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ZORBAX® Rapid Resolution Reversed-Phase Columns for Peptide and Protein Separation

Application Note 228-376

High Performance Liquid Chromatography

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Abstract

ZORBAX® Rapid Resolution reversed-phase columns reduce analysis time and solvent usage by 40 to 66 percent (depending on column size) while maintaining equivalent resolution to 5-µm particle sized columns. Utilizing 3.5-µm particles in short column lengths (150, 75, and 50 mm), Rapid Resolution columns provide the same separating power as 250- and 150-mm columns using 5-µm particles. They have the same physical characteristics (surface area, pore size, and surface coverage) as 5-µm particles, resulting in the same selectivity and retention times.

ZORBAX StableBond Rapid Resolution columns, with both 300 Å and 80 Å pore sizes, use a proprietary bonding technology for stable operation at low pH and high temperature. This makes them ideal for protein and peptide separations.

This application note demonstrates the use of Rapid Resolution columns for process monitoring, high-throughput sample testing, highly complex tryptic digests resolution, and protein and peptide separation.

A discussion on the chromatographic theory behind Rapid Resolution columns is included.

Introduction

Rapid Resolution columns are suitable for almost any application, including high-throughput QA/QC separations as well as basic research. Rapid Resolution columns allow you to:

- Reduce analysis time and solvent use while maintaining resolution
- Improve resolution for difficult separations
- Reduce plugging compared to other columns with 3-µm particles
- Use stable bonded-phase chemistries for a wide range of applications
- Separate a wide range of molecular weight compounds with both 300 Å and 80 Å pore sizes

Save Time and Solvent

ZORBAX Rapid Resolution reversed-phase columns reduce analysis time and solvent usage by 40 to 66 percent (depending on column size) while maintaining equivalent resolution to 5-µm particle sized columns, as shown in figure 1, chromatograms A and B. The same sample is separated on a comparable 150-mm Rapid Resolution column in 24 minutes (figure 1, chromatogram B) versus 40 minutes for the 250-mm column (figure 1, chromatogram A). The more efficient 3.5-µm particles provide equivalent plates for the shorter column. Thus, gradient time and solvent usage can be reduced without compromising resolution.

Figure 1, chromatogram C, shows the same separation done on a 50-mm Rapid Resolution column in only 9 minutes. This is a *total time reduction of 75 percent* with only a 10 percent loss in resolution. Use of a 50-mm rather than the 150-mm column length was possible because the resolution of peptides in chromatogram B was greater than required. Table 1 provides information on choosing the best Rapid Resolution column to replace comparable 5-µm columns.

How Smaller Particles and Short Columns Can Improve Resolution

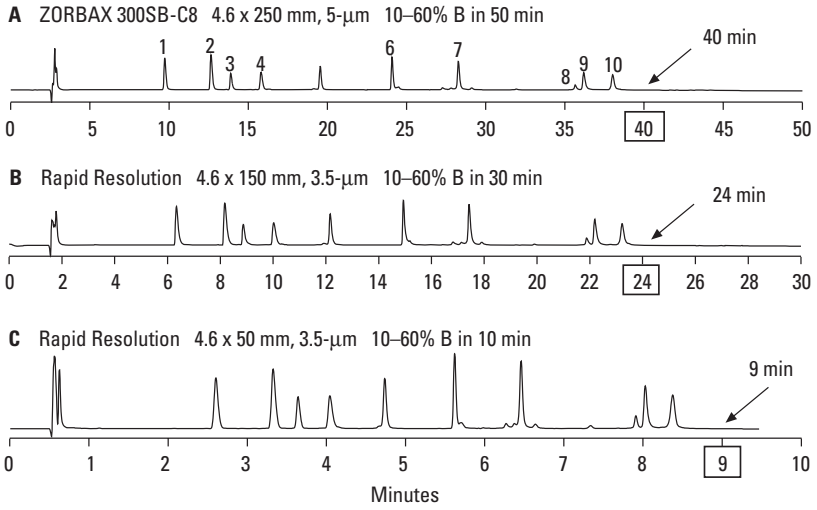
Compared to columns of the same length, Rapid Resolution columns increase efficiency, thereby improving resolution, by using smaller particles (as shown in figure 2, chromatograms A and B). Table 2 shows plate numbers for both 5-µm and 3.5-µm columns.

