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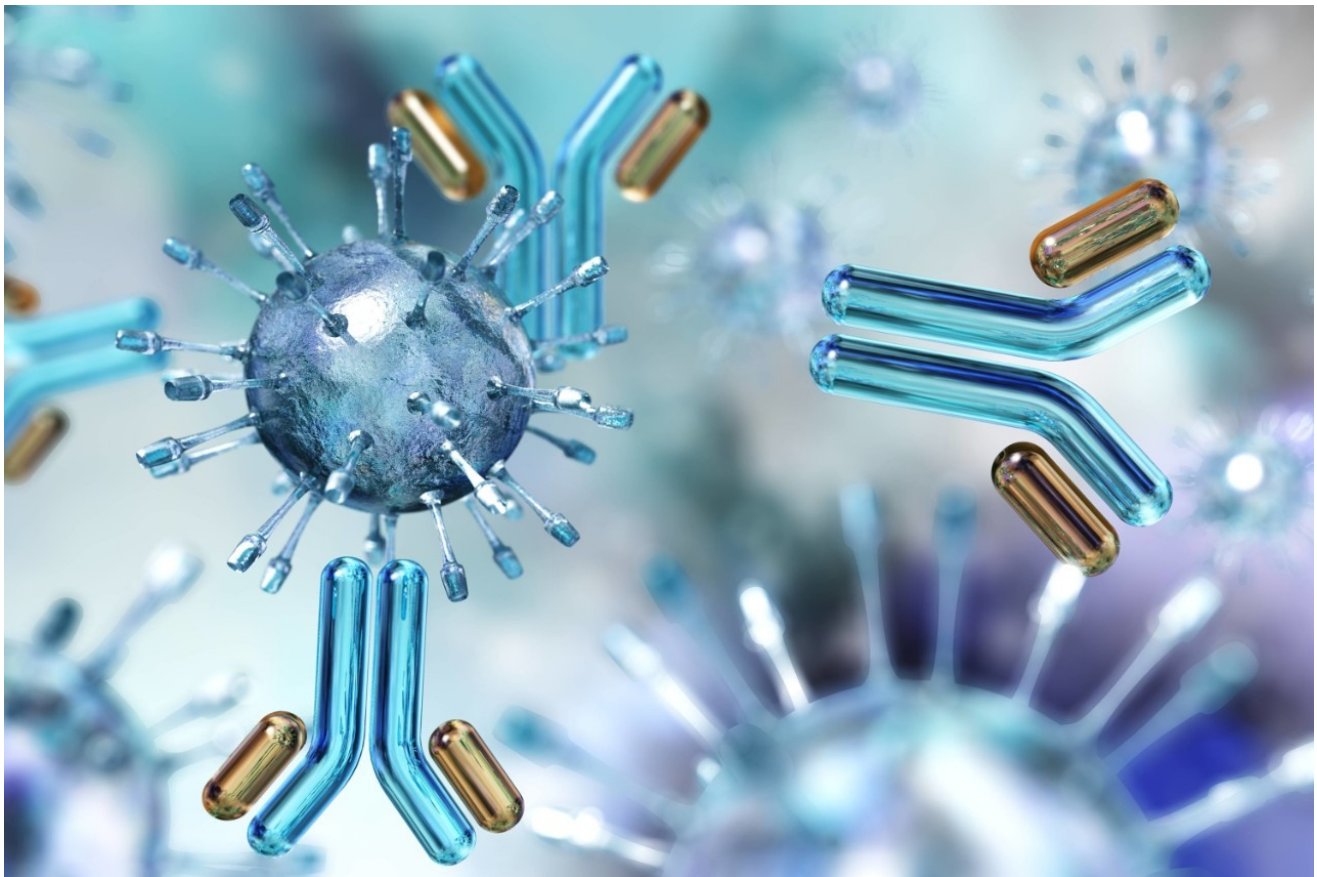
應用手冊

## Small Scale Peptide Isolation Using UPLC with Mass-Directed Purification

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### Abstract

The ACQUITY UPLC H-Class System configured with the Waters Fraction Manager-Analytical (WFM-A), with its

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very low system dispersion, exact control of solvent composition, accurate sample injection scheme, and precise fraction collection, allows scientists to perform small scale peptide and impurity isolation with assurance. This application note illustrates the utility of the ACQUITY UPLC H-Class and Waters Fraction Manager-Analytical (WFM-A) systems for the analysis and isolation of a synthetic peptide at the small scale.

