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Improving Assay Robustness and Increasing Sensitivity with Trap and Back-Flush Elution using an ACQUITY UPLC System with 2D Technology

INTRODUCTION

Assay robustness can be improved, and sensitivity increased, by performing a trap and back-flush elution using a Waters ACQUITY UPLC® System with 2D Technology. This technique involves injecting a sample onto a small-volume column where analytes of interest are concentrated before elution onto an analytical column where they are separated prior to detection. The trapping process reduces sample matrix interferences from reaching the analytical column and detector, optimizing the sensitivity and selectivity of the assay and improving robustness. In addition, concentrating the sample onto the trapping column allows for larger injection volumes compared to a standard one dimensional experiment, thus increasing sensitivity.

This document provides a step-by-step approach to creating a two-dimensional reverse phase/reverse phase trap and back-flush elution method, from a standard one-dimensional method, involving just the analytical column.

PROCEDURE

The typical steps of a trap and elute analysis are shown below:

- Load sample
- Trap sample
- Wash sample on trapping column
- Valve switch
- Elute sample in reverse direction to analytical column
- Analyze sample
- Valve switch
- System Reset (Regenerate system, Re-equilibrate system)
- Repeat

The sample is first loaded onto the system, analytes are trapped on the trapping column and the column is washed. At the end of the wash, the valve switches, and the compound(s) of interest are eluted in the reverse direction, onto the head of the analytical column. The separation is then performed, the samples are analyzed, and then the valve is switched to its original position allowing the pumps and columns to regenerate and re-equilibrate. At this point the system is ready for another sample.

MATERIALS

System configuration

ACQUITY UPLC System with
2D Technology comprised of:

- Waters HP Direct Connect 2.1 x 30 mm trapping column, 10 or 20 µm particle size depending on the chemistry (XBridge™ C_{18}, XBridge C_8 or Oasis® HLB) – a small-volume column where the analytes of interest are concentrated
- Alpha pump – Synchronized with the Autosampler and can be either an ACQUITY UPLC Quaternary Solvent Manager (QSM) or an ACQUITY UPLC Binary Solvent Manager (BSM)
- Beta pump – Performs the analytical separation and is always a BSM
- ACQUITY UPLC Sample Manager with Flow-Through Needle (SM-FTN)
- ACQUITY UPLC Column Manager with Active Preheating (CM-A) with two 6-port, 2-position valves
- Xevo® TQ MS

The flow path

Following the flow path of a system configured to perform a trap and back-flush elution method is critical to conceptually understanding the process. Figures 1 and 2 are plumbing diagrams of an ACQUITY UPLC System with 2D Technology configured for a trap and back-flush elution method. The system pictured has two pumps, Alpha and Beta, an ACQUITY UPLC Sample Manager with Flow through Needle (SM-FTN), an ACQUITY UPLC Column Manager with Active preheating (CM-A) with two 6 port, 2-position valves and a Xevo TQ MS. In each figure the flow path of the Alpha pump is highlighted in red and that of the Beta pump in blue. For plumbing and tubing directions, consult the ACQUITY UPLC with 2D Technology Capabilities Guide.

Figure 1 displays the flow path of the system in the trap state. Flow from the Alpha pump passes through the SM-FTN, into the valve, then out to the trapping column. After passing through the trapping column, the flow returns to the CM-A valve and is then diverted to waste. At this same time, the Beta pump flow moves from the Beta pump to the valve, through the analytical column and into the detector.

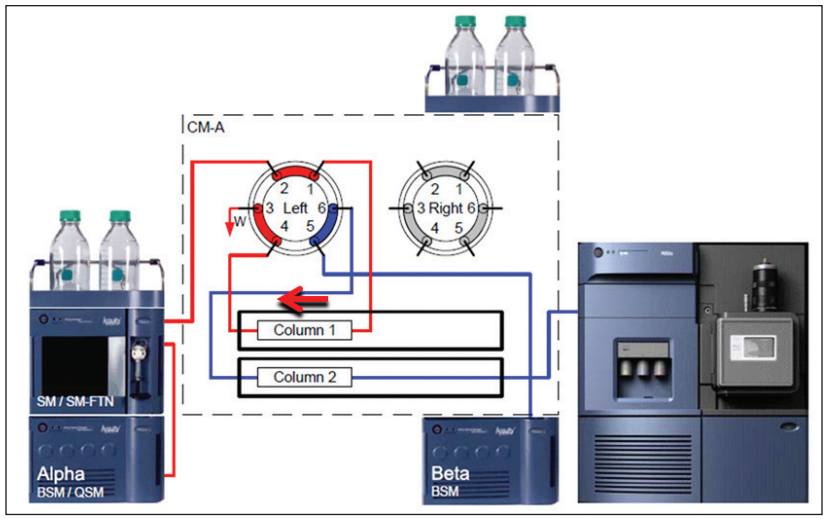

Figure 1. Trap and back-flush elution plumbing diagram. The red highlight is the flow path from the alpha pump when the valve is in the load position. The blue highlight is the flow path from the beta pump.

