LC Column Troubleshooting

Isolating the Source of the Problem

Rita Steed

December 12, 2013

What Do We Troubleshoot

The typical LC troubleshooting approach asks the questions:

- What's wrong with the column?
- What's wrong with the instrument?

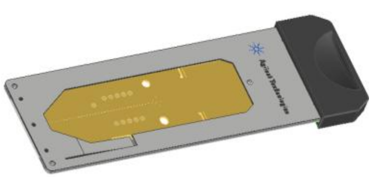
- But separations are controlled by more than just the column or instrument.

The better question is "Why doesn't my separation work as expected?

- *And* the answer could be there is a problem with the column, the instrument or something else (sample, mobile phase etc.).

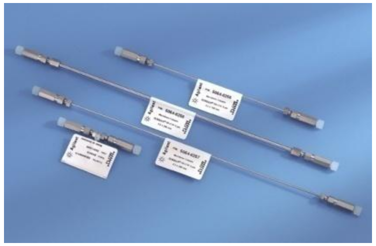
Presentation Goals

- Introduce the most commonly observed column related problems in HPLC.
- Explore the reasons for these column problems.
- Propose preventative maintenance and method development/optimization approaches to minimize HPLC column problems and increase column lifetimes.



Chip LC

Nano LC



Capillary LC

Analytical LC

Prep LC

Major Areas of Column Problems - Dramatic Changes in 3 Key Areas:

1. U/HPLC System Pressure

2. Chromatogram - Peak Shape

3. Chromatogram - Peak Retention/Selectivity

1. Pressure Issues

Column Observations

- Large Pressure Increase

Potential Problems

- Plugged Inlet Frit
- Column Contamination
- Plugged Packing

Note: Low pressure is typically a connection or LC issue; unless the column has been improperly used and disassembled or lost all its packing.

Determining the Cause and Correcting High Back Pressure

- Check pressure with/without column - many pressure problems are due to blockages elsewhere in the system

If column pressure remains high:

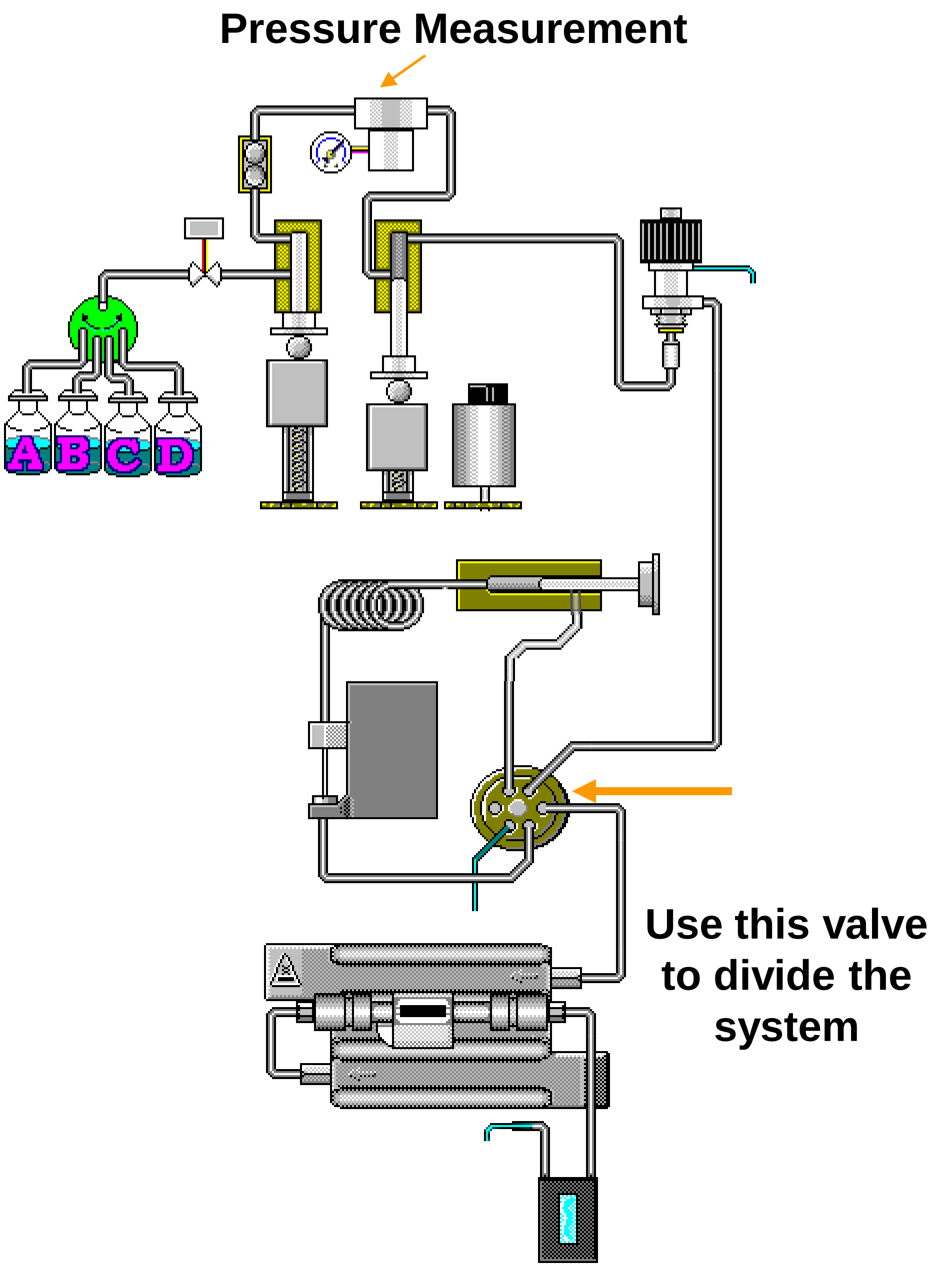
- Rinse column (remove detector from flow path!)
  - Eliminate column contamination and plugged packing
  - high molecular weight/adsorbed compounds
  - precipitate from sample or buffer
- Back flush column – may clear plugged column inlet frit, may not be possible, check column info
- Change column inlet frit (not possible on many newer columns due to loss in efficiency, so discard column)

Tip: Eliminate pressure issues – add a 0.5 or 2um in-line filter to system

Pressure Problem

Pressure Too High

- Column inlet frit contaminated
- Frit in purge valve contaminated
- Column contaminated
- Blockage in a capillary, particularly needle seat capillary
- Rotor in injection valve plugged
- Injection needle or needle seat plugged



Agilent Technologies

Column Cleaning

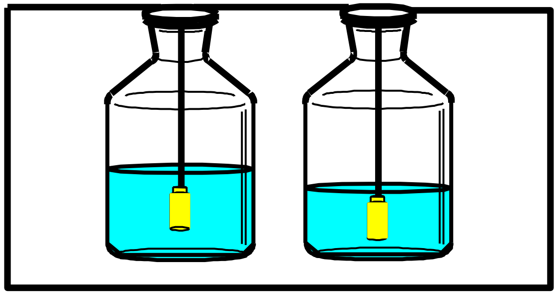
Flush with stronger solvents than your mobile phase
Make sure detector is taken out of flow path

Reversed-Phase Solvent Choices in Order of Increasing Strength
Use at least 10 x V_m of each solvent for analytical columns

1. Mobile phase without buffer salts (water/organic)
2. 100% Organic (MeOH or ACN)
3. Is pressure back in normal range?
4. If not, discard column or consider more drastic conditions:
   75% Acetonitrile:25% Isopropanol, then
5. 100% Isopropanol
6. 100% Methylene Chloride*
7. 100% Hexane*

* When using either Hexane or Methylene Chloride the column must be flushed with Isopropanol before returning to your reversed-phase mobile phase.

Preventing Column Back Pressure Problems



- Filter mobile phase
  - Filter non-HPLC grade solvents
  - Filter buffer solutions
  - Install an in-line filter between auto-sampler and column (removes pump seal debris, ALS rotor debris, and sample particulates). Use 2 um frit for 3.5 um/5um columns, use 0.5 um frit for 1.8 um/2.7 columns.
- Filter all samples and standards
- Perform sample clean-up (i.e. SPE, LLE) on dirty samples
- Appropriate column flushing – flush buffers from entire system with water/organic mobile phase
- Replace buffers every 24-48 hours
  - Never add to the bottle – always use a new one

2. Peak Shape Issues in HPLC

- Split peaks
- Peak tailing
- Broad peaks
- Poor efficiency (low N)
- Inconsistent response

➢ Many peak shape issues are also combinations – i.e. broad and tailing or tailing with increased retention

Split Peaks

Can be caused by:

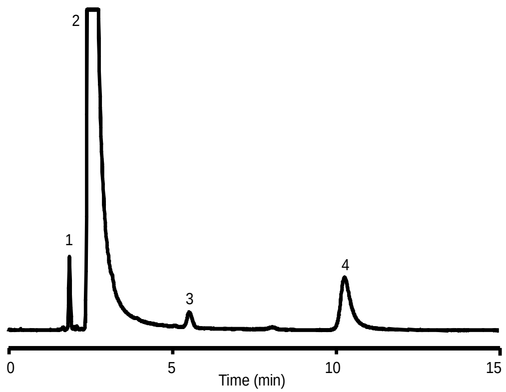
- Column contamination
- Partially plugged frit
- Column void (gap in packing bed)
- Injection solvent effects

Split Peaks

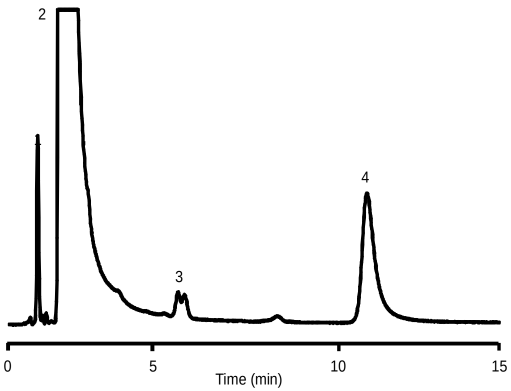
Column Contamination

Column: StableBond SB-C8, 4.6 x 150 mm, 5 mm   Mobile Phase: 60% 25 mM Na_2HPO_4, pH 3.0 : 40% MeOH   Flow Rate: 1.0 mL/min
Temperature: 35°C   Detection: UV 254 nm   Sample: Filtered OTC Cold Medication: 1. Pseudoephedrine 2. APAP 3. Unknown 4. Chlorpheniramine

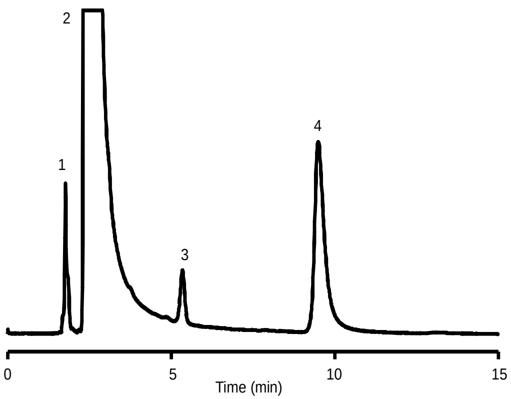
Injection 1



Injection 30



Injection 1
After Column Wash with 100% ACN



Column washing eliminates the peak splitting, which resulted from a contaminant on the column.

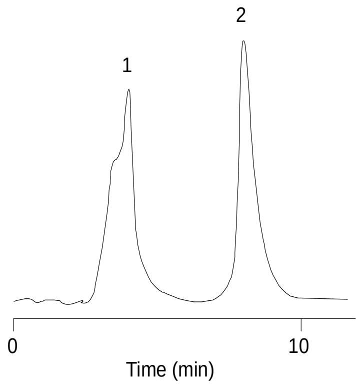
Agilent Technologies

Split Peaks

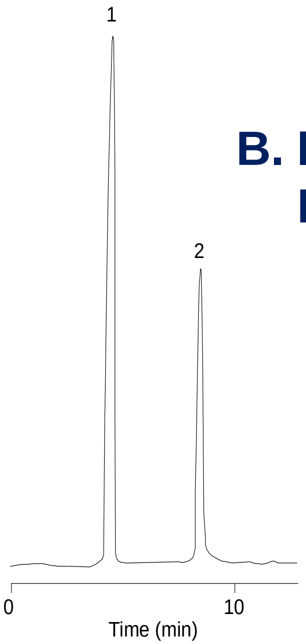
Injection Solvent Effects

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

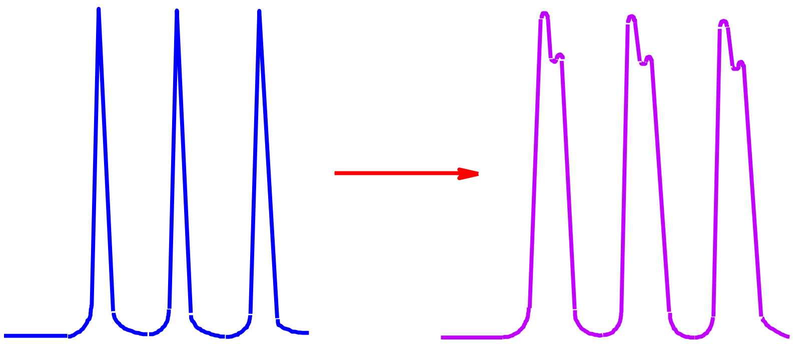


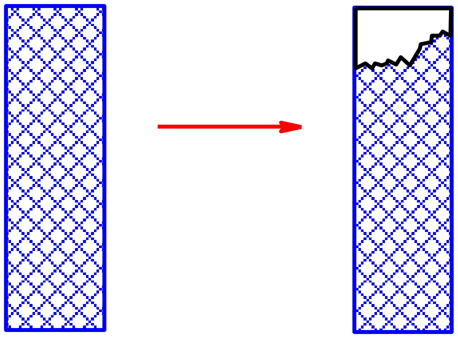
B. Injection Solvent Mobile Phase



- Injecting in a solvent stronger than the mobile phase can cause peak shape problems, such as peak splitting or broadening
- Note : earlier peaks (low k) most affected by splitting, later peaks broader

Peak Shape Problems –
Doublets (A Form of Split Peaks)





- Void Volume in Column or Poor Fitting
- Partially Blocked Frit
- Only One-Peak a Doublet - Coeluting Components or one peak overloaded
- Early (low k) peaks most affected

Determining the Cause of Split Peaks

1. Complex sample matrix or many samples analyzed - likely column contamination or partially plugged column frit.

2. Mobile phase pH > 7 - likely column void due to silica dissolution (unless specialty column used, polymer base - PLRP-S, ZORBAX Extend-C18 stable to pH 11.5)

3. Injection solvent stronger than mobile phase - likely split *and* broad peaks, shape dependent on injection volume and k value.

