

# Separation Modes in HPLC

## Short Overview

### Separation Modes in Liquid Chromatography

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

### REVERSED PHASE the most widely Used Separation Mode

Apolar Stationary Phase



SOLUTES: סוגי חומרים

MOST OF THE  
BIOMEDICAL SUBSTANCES  
All substances with organic backbone

CONDITIONS: תנאי עבודה



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

### NORMAL PHASE

ADSORPTION on Polar  
Stationary Phase

SOLUTES



LIPOPHYLIC:  
OILS, FATS, LIPIDS

CONDITIONS



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

### Hydrophilic Interaction (HILIC)

ADSORPTION on Polar  
Stationary Phase

SOLUTES



Polar Compounds  
(saccharides, Peptides)

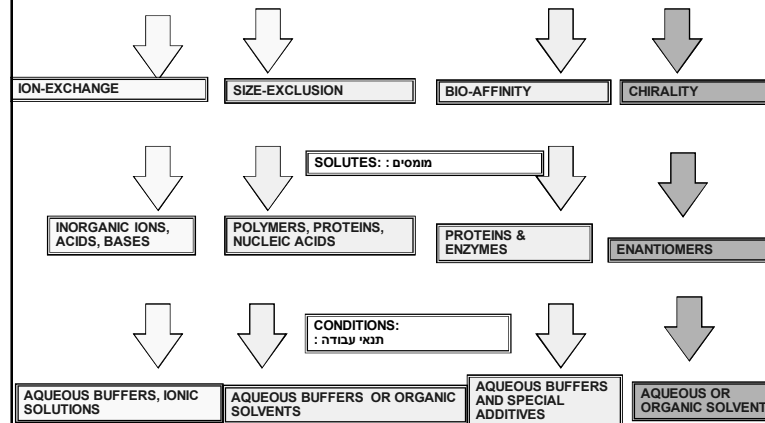
CONDITIONS



ACETONITRILE/WATER AND ORGANIC  
BUFFERS

### High Performance Liquid Chromatography: Biomedical Mostly

PRINCIPLE OF SEPARATION: עקרון ההפרדה:



## Short Overview

## REVERSED PHASE

the most widely Used Separation Mode

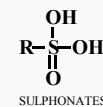
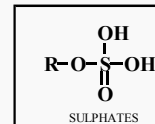
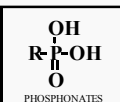
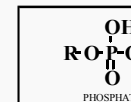
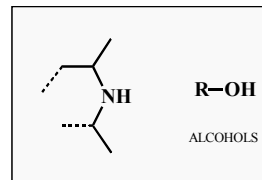
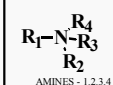
### Apolar Stationary Phase

**SOLUTES:** סוגי חומרים

**MOST OF THE  
BIOMEDICAL SUBSTANCES**  
All substances with organic backbone

**CONDITIONS: תנאי עבודה**

## Ionizable Molecules



### Chromatographic Process: התהליך הכרומטוגרפי

Mobile phase: פאזה נעה  
Water, Buffers  
MeOH, Acetonitril, IPA

Hydrophobic  
Stationary  
Phase

Distribution:  
 $K = C_s/C_m$

A

1

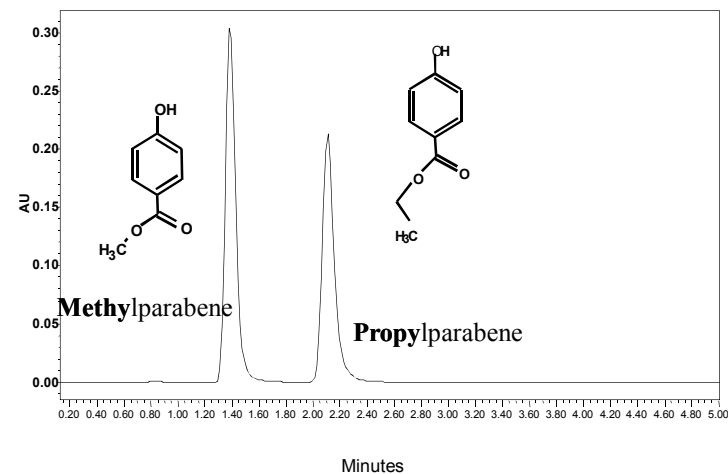
Chron

Chron

Migration through the Column  
מסע דרך העמודה

Chromatogram כרומטוגרמה

## Reversed Phase Elution Order סדר יציאה



# Separation Modes in HPLC

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### Clinical Applications יישומים רפואיים



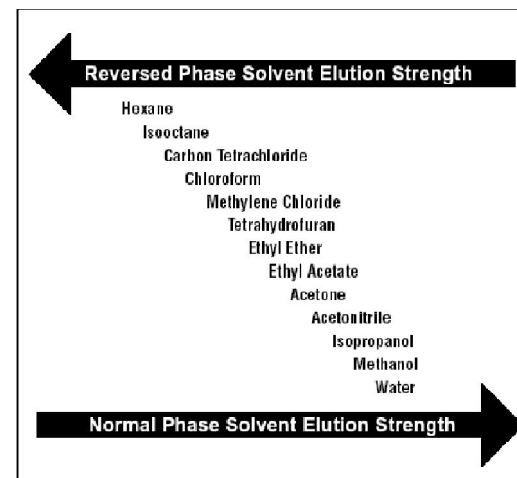
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### MOBILE PHASE

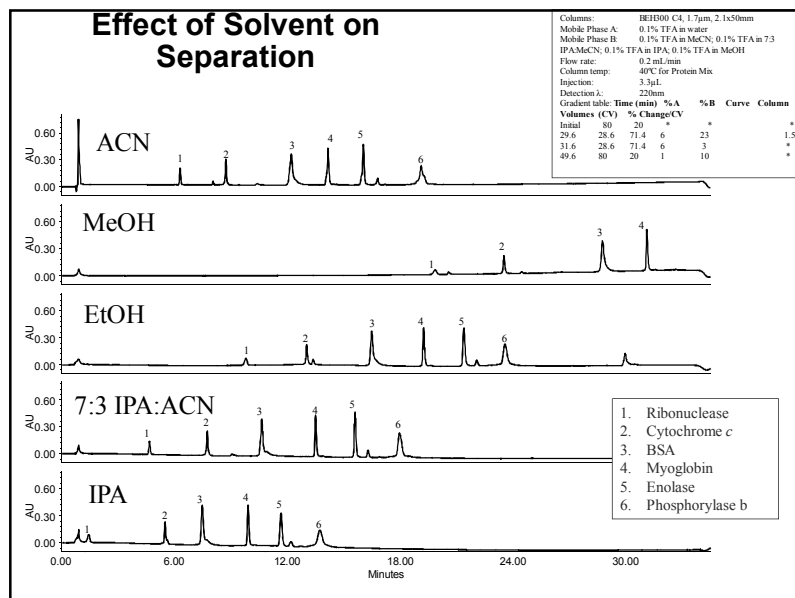
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### Elution Strength



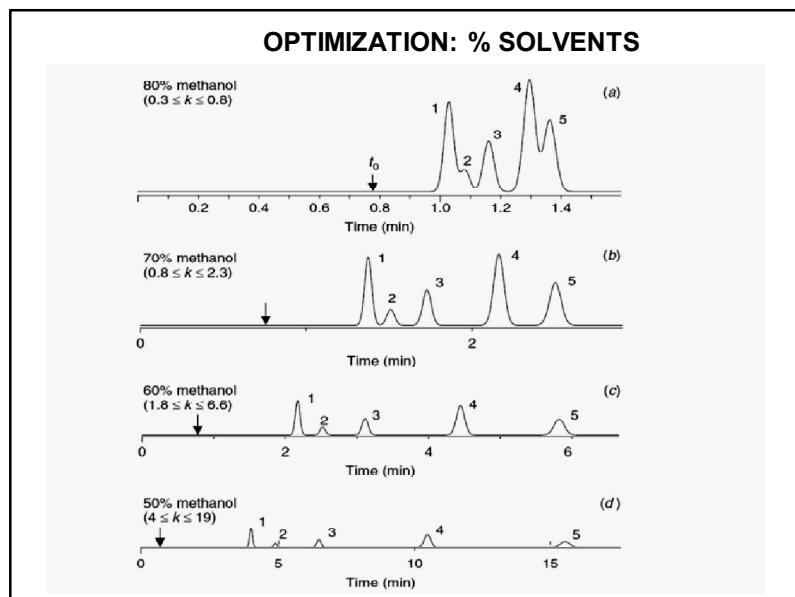
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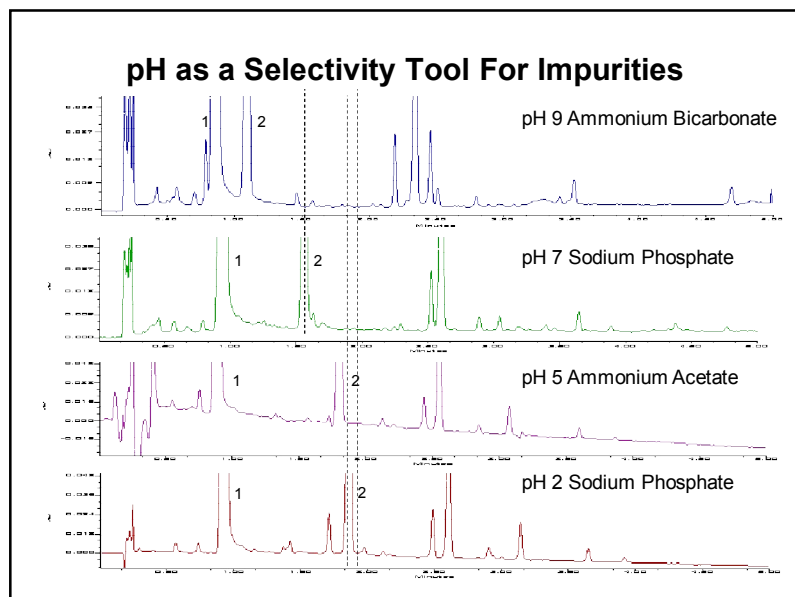


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# Separation Modes in HPLC

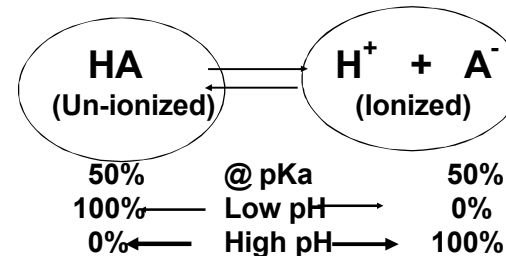
## Short Overview



## Ionization of Acids and Bases

### Dissociation of Molecule

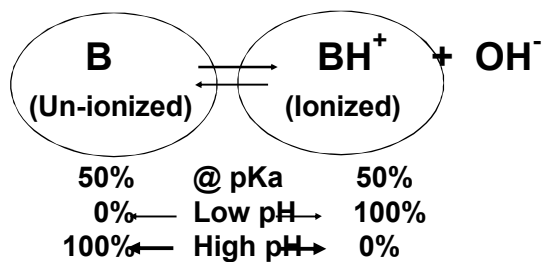
#### Acid



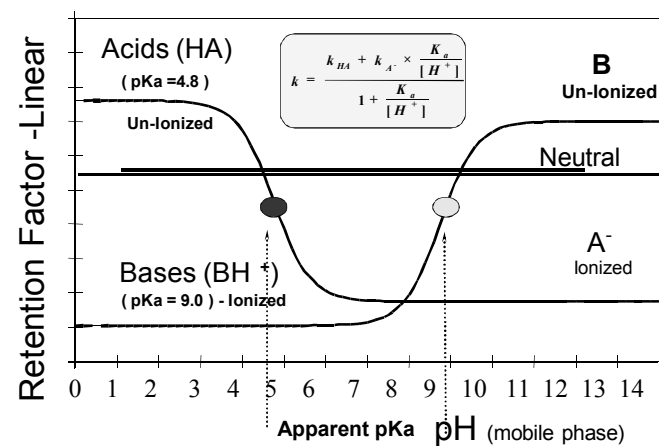
## Ionization of Acids and Bases

### Dissociation of Molecule

#### Base



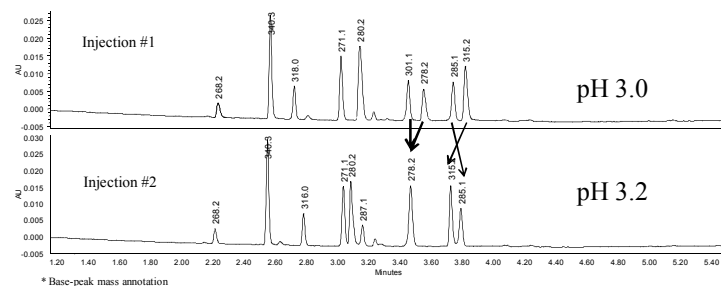
### Retention Factor versus pH for Acids, Bases and Neutrals



