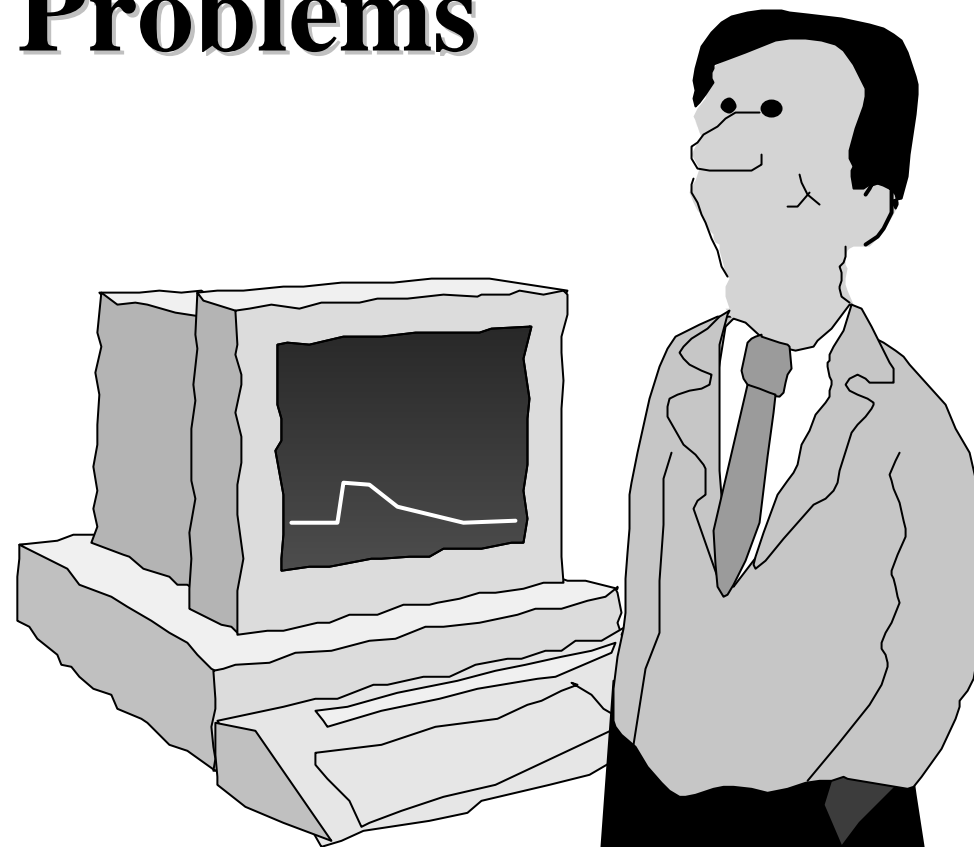
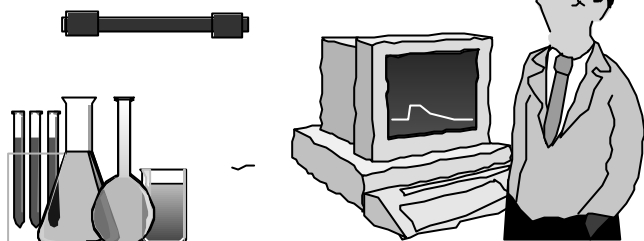


# Troubleshooting Common HPLC Problems



<http://www.hplc1.com/shodex/english/dd.htm>

## Troubleshooting Common HPLC Problems



<http://www.hplc1.com/shodex/english/dd.htm>

# HPLC Troubleshooting

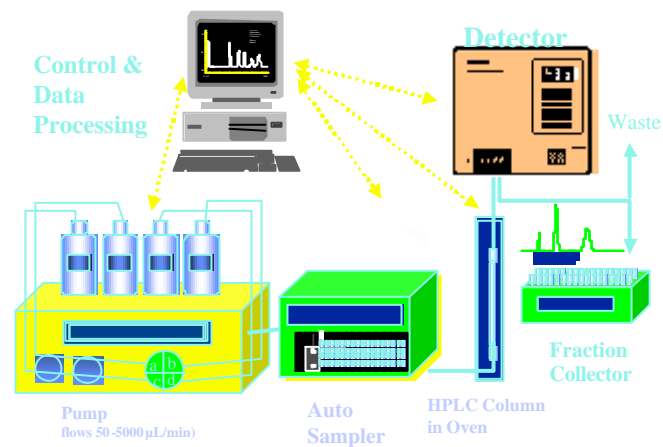


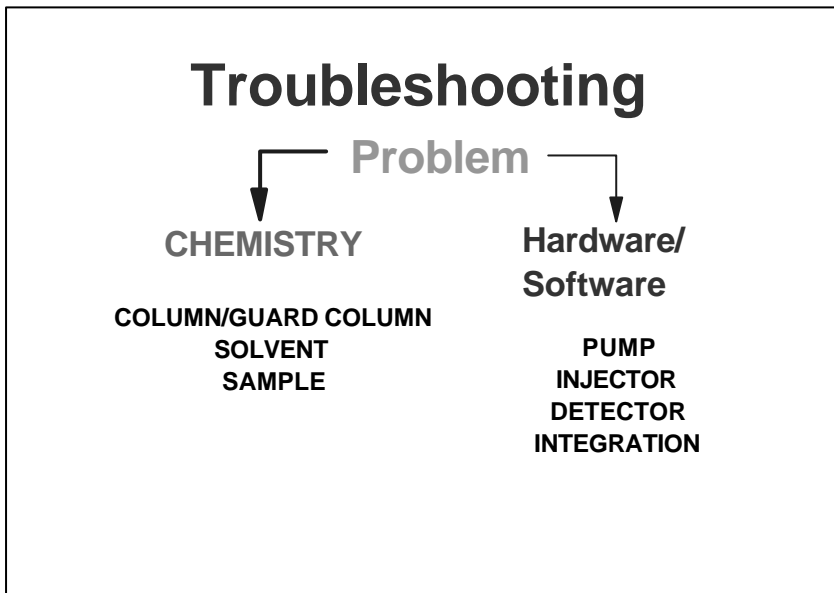
## Performance Monitoring

Use Your Test Method  
(Known Performance)

