Performance Characterizations and Comparisons of HPLC Column Options for Ultra High-Speed and High-Resolution HPLC Separations.

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What Are the Column Options for High Speed and High Resolution Analysis?

Columns with different particle sizes – 5, 3.5, 2-2.5, <2um

- **The current dominant approach is to select columns with smaller particle sizes to maintain resolution in a shorter column - we will focus on this approach**
- **Benefit of this approach is that separations can be matched on different particle sizes for flexibility in a lab or organizations**
- **Another benefit is that many selectivity choices are available to optimize methods**

Monoliths – will be considered here

Superficially porous particles – new area and more study is needed for comparison on use with small molecules

Introduction

1. **Why Choose Smaller Particles?**
   1. High speed separations with good resolution
   2. High resolution separations
2. **Performance Characterizations**
   1. Van Deemter plots – a variety of different types of plots are compared to take into account porosity of the materials packed in the column
   2. Performance vs. Theoretical – takes into account LC impact on the column/separation
   3. Resolution vs. Analysis Time
   4. Practical use characterizations – pressure, reproducibility, results across different particle sizes
3. **Is the smallest particle size the best?** Efficiency vs. Analysis Time

1. Why Choose Smaller Particle Sizes? - Efficiency and Resolution in Shorter Columns

This is the basic premise from which we operate.

Plates	Selectivity	Retention

$$R_s = \frac{\sqrt{N}}{4} \bullet (\alpha - 1) \bullet \frac{k'}{k'+1}$$

$$N \propto \frac{L}{d_p}$$

To Maintain Rs:

e.g.: L/2 → $d_p/2$

↓ Column Length	=	↓	N
↓ Particle Size	=	↑	N

Sub 2-Micron Columns Provide the Efficiency of Longer Columns for More Productivity

Column Length (mm)	Resolving Power N(5 µm)	Resolving Power N(3.5 µm)	Resolving Power N(1.8 µm)	Typical Pressure Bar (1.8 µm)	Analysis Time*
150	12,500	21,000	32,500	580	
100	8,500	14,000	24,000	420	-33%
75	6000	10,500	17,000	320	-50%
50	4,200	7,000	12,000	210	-67%
30	N.A.	4,200	6,500	126	-80%
15	N.A.	2,100	2,500	55	-90%

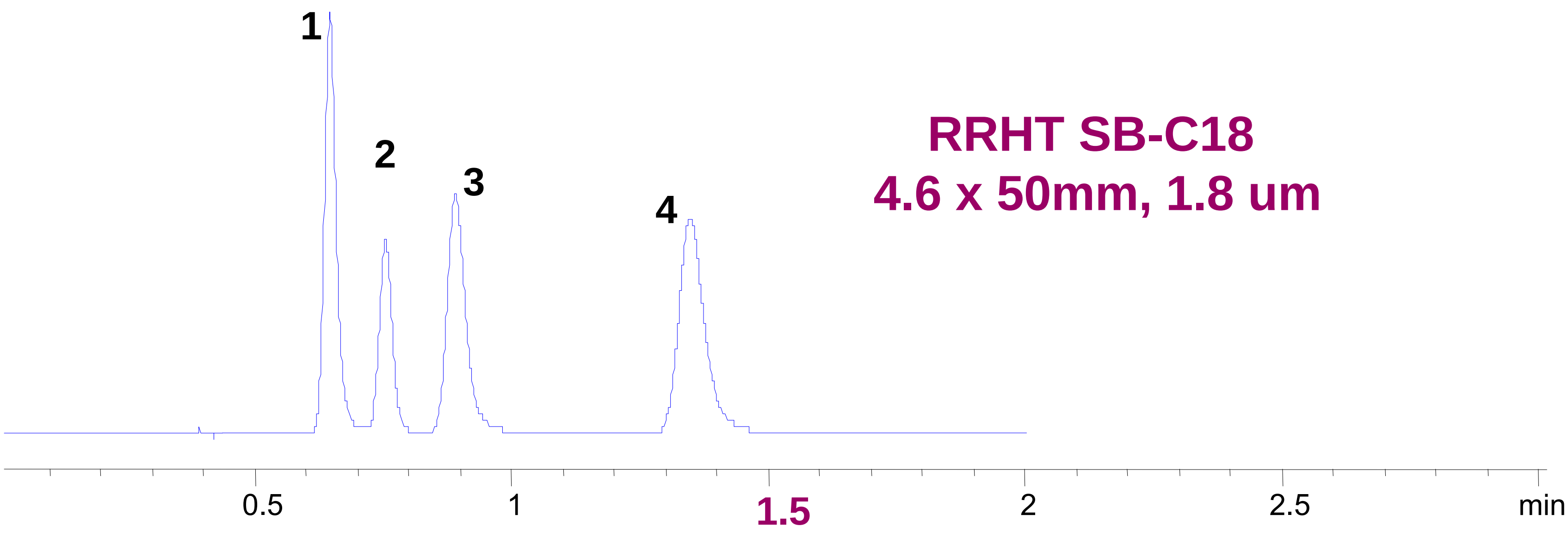
Analysis Time
Peak Volume
Solvent Usage

* Reduction in analysis time compared to 150 mm column

- pressure determined with 60:40 MeOH/water, 1ml/min, 4.6mm ID

High Speed Separations with Good Resolution

Column: 4.6 x 50mm, 1.8um Mobile Phase: A= 0.1% Formic Acid, B=ACN + 0.1%Formic Acid (95:5) Flow rate: 1.5 mL/min
Inj. Vol: 2 ul Sample: Xanthines: 1. 1-methylxanthine, 2. 1,3-dimethyluric acid, 3. 3,7-dimethylxanthine, 4. 1,7-dimethylxanthine



➢The short 4.6 x 50mm, 1.8um column allows complete resolution of multiple analytes in a minimum amount of time.

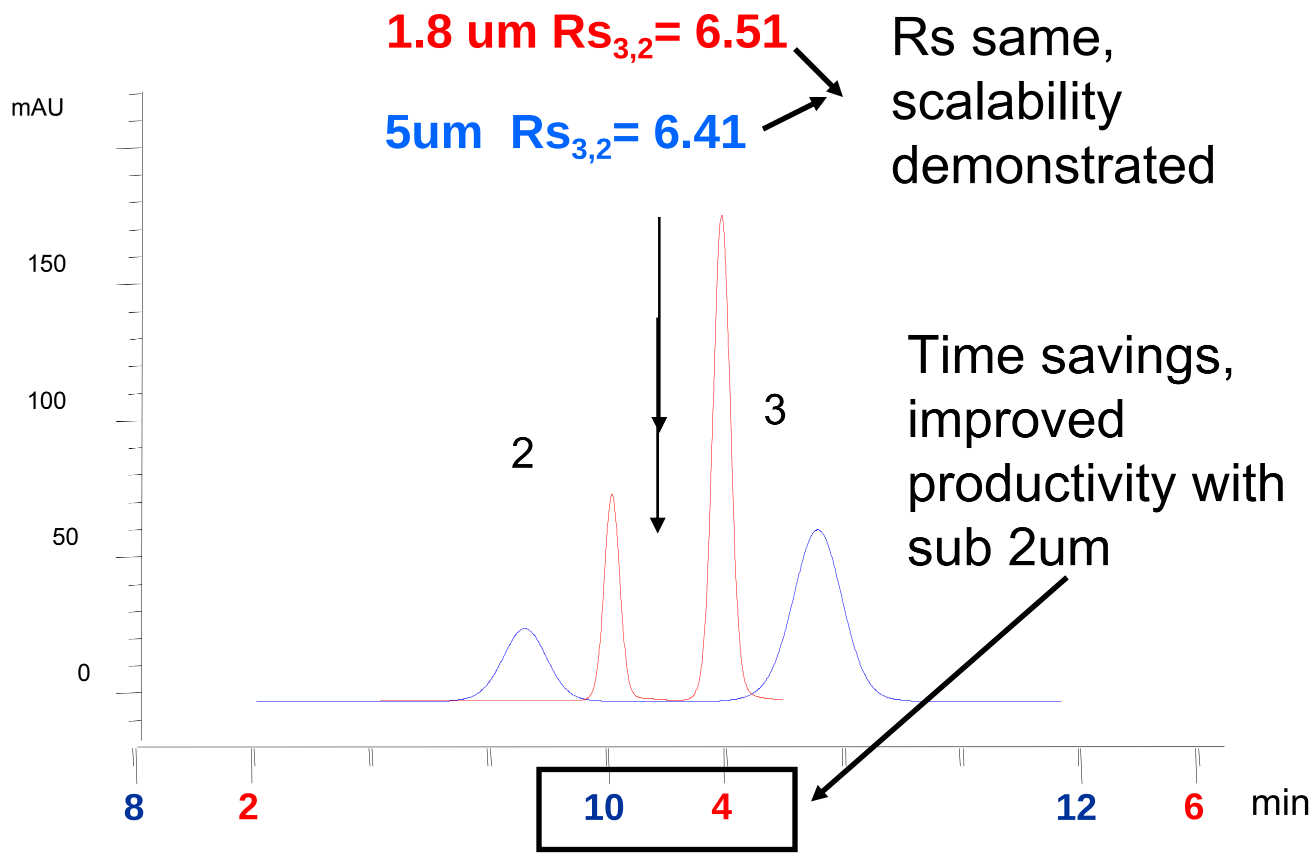
Time Savings, Scalability and Improvement in Sensitivity with Sub 2-um Particles

Columns: Eclipse Plus C18, as described below. Mobile Phase: A: water, B: MeOH, (15:85)
Temperature: 25°C Flow: 1 mL/min. Detection: 310, 4 nm, 0.5 s response time, semi-micro flow cell, Sample: Sunscreens

4.6 x 150 mm, 5 µm
P=82 bar

4.6 x 50 mm, 1.8 µm
P=208 bar

1. 2-hydroxy-4-methoxybenzophenone
2. Padimate-O
3. 2-ethylhexyl trans-4-methoxycinnamate
4. 2-ethylhexyl salicylate