Figure 2 displays the flow path of the system in the elute state. The Alpha pump flow now passes through the SM-FTN, to the CM-A valve and then to waste. The Beta pump flow moves through the CM-A valve, through the trap column in the reverse direction, through the analytical column and finally into the detector.

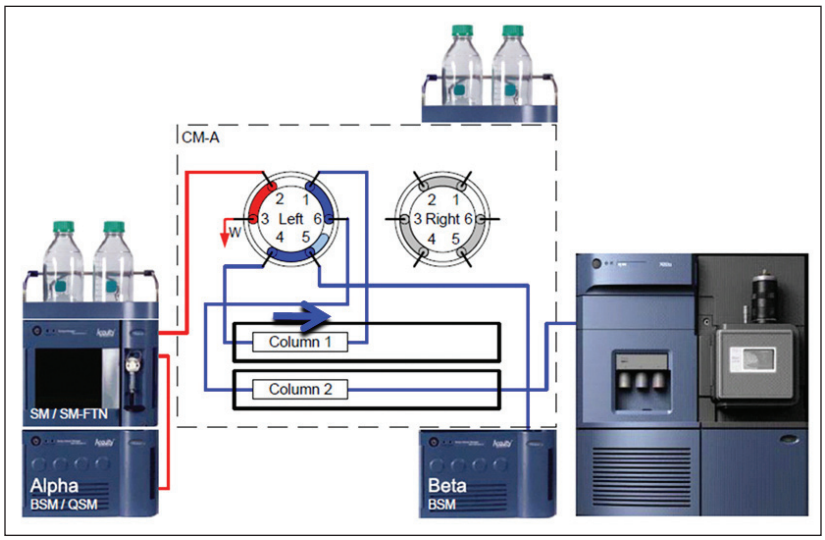

Figure 2. Trap and back-flush elution plumbing diagram. The red highlight is the flow path from the alpha pump when the valve is in the elute position. The blue highlight is the flow path from the beta pump. Flow is reversed through the trapping column at this point.

SYSTEM PROCEDURES

Following is a step-by-step look at the specifics of each of the trap and back-flush elution procedures, which sets the stage for purposefully development of effective methods.

Load and trap sample

Rapid loading and trapping of a sample occurs in the Alpha pump flow path. The particle sizes of Waters HP Direct Connect 2.1 x 30 mm trapping columns are 10 and 20 µm depending on the chemistry (XBridge C_{18}, XBridge C_8, or Oasis HLB), so high flow rates can be used.

In addition, the small column volume of roughly 90 µL allows for short loading times. For example, at a flow rate of 1 mL/min with a reverse phase 2.1 x 30 mm column, passing five column volumes through will take roughly 30 seconds. To best facilitate the trapping process, the column, mobile phase organic strength, and pH must maximize retention of the desired analyte(s). If the organic strength of the mobile phase or sample diluent is too high, or too many column volumes are passed through the column, the analytes may begin to separate and elute from the trap.

The best conditions are optimized per experiment. In addition, the small column volume produces sharp peaks after concentrating larger injection volumes when compared to a standard one-dimensional method, as shown in Figure 3.

