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Application Note

ACQUITY UPLC System-to-System Reproducibility for Peptide Mapping

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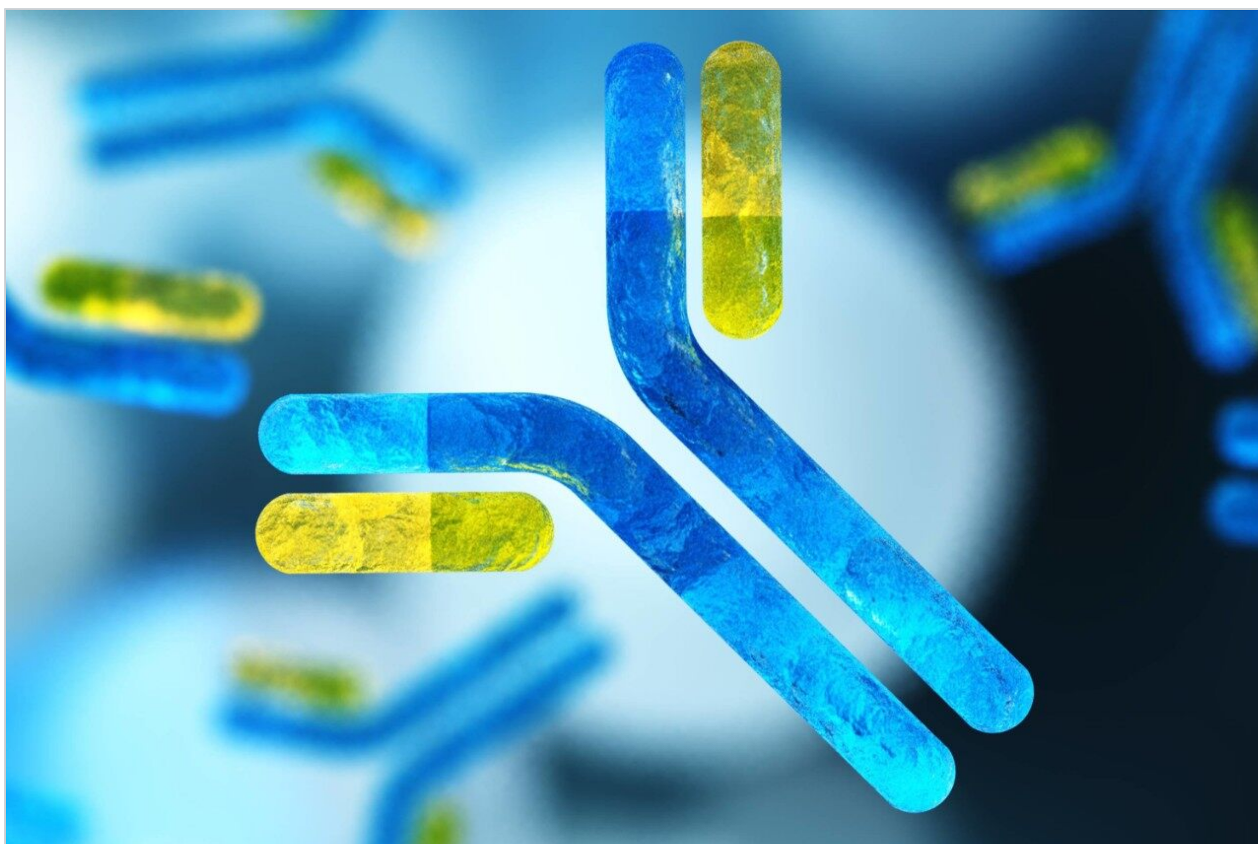

Abstract

In this application note, 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.

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 are 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, ACQUITY UPLC 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 application note, 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.

