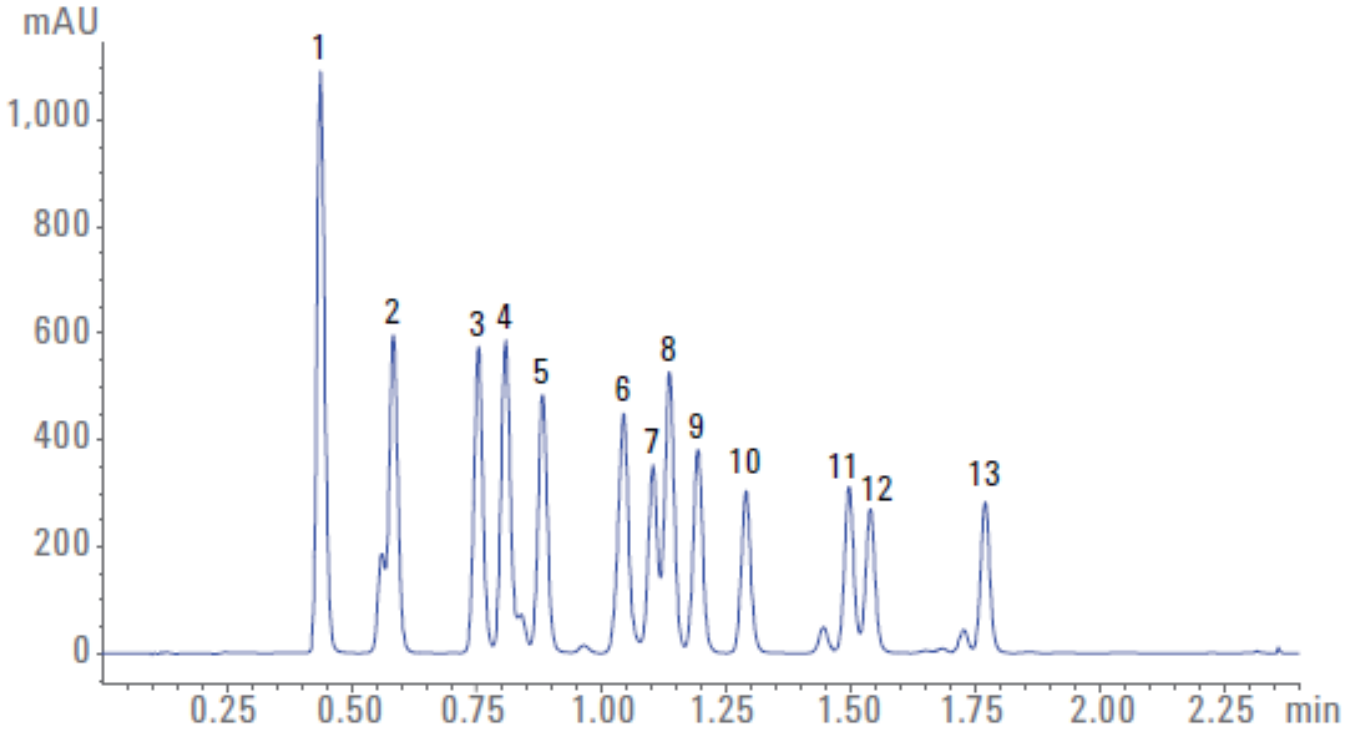
The Secrets to Better HPLC Methods

Carl Griffin

ASTS-Quebec City

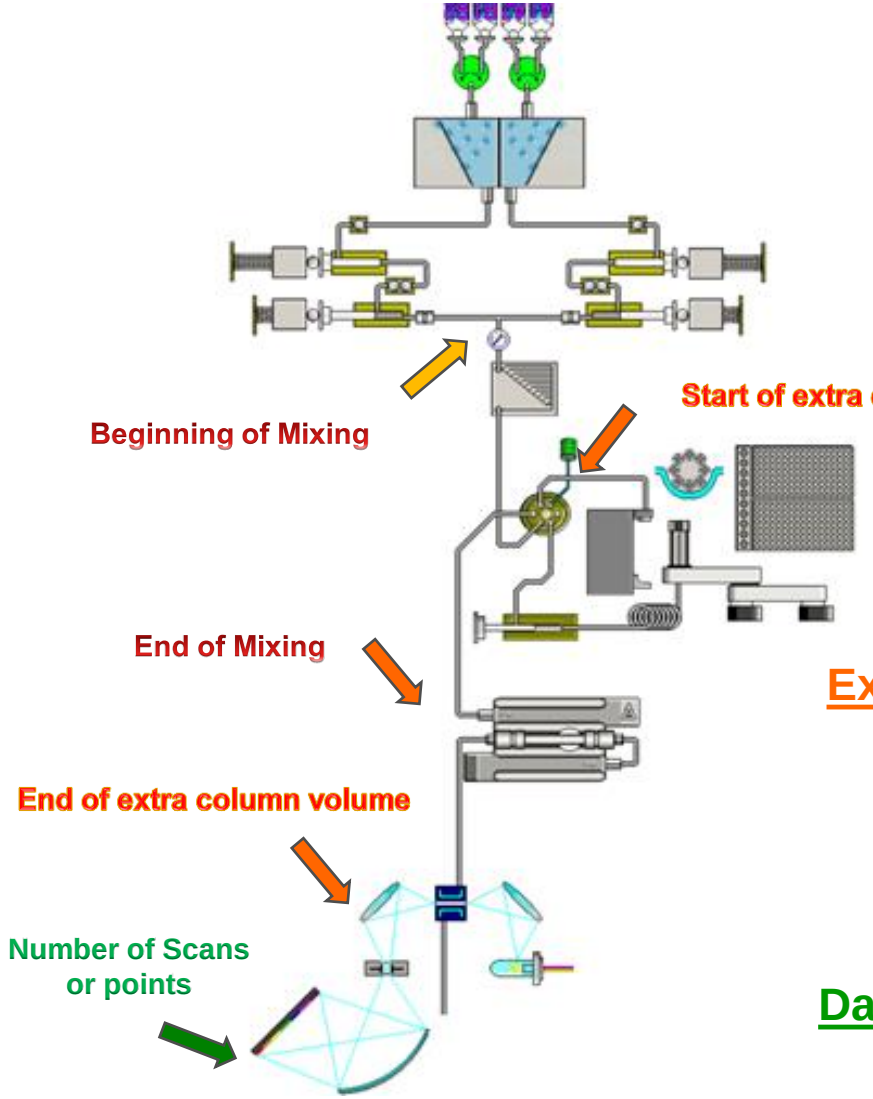
Have You Ever Had Peaks That You Can't Resolve?