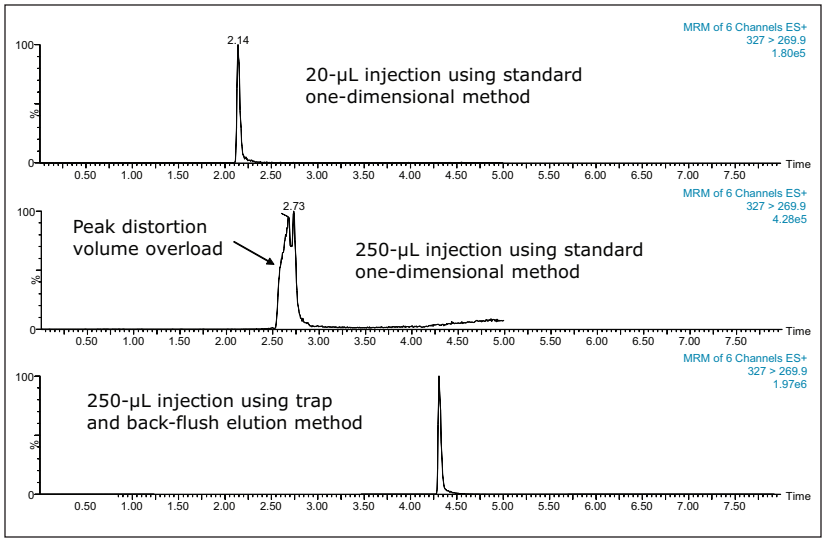

Figure 3. MRM chromatograms comparing a 20-µL injection using a standard one-dimensional method, a 250-µL injection using a standard one-dimensional method, and a 250-µL injection using a trap and back-flush elution method.

Washing the trapping column

When the trapping column is washed, the desired result is to retain the analyte(s) of interest while allowing the hydrophilic contaminants to flow to waste. For reversed phase trap and back-flush elution, the general approach is to load, trap, and wash at low organic strength, such as 100 to 95% aqueous for five to 10 column volumes. In situations where an analytical method is available, another approach can be to load and trap at 100 to 95% aqueous, then step to the initial conditions of the established method and flush for five to 10 column volumes. This step can be optimized by experimentation.

Elute sample

The valve is switched and now both the trapping column and analytical column are in the Beta pump flow path. The direction of flow through the trapping column is the reverse of that during loading, trapping, and washing. The valve switch occurs after the washing step is complete. Prior to the valve switch, both the trapping column and the analytical column must be in initial strength mobile phase. The Alpha pump is running only to waste at this time, so lowering the flow rate will conserve mobile phase.

Analyze sample

Once the valve is turned, the analyte(s) of interest will elute off of the trapping column onto the head of the analytical column. If an established reversed phase MS-friendly method is available, this is when it will start. If no method is available, a conservative approach would be to run a 5 to 95% organic gradient over five column volumes (for a 2.1 x 50 mm column, this value would be roughly 0.85 mL). Whatever the method, this is where the analyte(s) of interest are separated from any matrix effects. If needed, a typical LC/MS method development might have to occur prior to the trap and back-flush elution method development.

Regenerate and re-equilibrate the system

At the end of the separation method, the valve will turn and the system will return to the flow condition of Figure 1. The Alpha pump flow will include the trapping column and the Beta pump flow, the analytical column. Each flow path can be regenerated and re-equilibrated independently to their respective initial conditions. At the completion of this step, the system is now ready for another sample.

PLANNING THE TRAP AND ELUTE EXPERIMENT

For those who have little to no experience with the setup of trap and back-flush elution or other 2D scenarios, a conservative and systematic approach is suggested. By following this approach, the correct system plumbing, the appropriate process steps, and properly-timed event and gradient tables will result.

The storyboard

The first step in planning a trap and back-flush elution experiment involves the use of the storyboard. Figure 4 shows a blank example of a worksheet that can be used as part of the storyboard. The three column segments are: time, action (or step in the process), and connections and position, which shows a six-port, two-position valve and schematic of the column positions of the CM-A.

