Tips and Tricks of Faster LC Analysis Without Capital Investment

It may be easier than you think!

Agilent Technologies

Tips and Tricks of HPLC System Troubleshooting

Agilent Technologies

Trouble Shooting Steps

You Have Recognized There is a Problem!

How Do You Fix It?

- 1st Did System Suitability or Calibration Sample Fail?
  - Make Blank Runs
- 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 Problems From *LC GC User Survey*

Column Problem	Percentage of Respondents
Back Pressure, Plugged Frits	24%
Poor Reproducibility	16%
Sample Recovery	14%
Loss of Resolution	13%
Instability	11%
Voids	8.1%
Leaks, Fittings	4.8%
pH Range	2.0%
Low Plate Count	2.0%
Column Overload	2.0%
Cost	1.7%
Miscellaneous	15%

Categories of 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!***

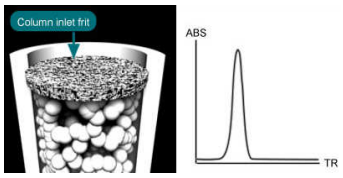
Correcting Overpressure

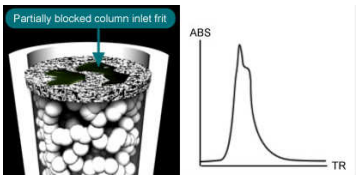
Determining the Cause and Correcting High Back Pressure

Many pressure problems relate to blockages in the system.
Check system pressure with / without column

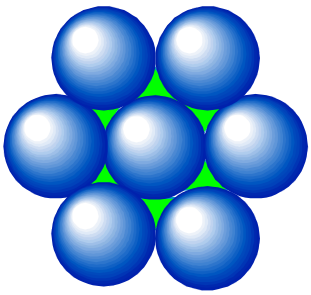
If column pressure is high:

- Back flush column (care regarding future performance)
- Clear blocked frit (reverse flush with strong solvent)
- Wash column
  Eliminate column contamination and clear blocked packing
  Remove high M_w / adsorbed compounds
  Clear precipitate introduced from the sample or buffer





Plugged Packing



Particle Size	Area (um^2)	Diameter (um)	Frit Porosity (um)
5.0	2.7	**0.9**	2.0
3.5	1.3	**0.6**	2.0
3.0	1.0	**0.6**	0.5
2.5	0.7	**0.5**	
1.8	0.4	**0.4**	0.5
1.7	0.3	**0.3**	

Beware of buffered mobile phases

Buffers usually contain insoluble material – filter

Buffer solubility decreases with increasing % organic* - avoid 100%B with buffer salts

***Schellinger,A.P. and Carr,P.W., LC-GC North America, 22, 6, 544-548 (2004)**

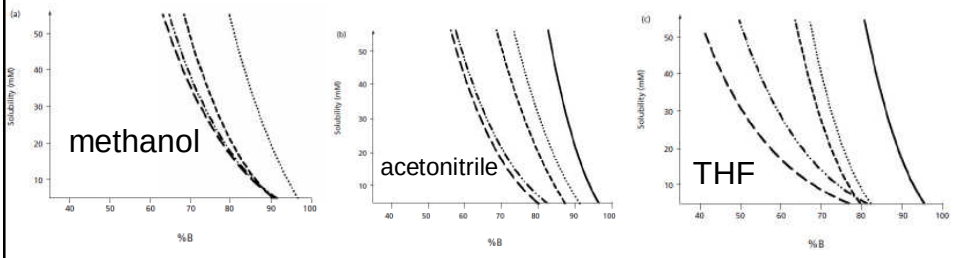
Buffer solubility

Table I: Summary of buffer solubility

	Solubility (mM)		
%B	Methanol	Aetonitrile	Tetrahydrofuran
0	>50	>50	>50
10	>50	>50	>50
20	>50	>50	>50
30	>50	>50	>50
40	>50	>50	50
50	>50	>50	25
60	>50	45	15
70	35	20	10
80	15	5	<5
90	5	0	0

Table I presents an estimate of the soluble concentration of the least soluble buffer (potassium phosphate at pH 7.0) in three organic cosolvents

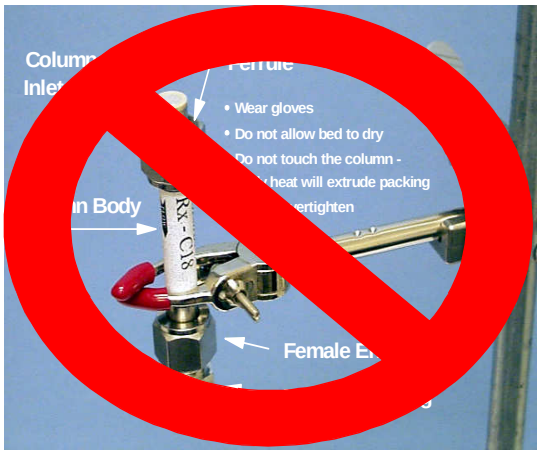
Solubility of five buffers in mixtures with (a) methanol, (b) acetonitrile, and (c) tetrahydrofuran



Solubility of five buffers in mixtures with (a) methanol, (b) acetonitrile, and (c) tetrahydrofuran, where **— represents ammonium acetate at pH 5.0, •••• represents ammonium phosphate** at pH 3.0, **--- represents potassium phosphate at pH 3.0, –••– represents ammonium phosphate** at pH 7.0, and **– – represents potassium phosphate at pH 7.0. The 95% confidence level** intervals are 3.3 mM.

