Method Development for Peptide Purification – How to Create a Step Gradient

Summary

Synthetic peptide purification represents the most significant bottleneck of the entire peptide workflow. Reversed-phase flash chromatography is gaining traction as a strategy to reduce this bottleneck given the significant increase in loading capacity and short purification times. Presented herein are guidelines for creating a step gradient for peptide purification, ultimately enabling complete separation of a 20 amino acid peptide from a 19 amino acid peptide impurity.

Introduction

As interest in peptides for a variety of applications continues to grow, so does the demand for workflow efficiency – i.e. the delivery rate of purified peptide samples for experimental evaluation. Automated synthesis platforms enable rapid synthesis of tens to hundreds of compounds simultaneously, creating 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 acceptance as a technique for peptide purification, improving overall peptide workflow efficiency. Despite the increased loading capacity, HPFC can be hampered by reduced resolution when compared to HPLC, increasing the need for specialized gradient method development. One such strategy is the use of a step gradient, where an acetonitrile concentration is held isocratically, then "stepped" to a new concentration and repeated until the desired compound is eluted from the stationary phase, Figure 1. This approach, common in normal-phase purification, can increase resolution between individual compounds in the sample without causing significant peak broadening when using reversed-phase chromatography as well.

The most difficult, and often most critical, peptide separations involve removing a single amino acid deletion or insertion product from the desired peptide. In this series of purifications, the crude sample contains a 19 amino acid deletion sequence, among other impurities, that negatively impacts the performance of the desired 20 amino acid peptide product and must be removed.

Purification Using a Standard Linear Gradient

Before any purification efforts are initiated, an analytical separation should be performed to confirm the desired product was, in fact, synthesized and to approximate sample purity. With this information, the concentration of acetonitrile required to elute the compound can be easily calculated. Importantly, this calculated acetonitrile elution concentration remains consistent when comparing retention by the analytical HPLC column and the Biotage® Sfär Bio C18 column, simplifying the transition to HPFC. In general, using the crude analytical HPLC data as a reference, a linear gradient can be designed based on the following parameters:

- Equilibration: use 3 column volumes (CVs) at the initial condition
- Initial condition: subtract 20 percent from the elution concentration calculated from the crude analytical HPLC
- Linear gradient: increase acetonitrile concentration a total of 50 percent over 10 CVs from the initial condition
- Column wash: increase from final acetonitrile concentration to 100% acetonitrile over zero CVs and hold for two CVs

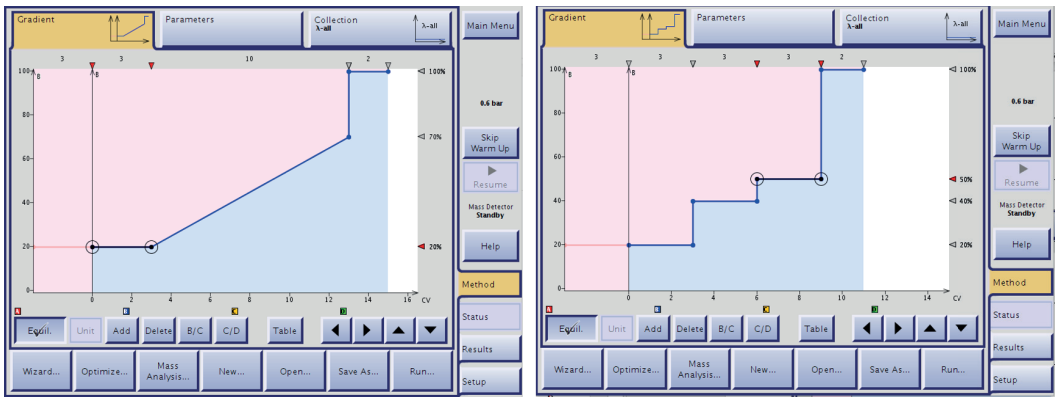

Figure 1. Representative gradients for peptide purification. Linear gradients (left) are commonly used in reversed phase purification whereas step gradients (right) are much more commonplace in normal phase purifications. A step gradient can be used in place of a very long, shallow linear gradient employed to improve reversed phase separations.