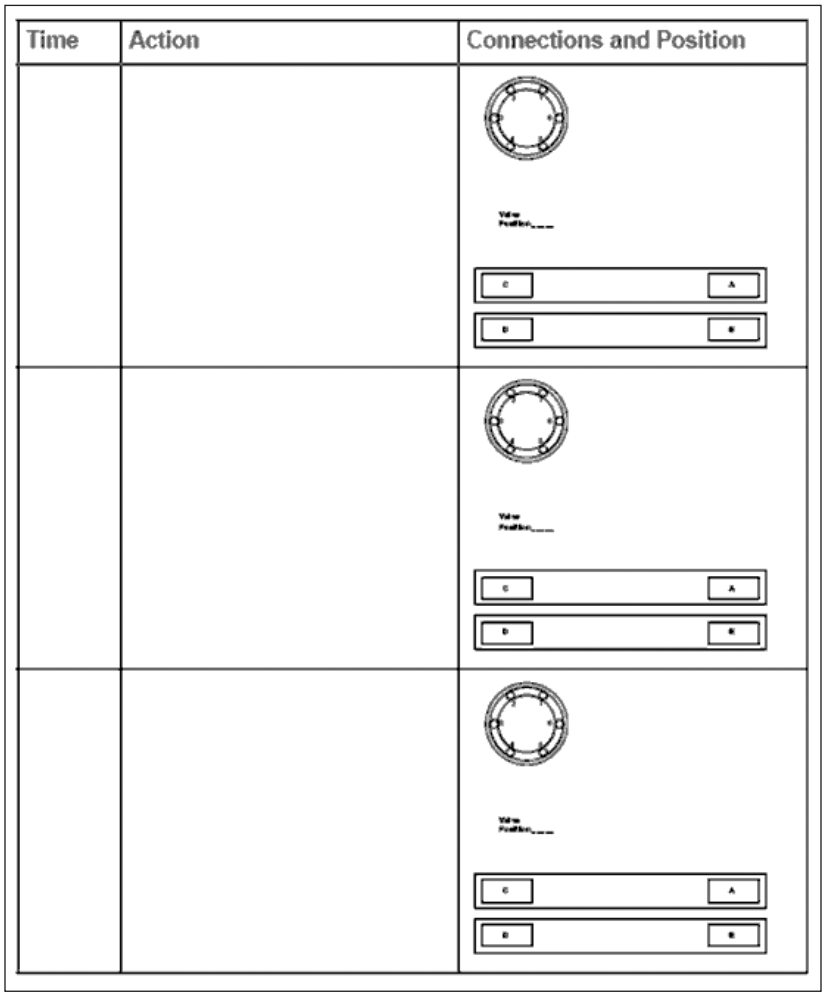

Figure 4. Trap and back-flush elution storyboard worksheet.

