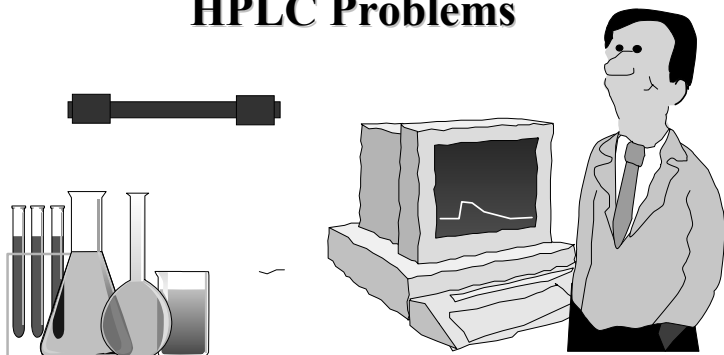


Troubleshooting Common HPLC Problems

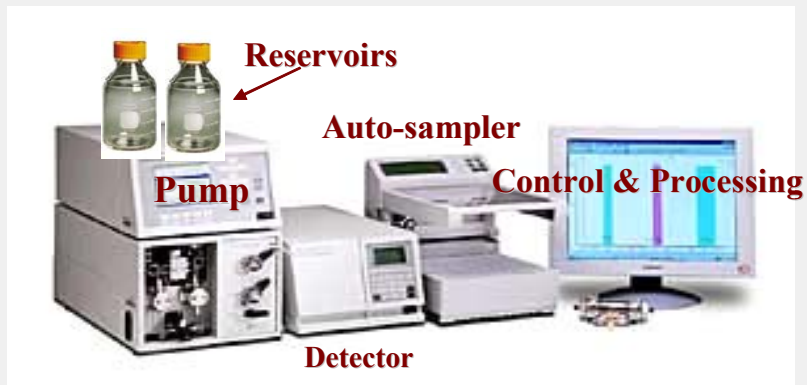


<http://www.hplc1.com/shodex/english/dd.htm>

HPLC Troubleshooting



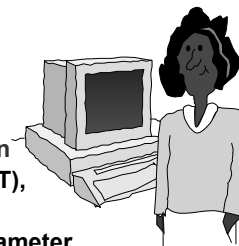
HPLC System



Performance Monitoring

Parameters Tested Before any Result is reported:

- * 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)



Test of Blank

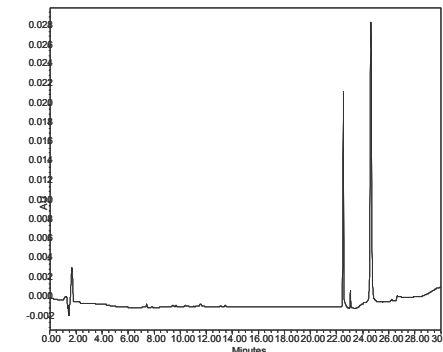
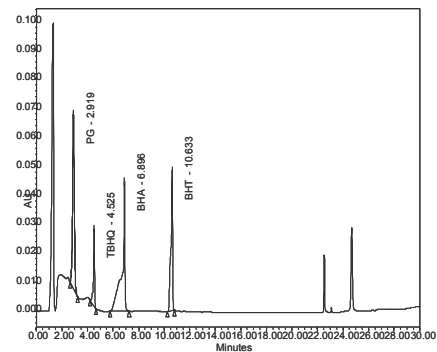
Quality Control

VIAL	SAMPLE NAME	INJ VOL	No of Inj	Function	Method	Run Time	Sample Weight	Dilution
1	Blank	20.0	1	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
2	System Suitability	20.0	1	Inject Samples	SST Method Set	10.00	1.00000	1.00000
				Clear Calibration	LC Demo Method Set			
3	Std1	20.0	5	Inject Standards	LC Demo Method Set	10.00	1.00000	1.00000
4	Std2	20.0	2	Inject Standards	LC Demo Method Set	10.00	1.00000	1.00000
				Report	LC Calibration Report			
				Report	Standard Comparison			
				Clear Calibration	LC Demo Method Set			
1	Std1	20.0	1	Inject Standards	LC Demo Method Set	10.00	1.00000	1.00000
2	Unk.1	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
3	Unk.2	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
4	Unk.3	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
5	Unk.4	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
6	Unk.5	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
7	Unk.6	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
1	Std1	20.0	1	Inject Standards	LC Demo Method Set	10.00	1.00000	1.00000
				Clear Calibration	LC Demo Method Set			
				Calibrate	LC Demo Method Set			

