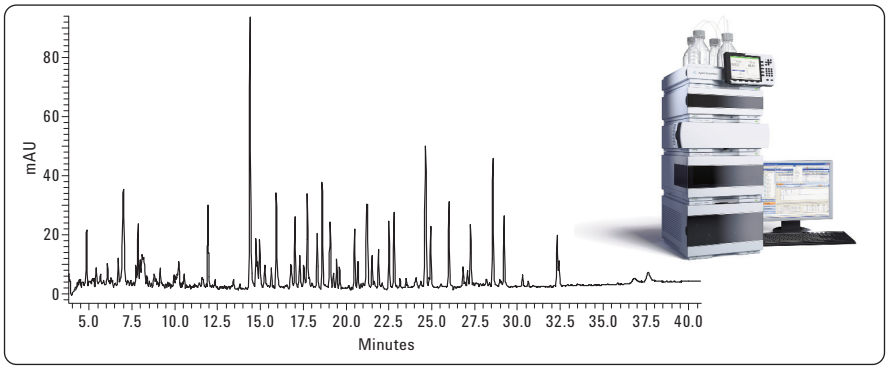
Increased peak capacity for peptide analysis with the Agilent 1290 Infinity LC System

Application Note

Biopharmaceuticals



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Abstract

This Application Note describes the separation of a trypsin-digested protein sample, using the Agilent 1290 Infinity LC System with Agilent ZORBAX Eclipse Plus C18, 150 mm × 2.1 mm, 1.8 µm particles column. Peak capacity variation with change in flow rate and time for various gradients were evaluated. Increase in flow rate from 0.2mL/min to 1.2mL/min effectively improved the peak capacity value to double and increase in gradient time from 5minutes to 50 minutes enhanced a 3.4 fold improvement in peak capacity value. The unique design of the Agilent 1290 Infinity LC System tolerates 1200 bar column back pressure, allowing higher flow rates, and reducing the run time.

Introduction

Peptide mapping is one of the important steps in QA/QC analysis of protein biologics in the biopharmaceutical industry. A significant challenge is the chromatographic separation of complex protein digests for the characterization of proteins. In addition to the large number of peptide fragments, side reactions such as oxidation can happen during enzymatic protein digestion, making the sample more challenging for an efficient separation. Adequate separation for peptide mapping using high performance liquid chromatography (HPLC) with 5-µm columns, often requires long run

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times. Ultra high performance liquid chromatography (UHPLC) separation using sub-2-µm particles improves resolution per time and sensitivity, shortens run times and makes peptide mapping less challenging. The power range of the Agilent 1290 Infinity LC System (max pressure versus flow) overcomes the challenge of increased back pressure caused by smaller particle columns, which often restricts the use of higher flow rates.

Experimental

System Configuration

An Agilent 1290 Infinity LC System with the following configuration was used for the study.

- Agilent 1290 Infinity Binary Pump with integrated vacuum degasser (G4220A)
- Agilent 1290 Infinity Autosampler (G4226A)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1290 Infinity Diode Array Detector (G4212A)

LC Method parameters are tabulated in Table 1 and the gradient used for the study was tabulated in Table 2.

Trypsin was added in an enzyme/substrate ratio of 1/20 and the mixture was incubated overnight at 37 °C. Prior to injection, the sample solution was acidified with mobile phase A in the ratio 3:1. A peptide standard mix from Aldrich which contains five pure standard peptides was used to calculate the peak capacity. The individual components of the standard mix were 1) GLY-TYR, 2) VAL-TYR-VAL, 3) methionoine enkephalin acetate, 4) leucine enkephalin and 5) angiotensin II acetate. The standard sample was diluted with mobile phase A to a level where the individual peak heights were almost matching with that of peaks in the tryptic digested BSA. Both the sample and standard mix were injected in ten different gradient (elution time) trials and each gradient was tried with six different flow rates resulting in 60 different gradients. The average peak widths for five peptides were calculated for each gradient, and these values were used for the calculation of peak capacity for each gradient.

