Preparative Chromatography Focused Gradients, pH Control, and Ionizable Compounds 2: Peptides

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Chromatography Application Note AN141

Abstract

Peptides can be purified using the Focus Gradient Generator, but the calculation of the focused gradient is affected by the solvent modifiers used, the concentration of the peptide, and the peptide's isoelectric point. Replacing trifluoroacetic acid with a buffer improves peptide recovery, provided the mobile phase is buffered at least 2 pH units from the isoelectric point. A mass spectrometer helps to verify peptide elution and elution of the desired peptide in single or multiple peaks.

Background

Peptides are commonly purified on reverse phase using 0.1% trifluoroacetic acid (TFA) as a solvent modifier. While this works well for analytical liquid chromatography due to the low concentration of the peptide analyte, scaling the purification to a preparative sample load often causes the concentration of peptide within the volume of a peak to match or exceed the concentration of TFA in the mobile phase.

Ionizable compounds such as peptides can convert between a polar form and a less polar form in solution (Figure 1). When running reverse phase columns, the ionized form elutes earlier, while the unionized moiety has later elution. A compound may elute as a narrow peak, a broad peak, or as multiple peaks depending on the number of ionizable groups on the molecule, their ionization constants, and whether the ionized and unionized forms are readily interconvertible.

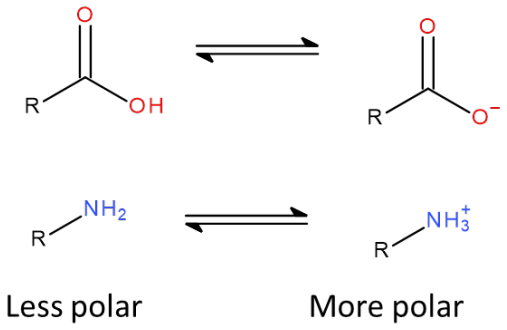

Figure 1. Examples of ionizable compound equilibrium between ionized and unionized forms.

Experimental and Results

All runs below used a Teledyne ISCO ACCQ*Prep*® HP150 preparative HPLC system with a PurIon™ L mass spectrometer and Redi*Sep*® Prep 20x150 mm C18 column. Both scouting and focused gradient runs were run on the ACCQ*Prep* HPLC system.

Acetonitrile and TFA

The most common solvent system used for peptides is water/acetonitrile containing 0.1% trifluoroacetic acid (TFA). The TFA solution is made by adding 1.0 mL (1.48 g) of TFA to the solvent to make a 13 mMol solution. Analytical HPLC uses very small amounts of sample, so the analyte concentration is very low compared to that of the solvent modifier. Likewise, scouting runs using the preparative LC system use the smallest amount of sample needed to detect the desired compound, so the concentration of the sample is still small compared to the modifier concentration. However, preparative chromatography uses a much higher sample concentration because the goal of preparative LC is to purify as much compound as possible within a single run. A compound will often elute within the volume of a single fraction tube. If 100 milligrams of a 300g/mol sample elutes within the volume of a 20 mL fraction, the concentration of that sample is ~17 mMol, which exceeds the 13 mMol concentration of the TFA. The sample is no longer buffered at all. The peptide EGFRviii is 1635 g/mol, so 100 mg in a 20 mL fraction is 3 mMol, about 1/4 the concentration of TFA. The peptide also has multiple sites that can ionize in the presence of an acid as indicated by the mass spectrum, which shows the $[M+2H]^{2+}$ (818 Da) and $[M+3H]^{3+}$ (546 Da) ions. The $[M+2H]^{2+}$ ion was used for detection because it was the most intense ion (Figure 2).

