High Performance Liquid Chromatography (HPLC) Overview

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Homepage: http://www.forumsci.co.il/HPLC

HPLC COURSE LAYOUT

- **Introduction & Applicability**
- **Modes of Chromatography**
- **Quantitative work and System Qualification.**

What does HPLC mean?

High pressure liquid chromatography

High priced liquid chromatography

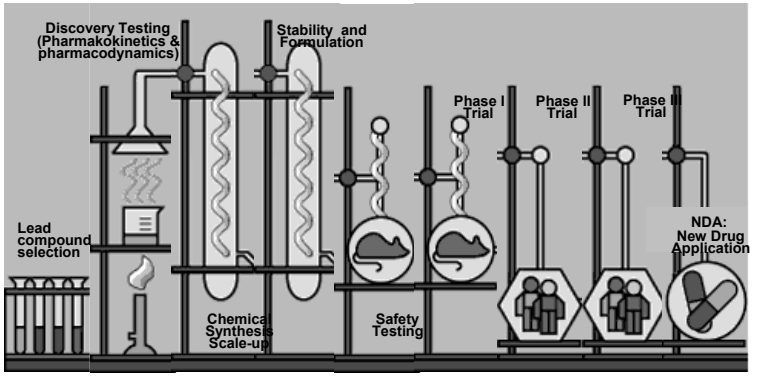
Hewlett-Packard liquid chromatography

High performance liquid chromatography

Hocus pocus liquid chromatography

High patience liquid chromatography

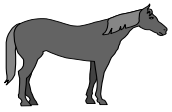
HPLC in Pharmaceutics Technique No 1



High Performance Liquid Chromatography – HPLC - Overview

APPLICATIONS OF HPLC

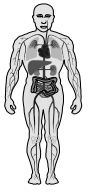
Veterinary



Environmental

Chemistry

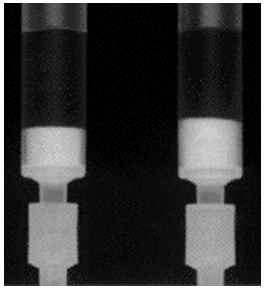
Agriculture & Food

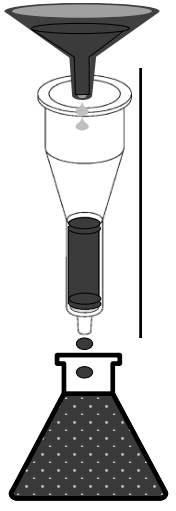


Biomedical and Clinical

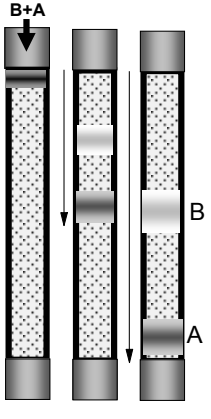
The beginning:

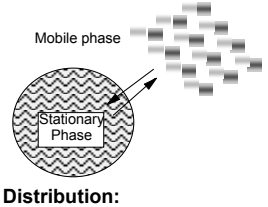
Gravitational Chromatography





Chromatographic Process

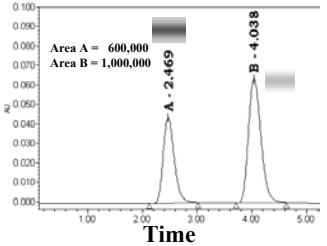




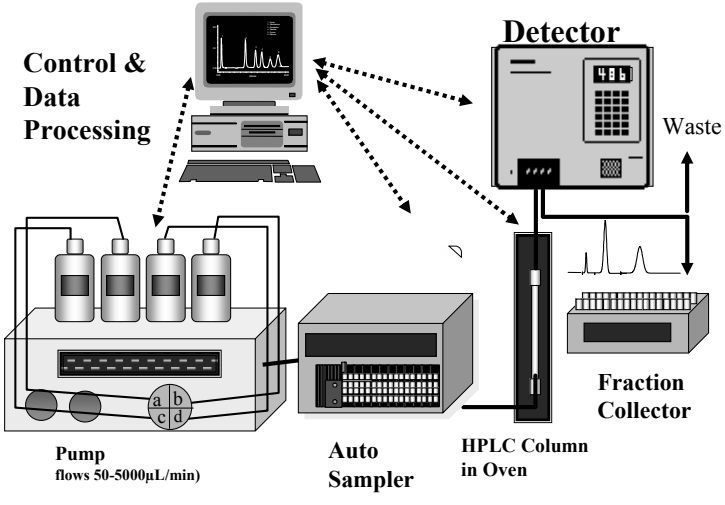
Distribution:

$K = C_s / C_m$

Chromatogram

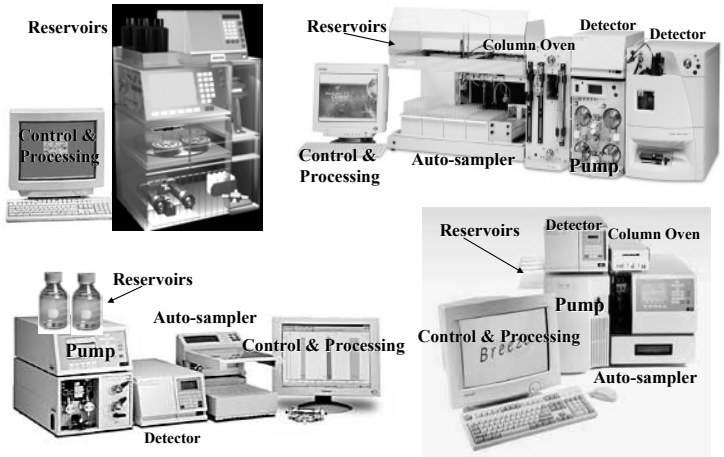


Elution through the Column-movie

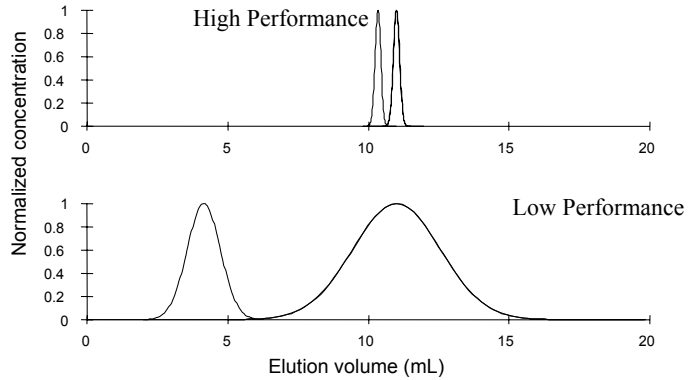


High Performance Liquid Chromatography – HPLC - Overview

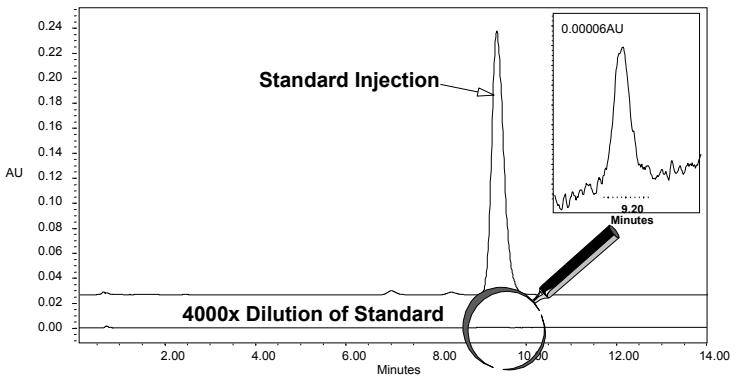
HPLC Systems



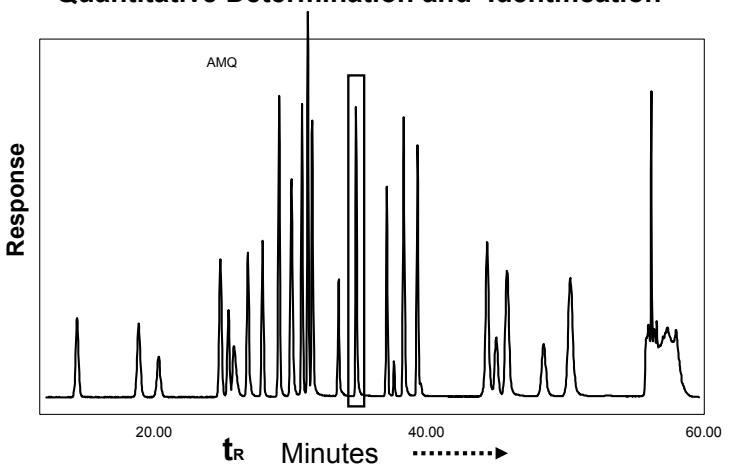
Comparison of Performance



Sensitivity



Quantitative Determination and Identification



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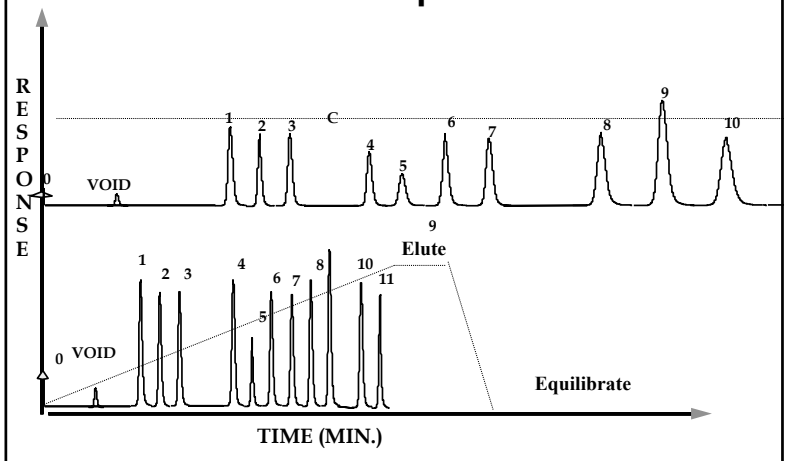
Gradient Operation



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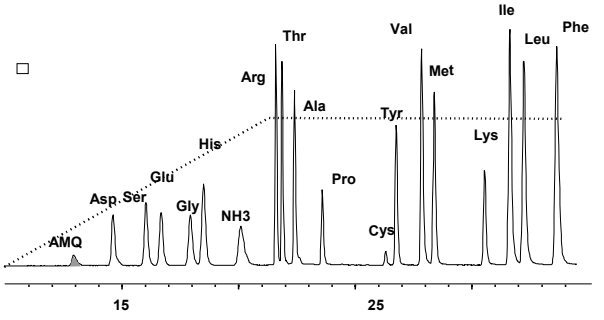
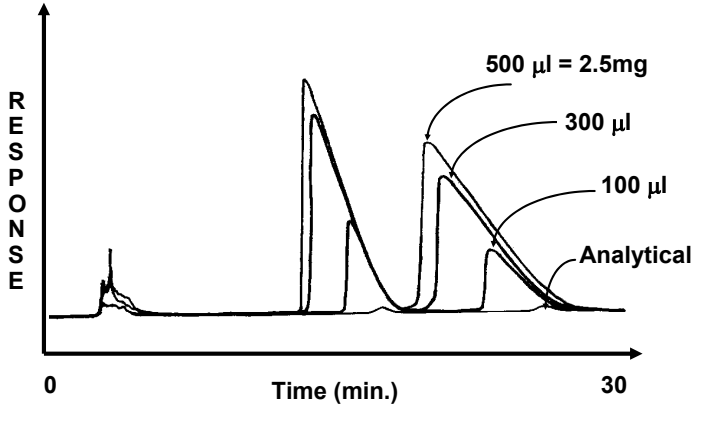
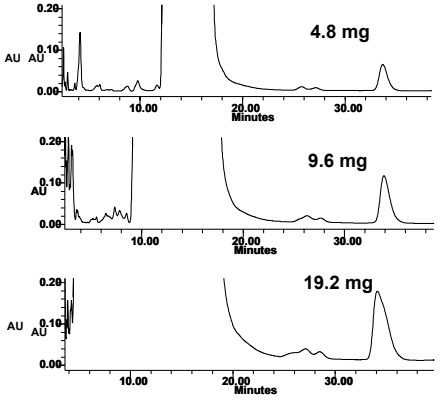


Optimization of the throughput

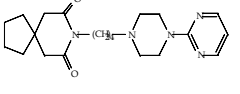


Buspirone: Effect of Increasing Load on the Separation of Impurities



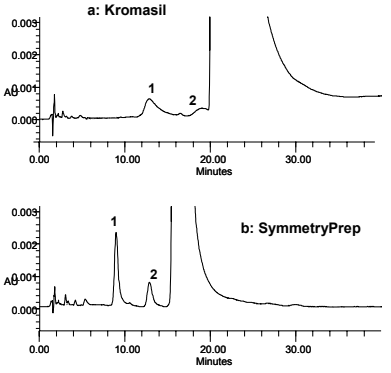
Column: SymmetryPrep C18, 7µm (3.9 x 150) mm
Mobile Phase: 28% acetonitrile / 72% 0.18% TETA-MeCOOH pH 7.0
Flow Rate: 1.0 mL/min
Detection: UV at 360 nm
Sample: 12 mg/mL of Buspirone
Injection: from 0.4 to 1.6 mL

Structure of Buspirone

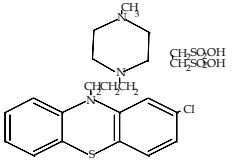


High Performance Liquid Chromatography – HPLC - Overview

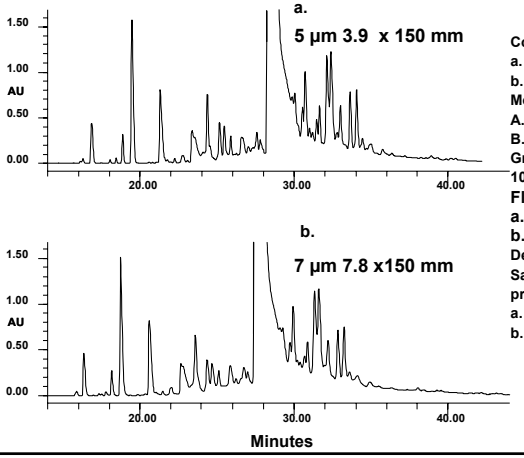
Prochlorperazine: Effect of Loading Capacity on the Separation of Impurities



