Achieve Highly Pure Peptides with High Performance Flash Chromatography

Biotage®

Executive Summary

Peptides have garnered much attention in a variety of applications over the last few years, and interest only continues to grow. With improved synthetic strategies, peptides are now being synthesized in greater length and complexity than ever before. However, very few changes have been made when it comes to peptide purification, leading to a bottleneck in the overall peptide workflow. Peptide purification via flash chromatography has recently been demonstrated as a viable alternative to the more standard HPLC methods currently utilized. Flash chromatography offers peptide chemists the advantage of significantly greater loading capacity reducing the overall purification time but with a compromise of decreased peak resolution. Herein we present several strategies that, when implemented, allow for very high purity peptide samples purified by flash chromatography.

Introduction

Significant efforts have been invested in peptide research for a variety of applications. From a therapeutic standpoint, peptides fall somewhere between traditional small molecules and biologics with the potential advantage of high selectivity and specificity realized by biologics, but intracellular accessibility associated with traditional small molecule therapeutics[1]. Peptides though, have gone beyond the therapeutic entities and found utility in conjugated targeting moieties[2], intracellular delivery sequences[3], or even as the principle component of an encapsulation strategy[4], among others.

Given the ever broadening landscape of peptide-based applications and associated compound complexity, improved synthesis strategies have evolved[5,6,7]. Despite such synthesis improvements, synthetic peptides still require purification. As the number of compounds synthesized increases, purification quickly becomes a bottle neck in the overall synthesis workflow – significantly hampering progress from initial building-block starting materials to delivering the final purified peptide product.

Particle Size Impacts Resolution During Purification

Traditionally, peptides are purified using reversed phase chromatography. Separation occurs via a partitioning mechanism as compounds pass through a chromatographic column, in which an equilibrium interaction with the hydrophobic stationary phase and hydrophilic mobile phase is established with the peptide continually sampling interactions between each phase throughout the purification process. The equilibrium can be perturbed, yielding compound elution, only when the mobile phase composition changes to contain a sufficient percentage of organic solvent, Figure 1.

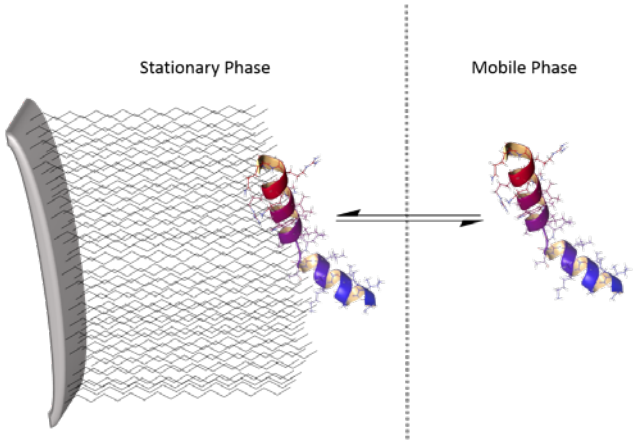


Figure 1. Peptides are purified via a partitioning mechanism in which sample components are constantly sampling interactions with the stationary phase alkyl chains and the mobile phase. Peptides are eluted from the stationary phase when the mobile phase contains a sufficiently high organic solvent concentration that the mobile phase interaction is more favorable than the interaction with the stationary phase.

The partitioning mechanism describes the process by which compounds interact with the stationary and mobile phases, but ultimately the purification depends on several other factors. Resolution, or the separation between two neighboring peaks, is affected by three critical factors: selectivity, efficiency, and retention, Equation 1.

$$R_S = \underbrace{\frac{\sqrt{N}}{4}}_{\text{Efficiency}} \times \underbrace{\frac{\alpha - 1}{\alpha}}_{\text{Selectivity}} \times \underbrace{\frac{k}{1+k}}_{\text{Retentivity}}$$

Equation 1. The resolution equation describes the relationship between column efficiency, selectivity, and retention where N is plate count, α is selectivity factor and k is the retention factor.

Both selectivity and retentivity factors can be modulated chemically by varying a number of parameters to improve resolution (see further discussion below), but the efficiency factor relates physically to the specific column and stationary phase particles, making it much more difficult to alter when seeking to improve sample resolution. The simplest approach to physically alter resolution is to increase the total plate count within a column. Plate count is inversely proportional to particle size, motivating the community to seek smaller and smaller stationary phase particles to increase separation capability through increased resolution. While extremely small particles serve analytical chemists well, making sample impurities readily identifiable and easily calculating purity, peptide chemists striving to purify and recover large sample quantities find themselves with a purification bottle neck, for with small particle size comes decreased sample loading capacity, Figure 2.

