

High Performance Liquid Chromatography - HPLC

Introduction

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

Dr. Shulamit Levin

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

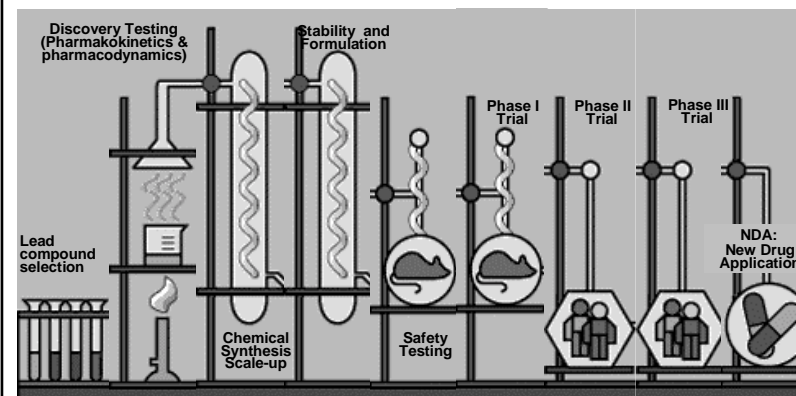
Hewlett-Packard liquid chromatography

High performance liquid chromatography

Hocus pocus liquid chromatography

High patience liquid chromatography

HPLC in Pharmaceuticals Technique No 1



Dr. Shulamit Levin

High Performance Liquid Chromatography - HPLC

Introduction

APPLICATIONS OF HPLC

Veterinary



Environmental



Agriculture &
Food



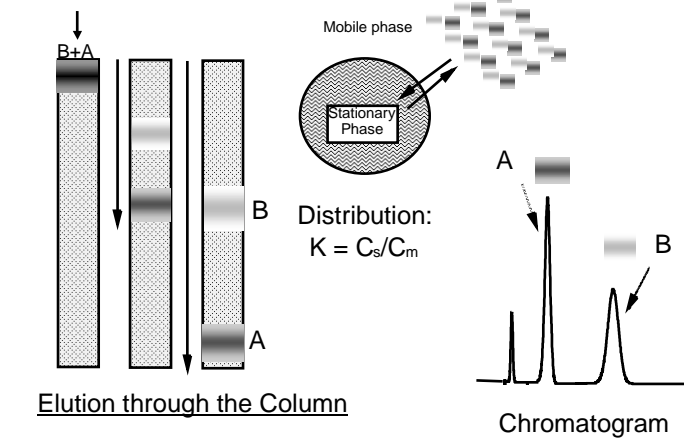
Biomedical and Clinical



Chemistry

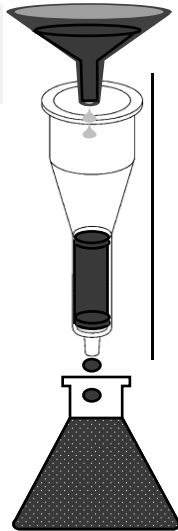


Chromatographic Process



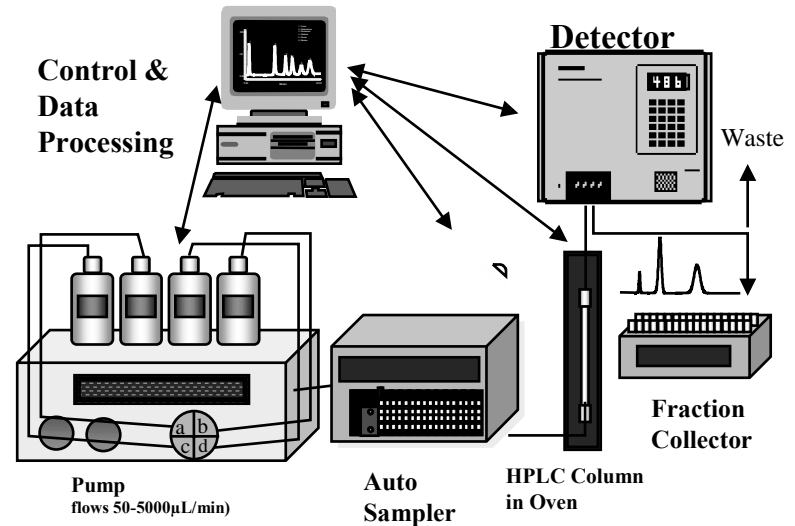
The beginning:

Gravitational chromatography



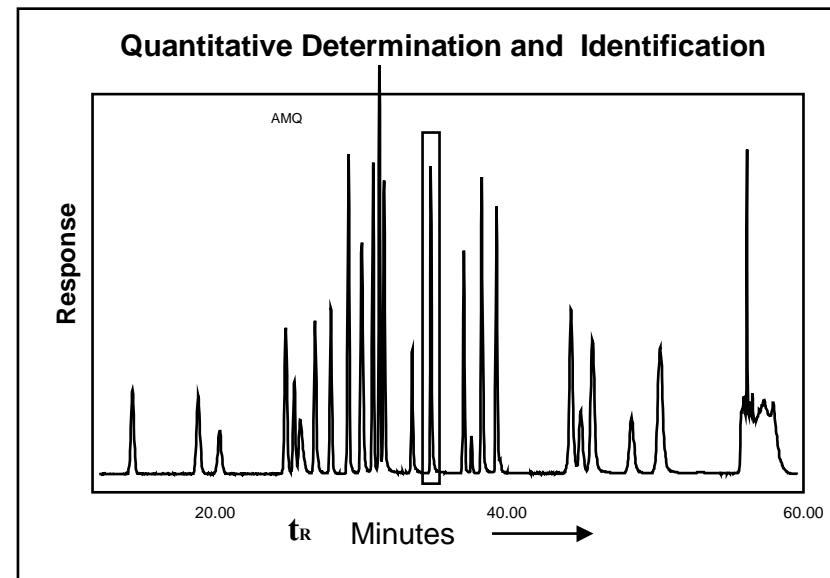
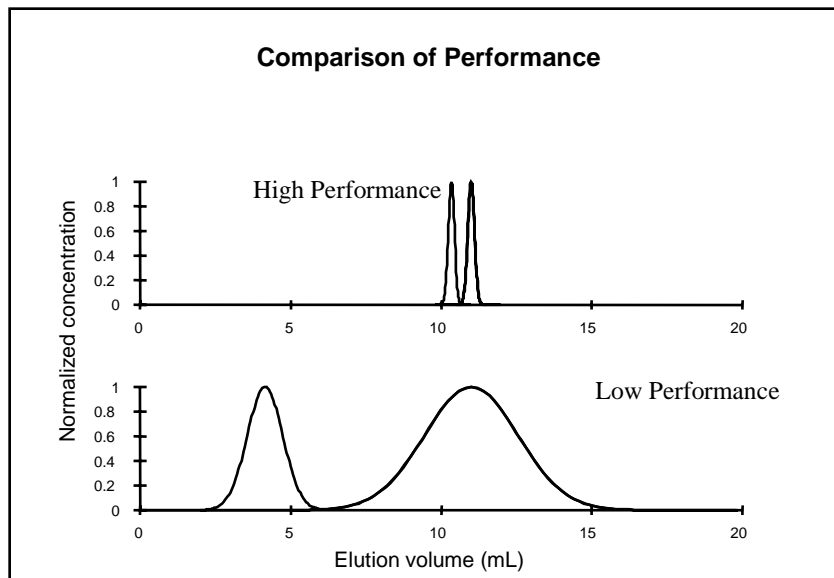
Control &
Data
Processing

