The Secrets to Better HPLC Methods

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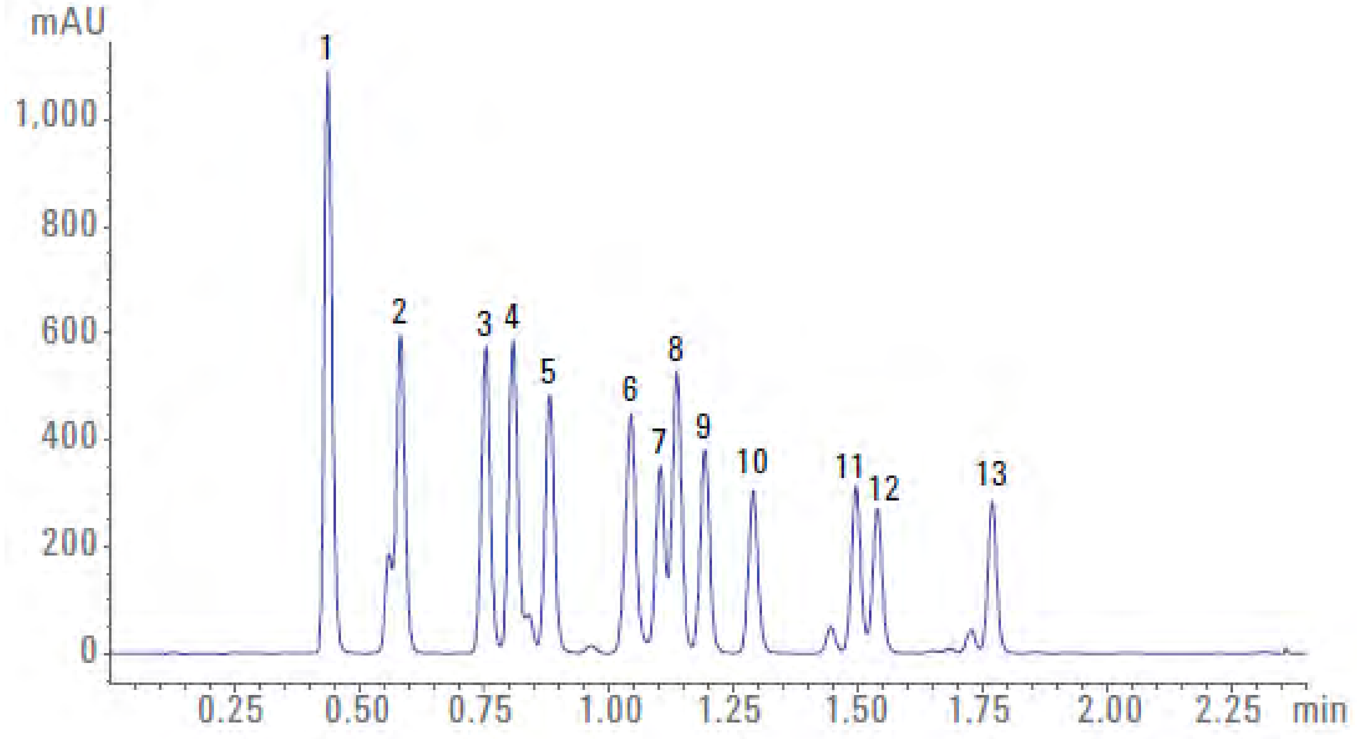
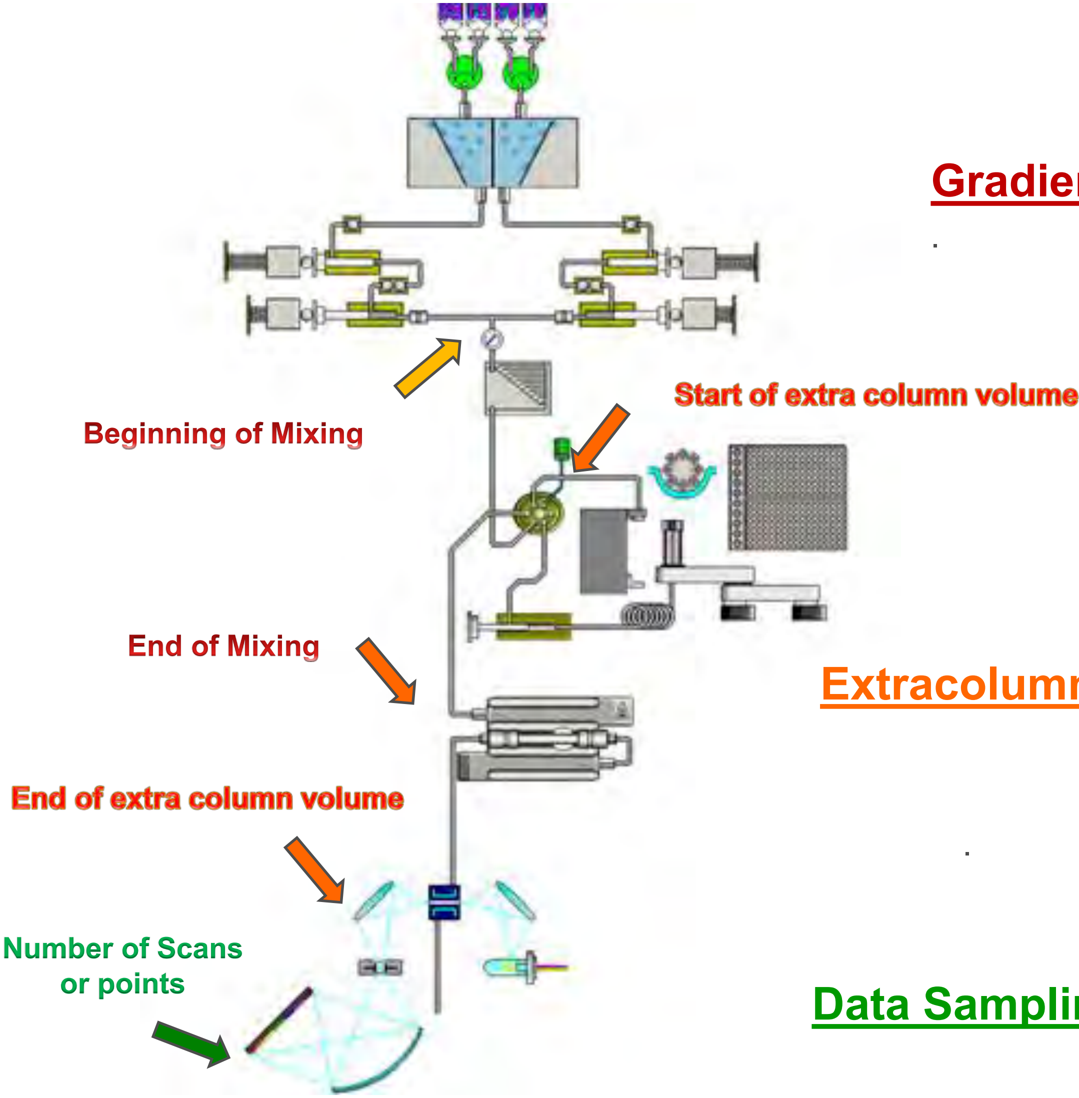
Have You Ever Had Peaks That You Can't Resolve?



