Improving Efficiency for Peptide Purification: Application of Heat and Focused Gradients at Preparative Scale

Peptide purification is recognized as a major challenge due to the subtle differences between the desired target and synthetic impurities. Therefore, new tools to improve purification performance and efficiency have the potential to provide significant benefits.

Below, we explain the benefits of heating combined with focused gradients for preparative-scale chromatography of peptides.

Why use heating for chromatography?

Most analytical chromatography systems have incorporated column heating for many years. This is largely due to the recognized benefits that heating can often bring to chromatography for improving resolution. By improving chromatographic resolution, additional benefits can be potentially obtained, such as achieving faster purification times and generating less chromatographic waste.

At the smaller scale range of analytical chromatography, heating is relatively straightforward to apply due to smaller column IDs and relatively low flow rates. Efficient heating is generally implemented by heating the outside of the column as well as a short section of tubing on the inlet side of the column (in order to pre-heat the mobile phase). However, at preparative scales where larger column IDs and higher flow rates are used, sufficient heating of the mobile phase and internal dimensions of the column is more difficult and requires a more sophisticated means of mobile phase pre-heating.

Due to the added difficulty, heating is not often used in preparative chromatography, which contributes to decreased performance and efficiency. However, with the introduction of the Prodigy™ peptide purification system, which includes an integrated heating system (column oven and mobile phase pre-heater optimized for high flow rates), efficient heating at preparative scales is easy and straightforward.

Key Advantages of Heating

- Improved Resolution
  Elevated temperatures increase column efficiency and improve peak shapes, resulting in better resolution. This is especially useful for separating closely related compounds or resolving complex mixtures (shown in **Figure 1** on page 3).
- Faster Purification
  Improved column efficiency can allow for the use of shorter columns which results in significantly shorter chromatography methods. Additionally, reduced backpressure enables the use of higher flow rates, which further reduces method run times.
- Less Waste
  The ability to use shorter chromatography methods reduces the amount of solvent used.
- Improved Reproducibility
  Standardizing temperature conditions makes HPLC/UHPLC run results more reproducible, improving accuracy and precision in the analysis.

Why use a focused gradient at the preparative scale?

When purifying compounds at the preparative scale, it is desirable to load higher mass amounts onto the column. This can present increased challenges for overall resolution and make it more difficult to resolve closely eluting impurities. One option to address this is the use of a shallower gradient throughout the entire run, which can improve chromatographic resolution. However, this approach comes at the expense of both increased run times and waste generated.

Alternatively, the use of a focused gradient provides a shallower gradient only at the critical region of the chromatogram where the target peak is eluting. This improves resolution without increasing run times or waste generated (shown in **Figure 2** on page 3).

Purification Case Study

To illustrate a typical workflow using the Prodigy system, we synthesized a 12-mer antimicrobial peptide known as Holothuroidin I (sequence: HLGHHALDHLLK) on the Liberty Blue™ microwave peptide synthesizer.

We then dissolved the crude peptide to ~12 mg/mL in 10 % acetonitrile, and then filtered the sample using a regenerated cellulose syringe filter.

Next, we created a screening gradient (5 – 70% acetonitrile, with 0.1% TFA) on our analytical systems so that we can determine the overall purity and to help determine the best conditions for scale-up. For comparison, below are the results from equivalent gradients (and both operating at 60 °C) on both a Waters ACQUITY UPLC® system as well as an Agilent 1260 HPLC system (shown in **Figure 3** on page 3).

As expected, the chromatography appears identical, but the UPLC method is significantly shorter.

So now that we have completed the screening run, the next step is to create a focused gradient and scale-up the method to the Prodigy system. You could attempt to calculate the method manually, or try to find a gradient calculator from a third-party, but there is no need. Embedded in the Prodigy system software is a Focused Gradient Calculator that's simple to use and can help you create the optimal focused gradient method for purification.

Once you have opened the calculator (shown in **Figure 4** on page 4), follow these steps:

Step 1

Enter the approximate amount of the sample you want to purify. We will just put in 100 mg since that's the approximate crude recovery of a 0.1 mmol synthesis of our 12-mer peptide.

Step 2

Enter the retention time of the target peak from the analytical run. Looking at the UPLC screening run, we have determined the retention time to be 1.88 min.

