Using Focused Gradients to Improve Peptide Purity Using Reversed-Phase Flash Chromatography

Summary

Purification of synthetic peptides represents the largest bottleneck of the entire peptide workflow. Reversed-phase flash chromatography is gaining traction as a strategy to reduce this bottleneck but can be hindered by low resolution. Presented herein is data supporting the use of a focused gradient, which can be useful to overcome low resolution separations and enable recovery of highly pure peptides using reversed-phase flash chromatography, without significant method development efforts.

Introduction

As interest in peptides for a variety of applications continues to grow, so does the demand for workflow efficiency – ie) the delivery rate of purified peptide samples for experimental evaluation. Automated synthesis platforms enable rapid synthesis of tens to hundreds of compounds simultaneously which can create a purification bottleneck.

Peptides are traditionally purified using reversed-phase HPLC (RP-HPLC), but the use of reversed-phase High Performance Flash Chromatography (HPFC) is quickly gaining traction as a technique for peptide purification, thereby improving overall peptide workflow efficiency. Despite the high loading capacity, HPFC can be hampered somewhat by reduced resolution, thus increasing the need for specialized method development. Herein, we present data demonstrating that the use of a focused gradient can improve the recovered peptide purity while minimizing the need for complex method development.

Methods

Peptides were synthesized with a Biotage® Initiator+ Alstra™ microwave peptide synthesizer on Rink amide ChemMatrix® resin using DIC, Oxyma, and Fmoc-protected amino acids. The peptide was elongated using 5 equivalents of each, relative to resin loading, and reacted at 75 °C for 5 minutes. The *N*-terminal Fmoc protecting group was removed with 20% piperidine in DMF with two reactions, the first for 3 minutes and the second for 10 minutes at room temperature.

The peptide was cleaved from the resin using a cocktail of 95% TFA, 2.5% TIS and 2.5% H_2O for 2 hours at room temperature and rapidly evaporated using the Biotage® V-10 evaporation system. The resulting residue was washed with cold diethyl ether for further purification.

The crude peptide sample was dissolved in DMSO and purified using a Biotage® Isolera™ Dalton 2000 flash purification system equipped with a 10 gram Biotage® Sfär Bio C18 column (20 µm particles with 300 Å pores) and eluted with mobile phase solvents consisting of water + 0.1% TFA (Solvent A) and acetonitrile + 0.1% TFA (Solvent B). Purified peptides were dissolved in methanol and analyzed with an Agilent 1260 Infinity series HPLC and AB Sciex MS equipped with Restek Raptor C18 column (2.7 µm particles, 2.1 x 50 mm).

Results and Discussion

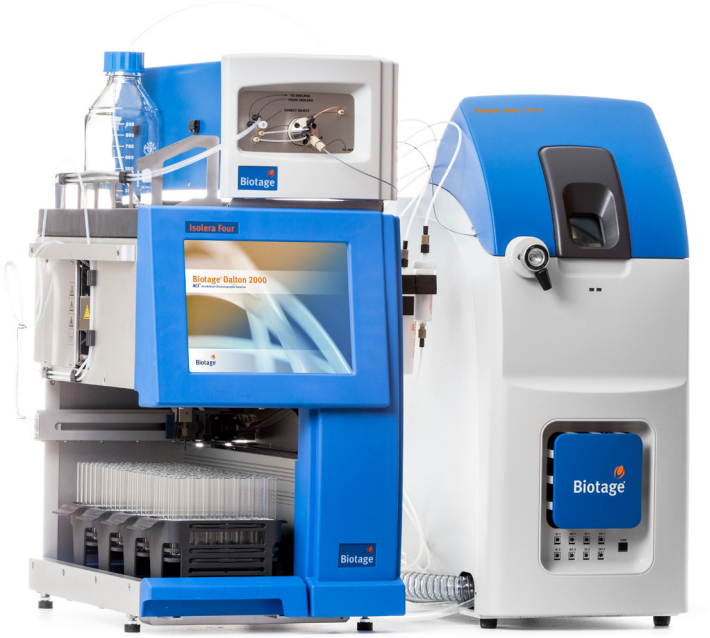
Purification Using a Standard Linear Gradient

