What's Wrong With My Chromatography ?

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Applications Engineer

LC Columns & Consumables

Technical Support

October 14, 2015

Outline of Presentation

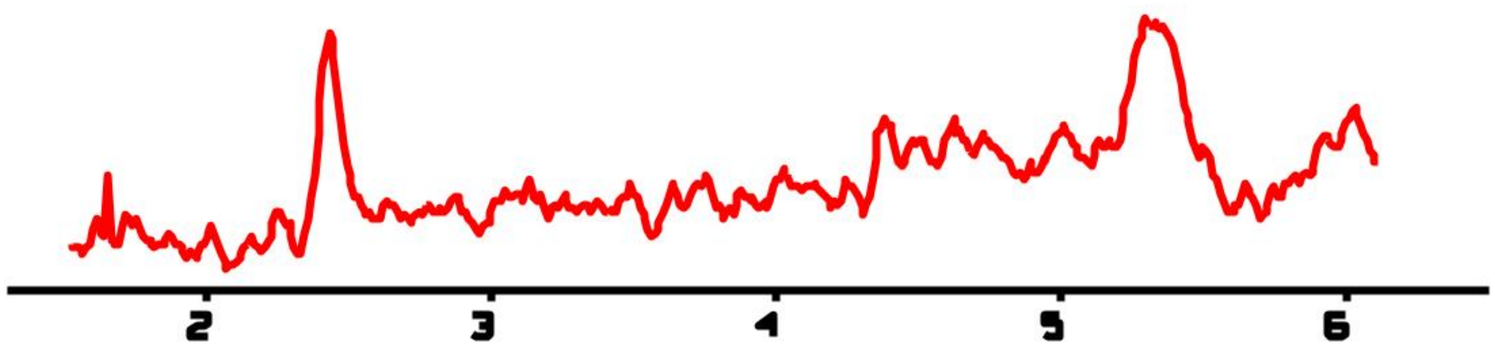
I. Common Problems, Symptoms, and Solutions
   - Baseline Shifts/Noise
   - Peak Shapes
   - Pressure
   - Retention Shifts

II. Preventative Maintenance/Good Practices
   - In-line Devices
   - Sample Preparation
   - Operational Limitations

Common Problems
Noisy Baselines

Possible Causes

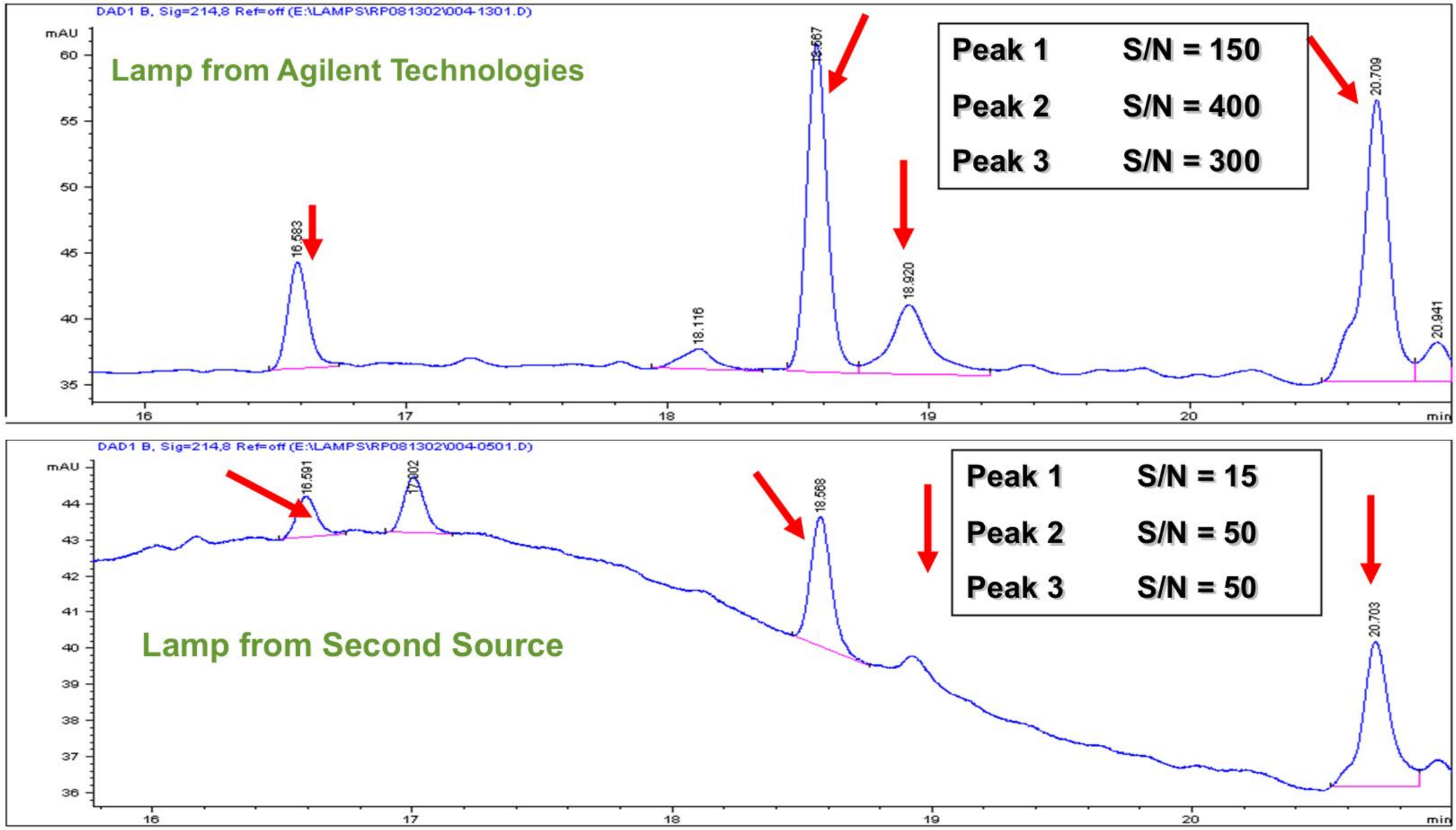
- Dirty flow cell
- Lamp failing
- Pulses from pump (if periodic)
- Temperature effects on detector
- Air bubbles passing through detector



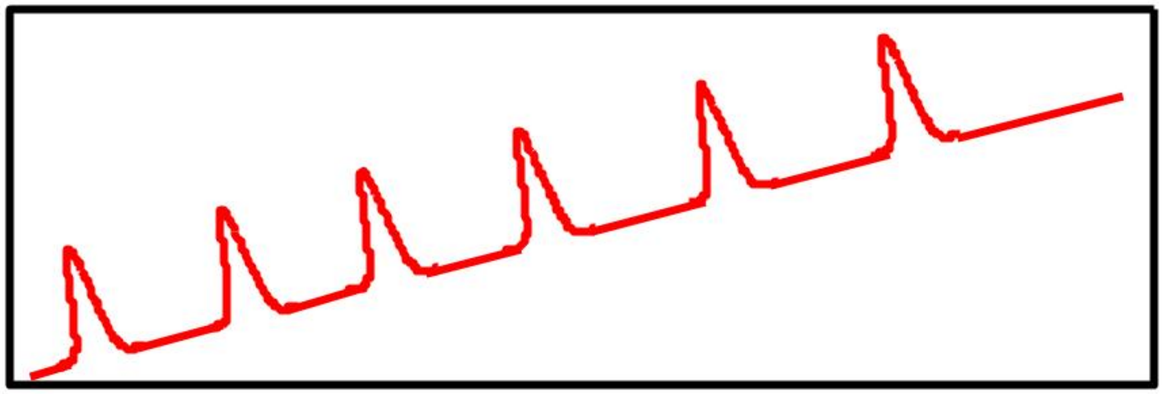
Time (min.)

Non-spec Lamp

Chromatographic Results with Second Source Lamp at 214 nm



Drifting Baseline



Possible Causes

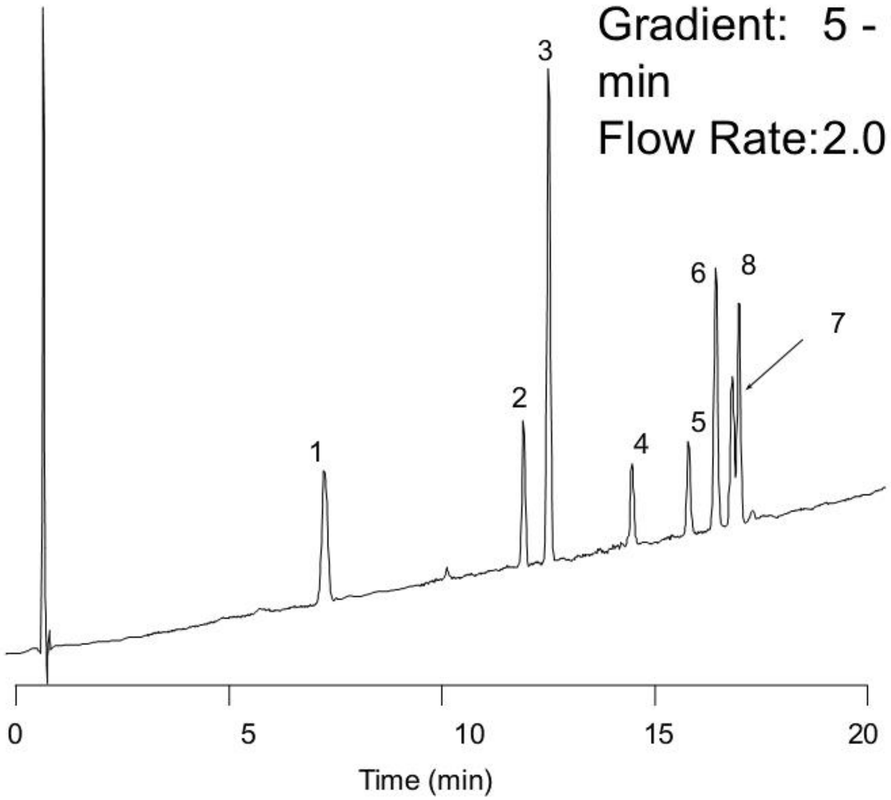
- Gradient elution
  - Mobile phase component
- Temperature unstable (RID)
- Contamination in mobile phase
- Mobile phase not in equilibrium with column
- Contaminant bleed in system
  - Hardware chemical compatibility

Effect of TFA on Baseline

A: 0.1% TFA in H_2O B: 0.1% TFA in ACN Temperature: 35°C
Sample: 1. Phenacetin 2. Tolmetin 3. Ketoprofen 4. Fenoprofen 5. Ibuprofen 6. Phenylbutazone 7. Mefenamic acid 8. Flufenamic acid