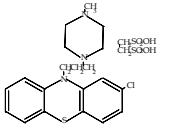
Column:
a: SymmetryPrep C18 7µm (4.6x150) mm
b: Kromasil C18 7µm (4.6x150) mm
Mobile Phase: 75% methanol / 25% 20 mM phosphate buffer pH 7.0
Flow Rate: 1 mL/min
Detection: UV at 254 nm
Sample: 10 µL of 0.97 mg/mL solution



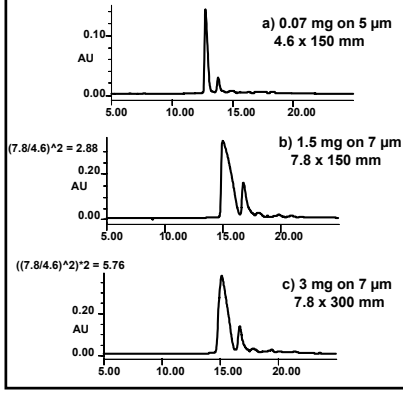
Degradation Products of Prochlorperazine
Scale-up to 7 µm SymmetryPrep Column



Columns:
a. Symmetry C_{18}
b. SymmetryPrep C_{18}
Mobile Phase:
A. 0.1% TFA aqueous;
B. acetonitrile
Gradient:
10% to 60% B in 50 minutes
Flow Rates:
a. 0.7 mL/min
b. 2.8 mL/min
Detection: UV at 280 nm
Sample:
prochlorperazine edisylate
a. 0.8 mg
b. 3.2 mg



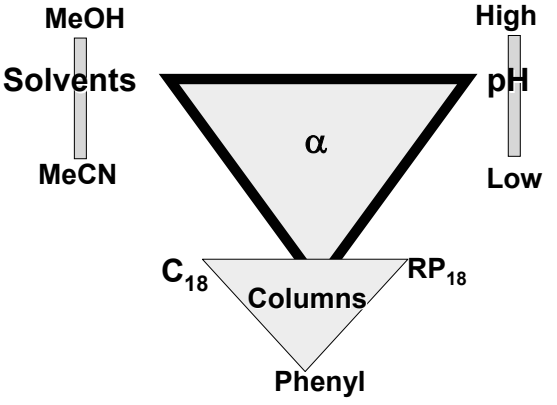
Scale-up of Insulin Impurity Isolation to 7.8 mm SymmetryPrep C_{18} Columns



Mobile Phases:
A. 0.1% TFA aqueous
B. 0.1% TFA in acetonitrile
26% B to 33% B in 14 minutes
Sample:
Bovine Pancrease Insulin, 10 mg/mL in 0.01N HCl

HPLC System:
a. analytical system
b. and c. system modified for prep analysis, larger syringe and loop in injector and 0.04 in. i.d. tubing

Streamlined Method Development Strategy



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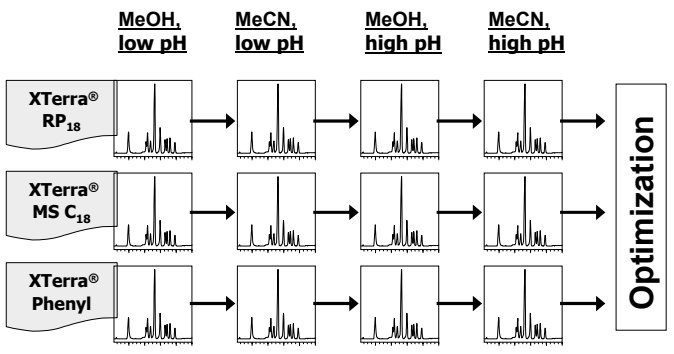
Method Development



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- Modes of Chromatography
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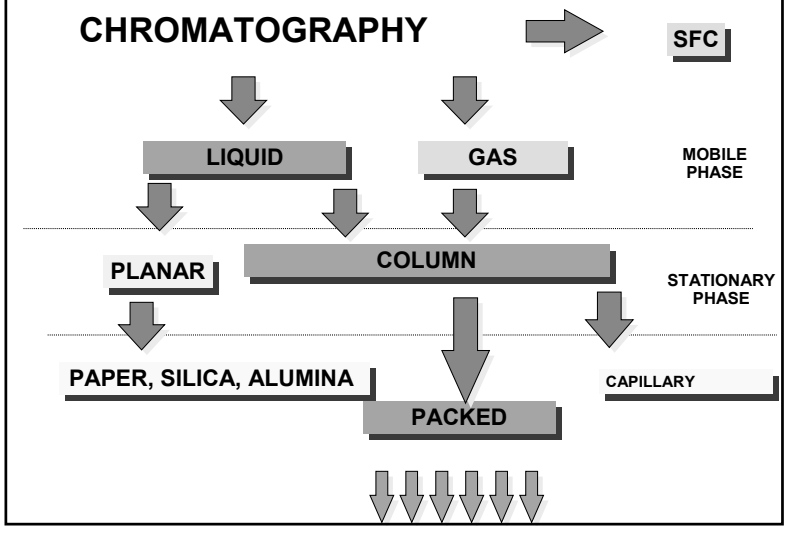
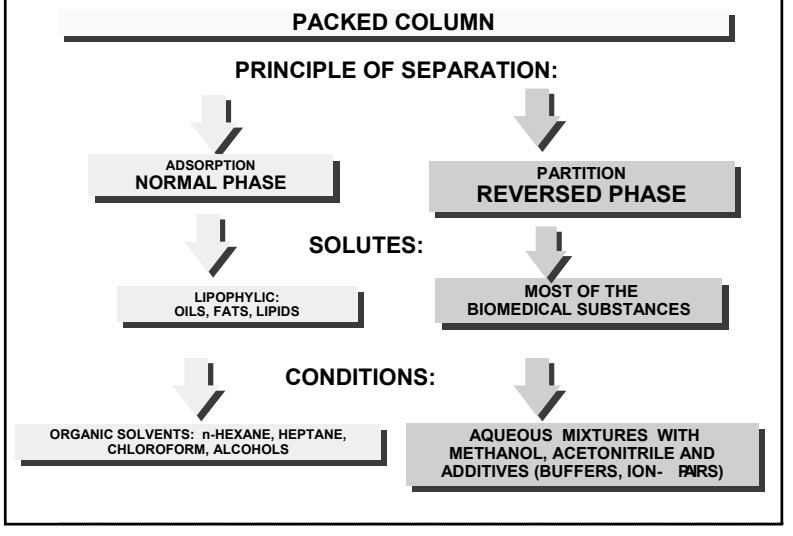




High Performance Liquid Chromatography – HPLC - Overview

High Performance Liquid Chromatography

PRINCIPLE OF SEPARATION:

ION EXCHANGE	SIZE EXCLUSION	BIO AFFINITY	CHIRALITY
SOLUTES:			
INORGANIC IONS, ACIDS, BASES	POLYMERS, PROTEINS, NUCLEIC ACIDS	PROTEINS & ENZYMES	ENANTIOMERS
CONDITIONS:			
AQUEOUS BUFFERS, IONIC SOLUTIONS	AQUEOUS BUFFERS OR ORGANIC SOLVENTS	AQUEOUS BUFFERS AND SPECIAL ADDITIVES	AQUEOUS OR ORGANIC SOLVENTS

REVERSED PHASE

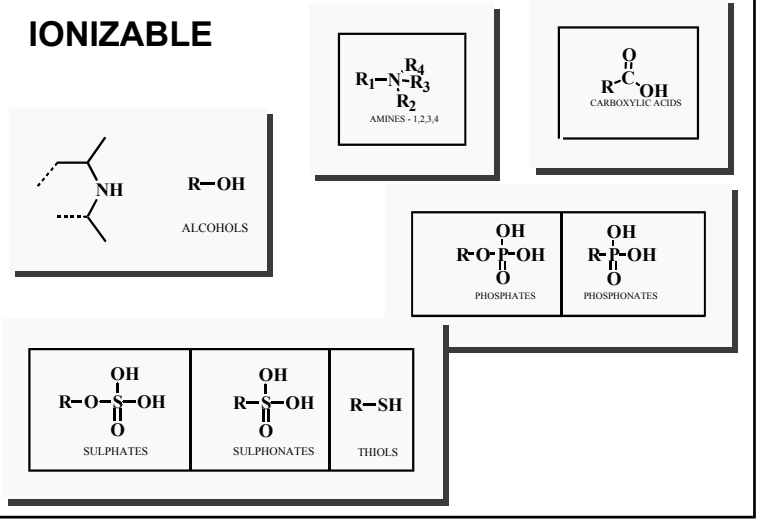
SOLUTES:

MOST OF THE BIOMEDICAL SUBSTANCES

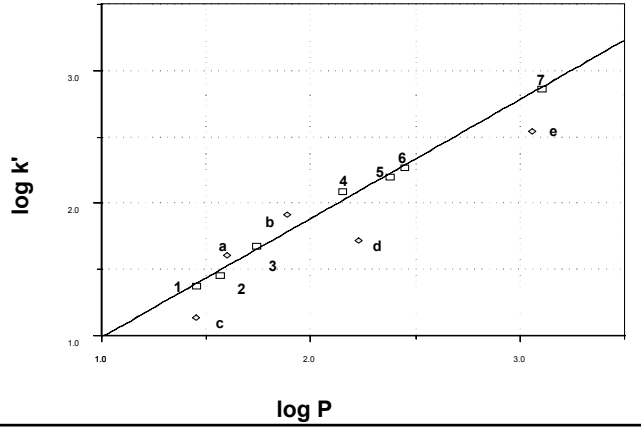
CONDITIONS:

AQUEOUS MIXTURES WITH METHANOL, ACETONITRILE AND ADDITIVES (BUFFERS, ION-PAIRS)

IONIZABLE



Hydrophobicity



High Performance Liquid Chromatography – HPLC - Overview

MOBILE PHASE

SOLVENTS:
water, methanol, acetonitrile

ADDITIVES:
buffers, salts, ion pairing reagents, complexants.

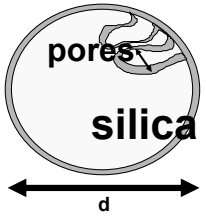
STATIONARY PHASE

CHEMISTRY:

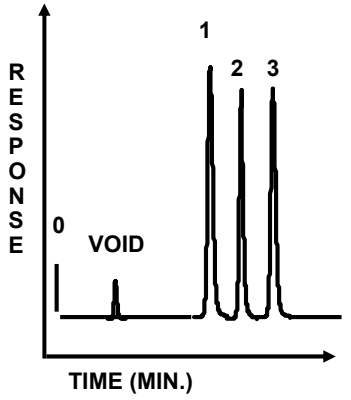
* BONDED HYDROCARBON: C-18, C-8, C-4, C-1
* % COVERAGE
* ADSORBED SURFACTANTS
* TYPE OF SILICA GEL