In general, using the analytical HPLC data as a reference, a linear gradient is designed centering on the calculated acetonitrile elution concentration with a linear slope of 50% acetonitrile change over 10 column volumes. Importantly, the calculated acetonitrile elution concentration remains consistent between the analytical HPLC column and the Sfär Bio C18 column, simplifying the transition to HPFC.

In this example, the crude analytical chromatogram of peptide 18A, an 18 amino acid amphipathic peptide (sequence: DWLKAFYDKVAEKLQEAF-NH2) was first collected to evaluate crude purity and determine the acetonitrile concentration required for elution. See Figure 1.

A 300 µL aliquot of the peptide, dissolved in DMSO, was purified using a linear gradient starting at 20% acetonitrile and ending at 70% acetonitrile, in correspondence to an approximate 50% acetonitrile elution concentration as measured by analytical HPLC. See Figure 2.

This initial gradient provided reasonable separation of the desired product (peak 3) from the other significant impurities despite the steep gradient slope (8.33% per minute at 25 mL/min). The product peak does tail somewhat, suggesting co-eluting impurities may still be present and are evident in the follow up analytical HPLC. See Figure 3.



Isolera™ Dalton 2000 flash purification system with mass detection.

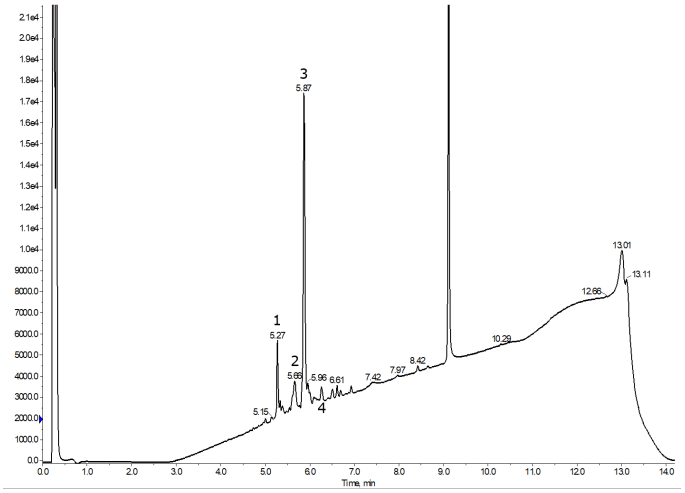


Figure 1. Analytical HPLC chromatogram for crude 18A. The desired peptide is contained in the peak labeled 3 and constitutes the major peptide product. Three other significant impurities are also labeled. The large peak eluting at approximately 9 min is attributed to residual protecting groups in the sample as no peptidic masses are associated with this peak.

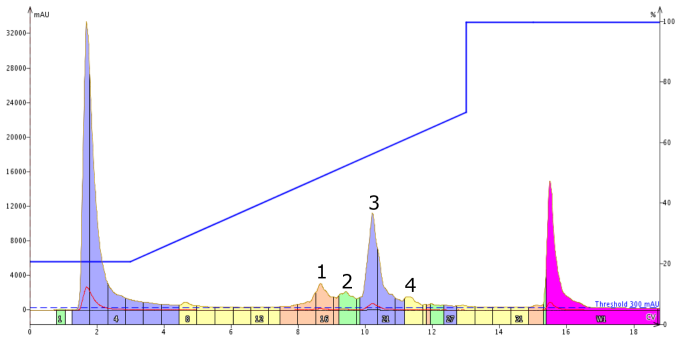


Figure 2. Purification of 18A using a standard linear gradient. The linear gradient has a total of 50% change in acetonitrile concentration (20-70%) over 10 column volumes. The desired peptide is contained in the peak labeled 3.

