HPLC Column Troubleshooting:

Is It Really The Column?

Agilent Technologies, Inc.
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January 22, 2010

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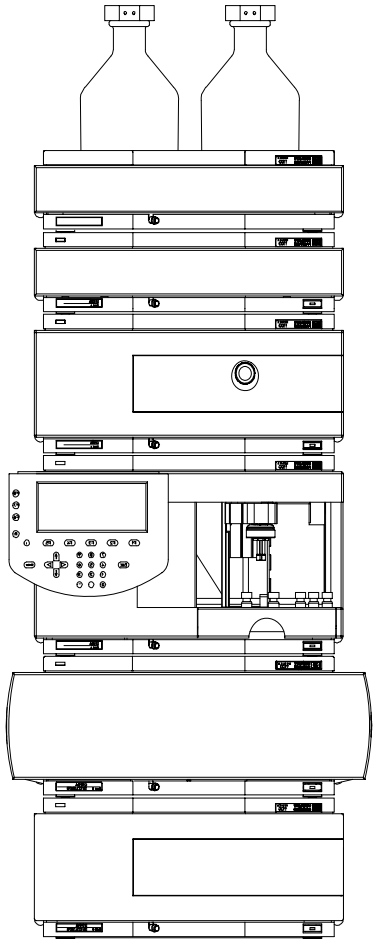
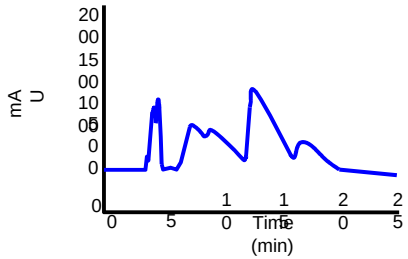
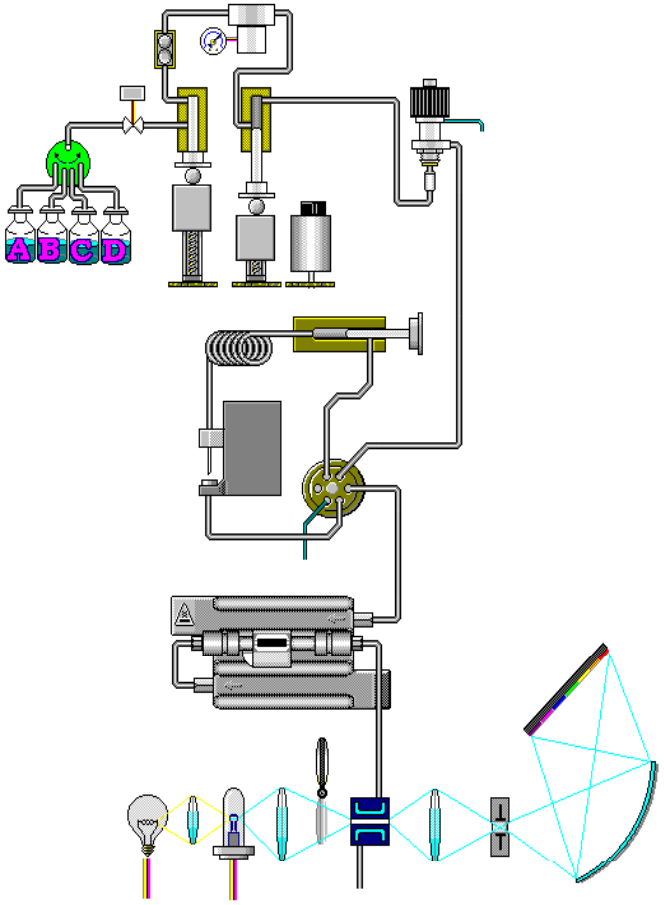
Troubleshooting in HPLC







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HPLC Components

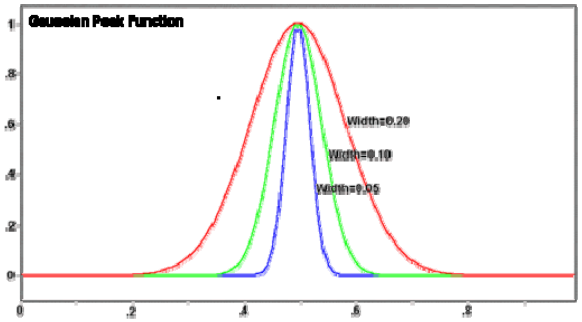
- Pump
- Injector/Autosampler
- Column
- Detector
- Data System/Integrator

All of these components can have problems and require troubleshooting.

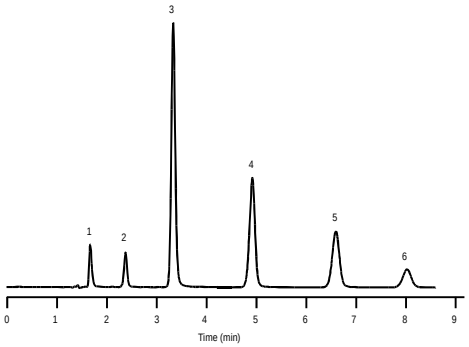
Categories of Column Problems

A. Pressure

B. Peak shape



C. Retention



1. Pressure Issues

Observation	Potential Problems
Large pressure change	Plugged inlet frit Column contamination Plugged packing

Determining the Cause and Correcting High Back Pressure

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

If Column pressure remains high:

- **Rinse column (remove detector from flow path!)**
  - **– Eliminate column contamination and plugged packing**
  - **– high molecular weight/adsorbed compounds**
  - **– precipitate from sample or buffer**
- **Back flush column – may clear plugged column inlet frit**
- **Install New column**

Column Cleaning:

