

Advanced Topic:

**Tips and Tricks of
HPLC Separations
and Troubleshooting**

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LC Advanced Topics Seminar Series

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Troubleshooting Steps

You Have Recognized There is a Problem! How Do You Fix It?

- 1st Did System Suitability or Sample Fail?
- 2nd Review Method for Compliance
 - Is The Procedure Being Followed Properly?
 - Are Instrument Settings Correct?
- 3rd Ask More Questions!
 - When Did the System Last Function Properly?
 - Has Anything Been Changed?
- 4th Review ALL parameters!
 - The Obvious Is Not Always the Cause
 - Was There More Than One Change?

HPLC System Components

- Pump
- Injector/Autosampler
- Column
- Detector
- Data System/Integrator

Problems Can Be Related to All Components in the System

Column and System Problems

A. Pressure

B. Peak shape

C. Retention

Pressure Issues

Column Observations

Potential Problems

High Pressure

- Plugged frit
- Column contamination
- Plugged packing

Low Pressure

- Leak
- Flow Incorrect

Determining the Cause and Correcting High Back Pressure

Check pressure with/without column - many pressure problems are due to blockages in the system or guard col.

- Remove Column - Pressure Still High?
 - Remove Guard – Pressure Still High?
 - **If Column pressure is high:**
 - Back flush column – Clear “dirty” frit surface
 - Wash column – Eliminate column contamination and plugged packing
 - High molecular weight/adsorbed compounds
 - Precipitate from sample or buffer
-  Change frit – Clear plugged frit **PREVENT THIS!**

Column Cleaning

Flush with stronger solvents than your mobile phase

Reversed Phase Solvent Choices In Order of Increasing Strength

Use at least 25 mL of each solvent for analytical columns

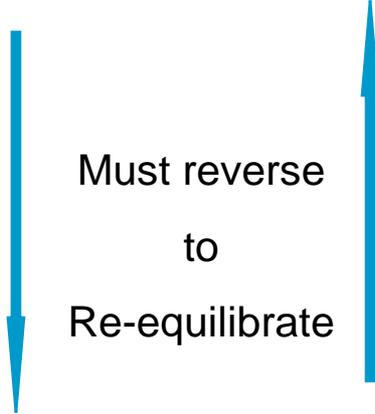
- Mobile phase without buffer salts
- 100% Methanol
- 100% Acetonitrile

- 75% Acetonitrile:25% IPA
- 100% Isopropanol

- 100% Methylene Chloride*
- 100% Hexane*

This Is time consuming
Often performed offline

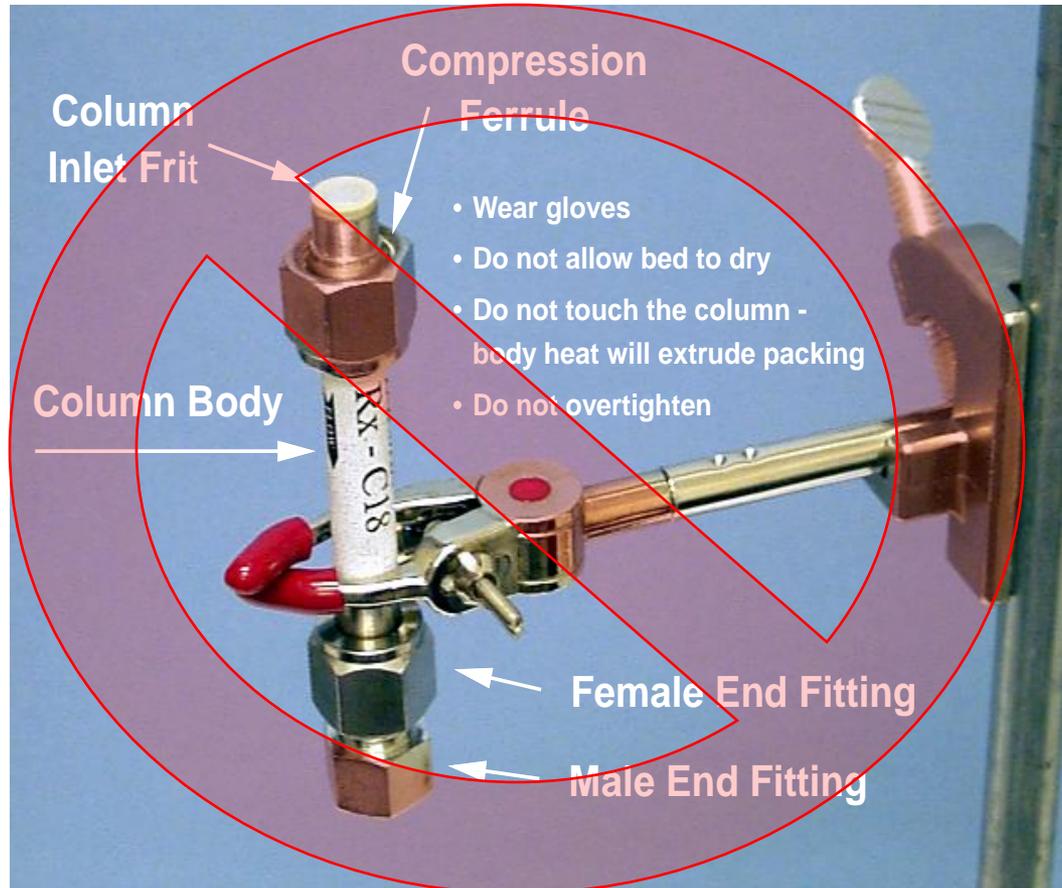
Must reverse
to
Re-equilibrate



Tip: When using either Hexane or Methylene Chloride; The column must be flushed with Isopropanol before returning to your reverse phase mobile phase.

Changing a Frit May Not Be a Good Idea

May not be possible with new generation columns
May damage high performance columns



Tip: Prevention is a Much Better Idea!

The Trick

Prevention Techniques - A Better Choice!

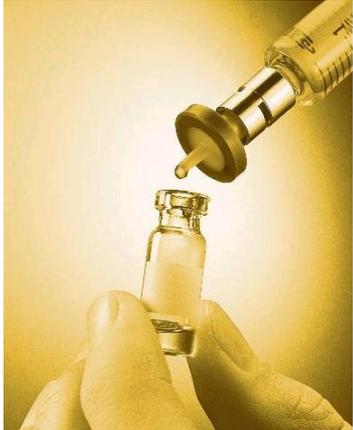
- Use column protection
 - In-line filters
 - Guard columns
- Filter samples
- Filter buffered mobile phases

Easy

- Sample clean-up (i.e. SPE)
- Appropriate column flushing

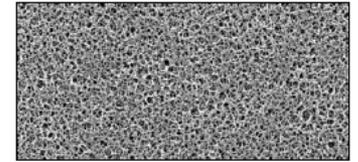
Not as Easy

Inexpensive Filters Prevent Column Frit Plugging



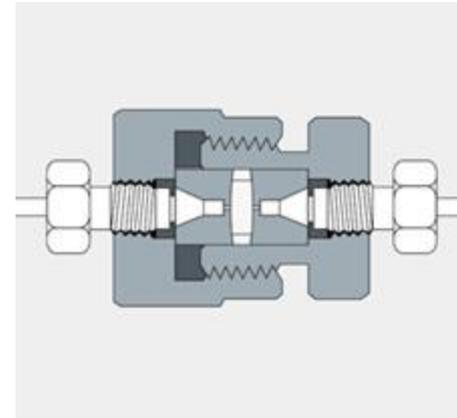
Regenerated Cellulose (RC) Recommended

- Universal hydrophilic membrane; Compatible with most solvents - aqueous and organic
- High purity, extremely low extractables and binding
- More Uniform Surface
- ***Different than Other Cellulose Filters!!***



In-line Filters

- Easy to use and replace
- Frits available in 0.2, 0.5 and 2.0 μ porosity
- Much less expensive than a column
- Easier and faster to replace than a column frit



What Are Common Peak Shape Issues?

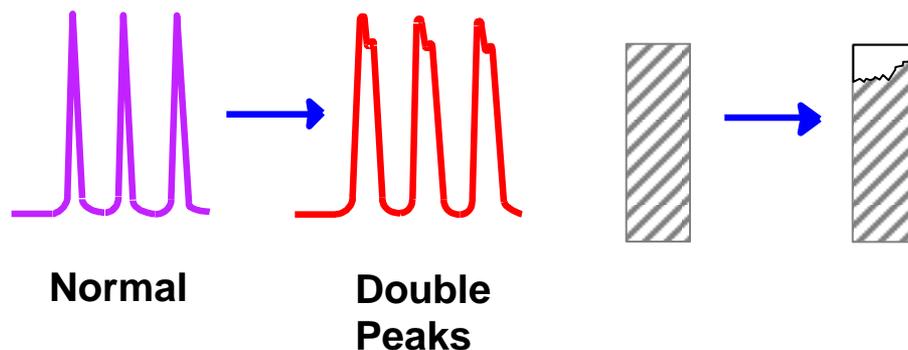
1. Split peaks
2. Peak tailing
3. Broad peaks

- Many peak shape issues are also combinations - i.e., broad and tailing or tailing with increased retention
- Symptoms do not necessarily affect all peaks in the chromatogram
- Each of these problems can have multiple causes



Peak Splitting Caused By Disrupted Sample Path

- Flow path disrupted by void
- Sample allowed to follow different paths through column
- Poorly packed bed settles in use
- High pH dissolves silica

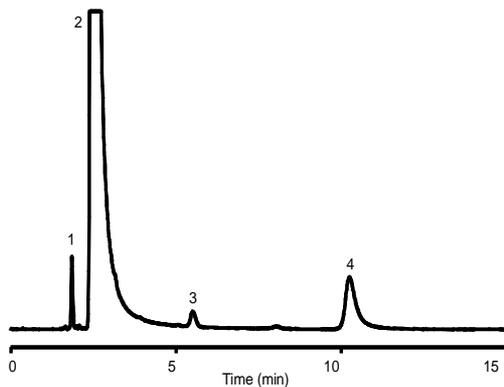


Tip: Similar Effect Can be Caused by Partially Plugged Frit

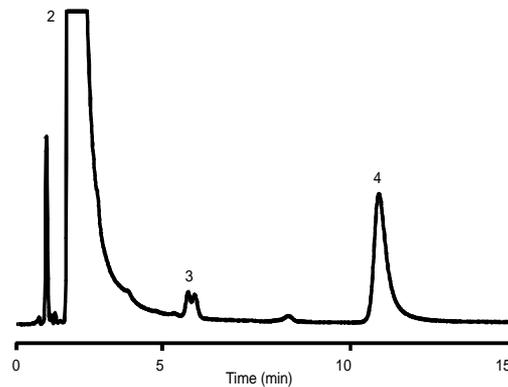
Split Peaks from Column Contamination

Column: StableBond SB-C8, 4.6 x 150 mm, 5 μ m Mobile Phase: 60% 25 mM Na₂HPO₄, 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