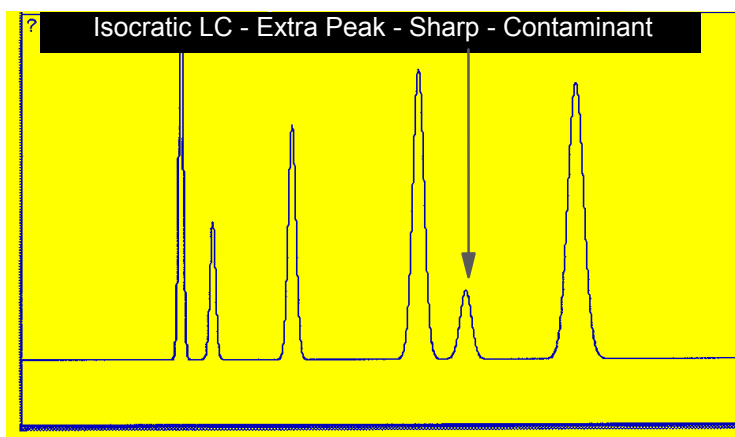
Test for Extraneous Peaks

Sample

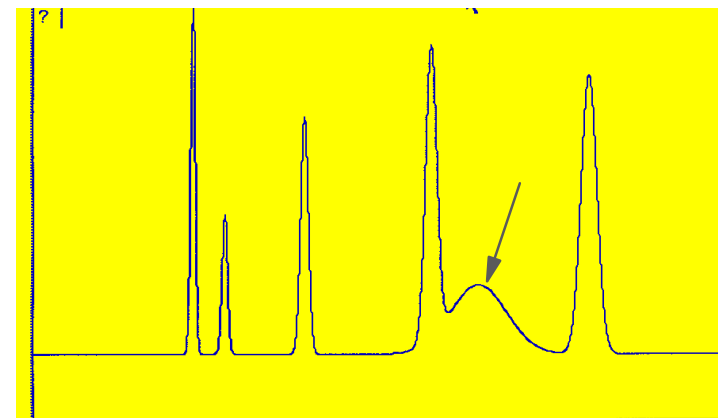
Blank



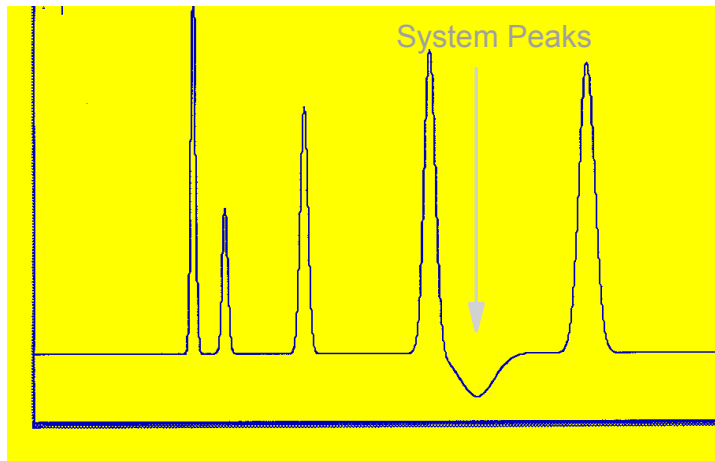
Extraneous Peaks



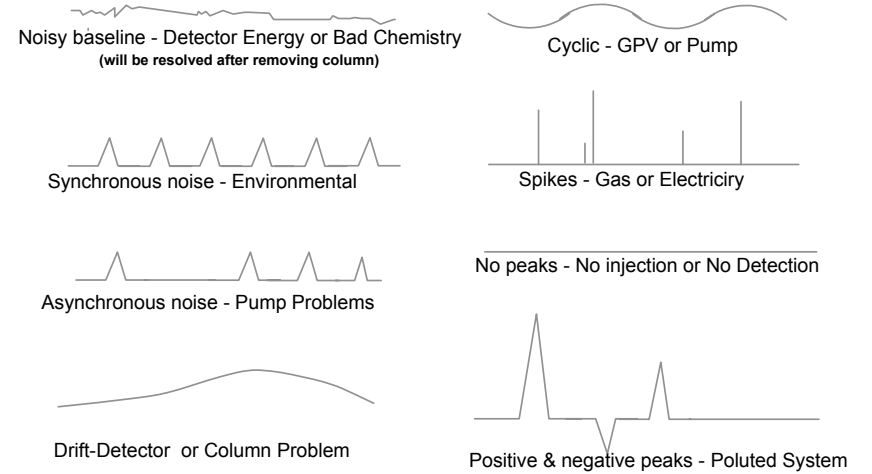
Extraneous Peaks



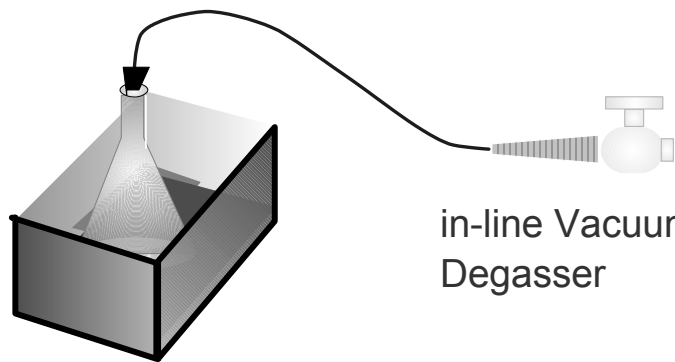
**Isocratic LC - Negative Peak
often occurs in Ion-Pairing -- Sample Solvent**



BASELINE TROUBLESHOOTING



Degas Solvents



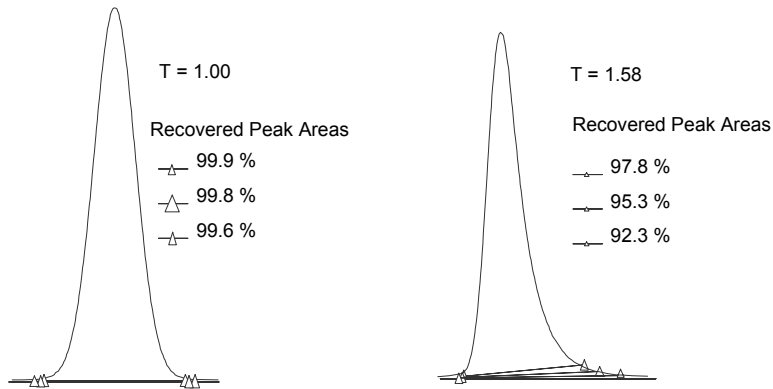
or Helium Sparge
or Ultrasonic bath

System Suitability

Quality Control

VIAL	SAMPLE NAME	INJ VOL	No of inj	Function	Method	Run Time	Sample Weight	Dilution
1	Blank	20.0	1	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
2	System Suitability	20.0	1	Inject Samples	SST Method Set	10.00	1.00000	1.00000
				Clear Calibration	LC Demo Method Set			
3	Std1	20.0	5	Inject Standards	LC Demo Method Set	10.00	1.00000	1.00000
4	Std2	20.0	2	Inject Standards	LC Demo Method Set	10.00	1.00000	1.00000
				Report	LC Calibration Report			
				Report	Standard Comparison			
				Clear Calibration	LC Demo Method Set			
1	Std1	20.0	1	Inject Standards	LC Demo Method Set	10.00	1.00000	1.00000
2	Unk.1	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
3	Unk.2	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
4	Unk.3	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
5	Unk.4	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
6	Unk.5	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
7	Unk.6	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
1	Std1	20.0	1	Inject Standards	LC Demo Method Set	10.00	1.00000	1.00000
				Clear Calibration	LC Demo Method Set			
				Calibrate	LC Demo Method Set			

Integration Errors Caused by Tailing



System Suitability Tests: Peak Shape & Symmetry

- ▶ Causes of Bad Peak Shapes:
 - ▶ Instrumental
 - ▶ Chemical

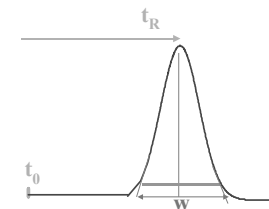
Plate Count - Efficiency of the Separation

