Chromatography

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Separation techniques

Method	Basis of Method
Mechanical phase separation	
precipitation and filtration	Difference in solubility of compound formed
distillation	Difference in volatility of compound formed
extraction	Difference in solubility two immiscible liquids
ion exchange	Difference in interaction of reactant with ion exchange resin
Chromatography	Difference in rate of movement of solute through a stationary phase
Electrophoresis	Difference in migration rate of charged species in an electric filed
Filed-flow fractionation	Difference in interaction with a field or gradient applied perpendicular to transport direction

Introduction to Chromatography

Chromatography

- Chroma
  - Color
- graphy
  - To write

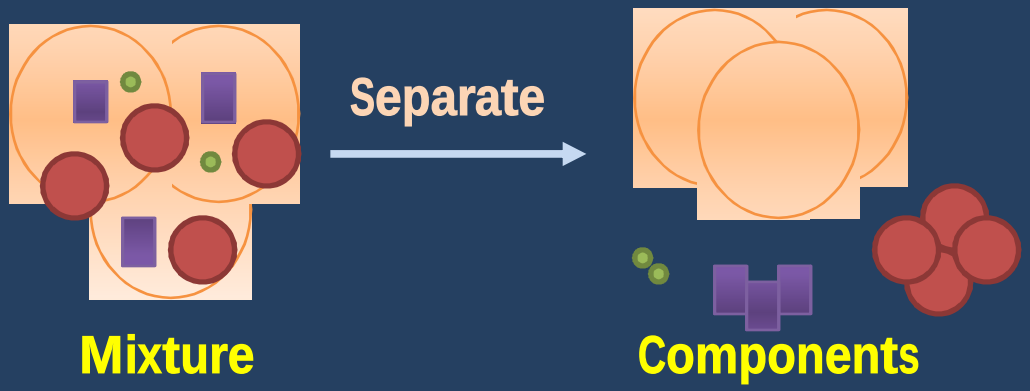
Chromatography is a technique which the components of a mixture are separated based on differences in the rate at which they carried though a stationary phase by mobile phase

There are two type of chromatography

- Preparative
  - Separates the components of a mixture For further use (Purification)
- Analytical
  - Measure the relative proportions components of a mixture (small amount)

Introduction to Chromatography

Chromatography is a physical method of separation in which the components to be separated are distributed between 2 phases; mobile phase and stationary phase.



- **Analyze**
- **Identify**
- **Purify**
- **Quantify**

The two phase of chromatography are:

Mobile Phase

Phase that does move

A gas, a liquid or a supercritical fluid

Stationary Phase

Phase that doesn't move

A solid or a liquid held on a solid support

The phases are chosen such that components of the sample have different solubilities in each phase

Chromatographic Terms

Sample

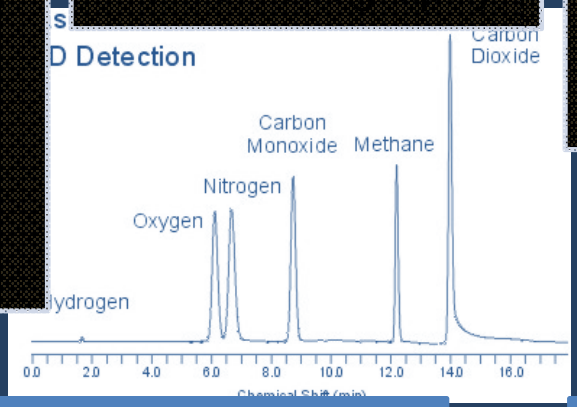
- The matter which is analyzed in chromatography. It can be a single component or a mixture.

Analyte

- substance which is to be separated in chromatography

Elution

- Separation of analyte from the stationary phase by the mobile phase



Chromatogram

Chromatograph

Principle of Chromatography

Difference in the behavior of analyte towards mobile phase and stationary phase Component interact with the stationary phase based on

1. **Charge: ion-ion interaction or ion-dipole interaction**
2. **Van der Waals‘ forces**
3. **Relative solubility**
4. **Adsorption: hydrophobic interactions or specific affinity**

The rate of migration of solute through a stationary phase

$$K_c = \frac{C_S}{C_m}$$

C_s = Total solute concentration in stationary phase

C_m = Total solute concentration in mobile phase

Large K_c means slow solute migration
Small K_c value shows rapid solute migration

Usually retention time and retention factor are measured instead of distribution constant

Retention Time

A measure of speed with which a substance moves though chromatographic system

Continuous development system

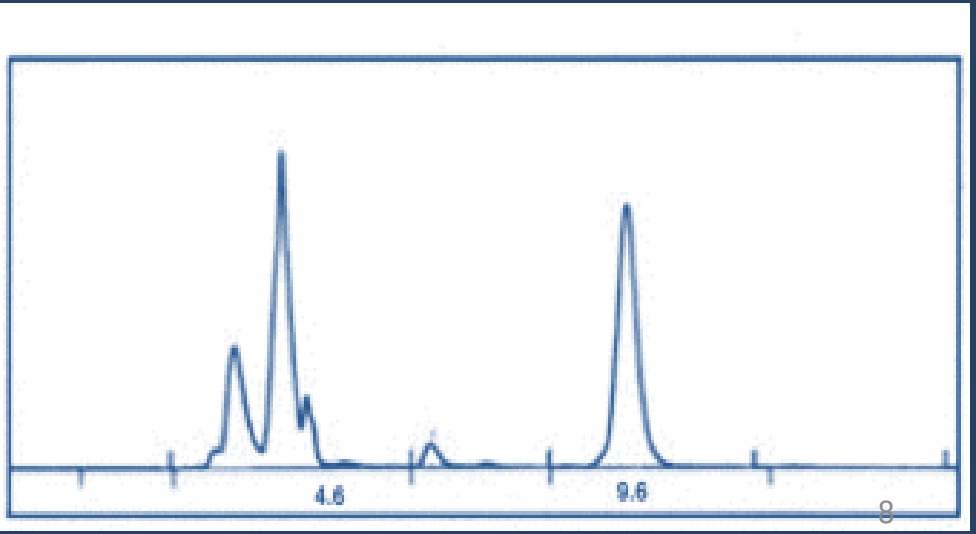
- eg. HPLC, GC
- R-t or t_R, the time between injection and detection