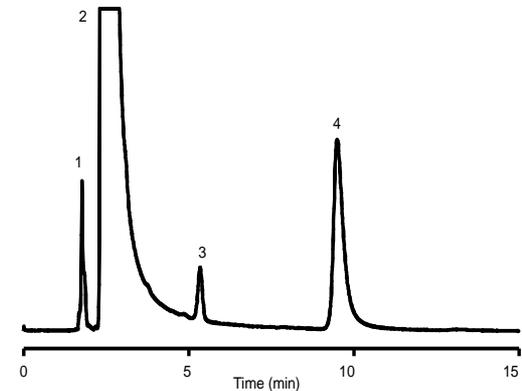
Injection 1



Injection 30



Injection 1
After Column Wash
with 100% ACN

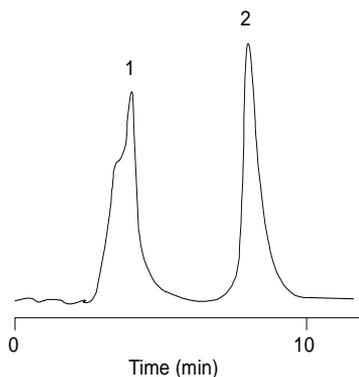


Tip: Column washing eliminates the peak splitting, which resulted from a contaminant on the column
How could this be prevented? (Guard Column, SPE clean up of samples, Periodic column wash)

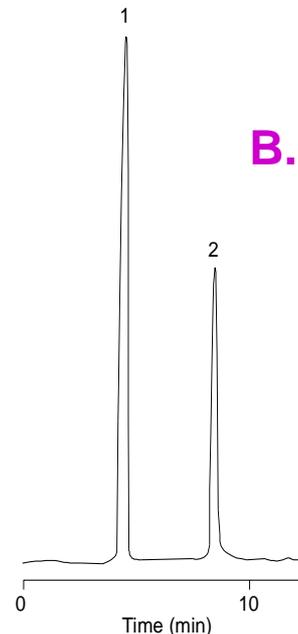
Split Peaks from Injection Solvent Effects

Column: StableBond SB-C8, 4.6 x 150 mm, 5 μ m Mobile Phase: 82% H₂O : 18% ACN
Injection Volume: 30 μ L Sample: 1. Caffeine 2. Salicylamide

**A. Injection Solvent
100% Acetonitrile**



**B. Injection Solvent
Mobile Phase**



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

Trick: Keep Organic Concentration in Sample Solvent \leq Mobile Phase

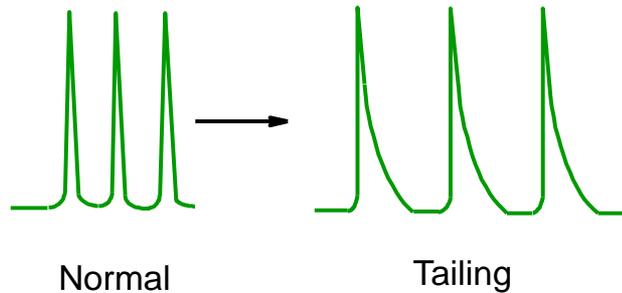
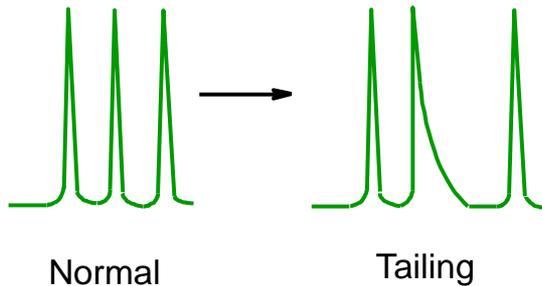
Peak Tailing, Broadening and Loss of Efficiency

May be caused by:

- Column “secondary interactions”
- Column contamination
- Column aging
- Column loading
- Extra-column effects

Peak Shape: Tailing 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.
- Build up of Contamination on Column Inlet.
- Heavy Metals.
- Bad Column.

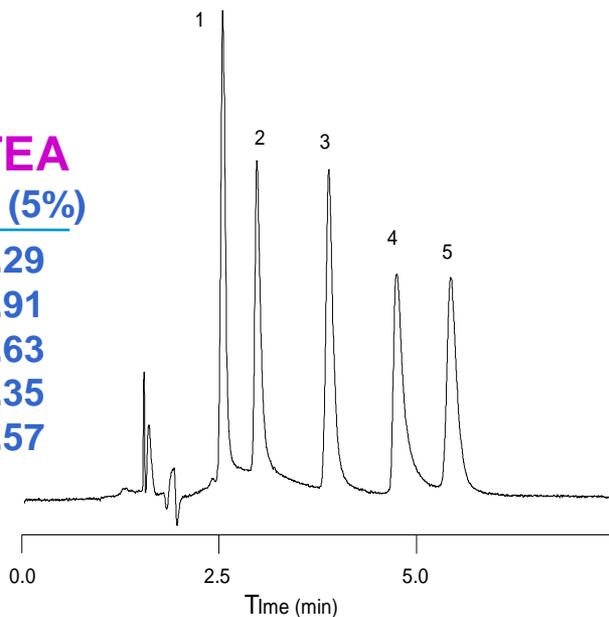
Peak Tailing

Identifying Column “Secondary Interactions”

Column: Alkyl-C8, 4.6 x 150 mm, 5 μ m Mobile Phase: 85% 25 mM Na₂HPO₄ pH 7.0 : 15% ACN Flow Rate: 1.0 mL/min
Temperature: 35°C Sample: 1. Phenylpropanolamine 2. Ephedrine 3. Amphetamine 4. Methamphetamine 5. Phenteramine

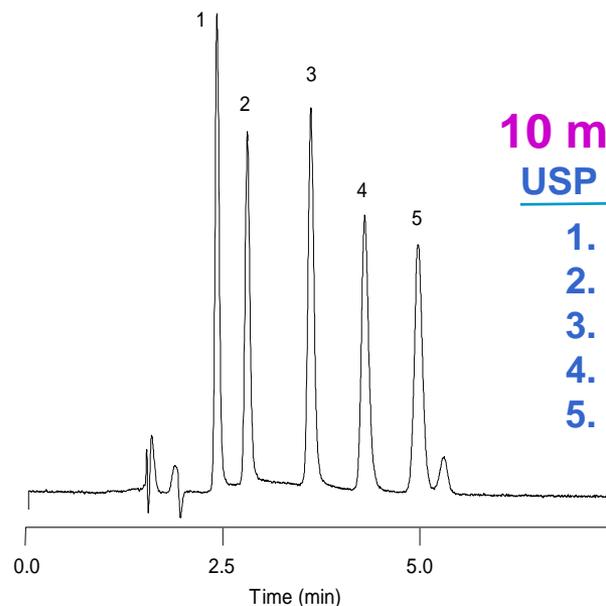
No TEA
USP T_f (5%)

- 1. 1.29
- 2. 1.91
- 3. 1.63
- 4. 2.35
- 5. 1.57



10 mM TEA
USP T_F (5%)

- 1. 1.19
- 2. 1.18
- 3. 1.20
- 4. 1.26
- 5. 1.14



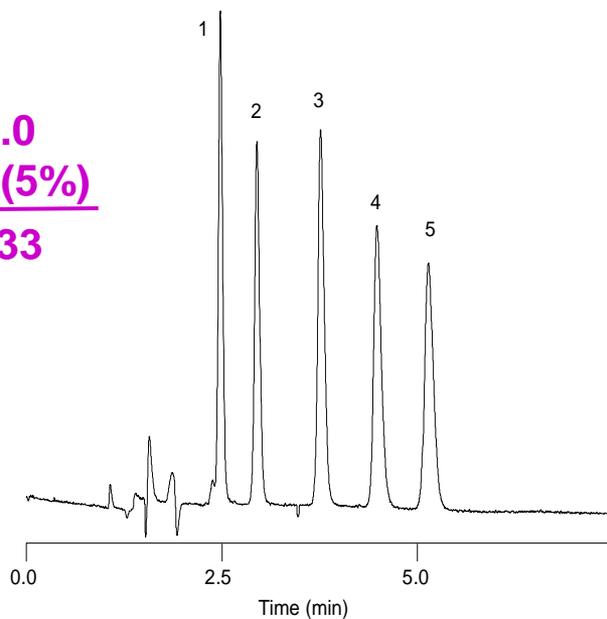
Tip: Mobile phase modifier (TEA) competes with sample for surface ion exchange sites at mid-range pH values

Peak Tailing

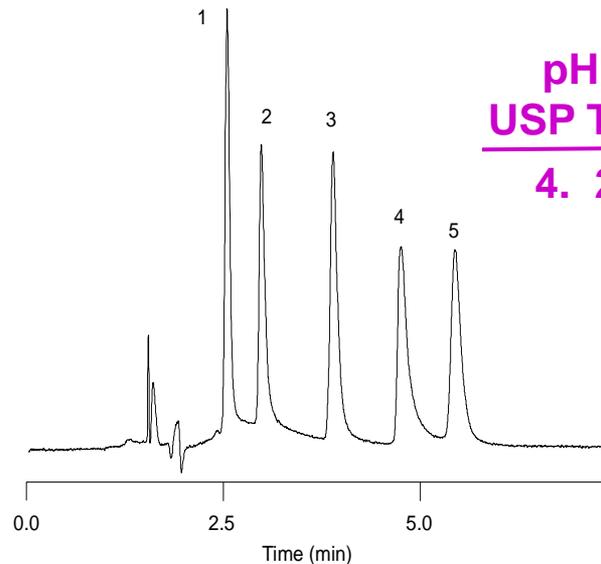
Low pH Minimizes “Secondary Interactions” for Amines

Column: Alkyl-C8, 4.6 x 150 mm, 5 μ m Mobile Phase: 85% 25 mM Na₂HPO₄ : 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 T_f (5%)
4. 1.33



pH 7.0
USP T_f (5%)
4. 2.35



Tip: Reducing mobile phase pH reduces silanol interaction and peak tailing.

Peak Tailing

High pH Eliminates “Secondary Interactions” for Amines

Column: ZORBAX Extend-C18, 4.6 x 150 mm, 5 m m

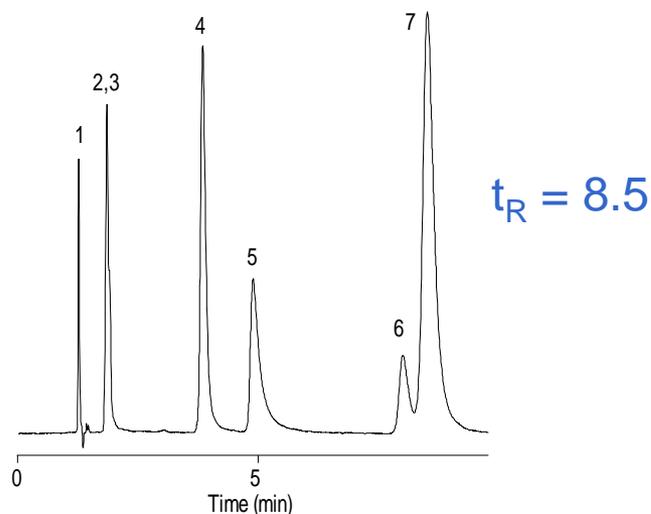
Mobile Phase: See Below , Flow Rate: 1.0 mL/min , Temperature: RT, Detection: UV 254 nm

Sample 1. Maleate 2. Scopolamine 3. Pseudoephedrine 4. Doxylamine 5. Chlorpheniramine 6. Triprolidine 7. Diphenhydramine

pH 7

30% 20 mM Na_2HPO_4

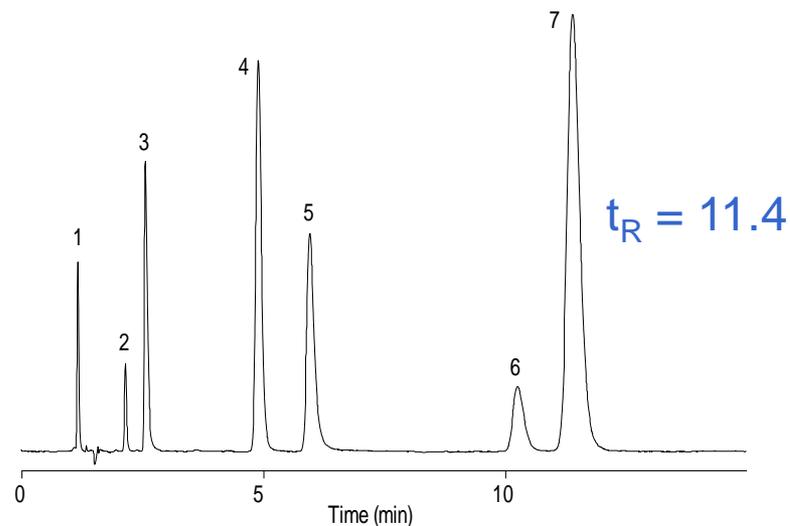
70% MeOH



pH 11

30% 20 mM TEA

70% MeOH



Peak shape and retention of this sample of basic compounds improves at high pH where column has high IEX activity. Why?

