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アプリケーションノート

High Sensitivity Peptide Analysis with the ACQUITY UPLC System

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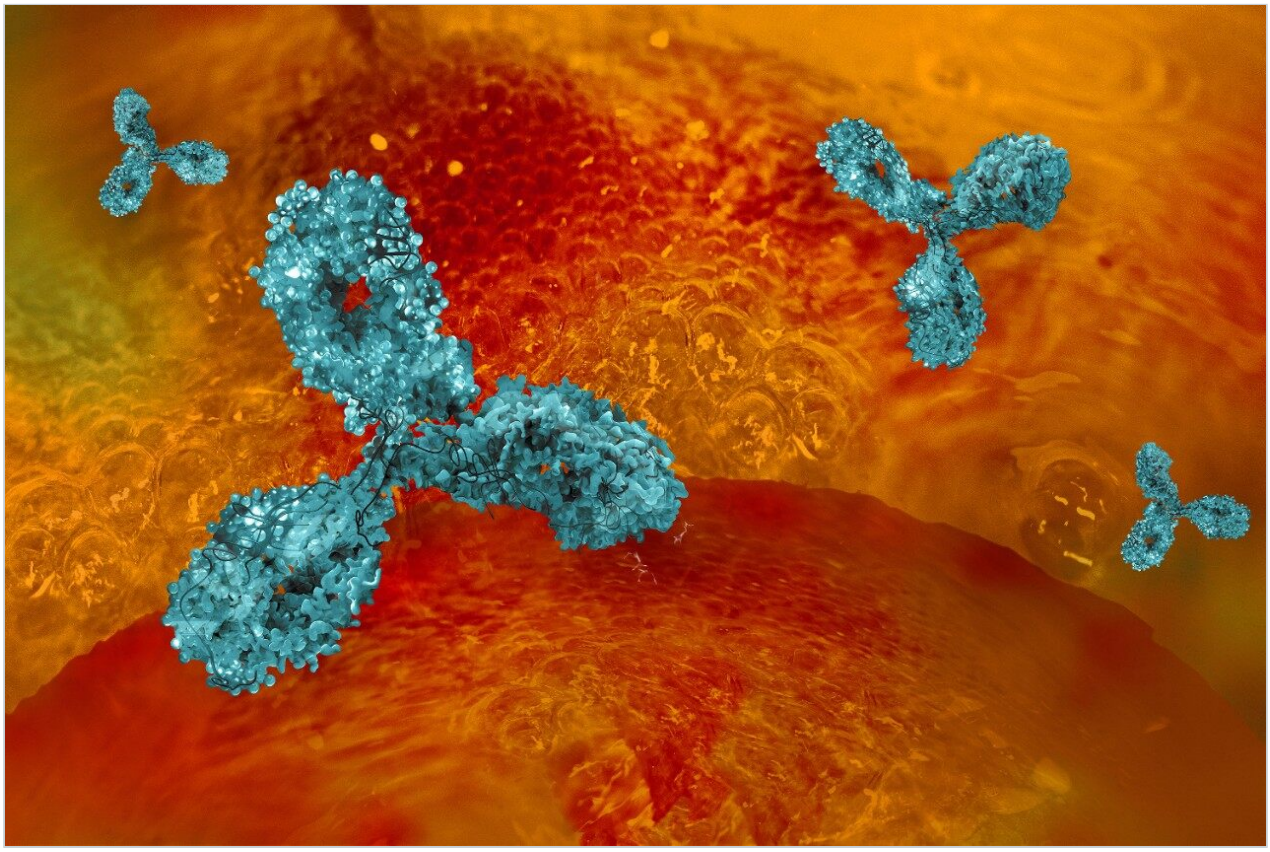

Abstract

In this application note, we demonstrate sensitive analyses for selected peptides with the ACQUITY UPLC

System.

Introduction

With the increased use of monoclonal antibodies and recombinant proteins for therapeutic purposes, peptide mapping and peptide characterization methods are gaining a prominent role in biopharmaceutical analysis field. The sensitive detection of impurities and modifications is a crucial requirement in clinical studies and the biopharmaceutical product approval process. Liquid chromatography (LC) is a useful tool for confirmation of the primary structure and purity of protein-based therapeutics.