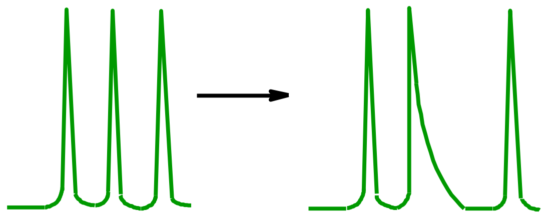
Peak Tailing, Broadening and Loss of Efficiency (N, plates)

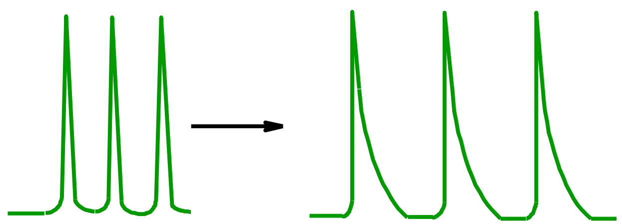
May be caused by:

1. Column “secondary interactions”
2. Column packing voids
3. Column contamination
4. Column aging
5. Column loading
6. Extra-column effects

Peak Shape: Tailing Peaks
First Question: All Peaks or Some Peaks?

Symmetry > 1.2





Causes

Some Peaks Tail:

- Secondary - retention effects.
- Residual silanol interactions.
- Small peak eluting on tail of larger peak

All Peaks Tail:

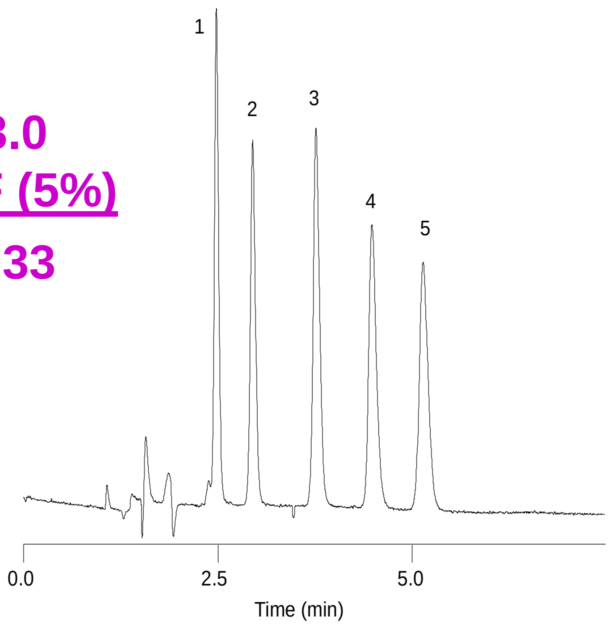
- Extra-column effects i.e. poor connections, too much volume
- Build up of contamination on column inlet (partially plugged frit)
- Bad column.

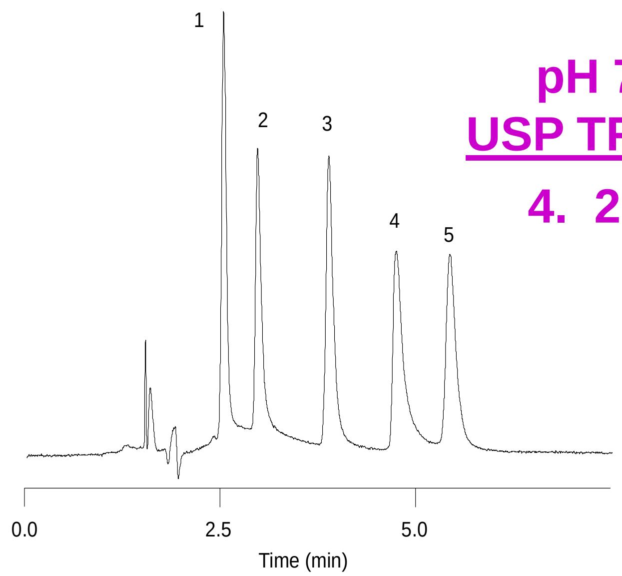
Peak Tailing

Column "Secondary Interactions"

Column: C8, 4.6 x 150 mm, 5µm Mobile Phase: 85% 25 mM Na_2HPO_4 : 15% ACN Flow Rate: 1.0 mL/min
Temperature: 35°C Sample: 1. Phenylpropanolamine 2. Ephedrine 3. Amphetamine 4. Methamphetamine 5. Phenteramine

pH 3.0
USP TF (5%)
4. 1.33





pH 7.0
USP TF (5%)
4. 2.35

- Reducing the mobile phase pH reduces interactions with silanols that can cause peak tailing; No additional mobile phase modifiers required
- Consider bonded phase with more endcapping, designed for good pH 7 performance

Agilent Technologies

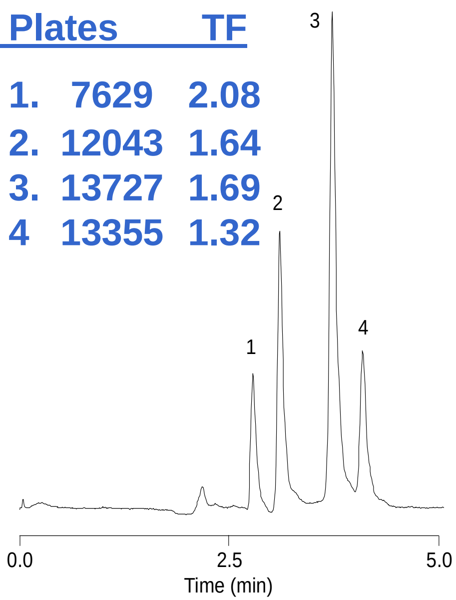
Peak Tailing

Column Contamination

Column: StableBond SB-C8, 4.6 x 250 mm, 5µm Mobile Phase: 20% H_2O : 80% MeOH Flow Rate: 1.0 mL/min
Temperature: R.T. Detection: UV 254 nm Sample: 1. Uracil 2. Phenol 3. 4-Chloronitrobenzene 4. Toluene

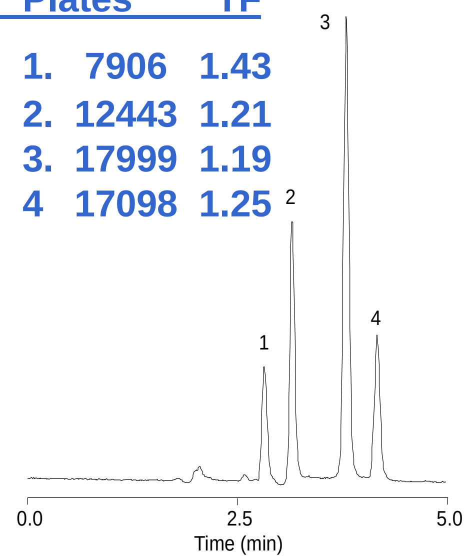
QC test forward direction

	Plates	TF
1.	7629	2.08
2.	12043	1.64
3.	13727	1.69
4	13355	1.32



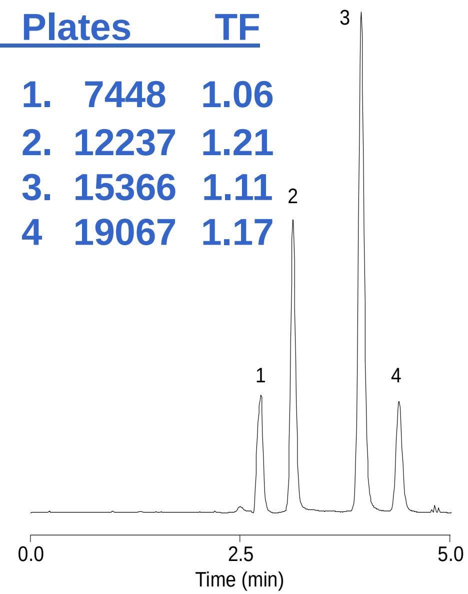
QC test reverse direction

	Plates	TF
1.	7906	1.43
2.	12443	1.21
3.	17999	1.19
4	17098	1.25

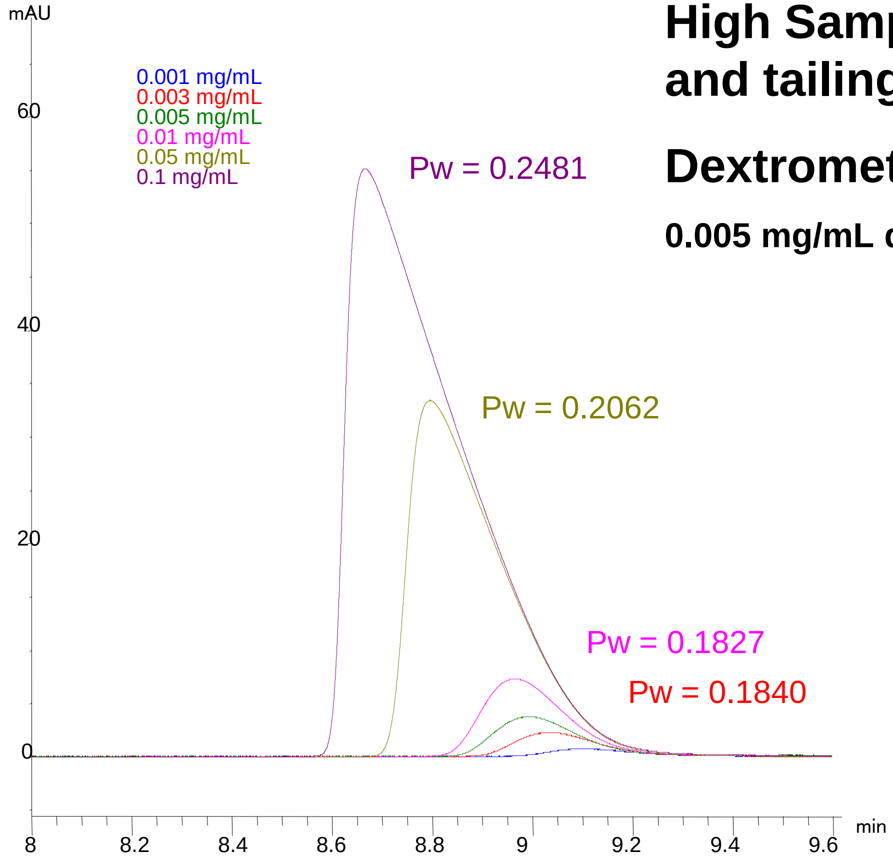


QC test after cleaning 100% IPA, 35°C

	Plates	TF
1.	7448	1.06
2.	12237	1.21
3.	15366	1.11
4	19067	1.17



Comparison of Peak Shape at Low and High Loads
Broadening and Tailing



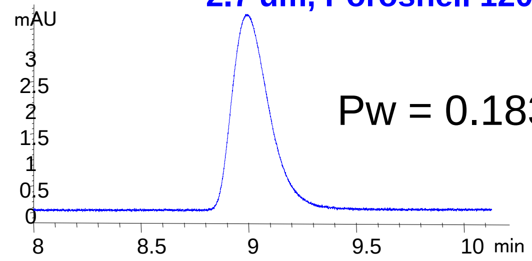
High Sample Loads can give broad or broad and tailing peaks

Dextromethorphan is 35% broader at high load

0.005 mg/mL dextromethorphan (4.1 uL injection volume)

