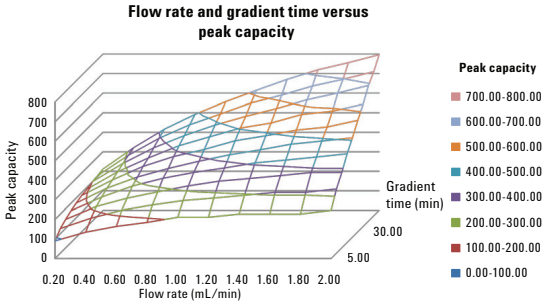
Maximizing chromatographic peak capacity with the Agilent 1290 Infinity LC system using gradient parameters

Application Note

Pharmaceutical and Chemical



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Abstract

Peak capacity is one of the parameters used to evaluate the performance of a chromatographic separation. In this Application Note, effects of gradient parameters, such as flow rate on peak capacity, are measured and discussed in the context of current literature. The study was performed using a typical standard spike mix of small molecules. Agilent ZORBAX Eclipse Plus columns with an inner diameter of 2.1 mm, 1.8 µm particle size and various column lengths (50 mm, 100 mm and 150 mm) were used for this evaluation.

An Agilent 1290 Infinity LC was used for the study because it can deliver a broad power range, which is the integral on flow rate and pressure. The unique design of the Agilent 1290 Infinity LC Binary Pump tolerates up to 1200 bar column backpressure.

This Application Note discusses the variation of peak capacity values with change in gradient parameters such as flow rate, gradient time, column length and slope of the gradient.

Agilent Technologies

Introduction

The concept of reverse phase liquid chromatography including gradient elution was introduced in the late 1970s and the technology, knowledge and skills are still improving for separation techniques. After 40 years most parameters in chromatographs have been modified. The launch of various column dimensions, different particles sizes and the concept of gradient elution are some of the key milestones in the field of separations.

The most commonly used measure for the performance of a chromatographic separation is plate counts. This value mainly depends on the particle size, column length and the packing quality of the column. However, plate counts are not an effective indicator of gradient separation because it is defined by the width of the chromatographic peak. Since the peak width is determined solely by diffusion processes, according to *van Deemter* in isocratic analyses, it is also influenced by focusing the compound on the column head in gradient analysis. This caused the introduction of the concept of peak capacity[1,2]. Peak capacity is a performance measure that describes the number of peaks that can be separated during a gradient run with a certain resolution[3]. Higher peak capacity values are important because of the increasing demand for high throughput gradients for the separation of complex samples with an unknown number and variety of analytes. Generally speaking, the higher the peak capacity, the higher the probability to separate all peaks in different samples. For example, the peak resolution is compromised when the number of components exceeds 1/3 of the peak capacity[4]. In other words, the peak capacity must exceed the number of components by a factor of 100, in order to resolve 98% of the components [5].

The concept of peak capacity impacts:

i) Starting point in method development, because a method with higher peak capacity is able to separate compounds in an unknown sample with higher probability.

ii) Impurity scouting, because a method with higher peak capacity unravels all impurities with higher probability.

iii) A generic method, because the higher peak capacity is able to separate compounds in an unknown sample with higher probability.

The following equation was used to calculate peak capacity:

$$P = 1 + \frac{t_G}{\frac{1}{n}\sum_{1}^{n} w_P}$$

Where, n is the number of peaks used for the calculation, t_G is the gradient time, w_P is the average peak width measured at 4σ peak height[1].

This Application Note discusses the variation of peak capacity values with change in gradient parameters such as flow rate, gradient time, column length and slope of the gradient.

In this study, the peak width is uniform throughout the gradient chromatogram and as a mideluting peak, the peak width of N, N-diethyl-m-toluamide was very close to the observed average peak width value. Therefore, the peak width measured at 4σ of N, N-diethyl-m-toluamide was selected for the peak capacity calculation because it was representative of the distribution of the peak width throughout the chromatogram.

A more practical guide on how to achieve the maximum peak capacity is presented in Reference 6.

