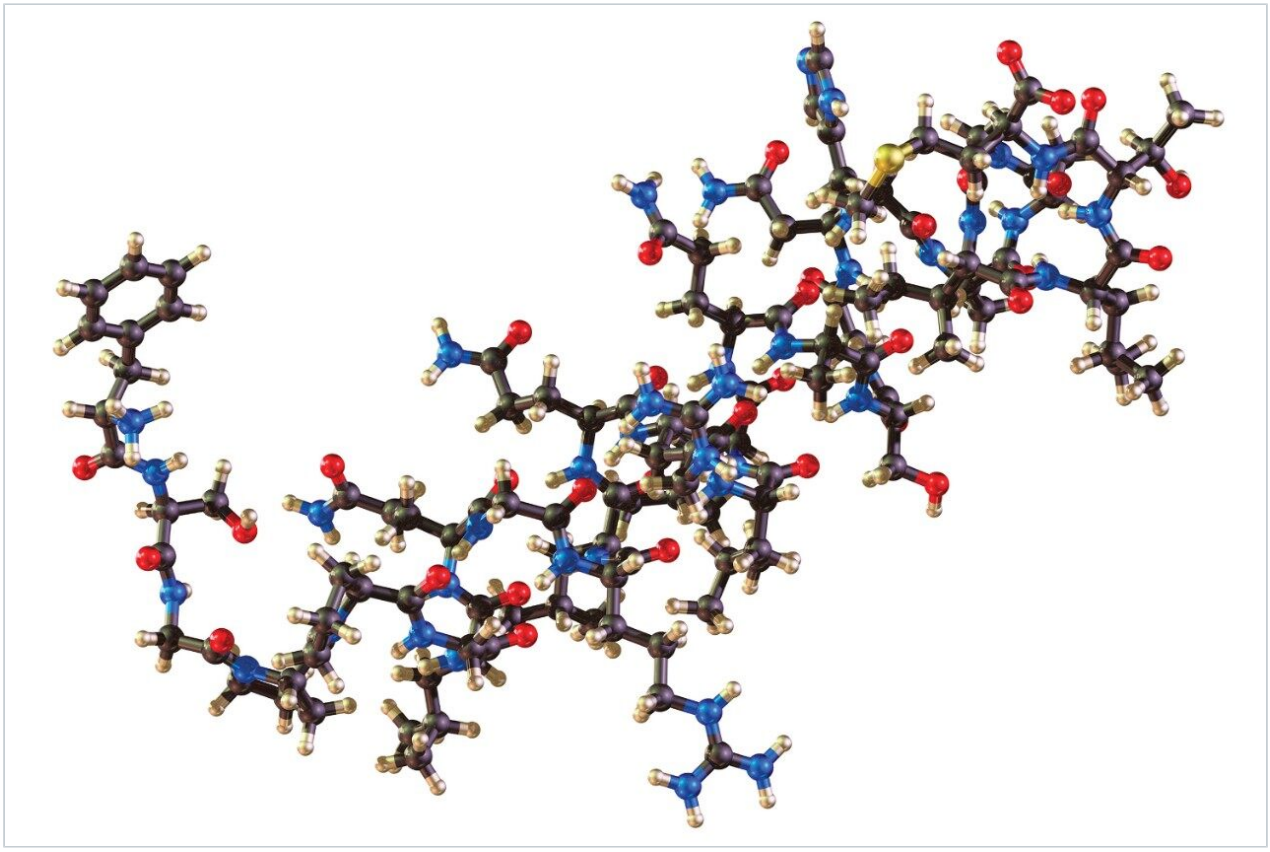
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Applikationsbericht

Quantitative Aspects of UPLC Peptide Mapping

Thomas E. Wheat, Ziling Lu, Beth L. Gillece-Castro, Jeffrey R. Mazzeo

Waters Corporation



Abstract

In this application note, we focus on the quantitative aspects of UPLC peptide mapping with UV detection. The technique is evaluated with respect to both chromatographic and detection linearity since the altered and normal peptides occur at extreme molar ratios. Reproducibility of the area measurement at these extreme ratios is also examined. Results from a mixture of peptide standards and from a digest spiked with a known amount of peptide are shown.

Benefits

The technique can be used to measure the specific variants of protein structure that may be observed in the development of a biopharmaceutical protein.

