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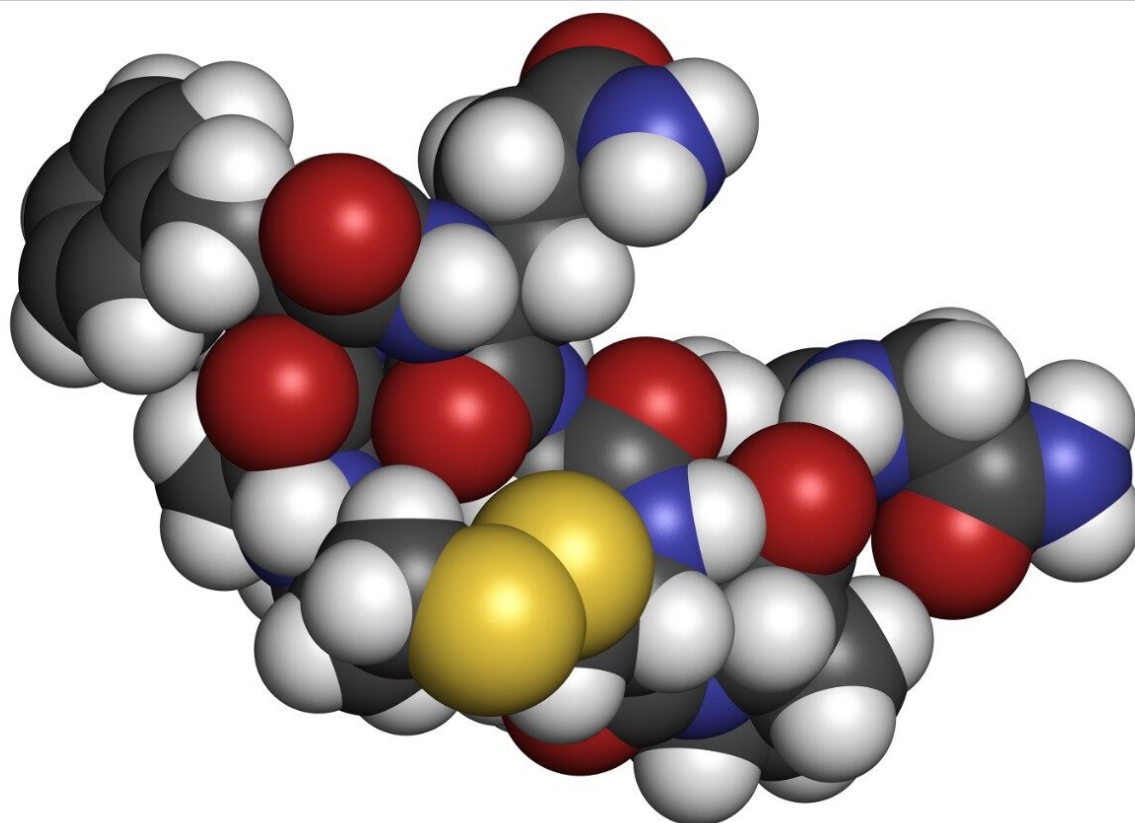
# Synthetic Peptide Impurity Analysis on Waters Reversed-Phase Columns

Hua Yang, Stephan M. Koza, William J. Warren, Weibin Chen

Waters Corporation

## Abstract

In this Application note, Waters reversed phase columns were screened to separate a series of commercially available and relevant synthetic peptide sequences under different gradient-based separation conditions. Based on the screening results, a general guidance for column selection is provided.

## Benefits

- Guidance behind selecting an appropriately diverse set of reversed phase columns for separating relevant biotherapeutic peptides under generic gradient conditions

- While all reversed-phase columns offer subtle peptide separation selectivity differences, a rational approach can be developed by selecting only four Waters Peptide Separation Columns for method screening purposes

- Effective use of mass spectrometry in the analysis of complicated synthetic peptide reaction mixtures

# Introduction

Peptides are gaining more and more attention as potential biotherapeutics. Currently, more than 100 peptides are marketed worldwide. Therapeutic peptides have been derived from three sources: natural sources, recombinant technology, and chemical synthesis. The synthetic peptide approach has the advantage over the other two approaches in that the synthetic peptide can be generated in a quick and well-controlled way using solid phase peptide synthesis (SPPS). In addition, chemical modifications that can extend the chemical and structural diversity and stability of the peptides can be introduced fairly easily.[1,2]

Impurities in the final desired synthetic peptide product can be produced both during manufacturing and upon storage. Since these impurities can potentially affect the safety and efficacy of the therapeutic peptide, they need to be monitored and characterized.

Selecting optimal columns and separation methods for synthetic peptide impurity analysis is often not straightforward. In this study, Waters reversed phase columns were screened to separate a series of commercially available and relevant synthetic peptide sequences under different gradient-based separation conditions. Based on the screening results, a general guidance for column selection is provided.