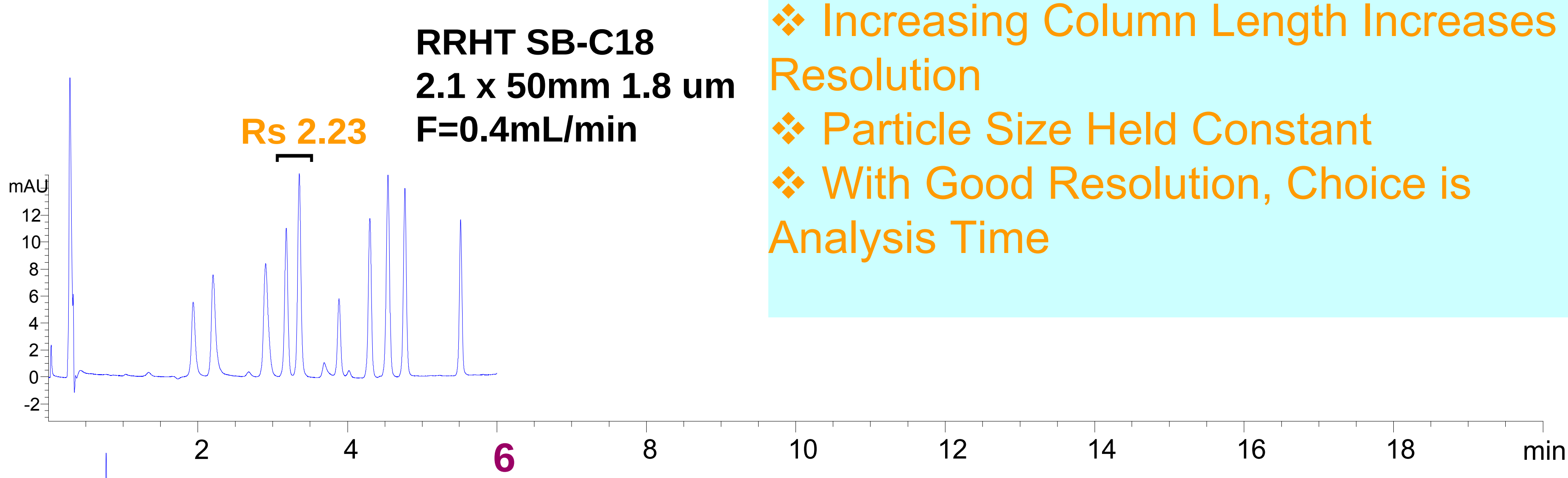


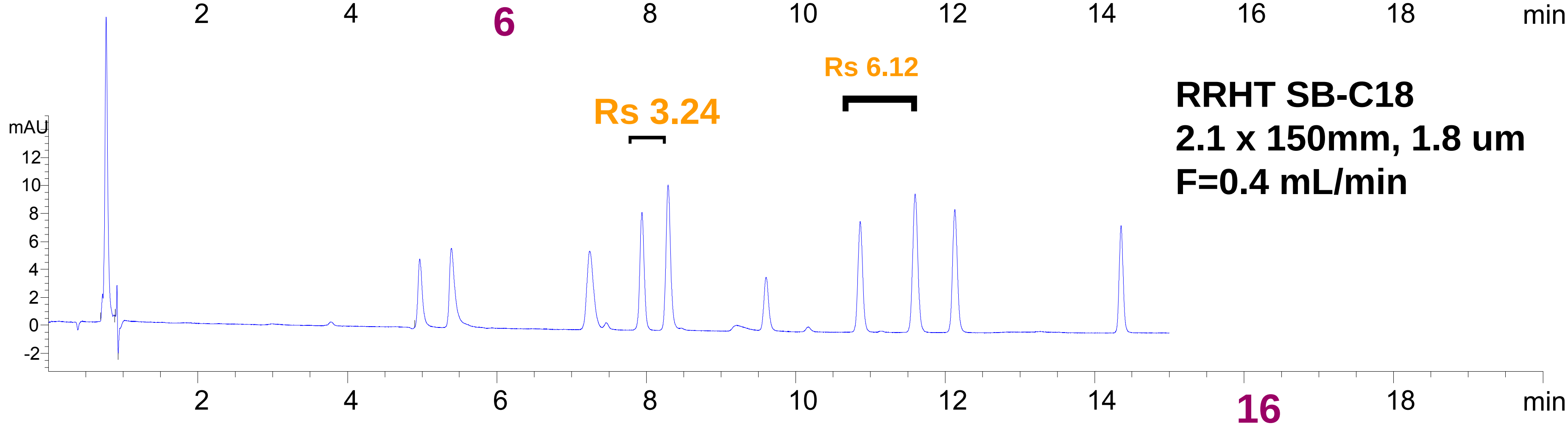
This blow-up of a chromatogram shows the added sensitivity achieved with a 1.8um column. The chromatograms are on the same scale and we are looking at the same 2 peaks in each.

Increasing Resolution by Using Long RRHT, 1.8um Columns

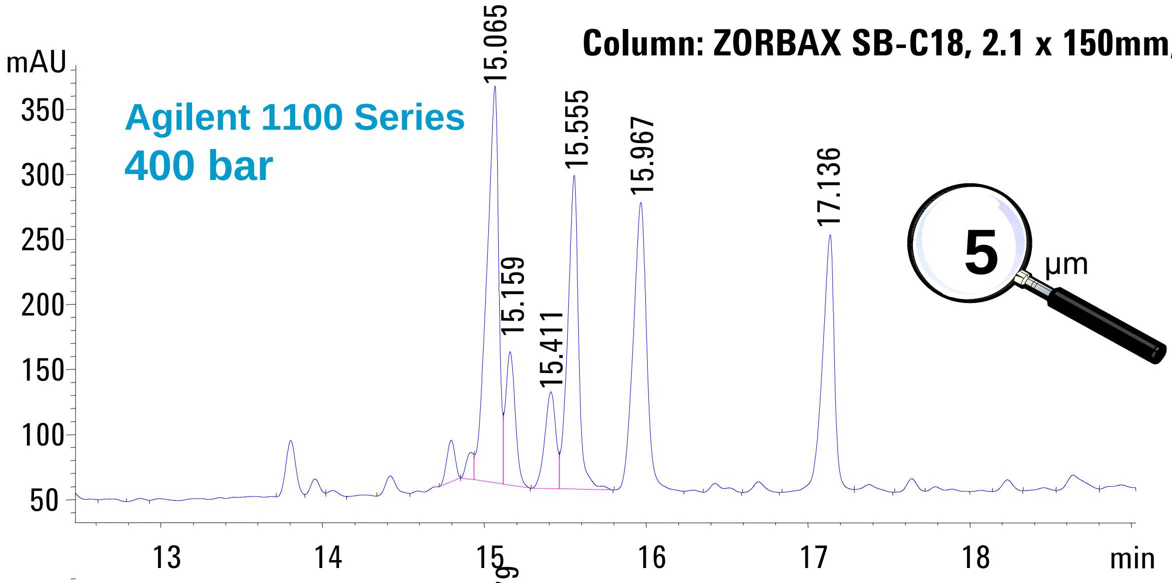
Sample: Dexamethasone & Impurities

- Increasing Column Length Increases Resolution
- Particle Size Held Constant
- With Good Resolution, Choice is Analysis Time

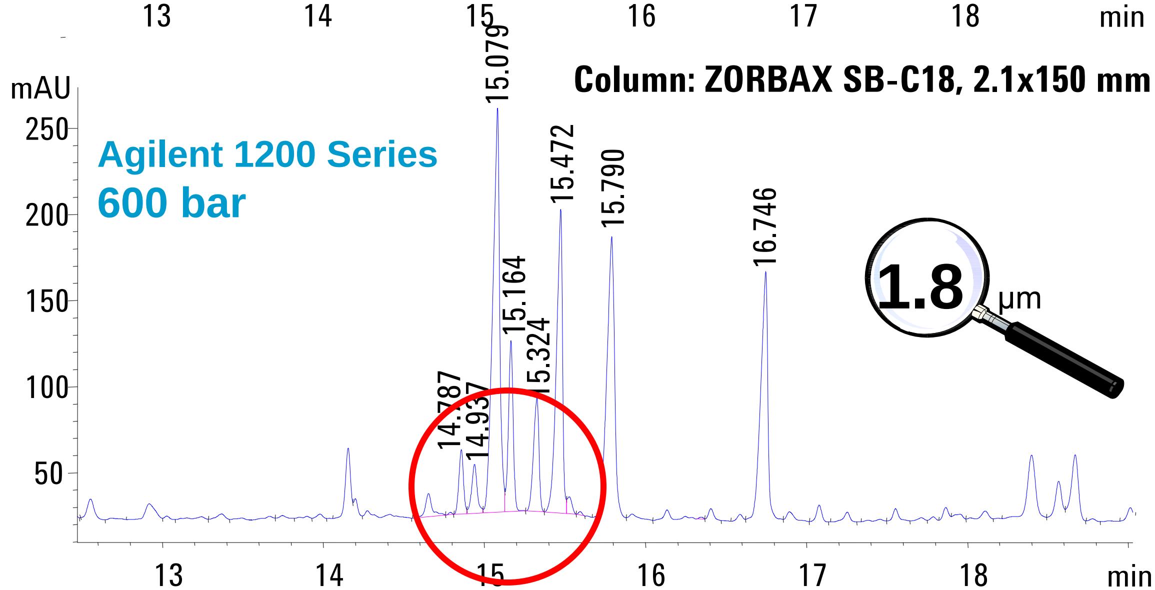




High Resolution – Separate More Peaks with 1.8um



Mobile phases: A = H2O + 0.1% TFA
B = ACN + 0.1% TFA
Gradient: 10% to 95% ACN in 40min, hold for 1min
Flow: 0.4 mL/min
Inj. volume: 3 µL, partial loop filling
DAD: 220 nm (20 Hz)
2µl flowcell



Improved Resolution

Agilent Application Note:
Analysis of a complex natural product extract from ginseng – Part I
Publication Number 5989-4506EN

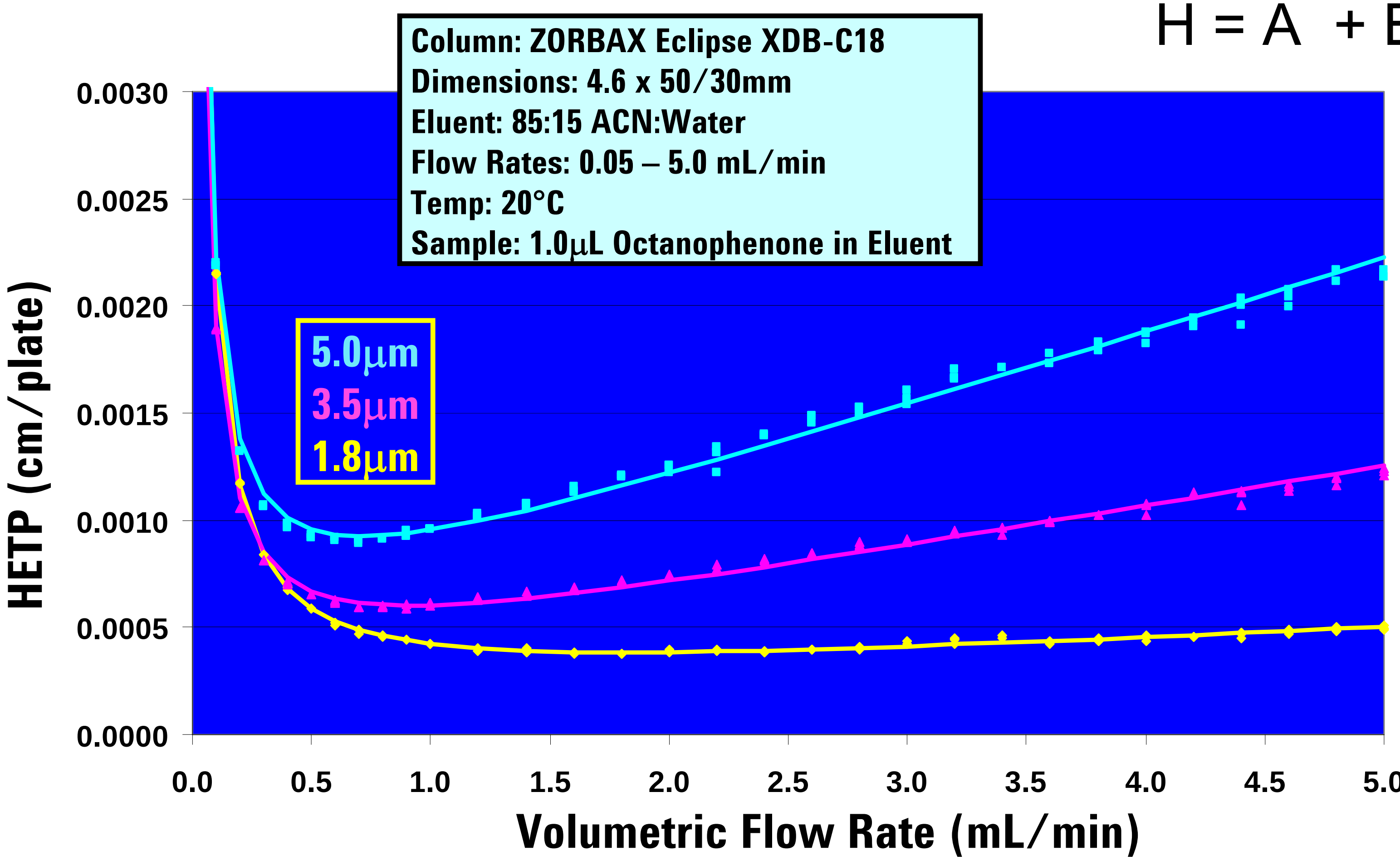
Performance Characterizations

1. Van Deemter plots
2. Performance vs. Theoretical – takes into account LC effects
3. Resolution vs. Analysis Time
4. Practical use characterizations – pressure, efficiency, reproducibility, results across different particle sizes