- \* Monitor at least One Peak in one injection
  - Plate Count (Peak width relative to RT),
  - Peak Asymmetry,
  - Retention Time and/or Retention parameter
  - Relative Retention Time for Critical Pair of Analytes.
  - Peak Response

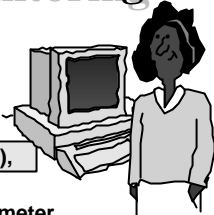
- \* Inject Multiple Runs
  - Precision (at least 5 injections)
  - Accuracy (Use Control Samples)





## Performance Monitoring

Use Your Test Method  
(Known Performance)



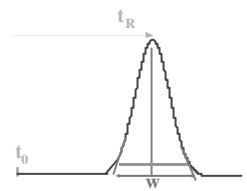
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  - Relative Retention Time for Critical Pair of Analytes.
  - Peak Response
- \* Inject Multiple Runs
  - Precision (at least 5 injections)
  - Accuracy (Use Control Samples)

## Plate Count - Efficiency of the Separation

\* A "Plate Count" Actually Is a Determination Of Both The Column AND Instruments' Performance

## Performance Monitoring

Column Efficiency:  
 N = the number of Theoretical Plates  
 a = is a constant depending on the Method used  
 t<sub>r</sub> = retention time of peak  
 W = the peak width (time units) at a given peak height

$$N = a \left( \frac{t_r}{W} \right)^2$$


METHOD	a
Peak Width at Half Height	5.54
Peak Width at 4.4% Peak Height (5 Sigma)	25.0
Tangent	16.0

## Performance Monitoring

### Band Spreading

- \* Band Spreading Impacts Chromatographic Performance -- The Greater The Band Spreading, The Poorer The Performance (ie; Resolution)
- \* Band Spreading Contains Both An Instrument AND A Column Contribution

## Extra-Column Band Spreading

The Observed Bandwidth (TOT)

- \* Sum of the Bandspreading Contributions
  - Column (COL)
  - Extra-Column (EC) Instrument components

$$S_{TOT}^2 = S_{COL}^2 + S_{EC}^2$$

## Band Spreading

- \* Column Contribution

$S_{COLUMN}^2$  = optimized by choosing the correct column and conditions

- \* Instruments Contribution = Extra-Column

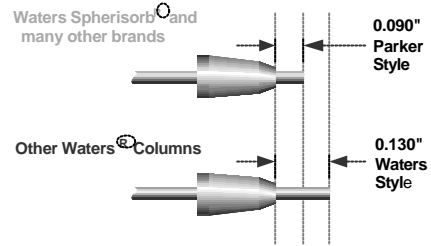
$$S_{EC}^2 = S_{TUBING}^2 + S_{CONNECTIONS}^2 + S_{INJECTORS}^2 + S_{DETECTORS}^2$$

## Performance Monitoring

Extra-Column Band Spreading  
(Instruments' Contribution)

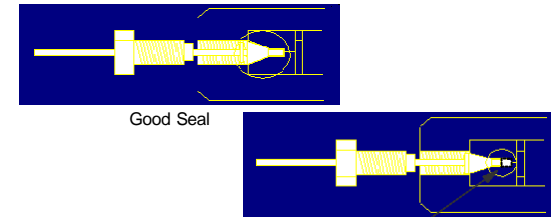
1. Injection Volume
2. Injector
3. Connection Tubing
  - a. from Injector to Column
  - b. from Column to Detector
  - c. Endfittings and Frits
4. Detector Volume

### Connectors



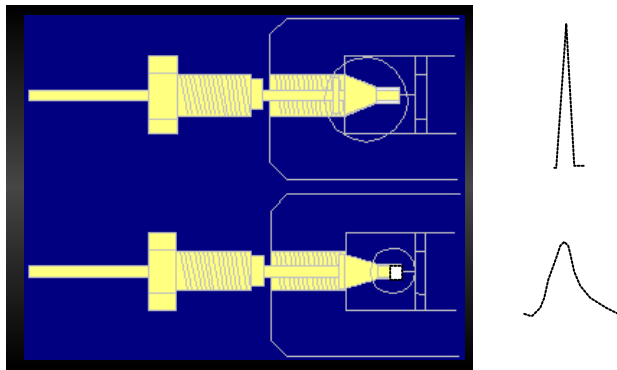
### Installation and Equilibration

- ✓ Make sure column inlet connected correctly
- ✓ Make sure nut and ferrule are seated



NOTE: column inlet connector not seated properly  
(PEEK Connectors Easier to Use -- THF makes PEEK brittle)

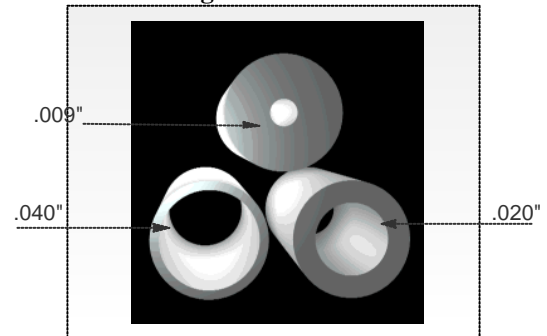
### Extra-Column Band Spreading



Column Connection Contribution

### Extra-Column Band Spreading

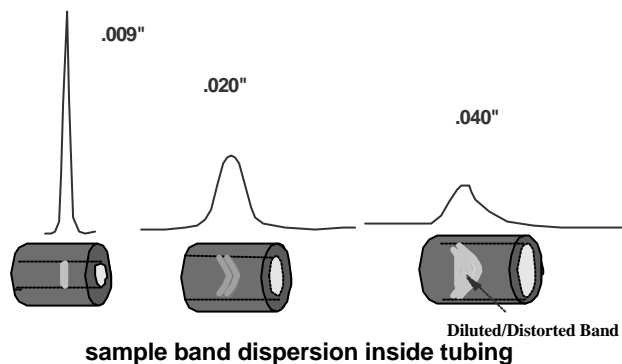
#### Tubing Contribution



note the differences of the inner diameter of this tubing

## Performance Monitoring

Effect of Connecting Tubing on System Bandspreading



## Measuring The Instruments Contribution

\* Perform An Instrument Band Spreading Test

## Performance Monitoring

To perform a measurement:

- disconnect column from system
- connect injector directly to detector

Parameter	Setting
Flow Rate	1.0 mL/min
Chart Speed	20 cm/min
Detector Sensitivity	0.5 - 1.0 AUFS
Time Constant	0.2 seconds or less

dilute test mixture 1 to 10 in mobile phase  
inject 2 to 5  $\mu$ l of this solution

## Performance Monitoring

Using 5 sigma efficiency method, measure the peak width at 4.4% of peak height

Convert to microliters using the following equation:

$$\left( \frac{2\text{cm}}{\text{PW}} \right) \left( \frac{1\text{min}}{20\text{cm}} \right) \left( \frac{1\text{mL}}{\text{min.}} \right) \left( \frac{1000\text{mL}}{\text{mL}} \right) = 100 (\mu\text{L})$$

where:

1min/20cm = chart speed

1 mL/min = flow rate

1000  $\mu$ L/mL = volume correction factor

Typical LC System should be 100 $\mu$ L +/- 30 $\mu$ L

Microbore System should be no greater than 20 $\mu$ L

## Performance Monitoring

### Impact of System Band Spread on a Plate Count:

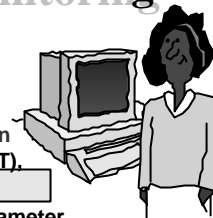
- System with 70 $\mu$ l Band Spread >> 10,000 plates
- System with 130ml Band Spread >> ~8,000 plates

### On the Same Column!

Assumption: <40% loss in resolution at  $k' = 5$  and  $N = 10,000$  and <20% loss in resolution at the preferred value

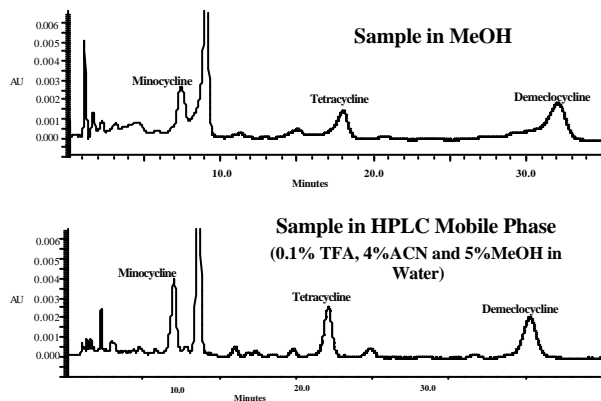
## Performance Monitoring

### Use Your Test Method (Known Performance)



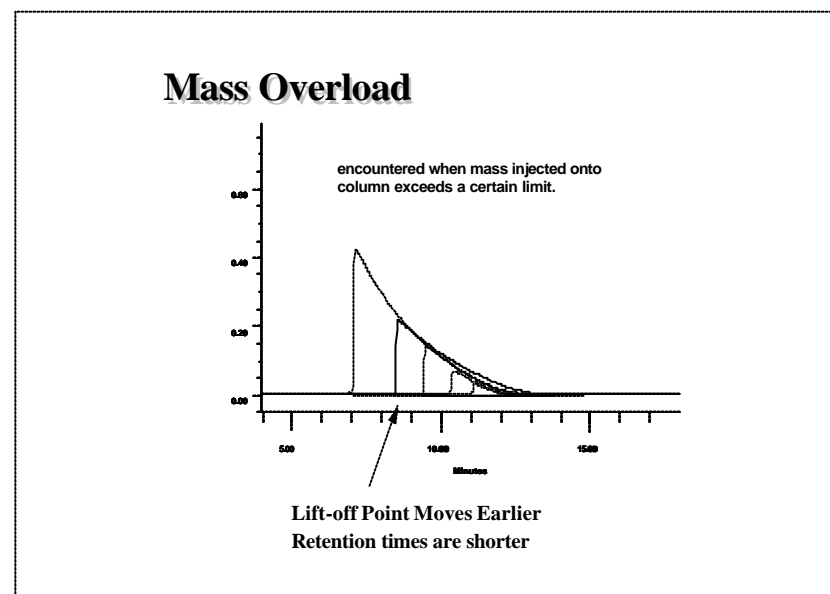
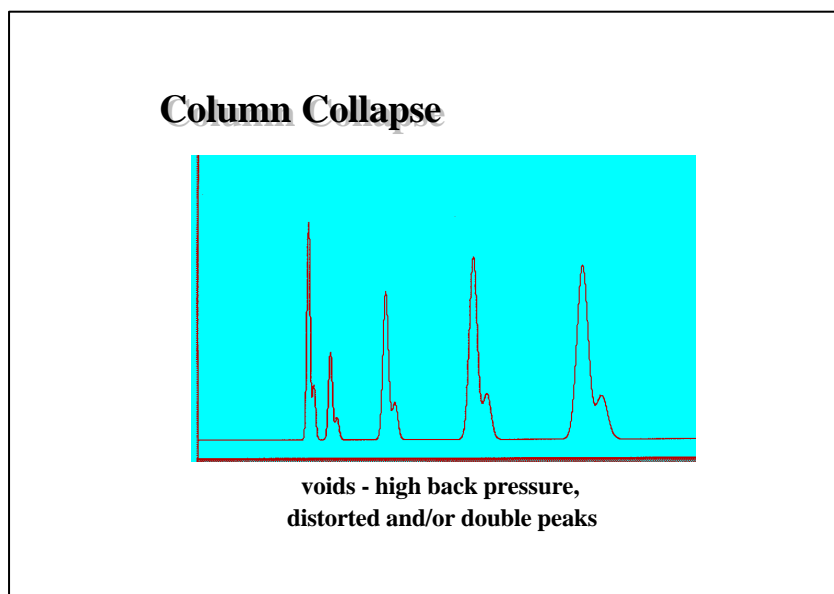
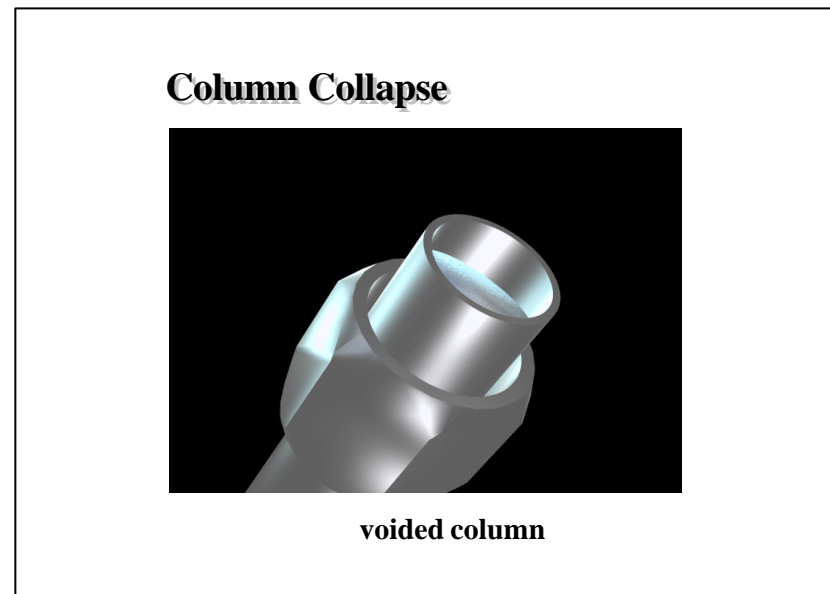
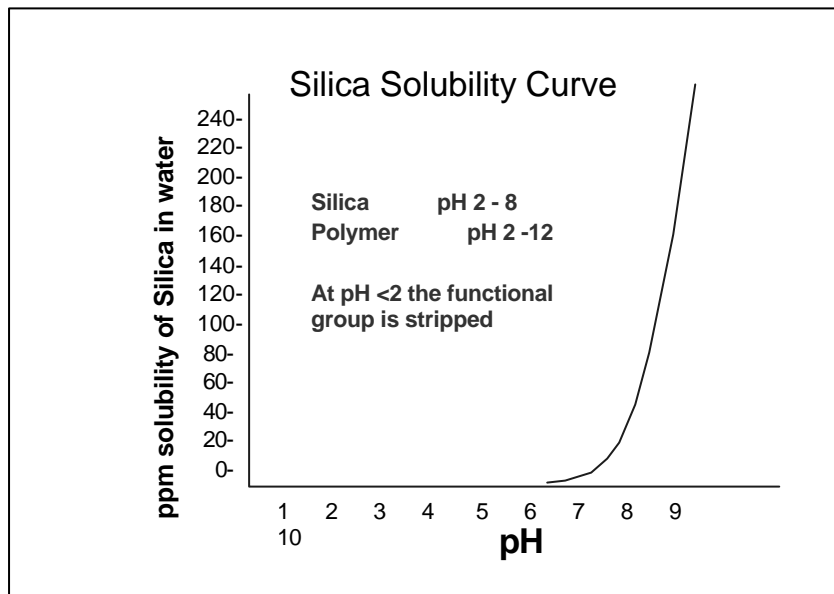
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  - Plate Count (Peak width relative to RT).
  - Peak Asymmetry,
  - Retention Time and/or Retention parameter
  - Relative Retention Time for Critical Pair of Analytes.
  - Peak Response
- \* Inject Multiple Runs
  - Precision (at least 5 injections)
  - Accuracy (Use Control Samples)