Introduction

Throughout the development of a biopharmaceutical protein, peptide mapping is used to demonstrate genetic stability and to confirm the integrity of the protein. Changes in retention time, often in combination with MS or MS/MS detection, reveal changes in the primary structure of the protein.

Modifications such as oxidation, deamidation, deletions, sequence clips, and glycosylation all affect chromatographic behavior. The modified peptides must be separated from the native peptides for a peptide mapping method to be useful. The presence of a modified peptide in the map of a sample reflects the presence of modified protein in the original sample.

In the initial characterization of a protein, it is important to develop a peptide mapping method that resolves modified peptides from native peptides so that all possible modifications may be detected. As development of the biopharmaceutical advances, these peptides must be quantitated. Quantitation is generally expressed as area or height percent of the native peptide.

In this way, the peptide map can provide information on the mixture of protein forms in each sample so that the safety and efficacy of the preparation may be assured. Methods must, therefore, exhibit excellent sensitivity and linearity for quantitative work.

UltraPerformance LC (UPLC) has demonstrated significant advantages compared to HPLC for peptide mapping. UPLC gives increased resolution, higher sensitivity, excellent peak shapes for glycopeptides, and the potential to increase throughput (Reference 1 and 2).

In this application note, we focus on the quantitative aspects of UPLC peptide mapping with UV detection.

The technique is evaluated with respect to both chromatographic and detection linearity since the altered and normal peptides occur at extreme molar ratios. Reproducibility of the area measurement at these extreme ratios is also examined. Results from a mixture of peptide standards and from a digest spiked with a known amount of peptide are shown.

Experimental

Materials and Methods

Samples:	Waters MassPREP Peptide Standard Mixture Part Number: 186002337 Waters MassPREP Hemoglobin tryptic digest Part Number: 186002327
Sample Buffer:	95% Buffer A/5% Buffer B
Instrument:	Waters ACQUITY UPLC System
UPLC Column:	Waters Peptide Separation Technology ACQUITY UPLC BEH 130 C_{18}, 1.7 µm, 2.1 x 100 mm Part Number:186003555
Flow Rate:	0.2 mL/minute
Mobile Phase:	A: 0.1% TFA in Milli-Q water B: 0.08% TFA in acetonitrile
Gradient:	0 to 50% B in 29 minutes (peptide mixture) 0 to 50% B in 58 minutes (hemoglobin digest)
Temperature:	40 °C

Detection:	UV at 214 nm with 10 mm path length cell at 10 Hz
Quantitation:	Waters MassLynx Software 4.1's QuanLynx Application Manager
Peptide ID:	Waters LCT Premier oa-TOF Mass Spectrometer
Ionization Mode:	Electrospray, positive
Scan:	400 to 1800 *m/z* at a rate of 2 scans per second

Results and Discussion

A mixture of peptides, 20 pmoles of each, was analyzed on a Peptide Separation Technology ACQUITY UPLC BEH 130, C_{18}, 1.7 µm, 2.1 x 100 mm column. The separation was monitored at 214 nm. Six replicate injections are overlaid in Figure 1, demonstrating the reproducibility of UPLC for peptide mapping. The same sample was subsequently injected at different levels to test linearity and sensitivity.

