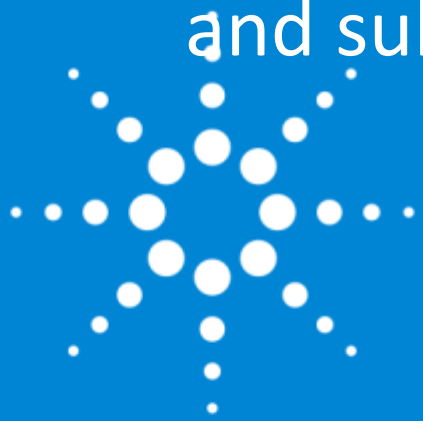
UHPLC and HPLC Method Optimization: Secrets You Can Put In Use Today

Agilent Technologies, Inc.

So … You Think You Know How to Run Solid Core-Superficially Porous and sub-2u LC Columns



Agilent Technologies, Inc.

WHAT IS HPLC?
WHAT IS UHPLC?

- High Pressure Liquid Chromatography?
- High Price Liquid Chromatography?
- I Have No Idea?
- **High PERFORMANCE Liquid Chromatography!**
- **ULTRA High PERFORMANCE Liquid Chromatography!!**

What Worked in the Past
May Not Be a Best Practice Today!

10 µm particles

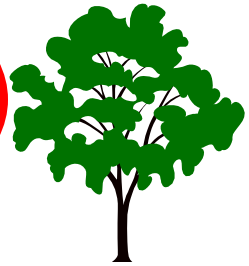
250 mm length

5 µm particles

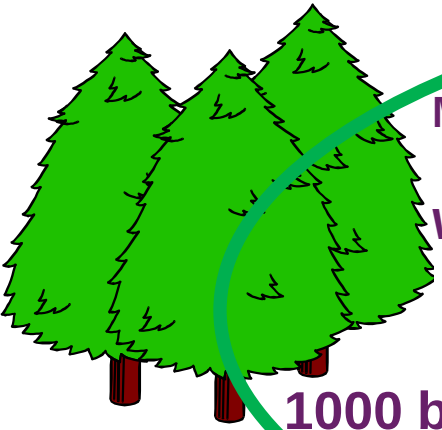
400 bar Instruments

150 mm length

3.5 µm particles



Non-Porous Particles



Micro-Bore

Narrow-Bore

Capillary Columns

Well-Plate Samplers

1.8 µm particles

1000 bar Instruments

Superficially Porous Particles – 300A, 5µm

15 mm length

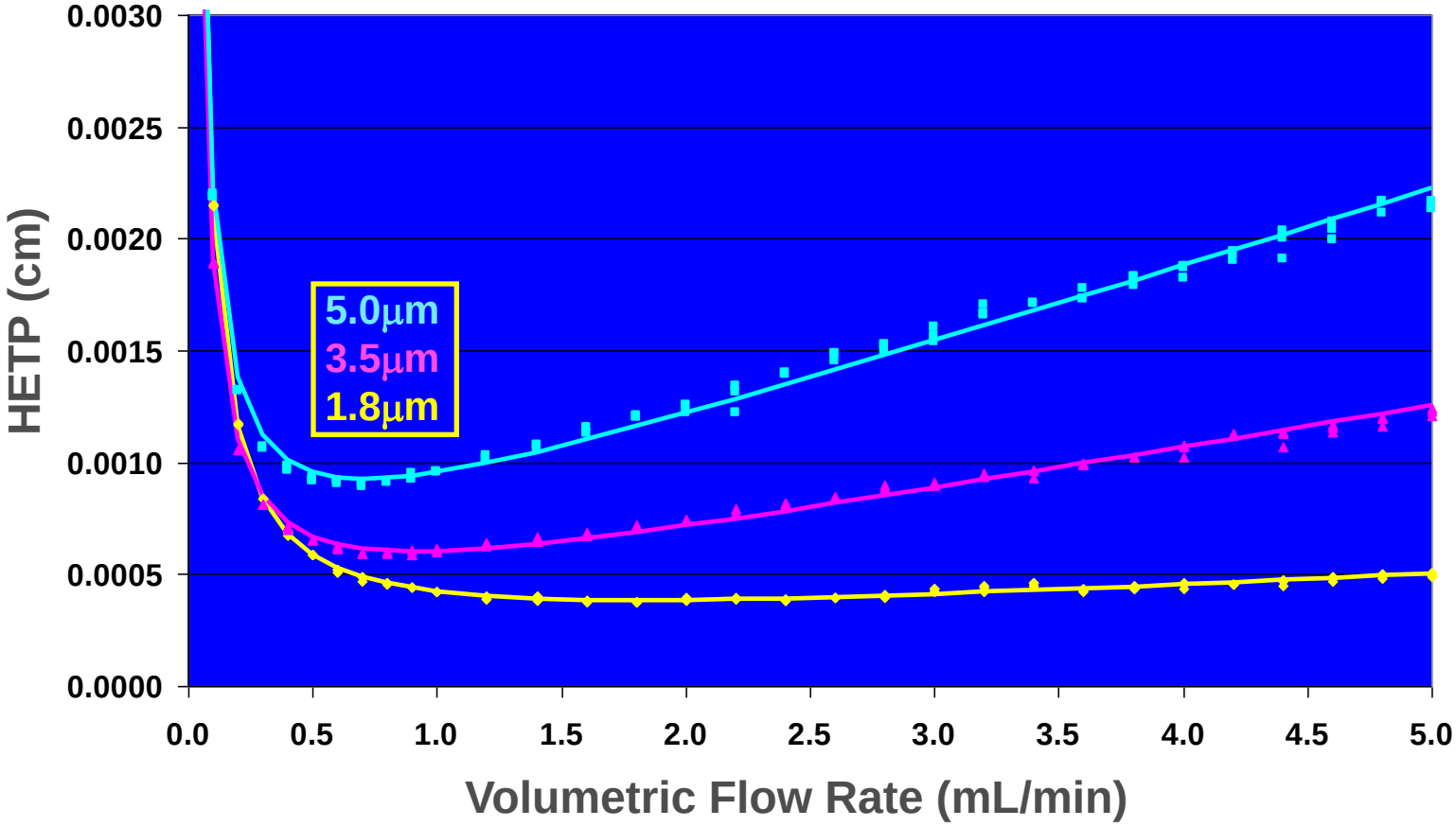
Overlapped Injections

Superficially Porous Particles – 120A, 2.7µm

1300 bar Instruments

LC Separation Improvement

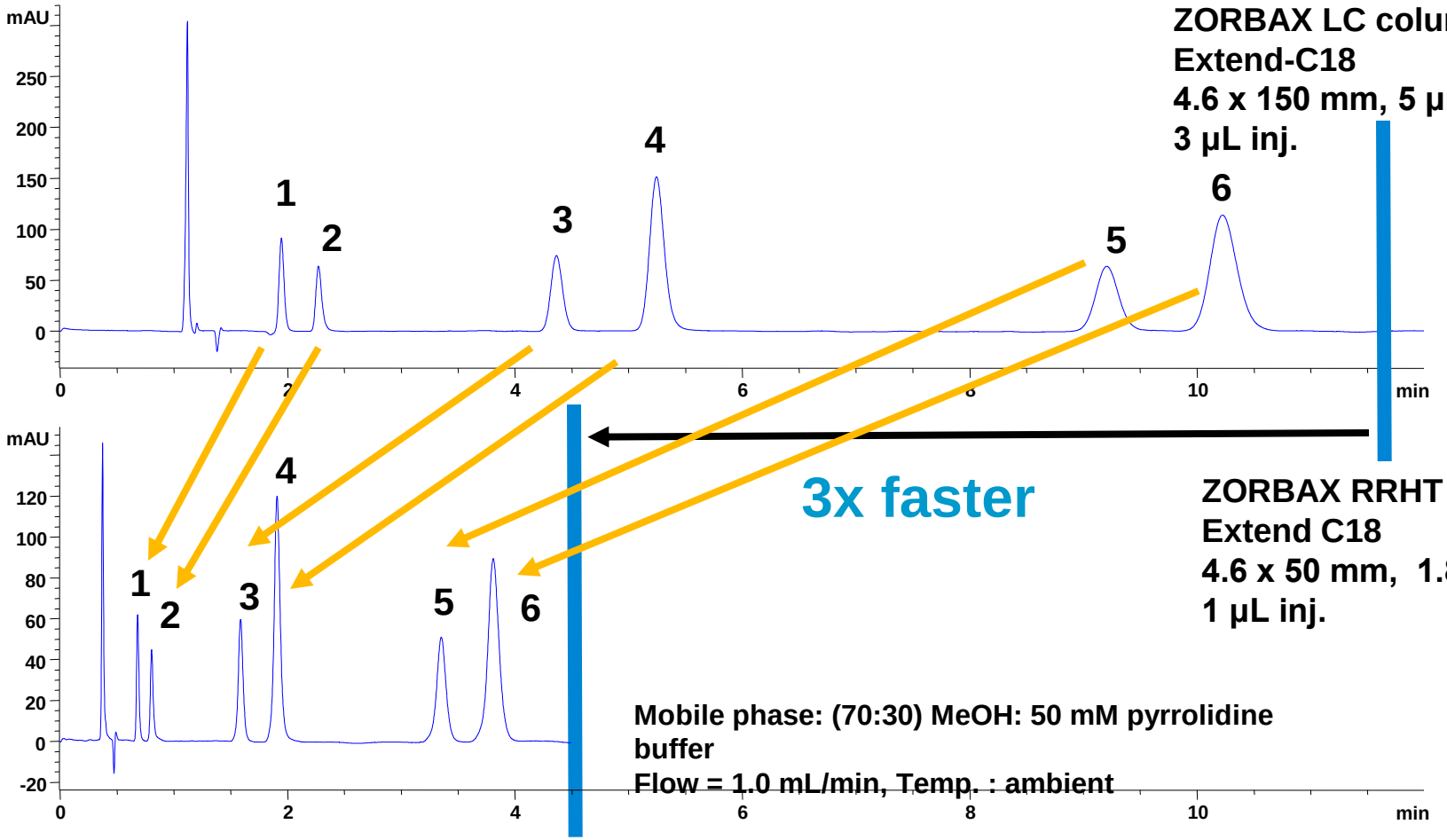
Why Develop Smaller Particles?
Better Performance!



Smaller particle sizes yield flatter curves, minima shift to higher flow rates

Page 6

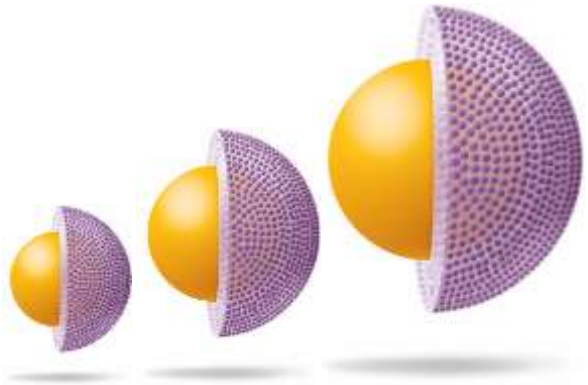
One Benefit of UHPLC
Faster Analysis

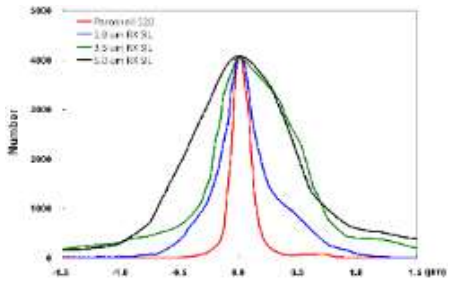


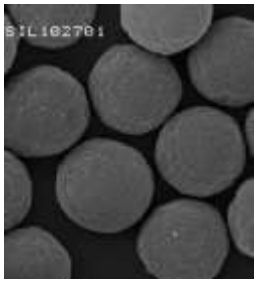
Higher efficiencies using SPP

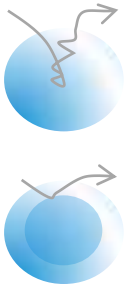
Additional efficiency can be generated through the use of superficially porous particles (SPP) rather than a totally porous particle (TPP)

SPP particle	For	Maximum pressure	Typical pressure	Efficiency
1.9 µm	Highest UHPLC performance	1300 bar	Similar to sub-2 µm totally porous	~120% of sub-2 µm totally porous
2.7 µm	UHPLC performance at lower pressures	600 bar	50% of sub-2 µm totally porous	~90% of sub-2 µm totally porous
4 µm	Improved HPLC performance	600 bar	Typically < 200 bar	~200% of 5 µm totally porous





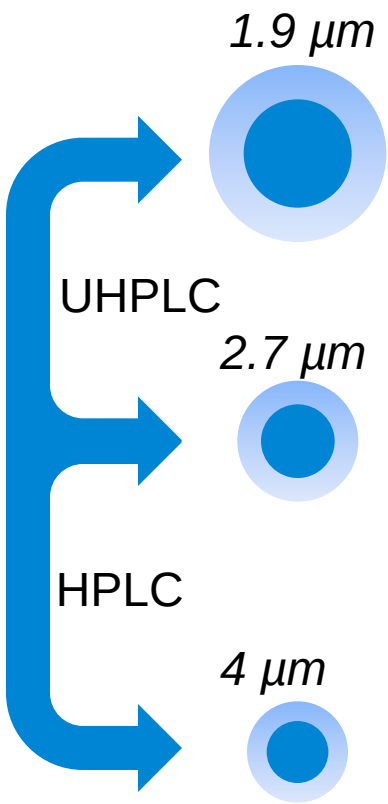




An SPP column for everyone!

Technique / product

Performance



Highest UHPLC performance
Pressure rating: 1300 bar
Typical pressure: Similar to sub-2 µm totally porous
Efficiency: ~120% of sub-2 µm totally porous

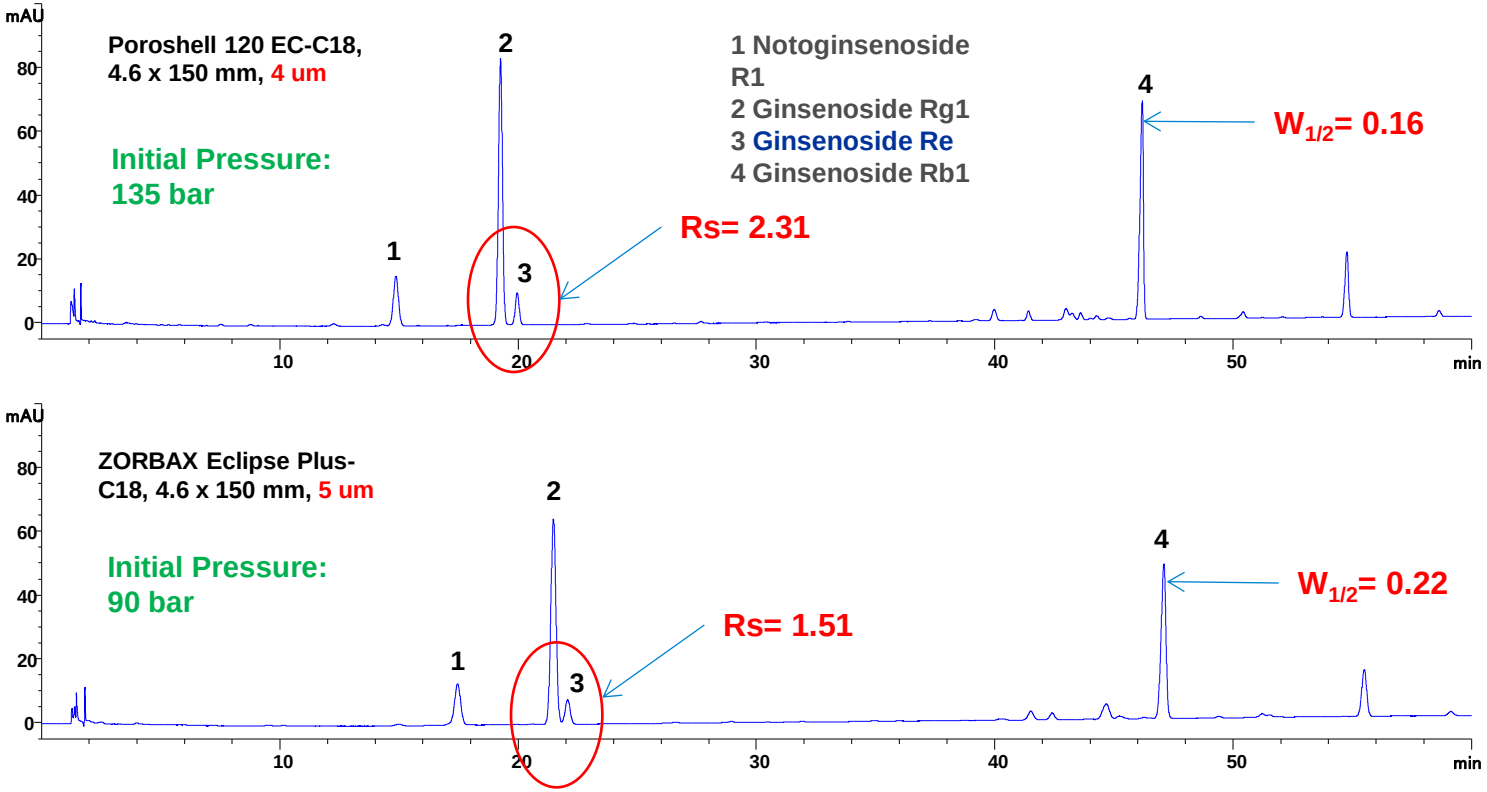
UHPLC performance at lower pressure
Pressure rating: 600 bar
Typical pressure: 50% of sub-2 µm totally porous
Efficiency: ~90% of sub-2 µm totally porous

