Taking the Trouble Out of Troubleshooting Is It the Column, Method, or Instrument

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What Do We Troubleshoot

Typical LC troubleshooting approach asks:

– What's wrong with my column?

– What's wrong with my instrument?

But our separations are controlled by more than just the column or instrument. The **better question** to ask is -
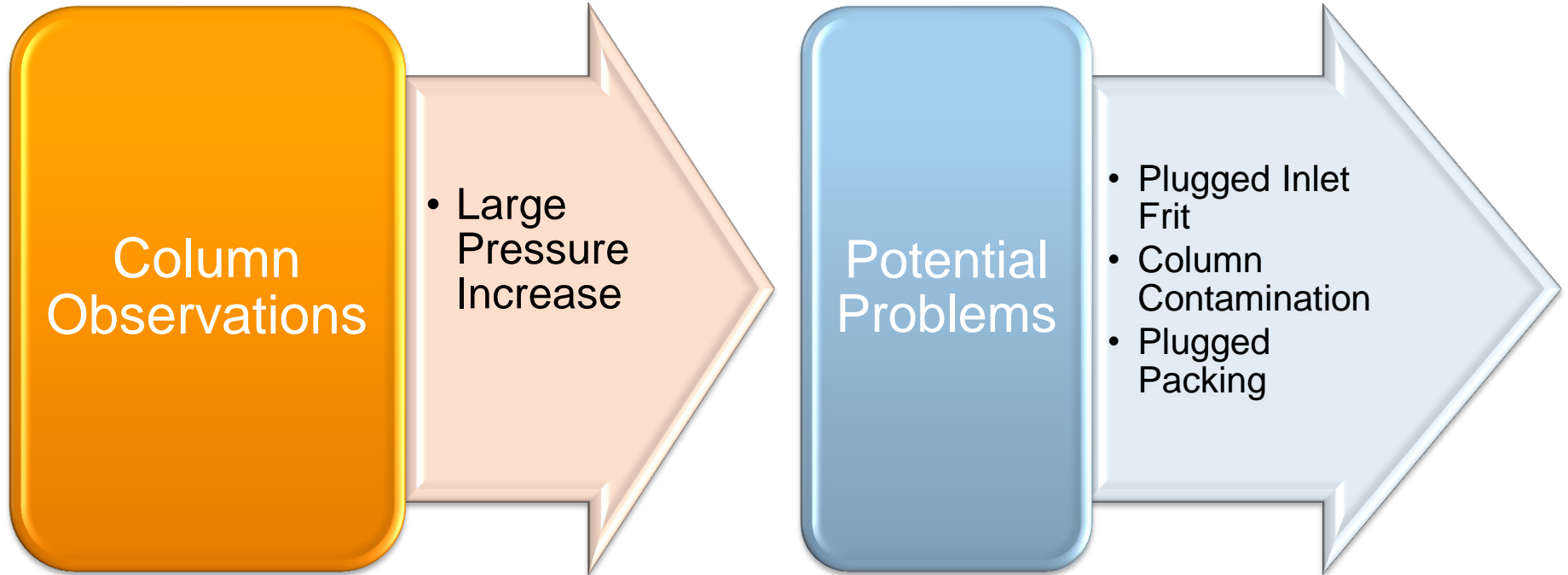
Why doesn't my separation work as expected?

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

Why Doesn't My Separation Work as Expected?
Finding the Answer

➢ Discuss some of the most commonly observed column related issues in HPLC

- System pressure
- Peak shape
- Retention/Selectivity

➢ Explore reasons for problems

➢ Preventive measures

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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.

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Determine the Cause and Correct

Possible Causes

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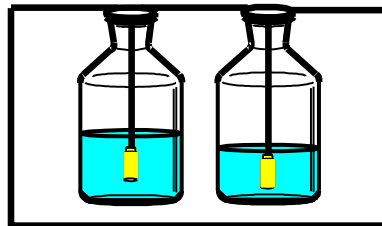- Guard or in-line filter

Check pressure with/without column

If Column pressure is high -

- Wash column (see Appendix)
  - ✓ Eliminate column contamination and plugged packing
  - ✓ high molecular weight/adsorbed compounds
  - ✓ precipitate from sample or buffer
- Back flush column
  - ✓ Clear plugged frit (check column info)

Preventing Column Back Pressure Problems

RRLC in-line filter, 0.2 µm filter
4.6 mm ID, 5067-1553
2.1 mm ID, 5067-1551

1290 Infinity in-line filter, 0.3 µm, 1200 bar, 5067-4638

- Filter mobile phase
  - Filter non-HPLC grade solvents
  - Filter buffer solutions
- Install in-line filter between auto-sampler & column (removes pump seal debris, ALS rotor debris, & sample particulates)
- Filter all samples and standards
  - Syringe; in-line
- Perform sample clean-up (i.e. SPE, LLE) on dirty samples
  - Analyte Adsorption/Matrix Adsorption
- Appropriate column flushing – flush buffers from entire system with water/organic mobile phase
- Best practice, replace buffer every 24-48 hours
  - Never add to the bottle – always use a clean new one
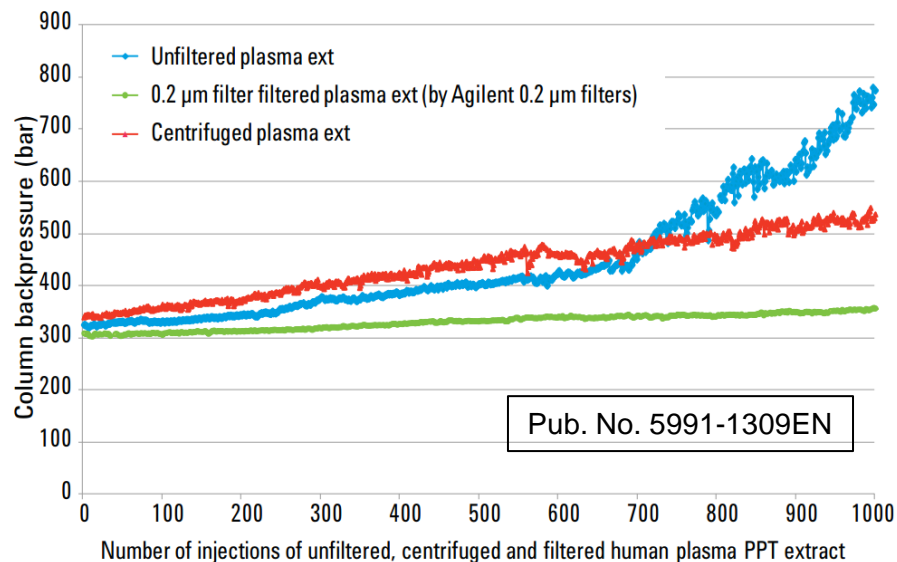
Captiva Filtration and it's Benefits

Filtration is basic sample preparation method for all kinds of samples

Physically removes particulates from the sample

Prevents blocking of capillaries, frits, and column inlet (especially for UHPLC)

Results in less downtime of the instrument for repairs

Results in less wear and tear on the critical moving parts of the injection valves

Legend:
- Unfiltered plasma ext
- 0.2 μm filter filtered plasma ext (by Agilent 0.2 μm filters)
- Centrifuged plasma ext

Y-axis: Column backpressure (bar)
X-axis: Number of injections of unfiltered, centrifuged and filtered human plasma PPT extract

Pub. No. 5991-1309EN

Unfiltered, centrifuged, and filtered plasma extracts
Zorbax RRHD Eclipse Plus C18, 2.1 x 50 mm, 1.8 μm column, PN 959757-902

Captiva Syringe Filters Guide 5991-1230EN

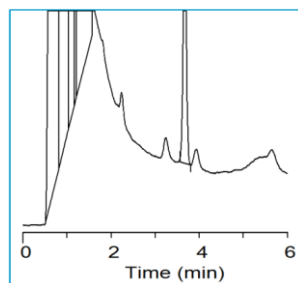Syringe Filter Selection Tool

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Why perform Sample Preparation?

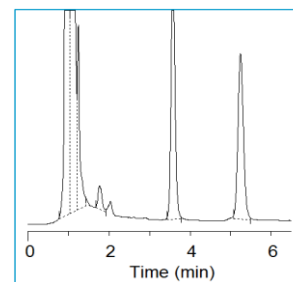To acquire desired sensitivity/selectivity

To reduce contamination/carryover issues

Use of sensitive and expensive instruments: _Protect your investment!!!_

Pesticides in Avocado _without_ SP

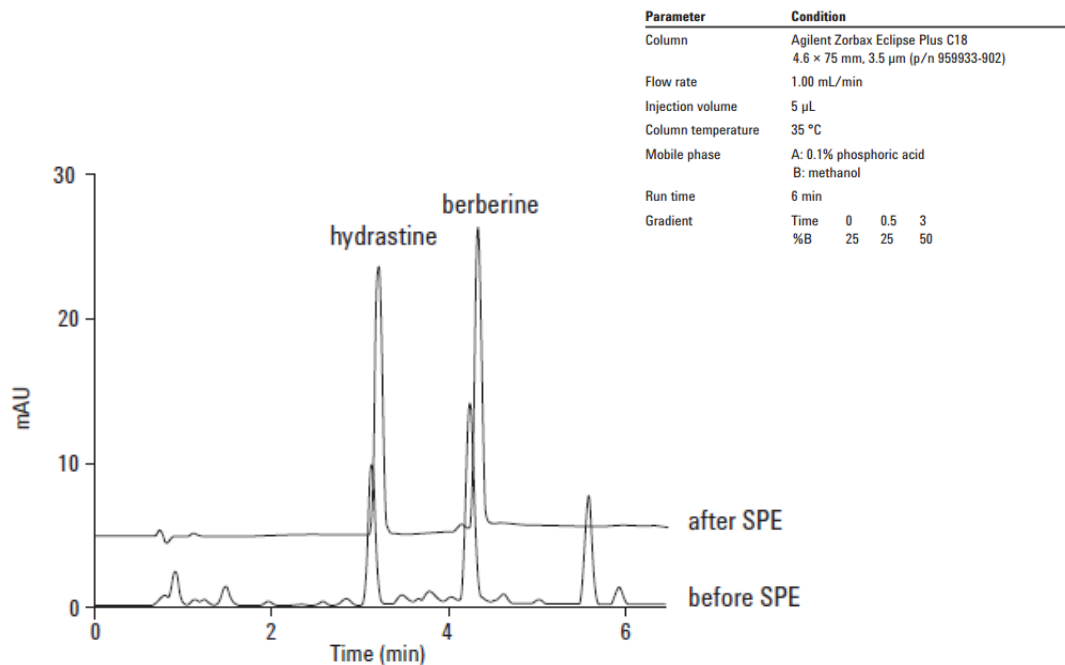Pesticides in Avocado with SP

Contaminant Peaks
Bond Elut SPE and its Benefits

In this example, results after SPE show a decrease in the number of small peaks/removal of interferences and an increase in the intensity of the peak heights