## Benefits

- Mass-directed small scale purification is a useful technique for isolating target peptides from crude mixtures and simplifies the process of obtaining compounds for preliminary diagnostic studies from samples with limited availability.
- Very low system dispersion, exact control of solvent composition, accurate sample injection, precise fraction collection, and innovative column chemistries improve the isolation of target peptides from complicated sample matrices, facilitating the timely procurement of product for exploratory experimental investigations.
- ACQUITY UPLC H-Class Systems configured with the ACQUITY QDa Detector, the Waters Fraction Manager-Analytical (WFM-A), and FractionLynx Software provide clear identification of target compounds in complex matrices, customized fraction collection and tracking, and automatic sample processing, which improve the purification process efficiency.

## Introduction

Peptides and other biological molecules such as proteins and monoclonal antibodies are becoming increasingly popular in many therapeutic areas such as drug discovery, medical diagnostics, and precision medicine.<sup>1,2</sup> Because the initial stages of drug discovery often require only small amounts of target compound, fast and efficient product isolation is a key element to meeting aggressive development timelines. While traditional peptide isolation is generally performed using UV detection, mass-directed isolation makes the purification process easier by providing better differentiation between target peptide and the contaminants formed during synthesis and cleavage. In addition, developing a separation that utilizes both mass and UV detections ensures a more complete chromatographic profile. Compounds that do not ionize, or ionize poorly, will often be detected with low wavelength UV. Conversely, peptides with very low UV extinction will usually be detected with mass analysis. The fluidically-optimized flow path of the UPLC combined with a specially-designed low dispersion fraction collector enable the mass-directed isolation of sharp, narrow product peaks. In this study, we illustrate the utility of the ACQUITY UPLC H-Class and Waters Fraction Manager-Analytical (WFM-A) systems for the analysis and isolation of a synthetic peptide at the small scale. Fast, targeted isolation increases purification

efficiency by reducing unnecessary sample handling while generating just enough product for future experiments.

## Experimental

### Instrumentation

UPLC System controlled by MassLynx Software with FractionLynx Application Manager: ACQUITY UPLC H-Class, ACQUITY Sample Manager, ACQUITY PDA Detector, ACQUITY QDa Detector, ACQUITY Isocratic Solvent Manager (ISM), Waters Fraction Manager-Analytical (WFM-A)

### LC conditions

Column:	ACQUITY UPLC Peptide BEH C <sub>18</sub> , 2.1 × 100 mm, 1.7 μm, 130Å, (p/n: 186003555)
Flow rate:	0.5 mL/min
Mobile phase A:	0.1% trifluoroacetic acid in water
Mobile phase B:	0.1% trifluoroacetic acid in acetonitrile
Column temp.:	30 °C
Makeup solvent:	9:1 methanol:water, 0.01% formic acid
ISM flow rate:	0.6 mL/min
Wavelength:	214 nm

Gradients and injection volumes: as noted in figures

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### QDa Conditions

MS scan, 100–1250  $m/z$ , ES+, continuum

Sampling frequency: 2 Hz

Cone voltage: 10 V

Probe temp.: 500 °C

ESI capillary: Positive mode, 1.5



Figure 1. UPLC system controlled by MassLynx Software with FractionLynx Application Manager: ACQUITY UPLC H-Class, ACQUITY Sample Manager, ACQUITY PDA Detector, ACQUITY QDa Detector, ACQUITY Isocratic Solvent Manager (ISM), and Waters Fraction Manager-Analytical (WFM-A).

## Sample

Peptide comprised of 11 amino acids (6 nonpolar, 2 polar, 2 acidic, 1 basic); 1.3 and 5.4 mg/mL, dissolved in water and filtered

<b>Monoisotopic peptide mass: 1188.6</b>	
<b>Target ions for charge states</b>	<b><i>m/z</i></b>
$[M+H]^+$	1189.6
$[M+2H]^{2+}$	595.3

## Results and Discussion

The biopharmaceutical peptide drug candidate, with its inherent complexities, often requires isolation from complicated sample matrices. Whether the target peptide is synthesized chemically, or isolated from natural sources, initial experimental studies to determine efficacy in drug discovery may only require small amounts for testing. Undeniably, in some cases, only limited amounts of the potential candidate are available in the sample mixture at the outset of the investigation. The high specificity and sensitivity of mass analysis, combined with the improvements in UPLC and fraction collection technology, ensure the successful implementation of mass-directed purification at the small scale. For the studies described, the ACQUITY UPLC H-Class and Waters Fraction Manager-Analytical systems were employed for the analysis and isolation of a synthetic peptide at the small scale. Expedious, unambiguous identification and purification of the peptide yielded high purity product with an efficient, streamlined workflow.

