Application Note

Biopharma/Pharma

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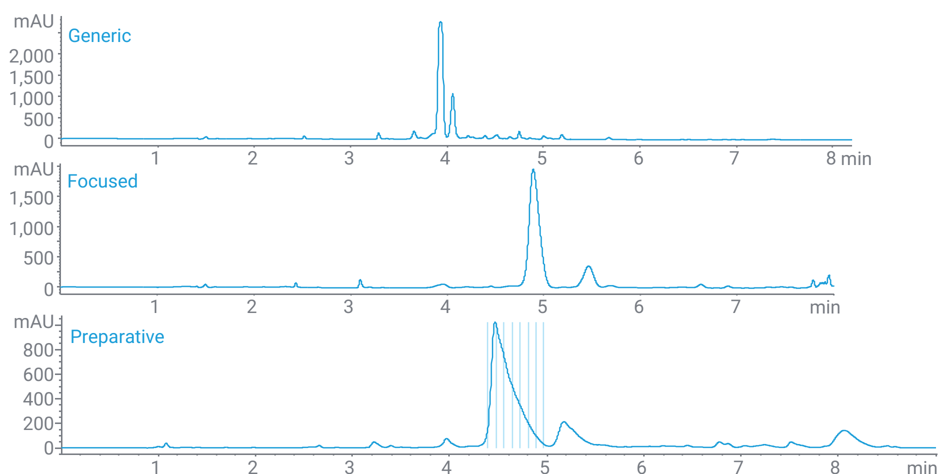
Efficient Purification of Synthetic Peptides at High and Low pH



Author

Florian Rieck

Agilent Technologies, Inc.

Abstract

Synthetic peptides play an increasingly important role as therapeutics. To analyze and isolate pure products from a synthesis mixture, high-performance liquid chromatography (HPLC) at low pH is an established tool. Some peptides, however, do not dissolve at low pH and thus must be separated under basic conditions. This application note presents a workflow for analytical method development and preparative purification using a single HPLC system. Valve automation enables fast and easy switching between pH modes and scales by a simple method parameter. A crude synthetic peptide is purified using both high and low pH, with automated fraction reanalysis for purity assessment. Fraction slices with purity of typically more than 95% were collected, enabling fraction pooling depending on purity requirements.

Introduction

Since the introduction of insulin as the first therapeutic peptide in the 1920s, the number of peptide-based drugs approved by the FDA has constantly increased to about 80 in 2021.[1] Already in the early development of new therapeutic peptides, potential drug candidates undergo multiple tests for activity, side effects, solubility, and more. For these tests, the compounds need to be clean from any residues from the synthesis, as well as product-related impurities. This cleanup can be done using preparative HPLC.

Owing to the amphoteric nature of amino acids, the building blocks of peptides, each synthetic peptide has a pH at which the net charge is zero. At this so-called isoelectric point, the peptide is least soluble. In solution, depending on the pH, amino acid side chains are dissociated and charged. In HPLC analyses, this behavior may cause poor peak shape due to ionic interactions with free silanols, in addition to the hydrophobic interactions with the nonpolar stationary phase. To achieve sharp signals, a low pH is typically chosen to protonate all carboxylate groups of the peptide. Many HPLC methods use trifluoroacetic acid for this purpose. Depending on the amino acid sequence of a peptide, however, it can be beneficial to adjust the pH to higher levels, for example by adding ammonia or an ammonium bicarbonate buffer.

This application note demonstrates the workflow of a peptide purification under both acidic and basic conditions. A crude desalted synthetic peptide is analyzed using an HPLC system with UV and mass selective detection (MSD). Valve automation enables automated switching between solvents and columns to run analyses in two pH ranges without hardware changes. The Agilent 1290 Infinity II Autoscale Preparative LC/MSD System further allows seamless transfer from analytical to preparative conditions. The analytical method can thus be developed on analytical scale and then transferred to preparative scale without moving to another instrument.