Five Things That You Can Do To Improve The Separation

#1 System Tubing

HPLC Instrument Components



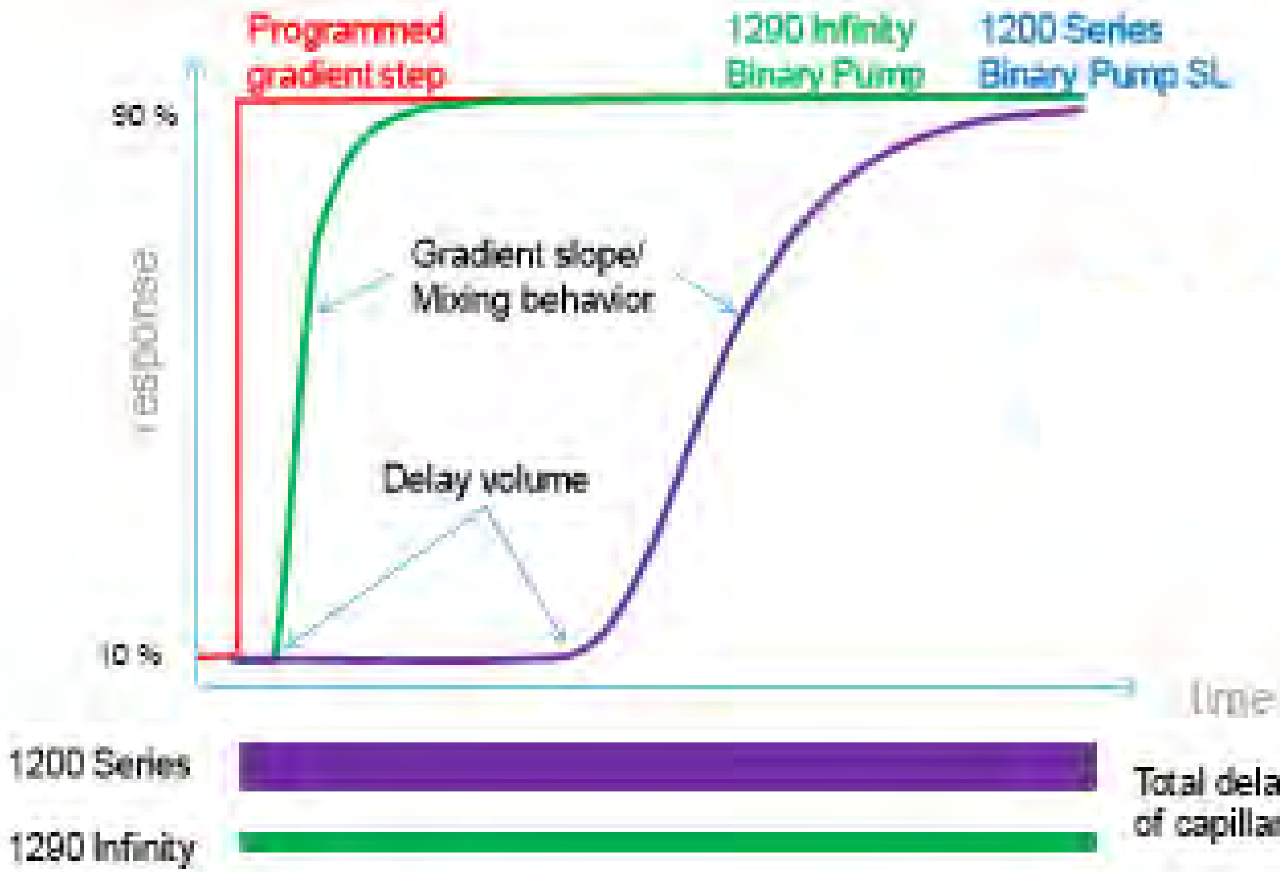
Gradient Delay or Dwell Volume

Extracolumn Volume

Data Sampling or Acquistion Rate

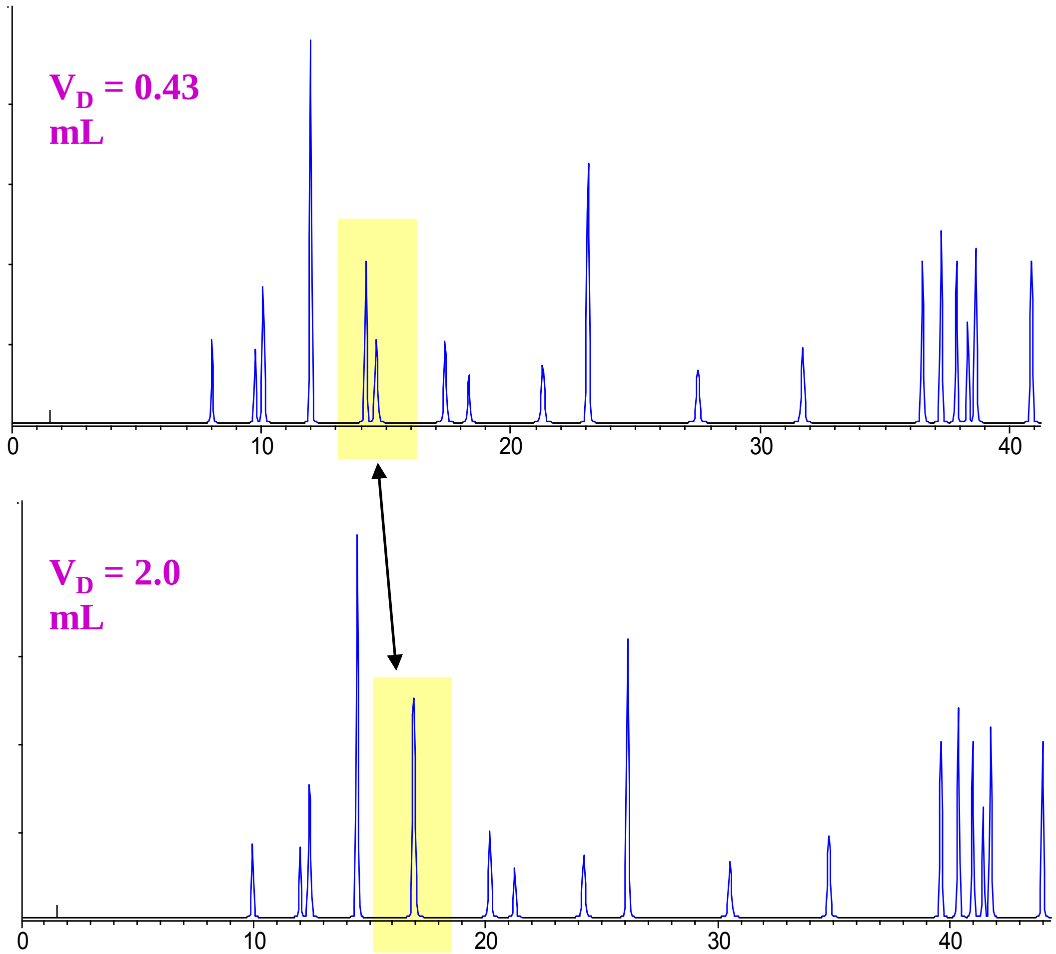
Method Transferability

Impact of delay volume and mixing behavior...



Total delay volume of the system (sum of capillaries, mixer, cells, valves..)

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

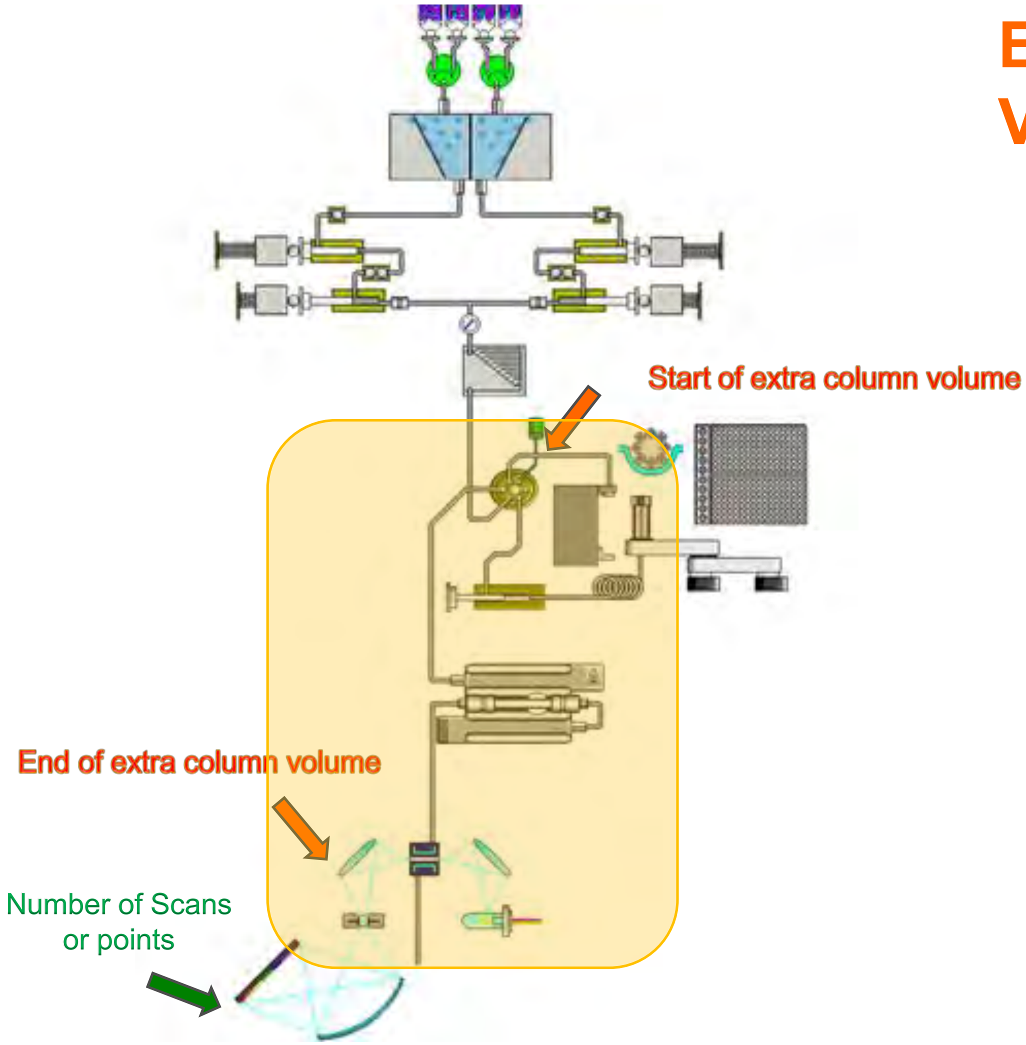
Sample: Mixture of antibiotics and antidepressants

Upper trace simulates actual run data entered into DryLab® 3.0 software

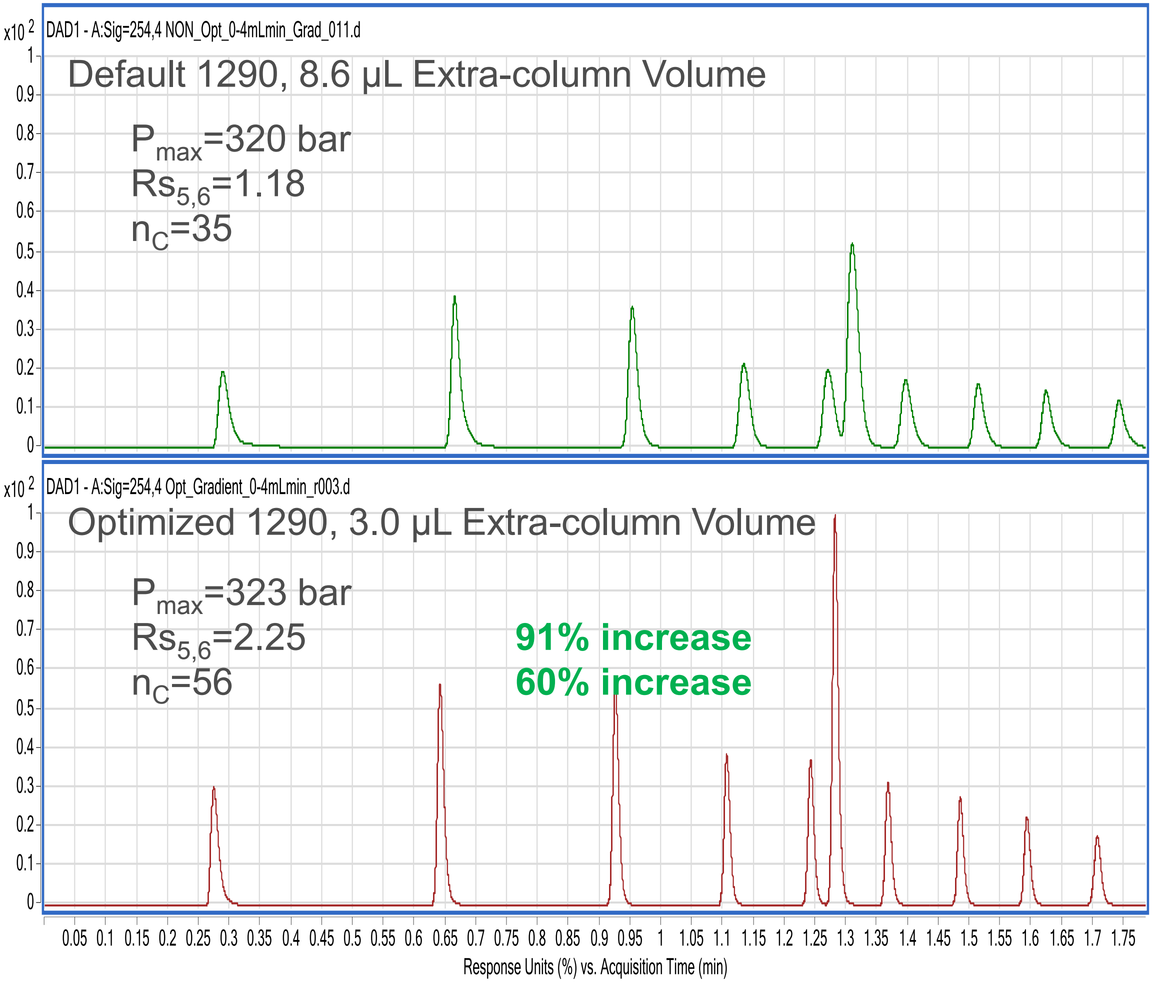
Lower trace is simulated chromatogram for larger V_D

