

Welcome to our E-Seminar:

Choosing HPLC Columns for Faster Analysis Smaller and Faster



Agilent Technologies

High Throughput/Fast LC Requires....

Short columns – 50 mm or shorter

Small particle sizes – 3.5 μm Rapid Resolution or new 1.8 μm Rapid Resolution HT for maximum resolution rather than traditional 5 μm or 10 μm particle size columns

Optimized HPLC equipment – to maintain resolution and eliminate band broadening due to extra column volume

And can be used for...

Faster method development

Faster analysis

Solvent savings

Zorbax High Throughput and High Resolution HPLC with Rapid Resolution and Rapid Resolution HT Columns

Rapid Resolution Columns

3.5 μm particle size

150 mm, 100 mm, 75 mm, 50 mm, 30 mm, 15 mm column lengths

Lower efficiency than Rapid Resolution HT

Lower pressure than Rapid Resolution HT

Rapid Resolution HT Columns

1.8 μm particle size

50 mm, 30 mm and 15 mm column lengths

75 – 100% greater efficiency than Rapid Resolution

Higher pressure than Rapid Resolution

Rapid Resolution Chromatography Increases Sample Throughput for Isocratic or Gradient Elution

Decrease Column Length



decreases)

+

Decrease in Particle Size



=

proportional decrease
in analysis time (time~length, L)

proportional decrease
in solvent usage (~L)

N, plates~ L and thus N

maintain resolution, R

$N \sim 1/d_p^{1.8}$ and thus N increases
 $R \sim N^{1/2}$

ΔP , pressure $\sim 1/d_p^2$; ΔP

ZORBAX Rapid Resolution and Rapid Resolution HT Column Lengths and Typical Efficiencies

	Column Length	Resolving Power	Resolving	Efficiency (N)	Analysis Time
		N (3.5 μm)*	N (1.8 μm)		
High Resolution	150	21,000	N.A.	↑ Pressure ↓	↓ Peak Volume
	100	14,000	N.A.		
	75	10,500	N.A.		
High Throughput	50	7000	11,500		
	30	4200	6500		
	15	2100	2500		

Resolution $\propto N^{1/2}$

*5 μm HPLC columns of the same length have 40% fewer plates (N-value); 4.6-mm i.d.

N.A. – Not currently available

ZORBAX Rapid Resolution and Rapid Resolution HT Column Lengths

	Column Length (mm)	Resolving Power N(3.5 μ m)	Resolving Power N(1.8 μ m)		Analysis Time*	
High Resolution	150	21,000	N.A.	Efficiency (N) ↑	-	
	100	14,000	N.A.		Analysis Time	-33%
	75	10,500	N.A.		Peak Volume	-50%
High Throughput	50	7,000	11,500	Pressure ↓	-67%	
	30	4,200	6,500		Solvent Usage	-80%
	15	2,100	2,500			-90%

* Reduction in analysis time compared to 150mm column

Reduce Analysis Time With Rapid Resolution Columns (3.5- μm)

Conditions:

Columns: ZORBAX SB-C18

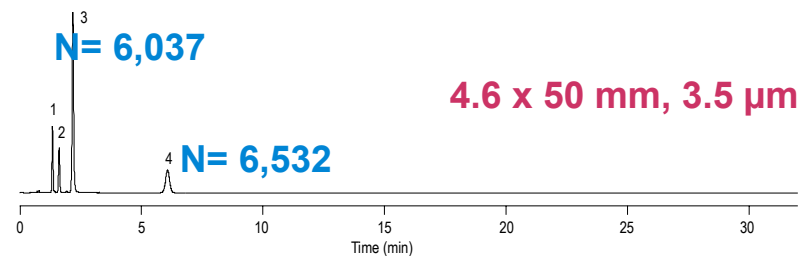
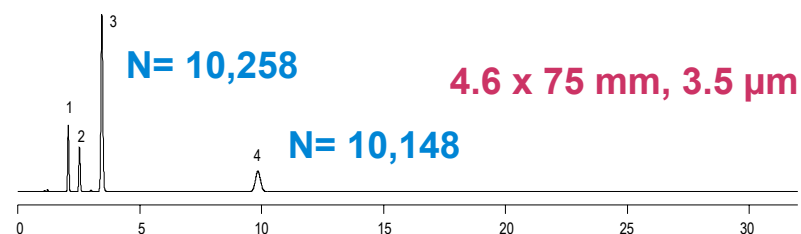
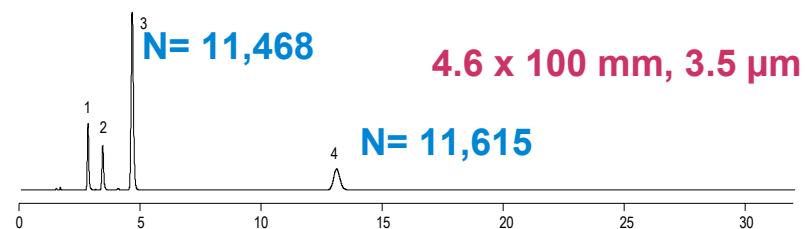
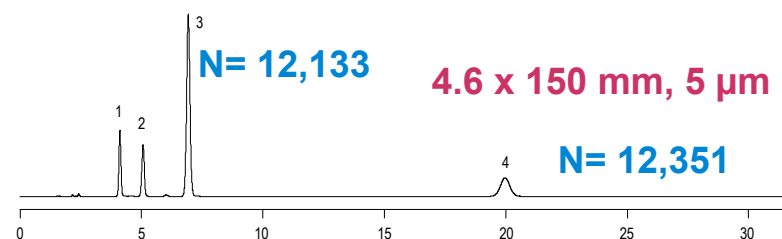
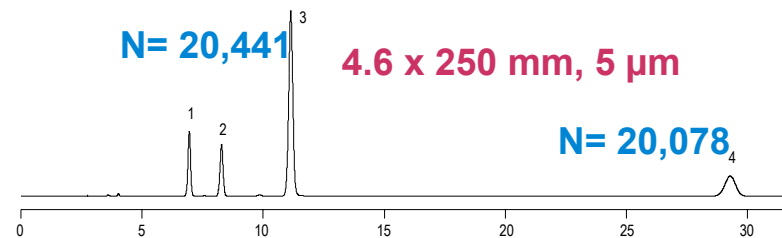
Mobile Phase: A: 50% 20 mM NaH_2PO_4 , pH 2.8