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

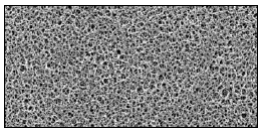
- Sample clean-up (i.e. SPE)
- Appropriate column flushing

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
- High recovery
- More Uniform Surface
- ***Different than Other Cellulose Filters!!***

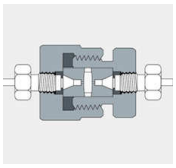


In-line Filters **Easy to Use and replace**

Frits Available in 0.2,0.5 and 2.0µ Porosity

Much Less expensive than a Column

Easier and Faster to Replace than a Column Frit



Mini-UniPrep Vials

UniPrep vials contain an integral filter

(PTFE, PP, RC or Nylon - 0.2 or 0.45µm porosity)

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

This Is Time Consuming

Often Performed Offline

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

Must Reverse to Re-Equilibrate

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

Column Cleaning - Normal Phase

Use at least 50mL or 20–30 column volume changes for analytical columns
- Typical normal phase solvent choices in order of increasing strength:

Solvent	Composition
Methanol:Chloroform	50:50%
Ethyl Acetate	100%

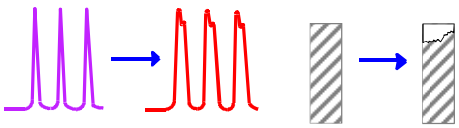
What Are Common Peak Shape Issues?

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**



Normal **Double Peaks**

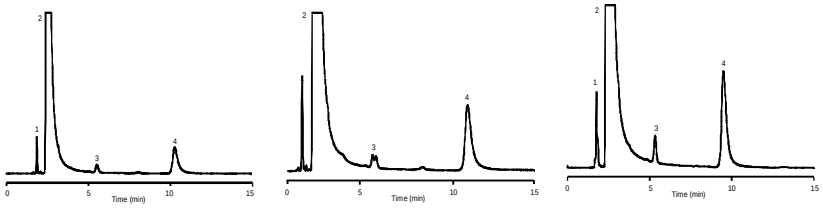
Split or Double Peaks

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 µm Mobile Phase: 60% 25 mM Na_2HPO_4, pH 3.0 : 40% MeOH Flow Rate: 1.0 mL/min
Temperature: 35°C Detection: UV 254 nm Sample: Filtered OTC Cold Medication: 1. Pseudoephedrine 2. APAP 3. Unknown 4. Chlorpheniramine

Injection 1 **Injection 30** **Injection 1 After Column Wash with 100% ACN**

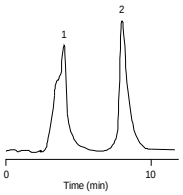


Tip: Column washing eliminates the peak splitting, which resulted from a contaminant on the column
How could this be prevented? (Guard Column, SPE clean up of samples, Periodic column wash)

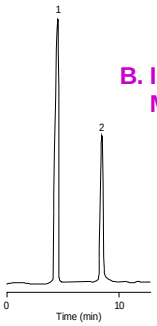
Split Peaks from Injection Solvent Effects

Column: StableBond SB-C8, 4.6 x 150 mm, 5 µm Mobile Phase: 82% H_2O : 18% ACN
Injection Volume: 30 µL Sample: 1. Caffeine 2. Salicylamide

A. Injection Solvent 100% Acetonitrile



B. Injection Solvent Mobile Phase



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 ≤ Mobile Phase

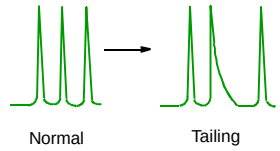
Peak Tailing, Broadening and Loss of Efficiency

May be caused by:

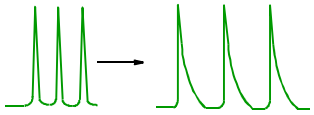
- Column "secondary interactions"
- Column contamination
- Column aging
- Column loading
- Extra-column effects

Peak Shape: Tailing Peaks

Symmetry > 1.2



Normal Tailing



Normal Tailing

Causes

Some Peaks Tail:

- Secondary - Retention Effects.
- Residual Silanol Interactions.
- Small Peak Eluting on Tail of Larger Peak.

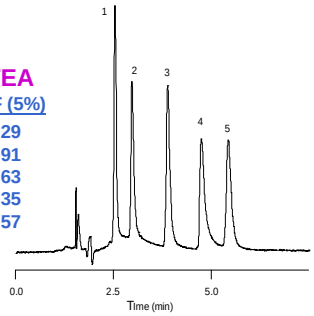
All Peaks Tail:

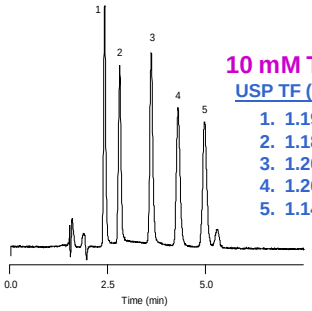
- Extra-Column Effects.
- Build up of Contamination on Column Inlet.
- Heavy Metals.
- Bad Column.