Eclipse XDB-C8
4.6 x 150 mm, 5 µm

Gradient: 5 - 100% B in 30 min
Flow Rate:2.0 mL/min



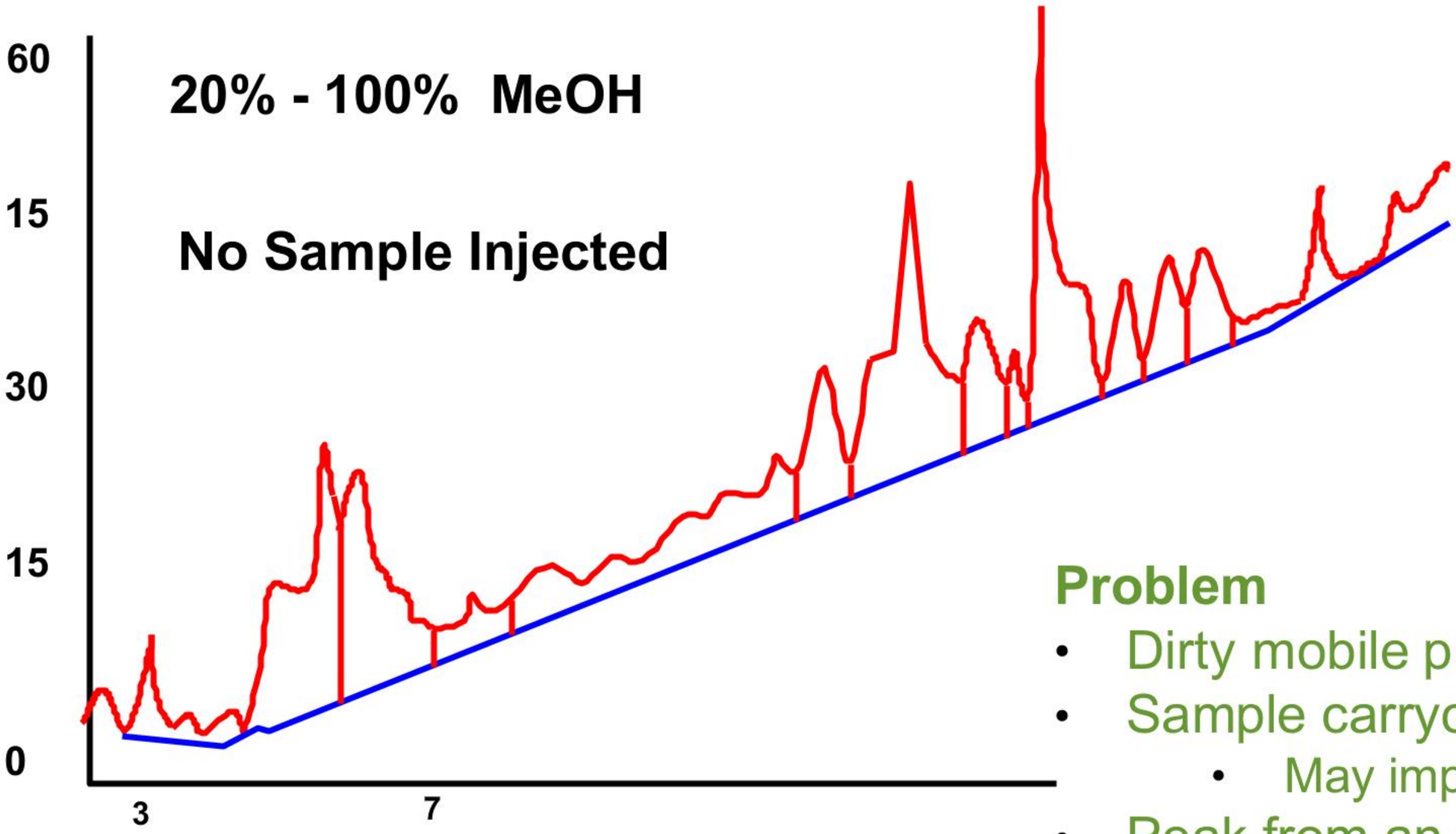
✓Wavelength
- •215nm
  - •254nm less impact

✓Adjust TFA concentration in Solvent B to level baseline

➢ Know the UV Cutoff of your mobile phase components

Peak Shape

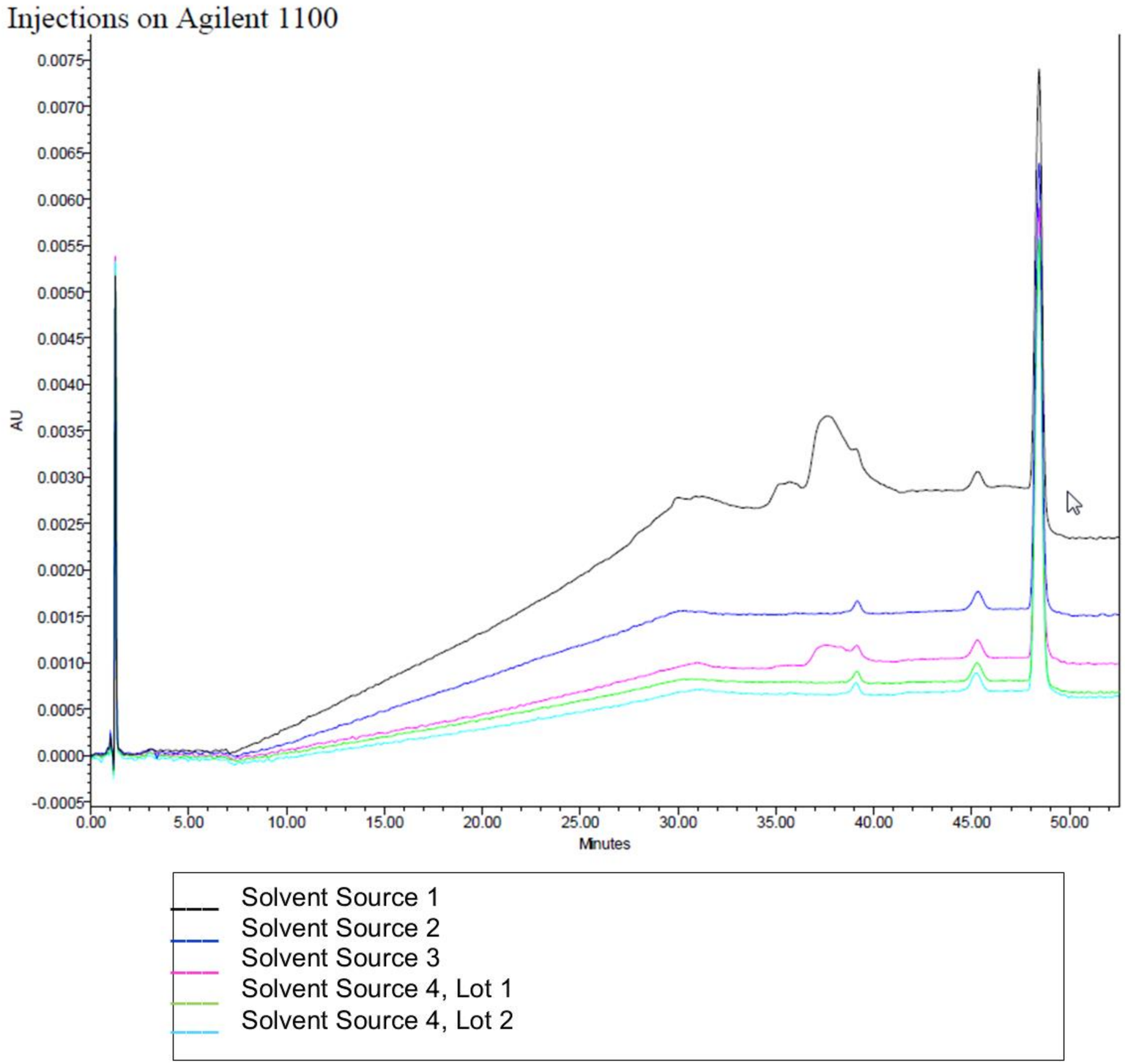
Ghost Peaks – Appear When No Sample is Injected



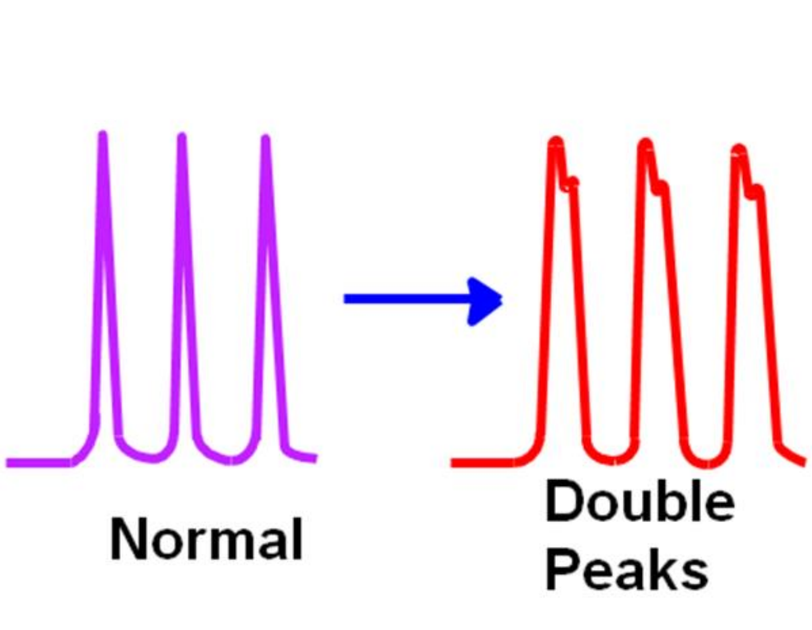
Problem

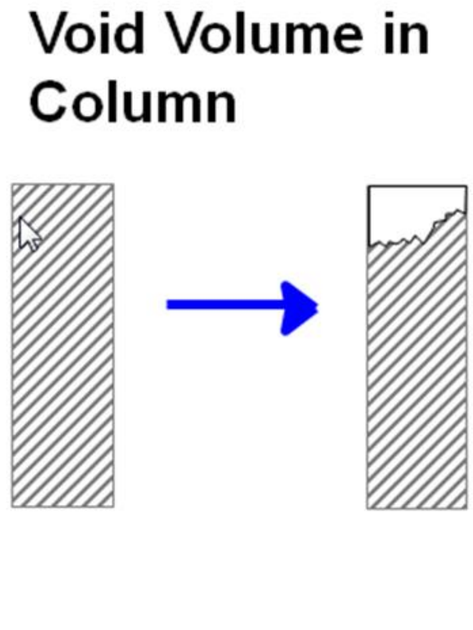
- Dirty mobile phase
- Sample carryover
  - May imply poor recovery
- Peak from an early run (isocratic)

Solvent Contamination



Peak Shape
Double Peaks





Possible Causes

- Void in column
- Partially plugged frit
- Only one peak
- Sample solvent mismatch

Strong Sample Solvent Can Compromise Peak Shape

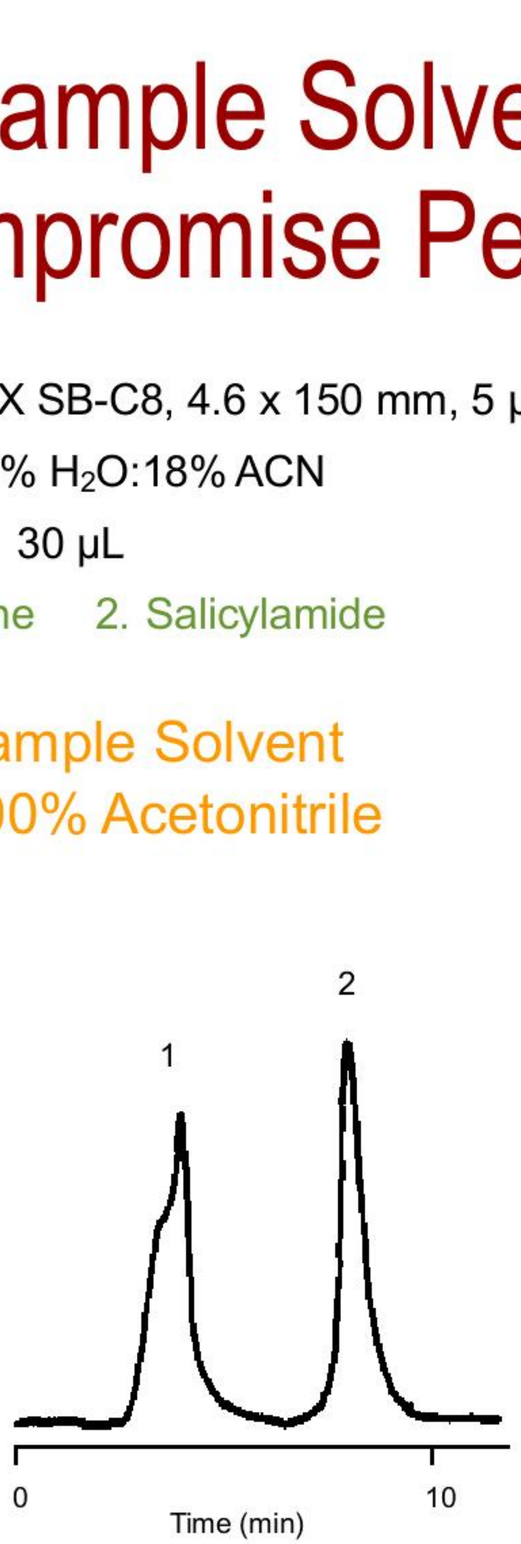
Column: ZORBAX SB-C8, 4.6 x 150 mm, 5 µm