Low sample loads provide symmetrical, non-tailing peaks with narrow peak widths

2.7 um, Poroshell 120 EC-C18



Agilent Technologies

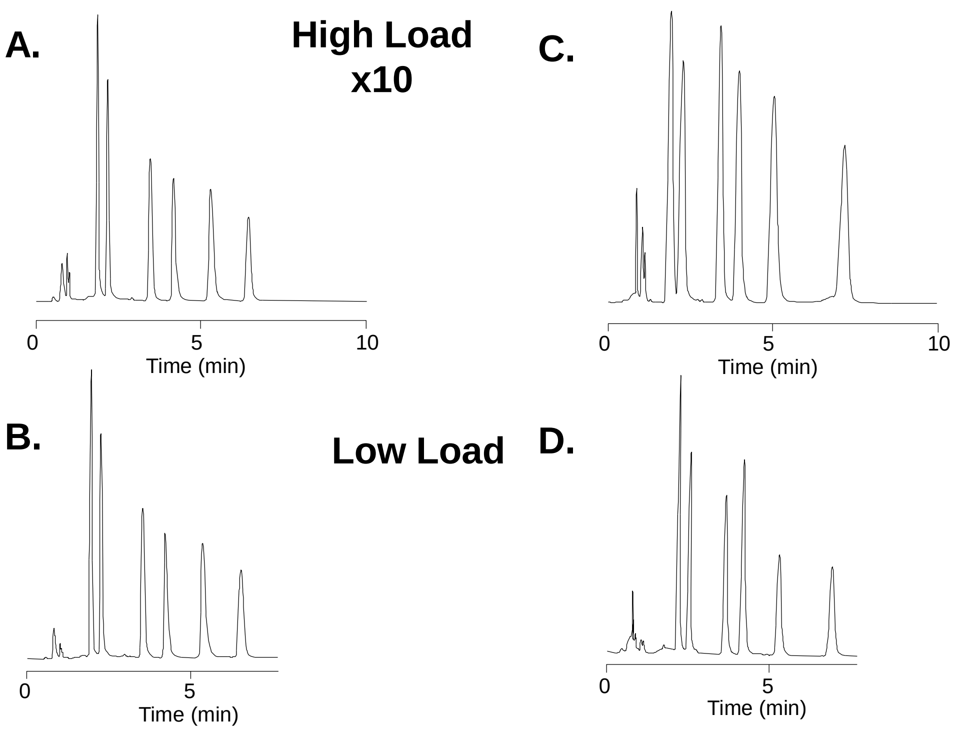
Peak Tailing/Broadening
Sample Load Effects

Columns: 4.6 x 150 mm, 5µm Mobile Phase: 40% 25 mM Na_2HPO_4 pH 7.0 : 60% ACN Flow Rate: 1.5 mL/min
Temperature: 40°C Sample: 1. Desipramine 2. Nortriptyline 3. Doxepin 4. Imipramine 5. Amitriptyline 6. Trimipramine

Tailing

Eclipse XDB-C8
USP TF (5%)

	A	B
1.	1.60	1.70
2.	2.00	1.90
3.	1.56	1.56
4.	2.13	1.70
5.	2.15	1.86
6.	1.25	1.25



Broadening

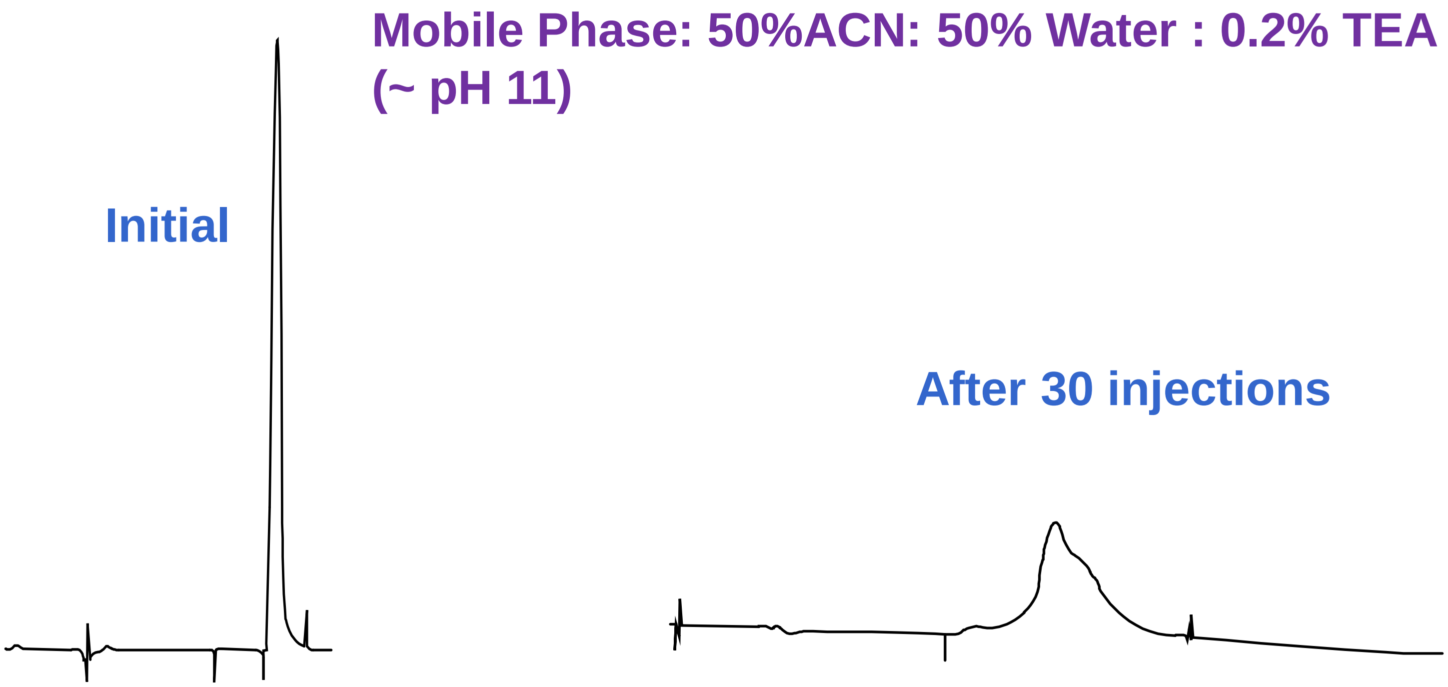
Competitive C8
Plates

	C	D
1.	850	5941
2.	815	7842
3.	2776	6231
4.	2539	8359
5.	2735	10022
6.	5189	10725

Agilent Technologies

Peak Broadening, Splitting

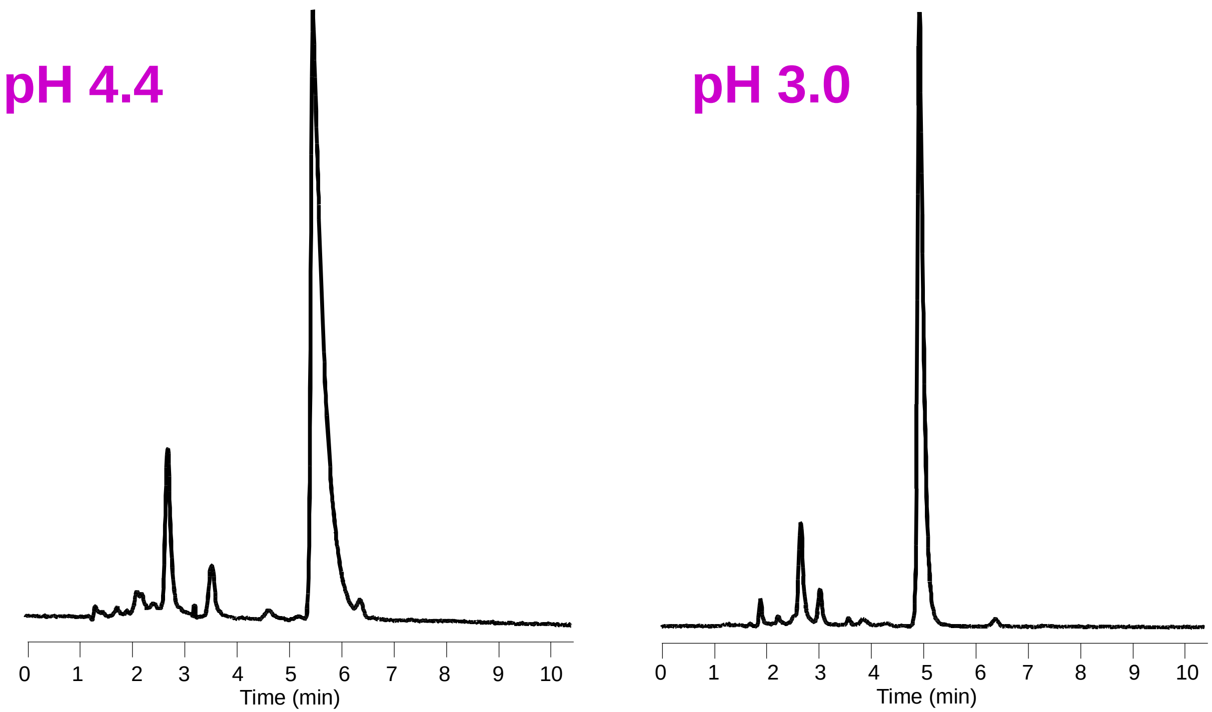
Column Void

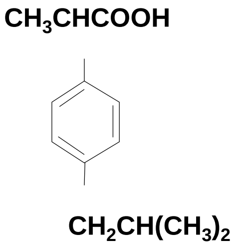


- Multiple peak shape changes can be caused by the same column problem.
- In this case a void resulted from silica dissolved at high pH

Effect of pH on Peak Shape
What Happens Near the Sample pK_a

Column: ZORBAX SB-C8 4.6 x 150 mm, 5 um Mobile Phase: 40% 5 mM KH_2PO_4: 60% ACN
Flow Rate: 1.0 mL/min. Temperature: RT





Ibuprofen
pK_a = 4.4

- Inconsistent and tailing peaks may occur when operating close to an analyte's pKa; mobile phase pH should be selected to avoid this.

Determining the Cause of Peak Tailing

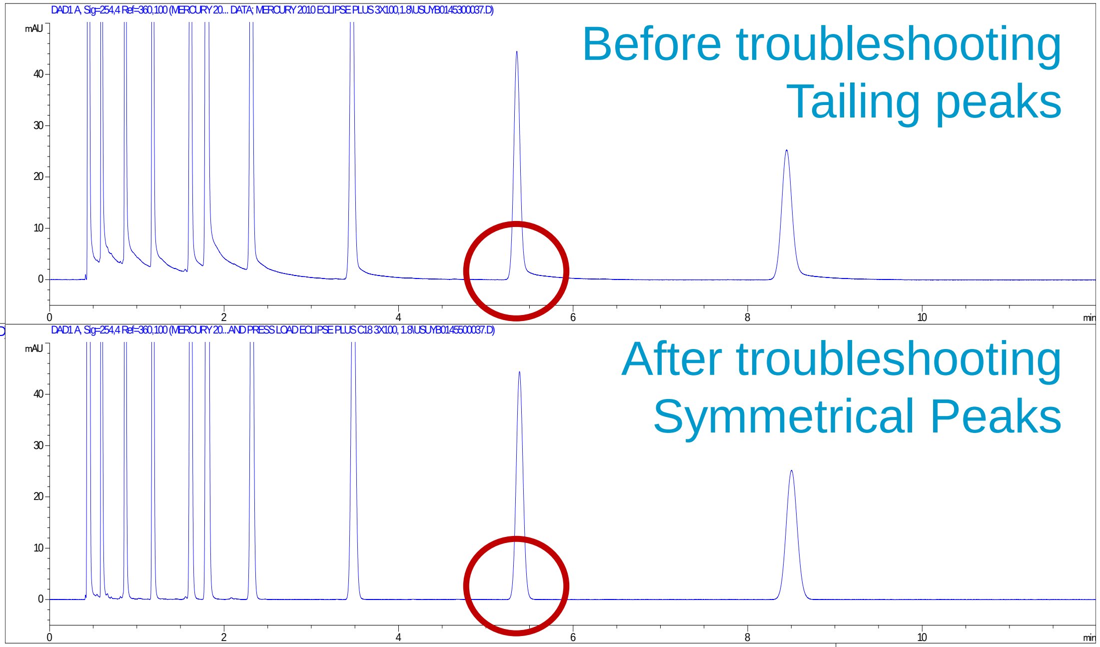
- Evaluate mobile phase effects - alter mobile phase pH and/or additives to eliminate secondary interactions
- Evaluate column choice - try column with high purity silica or different bonding technology
- Reduce sample load – injection volume and concentration
- Flush column and check for aging/void
- Eliminate extra-column effects – tubing, fittings, UV cell
  - ✓ This is even more critical for today's UHPLC separations and with 2.1 mm ID columns

Peak Shape and Related Problems Due to Extra Column Volume from Connections and Fittings

- ECV is volume in the LC system outside of the column.
- There will always be some in the flow path and the LC system is designed to minimize the impact of this.
- Connections and fittings, if made improperly, result in areas where the flow does not move smoothly.
  - These can be fittings swaged incorrectly, to the wrong depth or incompatible fittings being used.
- These unswept or poorly swept areas will cause <u>tailing, broadening and loss of column efficiency</u>