Improved HPLC performance
Pressure rating: 600 bar
Typical pressure: Often < 200 bar
Efficiency: ~200% of 5 µm totally porous

Higher Performance for Any LC

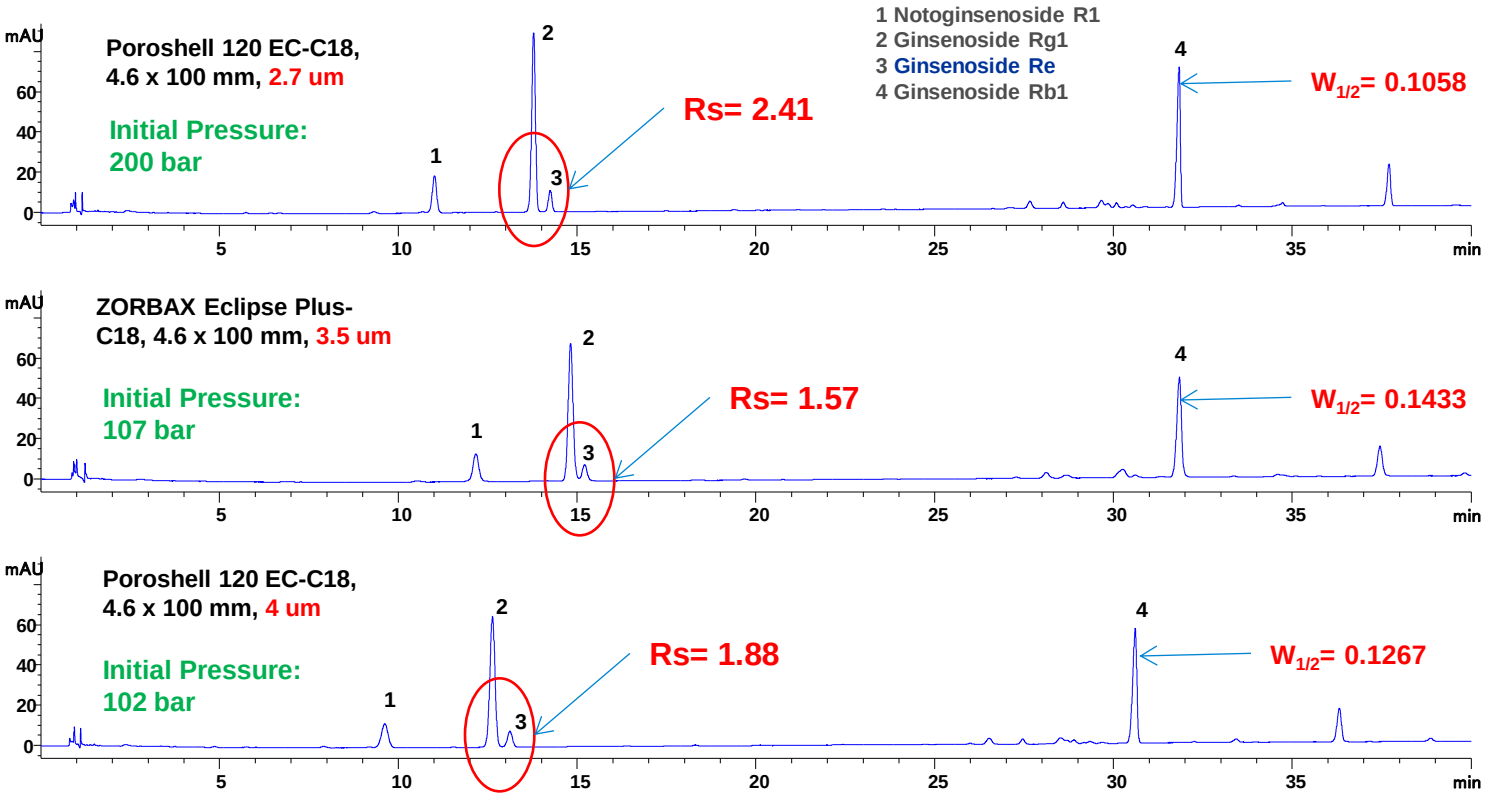
Instruments		We recommend…
	UHPLC only **Maximum pressure:** High **(> 600 to 1000+ bar)** **Dispersion volume:** Very low	InfinityLab Poroshell 120 1.9 µm InfinityLab Poroshell 120 2.7 µm
	HPLC and UHPLC **Maximum pressure:** Low to high **(400 to 1000+ bar)** **Dispersion volume:** Medium to very low	InfinityLab Poroshell 120 2.7 µm InfinityLab Poroshell 120 4 µm
	HPLC only **Maximum pressure:** Low to mid **(400 to 600 bar)** **Dispersion volume:** High to low	InfinityLab Poroshell 120 4 µm InfinityLab Poroshell 120 2.7 µm

Improved Performance by Using Poroshell 120 4µm
and Totally Porous 5um columns



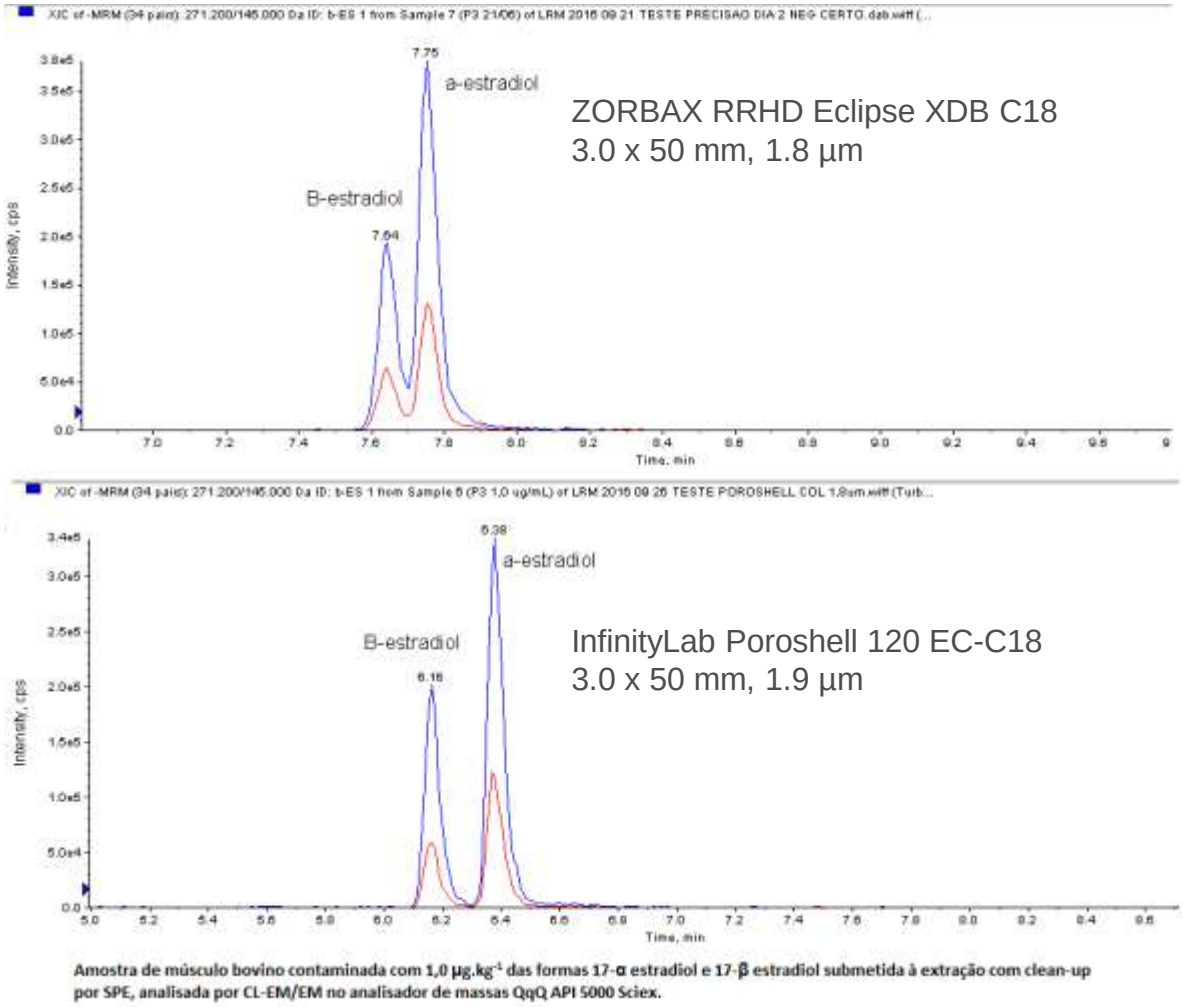
- 50% improvement in resolution over the 5um totally porous column
- Backpressure still below 200 bar

Comparison of 3.5u and Poroshell 2.7u and 4.0u



- Poroshell 120 4µm offers higher performance than totally porous 3.5µm columns with 600 bar stability
- Poroshell 120 2.7µm offers the highest performance

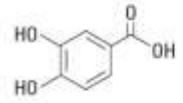
Higher Efficiencies: TPP vs. SPP
Seed column feedback



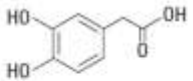
Amostra de músculo bovino contaminada com 1,0 µg.kg^{-1} das formas 17-α estradiol e 17-β estradiol submetida à extração com clean-up por SPE, analisada por CL-EM/EM no analisador de massas QqQ API 5000 Sciex.

"…the efficiency of the new Poroshell column was superior to Zorbax once achieved a good resolution in the separation of isomers of estradiol, essential for the validation of a method for monitoring such analytes. Thus, we will start to use the new Poroshell column in the ongoing validations in anabolic"
- Residue Laboratory Veterinary Medication LANAGRO / MG

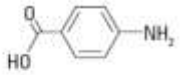
Fast LC
Aromatic acids



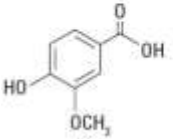
Protocatechuic acid



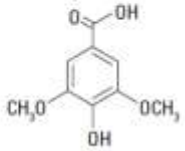
3,4-Dihydroxyphenylacetic acid (DOPAC)



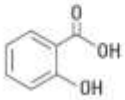
4-aminobenzoic acid (PABA)



Vanillic acid



Syringic acid



Salicylic acid

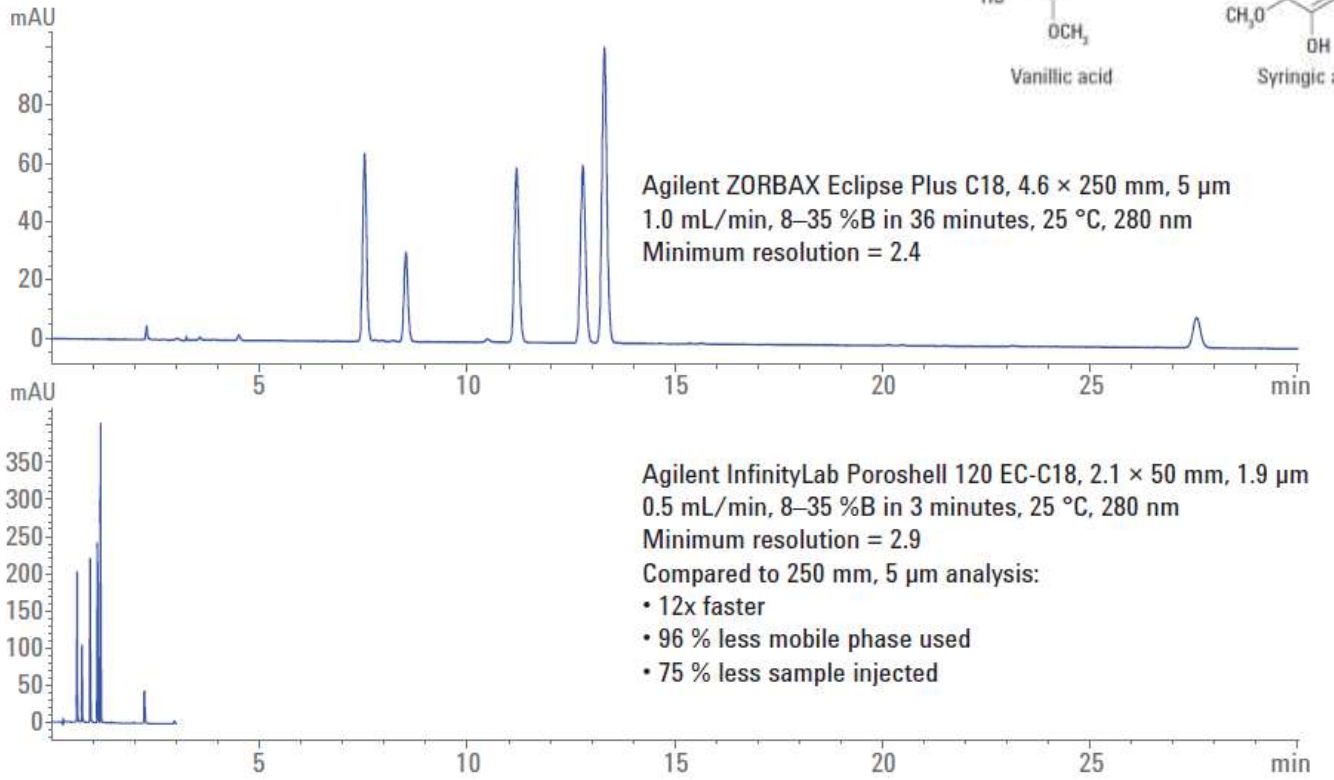


Figure 2. A 250 mm, 5 µm Agilent ZORBAX analysis of aromatic acids is improved by transferring to a high-performance 50 mm, 1.9 µm Agilent InfinityLab Poroshell column; minimum resolution is improved, while saving significant time, sample, solvent, and money.

5991-7518EN

Ultra-fast LC
Aromatic acids

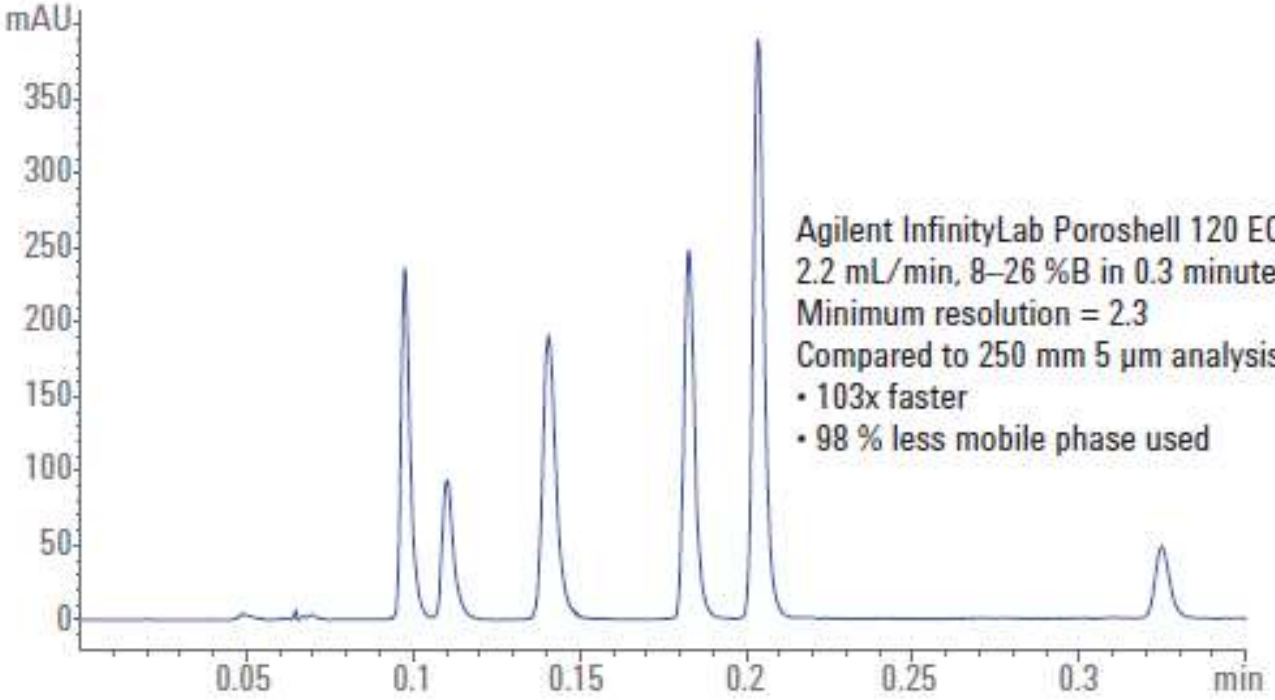


Figure 4. Additional time, solvent, and money can be saved by operating the highly performing 50 mm, 1.9 µm Agilent InfinityLab Poroshell column near its pressure limit without compromising method performance.