Peak Tailing
Identifying Column "Secondary Interactions"

Column: Alkyl-C8, 4.6 x 150 mm, 5µm Mobile Phase: 85% 25 mM Na_2HPO_4 pH 7.0 : 15% ACN Flow Rate: 1.0 mL/min
Temperature: 35°C Sample: 1. Phenylpropa nolamine 2. Ephedrine 3. Amphetamine 4. Methamphetamine 5. Phenteramine

No TEA
USP TF (5%)
1. 1.29
2. 1.91
3. 1.63
4. 2.35
5. 1.57





10 mM TEA
USP TF (5%)
1. 1.19
2. 1.18
3. 1.20
4. 1.26
5. 1.14

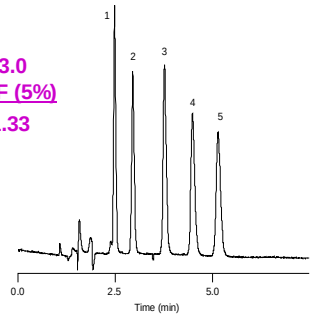
Tip: Mobile phase modifier (TEA) competes with Sample for surface ion exchange sites at mid-range pH values

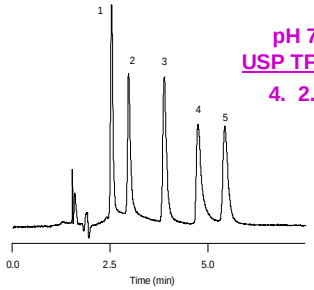
Peak Tailing

Low pH Minimizes "Secondary Interactions" for Amines

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

pH 3.0
USP TF (5%)
4. 1.33





pH 7.0
USP TF (5%)
4. 2.35

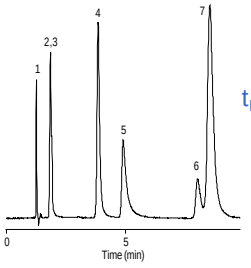
Tip: Reducing mobile phase pH reduces interactions with silanols and peak tailing.

Peak Tailing

High pH Eliminates "Secondary Interactions" for Amines

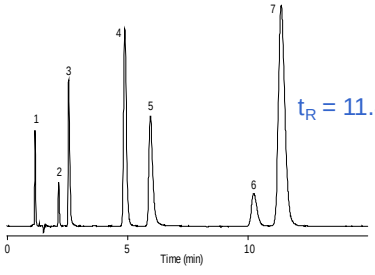
Column: ZORBAX Extend-C18, 4.6 x 150 mm, 5 m m Mobile Phase: See Below Flow Rate: 1.0 mL/min Temperature: RT
Detection: UV 254 nm
Sample 1. Maleate 2. Scopolamine 3. Pseudoephedrine 4. Doxylamine 5. Chlorpheniramine 6. Triprolidine 7. Diphenhydramine

pH 7
30% 20 mM Na_2HPO_4
70% MeOH



t_R = 8.5

pH 11
30% 20 mM TEA
70% MeOH



t_R = 11.4

Peak Shape and Retention of this sample of basic compounds improves at high pH where column has high IEX activity. *Why?*

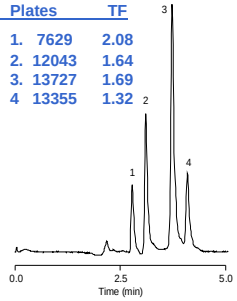
Peak Tailing - Column Contamination

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

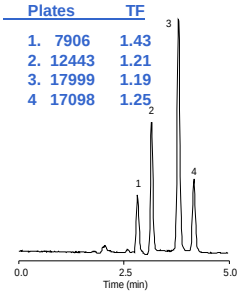
QC test forward direction

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



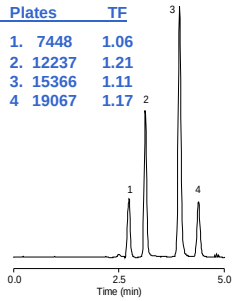
QC test reverse direction

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



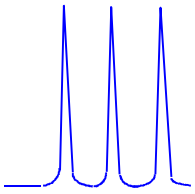
QC test after cleaning 100% IPA, 35℃

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

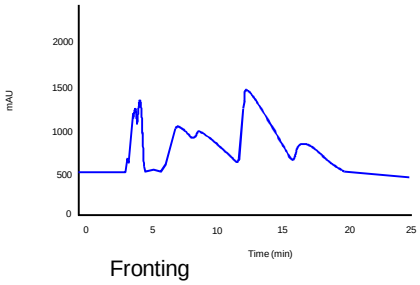


Column: StableBond SB-C8, 4.6 x 250 mm, 5µm Mobile Phase: 20% H_2O : 80% MeOH Flow Rate: 1.0 mL/min
Temperature: R.T. Detection: UV 254 nm Sample: 1. Uracil 2. Phenol 3. 4-Chloronitrobenzene 4. Toluene

Peak Shape: Fronting Peaks



Normal



Fronting

Symmetry < 0.9

Causes:

- Column Overload

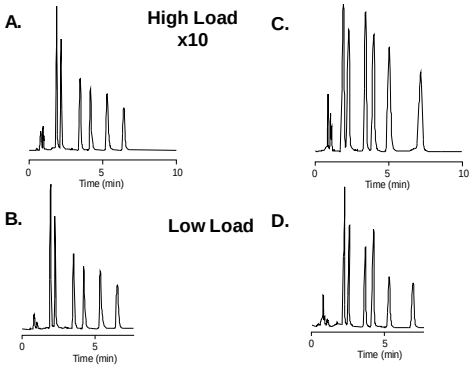
Peak Tailing/Broadening
Sample Load Effects