B: 50% ACN

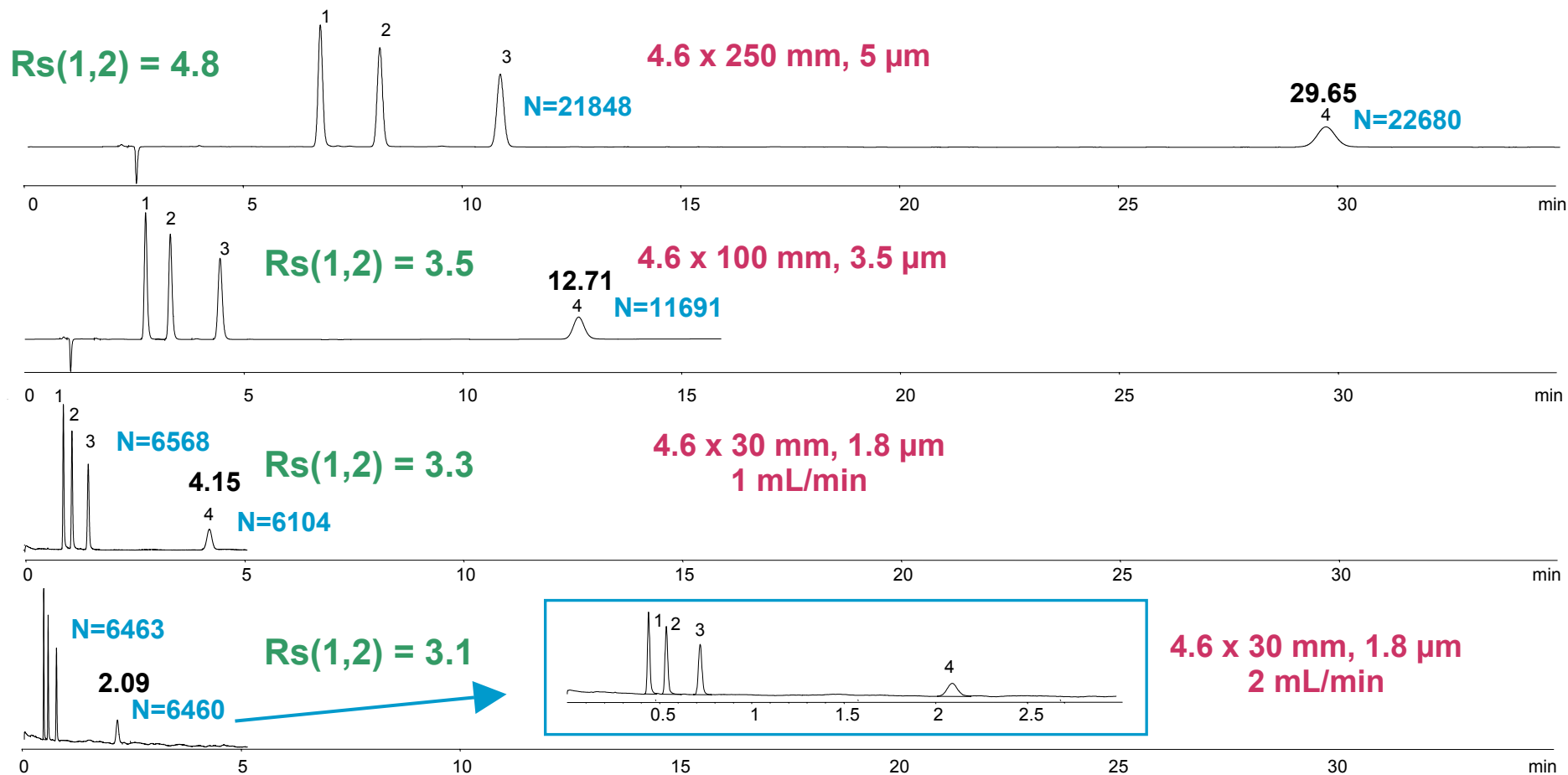
Flow Rate: 1 mL/min

Temperature: 25°C

Sample: 1. Estradiol
2. Ethynylestradiol
3. Dienestrol
4. Norethindrone

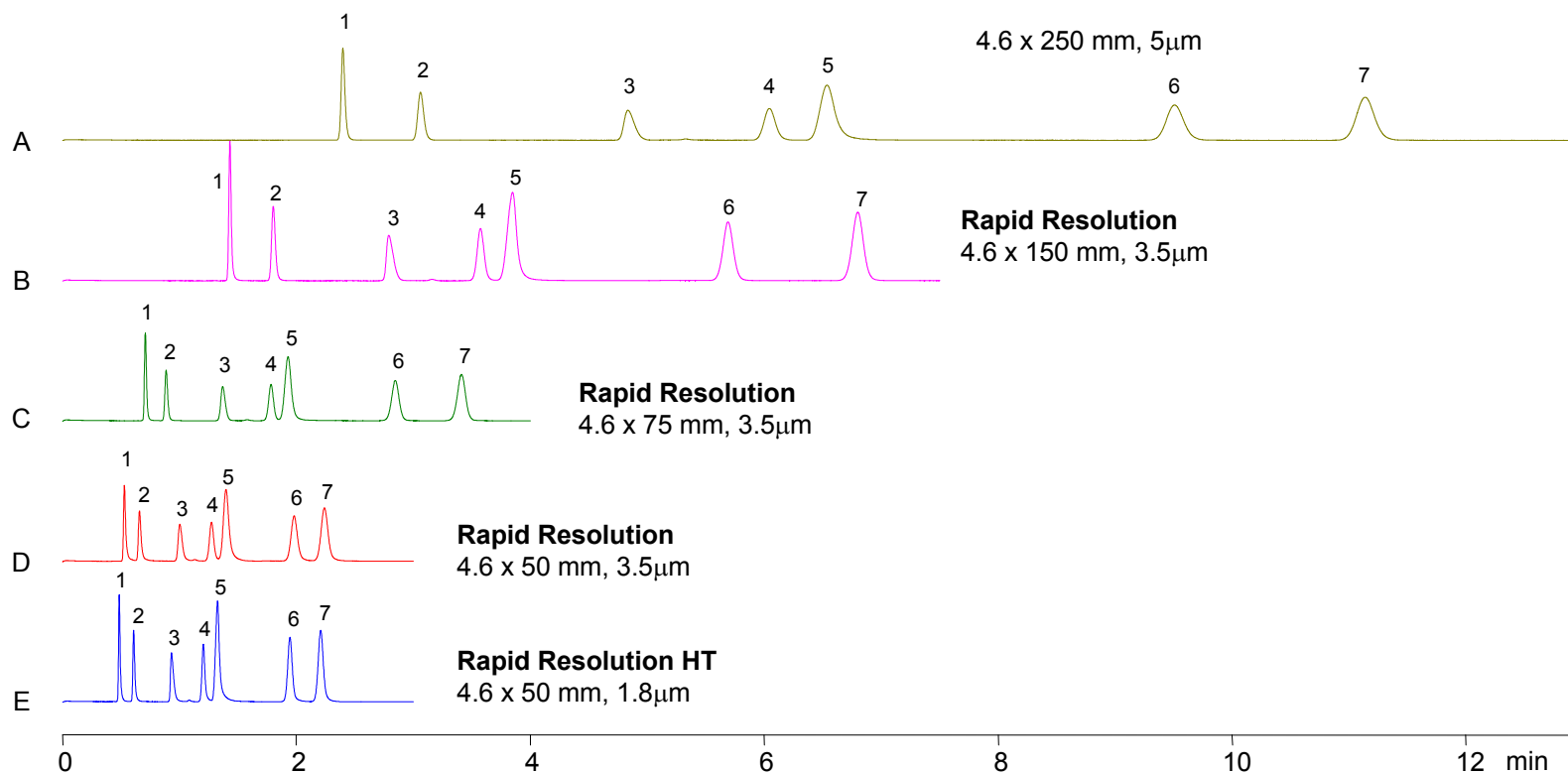


Reduce Analysis Time Even Further by using Rapid Resolution HT (1.8- μm) Columns



Columns: ZORBAX SB-C18 Mobile Phase: 50% 20 mM NaH_2PO_4 , pH 2.8: 50% ACN Flow Rate: 1 mL/min Temperature: RT
 Detection: UV 230 nm Sample: 1. Estradiol 2. Ethynylestradiol 3. Dienestrol 4. Norethindrone

Reduce Analysis Time by 80% Using Rapid Resolution HT Columns



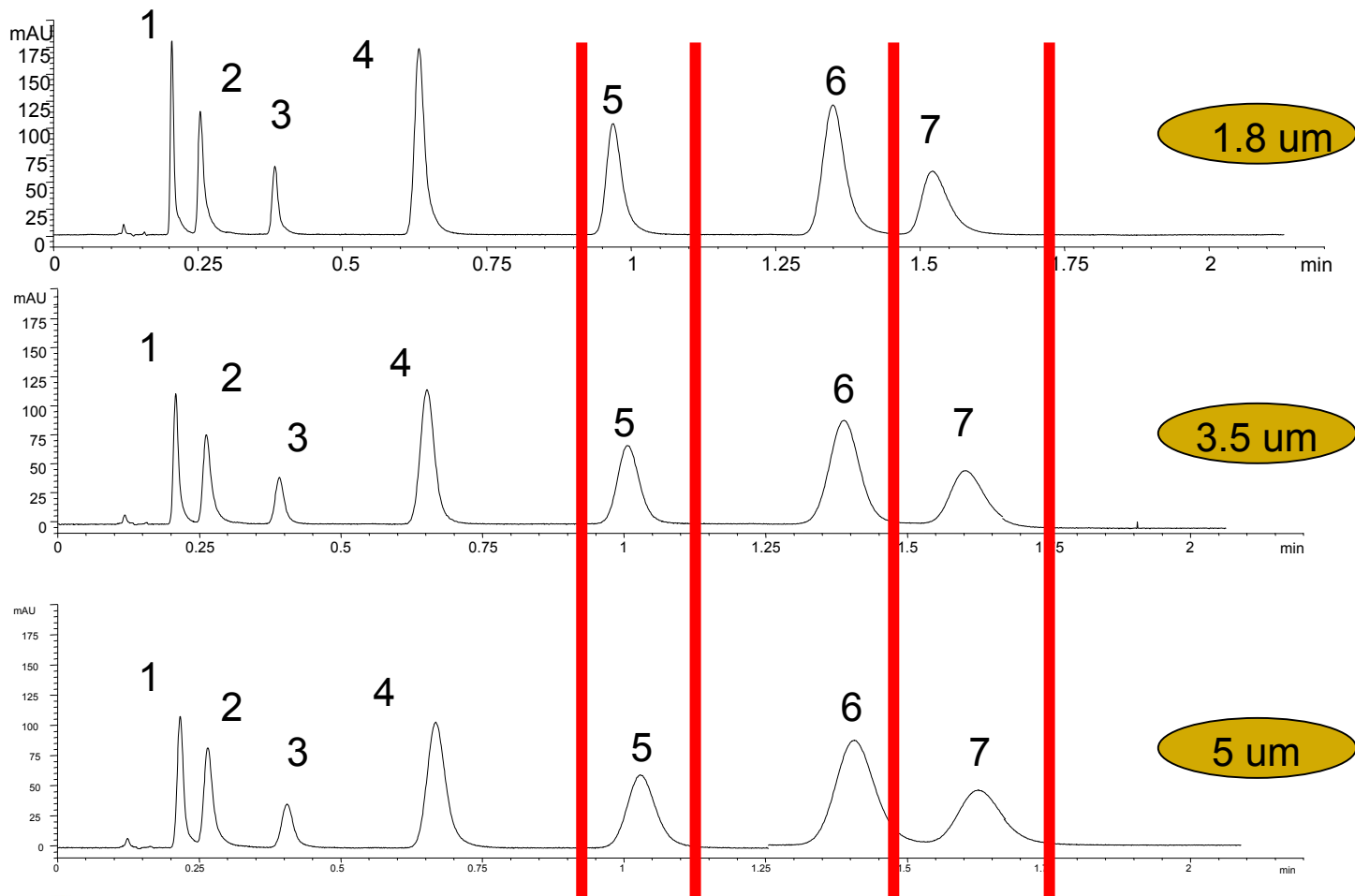
Columns: ZORBAX Eclipse XDB-C18
 Part Numbers: A) 990967-902
 B) 963967-902
 C) 966967-902
 D) 935967-902
 E) 925975-902

Mobile Phase: 73% MeOH, 27% 20mM Phosphate Buffer, pH 7.0
 Flow Rate: 1 mL/min
 Temperature: Ambient
 Detection: UV 254 nm
 Sample: 1) uracil, 2) naproxen, 3) mefanamic acid, 4) butyl paraben,
 5) propranolol, 6) naphthalene, 7) dipropyl phthalate



High Speed Separation of Analgesics

Impact of Particle Size on Resolution



- 1 4-Acetamidophenol
- 2 Caffeine
- 3 2-Acetamidophenol
- 4 Acetanilide
- 5 Acetylsalicylic Acid
- 6 Phenacetin
- 7 Salicylic Acid

LC Conditions

Column: SB-C18, 4.6 x 30 mm

Detector: 254 nm

Injector: 1 μl ,

Mobile Phase: 1% Formic Acid
Acetonitrile
(82:18)

Flow: 2.0 ml/min

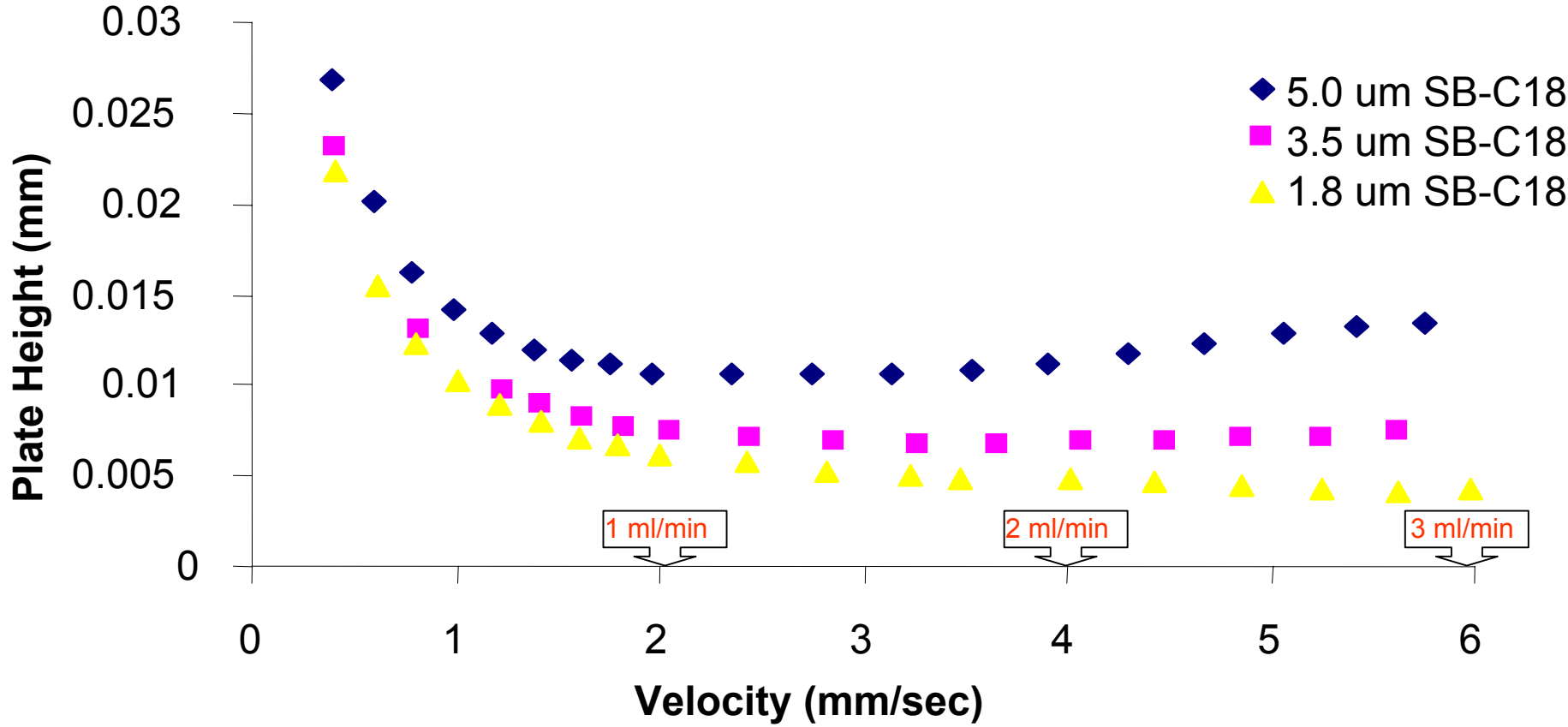
Chromatographic Comparisons on Columns with Different Particle Sizes