Parameter	Condition			
Column	Agilent Zorbax Eclipse Plus C18 4.6 × 75 mm, 3.5 µm (p/n 959933-902)			
Flow rate	1.00 mL/min			
Injection volume	5 µL			
Column temperature	35 °C			
Mobile phase	A: 0.1% phosphoric acid B: methanol			
Run time	6 min			
Gradient	Time	0	0.5	3
	%B	25	25	50

HPLC-DAD chromatograms of hydrastine and berberine from goldenseal roots extract before and after SPE

Determination of Alkaloids in Goldenseal using Agilent Bond Elut Plexa (5990-9563EN)

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Prevent Pressure Problems *by* Preventing Microbial Growth

➢ Potential problems

- **Increased system pressure or pressure fluctuations**
- **Increased column pressure, premature column failure**
- Can mimic application problems
- Gradient inaccuracies
- Ghost peaks

➢ Prevent and/or Reduce Microbial Growth

- Use freshly prepared mobile phase
- Filter
- Do not leave mobile phase in instrument for days without flow
- Always discard "old" mobile phase
  - o Do not add fresh mobile phase to old
- Use an amber solvent bottle for aqueous mobile phase
- If possible, can add
  - o 5% organic added to water can be used to reduce bacterial growth
  - o Few mg/l sodium azide

Check your instrument manual for guidelines

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Troubleshooting Pressure
Things to Remember

I. Continuously increasing pressure even _with no_ injections

- Pump Seals

- Mobile Phase Particulates

- Mobile Phase Solubility

- Mobile Phase Unstable (polymerization)

- Column Void Formation (use condition dependent)

- Microbial Growth

II. Increasing pressure _with_ sample injections

- Sample Particulates

- Sample Not Soluble in Mobile Phase

- Sample Components Irreversibly Bound to Stationary Phase

2. Peak Related Issues

- Split peaks

- Peak tailing

- Broad peaks

- Poor efficiency (low N)

- Inconsistent response

- Ghost Peaks

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

Split Peaks

Can be caused by:

- Column contamination

- Partially plugged frit
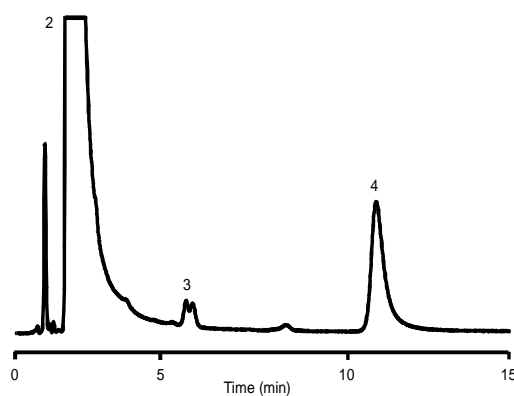
- Column void

- Injection solvent effects

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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
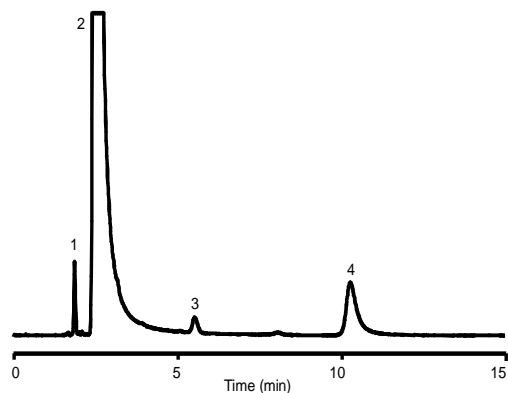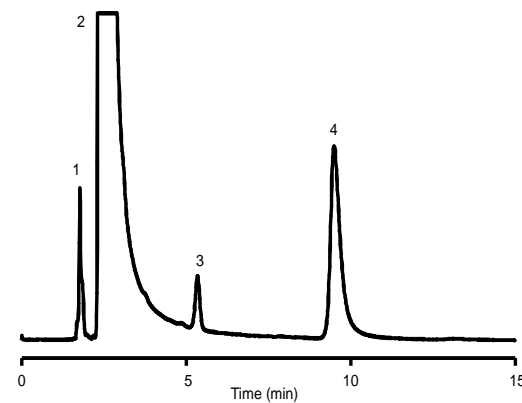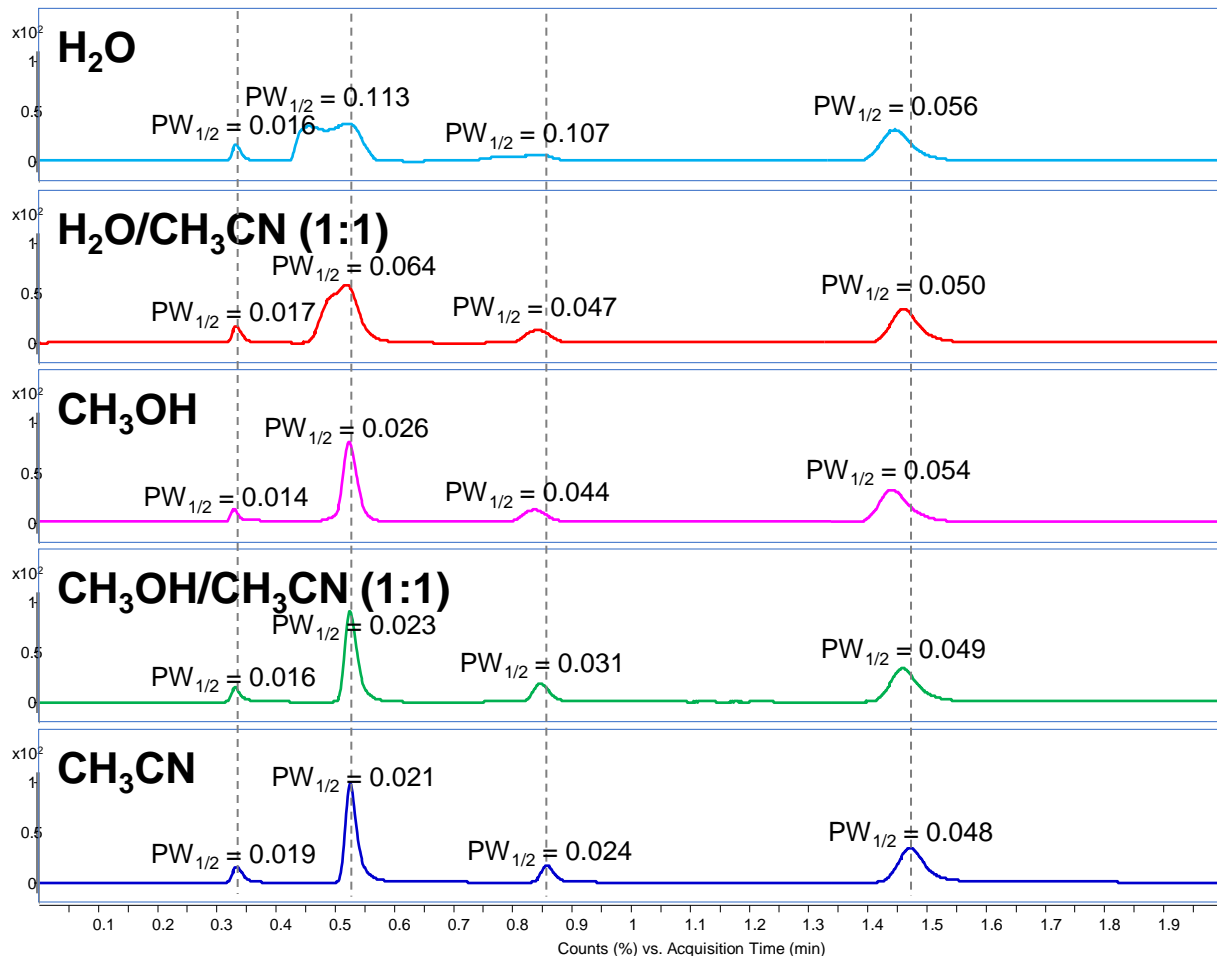

- Column washing eliminates the peak splitting, which resulted from a contaminant on the column.

