# Waters

Paul Rainville, Himanshu Gadgil, Da Ren, Reb Russell and Jeff Mazzeo Waters Corporation, Chemical Applied Technology, 34 Maple Street, Milford, MA, 01757

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

Prototype BioSuite™ C<sub>18</sub>, 5 µm, 100A PA-A, 2.1 x 250mm Enolase digest was obtained by using Waters MassPREP™ enolase Column(s): Waters Biosuite<sup>™</sup> C<sub>18</sub>, 3 µm, 100A PA-A, 2.1 x 250mm Waters Biosuite<sup>™</sup> C<sub>18</sub>, 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<sub>18</sub>, 5 µm, 300A, 2.1 x 250 mm

BioSuite, Alliance, and Waters are trademarks of Waters Corporation. © 2003 Waters Corporation Waters Corporation Milford, Massachusetts 01757-3696 U.S.A. 508-478-2000 www.waters.com

eparation Condition	s:
olumn temperature:	40.0°C
ow rate:	0.2 mL/min
radient elution:	refer to figure legend
obile phase:	A: 0.02% TFA
	B: 0.016%TFA/MeCN
	or
	A: 0.1% formic acid
	B: 0.1%formic acid/ N
<b>o</b>	
S conditions:	Mode:
	Capillary:

Cone: Desolvation gas flow: Cone gas flow: Source temperature: Desolvation temperature:

Sample preparation:

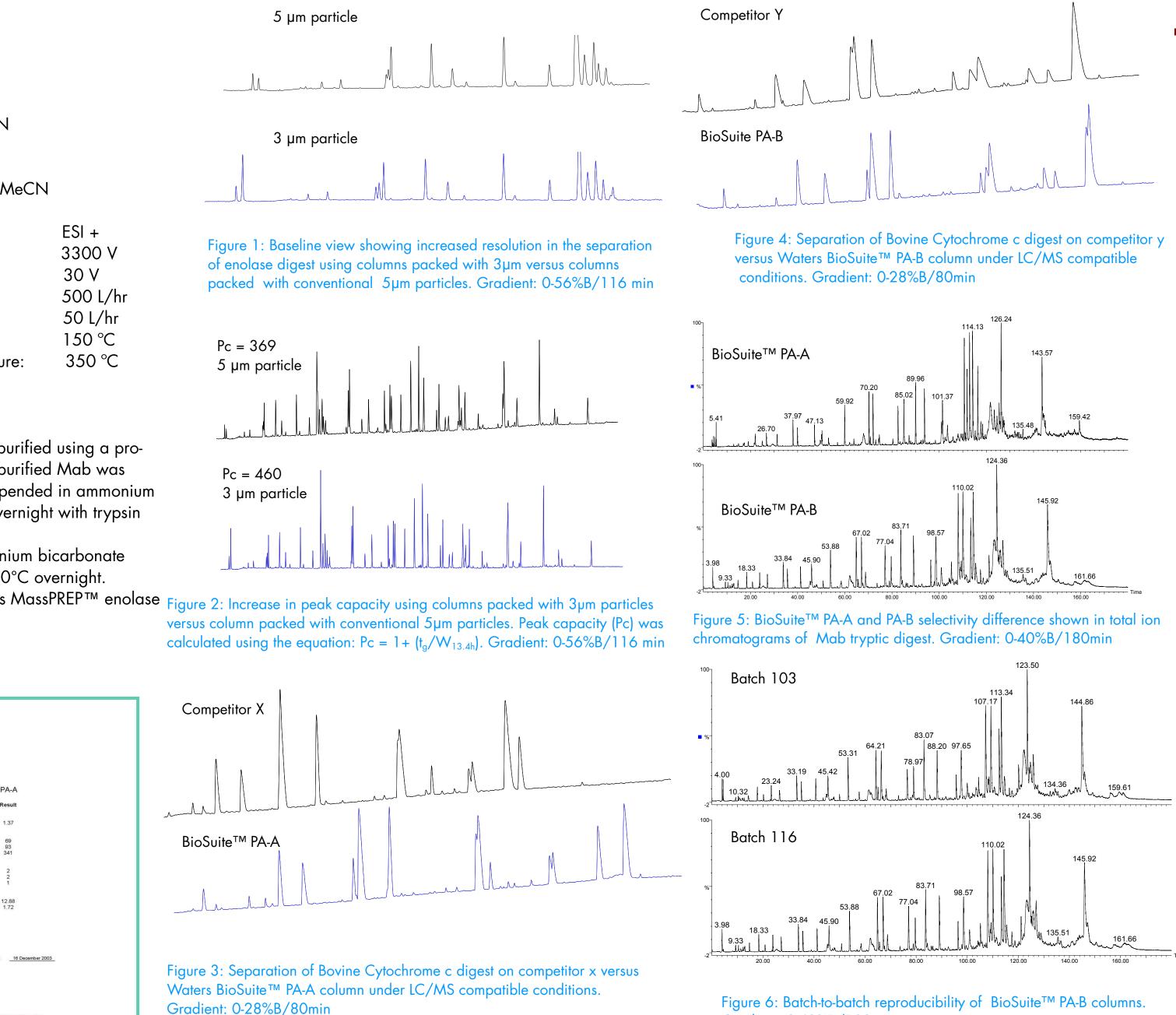
IgG1 was obtained from mouse ascites and purified using a protein G column (Amersham Biosciences). The purified Mab was concentrated by TCA precipitation and resuspended in ammonium bicarbonate buffer, pH 7.8, and digested overnight with trypsin (Promega) at 37.0°C.