Experimental

Instrument configuration

An Agilent 1290 Infinity LC controlled by Chemstation (Version B.04.02) and equipped with an Agilent 1290 Infinity Binary Pump with integrated vacuum degasser, Agilent 1290 Infinity Autosampler, Agilent 1290 Infinity Thermostatted Column Compartment and an Agilent 1290 Infinity Diode Array Detector with 10 mm flow cell was used for data acquisition.

Chemicals and standards

Super gradient grade acetonitrile (ACN) was purchased from Lab-Scan (Bangkok, Thailand) and the modifier formic acid was purchased from Sigma Aldrich (India). HPLC grade water was freshly taken from a Milli-Q water purification system. Samples for analysis were prepared by mixing the HPLC gradient system diagnostic mix (Supelco, USA. Cat No: 48271) and reverse phase test mix (Supelco, USA. Cat No: 47641-U). The individual components of the sample were uracil, phenol, methyl paraben, ethyl paraben, propyl paraben, butyl paraben, heptylparaben, toluene and N, N-diethyl-m-toluamide.

Columns

Agilent ZORBAX Eclipse Plus C18, 50 mm × 2.1 mm, 1.8 µm

Agilent ZORBAX Eclipse Plus C18, 100 mm × 2.1 mm, 1.8 µm

Agilent ZORBAX Eclipse Plus C18, 150 mm × 2.1 mm, 1.8 µm

LC Parameters

The LC method parameters are tabulated in Table 1 and the gradient used for the study is tabulated in Table 2.

The binary pump was operated at various flow rates and gradient times with

a gradient span from 20 to 100% B. The detector was operated at a sampling acquisition rate of 80 Hz for all runs in order not to compromise the peak shape or number of data points (response time 0.062 seconds, >0.003 m).

Procedure

Samples were injected and separated using 10 different gradients (Table 2) and each gradient was run with 10 different flow rates (Table 1). High backpressure at the limit of 1200 bar with 1.8 µm particles sized longer columns restricted the use of higher flow rates. An Agilent ZORBAX Eclipse Plus 50 mm × 2.1 mm, 1.8 µm column was used for a flow rate range of 0.2 mL/min to 2.0 mL/min (10 different trials), a 100 mm × 2.1 mm, 1.8 µm column was used up to 1.4 mL/min (seven trials) and a 150 mm x 2.1 mm, 1.8 µm column was used up to 1.0 mL/min (5 different trials).

Parameter	Details
Mobile phase A	0.1% formic acid in water
Mobile phase B	100% 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, 1.4 mL/min, 1.6 mL/min, 1.8 mL/min or 2.0 mL/min.
Injection volume	2 µL
Needle wash	Flush port activated for 6 seconds using mobile phase B
Column temp.	50 °C
Detection	254/4 nm; Reference off
Post run time	3 minutes

Table 1
LC method details for experiment.

%B	Time (min)
20 to 100	Variable (5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 min) -gradient time
100	For 2 minutes – column rinsing
20	For 3 minutes – column reconditioning

Table 2
Gradient used for experiment.

Results and Discussion

Effect of flow rate on peak capacity

A representative chromatogram for the analyte using a ZORBAX Eclipse Plus C18, 100 mm × 2.1 mm, 1.8 µm column is shown in Figure 1, with peak A highlighted. In order to assess the effect of flow rate on peak capacity, the analyte mix was first separated using several flow rates. The peak capacity values are smaller for lower flow rates and increase dramatically with higher flow rates, because peak widths decrease with increasing flow rates. Representative results for peak A (ZORBAX Eclipse Plus, 50 mm × 2.1 mm, 1.8 µm) are shown in Figure 2. A peak capacity of about 100 was achieved for the lower end of the flow rate range. A peak capacity of about 250 was achieved for the short 5 minutes gradients by increasing the flow rate up to 2 mL/min. A flow rate of 2 mL/min using a 5 min gradient returned about the same peak capacity as a 10 min gradient with 0.8 mL/min flow rate (Figure 2). This proves the efficiency of short gradients at higher flow rates to resolve the compounds in a sample by increasing the corresponding peak capacity.

