PRACTICAL APPLICATION OF UPLC FOR IMPROVED PEPTIDE MAPPING

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Beth L. Gillece-Castro, Thomas E. Wheat and Jeffrey R. Mazzeo
Waters Corp., Milford MA

INTRODUCTION

The chromatographic methods for peptide mapping must be highly resolving and reproducible. UltraPerformance LC® (UPLC®) has been applied to this analytical problem. This technology takes advantage sub-2µm particles with a novel fluidics design optimized for such columns. Numerous experiments have shown higher resolution peptide maps than comparable HPLC separations.[1] Several fundamental investigations show the mechanisms associated with the physical and chemical properties of the column that are responsible for improving resolution. With this understanding, we can approach the practical problem using the inherently better resolution to improve the process of using peptide mapping.

The objectives of an improved peptide map can be divided into three general categories. First, improved resolution could be required without regard to run time. Second, at the other extreme, an extremely fast separation, perhaps focused on one particular peptide, could be required. Third, the enhanced resolution of UPLC® could be used to reduce the run time without compromising resolution. We have developed step-wise examples showing the implementation of UPLC® to approach each objective.

METHODS

Chromatographic Conditions

Instrument: Waters AQUITY UPLC® System
Mixer: High Sensitivity Peptide Analysis
Columns: Peptide Separation Technology
ACQUITY UPLC® BEH 130 C18, 1.7 µm 2.1 x 50mm
ACQUITY UPLC® BEH 130 C18, 1.7 µm 2.1 x 150mm
ACQUITY UPLC® BEH 300 C18, 1.7 µm 2.1 x 150mm
Temperature: 40°C
Flow Rate: 200 µL/min (unless otherwise noted)
Mobile Phase A: 0.02% TFA in water
Mobile Phase B: 0.018% TFA in acetonitrile
Example Gradient Table:

Time	%A	%B	Curve
0	100	0	initial
5	50	50	6
5.5	20	80	1
6	100	0	1

UV Detection: 214 nm
Detection Rate: 10 scans/sec
MS Detection: m/z 400—1800scan, 1/sec
Test samples were Waters MassPREP™ Digest Standards enolase or phosphorylase dissolved in 0.5 mL of 95% water and 5% Acetonitrile with 0.1% TFA (unless otherwise noted), resulting in a concentration of 2 pmoles/µL.
Injection Amount: 10 µL in Partial Loop Mode

RESULTS

Figure 1: Effects of Gradient Time on Peptide Resolution

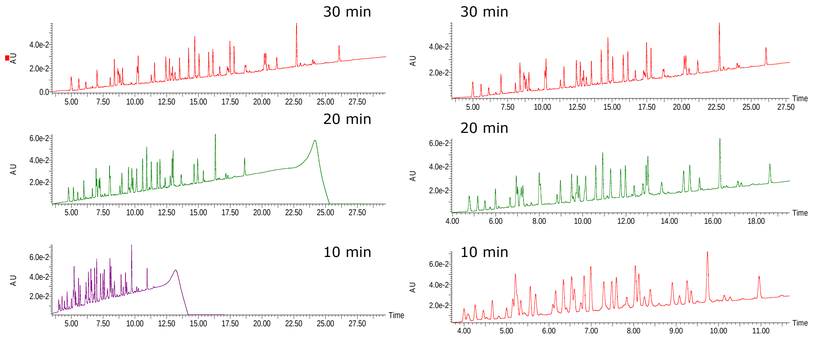

Figure 1. Chromatographic separation of peptides on a 2.1 x 50 mm UPLC column for the purpose of increasing the speed of a peptide map. Using a flow rate of 200 µL/min, the gradient length was decreased from 30 minutes to 20 minutes to 10 minutes. Resolution of closely-spaced peptides was lost, but some single peptides can be quantitated. In the right hand panels peaks are aligned to show the resolution changes.

Figure 2: Increasing Speed by Increasing the Flow Rate

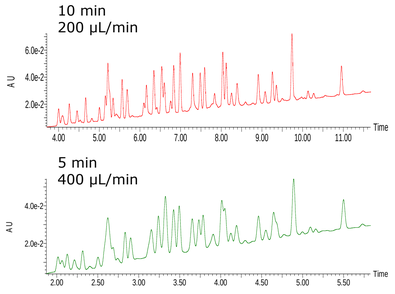

Figure 2. Comparison of speed at double the flow rate. Under fast chromatography conditions a mass spectrometer can be used as the detector for qualitative and quantitative peptide analysis

Figure 3: Increasing Resolution by Decreasing the Flow Rate

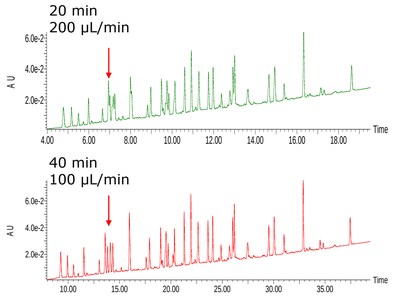

Figure 3. Comparison of the resolution at half the flow rate. Improved resolution with the accompanying increased gradient duration is the result of better optimization of the mobile phase linear velocity with the diffusion coefficient of peptides. Important closely-spaced peptides have been resolved.