Five Things That You Can Do To Improve The Separation

#1 System Tubing

HPLC Instrument Components

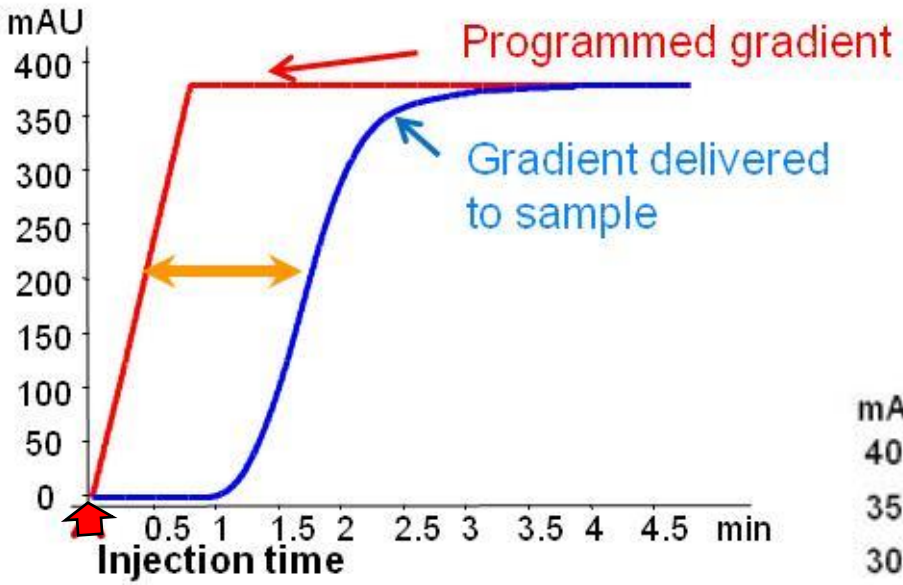


Gradient Delay or Dwell Volume

Extracolumn Volume

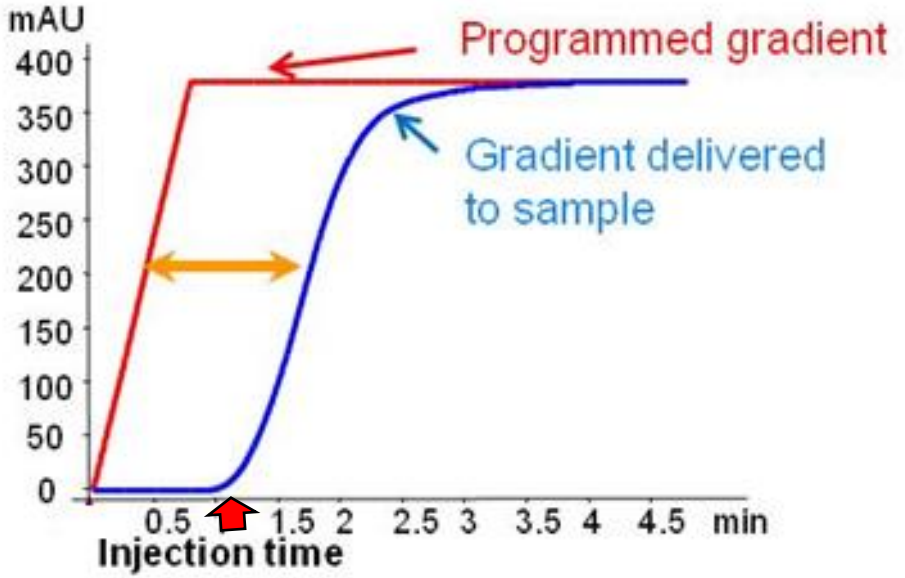
Data Sampling or Acquistion Rate

Effects of Delay Injection Program

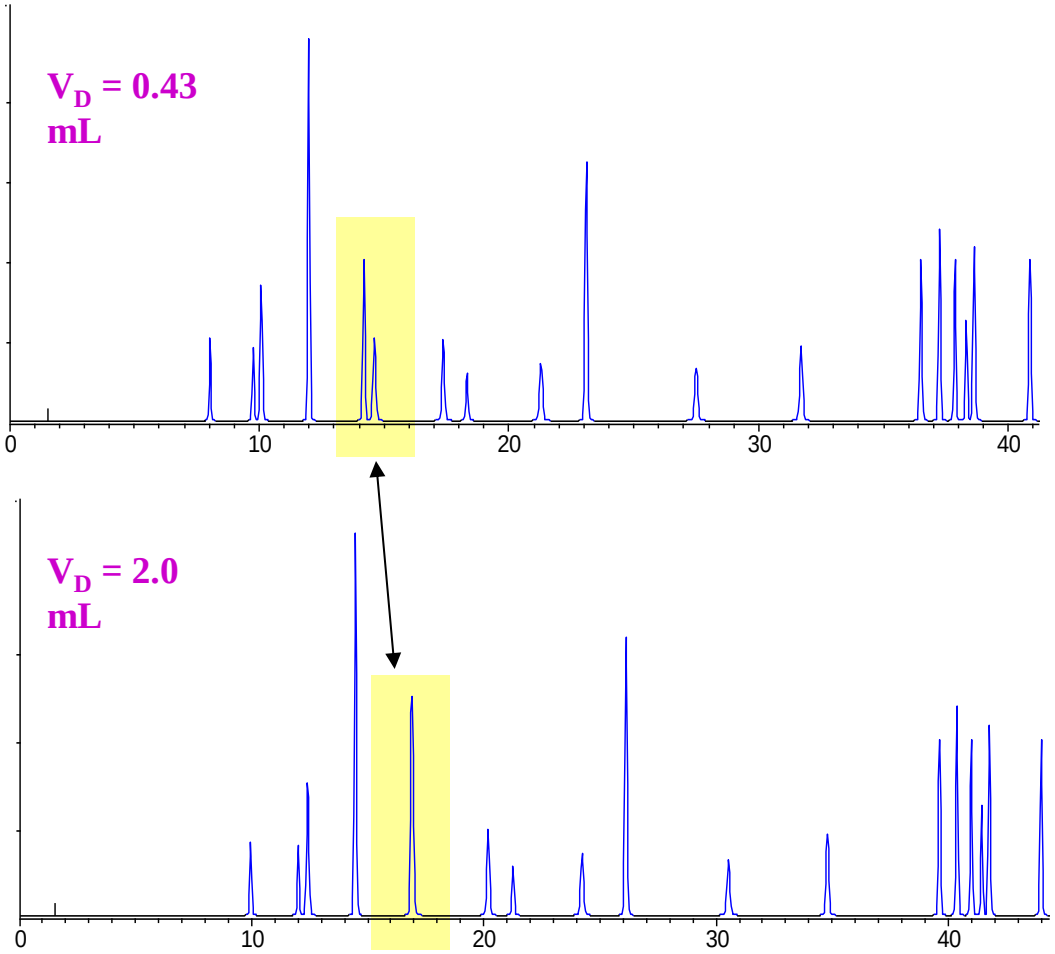


Standard Injection

Delayed Injection



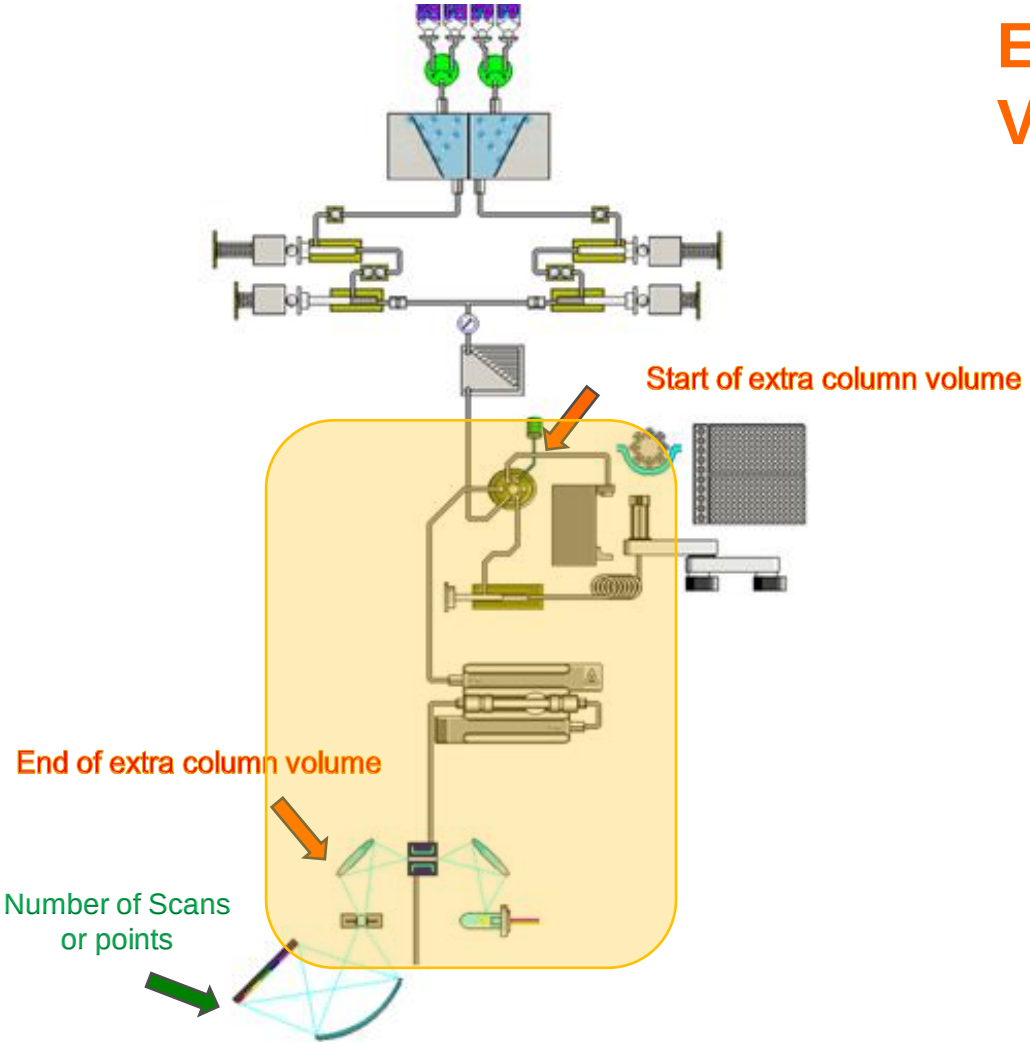
Minor Dwell Volume Differences Can Change Resolution