Mobile Phase: 82% H_2O:18% ACN

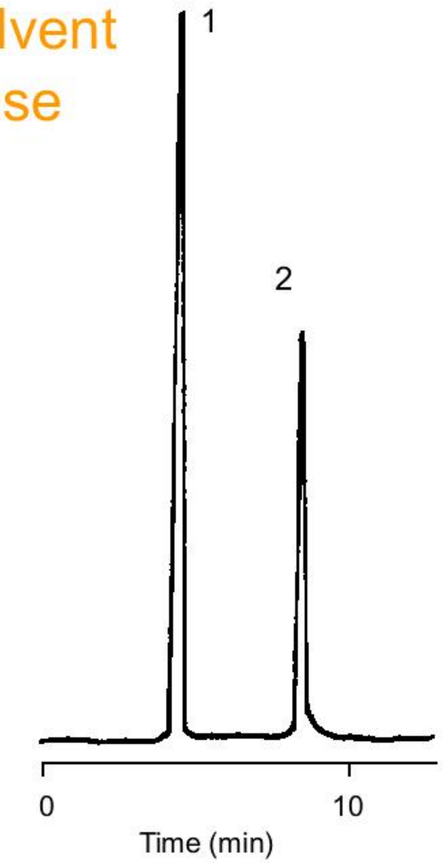
Injection Volume: 30 µL

Sample: 1. Caffeine 2. Salicylamide

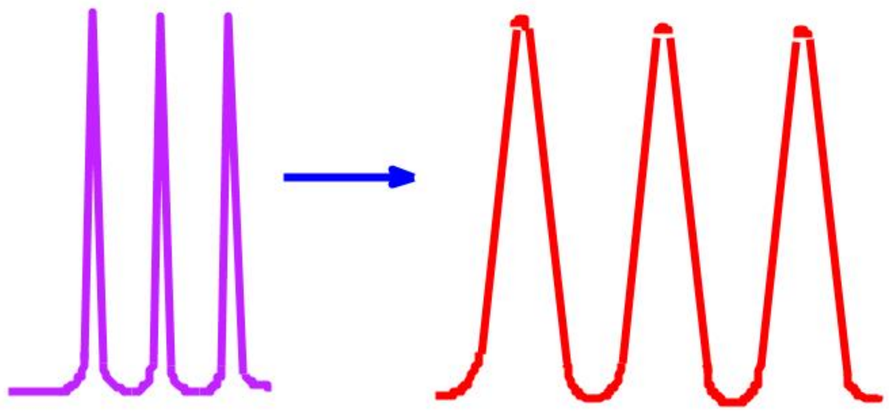
A. Sample Solvent
100% Acetonitrile

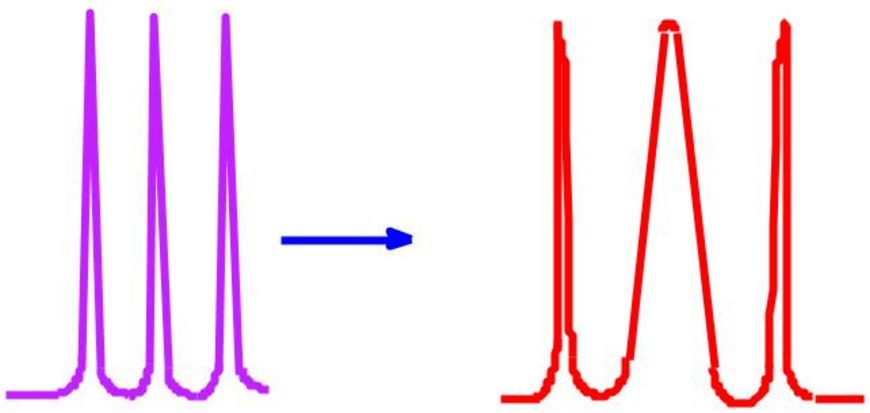


B. Sample Solvent
Mobile Phase



Peak Shape
Broad Peaks





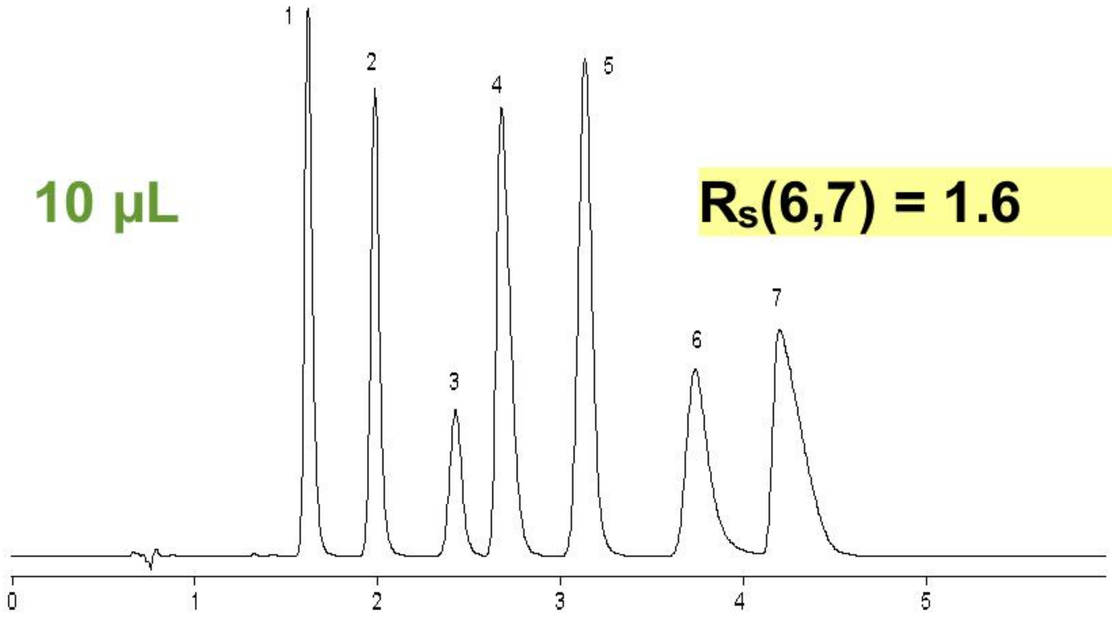
All Peaks Broadened

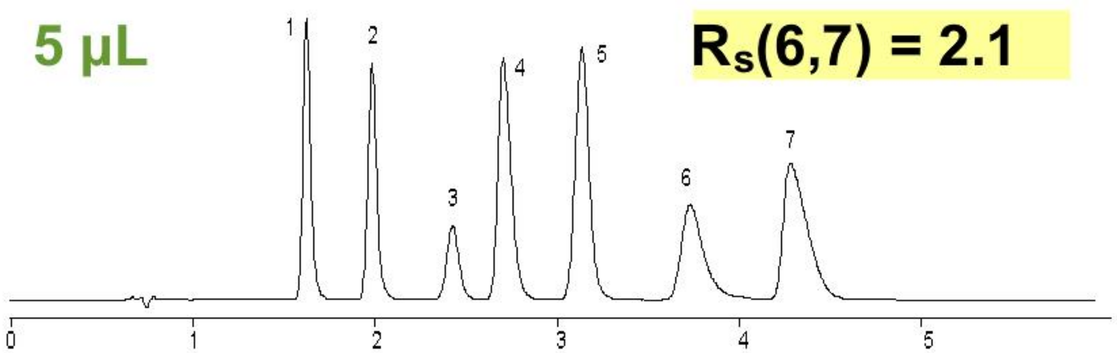
- Loss of column efficiency
- Large injection volume/mass
- High viscosity mobile phase
- Sample solvent mismatch

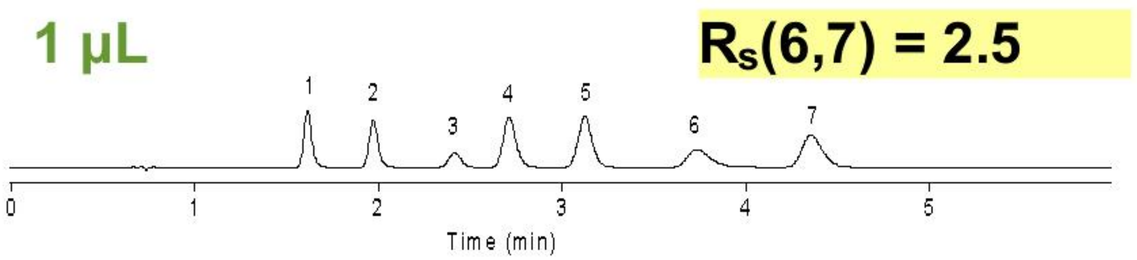
Some Peaks Broadened

- Late elution from previous sample (isocratic)
- High MW sample – Protein or polymer

Injection Volume Robustness







Column: ZORBAX Rapid Resolution Eclipse XDB-C8
4.6 x 75 mm, 3.5 µm

Mobile Phase: 44% 25 mM phosphate, pH 7.00
56% methanol

Flow Rate: 1.0 mL/min

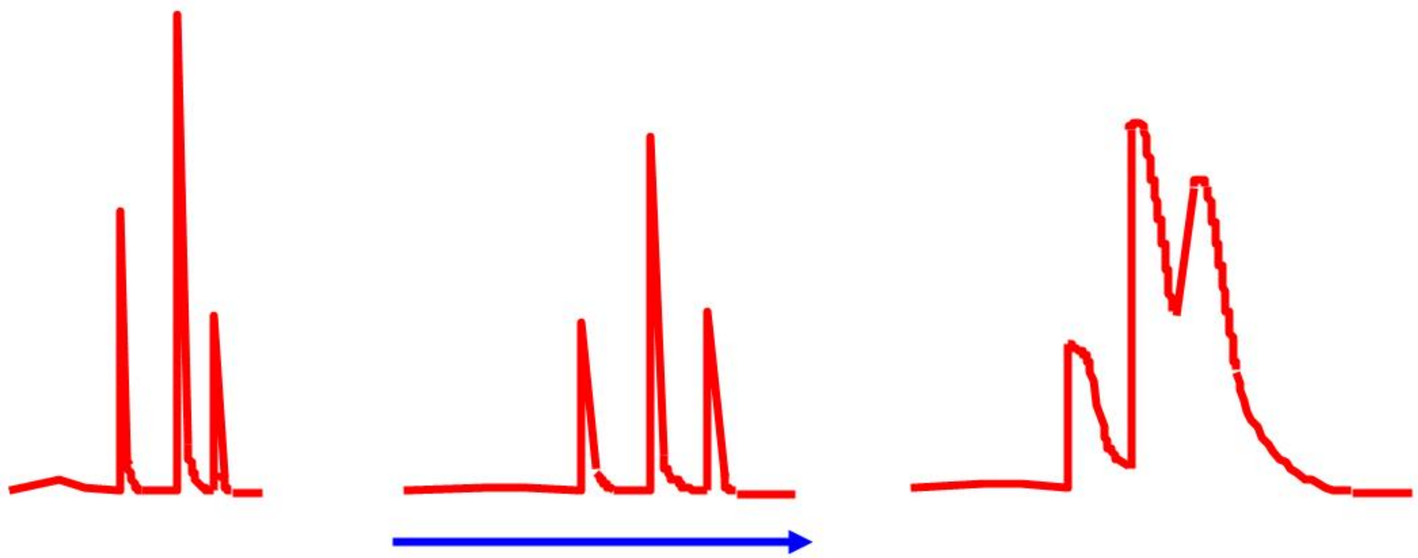
Temperature: 25°C

Detection: UV 250 nm

Sample:
1. ketoprofen
2. ethyl paraben
3. hydrocortisone
4. fenoprofen
5. propyl paraben
6. propranolol
7. ibuprofen

✓ Varying injection volume can sometimes reveal lack of robustness for resolution and peak shape.