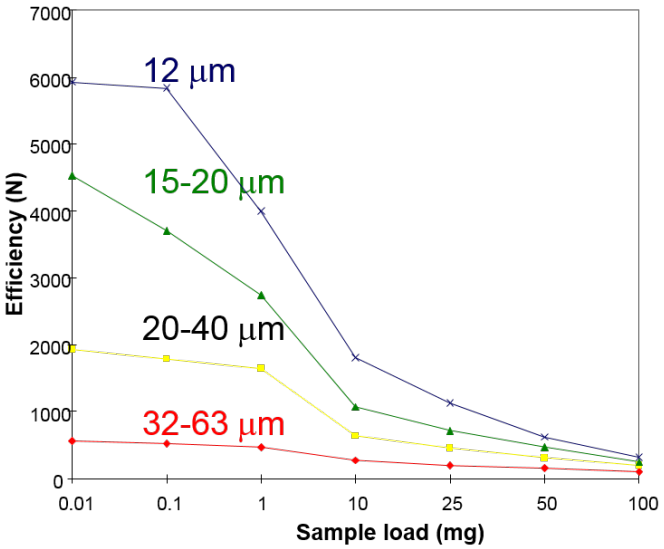


Figure 2. Separation efficiency, or plate count, with respect to sample load for a variety of different sorbent particle sizes. Although the plate count is extremely high for small sorbent particles, suggesting high resolution between sample peaks, with increasing sample loads plate count drops dramatically. Importantly, plate count is approximately equivalent, regardless of sorbent particle size, for high sample loads.

An alternative to traditional Reversed-Phase High Performance Liquid Chromatography (RP–HPLC) taking hold in the peptide community is High Performance Flash Chromatograph (HPFC). Reversed-Phase HPFC utilizes larger sorbent particles (approximately 20 µm vs. 5–10 µm), enabling significantly larger crude sample loads and reducing overall purification time. Despite the decrease in peak-to-peak resolution, HPFC has already been demonstrated to yield high purity peptides. For particularly difficult peptide separations, or when extremely high purities (›99%) are necessary, several unique approaches are discussed below that can be implemented to achieve highly pure peptide samples despite the loss in resolution.

Exploit the Peptide's Physiochemical Characteristics

The diversity of amino acid side chain functionality imbues peptides with their affinity, activity and even selectivity for a particular target. This great diversity however, can significantly complicate the purification effort if not carefully managed. Peak broadening, or even in extreme cases, complete peak splitting, has been observed during peptide purification primarily due to the equilibrium protonation states of the acidic or basic amino acid side chains present in the peptide product. For this reason, the mobile phase solvents contain a pH modifier that drives the peptide sample into a single protonation state.

While most peptide purifications occur with a mobile phase modified with a low concentration of either trifluoroacetic acid (TFA) or formic acid (FA) to achieve a mobile phase pH of 2–2.5, there are certainly alternative mobile phase additives that could be used as well. Take for example a peptide mixture containing oxidized (cyclic) and reduced (linear) oxytocin, a nine amino acid peptide cyclized via a disulfide bond. Although "standard" conditions with acidified mobile phase indicated that separation should be possible for this mixture by analytical HPLC, the loss of resolution due to the larger stationary phase particles resulted in an HPFC purification that was completely unsuccessful, Figure 3.

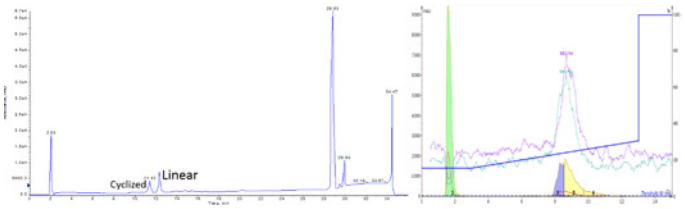


Figure 3. Analytical HPLC chromatogram for a sample containing both linear and cyclized oxytocin using standard, acidified mobile phase solvents (left) suggests a simple separation of the two components. Purification by HPFC using acidified mobile phase solvents and an optimized linear gradient however, proved unsuccessful (right).

Upon closer examination, it is clear that under acidic conditions, where the Cys sidechain thiol is protonated, the difference in apparent hydrophobicity between linear and cyclic oxytocin is small, leading to the poor separation. In order to improve the resolution, the selectivity of the stationary phase for either of the two compounds in the solution must change. Changing the mobile phase content, specifically the modifier, is a simple yet effective strategy to alter the selectivity of the stationary phase.

In this case, the presence of two reduced Cys thiols present an opportunity that can be exploited chromatographically. The pKa of the side chain thiol is about 8.34 which can be deprotonated with elevated pH, dramatically altering the net charge and chromatographic behavior of the linear peptide, Figure 4.