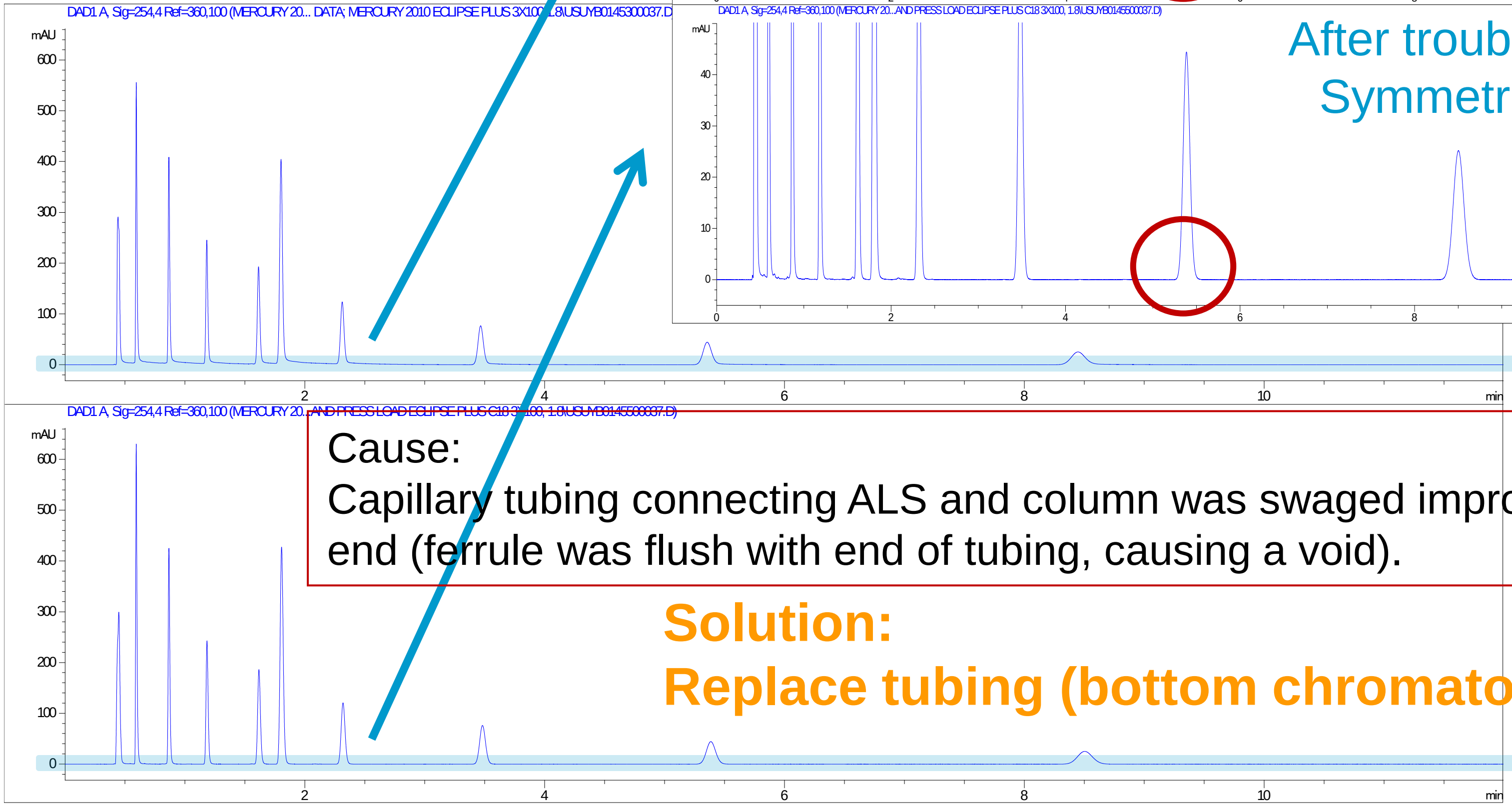
Peak Tailing – Extra Column Effects
Poor Fitting

Problem:
All peaks tail
(top chromatogram).



Before troubleshooting
Tailing peaks

After troubleshooting
Symmetrical Peaks

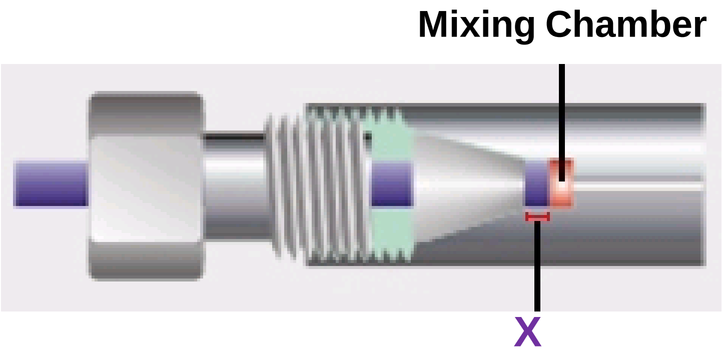


Cause:
Capillary tubing connecting ALS and column was swaged improperly on the ALS end (ferrule was flush with end of tubing, causing a void).

Solution:
Replace tubing (bottom chromatogram).

Peak Tailing/Fronting
What Happens If the Connection is Poorly Made ?

Wrong … too short



- If Dimension X is too short, a dead-volume, or mixing chamber, will occur
- This will broaden or split peaks or cause tailing.
- It will typically affect all peaks, but especially early eluting peaks.

Stainless Steel and Polymer Fittings

Agilent uses Swagelok type fittings with front and back ferrules, which give best sealing performance throughout our LC system (use this on the instrument connections, i.e. valves, heaters etc)

Stainless steel fittings – can be used anywhere and are especially popular for higher pressure connections

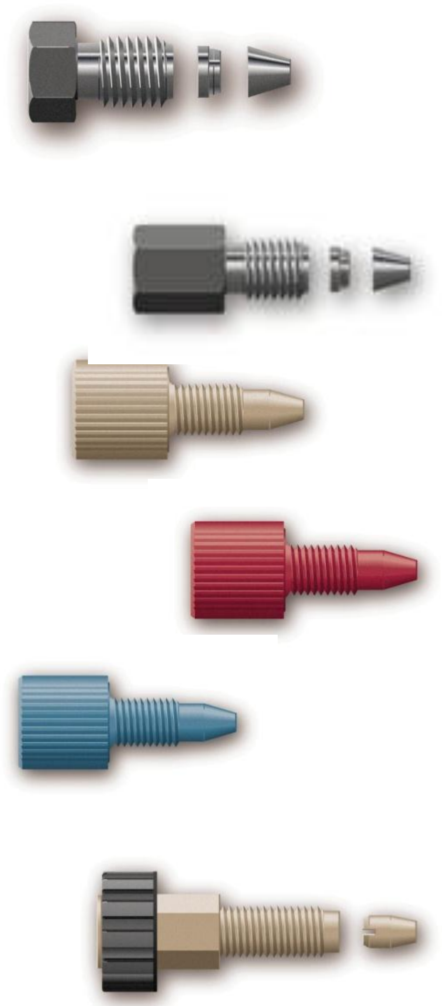
PEEK fittings (< 400 bar) are most popular when:

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

Polyketone fittings can be used up to 600 bar

- Use this fitting on column connections with Poroshell 120 (PN 5042-8957)

1200 bar removable fittings are available for UHPLC type systems



Some typical column connectors shown here

1200 Bar Removable Fittings

Part Number	Description	Picture
5067-4733	1200 Bar Removable Fitting	
5067-4738	1200 Bar Removable Long Fitting	
5067-4739	1200 Bar Removable Extra Long Fitting	

Fitting Description: Stainless steel screw, internal stainless steel ferrule, and a front ferrule in PEEK

Where to Use It: Anywhere in the flow path

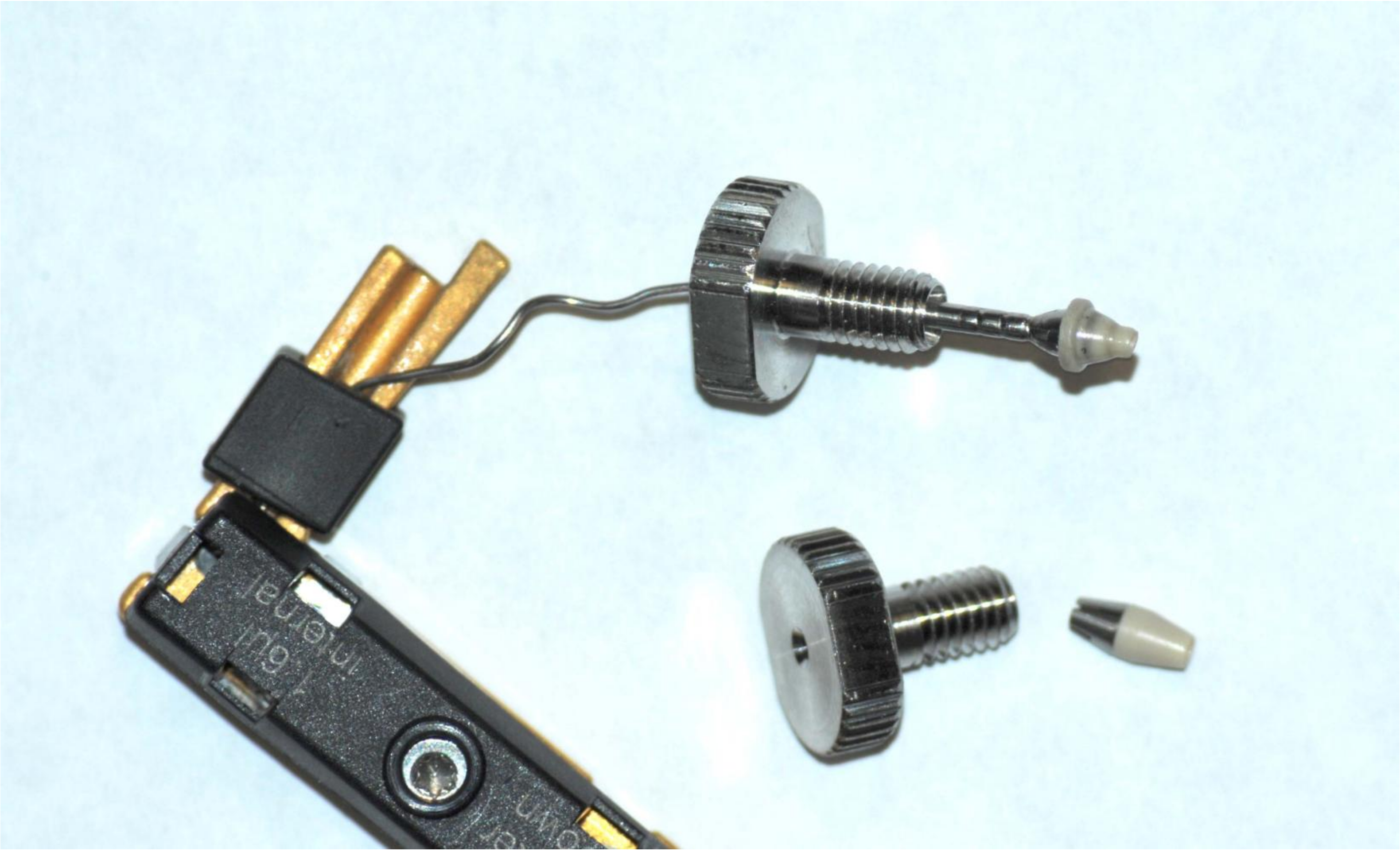
Ideal for the connection between the heat exchanger and the column because it can be re-used without losing tightness.

On competitive instruments

What Does it Replace: The standard stainless steel Swagelok fitting

Why: Because the heat exchanger has to be replaced when changing the column if a non-removable Swagelok fitting was used

Badly Made Hybrid (SS/Polymer Ferrule) Fitting Repeatedly, Poorly Made



Column Datasheet - Failure to Achieve Efficiency

Each Column is individually tested

LC Column Performance Report — **Agilent Technologies**

SERIAL NUMBER: USCFX01077

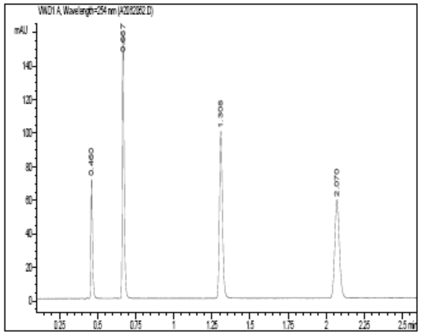
PART NUMBER: 695975-302
COLUMN TYPE: ZORBAX Poroshell 120 EC-C18 3 x 100 mm, 2.7 µm
PACKING LOT #: B10034

TEST CONDITIONS

MOBILE PHASE	=	60% Acetonitrile / 40% Water
COLUMN PRESSURE	=	239.8 Bar
COLUMN FLOW	=	0.80 ml / min
LINEAR VELOCITY	=	0.362 cm / sec
TEMPERATURE	=	AMBIENT (Nominally 23 °C)
INJECTION VOLUME	=	2 µl

QUALITY CONTROL PERFORMANCE RESULTS FOR NAPHTHALENE

	TEST VALUES	SPECIFICATIONS
THEORETICAL PLATES =	23259	MIN = 20000
SELECTIVITY =	1.90	RANGE = 1.84 - 1.94
USP TAILING FACTOR = (@ 5% Peak Height)	1.12	RANGE = 0.98 - 1.20
k' =	3.50	



Sample components with concentrations diluted in mobile phase in the following elution order.

Peak #	Conc (ug/ml)	Sample Component
1	10	Uracil
2	400	Phenol
3	50	4-Chloro Nitrobenzene
4	80	Naphthalene

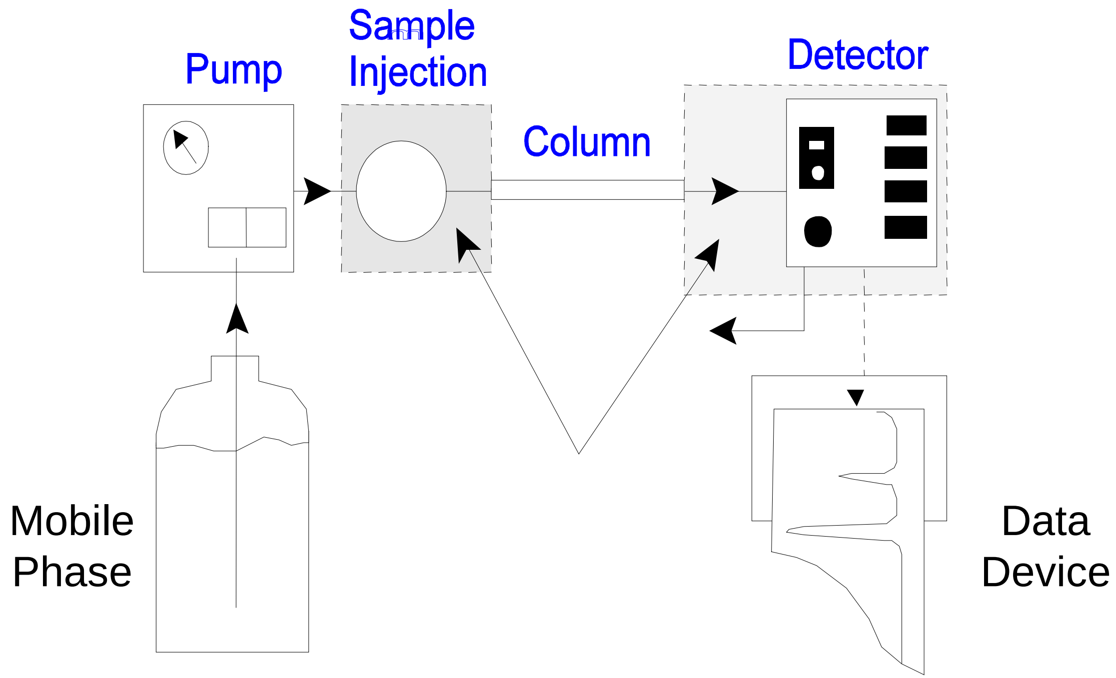
THIS COLUMN WAS SHIPPED CONTAINING ACETONITRILE AND WATER.
MATERIAL SAFETY DATA SHEETS ARE AVAILABLE UPON REQUEST.