Separation of Analgesics

Chromatographic Parameter	Particle Size		
	5 μm	3.5 μm	1.8 μm
Peak width, μL – Acetylsalicylic acid	0.056	0.043	0.028
Peak width, μL – Salicylic acid	0.077	0.059	0.049
Resolution – Acetylsalicylic acid/ Phenacetin	4.7	6.0	8.5
Resolution – Phenacetin/Salicylic acid	1.61	2.07	2.25
Efficiency (plates)– Acetylsalicylic acid	1980	3170	6474
Efficiency (plates)– Salicylic acid	1917	3430	6049

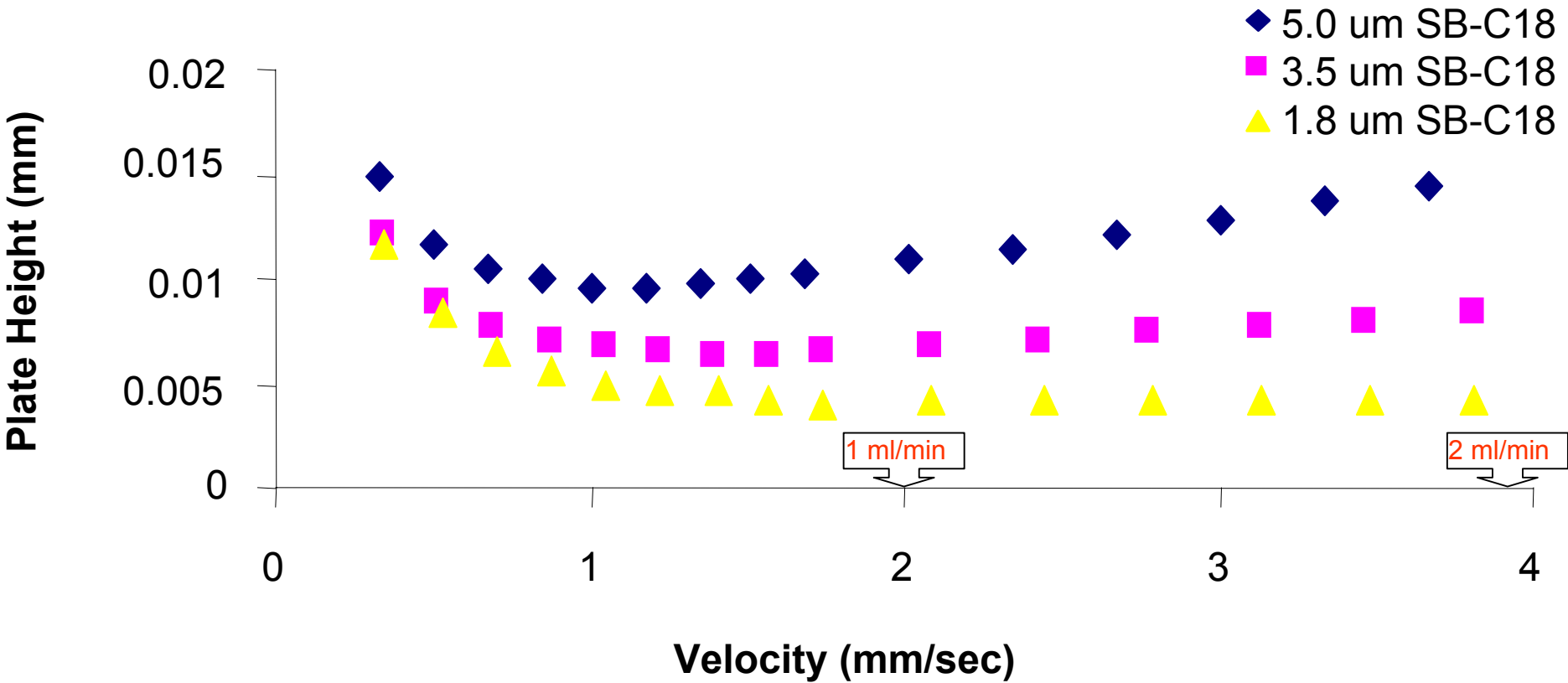
- The Rapid Resolution HT column with 1.8 μm particles provides the best results, measured by any parameter.

Column Particle Size Comparison - Van Deemter Curve for Acetonitrile/Water



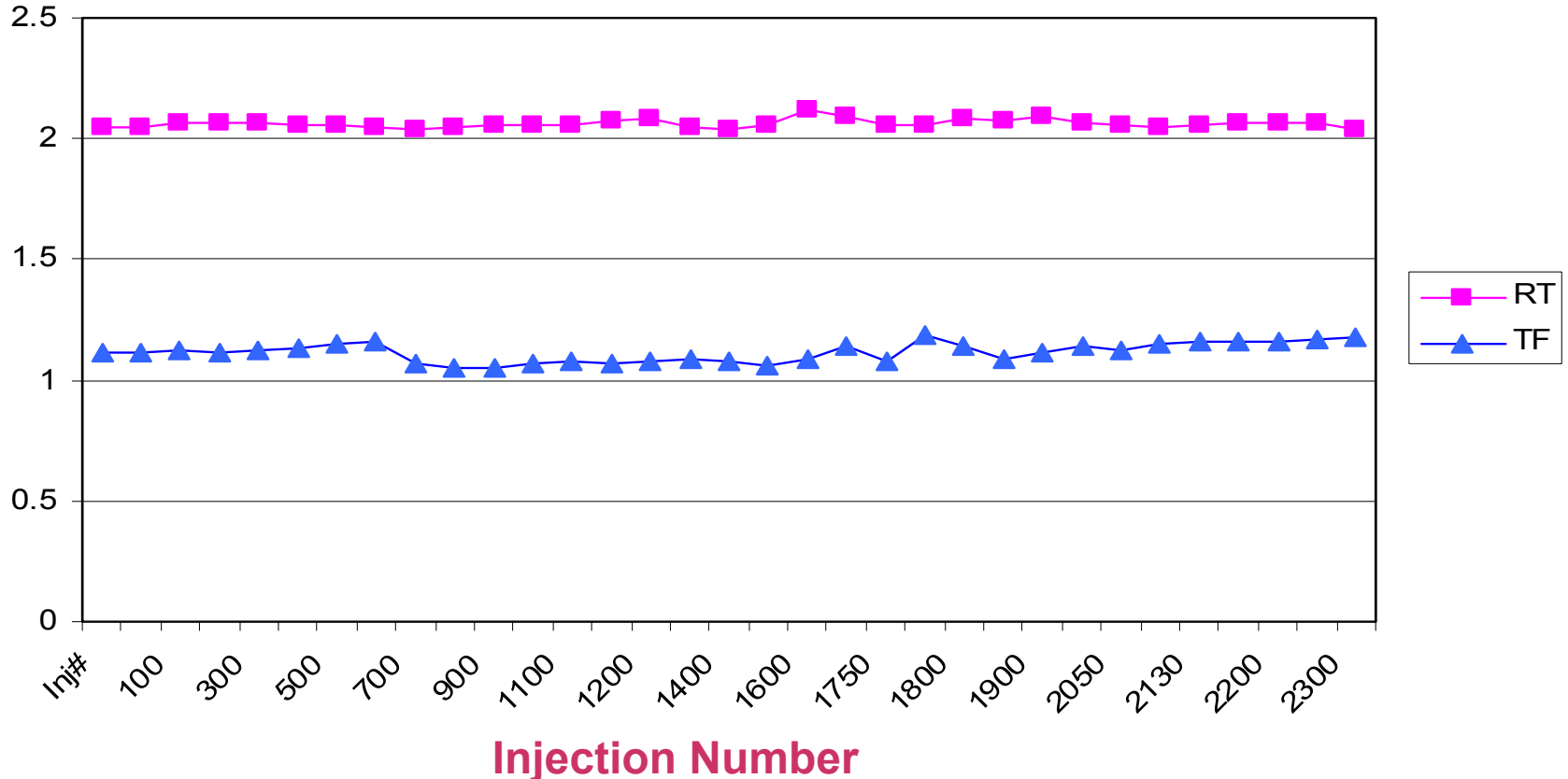
High flow rates can be used with acetonitrile/water mobile phases.

Column Particle Size Comparison - Van Deemter Curve for Methanol/Water



Columns with Smaller Particle Sizes are Reliable

Long Lifetime with Zorbax Rapid Resolution HT Columns



Both retention time and tailing factor are consistent for many injections – indicating long column lifetime.

Break Number 1

Agilent Technologies

Presentation

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Question for Presenter:

There are no questions pending.

Question & Answer Session

Please type your question into the Question Box at any time during the presentation.