Step 3

Choose the appropriate preparative column from the available list. We'll choose a 19 x 150 mm column dimension since that tends to work well for loadings up to 100 – 150 mg. Also, it is important to remember that the preparative column phase must match that of the analytical column.

Note

The % Loading (this is a value related to the injection amount relative to the preparative column size) will be calculated and can help guide the proper selection of column size. For initial injections and particularly for injections of lower purity peptides, it is not recommended to exceed ~0.5% loading.

Step 4

Select Calculate. The calculator will now display the ideal run conditions at the preparative scale. We can see that the ideal focused gradient should go from 13.6 to 20.6 %B over a period of 14 minutes, and at a flow rate of 27.8 mL/min.

The Settings tab on the calculator is where information about the analytical system (dwell volume, column information) and methods is located. There are also options to add additional inputs in order to generate even more detailed preparative method conditions.

After creating a method on the Prodigy using the ideal run conditions, and equilibrating the system at 60 °C, we injected a small amount of sample (~6 mg) in order to demonstrate the improved resolution of the focused gradient run as compared to the screening gradient on the analytical system. As shown in **Figure 5** on page 4, you can see improved resolution of the three main groups of impurities.

Next, we made two larger injections of 45 mg and 90 mg (shown in **Figure 6** on page 5). With the larger injections, there is a loss of some resolution between the main peak and the impurity peaks. But to determine the ideal loading for this peptide, fractions across the target peak should be analyzed using the analytical system.

The fraction analysis revealed similarly high purity fractions for both injection amounts, suggesting that the loading could likely be increased further.

When the fractions were pooled, we found that the overall purity for both injections was 94% and the recoveries were 86% (45 mg injection) and 70% (90 mg injection).

You can see that by utilizing the Prodigy approach, which combines heated chromatography with an intuitive way to determine ideal focused gradients, you can easily and efficiently accomplish improved peptide purifications.

Conclusion

Throughout this application note, we delved into the advantages of heated chromatography, such as improved separation, increased column efficiency, faster separation, and improved reproducibility. We also examined the process of transitioning from analytical to preparative chromatography using heated systems like Prodigy and how focused gradient calculators play a crucial role in optimizing preparative runs by providing valuable method parameters.

As you continue to explore chromatography techniques, consider the benefits of implementing heated preparative systems and focused gradient calculators to maximize your preparative purification efficiency. If you have any questions about the Prodigy system, focused gradient calculator, or need further assistance, please feel free to contact our support team at synthesis.support@cem.com or visit our instrument page at cem.com/prodigy.