Parameter	Details
Column	Agilent ZORBAX Eclipse Plus C18, 150 mm × 2.1 mm, 1.8 µm. (p/n 959759-902)
Column oven	50 °C
Mobile phase A	0.1% Trifluoroacetic acid in water
Mobile phase B	0.08% Trifluoroacetic acid in acetonitrile
Flow rate	Variable (0.2 mL/min, 0.4 mL/min, 0.6 mL/min, 0.8 mL/min, 1.0 mL/min, 1.2 mL/min)
Needle wash	Flush port activated for 3 sec using mobile phase B
Injection volume	4 µL (Autosampler thermostat was maintained at 5 °C)
Detection	214/4 nm; Reference 400/60 nm
Peak width	> 0.062 s response time (0.003 min)
Data acquisition rate	80 Hz
Post run time	2 min

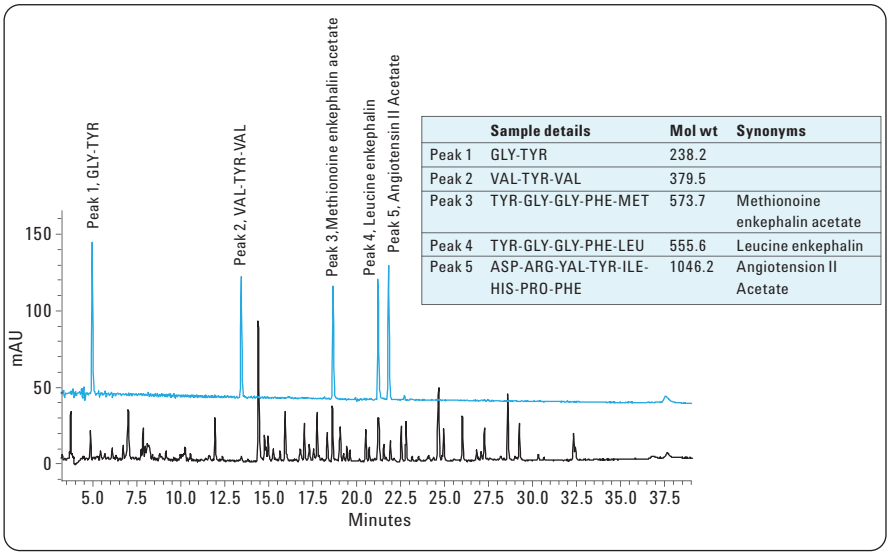
Table 1
LC method details.

%B	Time (min)
0 to 50	Variable (5, 10, 15, 20, 25, 30, 35, 40, 45, 50) -gradient elution
50 to 75	For 5 minutes-column rinsing
75	For 5 minutes-column rinsing
3	For 5 minutes-column reconditioning

Table 2
Gradient used for the study.

Results and disucussion

Representative chromatograms of the BSA digest and standard mix using a single method are as shown in Figure 1.



	Sample details	Mol wt	Synonyms
Peak 1	GLY-TYR	238.2	
Peak 2	VAL-TYR-VAL	379.5	
Peak 3	TYR-GLY-GLY-PHE-MET	573.7	Methionoine enkephalin acetate
Peak 4	TYR-GLY-GLY-PHE-LEU	555.6	Leucine enkephalin
Peak 5	ASP-ARG-YAL-TYR-ILE-HIS-PRO-PHE	1046.2	Angiotension II Acetate

Figure 1
Representative chromatograms of the BSA digest (black trace) and standard mix (blue trace). Individual components of the standard mix are included in the inserted table.