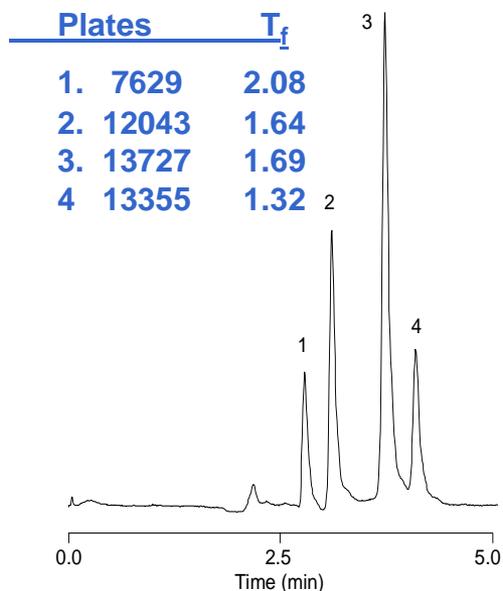
Peak Tailing - Column Contamination

Tip: Quick test to determine if column is dirty or damaged

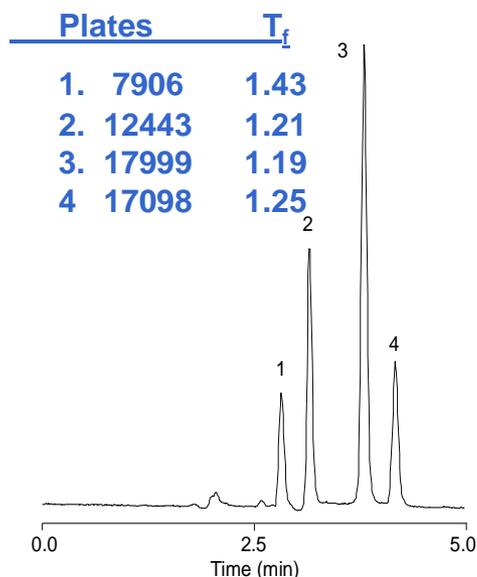
Trick: Reverse column and run sample

- If improved; Possible cleaning will help
- No improvement; Column damaged and needs to be replaced

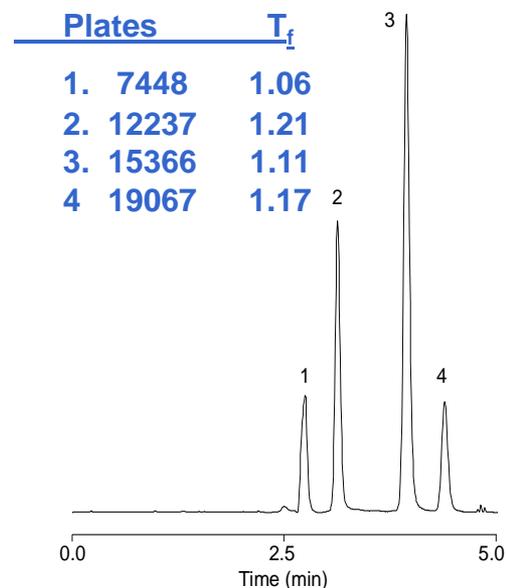
QC test forward direction



QC test reverse direction



QC test after cleaning 100% IPA, 35°C



Column: StableBond SB-C8, 4.6 x 250 mm, 5 μ m
Temperature: R.T. Detection: UV 254 nm

Mobile Phase: 20% H₂O : 80% MeOH Flow Rate: 1.0 mL/min
Sample: 1. Uracil 2. Phenol 3. 4-Chloronitrobenzene 4. Toluene

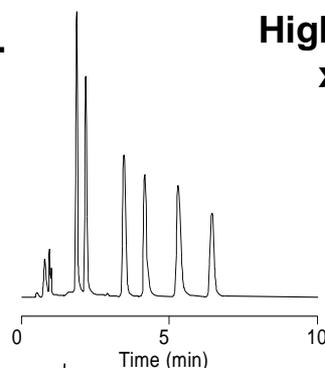
Peak Tailing/Broadening Sample Load Effects

Columns: 4.6 x 150 mm, 5 μ m Mobile Phase: 40% 25 mM Na₂HPO₄ 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

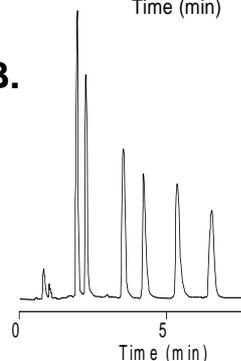
Tailing
 Eclipse XDB-C8
 USP T_f (5%)

	<u>A</u>	<u>B</u>
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

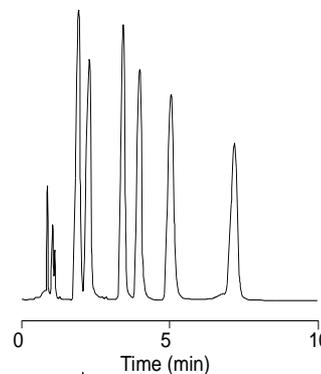
A. High Load x10



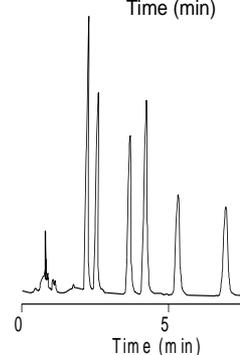
B. Low Load



C.



D.



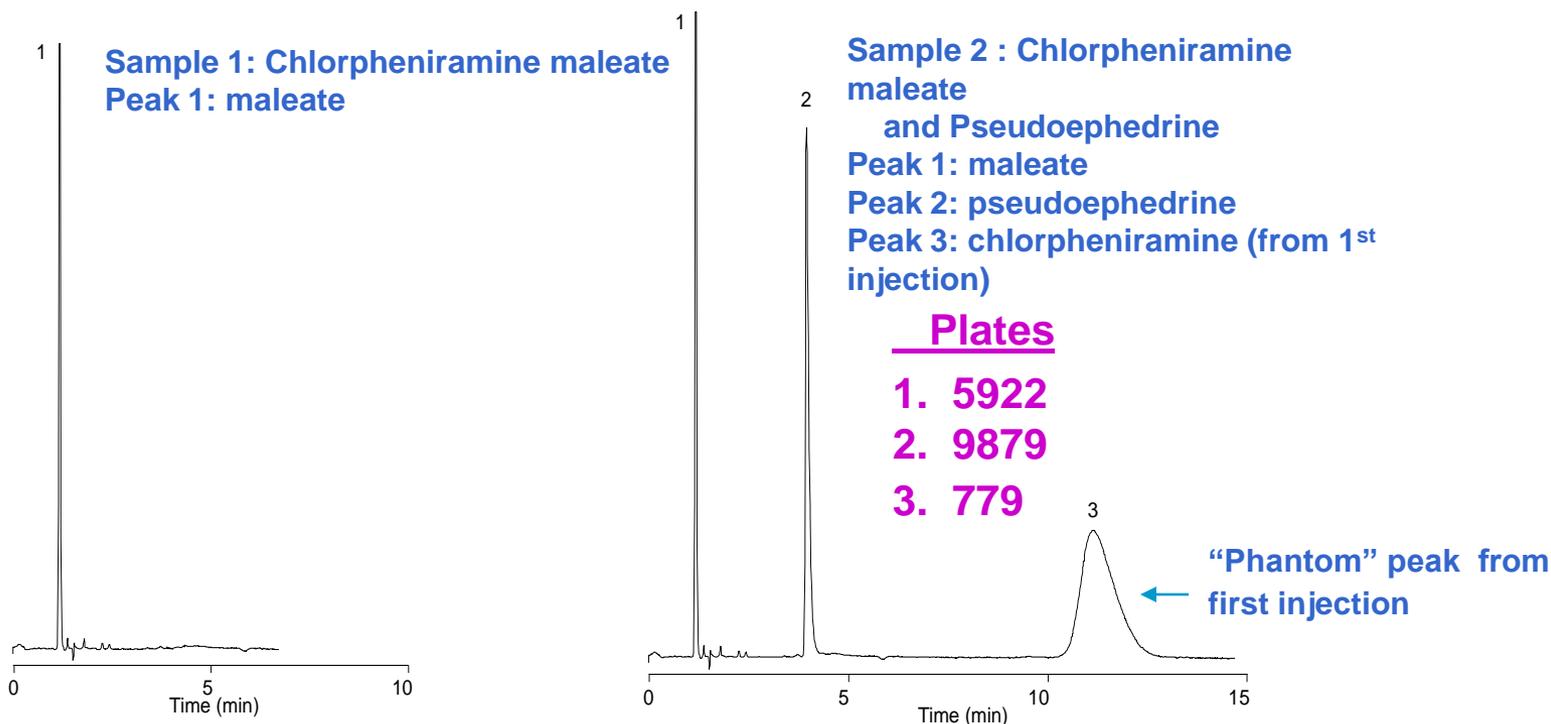
Broadening
 Competitive C8
 Plates

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

Tip: Evaluate Both Volume and Mass Loading

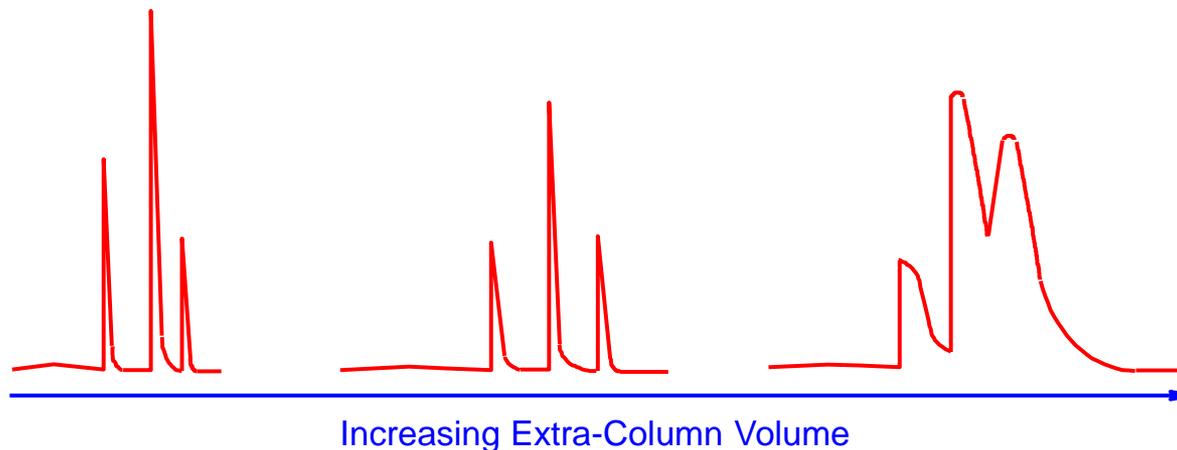
Unknown “Phantom” Peaks

Column: Extend-C18, 4.6 x 150 mm, 5 μ m Mobile Phase: 40% 10 mM TEA, pH 11 : 60% MeOH Flow Rate: 1.0 mL/min
Temperature: R.T. Detection: UV 254 Sample: 1. Maleate 2. Pseudoephedrine 3. Chlorpheniramine



Tip: The extremely low plates for moderately retained peaks are an indication of a very late eluting peak from a preceding run.