- In order to achieve the performance as shown in the data sheet (UHPLC column):
  - Collect data at proper rate
  - Use correct flow cell
  - Minimize extra column volume
  - Use the correct sample and method

Note: The LC column performance report is generated under ideal conditions minimizing the system effect; isolating the column contribution

Agilent Technologies

Extra Column Volume = sample volume + connecting tube volume + fitting volume + detector cell volume



The instrument schematic above depicts where extra-column volume can occur, thus effecting instrument and column performance.

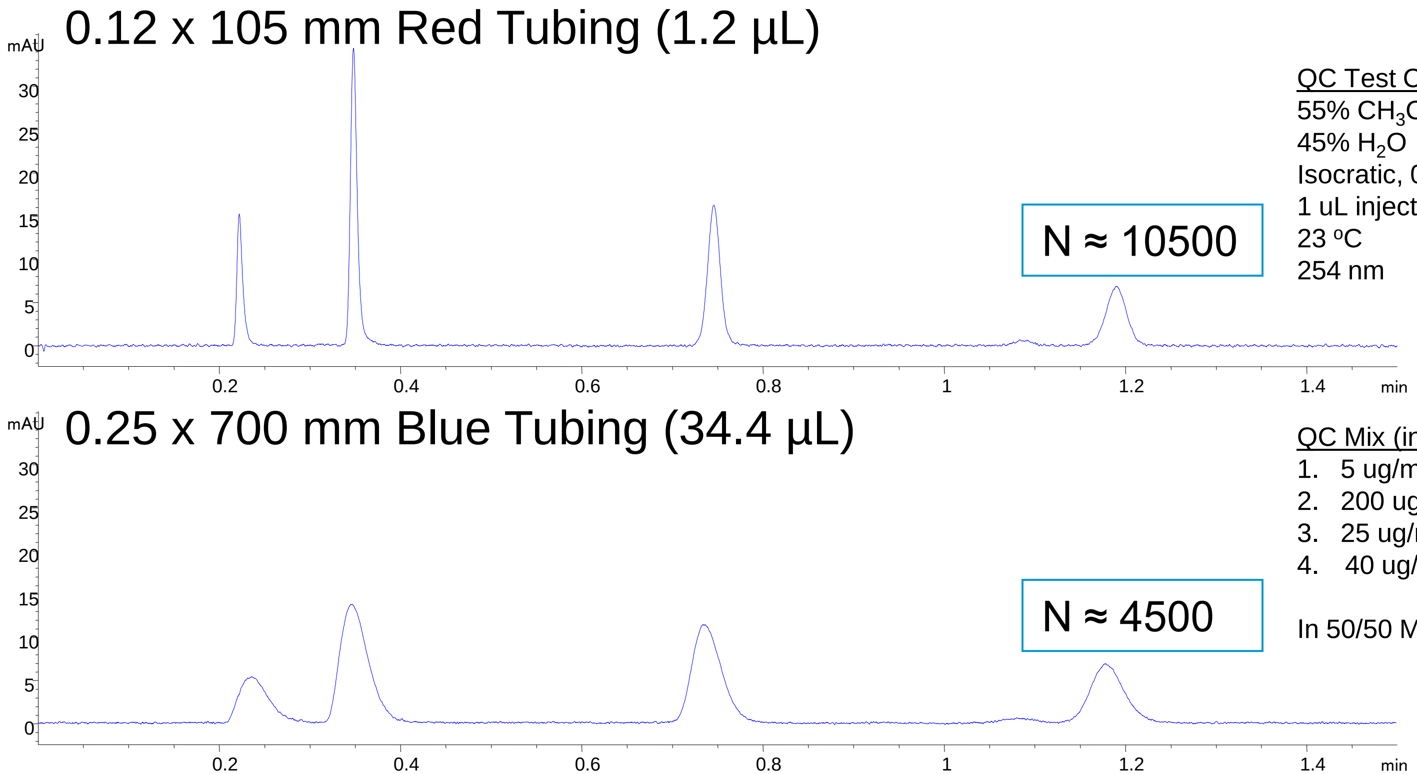
Use 0.12 mm Tubing Instead of 0.17 mm Tubing

Inside Diameter (mm)	Length (mm)	Material	Color	Connections	Part Number	Volume (ul)
0.12	180	SS	Red	1 end pre-swaged	G1313-87304	2.0
0.12	280	SS	Red	1 end pre-swaged	01090-87610	3.2
0.12	105	SS	Red	1 end pre-swaged	01090-87611	1.2
0.12	150	SS	Red	pre-swaged	G1315-87312	1.7
0.12	105	SS	Red	Without fittings	5021-1820	1.2
0.12	150	SS	Red	Without fittings	5021-1821	1.7
0.12	280	SS	Red	Without fittings	5021-1822	3.2
0.12	400	SS	Red	Without fittings	5021-1823	4.5
0.17	180	SS	Green	1 end pre-swaged	G1313-87305	4.1
0.17	280	SS	Green	1 end pre-swaged	01090-87304	6.4
0.17	130	SS	Green	1 end pre-swaged	01090-87305	2.9
0.17	90	SS	Green	1 end pre-swaged	G1316-87300	2.0
0.17	105	SS	Green	Without fittings	5021-1816	2.4
0.17	150	SS	Green	Without fittings	5021-1817	3.4
0.17	280	SS	Green	Without fittings	5021-1818	6.4
0.17	400	SS	Green	Without fittings	5021-1819	9.1

Use lower volume *RED* tubing when possible

GREEN tubing has 2x volume of RED tubing of same length

Efficiency is greatly reduced when extra-column volume increases



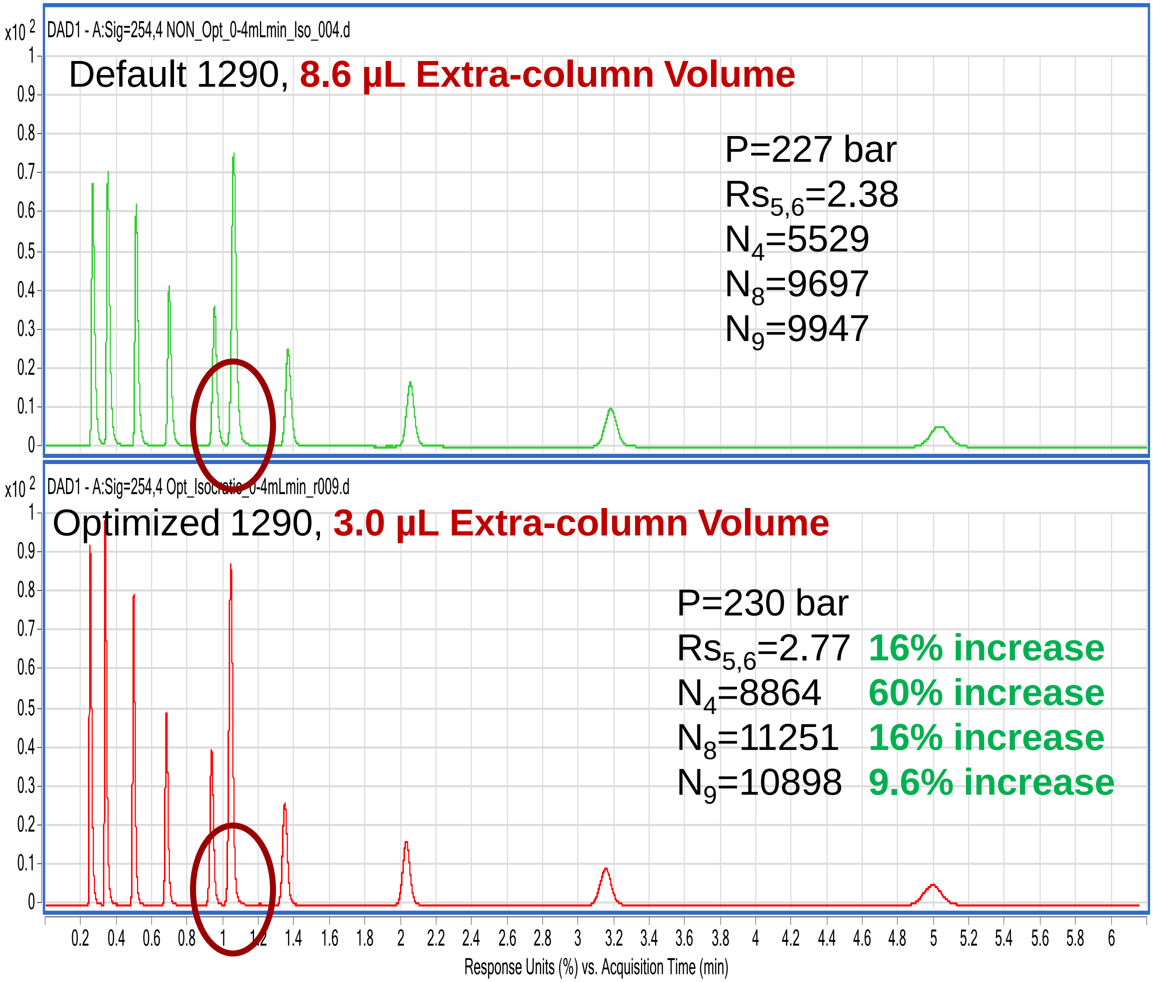
<u>QC Test Conditions</u>:
55% CH_3CN
45% H_2O
Isocratic, 0.6 mL/min
1 uL injection of QC Mix
23 ^oC
254 nm

<u>QC Mix (in elution order)</u>:
1. 5 ug/mL uracil
2. 200 ug/mL phenol
3. 25 ug/mL 4-chloro-nitrobenzene
4. 40 ug/mL naphthalene

In 50/50 MeCN/Water

- **QC test of a 2.1 x 50 mm, 1.8-µm Eclipse Plus C18 showing the peak broadening when larger volume tubing is installed between the autosampler and column. 43% of the efficiency is lost with too much extra column volume**

Effect of Extra Column Volume on an Isocratic Analysis of Alkylphenones – Efficiency



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

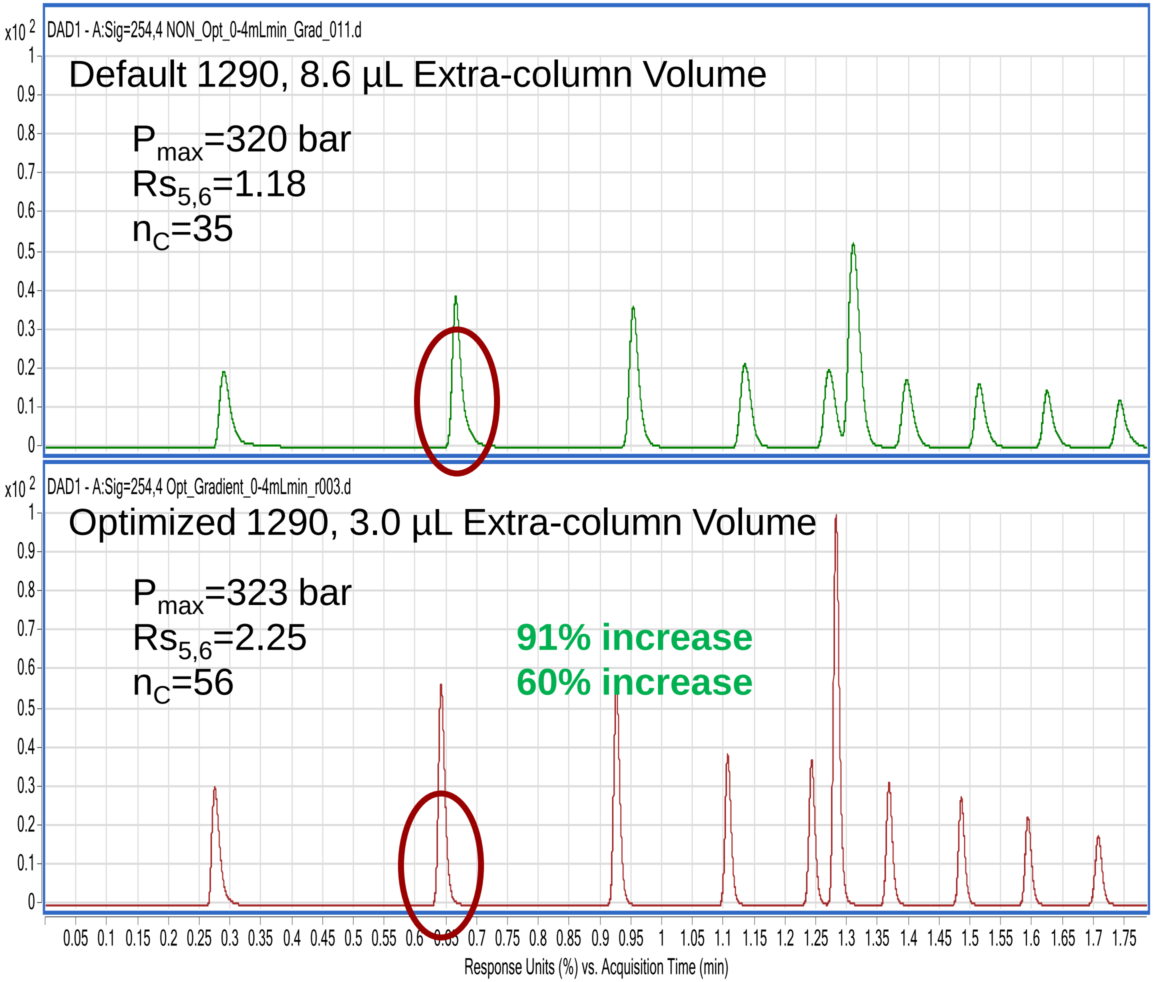
LC Rack System, 5001-3726

0.08 x 220 mm Capillary Tubing,

V(σ)0.6 µL Flow Cell,

A: H_2O; B: CH_3CN
0.4 mL/min
Isocratic, 60% B
1 µL injection of RRLC Checkout Sample (PN 5188-6529) spiked w/ 50 µL 2 mg/mL Thiourea in water/acetonitrile