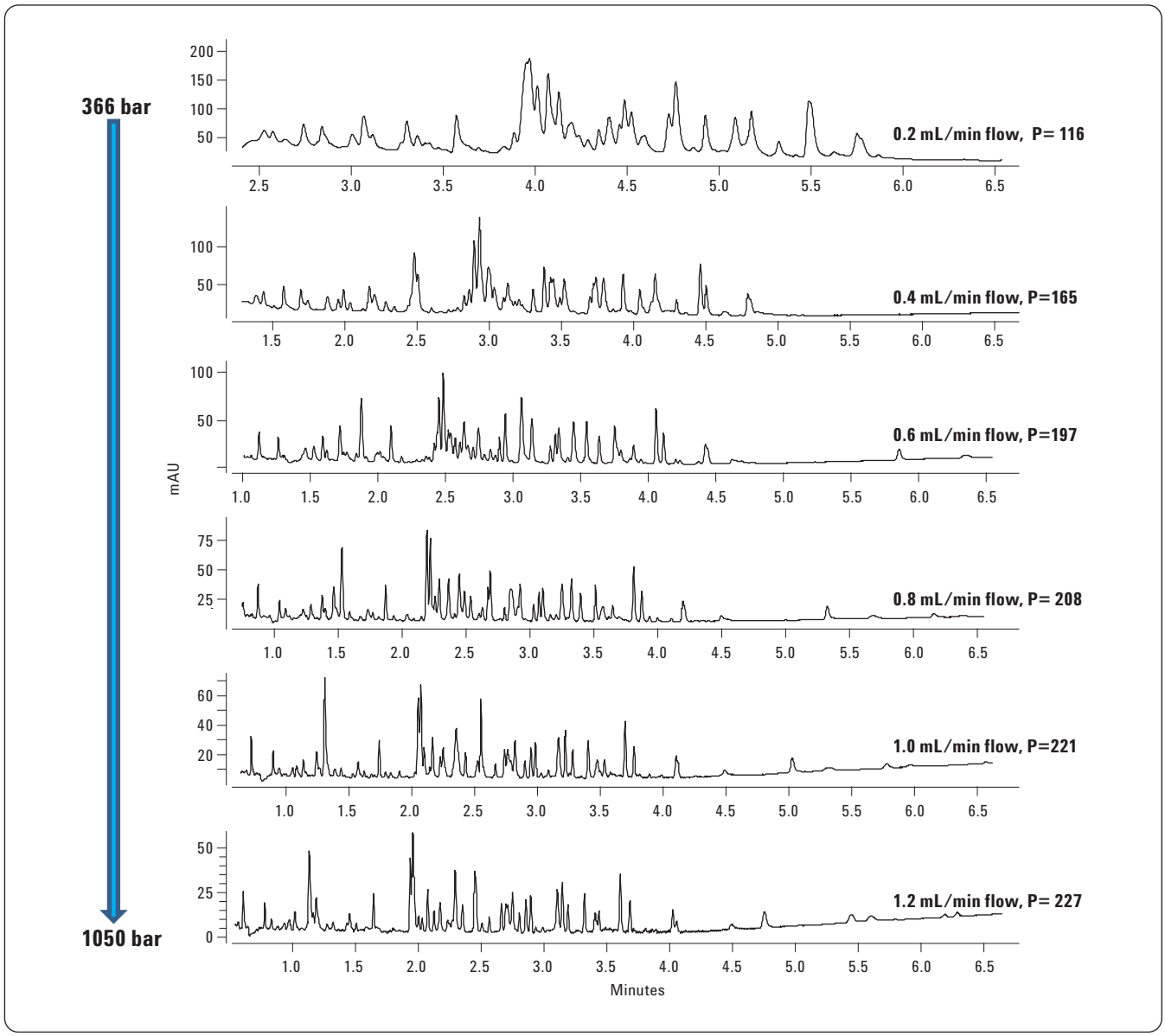


Figure 2
Peak capacity (P) increment with changes in flow rate. Run time is constant.

Peak capacity change with flow rate

Increasing peak capacity (P) while maintaining the column length can be achieved by varying the flow rate, gradient slope or a combination of both. Increasing the flow rate from 0.2 mL/min to 1.2 mL/min leads to an increase of twice the peak capacity values (from approximately 116 to 227) for a 5-min gradient. The equation used for peak capacity was P= 1+ (gradient time/average of 5 peak widths). The observed pressure increment was from approximately 366 bar to 1050 bar. The results are as shown in Figure 2. The data underline the advantages of the large pressure range of the Agilent 1290 Infinity LC System.

