Improving Sensitivity in LC/MS with Multi-Dimensional Chromatography

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OVERVIEW

- **MS detection can be improved with more complete chromatographic resolution for sample introduction**
- **Higher peak capacities in liquid chromatography require combining orthogonal modes of chromatography**
- **Full resolution multi-dimensional chromatography requires adjustment of mobile phase between the two separation modes**
- **Complex samples require multi-dimensional separation of multiple time segments**
- **An automated system has been assembled to meet all these objectives**

INTRODUCTION

Mass spectroscopic analyses are often combined with chromatographic inlets to improve the spectral data by eliminating ion suppression, by increasing sensitivity through sample concentration, by simplifying spectral interpretation through reducing mixtures, and by increasing robustness of the method and the instrument. For many samples, these benefits require high resolution separations that require in turn method development. All such development experiments reach a fundamental limit in possible resolution. In this presentation, we explore the value of multi-dimensional orthogonal chromatography to substantially exceed this limit.

Figure 1. System Configuration. See "Methods" for abbreviations

METHODS

Instrumentation (See Figure 1)

QSM - ACQUITY UPLC H-Class Bio System
SM-FTN -ACQUITY UPLC Sample Manager Flow Through Needle
ISM - Isocratic Solvent Manager
BSM - ACQUITY UPLC Binary Solvent Manager
CM - ACQUITY UPLC Column Manager, 9 port, 8 position
CM-Aux - ACQUITY UPLC Column Manager Auxillary
ACQUITY QDa Divert Valve
QDa - ACQUITY QDa Mass Detector (used for reversed-phase)
PDA - ACQUITY Photodiode Array Detector
Synapt Xevo QToF (not shown)(used for proteins)

All from Waters, Milford, MA

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,2.1 X 30 mm

For Protein A > Reversed Phase of Antibodies

Poros Protein A 2.1mm x 30mm
MassPREP Micro Desalting Column
ACQUITY UPLC BEH300 C_4 1.7 µ m 2.1mm x 50mm

For Size Exclusion > Revered-phase of proteins

ACQUITY UPLC Protein BEH SEC Column 200Å, 4.6mm x 150mm
MassPREP Micro Desalting Column
ACQUITY UPLC BEH300 C_4 1.7 µ m 2.1mm x 50mm

Samples

Infliximab Formulation, diluted to 1 mg/mL with PBS

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

RESULTS

System Functions

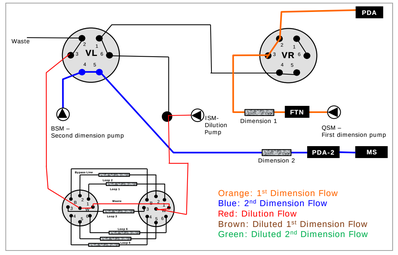


Figure 2. Sample Injection.

- *Sample is delivered to first chromatographic column and eluted with flow from QSM*
- *Second chromatographic is regenerated and equilibrated with initial mobile phase*
- *Flow from At-column dilution pump flushes holding valve*

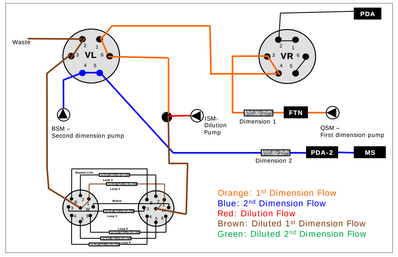
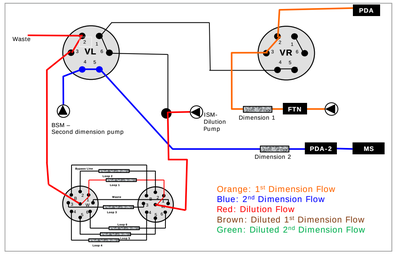


Figure 3. Isolation of Segment 1

- *Valve Right moves to position 2*
- *Eluate of column 1 driven toward Valve Left by flow from QSM*
- *Eluate of Column diluted with flow from At-column dilution pump*
- *Components of chromatographic Segment 1 held in Loop or Trap 1*
- *Equilibration of second chromatographic column continues*

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. This usually includes dilution with water, and it may also adjust pH or add ion pairing. For most reversed phase applications, we add trifluoroacetic acid with the diluent to maximize binding. The TFA is washed away before beginning elution to the second dimension.



