

# High Performance Liquid Chromatography - HPLC Introduction

## 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

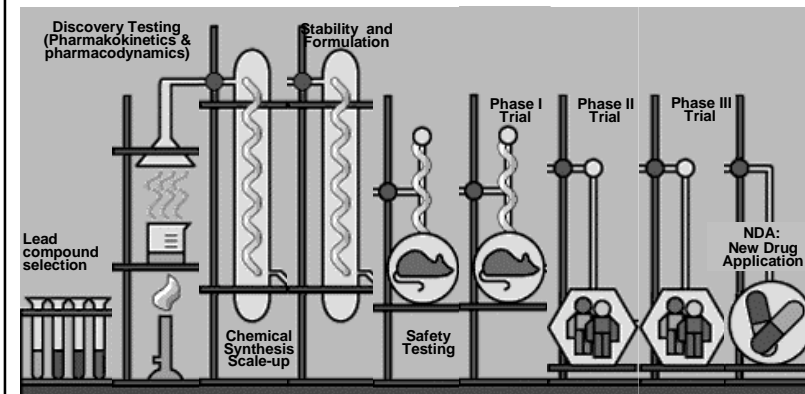
Hewlett-Packard liquid chromatography

High performance liquid chromatography

Hocus pocus liquid chromatography

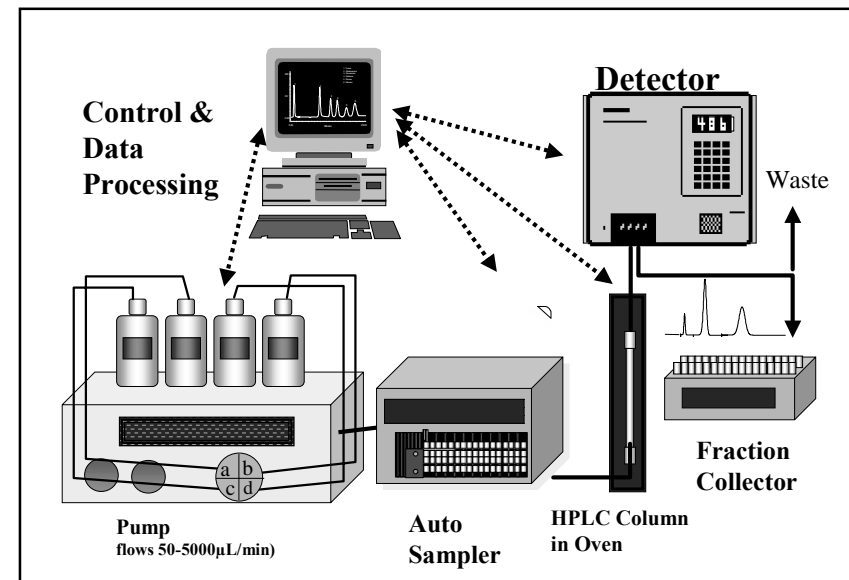
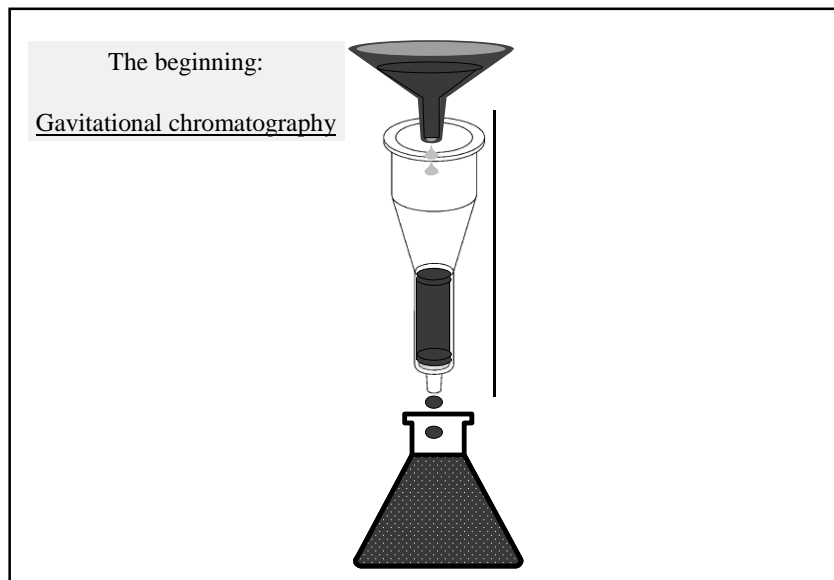
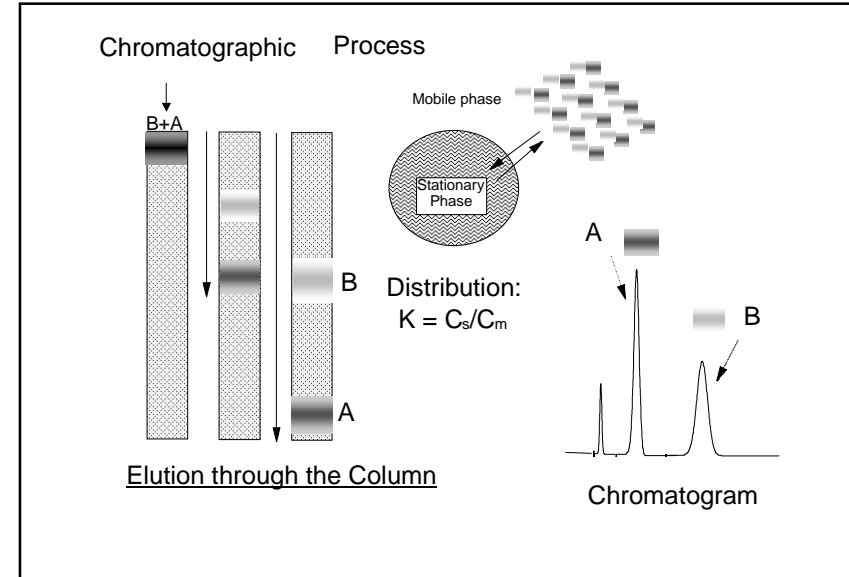
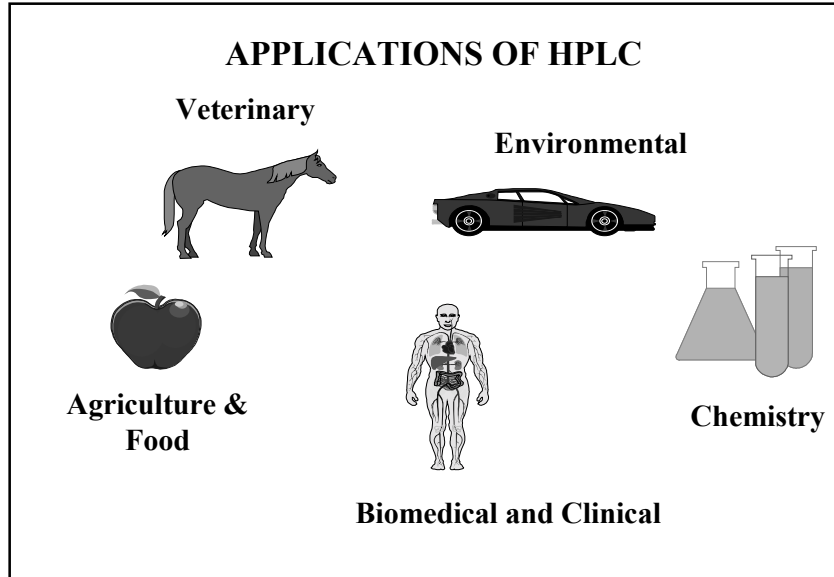
High patience liquid chromatography

## HPLC in Pharmaceutics Technique No 1



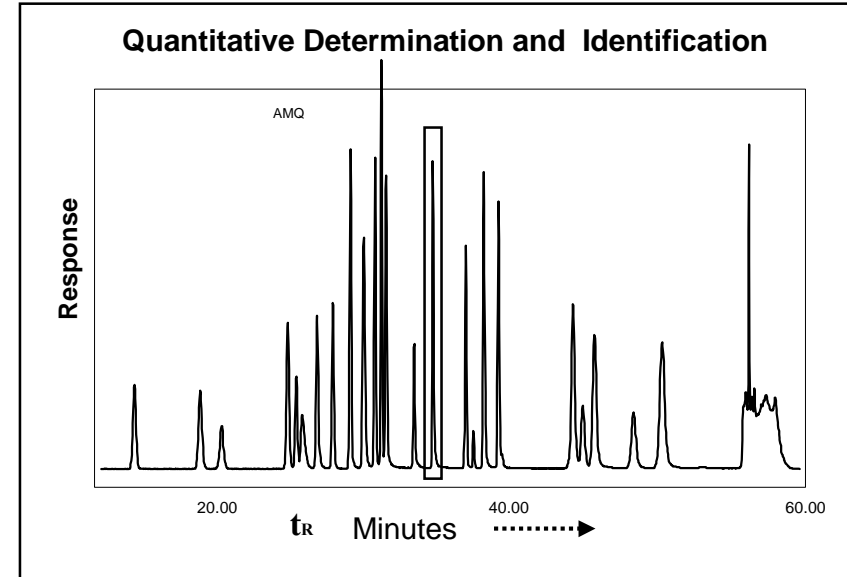
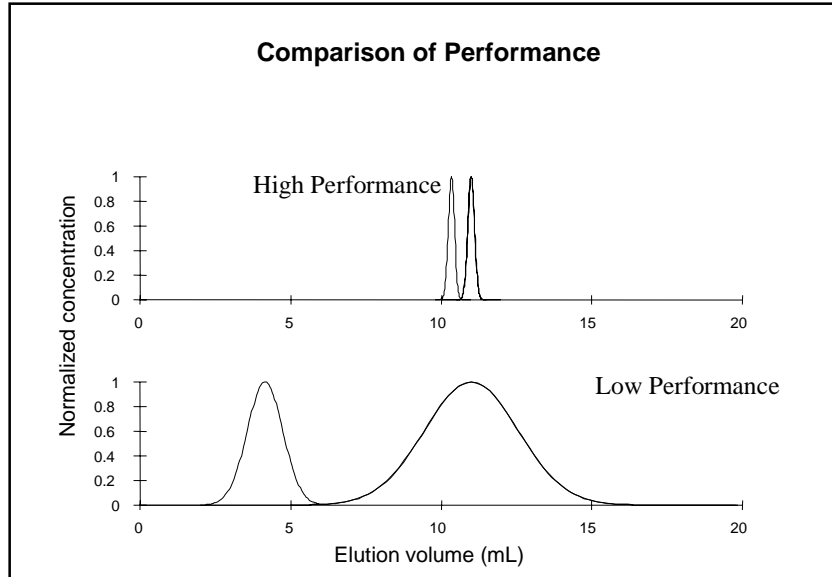
# High Performance Liquid Chromatography - HPLC

## Introduction



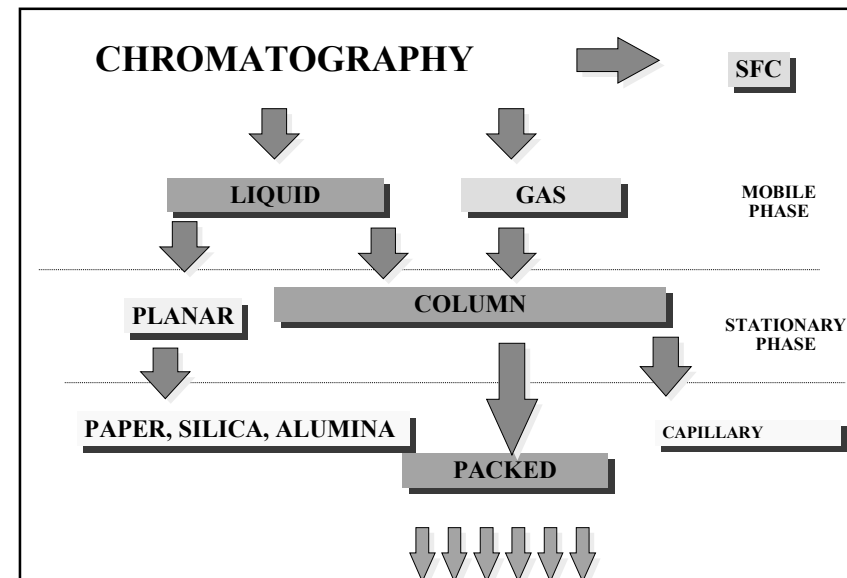
# High Performance Liquid Chromatography - HPLC