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High Resolution HPLC Applications with Rapid Resolution and Rapid Resolution HT

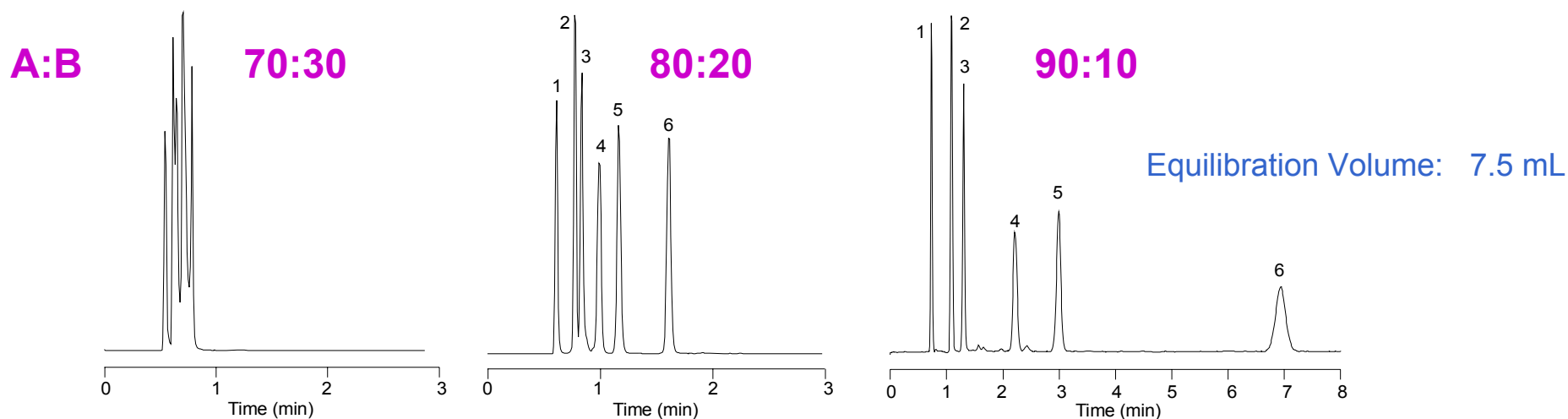


Save time by using ZORBAX Rapid Resolution and Rapid Resolution HT HPLC columns to:

- Use more efficient method development techniques
- Develop rapid separations
- Upgrade methods

Run Method Development Scouting Chromatograms on ZORBAX Rapid Resolution Columns

Nucleotides-Isocratic Conditions



Column: Rapid Resolution Eclipse XDB-C8, 4.6 x 75 mm, 3.5 μ m

B: Acetonitrile / 10 mM TBA

Flow Rate: 1.0 mL/min

Mobile Phase: A: 50 mM Na_2HPO_4 / 10 mM TBA (pH 7)

Temperature: 35°C

Detection: UV 254 nm

Sample: 1. NAD 2. GMP 3. AMP 4. NADH 5. ADP 6. ATP

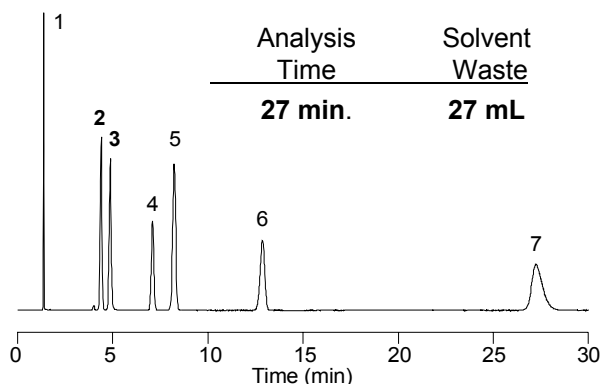


Develop More Rapid Isocratic Separations using Rapid Resolution 3.5-mm Columns

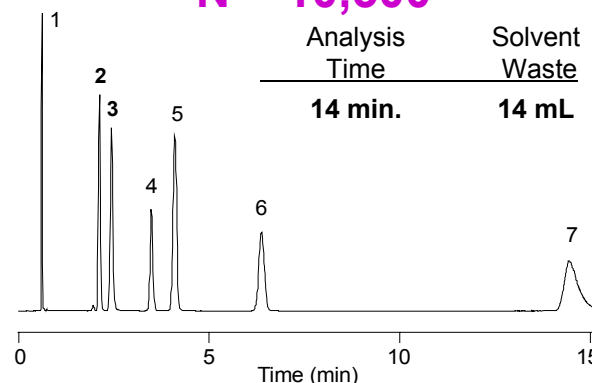
Resolution of Peaks 2 and 3 Determine Method Analysis Time

Isocratic HPLC - Separation of Test Mix

Eclipse XDB-C8
4.6 x 150 mm, 5 μ m
N = 12,000



Rapid Resolution Eclipse XDB-C8
4.6 x 75 mm, 3.5 μ m
N = 10,500



Mobile Phase: 35% 20 mM NaH₂PO₄, pH 7: 65% Methanol Temperature: 35°C

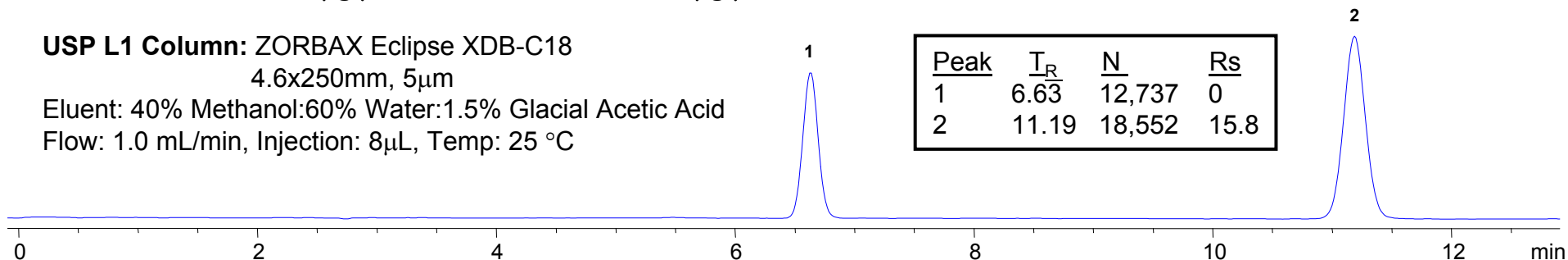
Sample: 1. Uracil 2. Butyl Paraben 3. Propranolol 4. Naphthalene 5. Dipropyl Phthalate 6. Acenaphthene 7. Amitriptyline

Update Existing Methods with Rapid Resolution HT Columns

USP Analysis of Guaifenesin

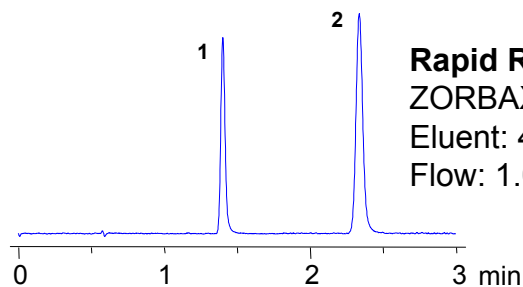
Sample: 1. Guaifenesin – 0.04 $\mu\text{g}/\mu\text{L}$, 2. Benzoic Acid – 0.10 $\mu\text{g}/\mu\text{L}$

USP L1 Column: ZORBAX Eclipse XDB-C18
4.6x250mm, 5 μm
Eluent: 40% Methanol:60% Water:1.5% Glacial Acetic Acid
Flow: 1.0 mL/min, Injection: 8 μL , Temp: 25 $^{\circ}\text{C}$



Rapid Resolution HT Column (L1)

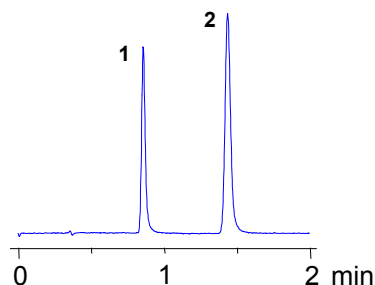
ZORBAX Eclipse XDB-C18, 4.6x50mm, 1.8 μm
Eluent: 40% Methanol:60% Water:1.5% Glacial Acetic Acid
Flow: 1.0 mL/min, Injection: 2 μL , Temp: 25 $^{\circ}\text{C}$



Peak	T_R	N	R_s
1	1.40	11,421	0
2	2.33	12,909	12.3

Rapid Resolution HT Column (L1)

ZORBAX Eclipse XDB-C18, 4.6x30mm, 1.8 μm
Eluent: 40% Methanol:60% Water:1.5% Glacial Acetic Acid
Flow: 1.0 mL/min, Injection: 2 μL , Temp: 25 $^{\circ}\text{C}$



Peak	T_R	N	R_s
1	0.85	5,855	0
2	1.43	7,300	8.6

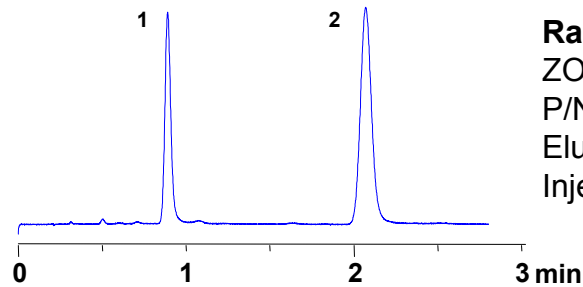
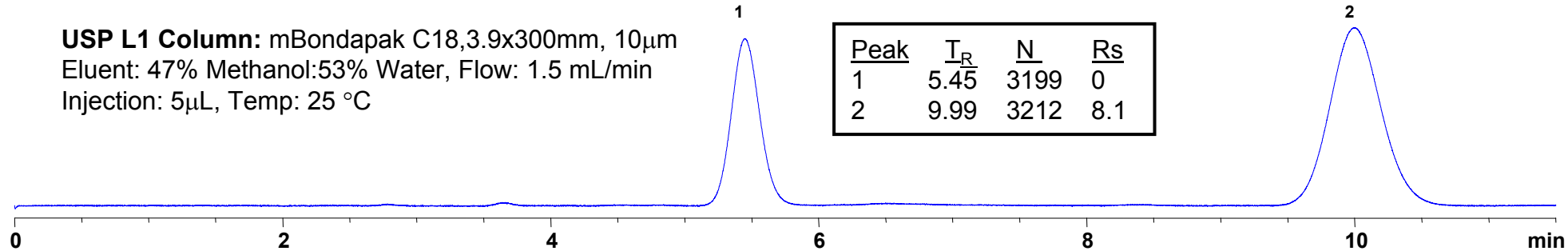


Updating Existing Methods with Rapid Resolution HT Columns

USP Analysis of Triamcinolone

Sample: 1. Triamcinolone - 0.2 µg/µL, 2. Hydrocortisone - 0.3 µg/µL Minimum Resolution Required = 3.0

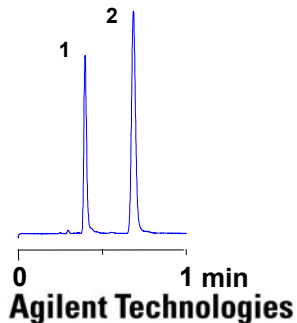
USP L1 Column: mBondapak C18, 3.9x300mm, 10µm
Eluent: 47% Methanol:53% Water, Flow: 1.5 mL/min
Injection: 5µL, Temp: 25 °C



Rapid Resolution HT Column (L1)

ZORBAX Eclipse XDB-C18: 4.6x30mm, 1.8µm
P/N 923975-902
Eluent: 47% Methanol:53% Water, Flow: 1.5 mL/min
Injection: 1µL, Temp: 25 °C

Peak	T_R	N	R_s
1	0.89	3256	0
2	2.07	4851	11.8



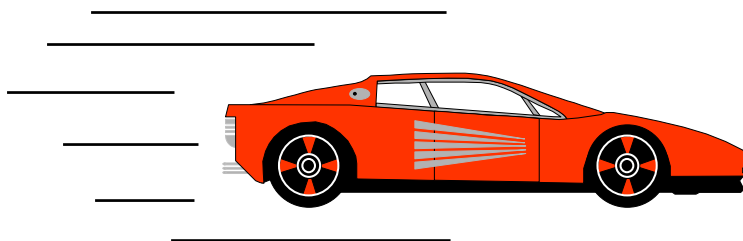
Rapid Resolution HT Column (L1)

ZORBAX Eclipse XDB-C18: 4.6x30mm, 1.8µm
P/N 923975-902
Eluent: 60% Methanol:40% Water, Flow: 1.5 mL/min
Injection: 1µL, Temp: 25 °C

Peak	T_R	N	R_s
1	0.40	2991	0
2	0.69	4025	6.9

High Throughput HPLC Applications

High Speed Separations



Isocratic

Gradient

Analytical (4.6-mm i.d.)

