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# Increasing Sample Throughput Using Parallel Column Regeneration for the Analysis of Water Soluble Vitamins by Hydrophilic Interaction Liquid Chromatography (HILIC)

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## Abstract

In this study, we use an ACQUITY UPLC System with 2D LC Technology for the Analysis of Water Soluble Vitamins by Hydrophilic Interaction Liquid Chromatography (HILIC).

### Benefits

- Increasing productivity using parallel column regeneration
- ACQUITY UPLC PLUS System with 2D LC Technology can be configured to perform parallel column regeneration


## Introduction

Analytical laboratories are constantly challenged to analyze more samples in less time to increase throughput. For typical gradient liquid chromatography (LC) methods, there are two segments. The first is the gradient itself during which the separation occurs. The second is the reconditioning step, also called column regeneration, which is used to wash and re-equilibrate the column. The reconditioning segment is essential for data consistency as well as increased column life. Depending on the gradient method, the duration of the reconditioning step may vary. Usually up to 60% of the total run time can be used for this process. To reduce the time needed for sample analysis, some common practices include increasing the flow rate, using a shorter column and/or reducing column re-equilibration time. However, there are limits to the extent that these parameters can be modified without impacting chromatographic performance.

Parallel column regeneration is a solution where the total analysis time is reduced with no impact on the separation. By adding an additional pump and utilizing the switching valves of the column compartment, a standard LC system can be configured to perform parallel column regeneration. In this study, we will use an ACQUITY UPLC System with 2D LC Technology which is equipped with two binary pumps and switching valves within the column manager. A hydrophilic interaction liquid chromatography (HILIC) method for water soluble vitamins will be used. The analysis will alternate between two identical columns with identical flow paths. Specifically, one sample is injected and separated on the first column, while the second column undergoes reconditioning. Once the separation is complete on the first column, the valves switch positions. The subsequent injection is directed onto the second column for analysis, while the first column undergoes

reconditioning. With this configuration, the reconditioning step can be excluded from the cycle time, while preserving the separation. This allows for increased throughput and reduced analysis time.

## Experimental

### Method conditions

LC conditions

| | |
|---|---|
| System: | ACQUITY UPLC PLUS System with 2D LC Technology |
| Gradient pump: | ACQUITY UPLC I-Class PLUS Binary Solvent Manager (BSM) |
| Reconditioning pump: | ACQUITY UPLC I-Class PLUS Binary Solvent Manager (BSM) |
| Injector: | ACQUITY UPLC H-Class PLUS sample manager with flow through needle (SM-FTN) |
| Column compartment: | ACQUITY UPLC Column manager (CM) |
| Detector: | Photodiode Array eλ (PDA) |
| Column: | ACQUITY UPLC BEH Amide, 130 Å, 1.7 µm, 2.1 mm × 50 Column (p/n: 186004800) |
| Column temp.: | 30 °C |
| Sample temp.: | Ambient |
| Injection vol.: | 1 µL |

LC conditions

| | |
|---|---|
| Flow rate: | 0.4 mL/min |
| Mobile phase A: | 50/50 acetonitrile/10 mM ammonium acetate and ammonium hydroxide, pH 9.0 |
| Mobile phase B: | 90/10 acetonitrile/10 mM ammonium acetate and ammonium hydroxide, pH 9.0 |
| Wavelength: | 265 nm |
| Sampling rate: | 20 points/sec |
| Data management: | Empower 3, Feature Release 3 |

## Gradient table:

Gradient pump

| Time (min) | Flow (mL/min) | %A | %B |
|---|---|---|---|
| Initial | 0.4 | 0.1 | 99.9 |
| 3.50 | 0.4 | 50.0 | 50.0 |
| 3.51 | 0.4 | 0.1 | 99.9 |
| 4.00 | 0.4 | 0.1 | 99.9 |

## Reconditioning pump

| Time (min) | Flow (mL/min) | %A | %B |
|---|---|---|---|
| Initial | 0.4 | 0.1 | 99.9 |
| 4.00 | 0.4 | 0.1 | 99.9 |

## Sample description

Six individual water soluble vitamins were purchased from Sigma-Aldrich as powders. Stock solutions at a concentration 2 mg/mL were prepared for each vitamin (except for riboflavin) in a sample diluent of 75/25 acetonitrile/methanol with 0.2% acetic acid. Riboflavin was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 2 mg/mL.

