# Developing Strategies for the Isolation and Purification of Synthetic Peptides

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## INTRODUCTION

The purification of synthetic peptides is important for many kinds of research and development. The required peptides present a problem in isolation because they can have a wide range of chemical and physical properties. Such differences often lead to specialized isolation methods that are adjusted for each sample. We have investigated the variables that can be manipulated to control a peptide separation so that an efficient isolation protocol can be developed. This includes selection of a column based on the properties of the peptides. The effects of column chemistry, pore size and particle size have been considered. Modifying method parameters, such as gradient slope, will also be described. These considerations can be used to suggest a general protocol for high throughput purification. Some samples still require optimized separations, either because of the requirements of subsequent experiments or because of the complexity of the synthetic mixture. These separations are optimized on smaller, pilot-scale columns. We will demonstrate an efficient process for scaling the optimized separation to a larger sample load. The combination of an optimized general protocol and systematic scaling procedures can improve efficiency in the isolation of synthetic peptides.

## MATERIALS AND METHODS

### LC Conditions

LC System: Waters 2796 Separation Module
UV Detection: Waters 2487 Dual Wavelength Absorbance Detector
Wavelength 214 nm
MS System: Waters ZQ™ Mass Spectrometer
Electrospray Ionization (+)
Mobile Phase: With "TFA" modifier:
A = 0.1% Trifluoroacetic Acid in Water
B = 0.1% Trifluoroacetic Acid in Acetonitrile
With "FA" modifier
A = 0.1% Formic Acid in Water
B = 0.1% Formic Acid in Acetonitrile

### Gradient for Column Evaluation

| Time | %A | %B | Flow | Curve |
|---|---|---|---|---|
| 0 | 95 | 5 | 1.5 | * |
| 50 | 50 | 50 | 1.5 | 6 |
| 52 | 10 | 90 | 1.5 | 6 |
| 54 | 10 | 90 | 1.5 | 6 |
| 55 | 95 | 5 | 1.5 | 6 |
| 72 | 95 | 5 | 1.5 | 6 |

### Columns for Evaluation

Peptide Separation Technology
XBridge™ BEH130 $C_{18}$ 4.6mm x 150mm 3.5µm
XBridge™ BEH130 $C_{18}$ 4.6mm x 150mm 5µm
XBridge™ BEH130 $C_{18}$ 4.6mm x 150mm 5µm Prep
XBridge™ BEH130 $C_{18}$ 4.6mm x 150mm 10µm
XBridge™ BEH300 $C_{18}$ 4.6mm x 150mm 3.5µm
XBridge™ BEH300 $C_{18}$ 4.6mm x 150mm 5µm
XBridge™ BEH300$C_{18}$ 4.6mm x 150mm 5µm Prep
XBridge™ BEH300 $C_{18}$ 4.6mm x 150mm 10µm

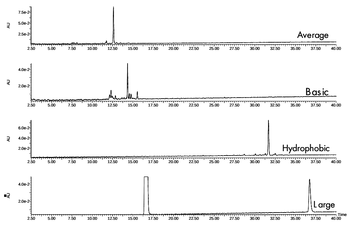
### Test Peptides

Neutral and "Average
ISQAVHAAHAEINEAGR
17 residues; 1772.89Da
pI 6.4; B&B 5580; HPLC 26.5
Basic
AIKEVKQTIVKHPRY
15 residues; 1809.06Da
pI 10.3; B&B -860; HPLC 21.7
Hydrophobic
WLTGPQLADLYHSLMK
16 residues; 1871.96Da
pI 7.3; B&B -5200; HPLC 113.3
Large
Released Biopharmaceutical Dosage Form
Approximately 40 residues; Over 4000 Da
pI 4.7; B&B 2540; HPLC 89.6

