Separation by Chromatography Methods

Analytical Biochemistry

3.1 Principle of Separation techniques

3.2 Methods Based on Polarity (3.2.1-3.2.3)

Biochemistry and Molecular Biology

11.5 Partition Chromatography

11.6 Ion Exchange Chromatography

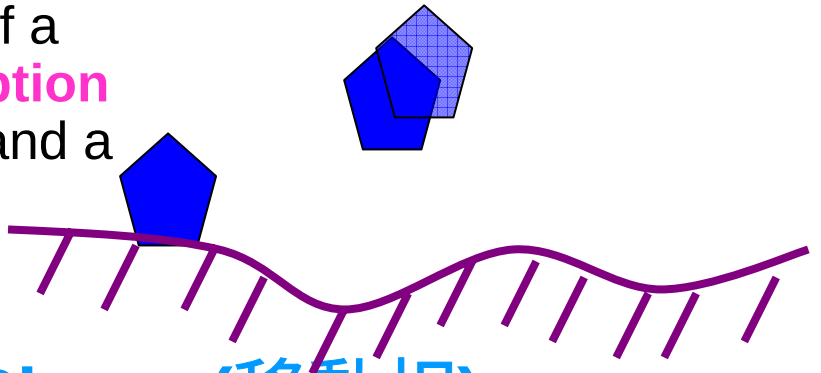
11.7 Gel Filtration Chromatography

11.8 Affinity Chromatography

http://www.waters.com

How Does Chromatography Work?

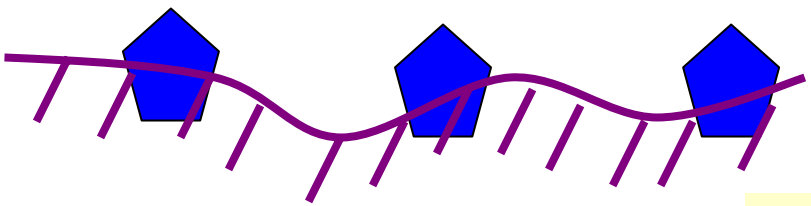
Chromatography is a method for separating the components of a mixture by differential **adsorption** between a stationary phase and a mobile (moving) phase



Mobile Phase (移動相)

Liquid

Liquid



Liquid

Solid

Partition chromatography

Stationary Phase (固定相)

Adsorption chromatography

Principles of Separation Techniques

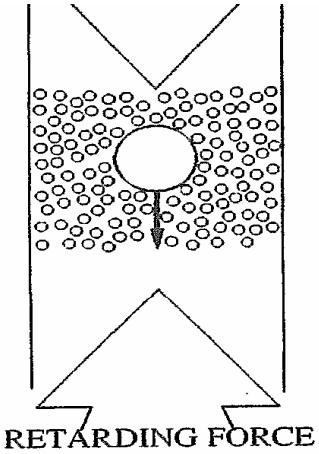
AB 3.1

Molecular Characteristic	Physical property	Separation Technique
Polarity	Volatility	Gas-liquid chromatography
	Solubility	Liquid-liquid chromatography
	Adsorptivity	Liquid-solid chromatography
Ionic	Charge	Ion-exchange chromatography
		Electrophoresis
Size (mass)	Diffusion	Gel permeation chromatography
		Dialysis
Shape	Sedimentation	Ultracentrifugation
	Liquid binding	Affinity chromatography

Factors Involved in Separation

Impelling Force

- Gravitational (Ultracentrifugation)
- Electrokinetic (Electrophoresis)
- Hydrodynamic (Chromatography, 沖堤液驅動力)

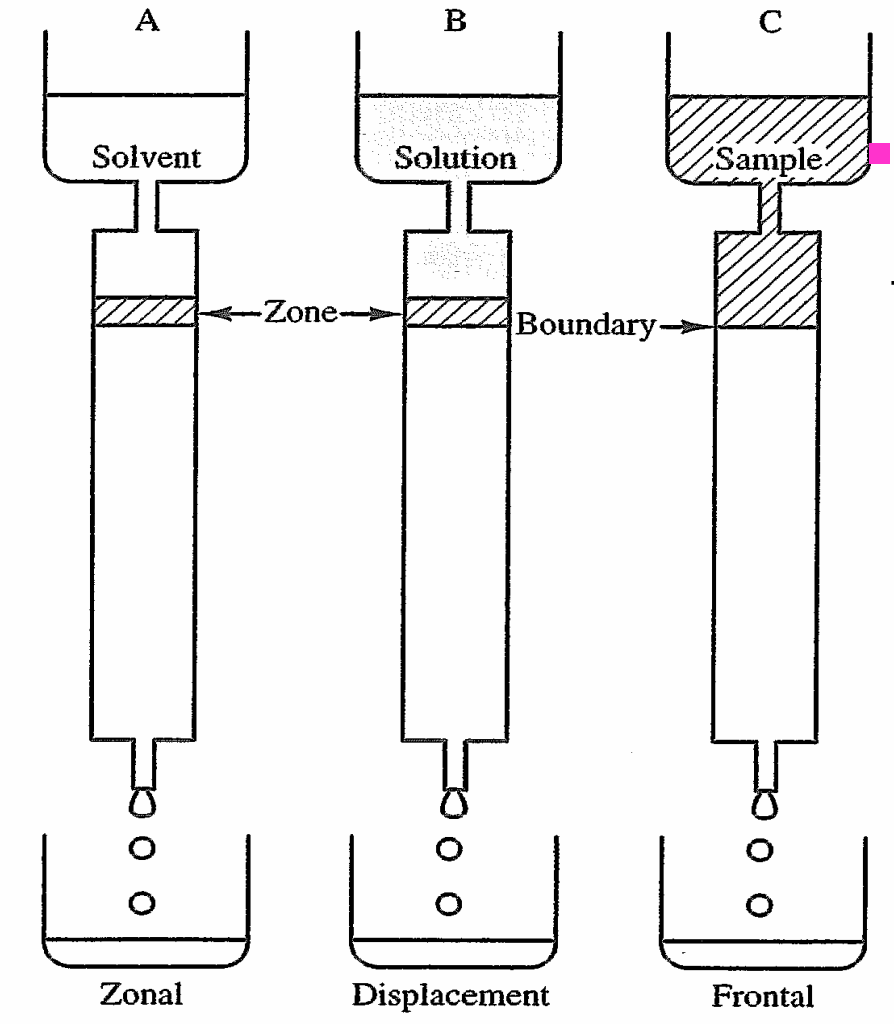


Retarding Force

- Single phase technique
- Molecular friction
- Electrostatic

- Dual phase technique
- Adsorption
- Binding
- Ionic interaction

Three Major Methods in Chromatography



- **Zonal (區帶)**

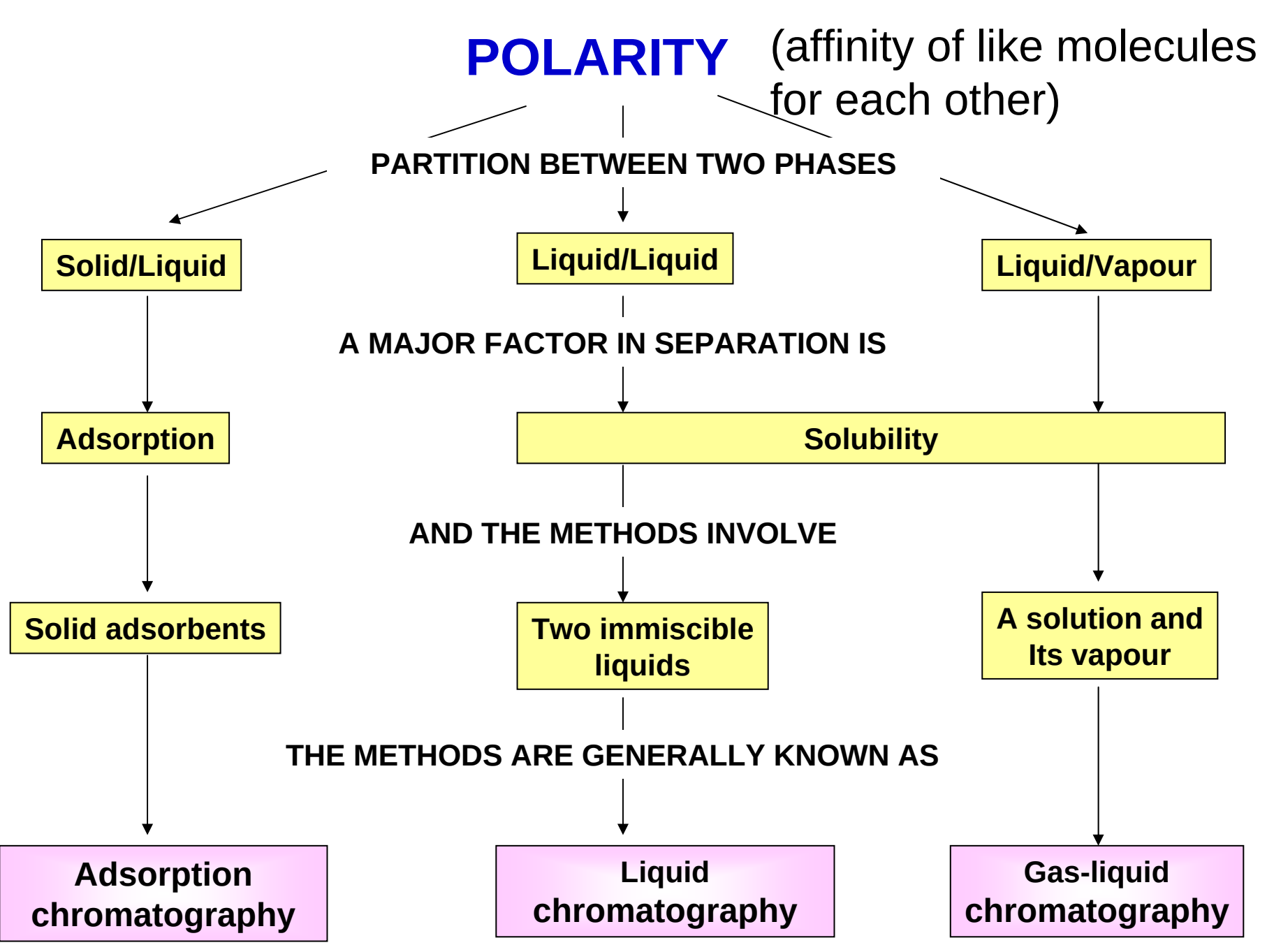
  A band (zone) in a solvent system
  -- Change in pH, size…etc

- **Displacement**

  A band (zone) in two-phase solvent system
  ---Different affinity for the solid support (stationary/mobile phase)

- **Frontal (前端)**

  Large sample containing in mobile phase


POLARITY
(affinity of like molecules for each other)
PARTITION BETWEEN TWO PHASES
Solid/Liquid
Liquid/Liquid
Liquid/Vapour
A MAJOR FACTOR IN SEPARATION IS
Adsorption
Solubility
AND THE METHODS INVOLVE
Solid adsorbents
Two immiscible liquids
A solution and Its vapour
THE METHODS ARE GENERALLY KNOWN AS
Adsorption chromatography
Liquid chromatography
Gas-liquid chromatography

Different Kinds of Chromatography (characterized by the mobile phase)

- Liquid chromatography (includes column chromatography, thin-layer, and HPLC)
  - Stationary phase: silica, alumina, etc.
  - Mobile phase (moving phase): organic solvents
  - Important properties: polarity
- Gas chromatography
  - Stationary phase: a film of a polymer or a wax. The film must have a high boiling point
  - Mobile phase: gas (Helium is the usual carrier gas)
  - Important properties: boiling point

Modes of Chromatography

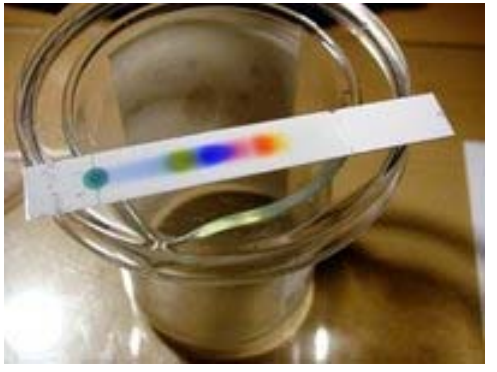
(characterized by shape of stationary phase

- Column chromatography

- Stationary phase is packed into a column

- Thin-Layer chromatography

- Stationary phase is coated onto glass, metallic or plastic plate.



Liquid-Solid Chromatography (Adsorption)

AB 3.2.1

Adsorption (吸附):

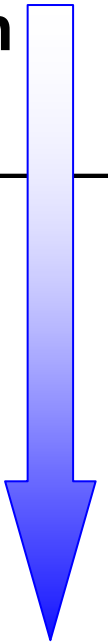
Some substances physically bind to the surface of a solid polar substances

- Polar compound
- Large surface for adsorption
- Often by OH (hydroxy group) to form H-bonding

Polarity of Selected Solutes and Solvents

Silica

Solute	Adsorption Energy	Solvent	Solvent Strength
Hydrocarbon	0.07	Hexane	0.01
Halogen Derivativ	1.74	Benzene	0.32
Aldehyde	4.97	Chloroform	0.4
Ester	5.27	Acetone	0.55
Alcohols	6.5	Pyridine	0.71
Acids/Bases	7.6	Methanol	0.95



Increasing Polarity

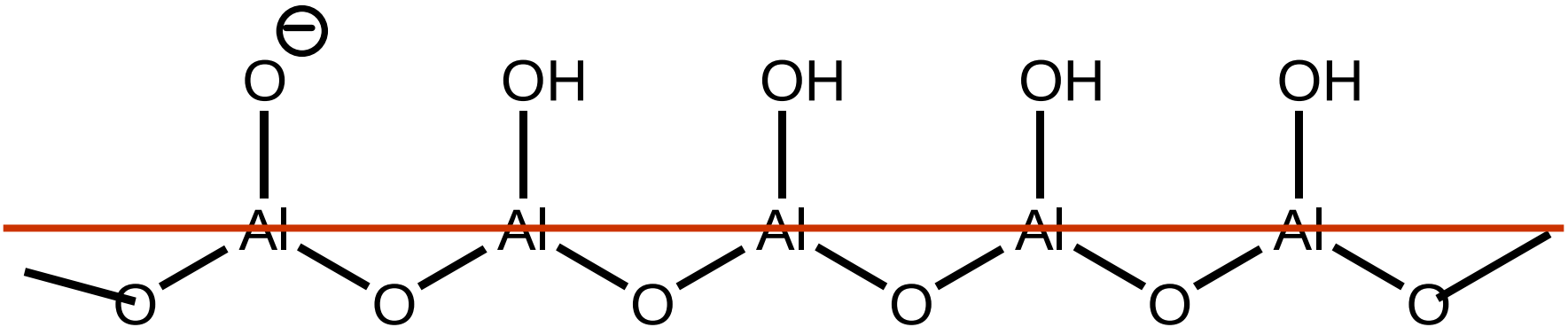
Adsorption Energy

the affinity of a solute with an adsorbent (vary with adsorbant)

Solvent Strength

the affinity of a solvent with an adsorbent (vary with adsorbant)

Stationary Phase: Alumina



Acidic: -Al-OH

Neutral: -Al-OH + -Al-O$^-$

Basic: -Al-O$^-$

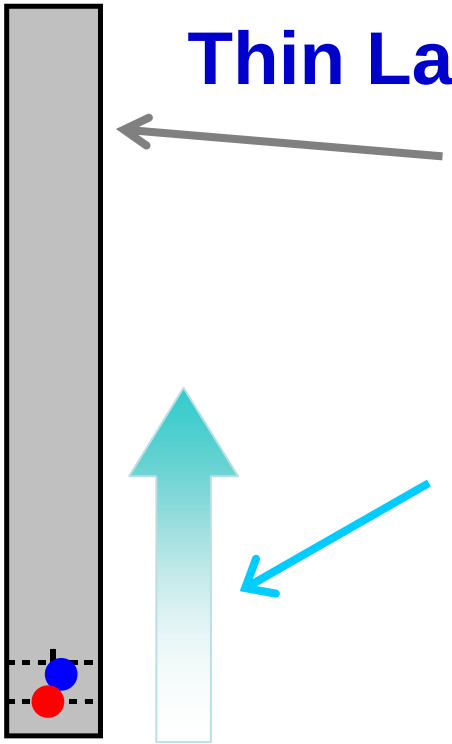
Examples of Absorbents and Applications

Adsorbent	Strength	Application
Silicic acid(silica gel)	Strong	Steroids,amino acids,lipids
Charcoal	Strong	Peptides,carbohydrates
Aluminium oxide	Strong	Steroids,esters,alkaloids
Magnesium carbonate	Medium	Porphyrins
Calcium phosphate	Medium	Proteins,polynucleotides
Cellulose	Weak	Proteins

Thin-layer chromatography and column chromatography are different types of **liquid chromatography**. The principle of operation is the same!

- The mobile (moving) phase is a liquid.
- The stationary phase is usually silica or alumina.---→ a *very polar* layer of adsorbent on an inert, flat support.