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

Performance Monitoring

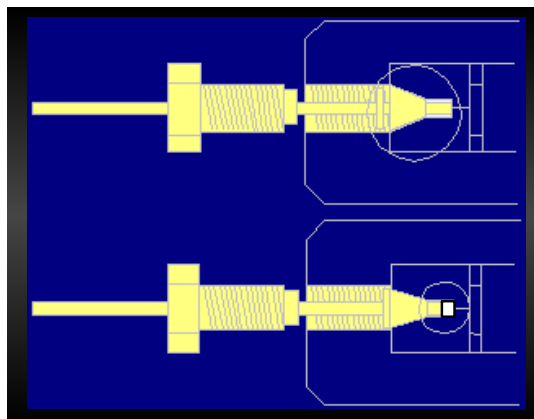
Column Efficiency:
N = the number of Theoretical Plates
a = is a constant depending on the Method used
t_r = retention time of peak
W = the peak width (time units) at a given peak height

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



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

Extra-Column Band Spreading

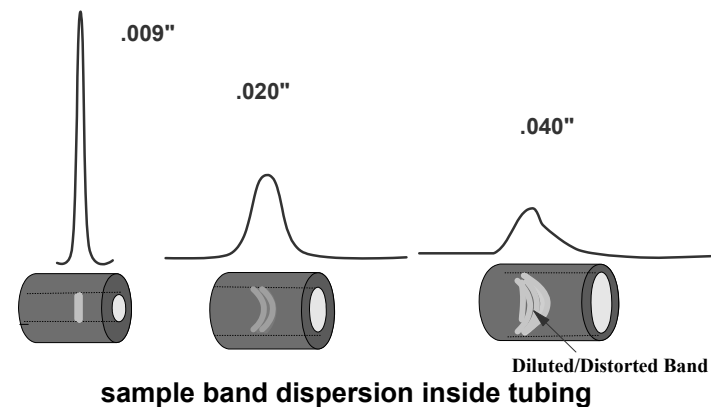


Column Connection Contribution



Performance Monitoring

Effect of Connecting Tubing on System Bandspreading

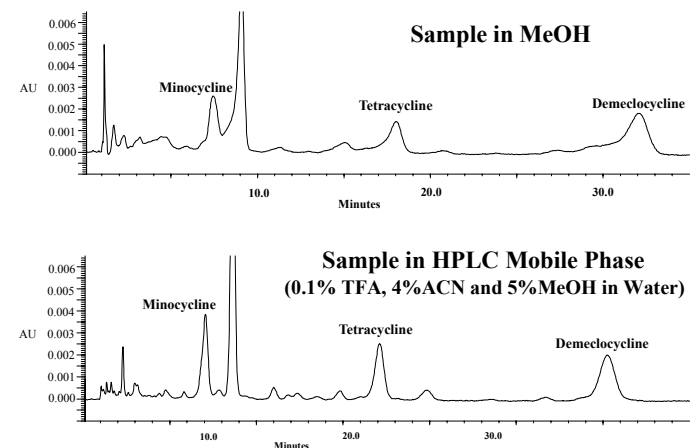


System Suitability Tests:

Peak Shape & Symmetry

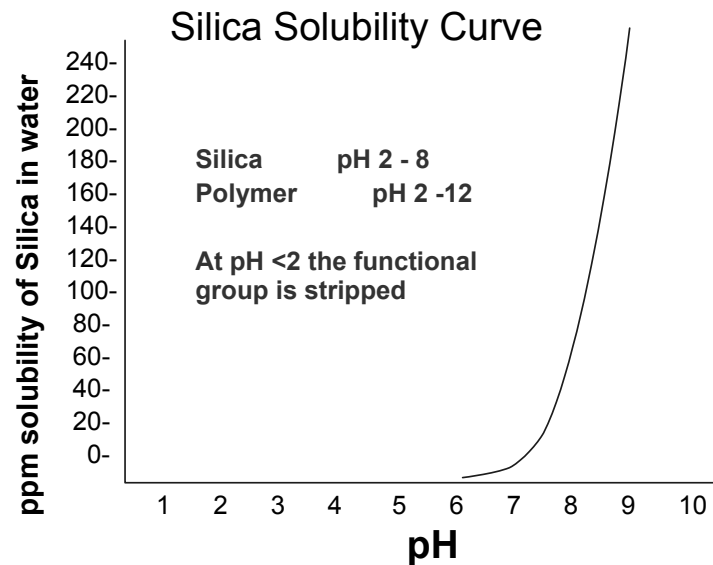
- ▶ Causes of Bad Peak Shapes:
 - ▶ Instrumental
 - ▶ Chemical

Incorrect Sample Solvent

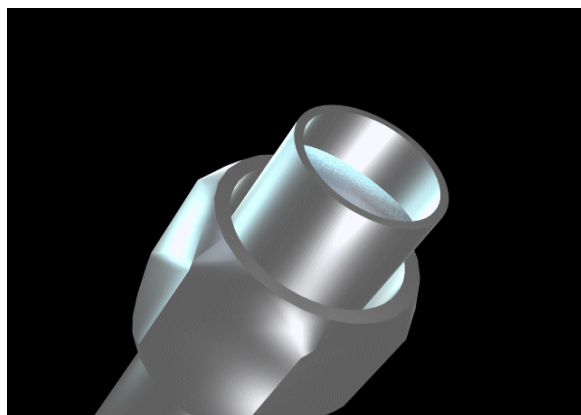


Column Use

- ✓ 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

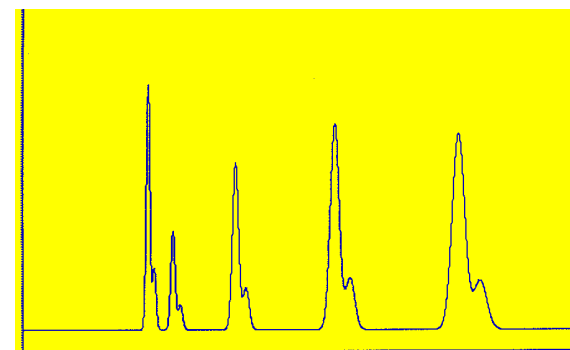


Column Collapse



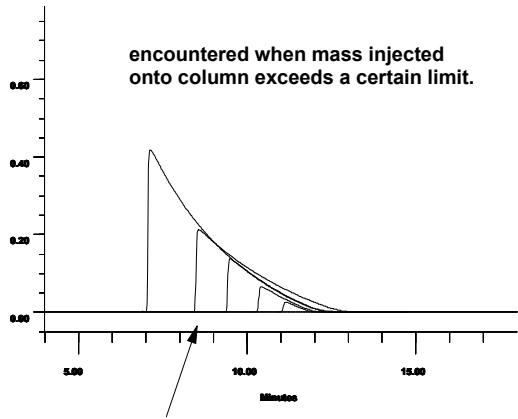
voided column

Column Collapse



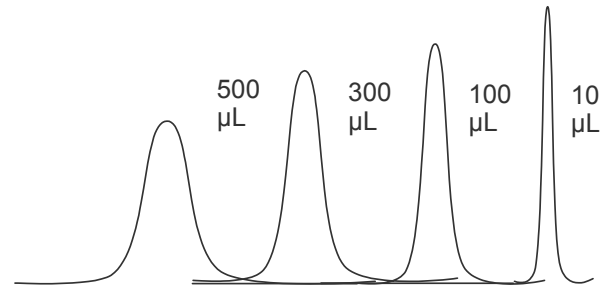
voids - high back pressure,
distorted and/or double peaks