To analyze product purity and distinguish minor modification, methods offering both high resolution and sensitivity are necessary. Peptide mapping and purity profiling should be robust, precise, and suitable for detection of minor modifications. Waters UltraPerformance LC (UPLC) technology provides capabilities for high resolution and high signal-to-noise (S/N) detection, which are very useful for fast method development of complex mixtures such as peptide maps.

In this application note, we demonstrate sensitive analyses for selected peptides with the ACQUITY UPLC System. A high S/N ratio was demonstrated for both ACQUITY UPLC Tunable Ultraviolet (TUV) and Photodiode Array (PDA) Detectors at a 4 picomole (pmol) peptide injection level.

Experimental

Sample

Waters MassPREP Peptide Standard Mixture was reconstituted in 1000 µL of 0.02% trifluoroacetic acid (TFA) aqueous solution containing 5% acetonitrile. The MassPREP Standard Mixture contained nine peptides, including:

- RASG-1
- Angiotensin fragment 1-7
- Bradykinin
- Angiotensin II
- Angiotensin I

- Renin substrate
- Enolase T35
- Enolase T37
- Melittin

The concentration of each peptide was approximately 1 pmol/µL. Samples were prepared immediately prior to use to prevent oxidation.

The peptide mixtures were separated on a Waters ACQUITY UPLC Peptide Separation Technology Column.

LC Conditions

LC system:	Waters ACQUITY UPLC System
Column:	ACQUITY UPLC Peptide Separation Technology, BEH300 C_{18} 1.7 um, 21 x 50 mm
Column temp.:	40 °C
Flow rate:	0.2 ml/min
Mobile phase A:	0.02% TFA in water
Mobile phase B:	0.018% TFA in acetonitrile
Gradient:	0 to 50% B in 30 min
Detection:	214 nm

Results and Discussion

Figure 1 shows the separation of 9-peptide MassPREP sample. Various picomole amounts of sample were injected on column, followed by a blank injection. Less than 1 pmol amount of peptides can be easily detected with a good S/N ratio, which is consistent with previously published data illustrating the limits of UV detection for peptides to be well below 250 fentomoles.[1]