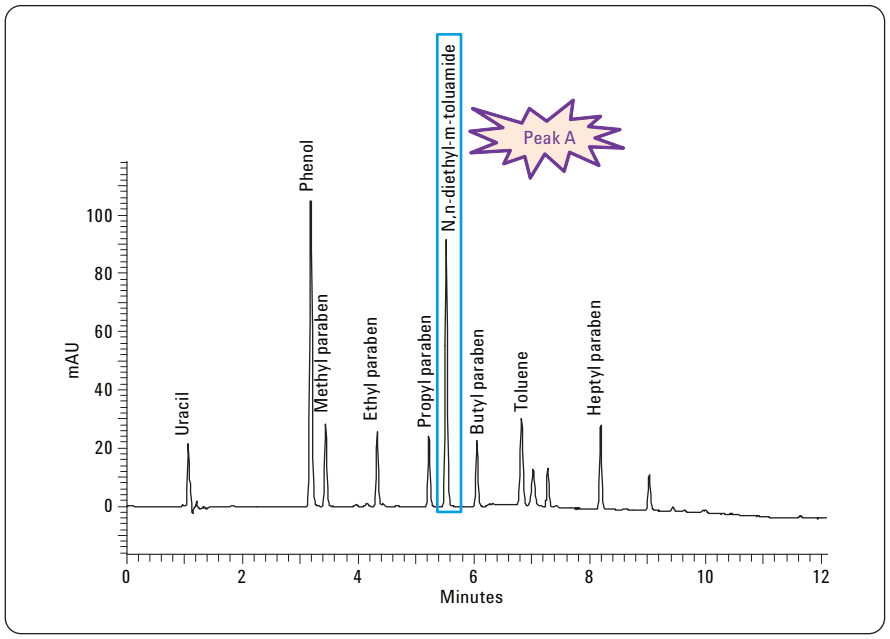

Figure 1
Chromatographic representation of analyte separation using an Agilent ZORBAX Eclipse Plus C18, 100 mm × 2.1 mm, 1.8 µm column. Gradient time: 10 min and flow rate: 0.2 mL/min.

Effect of gradient time on peak capacity

Peak capacity versus gradient time was determined for different flow rates on a ZORBAX Eclipse Plus 50 mm × 2.1 mm column with 1.8 µm particles. Representative data for peak A using flow rates of 0.4 mL/min and 2.0 mL/min are shown in Figure 3. The results illustrate that a 2.0 mL/min flow rate gave a peak capacity value of about 250 when using a 5-min gradient while the peak capacity value was 500 when the gradient time was increased to 25 min. Increased peak capacity with an increase in the gradient time for a selected flow rate using sub-2-µm columns are consistent with existing literature [2]. A peak capacity similar to a 25-min gradient at 0.4 mL/min can be achieved by using the same column with a 5-min gradient at 2.0 mL/min.

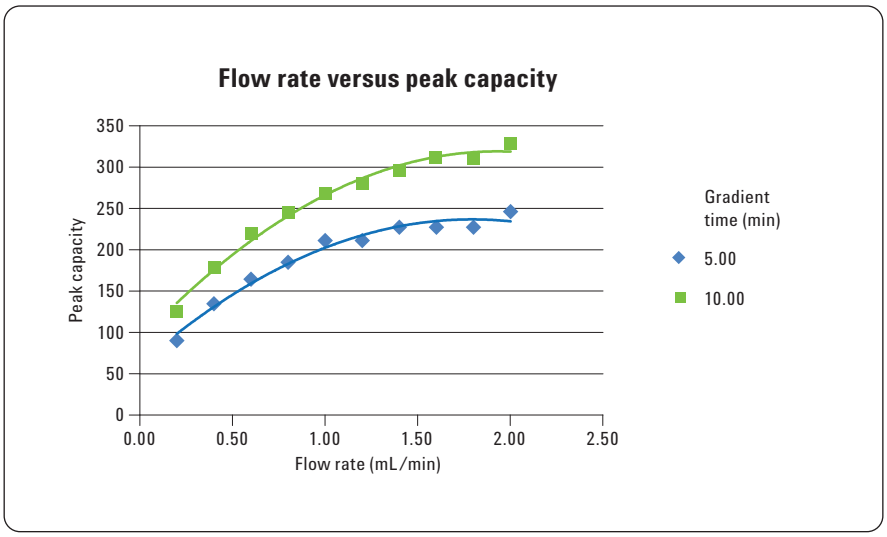

Figure 2
Variation of peak capacity with change in flow rate at two selected gradient times for peak A using an Agilent ZORBAX Eclipse Plus 50 mm × 2.1 mm, 1.8 µm column.