Thin Layer Chromatography (薄層層析法)



1. The surface of the plate consists of a very thin layer of silica on a plastic or aluminum backing. The silica is very polar— the **stationary phase**.

2. Spot the material at the origin (bottom) of the TLC plate.

3. Place the plate into a glass jar with a small amount of a solvent in the glass jar.— the **moving phase**.

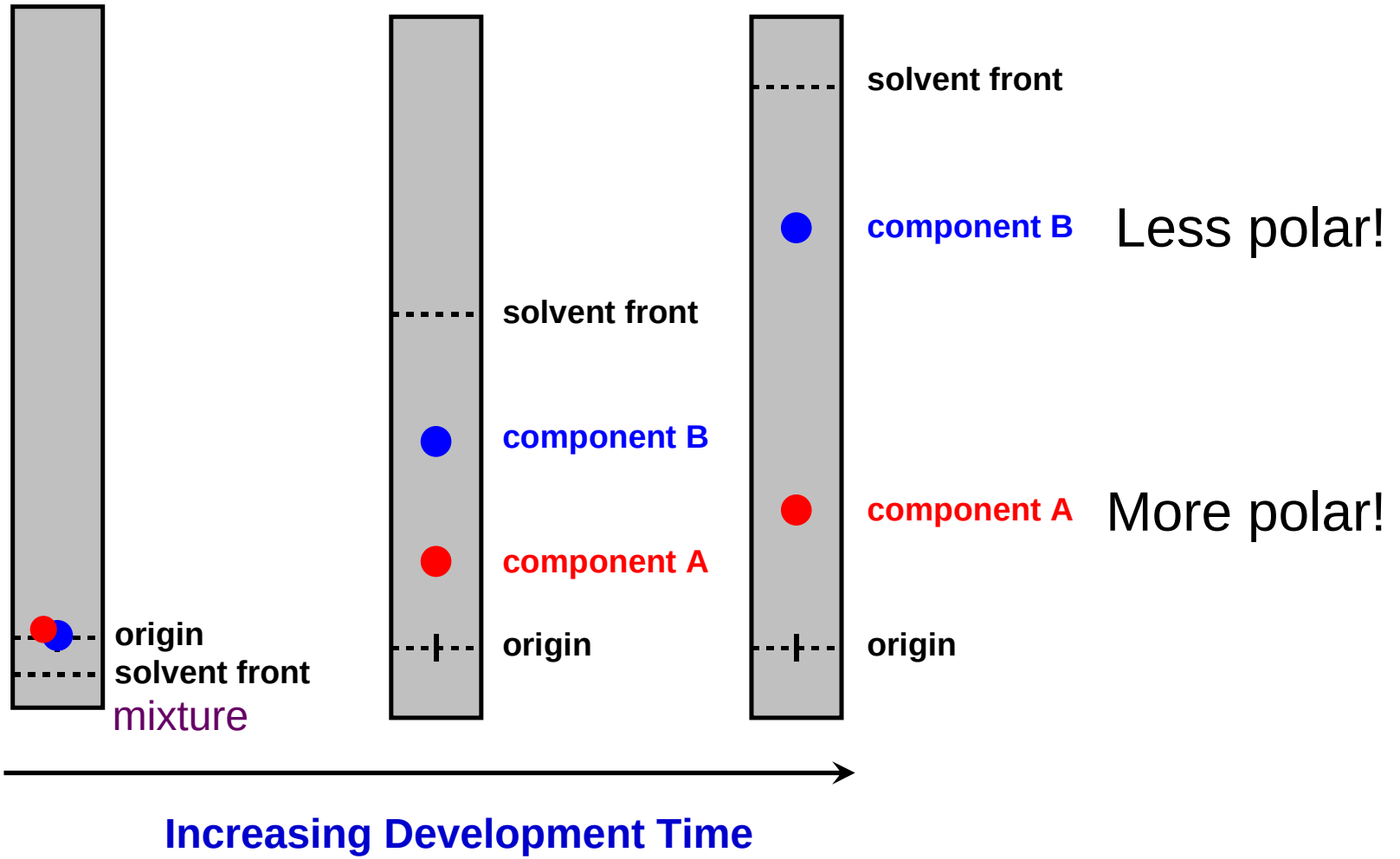
4. Remove the plate from the bottle when the solvent is close to the top of the plate.

5. Visualize the spots (Ultraviolet light, color reagent…etc)

Non-polar compounds will be **less strongly** attracted to the plate and will spend more time in the moving phase. This compound will move faster and will appear closer to the top of the plate.

Polar compounds will be **more strongly** attracted to the plate and will spend less time in the moving phase and appear **lower on the plate**.

Thin-Layer Chromatography: A Two-Component Mixture



Determination of R_f Values (Rate of Flow)

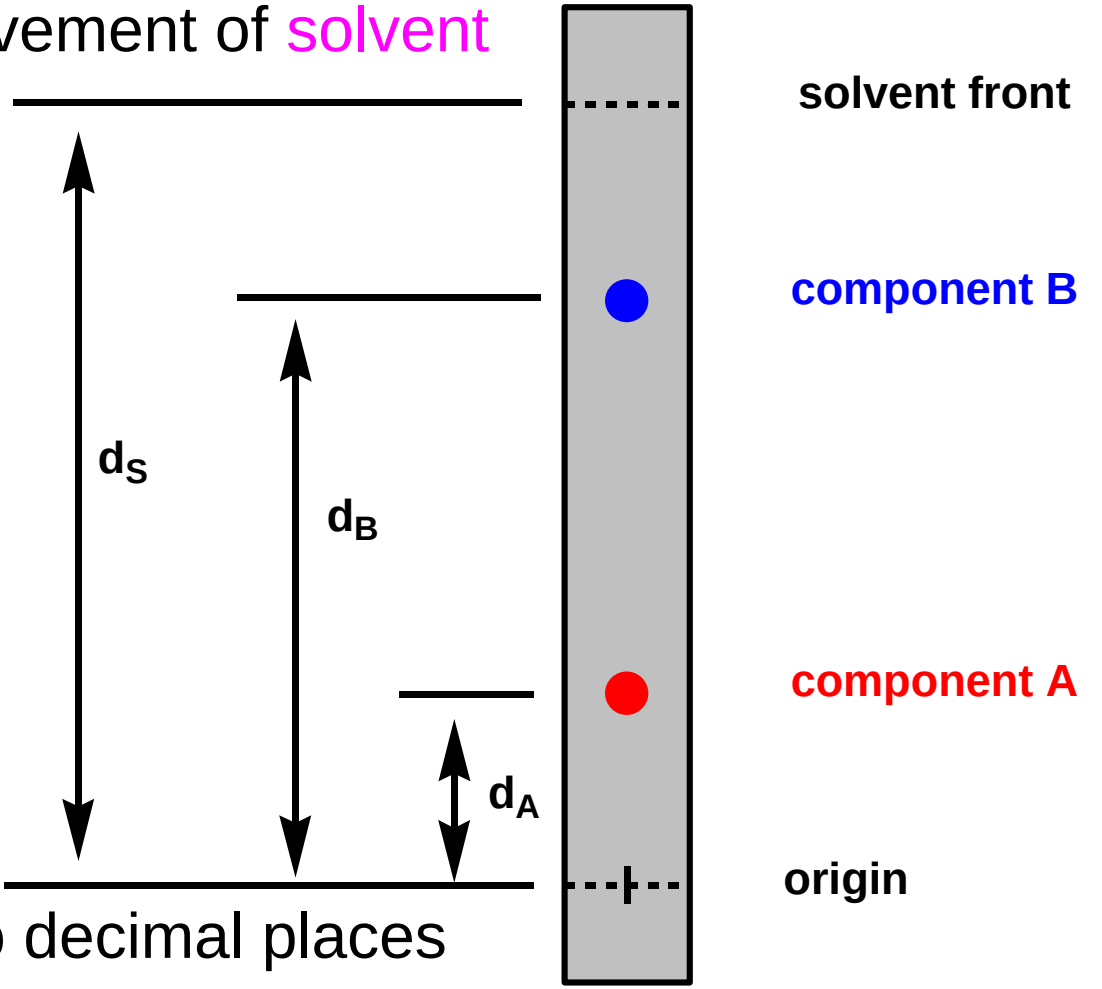
A measure of the movement of a compound compared with the movement of solvent

R_f of component A =

$$\frac{d_A}{d_S}$$

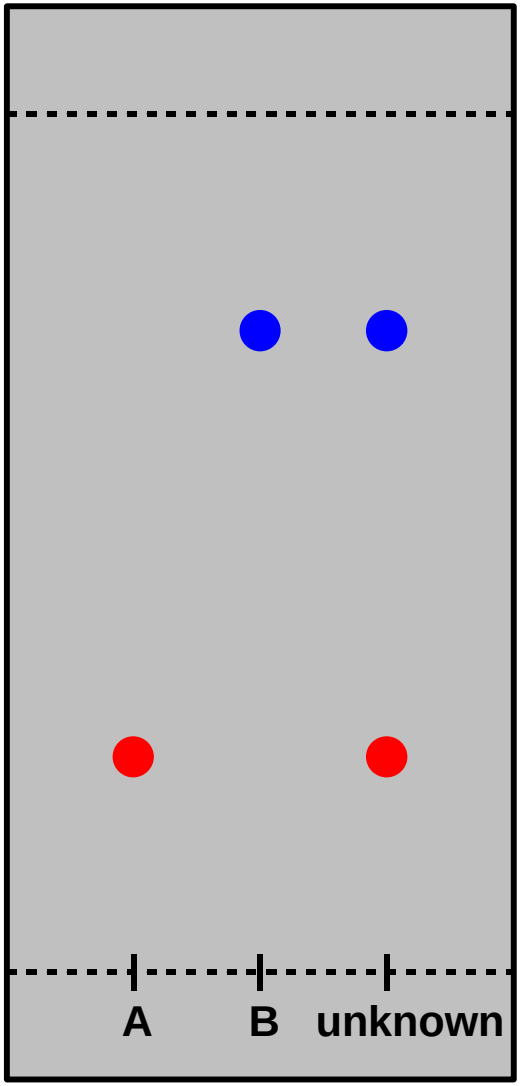
R_f of component B =

$$\frac{d_B}{d_S}$$



1. Only reported to two decimal places

Thin-Layer Chromatography: Qualitative Analysis



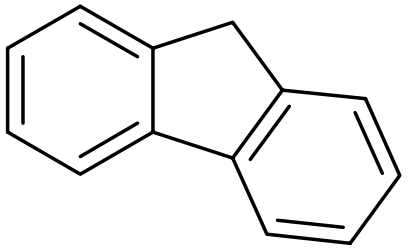
Ideally, the R_f value should be the same of a given compound using the same solvent

(Practically, the movement depends on the structure and thickness of the layer, the amount of water remaining and effect of the binding agents.

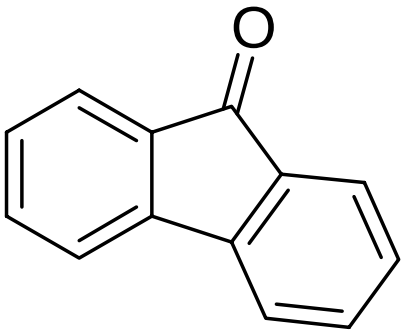
Advantages

- Simple
- Rapid
- Cheap

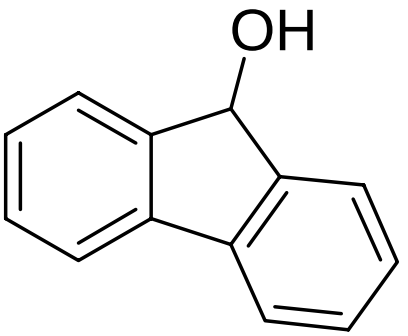
Example: Thin-Layer Chromatography



Fluorene



Fluorenone



Fluorenol

a) Which one of these compounds is the least polar?

b) Which one of these compounds is the most polar?

c) What would be the relative order of separation on the TLC plate remembering that CH_2Cl_2 is not very polar?

Liquid-Liquid Chromatography

Partition of a solute between two immiscible liquid phases

Example:

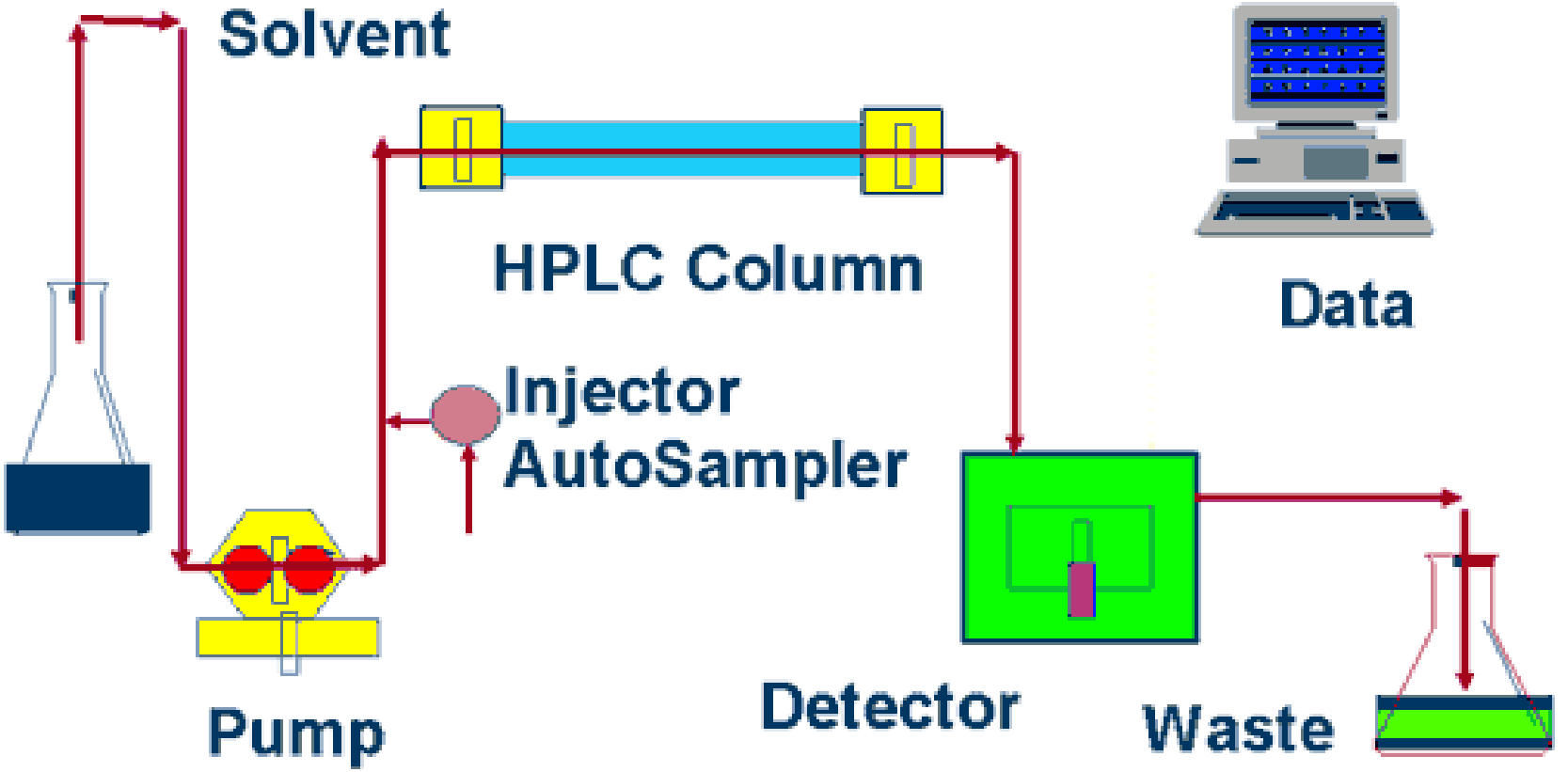
High Performance Liquid Chromatography

- Partition chromatography
- Adsorption chromatography
- Gel filtration chromatography
- Affinity chromatography
- Ion-exchange chromatography

High Performance Liquid Chromatography

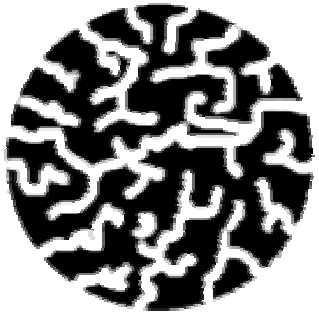
Small and regular support media with stationary phase

AB 3.2.2

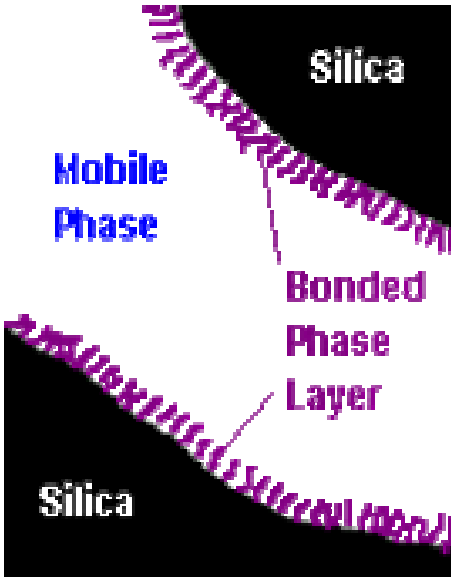


Provide steady solvent flow rate for isocratic or gradient mobile phase

HPLC Column

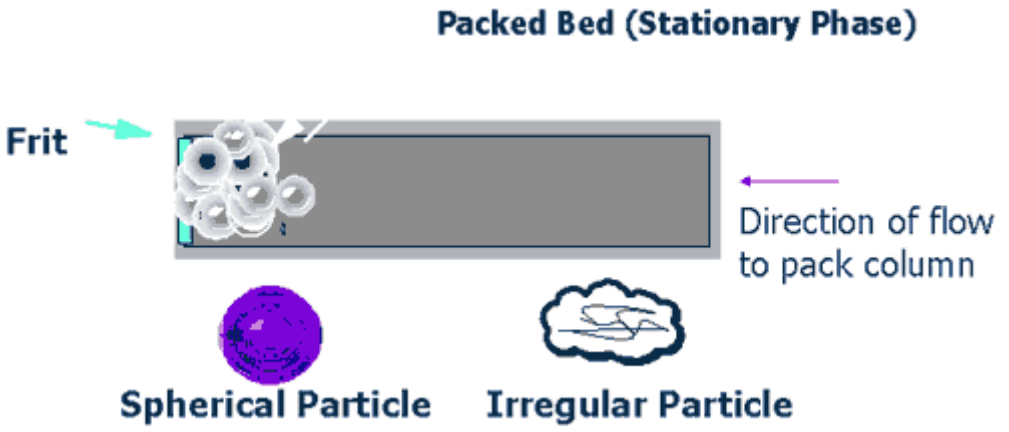


porous.