Van Deemter Curve 1: HETP vs. Volumetric Flow Rate

$$H = A + B/u + \boxed{Cu}$$

Mass transfer term

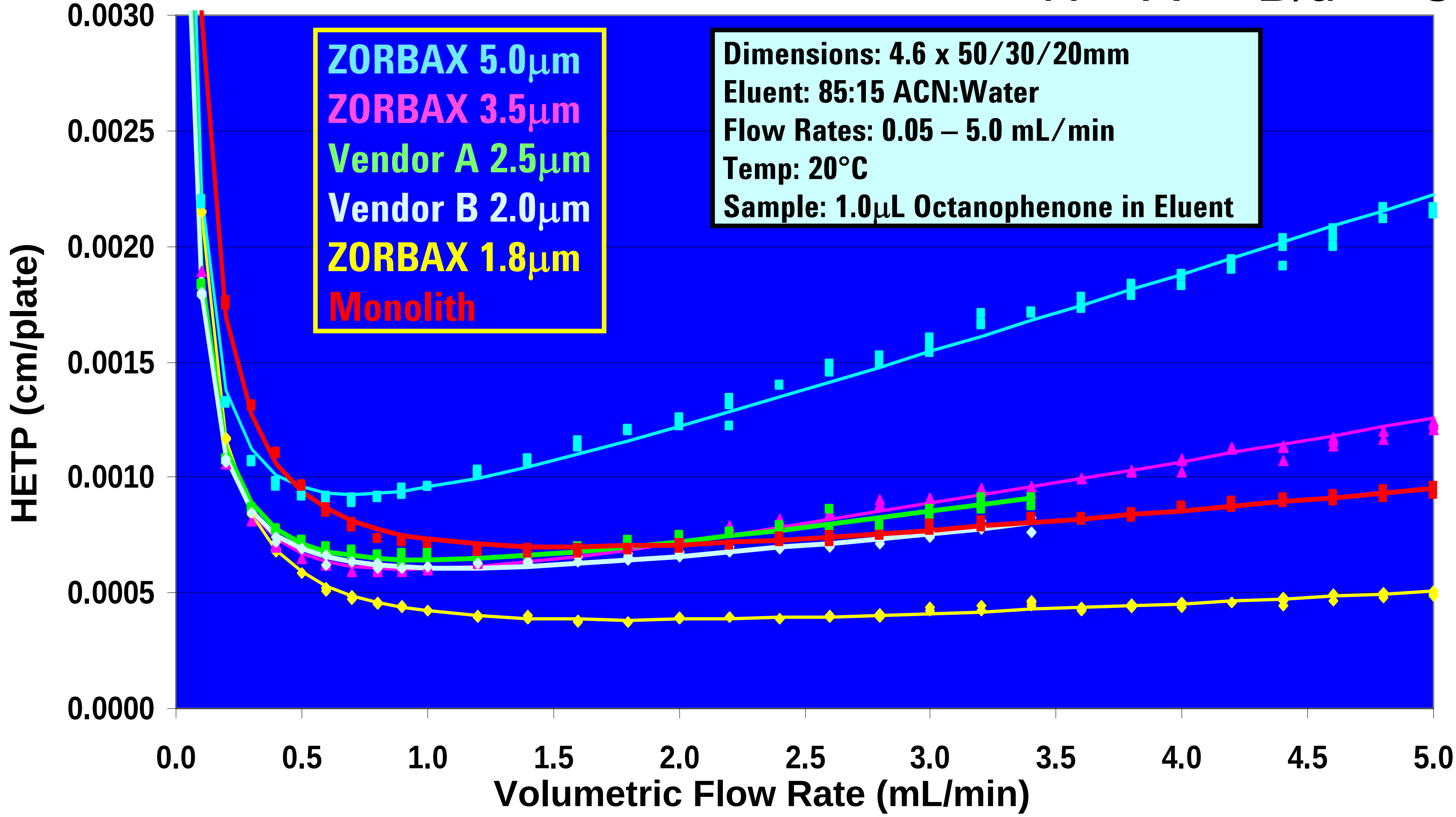


- ➢Smaller particle sizes should have flatter curves, minima shift out slightly
- ➢Smaller particles have more rapid mass transfer

Van Deemter Plot 1: HETP vs. Volumetric Flow Rate

Small Particle Columns including Monolith

$$H = A + B/u + Cu$$



- Considering more particle choices, 1.8um generates the best results
- Monolith appears similar to 2-2.5 um particles

Application – Independent Measures of Relative Column Performance

1. HETP vs. Interstitial Linear Velocity (u_e)

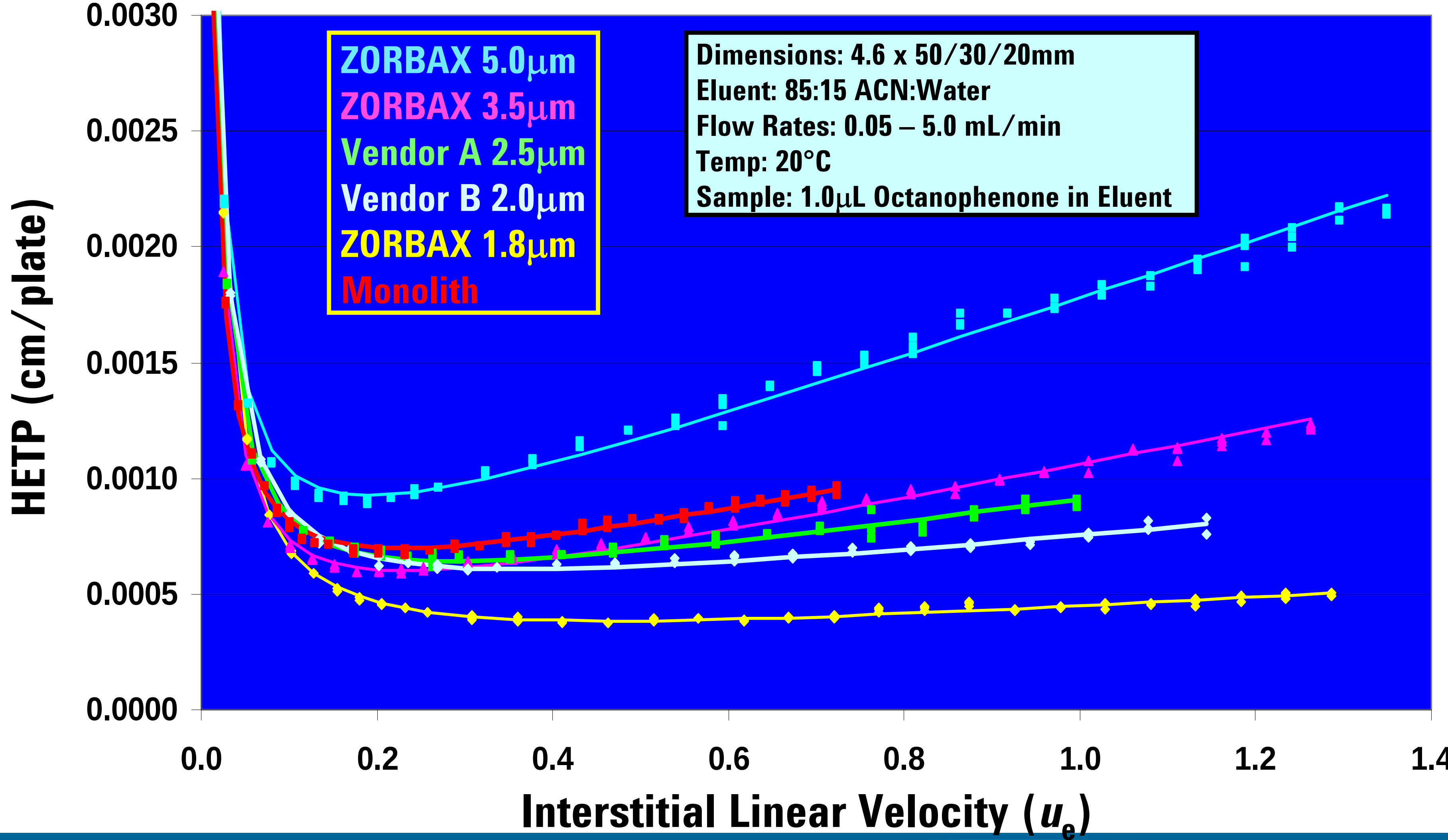
- Shows relative efficiency differences as a function of d_p
- Must be used to compare monolith columns to porous particle columns

2. Dimensionless Parameters

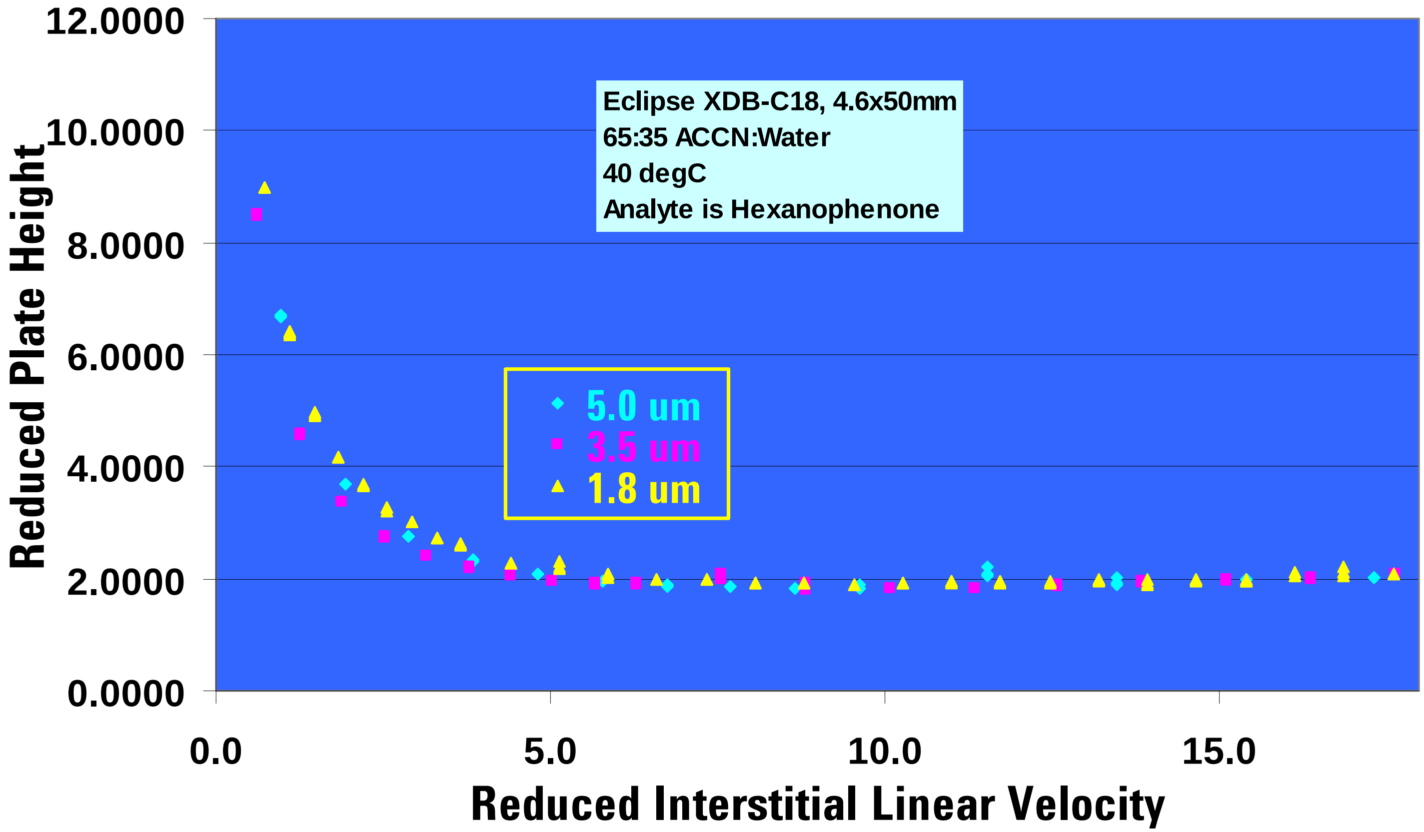
- Reduced Plate Height (h) vs. Reduced Linear Velocity (υ)
- Van Deemter Coefficients: $h = A + B/\upsilon + C\bullet\upsilon$
- Knox Equation Coefficients: $h = A\,\upsilon^{0.33} + B/\upsilon + C\bullet\upsilon$
  - A or $A\,\upsilon^{0.33}$ is a measure of packing quality
  - B/υ is the result of longitudinal (axial) diffusion in both moving and stationary zones
  - $C\upsilon$ is the contribution from slow mass transfer within the entire stationary zone.
  - $H = L/N$ $h = H/d_p$ $\upsilon = u_e d_p/Dm$ $u_e = F/(\pi r^2 \varepsilon_e)$ $\varepsilon_e = V_e/V_g$