# Experimental

## Sample description

All synthetic peptide samples were purchased from Bachem. Lyophilized materials were reconstituted in water to a concentration of 2 mg/mL based on the mass of the lyophilized peptide. The samples were further diluted to lower concentrations in 0.1% formic acid. Table 1 lists the peptides and their properties.

| Peptide | Peptide sequence | Length | MW | pI* | Comment |
|---|---|---|---|---|---|
| Bivalirudin trifluoroacetate hydrate | H-D-Phe-Pro-Arg-Pro-Gly-Gly-Gly-Gly-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-OH, trifluoroacetate hydrate | 20 aa | 2180.29 | 3.87 | deamidation |
| Ceruletide diethylamine | Pyr-Gln-Asp-Tyr(OSO3H)-Thr-Gly-Trp-Met-Asp-Phe-$NH_2$, diethylamine | 10 aa | 1352.42 | 3.34 | chemical modification |
| Desmopressin acetate salt | 3-Mercaptopropionyl-Tyr-Phe-Gln-Asn-Cys-Pro-D-Arg-Gly-NH□ acetate salt | 8 aa | 1069.22 | 8.61 | chemical modofication disulfide bond |
| Lanreotide acetate | H-2Nal-c[Cys-Tyr-D-Trp-Lys-Val-Cys]-Thr-$NH_2$, acetate | 8 aa | 1096.33 | 8.40 | cyclic chemical modification |
| Secretin (porcine) | H-His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Glu-Leu-Ser-Arg-Leu-Arg-Asp-Ser-Ala-Arg-Leu-Gln-Arg-Leu-Leu-Gln-Gly-Leu-Val-$NH_2$ | 27 aa | 3055.45 | 9.85 | aspartimide formation |
| Salmon calcitonin | H-c[Cys-Ser-Asn-Leu-Ser-Thr-Cys]-Val-Leu-Gly-Lys-Leu-Ser-Gln-Glu-Leu-His-Lys-Leu-Gln-Thr-Tyr-Pro-Arg-Thr-Asn-Thr-Gly-Ser-Gly-Thr-Pro-$NH_2$ | 32 aa | 3431.90 | 9.00 | cyclic |
| PTH (1-34) | H-Ser-Val-Ser-Glu-Ile-Gln-Leu-Met-His-Asn-Leu-Gly-Lys-His-Leu-Asn-Ser-Met-Glu-Arg-Val-Glu-Trp-Leu-Arg-Lys-Lys-Leu-Gln-Asp-Val-His-Asn-Phe-OH | 34 aa | 4117.74 | 9.10 | oxidation, large |

Table 1. List of synthetic peptides and their properties.

*: pIs are obtained from BioLynx without chemical modifications.

## LC conditions

System:                                   ACQUITY UPLC H-Class Bio

Sample temp.:                             4 °C

Analytical column temp.:                  60 °C

Flow rate:                                0.3 mL/min (general gradient)

| | |
|---|---|
| Injection volume: | 2.5–10 µL |
| Columns: | ACQUITY UPLC Peptide BEH $C_{18}$, 300A, 1.7 µm, 2.1 x 150 mm (p/n: 186003687) |
| | ACQUITY UPLC Peptide BEH $C_{18}$, 130A, 1.7 µm, 2.1 x 150 mm (p/n: 186003556) |
| | ACQUITY UPLC Peptide CSH $C_{18}$, 130A, 1.7 µm, 2.1 x 150 mm (p/n: 186006938) |
| | ACQUITY UPLC Peptide HSS T3, 100A, 1.8 µm, 2.1 x 150 mm (p/n: 186008756) |
| | ACQUITY UPLC CSH Phenyl-Hexyl, 130A, 1.7 µm, 2.1 x 150 mm (p/n: 186005408) |
| | ACQUITY UPLC CSH Fluoro-Phenyl, 130A, 1.7 µm, 2.1 x 150 mm (p/n: 186005353) |
| | ACQUITY UPLC BEH $C_8$, 130A, 1.7 µm, 2.1 x 150 mm (p/n: 186003377) |
| Detection: | ACQUITY UPLC TUV Detector with 5 mm titanium flow cell, 214 nm |
| Sample collection/vials: | LCGC Certified Clear Glass 12 x 32 mm Screw Neck Total Recovery Vial, with cap and Preslit PTFE/Silicone Septa 1 mL volume (p/n: 186000385C) |
| Mobile phase A: | 0.1 % (v/v) trifluoroacetic acid (TFA) or 0.1% (v/v) formic acid (FA) or 20 mM ammonium formate, pH 10 in water |
| Mobile phase B: | 0.1 % (v/v) trifluoroacetic acid (TFA) or 0.085% (v/v) formic acid (FA) or 20 mM ammonium formate, pH 10 in acetonitrile |

Data management: MassLynx v4.1

## Typical gradient:

| Time | Flow Rate(mL/min) | %A | %B | Curve |
|------|------|------|------|------|
| 0.00 | 0.3 | 74 | 26 | - |
| 2.00 | 0.3 | 74* | 26* | 11 |
| 22.00 | 0.3 | 54 | 46 | 6 |
| 24.00 | 0.3 | 5 | 95 | 6 |
| 24.01 | 0.3 | 74 | 26 | 11 |
| 34.00 | 0.0 | 74 | 26 | 11 |

*The %A and %B composition changes with different mobile phases and different peptides. However, the gradient slope was kept constant as Δ20%B per 20 mins.*

# Results and Discussion

## Column Screening

Solid phase peptide synthesis (SPPS) is a common technique used for chemical synthesis, and offers a number of advantages over solution phase synthesis. The general principle of SPPS is one of repeated cycles of deprotection-wash-coupling-wash. This technique uses a solid support and adds amino acids in a sequential fashion. All soluble reagents and by-products are washed away. After the desired peptide has

been synthesized, it is cleaved from the support with all amino acid blocking groups removed.