Analysis of the crude analytical indicates the desired compound elutes with approximately 35% acetonitrile (data not shown). The initial gradient was programmed on the Isolera™ Dalton 2000 to run from 10% acetonitrile to 60% acetonitrile over 10 column volumes, Figure 2.

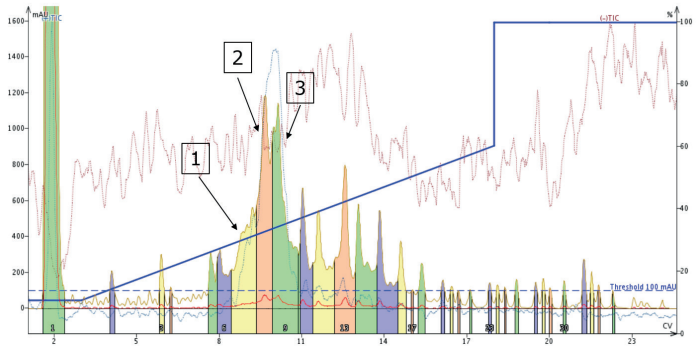

Figure 2. Purification of a 20 amino acid peptide using the template linear gradient. The desired product is contained in the orange fraction (labeled 2). Critical impurities are contained in both the yellow fraction (labeled 1) and green fraction (labeled 3).

This standard gradient template often yields reasonable purity of the desired peptide. In this case, however, the desired compound essentially coelutes with both the most important impurity (labeled 1) and a trailing impurity (labeled 3). While the purity level achieved here is unacceptable, the linear gradient purification provides a reference from which to build a step gradient.

Using the "Optimize" Feature to Design a Step Gradient

The Isolera™ Dalton 2000 is equipped with a software feature, Optimize, that predicts the specific acetonitrile concentration required to elute compounds contained within a selected peak using a reference linear gradient purification. This concentration, coupled with the required duration of each isocratic hold, enables construction of a step gradient to use for purification.

Using the previous linear gradient purification as a reference, the Optimize software tool is used to predict the acetonitrile concentrations required to elute each of the three compound peaks of interest, Figure 3.

The Optimize software feature was designed for use with normal phase purifications, so some additional care must be taken when applying the suggested gradient predictions to reversed phase peptide purifications. First, note the initial conditions for the gradient (first isocratic hold). The software typically suggests a gradient starting point that is much too close to the desired compound elution concentration, preventing compound retention by the column. An acetonitrile concentration similar to that of the initial condition for the linear gradient is sufficient. Second, note the duration of each isocratic hold. Optimize predicts that the compound contained within the selected peak will elute in a single CV. Previous experience has shown that a single CV will not fully elute the selected compound. When constructing the actual gradient, a minimum of three CVs should be used for each isocratic step to ensure full column equilibration and subsequent elution.

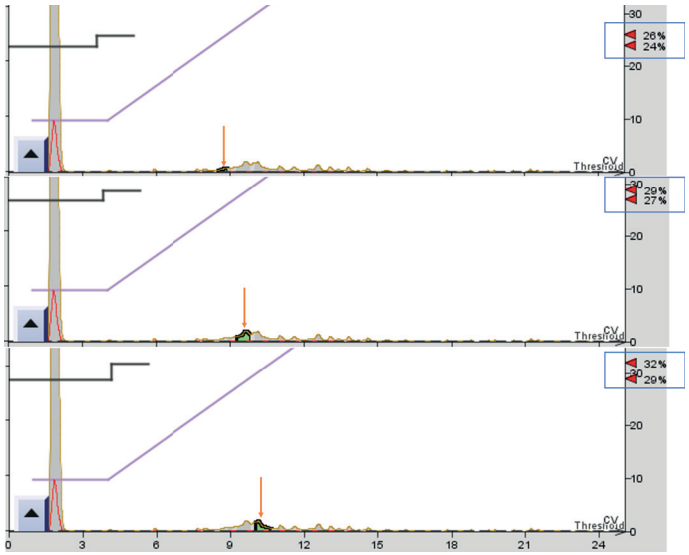

Figure 3. Using the Optimize software tool to predict acetonitrile concentrations for a step gradient purification. The earliest eluting impurity is selected (top) and corresponding acetonitrile concentration for elution is predicted to be 26%. This is repeated for the product peak (middle) and late eluting impurity (bottom) with corresponding acetonitrile concentrations of 29% and 32% respectively.

