YouHPLC/MS

Solutions for your instrument

Understanding what YOU can do to get faster, better chromatography from your HPLC system

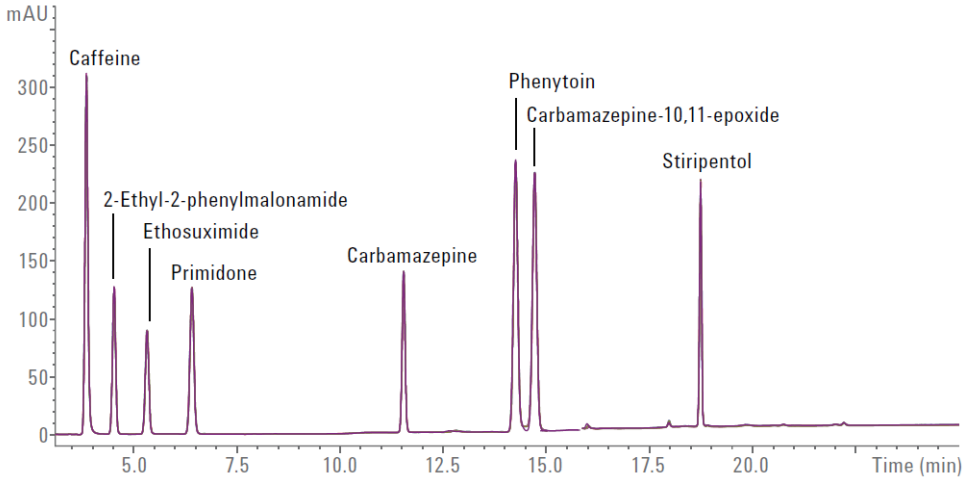
Agilent

Trusted Answers

Outline of Today's talk

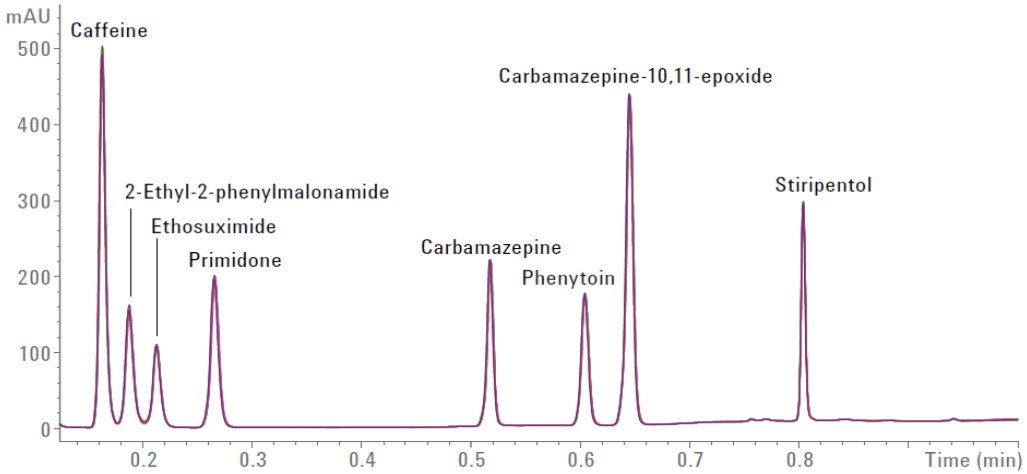
- Why do people move to UHPLC
  - Advantages
  - Side effects/challenges
  - Math behind the move
- Adding LCMS to the mix
  - Solvent volume and Drying Effects
- Dispersion – the enemy of sharp peaks
  - Connections and mixing
  - Extra-column volume – LD Heat Exchanger, tubing

Why move from HPLC to UHPLC?



	RT RSD (%)	Area RSD (%)	Resolution
Caffeine	0.040	0.153	9.0
2-ethyl-2-phenylmalonamide	0.043	0.083	4.8
Ethosuximide	0.041	0.248	5.2
Primidone	0.046	0.143	6.3
Carbamazepine	0.018	0.203	32.1
Phenytoin	0.019	0.163	16.4
Carbamazepine-10,11-epoxide	0.015	0.648	2.3
Stiripentol	0.007	0.533	7.4

Figure 1. HPLC Analysis of seven antiepileptic drugs and caffeine (overlay of six consecutive runs) on an Agilent ZORBAX SB-C18, 4.6 × 150 mm, 5-µm column, using the Agilent 1290 Infinity LC system.



	RT RSD (%)	Area RSD (%)	Resolution
Caffeine	0.166	0.295	5.3
2-ethyl-2-phenylmalonamide	0.302	2.527	2.5
Ethosuximide	0.323	1.459	2.4
Primidone	0.318	0.229	5.0
Carbamazepine	0.057	0.265	25.7
Phenytoin	0.027	0.259	9.3
Carbamazepine-10,11-epoxide	0.017	0.206	5.0
Stiripentol	0.006	0.527	2.9

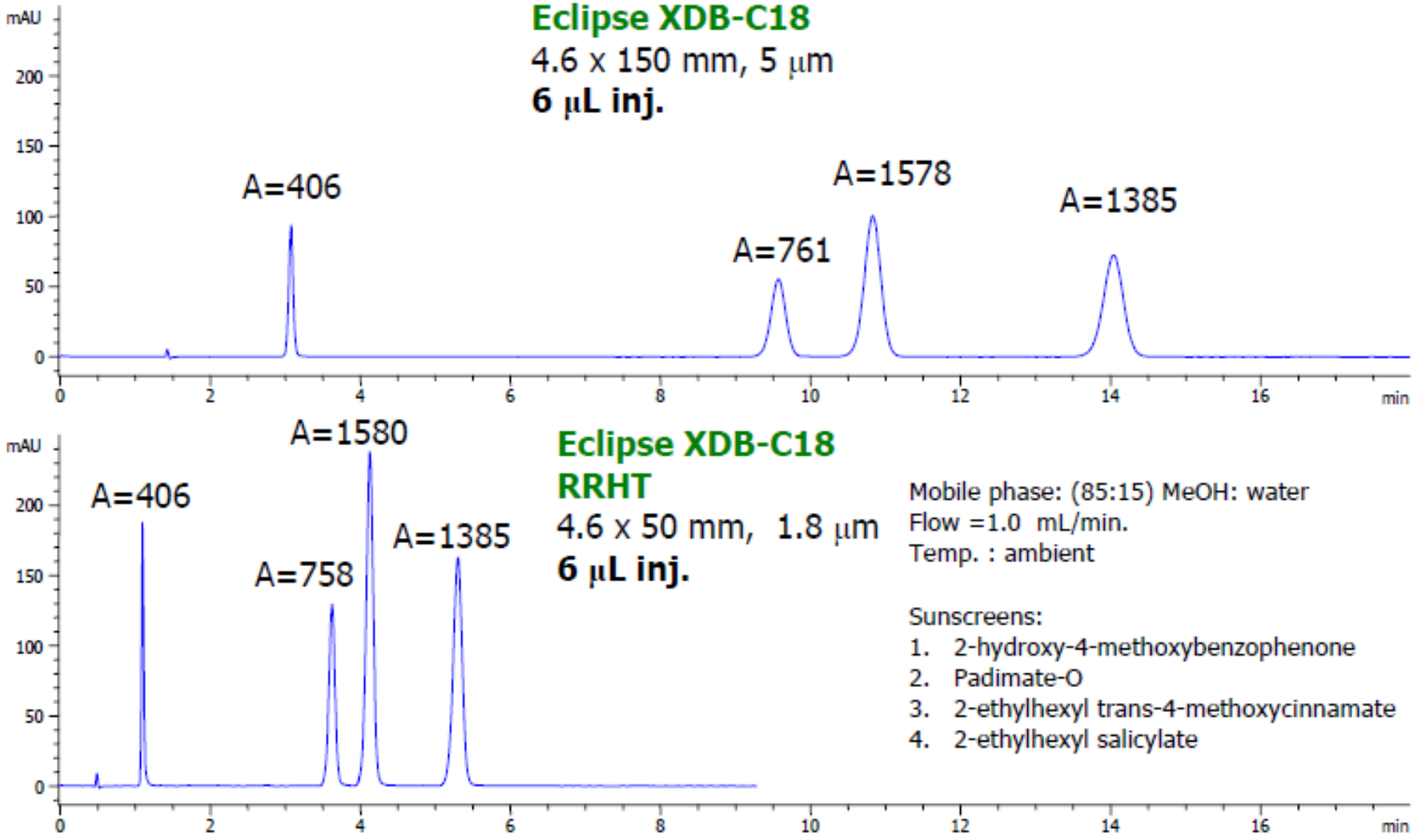
Figure 5. UHPLC analysis (optimized for speed) of seven antiepileptic drugs and caffeine (overlay of six consecutive runs) on an Agilent ZORBAX SB-C18, 2.1 × 50 mm, 1.8-µm column, using the Agilent 1290 Infinity LC system.