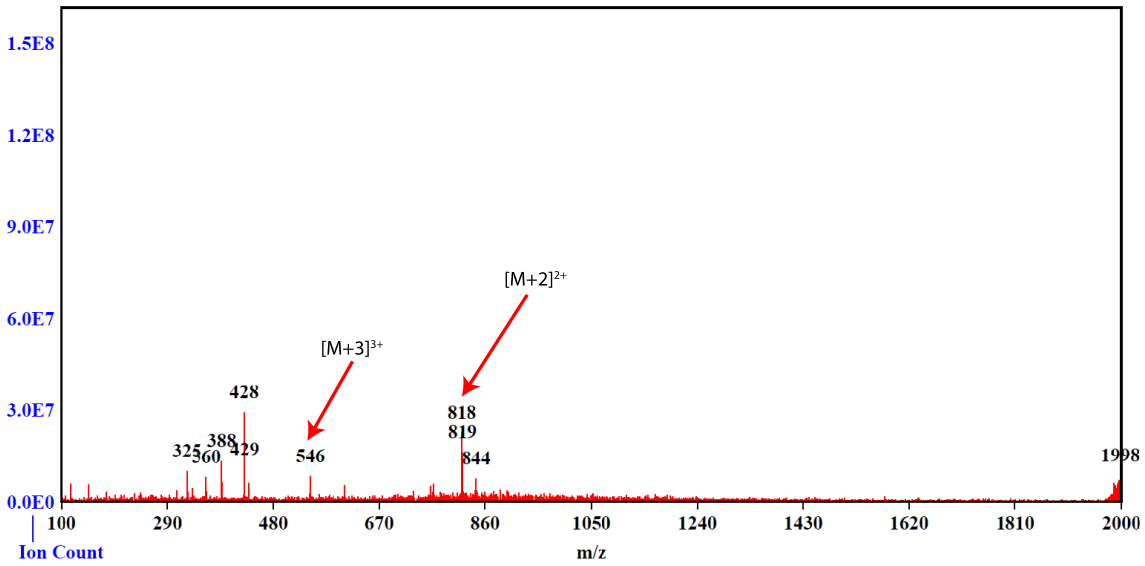

Figure 2. Mass spectrum of EGFRviii showing $[M+2]^{2+}$ at 818 Da and $[M+3]^{3+}$ at 546 Da.

A scouting run with an acetonitrile/water gradient containing 0.1% TFA was run using ~2 mg sample dissolved in DMSO (Figure 3). The calculated focused gradient resulted in the compound eluting during the wash portion of the run, after the focus gradient segment ended. Increasing the start and end point of the focused gradient by ~8% acetonitrile moved the main peak to the center of the gradient, but the mass trace showed that the peptide was eluting as at least three peaks (Figure 3). If only the UV detection was used, much of the peptide would be lost, as only the material eluting from 6 to 7 minutes would be collected as the largest peak.

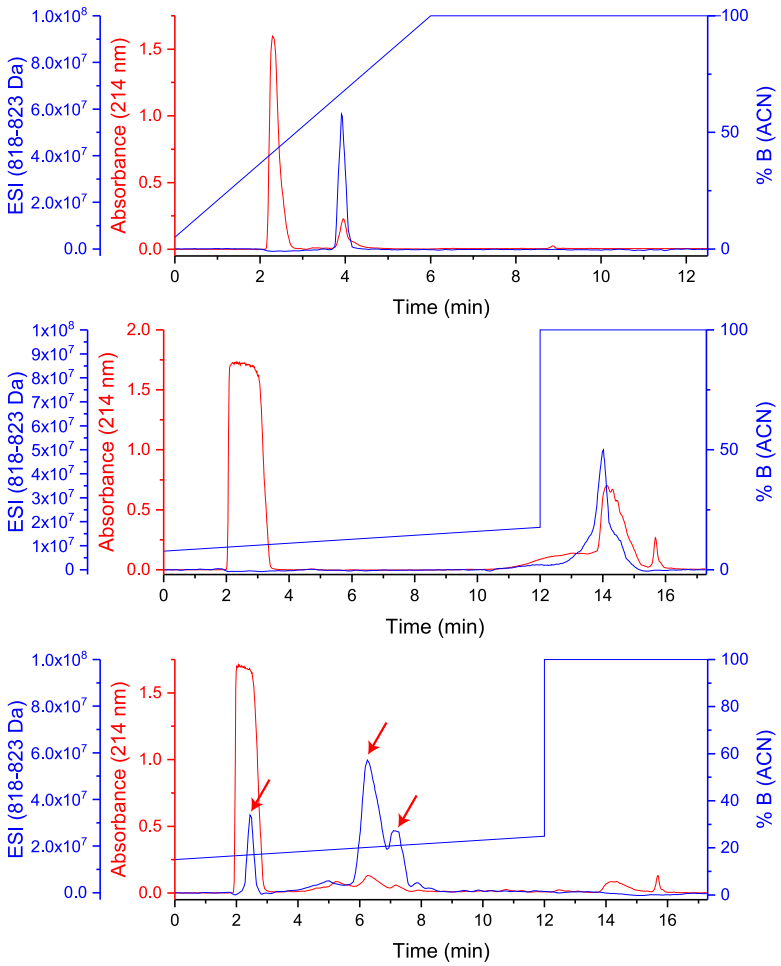

Figure 3. Scouting run with water/acetonitrile with 0.1% TFA, calculated focused gradient, and adjusted gradient.

Methanol and ammonium acetate (pH 4.5)

The solvent system was changed to 50 mMol ammonium acetate with the pH adjusted to 4.5. Ammonium acetate was chosen because it is a volatile salt, making it friendly for mass spectrometer detection after the fluid interface reduced the amount of salt to below 1 mMol in carrier solvent. The change to 50 mMol buffer also required a change from acetonitrile to methanol so that the buffer could remain in solution as the gradient was changed.