Using the information obtained with the Optimize feature as a guide, a second aliquot of the crude peptide sample was purified using a designed step gradient, Figure 4.

%B	Duration of hold (CV)
10	3
26	3
30	4
100	2

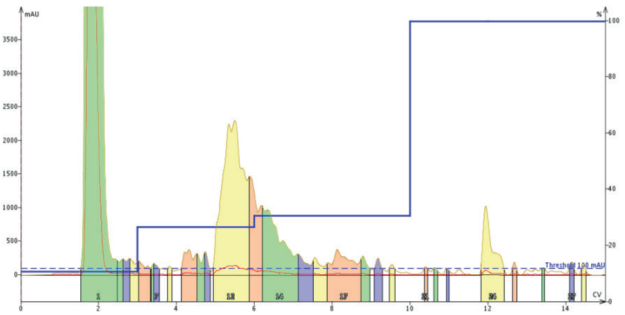

Figure 4. Purification of a 20 amino acid peptide using a step gradient. The concentration of acetonitrile and duration used for each isocratic hold (left) was created referencing the Optimize software feature suggestions on a Isolera™ Dalton 2000. Unfortunately, these conditions caused all three compounds of interest to coelute.

There are only 2 "steps" in our purification despite the presence of three key peaks in our sample. The reasoning behind this is simple – if properly designed, the step gradient will resolve the early eluting impurity from the desired product (first step) and elute the desired compound (second step) while retaining the trailing impurity until the final column flush.

There are several apparent differences when we compare this purification to that performed with the standard linear gradient. The most distinct is that our product peak is much sharper than observed with the linear gradient. This is expected given the differences in gradient design. With a step gradient, retained compounds concentrate on the stationary phase and then elute in tighter bands when %B is increased. Upon closer inspection though, the peak sharpness is due to the fact that the three compounds of interest are simply coeluting, suggesting that the acetonitrile concentration is simply too high (data not shown).

In order to improve the purity, there are three aspects to consider:

1. Adjust the acetonitrile concentration for some or all isocratic holds
2. Increase the isocratic hold duration
3. Increase the number of isocratic hold steps

It's likely that a combination of these three will be used for the optimal separation.

Adjusting Acetonitrile Concentration

Given that the critical impurity still coelutes with the desired product, the acetonitrile concentration was decreased for each of the two steps but the isocratic hold duration held constant.

The intent was to determine the ideal acetonitrile concentration to elute each compound individually, Figure 5.

Upon initial inspection, the changes seem to have improved the purification. An early peak is fully resolved from the major peak containing the desired compound. The major peak, however, is significantly broader than in the previous purification. After closer inspection with the Isolera™ Dalton 2000 mass spectral data, it is apparent that the critical impurity elutes very tightly at the very beginning of the peak (see Figure 7), while the masses corresponding to the desired product essentially bleed off the column. This suggests that the 25% acetonitrile is still stronger than ideal for the critical impurity, but slightly weaker than ideal for the desired product.

Increasing Isocratic Hold Duration

Given the above observations, two changes were made to the step gradient prior to the next purification attempt, Figure 6. The first was to increase the concentration of acetonitrile held at each step slightly. By increasing from 20% acetonitrile to 22% acetonitrile for the first step the goal is to draw out the critical impurity while still retaining the desired peptide and ultimately increasing the resolution between the two compounds. Increasing the second isocratic hold from 25% to 27% acetonitrile should sharpen the desired product elution from the column. The second change was to increase the duration of each isocratic hold step. In the previous two step gradient purifications, the peaks begin to elute after a duration of 2 column volumes, suggesting that duration as a minimum for column equilibration. However, a longer duration enables compounds to fully partition off the stationary phase and into the mobile phase for elution.