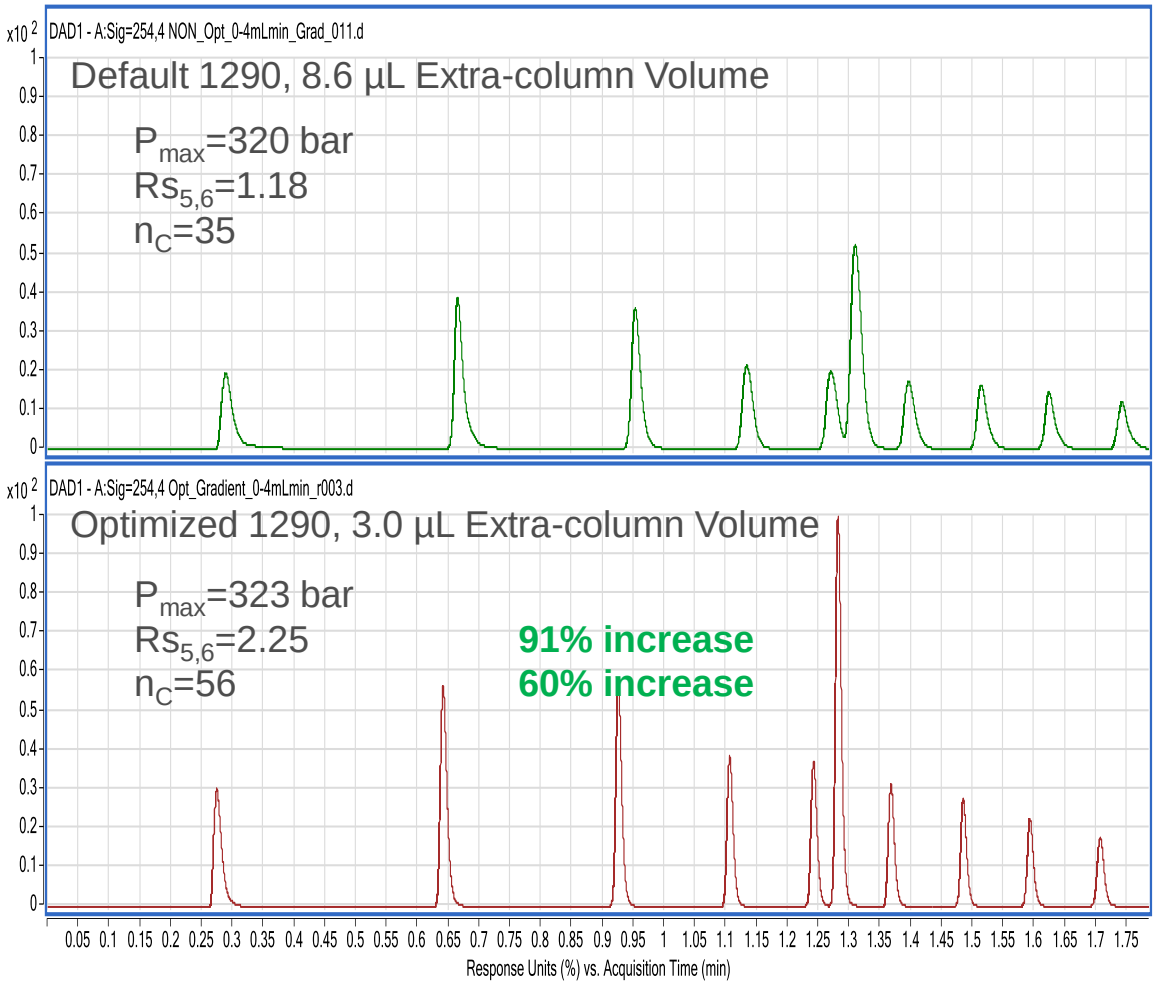
Column:	**ZORBAX Rapid Resolution Eclipse XDB-C8 4.6 x 75 mm, 3.5 µm**
Mobile Phase:	Gradient, 0 - 100 %B in 52.5 min.
	A: 5/95 methanol/ 25 mM phosphate pH 2.50
	B: 80/20 methanol/25 mM phosphate pH 2.50
Flow Rate:	0.5 mL/min
Temperature:	25°C
Injection:	5 µL
Detection:	250 nm
Sample:	Mixture of antibiotics and antidepressants
	Upper trace simulates actual run data entered into DryLab® 3.0 software
	Lower trace is simulated chromatogram for larger V_D

2. Gradient Separations- Instrument Impact on Column Performance

Extra-column Volume



Effect of Extra-column Volume on a Gradient Analysis of Alkylphenones



A: H_2O; B: CH_3CN
0.4 mL/min

t (min)	0	1.2
%B	25	95

1 µL injection of RRLC Checkout Sample (PN 5188-6529) spiked w/ 50 µL 2 mg/mL Thiourea in water/acetonitrile
TCC: ambient
DAD: Sig=254,4nm; Ref=Off
Sample:

1. Thiourea (v_0 marker)
2. Acetanilide
3. Acetophenone
4. Propiophenone
5. Butyrophenone
6. Benzophenone
7. Valerophenone
8. Hexanophenone
9. Heptanophenone
10. Octanophenone

