PEPTIDE IMPURITY QUANTITATION

Beth L. Gillece-Castro, Thomas E. Wheat and Jeffrey R. Mazzeo
Waters Corp., Milford MA

Waters
THE SCIENCE OF WHAT'S POSSIBLE.™

INTRODUCTION

Validated assays for protein-related impurities are commonly based on peptide maps. The biological properties of a protein may be affected by modification, for example, oxidation or deamidation, at a particular residue. To assess the incidence of modification in a protein sample, the objective may be described as measuring a small amount of a peptide that contains the modified amino acid in the presence of a large amount of the same peptide with the unmodified amino acid. This measurement requires that the myriad of peptides must be sufficiently resolved for quantitation based on UV absorbance using reversed phase chromatography. The method must, therefore, be both highly resolving and reproducible. Both the detector and the column must accommodate a wide range of mass loading for successful trace analysis. UltraPerformance LC® (UPLC®) has been applied to this analytical problem.

METHODS

Chromatographic Conditions

Instrument:	Waters AQUITY UPLC® System with TUV
Mixer:	High Sensitivity Peptide Analysis
Columns:	Peptide Separation Technology ACQUITY UPLC® BEH 130 C_{18}, 1.7 µm In 2.1 x 50mm, 2.1 x 100 mm sizes
Temperature:	40 °C
Flow Rate:	200 µL/min (unless otherwise noted)
Mobile Phase A:	0.02 or 0.1% TFA in water
Mobile Phase B:	0.018 or 0.09% TFA in acetonitrile

Reproducibility Gradient Table:

(2.1 x 50mm)	Time	%A	%B	Curve
	0	100	0	initial
	10.0	50	50	6
	11	20	80	1
	11.1-17	100	0	1

Linearity Gradient Table:

(2.1 x 100 mm)	Time	%A	%B	Curve
	0	100	0	initial
	28.8	50	50	6
	32.6	10	90	1
	33	100	0	1

UV Detection:	214 nm
Detection Rate:	10 scans/sec
Injection Amount:	10 µL in Partial Loop Mode 20 µL in Full Loop Mode

Test samples were Waters MassPREP™ Digest Standard hemoglobin and MassPREP™ Peptide Mixture. Dissolved in 0.5 mL of 95% water and 5% Acetonitrile with 0.1% TFA (unless otherwise noted), resulting in a concentration of 2 pmoles/µL.

RESULTS

Figure 1: Retention Time Reproducibility

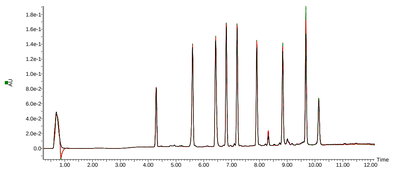

Figure 1. Chromatographic separation of peptides on a 2.1 x 50 mm UPLC column. Overlay of nine partial loop injections made over a four hour period. Sample was dissolved in water:acetonitrile 95/5 with 0.02% TFA. Retention time standard deviations were less than a second for all peptides.

Figure 2: Limits of Quantitation

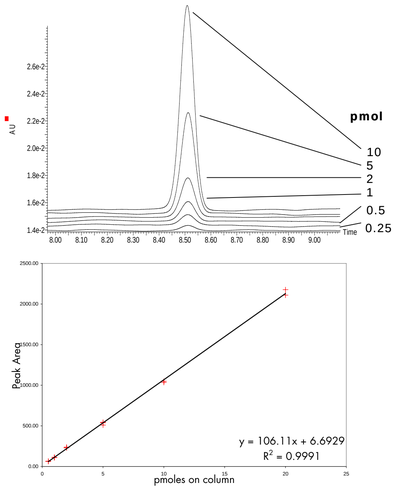

Figure 2. Linearity and detection limits were assessed for the separation on a 2.1 x 100 mm BEH UPLC column. 250 fmol could be detected, and the linearity from 500 fmol to 200 pmol was excellent. Linearity is shown from 500 fmol to 20 pmol.

Figure 3: Detection of Trace Contaminants

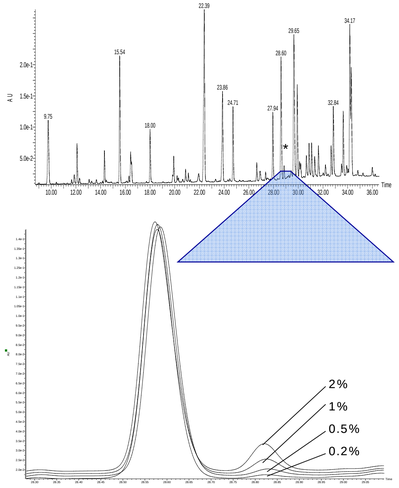

*Figure 3. A peptide map of bovine hemoglobin with a spiked trace contaminant designated *. The peptide mixture was added to the digest from 0.2 to 2% on a molar basis. The linear gradient from 0 – 50% buffer B occurred over 58 minutes. The region at 28 minutes is highlighted. This successful trace analysis requires the high resolution of UPLC for peaks that are only 15 seconds apart, center-to-center. The symmetrical peak shapes at this extreme concentration ratio permits the quantitation of the small peak eluting so closely after the large peak.*

Sources of Variability in Peptide Quantitation

Quantitative analysis of peptide maps is subject to all the variability of any chromatographic assay, including such things as injector performance, signal-to-noise, integration, and so on. Peptides, however, have additional complicating factors rooted in their chemical properties. A given sample will contain species over a wide range of size, hydrophobicity, isoelectric point, and solubility. That means that some peptides may differentially precipitate or adsorb to surfaces. A panel of peptides was selected to investigate strategies to mitigate this general problem. The sample diluent and mode of injection were examined as important variables.