Figure 4 shows the peptide eluted later in the scouting run compared to Figure 3, corresponding to the increased B solvent (methanol) concentration at the start and end of the focused gradient. The peptide eluted as two peaks within the focused gradient. At this point, the pI of the peptide was calculated to be 5.5; peptides should be purified at more than one pH unit from the pI. The isoelectric point (pI) is the pH at which a molecule has an overall neutral charge, although parts of the molecule may be ionized.

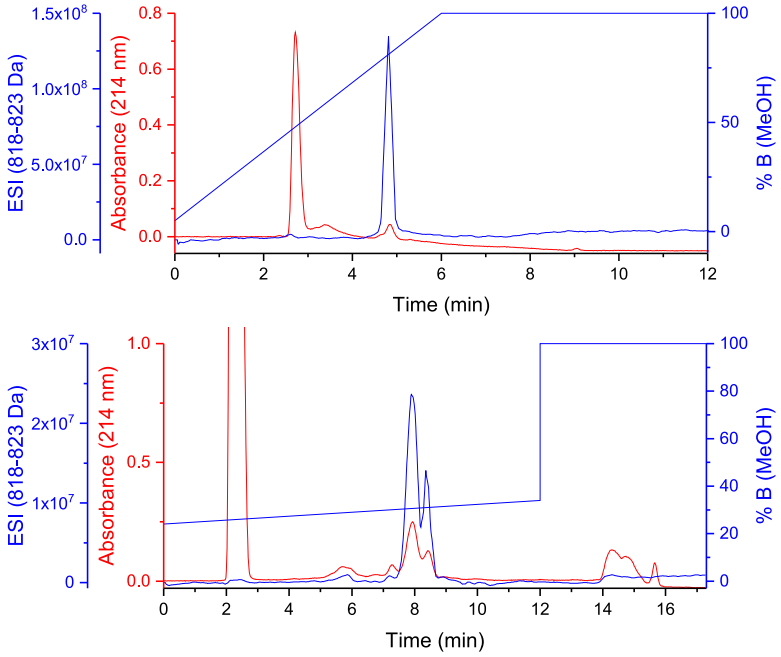

Figure 4. EGFRviii in a water/methanol gradient buffered with ammonium acetate at pH 4.5.

Methanol and Ammonium formate (pH 3.5)

Moving the pH to 3.5 was done by using ammonium formate. As the buffer was changed, a new scouting run was used to calculate a new focused gradient.

The peptide eluted as a single peak, while other UV absorbing compounds were resolved from the peptide. The peptide was run using an efficient gradient method with good resolution from impurities. As the peptide eluted in a single peak, the recovery was improved. The use of the PurIon mass spectrometer confirmed where the desired compound was eluting during the purification.

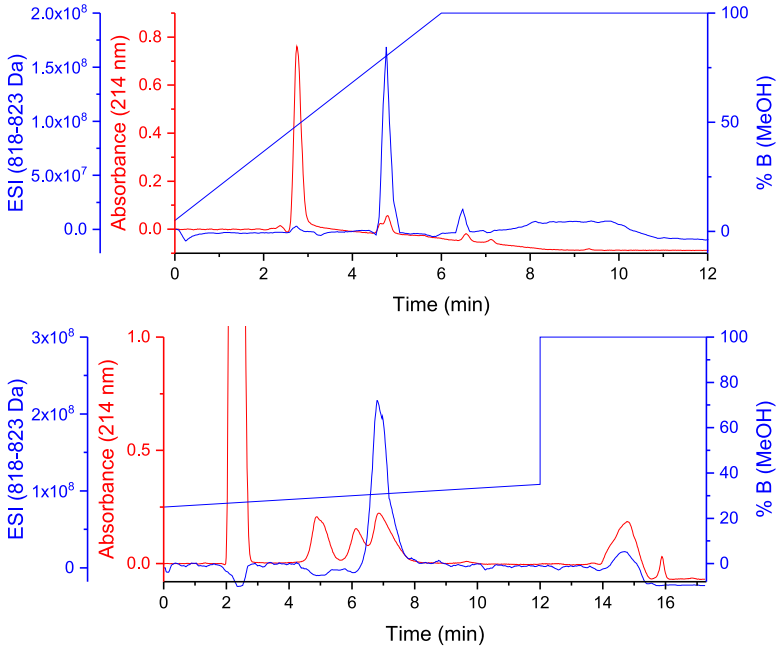

Figure 5. EGFRviii in a water/methanol gradient buffered with ammonium formate at pH 3.5.

Conclusion

The PeakTrak Focus Gradient Generator allows fast method development and purification of peptides when the peptide pI is considered, and when the solvent system is buffered to allow for the higher concentration of peptides compared to analytical chromatography of the same peptide. The PurIon mass spectrometer aids both method development and validates that the peptide is eluting from the gradient method as expected. The mass spectrometer also confirms collection of the correct peak by using the correct ion—whether it is the molecular ion of one of the multiply charged ions.

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