Extra Column Volume



Increasing extra-column volume

- 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

Peak Broadening

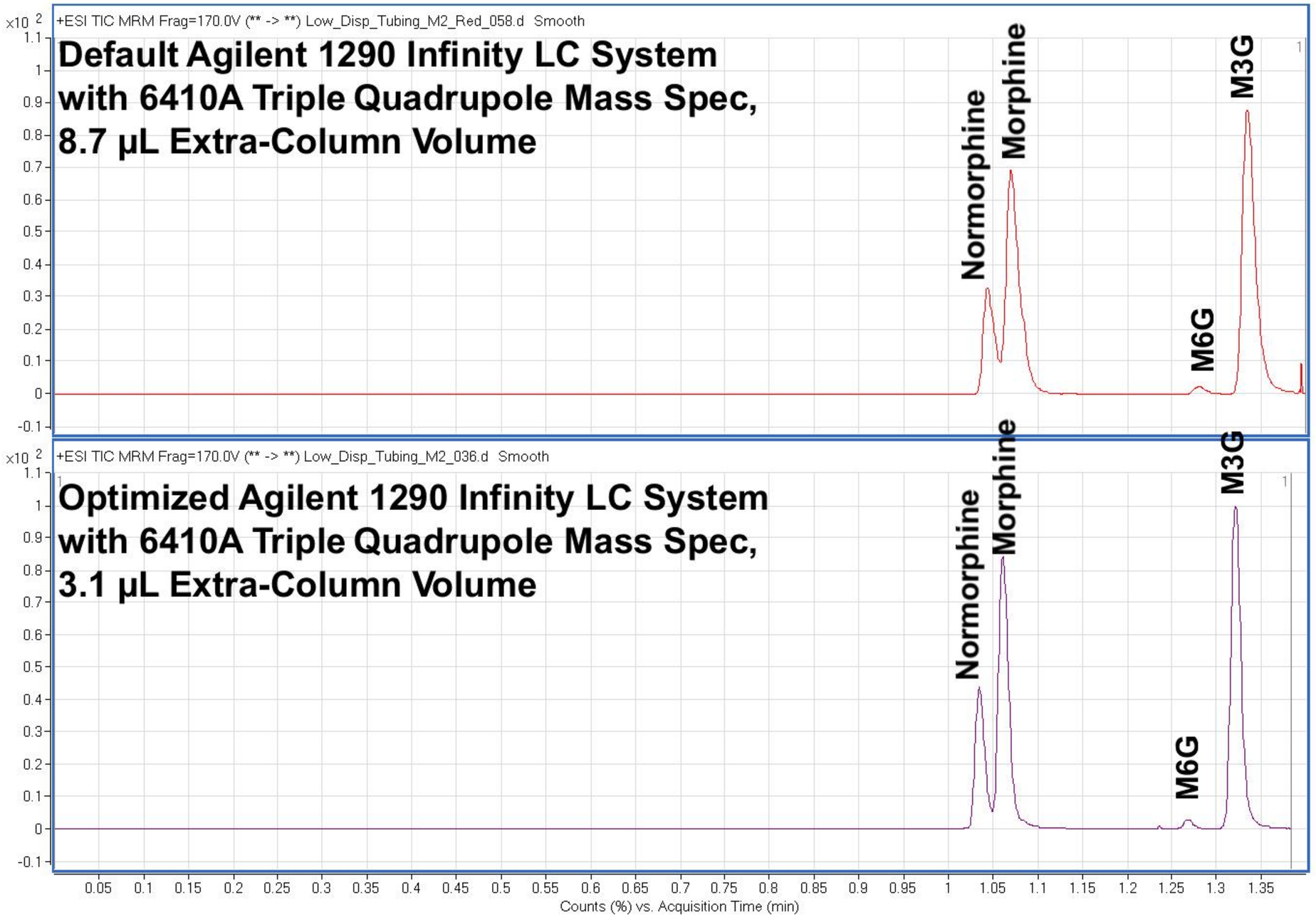
Differences in Detector Flow Cell Volume Can Affect N and R_s

Scenario: ZORBAX Rapid Resolution Column: 75 mm, 3.5-µm; Flow Rate: 1mL/min; k = 3

Flow Cell Volume	Band Broadening* (4.6 mm)	Band Broadening* (2.1 mm**)
1.7 µL	0.3%	6%
8 µL	6%	138%
14 µL	19%	423%

*Versus 8571 theoretical plates (HPLC Calculations Assistant, Version 2.1, Savant Audiovisuals)
**Flow Rate, 0.2 mL/min

Analysis of Morphine and Metabolites by LC/MS/MS using an RRHD HILIC Plus Column



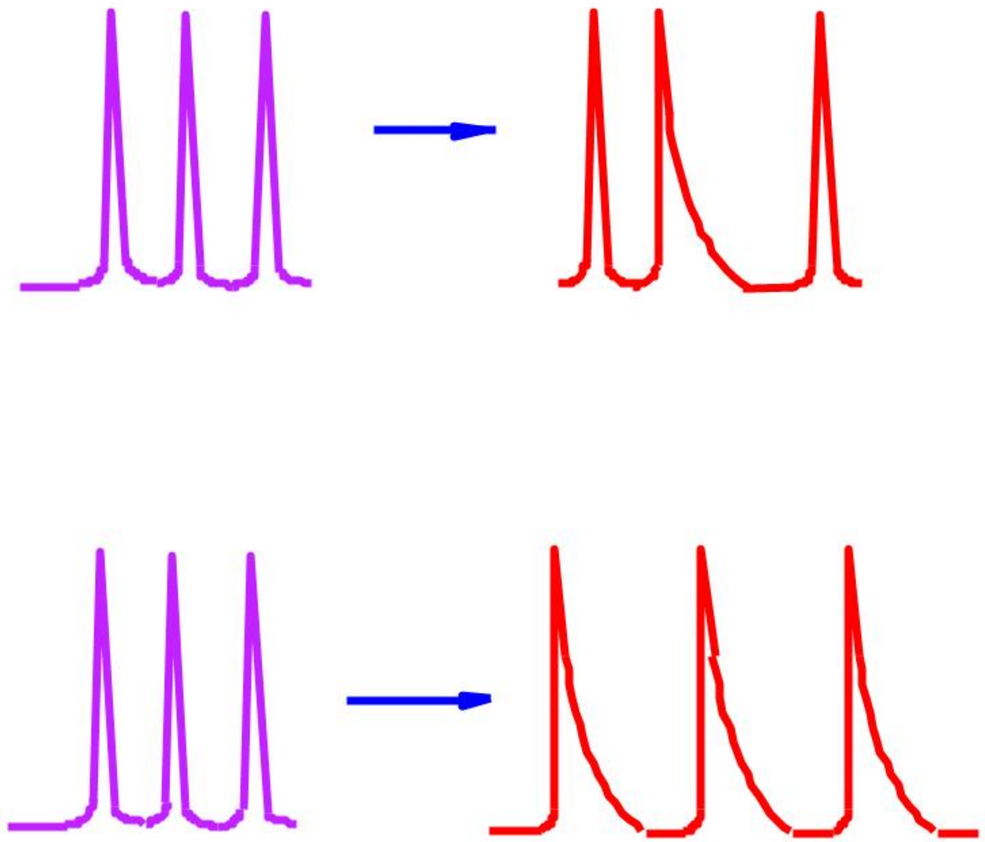
A: 10mM ammonium formate, pH 3.2
B: acetonitrile/100mM ammonium formate, pH 3.2 (9:1)
0.4 mL/min

t(min)	0.0	0.25	1.00
%B	100	100	55

0.1 µL injection
TCC: 25 ºC
MS: ESI+, dMRM, 250 C, 11 L/min, 30 psi, 4000 V, 200 V delta EMV, 40 ms cycle time
Agilent ZORBAX RRHD HILIC Plus, 2.1 mm x 50 mm, 1.8 µm
Sample:

1. Normorphine
2. Morphine
3. Morphine-6-b-D-glucuronide (M6G)
4. Morphine-3-b-D-glucuronide (M3G)

Peak Shape
Tailing Peaks, >1.5



Causes

Some Peaks Tail

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

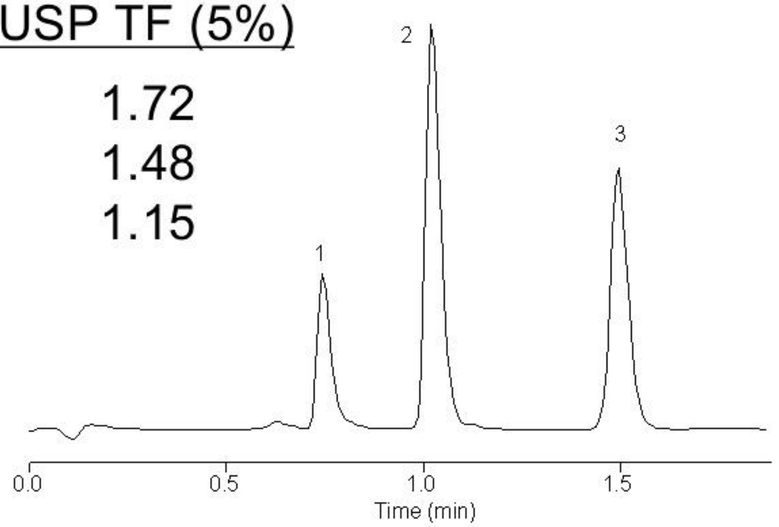
All Peaks Tail

- Extra column effects
- Bad column
- Contamination
  - Column
  - Frit
- Metals
- Inappropriate sample size or solvent

Peak Tailing
Injector Seal Failure

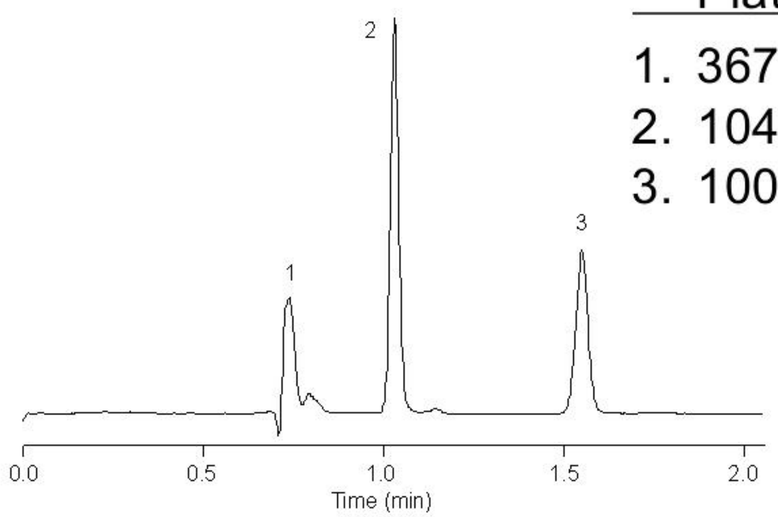
Column: Bonus-RP, 4.6 x 75 mm, 3.5 mm Mobile Phase: 30% H_2O : 70% MeOH Flow Rate: 1.0 mL/min
Temperature: R.T. Detection: UV 254 nm Sample: 1. Uracil 2. Phenol 3. N,N-Dimethylaniline

	Plates	USP TF (5%)
1.	2235	1.72
2.	3491	1.48
3.	5432	1.15



Before

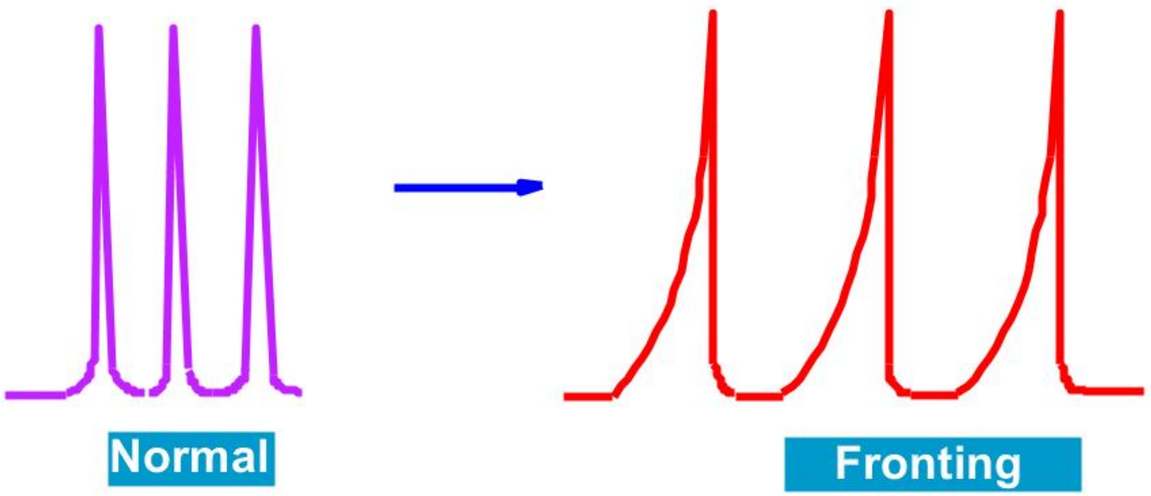
	Plates	USP TF (5%)
1.	3670	1.45
2.	10457	1.09
3.	10085	1.00



After replacing rotor seal and isolation seal

- Overdue instrument maintenance can cause peak shape problems.

