

Care, Maintenance, and Troubleshooting of HPLC Columns

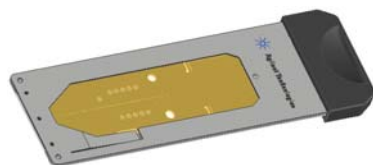
Columns and Consumables

Edward Kim
Applications Engineer
January 17, 2008



Goals for this presentation:

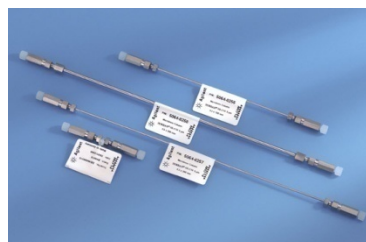
1. Introduce the most commonly observed column related problems in HPLC.
2. Explore the reasons for these column problems.
3. Propose preventative maintenance and method development/optimization approaches to minimize HPLC column problems and increase column lifetimes.



Chip LC



Nano LC



Capillary LC



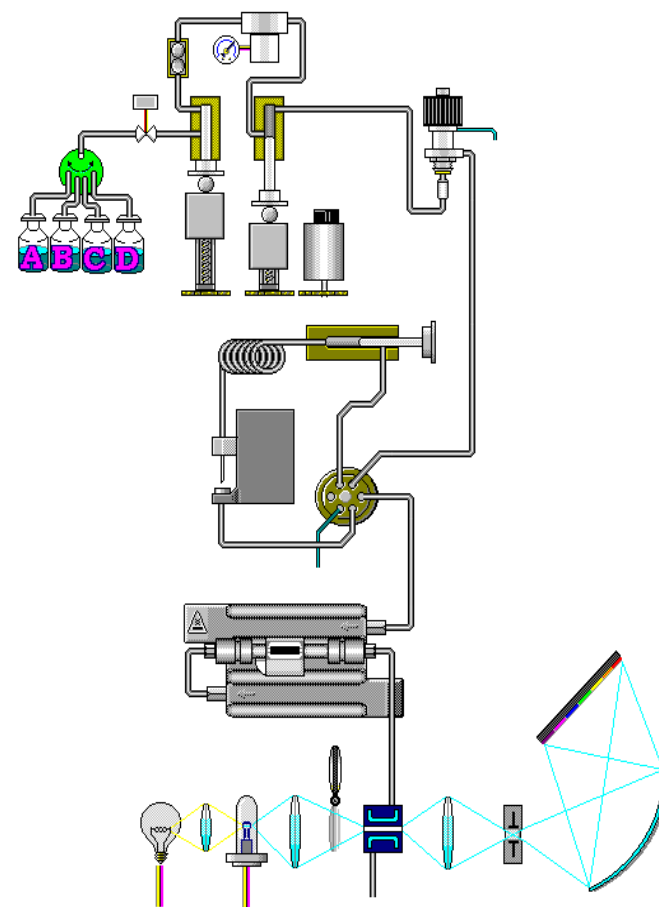
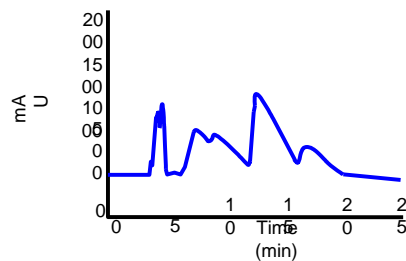
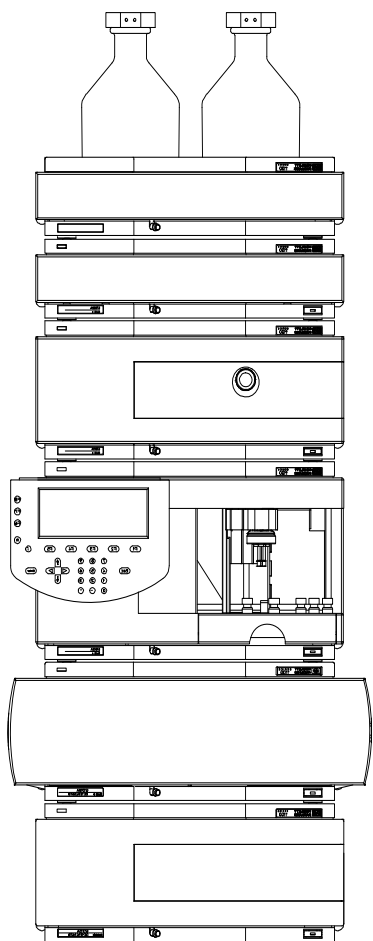
Analytical LC



Prep LC



Troubleshooting in HPLC



Major Areas of Column Problems - Dramatic Changes in 3 Key Areas:

- 1. HPLC System Pressure**
- 2. Chromatogram - Peak Shape**
- 3. Chromatogram - Peak Retention/Selectivity**



1. Pressure Issues

Column Observations

Large pressure change

Potential Problems

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
- Change column inlet frit (... or discard column)

Eliminate pressure issues – add a disposable 0.5 or 2 um in-line filter to system.