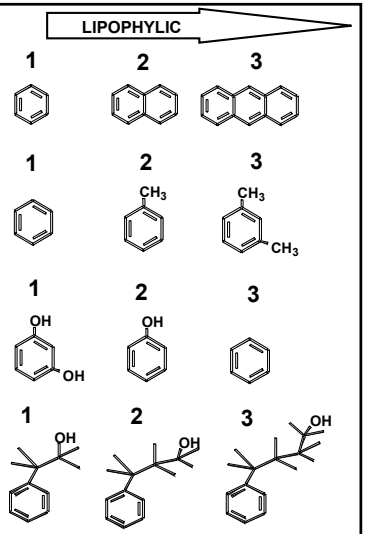
GEOMETRY

* SPHERE- IRREGULAR
* PARTICLE DIAMETER
* POROSITY

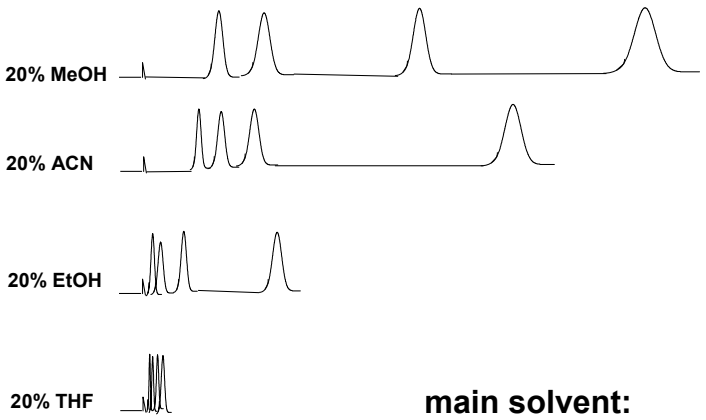


ELUTION ORDER IN REVERSED PHASE

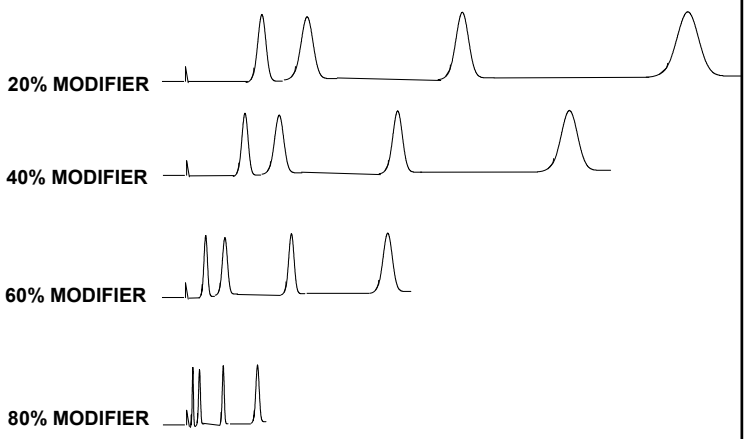




OPTIMIZATION: CHOICE OF SOLVENTS REVERSED PHASE



OPTIMIZATION: % SOLVENTS



IONIZATION and RETENTION

WEAK ACIDS

$HA \rightleftharpoons H^+ + A^-$

pKa~ 4-5

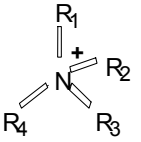
At pH >4-5 the main species is A^-

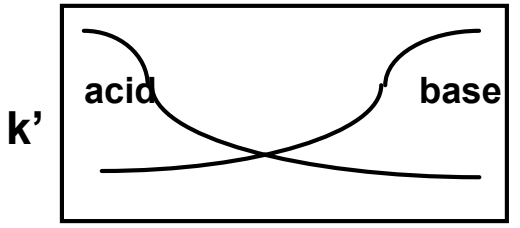
WEAK BASES

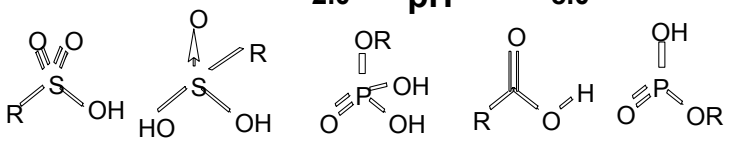
$HB^+ \rightleftharpoons H^+ + B$

At pH < 7-8 the main species is BH^+

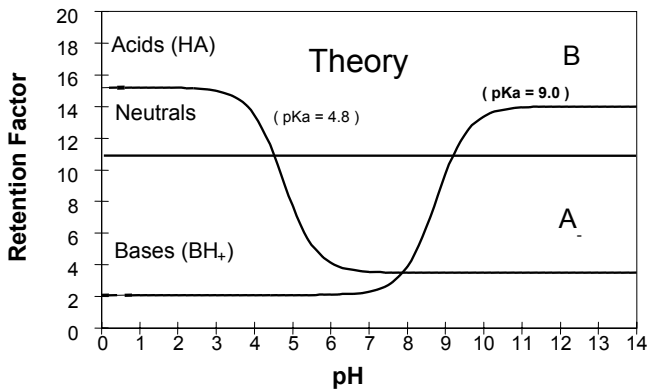
pH DEPENDENT RETENTION



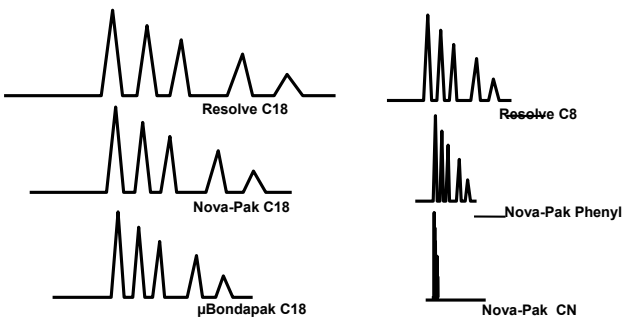




Retention Factor versus pH for Acids, Bases, and Neutrals



Types of Reversed Phase Columns



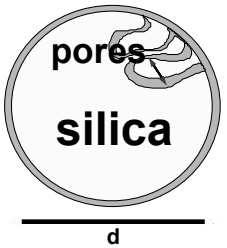
Stationary Phase Properties

CHEMISTRY:

* **BONDED HYDROCARBON:**
  C-18, C-8, C-4, C-1, CN, phenyl
* **% COVERAGE**
* **TYPE OF SILICA GEL**

GEOMETRY

* **SPHERE IRREGULAR**
* **PARTICLE DIAMETER**
* **POROSITY**



Stationary Phase Supports

Stationary phase	Functionality
C_{18}	$-Si(CH_3)_2C_{18}H_{37}$
C_8	$-Si(CH_3)_2C_8H_{17}$
tC_2	$-SiC_2H_5$
Aminopropyl	$-Si(CH_2)_3NH_2$
Cyanopropyl	$-Si(CH_3)_2(CH_2)_3CN$
Diol	$-Si(CH_2)_3OCH_2CH(OH)CH_2OH$

Retention time

Chain length CN Phenyl NH2 C4 C8 C18

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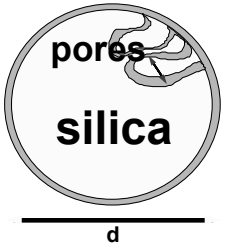

CARBON LOAD

Increasing carbon load on a particles increases retention. **similar geometrical shaped**

Retention time

Carbon load 5% 7% 9% 12% 15% 17%

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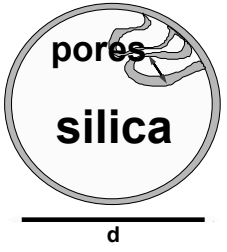
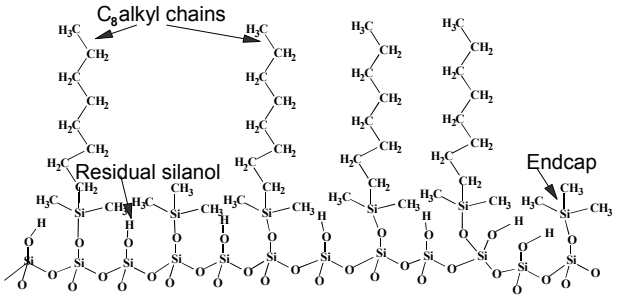


Surface of a Silica Gel Bonded-Phase Packing Material

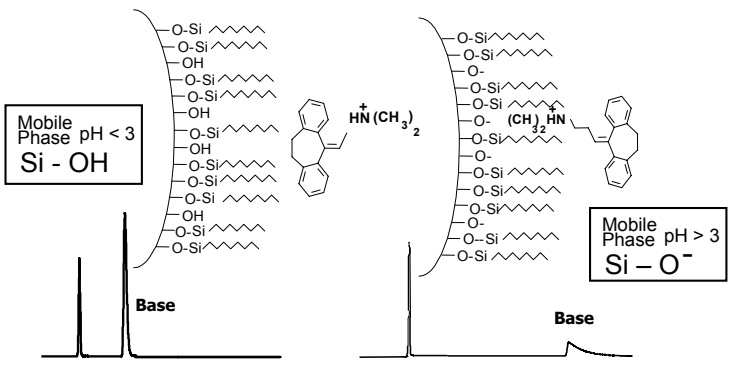


Note: ~50% of the surface silanols remain even with high ligand bonding densities

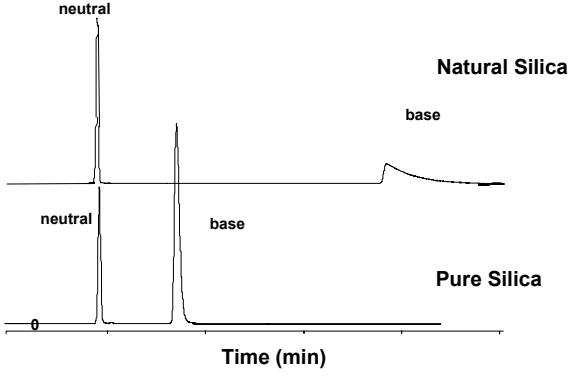
Mixed-Mode Retention:

Hydrophobic Interaction with Bonded Phase

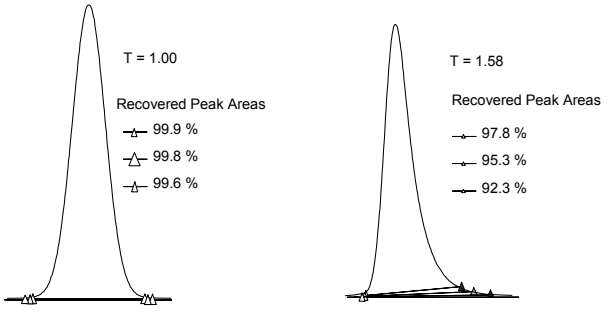
Ion exchange Interaction with Charged Sites



Quality of Columns Performance



Integration Errors Caused by Tailing



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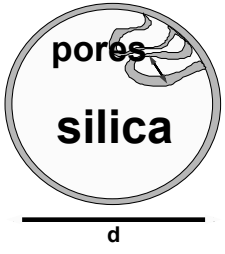
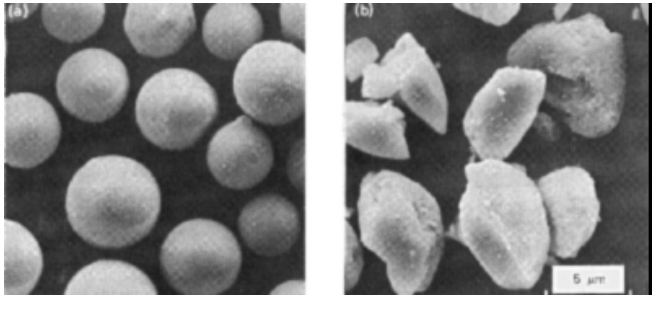
* **PARTICLE DIAMETER**
* **POROSITY**



Spherical and Irregular particles

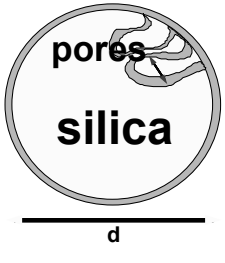


- **Electron microphotograph of spherical and irregular silica particles. [W.R.Melander, C.Horvath, Reversed-Phase Chromatography, in HPLC Advances and Perspectives, V2, Academic Press, 1980]**

Stationary Phase Properties

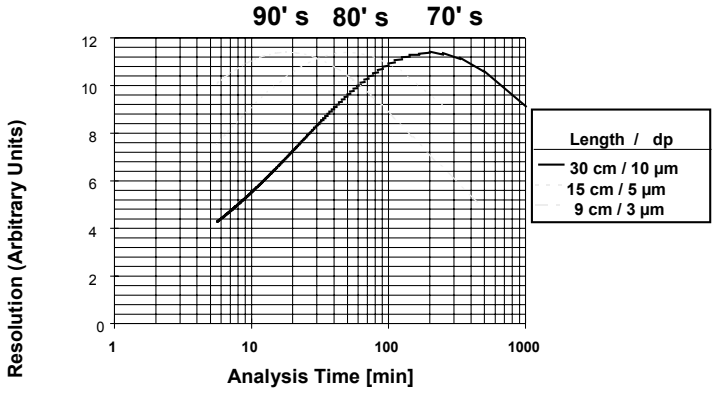
CHEMISTRY:
* **BONDED HYDROCARBON:**
  C-18, C-8, C-4, C-1, CN, phenyl
* **% COVERAGE**
* **TYPE OF SILICA GEL**

GEOMETRY
* **SPHERE IRREGULAR**
* **PARTICLE DIAMETER**
* **POROSITY**



High Performance Liquid Chromatography – HPLC - Overview

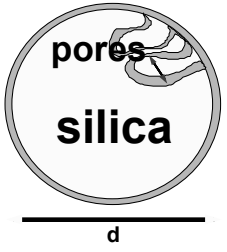
Resolution - Time Diagram



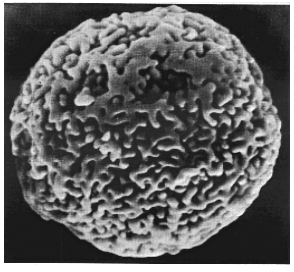
Stationary Phase Properties

CHEMISTRY:
* BONDED HYDROCARBON: C-18, C-8, C-4, C-1, CN, phenyl
* % COVERAGE
* TYPE OF SILICA GEL

GEOMETRY
* SPHERE - IRREGULAR
* PARTICLE DIAMETER
* POROSITY



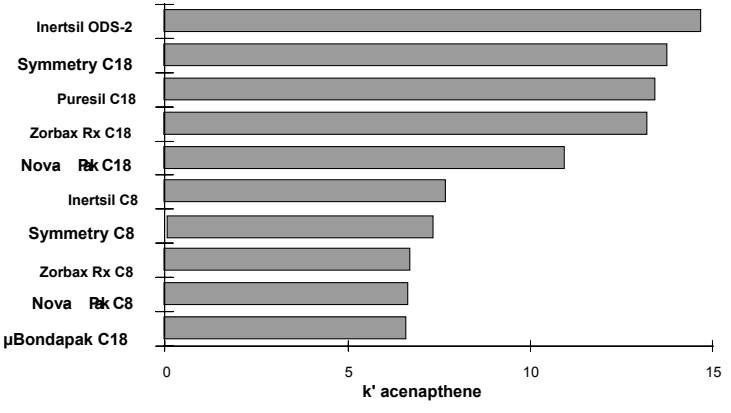
Pore size, shape and distribution



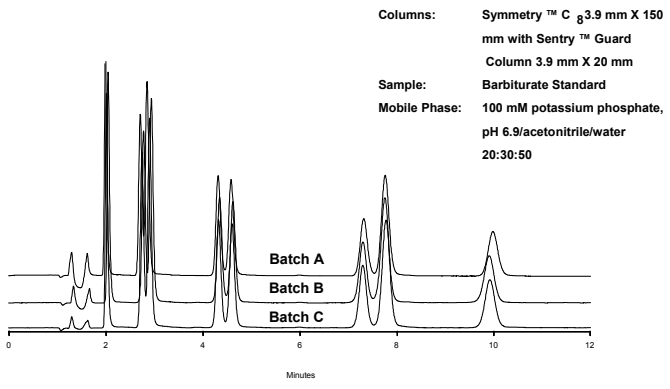
Macroporous spherical silica particle. [K.K.Unger, Porous silica, Elsevier, 1979]

Pore size defines an ability of the analyte molecules to penetr inside the particle and interact with its inner surface. This is especially important because the ratio of the outer particle surfa to its inner one is about 1:1000. The surface molecular interacti mainly occurs on the inner particle surface.