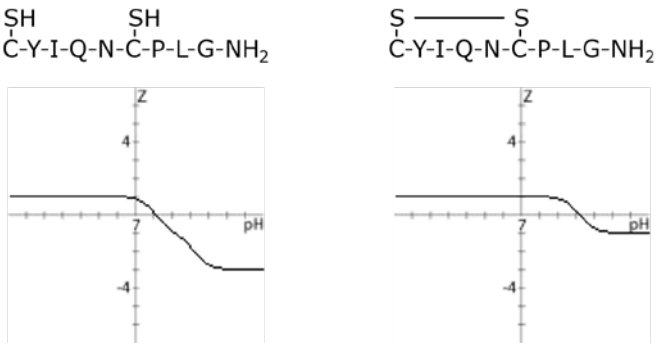


Figure 4. Net charge of linear oxytocin (left) or cyclized oxytocin (right) at varying pH levels. Under acidic conditions (pH 2-2.5), the compounds carry the same net charge, making separation very difficult with large sorbent particles. Under basic conditions (pH ~10) though, a difference in net charge is observed for the linear peptide (Z = -2) when compared to the cyclized peptide (Z = -1) which alters selectivity for the stationary phase and improves the separation.

When mobile phase solvents were modified with 0.1% ammonium hydroxide, to a final pH of approximately 10, the previously unresolvable linear and cyclic peptide components are now separated by more than ten column volumes, Figure 5.

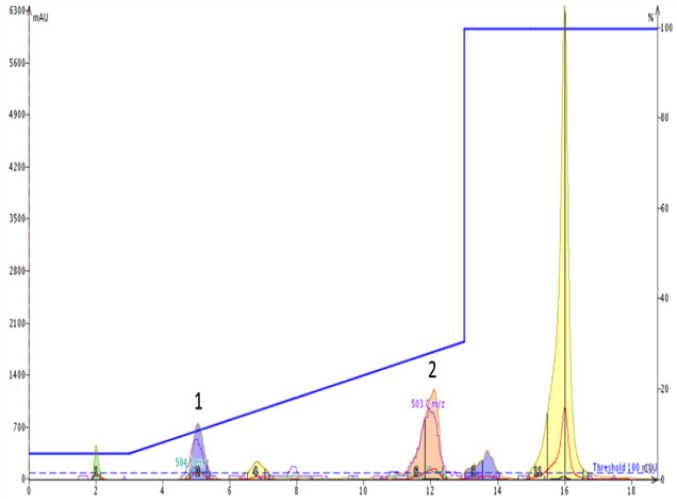
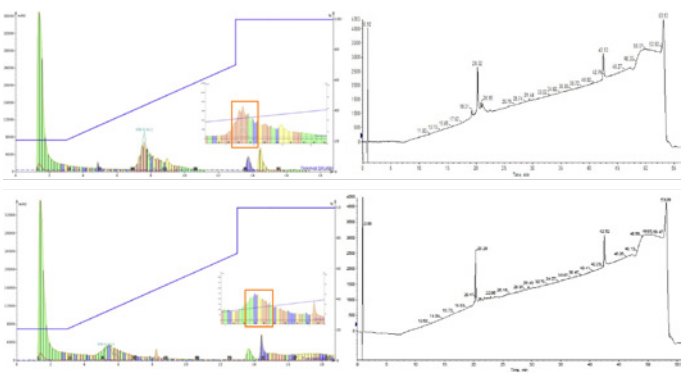


Figure 5. HPFC chromatogram generated during the separation of linear oxytocin (peak 2) from cyclized oxytocin (peak 1) using mobile phase modified with 0.1% ammonium hydroxide, bringing the solution to pH = 10. Under basic conditions, the two peptides are separated by greater than 10 column volumes as predicted by the net charge vs. pH plots.

Interestingly, the linear, deprotonated, peptide is retained by the C18 stationary phase to a greater extent than the cyclized peptide. This is likely due to an ion-pairing interaction between the Cys thiolate anions and ammonium cation counter ions present in the mobile phase.

The case of oxytocin is rather extreme, but the same strategy can also be applied to peptides of greater length with similar results. For example, when purifying GLP-1, a 37 amino acid peptide, by HPFC with acidified mobile phase solvents, the peptide is eluted in approximately eight column volumes to 64% final purity after combining fractions (top inset, orange box). However, if the same peptide is purified using basic mobile phase solvents, elution occurs within five column volumes and, after combining fractions, to greater than 85% final purity (bottom inset, orange box), Figure 6.



The most critical factor to consider for successfully improving the purification by altering stationary phase selectivity with mobile phase modifiers in peptides of significant length like GLP-1 is the isolectric point (pI). In the case of GLP-1 (pI = 5.35), adjusting the pH to 2 with acidic modifiers ensures a fully protonated peptide while a pH of 10 with basic modifiers ensures a deprotonated peptide, Figure 7.

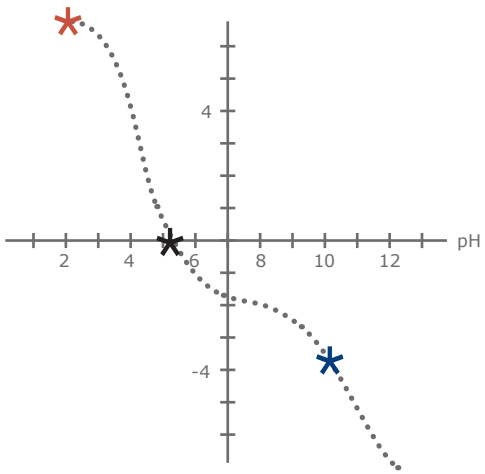


Figure 7. Net charge of GLP-1 (1-37) under varying pH levels. Net charge at pH 2 (red asterisk), pH 10 (blue asterisk) and zero net charge (black asterisk) are highlighted.

