High Performance Liquid Chromatography (HPLC) for Clinical and Biomedical Applications

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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

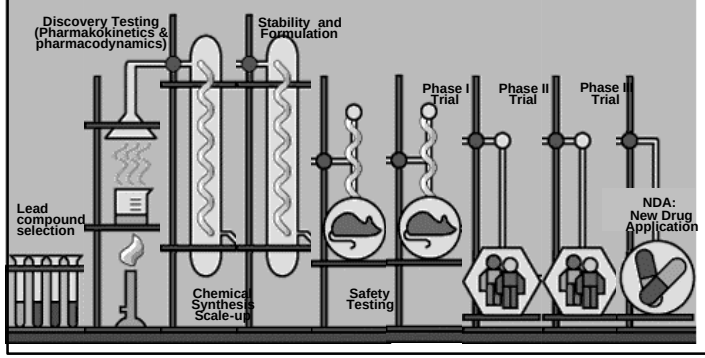
Hewlett-Packard liquid chromatography

High performance liquid chromatography

Hocus pocus liquid chromatography

High patience liquid chromatography

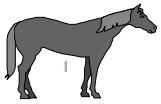
HPLC in Pharmaceutics Technique No 1



High Performance Liquid Chromatography – HPLC - Overview

APPLICATIONS OF HPLC

Veterinary



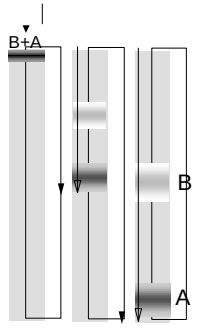
Environmental

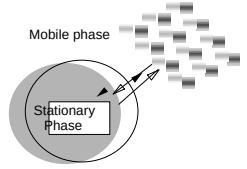
Agriculture & Food

Chemistry

Biomedical and Clinical

Chromatographic Process

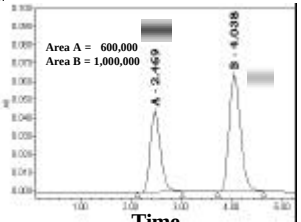




Distribution:
$K = C_s/C_m$

Elution through the Column

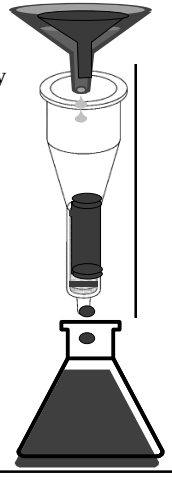
Chromatogram

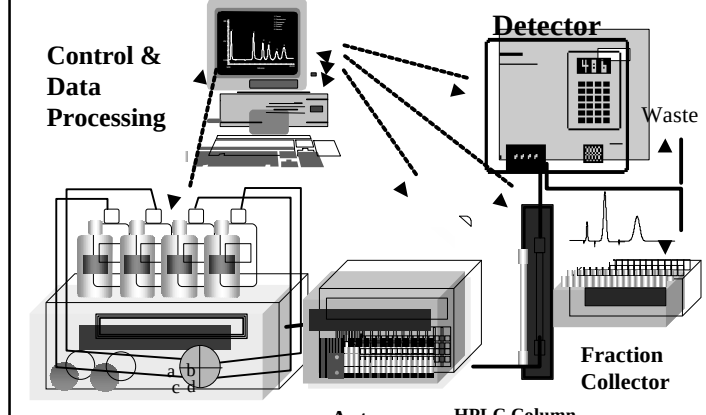


Time

The beginning:

Gravitational Chromatography





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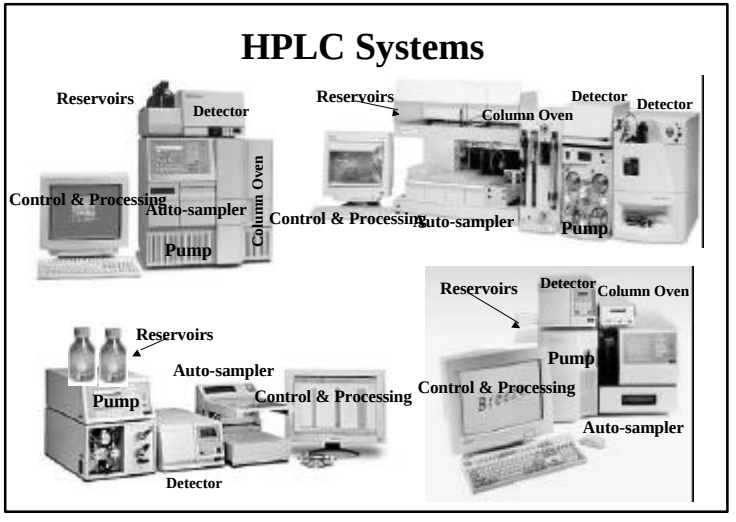