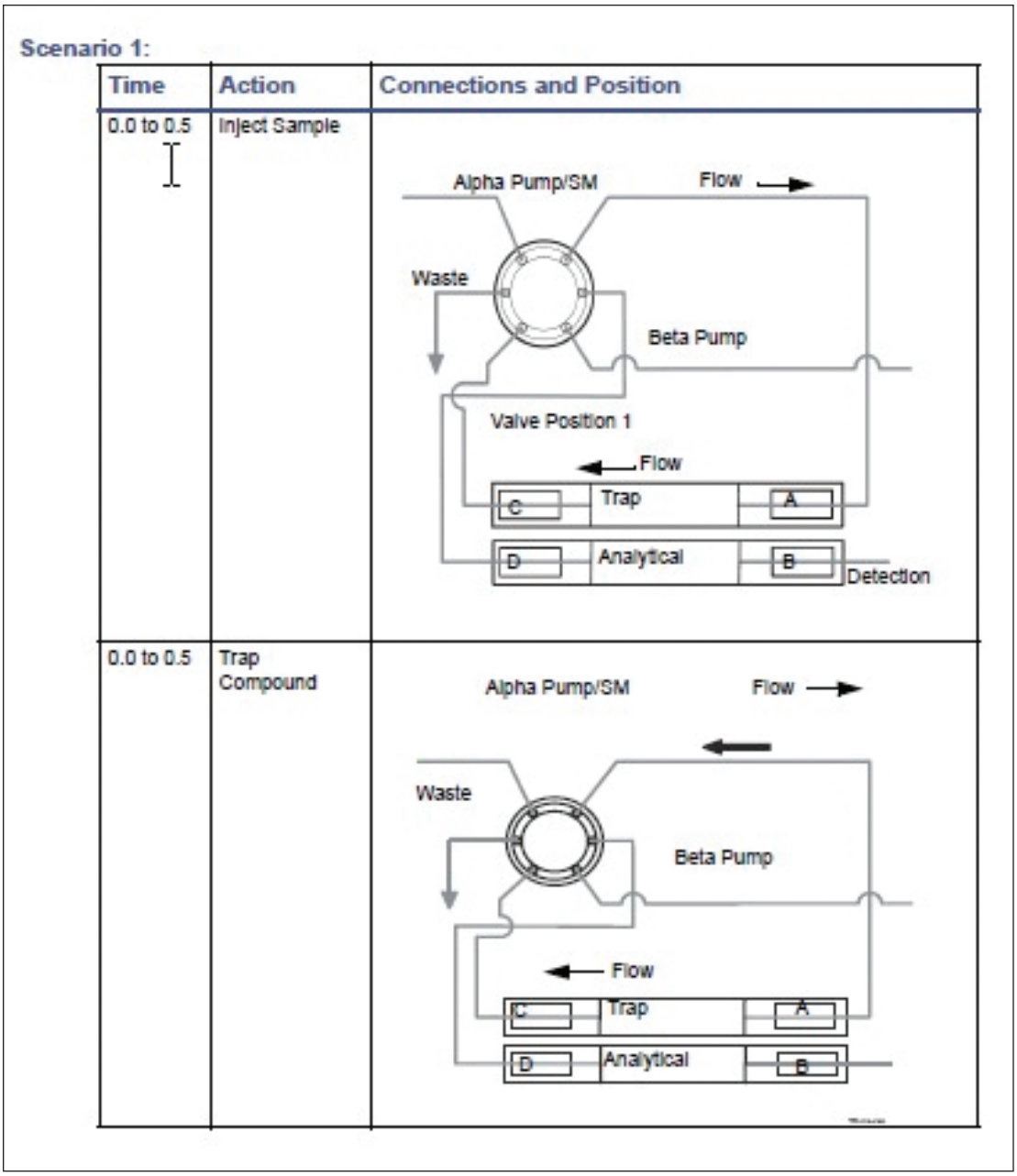

Figure 5. One page of a completed storyboard.

The steps of the trap and elute are qualitatively described in the action column and the associated flow path plumbing and valve state are drawn in the connections and position column. Time is less important at this step in the planning.

An example of a completed storyboard is shown in Figure 6. Note the complete plumbing information of the flow paths and flow directions as well as the respective column placement. The storyboard step can be crucial in the effective conception, set up and execution of a trap and back-flush elution or other 2D experiment.

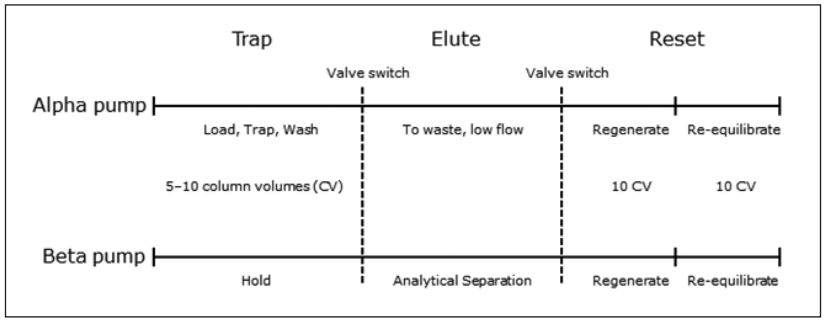

Figure 6. Parallel timelines for the alpha and beta pumps during a trap and back-flush elution method.

The trap and back-flush elution timeline

Proper event timing is critical to an effective trap and back-flush elution method. The timelines in Figure 6 display the parallel activities of the pumps and valve during a typical trap and back-flush elution experiment. It is divided into three phases covering the entire process: trap, elute, and a reset of the system. While the individual steps of the process have been described above, this figure displays their relation in time.

In the trap phase, the Alpha pump is involved in loading, trapping, and washing. Simultaneously, the Beta pump is holding at initial conditions of the analytical separation. During the elute phase, as the Beta pump drives the analytical separation gradient, the Alpha pump is flowing to waste.

During the reset phase, both pumps are separately regenerating and re-equilibrating. Valve switching occurs at the end of the wash of the Alpha pump and at the end of the separation gradient of the Beta pump. To help derive the actual times associated with the events portrayed in the figure, the suggested column volume information from the earlier sections is included.

The overall development will involve three general steps:

1. Complete the storyboard. Outline the steps of the trap and back-flush elution protocol. Clearly plan and draw the plumbing for the configuration. Refer to Figures 4 and 5 for the storyboard and Figures 1 and 2 for plumbing.
2. Design the experiment. Refer to the trap and back-flush elution timelines. Select mobile phase composition, flow rate, and calculate the length of each segment of the process for each of the pumps.
3. Transfer to software. Populate the gradient tables in MassLynx™ Software and column manager events table with the calculated values.

EXAMPLE

A user wishes to achieve better assay robustness and sensitivity with a standard one-dimensional method and will go through the process of converting the method to a trap and back-flush elution method. The gradient table of the standard method is shown in Figure 6. This method contains a 2-minute gradient from 5 to 70% organic, with a minute hold and 3-minute re-equilibration. Mobile phase A is water with 0.1% formic acid, and mobile phase B is acetonitrile with 0.1% formic acid. The analysis uses an ACQUITY UPLC BEH C_{18} 2.1 x 50 mm, 1.7-µm column.