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

Reversed-Phase Solvent Choices in Order of Increasing Strength

Use at least 10 x V_m of each solvent for analytical columns

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

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

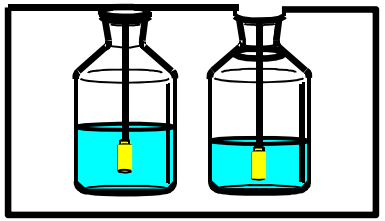
Column Cleaning

Normal Phase Solvent Choices

In Order of Increasing Strength

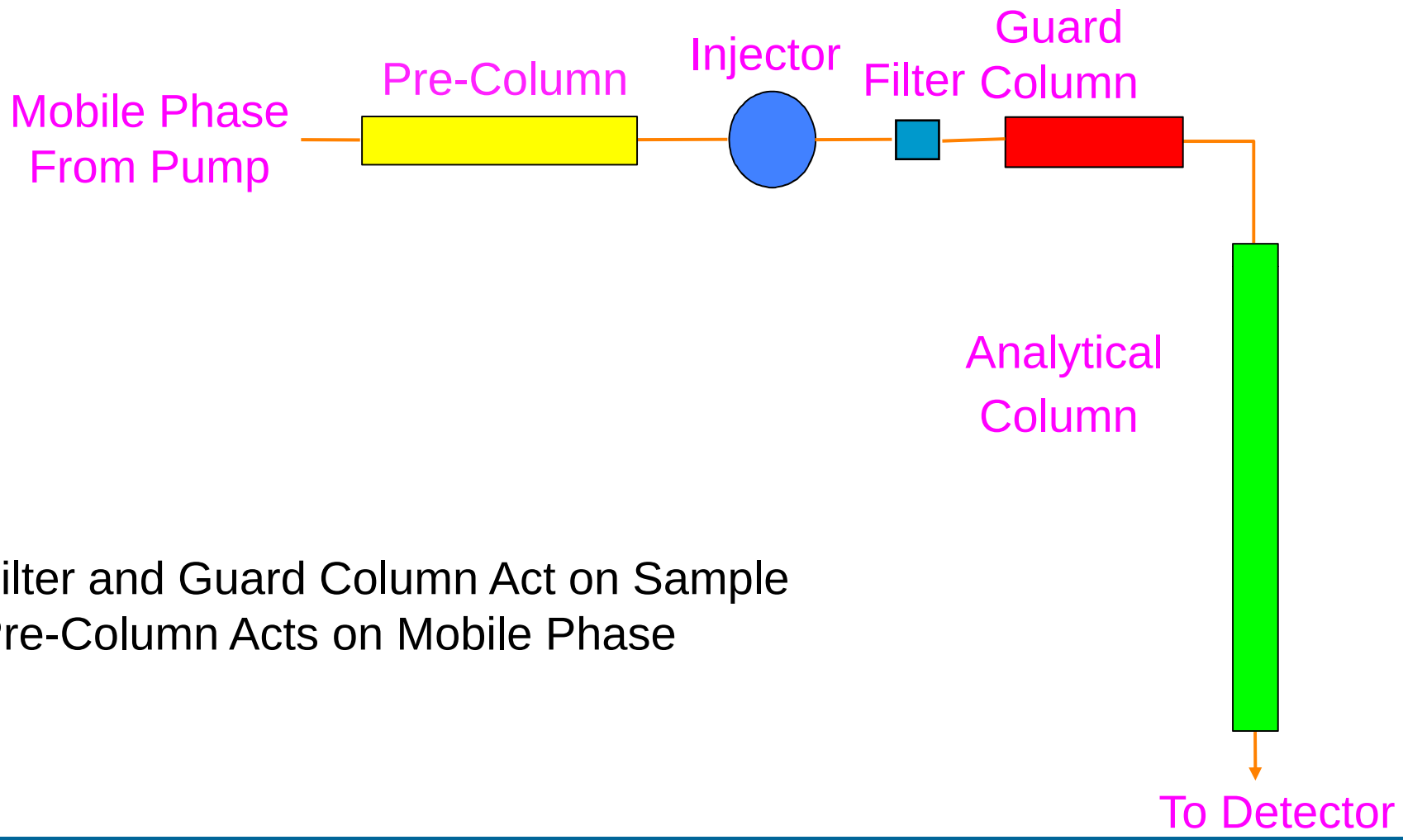
- Use at least 50 mL of each solvent
- 50% Methanol : 50% Chloroform
- 100% Ethyl Acetate

Preventing Column Back Pressure Problems



- Filter mobile phase:
  - Non-HPLC grade solvents
  - Buffer solutions
- Install an in-line filter between auto-sampler and column
  - Use 2 um frit for 3.5 um columns, use 0.5 um frit for 1.8um columns.
- Filter all samples and standards
- Perform sample clean-up (i.e. SPE, LLE) on dirty samples.
- Appropriate column flushing –
  - Flush buffers from entire system at end of day with water/organic mobile phase
- Use Mobile Phase Miscible Sample Solvents

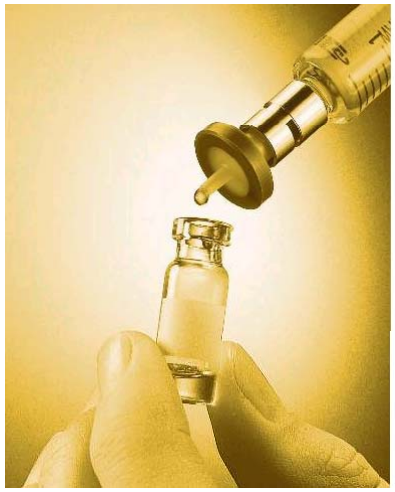
Preventing Back Pressure Problems: In-Line Devices



Filter and Guard Column Act on Sample
Pre-Column Acts on Mobile Phase

Why Filter the Sample?

Extreme Performance Requires Better Sample “Hygiene”



- Prevents blocking of capillaries, frits, and the column inlet
- Results in less wear and tear on the critical moving parts of injection valves
- Results in less downtime of the instrument for repairs
- Produces improved analytical results by removing potentially interfering contamination

Mini-UniPrep Syringeless Filters

New

from Agilent!

Mini-UniPrep Syringeless Filters are preassembled filtration devices for removing particulate matter from samples.

A single disposable unit can replace the combination of syringe filters, syringes, auto-sampler vials, transfer containers, septa and caps.

Mini-UniPrep provides a quick, economical and environmentally conservative way to filter samples prior to HPLC analysis.

Now you can buy them from the same source as your HPLC columns - Agilent!

Manufactured by Whatman, a division of GE Healthcare

Key Reminders

1. As column particle size shrinks, column frit porosity is reduced
   - 5µm - 2µm frit ✧ 3-3.5µm - 0.5µm-2um frit ✧ 1.8µm - 0.2µm frit
2. Mobile phase filtering reduces wear on instrument parts (Check valves, Piston seals, Autosampler)
3. Sample filtering reduces wear on instrument and prevents column plugging due to particulates

A Little Prevention Reduces Downtime and Maintenance Costs

2. Peak Shape Issues in HPLC

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

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

Split Peaks

Can be caused by:

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

Determining the Cause of Split Peaks

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

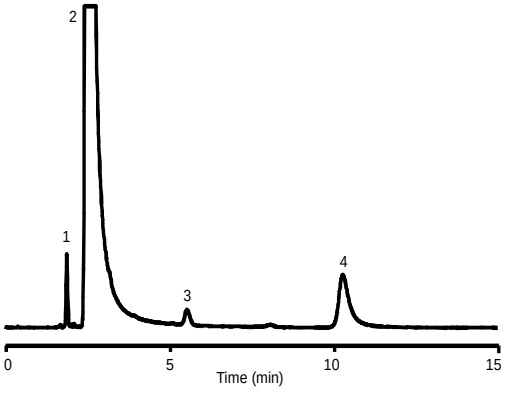
2. **Mobile phase pH > 7 - likely column void due to silica dissolution (unless specialty column used, Zorbax Extend-C18 stable to pH 11)**