Peak Shape
Fronting Peaks, Symmetry <0.9



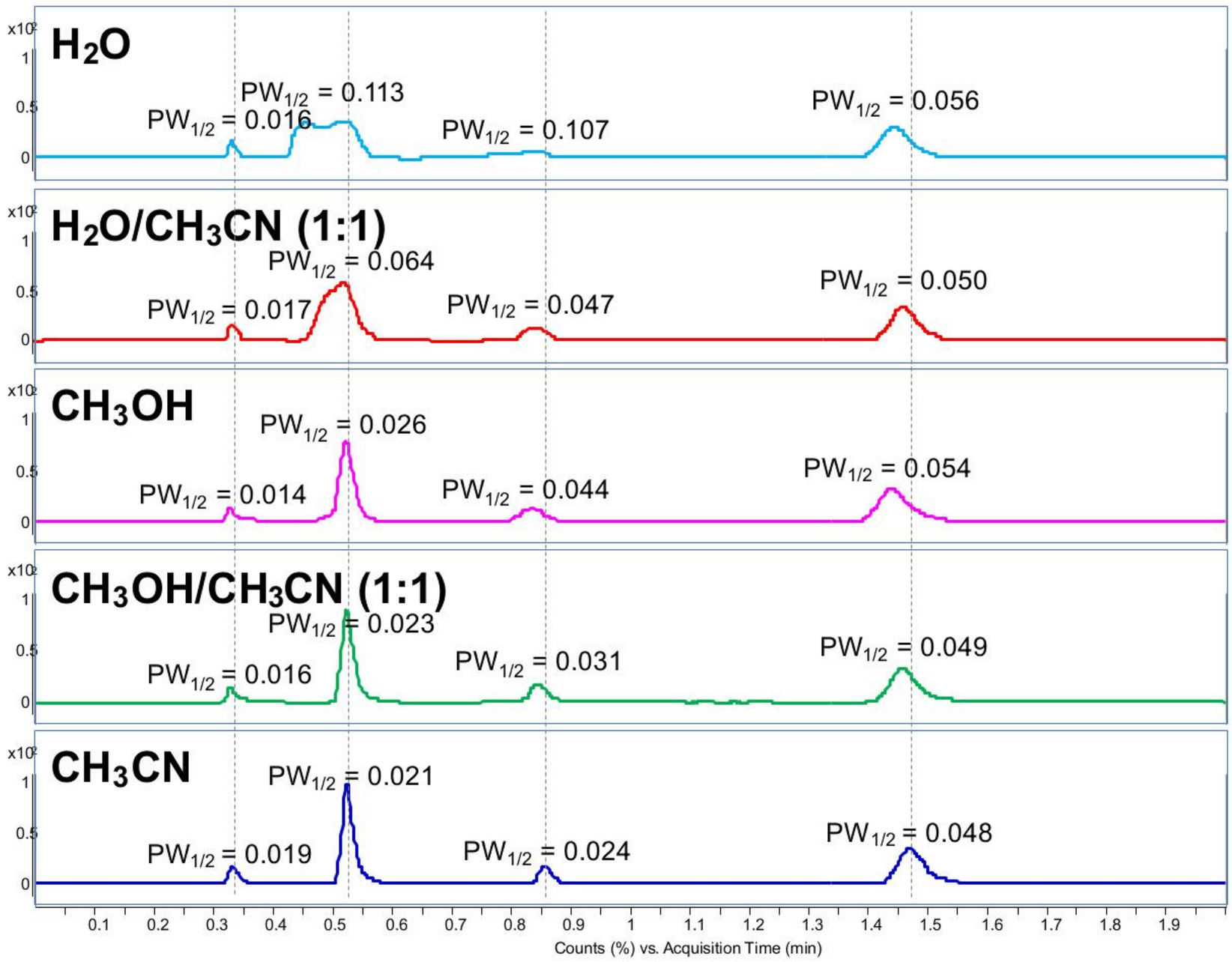
Causes

- Small band eluting before large band
- Column void
- Inappropriate sample solvent

Injection Solvent Effects - HILIC

H_2O, CH_3OH, CH_3CN

LC/MS Separation of Vitamin B Related Compounds



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

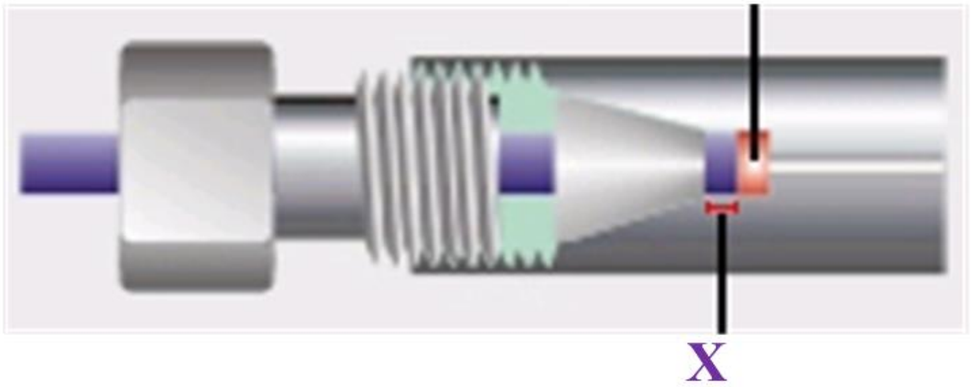
Acetonitrile / 100 mM ammonium formate pH 3.2 (9:1)
0.4 mL/min, Pressure: 135 bar
Isocratic elution
Injection Volume: 1 µL of 5 µg/mL sample
Column: 25 ◦C
MS: ESI+, SIM, 200 ◦C, 10 L/min, 30 psi, 4000 V, 15 ms dwell time

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 solvents negatively affect peak shape and retention**

Poorly Made Connections = Peak tailing/fronting

Wrong … too short

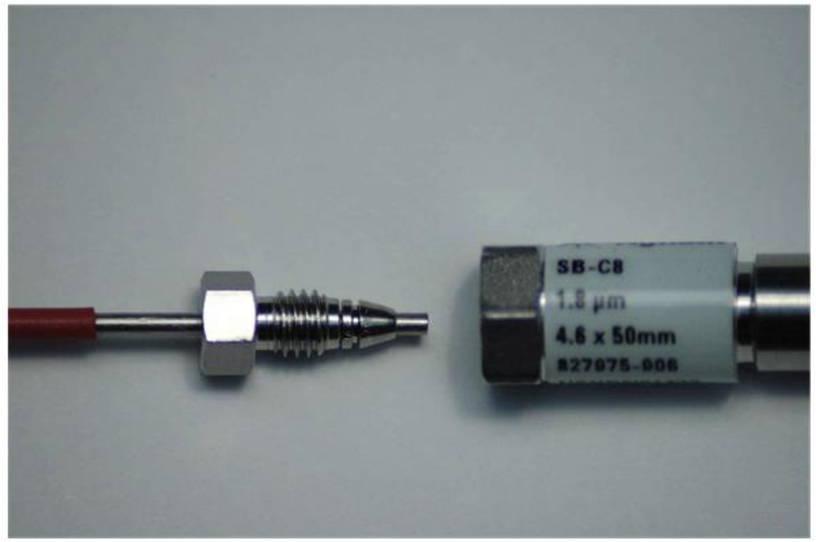


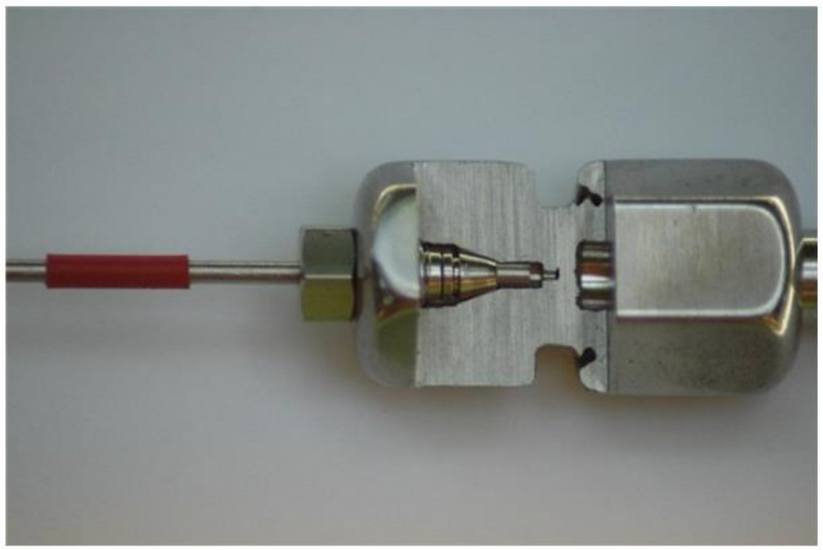
If Dimension X is too short, a dead-volume, or mixing chamber, will occur.

This can broaden or split peaks and/or cause tailing.

It will typically affect all peaks, but especially early eluting isocratic peaks

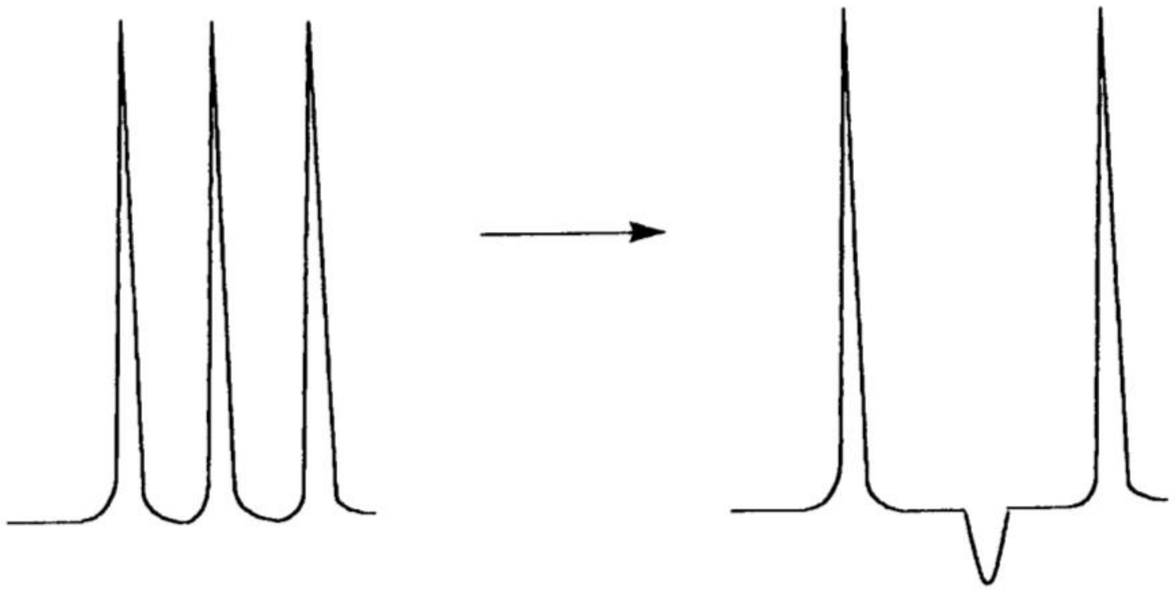
For information on making proper connections check out The LC Handbook, Pub. No. 5990-7595EN





- ***New A-Line fittings and capillaries can prevent this***

Peak Shape
Negative Peaks



Normal **Negative**

Causes

- Absorbance of sample is less than the mobile phase
- Equilibrium disturbance when sample solvent passes through the column
- Normal with Refractive Index Detectors
- Indirect UV detection