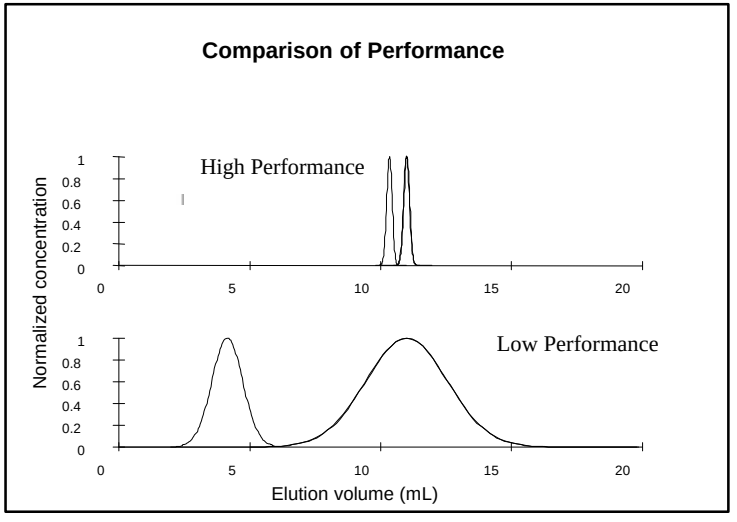

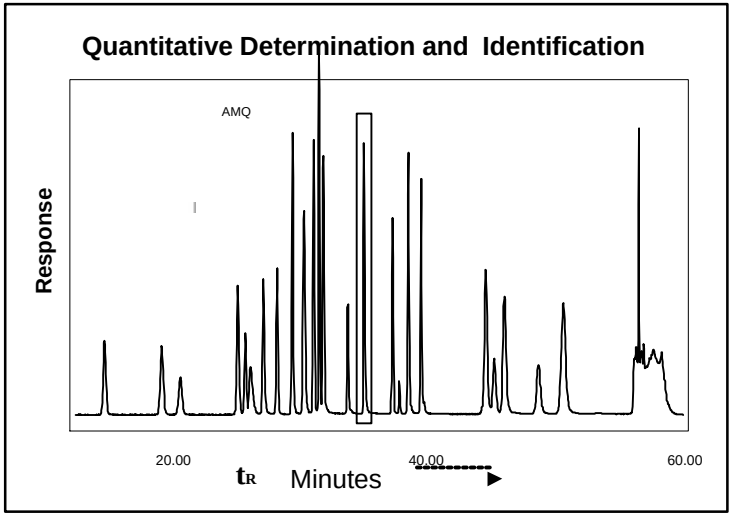

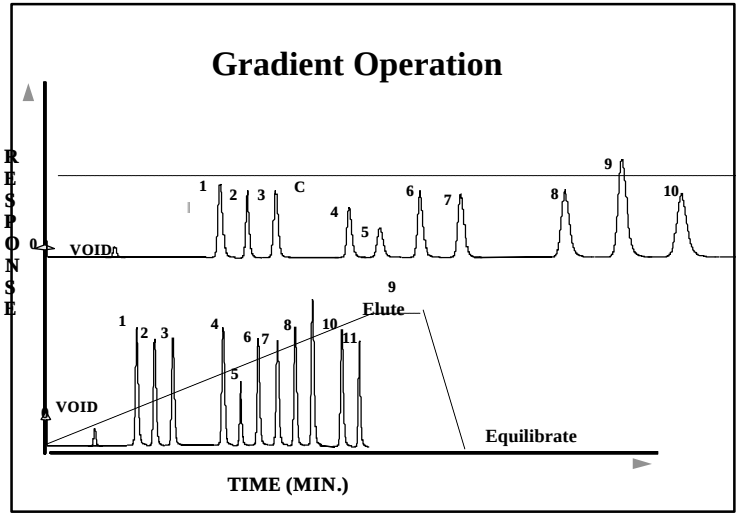

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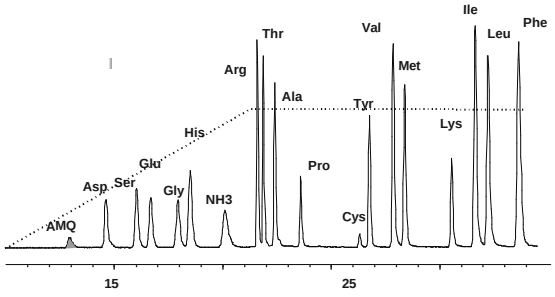
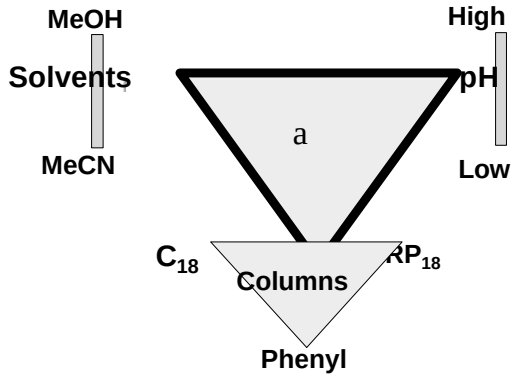
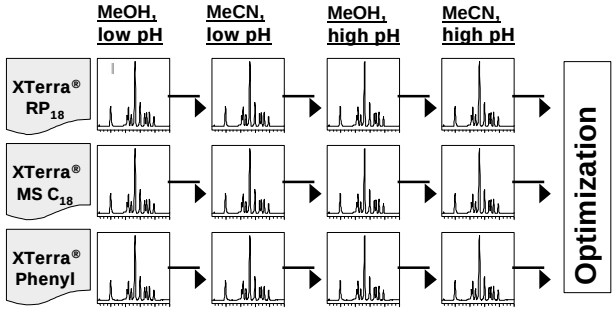
Gradient Operation



Streamlined Method Development Strategy



Method Development



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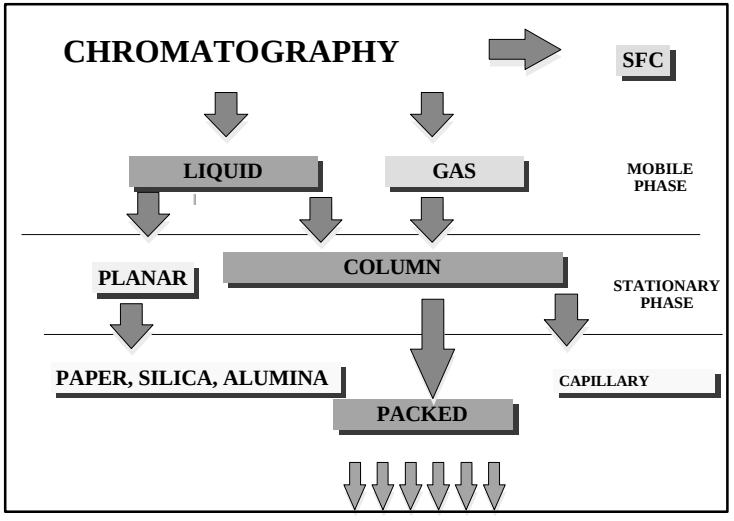
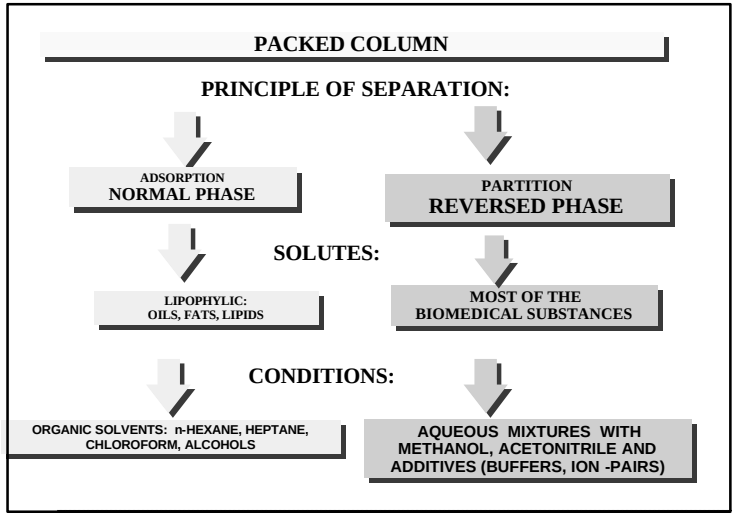
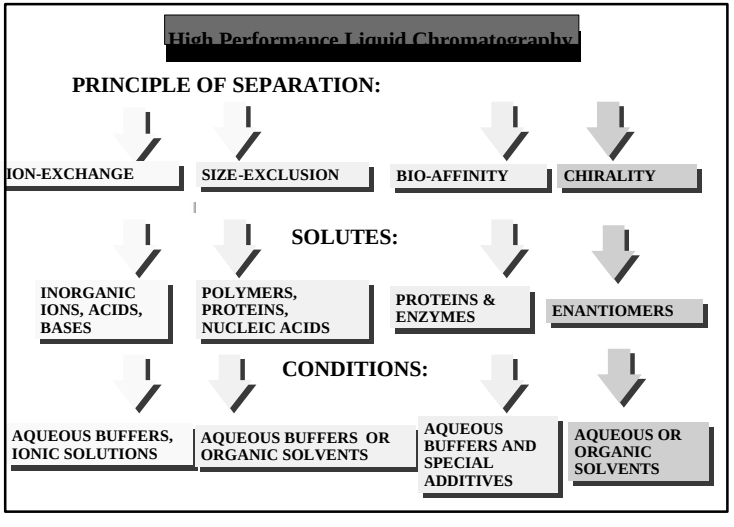
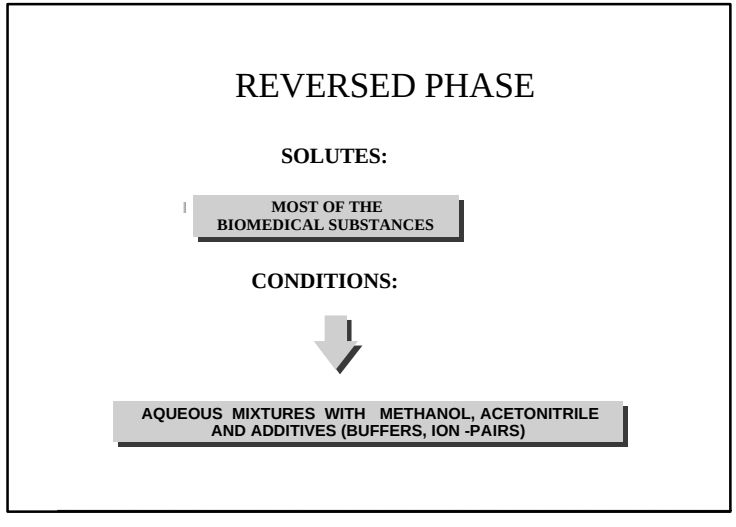
High Performance Liquid Chromatography – HPLC - Overview



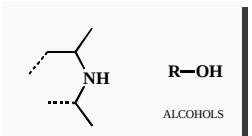


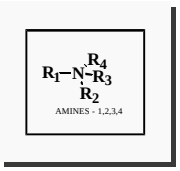


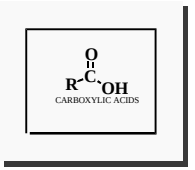


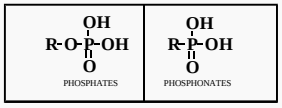
High Performance Liquid Chromatography – HPLC - Overview

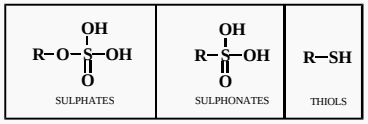
IONIZABLE



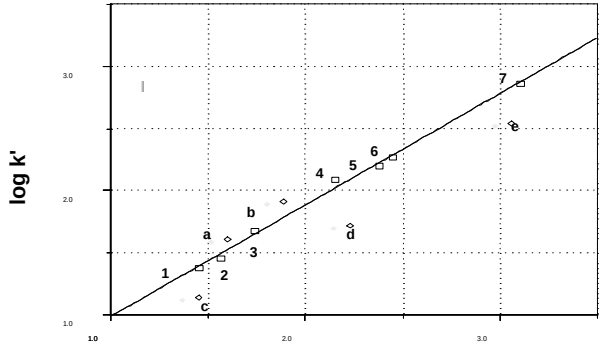








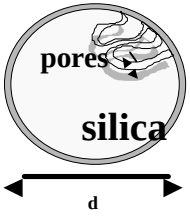
Hydrophobicity



MOBILE PHASE

SOLVENTS:
water, methanol, acetonitrile

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



STATIONARY PHASE

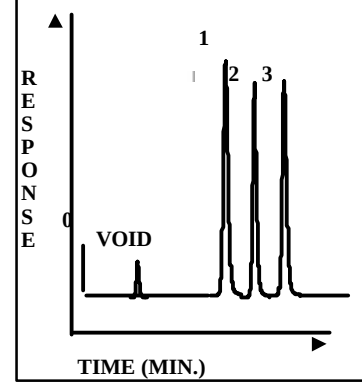
CHEMISTRY:

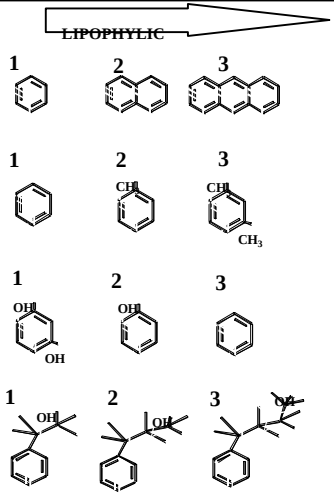
* **BONDED HYDROCARBON: C-18, C-8, C-4, C-1**
* **% COVERAGE**
* **ADSORBED SURFACTANTS**
* **TYPE OF SILICA GEL**

GEOMETRY

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

ELUTION ORDER IN REVERSED PHASE





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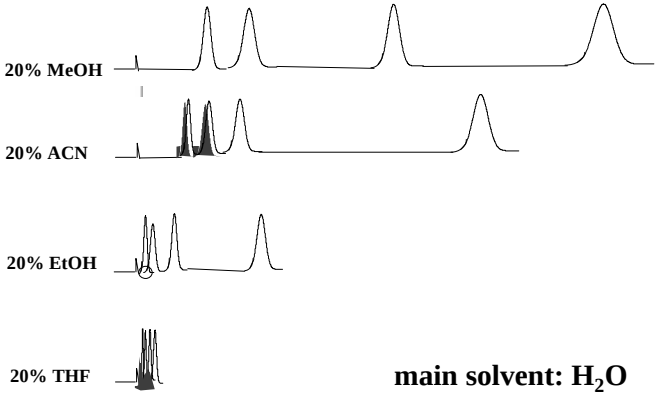
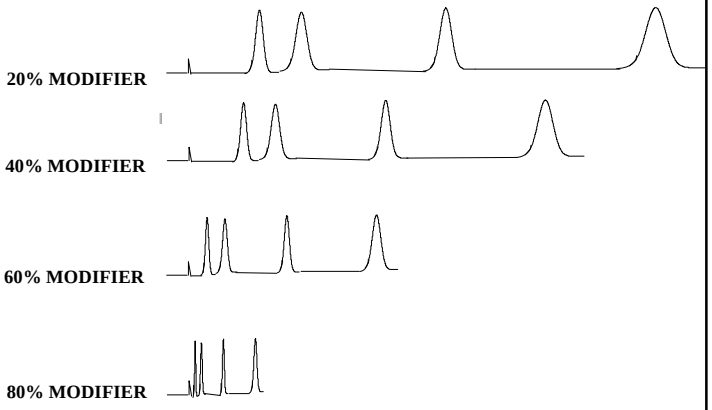
OPTIMIZATION: CHOICE OF SOLVENTS REVERSED PHASE



main solvent: H_2O

OPTIMIZATION: % SOLVENTS



IONIZATION and RETENTION

WEAK ACIDS

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

pKa~ 4-5

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

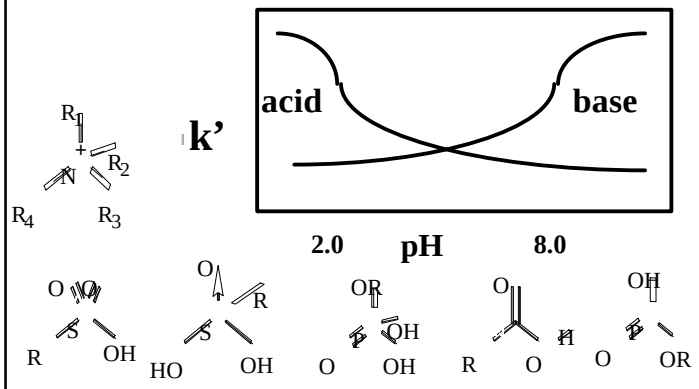
WEAK BASES

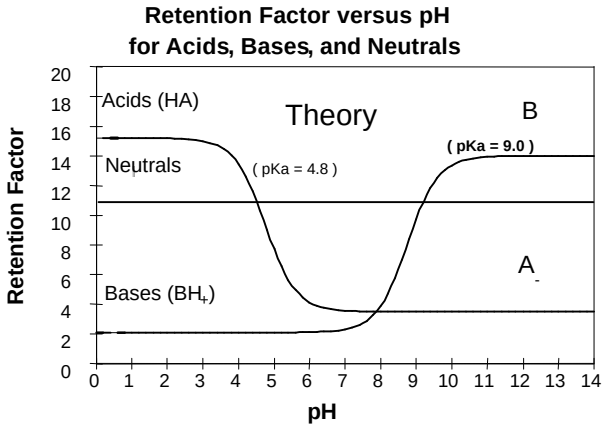
$HB^+ \rightleftharpoons H^+ + B$

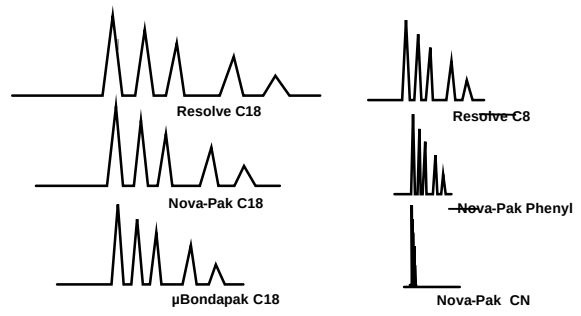
pKa ~ 7-8

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

pH DEPENDENT RETENTION







Stationary Phase Properties

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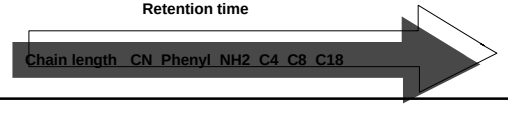
Stationary Phase Supports

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	$-Si(CH_2)_3OCH_2CH(OH)CH_2OH$



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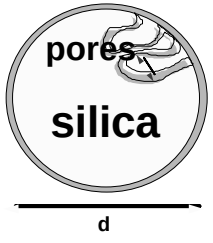

CARBON LOAD

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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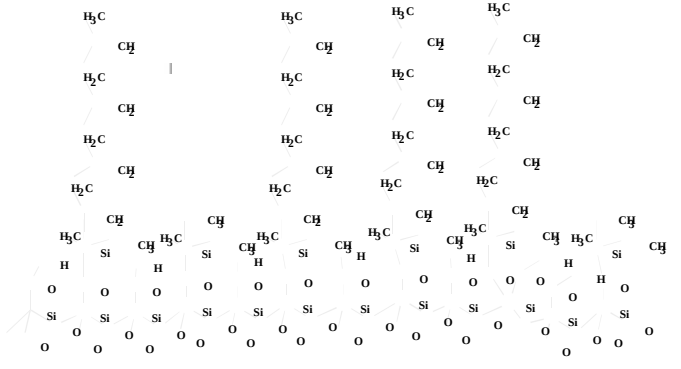
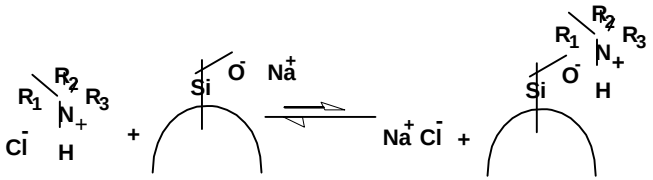
Surface of a Reversed- Phase Packing

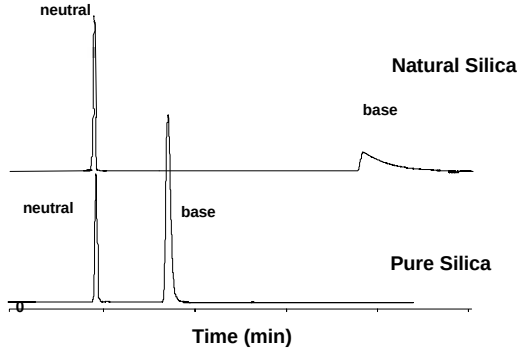


What Causes Tailing?

