DIONEX

Technical Note 80

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Reduce Eluent Consumption by Optimizing UltiMate 3000 Quaternary Analytical Systems for Small Column Volumes

INTRODUCTION

The reduction of eluent consumption in reversed-phase HPLC separations has gained in importance due to growing ecological concerns and the current worldwide acetonitrile shortage. One strategy to reduce eluent consumption is the use of small particle columns. These columns can maintain separation efficiency with shorter run times. This reduces not only eluent consumption, but also increases productivity of the HPLC system. Currently, 5 µm is the most commonly used size, but 3 µm, 2 µm, or sub-2 µm particles are becoming more and more popular. The figure below clearly indicates why.

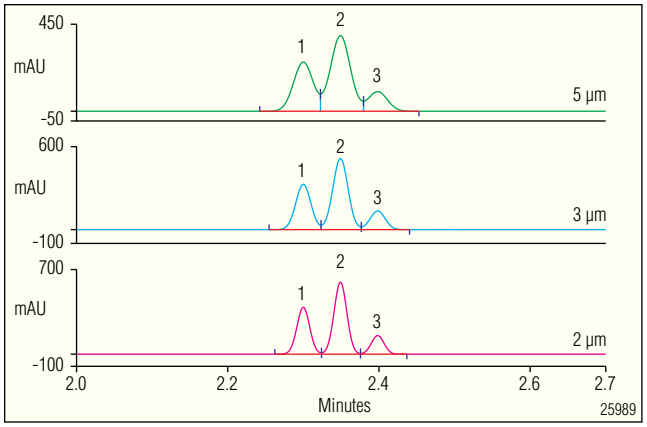

Figure 1. Comparison of three separations obtained with 5 µm, 3 µm, and 2 µm particulate columns. All other parameters are kept constant.

All separations were performed under identical conditions such as column dimension, mobile phase composition, flow, and stationary phase; only the particle size decreases top to bottom, from 5 µm to 3 µm to 2 µm. The resolving power of the column increases with the reduction of the particle size. The main drawback of using smaller particles is the increase in pressure. The pressure is inversely proportional to the square of the reduction in particle size. Starting with 5 µm material, this means for the separation above a pressure increase from 110 bar (5 µm) to 305 bar (3 µm) to 690 bar (2 µm).

For the 2 µm material, the pressure exceeds the upper pressure limit of a conventional HPLC instrument. With equipment of this kind, a common strategy is therefore to shorten the column length when reducing the particle size. But which column length provides a comparable resolving power when changing to smaller particles? The L/dp ratio (length/particle size) gives a direct measure, as long as the optimal linear velocity is considered (van Deemter theory). For example, a 2 µm, 50 mm long column (L/dp=25,000) provides more than 80 % of a 5 µm, 150 mm length column (L/dp=30,000), which is mostly enough for a method transfer to this format. A 3 µm, 100 mm column (L/dp=33,333) provides even more resolving power than the 5 µm column. Small particle columns generate the same peak resolution on a shorter column length and at higher mobile phase linear velocities. Hence, they support markedly shorter analysis times and reduced eluent usage, particularly when packed in small bore columns.

UltiMate® 3000 RSLC systems are the best choice for operating small bore/ small particle columns. They feature a maximum operating pressure of 800 bar (11,600 psi) in combination with a flow rate of up to 5 mL/min. However, not everyone can easily afford investing in a new system. This Technical Note therefore demonstrates how existing equipment can be improved for high performance with small volume columns. The optimization is based only on the system fluidics; the upper pressure limit of the system remains unchanged.

The Quaternary Analytical System is the most common UltiMate 3000 system configuration. Its fluidic design is optimized for 3–4 mm bore columns with 10–25 cm lengths for highest chromatographic performance, reliability, and ease-of-use. The easiest way to optimize these fluidics for small column volumes is to install the Viper Capillary Kit, RS system. Viper is a new stainless steel fingertight fitting system, that provides ease-of-use and guaranteed zero-dead-volume plumbing.