Mass Overload



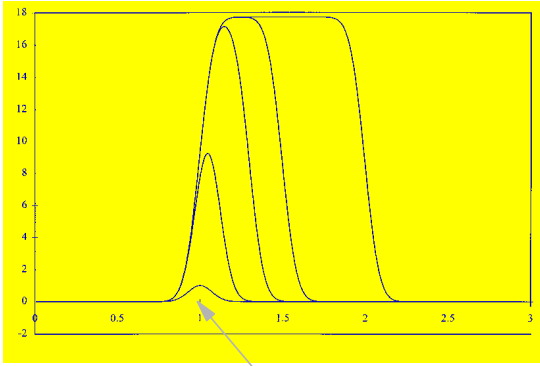
Lift-off Point Moves Earlier
Retention times are shorter

Column/Volume Overload



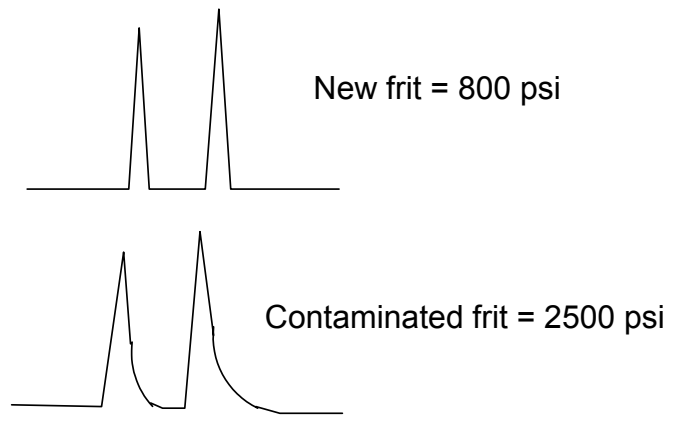
EFFECT OF INJECTION VOLUME
ON PEAK DISTORTION

Volume Overload

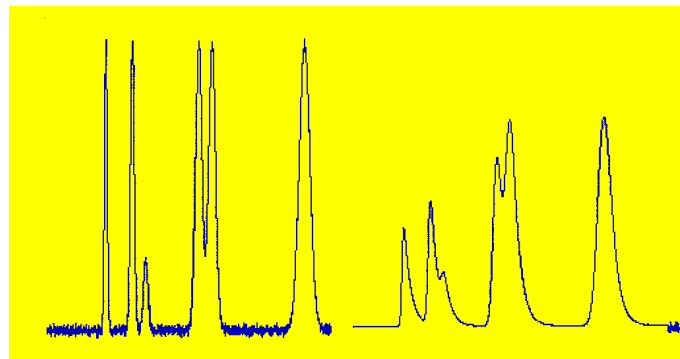


Lift-off Point Remains Constant
Retention times are longer

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

Test of Precision

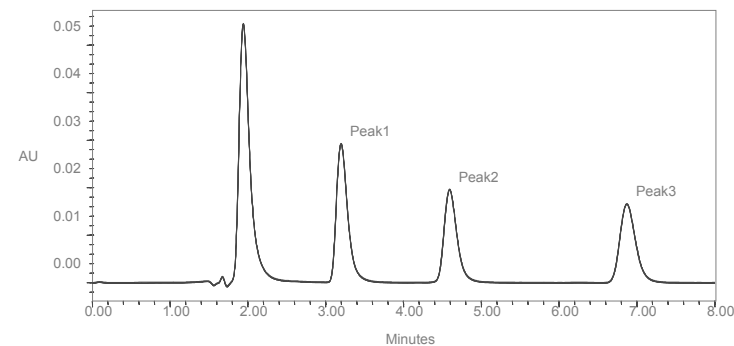
Quality Control

VIAL	SAMPLE NAME	INJ VOL	No of Inj	Function	Method	Run Time	Sample Weight	Dilution
1	Blank	20.0	1	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
2	System Suitability	20.0	1	Inject Samples	SST Method Set	10.00	1.00000	1.00000
				Clear Calibration	LC Demo Method Set			
3	Std1	20.0	5	Inject Standards	LC Demo Method Set	10.00	1.00000	1.00000
4	Std2	20.0	2	Inject Standards	LC Demo Method Set	10.00	1.00000	1.00000
				Report	LC Calibration Report			
				Report	Standard Comparison			
				Clear Calibration	LC Demo Method Set			
1	Std1	20.0	1	Inject Standards	LC Demo Method Set	10.00	1.00000	1.00000
2	Unk.1	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
3	Unk.2	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
4	Unk.3	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
5	Unk.4	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
6	Unk.5	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
7	Unk.6	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
1	Std1	20.0	1	Inject Standards	LC Demo Method Set	10.00	1.00000	1.00000
				Clear Calibration	LC Demo Method Set			
				Calibrate	LC Demo Method Set			

Troubleshooting Reproducibility

Retention time

Overlaid Chromatograms Good Precision



SampleName 2690_Eau40_60CH3OH_2mm_D Vial 4 Injection 1
 SampleName 2690_Eau40_60CH3OH_2mm_D Vial 4 Injection 2
 SampleName 2690_Eau40_60CH3OH_2mm_D Vial 4 Injection 3
 SampleName 2690_Eau40_60CH3OH_2mm_D Vial 4 Injection 4
 SampleName 2690_Eau40_60CH3OH_2mm_D Vial 4 Injection 5
 SampleName 2690_Eau40_60CH3OH_2mm_D Vial 4 Injection 6