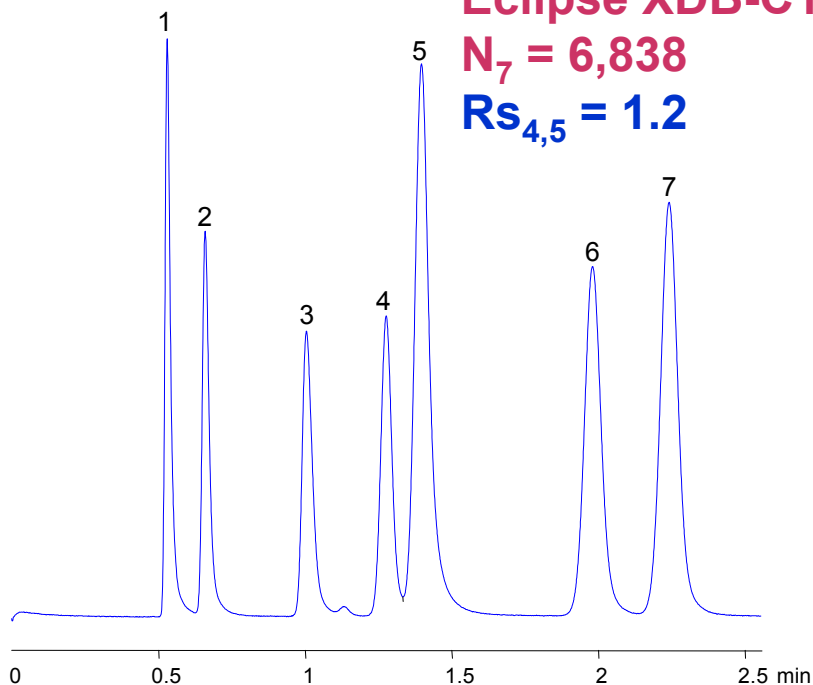
Narrow-Bore (2.1-mm i.d.)



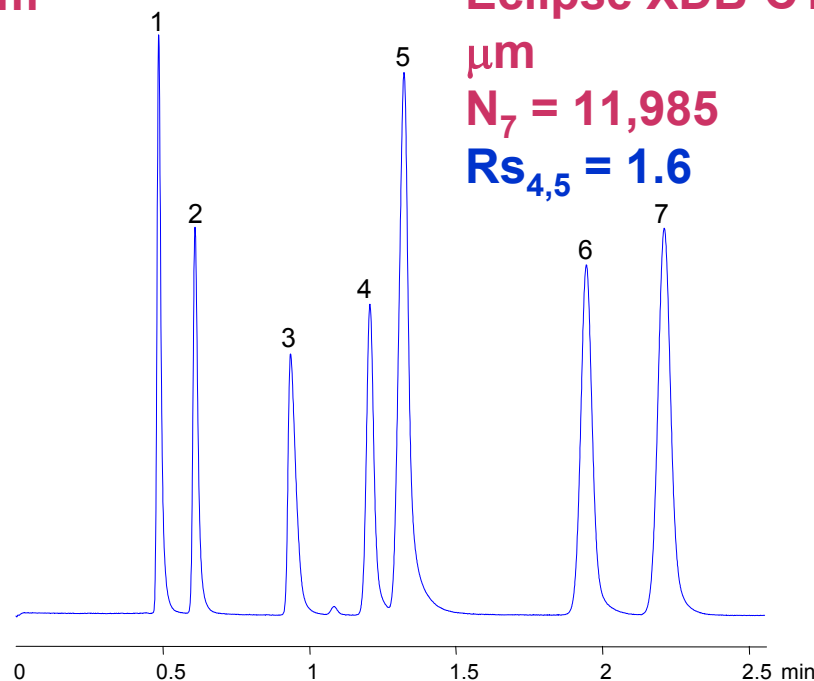
Improved Efficiency and Resolution on Rapid Resolution HT- Particle Size Comparison

Conditions: Column: Eclipse XDB-C18, 4.6 x 50mm Mobile Phase: 73% Methanol, 23% 20mM Phosphate Buffer, pH 7
Flow Rate: 1 mL/min, Temperature: Ambient Injection Volume: 1.0 μ L Inj., Detection: UV 254nm Sample: 1) Uracil, 2) Naproxen,
3) Mefanamic Acid, 4) Butyl Paraben, 5) Propranolol, 6) Dipropyl Phthalate, 7) Naphthalene.

Rapid Resolution
Eclipse XDB-C18, 3.5 μ m
 $N_7 = 6,838$
 $Rs_{4,5} = 1.2$



Rapid Resolution HT
Eclipse XDB-C18, 1.8 μ m
 $N_7 = 11,985$
 $Rs_{4,5} = 1.6$

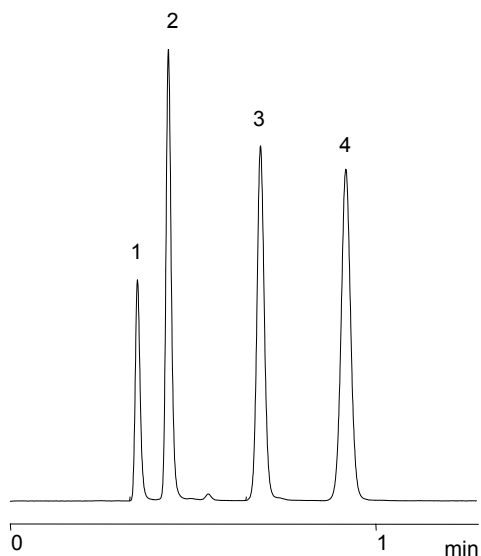


Resolution improves and efficiency nearly doubles using the Rapid Resolution HT column.

Rapid Resolution Columns Provide Very High Efficiency and Very Short Analysis Times

Columns: ZORBAX SB-C18, 4.6 x 50 mm Mobile Phase: 25% Water: 75% MeOH Flow Rate: 1.5 mL/min Temperature: RT
Detection: UV 254 nm Sample: QC: 1. Uracil 2. Phenol 3. 4-Cl-Nitrobenzene 4. Toluene

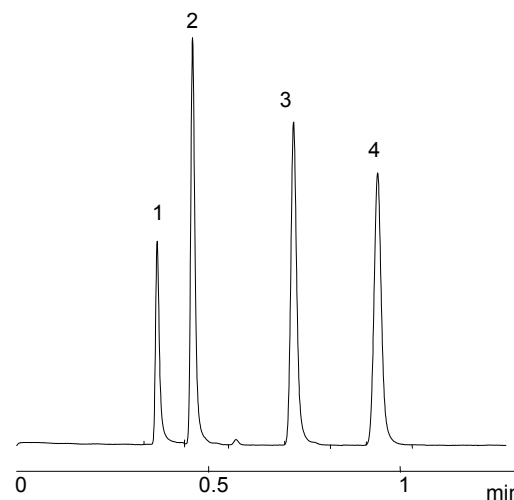
Rapid Resolution 4.6 x 50 mm, 3.5 μm



Plates (N)

1. 3476
2. 4585
3. 5673
4. 6180

Rapid Resolution HT 4.6 x 50 mm, 1.8 μm



Plates (N)

1. 6560
2. 8958
3. 11508
4. 12266

Analysis time of < 1 minute for high throughput analysis with very high efficiency on Rapid Resolution HT.



Isocratic High-Throughput HPLC

Increase Flow Rate to Reduce Analysis Time

Column: Rapid Resolution StableBond SB-C18, 3.5 μm , 4.6 x 30 mm

Sample: 1. Acetaminophen (4-acetamidophenol) 2. Caffeine 3. 2-Acetamidophenol
4. Acetanilide 5. Acetylsalicylic acid 6. Salicylic acid 7. Acetophenetidin

Pressure

90 bar

2 mL/min

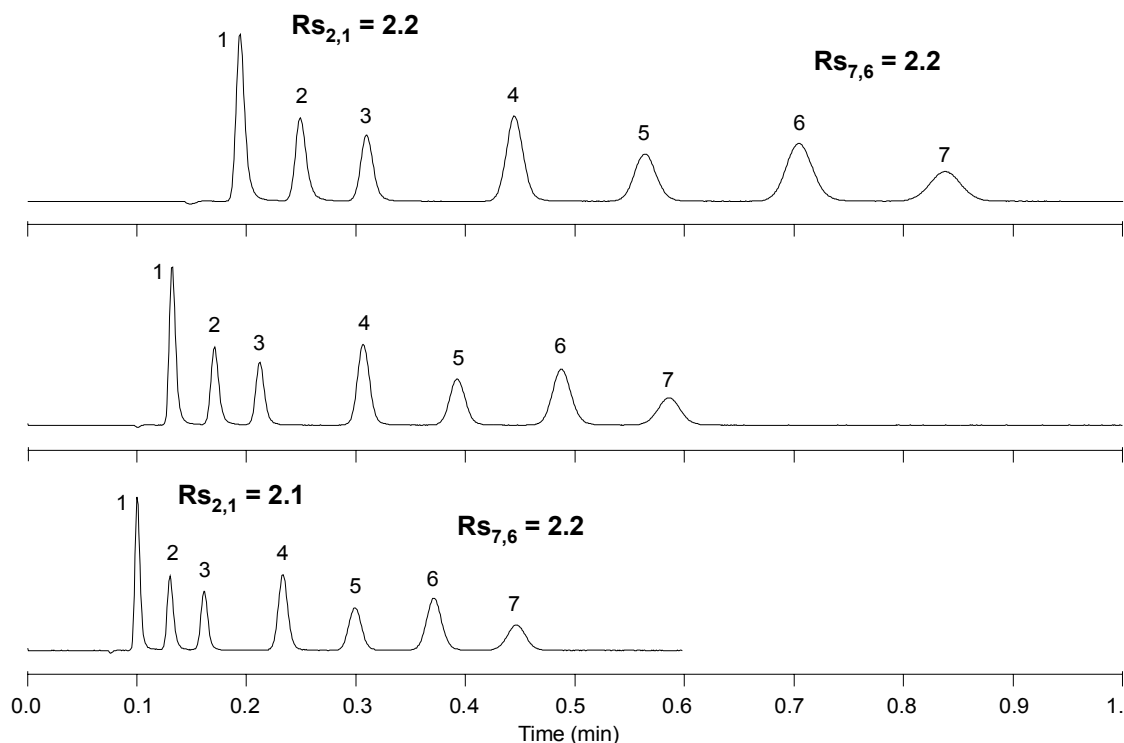
133 bar

3 mL/min

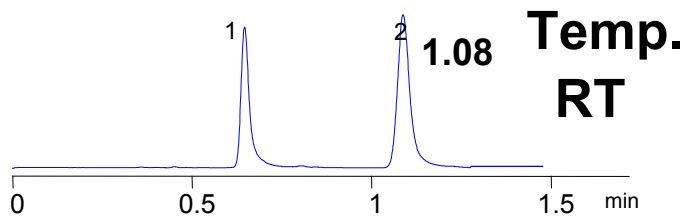
181 bar

4 mL/min

2650 psi



Increase Temperature to Reduce Analysis Time on Rapid Resolution and RR HT Columns



