SYSTEMATIC DEVELOPMENT OF PEPTIDE MAPS FOR PROTEIN CHARACTERIZATION

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.

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- Symmetry™ C_{18}, 2.1 x 150 mm 5 µm particles, 300 Å pores
C- Prototype BEH™ (Bridged-Ethyl-Hybrid)C_{18}, 2.1 x 150 mm, 4.5 µm, 300 Å
D- Prototype BEH™ (Bridged-Ethyl-Hybrid)C_{18}, 2.1 x 150 mm 3.5 µm, 130 Å

RESULTS

Effect of Peptide Properties on Retention

In an effort to better define the basis for column selection for peptide mapping, the MassPREP™ Enolase Digestion Standard was used 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.1: Effect of Pore Size on Specific Peptides

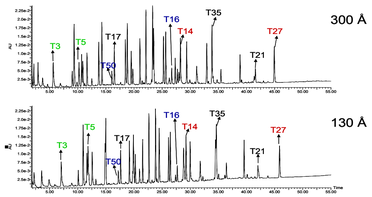


Figure 4: The small and large pore size materials were compared using the MassPREP™ Enolase Digestion Standard, and the diagnostic peptides are labeled. This test reveals little difference between the pore sizes. Only the hydrophilic peptides near T50 show a useful difference.

Fig.2: Effect of Bonded Phase on Specific Peptides

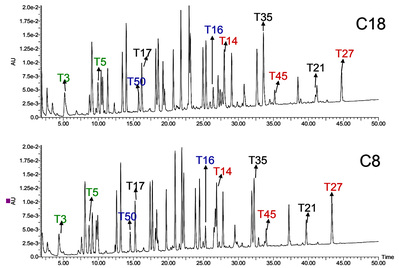


Fig.3: Effect of Gradient Slope on Selectivity

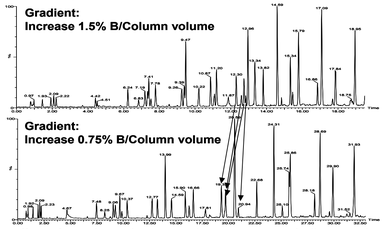


Figure 3: Effect of Gradient Slope on Selectivity: More shallow gradients are usually used to increase resolution. In this example of the map of the tryptic digest of enolase, rearrangements in selectivity often occur with such changes. Two peaks that are separated in the steep gradient merge in the shallow while two other peaks reverse elution order.

Fig.4: Effect of Mobile Phase Modifier

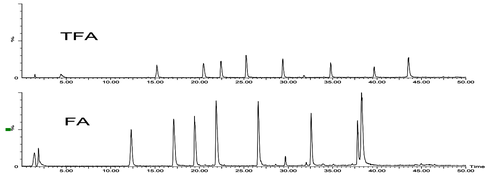


Figure 4: The MassPREP™ Peptide Standard was separated in the presence of either trifluoroacetic acid or formic acid. As expected, ESI-MS sensitivity is increased, in this case by about a factor of 3x in formic acid. The absence of ion pairing is responsible for this increased ionization efficiency. 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.5: Column Differences with TFA

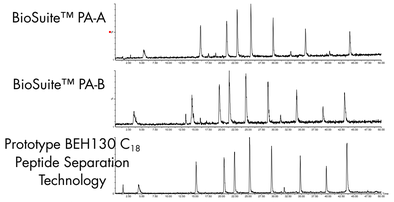


Figure 5: The MassPREP™ Peptide Standard was separated in the presence of 0.02% TFA. This relatively low TFA concentration is often used to maximize ESI-MS sensitivity while preserving the separation selectivity observed with ion pairing. The columns differ somewhat in peak shapes.

Fig.6: Column Differences with Formic Acid

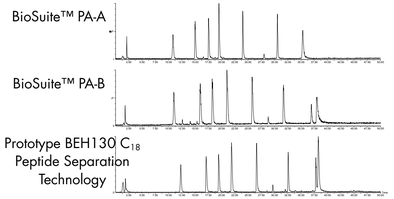


Figure 6: The MassPREP™ Peptide Standard was separated in the presence of 0.10% Formic Acid. The differences among the columns follow the same patterns observed with TFA as a modifier. The other three columns do differ from one another in ways that may be of benefit for different samples.

