Rapid Peptide Mapping Method with High Resolution Using a sub 2-µm Column

Application Note

Biopharmaceuticals

Authors

Laurie Jones, Robert W. Bailey and Sururat Coulibaly
Amgen Inc.
1201 Amgen Court West
Seattle, WA 98119
USA

Judy Berry
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19809-1610
USA

Abstract

A high-speed method was developed for peptide mapping that offers a high degree of column resolution and excellent retention time and peak area reproducibility. The method uses a sub 2-µm column that gives complete sequence coverage and a reduction in run time from 214 min obtained with conventional 5-µm particle columns, to 60 min with no loss in peak capacity. A flow rate of 0.5 mL/min allows the use of MS detection to yield quality mass spectral data for peptide identification and gives the analyst the versatility of using conventional HPLC instrumentation without the need for high-pressure pumps.

Agilent Technologies

Introduction

Peptide mapping is one of the most important and widely used techniques in protein characterization and is used in various stages of the development and production of biopharmaceutical proteins to ensure product integrity and stability. The sensitivity of the peptide map to even the smallest change in the covalent structure of the protein makes it a valuable 'fingerprint' for identity testing and process monitoring. Peptide maps are particularly useful for comparison of different lots of a therapeutic protein for quality control. This technique also provides a wealth of information about newly discovered proteins that includes expression errors, mutations, location of glycosylation, disulfide linkages, and post translational modifications. Because peptide mapping can easily detect degradation products derived from deamidation, oxidation, and a host of other covalent modifications, it is an important procedure for determining the shelf life of a product during stability studies (1-2).

A protein is selectively cleaved by enzymes (proteases) or by chemical digestion into smaller fragments in peptide mapping. After the protein is digested, the peptide fragments are typically analyzed by gradient reversed-phase high performance liquid chromatography (HPLC) using an acetonitrile (ACN)/water gradient with added trifluoroacetic acid (TFA). Retention time precision and peak area precision are critical attributes of effective peptide mapping analyses (3).

A high-speed method for peptide mapping that uses a sub 2-µm column was developed for a protein digest that gave a significant reduction in run time from 214 to 60 min, as compared to peptide mapping with conventional 5-µm particles, with no loss in sequence coverage and critical mass spectral data. A high level of resolution and peak capacity with excellent peak retention time and peak area precision was achieved. This work shows that the use of smaller particle HPLC columns can enhance laboratory productivity while maintaining a high level of data integrity with conventional HPLC instrumentation.

Experimental

A reduced and alkylated protein was deglycosylated with PNGase F and then digested with either trypsin or Lys-C endopeptidase. The protein digest was evaluated using three reversed-phase columns with slightly different bonded-phase chemistries and column dimensions. Two of the columns had standard 5-µm particles. The third column was shorter with 1.8-µm particles. The columns tested are included in Table 1.

Table 1. Columns Used in Peptide Mapping

1. Agilent ZORBAX 300SB-C18, 2.1 mm × 150 mm, 5 µm
2. Phenomenex Jupiter C5, 2.0 mm × 250 mm, 5 µm
3. Agilent ZORBAX Solvent Saver High Throughput (HT) SB-C3, 3.0 mm × 100 mm, 1.8 µm, 600 bar (p/n 828975-309)

The protein digests were analyzed using gradient reversed-phase HPLC with an acetonitrile/water mobile phase modified with trifluoroacetic acid (TFA) on Agilent 1100 LC and 1200 RRLC Series HPLC systems with a binary pump. Peptides were detected using a diode array detector (DAD) at 214 nm and identified by ion trap MS. The analysis time of the deglycosylated Lys-C protein digest on the columns with the conventional 5-µm particles was 214 min. Experimental conditions for the 214-min method are included in Table 2.