5991-7518EN

High resolution LC

Tanshinones in Danshen (Salvia miltiorrhiza)

$$n_c = (t_{R,n} - t_{R,1})/w$$

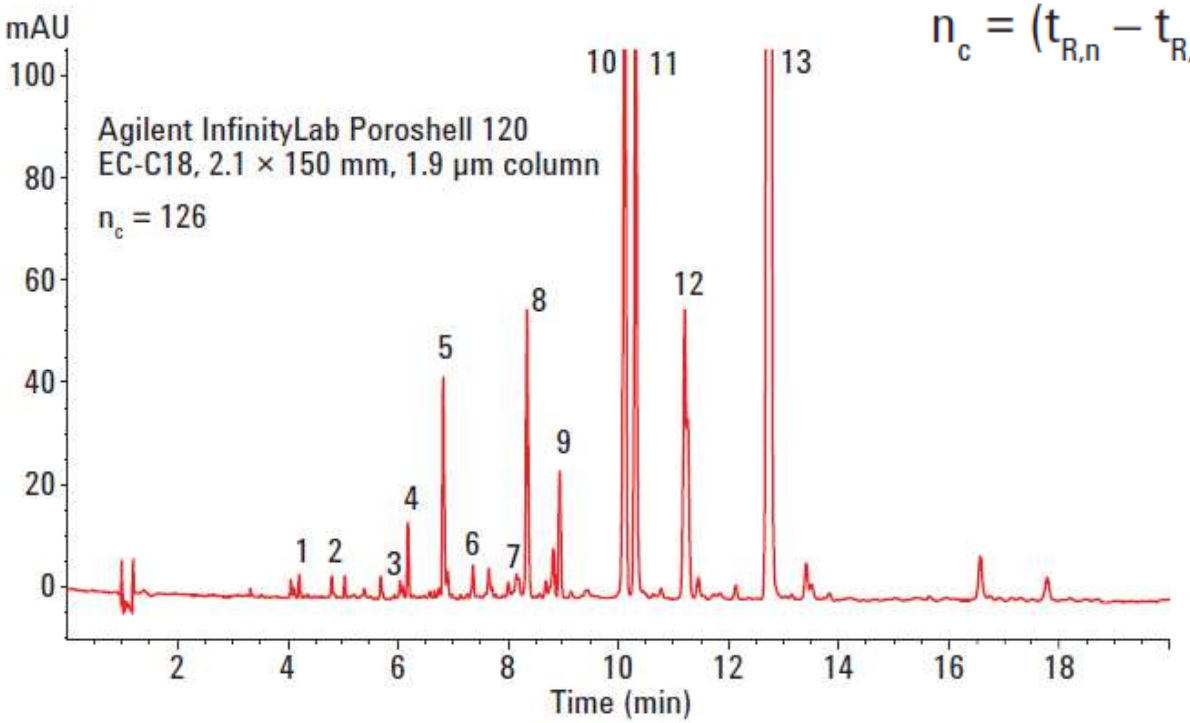


Figure 3. Tanshinones fingerprint profiling on an Agilent InfinityLab Poroshell 120 EC-C18, 2.1 × 150 mm, 1.9 µm column. Peaks 10 (cryptotanshinone) and 13 (tanshinone IIA) were identified using reference standards.

High efficiency column increases the number of peaks that can be resolved (n_c) and Improves the accuracy of fingerprinting

5991-7559EN

The advantage of longer columns

Total phenolic acids in Danshen (Salvia miltiorrhiza)

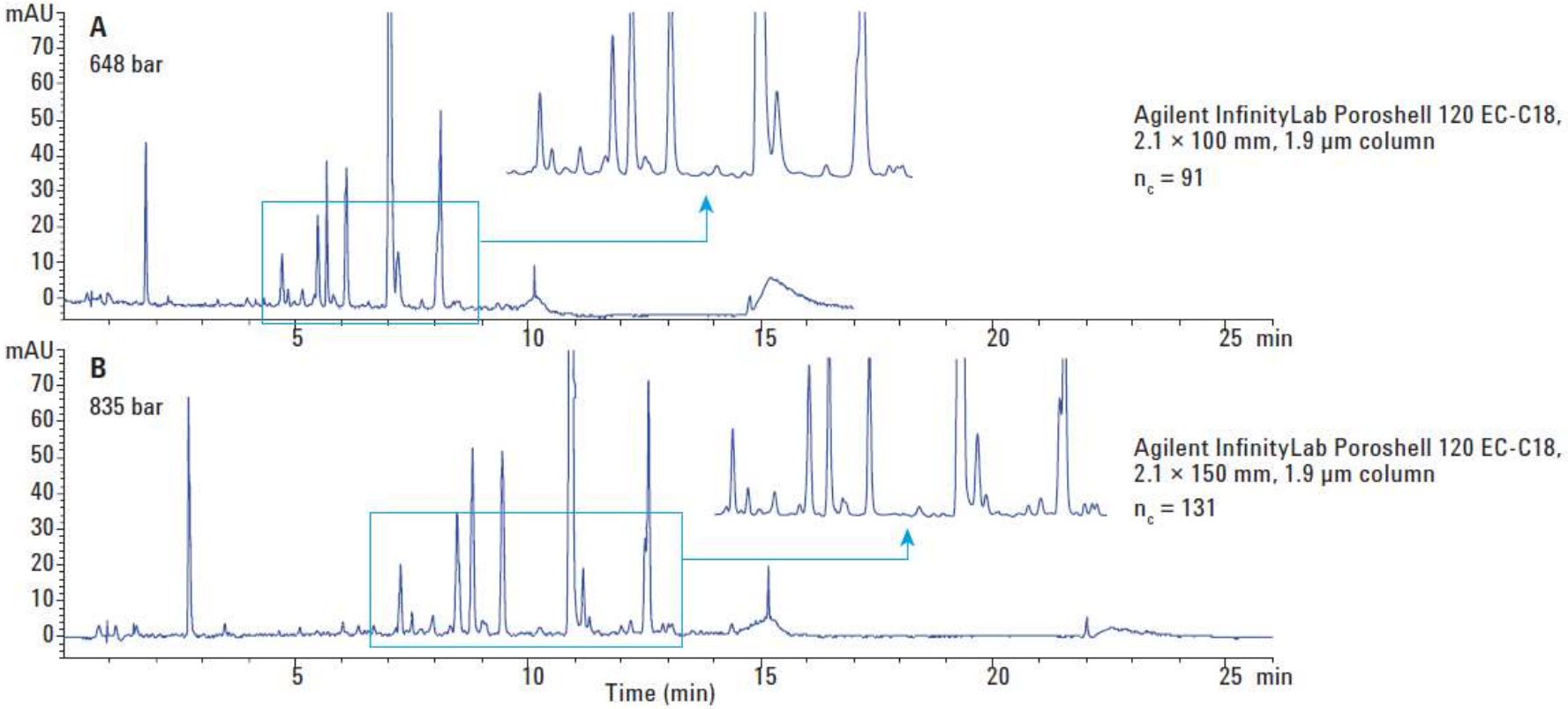


Figure 2. Comparison of the Salvia Total Phenolic Acids fingerprint profiling on Agilent InfinityLab Poroshell 120 EC-C18, 2.1 × 150 mm, 1.9 µm and 2.1 × 100 mm columns.

5991-7559EN

The advantage of a scalable family of particles
Aromatic acids

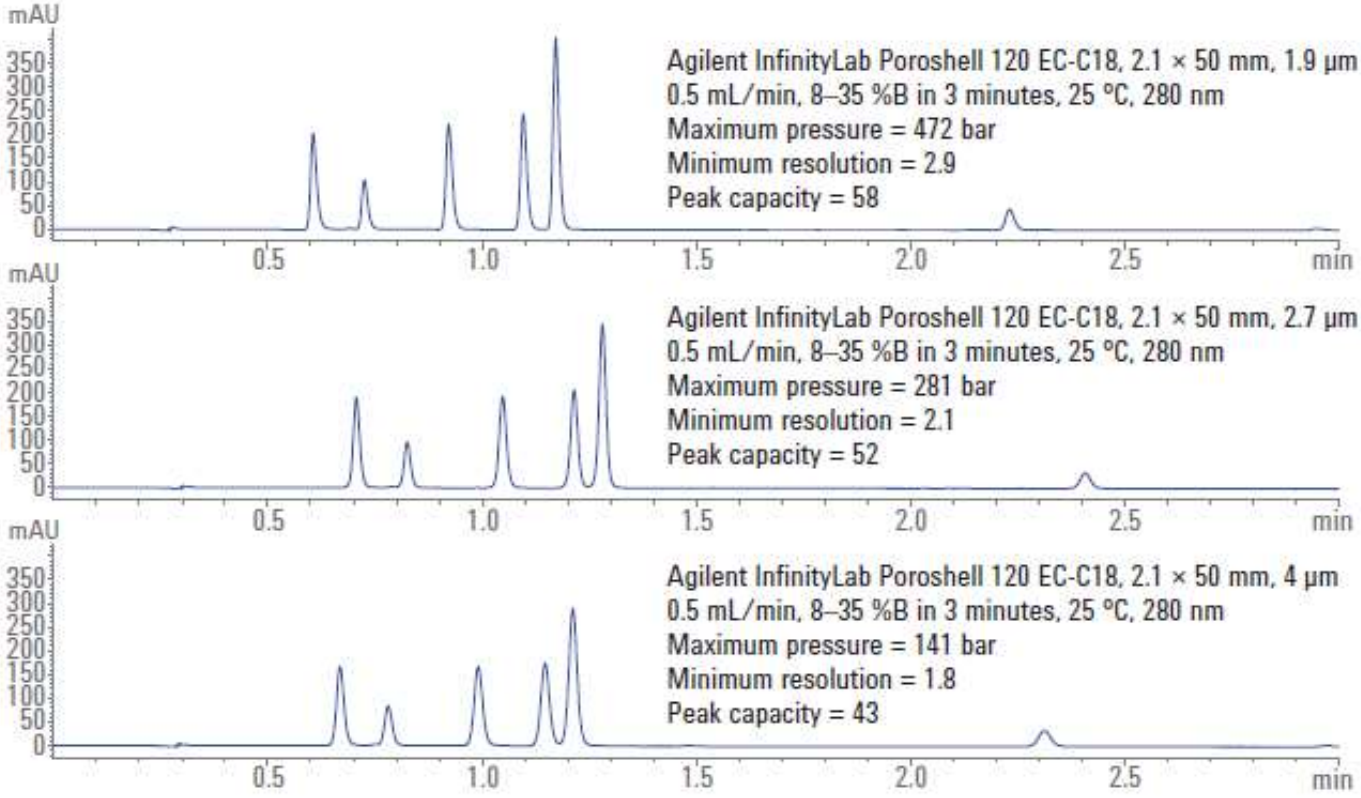
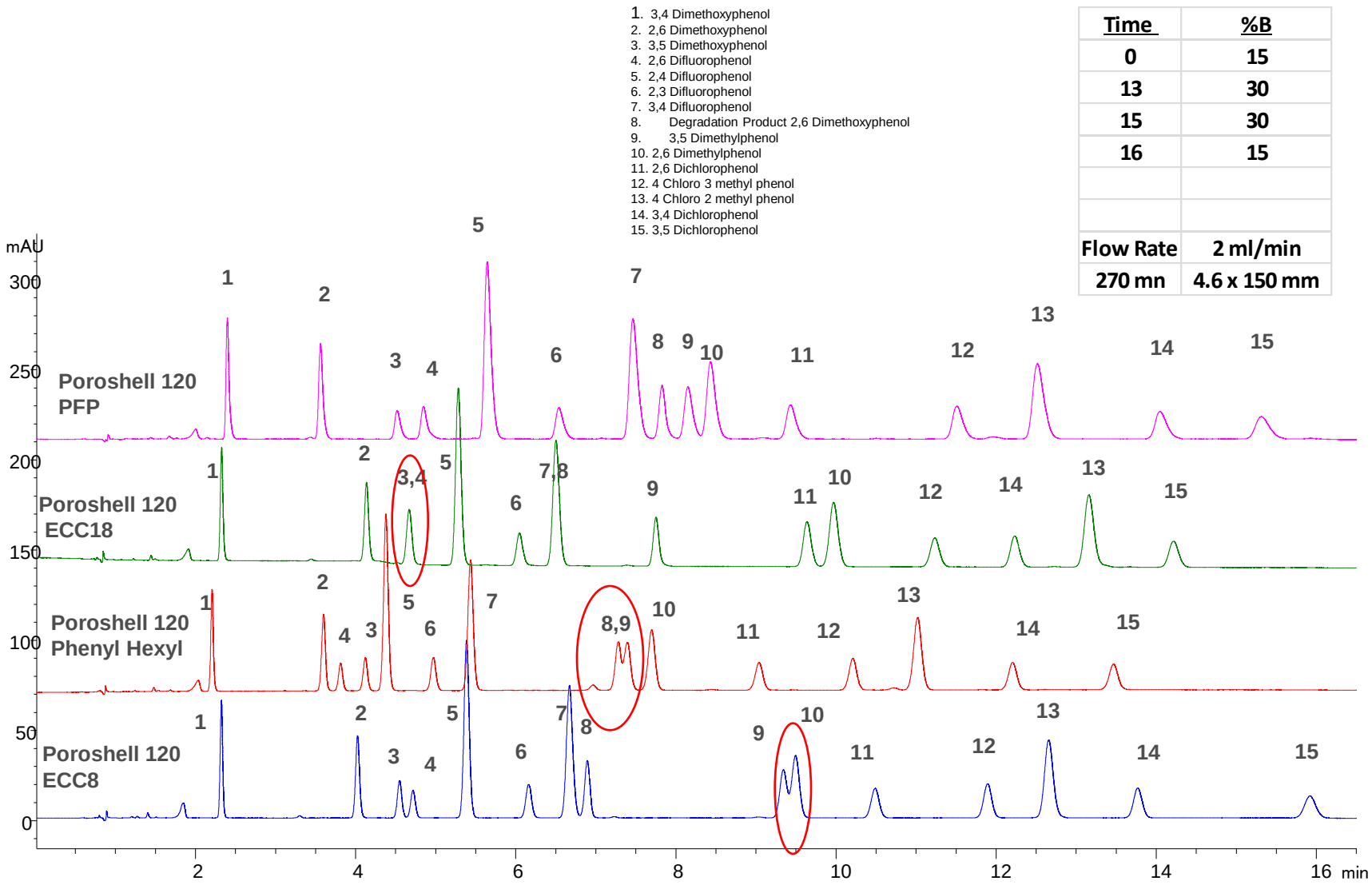


Figure 3. Similar selectivity among Agilent InfinityLab Poroshell particle sizes allows analysts to choose their column configuration based on instrument pressure limits or method performance requirements without needing to do additional method development.

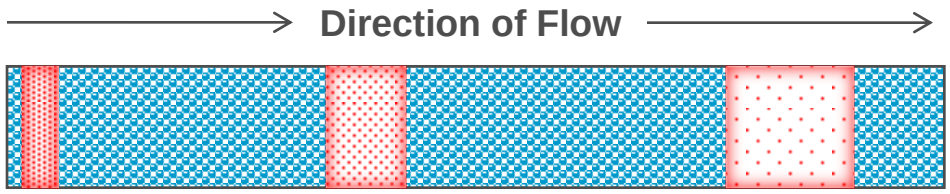
5991-7518EN

No Substitute for Chemistry Improved Resolution!