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

Tailing

Eclipse XDB-C8
USP TF (5%) i

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



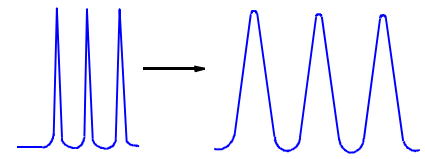
Broadening

Competitive C8
Plates

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

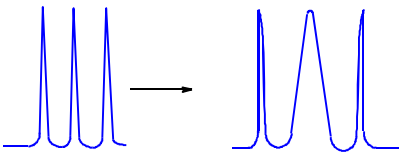
Tip: Evaluate Both Volume and Mass Loading

Peak Shape: Broad Peaks



All Peaks Broadened:

- Loss of Column Efficiency.
- Column Void.
- Large Injection Volume.

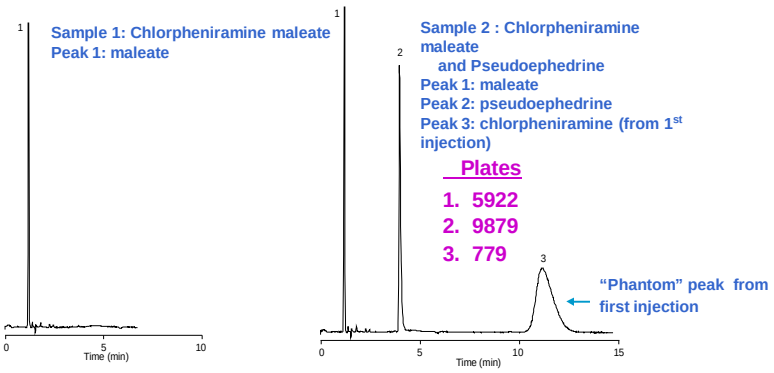


Some Peaks Broadened:

- Late Elution from Previous Sample (Ghost Peak).
  - High Molecular Weight.
  - Sample - Protein or Polymer.

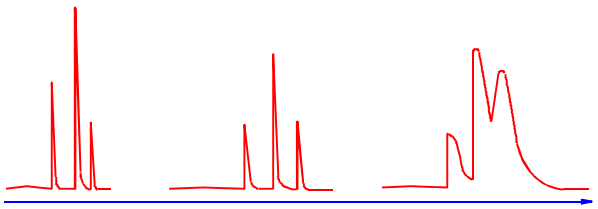
Unknown “Phantom” Peaks

Column: Extend-C18, 4.6 x 150 mm, 5 µm 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.

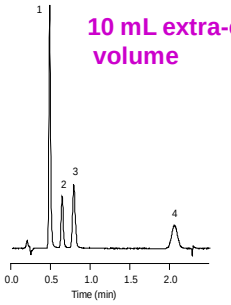
Extra-Column Dispersion



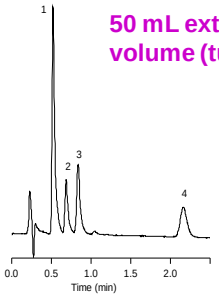
- Use short, small internal diameter tubing between the injector and the column and between the column and the detector.
- Make certain all tubing connections are made with matched fittings.
- Use a low-volume detector cell.
- Inject small sample volumes.

Peak Broadening
Extra-Column Volume

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



10 mL extra-column volume



50 mL extra-column volume (tubing)

Tip: Poorly Made HPLC System Connections Can Cause Peak Broadening

The System Has Been Optimized and :

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

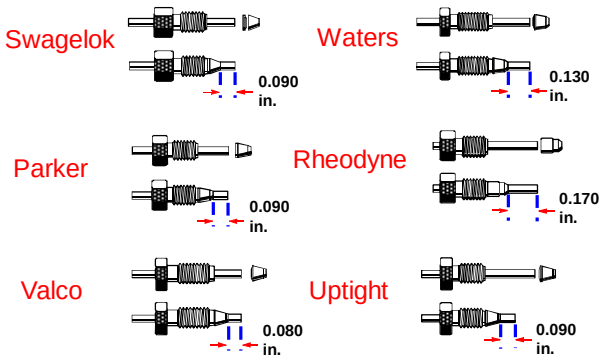
Symptom Still Seems to Have Too Much Extra-Column Volume

What Is Wrong?

Have You Made the Connections Properly?

Column Connectors Used in HPLC

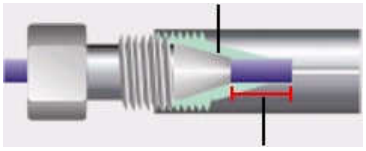
Troubleshooting LC Fittings, Part II. J. W. Dolan and P. Upchurch. LC/GC Magazine 6:788 (1988)



What Happens If the Connections Poorly Made ?

