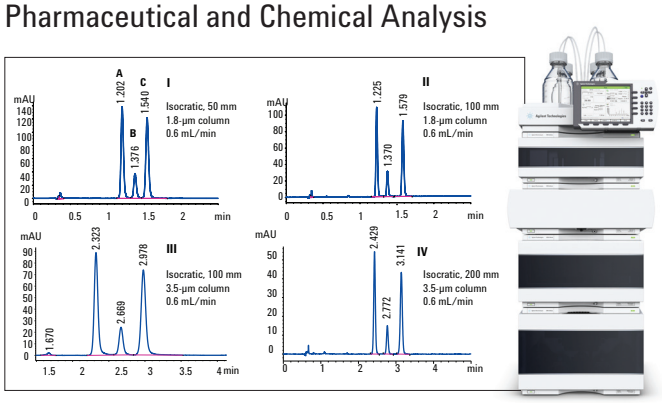
Increasing resolution using longer columns while maintaining analysis time

Advantages of the wide power range of the Agilent 1290 Infinity LC System

Application Note

Pharmaceutical and Chemical Analysis



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Abstract

Method development involves optimizing various parameters such as mobile phase, stationary phase and column chemistry[1]. Even with an already developed method, sometimes increased resolution is required for a critical peak pair. Without having to redevelop the method, the increased resolution can readily be achieved by increasing the column length. A disadvantage of using longer columns is that the analysis time has to be increased accordingly. However, when using UHPLC columns with sub-2 µm or superficially porous particles the flow rate can be increased without losing too much separation efficiency and, therefore, the analysis time can be reduced again to the analysis time of the original method. This Application Note evaluates the resolution efficiency on longer columns while maintaining analysis time. Gradient and isocratic conditions were applied on three different particle size columns (totally porous 1.8 µm and 3.5 µm, superficially porous 2.7 µm) of various lengths using three pharmaceutical compounds. The results of the isocratic runs show that increasing the column length significantly increases theoretical plates for all particle size columns. In gradient runs, all columns show an increase in peak capacity with the increase in flow rate. There is a significant gain in resolution with high flow rates on longer columns under both isocratic and gradient conditions.

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Introduction

Out of specification (OOS) studies often require an increase in resolution. Improved resolution is possible without changing other method parameters such as stationary phase, mobile phase, or temperature but increasing only the column length or decreasing the particle size (equation 1)[2].

$$R_S \approx \frac{1}{4}\sqrt{\frac{L}{d_p}}$$

R_s = resolution

L = length of the column

d_p = diameter of the particle

The advantage of switching to longer columns is that the theoretical plates will increase and so will the resolution. The number of theoretical plates (N) is directly proportional to the length of the column and inversely proportional to the particle diameter[2].

An increase in the column length also increases the analysis time as shown in equation 2. Equation 2 is applicable for columns lengths that have the same internal diameter and porosity.

$$T_2 = T_1 \frac{L_2}{L_1} \frac{F_1}{F_2}$$

T_1 = analysis time for column 1

T_2 = analysis time for column 2

L_1 = length of column 1

L_2 = length of column 2

F_1 = flow rate of column 1

F_2 = flow rate of column 2

Equation 2 shows that the change in flow rate is directly proportional to the change in column length. For example, if the column length is doubled, the analysis time also doubles. However, to maintain constant analysis time, the flow rate must also be doubled.

An Agilent 1290 Infinity LC System was used because of the wide power range (maximum pressure versus flow). This is important since the use of longer columns in combination with higher flow rates leads to a significant increase in backpressure.

Experimental

Instruments

The Agilent 1290 Infinity LC System consisting of the following modules was used:

- Agilent 1290 Infinity Binary Pump (G4220A), with Jet Weaver V35 mixer.
- Agilent 1290 Infinity Autosampler and Thermostat (G4226A, G1330B)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1290 Infinity Diode Array Detector (G4212A) equipped with 1 µL, 10-mm flow cell.

Software

- Agilent Chemstation B.04.03

Reagents and materials

All chemicals and solvents of HPLC grade or better were used. Purified water was used from a Milli Q water purification system (Millipore, USA). Acetonitrile super gradient was purchased from Lab-Scan (Bangkok, Thailand), monobasic sodium phosphate, glacial acetic acid, ortho phosphoric acid were purchased from Sigma-Aldrich (India). Simvastatin, clopidogrel and lovastatin were purchased from VARDA Biotech (Mumbai, India).

