APPROACHES FOR IMPROVING PEPTIDE ISOLATION AND PURIFICATION

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INTRODUCTION

Peptides play important roles in the physiology of organisms. Pure peptides are required to effectively study biochemical interactions in organisms as well as to understand structure-activity relationships in the development of peptide therapeutics. Many types of impurities complicate the isolation and purification of peptides. Failure, damaged, and deleted sequences, in addition to those sequences modified by incomplete peptide cleavage and deprotection require purification techniques that efficiently isolate the peptide product. Hydrophobic peptides that exhibit limited solubility are among the most difficult samples to purify. Solvents that dissolve crude peptide mixtures often jeopardize the chromatographic separation. Focused gradients and temperature control help to improve resolution between the product of interest and the impurities and increase sample solubility in the mobile phase. Optimized at-column dilution steps increase mass loading on the column and prevent high pressure problems associated with sample solubility. In this study, we illustrate the implementation of these three separation optimization techniques applied to Waters® Peptide Separation Technology Columns to improve the isolation of peptides with large numbers of nonpolar amino acid residues.

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. Peaks eluting earlier or later than the focused segment of the gradient are 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 improves the resolution between closely eluting peaks.

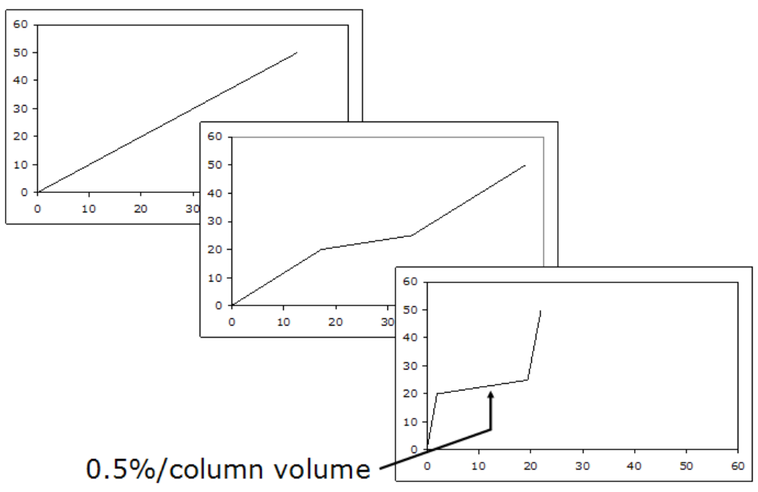

Figure 1. From top to bottom, a graphical representation of a linear gradient, a segmented gradient, and a focused gradient.

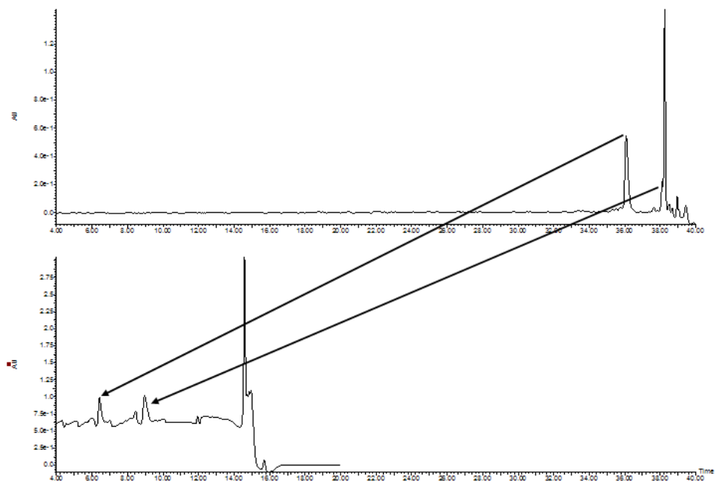

Figure 2. Focusing the gradient improves the resolution in reduced run time. The top trace is the linear gradient; the bottom trace is the focused gradient.