Van Deemter Plot 2: HETP vs. Linear Velocity

Small Particle Columns including Monolith



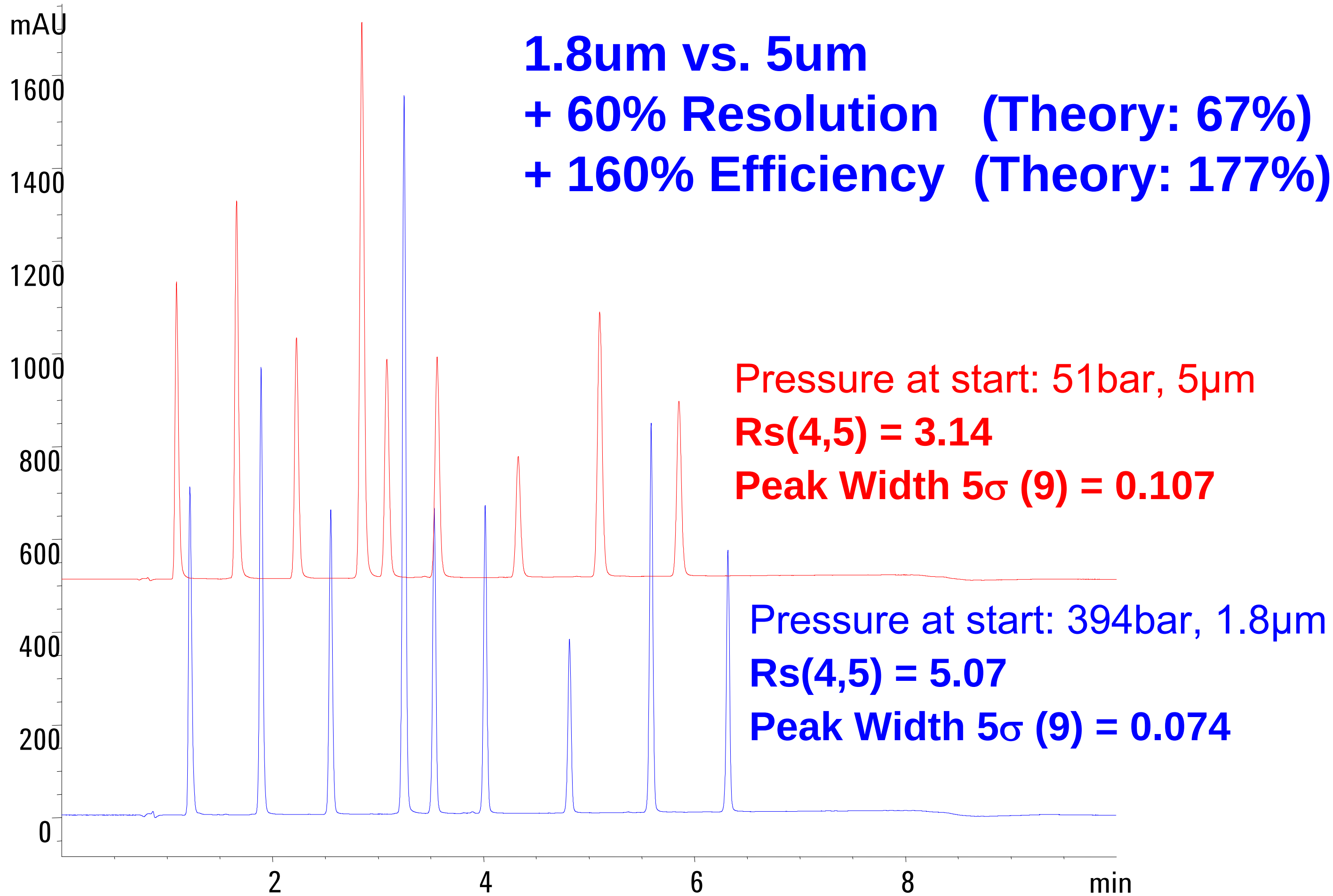
Van Deemter Curve 3: Dimensionless Plots of a Neutral Analyte on 3 particle sizes, same column



- Looking for all curves to overlay and reduced plate height to be about 2
- This indicates a well packed column independent of particle size

2. Actual Performance vs. Theoretical

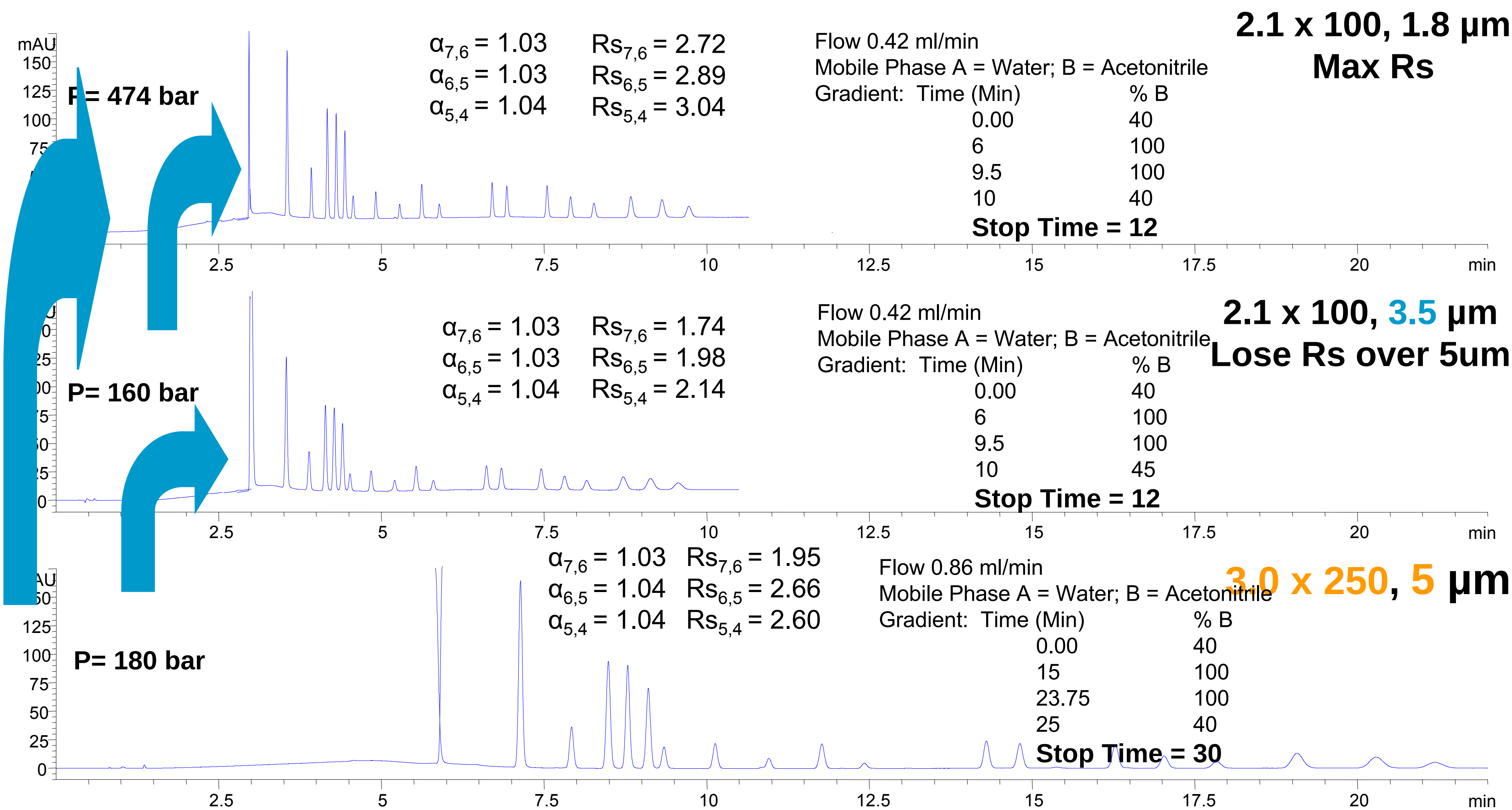
2.1 x150mm SB-C18 RRHT - 5µm vs. 1.8µm



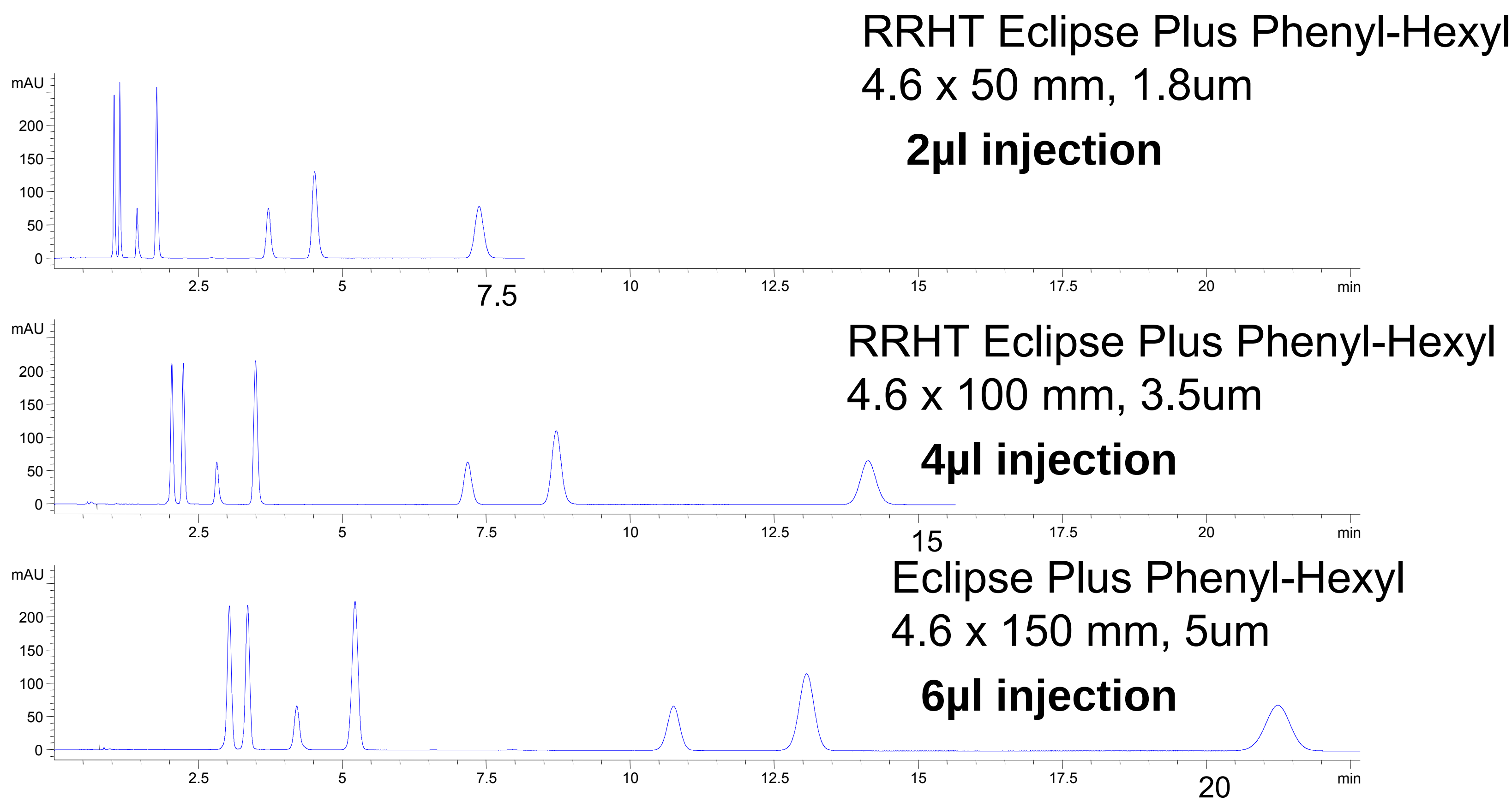
➢ **Resolution gains are close to theoretical – results can be achieved!**