Wrong … too long

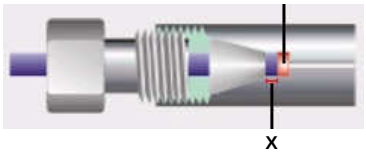
Ferrule cannot seat properly



If Dimension X is too long, leaks will occur

Wrong … too short

Mixing Chamber



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

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

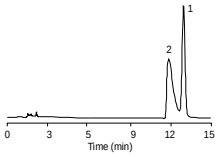
Changes in Retention Can Be Chemical or Physical

May be caused by:

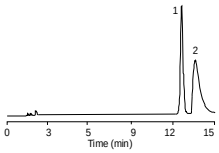
- Column aging
- Column contamination
- Insufficient equilibration
- Poor column/mobile phase combination
- Change in mobile phase
- Change in flow rate
- Different Gradient Delay Volumes

Column Aging/Equilibration Causes Retention/Selectivity Changes

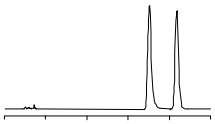
Column 1 - Initial



Column 1 - Next Day



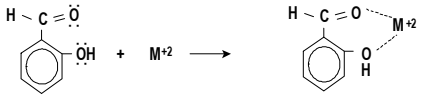
Column 1 - After Cleaning with 1% H_3PO_4 /Equilibration



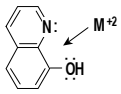
- The primary analyte was sensitive to mobile phase aging/ conditioning of the column
- The peak shape was a secondary issue (metal chelating compound) resolved by "de-activating" the active metal contamination

Metal Sensitive Compounds Can Chelate

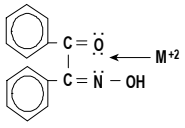
Hint: Look for Lone Pair of Electrons on :O: or N Which Can Form 5 or 6 Membered Ring with Metal



Salicylaldehyde **6-membered ring complex**



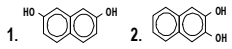
8-hydroxyquinoline
5-membered ring complex



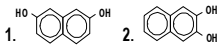
a-**benzoinoxomine**
5-membered ring complex

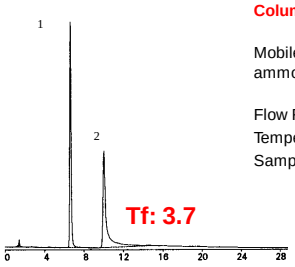
Acid Wash Can Improve Peak Shape

Before Acid Wash

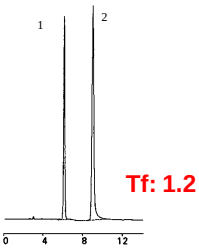


After Acid Wash
50 – 100 mLs 1% H_3PO_4





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



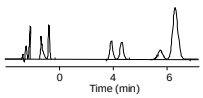
• A 1% H_3PO_4 solution is used on SB columns, 0.5 % can be used on endcapped columns.

Example: Change in Retention/Selectivity

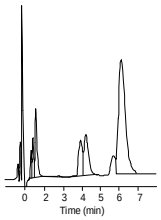
Unintended Mobile Phase Variation

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

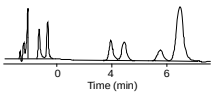
Column 1



Column 2

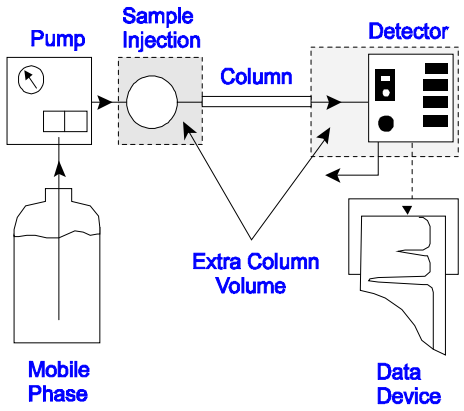


Column 2 - Fresh mobile phase



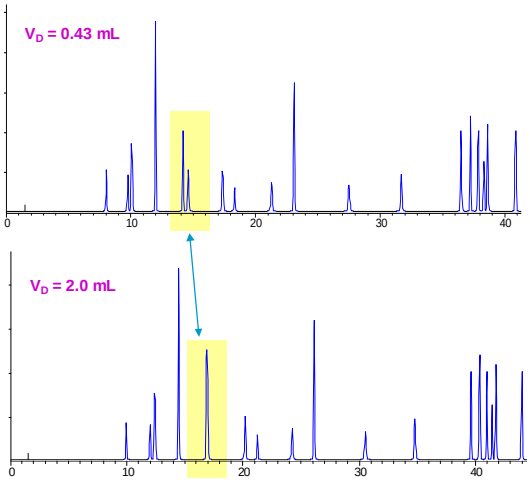
"I have experimented with our mobile phase, opening new bottles of all mobile phase components. When I use all fresh ingredients, the problem ceases to exist, and I have narrowed the problem to either a bad bottle of TEA or phosphoric acid. Our problem has been solved."

Maintain Resolution for Low Volume Peaks by Minimizing Extra-Column Volume



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

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℃
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} - delay = V_{D1}

Mobile Phase pH and pH 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

Minimize Change in Retention/Selectivity
Lot-to-Lot

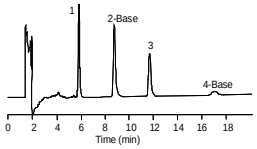
Evaluate:

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

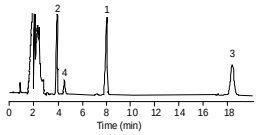
*All causes of column-to-column change should be considered first, especially when only one column from a lot has been tested.