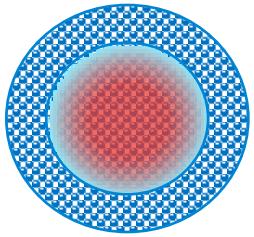


SmallerParticles in Smaller Volume Columns Reduces Dispersion Within the LC Column

a) Longitudinal Diffusion (dispersion)



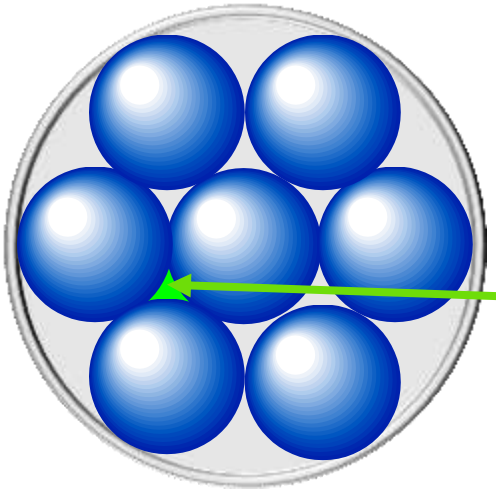
b) Radial Diffusion (dispersion)



Effect of Water Contaminants on LC

PARTICLES	BACTERIA	ORGANICS	IONS
- **Damage pump** and **injector** - **Plug column** and **frits** - **Increase** back **pressure**	- **Plug column** and **frits** - **Increase** back **pressure** - **Release ions** and **organics** *(see effect of ions and organics)	- Lead to **ghost peaks, baseline drift, poor repeatability** - **Interfere** with **analytes** - **Reduce column lifetime** - **Increase MS background** - **Suppress signal**	- Form **adducts** - **Suppress** signal - **Complicate mass spectra**

Plugged Packing



Particle Size	Area (um^2)	Diameter (um)	Frit Porosity (um)
5.0	2.7	**0.9**	2.0
3.5	1.3	**0.6**	2.0
2.7	0.7	**0.5**	2.0
1.8	0.4	**0.4**	0.2
1.7	0.3	**0.3**	0.2

Beware of buffered mobile phases

Buffers usually contain insoluble material – filter

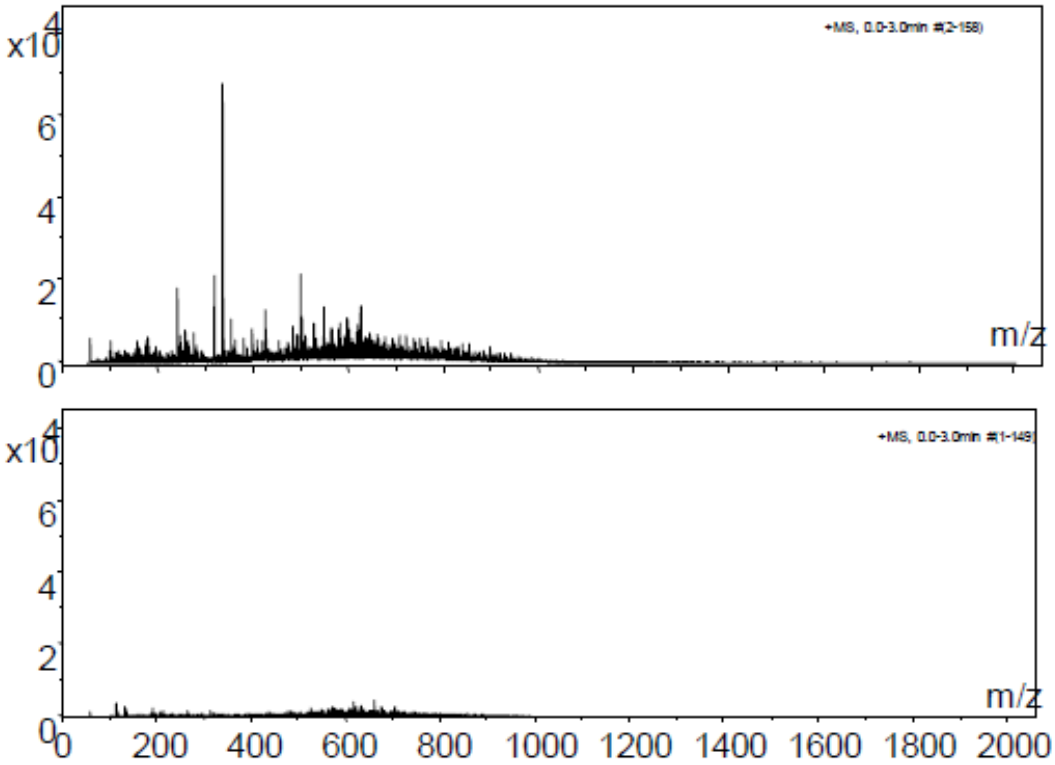
Buffer solubility decreases with increasing % organic* - avoid 100%B with buffer salts

*Schellinger,A.P. and Carr,P.W., LC-GC North America, 22, 6, 544-548 (2004)

Effect of Flushing on MS background

Milli-Q® water on Monday
(after weekend)

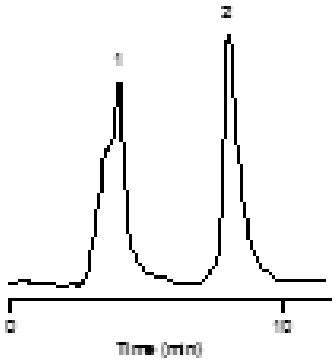
Fresh Milli-Q® water after discarding several liters



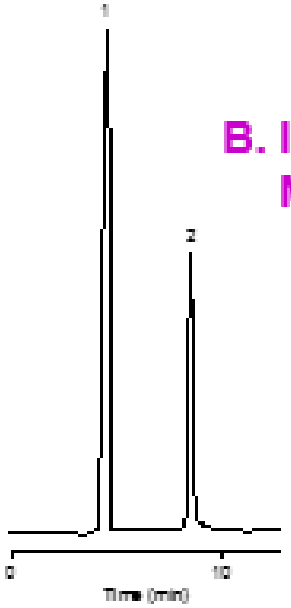
Split Peaks from Injection Solvent Effects

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

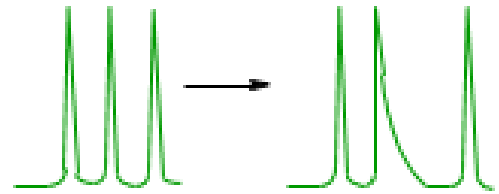


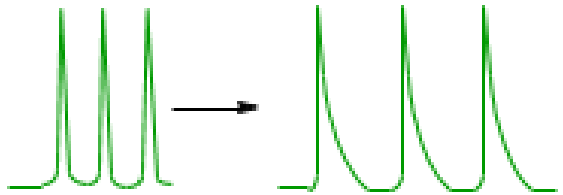
Tip: Injecting in a solvent stronger than the mobile phase can cause peak shape problems such as peak splitting or broadening

Trick: Keep Organic Concentration in Sample Solvent ≤ Mobile Phase

Peak Tailing

Symmetry > 1.2





Causes

Some Peaks Tail:

- Secondary - Retention Effects.
- Residual Silanol Interactions.
- Small Peak Eluting on Tail of Larger Peak.

Chemistry Problem

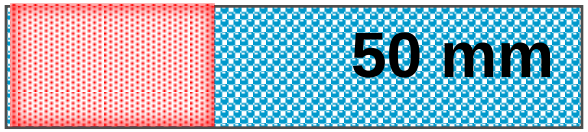
All Peaks Tail:

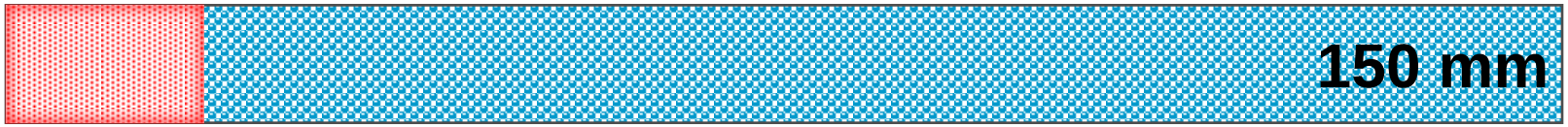
- Extra-Column Effects.
- Build up of Contamination on Column Inlet.
- Heavy Metals.
- Bad Column.

System Problem

Effect of Strong Sample Solvent

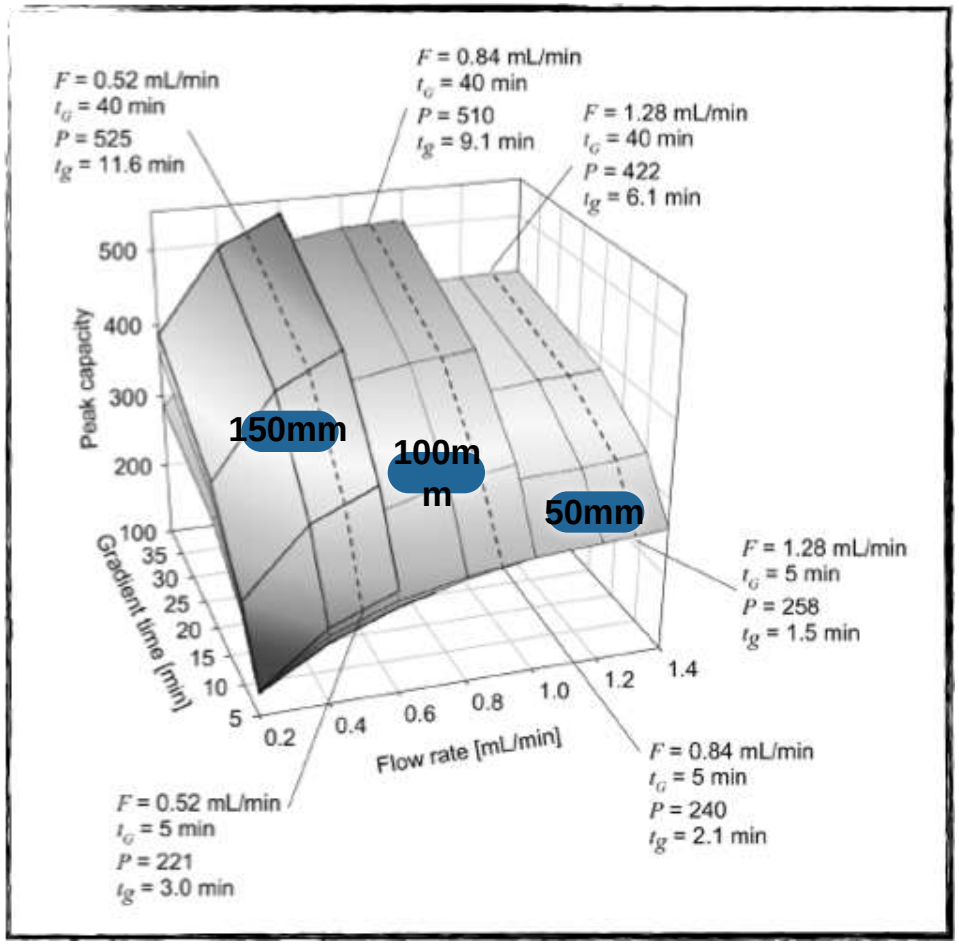
- **2.1mm I.D.**
- **35uL injection**
- **Sample Solvent Strength (50%) > Mobile Phase**





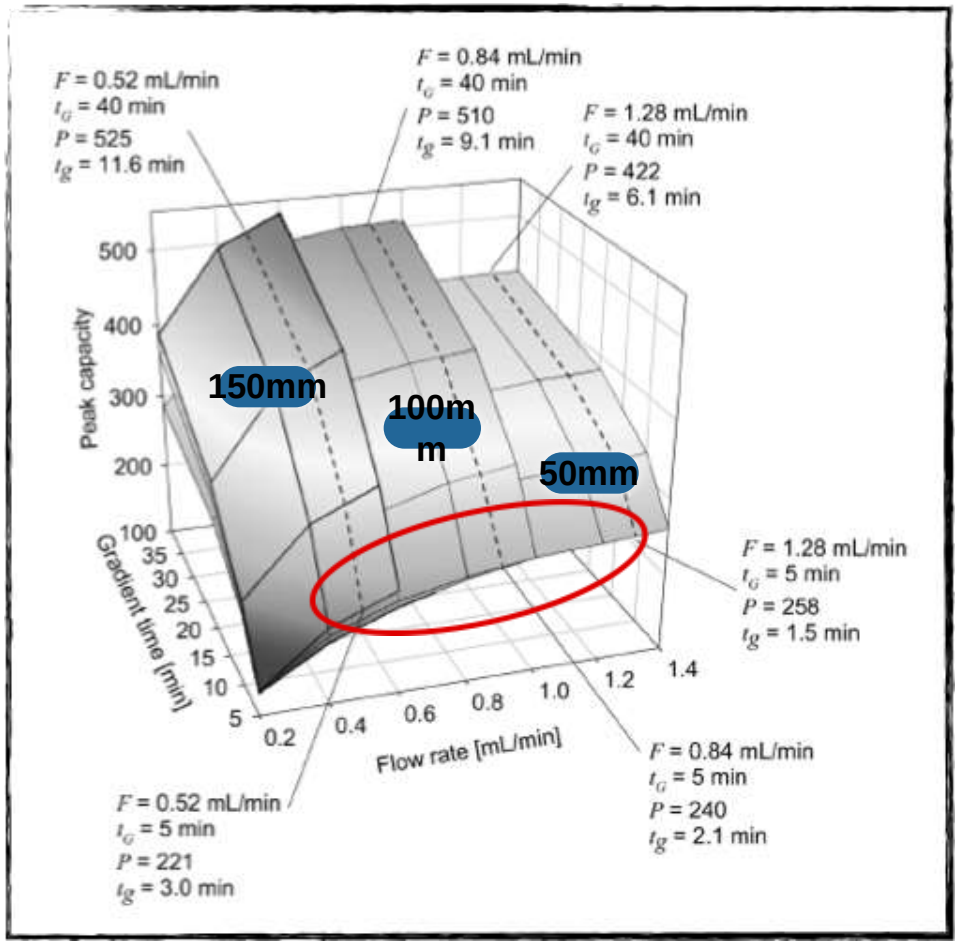
Direction of Flow

What Length Column for New Methods?



- **Flow rate vs. Gradient time vs. Peak capacity**
- **For small molecules(MW < ~1000)**
- **Different Column Lengths**
- **Broken lines are isobar (800 bar)**

Shorter Columns with Fast Gradients Yield Higher Peak Capacity

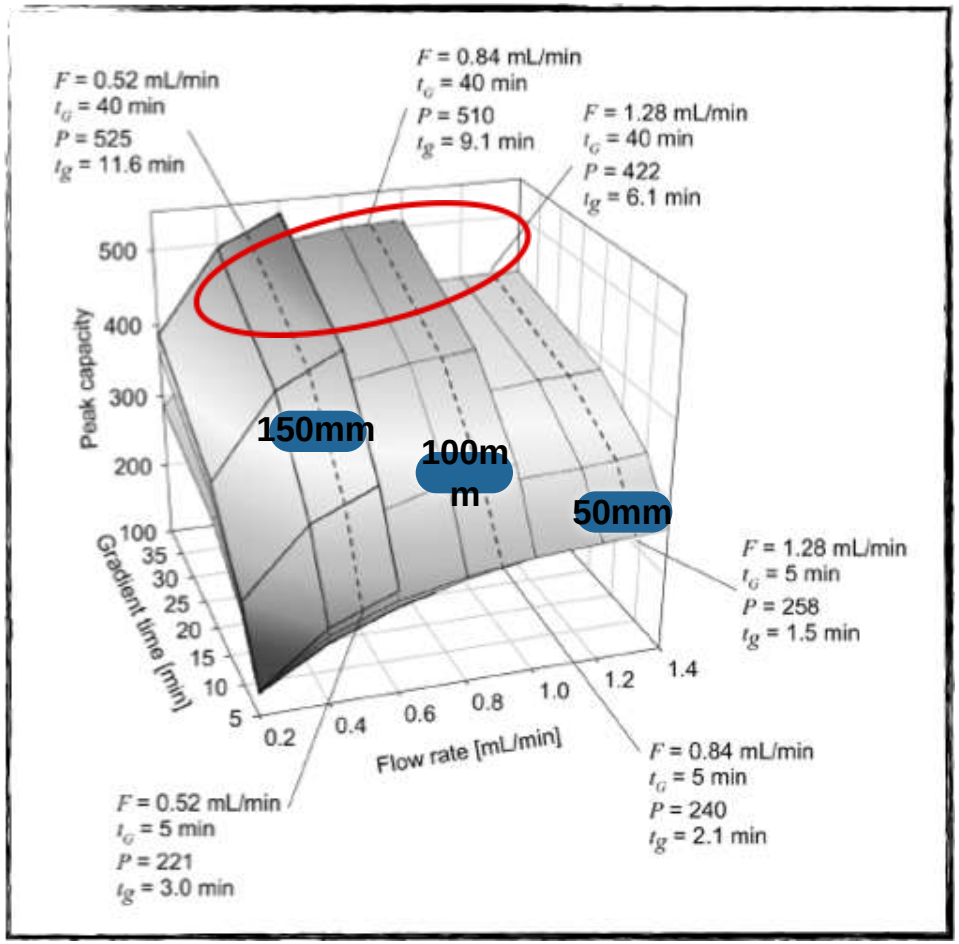


Shorter Gradient (5 min)