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

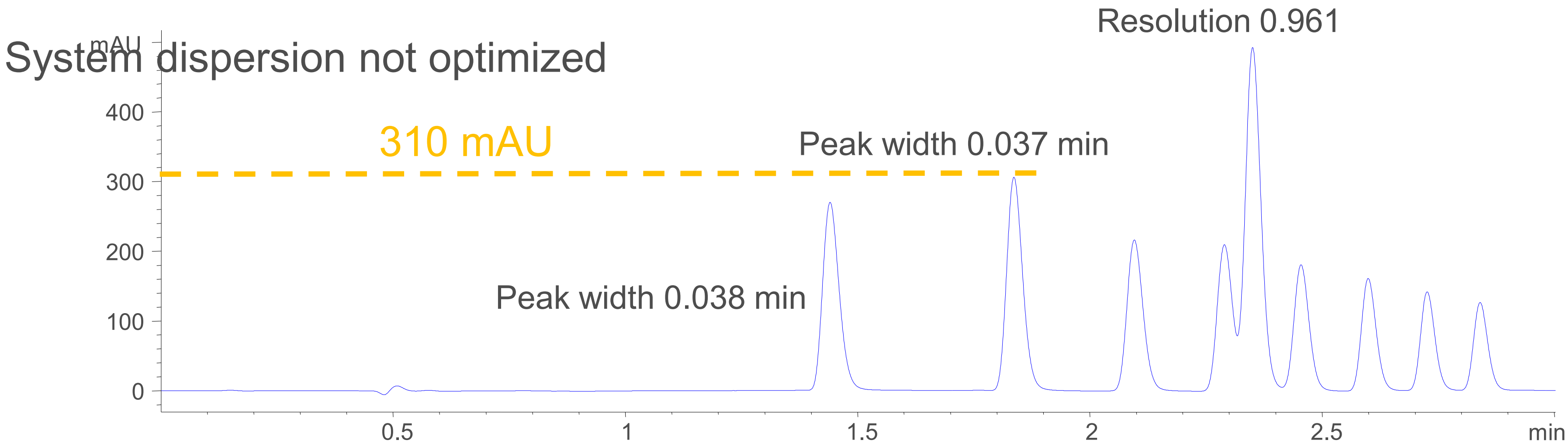
LC Rack System, 5001-3726

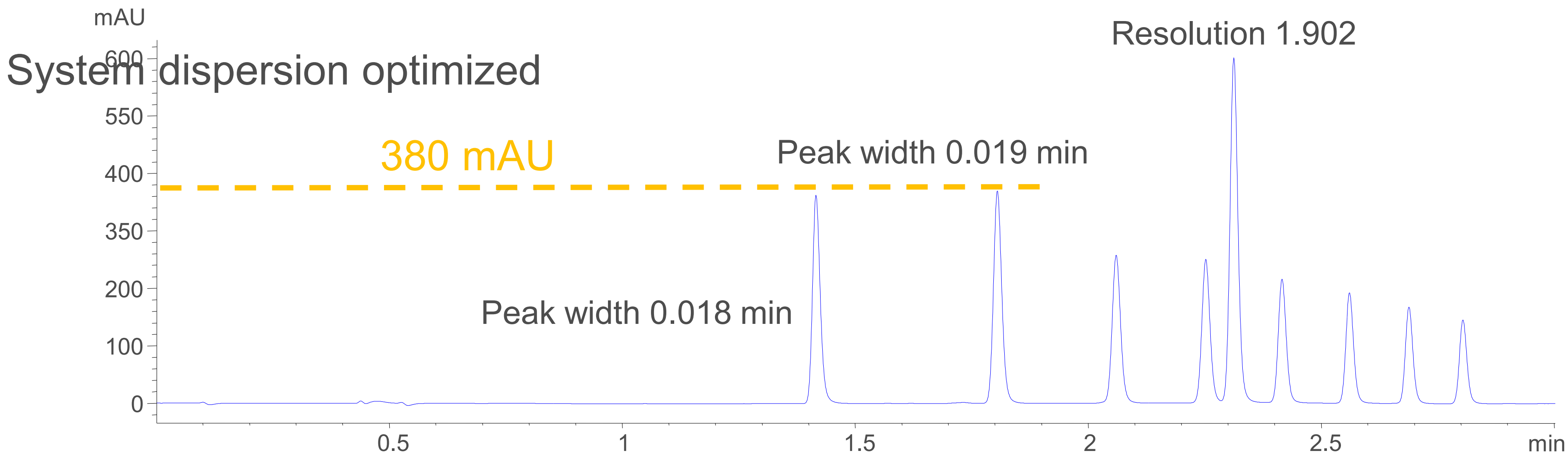
0.08 x 220 mm Capillary Tubing

V(σ)0.6 µL Flow Cell

System – Signal Height

System volumes – System dispersion

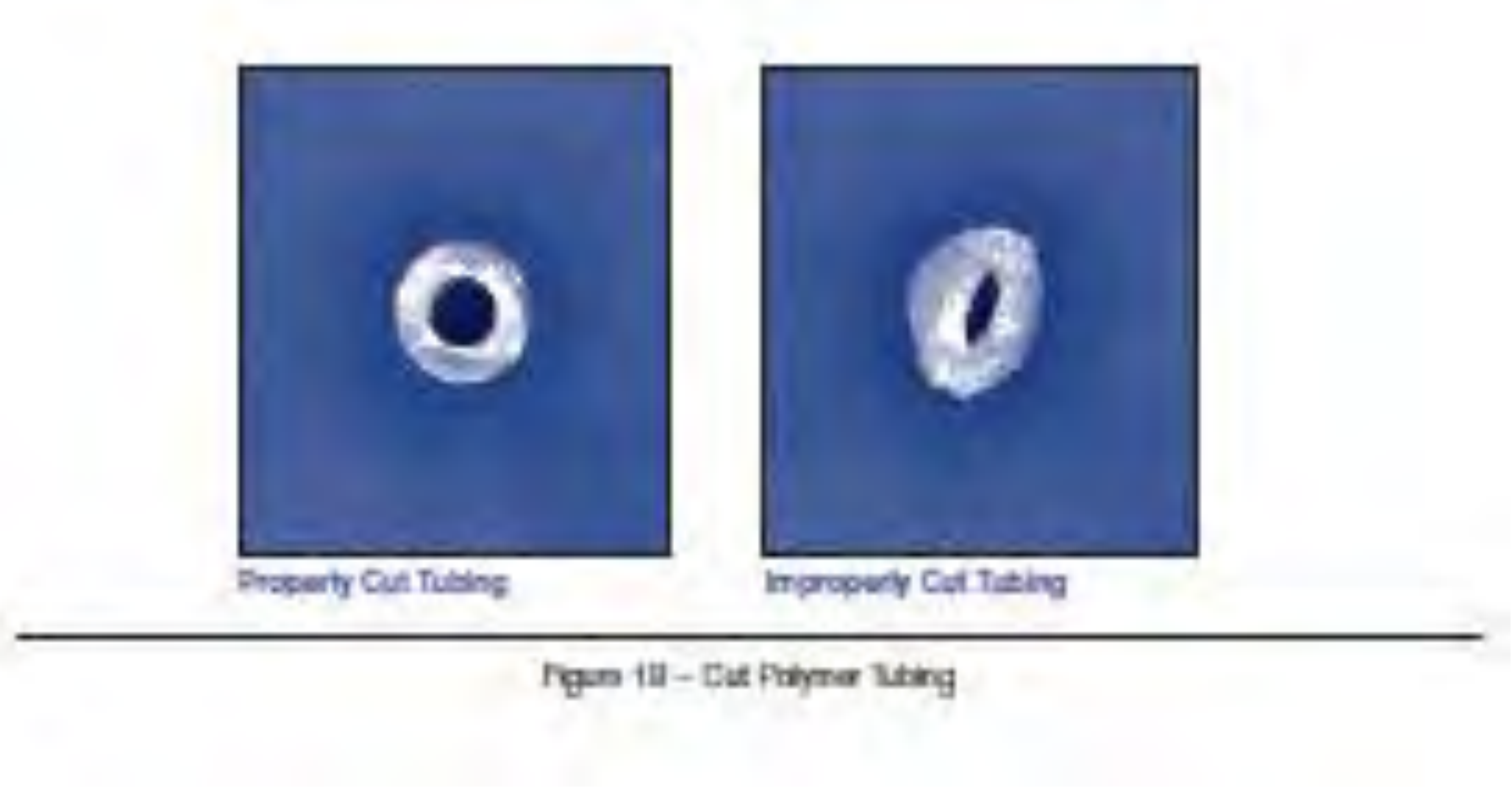




Tubing After Cutting

Even “properly cut” Tubing Has Problems

- Best Practice is to use “pre-cut” tubing (capillary in Agilent speak)
- Second Best – use a high quality tubing cutter
- DO NOT USE Side Cut Pliers or Scissors” (You Laugh, but it has happened)



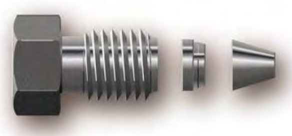
Five Things That You Can Do To Improve The Separation

#2 Column Connections

Stainless Steel and Polymer Fittings

Which type is used and when?

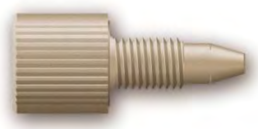
Stainless Steel (SS) fittings are the best choice for reliable high pressure sealing



- Agilent uses Swagelok type fittings with front and back ferrules – which give best sealing performance – throughout all our LC systems

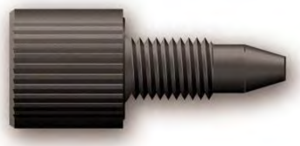
PEEK (<400 bar System Pressure) fittings are ideal where:

- Connections are changed frequently, i.e. connecting columns
- Pressure is less critical



PolyKetone

- Easy, hand tighten column connection
- **600 bar Pressure Rating** PN: 5042-8957 (10/pk)
- Fits to SS Tubing