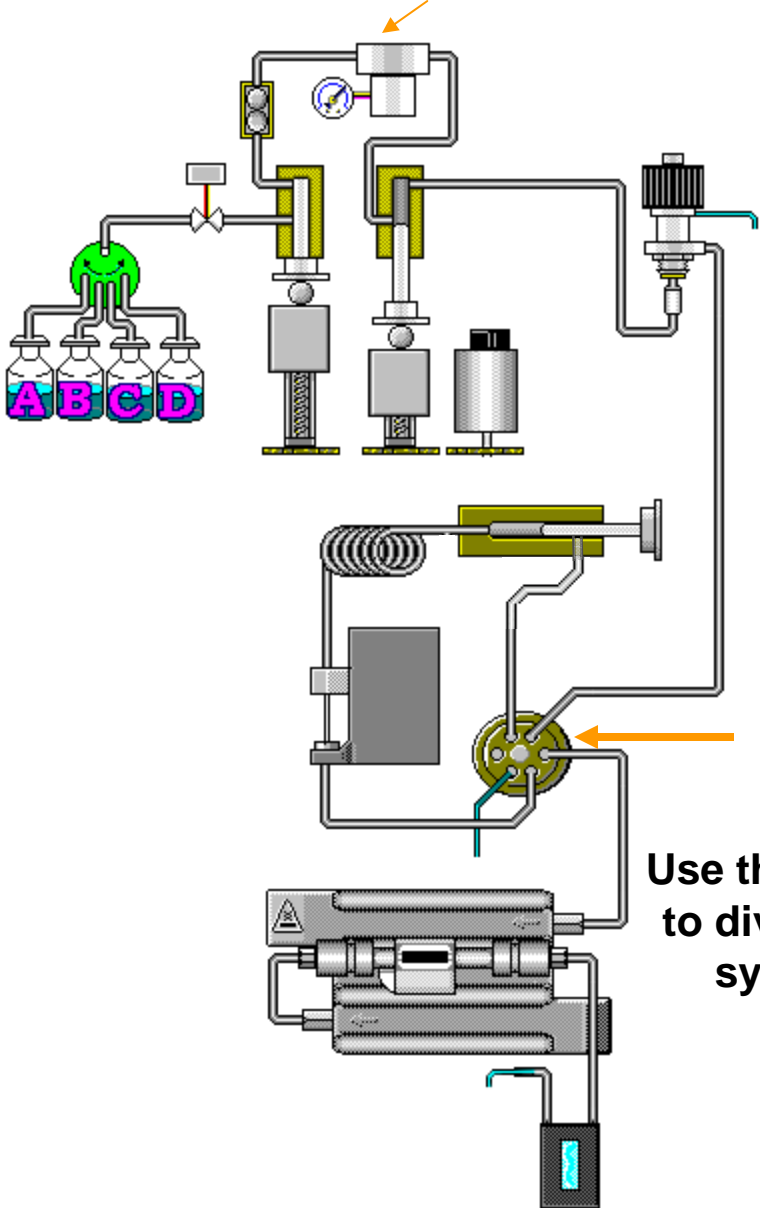


Pressure Problem I

Pressure Too High

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

Pressure Measurement

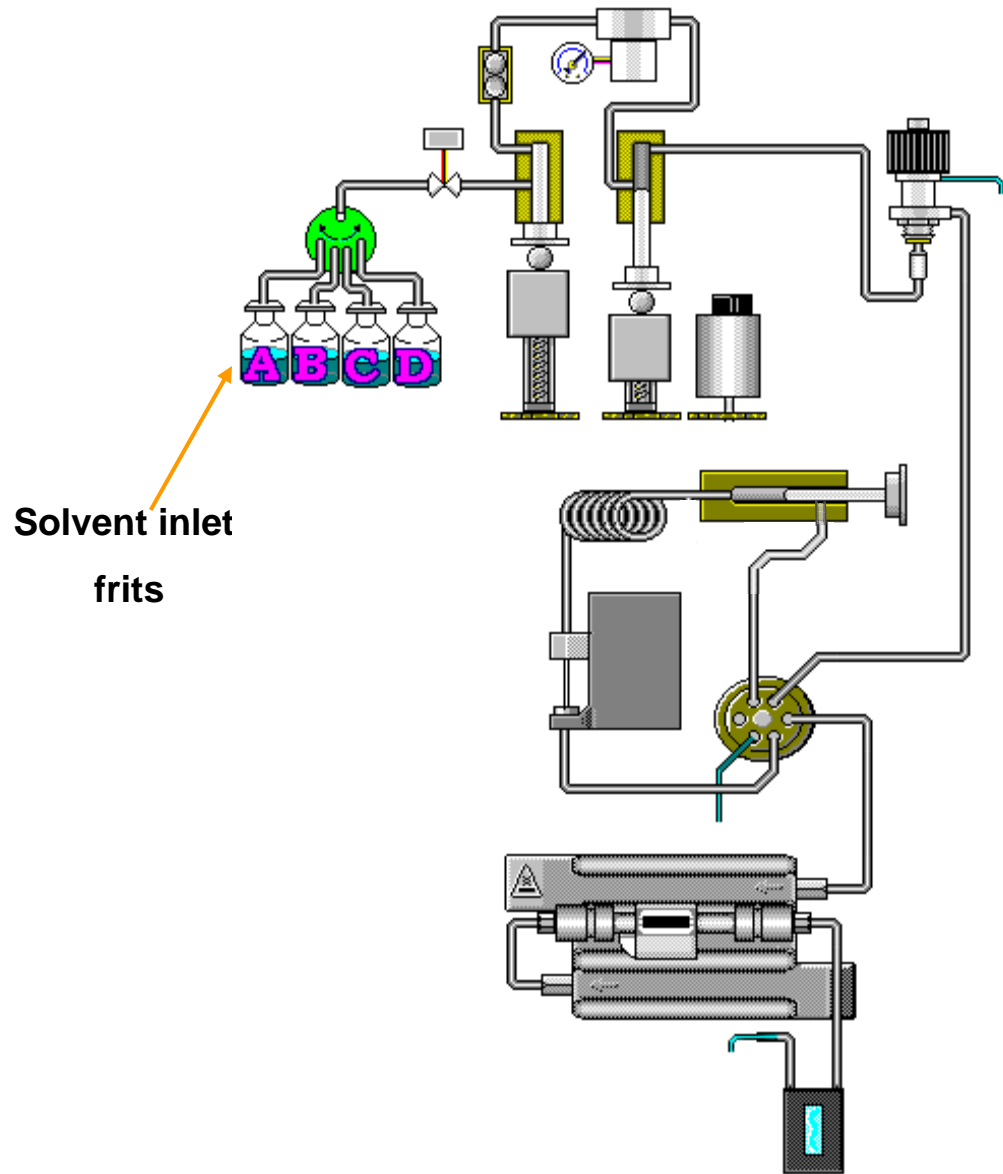


Pressure Problem II

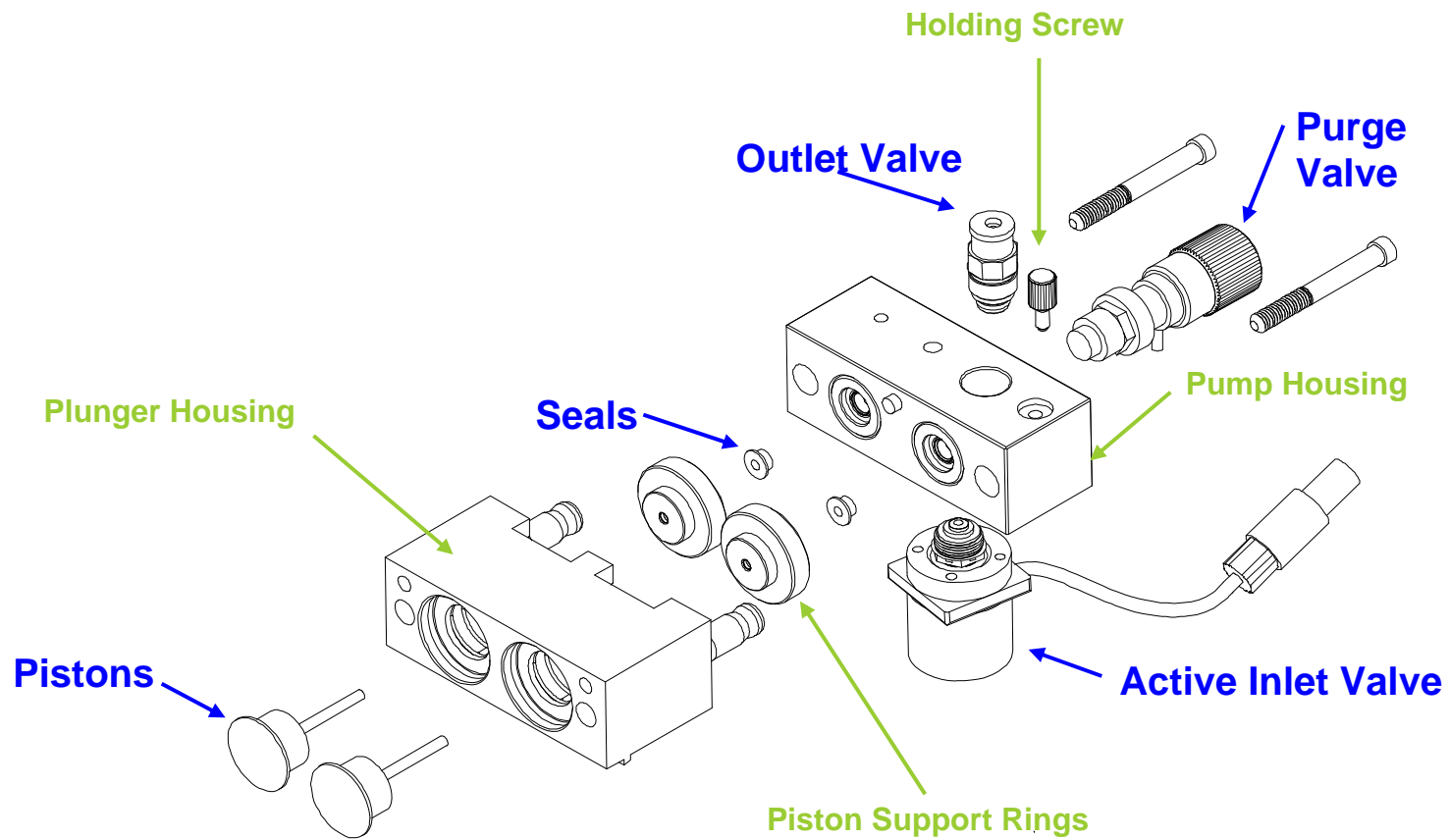
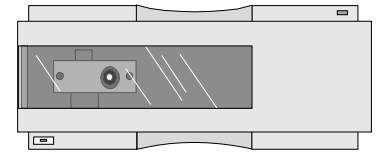
Pressure Too Low

- Solvent inlet frit plugged
- Leak in a capillary connection or other part (pump seals)
- Wrong solvent or flow rate
- AIV (Active inlet valve) defective
- Multichannel Gradient valve incorrectly proportioning
- Ball valve defective
- Column defective (stationary phase)

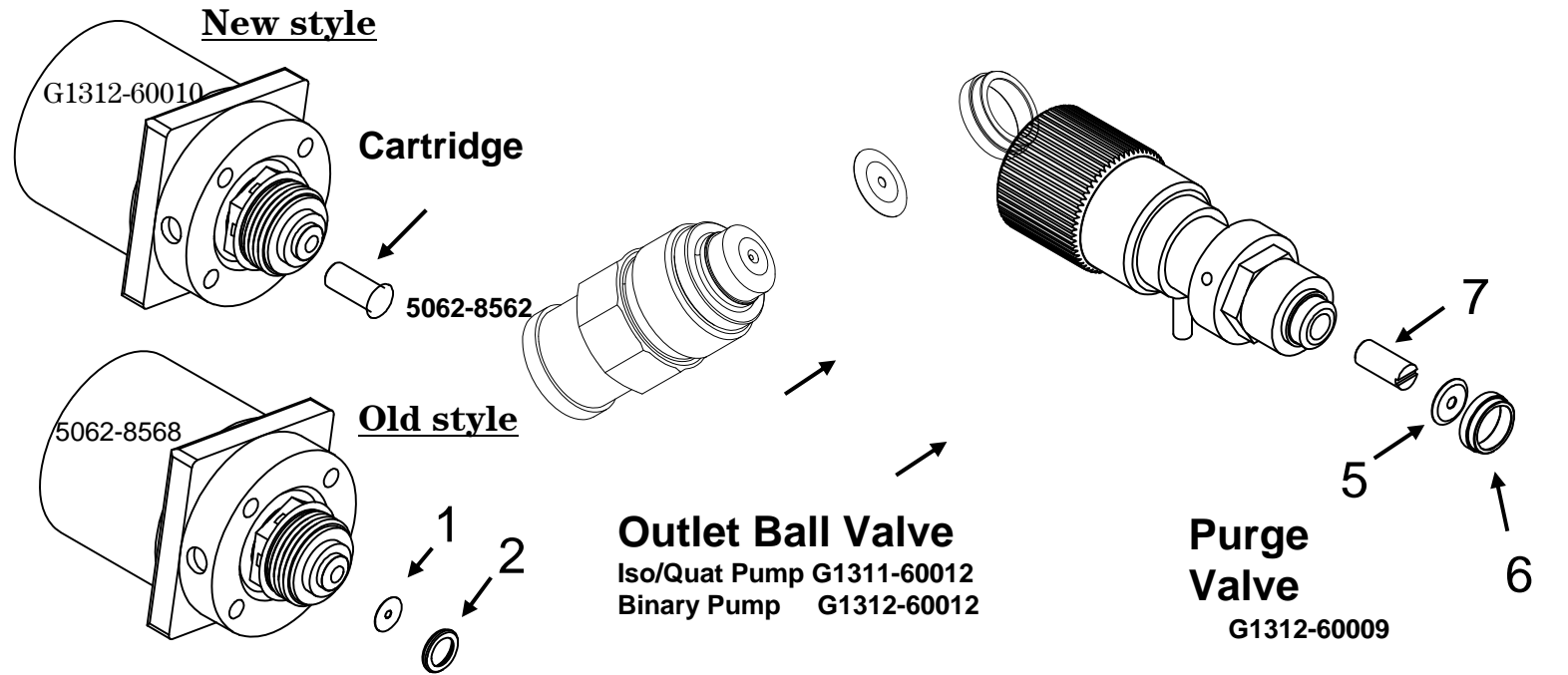
Pressure Measurement



1100 and 1200 Pumps Exploded View



Pump Check Valves



Active Inlet Valve
(common to all)

- 1. Gold Washer 5001-3707
- 2. Plastic cap 01018-21207
- 3. Gold Seal 5001-3707
- 4. Cap(4pk) 5062-2485

- 5. Gold Seal 5001-3707
- 6. Cap(4pk) 5062-2485
- 7. PTFE (5pk) 01018-22707

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.