## Incorrect Sample Solvent

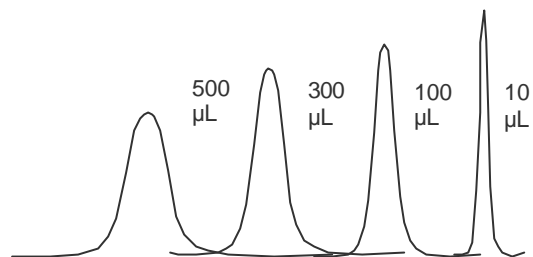


## Column Use

- ✓ Silicas hydrolyze at high pH
- ✓ Instability of bonded phase at low pH
- ✓ Elevated temperatures decrease column lifetime
- ✓ C18 approximately 1000 times more stable than CN

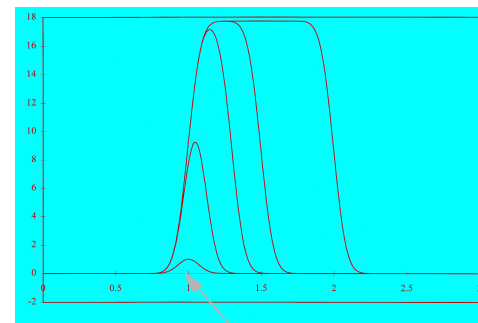


### Column/Volume Overload



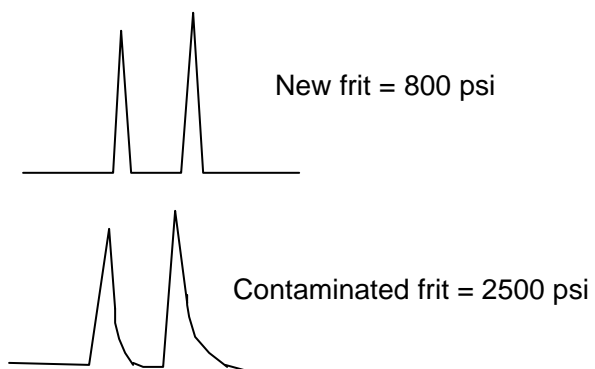
EFFECT OF INJECTION VOLUME ON PEAK DISTORTION

### Volume Overload



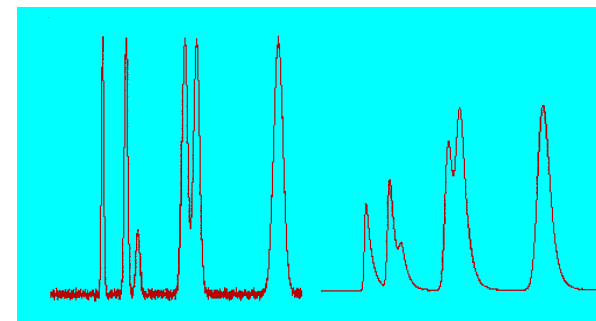
Lift-off Point Remains Constant  
Retention times are longer

### Contaminated In-Line Filter



### Extra Column Effects

Isocratic LC - Time Constant Differences  
(Detector setting)



left is 0.1 secs  
right is 10 secs  
note the noisy baseline on left chromatogram

## Performance Monitoring

Use Your Test Method  
(Known Performance)