EQUIPMENT

Initial System:

UltiMate 3000 Quaternary Analytical System consisting of the following modules:

-Solvent Rack SR-3000
-Quaternary Analytical Pump LPG-3400A
-Analytical Wellplate Sampler WPS-3000SL Analytical
-Thermostatted Column Compartment TCC-3000
-Variable Wavelength Detector VWD-3400RS

Initial Accessories:

Inter-module connection tubings with 0.010” (0.25 mm) inner diameter, stainless steel

Analytical flow cell (11 µL, stainless steel)

Initial LC Conditions:

Columns: Acclaim® 120, C18, 5 µm 4.6 x 100 mm
Acclaim 120, C18, 3 µm 3 x 75 mm
Mobile Phase A: Water
Mobile Phase B: Acetonitrile
Gradient:

Time (min)	%B
0	75
3	95
3.5	95
4	75
7	75

Initial Injection Volume: 15 µL (4.6 x 100 mm column)
Column temperature: 40 °C
Detection: 251 nm
Initial Data Collection Rate: 10 Hz
Initial Time Constant: 0.1
Sample:

1. Uracil
2. Impurity 1
3. Naphthalene
4. Biphenyl
5. Fluorene
6. Anthracene
7. Fluoranthene
8. Impurity 2

Analyte Concentrations:
6–82 ng/µL in acetonitrile/methanol/water 2/1/1 (v/v/v)

EXPERIMENTAL

Step 1: Method Transfer to Faster Column

The top chromatogram of Figure 1 shows a separation of eight peaks on a 4.6 × 100 mm Acclaim 120, C18, 5 µm column. All peaks are well baseline separated and elute within 3.4 min. The total run time is 7 min. This relatively fast analytical HPLC separation is transferred to a 3 × 75 mm Acclaim 120, C18, 3 µm RSLC column using the Dionex Speed-Up Calculator. This multi-language tool is based on Snyder’s gradient volume principle,[1] which defines the requirements for downscaling a gradient method to a smaller column. It is

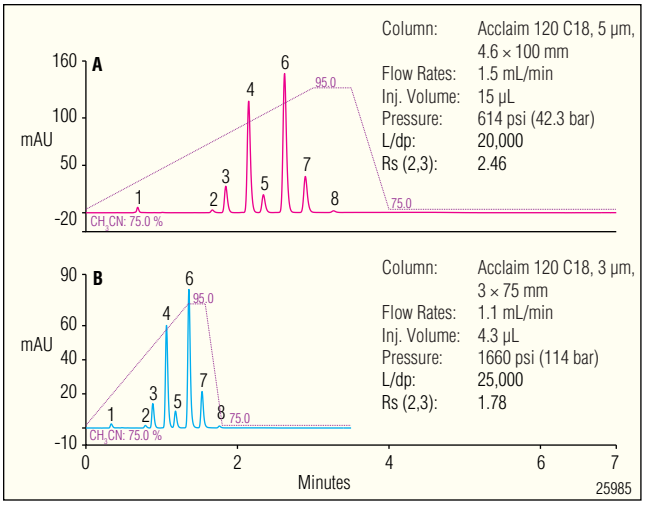

Figure 2. Comparison of chromatograms obtained on A) a 4.6 × 100 mm, 5 µm column and B) a 3 × 75 mm, 3 µm smaller volume column. Both separations were performed on a quaternary analytical system.

available free of charge from your Dionex representative, and makes method transfer intuitive without requiring in-depth knowledge of chromatography fundamentals.[2] It automatically adapts parameters, such as flow rate, gradient profile, and injection volume, and estimates backpressure and the resolution of the critical peak pair.

The 3 µm, 75 mm length column (L/dp = 25,000) should provide more resolving power than the initial 5 µm, 100 mm length column (L/dp = 20,000). Consequently, in addition to the shorter run time, a better peak separation can be expected. The actual data, however, show that the separation of the peaks is acceptable (Rs(2,3) = 1.78), but it is worse than for the initial separation on the 5 µm column (Rs(2,3) = 2.46). Typically, a resolution of Rs ≥1.5 is considered a baseline separation.

This discrepancy between the theoretical and practical resolving power can again be explained by Snyder's gradient volume principle. In addition to the settable system parameters, this rule applies to other system volumes such as the gradient delay volume (GDV) and the extra-column volume (ECV), too. In practice, when downscaling a gradient application to small volume/small particle fast LC columns, GDV and ECV have to be downscaled accordingly. Relative to the analytical method, the adaption of the GDV preserves the retention factors of analyte peaks. The adaption of the ECV maintains the chromatographic performance, which can be expected from the particle size and dimensions of the fast column.