Detector



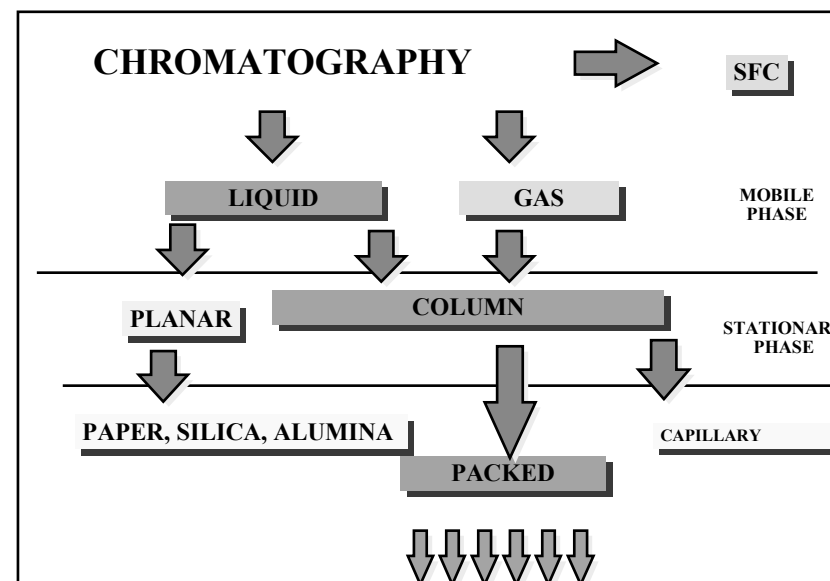
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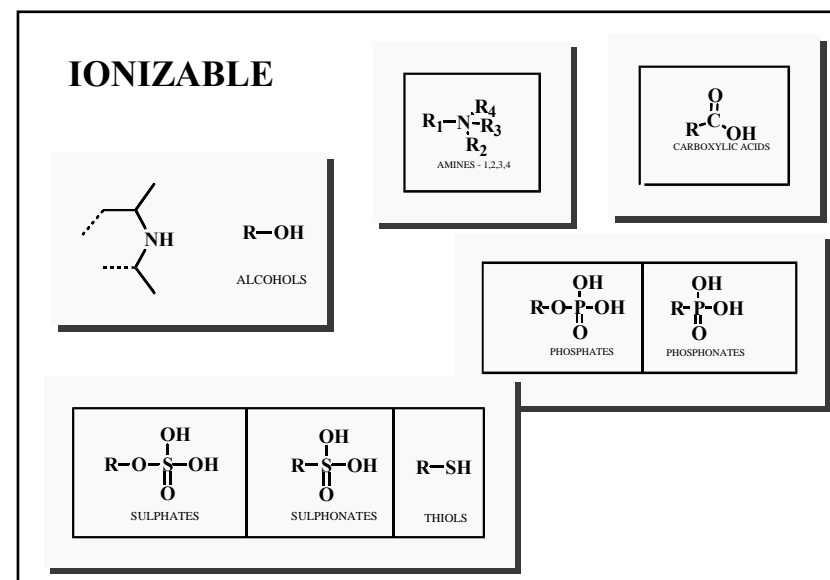
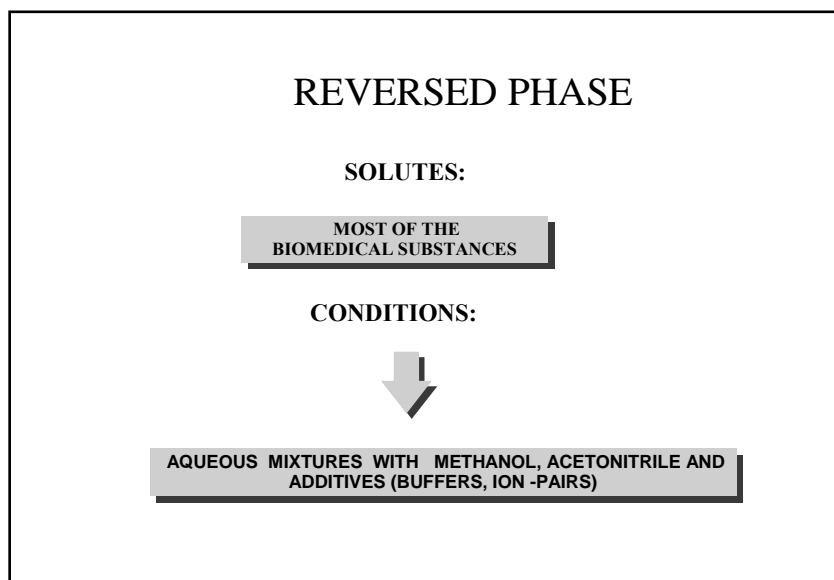
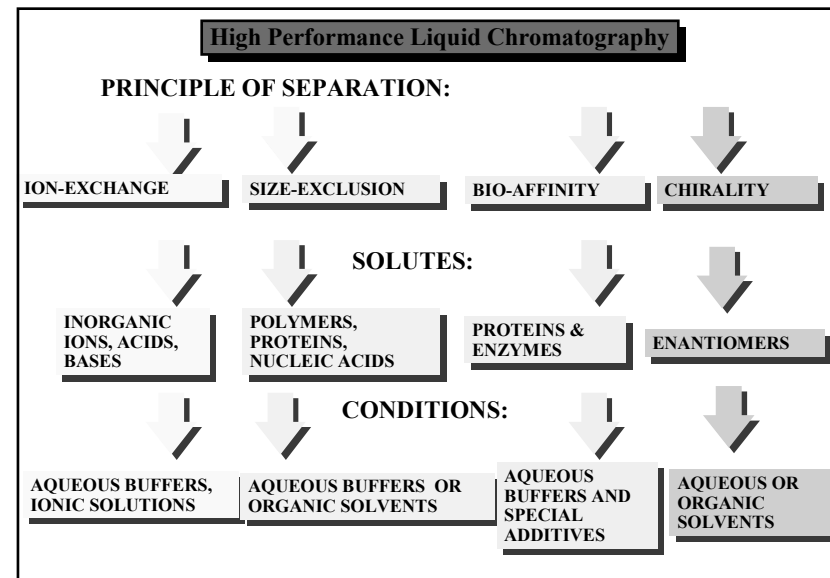
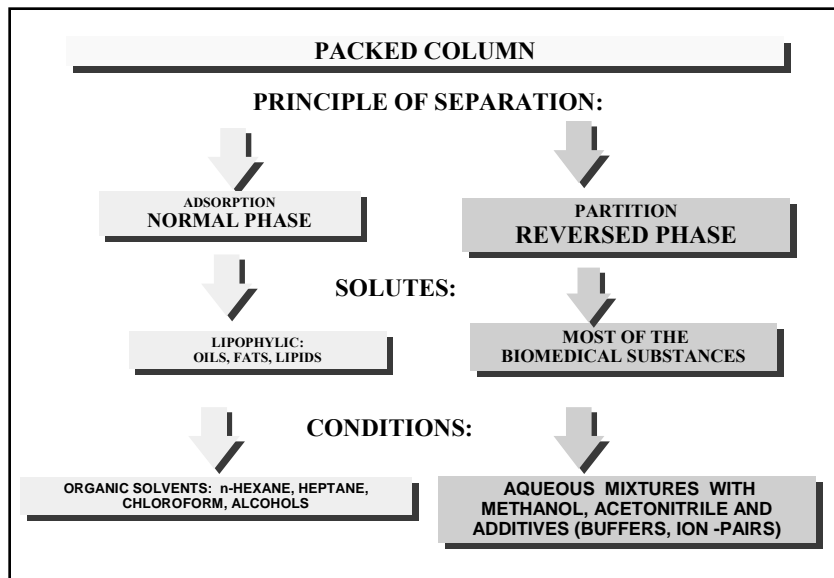
HPLC COURSE LAYOUT