The six vitamin stock solutions were combined at specified volumes and diluted using 75/25 acetonitrile/methanol with 0.2% acetic acid to a total volume of 2 mL. Final concentrations are listed below (Table 1).

| Label | Water soluable vitamins | Volume of stocking solution (µL) | Conc. (mg/mL) |
|---|---|---|---|
| 1 | Nicotinamide (B3) | 200 | 0.20 |
| 2 | 4-Aminobenzoic acid/PABA (B10) | 80 | 0.08 |
| 3 | (-) – Riboflavin (B2) | 80 | 0.08 |
| 4 | Nicotinic acid (Anhydrous) (B3) | 150 | 0.15 |
| 5 | Thiamine hydrochloride (B1) | 300 | 0.30 |
| 6 | Cyanocobalamin (B12) | 350 | 0.35 |
| | Sample diluent | 840 | |
| | Total volume (µL) | 2000 | |

*Table 1. Preparation of a mix solution of six water soluble vitamins.*

## Results and Discussion

### Strategy for parallel column regeneration

For a typical gradient LC method, both the gradient and the reconditioning segments are delivered using a single pump. Alternatively, parallel column regeneration requires the use of two pumps and two identical columns. While one pump delivers the gradient for the separation to one column, the other pump reconditions the second column. The concept is illustrated in Figure 1.