If the peptide's pI is less than two pH units removed from the mobile phase pH, an equilibrium distribution of protonation/deprotonation states for each ionizable side chain will occur as discussed above, increasing the purification difficulty rather than simplifying it as desired.

NOTE

Before exploring the utility of this technique, it is critical to understand the bonding chemistry used for the manufacture of your specific HPFC cartridge. As with most HPLC columns, the bonding chemistry may (or may not) be stable to all pH ranges. If exposed to a pH outside the stable range, you risk compromising the integrity of the stationary phase. Practically, a release of the silica-bonded alkyl chains occurs, causing decreases in loading capacity, decline in peak-to-peak resolution and potential for contaminating the purified final peptide sample with the released alky chain (impossible to detect by UV). Biotage® SNAP Bio cartridges are tested at high and low pH for alkyl chain stability as part of the quality assurance process. If the cartridges are properly stored after use with high concentrations of un-modified organic solvent (acetonitrile or methanol), switching between mobile phase pH extrema as described above will not harm the integrity of the stationary phase.

Figure 6. Purification of GLP-1 (1–37) with crude purity of approximately 26% by HPFC. The mobile phase solvents were modified to either acidic conditions (top panels) or basic conditions (bottom panels). Under equivalent sample loads and using the same linear gradient, greater purity is achieved with a base-modified mobile phase.

Utilize Multiple Stationary Phases Simultaneously

An alternative to changing the mobile phase additive composition is to include a second stationary phase for the purification, similar to strategies used for shotgun proteomics experiments. In shotgun proteomics, a protein sample (sometimes as complex as whole cell lysate), is subjected to proteolytic digestion, yielding a multitude of peptide fragments, followed by multidimensional chromatography directly coupled to mass spectrometry for individual peptide/protein identification. In a strategy pioneered by Yates and colleagues, MUDPit incorporates both strong cation exchange and reversed phase sorbents to alter the stationary phase selectivity. This decreases the complexity of the "sample mix" entering the mass spectrometer and simplifies the identification process.

Unfortunately, I don't have access to two, truly orthogonal stationary phases compatible with the Isolera™ purification system, but there are two reversed-phase stationary phases available with significantly different functionality – a C4- and a C18-based sorbent – and fortunately the cartridges can be connected via the outlet and inlet luer fittings, Figure 8.

The alkyl chain length impacts stationary phase selectivity in two principles ways: 1) the longer the alkyl chain, the greater the apparent hydrophobicity presented to the sample during purification and 2) a shortened alkyl allows for greater influence of the polar, silica stationary phase. Although C18 is among the most common stationary phase choice for peptide purification, C4 is also used, particularly for hydrophobic peptides.

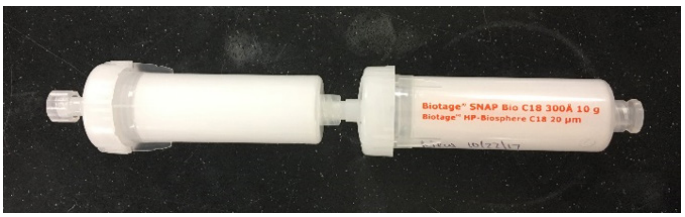


Figure 8. Rather than constructing a whole new column, two Biotage® SNAP cartridges can be connected via the outlet and inlet luer fittings.

The differences in stationary phase selectivity becomes clear when purifying 18A, an amphipathic 18 amino acid peptide. Purifying an aliquot of crude 18A using a Biotage® SNAP Bio C18 cartridge was quite successful, Figure 9.