Agilent ZORBAX Eclipse Plus RRHD C18
2.1 mm x 50 mm, 1.8 µm, 959757-902

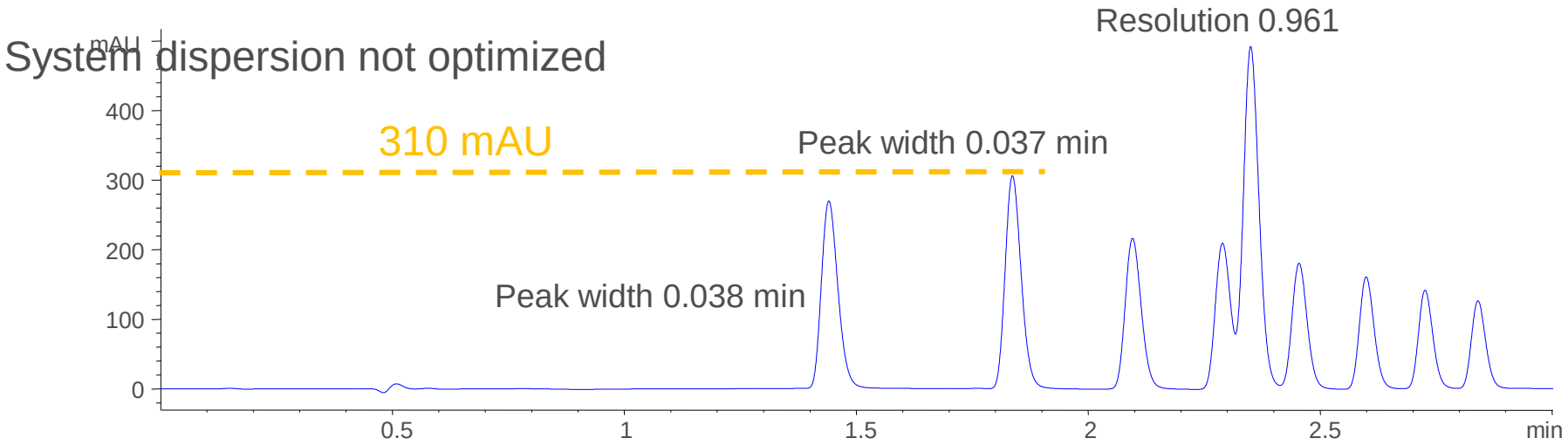
LC Rack System, 5001-3726

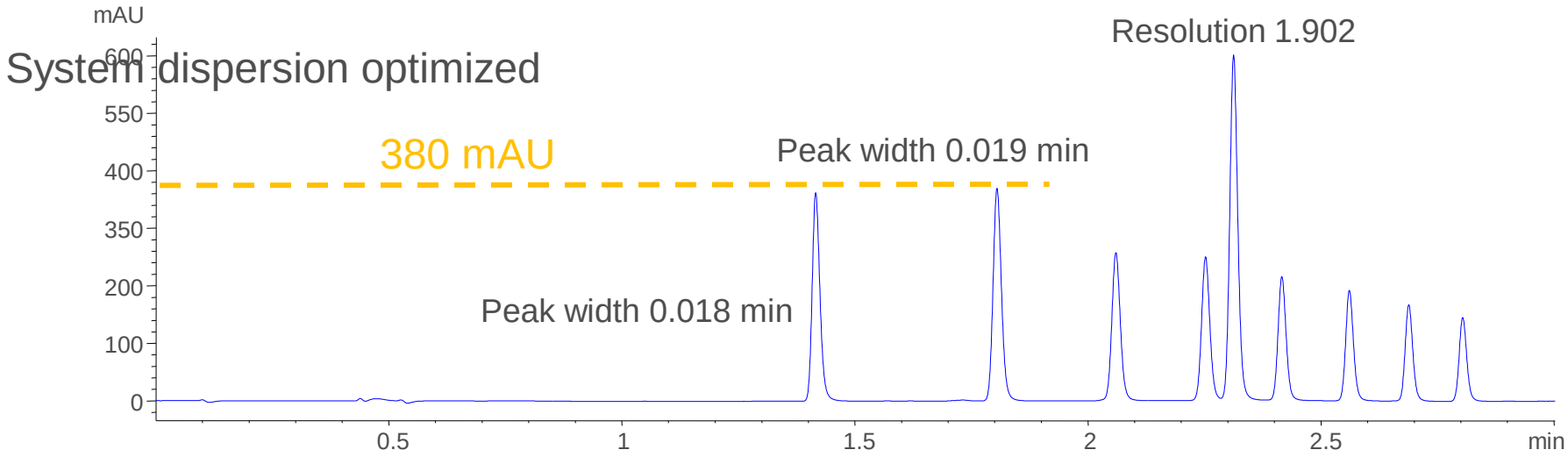
0.08 x 220 mm Capillary Tubing

V(σ)0.6 µL Flow Cell

System – Signal Height

System volumes – System dispersion

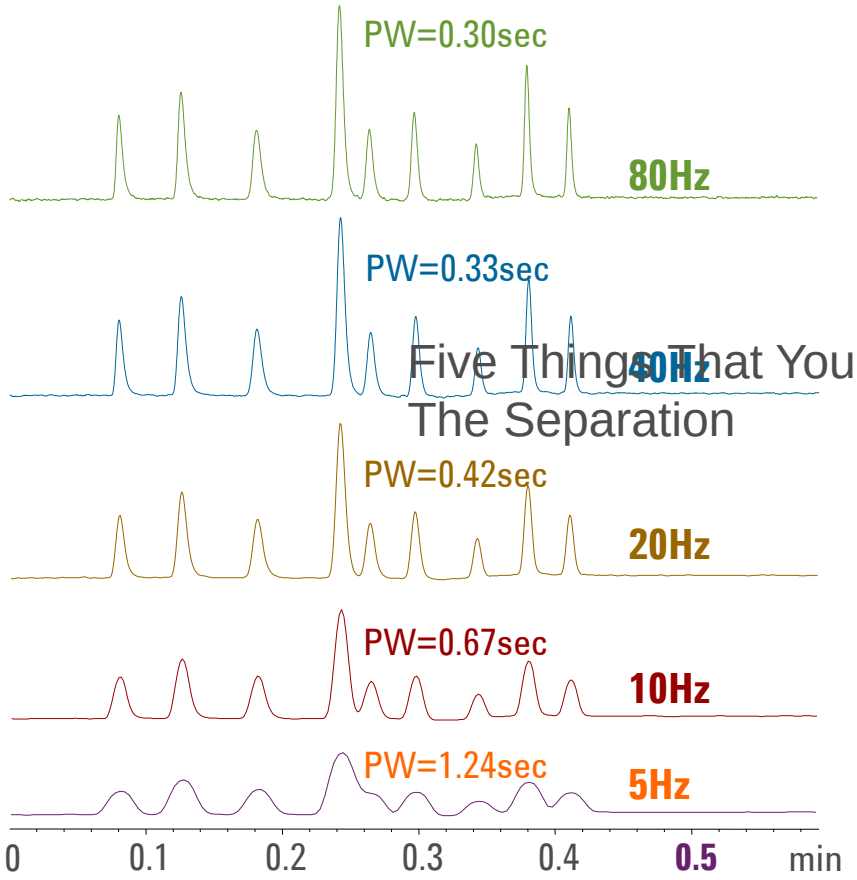




Five Things That You Can Do To Improve The Separation

#2 Sample Data Rate

Detectors
For narrow peaks, high data rates!!!
Maintaining Resolution at High Analysis Speed



Five Things That You Can Do To Improve The Separation

80Hz versus 10Hz (20Hz) Data Rate

- Peak Width: – 55% (– 30%)
- Resolution: + 90% (+ 30%)
- Peak Capacity: + 120% (+ 40%)
- App. Column Eff.: + 260% (+ 70%)

Data Rate	Peak Width	Resolution	Peak Capacity
80 Hz	0.300	2.25	60
40 Hz	0.329	2.05	55
20 Hz	0.416	1.71	45
10 Hz	0.666	1.17	29
5 Hz	1.236	0.67	16

Sample: Phenones Test Mix
Column: Zorbax SB-C18, 4.6x30, 1.8um
Gradient:: 50-100%ACN in 0.3min
Flow Rate: 5ml/min

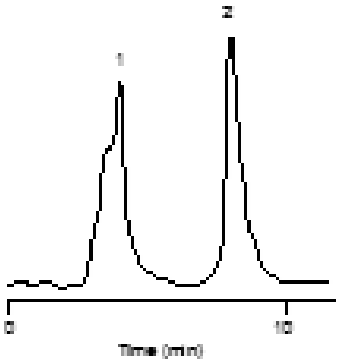
Five Things That You Can Do To Improve The Separation

#3 Sample Solvent

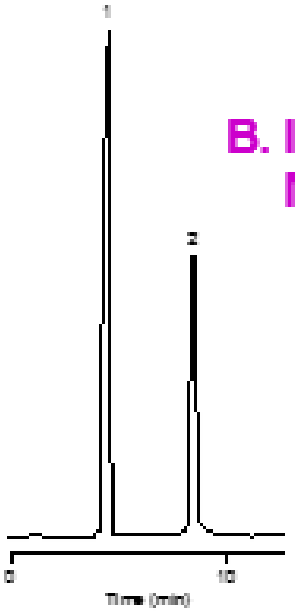
Split Peaks from Injection Solvent Effects

Column: StableBond SB-C8, 4.6 x 150 mm, 5 µm Mobile Phase: 82% H_2O : 18% ACN
Injection Volume: 30 µL Sample: 1. Caffeine 2. Salicylamide

A. Injection Solvent 100% Acetonitrile



B. Injection Solvent Mobile Phase



Tip: Injecting in a solvent stronger than the mobile phase can cause peak shape problems such as peak splitting or broadening

Trick: Keep Organic Concentration in Sample Solvent $\leq$ Mobile Phase

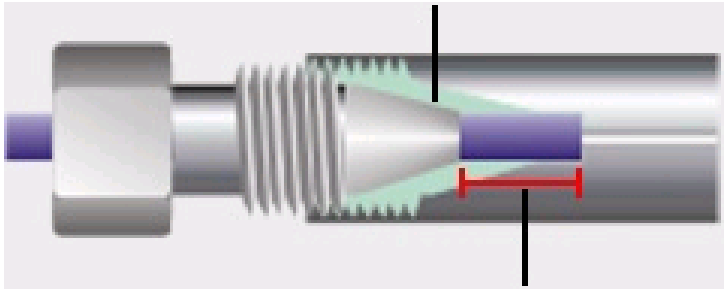
Five Things That You Can Do To Improve The Separation

#4 Column Connections

What Happens If the Connections Poorly Made ?