Peak Capacity:

- **258 for 50 mm**
- **240 for 100 mm**
- **221 for 150 mm**

Longer Columns with Long Gradient Times Yield Greater Peak Capacity



Long Gradient (40 min)

Peak Capacity:

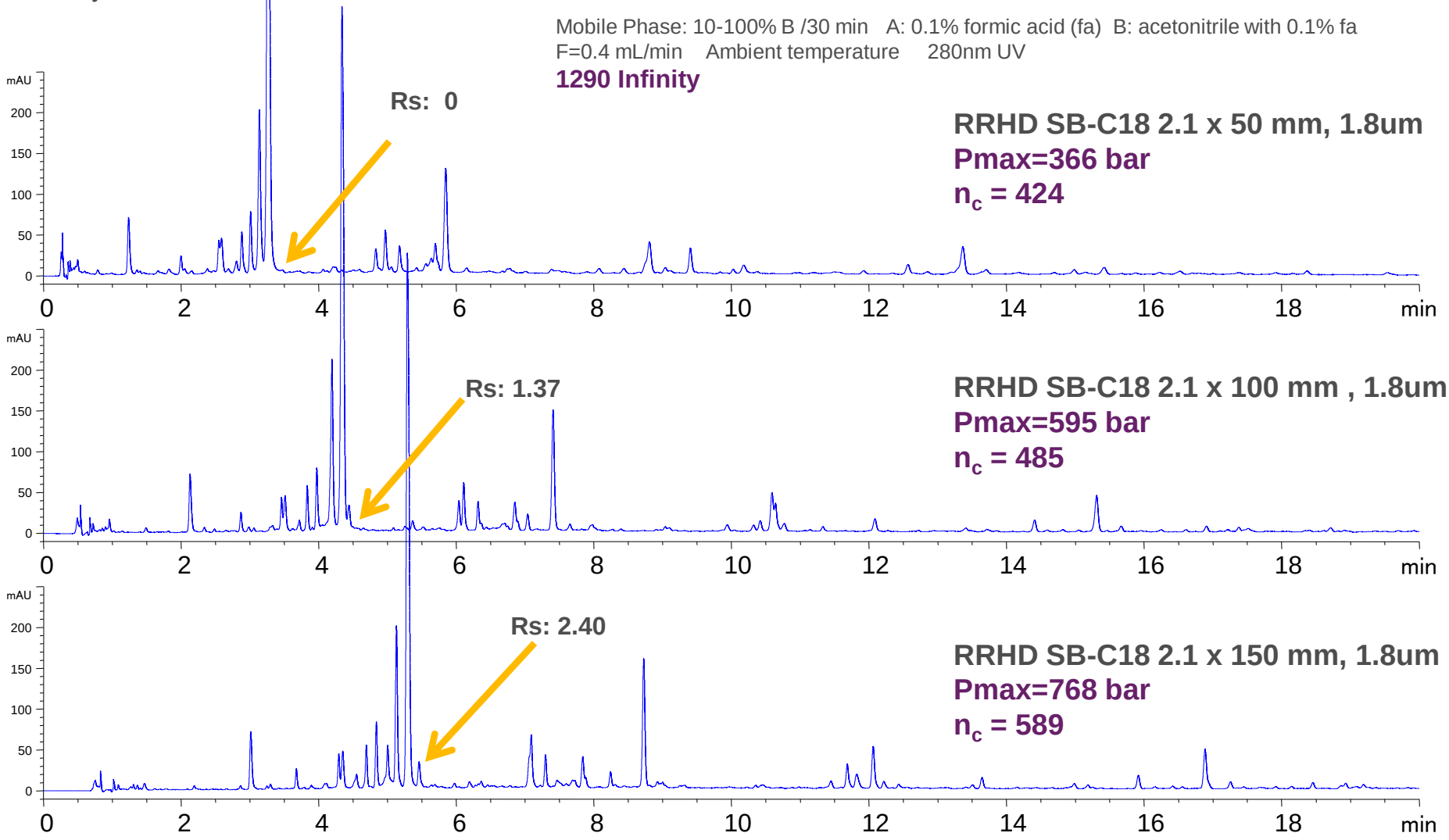
- **422 for 50 mm**
- **510 for 100 mm**
- **525 for 150 mm**

Separation of Licorice Root on RRHD Columns –

3X Column Length Produces Moderate increase in Retention with Major Improvement in Resolution – Why?

Mobile Phase: 10-100% B /30 min A: 0.1% formic acid (fa) B: acetonitrile with 0.1% fa
F=0.4 mL/min Ambient temperature 280nm UV

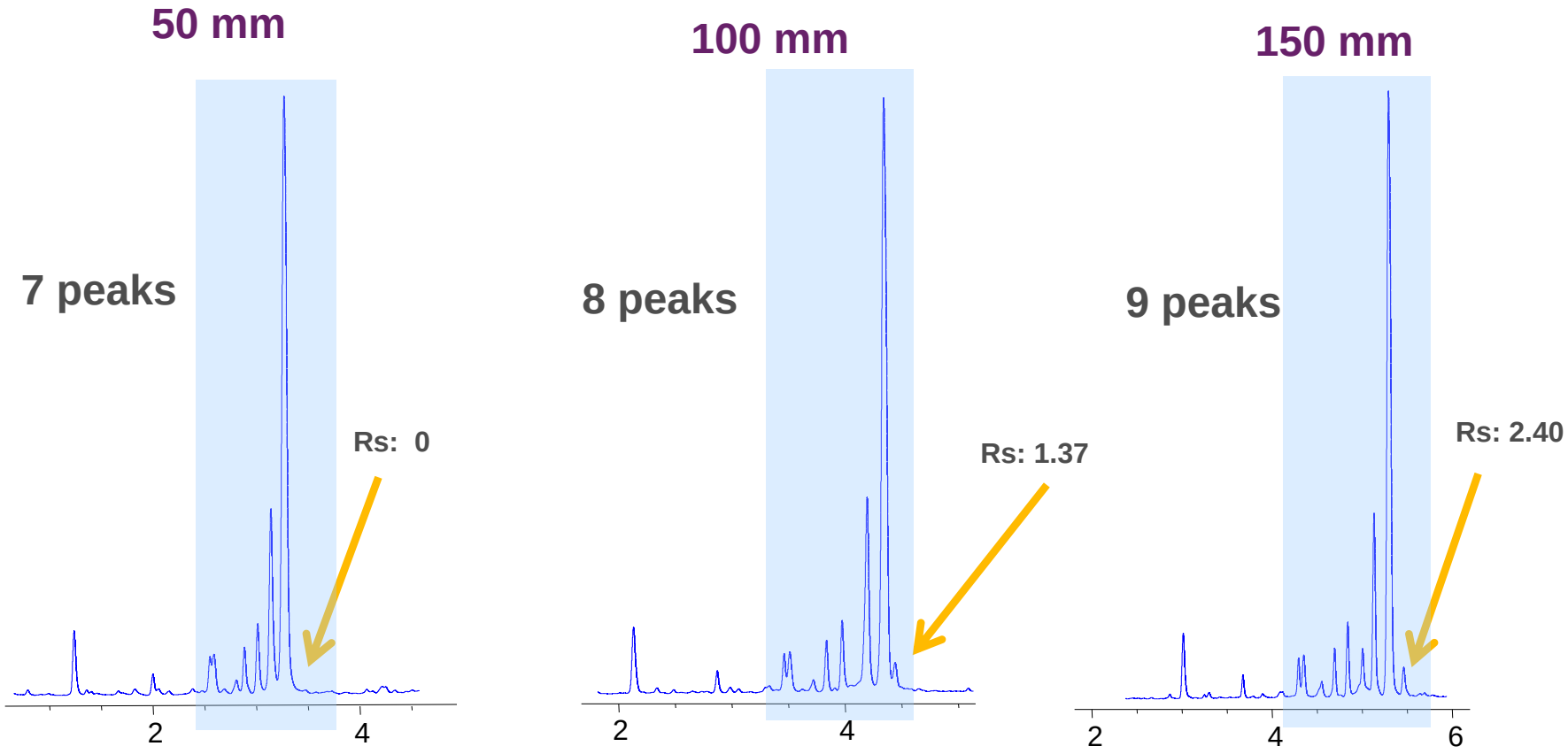
1290 Infinity



Increased Peak Capacity and *Change in Gradient Retention* Yields More Information

Mobile Phase: 10-100% B /30 min A: 0.1% formic acid (fa) B: acetonitrile with 0.1% fa F=0.4 mL/min Ambient temperature 280nm UV

1290 Infinity



Increasing peak capacity led to increased Rs for this sample.

Agilent UHPLC Column Selection Guidelines

	Fast Analysis	High Rs (N)	400 bar LC "Fitness"	1000+ bar UHPLC "Fitness"	ID's 4.6, 3.0 & 2.1 mm	Dirty Samples?
Poroshell 120, 600 bar	✓	✓	✓	✓	✓	✓ 2um frit
RRHT, 600 bar	✓	✓	✓ 50mm	✓	✓	✓
RRHD, 1200 bar	✓	✓	X	✓	X (3.0 & 2.1)	✓

Today's Chromatography - Higher Performance Requires More Attention to Details

- Higher Performance Column Capabilities
  - Higher Theoretical Plates (N) Yield Narrower Peaks
  - Need Better Control of Flow Path and Lower Dispersion
  - Need Higher Data Acquisition Rate
- New Instrument Capabilities for More Efficiency
  - Better Flow Control
  - Lower Extra Column Volume
  - Lower Dispersion
- Need to Optimize Older Instruments
  - Decrease Extra Column Volume
  - Improve Connections
  - Increase Data Rate

Which Instruments May Need to be Adapted?

1290 Infinity II LC

READY TO GO

1100 through 1260

MAY NEED OPTIMIZATION

Optimize LC for Lower Volume and Sharp, Efficiency Peaks

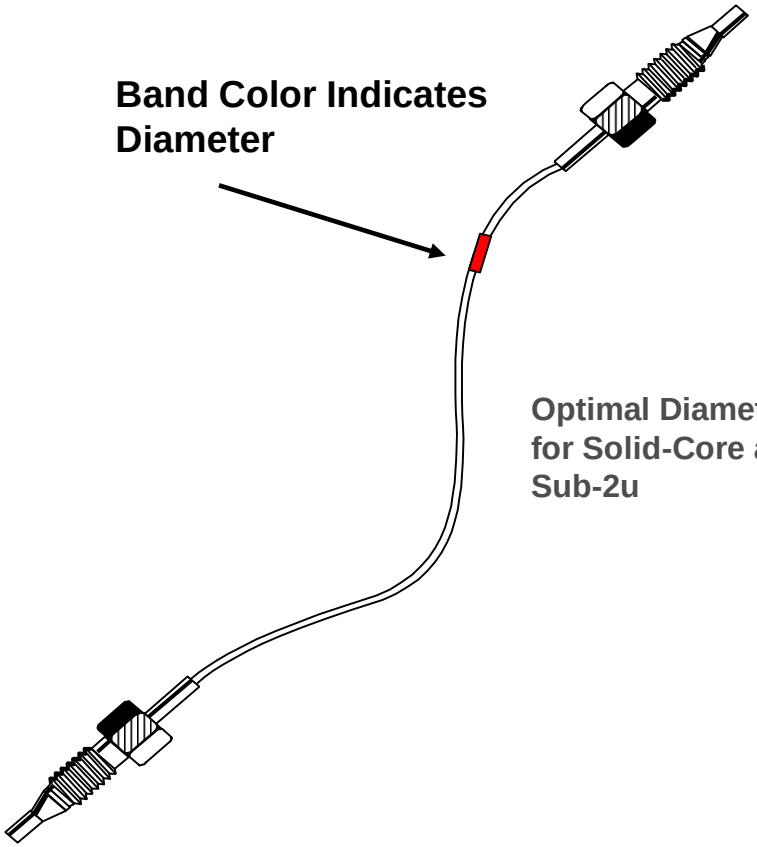
Adjust Data Collection Rate

- Set to fastest setting that does not compromise S/N
- Most often the fastest setting will not be necessary

Reduce extra column volume

- With Agilent LC's change "green" tubing (0.17mm) to "red" tubing (0.12 mm)
- Change needle seat to lower volume
- Choose a lower volume flow cell
- Choose lower volume for column heater

Lower Tubing Volume Lowers Dispersion
Easiest Path is to Decrease Diameter



Color	i.d.
Black	0.075 mm (0.003 inches)
Red	0.12 mm (0.005 inches)
Green	0.17 mm (0.007 inches)
Blue	0.25 mm (0.01 inches)
Clear	0.50 mm (0.02 inches)

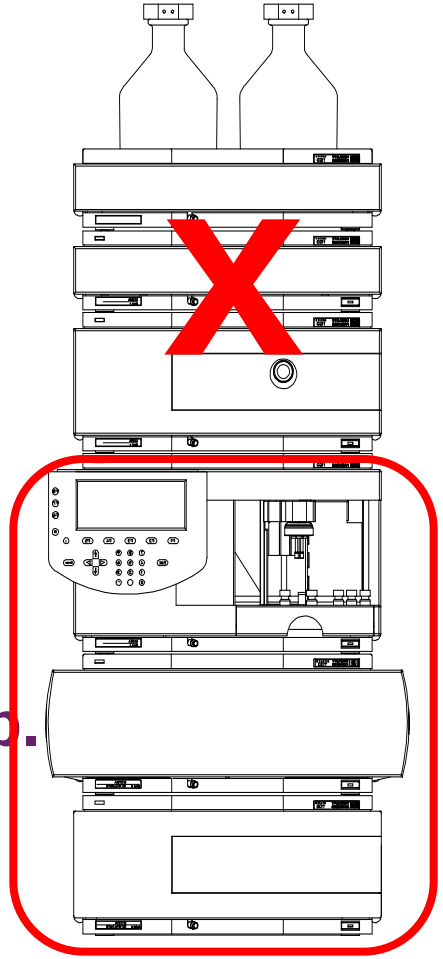
Where May Instrument Modifications Be Necessary?

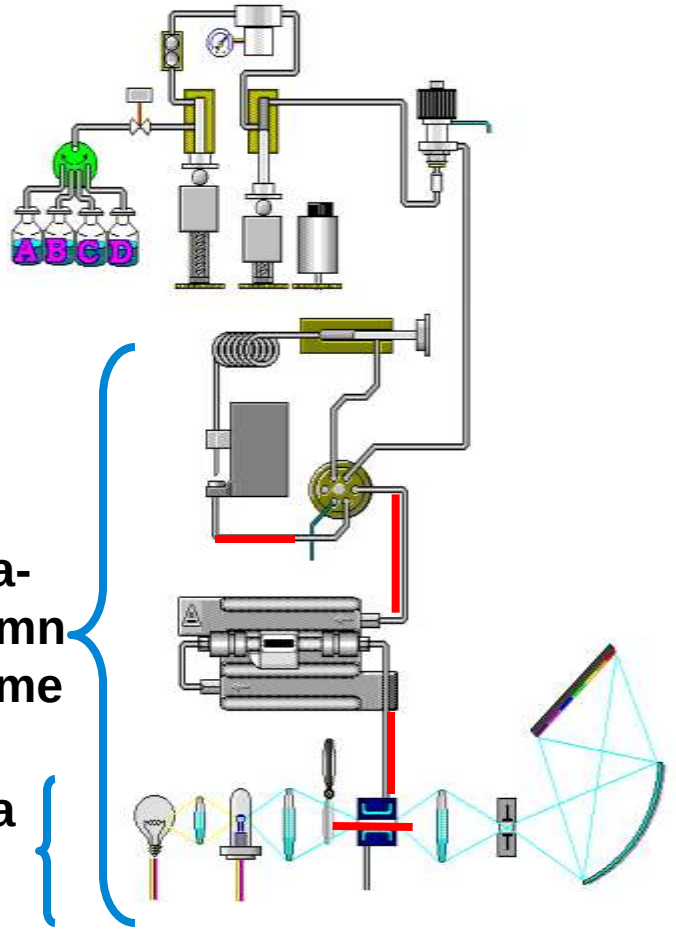
Pump

Autosampler

Column Comp.