- *Figure 34 from first chromatographic column continues with with VR diversion to waste. When the next desired time segment is reached, the configuration shown in Figure 2 is repeated.*
- *Cartridge with trapped analyte is flushed to waste with ACD pump. This removes mobile phase components of Dimension 1 that would interfere with dimension 2.*
- *Equilibration of second chromatographic column continues*

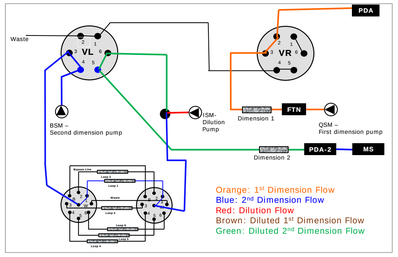


Figure 5. Elution of Segment 1 Through Dimension 2 Column

- *Valve Left moves to position 2*
- *BSM, dimension 2 pump, delivers gradient of increasing organic to elute trapped analytes from reversed phase cartridge*
- *As analytes elute from the cartridge, they are diluted by flow from the ISM dilution pump. This diluent is water with formic acid to ensure binding as a narrow band to the head of the second chromatographic column. The same At-column Dilution pump is used for both steps, but different solvents are used, drawing from different ports on the built-in solvent select valve.*
- *The gradient continues to elute the analytes from the Dimension 2 reversed-phase column into the PDA and MS detectors.*

Small Molecule Example

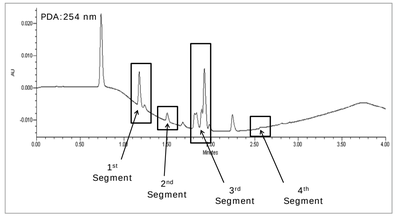


Figure 6. Reversed-phase Separation at Low pH. These chromatographic conditions do not resolve all the components of this mixture. The four outlined segments were selected for a second chromatographic dimension at high pH.

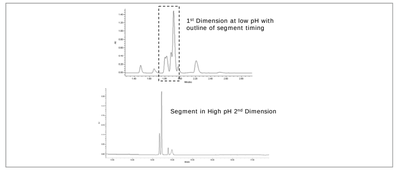


Figure 7. Chromatographic Selectivity Differences between the Two Dimensions. The two chromatographic dimensions were formic acid and ammonium hydroxide as mobile phase additives. The column and gradient were held constant, and the same column was used in both dimensions. The extremes of pH give useful differences in selectivity.

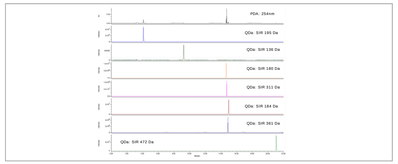


Figure 8. MS Identification in Second Dimension. The analytes as selected in Figure 5 were eluted from the reversed-phase column directly into the electrospray source of the QDa. Each analyte was readily identified using the SIR channels corresponding to the known components. The peaks in the final analysis are narrow and symmetrical, reflecting the useful effects of At-column Dilution for re-focusing the analytes after each chromatographic step.

Protein Example

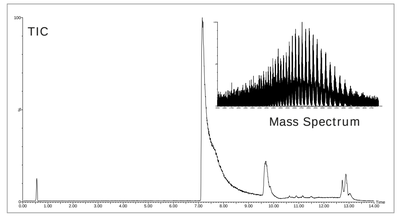


Figure 9. Analysis of Infliximab. First Dimension is Protein A isolating antibody from formulation. Acid eluate trapped in reversed-phase. Second Dimension is full reversed-phase into MS.

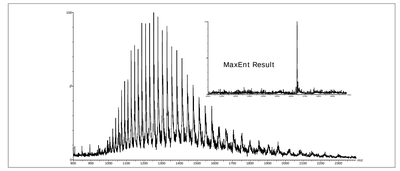


Figure 10. Analysis of BSA. First Dimension is Size Exclusion separation of complex protein mixture. High ionic strength eluate (phosphate-buffered saline) trapped in reversed-phase. Second Dimension is full reversed-phase into MS.

CONCLUSION

- **An automated multi-dimensional UPLC system interfaced to electrospray MS is described and demonstrated**
- **Orthogonal separation modes are combined**
- **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 and Quadrupole Time of Flight Mass Spectrometers**

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