

High Performance Liquid Chromatography - HPLC

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

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Medtechnica

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

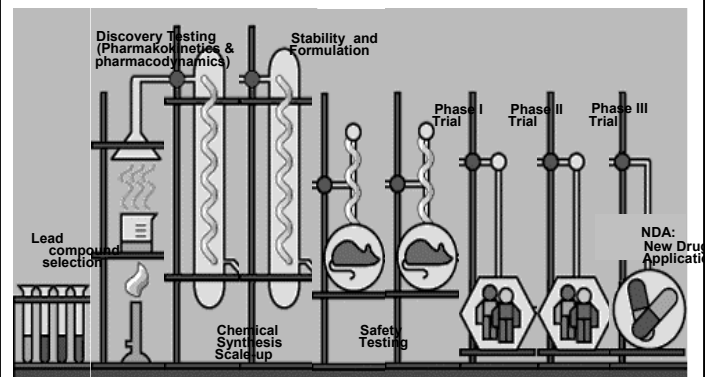
Hewlett-Packard liquid chromatography

High performance liquid chromatography

Hocus pocus liquid chromatography

High patience liquid chromatography

HPLC in Pharmaceuticals Quantitative Analytical Technique No 1





Dr. Shulamit Levin, Medtechnica

High Performance Liquid Chromatography - HPLC


APPLICATIONS OF HPLC


Veterinary






Environmental






Biomedical and Clinical

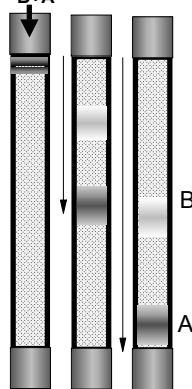


Chemistry

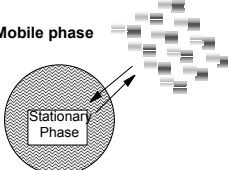


Agriculture & Food

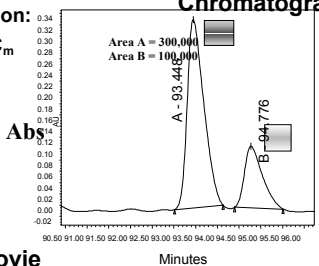
התהליך הכרומטוגרפי



Elution through the Column-movie



Distribution:
 $K = C_s / C_m$



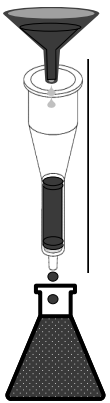
Chromatogram

Area A = 300,000
Area B = 100,000

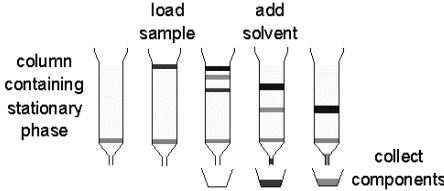
A - 93.44
B - 94.776

Absorbance vs. Time (Minutes)

Low Performance Liquid Chromatography



column containing stationary phase




load sample add solvent collect components


Gravitational Chromatography

HPLC System Components

Control & Data Processing

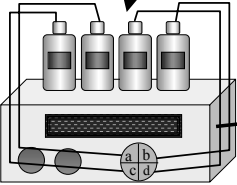


Detector
גלאי

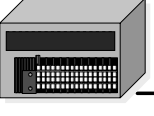


Waste

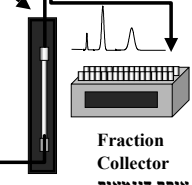
Pump - משאבה
flows 50-5000µL/min



Auto Sampler
דוגם אוטומטי

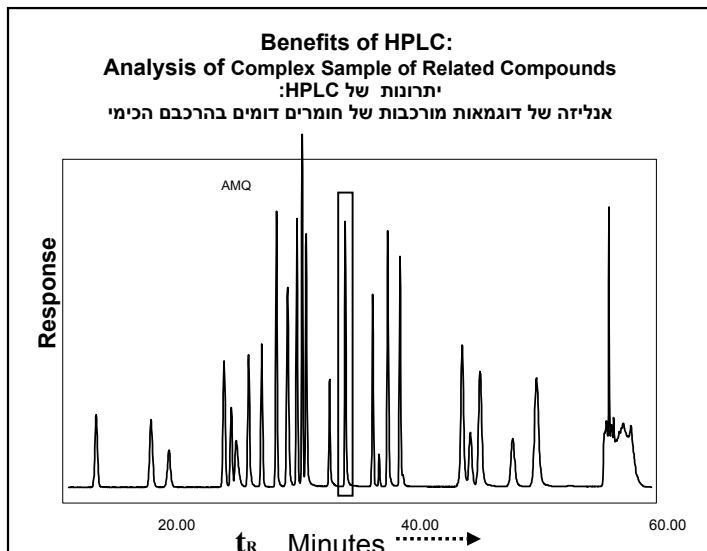
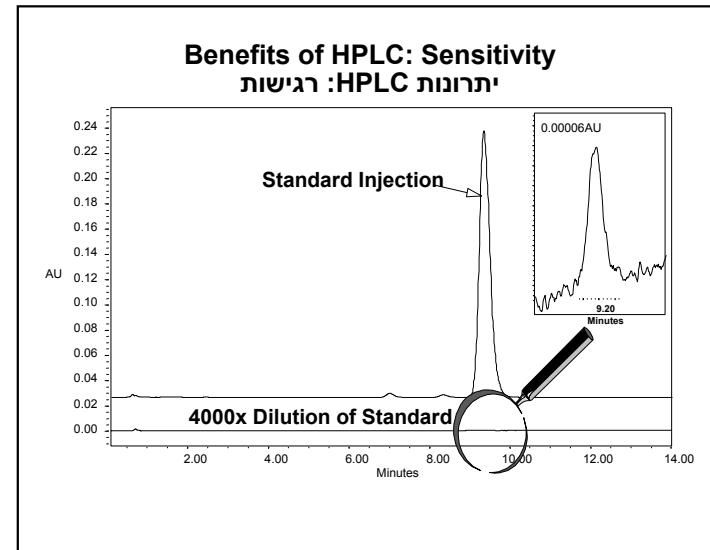
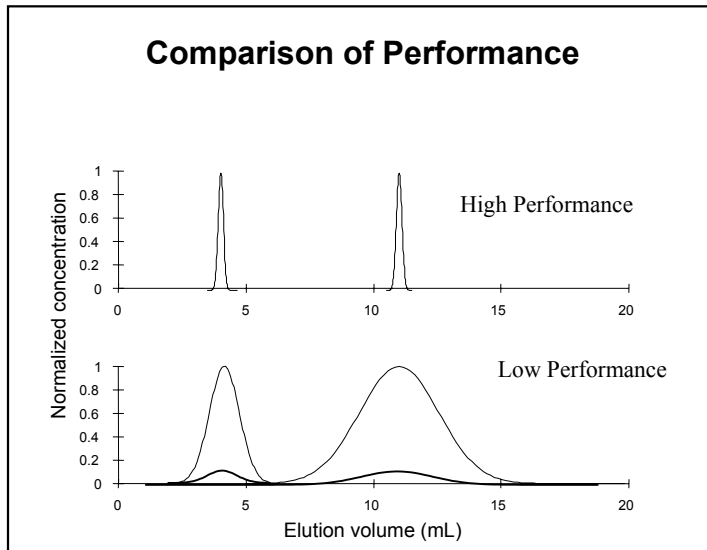