3. Choose column length and particle size for Rs and Time – Note Pressure choices

Sample: 16 PAH's



3. Smaller Particle Sizes and Shorter Columns Maintain Resolution but Reduce Analysis Time



Mobile Phase 40 % ACN 60 % 25 mM Sodium Phosphate Buffer pH= 2.4 Flow Rate= 1.5 ml/min UV 210 nm 2µl Elution order for Eclipse Plus Phenyl Hexyl: (1) Piroxicam, (2) Sulindac,(3) Tolmetin, (4) Naproxen, (5) Ibuprofen, (6) Diclofenac, (7) Celebrex (equal portions of approximately 1 mg/ml solutions

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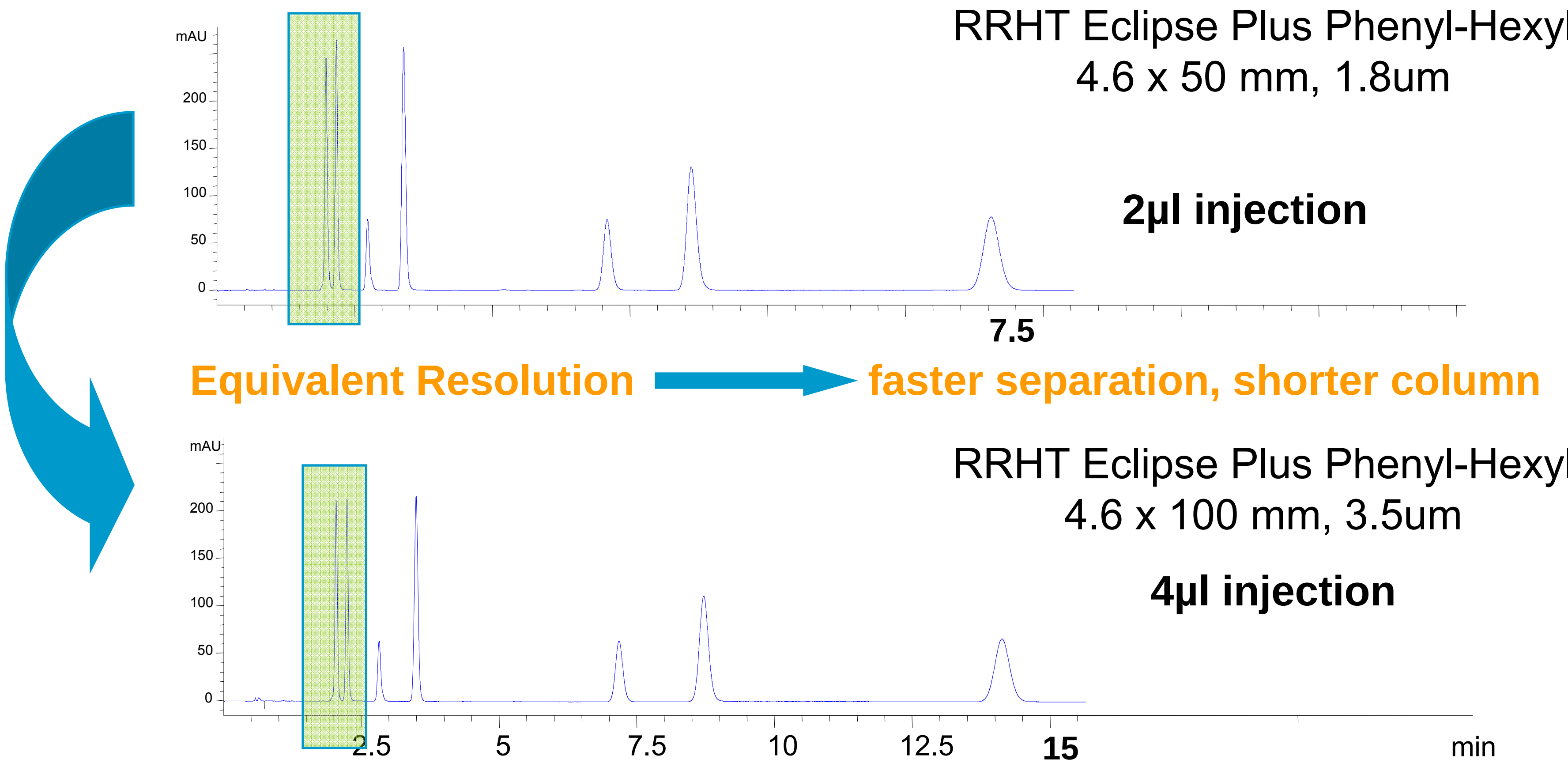

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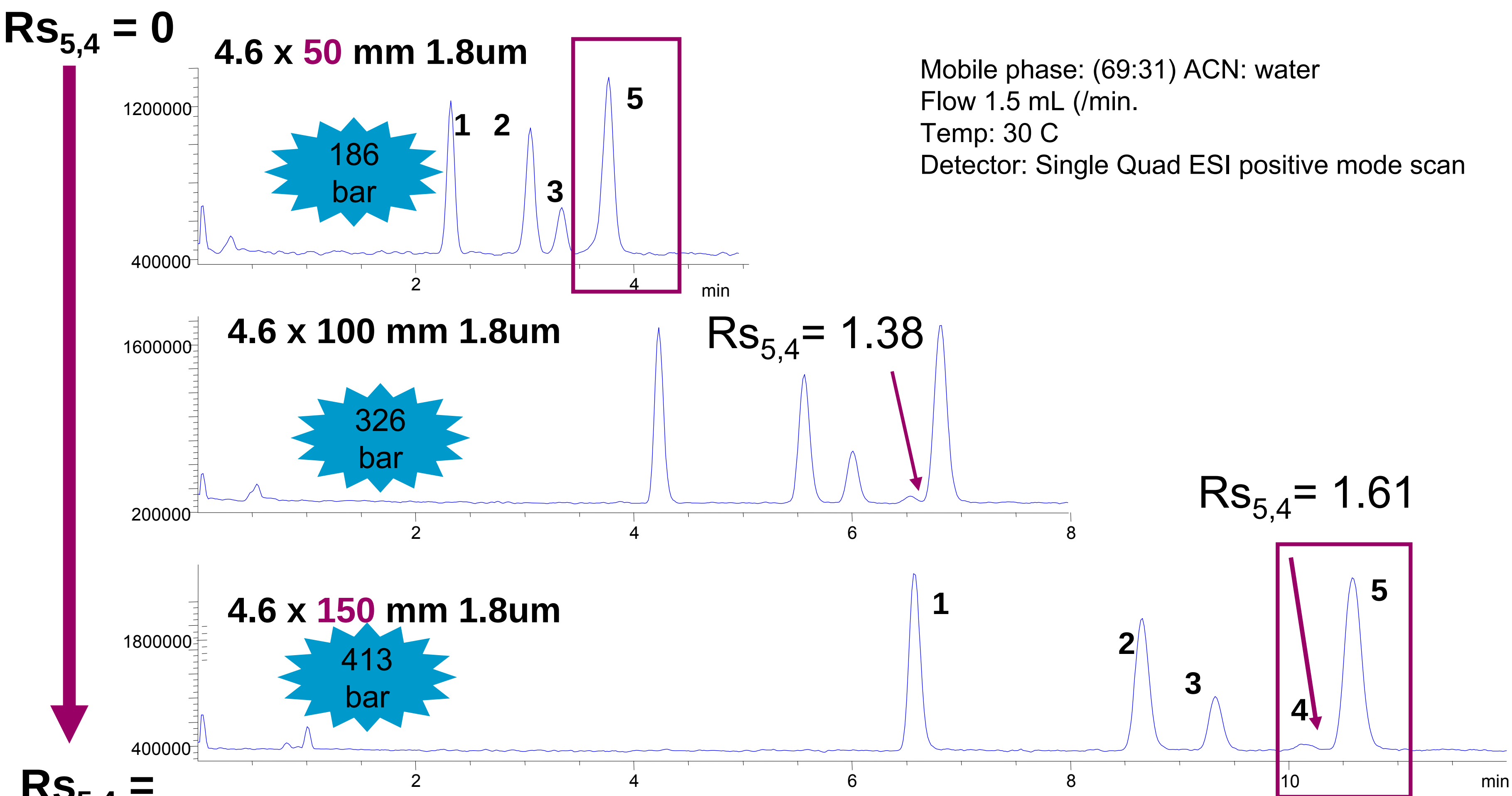
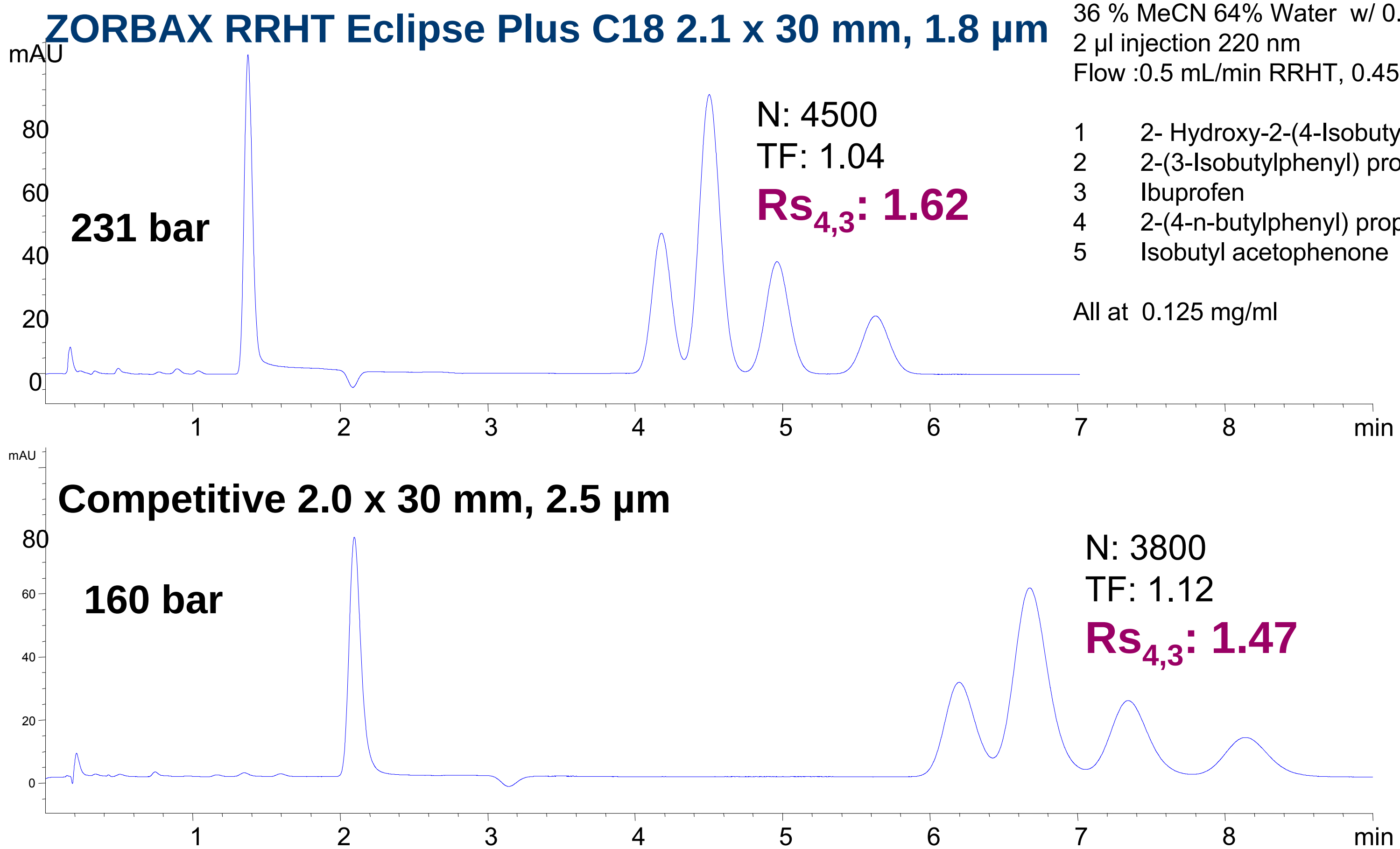
4. Longer RRHT Columns Improve Efficiency and Rs
Pressure Increases with Length