Test of Precision

Component Summary Table

Name: Peak2

	SampleName	Vial	Inj	Name	RT	Area	Height	Area_Amount
1	1mL/min Grad	1	1	Peak2	8.778	128107	22379	128107
2	1mL/min Grad	1	2	Peak2	8.775	128062	22490	128062
3	1mL/min Grad	1	3	Peak2	8.779	129036	22614	129036
4	1mL/min Grad	1	4	Peak2	8.774	128060	22443	128060
5	1mL/min Grad	1	5	Peak2	8.786	128649	22521	128649
Mean						128383	22489	128383
Std. Dev.						442	88	442
% RSD						0.3	0.4	0.34

Component Summary Table

Name: Peak3

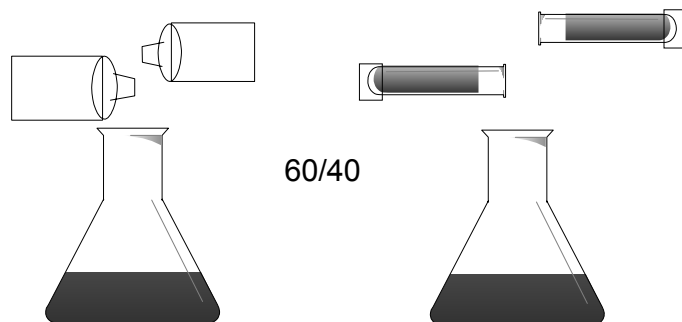
	SampleName	Vial	Inj	Name	RT	Area	Height	Area_Amount
1	1mL/min Grad	1	1	Peak3	14.767	161402	18346	161402
2	1mL/min Grad	1	2	Peak3	14.768	159816	18278	159816
3	1mL/min Grad	1	3	Peak3	14.765	162456	18294	162456
4	1mL/min Grad	1	4	Peak3	14.760	160979	18243	160979
5	1mL/min Grad	1	5	Peak3	14.773	160820	18178	160820
Mean						161094	18268	161094
Std. Dev.						958	62	958
% RSD						0.6	0.3	0.59

Reasons for Problems with Retention Time and Area Reproducibility

- Chemistry of the Separations
 - ▶ Solvent Composition
 - ▶ Temperature
 - ▶ pH-Control
 - ▶ Equilibration
 - ▶ Stationary Phase Stability
 - ▶ Column Contamination
 - ▶ Hydrophobic Collapse
 - ▶ Ion Pairing

Solvent Composition

- Clearly specify HOW the Mobile Phase is to be prepared



pH Reminder: Measure pH Before the organic is added

Compositional Ripple



Proportioned Solvents
A B A B A B

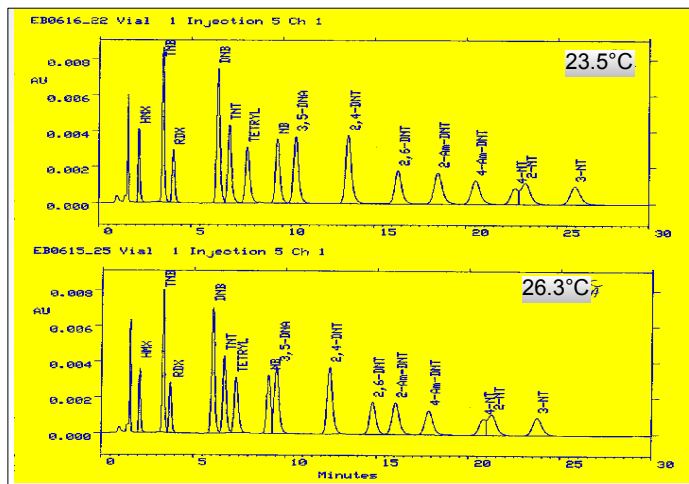
- ▶ Compositional ripple is change in solvent composition over time



Incomplete Mixing

- ▶ Ripple is the results of incomplete mixing

Temperature Control



Retention Time Reproducibility

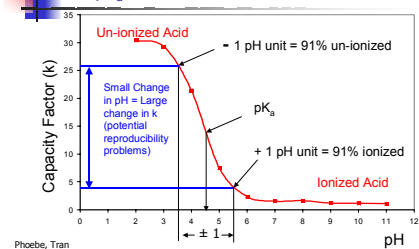
Non-Column Influences:

pH

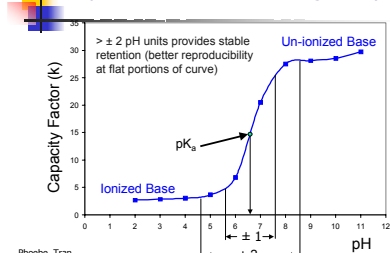
- Neutrals: No Influence
- Acids: Reduced Retention with Increasing pH
- Bases: Increased Retention with Increasing pH
- 10% Change in Retention per 0.1 pH Units

Retention Times as Function of pH of Mobile Phase

Reversed-Phase Retention Behavior of **Acidic** Compounds Relative to Changes ± 1 pH Unit from pK_a

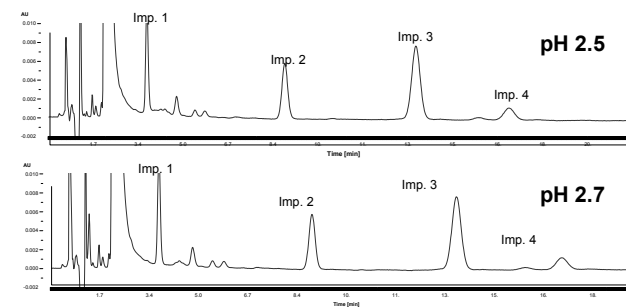


Reversed-Phase Retention Behavior of **Basic** Compounds Relative to Changes in pH



pH Control AZT: Robustness Testing

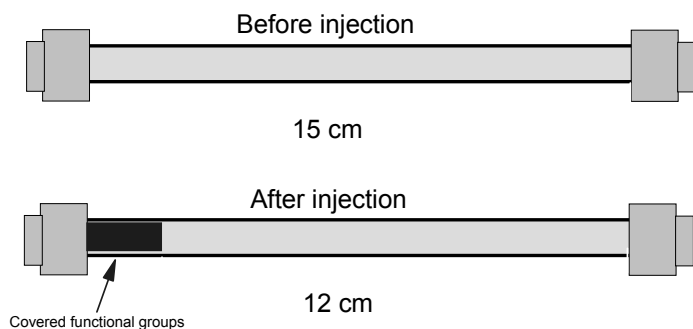
6% Methanol, 6% THF



Column Contamination

Retention times getting shorter after each injection?

