Flash Peptide Purification and Method Development: EGFRvii

TELEDYNE ISCO
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Chromatography Application Note AN124

Abstract

This application note continues the exploration of the purification of peptides using flash chromatography. In our previous Application Note, AN113, proper column selection was shown to play an important role in peptide purification for preparative HPLC. As flash chromatography has historically been used for small molecules, purification of small peptides can be anticipated. Consequently, EGFRvii (a small peptide with a molecular weight of 1635 Da) can be expected to be successfully purified using flash chromatography. Redi*Sep* Gold® reverse phase columns purified the peptide very well. Method development was performed concurrently with column screening, saving time, and was completed within 30 minutes. The purified peptide was clean, as verified by analytical HPLC, and could be used for other applications without further purification by HPLC.

Background

Peptides, especially those with larger chain lengths, are traditionally purified on preparative HPLC systems. Synthesized peptides typically contain several impurities, most commonly deletions, which are chromatographically similar to the desired compounds. Flash chromatography traditionally doesn't have the resolution needed to resolve these compounds, especially as the peptides become larger and the proportion of impurities increases, and as single amino acid deletions are similar to the desired compounds. Smaller peptides can be purified like other small molecules. EGFRVii used here has the sequence H2N-LEEKKGNYVVTDHC-OH, and so is a relatively small peptide. The use of nearly isocratic gradients and Redi*Sep* Gold reverse phase columns allows rapid flash purification of peptides.

Experimental and Results

Scouting runs were used to determine if the peptide would elute and to evaluate the peak shape and resolution from impurities. Using the "Time-on-Target" algorithm, it is possible to use the retention time of the desired peptide from the scouting run to calculate an efficient solvent method using only a milligram of this peptide. See Technical Note 65 on the Teledyne ISCO web site.

Method Development

Method development consists of two parts: determining the best column and determining the elution conditions. The pI of this peptide was estimated to be ~5.98, so the solvent system was buffered to pH 3.8 with 50 mMol ammonium formate to keep the peptide in a single ionization state. The organic solvent methanol, contained no additives. Ammonium formate is volatile, and so may be used with a mass spectrometer; the fluid interface reduces the buffer concentration to a level compatible with the mass spectrometer.

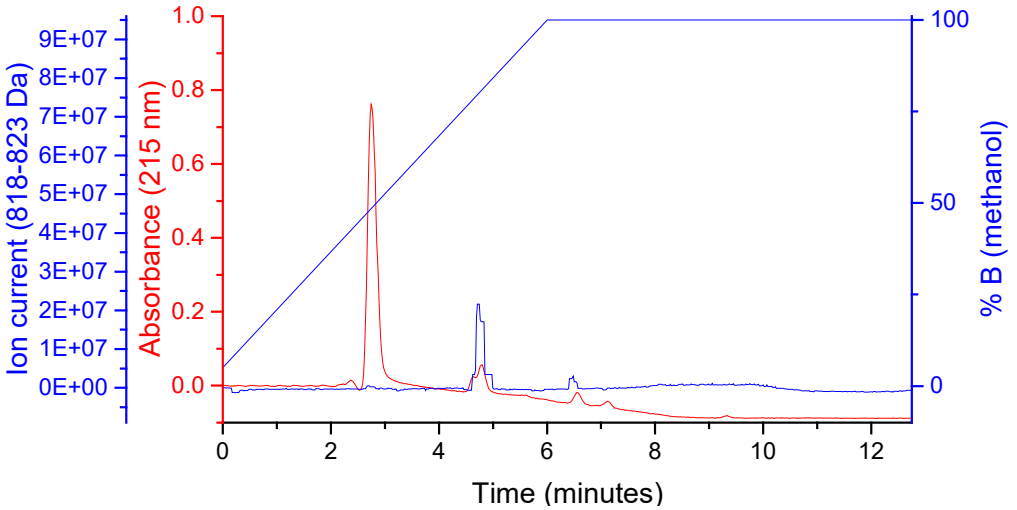

Figure 1—This first scouting gradient was run with a RediSep® Prep C18 column with an HP150; the focused gradient was calculated with the HP150 focus gradient generator for the flash C18 run in Figure 4.

