UNIVERSITY OF PATRAS

INSTRUMENTAL ANALYSIS OF BIOLOGICAL MACROMOLECULES

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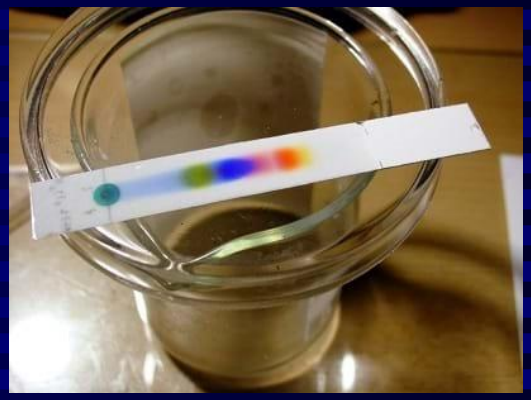
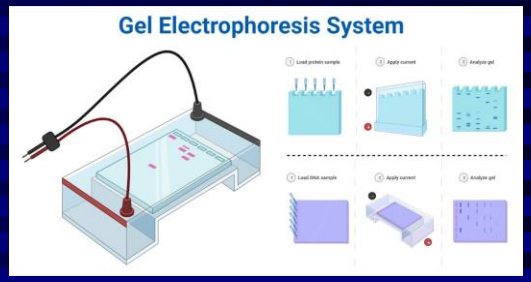
PATRAS 2026

Instrumental analysis of biomacromolecules

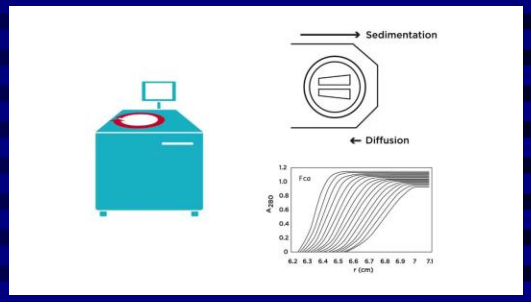
- Chromatography



- Electrophoresis

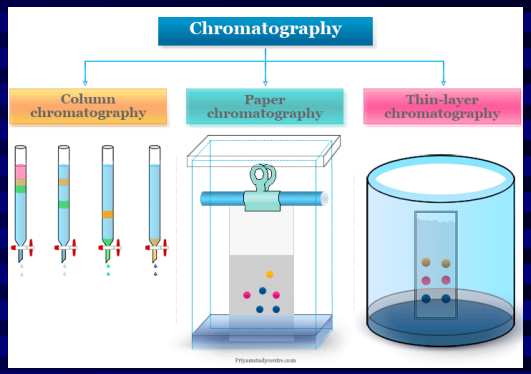


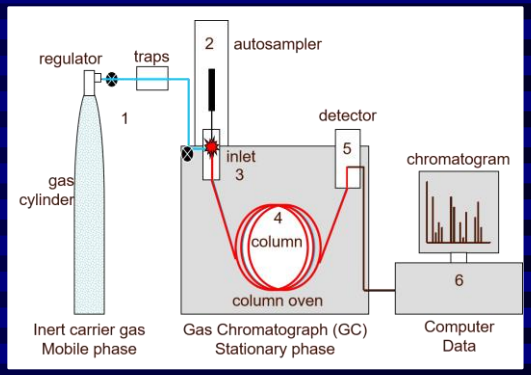
- Ultracentrifugation



Chromatography – Classification

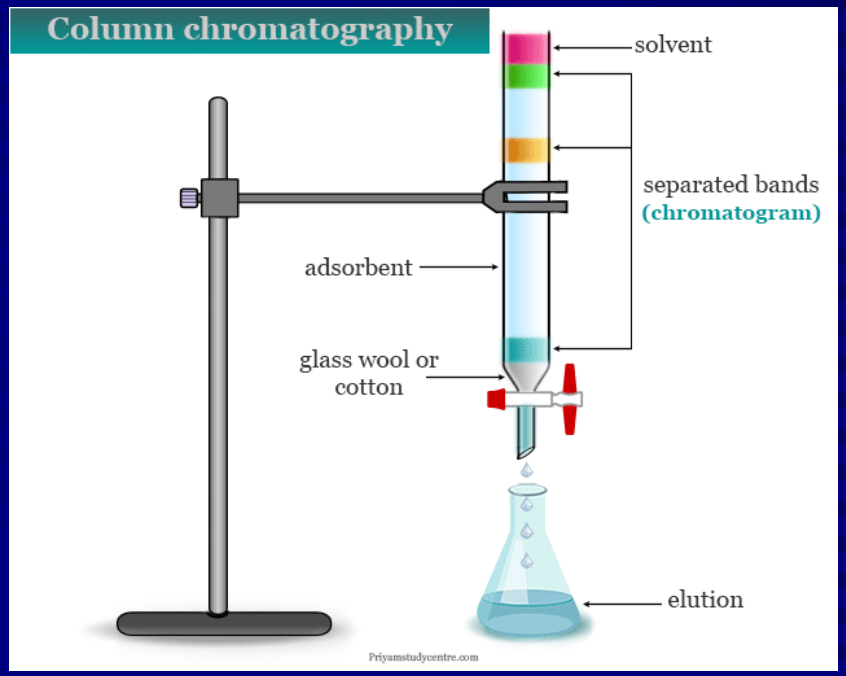
- **Physical state of mobile phase**
  - Liquid chromatography
  - Gas chromatography

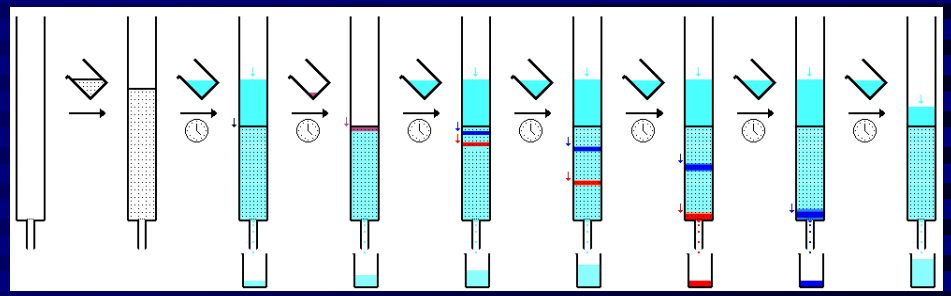




Chromatography – Classification

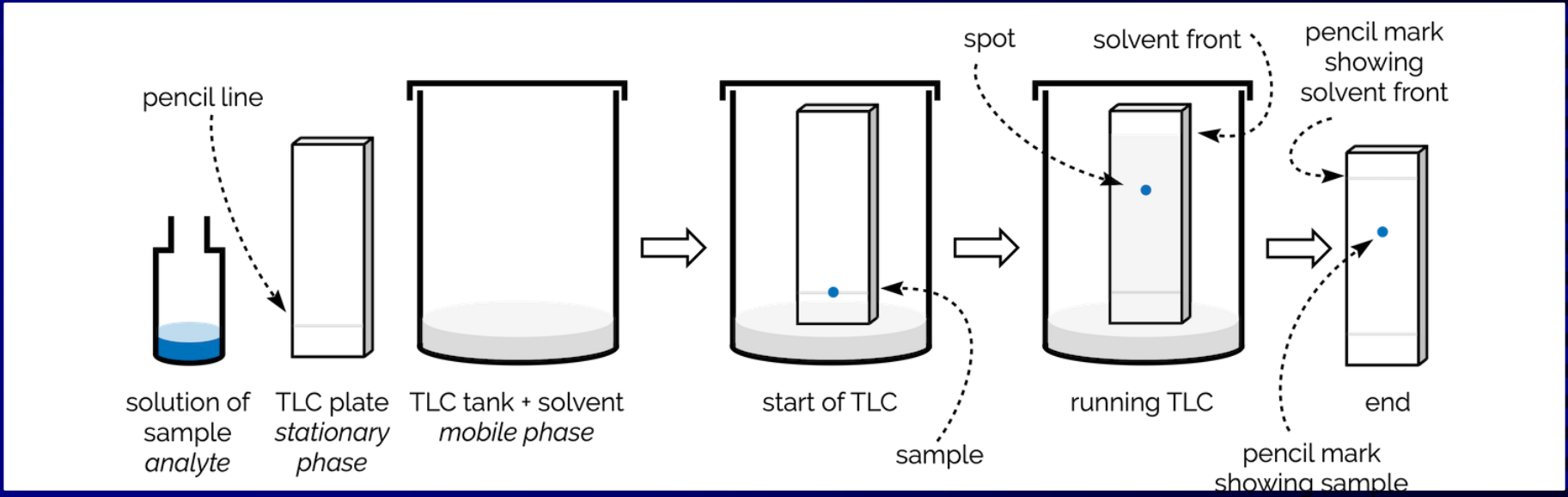
- **Physical state of stationary phase**
  - Column chromatography





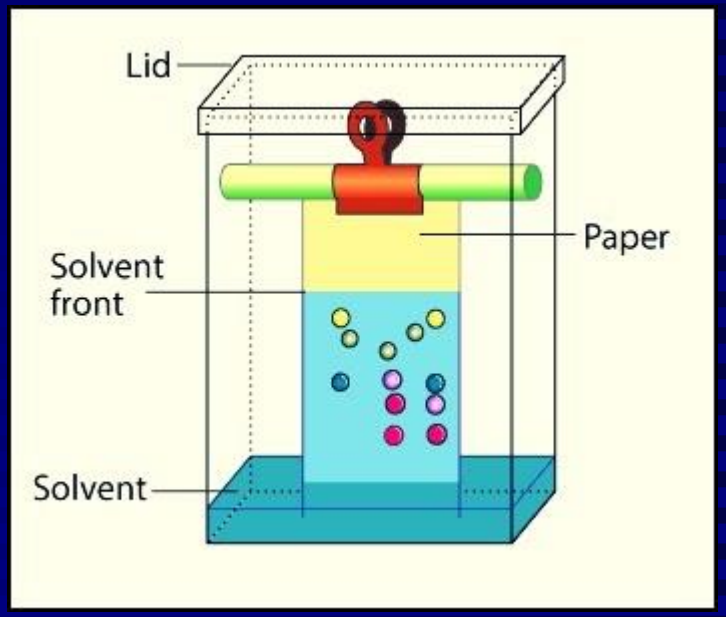
Chromatography – Classification

- **Physical state of stationary phase**
  - Thin layer chromatography [flat chromatography]



Chromatography – Classification

- **Physical state of stationary phase**
  - Paper chromatography [flat chromatography]



Chromatography – Classification

- **Depending on the phenomenon observed between the stationary phase and the molecules to be separated**
  - Adsorption chromatography
    - Stationary phase has adsorptive properties
  - Partition chromatography
    - Stationary phase has not adsorptive properties, but it possesses different properties to the solvent
  - Ion exchange chromatography
    - Stationary phase has a charge [positive or negative], therefore an ion exchange takes place between the stationary phase and the ions of the opposite charge of the sample

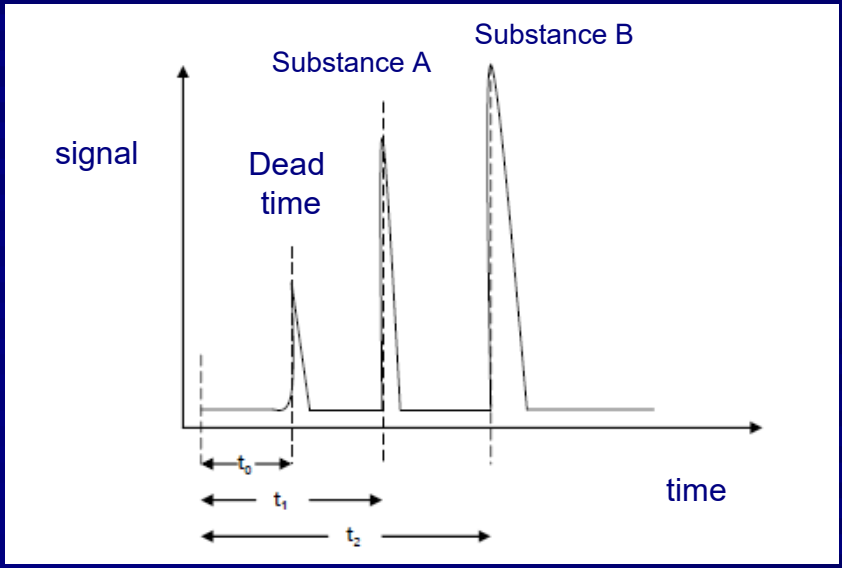
Chromatographic separation

- Each component of the sample is distributed between the two phases (mobile and stationary, which do not mix) and an equilibrium is established
- The distribution of each component in the two phases depends on the properties of the component in relation to the two phases
- The continuous flow of the mobile phase (solvent) causes the components to move along the stationary phase
- The separation of the individual components of the mixture is achieved due to the different speed of movement of each component

Chromatography

- Thin layer
  - Separation of molecules of low molecular mass
  - Movement through a bed of inert material under the influence of a solvent specific for the molecules to be separated
    - Silicon dioxide [silica], cellulose (simple or modified)
- Paper
  - Separation of molecules of low molecular mass
  - Movement through a bed of inert material under the influence of a solvent specific for the molecules to be separated
- Column
  - Separation of molecules of a wide range of molecular masses
  - Separation of molecules of different charge
  - Separation of molecules with specialized properties (enzymes, antibodies, etc.)
- HPLC
- Gas
  - Any constituents can be vaporized

Sizes and Definitions in Chromatography



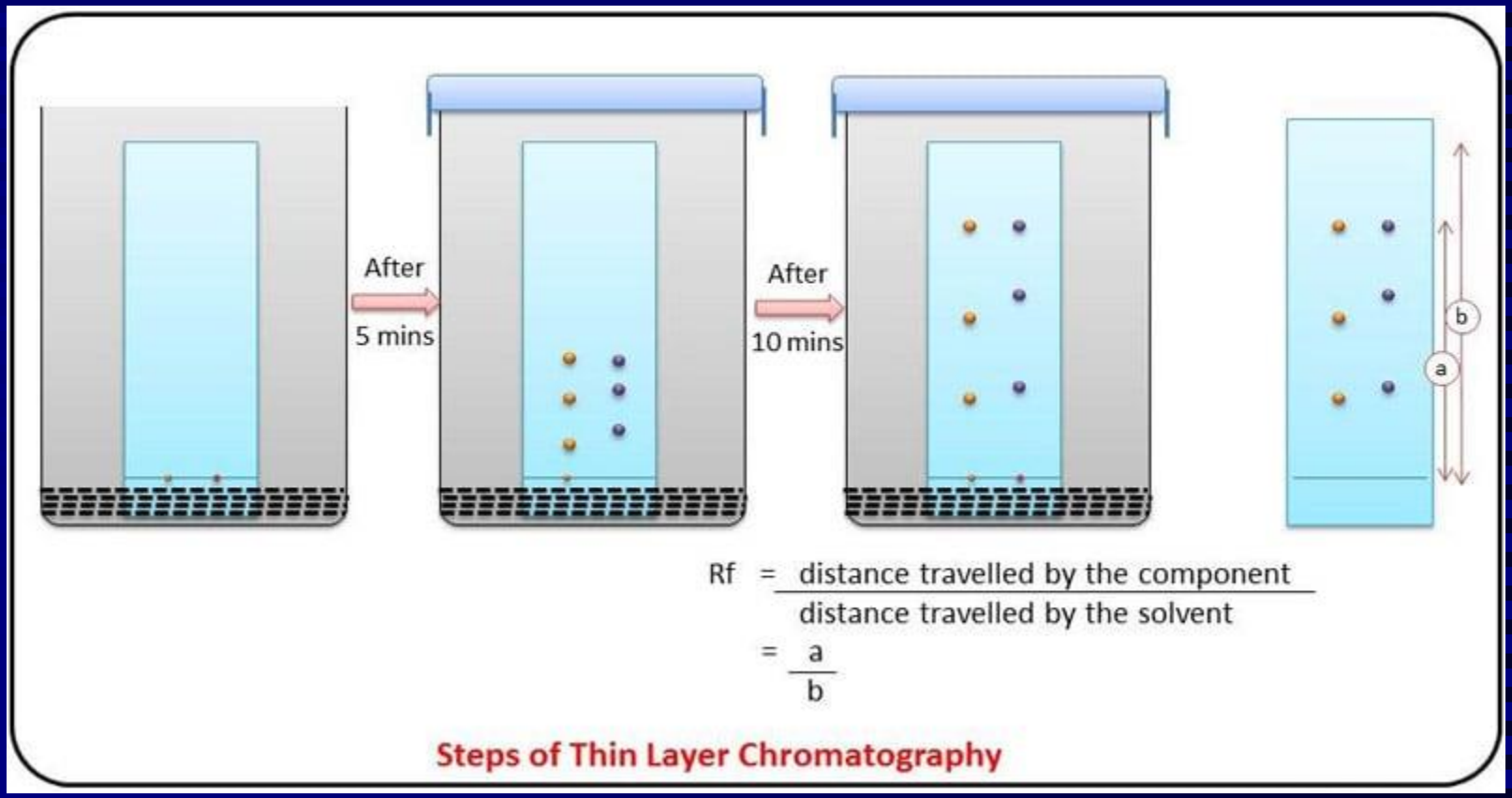
- **Retention time:** the time required for the analyte to exit or be recorded
- **Dead/Void time:** the time required for the component that is not retained to exit or be recorded
- **Resolution coefficient of two components:** expresses the measure of the ease or difficulty of separation under the given conditions

$$\alpha = \frac{t_2 - t_0}{t_1 - t_0}$$

- **Resolution:** expresses the measure of separation of two components and is related to the width of the base of each peak – when Rs=1.5 there is complete separation

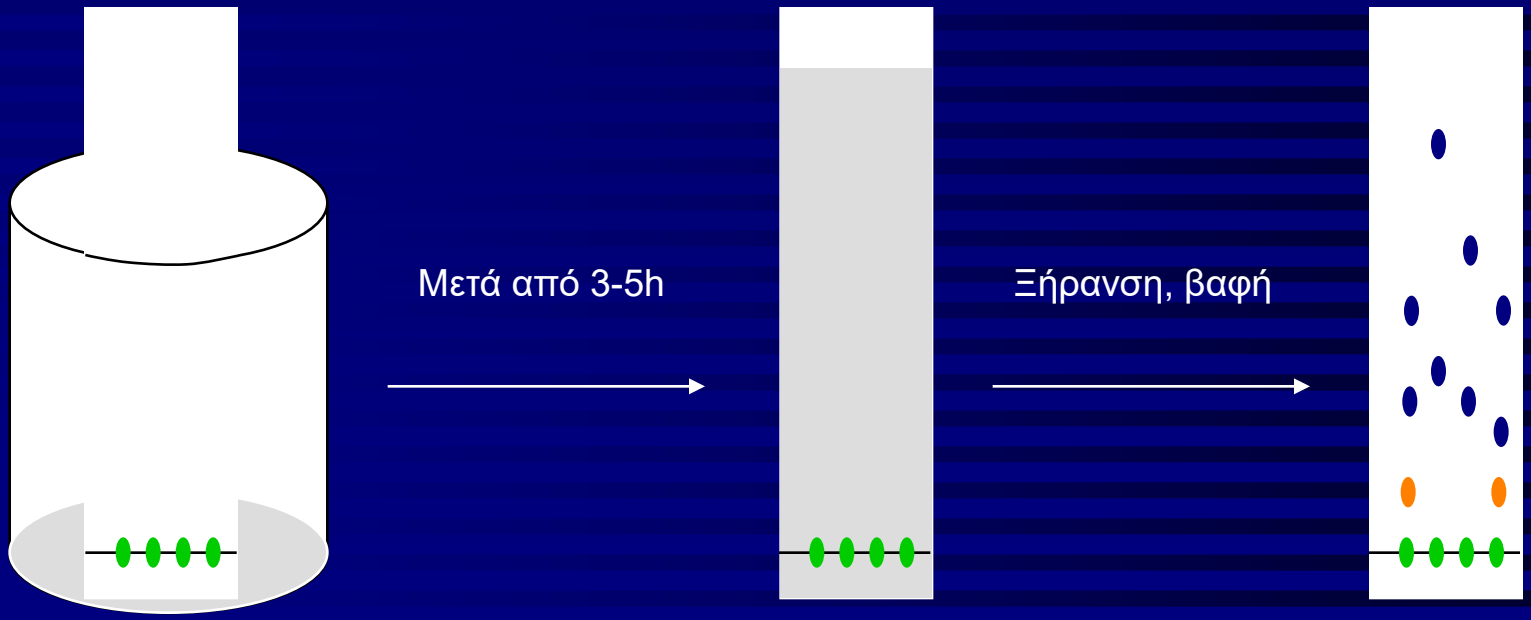
$$R_s = \frac{2 \cdot (t_2 - t_1)}{w_1 + w_2}$$

Thin layer/Paper chromatography



Thin layer/Paper chromatography

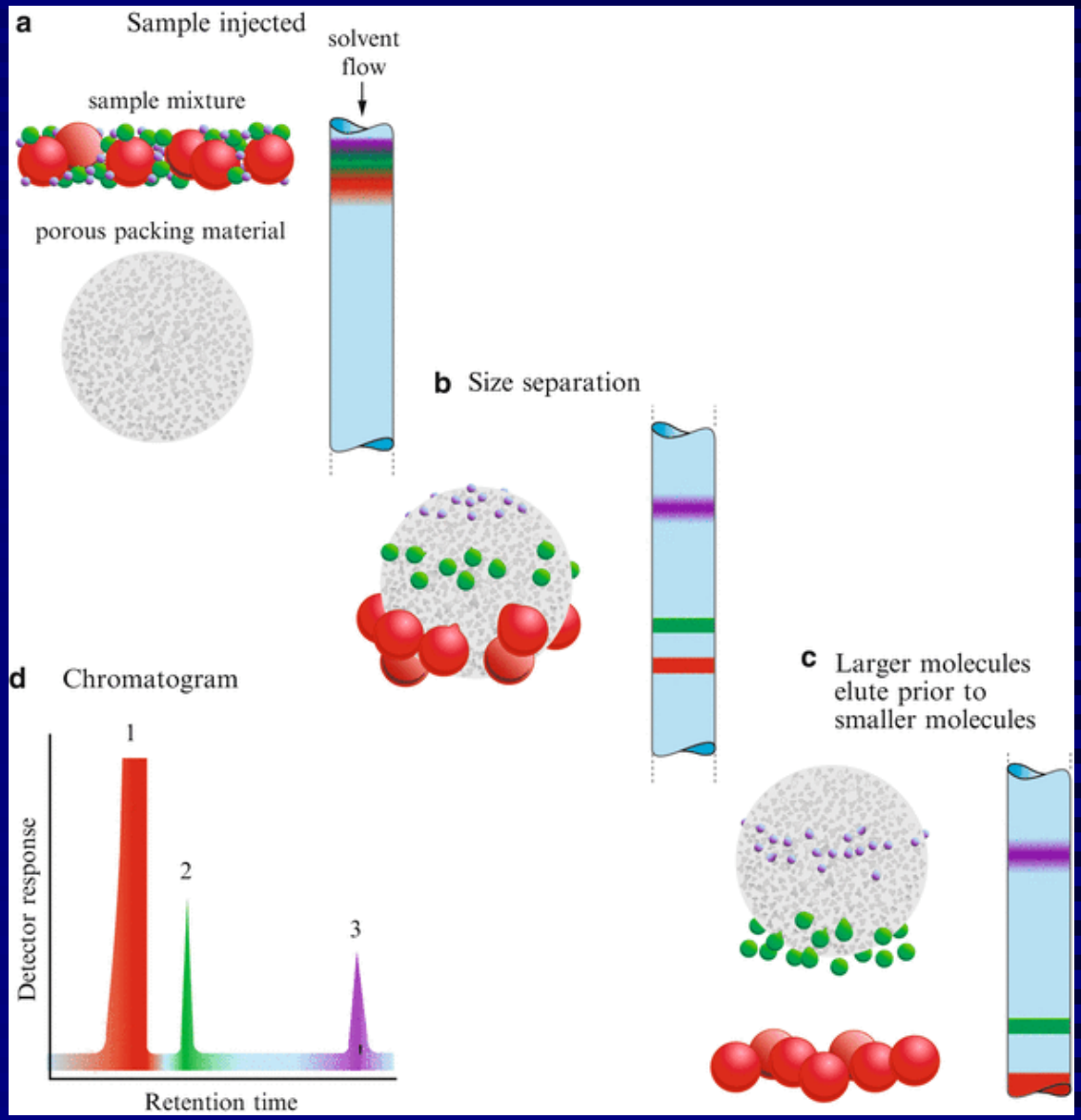
Applications: Detection of aminoacids in protein hydrolysates,
i.e. Detection of Val instead of Glu in σickle cell ανεμια patients