Wrong … too long

Ferrule cannot seat properly

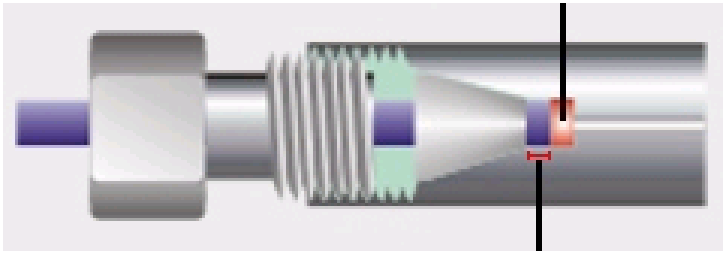


X

If Dimension X is too long, leaks will occur

Wrong … too short

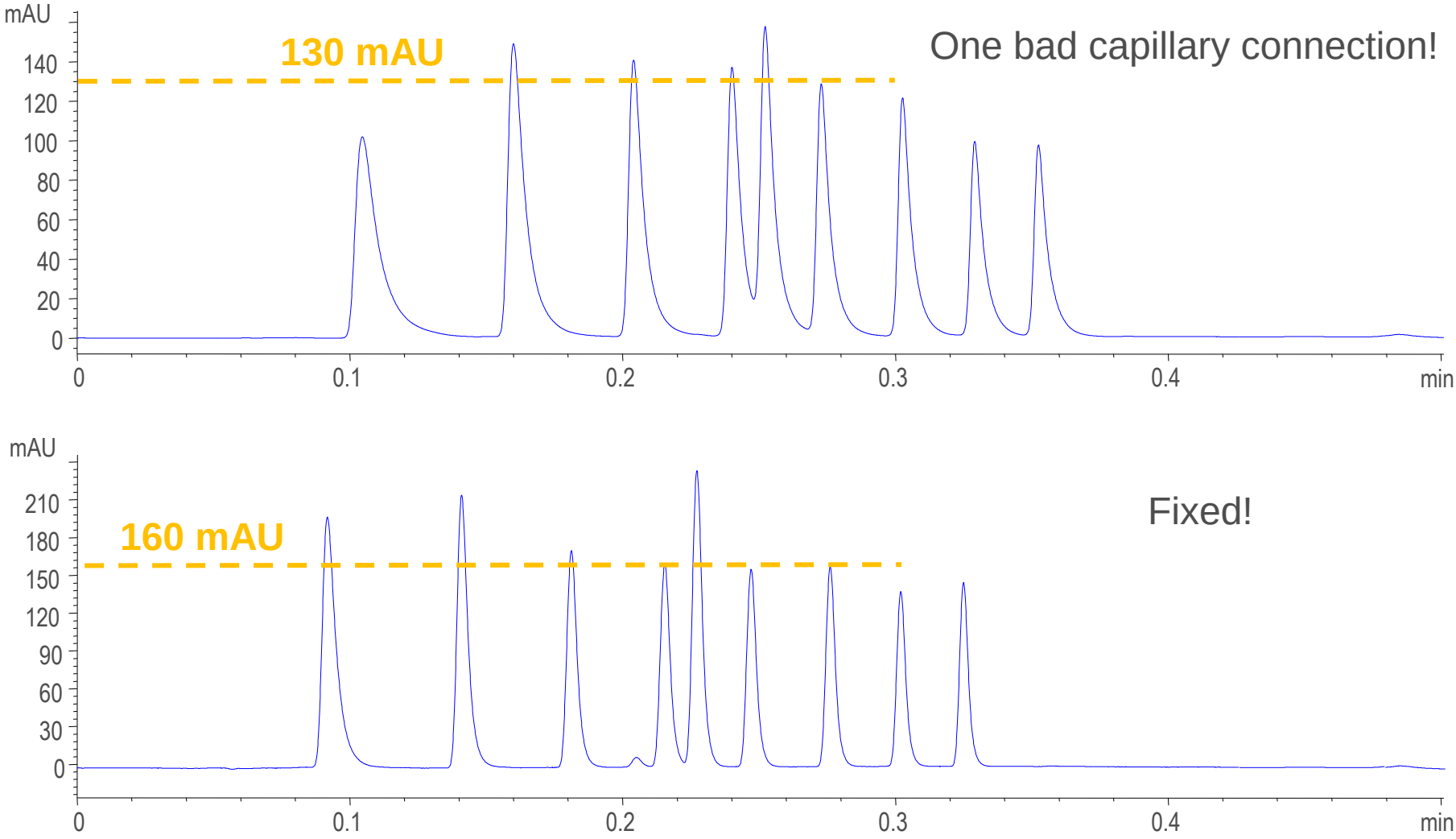
Mixing Chamber



X

If Dimension X is too short, a dead-volume, or mixing chamber, will occur

Influence post-column capillary connections



A-Line Fitting

Prefect Fitting Every Time

1300 Bar Rating

Finger Tight

600 Bar Finger Tight
1300 Bar with a ¼ turn

Five Things That You Can Do To Improve The Separation

#5 High Efficiency Columns

Benefits of High Efficiency

Use of smaller columns

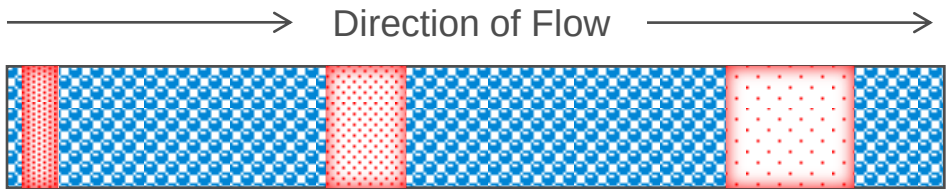
Faster Methods

Higher Sensitivity

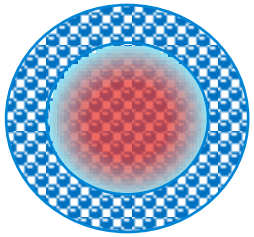
Lower Limitations of Detection

More Efficient Particles in Smaller Volume Columns Reduces Dispersion Within the LC Column

a) Longitudinal Diffusion (dispersion)