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

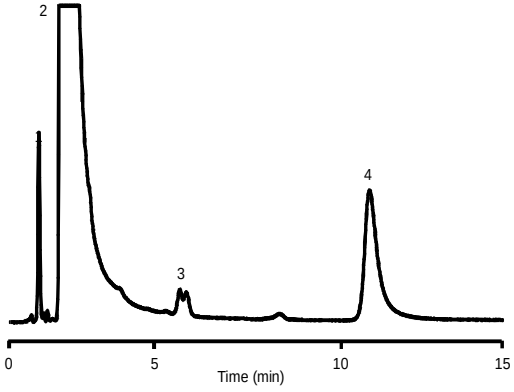
Split Peaks
Column Contamination

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

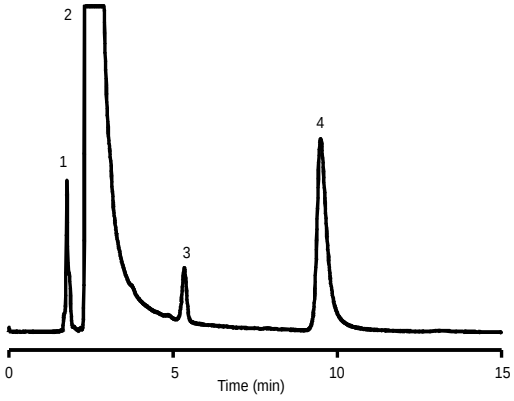
Injection 1



Injection 30



Injection 1 After Column Wash with 100% ACN

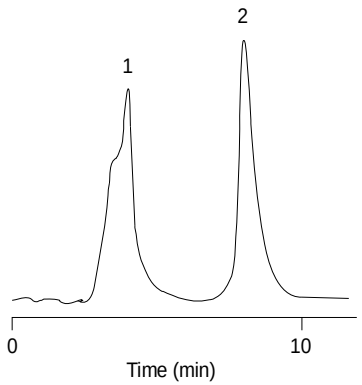


- Column washing eliminates the peak splitting, which resulted from a contaminant on the column.

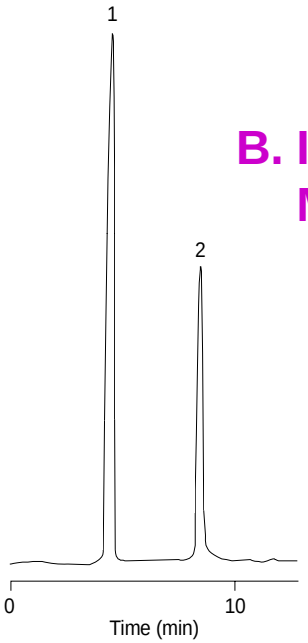
Split Peaks
Injection Solvent Effects

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

A. Injection Solvent 100% Acetonitrile



B. Injection Solvent Mobile Phase



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

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

May be caused by:

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

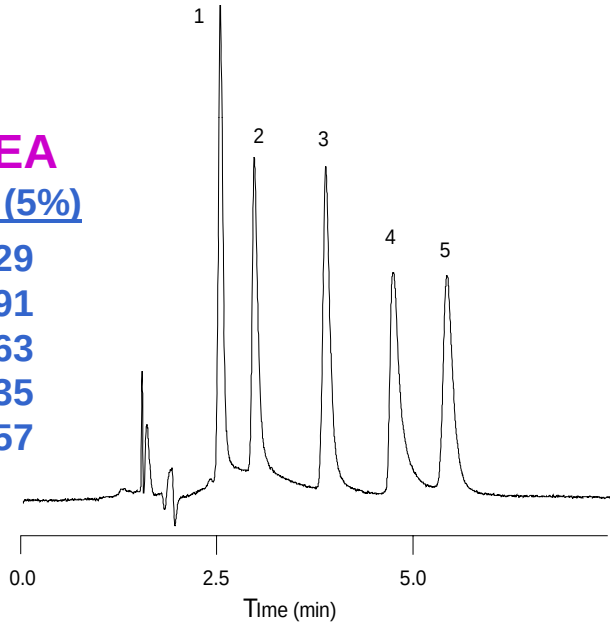
Peak Tailing
Column “Secondary Interactions”

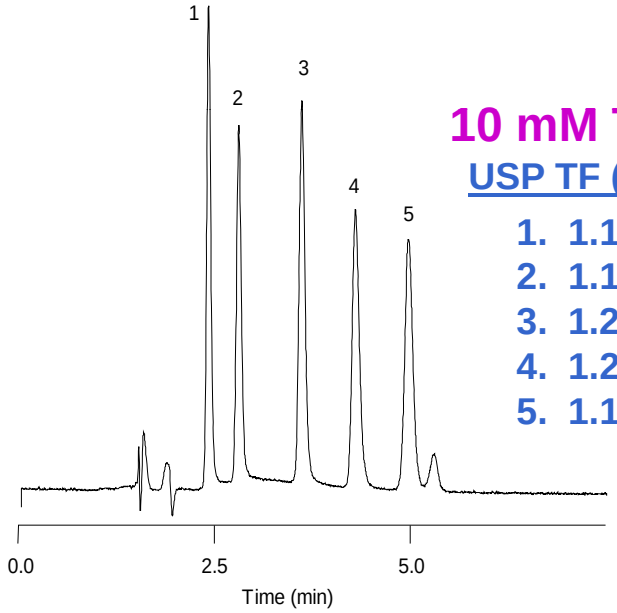
Column: Alkyl-C8, 4.6 x 150 mm, 5µm Mobile Phase: 85% 25 mM Na_2HPO_4 pH 7.0 : 15% ACN
Flow Rate: 1.0 mL/minTemperature: 35°C Sample: 1. Phenylpropanolamine 2. Ephedrine 3. Amphetamine 4. Methamphetamine 5. Phenteramine

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

- Peak tailing of amine analytes eliminated with mobile phase modifier (TEA, triethylamine) at pH 7