To start the SPPS, C-terminus of the first amino acid is coupled to an activated solid support, commonly a chemically unreactive polystyrol. The first amino acid, C-terminal, is coupled to a resin (there is a linker between the amino acid and the support). The resin acts as the C-terminal protecting group, the immobilized peptide can be retained during a filtration process while liquid-phase reagents and by-products of synthesis are flushed away.[3]

After completion of the synthesis, the desired peptide is cleaved from the resin. During each step of the synthesis, impurities will be introduced, which include amino acid deletion, amino acid insertion, truncated peptides, racemerization, by-products formed during synthesis, etc. Some impurities can still be present even after initial single step purification and isolation. In addition, peptide product degradation could occur during storage.[4,5]

Chromatographic methods are often employed to assess the levels of these impurities in synthetic peptide preparations, and reversed-phase chromatography is the most commonly used chromatographic technique.

In 2017, an application note was published by Waters, in which the performance of 10 reversed-phase columns were compared side-by-side for a peptide mapping application.[6] While all of the columns that were evaluated were effective in this application, four columns were the most recommended for peptide mapping for the purpose of protein characterization and quality testing. These four columns are: Peptide BEH $C_{18}$, 130Å; Peptide BEH $C_{18}$, 300Å; Peptide CSH $C_{18}$, 130Å; and Peptide HSS T3, 100Å.

In this study, a synthetic peptide impurity analysis was carried out by screening the four above mentioned columns, as well as three other columns based on the differences in selectivity and retentivity. Figures 1–7 show chromatograms of seven synthetic peptides separated on Waters' reversed-phase columns under different mobile phase conditions. The results for the individual peptides are summarized below.

## Bivalirudin (Figures 1a, 1b, and 1c; Table 2)

Bivalirudin (Brand name: Angiomax, Angiox) is a specific and reversible direct thrombin inhibitor.[7] It is used with aspirin to decrease the clotting ability of the blood and to help prevent harmful clots from forming in blood vessels. Specifically, it is used in patients who are having certain heart and blood vessel procedures, such as coronary angioplasty.[8]

Bivalirudin is a 20 amino acid peptide, with a monoisotopic mass of 2178.986 Da. It has an asparagine (Asn) residue, which could undergo deamidation. In addition, the poly-glycine motif in the sequence is of great interest.