Table 2. Experimental Conditions for 214-Min Run on 5-um Columns

LC:	Agilent Series 1100 and 1200 Series binary pump SL for rapid resolution	
Mobile phase:	Solvent A: H_2O (0.12% w/v TFA) Solvent B: 80/20 ACN (0.11% w/v TFA)/H_2O (0.11% w/v TFA)	
Gradient:	t(min)	%B
	0	1.0
	5.0	1.0
	165.0	65.0
	175.0	100.0
	177.0	100.0
	182.0	1.0
Post time	32 min re-equilibration	
Column temperature:	40 °C	
Flow rate:	0.2 mL/min	
Detection:	DAD at 214 nm and Agilent LC/MSD Trap SL	
Injection volume:	50 µL	
Sample:	Lys-C protein digest	

Trypsin and Lys-C digests were analyzed on a ZORBAX Solvent Saver HT SB-C3 with 1.8-µm particles that resulted in a shorter run time of 60 min. The experimental conditions for the 60-min run are listed in Table 3.

Table 3. Experimental Conditions for 60-Min Run on 1.8-um Column

LC:	Agilent 1100 and 1200 Series Binary Pump SL for Rapid Resolution	
Mobile Phase:	Solvent A: H_2O (0.12% wt/v TFA) Solvent B: 80/20 ACN (0.11% w/v TFA)/H_2O (0.11% w/v TFA)	
Gradient	t(min)	%B
	0	1
	3	1
	53	57
	58	100
	60	100
	60.1	1
Post Time	5 min re-equilibration	
Column Temp.	60 °C	
Flow Rate	0.5 mL/min	
Detection	DAD at 214 nm and Agilent LC/MSD Trap SL	
Injection Volume	50 µL	
Sample	Lys-C and trypsin protein digests	

Results and Discussion

A Lys-C endopeptidase protein digest was analyzed on two different reversed phase 5-µm columns with slightly different bonded phase chemistries using a run time of 214 minutes. This method gave good sequence coverage but had a lengthy analysis time. The same protein digest was also run on a shorter ZORBAX Solvent Saver HT SB-C3 column with 1.8-µm particles that resulted in a significantly shorter run time of 60 minutes with no loss of sequence coverage. A comparison of the peptide maps obtained from all three columns using UV detection at 214 nm is shown in Figure 1.

The 60-min method results show that the significant decrease in analysis time did not reduce resolution or peak capacity. In fact, more peaks were resolved in the second half of the chromatogram on the Solvent Saver HT SB-C3 column, compared to the C18 and C5 columns, due to fewer co-eluting peptides. Also noteworthy is the improvement in peak shape of the peak circled at 26 min. The high-speed method also offered