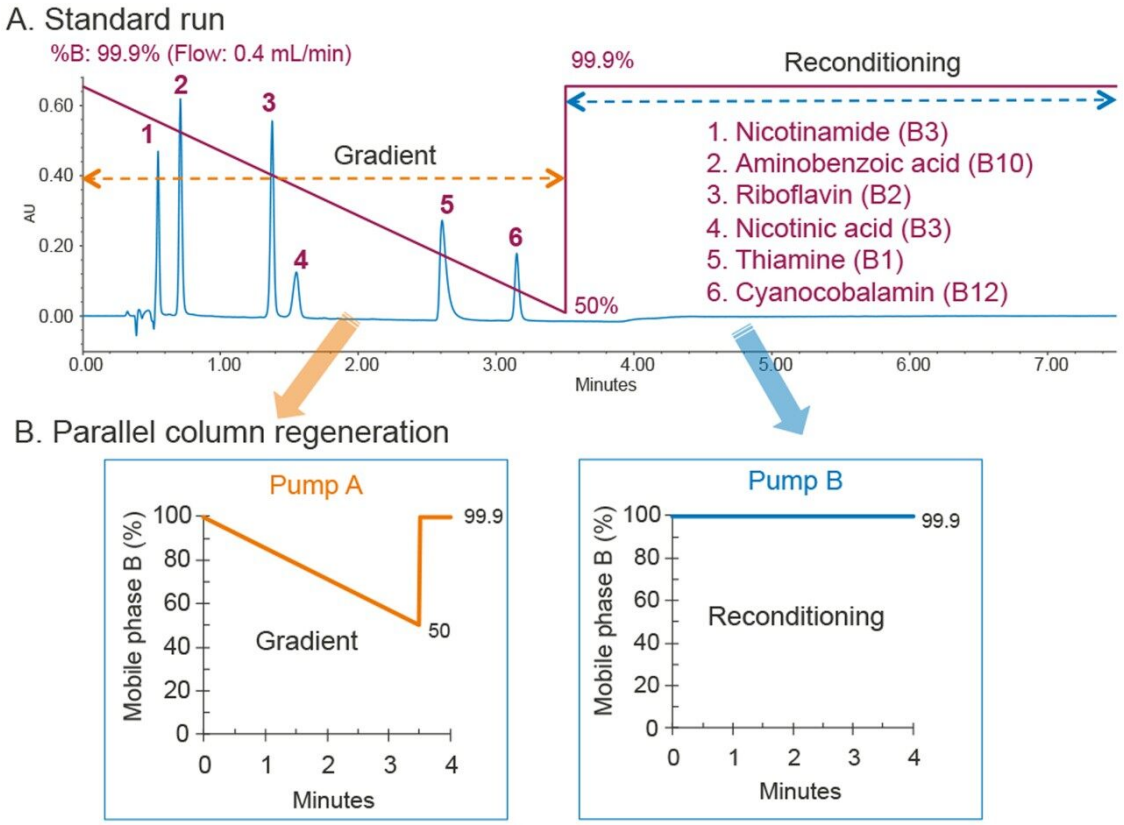


*Figure 1. Illustration of parallel column regeneration strategy for the analysis of water soluble vitamins. A) Standard run (single pump): the HILIC separation of water soluble vitamins consists of two segments, gradient and reconditioning (LC method was modified based on reference [1]; B) Parallel column regeneration (two pumps): one pump (pump A) delivers gradient to one column, while the other pump (pump B) reconditions the other column.*

In parallel column regeneration, the reconditioning segment is excluded from the injection cycle. Thus, the analysis time is shortened while maintaining the separation. For the HILIC separation of water soluble vitamins, as shown above, the run time is 7.5 min using a single pump. With parallel column regeneration, the run time can be shortened to 4.0 min by excluding the reconditioning step from the cycle time. The total