Bovine cytochrome c was digested in ammonium bicarbonate buffer, pH 7.8, with trypsin (Promega) at 37.0°C overnight. digestion standard.

<b>Bio</b> Suite <sup>™</sup>		<b>Bio</b> Suite <sup>™</sup>
	Review of Quality Tests for BioSuite™ C <sub>18,</sub> 3µm PA-A Columns	Certificate of BioSuite™ C <sub>18</sub> .3
Certificate of Analysis BioSuite™ C <sub>18</sub> , 3 μm PA-A	Waters BioSulte™ Cr <sub>63</sub> 3µm PA-A Certificate of Analysis covers all the key physical and chemical properties of the packing material batch contained in your column and contains some of the most exhaustive tests in the industry. The following is a brief explanation of Waters' quality control tests.	BioSulie ····· C <sub>18</sub> , 3 Batch #
Batch #109	Analysis of Unbonded Silica Particles Physical Properties	
Cytochrome <i>c</i> Tryptic Digest HPLC Test Results*	Particle size distribution is measured with an electrozone-sensing particle size analyzer for maximum resolution. The particle size distribution affects column efficiency and back-pressure. The 90%/10% volume-weighted diameter ratio is a measure of the particle size distribution.	Analytical Results for BioSuit
0.25- PP 52- 04- 22- 14- 22- 14-	Multipoint nitrogen sorption measures the specific surface area, pore-volume and pore-size distribution. The particle porosity is derived from the pore-volume and is important for packed bed density. The specific surface area	Analysis of Unbonded Silica Particles
830 ⊋ <sup>815</sup> ₽∞	particle processly is derived in on the processioning and is important for packed used density. The specific surface area affects the retention characteristics of a packing material and the pore-size distribution determines the type of analyses the material can perform.	Particle Size Distribution 90% /10% Diameter Ratio
111 14 10 10 10 10 10 10 10 10 10 10 10 10 10	<u>Chemical Properties</u>	Multipoint Nitrogen Sorption
200 <u><u><u></u></u> <u><u></u> <u></u> <u></u> <u></u> <u></u> <u></u> <u></u> <u></u> <u></u> <u></u> <u></u></u></u>	Trace Metal Impurities are measured by inductively coupled plasma atomic emission spectroscopy and are expressed in parts per million (ppm). The concentration of trace metals ( $e_{J,A}$ atominum) must be minimized since they can increase the acidity of residual silanois. These silanois can affect retention and cause tailing peaks for basic compounds. Further, some analytees may coordinate strongly with Lewis acid impurities such as Fe <sup>2+</sup> .	% Porosity Median pore diameter (Å) Surface Area (m²/g)
Note that subtle changes in chromatography may be observed due to the use of a biological standard for this test.	Analysis of Bonded Phase	Trace Metal Impurities (ppm)
T1 N-AcGDVEK T8 TGPNLHGLFGR	Total Carbon content is determined using a coulometric carbon analyzer. Total carbon content is the elemental	Fe Na
T13-T14 KYIPGTK T15 MIFAGIK T14 YIPGTK T19 EDLIAYLK	carbon content after all bonding steps. Surface Coverage is the amount of bonded phase per unit surface area. It is derived from the carbon content and	AI
T4 IFVQK T5 CAQCHTVEX (heme attached) T9-T10 KTGQAPGFSYTDANK T12-T13 GTWGEETLMEYLENPKK T10 TGQAPGFSYTDANK T12 GTWGEETLMEYLENPK	the specific surface area.	Analysis of BioSuite™ C <sub>18</sub> , 3 µm PA-A
T19C EDLIAY	Chromatographic Characterization	Total Carbon (%) C <sub>18</sub> Surface Coverage (μmoles/m²)
Test Criteria Result   Retention Time 15, minutes 36.05   Critical Pair Separation, minutes 10.09   T14 / T13-T14 0.580   T10 / T9-T10 1.09   T5/ T19 0.847   T12 / T12-T13 2.03   * Chromatographic Conditions: Column, 3.9 mm x 150 mm; Flow Rate, 0.7mL/min; Temperature, 35° C; Linear Gradient fom 10 b 39%. B nd 5 min; Eluent A=0.025% TFA in water; Eluent B=0.05% TFA in action/trije;	The <b>Cytochrome c Tryptic Digest Test</b> is employed to verify reproducible performance for biopharmaceutical applications. The sample is a tryptic digest of bovine cytochrome c. The peak assignments were confirmed by electrospray LC/MS. The chromatographic operating conditions are described below the chromatographic results. Note that in order to achieve the highest test precision, the gradient profile and test temperature are strictly controlled. Note that subtle changes in chromatography may be observed due to the use of a biological standard for this test. We specify the retention time of the fragment which bears the covariently bound heme group (T5) because it is found to be highly sensitive to both the surface area of the base silica and the surface coverage of the bonded phase. We also specify the separation (retention time of the order (for four critical paties: Note that the fragments giving rise to three of these pairs (T14/T13-T14, T10/T9-T10 and T12/T12-T13) differ by the presence of a single lysine residue.	Material Approved: JEan P. Backman
Instrument: Waters Alliance <sup>™</sup> System; Detection, Absorbance at 220 nm.	BioSuite and Waters are trademarks of Waters Corporation.	Material Approved:Quality Control
Material Approved: hear f. Backer Date: 16 December 2003		