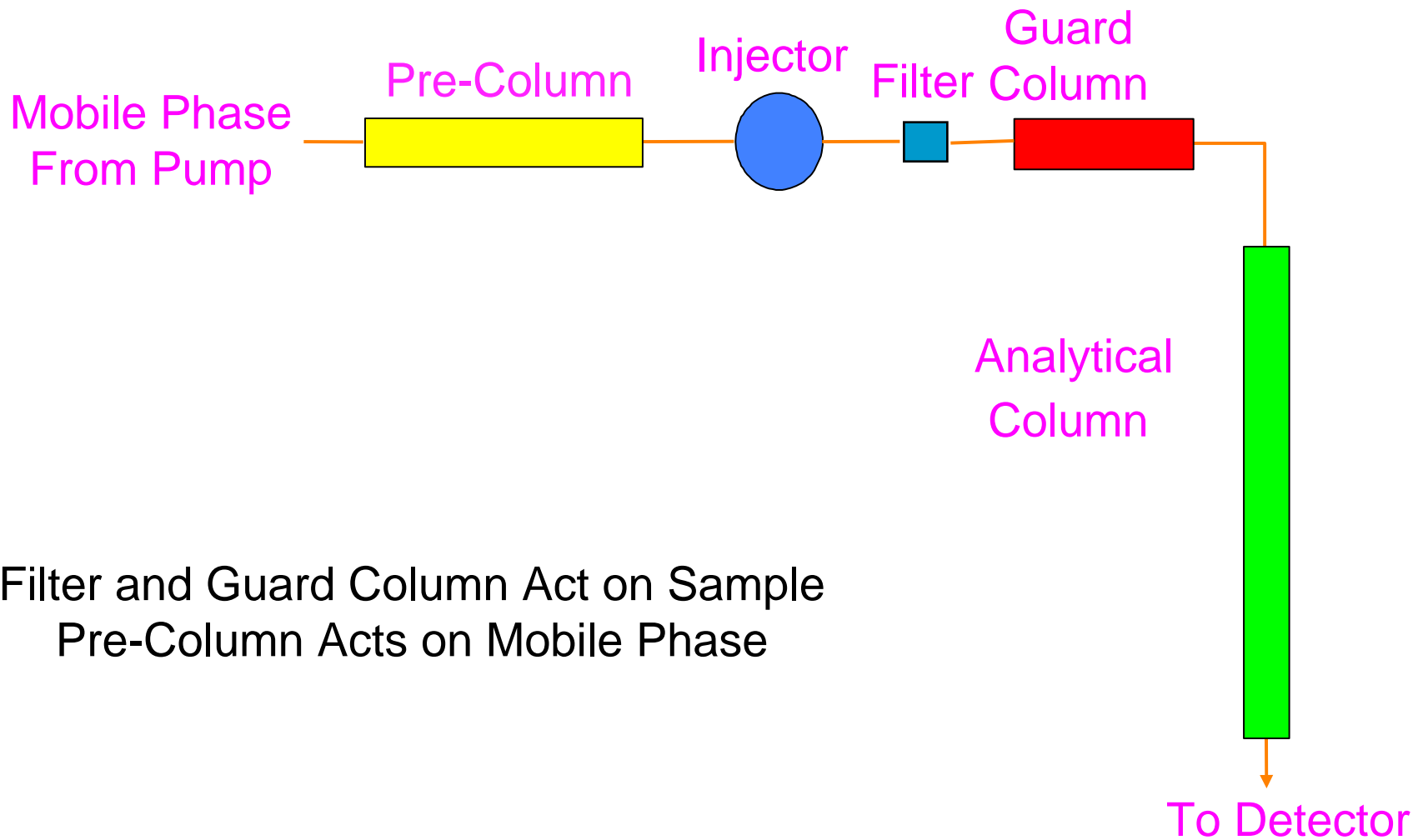
Column Cleaning

Normal Phase Solvent Choices in Order of Increasing Strength

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

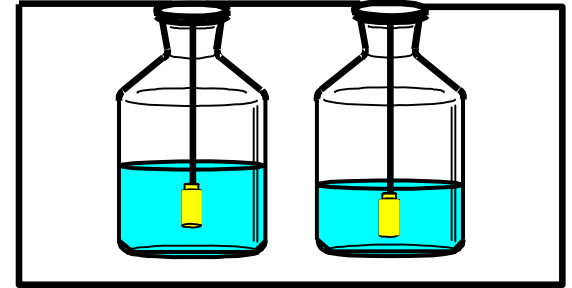


Preventing Back Pressure Problems: In-Line Devices



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

Preventing Column Back Pressure Problems



1. Filter mobile phase:
 - filter non-HPLC grade solvents
 - filter buffer solutions
 - Install an in-line filter between auto-sampler and column (removes pump seal debris, ALS rotor debris, and sample particulates). Use 2 μm frit for 3.5 μm columns, use 0.5 μm frit for 1.8 μm columns.
2. Filter all samples and standards
3. Perform sample clean-up (i.e. SPE, LLE) on dirty samples.
4. Appropriate column flushing – flush buffers from entire system at end of day with water/organic mobile phase.

2. Peak Shape Issues in HPLC

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

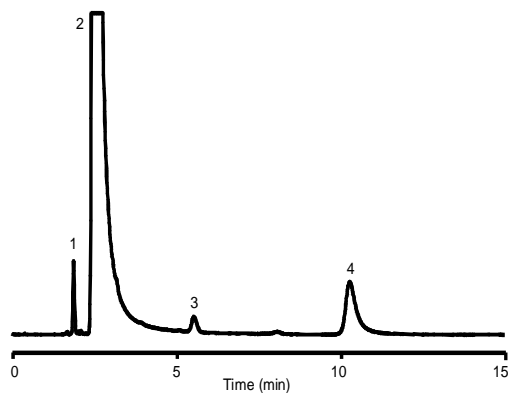


Split Peaks

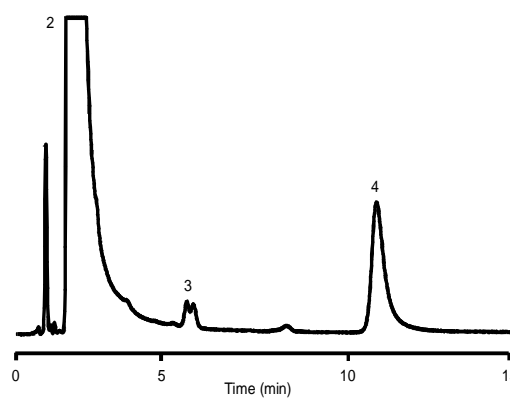
Column Contamination

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

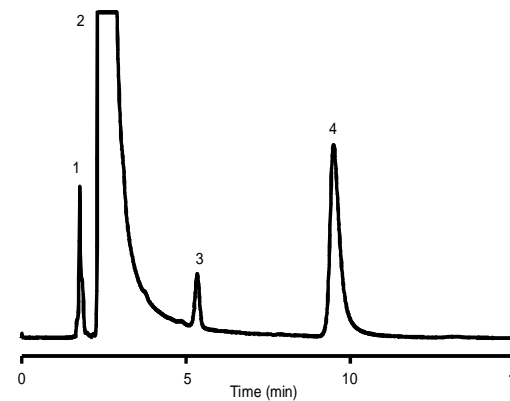
Injection 1



Injection 30



Injection 1 After Column Wash with 100% ACN



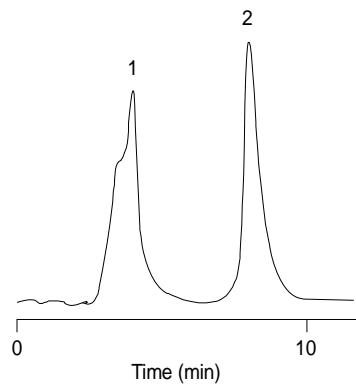
- Column washing eliminates the peak splitting, which resulted from a contaminant on the column.