# Separation Modes in HPLC

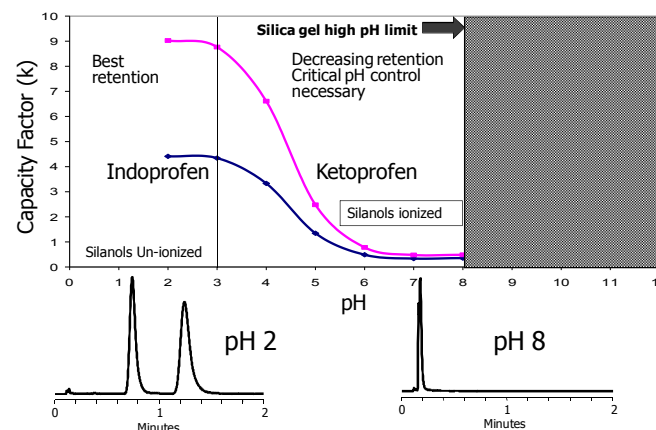
## Short Overview

My system seems OK but I am getting strange results...

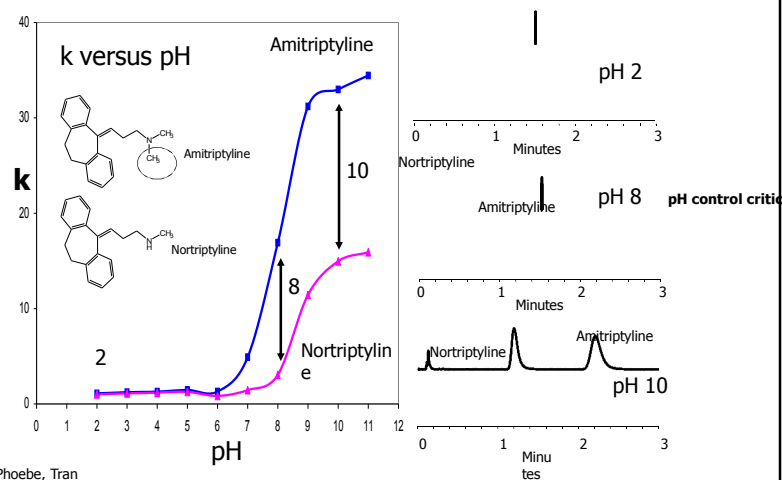


Slight changes in mobile phase preparation can affect method robustness!

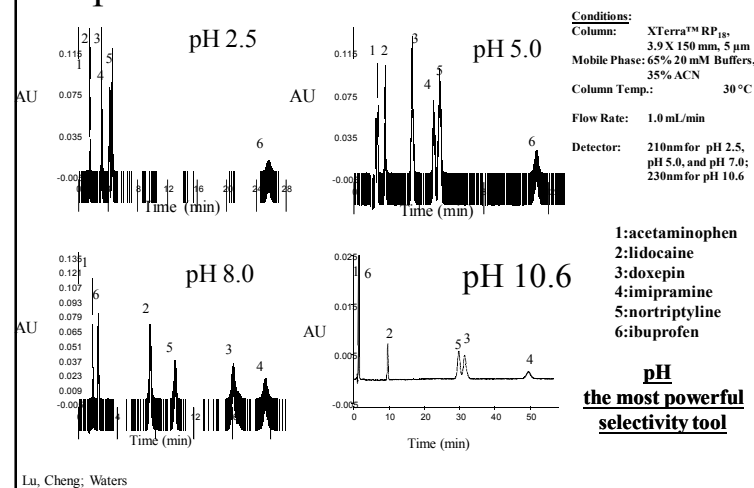
## Resolution of Two Acidic Compounds at Different Mobile Phase pH's



## Enhanced Resolution of Basic Compounds at High pH



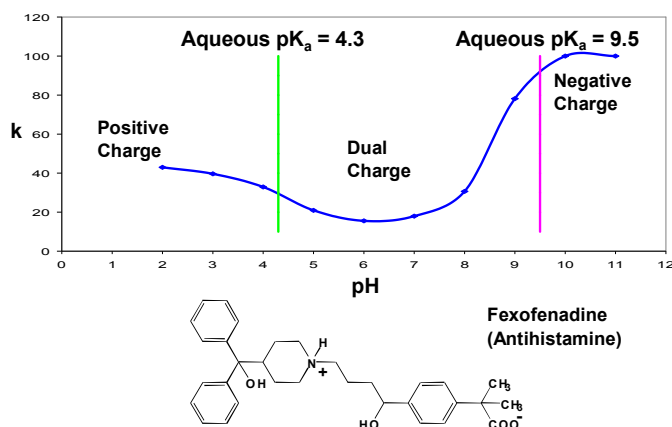
## Dependence of Selectivity on pH



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### Impact of pH on the Retention of a Zwitterionic Compound



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### Recommended Buffers for pH's 2-7 (Silica and Hybrid Packing Materials)

Additive or Buffer	pK <sub>a</sub>	pH range (± 1 pH unit)	Volatile or Non-Volatile	Recommended Concentration Ranges and Common Counter-Ions
TFA	0.3		Volatile	(0.02-0.1%)
Acetic Acid	4.76		Volatile	(0.1-1.0%)
Formic Acid	3.75		Volatile	(0.1-1.0%)
Acetate	4.76	3.76 – 5.76	Volatile/Non-volatile	(1-10mM) NH <sub>4</sub> , Na, K
Formate	3.75	2.75 – 4.75	Volatile/Non-volatile	(1-10mM) NH <sub>4</sub> , Na, K
Phosphate	2.15	1.15 – 3.15	Non-volatile	
	7.20	6.20 – 8.20	Non-volatile	Not for pH's >7.0 (reduce the temperature & conc. for longer column lifetime)

### Non-Recommended Buffers for pH's >7

Buffer	pK <sub>a</sub>	pH range (± 1 pH unit)	Volatile or Non-Volatile	Effect if buffer used (Dissolution)
Phosphate	12.3	11.3 – 13.3	Non-Volatile	Short Column Lifetime
Borate	9.2	8.2 – 10.2	Non-Volatile	Short Column Lifetime

Not recommended for either hybrid (XTerra) or silica gel based columns.

# Separation Modes in HPLC

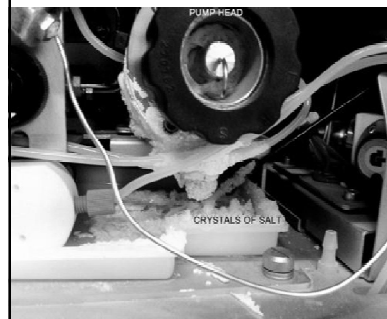
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### Types of Buffers and Ionic Strength

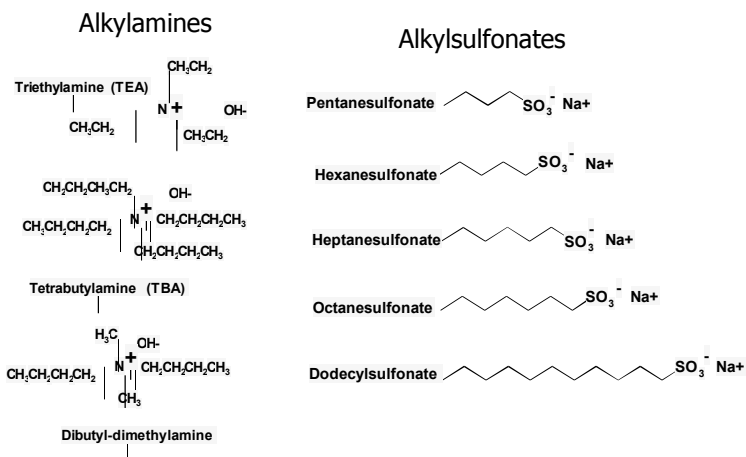
- pH 10: Borate
  - 20 mM  $\text{H}_3\text{BO}_3$
- pH 7: Phosphate
  - 20 mM  $\text{K}_2\text{HPO}_4$
- pH 4-5: Acetate
  - 10 mM  $\text{CH}_3\text{COONH}_4$
  - 100 mM  $\text{CH}_3\text{COOH}$
- pH 2-3.5: Phosphate
  - 20 mM  $\text{H}_3\text{PO}_4$  -  $\text{KH}_2\text{PO}_4$



### MOBILE PHASE

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### Ion Pair Reagent



# Separation Modes in HPLC

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### Examples of Ion Pair Reagents

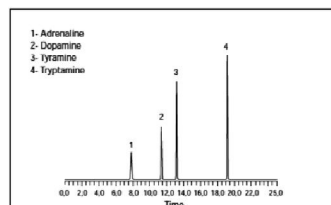


Figure 4. Mixture of biogenic amines resolved by IPC

**Columns:** Discovery™ C18 Column (250 x 4.0 mm) ID, 5 µm (Cat. No 04971-40)  
**Eluent:** acetonitrile: heptanesulfonic acid buffer pH 2.4:  
**Buffer concentration:** 0.005 M heptanesulfonic acid sodium salt (Cat. No 51832) + 0.01 M phosphoric acid (Cat. no 79506)  
**Weigh-in:** ~ 2 mg in 10 ml acetonitrile/phosphoric acid (0.01 M) 1:9  
**Acetonitrile gradient:** t=0 min : 6%, t=5 min : 6%, t=18 min : 25%  
**Flow:** 1.5 ml/min  
**Detection:** 220 nm  
**Injection volume:** 20 µl  
**Temperature:** ambient  
**Detector:** UV 1000  
**Pump:** P 4000

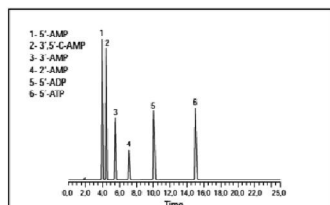
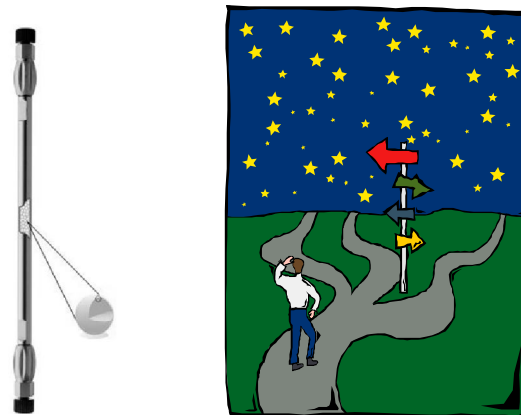


Figure 5. Mixture of nucleotides resolved by IPC

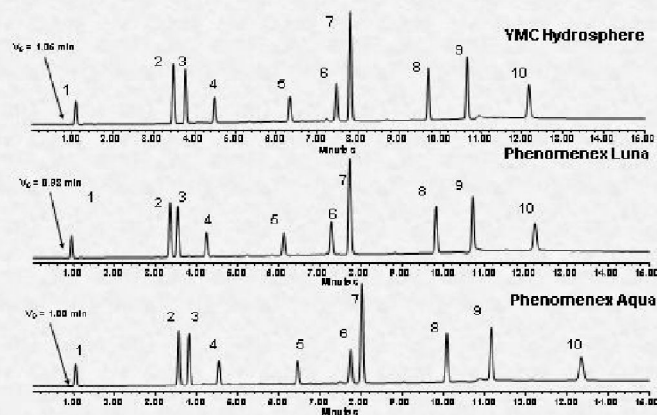
**Column:** Discovery™ C18 Column (250 x 4.0 mm) ID, 5 µm (Cat. No 504971-40)  
**Eluent:** acetonitrile: tetrabutylammonium buffer pH 7.0  
**Gradient Buffer concentration:** 0.005 M tetrabutylammonium hydrogensulfate (Cat. No 86853) + 0.01 M Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O (Cat. No 71649).  
**Weigh-in:** ~ 4 mg in 10 ml acetonitrile / water 1:9  
**Acetonitrile gradient:** t=0: 10%; t=4 min 10%; t=14 min: 25%  
**Flow:** 1.5 ml/min  
**Detection:** 254 nm  
**Injection volume:** 20 µl  
**Temperature:** ambient  
**Detector:** UV 1000  
**Pump:** P 4000