run time is then reduced by 47% (Figure 2). Parallel column regeneration is a tool to increase sample throughput, especially for legacy and/or validated methods.

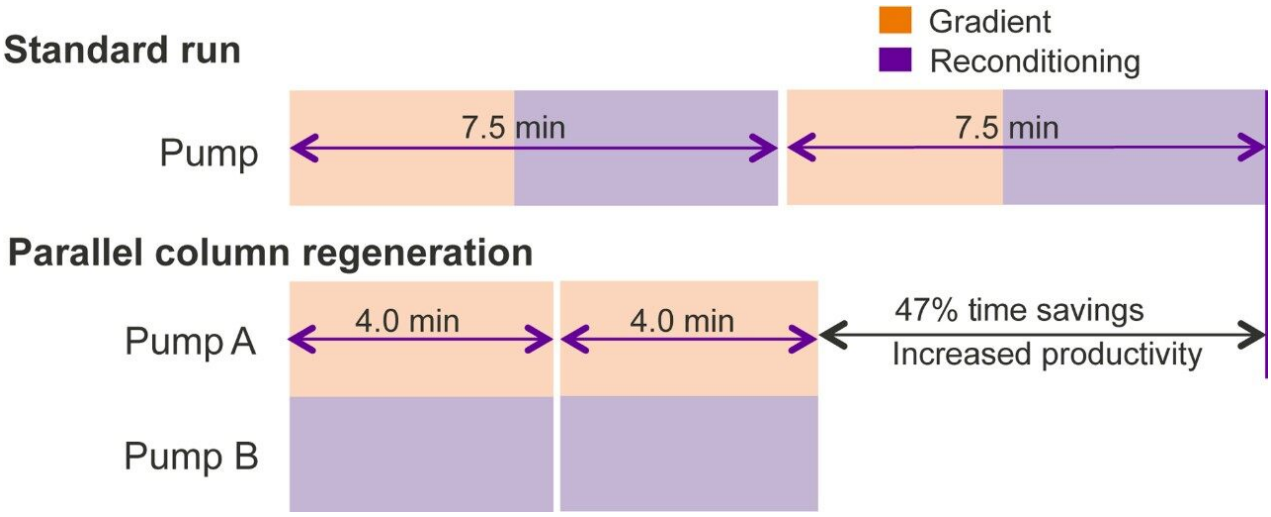


*Figure 2. Demonstration of time savings using two pumps in parallel column regeneration as compared with standard analysis using one pump for the analysis of water soluble vitamins.*

## Parallel column regeneration configuration and workflow

In order to perform parallel column regeneration, an LC system must be equipped with 1) two pumps; 2) two identical columns; 3) switching valves, and 4) identical flow paths for each column. One pump serves as the gradient pump to deliver the mobile phase for separation of the analytes on both columns. The second pump serves as the reconditioning pump for both columns. Figure 3 illustrates the configuration and workflow of the parallel column regeneration.

