REPRODUCIBILITY IN UPLC PEPTIDE MAPPING: SYSTEM-TO-SYSTEM ROBUSTNESS

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INTRODUCTION

Recombinant proteins and monoclonal antibodies are developed for therapeutic purposes. Peptide mapping is used to confirm the primary structure of a protein, identify post-translational modification (PTMs), and analyze potential impurities. Any difference in structure of a protein should be reflected in a change in retention time for the peptide containing the modification. The relative amounts of the peptide with and without a particular modification is used to measure the fraction of the protein in the particular sample that carries that modification. Changes in area proportions correspond to the fraction of the protein molecules in the sample having a particular modification.

UltraPerformance LC® (UPLC®) shows greater resolution and higher sensitivity for peptide mapping as compared to HPLC. To achieve maximum resolution, all elements of the analysis, including the instrument, column, solvents, and sample, must be optimized to work together as a complete system. Using the UPLC Peptide Analysis Solution Kit, the Waters ACQUITY UPLC® System has been shown to give consistent chromatographic separations and reproducible quantitation for peptide mapping.[1]

When a completely satisfactory peptide map has been developed, it will be used on multiple systems within a department as well as transferred to another department, laboratory, or CRO. Reproducibility of retention time and relative area must be consistent from one ACQUITY UPLC System to another.

In this presentation, we demonstrate reproducibility of the peptide map of a protein digest run on three identical ACQUITY UPLC systems. Retention time, area and relative area reproducibility of selected peaks were evaluated.

MATERIALS AND METHODS

Experimental Design
Three identical ACQUITY UPLC Systems (ACQUITY UPLC 1, 2, and 3) were configured according to the instructions found in the UPLC Peptide Analysis Solution Kit.[2] Briefly, a core ACQUITY UPLC System consisting of an ACQUITY UPLC Binary Solvent Manger, ACQUITY UPLC Sample Manager with Column Heater and ACQUITY UPLC TUV Detector was modified to be compatible with peptide analysis. A high sensitivity peptide mixer was installed on the pump. The Autosampler was equipped with a 20 µL loop and a 15 µL PEEK/Sil ACQUITY UPLC peptide needle. One Waters Peptide Separation Technology Column was used on all three systems. A shallow gradient of 0.5%/column volume was selected as typical of peptide mapping gradients. ACQUITY UPLC systems 1, 2, and 3 were run on day 1, 2, and 3, respectively. Six consecutive runs were completed on each system before moving the column to the next system. The mobile phase was prepared fresh on day 1 and divided among the instruments. MassPREP™ Enolase Digestion Standard was reconstituted with sample buffer to 10 pmol/µL on day 1. Aliquots of 100 µL were frozen in a -80 °C freezer. On day 1, a fresh, unfrozen aliquot was loaded on ACQUITY UPLC 1. A frozen aliquot was defrosted and loaded on ACQUITY UPLC 2 and 3 just before the start of the injections. Data was processed using Empower™ 2 Software. The peaks in the chromatograms were integrated using the ApexTrack™ integration algorithm. The first injection of each day was a system blank run.

Samples: MassPREP Enolase Digestion Standard (3 vials of 1 nmol tryptic digest of protein, 8 pmol/µL)
Sample Buffer: 0.2% TFA in 95:5 water/acetonitrile (100 µL per vial of digestion standard)
Instrument: ACQUITY UPLC configured for peptide analysis
Column: Peptide Separation Technology ACQUITY UPLC BEH 300 C_{18}, 1.7 µm, 2.1 x 100 mm
Flow Rate: 200 µL/min
Mobile Phase: A: 0.02% TFA in water
B: 0.018% TFA in acetonitrile

Gradient:

Time (min)	%A	%B	Curve
0	98	2	
5	98	2	6
206	40	60	6
206.1	10	90	6
208.1	10	90	6
208.2	98	2	6
234.2	98	2	6

Column Temp.: 40 °C
Injection Vol.: 8 µL of 10 pmol/µL of reconstituted MassPREP Enolase Digestion Standard
Mode: Partial Loop
Weak Wash: 600 µL of 95:5 $H_2$0:ACN 0.2% TFA
Strong Wash: 200 µL of 20% Mobile Phase A: 80% Mobile Phase B
Sample Temp.: 4 °C
Detection: Wavelength: 214 nm
Sampling Rate: 10 pts/sec
Filter Time Constant: Normal 0.2 sec

RESULTS

The peptide map of the MassPREP Enolase Digestion Standard is shown in Figure 1. Empower 2 using ApexTrack integration was used to integrate all chromatograms. The software generated integrated chromatogram showed over 300 peaks of which three peaks were compared in this study. An early eluting (peak A), middle eluting (peak B) and later eluting (peak C) peak were selected as representative peaks in the chromatogram.

Figure 2 illustrates the overlay of five consecutive runs of the MassPREP Enolase Digestion Standard for ACQUITY UPLC 1 run on day 1. There is no observable shift in retention time that compromises the identification of a peak. For all peaks, retention time reproducibility with in a single system is better than 0.3% RSD.

Inter-system reproducibility is shown by the overlay of the chromatograms of the MassPREP Enolase Digestion Standard peptide map from injection 3 on ACQUITY UPLC 1, 2, and 3 run over three days in Figure 3. The peaks detected from the three systems were identified and counted without any manual manipulation. The same number of peaks were found in all chromatograms. Additionally, peaks A, B, and C were correctly identified in all chromatograms.