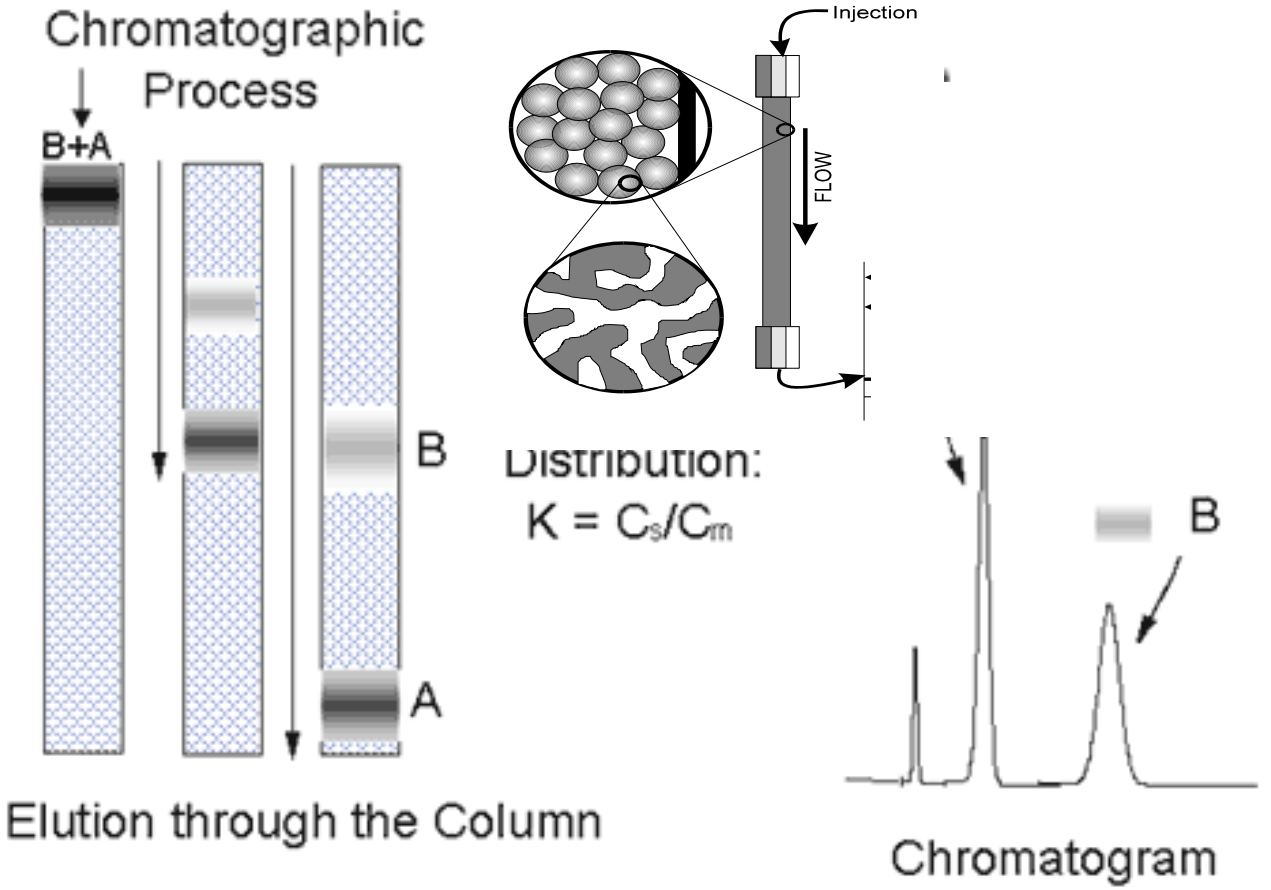
Most HPLC packings are porous. Most of the stationary phase surface area is on the inside of the particles

A layer of alkyl chains bonded to the silica surface



The composition of the mobile phase provides the chemical environment for the interaction of the solutes with the stationary phase.

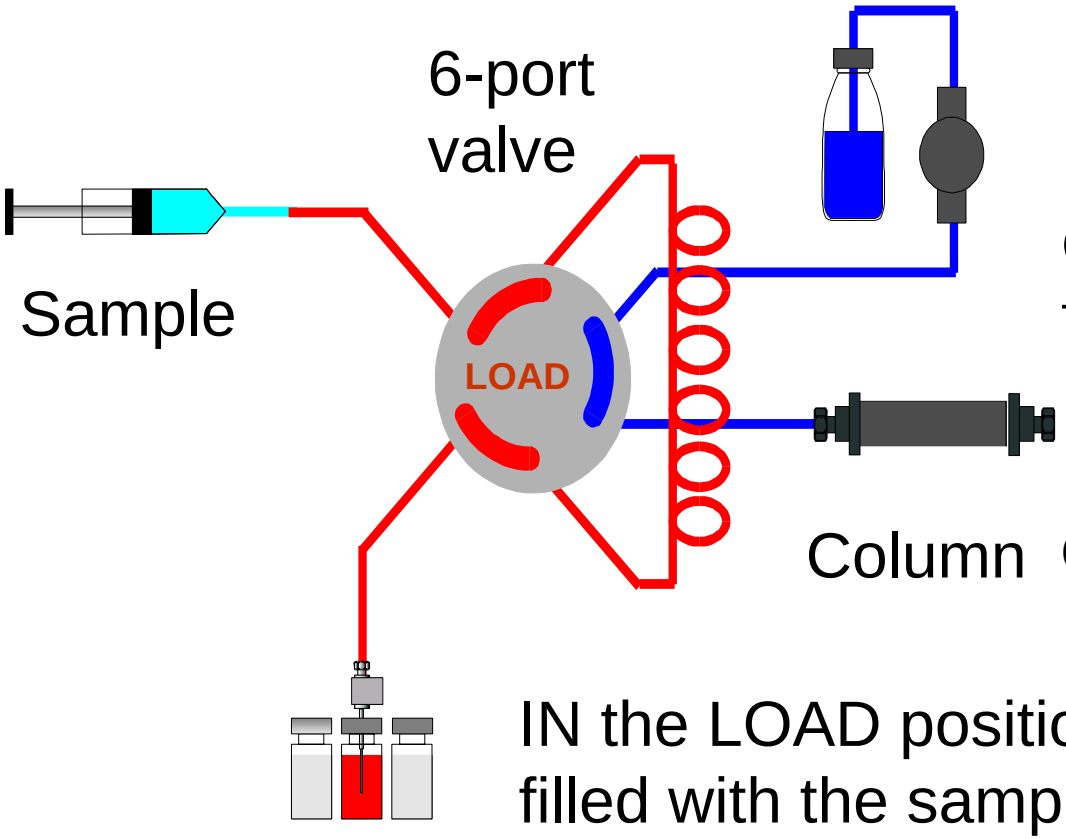
Chromatographic Separation



In a liquid chromatographic process a liquid permeates through a porous solid stationary phase and elutes the solutes into a flow-through detector

Sample Injector (AutoSampler)

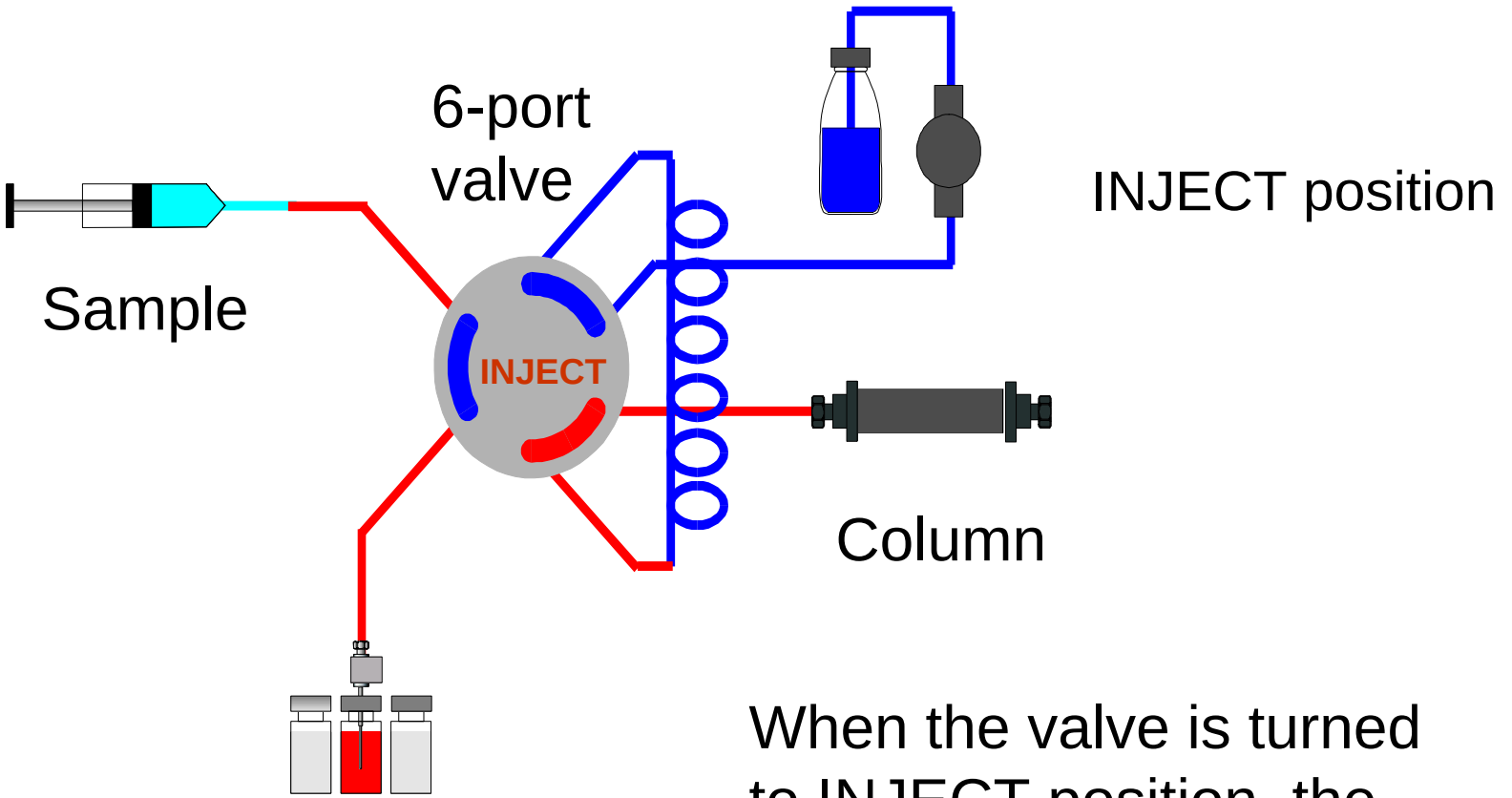
Full Loop Injection (cont'd)



IN the LOAD position, the loop can be filled with the sample using a simple syringe or an autosampler

Sample Injector (AutoSampler)

Full Loop Injection (cont'd)

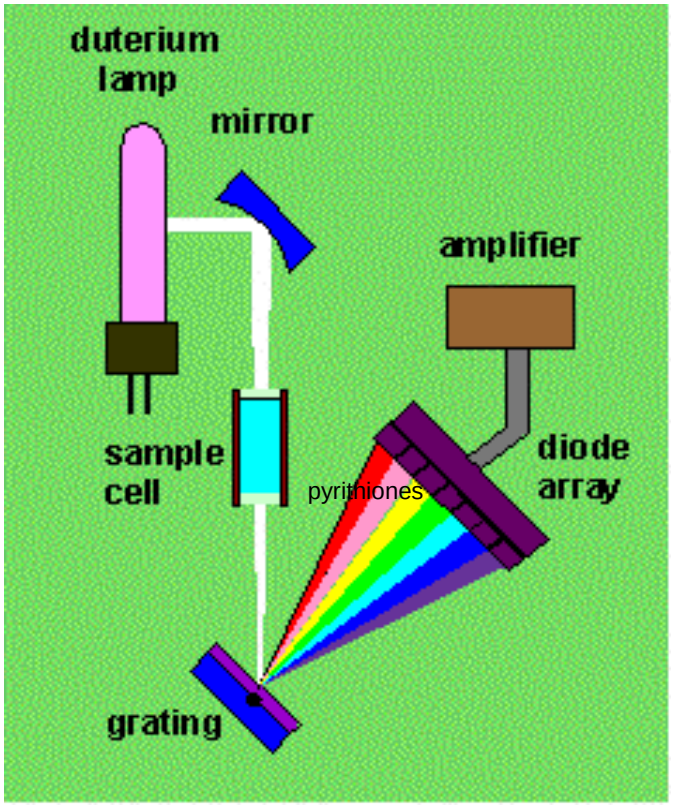


When the valve is turned to INJECT position, the sample is washed into the column.