Figure 4: Narrow Shallow Gradient to Separate a Pair

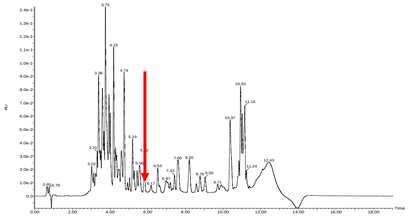

Figure 4. In this trypsin digest map a critical pair of peptides eluted at approximately six minutes.

Figure 5: Stepped Gradient Slope to Separate a Pair

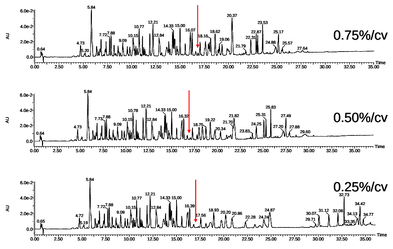

Figure 4. Creating a shallow gradient in that region can separate the pair without lengthening the gradient time.

Figure 4—Improving Resolution with a longer Column

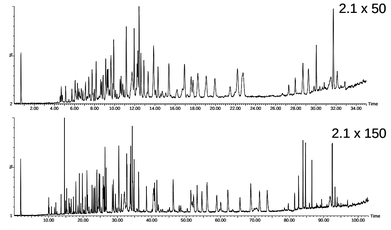

Figure 4. When a critical pair requires separation, a longer column may provide useful brute resolution. In this case a trypsin digest of phosphorylase b (97 kDa) was analyzed on a 50 mm and a 150 mm column.

Figure 7—Excellent Resolution in Half the Time

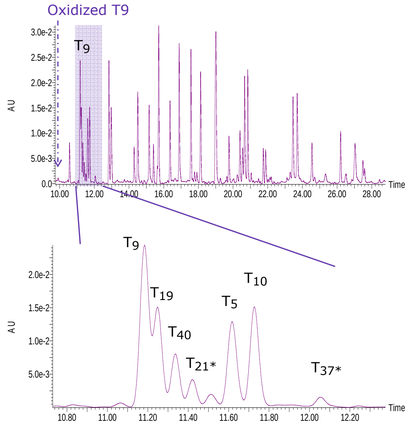

Figure 7. The resolving power of the 1.7 micron particles provides resolution comparable to traditional HPLC methods in half the time. In this case the peak of interest is T9 (sequence WMGK). To quantitate the oxidized fraction both the oxidized and non-oxidized peaks must be baseline separated from their neighboring peaks. The asterisk (*) indicates minor chymotryptic/tryptic peptides.

Figure 8—Change Start Composition

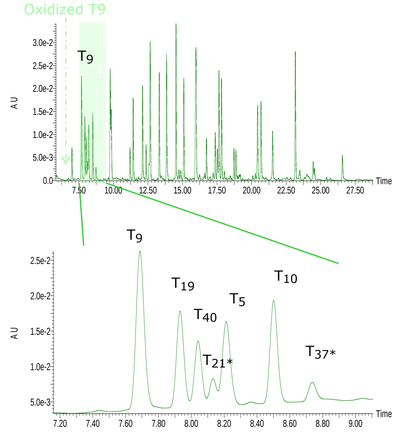

Figure 8. Changing the start composition of the gradient from 0% acetonitrile to 5% acetonitrile has selectivity repercussions. In this example the peak of interest, T9 has been resolved from the surrounding peptides under these conditions. The asterisk (*) indicates minor chymotryptic/tryptic peptides.

DISCUSSION

The inherently high resolution associated with UPLC® can improve many aspects of peptide mapping. For such complex samples, increasing separation power helps to ensure that no trace peptide will be overlooked in the sample analysis. It has, however, long been common to fully develop a peptide map, often in conjunction with MS detection, to meet this objective. If resolution is already sufficient, the separation power of UPLC can still be used to improve the assay. It is possible to maintain the same or comparable resolution in a shorter runtime by transferring the separation to a small particle-size column. The same approaches can be used to focus a separation on much more rapid analysis for a single peptide peak that is diagnostic of particular properties of a protein. In the course of these experiments, it has also been demonstrated that the same techniques for controlling selectivity in peptide mapping apply in both HPLC and UPLC.

CONCLUSION

- **Peptide mapping on small particle-size packing materials gives improved resolution and sensitivity.**
- **The enhanced resolution of UPLC® can be used to gain more information about a protein digest sample.**
- **The enhanced resolution of UPLC can be "traded" for reduced run time while keeping resolution constant**
- **The same techniques for controlling peptide separations apply in HPLC and in UPLC**

References Mazzeo, J.R.; Wheat, T.A., Gillece-Castro, B.L. and Lu, Ziling *BioPharm International*, 2006, January, 1-9.

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