Experimental

Three identical ACQUITY UPLC Systems (ACQUITY UPLC 1, 2, and 3) were configured according to the instructions found in the UPLC Peptide Analysis Application Solution.[2] Briefly, a coreACQUITY UPLC System,

consisting of an ACQUITY UPLC Binary Solvent Manger, ACQUITY UPLC Sample Manager with Column Heater Module, 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 days 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. Waters 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 System 1. A frozen aliquot was defrosted and loaded on ACQUITY UPLC Systems 2 and 3 just before the start of the injections. Data were processed using Empower 2 Software. The peaks in the chromatograms were integrated using the ApexTrack integration alogorithm. The first injection of each day was a system blank run.

LC Conditions

Samples:	Waters 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)
LC system:	Waters ACQUITY UPLC, configured for peptide analysis (Details in experimental design section)
Column:	Waters 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:	0.020% TFA in water

Mobile phase B:	0.018% TFA in acetonitrile
Column temp:	40 °C
Injection volume:	8 µL of 10 pmol/µL of reconstituted MassPREP Enolase Digestion Standard
Mode:	Partial Loop
Weak wash:	600 µL of 95:5 H_20/ACN 0.2% TFA
Strong wash:	200 µL of 20:80 Mobile phase A/mobile phase B
Sample temp.:	4 °C
Detection:	Wavelength: 214 nm Sampling rate: 10 pts/sec Filter time constant: Normal

Gradient:	Time (min)	%A	%B	Curve
	0.0	98	2	NA
	5.0	98	2	6
	206.0	40	60	6
	206.1	10	90	6
	208.1	10	90	6
	208.2	98	2	6
	234.2	98	2	6

Results and Discussion

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

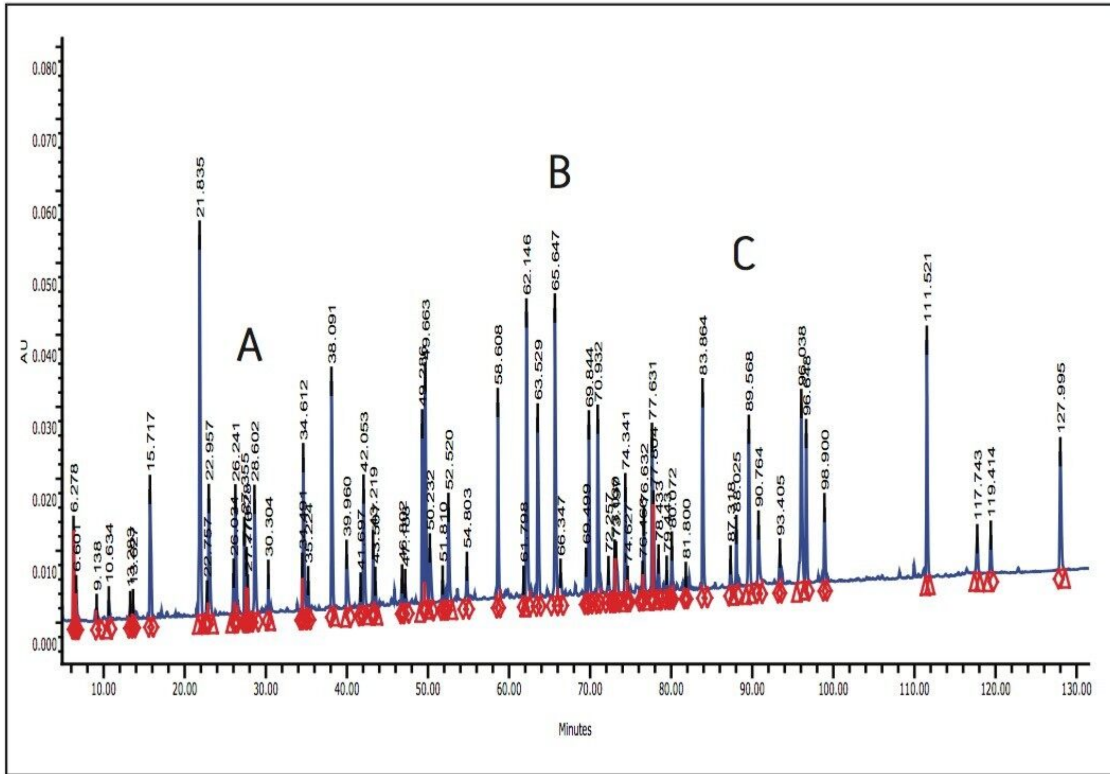

Figure 1. UV chromatogram of the peptide map of MassPREP Enolase Digestion Standard.