Figures 1a, 1b, and 1c show separations of bivalirudin and its impurities using formic acid, TFA, and ammonium formate (pH 10) mobile phases, respectively. A few observations were made upon close examination of the chromatograms:
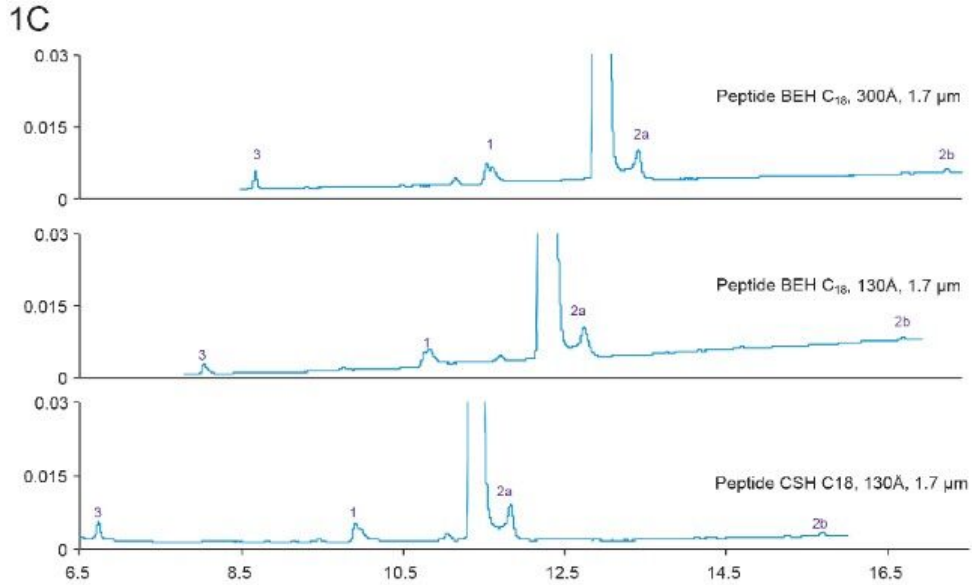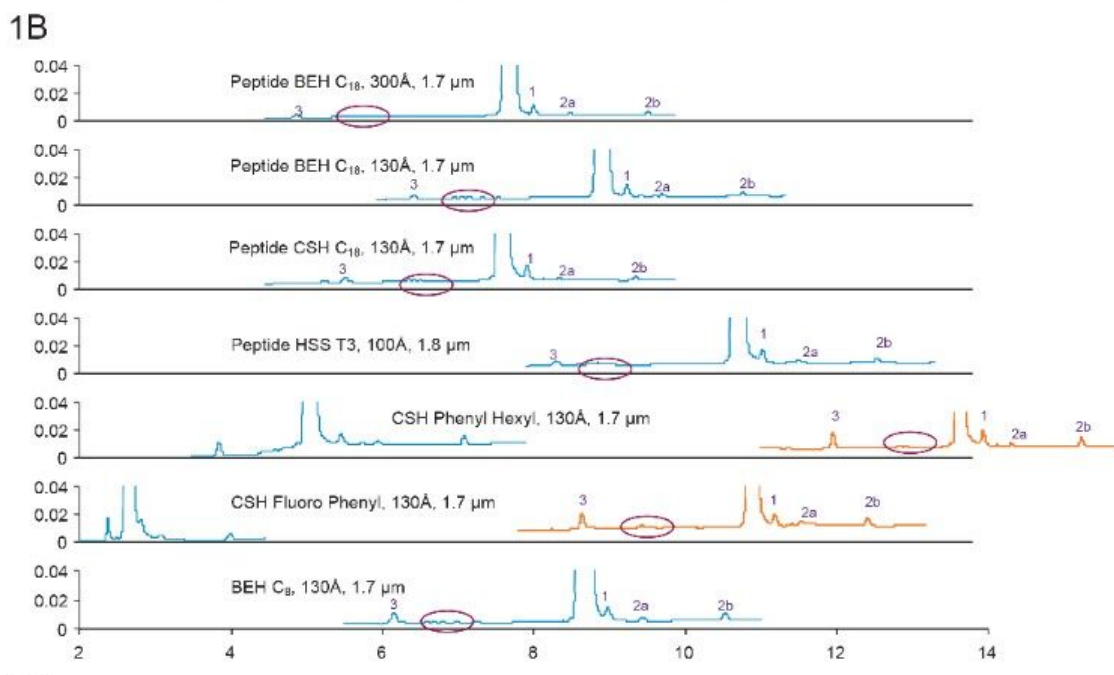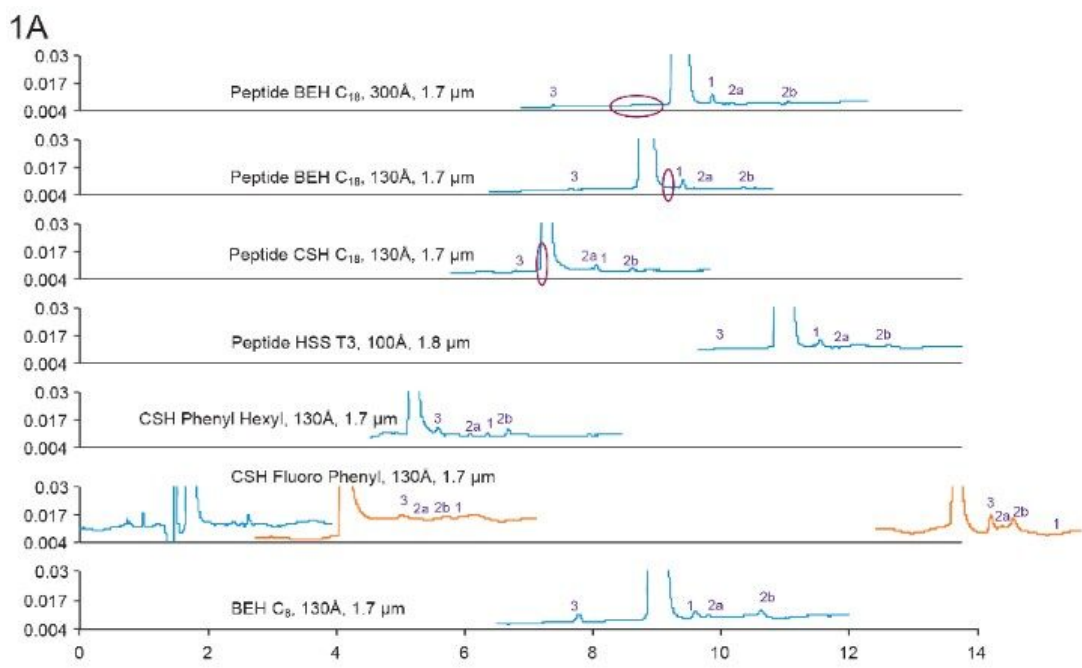
**1A**

**1B**

**1C**

*Figure 1. 1A: Separation of bivalirudin with formic acid mobile phases. Gradient: 18–38% B in 20*

, 300Å Column were identified as fragments [6–20], [7–20], [8–20], [9–20], [10–20], with shorter fragments eluting out later (Figure 1A). Interestingly, all of these fragment peaks were observed under TFA-mobile phase conditions on all of the columns (Figure 1B).