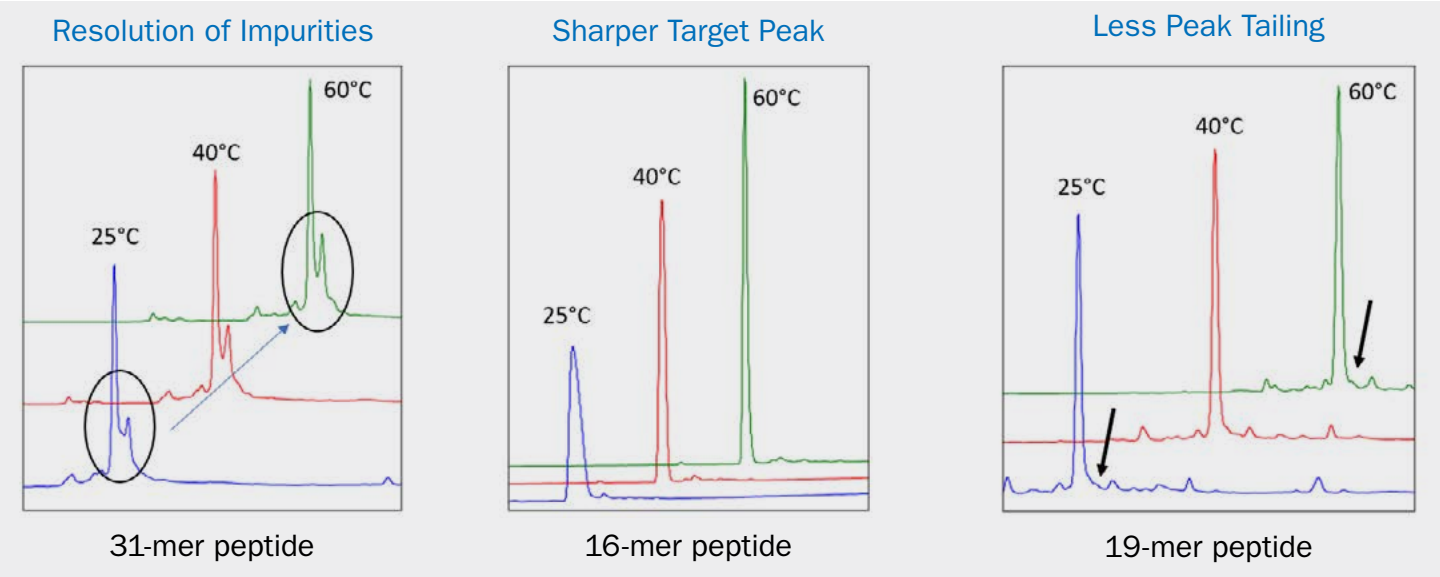

Figure 1. Impact of Increased Temperature on Overall Peak

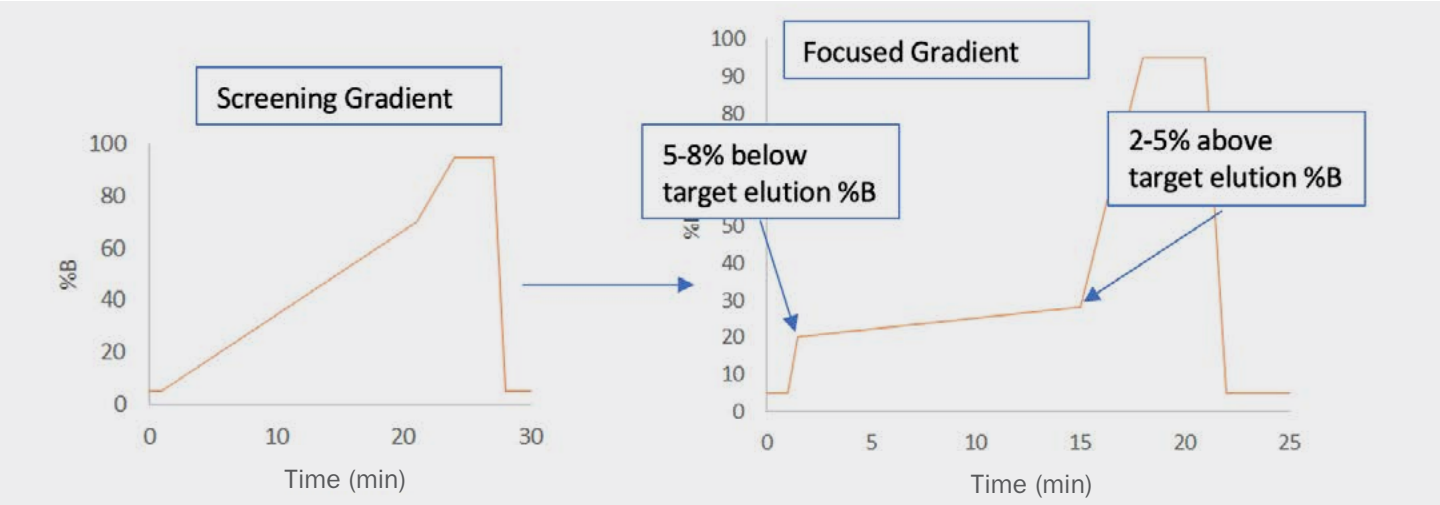

Figure 2. A Comparison between Screening Gradient and Focused Gradient