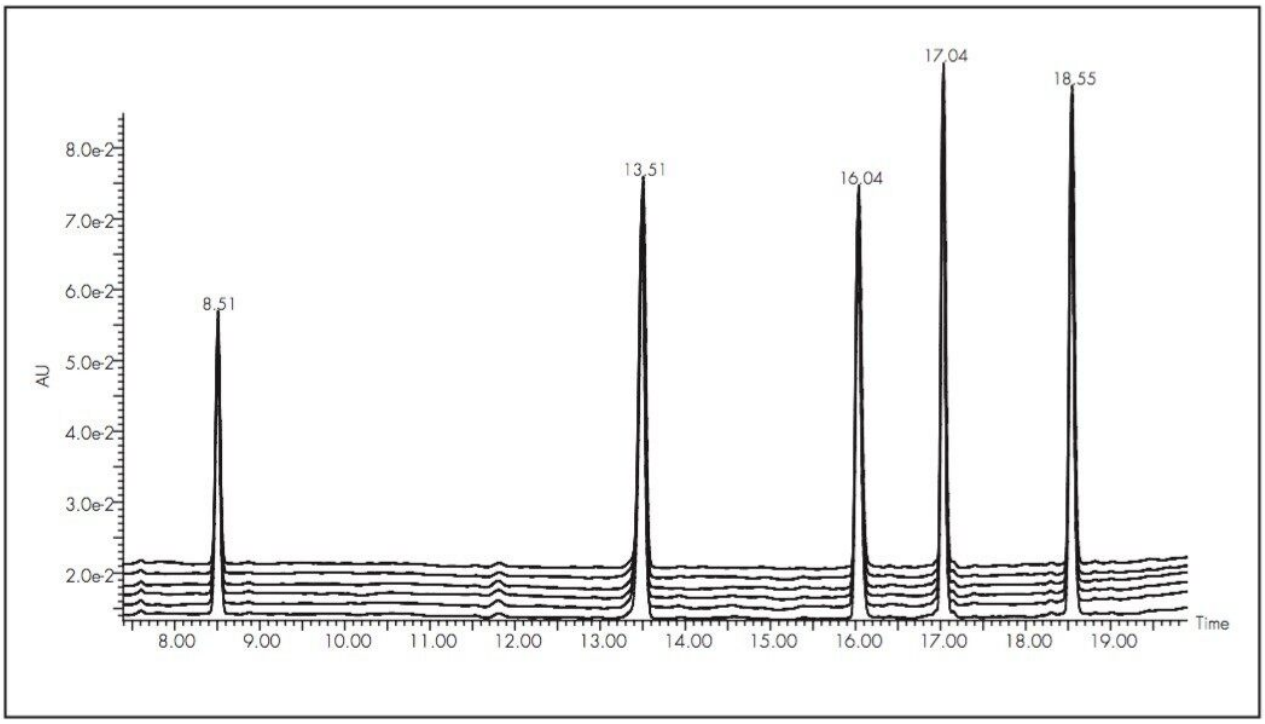


Figure 1. Six replicate 20 pmol injections of peptide mixture standard were made on a Peptide Separation Technology ACQUITY UPLC BEH 130 C_{18} 1.7 µm, 2.1 x 100 mm column. Retention time, peak height, and peak area reproducibility were measured for a peptide mapping gradient.

Figure 2 shows the analysis of peptide standards from 250 fmol to 100 pmol injected on-column. There is no significant shift in retention or deterioration in peak shape from low to high levels. This confirms that the dynamic range of the chromatographic material and gradient method is sufficient for the analysis of small amounts of one peptide in the presence of much larger amounts of another.

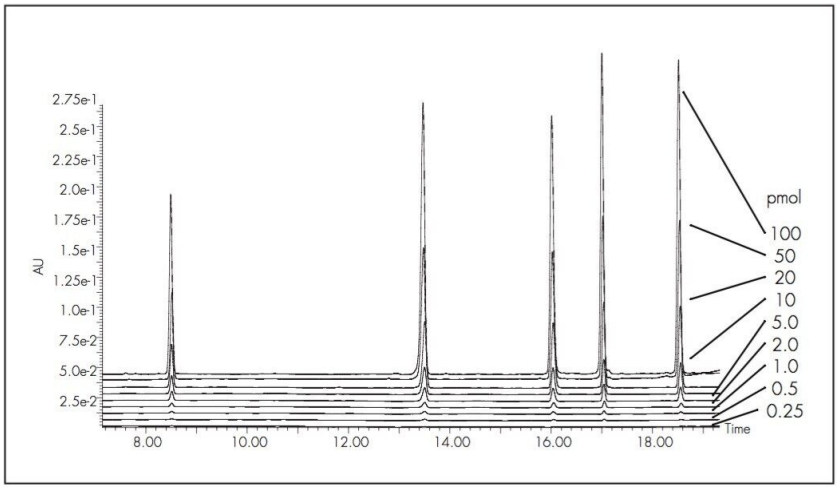


Figure 2. The peptide mixture was analyzed from 250 fmol to 100 pmol on column.

The lower limit is shown in Figure 3, where the six lowest levels are overlaid. The signal-to-noise ratio should be sufficient for quantitation at 250 fmol, and this is consistent with the area reproducibility summarized in Table 1. Peak area response is linear over two-and-a-half orders of magnitude, as shown in Figure 4.