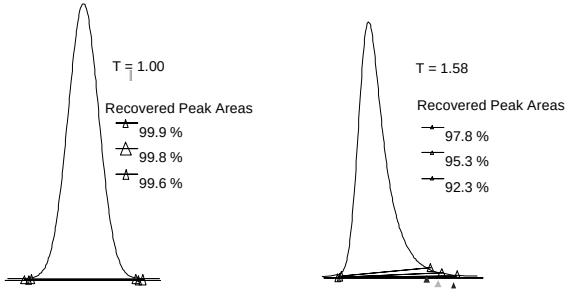
- Mixed-mode retention:
  - hydrophobic - interaction with bonded phase
  - ion exchange - interaction with charged sites



Quality of Columns Performance



Integration Errors Caused by Tailing



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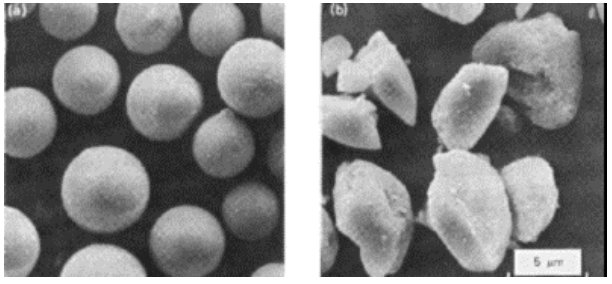
Spherical and Irregular particles



- 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]

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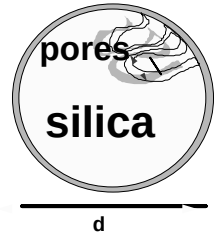
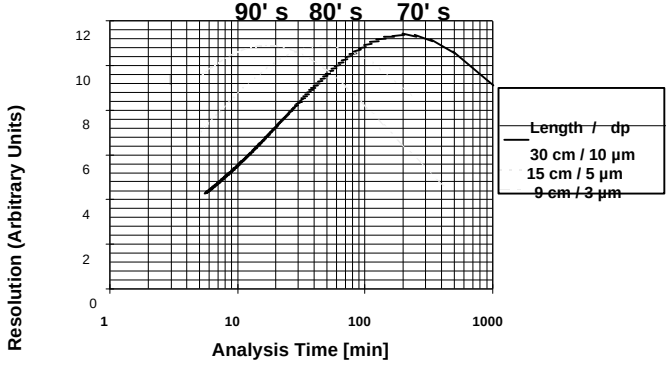
GEOMETRY
* SPHERE- IRREGULAR
* PARTICLE DIAMETER
* POROSITY



Resolution - Time Diagram



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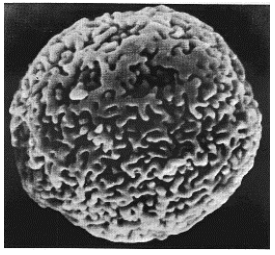
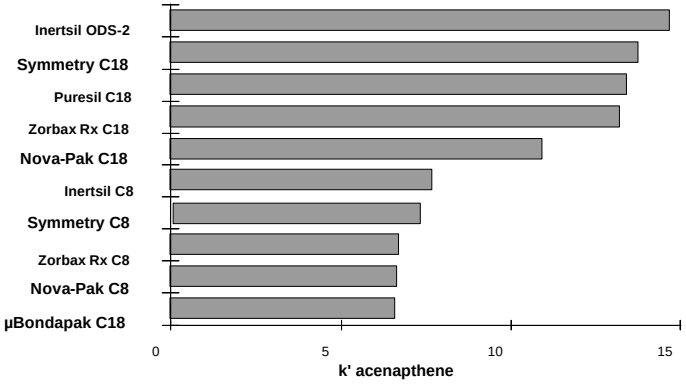
Pore size, shape and distribution



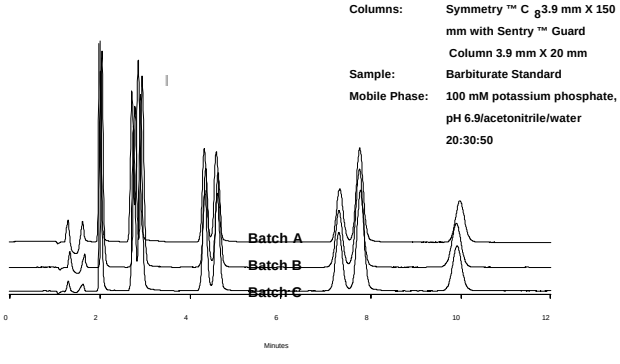
Macroporous spherical silica particle. [K.K.Unger, Porous silica, Elsevier, 1979]

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

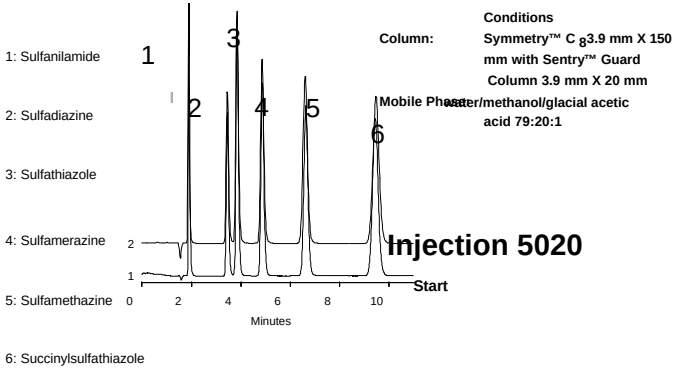
Relative Hydrophobicities of General Purpose Analytical Packings



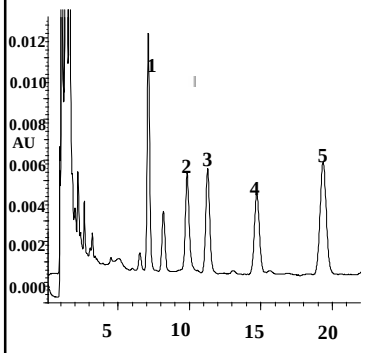
Batch-to-Batch Reproducibility of Columns



Chromatogram of lifetime test



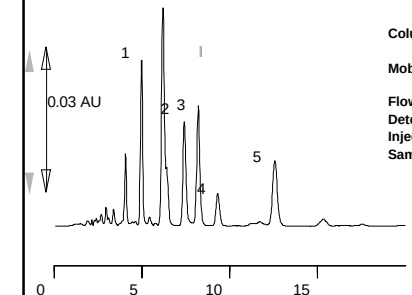
Example: Barbitorates



Cheng

Column: Symmetry® C18, 5 µm, 3.9 x 150 mm
Mobile Phase: 20 mM Phosphate: pH 7: Acetonitrile:Methanol (56:21:23)
Detection: UV at 240 nm
Flow Rate: 1.0 mL/min.
Injection Volume: 20 µL
Sample Identification:
Peak 1: Norchlordiazepoxide
Peak 2: Oxazepam
Peak 3: Desmethyldiazepam
Peak 4: Chlordiazepoxide
Peak 5: Demoxepam (I.S.)

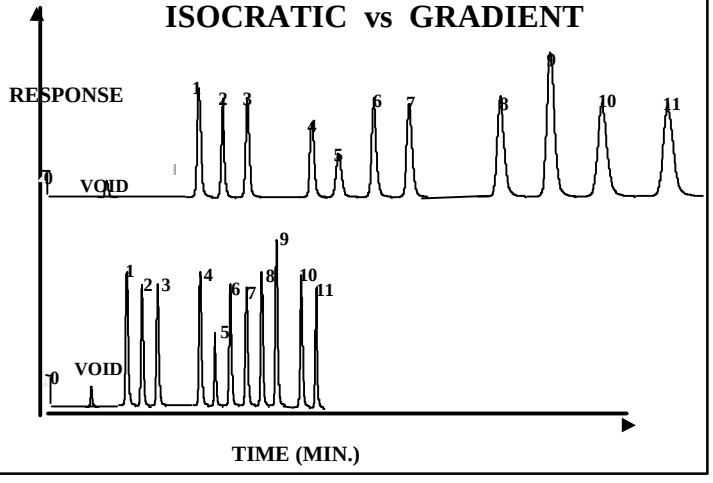
Example: Caffeine and Metabolites



Phillips

Column: SymmetryShield™ RP8, 5 µm, 3 mm x 150 mm
Mobile Phase: 20 mM Ammonium Acetate pH 5: Acetonitrile (95:5)
Flow Rate: 0.6 mL/min
Detection: UV at 254 nm
Injection Volume: 20 µL
Sample Identification:
Peak 1:Theobromine
Peak 2: Paraxanthine
Peak 3: Theophylline
Peak 4: Hydroxyethyltheophylline (I.S.)
Peak 5: Caffeine