Split Peaks

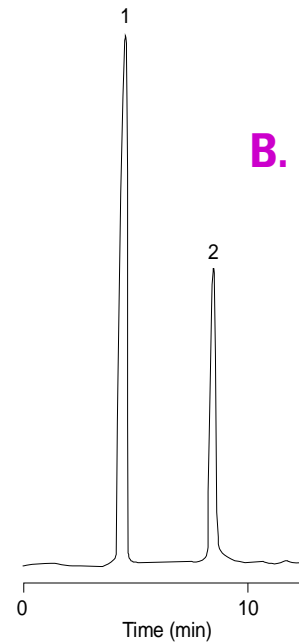
Injection Solvent Effects

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

**A. Injection Solvent
100% Acetonitrile**

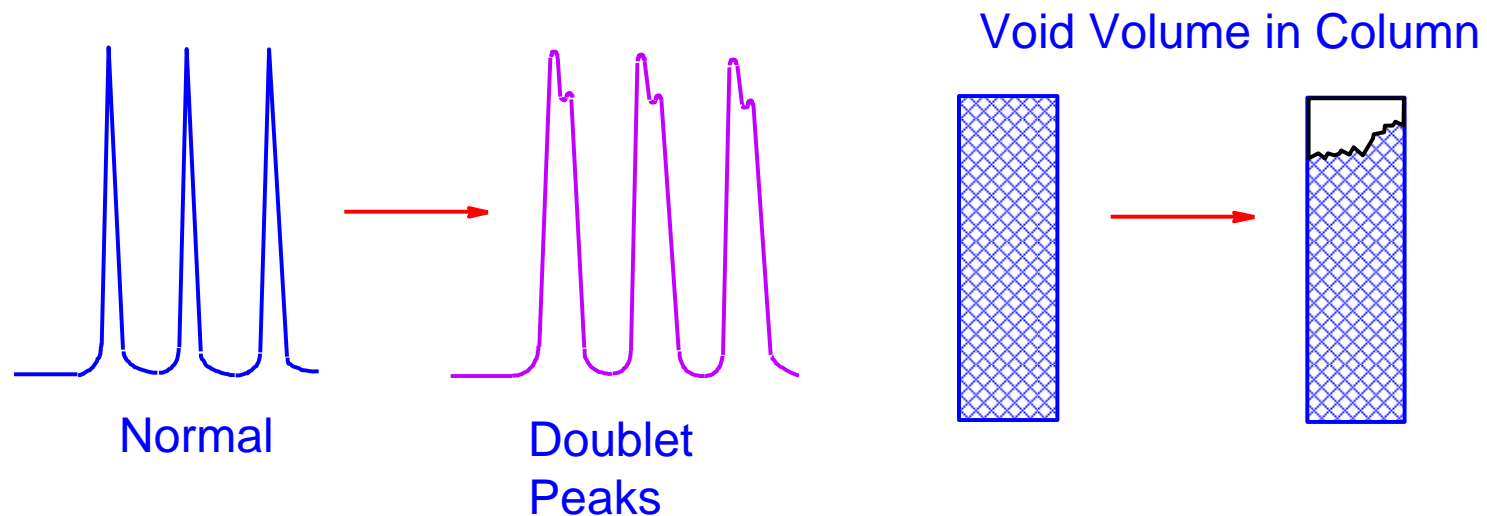


**B. Injection Solvent
Mobile Phase**



- 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 Shape Problems - Doublets



- Void Volume in Column
- Partially Blocked Frit
- Only One-Peak a Doublet- Coeluting Components
- Early (low k) peaks most affected

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.**

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**

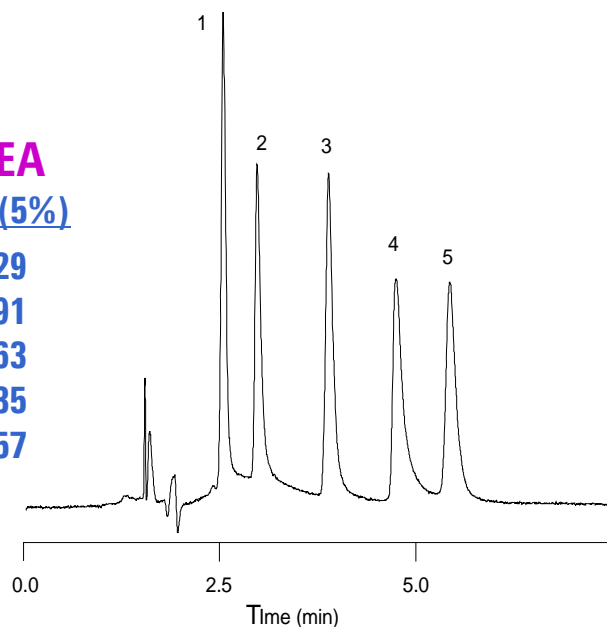


Peak Tailing Column “Secondary Interactions”

Column: Alkyl-C8, 4.6 x 150 mm, 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

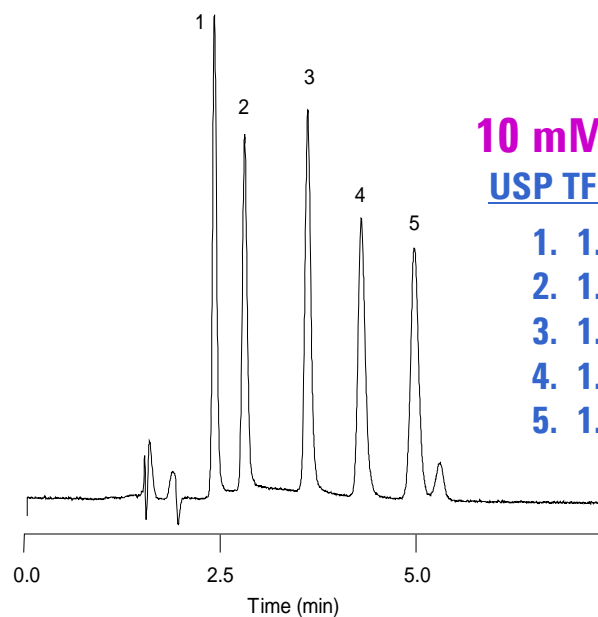
No TEA
USP TF (5%)

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

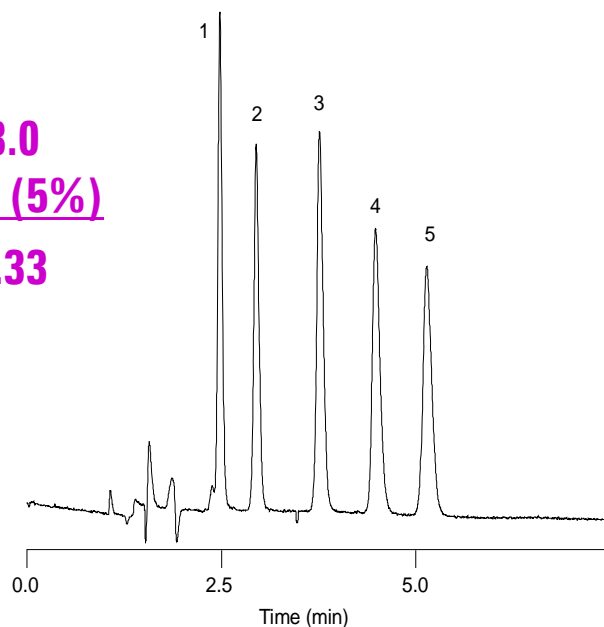
Peak Tailing

Column “Secondary Interactions”

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

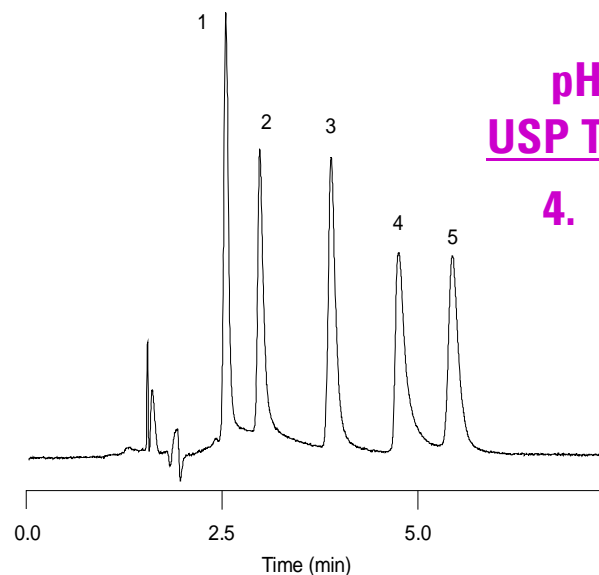
pH 3.0
USP TF (5%)

4. 1.33



pH 7.0
USP TF (5%)

4. 2.35



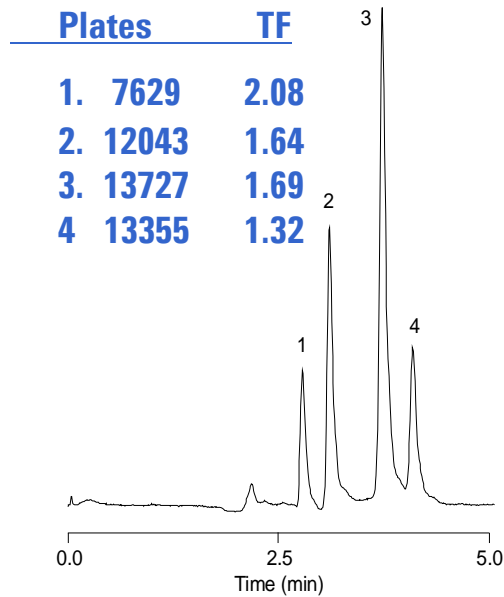
- Reducing the mobile phase pH reduces interactions with silanols that cause peak tailing. No TEA modifier required.