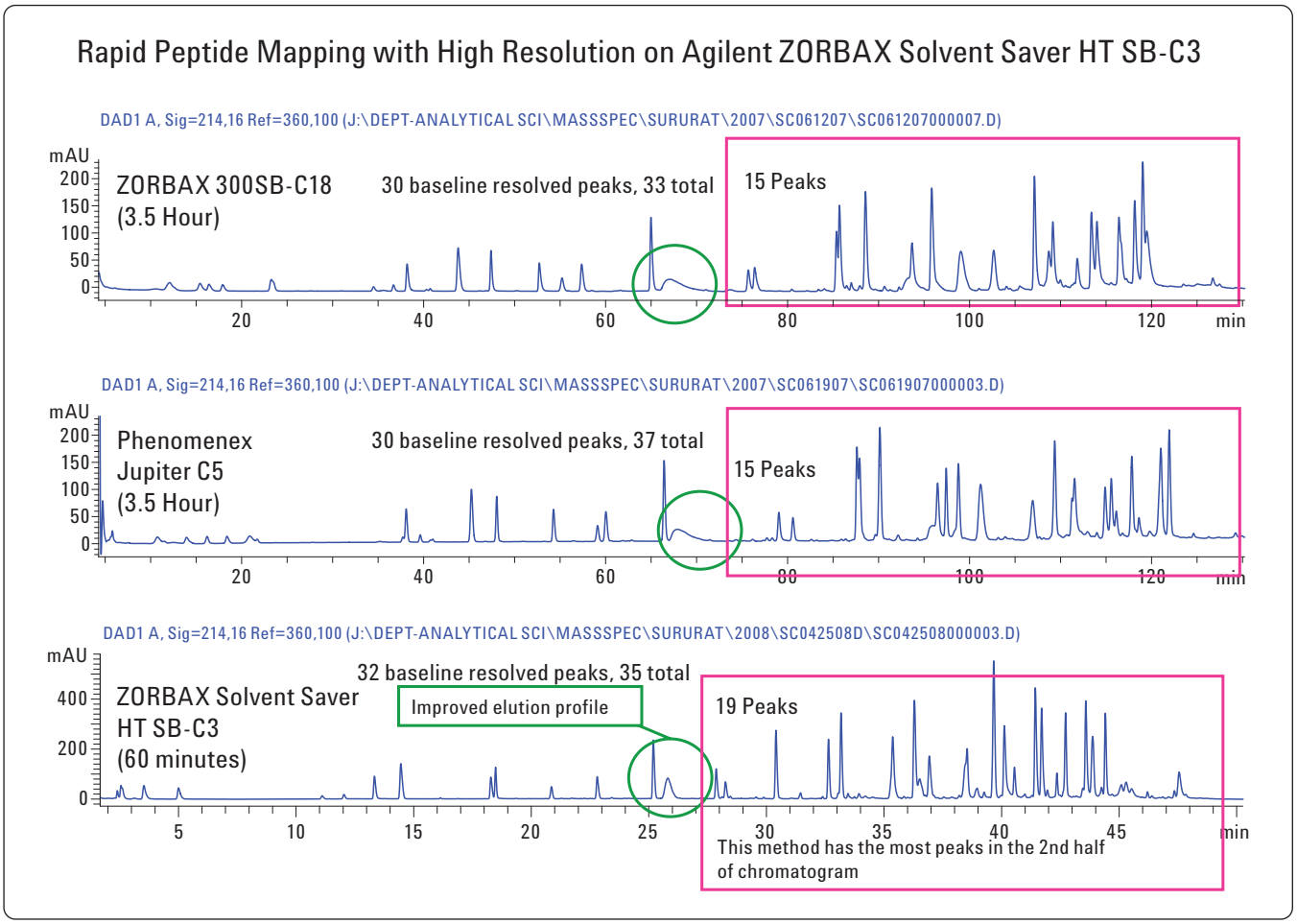

Figure 1. Comparison of chromatograms of Lys-C digest on three different reversed-phase columns. Experimental conditions listed in Tables 2 and 3.

the same sequence coverage of 99.1%, compared to the longer method as verified by ion trap MS analysis. An additional benefit is minimal run-to-run sample carryover. A comparison of the three methods is shown in Table 4 highlighting the benefits of the shorter run time on the 1.8-µm column.

Table 4. Method Comparison of Three Reversed-Phase Columns Based on Essential Peptide Mapping Criteria

Column	Run Time (min)	Ion trap ms/ms	Sequence coverage	% Sample carryover	No. of peaks
ZORBAX 300SB-C18, 5 µm	214	Good	99.1	6.1	33
Phenomenex Jupiter C5, 5 µm	214	Good	99.1	2.9	37
ZORBAX Solvent Saver HT SB-C3, 1.8 µm	60	Good	99.1	0.8	35

Reproducibility of retention time (RT), peak area and % peak area were determined for within-day and between-day analysis of a tryptic digest. Figure 2 shows the overlay of six injections of a tryptic digest on two different days with Peaks A and B representing early and late eluting peaks, respectively. Visually, the overlays for each day are identical and show excellent within-day reproducibility. Table 5 includes the statistics for standard deviation (SD) and % relative standard deviation (%RSD) for RTs, peak areas, and % peak area for Peaks A and B on Day 1. Similar data were obtained for Day 2. Table 6 shows the combined data of six injections made on two different days for Peaks A and B. Precision data were obtained indicating that the method has excellent between-day reproducibility. Overall, the method has the necessary reproducibility that is critical to peptide mapping, so that any differences in chromatograms of seemingly identical protein digests can be attributed to the samples themselves, not the chromatographic system or method.