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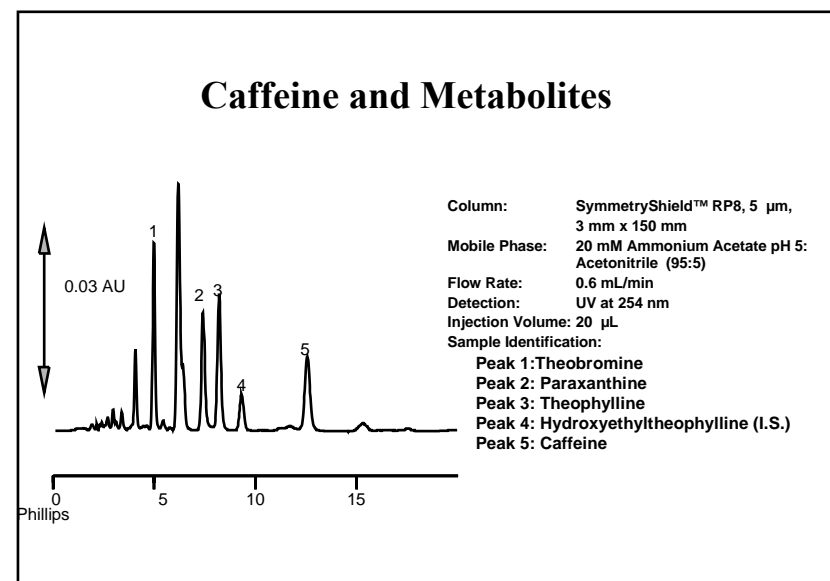
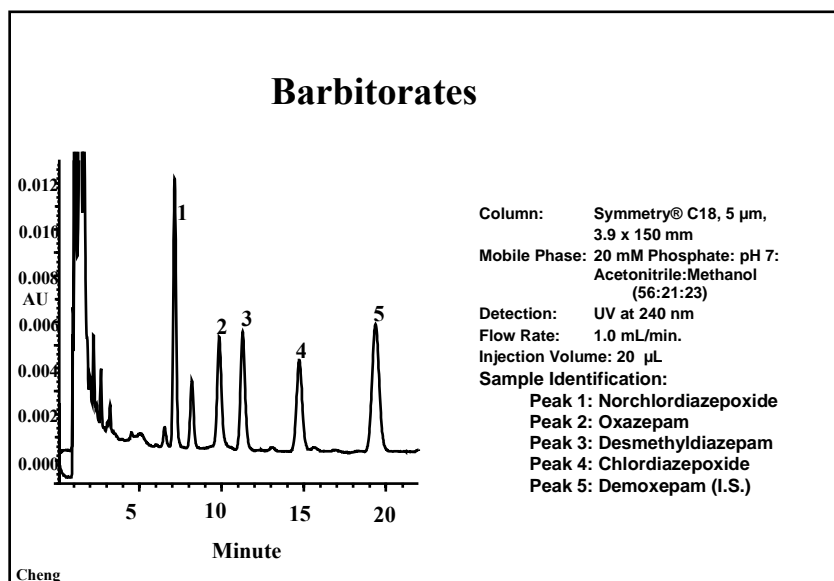
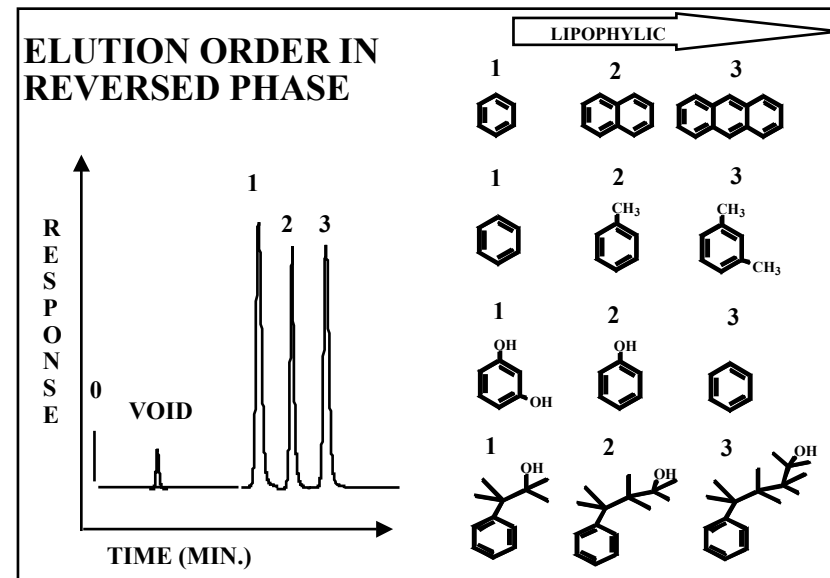
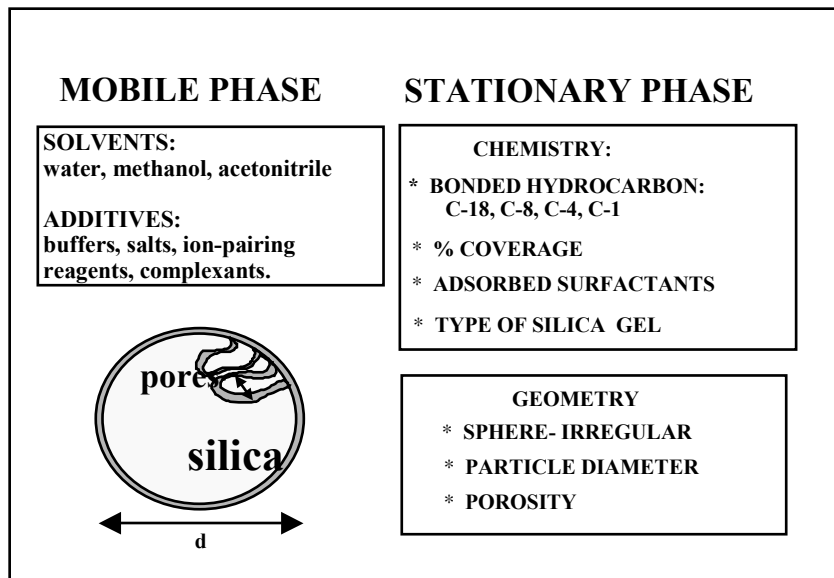
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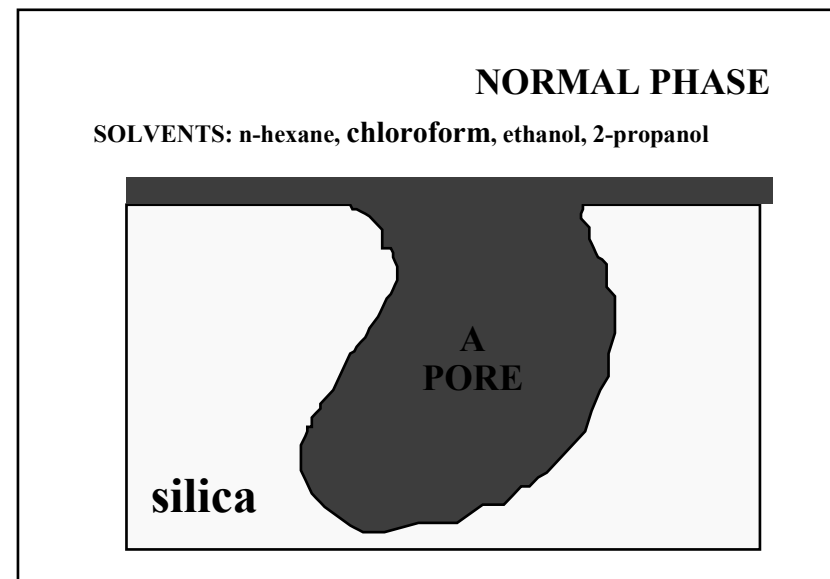
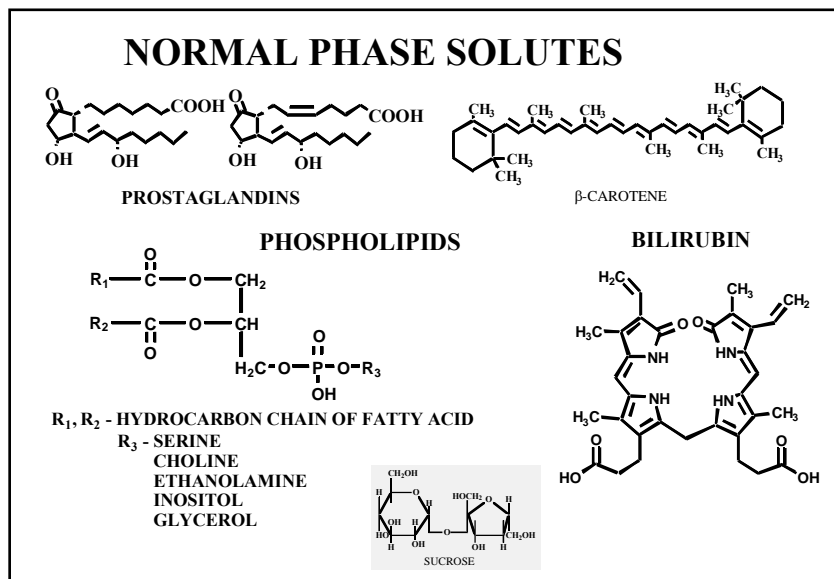
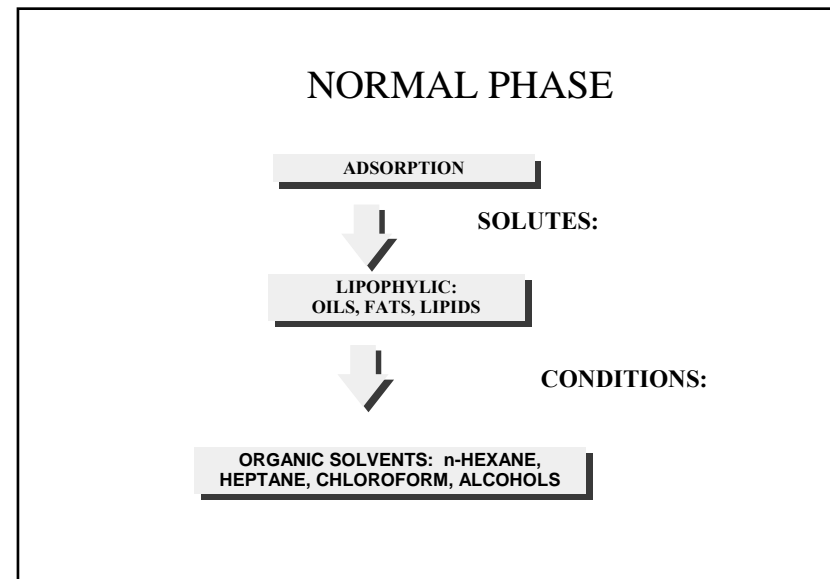
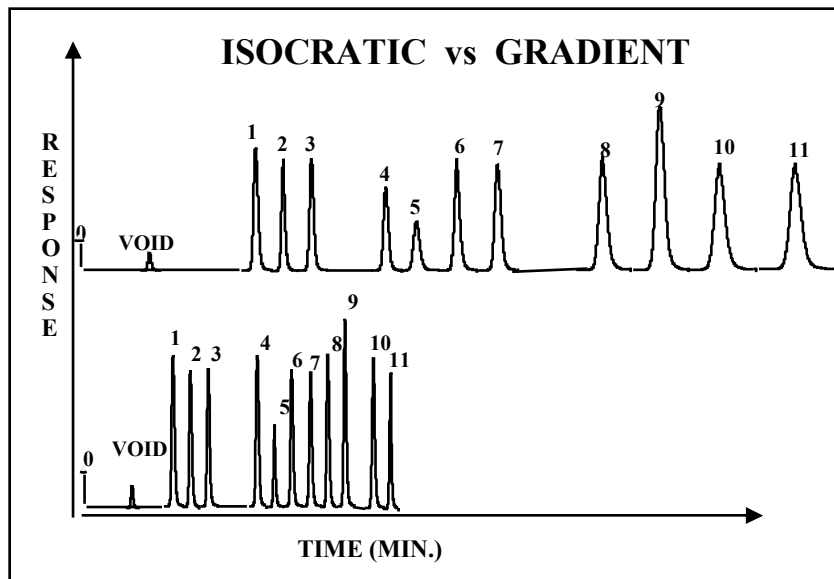
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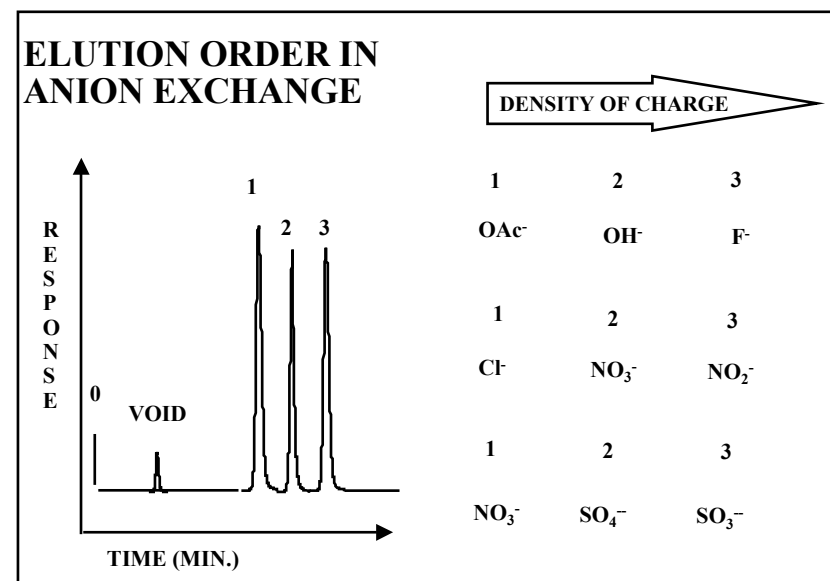
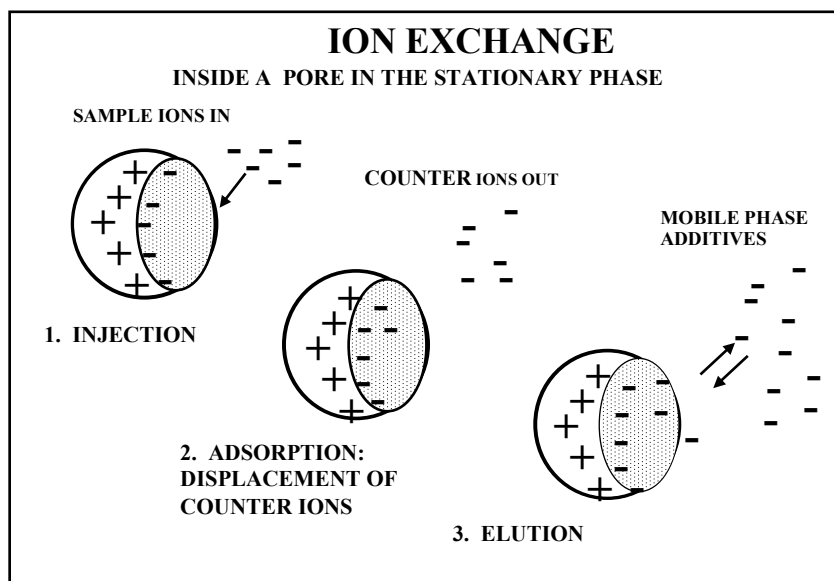
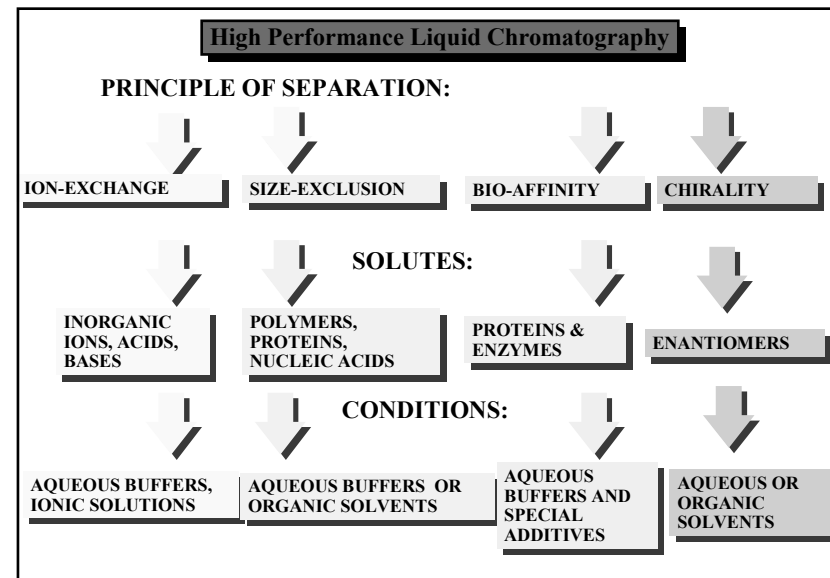
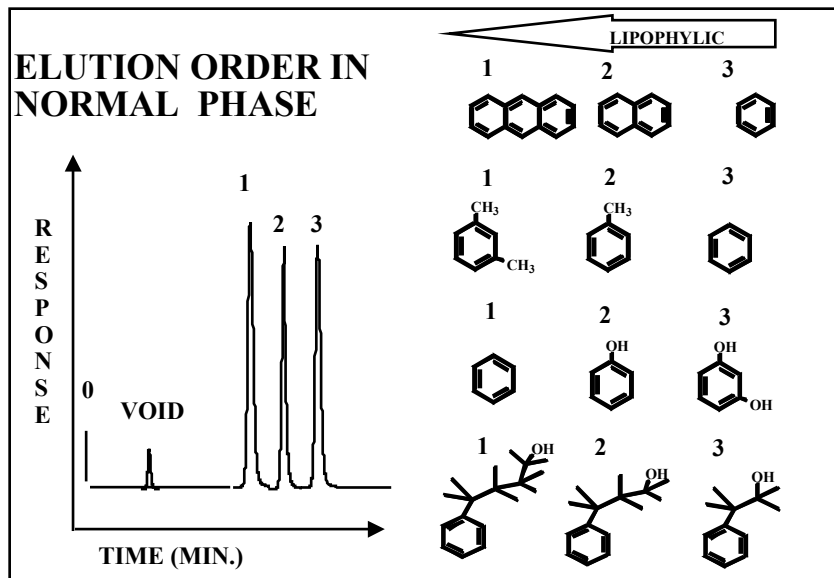
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High Performance Liquid Chromatography - HPLC