\* Monitor at least One Peak in one injection  
- Plate Count (Peak width relative to RT),  
- Peak Asymmetry,

- Retention Time and/or Retention parameter
- Relative Retention Time for Critical Pair of Analytes.
- Peak Response

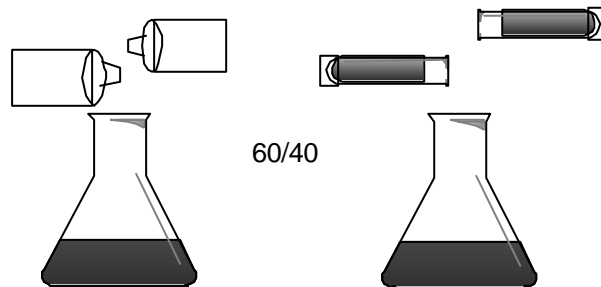
\* Inject Multiple Runs  
- Precision (at least 5 injections)  
- Accuracy (Use Control Samples)

## Retention Time Problems

- Reproducibility
  - ▶ Solvent Composition
  - ▶ Temperature
  - ▶ pH-Control
  - ▶ Ion Pairing
- Drifting Retention
  - ▶ Equilibration
  - ▶ Stationary Phase Stability
  - ▶ Column Contamination
  - ▶ Hydrophobic Collapse

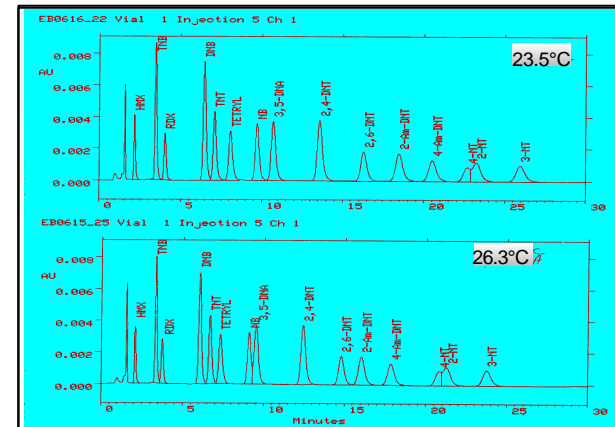
## Solvent Composition

■ Clearly specify HOW the Mobile Phase is to be prepared



pH Reminder: Measure pH Before the organic is added

## Temperature Control

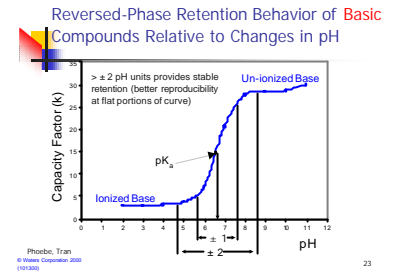
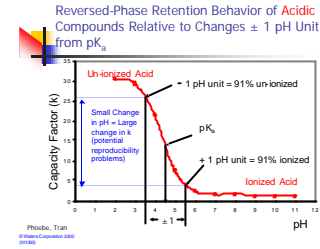


## Retention Time Reproducibility

Non-Column Influences:

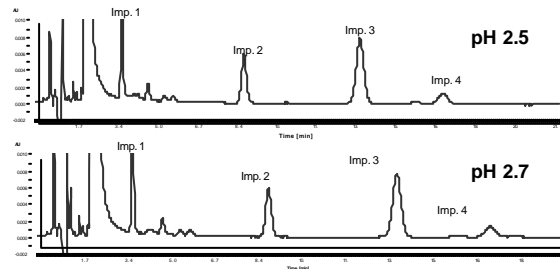
pH

- **Neutrals: No Influence**
- **Acids: Reduced Retention with Increasing pH**
- **Bases: Increased Retention with Increasing pH**
- **10% Change in Retention per 0.1 pH Units**



## pH Control AZT: Robustness Testing

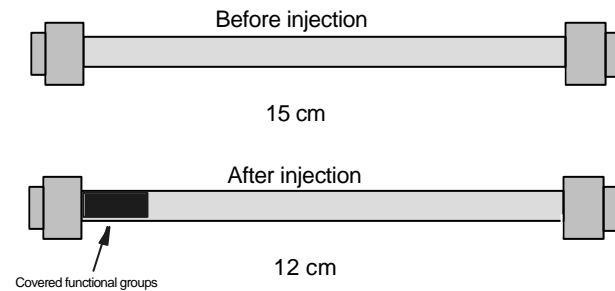
6% Methanol, 6% THF



## Changing Retention Times

Retention times getting shorter after each injection?

Sample analytes can adhere to and cover active functional group sites making a shorter column



## COLUMN REGENERATION

### REVERSE PHASE

1. Wash with unbuffered mobile phase
2. Wash with 100% water
3. Wash with methanol (or ACN)
4. Wash with THF or IPA
5. Wash with methylene chloride
6. Wash with N-Heptane
7. Wash with methylene chloride
8. Wash with methanol (or ACN)
9. Wash with water
10. Return to solvent

## Installation and Equilibration

- ✓ Purge column with 10 column volumes of mobile phase to be used in analysis  
(>>> 4.6x150mm = 25mL)
- ✓ Reversed-Phase (C18 etc.) columns equilibrate quicker than Normal Phase columns  
→ (magnitude of ten)
- ✓ Normal phase columns (silica or alumina) may take several DAYS at flow rates of 1.0 ml/min

## Installation and Equilibration

Internal Diameter (mm)	Length (mm)	Column Volume (mL)
2.0	150	.47
2.0	300	.94
3.9	50	.6
3.9	75	.9
3.9	100	1.2
3.9	150	1.8
3.9	300	3.6
4.6	150	2.5
4.6	250	4.2
5	100	2.0
8	100	5.0
7.8	300	4.3
19	150	43
25	100	49
30	300	212
40	100	125
47	300	520
50	300	589

## Solvent Viscosities

Solvent	Viscosity [cP] at 20° C
Acetone	0.32
Acetonitrile	0.37
Cyclohexanone	0.98
Diisopropylether	0.37
Diethylether	0.23
Dimethyl acetamide	2.1
Dimethyl formamide	0.92
Dimethyl sulfoxide	2.2
Dioxane	1.54
Ethanol	1.2
Ethylacetate	0.45
Hexafluoroisopropanol	1.0
iso-Propanol	2.5
Isooctane	0.5
Methanol	0.6