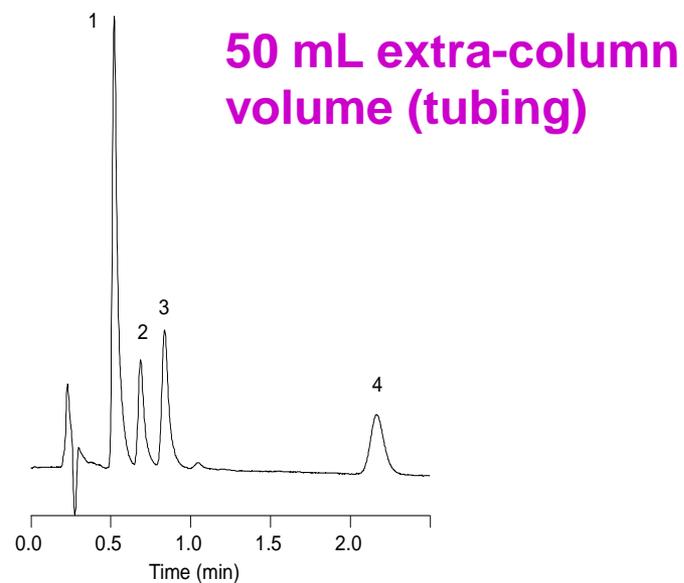
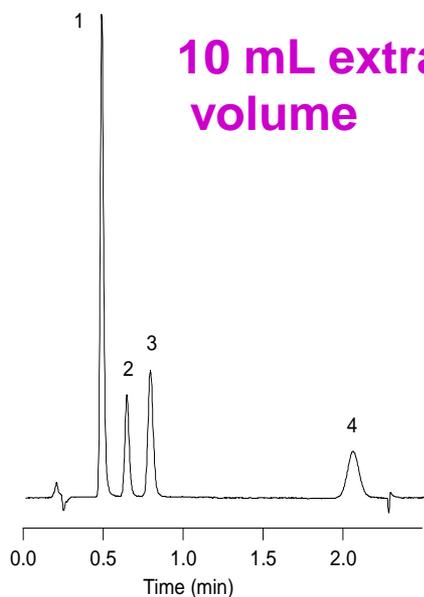
Extra-Column Dispersion



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

Peak Broadening Extra-Column Volume

Column: StableBond SB-C18, 4.6 x 30 mm, 3.5 μ m Mobile Phase: 85% H₂O with 0.1% TFA : 15% ACN Flow Rate: 1.0 mL/min
Temperature: 35°C Sample: 1. Phenylalanine 2. 5-benzyl-3,6-dioxo-2-piperazine acetic acid 3. Asp-phe 4. Aspartame



System Connections Can Cause Peak Broadening

The system has been optimized and :

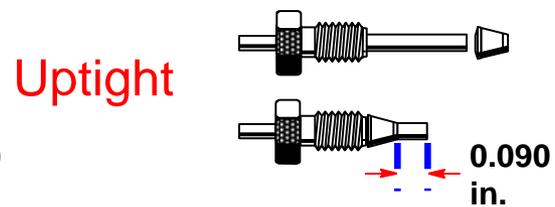
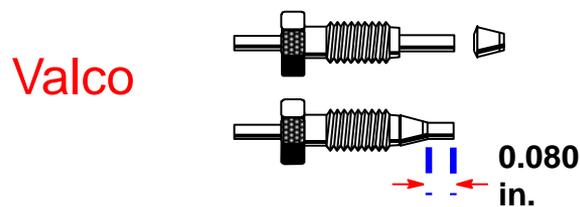
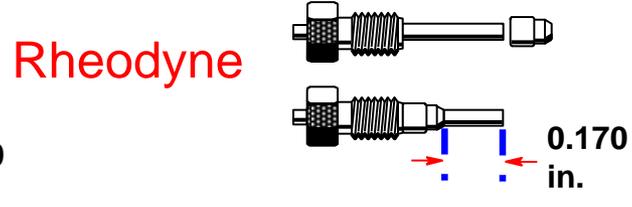
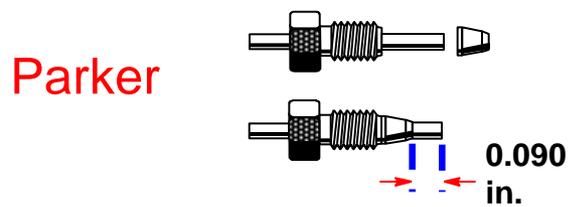
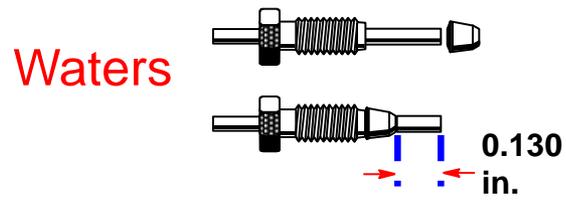
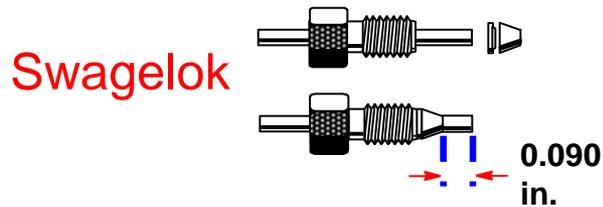
- All tubing lengths are minimum
- Smallest diameter tubing used
- Proper flow cell column

Symptom: Still seems to have too much extra column volume

What is wrong?

Have you made the connections properly?

Column Connectors Used in HPLC

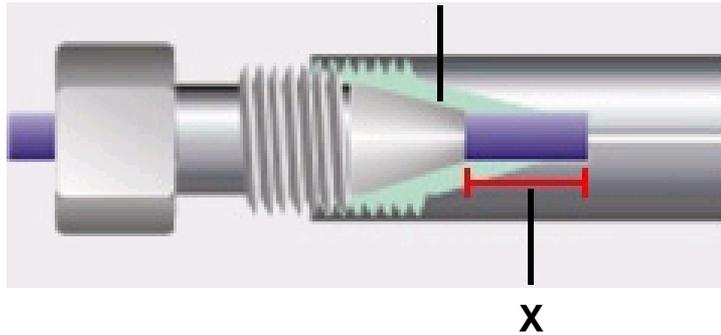


Troubleshooting LC Fittings, Part II. J. W. Dolan and P. Upchurch, LC/GC Magazine 6:788 (1988)

What Happens If Connections Are Poorly Made?

Wrong ... too long

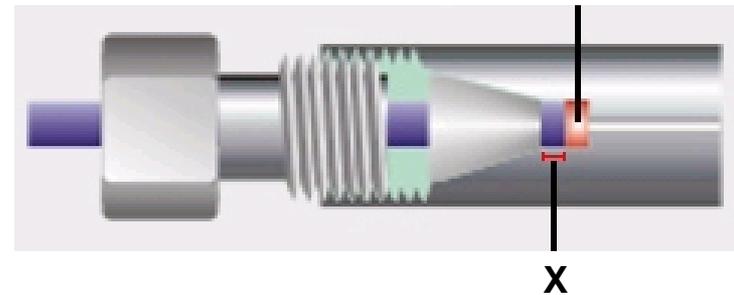
Ferrule cannot seat properly



If Dimension X is too long, leaks will occur

Wrong ... too short

Mixing Chamber



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

Stainless Steel and Polymer Fittings

Which type is used and when?

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



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

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



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

PolyKetone

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



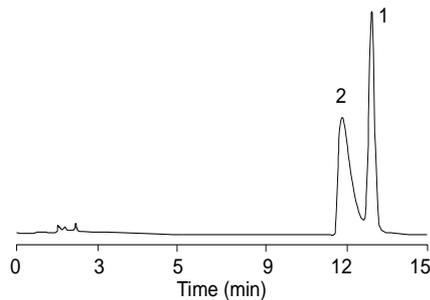
Changes in Retention Can Be Chemical or Physical

May be caused by:

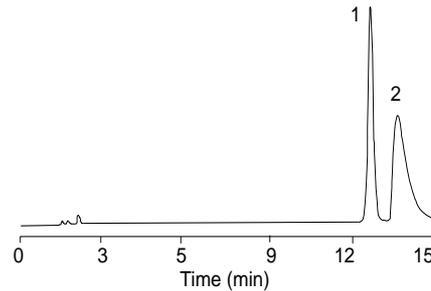
- Column aging
- Column contamination
- Insufficient equilibration
- Poor column/mobile phase combination
- Change in mobile phase
- Change in flow rate
- Different gradient delay volumes

Column Aging/Equilibration Causes Retention/Selectivity Changes

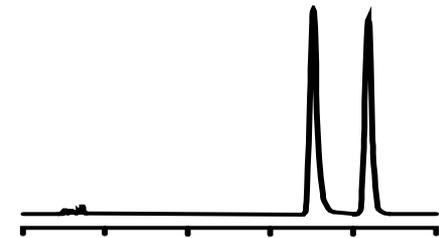
Column 1 - Initial



Column 1 - Next Day



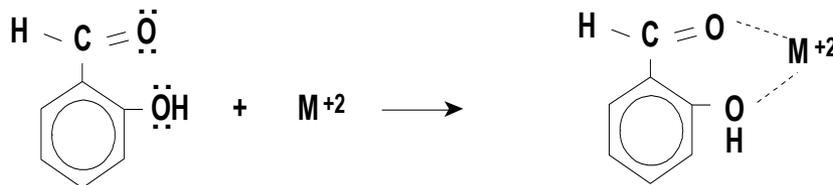
Column 1 - After Cleaning with 1% H_3PO_4 /Equilibration



- The primary analyte was sensitive to mobile phase aging/conditioning of the column
- The peak shape was a secondary issue (metal chelating compound) resolved by “de-activating” the active metal contamination

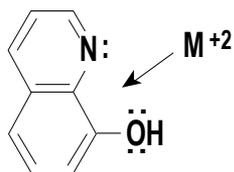
Metal Sensitive Compounds Can Chelate

Hint: Look for lone pair of electrons on :O: or N which can form 5- or 6-membered ring with metal

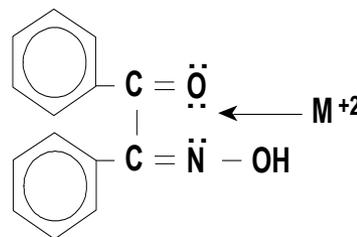


Salicylaldehyde

6-membered ring complex



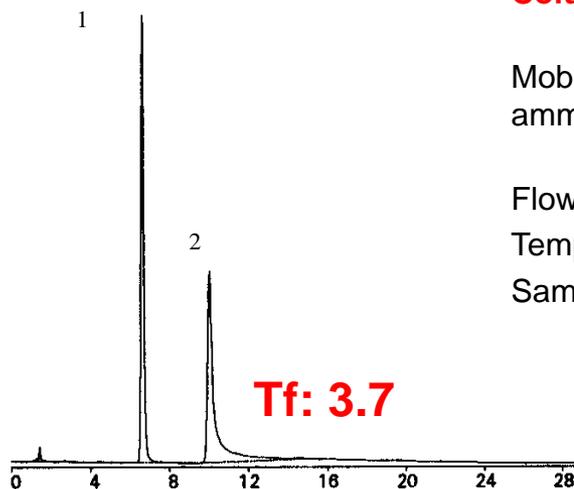
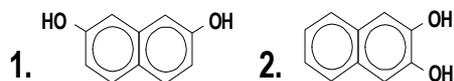
8-hydroxyquinoline
5-membered ring complex



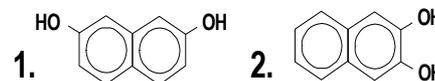
α -benzoinoximine
5-membered ring complex

Acid Wash Can Improve Peak Shape

Before Acid Wash



After Acid Wash
50 – 100 mLs 1% H₃PO₄



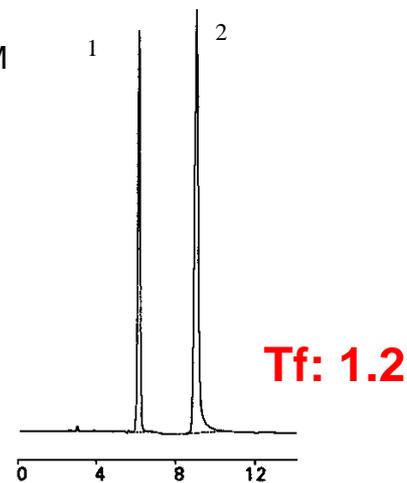
Columns: ZORBAX SB-Phenyl
4.6 x 150 mm

Mobile Phase: 75% 25 mM
ammonium phosphate buffer
25% ACN

Flow Rate: 1.0 mL/min.