Advantages of moving to UHPLC

- Faster run times
- Less solvent consumption – savings on both the cost to purchase and the cost to dispose of
- Higher throughput – shorter run times means more samples per work day
- More resolving power – if we use longer columns with smaller particles, we increase the surface area of bonded phase and therefore increase our ability to resolve analytes
- Sharper peaks also mean better sensitivity – The area under the peak should remain constant, so as the width decreases the height increases. Assuming the noise remains fairly constant, this works out to better S/N

Another Benefit is Increased Sensitivity (Signal/Noise) Comparing Same Injection Volume:

4.6 x 150 mm, 5 µm vs. 4.6 x 50 mm, 1.8 µm RRHT



Eclipse XDB-C18
4.6 x 150 mm, 5 µm
6 µL inj.

Eclipse XDB-C18
RRHT
4.6 x 50 mm, 1.8 µm
6 µL inj.

Mobile phase: (85:15) MeOH: water
Flow =1.0 mL/min.
Temp. : ambient

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

Side effects or Challenges of the Switch

- Pressure
  - UHPLC is based upon the idea of switching to smaller particle sizes to increase resolution per unit of column length. The major side effect of using these smaller particles is a significant increase in back pressure
  - *Ex*: 3.0mm x100mm column with 5um particle size at 1.0 ml/min we expect a pressure of ***90-100 bar***

    3.0mm x100mm column with 1.8um particle size at 1.0 ml/min we expect a pressure around ***725 bar***.
- Extra-column and delay volumes have a much higher impact on our results
  - Sharper peaks from smaller columns have much smaller peak volumes – peak width by flowrate gives peak volume.
  - Smaller peak volumes for constant amount of sample means higher sample concentration when in the flowpath

The Math behind the Move:

Flow rate & linear velocity

Flow rate is a measure of how fast the pump is delivering mobile phase, typically in mL/min

Linear velocity is a measure of the rate that the mobile phase travels through your column, typically in mm/min

Linear velocity is calculated as the flow rate divided by the column volume:length ratio

The column volume is either estimated as 50%-60% of the geometric volume a cylinder or it can be calculated using a void volume marker. When using a void volume marker, you simply multiply the retention time of the marker (the void time) by the flow rate to get a void or column volume.

When transferring methods to smaller columns, we maintain separation characteristics by keeping linear velocity constant. We do this by scaling to the ratio of the squared diameters:

Linear velocity of a 4.6 x 150mm column with 1.0 ml/min flowrate = 100mm/min

Scale this to 2.1mm ID:

$(2.1^2/4.6^2) \times 1.0\, ml/min = 0.208 \approx 0.2\, ml/min$ ***maintains linear velocity***

The Math behind the Move:

Rescaling the gradient

Once we calculate the new flowrate, for constant linear velocity, we then have to recalculate the gradient times.

$$t_{G,new} = t_{G,old} \cdot \frac{F_{old}}{F_{new}} \cdot \frac{L_{new}}{L_{old}} \cdot \left(\frac{d_{c,new}}{d_{c,old}}\right)^2$$

Where:

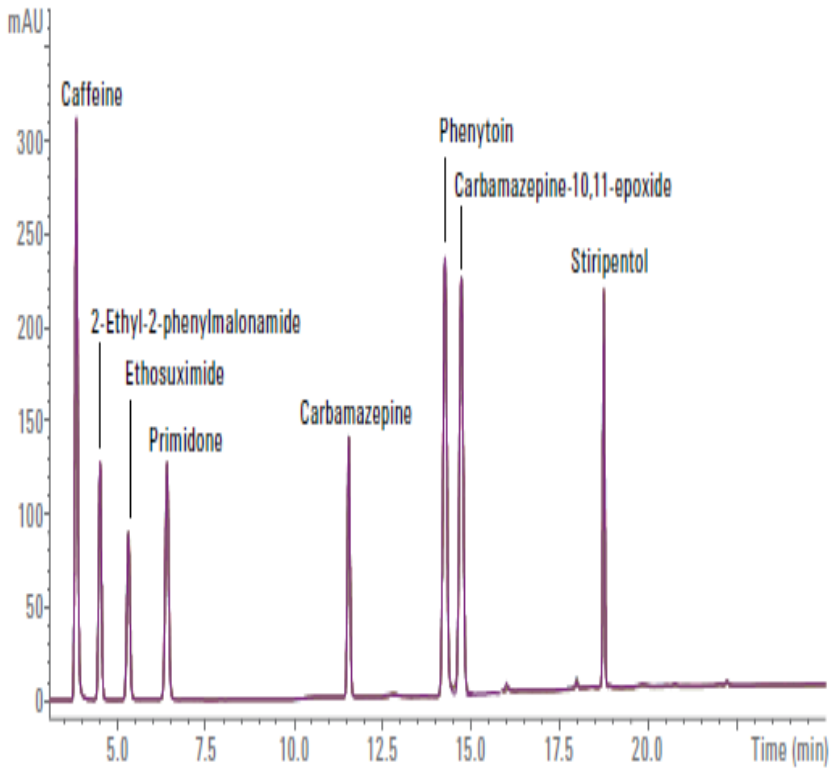
t_G is the gradient time

F is the flow rate

d_c is the column diameter

The Math behind the Move:

Column Volume and Re-equilibration time



Column volume is the amount of space in your column after the stationary phase, which is to say the amount of solvent that your column will hold.

When we re-equilibrate a column we typically want 6 column volumes.

Consider the amount of time that would add to the various chromatograms:

4.6mm x 150mm has approximately 1.5mL of column volume

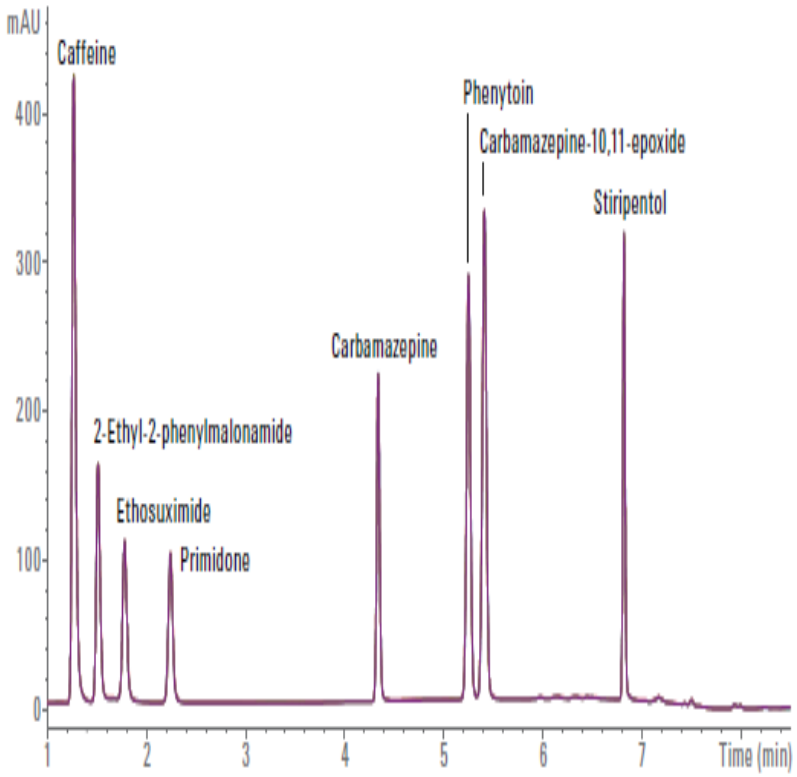
6 column volumes to re-equilibrate = 9mL to re-equilibrate

9ml at 1 mL/min means 9 minutes of re-equilibration

20 minute run time + 9 minute re-equilibration = 29 minutes total

The Math behind the Move:

Column Volume and Re-equilibration time



Here we scale the 4.6mm x150mm down to a 2.1mm x 50mm.