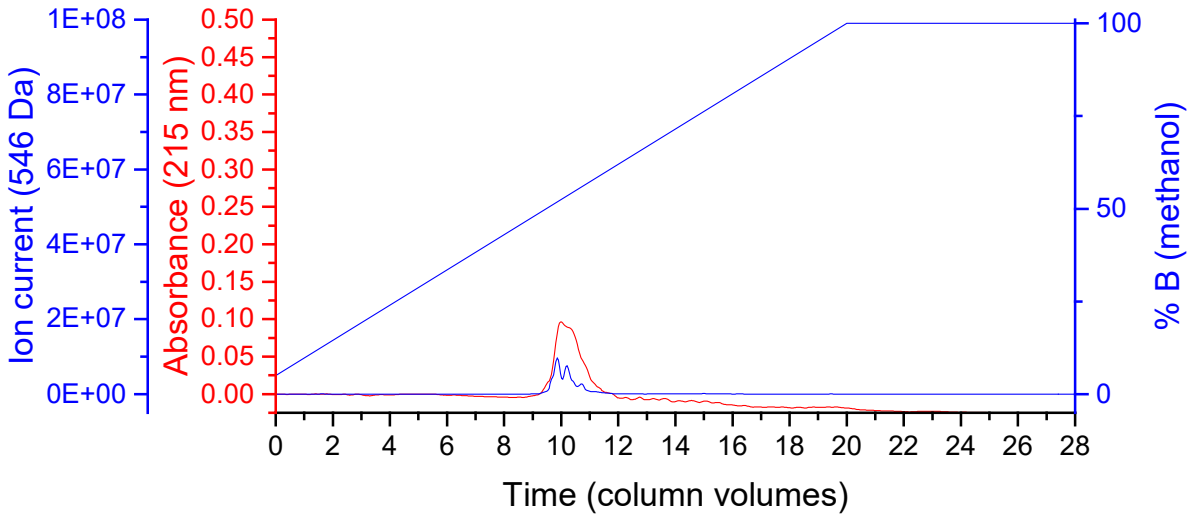

Figure 2—Scouting gradient using RediSep Gold C18Aq column.

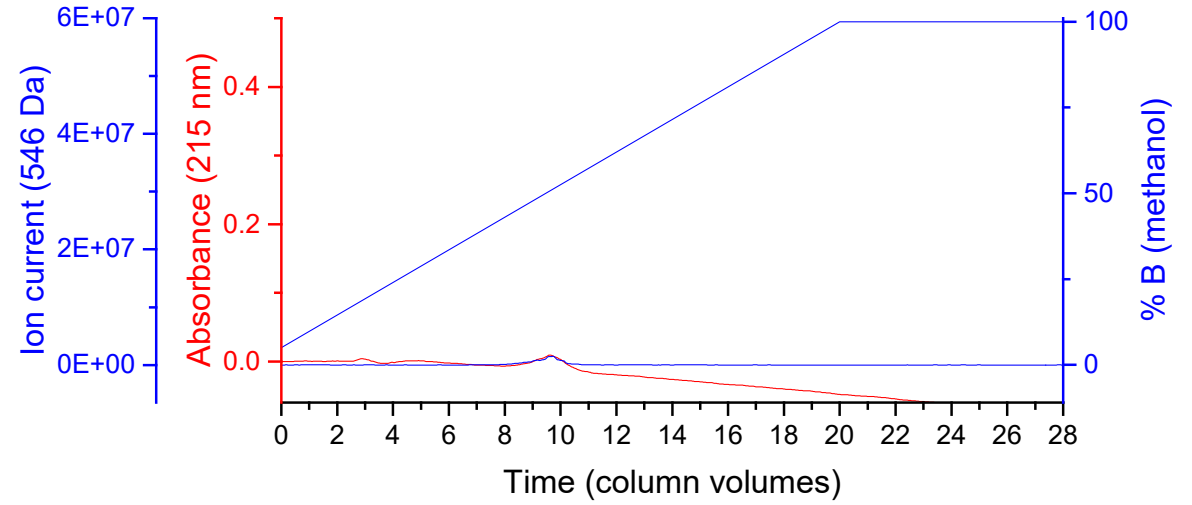

Figure 3—Scouting gradient using RediSep Gold C8 column.

With the exception of the first scouting run, the runs all used the same scouting gradient, solvent, and sample. Only one milligram of sample was injected for each run. The first scouting run was performed with an ACCQ*Prep*® HP150 and a Redi*Sep*® Prep C18 column which matches the chemistry of the flash column. The Focus Gradient Generator was used to determine the focused gradient of the NextGen 300+ as described in Application Note 119, which describes how to calculate focused gradients for flash systems using the ACCQ*Prep*. As the Redi*Sep* Prep C18 columns match the chemistry of the Redi*Sep* Gold C18 columns, the use of the ACCQ*Prep* is another way to calculate flash focused gradients. The C18Aq and C8 flash focused gradients were determined using the Flash Focus Gradient Generator in PeakTrak. Although the peptide itself is soluble in water, there was non-water-soluble material in the peptide. Neat trifluoroacetic acid dissolved the sample very well, so the injection was 1 mg of sample dissolved in 0.2 mL TFA. The focused gradient for each purification was calculated using the "Time-on-Target" algorithm from the scouting runs.