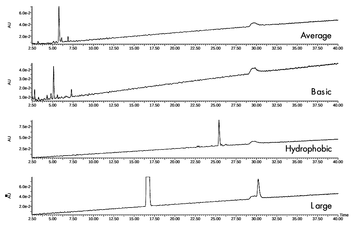
## RESULTS

### FIGURE 1: COLUMN COMPARISON

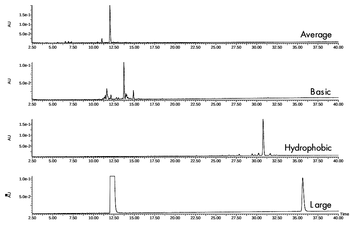
#### FIGURE 1A: BEH130; 3.5µm; TFA




#### FIGURE 1B: BEH130; 3.5µm; FA




#### FIGURE 1C: BEH300; 3.5µm; TFA




#### FIGURE 1D: BEH300; 3.5µm; FA

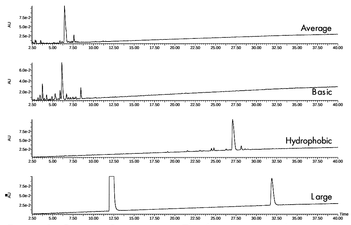



Figure 1: Column Comparison. The four test peptides representing a wide range of chemical and physical properties gave symmetrical peaks on the large and small pore size columns in both mobile phase modifiers. Selectivity differences are observed both between the pore sizes and, as expected, with the different modifiers. This small set of columns can, therefore, provide the basis for developing methods for isolation.

### FIGURE 2: Effect of Particle Size

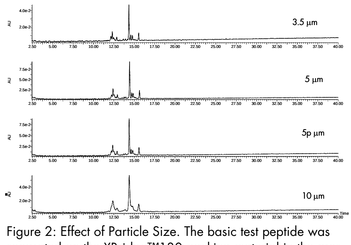
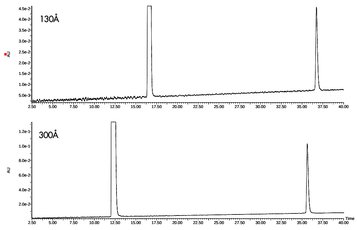



Figure 2: Effect of Particle Size. The basic test peptide was separated on the XBridge™130 packing material in the presence of 0.1%TFA. The four different particle sizes were packed into identical columns and separated with the same method. The selectivity is identical in all four experiments so methods can be scaled. Resolution is nearly the same on the 3.5µm and the two 5µm particles. Larger resolution losses are observed with the 10µm.

### FIGURE 3: PORE SIZE EFFECTS

#### FIGURE 3A: LARGE PEPTIDE




#### FIGURE 3B: HYDROPHOBIC PEPTIDE

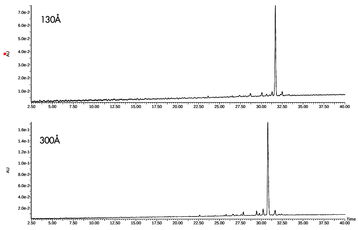



Figure 3: Effect of Pore Size. Large pore size packings are usually selected for peptide separations to minimize steric effects. With the largest peptide in the test set, narrow, symmetrical peaks are observed on both the small and large pore materials. It is also commonly reported that hydrophobic peptides behave better with larger pores, but no significant effect is observed with these peptides. While the 130Å is more retentive than the 300Å, no clear size or hydrophobicity limit is found in this test set. Steric effects certainly exist but probably only become important for significantly larger peptides.

### FIGURE 4: Effect of Modifier

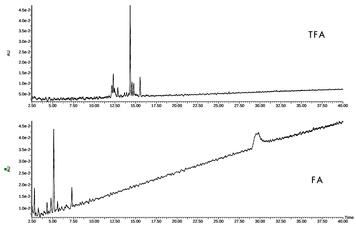



Figure 4: Effect of Modifier. The basic test peptide was separated on the XBridge™130 packing material in the presence of 0.1%TFA or 0.1%formic acid. As expected, the elimination of ion pairing reduces retention for all the peptides in the sample. Some peptides are affected more than others resulting in selectivity changes that should be useful in developing purification protocols.

## CONCLUSIONS

- The BEH Technology™ packing material used as the basis for Peptide Separation Technology columns gives good peak shape for peptides over a wide range of properties.
- When TFA is replaced with formic acid as modifier, retention is reduced, as expected, but symmetrical peak shape is retained.
- Retention and selectivity are identical across a range of particle sizes, as required for scalability.
- Peaks are broader with larger particle size packings.
- There is no clear correlation between pore size and peptide molecular weight for this set of test peptides covering up to about 4kD.
- There is no clear correlation between pore size and peptide hydrophobicity for this set of test peptides covering up to about 40% acetonitrile.
- Changes in mobile phase modifier affect retention and selectivity.
- Secondary interactions between peptides and the stationary phase are minimized with Peptide Separation Technology columns.

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