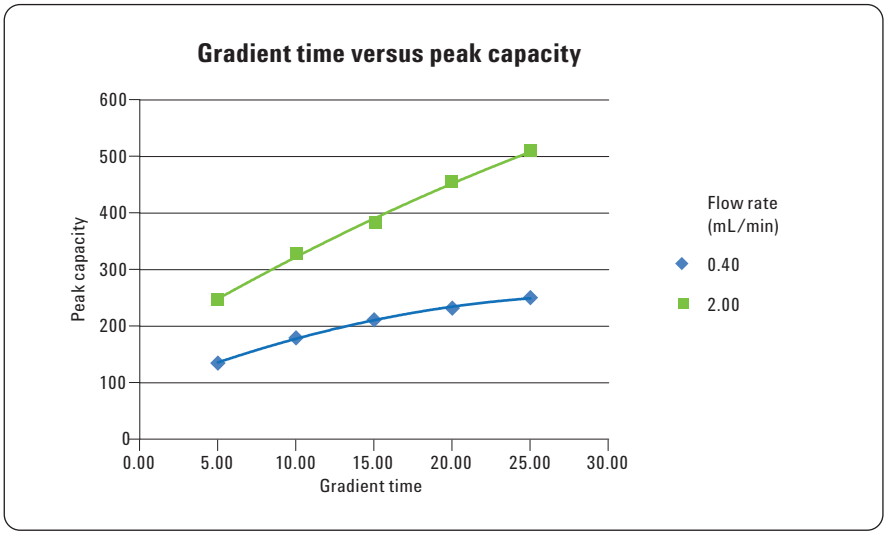

Figure 3
Variation of peak capacity with change in gradient time at a fixed flow rate for an Agilent ZORBAX Eclipse Plus 50 mm × 2.1 mm, 1.8 µm column.

Combined effects of flow rate and gradient time on peak capacity

The combined effects of gradient time and flow rate are illustrated in the three-dimensional plot shown in Figure 4. The observed peak capacity values for peak A (N, N-diethyl-m-toluamide) with changing gradient time and flow rate are tabulated in Table 3. The results show that the highest peak capacity values are observed for the higher flow rates for each examined gradient time. Typical gradients for 50 mm × 2.1 mm, 1.8 µm columns are about 5 or 10 minutes long and peak capacities of about 250 and 330 can be achieved, respectively. Peak capacity values are higher using a shorter gradient with higher flow rates than a longer gradient at lower flow rates for the given gradient volume. For example, the peak capacity value for a 50-min gradient at 0.2 mL/min flow rate was 211, whereas for a 5-min gradient with a 2.0 mL/min flow rate the value was 246 (~17% more). This shows that 45 minutes can be saved for a particular analysis with the same or even better peak capacity performance. The challenge in this case is the high column backpressure with sub-2-µm columns at higher flow rates. The Agilent 1290 Infinity LC can use flow rates of up to 2 mL/min at column backpressures of up to 1200 bars.

Effect of column length on peak capacity

Experimental data were collected for peak A at various flow rates and gradient times on 50 mm, 100 mm and 150 mm × 2.1 mm, 1.8 µm ZORBAX Eclipse Plus columns. The results are tabulated in Tables: 3A, 3B and 3C.