For example, a 150-mm, 3.5-µm column has 20,000 plates versus 12,000 plates for a 5-µm column of the same length. In crucial separations, such as quantitating a minor component next to a much larger peak, even small resolution increases may help separate compounds of interest.

Placing two 150-mm, 3.5-µm Rapid Resolution columns in series further improves resolution. The resultant run time and gradient time are approximately the same as for a 250-mm, 5-µm column. Backpressure can be lowered significantly and resolution further improved by using higher operating temperatures.[1, 5]

When using a relatively steep gradient (for example, 2 to 75 percent B/30 minutes) with a 150-mm, 3.5-µm Rapid Resolution column, changing to a shorter 50-mm, 3.5-µm column with the *same gradient time* can often improve resolution (as illustrated in figure 3). This effect is lessened when a shallower initial gradient is used.

Compared to 150-mm columns that use 5-µm particles, the shorter, 50-mm, 3.5-µm Rapid Resolution column also improves resolution with the same run time (shown in figure 4). Resolution can also be improved by other factors, such as bonded phase and mobile phase types and temperature changes.[8, 9]



Mobile Phase:	A: 95 : 5% Water : ACN with 0.1% TFA B: 5 : 95% Water : ACN with 0.085% TFA
Flow Rate:	1.0 mL/min
Detection:	UV-215 nm
Sample:	1–10-µg protein

Peaks	
1. Met-enkephalin	6. Insulin (Bov)
2. Leu-enkephalin	7. Lysozyme
3. Angiotensin II	8. Calmodulin
4. Neurotensin	9. Myoglobin
5. RNase	10. Carbonic anhydrase

Figure 1. Rapid Resolution with Reduced Particle Size[6]

Table 1. Conversion of 5-µm Columns to 3.5-µm Rapid Resolution Columns

Initial 300 Å or 80 Å 5-µm Column	300 Å, 3.5-µm Rapid Resolution Column	Time, Solvent Reduction	80 Å, 3.5-µm Rapid Resolution Column	Time, Solvent Reduction
4.6 x 250 mm	4.6 x 150 mm	40%*	4.6 x 150 mm	40%*
4.6 x 250 mm	4.6 x 50 mm	75%**	4.6 x 75 mm	66%**
4.6 x 150 mm	4.6 x 50 mm	66%*	4.6 x 75 mm	50%*

* Resolution equivalent to that of initial column

** Approximately 10 percent loss in resolution from 4.6 x 150 mm 3.5-µm column, with optimized gradient time

Table 2. Approximate Plate Number (N) and Void Volume (Vm) for 5-µm and 3.5-µm Columns

Column Size	Particle Size	Plate Number (N)	Void Volume (Vm)
4.6 x 250 mm	5-µm	20,000	2.5 mL
4.6 x 150 mm	5-µm	12,000	1.5 mL
4.6 x 150 mm	3.5-µm Rapid Resolution	20,000	1.5 mL
4.6 x 75 mm	3.5-µm Rapid Resolution	10,000	0.75 mL
4.6 x 50 mm	3.5-µm Rapid Resolution	6,000	0.5 mL

Precisely Sized Packing Material Maximizes Column Life

Particle-size distribution of the 3.5-µm ZORBAX Rx-SIL silica particles used in Rapid Resolution columns is closely controlled to narrow tolerances.[4] This allows use of 2-µm porosity inlet frits to maximize column life by minimizing plugging by particle "fines." By contrast, columns of broad particle-size distributions for other 3-µm particles contain more fines, requiring the use of 0.5-µm porosity inlet frits that plug easily with sample debris.

