Systematic Strategies for Developing Peptide Maps

Waters

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INTRODUCTION

Peptide maps that are used in the characterization of biopharmaceuticals must completely resolve all possible peptides derived from the sample, including those representing a variety of minor chemical modifications. Development of such separations is a time-consuming and often labor-intensive procedure. It is greatly influenced by the experience of the scientists involved. In making this procedure more efficient, various columns are evaluated. Those columns are selected because they have properties that are expected to interact with peptides in useful ways. These variables include column dimensions and particle size, but the greatest significance is often ascribed to bonded phase chain length, pore size, and base material. The relative importance of these properties is difficult to estimate since there are few examples that compare columns that differ in only one parameter. Such comparisons are developed in these experiments. Where necessary, small amounts of prototype packing materials were synthesized to permit evaluation of each relevant property.

MATERIALS AND METHODS

LC System: Waters 2796 Separation Module
UV Detection: Waters 2487 Dual Wavelength Absorbance Detector. Wavelength 214 nm
MS System: Waters Micromass ZQ™ Mass Spectrometer Electrospray Ionization (+)
Mobile Phase: With "TFA" modifier:
A = 0.02% Trifluoroacetic Acid in Water
B = 0.018% Trifluoroacetic Acid in Acetonitrile
With "FA" modifier
A = 0.1% Formic Acid in Water
B = 0.1% Formic Acid in Acetonitrile
Flow Rate: 0.3 mL/min
Injection Volume: 20 µL
Columns: Waters
A- BioSuite™ C_{18} PA-A, 2.1 x 150 mm 3.0 µm particles, 120 Å pores
B- BioSuite™ C_{18} PA-B, 2.1 x 150 mm 3.5 µm particles, 300 Å pores
C- Prototype BEH™ (Bridged-Ethyl-Hybrid) C_{18}, 2.1 x 150 mm, 4.5 µm particles, 300 Å pores
D- XBridge™ C_{18}, 2.1 x 150 mm 3.5 µm particles, 130 Å pores
E- Symmetry™ C_{18}, 2.1 x 150 mm 5 µm particles, 300 Å pores
F- BioSuite™ C_{18} PA-B, 2.1 x 250 mm 3.5 µm particles, 300 Å pores
G- Prototype C_8, 2.1 x 150 mm, 5 µm particles, 300 Å pores
Grace Vydac
H- C_{18}, 2.1 x 150 mm, 3 µm particles, 300 Å pores

RESULTS

Fig.1: Effect of Particle Size

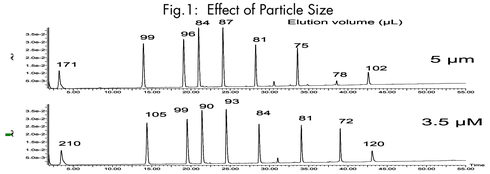

Figure 1: The MassPREP™ Peptide Standard was separated on two columns differing only in particle size. The potential for improved resolution was judged by measuring the elution volume of each peptide. On this basis, there is little benefit in replacing 5µm particles with 3.5µm packings.

Fig.2: Effect of Column Length

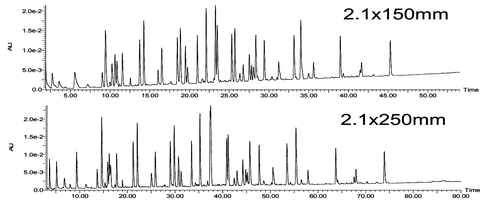

Figure 2: The MassPREP™ Enolase Digestion Standard was separated on the same packing material in 150mm and 250mm column lengths. The gradient duration was proportional to length. When separation conditions are controlled, only small resolution benefits are associated with the longer column.

Fig.3: Effect of Pore Size

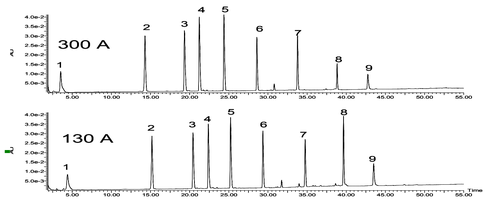

Figure 3: The MassPREP™ Peptide Standard was separated on two columns differing only in pore size. For the range of peptides in this mixture, up to about 2800da or 26 residues, pore size has little effect on retention or selectivity. This parameter may be more significant for larger peptides if they have a larger radius in solution.

Effect of Peptide Properties on Retention

It is difficult to demonstrate the importance of certain common preferences in developing peptide maps. In an effort to better define the basis for these preferences, the larger set of peptides in the MassPREP™ Enolase Digestion Standard was tested as a model system. The peptides in this mixture were categorized based on those chemical properties most likely to affect retention and selectivity. These include relative hydrophobicity and hydrophilicity based on common models, calculated isoelectric point, pI, and size. These categories are tabulated below. Note that a peptide may appear in more than one category, sometimes with unexpected results. For example, the largest peptide is the most hydrophobic, but the second largest is among the most acidic.

Size (mass)	Hydrophobic	Hydrophilic	Basic (pI = 10.1)	Acidic (pI = 3.6 to 3.9)
T21 (3737)	T21	T3	T5	T27
T27 (3257)	T35	T5	T16	T45
T35 (1872)	T16		T50	T14
				T37

Fig.4: Effect of Pore Size on Specific Peptides

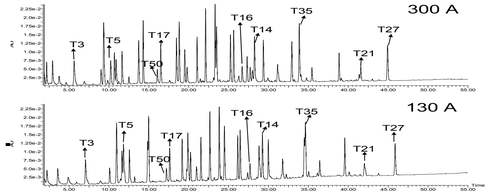

Figure 4: The small and large pore size materials were compared using the MassPREP™ Enolase Digestion Standard, and the diagnostic peptides are labeled. This test reveals little difference between the pore sizes. Only the hydrophilic peptides near T50 show a useful difference.