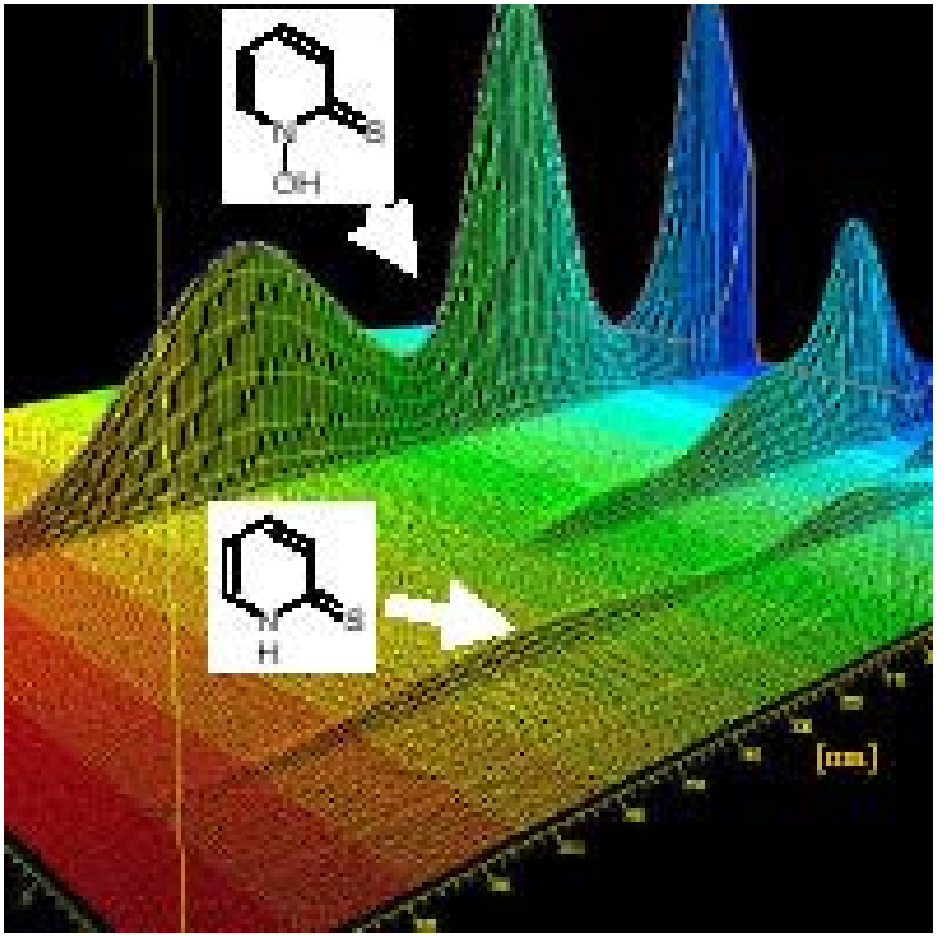
Detection Methods

- **UV – Ultraviolet light---** most popular
  - Lamp
  - Grating/Lens - Wave length 190-350 nm
  - FlowCell
  - PhotoDiode - Differential Light Output
- **RI – Refractive Index**
  - Universal analyte detector
  - Solvent must remain the same throughout separation
  - VERY temperature sensitive
  - Sometimes difficult to stabilize baseline
- **FD – Fluorescence-**greater sensitivity, not so popular
  - Excitation wavelength generates fluorescence emission at a higher wavelength
  - Analytes must have fluorophore group---not very common
  - Very sensitive and selective
- **MS – Mass Spectrometry**
  - Mass to charge ratio (m/z)
  - Allows specific compound ID

HPLC Diode Array Detection Analysis -



Elution Time

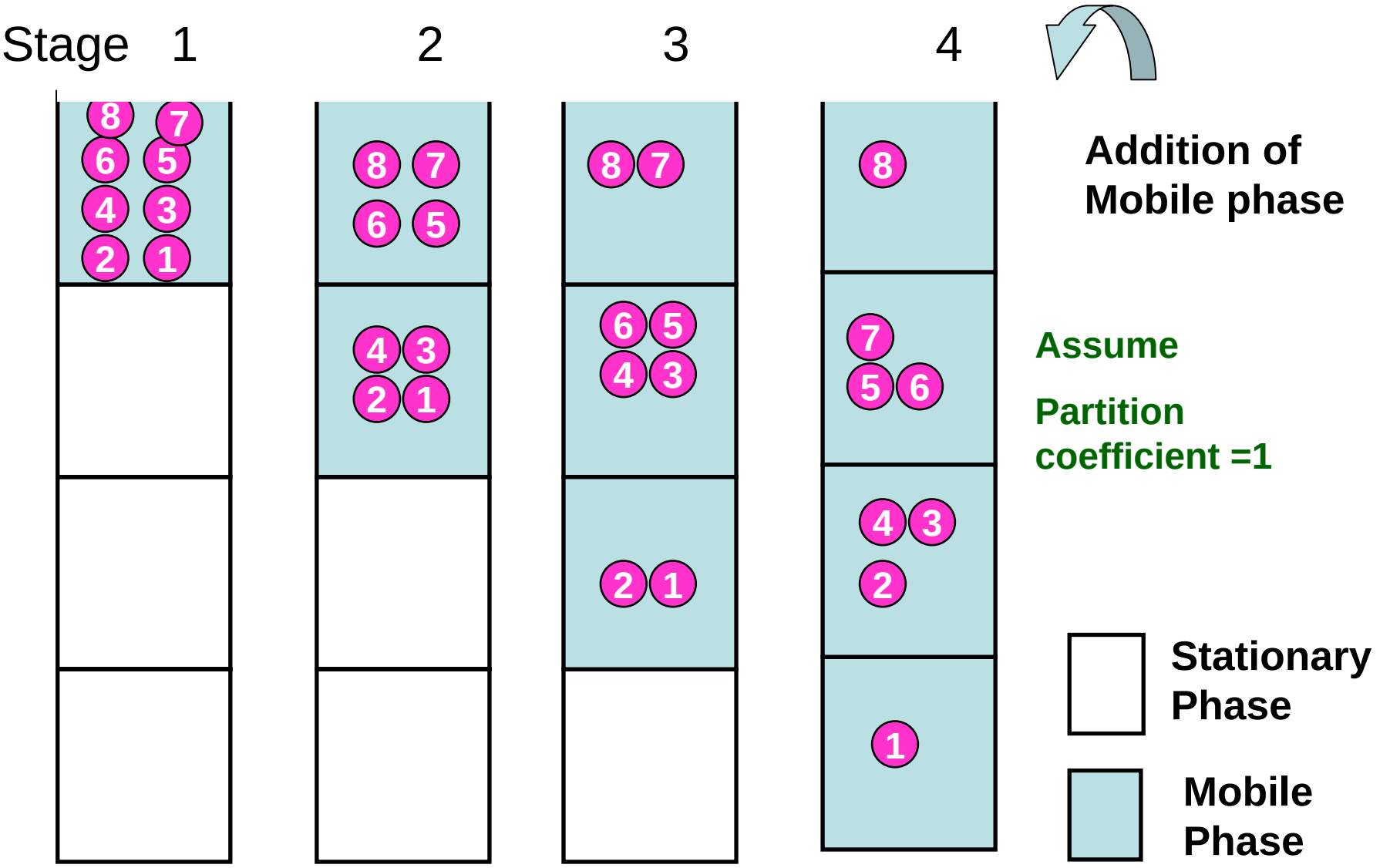


Absorption Wavelength

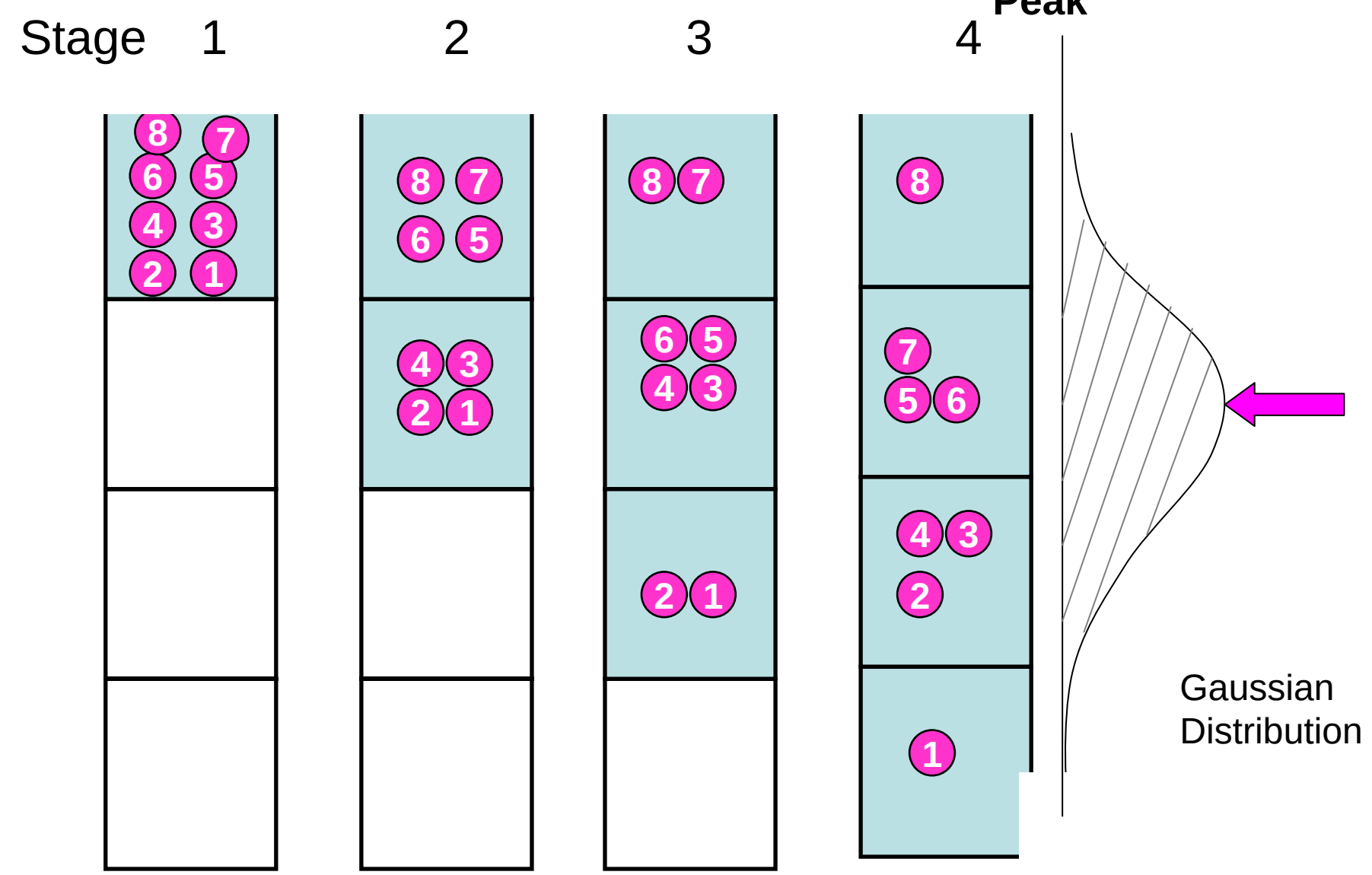
Absorbance is measured at two or more wavelengths

Pyrithione is an anti-oxidant

Column Chromatographic Separation

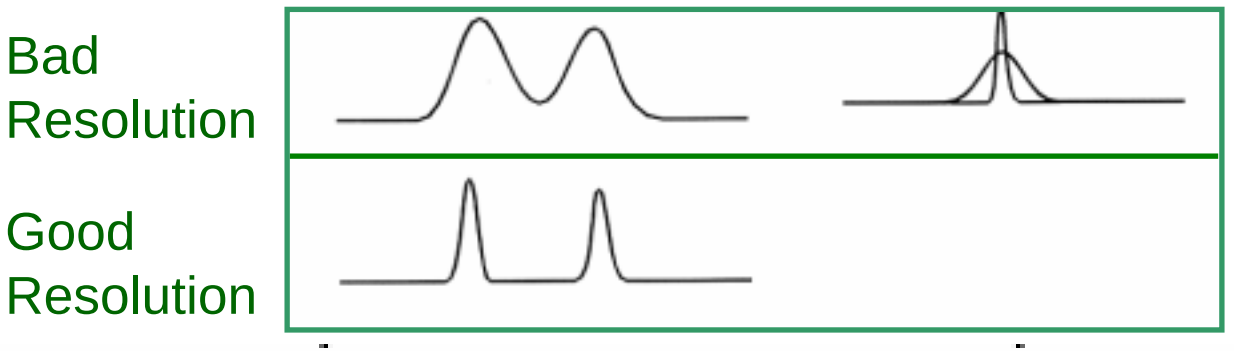


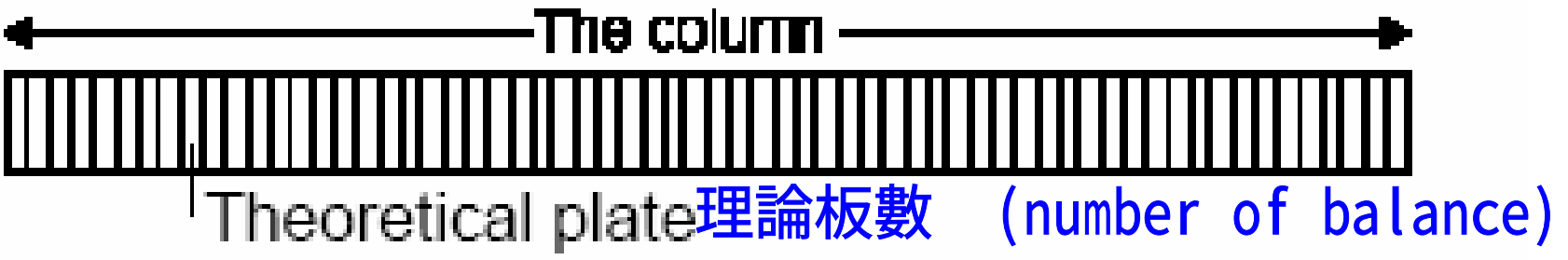
Band Broadening



Separation Efficiency: Plate Theory

The plate theory suppose that the chromatographic column contains a large number of separate layers, called **theoretical plates**. Separate equilibrations of the sample between the stationary and mobile phase occur in these "**plates**". The analyte moves down the column by transfer of equilibrated mobile phase from one plate to the next.

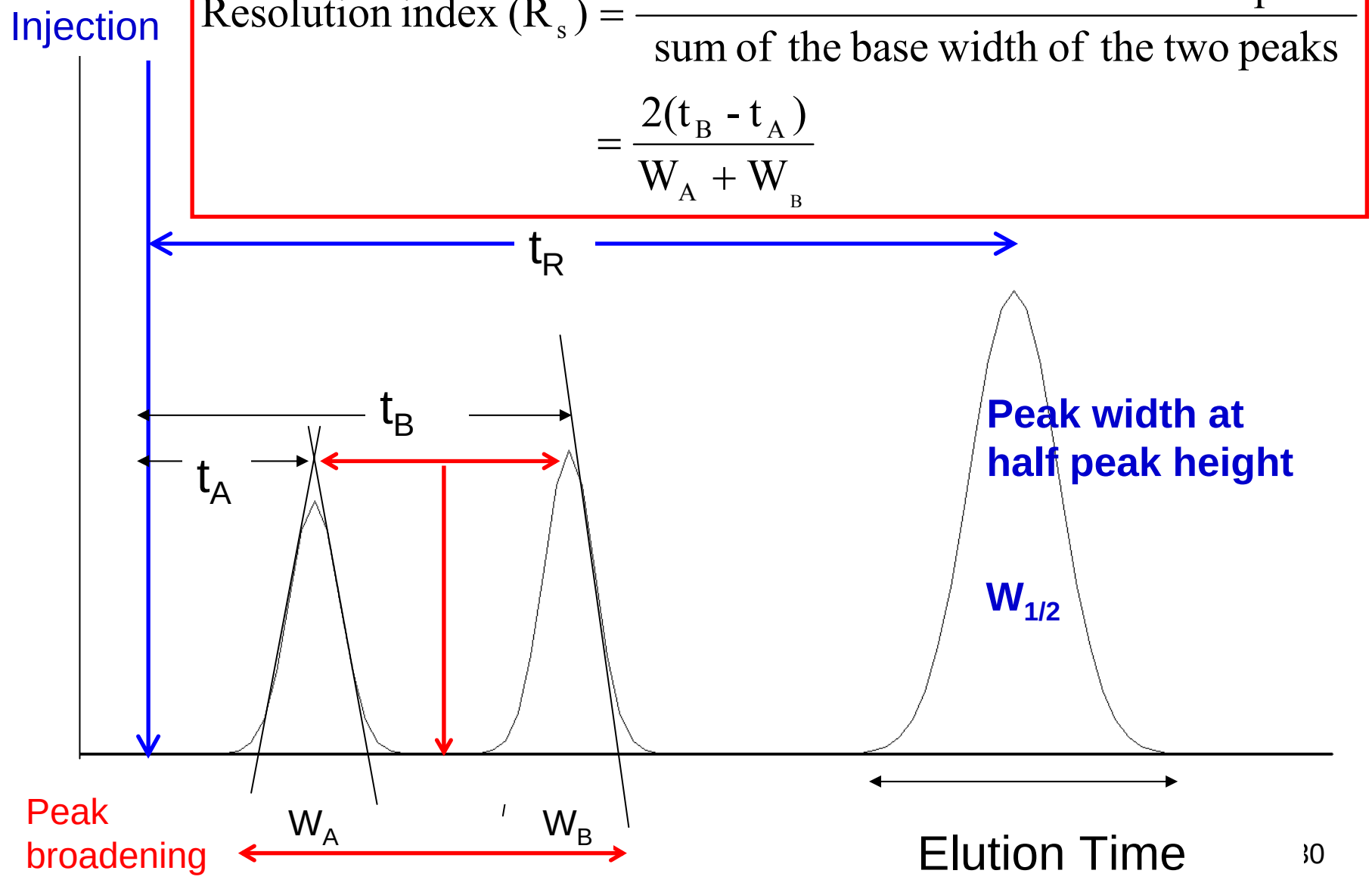




Greater theoretical plates → Better separation resolution

Assessment of Column Efficiency

$$\text{Resolution index } (R_s) = \frac{\text{twice the distance between the two peaks}}{\text{sum of the base width of the two peaks}} = \frac{2(t_B - t_A)}{W_A + W_B}$$



Theoretical Plate Number—Resolution

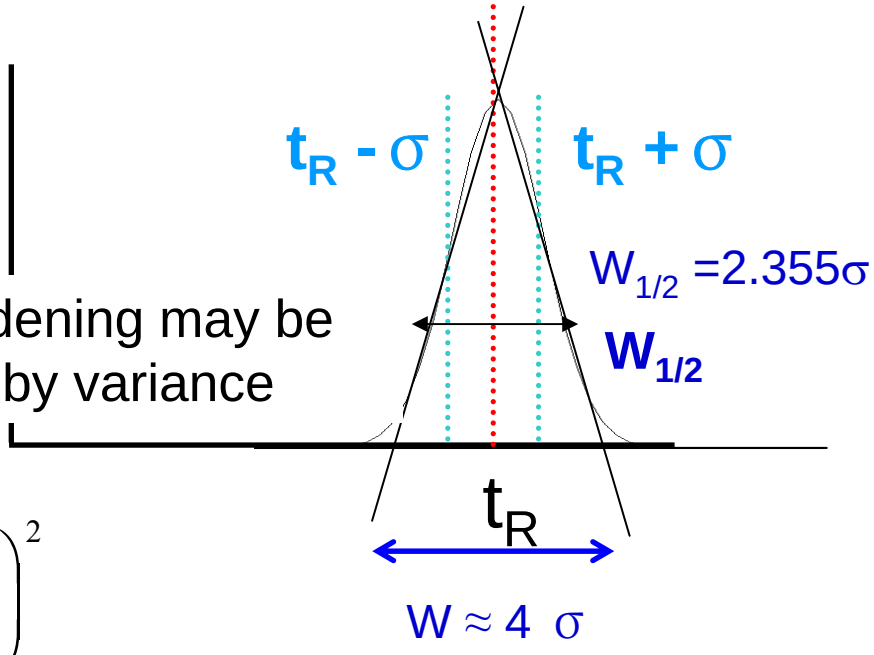
A measure of separation efficiency: How many times the