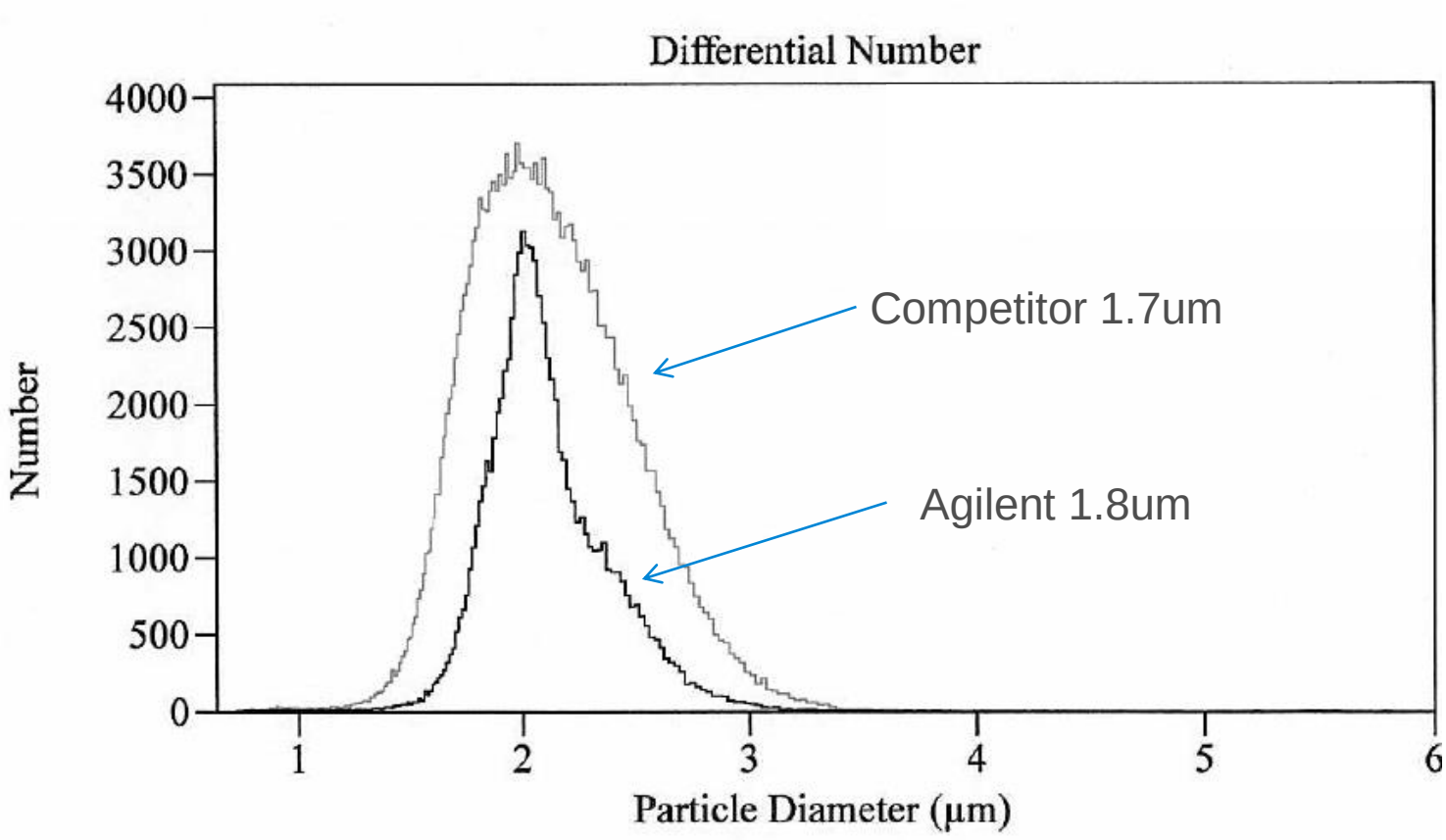


b) Radial Diffusion (dispersion)



Greater Control of Particle Size Distribution

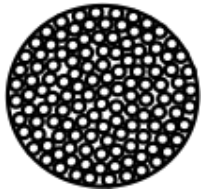
A Little Known Secret for Better High Pressure Performance

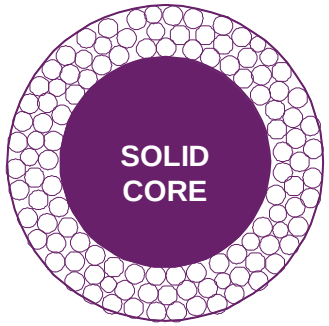


(Packing from first generation 2.1 x 100mm, 1.7um column)

Agilent Particle Choices for UHPLC

Longest History of UHPLC Particle Innovation and Manufacture





Sub 2um Totally Porous Particle

Superficially Porous Particle

High Efficiency Chromatographic Media

Zorbax RRHD RRHT 1.8 um

Poroshell 120 2.7 um

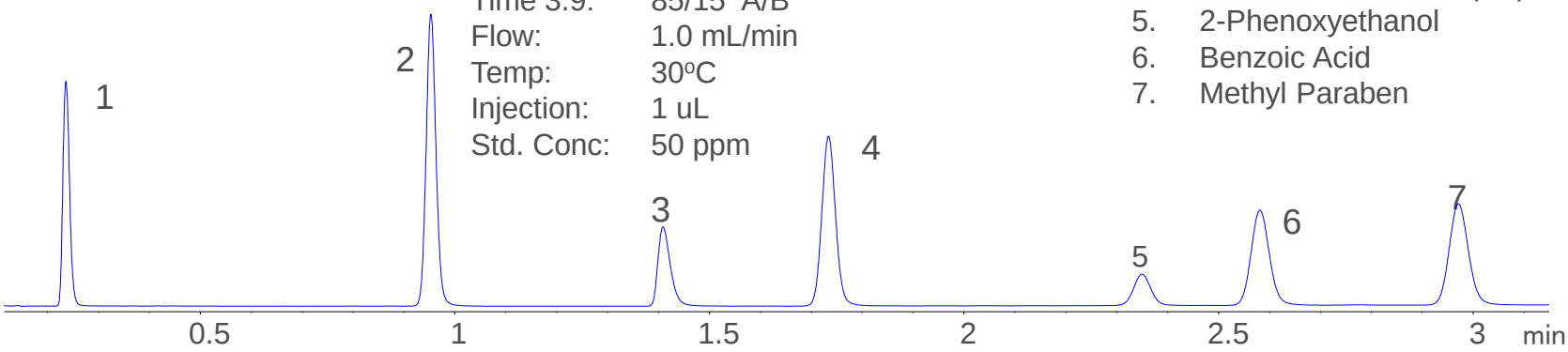
Comparable Performance Under Moderate UHPLC Conditions

RRHD Eclipse Plus C18 and Acquity BEH C18 (2.1 x 50mm)

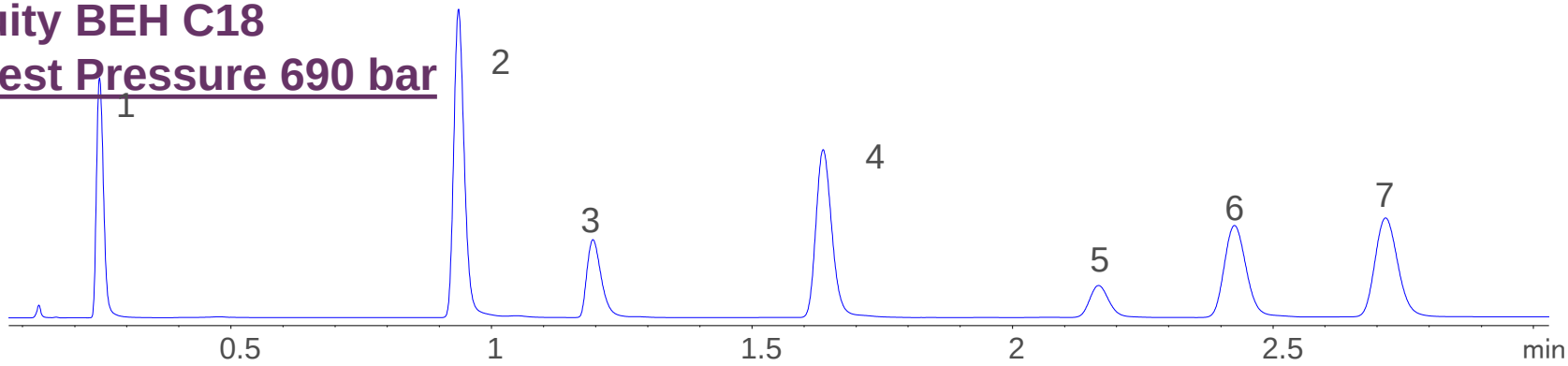
RRHD Eclipse Plus C18
Highest Pressure 630 bar

A: H_2O (0.05 v% TFA) B: ACN (0.04 v% TFA)
Time 0: 95/5 A/B
Time 2.9: 85/15 A/B
Time 3.9: 85/15 A/B
Flow: 1.0 mL/min
Temp: 30°C
Injection: 1 uL
Std. Conc: 50 ppm

1. Kathon 1A
2. Kathon 1B
3. Carbendazim
4. 1,2-Benzisothiazol-3(2H)-one
5. 2-Phenoxyethanol
6. Benzoic Acid
7. Methyl Paraben