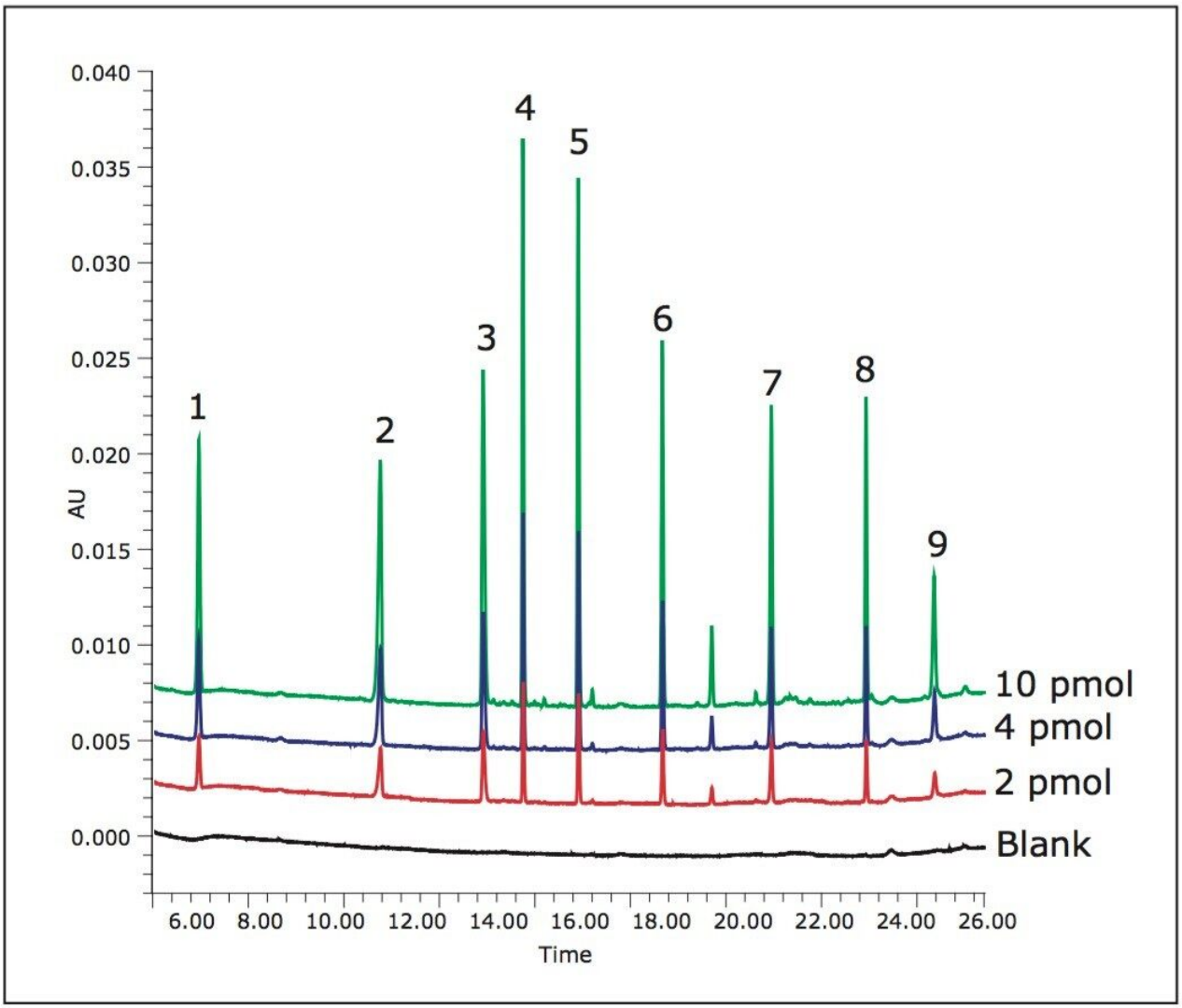

Figure 1. Chromatograms of MassPREP Peptide Standard Mixture. Peaks were detected with a TUV Detector at 214 nm.

The UV S/N in gradient elution depends strongly on the mobile phase additives. Since the peptides are typically detected at short UV wavelengths, the ion-pairing agents such as TFA contribute significantly to the background, often causing baseline drift and increased baseline noise. Understandably, the baseline noise is detrimental for the trace analysis of impurities.

Choikhet *et al*.[2] recently studied the impact of TFA on baseline noise. Small imperfections in mobile phase mixing add to the baseline noise and can interfere with the detection of minor peaks.[2]

In the experiment shown in Figure 1, we selected 0.02% TFA in water as mobile phase A and 0.018% TFA in acetonitrile as mobile phase B. The TFA balancing in mobile phase B (0.018% versus 0.02% in A) is important to minimize the baseline drift during thegradient. Although the peptide separations are typically

performed with ~0.1% TFA mobile phase concentrations, the lower TFA buffers are also used by many laboratories, with two main advantages:

- Decreasing TFA concentration in mobile phases reduces the baseline drift and mixing related noise.
- The mobile phase is more suitable for LC-MS detection.

Unlike some other columns on the market, Waters ACQUITY UPLC Peptide Separation Technology Columns perform well with reduced TFA concentrations. Little or no evidence of peak broadening or tailing is observed with 0.02% TFA concentration. ACQUITY UPLC Peptide Separation Technology Columns packed with 1.7 µm sorbent provide increased resolution and narrower peaks than achievable with conventional LC cColumns. This in turn improves the detection S/N, since the peaks are less diluted in the detector cell.[3,4]

Figure 2 illustrates an analysis of 9-peptide mixture monitored by PDA or single wavelength TUV detection. The S/N ratios for the selected peptides were calculated according to European Pharmacopeia (EP) definition as shown in Figure 3.