## Introduction



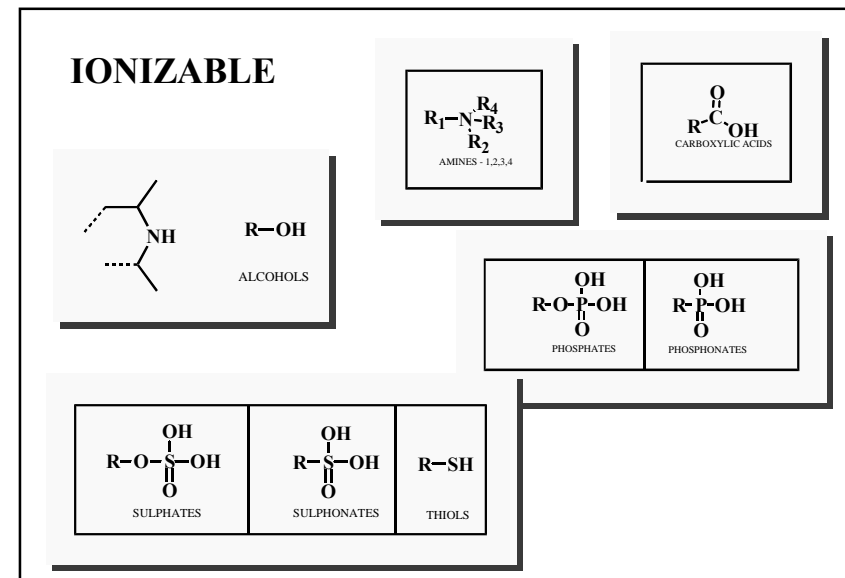
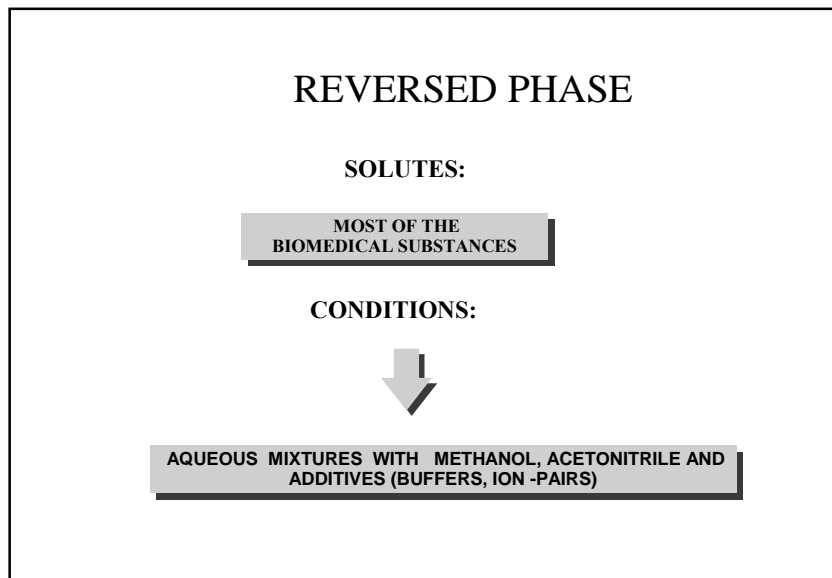
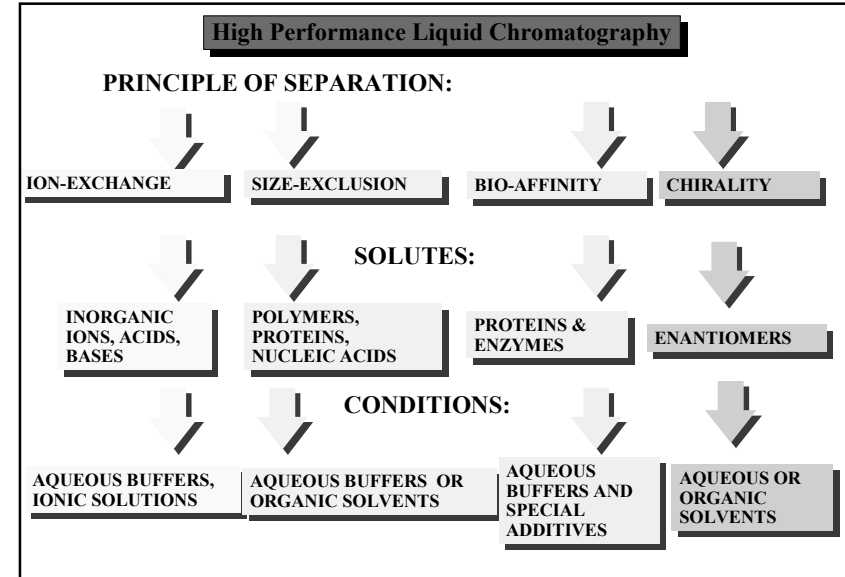
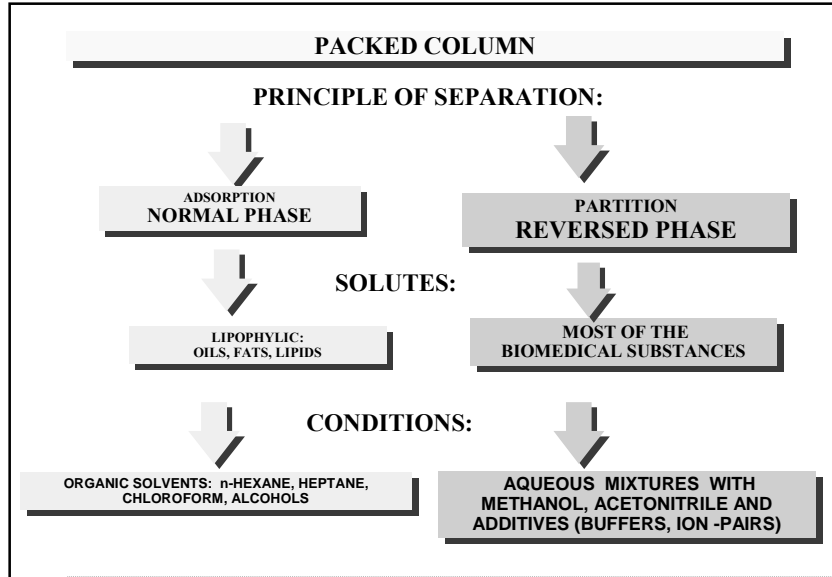
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- Introduction & Applicability
- Modes of Chromatography
- Quantitative work and System Qualification.



# High Performance Liquid Chromatography - HPLC

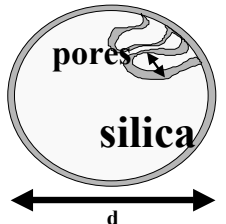
## Introduction



# High Performance Liquid Chromatography - HPLC

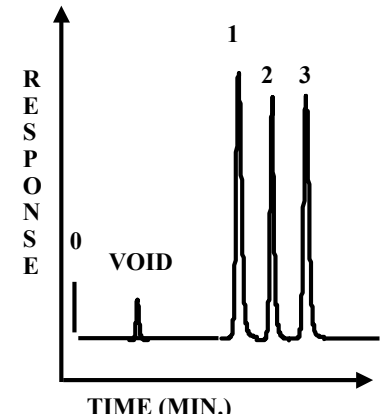
## Introduction

MOBILE PHASE	STATIONARY PHASE
<b>SOLVENTS:</b> water, methanol, acetonitrile	<b>CHEMISTRY:</b> * BONDED HYDROCARBON: C-18, C-8, C-4, C-1 * % COVERAGE * ADSORBED SURFACTANTS * TYPE OF SILICA GEL
<b>ADDITIVES:</b> buffers, salts, ion-pairing reagents, complexants.	<b>GEOMETRY</b> * SPHERE- IRREGULAR * PARTICLE DIAMETER * POROSITY



The diagram shows a circular silica particle with a diameter labeled 'd'. Inside the particle, there are irregular, interconnected spaces labeled 'pores'.

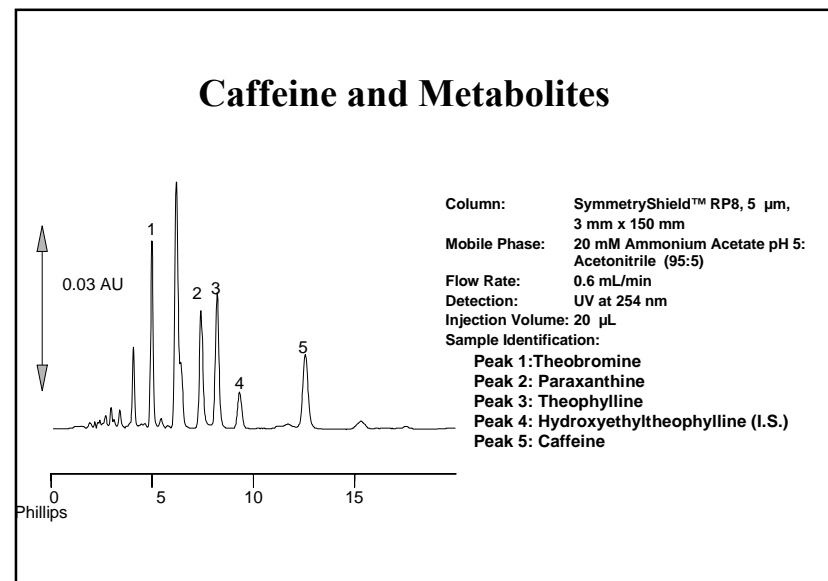
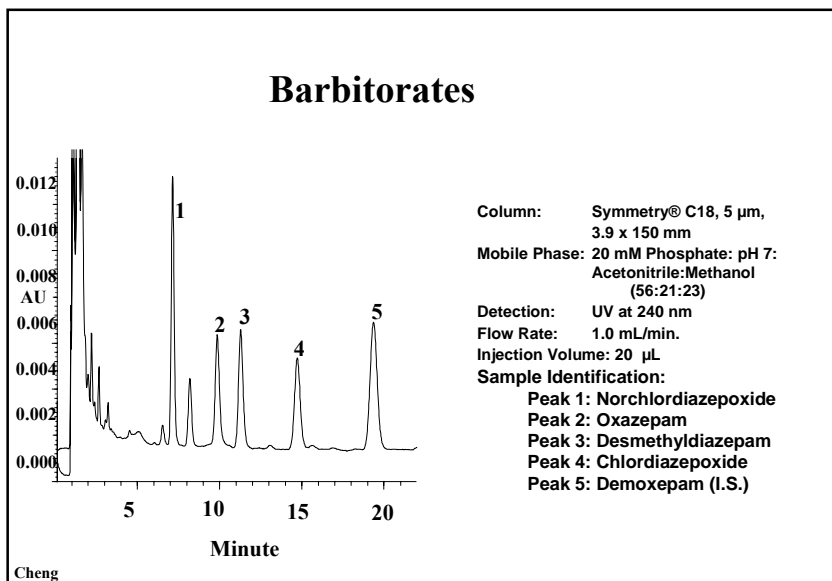
### ELUTION ORDER IN REVERSED PHASE



The chromatogram plots RESPONSE against TIME (MIN.). It shows a 'VOID' peak at the beginning, followed by three main peaks labeled 1, 2, and 3. The elution order is from least lipophilic (1) to most lipophilic (3).

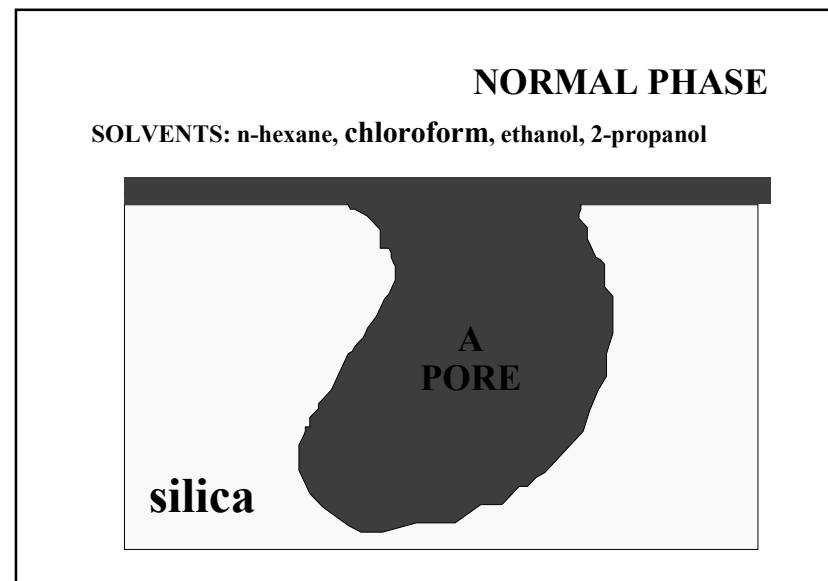
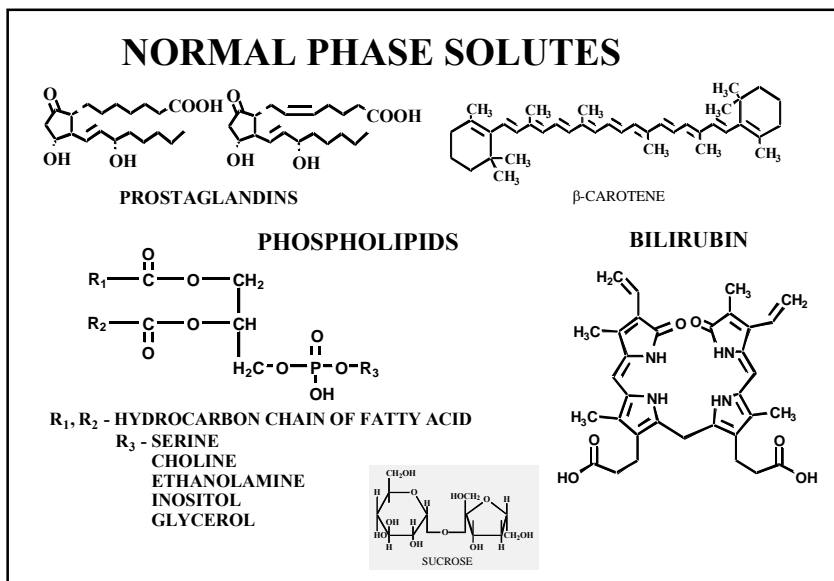
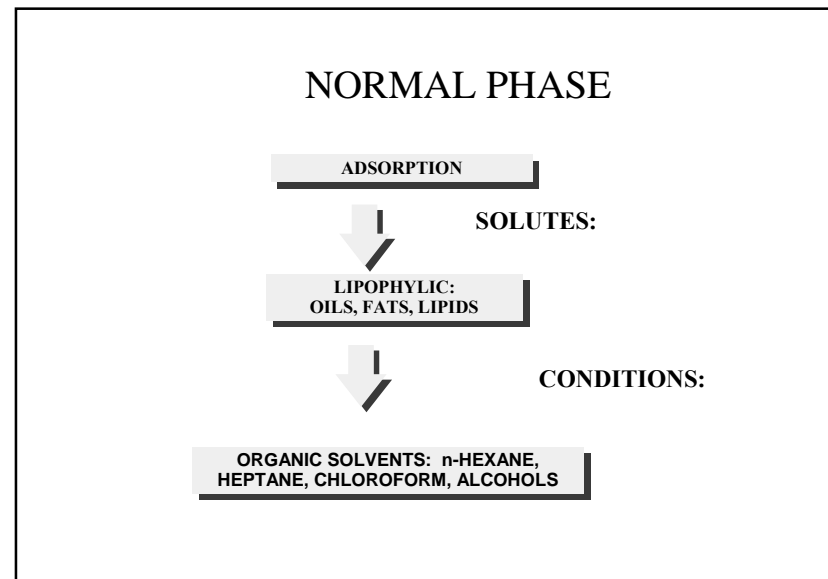
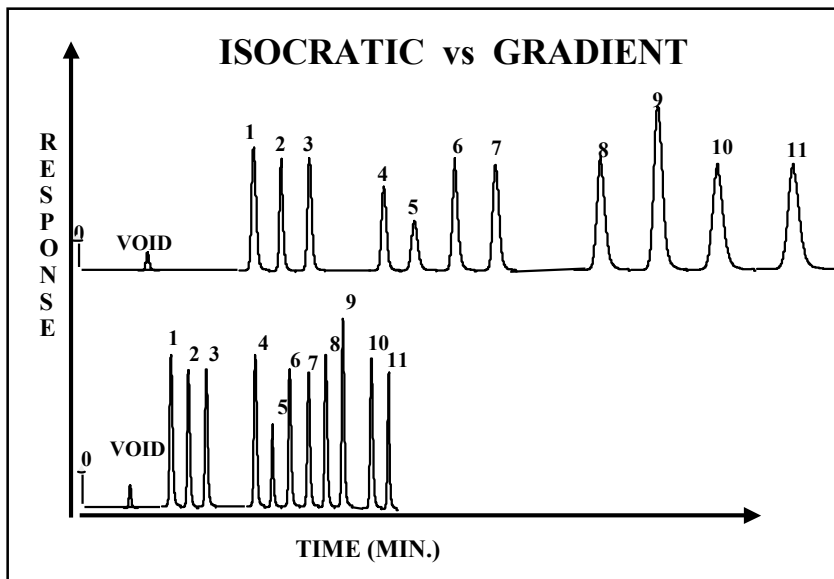
**LIPOPHYLIC** (indicated by an arrow pointing right)