### Stationary Phase Characterization

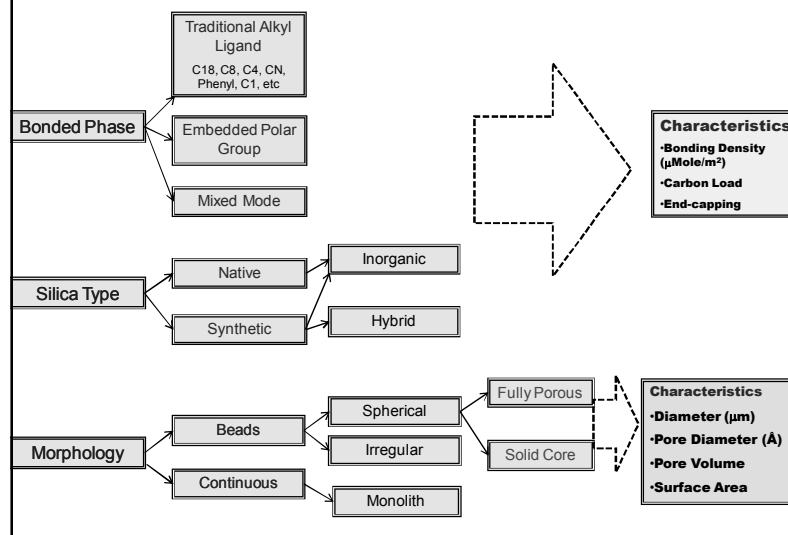


### Various C18 Columns

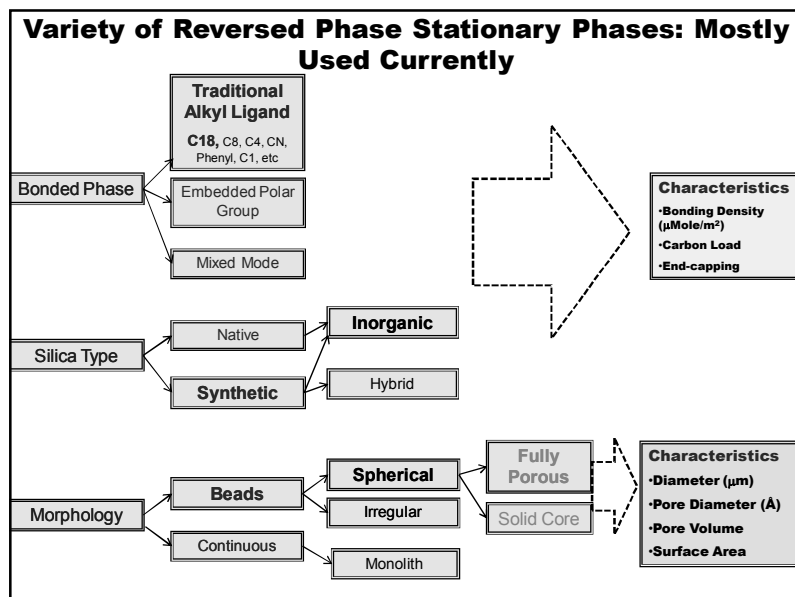
#### Different Columns – Different Chromatograms



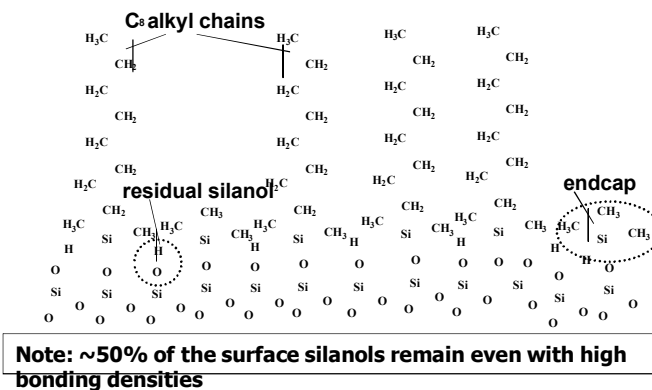
### Variety of Reversed Phase Stationary Phases



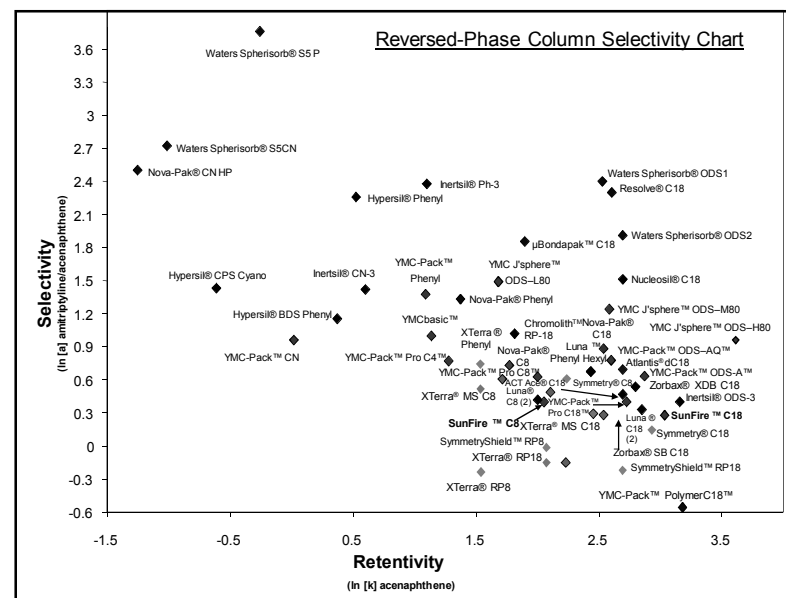
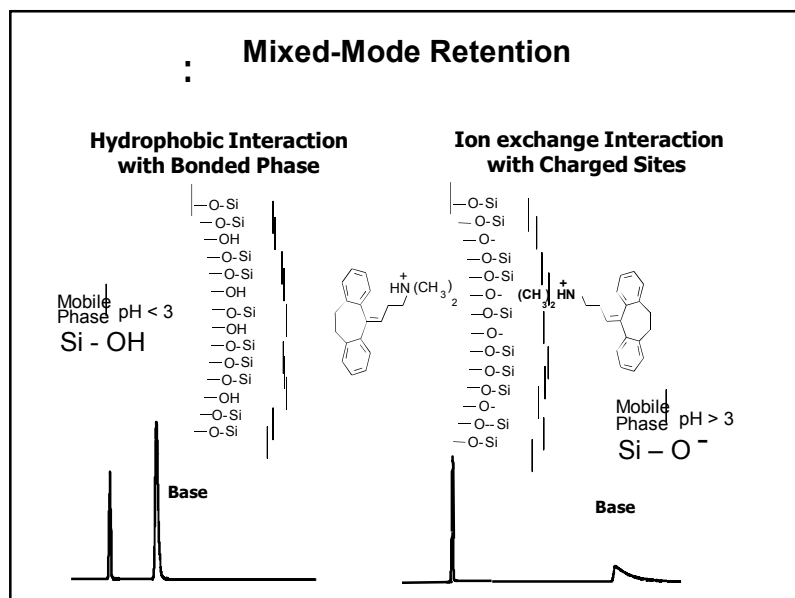
## Short Overview



## Surface of a Silica Gel Bonded-Phase Packing Material

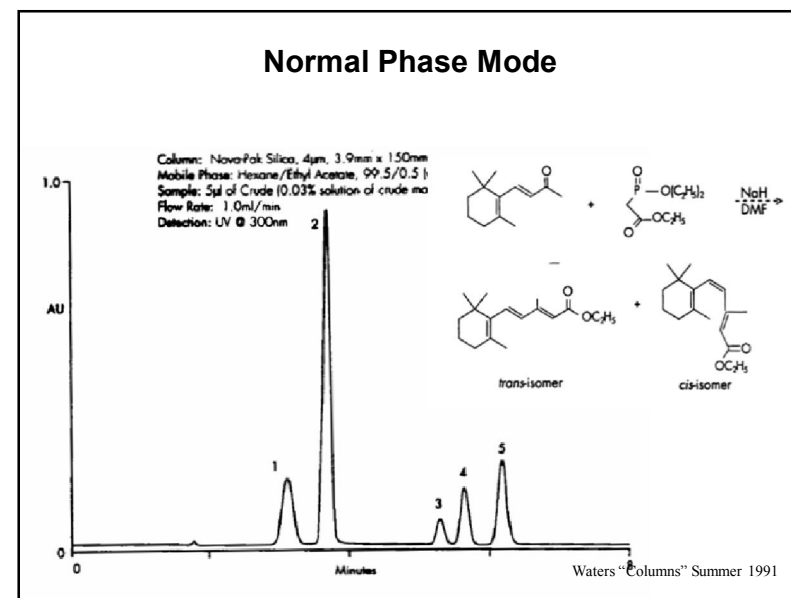
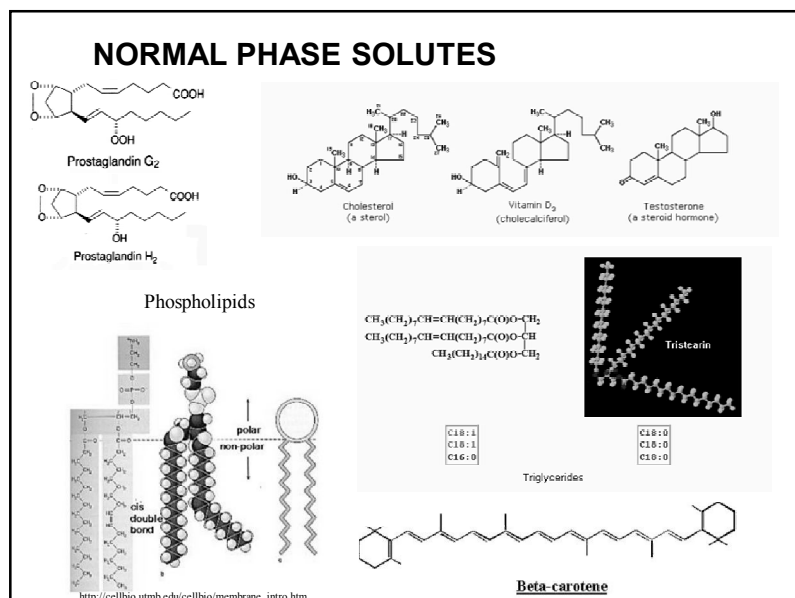
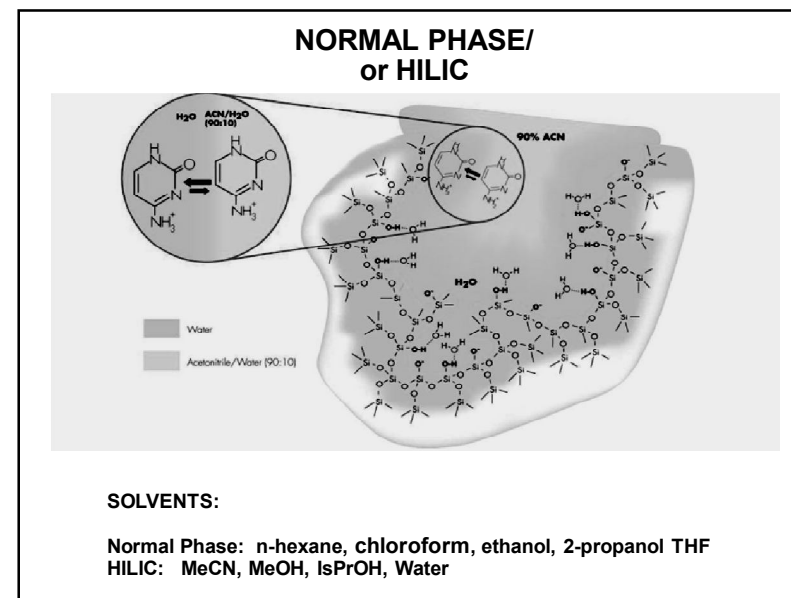
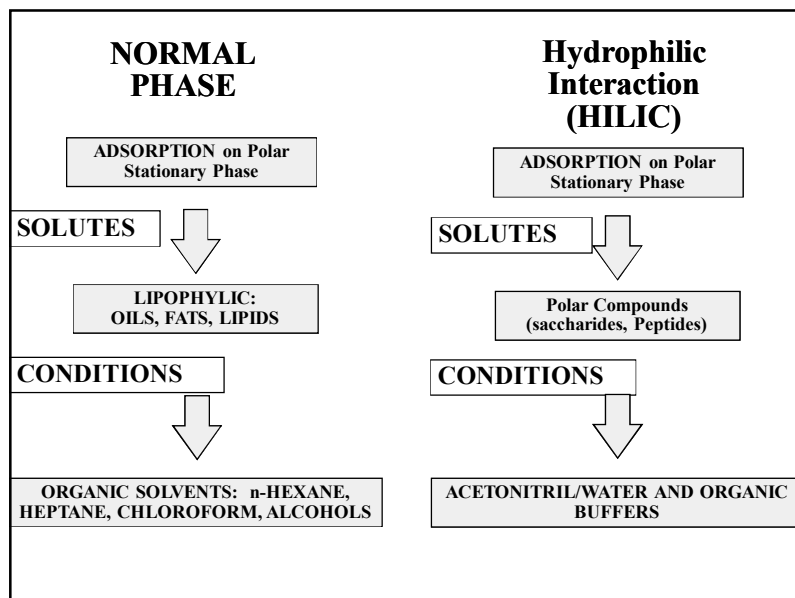