### Crude peptide screening and gradient focusing<sup>3</sup>

In most preparative workflows, crude sample analysis and method development are usually performed to optimize the separation before isolation. The process for small scale mass-directed purification is no different. The ACQUITY UPLC H-Class System configured with the WFM-A can be used with, or without fraction collection, depending upon the objective of the experiment. The selection of a specific fraction method in the sample list turns collection on or off.<sup>3</sup>

~~A chromatographic method with a gradient slope of 2.06% change per column volume on a 2.1 × 100 mm~~

reversed-phase column was used to screen the crude peptide. The large peptide product peak and three closely-eluting impurity peaks were present in the initial analysis of the sample mixture. Although this separation was almost baseline-resolved (Figure 2A), focusing the gradient and using a slope of 0.41% change per column volume altered the chromatographic profile and improved the peak-to-peak resolution even further (Figure 2B).

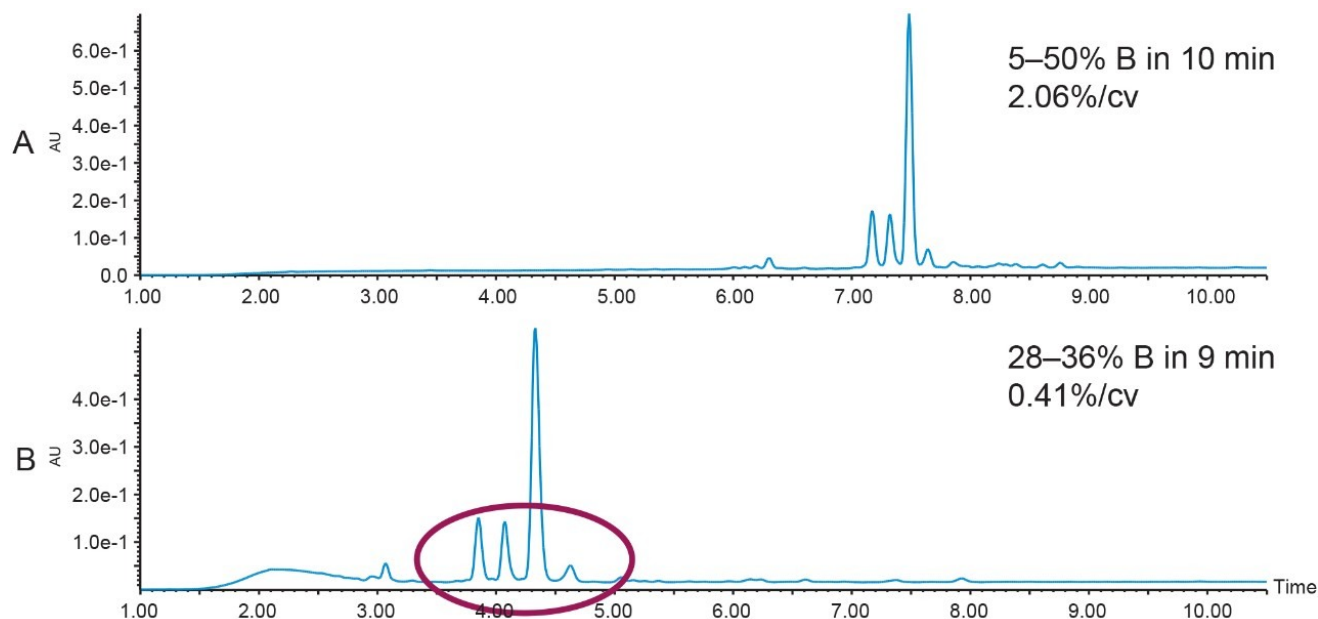


Figure 2. (A) Crude peptide screening run; 5–50% B in 10 min, 2.06% change per column volume. (B) Crude peptide separation with the focused gradient; 28–36% B in 9 min, 0.41% change per column volume. Peptide concentration, 1.3 mg/mL; inj. vol. 3  $\mu$ L.

Enhanced resolution between impurities and the target product suggests the possibility of achieving higher product purity values. A loading study was performed to determine the maximum sample load while maintaining the resolution between the desired peptide and its contaminants (Figure 3). While the separation at the higher loadings of 32.4 and 43.2  $\mu$ g (Figures 3B and 3C) were satisfactory, the more conservative loading at 16.2  $\mu$ g (Figure 3A) was chosen for prep, since the objective of the isolation was to obtain product with high purity.