1	2	3
<chem>c1ccccc1</chem>	<chem>c1ccc2ccccc2c1</chem>	<chem>c1ccc2cc3ccccc3cc2c1</chem>
<chem>c1ccccc1</chem>	<chem>Cc1ccccc1</chem>	<chem>Cc1cc(C)cc(C)c1</chem>
<chem>Oc1ccc(O)cc1</chem>	<chem>Oc1ccccc1</chem>	<chem>c1ccccc1</chem>
<chem>CC(C)(O)c1ccccc1</chem>	<chem>CC(C)(O)C(C)C1=CC=CC=C1</chem>	<chem>CC(C)(O)C(C)C(C)C1=CC=CC=C1</chem>



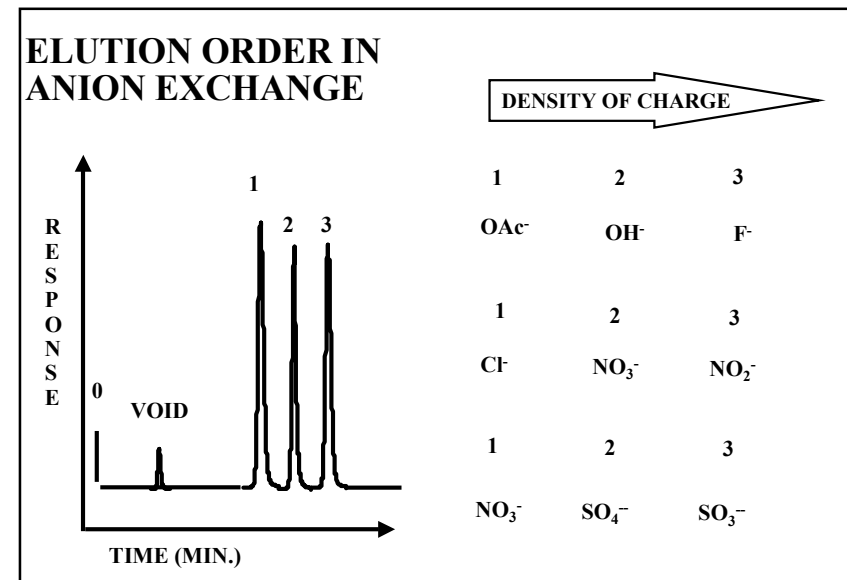
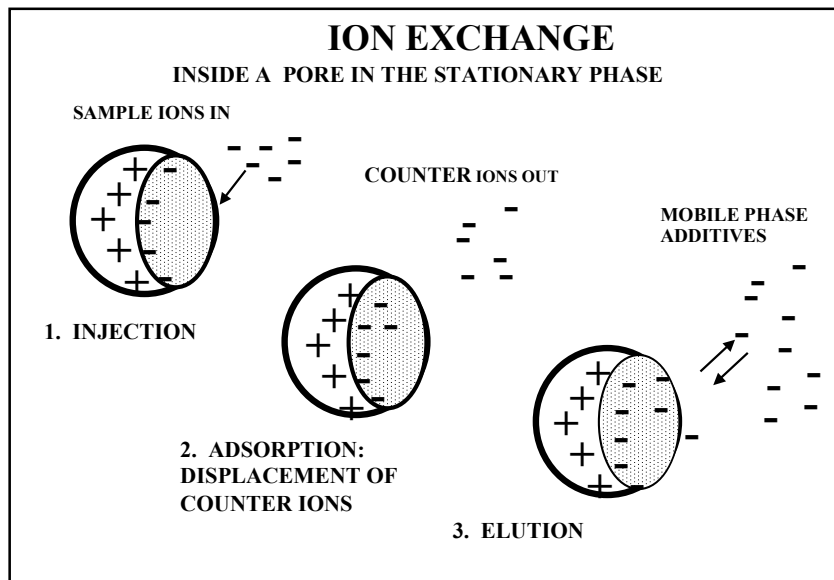
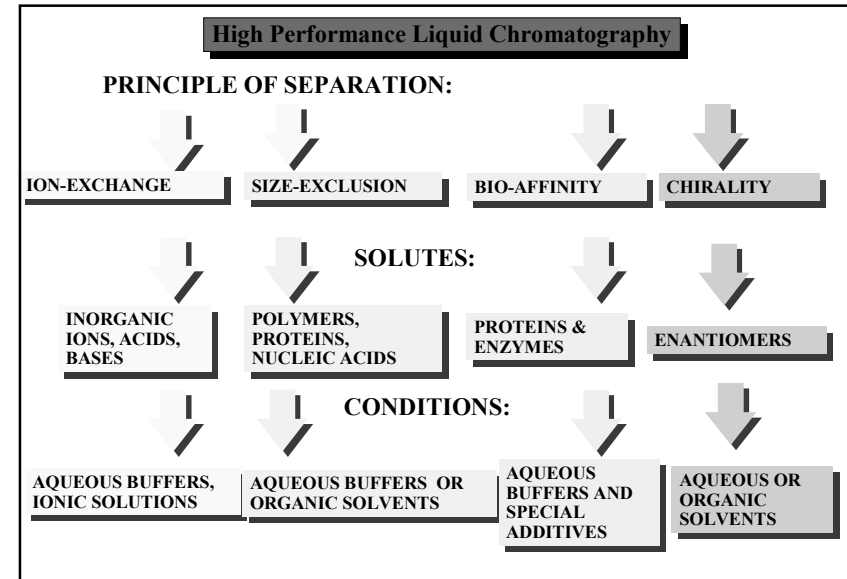
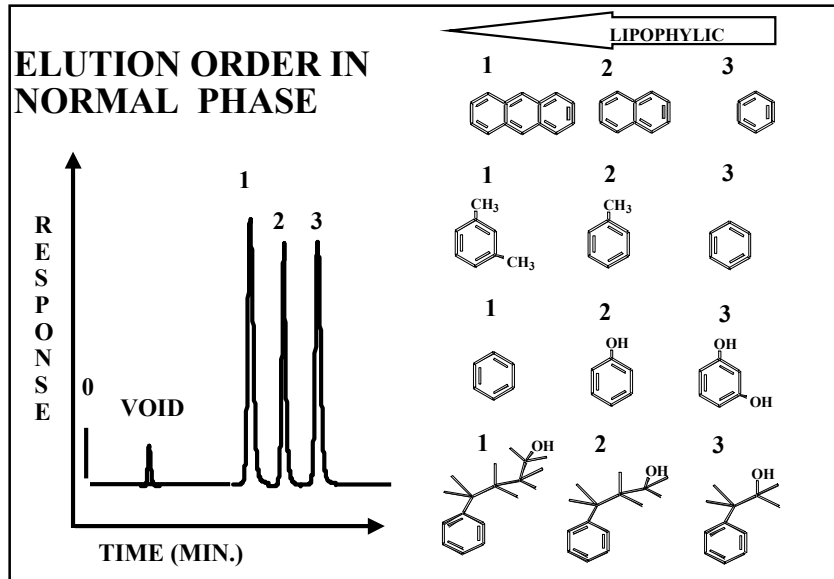
# High Performance Liquid Chromatography - HPLC

## Introduction



# High Performance Liquid Chromatography - HPLC

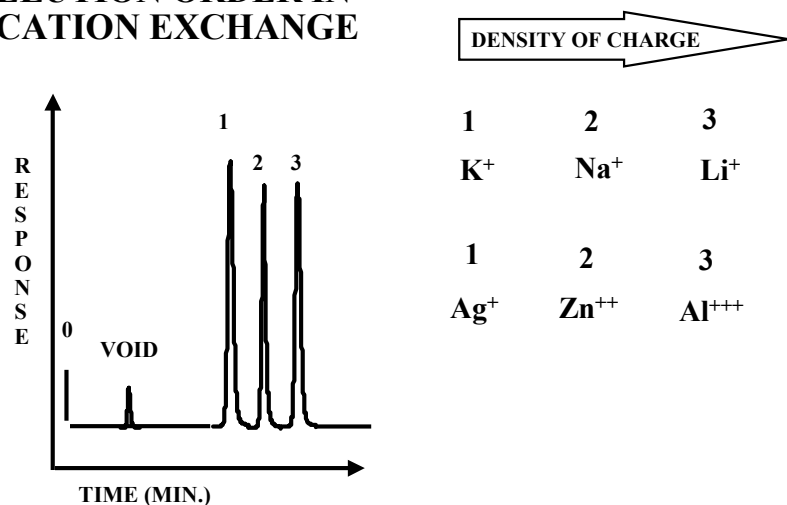
## Introduction



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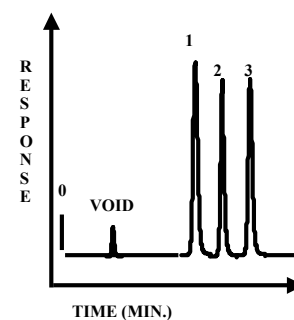
### ELUTION ORDER IN CATION EXCHANGE