Chromatographic parameters

Chromatographic parameters for reverse phase liquid chromatography are shown in Table 1.

Parameters	Conditions
Temperature	
Thermostatted Column Compartment	45 °C
Mobile phase A	Sodium phosphate (monobasic) buffer pH adjusted to 4.5 using ortho phosphoric acid.
Mobile phase B	100% acetonitrile
Isocratic runs	Mobile phase A: Mobile phase B (30:70)
Gradient runs	See Table 2
Injection Volume	5 µL
UV, data rate	238 nm, 40 Hz

Table 1
Chromatographic parameters used for the separation of standards.

Columns - Isocratic and gradient run times

	Isocratic runs		Gradient runs							
1.8 µm column	**Run time**	**Flow rate**	**Run time**	**Flow rate**	**Gradient condition**					
Agilent ZORBAX RRHT Eclipse Plus C18, 3.0 × 50 mm, 1.8 µm (p/n 959941-302)	2.5 min	0.6 mL/min	7 min	0.6 mL/min	Time (min)	0	5	5.5	5.6	7
					% B	5	95	95	5	5
Agilent ZORBAX RRHD Eclipse Plus C18, 3.0 × 100 mm, 1.8 µm (p/n 959758-302)	5 min	0.6 mL/min	14 min	0.6 mL/min	Time (min)	0	10	11	11.2	14
					% B	5	95	95	5	5
Agilent ZORBAX RRHD Eclipse Plus C18, 3.0 × 100 mm, 1.8 µm (p/n 959758-302)	2.5 min	1.2 mL/min	7 min	1.2 mL/min	Time (min)	0	5	5.5	5.6	7
					% B	5	95	95	5	5
	Isocratic runs		**Gradient runs**							
Poroshell 2.7 µm column	**Run time**	**Flow rate**	**Run time**	**Flow rate**	**Gradient condition**					
Agilent Poroshell 120 EC-C18, 3.0 × 50 mm, 2.7 µm (p/n 699975-302)	2.5 min	0.6 mL/min	7 min	0.6 mL/min	Time (min)	0	5	5.5	5.6	7
					% B	5	95	95	5	5
Agilent Poroshell 120 EC-C18, 3.0 × 100 mm, 2.7 µm (p/n 695975-302)	5 min	0.6 mL/min	14 min	0.6 mL/min	Time (min)	0	10	11	11.2	14
					% B	5	95	95	5	5
Agilent Poroshell 120 EC-C18, 3.0 × 100 mm, 2.7 µm (p/n 695975-302)	2.5 min	1.2 mL/min	7 min	1.2 mL/min	Time (min)	0	5	5.5	5.6	7
					% B	5	95	95	5	5
	Isocratic runs		**Gradient runs**							
3.5 µm column	**Run time**	**Flow rate**	**Run time**	**Flow rate**	**Gradient condition**					
Agilent ZORBAX Eclipse Plus C18, 3.0 × 100 mm, 3.5 µm (p/n 959961-302)	5 min	0.6 mL/min	14 min	0.6 mL/min	Time (min)	0	10	11	11.2	14
					% B	5	95	95	5	5
Agilent ZORBAX Eclipse Plus C18, 3.0 × 200 mm, 3.5 µm: coupling two 100-mm columns	10 min	0.6 mL/min	28 min	0.6 mL/min	Time (min)	0	20	22	22.4	28
					% B	5	95	95	5	5
Agilent ZORBAX Eclipse Plus C18, 3.0 × 200 mm, 3.5 µm: coupling two 100-mm columns	5 min	1.2 mL/min	14 min	1.2 mL/min	Time (min)	0	10	11	11.2	14
					% B	5	95	95	5	5

Table 2
Run times and flow rates used for 3.0-mm id columns of various lengths and particle sizes used in the experiment.

Simvastatin, clopidogrel, and lovastatin were used as samples. The USP simvastatin assay method suggests an isocratic run of 65% mobile phase B at 1.5 mL/min flow rate for a 4.6 × 250 mm, 5-µm column. This method was adopted for a 3.0-mm id column. The flow rate for the initial method was kept at 0.6 mL/min. To achieve acceptable resolution, the mobile phase percentage composition was modified from the original method to 70% mobile phase B to achieve acceptable resolution. The three standards were well separated using this method. The same samples were also run under gradient conditions (Table 2).