Mobile phase: (69:31) ACN: water
Flow 1.5 mL (/min.
Temp: 30 C
Detector: Single Quad ESI positive mode scan



Sample: 1. Anandamide (AEA) 2. Palmitoylethanolamide (PEA) 3. 2-arachinoylglycerol (2-AG)
4. 1(3)-arachidonylglycerol5. Oleoylethanolamide (OEA)

4. Comparison of 1.8um and 2.5 um Columns



36 % MeCN 64% Water w/ 0.7 ml/L phosphoric acid
2 µl injection 220 nm
Flow :0.5 mL/min RRHT, 0.45 mL/min. (Luna)

1 2- Hydroxy-2-(4-Isobutylphenyl) proprionic acid
2 2-(3-Isobutylphenyl) proprionic acid
3 Ibuprofen
4 2-(4-n-butylphenyl) proprionic acid
5 Isobutyl acetophenone

All at 0.125 mg/ml

RRHT, 1.8um has at least 18% more efficiency than 2.5um
That means greater than baseline resolution can be achieved when needed.

Is the Smallest Particle Size the Best?

Kinetic plots – comparison of 1.8, 3.5, 5um and monoliths

Very high efficiency separations with small particle size

Comparison of particle sizes – as a function of analysis time

Bonded phase is always worth considering

What is a Kinetic Plot – What can it show?

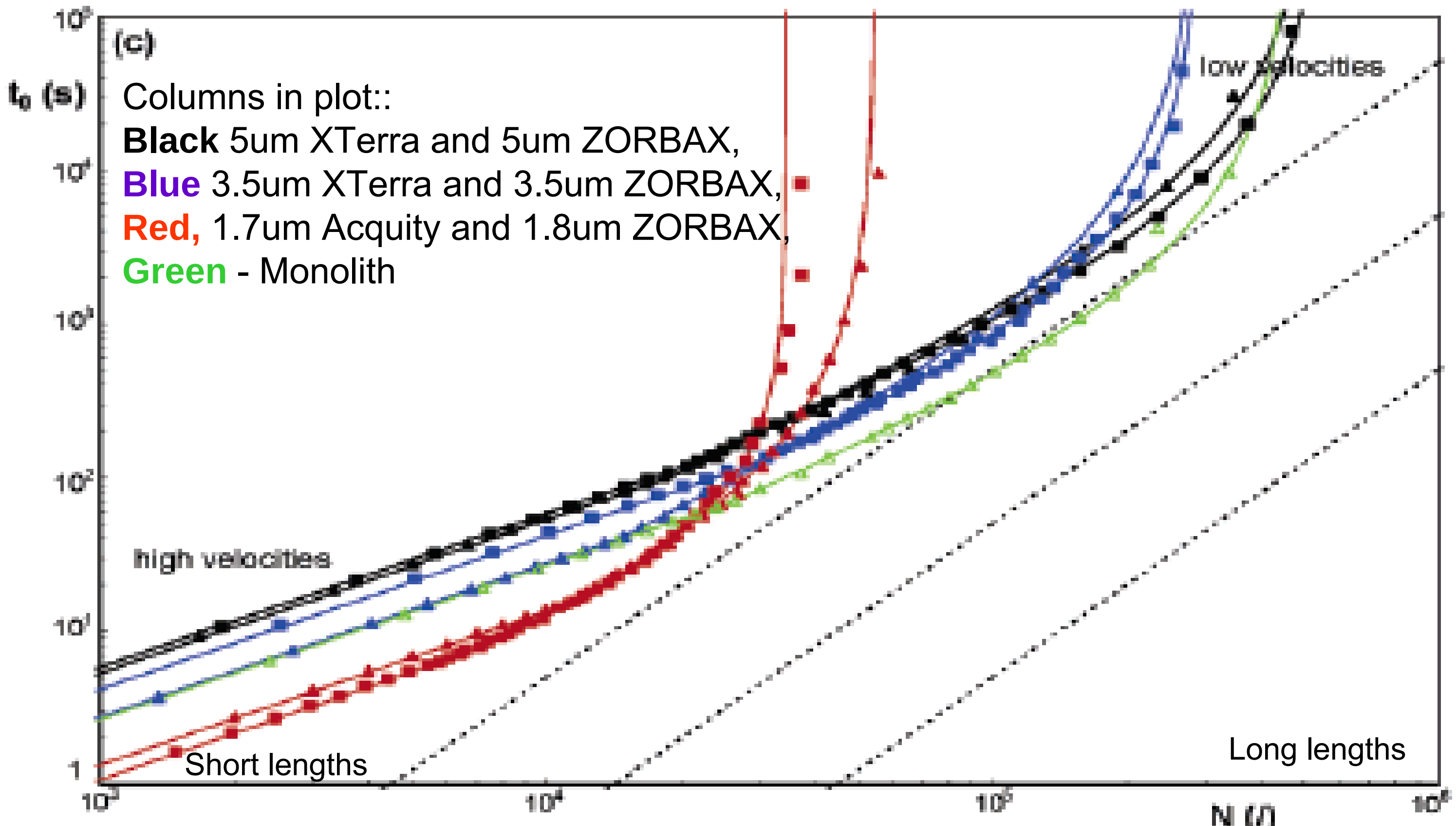
Convert Van Deemter Plots to those based on t_0 vs. N in order to characterize column packing materials over a range of N values, practically you can compare the performance of different length columns and compare the analysis time needed to achieve a certain level of efficiency

- Free kinetic plots apply no column length constraints
- Constrained kinetic plots, i.e. constrained by column length, pressure can help choose an optimum column based on real choices

Can look at practical constraints on column length and particle size to choose an optimum configuration for the needed efficiency or analysis time

Kinetic Plot for Comparison of Different Particle Sizes, based on u_{max}

Desmet et. al. Anal. Chem. **2006,** 78, 2150-2162



Smallest, sub 2um, particles generate greater efficiency in short columns at high velocities than other particle sizes, larger particle sizes in longer columns can generate more efficiency

Is the Smallest Particle Size the Best? It Depends

Kinetic plots show that smallest particle size is best for fast separations – under 15 – 40 minutes

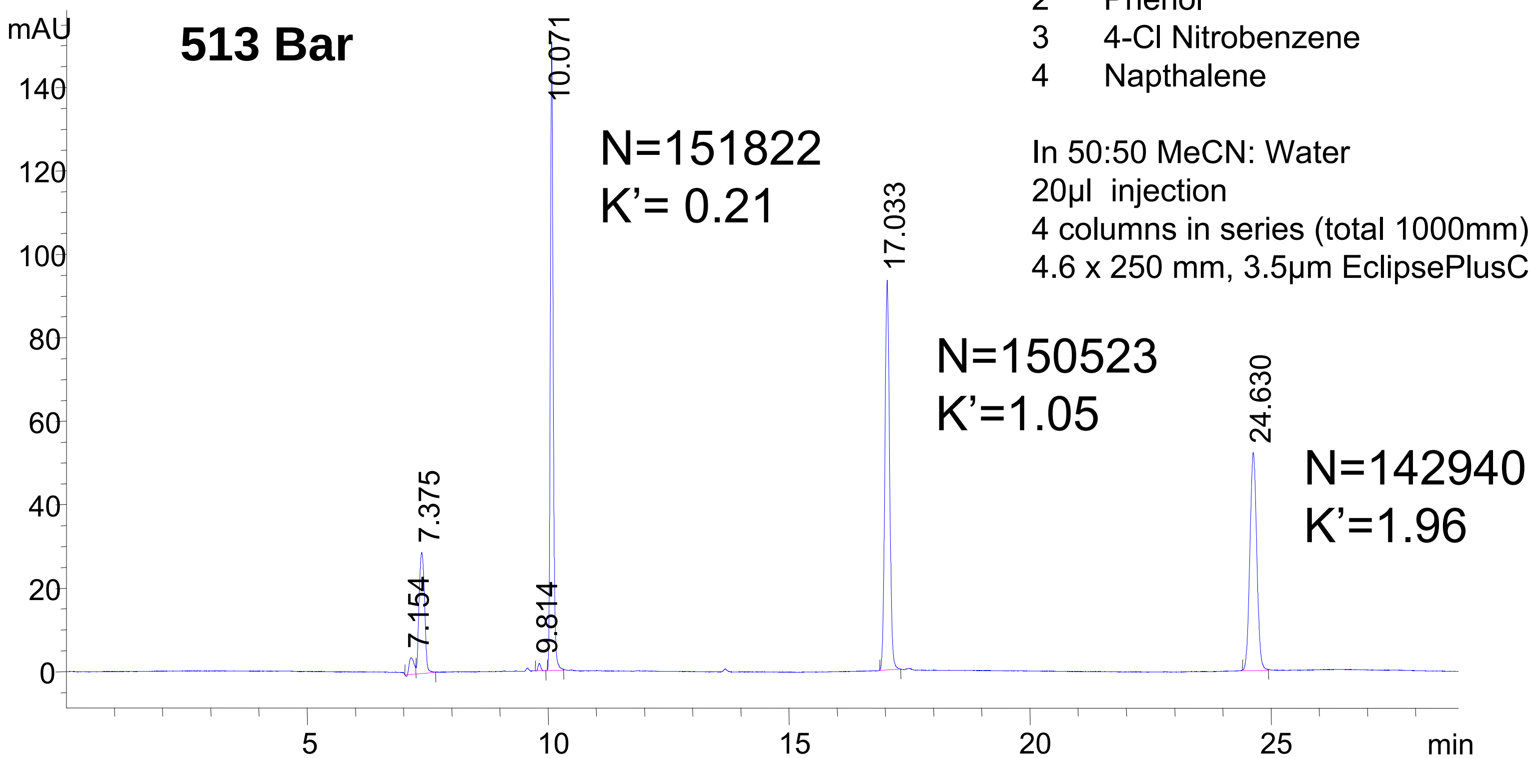
For a mid time frame – 40 – 60 minutes, a 3.5um particle size can be a better choice for maximizing efficiency if you are willing to increase analysis time (1.8um can still be a good choice for high efficiency)

For a long time frame – 1 hour or more a larger, 5um particle size can generate the most efficiency

4 Eclipse Plus C18, 3.5um in Series for Maximum Efficiency – Cost is Analysis Time

70:30 MeCN :Water
1.2 ml/min 254 nm 6 mm flowcell
40 C
1 Uracil
2 Phenol
3 4-Cl Nitrobenzene
4 Napthalene