Figure 2 shows the overlay of five consecutive runs of the MassPREP Enolase Digestion Standard for the ACQUITY UPLC System 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 within a single system is better than 0.3% RSD.

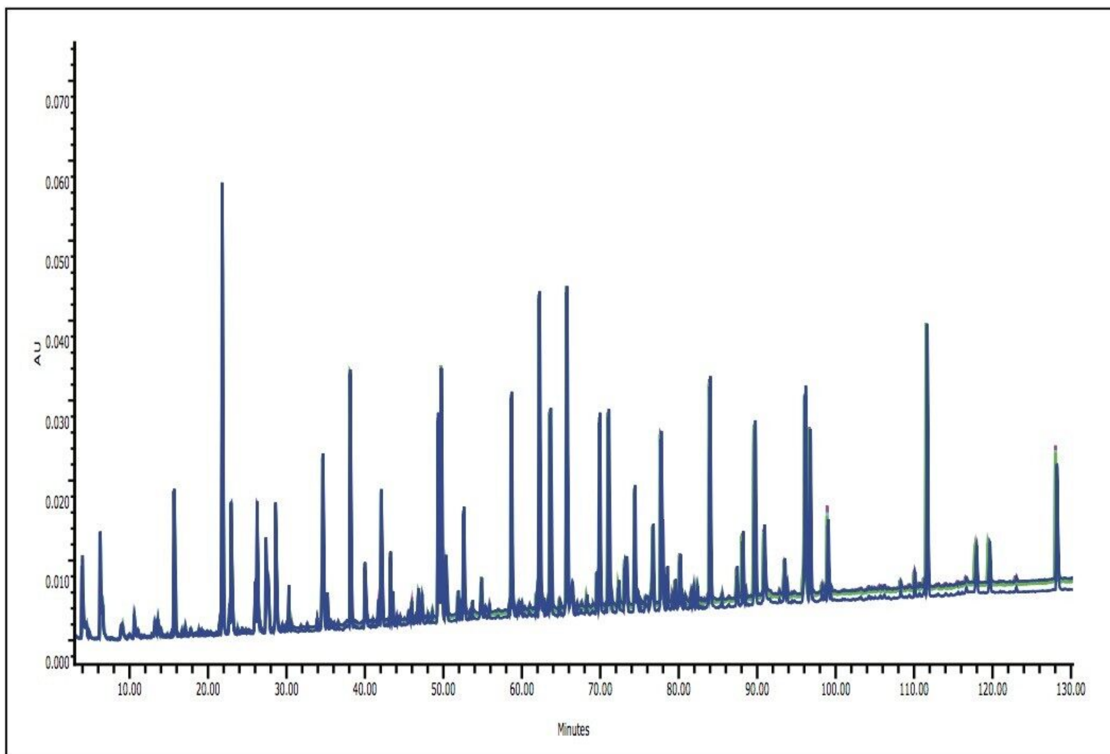

Figure 2. Overlay of five consecutive runs of the MassPREP Enolase Digestion Standard peptide map on ACQUITY UPLC 1.

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 Systems 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 was found in all chromatograms. Additionally, peaks A, B, and C were correctly identified in all chromatograms.