Experimental

Instrumentation

All experiments were conducted on an Agilent 1290 Infinity II Autoscale Preparative LC/MSD System:

- Agilent 1260 Infinity II Quaternary Pump (G7111B) with active seal wash (option #030) and active inlet valve (option #032)
- Agilent 1290 Infinity II Preparative Binary Pump (G7161B) with 200 mL pump heads (option #206)
- Agilent 1290 Infinity II Preparative Open-Bed Sampler/Collector (G7158B)
- Agilent 1260 Infinity II Variable Wavelength Detector (G7114A) with 0.3 mm preparative flow cell (option #024)
- Agilent 1260 Infinity II Diode Array Detector WR (G7115A) with 10 mm standard flow cell (option #018)
- Agilent 1290 Infinity II MS Flow Modulator (G7170B)
- Three Agilent 1290 Infinity II Valve Drives (G1170A), equipped with:
  - 2-position/14-port preparative-scale valve (G4738A)
  - 4-position/10-port valve (G4237A)
  - 6-position/14-port preparative-scale valve (G4734B)
- Agilent 1290 Infinity II Preparative Column Compartment (G7163B)
- Agilent 1260 Infinity II Delay Coil Organizer (G9324A) with delay coils for 15–40 mL/min flow (option #210)
- Agilent InfinityLab LC/MSD XT (G6135B)

Columns

Analytical columns:

- Agilent InfinityLab Poroshell 120 SB-C18, 4.6 × 150 mm, 4 µm (part number 683970-902)
- Agilent InfinityLab Poroshell HPH-C18, 4.6 × 150 mm, 4 µm (part number 693970-702)

Preparative columns:

- Agilent InfinityLab Poroshell 120 SB-C18, 21.2 × 150 mm, 4 µm (part number 670150-902)
- Agilent InfinityLab Poroshell 120 HPH-C18, 21.2 × 150 mm, 4 µm (part number 670150-702)

Software

Agilent OpenLab CDS ChemStation edition for LC and LC/MS Systems, Rev. C.01.10 [272] or later versions

Solvents

HPLC gradient grade acetonitrile (ACN) was purchased from VWR (Darmstadt, Germany). Ammonia solution 25%, ammonium bicarbonate >99.5%, LC/MS grade formic acid, and reagent grade trifluoroacetic acid were obtained from Merck (Darmstadt, Germany). Agilent InfinityLab Ultrapure LC/MS methanol (part number 5191-4497) was used to prepare make-up solvent for the MSD. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak).

Sample

Angiotensin I (sequence DRVYIHPFHL) was purchased from Proteogenix (Schiltigheim, France) with quality grade "desalted, crude" (purity approximately 51%, according to delivery note). Sample solutions were created with acetonitrile:water 15:85 (v:v).

Method settings

Table 1. Chromatographic conditions of analytical and preparative runs at low pH.

Parameter	Analytical Runs	Preparative Runs
Mobile Phase	A1) 0.1% trifluoroacetic acid in water B1) 0.1% trifluoroacetic acid in ACN	
Flow Rate	1.5 mL/min	32 mL/min
Gradient	Time (min) %B 0 9 1 25 7 35 8 99 10 min stop time 2.5 min post time	Time (min) %B 0 9 1 9 2 25 7 35 7.1 99 8.1 99 8.2 9 9 min stop time
Injection Volume	5 µL	1,000 µL
Sampler Method Preset	Preset 1: Polar sample matrix	Preset 1: Polar sample matrix
Temperature	Ambient	Ambient
UV Detection	Signal A: 220 nm 10 Hz data rate	Signal A: 220 nm 5 Hz data rate
MS Detection	Signal 1: positive scan *m/z* 300 to 1,500 Signal 2: negative scan *m/z* 300 to 1,500	Signal 1: positive scan *m/z* 300 to 1,500 Signal 2: negative scan *m/z* 300 to 1,500
Split Ratio to MSD	Full flow	10,000:1 (mode M7) Turn on after 1.00 min
Fraction Collection	Not applicable	Peak-based, UV and MSD UV threshold: 20 mAU UV upslope: 70 mAU/s UV downslope: 2 mAU/s MSD threshold: 5,000 cps

Table 2. Chromatographic conditions of analytical and preparative runs at high pH.