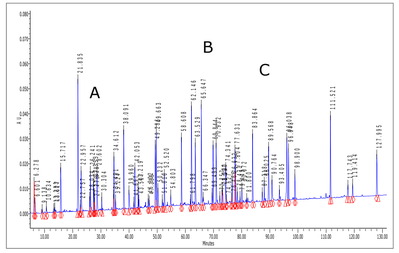

Figure 1. UV chromatogram of the peptide map of MassPREP Enolase Digestion Standard. Marker peaks labeled as A, B, and C.

Table 1 compares the average and standard deviation for retention time for each system and all of the runs for the three selected representative peaks. As expected, there is more retention time variability in the inter-system runs when compared to runs within a single system. The standard deviation for all of the runs across the three marker peaks is better than 0.2 minutes. As with the runs within a system, there is no shift in retention time for the inter-system runs to compromise the identification of a peak.

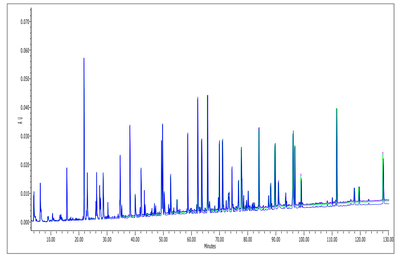

Figure 2. Overlay of 5 consecutive runs of the MassPREP Enolase Digestion Standard peptide map on ACQUITY UPLC 1.

The expanded view of a pair of closely resolved peptides that includes peak A is shown in Figure 4. The shape of the peaks and the valley between them is sensitive to all aspects of the separation, including flow rate, gradient, and temperature. The consistency of this separation is a measure of the similarity among the three ACQUITY UPLC systems.

Many factors contribute to judging quantitative behavior. Different peptides have distinctive properties resulting in more or less variability in area. Optimization of the diluents and injection mode will influence the reproducibility of the peak areas. Table 2 compares the average and percent relative standard deviation of the peak area for the three marker peaks using all of the runs from each of the three ACQUITY UPLC systems. The peak area %RSD for the three peaks within a system is better than 3.3%. The peak area %RSD for all runs is between 5-6.3%.

For quantitative characterization of a protein sample, the amount of the modified structure is often reported as a percentage of the native structure. Area ratios are a useful measure of the reliability of quantification across three systems. The area ratios shown in Table 3 for the three marker peaks are essentially identical.

	Peak A Ret. Time		Peak B Ret. Time		Peak C Ret. Time	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
ACQUITY UPLC 1	26.21	0.085	65.69	0.048	87.38	0.068
ACQUITY UPLC 2	26.28	0.019	66.01	0.107	87.53	0.071
ACQUITY UPLC 3	26.28	0.023	65.89	0.062	87.49	0.063
ALL	26.54	0.050	65.90	0.185	87.55	0.200

Table 1. Retention time average and standard deviation of peaks A, B, and C for ACQUITY UPLC 1, 2, 3 and all runs.

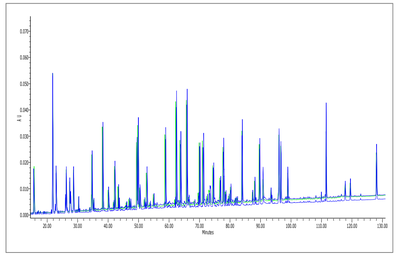

Figure 3. Overlay of injection 3 of the peptide maps of the MassPREP Enolase Digestion Standard on ACQUITY UPLC 1, 2, and 3.

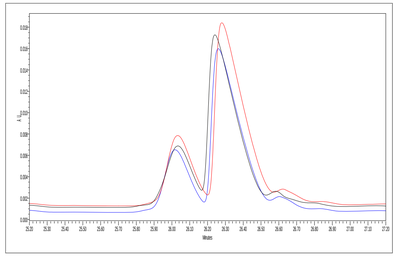

Figure 4. Overlay of injection 3 of peak A on ACQUITY UPLC 1, 2 & 3.

	Peak A Area		Peak B Area		Peak C Area	
	Mean	%RSD	Mean	%RSD	Mean	%RSD
ACQUITY UPLC 1	172802	0.80	430452	0.67	40855	2.14
ACQUITY UPLC 2	187035	1.00	467935	1.28	44650	2.19
ACQUITY UPLC 3	165696	0.38	415678	0.75	39062	3.27
ALL	175178	5.29	438021	5.28	41522	6.29

Table 2. Area average and %RSD of peaks A, B and C for ACQUITY UPLC 1, 2, 3 and all runs.

	ACQUITY UPLC 1	ACQUITY UPLC 2	ACQUITY UPLC 3
Ratio			
A/B	0.401	0.400	0.399
C/A	0.236	0.239	0.236
C/B	0.095	0.095	0.094

Table 3. Area ratio of peaks A, B, and C for ACQUITY UPLC 1, 2, and 3.

DISCUSSION

Peptide mapping is used to confirm the primary structure of a protein, identify post-translational modification (PTMs) and analyze potential impurities. ACQUITY UPLC peptide mapping provides the high resolution required by these applications. A peptide mapping method can be developed and consistently observed on a single system.

With careful attention to detail, the same separation can be transferred to additional ACQUITY UPLC systems. The quantitative reliability within such a transfer is more than satisfactory for relative quantitation. The total system solution including the instrument, column and solvents is essential to achieving these results. Protein characterization laboratories can develop fully defined peptide maps on ACQUITY UPLC. The peptide mapping method can be transferred to another department, laboratory, or CRO using the same instrument and column chemistry.

CONCLUSIONS

- **UPLC provides high resolution peptide maps. Retention and resolution are reproducible within a series of runs on a single system.**
- **Retention and resolution are consistent across multiple ACQUITY UPLC systems.**
- **Relative quantitation is consistent across multiple ACQUITY UPLC systems.**
- **Well-developed peptide mapping methods can be directly transferred to other laboratories using the same ACQUITY UPLC systems.**

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