ISOCRATIC vs GRADIENT



NORMAL PHASE

ADSORPTION

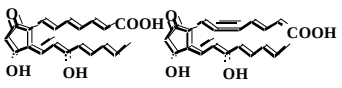
SOLUTES:

LIPOPHYLIC: OILS, FATS, LIPIDS

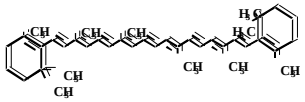
CONDITIONS:

ORGANIC SOLVENTS: n-HEXANE, HEPTANE, CHLOROFORM, ALCOHOLS

NORMAL PHASE SOLUTES

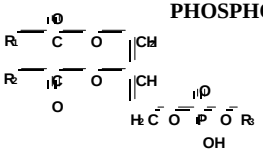


PROSTAGLANDINS



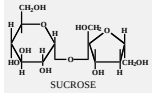
β -CAROTENE

PHOSPHOLIPIDS



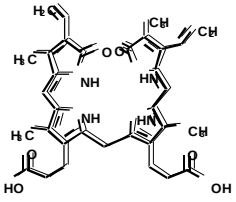
R_1, R_2 - HYDROCARBON CHAIN OF FATTY ACID

R_3 - SERINE
CHOLINE
ETHANOLAMINE
INOSITOL
GLYCEROL



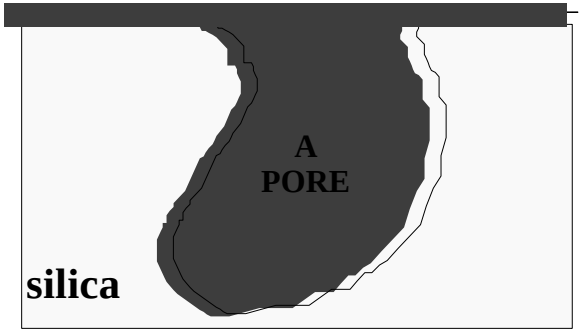
SUCROSE

BILIRUBIN

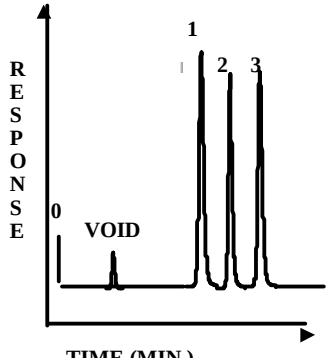


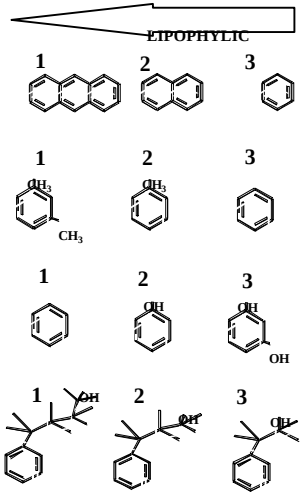
NORMAL PHASE

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



ELUTION ORDER IN NORMAL PHASE





High Performance Liquid Chromatography

PRINCIPLE OF SEPARATION:

- ION-EXCHANGE
- SIZE-EXCLUSION
- BIO-AFFINITY
- CHIRALITY

SOLUTES:

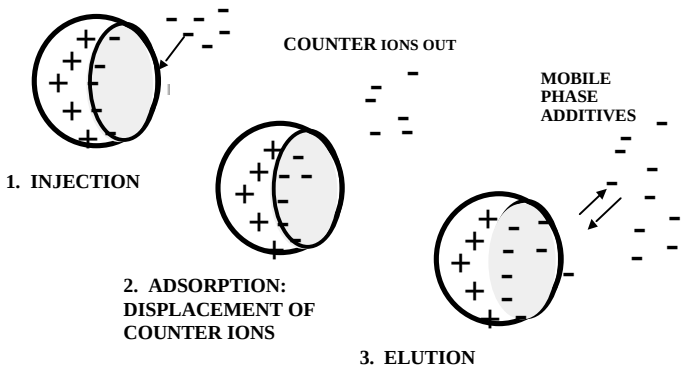
- INORGANIC IONS, ACIDS, BASES
- POLYMERS, PROTEINS, NUCLEIC ACIDS
- PROTEINS & ENZYMES
- ENANTIOMERS

CONDITIONS:

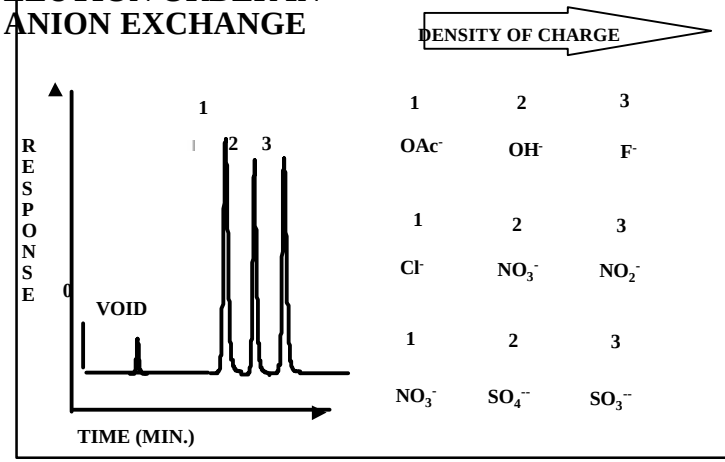
- AQUEOUS BUFFERS, IONIC SOLUTIONS
- AQUEOUS BUFFERS OR ORGANIC SOLVENTS
- AQUEOUS BUFFERS AND SPECIAL ADDITIVES
- AQUEOUS OR ORGANIC SOLVENTS

ION EXCHANGE

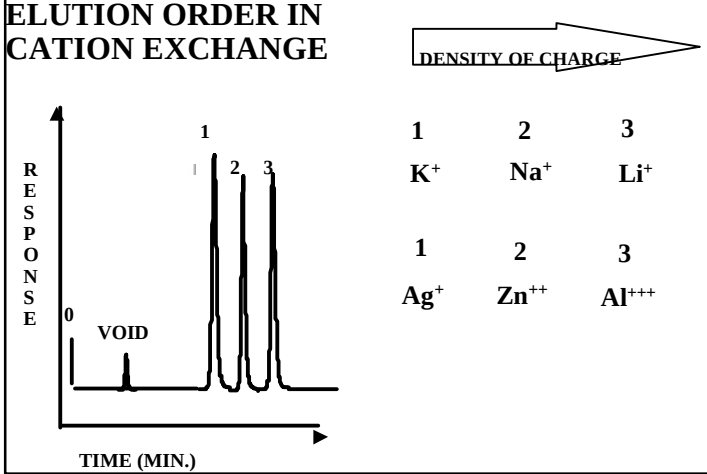
INSIDE A PORE IN THE STATIONARY PHASE



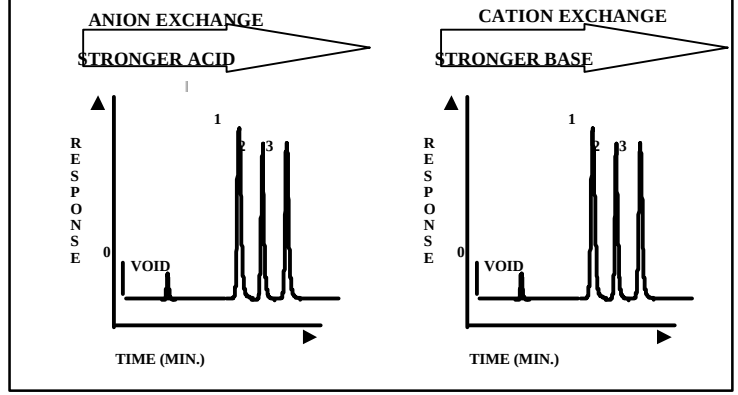
ELUTION ORDER IN ANION EXCHANGE



ELUTION ORDER IN CATION EXCHANGE



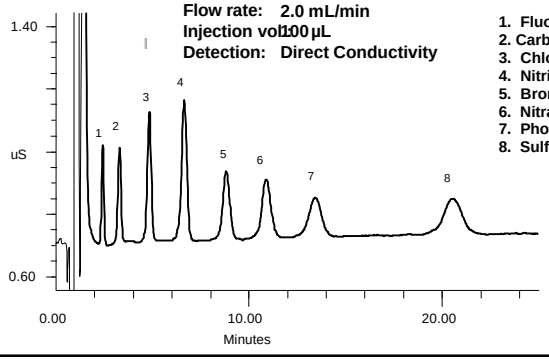
ELUTION ORDER IN ION EXCHANGE



Analysis of Ions

Column: Waters IC-Pak Anion HC
Eluent: Borate/Gluconate
Flow rate: 2.0 mL/min
Injection vol: 100 µL
Detection: Direct Conductivity