Parameter	Analytical Runs	Preparative Runs
Mobile Phase	A2) 10 mM ammonium bicarbonate, pH 9.8 in water B2) 10 mM ammonium bicarbonate, pH 9.8 in ACN:water 90:10 (v:v)	
Flow Rate	1.5 mL/min	32 mL/min
Gradient	Time (min) %B 0 9 1 22 6 32 7 99 8 min stop time 2.5 min post time	Time (min) %B 0 9 1 9 2 22 7 32 7.1 99 8.1 99 8.2 9 9 min stop time
Injection Volume	5 µL	1,000 µL
Sampler Method Preset	Preset 1: Polar sample matrix	Preset 1: Polar sample matrix
Temperature	Ambient	Ambient
UV Detection	Signal A: 220 nm 10 Hz data rate	Signal A: 220 nm 5 Hz data rate
MS Detection	Signal 1: positive scan *m/z* 300 to 1,500 Signal 2: negative scan *m/z* 300 to 1,500	Signal 1: positive scan *m/z* 300 to 1,500 Signal 2: negative scan *m/z* 300 to 1,500
Split Ratio to MSD	Full flow	10,000:1 (mode M7) Turn on after 1.00 min
Fraction Collection	Not applicable	Peak-based, UV and MSD UV threshold: 20 mAU UV upslope: 70 mAU/s UV downslope: 10 mAU/s MSD threshold: 10,000 cps

Table 3. MSD spray chamber and fraction collection settings.

Parameter	Value
Make Up Solvent	0.1% formic acid in methanol/water 70/30
Make Up Flow	1.5 mL/min
Ionization Source	Agilent Jet Stream Electrospray
Nebulizer Pressure	35 psig
Drying Gas Temperature	300 °C
Drying Gas Flow	13.0 L/min
Sheath Gas Temperature	350 °C
Sheath Gas Flow	12.0 L/min
Capillary Voltage	±2,500 V
Nozzle Voltage	±600 V
Target Mass	1,295.7 *m/z*
Ion Species	$[M+H]^+$, multiple charge states: 2, 3

Results and discussion

Diluted samples were analyzed with separation methods using generic gradients from 9 to 99% B, both with low and high pH eluents (Tables 1 and 2). Method parameters for the MSD were the same for both pH ranges (Table 3). The retention time of the target peak was used to calculate shallow, focused gradients to enhance the resolution around the target peak.[2] Figure 1 shows chromatograms of the separation by a generic and a focused gradient at high pH. The resolution between the target peak (1) and the major impurity (2) increased noticeably when the focused gradient was applied. High resolution of the target peak is a prerequisite to increase the sample load on the column on preparative scale. A similar increase in resolution was achieved by optimizing the low pH method (Figure 2).

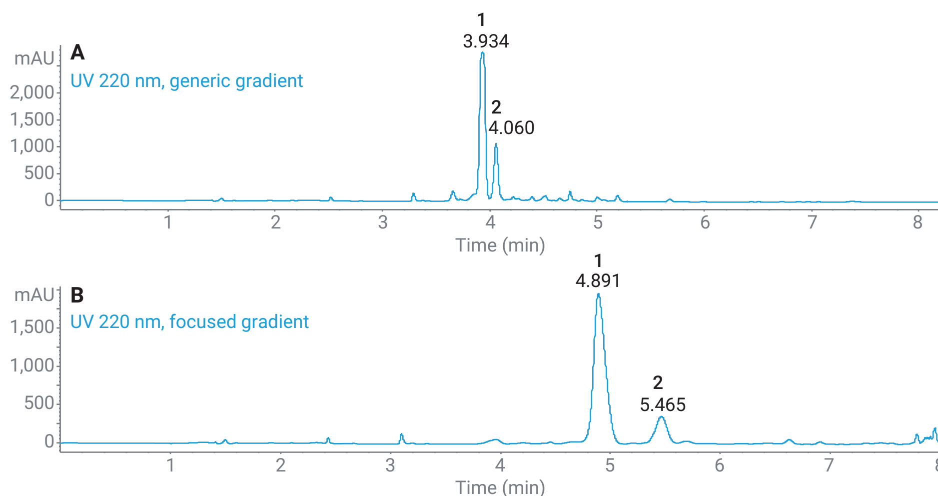


Figure 1. Separation of crude angiotensin I (1) with generic (A) and focused gradient (B) at high pH. Note the increase in resolution between angiotensin and the main impurity (2).