To maintain linear velocity we adjust flowrate to 0.2 mL/min

If the gradient resets at 7 minutes how much time to re-equilibrate?

2.1mm x 50mm has approximately 0.1mL of column volume

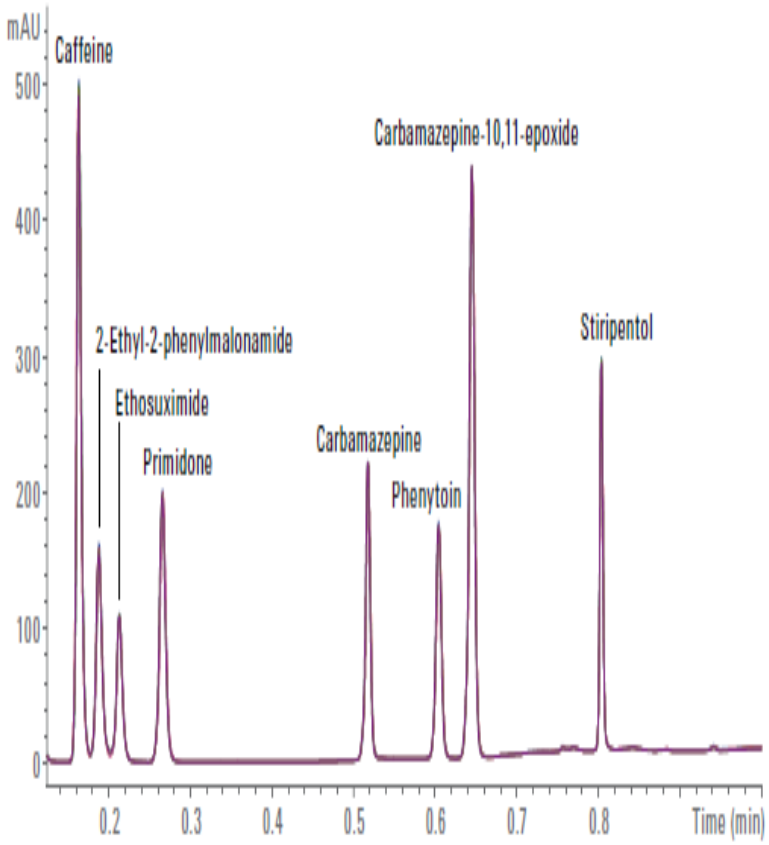
6 column volumes to re-equilibrate = 600uL to re-equilibrate

600uL at 200 uL/min means 3 minutes of re-equilibration

7 minute run time + 3 minute re-equilibration = 10 minutes total

The Math behind the Move:

Column Volume and Re-equilibration time



Ultimately, we have excess resolution and can increase linear velocity to shorten run time.

For a one minute run time and a flowrate of 1.5 mL/min how long do we re-equilibrate?

2.1mm x 50mm has approximately 0.1mL of column volume

6 column volumes to re-equilibrate = 600uL to re-equilibrate

600uL at 1.5 mL/min means 0.4 minutes of re-equilibration

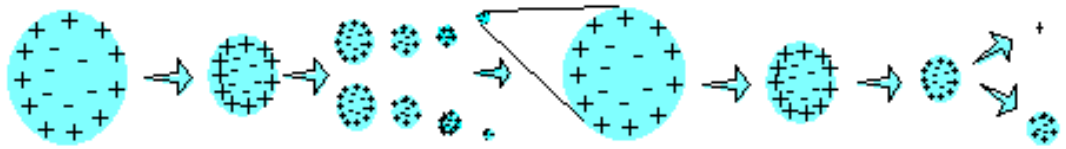
1 minute run time + 0.4 minute re-equilibration = 1.4 minutes total

Solvent Volume and Drying Effects:

The glass ceiling for LCMS method transfer

- Signal to Noise
- Solvent Volume and Drying Effects

Ion Evaporation Model



Evaporation

Coulomb Explosion

Rayleigh Limit

Analyte Ion Is ejected from the surface of the droplet due to field strength at the surface

Solvent Volume and Drying Effects:

What's the "sweet spot" for LCMS?

System Backpressure as it relates to column dimensions and solvent flow rate

Column Dimensions	Flow Rate	Particle Size	Backpressure
4.6 x 150mm	1.0 mL/min	5 um	60 bar
2.1 x 50mm	0.2 mL/min	1.8 um	150 bar
2.1 x 50mm	1.5 mL/min	1.8 um	1100+ bar
2.1 x 50mm	***0.4 mL/min***	***1.8 um***	***300 bar***

Dispersion

- We define the extracolumn volume as the system volume between the point of injection and the detector outlet.
- The major contributors to extracolumn volume are capillaries, heat-exchangers, connectors and fittings, and the detector flow cell.
- Large extracolumn volume causes dispersion of sample and band broadening of analytes, which result in decreased resolution and less sensitivity.
- Take special care with capillary connectors or when mounting columns into a system. Making compromises with connectors often results in the introduction of unintentional and often not easily visible dead volumes that can deteriorate a separation significantly.
- Extracolumn volume and small capillary inside diameters must be taken seriously under consideration, when using small column inside diameters.

Non-column Sources of Dispersion

- General:
  - Interconnecting tubing (i.d., length, internal surface)
  - Connectors (unions, tees, bulkhead fittings)
  - Guard columns and/or inline particle filters
  - Switching valves for autoSPE, column selection, column regeneration
- Sampler:
  - Diluent strength and injection volume
  - Sample aspirating needle and loading/transfer port
  - Sampler switching valve(s) contacting sample
- Detection:
  - Inlet heat exchangers, flow cell volume and geometry
- Data filtering effects in high speed applications

Tip: Poorly Made HPLC Connections Can Cause Peak Broadening

The System Has Been Optimized and :

- All Tubing Lengths Are Minimum
- Smallest Diameter Tubing Used
- Proper Flow Cell Volume

Symptom: Poor peak shape, broadening

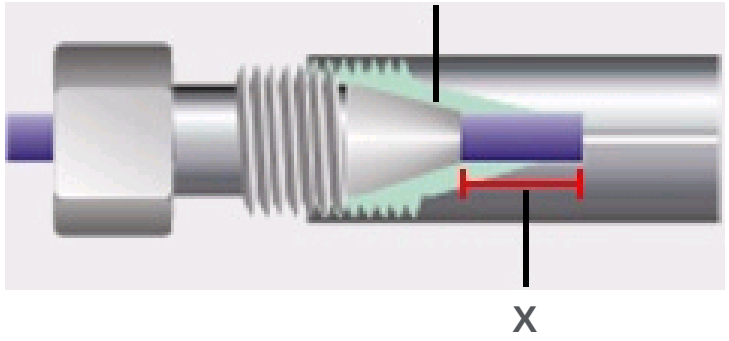
What Is Wrong?

Have You Made the Connections Properly?

Dispersion: What Happens If the Connections Poorly Made ?