## Mixed-Mode Retention



# Separation Modes in HPLC

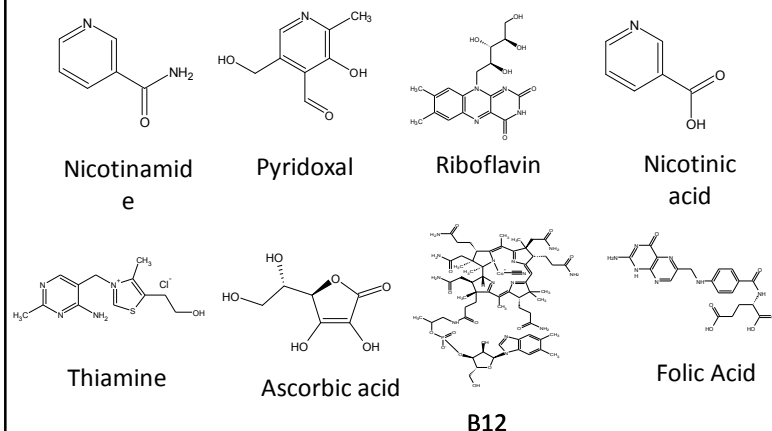
## Short Overview



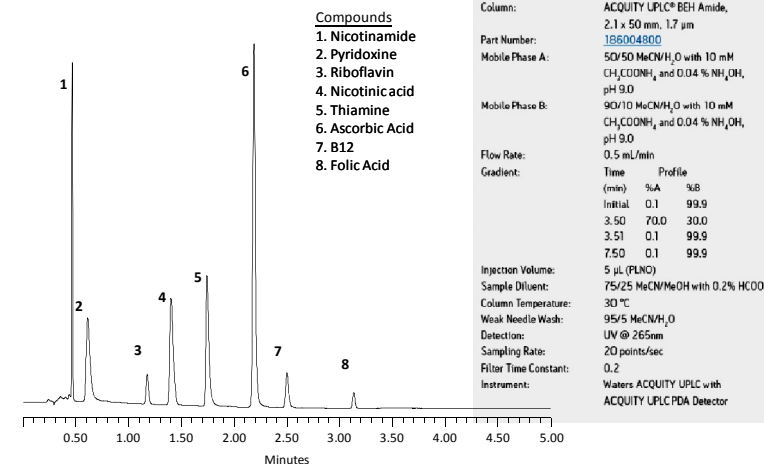
# Separation Modes in HPLC

## Short Overview

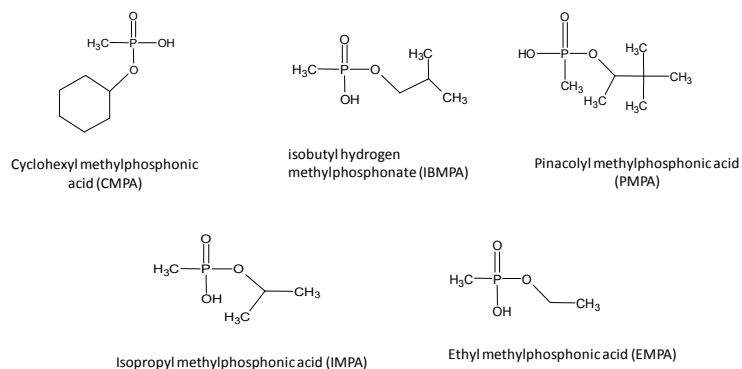
### Example 1, Water Soluble Vitamins



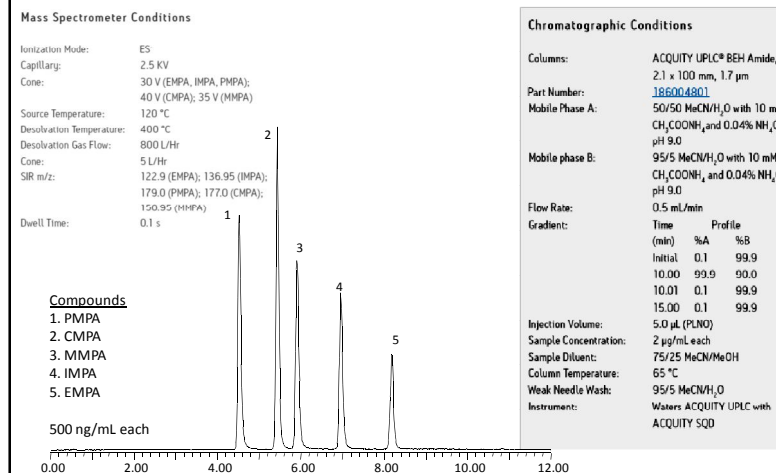
### Final Method: Water-soluble Vitamins



### Example 2, Organophosphonic Acid Nerve Agents

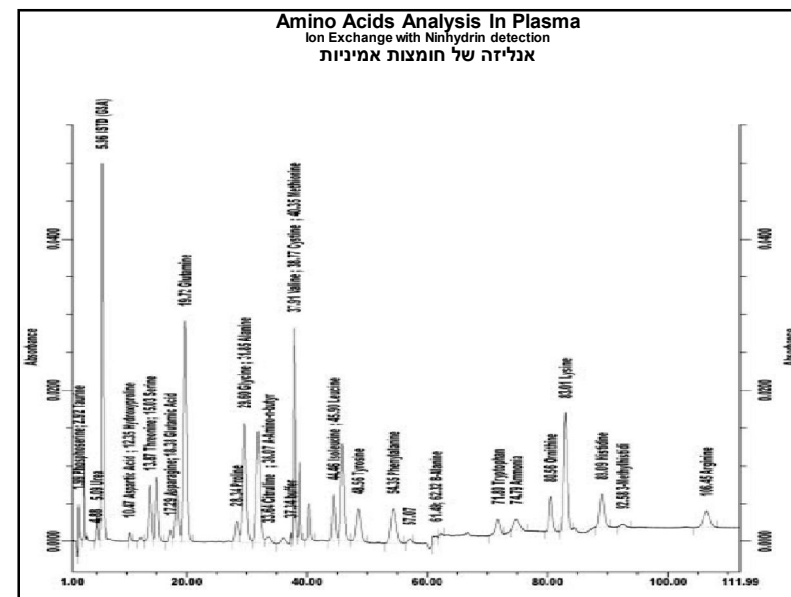
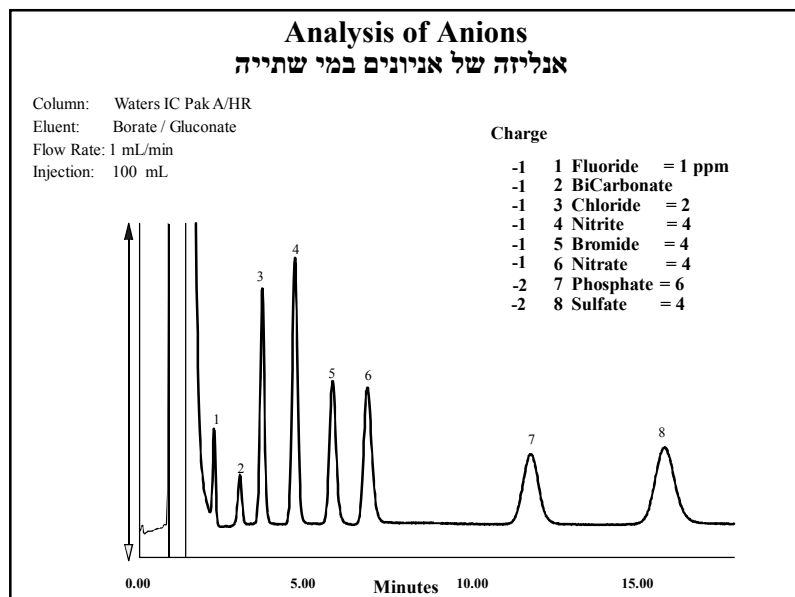
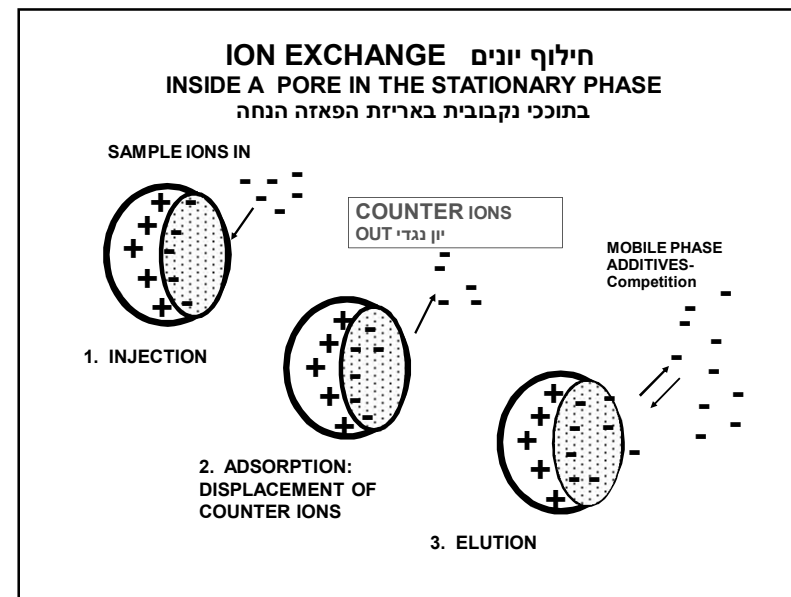
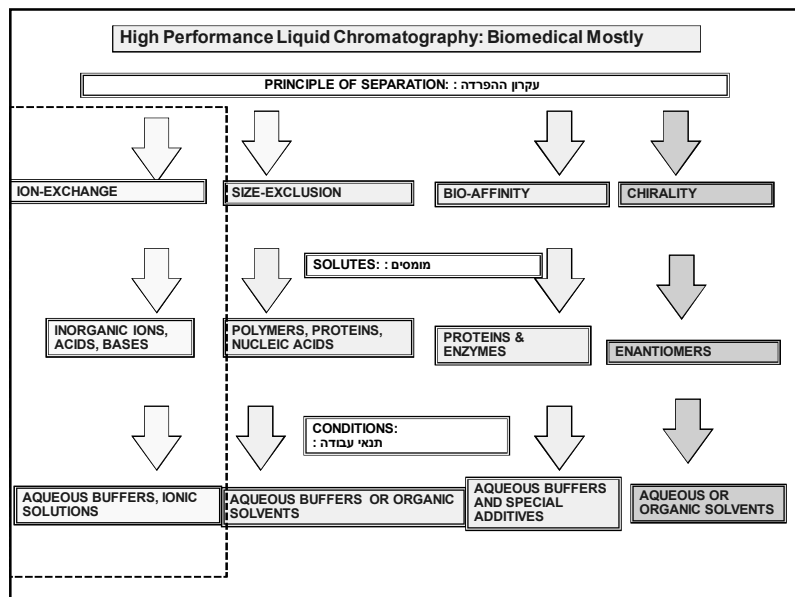