The step-by-step process to convert the standard method to a trap and back-flush elution method follows. Note that the method developed here is very conservative and can be optimized by careful experimentation once in place.

1. **Complete the method development storyboard**

   This will clarify setup of the system. Refer to Figures 5, 6, and 7. A complete storyboard will not be a part of this document.

2. **Design the experiment**

   Using the trap and back-flush elution method development timeline as a guide, the steps that follow will determine the parameters below:

   - Flow rates for both pumps
   - Mobile phase compositions
   - Segment times for each phase of the trap and back-flush elution method
   - Proper timing of valve switching

   a. ***Select the trap phase:*** Select mobile phase composition, flow rate, and calculate segment time.

   Alpha pump:

   Mobile phase composition: Two choices are available Either 1) load, trap, and wash at one low organic strength mobile phase composition; or 2) load and trap at a low organic then wash at the initial organic strength of the analytical method.

   - Load, trap, wash – 95% aqueous for 5 CV

   Or:

   - Load, trap – 100% aqueous for 5 CV
   - Wash – 95% aqueous for 5 CV

   Note: Column volume = π (column radius)^2 X column length. This can also be determined using the online ACQUITY UPLC Column Calculator.

   Flow rate: The larger particle sizes of the Waters HP Direct Connect trapping columns (10 and 20 µm) allow higher flow rates; try 1.0 mL/min.

   Segment time: To calculate, use the flow rate and suggested column volumes:

   - 5 CV ≈ 0.5 mL (rounding up)
   - Segment time = column volumes/flow rate = 0.5 mL/1.0 mL/min = 0.5 min

Final parameters for Alpha pump:

- Flow = 1.0 mL/min
- Mobile phase composition = Load, trap, and wash at 95% aqueous
- Segment time = 0.5 min

Beta pump:

The Beta pump is holding at the initial conditions of the analytical method (Figure 5).

Valve switch event:

The first valve switch occurs at the end of the wash step.

- Valve switch event time = 0.5 min

b. ***Select the elution phase:*** Again, select mobile phase composition, flow rate, and segment time.

Alpha pump:

At this phase the Alpha pump is running to waste. Flow can be lowered to conserve mobile phase.

- Mobile phase composition = 100% organic; can begin pump regeneration
- Flow = 0.1 mL/min
- Segment time = length of analytical separation gradient and hold in minutes

Beta pump:

The Beta pump delivers the separation gradient and hold. Refer to the original gradient table in Figure 7.

- Mobile phase composition = gradient from 5% to 70% organic with hold
- Flow = 0.65 mL/min
- Segment time = 2 min gradient + 1 min hold = 3 min

Valve switch event:

The second valve switch occurs at the end of the hold of the analytical separation and returns the valve to its initial position.

- Valve switch event time = 0.5 min (load, trap, wash) + 3.0 min (separation) = 3.5 min

Time	Flow	%A	%B	Curve
0.0	0.5	95	5	-
2.0	0.5	30	70	6
3.0	0.5	30	70	6
6.0	0.5	95	5	1
30	0.0	95	5	11

Figure 7. Standard one-dimensional method gradient table.

c. **System Reset:** In this phase there are two steps: regeneration and re-equilibration. The regeneration step removes hydrophobic compounds that may remain following the completion of the analytical separation gradient. The re-equilibration step returns the individual pumps and columns to initial conditions and readies them for the next injection. Ten column volumes for each phase is a good starting point. There are no valve switch events.

Regeneration

Alpha pump

- Mobile phase composition = use high organic, 95%
- Flow = same as the trap phase, 1.0 mL/min
- Segment time = start with 10 CV, 10 CV ≈1 min for the trap column

Beta pump

Since the separation gradient only goes up to 70% organic, a higher organic wash will be needed to remove residual hydrophobics from the analytical column.

