Tryptic digest analysis using the Agilent 1290 Infinity LC System

Application Note

Drug Development, Production QA/QC

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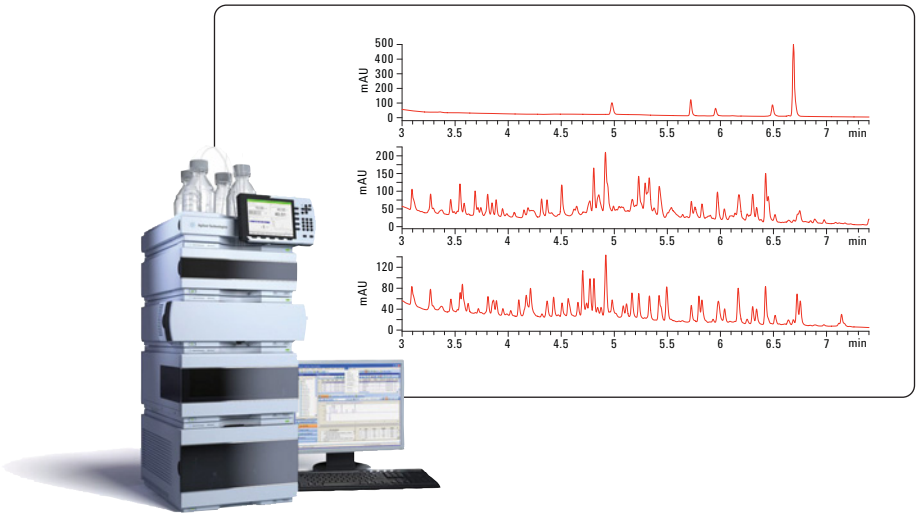

Abstract

This Application Note demonstrates:

- The applicability of the Agilent 1290 Infinity LC System to resolve peptide mix tures of higher complexity.
- A bovine serum albumin (BSA) tryptic digest was separated on a 250 mm × 2.1 mm id × 1.7 µm dp RP-LC column using different gradient slopes and flow rates.
- The maximum pressure applied was 900 bar. Peak capacities from 188 to 851 within total analysis times of 8 and 260 min, respectively, were obtained.

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Introduction

Peptide separations are of great importance in a variety of fields ranging from the characterization of recombinant, therapeutic proteins to proteomics-based biomarker discovery and verification. Sample complexity is enormous with typically hundreds of species encountered in biopharmaceutical preparations and many thousands of peptides in proteomics samples. Evidently, the chromatographer is confronted with an enormous separation challenge.

In this application note, the resolving power of ultra-high pressure LC (UHPLC) using the 1290 Infinity LC system is demonstrated. BSA tryptic digest was separated on a 250 mm × 2.1 mm × 1.7 µm d_p column. Peak capacity and peak capacity productivity, two powerful metrics to evaluate the separation, were determined at different gradient slopes and flow rates.

Note that an *in silico* digest of BSA generates approximately 150 peptides with one miscleavage allowed, and aspecific cleavages not taken into account.

Experimental

Instrumentation and method

An Agilent 1290 Infinity LC system with the following configuration was used:

Part number	Description
G4220A	Agilent 1290 Infinity Binary Pump with integrated vacuum degasser
G4226A	Agilent 1290 Infinity Autosampler
G1316C	Agilent 1290 Infinity Thermostatted Column Compartment
G4212A	Agilent 1290 Infinity Diode Array Detector

Method parameters:			
Column	C18 150 mm × 2.1 mm 1.7 µm C18 100 mm × 2.1 mm 1.7 µm		
Mobile phase	A = 0.10% TFA in water/acetonitrile 98/2 v/v B = 0.08% TFA in acetonitrile		
Flow rate	0.4 mL/min or 0.2 mL/min		
Gradient	0 to 50% B 65% B 0% B	variable time for 10 min for 5 min	(gradient elution) (column rinsing) (column reconditioning)
Temperature	60 °C		
Injection	10 µL		
Detection	DAD, Signal 214/4 nm, Reference 400/60 nm, 40 Hz		

Samples

Tryptic digestion of BSA was carried out in an ammonium bicarbonate buffer at pH 8. Trypsin was added in an enzyme/substrate ratio of 1/50 and the mixture was incubated overnight at 37 °C. Another BSA sample (called BSA RA) was reduced and alkylated prior to digestion. Both samples were acidified with mobile phase A to a concentration of 3 nmol/mL prior to injection. A peptide standard mixture, used to aid in the calculation of the peak capacity, was dissolved in mobile phase A and contained bradykinin 1–5 (5 nmol/mL), angiotensin II (3 nmol/mL), neurotensin (2 nmol/mL), ACTH clip [18-39] (2.5 nmol/mL), and bovine insulin chain B (12.5 nmol/mL).