### Final Method: Organophosphonic Acids



# Separation Modes in HPLC

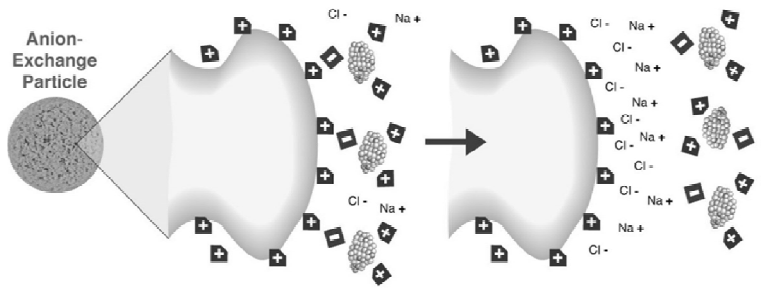
## Short Overview



# Separation Modes in HPLC

## Short Overview

### Ion-Exchange Chromatography of Proteins

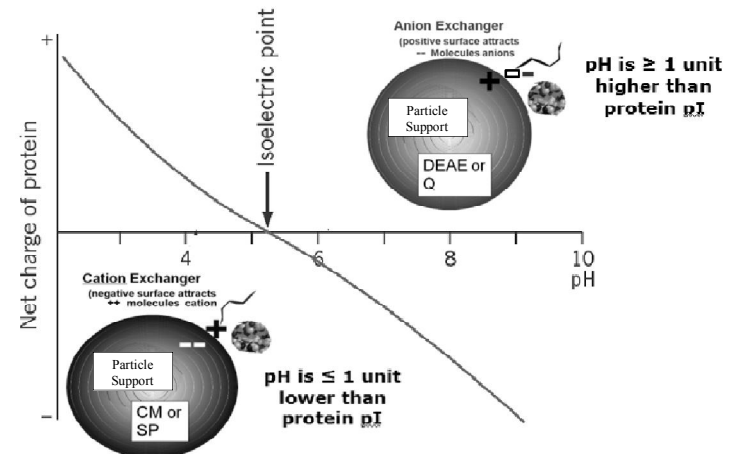


**Binds at Low Ionic Strength**

**Elute with Step or Continuous Gradients of Increasing Ionic Strength**

- Separations are based on net surface charge on protein with oppositely charged groups on ion-exchanger
- Proteins elute from column using either a gradient of increasing salt concentration (most common) or changing pH (less common)

### Protein Isoelectric Points and IEX



### Separation of Proteins by High Performance Cation-Exchange Chromatography

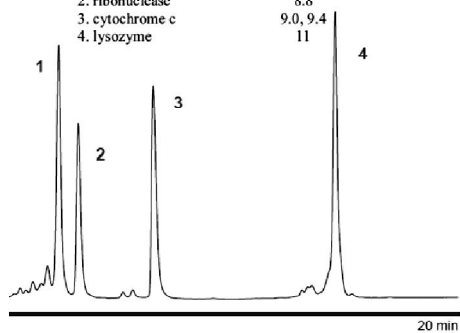
#### Conditions

**Column:** 400VHP575 (5  $\mu$ m, 'S' type cation exchange, 7.5 x 50 mm)

**Eluent:** 10 mM phosphate, pH 6.5 with a gradient from 0 to .5 M NaCl in 50 min.

#### Proteins:

- |                                 |               |
|---------------------------------|---------------|
| 1. $\alpha$ -chymotrypsinogen A | 8.8, 9.2, 9.6 |
| 2. ribonuclease                 | 8.8           |
| 3. cytochrome c                 | 9.0, 9.4      |
| 4. lysozyme                     | 11            |



### Separation of Peptides by Cation Exchange

#### Separation by Charge/Hydrated size

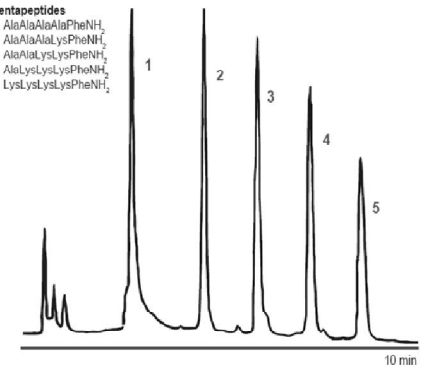
#### Separation of Peptides by Cation Exchange

Five pentapeptides with one to five positive charges (at low pH) were synthesized and separated on a 400VHP575 column. Separation between adjacent peptides and peak shape were excellent.

#### Conditions

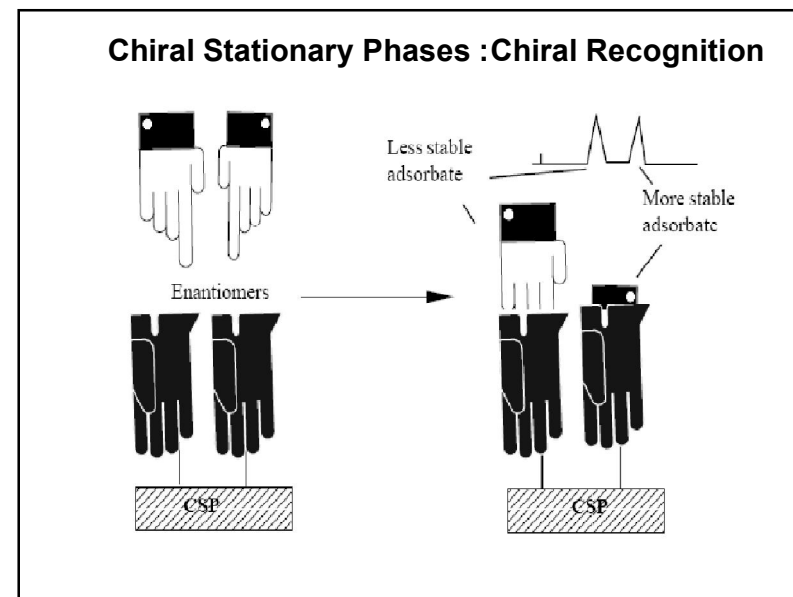
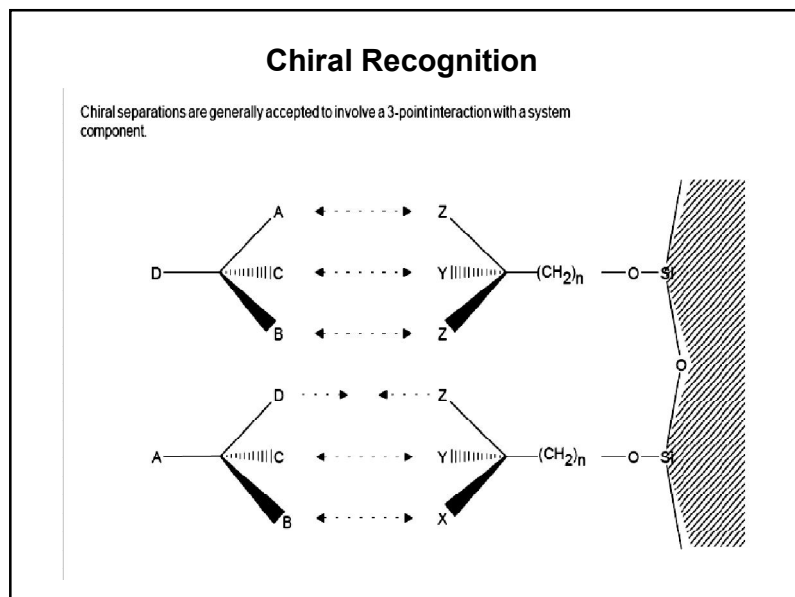
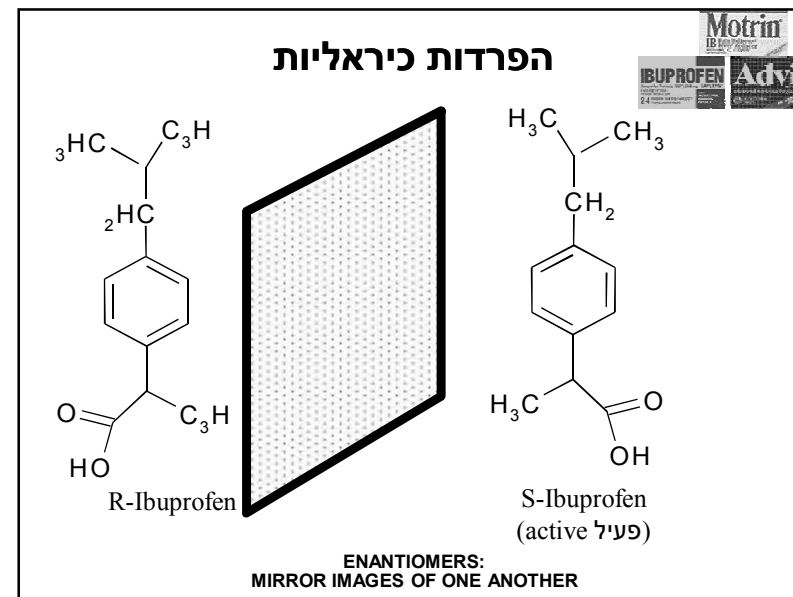
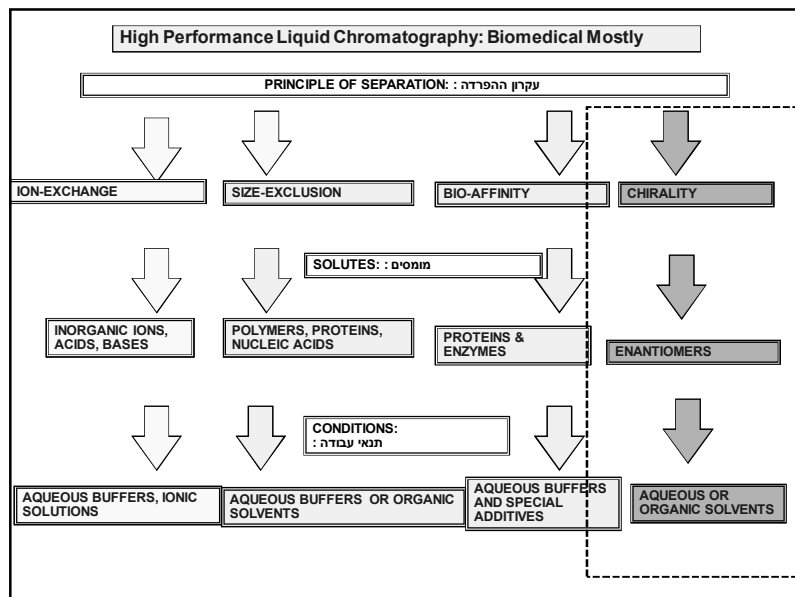
**Column:** Vydac 400VHP575, (S-type Cation Exchange, 5  $\mu$ m, 7.5 x 50 mm).  
**Buffer A:** 8 mM phosphate in 20% acetonitrile/water, pH 4.0  
**Buffer B:** Buffer A with 0.4 M sodium chloride  
**Gradient:** 1 min hold at 0% B, 0-100% B over 10 min.  
**Flow rate:** 2.5 ml/min  
**Detection:** UV at 220 nm  
**Sample:** about 50 mgrams of each pentapeptide  
*Data courtesy of Mike Giles, Zeneca Pharmaceuticals*