The GDV is the volume between the first point of mixing and the head of the column. Thus the gradient delay volume determines the time the gradient needs to take effect on the column. The ECV is the volume between the sample injection and the detector cell minus the column volume. This value has a significant impact on the quality of HPLC results. If the ECV is too large, it negatively affects parameters such as the chromatographic efficiency or the asymmetry of analyte bands and thus the peak resolution.

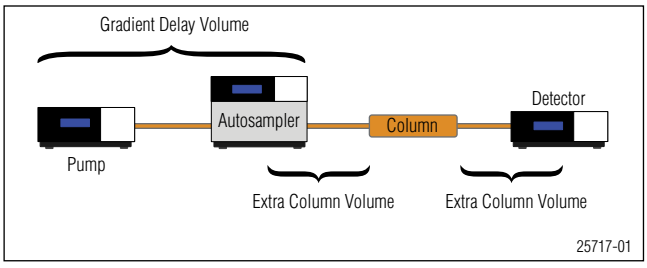

Figure 3. Gradient delay volume and extra column volume of an HPLC system.

Step 2: Viper RS Capillary Kit

The Viper RS capillary kit is designed for use with small volume columns and is the default plumbing kit for UltiMate RSLC systems. When installed with a quaternary analytical system, small volume columns can be operated at high performance. Two optimizations are done in addition. First, a 40 µL sample loop was installed in the autosampler, to reduce the GDV volume of the sampler. Second, a 2 µL eluent pre-heater was installed in front of the column. Figure 4 compares the separation of the sample, using the standard system configuration (magenta trace) and another configuration optimized by the kit (blue trace).

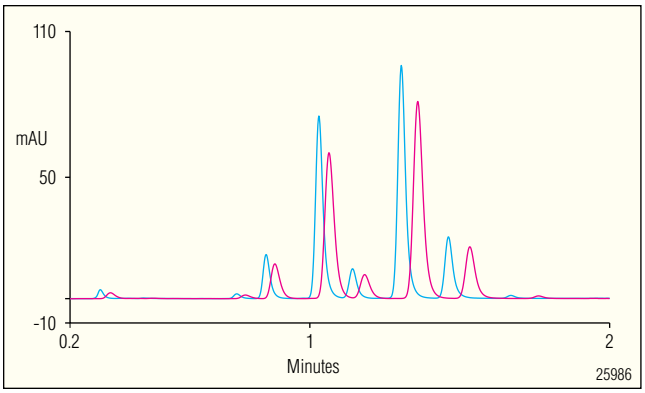

Figure 4. Separation without (magenta trace) and with RSLC conversion kit (blue trace).

Optimization includes reduced gradient delay and extra column volumes. The gradient takes effect earlier on the column, and analytes are transferred more quickly to the detector. Both cause retention times to decrease. At the same time, they are narrower, increasing amount sensitivity and peak resolution. The table below compares resolution between all analyte peaks before and after installing the kit.

Table 1. Peak Resolution with and without Viper RS Capillary Kit

Peaks	Resolution without Kit (3 × 75 mm Column)	Optimized Resolution (3 × 75 mm Column)
2, 3	1.78	2.29
3, 4	3.19	4.05
4, 5	2.08	2.46
5, 6	3.04	3.53
6, 7	2.96	3.29
7, 8	4.01	4.31

The resolution between all analyte peaks is significantly better using the optimized system. Now, how does the optimized column/system combination compare against the initial, large volume column setup? The table below compares the resolutions.

Table 2. Peak Resolution before and after Column/System Optimization

Peaks	Original Method (4.6 × 100 mm Column)	Optimized Method (3 × 75 mm Column)
2, 3	2.46	2.29
3, 4	4.09	4.05
4, 5	2.50	2.46
5, 6	3.47	3.53
6, 7	3.25	3.29
7, 8	4.31	4.31

The optimized method shows a trend of slightly reduced resolution towards early eluting peak pairs. This is a consequence of the non-constant ratio of gradient delay volume to column volume. With the small volume column, the gradient takes effect later, i.e., the initial isocratic phase of the gradient is expanded. The peaks' elution mechanisms become more isocratic, slightly delaying and broadening them. This effect is pronounced towards earlier elution times and therefore influences early peak pairs the most.