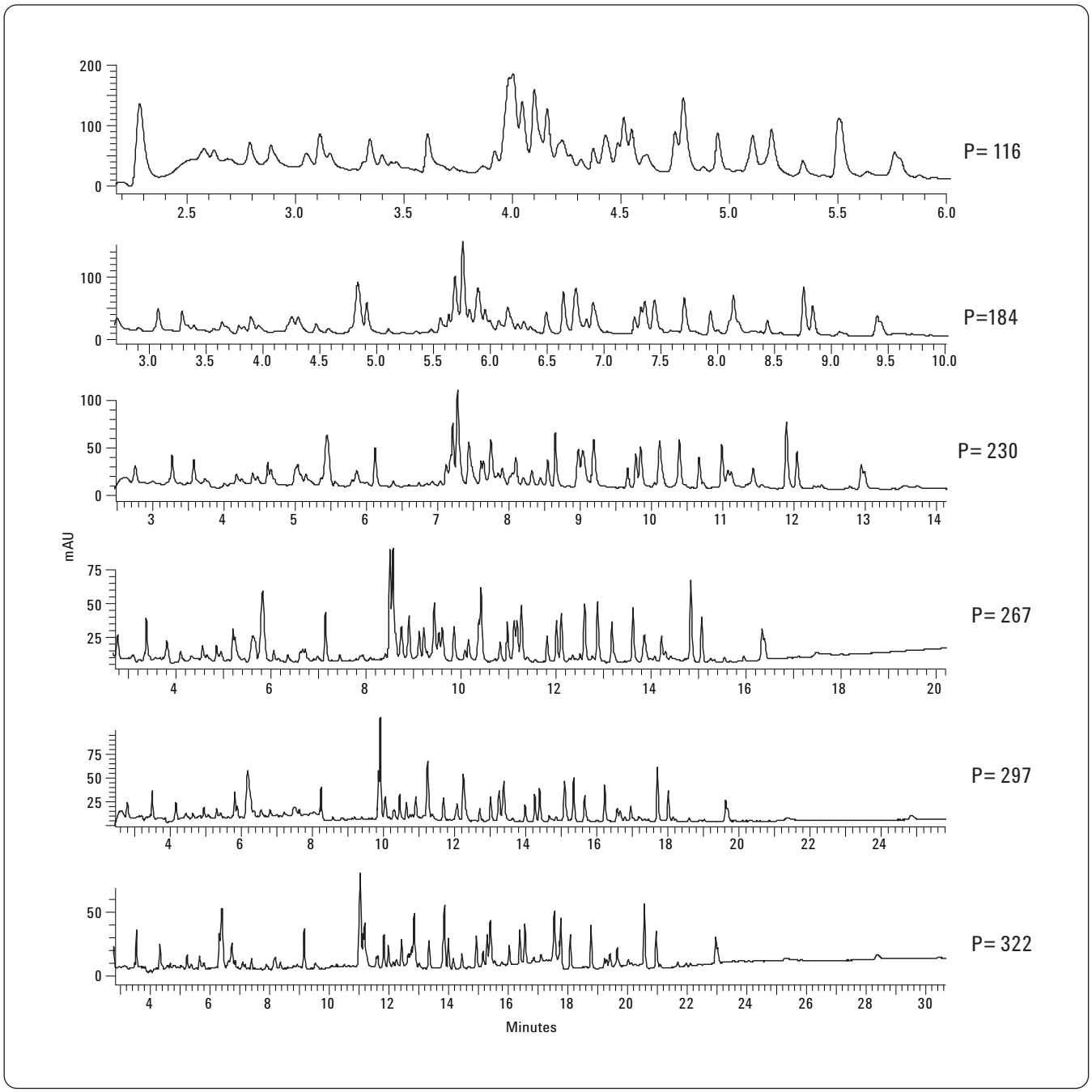


Figure 3
Peak capacity increment with change in gradient time. Observed peak capacity values (P) are marked. A constant (0.2 mL/min) flow rate was used. (continued)

