

# Using Empower 3 Software for Monitoring Synthetic Peptide Impurities with an ACQUITY QDa Detector for Improved Confidence in Analysis

Brooke M. Koshel, Robert E. Birdsall, and Ying Qing Yu  
Waters Corporation, Milford, MA, USA

## APPLICATION BENEFITS

- Incorporating mass detection of synthetic peptide impurities using compliant-ready Empower 3 Software
- Integrated peak purity functionality for readily assessing product quality

## WATERS SOLUTIONS

[ACQUITY UPLC® H-Class Bio System](#)

[ACQUITY® QDa® Detector](#)

[ACQUITY UPLC Tunable Ultra-Violet \(TUV\) Detector](#)

[ACQUITY UPLC Peptide CSH C<sub>18</sub> Column](#)

[Empower 3 Chromatography Data Software](#)

## KEYWORDS

Synthetic peptide, Empower, impurities, impurity tracking, compliance

## INTRODUCTION

Peptide-based therapeutics have recently seen a renewed interest due in part to overcoming some of the early challenges imparted by their physicochemical properties.<sup>1,2</sup> Therapeutic peptides can be produced through chemical synthesis, recombinant strategies, or through extraction from a natural source.<sup>1</sup> Although each route can offer advantages, an increasing number of these drugs are now being made synthetically. When following a synthetic approach, the raw materials as well as the manufacturing process can both contribute to process-related impurities, where many of the modifications or deletions can be pre-determined,<sup>3</sup> and are generally considered to be safe at low levels.<sup>4</sup>

Most often, determining synthetic peptide purity is an LC-UV-based assay.<sup>5,6</sup> Recent work by Zeng and colleagues reported that their LC-HRMS method could identify additional impurities not reported through the USP HPLC-UV method for a currently marketed product.<sup>7</sup> The notion that manufacturers should continually strive to improve product knowledge with the interest of public health in mind has global implications. The International Council for Harmonisation (ICH), which brings together regulators and the pharmaceutical industry from Europe, Japan, and the United States, addresses this need with ICH Q10, a quality document that supports innovation and continual improvement throughout product lifecycle.<sup>8</sup> In the current study, we show that by incorporating the ACQUITY QDa Detector, a cost effective mass detector, impurities can be identified without the need for expensive instrumentation. Because impurities that are not qualified generally have tighter acceptance criteria,<sup>6</sup> added mass detection can more readily confirm impurity identification than relying on relative retention time from an optical-based assay.

In this study, eledoisin, a vasodilator, is used as a clinically relevant sample to demonstrate how the ACQUITY QDa Detector with Empower 3 Software can be used for synthetic peptide impurity monitoring. Eledoisin impurities can be documented via traditional optical-based methods, but by incorporating the ACQUITY QDa Detector, co-elutions can be realized. This strategy demonstrates the ability to improve confidence in a manufacturing environment by combining optical detection and orthogonal mass detection into a single workflow.

## EXPERIMENTAL

### Sample description

The synthetic peptide eledoisin (pE-PSKDAFIGLM-amide) was purchased from New England Peptide Inc. (Gardner, MA) at  $\geq 95\%$  purity by HPLC percent area. A stock solution of 2 mg/mL eledoisin in water was further diluted to a working concentration of 0.4 mg/mL.

### LC conditions

|                 |  |
|-----------------|--|
| LC system:      | ACQUITY UPLC H-Class Bio System  |
| Detector:       | ACQUITY UPLC Tunable Ultra-Violet (TUV) Detector   |
| Wavelength:     | 215 nm   |
| Vials:          | LCMS Certified Clear Glass 12 x 32 mm Screw Neck Total Recovery Vial<br><a href="#">(p/n 600000750cv)</a>                |
| Column:         | ACQUITY UPLC Peptide CSH C <sub>18</sub> 130 Å<br>1.7 $\mu\text{m}$ , 2.1 mm x 100 mm<br><a href="#">(p/n 186006937)</a> |
| Column temp.:   | 60 °C  |
| Sample temp.:   | 10 °C  |
| Injection vol.: | 5 $\mu\text{L}$  |
| Mobile Phase A: | H <sub>2</sub> O with 0.1% (v/v) FA  |
| Mobile Phase B: | Acetonitrile with 0.1% (v/v) FA  |
| Gradient:       | Original: 15–45% B over 20 minutes<br>Optimized: 16–24% B over 30 minutes  |