In Figure 3A, when both switching valves are at position "2", the sample is injected and separated on column 1, while column 2 undergoes reconditioning. At the end of the programmed gradient, the valves switch to position "1" (Figure 3B). Now, the sample is injected and separated on column 2, while column 1 undergoes reconditioning. In order to obtain comparable chromatography on both column 1 and 2, the flow paths from the injector valve to the column and from column to detector need to be identical. This means the capillaries and preheaters need to be the same inner diameter and length. In this study, Waters ACQUITY UPLC System with 2D LC Technology is configured for parallel column regeneration since it can meet all the requirements.[2] All required additional capillaries and parts for this setup are listed in Table 2.

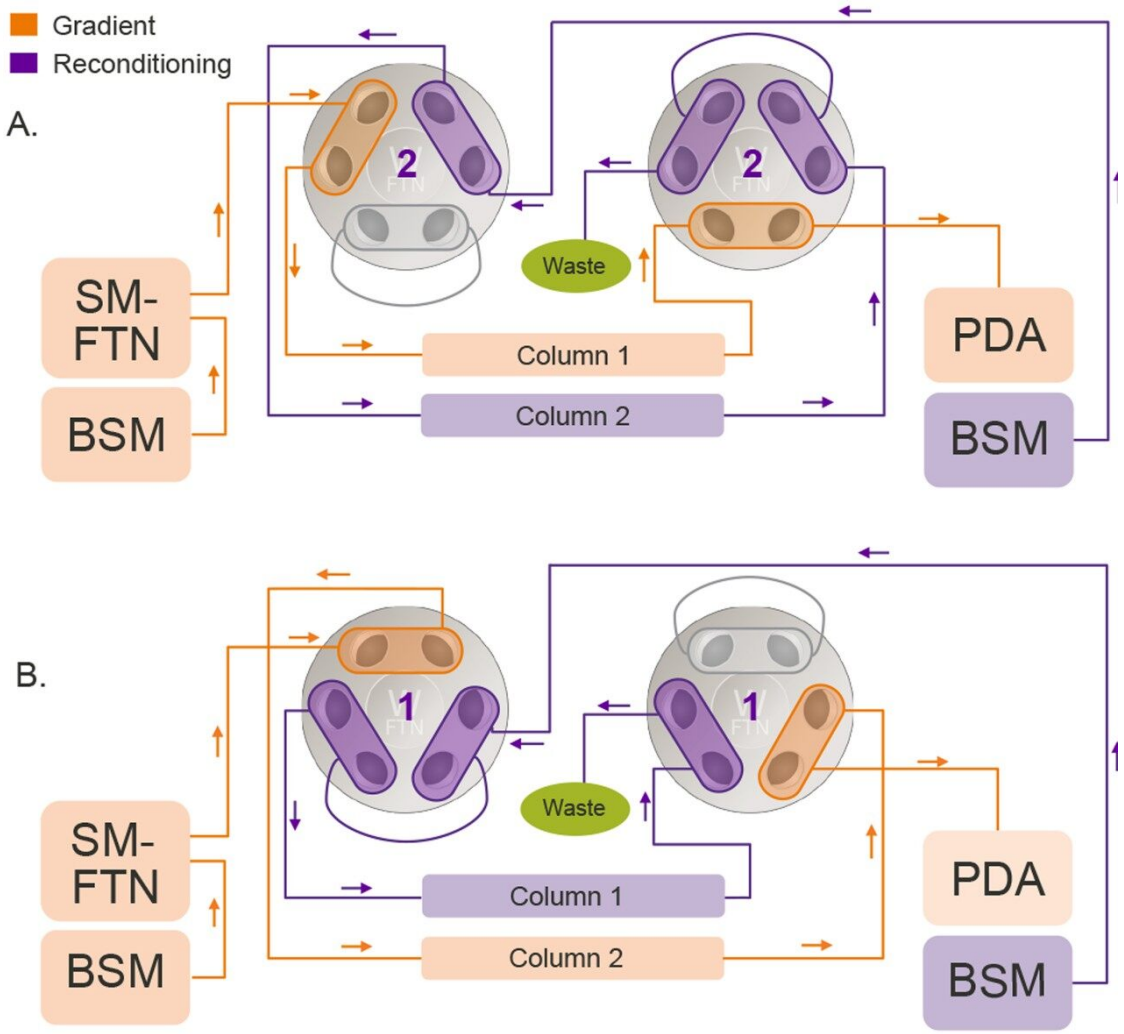


*Figure 3. System configuration and flow schematic for parallel column regeneration. A) Sample is being injected on column 1 while column 2 is being reconditioned; B) At the end of gradient separation on column 1, valves switch positions, sample is being injected on column 2 while column 1 is being reconditioned.*

| Amount | Description | P/N |
|---|---|---|
| 2 | Welded tube, stainless steel, .007 × 6.0 in. (HP/HP) | 430003008 |
| 2 | Welded tube, stainless steel, .004 × 14.5 in. (LP/LP) | 430002681 |
| 2 | ACQUITY UPLC Active Preheater, stainless steel, 12.5 in. – PLUS | 205001774 |

*Table 2. List of additional parts needed for parallel column regeneration.*

## Consideration for column switching

As described above, the valves switch at the end of the gradient of column 1 to perform the next analysis on column 2, or vice versa. Since the same capillaries from the gradient pump to the switching valve are used, this part of flow path must be equilibrated with the initial conditions of the gradient prior to the next injection. If this does not occur, the final gradient composition from previous injections will remain in the capillaries and subsequently be transferred to the freshly equilibrated column, causing poor peak shape and inconsistent retention times (Figure 4A). To avoid this phenomenon, a short equilibration segment can be added at the end of the gradient, allowing for the capillaries to be filled with the initial mobile phase. The time required is dependent on the inlet capillary dimensions and the flow rate. For this analysis, a 0.5 min segment of initial gradient composition was added to the end of the programmed gradient to obtain reproducible results (Figure 4B).