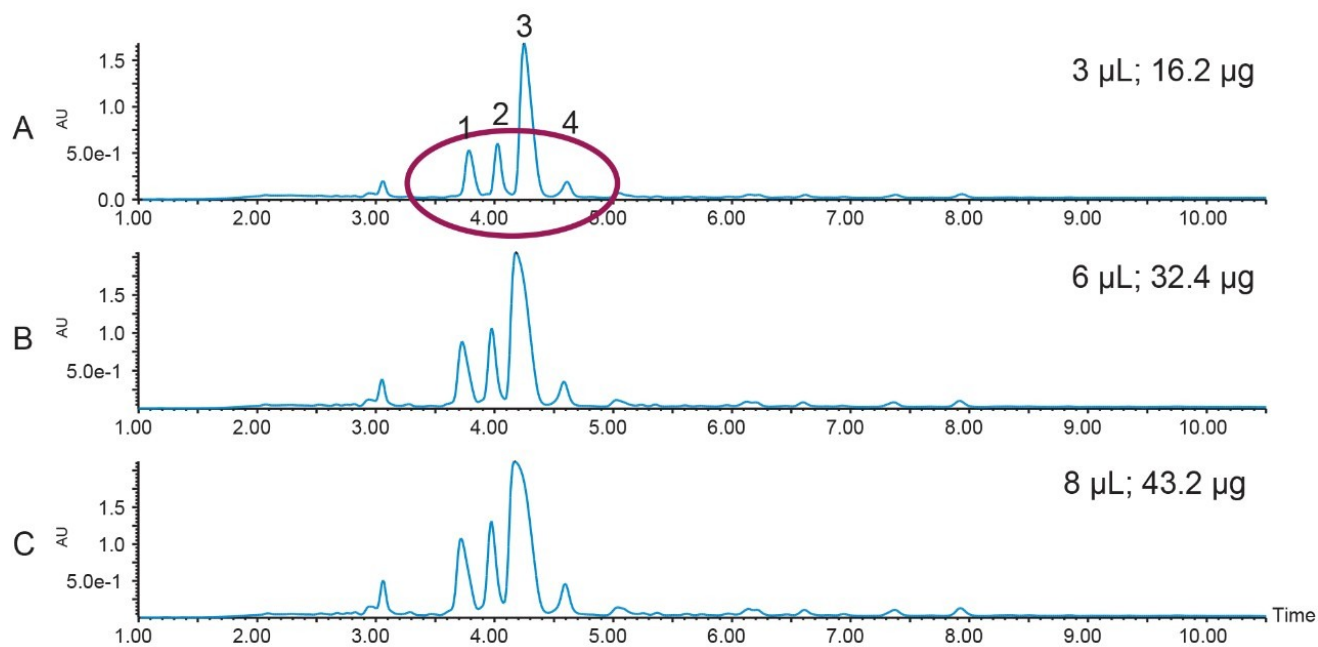


Figure 3. Loading study to determine how much crude peptide could be loaded on the column in each chromatographic run; 28–36% B in 9 min, 0.41% change per column volume; total run time, 15 min. Peptide concentration, 5.4 mg/mL.

### Gradient modification to shorten run time

Although the resolution between the peptide and its contaminants was excellent, the total run time of the focused gradient was 15 minutes. Since all of the peaks of interest eluted within the first 5 minutes of the focused gradient, the elution percentage for each of the four peaks of interest in the crude sample chromatogram was calculated (Table 1) and the focused gradient was modified (Table 2) to shorten the total chromatographic run time.



Peak	Retention time (min)	%B elution
1	3.85	29.4
2	4.08	29.6
3	4.32	29.8
4	4.67	30.2

Table 1. Peak retention time and elution percentages calculated from the 28–36% B focused gradient.

Time	Flow rate	%A	%B
0.00	0.5	95	5
1.00	0.5	72	28
2.00	0.5	72	28
5.35	0.5	69	31
5.40	0.5	5	95
6.40	0.5	5	95
6.50	0.5	95	5
10.00	0.5	95	5

Table 2. Modified focused gradient used for peptide and impurity mass-directed isolation; 28–31% B in 3.4 min; 0.41% change per column volume.

### Example



Calculation of peak elution percentage:

System volume estimated at 0.400 mL

Column volume = 0.229 mL

Retention time for impurity 1 = 3.85 min

Offset between the point of gradient formation and the detector = system volume + column volume

Offset = 0.400 mL + 0.229 mL = 0.629 mL

Time for the solvent to reach the detector = Offset/Flowrate

0.629 mL/0.5 mL/min = 1.258 min

Time when the peak elution concentration was formed = Peak retention time - Time to detector - Gradient hold

3.85 min - 1.258 min - 1 min = 1.592 min

Peak elution % = (Time elution concentration was formed/Length gradient segment) × %Change + Initial gradient

(1.592 min/8.94 min) × 8% + 28% = 29.4%

Although the elution percent for the peptide and each related impurity differed by only 0.2%, the resolution was maintained between each of the chromatographic peaks. The ACQUITY UPLC H-Class System, with its low system dispersion, exact control of solvent composition, and accurate sample injection scheme,<sup>4-6</sup> provided excellent chromatographic reproducibility, as shown in Figure 4, where ten peptide sample injections overlaid exactly.