Column chromatography

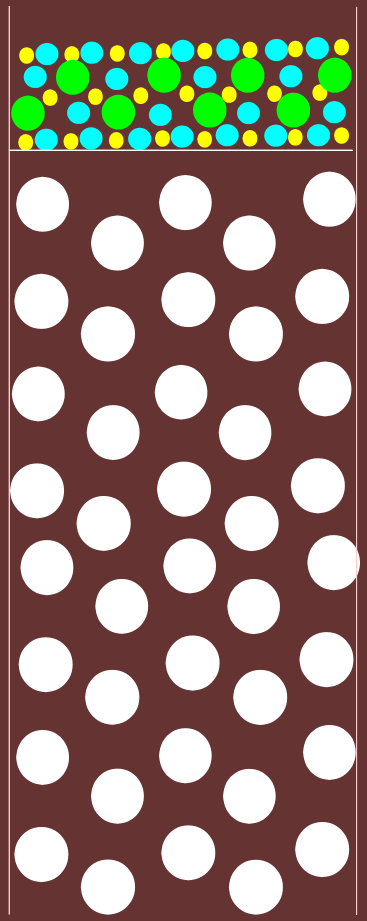
- These are the best and most effective methodologies:
  - Molecular filtration or molecular sieve or gel
    - Using poorly soluble porous polymers
  - Ion exchange
    - Mainly substituted celluloses or dextrans
    - Proper adjustment of pH and ionic strength is necessary
  - Adsorption
    - Usually hydroxyapatite
  - Affinity
    - The solid substrate carries as a ligand a substance that interacts (receptor/ligand, substrate analogue, inhibitor, etc.) with the molecules to be analyzed

Gel chromatography



Gel chromatography

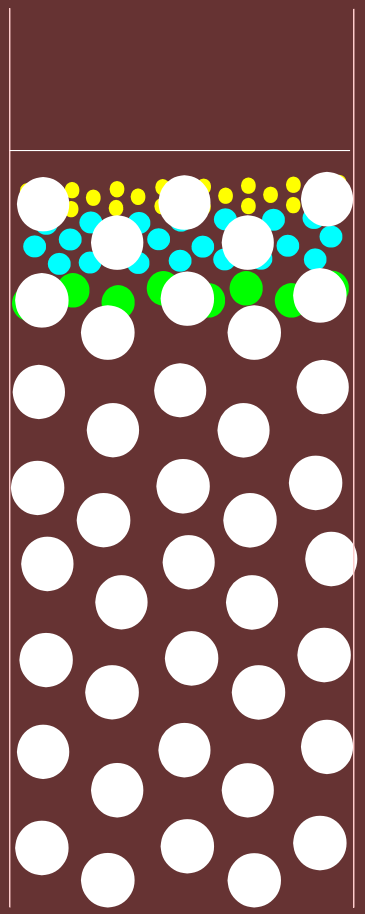
Gel chromatography



The sample (a mixture of molecules of different hydrodynamic size) is placed on the top of the column

Gel chromatography

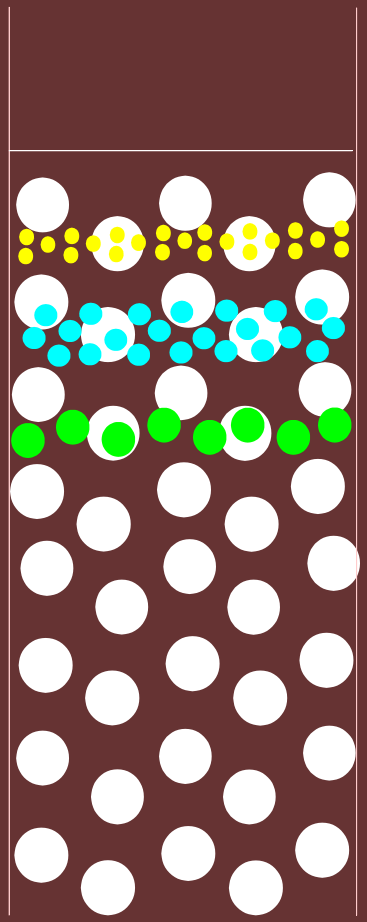
Gel chromatography



The chromatography is developed and separation of molecules begins

Gel chromatography

Gel chromatography



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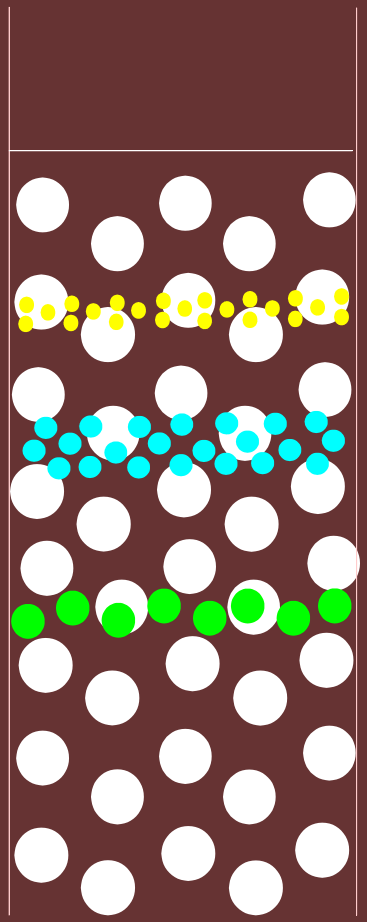
Gel chromatography

Gel chromatography

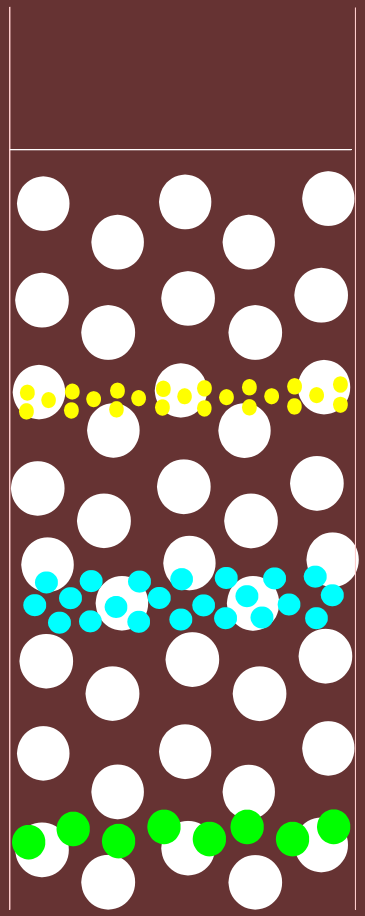


As the chromatography develops, the separated molecules are moved as a band

The molecules of the higher hydrodynamic size are moved rapidly, followed by the smaller ones according to their decreased size

Gel chromatography

Gel chromatography



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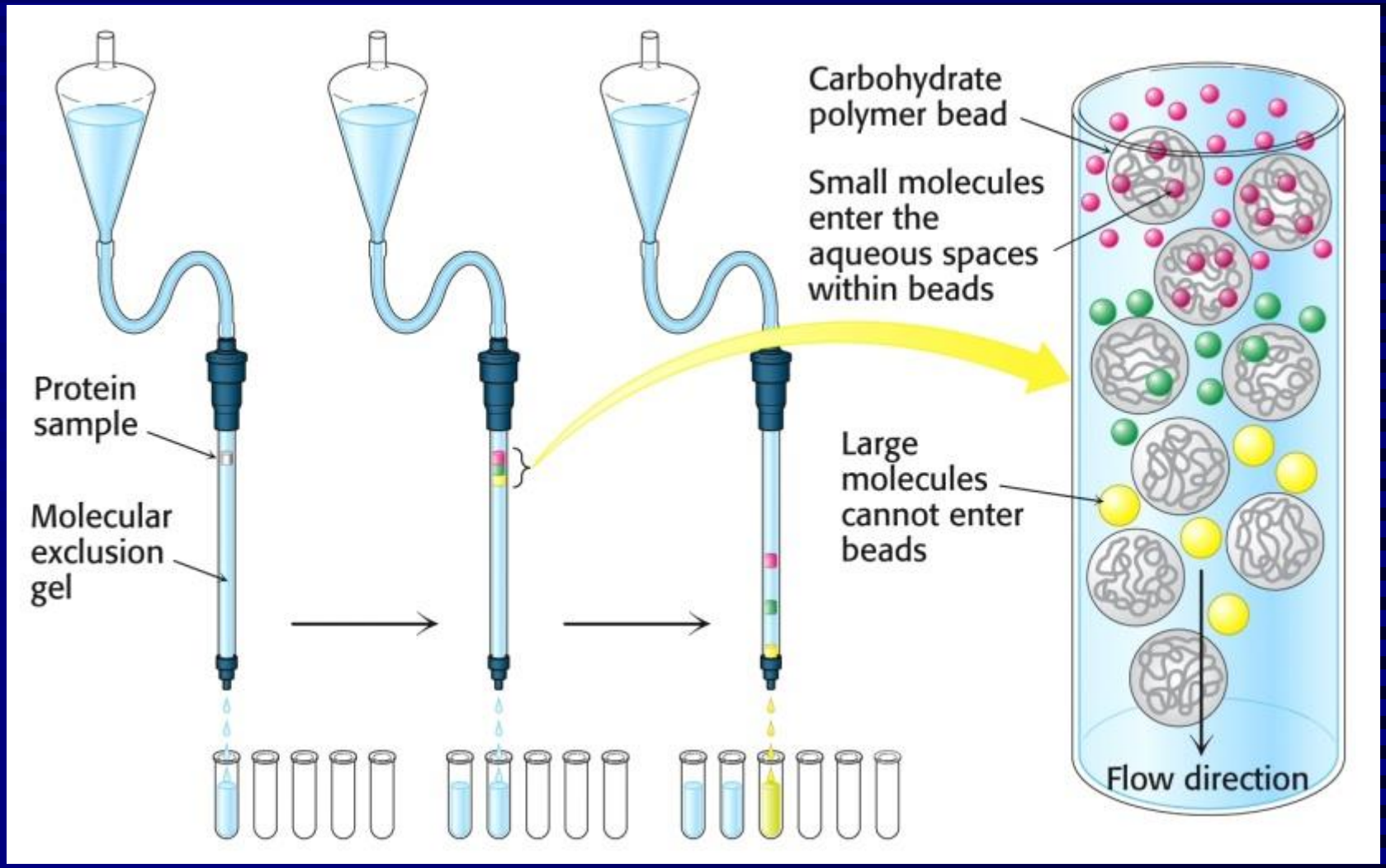
Gel chromatography



Gel chromatography

Stationary phase

Natural or modified dextrans – Sephadex

Natural or enhanced agarose – Sepharose [CL]

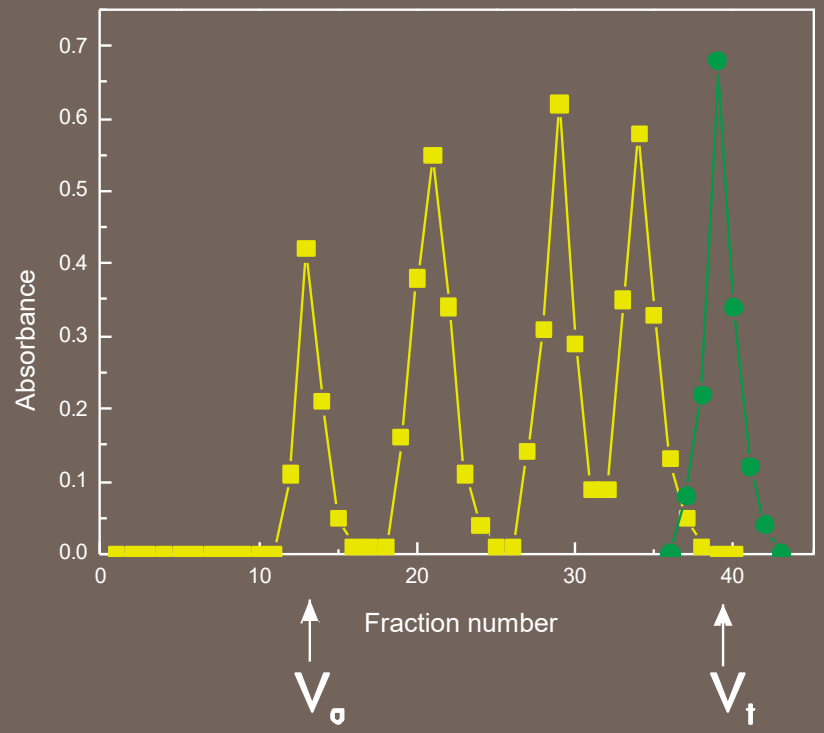
Polyacrylamide – BioGel

Dextran-polyacrylamide combination – Sephacryl

Porous glass

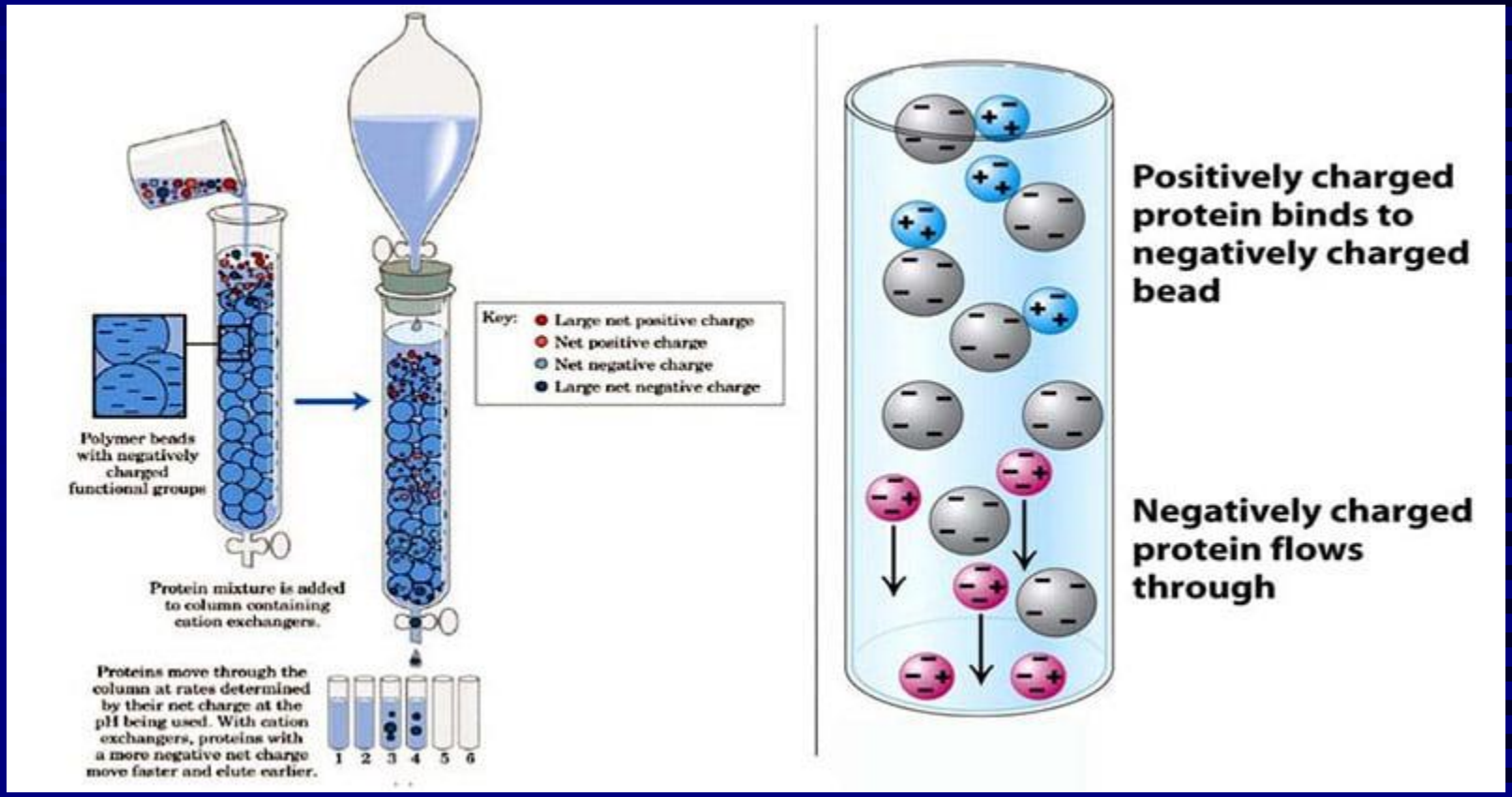
Gel chromatography

Gel chromatography



The graphic presentation of results follows

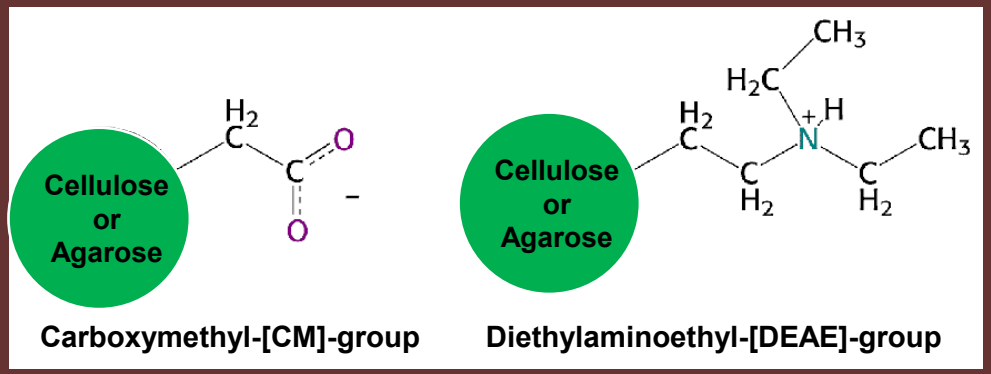
Ion exchange chromatography



Ion exchange chromatography

Ion exchange chromatography

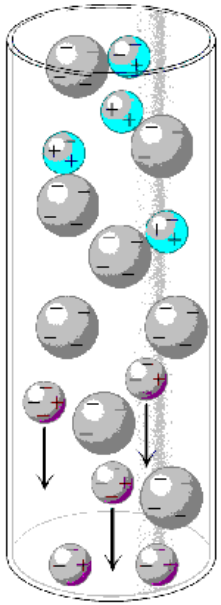
Typical examples of ion exchangers in Biochemistry



Ion exchange chromatography

Ion exchange chromatography

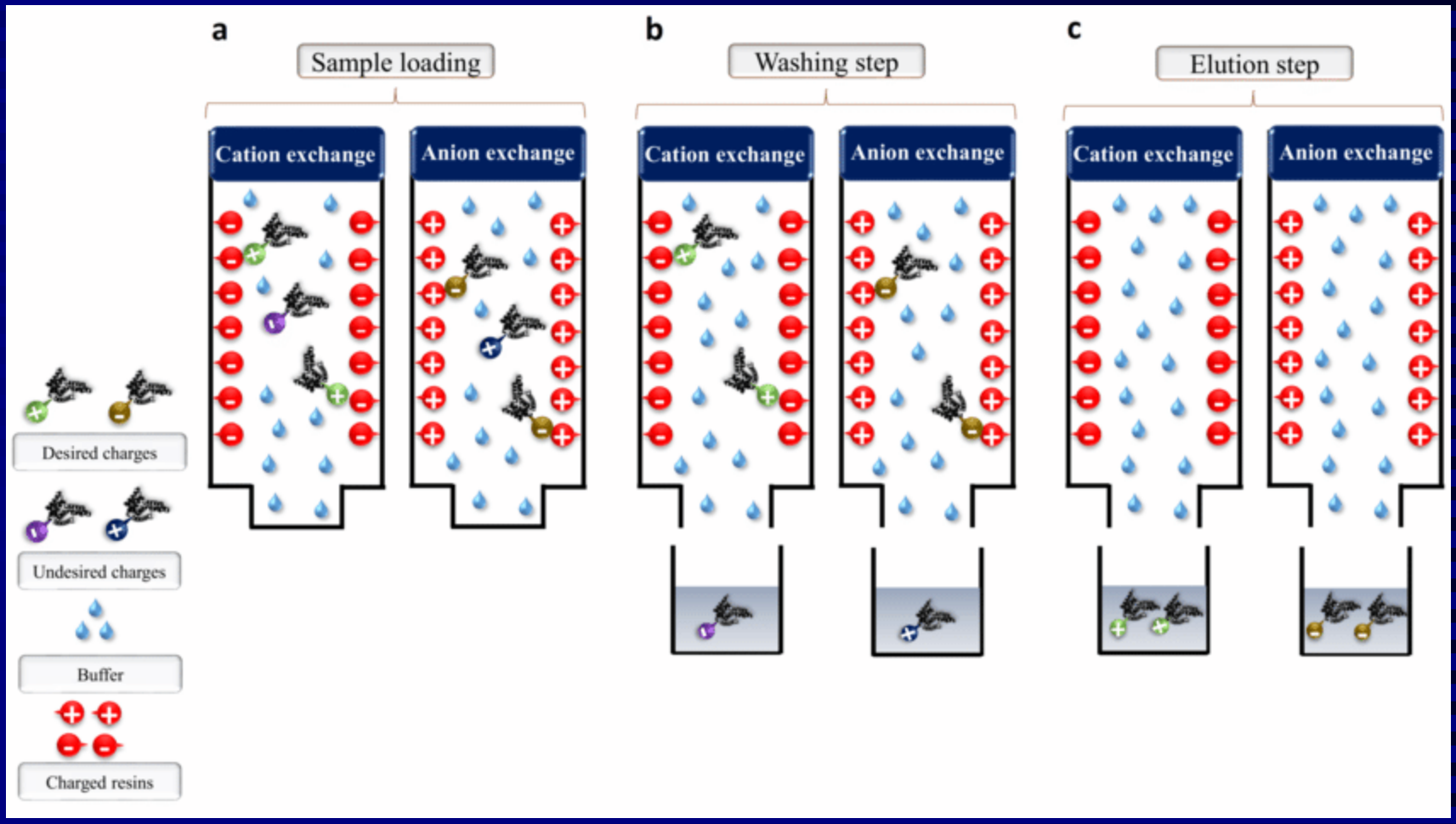
The principle of the separation is based on the different charge of each one of the constituents to be separated



Positively charged components are bound to negative charges of the stationary phase