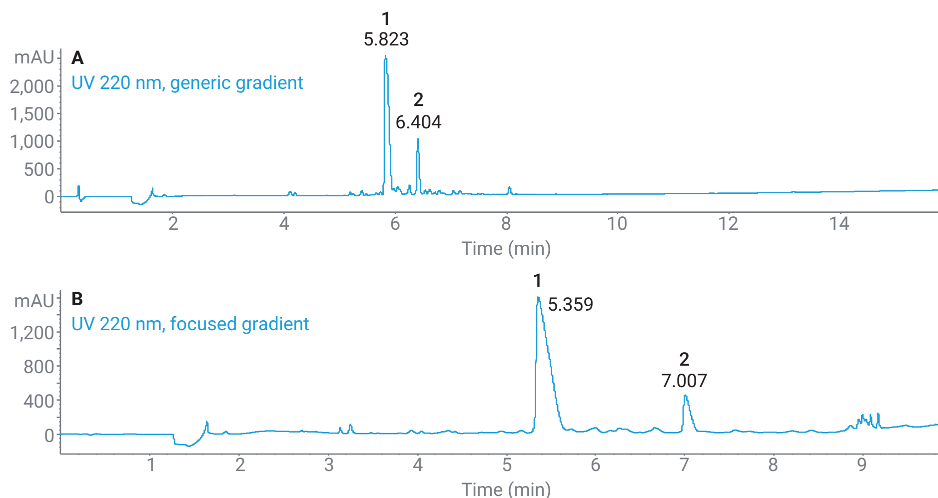


Figure 2. Separation of crude angiotensin I (1) with generic (A) and focused gradient (B) at low pH. Note the increase in resolution between angiotensin and the main impurity (2).

To identify the target peak and confirm the trigger masses and ion species, a mass spectrum was extracted from the main peak. Using the integrated deconvolution tool in Agilent ChemStation, three prominent signals of the mass spectrum were successfully assigned to the target molecule. The software identified the signals as single, double, and triple charged species of the angiotensin I ion (Figure 3).

The optimized analytical methods and focused gradients were then transferred to preparative scale using the Agilent HPLC Advisor App. The gradient method translation tool recalculated the flow rate and gradient to account for the wider column diameter and larger dwell volume of the preparative system path. Sample load on column was scaled up 200-fold to between 20 and 30 mg per run. Fraction collection was enabled and set to a peak-based trigger with collection of time slices to facilitate selection of the purest part of the peak.

Figure 4 shows the preparative chromatogram of the fraction collection run at high pH. The focused gradient enabled separation of the target peak from the impurities despite the high sample load. An overlay of the extracted ion chromatogram (EIC) of the $[M+2H]^{2+}$ target mass underlines the specificity gained by the mass selective detector. Seven fractions of six seconds each were collected, based on a combination of the UV and MSD signals.