- Pentapeptides**
1. AlaAlaAlaAlaPheNH<sub>2</sub>
  2. AlaAlaAlaLysPheNH<sub>2</sub>
  3. AlaAlaLysLysPheNH<sub>2</sub>
  4. AlaLysLysLysPheNH<sub>2</sub>
  5. LysLysLysLysPheNH<sub>2</sub>



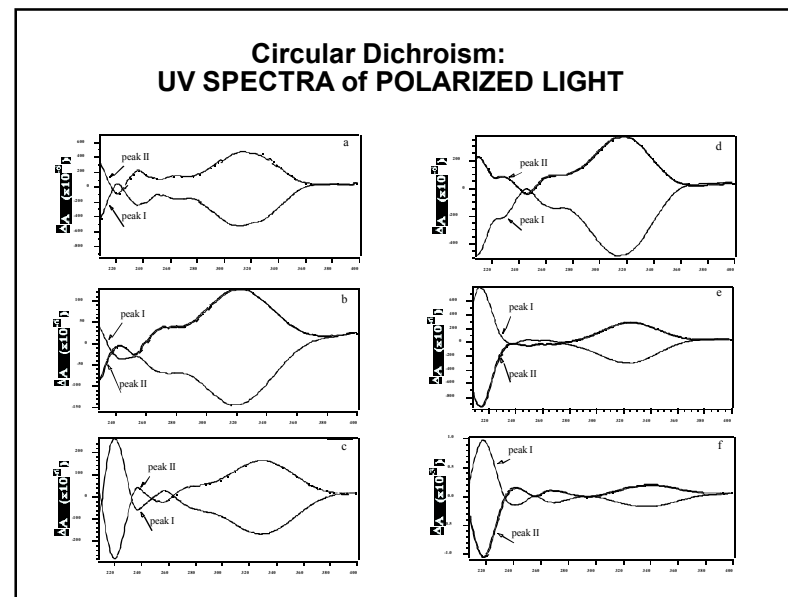
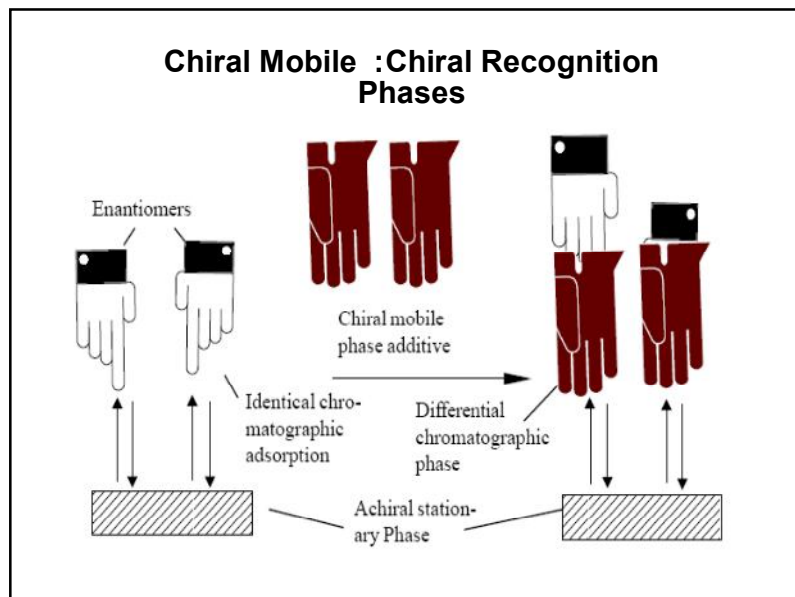
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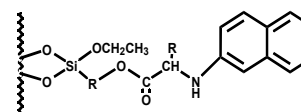


### Types of Chiral stationary phases:

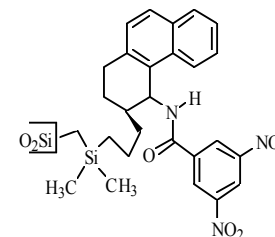
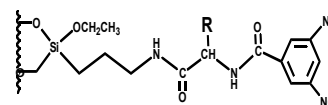
- Ligand exchange
- $\pi$ -Donnor  $\pi$ -acceptor (Pirkle)
- Chiral Host-guest (cyclodextrin)
- Immobilized proteins
- Immobilized polysaccharides
- Macrocyclic Antibiotics
- Crown Ethers



### $\pi$ - Donor $\pi$ - acceptor (Pirkle) Type Naphthyl amino acids



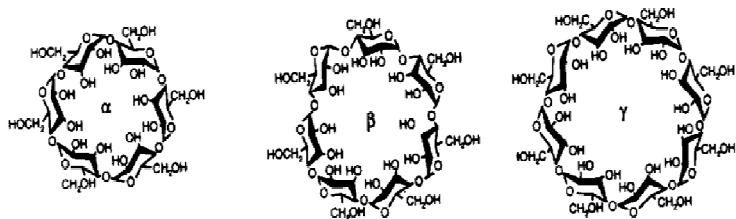
### Dinitrobenzoyl amino acids



# Separation Modes in HPLC

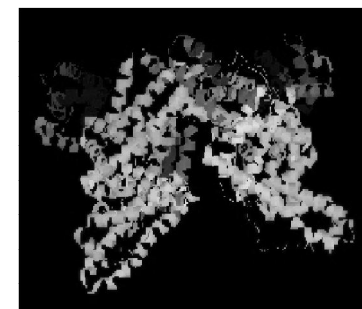
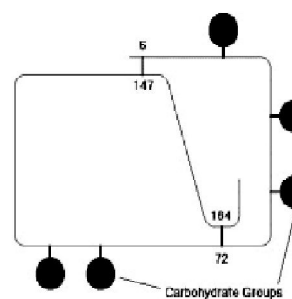
## Short Overview

### Chiral cavity by cyclodextrins



CD	No.Of units	size Å	Molecules included	Chiral centres
alpha	6	4.5-6.0	5-6 membered aromatic	30
beta	7	6.0-8.0	biphenyl or naphthalene substituted pyrenes and	35
gamma	8	8.0-10.0	Steroids	40

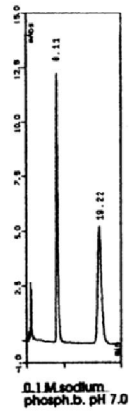
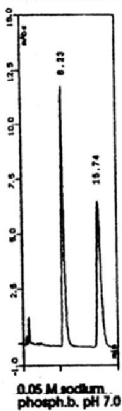
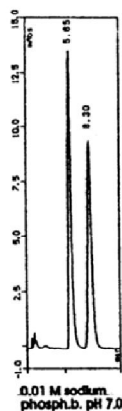
### Protein Columns



### Effect of ionic strength in Immobilized Protein Columns

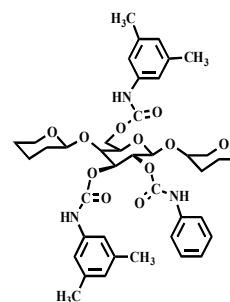
Changing the buffer concentration: By changing the buffer concentration in the mobile phase, it is possible to affect both the retention ( $k'$ ) and the enantioselectivity ( $\alpha$ ). These effects have been observed for acids and in special cases also for some amines. The chromatograms below demonstrate the effect of changing the buffer:

#### NAPROXEN

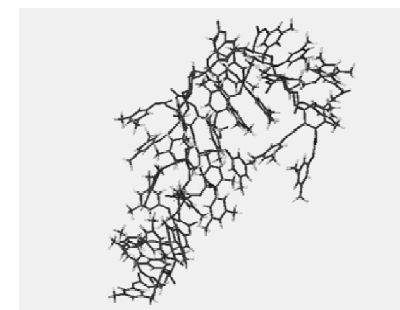


### Immobilized polysaccharides:

Amylose  
or  
Cellulose



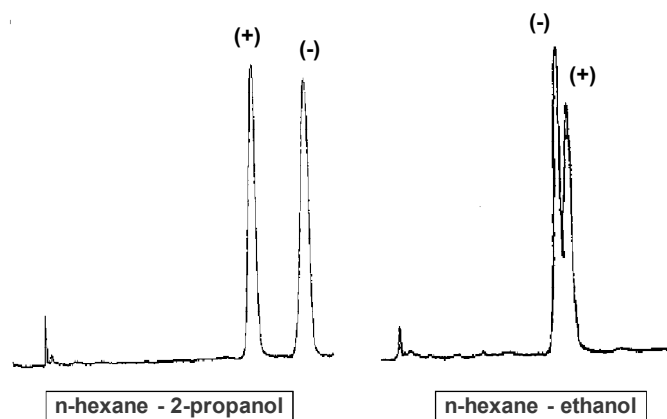
tribenzoate  
tris phenylcarbamate  
triacetate



# Separation Modes in HPLC

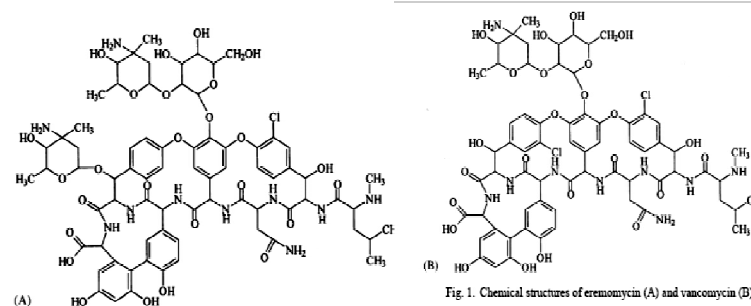
## Short Overview

(+) and (-)-  $\Delta^6$ -THC IN TWO TYPES OF MOBILE PHASES:  
REVERSAL OF ELUTION ORDER WITH THE CHANGE OF MODIFIER



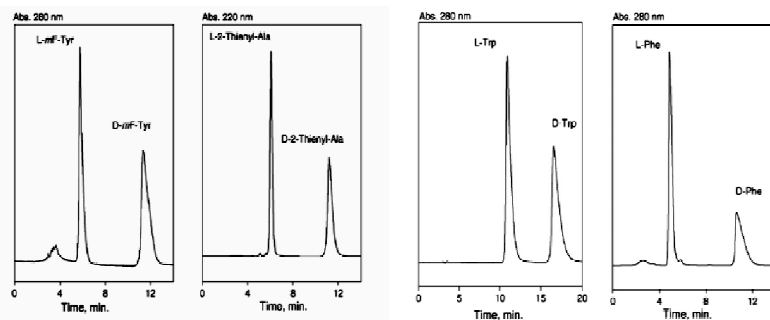
Immobilized Amylose Column

macrocyclic glycopeptide  
antibiotic eremomycin chemically bonded to silica



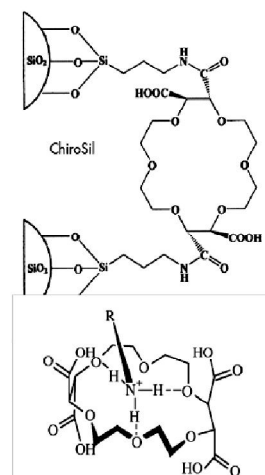
Journal of Chromatography A, 1108 (2006) 263–266

Separation of enantiomers of amino acids on eremomycin CSP. Column



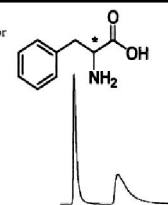
250mm4.0' mm. Eluent: methanol-0.1M NaH<sub>2</sub>PO<sub>4</sub> (20:80, v/v), 0.7 ml/min.