Preparation of standards

Prediluting solution: 3.0 mL of glacial acetic acid was added to 900 mL of water. The pH of this solution was adjusted using 5N sodium hydroxide solution to pH 4.0 and then diluted with water to 1,000 mL.

Diluting solution: 200 mL of prediluting solution was added to 800 mL of acetonitrile to obtain the diluting solution.

Mobile phase A preparation:
3.9 g of monobasic sodium phosphate was dissolved in 900 mL of water. The pH was adjusted to 4.5 using 85% ortho-phosphoric acid and diluted to 1,000 mL to obtain the buffer solution.

Mobile phase B preparation:
100% acetonitrile was used for mobile phase B.

Preparation of isocratic samples:
1 mg of simvastatin, clopidogrel and lovastatin standards were dissolved in 2 mL of acetonitrile and diluted to 20 mL using the diluting solution.

Preparation of gradient samples:
1 mg of simvastatin, clopidogrel and lovastatin standards were dissolved in 2 mL of acetonitrile and diluted to 20 mL using the prediluting solution.

Terminology

Starting method: Isocratic or gradient method, short column, low flow rate, short analysis time

Modified method A: Isocratic or gradient method, increased column length, low flow rate, increased analysis time

Modified method B: Isocratic or gradient method, increased column length, increased flow rate, short analysis time

Procedure

The columns with various particle sizes and lengths were run first under isocratic conditions (starting method), followed by two methods, one method with a longer analysis time (modified method A) and the other method with a shorter analysis time but increased flow rate (modified method B) (Table 2).

Resolution and the number of theoretical plates were calculated for the isocratic run. The same procedure was repeated for gradient runs, where resolution and peak capacity were calculated. Peak capacity was calculated according the following equation:

$$1 + tg/Pw$$

tg = time of the gradient

Pw = 5 sigma peak width.

The average of six replicates injections was used to calculate the number of theoretical plates, peak capacity, and resolution (tangent method) for clopidogrel.

Results and discussion

Chromatograms of isocratic runs

Improved resolution and higher number of theoretical plates were obtained when changing from the starting method to the modified method B, while similar RT values were obtained (Figure 1).

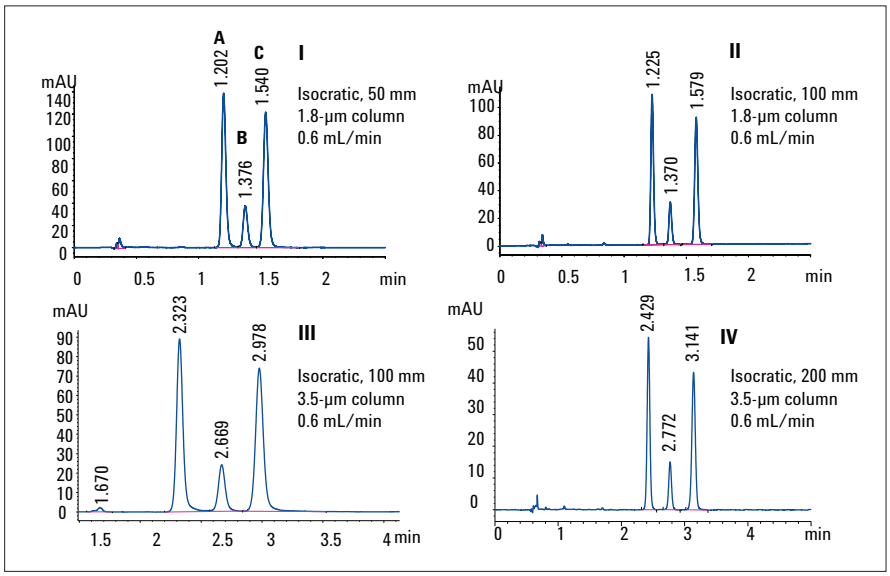

Figure 1
Isocratic chromatographic runs on: 50 mm, 1.8-µm column (I); 100 mm, 1.8-µm column (II); 100 mm, 3.5-µm column (III) and 200 mm, 3.5 µm (IV) columns. 1.2 mL/min runs represent the constant analysis time achieved due to increased flow rate on longer columns. A – lovastatin, B – clopidogrel and C – simvastatin.