Detector



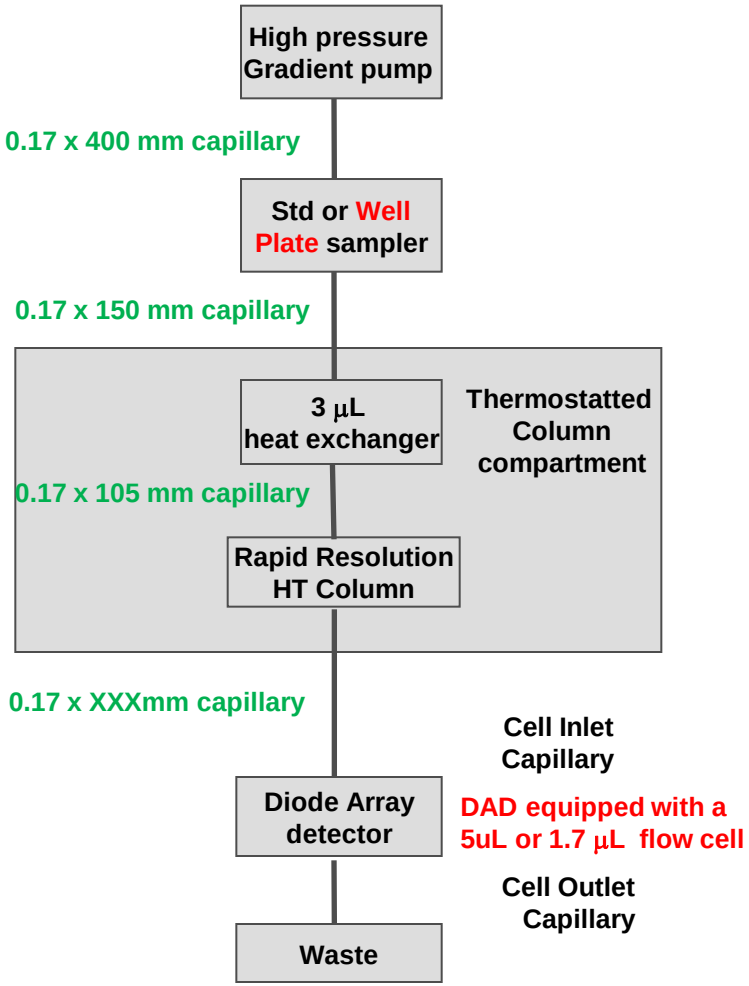


Extra-column volume

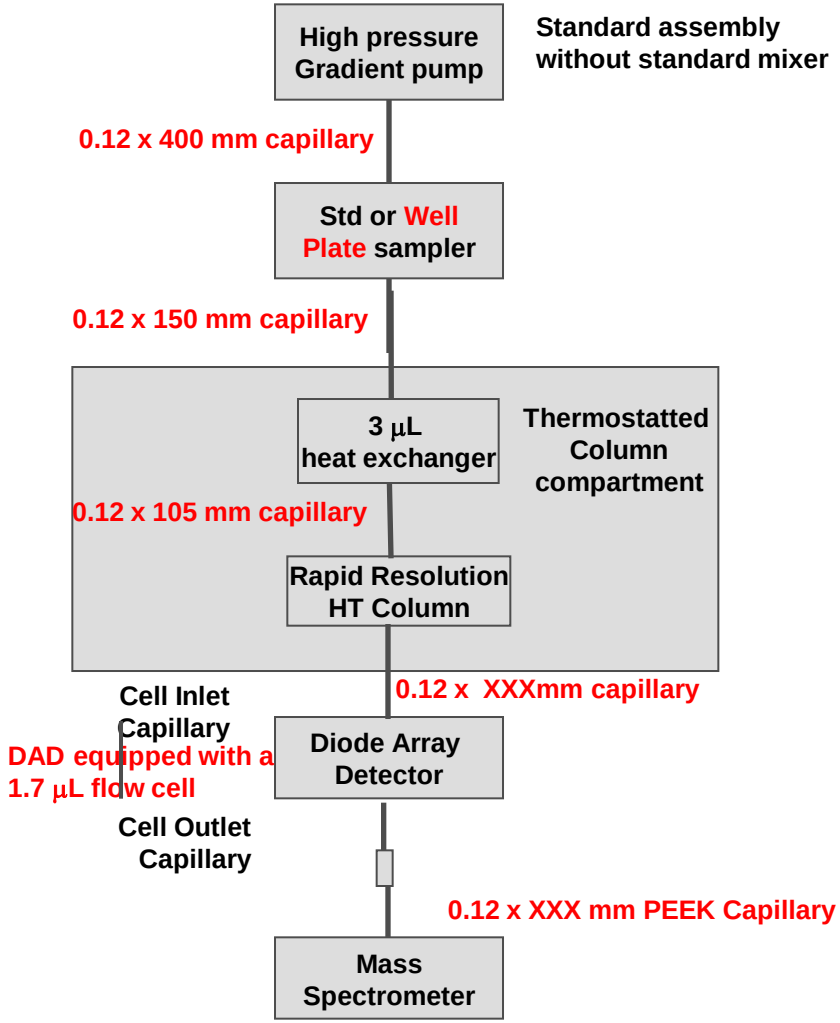
Data rate

Tubing Choices based on Column I.D.

4.6 mm ID columns



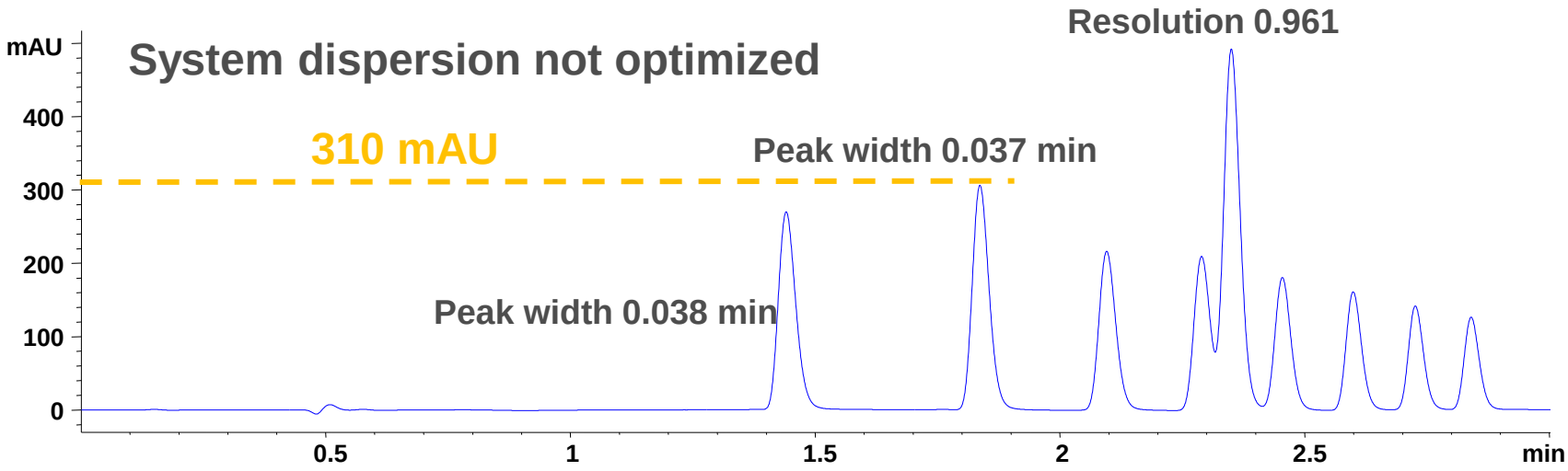
2.1 mm ID columns

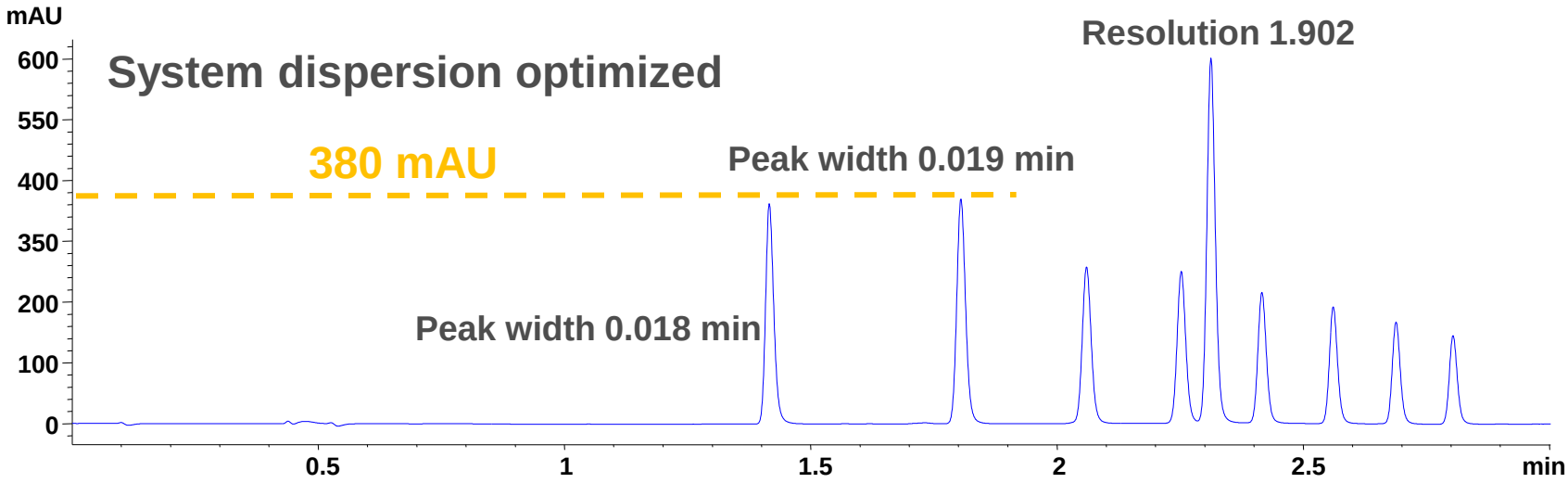


* Pieces to upgrade, in kits

System – Signal Height

System volumes – System dispersion

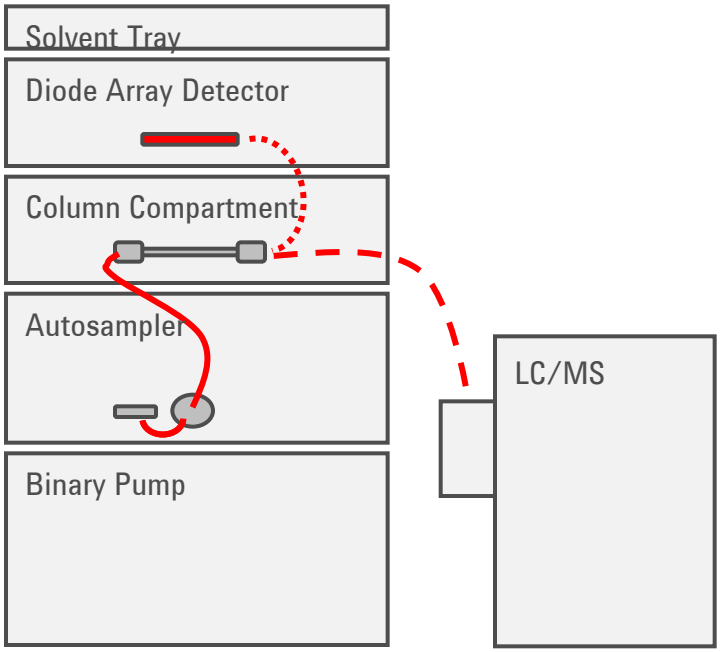




Comparison of 1290 Gradient Performance

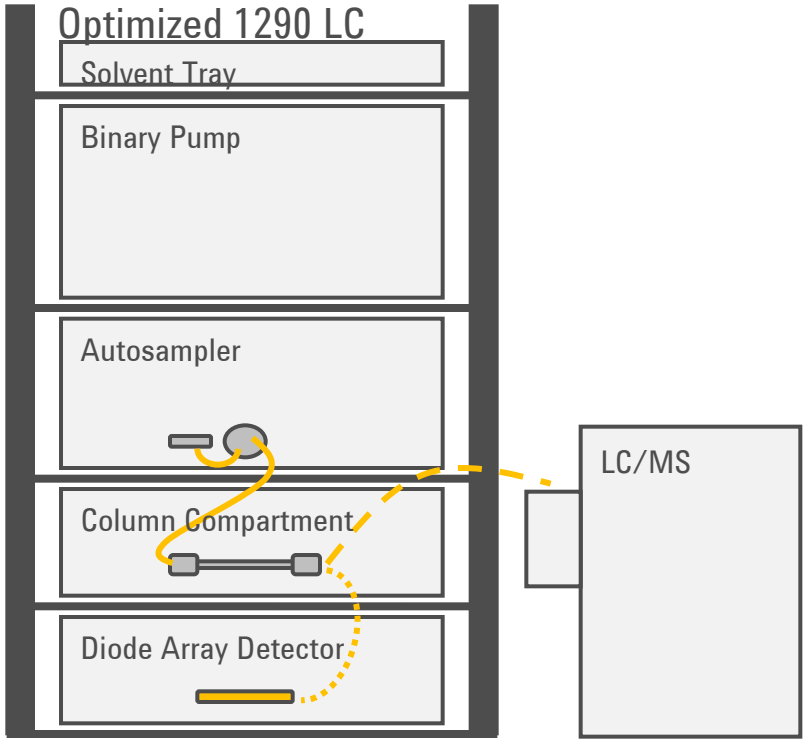
LC/UV systems extra column volume is reduced by 60% (from 9.7 to 3.9uL)
LC/MS system extra column volume is reduced by 64% (from 8.7 to 3.1 uL)

Default 1290 LC



Needle Seat Capillary: 0.12 x 100 mm = 1.1 µL
ALS→TCC Capillary: 0.12 x 340 mm = 3.8 µL
TCC→DAD Capillary: 0.12 x 220 mm = 2.5 µL
Flow Cell V(σ)1.0 µL = 2.3 µL
TCC→MS Capillary: 0.12 x 340 mm = 3.8 µL
2.1 x 50 mm Column = 172.3 µL
Void Volume of Column = 103.9 µL

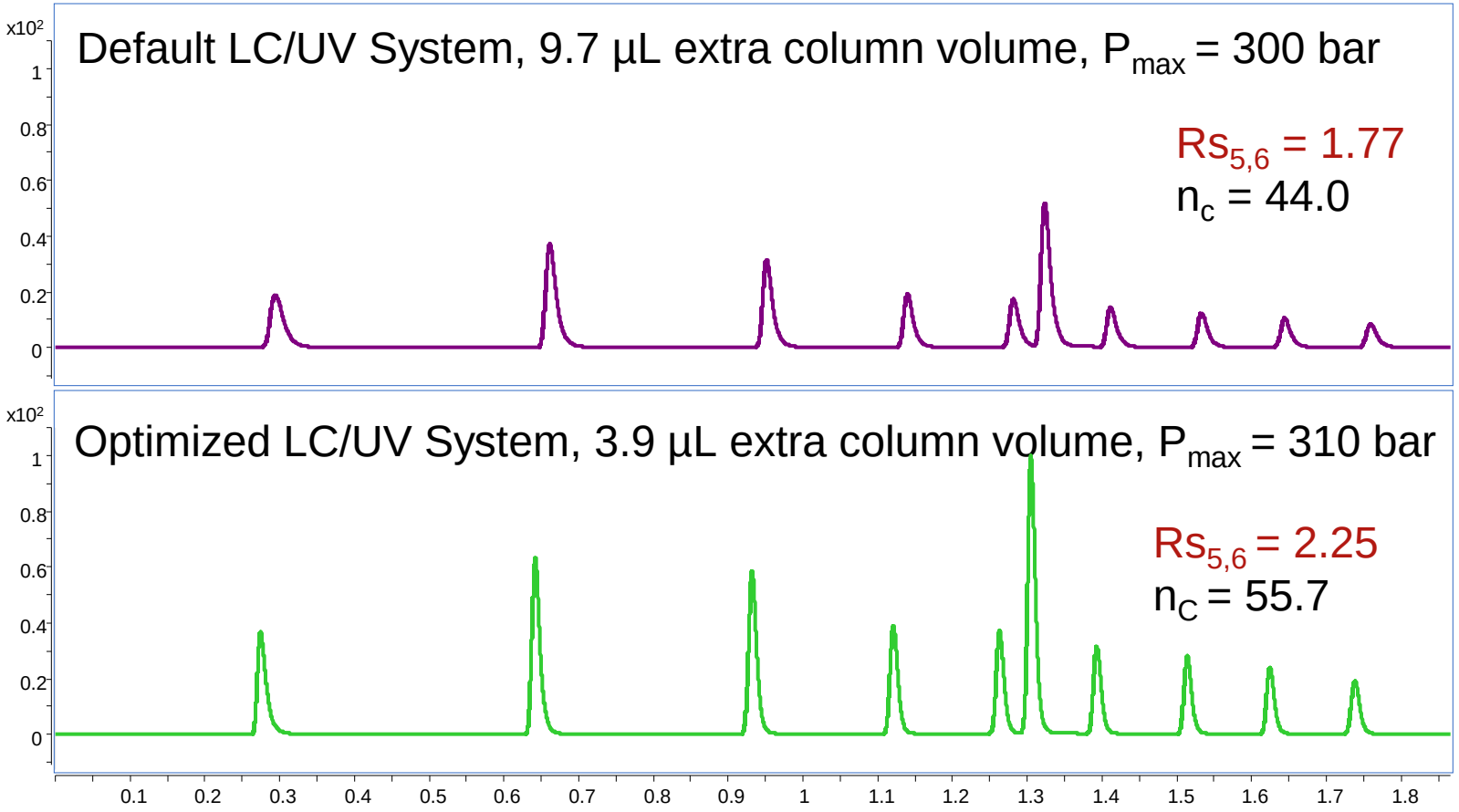
Optimized 1290 LC



Needle Seat Capillary: 0.11 x 100 mm = 0.9 µL
ALS→TCC Capillary: 0.08 x 220 mm = 1.1 µL
TCC→DAD Capillary: 0.08 x 220 mm = 1.1 µL
Flow Cell V(σ)0.6 µL = 0.8 µL
TCC→MS Capillary: 0.08 x 220 mm = 1.1 µL
2.1 x 50 mm Column = 172.3 µL
Void Volume of Column = 103.9 µL