$\text{Analyte}_{\text{mobile}} \rightarrow \text{Analyte}_{\text{stationary}}$ equilibrium is achieved

$$N = \frac{t_R^{\,2}}{\sigma^2} = \left(\frac{t_R}{\sigma}\right)^2$$

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

Peak broadening may be expressed by variance



$$N = \left(\frac{2.355 \times t_R}{\text{peak width at half peak height}}\right)^2$$

$$= \left(\frac{\text{retention distance}}{\text{width at half peak height}}\right)^2 \times 5.54$$

$t_R^{\,2}$ Retention time: measure of effective column volume for analyte

W Base width

Height Equivalent to a Theoretical Plate (HETP)

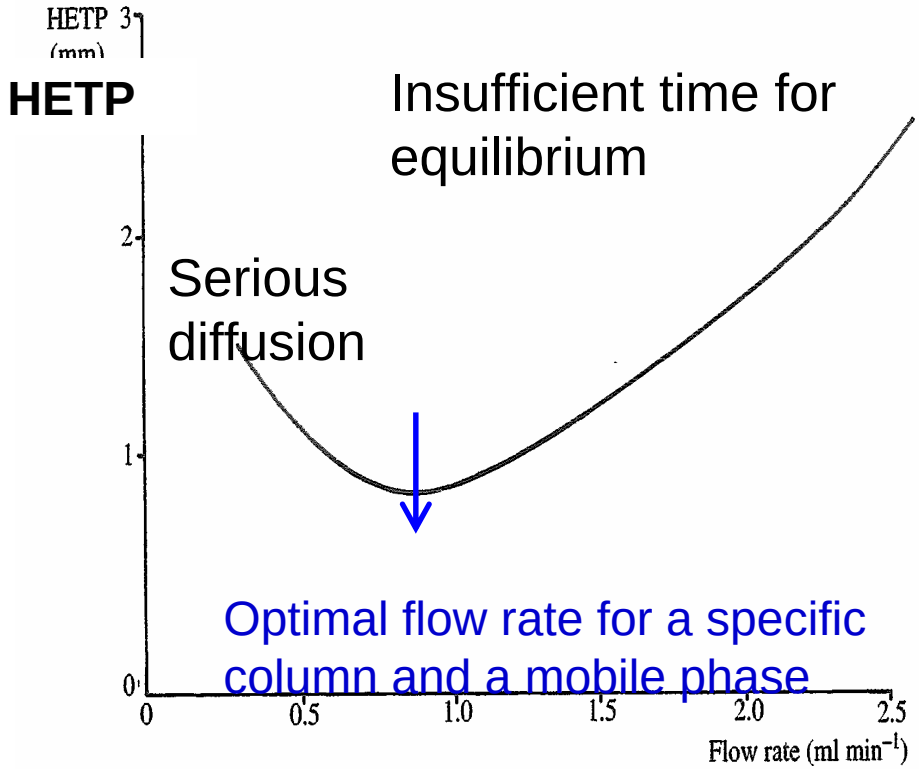
- Length of a column necessary for the attainment of compound distribution equilibrium (measure the efficiency of the column)

$$\mathrm{HETP} = \frac{\text{length of the column}}{\mathrm{N}}$$

h: Reduced plate height

$$\mathrm{h} = \frac{\mathrm{HETP}}{\text{partical diameter}(\mu\mathrm{m})}$$

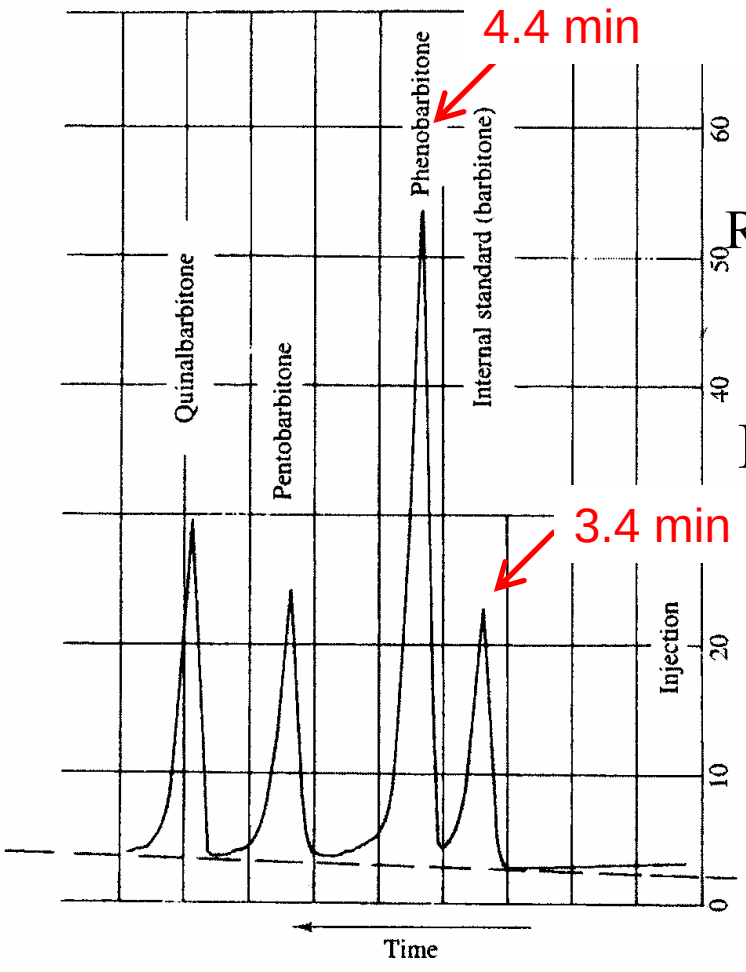
Illustrate the effect of varying the flow rate of the mobile phase on the efficiency of separation process



Flow Rate (ml/min)

Qualitative Analysis

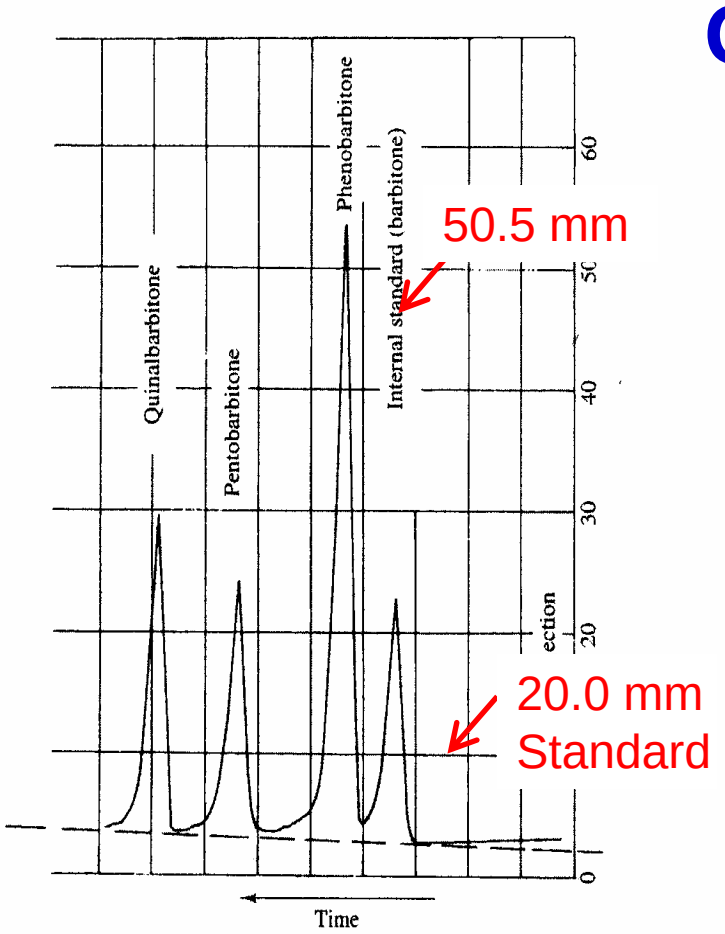
- By comparison with known components, retention time (Distance) is used for identification of a component of a mixture.



$$\text{Relative retention} = \frac{\text{test retention time}}{\text{reference retention time}}$$

$$\text{Relative retention} = \frac{4.4}{3.4} = 1.3$$

Quantitative Analysis



Separation took approximately 15 minutes.

Component	Peak height (mm)
Standard	20.0
Phenobarbitone	50.5
Pentobarbitone	21.0
Quinalbarbitone	26.0

Calculation

Component	Concentration ($mmol\,l^{-1}$)
Phenobarbitone	$\frac{50.5}{20.0} \times \frac{1.1}{1.0} \times 5.0 \times \frac{0.1}{1.1}$

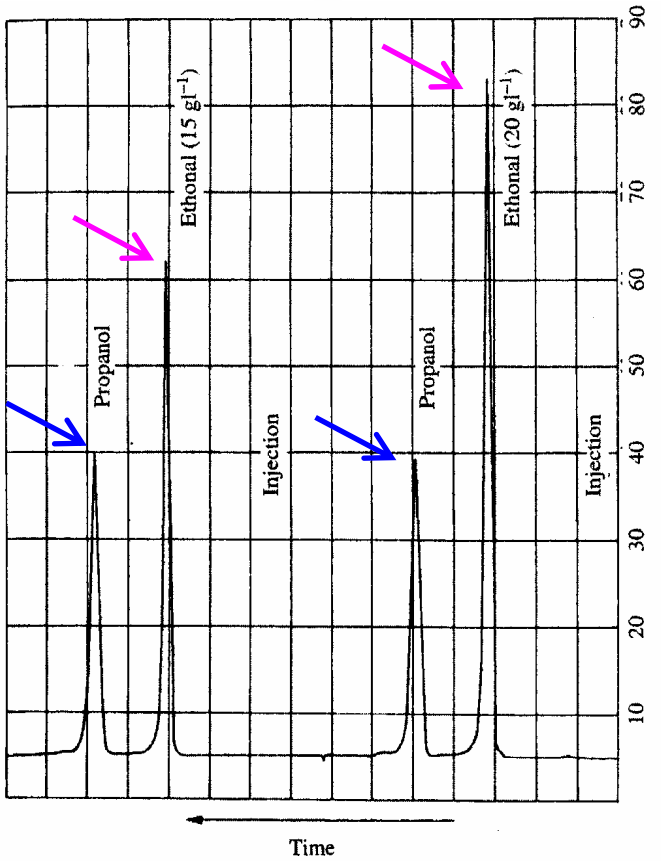
0.1 ml of the internal standard (barbitone, 5.0 mmol/l) was added to 1.0 ml of sample.

20 uL of the mixture was injected

What is the concentration of phenobarbitone?

Injection 2

Injection 1



What is the concentration of test sample?

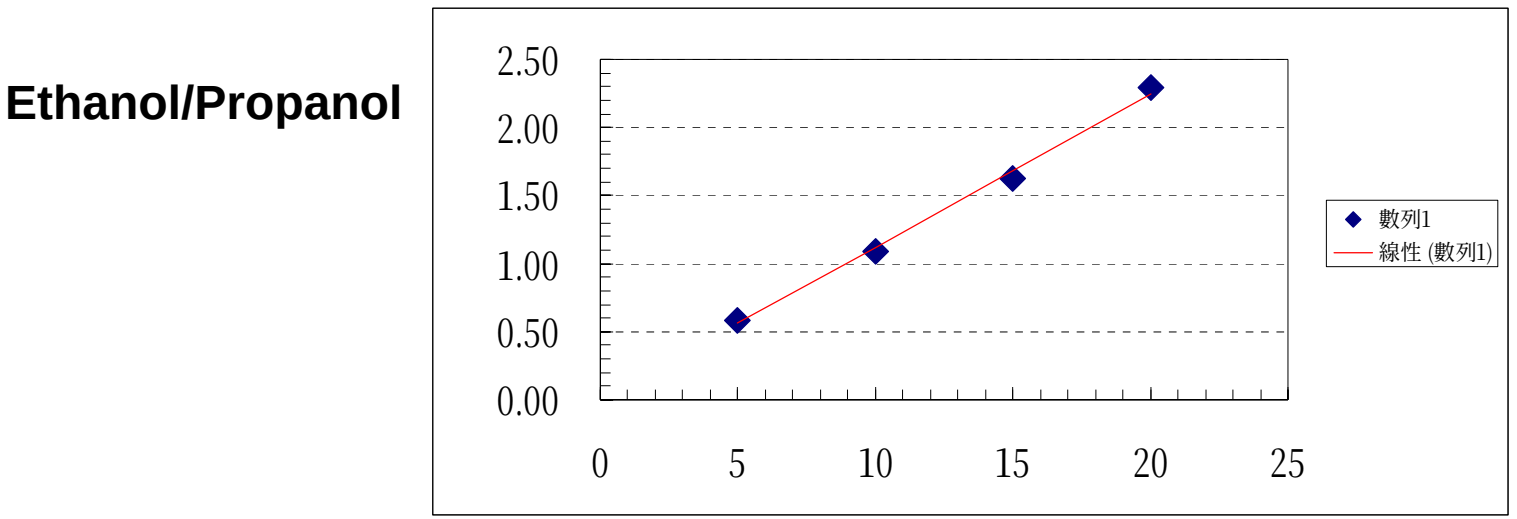
Reference: Propanol

Standard: Ethanol

Injection number	Component 1	Peak height (mm)	Component 2	Peak height (mm)	Peak heights ratio
1	Ethanol (20 gl^{-1})	78	Propanol (1.0 gl^{-1})	34	2.29
2	Ethanol (15 gl^{-1})	57	Propanol (1.0 gl^{-1})	35	1.62
3	Ethanol (10 gl^{-1})	37	Propanol (1.0 gl^{-1})	34	1.08
4	Ethanol (5 gl^{-1})	21	Propanol (1.0 gl^{-1})	36	0.55
5	Test sample	45	Propanol (1.0 gl^{-1})	35	1.28

Reference:

Ethanol Conc.	Peak height	Propanol	Peak height	Ethanol/Propanol
20	78	1	34	2.29
15	57	1	35	1.63
10	37	1	34	1.09
5	21	1	36	0.58
X	45	1	35	1.29



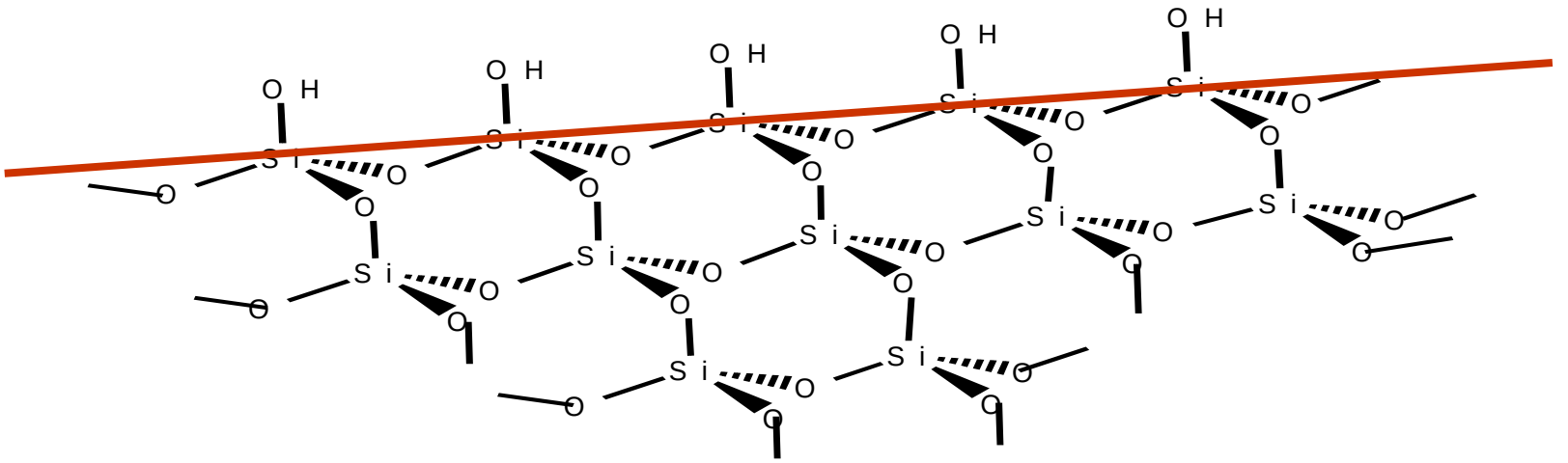
Partition Chromatography BMB 11.5

Partition chromatography is based on differences in capacity factors and distribution coefficients of the analytes using liquid stationary and mobile phases.