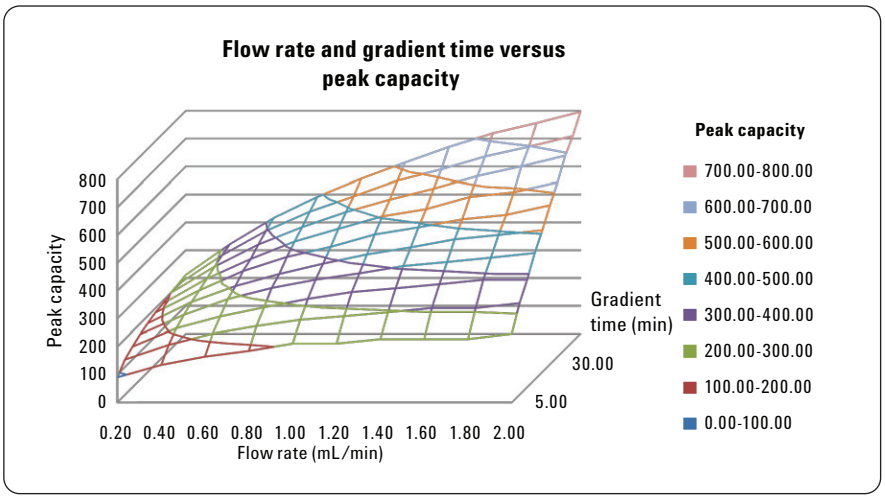

Figure 4
Variation of peak capacity with change in gradient time and flow rate for N, N-diethyl-m-toluamide using Agilent ZORBAX Eclipse Plus 50 mm × 2.1 mm, 1.8 µm column.

A) Column: 50 mm × 2.1 mm, 1.8 µm particles

Flow rate (mL/min) / Gradient time (min)	0.20	0.40	0.60	0.80	1.00	1.20	1.40	1.60	1.80	2.00
5.00	90.20	134.80	164.50	185.00	211.30	211.30	227.40	227.40	227.40	246.30
10.00	126.30	179.40	219.10	246.30	268.60	281.40	295.40	310.90	320.90	328.10
15.00	145.80	211.30	253.30	285.90	316.40	340.70	354.30	369.00	385.00	385.00
20.00	162.30	231.90	288.20	328.10	357.80	380.80	407.00	421.50	437.10	453.90
25.00	174.20	250.50	307.60	351.10	388.30	421.50	447.00	475.80	491.60	508.50
30.00	185.00	264.60	334.30	385.00	421.50	465.80	491.60	520.50	536.20	570.80
35.00	190.00	279.40	350.30	413.10	458.90	503.60	529.40	573.40	589.80	625.40
40.00	200.60	295.40	374.80	437.80	491.60	536.20	575.40	620.70	655.20	693.60
45.00	204.80	309.10	396.40	465.80	520.50	577.00	617.10	663.30	698.20	736.90
50.00	211.30	321.00	415.60	491.60	556.40	614.30	670.00	719.00	755.80	796.60

B) Column: 100 mm × 2.1 mm, 1.8 µm particles

Flow rate (mL/min) / Gradient time (min)	0.20	0.40	0.60	0.80	1.00	1.20	1.40
5.00	102.50	164.50	197.30	211.30	227.40	246.30	246.30
10.00	144.60	219.10	268.60	295.40	310.90	310.90	328.10
15.00	174.20	253.30	305.50	340.70	354.30	369.00	385.00
20.00	194.00	281.40	337.40	369.00	393.50	407.00	437.10
25.00	211.30	301.40	360.00	398.80	433.90	447.00	461.00
30.00	224.60	322.10	385.00	431.80	465.80	491.60	505.60
35.00	237.90	338.80	405.00	449.00	491.60	516.20	543.30
40.00	246.30	352.50	429.20	472.00	513.00	548.70	575.40
45.00	255.70	369.00	442.60	500.90	541.70	577.00	617.10
50.00	263.80	383.00	416.00	517.40	567.10	601.80	640.90

Table 3
Tabulated data for peak capacities dependend on flow rates, gradient times and change in column length.
A: Agilent ZORBAX Eclipse Plus, 50 mm × 2.1 mm,1.8 µm column. Some examples with the same gradient slope are color coded.
B: Agilent ZORBAX Eclipse Plus, 100 mm × 2.1 mm, 1.8 µm column. (Continued)

Peak capacity increases with an increase in column length. However at low flow rates (for example, 0.2 mL/min), peak capacity values with a 150-mm column for a short gradient time (5 min) are marginally better than for a 50 mm column. The longer column delivers higher peak capacities with longer gradient times. For example, a 150-mm column (50-min gradient at 0.2 mL/min) gave a peak capacity value of 308 while a 100-mm column gave a value of 264, and a 50-mm column gave a peak capacity value of only 211.