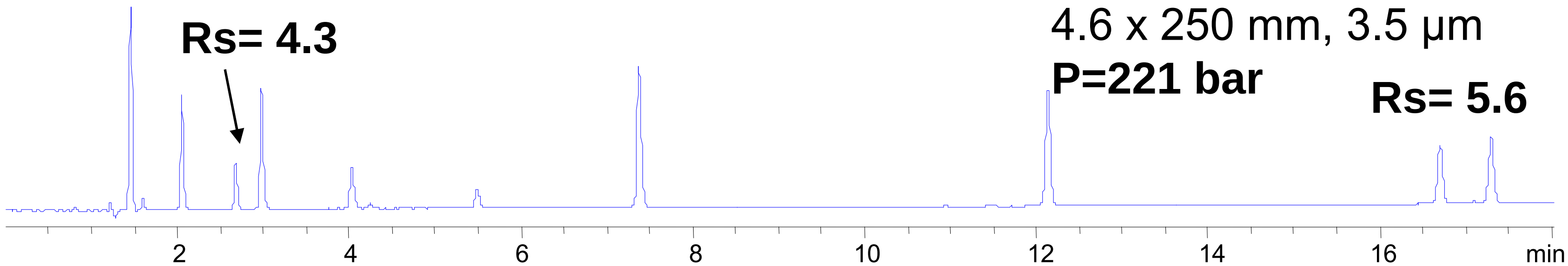
In 50:50 MeCN: Water
20µl injection
4 columns in series (total 1000mm)
4.6 x 250 mm, 3.5µm EclipsePlusC18

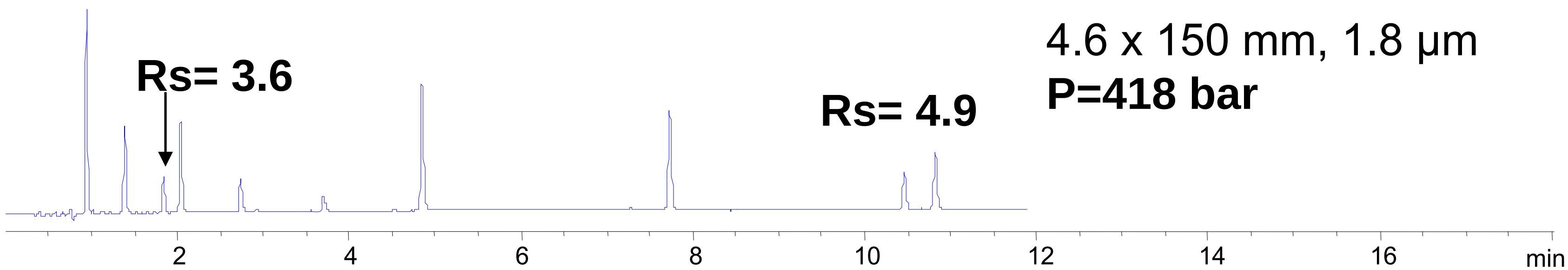


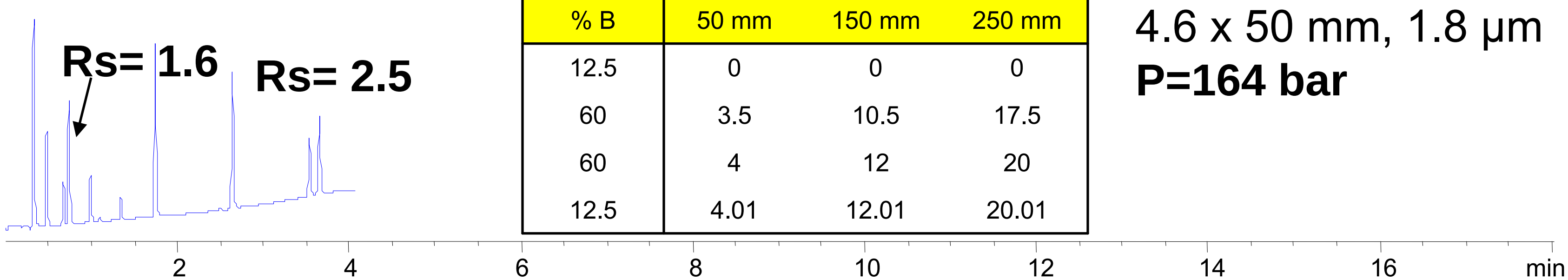
High pressure LC can be used for very high efficiency separations with multiple columns in series.

Different Particle Sizes May Be an Appropriate Choice for a Separation

Pressures:	Room Temp	70ºC
4.6 x 250mm, 3.5um	399 bar	221 bar
4.6 x 150mm, 1.8um		418 bar

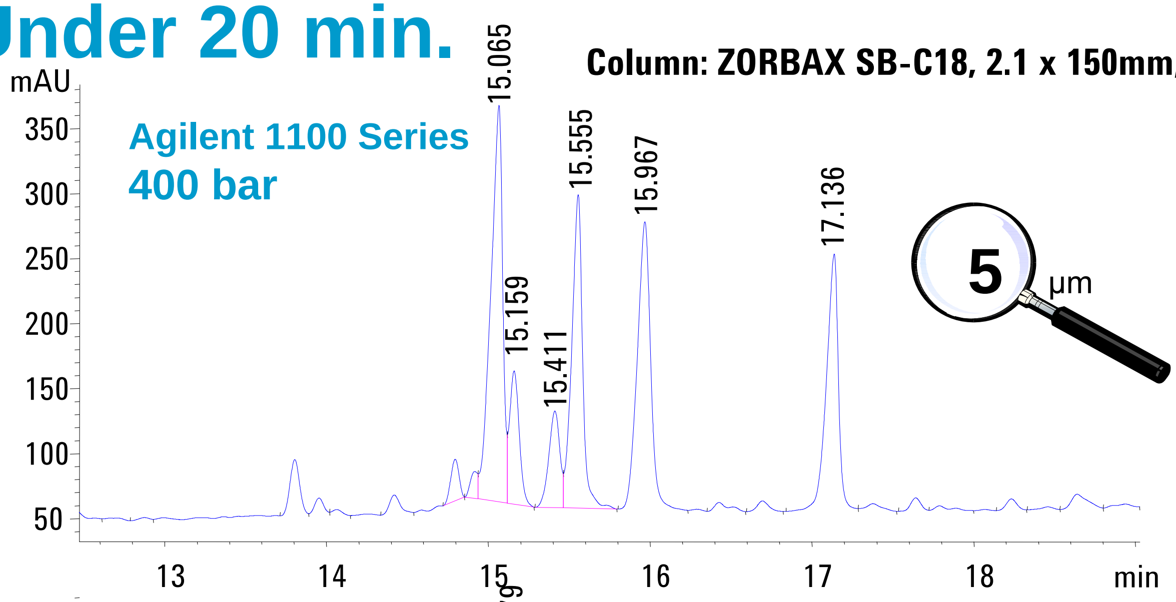


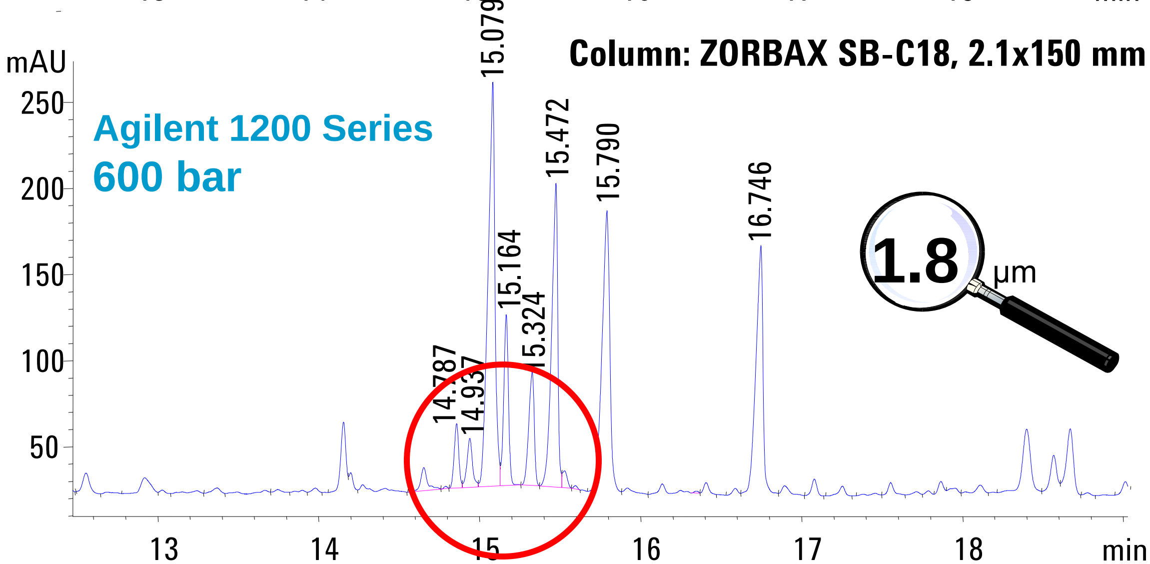




% B	50 mm	150 mm	250 mm
12.5	0	0	0
60	3.5	10.5	17.5
60	4	12	20
12.5	4.01	12.01	20.01

Separate More Peaks with 1.8um – Analysis Time Under 20 min.





Mobile phases:	A = H2O + 0.1% TFA
	B = ACN + 0.1% TFA
Gradient:	10% to 95% ACN in 40min, hold for 1min
Flow:	0.4 mL/min
Inj. volume:	3 µL, partial loop filling
DAD:	220 nm (20 Hz)
	2µl flowcell

Improved Resolution

Agilent Application Note:
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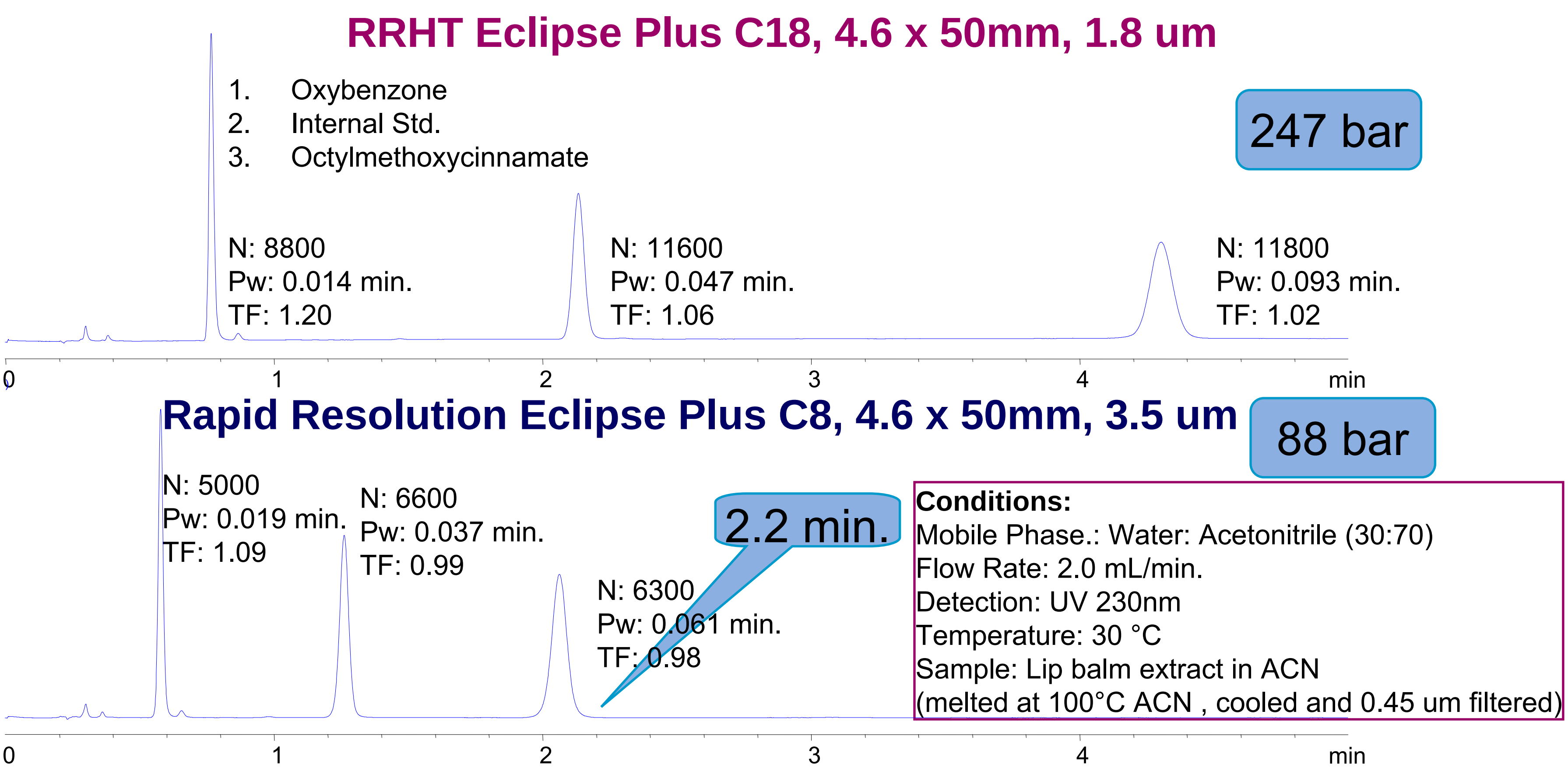
Publication Number 5989-4506EN

An Optimum Bonded Phase Can Always Be the Best Choice



Less retention can save significant time – the C8 is a good choice here.