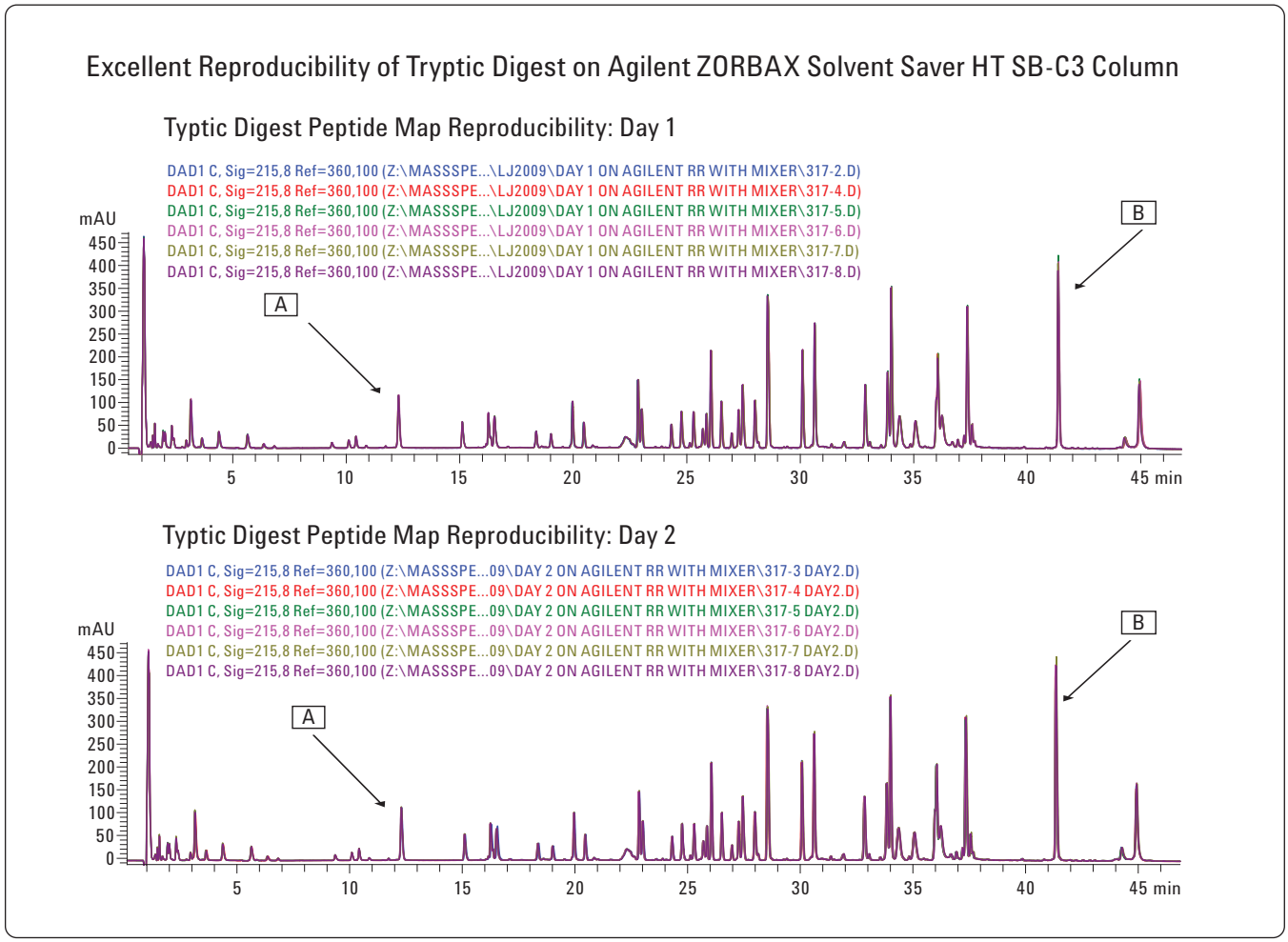

Figure 2. Six overlaid injections of tryptic digest analyzed on two different days.