Remember: Some mixtures are more viscous than either pure solvent -- 50/50 MeOH/H2O is almost 2x

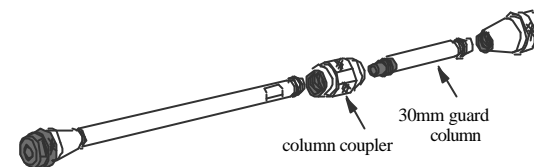
## Solvent Viscosities

Solvent	Viscosity [cP] at 20° C
Methyl acetate	0.37
Methylene chloride	0.44
Methylethyl ketone	0.4
n-Heptane	0.42
n-Hexane	0.33
N-Methylpyrrolidone	1.67 (25° C)
n-Pentane	0.235
n-Propanol	2.3
o-Dichlorobenzene	1.41
Tetrahydrofuran	0.46
Toluene	0.59
1,2,4-Trichlorobenzene	1.89 (25° C)
Water	1.0
m-Xylene	0.62
o-Xylene	0.81

Remember: Some mixtures are more viscous than either pure solvent -- 50/50 MeOH/H<sub>2</sub>O is almost 2x

## Column Protection

Major cause of column deterioration is contamination.  
Use of guard columns may increase column life-time to > 10,000 analyses

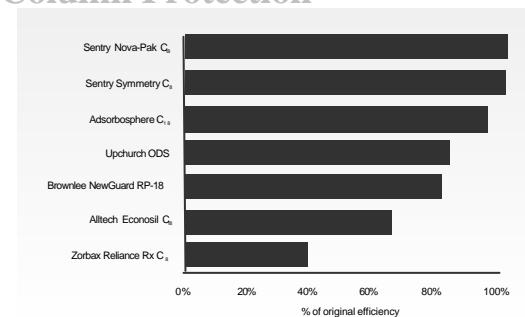


## Column Protection

1. Guard column should be regarded as a cost-effective sacrifice to extend analytical column life-time
2. Should contain IDENTICAL packing material as the analytical column  
e.g. using a different C18, with different retention properties could actually destroy the separation

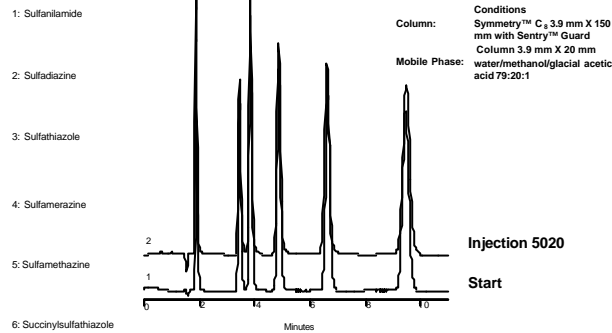
Well designed, well packed guard column will actually IMPROVE the analytical separation efficiency

## Column Protection



Effect of guard column on HPLC columns efficiencies  
Analytical column Nova-Pak C<sub>8</sub> (150 x 3.9mm or 4.6mm) except Zorbax Rx C<sub>8</sub> (150 x 4.6mm)  
Sample was 0.5<sup>mL</sup> injection acenaphthene (2.9 mg/mL) and acetone (34 <sup>mL</sup>/mL) in ACN/Water

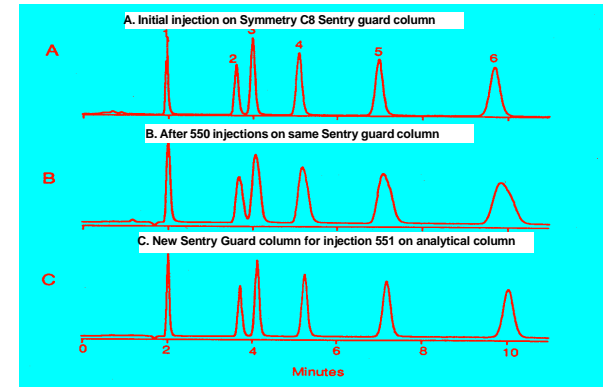
## Column Protection



Chromatogram of Life-time Test

\* Guard Column Changed Every 500 Injections

## Column Protection



Extension of column lifetime with Guard Column using a mixture of sulfa drugs as the sample

## Performance Monitoring

Use Your Test Method  
(Known Performance)



- \* Monitor at least One Peak in one injection
  - Plate Count (Peak width relative to RT),
  - Peak Asymmetry,
  - Retention Time and/or Retention parameter
  - Relative Retention Time for Critical Pair of Analytes.
  - Peak Response

- \* Inject Multiple Runs
  - Precision (at least 5 injections)
  - Accuracy (Use Control Samples)

## Variable Reported Concentrations Problems with Peak Response

### Linearity Test of Concentrations

#### - Check Injector (Use Standards)

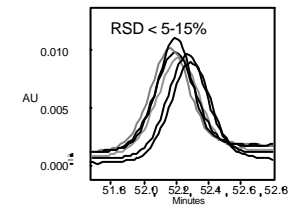
- \* Multiple Injections - Same Vial -- Syringe Problem  
or If Only 1st Injection Low -- Septa Problem
- \* Different Vials -- Evaporation -- Degradation
- \* Injection Volume Test (Weight before and after injection)

#### - Integration Software

- \* Electronic Peak Generator
- \* Poor Peak Shape

#### - Detector

- \* Cell Problem
- \* Lamp Failing



## Performance Monitoring

Use Your Test Method  
(Known Performance)



- \* Monitor at least One Peak in one injection
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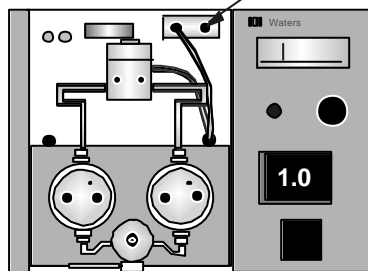
## Troubleshooting your Injector

Make repetitive injections of the same volume to check reproducibility.

Make injections of varying volumes to check linearity.