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Injection Solvent Effects - HILIC
H_2O, CH_3OH, CH_3CN

Agilent 1290 Infinity LC System
Agilent 6410A LC/MS
Agilent ZORBAX RRHD HILIC Plus 2.1 x 50 mm, 1.8 µm

Acetonitrile / 100 mM ammonium formate pH 3.2 (9:1)
0.4 mL/min, Pressure: 135 bar
Isocratic elution
Injection Volume: 1 µL of 5 µg/mL sample
Column: 25 ^{o}C
MS: ESI+, SIM, 200 ^{o}C, 10 L/min, 30 psi, 4000 V, 15 ms dwell time

Sample:
4-Aminobenzoic acid, m/z 138 (Frag 110 V)
Nicotinamide, m/z 123 (Frag 130 V)
Riboflavin, m/z 377 (Frag 160 V)
Nicotinic acid, m/z 124 (Frag 130 V)

➢ **Strong injection solvent can affect peak shape and retention**

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 – may have a column void due to silica dissolution (unless specialty column used; Poroshell 120 HpH, Zorbax Extend C18, or polymer based like PLRP-S)**

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

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

Symmetry > 1.2

Normal   Tailing

Normal   Tailing
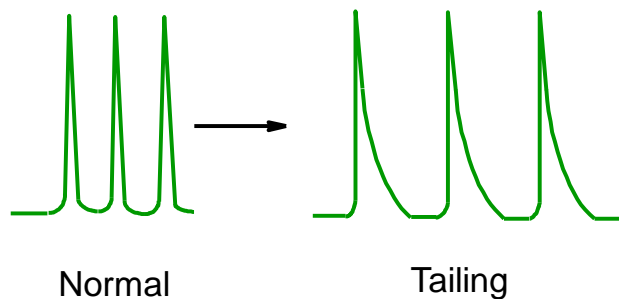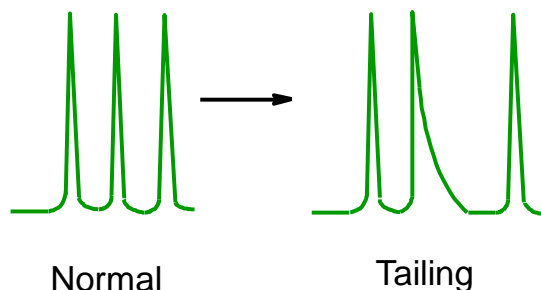
Causes

Some Peaks Tail:

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

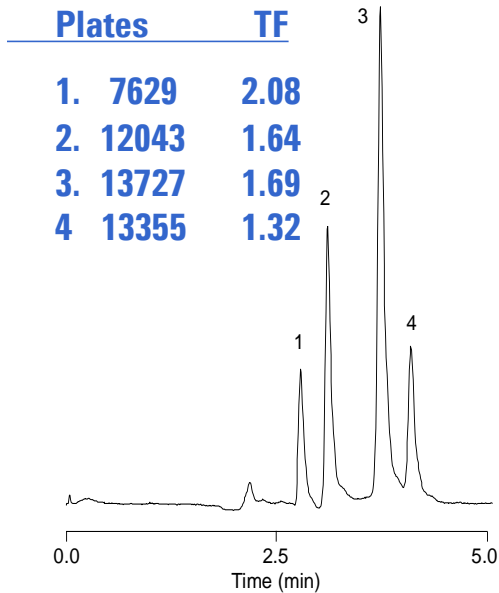All Peaks Tail:

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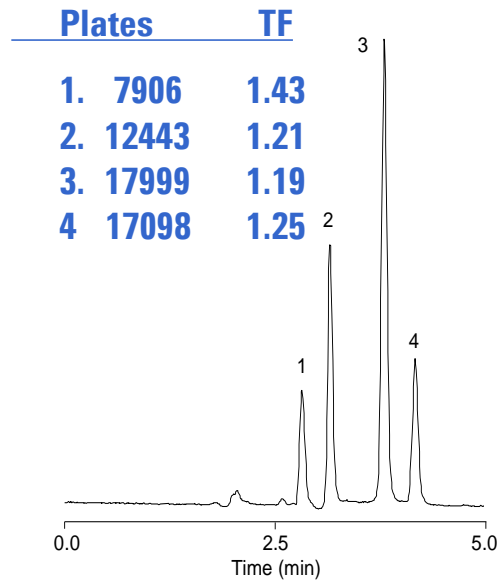- Bad column or bad choice of column

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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
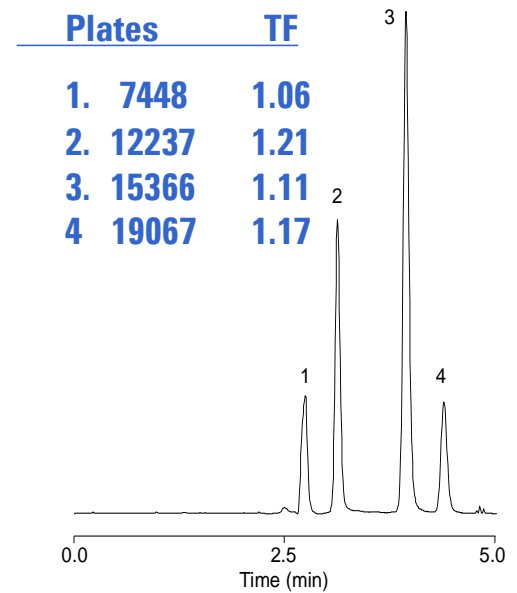
QC test forward direction

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

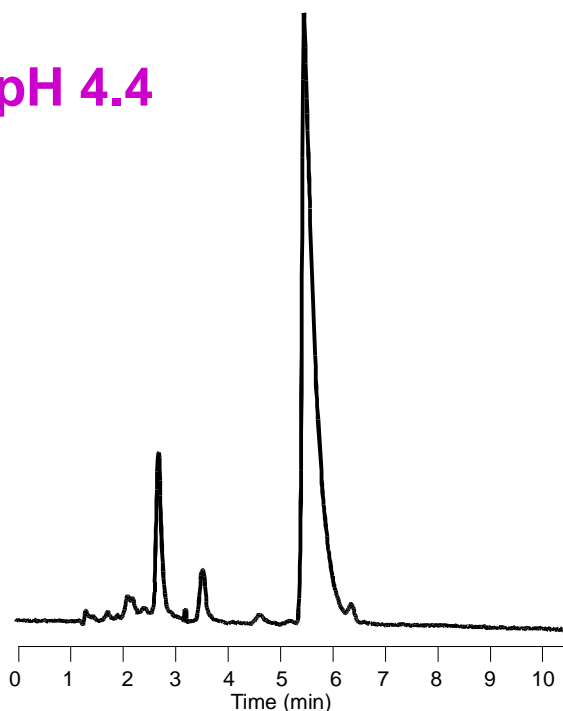| Plates	TF
1. 7448	1.06
2. 12237	1.21
3. 15366	1.11
4 19067	1.17

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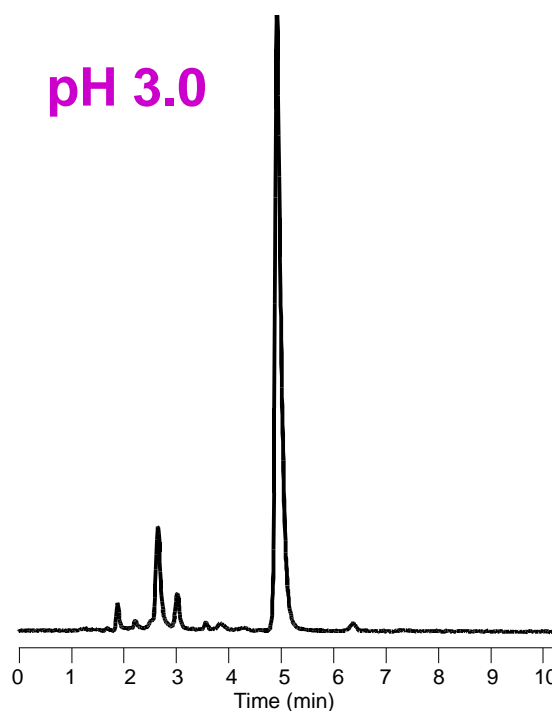
Effect of pH on Peak Shape
What Happens Near the Sample pK$_a$

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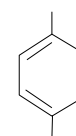Flow Rate: 1.0 mL/min.   Temperature: RT

pH 4.4

pH 3.0

$CH_3CHCOOH$

$CH_2CH(CH_3)_2$

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.

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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

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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

  o 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.

  o These can be fittings swaged incorrectly, to the wrong depth or incompatible fittings being used

- These unswept or poorly swept areas will cause tailing, broadening and loss of column efficiency

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

**Wrong … too short**

**Mixing Chamber**

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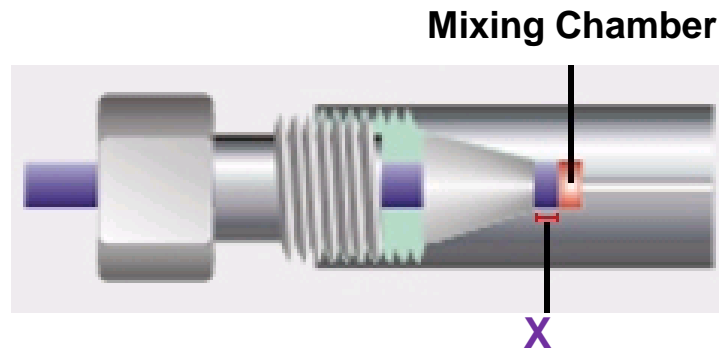
➢ **If Dimension X is too short, a dead-volume, or mixing chamber, will occur**

➢ **This can broaden peaks and/or cause them to split or tails**

➢ **It will typically affect all peaks, but especially early eluting peaks**

Peak Tailing – Extra Column Effects
Poor Fitting

Problem:
All peaks tail
(top chromatogram).