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 3-6 illustrate the fundamental principles of at-column dilution. *U.S. Patent No. 6,790,361 B2; Application Note 71500078010; www.waters.com/library

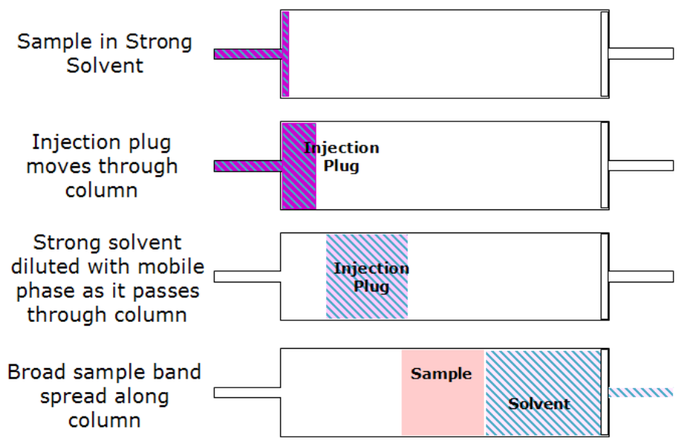

Figure 3. Sample is not retained until the strong solvent is diluted in the column.

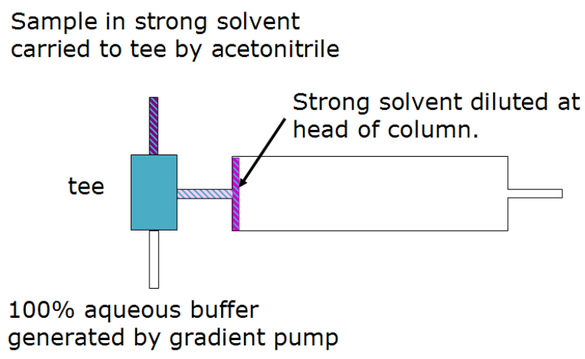

Figure 4. The peptide sample in strong solvent is carried to one side of the tee placed at the head of the column. The mobile phase at the gradient initial condition is carried to the other side of the tee. The sample is quickly diluted and deposited on the head of the column.

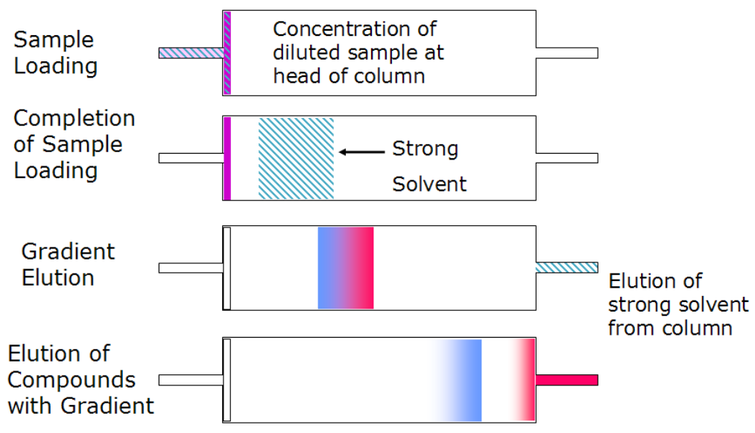

Figure 5. The diluted peptide sample is concentrated at the head of the column and the strong solvent begins moving down the column, leaving the peptide sample behind. The peptide elution begins when the gradient is started.