Temp. RT
Pressure 245 bar

Column: Rapid Resolution HT
SB-C18
4.6 x 30 mm, 1.8 μ m

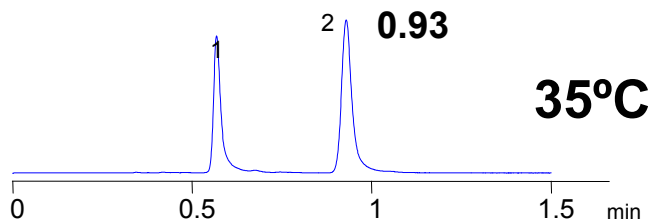
Mobile Phase: 40% water: 60% methanol

Flow Rate: 1 mL/min

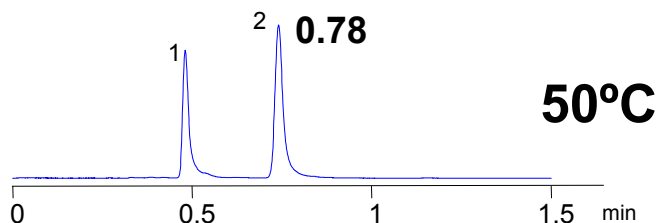
Detection: UV 254 nm

Temperature: As noted

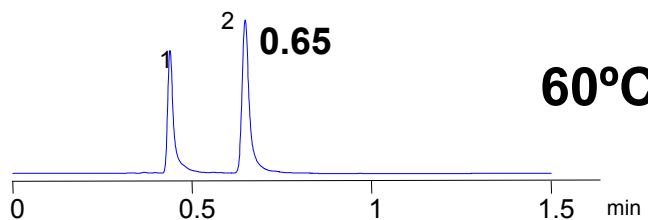
Sample: 1. Triamcinolone
2. Hydrocortisone



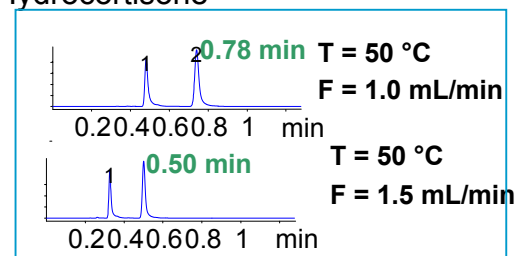
35°C
225 bar



50°C
200 bar



60°C
189 bar



Can increase flow rate to decrease analysis time even further

• Increasing temperature reduces analysis time by 40% or more and pressure by 25%.

Break Number 2

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Presentation

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Question for Presenter:

There are no questions pending.

Question & Answer Session

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How To Use Small Volume Columns Effectively for Isocratic and Gradient Separations

Critical instrument parameters include:

- | | |
|-------------------------------|--|
| Isocratic and Gradient | Data rate |
| | Flow cell size |
| | Tubing |
| Gradient only | Mixing volume (part of gradient delay volume) |
| | Gradient delay volume (dwell volume) |

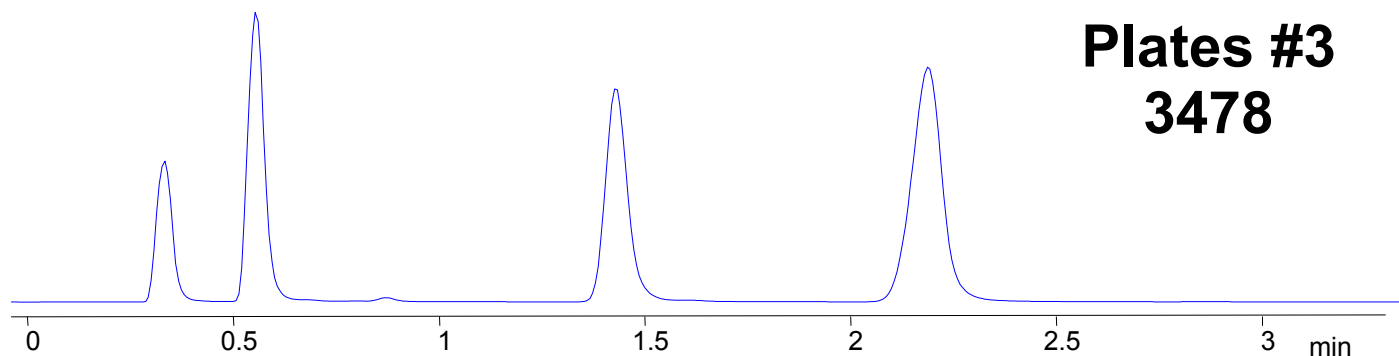
Optimization of Results with Rapid Resolution HT Columns - Data Acquisition Rate Comparison

Column: ZORBAX Rapid Resolution HT SB-C18 4.6 x 30 mm, 1.8 μ m Mobile Phase: 60% Methanol: 40 Water Flow Rate: 1mL/min
Temperature: RT Detection: UV 254 nm Sample: QC Test 1. Uracil 2. Phenol 3. 4-Cl-Nitrobenzene 4. Toluene

Data Acquisition Rate = 2 sec

**Plates #3
3478**

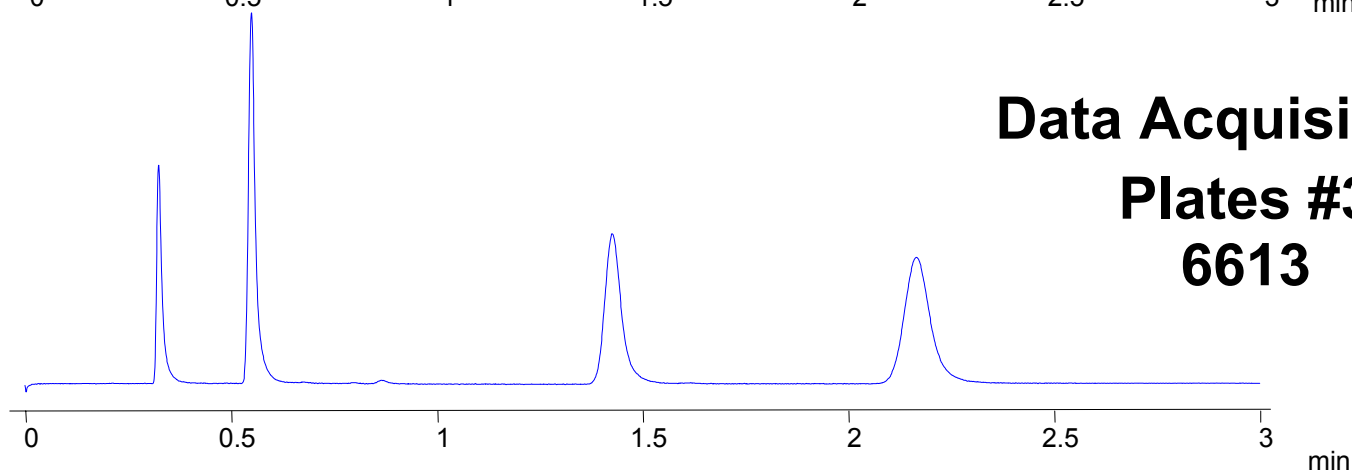
**Plates #4
4384**



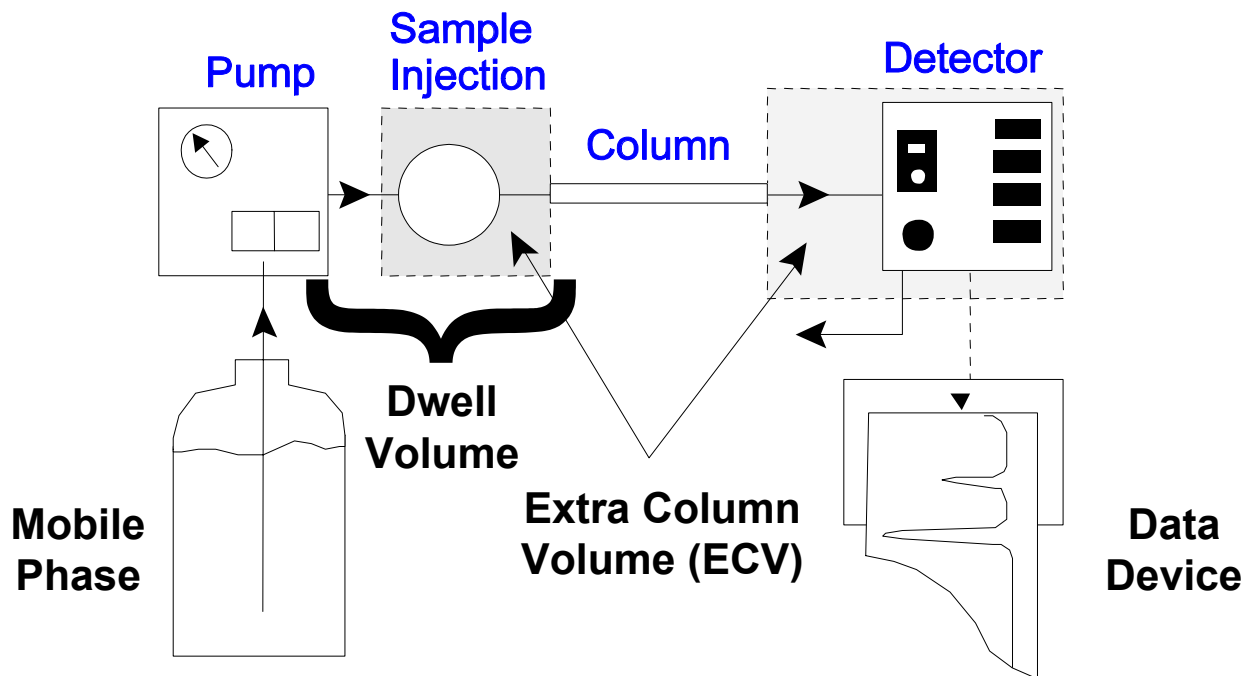
Data Acquisition Rate = 0.1 sec

**Plates #3
6613**

**Plates #4
6138**



HPLC Instrumentation Contribution to Isocratic and Gradient Performance



**ECV = sample volume + connecting tube volume
+ fitting volume + detector cell volume**

**Dwell Volume = volume from formation of gradient to top
of column**

How to Use Rapid Resolution and Rapid Resolution HT Columns Effectively

LC A = standard LC, plumbed with 0.17 mm (green) tubing, semi-micro flow cell (8 uL) and 80 uL mixer for gradient runs.

