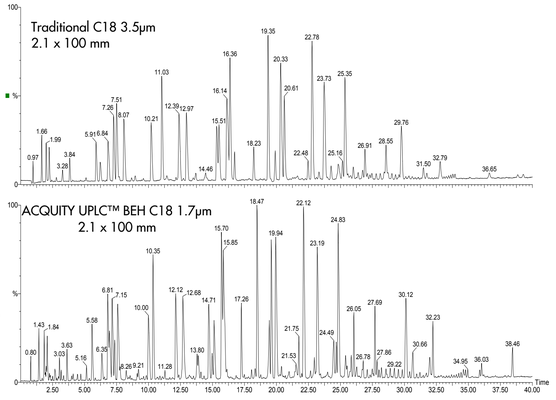
Ultra Performance Liquid Chromatography: Faster and Higher Resolution Peptide Maps

Jeffrey R. Mazzeo, Paul D. Rainville, Thomas E. Wheat, Eric S. Grumbach and Diane M. Diehl
Chemical Applied Technology, Waters Corporation, 34 Maple Street, Milford, MA 01757

Abstract

Continuously improving peptide mapping to give better resolution without sacrificing speed or sensitivity is fundamental to developing well-characterized biopharmaceuticals. This technique is used for protein characterization, whether for identity testing, purity assays, or sequence confirmation with LC/MS. It is among the most demanding analytical techniques because of the extreme resolution requirements. Large proteins can generate hundreds of peptides from a proteolytic digestion, many of which occur in modified forms reflecting oxidation, deamidation, and other modifications. To achieve adequate resolution, HPLC peptide maps usually employ relatively long columns, 150 to 250mm, with small particle packing materials, 2.5 to 3.5µm. Very shallow gradients are used to effect the separations, often 1-3hours. Concerns often remain about whether all variants have been separated. There is, therefore, always a need for greater resolution. Recently, a new category of separation science, Ultra Performance LC™ (UPLC™), was introduced. This technology takes advantage of the chromatographic benefits afforded by sub-2µm particles using a completely redesigned instrument platform. UPLC improves resolution, speed and sensitivity for many HPLC methods. The application of UPLC to peptide mapping is described here. Flow rate and gradient slope are optimized for resolution of a wide range of peptides of different sizes and chemical properties. The optimized UPLC peptide maps are compared to separations of the same samples using conventional peptide mapping systems. For comparison purposes, the separations are monitored with electrospray orthogonal time-of-flight mass spectrometry to track the elution of individual peaks. Ultra Performance LC does improve resolution for peptide mapping.

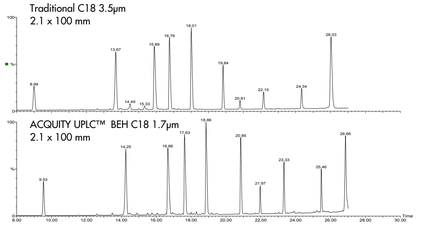
Figure 1: Separation of tryptic peptides on a conventional wide pore C18 RP-HPLC column and an ACQUITY UPLC™ BEH C18 1.7 µm column



The tryptic peptides generally appear as narrower, better resolved peaks on UPLC
Conditions: Flowrate 300µL/min Gradient 0-50%B/54 min.

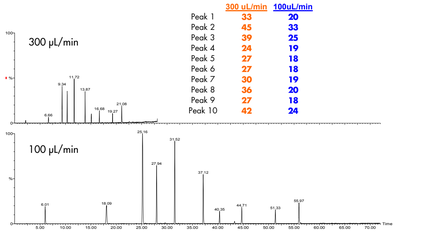
Chromatographic Variables In Peptide UPLC

Figure 2: Selectivity



Comparison of selectivity of peptides separated on a column packed with traditional packing material to a column packed with ACQUITY UPLC™ BEH C18 1.7µm
Conditions: Flow Rate 300µL/min Gradient 0-50%B/27 min.

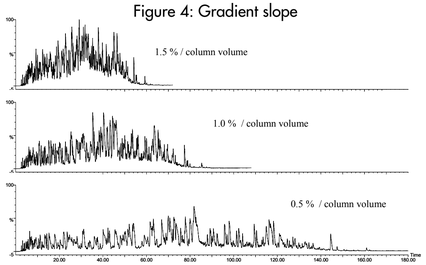
Figure 3: Flow Rate



	300 uL/min	100uL/min
Peak 1	33	20
Peak 2	45	33
Peak 3	39	25
Peak 4	24	19
Peak 5	27	18
Peak 6	27	18
Peak 7	30	19
Peak 8	36	20
Peak 9	27	18
Peak 10	42	24