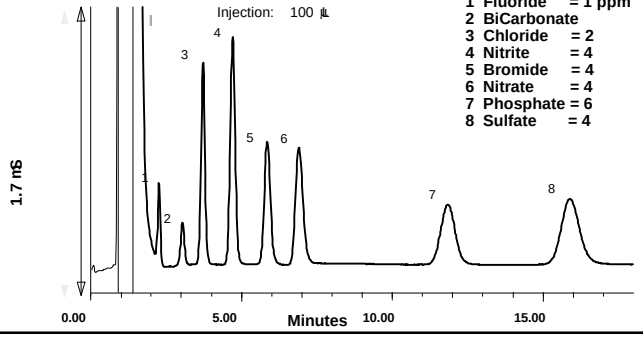
1. Fluoride 1 ppm
2. Carbonate --------
3. Chloride 2 ppm
4. Nitrite 4 ppm
5. Bromide 4 ppm
6. Nitrate 4 ppm
7. Phosphate 6 ppm
8. Sulfate 4 ppm



Analysis of Anions

Column: Waters IC Pak A/HR
Eluent: Borate / Gluconate
Flow Rate: 1 mL/min
Injection: 100 µL

1 Fluoride = 1 ppm
2 BiCarbonate
3 Chloride = 2
4 Nitrite = 4
5 Bromide = 4
6 Nitrate = 4
7 Phosphate = 6
8 Sulfate = 4

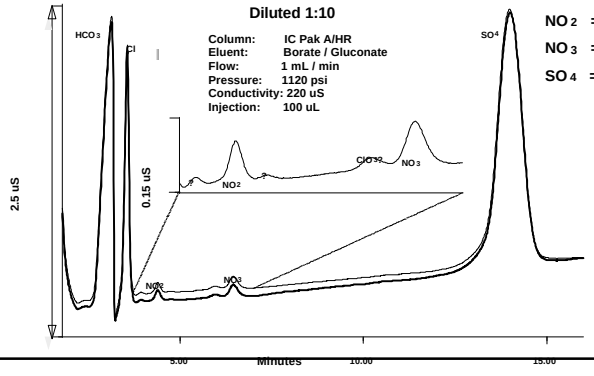


Analysis of Anions in Waste Water

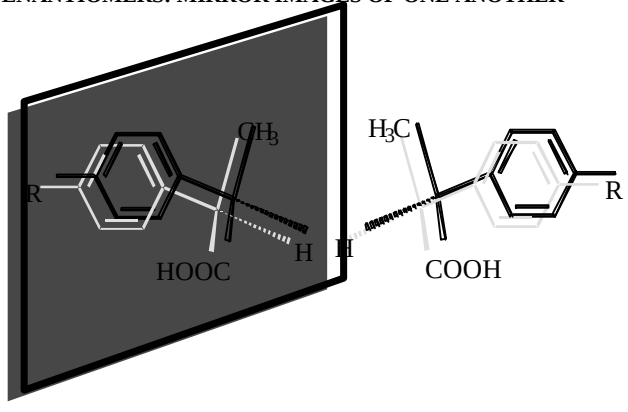
Duplicate Injections of Wastewater
Diluted 1:10

Column: IC Pak A/HR
Eluent: Borate / Gluconate
Flow: 1 mL / min
Pressure: 1120 psi
Conductivity: 220 uS
Injection: 100 uL

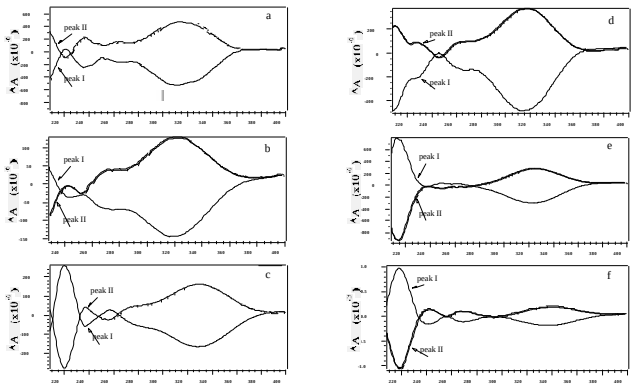
Original Sample
Cl = 34.2 ppm
NO_2 = 3.0
NO_3 = 5.1
SO_4 = 258



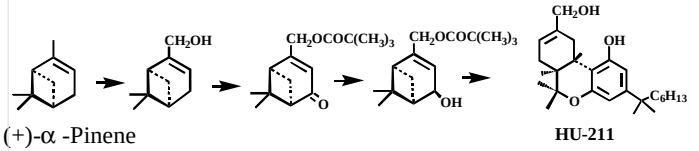
ENANTIOMERS: MIRROR IMAGES OF ONE ANOTHER



Circular Dichroism SPECTRA

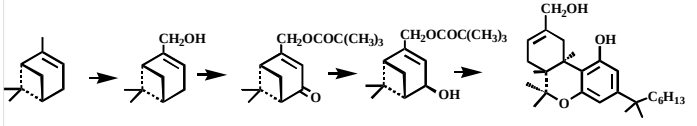


Asymmetric Synthesis



(+)-α -Pinene

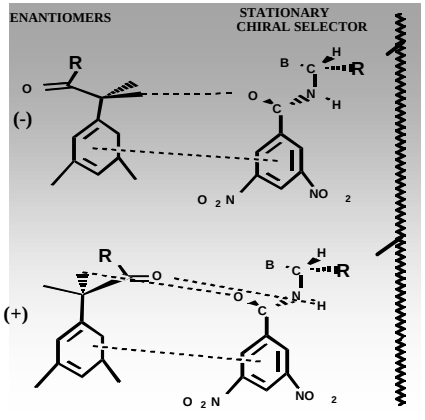
HU-211

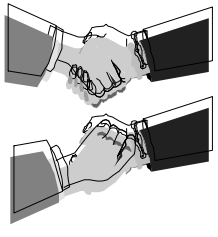


(-)-α -Pinene

HU-210

BASIS FOR SEPARATION: CHIRAL RECOGNITION



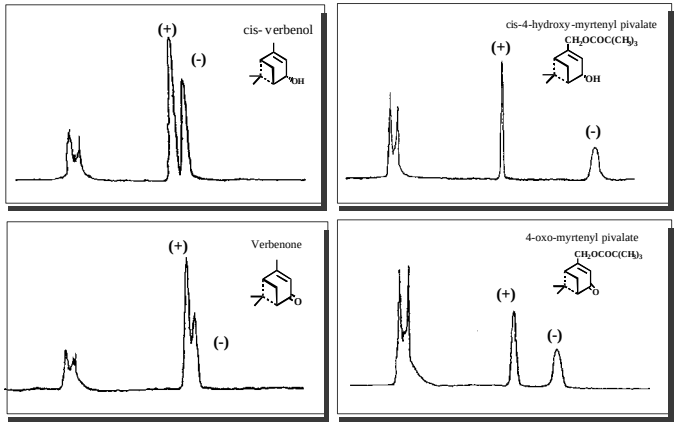


Chiral stationary phases:

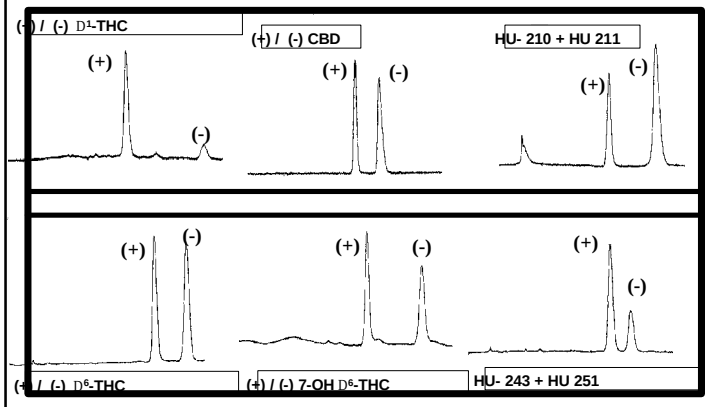
- **Ligand exchange**
- **p-Donor p-acceptor (Pirkle)**
- **Chiral Host-guest (cyclodextrin)**
- **Immobilized proteins**
- **Immobilized polysaccharides**