Table 5. Within-Day Precision Data for Day 1 of Peaks A and B in Peptide Map

Injection	Peak A area	Peak A RT	Total area	% Peak A area
1	687.2	12.300	31305	2.20
2	686.4	12.296	30657	2.24
3	686.4	12.296	30657	2.24
4	686.4	12.303	31671	2.16
5	691.1	12.312	32348	2.14
6	688.6	12.293	31597	2.18
Average:	687.35	12.30	31372.50	0.02
Std Dev:	2.29	0.01	651.08	0.00
% RSD:	0.33	0.06	2.08	1.90
Injection	**Peak B area**	**Peak B RT**	**Total area**	**% Peak B area**
1	2073.8	41.381	30833	6.73
2	2131.3	41.36	31305	6.81
3	2000.7	41.36	30657	6.53
4	2173.6	41.366	31671	6.86
5	2117.8	41.377	32348	6.55
6	2078.4	41.346	31597	6.58
Average:	2095.93	41.37	31401.83	0.07
Std Dev:	59.38	0.01	615.39	0.00
% RSD:	2.83	0.03	1.96	2.16

Table 6. Inter-day Precision Data for Peaks A and B in Peptide Map

Day	Injection	Peak A area	Peak A RT	Total area	% Peak A area
1	1	687.2	12.3	31305	2.20
1	2	686.4	12.296	30657	2.24
1	3	686.4	12.296	30657	2.24
1	4	684.4	12.303	31671	2.16
1	5	691.1	12.312	32348	2.14
1	6	688.6	12.293	31579	2.18
2	1	685.4	12.317	31619	2.17
2	2	680.4	12.298	31663	2.15
2	3	685.2	12.292	31642	2.17
2	4	687.3	12.286	31512	2.18
2	5	693.9	12.302	32255	2.15
2	6	675.8	12.303	31403	2.15
Average:		686.01	12.30	31527.42	0.02
Std Dev:		4.65	0.01	508.88	0.00
% RSD:		0.68	0.07	1.61	1.53
Day	**Injection**	**Peak B area**	**Peak B RT RT**	**Total area**	**% Peak B area**
1	1	2073.8	41.381	30833	6.73
1	2	2131.3	41.36	31305	6.81
1	3	2000.7	41.36	30657	6.53
1	4	2173.6	41.366	31671	6.86
1	5	2117.8	41.377	32348	6.55
1	6	2078.4	41.346	31597	6.58
2	1	2139	41.327	31619	6.76
2	2	2108.4	41.317	31663	6.66
2	3	2088.9	41.322	31642	6.60
2	4	2149.5	41.329	31512	6.82
2	5	2271.9	41.368	32255	7.04
2	6	2200.6	41.359	31403	7.01
Average:		2127.83	41.35	31542.08	0.07
Std Dev:		68.93	0.02	483.41	0.00
% RSD:		3.24	0.05	1.53	2.55

Conclusion

Peptides can be rapidly analyzed in 60 min, providing a high degree of resolution with excellent peak shapes using a 1.8-µm column. The method offers exceptional reproducibility and sequence coverage with minimal run-to-run sample carry-over. A flow rate of 0.5 mL/min allows the use of a mass spectrometer for accurate peptide identification with back pressures that permit the use of optimized conventional HPLC systems. This peptide mapping method demonstrates how laboratory productivity can be improved by using shorter columns with smaller, more efficient particles to analyze complex samples in less time, while increasing resolution and peak capacity.

References

1. Advances in Chromatography Volume 37 by Phyllis R. Brown, Eli Grushka CRC 1 edition 1997 p. 439
2. New Methods in Peptide Mapping for the Characterization of Proteins (CRC Series in Analytical Biotechnology) by William S. Hancock published by CRC: 1 edition (Oct. 1995).
3. Maria Serwe and Bernd Glatz, "Peptide Separations by Microbore Reversed-Phase HPLC," Agilent Technologies publication 5964-9935E, 1996.

For More Information

For more information on our products and services, visit our Web site at www.agilent.com/chem.

www.agilent.com/chem

Agilent shall not be liable for errors contained herein or for incidental or consequential damages in connection with the furnishing, performance, or use of this material.

Information, descriptions, and specifications in this publication are subject to change without notice.

© Agilent Technologies, Inc., 2009

Printed in the USA
October 13, 2009
5990-4712EN

Agilent Technologies