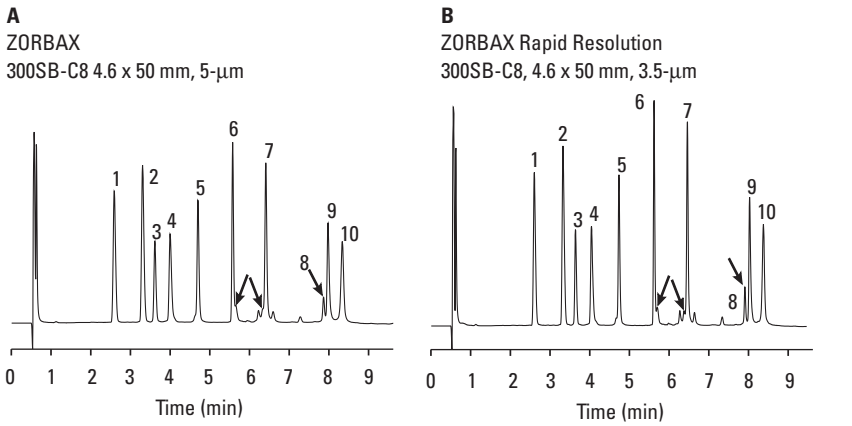
Rapid Resolution columns readily operate without plugging providing rugged methods at operating pressures compatible with standard HPLC systems.

Reversed-Phase Surface Chemistry Specific for pH Range Ensures Stability

Designed for use at low pH, ZORBAX StableBond columns use a patented bonding chemistry that ensures long column life. This chemistry uses unique silanes to protect the siloxane bond sterically at low pH (as shown in figure 5).

StableBond phases are extremely acid-stable, even for short-chain CN and C3, allowing a wide range of reliable selectivity.[2] These stable, shorter-chain phases are often effectively used to perform separations not possible using either C18 or C8 phases. StableBond columns can also be used at high temperatures—up to 90 °C for C18, and up to 60 °C for C8, C3, and CN.

High temperatures are useful for separating hydrophobic and aggregating peptides[3, 9] as well as for general protein and peptide separations. ZORBAX StableBond Rapid Resolution columns are available in both 300 Å and 80 Å pore sizes.

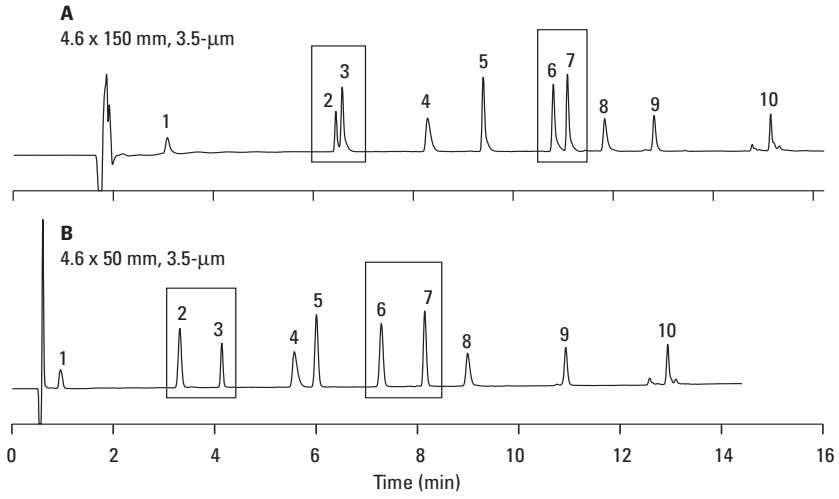


• Resolution improved with 3.5-µm particles

Mobile Phase:	A: 95% Water : 5% ACN, 0.1% TFA B: 5% Water : 95% ACN, 0.085% TFA
Gradient:	10–60% B over 30 min
Flow Rate:	1.0 mL/min
Temperature:	Room temperature

Peaks	
1. Gly-tyr	6. Leu-enk
2. Val-tyr-val	7. Angiotensin II
3. [Glu]-ß protein amyloid fragm 1-16	8. Kinetensin
4. [Tyr⁸] bradykinin	9. RNase
5. Met-enk	10. Ins(equine)

Figure 2. Rapid Resolution Separation of Peptides[6] Improved with 3.5-µm Particles