Before troubleshooting
Tailing peaks

After troubleshooting
Symmetrical Peaks
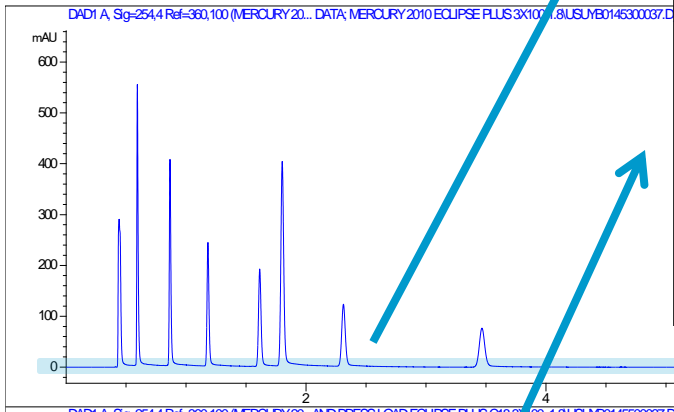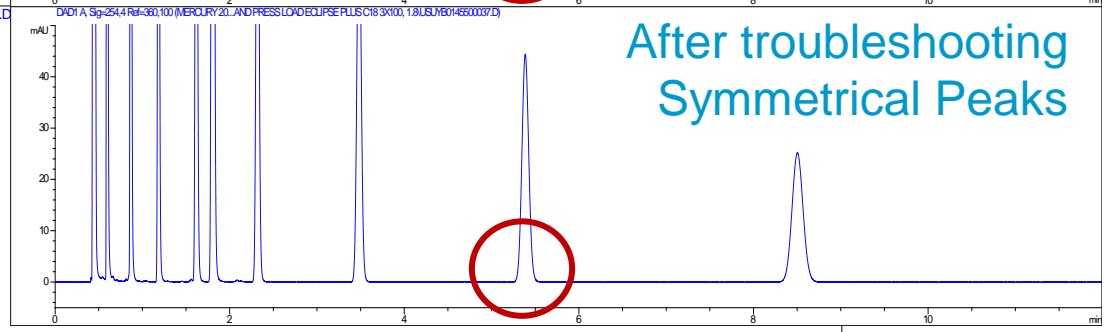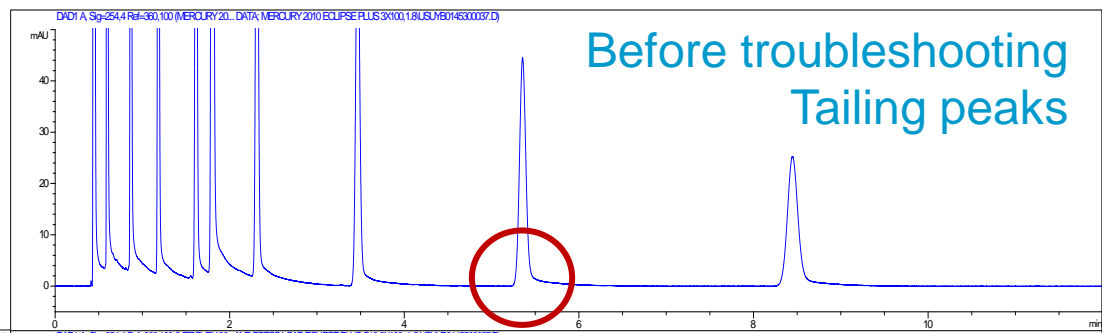
Cause:
Capillary tubing connecting ALS and column was swaged improperly on the ALS end (ferrule was flush with end of tubing, causing a void).
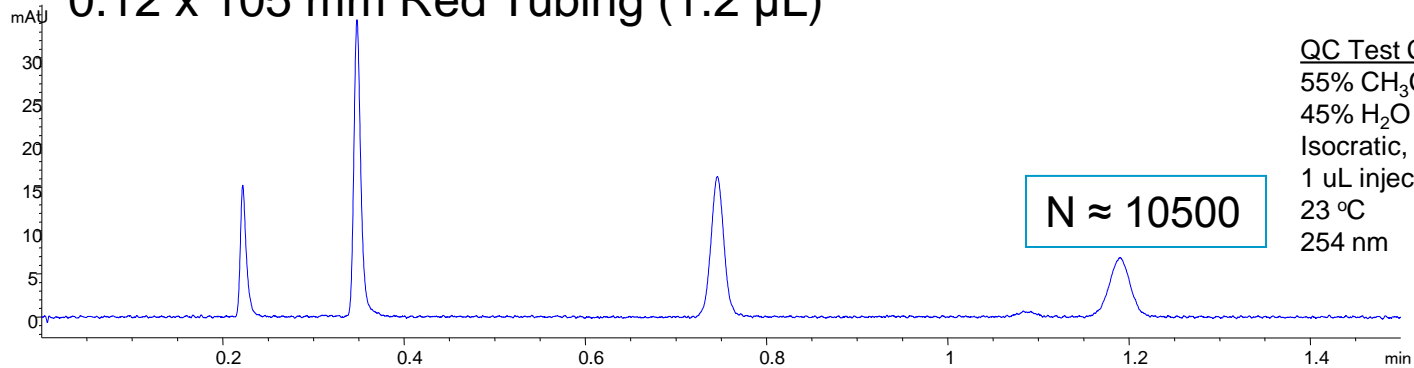
Solution:
Replace tubing (bottom chromatogram).
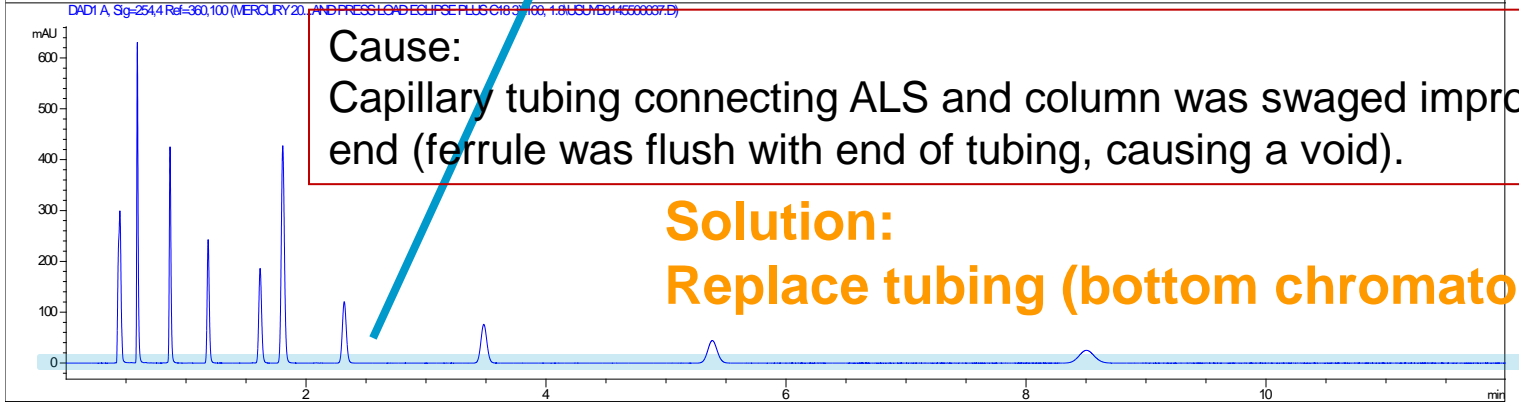
Peak Shape - Broadening
Efficiency is greatly reduced when extra-column volume increases

0.12 x 105 mm Red Tubing (1.2 μL)

N ≈ 10500

QC Test Conditions:
55% CH_3CN
45% H_2O
Isocratic, 0.6 mL/min
1 uL injection of QC Mix
23 °C
254 nm

0.25 x 700 mm Blue Tubing (34.4 μL)

N ≈ 4500

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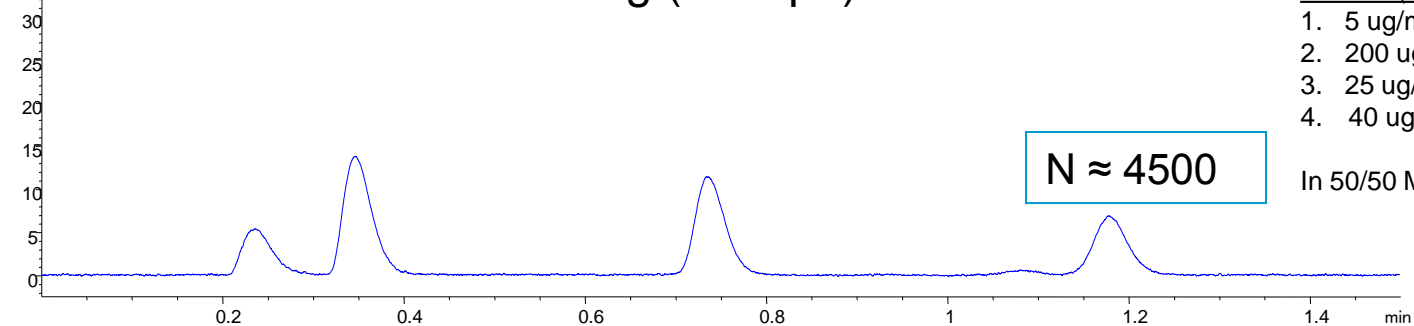
QC test of a 2.1 x 50 mm, 1.8-μm Eclipse Plus C18 showing the peak broadening when larger volume tubing is installed between the autosampler and column.
43% of the efficiency is lost with too much extra column volume

Extra Column Volume = sample volume + connecting tube volume + fitting volume + detector cell volume
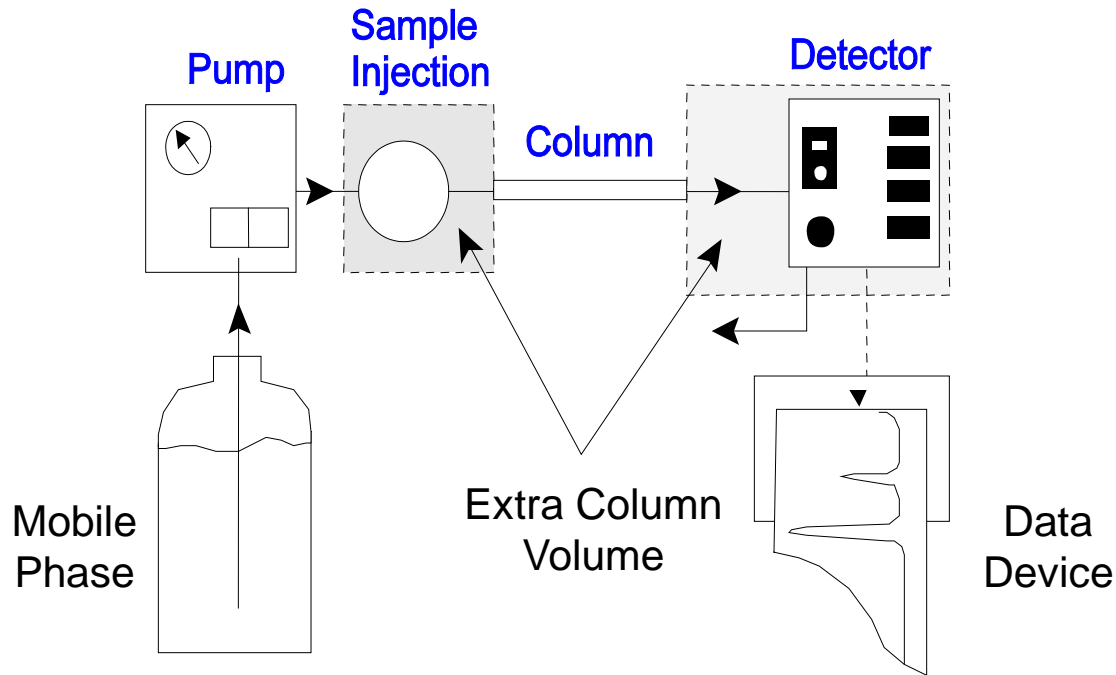
The instrument schematic above depicts where extra-column volume can occur, thus effecting instrument and column performance.