- Mobile phase composition = 95% organic
- Flow = 0.65 mL/min, that of the analytical separation
- Segment time = 10 CV at 0.65 mL/min
- 1 CV of the 2.1 x 50 mm BEH column ≈ 0.17 mL, so 10 CV ≈1.7 mL
- Segment time = 1.7 mL/ 0.65 mL/min = 2.6 min

Re-equilibration

Alpha pump

- Mobile phase composition = 100% aqueous, initial conditions of the Alpha pump and trap column
- Flow rate = 1.0 mL/min
- Segment time = 1 min (same as regeneration)

Beta pump

- Mobile phase composition = 95% aqueous, initial conditions of the Beta pump and analytical column
- Flow rate = 0.65 mL/min
- Segment time = 10 CV = 2.6 min (the same as regeneration above)

3. **Transfer to software**

The timing of all segments and events is summarized in Figure 8. These values, with mobile phase and flow rate information, can now be transcribed into the gradient tables and column manager events table of MassLynx Software. Figure 9 displays the completed gradient and events table. Note that the parameters resulting from this process are very conservative and can be optimized through thoughtful experimentation.

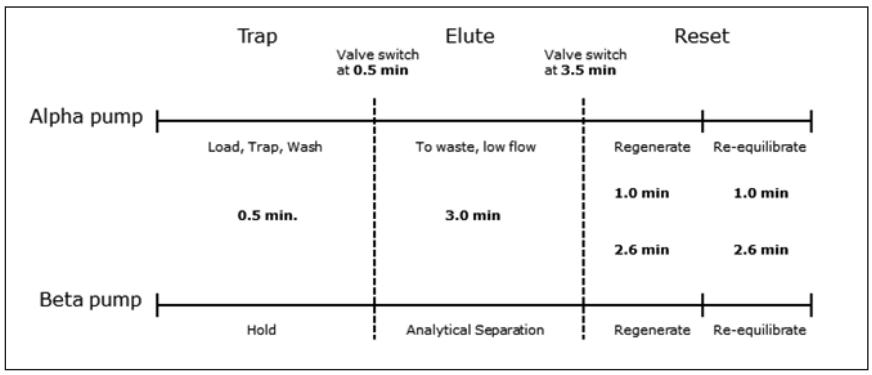

Figure 8. Trap and back-flush elution timeline with calculated times for each segment and valve switch.

Time	Flow	%A	%B	Curve
0	10	100	0	–
0.5	10	100	0	11
0.6	0.1	0	100	11
3.5	0.1	0	100	11
8.7	10	100	0	1
30	0.0	100	0	11

Time	Flow	%A	%B	Curve
0	0.5	95	5	–
0.5	0.5	95	5	6
2.5	0.5	30	70	6
3.5	0.5	30	70	6
6.1	0.5	5	95	1
8.7	0.5	95	5	1
30	0.0	100	0	11

Time	Event	Action
0.5	Left valve	Position 2
3.5	Left valve	Position 1

Figure 9. Completed gradient and event tables for the trap and back-flush elution method.

Results

Chromatograms of both the standard one-dimensional method and the trap and back-flush elution method are shown in Figure 10. The one-dimensional standard method was run on a system containing a Quaternary Solvent Manager (QSM) while the trap and back-flush elution method used a Binary Solvent Manager (BSM) as the analytical pump (Beta pump). The observable difference in the retention times is a reflection of the decreased dwell volume of the BSM. Precursor scans for the most abundant interferences of the standard one dimensional method and the trap and back-flush elution method are shown in Figure 11. The trap and back-flush elution method reduces very hydrophobic interferences and contaminants from entering the analytical column and the detector, improving assay robustness.