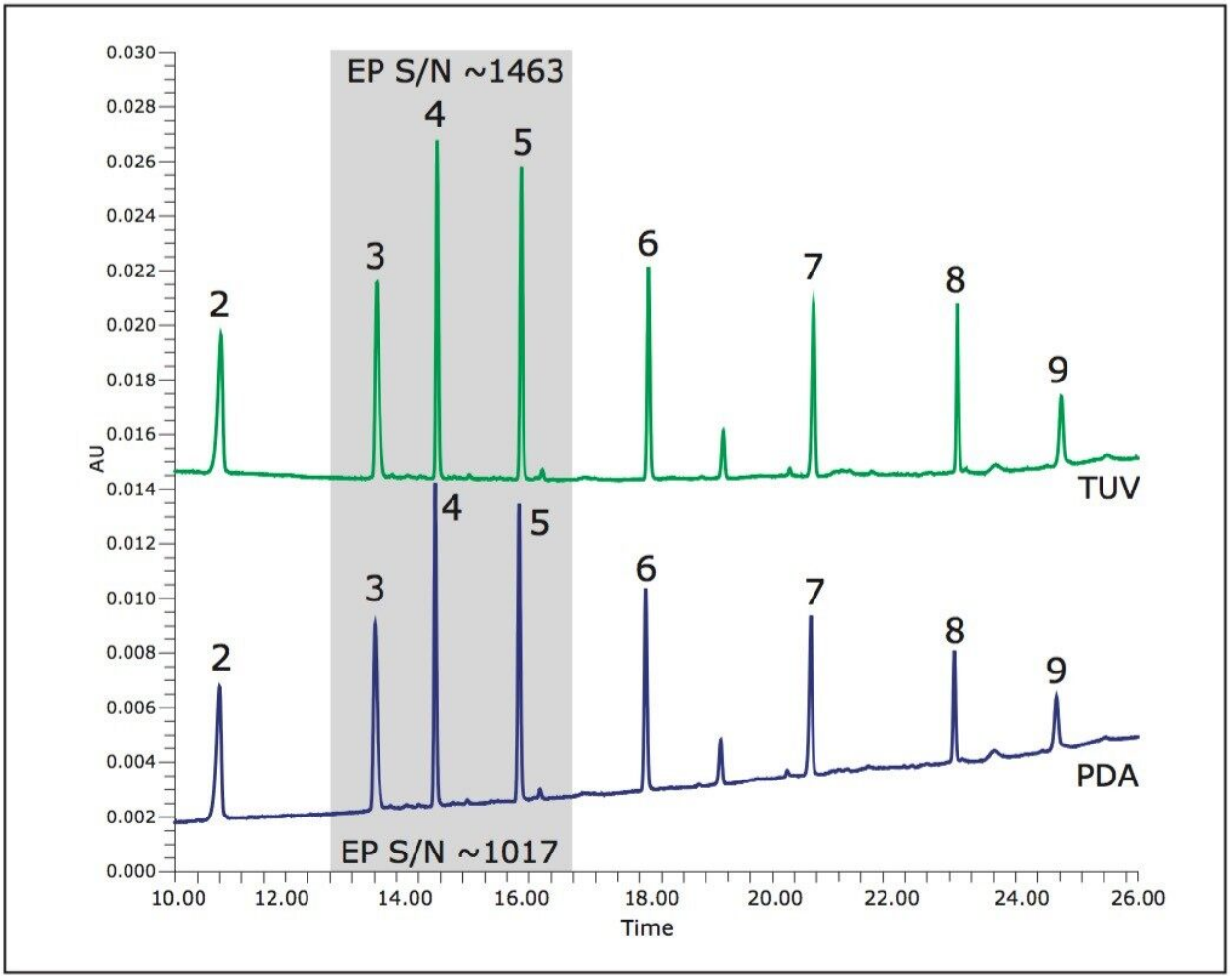

Figure 2. The MassPREP Peptide Standard Mixture was analyzed at 4 pmol and detected by either TUV or PDA at 214 nm. The EP S/N ratios were calculated based on peaks 3, 4, and 5.