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

Troubleshooting Pressure

I. Continuously Increasing Pressure *With No* Injections
   - Pump Seals
   - Mobile Phase Particulates
   - Mobile Phase Solubility
   - Mobile Phase Unstable (polymerization)
   - Column Void Formation (use condition dependent)

II. Increasing Pressure *With* Sample Injections
   - Sample Particulates
   - Sample Not Soluble in Mobile Phase
   - Sample Components Irreversibly Bound to Stationary Phase

Determining the Cause and Correcting

- Check pressure with/without column - many pressure problems are due to blockages in the system or at the guard or in-line filter

If Column pressure is high:

- Wash column
  - ✓ Eliminate column contamination and plugged packing
  - ✓ high molecular weight/adsorbed compounds
  - ✓ precipitate from sample or buffer
- Back flush column
  - ✓ Clear plugged frit
- Change frit – Not recommended

Column Cleaning

Flush with stronger solvents than your mobile phase

Reversed-Phase Solvent Choices
in Order of Increasing Strength

- Mobile phase without buffer salts
- 100% Methanol
- 100% Acetonitrile
- 75% Acetonitrile:25% Isopropanol
- 100% Isopropanol
- 100% Methylene Chloride*
- 100% Hexane*

Use at least 10 column volumes of each solvent for analytical columns

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

Protein/Peptide Samples

If precipitate might be present (i.e., aggregated protein, cellular material, polymer)

- Attempt to clear blockage with appropriate solubilizing solvent
  - 0.1% TFA / 80% acetonitrile, 6M Guanidine HCl, THF, HFIP
  - See Appendix for other suggestions

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
    - Do not add fresh mobile phase to old
  - Use an amber solvent bottle for aqueous mobile phase
  - If possible, can add
    - 5% organic added to water can be used to reduce bacterial growth
    - Few mg/l sodium azide

Check your instrument manual for guidelines

Column Troubleshooting
Retention Shifts

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)
- Column Change (bonded phase or contamination)

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**

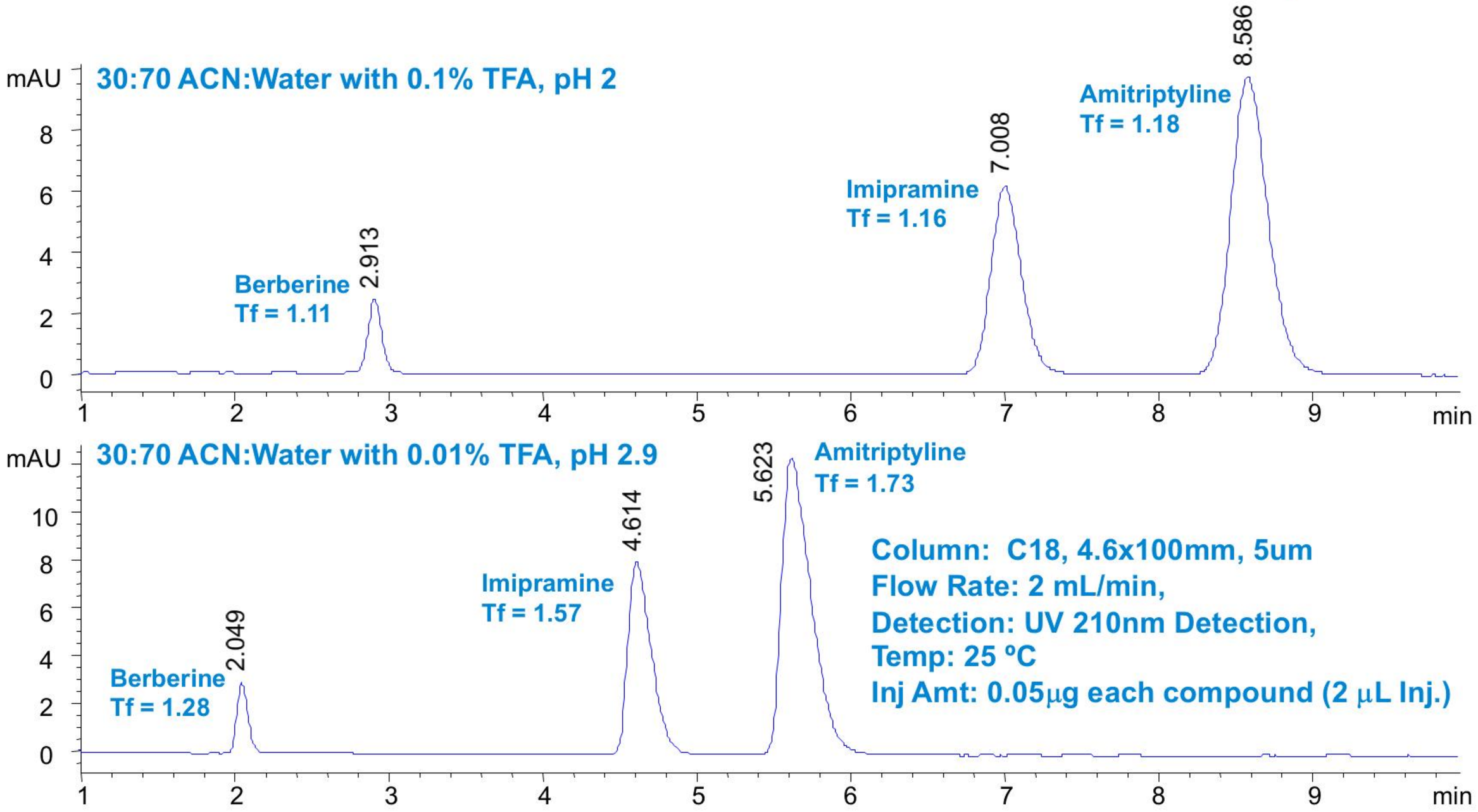
Separation Conditions That Can Cause Changes in Retention*

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.

Change in Volatile Buffer Concentration

Can Cause Shifts in Retention Time and Peak Shape



Agilent Technologies

Mobile Phase Preparation

➢ Small changes in mobile phase strength can have a large effect on retention

- ✓ HPLC grade or better
- ✓ Buffer prep procedure
  - Be consistent
  - Document process
- See appendix

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

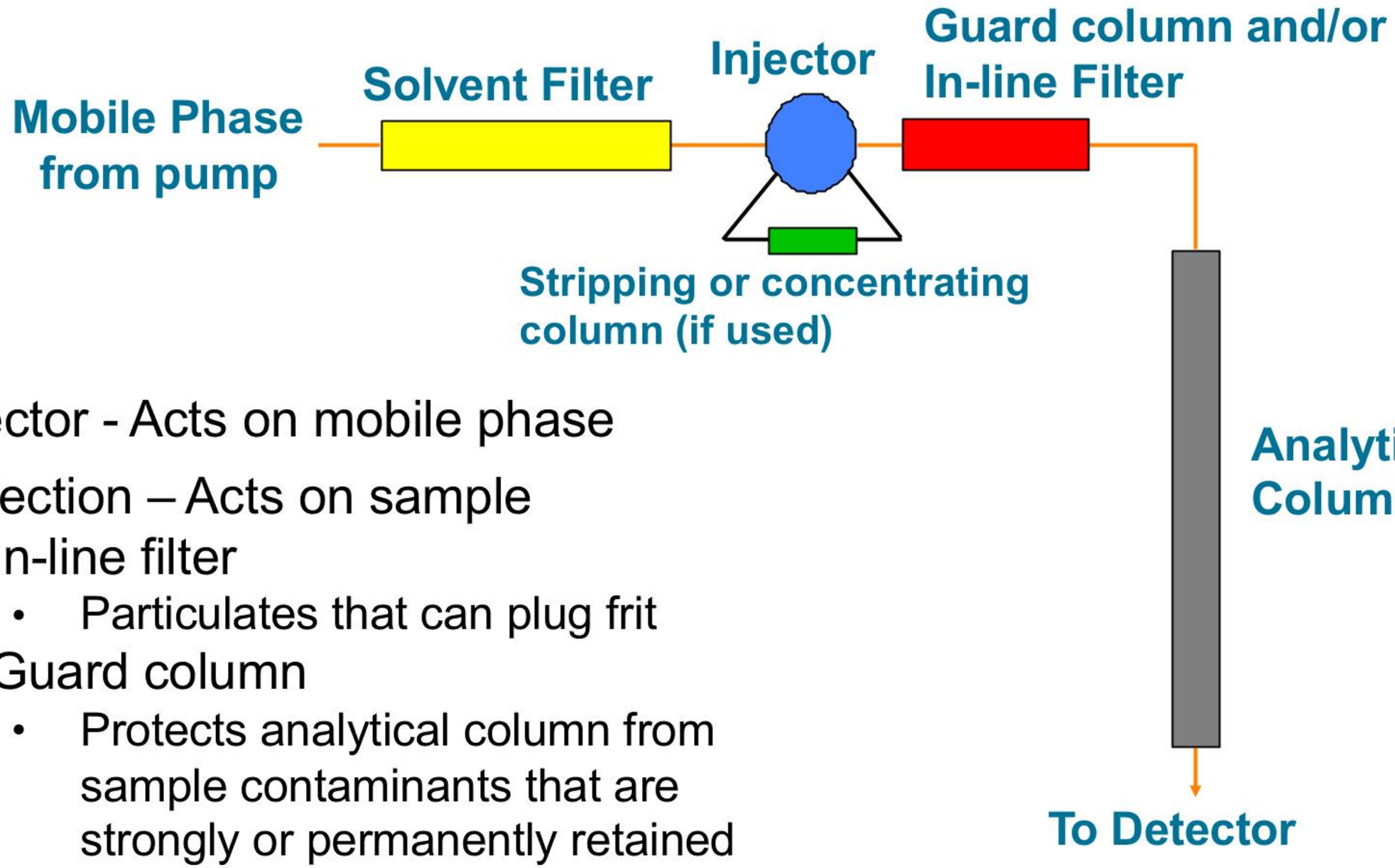
II. Preventing HPLC Column Problems

- ✓ **In-line devices**
  - **Pre-column**
  - **In-line Filter**
  - **Guard Column**

- ✓ **Sample Preparation**

- ✓ **Operational Conditions**

In-line Filters & Guard Columns



Pre-injector - Acts on mobile phase

Post Injection – Acts on sample

- In-line filter
  - Particulates that can plug frit
- Guard column
  - Protects analytical column from sample contaminants that are strongly or permanently retained

II. Preventing HPLC Column Problems

✓ In-line devices

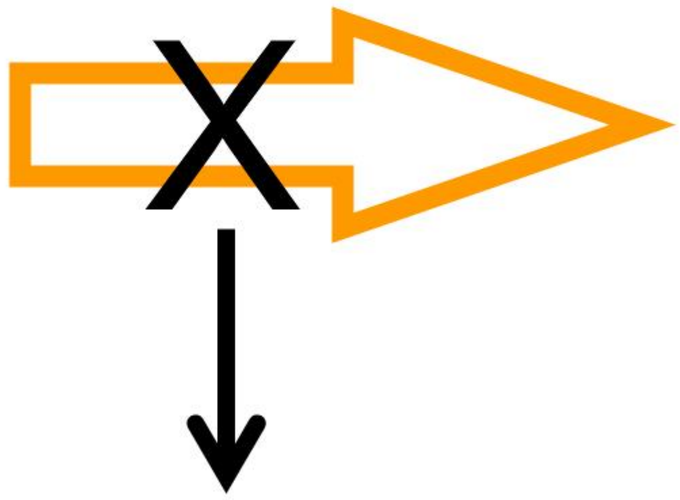
✓ **Sample Preparation**

- **SPE**
  - **Analyte Adsorption (Bind-Elute)**
  - **Matrix Adsorption (Interference Removal)**
- **Filtration**
  - **Syringe**
  - **In-line**

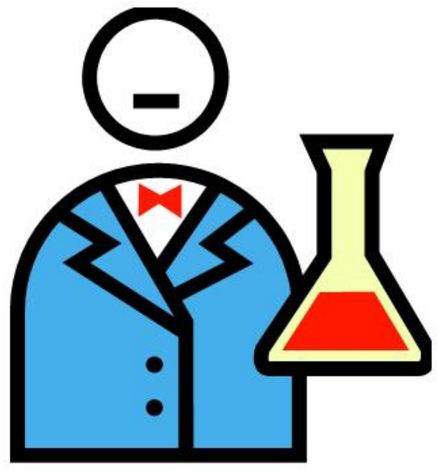
✓ Operational Conditions

Why is Sample Prep Required?