Negatively charged components are eluted without binding to the stationary phase

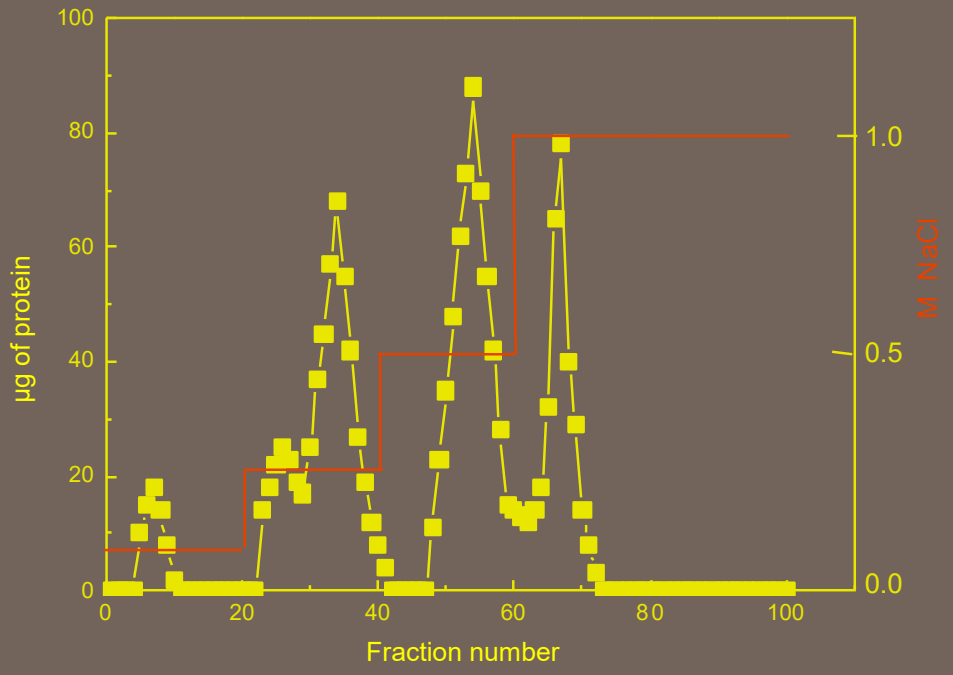
Ion exchange chromatography



Ion exchange chromatography

Ion exchange chromatography

Protein separation on DEAE-cellulose

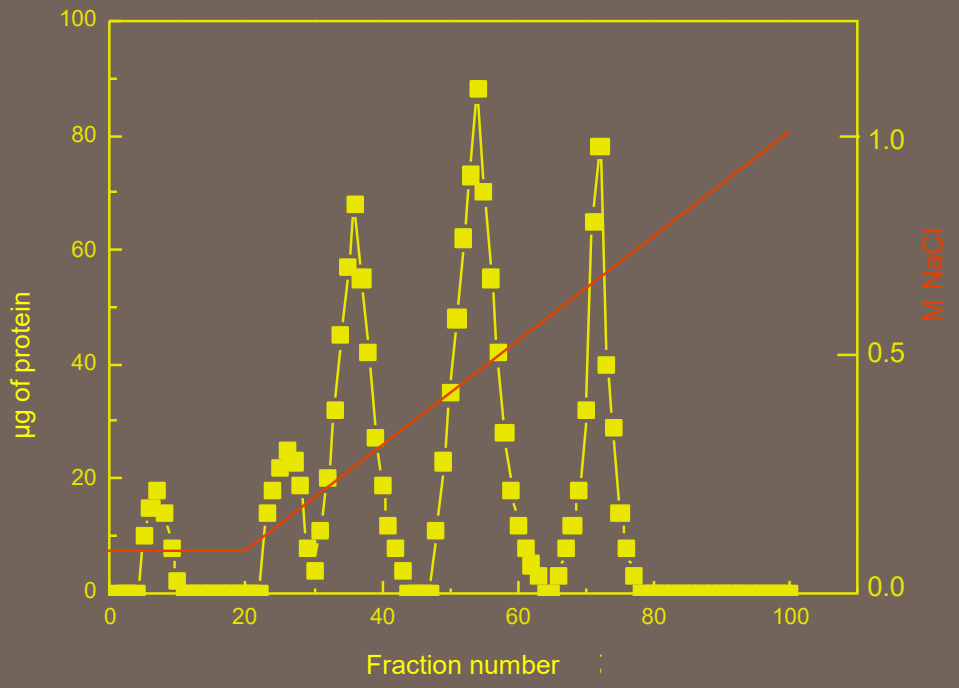


1. Elution is done with stepwise increase of ionic strength

Ion exchange chromatography

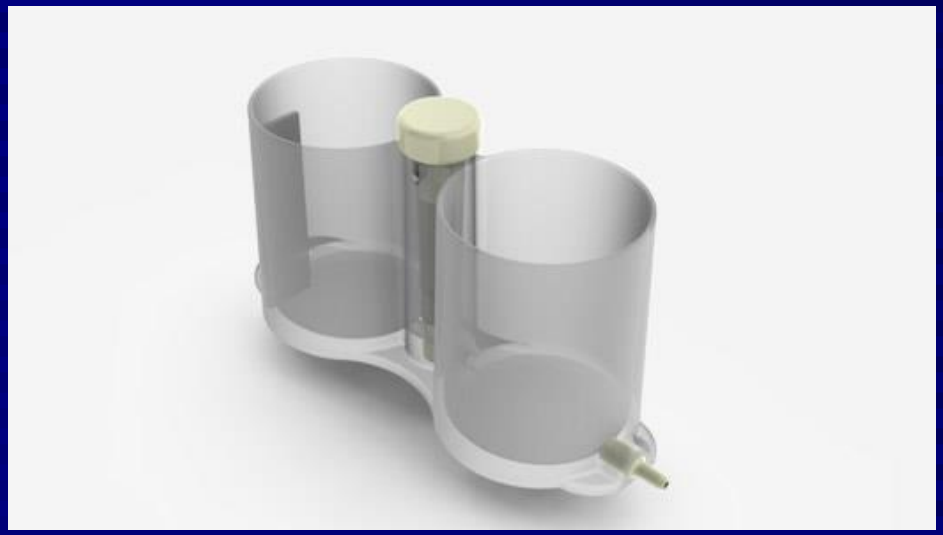
Ion exchange chromatography

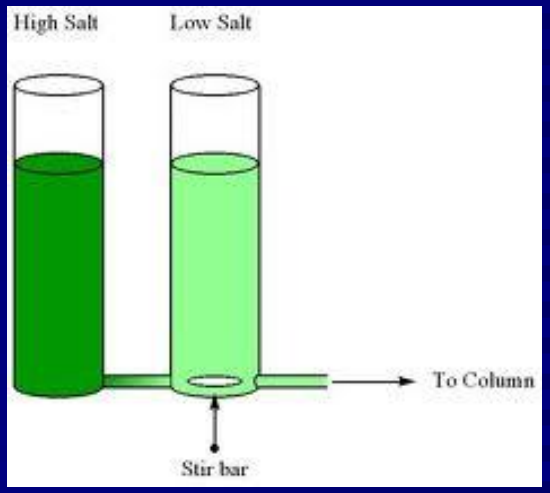
Protein separation on DEAE-cellulose



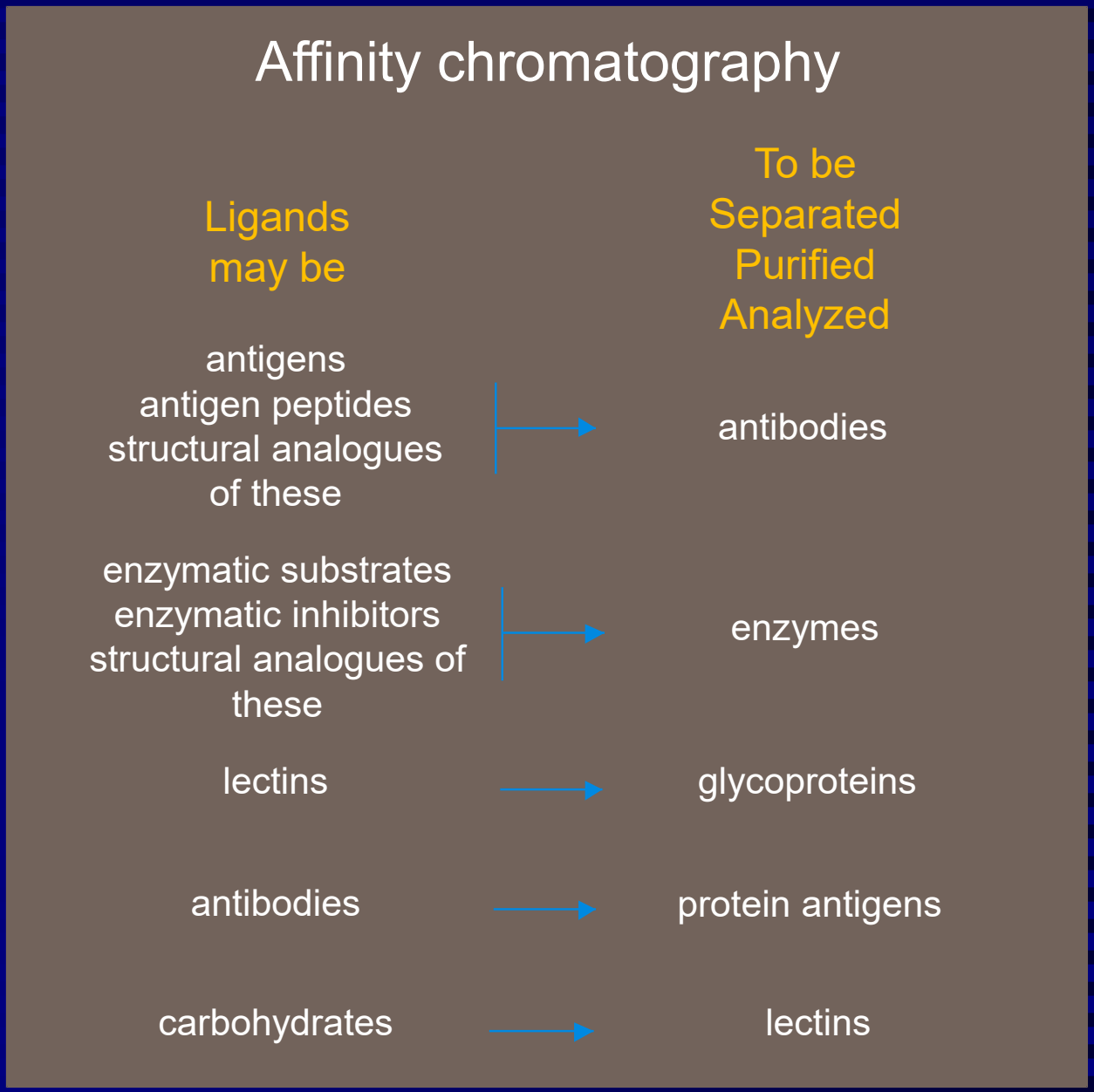
2. Elution is done with linear increase of ionic strength

Preparation of linear gradient





Affinity chromatography



Affinity chromatography

Affinity chromatography

Elution of bound molecules is done

Usually using a specific compound to the eluent that specifically recognizes the prosthetic group of the stationary phase and elutes the bound constituent

Sometimes by changing the pH or the ionic strength of the eluent

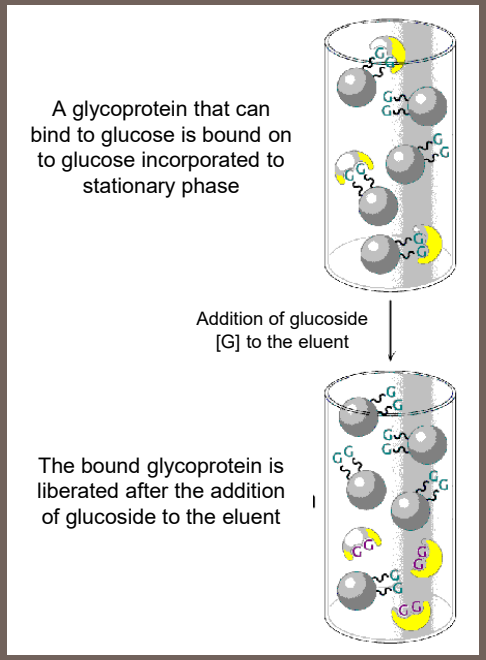
Affinity chromatography

Affinity chromatography

Bound constituent	Elution or additive to the eluent
antigens antigen peptides structural analogues of these	→ structural analogue of antigen or pH 2.2 or increase of ionic strength
enzymatic substrates enzymatic inhibitors structural analogues of these	→ soluble [free] substrate
lectin	→ carbohydrate or glycoside
antibody	→ pH 2.2 or increase of ionic strength
carbohydrate	→ carbohydrate or glycoside

Affinity chromatography

Affinity chromatography



Separation of glycoproteins using a stationary phase carrying a lectin as ligand

HPLC

High

Performance

Liquid

Chromatography

HPLC

High

Pressure

Liquid

Chromatography

HPLC

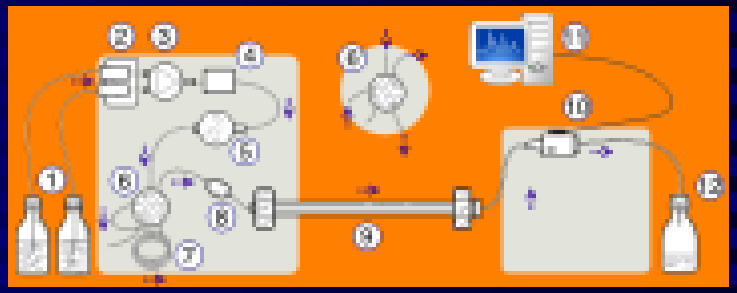
High

Priced

Liquid

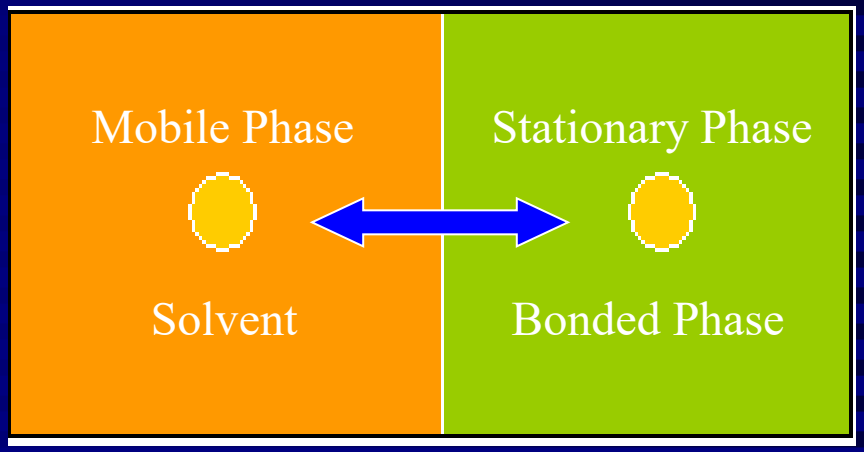
Chromatography

HPLC



Partition HPLC

- Separation is based on the differences in solubility of the substance under analysis to two different phases

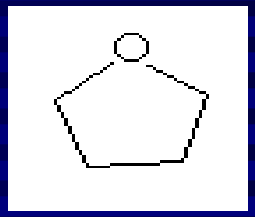


HPLC - Types

- Normal phase
  - polar stationary phase and non-polar eluent

- Reversed phase
  - non-polar stationary phase and polar eluent

Common eluents/solvents in reversed phase HPLC

- methanol CH_3OH
- acetonitrile CH_3CN
- tetrahydrofuran 
- water H_2O

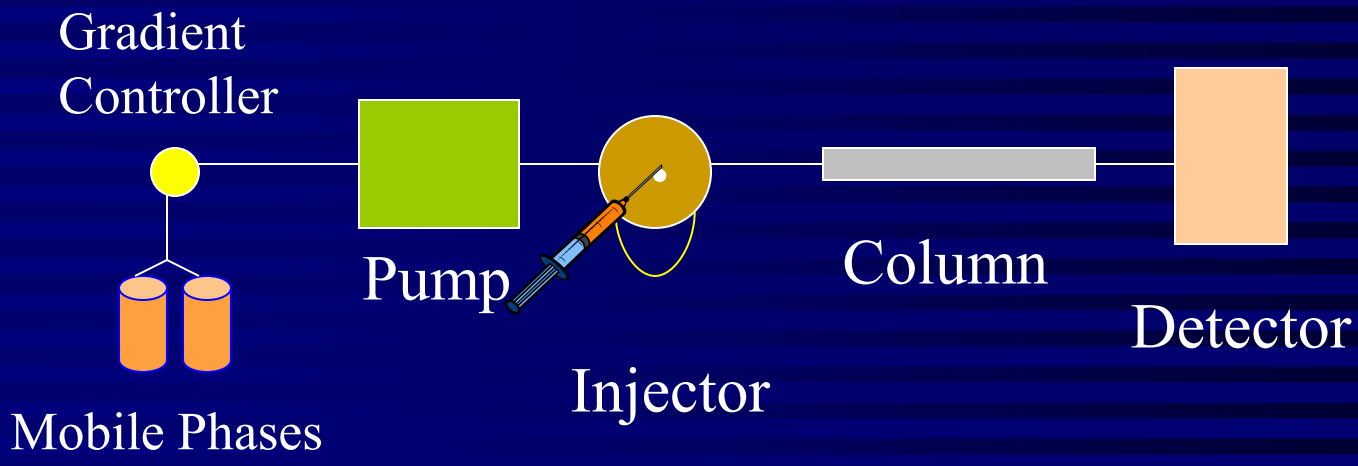
HPLC columns

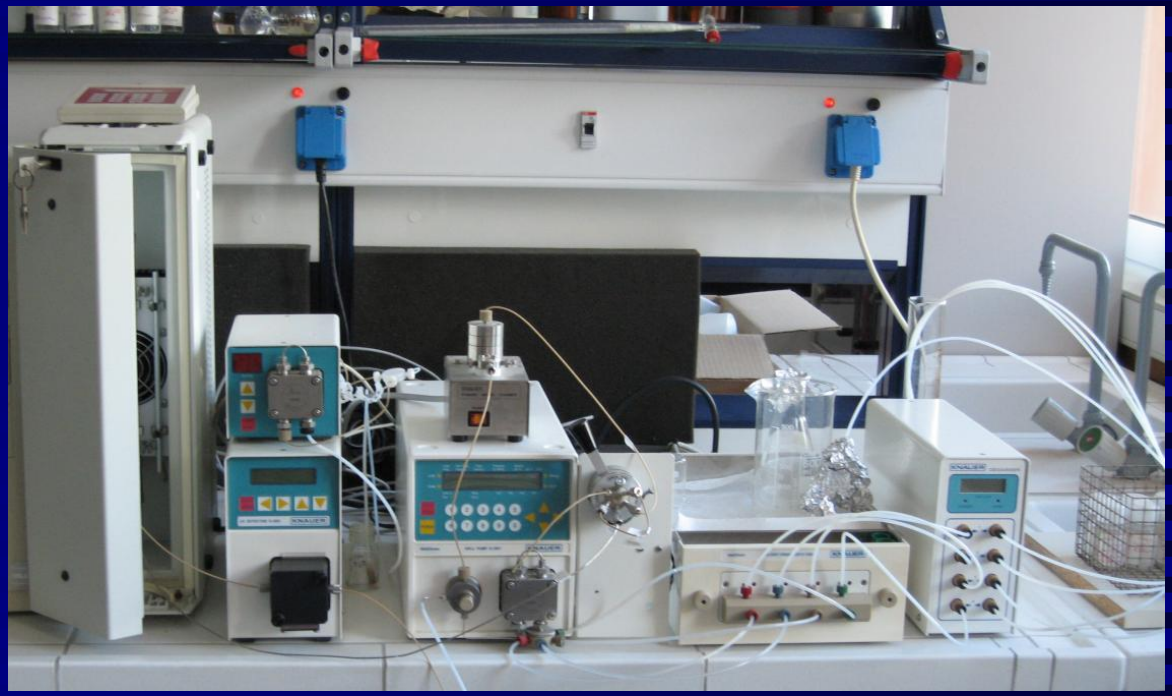
- Analytical column – separation of compounds
- Guard column – protects the analytical column
  - Retains particles
  - Removes substances that interfere with separation
  - Increases analytical column life
- Stationary phase – usually substituted
  - In most of the cases 10μ, 5μ or 3μ silica or polymer
- Substituted phases – the functional groups are incorporated via chemical reaction to stationary phase
  - Extended stability
  - Reproducibility

Substituted phases

- C-2 Ethyl Silyl -Si-CH_2-CH_3
- C-8 Octyl Silyl -Si-$(CH_2)_7$-CH_3
- C-18 Octadecyl Silyl -Si-$(CH_2)_{17}$-CH_3
- CN Cyanopropyl Silyl -Si-$(CH_2)_3$-CN

Instrumentation





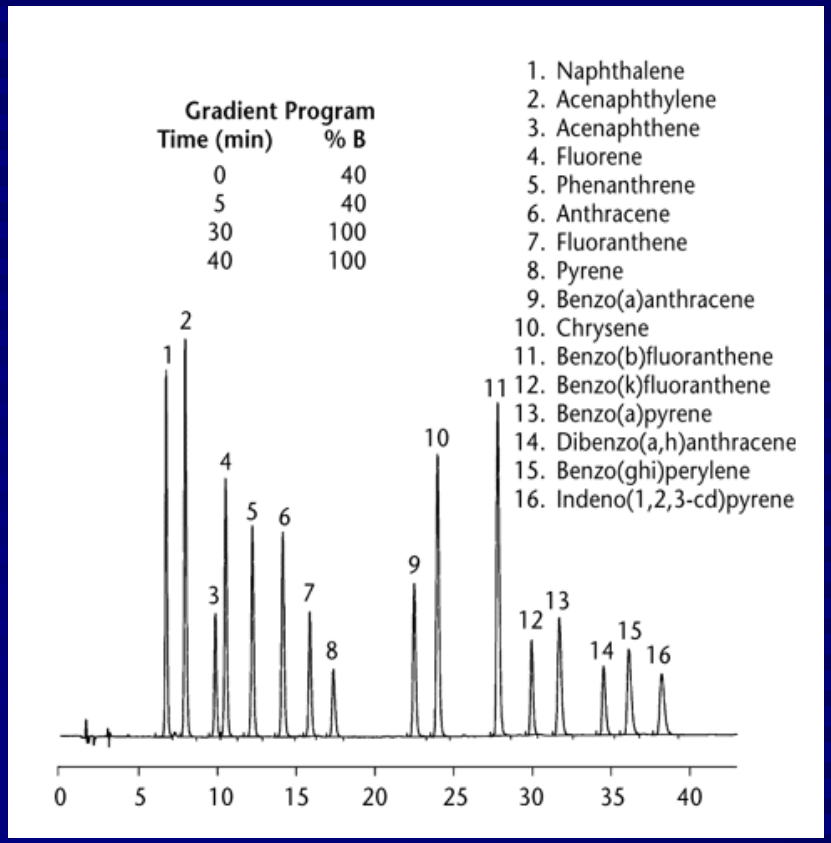
Detectors

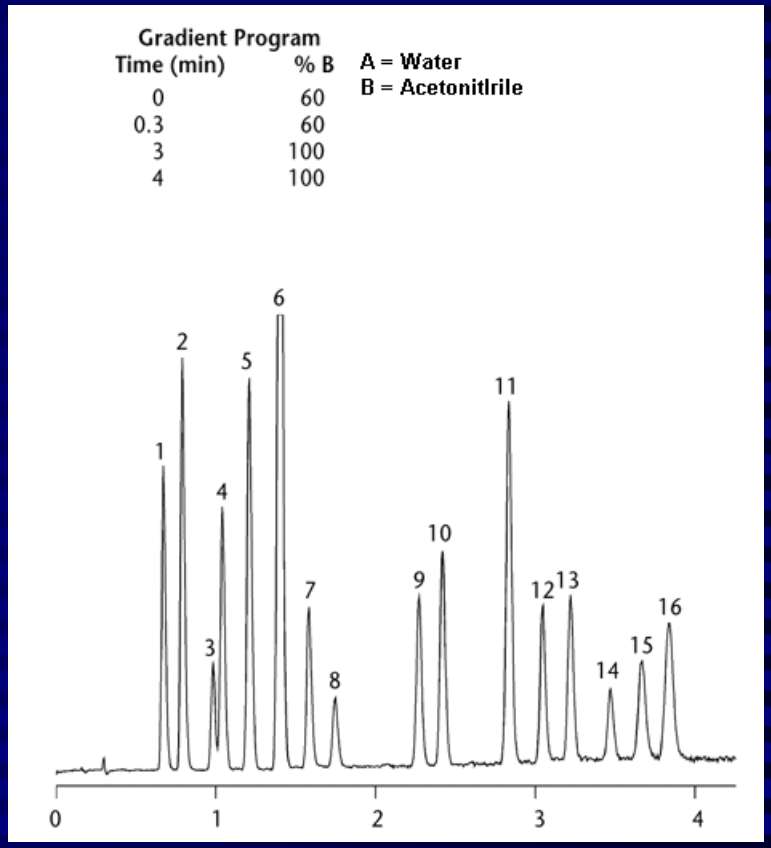
- UV
  - Single wavelength
  - Each wavelength (monochromator)
  - Multiple wavelengths
- Fluorescence
- Electrochemical
- Mass spectrometry

Applications

- Quantitative analysis of drugs in preparations
  - anti-inflammatory
  - Anxiolytics/Anti-anxiety agents
  - Narcotics/Illegal drugs
- Determination of preservatives in cosmetics, food and beverages
- Determination of pesticides, insecticides, dioxins or other toxic substances in environmental samples
- Determination of anabolics and their metabolites in biological fluids
- Determination of drugs (narcotics, cardiotonics) in biological fluids of patients
- Separation of substances at the preparative level

Chromatograms





A Supelcosil LC-PAH Columns. B

Conditions: A: 150mm x 4.6mm, 5µ.

Flow Rate: 1.5 mL/min

Conditions: B: 50mm x 4.6mm, 3µ.

Flow Rate: 3.0 mL/min

Gas chromatography

- It allows the separation of
  - gas mixtures
- in a sequential order that is established between
  - a gaseous mobile phase
  - and a liquid or solid phase
- contained inside the column

- If the stationary phase is liquid, it is
  - partition chromatography
- If the stationary (static) phase is solid, it is
  - adsorption chromatography

Gas chromatography

- Gas chromatography separates
  - Substances that are naturally gaseous
  - Substances that are naturally solid or liquid, but that easily convert to gases with increasing temperature
    - As long as they are not destroyed by increasing temperature
  - Substances that can, by derivatization, be converted to gases or volatile compounds
- Converting compounds into others increases analysis time and analysis error

Gas chromatography Instrumentation

The compartments of a gas chromatography instrument are:

Gas source [mobile phase]: The gas selected should be of very high purity, chemically inert and compatible with the detector. Argon, Helion, Hydrogen and Nitrogen are more commonly used.