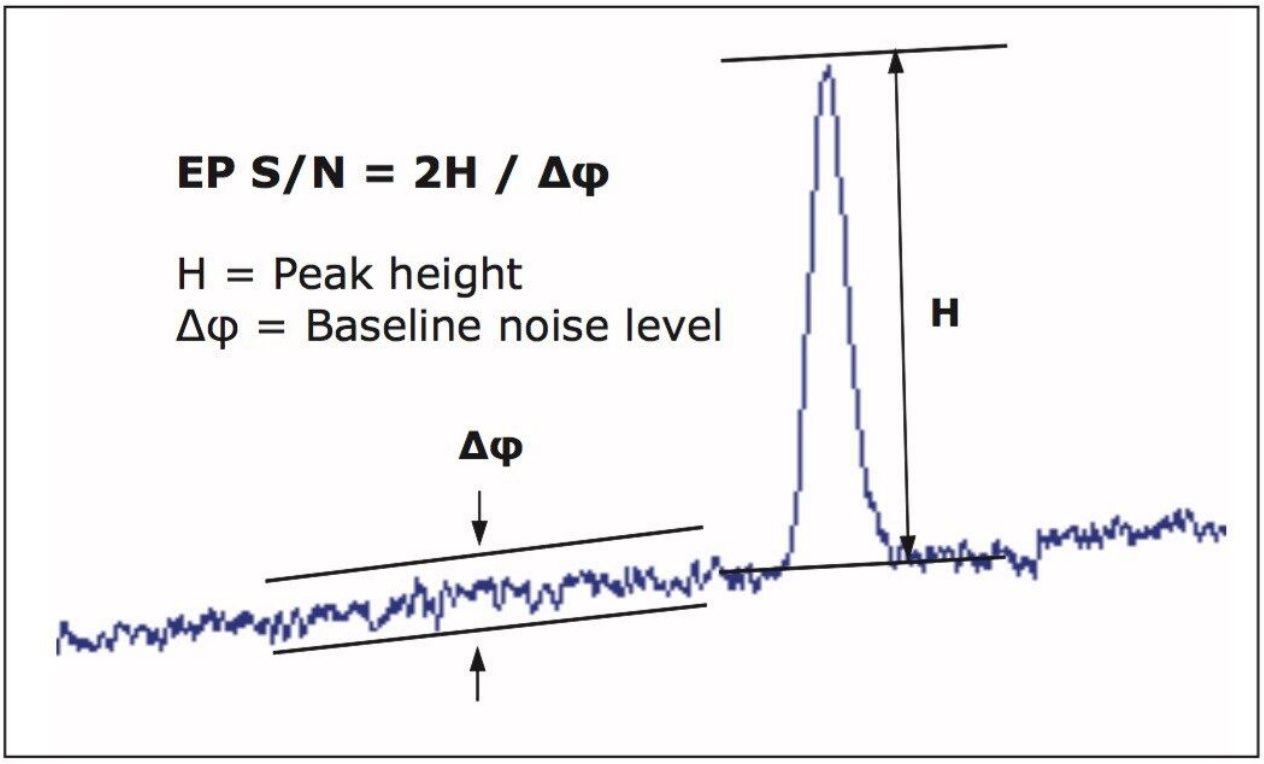

Figure 3. An illustration for the calculation of European Pharmacopeia signal-to-noise ratio.

Using detector settings as outlined in Table 1, the measured S/N ratios were about 1017 for PDA and 1463 for TUV detector at injection of ~4 pmol of each peptide (Figure 2). The resolution (optical bandwidth) on the PDA can be increased, such that a resolution of 4.8 nm on the PDA will improve S/N ratios comparable to TUV. Naturally, the PDA spectral resolution is reduced when using larger slit widths for detection.

Detector	TUV	PDA
Flow cell volume	0.5 µL	0.5 µL
Flow cell flow path	10 mm	10 mm
Sampling wavelength	214 nm	214 nm
Resolution (optical bandwidth)	5 nm	1.2 nm
Sampling rate	20 Hz	20 Hz
Filter time constant	Fast (0.2 sec)	Normal (0.2 sec)

Table 1. Detector settings.

Conclusion

The results from peptide mixture analysis with ~0.02% TFA mobile phases demonstrate several advantages of using the UPLC system for analysis and characterization of peptides.

- Peptide Separation Technology columns perform well with 0.02% TFA mobile phases. Good peak shape and high peak capacity is obtained.
- Reducing the amount of TFA ion-pairing agent in the mobile phase improves the detection S/N ratios for both TUV and PDA detectors.
- The S/N levels are in order of ~1000 at 4 pmol injection levels, which allow for detection of peaks from minor impurities. Tens to hundreds fentomoles of impurities are readily detectable.[1]
- When using 0.1% TFA mobile phases, the sensitivity of UV detection does not change. However, the noise level increases moderately, reducing the S/N by factor of 2-3.
- The high S/N ratio is crucial when analyzing impurities and minor peptide modification as is often case in the peptide mixture analyses.

This application note demonstrates the ability of the ACQUITY UPLC System with UV detection for high sensitivity and high S/N peptide analysis. This capability is critical for biopharmaceutical drug development, characterization, and validation: the quality of analysis has a direct impact on fulfilling regulatory

requirements for new biopharmaceutical compounds.

Early detection of minor structural variants will make the drug development safer and robust and may significantly reduce the cost of drug development. Similarly to proteins, the therapeutic synthetic peptides can be analyzed and characterized with UPLC and UPLC-MS tools. The ACQUITY UPLC System with Peptide Separation Technology Columns allow for fast characterization of peptides and peptide mixtures with speed that is typically four to eight times greater compared to conventional HPLC systems using columns packed with 5 µm sorbents.

References

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