Theoretical plates and resolution for isocratic runs

For all particle sizes, an increase of resolution and the number of theoretical plates could be observed as expected when changing from the starting method to the modified method A. When changing to the modified method B, the resolution and number of theoretical plates decreased compared to modified method A, but was still higher (151% in theoretical plates and 32% in resolution) than using the starting method (Figure 2 and Table 3). It can also be seen that the increase in resolution and number of theoretical plates is higher for sub-2 µm and Agilent Poroshell 120 EC-C18 columns than for 3.5-µm columns when changing from the starting method to the modified method B as expected due to the flatter *van Deemter* curves.

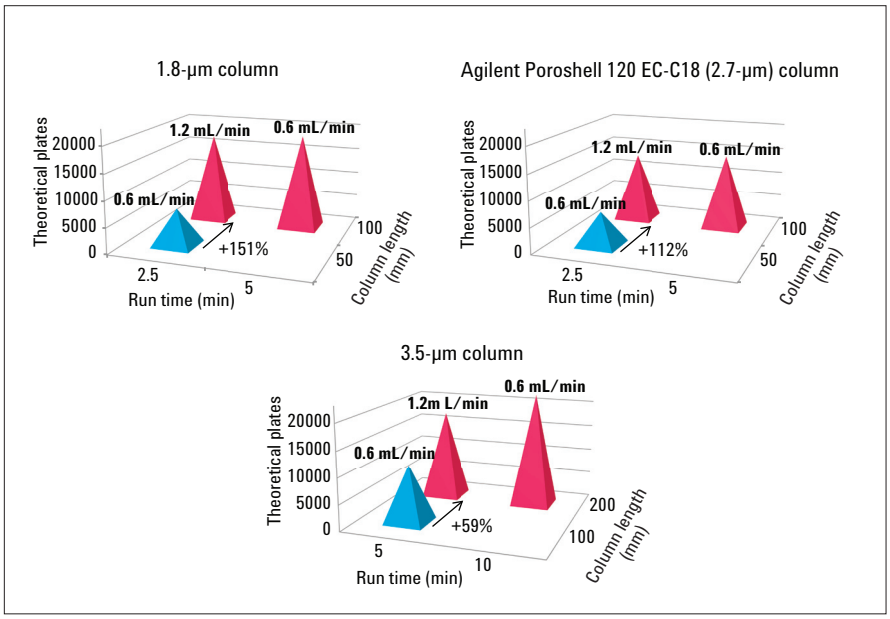

Figure 2
Isocratic runs of various particle sizes showing the increase in theoretical plates for clopidogrel while maintaining the run time.

Column type	Resolution	Resolution (modified method A)	Resolution (modified method B)	Theoretical plates	Theoretical plates (modified method A)	Theoretical plates (modified method B)
1.8-µm column	2.7	4.18	3.56	6936	18606	17399
Agilent Poroshell 120 EC-C18 column	2.11	3.27	2.83	6339	14600	13418
3.5-µm column	3.5	4.9	4.1	10949	22207	17416

Table 3
The resolution and theoretical plates with an increase in the column length.

Chromatograms of gradient runs

For the gradient runs improved resolution and peak capacity could be observed when changing from the starting method to the modified method B, while similar RT values were obtained. This is due to the narrower peak widths as shown in Figure 3: II and IV.

Peak capacity and resolution for gradient runs

Peak capacity and resolution for clopidogrel was improved when changing from the starting method to the modified method B. On the 1.8-µm columns an increase of 9% in resolution and 62% in peak capacity could be observed. The increase in peak capacity and resolution was also evident for all particle size (Figure 4 and Table 4), however the 3.5-µm particle columns showed only marginal increase in peak capacity. These results are in line with the results obtained by *Petersson et al*, where the peak capacity for sub-2 µm, 3.0 µm, and 2.7 µm were compared. Although 2.7-µm and sub-2 µm columns showed increase in peak capacity, the 3-µm particle columns showed only marginal increase from 0.4 to 0.6 mL/min[3].