Sample injection system: The sample is injected via a specific syringe to a specific membrane.

Chromatography column: It is found into a **heated chamber** [furnace]. The gas chromatography column contains, as in the liquid chromatography, a stationary phase, but it is of smaller diameter and of higher length [usually 1-4 meters or higher], and it is of coiled coil form to fit into the heated chamber. The furnace is able to work in a wide range of temperatures, and the personnel select the temperature according the substances to be analyzed.

Detector: It is connected to a computer for receiving and processing the measurements.

Applications of gas chromatography

- Gas analyses of all types
  - Fuels
  - gases produced from landfills
  - engine exhaust gases
  - pyrolysis kinetics of various substances, etc
- Control of atmospheric pollution
  - hydrocarbons
  - CO, CO_2, H_2S, NO_x, etc
- Human breath analysis
- Determination of alcohols (ethanol, methanol) in blood

Applications of gas chromatography

- Analyses of colognes and perfumes
- Control of pollution of water, fruits, vegetables, soils (after appropriate extraction and treatment) by pesticides and insecticides
- Quality control of essential oils
- Separation and isolation of compounds in very pure form and large quantities (preparative chromatography)

Comparison of GC and HPLC

Quantitative Analysis in Chromatography

- The % area of the peak of the substance, relative to the sum of the areas of all peaks of the separated substances, expresses the % percentage of the substance in the mixture
- Calculation of signal-concentration correlation: A = f∗C for each substance and application of the relationship

$$C_x\% = \frac{f_x \cdot C_x}{f_1 \cdot C_1 + f_2 \cdot C_2 + ... + f_x \cdot C_x}$$

- External standard method
  - A standard solution of the analyte is compared with the analyte in the separated mixture

$$C_x = C \cdot \frac{A_x}{A}$$

- Internal standard method
  - A substance that is NOT present in the analyzed mixture is added to it and analyzed together with the other substances, e.g., Norleucine in an amino acid mixture

Electrophoresis

For more information read
https://microbenotes.com/gel-electrophoresis-system-apparatus-parts-types-examples/

- Electrophoresis is a technique used to separate, characterize and analyze biomolecules
- It is based on the fact that macromolecules, such as, DNA, RNA and proteins carry electric charges that enable them to migrate in an electric field
- Migration of the biomolecules during electrophoresis is done onto a solid support
- This is needed because electric current produces heat, that diffuses and overlaps the bands in its absence

Electrophoresis

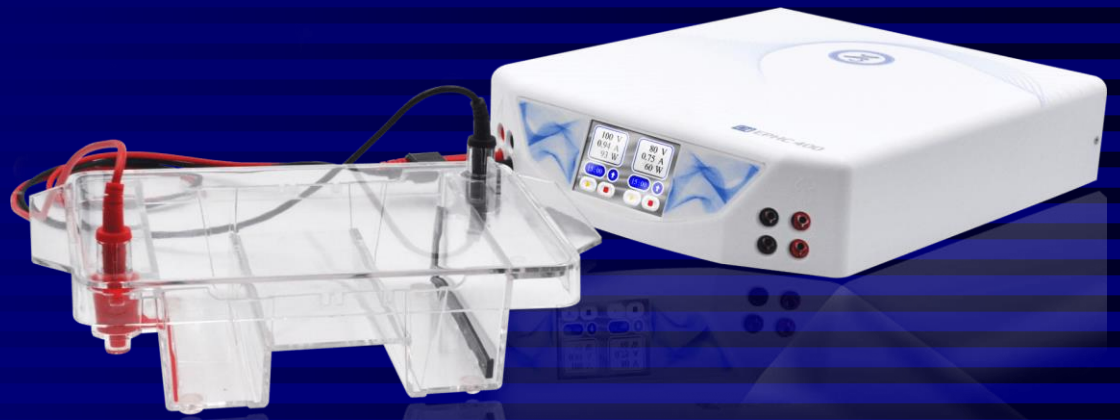
The solid support may be

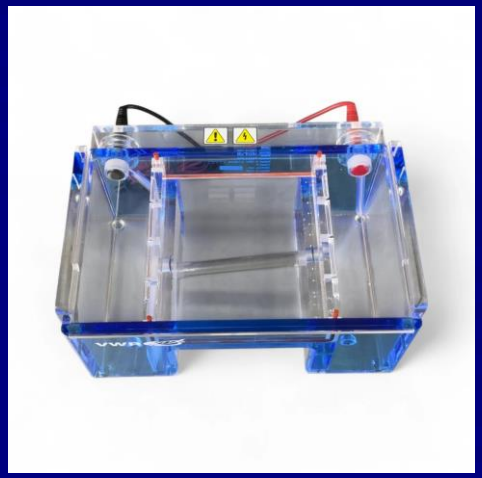
- Paper
- Cellulose acetate
- A gel made up of agarose, polyacrylamide, etc

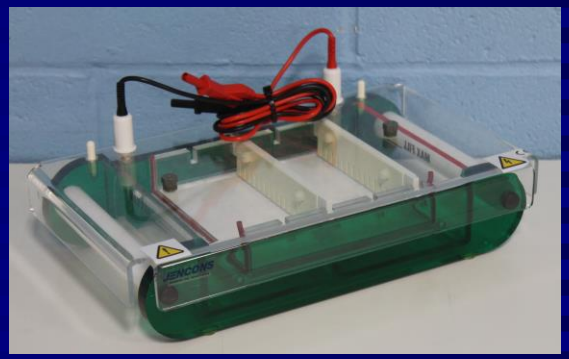
Electrophoresis

Electrophoresis may be proceeded

- Horizontally



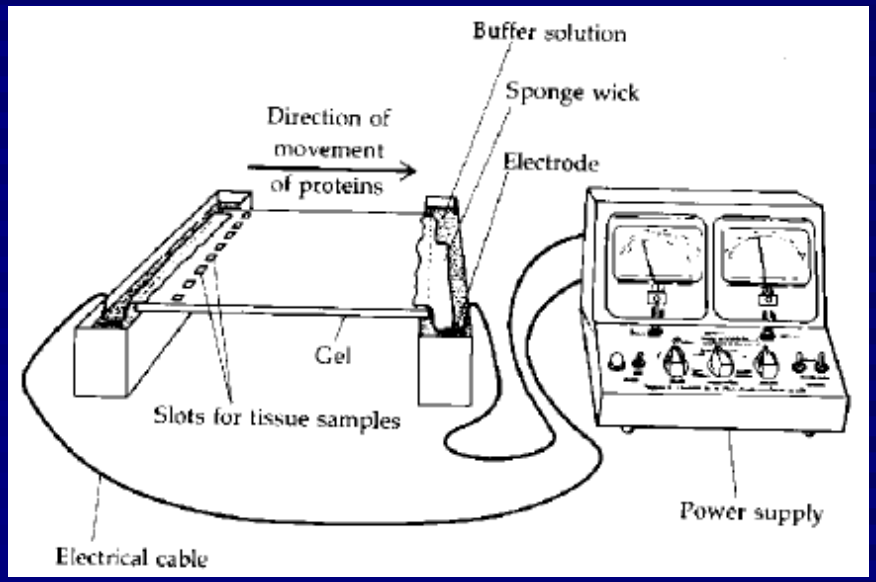


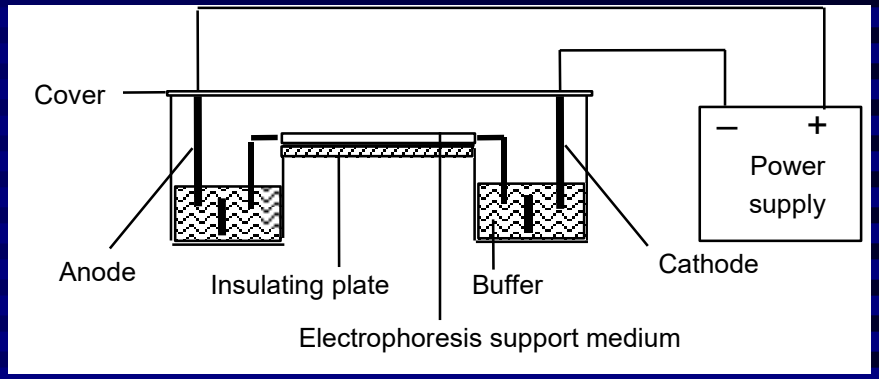


DNA electrophoresis

- It proceeds always in agarose
- Agarose concentration depends on the size of polynucleotides under analysis
  - RFLP (anemias, new types of viruses)
  - Restriction enzymes fragments (plasmids, genetic engineering)
  - PCR products

Cellulose acetate electrophoresis

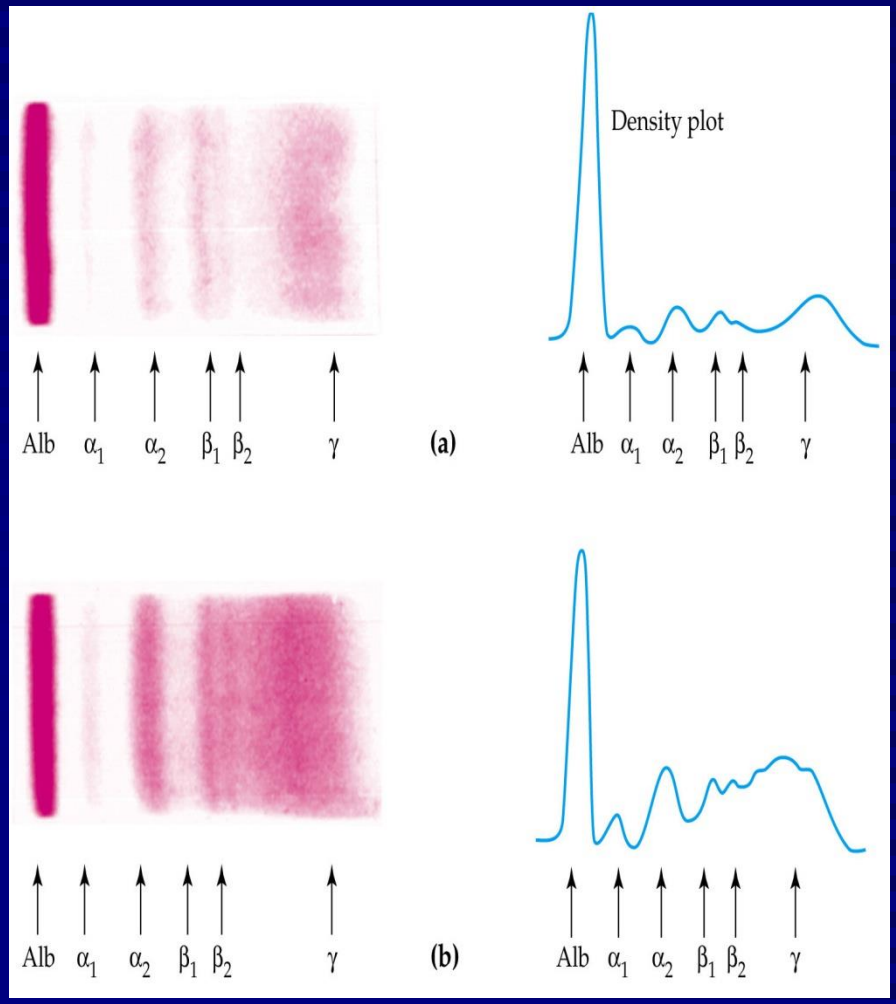




Cellulose acetate electrophoresis

- This is followed by the application of current – initially with a gradual increase so as not to cause overheating – for 45 minutes
- After the time has elapsed, the strips are removed from the device and placed in the protein development liquid

Cellulose acetate electrophoresis

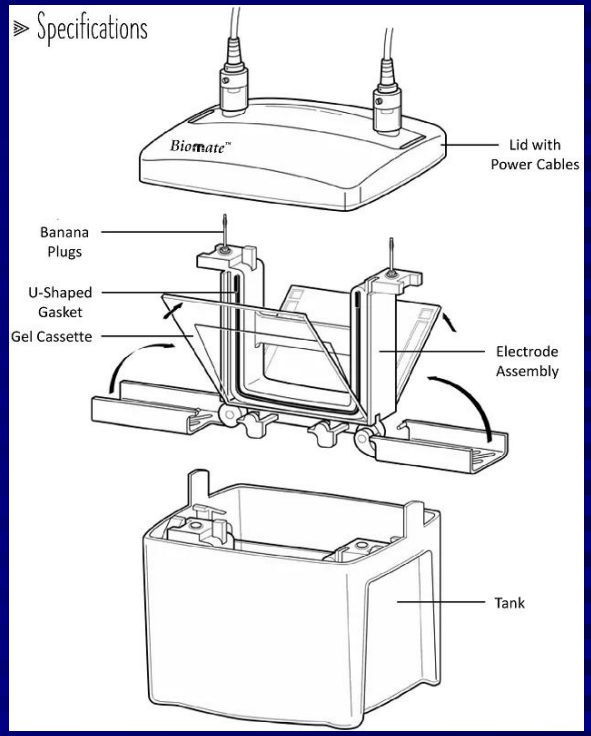


Serum electrophoresis in cellulose acetate results to the separation of five protein bands

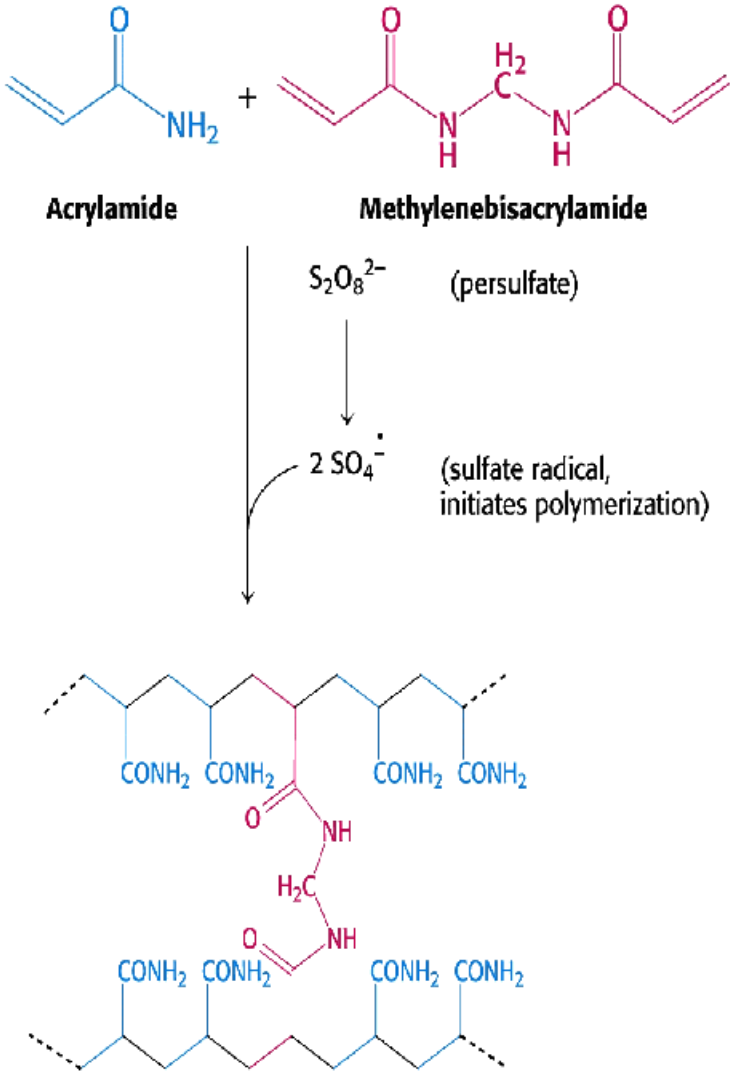
Electrophoresis

Electrophoresis may be proceeded

- Vertically, usually in polyacrylamide gels

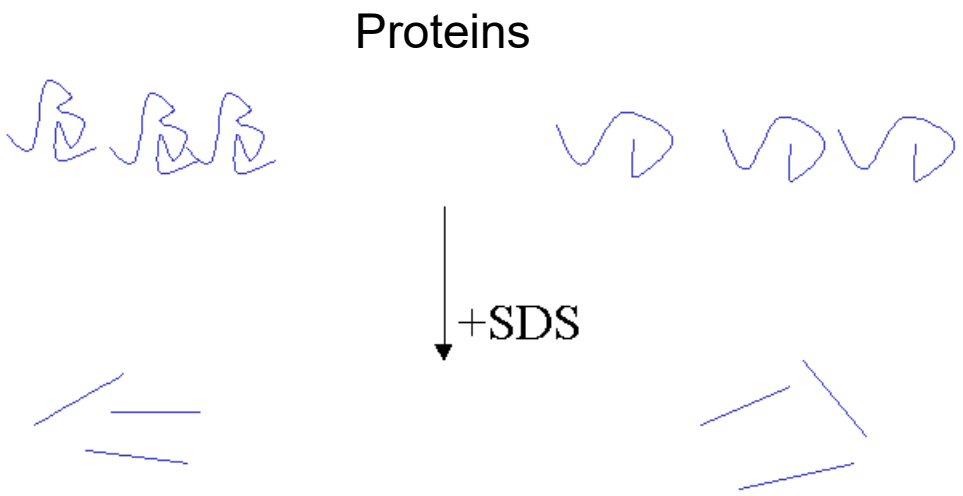


Protein electrophoresis
Polymerization of acrylamide



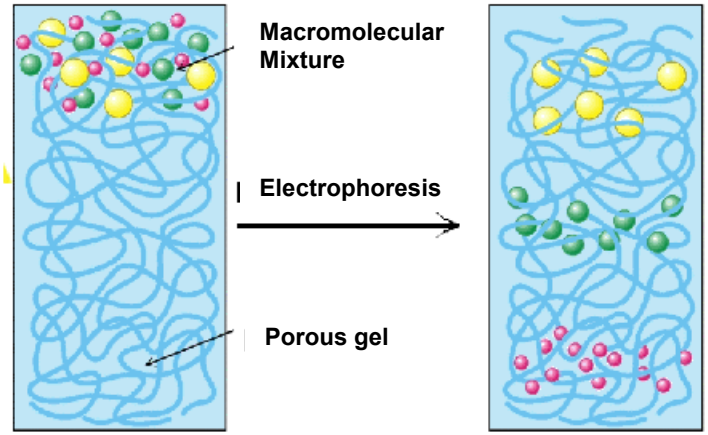
Protein electrophoresis in polyacrylamide gels in the presence of SDS

The anionic detergent sodium dodecyl sulphate [SDS] acts as a denaturing/chaotropic agent. It binds to proteins and denatures them.



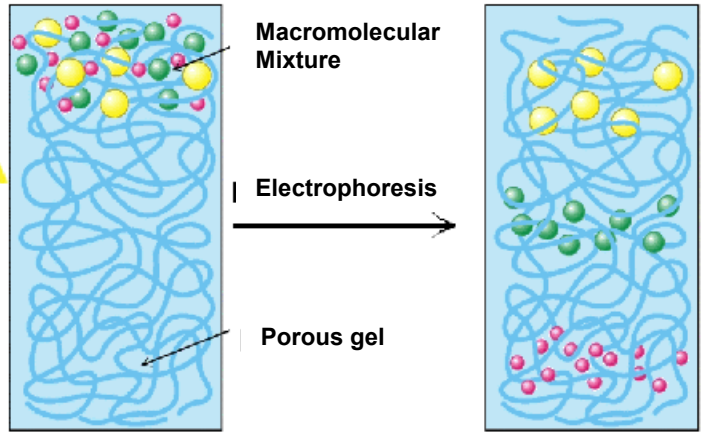
Protein electrophoresis in polyacrylamide gels in the presence of SDS

Because the amount of SDS that binds to the great majority of the known proteins is constant per g of protein, the peptide chains obtain the same charge density and the same shape, therefore their migration in the gel is exclusively related to their molecular mass.
So, the small proteins are rapidly moved within the pores of the gel.

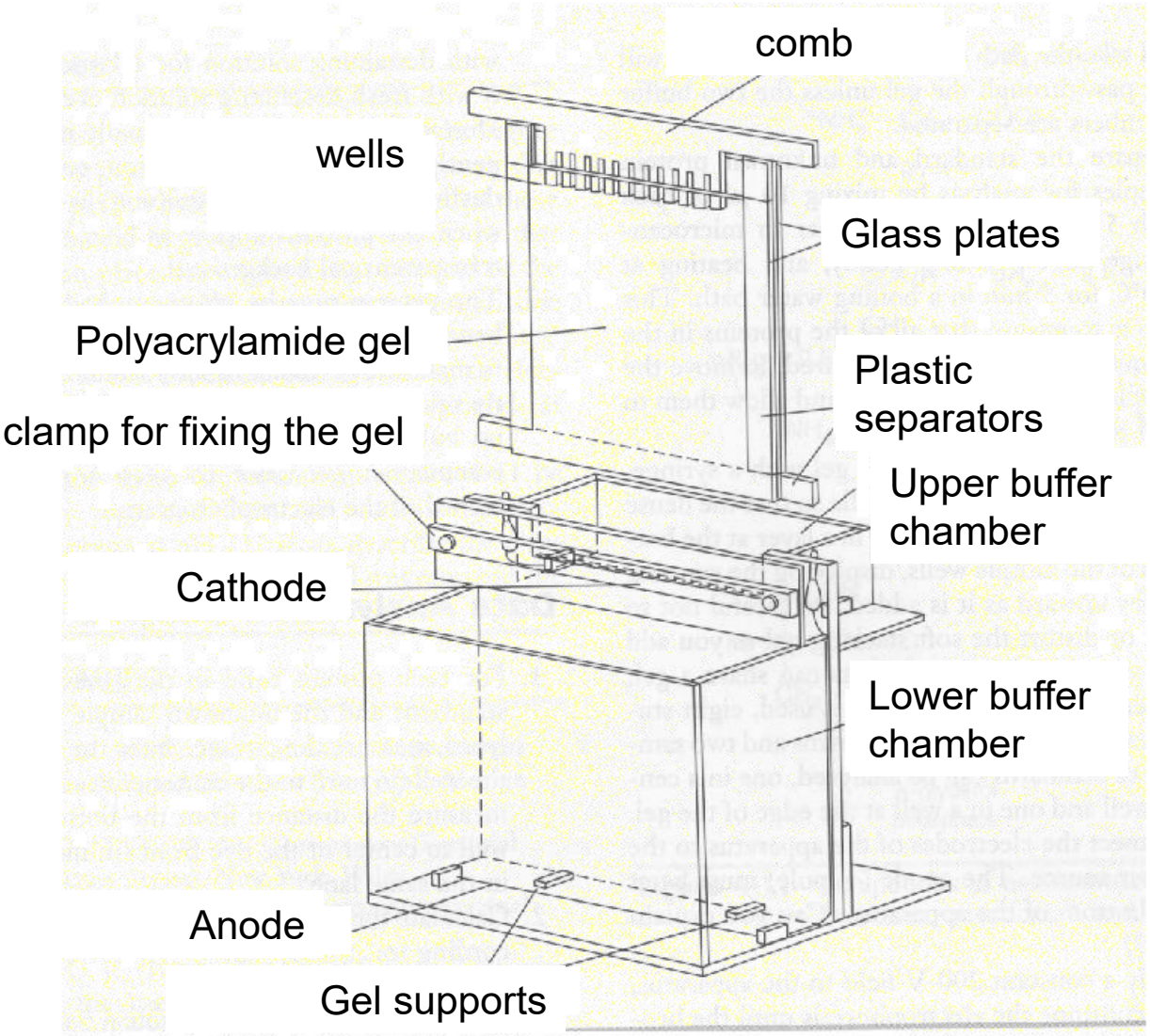


Protein electrophoresis
The role of glycine

The buffer solutions used in electrophoresis contain three components (tris-hydroxymethyl-amino-methane, glycine, HCl) that are responsible for the concentration of protein bands. The proteins possess high negative charge, due to SDS bound, but lower to Cl^- and higher to glycinate anions. Since Cl^- and glycinate are of very small size they move rapidly and so, the protein bands migrating between them they are subjected to concentration.