Interrupted development system

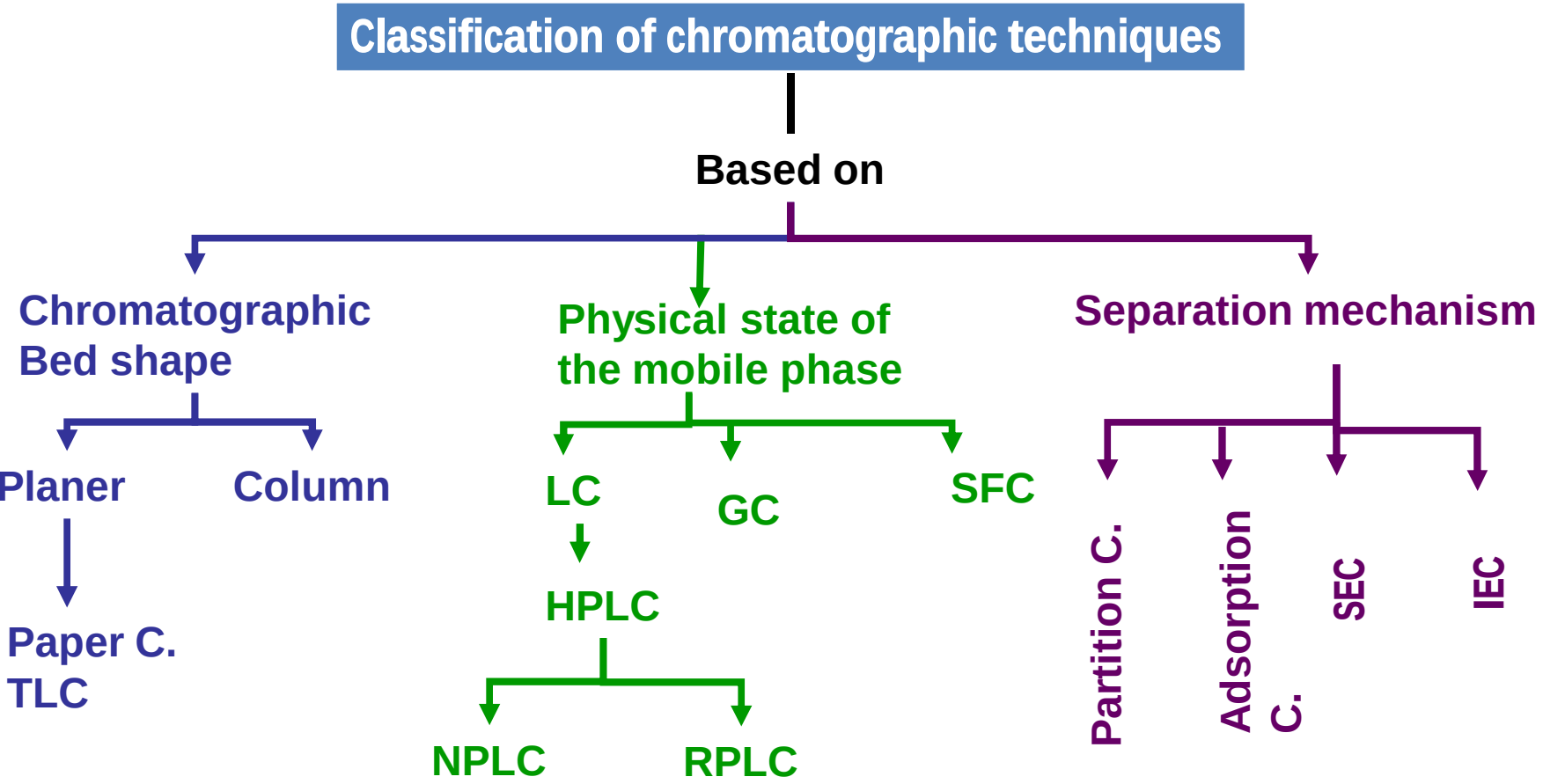
- eg. TLC, PC
- R_f = Distance moved by the compound / Distance moved by the solvent

Factors effecting retention time:

- Variation of the eluent
- The stationary Phase
- Temperature
- Sample Matrix
- The set up



Classification of Chromatographic Techniques



Classification of Chromatographic Techniques Based on Bed Shape

Planer Chromatography

Package

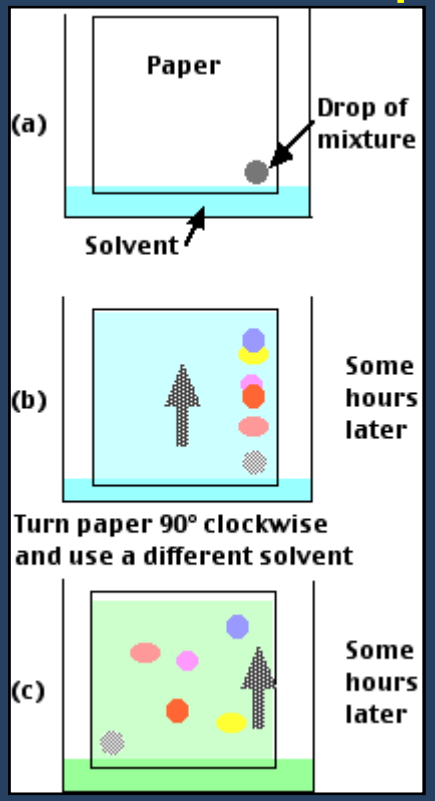
The stationary phase is present as or on plane

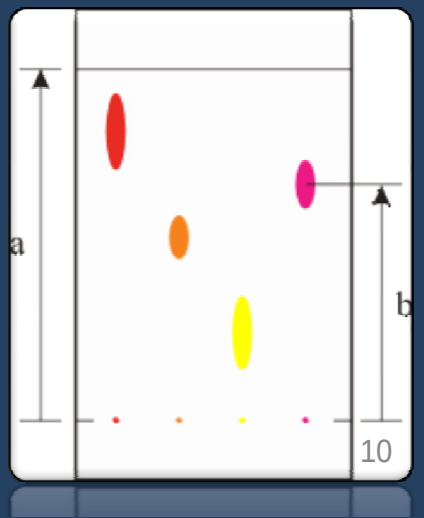
Paper Chromatography

A form of chromatography in which a sheet of special paper is used for adsorption

Thin Layer Chromatography (TLC)

Involves a stationary phase of a thin layer of adsorbent like silica gel, alumina, or cellulose on a flat, inert substrate





TLC advantage over the paper chromatography

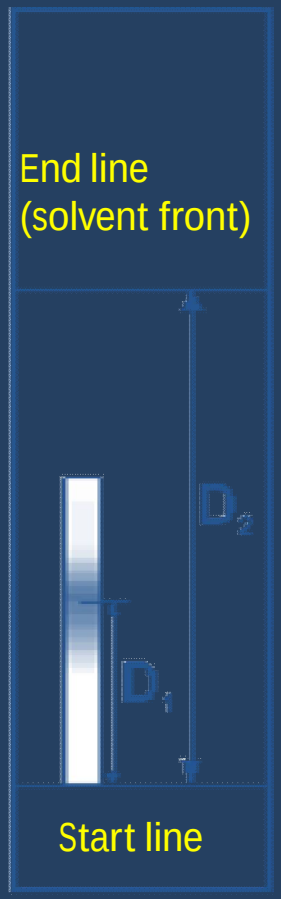
- Faster run
- Better separation
- choice between different adsorbents

Retention Factor (R_f) for TLC

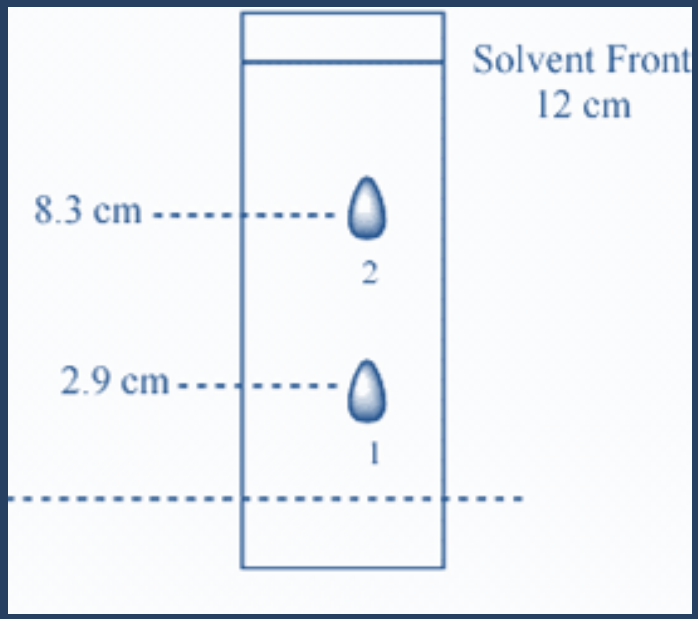
R_f= distance migrated by the component (a) / distance migrated by the solvent (d)