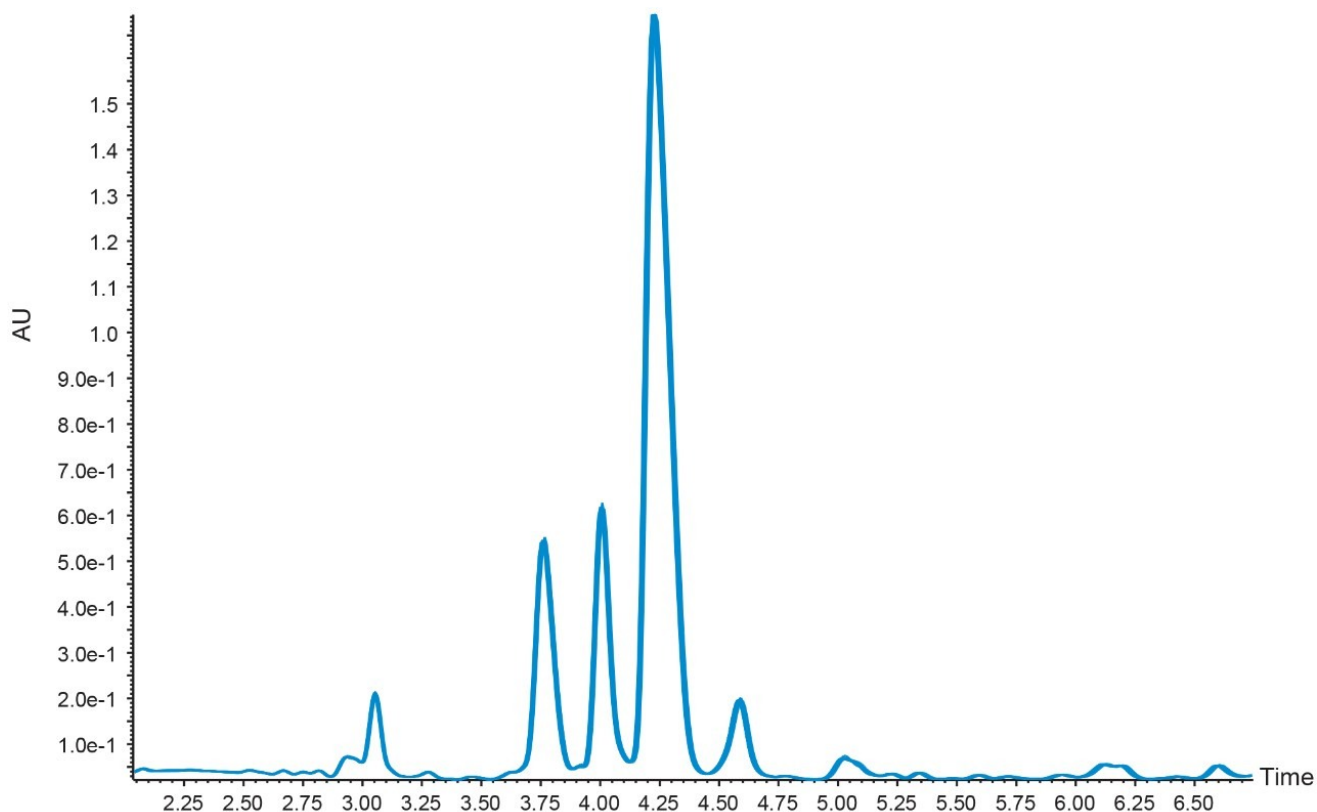


Figure 4. Overlay of 10 crude peptide sample injections; 28–31% B in 3.4 min; 0.41% change per column volume; peptide concentration, 5.4 mg/mL; inj. vol. 3  $\mu$ L.

Since small-scale compound isolation can produce collections with very small volumes, the WFM-A was specifically designed to address the challenge of peak dispersion. Figure 5 illustrates the superior peak shape for compounds collected using the low dispersion WFM-A, as compared to the same sample collected using a traditional fraction collector. Fast valve switching, and movement between collection vessels, enables the collection of narrow UPLC peaks. Collectively, these attributes improve the probability of obtaining high recovery with limited sample amounts. Precision control in the UPLC system drives the predictability of the chromatography, as well as the success of the isolation and fraction collection.