Temperature: RT

Sample Size: 5 mL

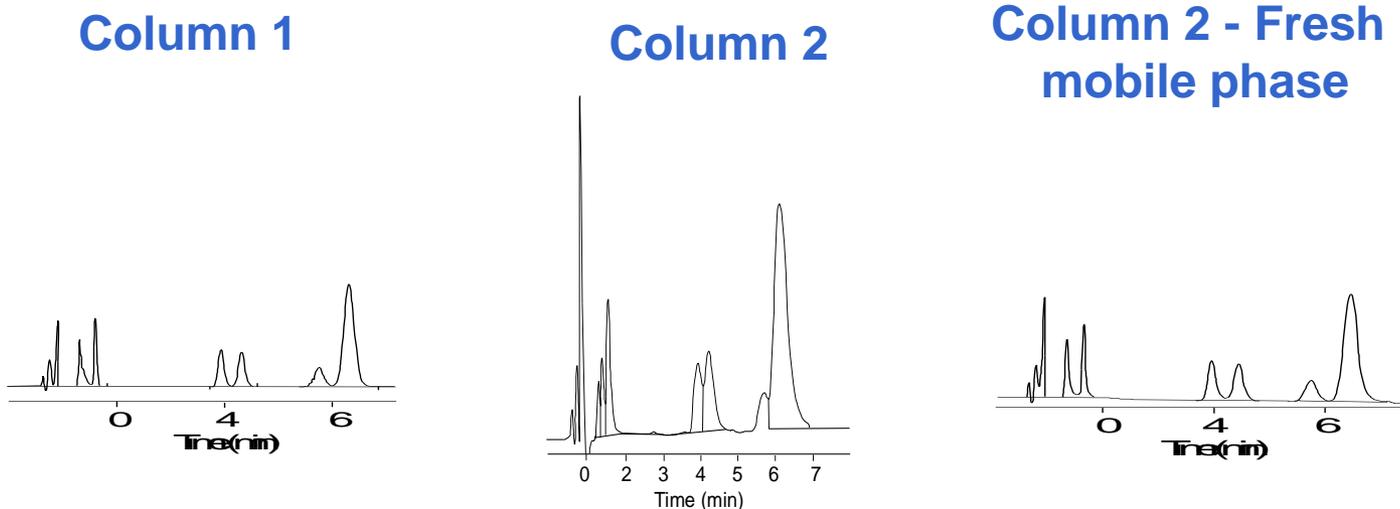


- A 1% H₃PO₄ solution is used on SB columns, 0.5 % can be used on endcapped columns.

Example: Change in Retention/Selectivity

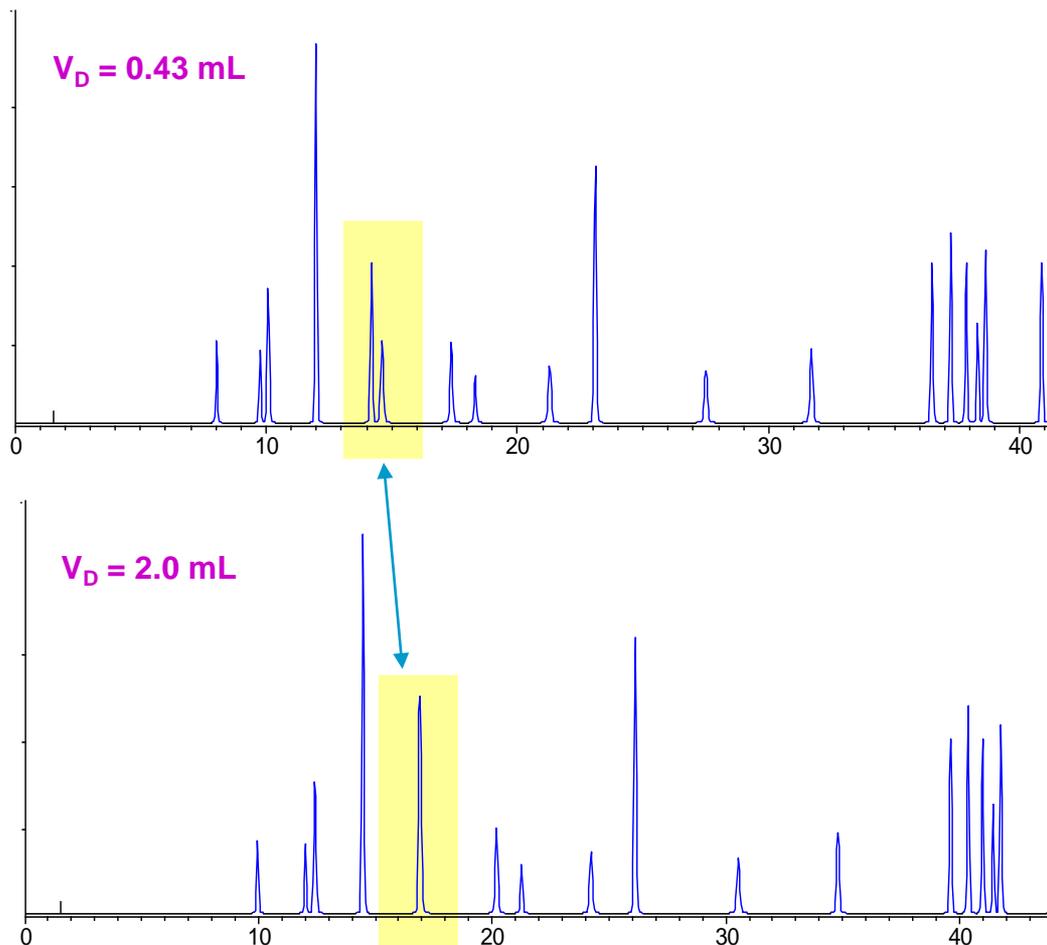
Unintended Mobile Phase Variation

Tip: The Source of the Problem is Often Not the Obvious Change



“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.”

Tip: 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

Trick:

Measure and Correct for Dwell Volume (V_D)

If $V_{D1} > V_{D2}$

**Compensate for longer V_{D1} by adding an isocratic hold to V_{D2} , such that
Hold + $V_{D2} = V_{D1}$**

If $V_{D1} < V_{D2}$

Delay injection, such that $V_{D2} - \text{delay} = V_{D1}$

Mobile Phase pH and Buffers

Why Are These So Important in HPLC?

- pH affects ionization
 - Silica surface of column
 - Sample components of interest
- Buffers
 - Resist changes in pH and maintain retention
 - Improve peak shape for ionizable compounds
- Affects Column Life
 - Low pH strips bonded phase
 - High pH dissolves silica

Minimize Change in Retention/Selectivity Lot-to-Lot

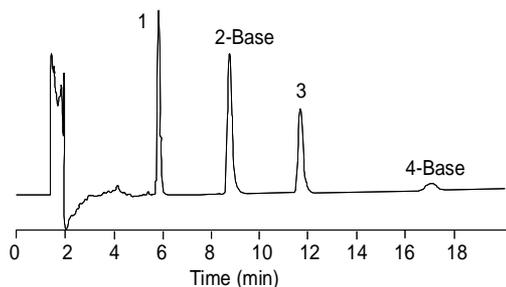
Evaluate:

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

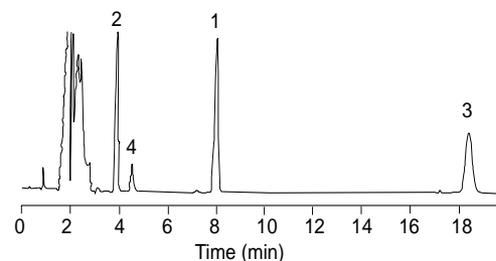
*All causes of column-to-column change should be considered first, especially when only one column from a lot has been tested.

Lot-to-Lot Selectivity Change Related to pH Choice

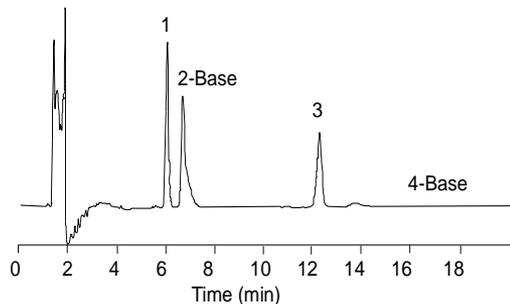
pH 4.5 - Lot 1



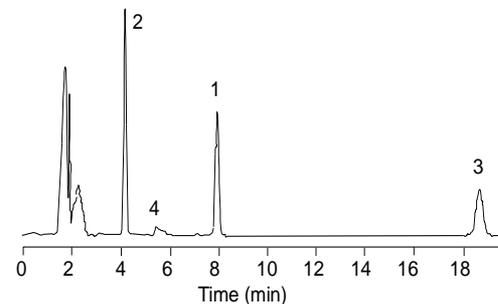
pH 3.0 - Lot 1



pH 4.5 - Lot 2



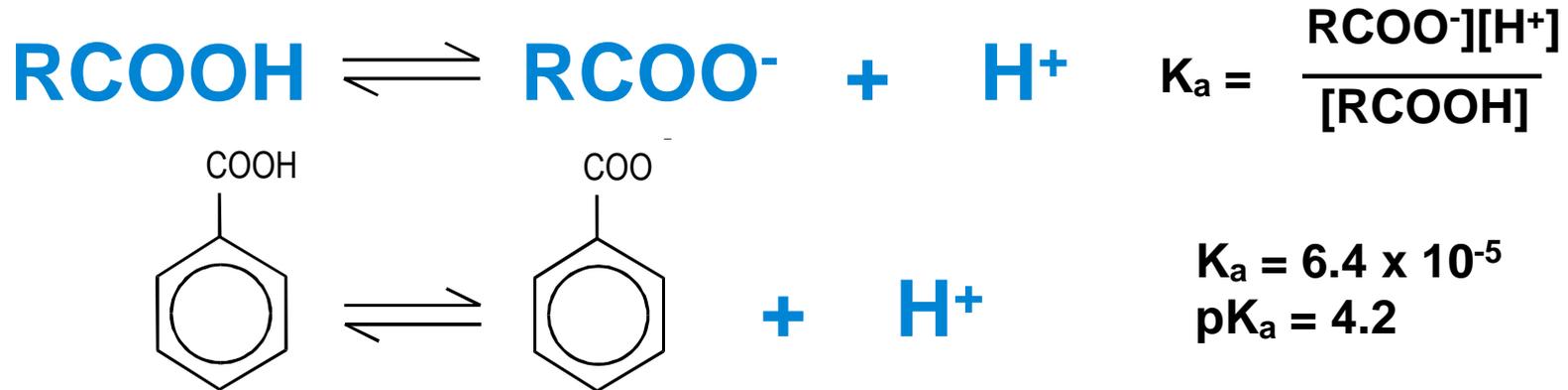
pH 3.0 - Lot 2



- pH 4.5 shows selectivity change from lot-to-lot for basic compounds
- pH 3.0 shows no selectivity change from lot-to-lot

Why Worry About pH?

pH, pKa and Weak Acids



At pH 4.2 – the sample exists as benzoic acid and the benzoate ion in a ratio of 1:1. Peak shape can be poor

At pH 5.2 – 91% of the sample exists as the benzoate ion. RP retention decreases.