Acquity BEH C18
Highest Pressure 690 bar



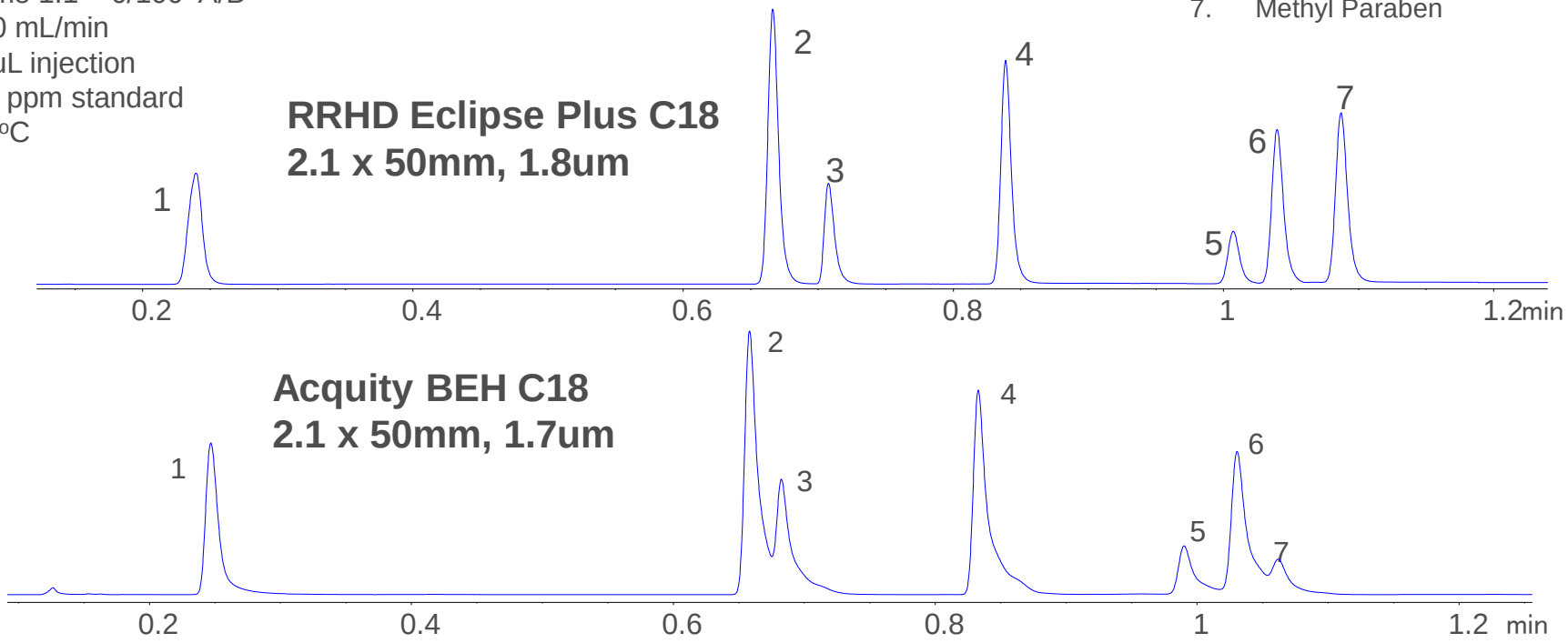
Agilent has lower pressure due to better control over particle size and distribution

Particle Durability Needed to Maintain Performance at Consistently High Pressure

A: H_2O (0.05 v% TFA)
Time 0.0 95/5 A/B
Time 0.1 95/5 A/B
Time 0.25 90/10 A/B
Time 1.0 65/35 A/B
Time 1.1 0/100 A/B
1.0 mL/min
1 uL injection
50 ppm standard
30°C

B: ACN (0.04 v% TFA)
DAD: 275 nm (0 min)
225 nm (0.8 min)
255 nm (1.08 min)

1. Kathon 1A
2. Kathon 1B
3. Carbendazim
4. 1,2-Benzisothiazol-3(2H)-one
5. 2-Phenoxyethanol
6. Benzoic Acid
7. Methyl Paraben



Nominal High Pressure 800 bar on Both Columns

RRHD Performance With Ballistic Gradients

Opens New possibilities for Resolution AND Speed

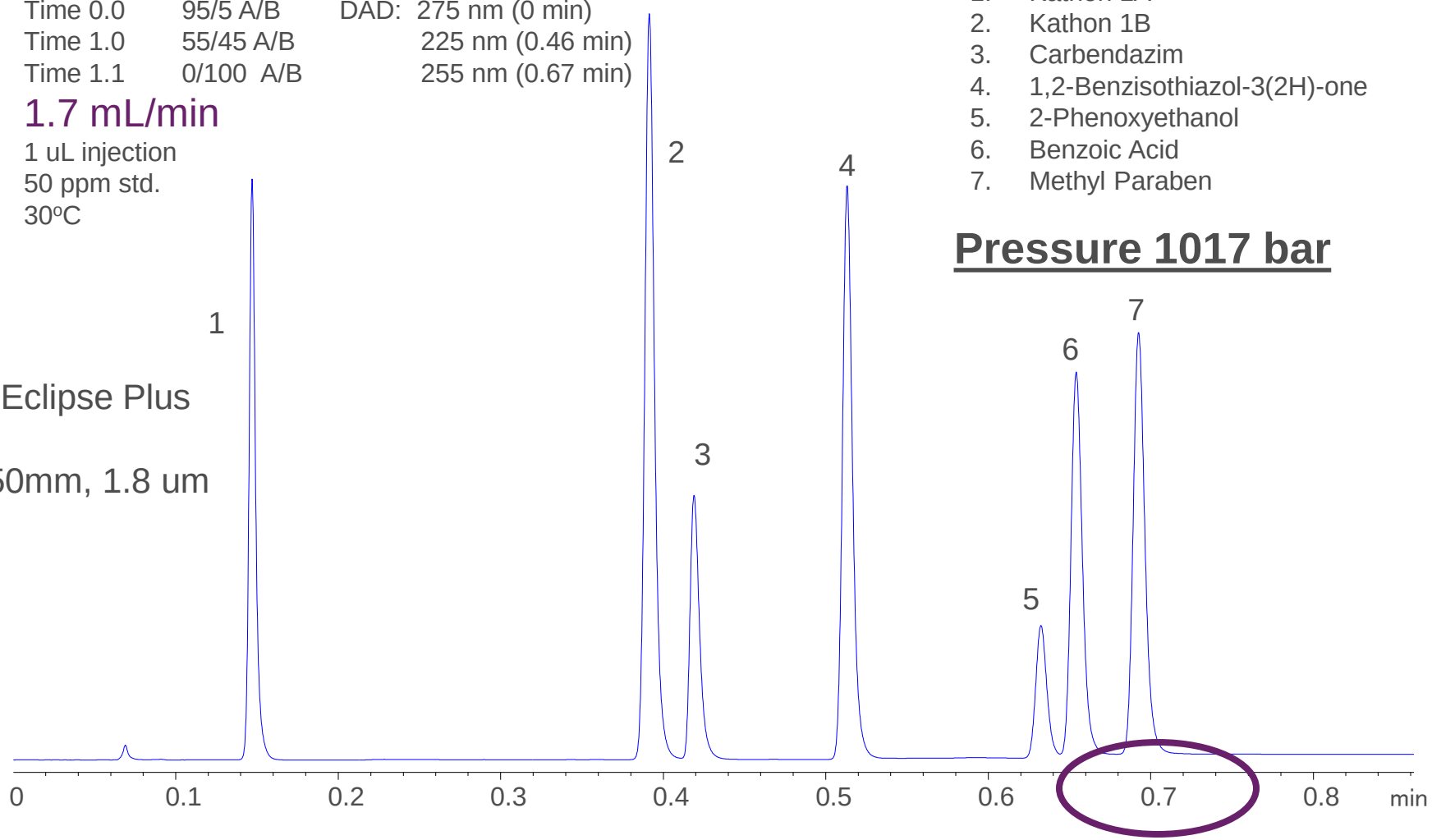
A: H_2O (0.05 v% TFA) B: ACN (0.04 v% TFA)
Time 0.0 95/5 A/B DAD: 275 nm (0 min)
Time 1.0 55/45 A/B 225 nm (0.46 min)
Time 1.1 0/100 A/B 255 nm (0.67 min)

1.7 mL/min
1 uL injection
50 ppm std.
30°C

1. Kathon 1A
2. Kathon 1B
3. Carbendazim
4. 1,2-Benzisothiazol-3(2H)-one
5. 2-Phenoxyethanol
6. Benzoic Acid
7. Methyl Paraben