$$R_f = D_1 / D_2$$

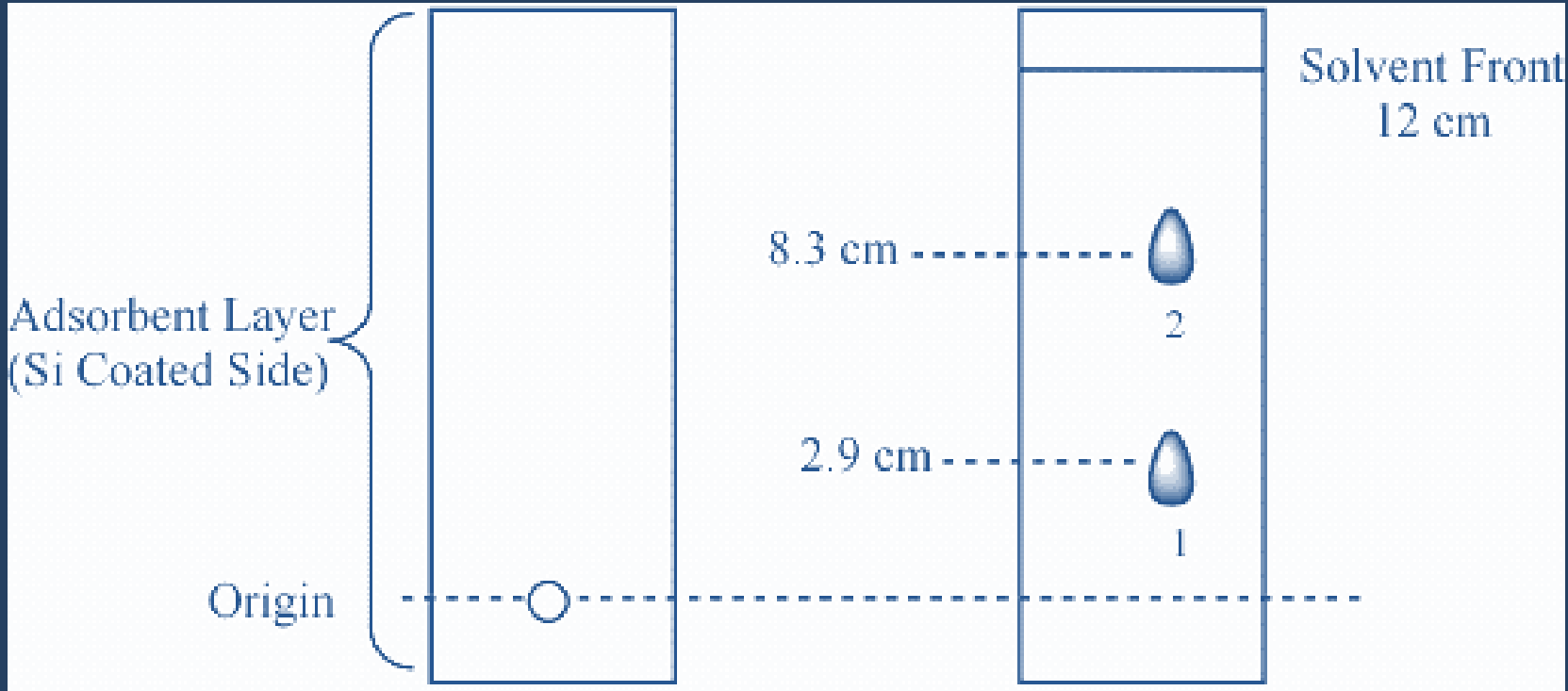
D1 = distance that band traveled, measured from center of the band of color to the point where the spot color was applied
D2 = total distance that solvent traveled
R_f value should be between 0.0 and 1.0 and is unit less.



Example
A mixture of two component were separated using TLC , and the following result fond .Find out retention factor for both component1, 2



Retention Factor (R_f) for TLC: Cont. Example



R_f = Distance Traveled by Spot / Distance Traveled by Solvent

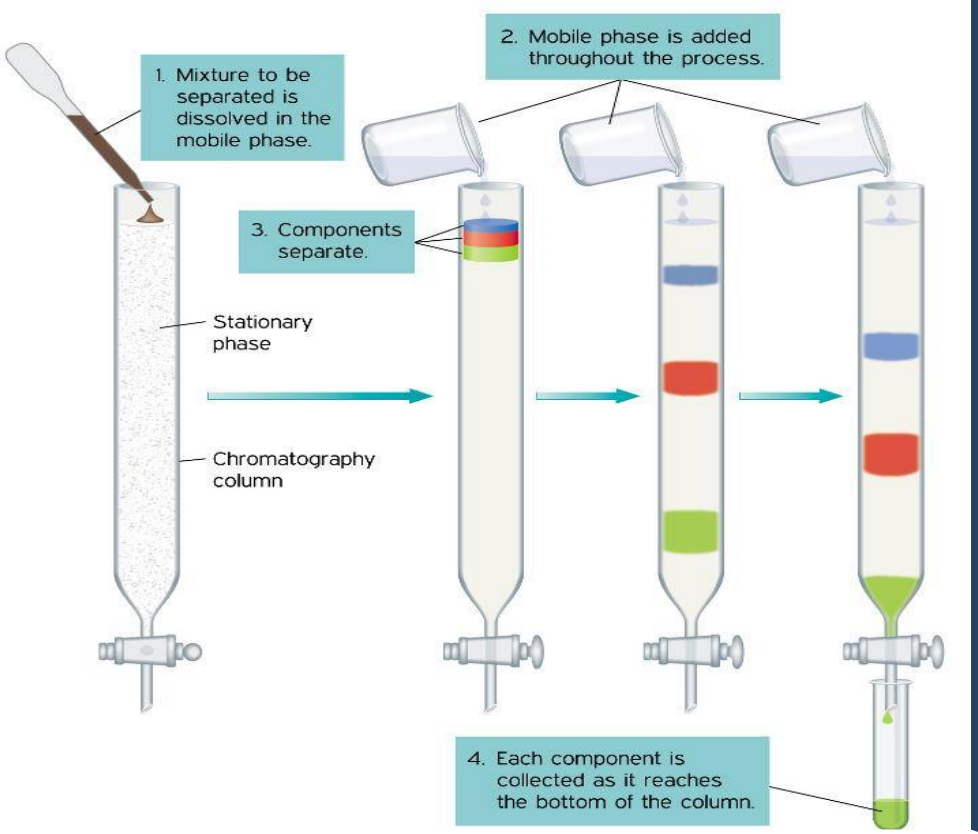
$R_f(1) = 2.9 \text{ cm} / 12 \text{ cm} = 0.24$

$R_f(2) = 8.3 \text{ cm} / 12 \text{ cm} = 0.69$

Note: R_f Values Are Always Less Than 1

Classification of Chromatographic Techniques Based on Bed Shape

Column Chromatography



- Probably the most common form of Chromatography
- Consists of a tightly packed bead matrix in a tube
- Mobile phase enters the column and flows out at a constant rate
- Usually coupled to a detection device
- Column can be used manually or in instruments

Types of column chromatography

1. liquid chromatography (LC)..... mobile phase is liquid
2. gas chromatography (GC) ..... mobile phase is gas
3.supercritical chromatography (SFC). ..... mobile phase is supercritical fluid

General Classification	Specific Method	Stationary Phase	Type of Equilibrium
Gas chromatography (GC)	a. Gas-liquid chromatography (GLC)	Liquid adsorbed or bonded to a solid surface	Partition between gas and liquid
	b. Gas-solid	solid	Adsorption
Liquid Chromatography (LC)	a. Liquid-liquid or partition	Liquid adsorbed or bonded to a solid surface	Partition between immiscible liquid
	b. Liquid-solid or adsorption	Solid	Adsorption
	c. Ion exchange	Ion exchange resin	Ion exchange
	d. Size exclusion	Liquid in interstices of a polymeric solid	Partition
	e. Affinity	Group specific liquid bonded to a solid surface	Partition between supercritical fluid and bonded surface
Supercritical fluid chromatography(SFC)		Organic species bonded to a solid surface	Partition between supercritical fluid and bonded surface