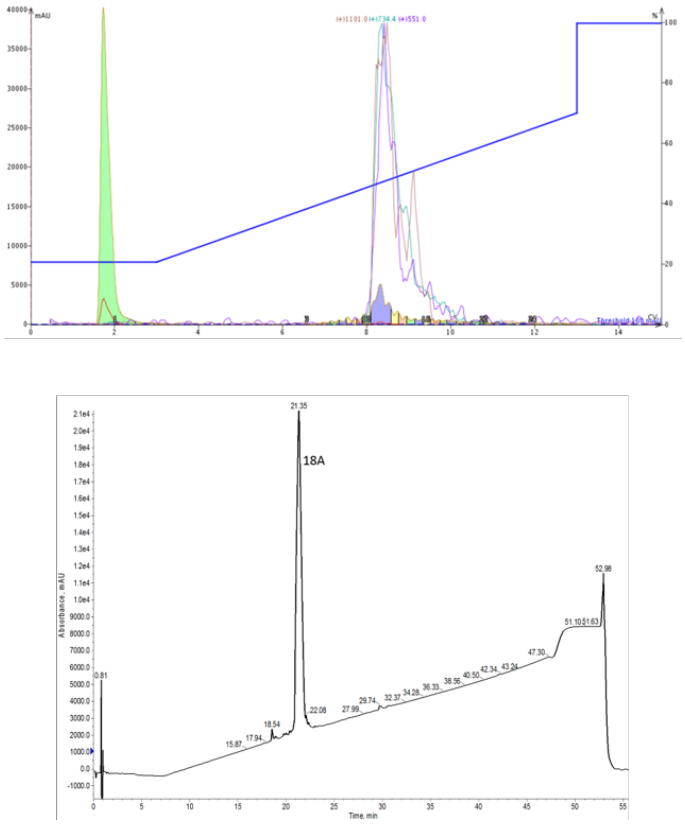


Figure 9. Purification of crude 18A using C18-functionalized SNAP Bio cartridge (top). Concentrating the main fraction (purple) yielded peptide >95% pure by analytical HPLC (bottom).

The principle impurity remaining in the analytical HPLC elutes earlier than the desired 18A peptide, indicating that the impurity is more hydrophilic that the desired 18A peptide. Purifying and equivalent aliquot of crude 18A using a Biotage® SNAP Bio C4 cartridge using the exact same gradient as that used for the C18-mediated purification was also relatively successful, Figure 10.

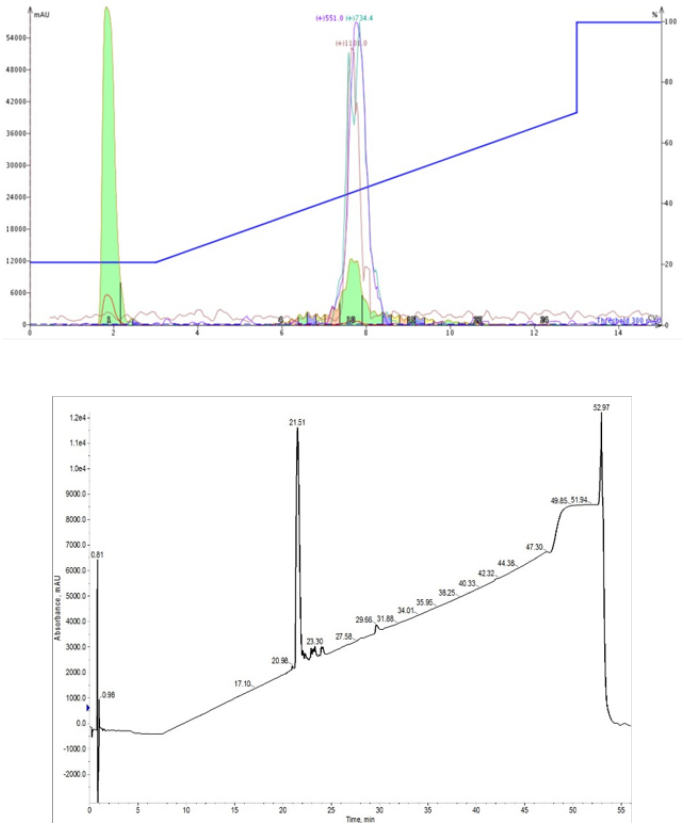


Figure 10. Purification of crude 18A using C4-functionalized SNAP Bio cartridge (top). Concentrating the main fraction (green) yielded peptide >90% pure by analytical HPLC (bottom).

In this case, the remaining impurities in the analytical HPLC elute later than the desired 18A peptide, indicating greater hydrophobic content when compared to the peptide product.

A closer examination of the individual purification chromatograms highlights these differences further, Figure 11.

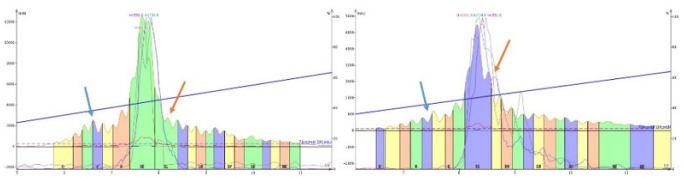


Figure 11. Comparing purification efficiency for crude 18A when purified using C4 (left) or C18 (right) functionalized stationary phase. An early eluting (hydrophilic) impurity is observed for the C4-mediated purification by is absent in the C18-mediated purification, highlighting the differences in stationary phase selectivity.

The first and most obvious difference is the change in retention of the product peak. As expected, 18A is less retained by the C4-functionalized stationary phase than by the C18-functionalized stationary phase by approximately 1 column volume. Despite the earlier elution of 18A, the hydrophilic impurities co-eluting with 18A when purified with SNAP Bio C18 are clearly resolved by the SNAP Bio C4 stationary phase (blue arrows). The more hydrophobic, later eluting impurities though, are more clearly resolved by the SNAP Bio C18 (orange arrows).

Although both cartridges perform well individually, the noted differences in selectivity may allow for improvements in the final purity. The question is, which order to connect the cartridges for the best purification outcome? To determine this, the cartridges were connected in-line by way of the luer fitting cartridge inlet and outlet and an equivalent aliquot, using the same gradient as above, was purified with the new “dual cartridge” with either the SNAP Bio C18 cartridge or the SNAP Bio C4 cartridge first.