HPLC Column in Oven
עמודה כרומטוגרפית בתוך תנור



Fraction Collector
איסוף דוגמאות

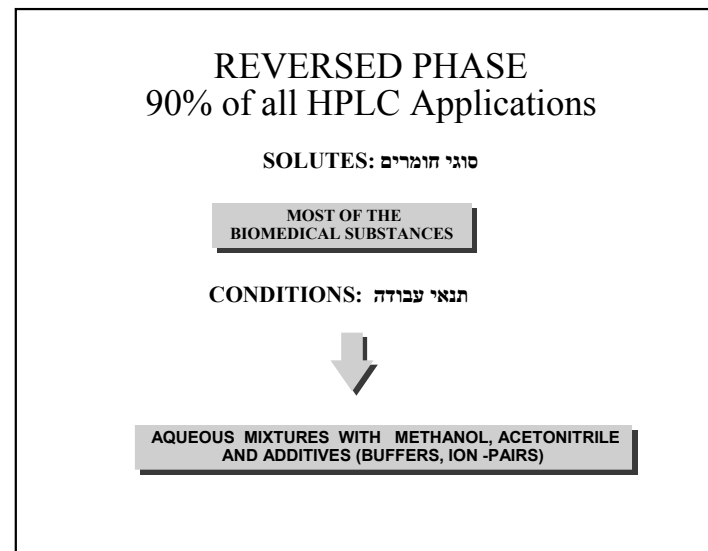
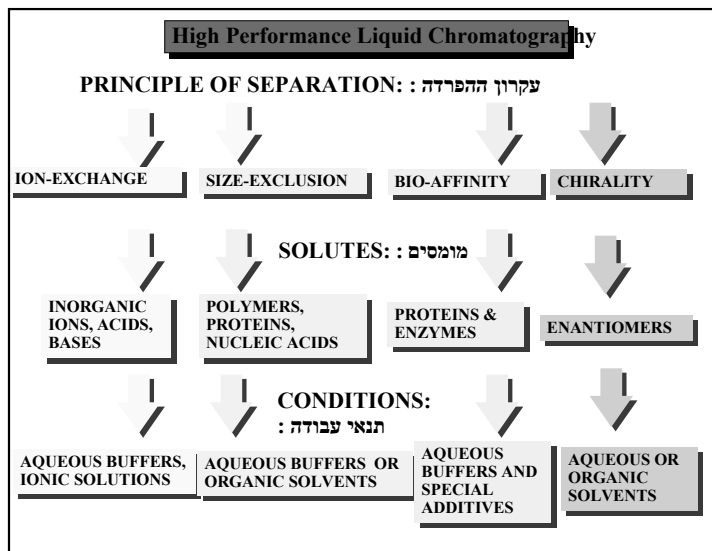
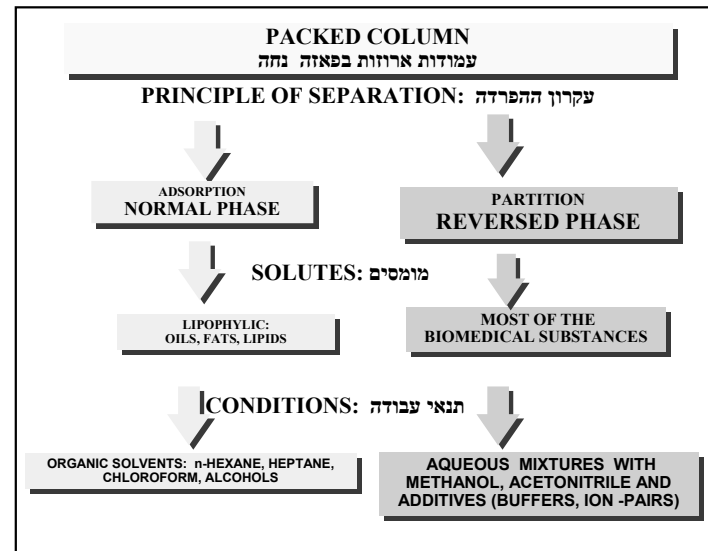
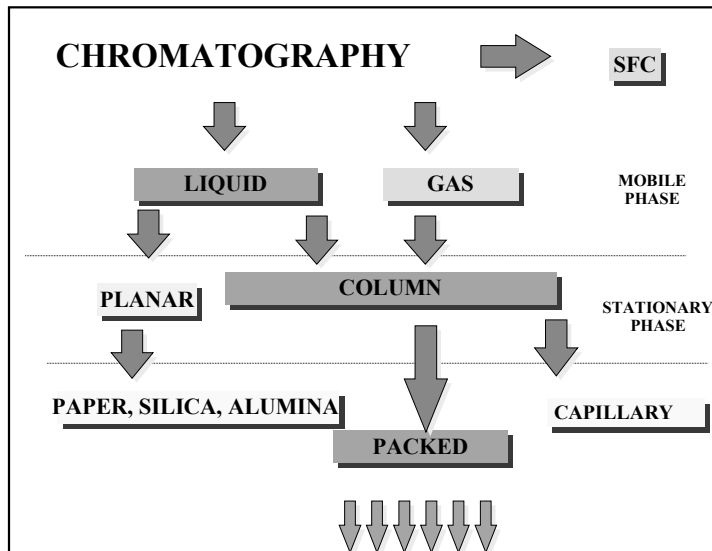
High Performance Liquid Chromatography - HPLC



HPLC COURSE LAYOUT

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

High Performance Liquid Chromatography - HPLC



High Performance Liquid Chromatography - HPLC

Chromatographic Process: התהליך הכרומטוגרפי
Reversed Phase

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

Hydrophobic Stationary Phase

Distribution:
 $K = C_s / C_m$

Migration through the Column
 מסע דרך העמודה

Chromatogram כרומטוגרמה

Ionizable Molecules מולקולות מיוננות

AMINES - 1,2,3,4

CARBOXYLIC ACIDS

ALCOHOLS

PHOSPHATES

PHOSPHONATES

SULPHATES

SULPHONATES

THIOLS

Clinical Applications יישומים רפואיים

Chronic Alcohol-Abuse

Monitoring Oxidative Stress

Vitamin Profiling

Risk Factor for Arterio-sclerosis

Osteoporosis Diagnosis

Biogenic Amines

Therapeutic Drug Monitoring (TDM)

Occupational Medicine

Porphyrin Profiling

Hemoglobin-Testing

6PLUS1 Multilevel Calibrator Sets

HPLC-Instruments & Software

MOBILE PHASE מאזה נעה

SOLVENTS:
 water, methanol, acetonitrile

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

STATIONARY PHASE מאזה נחה

CHEMISTRY:

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

GEOMETRY

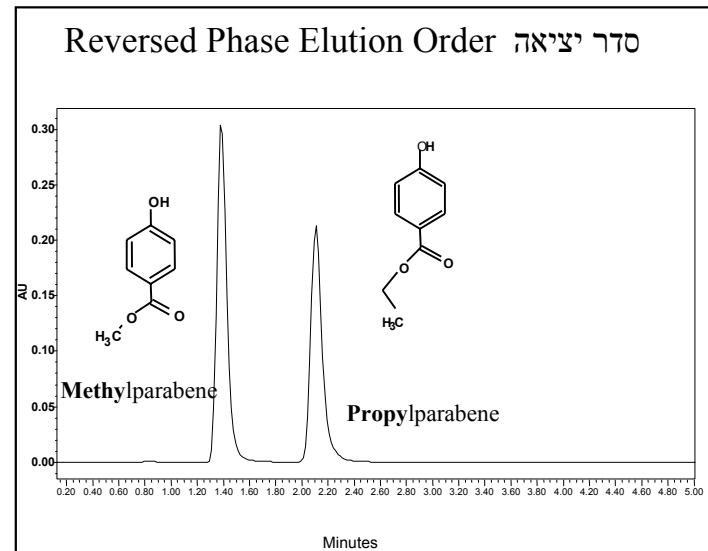
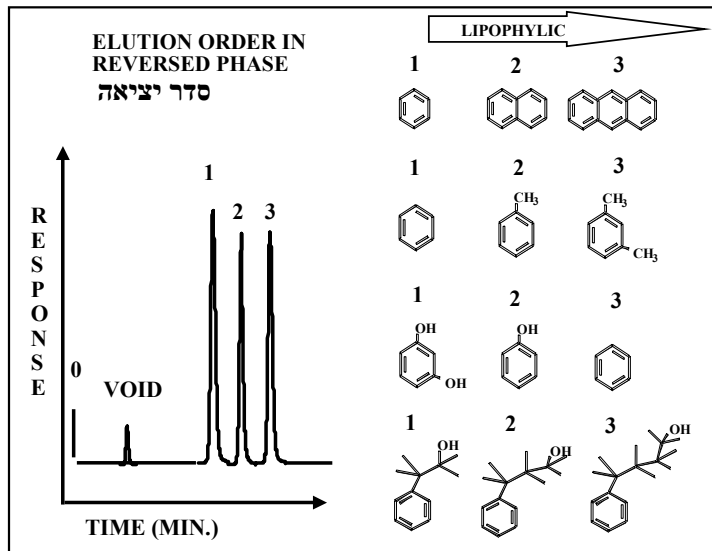
- * SPHERE-IRREGULAR
- * PARTICLE DIAMETER
- * POROSITY

silica

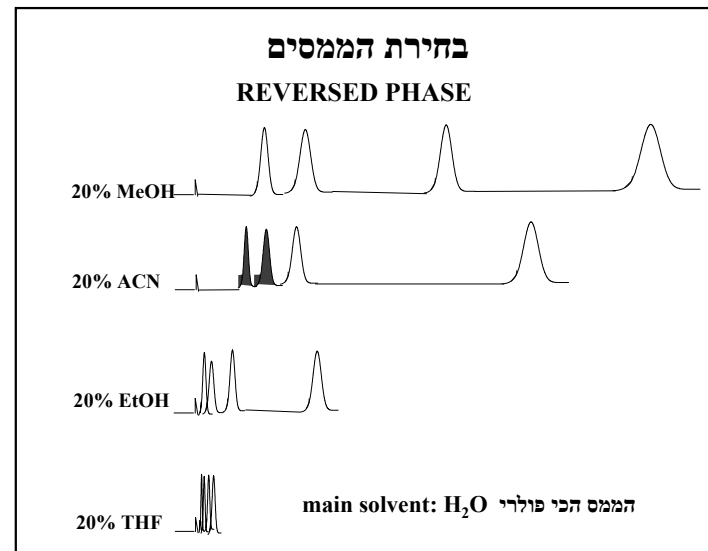
pores

d

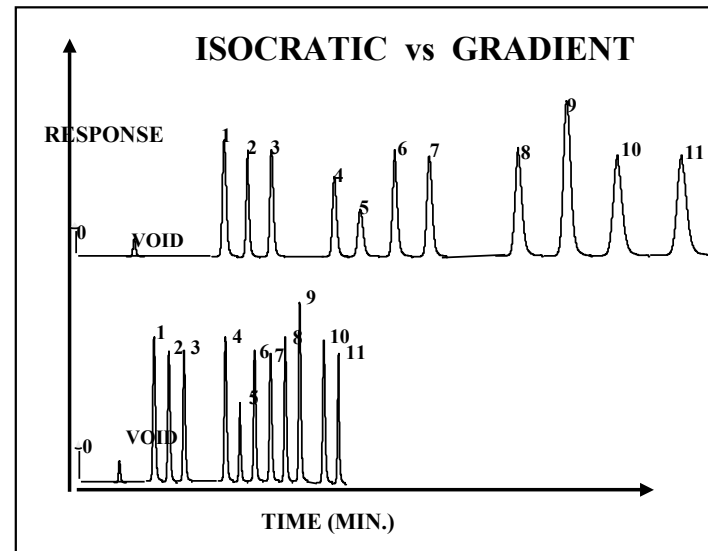
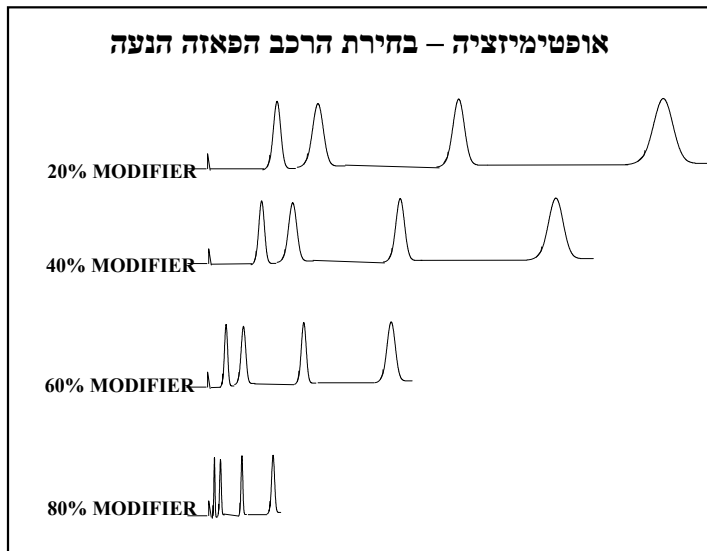
High Performance Liquid Chromatography - HPLC



- MOBILE PHASE**
פאזה נעה
1. TYPE OF MODIFIER (MeOH, ACN)
 2. MOBILE PHASE COMPOSITION (% modifier)
 3. pH
 4. TYPE OF BUFFER (phosphate, acetate)
 5. IONIC STRENGTH (Salts, buffer concentration)
 6. ION-PAIRING REAGENTS (alkyl-amines, -sulfonates)



High Performance Liquid Chromatography - HPLC



Stationary Phase Properties
תכונות הפאזה הנחה

CHEMISTRY:

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

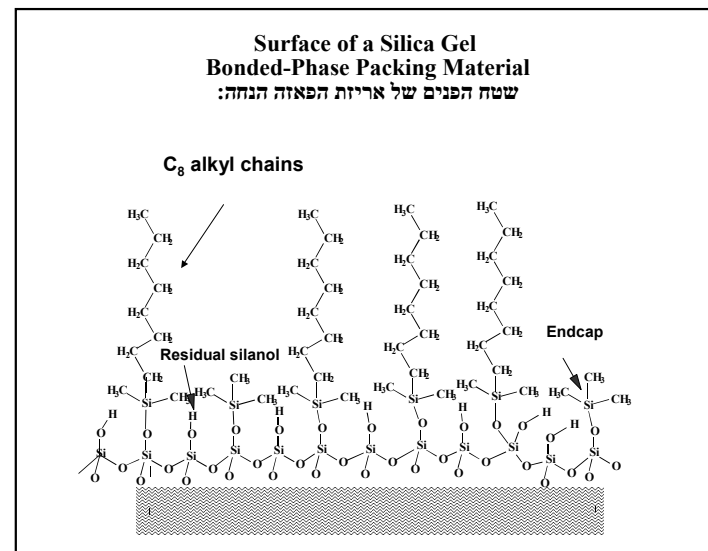
GEOMETRY

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

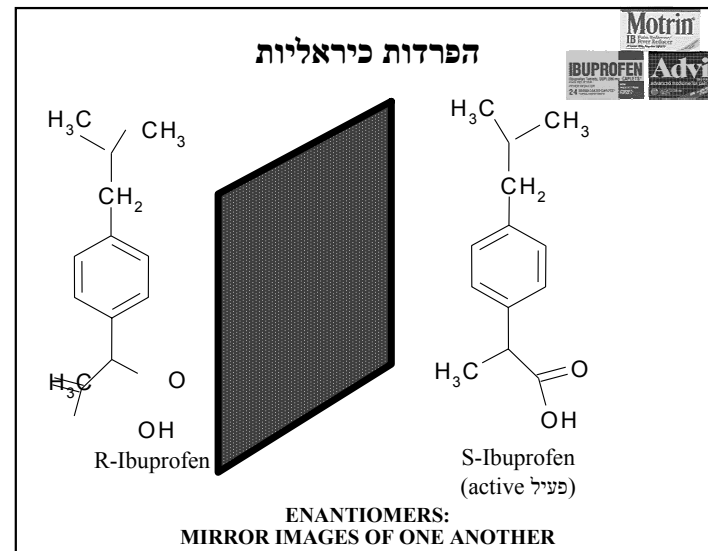
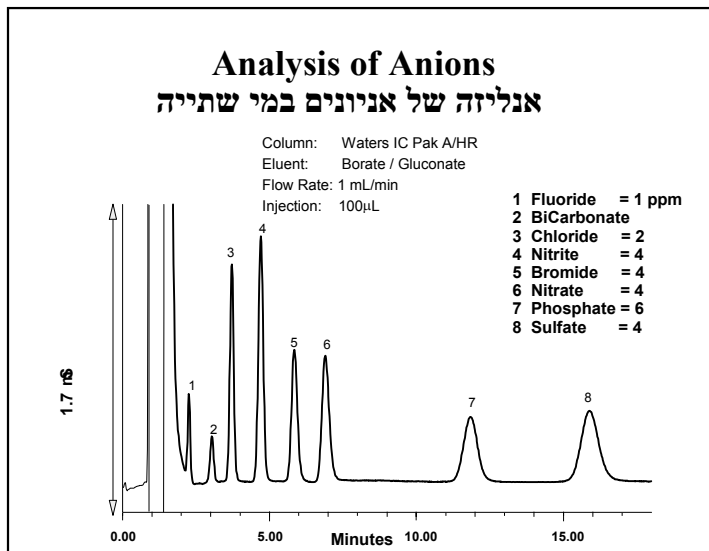
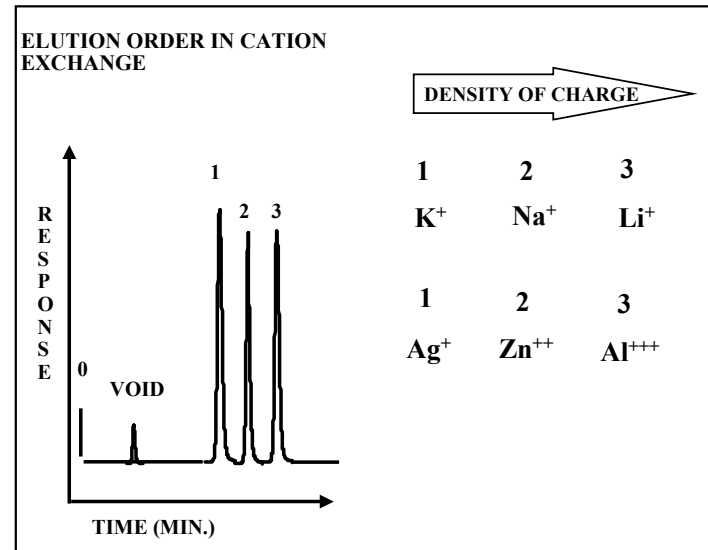
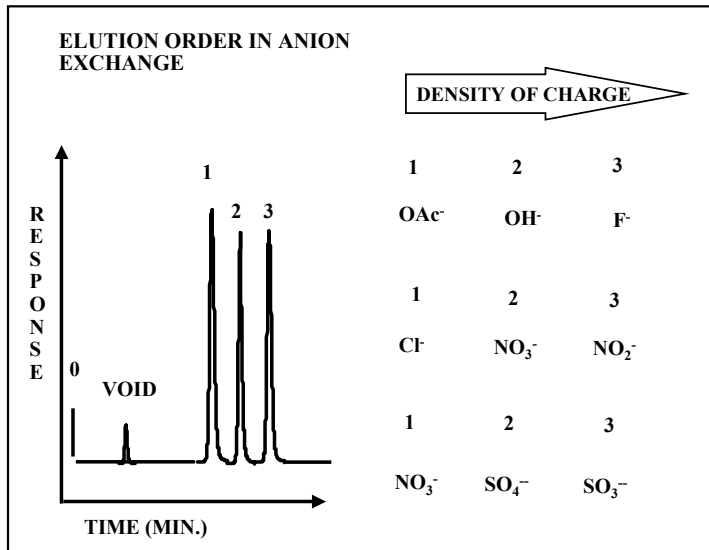
pores

silica

d

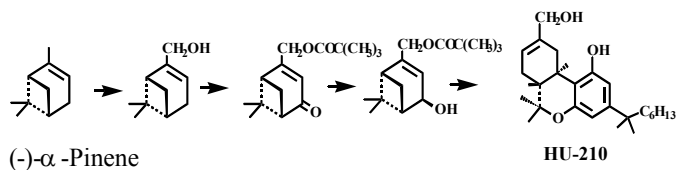
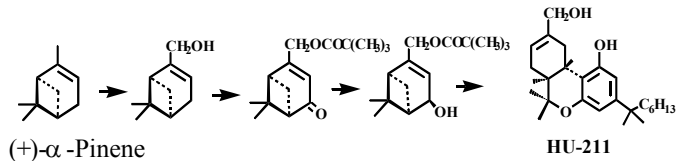


High Performance Liquid Chromatography - HPLC



High Performance Liquid Chromatography - HPLC

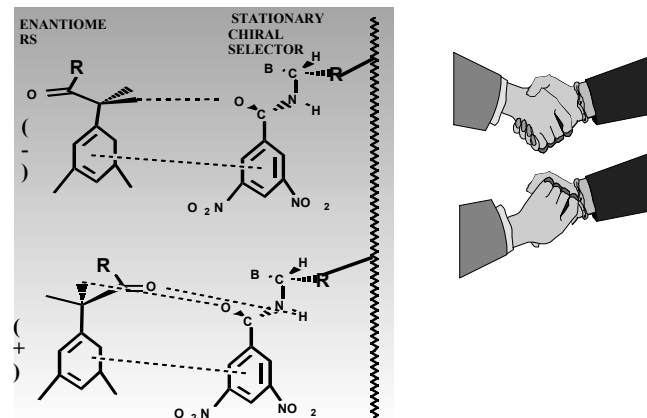
Asymmetric Synthesis סינתזה שומרת-כיראליות