The RRHT column is delivering the efficiency and resolution expected, but the C8 bonded phase may be the best choice.

Conclusions

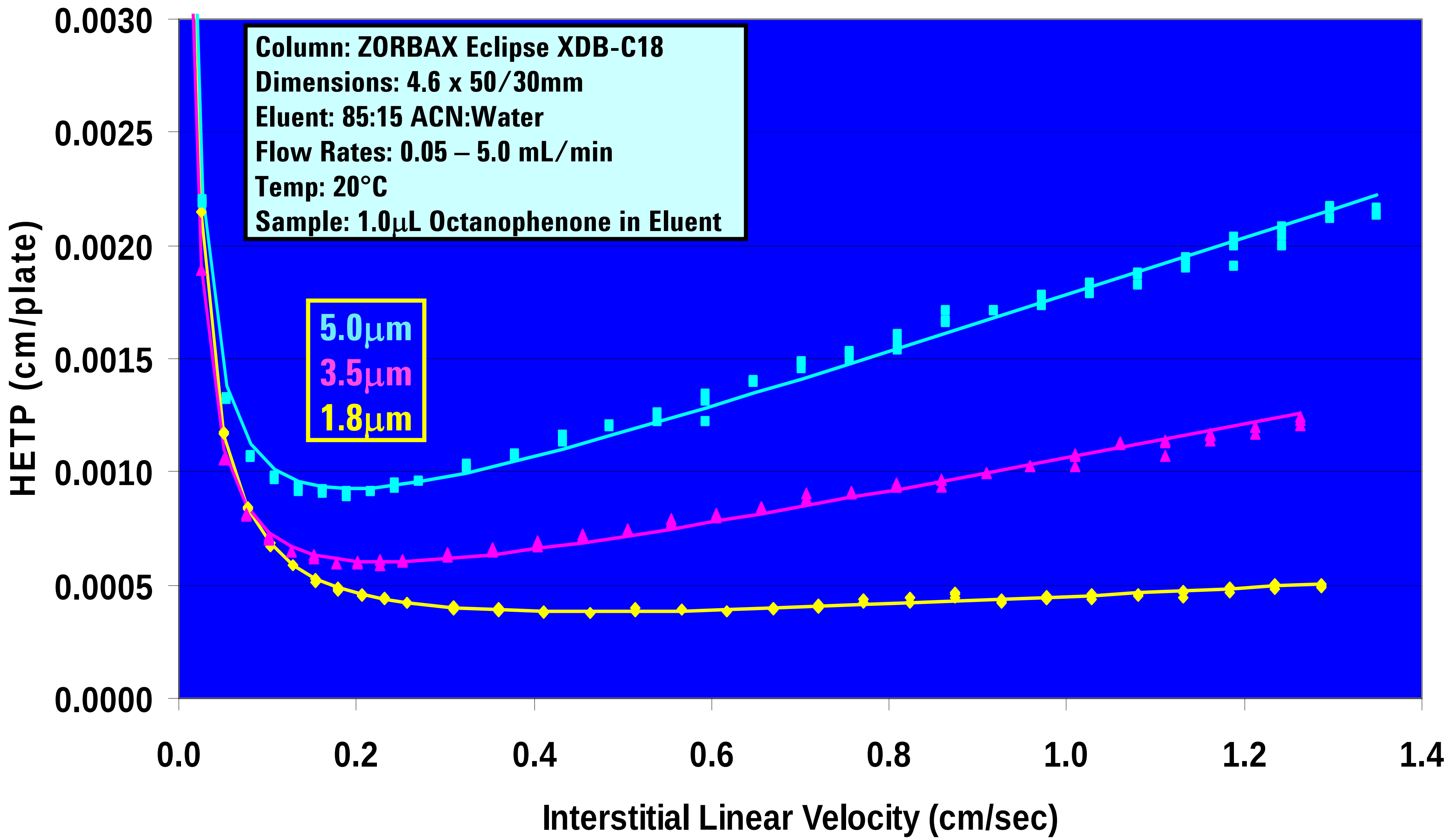
Small particle sizes, sub 2-micron, deliver the highest efficiency for fast separations – those basically less than 30 minutes long

It is possible to achieve close to theoretical performance with an optimized LC.

Larger particle sizes can generate very high efficiency separations, but the highest efficiency will always require more analysis time.

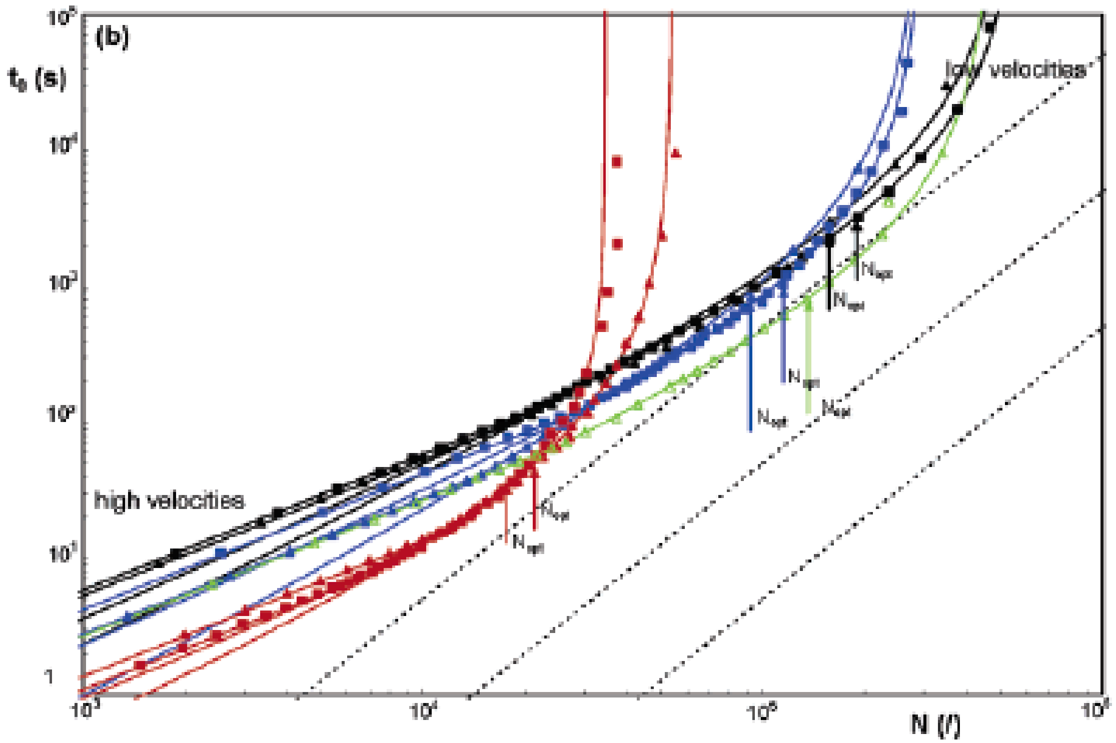
An optimum bonded phase for the separation can be equally important.

HETP vs. Interstitial Linear Velocity



Free Kinetic Plot

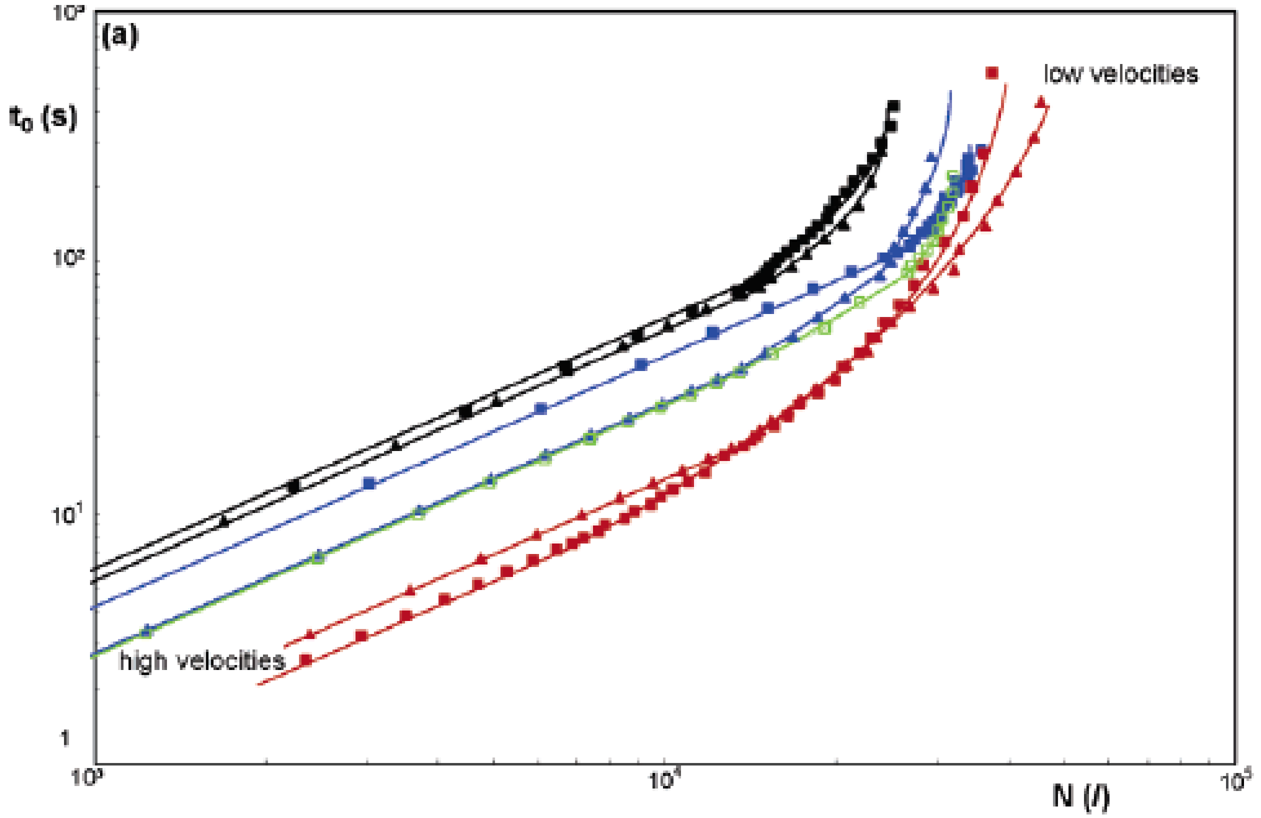
Desmet et. al. Anal. Chem. **2006,** 78, 2150-2162



Columns in plot: **Black** 5um XTerra and 5um ZORBAX, **Blue** 3.5um XTerraand 3.5um ZORBAX, **Red,** 1.7um Acquity and 1.8um ZORBAX, **Green** - Monolith

In the free kinetic plot the monolith and 5um particles appear to offer the optimum efficiency.

Constrained Kinetic Plot



Achieve more gain in analysis time with sub 2um