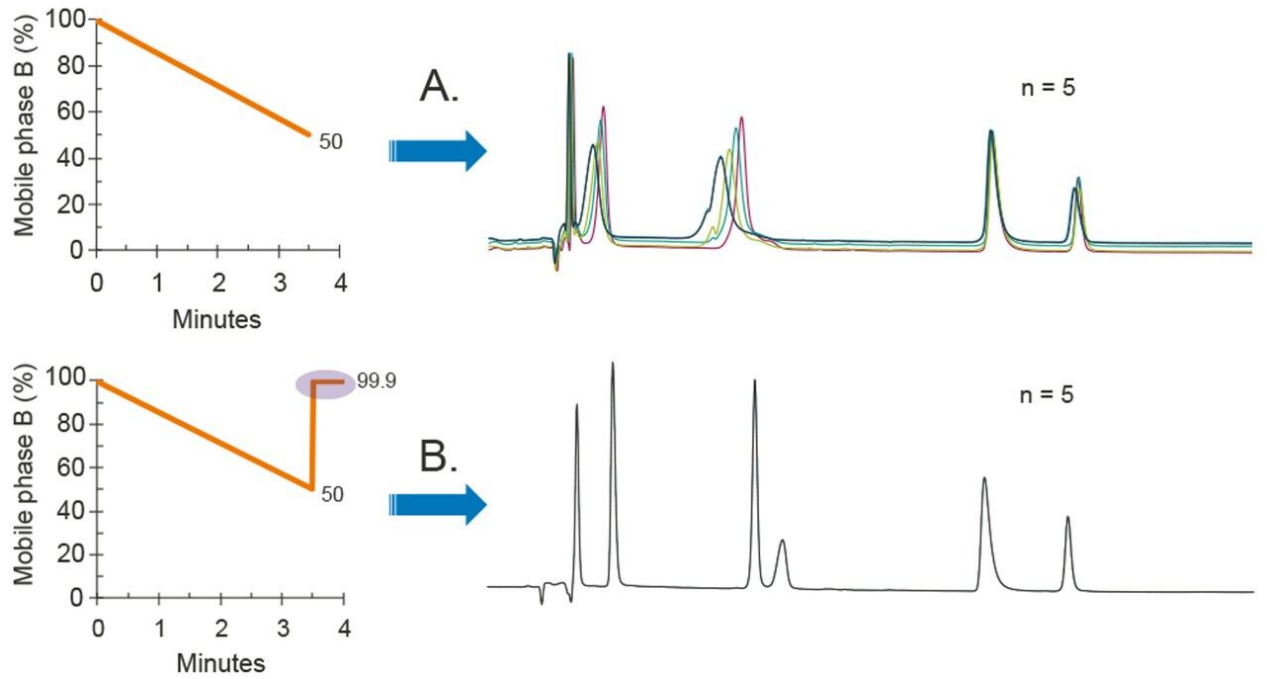


*Figure 4. Impact of a short re-equilibration of initial gradient conditions prior to column switching on data consistency. A) Gradient table ends at 3.5 min and no initial gradient condition is added, resulting in poor chromatograms; B) A 0.5 min initial condition of gradient is added to the end of gradient table and the peak shape and the repeatability of retention times greatly improved.*

## Data comparison of standard run vs. parallel column regeneration

To illustrate the data consistency between a standard 7.5 min run and a 4.0 min run using parallel column regeneration, water soluble vitamins were analyzed on the same column. Analysis of five replicates under both configurations shows the consistency of the data in Figure 5. The data repeatability for retention time and peak area are summarized in Table 3.

For the standard 7.5 min run, the retention time relative standard deviation (RSD) was 0.05%, while the 4.0 min run using parallel column regeneration, the retention time RSD was 0.11%. For peak area, the RSD was identical (0.56%) for both the standard run and the run with parallel column regeneration. There was no significant difference in the data quality observed between these two configurations. Parallel column regeneration demonstrated the same level of reproducibility as standard runs.