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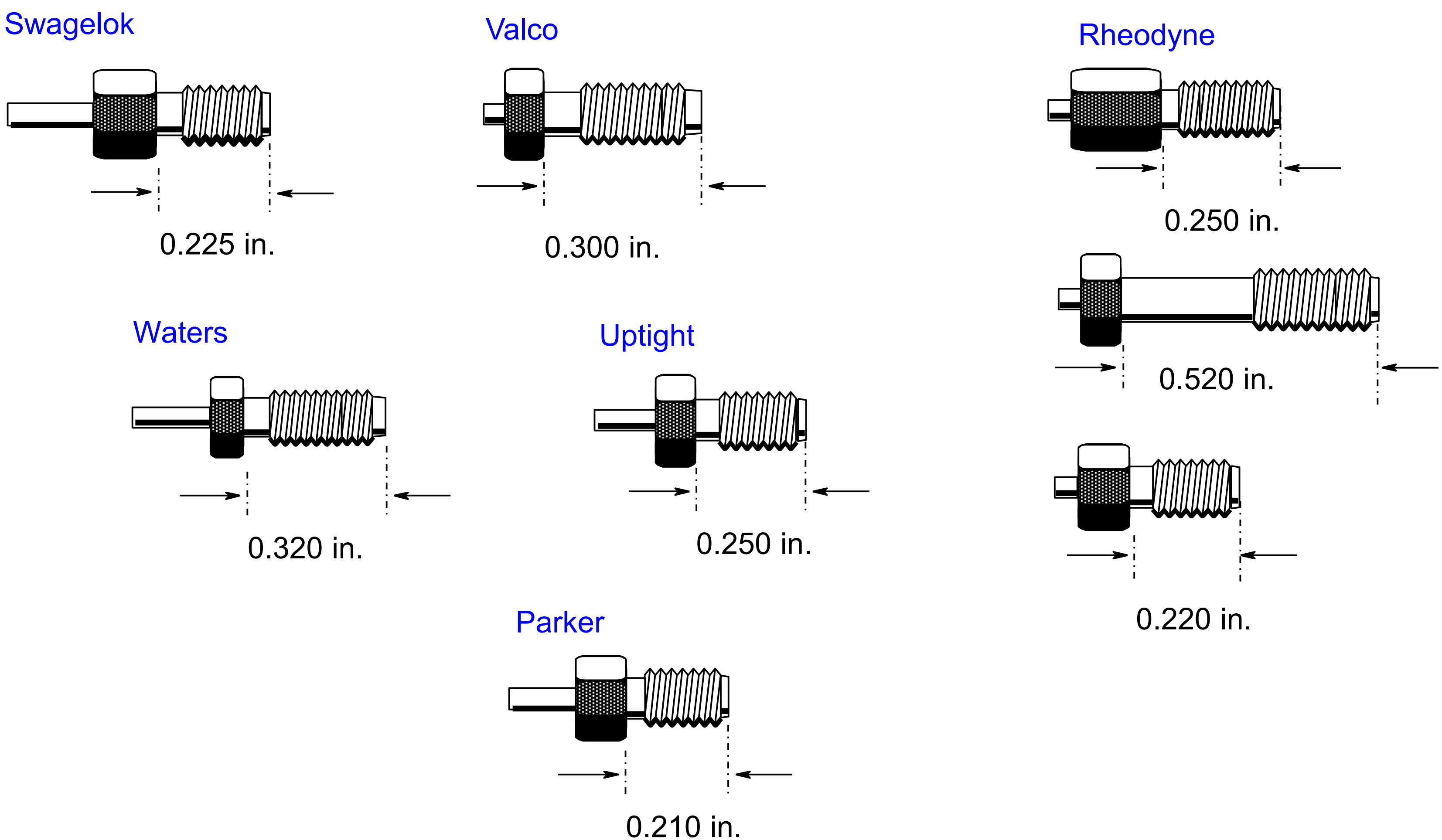
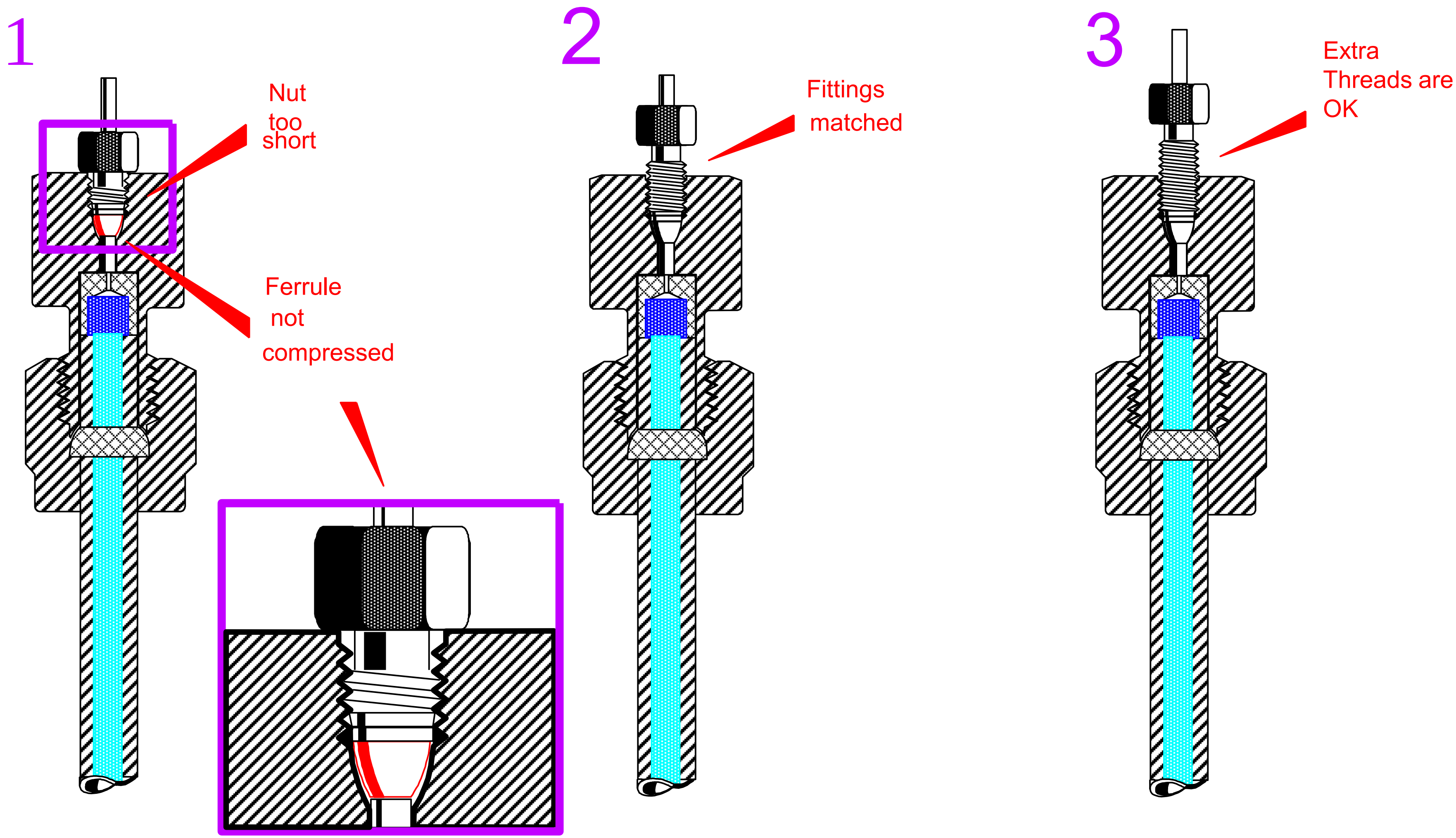
Compression Screws from Various Manufacturers



Troubleshooting LC Fittings, Part I. J. W. Dolan and P. Upchurch. LC/GC Magazine 6:788 (1988)

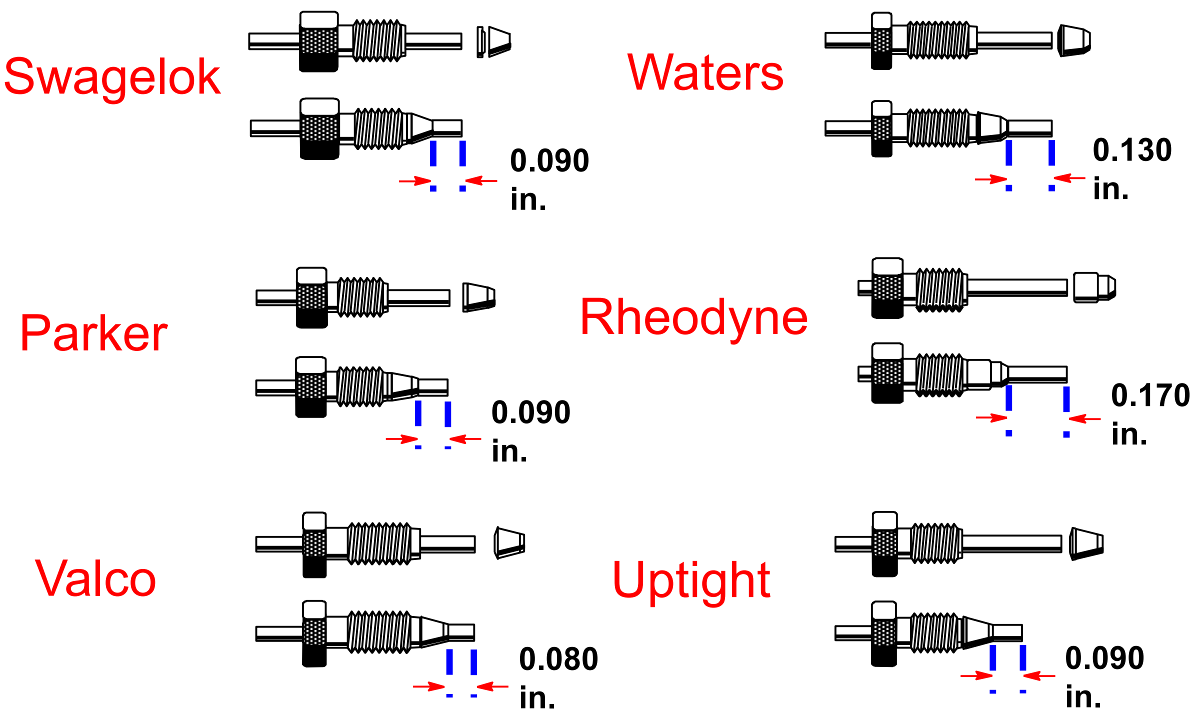
STEVE GEORGE

Mismatched Compression Screws



Column Connectors Used in HPLC

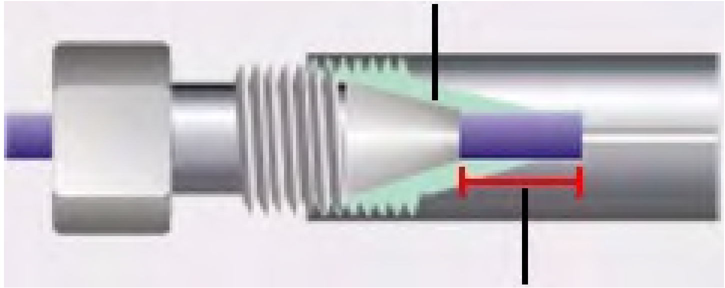
Troubleshooting LC Fittings, Part II. J. W. Dolan and P. Upchurch. LC/GC Magazine 6:788 (1988)



What Happens If the Connections Poorly Made ?

