USING CHROMATOGRAPHIC RESOLUTION TO INCREASE SENSITIVITY AND SPECTRAL INFORMATION IN LC/MS PEPTIDE MAPPING

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INTRODUCTION

The combination of MS detection with LC separation as a peptide mapping technique is fundamental to the characterization of proteins. Tandem MS techniques are often useful for extracting structural information from incompletely resolved peptides. The best sensitivity and the most readily interpreted spectra are, however, observed with chromatographically resolved single peptides. This resolution for a typically complex protein digest requires the best possible chromatographic techniques. We describe here the use of very small particle packings to maximize chromatographic resolution and sensitivity. This approach is evaluated with respect to the usability of the mass spectra obtained.

METHODS

MS Conditions

Waters Q-Tof *micro*™ Hybrid Tandem Mass Spectrometer

Capillary:	3500 V
Cone:	30 V
Source Temperature:	150°C
Desolvation Temperature:	300°C
Collision Energy:	5.0 eV
Scan Range:	400 to 1800 *m/z*
Scan Rate:	2 scans/sec

Chromatographic Conditions

Waters AQUITY UPLC™ System
Columns: (A) Peptide Separation Technology
ACQUITY UPLC™ BEH 130 C18, 1.7 µm 2.1 x 100mm
ACQUITY UPLC™ BEH 300 C18, 1.7 µm 2.1 x 100mm
(B) Conventional 300 Å C18, 3.5 µm 2.1 x 100mm

Injection Volume:	10 µL
Temperature:	40°C
Flow Rate:	100 µL/min (unless otherwise noted)
Mobile Phase A:	0.02, 0.05 or 0.1% TFA in water
Mobile Phase B:	0.018, 0.05 and 0.1% TFA in acetonitrile Respectfully.
Mixer:	High Sensitivity Peptide Analysis (P/N 205000403)
Gradient Slope:	1.5%/column volume

Gradient Table:	Time	%A	%B	Curve
	0	100	0	initial
	115.4	50	50	6
	124.4	10	90	1
	160	100	0	1

UV Detection:	214 nm
Detection Rate:	10 scans/sec

Test sample was Waters MassPREP™ Digest Standard Phosphorylase b . Dissolved in 0.5 mL of 95% water and 5% Acetonitrile with 0.02% TFA, resulting in a concentration of 2 pmoles/µL.

RESULTS

Figure 1A— Effect of Particle Size on Peptide Separations

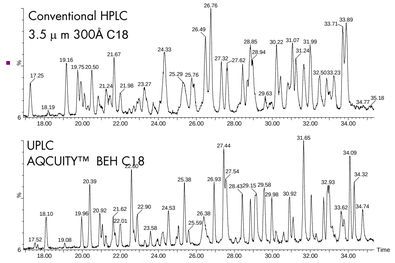

Figure 1A. Comparison of the separation of tryptic peptides of Phosphorylase b with HPLC and UPLC. ACQUITY UPLC™ BEH130 1.7 µm Peptide Separation Technology Column provides narrower and sharper peaks, resulting in better resolution and better sensitivity. More peaks, especially low abundance peptides, can be identified.

Figure 1B— Band Broadening in Peptide Mapping

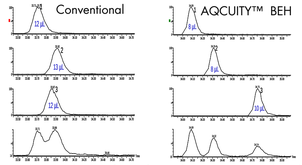

Figure 1B. Elution volumes of specific peaks were measured on the HPLC and UPLC materials. The improved resolution and sensitivity is a direct result of peaks that elute in a smaller volume because less band broadening occurred during the separation.

Figure 1C Chromatographic Resolution and Mass Spectra

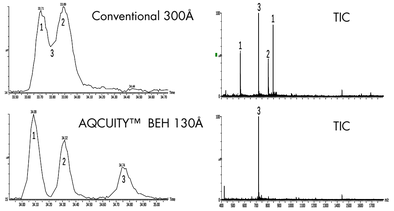

Figure 1C. Mass Spectra of Separated Peptides. Spectra were extracted from chromatographic peaks in the UPLC and in the HPLC separations. As expected, the overlapping HPLC peaks leading strong signals for multiple masses while the well-resolved UPLC peaks show the simple spectrum of a single peptide