At pH 3.2 – 91% of the sample exists as benzoic acid. RP retention increases.

Effect of pH on Peak Shape at or Near the Sample pK_a

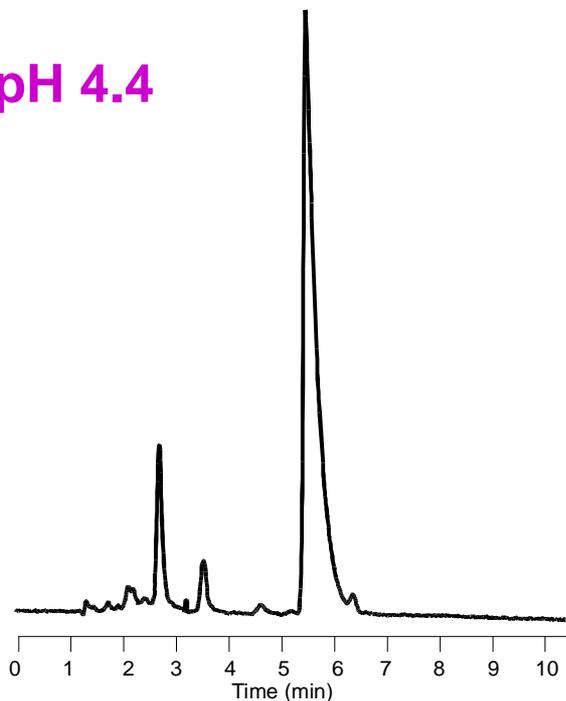
Column: ZORBAX SB-C8 4.6 x 150 mm, 5 mm

Mobile Phase: 40% 5 mM KH₂PO₄: 60% ACN

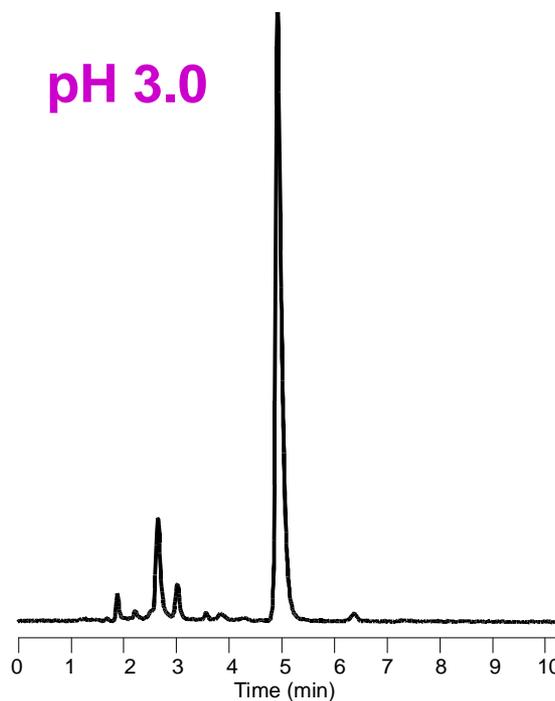
Flow Rate: 1.0 mL/min

Temperature: RT

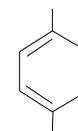
pH 4.4



pH 3.0



CH₃CHCOOH



CH₂CH(CH₃)₂

Ibuprofen
pK_a = 4.4

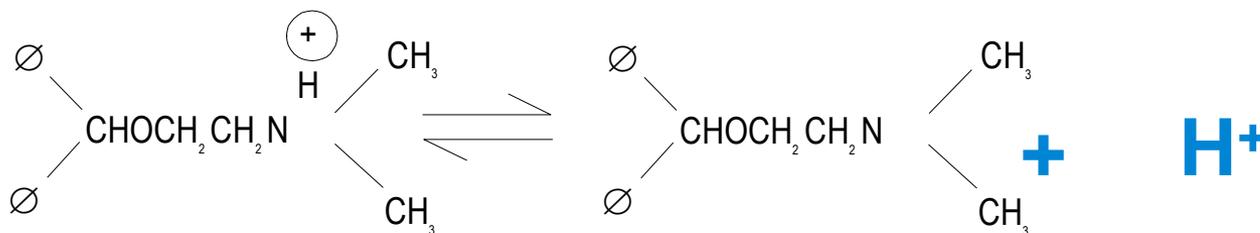
- Inconsistent and tailing peaks may occur when operating close to an analyte's pK_a and should be avoided.

Why Worry About pH?

pH, pKa and Weak Bases



$$K_a = \frac{[R_3N][H^+]}{[R_3NH^+]}$$



$$K_a = 1 \times 10^{-9}$$

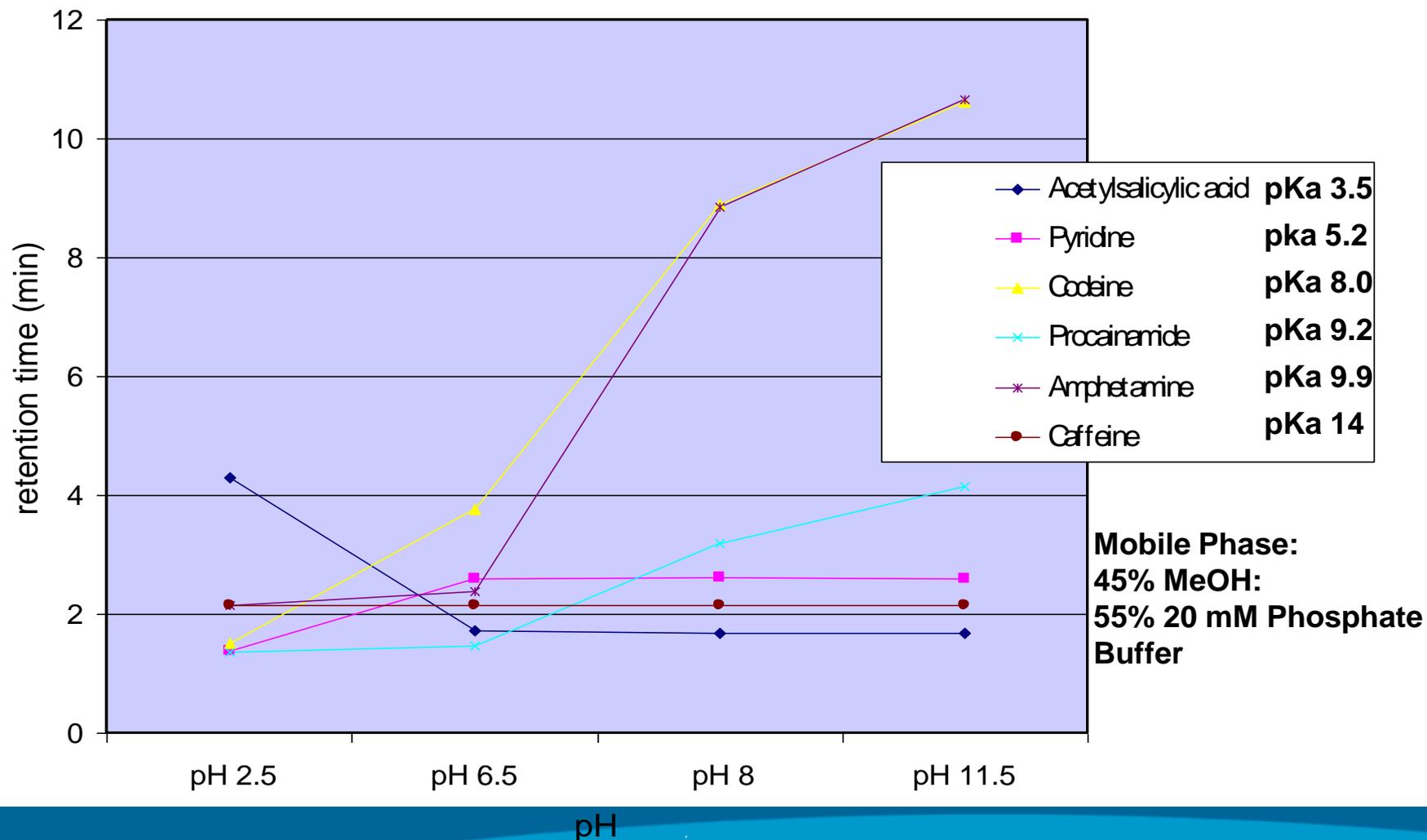
$$pK_a = 9$$

At pH 9 – the sample exists as protonated and unprotonated diphenhydramine in a ratio of 1:1. Peak shape can be poor.

At pH 10 – 91% of the sample exists as unprotonated diphenhydramine.

At pH 8 – 91% of the sample exists as protonated diphenhydramine.

Change in Retention with pH for Ionizable Compounds is Compound Dependent



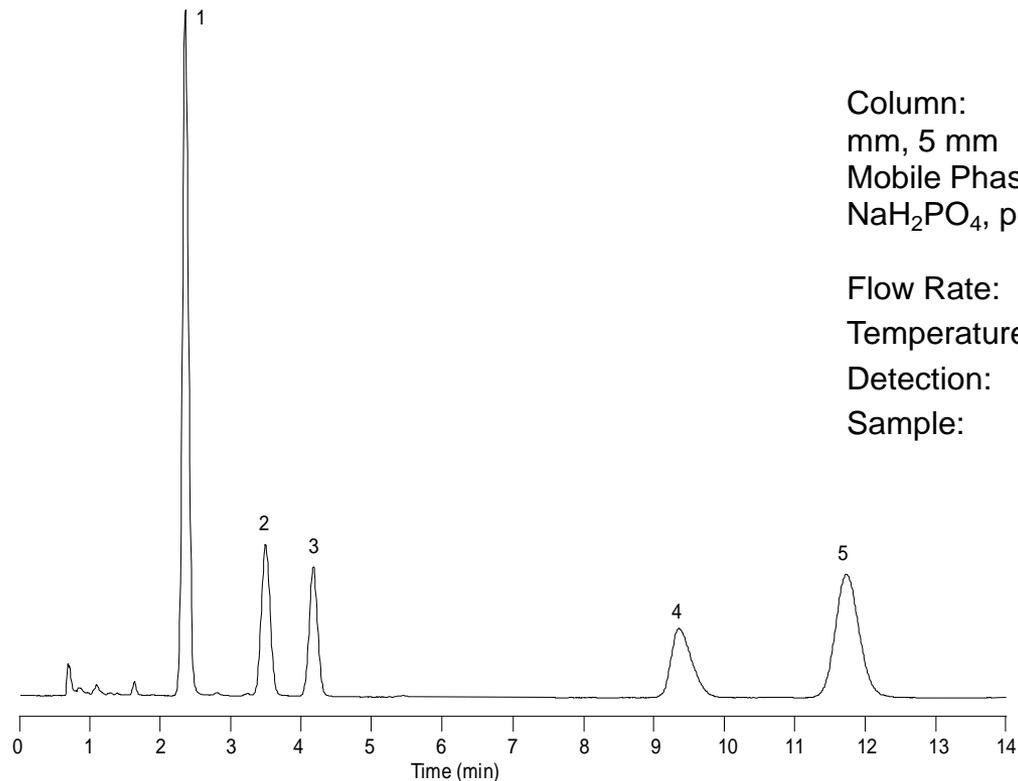
Importance of pH and Buffers

A Practical Example

- Why the Sample Dictates Use
- What Happens When Buffer Used Effectively
- What Happens When Buffer Ignored or Used Improperly

Importance of pH and Buffers - A Practical Example

Optimized Isocratic Conditions for Cardiac Drugs



Column: StableBond SB-C18, 4.6 x 150 mm, 5 mm
Mobile Phase: 45% 25 mM NaH₂PO₄, pH 3.0
55% MeOH
Flow Rate: 2.0 mL/min.
Temperature: 35°C
Detection: UV 254 nm
Sample: Cardiac Drugs
1. Diltiazem
2. Dipyridamole
3. Nifedipine
4. Lidoflazine
5. Flunarizine

I Don't Have Time to Make Buffers or Adjust pH ...