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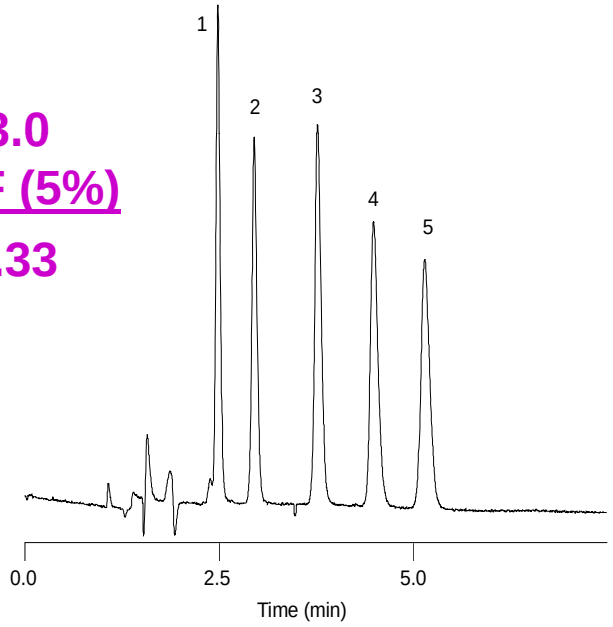
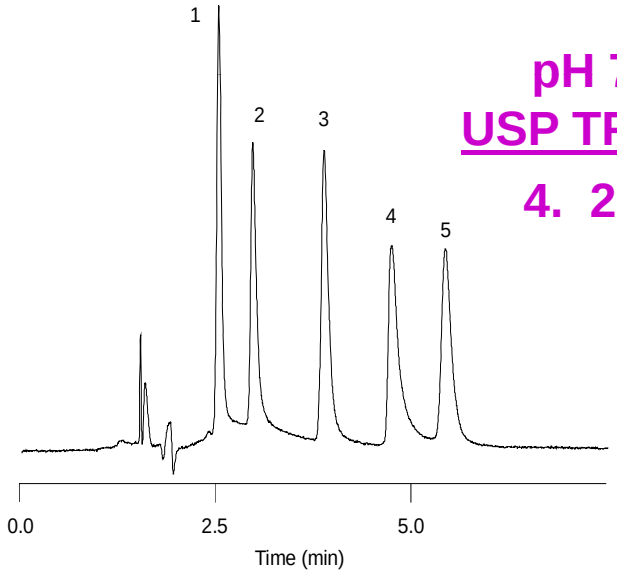
Peak Tailing
Column “Secondary Interactions”

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

- Reducing the mobile phase pH reduces interactions with silanols that cause peak tailing. No TEA modifier required.

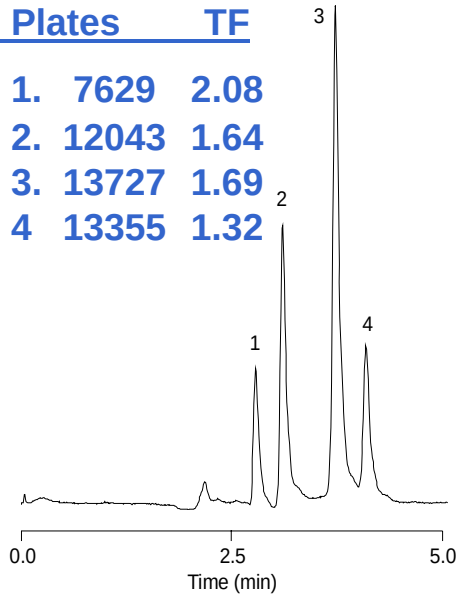
Peak Tailing

Column Contamination

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

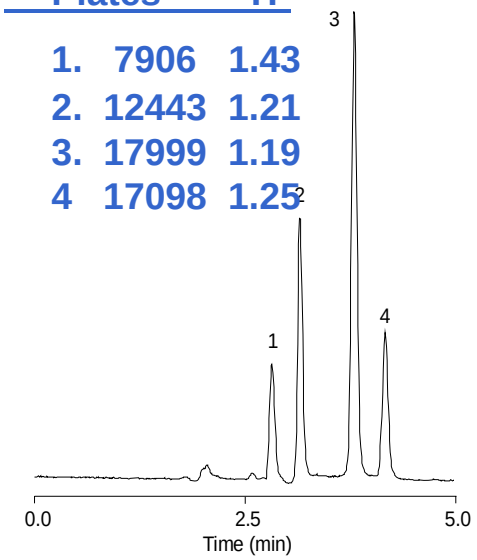
QC test forward direction

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



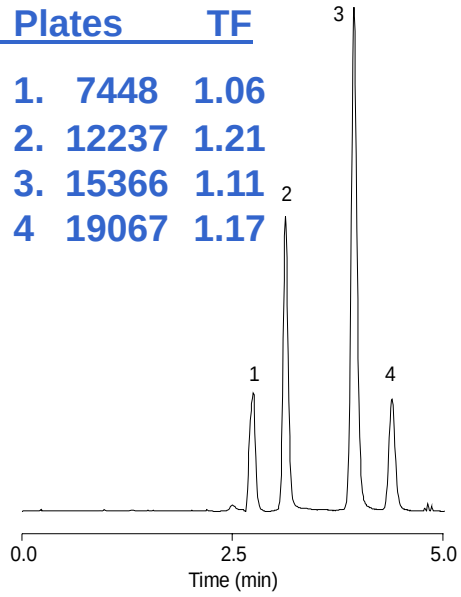
QC test reverse direction

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



QC test after cleaning 100% IPA, 35°C

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



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Peak Tailing/Broadening
Sample Load Effects

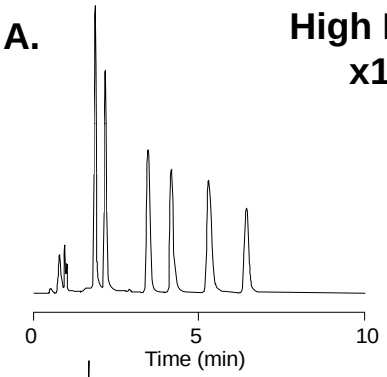
Columns: 4.6 x 150 mm, 5µm Mobile Phase: 40% 25 mM Na_2HPO_4 pH 7.0 : 60% ACN Flow Rate: 1.5 mL/min
Temperature: 40°C Sample: 1. Desipramine 2. Nortriptyline 3. Doxepin 4. Imipramine 5. Amitriptyline 6. Trimipramine

Tailing

Eclipse XDB-C8
USP TF (5%)

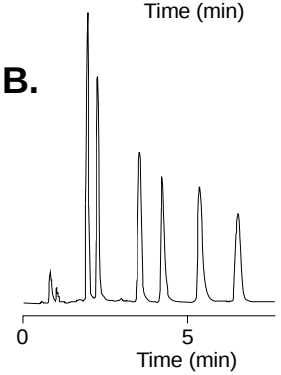
	A	B
1.	1.60	1.70
2.	2.00	1.90
3.	1.56	1.56
4.	2.13	1.70
5.	2.15	1.86
6.	1.25	1.25

A.



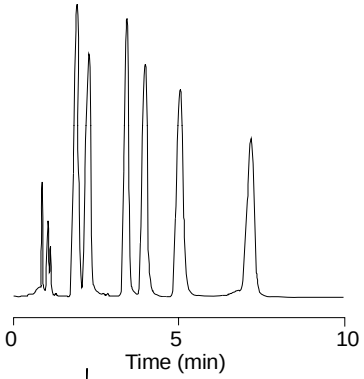
High Load x10

B.

