OPTIMIZING THE COMBINATION OF MULTI-SEGMENT, MULTI-DIMENSIONAL SEPARATIONS WITH PHOTODIODE ARRAY AND MASS SPECTROMETRY DETECTION

Thomas E. Wheat, Amanda B. Dlugasch, Patricia R. McConville
Waters Corporation, Milford, Massachusetts, USA

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INTRODUCTION

Multi-dimensional separations can provide a solution to the fundamental limits on chromatographic resolution, particularly when the separation steps use orthogonal mechanisms for selectivity. This approach can be particularly powerful when combined with information-rich detection. The detection becomes more useful when the multiple detectors respond to different properties of the analytes. For the system to provide the greatest resolution and information, both the chromatographic steps and the detection must be chemically compatible and must not impose time constraints. We describe an instrument configuration to approach these objectives. The system includes two UPLC gradient pumps, and a pair of valves that are used to divert one or more segments of a first dimension chromatogram to a holding facility, either a chromatographic trap or an empty loop. The system includes the capacity to hold multiple segments before beginning the second stage of analysis. Each trap cartridge is eluted in turn onto a second column and into the detectors. In this arrangement, maximum resolution is possible in both dimensions because the cycles and timing of the two separations are independent. At-column dilution is available at each chromatographic step to establish compatibility between the successive chromatographic modes and to ensure optimum detectability. The system function is illustrated with separation of small molecules and with monoclonal antibodies. The small molecules were separated using low pH reversed-phase followed by high pH reversed-phase with a set of C18 trap cartridges as the holding facility. The monoclonal antibodies were separated using ion exchange followed by SEC. Between dimensions, the protein peaks were isolated in lengths of empty tubing to ensure good recovery of the proteins. This system reliably combines multiple modes of chromatography and detection.

Samples:

Reversed Phase Test Mix:
0.45 mg/mL Sulfadimethoxine, 0.45 mg/mL Terfenadine, 0.45 mg/mL Reserpine, 0.45 mg/mL Acetaminophen, 0.45 mg/mL Caffeine, 90ug/mL Acetamidophenol, 90ug/mL Acetanilde, 90ug/mL Acetylsalicylic Acid, 90ug/mL Phenacetin, 0.20 mg/mL Salicylic Acid, 0.20 mg/mL 3-benzoylpyridine, 0.20 mg/mL Cortisone, 0.20 mg/mL 4-nitroaniline,0.20 mg/mL 4,4'-biphenol

Monoclonal antibody:
Infliximab Formulation, diluted to 1 mg/mL with PBS

Columns:

For Small Molecule Reversed-phase:

ACQUITY UPLC BEH C18, 130Å, 1.7 µm, 2.1 mm X 50 mm
XBridge BEH C18 Direct Connect HP Column, 130Å, 10µm, 2.1mm X 30 mm

For Protein A > Size Exclusion:

Poros Protein A 2.1mm x 30mm
MassPREP Micro Desalting Column
ACQUITY UPLC Protein BEH SEC 200Å, 4.6mm x150mm