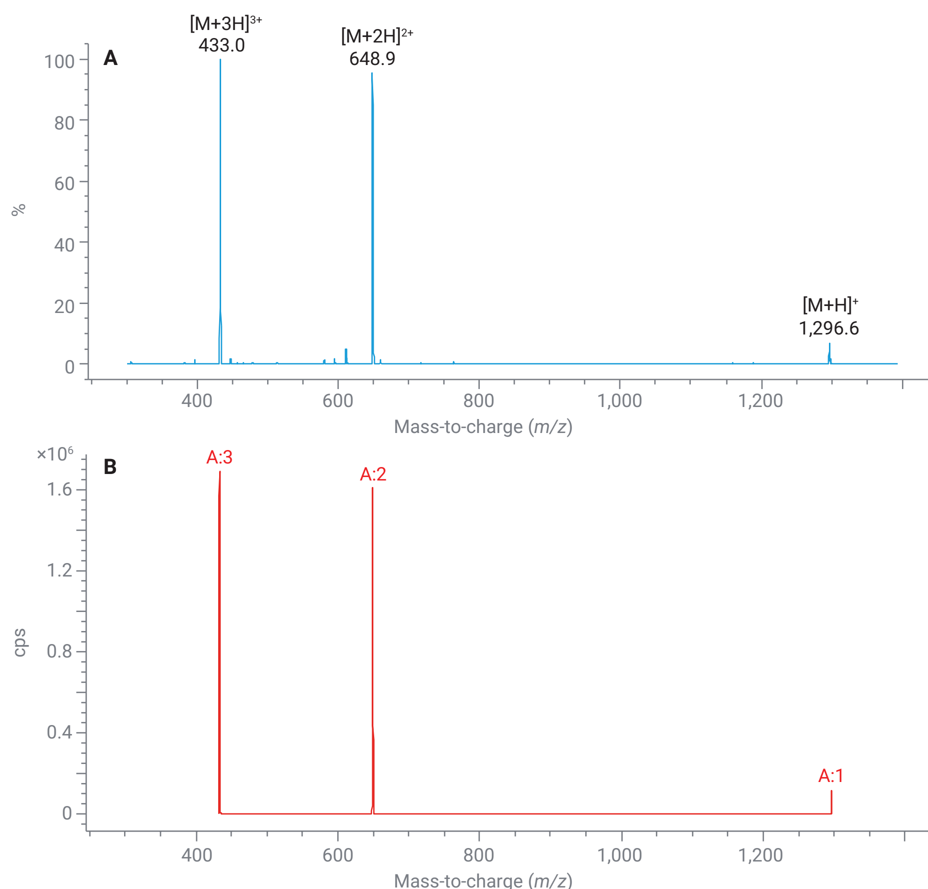


Figure 3. Mass spectrum and deconvolution of the main peak. The three most abundant signals are different charge states of the angiotensin I ion.

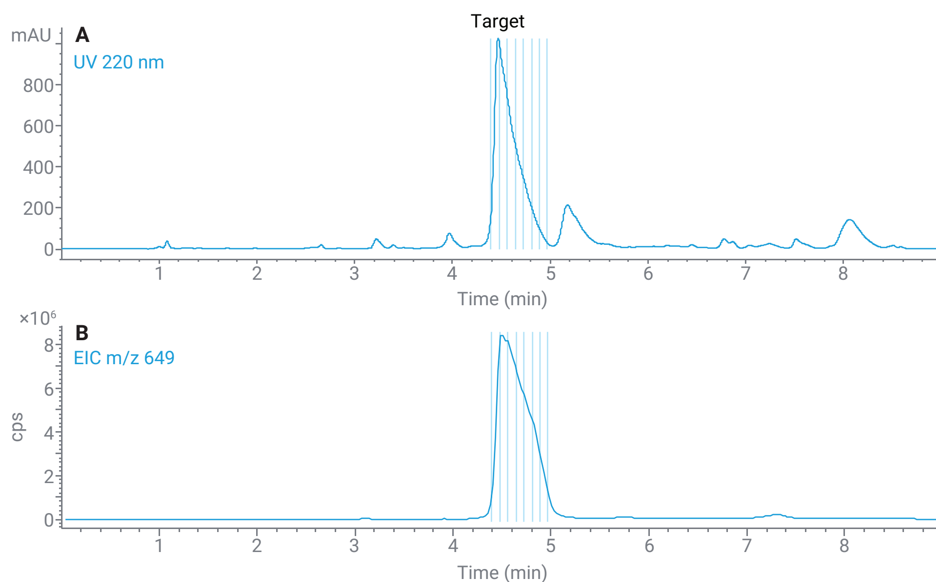


Figure 4. Chromatogram of the preparative purification at high pH. The target peak was collected into seven fractions, triggered by the UV signal and a target mass of *m/z* 649.

The separation at low pH was also successfully scaled up. Figure 5 displays a chromatogram overlay of the UV and MSD signals. Like on analytical scale, the target peak is wider and less Gaussian-shaped compared to the separation at high pH. Judging by the broad shoulder, the UV peak appears to be coeluting with another compound. The EIC, however, shows that the target mass was present throughout the entire peak. Therefore, 12 fractions of 6 seconds each were collected.

Finally, all collected fractions were reanalyzed by switching back to the analytical path of the system and using the optimized analytical methods. Depending on workflow requirements, a minimum purity of the final product must be observed. By collecting slices of the target peak, only the fractions that meet purity requirements can be picked and pooled. Fractions containing impurities can be repurified without having to discard the entire collection.