Column:	ZORBAX 300SB-C8, 3.5-µm
Mobile Phase:	A:5:95, ACN:water 0.1% TFA B:95:5, ACN:water 0.085% TFA
Gradient:	2–75% B in 30 min
Flow Rate:	1.0 mL/min
Injection:	10-µL, 2.6-µg each
Temperature:	35 °C
Detection:	UV-210 nm

Peaks	
1. Gly-tyr	6. Leu-enk
2. Val-tyr-val	7. Angiotensin II
3. [Glu]-ß protein amyloid fragm 1-16	8. Kinetensin
4. [Tyr⁸] bradykinin	9. RNase
5. Met-enk	10. Ins(equine)

Figure 3. For Steep Gradients, Improved Resolution with Shorter Column Length[7]

Eclipse XDB-C8 columns use proprietary bonding chemistry that gives superb peak shape and high stability for applications in the mid-pH range. To achieve these benefits, Eclipse bonding chemistry uses a dimethyl-C8 bonded phase that is end-capped with two different reagents to deactivate the surface. Eclipse XDB-C8 columns are available in the 80 Å pore size that is useful for many high-resolution peptide separations.

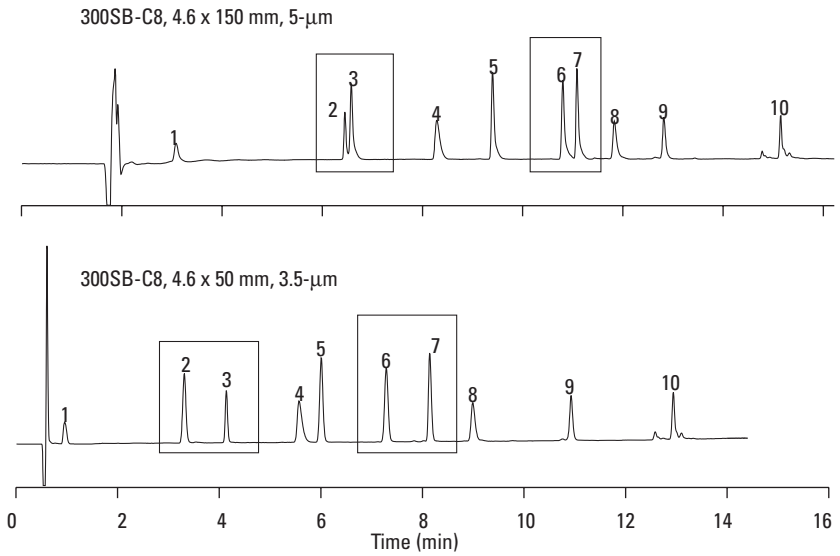
Applications

These columns are particularly effective in resolving small impurities, monitoring high throughput processes, resolving highly complex tryptic digests, and separating a range of proteins.

Resolving Small Impurities in Pharmaceutical Samples

One of the most demanding applications in modern protein chemistry is quantitating low-level impurities in protein or peptide samples. For these separations, small particles offer improved resolution and sensitivity. Figure 6, chromatograms A and B, shows the improved resolution of minor components a and b in a tryptic digest using a Rapid Resolution column (R_s = 1.3 vs 1.0).

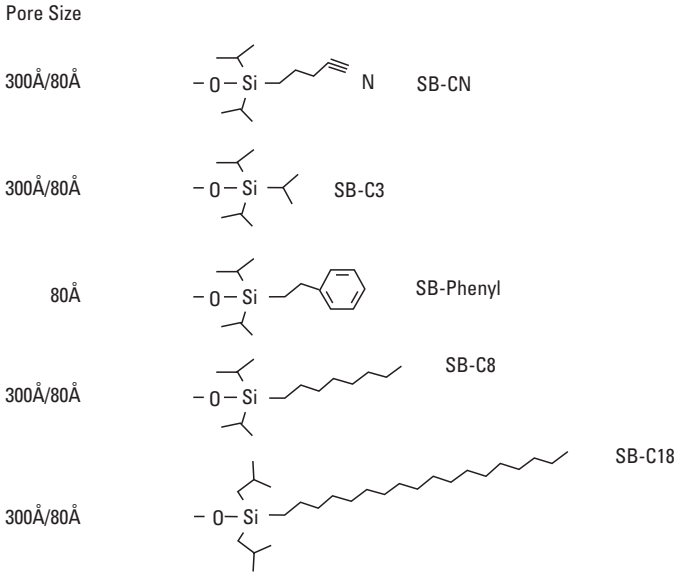
A further improvement in resolution (R_s = 1.5) is achieved by using the column at a higher temperature (figure 6, chromatogram C). The higher temperature even resolves an additional, new minor component (as shown in the inset). Using ZORBAX StableBond reversed-phase packings ensures long column life during this high-temperature operation.