For an amphipatic peptide like 18A, the cartridge order absolutely impacts the final purity. When the peptide was purified using the C4-C18 cartridge configuration, the leading impurities resolved by the C4-functionalized sorbent were again poorly resolved, reminiscent of the C18-mediated purification. Importantly though, the trailing hydrophobic impurities were still resolved, even to a slightly greater extent, than with the C18-functionalized sorbent alone, Figure 12.

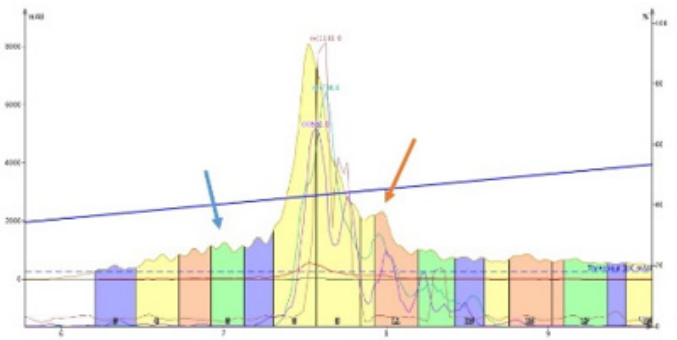


Figure 12. Mixed stationary phase purification of 18A using two cartridges connected C4-to-C18 in line. The early eluting impurity previously observed by the C4 alone mediated purification is no longer observed (orange arrow), suggesting that the selectivity improvement is not sufficient to overcome the resolution loss observed in the C18 alone purification. Importantly, resolution of the hydrophobic impurity is retained (blue arrow)

This suggests that the selectivity of the C4 cartridge is not sufficient to overcome the poor selectivity for the hydrophilic impurities with the C18 stationary phase when encountered first by the sample. However, if the cartridge order is reversed, with a C18-C4 configuration, resolution of the hydrophilic impurities is restored to that reminiscent of the C4-containing cartridge alone. Contrary to the above pattern, the hydrophobic impurity is also still resolved, Figure 13.

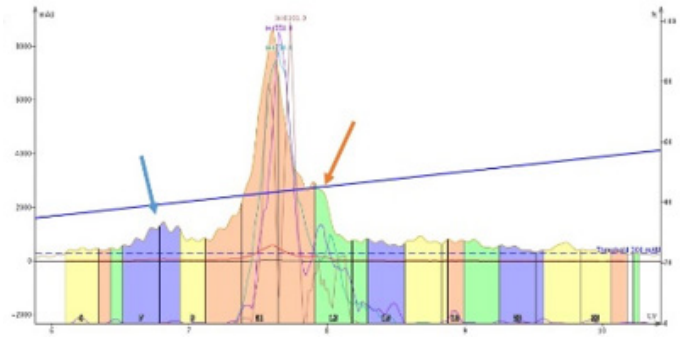


Figure 13. Mixed stationary phase purification of 18A using two cartridges connected C18-to-C4 in line. Resolution of the hydrophilic impurity has been restored (orange arrow). Interestingly, resolution of the hydrophobic impurity is also retained (blue arrow), suggesting that retention of the hydrophobic impurity by the C18 stationary phase is sufficient for efficient separation, regardless of cartridge order.

The combined difference in selectivity, paired with a proper cartridge orientation yielded 18A with >98% final purity, despite the large media particle size and low pressure system.

A Step Gradient Alternative to the Linear Gradient

The most common strategy for altering resolution during a purification, regardless of the purification strategy, is to change the gradient slope. More often than not, changing the gradient involves decreasing the slope of the linear gradient, increasing the time spent at each specific concentration of strong solvent (acetonitrile in this case) during the purification. While this strategy works pretty well for RP-HPLC methods, it doesn't work well when applied to RP-HPFC, Figure 14.

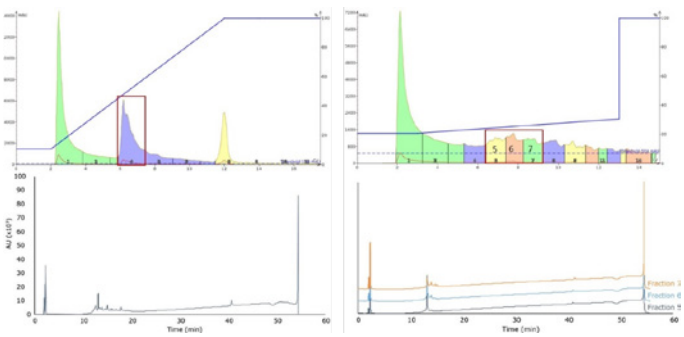


Figure 14. Purification efficiency is also dictated by gradient design. Efficiency is often poor if too steep of a linear gradient is used for the purification (left panels), but decreasing the gradient slope often results in peak broadening, decreasing the ability to correctly identify the desired peptide peak and compromising total peptide recovery from the purification (right panels).