- Normal/Reverse Phase Chromatography
- Ion-Exchange Chromatography
- Gel Filtration Chromatography
- Affinity Chromatography

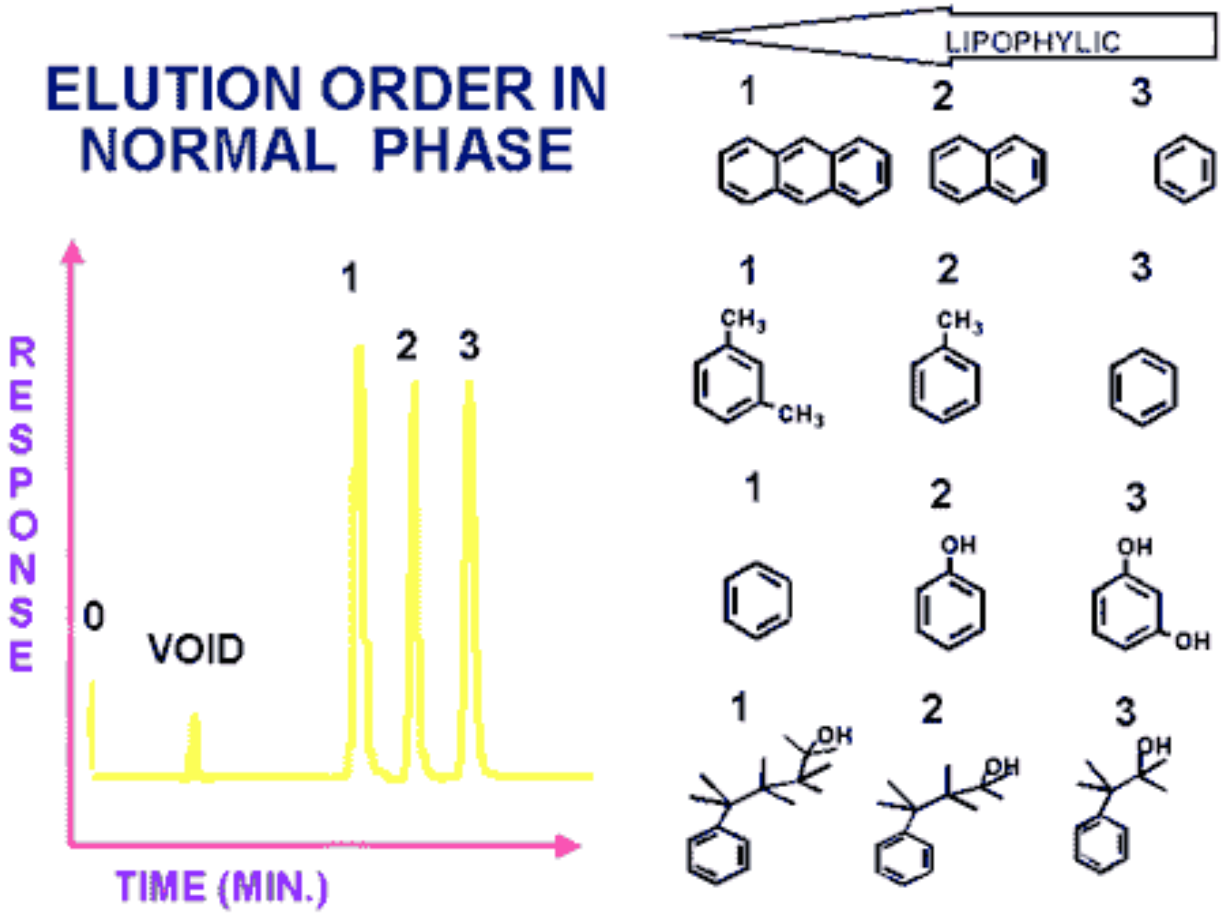
Normal-Phase HPLC

Adsorption of analytes on the polar, weakly acidic surface of silica gel



- Stationary Phase.: Silica (pH 2-8), Alumina (pH 2 - 12), Bonded Diol, and NH_2
- Mobile Phase: Non-polar solvents (Hexane, $CHCl_3$)
- Applications: Non-polar and semi-polar samples; hexane soluble; positional isomers.

Normal Phase Liquid Chromatography

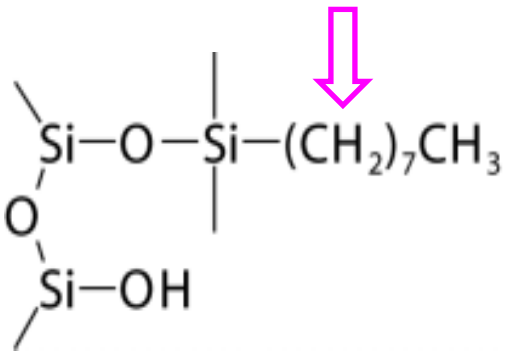


Polar solutes elute later than non-polar lypophilic ones.

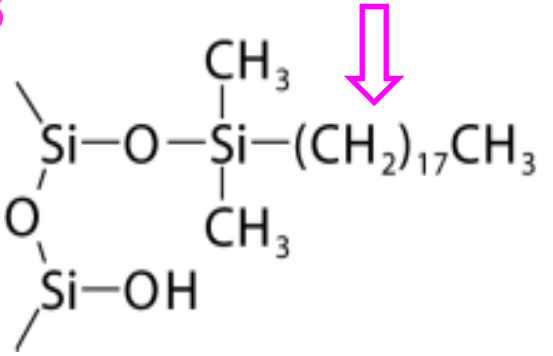
Reversed-Phase HPLC

Partition of analytes between mobile phase and stagnant phase inside the pore space + adsorption on the surface of bonded phase

C8

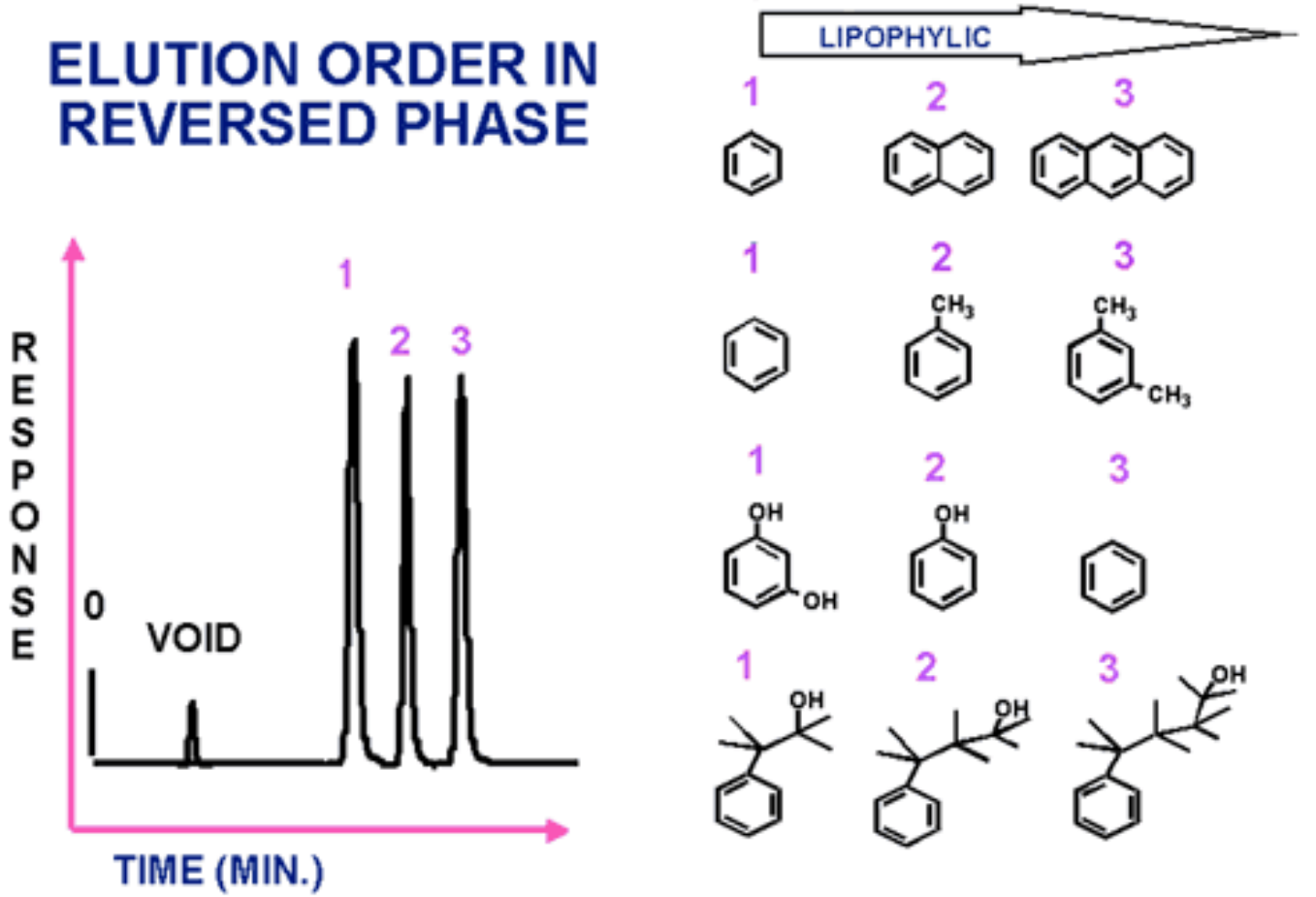


C18



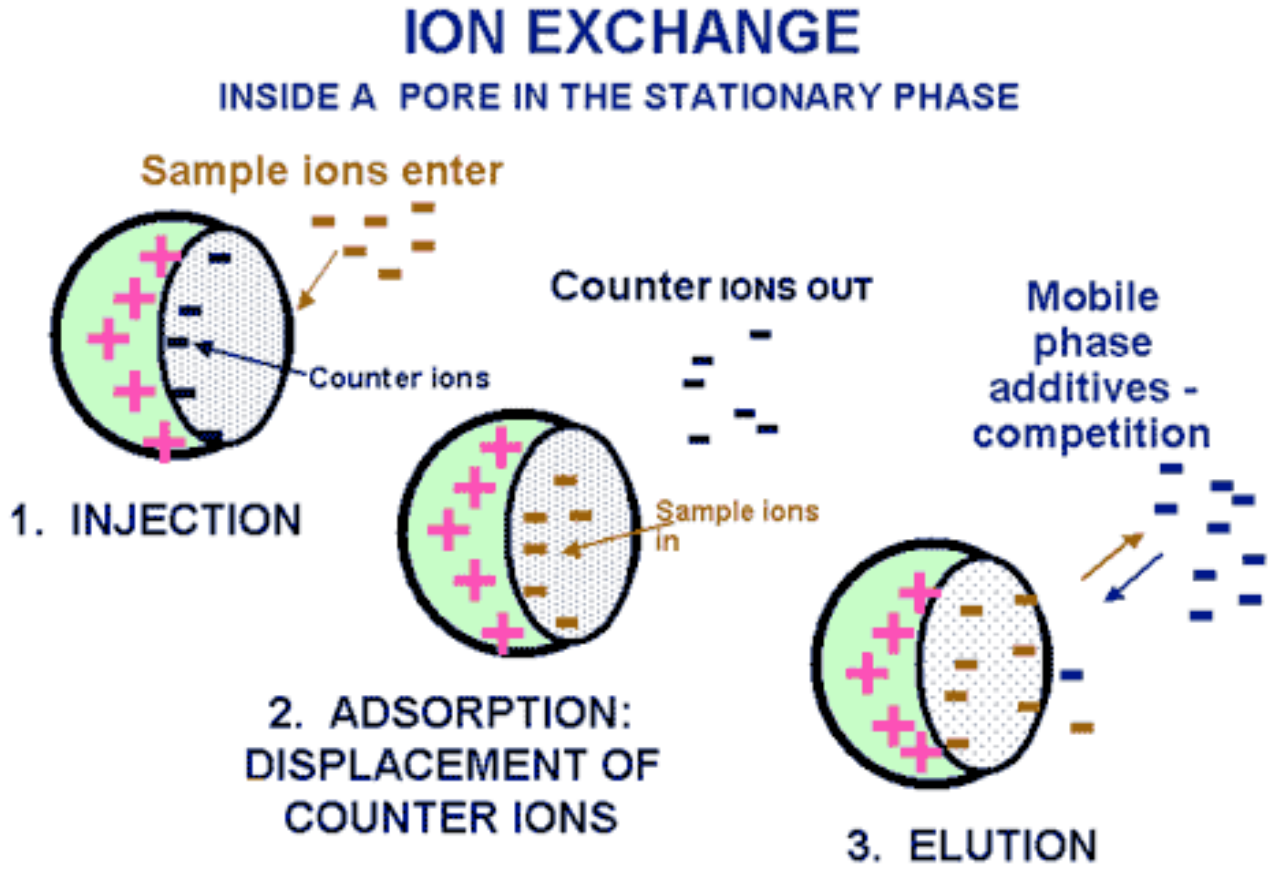
- Stationary Phase: **Hydrophobic surfaces** of moieties bonded on silica (C18, C8, C5, Phenyl, CN)
- Mobile phase: Methanol or Acetonitrile and Water.
- Applications: ~80% of all separations done on RP HPLC.

“Reverse” Phase Liquid Chromatography



In Reversed Phase separations organic molecules are separated based on their degree of hydrophobicity. There is a correlation between the degree of lipophylicity and retention in the column.

Ion Exchange Liquid Chromatography



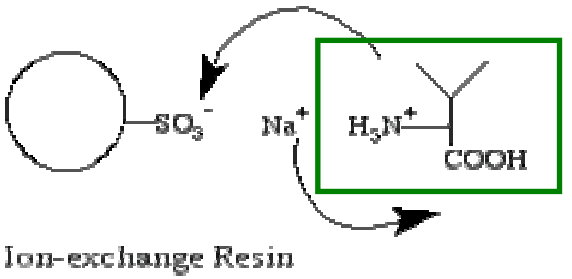
Elution order in ion exchange chromatography is determined by the charge density (charge/radius) of the hydrated ion. In organic acids and bases the elution order is determined by their pKa or pKb (strength of acid or base).

Different Types of Ion Exchange Resins

Cation exchanger

Charge of Analyte

+ charge



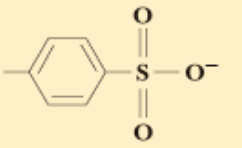
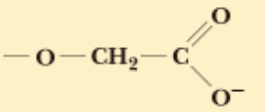
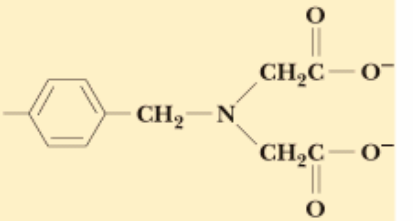
Ion-exchange Resin

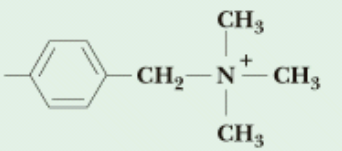
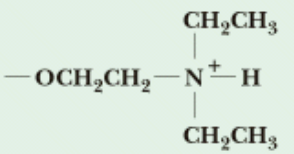
pH2

pH4.5

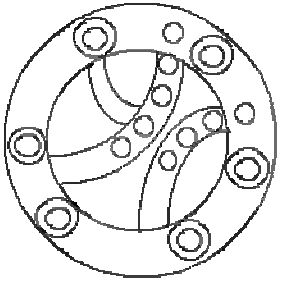
- charge

Anion exchanger.