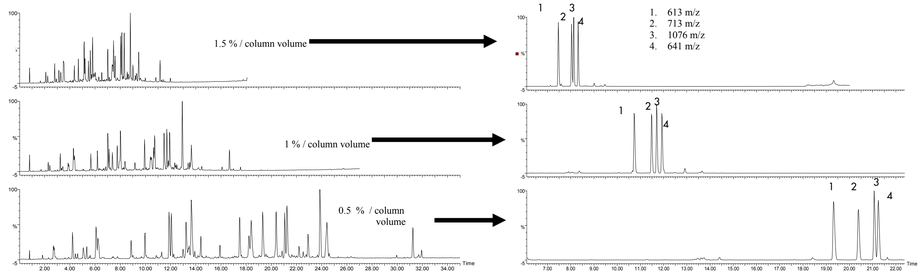
Running the separation at 100 µL/min decreases the elution volume compared to 300 µL/min. Increased resolution and sensitivity are the consequences of reduced peak volume.
Conditions: Flow Rate 300µL Gradient 0-50% B/24 min.
Flow Rate 100µL Gradient 0-50% B/72 min.

Figure 4: Gradient slope



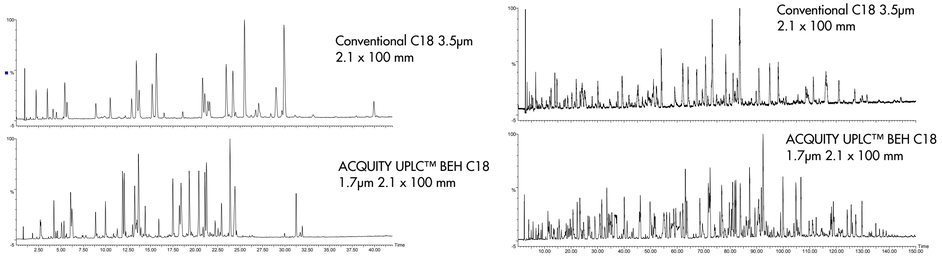
The tryptic peptides of Phosphorylase b are better resolved with a more shallow gradient.
Conditions: Flow Rate 100 µL Gradient 0-50% B/ 54 min, 108 min and 216 min.

Figure 5: Chromatographic selectivity of peptides as a function of gradient slope



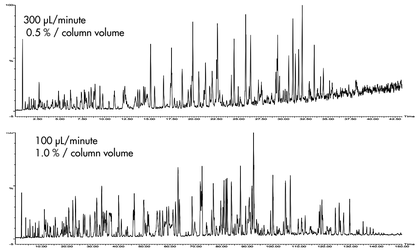
As shown for the Phosphorylase b peptides, Bovine Hemeglobin tryptic peptides are better resolved with a more shallow gradient. Using MS data the resolution of the individual peptides eluting near 7-8 minutes in the steepest gradient can be tracked over the series of experiments. This shows a change in selectivity for peak "3" over the series of experiments.
Conditions: Flow Rate: 300 µL/min. Gradient 0–50% B / 18, 36, 54 minutes

Figure 6: Improved separation of complex tryptic digests



The separation of a tryptic digest of bovine hemoglobin (left) and phosphorylase b (right) on a conventional column and on an ACQUITY UPLC™ BEH C18 column. UPLC separation gives better resolution and sensitivity for both samples.

Figure 7: Relative Impact of flow rate and gradient slope



The lower flow rate with steeper gradient gives better resolution than the higher flow rate, more shallow gradient.

Experimental

Sample preparation:

MassPREP™ Enolase, Phosphorylase b, and Bovine Hemeglobin digestion standards and MassPREP™ peptide mixture were dissolved in 100 µL of mobile phase A.

Instrumentation:

LC System:	ACQUITY Ultra Performance LC™ (Waters Corp.)
Mass Spectrometer:	Q-Tof micro (Waters Corp.)
Ionization mode:	ES +
Capillary voltage:	3300 V
Cone voltages:	35 V
Source temp:	150 °C
Desolvation temp:	350 °C
Gas flow:	500 L/Hr

Method parameters:

Mobile phase A:	0.02%
Mobile phase B:	0.018 % in ACN
Flow Rate:	refer to figure legend
Column temperature:	40.0° C
Gradient elution:	refer to figure legend

Columns

Symmetry 300® C18 3.5 µm 2.1 x 100mm (Waters Corp.)
ACQUITY UPLC™ BEH C18 1.7µm 2.1 x 100 mm (Waters Corp.)

CONCLUSIONS

UPLC can provide better resolution and sensitivity for typical peptides, up to 20-25 residues, from tryptic peptide maps.

Chromatographic selectivity of ACQUITY UPLC™ BEH C18 columns is similar to that observed with conventional reversed-phase columns for a population of peptides within a tryptic peptide map.

Optimizing flow rate and gradient slope gives better resolution and sensitivity.

UPLC peptide maps can be directly coupled to tandem mass spectrometry for identification and peak purity.