Maximizing chromatographic peak capacity with the Agilent 1290 Infinity LC system

A practical guide on how to use parameters to increase peak capacity

Application Note

Pharmaceutical and Chemical

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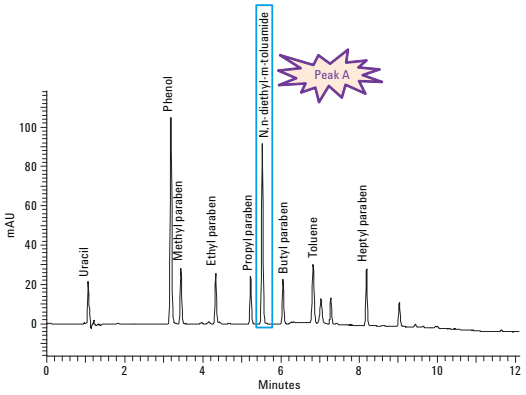

Abstract

Peak capacity can be used to evaluate the performance of a chromatographic separation. In this Application Note, the effects of gradient parameters, such as flow rate on peak capacity are measured and discussed to provide UHPLC instrument users with guidance for easily maximizing peak capacity.

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.

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Introduction

Peak capacity is a performance measure describing the number of peaks that can be separated during a gradient run with a certain resolution[1]. The importance of higher peak capacity values is highlighted by the increasing demand for high throughput gradients for the separation of complex samples with an unknown number of various types of analytes. Generally speaking, the higher the peak capacity the higher the probability of separating all peaks in different samples. For example, the peak resolution is compromised when the number of components exceeds 1/3 of the peak capacity. Specifically, 98% of the components can only be resolved if the peak capacity exceeds the number of components by a factor of 100.[2,3]

Peak capacity has an impact on:

- Starting point in method development, because a method with higher peak capacity can separate compounds in an unknown sample with higher probability.
- Impurity scouting, because a method with higher peak capacity unravels all impurities with higher probability.
- Generic method, because higher peak capacity can 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.

This Application Note, evaluates 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 addition, practical guidelines for the maximization of peak capacity are provided. A more detailed discussion of this topic is presented in Reference 4.

Experimental

Instrument configuration

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 formic acid was purchased from Sigma-Aldrich (India) as a modifier. 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.

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.

The binary pump was operated at variable flow rates and gradient times with a gradient range of 20 to 100 % B. The detector was operated at a sampling acquisition rate of 80 Hz for all runs in order to avoid compromise of peak shape and number of data points (response time 0.062 seconds, >0.003 m).

Procedure

Samples were injected and separated using 10 different gradients (Table 2). Each gradient was run with 10 different flow rates (Table 1). The use of higher flow rates was restricted by a high backpressure limit of 1200 bar when longer 1.8 µm columns were used. An Agilent ZORBAX Eclipse Plus 50 mm × 2.1 mm, 1.8 µm column was used for a flow rate range of 0.2 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 (five different trials).

4σ peak width for N,N-diethyl-m-toluamide (peak A) was measured for each gradient and used for peak capacity calculation. A representative chromatogram for the analyte using an Agilent ZORBAX Eclipse Plus C18, 100 mm × 2.1 mm, 1.8 µm column is shown in Figure 1, with peak A highlighted.

Results and discussion

General considerations for peak capacity

In order to assess the effect of flow rate on peak capacity the analyte mix was first separated using several flow rates. For the lower end of the flow rate range a peak capacity of about 100 was achieved. By increasing the flow rate up to 2 mL/min a peak capacity of about 250 was achieved for the short 5-min gradient. A flow rate of