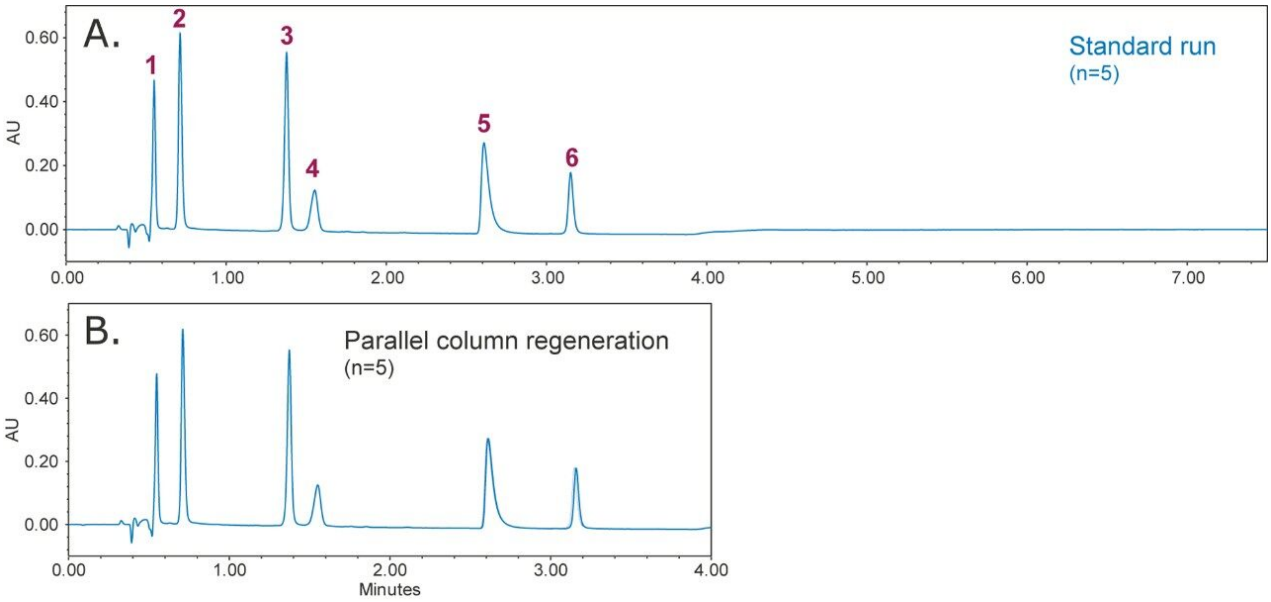


*Figure 5. Overlay of the separation of water soluble vitamins on the same column (n=5). A) 7.5 min standard run; B) 4.0 min in parallel column regeneration.*

| Peak label | Standard (n=5) | | | Column regeneration (n=5) | | | Abs. RT Δ (min) |
|---|---|---|---|---|---|---|---|
| | RT (min) | RT RSD (%) | Area RSD (%) | RT (min) | RT RSD (%) | Area RSD (%) | |
| 1 | 0.550 | 0.00 | 0.58 | 0.547 | 0.10 | 0.70 | 0.003 |
| 2 | 0.712 | 0.06 | 0.78 | 0.710 | 0.08 | 1.08 | 0.001 |
| 3 | 1.378 | 0.08 | 0.11 | 1.372 | 0.10 | 0.08 | 0.006 |
| 4 | 1.553 | 0.08 | 0.40 | 1.548 | 0.11 | 0.46 | 0.005 |
| 5 | 2.610 | 0.03 | 0.62 | 2.609 | 0.11 | 0.27 | 0.002 |
| 6 | 3.151 | 0.07 | 0.86 | 3.153 | 0.18 | 0.74 | 0.002 |
| Average | | 0.05 | 0.56 | | 0.11 | 0.56 | 0.003 |

*Table 3. Data repeatability (retention time and peak area) comparison between standard 7.5 min run and 4.0 min run in parallel column regeneration for the same column.*

## Data comparison from columns 1 and 2 in parallel column regeneration

Data consistency across both columns in parallel column regeneration configuration is critical for routine assays. Overlaid chromatograms from column 1 and column 2 using the parallel column regeneration setup are displayed in Figure 6.

The data repeatability of retention time and peak area for individual columns (intra-column) and across

columns (inter-columns) are summarized  in Tables 4 and 5.