There seems to be a fine balance between a steep gradient slope - yields sharp peaks, but poor resolution and purification efficiency – and a shallow gradient slope – yielding significant peak broadening, compromised sample recovery and difficulty identifying the product peak. So then where do you begin when purifying something using HPFC?

After purifying hundreds of different peptides with varying length, amino acid content, and crude purity I have settled on a basic preliminary gradient framework as follows:

1. Run an analytical HPLC of the crude peptide. This serves two purposes:
   a. Lets you know if the desired peptide is actually present in the crude mix and
   b. Gives you an idea of the acetonitrile concentration at which the desired peptide elutes
2. Build a gradient that starts at least 15% acetonitrile below the elution concentration and runs a total of 40-50% acetonitrile (total change) over 10 column volumes.

As an example, crude ACP(65-74) elutes approximately 25% acetonitrile, so the gradient I use when purifying this peptide by RP-HPFC runs 10% to 50% over 10 column volumes. This gradient is somewhat steeper than the more standard HPLC gradients, but it seems to balance nicely with the resolving power of the HPFC particles and generally yields pretty pure peptides.

There are certainly cases though where this type of gradient does not provide sufficient purity in a single purification, Figure 15.

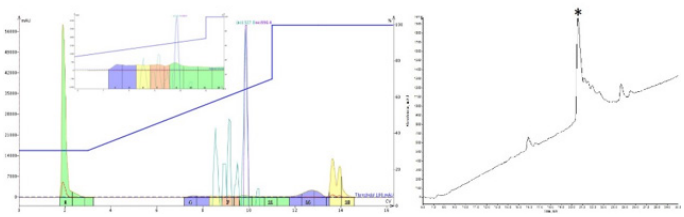


Figure 15. Purification of a 33 residue cationic peptide using a linear gradient constructed using data from the analytical HPLC evaluation. Using this unoptimized gradient, a final purity of only 60% was achieved.

Rather than decreasing the slope of the initial gradient, I decided to use the selectivity information gleaned from the linear gradient to build a step gradient. Step gradients are commonly used in normal phase chromatography as a strategy to decrease the purification time and total solvent consumption.

There are several questions that immediately arise when the decision is made to program a step gradient for peptide purification.

- What initial acetonitrile concentration should I use?
- What acetonitrile concentration will allow me to elute hydrophilic impurities, but not my peptide?
- What acetonitrile concentration will elute my peptide, but not any hydrophobic impurities?
- How long should each step acetonitrile concentration step be held to ensure complete elution?

Fortunately, the Isolera™ or Isolera™ Dalton 2000 provide answers to these above questions using the Optimize feature together with a linear gradient result, Figure 16.

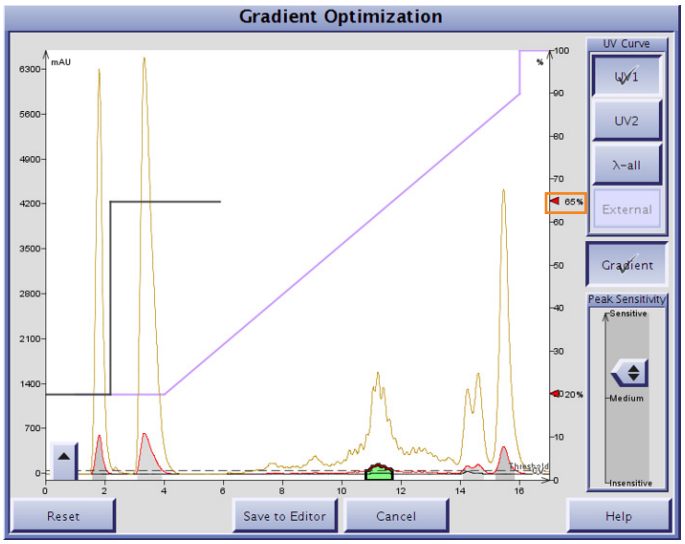


Figure 16. The Optimize feature of within the Isolera™ Dalton 2000 software allows a user to select a peak of interest (product or impurity) and then automatically creates an optimal step gradient for purification of the selected peak.

From the Optimize window, you can select the peak of interest, whether it be your desired product or one of the neighboring impurity peaks, using any of the wavelengths defined in the linear gradient, and increase or decrease the sensitivity, allowing the software to populate an elution acetonitrile concentration (Figure 16, orange box). The step gradient information can then be imported directly into the method builder for the next purification.

Selecting the desired peptide peak and programming a single step purification (naively) as suggested by the software, does show some improvement in final purity over the linear gradient, Figure 17.