Purification

The solvents for the purification were the same as those used in the scouting gradients. The focused gradient started at approximately 30% methanol and ran to approximately 40% B solvent, followed by a step to 100% methanol to wash any out late-eluting compounds. The exact focused gradient calculated and used is listed in the chromatograms. Mass detection (PurIon™ L mass spectrometer with fluid interface) was used for these runs. Each column was loaded with 100 mg sample.

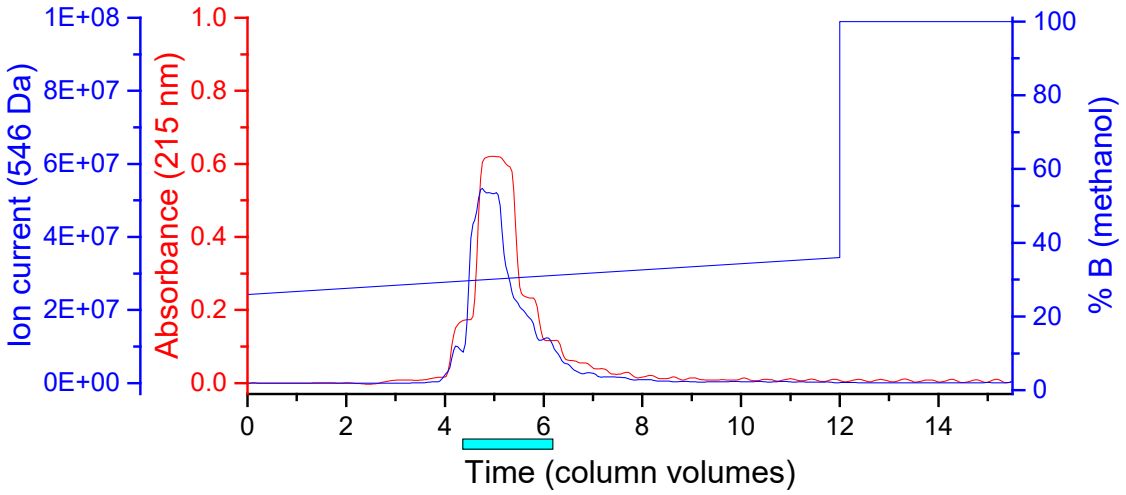

Figure 4—The C18 focused gradient was calculated to be 26 to 35 % methanol. Fractions were combined as indicated to yield 70 mg.

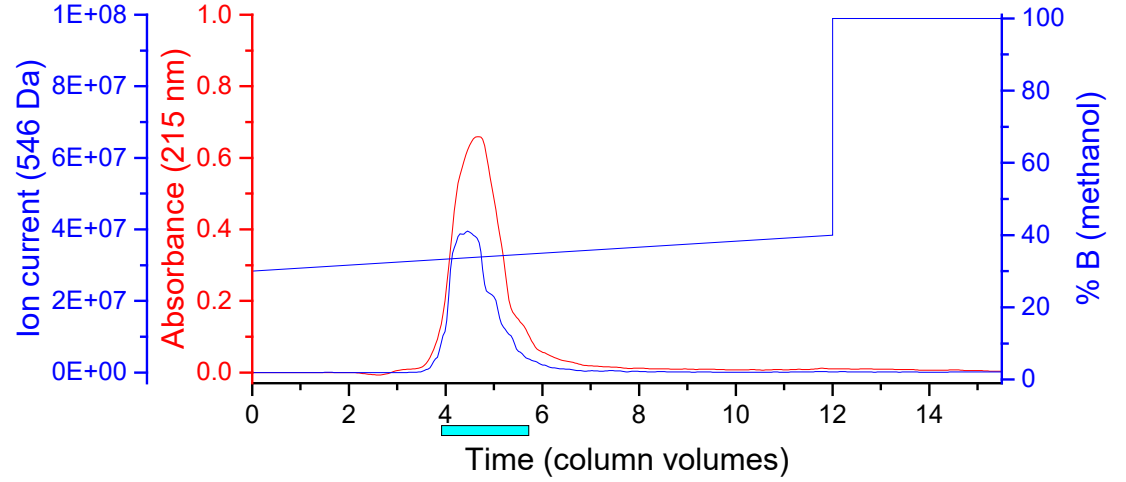

Figure 5—The C18AQ focused gradient was determined to be 30 to 40 % methanol, showing how the C18AQ column has improved retention for polar compounds than regular C18. This run recovered 75 mg of peptide.