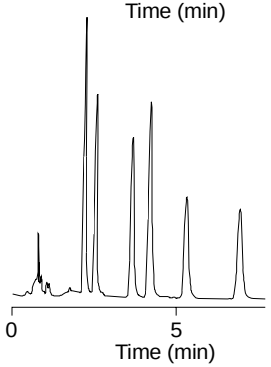


Low Load

C.



D.



Broadening

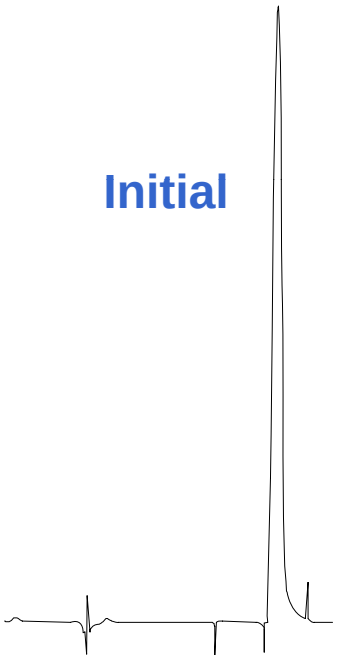
Competitive C8
Plates

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

Peak Broadening, Splitting
Column Void

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

Initial

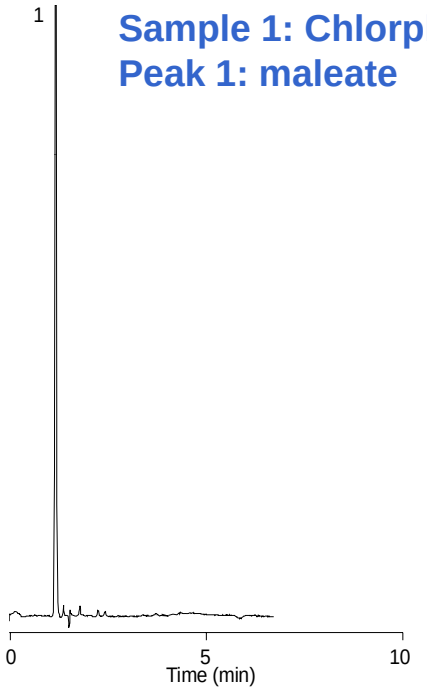


After 30 injections

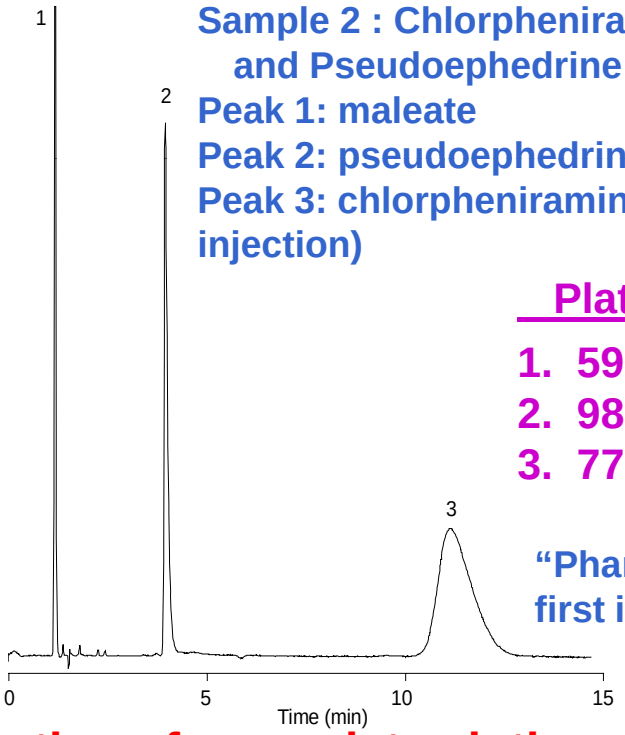
- Multiple peak shape changes can be caused by the same column problem. In this case a void resulted from silica dissolved at high pH.

Broad Peaks
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



Sample 1: Chlorpheniramine maleate
Peak 1: maleate



Sample 2 : Chlorpheniramine maleate and Pseudoephedrine
Peak 1: maleate
Peak 2: pseudoephedrine
Peak 3: chlorpheniramine (from 1st injection)

Plates

1. **5922**
2. **9879**
3. **779**

“Phantom” peak from first injection

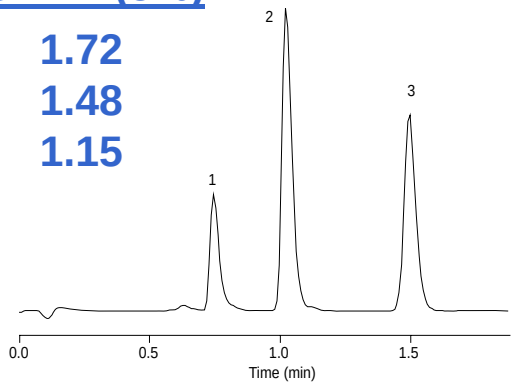
- **The extremely low plates are an indication of a very late eluting peak from the preceding run.**

Peak Tailing
Injector Seal Failure

Column: Bonus-RP, 4.6 x 75 mm, 3.5 µm 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