The Viper RS Capillary Kit supports high efficiency and fast separations on a standard quaternary analytical system. Typically, the system optimization is sufficient for the supported column formats. For very critical separations, however, additional optimizations can be done. These are described in the following sections.

Optional Flow Cell Changes

Another system optimization is performed by replacing the analytical flow cell (11 µL) with a semi-micro flow cell (2.5 µL). One benefit of this change is that the semi-micro flow cell improves the integration of the peak. A rule of thumb is that the flow cell volume should not exceed 1/10th of the peak volume. This is equivalent to replacing the cell volume 10 times over the course of the peak. The semi-micro flow cell achieves this even with column volumes smaller than recommended for this RSLC conversion kit. Semi-micro flow cells are available for the UltiMate VWD-3000, the DAD-3000, and the MWD-3000 detector series. The 5 µL semi-analytical flow cell is another useful cell format, available for the two latter detectors. Like the semi-micro flow cell, it features a light path of 7 mm, but supports a higher light transmission. This can be beneficial for the signal-to-noise ratio if the peak volume is around 50–100 µL. For the UltiMate PDA-3000, a 3.1 µL semi-micro flow cell is available.

Typically however, the performance achieved with a standard analytical flow cell is sufficient for most separation challenges with the recommended column formats. Installing a semi-micro flow cell further improves the performance if needed.

Figure 5 compares the separation of the peak pair (4, 5) after installation of the RSLC conversion kit (A, black), and after installation of a semi-micro flow cell (B, magenta). Note that the reduction in peak height is caused by the 7 mm light path of the semi-micro flow cell compared to the 10 mm light path of the analytical flow cell. The table below compares the peak widths of peak 4 at half height and the resolution Rs (4, 5).

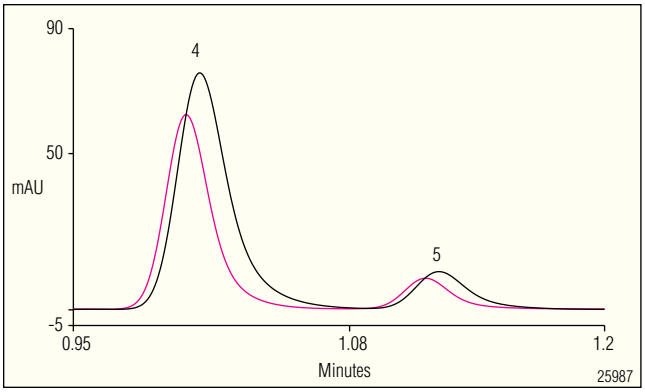

Figure 5. Overlays of separation of the peak pair (4, 5) with RSLC conversion kit (A, black), and further optimized with the semi-micro flow cell (B, magenta).

Table 3. Comparison of Peak 4 Width at Half Height with Resolution of Peak Pair 4, 5

Separation	Peak Width [sec] at Half Height, Peak 4	Resolution Rs (4, 5)
A	1.55	2.56
B	1.37	2.84

These parameters can be taken as a measure of the separation quality. The change of the flow cell reduces peak width and therefore improves peak capacity and resolution. In this example, the resolution is increased by 11% by installing the semi-micro flow cell.

Further Accelerating the Analysis

With the final system optimization described above, it took only 1.8 minutes to elute eight substances, all baseline separated. This fast analysis was performed at a flow rate of 1.1 mL/min at a maximum backpressure of only 160 bar. Following van Deemter theory, the flow rate of 1.1 mL/min provides the optimum linear velocity for 3 mm i.d. columns filled with 3 µm particles. One major advantage of small packing material is that they can be operated at flow rates above their optimum while maintaining most of their separation efficiency. Figure 6 compares the separation at 1.1 mL/min (A) and 2.2 mL/min (B).

