TECHNIQUES FOR IMPROVING ISOLATION OF SYNTHETIC PEPTIDES

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INTRODUCTION

Peptides have many biological functions and are essential for the research and development of pharmaceuticals. Even though the synthesis and cleavage of the peptide is carefully controlled, numerous impurities are generated. Impurities include deleted and truncated sequences, cleavage adducts, incomplete deprotections, and modified amino acids. All of these contaminants must be removed from the target peptide for unambiguous results in future experiments. Several approaches are available to adjust the purification process for improved yield of pure material. Some peptide mixtures are difficult to dissolve and keep in solution throughout the isolation process. Solvents like dimethylformamide or dimethylsulfoxide are good for dissolving samples but their use can jeopardize the chromatographic purification. The Waters patented at-column dilution technique, used in conjunction with temperature control and focused gradients, improves chromatographic resolution, column mass capacity, and purification system ruggedness by preventing sample precipitation. Controlling the temperature of the isolation at the large scale vastly improves the purification of the product by improving the peptide's solubility in the mobile phase and increasing efficiency. Focused gradients give better resolution of the peptide product from its closely-eluting contaminants without increasing run time. In this study, we illustrate the use of at-column dilution, temperature control and focused gradients for improving synthetic peptide isolation. Employing these techniques ultimately lead to improved process efficiency and peptide products with higher purity and increased yield.

EXPERIMENTAL DESIGN

Steps

- Use a focused gradient with temperature control to develop a separation for a hydrophobic peptide
- Apply optimized at-column dilution loading to increase the mass capacity on the column

Instrumentation

LC System: Waters® 2525 Binary Gradient Module, 2767 Sample Manager, Column Fluidics Organizer, 2996 Photodiode Array Detector, ZQ™ Mass Spectrometer, Water Bath

Column: XBridge™ BEH 130 PREP C_{18} OBD™ Column 19 x 100 mm, 5 µm

Mobile Phase A: 0.1% TFA in water
Mobile Phase B: 0.08% TFA in acetonitrile

FOCUSED GRADIENTS

Chromatographic separations for isolation and purification are governed by the same physical and chemical principles as analytical separations. In prep experiments, however, scientists isolate target sequences at higher mass loads, often on large columns, and require better resolution to enhance purity and recovery of the collected peptides. Although creating a shallower linear gradient is a good first approach to enhancing resolution, changing the gradient slope for the whole separation leads to broader peaks and an increase in total run time. Segmented gradients are very shallow where the target peptide elutes, yet the other segments of the separation are preserved with relatively little change from the linear gradient. Focused gradients decrease the gradient slope for only that portion of the chromatogram that needs increased resolution. The resolution of peaks eluting earlier or later than the focused segment of the gradient is sacrificed in the interest of increasing the resolution of the separation around the target peptide without increasing the total run time of the isolation. Figure 1 graphically illustrates the difference between linear, segmented, and focused gradients. Figure 2 shows how focusing the gradient decreases the run time.

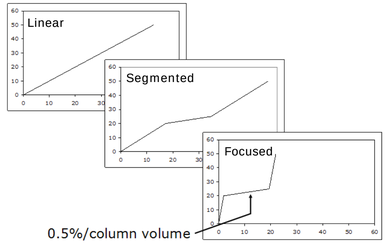

Figure 1. From top to bottom, a graphical representation of a linear gradient, a segmented gradient, and a focused gradient.

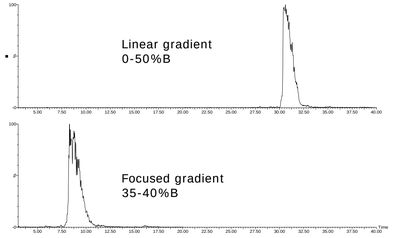

Figure 2. Focusing the gradient elutes the peptide in reduced run time. The top trace is the linear gradient; the bottom trace is the focused gradient.

TEMPERATURE CONTROL

Temperature control is most often used for delicate separations, separations where sample solubility is less than ideal, and in cases where the mobile phase viscosity is high, resulting in higher system pressure. It is difficult to heat large diameter columns from the outside and temperature gradients are generated inside the column. Preparative separations occur at the temperature of the incoming solvent. As shown in Figure 3, a 5 mL sample loop connected to the head of the column serves as a solvent preheater. Continuously introducing the solvent at 60°C brings the column to equilibrium internally while the water bath stabilizes the external column environment. The amount of band broadening attributed to a solvent preheating loop is negligible because the loop is made with narrow internal diameter tubing. Furthermore, temperature control is used most often with gradient methods that reconcentrate the sample at the head of the column. Figure 4 shows how increasing the temperature of the separation to 40°C improves the resolution and peak shape of the peptide product, making isolation and purification easier.

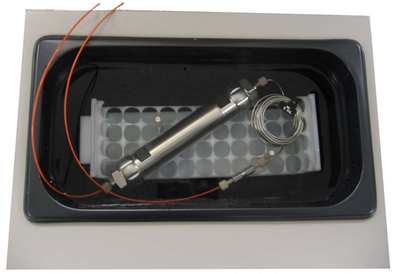

Figure 3. A 5 mL sample loop connected to the head of the column and submerged in the water bath acts as a solvent preheater. Approximately 2.5 min are required to bring the column to equilibrium.