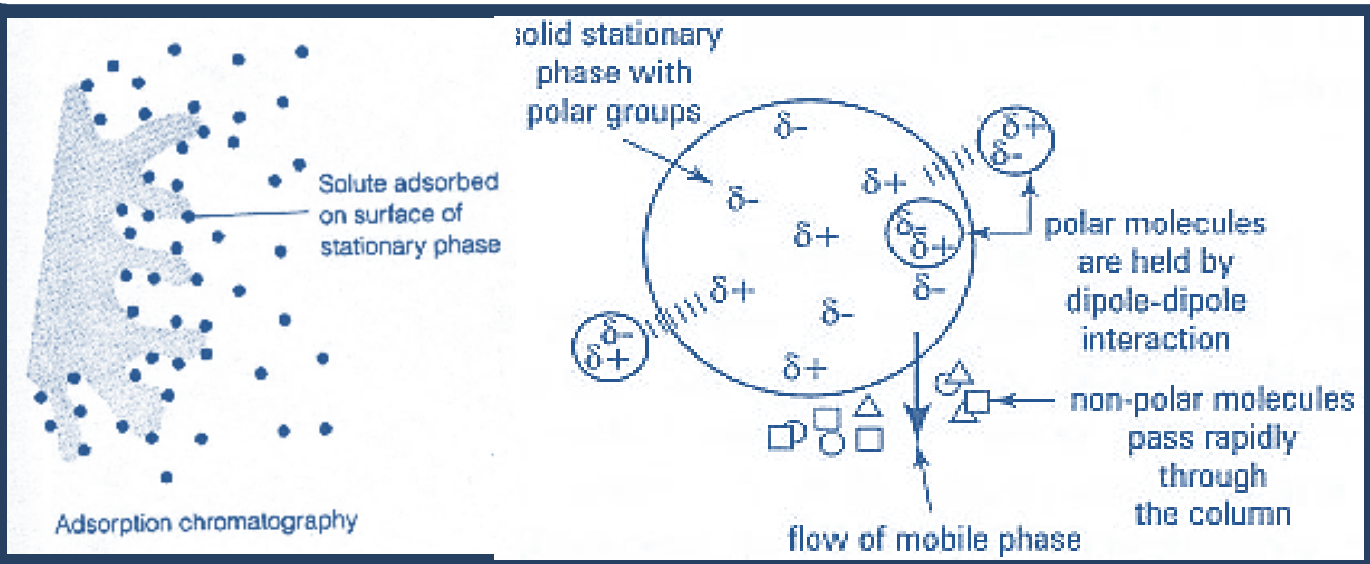
Mechanisms of separation in chromatography

- Adsorption Chromatography
- Partition Chromatography
- Ion-exchange Chromatography (IEC)
- molecular Exclusion Chromatography
- Affinity Chromatography

Adsorption Chromatography

Analyte species are adsorbed onto the surface of a polar stationary phase

The components of mixture interacts with the stationary phase due to dipole-dipole interaction



Application

Separation of relatively non-polar, water-insoluble organic compound

Mobile phase

- Liquid or gas
- Organic solvent or mixture of organic solvent

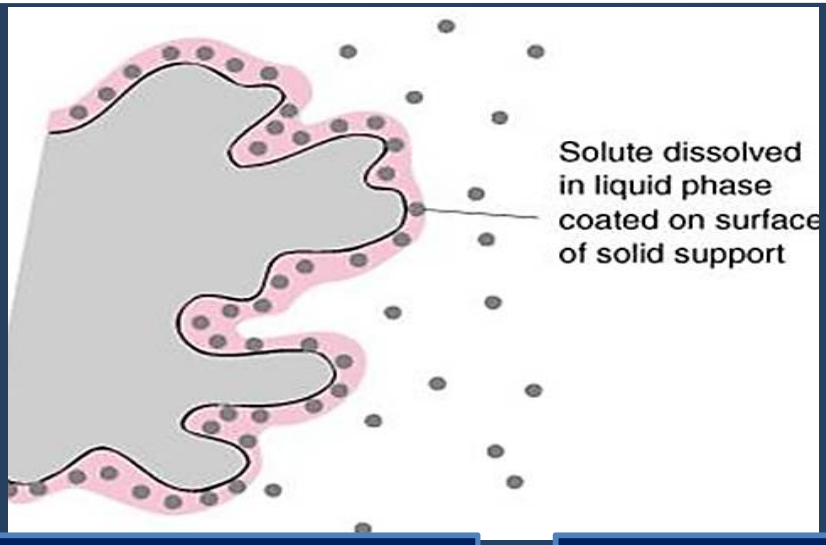
Stationary phase

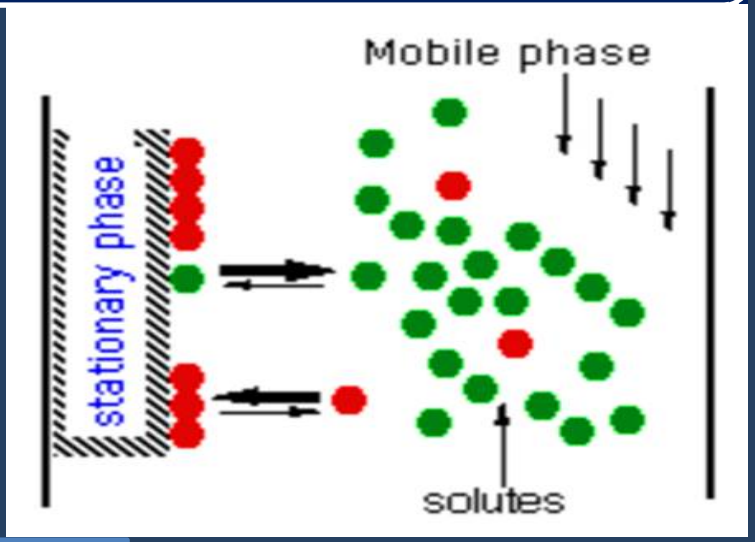
- Solid
- Finely divided polar solid that contains sites for retention of analyte
- Silica & alumina are the only used

Partition Chromatography

Components partition(dissolve) themselves to different degree between liquid stationary Phase & mobile phase

The components of mixture interacts with the stationary phase due to dipole-dipole interaction