Mobile Phase:	A: 95% Water : 5% ACN, 0.1% TFA B: 5% Water : 95% ACN, 0.085% TFA Gradient: 10–60% B over 30 min
Flow Rate:	1.0 mL/min
Temperature:	Room temperature

Peaks	
1. Gly-tyr	6. Leu-enk
2. Val-tyr-val	7. Angiotensin II
3. [Glu]-ß protein amyloid fragm 1-16	8. Kinetensin
4. [Tyr^8] bradykinin	9. RNase
5. Met-enk	10. Ins(equine)

Figure 4. Increased Gradient Resolution (R_s) and Retention (k*) by Decreasing Column Length and Particle Size[7]



Bulky diisobutyl (with C18) or diisopropyl (with C8, C3, CN, phenyl) side-chain groups are used to stabilize both long- and short-chain monofunctional ligands.

Figure 5. ZORBAX StableBond Structures

Process Monitoring for High-Throughput Sample Testing

Because of their fast re-equilibration, Rapid Resolution columns are ideal for process monitoring or high throughput sample testing requiring gradient elution (as illustrated in figure 7). For this series of three complete injections in less than 40 minutes, analysis time for this complex sample is less than 10 minutes and re-equilibration time is only 4 minutes.

Tryptic Digests

Rapid Resolution columns can be used effectively to resolve highly complex tryptic digests (shown in figure 6, chromatogram B). The combination of using high temperature (85 °C) with StableBond phases and a Rapid Resolution column successfully resolved the indicated peaks in a calmodulin digest (figure 6, chromatogram C).

At the same time, backpressure decreased because of the increase in temperature. The combination of high efficiency and stable operation at high temperature and low pH is a unique feature of ZORBAX StableBond columns.

General Protein and Peptide Separations

Rapid Resolution columns are available with a variety of pore sizes and stationary phases that can be used effectively for a wide range of protein separations. Complex peptide samples often are best separated with Rapid Resolution columns. These columns can be connected in series and operated at high temperatures for extremely difficult separations.

Chromatographic Theory Behind Rapid Resolution Columns

The fundamental resolution (R_s) equation for gradients is,

$$R_s \approx (^1/_4)\ \sqrt{N}\ \alpha^* k^*$$

where

$N = 16\ (t_r/t_w)^2$

$\alpha^* = k^*_2/k^*_1$

$k^* \cong\ t_g F/S\Delta\phi V_m$

where:

α^* = selectivity factor (band spacing)

N = plate number

k^* = gradient retention factor

t_g = gradient time (min)

t_w = peak baseline width time

F = flow rate (mL/min)