Lot-to-Lot Selectivity Change Related to pH Choice

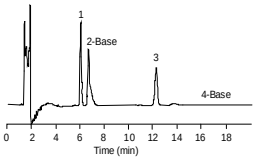
pH 4.5 - Lot 1



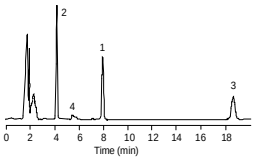
pH 3.0 - Lot 1



pH 4.5 - Lot 2



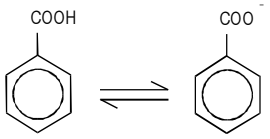
pH 3.0 - Lot 2



- pH 4.5 shows selectivity change from lot-to-lot for basic compounds
- pH 3.0 shows no selectivity change from lot-to-lot
- Indication of poorly controlled ionization

Why Worry About pH?
pH, pKa and Weak Acids

$$RCOOH \rightleftharpoons RCOO^- + H^+ \qquad K_a = \frac{[RCOO^-][H^+]}{[RCOOH]}$$



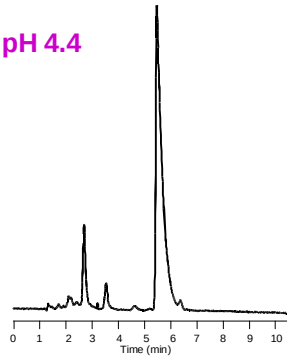
$K_a = 6.4 \times 10^{-5}$
$pK_a = 4.2$

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.

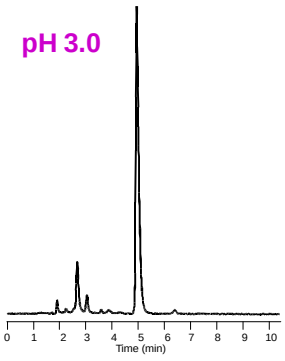
Effect of pH on Peak Shape at or Near the Sample pK_a

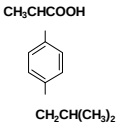
Column: ZORBAX SB-C8 4.6 x 150 mm, 5 mm Mobile Phase: 40% 5 mM KH_2PO_4: 60% ACN
Flow Rate: 1.0 mL/min. Temperature: RT

pH 4.4



pH 3.0





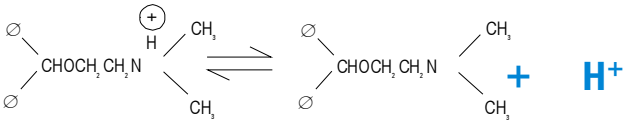
Ibuprofen
$pK_a = 4.4$

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

Why Worry About pH?
pH, pKa and Weak Bases

$$R_3NH^+ \rightleftharpoons R_3N + H^+$$

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



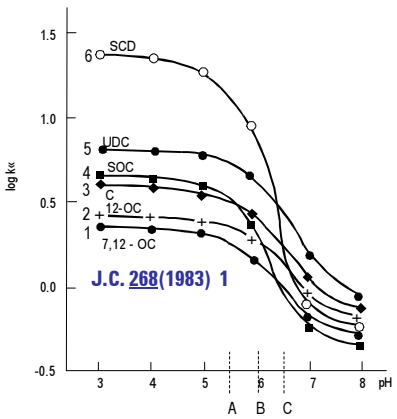
$K_a = 1 \times 10^{-9}$

$pK_a = 9$

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.

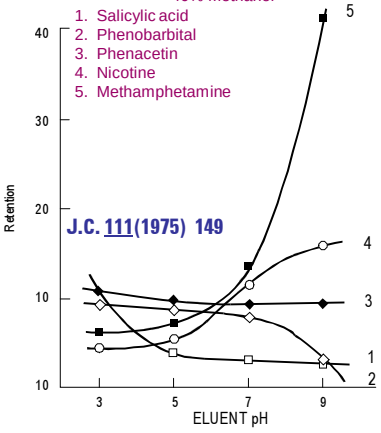
pH vs. Selectivity for Acids and Bases

Column: Nucleosil-C18
Mobile Phase: 45% ACN/55% phosphate buffer
Sample: Bile Acids



Column: mBondapak-C18
Mobile Phase: 60% 25 mM phosphate buffer
40% Methanol

1. Salicylic acid
2. Phenobarbital
3. Phenacetin
4. Nicotine
5. Methamphetamine

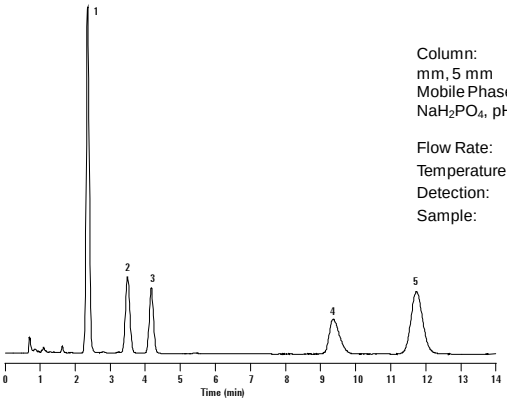


• **Retention and selectivity can change dramatically when pH is changed.**

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

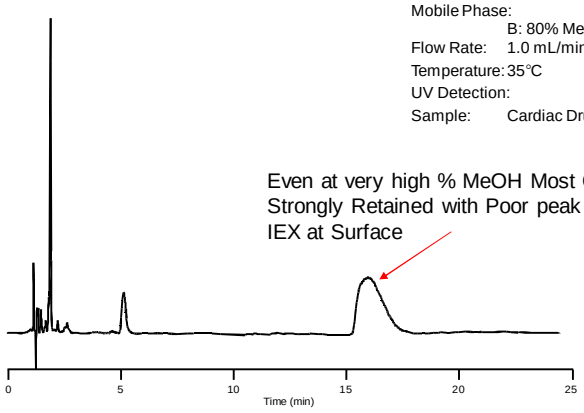


Column: StableBond SB-C18, 4.6 x 150 mm, 5 mm
Mobile Phase: 45% 25 mM NaH_2PO_4, pH 3.0
55% MeOH
Flow Rate: 2.0 mL/min.
Temperature: 35°C
Detection: UV 254 nm
Sample: Cardiac Drugs
1. Diltiazem
2. Dipyridamole
3. Nifedipine
4. Lidoflazine
5. Flunarizine