Figures 6 and 7 show chromatogram overlays of the reanalyses of the fraction collection at high and low pH, respectively. Of the seven fractions collected at high pH, the first one clearly contains an impurity coeluting with the front of the target peak. All other fractions are more than 95% pure (by UV signal), with a small impurity occurring at the later fractions. A peculiar impurity was present at fraction 4, eluting before the target peak, but not present in any other fraction. It seems like this compound was associated with angiotensin I during the preparative injection and could only be separated during fraction reanalysis. Mass spectral information on this peak suggests that the impurity is a deletion of valine or proline from the angiotensin I sequence.

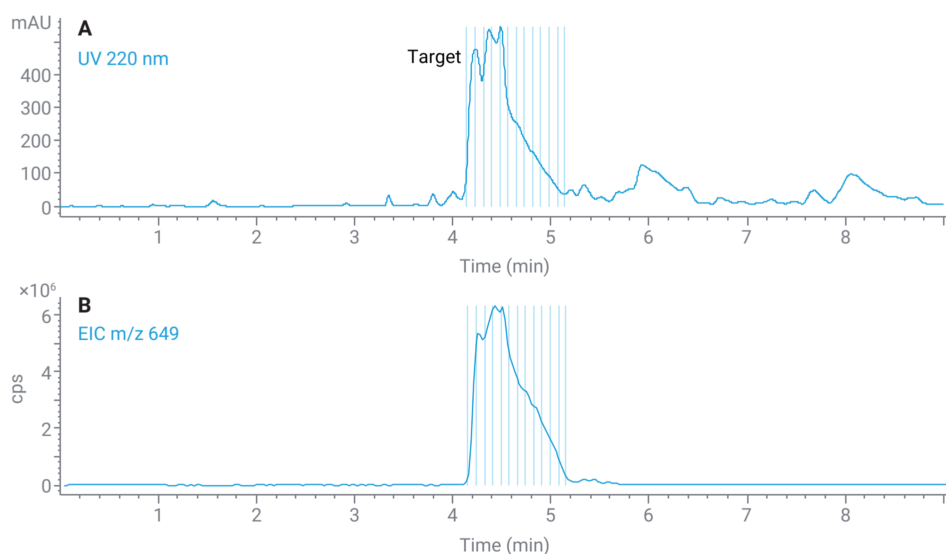


Figure 5. Chromatogram overlay of the preparative purification at low pH. The target peak was collected into 12 fractions, triggered by the UV signal and a target mass of *m/z* 649.

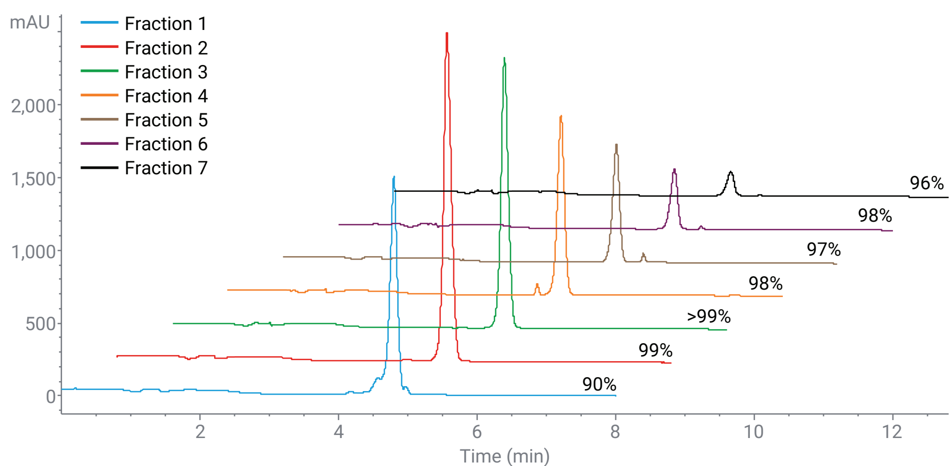


Figure 6. Reanalysis results of the fractions collected at high pH.

