SELECTION OF CHROMATOGRAPHIC MEDIA FOR HIGHER RESOLUTION PEPTIDE MAPPING 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.

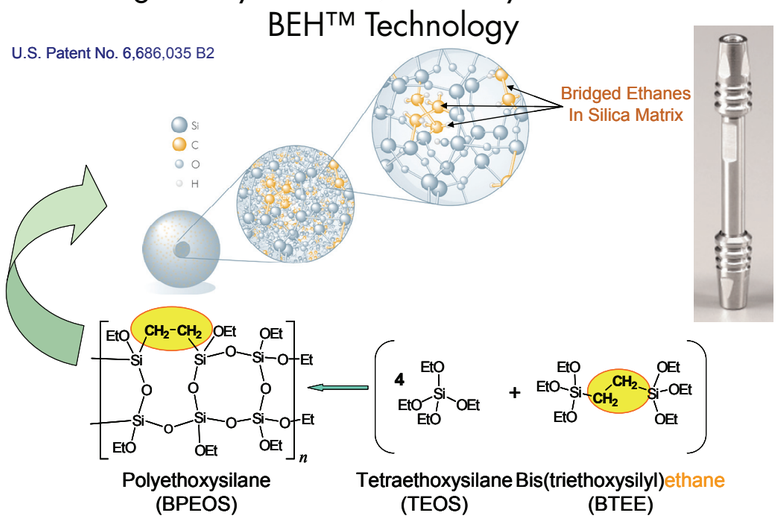
METHODS

Waters AQUITY UPLC® System
Columns: Peptide Separation Technology
ACQUITY UPLC BEH 130, C18, 1.7µm, 2.1 x 100/150/250mm
ACQUITY UPLC BEH 300, C18, 1.7µm, 2.1 x 100mm
ACQUITY UPLC BEH C8, 1.7µm, 2.1 x 100mm

Injection Volume:	10 µL (20 pmol on column)
Temperature:	40°C
Flow Rate:	200 µL/min (unless otherwise noted)
Mobile Phase A:	0.02, 0.05 or 0.1% TFA in water
Mobile Phase B:	0.018, 0.05 or 0.1% TFA in acetonitrile
Mixer:	High Sensitivity Peptide Analysis (P/N 205000403)
Gradient Slope:	1.5%B increase per column volume
UV Detection:	214 nm at 10 scans/sec
MS Detection	Waters Q-Tof *micro*™ Mass Spectrometer
	m/z 400-1800 at 2 scans/sec

Test samples were Waters MassPREP™ Digest Standards Phosphorylase b or Enolase. Dissolved in 0.5 mL of 95% water and 5% Acetonitrile with 0.02% TFA, resulting in a concentration of 2 pmoles/µL.

Bridged EthylSiloxane/Silica Hybrid Particles
BEH™ Technology



RESULTS

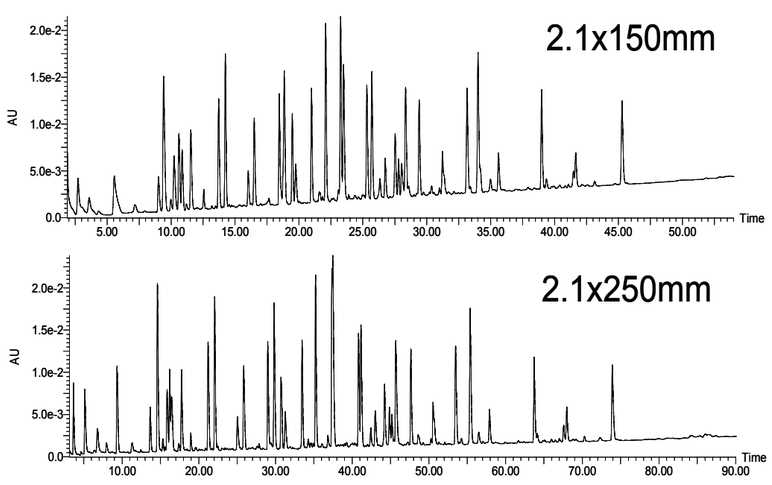


Figure 1. The MassPREP™ Enolase Digestion Standard was separated on the same packing material in 150mm and 250mm column lengths. The gradient duration was proportional to length. When separation conditions are controlled, only small resolution benefits are associated with the longer column.

