INCREASED RESOLUTION IN PEPTIDE SEPARATIONS USING UPLC™

Waters

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INTRODUCTION

A clear trend in the field of liquid chromatography has been the continued reduction in the particle diameter of the packing material used to perform the separation. Modern reversed phase LC began in the mid-1970's with the advent of 10 µm irregular particle size packing materials, and within the last 5 years 2.5 µm particles have become available. However, the smaller particles have been used in shorter columns. This leads to faster analysis times but relatively modest gains in resolving power. Column length has decreased with particle size because the system pressure required is inversely proportional to the particle diameter cubed. For example, reducing the particle size by a factor of two requires an increase of the operating pressure by a factor of eight. It is, therefore, necessary to use shorter columns at lower flow rates to remain within the capabilities of the system. Clearly, in order to take advantage of smaller particle size columns, both in terms of improved speed and improved resolution, instrumentation capable of higher pressure operation is required. In addition, system band-broadening must be reduced to observe the narrow peaks generated with small particle packings. In 2004, the first liquid chromatography system capable of operation up to 15,000 psi was introduced. The combination of a system capable of higher pressure operation and columns packed with sub-2 µm particles has been termed Ultra Performance Liquid Chromatography™ (UPLC), to differentiate it from HPLC. The benefits of UPLC were originally demonstrated for small molecules (<500 MW) with reversed phase columns. Improved resolving power (1.7X), sensitivity (3X) and separation speed (9X) were demonstrated for many applications.

More recently, we have been investigating the use of UPLC for reversed phase peptide mapping. Peptide mapping continues to be a workhorse technique in biopharmaceutical characterization. In a peptide map, it is necessary to separate every peptide into a single peak. Therefore, peptide mapping represents a significant chromatographic challenge. In addition to the large number of peptides that are generated from the enzymatic digest of a protein, the number of alternative peptide structures, e.g., post-translational modifications, oxidations, and so on, can be very large. The capabilities of UPLC should make higher resolution peptide mapping possible.

MATERIALS AND METHODS

Separations were performed using an ACQUITY UltraPerformance LC™, and monitored with a Q-Tof *micro*™ mass spectrometer (Waters Corp., Milford, MA). Linear velocity and gradient slope were varied as described in the figure legends. Results were evaluated for chromatographic peak volume and resolution well as intensity of mass signal. MassPREP™ Peptide and Protein Digestion Standards (Waters Corp.) were prepared in H_2O containing 0.1% TFA.

LC-MS Instrumentation and Columns

LC System: ACQUITY UPLC™ Solvent Delivery System Operating Pressures from 5000– 13000
ACQUITY UPLC™ Sample Manager

MS_MS System: Q-Tof *micro*™ Mass Spectrometer; Electrospray Ionization (+)

Mobile Phase: With "TFA" modifier: A = 0.02% Trifluoroacetic Acid in Water
B = 0.018% Trifluoroacetic Acid in Acetonitrile
With "Formic" modifier A = 0.1% Formic Acid in Water
B = 0.1% Formic Acid in Acetonitrile

Gradient Conditions

Flow Rate/Gradient Slope	0.1mL/min	0.3mL/min
0.75%/Col.Vol.	5-50%B in104min	5-50%B in35min
1.50%/Col.Vol.	5-50%B in208min	5-50%B in 70min

Columns:
Waters **A**- BioSuite™ C_{18} PA-A, 2.1 x 100 mm 3.0 µm particles, 100Å pores
B- BioSuite™ C_{18} PA-B, 2.1 x 100 mm 3.5 µm particles, 300Å pores
C- ACQUITY UPLC™ BEH™ (Bridged-Ethyl-Hybrid) C_{18}, 2.1 x 100 mm, 1.7 µm particles, 120Å pores

RESULTS

Figure 1: Chromatography Principles
Mass Transfer and Diffusion

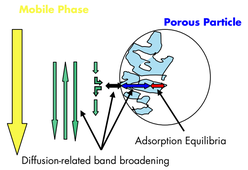


Figure 1. The chromatographic benefits of UPLC are largely derived from reduced band-broadening that is, in turn a consequence of reduced diffusion distances in small particles. When moving to 1.7 µm particle packing material one expects an increase in efficiency (**N**) with a concomitant increase in pressure.

