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IMPROVED SPECTRAL QUALITY IN LC-MS PEPTIDE ANALYSIS USING ULTRA SMALL PARTICLE CHROMATOGRAPHIC PACKINGS

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OVERVIEW

- Small particle size chromatographic packing materials can be used for peptide mapping
- This application of UPLC yields reduced peak volumes for better resolution and sensitivity.
- Better resolution results from reducing flow rate and gradient slope.
- TFA can be replaced with formic acid for increased sensitivity
- UPLC can reduce run time without compromising resolution

INTRODUCTION

Proteins are commonly identified and characterized using peptide mapping with mass spectral identification. Although Peptide Mass Fingerprinting of the unfractionated digest can be used for identification, the most complete structural information becomes available when the digest is fractionated. The most complete separation yields the best spectra. Recent advances in column chemistry and instrumentation permit the routine use of very small particle packings to enhance separations. The basis of this technique is described in the van Deemter equation and is explicitly related to diffusion. To apply ultra high resolution techniques to peptides, we have investigated operating conditions that could influence the application of UPLC to peptide mapping.
We have compared the packing material used for UPLC for to those more commonly used for peptide LC/MS to measure effect on resolution and selectivity. Those parameters that influence diffusion-related chromatographic equilibria, specifically linear velocity and gradient slope, have been measured. The suitability of the material with different modifiers to increase electrospray signal is demonstrated. The possibility of using intrinsically higher resolution to reduce run is explored.

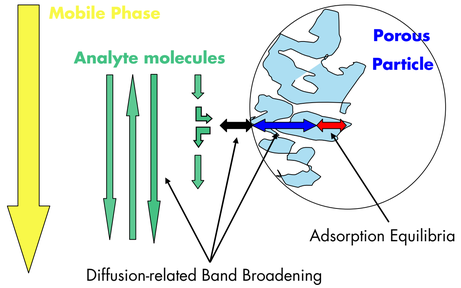
METHODS

All separations were performed using an ACQUITY UltraPerformance LC™, and monitored with a Q-Tof *micro*™ tandem mass spectrometer (Waters Corp., Milford, MA). The columns evaluated were selected to include particles that affect diffusion differently, including porous and superficially porous materials. The porous packing materials included different particle sizes and pore sizes. Linear velocity, gradient slope, and sample load were varied independently. Results were evaluated for chromatographic peak volume and resolution, intensity of mass signal, and homogeneity of MS and MS/MS spectra. MassPREP™ protein digests (Waters Corp., Milford, MA) were prepared in H_2O containing 0.1% TFA. The mobile phases were H_2O with 0.02% TFA (A) and acetonitrile with 0.018% TFA (B). The gradients were from 5 to 50% B with a flow rate of 0.3 mL/min. All column dimensions are 2.1x100 mm and 35 min gradient time was used except the noted. C_{18} packing materials - ACQUITY UPLC™ BEH, Biosuite™ PA-A and PA-B were compared.

LC-MS Instrumentation

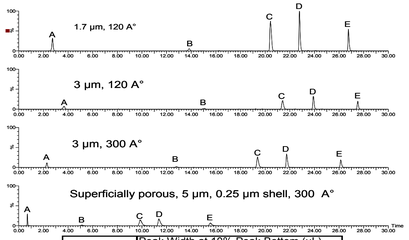
LC System:	ACQUITY UPLC™ Solvent Delivery System Operating Pressures from 5000– 13000 psi ACQUITY UPLC™ Sample Manager Dual-plate Autosampler
UV Detection:	ACQUITY UPLC™ UV Detector Wavelength 214 nm
MS_MS System:	Q-Tof *micro*™ Mass Spectrometer Electrospray Ionization (+)
Mobile Phase:	With "TFA" modifier: 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
Columns: Waters	**A**- BioSuite™ C_{18} PA-A, 2.1 x 100 mm 3.0 µm particles, 120 A pores **B**- BioSuite™ C_{18} PA-B, 2.1 x 100 mm 3.5 µm particles, 300 A pores **C**- ACQUITY UPLC™ BEH™ (Bridged-Ethyl-Hybrid) C_{18}, 2.1 x 100 mm, 1.7 µm particles, 120 A pores
Agilent	**D**- POROSHELL 300 SB C_{18}, 2.1 x 75 mm, 5 µm particles, 300 A pores

Chromatography Principles
Mass Transfer / Diffusion



RESULTS

Fig.1: UPLC Compared with Conventional Peptide Separation



	Peak Width at 10% Peak Bottom (µL)				
Column	A	B	C	D	E
BEH	66	81	78	54	57
PA-A	84	90	84	63	60
PA-B	57	90	90	66	69
POROSHELL	24	99	126	111	123

Fig1: The peptide standard mixture was separated on the four test media using identical conditions of linear velocity and gradient slope. Measured peak volumes were smallest on the BEH material. This gives better signal, as shown, and should improve resolution. (The superficially porous material was excluded from further testing because the peaks are so broad and asymmetrical.)

