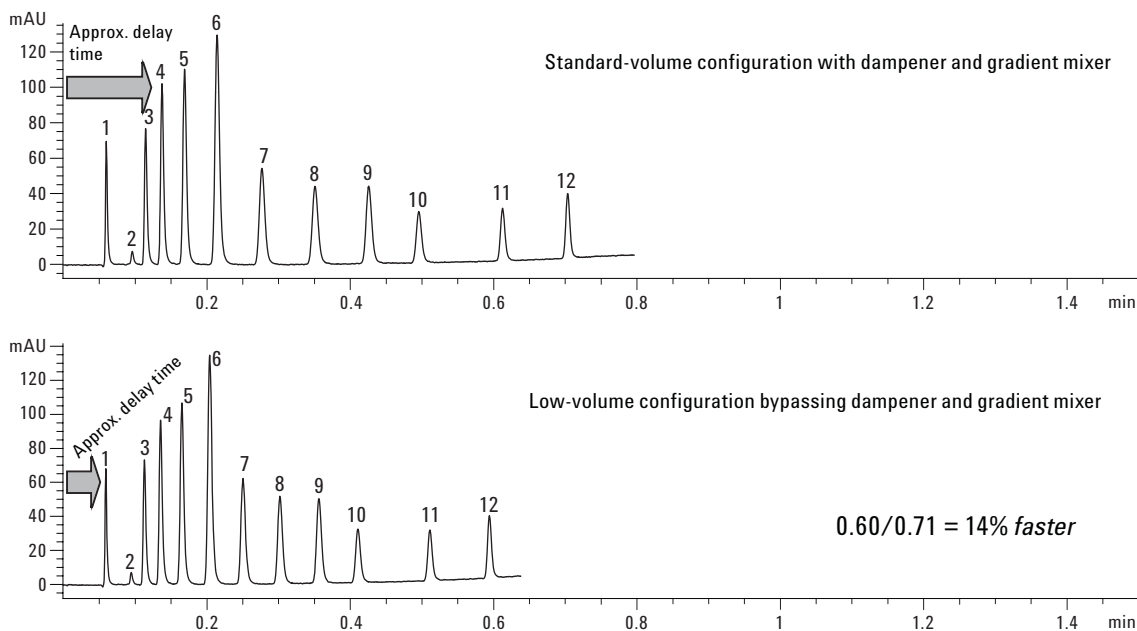
**Flow rate:** 1.5 mL/min

**Temperature:** 25 °C

**Detection:** UV 254 nm, micro flow cell, data rate: 0.5 s

**Sample:** Uracil (peak 1) and alkylphenones C3, C4, C5, C6, C7, C8, C9, C10, C12, C14 (peaks 3-12)

**Figure 2.** Smaller delay volume for narrow-bore RRHT columns yields higher throughput.



**Column:** ZORBAX RRHT Eclipse XDB-C8, 4.6 mm × 30 mm  
**Mobile phase:** A: 0.1% formic acid, B: 0.1% formic acid in ACN  
**Gradient:** 70 to 100% B/0.5 min  
**Flow rate:** 5 mL/min  
**Temperature:** 25 °C  
**Detection:** UV 254 nm, micro flow cell, data rate: 0.5 s  
**Sample:** Uracil (peak 1) and alkylphenones C3, C4, C5, C6, C7, C8, C9, C10, C12, C14 (peaks 3-12)

**Figure 3. Smaller delay volume for standard-bore RRHT columns has less effect.**

### Selectivity of Stationary Phase May Speed Throughout

The different stationary phase (Eclipse XDB-C8 vs. Eclipse Plus C18) also decreased retention time, despite operating at a lower mobile phase linear velocity compared to the 2.1 id Eclipse Plus C18. Comparing Figures 2 and 3, analysis time was reduced from 0.9 minute to 0.6 minute, so a significant increase in throughput was due to the shorter alkyl phase. Having a variety of stationary phases available is a valuable tool to quickly change retention, selectivity, and resolution. Currently there are large numbers of combinations of column lengths, diameters, and 1.8- $\mu$ m stationary phases to optimize high-throughput methods.