Peak Tailing

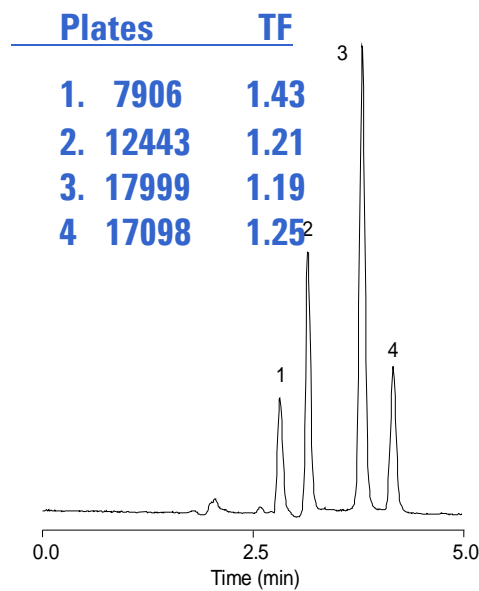
Column Contamination

Column: StableBond SB-C8, 4.6 x 250 mm, 5 μ m Mobile Phase: 20% H₂O : 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

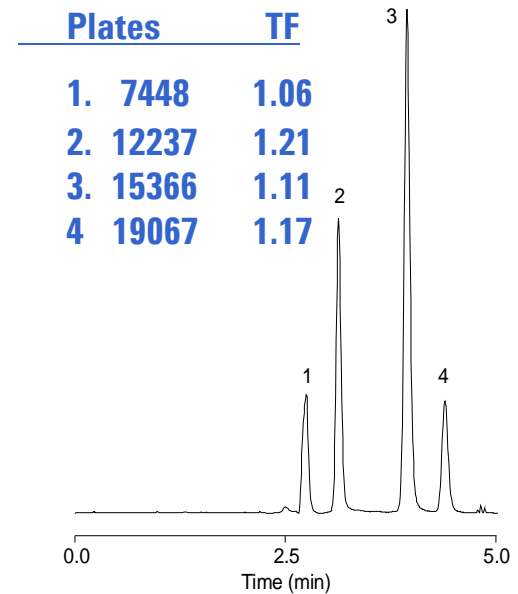
QC test forward direction



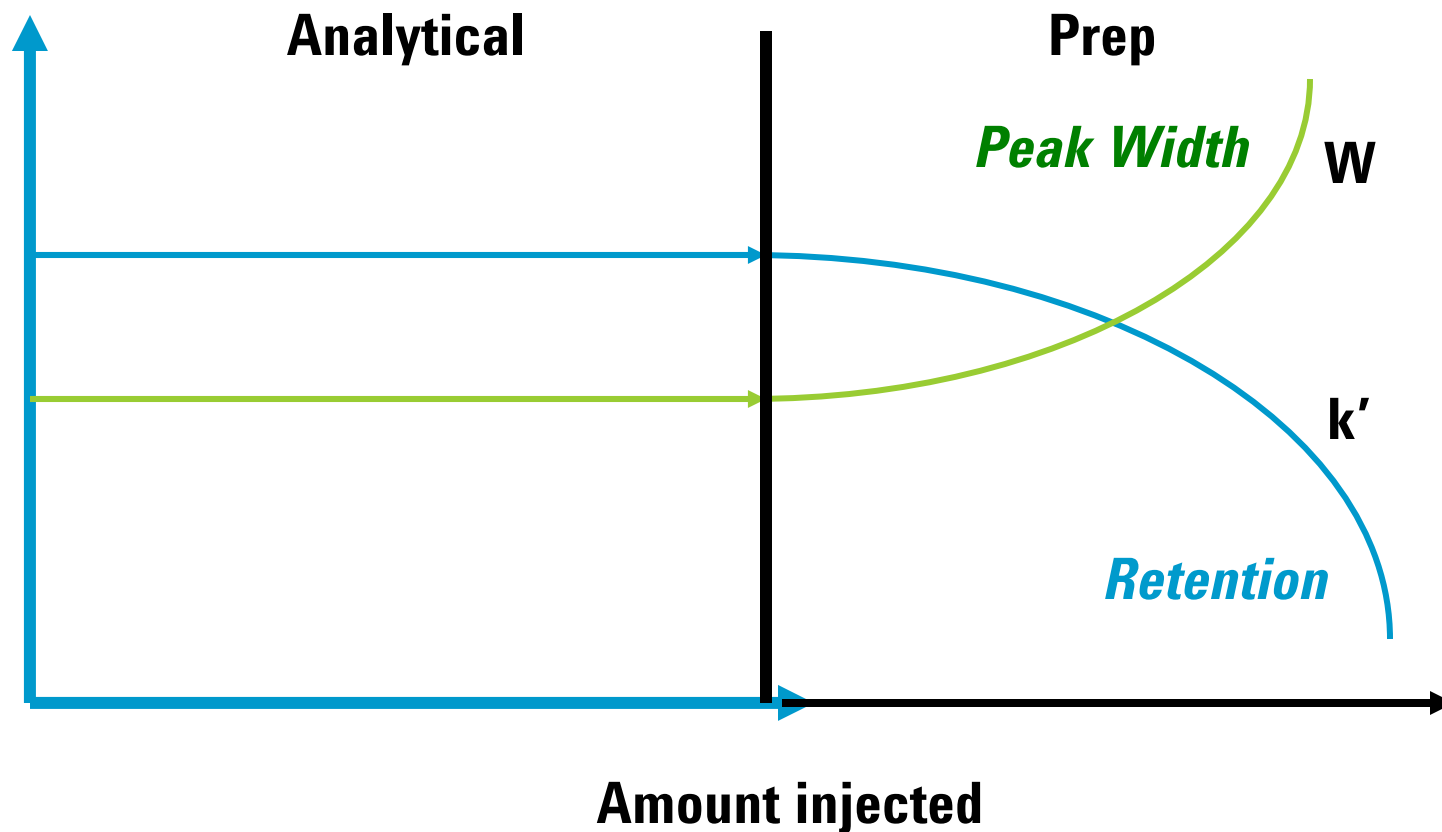
QC test reverse direction



QC test after cleaning 100% IPA, 35°C



Analytical vs. Preparative Scale HPLC. Non-linear Adsorption Isotherms, or Overload Conditions:

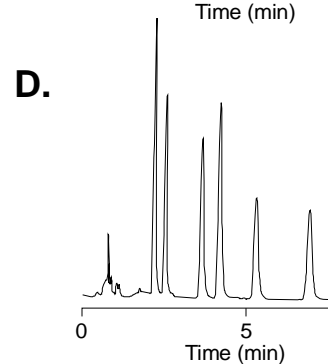
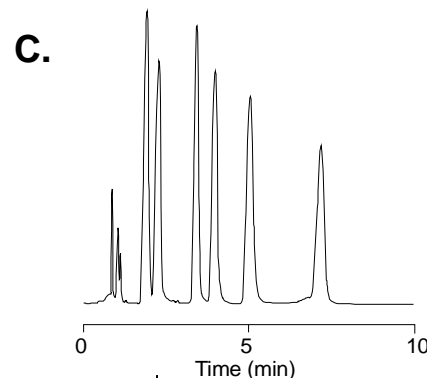
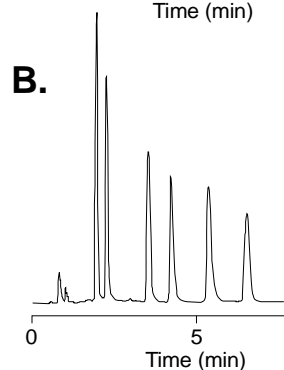
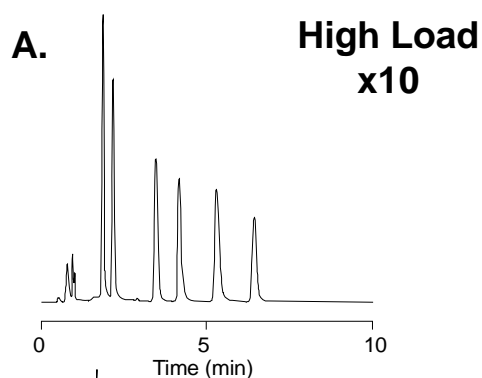


Peak Tailing/Broadening Sample Load Effects

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 TF (5%)

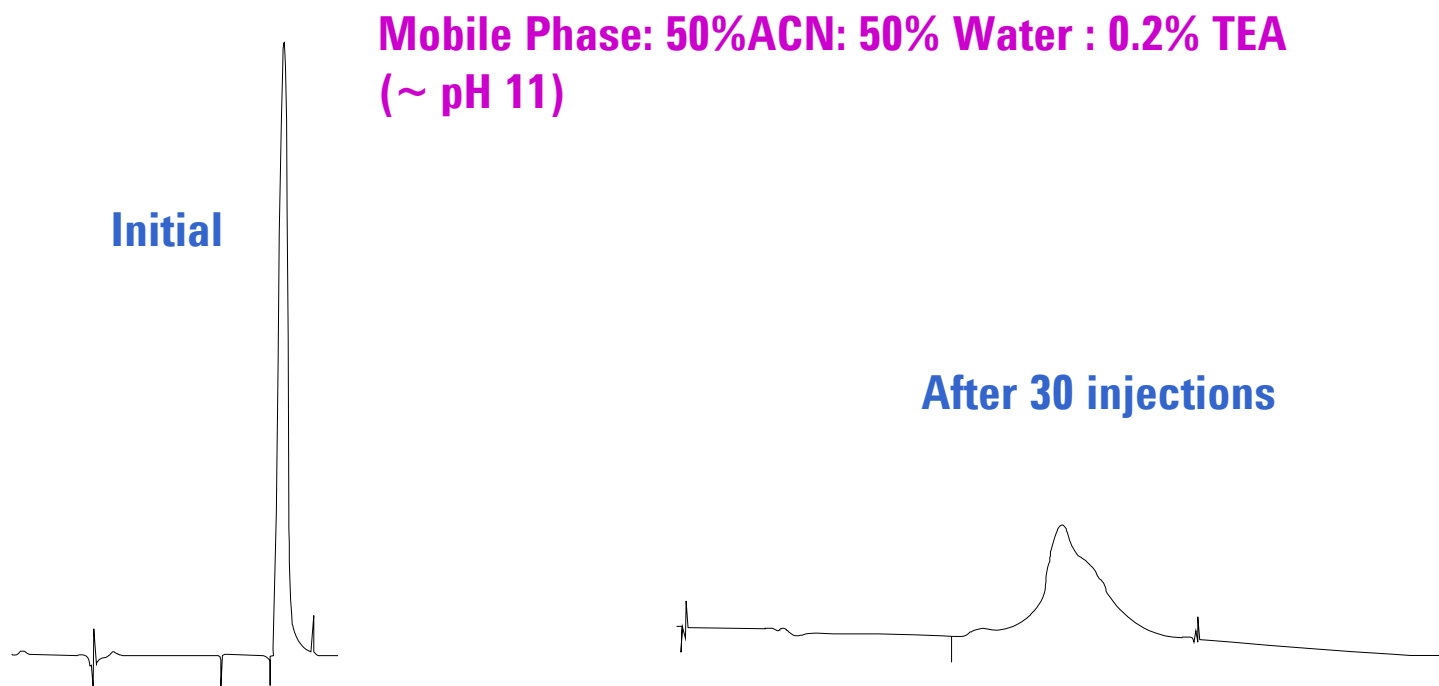
| | <u>A</u> | <u>B</u> |
|----|----------|----------|
| 1. | 1.60 | 1.70 |
| 2. | 2.00 | 1.90 |
| 3. | 1.56 | 1.56 |
| 4. | 2.13 | 1.70 |
| 5. | 2.15 | 1.86 |
| 6. | 1.25 | 1.25 |