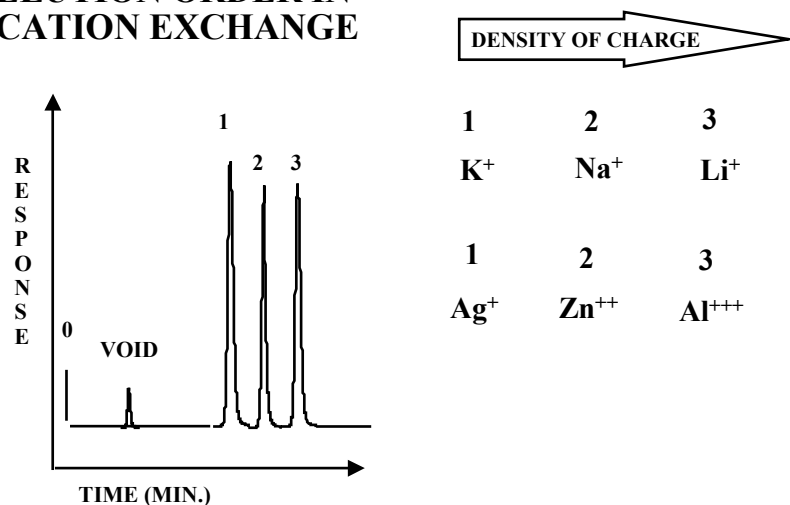
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High Performance Liquid Chromatography - HPLC

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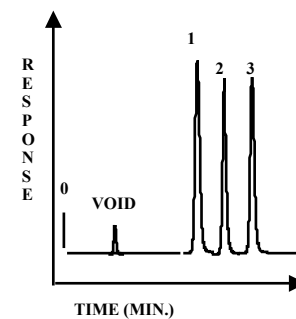
ELUTION ORDER IN CATION EXCHANGE



ELUTION ORDER IN ION EXCHANGE

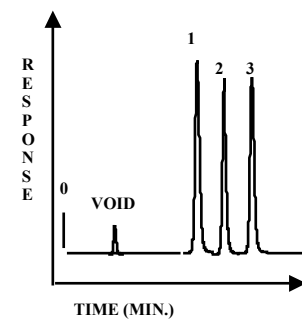
ANION EXCHANGE

STRONGER ACID →

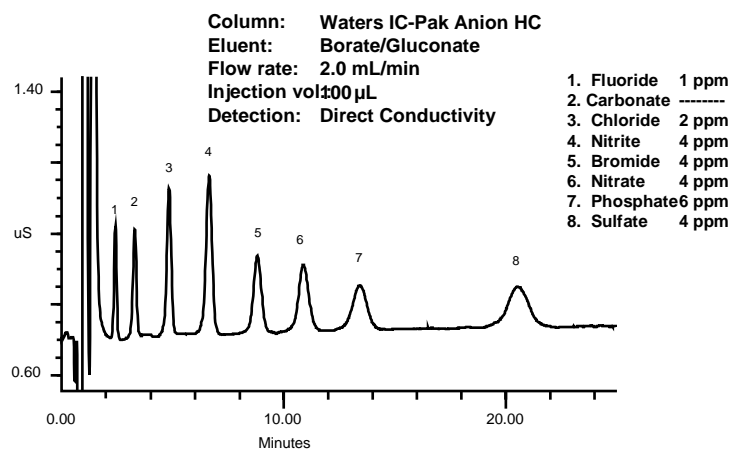


CATION EXCHANGE

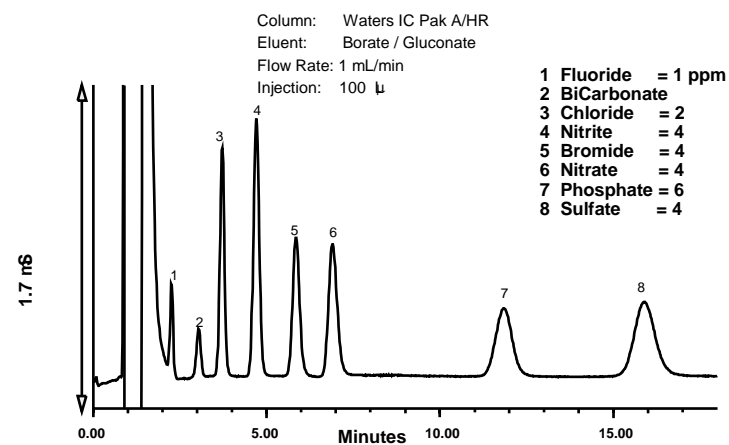
STRONGER BASE →



Analysis of Ions



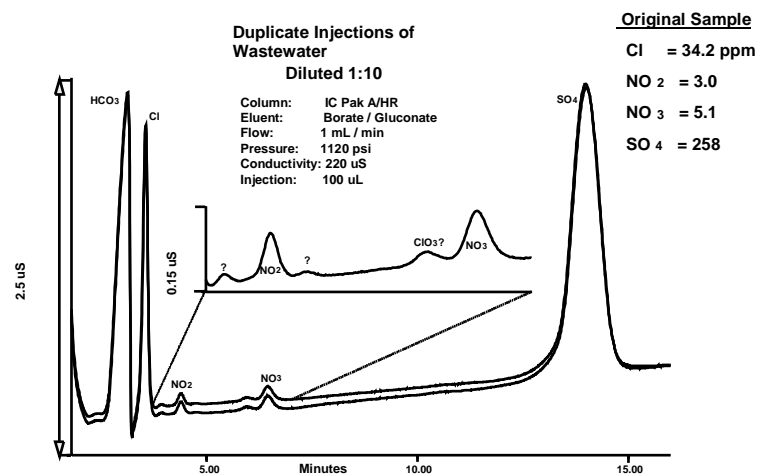
Analysis of Anions



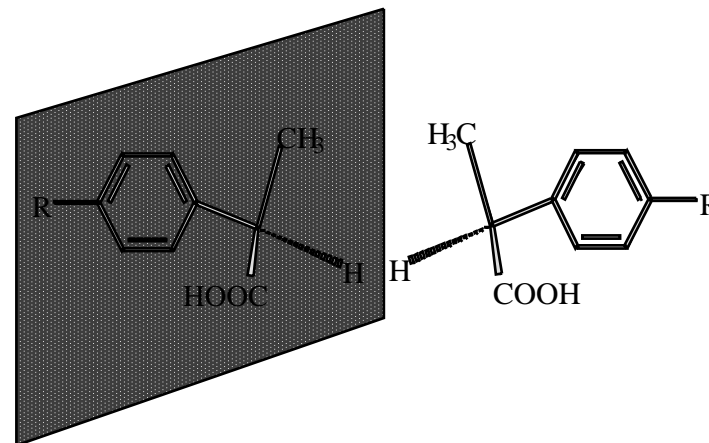
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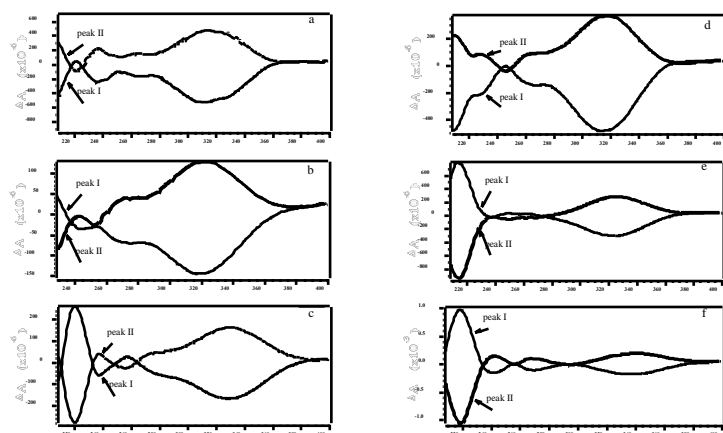
Analysis of Anions in Waste Water



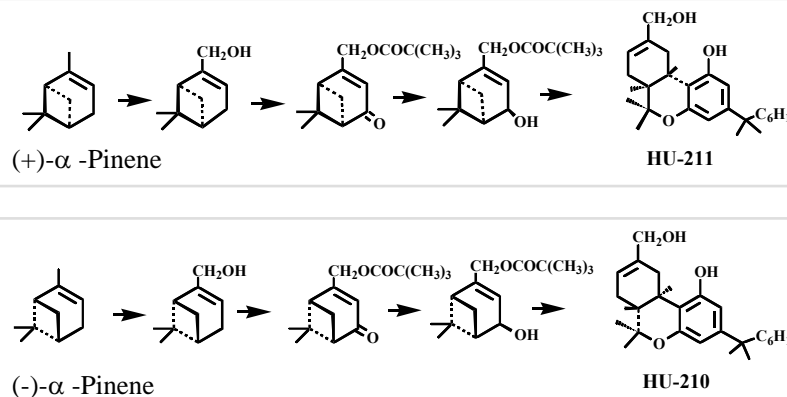
ENANTIOMERS: MIRROR IMAGES OF ONE ANOTHER