On an optimized UltiMate 3000 Quaternary Analytical system, eight analyte peaks could be eluted in less than 55 seconds. Baseline separation of all peaks is maintained even at double the optimum flow rate. To calculate the speed up factor, let's compare the elution time of the last peak for the initial and the fastest separation. With the initial separation, peak 8 eluted in 4.364 min, with the fastest separation in 0.834 min. Thus the method is 5.2 times faster. At the same time, eluent consumption is significantly reduced. The initial application required 1.5 mL/min × 7 min = 10.5 mL eluents. The fastest method requires 2.2 mL/min × 2 min = 4.4 mL, 60% less than the initial eluent consumption.

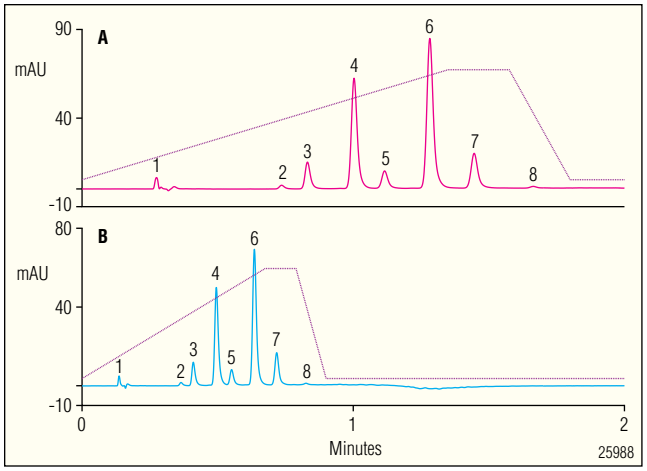

Figure 6. Separations on UltiMate 3000 Quaternary Analytical System optimized using the Viper RS Capillary Kit and semi-micro flow cell. Flow rate of separation A: 1.1 mL/min. Flow rate of separation B: 2.2 mL/min. Injection volume for both methods: 4.3 µL.

CONCLUSION

- The Viper RS Capillary Kit easily optimizes UltiMate 3000 Quaternary Analytical systems for small-volume columns. This kit optimizes the system's gradient delay and extra column volumes while the upper pressure limit of the system remains the same.
- Further optimization of the system is possible for achieving even higher separation power for challenging or boosted applications (see optional accessories next page).
- A speed-up factor of 5 and 60% less eluent consumption is achieved by utilizing a small volume column on an optimized analytical HPLC system.

REFERENCES

1) Snyder, L. R.; Dolan, J. W.; Grant, J. R. *J. Chromatogr.* **1979,** *165* (1), 3-30.

2) Dionex Corporation. *Easy Method Transfer from HPLC to RSLC with the Dionex Method Speed-Up Calculator*; Technical Note 75, LPN 2140-01. Sunnyvale, CA, 2009.

RSLC CONVERSION KIT FOR QUATERNARY ANALYTICAL SYSTEMS

The products below are recommended for analysts converting their UltiMate 3000 quaternary analytical systems to support Rapid Separation applications, as outlined in this technical note. In the U.S., call 1-800-346-6390, or contact the Dionex Regional Office nearest you. Outside the U.S., order through your local Dionex office or distributor. Refer to the following part numbers:

PRODUCT DESCRIPTION	PART NUMBER
Viper UHPLC Fingertight Fitting and Capillary Kit for UltiMate 3000 RSLC Systems, SST	6040.2301
Kit Includes:	
Viper Installation and Operation Guide	
Viper UHPLC Fingertight Fitting, i.d. 0.007 inch (0.18 mm), length 450 mm, SST	6040.2365
Viper UHPLC Fingertight Fitting, i.d. 0.005 inch (0.13 mm), length 350 mm, SST	6040.2335
Viper UHPLC Fingertight Fitting, i.d. 0.005 inch (0.13 mm), length 250 mm, SST	6040.2325
Optional Accessories:	
Sample Loop Micro, 25 µL, WPS-3000SL	6820.2415
Eluent Preheater, 2 µL	6722.0530
Semi-Micro Flow Cell, VWD-3x00(RS), 2.5 µL, stainless steel	6074.0360
Semi-Micro Flow Cell, DAD-3000(RS) and MWD-3000(RS), 2.5 µL, stainless steel	6082.0300
Semi-Analytical Flow Cell, DAD-3000(RS) and MWD-3000(RS), 5 µL, stainless steel	6082.0200
Semi-Micro Flow Cell, PDA-3000, 3.1 µL, stainless steel	6080.0230

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