%B	Duration of hold (CV)
10	3
20	3
25	4
100	2

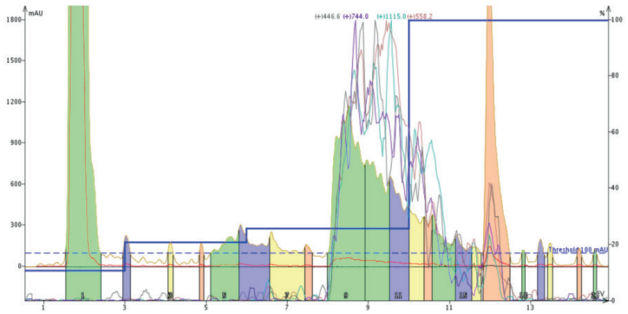

Figure 5. Conditions (left) for the attempted separation of a 20 amino acid peptide from a critical 19 amino acid impurity using a step gradient. Both the desired peptide and the critical impurity are still coeluting under these conditions.

%B	Duration of hold (CV)
10	3
22	4
27	5
100	2

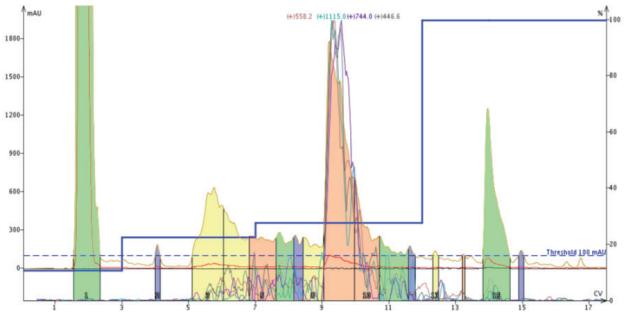

Figure 6. Conditions (left) for the attempted separation of a 20 amino acid peptide from a critical 19 amino acid impurity using a step gradient. With the gradient adjustments, the two compounds are fully resolved.

While adjusting the acetonitrile concentration appears to have the most significant role in the success of this purification, increasing the duration of each isocratic hold also contributes by allowing each of the two significant compounds to fully elute before another acetonitrile change occurs. This also serves to improve the recovery of highly pure peptide from the crude sample. Follow up analysis of the two fractions collected for the main peak (orange) indicate though, the presence of a trailing impurity not readily identifiable in the purification trace.

These results clearly demonstrate that even very small changes in acetonitrile concentration or step duration can have dramatic impact on the purification efficiency. Both the early eluting peak and the desired peak increase sharpness, suggesting that the acetonitrile has approached ideality for each compound. Further inspection of the mass spectrum within each peak confirms that the critical impurity, previously co-eluting, is now baseline resolved from the desired product, Figure 7.

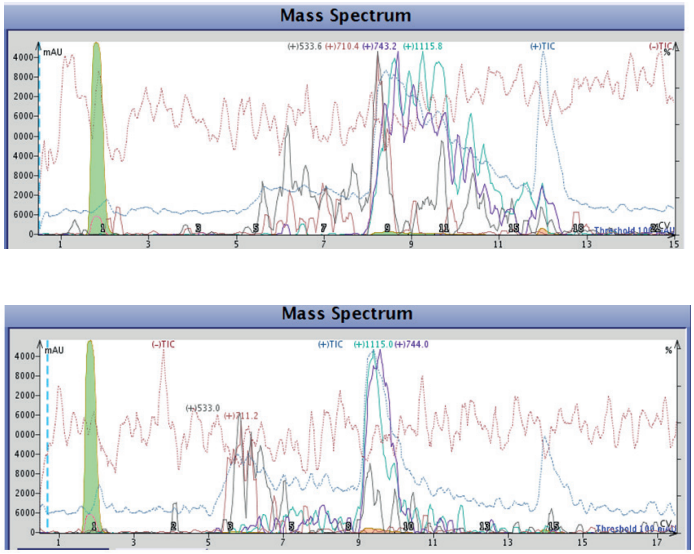

Figure 7. Mass spectral analysis for two step gradient-mediated purifications demonstrating how small changes in acetonitrile concentration can impact resolution. With concentrations held at 20% and 25% acetonitrile (top) the critical impurity, grey and red traces, coelute with a desired peptide, purple and teal traces, that bleeds off the column. Adjusting to 22% and 27% acetonitrile (bottom) enables sharp and full resolution of the critical impurity, red and grey traces, from the similarly sharply eluting desired peptide, purple and teal traces.