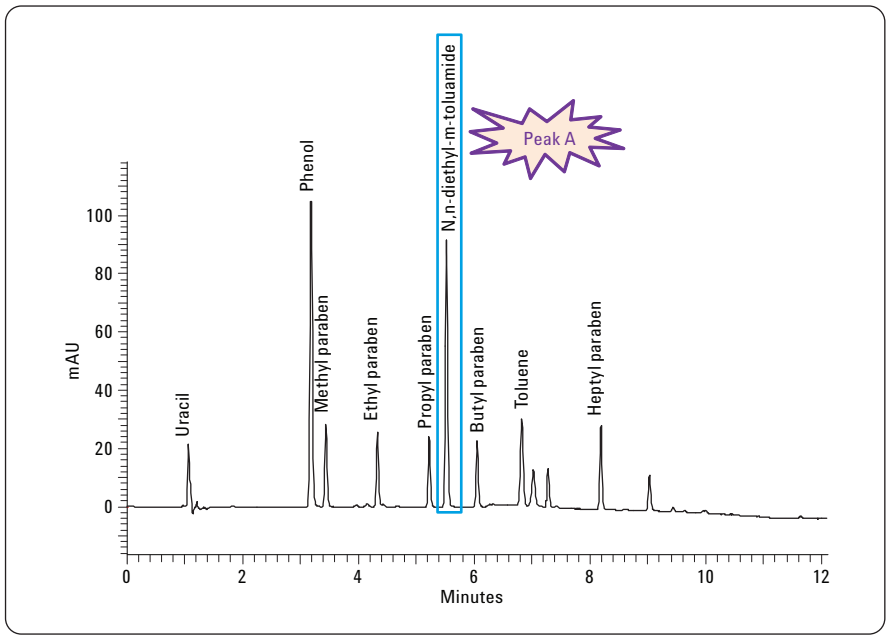

Figure 1
Chromatographic representation of analyte separation using an Agilent ZORBAX Eclipse Plus C18, 100 mm × 2.1 mm, 1.8 µm column. Gradient time: 10 min and flow rate: 0.2 mL/min.

2 mL/min using a 5-min gradient can return approximately the same peak capacity as a 10 min gradient with a 0.8 mL/min flow rate (Figure 2).

This pattern was also evaluated as peak capacity versus gradient time. The results shows that 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 30 min. Figure 2 illustrates that the peak capacity of a 30-min gradient at 0.4 mL/min can be similar to the peak capacity using 5-min gradient at 2.0 mL/min with the same column.

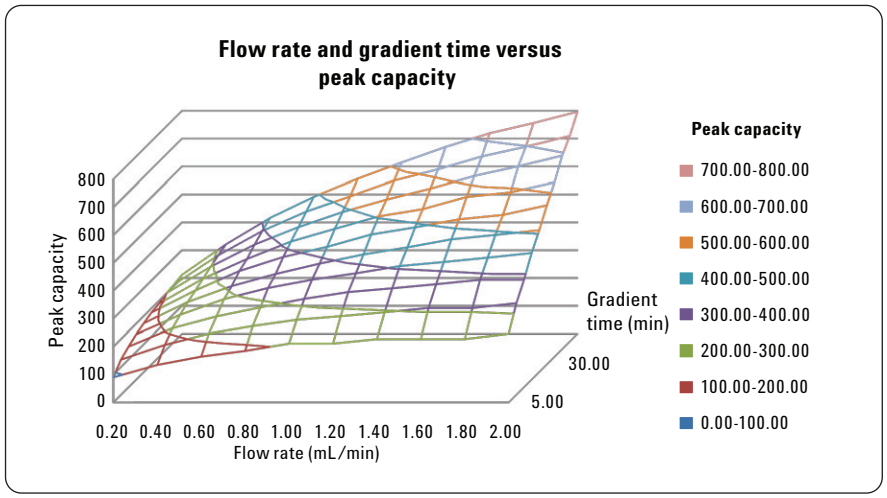

Figure 2
Variation of peak capacity with change in gradient time and flow rate for N, N-diethyl-m-toluamide using an Agilent ZORBAX Eclipse Plus 50 mm × 2.1 mm, 1.8 µm column.

The combined effects of gradient time and flow rate can be illustrated in a three-dimensional plot as shown in Figure 2. The 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 were 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 min long and peak capacities of about 250 and 330 can be achieved, respectively.

The effect of column length on peak capacity at the same flow rates but various gradient times on 50 mm, 100 mm and 150 mm × 2.1 mm, 1.8 µm ZORBAX Eclipse Plus columns shows that 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) was marginally better than that for a 50 mm column. With longer gradient times the longer columns achieved higher peak capacities. For example, a 150 mm column (50-min gradient at 0.2 mL/min) achieved a peak capacity value of 308 while a 100 mm column achieved a peak capacity of 264, and a 50 mm column achieved a peak capacity value of only 211. The results are tabulated in Tables 3A, 3B and 3C.

For a given gradient volume, peak capacity values are higher using a shorter gradient with higher flow rates than a longer gradient at lower flow rates. 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 (approximately 17% more). This proves that the efficiency for short gradients at higher flow rates can resolve the compounds in a sample by increasing the corresponding peak capacity.

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

C) Column: 150 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
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.
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.
C: Agilent ZORBAX Eclipse Plus, 150 mm × 2.1 mm, 1.8 µm column.

Adjusting the gradient time and flow rate obtains the same gradient slope and selectivity.