### ELUTION ORDER IN ION EXCHANGE

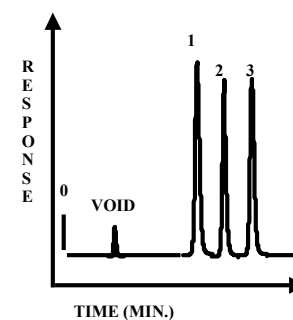
ANION EXCHANGE

STRONGER ACID

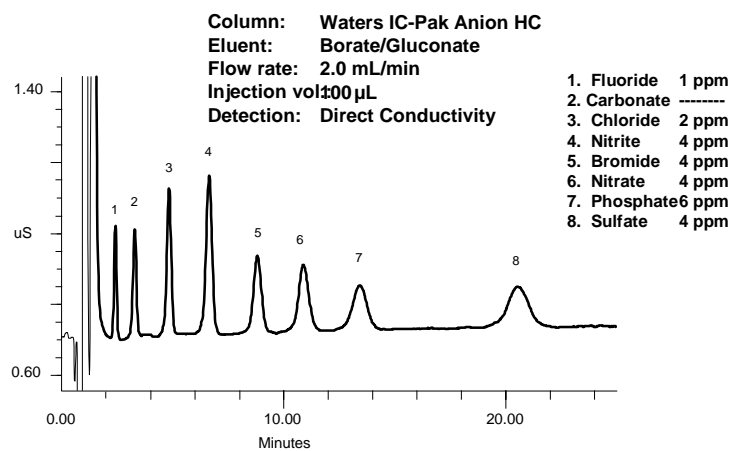


CATION EXCHANGE

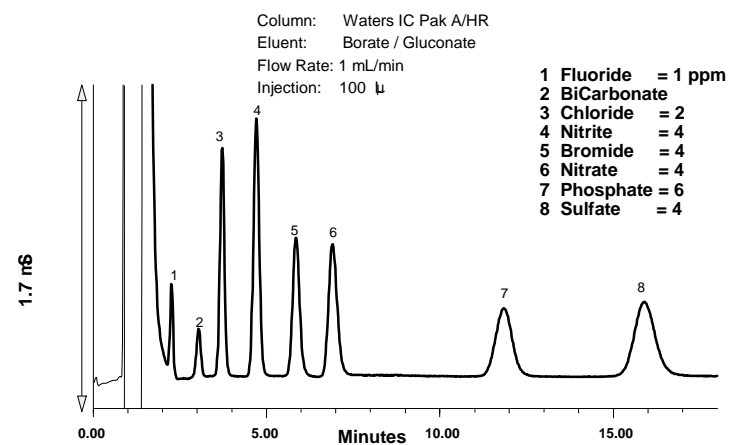
STRONGER BASE



### Analysis of Ions



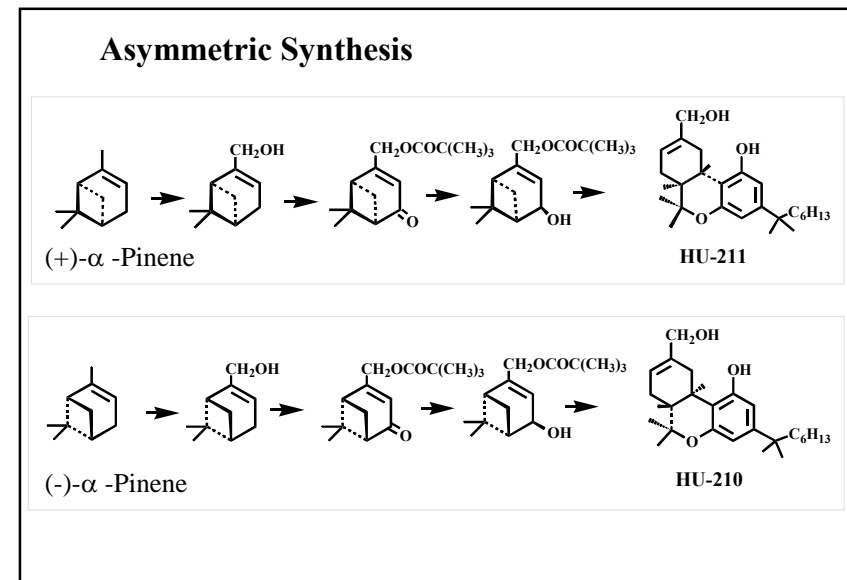
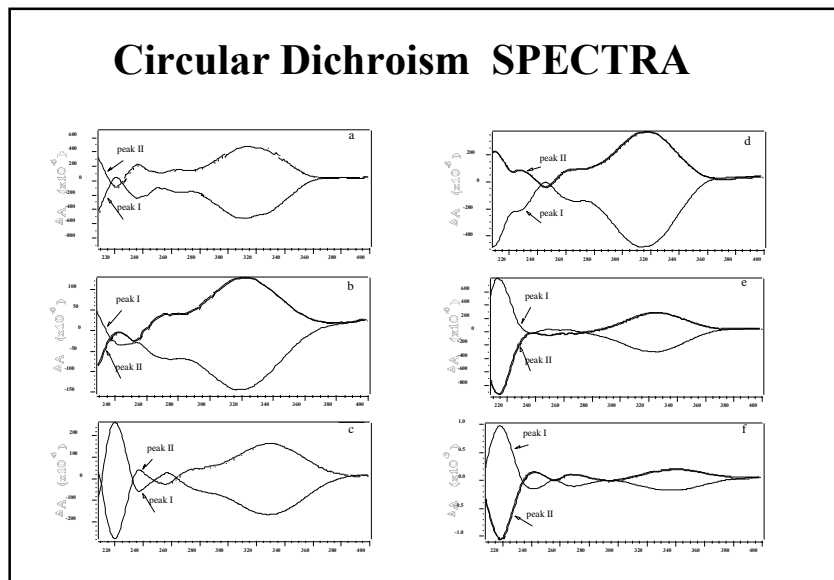
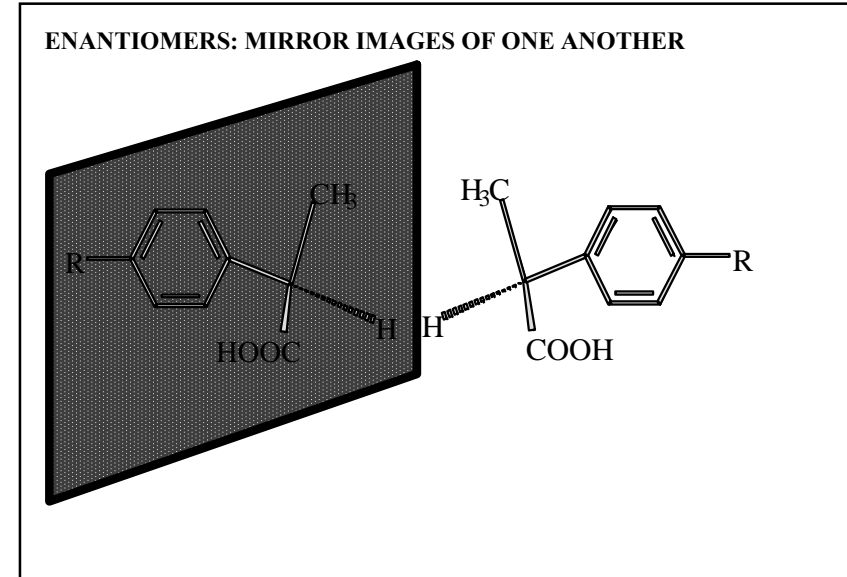
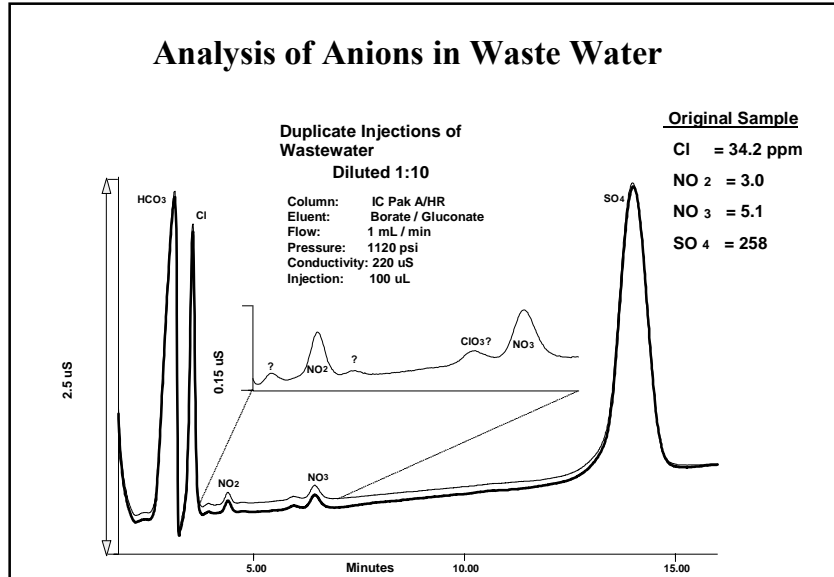
### Analysis of Anions





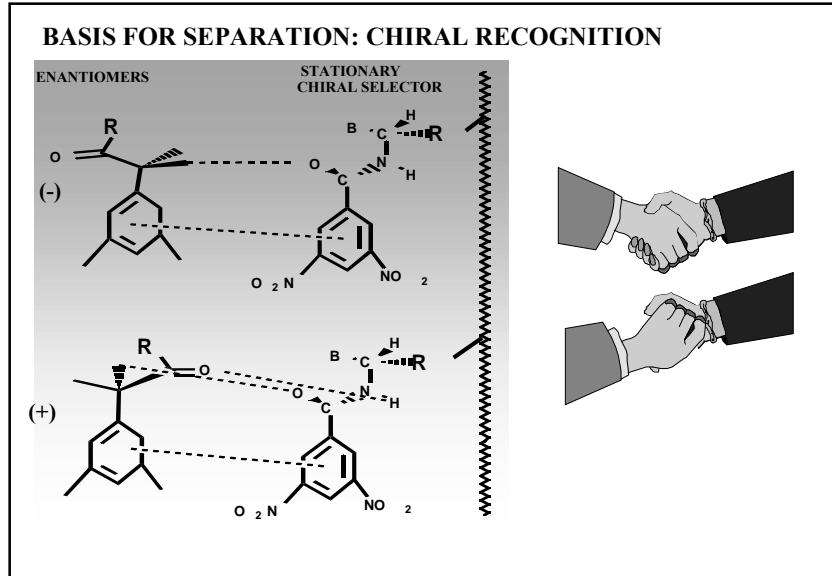
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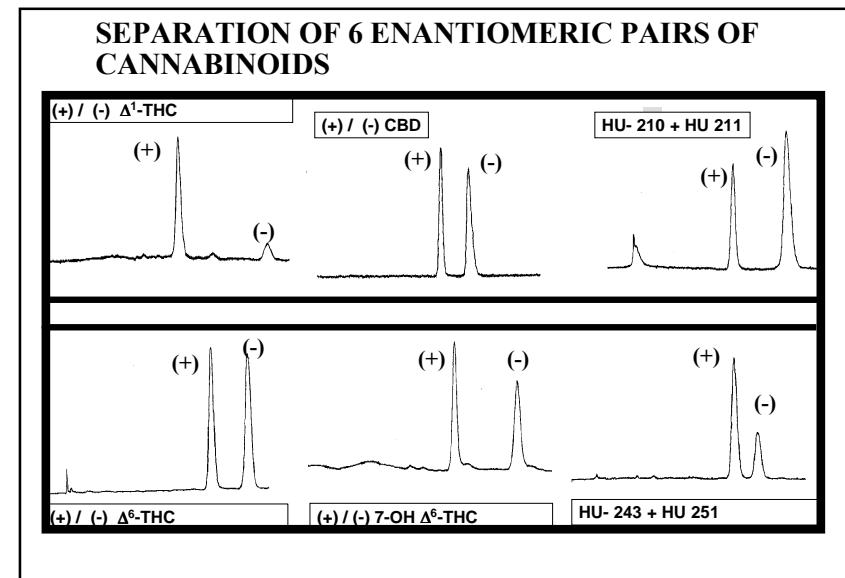
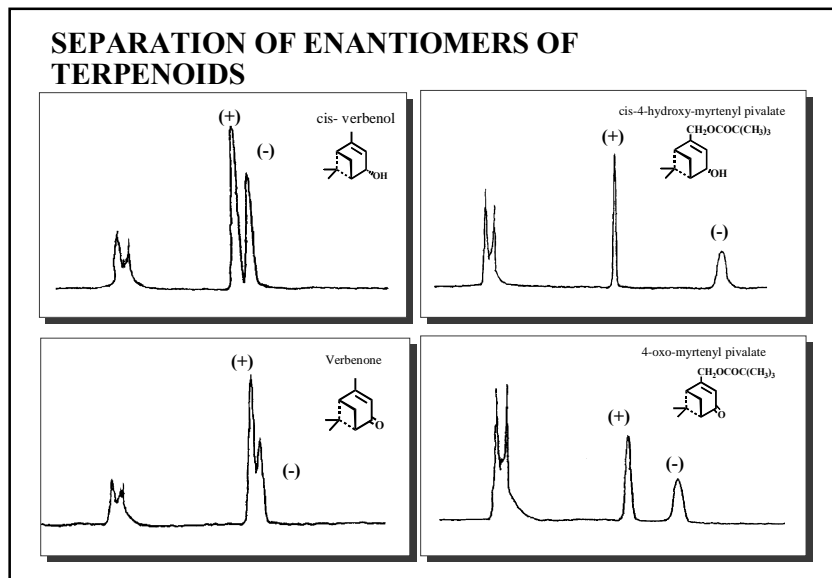
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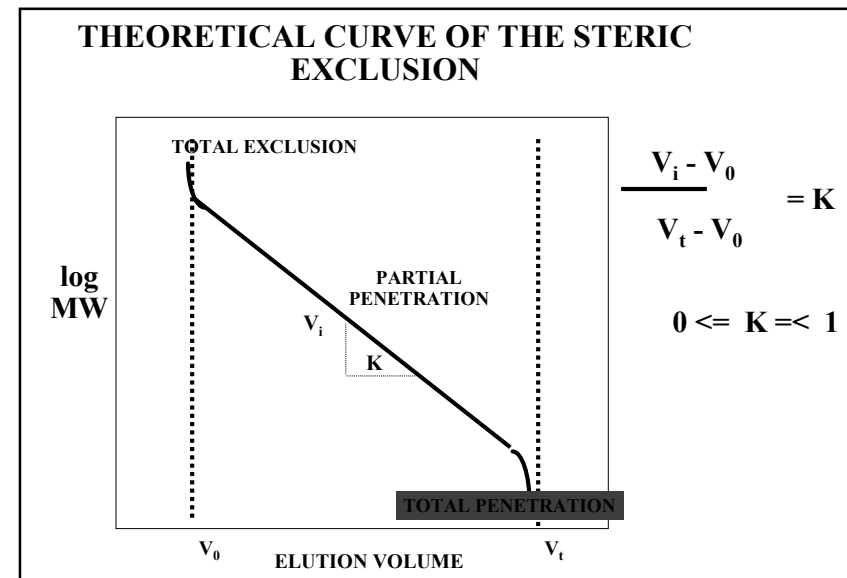
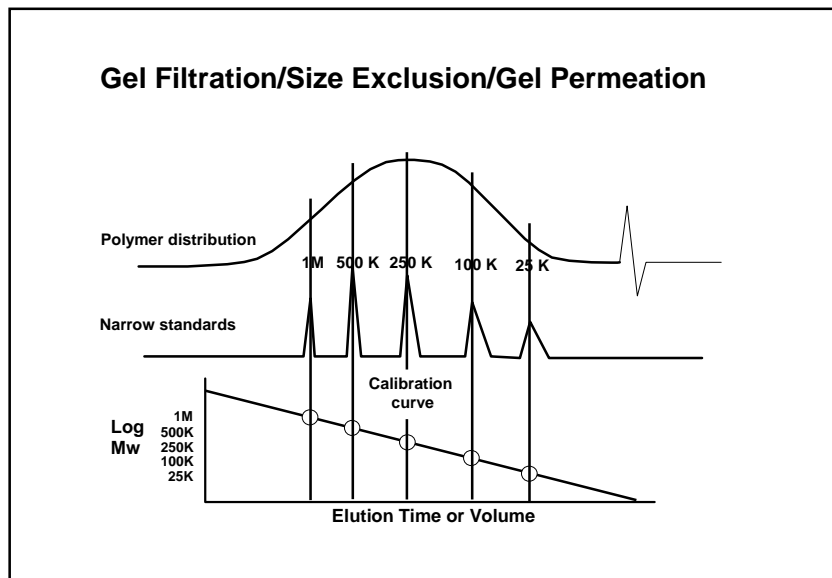
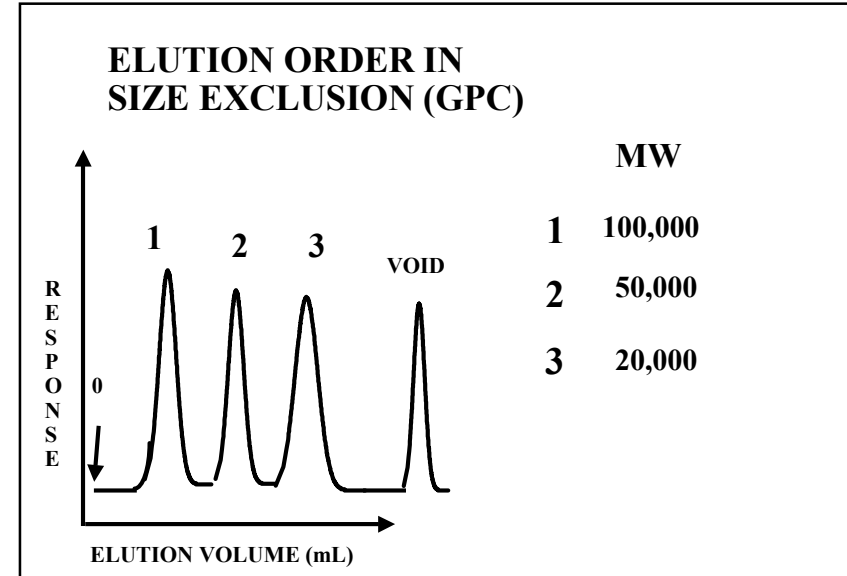
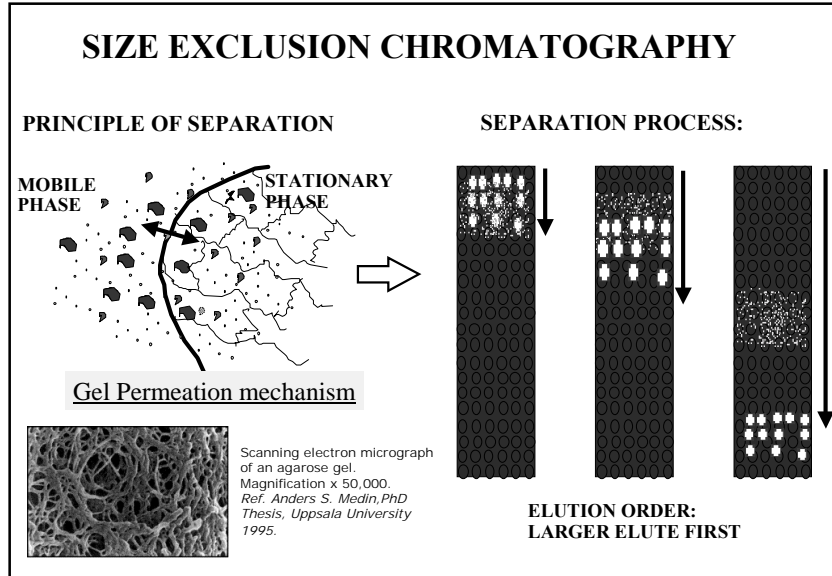
### Chiral stationary phases:

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



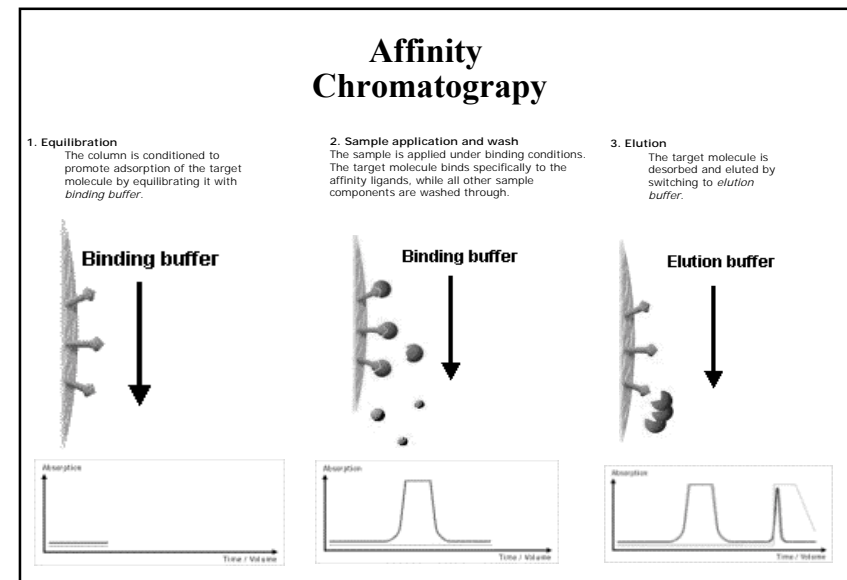
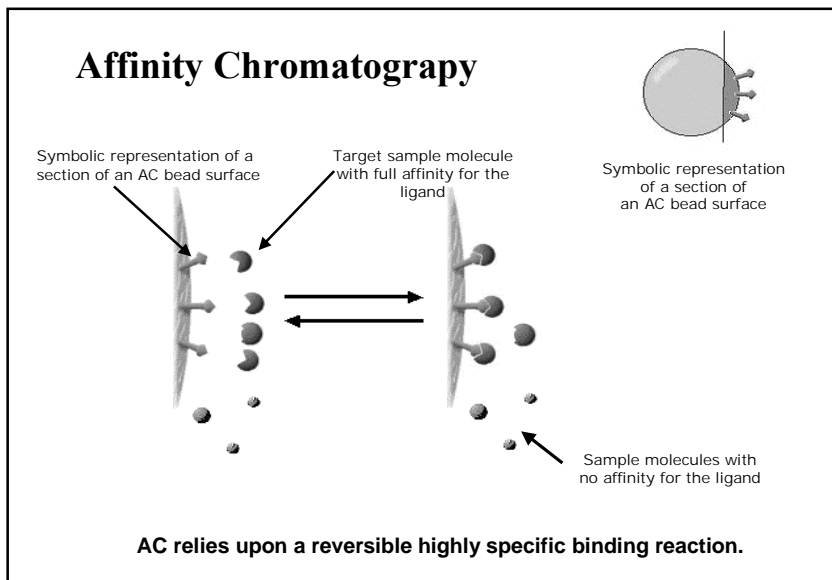
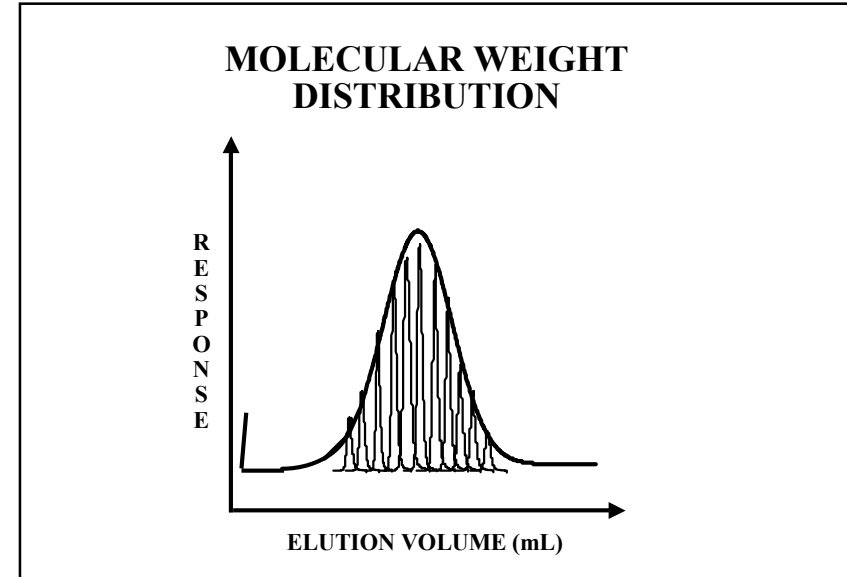
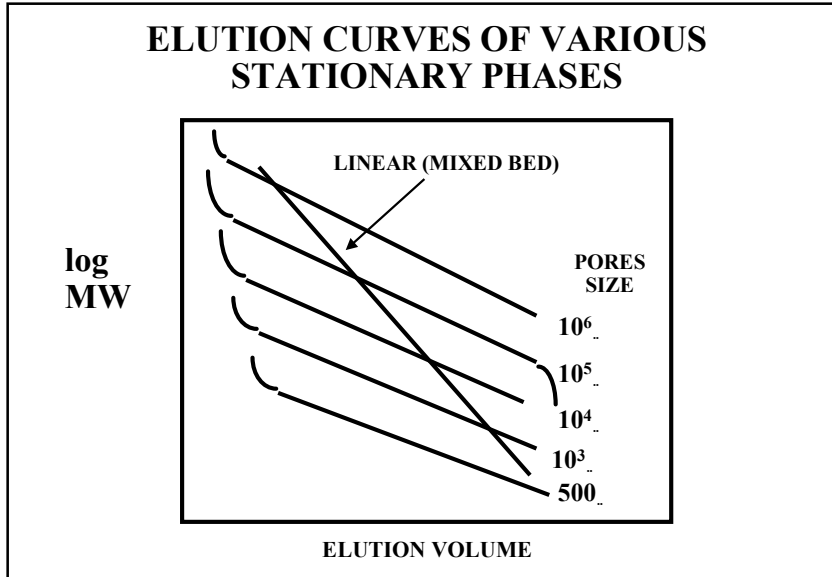
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# High Performance Liquid Chromatography - HPLC

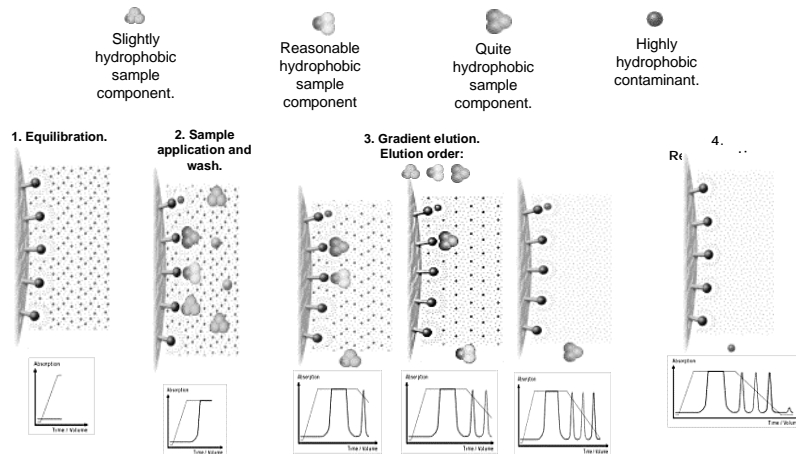
## Introduction



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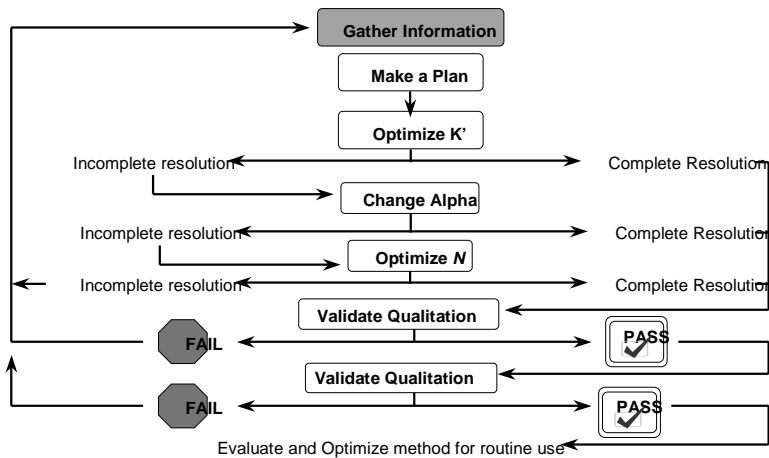
### Hydrophobic Interaction Chromatography (HIC)