Note: More on this in "Making LC Connections", October 24, 2016

Effect on Efficiency
Flow Cell Choice
2.1x100 mm Poroshell 120 EC-C18, PN 695775-902

Flow Cells

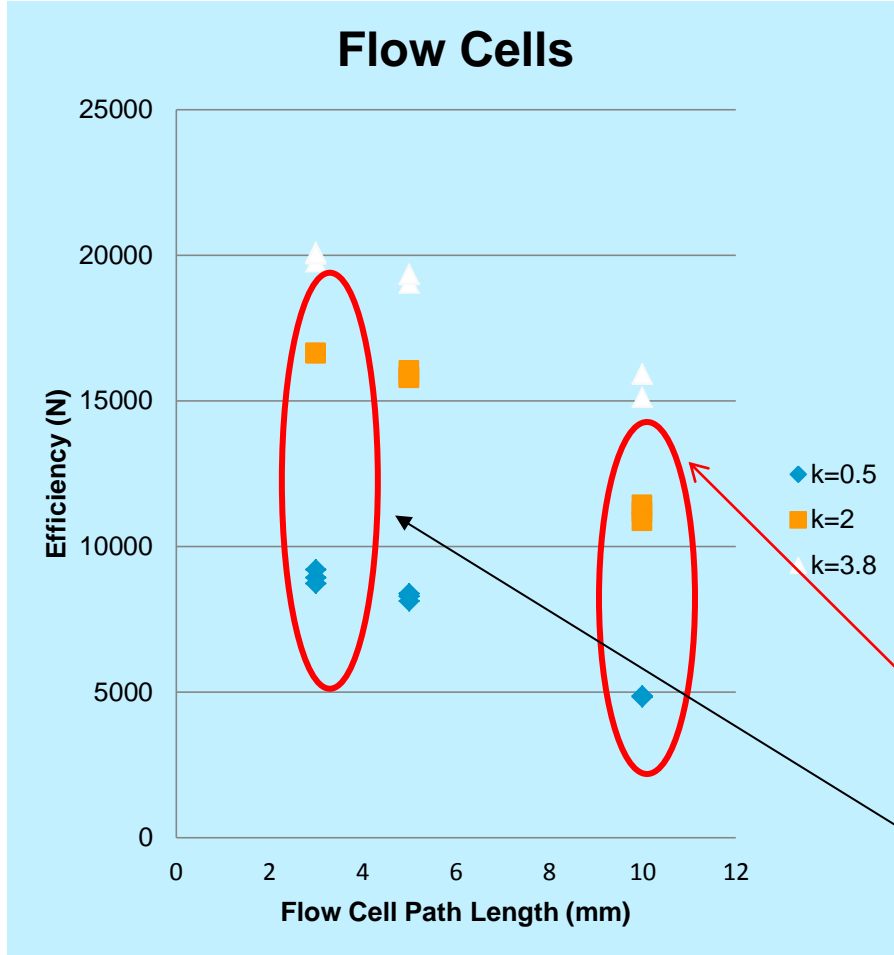Flow Cells are an integral part of HPLC instrumentation.
- Choose the best one for the column used
- Don't assume you have the best one for your column
- Peak broadening will compromise sensitivity and detection limits

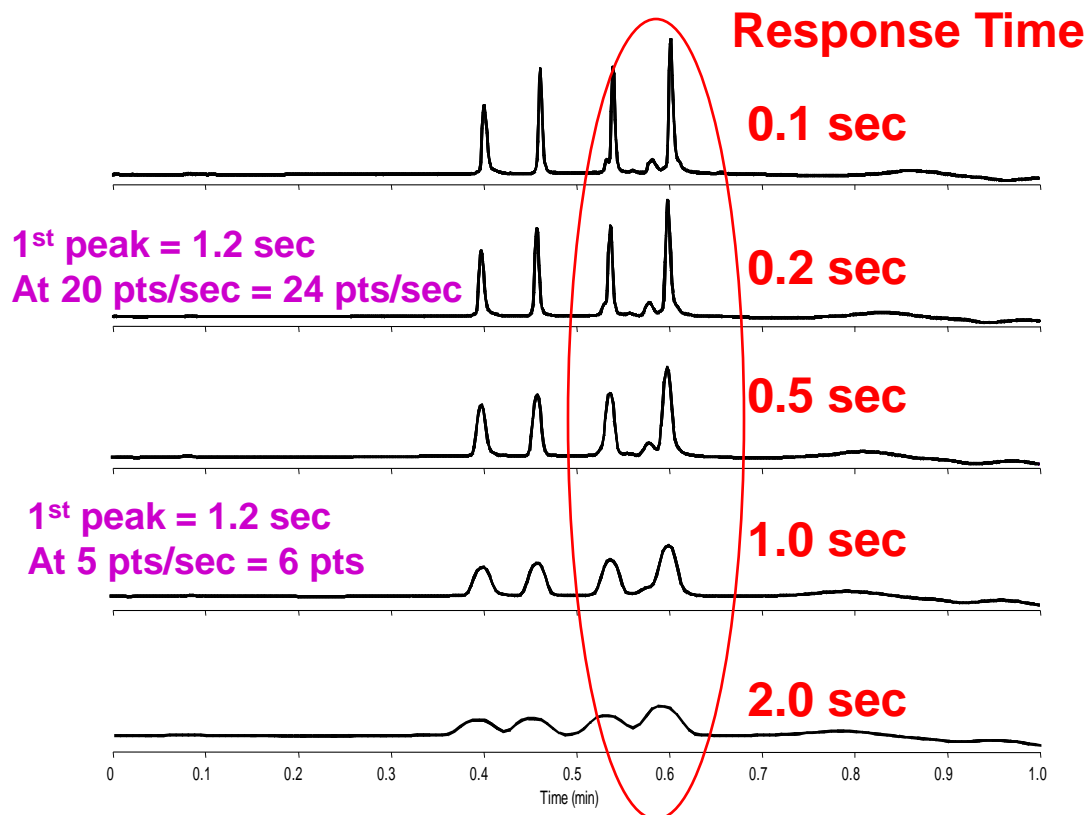*For best results, replace standard flow cells (10 µL) with 5 µL flow cells (2 µL when using 2.1 mm ID columns)*

• 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.

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

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Peak Shape
Effect of Detector Response Time

It may not be a "bad column"

Response Time

0.1 sec

0.2 sec

0.5 sec

1.0 sec

2.0 sec

1st peak = 1.2 sec
At 20 pts/sec = 24 pts/sec

1st peak = 1.2 sec
At 5 pts/sec = 6 pts

Time (min)

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 and shape

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Peak Shape
Ghost Peaks – Even with No Sample

20% - 100% MeOH

No Sample Injected

60

15

30

15

0

3   7
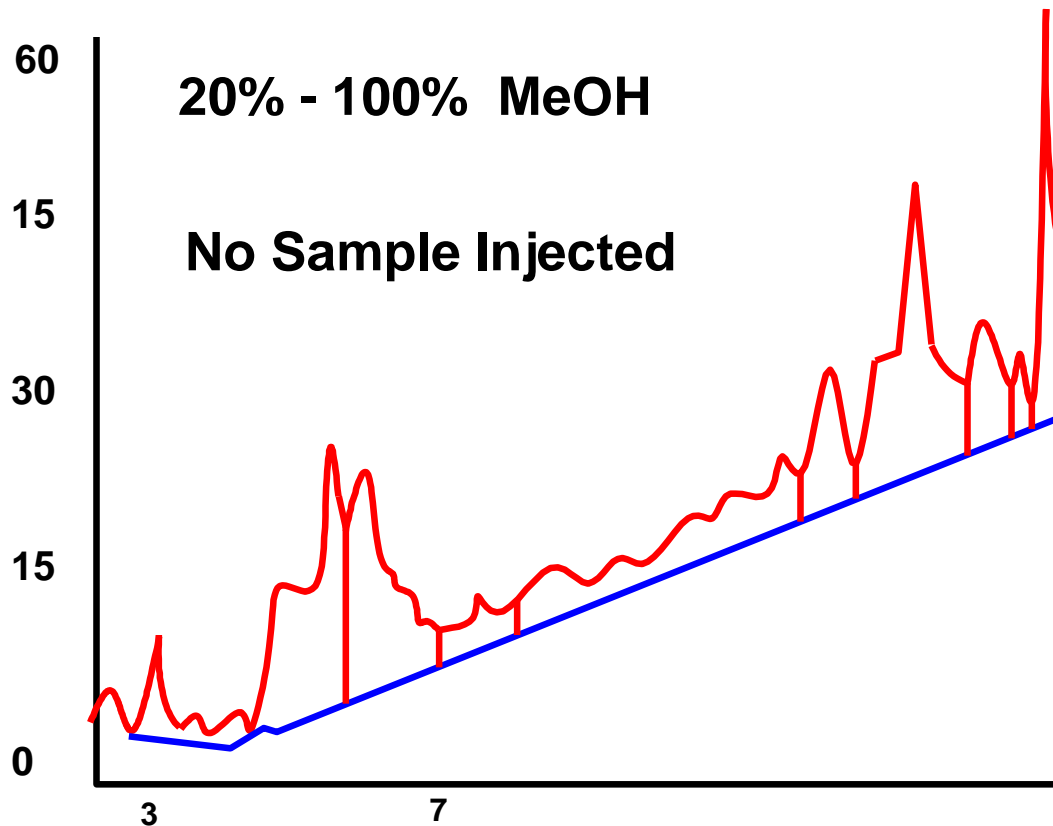
Problem
- Dirty mobile phase
- Sample carryover
  - May imply poor recovery
- Peak from an early run (isocratic)