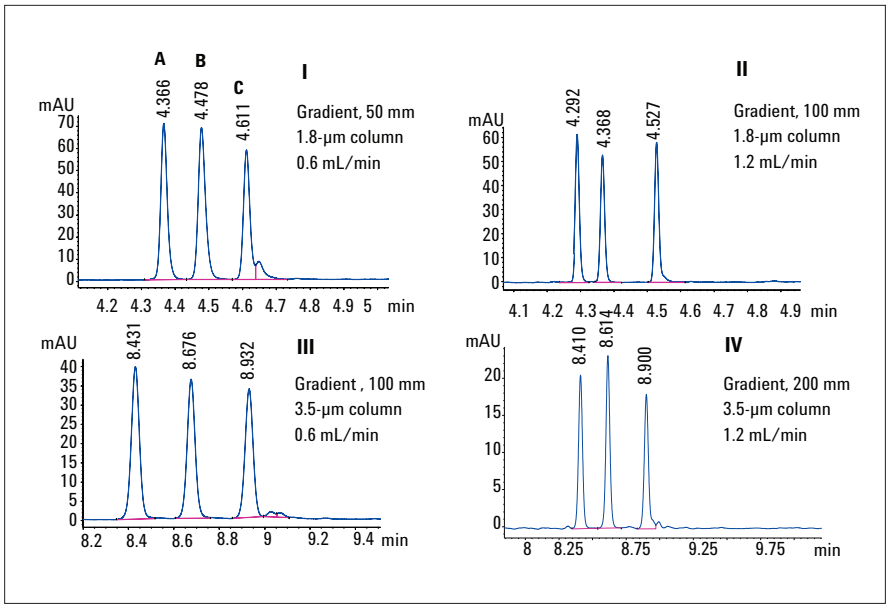

Figure 3
Gradient chromatographic runs on: 50 mm, 1.8-µm column (I); 100 mm, 1.8-µm column (II); 100 mm, 3.5-µm column (III) and 200 mm, 3.5-µm (IV) columns. 1.2 mL/min runs represent the constant analysis time achieved due to increased flow rate on longer columns. A – lovastatin, B – clopidogrel and C – simvastatin.

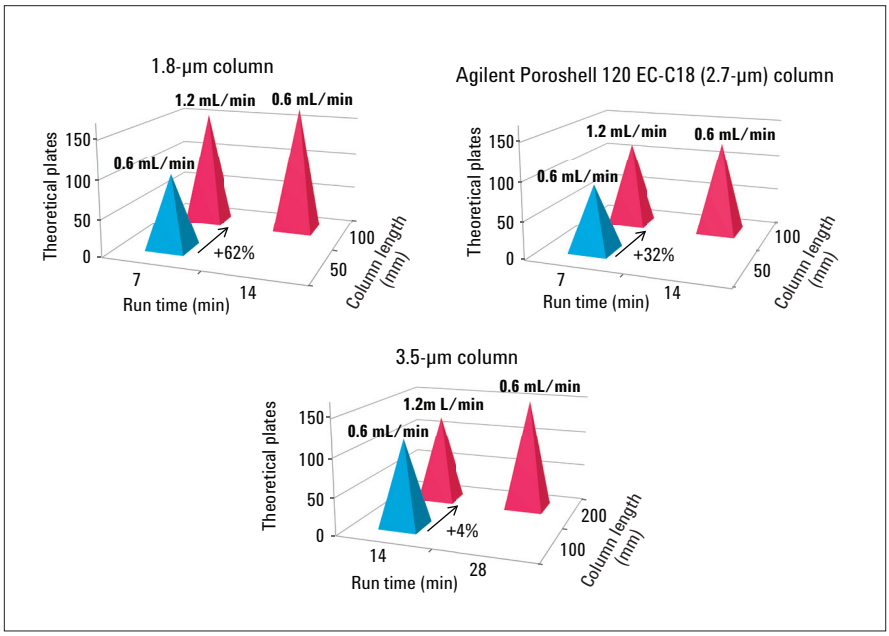

Figure 4
Gradient runs of various particle sizes showing increasing peak capacity with increasing flow rate.

Improvement in resolution for constant analysis runs

For the isocratic runs, increased resolution for clopidogrel be observed for all particle sizes when using modified method B. For the gradient runs, similar increase in resolution could be seen for all particle sizes except for the 3.5-µm columns, which show only marginal change in resolution. Figure 5 displays the increase in resolution when using modified method B while maintaining the shorter analysis time of the starting method.