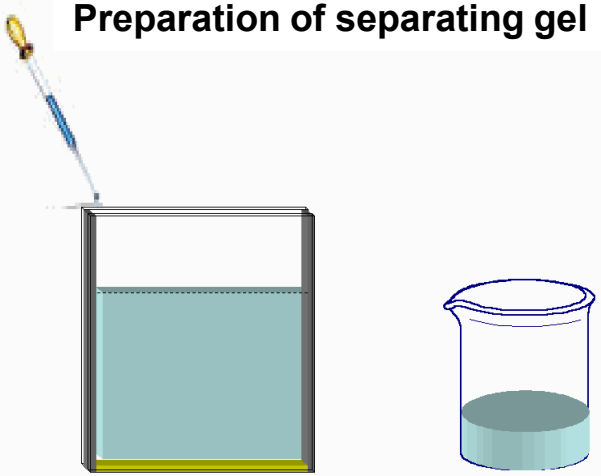


Preparation of the apparatus

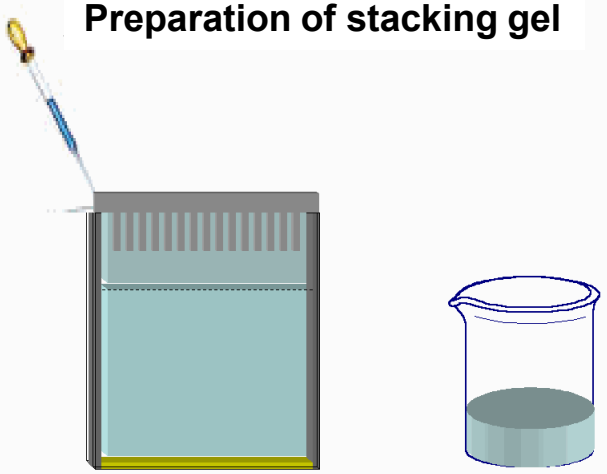


Experimental

Preparation of separating gel

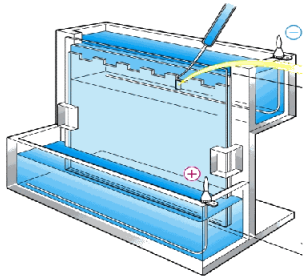


Preparation of stacking gel



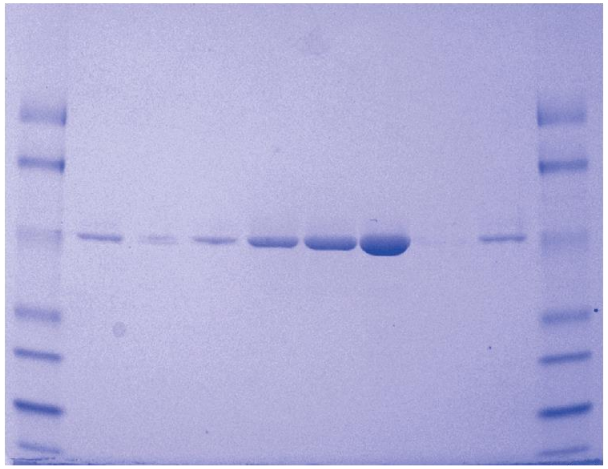
Electrophoretic process

The samples are heated for 5 min at 100 º C and applied to the stacking gel wells (15-20 µL/well)

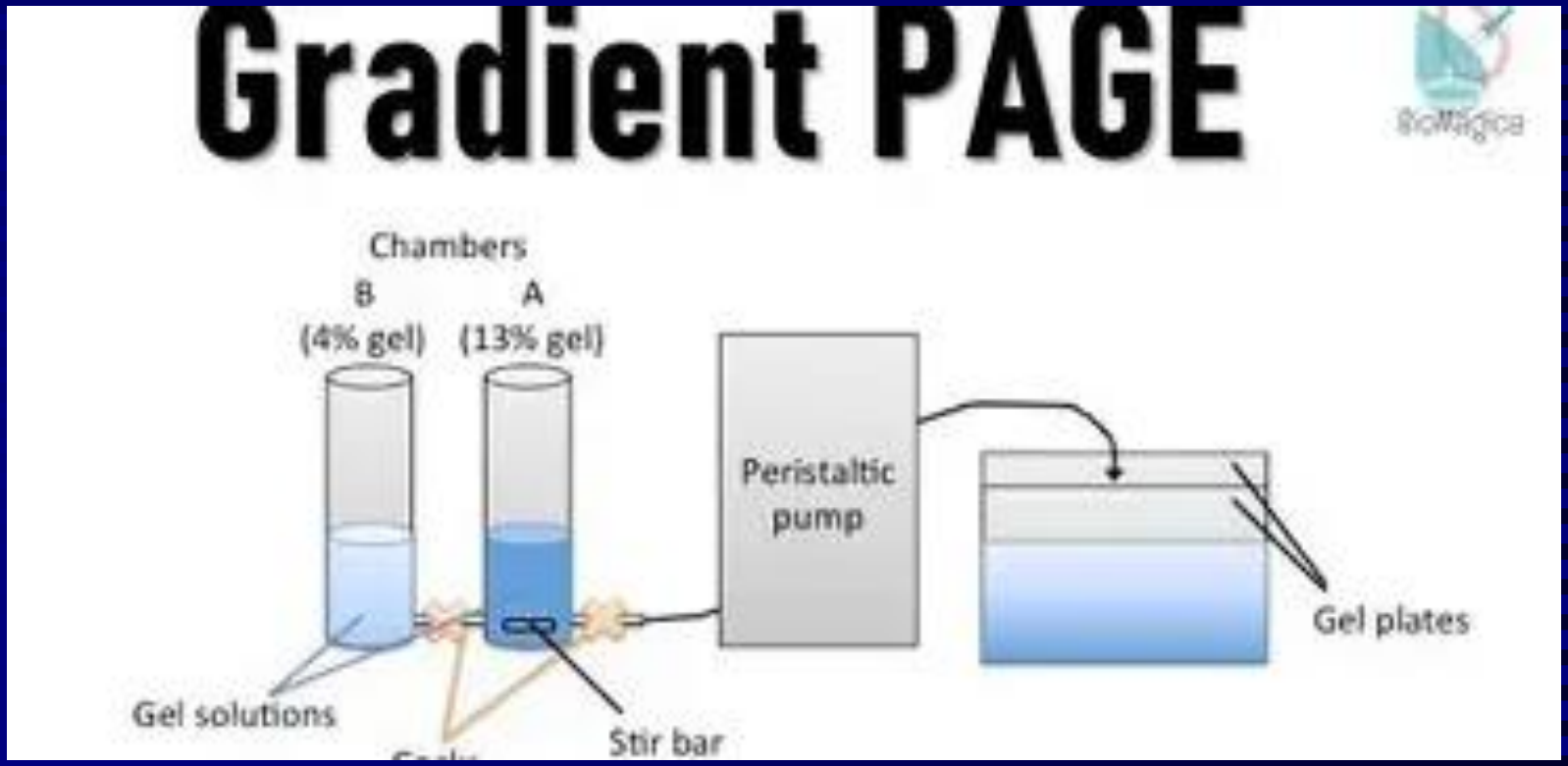


On the same run, a sample containing reference proteins [and a rapidly moved dye] is included to help in the calculation of molecular mass of the proteins under analysis

Staining of the separated protein bands



Experimental for gradient gels



After electrophoresis

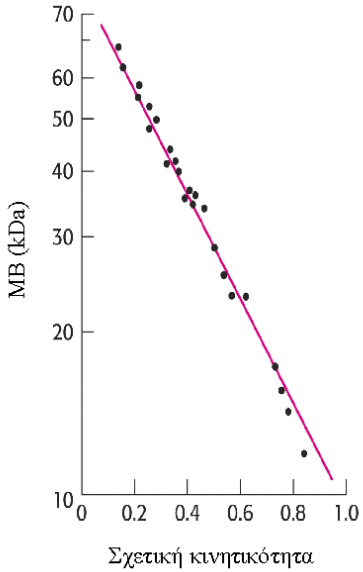
Calculation of molecular mass of the proteins under investigation

After staining and destaining of the gels, the migration [from the start of the separating gel] of each one of the protein bands appeared is measured. Similarly, the migration of the dye is measured, to calculate the migratory capacity of each protein

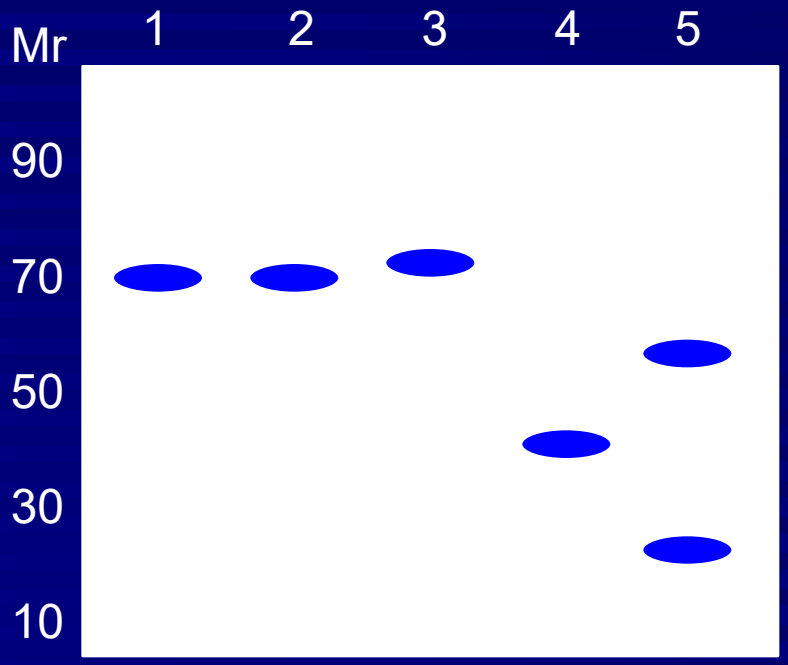
$$\mu = \frac{migration\ of\ protein\ band}{migration\ of\ dye}$$

Graphical representation of migration, μ, against log[molecular mass] produces a straight line

If a sample of unknown protein is subjected to electrophoresis UNDER THE SAME CONDITIONS, its molecular mass can be calculated by this plot



Discussion of electrophoretic results in the presence of SDS & MOH



1. sample – only SDS
2. sample – SDS & MOH
3. sample – SDS & MOH
4. sample – SDS & MOH
5. sample – SDS & MOH

EXPLANATION

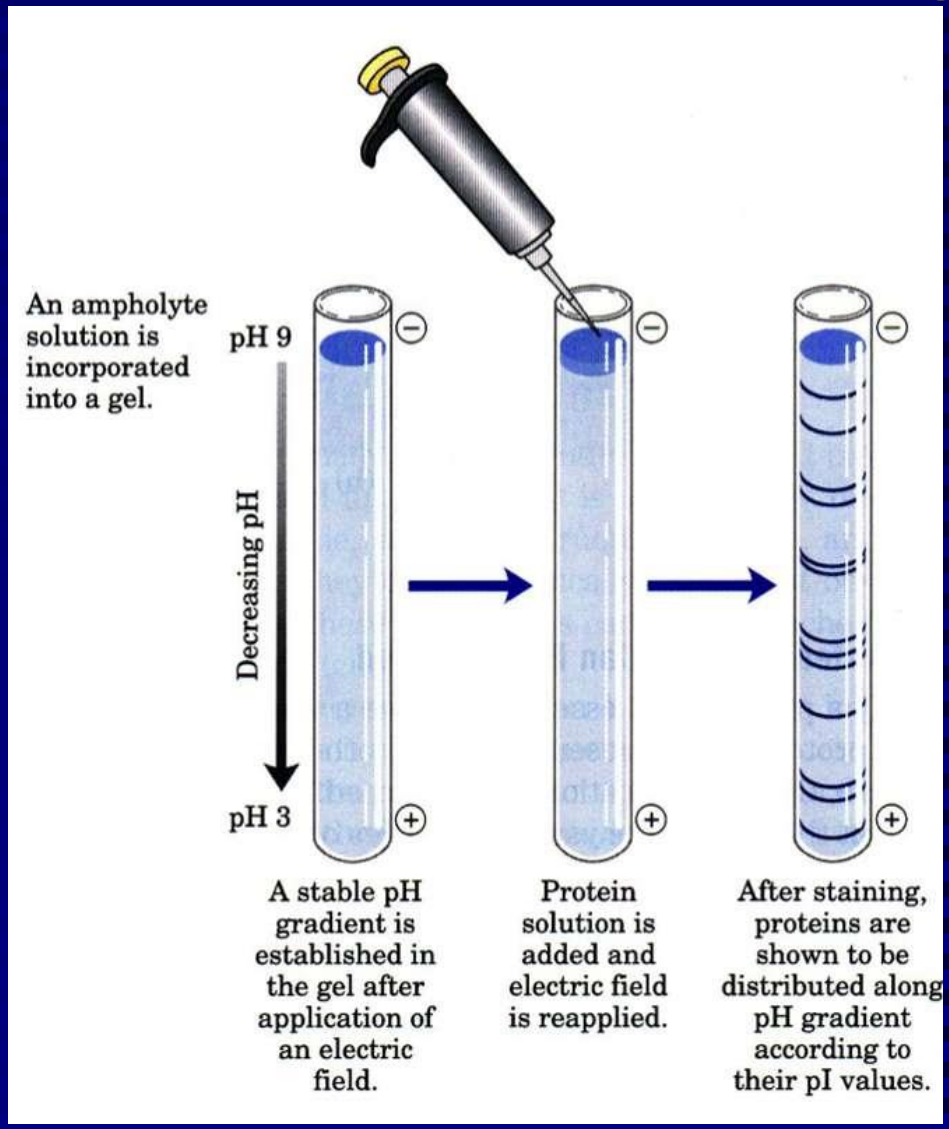
2. Absence of S-S bonds
3. S-S bonds in a single chain
4. Homodimeric protein
5. Heterodimeric protein

MOH: beta-mercaptoethanol
It is a reducing agent that reduces the S-S bonds to -SH

Isoelectric focusing

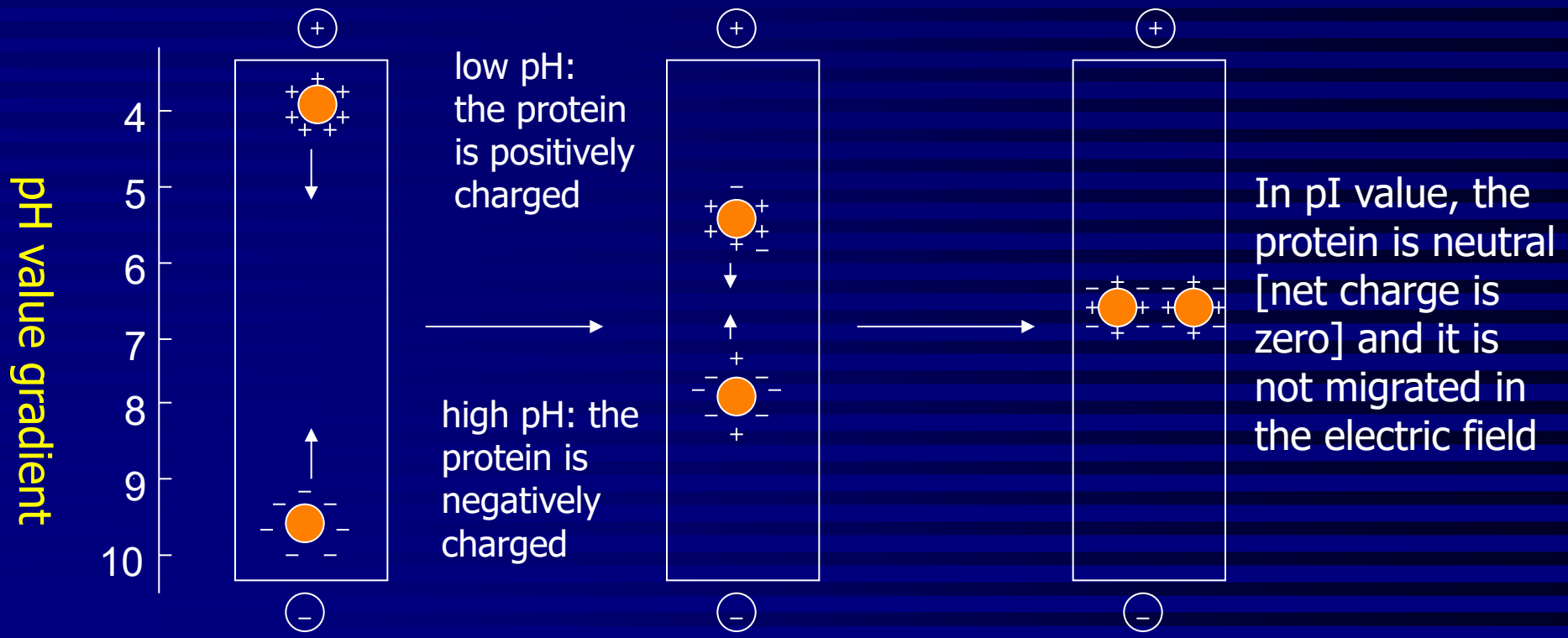
- This is a technique for analytical separation of proteins according to their isoelectric point (in the absence of SDS)
- A scale of different pH values is formed in the separation gel
- Under the influence of an electric field, proteins move and stop when they reach a region with a pH value = pI

Isoelectric focusing

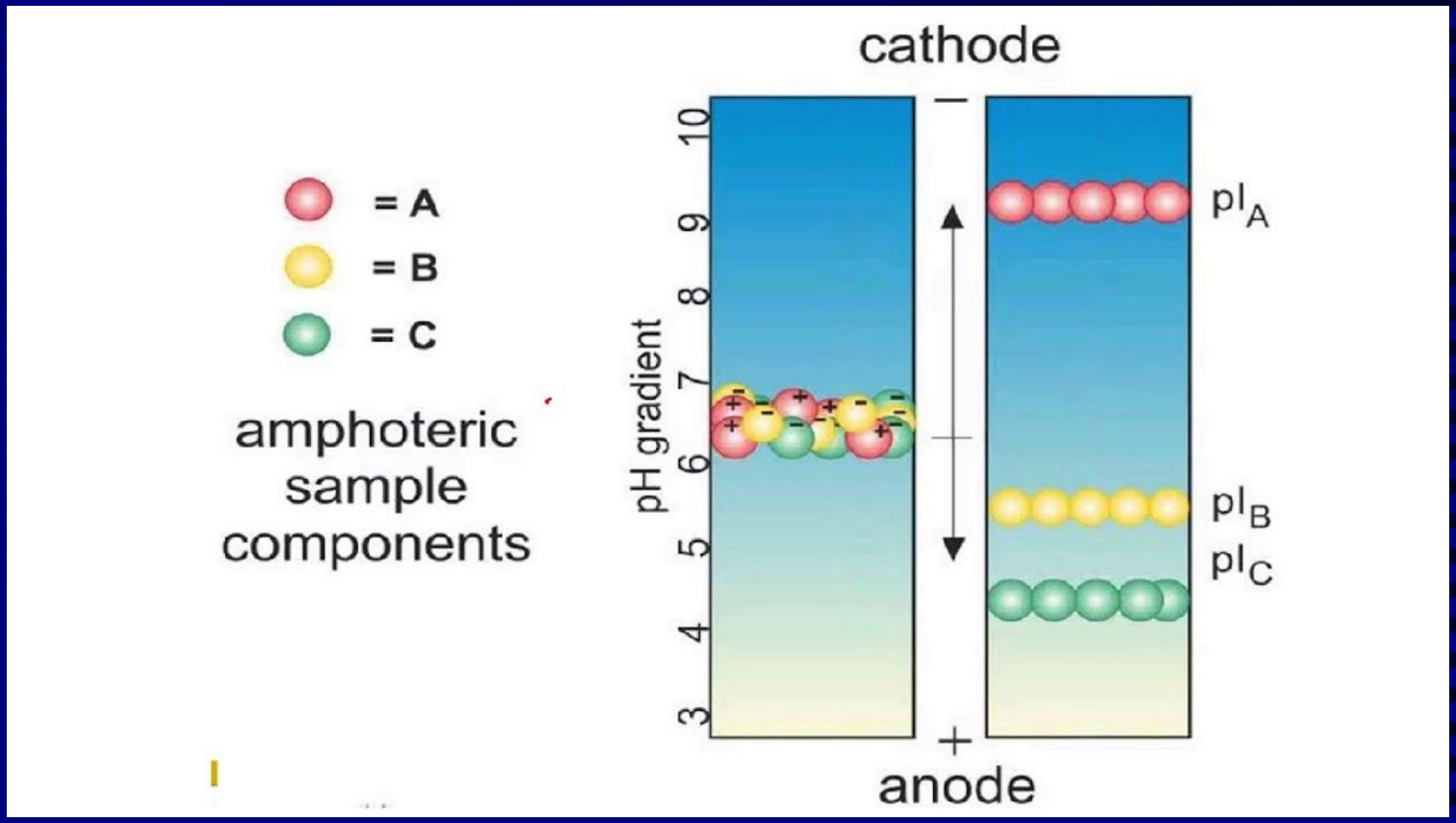


Isoelectric focusing

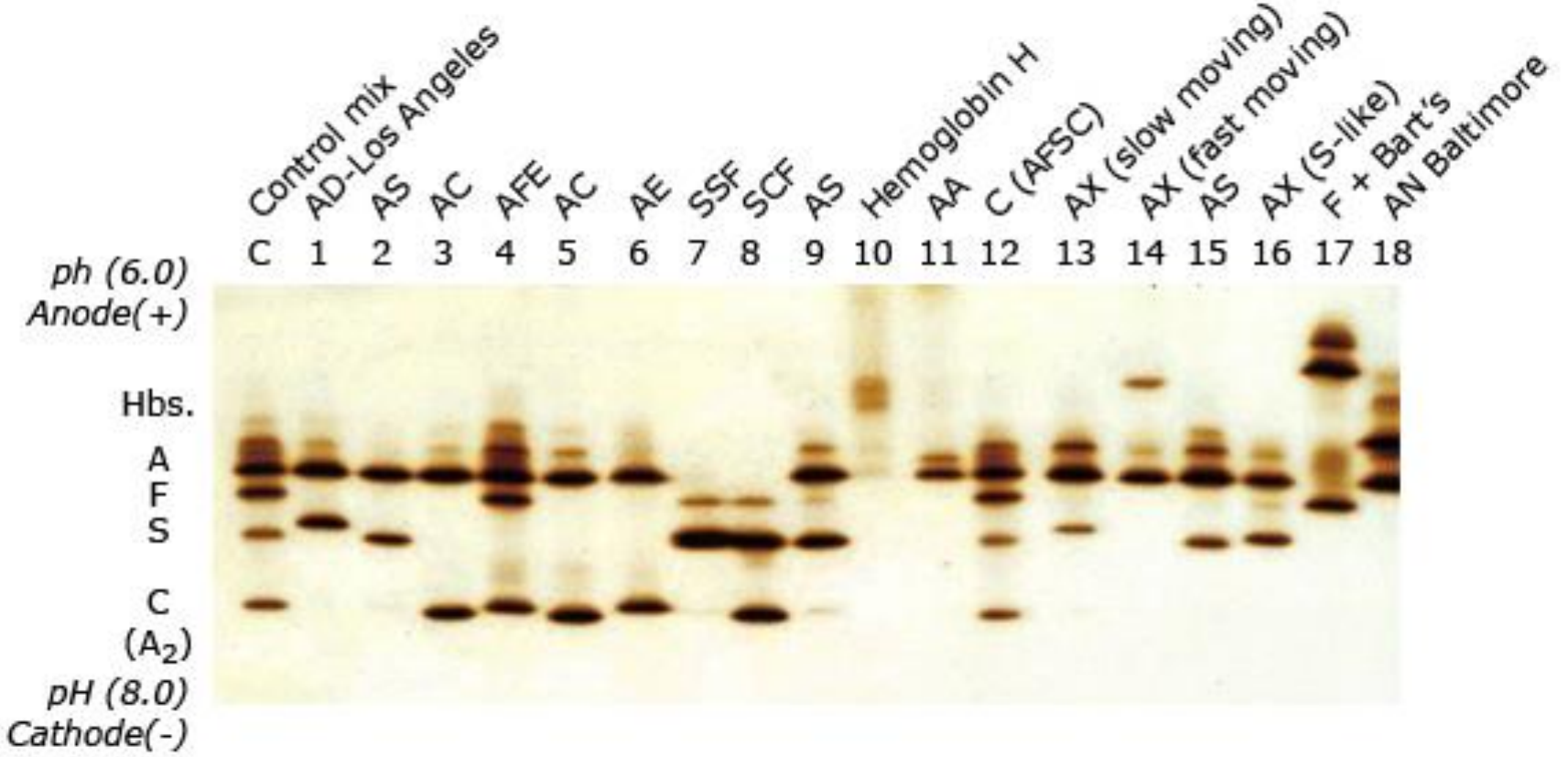
- Separation of protein bands according to their charge density