Adding Additional Steps

Given the presence of a late eluting, yet relatively significant impurity, a third isocratic hold step was installed for the final purification with the goal of maximizing pure peptide recovery. Each isocratic hold duration was maintained at five column volumes, carrying over the earlier observed peak elution start and ending points, Figure 8.

The acetonitrile hold concentration for the third step was informed from the initial step gradient purification where all compounds were eluted before the column could be equilibrated in 30% acetonitrile. Given that a one or two percent change in acetonitrile can have significant impact on the purification efficiency, 29% was chosen for the final step.

While there is not a third peak that separates distinctly from the other two peaks, there is a subtle transition in the peak shape, suggesting the beginning of a second compound eluting at the end of the major peak (Figure 8, labeled 7). This is further confirmed with analytical HPLC analysis of each isolated fraction, Figure 9. An alternative strategy could be to shorten the duration of the second isocratic hold step, forcing the late eluting impurity to elute during the column flush. Most importantly though, there is no evidence of the early eluting, critical impurity in any fraction collected here.

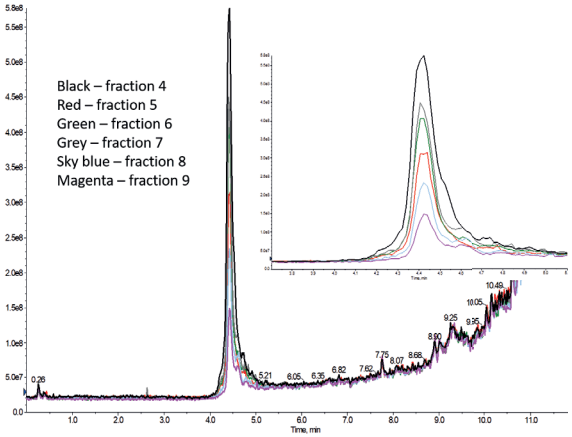

Figure 9. Analytical HPLC traces for fractions collected during purification of a 20 amino acid peptide sample using a step gradient containing three isocratic holds. Fractions 4–6 all contain peptide with >95% purity. The trailing impurity becomes evident starting with fraction 7 (grey trace), as suggested in the purification chromatogram. Inset shows an expanded view.

%B	Duration of hold (CV)
10	3
22	5
27	5
29	5
100	2

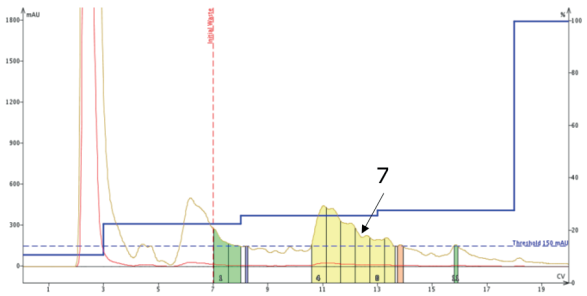

Figure 8. Purification of a crude 20 amino acid peptide using a step gradient containing three steps. Analytical HPLC conducted for each fraction indicates that the late eluting impurity begins to elute in fraction 7.

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Conclusion

The data presented herein demonstrates the use of step gradient to successfully separate a 19 amino acid impurity from the 20 amino acid desired product while maximizing recovery. Method development is often a time consuming, iterative process in which small changes are made, ultimately leading to a final optimal protocol. This optimization process required eleven total purification attempts, however, given the incredibly short purification times typically employed with flash chromatography, this was all completed in a single afternoon with the final analytical HPLC performed overnight. Importantly, the optimal step gradient identified can then be readily transferred for larger scale peptide purification. The short gradient times, high loading capacity, and now strategies to optimize a separation make flash chromatography an attractive tool for peptide purification.

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