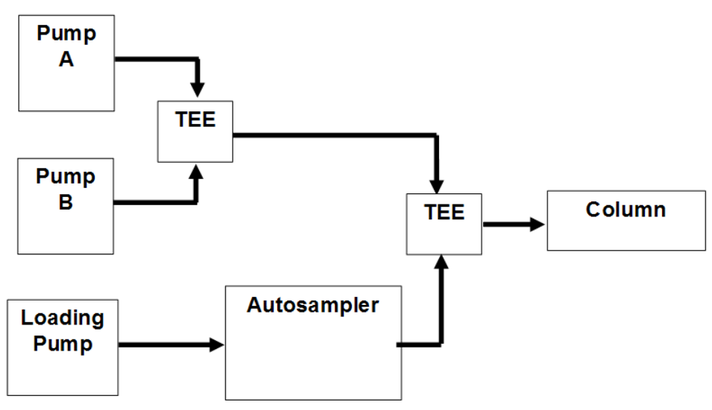

Figure 6. High pressure mixing gradient system plumbing diagram. The loading pump delivers acetonitrile directly to the injector on the autosampler, effectively carrying the peptide sample to the tee where it will be diluted and mixed with the mobile phase at the initial condition in the gradient.

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 10, the mass capacity is increased five fold using at-column dilution with 24% acetonitrile as the initial loading condition in the gradient.

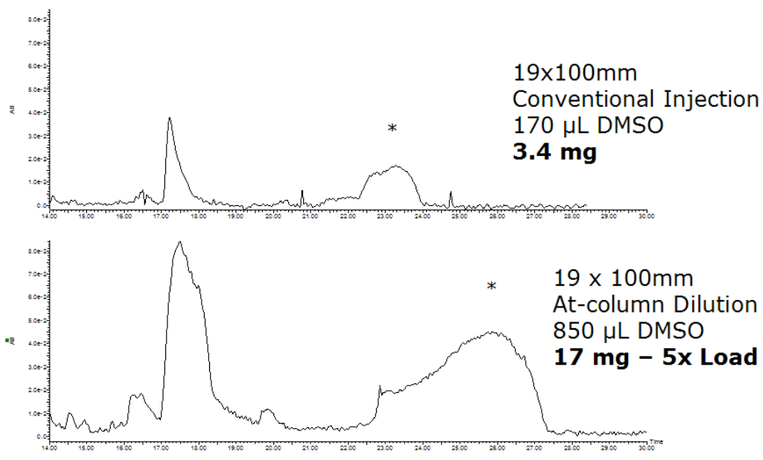

Figure 7. Top trace—conventional injection, 3.4 mg peptide load. Gradient: 48-53% B in 29 min with a slope of 0.25% per column volume at 60°C. Bottom trace—at-column dilution, 17 mg peptide load. Gradient: 48-53% B in 29 min with a slope of 0.25% per column volume at 60°C. Peptide sample loaded with 24% acetonitrile prior to start of gradient.

TEMPERATURE CONTROL

Temperature control is most often used for delicate separations, for those separations where sample solubility is less than ideal, and in those 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 loop plumbed at 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 inside diameter tubing. Furthermore, temperature control is used most often with gradient methods which reconstitute the sample at the head of the column. In the examples below, one synthetic peptide sample gives better resolution at 60°C, while a different synthetic peptide sample gives better resolution at 40°C.