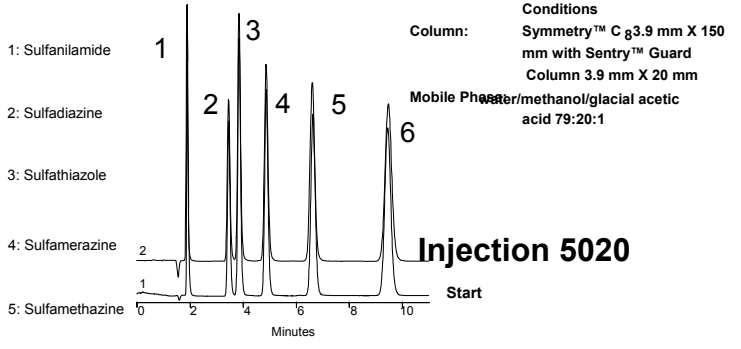
Relative Hydrophobicities of General Purpose Analytical Packings



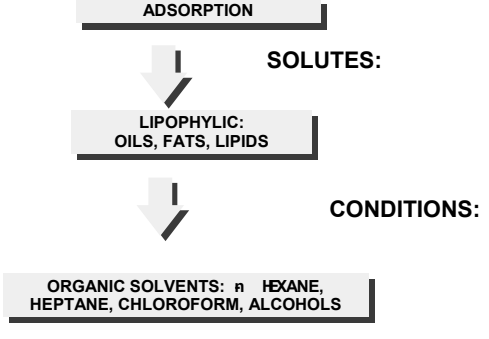
Batch-to-Batch Reproducibility of Columns



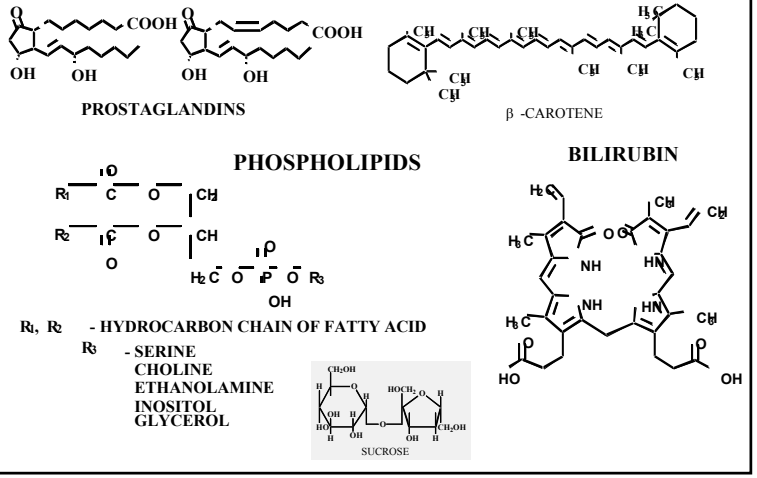
Chromatogram of lifetime test



NORMAL PHASE

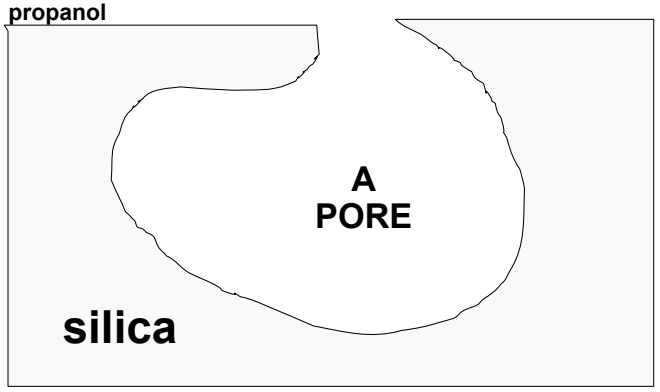


NORMAL PHASE SOLUTES

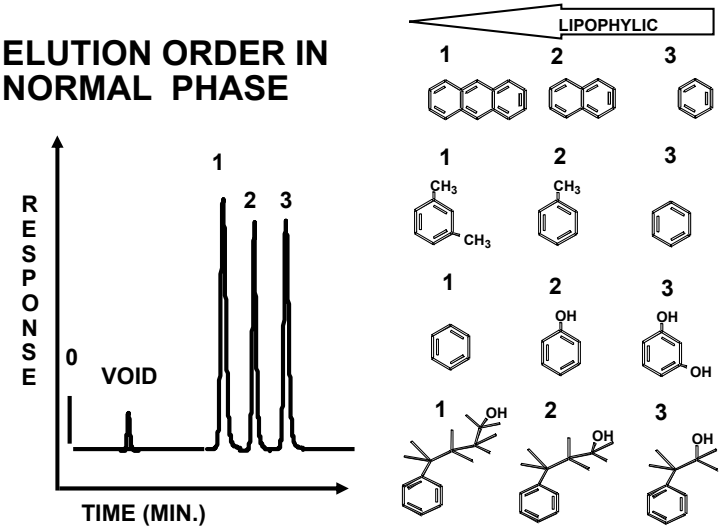


NORMAL PHASE

SOLVENTS: n-hexane, chloroform, ethanol, 2-propanol



ELUTION ORDER IN NORMAL PHASE

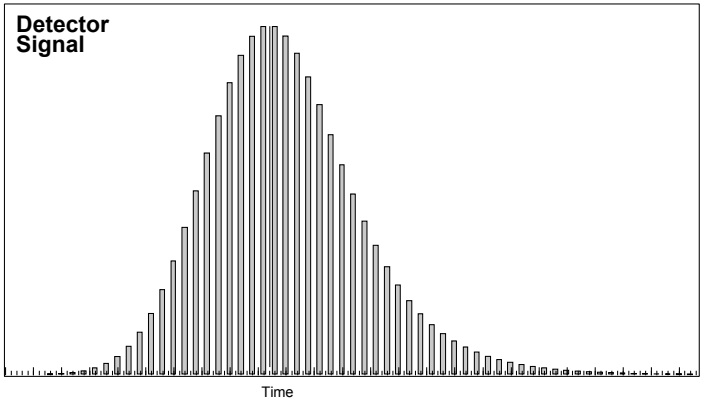


HPLC COURSE LAYOUT

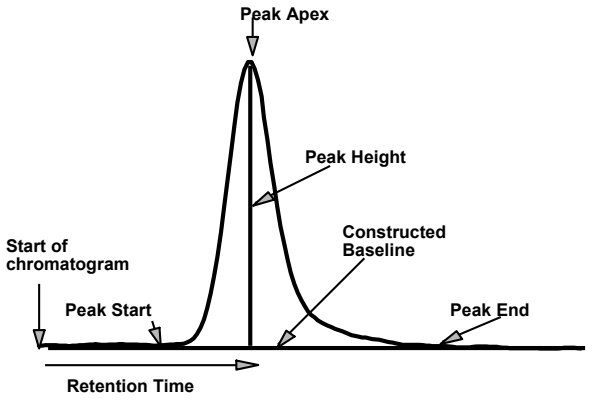
- Introduction & Applicability
- Modes of Chromatography
- Quantitative work and System Qualification.