BioSuite, Alliance, and Waters are trademarks of Waters Corporation. © 2003 Waters Corporation Waters Corporation Milford, Massachusetts 01757-3696 U.S.A. 508-478-2000 www.waters.com

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



Gradient: 0-40%B/180min

100 <b>4.39</b>	23.58	33.60	45.71 <sup>53.66</sup>	64.61	83.46	98.27 	114.05 <sup>124.11</sup>	145.6 135.05	3	
100 % 3.96 _2	23.60	33.59	45.7253.67	64.62	83.40	98.24 	113.98 <sup>124.02</sup>	145.5	3	
100 % 4.39	23.54	33.53	45.6953.62	66.69	83.42	98.21 	113.96 <sup>124.00</sup>	145.5	1 160.44	
100 % 4.39	23.52	33.51	45.6453.59	64.53	83.39	98.16 	113.93 <sup>123.93</sup>	145.4	5160.37	
100 % 4.41	23.49	33.47	45.6253.53	66.57	83.34 	98.09 	113.86 <sup>123.86</sup>	145.3 134.89	4 160.19	
100 % 4.0011.10	23.45	33.42	45.57 <sup>53.46</sup>	66.51	83.23	97.98 	113.68 <sup>123.77</sup>	145.2 134.72	1159.99	
100 % 4.39	23.40	33.35	45.5453.45	66.48	83.18	97.90	113.68 <sup>123.76</sup>	145.20 134.64	0 160.28	
100 <u>4.00</u> 11.03	23.34	33.31	45.48 <sup>53.42</sup>	64.29	83.11	97.81	113.50 <sup>123.62</sup>	145.00 134.57	8 159.80	
<sup>100</sup> % 4.0011.14	┯╇┯╇┯╇┯	┯┯ᡧᠰ᠋ᢩ᠇┯	45.48 <sup>53.36</sup>	66.36	83.10	97.72 	113.45 <sup>123.58</sup>	134.48	159.67	Time
	20.00	40	.00 60	).00	80.00	100.00	120.00	140.00	160.00	

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.