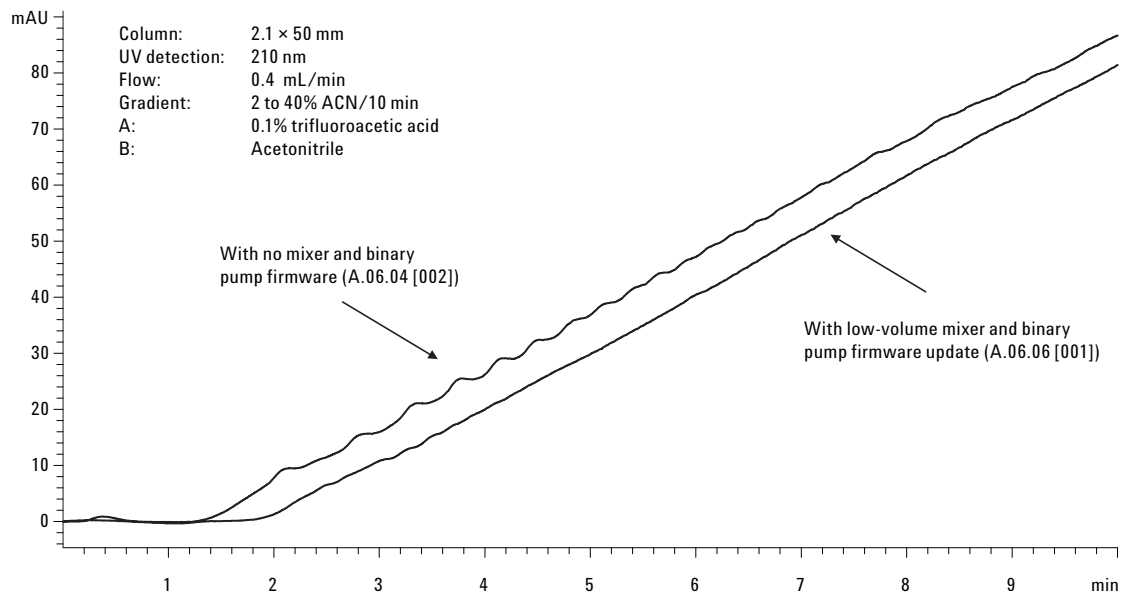
### Low-Volume Static Mixer Option

In a few cases, low percentage mixtures (< 20%) of organic solvent in the low delay volume mode might cause baseline anomalies due to differential UV absorbance of a mobile phase component. The symptom is a wavy baseline. Other types of detectors such as a mass spectrometer (MSD) are not affected. To minimize such possible effects, a 200- $\mu$ L static mixer (PN 5067-1565) is available, coupled with a Binary Pump SL firmware update (A.06.06 [001]). This firmware is optimized for