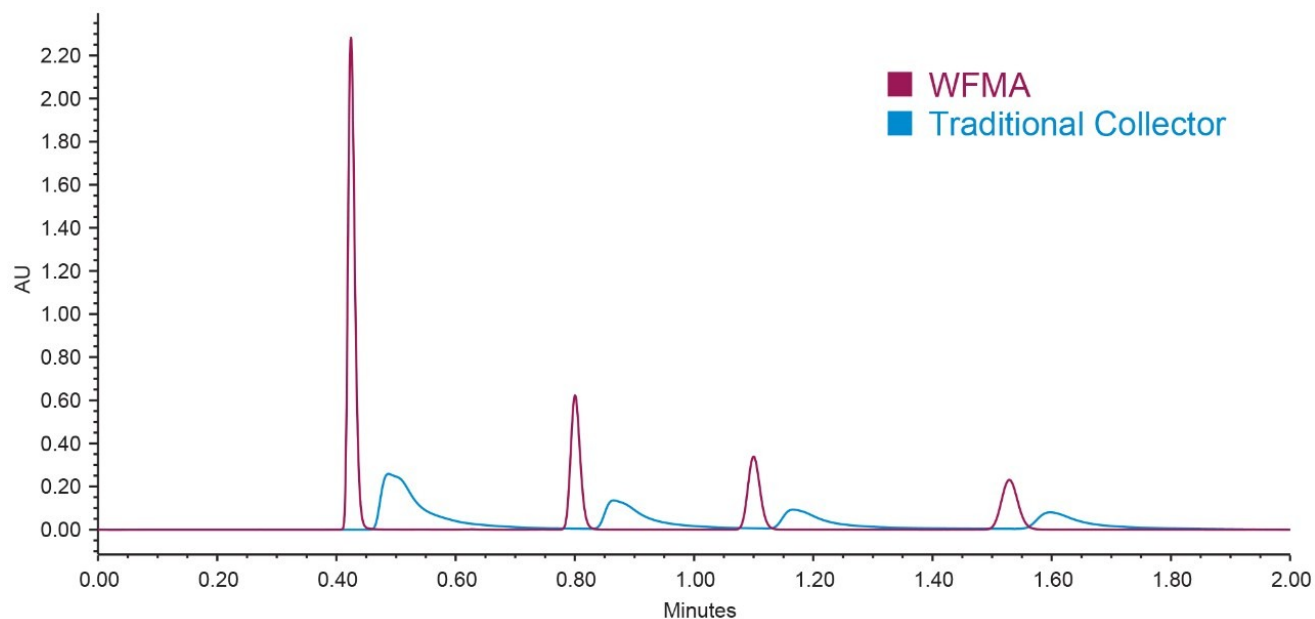


Figure 5. The WFM-A is specifically designed to address the challenge of peak dispersion. Comparison of the dispersion of a WFM-A and a traditional fraction collector.

### Mass-directed purification of the peptide and impurities

The target peptide and three other impurities were isolated by mass-directed purification on the  $2.1 \times 100$  mm column using the modified focused gradient (Table 2, Figure 6).