Figure 2A—Effects of Mobile Phase Modifier

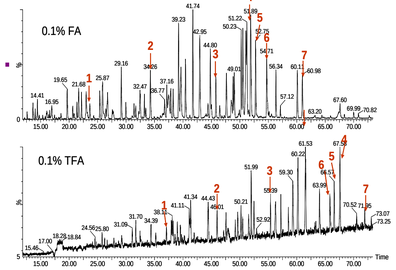

Figure 2a. Comparison of the separation of Phosphorylase b peptides with FA and TFA mobile phase modifiers. Formic acid is often preferred as the mobile phase modifier in LC-MS peptide mapping because sensitivity is better than with TFA. That substitution usually is best with specific columns that do not require ion-pairing for peptide separations. With UPLC BEH material, narrow symmetrical peaks are observed with either acid. Retention is greater with TFA and signal intensity is better with formic acid. Tracking of individual peaks as described in Table 1 shows the changes in selectivity associated with changing the modifier.

Figure 2B—Spectral Effects of Mobile Phase Modifier

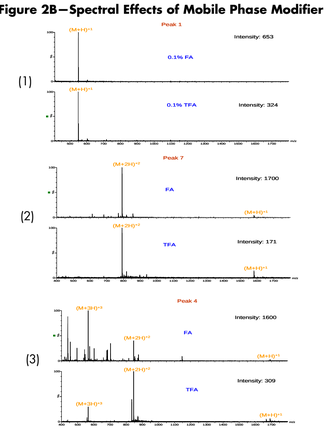

Figure 2c. Spectral effects of mobile phase modifier. Spectra were extracted from several peaks in both the TFA and FA maps, representing a singly, doubly, and triply charged peptides. As expected the signals are much more intense in formic acid than in TFA. The effect is greater for larger peptides. As expected the relative proportions of the different charge states also change, with the formic acid tending to give greater intensity for higher charge states.

Figure 3A—Effects of Modifier Concetration

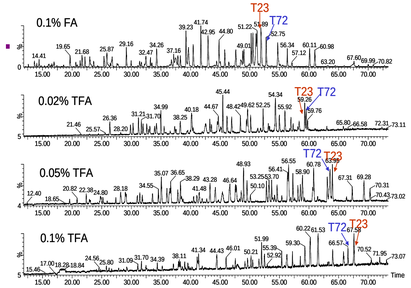

Figure 3A. Phosphorylase b peptides separated at different TFA concentrations. Altering a peptide separation to obtain better resolution is desirable but most often involves simple changes in gradient slope. Other options include adjustments of the mobile phase modifier for different slectivity. As shown in Figure 2A, FA and TFA five different sselectivity. Here we also see changes in selectivity with TFA concentration. Although the overall trend is toward lower retention with lower TFA concentration, we observe some specific large selectivity changes, e.g., the reversal of peptides T72 and T23.

Figure 3B—Spectral Effects of Modifier Concentration

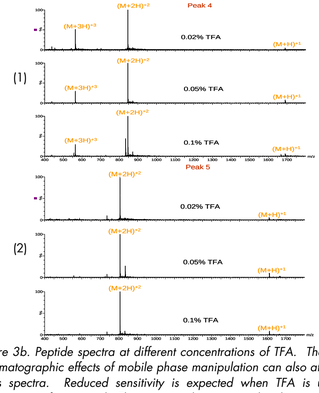

Figure 3b. Peptide spectra at different concentrations of TFA. The useful chromatographic effects of mobile phase manipulation can also affect the mass spectra. Reduced sensitivity is expected when TFA is used in preference to formic acid. The same mechanisms result in larger signal at lower concentrations of TFA than at higher. The relative proportions of the different charge states also show a dependednce on TFA concentration. The effect is not as profound as the difference between TFA and FA, but there is still a clear tendency for higher abundance of higher charge states at lower concentration of TFAQ. This is true for both doubly and triply charged peptides.

Table 1—Sensitivity Effects of Mobile Phase Modifier

Mobile Phase	Peak No.	Peak Width at 50% Peak Height	Peak Width at 10% Peak Height	UV Response Area	UV Response Height	MS Response Area	MS Response Height
0.1% FA	1	0.1161	0.2444	1220	10089	501	4171
	2	0.1349	0.2591	2361	17030	1839	13088
	3	0.1064	0.2549	2121	19859	1459	12241
	4	0.1743	0.3111	5398	31204	4754	27643
	5	0.1125	0.3879	1788	15861	2469	20418
	6	0.1195	0.3339	1837	12985	2465	16938
	7	0.1314	0.2551	1384	9870	1636	11864
0.1% TFA	1	0.1165	0.2123	1120	7829	86	719
	2	0.1293	0.2344	1604	11857	168	1278
	3	0.1106	0.2430	2205	18466	165	1826
	4	0.1296	0.2933	5782	39831	456	3467
	5	0.1221	0.2425	1837	15075	214	2041
	6	0.1369	0.3470	1897	11216	223	1566
	7	0.1013	0.1837	1083	7704	86	792