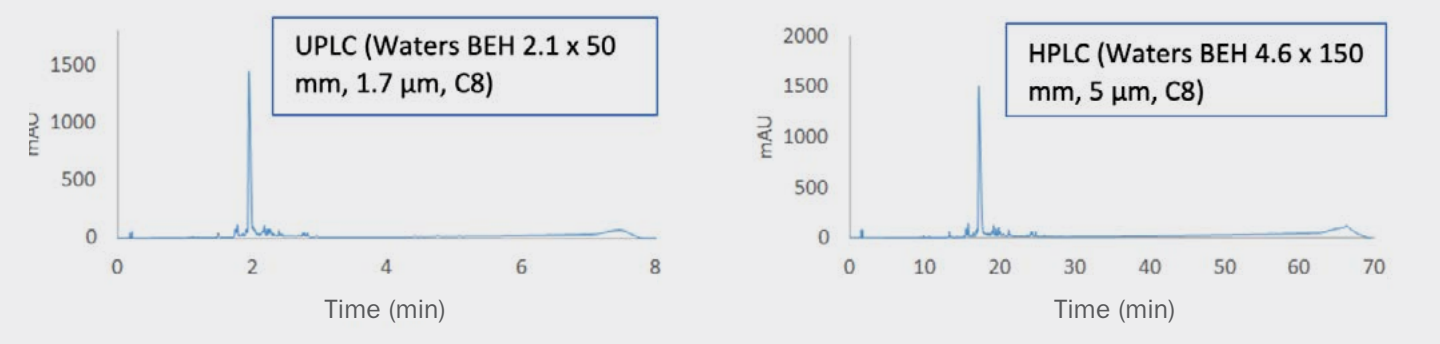

Figure 3. A Comparison of Equivalent Gradients Run on UPLC and HPLC

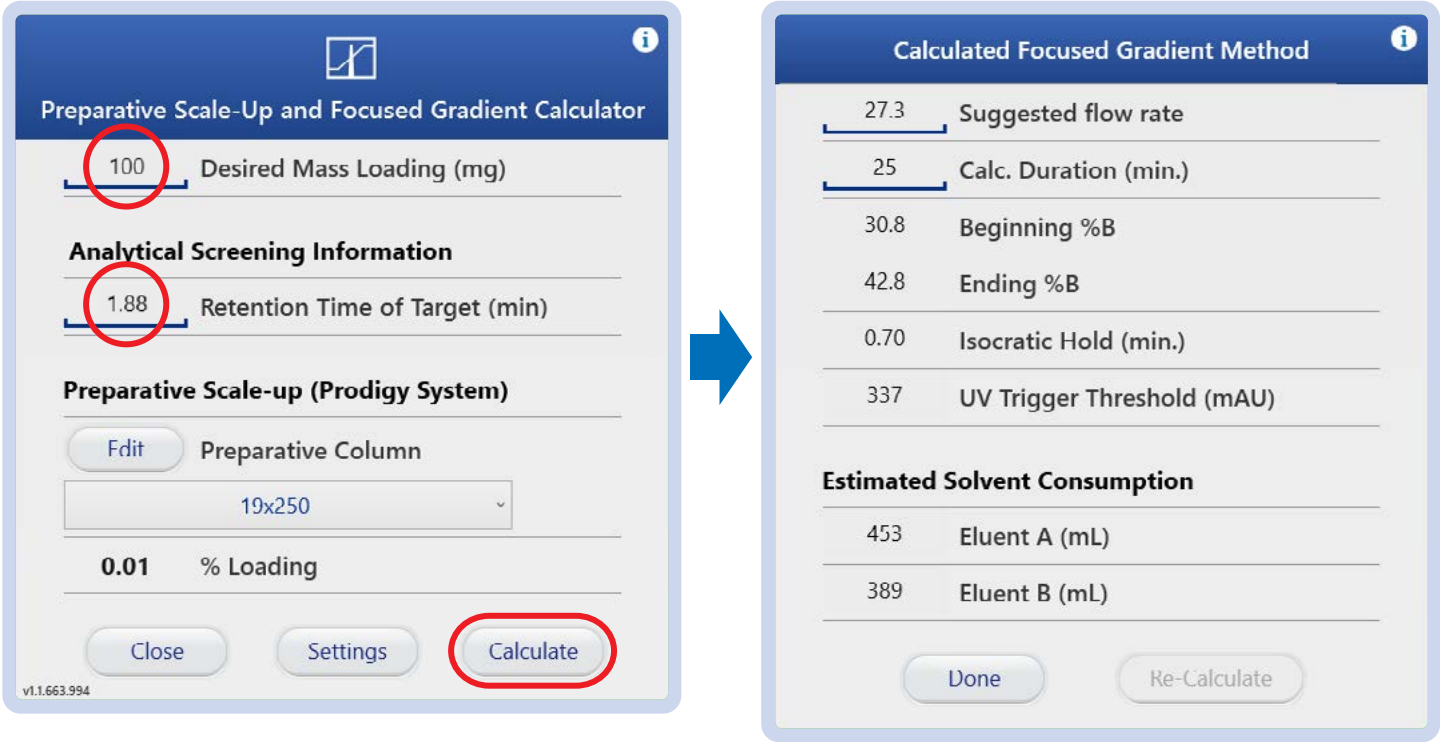

Figure 4. Screenshot from the Prodigy's Focused Gradient Calculator