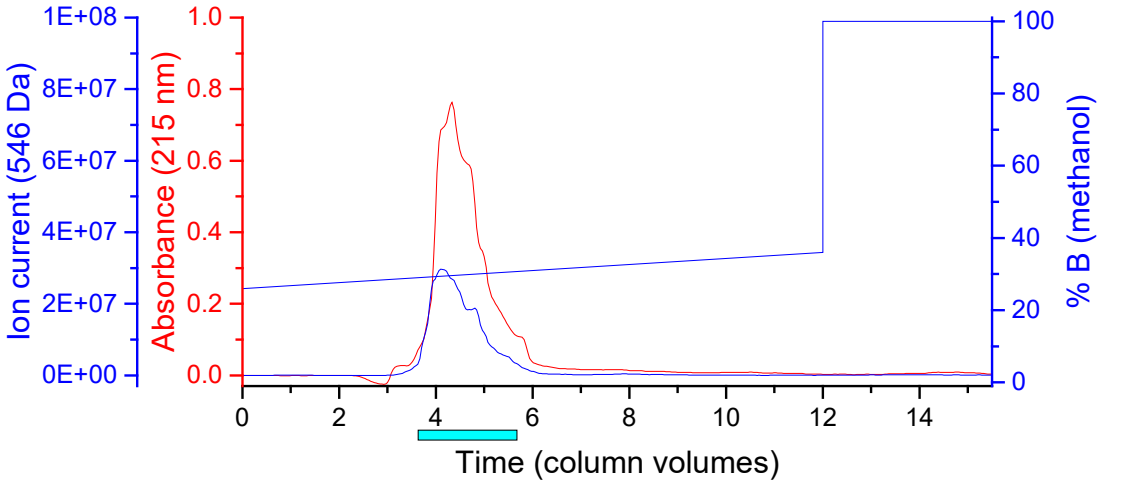

Figure 6—The RediSep C8 column was run with a calculated gradient of 26 to 36 % methanol. The recovery was 91 mg peptide due to reduced tailing.

The C8 column exhibited narrower peaks and showed a higher recovery of the peptide. As expected for a solvent system containing mostly water, the C18Aq column had improved retention, and required a slightly higher concentration of organic solvent to elute the peptide.

Analytical HPLC

Analytical HPLC was run on an Agilent 1290 with a Redi*Sep* Prep 2 x 50 mm C18 column in water/acetonitrile, both of which contained 0.1% TFA. The fractions were combined and analyzed prior to removal of methanol and freeze-drying. All three columns successfully purified the peptide and no additional purification was needed.

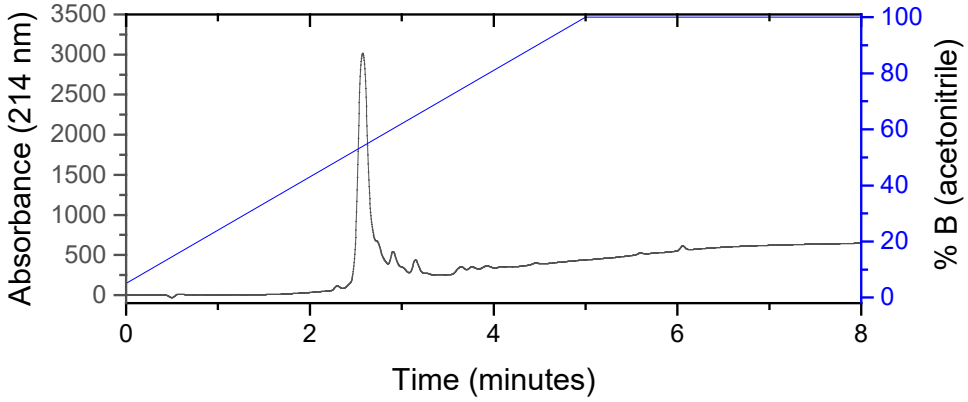

Figure 7—Analytical UHPLC of crude peptide.