Pressure 1017 bar

RRHD Eclipse Plus C18
2.1 x 50mm, 1.8 um



Poroshell 120

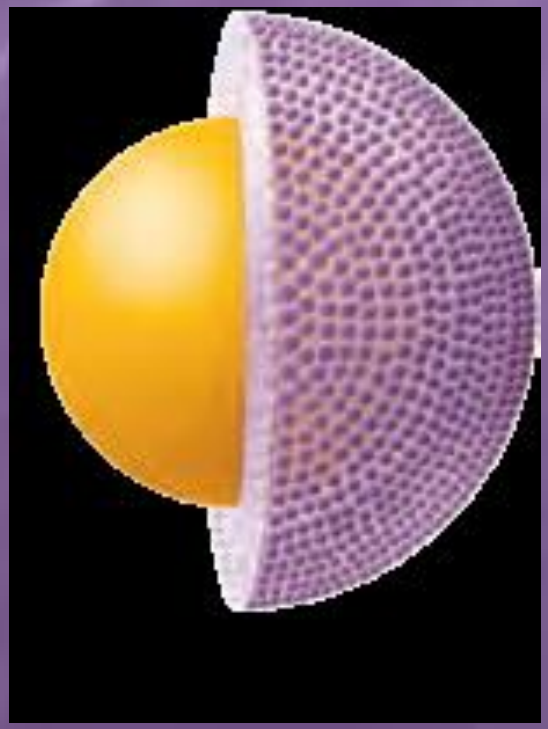
Superficially Solid Core

2.7 µm particle

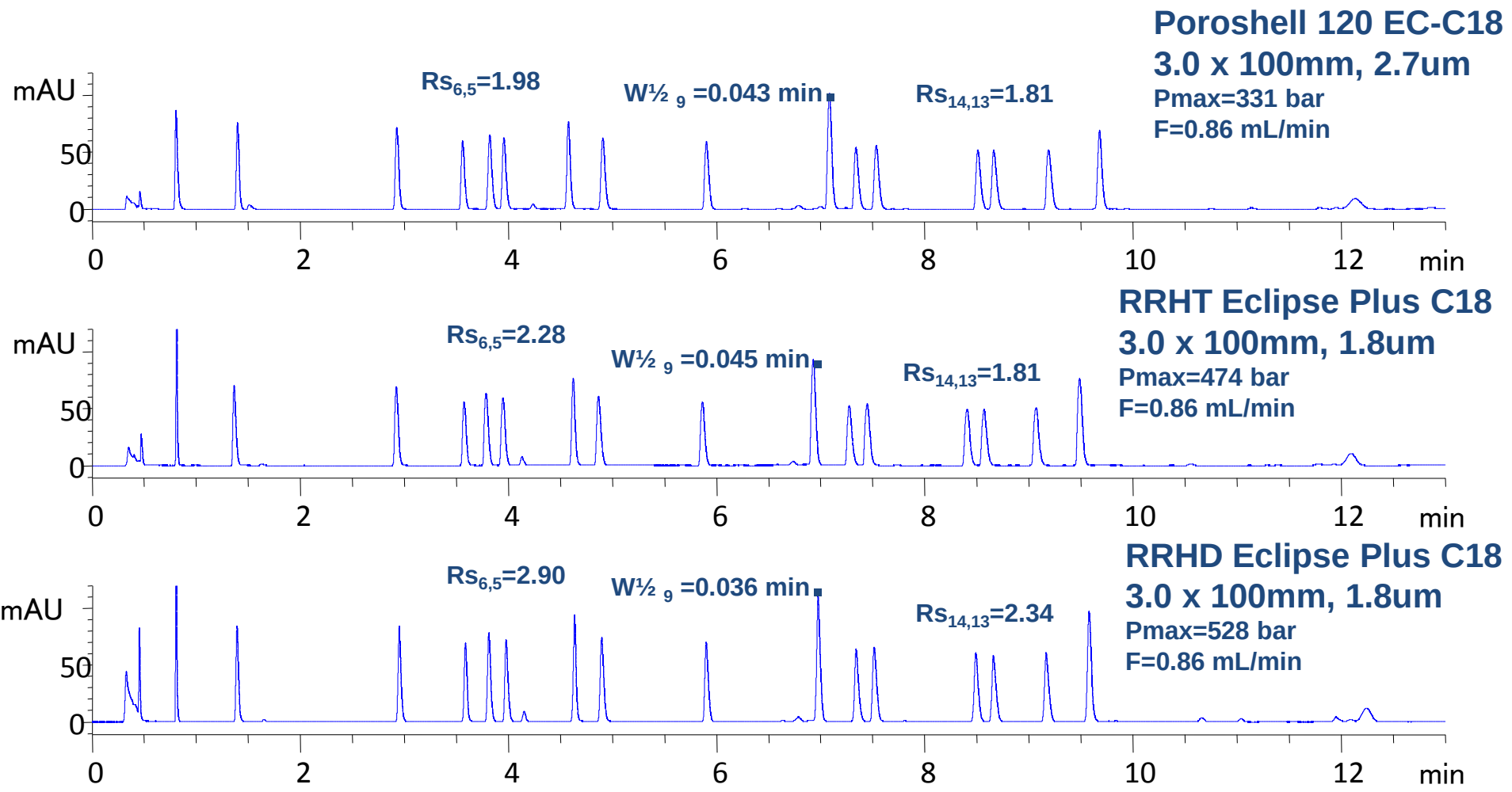
1.7 µm Solid Core

Highly efficient

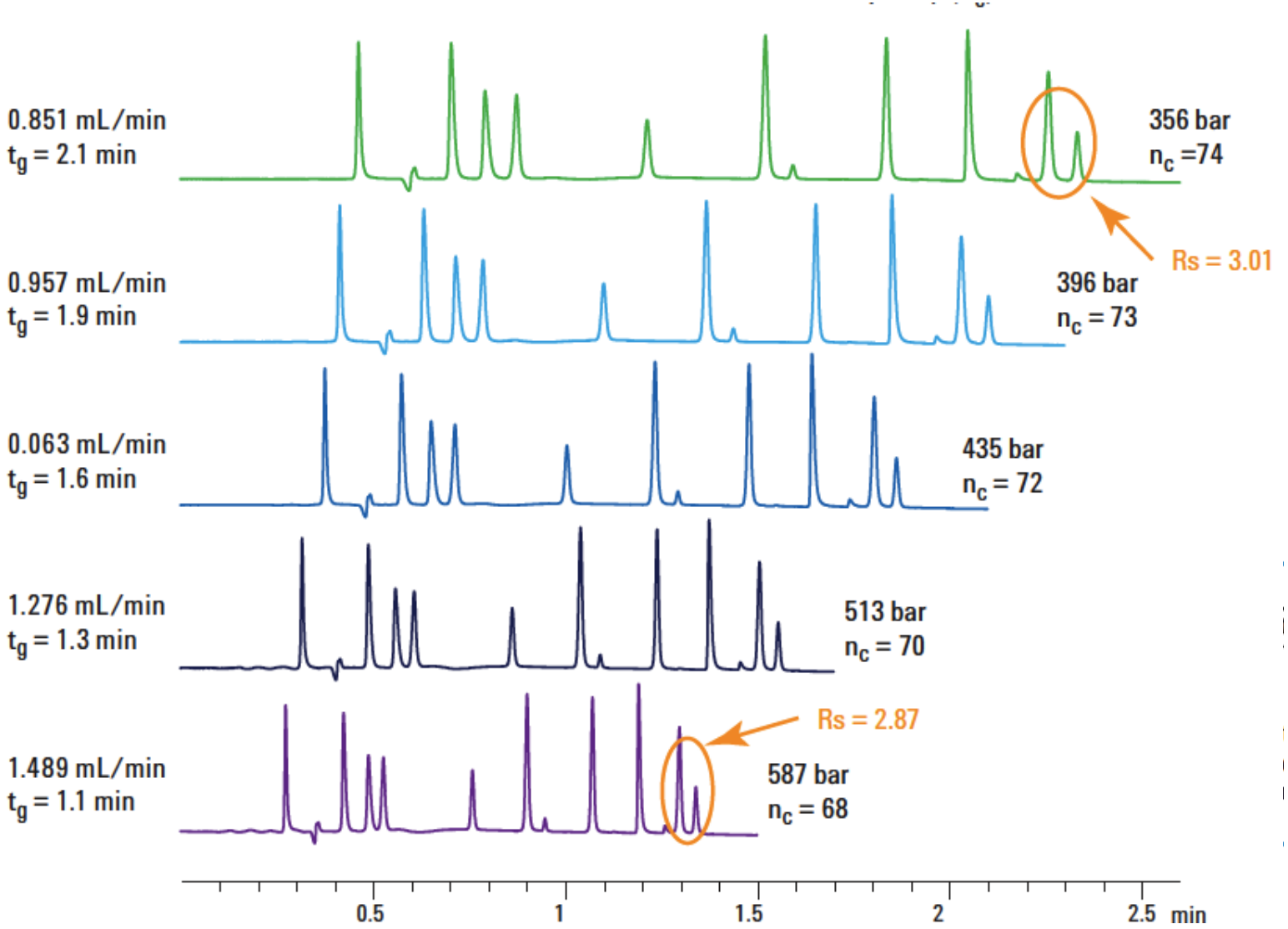
Lower Back Pressure Than Sub-2u Particles



RRHD and Poroshell Columns Provide Similar Performance 1290 Infinity



High Flow Rates Also Possible on Poroshell



In Summary: Optimize

- Tubing
- Connections
- Settings
- Sample Solvent
- Column Efficiency