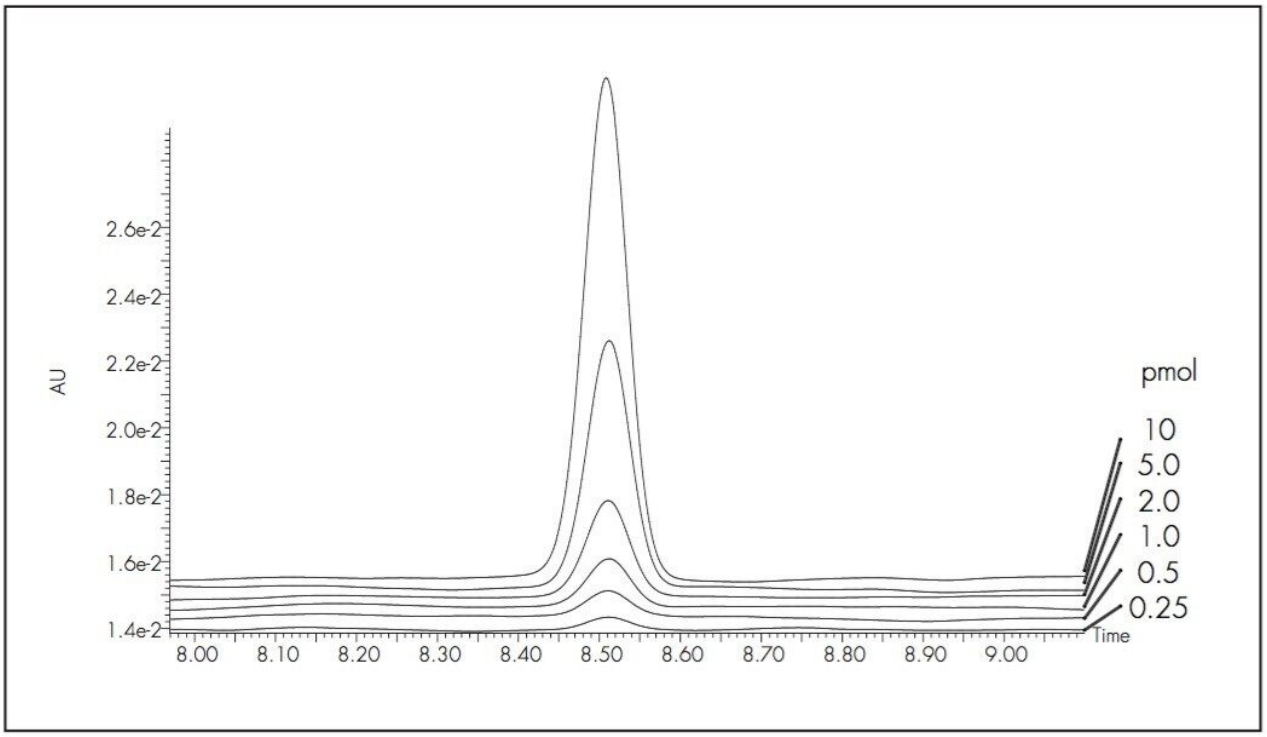


Figure 3. Chromatograms of one peptide from 250 fmol to 10 pmol show the high sensitivity region of the standard curve.