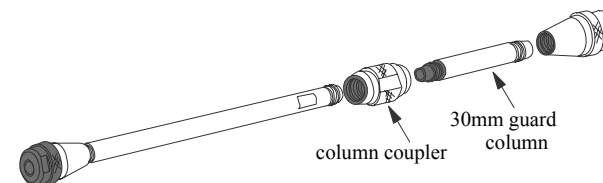
Sample analytes can adhere to and cover active functional group sites making a shorter column



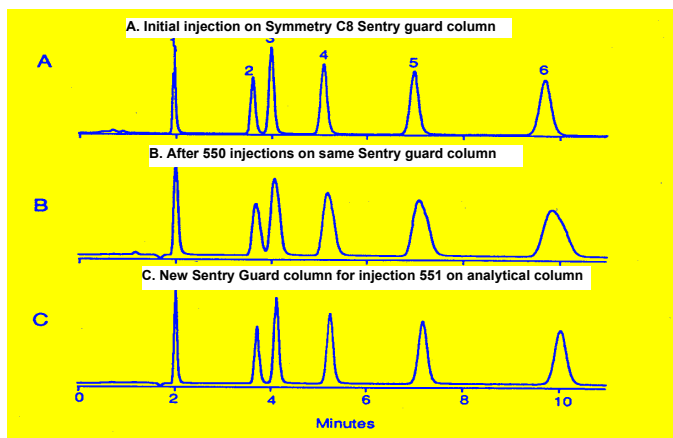
Column Protection

Major cause of column deterioration is contamination.

Use of guard columns may increase column life-time to > 10,000 analyses



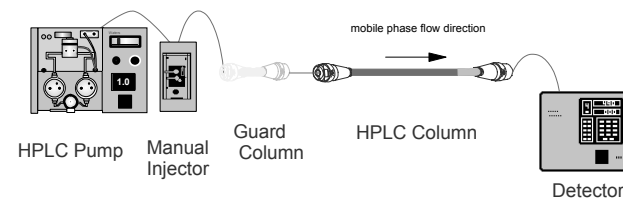
Column Protection



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

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)



Installation and Equilibration 20x Column Volumes

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

Troubleshooting Reproducibility

Area

Reasons for Problems with Area Reproducibility

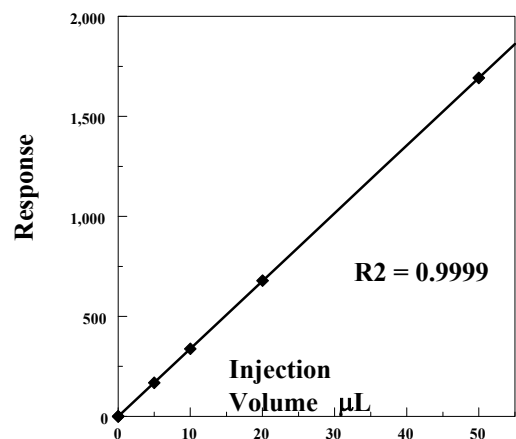
- Instrumental Parameters
 - ▶ Injection Volume
 - ▶ Detector Energy
 - ▶ Detector Impurity
 - ▶ Solvent Composition (GPV)

Troubleshooting your Injector

Make repetitive injections of the same volume to check reproducibility.

Make injections of varying volumes to check linearity.

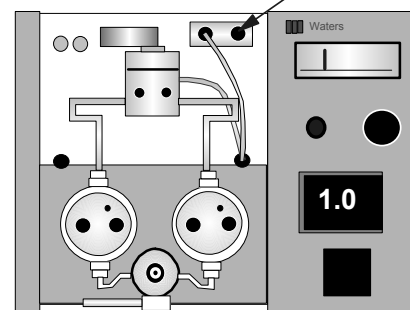
Injector Linearity



- ▶ Different volumes of the same standard
- ▶ Methods transfer

Troubleshooting your pump

Measure Flow Rate



6000 psi

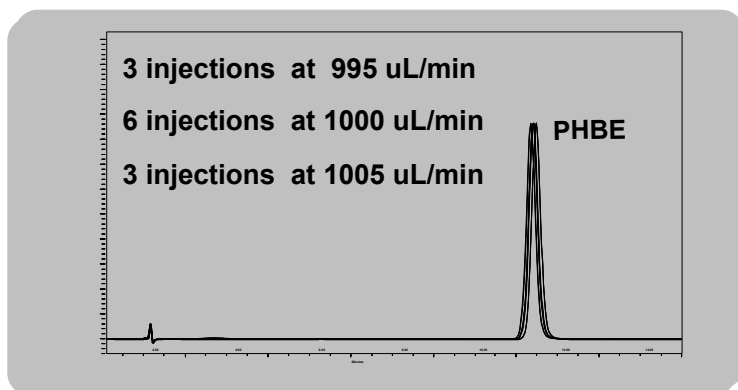
If pressure doesn't drop, outlet check is working

Right stroke

If pressure rises inlet check is working

Left stroke

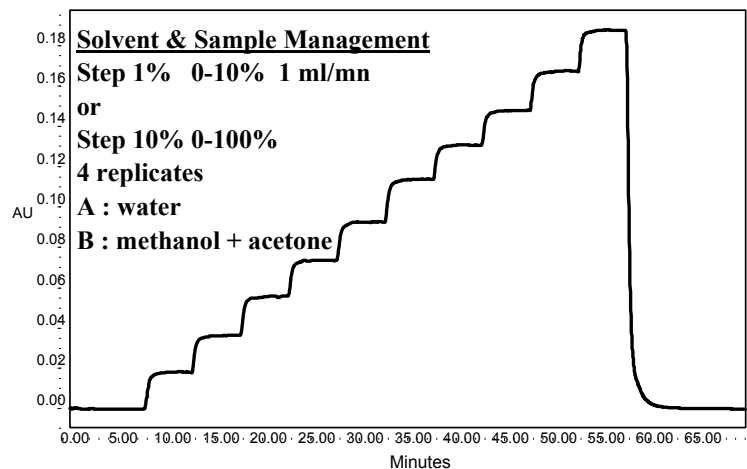
Area Changes with Flow Rate



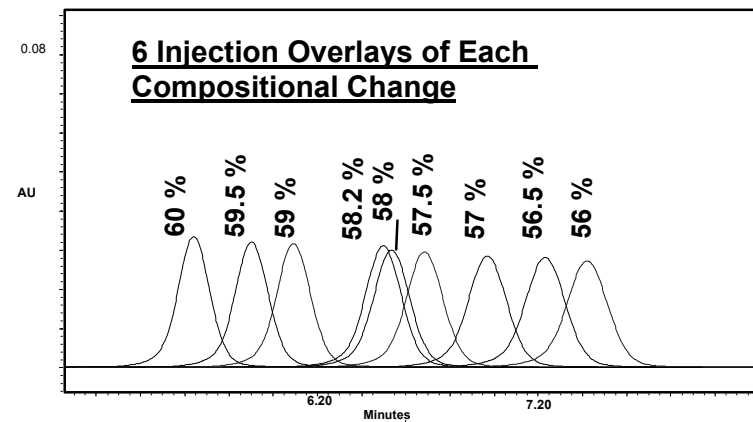
RSD is Affected by the Precision of the Pump Flow