Results and Discussion

A column length of 250 mm was obtained by coupling two columns (150 and 100 mm) using a stainless steel capillary of 70 mm with an internal diameter of 0.12 mm. Performing a relatively fast gradient analysis of 8% B/min resulted in a fast analysis of the digest (Figure 1). A peak capacity of approximately 190 was generated with this short gradient time (6.25 min). This corresponded to a peak capacity production rate of over 30 peaks/min. Peak capacity was calculated by dividing the gradient time with the average peak width at the base (4σ) determined for five standard peptides (Figure 1). The gradient applied in this note was longer than actually required to elute the last BSA fragments from the column. The reason for this is that this gradient is also applied for the analysis of other digests with more retentive peptides. When only the elution window (3 to 7.5 min) is taken into account for the calculation a peak capacity of 136 is obtained. The peak capacity productivity is not affected however; the number of peaks generated per minute remains the same.

Applying longer, more shallow gradients increases peak capacity and therefore the amount of detail visualized in the chromatogram. Evidently, the price to pay is analysis time. Figure 2 shows the result for the BSA digest analyzed with four different gradient slopes. The peak capacity tripled from 188 to 567 when the gradient time was increased from 6.25 min (8% B/min) to 50 min (1% B/min), respectively. If only the elution window of the BSA digest is taken into account for the 50-min gradient, the peak capacity is 375 in 39 min. It is clear that the chromatogram at the more shallow gradient reveals much more detail, while analysis time remains acceptable. Further increasing

the gradient time leads to a higher peak capacity, but the effect of the flatter gradient becomes less significant from a defined point and the peak capacity productivity becomes nearly fixed. This is summarized in Figure 3. Doubling the gradient time from 50 to 100 min increases the peak capacity by approximately 25% (567 to 711). However, an additional increase in the gradient time to 200 min, produces a gain in peak capacity of only approximately 15% (711 to 820). In the last situation, the analysis time is over 3 h and becomes less practical in routine operation. From Figure 3 it can be deduced that the best compromise between peak capacity and analysis time is obtained with a gradient time of 100 to 150 min.

When the flow rate and gradient slope are reduced to 0.2 mL/min and 0.5% B/min, respectively, the peak capacity increases from 567 to 645 compared to the analysis carried out at 0.4 mL/min and 1% B/min. However, the peak capacity production rate is reduced from 11.3 to 6.4 with this approach. When samples become more complex on the other hand, a moderate increase in resolution can become useful for detecting minor differences between related samples, especially when high-end qualitative detectors such as a mass spectrometer are applied.

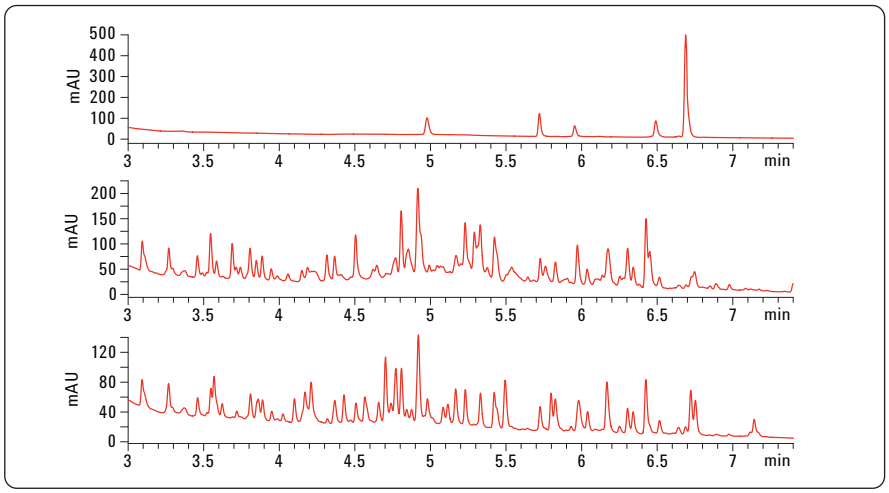


Figure 1
High speed analysis of the peptide standard mixture (upper trace), BSA digest (middle trace) and BSA RA digest (lower trace). Flow rate: 0.4 mL/min, gradient: 0–50% B in 6.25 min.