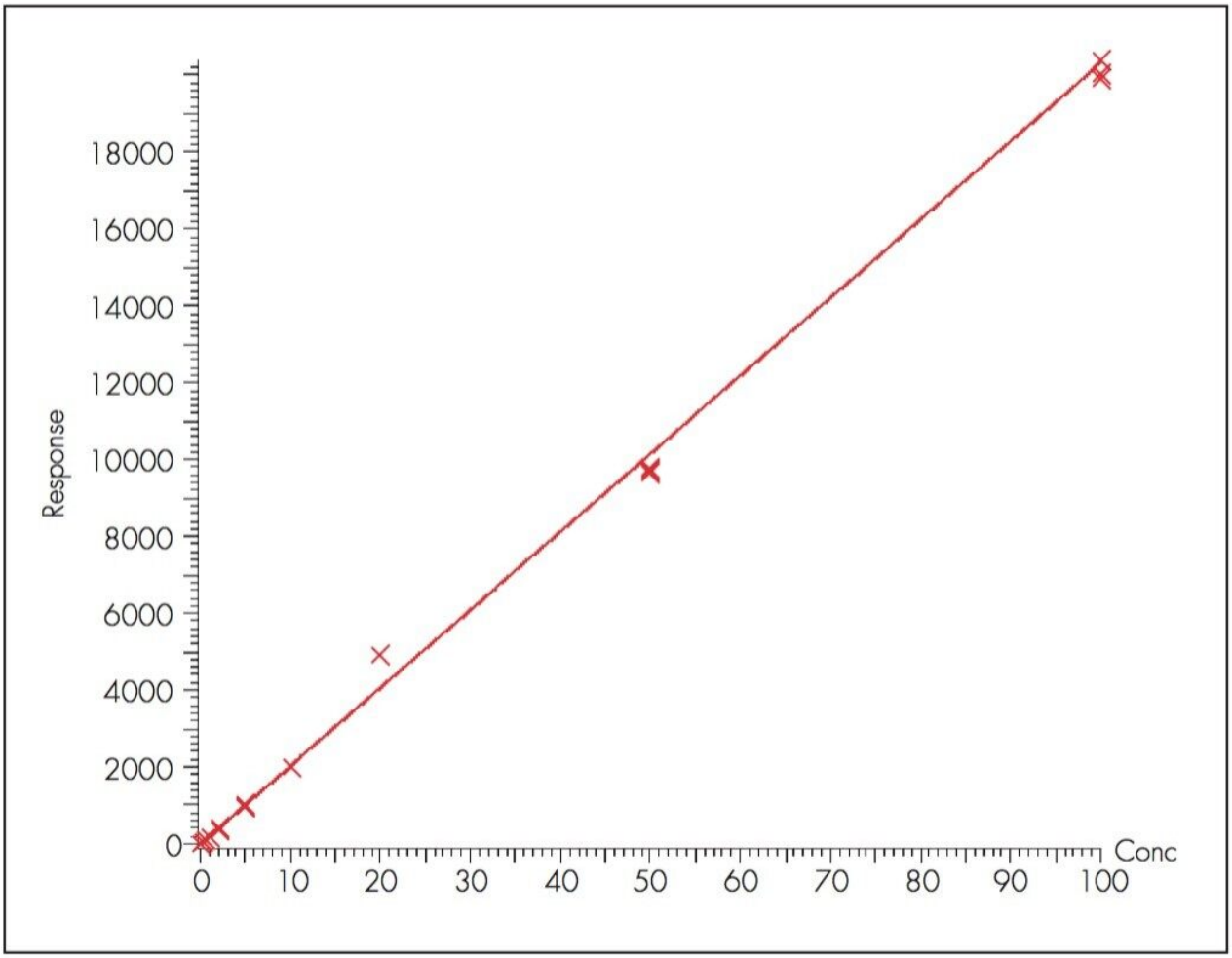


Figure 4. Linearity of UV detector response is shown for triplicate injections from 250 fmol to 100 pmol of the peptide standard mixture. This peptide eluted at 8.5 minutes, as shown in Figures 2 and 3 and Table 1.

Peptide Load (pmoles)	Mean Area	R.S.D. Area	% R.S.D. Area
0.25	18.98	0.70	3.7%
0.50	38.35	1.13	2.9%
1.00	74.48	1.10	1.5%
2.00	151.39	0.79	0.5%
5.00	392.83	2.50	0.6%
10.00	820.85	12.18	1.5%
20.00	1933.30	6.55	0.3%
50.00	4368.58	26.18	0.5%
100.00	9051.69	117.65	1.3%

Table 1. Reproducibility of peak area for the peptide eluting at 8.5 minutes. Triplicate injections from 250 fmol to 100 pmol were analyzed.

Given a typical injected amount of 100 pmol of digested protein, these quantities would correspond to 0.25% to 10% of a modified peptide. These results on standard peptides indicate that UPLC peptide mapping can be used to quantitate modified peptides in digests over a wide range of concentration.

A known amount of a specific peptide was added to an actual protein digest to test estimates of quantitation. This peptide serves as a surrogate, illustrating the behavior of modified peptides in the digest. A tryptic digest of hemoglobin (200 pmoles) was spiked with a peptide corresponding to a concentration range of 0.2 to 2% of the native material.