Crown Ether Type of Chiral Stationary Phase



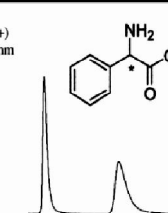
Phenylalanine

Phenylalanine  
Column: ChiroSil® RCA(+) or  
SCA(-) 15 cm x 4.6 mm  
Mobile Phase: (70/30)  
CH<sub>3</sub>OH/H<sub>2</sub>O  
+10 mM Acetic acid  
Flow Rate: 1.5 mL/min  
Detection: UV 210 nm  
Run Time: 8.9 min  
k<sub>1</sub>: 2.66  
 $\alpha$ : 2.57



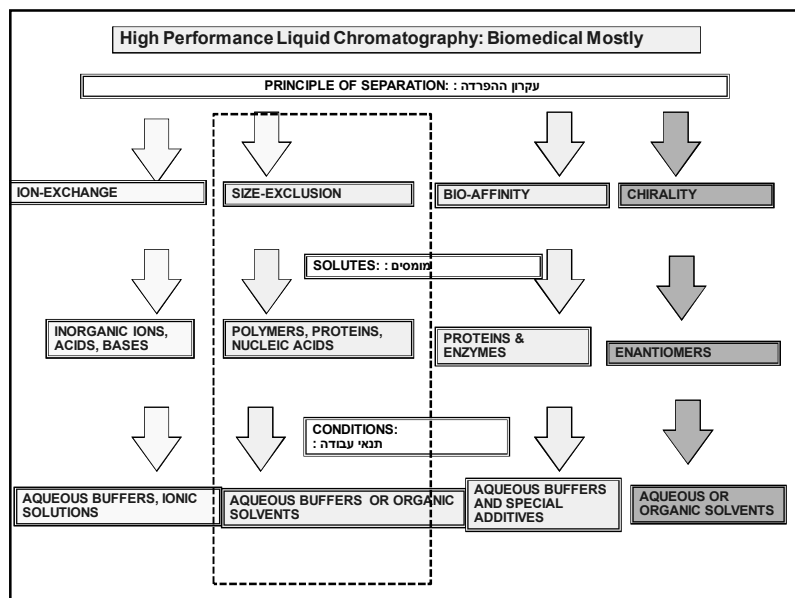
Phenylglycine

Phenylglycine  
Column: ChiroSil® RCA(+) or  
SCA(-) 15 cm x 4.6 mm  
Mobile Phase: (70/30)  
CH<sub>3</sub>OH/H<sub>2</sub>O  
+10 mM H<sub>2</sub>SO<sub>4</sub> and  
0.1% TEA  
Flow Rate: 1.0 mL/min  
Detection: UV 210 nm  
Run Time: 13.1 min  
k<sub>1</sub>: 3.14  
 $\alpha$ : 2.60



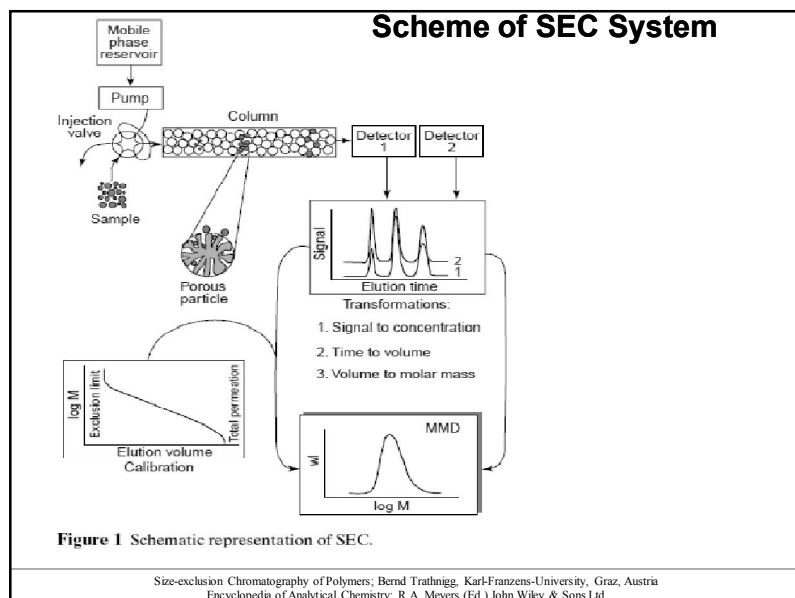
# Separation Modes in HPLC

## Short Overview



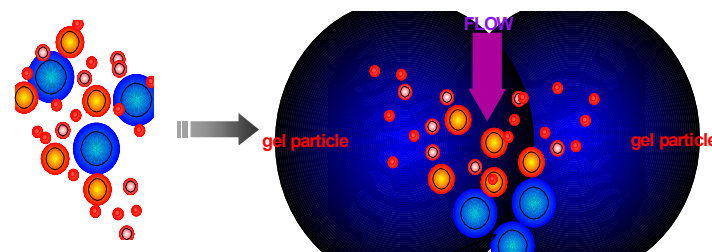
## Size Exclusion Chromatography Basics

- SEC known by many names
  - Gel Permeation Chromatography (GPC)
  - Size Exclusion Chromatography (SEC)
  - Gel filtration Chromatography (GFC)
- Separation is based on size exclusion
  - Actual behavior in solution, not molecular weight.
- All other interactions with the stationary phase (ion exchange, hydrophobic interaction) should be eliminated



## GPC Separation Process

- A dissolved polymer (comprised of a mixture of molecules) passes through a porous gel-based stationary phase
- The gel pores may be of uniform size or a blend of mixed sizes depending upon the column(s) chosen...

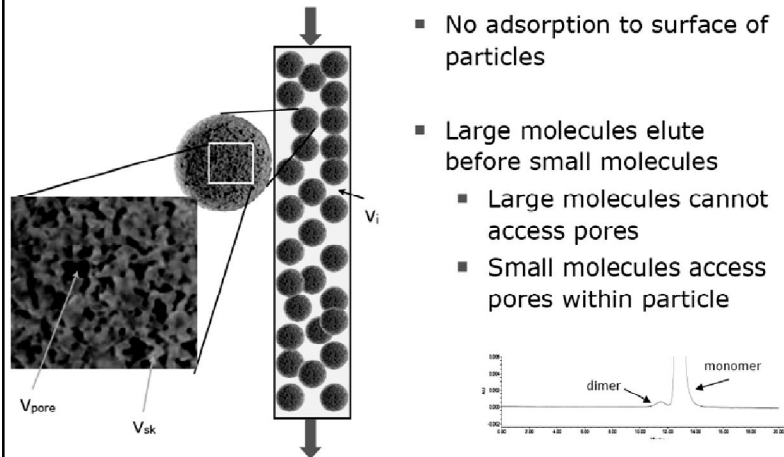


Larger macromolecules are not slowed down as they are too big to interact with the pores so elute first.

# Separation Modes in HPLC

## Short Overview

### Size Exclusion Separation of Proteins



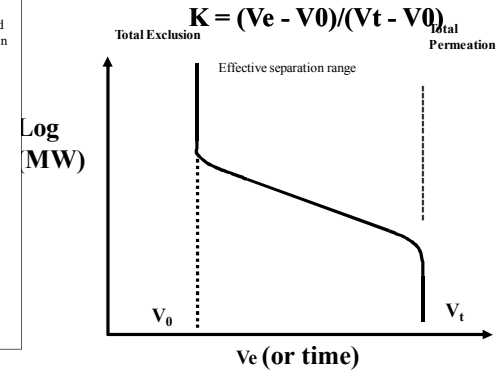
### Principles of Retention/Elution

- Simple relation between MW and elution volume/time.
- MW above the upper cutoff limit: all compounds are eluted at  $V_0$ 
  - -> Total Exclusion
- MW under the lower cutoff limit: all compounds are eluted at  $V_t$ 
  - -> Total Permeation

To calibrate a gel filtration column, a series of proteins with known molecular weights is passed through it, and the elution volume of each protein is measured.

A value,  $K$ , can be calculated for each, using the formula:  
 $K = (V_e - V_0)/(V_t - V_0)$   
 where  
 $V_e$  = elution volume  
 $V_t$  = total column void volume  
 $V_0$  = void volume outside the porous beads

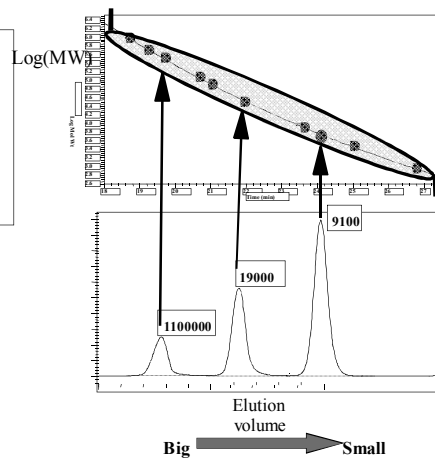
$K = 0$   
 for molecules too large to enter the pores  
 $K = 1$   
 for molecules that can enter the pores unhindered



### GPC Calibration Narrow Standards (Markers)

To build a calibration curve:

- Narrow dispersity standards (Polydispersity < 1.1)
- Elution volume at peak height
- Curve :  $\text{Log}(M) = f(V_e)$



### Liquid Chromatography Protein Separation Modes

#### Protein Structure

Primary,  
Secondary,  
Tertiary  
Structure

Carbohydrate  
Groups

Hydrophobic  
Regions

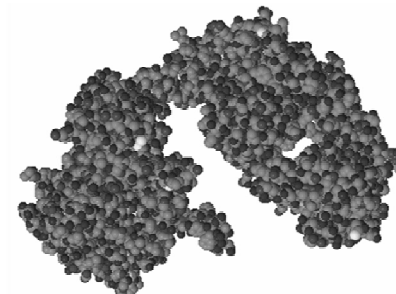
Disulfide  
Linkages

Net Charge

Hydrophilic  
Groups

Aromatic  
Groups

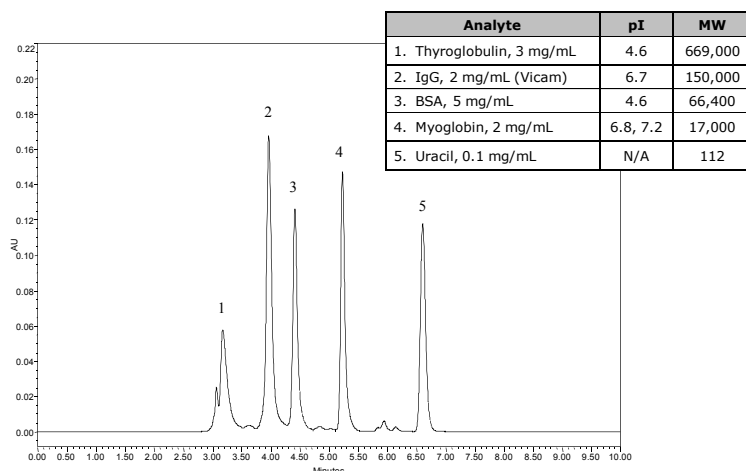
Hydrogen  
Bonding



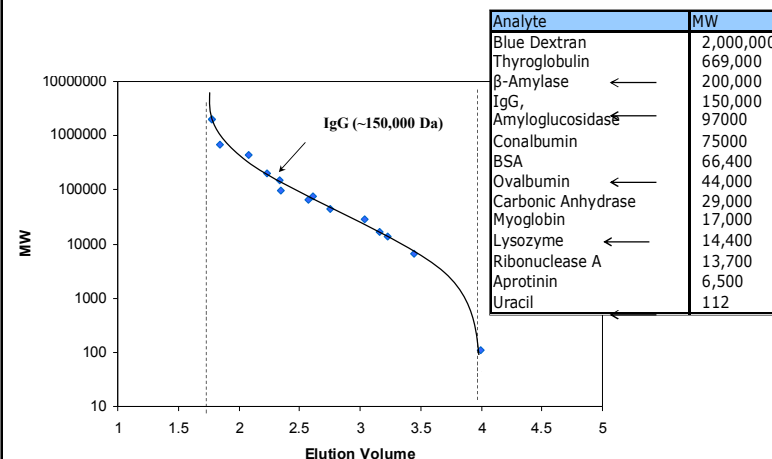
# Separation Modes in HPLC

## Short Overview

### Our Experiment: BEH200 SEC, 1.7um Column Markers

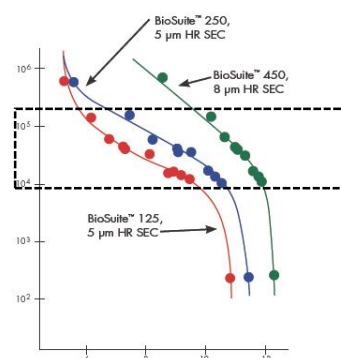


### BEH200 SEC, 1.7um Calibration Curve

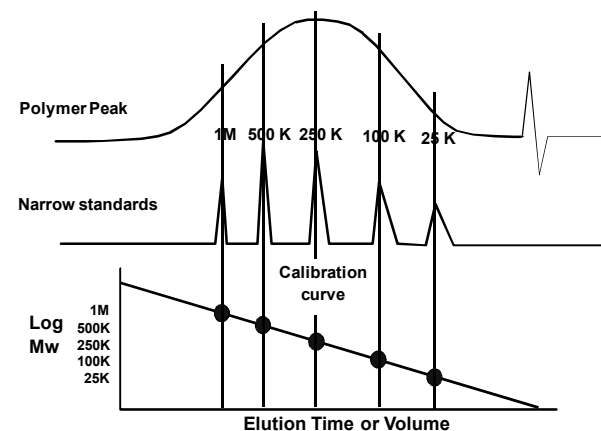


### Calibration Curves of Various Columns

- Typically log Molecular Weight versus retention volume
- Linear portion is where the best separation is achieved
- The boxed area shows the MW range for all three columns (some ranges may exceed this area)