Journal of Chromatography A, 664 (1994) 159-167

BASIS FOR SEPARATION: CHIRAL RECOGNITION

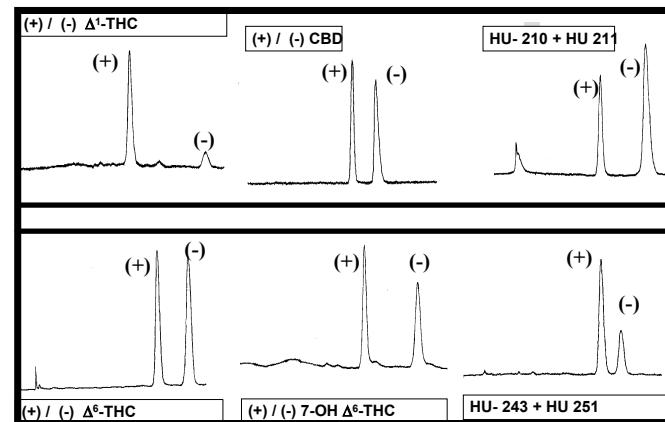
עקרונות ההפרדה: הכרה כיראלית על פני שטח הפנים של הפאזה הנחה



Chiral stationary phases:

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

SEPARATION OF 6 ENANTIOMERIC PAIRS OF CANNABINOIDS



High Performance Liquid Chromatography - HPLC

SIZE EXCLUSION CHROMATOGRAPHY Gel Permeation Chromatography -GPC

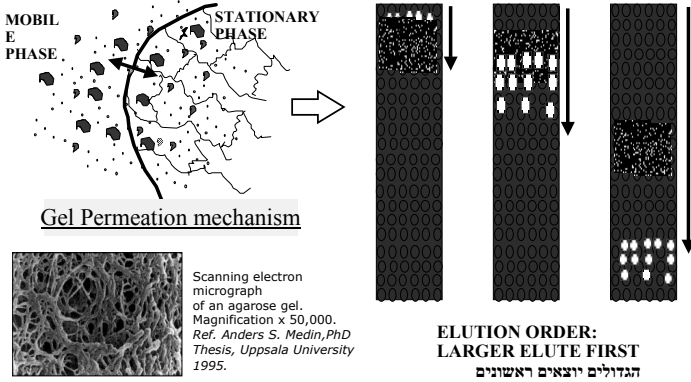
עקרון ההפרדה - דחיקה על פי גודל המומס

תהליך ההפרדה

MOBILE PHASE

STATIONARY PHASE

Gel Permeation mechanism



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

ELUTION ORDER:
LARGER ELUTE FIRST
הגדולים יוצאים ראשונים

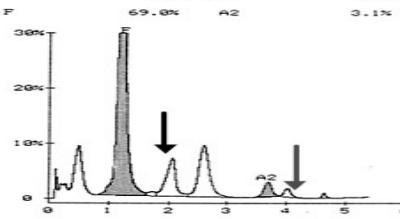
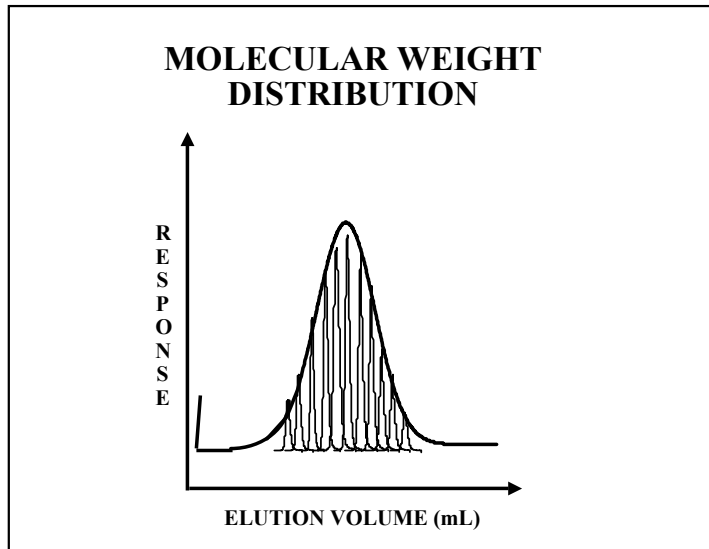
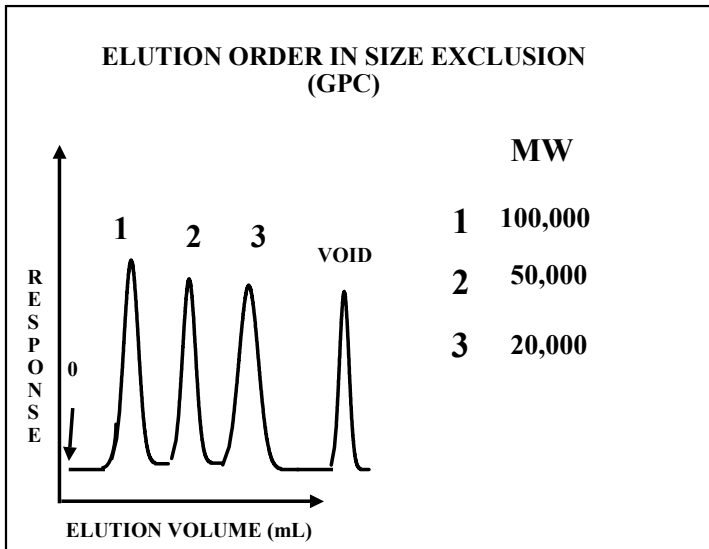
Clinical Applications

Test of haemoglobin variant for Thalassemia

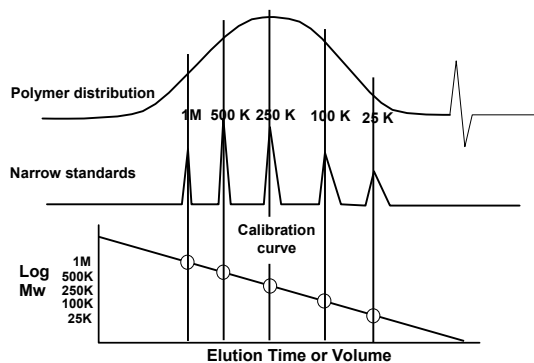
אנליזה של המוגלובין וריאנטים עבור טלסמיה

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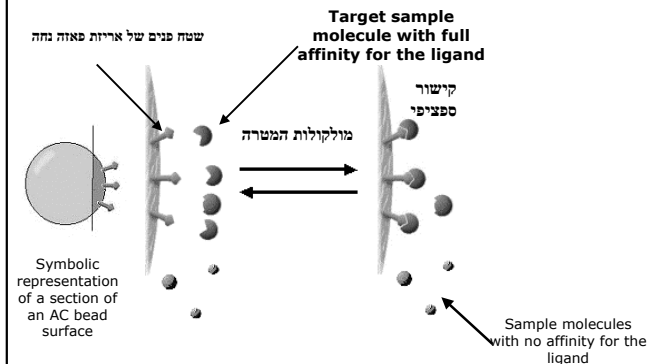
    **** Beta Thal Short 00405-A ****
    DATE:02/01/08          TIME:10:44:23
    TECH ID# 1
    VIAL# 0
    SAMPLE ID# 00000000000000000000
    REP # 01/03
    ANALYTE ID % TIME AREA
    F 69.0 1.22 886001
    P3 0.9 1.72 10725
    Unknown 1 9.1 2.86 111865
    A0 13.7 2.58 169225
    AC 3.1 3.68 34102
    D-WINDOW 1.3 4.01 15802
    TOTAL AREA 1227724
    F 69.0% A2 3.1%
    