The peak capacity using a 50 mm × 2.1 mm ZORBAX Eclipse Plus column is given in Table 3A. 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 provided a shorter analysis time at a higher flow rate compared to the first long gradient at a lower flow rate without a change in selectivity. However, the second gradient achieved a higher efficiency.

Practical considerations to achieve highest peak capacities

The following section explains how to develop a method with the maximum peak capacity in six steps.

1. Consider the number of peaks and required peak capacity.

The numbers of probable peaks (compounds) in the sample, that are to be separated require the peak capacity necessary to resolve all compounds as described in the introduction. As a rule of thumb the simple equation $P=n^{1.5}$ (where P is the sample peak capacity and n is the number of compounds) can be used to get an estimation of the required peak capacity. For example, a peak capacity of 300 would be required for 45 peaks[5]. This is roughly achieved within a 5-min gradient at 2 mL/min (Table 3A).

2. Consider the choice of gradient length.

The length of the gradient for a given separation problem depends on several factors. If high sample throughput is required shorter run times are chosen. The same is true if the results of the sample analysis is required in a short time to make a decision. Conversely, the required separation time depends on the complexity of the sample. In reality, a compromise between these requirements may be necessary.

3. Consider the choice of column.

The proper column length must be selected depending on the chosen run time and the sample complexity. If a short run time below 5 min is required, a column length of 50 mm is appropriate, and will allow separations with required peak capacities of up to 250. If a moderate separation time between 5 min and 30 min is chosen, a column length of 100 mm is the appropriate choice. This allows separations of moderately complex samples with required peak capacities of up to 500. For samples of highest complexity where the longest gradient times above 30 min are required, a column length of 150 mm is the best choice. This allows separations where a peak capacity of more than 500 is required. Tables 3A, 3B and 3C display the results of the described experiments.

4. Consider the choice of the right flow rate according to system and column pressure limitations.

The maximum flow rate that can give the maximum peak capacity for a chosen column and gradient length depends on the limitations of the HPLC or UHPLC system and the column used. For example, with a safety margin of 10%, the maximum available backpressure of the Agilent 1200 Series HPLC is 360 bar; the maximum achievable backpressure of the Agilent 1200 Series RRLC is 540 bar; and the maximum achievable backpressure of the Agilent 1290 Infinity LC is 1080 bar. This clearly demonstrates that the maximum peak capacity can be achieved with the Agilent 1290 Infinity LC. The column also limits the system pressure. For example, an Agilent RRHT 1.8 µm column can be used with a backpressure of up to 600 bar and an Agilent RRHD 1.8 µm column can be used for backpressures of up to 1200 bar. This makes the RRHT column the right choice for the standard Agilent 1200 Series HPLC and RRLC systems for highest resolution and peak capacity. The right solution for the Agilent 1290 Infinity LC is given with the RRHD column.

The change in pressure during the gradient must also be considered to choose the right flow rate. For example, the maximum viscosity and backpressure is reached for a mixture of 10% acetonitrile in water or for a mixture of 50% methanol in water. This is where the highest pressure occurs during the gradient.

5. Consider the choice of temperature on peak capacity.

The effect of temperature on peak capacity depends on the individual compound. In general, at elevated flow rates of more than 0.8 mL/min, elevated temperatures above 40 °C deliver higher peak capacities[1]. When elevating the temperature, the stability of the analyte and of the column phase must be considered. For example, ZORBAX Stable Bond (SB) phases are stable up to 90 °C and ZORBAX Eclipse phases are stable up to 60 °C.

6. Achieve the highest efficiency for the separation.

After these decisions are made, the flow rate can be increased to work at the maximum to achieve the best separation at the highest possible peak capacity. If selectivity is important it might be necessary to work at lower flow rates to separate a given set of peaks under a desired selectivity. In this case the flow rate can still increase to its possible maximum while reducing the gradient time to preserve the gradient and the required selectivity. This means that the most efficient separation can be achieved. Table 3A shows some examples of flow rate and gradient times with the same gradient slope and peak capacity using color coding.

Conclusion

The results discussed in this Application Note provide a course of action for maximizing the peak capacity and improving chromatographic separation performance by varying method parameters for small molecule separations. In general, peak capacity value increases with gradient time and flow rate. The highest separation efficiency can be achieved by increasing the flow rate using the Agilent 1290 Infinity LC. This system can operate up to 1200 bar, providing the highest possible flow rate for smaller particle sized columns. A simultaneous decrease in the gradient run time while maintaining the gradient and selectivity for a given separation can be easily achieved with the Agilent 1290 Infinity LC.

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