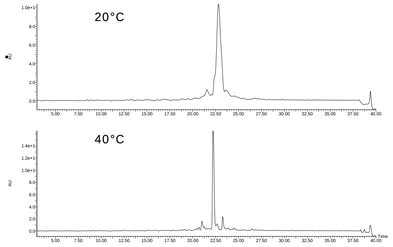

Figure 4. Temperature effect on peptide mixture with poor resolution at 20°C and better resolution at 40°C.

AT-COLUMN DILUTION*

Hydrophobic peptides with limited solubility are often very difficult to purify. Solvents that dissolve crude peptide mixtures may compromise the separation. Even when a sample is solubilized and applied to the column, elution may be problematic because of poor solubility in the mobile phase. At-column dilution, a patented technique that increases mass loading by improving sample solubility, has been optimized for peptides with large numbers of nonpolar amino acid residues. Figures 5-8 illustrate the fundamental principles of at-column dilution.

*U.S. Patent No. 6,790,361 B2; Application Note 71500078010; www.waters.com/library

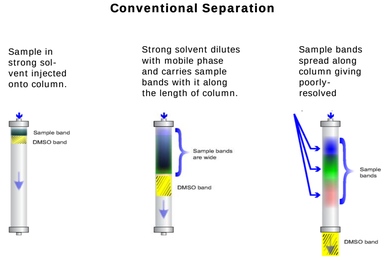

Figure 5. Sample is not retained until the strong solvent is diluted in the column.

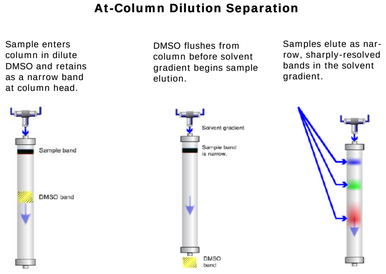

Figure 6. The diluted peptide sample is concentrated at the head of the column and the strong solvent begins moving down the column, leaving the peptide sample behind. The peptide elution begins when the gradient is started.

A hydrophobic peptide composed of 12 nonpolar and 3 polar, uncharged amino acid residues was dissolved in DMSO at a concentration of 20 mg/mL. A loading study at the pilot scale determined that upon scaling, the preparative chromatography injection volume should be 170 µL, or 3.4 mg. The mass capacity is dependent upon the solubility of the peptide in the mobile phase. Although the estimated peptide mass loading capacity for a 19 x 100 mm column is typically in the range of 9-18 mg, a 3.4 mg load for this particular peptide is reasonable because of its extremely limited solubility. As shown in Figure 8, the mass capacity is increased five fold using at-column dilution with 24% acetonitrile as the initial loading condition in the gradient.

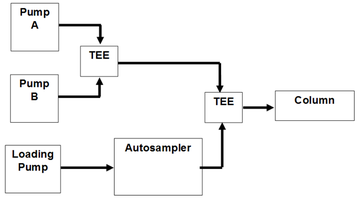

Figure 7. High pressure mixing gradient system plumbing diagram. The loading pump delivers acetonitrile directly to the injector on the autosampler, effectively carrying the peptide sample to the tee where it will be diluted and mixed with the mobile phase at the initial condition in the gradient.

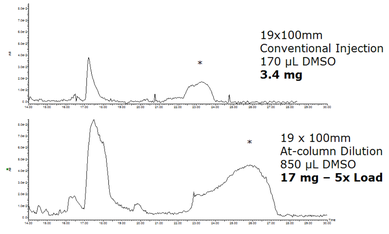

Figure 8. Top trace—conventional injection, 3.4 mg peptide load. Gradient: 48-53% B in 29 min with a slope of 0.25% per column volume at 60°C. Bottom trace—at-column dilution, 17 mg peptide load. Gradient: 48-53% B in 29 min with a slope of 0.25% per column volume at 60°C. Peptide sample loaded with 24% acetonitrile prior to start of gradient.

DETECTION MODE

Typical purification schemes employ UV detection, but some hydrophobic peptides or peptides with large numbers of uncharged residues may have very low extinction coefficients. For these samples, mass detection is useful for visualizing the product chromatographically. Figure 9 shows a peptide which has no UV absorption, but is visible with mass detection.

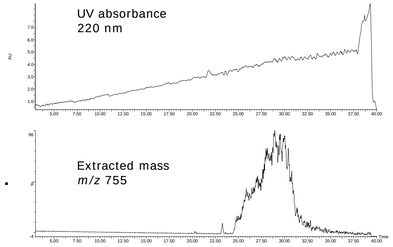

Figure 9. Additional modes of detection may be required for some peptides; same peptide with UV and mass detection.

CONCLUSIONS

- **Focused gradients can improve resolution without extending chromatographic run time.**
- **Temperature control can alter the selectivity and reduce peak volume for better resolution and recovery.**
- **At-column dilution can be used to maximize load and resolution, especially for very hydrophobic peptides.**
- **At-column dilution increases system ruggedness by reducing the occurrence of sample precipitation.**
- **Mass spectrometry may be used to detect hydrophobic peptides with low extinction coefficients.**
- **Isolation of high purity synthetic peptides can be improved using modified chromatographic operations.**

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