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Solvent Contamination

Injections on Agilent 1100

Legend:
- Solvent Source 1
- Solvent Source 2
- Solvent Source 3
- Solvent Source 4, Lot 1
- Solvent Source 4, Lot 2
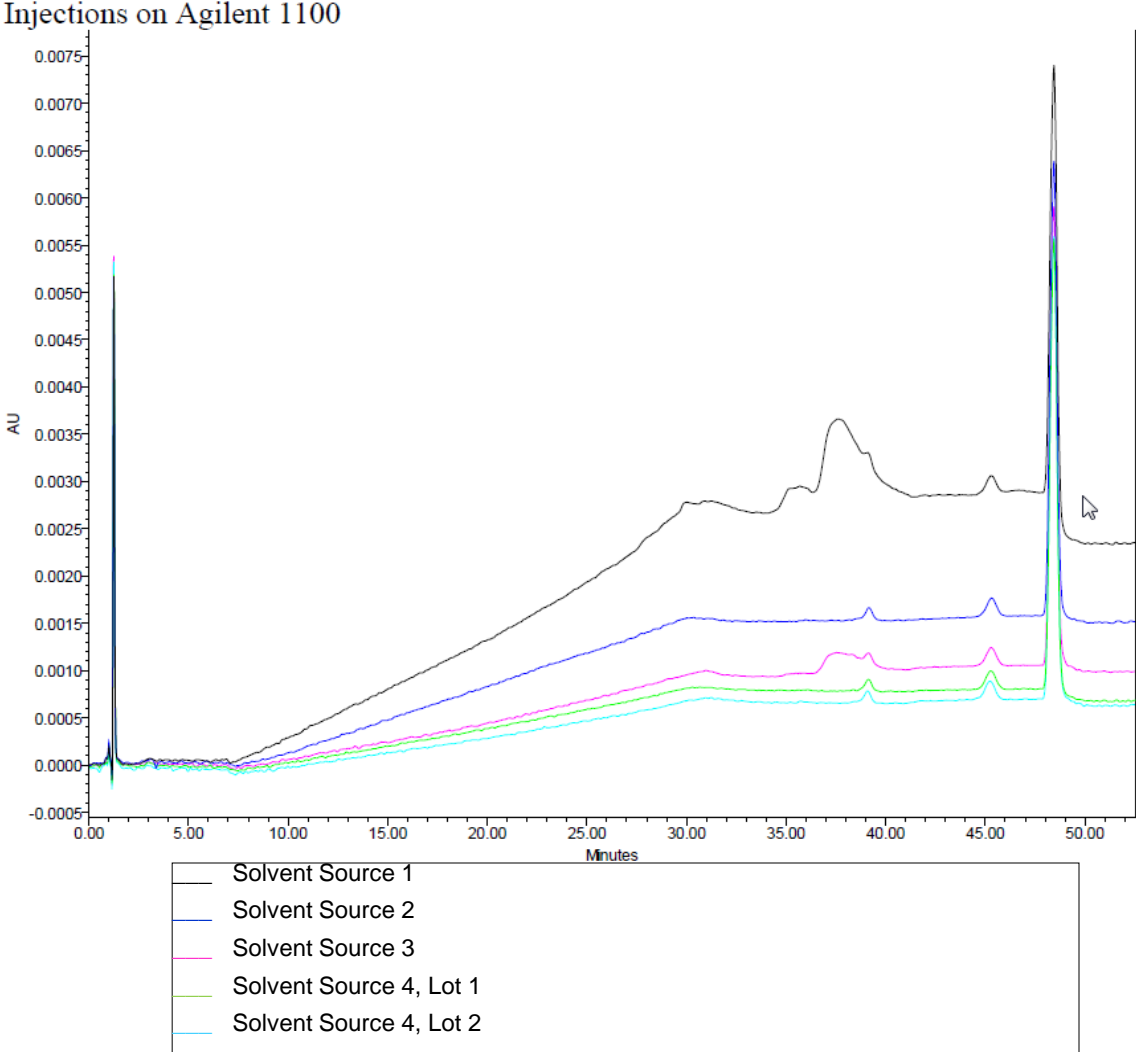
Troubleshooting Peak Shape Issues "Usual Suspects"

- ✓ Partially blocked inlet frit
- ✓ Sample solvent strength
- ✓ Injection volume
- ✓ Sample load
- ✓ Secondary interactions
- ✓ Hardware failure (rotor, stator)
- ✓ Ghost peaks
- ✓ Metal interactions/chelation
- ✓ No or insufficient mobile phase pre-heating
- ✓ Extra column volume
- ✓ Mobile Phase

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3. Retention Shifts/Selectivity Changes

All Peaks Shift to Lower Retention (acids, bases, neutrals)

- Loss of bonded phase
- Mobile phase unstable (less likely)
- Solvent delivery system (flow rate or mixing)

All Peaks Shift to Greater Retention

- Loss of organic solvent in aqueous/organic mix
- Column change (less likely)
- Solvent delivery system (flow rate or mixing)

Ionic Peaks Shift Retention

- Loss of volatile MP component (ionic strength, pH shift)

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)

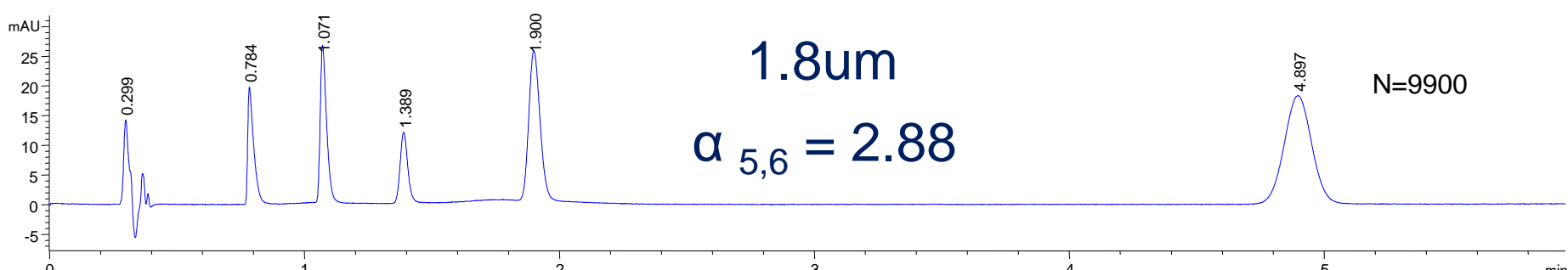- They were tested on the same day, on the same instrument, with the same mobile phase

Problem

- The retention/selectivity was different on each of the columns

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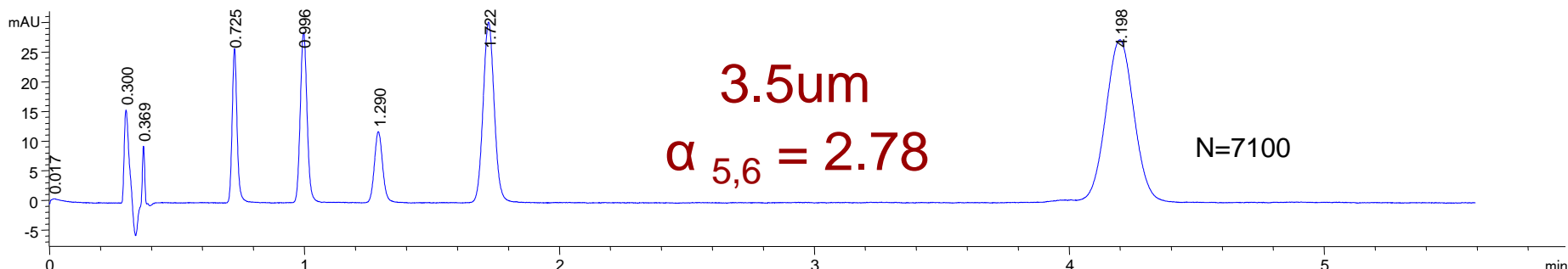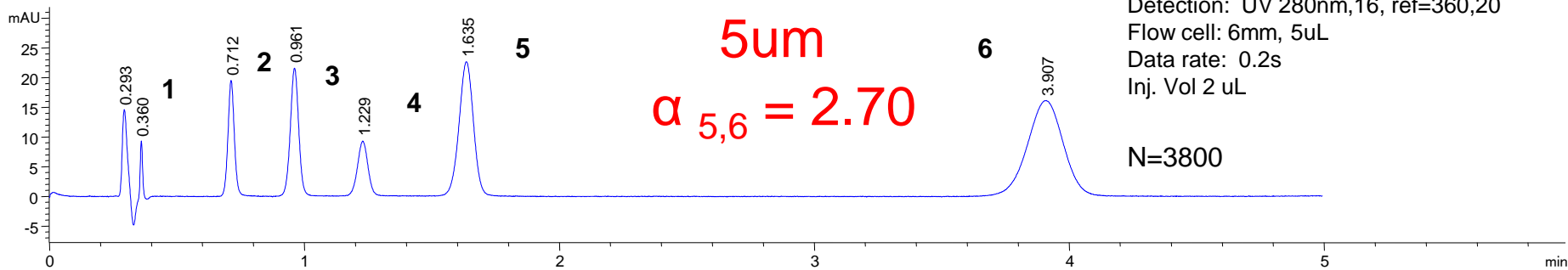
Problem with Proportioning Valve
One channel premixed mobile phase shows similar α