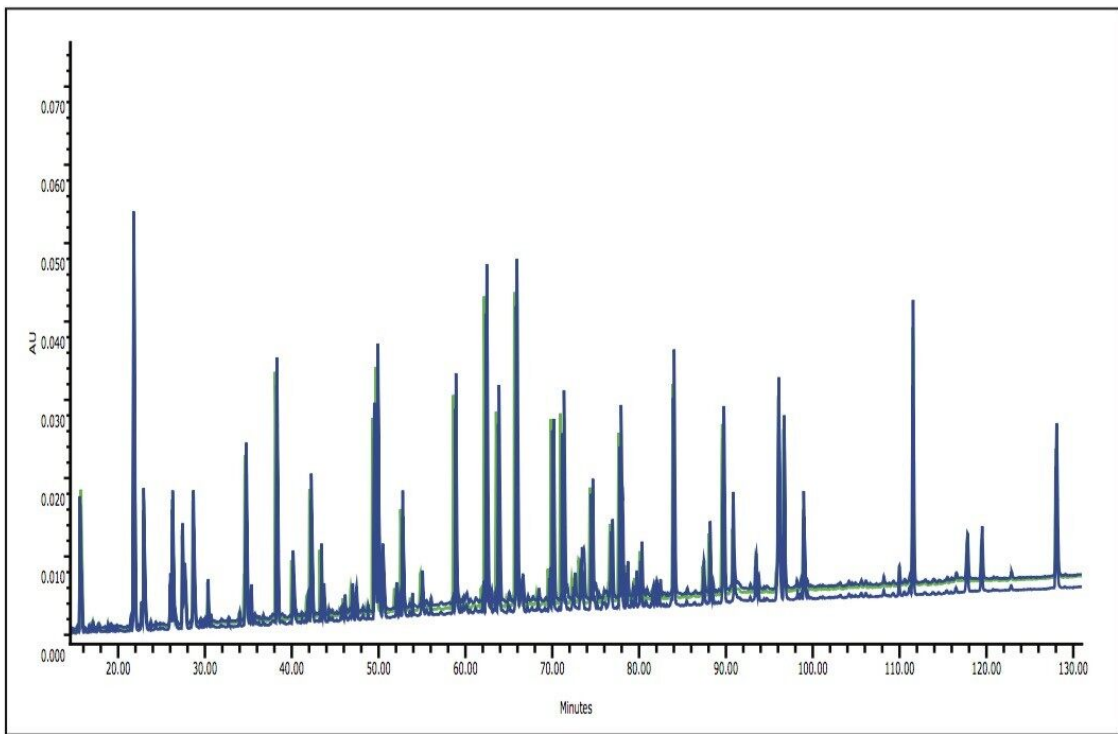

Figure 3. Overlay of injection 3 of the peptide map of the MassPREP Enolase Digestion Standard on ACQUITY UPLC Systems 1, 2, and 3.

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 was more retention time variability in the inter-system runs, compared to runs within a single system. The standard deviation for all of the runs across the three marker peaks was better than 0.20 minutes. As with the runs within a system, there was no shift in retention time for the inter-system runs to compromise the identification of a peak.

Retention time	Peak A		Peak B		Peak C	
	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation
ACQUITY UPLC System 1	26.242	0.007	65.686	0.048	87.377	0.068
ACQUITY UPLC System 2	26.284	0.005	66.007	0.120	87.522	0.077
ACQUITY UPLC System 3	26.266	0.006	65.867	0.026	87.474	0.045
All	26.264	0.019	65.900	0.185	87.458	0.088

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

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.