Optimized LC Improves Gradient Resolution

Column: RRHD Eclipse Plus C18, 2.1 x 50mm, 1.8um Gradient: 25-95% CH_3CN in 1.2 min, Flow Rate: 0.4 mL/min LC: Agilent 1290 Infinity Sample: Alkylphenones

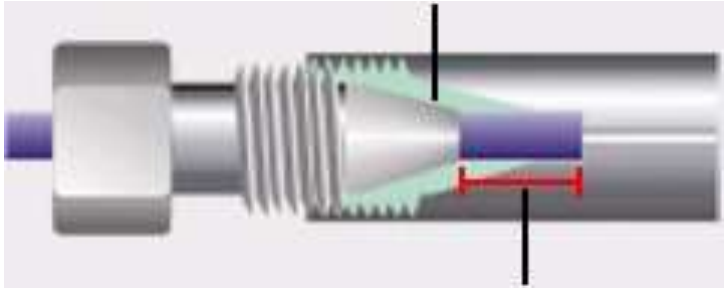


>20% improvement in gradient Rs and peak capacity with optimized LC

What Happens If the Connections Poorly Made ?

Wrong … too long

Ferrule cannot seat properly



X

If Dimension X is too long, leaks will occur

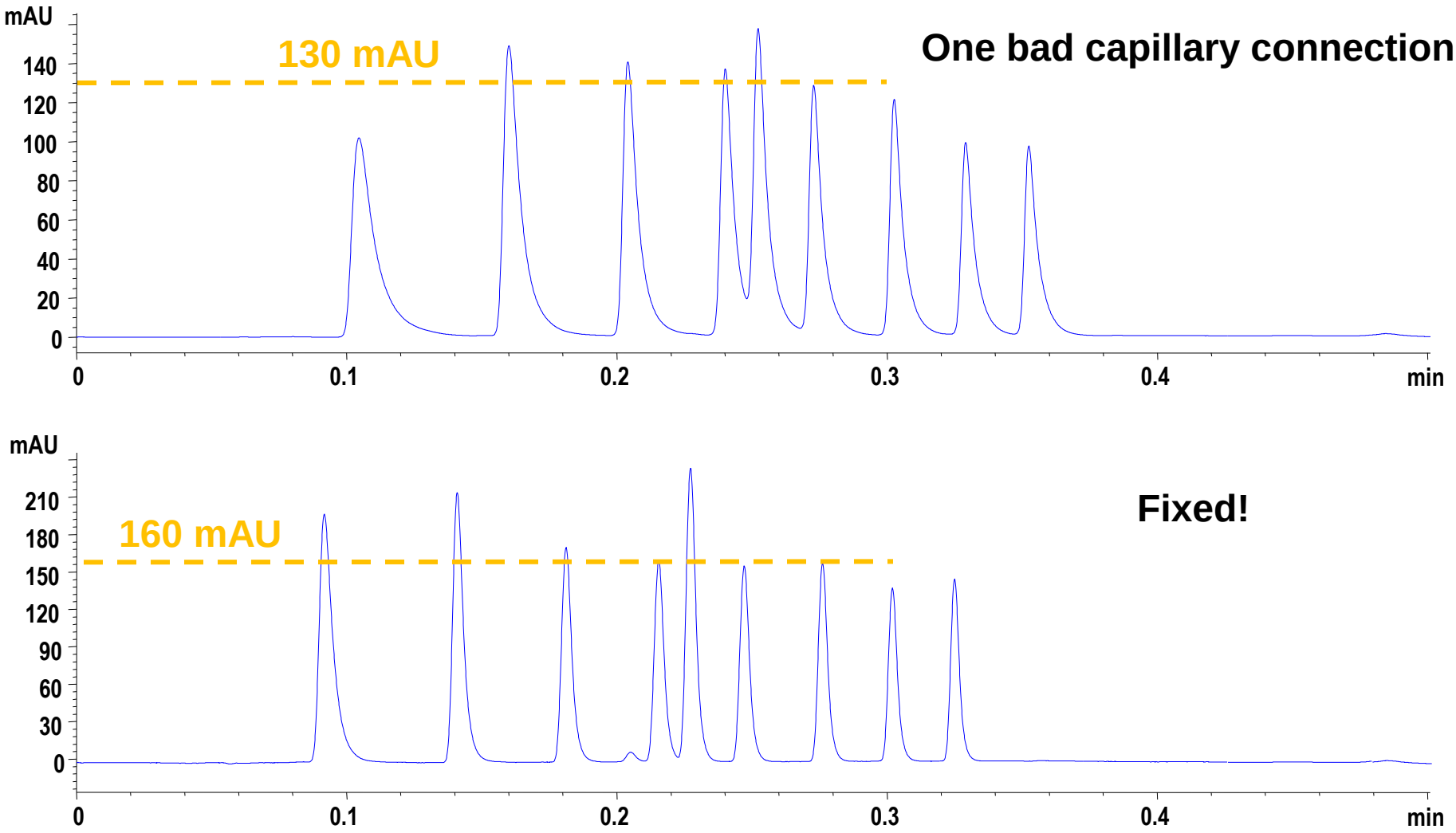
Wrong … too short

Mixing Chamber

X

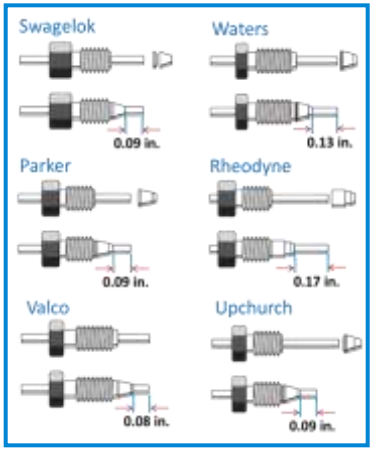
If Dimension X is too short, a dead-volume, or mixing chamber, will occur

Influence post-column capillary connections



The Agilent A-Line Fittings
Importance of the Spring Loaded Feature

Most commonly used fittings in UHPLC are non-adjustable 2-piece or 3-piece metallic fittings. Since different manufacturers of column hardware have different design in column end fittings, as shown in Figure 1, a new set of tubing and fittings needs to be installed for every brand of column to guarantee that the stem length, namely the length between the bottom of the ferrule and the end of tubing, fits the column end fitting.



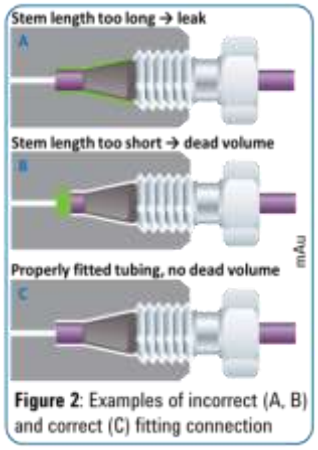


Figure 2: Examples of incorrect (A, B) and correct (C) fitting connection

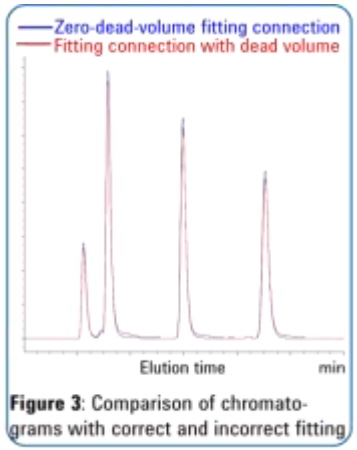
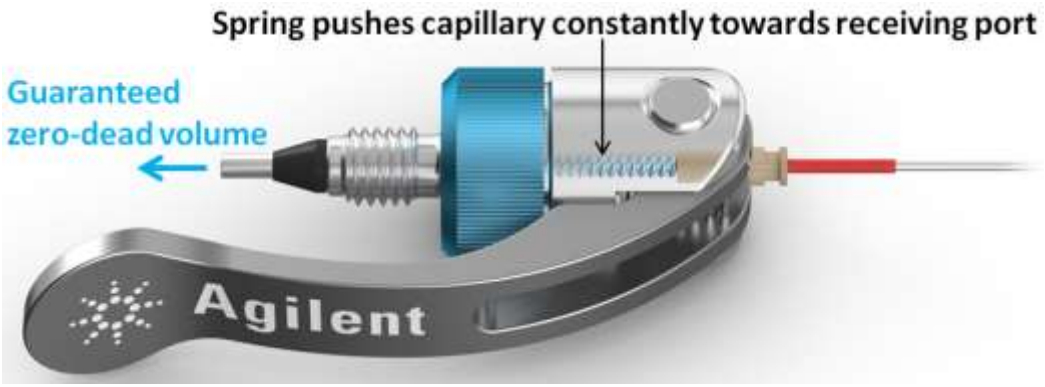


Figure 3: Comparison of chromatograms with correct and incorrect fitting

The spring-loaded design constantly pushes the tubing against the receiving port, delivering a reproducible connection with no dead volume for consistent chromatographic performance

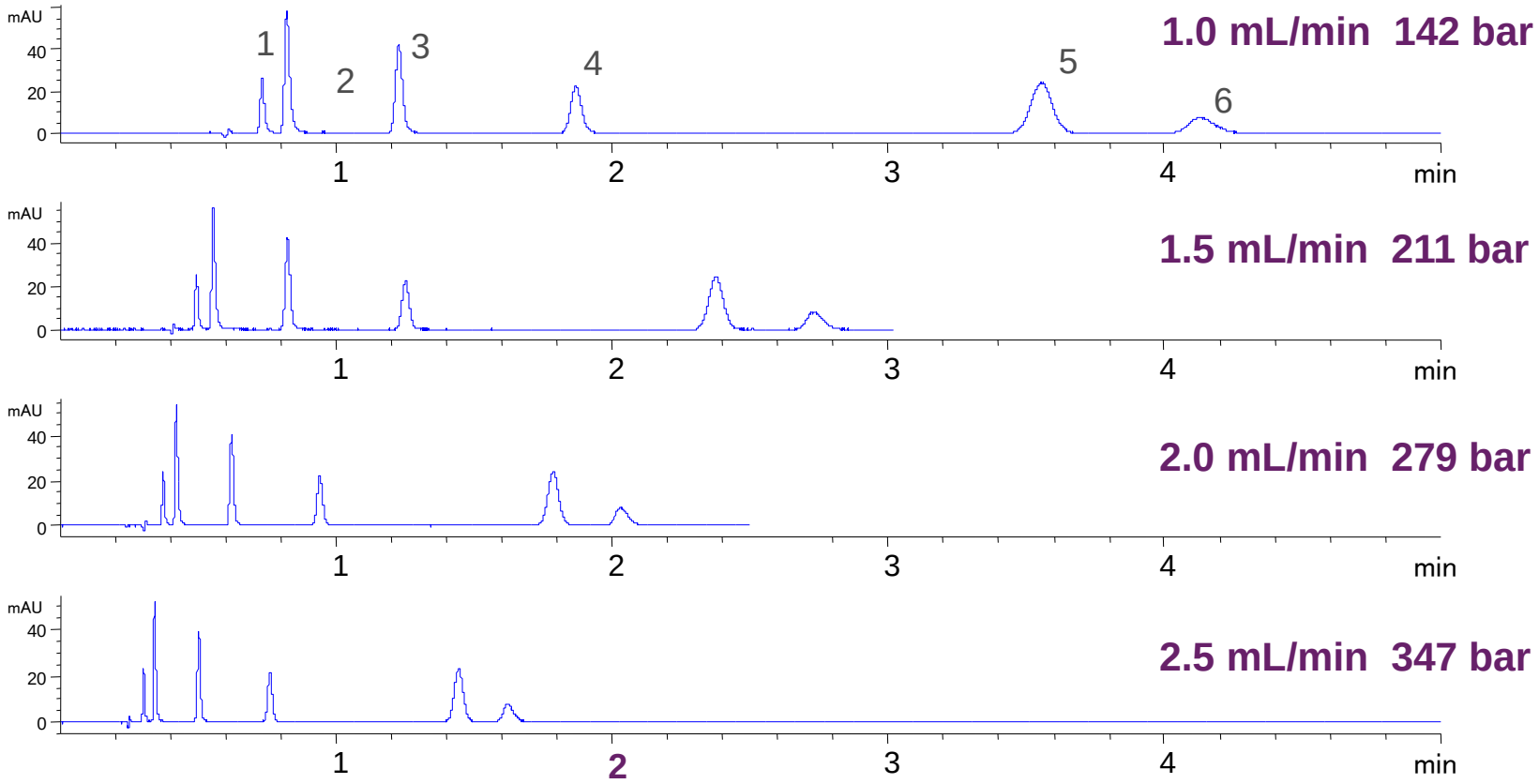
Stem length is adjustable through the spring, which makes the fitting compatible with all types of LC columns



Most Versatile, Secure High Pressure Fitting

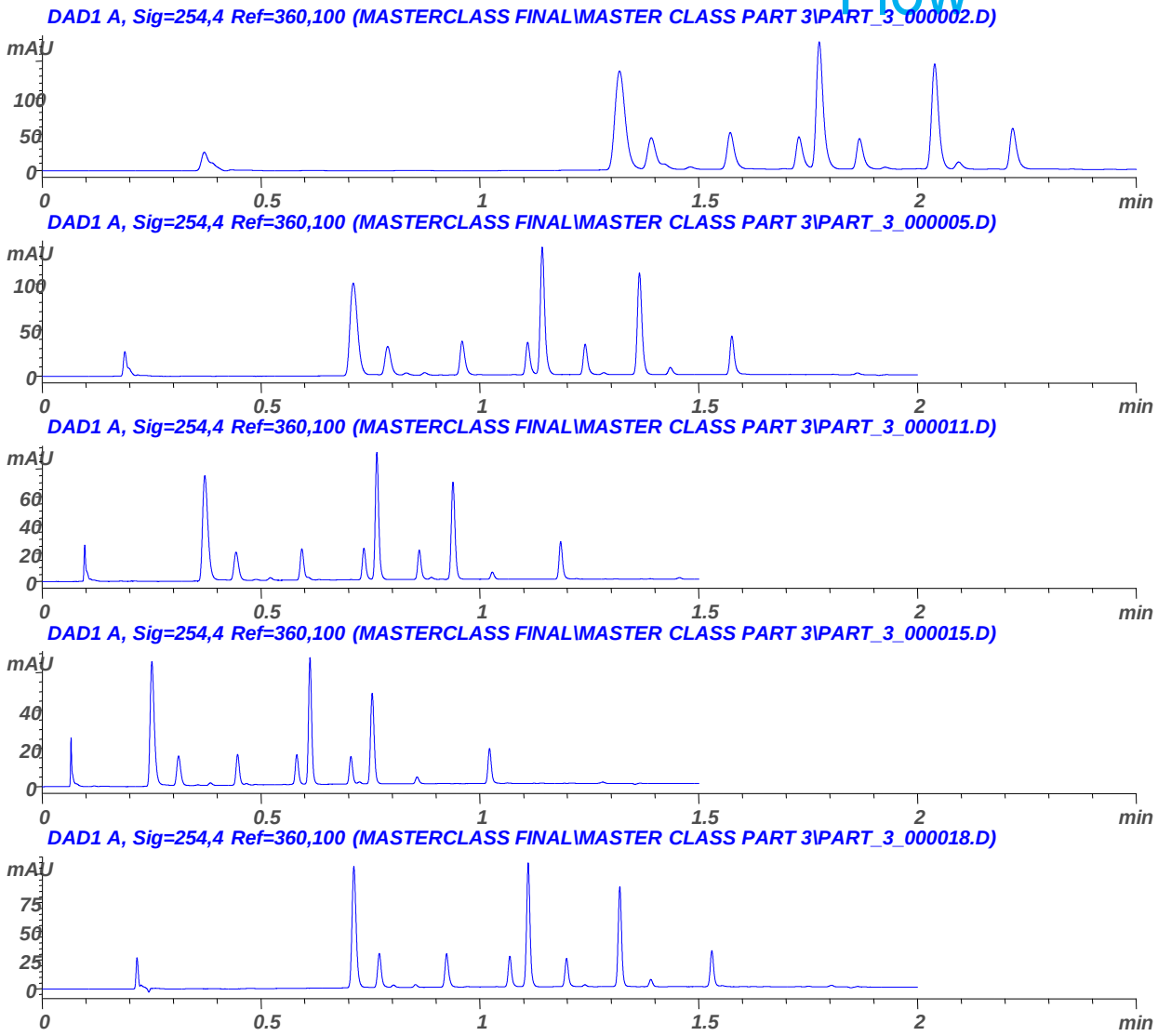
Fast Analysis of Analgesics on Poroshell 120 – Demonstrating “Fitness” for 400 bar LCs