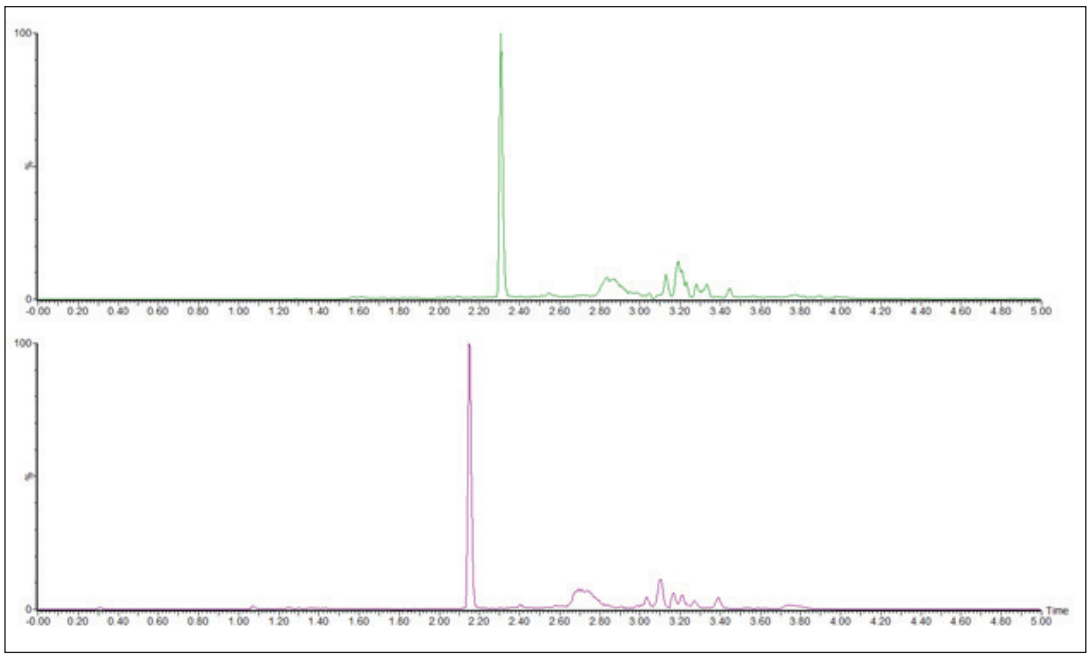

Figure 10. MRM chromatograms of the standard one-dimensional method (green) and the trap and back-flush elution method (purple).

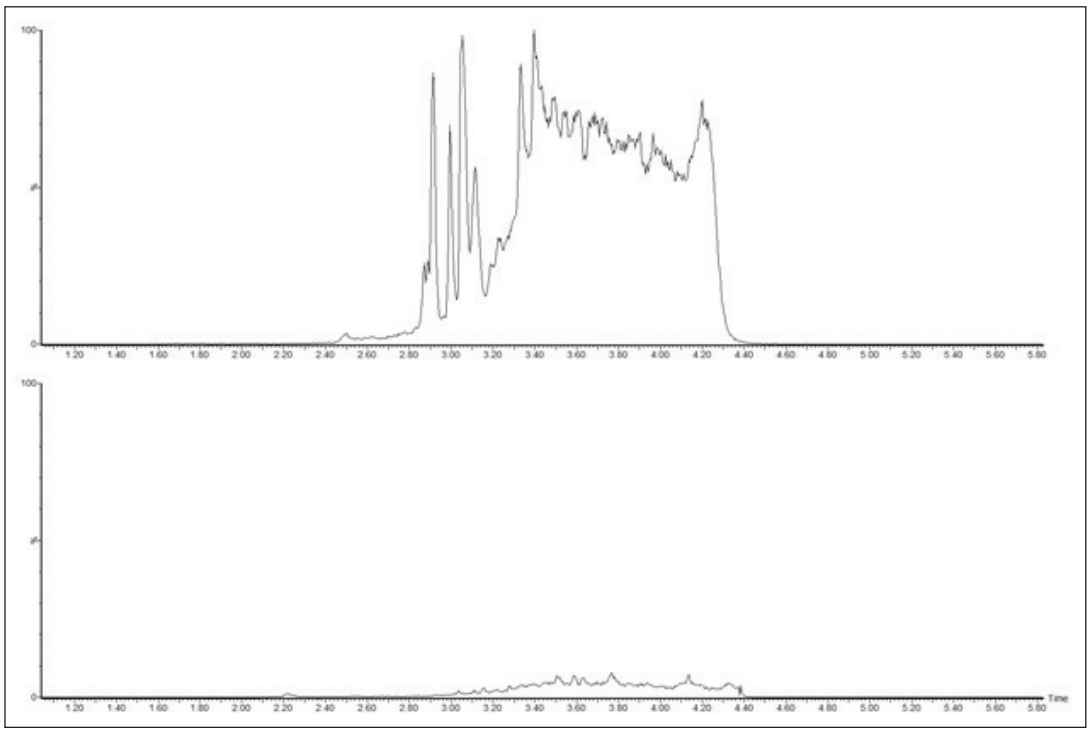

Figure 11. Precursor scans for the most abundant interferences for the standard one-dimensional method, above, and the tap and back-flush elution method below.

CONCLUSIONS

Assay robustness can be improved and sensitivity increased using a trap and back-flush elution method to increase the amount of analyte reaching the detector, which also prevents hydrophobic interferences and contaminants from entering the analytical column and the detector, improving assay robustness. This guideline is an effective tool to transfer a standard one-dimensional method to a trap and back-flush elution method using an ACQUITY UPLC System with 2D Technology.

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June 2012 720004396en AG-PDF

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