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

5 um, UXE01033, NEP0652003

46 bar

α= 2.71

1.8 um, UXG03882, B08021

246 bar

α= 2.74
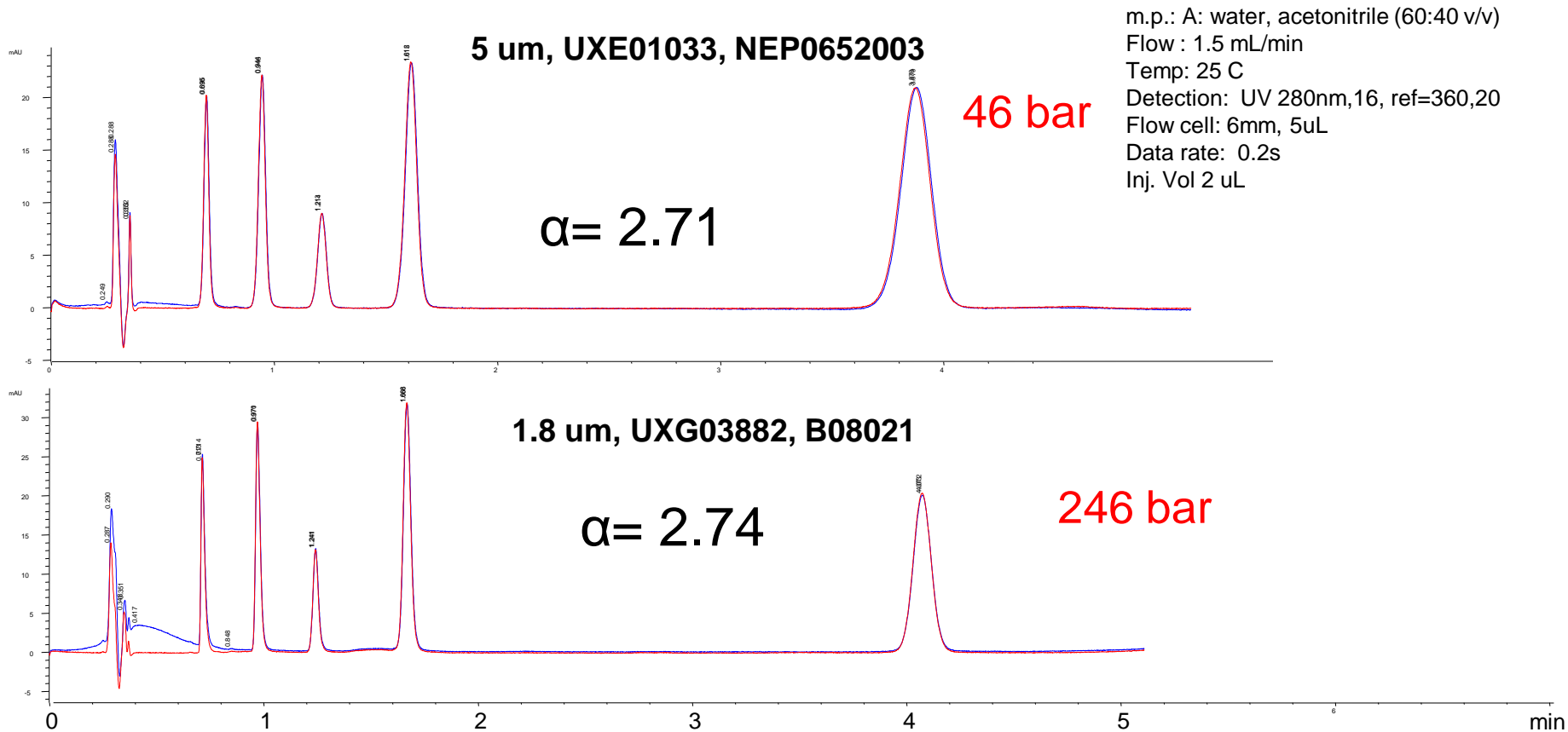
Compare alpha values from proportioned and premixed mobile phase

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 can change with pressure

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Retention Time Shifts and Peak Shape Change in Volatile Buffer Concentration

30:70 ACN:Water with 0.1% TFA, pH 2

Berberine
Tf = 1.11

2.913

Imipramine
Tf = 1.16

7.008

Amitriptyline
Tf = 1.18

8.586

30:70 ACN:Water with 0.01% TFA, pH 2.9

Berberine
Tf = 1.28

2.049

Imipramine
Tf = 1.57

4.614

Amitriptyline
Tf = 1.73

5.623

Column: C18, 4.6x100mm, 5um
Flow Rate: 2 mL/min,
Detection: UV 210nm Detection,
Temp: 25 °C
Inj Amt: 0.05μg each compound (2 μL Inj.)
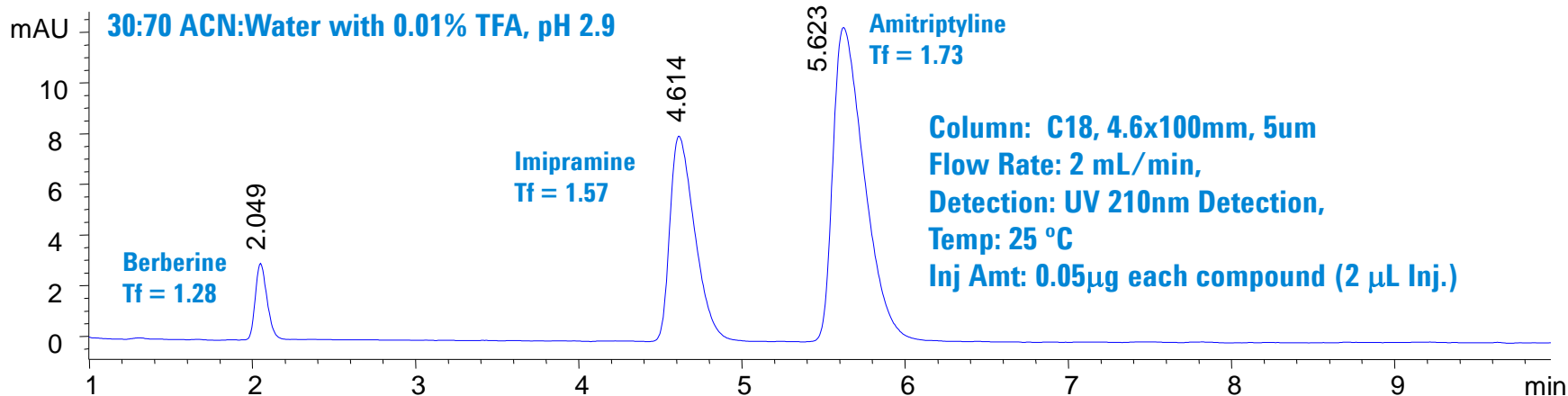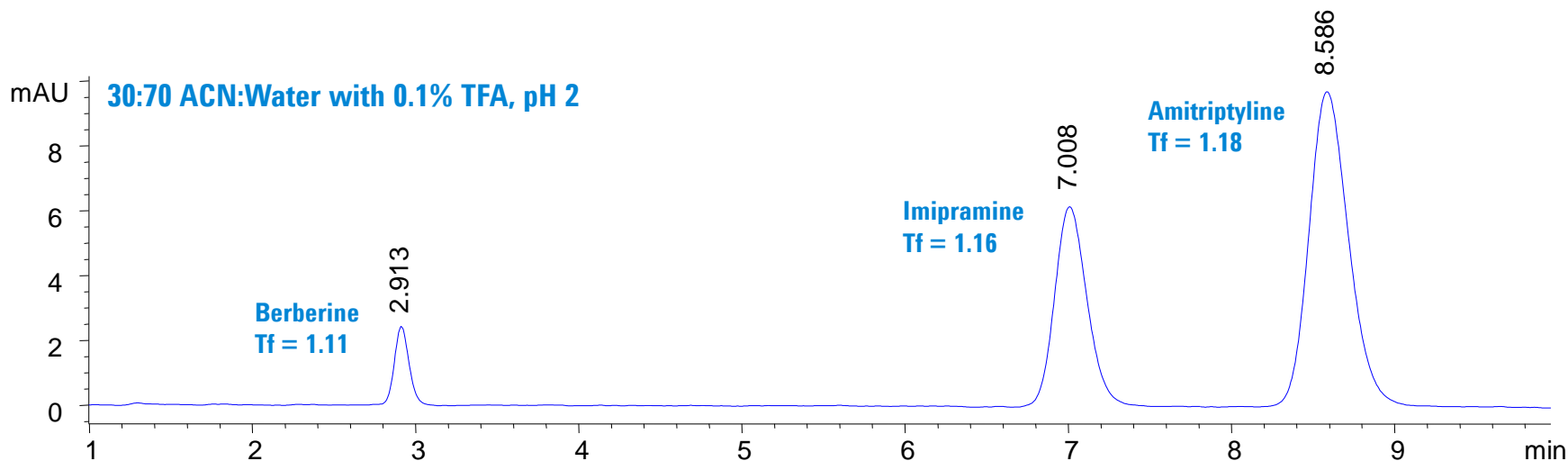
Mobile Phase Preparation

> ➤ Small changes in mobile phase strength can have a large affect on retention

✓ HPLC grade or better

✓ Buffer prep procedure

- Be consistent
  - ○ Document process

Volume % of solvents can depend on preparation

Specified volume ACN added to a 1 L volumetric and made to volume with H_2O

≠

Specified volume H_2O added to a 1 L volumetric and made to volume with ACN

≠

500 ml H_2O added to 500 ml ACN

✓ Degree of contraction is affected by the relative quantities of each

✓ Temperature

Retention
Separation Conditions That Can Cause Changes*

Condition	Change	Retention	Change
Flow Rate	+/- 1%	t_R	+/- 1%
Temperature	+/- 1 deg C	t_R	+/- 1 to 2%
% Organic	+/- 1%	t_R	+/- 5 to 10%
pH	+/- 0.01%	t_R	+/- 0 to 1%

*excerpted from "Troubleshooting HPLC Systems", J. W. Dolan and L. R. Snyder, p 442.

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Troubleshooting
Retention Shifts

Mobile Phase Related Problems

- **Make fresh, compare to aged**

  - **pH**

  - **conductivity**

  - **chromatographic test**

Column Related Problems

- **Test new column**

- **Test current column with test mixture or e.g., Toluene**

- **"Wash" column and retest**

- **Consider effect of sample matrix**

Why Doesn't My Separation Work as Expected?