LC B = standard LC, plumbed with 0.12 mm (red) tubing, semi-micro (8 uL) or micro high pressure flow cell (1.7 uL) and 80 uL or no mixer.

You can further optimize with the 500 nL flow cell for the 2.1 x 15 mm columns and no mixer for gradient separations.

Flow cells listed are for DAD

How to Use Short Rapid Resolution HT Columns Effectively for Isocratic and Gradient Separations

Column Length	Typical k	LC A		LC B	
		4.6	2.1	4.6	2.1*
50 mm	k = 1	-	-	+	+
	k = 5	+	-	+	+
	k = 10	+	-	+	+
30 mm	k = 1	-	-	+	-
	k = 5	+	-	+	+
	k = 10	+	-	+	+
15 mm	k = 1	-	-	+	-*
	k = 5	-	-	+	-*
	k = 10	-	-	+	+
Internal Diameter		4.6	2.1	4.6	2.1*

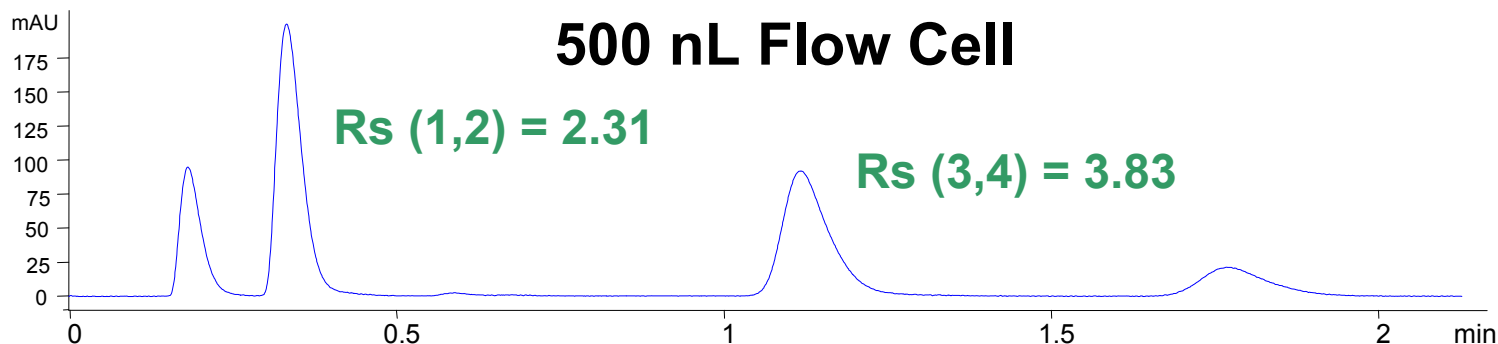
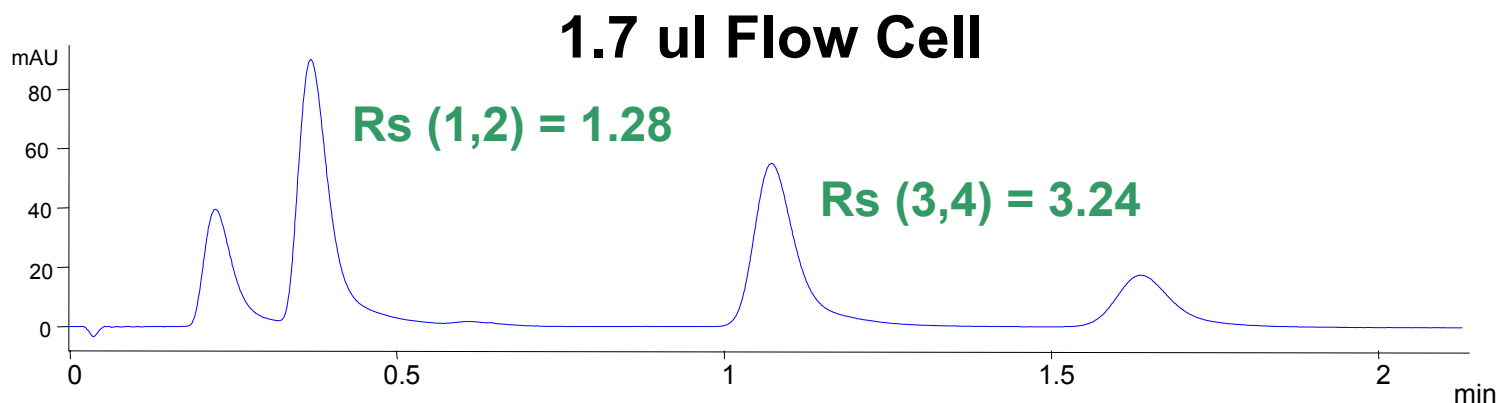
LC A = standard LC, with 0.17 mm (green) tubing, semi-micro flow cell (8 uL, DAD) and 80 uL mixer (5022-2165) for gradients.
 LC B = standard LC, with 0.12 mm (red) tubing, semi-micro (8 uL) or micro high pressure (1.7 uL) flow cell, and 80 uL (5022-2165) or no mixer for gradient analysis.

*the 500nL flow cell can be used with the 2.1 x 15 mm to improve results



Effect of Flow Cell Volume on Resolution— 2.1 x 15 mm Rapid Resolution HT Column

Column: Rapid Resolution HT SB-C18, 2.1 x 15 mm, 1.8 μm Mobile Phase: 50% Methanol:50% water Flow Rate 0.25 mL/min
Detection: UV 254 nm Sample: QC test 1. Uracil 2. Phenol 3. 4-Chloronitrobenzene 4. Toluene



• On the smallest volume column, resolution can be improved with the smallest flow cell.

Benefits of Using Gradient Elution for High Throughput HPLC

- Provides more efficiency because of gradient compression effects
- Preferred over isocratic elution when separating a number of unknown compounds
- Easily separates compounds that differ widely in polarity or have high molecular weight (i.e. peptides and proteins)
- Eluted peaks have essentially the same peak width, so sensitivity is similar throughout chromatogram
- Exposes a column continuously to solvents of increasing strength, minimizing sample and impurity build-up
- Equilibration time is not a limitation (small column volumes allows faster regeneration after gradient elution)

Gradient High-Throughput HPLC on Rapid Resolution 3.5- μm Columns

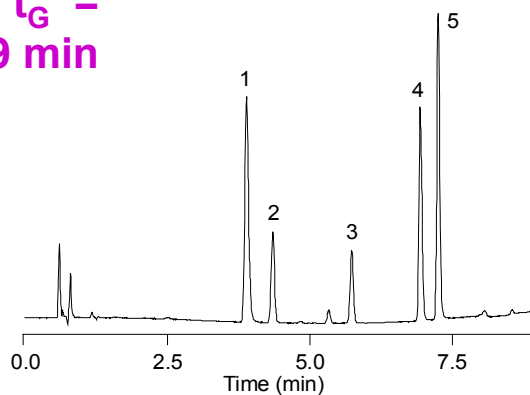
Reducing Column Length Reduces Run Time

Gradient Time (t_G) \sim Column Length

Column: Eclipse XDB-C8, 4.6 mm i.d. **Gradient:** 45-90% B in t_G minutes **Mobile Phase:** A: 25 mM Na_2HPO_4 , pH 3 B: Methanol **Temperature:** 35°C
Flow Rate: 1.0 mL/min **Sample:** Cardiac Drugs: 1. Diltiazam 2. Dipyridamole 3. Nifedipine 4. Lidoflazine 5. Flunarizine

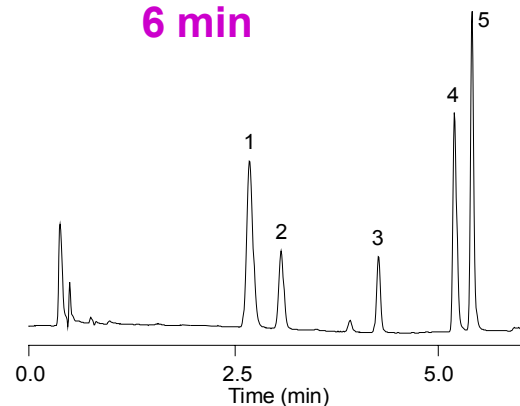
Length =
75 mm, 3.5 μm

t_G =
9 min



Length = 50 mm, 3.5 μm

t_G =
6 min



Gradient High-Throughput HPLC on Rapid Resolution 3.5- μm Columns

Increasing Flow Rate Reduces Gradient Run Time Even Further and Improves Sample Throughput

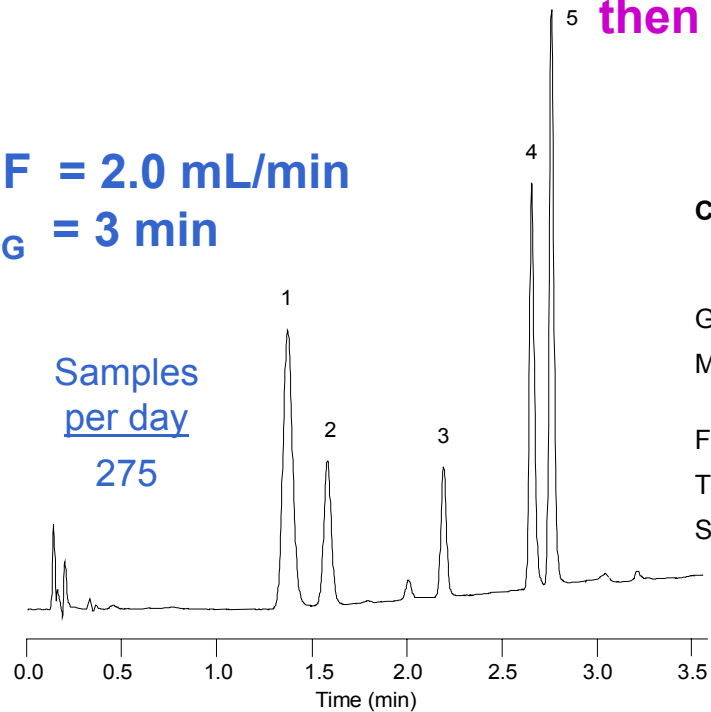
Note: If $t_G \times F = \text{constant}$,
then the elution pattern is unchanged