The resulting chromatograms are shown in Figure 5. The whole digest elutes between 7 and 53 minutes. Figure 5 focuses on the segment of quantitative interest and magnifies the elution profile of the surrogate peptide. The surrogate peptide, marked with a * in Figure 5, elutes just before minute 29. The peptide can be detected easily at the 0.2% level. Chromatographic resolution is maintained at this low level relative to the significantly larger peak that elutes 0.25 minutes earlier.

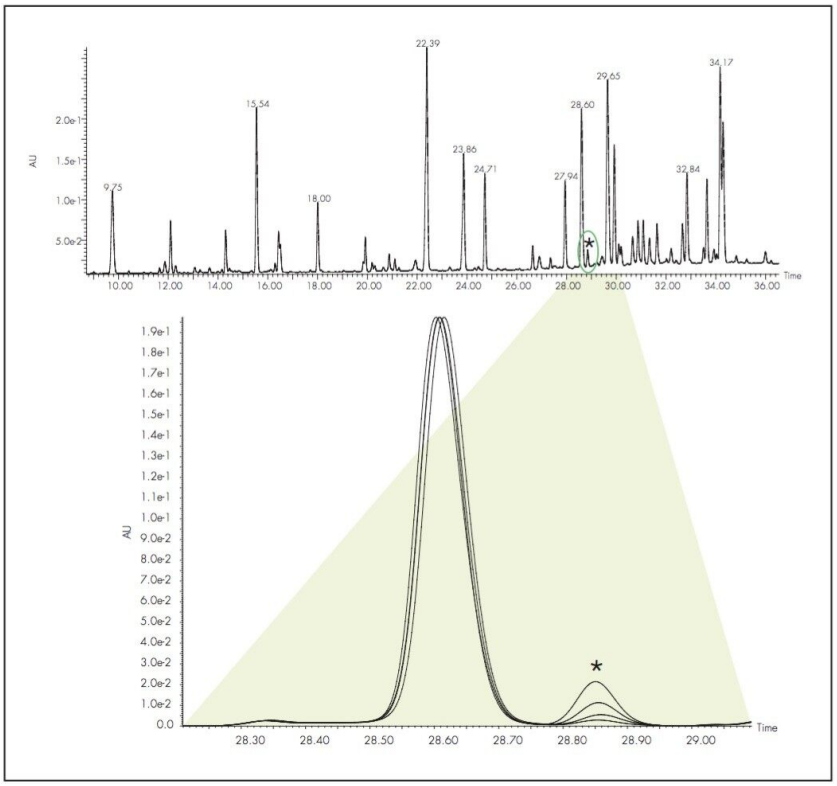


Figure 5. A tryptic digest of bovine hemoglobin was spiked with the peptide mixture as a surrogate to represent modified peptides. The surrogate peptide concentrations were 0.2 to 2% of the digest concentration on a molar basis. Panel A shows the peptide map of bovine hemoglobin with peptide standards spiked at 2% molar basis. A total injection on-column of 200 pmol of hemoglobin digest was made. Panel B focuses on the elution position of the surrogate.

Conclusion

UPLC yields qualitatively and quantitatively reproducible peptide maps. Chromatographic resolution and peak shape are constant over nearly three orders of magnitude of sample amount on column. Peak areas may be reliably and reproducibly quantitated to sub-picomole levels, as low as 250 fmoles on column.

This level of sensitivity is obtained with UV detection on 2.1 mm columns without resorting to special detection strategies. The detector response is linear with sample amount. This sensitivity in combination with

robust chromatographic behavior enables detection of low level peptides in a complex digest. In turn, the technique can be used to measure the specific variants of protein structure that may be observed in the development of a biopharmaceutical protein.

References

1. Enabling Significant Improvements in Peptide Mapping with UPLC, J. R. Mazzeo, T. E. Wheat, B. L. Gillece-Castro, Z. Lu, Waters Application Note (720001339EN < https://www.waters.com/webassets/cms/library/docs/720001339en.pdf>).
2. Next Generation Peptide Mapping with UPLC, J. R. Mazzeo, T. E. Wheat, B. L. Gillece-Castro, Z. Lu, *BioPharm International*, January 1, 2006.

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