Development of a High Resolution, Reproducible HPLC Column for Peptide Analysis

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Overview

The evaluation and selection of an HPLC column that provides the reproducibility and performance necessary to achieve reliable results is essential. Reproducibility of retention time and resolution of critical pairs are essential requirements of an effective peptide map. Modern packing and particle sizing technologies have provided the means to develop columns that exhibit high plate counts. Such columns are highly desirable when one is faced with the task of developing a method for separating peptides resulting from the enzymatic digestion of a complex protein pharmaceutical such as a monoclonal antibody (Mab). We have developed and designed two column chemistries intended for utilization in peptide separations, each column differing in pore size, silanol activity and hydrophobicity. This study shows data of column batch-to-batch consistency as well as the selectivity offered by each of the column chemistries. Columns evaluated in this study contained 3µm particles and were 25 cm in length. Results of the study showed that these columns produce very high resolution peptide maps and can be used in LC-UV or LC-MS methodologies.

Experimental

Waters BioSuite™ peptide mapping MS/MS system
Waters® 2796 Bioseparations Module
Waters Micromass® Q-TOF micro™
Waters® 2487dual wavelength detector, Abs 214 nm

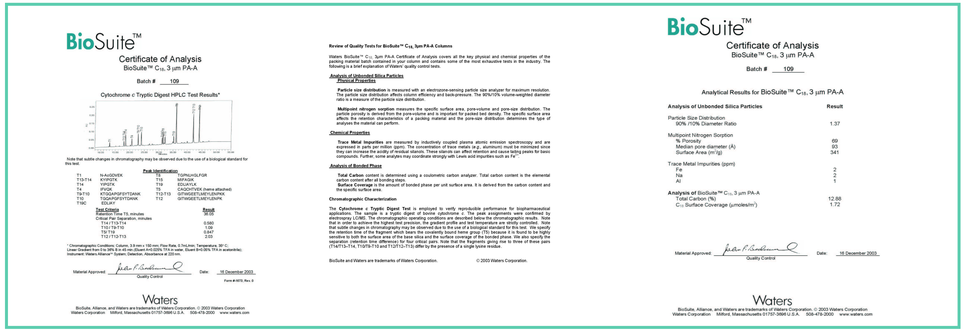
Column(s): Prototype BioSuite™ C_{18}, 5 µm, 100A PA-A, 2.1 x 250mm
Waters Biosuite™ C_{18}, 3 µm, 100A PA-A, 2.1 x 250mm
Waters Biosuite™ C_{18}, 3.5 µm, 300A, PA-B, 2.1 x250mm
Competitor X C_{18}/C_{12}, 4 µm, 90A, 2.0 x 250 mm
Competitor Y C_{18}, 5 µm, 300A, 2.1 x 250 mm

Separation Conditions:
Column temperature: 40.0°C
Flow rate: 0.2 mL/min
Gradient elution: refer to figure legend
Mobile phase: A: 0.02% TFA
B: 0.016%TFA/MeCN
or
A: 0.1% formic acid
B: 0.1%formic acid/ MeCN

MS conditions:

Mode:	ESI +
Capillary:	3300 V
Cone:	30 V
Desolvation gas flow:	500 L/hr
Cone gas flow:	50 L/hr
Source temperature:	150 °C
Desolvation temperature:	350 °C

Sample preparation:
IgG1 was obtained from mouse ascites and purified using a protein G column (Amersham Biosciences). The purified Mab was concentrated by TCA precipitation and resuspended in ammonium bicarbonate buffer, pH 7.8, and digested overnight with trypsin (Promega) at 37.0°C.
Bovine cytochrome c was digested in ammonium bicarbonate buffer, pH 7.8, with trypsin (Promega) at 37.0°C overnight.
Enolase digest was obtained by using Waters MassPREP™ enolase digestion standard.