Mobile phase

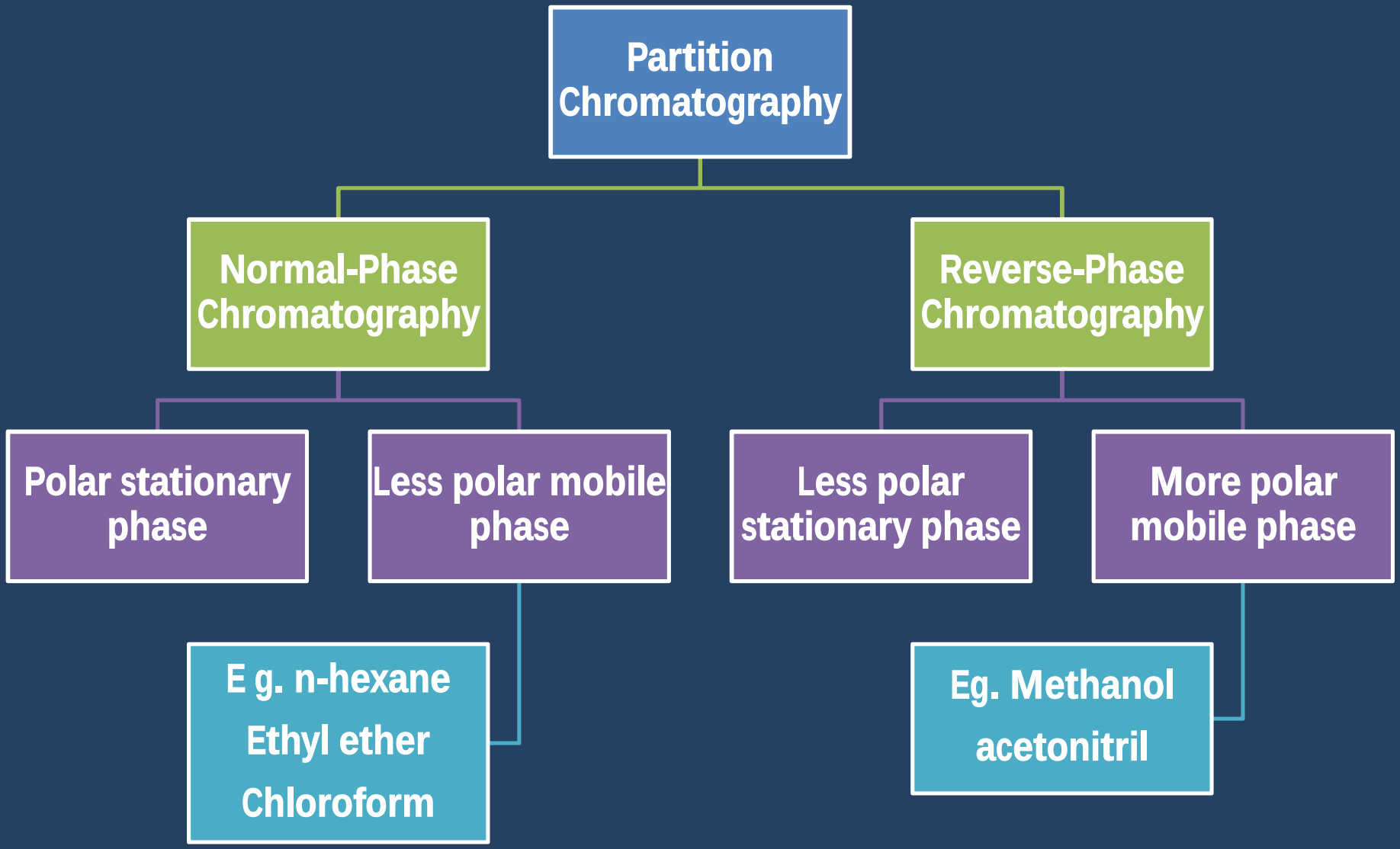
Stationary phase

- **Liquid or liquid adsorbed on a solid**

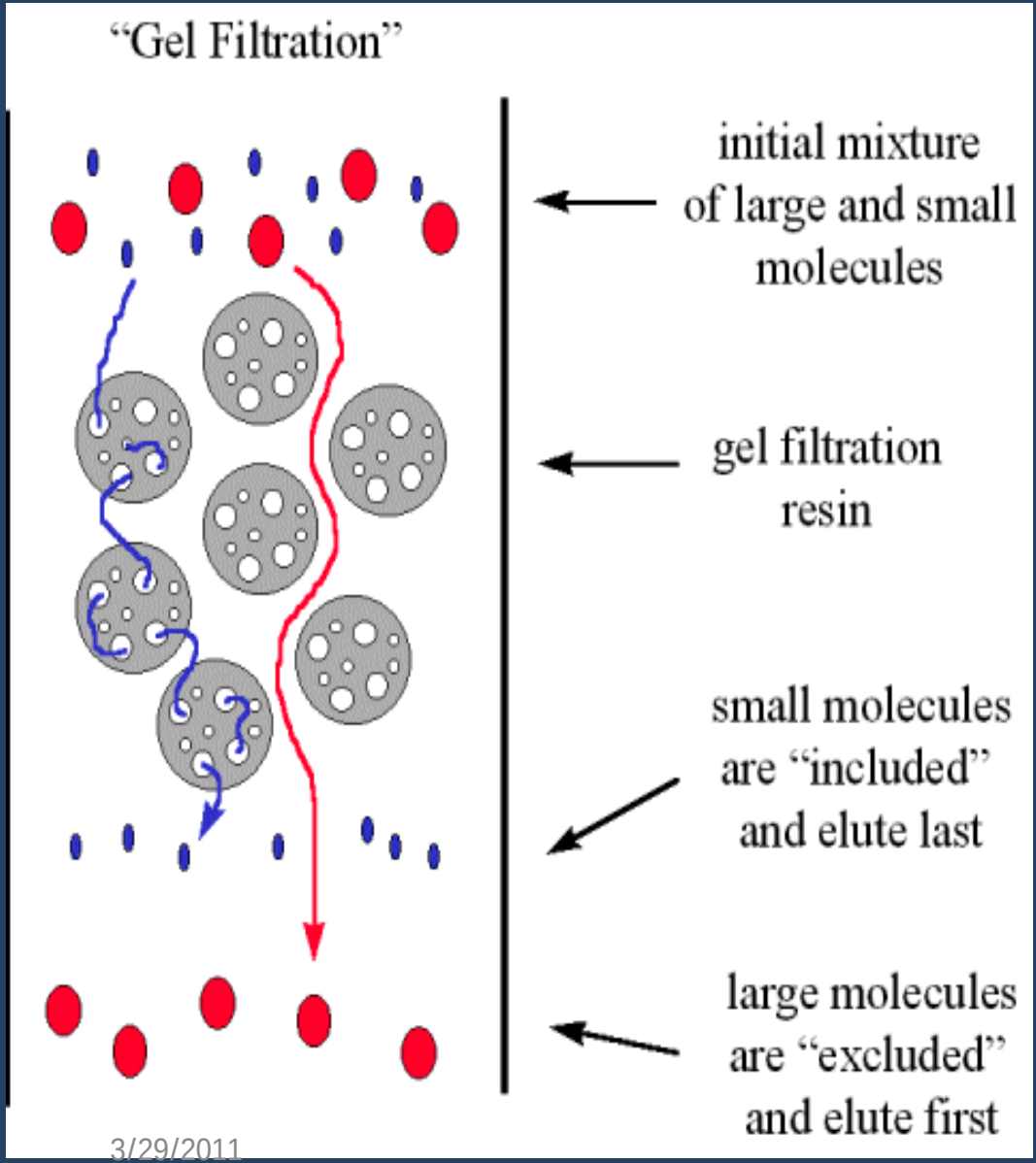
Application

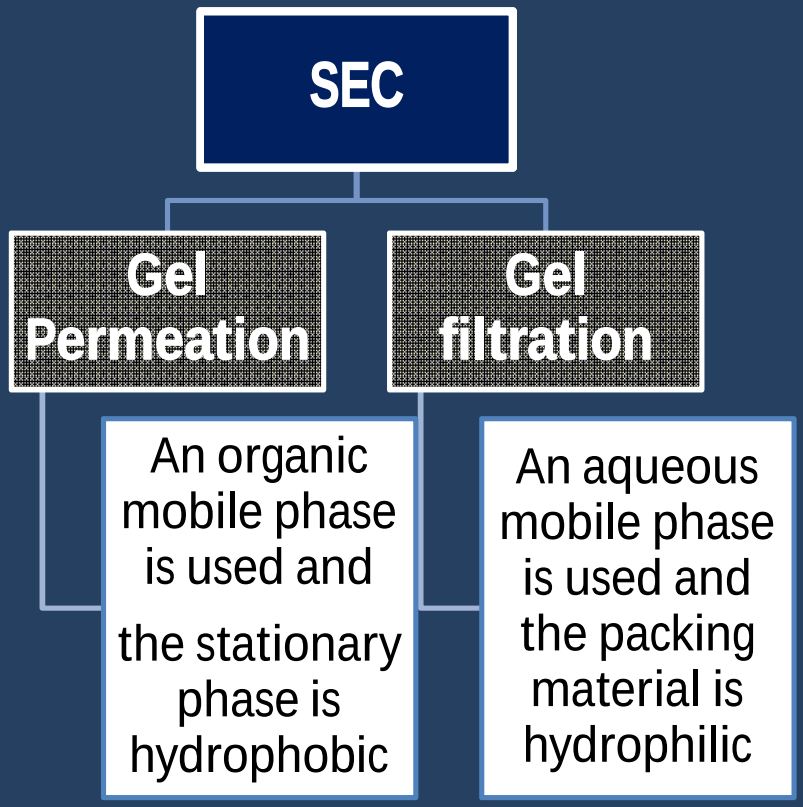
non-ionic compounds, polar compounds, and in certain cases ionic compounds

Partition Chromatography



Molecular Exclusion Chromatography





Gel Permeation

Principle	• Separates particles on the base of the size. • High molecular weight solutes (>10,000)
Separation	• The components of a mixture are separated according to their ability to penetrate into the pores of the stationary phase material
Packing Materials	• are wide-pore silica gel, polysaccharides, and synthetic polymers
Application	• the separation of large molecular weight biomolecules, and molecular weight distribution studies of large polymers and natural products

Size Exclusion Chromatography (SEC)