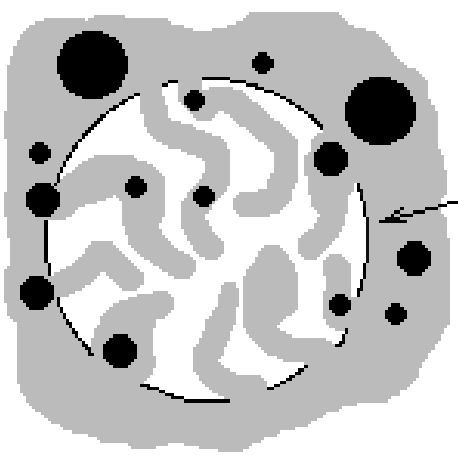
(a) Cation Exchange Media	Structure
Strongly acidic, polystyrene resin (Dowex–50)	
Weakly acidic, carboxymethyl (CM) cellulose	
Weakly acidic, chelating, polystyrene resin (Chelex–100)	

(b) Anion Exchange Media	Structure
Strongly basic, polystyrene resin (Dowex–1)	
Weakly basic, diethylaminoethyl (DEAE) cellulose	

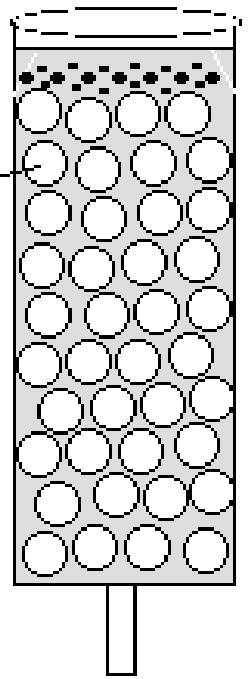
Gel Permeation Chromatography -- Molecular Sieve Chromatography

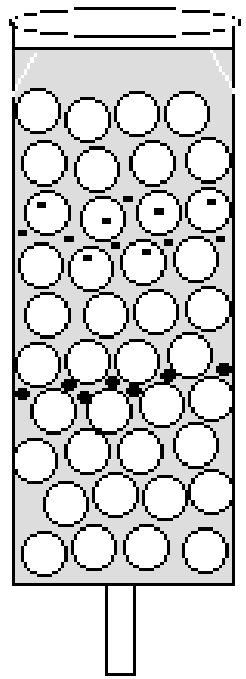


The separation is based on the molecule size and shape by the molecular sieve properties of a variety of porous material



Gel beads have pores in them of a defined size range which allows smaller molecules to enter but excludes molecules larger than the pore diameters.





Molecules smaller than gel bead pores

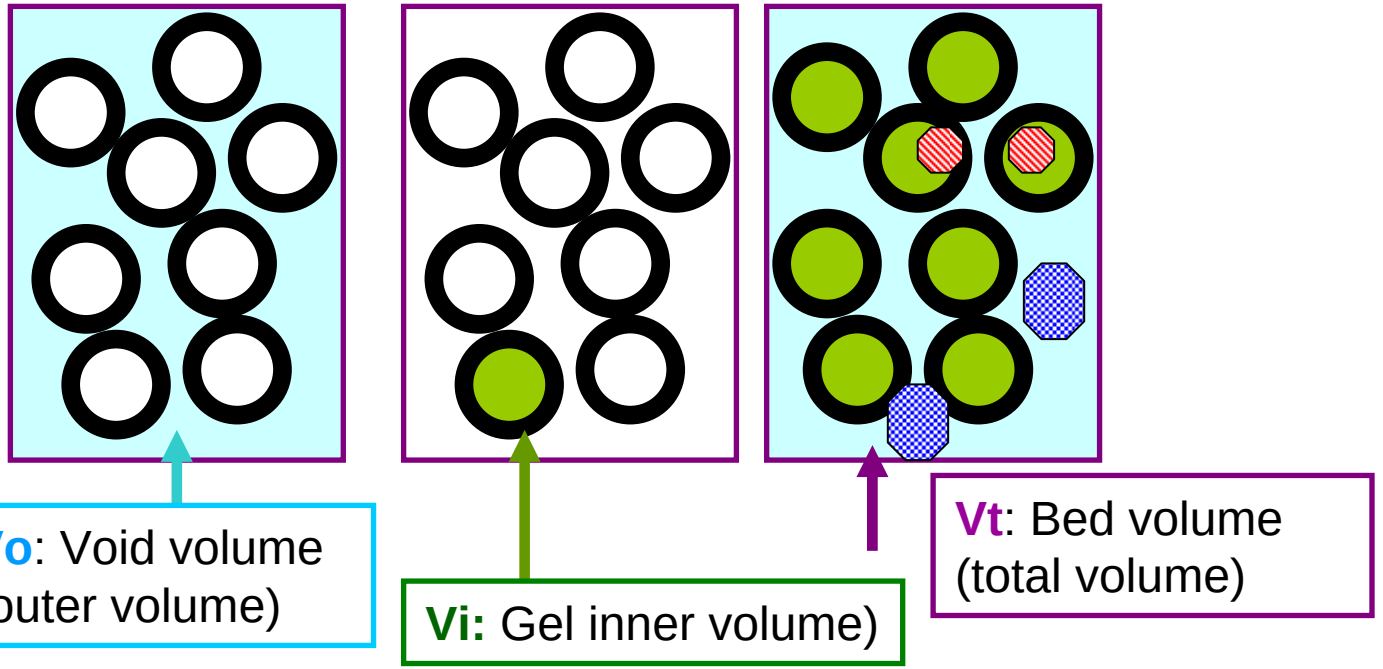
Molecules larger than gel bead pores

Gel Permeation Chromatography (GPC)

- Also known as ‘size exclusion chromatography’ and ‘gel filtration chromatography’
- Separates molecules on the basis of **molecular size**
- Separation is based on the use of a porous matrix. Small molecules penetrate into the matrix more, and their path length of elution is longer.
- Large molecules appear first, smaller molecules later

Mass measurement by Gel Permeation Chromatography

AB 3.4



Ve: Effluent volume (Elution volume of the desired protein)

$Ve = Vo + Kd \times Vi$

$Vi \approx Vt - Vo$

$$Kd = \frac{Ve - Vo}{Vt - Vo}$$

Kd: partition constant of solute between gel matrix and solvent

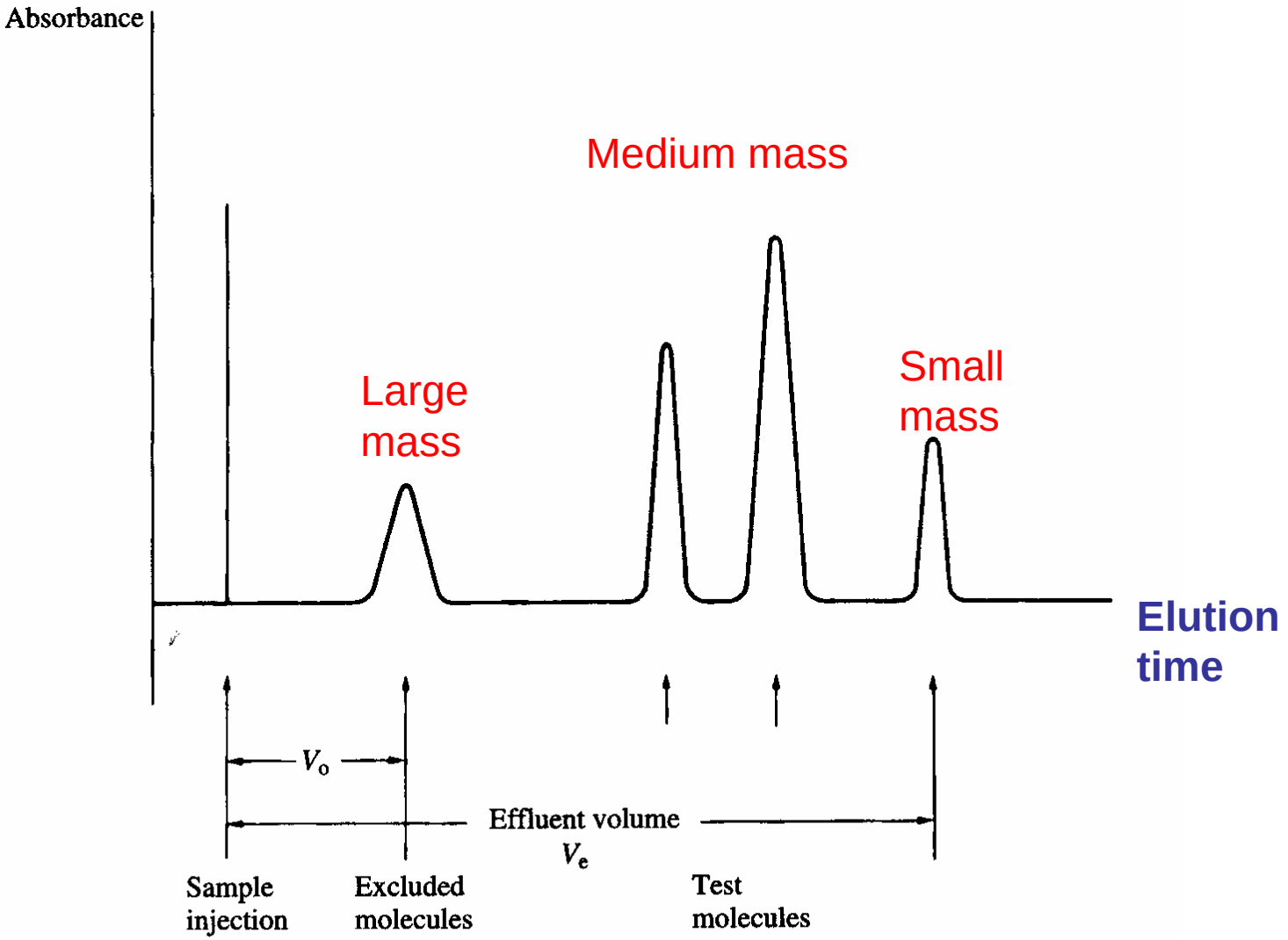
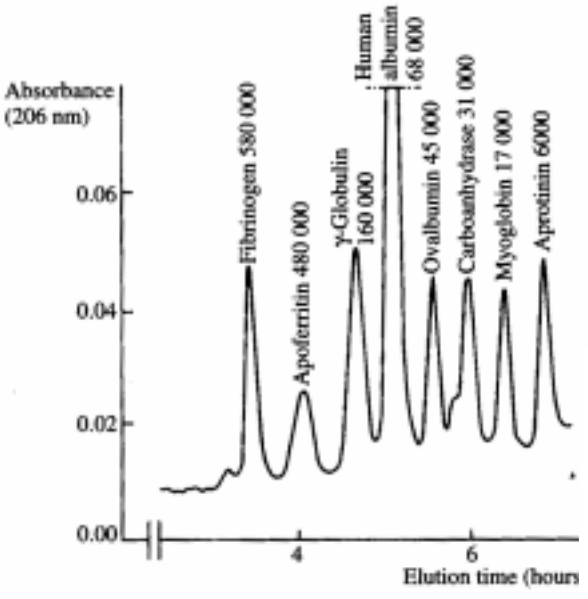
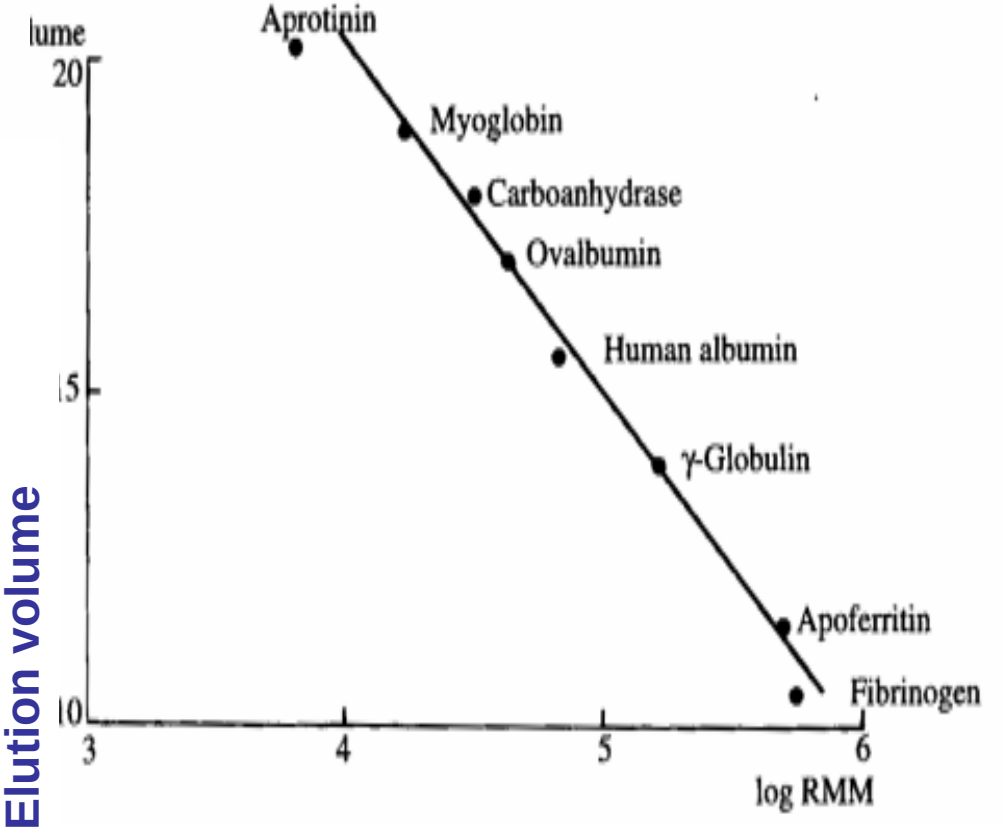


Figure 3.36 **Gel permeation chromatogram.** All molecules larger than the exclusion limit of the gel appear at V_o (the void volume). Molecules which can gain access to the gel structure to varying degrees are eluted in order of decreasing size.

Determination of Mass

The elution volume is approximately a linear function of the logarithm of the relative molecular mass



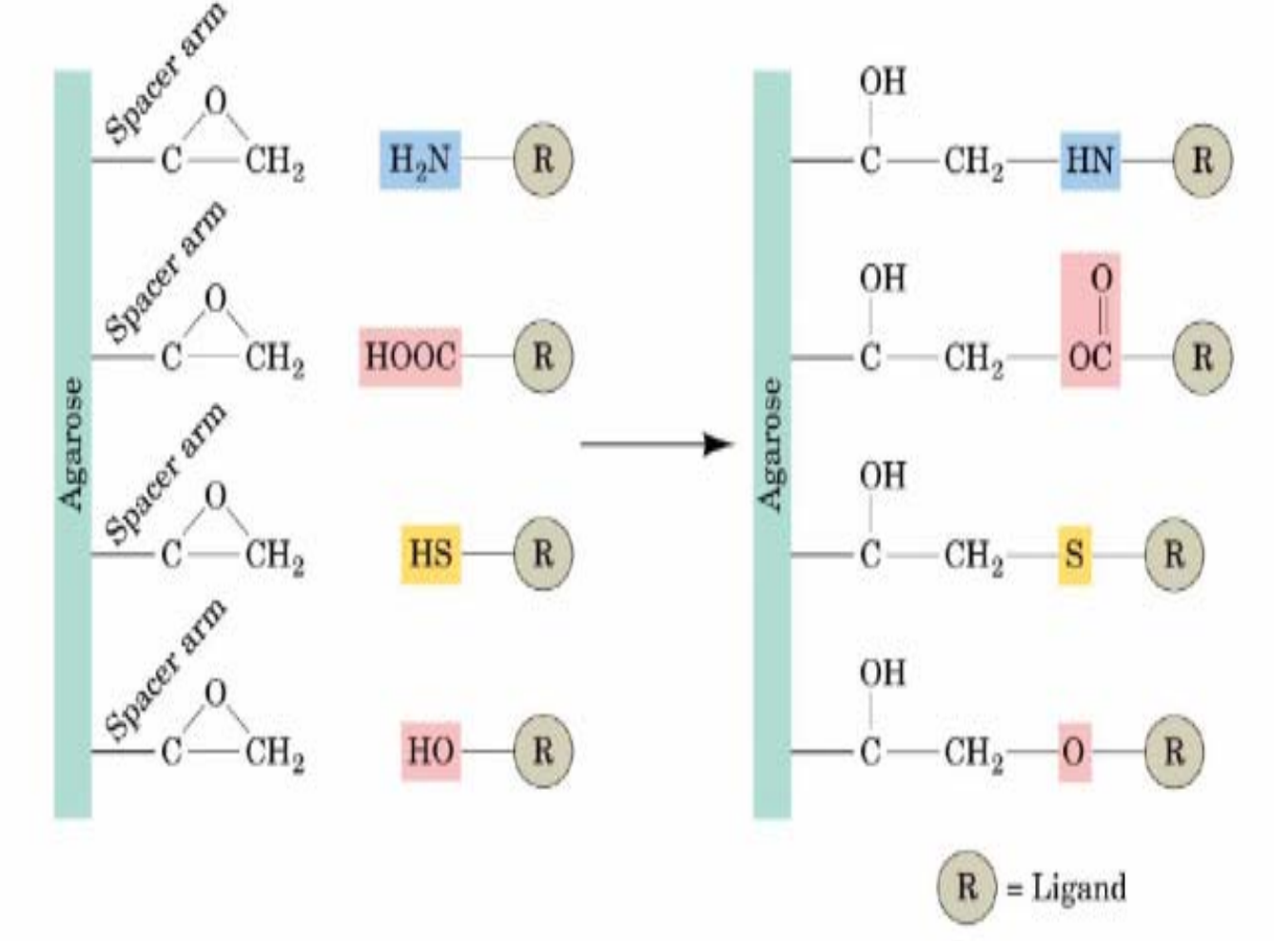


Affinity Chromatography

- Affinity chromatography is based on a (not necessarily biologically relevant) interaction between a protein of interest, and a ligand immobilized on a stationary phase substrate or product analogue
  - Antigen by Antibody:
  - Enzyme by Inhibitor /Substrate / Cofactor/coenzyme
- Specific protein is eluted by adding reagent which competes with binding