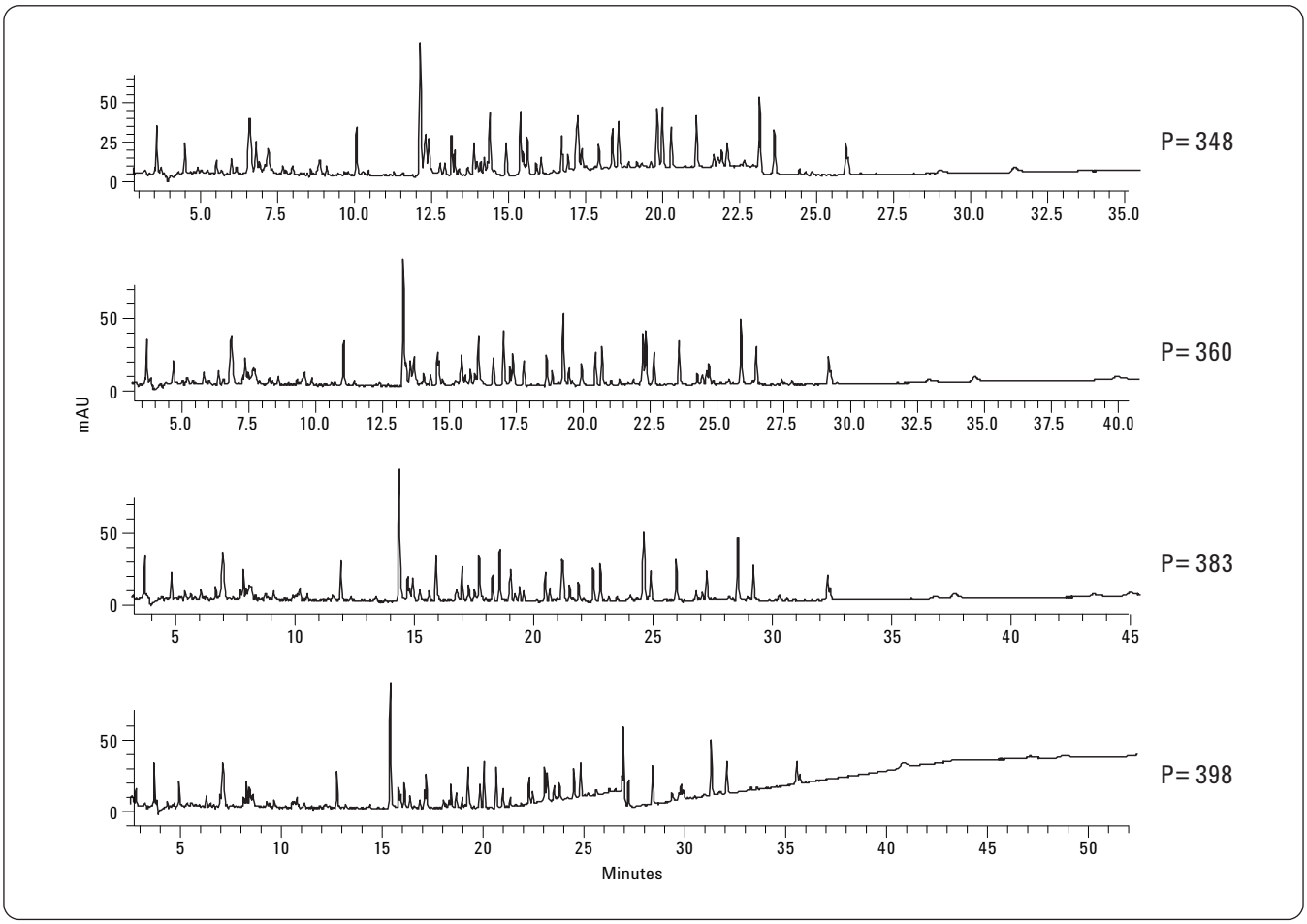


Figure 3
Peak capacity increment with change in gradient time. Observed peak capacity values (P) are marked. A constant (0.2 mL/min) flow rate was used.