### MS conditions

|                    |   |
|--------------------|---|
| MS system:         | ACQUITY QDa Detector<br>(Performance Model) |
| Ionization mode:   | ES+, centroid                               |
| Mass range:        | 350–1250 Da                                 |
| Capillary voltage: | 1.5 kV                                      |
| Cone voltage:      | 10 V  |
| Probe temp:        | 500 °C                                      |

### Data management

Empower 3 CDS, SR2

## RESULTS AND DISCUSSION

### MONITORING INDIVIDUAL IMPURITIES TO MEET QUALITY STANDARDS

Our previous work has shown how Empower 3 Software can be used for monitoring and reporting eledoisin impurities in accordance with ICH and USP guidelines with functionalities that are integrated into the software.<sup>9</sup> In brief, a 20 minute gradient from 15–45% acetonitrile in 0.1% (v/v) formic acid using CSH C<sub>18</sub> column chemistry was used to separate eledoisin from its process-related impurities (Figure 1A). To investigate the added benefit of mass detection, an ACQUITY QDa Detector was incorporated as an inline orthogonal detection method. The profile of the total ion chromatogram (TIC) (Figure 1B) shows a strong correlation with the optical data, suggesting that the ACQUITY QDa Detector is an appropriate detector for analysis.

The following criteria were used to determine if an eledoisin sample met acceptance criteria:

Any individual impurity: Not more than (NMT) 1.5%

Total impurities: NMT 5.0%

From Figure 1A, Impurity 3 was determined to be 1.6%, and thus is outside of specification. With added mass detection, we can further interrogate this impurity. Figure 2A shows a screen capture of the Mass Analysis Window within the Empower Software. The MS spectrum of Impurity 3 can be displayed for both the peak apex and the combined or average scan. From the combined spectrum, 638.5 and 595.0 are the most dominant  $m/z$  values. Having previous knowledge of what impurities are present in the eledoisin sample, these  $m/z$  values are known to represent impurities. Using these  $m/z$  values, extracted ion chromatograms (XICs) can be used to determine relative area percent of each of the two individual impurities that contribute to Impurity 3.