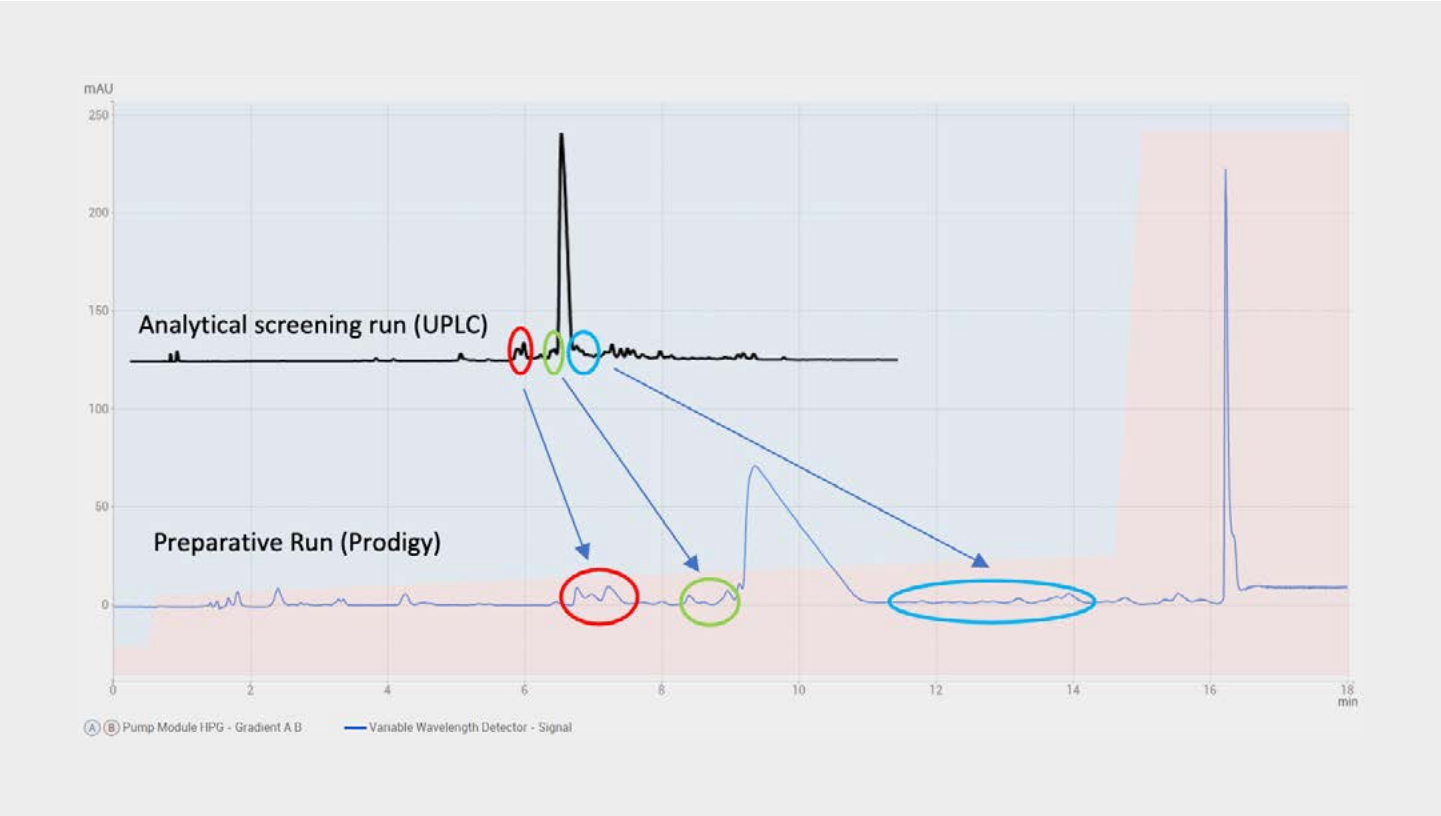

Figure 5. Enhanced Resolution through Focused Gradients and Heated Chromatography

Figure 6. Fraction Analysis of Higher Sample Injections

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