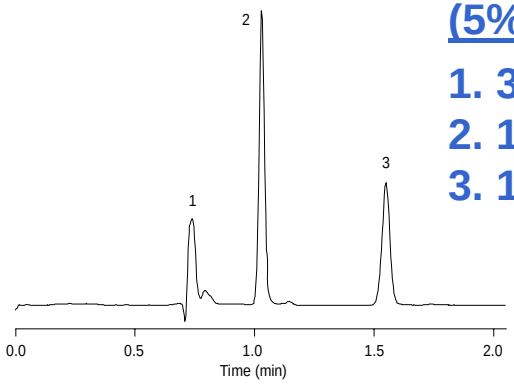
Before

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



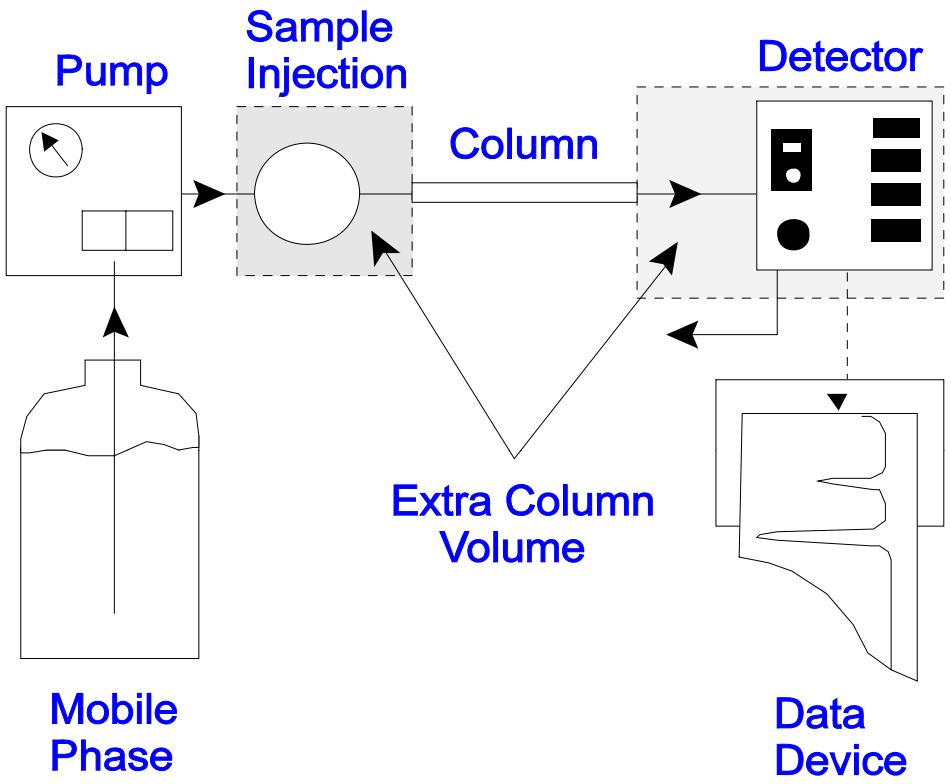
After replacing rotor seal and isolation seal

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



- **Overdue instrument maintenance can sometimes cause peak shape problems.**

Dwell Volume & Extra Column Volume



Dwell Volume = Volume of the Instrument before the column inlet

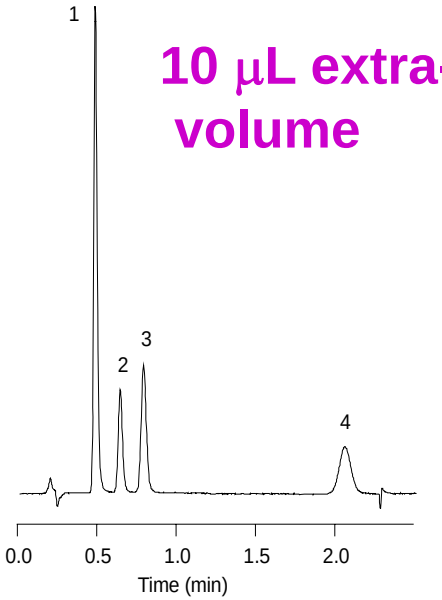
- High Pressure Mixing: V_D = mixing chamber + connecting tubing + injector
- Low Pressure Mixing:V_D = the above + pump heads + associated tubing

✓Behaves as isocratic hold at the beginning of gradient

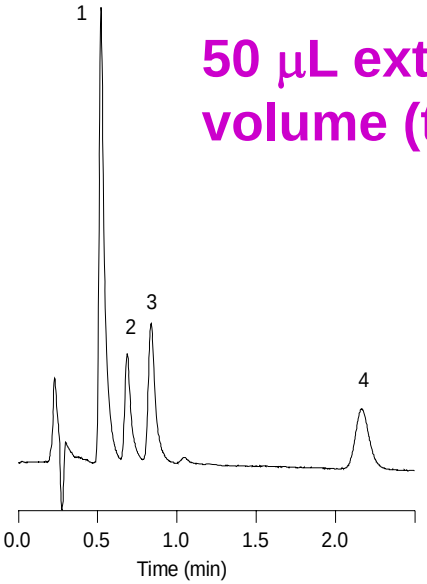
ECV= sample vol. + connecting tubing + fitting + detector cell

Peak Tailing
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. Phenylalanine 2. 5-benzyl-3,6-dioxo-2-piperazine acetic acid 3. Asp-phe 4. Aspartame



10 µL extra-column volume



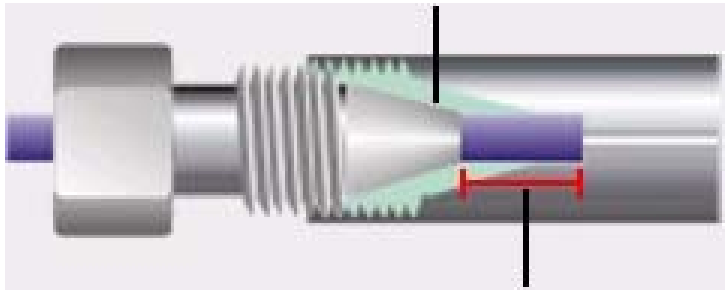
50 µL extra-column volume (tubing)

Peak tailing/fronting

What Happens If the Connections Poorly Made ?

Wrong … too long

Ferrule cannot seat properly

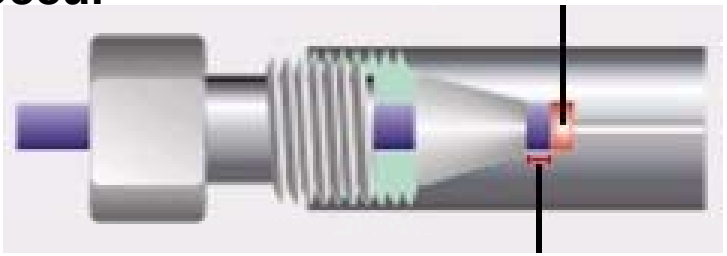


X

If Dimension X is too long, leaks will occur

Wrong … too short

Mixing Chamber



X

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

Determining the Cause of Peak Tailing

- **Evaluate mobile phase effects - alter mobile phase pH and additives to eliminate secondary interactions**
- **Evaluate column choice - try column with high purity silica or different bonding technology**
- **Reduce sample load – vol inj and concentration**
- **Eliminate extra-column effects**

  – tubing, fittings, UV cell
- **Flush column and check for aging/void**

3. Retention Issues

- **Retention time changes (t_r)**
- **Retention factor changes (k')**
- **Selectivity changes (α)**