Column: StableBond SB-C18
4.6 x 150 mm, 5 mm

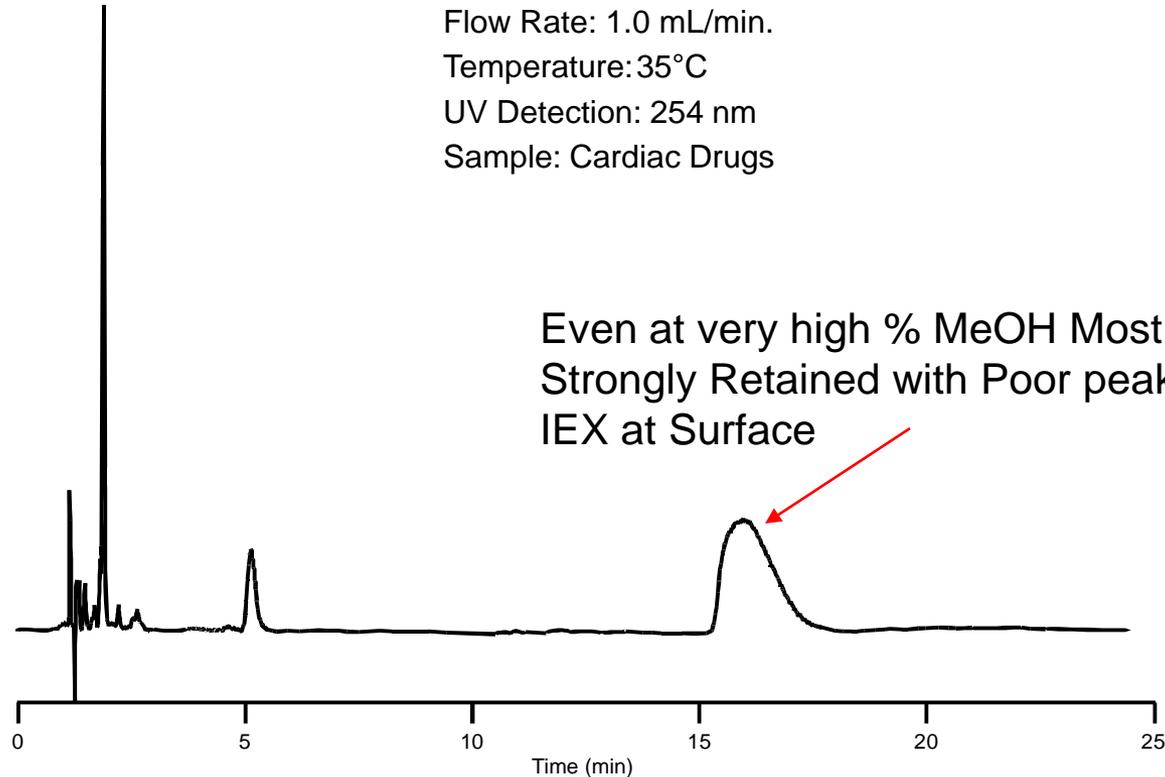
Mobile Phase: A: 20% H₂O
B: 80% MeOH

Flow Rate: 1.0 mL/min.

Temperature: 35°C

UV Detection: 254 nm

Sample: Cardiac Drugs



- **Buffers are critical to good retention and peak shape in many separations.**

What If You Work Outside the Buffer Range?

Columns: StableBond SB-C18
4.6 x 150 mm, 5 mm

Mobile Phase: A: 30% 25 mM NaH₂PO₄, pH 4.8 unbuffered
B: 70% MeOH

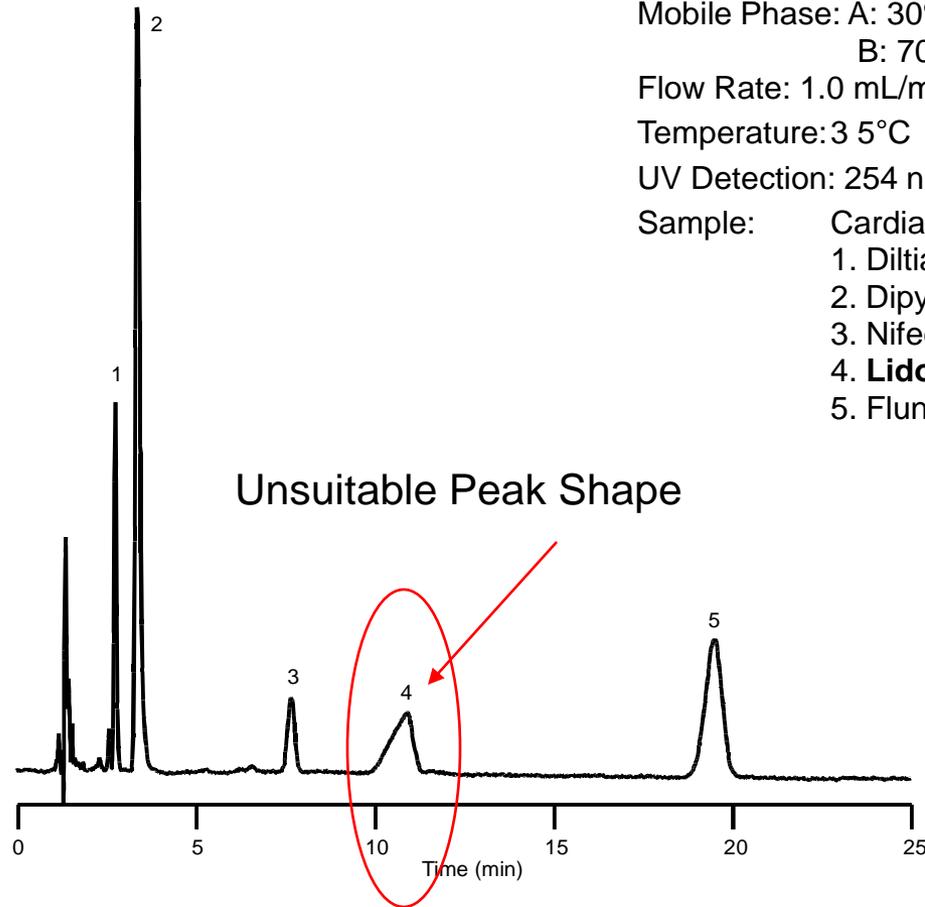
Flow Rate: 1.0 mL/min.

Temperature: 35°C

UV Detection: 254 nm

Sample: Cardiac Drugs

1. Diltiazem
2. Dipyridamole
3. Nifedipine
4. **Lidoflazine**
5. Flunarizine



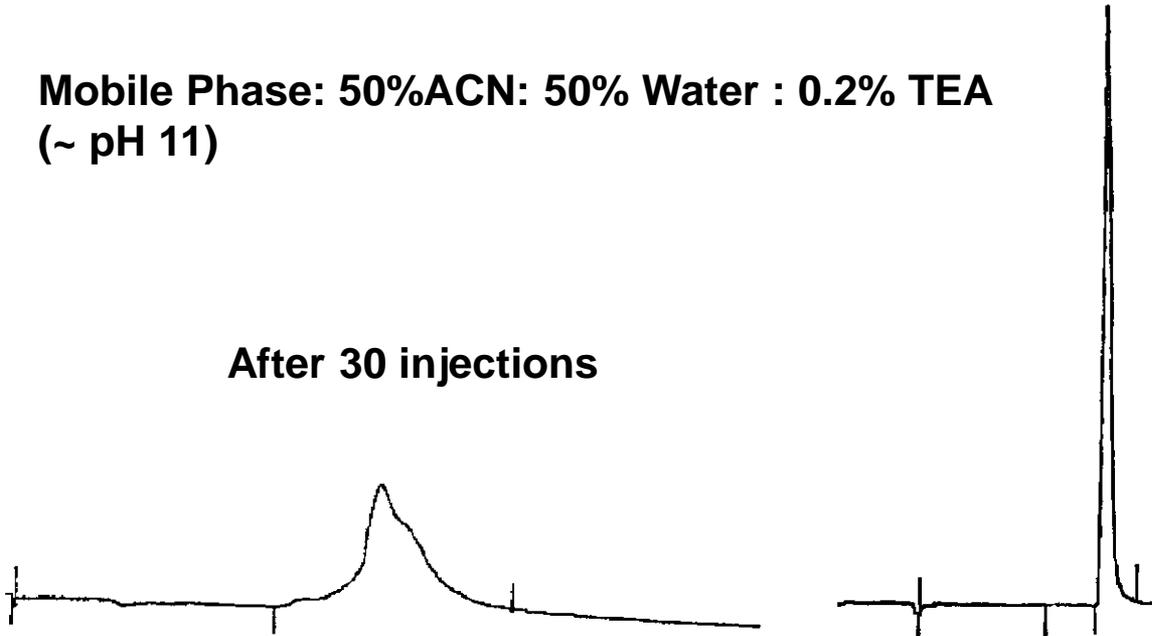
Don't Forget - Match Column to pH of Mobile Phase for Maximum Column Lifetime

High pH and Room Temperature (pH 11 RT)

Mobile Phase: 50%ACN: 50% Water : 0.2% TEA
(~ pH 11)