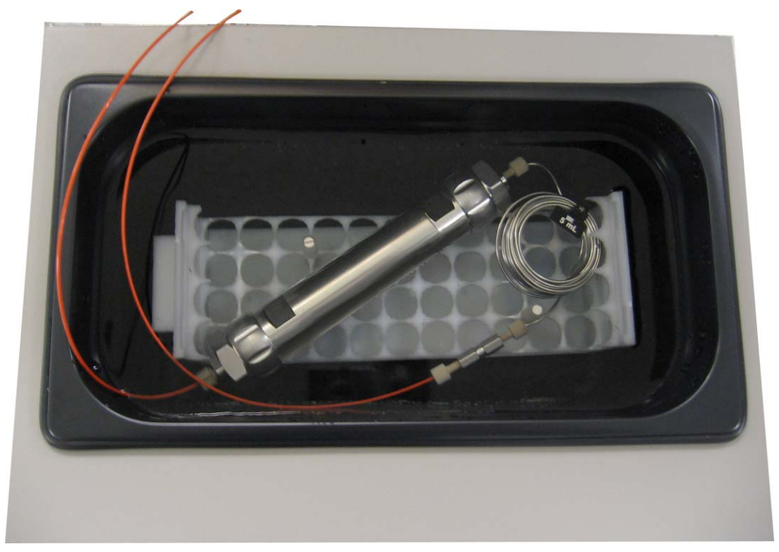

Figure 8. A 5 mL loop plumbed at the head of the column and submerged in the water bath acts as a solvent preheater. Approximately 2.5 min are required to bring the column to equilibrium.

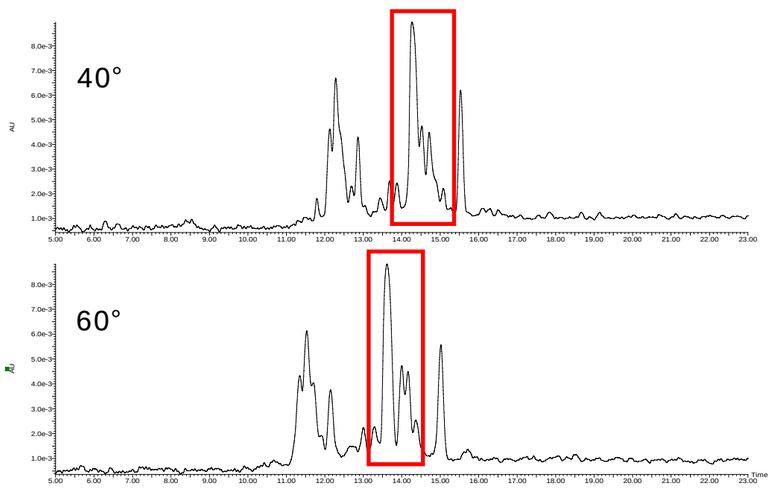

Figure 9. Temperature effect on peptide mixture with poor resolution at 40°C and better resolution at 60°C.

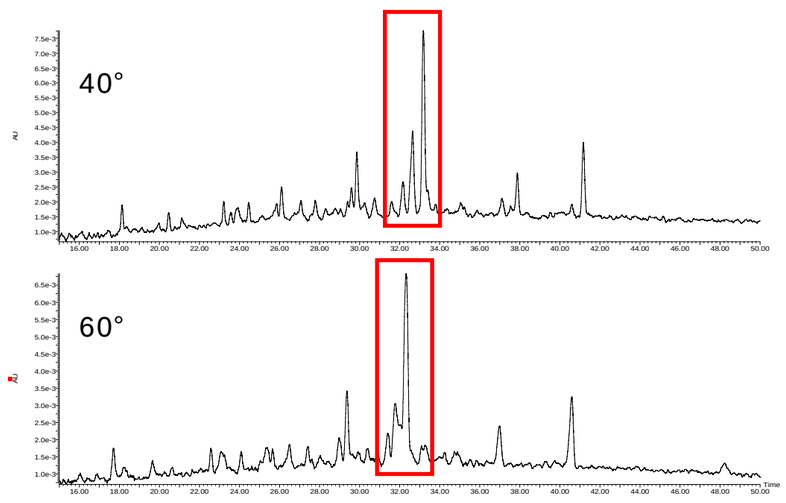

Figure 10. Temperature effect on peptide mixture with poor resolution at 60°C and better resolution at 40°C.

CONCLUSIONS

- **Isolation of high purity synthetic peptides can be improved using modified chromatographic operation.**
- **Focused gradients can give increased purity without extending chromatographic run time.**
- **Temperature control can alter the selectivity of the chromatogram for better resolution.**
- **Elevated temperature also can increase peptide solubility and yield.**
- **At-column dilution can be used to maximize load and resolution, especially for very hydrophobic peptides.**

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