The unique peak observed on the Peptide BEH $C_{18,}$ 130Å Column has a mass that is 129 Da lower than the main peak, while the unique peak was observed on the Peptide CSH $C_{18}$, 130Å Column is likely to be a glycine insertion (Figure 1A).

4. Additional peaks were also found under different mobile-phase conditions. For example, fragments [3–20] and [11–20] were identified under TFA conditions, and Fragment [1–11] was identified under ammonium formate (pH 10) conditions. Under ammonium formate (pH 10) conditions, Peak 1 split into two peaks with the same mass (Figure 1C). This behavior suggests that these two peaks are diasteromers.
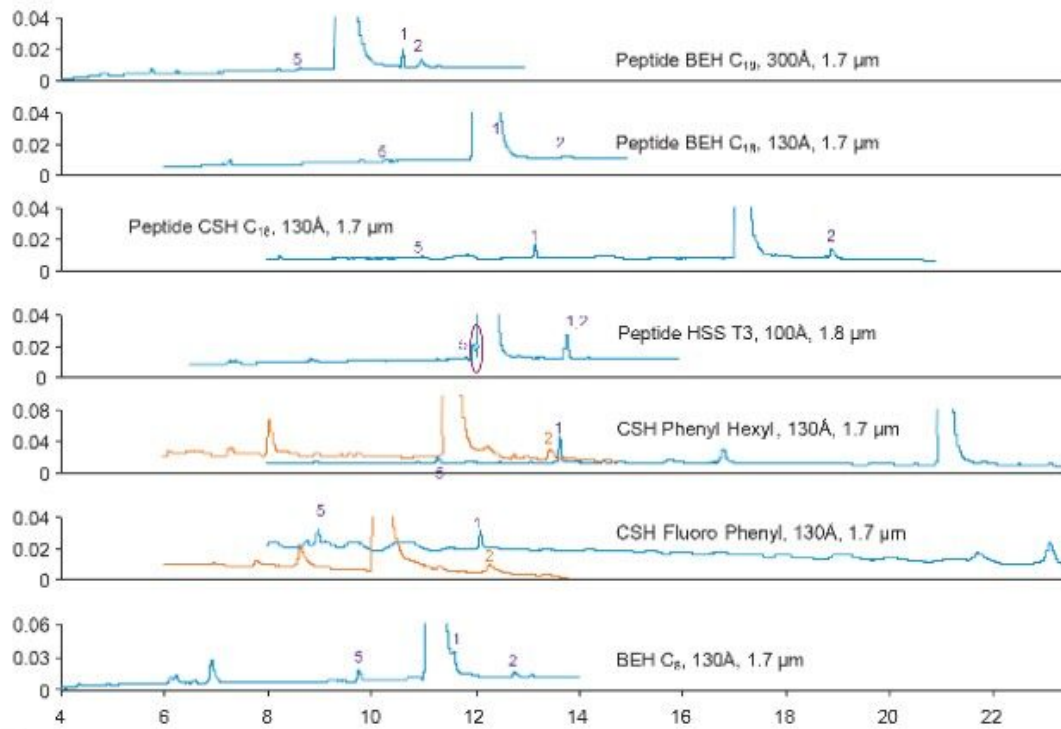
## Ceruletide (Figures 2a, 2b, and 2c; Table 3)

Ceruletide (Brand name: Takus, Tymtran) stimulates gastric, biliary, and pancreatic secretion and certain smooth muscle. It is used as a diagnostic aid in pancreatic malfunction.[9] Ceruletide is also known as cerulein or caerulein.
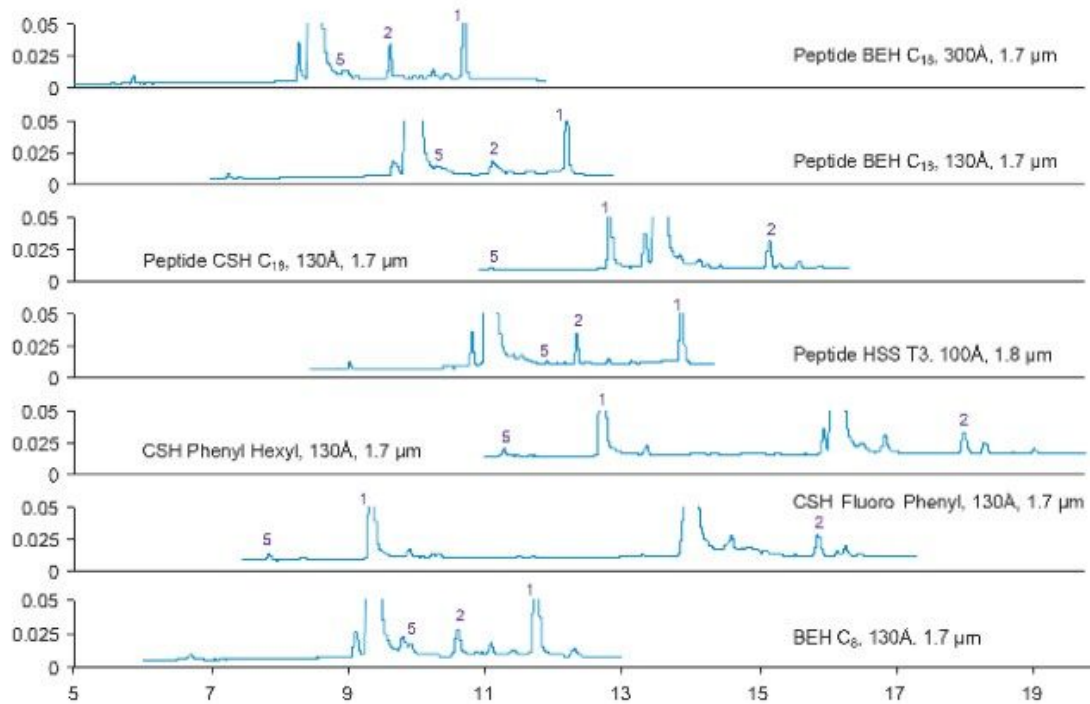
Ceruletide is a 10 amino acid peptide, with a monoisotopic mass of 1351.449 Da. It has a chemical modification – sulfonic acid – which is predicted to make the molecule extremely acidic. In addition, it has a pyroglutamic acid residue at the N-terminus, a methionine (Met) residue that could be prone to oxidation, and an amidated C-terminus.