Collected Sample



Analyze

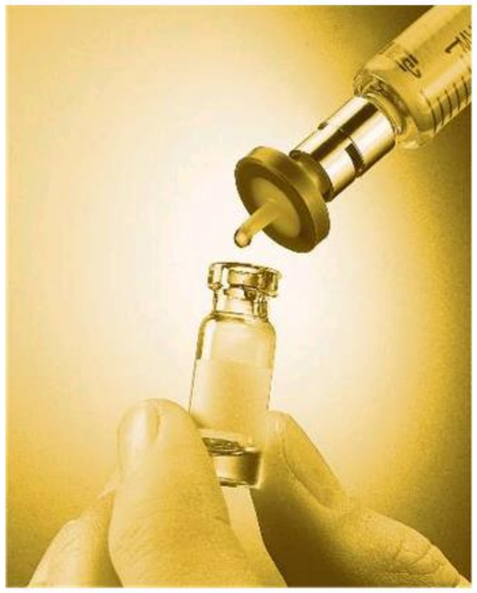


Current Sample - Unsuitable for further analysis

Why?

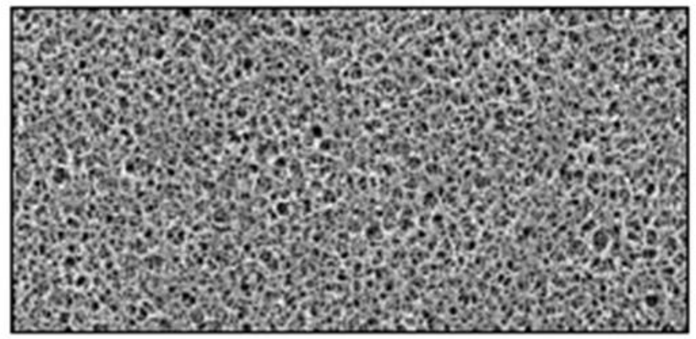
- Too dirty
  - Contains other sample matrix components that interfere with the analysis
- Too dilute
  - Analyte(s) not concentrated enough for quantitative detection, present in low level
- Too dangerous
  - Contaminants can be ‘column killers’

Inexpensive Filters Prevent Column Frit Plugging



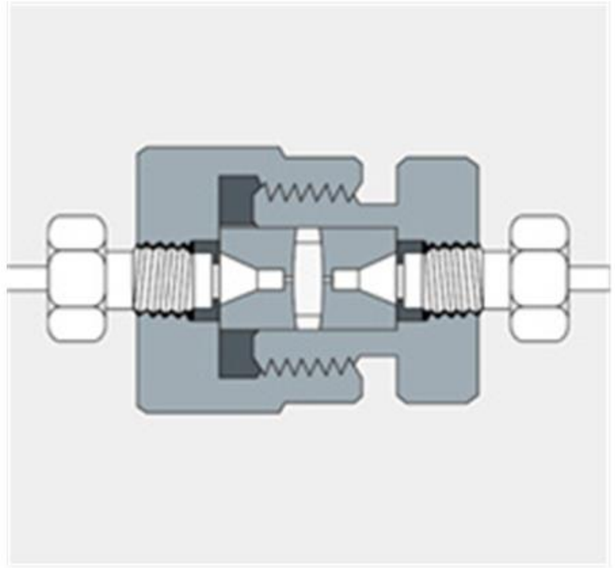
E.g., Regenerated Cellulose (RC)

- **Universal hydrophilic membrane, compatible with most solvents - aqueous and organic**
- **High purity, extremely low extractables and binding**
- **More uniform surface**
- **Choose appropriate pore size**



In-line filters

- **Easy to Use and replace**
- **Frits available in 0.2,0.5 and 2.0µ porosity**
- **Much Less expensive than a Column**
- **Easier and Faster to Replace than a Column Frit**



II. Preventing HPLC Column Problems

- ✓ In-line devices
- ✓ Sample Preparation
- ✓ **Operational Conditions**

Mobile Phase pH and Buffers
Why Are These So Important in HPLC?

- pH - Effects Ionization
  - Silica surface of column
  - Sample components of interest

- Buffers
  - Resist changes in pH and maintain retention
  - Improve peak shape for ionizable compounds

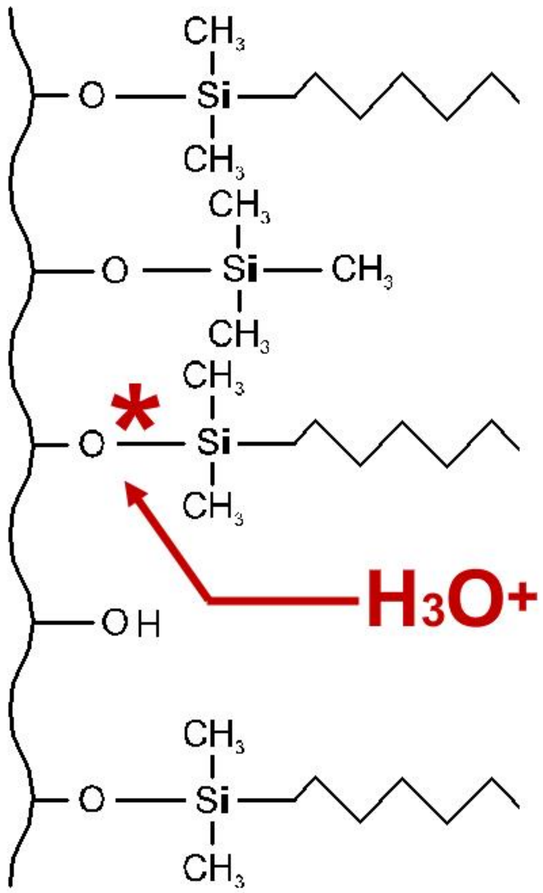
- Effects Column Life
  - Low pH strips bonded phase
  - High pH dissolves silica

Operational Conditions

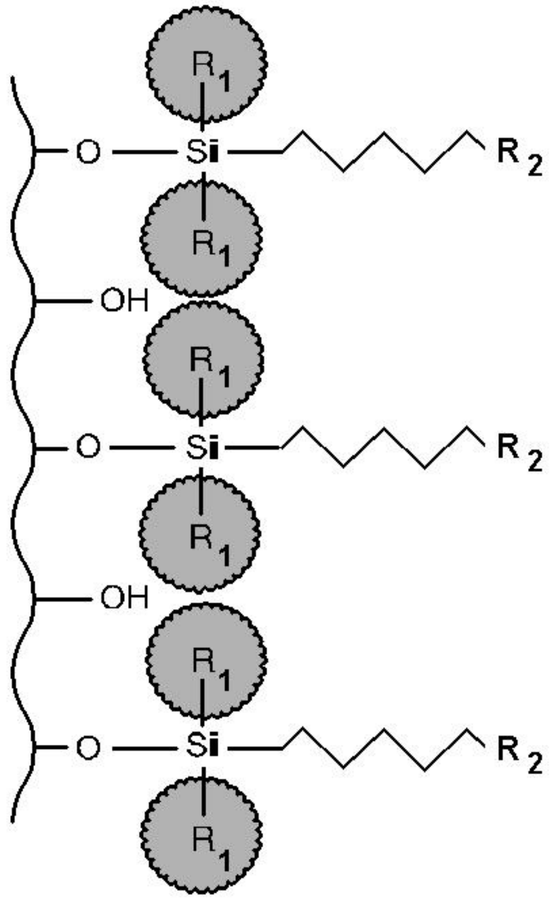
Mobile Phase Effects on Column Life

Low pH (1-3) - Bonded phase loss by acid catalyzed hydrolysis

Conventional



StableBond



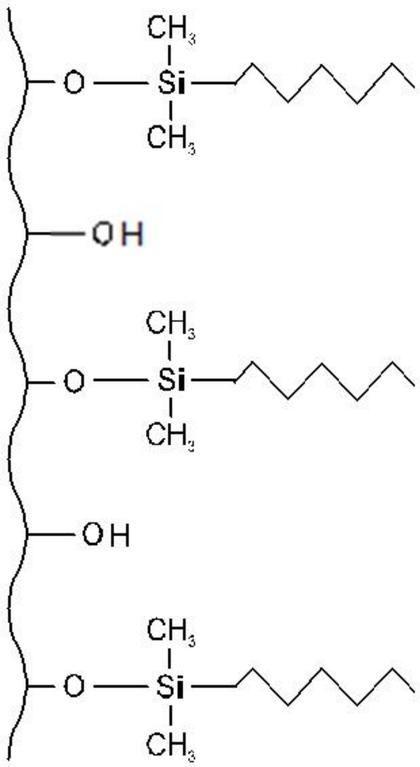
* Hydrolytically sensitive siloxane bond

Operational Conditions

Mobile Phase Effects on Column Life

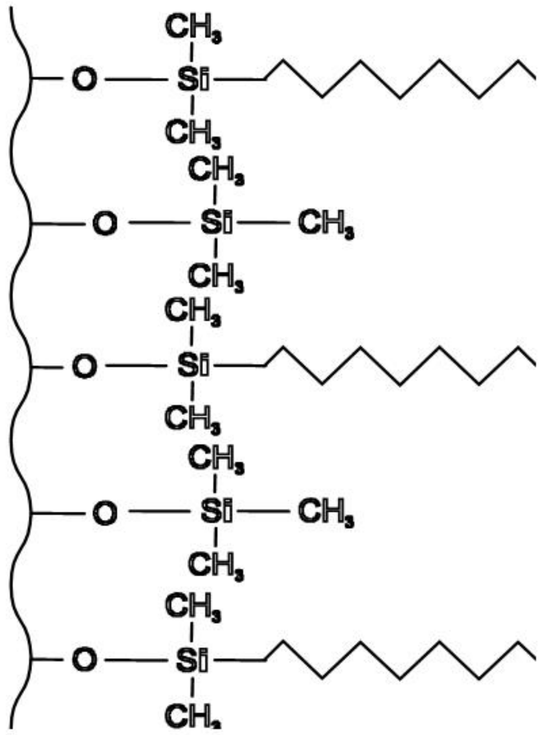
High pH >7- Silica Dissolution

Non-endcapped



Endcapped and/or modified surface

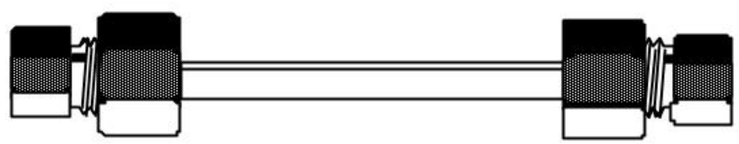
Eclipse Plus, Poroshell 120 EC, HpH



Dissolution Rate Increases With

- pH increase
- Temperature increase
- Phosphate>Borate>Organic Buffers

Good Column Practices



- ✓ Filter buffers.
- ✓ Investigate effects of sample solvent on solubility and separation.
- ✓ Pretreat samples which contain strongly retained components of no interest.
- ✓ Awareness of column packing limits
  - pH
  - Temperature
  - Chemical compatibility
- ✓ Use fresh aqueous solutions, consider use of a bio-stat (sodium azide).
- ✓ Flush column periodically with strong solvent
- ✓ To store column, purge buffers and leave in appropriate solvent (ACN)
- ✓ Avoid physically mishandling columns: banging, dropping, or over tightening fittings

Troubleshooting Summary

- Most HPLC column problems can be prevented provided proper precautions are taken
  - ✓ Mobile phase must be soluble and particulate free
  - ✓ Use guard columns and in-line filters; change as needed
  - ✓ Use appropriate sample preparation procedures
  - ✓ Use appropriate column cleaning procedure
  - ✓ Use appropriate columns for operating conditions (e.g., SB: pH 1-6, Poroshell 120 HpH: pH 3-11)
  - ✓ Keep record of column backpressure & important chromatographic parameters (e.g. R, N, k')
  - ✓ Store column in organic solvent (acetonitrile) or other recommended by manufacturer
- Problems are not always associated with the column and may be caused by instrument or experimental condition issues