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 large 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

Effect of Bonded Phase

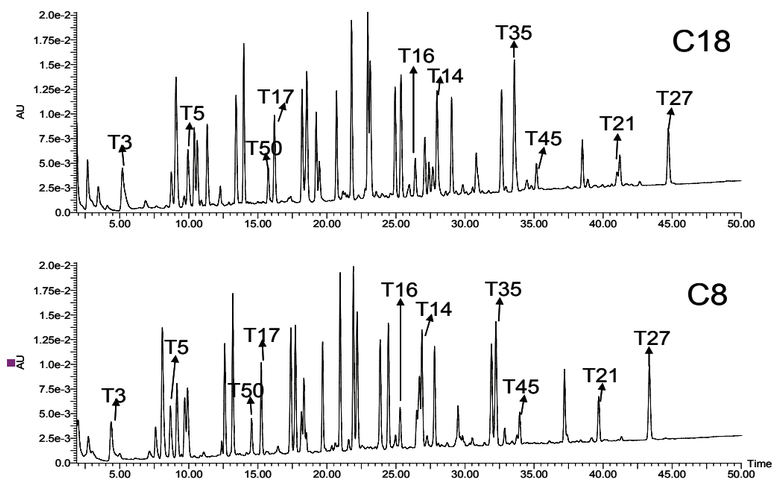


Figure 2. The C18 and C8 materials were compared using the MassPREP™ Enolase Digestion Standard, and the diagnostic peptides are labeled. In general, the entire map shows slightly longer retention on the C18 material. There are few changes in selectivity, even when examining the hydrophobic and hydrophilic peptides. Better resolution is observed on the C18 column for the peptides near the acidic T14 peptide.

Effect of Pore Size

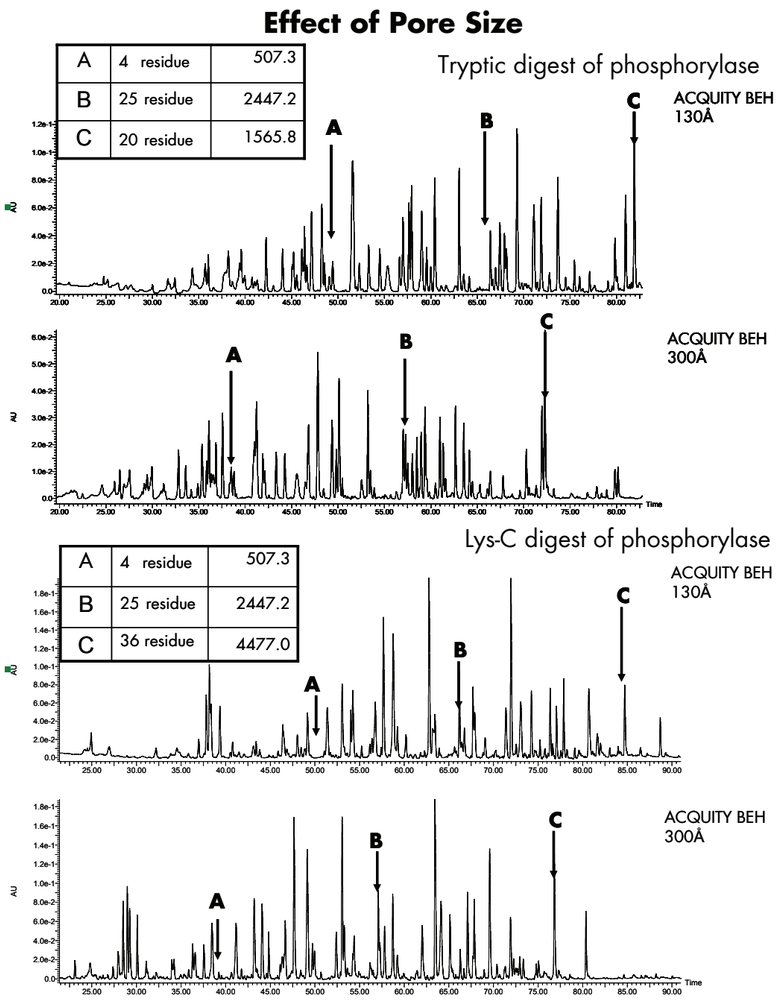


Figure 3. Comparison of the separation of tryptic and Lys-C digests of phosphorylase b on ACQUITY UPLC BEH130, 1.7mm and on ACQUITY UPLC BEH300, 1.7mm Peptide Separation Technology Columns. Larger pore size materials are often preferred for peptide separations. For tryptic peptides both columns work well, with the smaller pore size being more retentive. When the larger peptides produced by Lys-C digestion were tested both columns worked well. Slight differences in selectivity are detected in addition to the retentivity shift. When choosing a column, selectivity changes may dominate the decision.