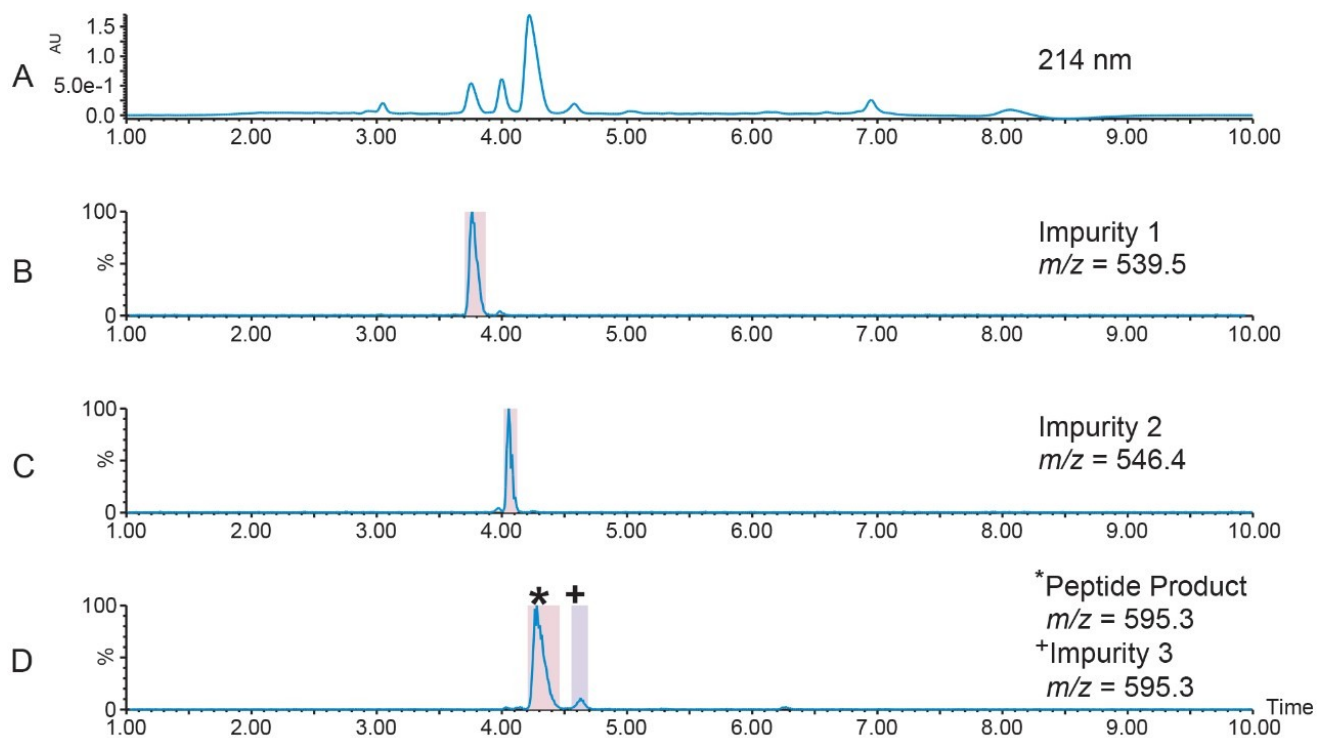


Figure 6. Mass-directed isolation of the target peptide and its related impurities: 28–31% B in 3.4 min; 0.41% change per column volume; peptide concentration, 5.4 mg/mL; inj. vol. 3  $\mu$ L.

## Fraction pooling

Fraction handling and processing is often challenging because small-scale compound isolation sometimes produces collections with very small volumes. Very narrow UPLC peaks are only a few seconds wide, with some fraction volumes less than 100  $\mu$ L. Limited fraction volumes containing only modest concentrations of target peak require more injections to ultimately isolate enough material for ensuing studies. Pooling, a feature available in FractionLynx Software, simplifies fraction handling by increasing the total volume of the collected product. Figure 7 shows the fraction collection and pooling for a set of three injections. Peak 1 from each injection was collected into tube 1, peak 2 from each injection was collected into tube 2, and so on. Pooling also increases the number of injections for the defined fraction collection plate. Unattended system operation also streamlines the process and improves efficiency.

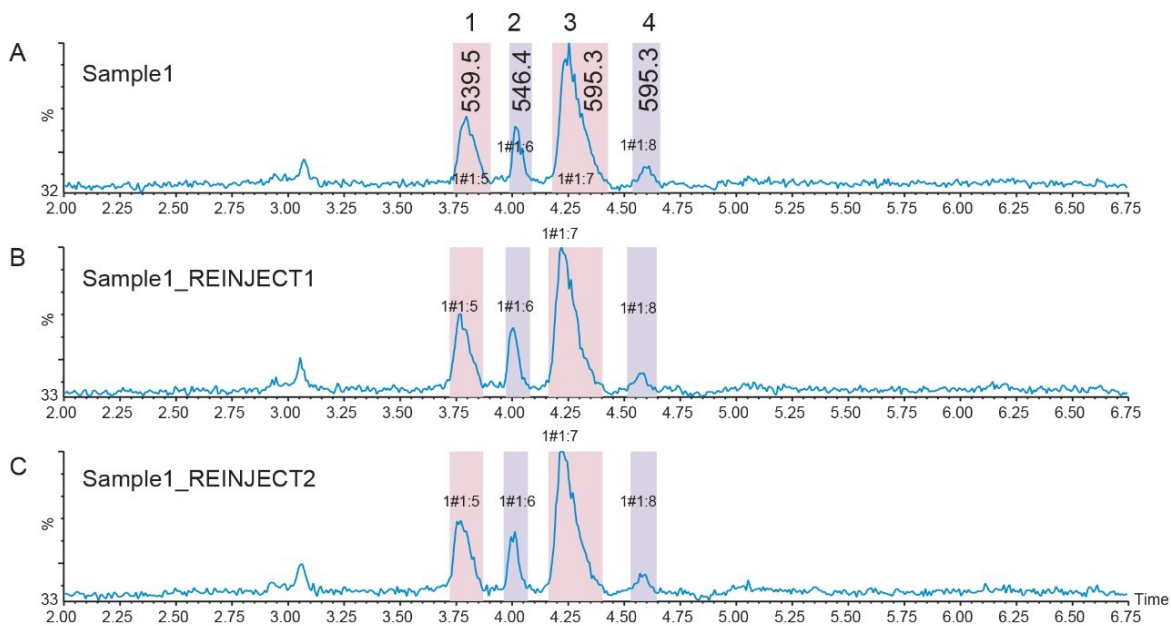


Figure 7. Total ion chromatograms for the mass-directed isolation of the target peptide and its related impurities with three injection fraction pooling; 28–31% B in 3.4 min; 0.41% change per column volume; peptide concentration, 5.4 mg/mL; inj. vol. 3  $\mu$ L.