Flow Rate uL/mn	RT Average	RT %RSD	Area Average (X1000)	Area %RSD	Injections Number
995	10.487	0.024	1405	0.026	3
1000	10.417	0.063	1398	0.061	6
1005	10.360	0.060	1383	0.049	3
All Data	10.420	0.456	1396	0.590	12

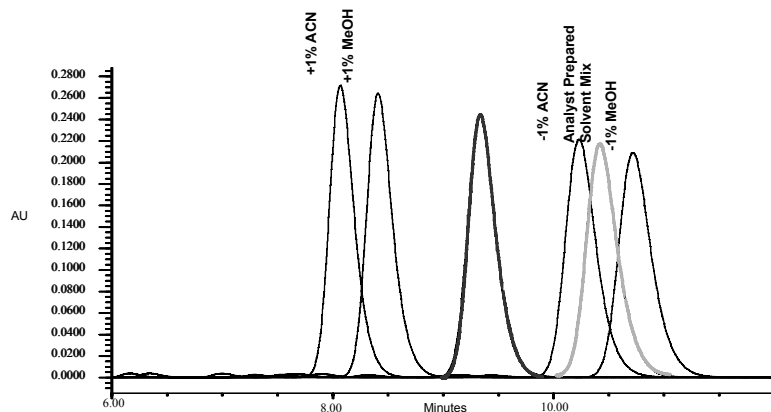
Troubleshooting the Gradient Proportioning Valve



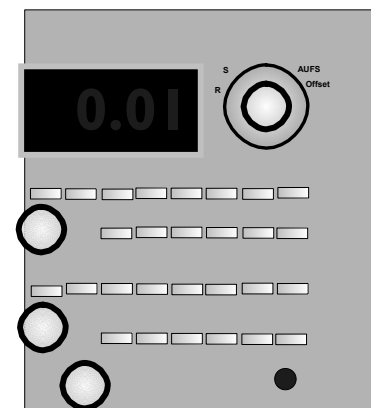
Overlays of Those Small Compositional Changes



Overlay of 5 Injections at each Compositional Change



The UV detector Can Also Effect Peaks Area



- ▶ Reference Energy
- ▶ Sample Energy
- ▶ Absorbance
- ▶ Offset
- ▶ Dirty Flow Cell

PROPERTIES OF DETECTORS

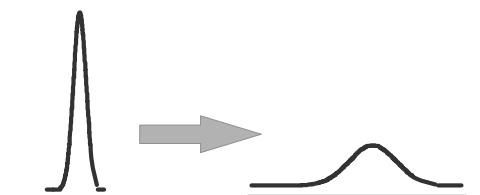
CONTRIBUTION TO BAND BROADENING



RESPONSE TIME



FLOW-CELL VOLUME



Detector Qualification

Wavelength Accuracy Test

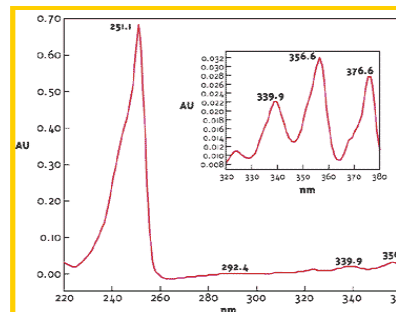


Figure 1. Spectrum of anthracene solution (1 mg/mL in acetonitrile) from Waters 996 PDA detector shows the annotations of λ_{max} . The inset shows an expanded view of the bands centered between 320 and 380 nm.

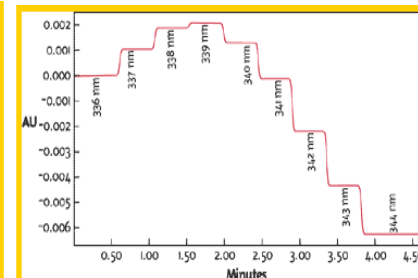


Figure 2. Calibrations of a UV-vis detector (Waters 2487) nm by incremental scanning from 336 to 344 nm. of anthracene is determined to be at 339 nm for this. The autozero on wavelength function in the detector nactivated for this test.

http://pubs.acs.org/subscribe/journals/tcaw/10/i02/html/02dong_tab1.html

Quality Control

VIAL	SAMPLE NAME	INJ VOL	No of Inj	Function	Method	Run Time	Sample Weight	Dilution
1	Blank	20.0	1	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
2	System Suitability	20.0	1	Inject Samples	SST Method Set	10.00	1.00000	1.00000
				Clear Calibration	LC Demo Method Set			
3	Std1	20.0	5	Inject Standards	LC Demo Method Set	10.00	1.00000	1.00000
4	Std2	20.0	2	Inject Standards	LC Demo Method Set	10.00	1.00000	1.00000
				Report	LC Calibration Report			
				Report	Standard Comparison			
				Clear Calibration	LC Demo Method Set			
1	Std1	20.0	1	Inject Standards	LC Demo Method Set	10.00	1.00000	1.00000
2	Unk.1	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
3	Unk.2	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
4	Unk.3	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
5	Unk.4	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
6	Unk.5	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
7	Unk.6	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
1	Std1	20.0	1	Inject Standards	LC Demo Method Set	10.00	1.00000	1.00000
				Clear Calibration	LC Demo Method Set			
				Calibrate	LC Demo Method Set			

Accuracy Test

Standards Comparison

Determination of a Control Standard: Test of Accuracy

Amount Std → Response Std

Amount Control → Response Unk

$$\text{Amount Control} = \frac{\text{Amount Std}}{\text{Response Std}} \times \text{Response Unk}$$

$$\text{Accuracy: } \frac{\text{Amount Control Determined}}{\text{Amount Control Prepared}} = 0.99-1.01$$

SOLUTION STABILITY

Time Point (Day)	Conditions	% Recovery	% Initial
Initial	Room Temp	99	N/A
1	Room Temp	101	102
	Refrigerated	101	102
2	Room Temp	99	100
	Refrigerated	99	100
3	Room Temp	98	99
	Refrigerated	98	99
7	Room Temp	98	99
	Refrigerated	98	99