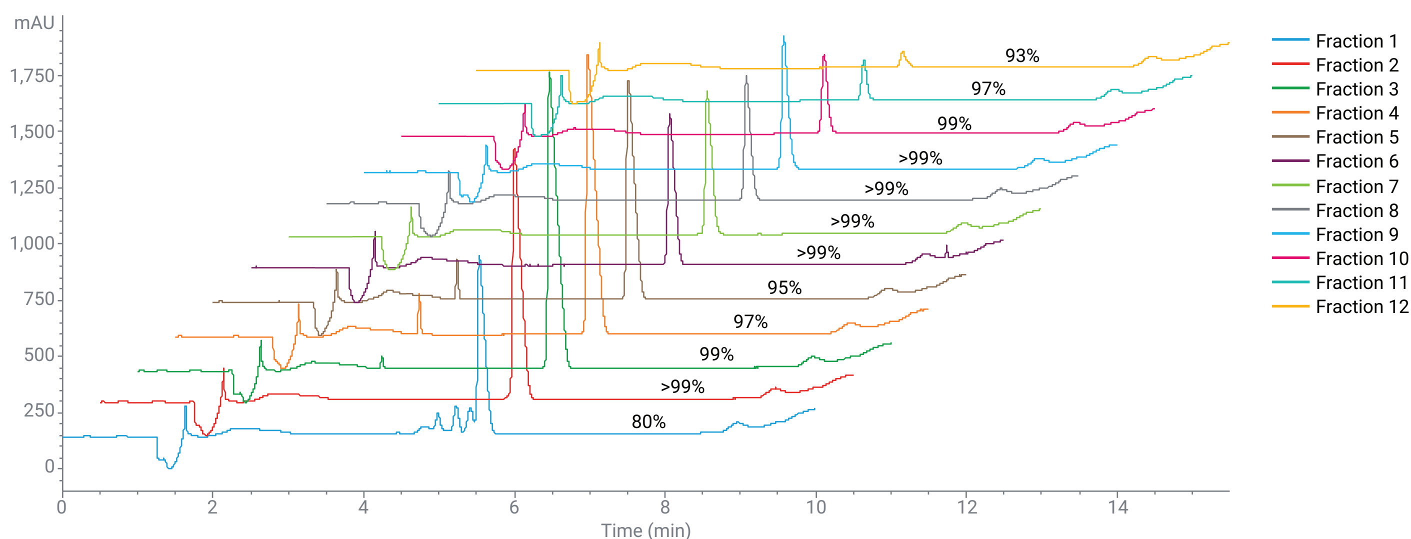


Figure 7. Reanalysis results of the fractions collected at low pH.

Why this molecule would be coeluting with the intact peptide, however, remains to be clarified. High-resolution MS analyses would be the next logical step to shed some light on this conundrum.

A similar pattern was observed for the fractions collected at low pH. All but the first and last fractions were 95% pure or higher. Fraction 2 already was more than 99% pure; in fractions 3 to 5, however, an impurity coeluting with angiotensin I was present like in the high pH fraction 4. Again, this impurity elutes with the heart of the target peak and can only be separated during reanalysis of the collected fraction. By collecting slices of the target peak, this suspected degradation product could now be separated from the pure fractions and investigated with high-resolution MS or another technique. Fractions that meet purity requirements may already be pooled and dried for later use in the downstream process.

Conclusion

A crude synthetic peptide was successfully purified using preparative HPLC under high and low pH conditions. Both methods were developed on an analytical scale and then transferred to preparative conditions using a method calculator. Switches between analytical and preparative scale, as well as low and high pH, were automated using built-in solvent and column selection valves. The target compound was successfully isolated using UV and MSD signals. Instead of a single fraction, time slices were collected and reanalyzed for purity. Purity was 95% or higher for all but the first and last slices, giving the option of pooling only the purest fractions. This workflow of method scale-up; column and solvent selection; and fraction reanalysis on a single system clearly facilitates the workflow for successful, confident purification of synthetic peptides.

References

1. Muttenthaler, M. *et al.* Trends in Peptide Drug Discovery. *Nat. Rev. Drug Discov.* **2021**, *20*, 309–325.
2. Penduff, P. Analytical to Preparative HPLC Method Transfer. *Agilent Technologies technical overview*, publication number 5991-2013EN, **2013**.

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