Wrong … too long

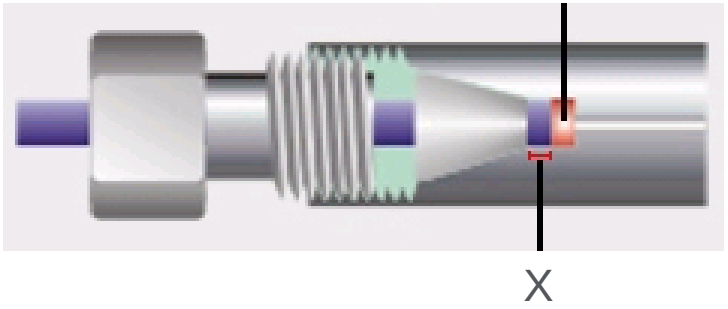
Ferrule cannot seat properly



If Dimension X is too long, leaks will occur

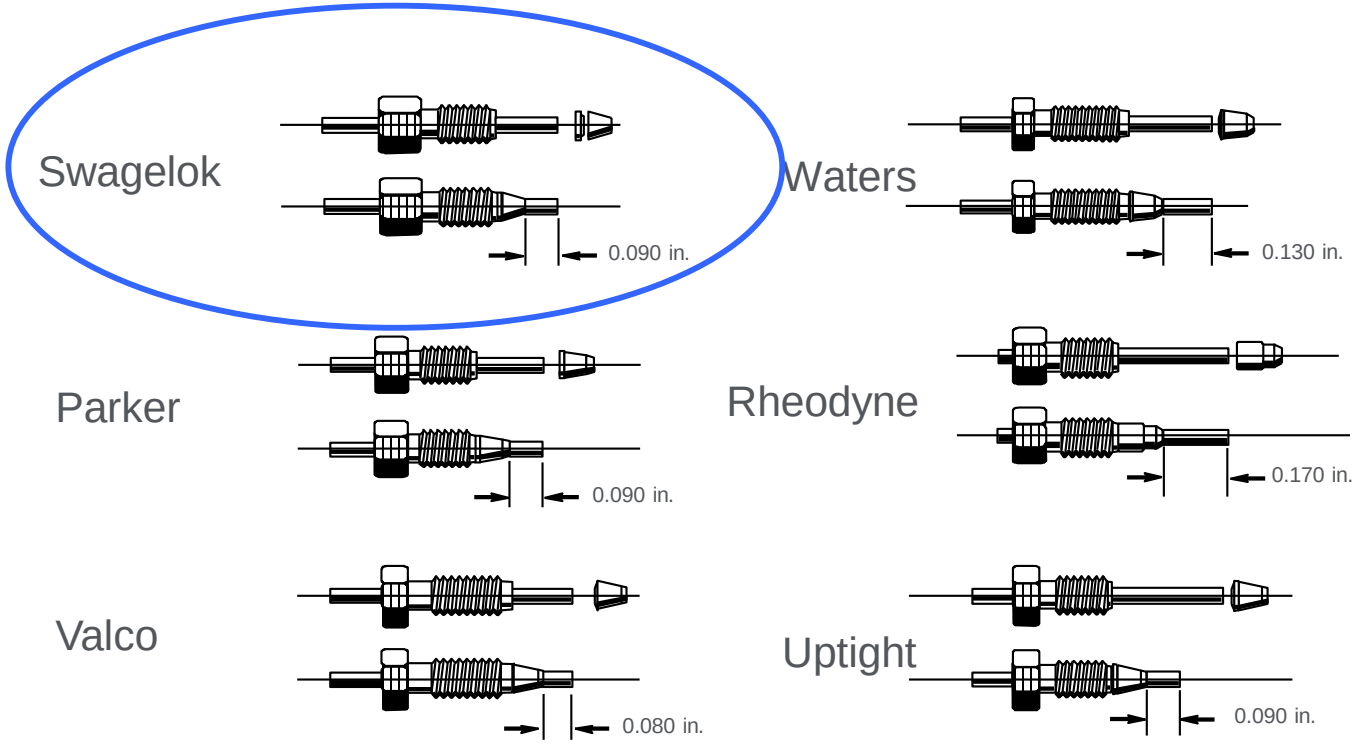
Wrong … too short

Mixing Chamber



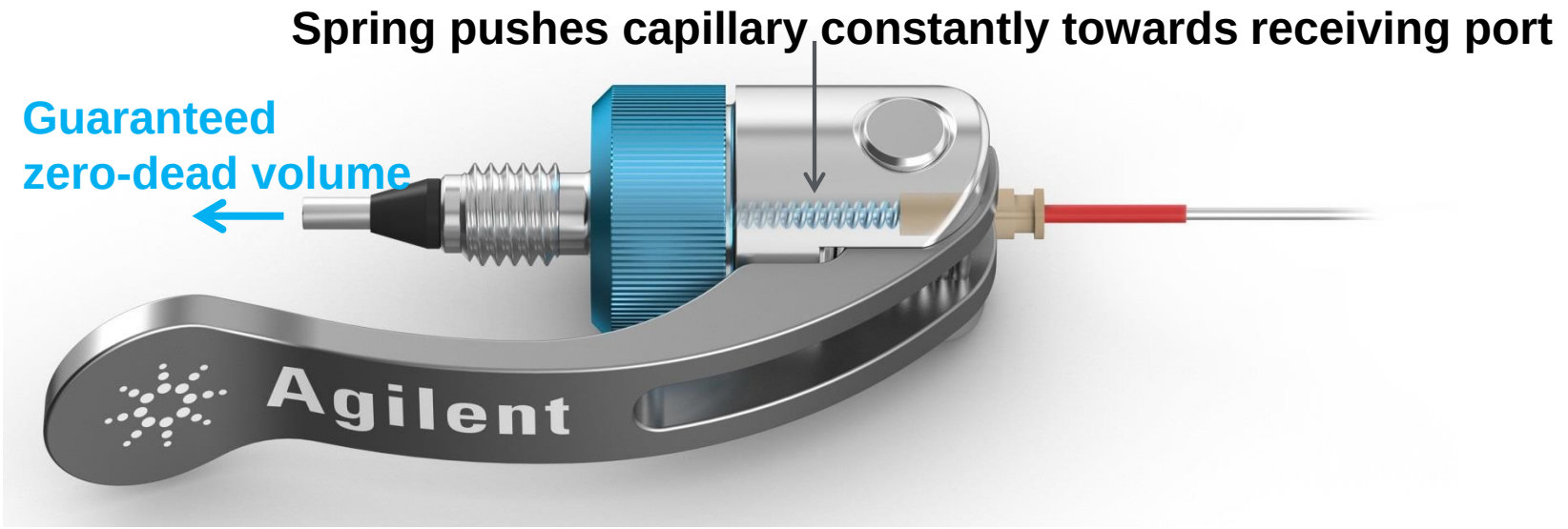
If Dimension X is too short, a dead-volume,
or mixing chamber, will occur