## Fraction analysis

Forty-five small scale peptide and impurity isolations were pooled by peak mass and analyzed with the original screening gradient. The first two eluting impurity peaks ( $m/z$  539.5 and  $m/z$  546.4), as well as the peptide product peak, were very pure. A co-eluting contaminant decreased the purity of the third impurity peak, which eluted after the peptide (Figure 8).

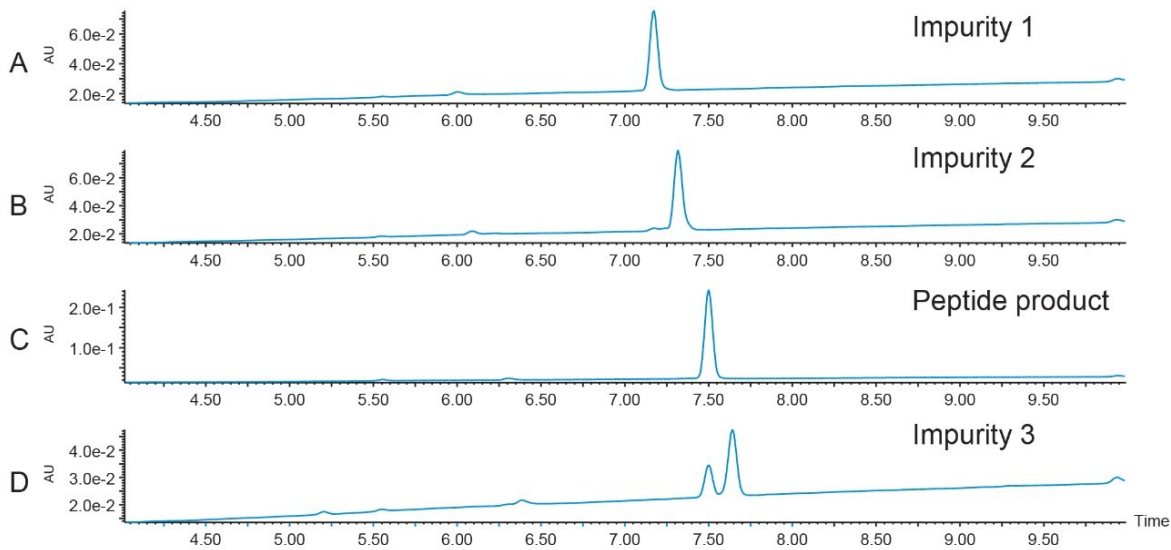


Figure 8. Fraction pool analysis for the collected impurity peaks and the peptide product; 5–50% B in 10 min; 2.06% change per column volume; inj. vol. 8  $\mu$ L; 214 nm.

## Recovery

Peptide recovery was calculated at 98.5%, based on the number of area counts in one injection of the pure fraction compared to the total number of area counts for all 45 injections in the total pool volume.

Area counts for one 8  $\mu$ L injection from the fraction pool = 12551

Total area counts in the fraction pool = 8684039

Total fraction pool volume = 5617  $\mu$ L

$8684039/5617 \mu\text{L} = 1546 \text{ counts}/\mu\text{L} \times 8 \mu\text{L} = 12368 \text{ counts}$

$12368 \text{ counts}/12551 \text{ counts} \times 100 = 98.5\% \text{ Peptide recovery}$

## Conclusion

Small scale mass-directed peptide isolation with fraction pooling reduces sample handling, which saves time and generates just enough product for additional analysis or future experiments.

The ACQUITY UPLC H-Class System configured with the Waters Fraction Manager-Analytical (WFM-A), with its very low system dispersion, exact control of solvent composition, accurate sample injection scheme, and precise fraction collection, allows scientists to perform small scale peptide and impurity isolation with assurance.

Fast valve switching and movement between vessels, as well as a fraction divert valve with very low dispersion volume, facilitates narrow target peak collection with high recovery and increases confidence in compound isolation.

Small scale peptide isolation saves sample, time, and resources, resulting in improved efficiency in the purification process.

## References

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