Isoelectric focusing



Isoelectric focusing

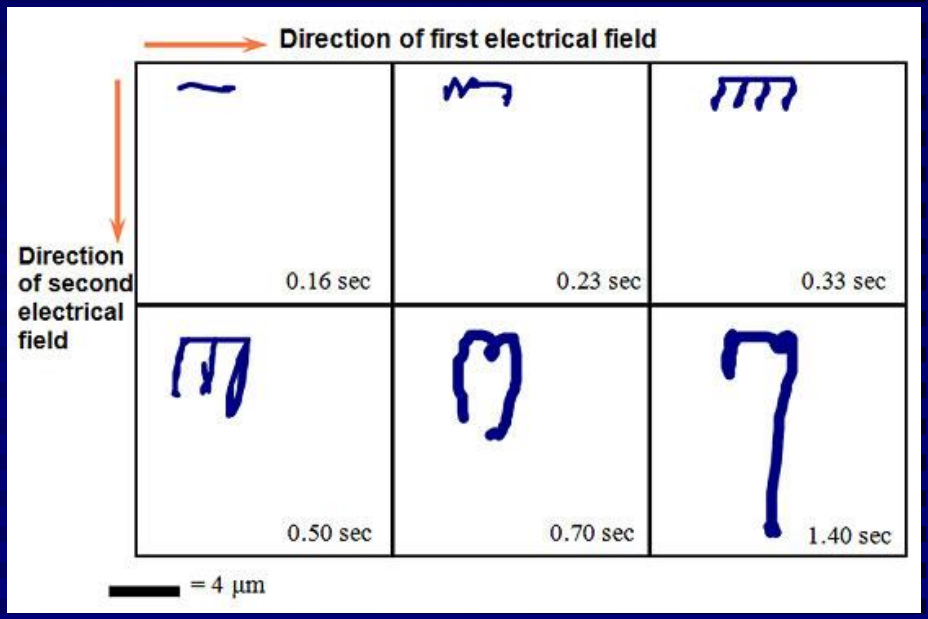


Pulse-Field Gel Electrophoresis

- In conventional electrophoresis, a simple electric field is applied to force biomolecules to move in the separation medium according to charge density, and their movement is characteristic of their molecular mass or size (Klotz and Zimm 1972)
- Separation of DNA fragments < 20 kb is achieved
- Larger fragments are not separated, but appear as a broad band at the top of the gel
- In 1984, Schwartz and Cantor invented pulsed field gel electrophoresis (PFGE) to solve the problem
- PFGE separates DNA by alternating the location of the electric field
- The result is the efficient separation of DNA fragments ~10 Mb by reorientation and movement at different speeds in the gel

Operation of PFGE

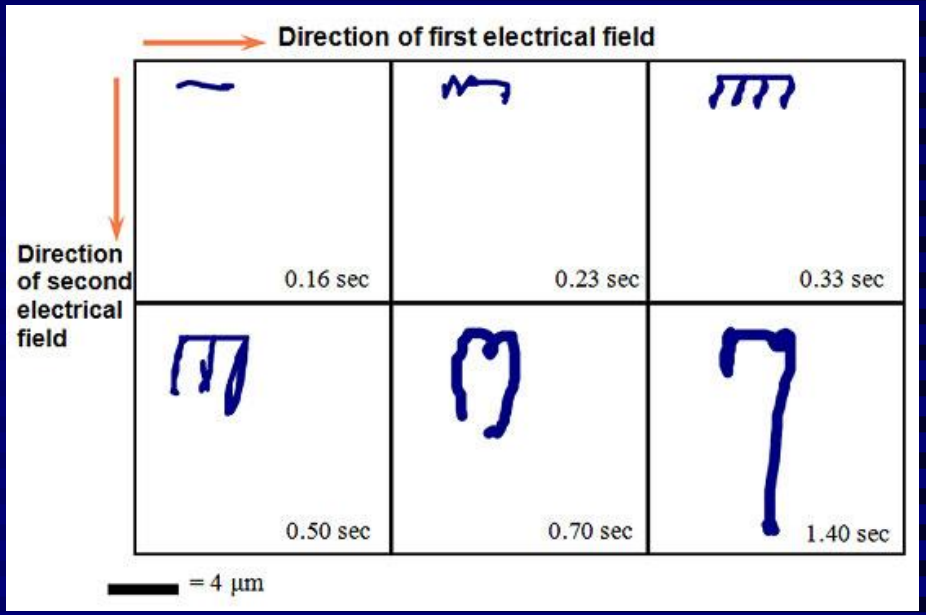
- PFGE arose from the observation that DNA molecules elongate upon application of an electric field and return to their original state upon removal of the electric field
- This relaxation rate depends on the size of the DNA



- When the orientation of the electric field changes during electrophoresis, DNA molecules must return to their elongated form before reorientation, thus affecting the migration rate
- This observation can be used to greatly expand the size range that DNA separation by electrophoretic techniques allows

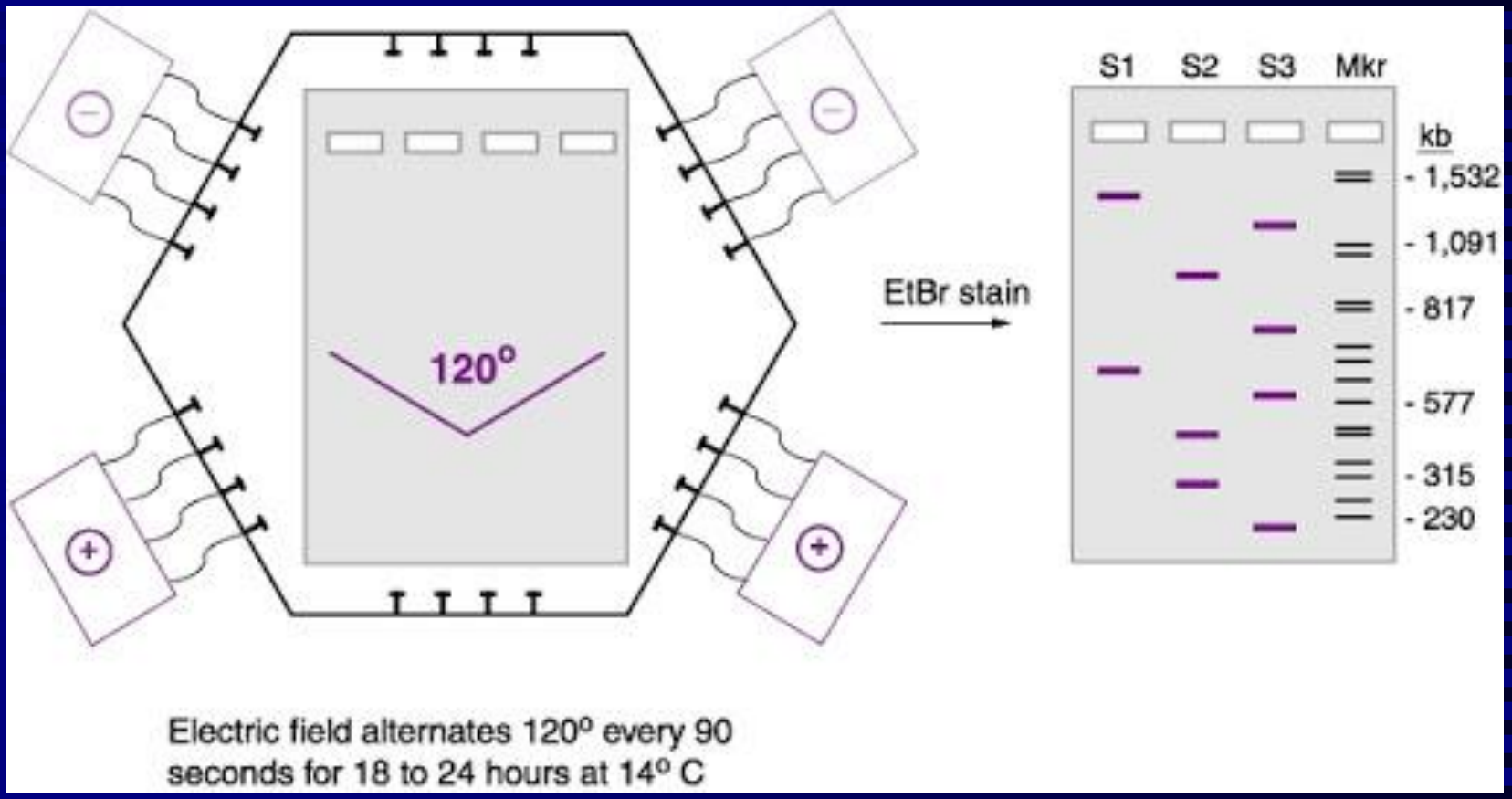
Operation of PFGE

- When the electric field is applied to the gel, the DNA molecules elongate in the direction of the electric field
- The first electric field is then transferred to the second field according to the operating specifications



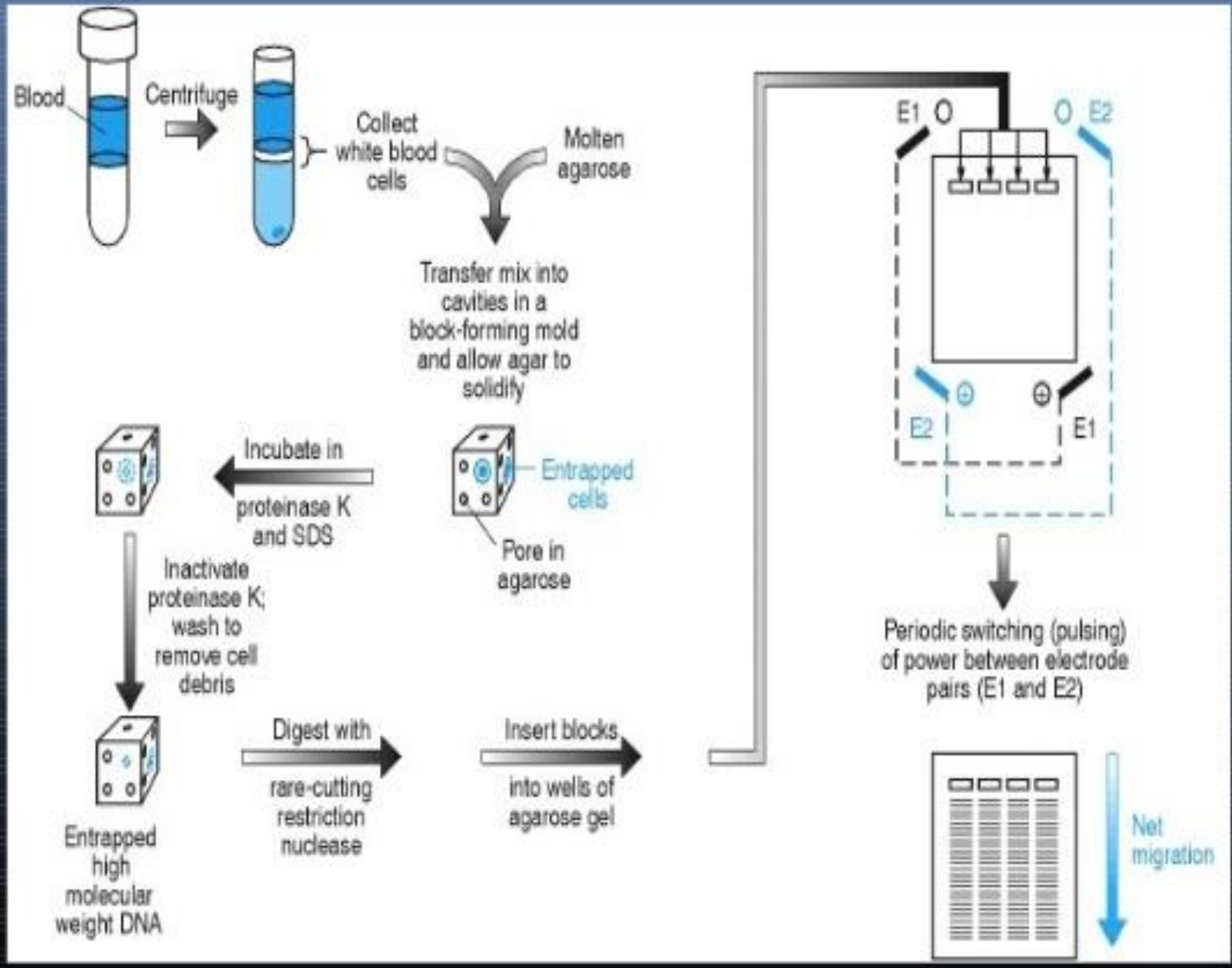
- DNA must change conformation and reorient itself before it can migrate in the direction of this field
- As long as the alternating fields are equal with respect to the voltage and pulse duration, the DNA will migrate in a straight path down the gel

Operation of PFGE



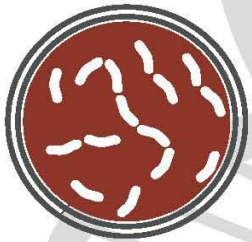
Sample preparation for PFGE

- The large size of DNA molecules to be separated by PFGE imposes certain limitations on sample preparation and handling
- Large DNA is easily disrupted by shear and imparts very high viscosity to the solution
- For these reasons, DNA samples for PFGE are generally prepared by embedding in a gel medium
- The cellular material is suspended in low-concentration agarose and the gelatinized suspension is poured into special molds
- All subsequent manipulations (cell lysis, protein removal, and restriction digestion) are performed by diffusion of reagents into the molds
- The treated molds are then carefully loaded into the wells of the PFGE agarose gel


Blood
Centrifuge
Collect white blood cells
Molten agarose
Transfer mix into cavities in a block-forming mold and allow agar to solidify
Entrapped cells
Pore in agarose
Incubate in proteinase K and SDS
Inactivate proteinase K; wash to remove cell debris
Entrapped high molecular weight DNA
Digest with rare-cutting restriction nuclease
Insert blocks into wells of agarose gel
E1
E2
E2
E1
Periodic switching (pulsing) of power between electrode pairs (E1 and E2)
Net migration

The Pulsed-Field Gel Electrophoresis Process

Bacterial Culture



1 The scientist takes bacterial cells from an agar plate.

Mix bacteria with Agarose

2 The scientist mixes bacterial cells with melted agarose and pours into a plug mold.

Plug Mold

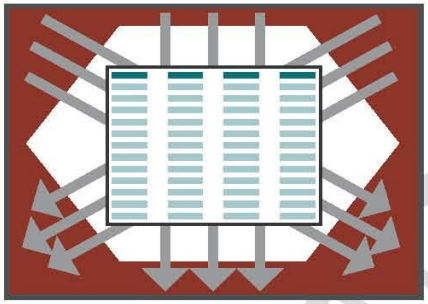
DNA is now in Plugs

Lyse Cells and Wash Plugs

3 The bacterial cells are broken open with biochemicals, or lysed, so that the DNA is free in the agarose plugs.

Cut DNA with Restriction Enzyme

Pulsed-field Gel Electrophoresis (PFGE)



4 The scientist loads the DNA gelatin plug into a gel, and places it in an electric field that separates DNA fragments according to their size.

Data Analysis (BioNumerics)

5 The gel is stained so that DNA can be seen under ultraviolet (UV) light. A digital camera takes a photograph of the gel and stores the picture in the computer.

Applications of PFGE

- Separation of mammalian DNA
- Molecular karyotype determination
- Comparison of DNA of microorganisms

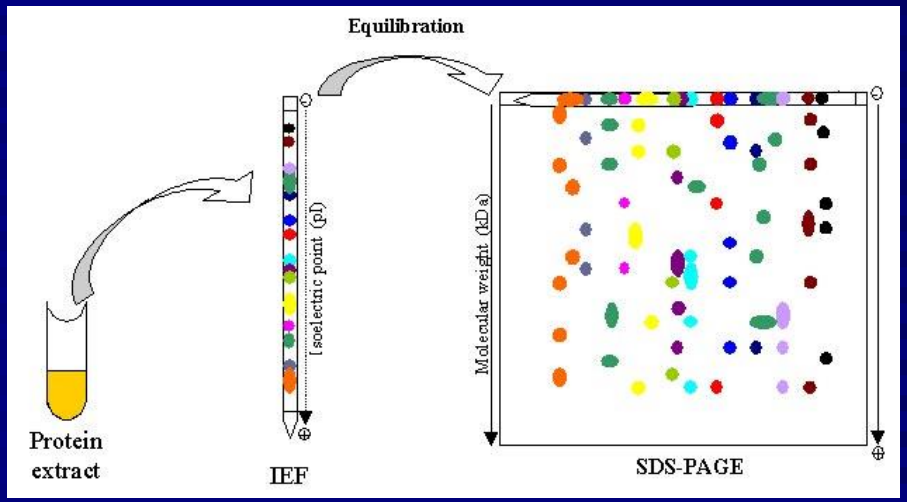
Combined electrophoretic techniques

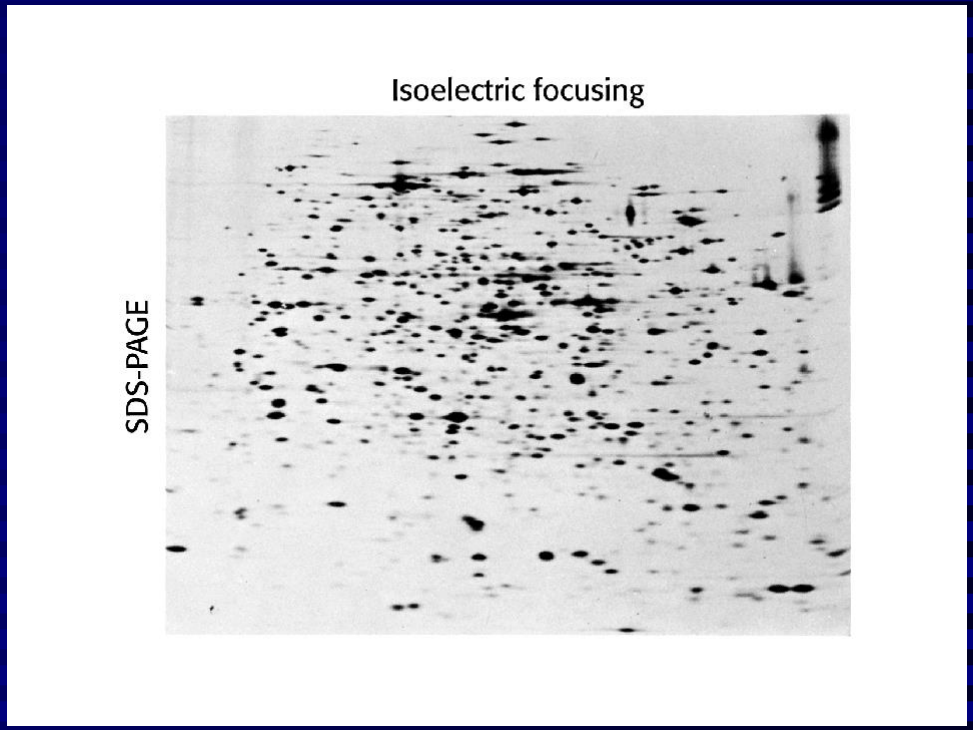
Two-Dimensional electrophoresis

- In SDS electrophoresis the proteins migrate in one dimension, therefore when two different polypeptide chains are of same or similar molecular mass cannot be distinguished

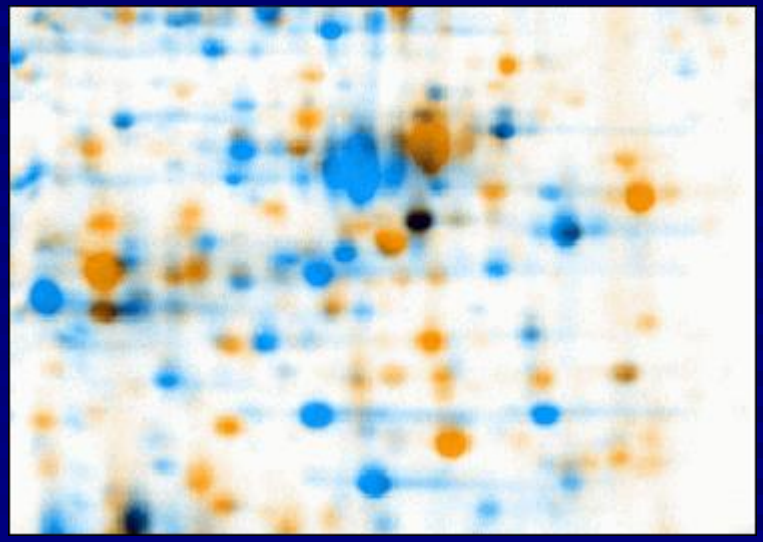
- To achieve their complete separation, a 2-D electrophoretic technique is applied
- This technique uses both isoelectric focusing [in the first dimension] and SDS PAGE [in the second dimension]
- Using this technique, more than 1000 protein dots can be distinguished in a cell extract that represent different polypeptides