Common Problems
- ✓ High pressure
- ✓ Undesirable peak shape
- ✓ Changes in retention/selectivity

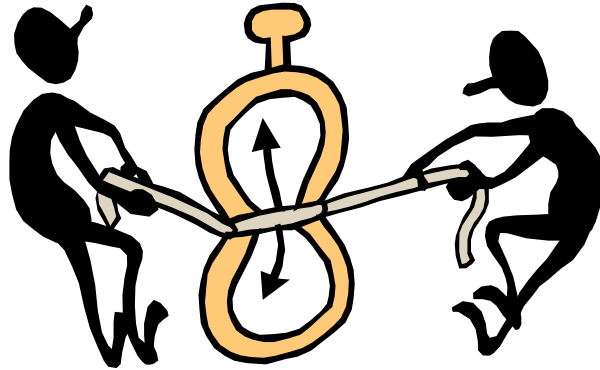Problems are not always associated with the column
- May be caused by instrument and experimental condition issues

Take the trouble out of troubleshooting
- Use proper precautions to prevent problems

The LC Handbook, pub # 5990-7595EN
Contact LC Column Tech Support

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The End – Thank You!

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Agilent Technical Support

800-227-9770 (Toll Free US & Canada)

- **For LC columns**

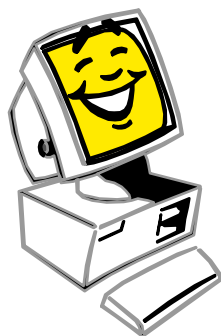- Select option 3, then option 3, option 2

  lc-column-support@agilent.com

  - *For GC Columns*

  Select option 3, then option 3, option 1

  gc-column-support@agilent.com

  - *For Sample Prep*

  Select option 3, then option 3, option 3

- spp-support@agilent.com

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Appendix

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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.

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Using Buffers Successfully
Initial Column and System Equilibration

In an appropriate vessel, test highest % organic/buffer ratio to verify that buffer will not precipitate. With stirring, add organic to buffer first, not vice versa.

Equilibrate column with, in order:

- 100% organic modifier (if brand new)
- mobile phase <u>minus</u> buffer
- buffered mobile phase containing highest % organic modifier (gradient high end)
- buffered mobile phase containing lowest % organic modifier (gradient low end).

Inject standard or sample several times until RTs stable, or for gradient methods, precede former with 1 or 2 blank gradients.

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Using Buffers Successfully
Shutdown State and Instrument Flushing

Shutdown State

Next day use—using same buffers

- Pump mobile phase very slowly (for example, 0.01 – 0.1mL/min).

When flushing column or for longer term column storage

- Flush with 20/80 organic/water, then 80/20 organic/water or 100% organic.

Instrument flushing

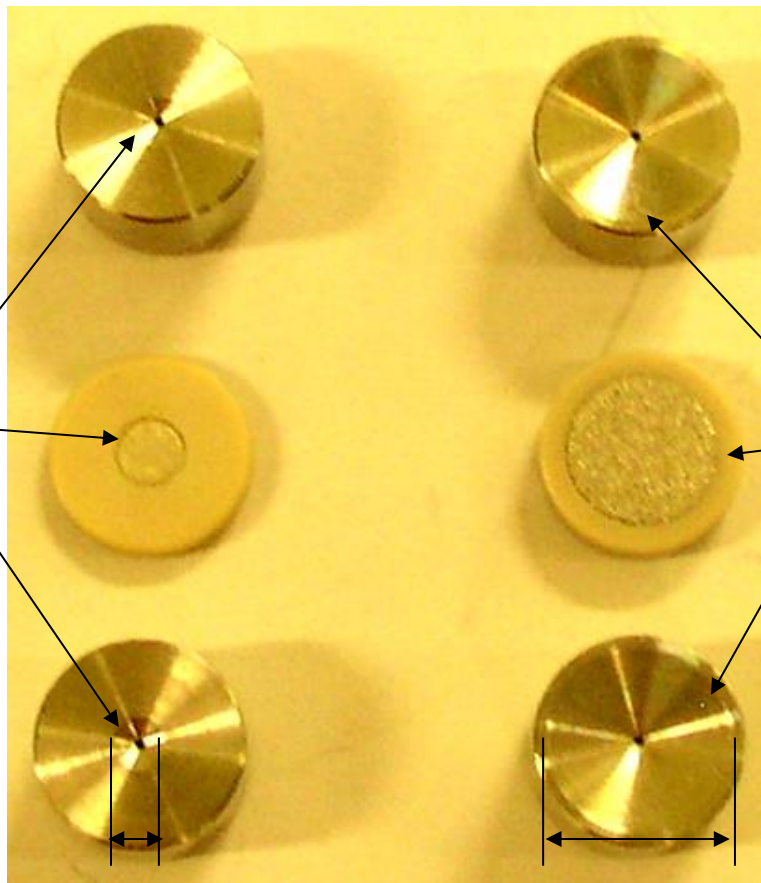Replace column with capillary tubing. Leave disconnected from detector.

Flush pumps with water, then connect capillary tubing to detector.

Inject water 2-3 times at maximum injection volume setting.

Flush all pumps with 100% organic for long term storage.

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High Pressure In-line Filter Kit

For **2.1mm Frits** use inserts with **small cone**

For **4.6mm Frits** use inserts with **big cone**

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Assembling the High Pressure Filter Kit

Put the first insert into the frit housing.
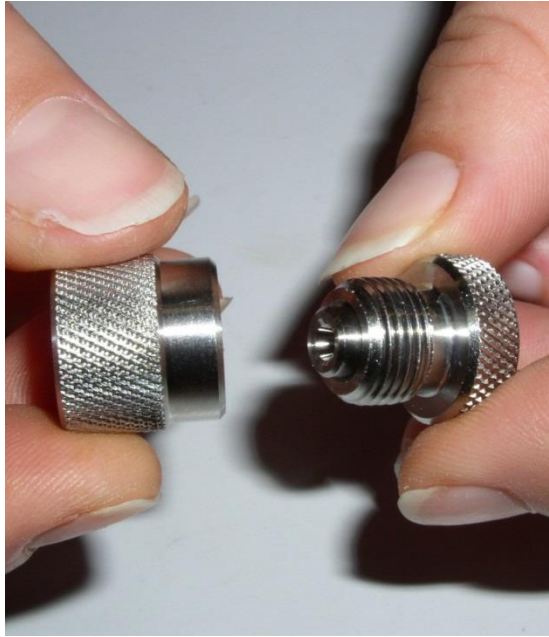
Place the frit on top of this insert

Then place the second insert on top of the frit
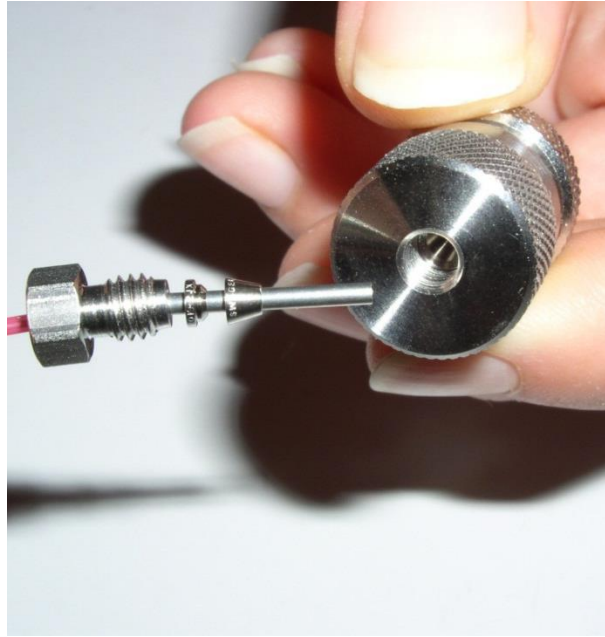
Assembling the High Pressure Filter Kit

Close the frit housing, screw finger tight

Slide the fitting, back and front ferrule onto the capillary. Insert the capillary into the frit housing bore, then tighten the fitting with your fingers.
Push the capillaries all the way in into the bore.
Connect the second capillary.

Ensure that both capillaries are still pushed all the way in into the bore. Then tighten both fittings at the same time with two ¼" spanners. This compresses the frit assembly and assures a leak tight connection.
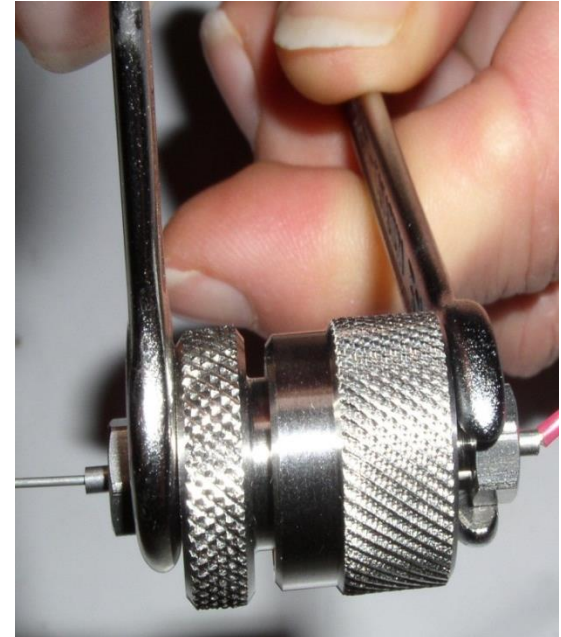
Use 0.12 mm Tubing Instead of 0.17 mm Tubing

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

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SPE Modes

Analyte Adsorption (Bind-Elute)

Analyte(s) retained ($K_D \gg 1$)

Matrix unretained ($K_D \sim 0$)
    and/or strongly retained ($K_D \gg 1$)

Pre-concentration factor

Cleaner extracts

Load at 1-3 drops/sec (recovery $\propto$ 1/flow)

Capacity issues may be more important

Matrix Adsorption (Interference Removal)

Analyte(s) unretained ($K_D \sim 0$)

Matrix retained ($K_D \gg 1$)

No pre-concentration advantage

Eluates may not be as clean

Sample loading may gravity fed

Used less often than analyte adsorption

If your sample needs further cleaning…

QuEChERS

Solid-supported liquid extraction (SLE)

Bond Elut Solid Phase Extraction (SPE)

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