Figure 2: Chromatography Principles
Van Deemter Plots for 1500Da Peptide

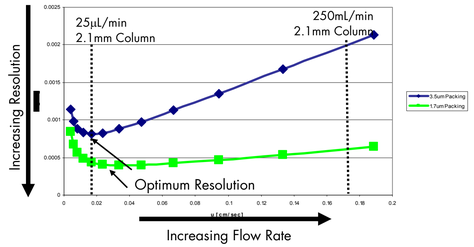


Figure 2: This is quantitatively described in the van Deemter equation that relates HETP (H) to linear velocity (u). This relationship is shown graphically for a peptide of 1500 molecular weight on 3.5µm and on 1.7µmpacking. The minimum in each curve corresponds to the maximum efficiency, and resolving power. The smaller particles have higher resolving power at a higher linear velocity. The 3.5µm particles have a minimum plate height of 0.811µm at 0.17mm/sec. Compared to 0.394µm at 0.33mm/sec with 1.7µm particles. UPLC could double the resolving power in peptide mapping while reducing the run time because the optimum is at a higher linear velocity. These principles have implications in flow rate. For the 3.5µm particle, the optimum linear velocity corresponds to a flow rate of about 24µL/min on a 2.1mm id column. This flow rate would not be used for a peptide map because the separation times would be too long. The common flow rate is 250µL/min on 2mm columns . This linear velocity of 1.7mm/sec gives to a plate height of about 2.1µm. This 2.6-fold loss of resolution with a 10-fold increase in separation speed has come to be an accepted compromise. For 1.7µm particles, the plate height at 250µL/min only increases to 0.645µm.

Figure 3: Effect of Flow Rate in Peptide Separations

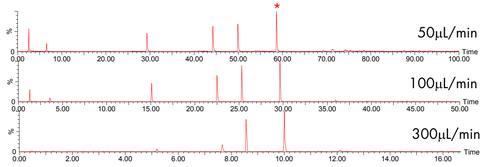


Figure 3: The MassPREP™ Peptide Standard was separated on an ACQUITY UPLC™ BEH C_{18} 1.7µm 2.1x100 column. Flow rates of 50, 100, and 300µL/min were compared at a constant gradient slope of 1%/column volume. The starred peak eluted in a volume of 17, 16, and 30 µL over the increasing flow rates. The signal intensities at the two lower flow rates were also comparable.

Figure 4: Effect of Flow Rate on Resolution

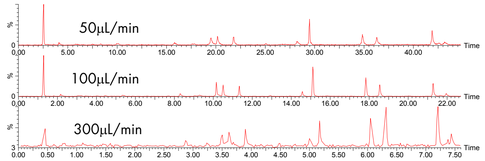
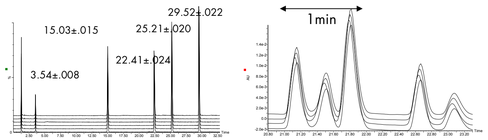


Figure 4: The MassPREP™ Enolase Digestion Standard was separated on an ACQUITY UPLC™ BEH C_{18} 1.7µm 2.1x100 column. Flow rates of 50, 100, and 300µL/min were compared at a constant gradient slope of 1%/column volume. The two lower flow rates have overtly better resolved peaks than observed at 300µL. The signal intensity is approximately 7-fold higher at 50 and 100µL/min.

The observations in Figures 3 and 4 are consistent with the expectations from the chromatographic principles outlined in Figures 1 and 2. With 1.7µm UPLC packings, there is little, if any change in resolution between 50 and 100µL/min so the higher flow was chosen for all subsequent experiments. It is, however, necessary to demonstrate that the system performance is adequate for use at these flow rates.