Fig.5: Effect of Bonded Phase on Specific Peptides

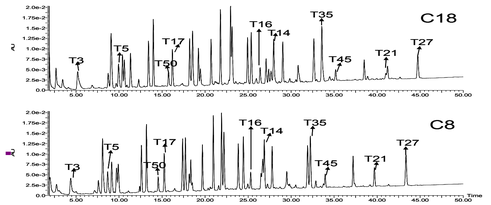

Figure 5: The C18 and C8 materials were compared using the MassPREP™ Enolase Digestion Standard, and the diagnostic peptides are labeled. In general, the entire map shows slightly longer retention on the C18 material. There are, however, few changes in selectivity, even when examining the most hydrophobic and hydrophilic peptides. Better resolution is observed on the C18 column for the peptides near the acidic T14 peptide.

Fig.6: Effect of Mobile Phase Modifier

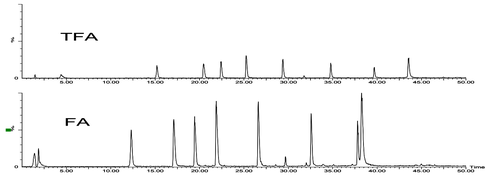

Figure 6: The MassPREP™ Peptide Standard was separated in the presence of either trifluoroacetic acid or formic acid. As expected, ESI-MS sensitivity is increased, in this case by about a factor of 3x in formic acid. The absence of ion pairing is responsible for this increased ionization efficiency. The same mechanism causes the reduction in retention and the broader peaks with formic acid. Some change in selectivity is also observed with the substitution.

Fig.7: Separations in Trifluoroacetic Acid on Different Columns

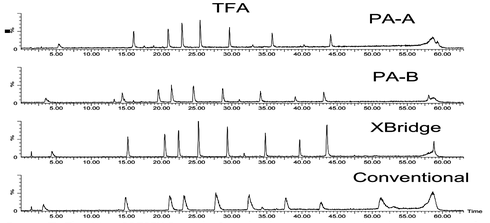

Figure 7: The MassPREP™ Peptide Standard was separated in the presence of 0.02% trifluoroacetic acid. This relatively low TFA concentration is often used to maximize ESI-MS sensitivity while preserving the separation selectivity observed with ion pairing. The different columns do differ significantly in peak shapes.

Fig.8: Separations in Formic Acid on Different Columns

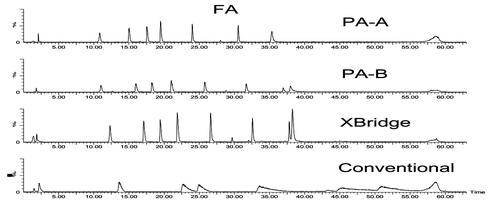

Figure 8: The MassPREP™ Peptide Standard was separated in the presence of 0.10% Formic Acid. The differences among the columns follow the same patterns observed with TFA as a modifier. The conventional 300Å C18 material does not give usable results with formic acid as a modifier. The other three columns do differ from one another in ways that may be of benefit for different

Fig.9: Effect of Gradient Slope

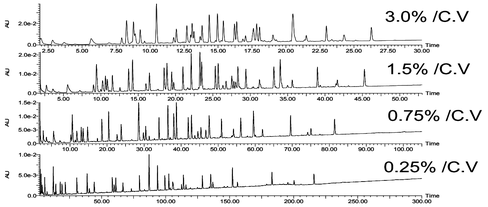

Figure 9: Separation of the MassPREP™ Enolase Digestion Standard was separated with progressively more shallow gradients. Resolution does clearly improve with more shallow gradients. Runtime, however, increases. Peak elution volumes also increase with a corresponding decrease in signal intensity. The use of gradient slope to optimize resolution is a compromise among these parameters.

Fig.10: Selectivity Effects of Gradient Slope

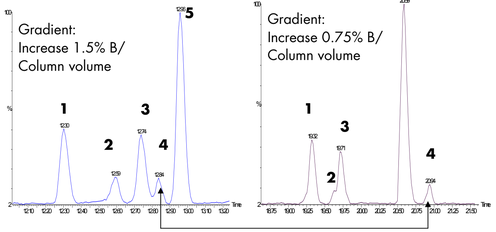

Figure 10: The enolase tryptic digest was separated on the BEH column at two different gradient slopes. The more shallow gradient gives better separation but with longer run time and somewhat lower sensitivity. While maps are similar, changes in selectivity including reversal of elution order can be identified by using MS-SIC to track peaks.

CONCLUSIONS

- There are several options for improving reversed phase peptide separations.
- Resolution in peptide mapping reflects the sum of all the properties of the packing material and the properties of all the peptides in the mixture.
  - With modern packing materials, the effect of each variable property is relatively small, in general.
  - Resolution of particular peptides can be significantly affected by the columns, but not in ways that can be readily anticipated from their properties.
- Adjustment of gradient slope is a compromise among resolution, sensitivity, and speed.
- There significant differences in the behavior of different columns with different mobile phase modifiers.

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