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 |

Peak Broadening, Splitting Column Void



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

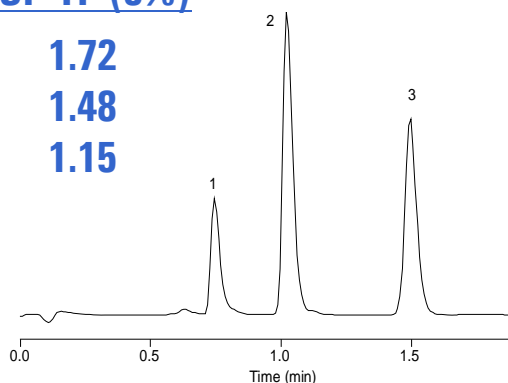
Peak Tailing

Injector Seal Failure

Column: Bonus-RP, 4.6 x 75 mm, 3.5 μm Mobile Phase: 30% H₂O : 70% MeOH Flow Rate: 1.0 mL/min
Temperature: R.T. Detection: UV 254 nm Sample: 1. Uracil 2. Phenol 3. N,N-Dimethylaniline

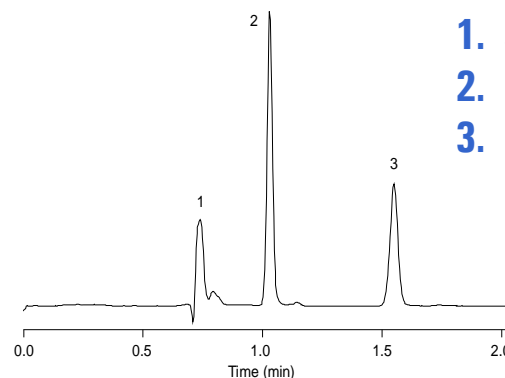
Before

| | <u>Plates</u> | <u>USP TF (5%)</u> |
|----|---------------|--------------------|
| 1. | 2235 | 1.72 |
| 2. | 3491 | 1.48 |
| 3. | 5432 | 1.15 |



**After replacing rotor seal
and isolation seal**

| | <u>Plates</u> | <u>USP TF (5%)</u> |
|----|---------------|--------------------|
| 1. | 3670 | 1.45 |
| 2. | 10457 | 1.09 |
| 3. | 10085 | 1.00 |

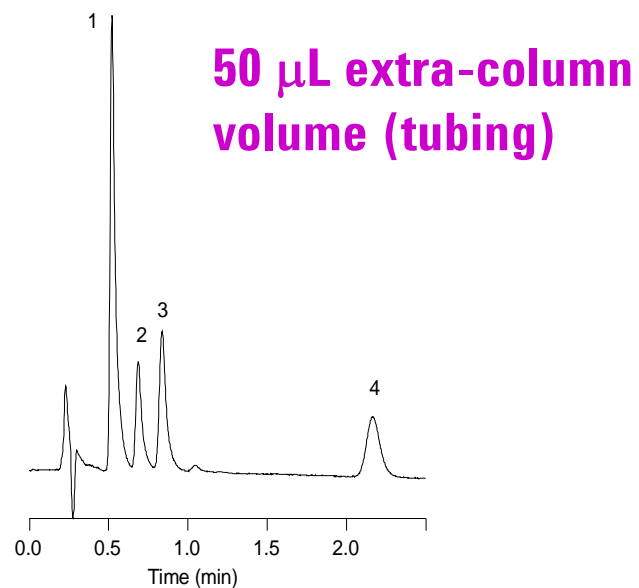
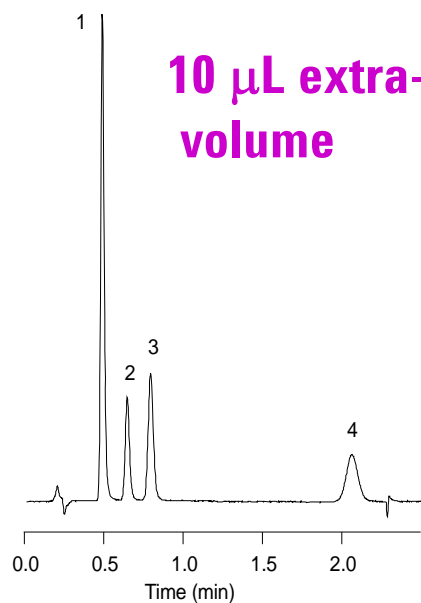


- **Overdue instrument maintenance can sometimes cause peak shape problems.**

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



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 – volume injection and concentration**
- **Eliminate extra-column effects – tubing, fittings, Uv cell**
- **Flush column and check for aging/void**

Reproducibility

Peak retention time precision:

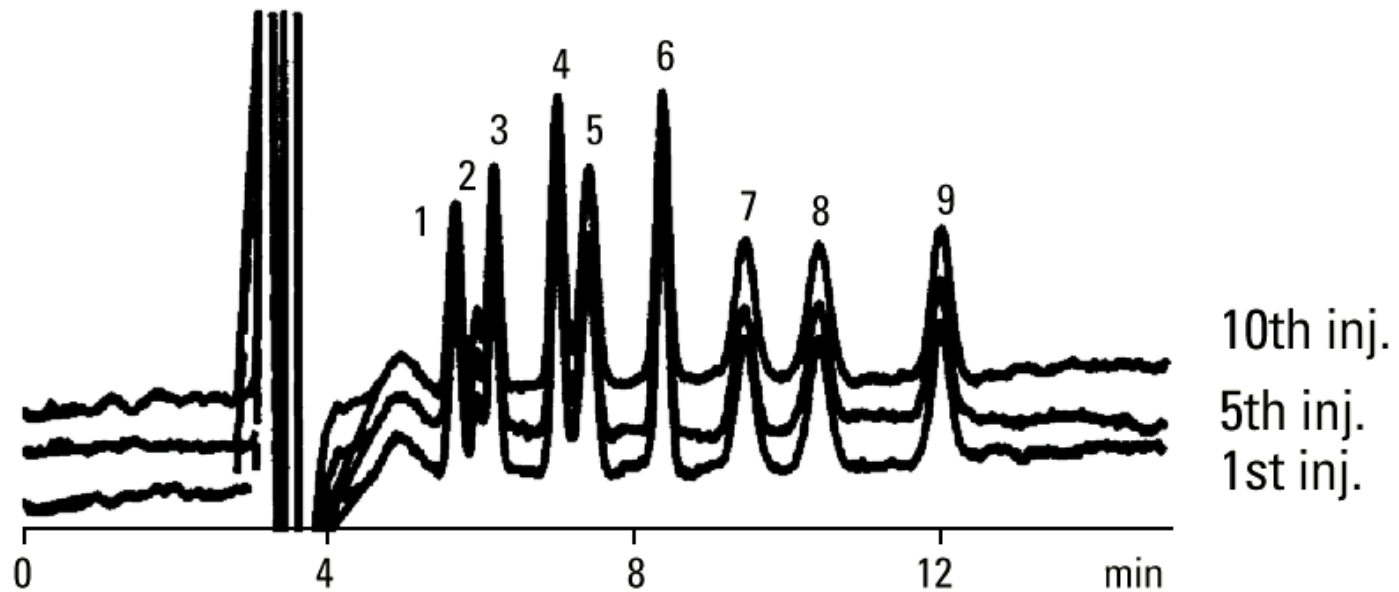
⇒ with oven: _____ < 0.3%

⇒ without oven: _____ < 0.7%

Peak area precision: ≤ 1.5%

Typically,

- Area and Peak Height problems together point to the autosampler system
- Area and Retention Time problems together point to the pump



Problems with Reproducibility – Peak Areas

Peak Areas not
Reproducible

With peak height

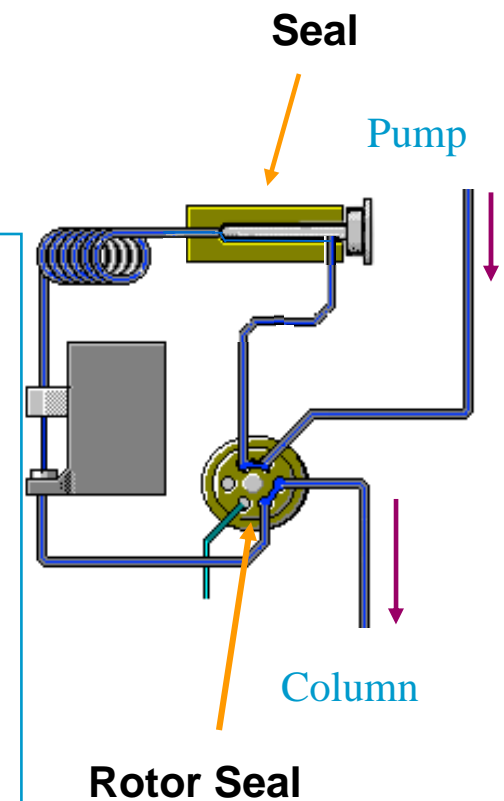
- Rotor seal cross-port leak or injection valve not tight
- Piston seal of metering unit leaking
- Needle partially blocked