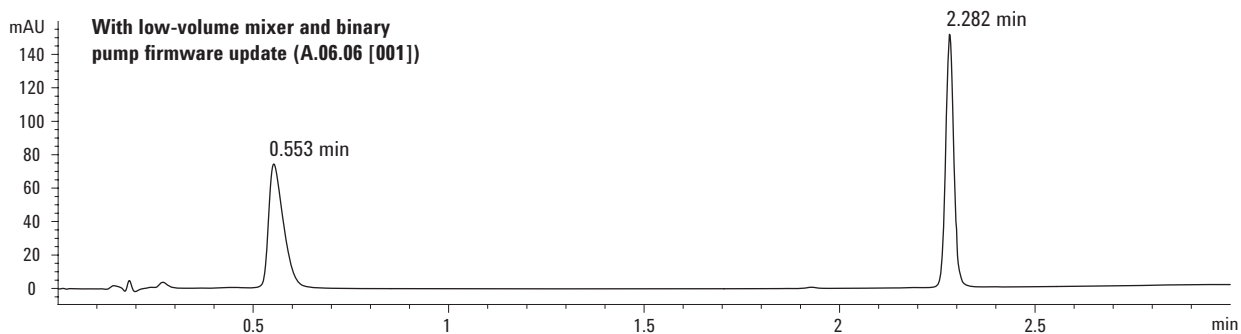
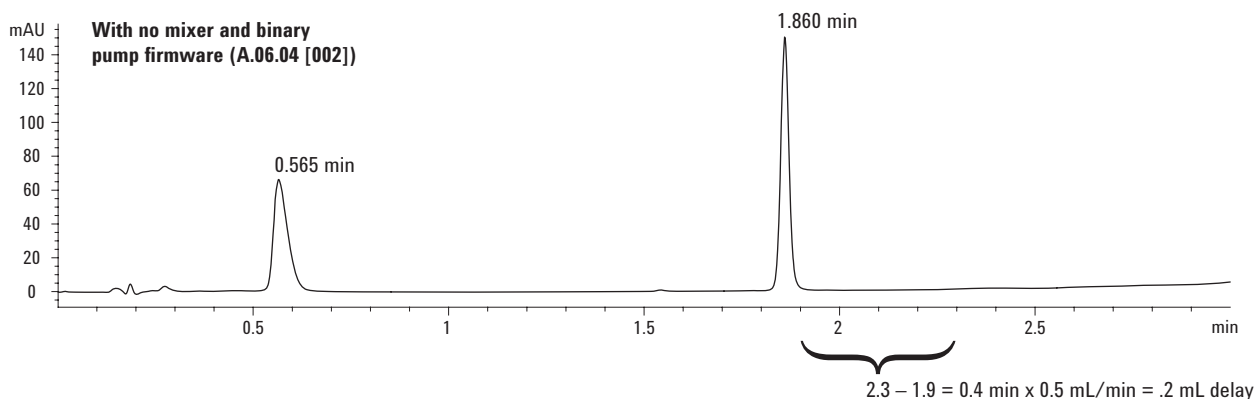
using the 200- $\mu$ L mixer without the dampener. The firmware reduces the pump stroke from 20 to 10  $\mu$ L.

An example of a mobile phase component that has a strongly different UV absorption in water and acetonitrile is trifluoroacetic acid (TFA). Figure 4 shows the effect of not using the mixer and firmware A.06.06 [001] with a low-volume column and low organic gradient. The baseline noise is much greater without the mixer/firmware at the high aqueous part of the gradient.

In many cases, there is no strong differential absorption in the mobile phase. Figure 5 compares different 1200 SL configurations running a separation of clindamycin antibiotic and its precursor, lincomycin. A low organic gradient (10 to 40% MeOH/2 min) is needed to elute them in a reasonable time, due to the very different hydrophobicity of the compounds, a low UV wavelength (205 nm) is used because of the lack of a chromophore, a low-volume column (2.1 x 30 mm) is chosen for fast analysis, and the micro-flow cell was used because of the low-volume column. The top chromatogram was obtained from a 1200 SL system in the low-volume mode with binary pump SL firmware (A.06.04 [002]), bypassing the mixer and dampener. Despite the similar conditions that produced