Table 1. Peptides of Particular Interest. Peptides 1-6 always had excellent area reproducibility. Peptides 7-9 had some variability dependent upon injection conditions and diluent.

	Sequence	#	MW	pI	HPLC Index
5	DRVYIHPFHL	10	1295.677	7.51	56.2
7	WLTGPQLADLYHSLMK	16	1871.960	7.34	113.3
8	YPIVSIEDPFAEDDWEAWSHFFK	23	2827.281	3.97	100.20
9	GIGAVLKVLTTGLPALIS-WIKRKRQQ	26	2845.738	12.06	124.40

Figure 4: Variability is Concentration Dependent

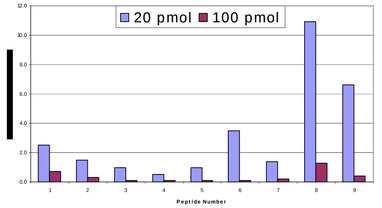

Figure 4. Variability measured as % standard deviation in area counts was first detected in full loop mode, and was found to be load/concentration dependent. In addition the variability was observed to be peptide dependent.

Figure 5 : Effect of Peptide Diluent

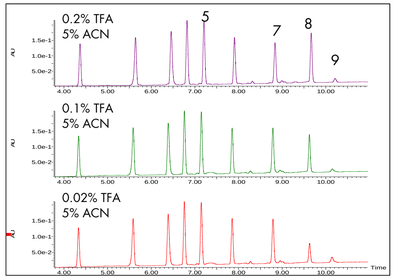

Figure 5. Sample diluent has effects on the solubility of individual peptides. Consequences of the peptide solubility are changes in peptide yield, and concomitant area count variability. Peptide area variability is shown in bar graphs Figures 6 A and B, and relate to the sequences above.

Figure 6: Effect of Peptide Diluent and Injection Mode

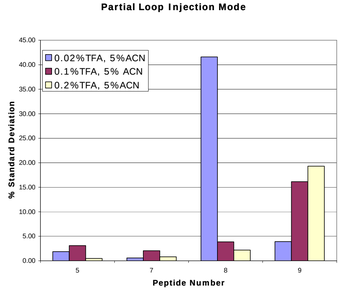

Figure 7A. Comparison of the area reproducibility of peptides 5, 7, 8, and 9 when injected in partial loop mode. Area reproducibility is linked to peptide yield. The hydrophobic peptide requires higher TFA concentrations to improve the solubility. The highly basic peptide becomes less soluble as the TFA concentration increases.

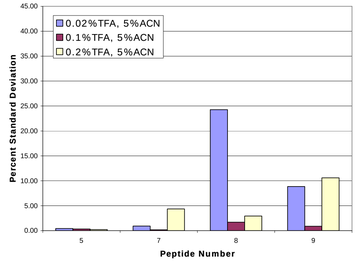

Figure 7B. Comparison of the area reproducibility of peptides 5, 7, 8, and 9 when injected in full loop mode with a 20 µL loop overfilled 3 times. Area reproducibility is linked to peptide yield. The hydrophobic peptide requires higher TFA concentrations to improve the solubility. The highly basic peptide becomes less soluble as the TFA concentration increases. The reproducibility is less sensitive to sample solvent in full loop mode.

DISCUSSION

Quantitative analysis in peptide mapping requires, like any HPLC assay, that the retention times be reproducible for peak identification and that the detector be sensitive enough for a good signal-to-noise ratio on low abundance peaks representing trace modifications of the protein. It is also essential that chromatographic column have sufficient dynamic mass capacity range to maintain good peak shape and resolution over the extreme concentration ratios found in trace analysis. The ACQUITY UPLC system used with Peptide Separation Technology columns meets these requirements.

Quantitation of peptide separations is complicated by the apparent variable yield of some sample components. While this is often a consequence of partial digestion, changes in concentration are often observed as a sample ages. This problem is often manifested as excessive variability in peak area. It is often caused by aggregation of peptides in the sample vial and by precipitation on surfaces. Various alternative sample diluents have been used to minimize the problem. When variability is judged in conjunction with peptide sequence, it could be observed that modest increases in TFA concentration in the sample diluent improved reproducibility for some hydrophobic peptides while decreasing the solubility of very basic peptides. The optimum diluent for a digest of given sample can be selected with a few tests of quantitative reproducibility.

CONCLUSION

- **Peptide mapping on small particle-size packing materials gives improved resolution and remarkable sensitivity was achieved a 214 nm on a 2.1 mm column.**
- **Quantitation with a 10mm, 500 nL flow cell proved useful to detect <0.5% impurities.**
- **Example shows the importance of high resolution for the quantitation of trace peptide variants.**
- **The sample diluent should be chosen to optimize peptide solubility for best quantitation and reproducibility.**
- **Better resolution and sensitivity yields high quality quantitation of sub-picomole amounts of peptide in mixtures.**

TO DOWNLOAD A COPY OF THIS POSTER, VISIT WWW.WATERS.COM/POSTERS

720002034EN