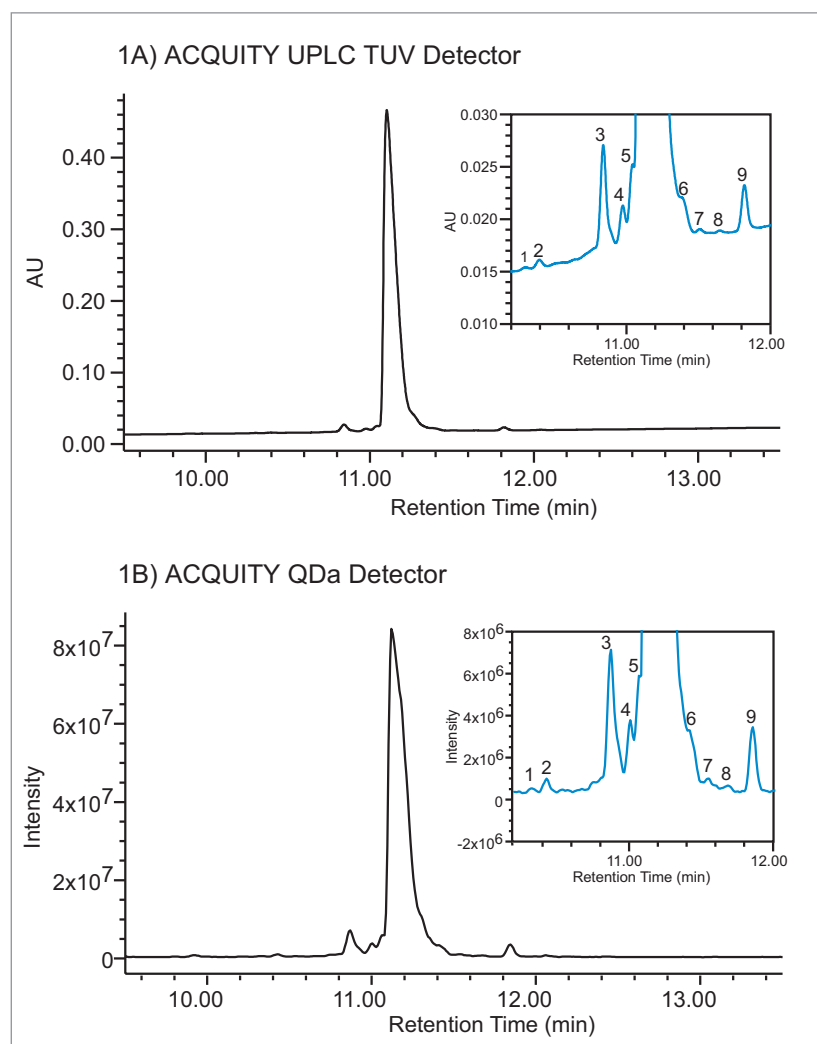


Figure 1. Synthetic peptide separation of eledoisin from its process-related impurities.

1A) Optical detection of eledoisin with inset showing impurities.

1B) Corresponding QDa data. Inset shows strong visual correlation to optical data.

From Figure 2B, Peak 3 is composed of 69.6% Impurity 3a and 30.4% Impurity 3b. With this in mind, if we now consider Impurity 3 as being composed of Impurity 3a and Impurity 3b, neither of these individual impurities exceeds the acceptance criteria of NMT 1.5% for any individual impurity. Being able to readily identify qualified peaks that are not critical attributes can avoid any further investigation, which can ultimately save both time and money by allowing operations to run more efficiently.