Peak capacity change with gradient time

A change in the gradient time has the largest impact on peak capacity increase. Figure 3 demonstrates that peak capacity values increased 3.5 times, when the gradient time changed from 5 minutes to 50 minutes, and the flow rate was held constant.

Optimizing both flow rate and gradient time leads to the highest peak capacities for an intended run time. For each gradient time there exists an optimum flow rate that provides the highest peak capacity. The observed peak capacity values calculated using the complete gradient run time for all experiments are shown in Table 3. In another approach, peak capacity values were calculated using the retention time (RT) window between the first and the last eluting peaks as tabulated in Table 4. This method is more realistic because it gives the peak capacity for the part of the gradient that is really used for the separation.

Behavior of the peak capacity for different gradients with same gradient volume

Gradient volume for a 50-min gradient at a flow rate of 0.2 mL/min will be equal to a 25-min gradient at a flow rate of 0.4 mL/min. Peak capacity values for different gradient times with the same gradient volume were compared and tabulated in Table 5. These results demonstrate the behavior of peak capacity values when varying the run time without changing selectivity by maintaining the same gradient volume. Figure 4 shows the similarity of two chromatograms with the same gradient volume.

Flow	Average peak width using 4 σ peak width Gradient time									
	50	45	40	35	30	25	20	15	10	5
0.2	397.7	382.8	360.0	347.9	322.1	296.6	267.4	229.8	183.8	116.0
0.4	491.6	472.4	448.7	428.1	398.8	370.8	333.6	295.4	242.3	164.5
0.6	538.2	512.5	491.6	473.6	440.4	409.9	378.4	335.5	278.7	197.3
0.8	552.3	522.5	499.9	478.0	453.9	426.4	393.5	348.7	295.4	208.3
1	538.2	530.9	495.7	482.5	461.0	433.9	396.1	354.3	301.4	220.7
1.2	567.1	518.5	485.6	461.0	444.8	424.0	396.1	351.4	301.4	227.4

Table 3
Tabulated peak capacity values for all gradient trials calculated using complete gradient run time.

Flow	Average peak width using 4 σ peak width Gradient time									
	50	45	40	35	30	25	20	15	10	5
0.2	271.4	262.7	249.4	243.6	229.0	214.5	197.5	174.8	145.1	99.8
0.4	320.9	310.0	297.1	286.3	270.2	255.2	234.2	212.7	181.1	132.3
0.6	343.1	328.9	318.4	309.8	291.6	275.6	259.4	235.6	203.0	153.4
0.8	347.0	330.7	319.5	308.4	296.4	282.7	265.7	241.1	211.6	159.1
1	334.9	333.1	313.9	308.5	298.3	285.0	264.9	242.4	213.4	166.3
1.2	350.5	322.8	304.9	292.6	285.6	276.2	262.8	238.6	211.6	169.6

Table 4
Tabulated peak capacity values for all gradient trials calculated using retention time window between first and last eluting peak.

Gradient time	Flow	Gradient volume	Peak capacity calculated using Complete gradient run time	Peak capacity calculated using RT window between first and last eluting peak
50	0.2	10	397.7	271.4
25	0.4	10	370.8	255.2
10	1	10	301.4	213.4
50	0.4	20	491.6	320.9
25	0.8	20	426.4	282.7
20	1	20	396.1	262.8
50	0.6	30	538.2	343.1
30	1	30	461.1	298.3
25	1.2	30	424.0	276.2
50	0.8	40	552.3	347.0
40	1	40	495.7	313.9

Table 5
Tabulated peak capacity values for different gradients with same gradient volume calculated in two different approaches.