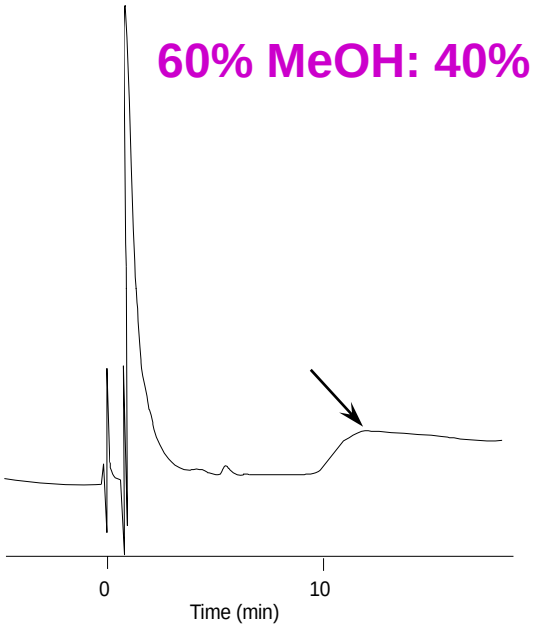
Changes in Retention (k)
Same Column, Over Time

May be caused by:

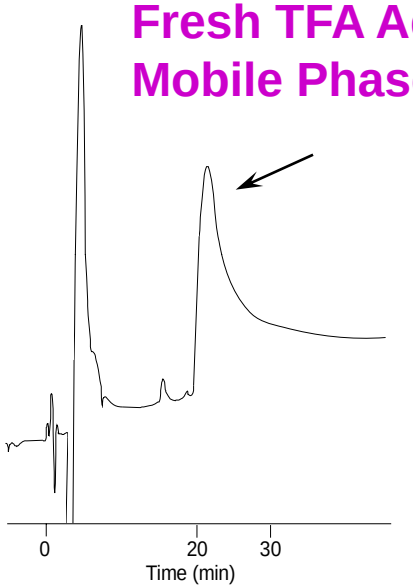
1. Column aging
2. Column contamination
3. Insufficient column equilibration
4. Poor column/mobile phase combination
5. Change in mobile phase
6. Change in flow rate
7. Change in column temperature
8. Other instrument issues

Mobile Phase Change Causes Change in Retention

60% MeOH: 40% 0.1%TFA



Fresh TFA Added to Mobile Phase



- **Volatile TFA evaporated/degassed from mobile phase. Replacing it solved problem.**
- **Chromatography is from a protein binding study and peak shape as expected.**

Separation Conditions That Cause Changes in Retention*

Flow Rate	+/- 1%	+/- 1% t_r
Temp	+/- 1 deg C	+/- 1 to 2% t_r
%Organic	+/- 1%	+/- 5 to 10% t_r
pH	+/- 0.01%	+/- 0 to 1% t_r

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

Determining the Cause of Retention Changes
Same Column

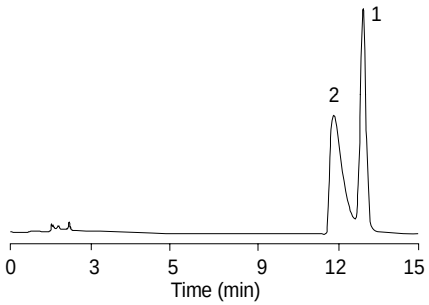
1. Determine k', α, and t_r for suspect peaks
2. Wash column
3. Test new column - note lot number
4. Review column equilibration procedures
5. Make up fresh mobile phase and test
6. Check instrument performance

Change in Retention/Selectivity
Column-to-Column

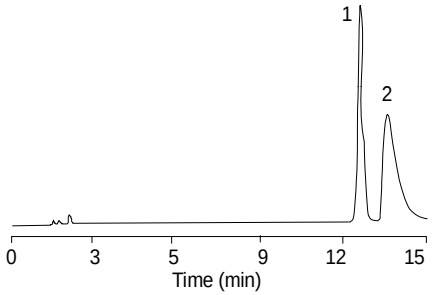
1. Different column histories (aging)
2. Insufficient/inconsistent equilibration
3. Poor column/mobile phase combination
4. Change in mobile phase
5. Change in flow rate
6. Other instrument issues
7. Slight changes in column bed volume (t_r only)

Column Aging/Equilibration Causes Retention/Selectivity Changes

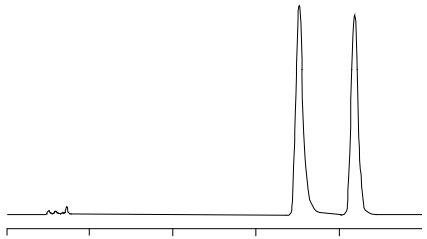
Column 1 - Initial



Column 1 - Next Day



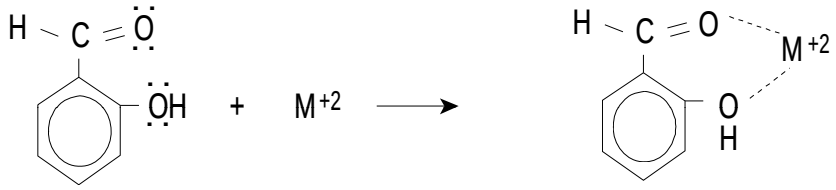
Column 1 - After wash with 1% H_3PO_4 /Equilibration



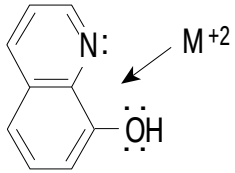
- The primary analyte was sensitive to mobile phase aging/ conditioning of the column
- The peak shape was a secondary issue (metal chelating compound) resolved by “de-activating” the active metal contamination

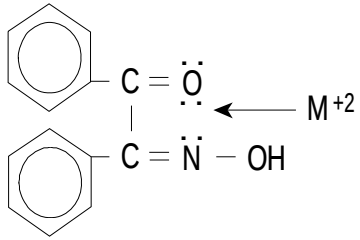
Metal Sensitive Compounds Can Chelate

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**



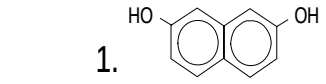


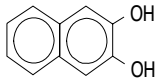
8-hydroxyquinoline
5-membered ring complex

a-benzoinoxomine
5-membered ring complex

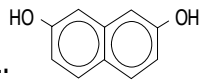
Acid Wash Can Improve Peak Shape

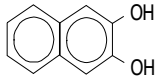
Before Acid Wash

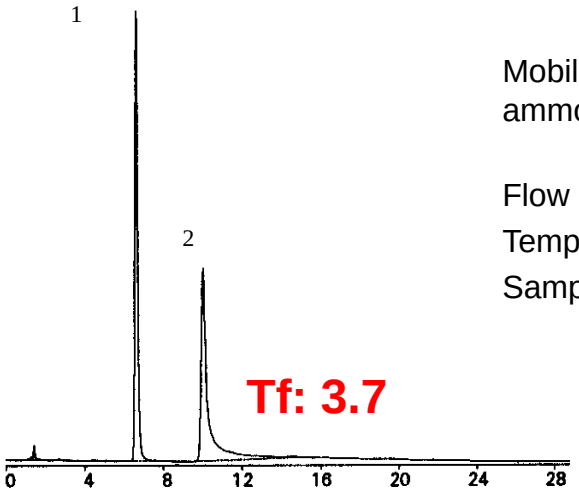
1. 