With retention time

- Variable pump flow rate

Other

- Capillary from injector to detector not tight
- Detector equilibration problems



3. Retention Issues

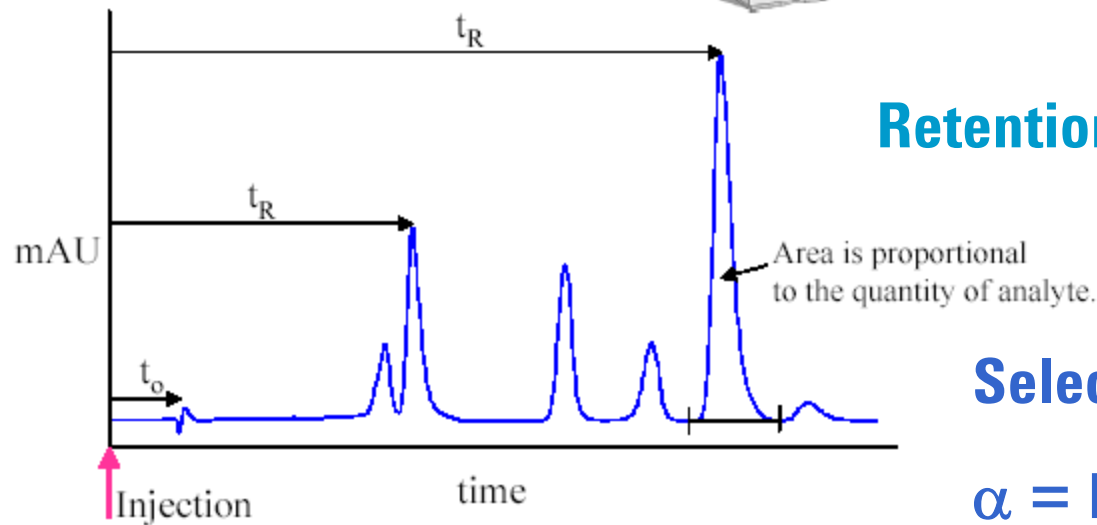
- Retention time changes (t_r)
- Retention factor changes (k')
- Selectivity changes (α)



Retention time t_R , Retention factor k' , and Selectivity factor α

The Chromatogram

t_0 - elution time of unretained peak
 t_R - retention time - determines sample identity



$$\text{Retention factor } k' = (t_R - t_0) / t_0$$

Selectivity factor α

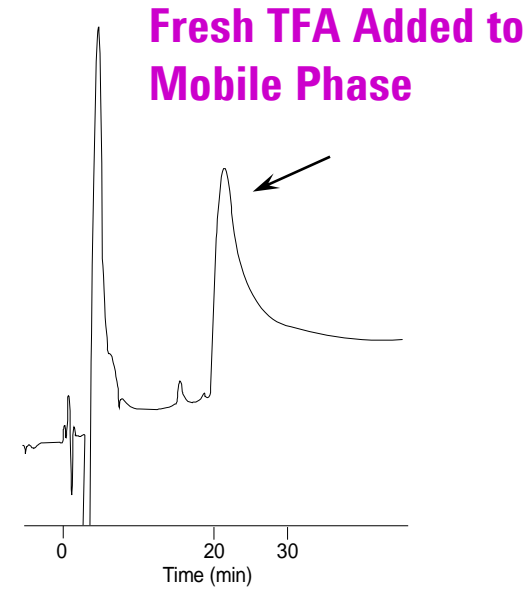
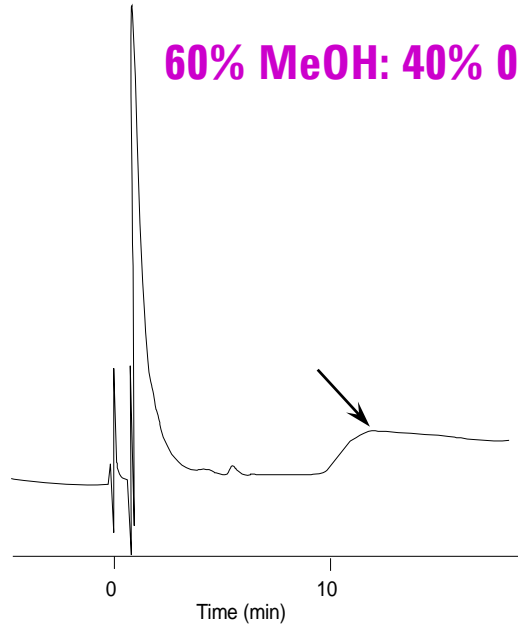
$$\alpha = k_2 / k_1$$

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



- **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° 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' , a , 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

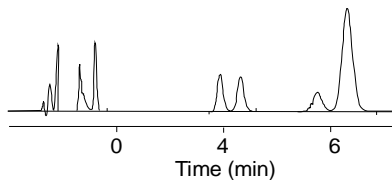
1. **Different column histories (aging)**
2. **Insufficient/inconsistent equilibration**
3. **Poor column/mobile phase combination**
4. **Change in mobile phase**
5. **Change in flow rate**
6. **Other instrument issues**
7. **Slight changes in column bed volume (t_r only)**

Example Change in Retention/Selectivity

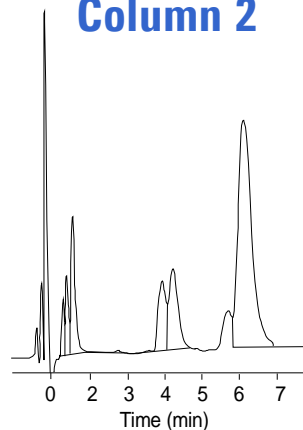
Column-to-Column

Mobile Phase Variation

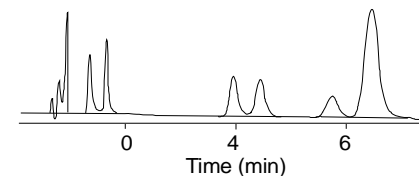
Column 1



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.”

Minimize Change in Retention/Selectivity

Lot-to-Lot

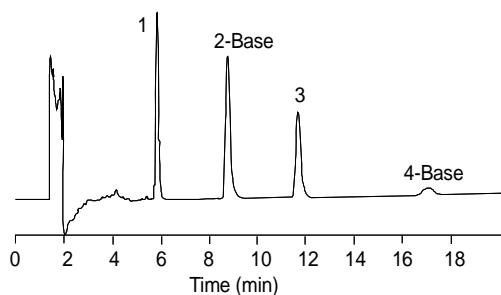
Evaluate:

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

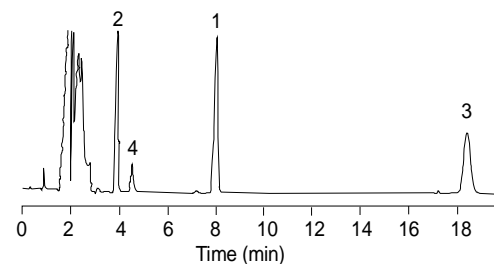
*All causes of column-to-column change should be considered first, especially when only one column from a lot has been tested.

Lot-to-Lot Selectivity Change - pH

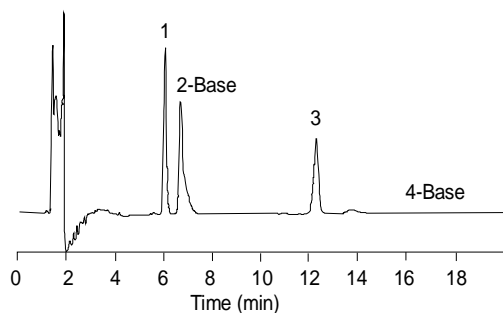
pH 4.5 - Lot 1



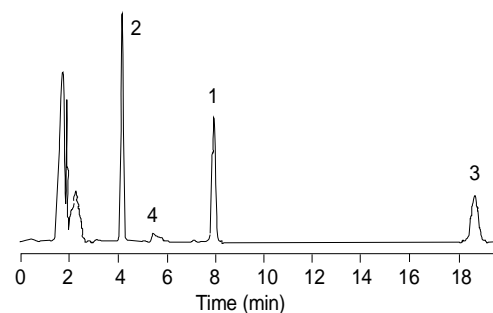
pH 3.0 - Lot 1



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

Problems with Reproducibility – Peak Areas

Peak Areas not Reproducible

With peak height

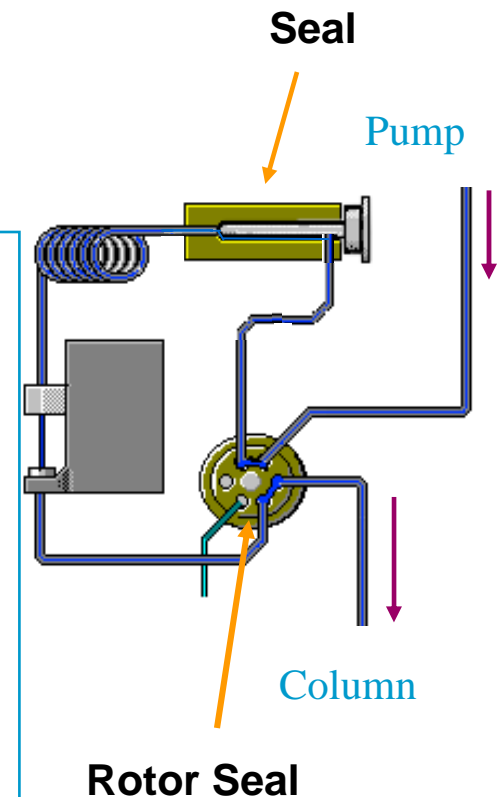
- Rotor seal cross-port leak or injection valve not tight
- Piston seal of metering unit leaking
- Needle partially blocked