## Troubleshooting your pump

Measure Flow Rate



6000 psi

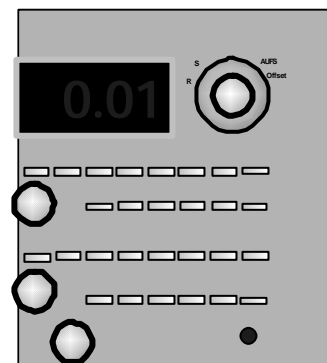
If pressure doesn't drop,  
outlet check is working

Right stroke

If pressure rises  
inlet check is working

Left stroke

## Troubleshooting your UV detector

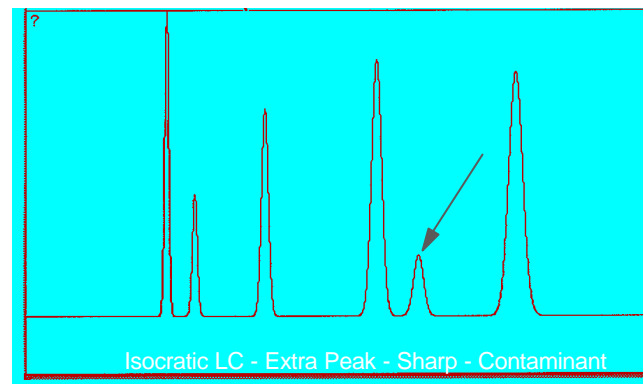


- ▶ Reference Energy
- ▶ Sample Energy
- ▶ Absorbance
- ▶ Offset

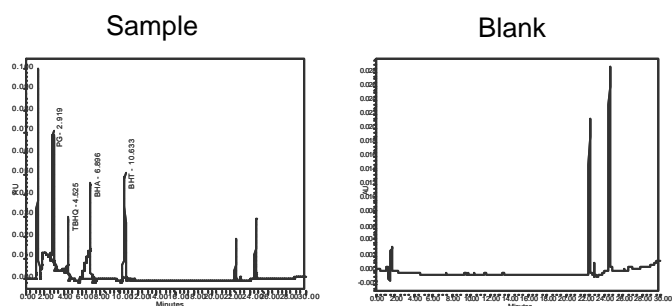
## Unusual Phenomena

- ▶ Extraneous Peaks
- ▶ Problems with Baseline

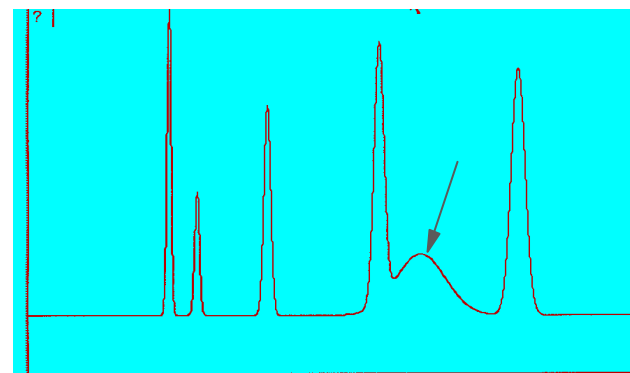
### Extraneous Peaks



### Extraneous Peaks

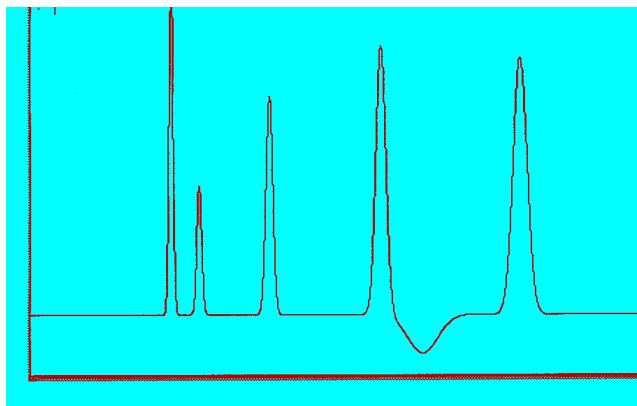


### Extraneous Peaks



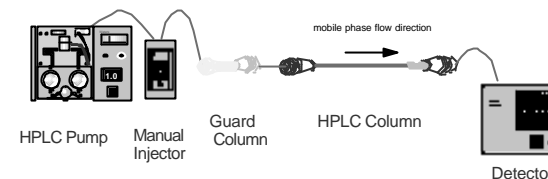
Isocratic LC - Broad -Peak from Previous Injection or Injector Contamination

**Isocratic LC - Negative Peak**  
often occurs in Ion-Pairing -- Sample Solvent

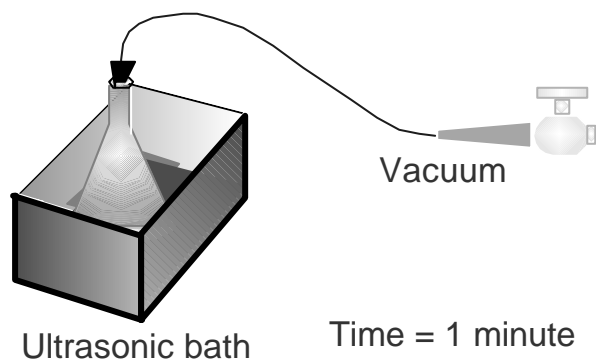


**Installation and Equilibration**

- ✓ Connect Column Inlet
- ✓ Purge Column at Low Flow Rate To Waste -- Then Connect to Detector  
(begin flow of analytical columns at 0.1 ml/min increase by 0.2 ml/min increments every 30 seconds until final analytical flow rate is reached)



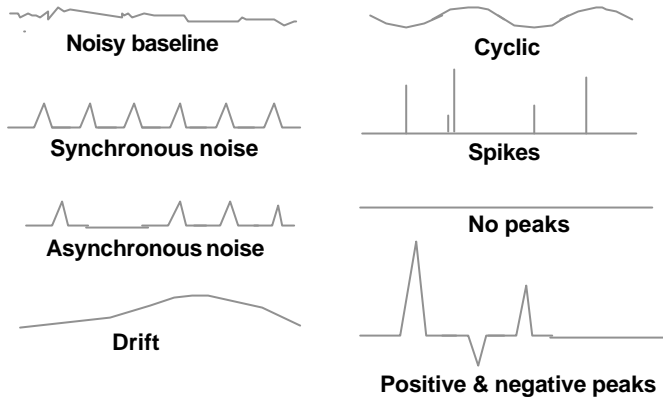
**Degas Solvents**



**Solvent Degassing Precautions**

1. Degas solvents prior to adding modifiers
2. Helium sparge is good, as long as solvent doesn't change due to volatility of solvents and/or additives
3. Solvents should be degassed daily