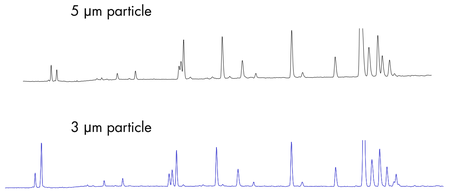


Figure 1: Baseline view showing increased resolution in the separation of enolase digest using columns packed with 3µm versus columns packed with conventional 5µm particles. Gradient: 0-56%B/116 min

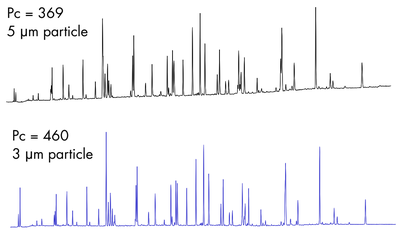


Figure 2: Increase in peak capacity using columns packed with 3µm particles versus column packed with conventional 5µm particles. Peak capacity (Pc) was calculated using the equation: $Pc = 1 + (t_g/W_{13.4h})$. Gradient: 0-56%B/116 min

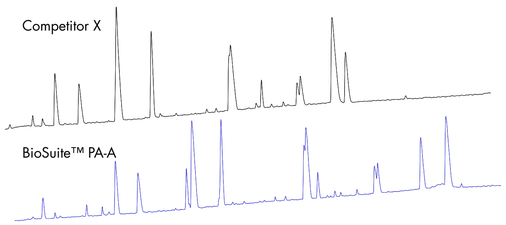


Figure 3: Separation of Bovine Cytochrome c digest on competitor x versus Waters BioSuite™ PA-A column under LC/MS compatible conditions. Gradient: 0-28%B/80min

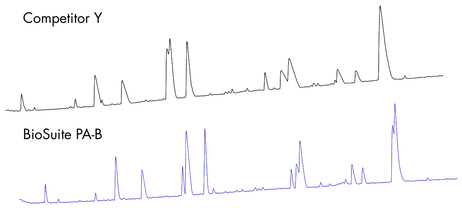


Figure 4: Separation of Bovine Cytochrome c digest on competitor y versus Waters BioSuite™ PA-B column under LC/MS compatible conditions. Gradient: 0-28%B/80min

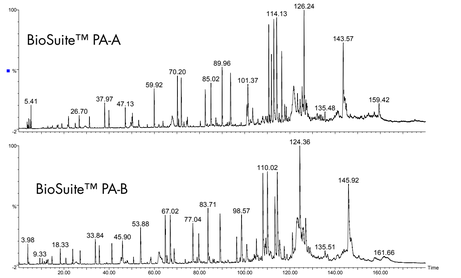


Figure 5: BioSuite™ PA-A and PA-B selectivity difference shown in total ion chromatograms of Mab tryptic digest. Gradient: 0-40%B/180min

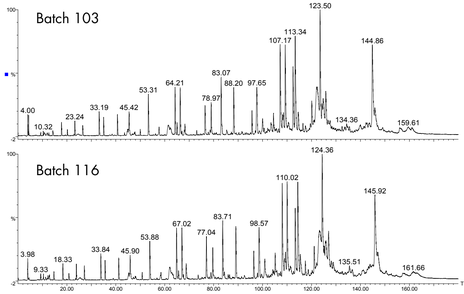


Figure 6: Batch-to-batch reproducibility of BioSuite™ PA-B columns. Gradient: 0-40%B/180min

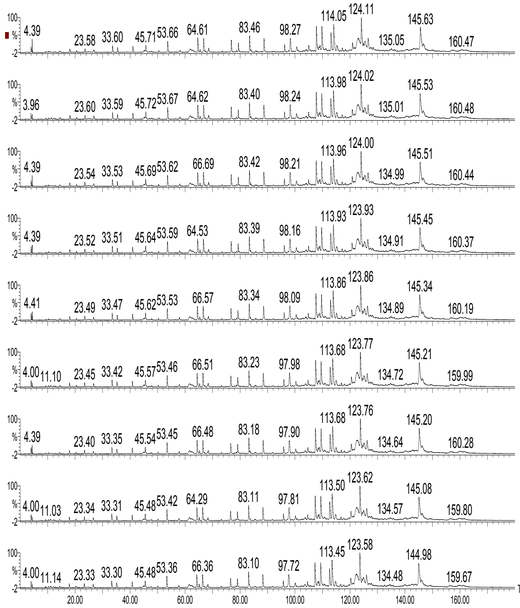


Figure 7: Run-to-run reproducibility in separation of Mab digest Gradient: 0-40%B/180min

Conclusions

Columns packed with 3µm particles offer better resolution and increased peak capacity as compared to columns parked with 5µm particles.

Excellent peak shape and resolving power was observed under LC/MS compatible conditions.

Reproducible peptide maps of a Mab digest was obtained using columns of different lots.