### 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



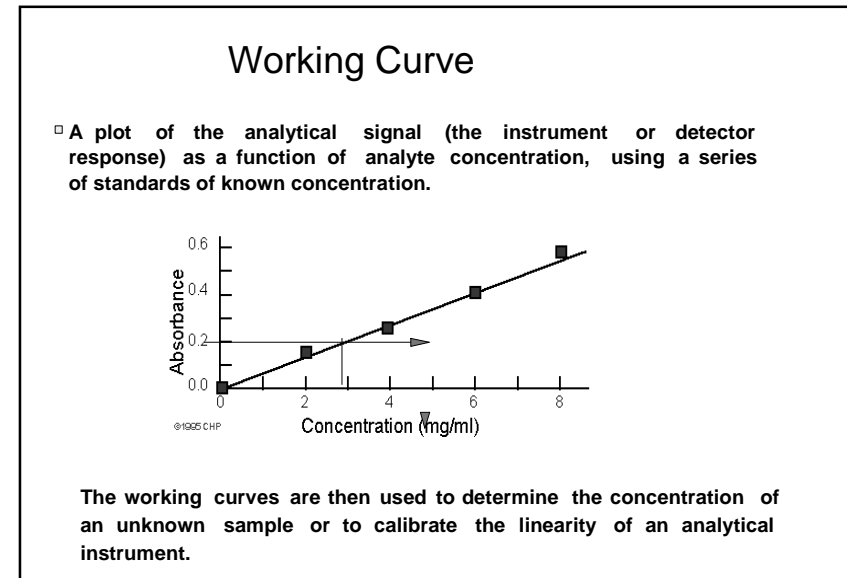
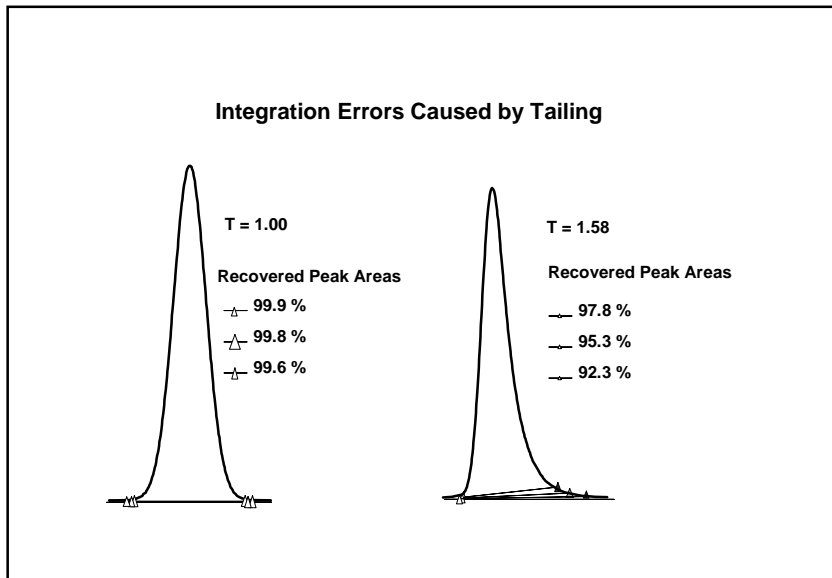
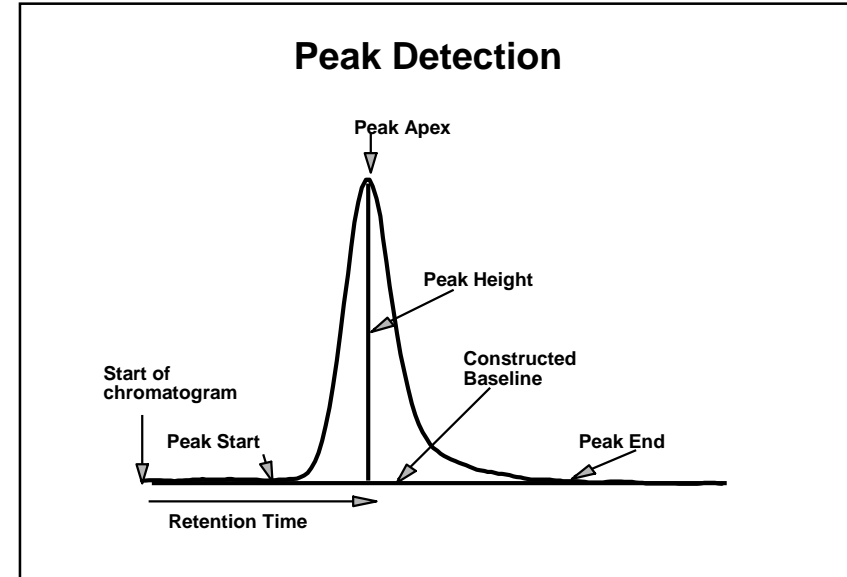
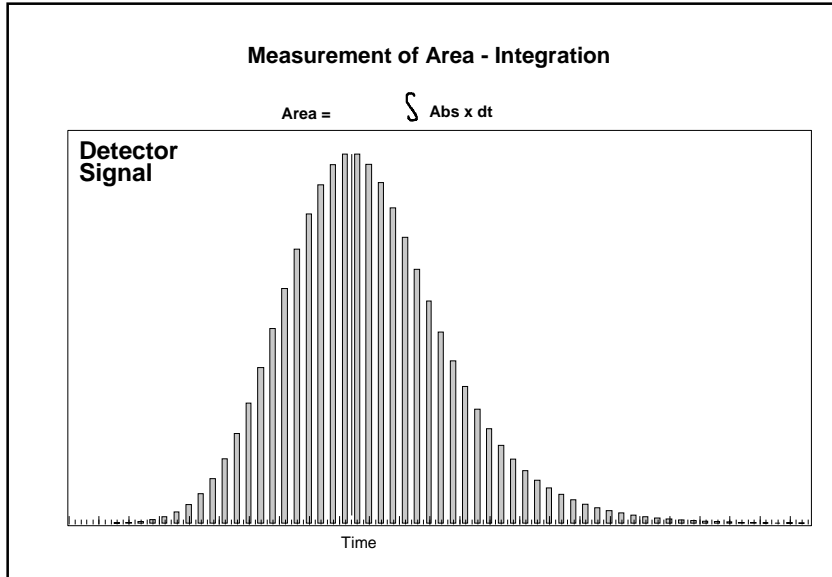
- Step by step method development strategy -

### HPLC COURSE LAYOUT

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

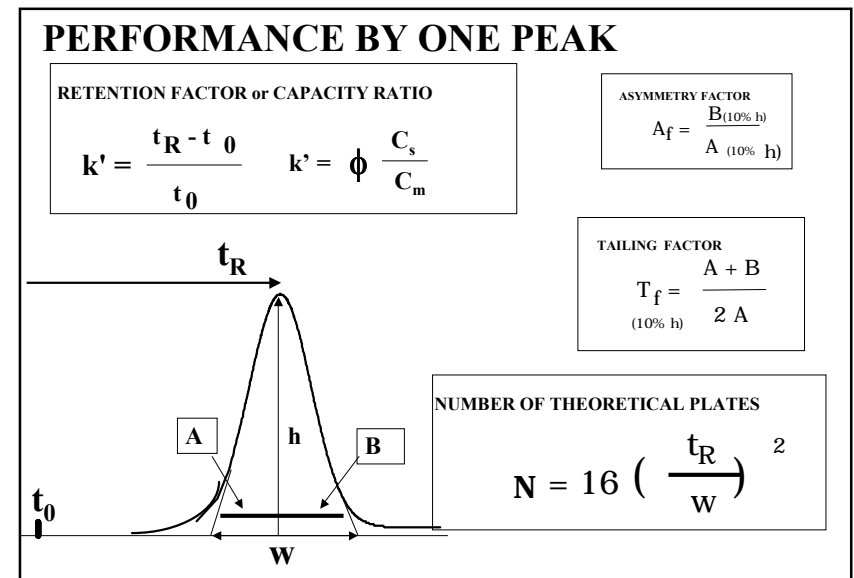
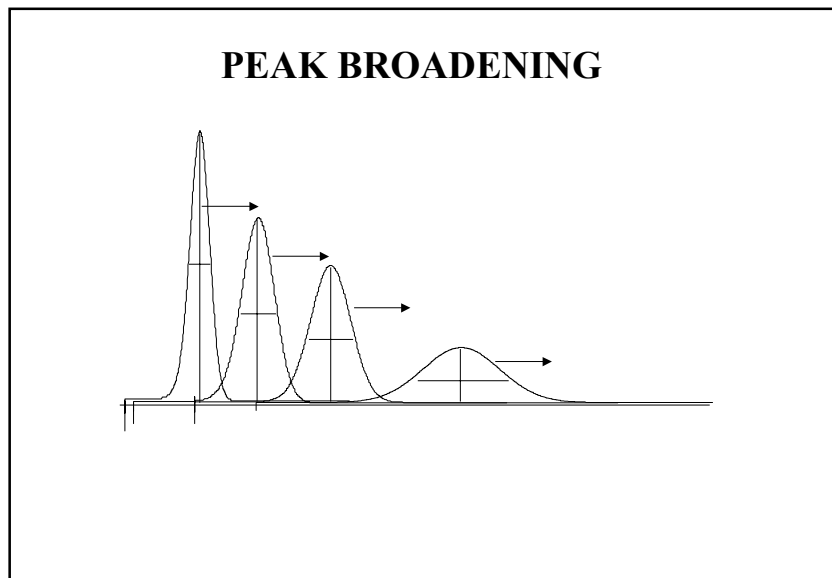
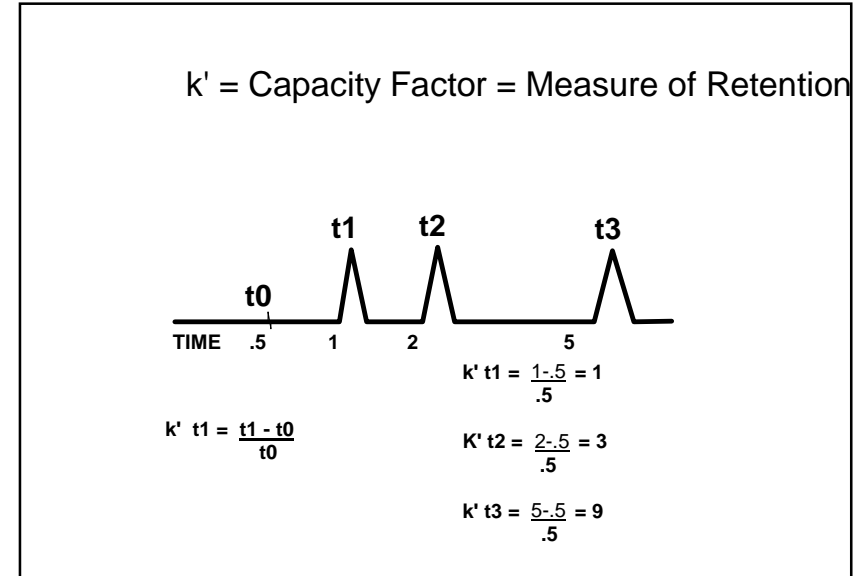
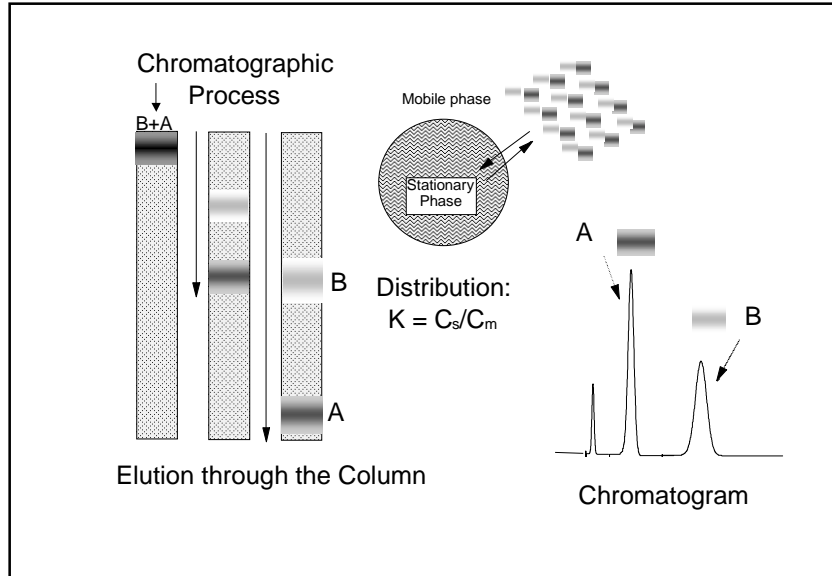
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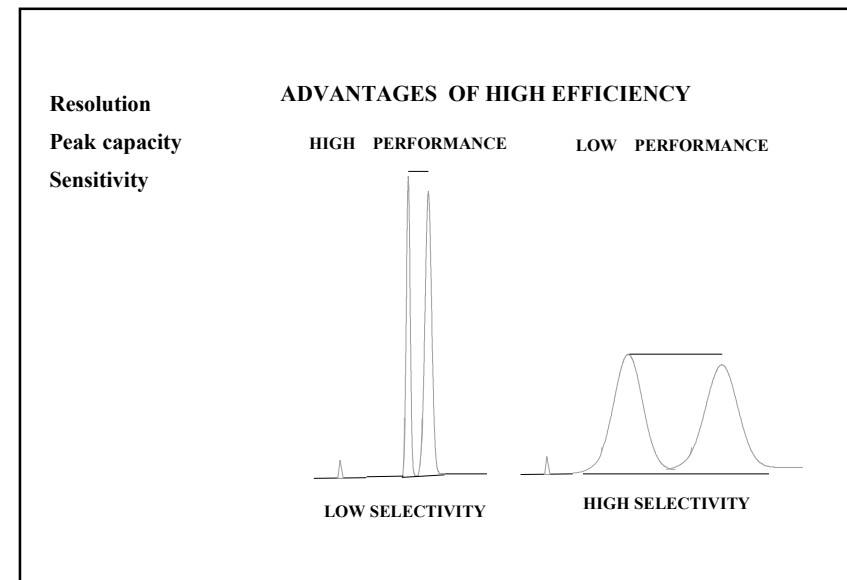
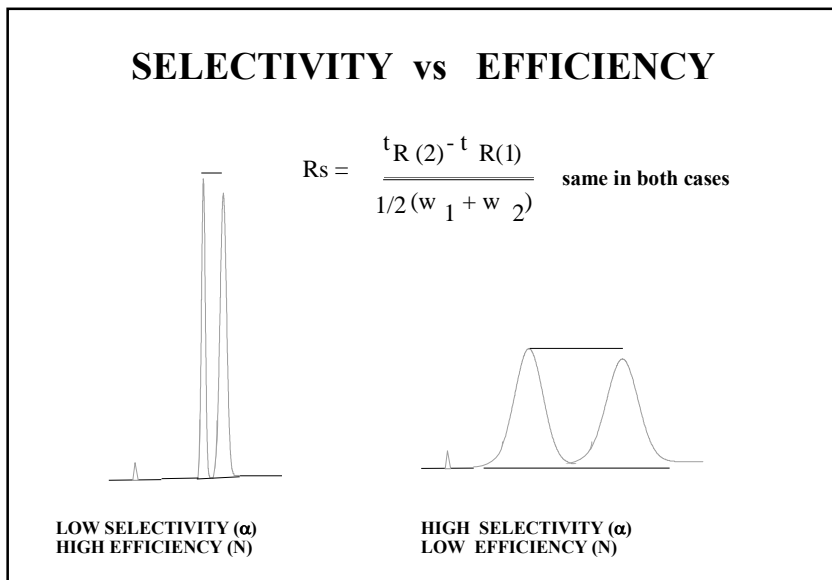
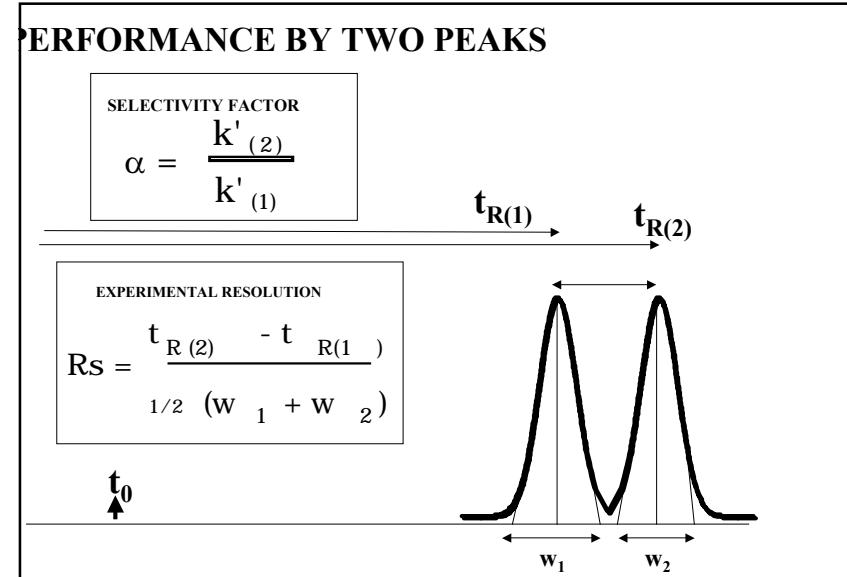
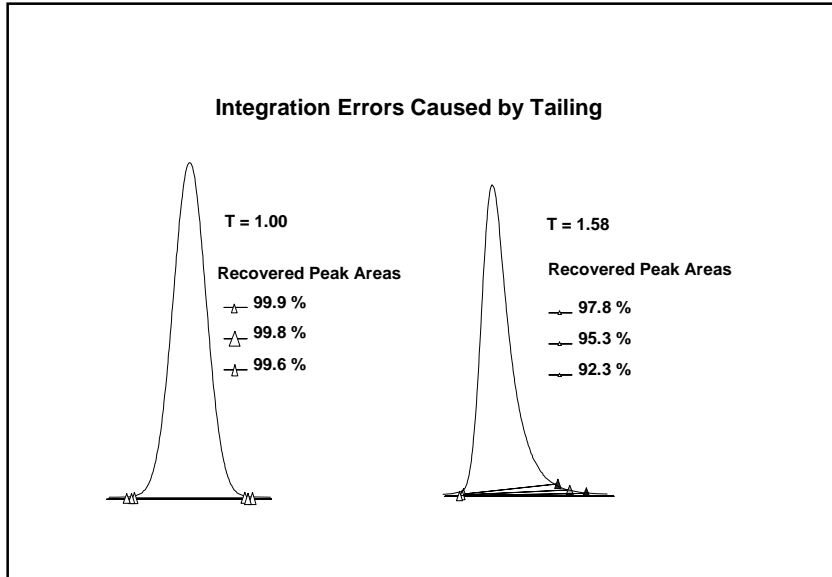
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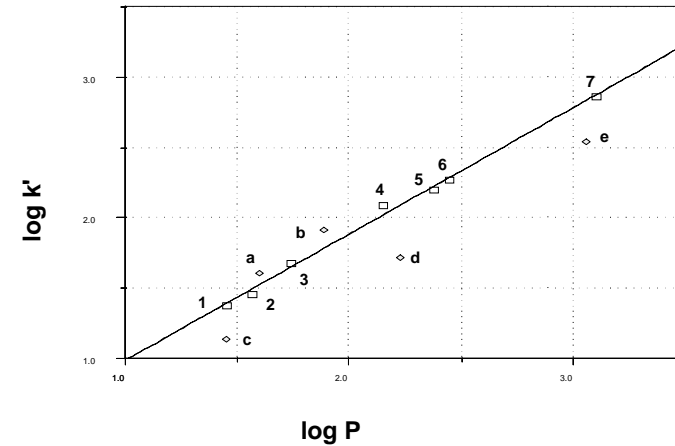
## Introduction