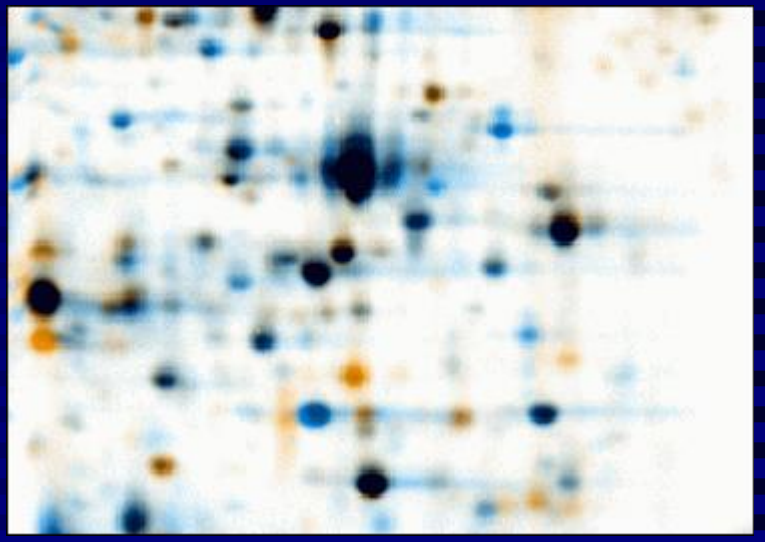
Combined electrophoretic techniques



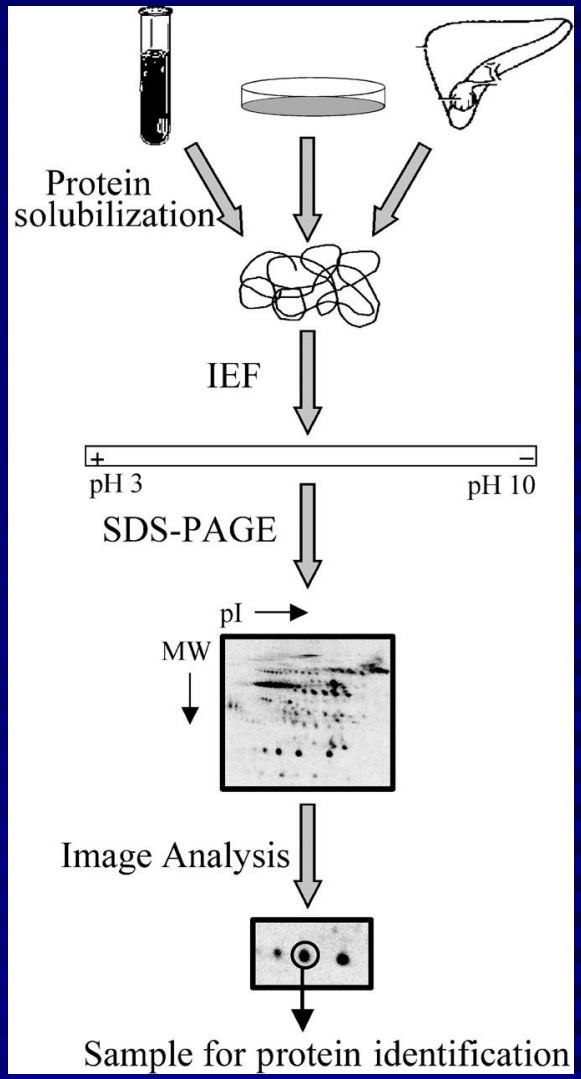


Combined electrophoretic techniques

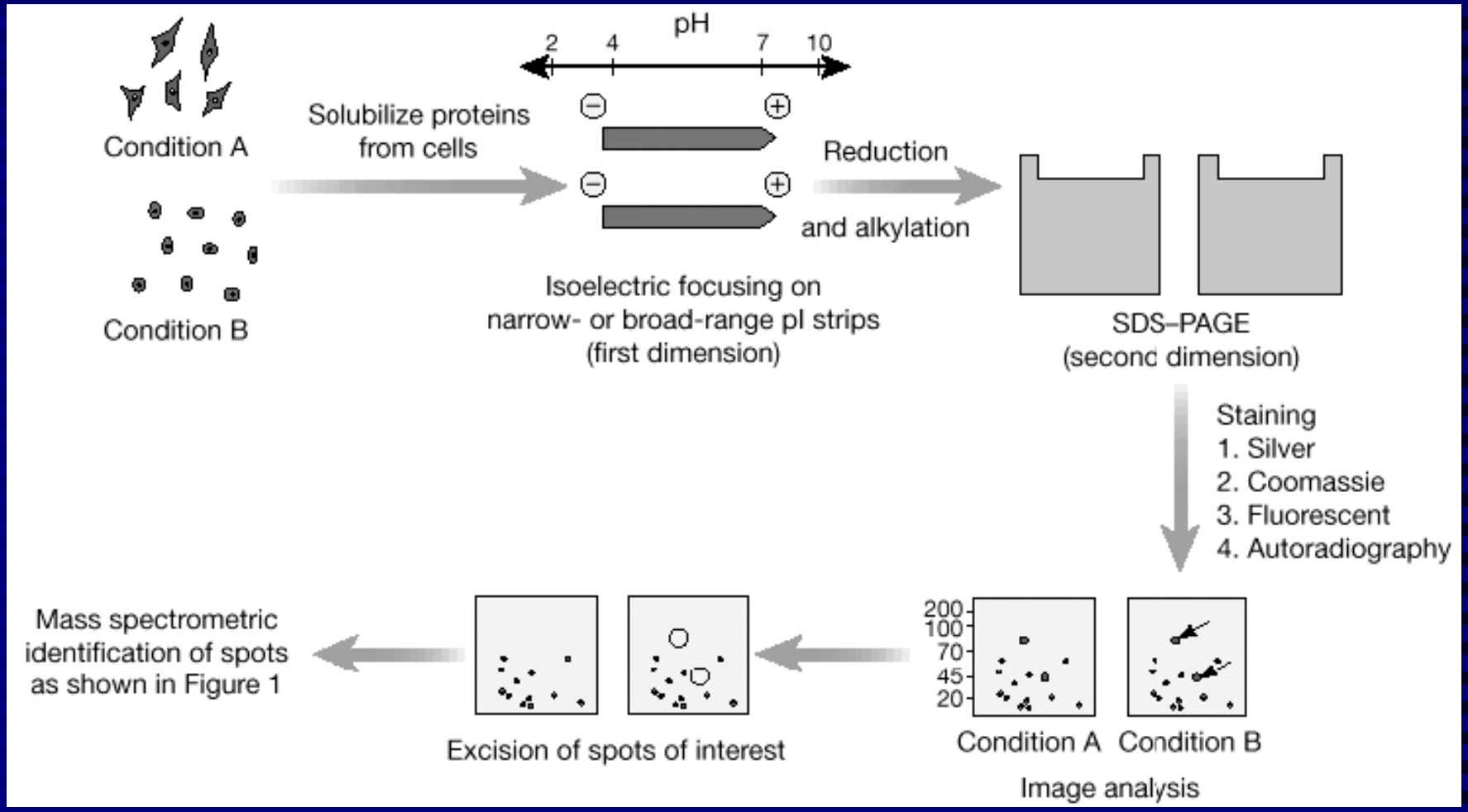




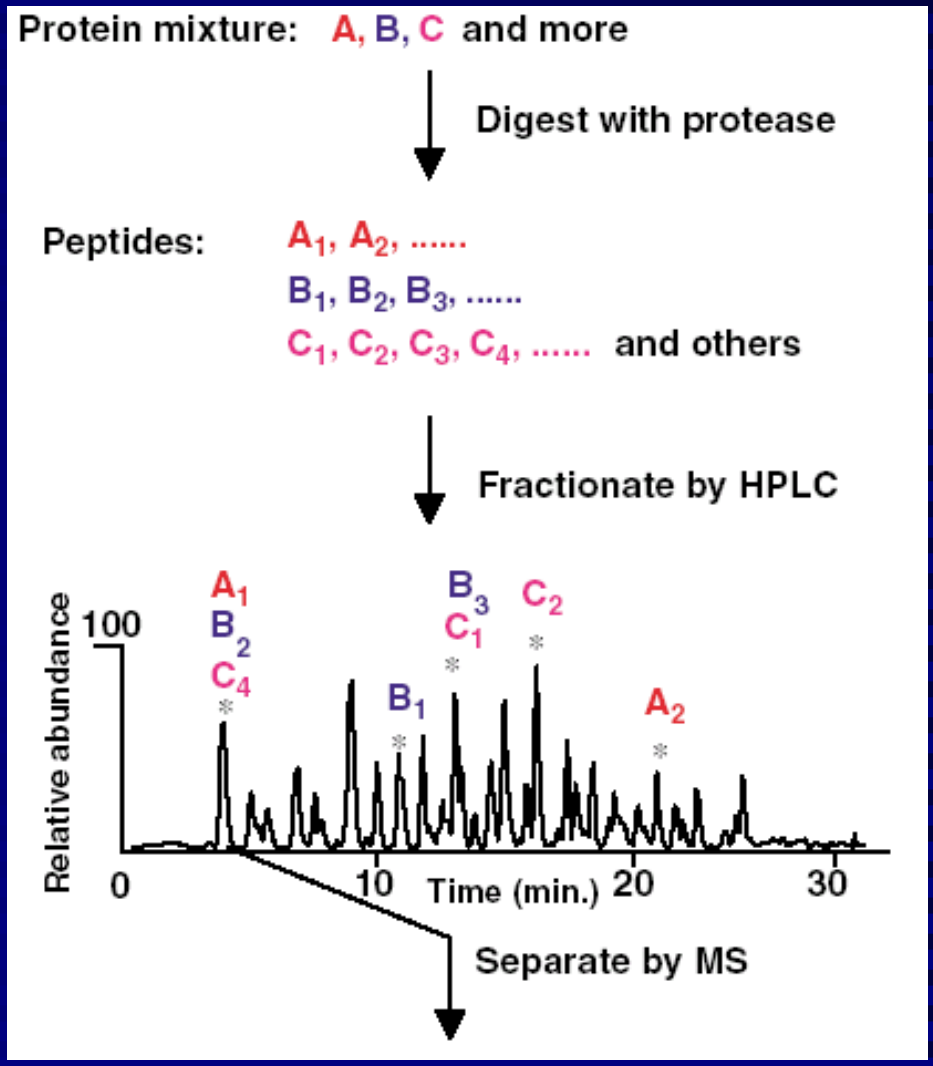
Combined electrophoretic techniques



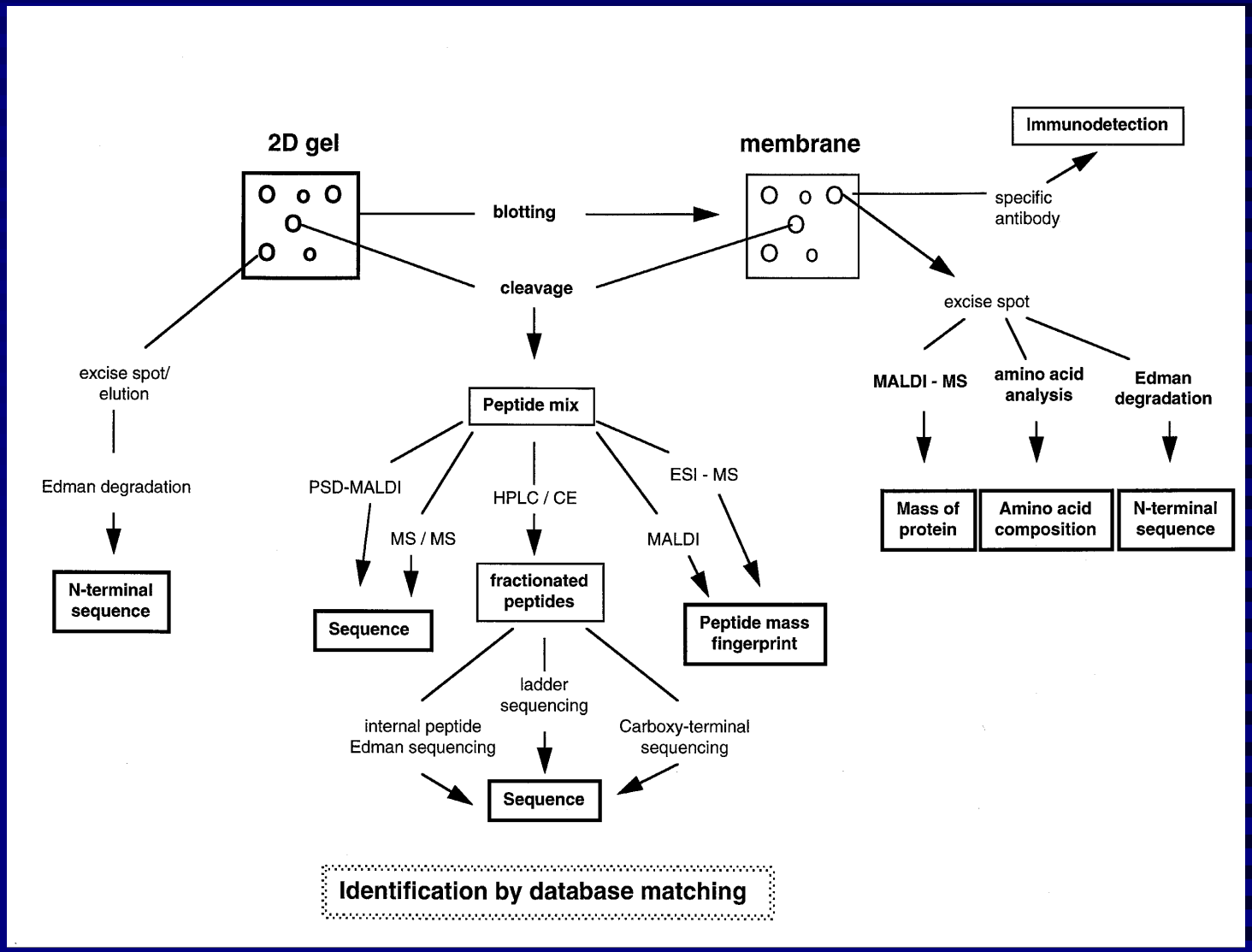
Combined electrophoretic techniques



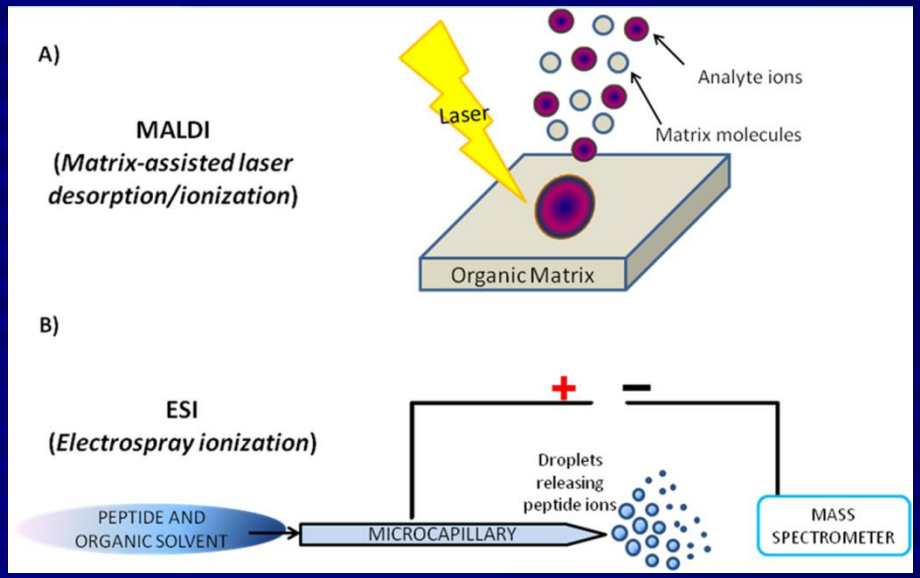
Hyphenated techniques

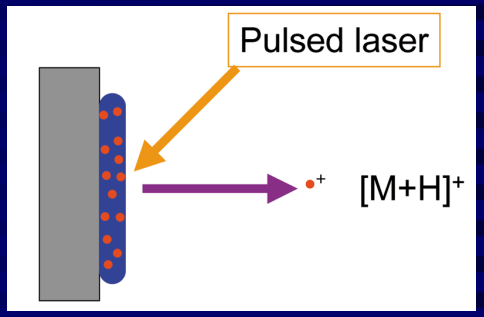


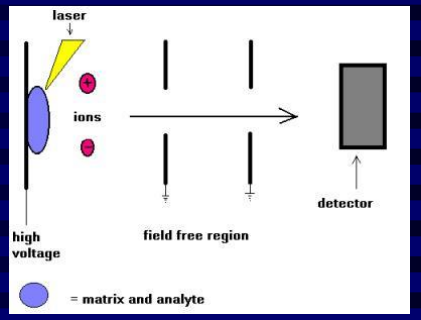
Hyphenated techniques shotgun - proteomics

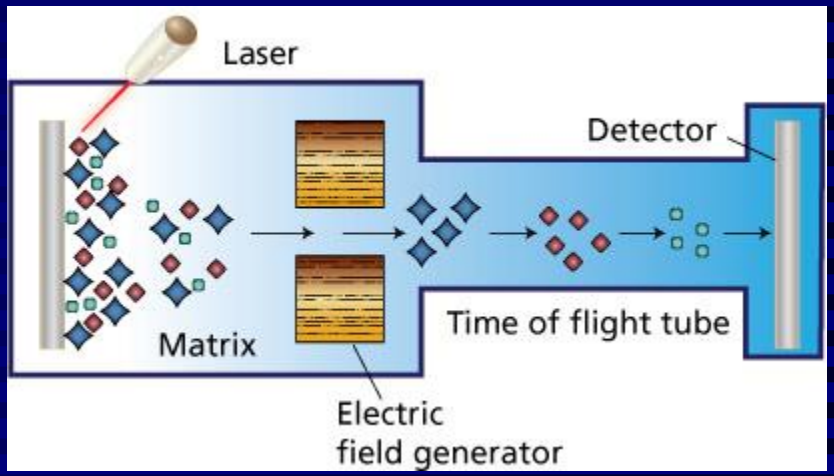


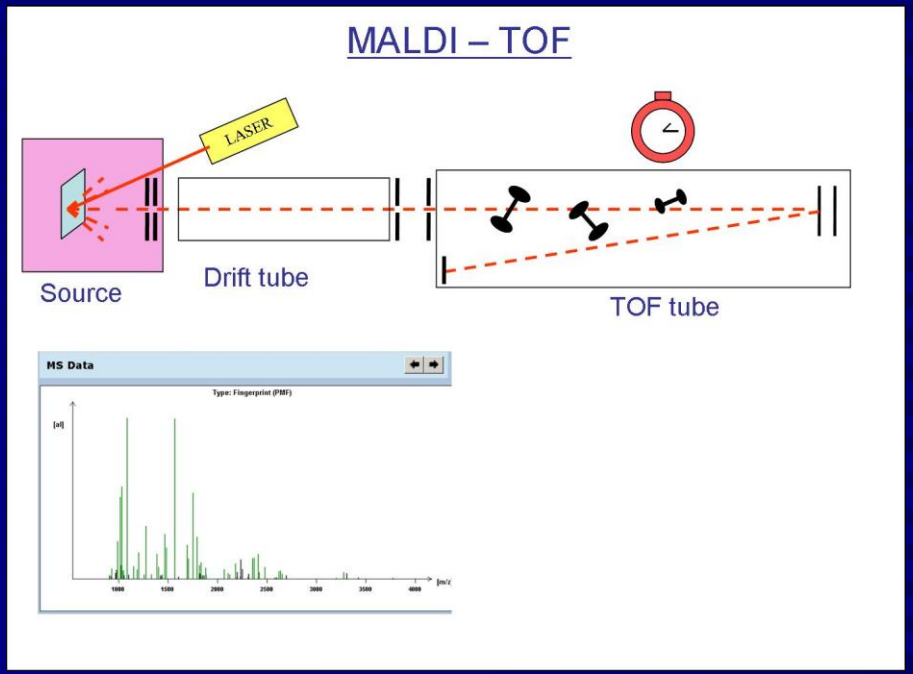
Detection using MS

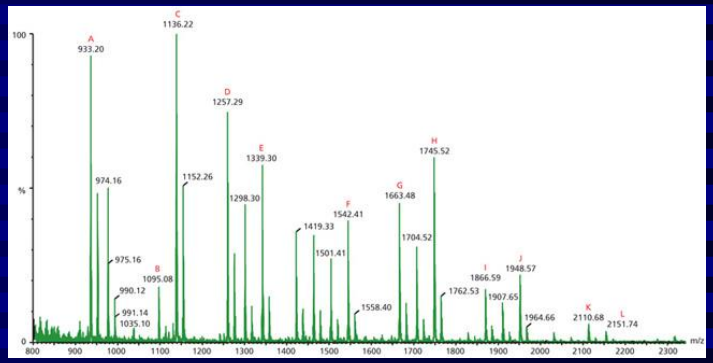












Hyphenated techniques

2D - system

MS

Ultracentrifugation

- Two different types
- Analytical
  - Determination of physicochemical constants (molecular mass, diffusion coefficient, sedimentation constant)
- Preparative
  - Isolation and purification of macromolecules

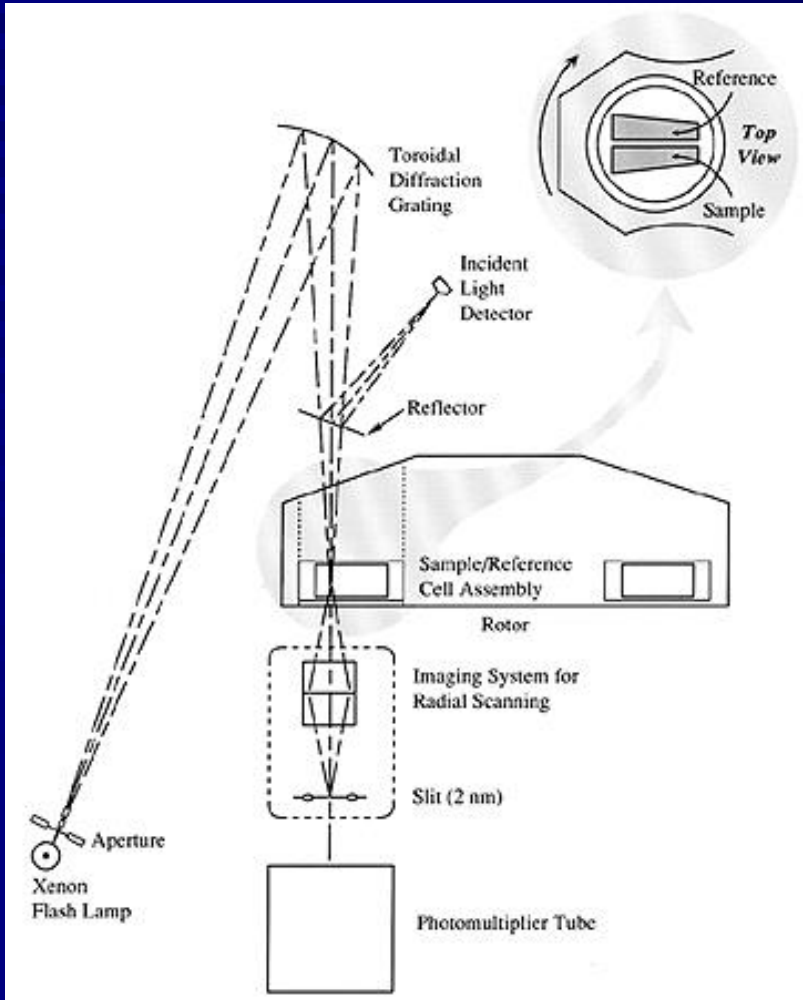
Analytical ultracentrifugation

- The aim is only to find the physicochemical constants of the molecule (molecular mass, diffusion coefficient, etc.)
- It is achieved by recording the movement of each particle during the centrifugation time
  - the absolute purity of the particles being centrifuged is necessary
  - to be carried out, the centrifuge tube must be made of glass or, better, quartz

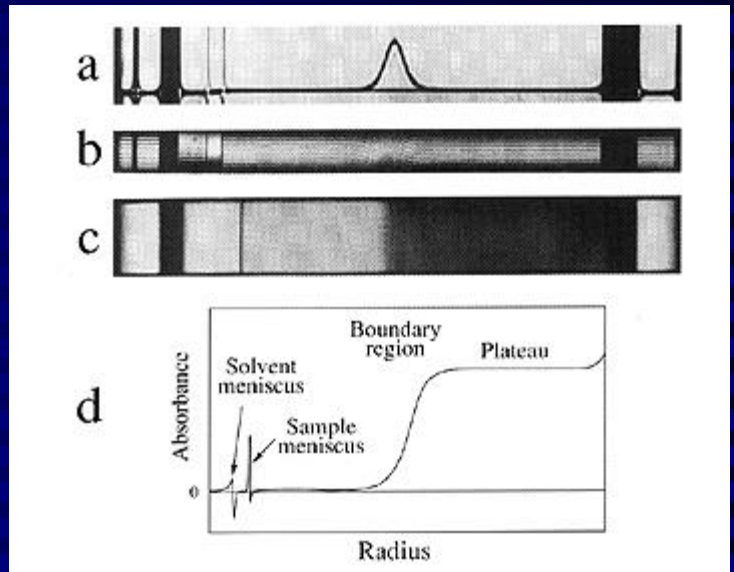
Analytical ultracentrifugation

- The recording of particle movement is carried out by measuring either UV or the refractive index and is recorded either on a recorder or on photographic film
- The development of AU has progressed in three areas:
  - Mechanical improvements of devices
    - development of higher speed, reduction of accident risks, etc.
  - Improvements of optical recording systems
  - Mathematical development

Analytical ultracentrifugation



Schematic diagram of the optical system of the Beckman Optima XL-A Analytical Ultracentrifuge From "Analytical Ultracentrifugation, Vol. 1", Beckman Instruments, Inc.



Comparison of the data obtained from the (a) schlieren, (b) interference, (c) photographic absorbance, and (d) photoelectric absorbance optical systems. ((a) (b) and (c) are taken from Schachman, 1959. Reprinted with the permission of Academic Press.)

From "Analytical Ultracentrifugation, Vol. 1", Beckman Instruments, Inc.