With retention time

- Variable pump flow rate

Other

- Capillary from injector to detector not tight
- Detector equilibration problems



Problems with Reproducibility – Retention Time

Retention Times not Reproducible

- **Pump Problems**

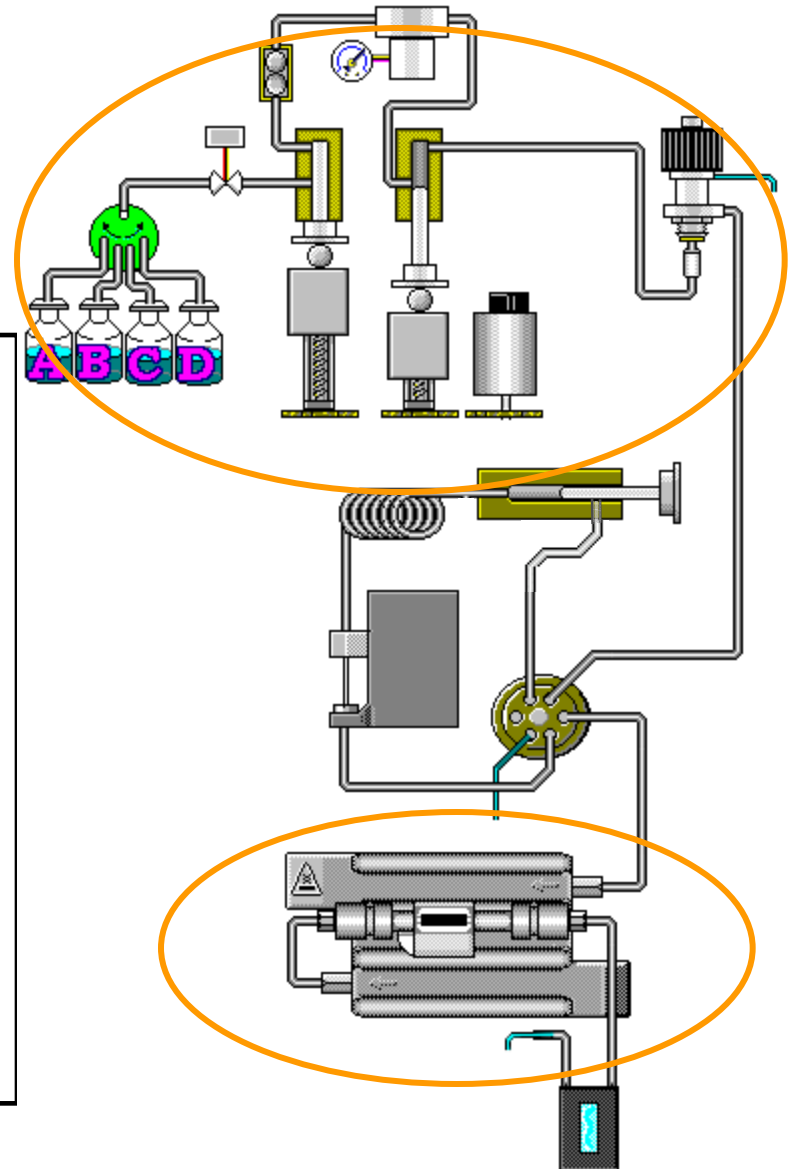
- Mobile phase composition problems
- Valves AIV, ball valve defective
- Flow rate problems

- **Column Oven Problems**

- Temperature fluctuations

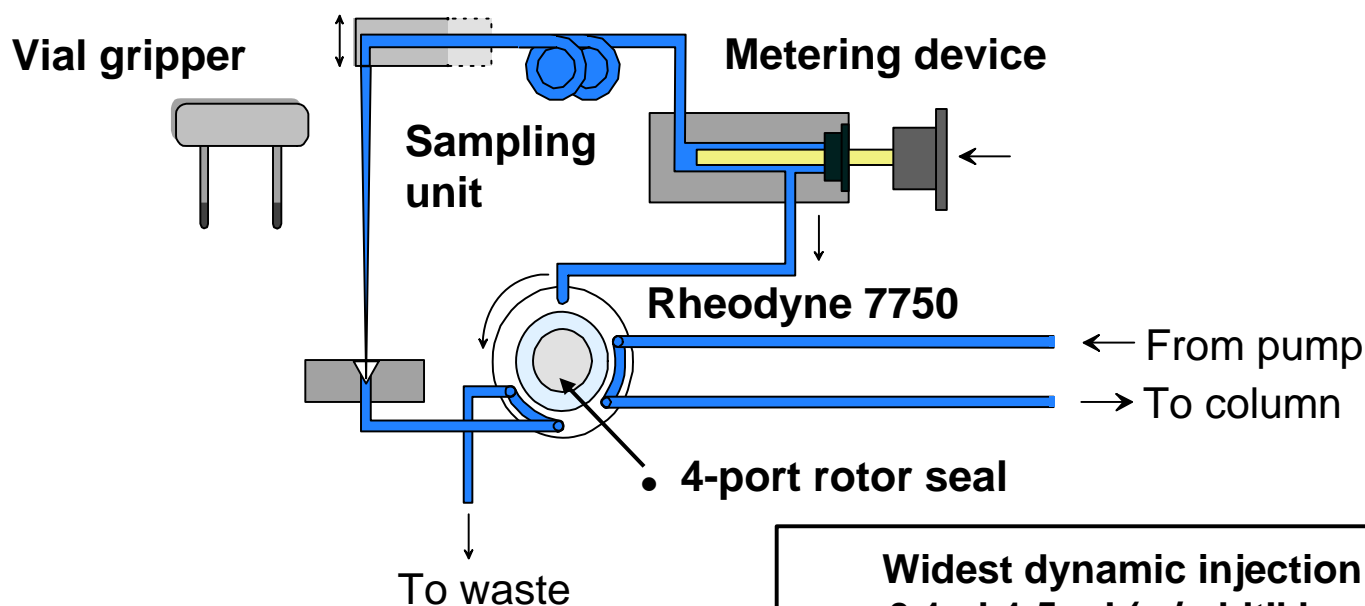
- **Other**

- Column equilibration
- Column deterioration



Autosampler Principle of Operation

Standard loop volume $300\mu\text{l}$
Total delay volume $300\mu\text{l} + V_{inj}$
Minimal (bypass) delay volume $6.2\mu\text{l}$



Widest dynamic injection range:
 $0.1\ \mu\text{l}$ - $1.5\ \text{ml}$ (w/addt'l hardware)

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, option 4, option 2 (LC columns)

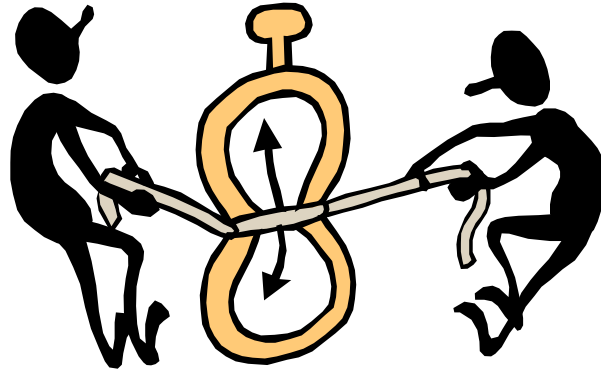


Conclusions

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.



The End – Thank You!

**Agilent LC Column Tech Support: 800-227-9770 #4, #2 Email:
Edward_kim@agilent.com**

Agilent LC Columns and Agilent J&W GC Columns Scientific Technical Support

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

302-993-5304 (phone)

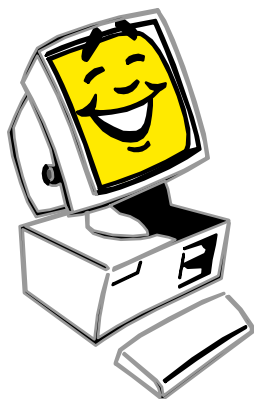
For LC columns

Select option 4, then option 2

For GC Columns

** Select option 4, then option 1.*

www.agilent.com/chem



Looking for More Information on Agilent's LC Systems and Software?

Agilent offers a full range of LC training courses including hands-on courses with the latest 1200 series equipment including Rapid Resolution, and additional 1100 series courses.

Each course includes a course manual for future reference and a certificate of completion. All courses are taught by industry experts.

Call 800.227.9770, Option 5 or visit www.agilent.com/chem/education to register today!

New On Demand Webinar: Utilizing Sub-Two Micron Particles to Optimize HPLC Methods

*A Four Part Workshop on Managing Chemistry and Pressure for Faster
and More Efficient HPLC Separations*

www.SeparationsNOW.com/agilentwebinar

Be sure to register after today's event.

Upcoming LC e-Seminars

New Technology for HPLC Environmental Assay

January 22, 2008 – 1:00 pm EST

A Look at Column Choices- Series 3

February 13, 2008 – 1:00 pm EST

Method Development – Series 4

March 18, 2008 – 2:00 pm EST