The effect of column length on peak capacity variations are shown in Figures 5 and 6. The trials with higher flow rates for longer columns are restricted because of the observed high column backpressure. Within the given pressure limit of 1200 bar, a 50-mm column delivers higher peak capacity values because it can permit higher flow rates when compared to longer columns of length 100 and 150 mm. At a flow rate of
2.0 mL/min, the 50-mm column delivers a peak capacity of about 330 for a short 10-min gradient (Figure 5). This value is comparable to the value delivered by the 100-mm column at 1.4 mL/min. The 50-mm column delivers a better peak capacity value than a long 150-mm column at a flow rate of 1.0 mL/min for

C) Column: 150 mm × 2.1 mm, 1.8 µm particles					
Flow rate (mL/min)	**0.20**	**0.40**	**0.60**	**0.80**	**1.00**
Gradient time (min)					
5.00	99.10	164.50	185.00	211.30	211.30
10.00	152.00	227.40	268.60	291.40	305.40
15.00	188.90	277.00	316.40	340.70	340.70
20.00	219.10	310.90	357.80	380.80	380.80
25.00	238.40	335.50	388.30	409.90	421.50
30.00	257.00	361.50	411.80	442.60	453.90
35.00	272.10	382.60	439.40	458.90	469.30
40.00	284.70	400.20	453.90	481.60	502.10
45.00	295.40	415.00	474.10	510.50	520.50
50.00	307.60	427.60	491.60	526.70	536.20

Table 3
Tabulated data for peak capacities dependend on flow rates, gradient times and change in column length.
C: Agilent ZORBAX Eclipse Plus, 150 mm x 2.1 mm, 1.8 µm column.

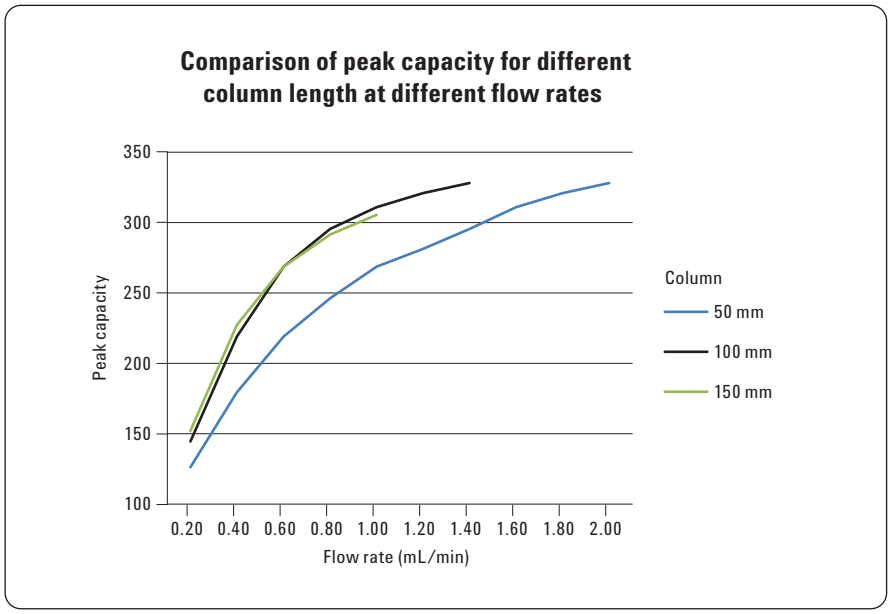

Figure 5
Variation of peak capacity with change in column length at the maximum achievable flow rate at the same short gradient length of 10 minutes.

the same gradient length. If more separation time can be spent for a given flow rate, the longer column achieves higher peak capacity (Figure 6).

The results show that one can achieve similar peak capacity values of a long column with a long gradient using short columns by adjusting the gradient time and flow rates. If the analysis time is important, a 5-min gradient can attain a higher peak capacity than a 50-min gradient under appropriate conditions.

Increasing the efficiency of a separation while maintaining the peak capacity

The elution pattern of the analyte at various flow rates for the 5-min gradient is shown in Figure 7. As the flow rate increases; the peak capacity increases by the observed decrease of peak width. In addition, the retention time for several peaks also decreases. Unfortunately, a change in selectivity is observed for peaks 7 and 8.

The selectivity between two compounds in a gradient separation is constant if the starting point, the span, and the dead volume of the system including column and the slope of the gradient are kept constant[3]. The slope of a gradient is defined as follows: Slope = Gradient span/(gradient time × flow rate).