Effect of Extra Column Volume on a Gradient Analysis of Alkylphenones – Efficiency and Tailing



Agilent ZORBAX RRHD Eclipse Plus 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

A: H_2O; B: CH_3CN
0.4 mL/min

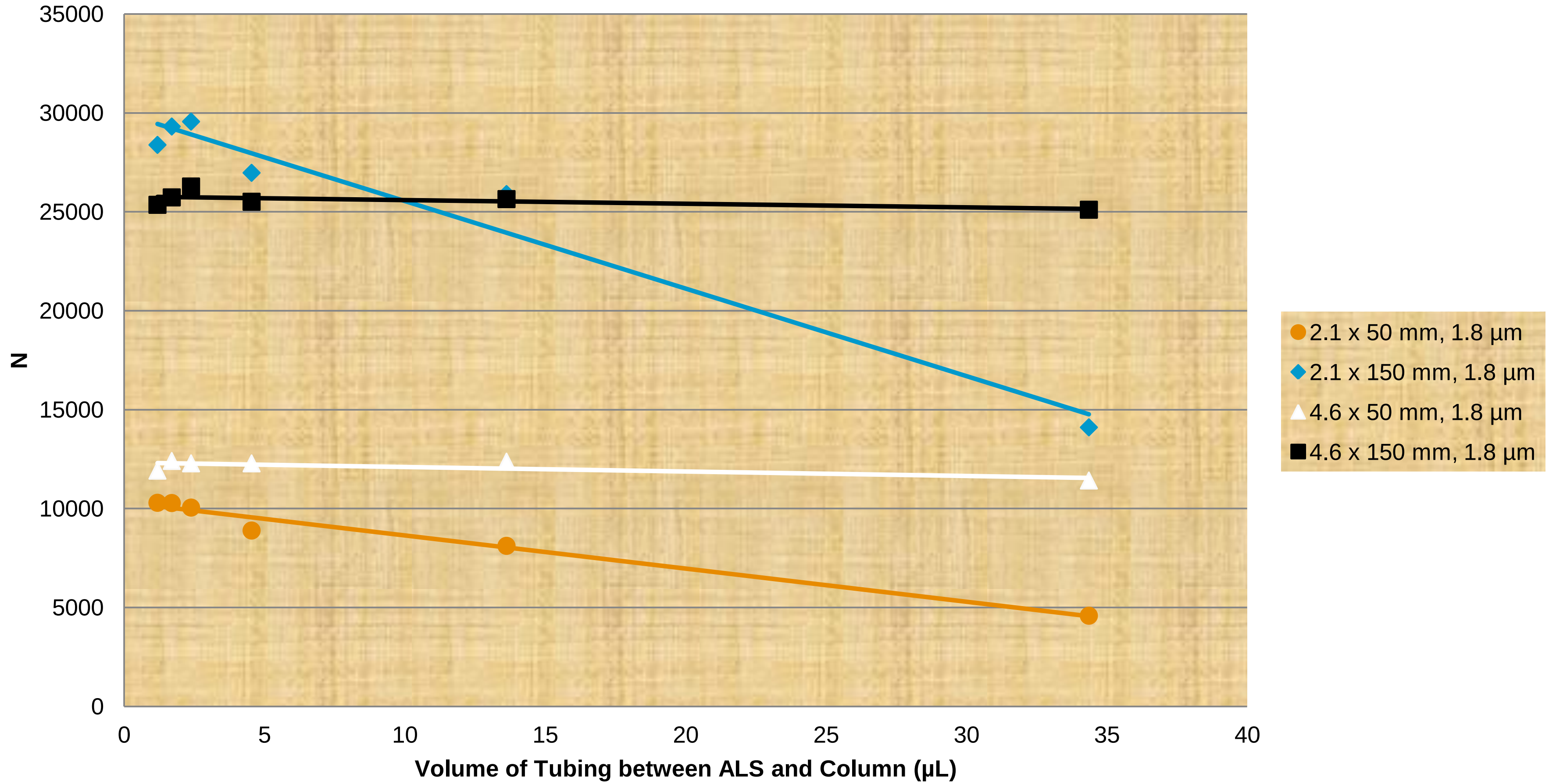
t (min)	0	1.2
%B	25	95

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

Extra-Column Volume Effect on 2.1 mm Observed Column Efficiency

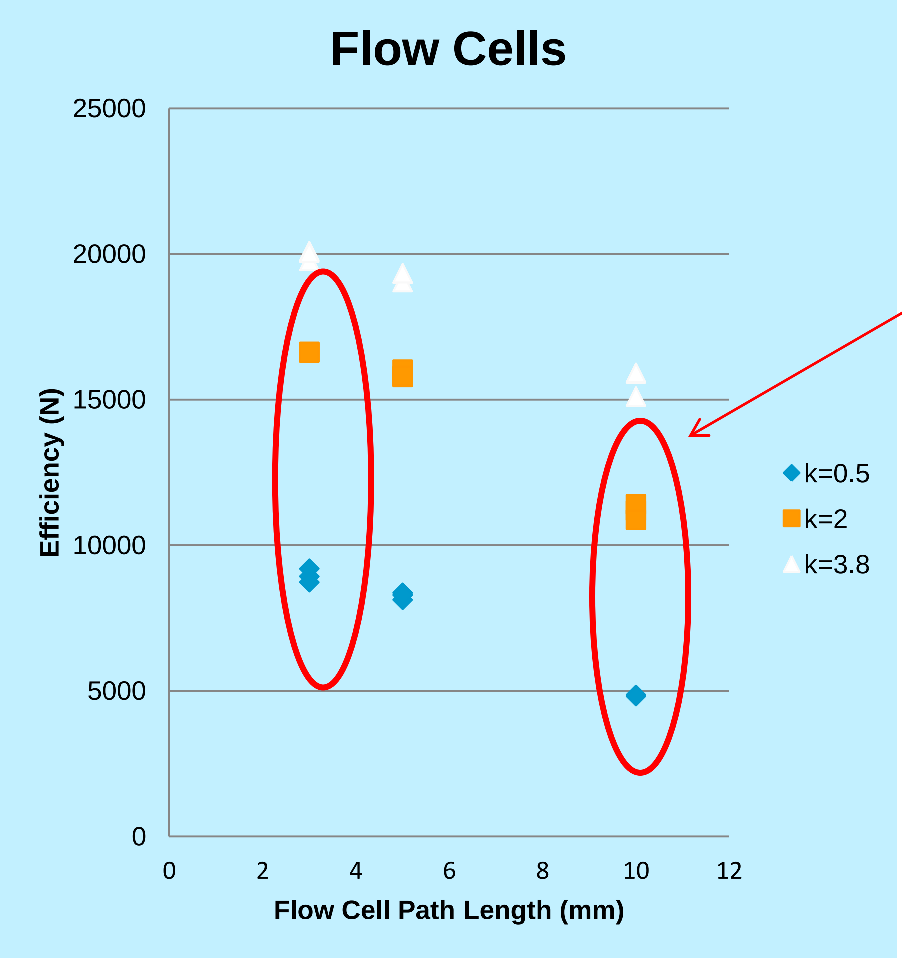


The above scatter plots compare the effects of column length and internal diameter on the ECV impact on efficiency; length has a lesser effect, while the column's internal diameter's effect is much greater

Flow Cell

- Flow Cells are an integral part of HPLC instrumentation.
  - Choose the best one for the column used
  - Don’t just use the largest one available
  - Peak broadening will compromise sensitivity and detection limits
- While detector speed can compensate for excessive flow cell dispersion, an appropriate flow cell should be used,
- The volume of a Standard flow cells for an Agilent 1100 or 1200 system is 10 µL.
- For best results, replace standard flow cells with 5 µL flow cells (2 µL when using 2.1 mm ID columns)

Flow Cell Choice With a 2.1 x100 mm Poroshell 120 EC-C18



- 30% loss of efficiency with a 10 mm standard flow cell
- With 2.1 mm columns, it is best to use a 3 mm flow cell.

Column part number 695775-902

1 ul QC Mix, Uracil, Phenol (k=0.5), 4-Chloronitrobenzene(k=2), Napthalene (k=3.8) 55% MeCN 45 % Water 0.55 ml/min micro flow cell

Detection Issues

Recognize where the problem originates

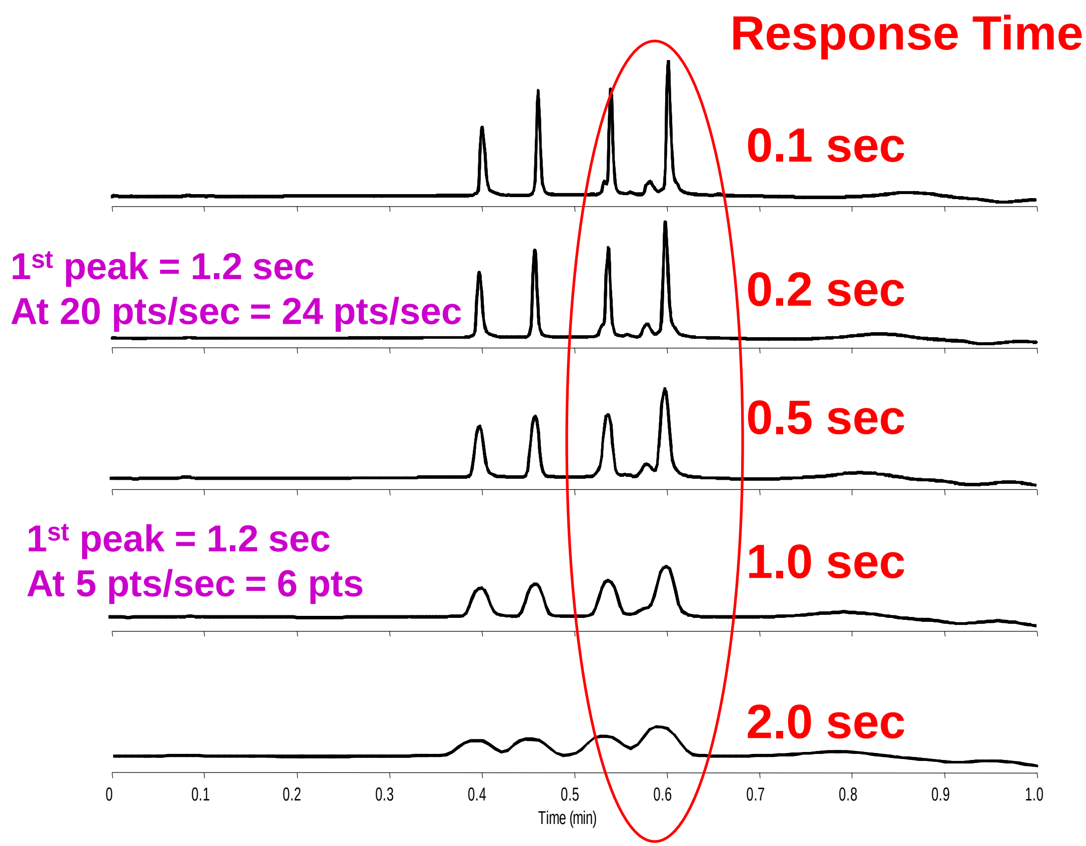
- Is it a consequence of technique?
- Is it expected due to certain mobile phase components?
- Can it be corrected by adjusting detector parameters?

Answers Will Help Find a Solution!

Effect of Detector Response Time

The System is operating well-the settings were poorly made!