```

Gel Filtration/Size Exclusion/Gel Permeation



Affinity Chromatography (AC) זיקה ביולוגית (AC)



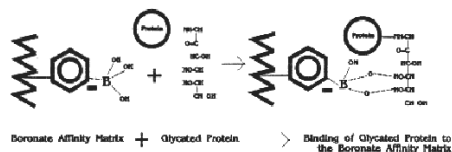
AC relies upon a reversible highly specific binding reaction.

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.

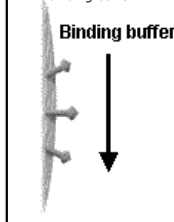
Affinity Binding of Glycated Protein



Affinity Chromatography

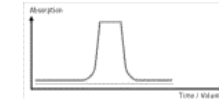
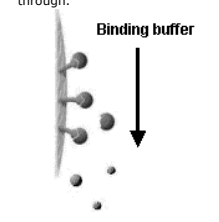
1. Equilibration

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



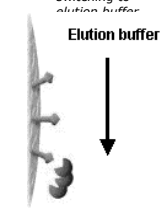
2. Sample application and wash

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.



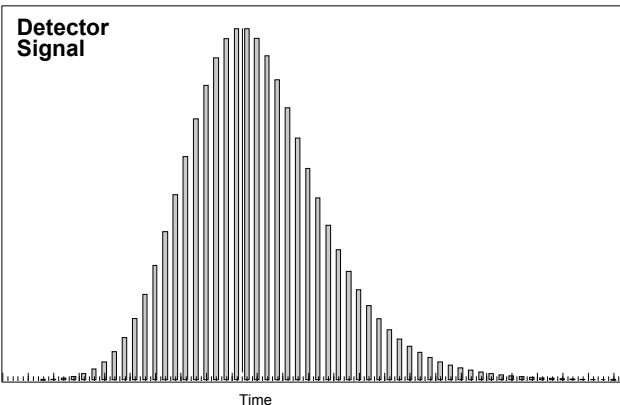
High Performance Liquid Chromatography - HPLC

HPLC COURSE LAYOUT

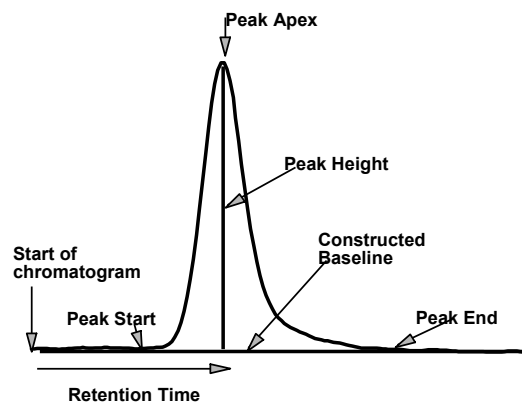
- Introduction & Applicability
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Measurement of Area - Integration

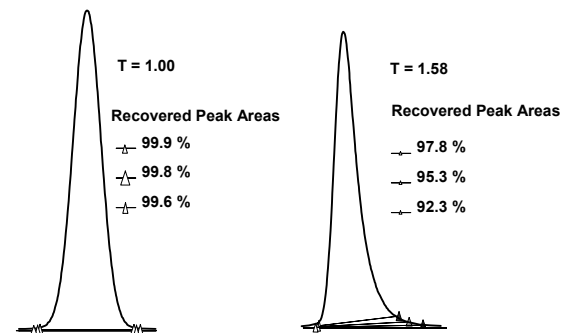
$$\text{Area} = \int \text{Abs} \times dt$$



Peak Detection



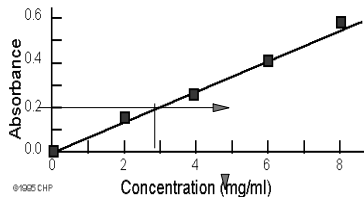
Integration Errors Caused by Tailing



High Performance Liquid Chromatography - HPLC

Working Curve

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

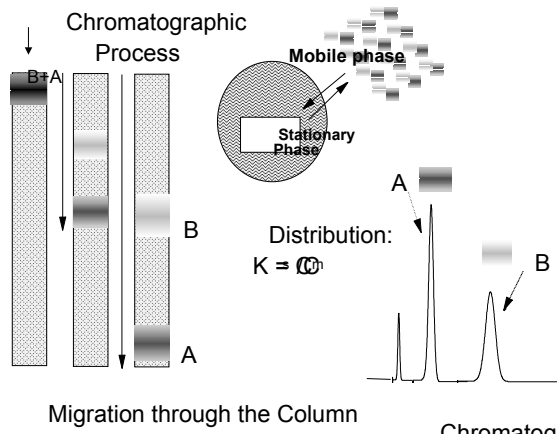


The working curve are then used to determine the concentration of an unknown sample to calibrate the linearity of an analytical instrument.

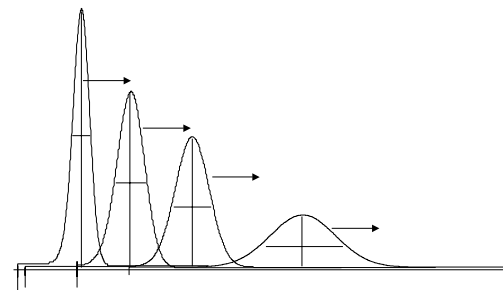
Quality Control – ביקורת איכות

VIAL	SAMPLE NAME	INJ VOL	No of Inj	Function	Method	Run Time	Sample Weight	Dilution
1	Blank	20.0	1	Inject Samples	.C Demo Method Set	10.00	1.00000	1.00000
2	System Suitability	20.0	1	Inject Samples	SST Method Set	10.00	1.00000	1.00000
				Clear Calibration	.C Demo Method Set			
3	Std1	20.0	5	Inject Standards	.C Demo Method Set	10.00	1.00000	1.00000
4	Std2	20.0	2	Inject Standards	.C Demo Method Set	10.00	1.00000	1.00000
				Report	.C Calibration Report			
				Report	Standard Comparison			
				Clear Calibration	.C Demo Method Set			
1	Std1	20.0	1	Inject Standards	.C Demo Method Set	10.00	1.00000	1.00000
2	Unk.1	20.0	2	Inject Samples	.C Demo Method Set	10.00	1.00000	1.00000