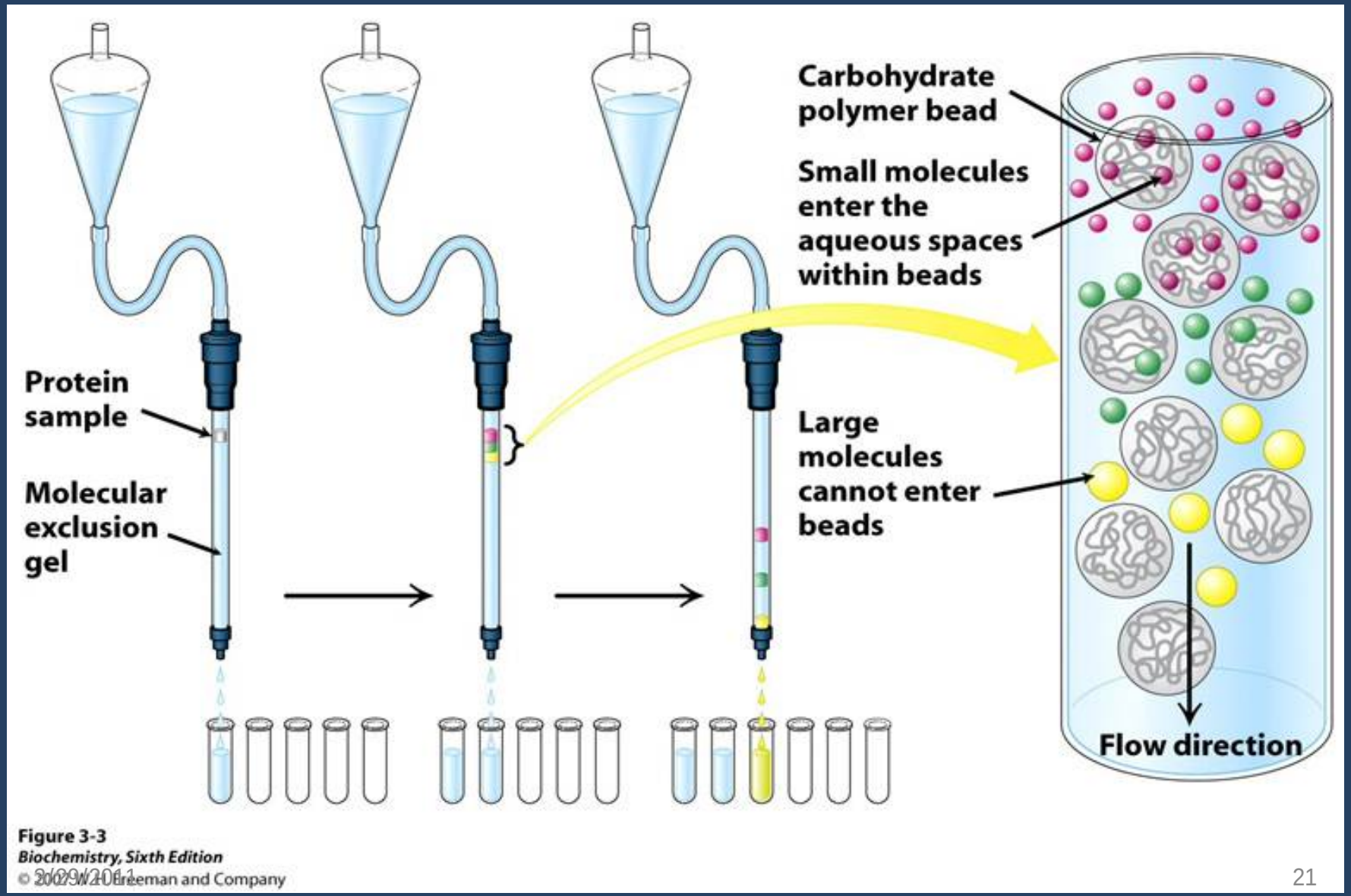


Figure 3-3
Biochemistry, Sixth Edition

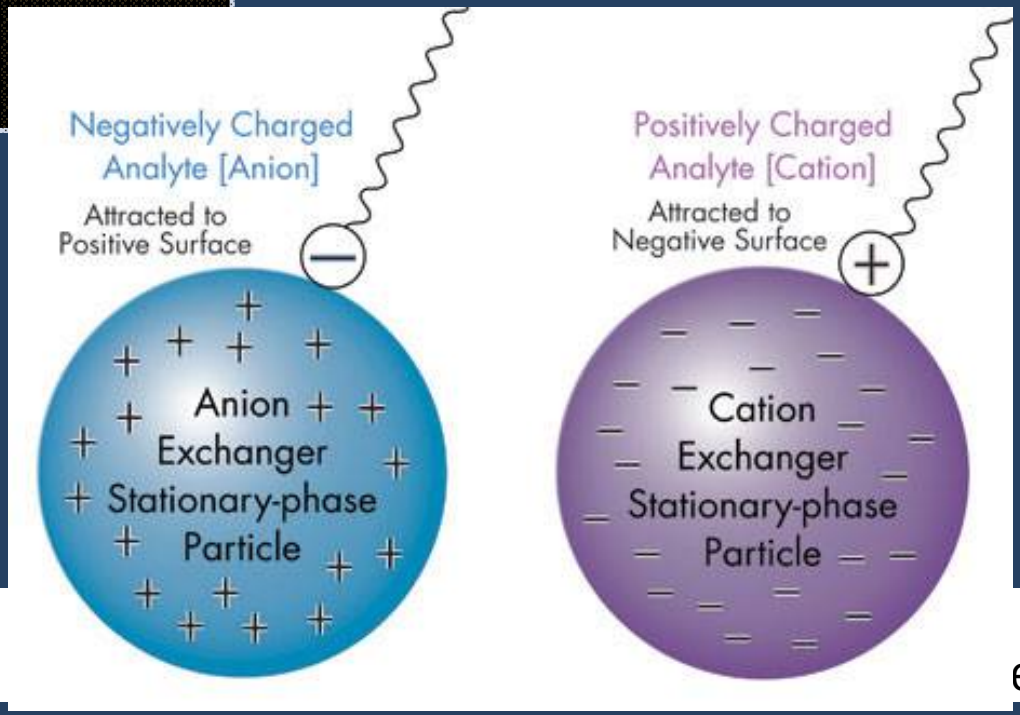
Ion-exchange Chromatography (IEC)

The stationary phase is small solid polymer resin beads that have ionic bonding sites on their surfaces.

The **resin** contains electrically charged sites at which one ion replace another