$F = 2.0 \text{ mL/min}$
 $t_G = 3 \text{ min}$

$F = 3.0 \text{ mL/min}$
 $t_G = 2 \text{ min}$

Samples
per day
275

Samples
per day
390



Column: Rapid Resolution
Eclipse XDB-C8
4.6 x 50 mm, 3.5 μm

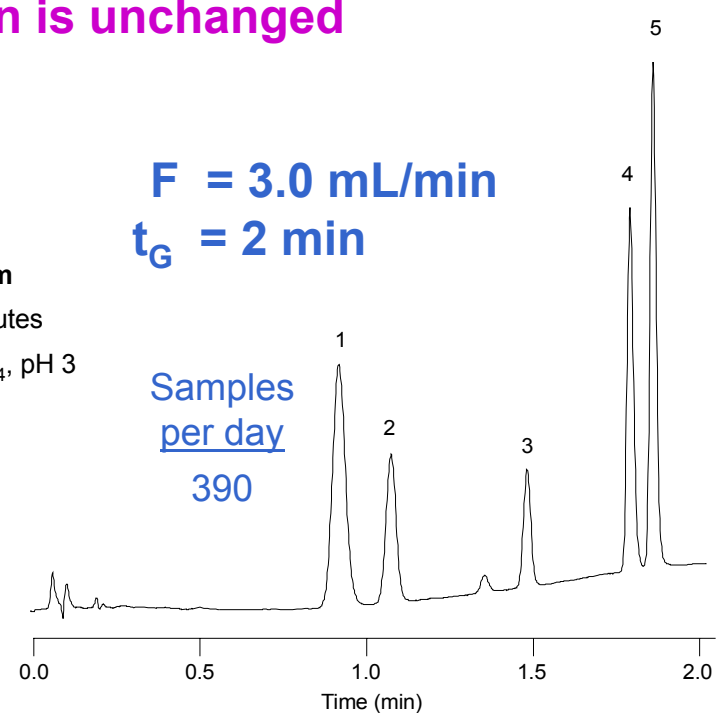
Gradient: 45-90% B in t_G minutes

Mobile Phase: A: 25 mM Na_2HPO_4 , pH 3
B: Methanol

Flow Rate: 1.0 mL/min

Temperature: 35°C

Sample: Cardiac Drugs
1. Diltiazam
2. Dipyradamole
3. Nifedipine
4. Lidoflazine
5. Flunarizine



Gradient Narrow-Bore HPLC on Rapid Resolution 3.5- μm Columns

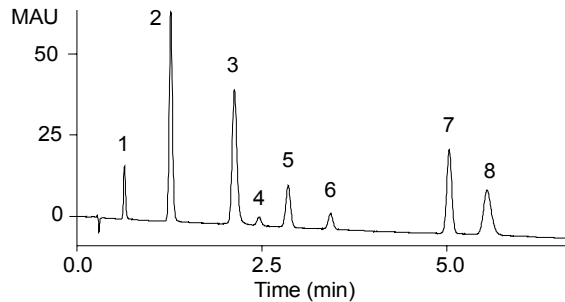
2.1-mm i.d. Column Reduces Solvent Waste and Improves Sensitivity

Mobile Phase: A: Water with 0.2% formic acid B: Methanol with 0.2% formic acid
Temperature: 35°C Inj: 2 μL Sample: Organic Acids: 1. Gallic 2. Protocatechuic
3. Hydrocaffeic 4. Gentisic 5. Vanillic 6. Syringic 7. Sinapinic 8. Salicylic

Rapid Resolution SB-C18 4.6 x 30 mm, 3.5 μm

Gradient: 5-35% B in 6 min.
Flow Rate: 1.0 mL/min

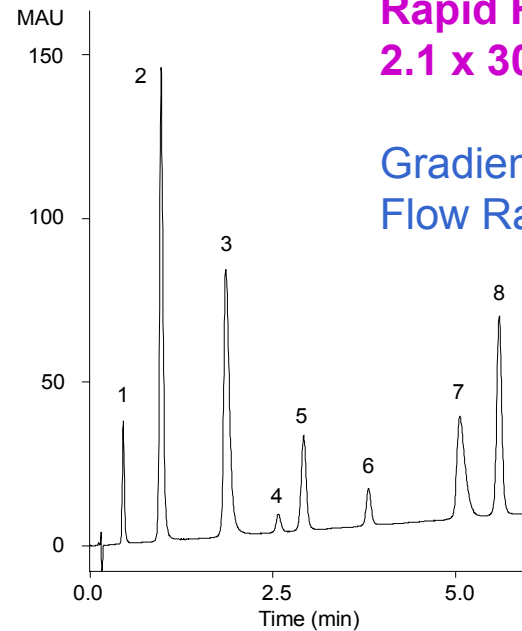
Solvent
Used: 5.6 mL



Rapid Resolution SB-C18 2.1 x 30 mm, 3.5 μm

Gradient: 5-35% B in 6 min.
Flow Rate: 0.4 mL/min

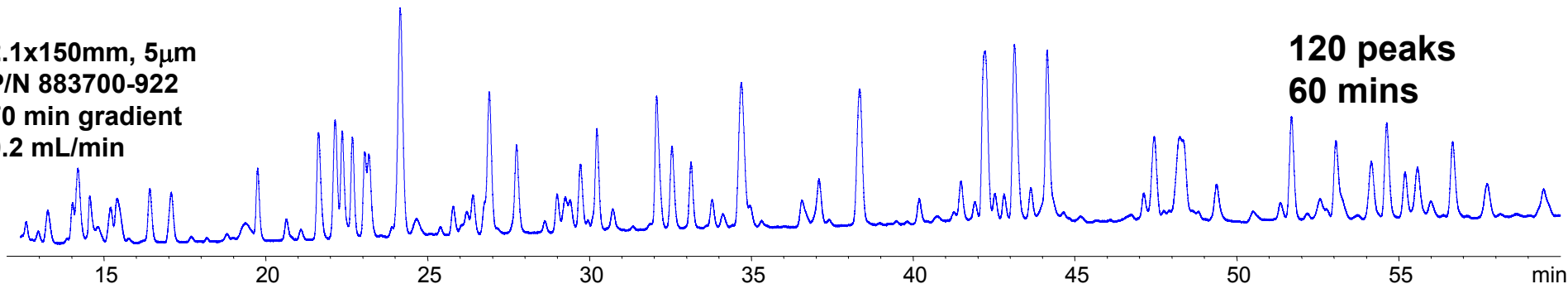
Solvent
Used: 2.2 mL



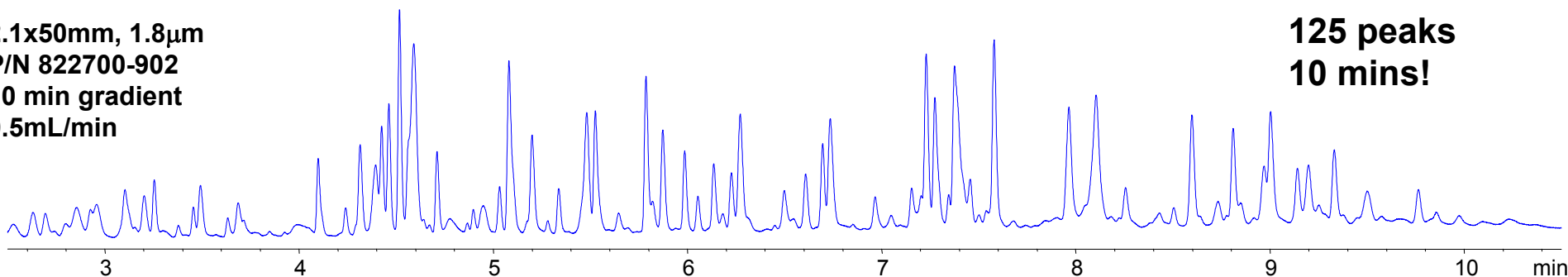
- Instrument is optimized for lowest dwell volume and extra-column-volume

Higher Resolution, Faster Analyses for Complex Samples – 2.1 mm i.d. Rapid Resolution HT

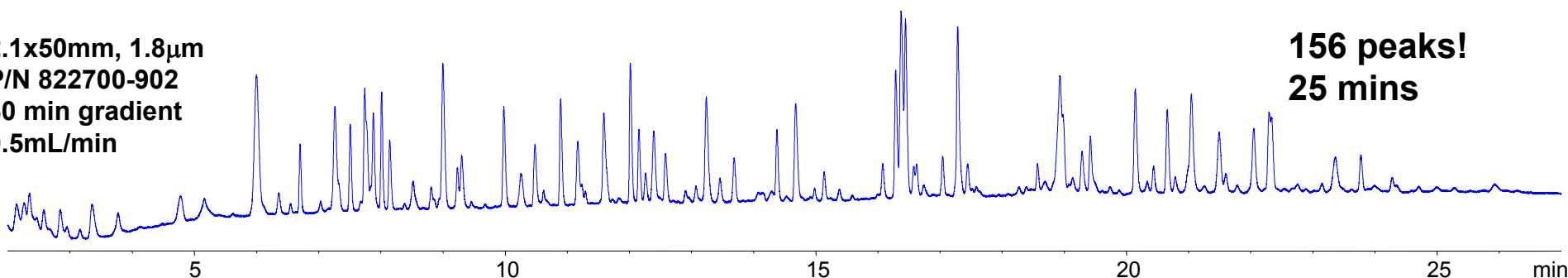
2.1x150mm, 5 μ m
P/N 883700-922
70 min gradient
0.2 mL/min



2.1x50mm, 1.8 μ m
P/N 822700-902
10 min gradient
0.5mL/min



2.1x50mm, 1.8 μ m
P/N 822700-902
30 min gradient
0.5mL/min



Conditions: Mobile Phase A: Water w/ 0.1% TFA, B: ACN w/0.1% TFA, Gradient 2%B to 50%B, Temperature: 50°C
Detection: UV 214 nm **Sample: HSA Tryptic Digest**

Conclusions

- **Shorter column lengths offer reduced analysis time whether using a 2.1-, 4.6- or 21.2-mm i.d. HPLC column under isocratic or gradient conditions.**
- **Rapid Resolution 3.5- μm particles provide good resolution for fast analysis.**
- **Smaller, 1.8- μm particles provide even more resolution for high throughput with the highest resolution available; for highest performance some instrument modification may be necessary.**
- **Gradient elution offers advantage over isocratic elution when using short, fast HPLC columns. Peaks elute with the same peak width and columns are protected against sample and mobile phase contamination as gradient displaces strongly retained materials. Gradient equilibration time is essentially a non-issue with very short, Rapid Resolution columns.**
- **Short, Rapid Resolution, narrow-bore columns offer very good resolving power in less than 60 seconds by using steep gradients and flow rates ≥ 1.0 mL/min.**



Rapid Resolution HT Application Notes

- 1. Increasing Sample Throughput Using ZORBAX Eclipse XDB-C18 Rapid Resolution HT Columns – pub no. 5988-9997EN**
- 2. The Influence of Sub Two Micron Particles on HPLC Performance – pub no. 5988-9251EN**
- 3. Fast Liquid Chromatography and Liquid Chromatography/ Mass Spectrometry Analysis of Antibiotics Using Rapid Resolution HT HPLC Columns with Sub Two-Micron (1.8 mm) Particles – pub no. 5989-0025EN**
- 4. Application Examples with Rapid Resolution HT HPLC columns – pub no. 5989-0026EN**
- 5. High Efficiency, High Throughput LC and LC/MS Applications Using ZORBAX Rapid Resolution HT Columns – pub no. 5989-0540EN**
- 6. ZORBAX Rapid Resolution HT Columns – A Breakthrough in High-Throughput HPLC Column Technology – pub no. 5989-XXXXEN**
- 7. Separation Times (Vol 16, No. 2, 2003 High Throughput HPLC with Short Columns and New Sub Two-Micron Particles – available as pub #5988-9615EN, Vol 16 No. 3, 2003)**