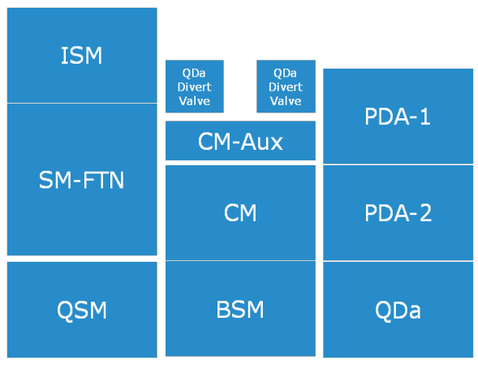

Figure 1. ACQUITY UPLC Multi-Dimensional System

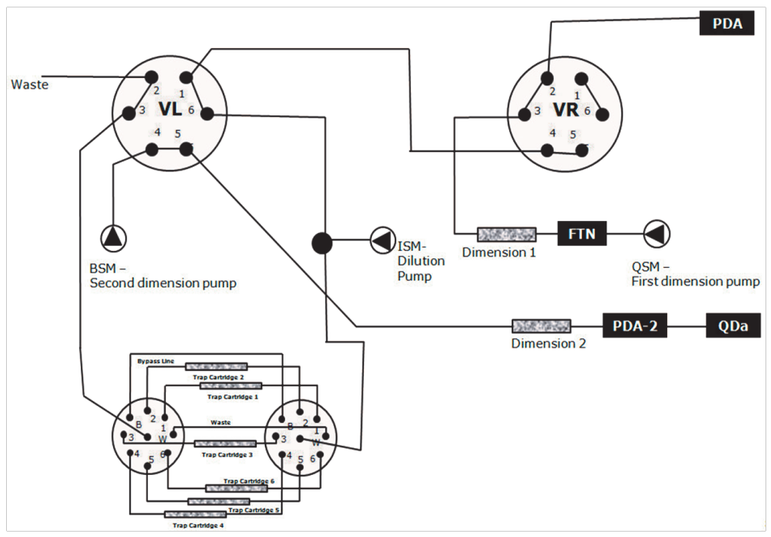

Figure 2. Fluid diagram for multiple segment with At-column Dilution system

The sample is delivered to the first chromatographic column and eluted with the first dimension mobile phase. At the time the desired peak is to be collected, the system valves are switched in order to direct this portion of the sample from the first dimension to a sample loop or trap cartridge. While being transferred, the eluant is diluted with flow from the At-column dilution pump and the components from the first dimension segment are held in the loop or trap cartridge. The At-column dilution function ensures that the selected sample components are retained as a tight band at the entrance to the trapping cartridge. The diluent is chosen to increase retention and usually includes dilution with water, which may also adjust the pH or add ion pairing. Once the peak is collected on the loop or cartridge, the valves switch back and the first dimension separation continues. As soon as the next desired peak is reached on the first dimension, the valves are switched again in which the second sample component is collected in a different sample loop or cartridge. The process is repeated for the number of desired peaks to be collected from the sample. Once all the peaks have been collected on the loop or cartridges, the system valves are switched so that the second dimension pump delivers the desired separation gradient of increasing organic to elute the trapped analytes. As the analytes elute from the cartridge, they are diluted by the flow from the dilution pump. The diluent is a composition that ensures the components are binding as a narrow band to the head of the second chromatographic column. The gradient continues to elute the analytes from the second dimension column into the PDA and MS detectors.

Monoclonal Antibody Example

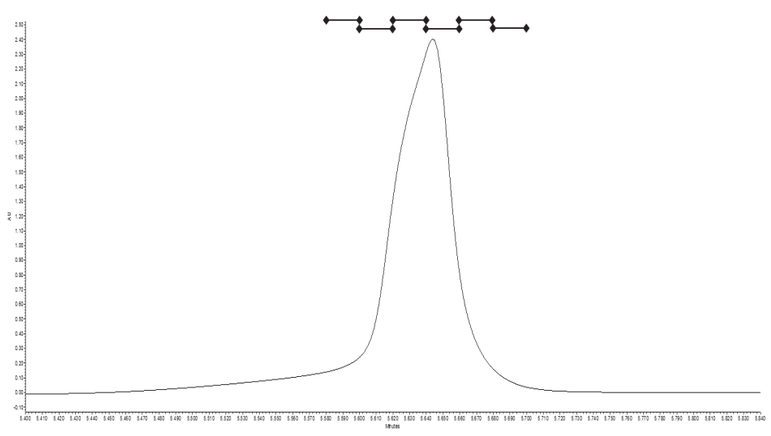

Figure 3. Infliximab first dimension separation. The monoclonal antibody elutes from the Protein A column. Six segments of the peak that were collected for second dimension analysis are shown. Each timed segment is 0.02 minutes. Each segment was analyzed on the second dimension SEC column as shown in Figure 4

RESULTS

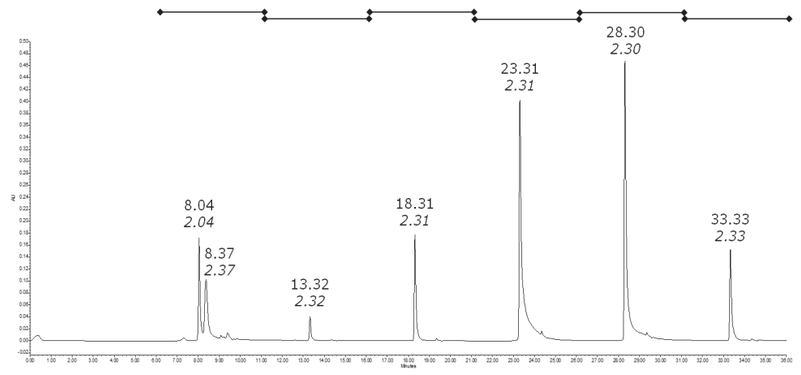

Figure 4. Second Dimension separation chromatogram of the six segments of the infliximab Protein A peak. Note that the analysis corresponding to the less retained material has a higher proportion of aggregates