Initial

After 30 injections



Tip: Use Columns Designed for chosen pH

Detection Issues

Recognize where the problem originates

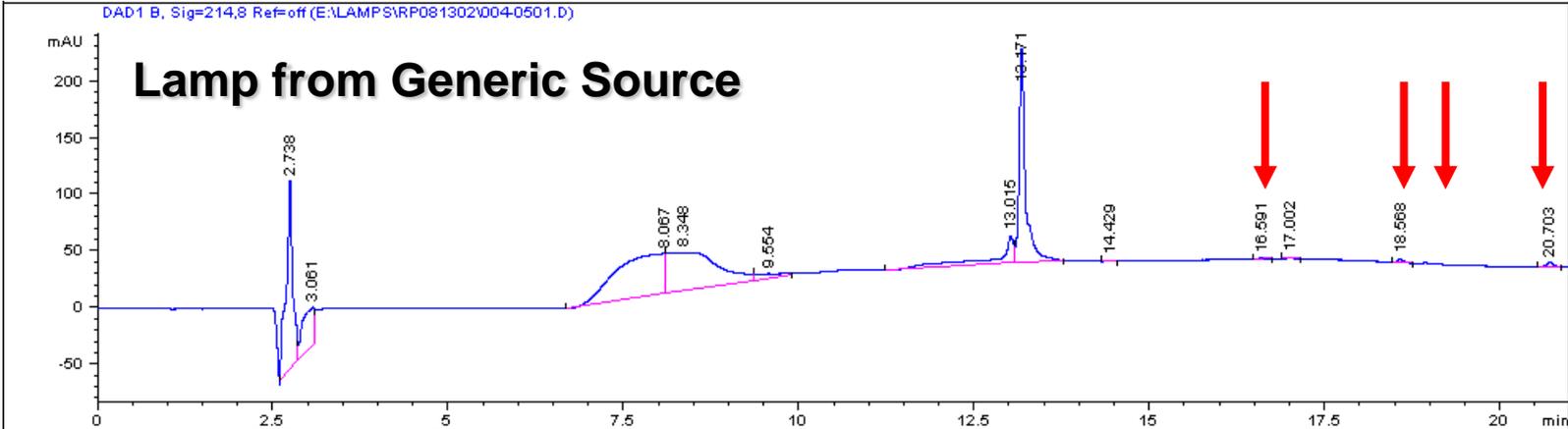
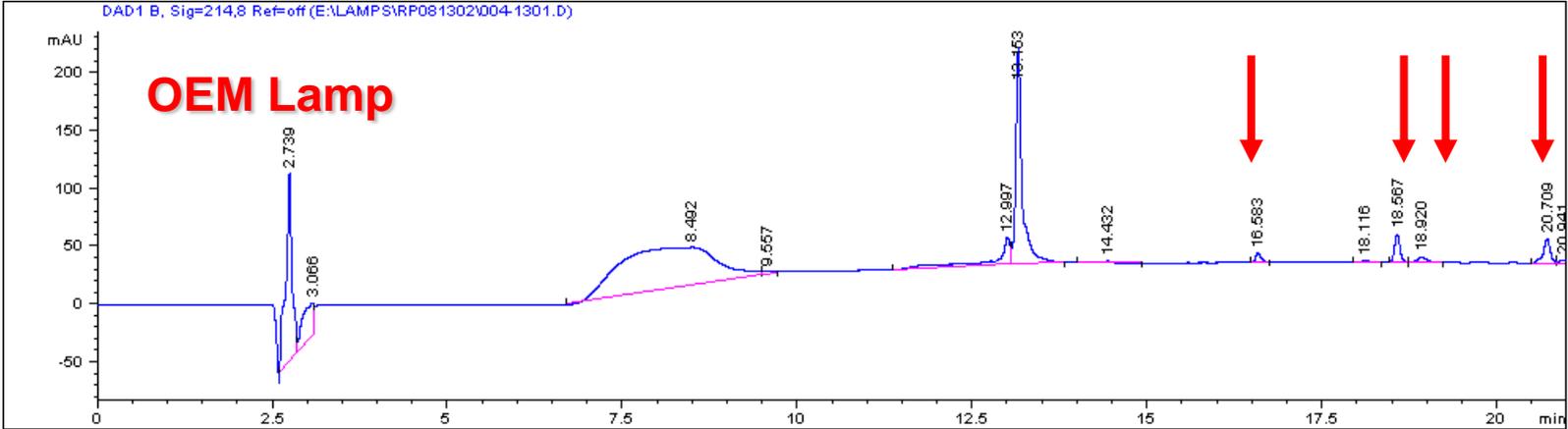
- Is it a consequence of technique?
- Is it expected due to use of certain mobile phase components?
- Can it be corrected by adjusting detector parameters?

Answers will help find a solution!

Let's explore some problems and solutions

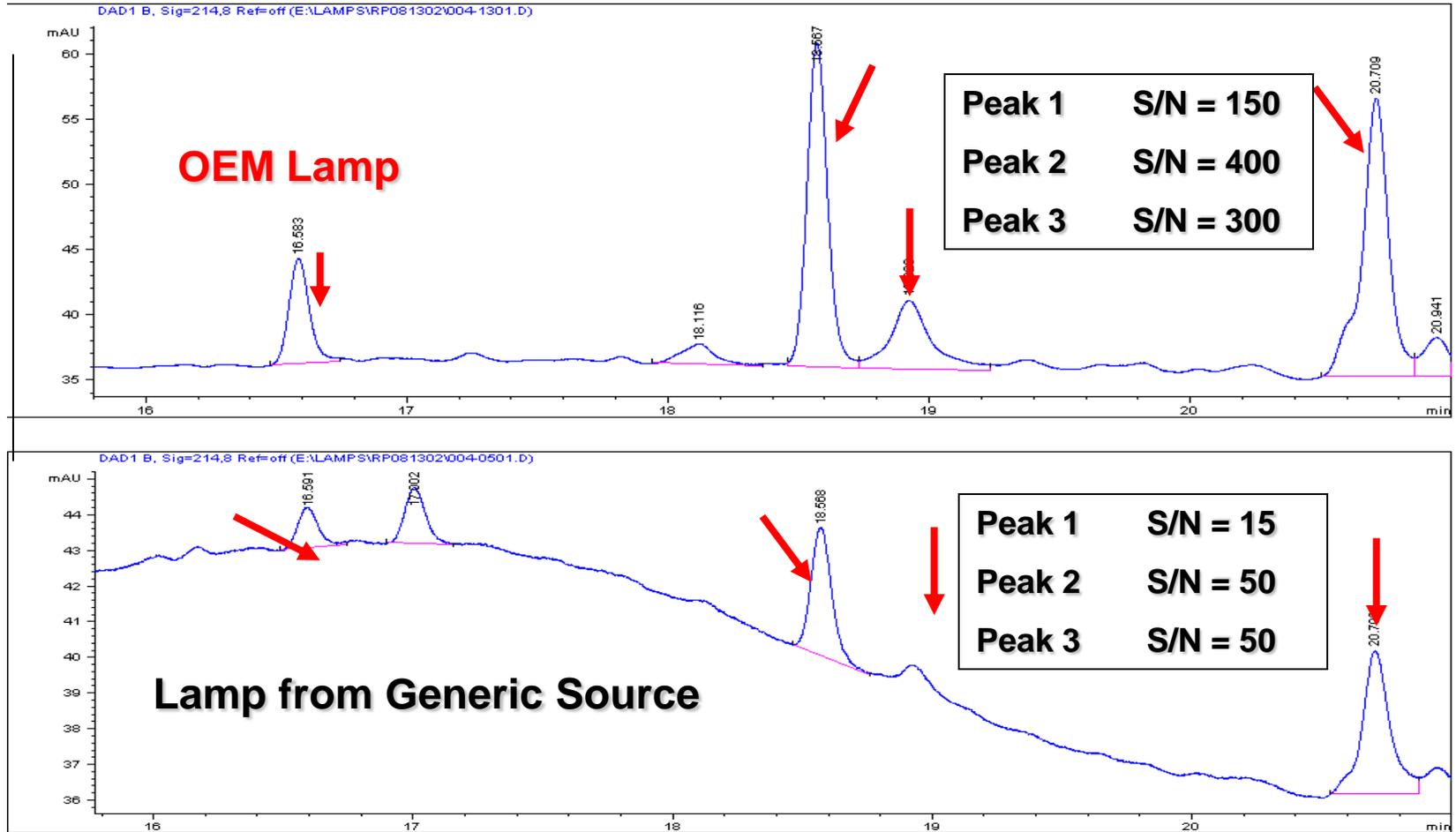


Chromatographic Results with “Wrong” Lamp at 214 nm Wavelength



Tip: Could also be a symptom of aging lamp

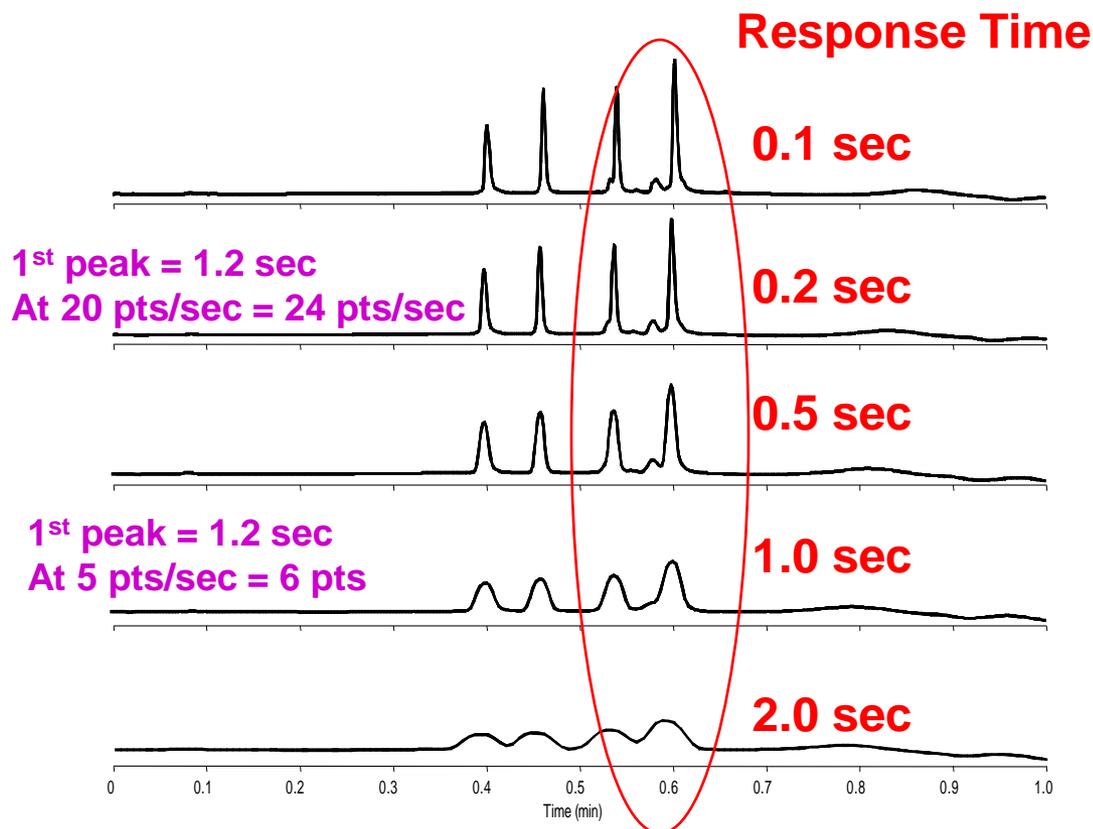
Expanded View of Chromatographic Results Generic Source Lamp at 214 nm Wavelength



Tip: Poor S/N makes it difficult to detect low level impurities

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% H₂O, 5% ACN with 0.1% TFA
B: 5% H₂O, 5% ACN with 0.1% TFA

Flow Rate: 2 mL/min

Temperature: 70°C

Detector: UV 215 nm

Piston stroke: 20

Sample:

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

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

Conclusions

HPLC column problems are evident as

- High pressure (prevention better than the cure)
- Undesirable peak shape
- Changes in retention/selectivity

Often these problems are not associated with the column and may be caused by instrument and chemistry issues.

- pH of mobile phase
- Instrument connections
- Detector settings
- Metal contamination

Start with the correct questions

- Find the answers
- Answers will lead to solutions