Wrong … too long

Ferrule cannot seat properly

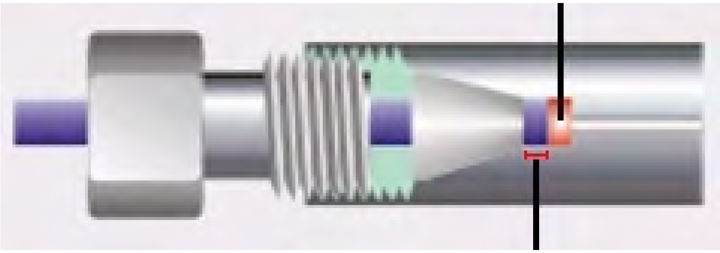


X

If Dimension X is too long, leaks will occur

Wrong … too short

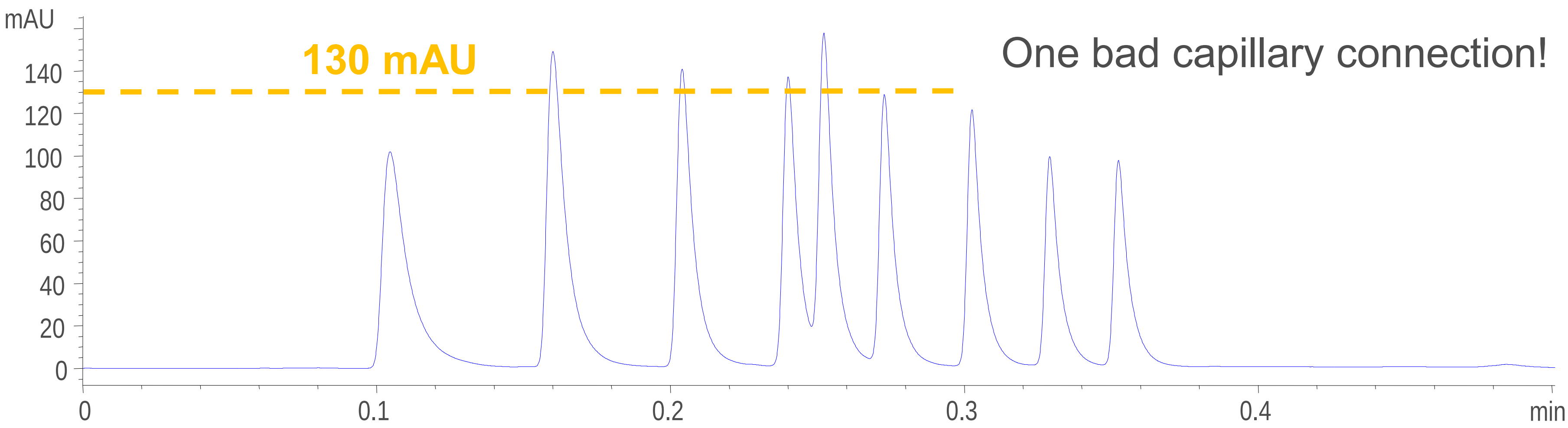
Mixing Chamber

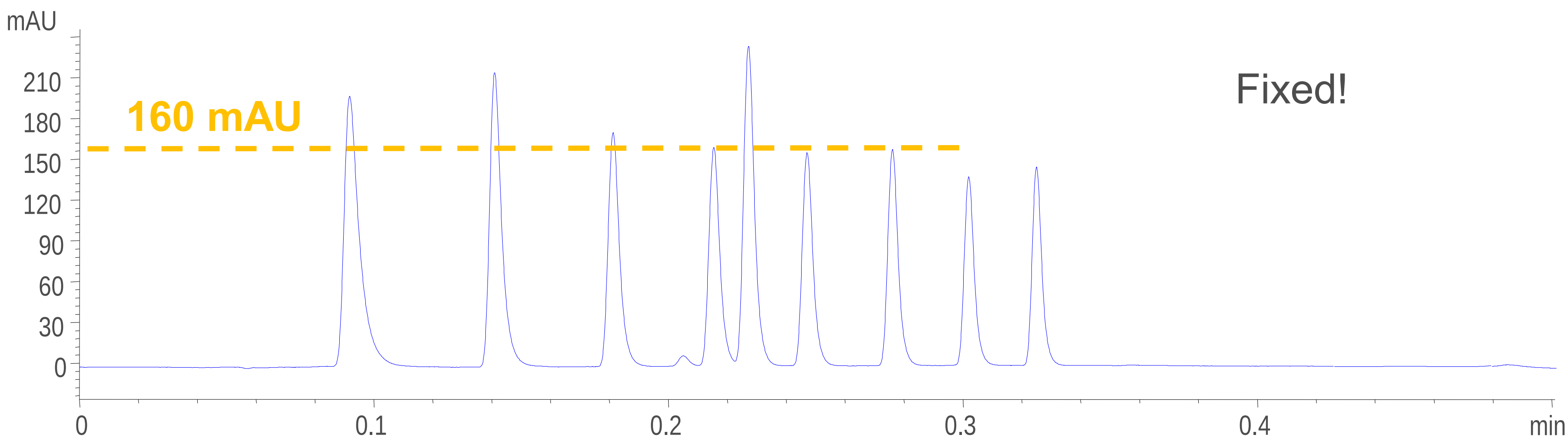


X

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

Influence post-column capillary connections

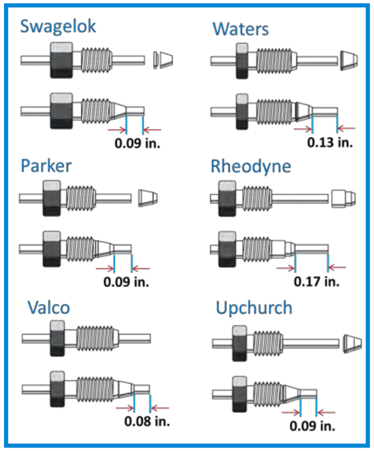




Agilent Technologies

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.



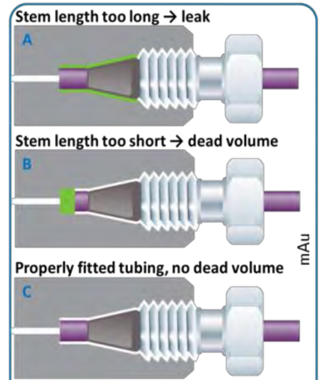


Figure 2: Examples of incorrect (A, B) and correct (C) fitting connection

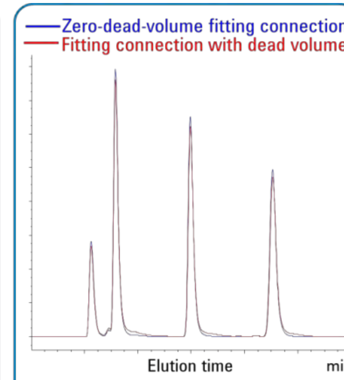
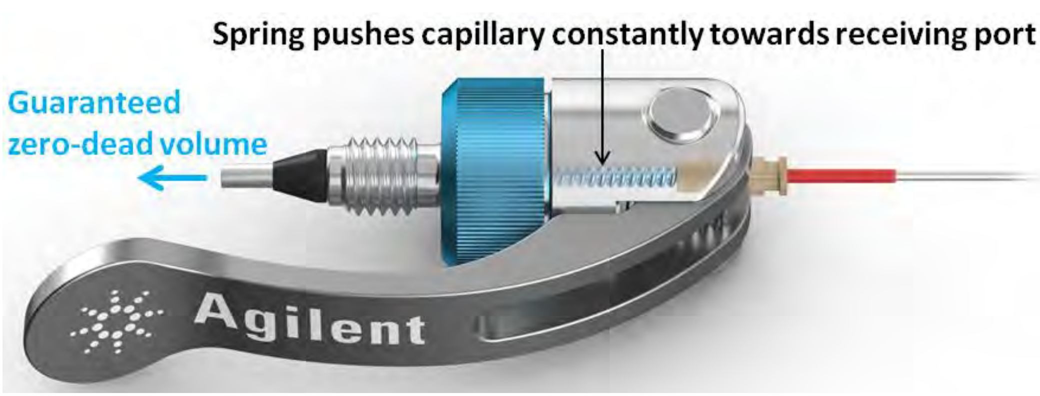


Figure 3: Comparison of chromatograms with correct and incorrect fitting

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

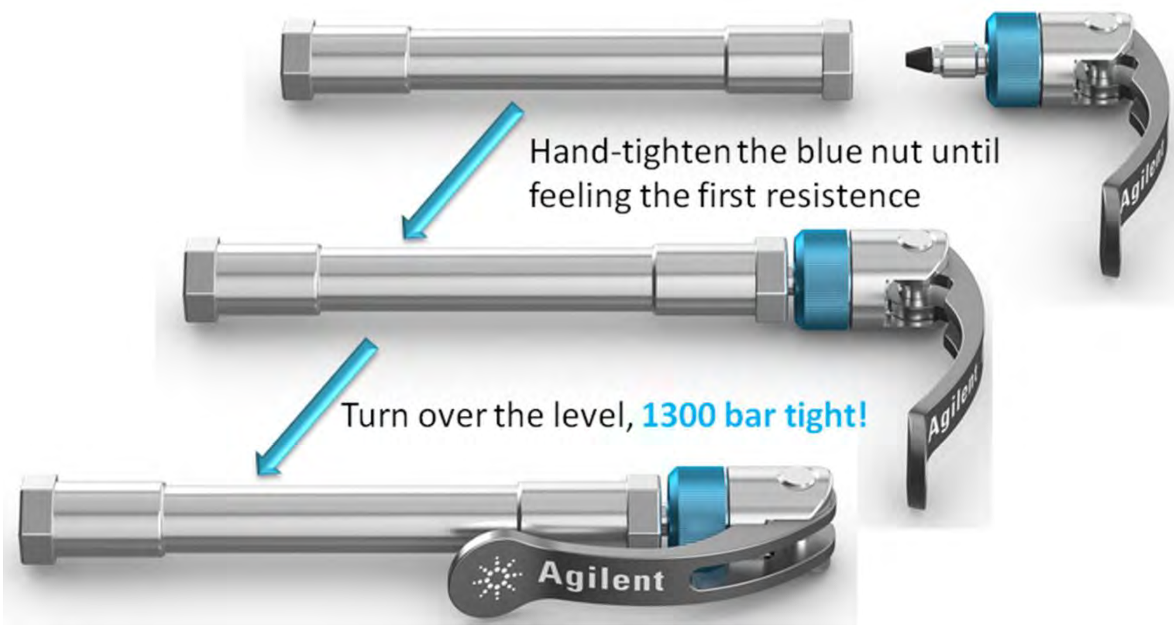


LESS STRESS MORE SUCCESS Agilent A-Line LC supplies

The Agilent A-Line Quick Connect Fitting

3 Single Steps to Connect a column

1
2
3

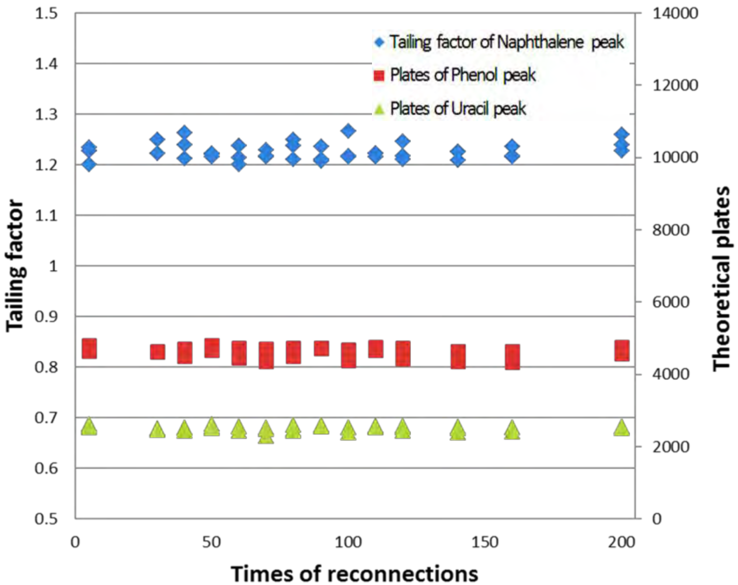


- We have successfully tested the fittings on UHPLC columns from Agilent, Waters, Supelco and Phenomenex.
- The fittings can be used in non-Agilent instruments on all Swagelok compatible connections.

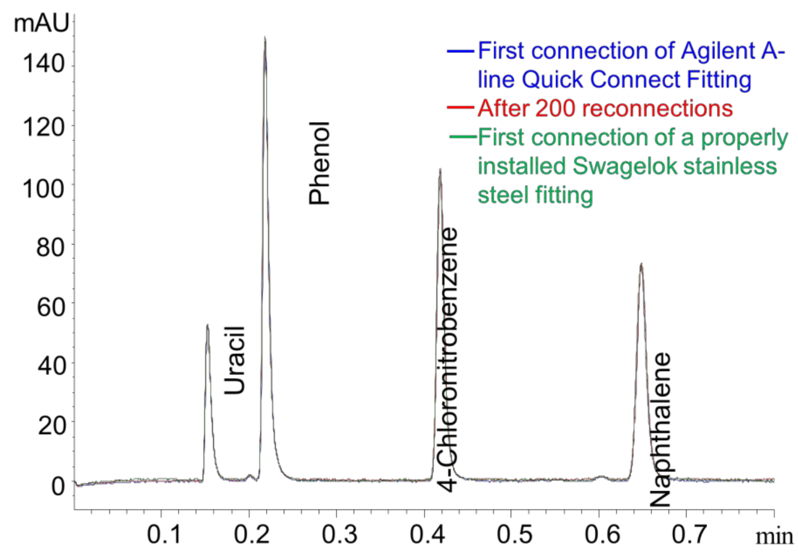
Note: *The fittings must be used with A-Line capillaries, which are not obligatory compatible with capillaries used in non Agilent instruments.*

The Agilent A-Line Quick Connect Fitting
Robustness

Monitoring of tailing factor and theoretical plates number over 200 reconnections of A-Line Quick Connect Fitting.