Slow Data Rates Can Hinder Impurity Detection and Reduce Sensitivity



Agilent 1100 DAD
Agilent 1100 WPS with ADVR

Column: **Poroshell 300SB-C18**
2.1 x 75 mm, 5 mm

Mobile Phase:
A: 95% H_2O, 5% ACN with 0.1% TFA
B: 5% H_2O, 5% ACN with 0.1% TFA

Flow Rate: 2 mL/min

Temperature: 70°C

Detector: UV 215 nm

Piston stroke: 20

Sample:
1. Neurotensin 3. Lysozyme
2. RNaseA 4. Myoglobin

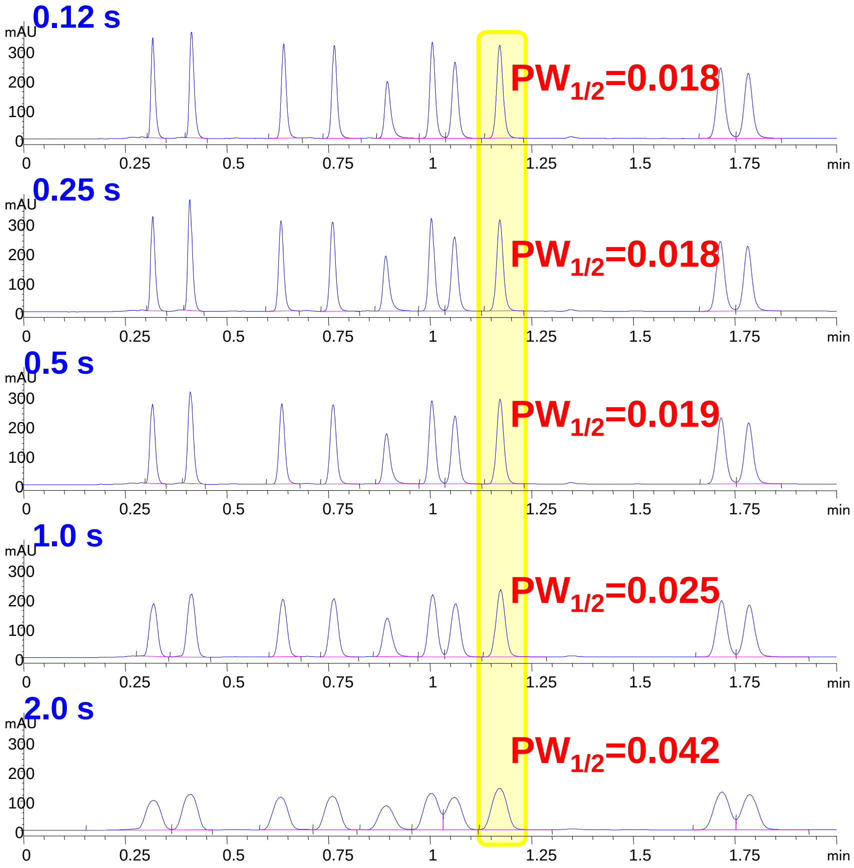
Adjust the response rate of your detector for best peak detection.

Agilent Technologies

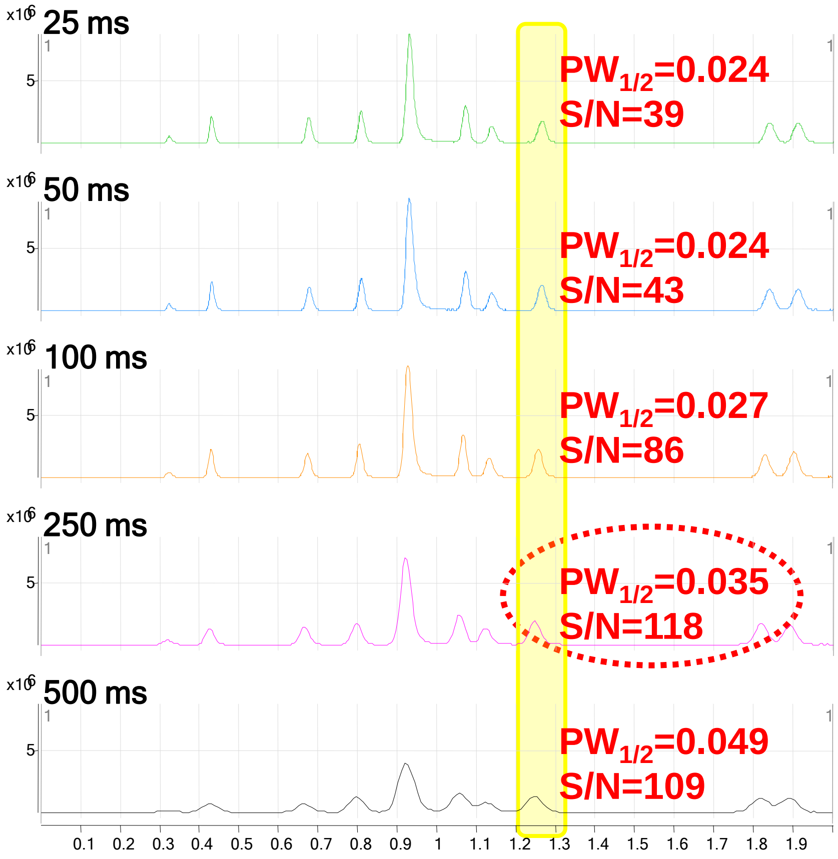
Different UV Data Collection Rates and MS Scan Rates in Scan Mode

Column: ZORBAX RRHD SB-C18, 2.1 x 100mm, 1.8um, 1200 bar
Sample: Green Tea

UV Data Collection Rate



MS Scan Rate



Optimize Detector

Optimize detector settings by:

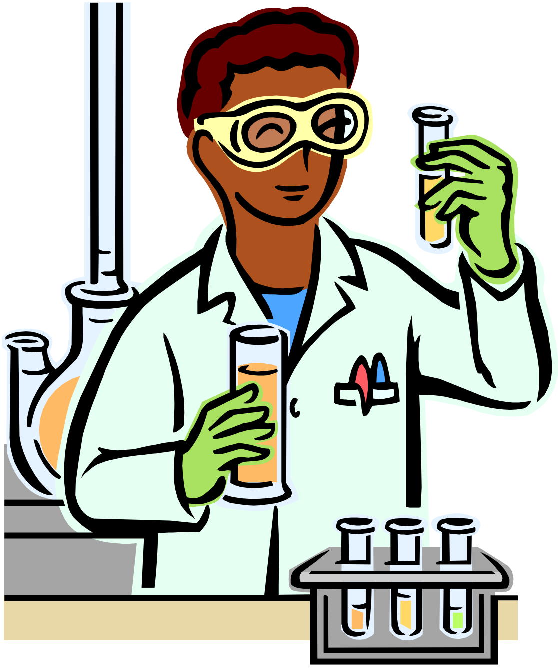
- Adjusting the scan rate and/or the time constant to the fastest possible settings
- Reduce if needed so that signal-to-noise (S/N) is not reduced

Peak width control in ChemStation lets you select the peak width (response time) for your analysis.

- The peak width (as defined in the ChemStation software) is the width of a peak at half height.
- Set the peak width to the narrowest expected peak in your sample.
- With Poroshell 120 column expect narrow peaks, like those on sub-2um
- Set the detector to the fastest setting, then to the second fastest setting and evaluate if the S/N is different

3. Retention Issues

- Retention time changes (t_r)
- Retention factor changes (k')
- Selectivity changes (alpha)



Changes in Retention (k) - Same Column, Over Time

May be caused by:

1. Column aging
2. Column contamination
3. Insufficient column equilibration
4. Poor column/mobile phase combination
5. Change in mobile phase
6. Change in flow rate
7. Change in column temperature
8. Other instrument issues

Change in Retention/Selectivity

Column-to-Column

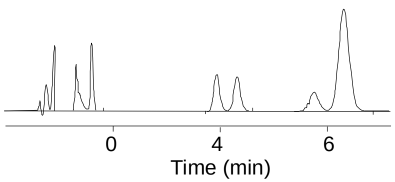
1. Different column histories (aging)
2. Insufficient/inconsistent equilibration
3. Poor column/mobile phase combination
4. Change in mobile phase
5. Change in flow rate
6. Other instrument issues
7. Slight changes in column bed volume (t_r only)

Example Change in Retention/Selectivity

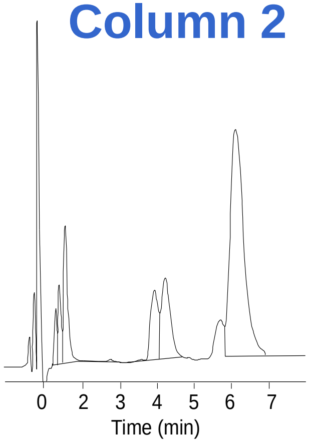
Column-to-Column

Mobile Phase Variation

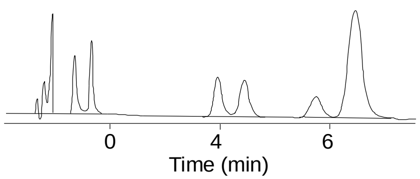
Column 1



Column 2



Column 2 - Fresh mobile phase



"I have experimented with our mobile phase, opening new bottles of all mobile phase components. When I use all fresh ingredients, the problem ceases to exist, and I have narrowed the problem to either a bad bottle of TEA or phosphoric acid. Our problem has been solved."

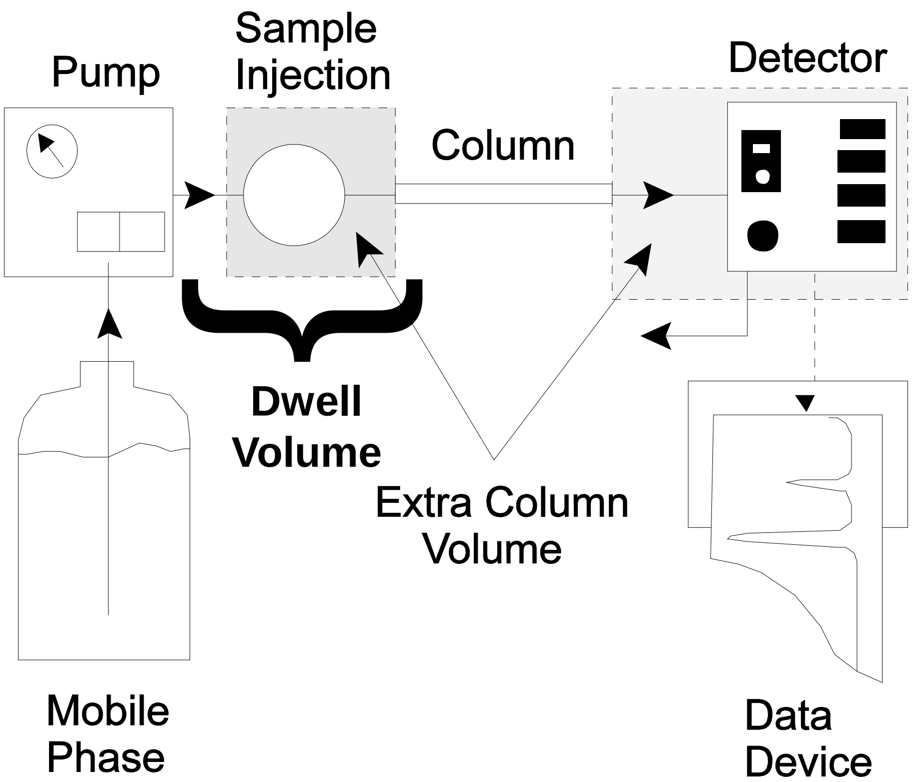
Minimize Change in Retention/Selectivity
Lot-to-Lot

Evaluate:

1. All causes of column-to-column change*
2. Method ruggedness (buffers/ionic strength)
3. pH sensitivity (sample/column interactions)

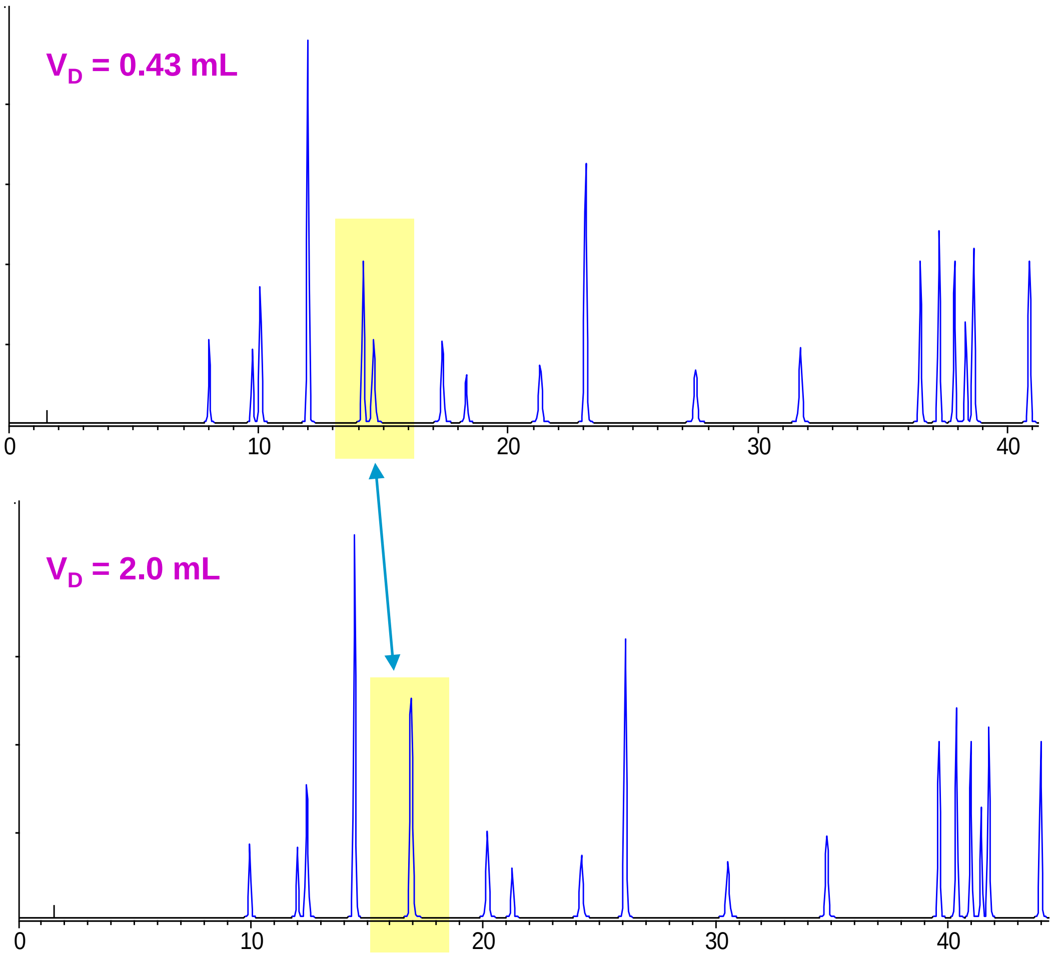
*All causes of column-to-column change should be considered first, especially when only one column from a lot has been tested.