Fig.2a: Complex Digest on UPLC and Conventional Media

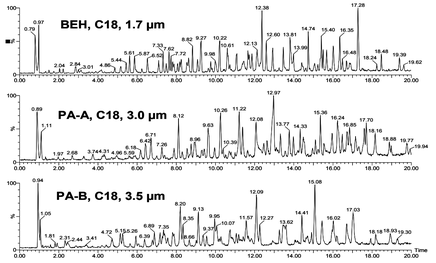


Fig.2b: Peak Tracking

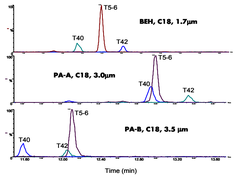


Figure 2: A tryptic digest of phosphorylase b was separated on the three media under identical conditions. The BEH material gives the most peaks. Although the separations are overtly similar, changes in selectivity can be identified using selected ion chromatograms for peak tracking.

Fig.3a: Effect of Gradient Slope on UPLC Peptide Mapping

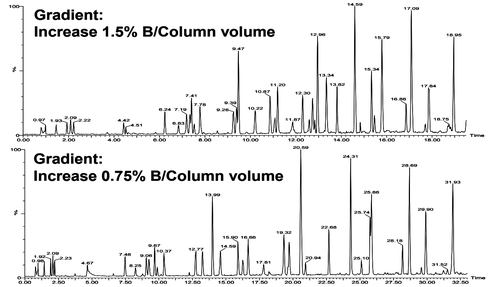


Fig.3b: Peak Tracking

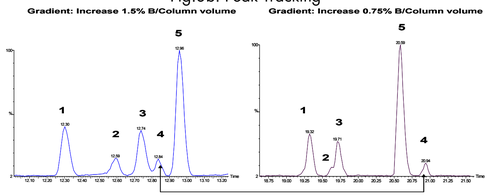


Figure 3: The enolase tryptic digest was separated on the BEH column at two different gradient slopes. The more shallow gradient gives better separation but with longer run time and somewhat lower sensitivity. While maps are similar, changes in selectivity including reversal of elution order can be identified using SIC to track peaks.

Fig.4: Effect of Flow Rate in UPLC Peptide Mapping

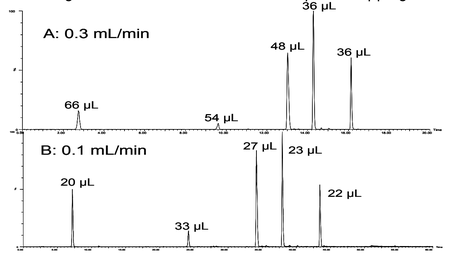


Figure 4: Since diffusional band-broadening is related to linear velocity, the peak widths for the peptide standard were measured at two flow rates with constant gradient slope. Peak volumes are significantly reduced with lower linear velocity, as predicted for the relatively slow diffusion of peptides.

Fig.5: Effect of Mobile Phase Modifier in UPLC Peptide Mapping

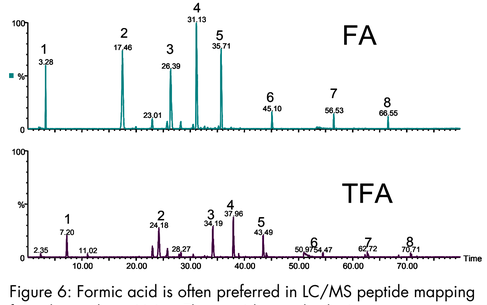


Figure 6: Formic acid is often preferred in LC/MS peptide mapping for enhanced sensitivity. The peptide standard mixture was separated with both modifiers. In the presence of formic acid, sensitivity is greatly enhanced with relatively small reduction in retention and increased peak width.

Fig.7: Reducing Column Length for Increased Speed

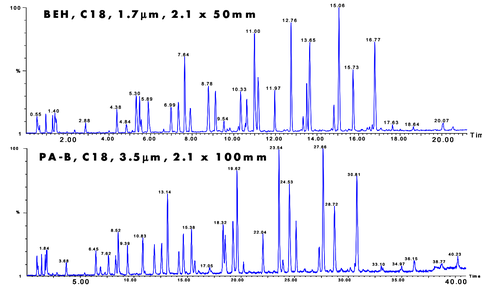


Figure 7: The same number of peaks can be separated in less time using UPLC as compared to more conventional HPLC.

CONCLUSIONS

- Peptide mapping can be performed using 1.7 µm particle packing material
- UPLC peptide mapping is characterized by smaller peak volumes for better resolution and sensitivity.
- The selectivity of the separation is dependent on the mobile phase modifier, the column packing chemistry, and the gradient slope
- Resolution is dependent on linear velocity
- The increased resolution of UPLC can be used to reduce run time without sacrificing speed or sensitivity.

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