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

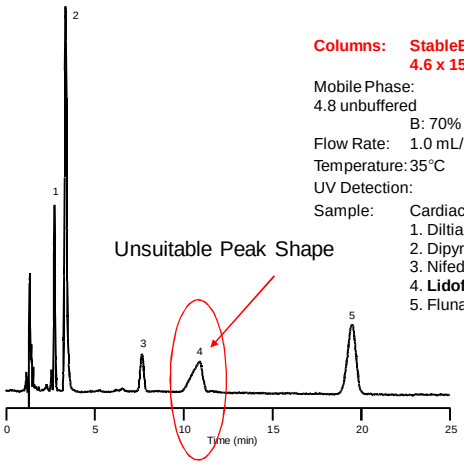
Column: **StableBond SB-C18**
4.6 x 150 mm, 5 mm
Mobile Phase: A: 20% H_2O
B: 80% MeOH
Flow Rate: 1.0 mL/min.
Temperature: 35°C
UV Detection: 254 nm
Sample: Cardiac Drugs

Even at very high % MeOH Most Components Strongly Retained with Poor peak Shape Due to IEX at Surface



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

What If You Work Outside the Buffer Range?



Unsuitable Peak Shape

Columns: **StableBond SB-C18**
4.6 x 150 mm, 5 mm
Mobile Phase: A: 30% 25 mM NaH_2PO_4, 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

Commonly Used Buffers for Reversed Phase HPLC

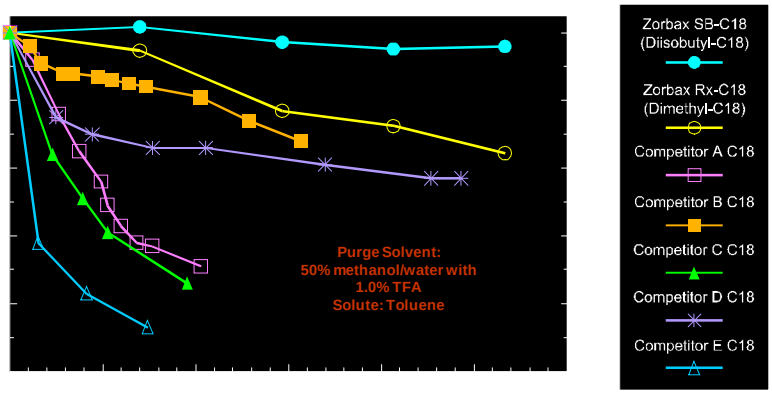
Buffer	pKa	Buffer Range	UV Cutoff (nm)
Phosphate	2.1	1.1-3.1	200
	7.2	6.2-8.2	
	12.3	11.3-13.3	
Formic acid*	3.8	2.8-4.8	210
Acetic acid*	4.8	3.8-5.8	210
Citrate	3.1	2.1-4.1	230
	4.7	3.7-5.7	
	5.4	4.4-6.4	
Tris	8.3	7.3-9.3	205
Triethylamine*	11.0	10.0-12.0	200
Pyrrolidine	11.3	10.3-12.3	200

* Volatile buffers for LC/MS applications

Optimum buffering capacity occurs at a pH equal to the pKa of the buffer. Most buffers provide adequate buffering capacity for controlling mobile phase pH only within ±1 unit of their pKa

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

low pH and high temperature (pH 0.8, 90°C)



Kirkland, J.J. and J.W. Henderson, Journal of Chromatographic Science, 32 (1994) 473-480.