Chromatogram overlap before and after 200 reconnections



- No visible change of chromatogram after 200 reconnections.
- Tailing factors and theoretical plates stayed constant within the experimental allowance through the reconnection procedures.

Fitting connection kept dead-volume free and leak-free after 200 times of reuses

LESS STRESS MORE SUCCESS

Agilent A-Line LC supplies

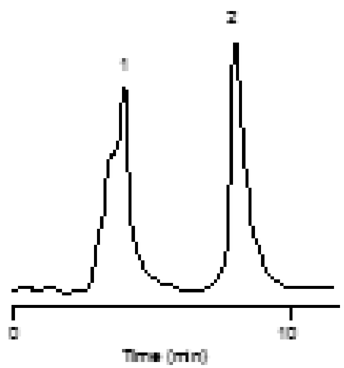
Five Things That You Can Do To Improve The Separation

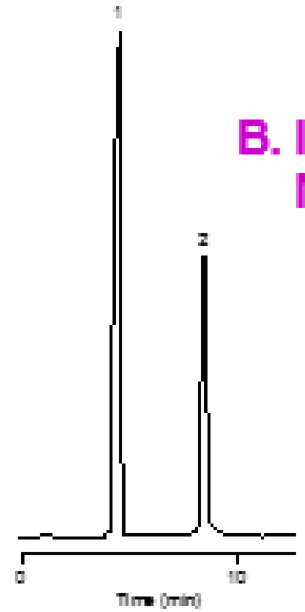
#3 Sample Solvent

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

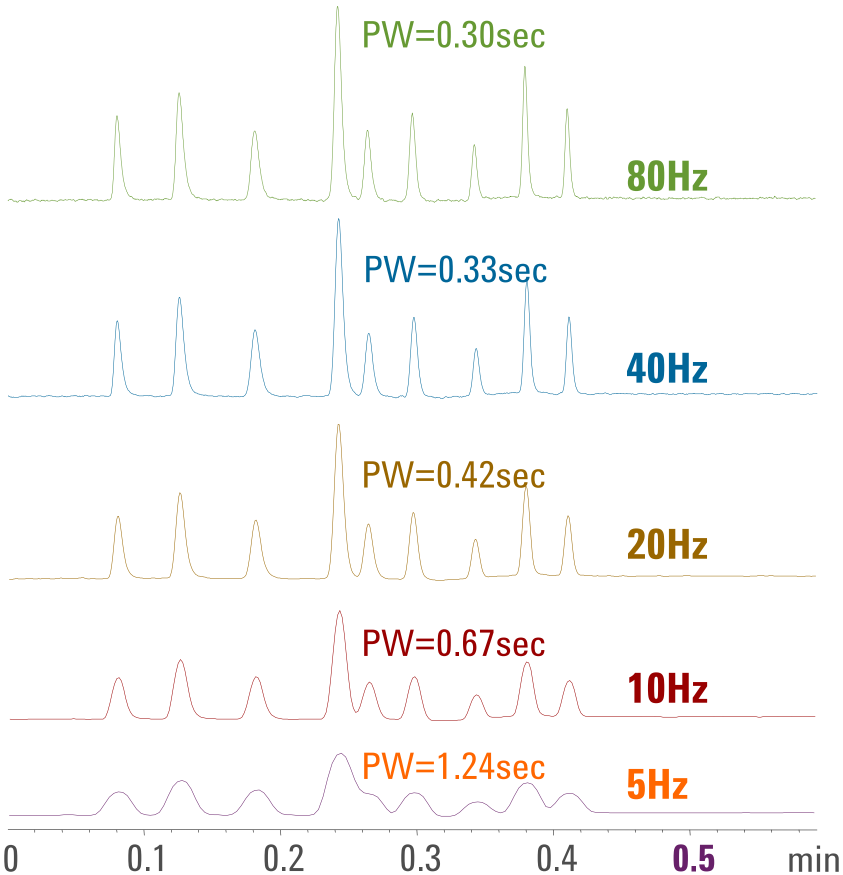
Tip: Injecting in a solvent stronger than the mobile phase can cause peak shape problems such as peak splitting or broadening

Trick: Keep Organic Concentration in Sample Solvent ≤ Mobile Phase

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



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

#5 High Efficiency Columns

Benefits of High Efficiency

Use of smaller columns

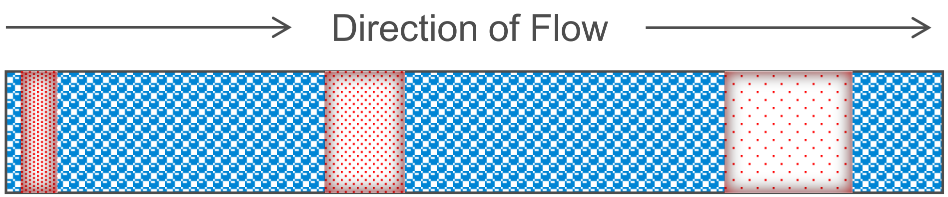
Faster Methods

Higher Sensitivity

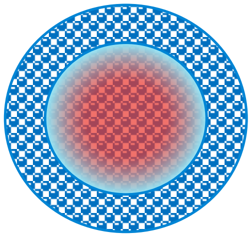
Lower Limitations of Detection

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

a) Longitudinal Diffusion (dispersion)

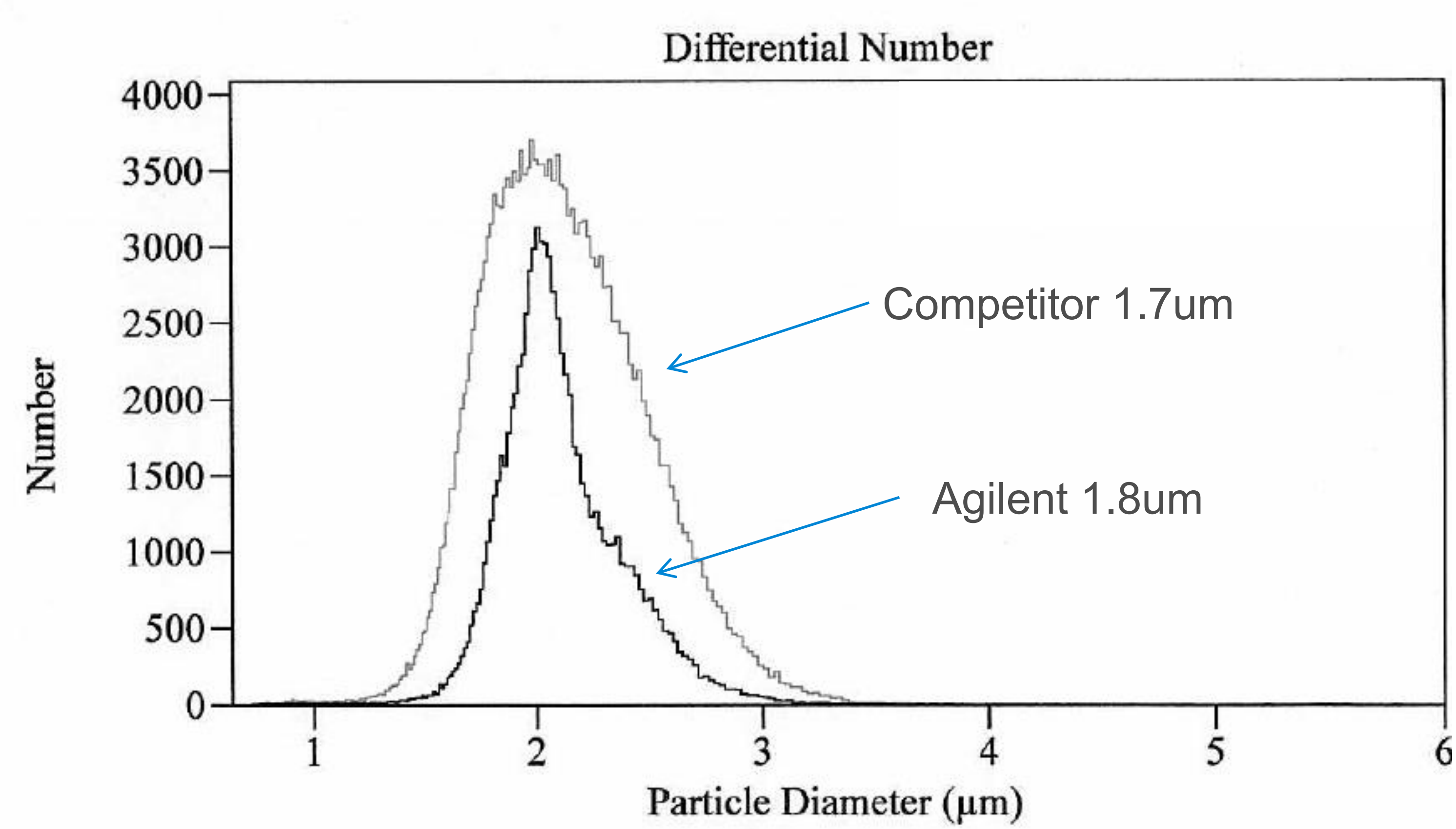


b) Radial Diffusion (dispersion)



Greater Control of Particle Size Distribution

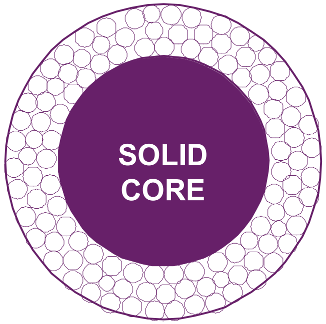
A Little Known Secret for Better High Pressure Performance

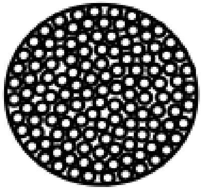


(Packing from first generation 2.1 x 100mm, 1.7um column)