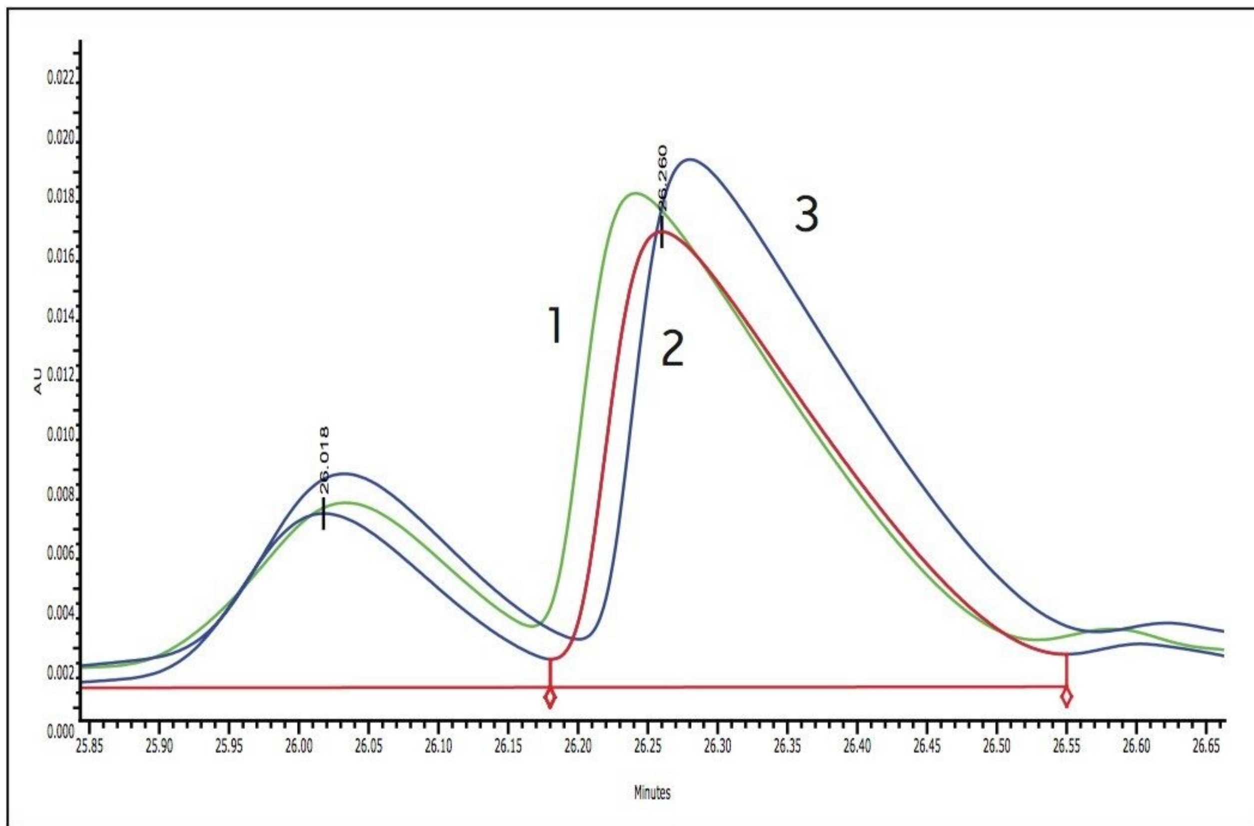

Figure 4. Overlay of injection 3 of peak A on ACQUITY UPLC Systems 1, 2, and 3.

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 modes 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 5.0% and 6.3%.

Area	Peak A		Peak B		Peak C	
	Mean	%RSD	Mean	%RSD	Mean	%RSD
ACQUITY UPLC System 1	172802	0.799	430452	0.670	40855	2.140
ACQUITY UPLC System 2	187035	0.999	467935	1.284	44650	2.186
ACQUITY UPLC System 3	165696	0.384	415678	0.752	39062	3.267
All	175178	5.294	438021	5.278	41522	6.292

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

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.

	ACQUITY UPLC System 1	ACQUITY UPLC System 2	ACQUITY UPLC System 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 Systems 1, 2, and 3.

Conclusion

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 the ACQUITY UPLC System. The peptide mapping method can be transferred to another department, laboratory, or CRO using the same instrument and column chemistry.

References

1. Wheat TE, Lu Z, Gillece-Castro B, Mazzeo JR. Quantitative Aspects of UPLC Peptide Mapping, Waters Application Note no. 720001839EN <https://www.waters.com/nextgen/us/en/library/application-notes/2008/quantitative-aspects-of-uplc-peptide-mapping.html> .
2. UPLC Peptide Analysis Application Solution Kit no. 715001381.

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ACQUITY UPLC System <https://www.waters.com/514207>

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