Conclusion

This Application Note demonstrates that higher resolution can be achieved by using longer columns without increasing the run time by simultaneously increasing the flow rate. The experiments were carried out on totally porous 1.8-µm and 3.5-µm particle columns and superficially porous 2.7-µm particle size columns. For isocratic runs, both the resolution and theoretical plates increased with an increase in column length, while maintaining the analysis time of the starting method. In gradient runs as well, the peak capacity could be increased on all columns. It could be shown that increasing the run time when using longer columns always leads to the best number of theoretical plates, resolution and peak capacity, however, the loss when increasing the flow rate was not too significant.

Column type	Resolution	Resolution (modified method A)	Resolution (modified method B)	Peak capacity	Peak capacity (modified method A)	Peak capacity (modified method B)
1.8-µm column	2.95	4.14	3.21	95	169	154
Agilent Poroshell 120 EC-C18 column	2.29	2.85	2.42	87	125	115
3.5-µm column	3.73	4.6	3.7	115	151	120

Table 4
The resolution and peak capacity with an increase in the column length for gradient runs.

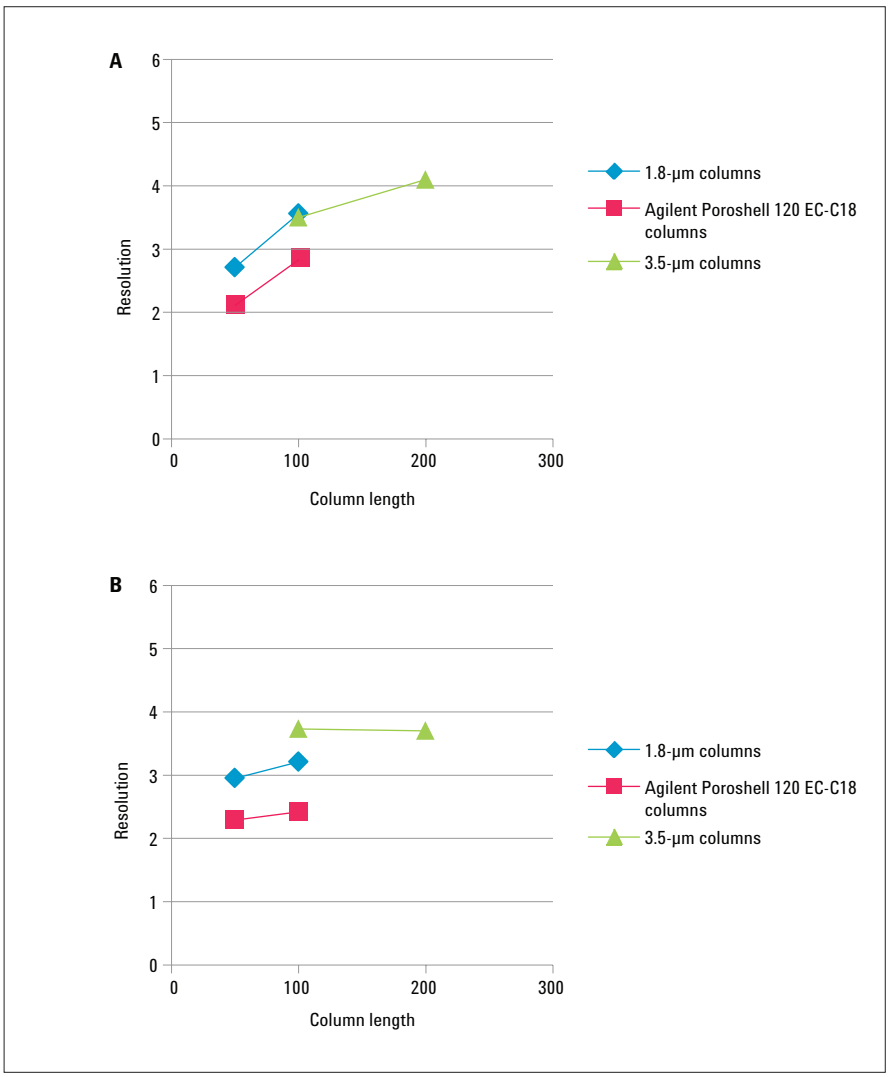

Figure 5
Increase in resolution while maintaining constant analysis time for 1.8-µm, Agilent Poroshell 120 EC-C18, and 3.5-µm columns with isocratic runs (A) and gradient runs (B).

References

1.
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