Fittings



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

Designed-In Supplies for 1290 Infinity II
Agilent A-Line Quick Connect Fitting



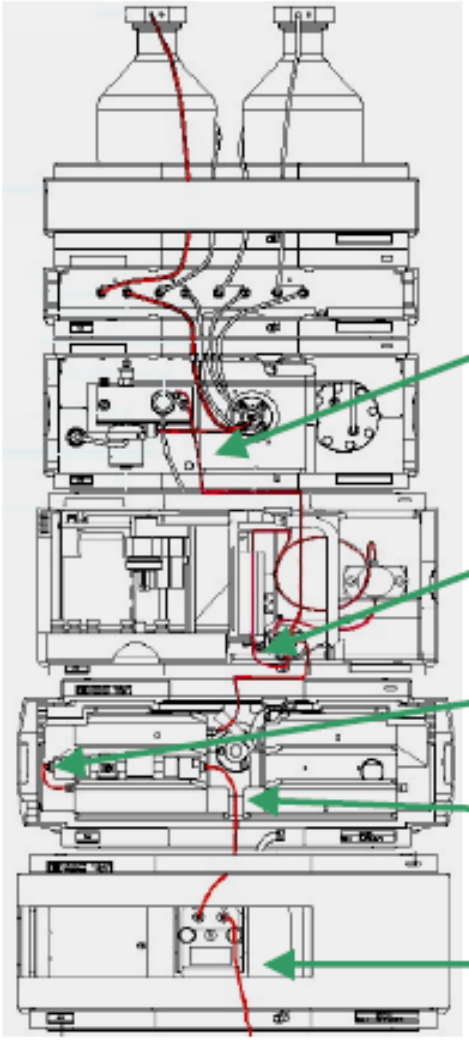
- Spring constantly pushes the tubing against the receiving port, delivering a reproducible connection with **no dead volume for consistent chromatographic performance**
- Spring assembly, including the lever, applies a constant force that presses the ferrule onto the tubing, so that **tubing slippage is avoided**
- **Compatible with all types of LC columns**
- **Little resistance** needed to tighten the fitting

Non-column Sources of Dispersion

- General:
  - Interconnecting tubing (i.d., length, internal surface)
  - Connectors (unions, tees, bulkhead fittings)
  - Guard columns and/or inline particle filters
  - Switching valves for autoSPE, column selection, column regeneration
- Sampler:
  - Diluent strength and injection volume
  - Sample aspirating needle and loading/transfer port
  - Sampler switching valve(s) contacting sample
- Detection:
  - Inlet heat exchangers, flow cell volume and geometry
- Data filtering effects in high speed applications

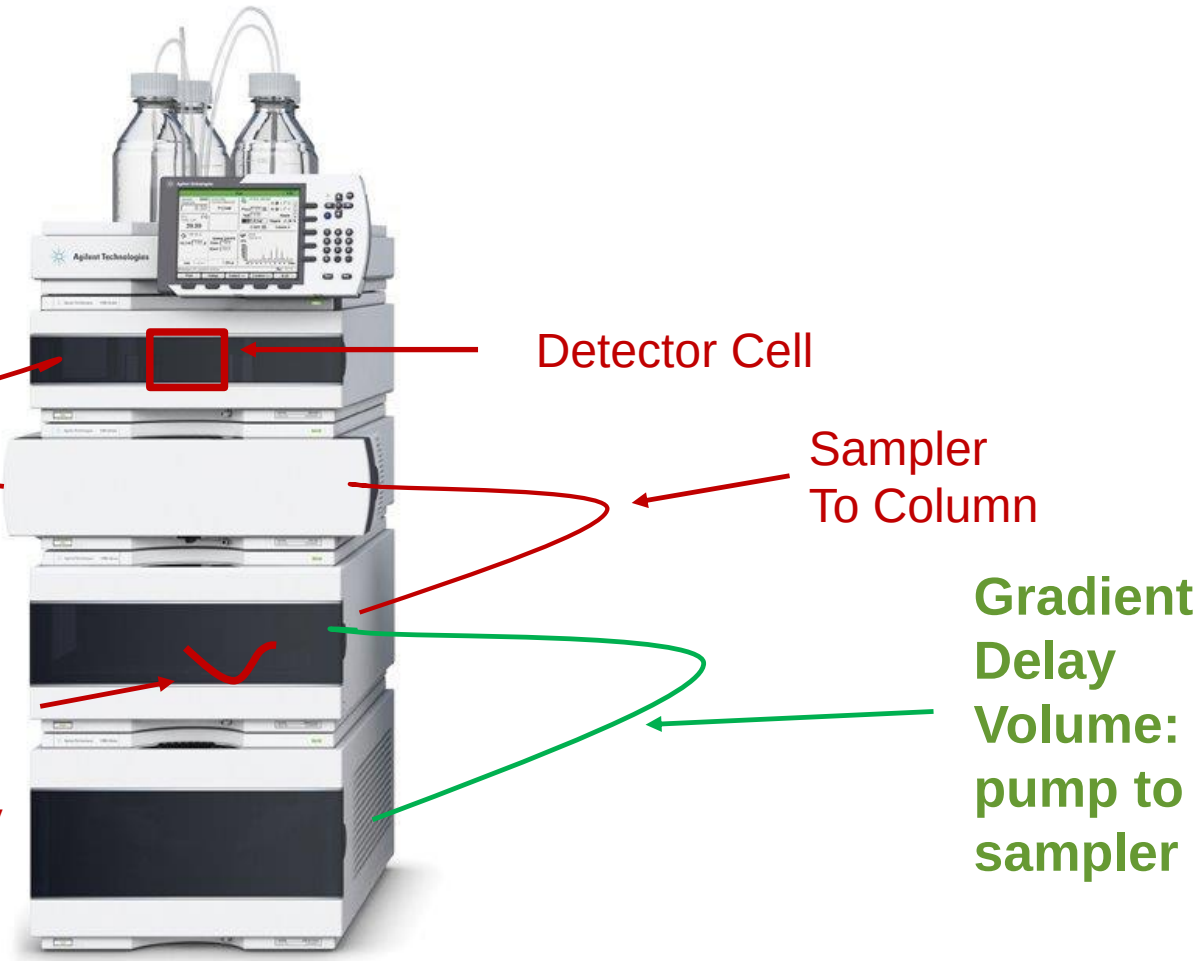
Recommended Capillary Connections

Transferring from HPLC to RRLC



Connection	HPLC	RRLC
Pump to Autosampler	60cm/0.17mm (G1312-67305)	60cm/0.17mm (G1312-67305)
Autosampler to column thermostat inlet	18cm/0.17mm (G1313-87305)	**18cm/0.12mm (G1313-87304)**
Column Thermostat to column	7cm/0.17mm (G1316-87300)	**7cm/0.12mm (G1316-87303)**
Column to Detector (DAD)	38cm/0.17mm (G1315-87311)	**15cm/0.12mm (G1315-87312)**
Detector to Waste (DAD)	PTFE, wide pore (0890-1713)	PTFE, wide pore (0890-1713)
Column to Detector (VWD)	PEEK/0.17 mm (5062-8522)	PEEK/0.17 mm (5062-8522)
Detector to Waste (VWD)	48 cm/0.25mm (5062-8535)	48 cm/0.25mm (5062-8535)

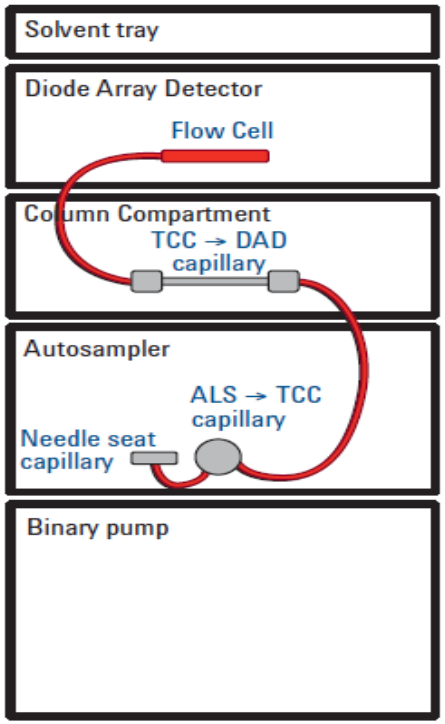
Variable Configurations for Dispersion Volume and Delay/Dwell Volume



System Configuration Options:

How to reduce extra column volume

Agilent 1290 Infinity LC System:
Default stacking and capillary tubing configurations