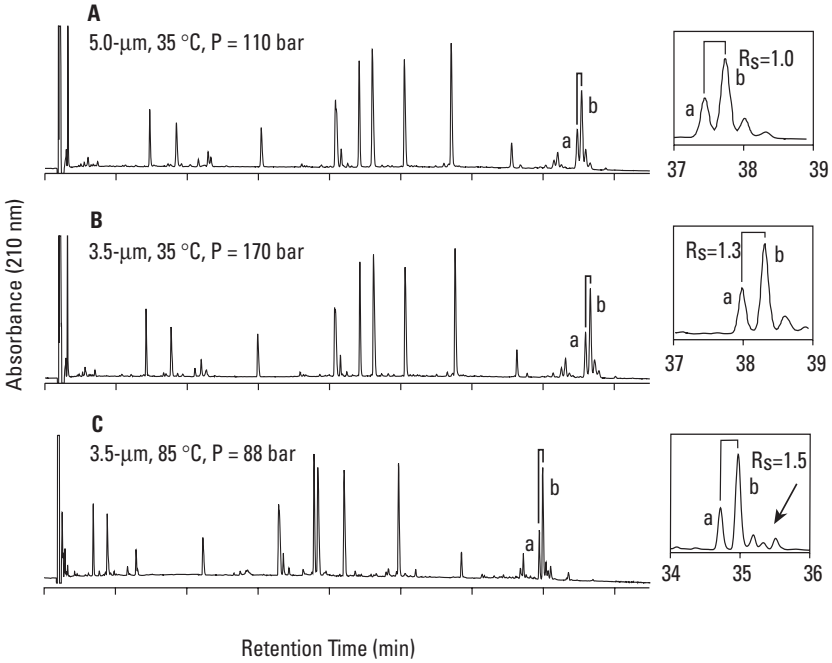
t_r = peak retention time

S = compound-dependent constant

$\Delta\phi$ = gradient range, or % change in organic content

V_m = column void volume

When changing columns from 250 mm to 150 mm in figure 1, chromatograms A and B, N (plate number) essentially remains constant because the loss of plates from shortening the column is offset by the increase of plates by the reduction in particle size. Figure 8 shows the relation between N, particle size, and flow rate. As particle size decreases, N increases. Note that higher flow



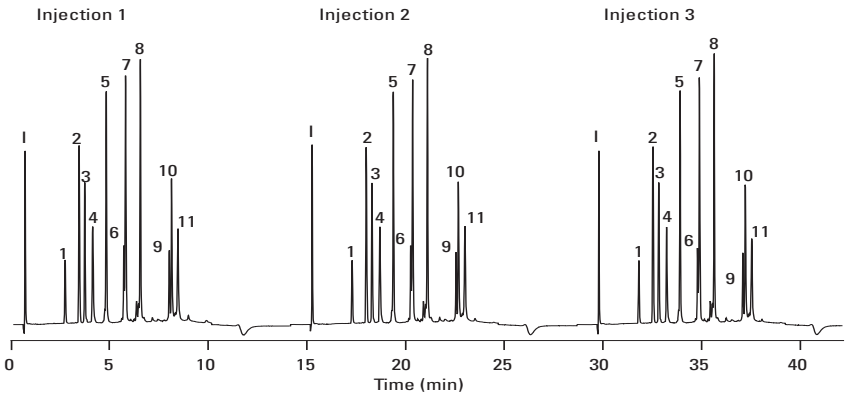
Column:	ZORBAX SB-C18, 4.6 x 150 mm
Mobile Phase:	Linear Gradient: 5–60% B in 45 min
	A: 0.1% TFA in water
	B: 0.095% TFA in 80% acetonitrile/20% water
Flow Rate:	1.5 mL/min

Figure 6. Effect of Particle Size and Temperature on Peak Width of Calmodulin Digest[1]

rates can be used with 3.5-µm columns with minimal loss in plates. In figure 1, chromatogram B, k* remains constant because V_m decreases by 40 percent as a result of the shorter column length and, simultaneously, t_g is reduced by 40 percent. As k* is constant, R_s is also unchanged, but gradient time and solvent consumption decrease.

Smaller particles increase N (plate number) in the fundamental resolution equation shown above, thus improving resolution by giving sharper peaks (figure 2). Because N is a square root function of resolution, a larger increase in N is required to equal the increase in resolution by optimizing the linear separation parameters α*, (selectivity or band spacing), and k* (retention factor). In contrast to α*, however, N is predictable.