Circular Dichroism SPECTRA



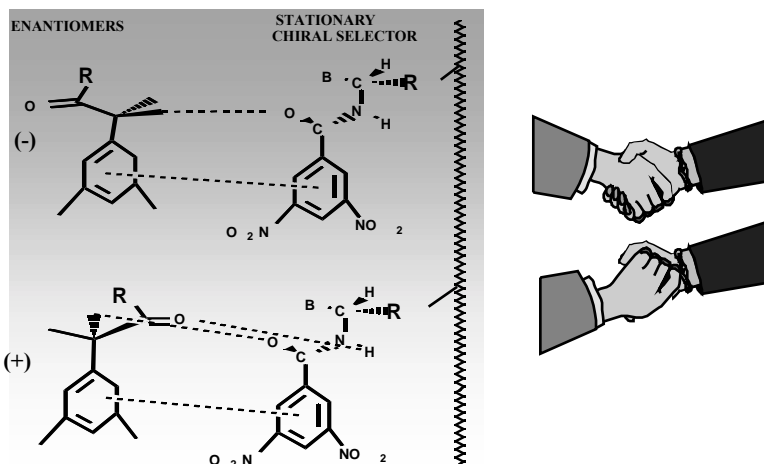
Asymmetric Synthesis



High Performance Liquid Chromatography - HPLC

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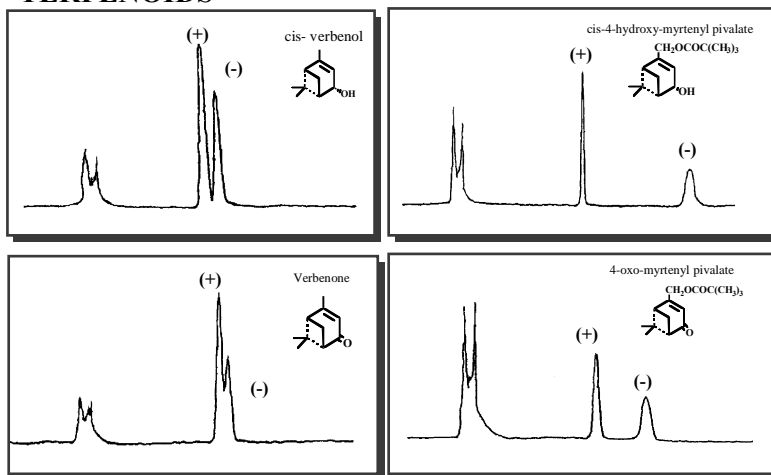
BASIS FOR SEPARATION: CHIRAL RECOGNITION



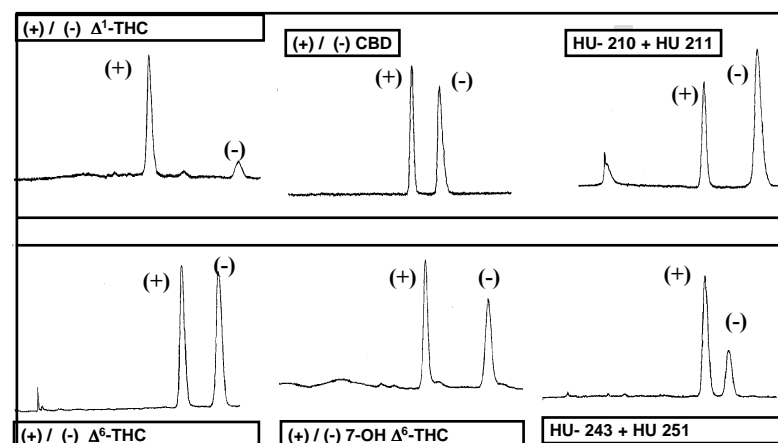
Chiral stationary phases:

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

SEPARATION OF ENANTIOMERS OF TERPENOIDS



SEPARATION OF 6 ENANTIOMERIC PAIRS OF CANNABINoids



High Performance Liquid Chromatography - HPLC

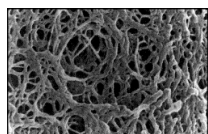
Introduction

SIZE EXCLUSION CHROMATOGRAPHY

PRINCIPLE OF SEPARATION

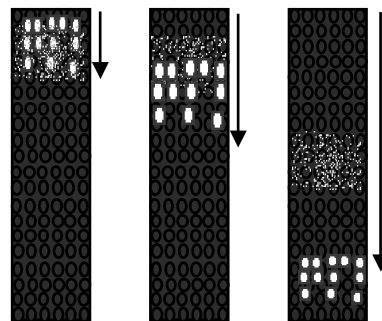


Gel Permeation mechanism



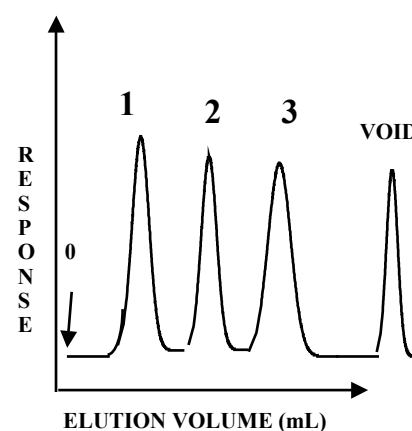
Scanning electron micrograph of an agarose gel.
Magnification x 50,000.
Ref. Anders S. Medin, PhD
Thesis, Uppsala University
1995.

SEPARATION PROCESS:



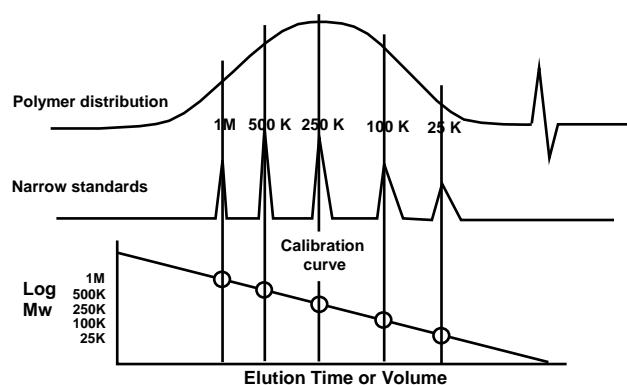
ELUTION ORDER:
LARGER ELUTE FIRST

ELUTION ORDER IN SIZE EXCLUSION (GPC)

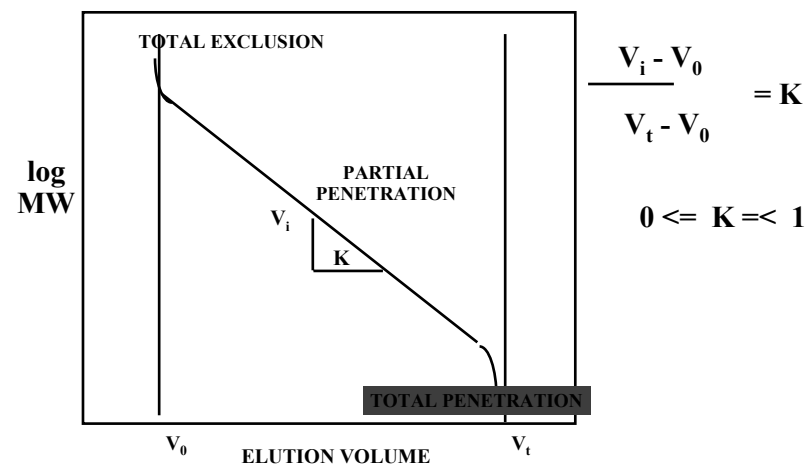


	MW
1	100,000
2	50,000
3	20,000

Gel Filtration/Size Exclusion/Gel Permeation



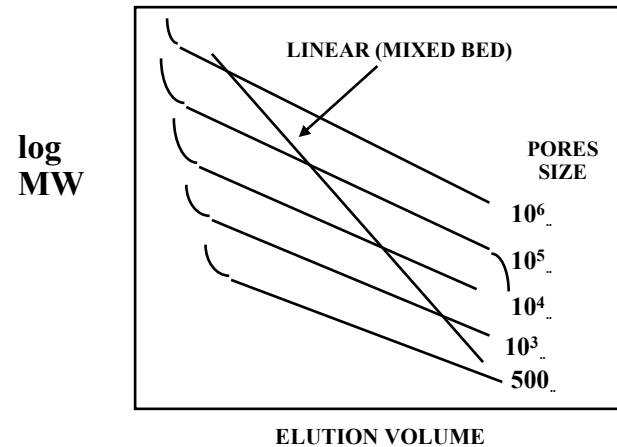
THEORETICAL CURVE OF THE STERIC EXCLUSION



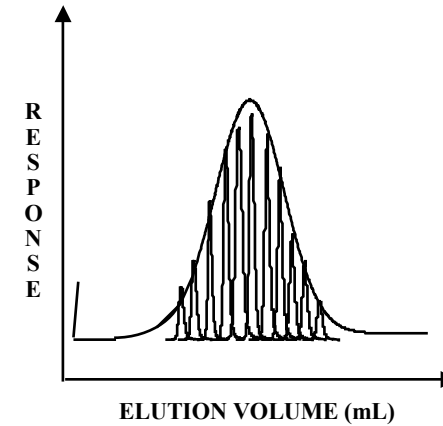
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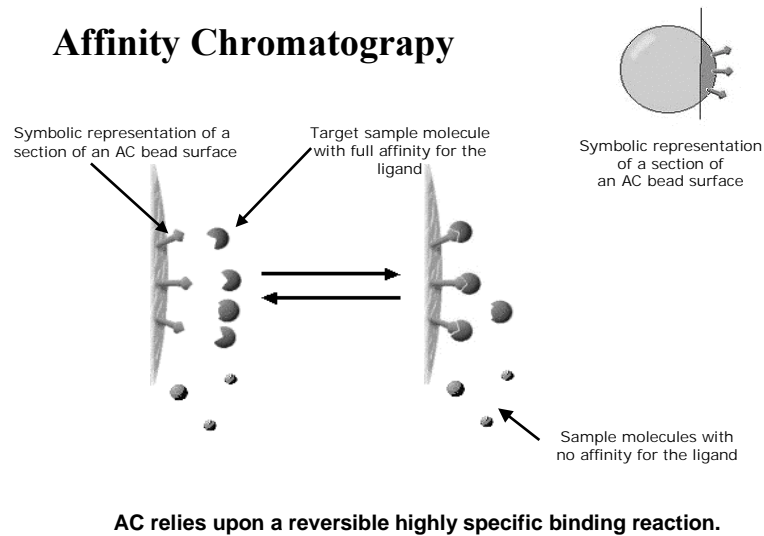
ELUTION CURVES OF VARIOUS STATIONARY PHASES