### Reversed Phase HPLC

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[http://www.Geocities.com/CapeCanaveral/8775/HPLC\\_guide\\_h.html](http://www.Geocities.com/CapeCanaveral/8775/HPLC_guide_h.html)

### Hydrophobicity

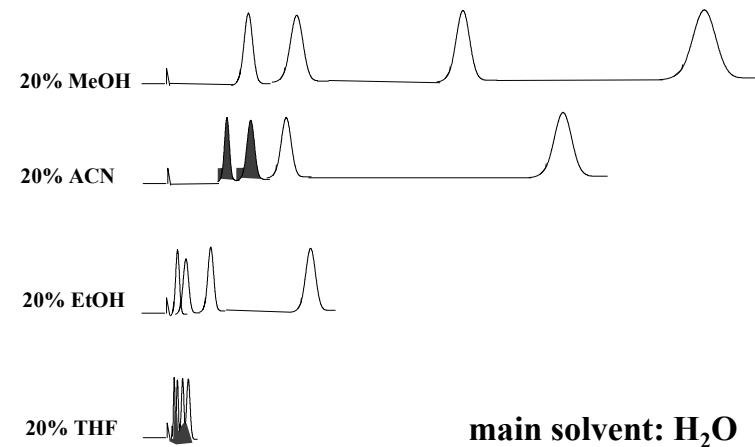


### MOBILE PHASE

- \* 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)

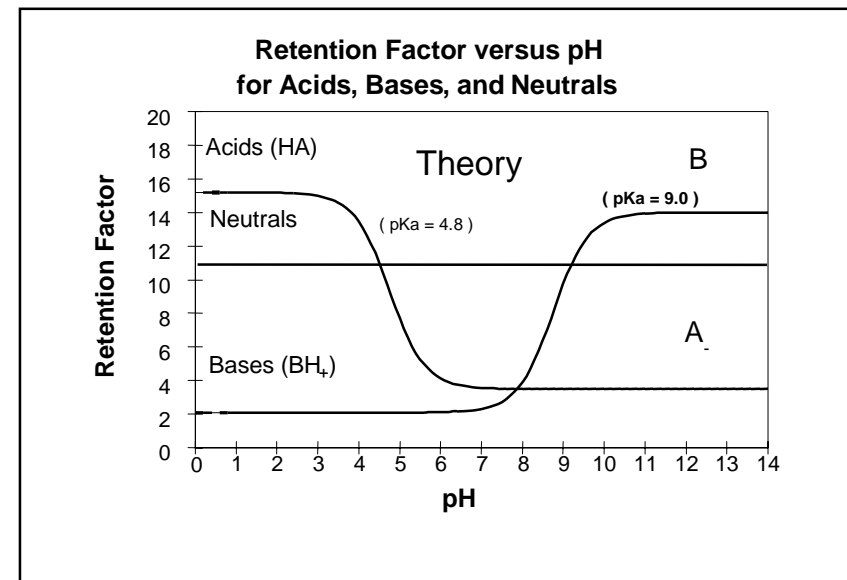
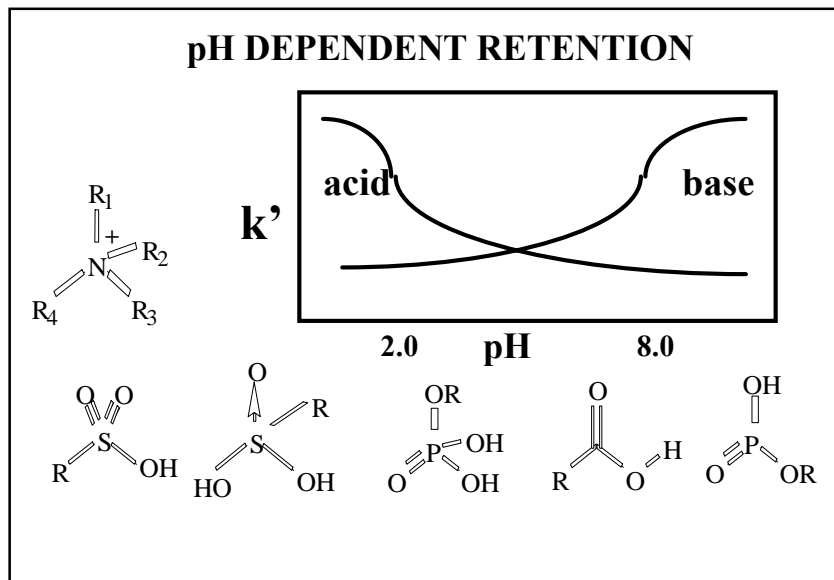
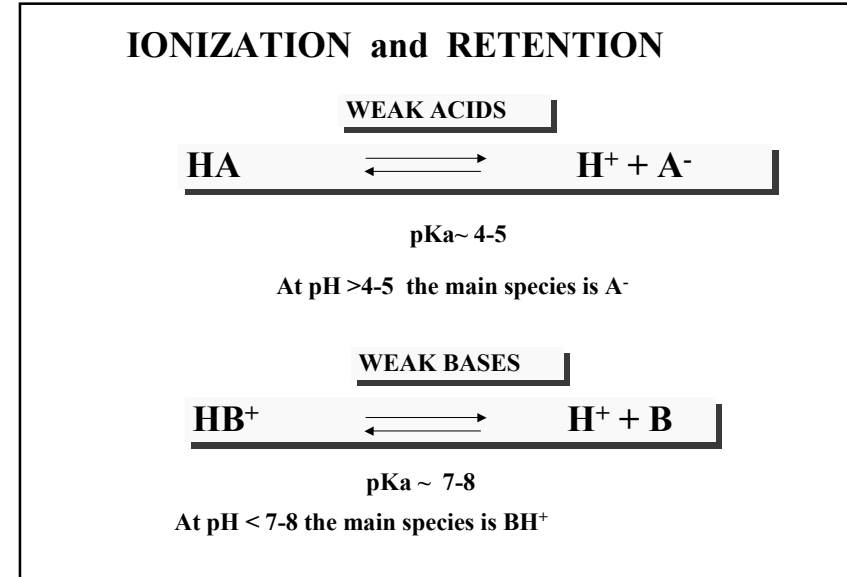
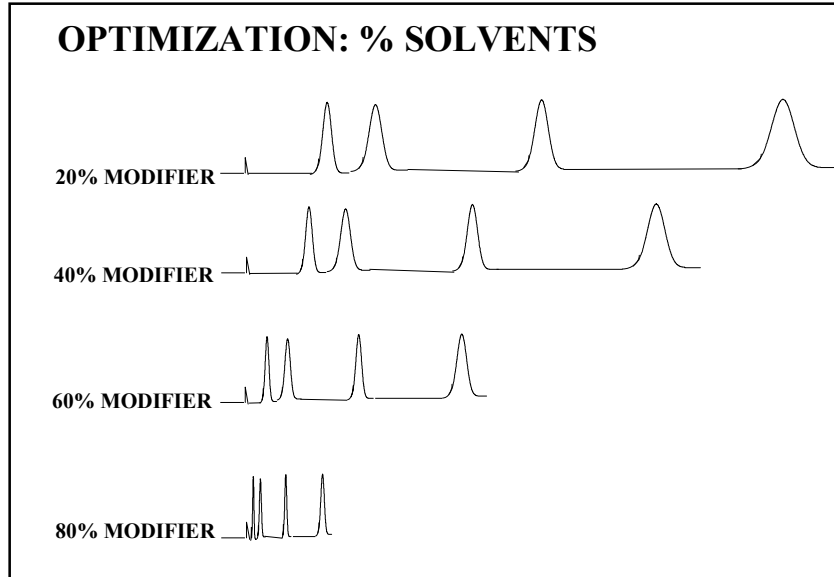
### OPTIMIZATION: CHOICE OF SOLVENTS