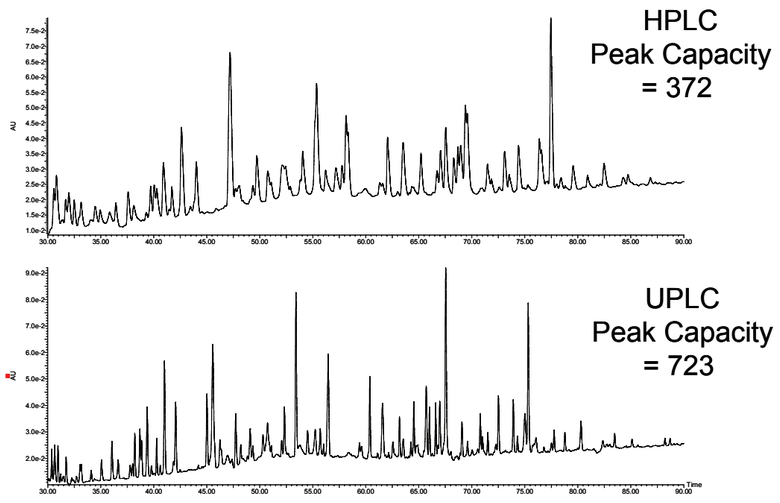


Figure 4. The peptide digest of phosphorylase b was analyzed under HPLC (3.5µm particles) and UPLC (1.7µm particles) conditions. The increased resolution due to short diffusion distances associated with the smaller particles translates into twice the peak capacity in the same length of time.

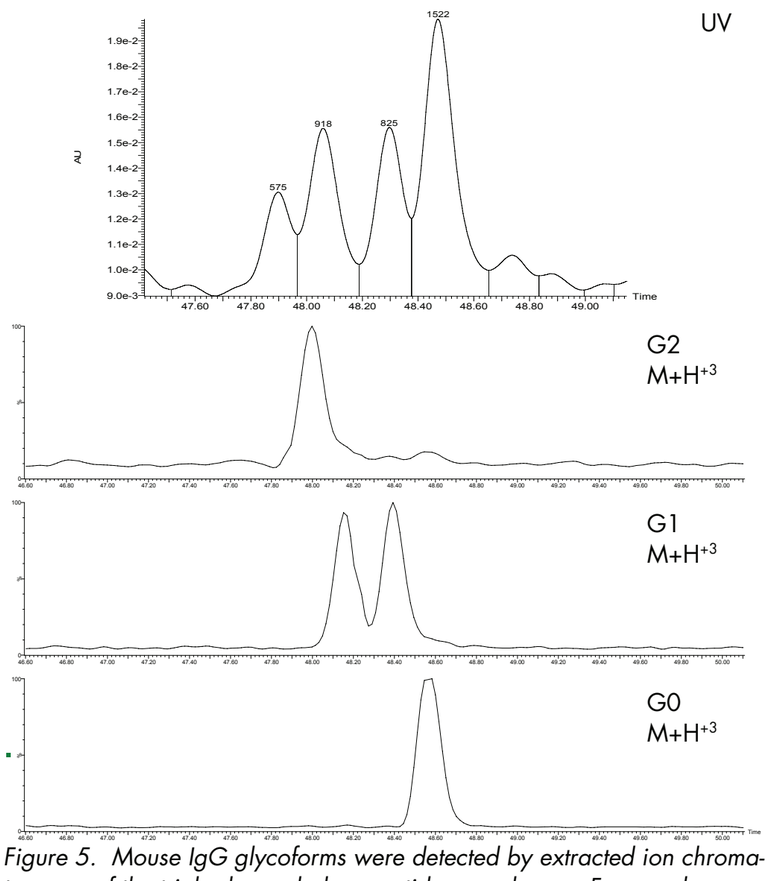
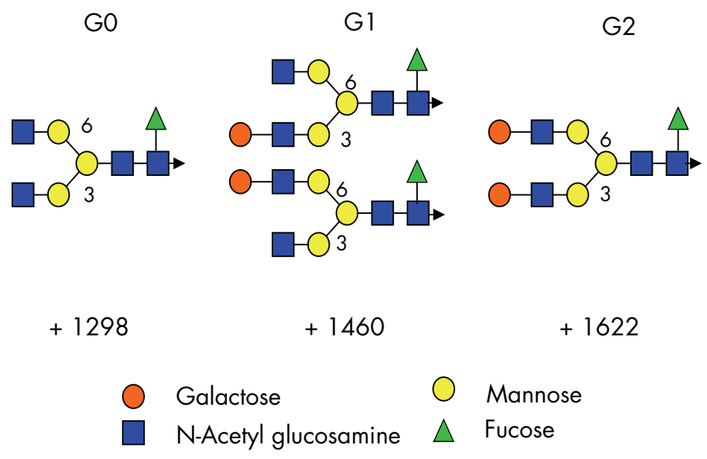


Figure 5. Mouse IgG glycoforms were detected by extracted ion chromatograms of the triply-charged glycopeptides are shown. Four peaks were observed for the three molecular weights indicating that the positional isomers of G1 have been separated by UPLC.

EEQFNSTFR = Peptide Sequence



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 BEH Columns show excellent resolution with different mobile phase modifiers.**

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