In figure 3, a 150-mm, 3.5-µm column is compared to a 50-mm, 3.5-µm Rapid Resolution column. In this case, N decreases with the shorter column. However, k* increases (smaller V_m term), while t_g is constant—unlike figure 1, chromatogram B, where t_g is decreased. Because the k* term increases *more* than N decreases, resolution increases. This improvement results from a flattening of the gradient (showing decreasing steepness) for the shorter column. More important, however, is the increase in band spacing (the larger α* value) between individual bands that often, but not always, occurs as a result of the less steep gradient generated. Values of k* (retention) increase with less steep gradients, but whether bands will move further apart or closer together (α*) is not predictable. Less steep gradients often result in improved resolution for mixtures of both peptides and proteins. With a shallower initial gradient range (lower [Δϕ/time]) and a longer column, the effect of improving resolution by using a shorter column with the same gradient time is reduced. References 8 and 9 provide a complete discussion of the effects of gradients and other operating parameters on HPLC separations.



Column:	ZORBAX 300SB-C8 4.6 x 50 mm, 3.5-µm
Mobile Phase:	A: 95% water : 5% ACN, 0.1% TFA B: 5% water : 95% ACN, 0.085% TFA Gradient: 10–60% B over 10 min
Flow Rate:	1.0 mL/min
Temperature:	35 °C

Peaks	
1. Met enkephalin	7. Cytochrome C
2. Leu enkephalin	8. Lysozyme
3. Angiotensin II	9. Calmodulin
4. Neurotensin	10. Myoglobin
5. RNase	11. Carbonic anhydrase
6. Insulin (Bov)	

Figure 7. Rapid Resolution Gradient Separation of Polypeptides[6]

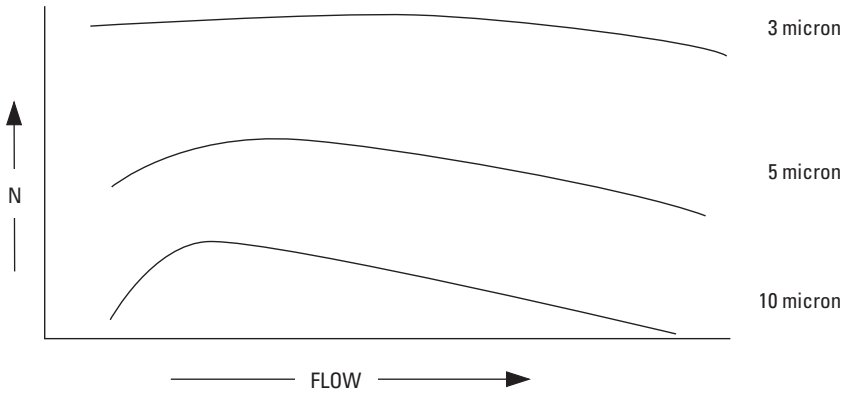


Figure 8. $N \propto 1/dp^2$

Although an increased gradient time decreases gradient steepness and can increase resolution (figure 9), run time increases. As detailed above, shortening the column with the same gradient time provides a less steep gradient, and has the advantage of a shorter run time.

Figure 4 compares a 150-mm, 50-µm column to a 50-mm, 3.5-µm Rapid Resolution column. Here, N remains similar (decreased because of column length, but balanced by the increase in N from using 3.5-µm particles). The k* term increases because V_m decreases and t_g is constant. As in figure 3, α* also increases. Thus, both k* and α* terms increase and N is only slightly less, improving resolution. Increases in α* are not predictable, but can significantly improve resolution.