High Performance Liquid Chromatography – HPLC - Overview

SEPARATION OF ENANTIOMERS OF TERPENOIDS

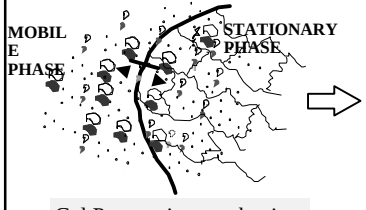


SEPARATION OF 6 ENANTIOMERIC PAIRS OF CANNABINOIDS

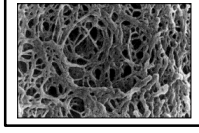


SIZE EXCLUSION CHROMATOGRAPHY

PRINCIPLE OF SEPARATION

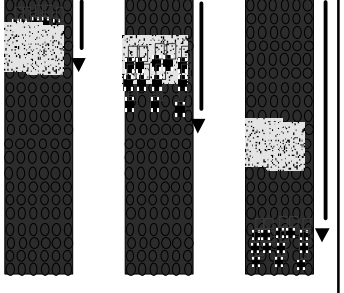


Gel Permeation mechanism



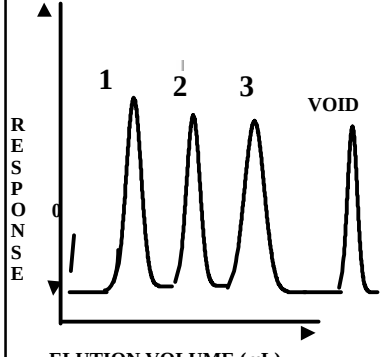
Scanning electron micrograph of an agarose gel. Magnification x 50,000. *Ref. Anders S. Medin,PhD Thesis, Uppsala University 1995.*

SEPARATION PROCESS:



ELUTION ORDER: LARGER ELUTE FIRST

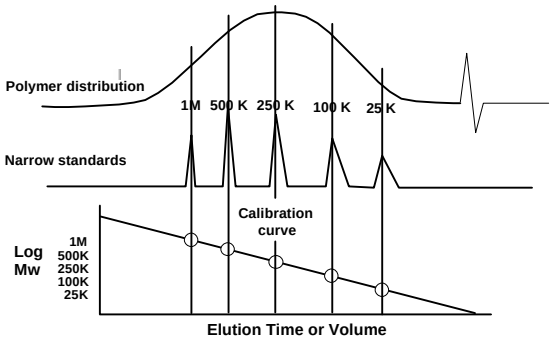
ELUTION ORDER IN SIZE EXCLUSION (GPC)



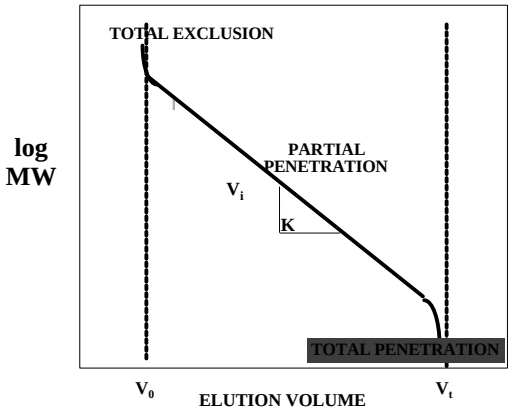
ELUTION VOLUME (mL)

	MW
1	100,000
2	50,000
3	20,000

Gel Filtration/Size Exclusion/Gel Permeation



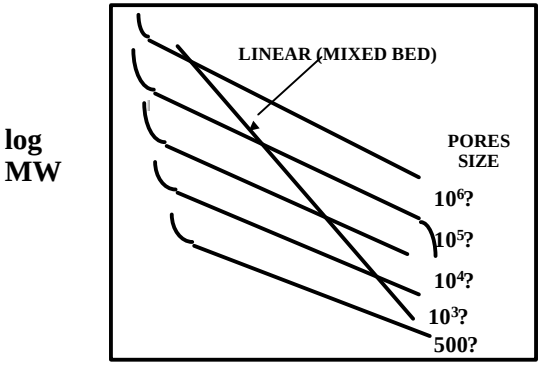
THEORETICAL CURVE OF THE STERIC EXCLUSION



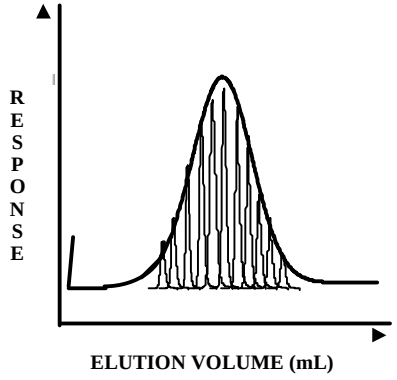
$$\frac{V_i - V_0}{V_t - V_0} = K$$

$$0 <= K =< 1$$

ELUTION CURVES OF VARIOUS STATIONARY PHASES

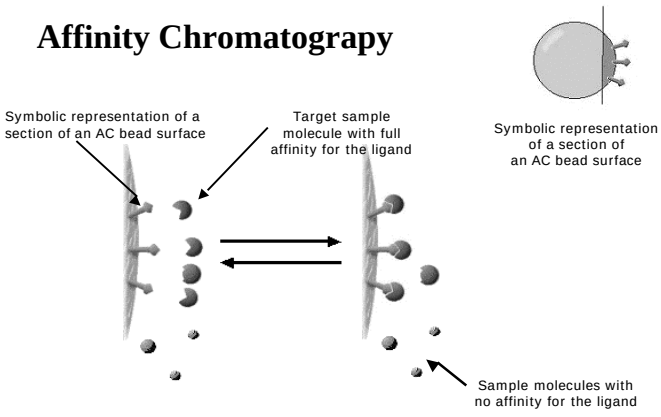


MOLECULAR WEIGHT DISTRIBUTION



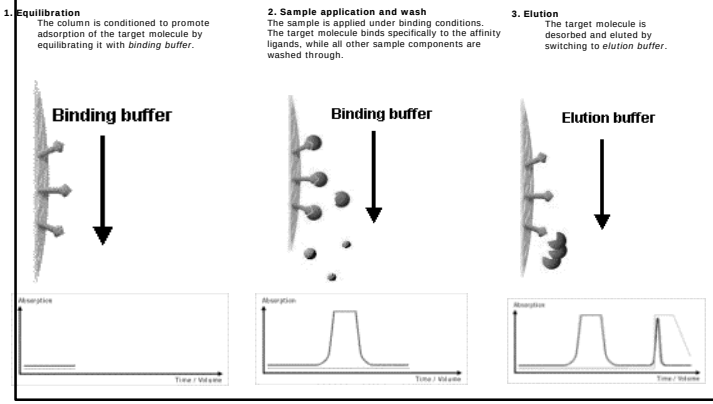
Dr. Shulamit Levin, Medtechnica (levins@medtechnica.co.il)

Affinity Chromatograpy

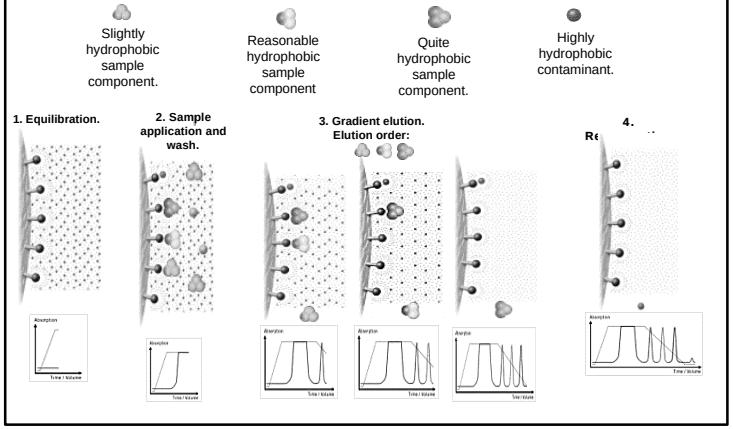


AC relies upon a reversible highly specific binding reaction.