Agilent Technical Support

800-227-9770 (US & Canada)

Options 3, 3, 2

Email: lc-column-support@agilent.com

www.agilent.com/chem

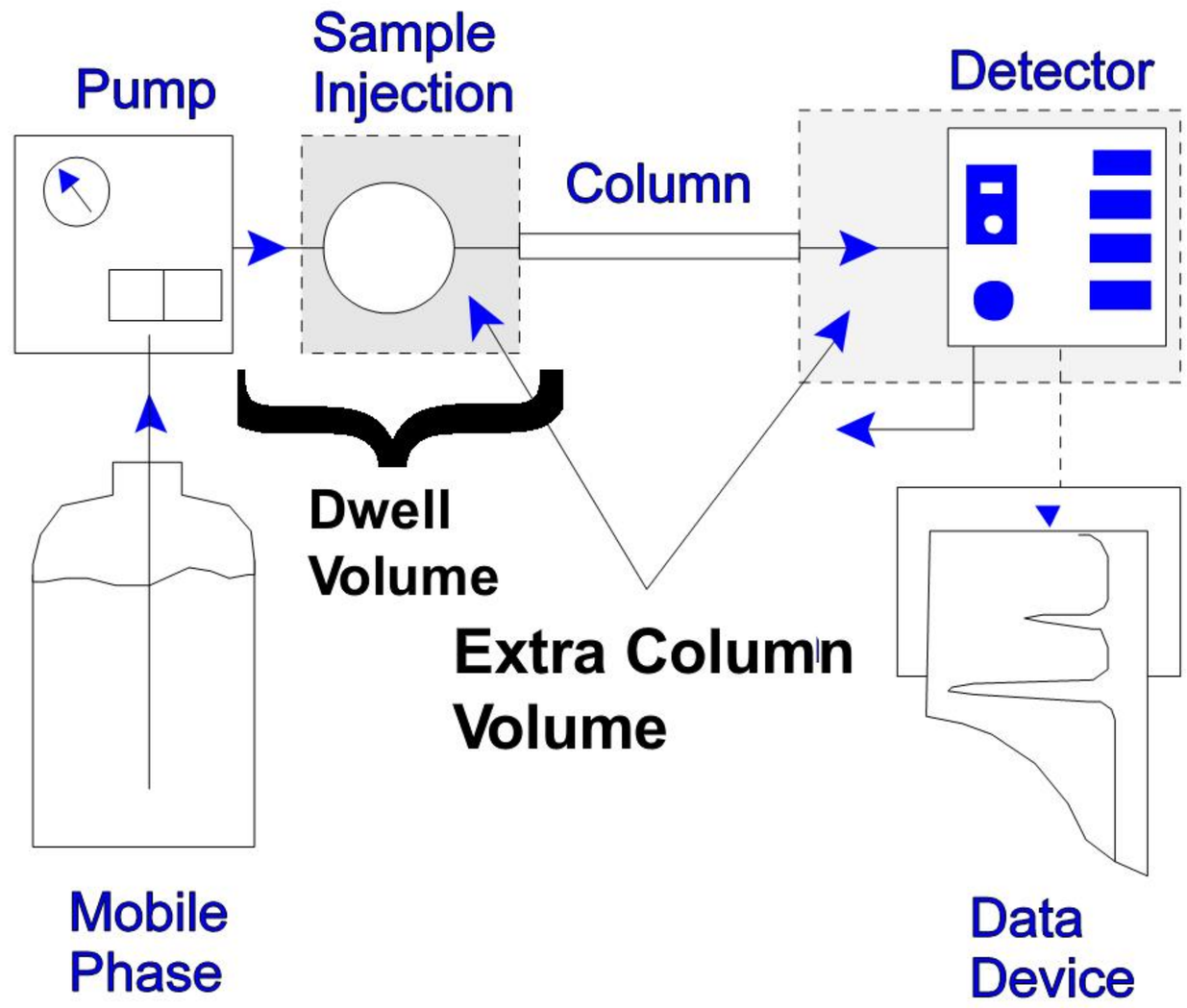
THANK YOU FOR ATTENDING

Appendix

Typical Column Problems

1. High back pressure
   a) Plugged frits
2. Reproducibility
3. Peak shape
4. Sample recovery
5. Loss of resolution
6. Lifetime
7. Leaks

Extra Column Volume and Dwell Volume



ECV = injection volume + connecting tube volume + fitting volume + detector cell volume

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

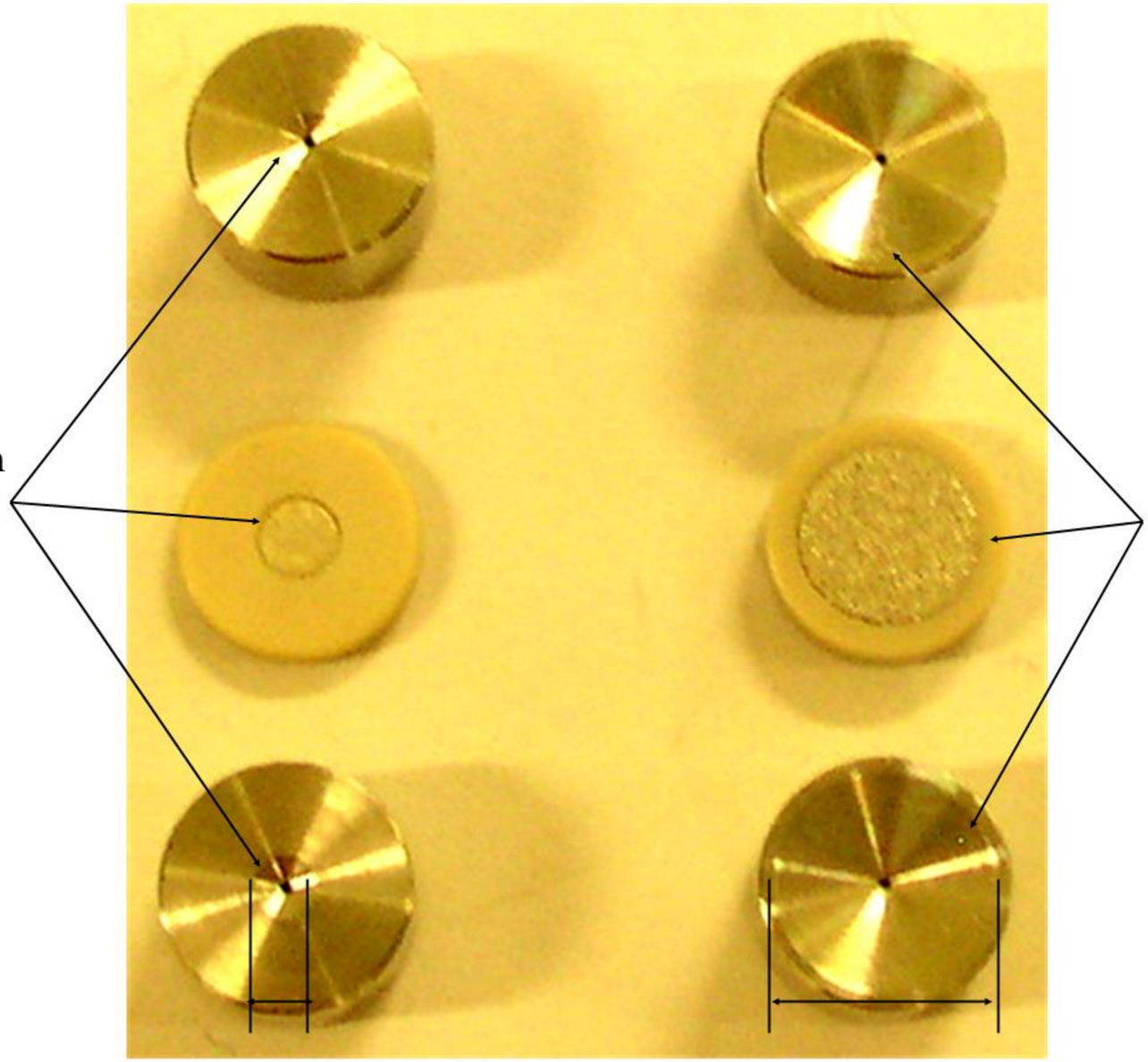
Solubilization Solvents for Proteins/Peptides

In Order of Weakest to Strongest

- Water / phosphate buffer
- Dilute Acid (TFA, HOAc or HCl)
- Neutral pH 6-8 M Guanidine-HCl or isothiocyanate
- 5 % HOAc / 6 M Urea
- Dilute Acid + aqueous / organic solvents (ACN, MeOH, THF)
- Dilute Base (Ammonium Hydroxide)
- Neat Organic Solvents- ACN, MeOH, THF
- 99 % Formic Acid
- HFIP or HFIP / aqueous mixtures
- 100 % TFA
- DMSO or 0.1 - 1 % TFA in DMSO
- Formamide

High Pressure In-line Filter Kit

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



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

Assembling the High Pressure Filter Kit

Put the first insert into the frit housing.

Place the frit on top of this insert

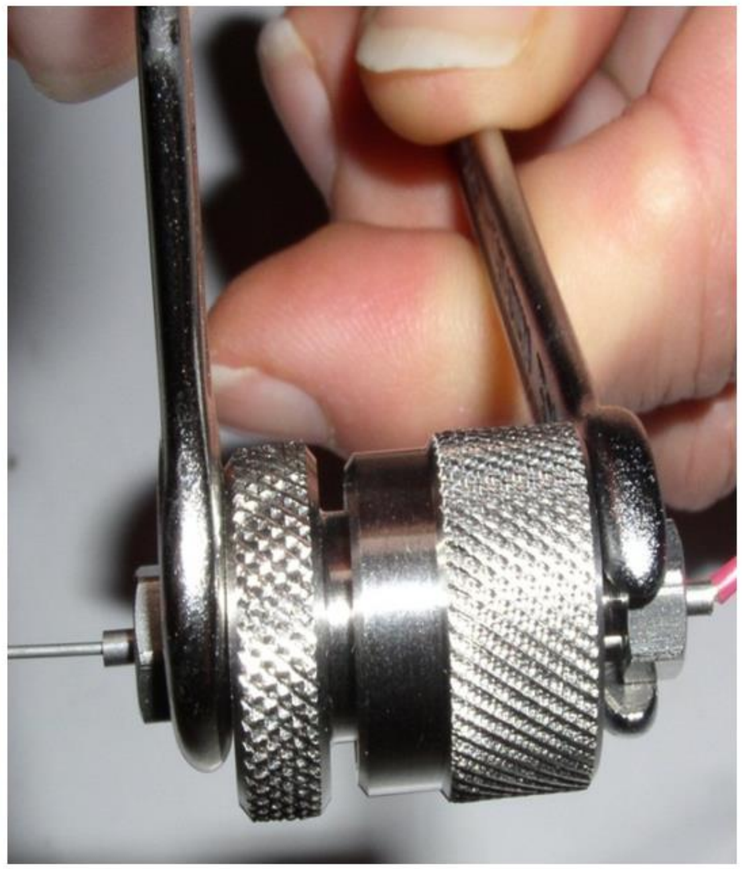
Then place the second insert on top of the frit

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.



SPE Modes

Analyte Adsorption (Bind-Elute)

Analyte(s) retained ($K_D >> 1$)

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

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 ($K_D >> 1$)

No pre-concentration advantage

Eluates may not be as clean

Sample loading may gravity fed

Used less often than analyte adsorption

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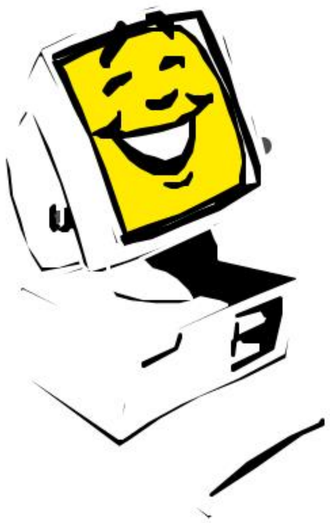
  - *For GC Columns*

  Select option 3, then option 3, option 1
  - 916-608-1964 (fax)
- www.agilent.com/chem



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