Agilent Particle Choices for UHPLC

Longest History of UHPLC Particle Innovation and Manufacture





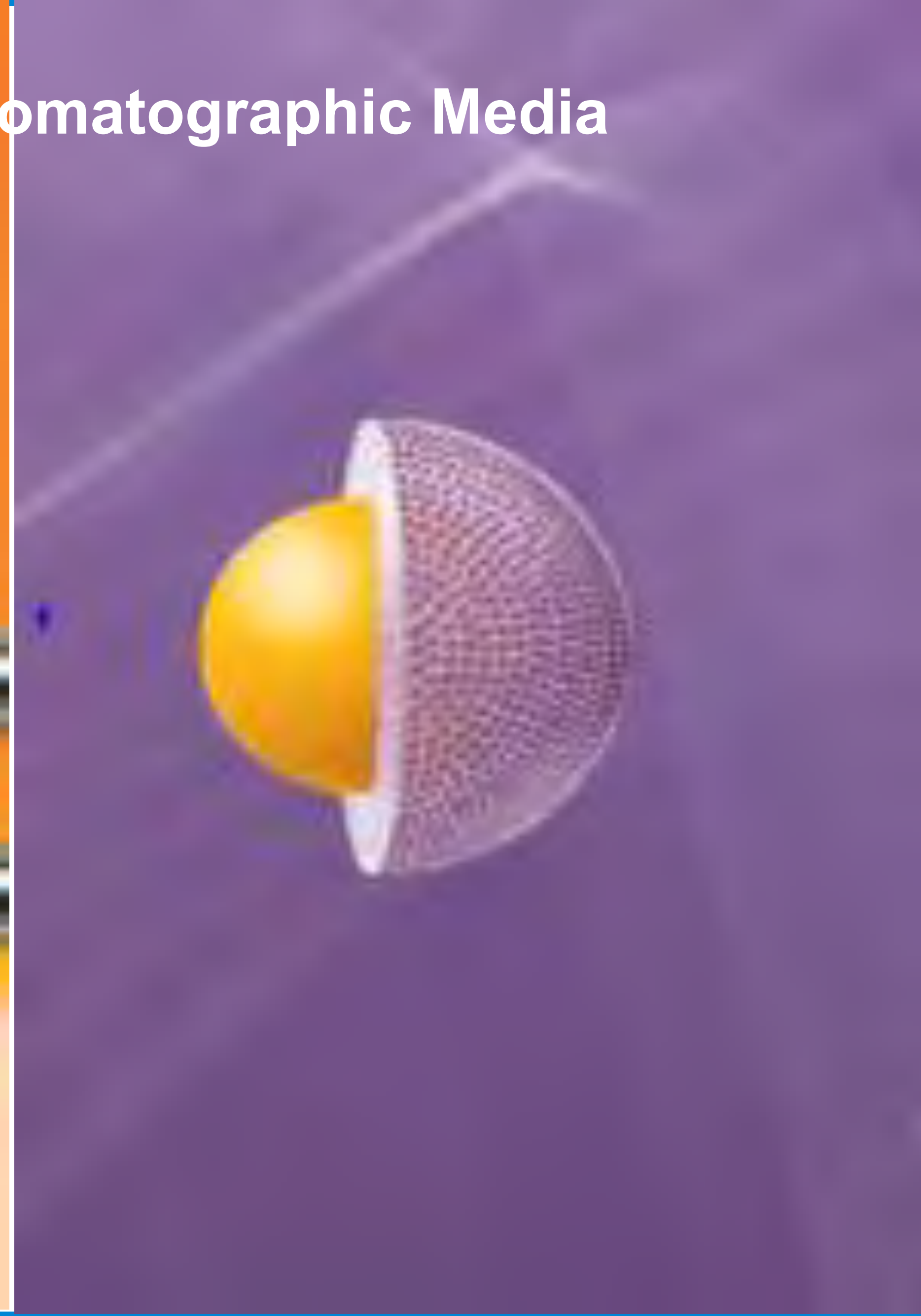
Superficially Porous Particle

Poroshell 300 Commercial Intro 2001

Sub 2um Totally Porous Particle

1.8um Commercial Intro 2003

High Efficiency Chromatographic Media



Comparable Performance Under Moderate UHPLC Conditions

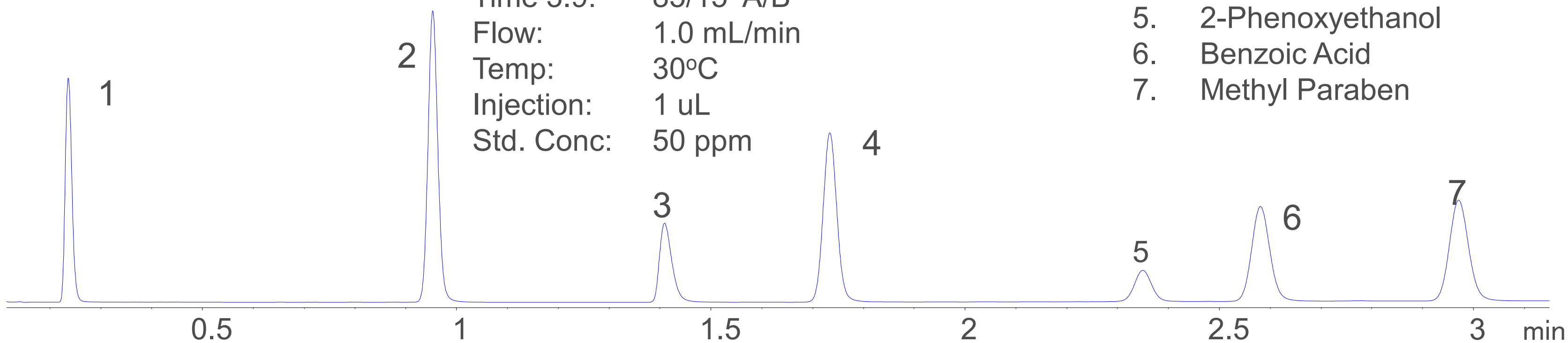
RRHD Eclipse Plus C18 and Acquity BEH C18 (2.1 x 50mm)

A: H_2O (0.05 v% TFA)   B: ACN (0.04 v% TFA)

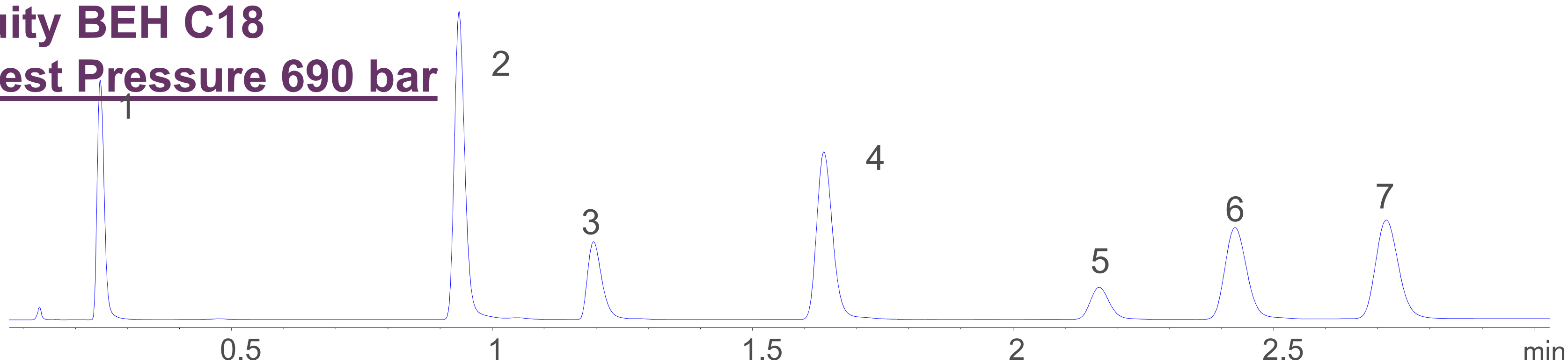
Time 0:	95/5 A/B
Time 2.9:	85/15 A/B
Time 3.9:	85/15 A/B
Flow:	1.0 mL/min
Temp:	30°C
Injection:	1 uL
Std. Conc.:	50 ppm

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

RRHD Eclipse Plus C18
Highest Pressure 630 bar



Acquity BEH C18
Highest Pressure 690 bar



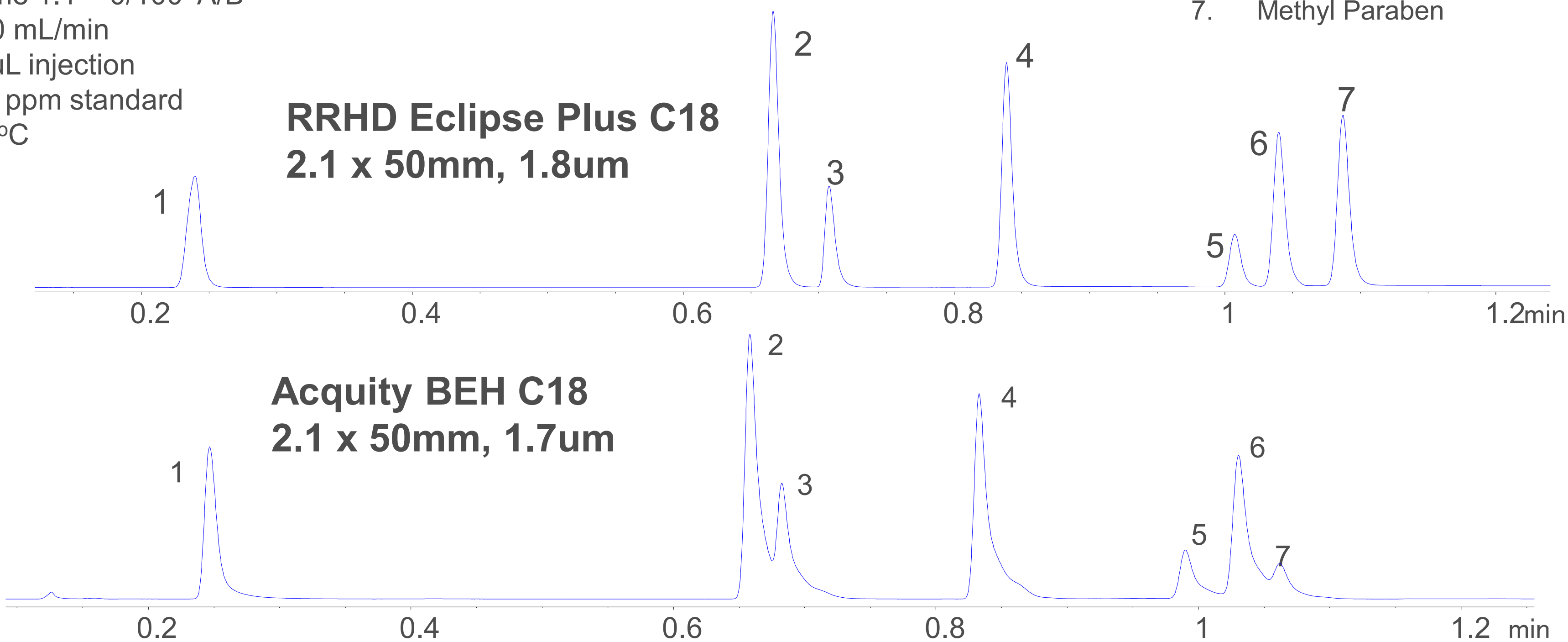
Agilent has lower pressure due to better control over particle size and distribution

Particle Durability Needed to Maintain Performance at Consistently High Pressure

A: H_2O (0.05 v% TFA) B: ACN (0.04 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

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)