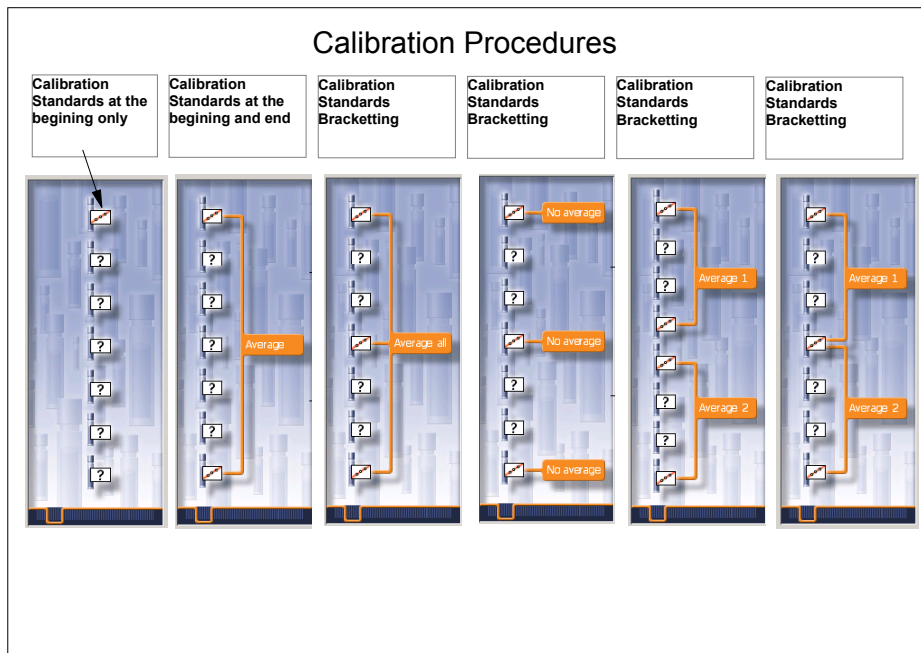
Criteria: %Recovery 98-102%; %Initial: 95-105%

Quality Control

VIAL	SAMPLE NAME	INJ VOL	No of Inj	Function	Method	Run Time	Sample Weight	Dilution
1	Blank	20.0	1	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
2	System Suitability	20.0	1	Inject Samples	SST Method Set	10.00	1.00000	1.00000
				Clear Calibration	LC Demo Method Set			
3	Std1	20.0	5	Inject Standards	LC Demo Method Set	10.00	1.00000	1.00000
4	Std2	20.0	2	Inject Standards	LC Demo Method Set	10.00	1.00000	1.00000
				Report	LC Calibration Report			
				Report	Standard Comparison			
				Clear Calibration	LC Demo Method Set			
1	Std1	20.0	1	Inject Standards	LC Demo Method Set	10.00	1.00000	1.00000
2	Unk.1	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
3	Unk.2	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
4	Unk.3	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
5	Unk.4	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
6	Unk.5	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
7	Unk.6	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
1	Std1	20.0	1	Inject Standards	LC Demo Method Set	10.00	1.00000	1.00000
				Clear Calibration	LC Demo Method Set			
				Calibrate	LC Demo Method Set			

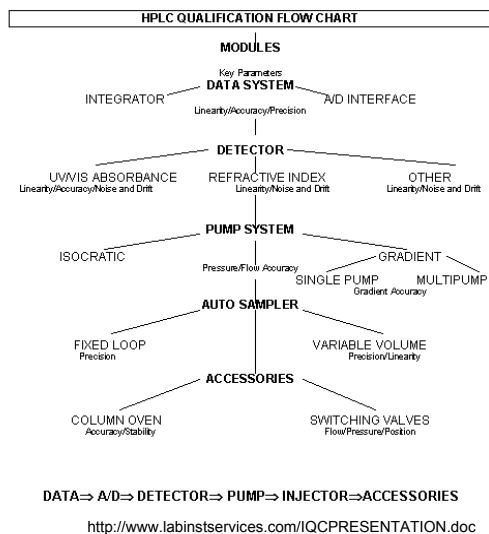
Quality Control

VIAL	SAMPLE NAME	INJ VOL	No of Inj	Function	Method	Run Time	Sample Weight	Dilution
1	Blank	20.0	1	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
2	System Suitability	20.0	1	Inject Samples	SST Method Set	10.00	1.00000	1.00000
				Clear Calibration	LC Demo Method Set			
3	Std1	20.0	5	Inject Standards	LC Demo Method Set	10.00	1.00000	1.00000
4	Std2	20.0	2	Inject Standards	LC Demo Method Set	10.00	1.00000	1.00000
				Report	LC Calibration Report			
				Report	Standard Comparison			
				Clear Calibration	LC Demo Method Set			
1	Std1	20.0	1	Inject Standards	LC Demo Method Set	10.00	1.00000	1.00000
2	Unk.1	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
3	Unk.2	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
4	Unk.3	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
5	Unk.4	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
6	Unk.5	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
7	Unk.6	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
1	Std1	20.0	1	Inject Standards	LC Demo Method Set	10.00	1.00000	1.00000
				Clear Calibration	LC Demo Method Set			
				Calibrate	LC Demo Method Set			



Periodic Tests of the Systems: Preventative Maintenance

Qualification of HPLC Systems



Typical Values

<http://www.geocities.com/HotSprings/Spa/6896/selpar.pdf>

Parameter	Procedure (*)	User Limit
Leak testing	Flow test by volume or weight/time	± 2.5%
Baseline drift	ASTM Method E19.09, 20 min	2 x 10 ⁻³ AU
Baseline noise	ASTM Method E19.09, 20 x 1 min	6 x 10 ⁻⁵ AU
Precision of injection volume	6 x injection of caffeine standard, RSD of peak areas	1 % RSD
Precision of flow rate	6 x injection of caffeine standard, RSD of retention times	1 % RSD
Detector linearity	inject 5 standards	1.0 AU, 5%
Wavelength accuracy	holmium oxide filter	± 2 nm
Temperature accuracy	comparison with external measuring device	± 1 °C
Temperature precision	monitoring temperature over 20 min	± 0.25 °C
Autosampler carry over	Injection of blank solvent after large concentration	< 0.3 %
Mobile phase composition accuracy	Step gradients from 4 to 7 % B, step heights relative to 100%, with acetone tracer	± 1 %
Mobile phase composition ripple	Peak to peak noise at 4, 5, 6 and 7% B	0.2 %