Needle seat capillary:
0.12 × 100 mm = 1.1 µL

ALS → TCC capillary:
0.12 × 340 mm = 3.8 µL

TCC → DAD capillary:
0.12 × 220 mm = 2.5 µL

Flow cell V(σ)1.0 µL = 2.3 µL

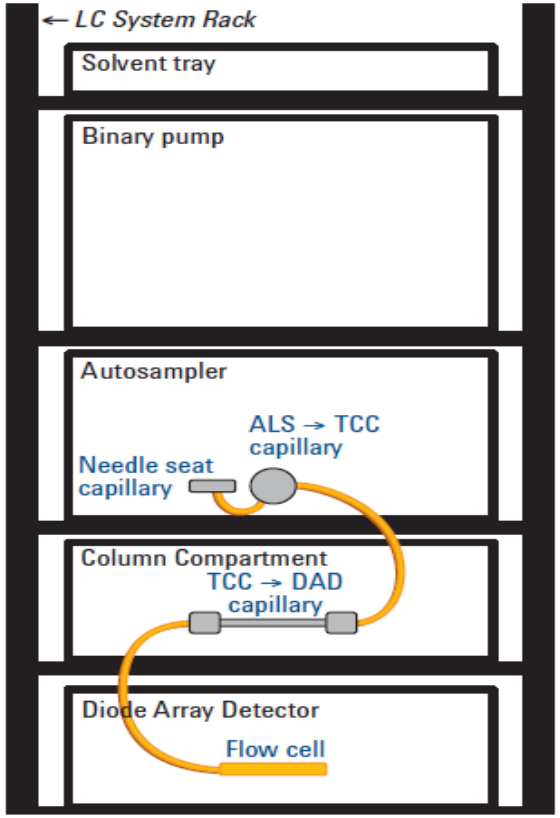
Total extra-column volume = 9.7 µL

Volume of 2.1 × 50 mm column = 172.3 µL
Void volume of column = 103.9 µL

Percent extra-column volume = 9.3%

60% Reduction in extra-column volume

Agilent 1290 Infinity LC System:
with LC System rack and ultra-low dispersion optimizations



Needle seat capillary:
0.11 × 100 mm = 0.9 µL

ALS → TCC capillary:
0.08 × 220 mm = 1.1 µL

TCC → DAD capillary:
0.08 × 220 mm = 1.1 µL

Flow cell V(σ)0.6 µL = 0.8 µL

Total extra-column volume = 3.9 µL

Volume of 2.1 × 50 mm column = 172.3 µL
Void volume of column = 103.9 µL

Percent extra-column volume = 3.7%

Figure 1. Comparison of extra-column volume on an Agilent 1290 Infinity LC System without (left) and with (right) ultra-low dispersion optimizations.

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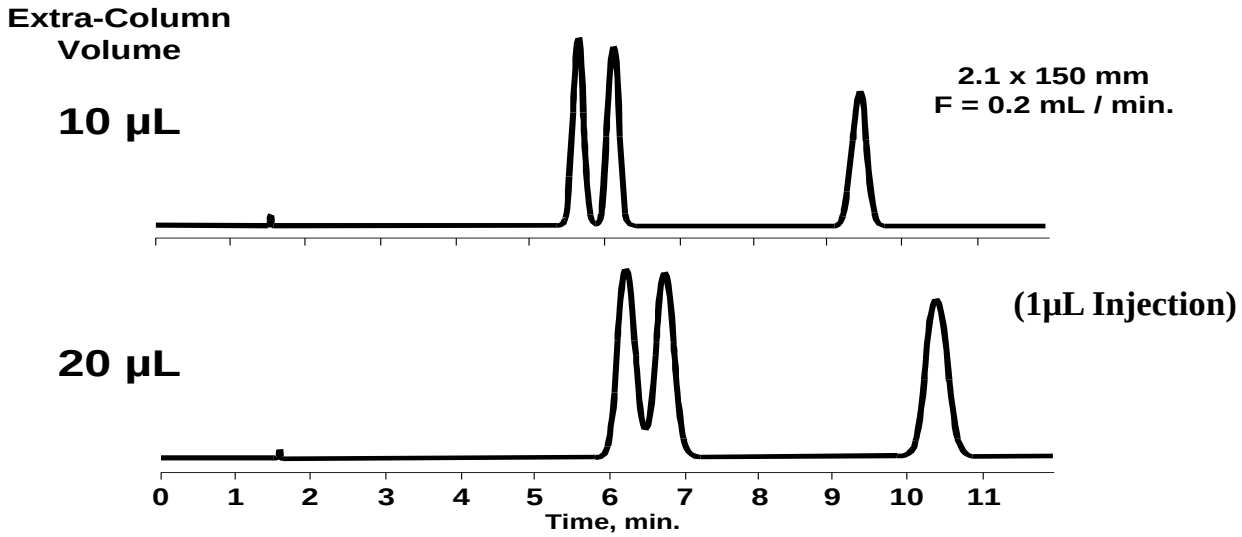
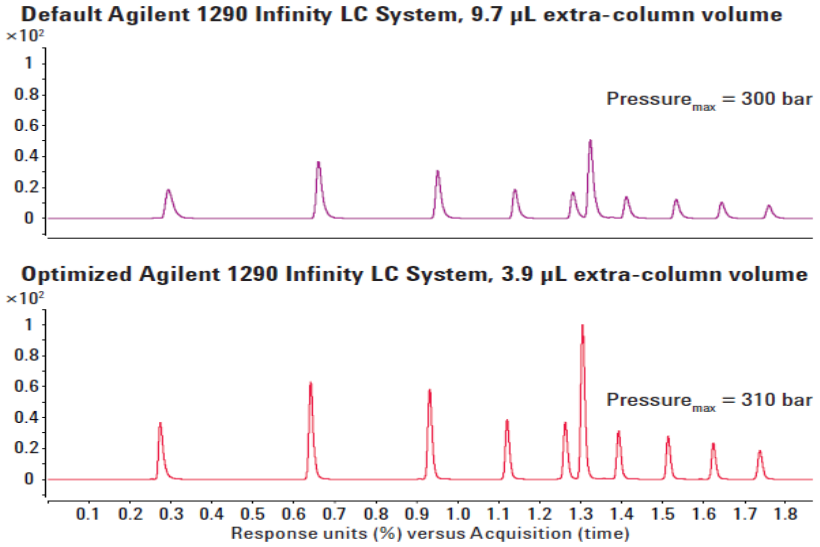
Effect of Extra-Column Volume:

Does an extra 10uL make my peakwidth look fat?

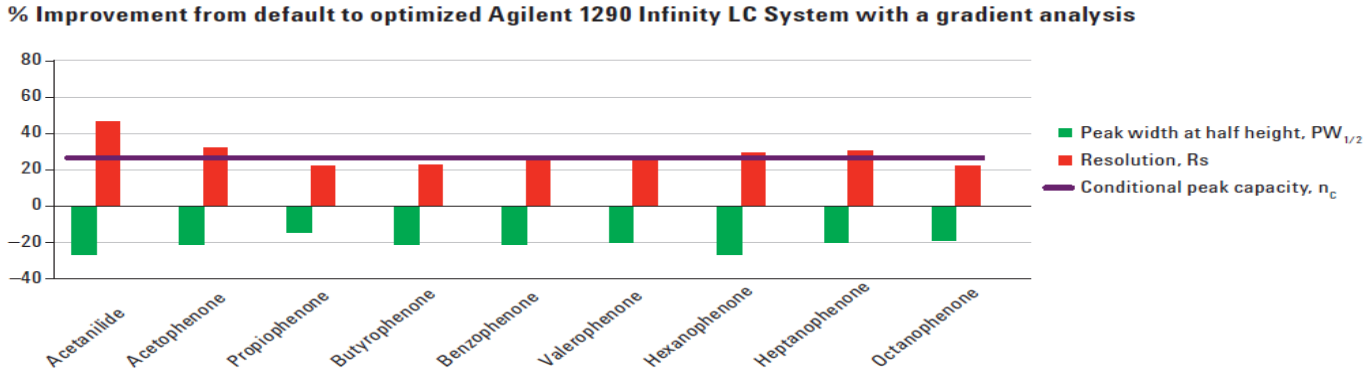


- Use short, small internal diameter tubing between the injector and the column and between the column and the detector.
- Make certain all tubing connections are made with matched fittings.
- Use a low-volume detector cell.
- Inject small sample volumes.