3	Unk.2	20.0	2	Inject Samples	.C Demo Method Set	10.00	1.00000	1.00000
4	Unk.3	20.0	2	Inject Samples	.C Demo Method Set	10.00	1.00000	1.00000
5	Unk.4	20.0	2	Inject Samples	.C Demo Method Set	10.00	1.00000	1.00000
6	Unk.5	20.0	2	Inject Samples	.C Demo Method Set	10.00	1.00000	1.00000
7	Unk.6	20.0	2	Inject Samples	.C Demo Method Set	10.00	1.00000	1.00000
1	Std1	20.0	1	Inject Standards	.C Demo Method Set	10.00	1.00000	1.00000
				Clear Calibration	.C Demo Method Set			
				Calibrate	.C Demo Method Set			

פיקים כרומוטוגרפיים מתרחבים תוך כדי המעבר דרך העמודה



PEAK BROADENING DUE TO DIFFUSION



High Performance Liquid Chromatography - HPLC

k' = Capacity Factor = Measure of Retention



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

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

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

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

PERFORMANCE CRITERIA BY ONE PEAK

RETENTION FACTOR or CAPACITY RATIO

$$k' = \frac{t_R - t_0}{t_0} = \frac{V_R - V_m}{V_m} = \frac{C - C_m}{C_m}$$

ASYMMETRY FACTOR

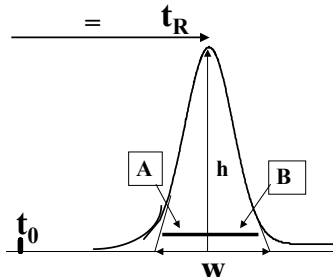
$$A_s = \frac{b}{a} = \frac{h_0}{h_1}$$

TAILING FACTOR

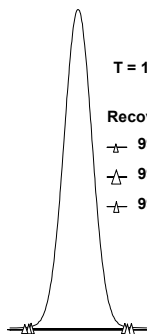
$$T_f = \frac{A}{B} = \frac{h}{h_0}$$

NUMBER OF THEORETICAL PLATES

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



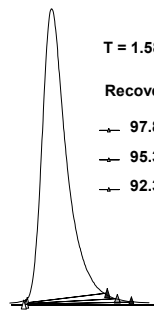
Integration Errors Caused by Tailing



$T = 1.00$

Recovered Peak Areas

- 99.9 %
- 99.8 %
- 99.6 %



$T = 1.58$

Recovered Peak Areas

- 97.8 %
- 95.3 %
- 92.3 %

PERFORMANCE BY TWO PEAKS

SELECTIVITY FACTOR

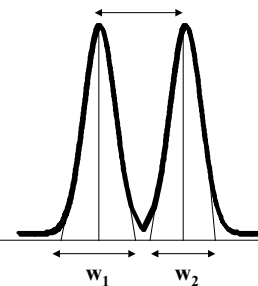
$$\alpha = \frac{k'(2)}{k'(1)}$$

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

EXPERIMENTAL RESOLUTION

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

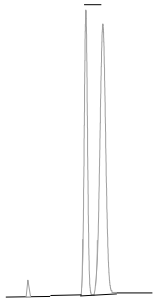
t_0



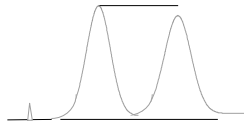
High Performance Liquid Chromatography - HPLC

SELECTIVITY vs EFFICIENCY

$$R_s = \frac{t_R(2)^t R(1)}{1/2(w_1 + w_2)} \text{ same in both cases}$$



LOW SELECTIVITY (α)
HIGH EFFICIENCY (N)



HIGH SELECTIVITY (α)
LOW EFFICIENCY (N)

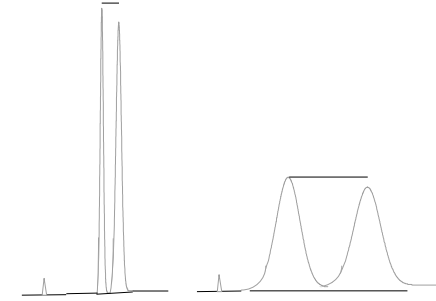
ADVANTAGES OF HIGH EFFICIENCY

Resolution