### Gel Filtration/Size Exclusion/Gel Permeation



# Separation Modes in HPLC

## Short Overview

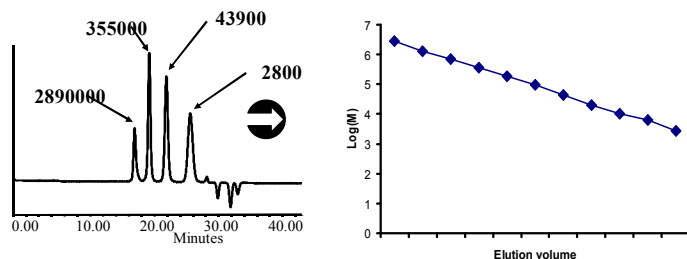
### Principles of Calculations

Step 1

**Processing standards :** ➡

Elution volume is obtained from data. –

Plot of log (M) versus f (elution volume). –

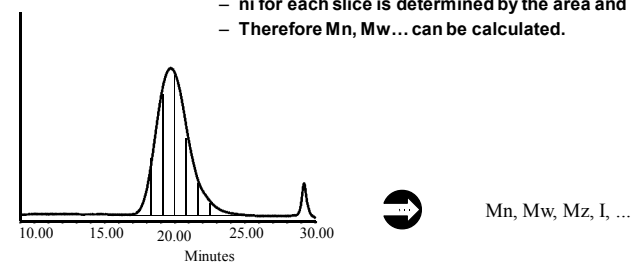


### GPC, Principles of Calculations

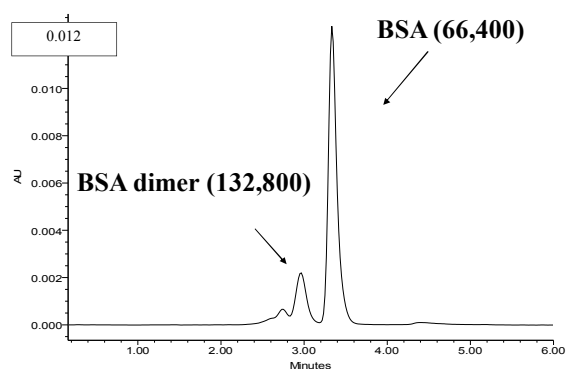
Step 2

➡ Processing unknowns :

- The signal is cut in slices (width dt).
- Each slice is characterized by :
  - » Elution volume,  $V_i$ .
  - » Area  $H_i \cdot dt = n_i M_i$ .
- With the calibration curve  $V_i$  gives the MW in each slice,  $M_i$ .
- $n_i$  for each slice is determined by the area and  $M_i$ .
- Therefore  $M_n, M_w, \dots$  can be calculated.



### Example for a Narrow Unknown



### Principles of Molecular Weight Distribution

• Definition of MW averages :

- A polymer is made of species (chains) of varying lengths. Each chain is characterized by its molecular weight,  $M_i$ , and its abundance  $n_i$ . Then :

$$M_n = \frac{\sum n_i M_i}{\sum n_i} \quad M_z = \frac{\sum n_i M_i^3}{\sum n_i M_i^2} \quad I = \frac{M_w}{M_n}$$

$$M_w = \frac{\sum n_i M_i^2}{\sum n_i M_i} \quad M_{z+1} = \frac{\sum n_i M_i^4}{\sum n_i M_i^3}$$

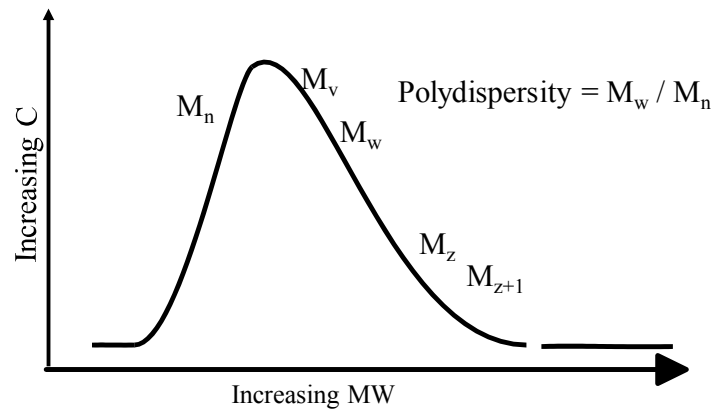
$M_n < M_w < M_z < M_{z+1}$

- Most of these values are necessary to characterize the polydispersity
- These values can be calculated by different techniques but SEC is the most convenient and widely used method.

# Separation Modes in HPLC

## Short Overview

### Molecular Weight Distribution



### Clinical Applications

Test of haemoglobin variant for Thalassemia

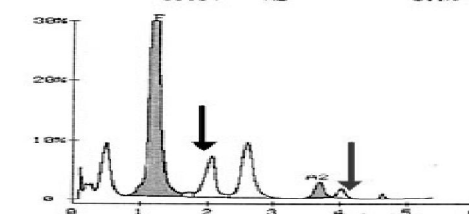
אנליזה של המוגלובין וריאנטים עבור תלסמיה

```

XXXX Beta Thal Short 00405-A XXXX
DATE:02-01-00 TIME:10:44:20

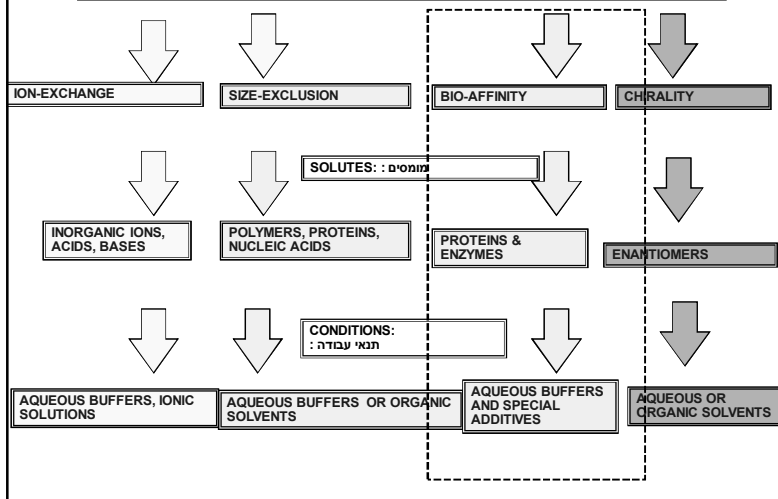
TECH ID# 1
VIAL# 0
SAMPLE ID# 00000000000000000000
REP # 01/03

ANALYTE ID % TIME AREA
F 69.0 1.22 886001
P3 0.9 1.72 10729
Unknown 1 9.1 2.06 111065
A0 13.7 2.58 169255
A2 3.1 3.68 34102
D-WINDOW 1.3 4.01 15002
TOTAL AREA 1227724
    
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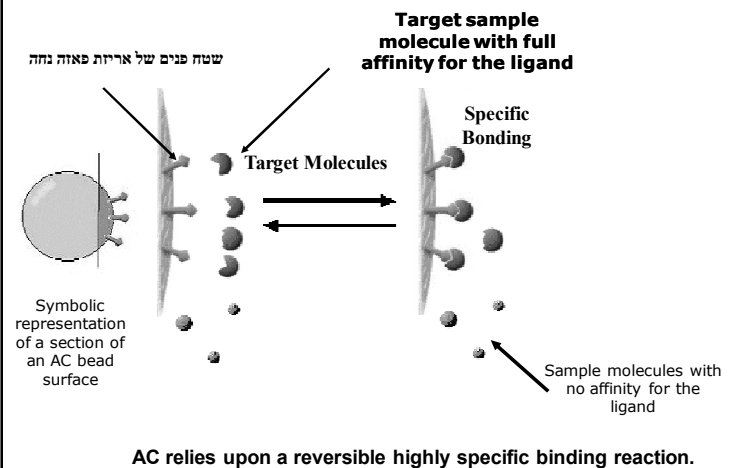


### High Performance Liquid Chromatography: Biomedical Mostly

PRINCIPLE OF SEPARATION: עקרון ההפרדה:



### Affinity Chromatography (AC)



# Separation Modes in HPLC

## Short Overview

### Affinity Chromatography

#### 1. Equilibration

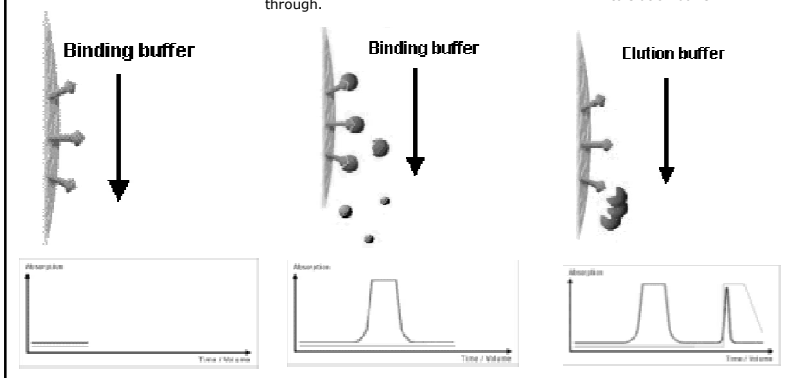
The column is conditioned to promote adsorption of the target molecule by equilibrating it with *binding buffer*.

#### 2. Sample application and wash

The sample is applied under binding conditions. The target molecule binds specifically to the affinity ligands, while all other sample components are washed through.

#### 3. Elution

The target molecule is desorbed and eluted by switching to *elution buffer*.

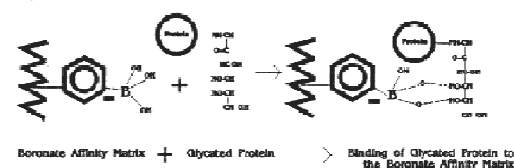


### Glycohemoglobin in Blood

Glycated proteins differ from non-glycated proteins by the attachment of a sugar moiety(s) at various binding sites by means of a ketoamine bond. Glycohemoglobin (GHb) thus contains 1,2-cis-diol groups not found in non-glycated proteins.

These diol groups provide the basis for separation of glycated and nonglycated components by boronate-affinity chromatography (1–3). In this analytical technique, a boronate such as phenylboronic acid is bonded to the surface of the column support.

#### Affinity Binding of Glycated Protein



### Hydrophobic Interaction Chromatography (HIC)

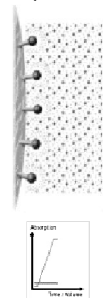
Slightly hydrophobic sample component.

Reasonable hydrophobic sample component.

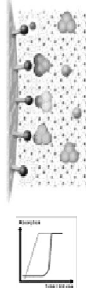
Quite hydrophobic sample component.

Highly hydrophobic contaminant.

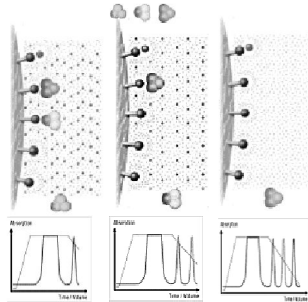
#### 1. Equilibration.



#### 2. Sample application and wash.



#### 3. Gradient elution. Elution order:



#### 4. Re-equilibration.



### 2 Dimension Chromatography