Small Molecule Example

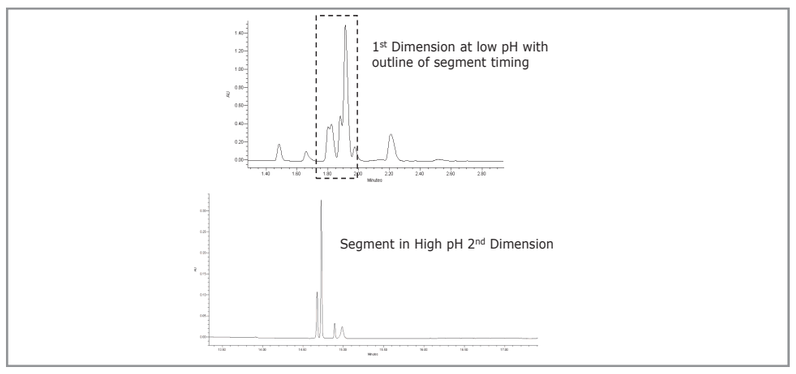

Figure 5. Chromatographic Selectivity Differences between the Two Dimensions. The two chromatographic dimensions were formic acid and ammonium hydroxide as mobile phase additives. The column and gradient were held constant, and the same column was used in both dimensions. The extremes of pH give useful differences in selectivity, but neither condition resolves all the components

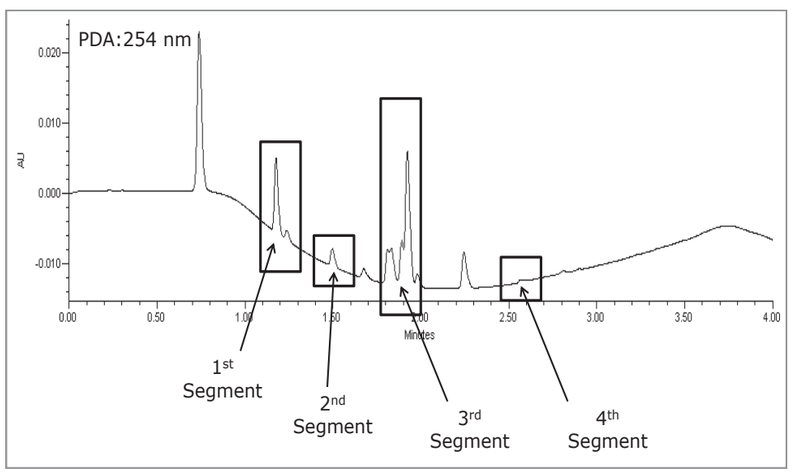

Figure 6. Reversed-phase Separation at Low pH. The four outlined segments were selected for a second chromatographic dimension at high pH.

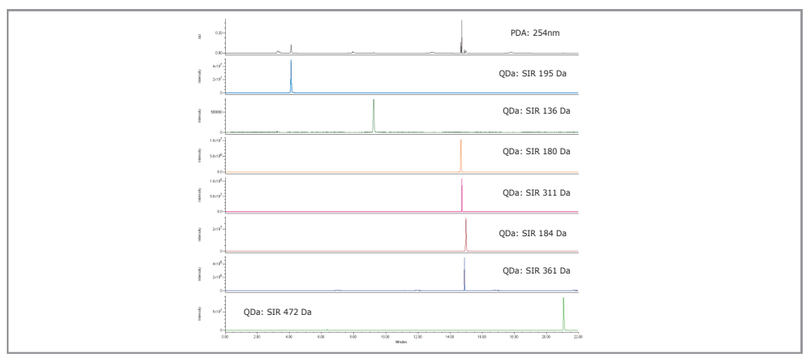

Figure 7. MS Identification in Second Dimension. The analytes as selected in Figure 6 were eluted from the high pH reversed-phase column directly into the electrospray source of the QDa. Each analyte was readily identified using the SIR channels corresponding to the known components. The peaks in the final analysis are narrow and symmetrical, reflecting the useful effects of At-column Dilution for re-focusing the analytes after each chromatographic step. The orthogonal second dimension provides resolution of coeluting peaks from the first dimension.

CONCLUSION

- **An automated multi-dimensional UPLC system interfaced to electrospray MS is described and demonstrated**
- **At-column dilution is implemented to establish chemical compatibility**
- **Multiple analytes are collected using a simple valve**
- **The system can be effectively used with both single quadrupole Mass Spectrometers**

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