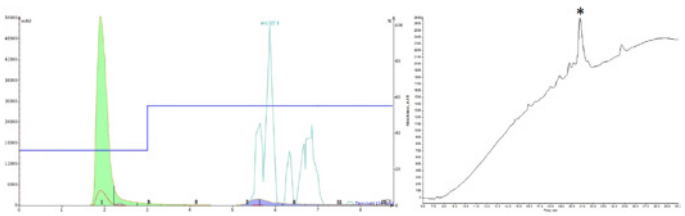


Figure 17. Purification of a 33 residue cationic peptide using a step gradient containing a single step, as directed by the Isolera™ Dalton 2000 Optimize software feature. Although the crude purity has increased when compared to the linear gradient purification, hydrophilic impurities now co-elute with the desired peptide product.

This level of purity is certainly not sufficient to move forward with experimentation though. What is clear though is that this type of gradient collapses any resolution between the desired product and any early eluting, hydrophilic impurities.

Incorporating an early intervening step will allow the early eluting, hydrophilic impurities to elute while retaining the desired peptide. The Optimize feature will allow a user to select any features in the reference gradient, providing guidance as to what concentration of strong solvent should be used for an early step. The subtly of gradient design and strong solvent concentration becomes clear as the optimization process proceeds. Changing the strong solvent concentration to elute the desired peptide only a few percentage points, guided by selecting the segment of the product peak where the peptide begins eluting, can increase the final purity significantly, Figure 18.

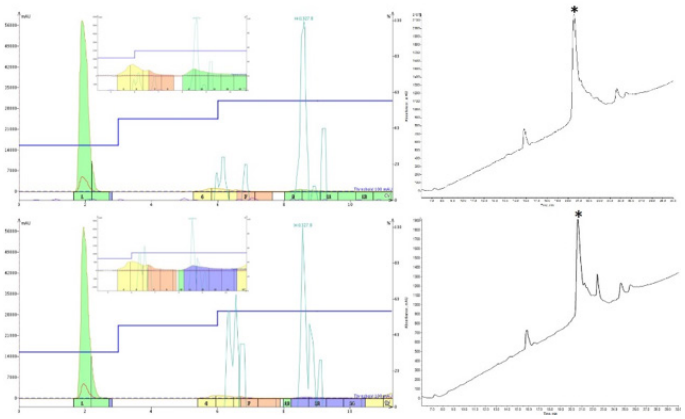


Figure 18. Improving purity by increasing the number of steps within the gradient. Incorporating an early step at 45% MeCN before the desired peptide elution concentration of 55% MeCN improved final purity of the 33 residue peptide from 60% (linear gradient) to 70% (top right panel). Decreasing the level of the final peptide elution step to 53% MeCN further improved final purity to 74% (bottom right panel).

The greatest improvement was observed after incorporating a third step, specifically targeting the hydrophobic, late eluting impurities, Figure 19.

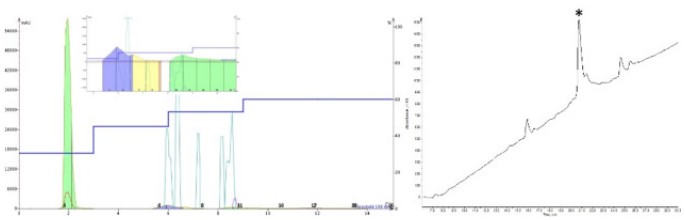


Figure 19. A final purity of 89% was achieved by incorporating an additional elution step, bringing the total to three steps and a total purification time equivalent to that required for linear gradient about 22 minutes). Importantly, adding the final step after the desired peptide's elution maintained well-defined peaks and prevented co-elution of the hydrophobic impurities with the desired peptide.

The extra step sharpens the elution of the desired peptide somewhat, increasing the overall recovery and preventing a long, slow elution from the cartridge which allowed the hydrophobic impurities to begin to co-elute. Ultimately, switching the gradient from an optimized linear gradient to an optimized step gradient enabled a significant increase in the final sample purity significantly from about 60% purity (linear gradient) up to about 89% final purity (step gradient). This final purity is now approaching purities sufficient for experimentation. Although this process required four purifications to identify an optimized step gradient, each purification required 20 minutes or less total time, increasing the peptide workflow efficiency.

The Optimize feature was originally designed for use in normal phase purifications of small molecules, so there are several things to keep in mind when programming a reversed phase gradient for peptide purification. First and foremost is the length of each step. I have found that a minimum of three column volumes is required for the cartridge to fully equilibrate in the solvent conditions and fully elute the compound(s) of interest. It is important to note that the Isolera has a tendency to use a starting condition that will not allow the peptide to be retained by the column. As with a linear gradient, it is recommended to use initial conditions 5% to 20% below the acetonitrile concentration required for elution.

Conclusions

Research interests involving the use of peptides for a wide variety of applications continues to grow. As research programs expand, the well-known purification bottle neck in peptide groups restricts progress. The use of High Performance Flash Chromatography (HPFC) can in fact reduce the total purification time, moving peptide-based projects forward more efficiently. Although larger spherical particles allow for high loading capacities, there is a compromise in resolution. Herein we demonstrate strategies that allow for high final purity of complex crude peptide mixtures requiring minimal additional effort and allowing users to take full advantage of the increased loading capacity and subsequent reduction in purification time.

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