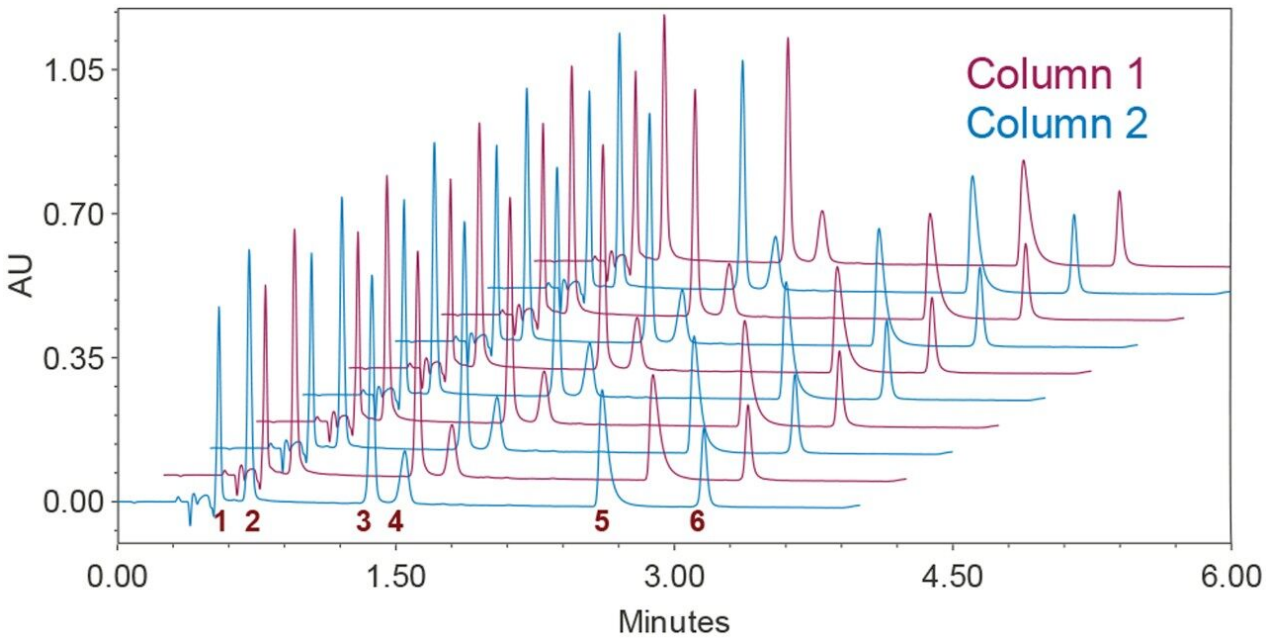


*Figure 6. Overlay (stacked view, Z-axis offset) chromatograms (alternating column 1 and 2) using parallel column regeneration setup.*

| Peak label | Column 1 (n=5) | | Column 2 (n=5) | | Abs. D (min) | Columns 1 and 2 (n=10) RSD (%) |
|---|---|---|---|---|---|---|
| | RT (min) | RSD (%) | RT (min) | RSD (%) | | |
| 1 | 0.548 | 0.08 | 0.547 | 0.10 | 0.001 | 0.12 |
| 2 | 0.704 | 0.12 | 0.710 | 0.08 | 0.007 | 0.50 |
| 3 | 1.368 | 0.08 | 1.372 | 0.10 | 0.003 | 0.16 |
| 4 | 1.552 | 0.07 | 1.548 | 0.11 | 0.004 | 0.17 |
| 5 | 2.634 | 0.10 | 2.609 | 0.11 | 0.025 | 0.52 |
| 6 | 3.146 | 0.17 | 3.153 | 0.18 | 0.007 | 0.20 |
| Average | | 0.10 | | 0.11 | 0.008 | 0.28 |

*Table 4. Intra- and inter-columns, retention time repeatability using parallel column regeneration.*

| Peak label | Column 1 (n=5) Area RSD (%) | Column 2 (n=5) Area RSD (%) | Columns 1 and 2 (n=10) RSD (%) |
|---|---|---|---|
| 1 | 0.73 | 0.70 | 1.36 |
| 2 | 0.06 | 1.08 | 0.72 |
| 3 | 0.15 | 0.08 | 0.59 |
| 4 | 0.45 | 0.46 | 1.61 |
| 5 | 0.61 | 0.27 | 0.52 |
| 6 | 0.48 | 0.74 | 0.61 |
| Average | 0.41 | 0.56 | 0.90 |

*Table 5. Intra- and inter-columns, peak area repeatability using parallel column regeneration.*

Retention time RSD values below 0.28% were achieved across columns in parallel column regeneration compared to 0.10% and 0.11% for each single column. The average absolute retention time shift between column 1 and column 2 was 0.008 min. For peak area, the RSD from column 1 was 0.41% and 0.56% for column 2, while across columns, the RSD of peak area was 0.90%. The chromatographic data quality is not compromised in parallel column regeneration, especially considering column-to-column variability. The low RSD of peak area demonstrates the suitability for quantitative data analysis using the parallel column regeneration setup.

## Conclusion

The ACQUITY UPLC System with 2D LC Technology, equipped with two pumps and switching valves in the column manager, can be configured to perform parallel column regeneration to increase sample throughput. In parallel column regeneration, the reconditioning step is excluded from the run time, while the original gradient separation method is not altered. Thus, the quality of the separation is maintained, and the analysis time is decreased.

For water soluble vitamins, the run time was decreased by 47% using parallel column regeneration, with no impact on the chromatographic data quality.

## References


1. ACQUITY UPLC BEH Amide Columns, Waters Product Solution, 720003122EN, 2009 Oct.
2. Root D and Claise P. Increasing Sample Throughput Using the ACQUITY UPLC System with 2D Technology and Parallel Column Regeneration. Waters Technology Brief, 720004598EN. 2013 Mar.


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