Figures 2a, 2b, and 2c showed chromatographic separation of ceruletide and its impurities on the columns tested under different mobile phases. Table 3 lists a few major impurities.
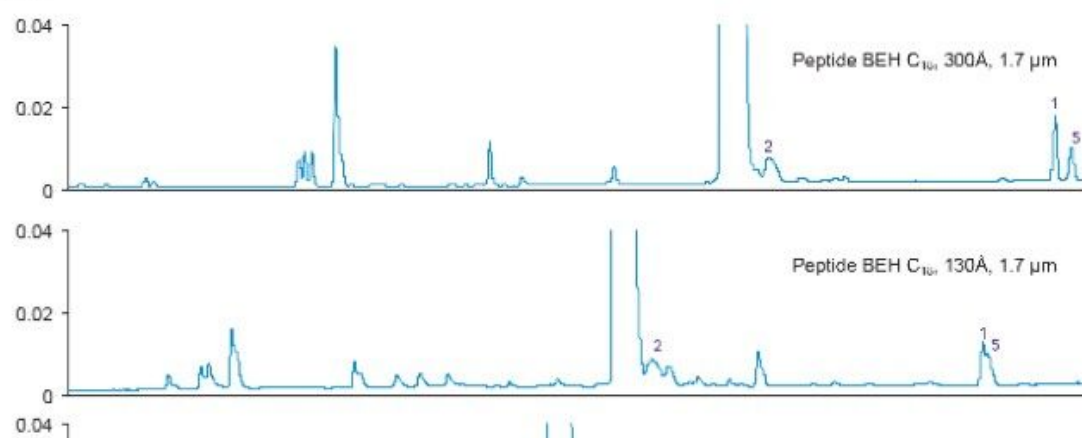
2A

Peptide BEH C₁₈, 300Å, 1.7 µm

Peptide BEH C₁₈, 130Å, 1.7 µm

Peptide CSH C₁₈, 130Å, 1.7 µm

Peptide HSS T3, 100Å, 1.8 µm

CSH Phenyl Hexyl, 130Å, 1.7 µm

CSH Fluoro Phenyl, 130Å, 1.7 µm

BEH C₈, 130Å, 1.7 µm

2B

Peptide BEH C₁₈, 300Å, 1.7 µm

Peptide BEH C₁₈, 130Å, 1.7 µm

Peptide CSH C₁₈, 130Å, 1.7 µm

Peptide HSS T3. 100Å, 1.8 µm

CSH Phenyl Hexyl, 130Å, 1.7 µm

CSH Fluoro Phenyl, 130Å, 1.7 µm

BEH C₈, 130Å. 1.7 µm

2C

Peptide BEH C₁₈, 300Å, 1.7 µm

Peptide BEH C₁₈, 130Å, 1.7 µm

2C. Separation of ceruletide with ammonium formate (pH 10) mobile phases. Gradient: 10–30% B in 20 min.

| Impurity | Retention time (min) for BEH C$_{18}$, 300 A | Mass (Da) | Δ Mass (Da) | Putative identity |
|---|---|---|---|---|
| Peak 1 | 10.69 | 1271.503 | -79.960 | SO$_3$ loss |
| Peak 2 | 11.06 | 1074.417 | -277.046 | (-262–15): fragment [1–8] - 15Da |
| Peak 5 | 8.69 | 917.381 | -434.082 | (-354–80): fragment [4–10] and SO$_3$ loss |

Table 3. Putative peak identification of Ceruletide impurities.