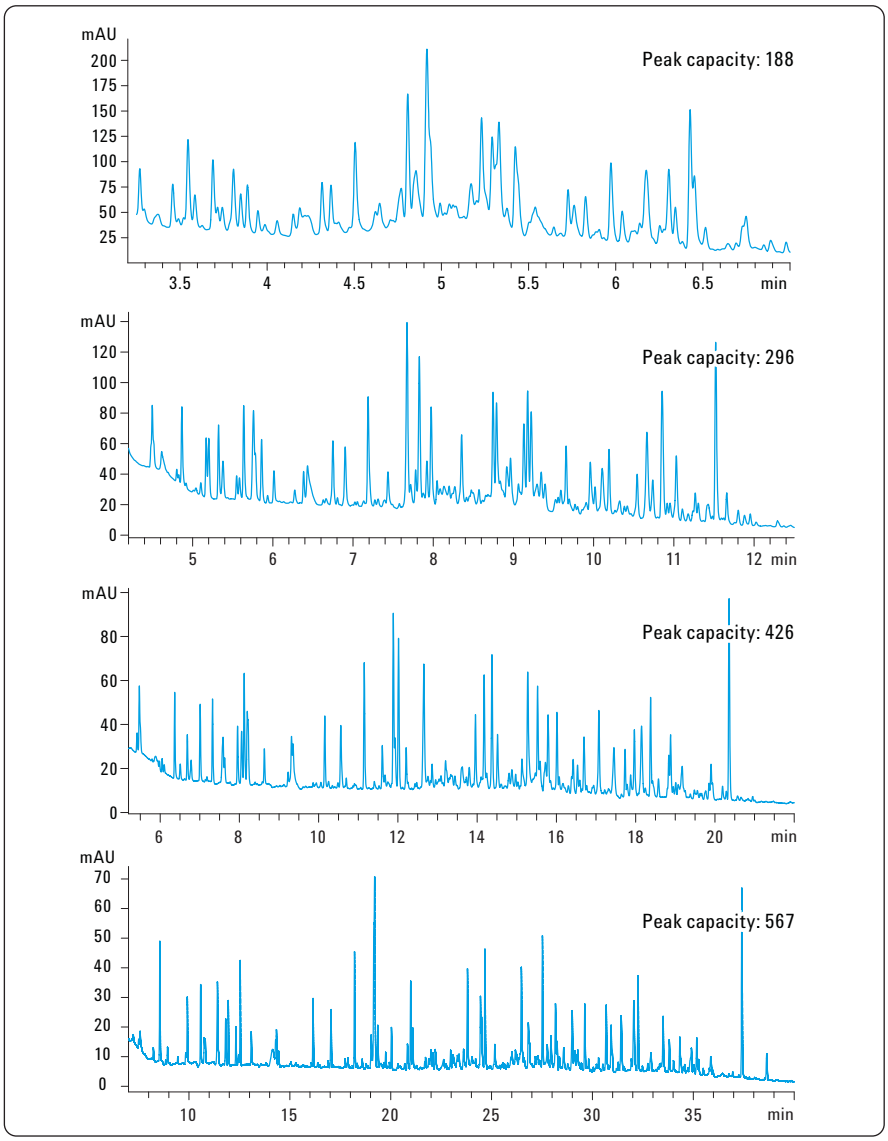
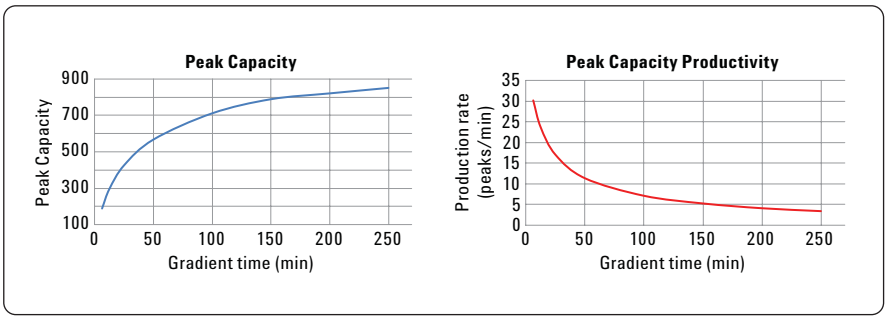


Figure 2
Analyses of the BSA digest with different gradients. Flow rate: 0.4 mL/min, gradient: 0-50%B in 6.25 min (8%/min), in 12.5 min (4%/min), in 25 min (2%/min), and in 50 min (1%/min).

Conclusion

This Application Note demonstrates the versatility of Agilent 1290 Infinity LC system for separating peptide mixtures of high complexity. Protein digests were analyzed on a 250 mm long column packed with 1.7-µm particles and operated at a pressure up to 900 bar. Depending on the need, high productivity (peak capacity of 188 in less than 10 min) or high resolution (peak capacity exceeding 800 in 3h) can be obtained.



Gradient time (min)	Gradient slope (% B/min)	Peak capacity	Peak capacity productivity (peaks/min)
6.25	8	188	30.1
12.5	4	296	23.7
25	2	426	17.0
50	1	567	11.3
100	0.5	711	7.1
150	0.375	788	5.3
200	0.25	820	4.1
250	0.2	851	3.4

Figure 3
Peak capacity and peak capacity production rate in function of gradient time.

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