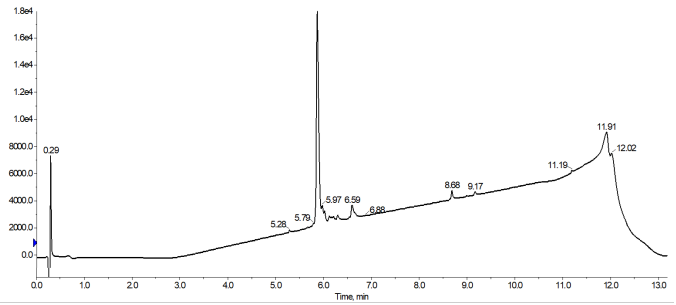


Figure 3. Analytical HPLC of combined 18A fractions collected during purification with a focused gradient. The purification yielded a product with 83% purity.

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Purification with a Focused Gradient

A focused gradient can simply be described as a gradient with regions of sharp change in mobile phase B concentrations and regions with a very shallow slope near where the desired peptide elutes. This strategy limits the peak broadening observed for extended, shallow gradients while improving the resolution between closely eluting compounds.

When designing a focused gradient, it is important to begin the purification at a low acetonitrile concentration to ensure compound retention by the column, especially if you are using a very strong solvent to dissolve the crude sample, like DMSO. Once loaded, and sufficiently retained, the acetonitrile concentration is quickly changed to slightly below the elution concentration and followed by a very shallow linear increase in acetonitrile. Once completed and the compound eluted, the acetonitrile concentration is quickly increased to clean the column as normal.

Using the linear gradient purification above as reference, the acetonitrile concentration at which the desired peptide begins eluting was approximated at 55% acetonitrile. See Figure 4.

With this information in hand, the focused gradient region was reprogrammed on the Biotage® Isolera™ Dalton 2000 for 50% to 60% acetonitrile over 10 column volumes and an additional aliquot of 18A was purified. See Figure 5.

The hypothesis that a shallower gradient (1.67% per minute at 25 mL/min), more reminiscent of gradients designed for HPLC-mediated purification, would enable increased resolution without compromising peak sharpness was realized. However, the focused gradient region starts with an acetonitrile concentration that is too high, compromising the product resolution from early eluting impurities.

Upon careful inspection, it is apparent that the compounds begin eluting within two column volumes of entering the focused gradient region. Previous work has shown that a minimum of two column volumes is required for a sample to fully traverse the column and enter the detector. This suggests that the desired compound, and the early eluting impurities, will elute more selectively from the stationary phase with less acetonitrile than previously approximated. With this result, the starting point for the focused region was reduced to 40% acetonitrile, maintaining the total duration and slope, and the purification was repeated. See Figure 6.

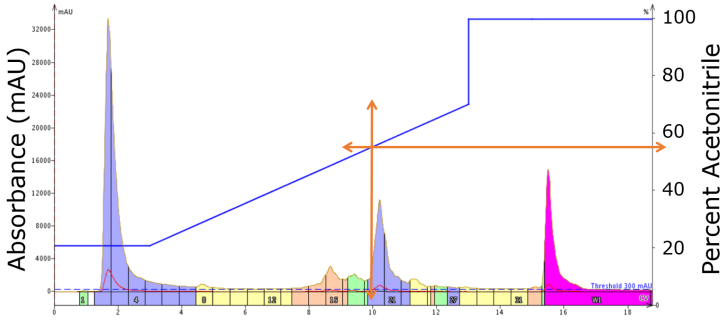

Figure 4. Approximating the acetonitrile concentration at which the desired compound elutes from the stationary phase. It is important to approximate the earliest acetonitrile concentration in order to minimize additional method development steps.