**Figure 4. Differential UV absorption effects on baseline are mitigated with low-volume mixer and firmware update.**

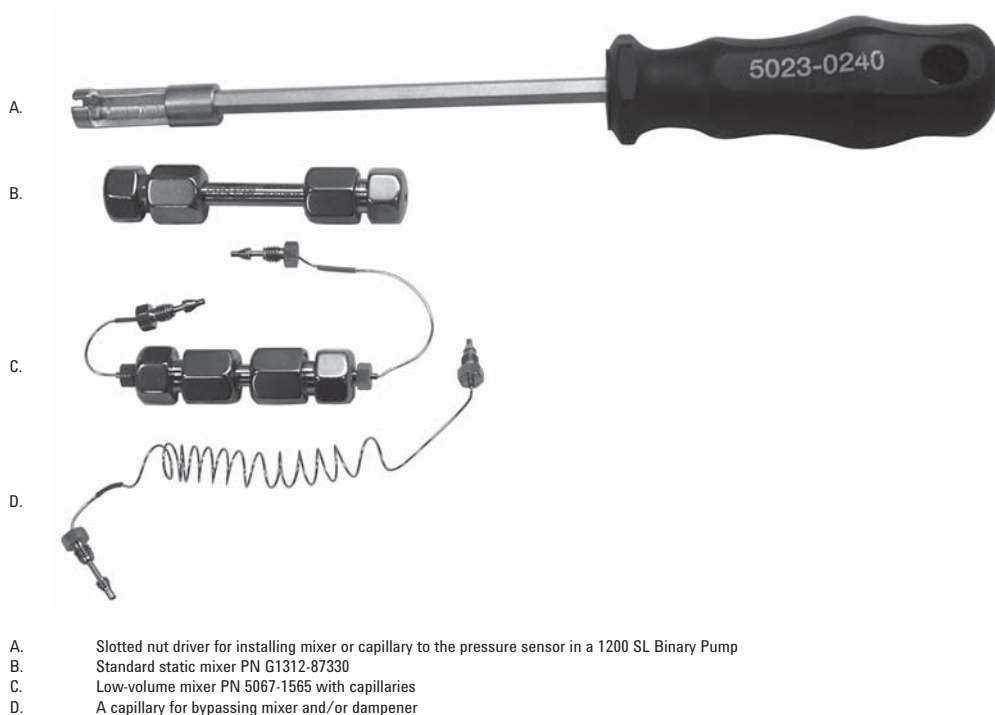


**Column:** ZORBAX RRHT SB-C18, 2.1 x 30 mm cartridge  
**Mobile phase:** A: 20 mM phosphate pH 2.8, B: ACN, Gradient: 10 to 40% B/2 min  
**Flow rate:** 0.5 mL/min  
**Temperature:** Ambient  
**Detection:** UV 205 nm, micro flow cell, Data rate: 0.5 s  
**Sample:** Lincomycin (peak 1) and clindamycin (peak 2)

**Figure 5. Low-volume mixer option minimizes any possible differential UV absorption effects of high aqueous gradients, only adds 0.2 mL delay.**

the wavy baseline in Figure 4 (low organic gradient, low-volume column, and low UV wavelength detection), no baseline anomaly is present. The bottom chromatogram has the 200- $\mu$ L mixer installed along with Binary Pump SL firmware (A.06.06 [001]). The delay volume increased by the volume of the mixer, as expected and calculated in the figure. The first peak's retention is the same in both instances because it elutes in the isocratic lag.

The ability to measure and manipulate the delay volume easily is a useful tool to transfer gradient methods between different HPLC instrument configurations and to scale down methods to faster ones using RRHT columns. Figure 6 shows several options for this. Additionally, the pulse dampener can be attached or not, resulting in multiple combinations for the best mixing and/or shortest or best matching delay volume for any specific gradient method.



**Figure 6. The delay volume can be adjusted with a variety of mixers and capillaries.**

## Conclusions

Small-volume RRHT columns are valuable for high-throughput gradient methods because rapid column re-equilibration times can improve throughput significantly compared to larger volume analytical-sized columns.

Reducing delay volume is another way to reduce analysis time and increase gradient throughput, especially for 2.1-mm id columns. Configuring the G1312B binary pump in its low delay volume flow path results in the gradient mobile phase composition reaching the column faster and minimizes the isocratic lag, or delay time. The end results are sharper peaks and shorter analyses.

Finally, the ability to substitute columns with different stationary phases is advantageous because it can significantly change selectivity, retention, and possibly further reduce analysis time, ultimately improving lab productivity.

## Reference

1. Optimizing Performance of the Agilent 1200 Rapid Resolution LC System, Agilent Publication G1312-90300, March 2006, p.159.

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