Dwell Volume



Dwell Volume = volume from formation of gradient to top of column

Dwell Volume Differences Between Instruments Can Cause Changes in Retention and 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

What Do We Troubleshoot?
Let's Look at a Complex Example and Troubleshoot

The typical LC troubleshooting approach asks the questions:

- What's wrong with the column?
- What's wrong with the instrument?

But separations are controlled by more than just the column or instrument.

The better question is "Why doesn't my separation work as expected?"

And the answer could be there is a problem with the column, the instrument or something else (sample, mobile phase, etc.)

Problem – Selectivity Does Not Appear the Same from Column to Column

Details:

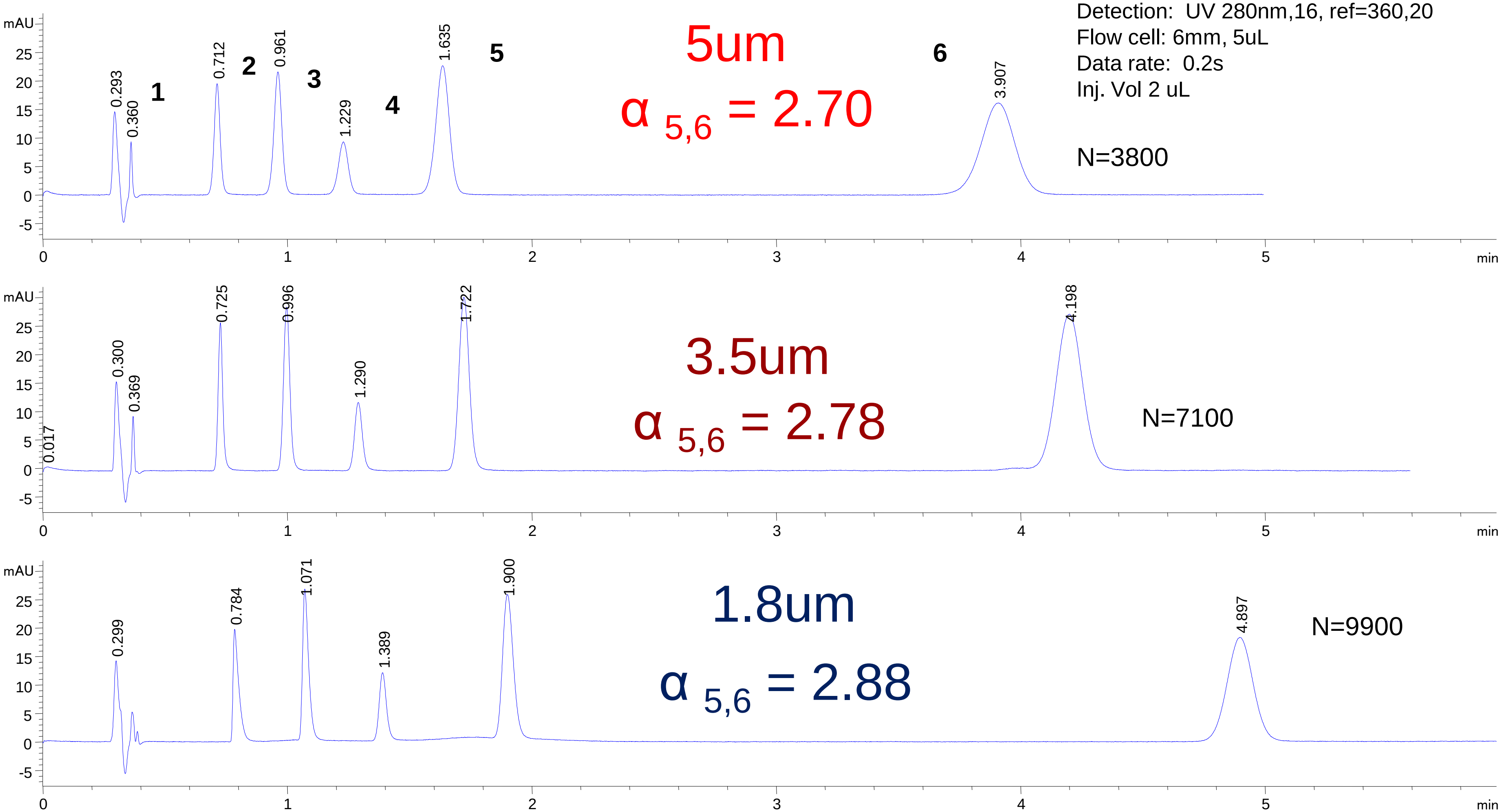
- 3 Columns with the *same bonded phase* were used
- They were the same dimensions, but with different particle sizes (and therefore different lots of material)
- They were tested on the same day, on the same instrument, with the same mobile phase

Problem:

- The selectivity was different on each of the columns

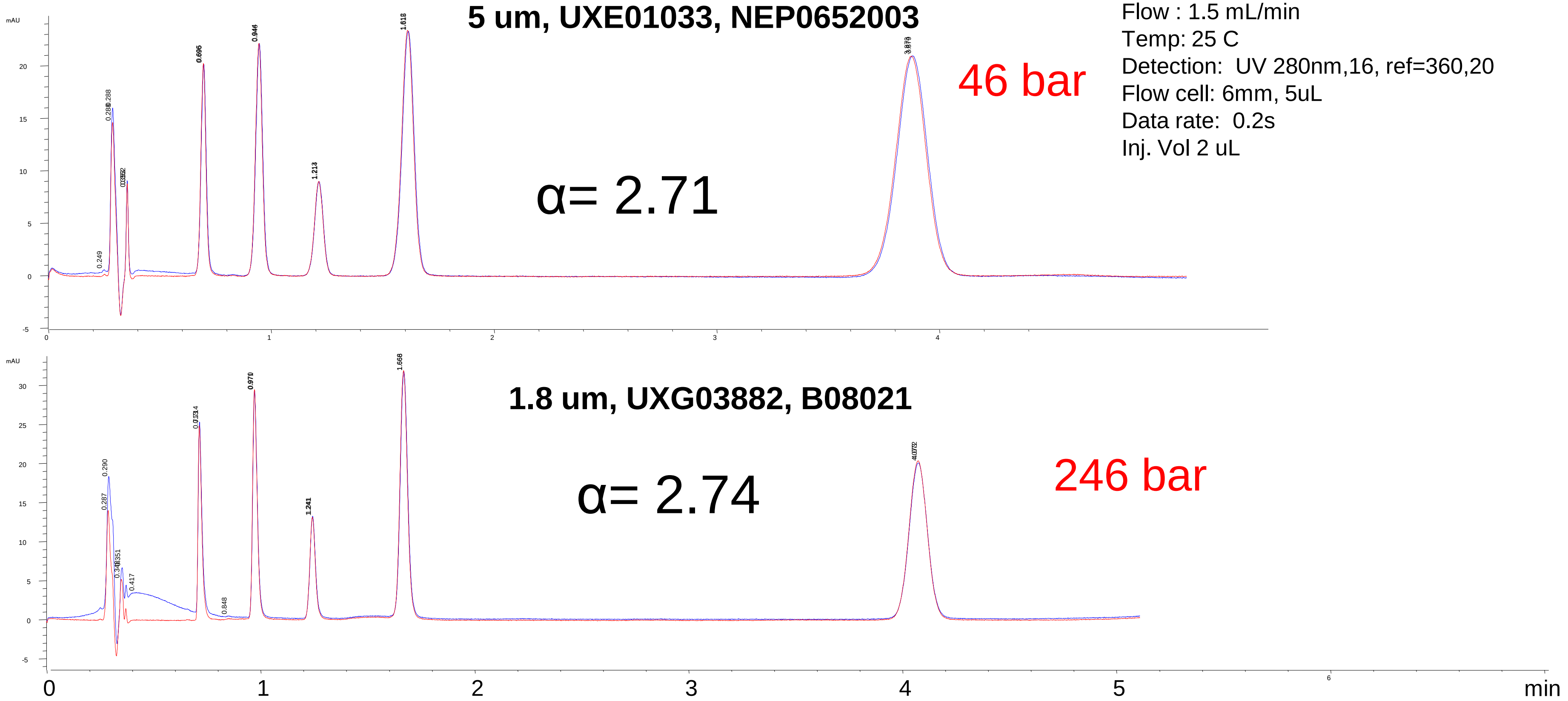
Inconsistent Selectivity between Particle Sizes of Eclipse Plus C18, 4.6 x 50 mm,

m.p.: A: water, B: acetonitrile (60:40 A:B)
Flow : 1.5 mL/min
Temp: 25 C
Detection: UV 280nm,16, ref=360,20
Flow cell: 6mm, 5uL
Data rate: 0.2s
Inj. Vol 2 uL



Problem with Proportioning Valve
One channel premixed mobile phase shows similar α

m.p.: A: water, acetonitrile (60:40 v/v)
Flow : 1.5 mL/min
Temp: 25 C
Detection: UV 280nm,16, ref=360,20
Flow cell: 6mm, 5uL
Data rate: 0.2s
Inj. Vol 2 uL



Comparison of alpha values from proportioned and premixed mobile phase

Column Type	Alpha of peaks 5,6 proportioned	Alpha of peaks 5,6 premixed
5um	2.70	2.71
3.5um	2.75	2.74
1.8um	2.88	2.74

- Selectivity changes from column to column and lot-to-lot are the hardest to resolve
- Problems can be more than just the column.
- Increasing pressure can make problems harder to troubleshoot.
- Proportion of mobile phase changes with pressure.

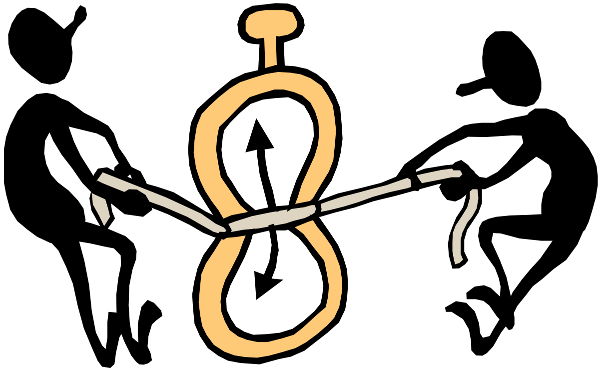
Conclusions:

Most HPLC column problems are evident as:

1. High pressure
2. Undesirable peak shape
3. Changes in retention/selectivity

These problems are not always associated with the column and may be caused by instrument and experimental condition issues.

The LC Handbook, pub # 5990-7595EN
Contact LC Column Tech Support, lc-column-support@agilent.com



The End – Thank You!

Agilent Technologies

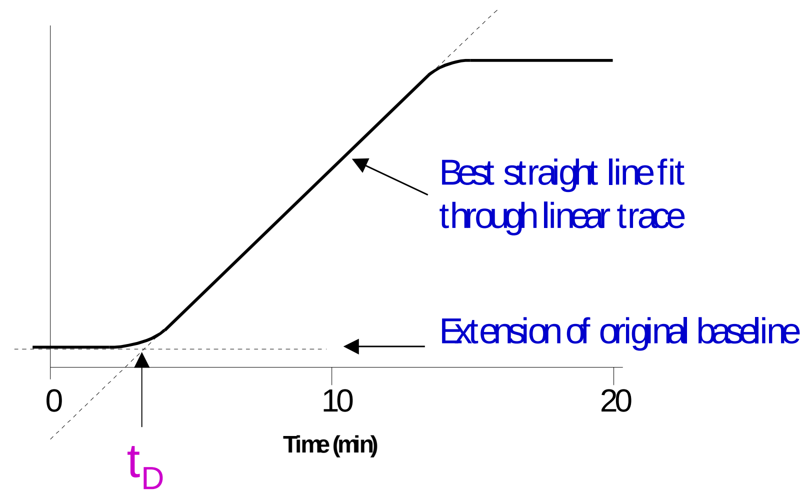
Determining the Dwell Volume (System Equilibration Volume) For Your HPLC System

- Replace column with Zero Dead Volume (ZDV) union
- Prepare mobile phase components

  A = methanol
  B = methanol with 0.2% v/v acetone

- Monitor at 254 nm
- Run gradient from 0 to 100%B in 10 minutes
- Use flow rate appropriate with column and HPLC system
  - Standard 1100 binary or quaternary system
    - 4.6 mm (e.g., 1 mL/min),
  - Capillary HPLC system plumbed for
    - 0.5 mm capillary column (10 - 20 µL/min)
    - 0.3 mm capillary column (3 – 5 µL/min)

$V_D = t_D \times F$



- Intersection of the two lines identifies dwell time (t_D)
- Dwell volume is equal to product of the flow rate and the dwell time