Smaller ID tubing can increase



Mobile phase A	H_2O
Mobile phase B	CH_3CN
Flow rate	0.4 mL/min
Gradient	t (min) 0 1.2 %B 25 95
Sample	A 1-µL injection of RRLC checkout sample (p/n 5188-6529) spiked with 50 µL 2 mg/mL thiourea in water/acetonitrile
TCC	26 °C
DAD	Sig = 254, 4 nm; Ref = Off
Column	Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 50 mm, 1.8 µm
Analytes	1. Thiourea (v_0 marker) 2. Acetanilide 3. Acetophenone 4. Propiophenone 5. Butyrophenone 6. Benzophenone 7. Valerophenone 8. Hexanophenone 9. Heptanophenone 10. Octanophenone



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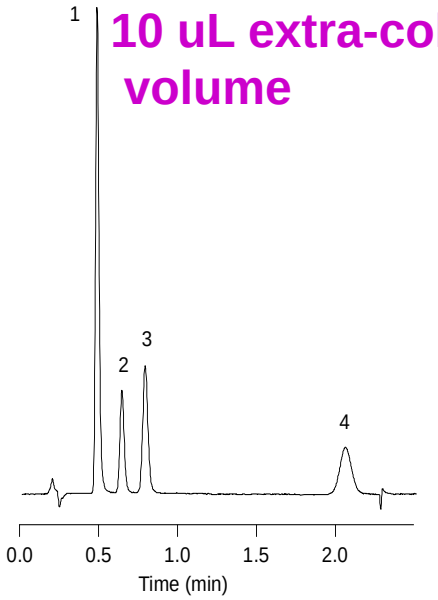
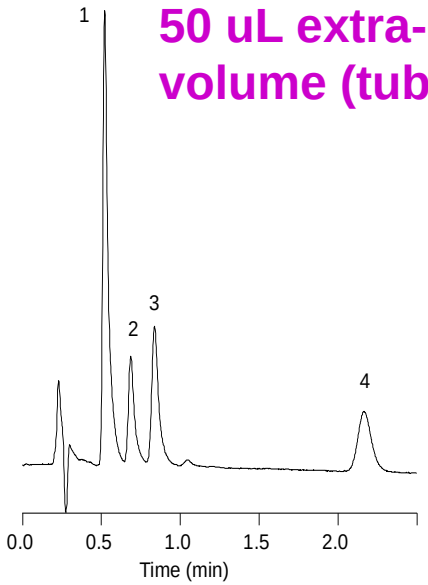
Dispersion: Peak Broadening Extra-Column Volume

10 uL extra-column volume

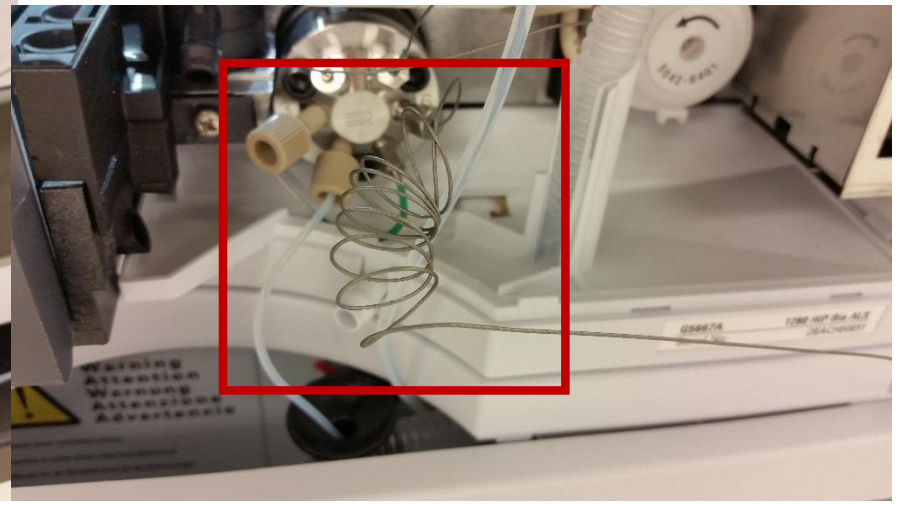


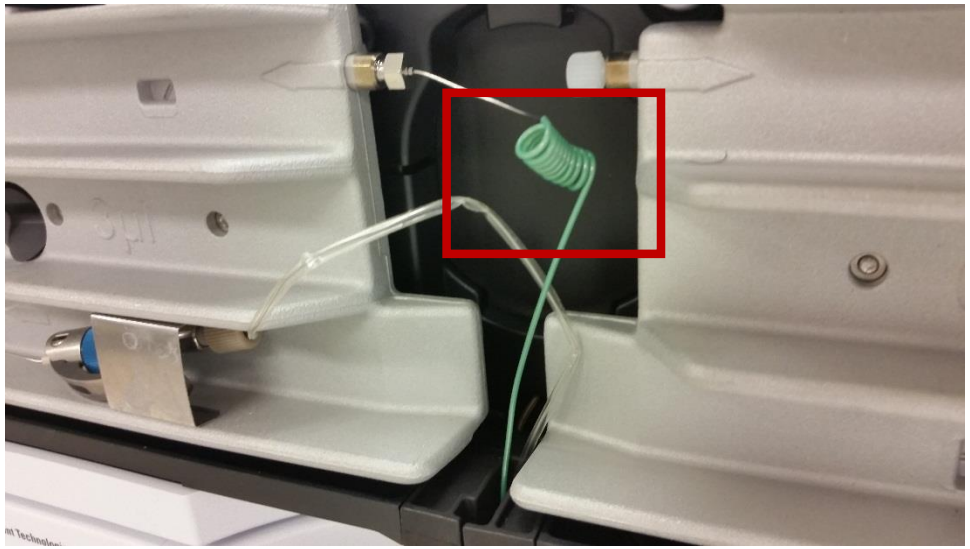
50 uL extra-column volume (tubing)



Column: StableBond SB-C18, 4.6 x 30 mm, 3.5 µm Mobile Phase: 85% H_2O with 0.1% TFA : 15% ACN Flow Rate: 1.0 mL/min
Temperature: 35°C Sample: 1. Phenylalanine 2. 5-benzyl-3,6-dioxo-2-piperazine acetic acid 3. Asp-phe 4. Aspartame