By adjusting gradient time and flow rate, the same gradient slope and selectivity can be obtained. Table 3A lists the peak capacity data using the ZORBAX Eclipse Plus 50 mm × 2.1 column. In this study, the slope of a long gradient with low flow rate (80%/(40 min ×0.2 mL/min) = 10%/mL) is equal to a short gradient with a high flow rate (80%/(5 min × 1.6 mL/min) = 10%/mL). The second gradient gave a shorter analysis time at higher flow rates compared to the first long gradient at lower flow rate without a change in selectivity. There are many gradients with the same gradient slope (Table 3A, see color coding).

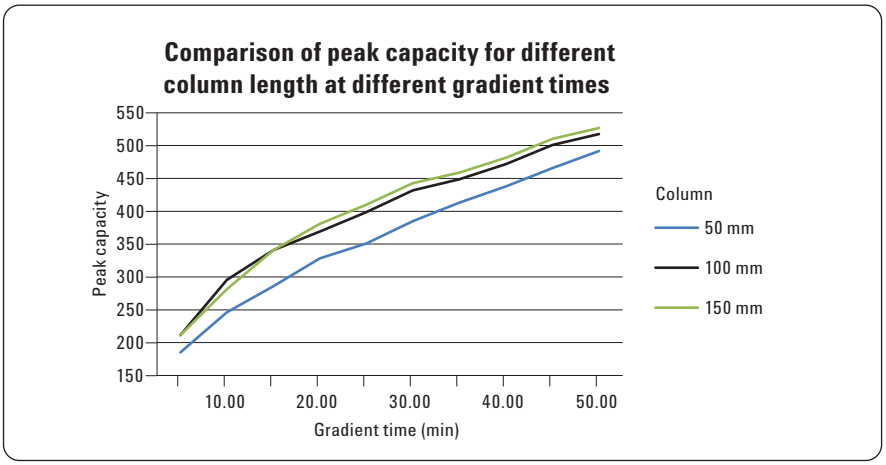

Figure 6
Variation of peak capacity with change in column length at different gradient times and constant flow rate of 0.8 mL/min.