Peak capacity

Sensitivity

HIGH PERFORMANCE LOW PERFORMANCE

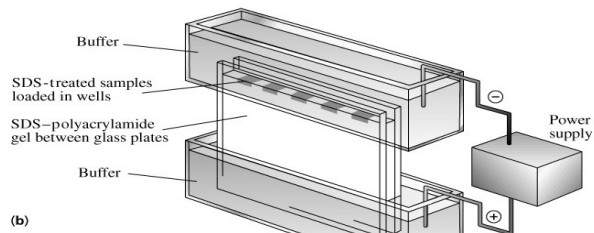


LOW SELECTIVITY

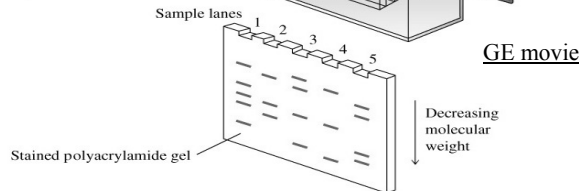
HIGH SELECTIVITY

Diagram of a typical electrophoresis apparatus.

(a)



(b)



[GE movie](#)

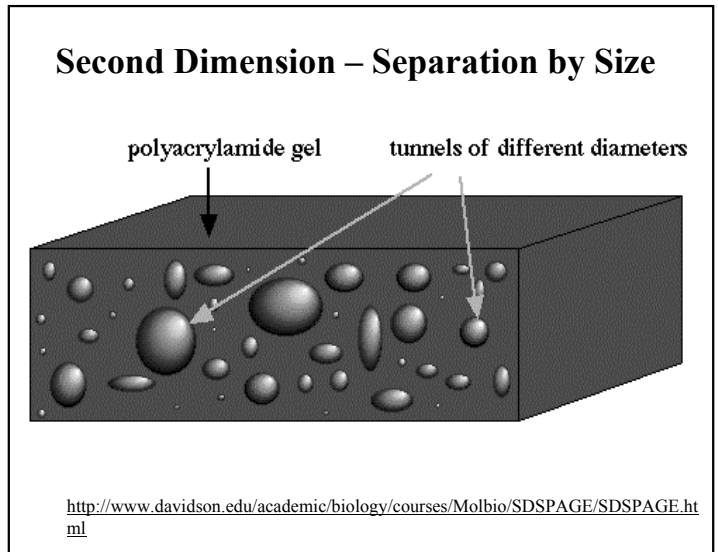
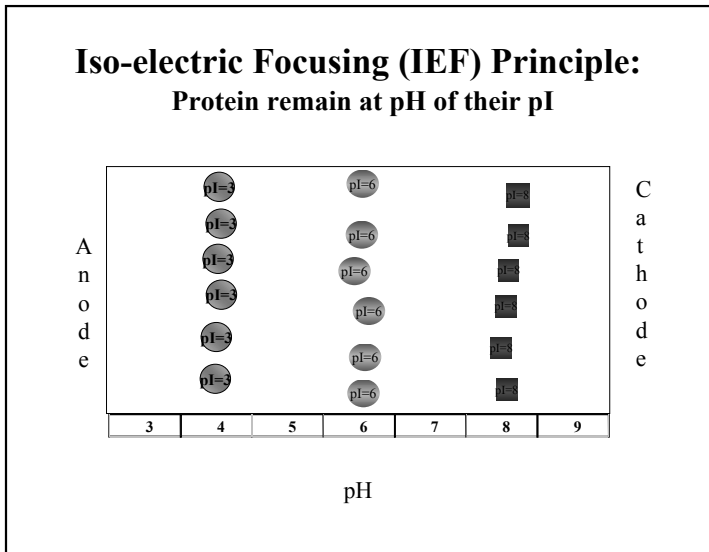
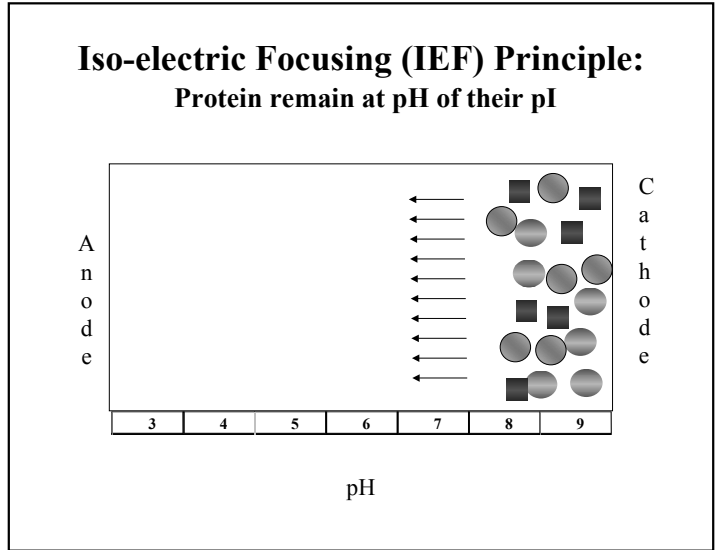
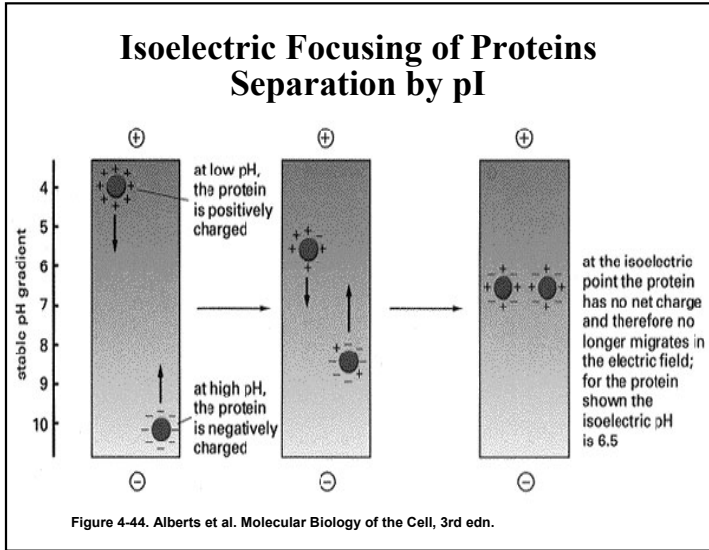
- Gels may be run either vertically or horizontally in slabs or tubes.

Electrophoretic Mobility מוביליות אלקטרופורטית

$$\mu = \frac{q}{6 \pi r \eta}$$

- q - charge (fixed for strong acids and bases
pH dependant for weak acid and bases)
- $6\pi r$ - effective ionic volume (*N.B. complexation and counter ion*)
- η - viscosity

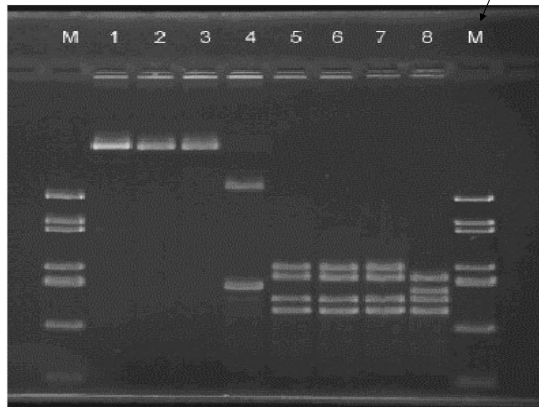
High Performance Liquid Chromatography - HPLC



High Performance Liquid Chromatography - HPLC

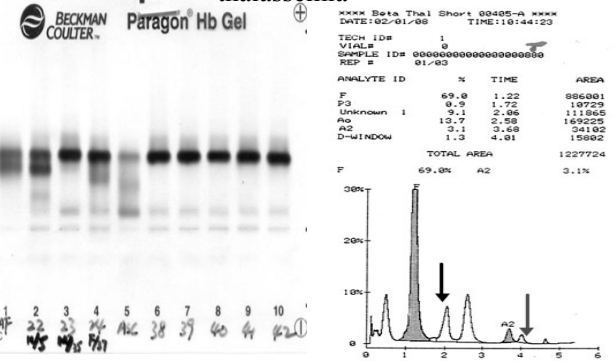
Samples Gels: Separation by Size

Markers-known MW



Clinical Applications

Test of haemoglobin variant for thalassemia

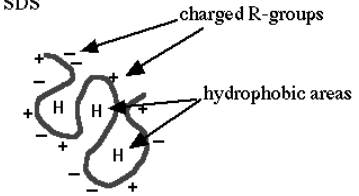


Denaturing Gel:
Sodium Dodecyl Sulfate (SDS)
PolyAcrylamide Gel (PAGE)

SDS-PAGE Electrophoresis of
Denatured Proteins

Denaturation of Proteins by SDS

BEFORE SDS



AFTER SDS

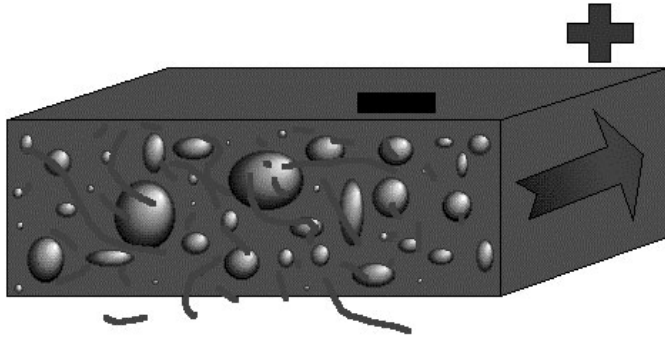


<http://www.davidson.edu/academic/biology/courses/Molbio/SDSPAGE/SDSPAGE.html>

High Performance Liquid Chromatography - HPLC

SDS – PAGE Electrophoresis

Migration of the Denatured Proteins Through the Gel Until Blocked

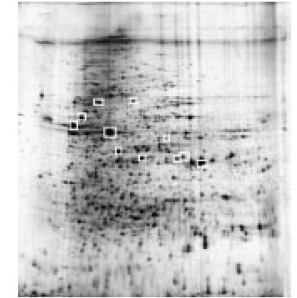
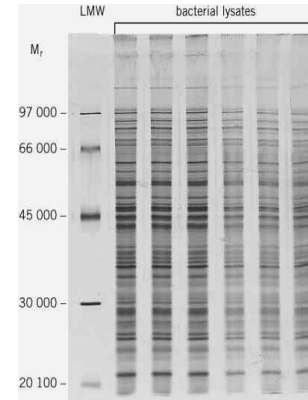


<http://www.davidson.edu/academic/biology/courses/Molbio/SDSPAGE/SDSPAGE.html>

Gel Electrophoresis

1D SDS Gel Electrophoresis

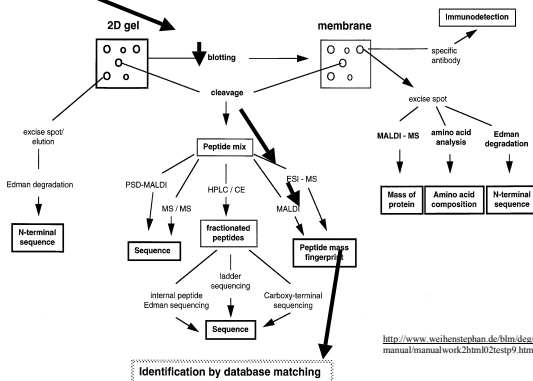
2D SDS Gel Electrophoresis



2-D gel of glioblastoma multiforme brain tumor cell culture extract stained with PlusOne Silver Staining Kit. Protein, using silver staining protocol 2 of the Processor Plus. p53 antigen that were detected on the 2-D blot above are boxed.

Application: Proteomics

Fig. 19. Spot identification



<http://www.wichemstephan.de/html/06/manual/manual/work/2em/02/step9.htm>