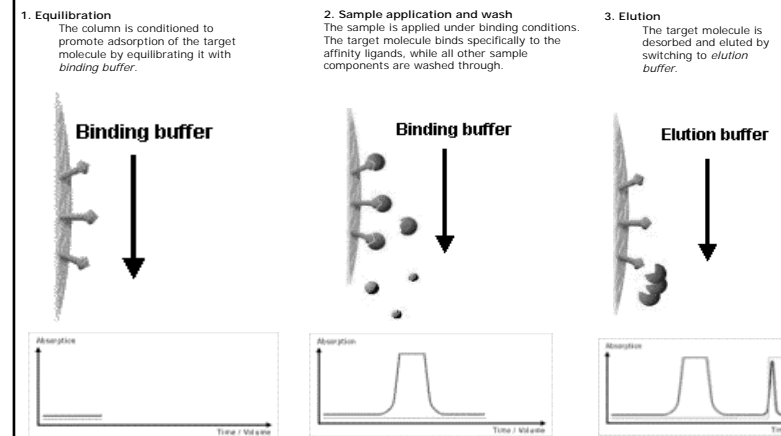
MOLECULAR WEIGHT DISTRIBUTION



Affinity Chromatography



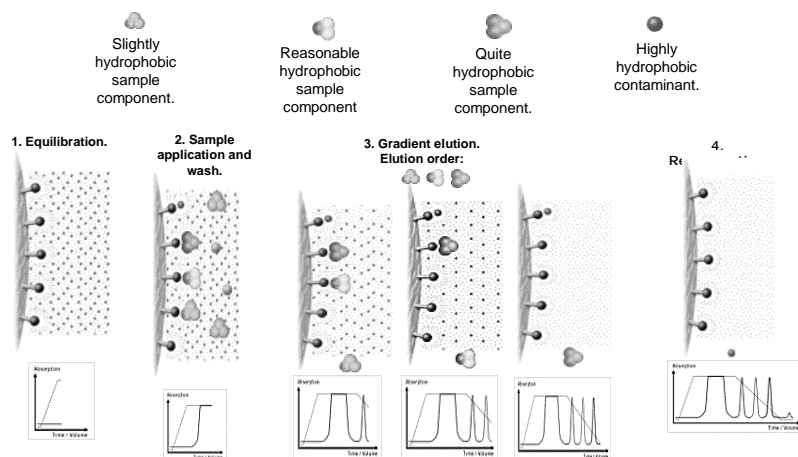
Affinity Chromatography



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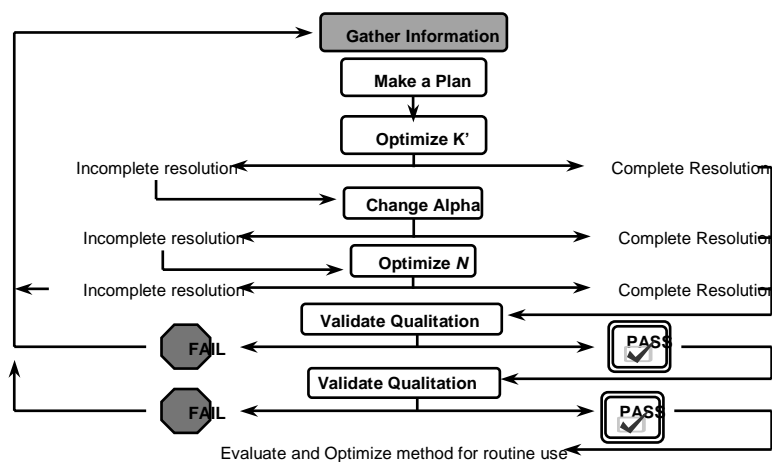
Hydrophobic Interaction Chromatography (HIC)



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 the separation, especially if compounds are similar in their structure.

Methods Development Strategy



- Step by step method development strategy -

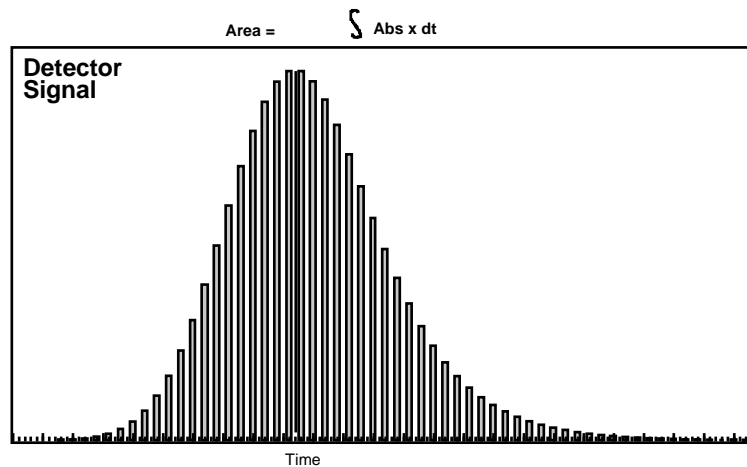
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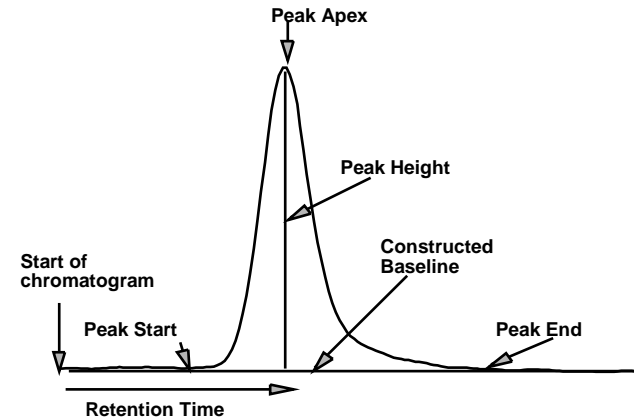
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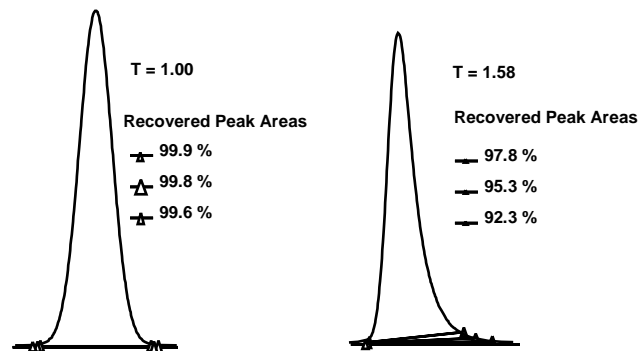
Measurement of Area - Integration



Peak Detection

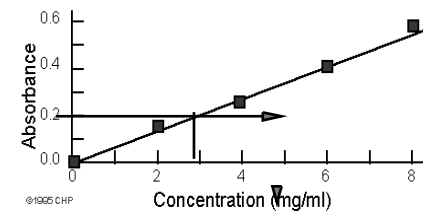


Integration Errors Caused by Tailing



Working Curve

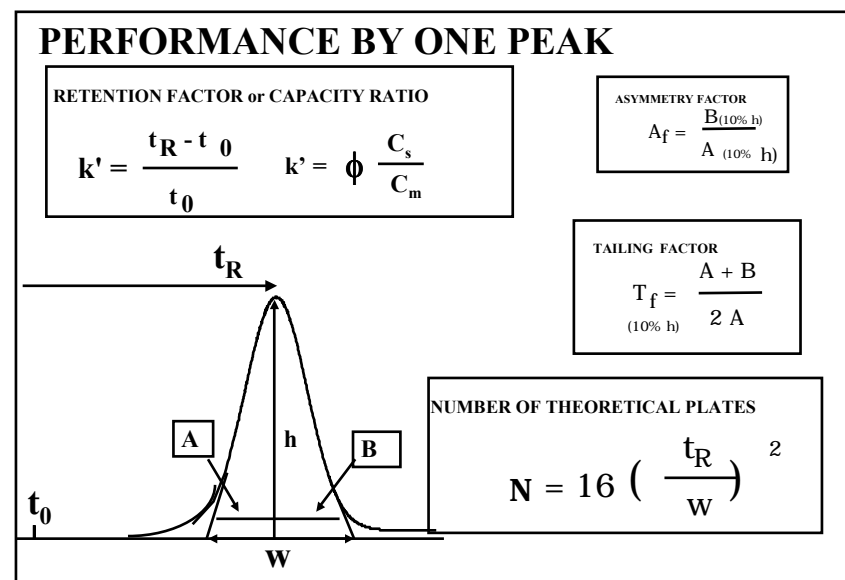
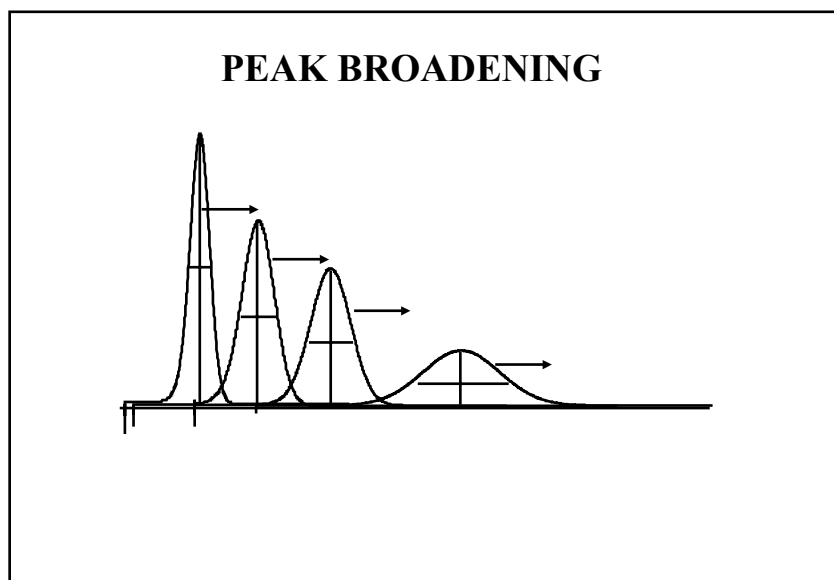
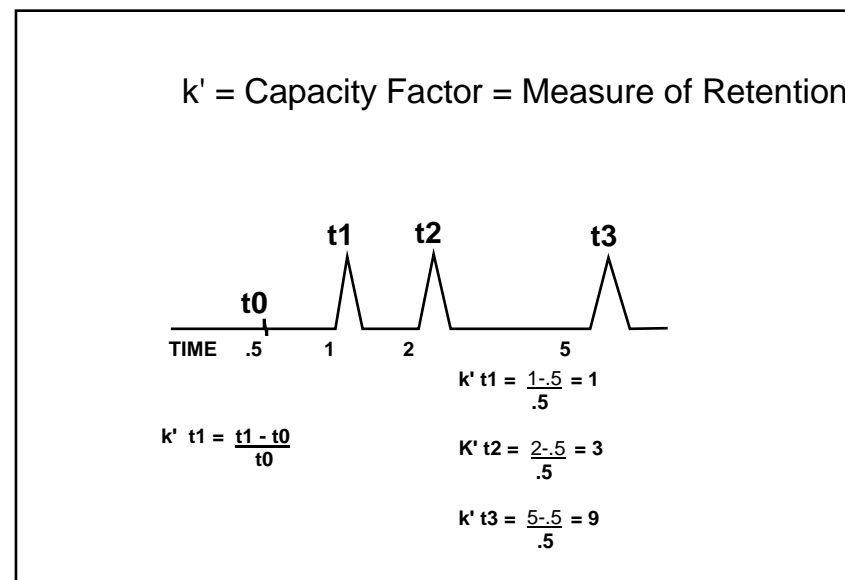
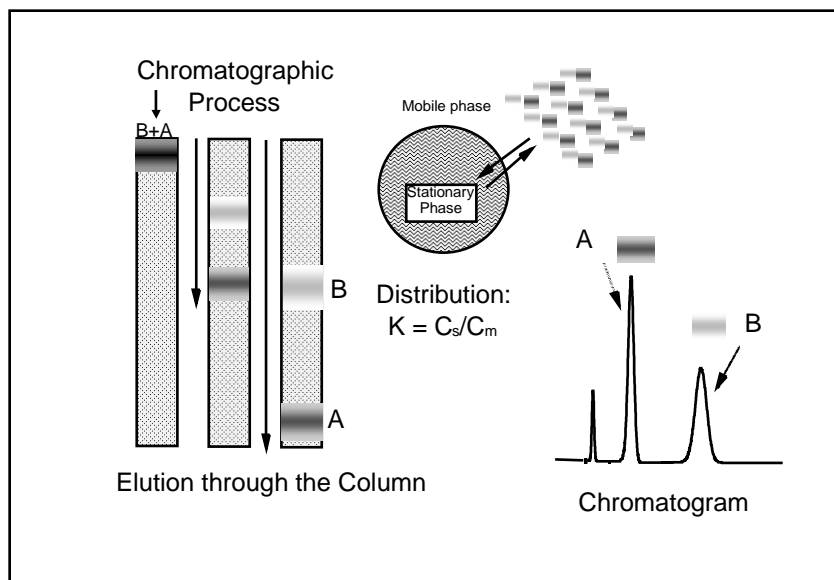
▣ A plot of the analytical signal (the instrument or detector response) as a function of analyte concentration, using a series of standards of known concentration.