Analytical ultracentrifugation

- As the centrifuge head rotates, centrifugal force is applied to each substance in the solution and it will sediment at a rate proportional to the centrifugal force
- The viscosity of the solution and the physical properties of the substance affect the rate of sedimentation
- Under certain values of centrifugal force and solution viscosity, the rate of sedimentation of a substance will be proportional to its size (molecular mass) and the difference in densities (substance and solution)

Analytical ultracentrifugation

- The movement of the substance will be stable when the centrifugal force and buoyancy are equal:

$$\frac{1}{6}\pi d^3(\rho_P - \rho_1)g = 3\pi d\mu v \qquad (1)$$

v = sedimentation rate of a substance
d = diameter [of sphere]
ρ_P = density of substance
ρ_1 = density of liquid
μ = viscosity of solution
g = gravity

- leading to:

$$v = \frac{d^2(\rho_P - \rho_1)}{18\mu} xg \qquad (2)$$

Analytical ultracentrifugation

- That is, the sedimentation rate:
  - It is proportional to the size (volume) of the substance
  - It is proportional to the difference in density between substance and solution (it becomes zero when they are equal)
  - It decreases with increasing solution viscosity
  - It increases with increasing centrifugal force

Analytical ultracentrifugation

Considering $\frac{dr}{dt} = v$ (in cm/sec)

per unit of centrifugal force, we obtain:

$$\frac{dr}{dt} = s\omega^2 r \qquad (3)$$

s = sedimentation coefficient (in sec)
r = distance of substance from the center (in cm)
ω = angular velocity

Analytical ultracentrifugation

- Combining the equations 2 and 3 we obtain a new one linking **sedimentation coefficient** and **molecular mass**

$$\frac{v}{\omega^2 r} = \frac{M(1-V\rho)}{f}$$

$$s = \frac{M(1-V\rho)}{Nf}$$

$$\frac{s}{D} = \frac{M(1-V\rho)}{RT} \qquad \textbf{(4)}$$

M = molecular mass
V = $1/\rho_P$
f = molecular coefficient of friction
N = Avogadro number
D = diffusion coefficient
R = gas constant
T = absolute temperature

- Equation 4 must be corrected for ideal solution and temperature conditions, in order to calculate $s_{20,w}$ that thereafter will be corrected for infinite dilution of the substance and thus the "real" value of $s^0_{20,w}$ will be obtained

R = 0,082 L·atm / mol·K ή R = 8,314 joule / mol·K.
The constant R or Rutherford constant is independent to gas and conditions used

Preparative ultracentrifugation

- Two different typs
- Differential: separation of particles
  - Separation of solid compounds
  - Separation of cells
  - Separation of subcellular organelles
- Ultracentrifugation of the separation of soluble macromolecules: there are two subtypes
  - Rate – sedimentation ultracentrifugation
  - Sedimentation – equilibrium centrifugation

Preparative ultracentrifugation

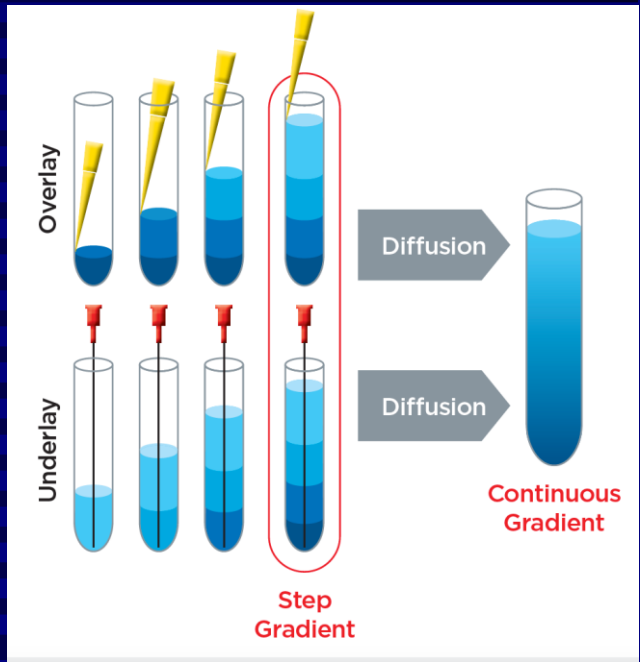
SELECTION OF THE SEPARATION APPROACH

- The method to be followed always depends on the type of molecules whose separation is requested
- For rate-sedimentation centrifugation to be successful, the density of the substance must be greater than the density of any point in the solution
- The centrifugation stops before any of the separated zones reaches the bottom
- In sedimentation-equilibrium centrifugation, the separation of the zones is based only on the different densities of the substances, regardless of the duration of centrifugation

Preparative ultracentrifugation

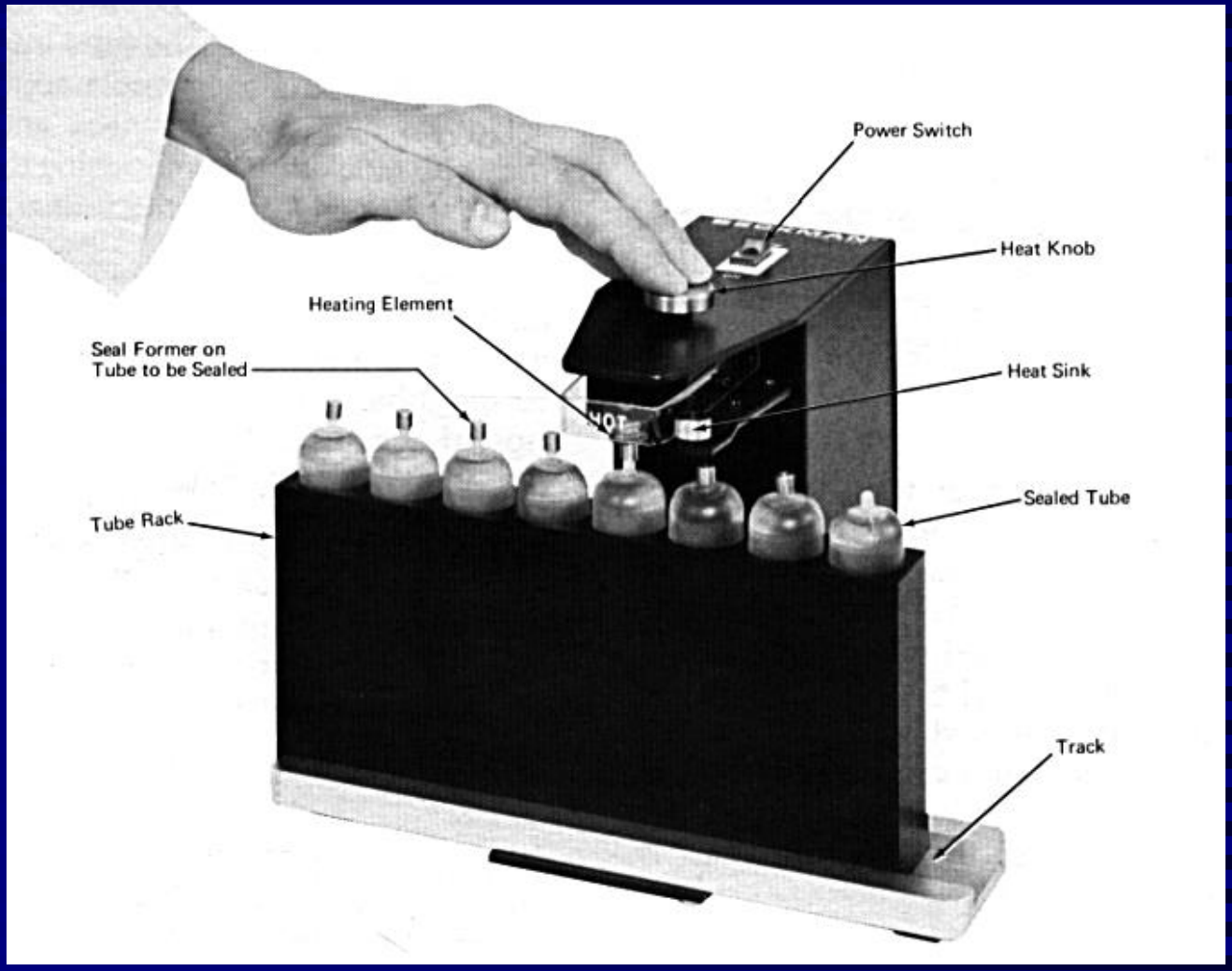
PREPARATION OF THE DENSITY GRADIENT

- Three methods
  - Specific apparatus
    - Linear gradient
  - Density gradients are commonly layered in steps using an underlay or overlay approach, where solutions (sucrose or CsCl) are added in order of increasing or decreasing density
  - Preparation of the density gradient during ultracentrifugation (in specified cases)



Preparative ultracentrifugation

FILLING OF THE TUBES

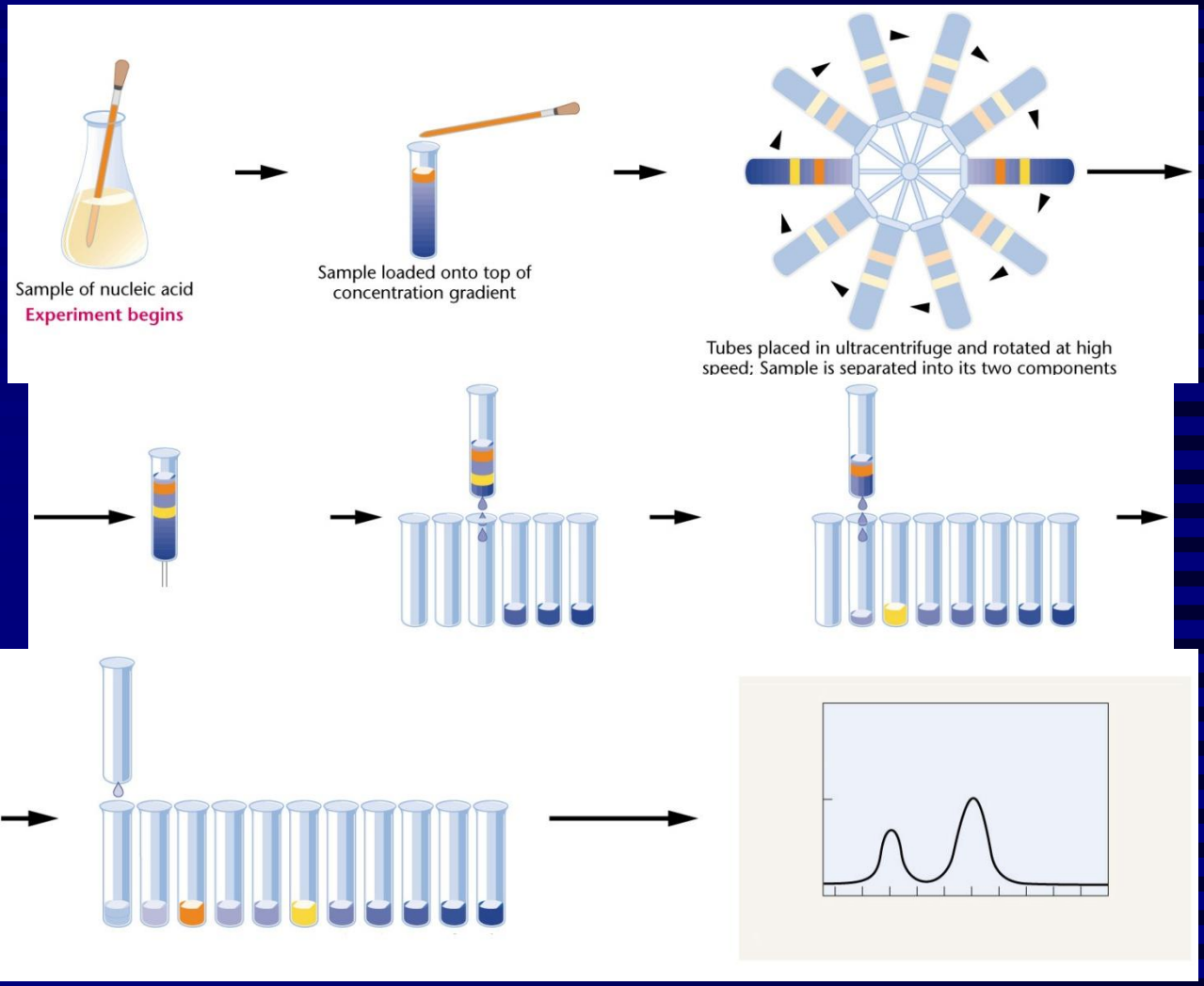


Preparative ultracentrifugation
RECOVERY OF SEPARATE ZONES

- Fractionation with:
- Flow of light, immiscible liquid (paraffin oil), from top to bottom
- Flow of heavy, immiscible liquid (fluorinert), from bottom to top (this is the most correct procedure)
  - In all cases, a fraction collector is used
- Cooling the tube and cutting of the bands with a specific saw
- The samples are immediately analyzed for the component to be studied, to determine whether or not the separation has occurred
- Also, the density is measured to confirm the perfection of the process followed

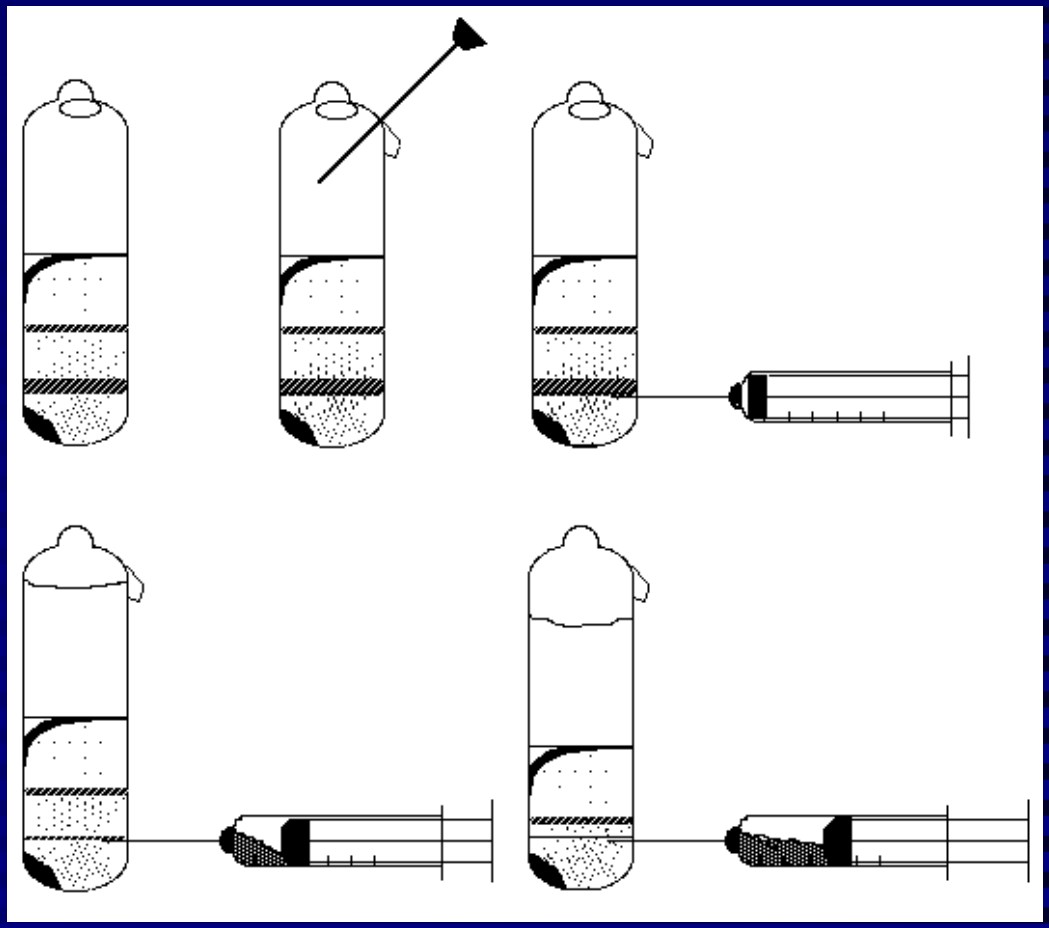
Preparative ultracentrifugation

RECOVERY OF SEPARATE ZONES



Preparative ultracentrifugation

RECOVERY OF SEPARATE ZONES



TO DECREASE THE DURATION OF PREPARATIVE ULTRACENTRIFUGATION

- Preparation of the density gradient in one of the two aforementioned ways
- Use of a fixed angle rotor (up to zero angle – near vertical rotor)
- Reduce the height of the centrifuge tube
- The factor that determines the duration of centrifugation (clearance factor, k and k”) is widely used for the direct comparison of the separation capacity of different centrifuge rotors and therefore finding the appropriate rotor, but also the time required to perform the specific separation

TO DECREASE THE DURATION OF PREPARATIVE ULTRACENTRIFUGATION

- The k factor gives an estimate of the time (in hours) needed for a particle of known sedimentation coefficient, s (in Svedberg units), to sediment (as a precipitate), at the higher velocity of the rotor

$$t = \frac{k}{s}$$

it is calculated by the equation

$$k = \frac{\ln(r_{max}/r_{min})}{\omega^2} \times \frac{10^{13}}{3600}$$

ω = 0.10472 X RPM
rmax = maximum radial distance (in cm) from the centrifuge axis
rmin = minimum radial distance (in cm) from the centrifuge axis

- The smaller the k factor, the shorter the centrifugation time

TO DECREASE THE DURATION OF PREPARATIVE ULTRACENTRIFUGATION

- The k” factor is used to determine the time required for a particle to reach the bottom of a centrifuge tube containing a gradient (5-20%) concentration of sucrose, below the maximum rotor speed

$$k' = \frac{(I_{Z_2} - I_{Z_1})}{\omega^2} \times \frac{10^{13}}{3600}$$

Z2 = higher concentration of sucrose

Z1 = lower concentration of sucrose

I = values obtained from charts after the calculation of Z0 by the equation

$$Z_0 = \frac{Z_1 r_{\max} - Z_2 r_{\min}}{r_{\max} - r_{\min}}$$

- The factors k and k” are used to find the centrifugation time in a rotor different from the one with which the separation is usually performed, through the relationship

$$t_1 = \frac{k_1 t_2}{k_2}$$

ZONAL ROTORS - CONTINUOUS FLOW ROTORS

- These are centrifuge rotors that do not hold tubes, but the entire rotor is the “centrifuge tube”
- There are two differences:
  - Their shape
  - The former are used only for rate-sedimentation centrifugation, while the latter can also be used for differential centrifugation

ZONAL ROTORS - CONTINUOUS FLOW ROTORS

QUESTIONS – A

1. What types of column chromatography do you know? What characteristic elements of the analyzed macromolecules are obtained by each of them?
2. Briefly state the differences between the techniques: analytical centrifugation, preparative centrifugation, differential centrifugation.
3. What are the similarities and differences and what are the advantages and disadvantages between normal phase and reversed phase HPLC? How is one chosen over the other?
4. What parameters affect the rate of sedimentation of a macromolecule during analytical centrifugation?
5. What is the role of dyes in the development of affinity chromatography? What are its applications in analysis?
6. You want to perform an analysis of cell membrane proteins via HPLC. What conditions will you apply and why? Briefly state.
7. In which cases will you use analytical centrifugation to determine the molecular mass of a macromolecule? What other characteristics of the macromolecule can you also determine? Explain.
8. What criteria would be used to decide whether to use agarose or polyacrylamide electrophoresis for the analysis of biological macromolecules? What is the role of SDS and ternary buffer in polyacrylamide electrophoresis?
9. By what methodologies or a combination of methodologies can you determine the molecular mass of a protein molecule? By what methodologies or a combination of methodologies can you determine the molecular mass of DNA? Explain.
10. What information can be obtained from analytical electrophoresis in the presence of SDS of a protein molecule? What additional information can be obtained from electrophoresis of the same molecule in two dimensions? Explain.

QUESTIONS – B

1. Why is the electrophoretic separation of nucleic acids usually done on agarose and proteins on polyacrylamide? Why is agarose electrophoresis done on a horizontal device, while the corresponding polyacrylamide electrophoresis is done on a vertical one? What could happen if it were done upside down?
2. You want to proceed with the analysis of a membrane enzyme. Which method would you prefer and why?
3. What is the preferred method for the separation and analysis of proteins in a cell extract. Explain.
4. You are asked to determine the presence of a specific protein molecule in a cell extract. Which method or combination of methods would you follow? Explain.
5. With which methodologies or combination of methodologies can you identify a protein? Explain.
6. The analysis of the proteins of a cell extract by electrophoresis showed the existence of a broad band of molecular mass of 50 kDa. What methodologies or combination of methodologies can you apply to determine whether the broad band corresponds to one or more proteins? Please describe in detail.
7. During the electrophoretic analysis of cellular proteins, you found that there is an accumulation of small molecular proteins at the front of the dye. What will you do to analyze these specific components? How will you think and which methodologies will you use?
8. You are given a commercial preparation of protein derived from blood. You suspect that it contains blood albumin as an impurity. What analytical methodologies can you apply to clarify your suspicion. Explain in detail.
9. Why is the analysis of serum proteins not performed by SDS-electrophoresis?
10. Give the definitions: a. retention time, b. separation coefficient, c. separability. What do these terms express and how are they used in chromatographic separations?

Written assignments - A

1. You suspect that a beef burger contains horse meat. What analytical methodologies can you use in combination or not to clarify your suspicion? Briefly refer to all the methodologies you have learned and you will develop in detail the methodologies related to the subject CHROMATOGRAPHY-ELECTROPHORESIS.
2. During the electrophoretic analysis of cellular proteins, you found that there is an accumulation of micromolecular proteins at the front of the dye. What will you do to analyze the specific components? Please refer in detail to the methodologies or combination of methodologies that you will follow.
3. What combination or combinations of mobile and stationary phases will you use in HPLC chromatography, in order to analyze steroids in the blood or urine of athletes and why? What would be the complete procedure to follow during the analysis, from the moment the biological fluid is obtained?
4. In a disease, extensive phosphorylation of the hydroxyamino acids of a protein molecule of blood cells has been observed, before the symptoms of the disease become visible. What analytical methodology or combination of methodologies would you apply in order to verify the normal or extensive phosphorylation of the protein, so that the disease can be diagnosed in a timely manner?
5. You are given a commercial preparation of protein derived from blood. You suspect that it contains blood albumin as an impurity. What analytical methodologies can you apply to clarify your suspicion. Explain in detail.
6. With what methodologies or combination of methodologies can you determine the molecular mass of a protein molecule? With what methodologies or combination of methodologies can you determine the number and type of subunits of a protein molecule? Explain in detail.
7. You want to perform an analysis of cell membrane proteins via HPLC. What conditions (mobile phase, stationary phase, etc.) will you apply and why? Explain in detail.

Written assignments - B

1. You are asked to determine the concentrations of pesticides in environmental samples (e.g. drinking water, soil, etc.). Which analytical methodology(s) would you recommend to use and why? What will be the process you will follow for the analysis from the moment you receive each different sample?
2. You are asked to determine the existence of a specific protein molecule in a cell extract. Which method or combination of methods will you follow? Briefly explain all the methods you can use and refer in detail to the methodologies related to the subject CHROMATOGRAPHY-ELECTROPHORESIS.
3. The analysis of the proteins of a cell extract by electrophoresis showed the existence of a broad band of molecular mass of 50 kDa. Which methodologies or combination of methodologies can you apply to determine whether the broad band corresponds to one or more proteins? Please refer in detail.
4. How is the phenomenon of the presence of many protein bands interpreted during the analysis by isoelectric focusing of a “pure” protein? Explain. Which methodology(ies) will you apply to confirm the explanation you will give?
5. What criteria will be used to decide whether to use agarose or polyacrylamide electrophoresis for the analysis of biological macromolecules? You will refer to all biological macromolecules. What is the role of SDS and ternary buffer in polyacrylamide electrophoresis? Explain in detail. What are the similarities and differences and what are the advantages and disadvantages between normal phase and reversed phase HPLC?
6. What types of column chromatography do you know? What characteristic elements (physicochemical, biochemical, biological, etc.) of the analyzed molecules or macromolecules are obtained from each of them?
7. What are the conditions for the application of gas chromatography in the analysis of molecules of biological interest? List at least five different examples (in terms of the type of components analyzed) of the application of gas chromatography methods for the analysis of molecules of biological interest.

Written assignments - C

1. What information can be obtained from analytical electrophoresis in the absence or presence of SDS of a protein molecule? What information can be obtained from analytical electrophoresis in the absence or presence of β-mercaptoethanol of a protein molecule? What additional information can be obtained from electrophoresis of the same molecule in two dimensions, in combination or not with other analytical methodologies? Explain in detail.
2. You are interested in isolating specific antibodies against a specific virus from the serum of an immunized animal. Which methodology or combination of methodologies will you choose? With which analytical methodology will you check the purity of the preparation? Explain in detail.
3. In a disease, extensive glycosylation of a protein molecule of blood cells has been observed, before the symptoms of the disease become visible. Which analytical methodology or combination of methodologies would you apply in order to verify the normal or extensive glycosylation of the protein, so that the disease can be diagnosed in a timely manner?
4. You are asked to determine ethanol in human blood. Which analytical methodology(ies) would you recommend to be used and why? Describe the entire analysis process from the moment of blood collection.
5. During the isolation of a heterologous protein from bacteria, you suspect that, due to the difference in the system of accompanying proteins, an intermolecular disulfide bond is created. Which analytical methodology would you apply and how to clarify your suspicion. [It is noted that you have the natural protein at your disposal as a model for the methodology you will propose].
6. Which analytical methodology(ies) would you recommend to be used for the quality control of essential oils and why? Explain in detail.
7. What is the procedure followed for the analysis of serum proteins? Why is this not performed by SDS-electrophoresis? Explain in detail with images and examples.
8. When analyzing a pure protein molecule by SDS-electrophoresis, its molecular mass was calculated to be 50 kDa. When electrophoresis was performed in the presence of β-mercaptoethanol, the mobility of the protein band corresponded to a molecular mass of 25 kDa. When analyzing another protein molecule by SDS-electrophoresis, its molecular mass was again calculated to be 50 kDa. However, in the second case, when electrophoresis was performed in the presence of β-mercaptoethanol, the mobility of the protein band corresponded to a molecular mass of 55 kDa. How are these results interpreted? What methodologies and how, to confirm the interpretation you propose?