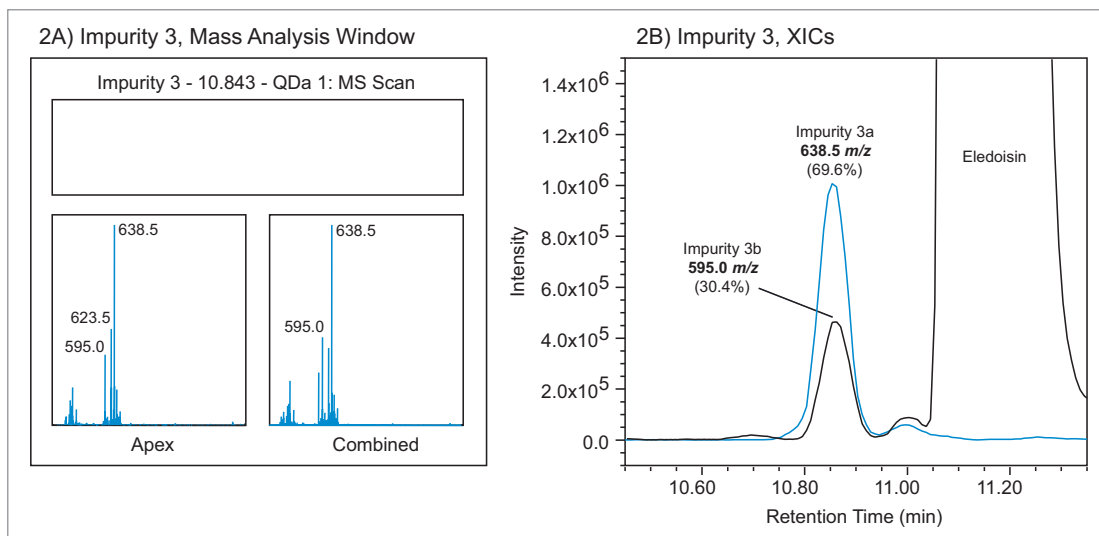


Figure 2. Determining the composition of the Peak 3 impurity. 2A) Screen capture from Empower Software of the Mass Analysis Window. Mass data is displayed for both the highest point (Apex) and the average (Combined) spectrum of Impurity 3. XICs of the two most dominant  $m/z$  values from the combined scan were used to determine the composition of Impurity 3, as a combined scan is more representative of the overall peak composition. (Note that the window above the MS spectra is blank because the TUV detector was used.) 2B) XICs of 638.5  $m/z$  (Impurity 3a) and 595.0  $m/z$  (Impurity 3b). From integration of the XICs, Impurity 3 is composed of 69.6% 3a and 30.4% 3b. By treating each of the impurities separately, the sample no longer contains peaks that exceed the criteria for the maximum allowable value for any individual impurity.

### ASSESSING INDIVIDUAL PEAK PURITY FOR DETERMINING PRODUCT QUALITY

It is not uncommon for synthetic peptide impurities to co-elute with the main peak of interest.<sup>10</sup> These co-eluting impurities may or may not be detrimental to patient safety, but their presence can lead to misinterpretation of overall product quality and purity. In addition to viewing mass data for the peak apex or combined peak, Purity View can be used to display mass data for the leading edge, apex, and trailing edge of the peak of interest. From the Apex and Combined scans of eleodoisin (Figure 3A), spectra appear clean and free of  $m/z$  values of unrelated species. This indicates that any impurities present are likely present at relatively low levels. However, from the Purity Spectrum View (Figure 3B), spectra from the leading edge (559.5  $m/z$ ) and trailing edge (630.6  $m/z$ ) of the main peak show  $m/z$  values that do not correspond to eleodoisin (595.1  $m/z$ ). This indicates that there are closely eluting peaks present that are not well resolved from the main peak of eleodoisin, which is also evidenced in Figure 1.

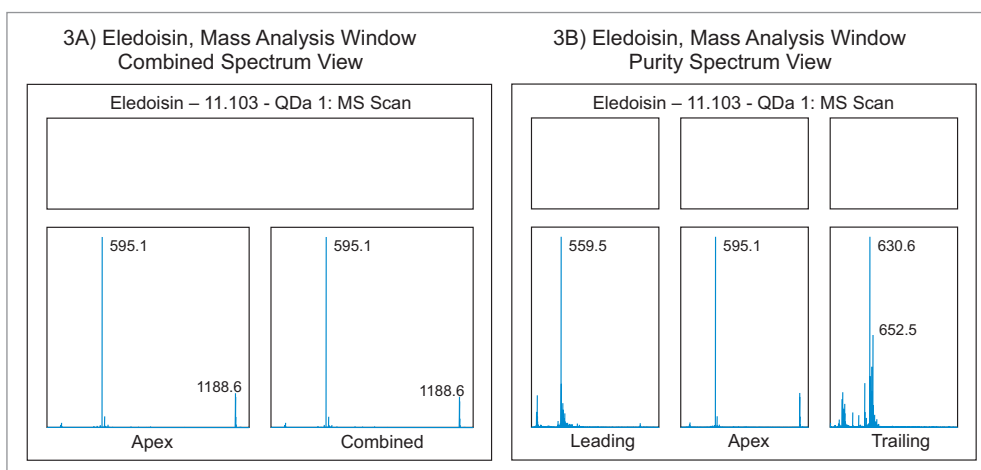


Figure 3. Screen captures from Empower Software Mass Analysis Window. 3A) Mass data is displayed for both the highest point (Apex) and the average (Combined) spectrum of eleodoisin. The  $m/z$  values shown in the screen captures match the values calculated for the  $[M+1H]^+$  and  $[M+2H]^{2+}$  charge states. 3B) Mass data is displayed for the leading edge, apex, and trailing edge of eleodoisin. The  $m/z$  values from the leading edge and trailing edge spectra are not charge states of eleodoisin, but instead are impurities that are not resolved from the main peak. (Note that the windows above the MS spectra are blank because the TUV detector was used.)