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

High Performance Liquid Chromatography - HPLC

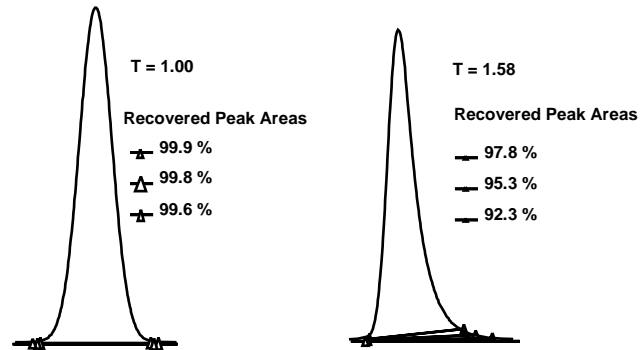
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High Performance Liquid Chromatography - HPLC

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Integration Errors Caused by Tailing



PERFORMANCE BY TWO PEAKS

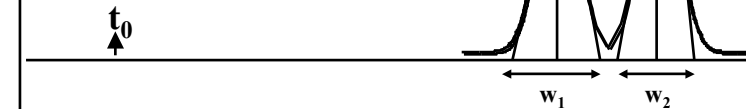
SELECTIVITY FACTOR

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

$t_{R(1)}$ $t_{R(2)}$

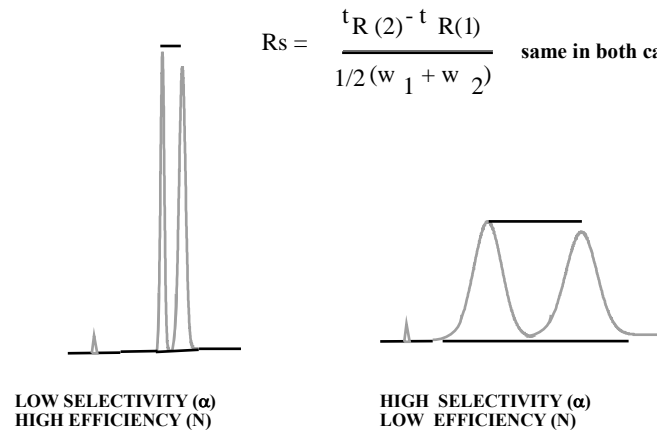
EXPERIMENTAL RESOLUTION

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



SELECTIVITY vs EFFICIENCY

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



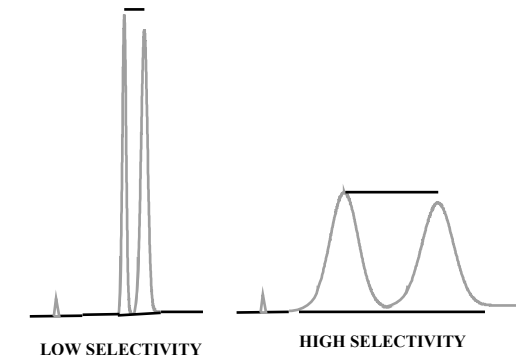
Resolution

Peak capacity

Sensitivity

ADVANTAGES OF HIGH EFFICIENCY

HIGH PERFORMANCE LOW PERFORMANCE



High Performance Liquid Chromatography - HPLC

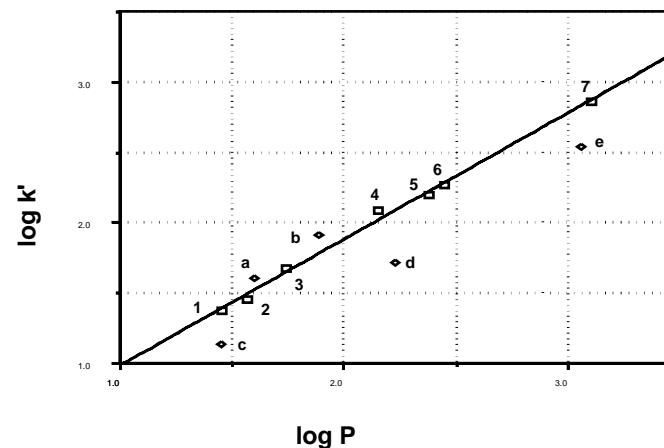
Introduction

Reversed Phase HPLC

Dr. Shulamit Levin
Medtechnica

http://www.Geocities.com/CapeCanaveral/8775/HPLC_guide_h.html

Hydrophobicity

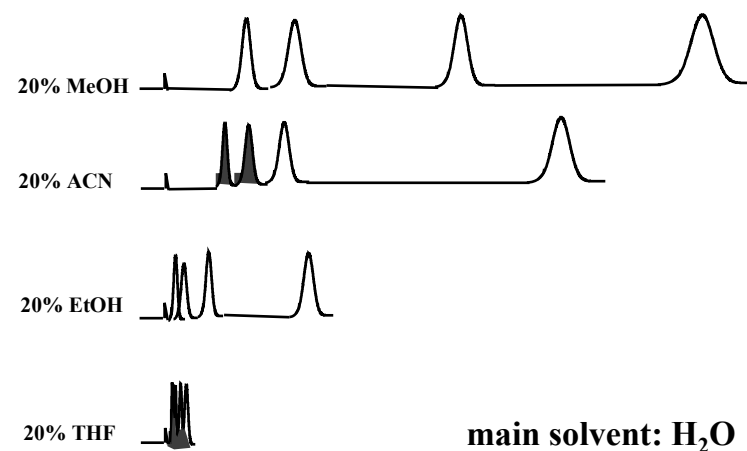


MOBILE PHASE

- * TYPE OF MODIFIER (MeOH, ACN)
- * SOLVENT STRENGTH (% modifier)
- * pH
- * TYPE OF BUFFER (phosphate, acetate)
- * IONIC STRENGTH (Salts, buffer concentration)
- * ION-PAIRING REAGENTS (alkyl-amines, -sulfonates)

OPTIMIZATION: CHOICE OF SOLVENTS

REVERSED PHASE



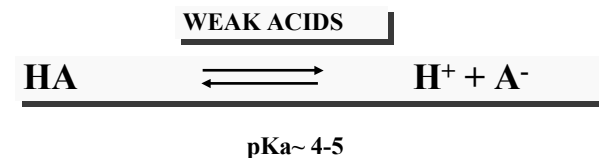
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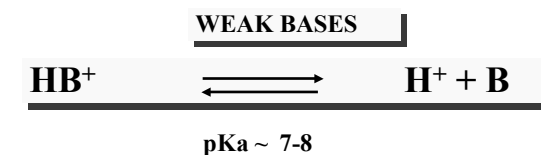
OPTIMIZATION: % SOLVENTS



IONIZATION and RETENTION

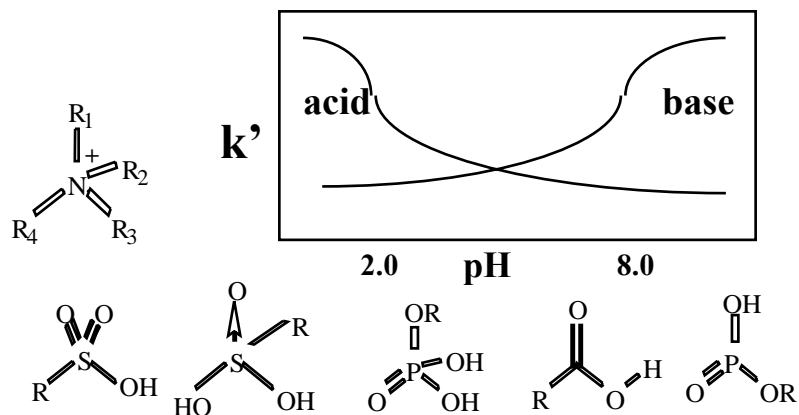


At pH > 4-5 the main species is A⁻

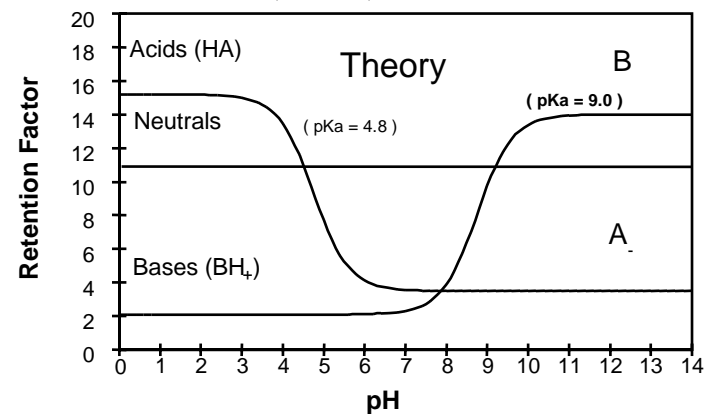


At pH < 7-8 the main species is BH⁺

pH DEPENDENT RETENTION



Retention Factor versus pH for Acids, Bases, and Neutrals



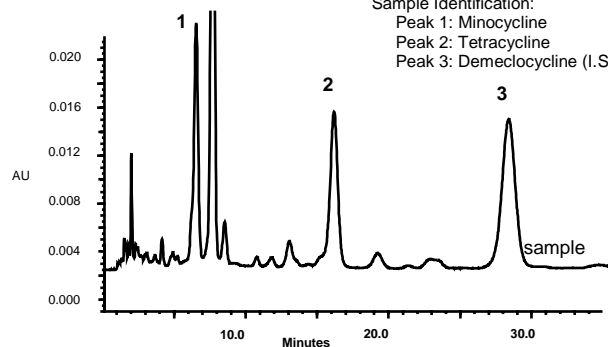
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Antibiotics

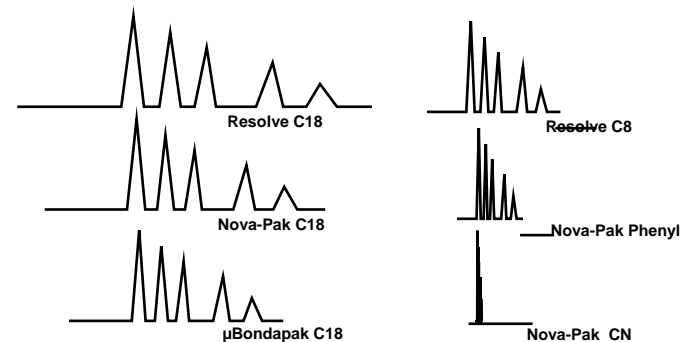
Column: SymmetryShield™ RP8, 5 µm,
3.0 x 150 mm
Mobile Phase: 0.1% TFA in Water : Acetonitrile:
Methanol (91:7:2)
Detection: UV at 270 nm
Flow Rate: 0.9 mL/min.
Injection Volume: 20 µL

Sample Identification:
Peak 1: Minocycline
Peak 2: Tetracycline
Peak 3: Demeclocycline (I.S.)