1. Retentivity

The most interesting observation is the retentivity of ceruletide on various columns. Typically, under the same gradient conditions, peptides have the lowest retentivity on CSH Columns and the highest retentivity on a Peptide HSS T3 Column. However, it is not the case for ceruletide. Instead, ceruletide has the highest retentivity on CSH Columns, and have similar retentivity on a Peptide HSS T3 column as the Peptide BEH C$_{18}$, 130Å Column. The main peak didn't elute on the CSH Fluoro Phenyl Column, while for the previous peptide, bivalirudin, the main peak came out at the void on the CSH Fluoro Phenyl Column. Indeed, the retentivity of Ceruletide on CSH Columns and a Peptide HSS T3 Column is reversed.

Our hypothesis is that since ceruletide is a very acidic peptide, it is still negatively charged even under the acidic conditions (FA and TFA) due to the sulfonic acid moiety. Since CSH particles have a controlled low level of positive charge, the negatively charged peptide will interact with positively charged CSH particles. As a result, the peptide eluted later on the CSH Columns. On the other hand, Peptide HSS T3 particles are silica-based. The negatively charged silanol group would repulse the negatively charged ceruletide. As a result, the retentivity is greatly decreased on the Peptide HSS T3 Column. It's worth noting that the retention time difference under FA conditions is much larger than that under TFA conditions.

Interestingly, the retentivity of ceruletide became "normal" under ammonium formate (pH 10) mobile phase conditions (Figure 2C) – it was the least retained on the Peptide CSH C$_{18}$ Column. Consistent with our hypothesis, under pH 10, the positive charges on the CSH particles are neutralized. Therefore, the ionic interaction between ceruletide and the CSH particles is dramatically reduced. As a result, ceruletide had the least retentivity on the Peptide CSH C$_{18}$ Column, which is typically observed for other peptides. Tests were not run on HSS T3 Columns because the particles are silica-based, which is not stable at pH 10.

2. Selectivity

Similarly to what has been seen on bivalirudin, column chemistry and mobile-phase conditions play critical roles in changing the selectivity of the separation. For example, under FA and TFA conditions, Peak 1 eluted before the main peak for all CSH Columns, while for other columns, it eluted after the main peak. Under pH 10 mobile phases, Peaks 1 and 5 eluted very closely, while under acidic conditions (FA and TFA), the two peaks were much more separated.

3. Under FA conditions, a unique peak was identified on the Peptide HSS T3, 100Å Column, which is likely to be pyroglutamic acid deletion and sulfur trioxide ($SO_3$) deletion. Under TFA condition, a new peak was observed right before the main peak on most of the columns, and it is likely an isomer of the main peak. Other impurities include amino acid deletion, dimer, +1 Da (presumably deamidation), -64 Da, +16 Da (presumably oxidation), with combination of $SO_3$ loss and fragments.

## Desmopressin (Figures 3a, 3b, and 3c; Table 4)

Desmopressin (Brand name: DDAVP, Minirin, Stimate) is a synthetic form of vasopressin. It is used to treat diabetes insipidus, bedwetting, hemophilia A, von Willebrand disease, and high blood urea levels.[10] Desmopressin has eight amino acids, with a monoisotopic mass of 1068.427 Da. It has a 3-mercaptopropionyl modification at N-terminus, and an amidation at C-terminus. Desmopressin is a cyclic peptide, with a disulfide bond formed between the modified moiety and cysteine (Cys) residue. It also has an asparagine (Asn) residue that could undergo deamidation.

Peaks indicated by the asterisk (*) are +1 Da peaks of the main peak, which could be deamidation of Asn. It was not clear at first whether these impurities occurred during chromatographic separation or during storage. When a new sample was diluted from the stock solution and injected onto one of the columns, the peak area reduced significantly (data not shown). Therefore, it is likely that these impurities are degradants that occurred during storage.