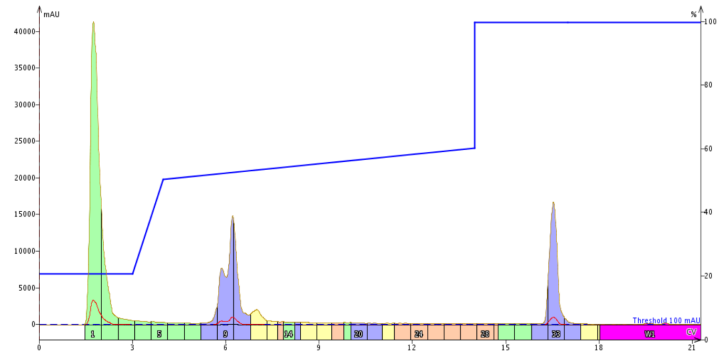

Figure 5. Purification of 18A using a focused gradient ranging from 50–60% acetonitrile. The focused region begins with an acetonitrile concentration that is too high for the early eluting impurities, causing co-elution with the desired product.

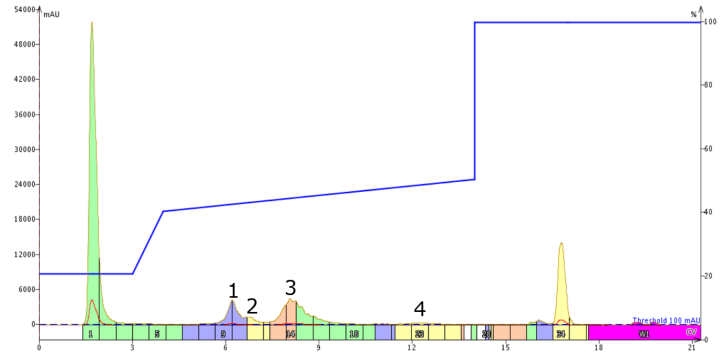

Figure 6. Purification of 18A using a focused gradient with a lower starting point. The three impurity peaks are well resolved from the desired product peak 3, with minimal peak broadening.

Although the slope of the gradient was the same as the previous purification, simply reducing the starting acetonitrile concentration resulted in full resolution of the four major compound peaks. When compared to the initial scouting gradient, the separation has improved, yielding baseline resolution between the product and the two closest eluting impurities. However, the assumption that a shallow, yet focused gradient, would limit peak broadening did not uphold. Compounds eluting later in the focused region exhibit peak broadening, whose severity increases with increased retention. Ideally, the desired compound would elute approximately halfway through the focused region to maximize resolution and minimize peak broadening. After combining the product peak fractions and evaporation, the final purity was in fact improved to 90% when compared to 83% from the initial, linear gradient. See Figure 7.

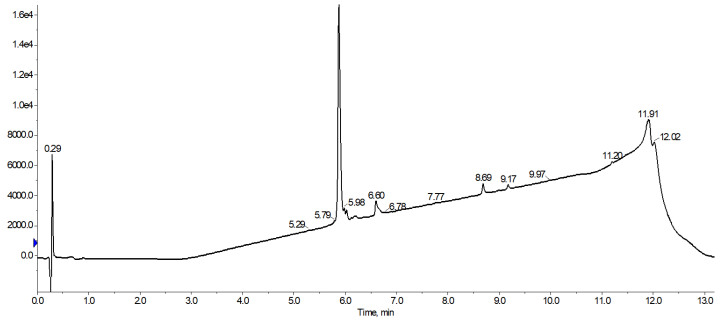

Figure 7. Analytical HPLC of combined 18A fractions collected during purification with a focused gradient. The purification yielded a final product with 90% purity.

Conclusion

The use of reversed-phase high performance flash chromatography (HPFC) is gaining traction as a strategy to reduce the overall purification time for synthetic peptides. Herein we present data demonstrating that a focused gradient, with a slope reminiscent to that commonly used for HPLC-mediated peptide purification, can indeed be used in HPFC to improve the final purity of a desired peptide with minimal time invested in method development.

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