Figure 5: Reproducibility of Retention Times and Separation



The MassPREP™ Peptide Standards (Left) and Enolase Digestion Standard (Right) were separated on an ACQUITY UPLC™ BEH C_{18} 1.7µm 2.1x100mm column at a flow rate of 100µL/min at 1%/column volume. The excellent retention time precision produces reproducible selectivity. In the digest, note the three peaks that elute in one minute.

Fig.6a: Complex Digest on UPLC and Conventional Media

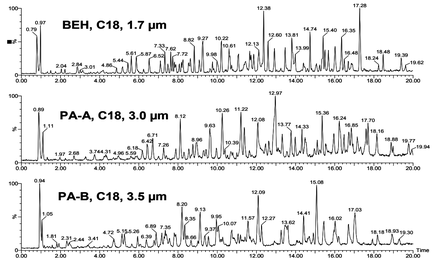


Fig.6b: Peak Tracking

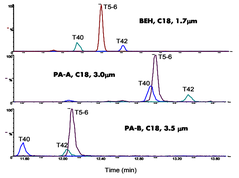


Figure 6: A tryptic digest of phosphorylase b was separated on the three media under identical conditions. The BEH material gives the most peaks. Although the separations are overtly similar, changes in selectivity can be identified using selected ion chromatograms for peak tracking.

Fig7: Effect of Mobile Phase Modifier in UPLC Peptide Mapping

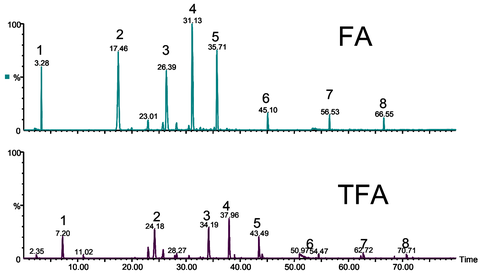


Figure 7: Formic acid is often preferred in LC/MS peptide mapping for enhanced sensitivity. The MassPrep™ Peptide Standards mixture was separated with both modifiers. In the presence of formic acid, sensitivity is greatly enhanced with relatively small reduction in retention and increased peak width. This enhanced sensitivity applies where ESI/MS is chosen as the detector. Since the changes are relatively small, it should prove relatively straightforward to switch modifiers without having to completely redevelop the map.

Figure 8: Separation of Native and Deamidated Peptides

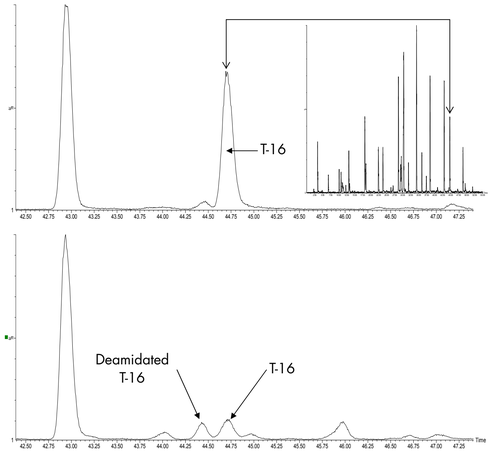


Figure 8: Enolase was incubated overnight at pH10.2 to induce deamidation of Asparagine residues. The separation of the native and deamidated forms of peptide T-16 is shown. This 15 residue peptide has a molecular weight of 1411.8.

CONCLUSIONS

- Chromatographic principles suggest that substantial improvements in resolution can be achieved when 1.7µm particles are used for reversed phase peptide mapping.
- Chromatographic principles suggest that current techniques for peptide mapping use flow rates far above the resolving power optimum.
- Peptide separations on 1.7µm particles offer a more optimal compromise between run time and resolution.
- Experimental analysis of peptide maps are consistent with these principles.
- The ACQUITY UPLC™ performs as required to exploit these principles.
- The selectivity of ACQUITY UPLC™ BEH C_{18} is similar to other materials often used for peptide separations.
- There is little loss of performance when formic acid is substituted for TFA with ACQUITY UPLC™ BEH C_{18}.
- UPLC is successful with difficult peptide separations.

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