Dispersion: Examples of Unnecessary Tubing/Plumbing



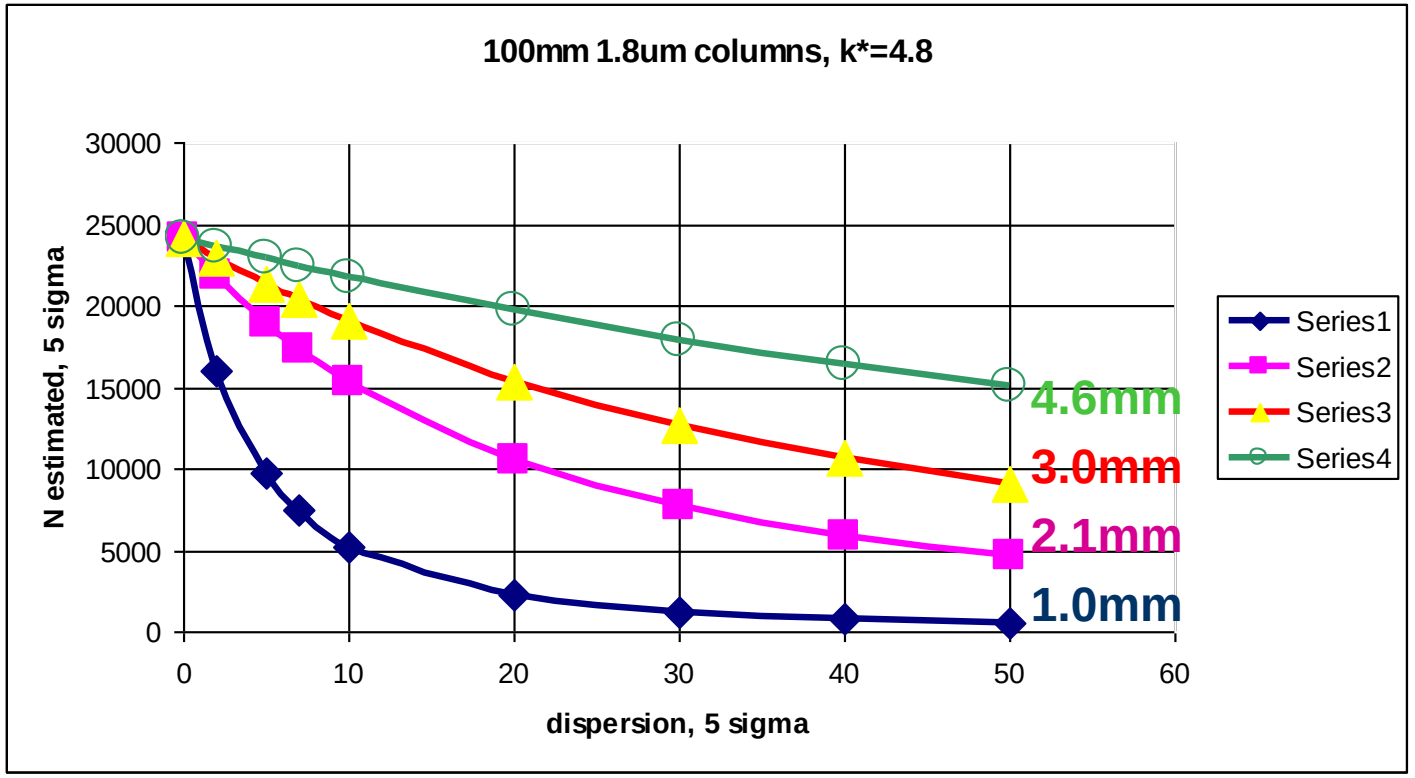


Column Compartment

- Temperature Settings
- Precolumn Heating
  - Needed for analyses done at non-ambient condition
  - ***Columns with smaller IDs (<4.6mm) and shorter length (<250mm) will see greater temperature disparities from front to back of column due to insufficient mobile phase heating***

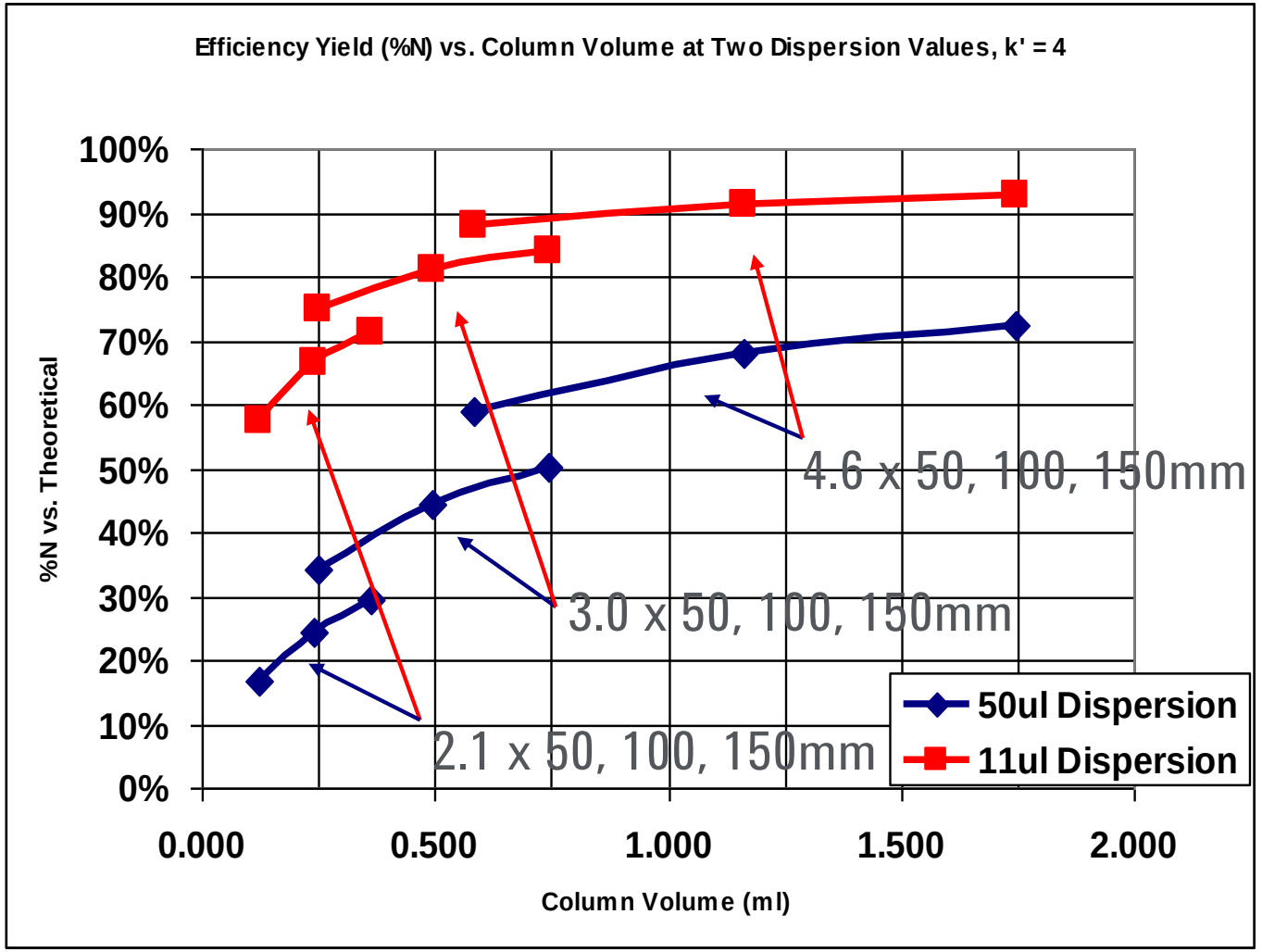
Use of a low dispersion heat exchanger is strongly recommended for LCMS

Efficiency Yield vs. Dispersion in LC systems



Use the largest columns suitable for the application requirements -- they are less affected by extracolumn contributions. At the same time, consider solvent consumption and detector (ELSD, MS, etc.) compatibility.

Calculated Yields, %N, for Column Diameter and Length with two system dispersion values



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Recap

- Translating methods from HPLC to UHPLC reduces run times, saves solvent costs and increases throughput
- These UHPLC columns use smaller columns with smaller particles (STM). They often use fast flowrates and leverage the ability of the pump to work at high pressure
- When switching to shorter run times with sharper peaks, dispersion and band broadening become much more significant considerations
- Reducing Extra Column Volume can significantly diminish the band broadening effects we experience
- We can use STM particle size short columns with HPLC/MS. The optimal flowrate regularly limits us before we exceed the 400bar limit of these systems.
- When using these small columns for HPLC/MS, the same concerns exist regarding band broadening and extra column volume

Thank you!!!!

Questions?

Contact Agilent Chemistries and Supplies Technical Support

1-800-227-9770 Option 3, Option 3:

Option 1 for GC/GCMS Columns and Supplies

Option 2 for LC/LCMS Columns and Supplies

Option 3 for Sample Preparation, Filtration and QuEChERS

Option 4 for Spectroscopy Supplies

Available in the USA & Canada 8-5 all time zones

gc-column-support@Agilent.com

lc-column-support@agilent.com

spp-support@agilent.com

spectro-supplies-support@agilent.com