Fig.7a: Selectivity Effects of Modifier Concentration

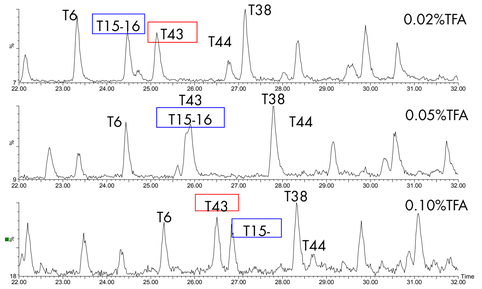
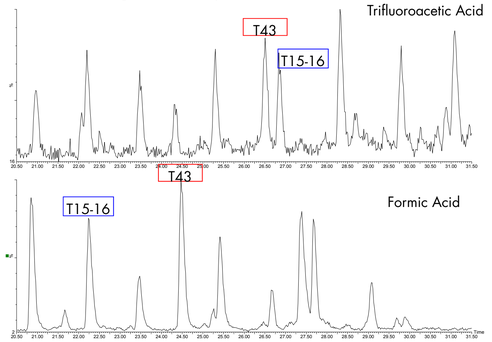


Fig.7b: Selectivity Effects of Modifier Choice



Figures 7a and 7b: Effects of Mobile Phase Modifier on Selectivity: In general, relative retention increases with the use of TFA instead of formic acid and with increasing concentrations of TFA. Within this principle, changes in selectivity are often observed, as shown here. Two mechanisms can be proposed. First, overall charge is reduced by higher concentrations of the stronger acid. Second, ion pairing is more complete. The side chains of the peptides showing altered selectivity can be identified as ion pairing sites (*) or ion suppression sites(#):

T43: VNQIGTLSE#SIK*
T15-16: SK*LGANAILGVSLAASR*
T38: TAGIQIVAD#D#LTVTNPK*
T44: AAQD#SFAAGWGVMVSH*R*

In these examples, the peptides with the greater change in retention are the species with the greater excess of ion pairing sites over ion suppression sites. The same mechanism can explain the highlighted change in selectivity between formic acid and TFA. Small changes in modifier can be used to improve the selectivity of the map and may be done in a planned way where the structure is known.

Fig.8: Effect of Temperature on Selectivity

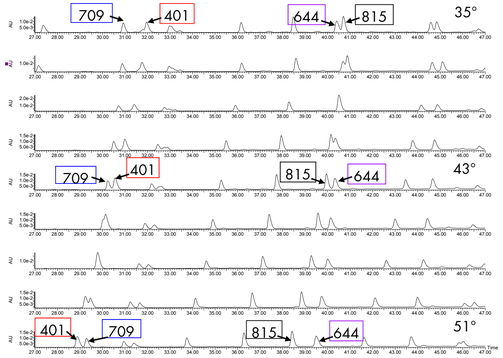


Figure 8: Effect of Temperature on Selectivity: The enolase digest was separated over the temperature range 35° to 51° in 2° increments. The elution of individual peptides was tracked by *m/z*, as shown. Profound selectivity changes, including reversal of elution order are observed over a small range.

DISCUSSION AND CONCLUSIONS

Stepwise Approach to Developing a Peptide Map

- Choose a Column
  - BioSuite™ PA-A
    - For MS detection with formic acid as modifier
    - For samples with many small, polar peptides
  - BioSuite™ PA-B
    - For UV detection with TFA as modifier
    - For generally best chromatographic peak shape with TFA
    - For samples with many peptides larger than 30 residues
  - Consider Peptide Separations Technology Columns
    - New BEH surface chemistry for best peak shape
    - Available in ACQUITY UPLC™ format today
    - New choices in continuous development
- Adjust Universal Operating Conditions
  - Start with gradient slope near 1% / column volume
  - Test effect of concentration of modifier over a 5-10x range
  - Test effect of temperature in small increments over a wide range
    - Temperatures from 30° to 60°
    - Changes of 1-2° can affect selectivity
- Adjust gradient slope for critical pairs
  - Estimate concentration where difficult pair elutes
  - Segments should extend ±2-3% around estimated concentration
  - Test both steeper and shallower slopes
    - Shallower than 0.25%/col. vol. generally degrades sensitivity
    - Steeper than 2-3%/col.vol. noticeably compromises resolution
- Final adjustments
- Revisit temperature and modifier concentration as needed
- Consider dividing into multiple shorter methods for specific modifications

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