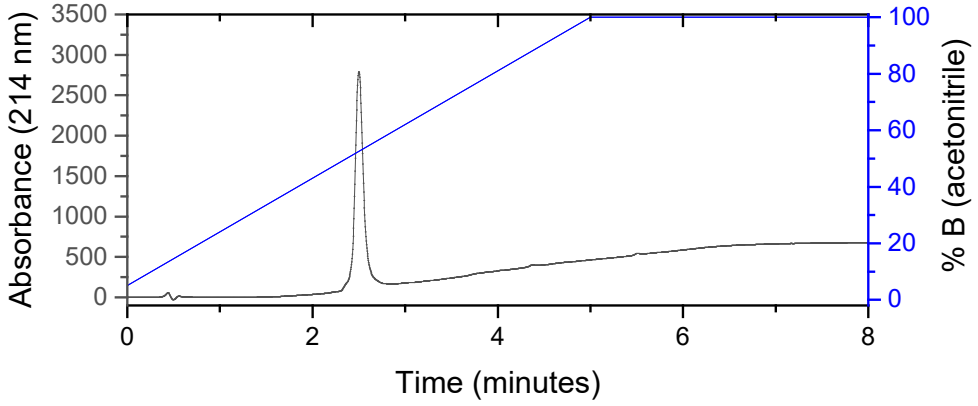

Figure 8—Analytical UHPLC of peptide purified on a RediSep C18 flash column.

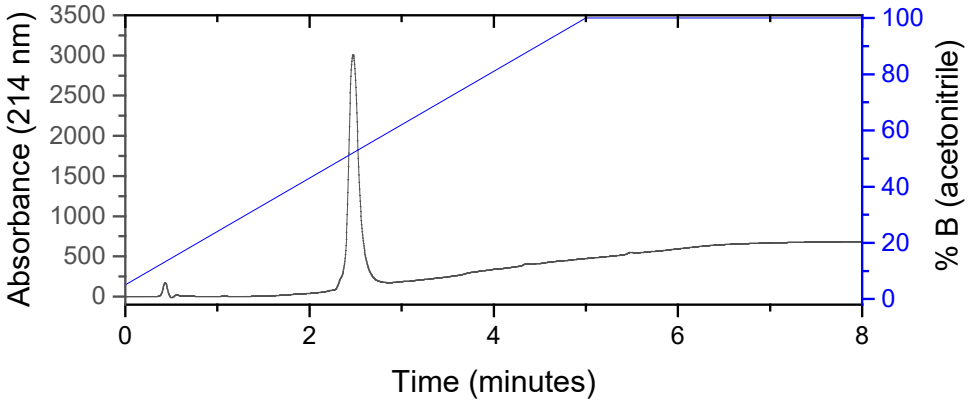

Figure 9—Analytical UHPLC of peptide purified on a RediSep C18AQ flash column.

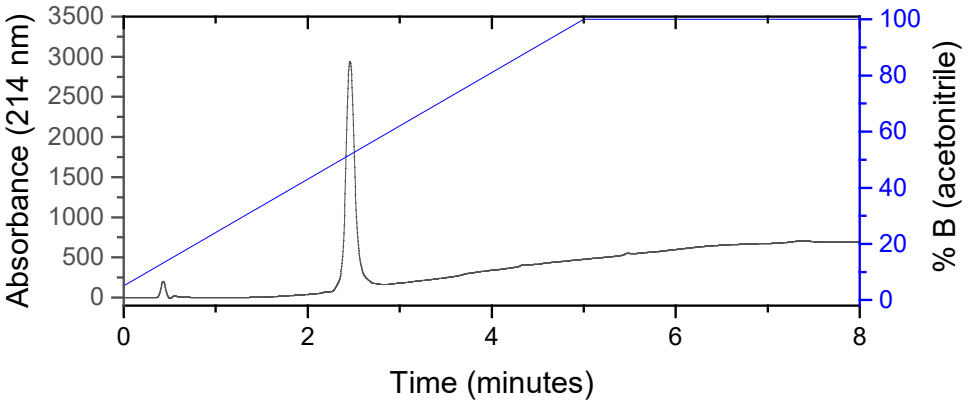

Figure 10—Analytical UHPLC of peptide purified on a RediSep C8 flash column.

Conclusions

Flash chromatography worked very well to purify peptides with a molecular weight of ~1000 Daltons. The use of the PurIon L mass spectrometer confirmed the eluting peaks were free from impurities. The total flash chromatography run time was approximately 9 minutes, with the desired peptide eluting within 5 minutes, and so the purification time was exceptionally fast.

[1] *Silver, J. Overview of Analytical-to-Preparative Liquid Chromatography Method Development.* ACS Combinatorial Science ***2019,*** 21 (9)*, 609–613.*

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