### BASELINE TROUBLESHOOTING



### NOISY BASELINE

#### INSTRUMENTAL

#### CHEMICAL

**WEAK DETECTOR LAMP**  
Replace lamp

**TRASH ELUTING OFF COLUMN**  
Flush column with strong solvent

**LEAKS**  
Stop leaks. Replace fittings

**DETECTOR CELL DIRTY**  
Flush with 6N nitric acid

**GAS IN MOBILE PHASE**  
Degas solvent

**GAS BUBBLE IN DETECTOR CELL**  
Put .009" tubing after detector (not RI!)

**ELECTRONIC NOISE**  
Remove source. Shield cables. Clean contacts

**SENSITIVITY TOO HIGH**  
Lower sensitivity. Adjust gain

### SYNCHRONOUS NOISE

**ALMOST ALWAYS CAUSED BY THE PUMP**

**Air in pump head - Prime pump and degas solvent**

**Check valve problem - Rebuild or replace**

**Broken plunger - Replace** (blame it on someone else)

**Mixing problem - Increase system volume**

**Electrical noise - Change circuits, remove source**

### ASYNCHRONOUS NOISE

#### BUBBLES

Degas mobile phase

#### GAS CAUGHT IN DETECTOR

Degas mobile phase. Put backpressure on cell.

#### LEAKS

Fix leaks, replace fittings

#### MIXING PROBLEMS

Increase system volume

#### PLUGGED LINES

Remove plug, flush system

#### ELECTRICAL PROBLEMS

Remove source, change circuits



## BASELINE DRIFT

### INSTRUMENTAL

#### GRADIENT - SOLVENT B ABSORBS MORE THAN SOLVENT A

Try a new mobile phase, use baseline subtraction

#### SOLVENT CHANGING (GAS ABSORPTION, EVAPORATION)

Helium sparge, enclose solvents

#### SOLVENT LEAKS

Tighten, replace fittings

#### THERMAL EFFECTS (ESPECIALLY RI, CONDUCTIVITY, ECD)

Cell temperature regulation

#### BACKPRESSURE CHANGES

Filter solvents and samples. Sample too viscous

#### SIPHONING (RI, CONDUCTIVITY, ECD)

Increase system volume

#### MIXING PROBLEMS

### CHEMICAL

#### COMPOUNDS ELUTING OFF COLUMN

Run strong solvent until baseline is stable

#### SOLVENTS IN GRADIENT ARE NOT PURE

Change the solvent batch or manufacturer.

Check if the solvents are gradient grade.



## CYCLIC BASELINE

### TEMPERATURE FLUCTUATIONS

Thermally insulate. Move away from ventilation. Increase cell temperature.

### MIXING PROBLEMS

Increase system volume

### GAS IN MOBILE PHASE

Degas solvents

### ELECTRICAL PROBLEMS

Change circuits, remove source

### ERRATIC PUMP

Repair pump

### PLUG

Remove obstruction, flush system



## SPIKES

### BUBBLES

Degas solvent

### POOR ELECTRICAL CONNECTION, LOOSE WIRING

Clean and tighten detector leads, check wiring, replace spade lugs.

### LAMP RELAY TRYING TO FIRE A DEAD LAMP

Replace lamp

### ELECTRICAL NOISE

Change circuits, remove source  
Common sources include switching valves, compressors, muffle furnaces, fraction collectors, power conditioners, lighting, poor power source.



## NO PEAKS

### INSTRUMENTAL

- Injector not making injections
- Pump not pumping
- Dead detector
- Integrator/recorder not wired correctly
- Gain setting too low
- Leaks

#### WHAT TO DO:

Inject acetone solution to make a peak

### CHEMICAL

- Column retaining all compounds
- Bad or wrong mobile phase
- Bad or wrong standard or sample
- Wrong guard column

#### WHAT TO DO:

Remove column and inject acetone solution to make a peak



## NEGATIVE & POSITIVE PEAKS

### INSTRUMENTAL

#### Air bubbles passing through cell

Degas mobile phase

#### You're using an RI detector

May be normal since peak direction is a function of refractive index differential from mobile phase

#### All peaks negative - polarity wrong

Reverse leads or change detector polarity

#### All peaks negative - You're using indirect UV

Change polarities or reverse leads

### CHEMICAL

#### Some eluting compounds absorb less than solvent

Use a different or cleaner solvent

## Strange things can happen!

Radio transmitters can cause baseline noise

Contaminated helium bottles and lines can cause noise

System components can get coated with impurities

Solvent vendors can misname solvent bottles

Some filters can introduce particulates

### Basic assumptions

1. The HPLC is plugged in and turned on
2. Solvent is in the reservoir
3. The pumps are primed and in good working order
4. The HPLC is plumbed and wired correctly
5. The detector has a good lamp in it
6. The solvent bottle doesn't have a vacuum on it
7. You're not using acetone for solvent at 195 nm
8. You're not injecting rocks
9. You're not doing a water to hexane gradient
10. You're not trying to detect sugars at 254 nm
11. You're not mixing ME OH and water without degassing
12. You're not sparging with nitrogen or air
13. You're not running water through a silica column
14. Solvent pH is not 13 on a silica base column
15. You're not running a 1M NaCl to 100% ACN gradient
16. You're not doing gradients with an RI detector
17. Your RI is not under the air conditioner vent
18. No buffer stalagmites on your pump heads
19. HCl vapors are not blowing onto your HPLC
20. You're having a wonderful time!



## Things not to do:

- \* Plug the outlet of your RI detector
- \* Flush your system with methanol after running buffer
- \* Inject samples that may precipitate in the eluent
- \* Run long durations with HCl on your stainless steel HPLC
- \* Filter organic solvents through aqueous filters
- \* Spill buffers onto HPLC electronics
- \* Try to change the column frits while it still has pressure in it
- \* Store THF on the shelf, uncapped, for weeks
- \* Pump cyclohexane above 2000 psi
- \* Tightly seal your mobile phase container
- \* Cut tubing with a wire cutter