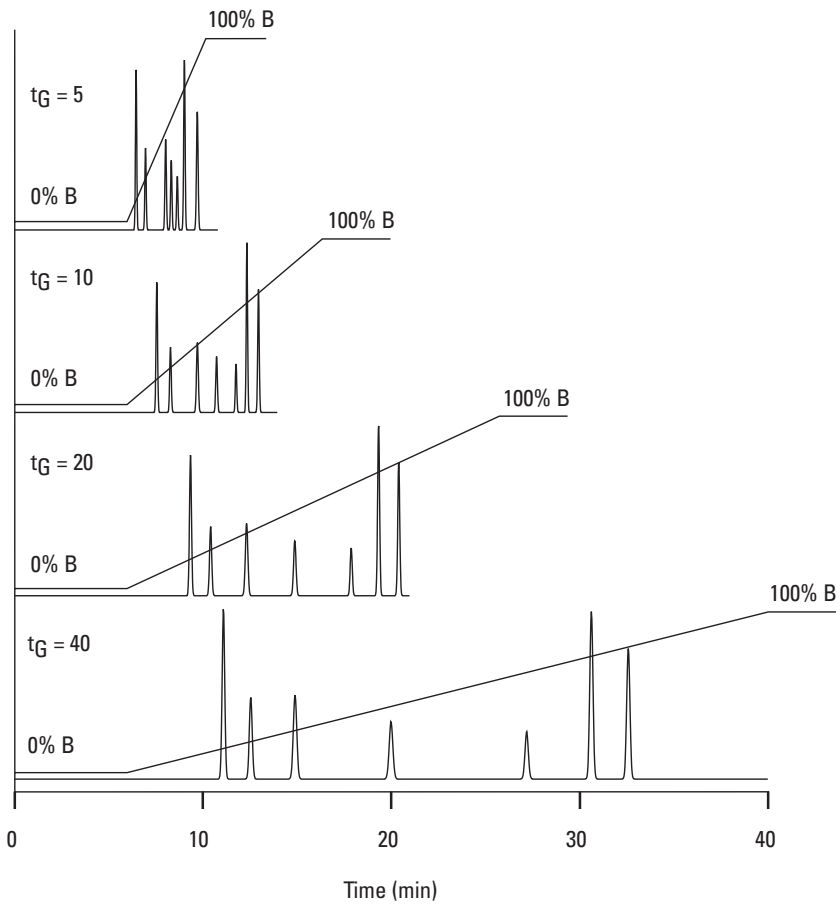


Figure 9. Gradient Steepness Effects on Retention (k*) and Resolution

Ordering Information for 3.5-µm ZORBAX Rapid Resolution Columns (Part Numbers)

300 Å ZORBAX StableBond Rapid Resolution Columns

Size (mm)	300SB-C18	300SB-C8	300SB-CN	300SB-C3
4.6 x 50	865973-902	865973-906	865973-905	865973-909
4.6 x 150	863973-902	863973-906	863973-905	863973-909
Guard Cartridges (4/pk)*	820950-921	820950-918	820950-923	820950-924

80 Å ZORBAX StableBond (SB) Eclipse and Rx Rapid Resolution Columns

Size (mm)	Eclipse XDB-C8	Eclipse XDB-C18	SB-C18	SB-C8	SB-CN	SB-Phenyl	Rx-C18
4.6 x 75	996967-906	966967-906	866953-902	866953-906	866953-905	866953-912	866967-902
4.6 x 150	963967-906	963967-902	863953-902	863953-906	863953-905	863953-912	863967-902
Guard Cartridges (4/pk)*	820950-926		820950-920	820950-915	820950-916	820950-917	820950-914

*Guard cartridges are 4.6 x 12.5 mm and require a reusable hardware kit, HP part number 820777-901.
Inquire for 2.1 mm and additional column configurations.

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4. Kirkland, J. J., "HPLC Method Development: Practical Aspects of Increasing Analysis Speed While Maintaining Separation Resolution," *Journal of Chromatographic Science*, 31, 1993, pp. 493-497.

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9. Snyder, L. R., Glajch, J. L., and Kirkland, J.J., "Practical HPLC Method Development," 2nd edition, John Wiley and Sons, New York, 1997.

For More Information

For more information on ZORBAX Rapid Resolution and StableBond columns, please see the following publications.

1. "ZORBAX Rapid Resolution HPLC Columns," Hewlett-Packard Company, Publication (23) 5965-7509E, March 1997

2. "ZORBAX StableBond Preparative Columns," Hewlett-Packard Company, Publication (23) 5965-7510E, March 1997.

3. Boyes, B. E and Coates, S. "Separation and Isolation of Hydrophobic Peptides Using HPLC," Hewlett-Packard Company, Publication (23) 5965-7507E, March 1997.

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