Ion exchange resin

- Zeolite
- Clay
- Synthetic resin

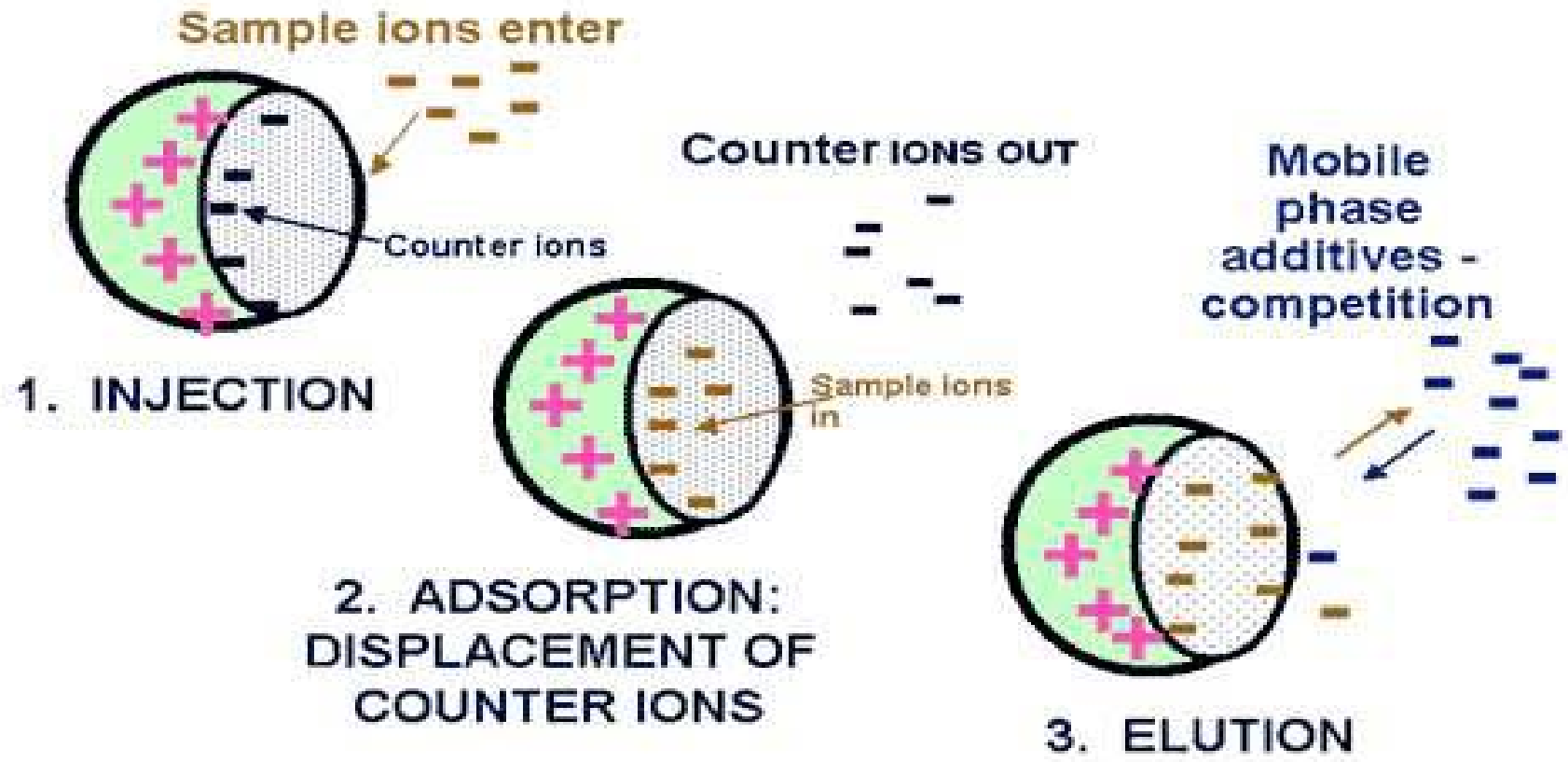


Cation exchange resin

Anion exchange resin

ION EXCHANGE

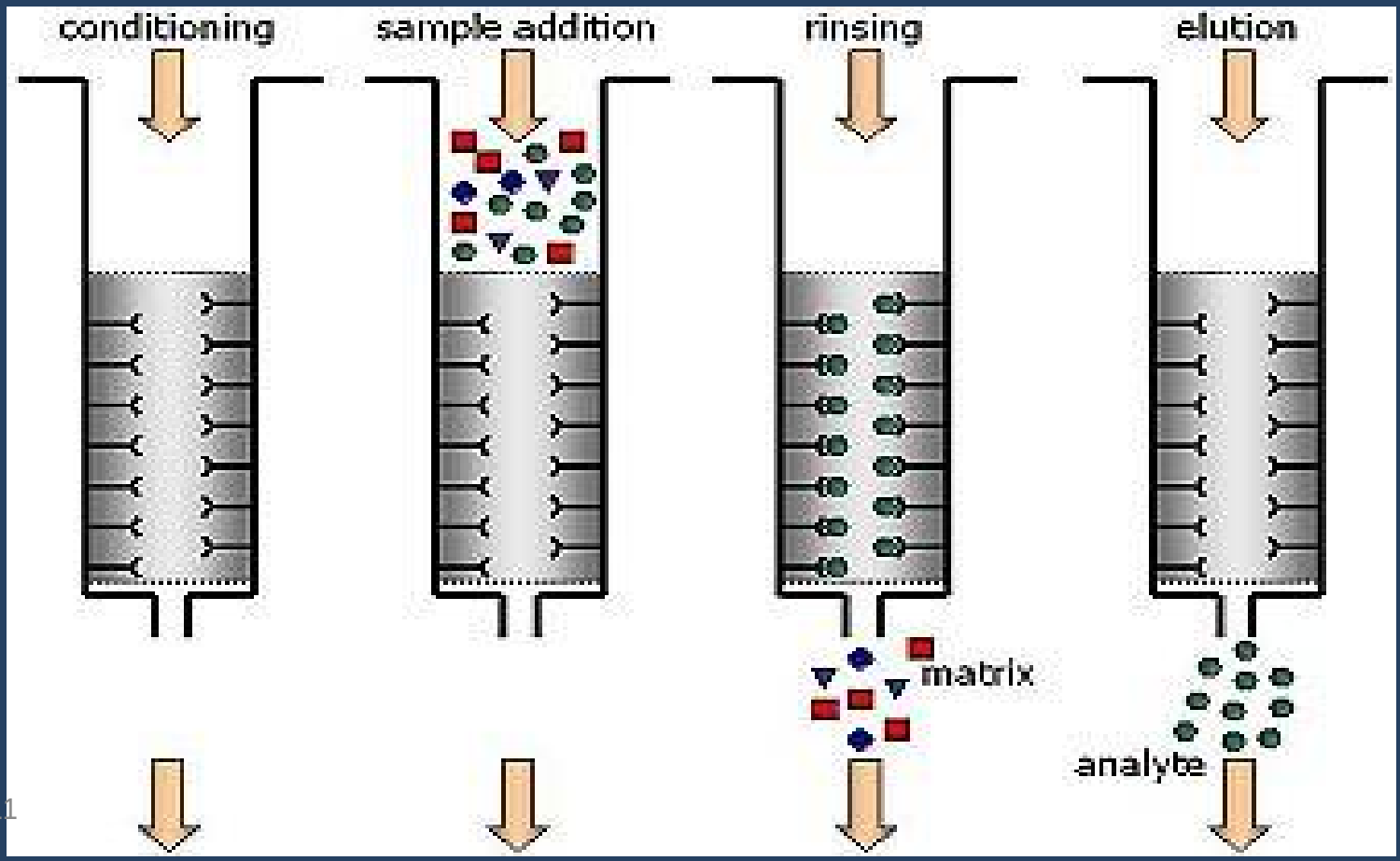
INSIDE A PORE IN THE STATIONARY PHASE



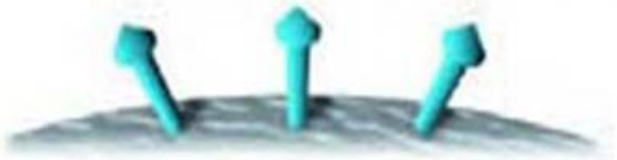
Affinity Chromatography

most selective kind of chromatography

specific interactions between one kind of solute molecule and a second molecule that is covalently attached (immobilized) to the stationary phase.