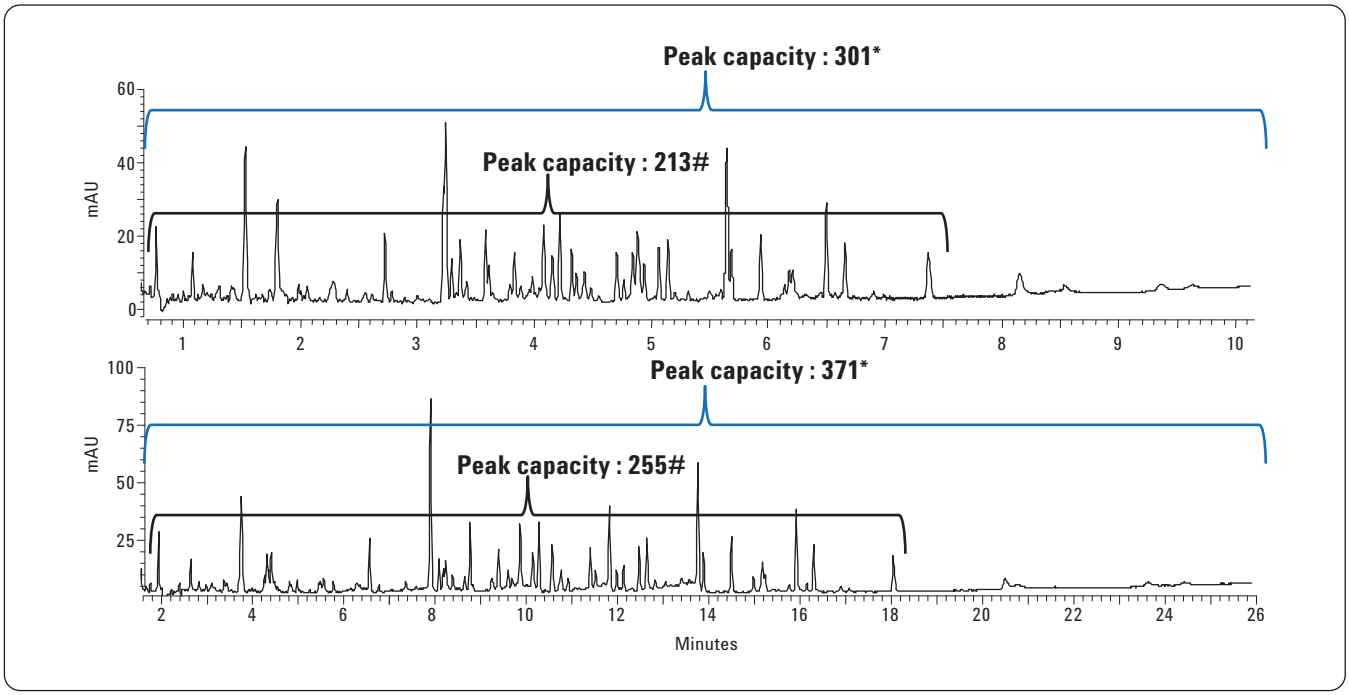


Figure 4

Examples of two chromatograms with same gradient volume.

Upper trace: 10 min gradient with 1 mL/min flow rate. **Lower trace:** 25-min gradient with 0.4 mL/min flow rate. (*calculated using total gradient run time, #calculated using RT window).

Conclusions

This Application Note uses the example of a tryptic BSA digest to demonstrate that optimizing flow rate and gradient time increases peak capacity to the level required for a given analysis and sample. However, this optimization needs a UHPLC system that offers the appropriate power range, for example, a high flow range at high pressures. The Agilent 1290 Infinity LC System offers an exceptional power range, separating complex samples. The use of 1.8 µm columns provides enhanced peak shapes. The optical design of the Agilent 1290 Infinity Diode Array Detector provides rapid and sensitive data acquisition.

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