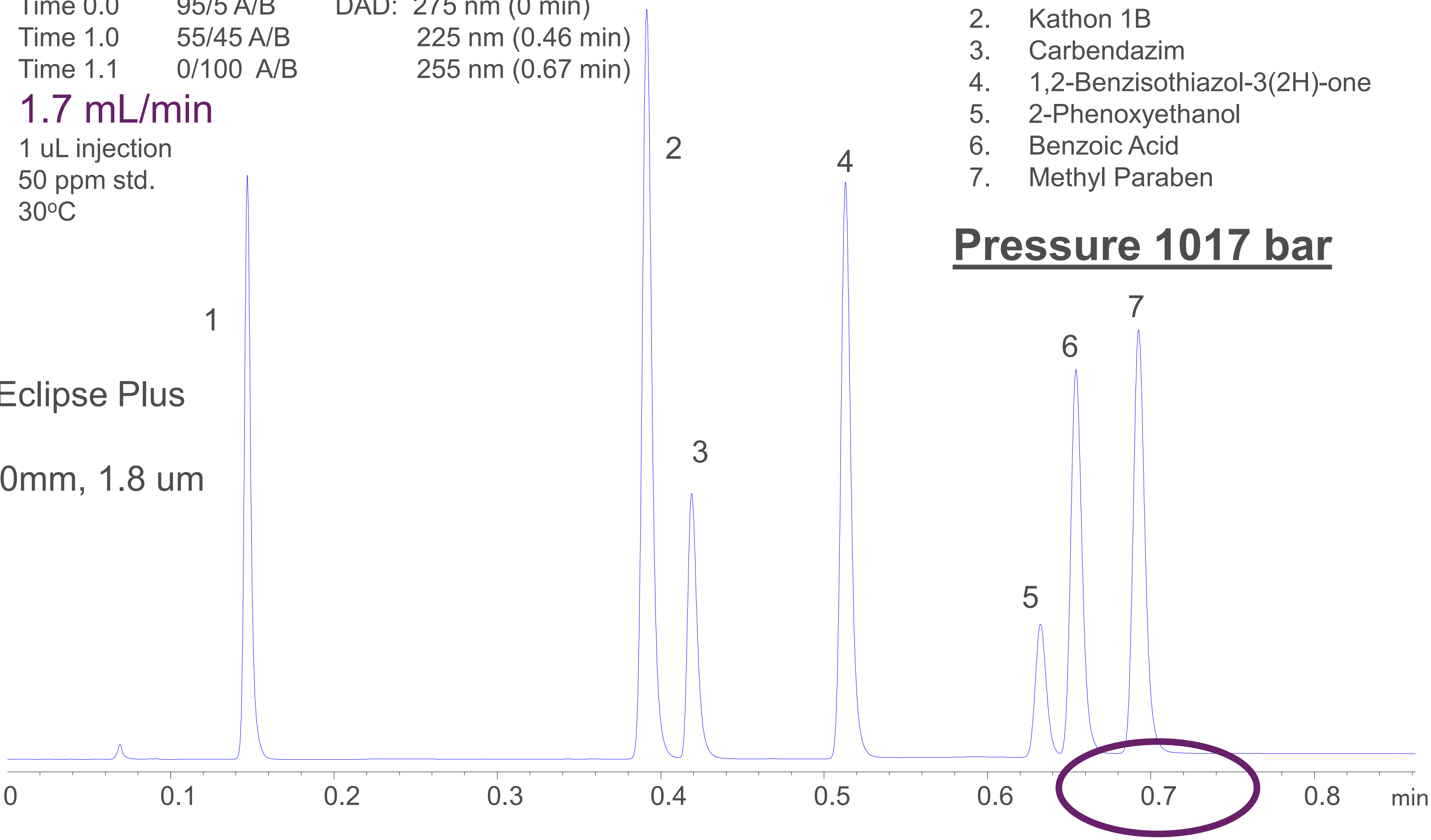
1.7 mL/min

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

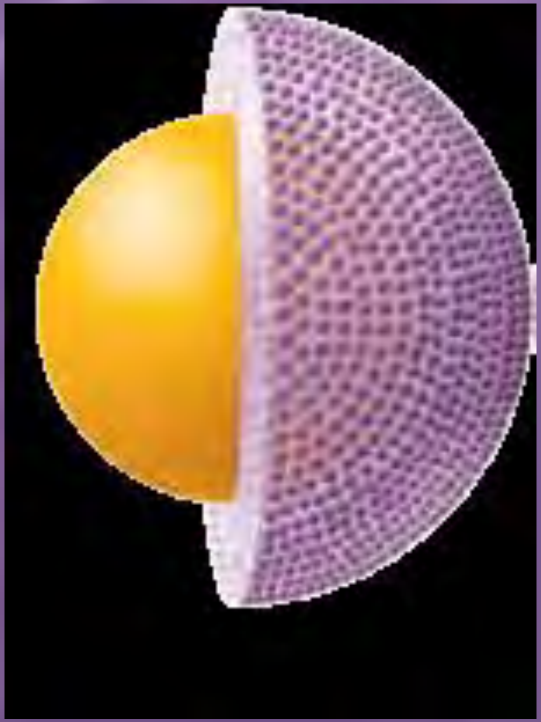
Superficially Solid Core

2.7 µm particle

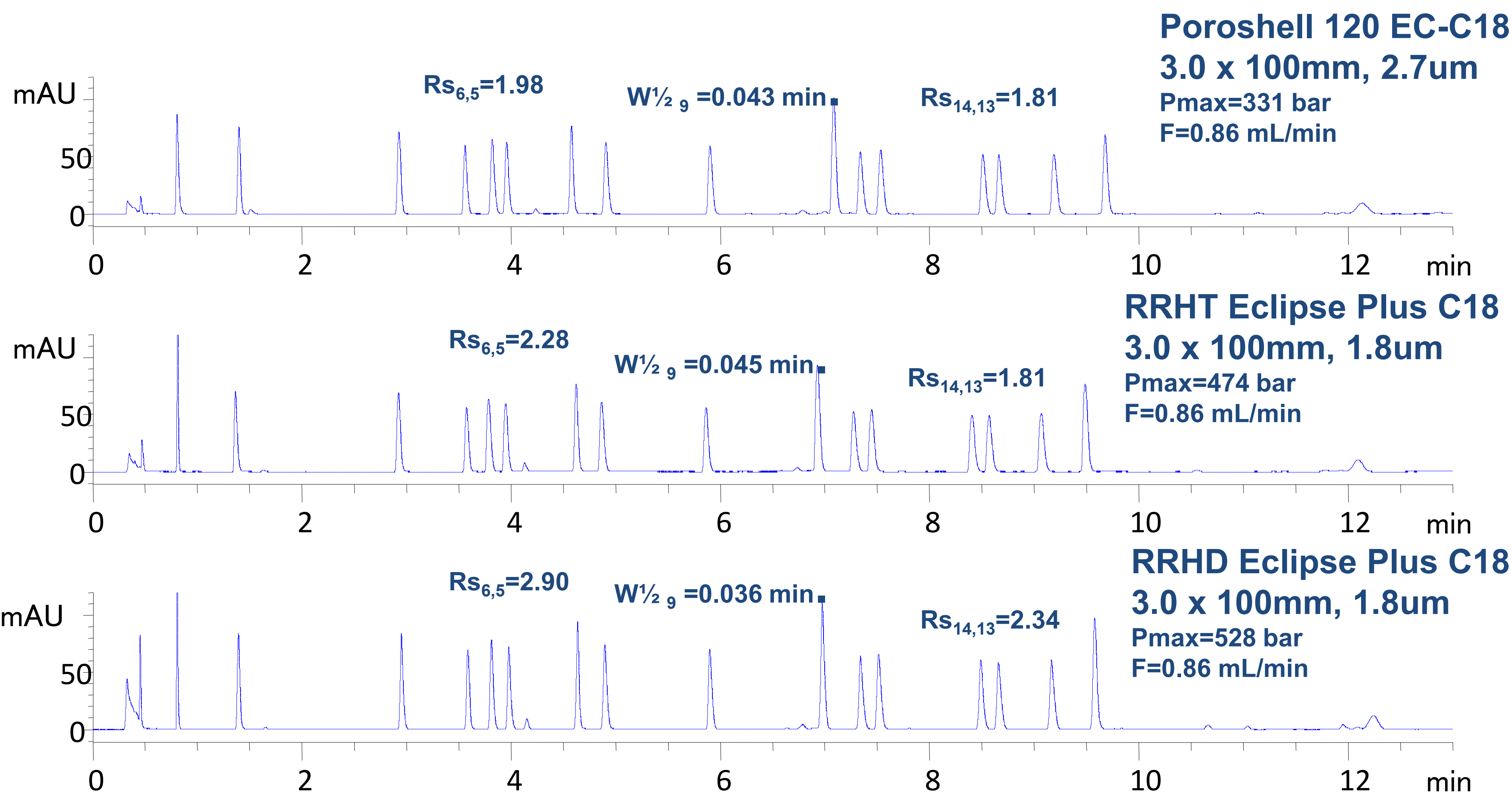
1.7 µm Solid Core

Highly efficient

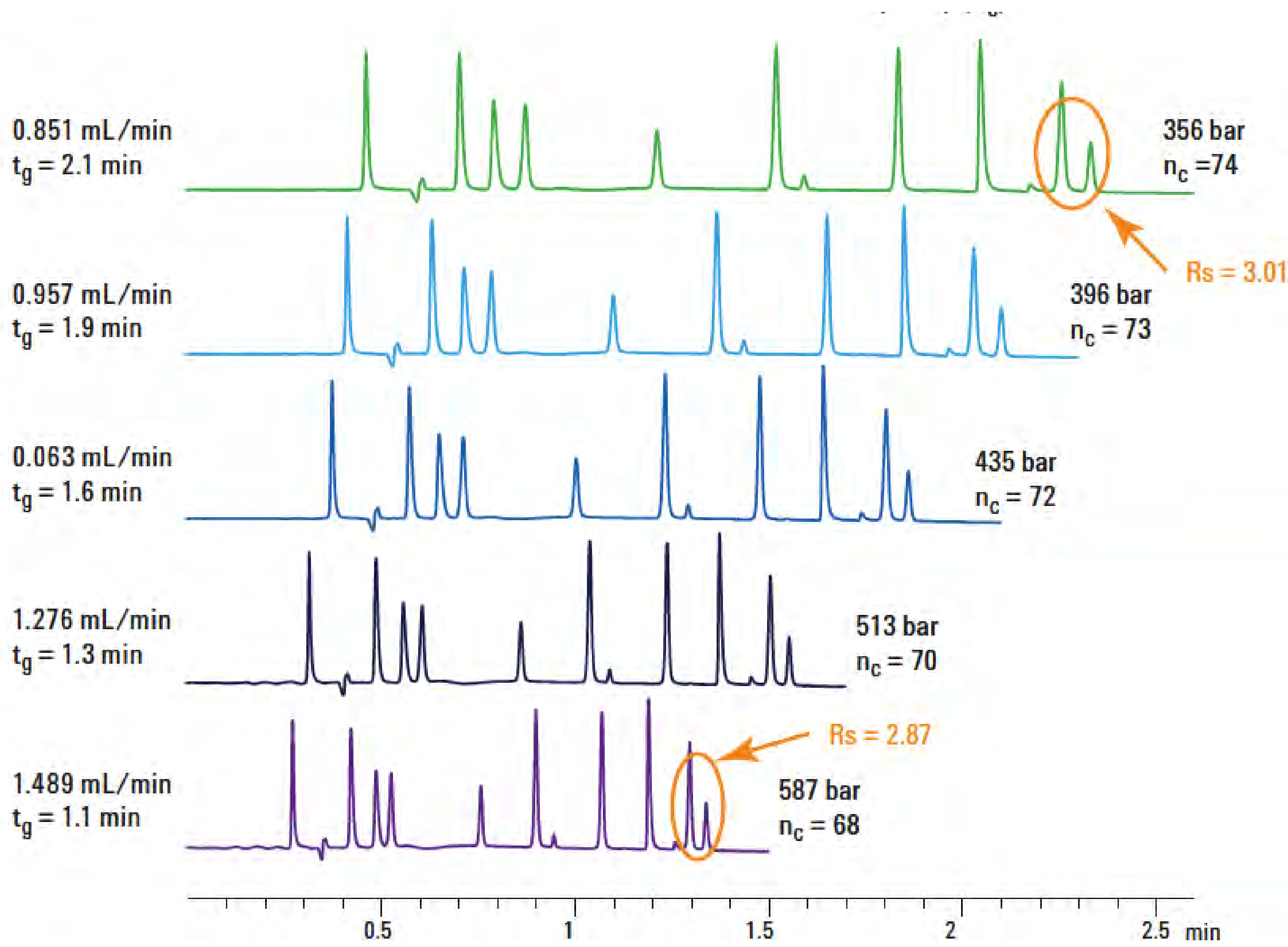
Lower Back Pressure Than Sub-2u Particles



RRHD and Poroshell Columns Provide Similar Performance 1290 Infinity



High Flow Rates Also Possible on Poroshell



In Summary: Optimize

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