1. Acetaminophen 2. Caffeine 3. 2-acetamidophenol 4. Acetanilide 5. Phenacetin 6. Salicylic acid



Conditions: Column: Poroshell 120 SB-C18, 4.6 x 50mm, 2.7um Mobile Phase: 0.2% Formic Acid 80% water:20% ACN
Temperature: 25 °C, Detection: 275 nm, Sample Injection: 2ul

UHPLC Durability Allows Enhanced Performance at High Flow



Column: 2.1x50Eclipse plus C18
Flow: 0.3ml/min
Peak Capacity: 35 (PW= 5sigma)
204bar

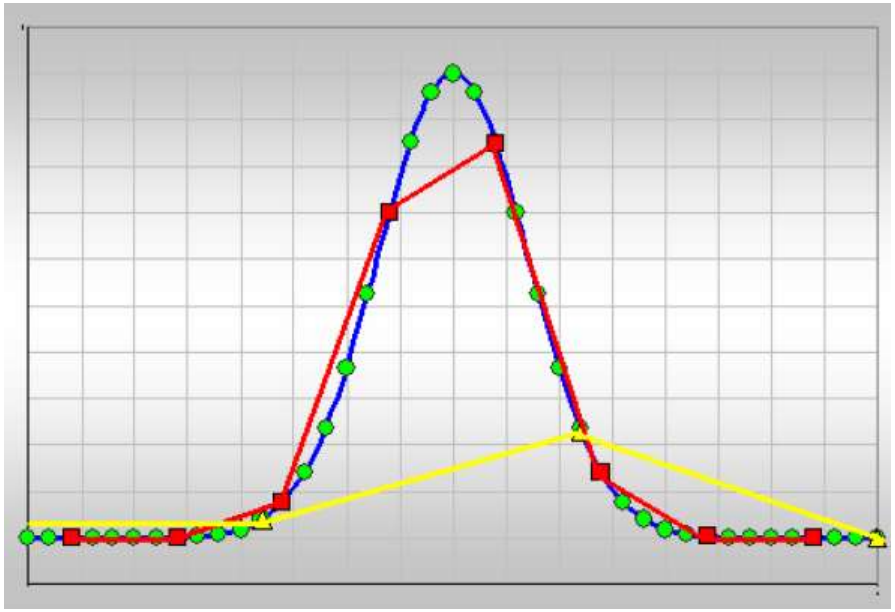
Column: 2.1x50Eclipse plus C18
Flow: 0.6ml/min
Peak Capacity: 53
395bar

Column: 2.1x50Eclipse plus C18
Flow: 1.2ml/min
Peak Capacity: 72
735bar

Column: 2.1x50Eclipse plus C18
Flow: 1.8ml/min
Peak Capacity: 84
1050bar

Column: 2.1x100Eclipse plus C18
Flow: 0.6ml/min
Peak Capacity: 69
862bar

Data Rate/Acquisition Frequency



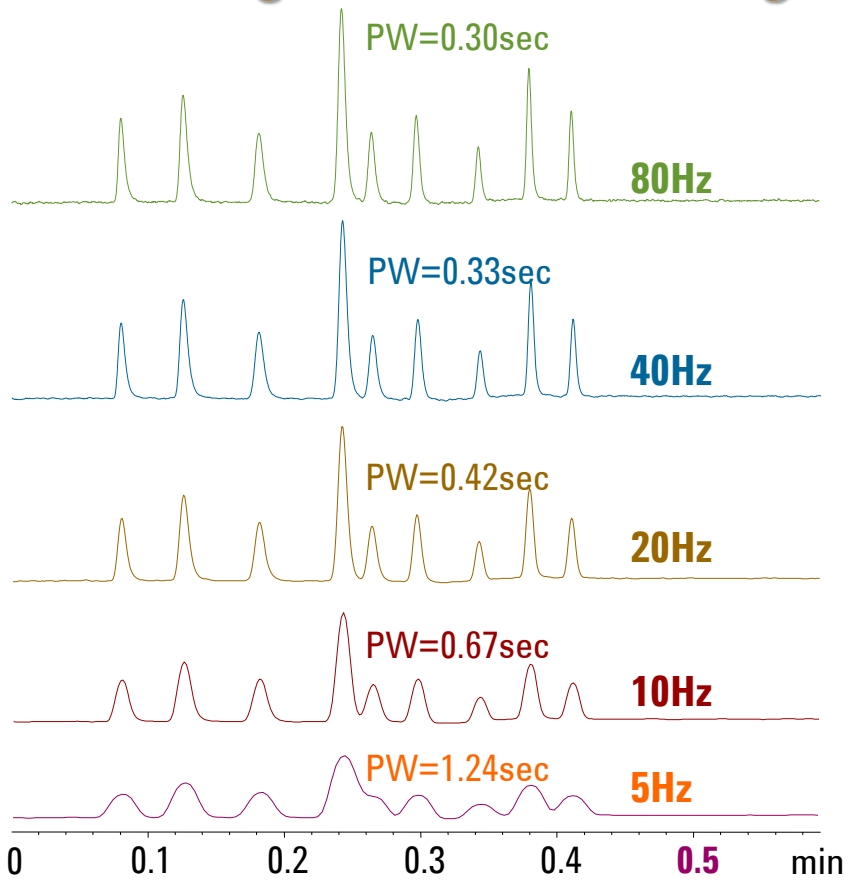
1. First the green dots: the peak is defined by 24 values and shows a well-integrated profile.
2. Second the red dots/line: the peak is only described by six measurements, the peak area is smaller (limit of detection is smaller) and the calculated efficiency is only 89% of the blue-lined peak.
3. Third the yellow dots/line: the peak is severely distorted, retention time and peak area are unreliable and a calculated efficiency shows only 39% of the reality.

- Savitzky-Golay (1964): 12-15 points per peak
- Dyson (1998): 100 points per peak

Detectors

For narrow peaks, high data rates!!!

Maintaining Resolution at High Analysis Speed



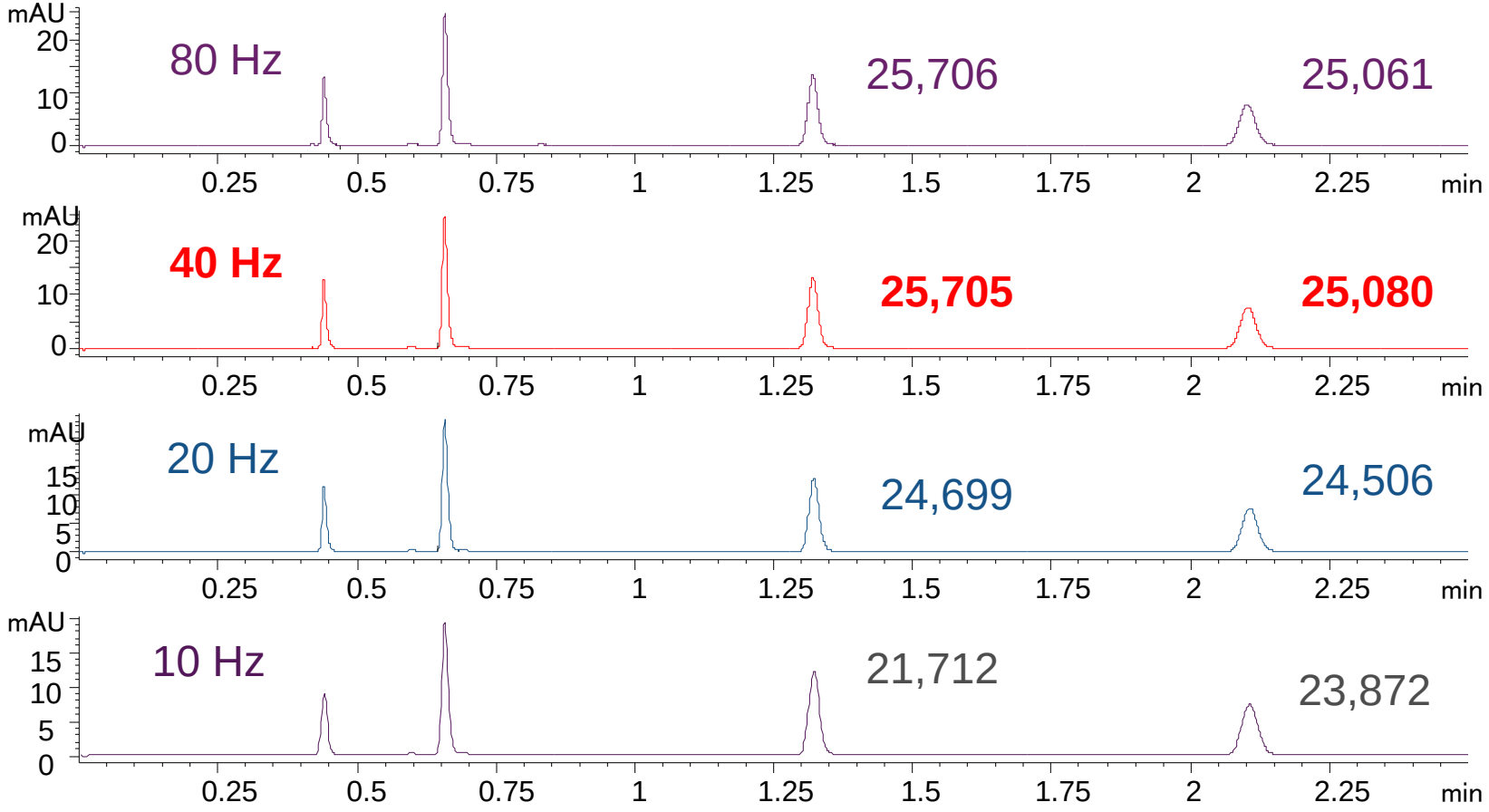
80Hz versus 10Hz (20Hz) Data Rate

- 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

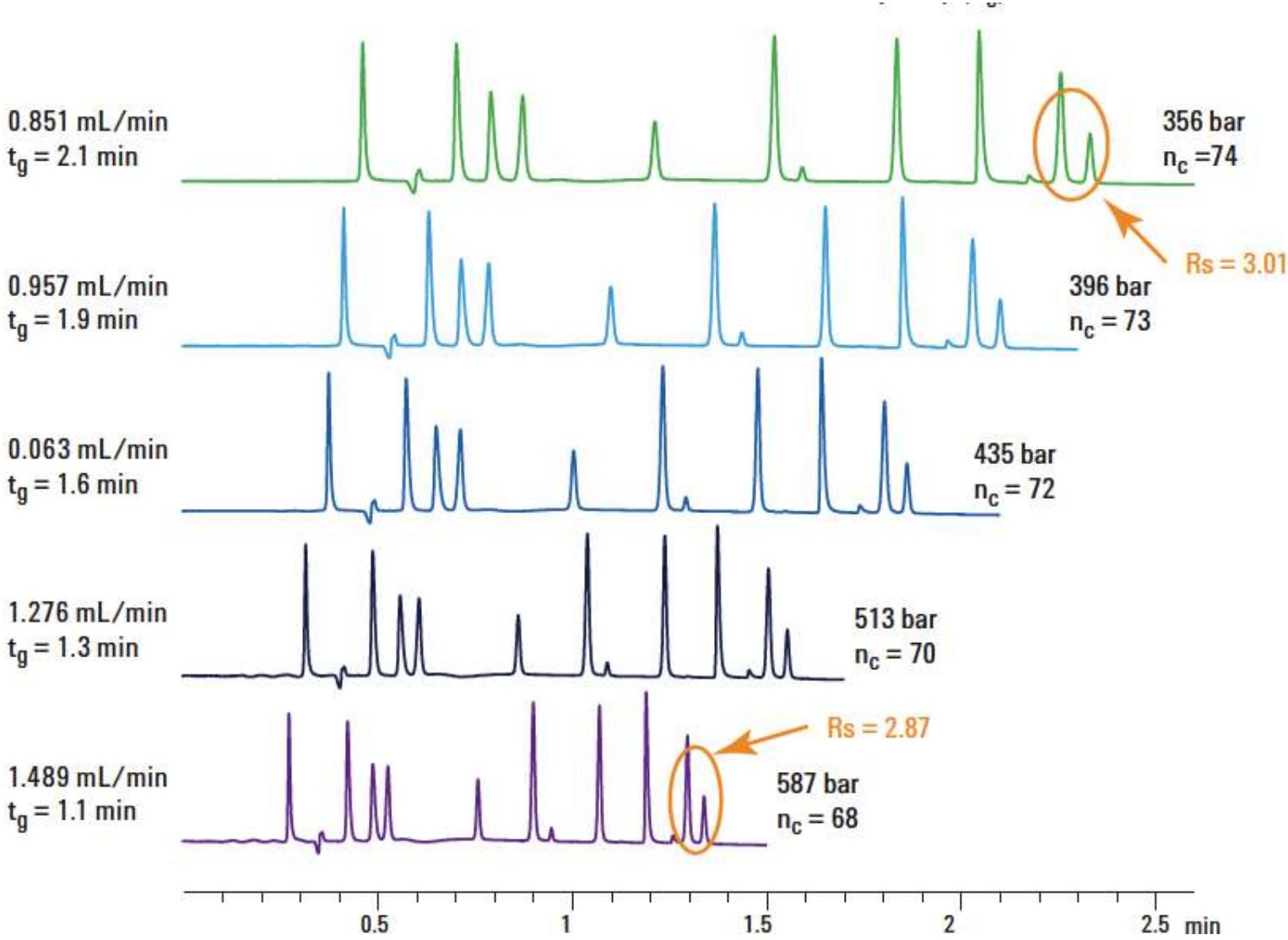
Sample: Phenones Test Mix
Column: Zorbax SB-C18, 4.6x30, 1.8um
Gradient:: 50-100%ACN in 0.3min
Flow Rate: 5ml/min

Comparison of Peak Efficiency on Poroshell 120 EC-C18 with Different Data Collection Rates



Column: Poroshell 120 EC-C18, 4.6 x 100mm Instrument: 1200 SL 2ul flow cell
Flow Rate: 2.00 ml/min Sample: 2ul injection of 3B Mobile Phase: 60:40 MeCN:Water

Increasing Flow Rate – Poroshell120



Summary

- The New Generation of UHPLC Instruments (1290 Infinity) are providing an Optimum Platform for Sub-2u and Poroshell Type Columns

- ZORBAX RRHD – Rapid Resolution High Definition – LC columns for the 1290 Infinity LC are designed for the demands of this UHPLC and any other UHPLC.

- RRHD columns are made from the same ZORBAX 1.8um particles and bonded phases in the ZORBAX RRHT columns for the RRLC.

- Both RRHT and RRHD Columns operate over the entire pressure range available in all UHPLC instruments.

- Superficially Porous (Poroshell) columns deliver Many Benefits of sub-2u particles at dramatically lower pressure

Basic Instructions for Using HPLC/ UHPLC Columns Effectively

1. Install and run the column only in the flow direction marked on the column
2. **Use only high quality, chromatography grade solvents**
3. Filter all aqueous buffers and all samples through a mobile phase appropriate 0.2um filter before use.
4. Replace bottles of mobile phase buffer every 24-48 hours – do not add mobile phase to the bottle.
5. Use an in-line filter to prolong column life.
    1. 5067-1551 for 2.1 or 3.0mm ID RRHT columns or 5067-1553 for 4.6 mm ID RRHT columns
    2. In-line filter ships with 1290 pump for use with RRHD columns and can be ordered separately – part number 5067-4638.

Contact Information

John Palmer

Agilent Technologies, Inc.

847-420-2838

john_palmer3@agilent.com