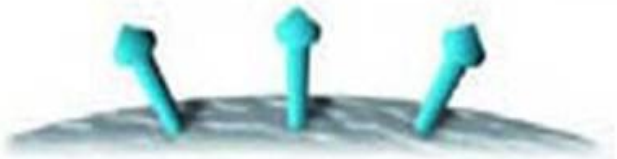
Affinity Chromatography



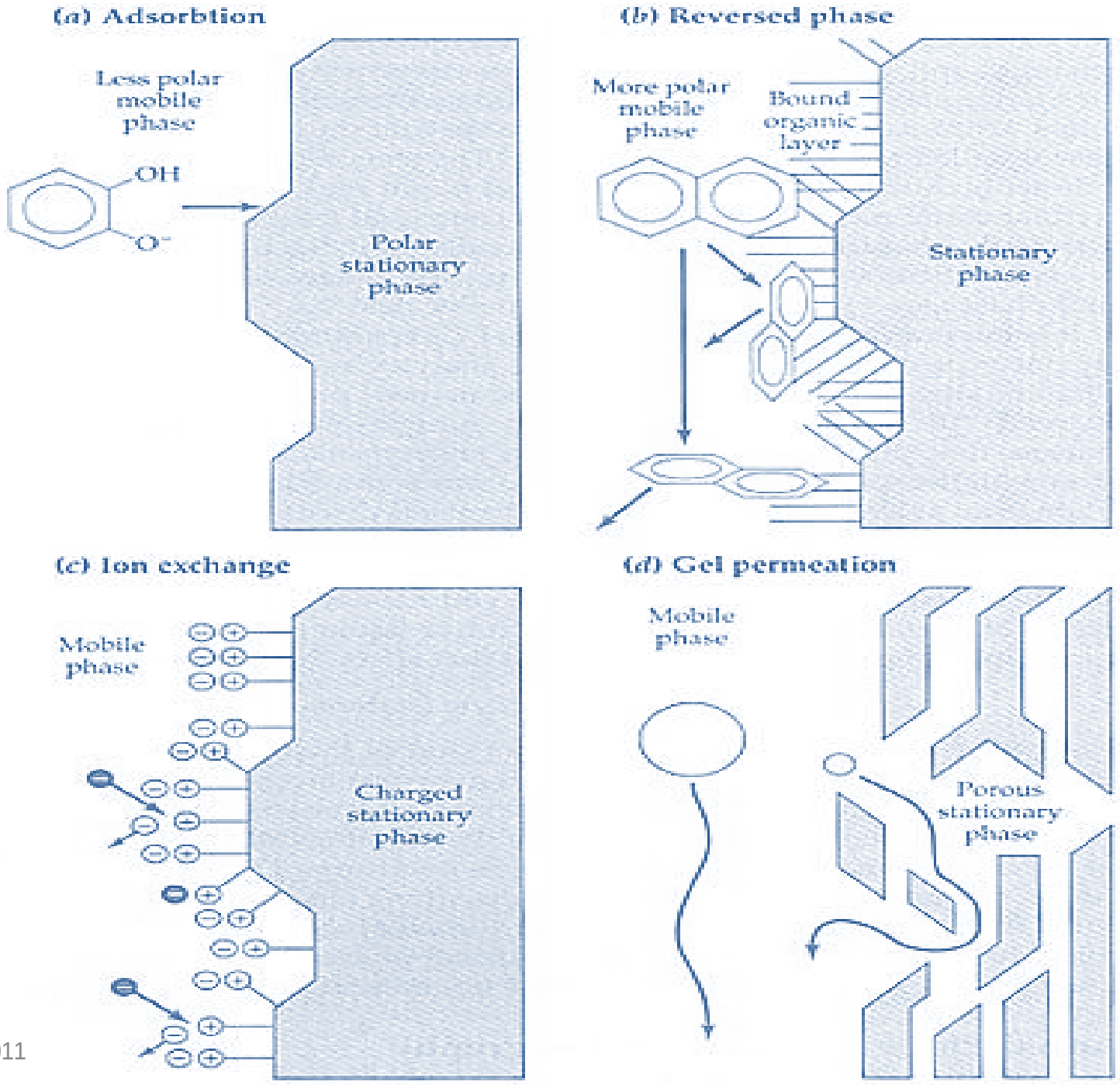
Affinity medium is equilibrated with binding buffer

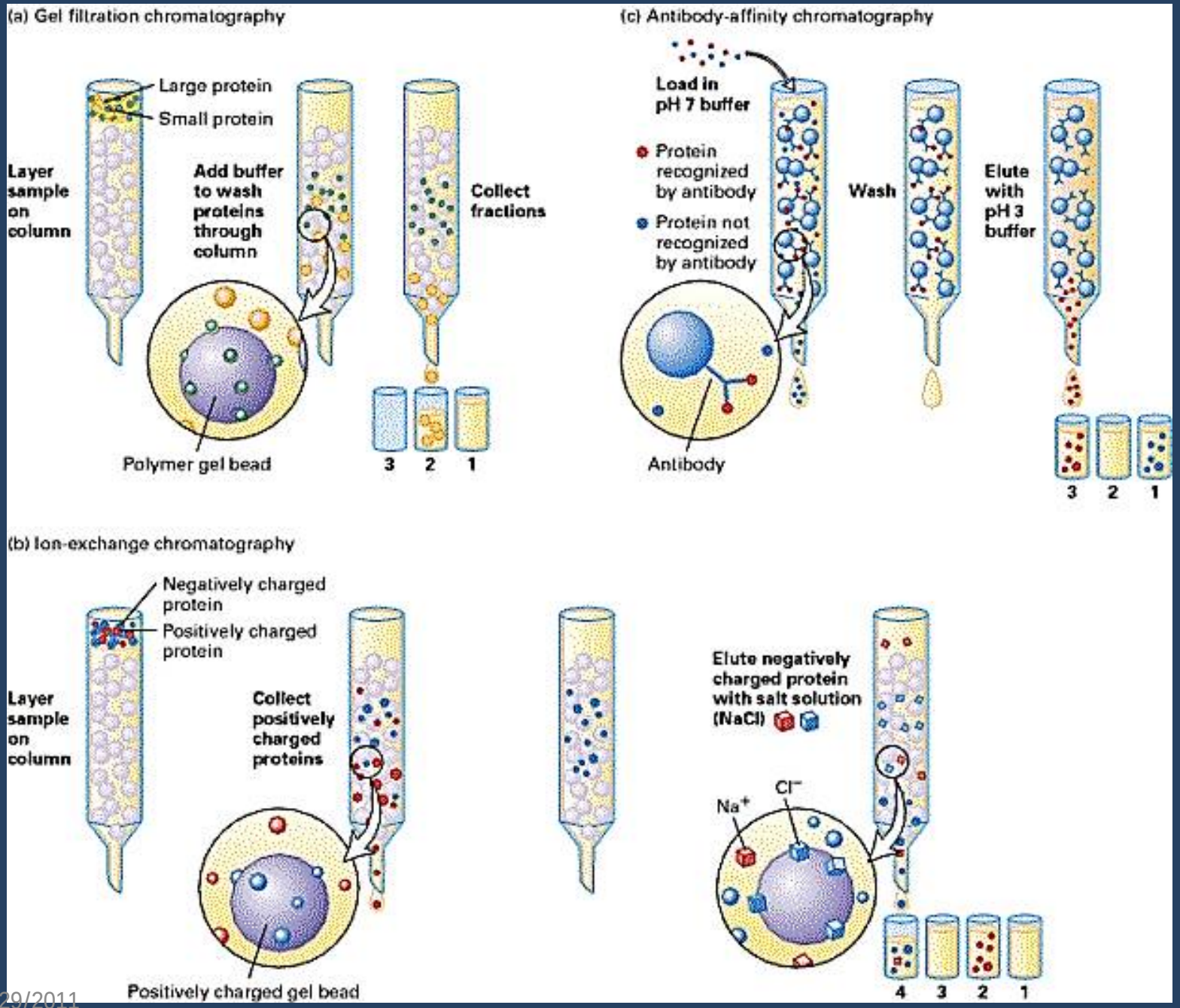
Sample is applied under optimum conditions that favor specific binding of the target molecule(s) to complementary binding molecules (the ligand). Desired molecules bind specifically, but reversibly, to the ligand and unbound material is washed through the column.

Target protein is recovered by changing conditions to favor elution of the bound molecules. Elution is performed specifically using a competitive ligand, or non-specifically, by changing the pH, ionic strength or polarity. Target protein is collected in a purified, concentrated form.



Affinity medium is re-equilibrated with binding buffer


(a) Adsorbtion
Less polar mobile phase
OH
O⁻
Polar stationary phase
(b) Reversed phase
More polar mobile phase
Bound organic layer
Stationary phase
(c) Ion exchange
Mobile phase
Charged stationary phase
(d) Gel permeation
Mobile phase
Porous stationary phase


(a) Gel filtration chromatography
Large protein
Small protein
Layer sample on column
Add buffer to wash proteins through column
Collect fractions
Polymer gel bead
3 2 1
(c) Antibody-affinity chromatography
Load in pH 7 buffer
Protein recognized by antibody
Protein not recognized by antibody
Wash
Elute with pH 3 buffer
Antibody
3 2 1
(b) Ion-exchange chromatography
Negatively charged protein
Positively charged protein
Layer sample on column
Collect positively charged proteins
Positively charged gel bead
Elute negatively charged protein with salt solution (NaCl)
Na+
Cl−
4 3 2 1