Affinity Chromatograpy



Hydrophobic Interaction Chromatography (HIC)



Seven Basic Considerations in Choosing HPLC Operating Parameters

1) **Solubility - Hexane, Chloroform, Methanol, Water (buffer pH), other?**

2) **Molecular Weight - Would GPC be useful in either the analysis or sample prep?**

3) **Functional Groups - Any ionizable groups? Acidic, Basic, or Neutral?**

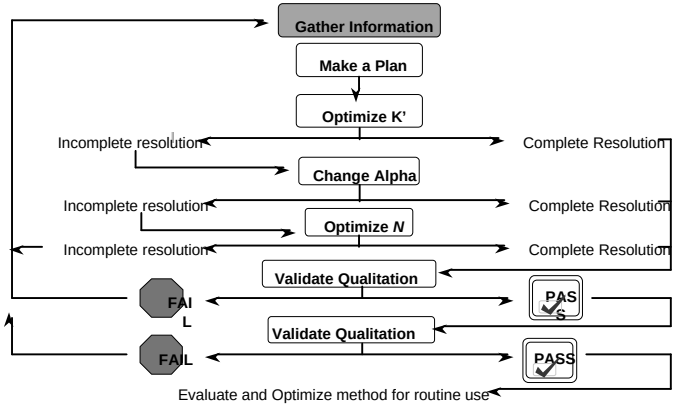
4) **Sample Matrix - What amounts are expected in matrix for either analytical or preparative isolation?**

5) **Levels in Matrix - What amounts are expected in matrix for either analytical or preparative isolation?**

6) **Detectability - Any chromophores or fluorophores? Consider Redox or derivatization. Together with point #5, an appropriate detector is chosen.**

7) **How Do Species Differ - An important clue to manipulate selectivity in the separation, especially if compounds are similar in their structure.**

Methods Development Strategy



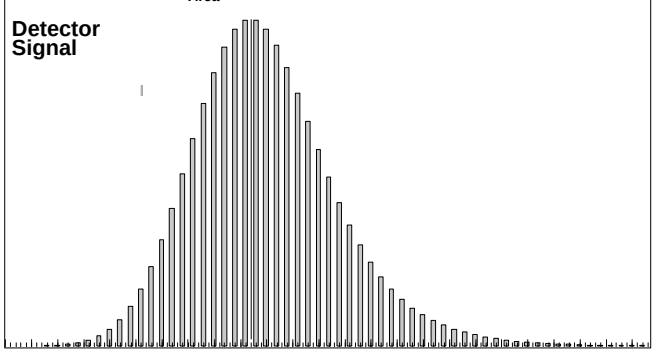
- Step by step method development strategy -

HPLC COURSE LAYOUT

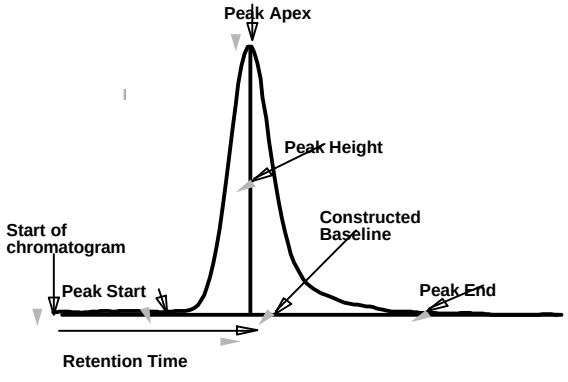
- **Introduction & Applicability**
- **Modes of Chromatography**
- **Quantitative work and System Qualification.**

Measurement of Area - Integration

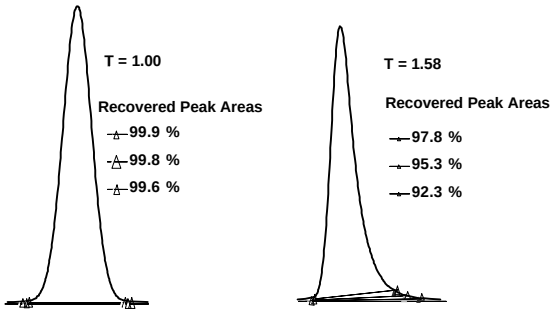
$$\text{Area} = \int \text{Abs} \times dt$$



Peak Detection

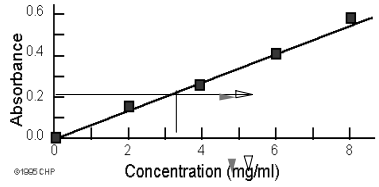


Integration Errors Caused by Tailing

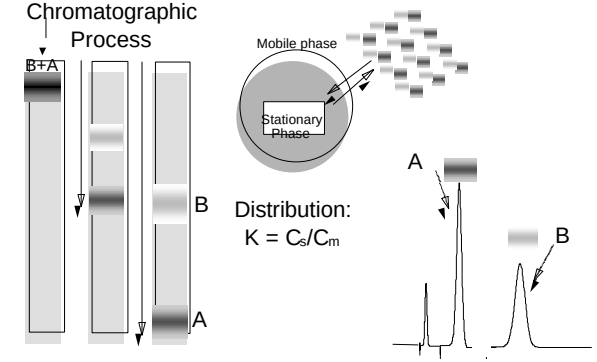


Working Curve

- **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

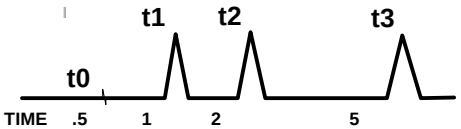
is

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



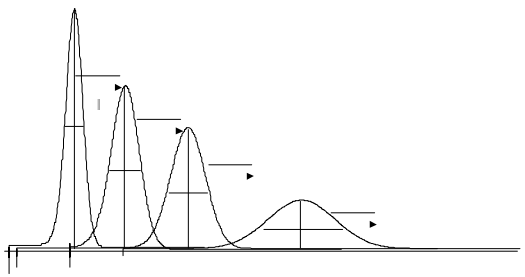
$k'\ t1 = \frac{t1 - t0}{t0}$

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

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

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

PEAK BROADENING



PERFORMANCE BY ONE PEAK

RETENTION FACTOR or CAPACITY RATIO

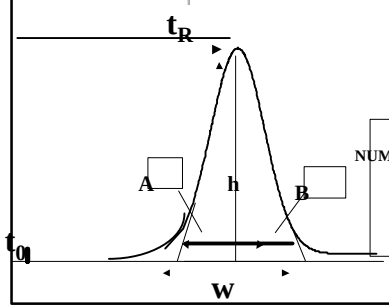
$$k' = \frac{t_R - t_0}{t_0} \qquad k' = f\,\frac{C_s}{C_m}$$

ASYMMETRY FACTOR

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

TAILING FACTOR

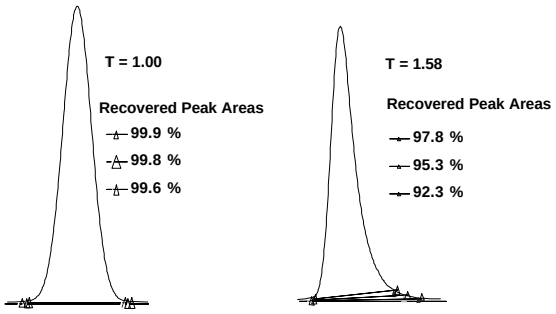
$$T_f = \frac{A + B}{2A}\ (10\%\ h)$$



NUMBER OF THEORETICAL PLATES

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

Integration Errors Caused by Tailing



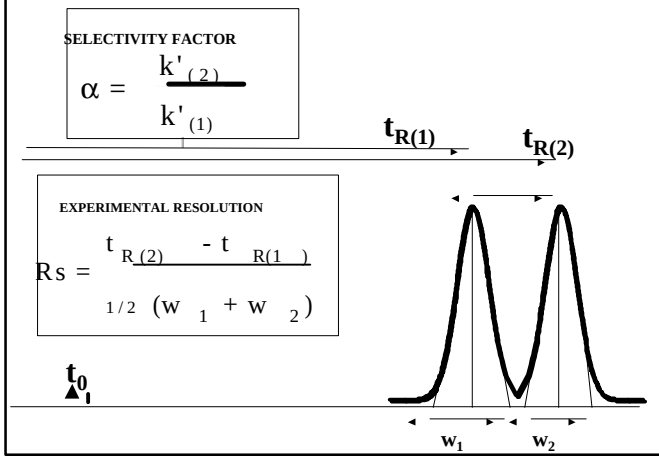
ERFORMANCE BY TWO PEAKS

SELECTIVITY FACTOR

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

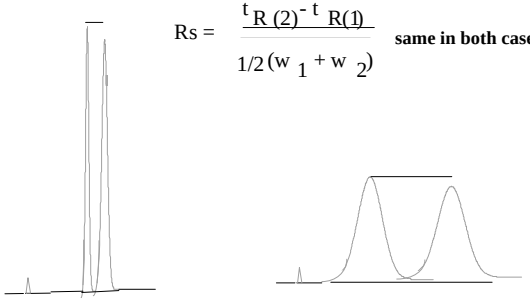
EXPERIMENTAL RESOLUTION

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



SELECTIVITY vs EFFICIENCY

$$Rs = \frac{t_{R(2)} - t_{R(1)}}{1/2\,(w_1 + w_2)}$$ same in both cases



LOW SELECTIVITY (a)
HIGH EFFICIENCY (N)

HIGH SELECTIVITY (a)
LOW EFFICIENCY (N)

ADVANTAGES OF HIGH EFFICIENCY

Resolution
Peak capacity
Sensitivity