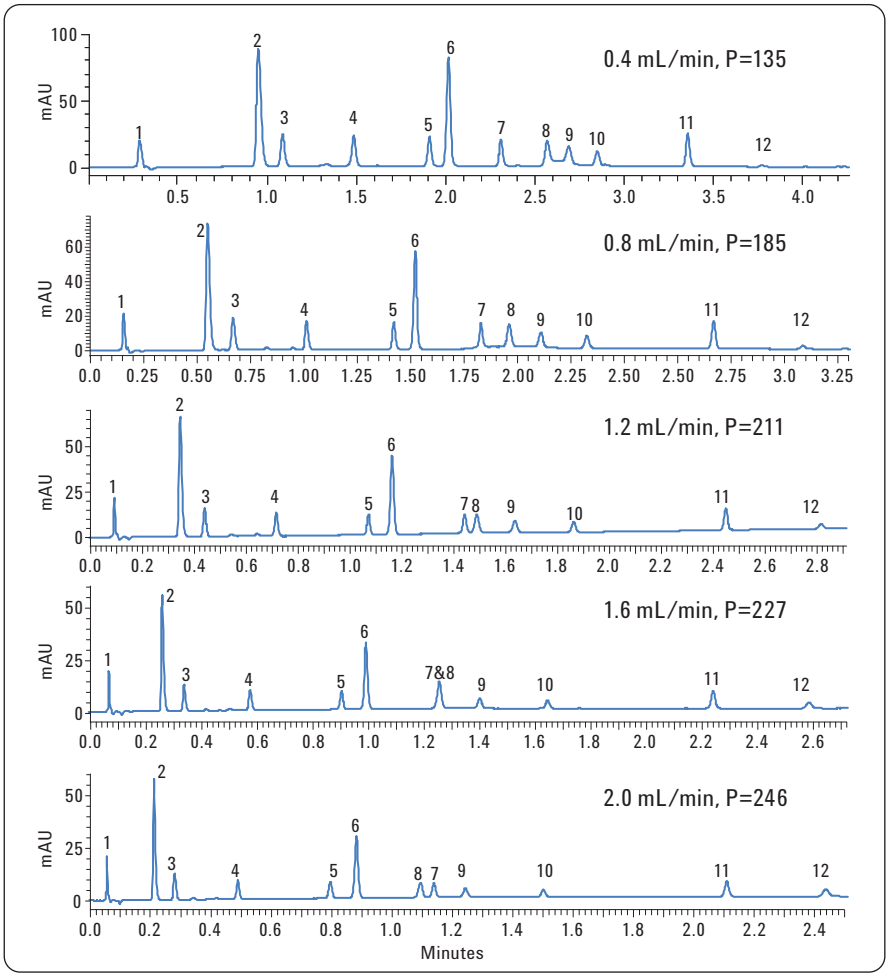

Figure 7
Elution pattern for the analyte using Agilent ZORBAX Eclipse Plus 50 mm × 2.1 mm, 1.8 µm column using various flow rates (0.4 mL/min, 0.8 mL/min, 1.2 mL/min, 1.6 mL/min and 2.0 mL/min) for a constant gradient time of 5 min. Peak capacity values are labeled.

A few example chromatograms with the same gradient slopes are shown in Figure 8. For a given gradient slope, the peak capacity value is higher for those gradients with higher flow rates. The data shows that a gradient with the same slope and similar selectivity is achieved for a given separation within only a tenth of the time if the flow rate is increased by a factor of ten. The Agilent 1290 Infinity LC, with pressure range up to 1200 bar makes this possible.

Conclusion

The results presented here show how to maximize the peak capacity and improve the chromatographic separation performance by varying the method parameters for small molecule separations. In general, peak capacity value increases with gradient time and flow rate. The highest separation efficiency is achieved by increasing the flow rate with the Agilent 1290 Infinity LC, which can go up to 1200 bar while providing the highest possible flow rate for smaller particle sized columns. The Agilent 1290 Infinity LC alllows a decrease in the run time, while maintaining the gradient and providing the same selectivity.

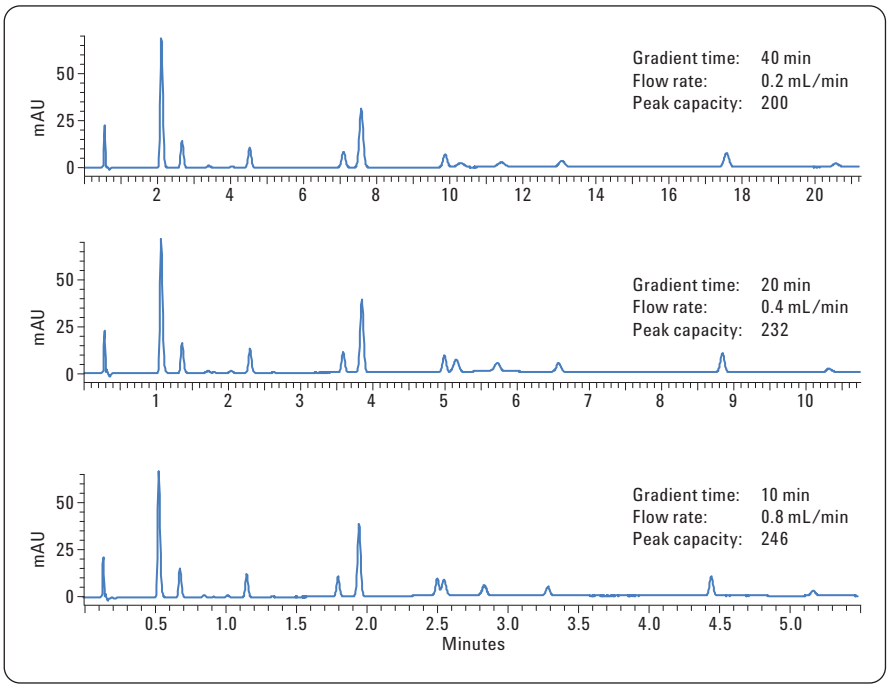

Figure 8
Variation of peak capacity among gradients with same slope, using the Agilent ZORBAX Eclipse Plus C18, 50 mm × 2.1 mm, 1.8 µm.

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