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 diluted 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 capacity 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 acids and residue.

**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® 2152 Binary Gradient Module, 2156 Spectrometer Manager, Column Fluidics Organizer, 2995 Photodiode Array Detector, ZQ™ Mass Selective Detector, Water Bath
  - Column: XBridge™ BEH 130 PREP C18 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, allowing for improved sample recoveries. Peptides of interest are eluted in the focused area of the gradient, while the other portions of the separation are preserved with relatively little change from the total run time of the isolation.

**AT-COLUMN DILUTION**

- The diluted peptide sample is concentrated at the head of the column and the strong solvent begins moving through the column (Figure 3). As shown in Figure 3, a 5 mL loop plumbed in the water bath acts as a solvent preheater. Approximately 2.5 min are required to bring the column to equilibrium.

**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 head large diameter columns effectively 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 40°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.

**CONCLUSIONS**

- Isolation of high purity synthetic peptides can be improved using at-column dilution 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.