Cheng

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	$-\text{Si}(\text{CH}_3)_2\text{C}_{18}\text{H}_{37}$
C ₈	$-\text{Si}(\text{CH}_3)_2\text{C}_8\text{H}_{17}$
tC ₂	$-\text{SiC}_2\text{H}_5$
Aminopropyl	$-\text{Si}(\text{CH}_3)_2\text{NH}_2$
Cyanopropyl	$-\text{Si}(\text{CH}_3)_2(\text{CH}_2)_3\text{CN}$
Diol	$-\text{Si}(\text{CH}_3)_2\text{OCH}_2\text{CH}_2\text{OH}$

Retention time

Chain length CN Phenyl NH₂ C₄ C₈ C₁₈

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Stationary Phase Properties

CHEMISTRY:

- * BONDED HYDROCARBON:
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- * % COVERAGE
- * TYPE OF SILICA GEL

GEOMETRY

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

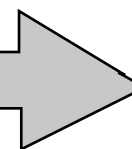


CARBON LOAD

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

Retention time

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



Stationary Phase Properties

CHEMISTRY:

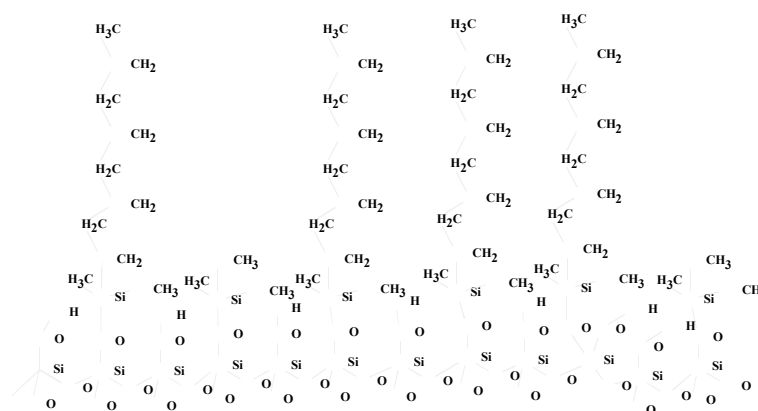
- * BONDED HYDROCARBON:
C-18, C-8, C-4, C-1, CN, phenyl
- * % COVERAGE
- * TYPE OF SILICA GEL

GEOMETRY

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



Surface of a Reversed- Phase Packing

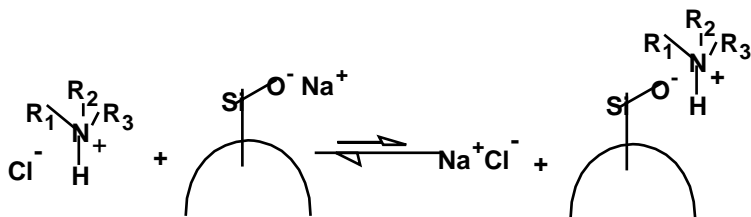


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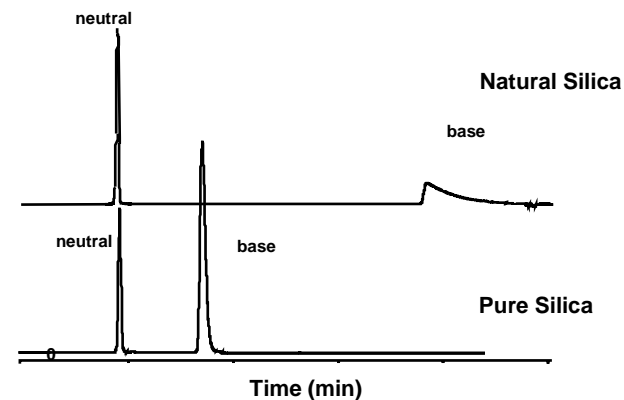
Introduction

What Causes Tailing?

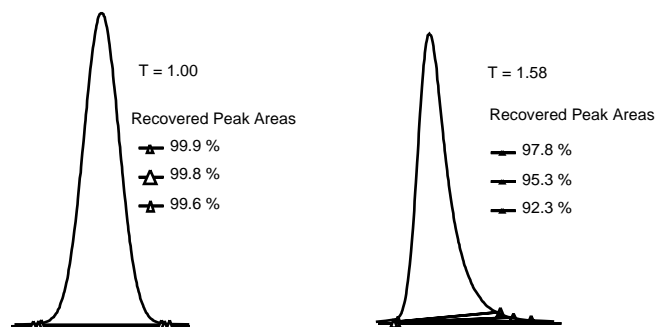
- Mixed-mode retention:
 - hydrophobic - interaction with bonded phase
 - ion exchange - interaction with charged sites



Quality of Columns Performance



Integration Errors Caused by Tailing



Stationary Phase Properties

CHEMISTRY:

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

GEOMETRY

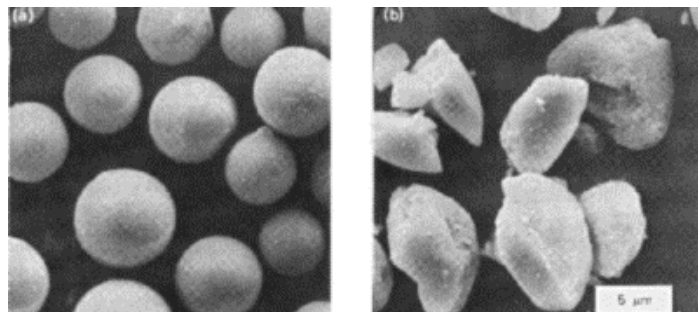
- * SPHERE-IRREGULAR
- * PARTICLE DIAMETER
- * POROSITY



High Performance Liquid Chromatography - HPLC

Introduction

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:

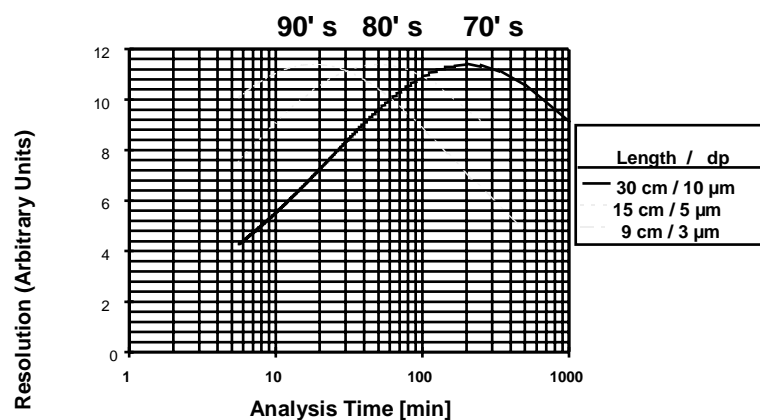
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C-18, C-8, C-4, C-1, CN, phenyl
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GEOMETRY

- * SPHERE- IRREGULAR
- * PARTICLE DIAMETER
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Resolution - Time Diagram



Stationary Phase Properties

CHEMISTRY:

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GEOMETRY

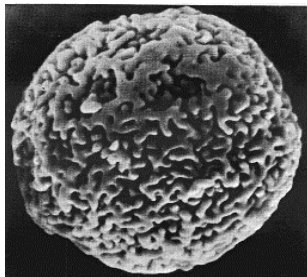
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- * PARTICLE DIAMETER
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High Performance Liquid Chromatography - HPLC

Introduction

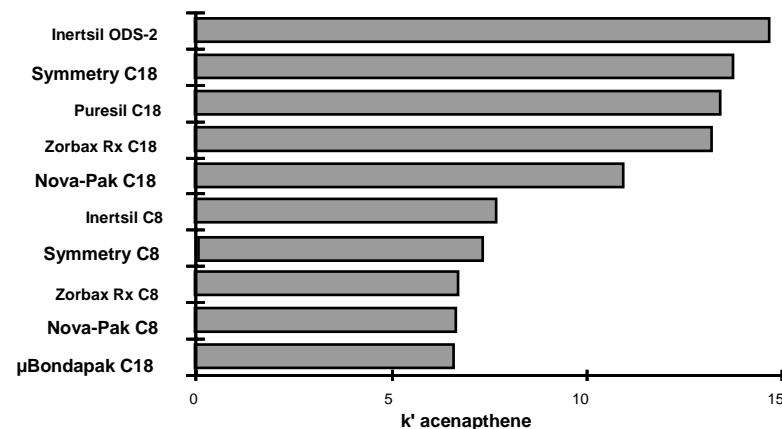
Pore size, shape and distribution



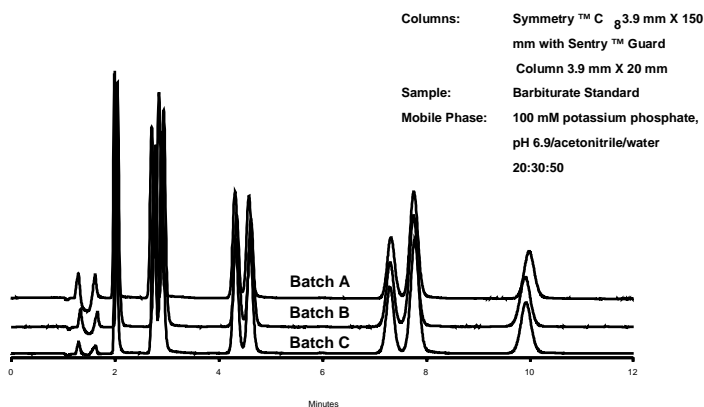
Macroporous spherical silica particle. [K.K.Unger, Porous silica, Elsevier, 1979]

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

Relative Hydrophobicities of General Purpose Analytical Packings



Batch-to-Batch Reproducibility of Columns



Chromatogram of lifetime test