Measurement of Area - Integration

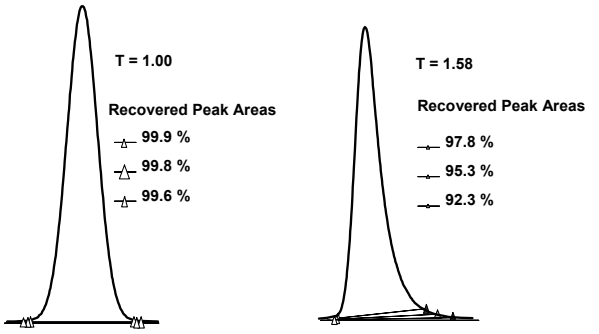
Area = $\int$ Abs x dt



Peak Detection

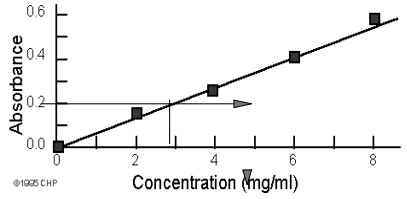


Integration Errors Caused by Tailing

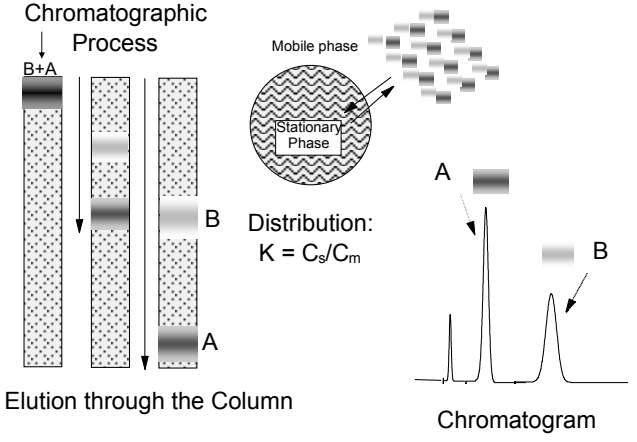


Working Curve

- A plot of the analytical signal (the instrument or detector response) as a function of analyte concentration, using a series of standards of known concentration.



The working curves are then used to determine the concentration of an unknown sample or to calibrate the linearity of an analytical instrument.



System Suitability

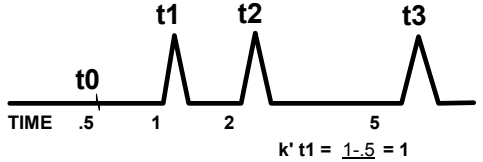
is

Based on the Theory of Chromatography

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			

k' = Capacity Factor = Measure of Retention



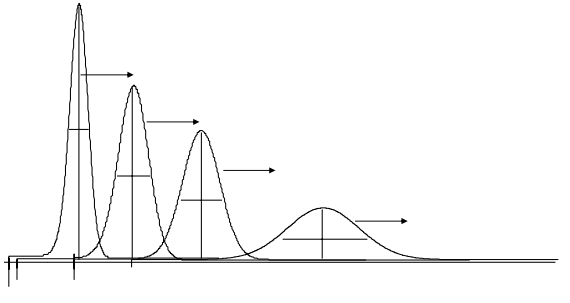
$$k' t1 = \frac{t1 - t0}{t0}$$

$$k' t1 = \frac{1-.5}{.5} = 1$$

$$K' t2 = \frac{2-.5}{.5} = 3$$

$$k' t3 = \frac{5-.5}{.5} = 9$$

PEAK BROADENING



High Performance Liquid Chromatography – HPLC - Overview

PERFORMANCE BY ONE PEAK

RETENTION FACTOR or CAPACITY RATIO

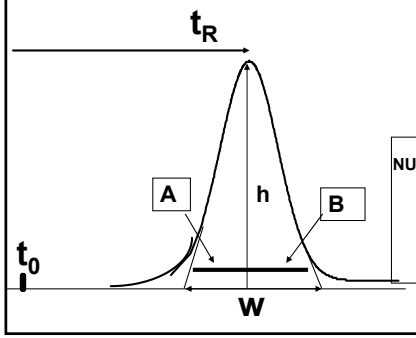
$$k' = \frac{t_R - t_0}{t_0} \qquad k' = \phi \frac{C_s}{C_m}$$

ASYMMETRY FACTOR

$$A_f = \frac{B_{(10\%\ h)}}{A_{(10\%\ h)}}$$

TAILING FACTOR

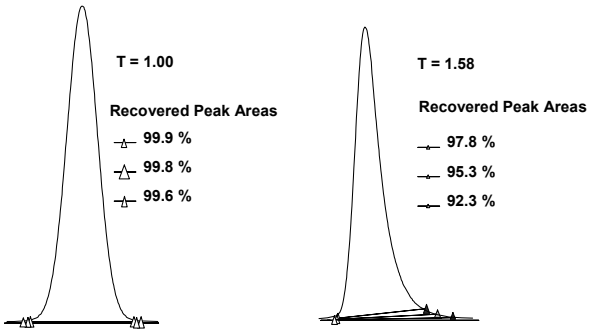
$$T_f = \frac{A + B}{2A}$$
$$(10\%\ h)$$



NUMBER OF THEORETICAL PLATES

$$N = 16 \left(\frac{t_R}{w} \right)^2$$

Integration Errors Caused by Tailing



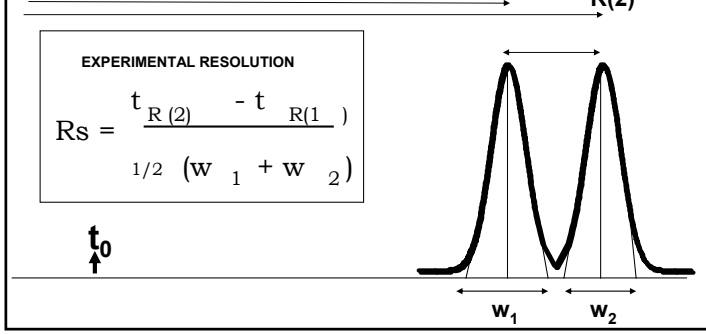
PERFORMANCE BY TWO PEAKS

SELECTIVITY FACTOR

$$\alpha = \frac{k'_{(2)}}{k'_{(1)}}$$

EXPERIMENTAL RESOLUTION

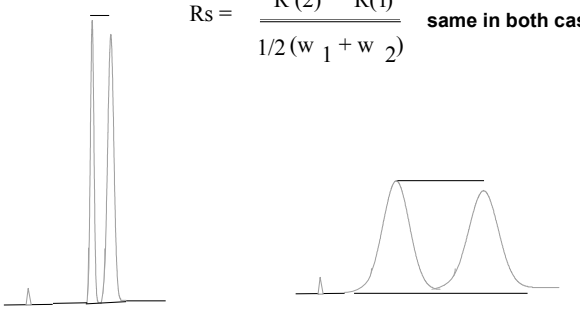
$$Rs = \frac{t_{R(2)} - t_{R(1)}}{1/2\ (w_1 + w_2)}$$



SELECTIVITY vs EFFICIENCY

$$Rs = \frac{t_{R(2)} - t_{R(1)}}{1/2\ (w_1 + w_2)}$$

same in both cases



LOW SELECTIVITY (α)
HIGH EFFICIENCY (N)

HIGH SELECTIVITY (α)
LOW EFFICIENCY (N)

High Performance Liquid Chromatography – HPLC - Overview

Resolution
Peak capacity
Sensitivity

ADVANTAGES OF HIGH EFFICIENCY