Table 1. Comparison of the Phosphorylase b peptide separations with FA and TFA mobile phase modifiers. For the peptides specifically tracked in the experiment, peak widths are similar in the two modifiers as a result of the surface chemistry of the BEH columns. From a sensitivity perspective, MS response is, of course much greater with formic acid. The uV responses, either height or area, do not show large differences and there is no consistent bias favoring one modifier.

Figure 4A Effect of Pore Size—Tryptic Peptides

A	4 residue	507.3
B	25 residue	2447.2
C	20 residue	1565.8

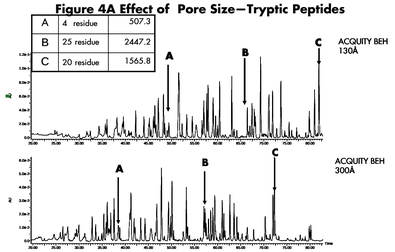

Figure 4A. Comparison of the separation of tryptic digest of phosphorylase b on ACQUITY UPLC™ BEH130, 1.7mm and on ACQUITY UPLC™ BEH300, 1.7mm Peptide Separation Technology Columns. Larger pore size materials are often preferred for peptide separations. For tryptic peptides, Both columns work well, with the smaller pore size being more retentive.

Figure 4B—Effect of Pore Size—LysC Peptides

A	4 residue	507.3
B	25 residue	2447.2
C	36 residue	4477.0

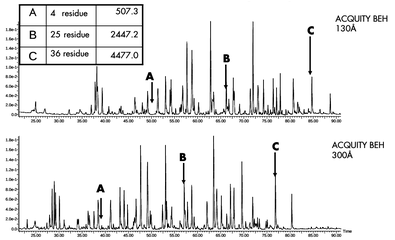

Figure 4B. The larger peptides derived with LysC digestion are expected to require larger pore sizes. In this comparison, however, both the 130Å and 300Å give narrow symmetrical peaks for peptides up to 4470Da. Certainly a size will exist where size exclusion effects become prominent.

DISCUSSION

The process of characterizing a protein with peptide mapping has come to rely heavily on MS detection. This approach can be limited by complex spectra including coeluting peaks. Such spectra can be particularly problematic when the peptides are found in disparate ratios as in oxidized or deamidated species or some specific post translational modifications. The large number of peptides observed in such a map include a very wide range of chemical and physical properties so very highly resolving chromatographic techniques are required to ensure anything approaching complete separation. The recent application of UltraPerformance Liquid Chromatography™ to peptide separations does enhance resolution. With this enhancement of resolution, it is more common that a single peptide enters the source of the mass spectrtometer. Under these conditions, it is easier to detect low abundance peptides because ion suppression is reduced and because it is less likely that the small signals will be overlooked. Even with abundant peptides, it is easier to interpret the spectrum of a single species, and the complexity of tandem mass spectrometry is less often required. This spectral simplification is shown above.

Further increases in spectral quality are possible by considering both MS and LC parameters. The routine use of formic acid as the modifier in LC/MS peptide mapping does affect the chromatography. Peptides usually give better resolution and peak shape with the ion pairing agents that reduce MS signal. The column chemistry used here has surface properties that give little tailing or band broadening in formic acid. Good signal intensity can, however, be observed with TFA in the mobile phase. The changes in selectivity associated with changing modifiers can be used to improve the utility of the mass spectra. Changes in concentration are as useful as changes in identity. The use of larger pore size packings will certainly extend the useful range peptide mapping, but they are not required for the kind of peptides observed in the digests used for protein characterization.

CONCLUSION

- **Peptide mapping on small particle-size packing materials gives improved resolution and sensitivity.**
- **Better resolution yields higher quality mass spectra that are easier to interpret in MS-only mode.**
- **Modifications of the mobile phase that improve chromatographic resolution can be applied while maintaining useful MS signal so that easily interpreted spectra are available.**
- **Both 130Å and 300Å pore size packings give useful peptide maps.**

References

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

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