Figure 4A shows XICs of 595.1  $m/z$  (eledoisin), 559.5  $m/z$  (Impurity 5), and 630.6  $m/z$  (Impurity 6). From this chromatogram, these two impurities are not well resolved from the main peak. By re-evaluating the original gradient used, a more focused gradient can be created in an effort to tease out additional impurities from the main peak. To generate a new gradient, it was determined what percentage of acetonitrile elutes the main peak under the first set of conditions. A narrower gradient centering on this elution percentage can then be used. Figure 4B shows XICs of these same  $m/z$  values using the optimized gradient, 16–24% acetonitrile in 30 minutes. From the optimized separation, Impurity 5 and Impurity 6 no longer co-elute with the main peak. The added mass detection in this case helped to determine a more optimal separation, which can lead to a more accurate determination of product purity.

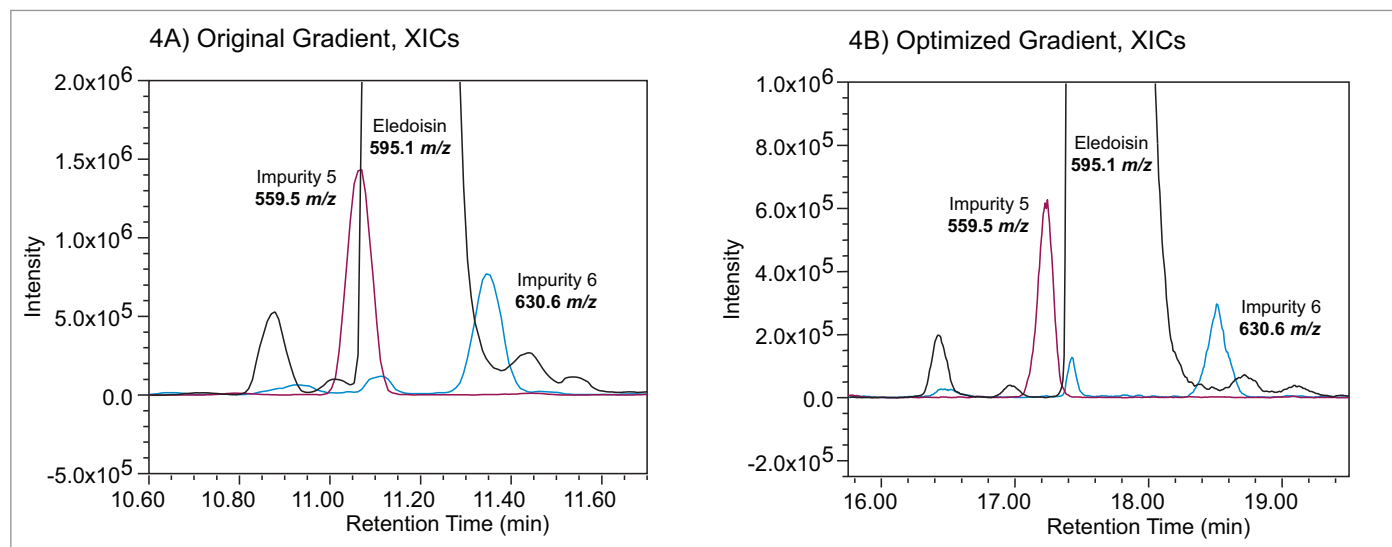


Figure 4. XICs of 559.5  $m/z$  (Impurity 5), 595.1  $m/z$  (eledoisin), and 630.6  $m/z$  (Impurity 6). 4A) Original gradient, 15–45% B in 20 minutes. From the chromatogram, Impurity 5 and Impurity 6 are not well resolved from the main peak. This creates inaccuracy in reporting the overall peak purity. 4B) Optimized gradient, 16–24% B in 30 minutes. The focused gradient further separates closely eluting impurities from the main peak, which can aid in determining product purity more reliably.