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

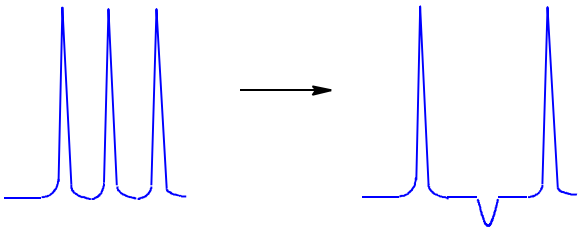
Detection Issues

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

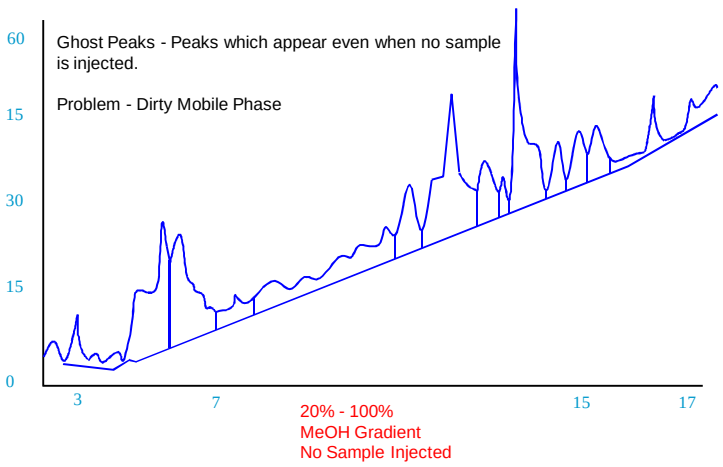
Peak Shape: Negative Peaks



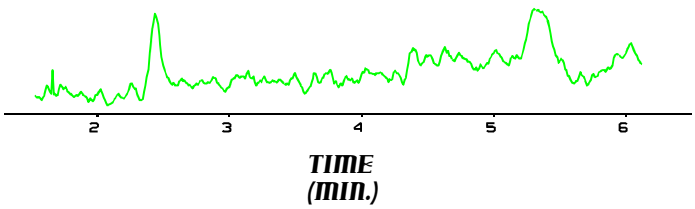
Causes:

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

Ghost Peaks



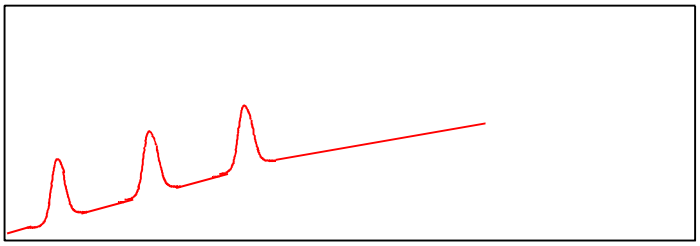
Noisy Baselines



Possible Causes:

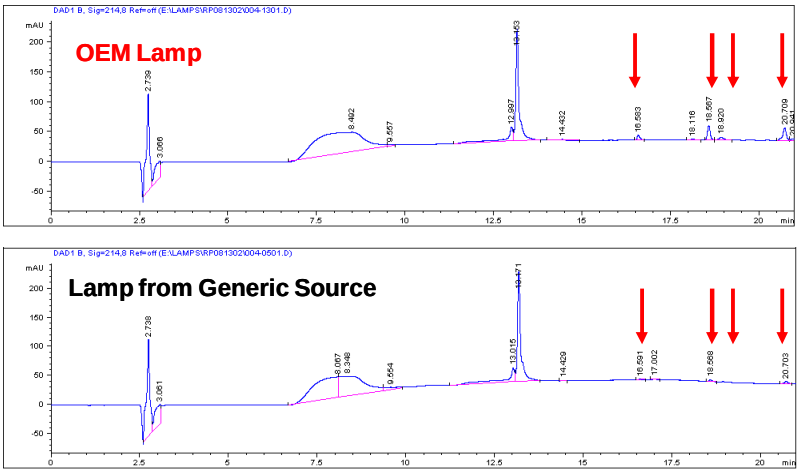
- Dirty Flow Cell
- Detector Lamp Failing
- Pulses from Pump if Periodic
- Temperature Effects on Detector
- Air Bubbles passing through Detector

Drifting Baselines



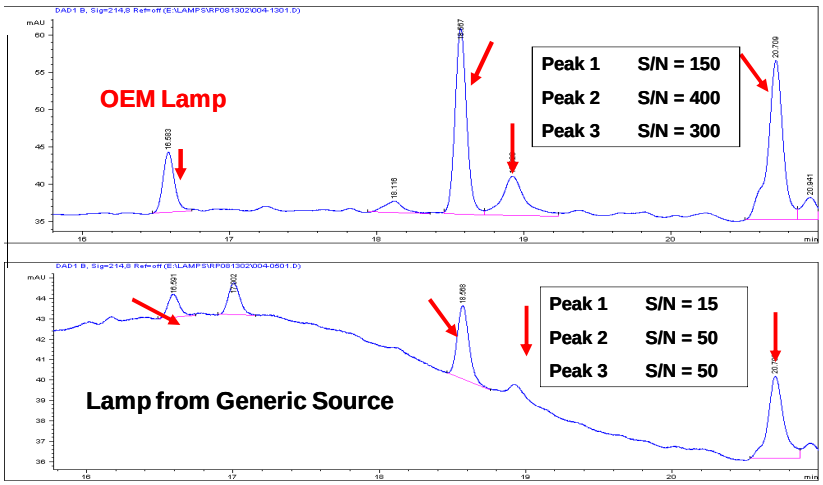
- Gradient Elution
- Temperature Unstable (Refractive Index Detector)
- Contamination in Mobile Phase
- Mobile Phase Not in Equilibrium with Column
- Contamination Bleed in System

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

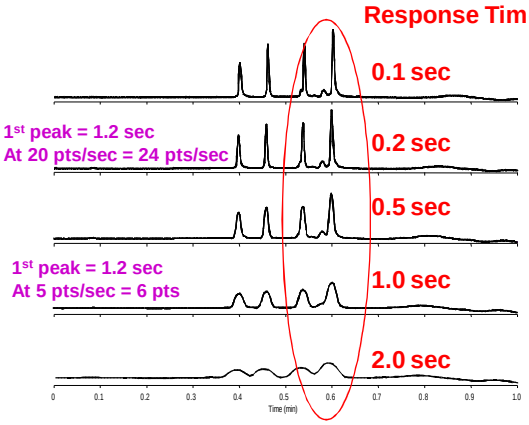


Tip: Poor S/N makes it difficult to detect low level impurities

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% 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

- 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
- The Answers will Lead to Solutions