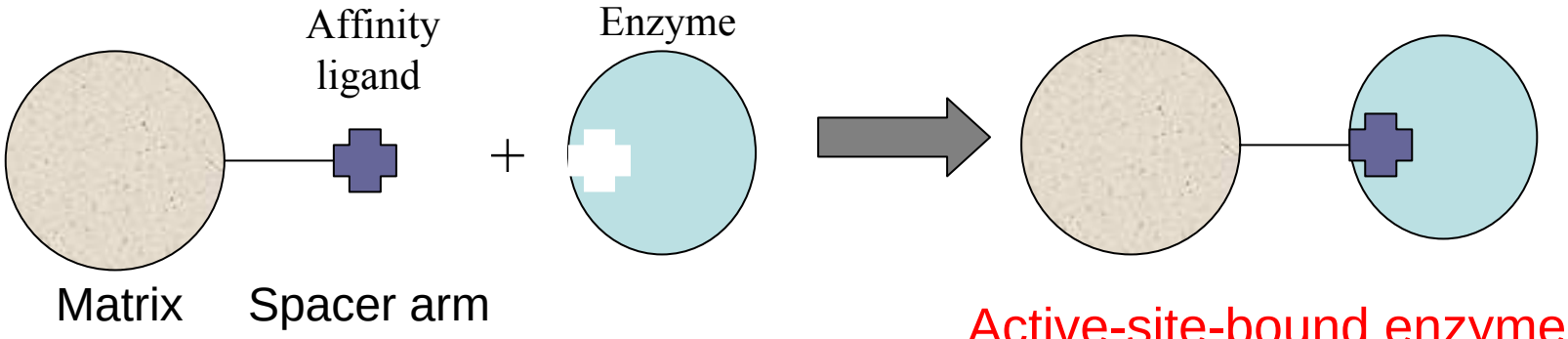
Covalent Attachment of Ligand to the Matrix

Derivation of Epoxy-Activated Agarose

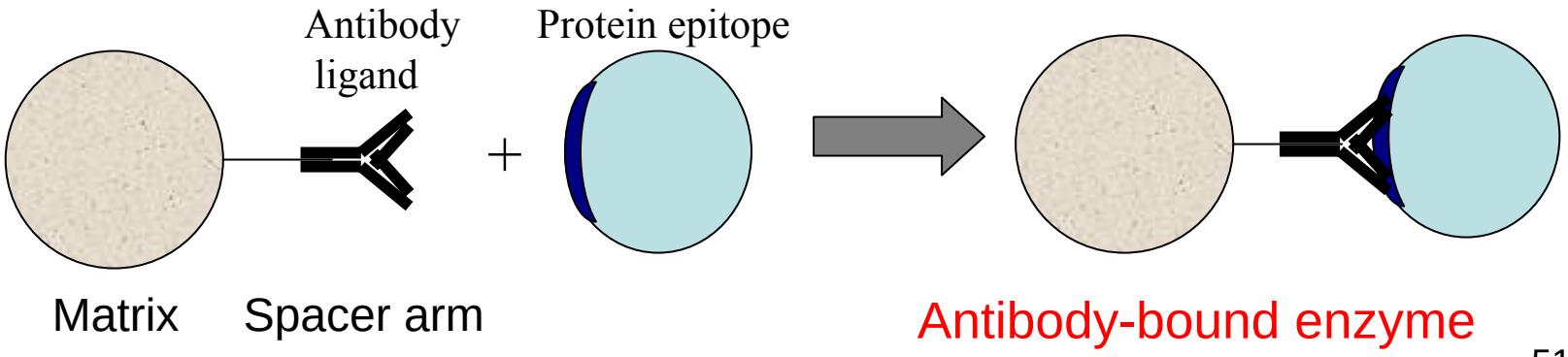


Affinity chromatography

- Substrate analogue affinity chromatography



- Immunoaffinity chromatography



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Journal of Chromatography B, 817 (2005) 35–47

www.elsevier.com/locate/chromb

Review

Multidimensional separation of peptides for effective proteomic analysis

Haleem J. Issaq*, King C. Chan, George M. Janini, Thomas P. Conrads, Timothy D. Veenstra

Laboratory of Proteomics and Analytical Technologies, SAIC-Frederick Inc., National Cancer Institute at Frederick,

It is generally accepted that no single chromatographic or electrophoretic procedure is capable of resolving the complex mixture of peptides. Therefore, combining two or more **orthogonal** (multimodal) separation procedures dramatically improves the overall resolution and results in a larger number of peptides being identified from complex proteome digests.

Chromatographic Modes of Protein Purification

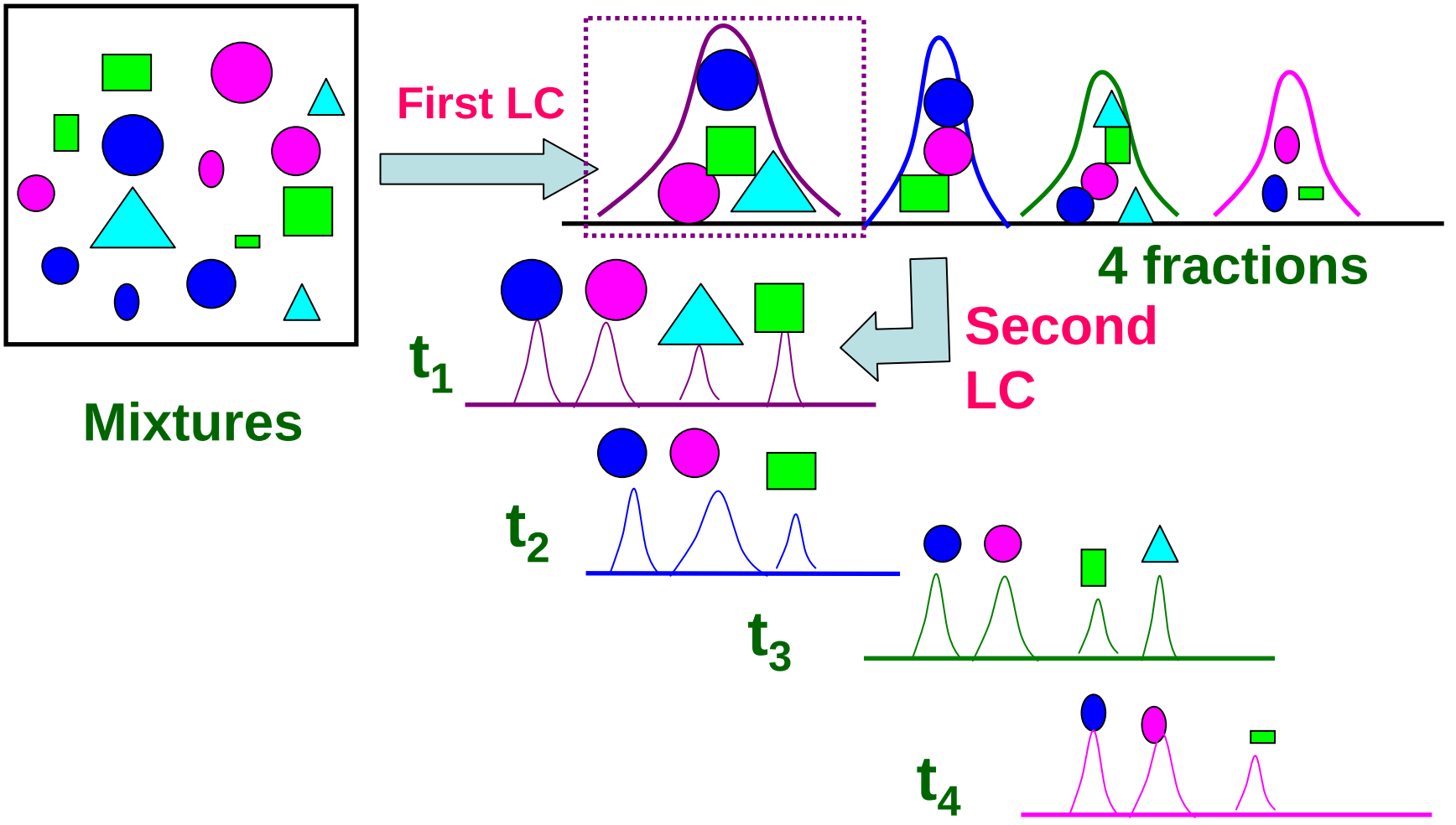
Chromatographic Mode	Acronym	Separation Principle
Non-interactive modes of liquid chromatography		
Size-exclusion chromatography	**SEC**	Differences in molecular size
Slalom chromatography (for DNA)	-	Diff. in length and flexibility
Interactive modes of liquid chromatography		
Ion-exchange chromatography	**IEC**	Electrostatic interactions
Normal-phase chromatography	**NPC**	Polar interactions
Reversed-phase chromtography	**RPC**	Dispersive interactions
Hydrophobic interaction chromatography	**HIC**	Dispersive interactions
Affinity chromatography	**AC**	Biospecific interaction
Metal interaction chromatography	**MIC**	Complex w/ an immobilized metal

(Christian G. Huber, Biopolymer Chromatography, Encylcopedia in analytical chemistry, 2000)

Multidimensional-Chromatography

- Transferring a fraction or fractions from one chromatographic medium (usually a column) to a secondary (or additional) chromatographic medium (column or columns) for further separation. The technique can be used for further resolution of complex mixtures that cannot be separated entirely on a single medium.
    - IEF-SCX
    - SCX-RP
    - SCF-Affinity

Two-dimensional Chromatography (2D-LC)



Complex Human Proteome

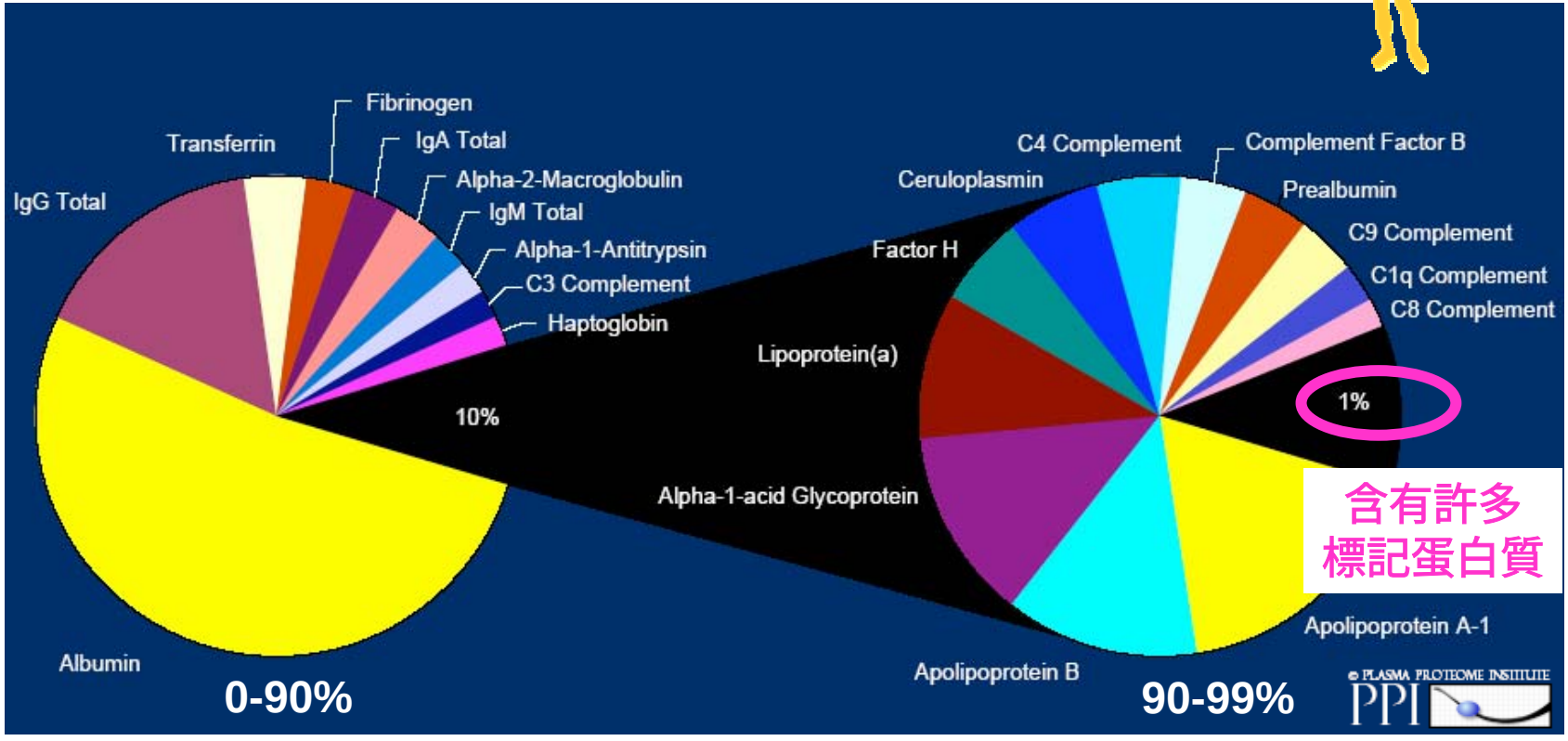
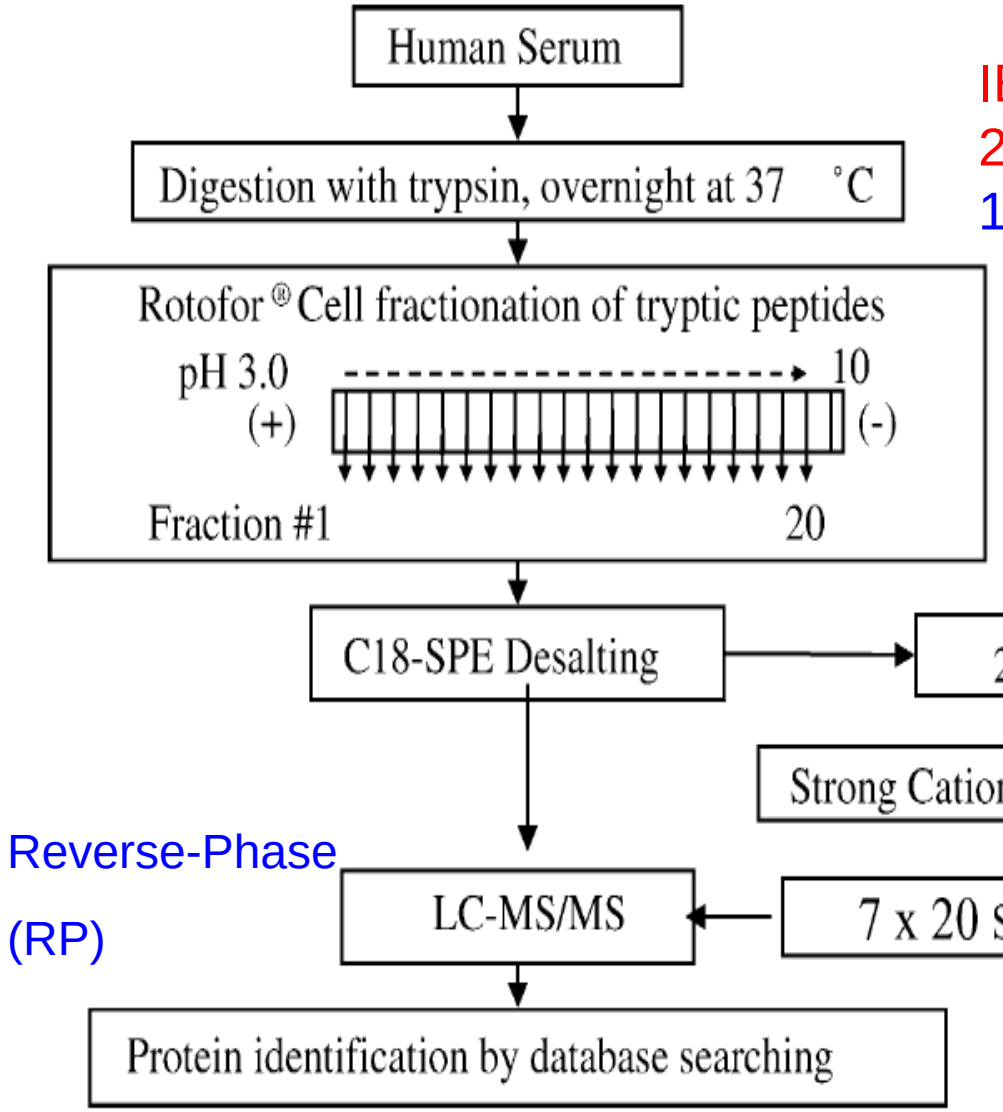


Fig. **Pie chart** representing the relative contribution of proteins within plasma.
Twenty-two proteins constitute 99% of the protein content of plasma

Ref: *www.plasmaproteome.org*
Molecular & Cellular Proteomics 2:1096–1103, 2003

3D LC for Global Analysis of Serum Proteome



IEF-SCX-RP
2071 peptides (identification of 1143 unique proteins).

Iso-electric Focusing

(IEF)

Strong-Cation Exchange

(SCX)

Reverse-Phase

(RP)

Clin. Proteomics, 1 (2004) 101.