## CONCLUSIONS

This work demonstrates how mass detection can be readily incorporated into an existing optical-based workflow for analysis of synthetic peptide impurities. Our previous work using a traditional LC-UV method was shown to benefit from added mass detection in accordance with ICH Q10, which identifies the need to enhance product quality through innovation and continual improvements in development and manufacturing activities. The ACQUITY QDa Detector was shown to identify the presence of co-eluting impurities and determine product purity for further method optimization. Overall, the added mass detection provides an efficient and cost-effective strategy for improving confidence in analysis.

## References

1. Uhlig, T. et al. The Emergence of Peptides in the Pharmaceutical Business: From Exploration to Exploitation. *EuPA Open Proteomics*. 2014; 4: 58–69.
2. Vlieghe, P., Lisowski, V., Martinez, J., Khrestchatsky, M. Synthetic Therapeutic Peptides: Science and Market. *Drug Discovery Today*. 2010; 15(1–2): 40–56.
3. Scanlon, D. B. and Finlayson, J. Prep/Semiprep Separations of Peptides. In *Methods in Molecular Biology* (vol. 251) HPLC of Peptides and Proteins: Methods and Protocols. Edited by: Aguilar, M.-I. Totowa, NJ: *Humana Press Inc.*, 2004.
4. Vergote, V., Burvenich, C., Van de Wiele, C., De Spiegeleer, B. Quality Specifications for Peptide Drugs: A Regulatory-Pharmaceutical Approach. *Journal of Peptide Science*. 2009; 15: 697–710.
5. Eggen, I., Gregg, B., Rode, H., Swietlow, A., Verlander, M., Szajek, A. Control Strategies for Synthetic Therapeutic Peptide APIs Part I: Analytical Considerations. *Pharmaceutical Technology*. 2014.
6. Eggen, I., Gregg, B., Rode, H., Swietlow, A., Verlander, M., Szajek, A. Control Strategies for Synthetic Therapeutic Peptide APIs Part II: Raw Materials Considerations. *Pharmaceutical Technology*. 2014.
7. Zeng, K., Geerlof-Vidavisky, I., Gucinski, A., Jiang, X., Boyne, M. T. Liquid Chromatography-High Resolution Mass Spectrometry for Peptide Drug Quality Control. *The AAPS Journal*. 2015; 17(3): 643–651.
8. ICH, ICH Q10 Pharmaceutical Quality System. 2008.
9. Koshel, B. M., Birdsall, R. E., Yu, Y. Q. LC-UV-Based Synthetic Peptide Impurity Tracking and Reporting with Compliant-Ready Empower 3 Software. Waters Application Note. 2017; 720005968EN.
10. Sanz-Nebot, V., Toro, I., Barbosa, J. Fractionation and Characterization of a Crude Peptide Mixture from the Synthesis of Eleodoisin by Liquid Chromatography-Electrospray Ionization Mass Spectrometry. *Journal of Chromatography A*. 1999; 846: 25–38.

# Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters, The Science of What's Possible, ACQUITY UPLC, QDa, and Empower are registered trademarks of Waters Corporation. All other trademarks are the property of their respective owners.

©2017 Waters Corporation. Produced in the U.S.A. April 2017 720005967EN AG-PDF

Waters Corporation  
34 Maple Street  
Milford, MA 01757 U.S.A.  
T: 1 508 478 2000  
F: 1 508 872 1990  
www.waters.com