#### REVERSED PHASE



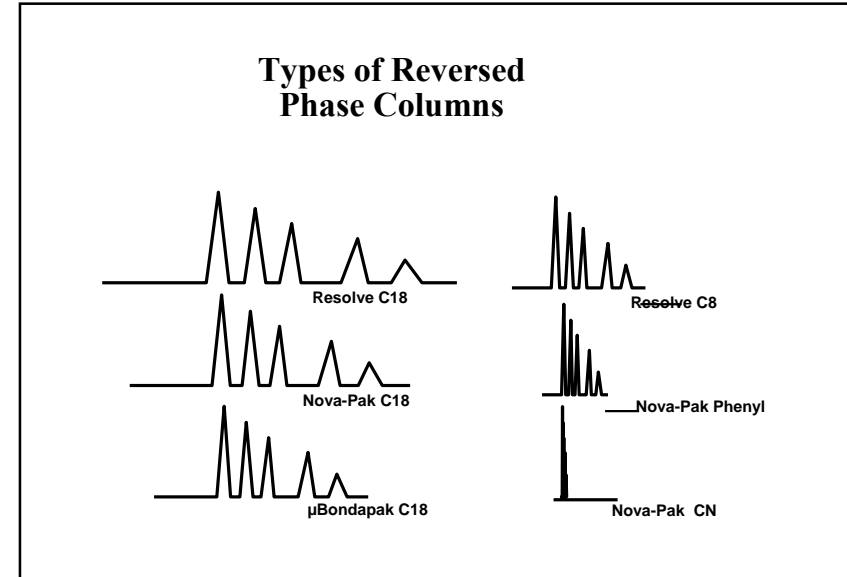
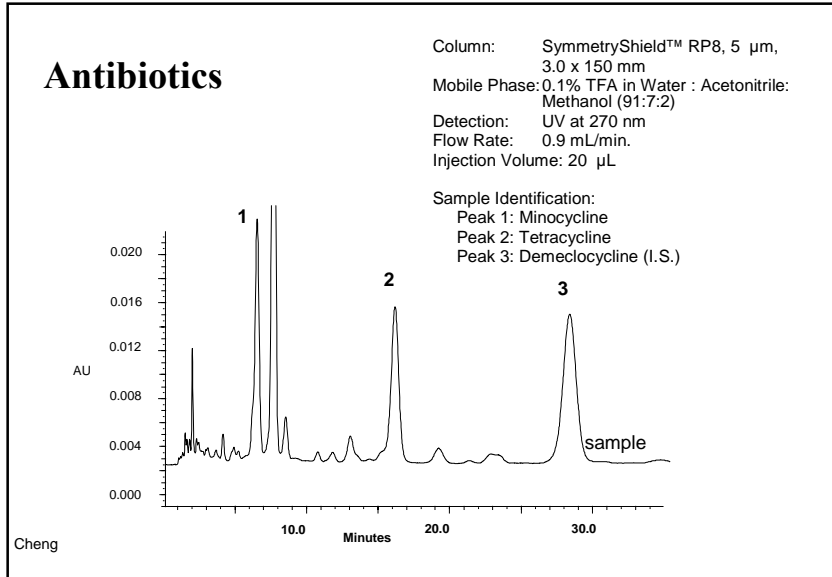
# High Performance Liquid Chromatography - HPLC

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# High Performance Liquid Chromatography - HPLC

## Introduction



### Stationary Phase Properties

**CHEMISTRY:**

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

pores

silica

d

**GEOMETRY**

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

### Stationary Phase Supports

Stationary phase	Functionality
C <sub>18</sub>	-Si(CH <sub>3</sub> ) <sub>2</sub> C <sub>18</sub> H <sub>37</sub>
C <sub>8</sub>	-Si(CH <sub>3</sub> ) <sub>2</sub> C <sub>8</sub> H <sub>17</sub>
tC <sub>2</sub>	-SiC <sub>2</sub> H <sub>5</sub>
Aminopropyl	-Si(CH <sub>3</sub> ) <sub>2</sub> NH <sub>2</sub>
Cyanopropyl	-Si(CH <sub>3</sub> ) <sub>2</sub> (CH <sub>2</sub> ) <sub>3</sub> CN
Diol	-Si(CH <sub>3</sub> ) <sub>2</sub> O CH <sub>2</sub> CH(OH)CH <sub>2</sub> OH

Retention time →

Chain length CN Phenyl NH<sub>2</sub> C<sub>4</sub> C<sub>8</sub> C<sub>18</sub>

# High Performance Liquid Chromatography - HPLC

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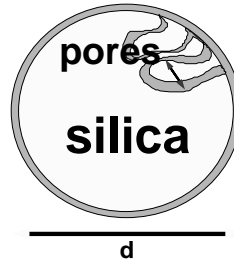
### Stationary Phase Properties

#### CHEMISTRY:

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

#### GEOMETRY

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

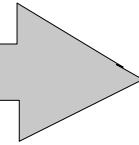


### CARBON LOAD

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

Retention time

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



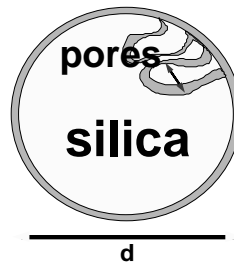
### Stationary Phase Properties

#### CHEMISTRY:

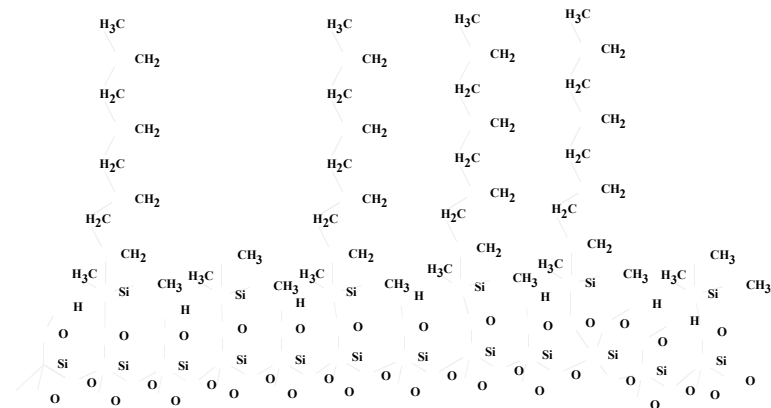
- \* BONDED HYDROCARBON:  
C-18, C-8, C-4, C-1, CN, phenyl
- \* % COVERAGE
- \* TYPE OF SILICA GEL

#### GEOMETRY

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



### Surface of a Reversed- Phase Packing

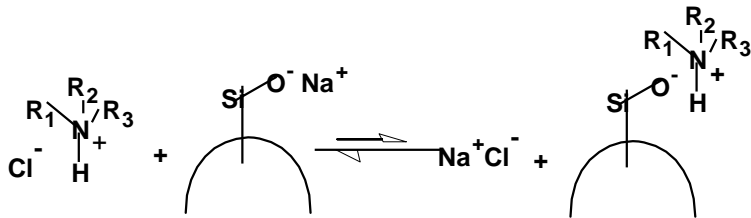


# High Performance Liquid Chromatography - HPLC

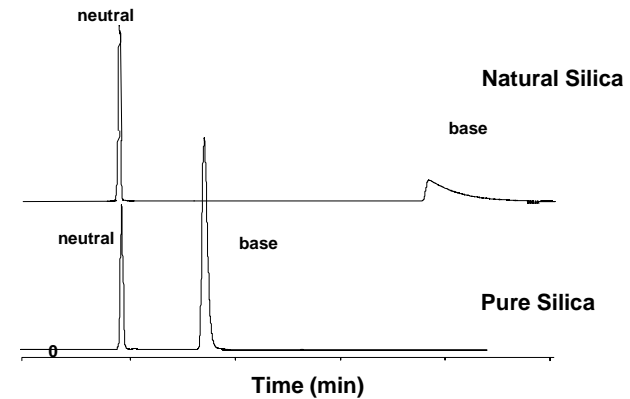
## Introduction

### What Causes Tailing?

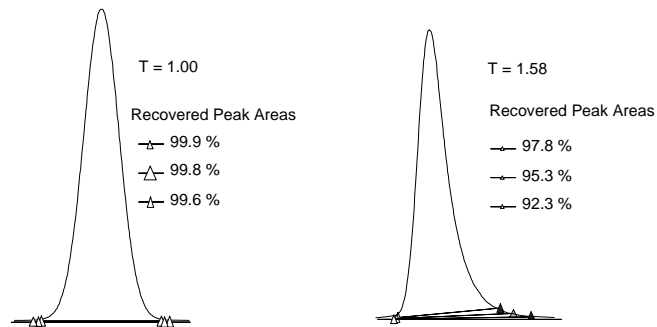
- Mixed-mode retention:
  - hydrophobic - interaction with bonded phase
  - ion exchange - interaction with charged sites



### Quality of Columns Performance



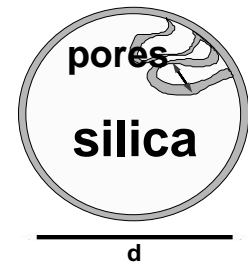
### Integration Errors Caused by Tailing



### Stationary Phase Properties

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

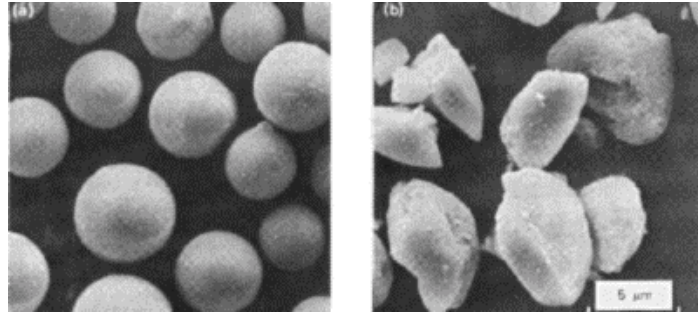
- GEOMETRY**
- \* SPHERE-IRREGULAR
  - \* PARTICLE DIAMETER
  - \* POROSITY



# High Performance Liquid Chromatography - HPLC

## Introduction

### Spherical and Irregular particles

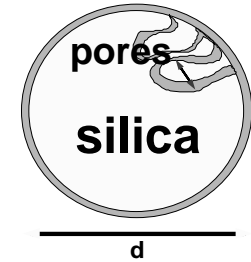


Electron microphotograph of spherical and irregular silica particles. [W.R.Melander, C.Horvath, Reversed-Phase Chromatography, in HPLC Advances and Perspectives, V2, Academic Press, 1980]

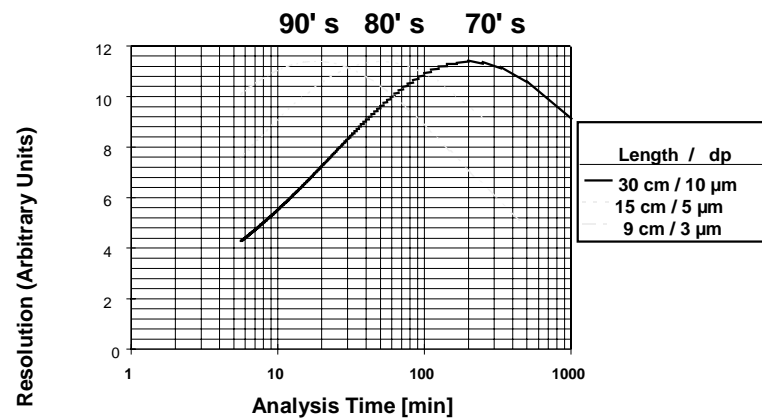
### Stationary Phase Properties

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

**GEOMETRY**  
 \* SPHERE- IRREGULAR  
 \* **PARTICLE DIAMETER**  
 \* POROSITY



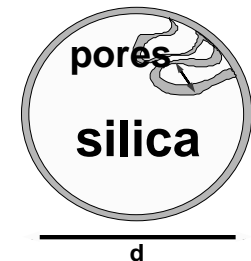
### Resolution - Time Diagram



### Stationary Phase Properties

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

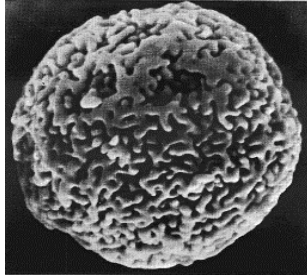
**GEOMETRY**  
 \* SPHERE- IRREGULAR  
 \* **PARTICLE DIAMETER**  
 \* POROSITY



# High Performance Liquid Chromatography - HPLC

## Introduction

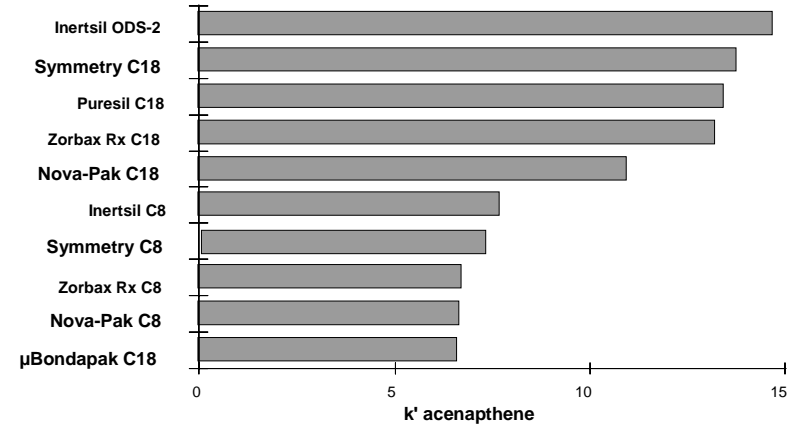
### Pore size, shape and distribution



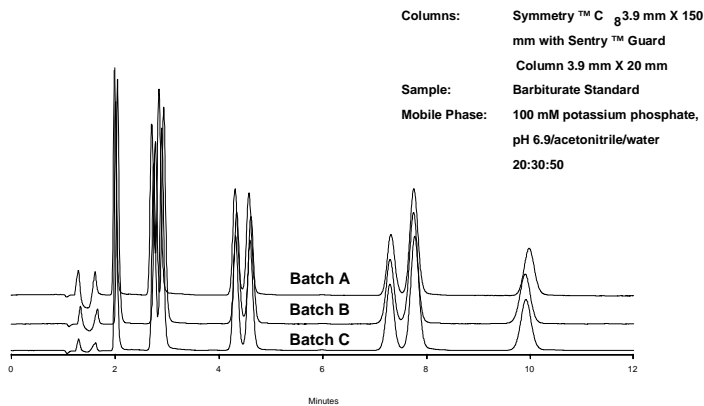
▣ Macroporous spherical silica particle. [K.K.Unger, Porous silica, Elsevier, 1979]

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