2. 

After Acid Wash
50 – 100 mLs 1% H_3PO_4

1. 

2. 



Tf: 3.7

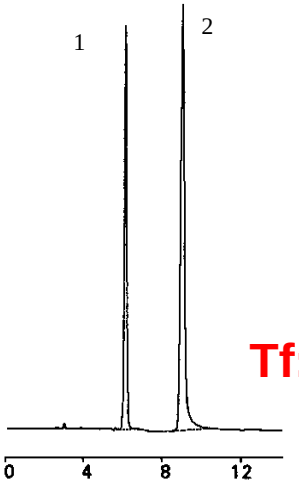
Columns: ZORBAX SB-Phenyl 4.6 x 150 mm

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

Flow Rate: 1.0 mL/min.

Temperature: RT

Sample Size: 5 mL



Tf: 1.2

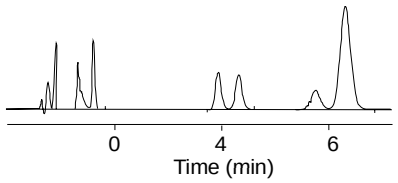
- A 1% H_3PO_4 solution is used on SB columns, 0.5 % can be used on endcapped columns.

Example Change in Retention/Selectivity

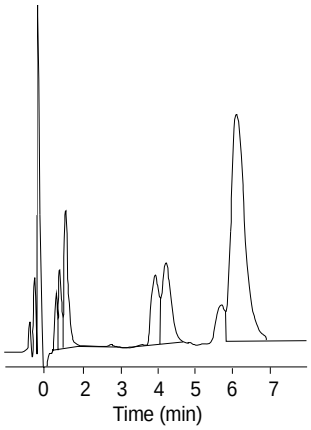
Column-to-Column

Mobile Phase Variation

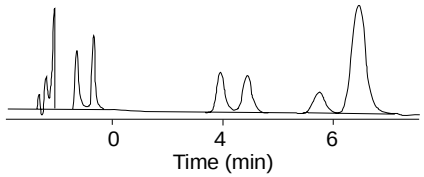
Column 1



Column 2



Column 2 - Fresh mobile phase



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

Determining the Cause of Retention Changes

Column-to-Column

1. Determine k', α, and t_r for suspect peaks
2. Test new column - note lot number
3. Determine column history of all columns
4. Review column equilibration procedures
5. Make up fresh mobile phase and test
6. Check instrument performance

Minimize Change in Retention/Selectivity
Lot-to-Lot

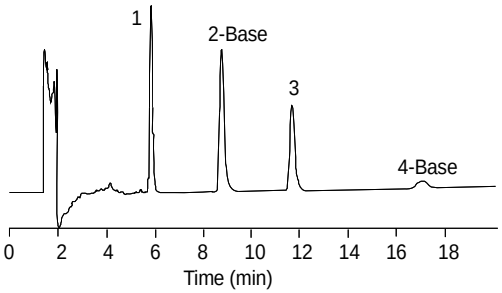
Evaluate:

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

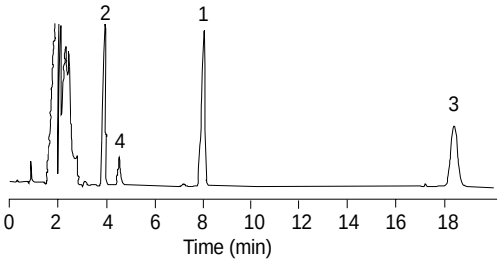
*All causes of column-to-column change should be considered first, especially when only one column from a lot has been tested.

Lot-to-Lot Selectivity Change - pH

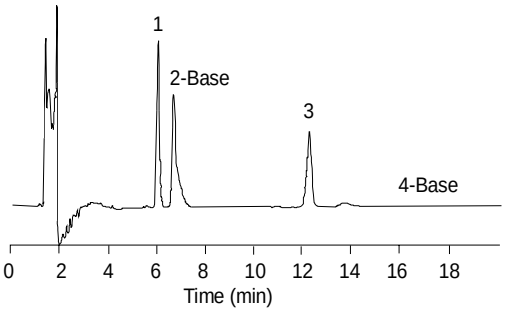
pH 4.5 - Lot 1



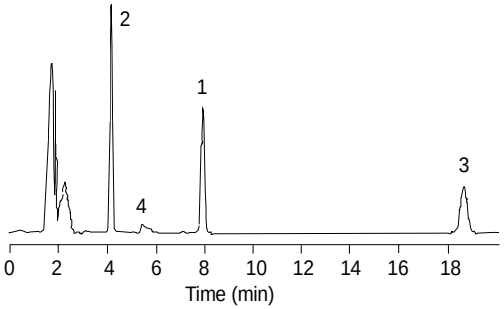
pH 3.0 - Lot 1



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, indicating silanol sensitivity at pH 4.5
- Evaluate several pH levels to establish most robust choice of pH

Evaluate Retention Changes
Lot-to-Lot

1. **Eliminate causes of column-to-column selectivity change**
2. **Re-evaluate method ruggedness - modify method**
3. **Determine pH sensitivity - modify method**
4. **Classify selectivity changes**
5. **Contact manufacturer for assistance***

***Agilent Column Support: 800-227-9770, opt.3, opt. 3, opt. 2(LC columns)**

Conclusions:

HPLC column problems are evident as:

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

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

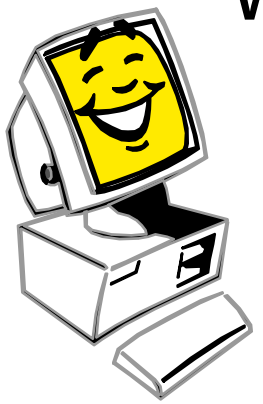
Agilent Technical Support

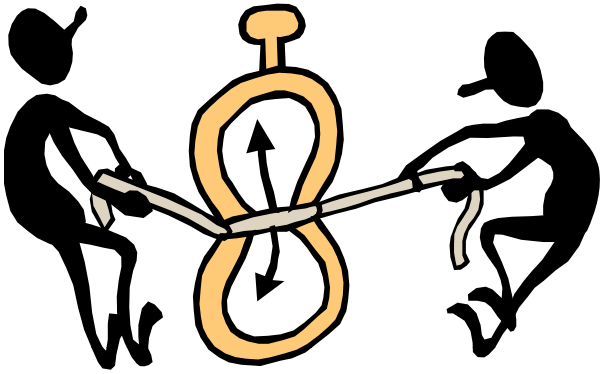
LC or GC Column Support

800-227-9770 (phone: US & Canada)

Select opt. 3, opt. 3, then option 1 for GC or option 2 for LC.

www.agilent.com/chem





The End – Thank You!