As shown in Table 4, multiple occasions of amino acid insertion were found as putative impurities.
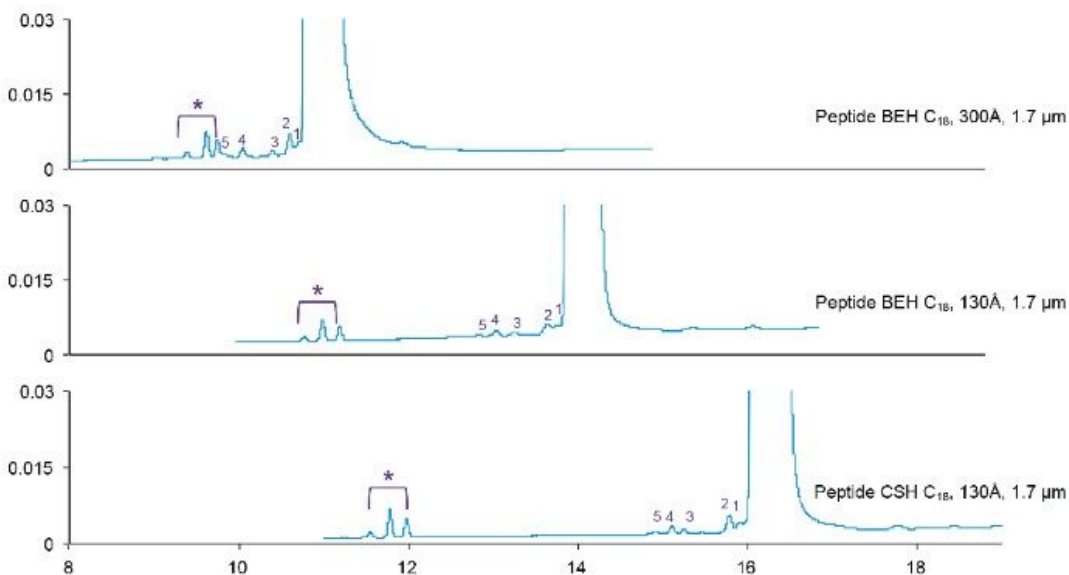
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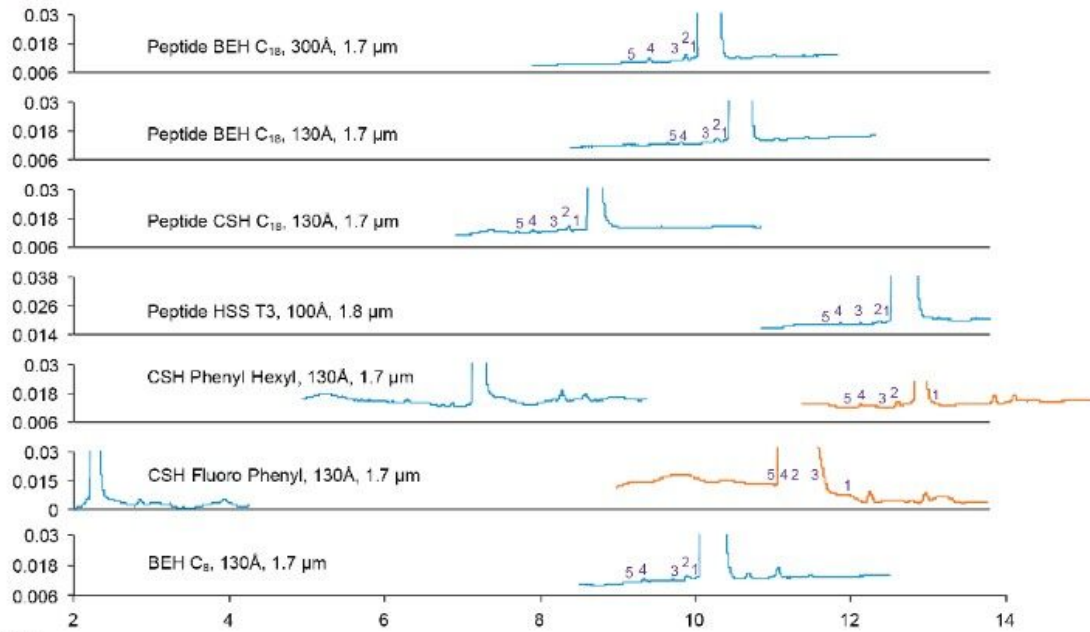
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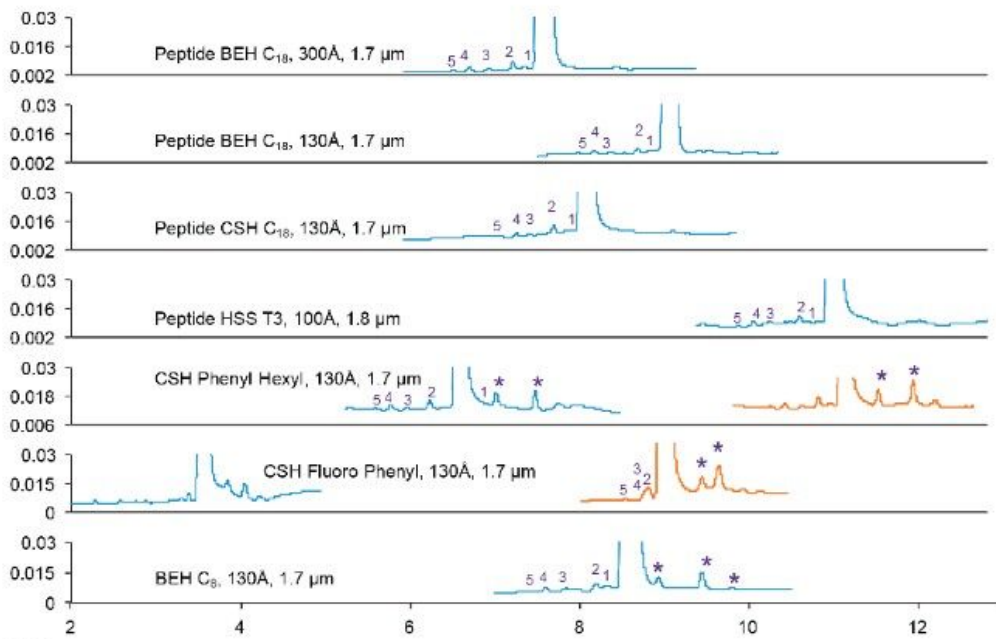
3C

*Figure 3A. Separation of Desmopressin with formic acid mobile phases. Gradient: 10–30% B in 20 min. Orange trace: 5.*

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