Biochemistry and Molecular Biology

Seminar 2

Biochemistry-based techniques

Degree in Medicine (academic year 2022-2023)

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Based on a presentation by Profesor Juan Saus and Dr. Jesús Salgado and Dr. Herminia González Navarro.

What is biochemistry?

What is biochemistry?

“A science that studies life using the language of chemistry".

"Biochemistry is the chemistry of life".

“A science that deals with the chemical basis of life".

“A science that deals with the study of living things at the molecular and sub-cellular level".

“A science that studies the different molecules and chemical reactions that occur in cells and living organisms".

What is biochemistry?

- **Biochemistry is the science that studies the chemical composition of living beings, paying special attention to the molecules that make up cells and tissues**.
  - nucleic acids
  - proteins
  - lipids
  - carbohydrates
  - Other small molecules that make up cells

- It also studies the **chemical reactions** these compounds undergo, which allow them to obtain energy and generate their own biomolecules. By doing so, we can understand the cellular metabolism and processes such as digestion, photosynthesis and immunity.

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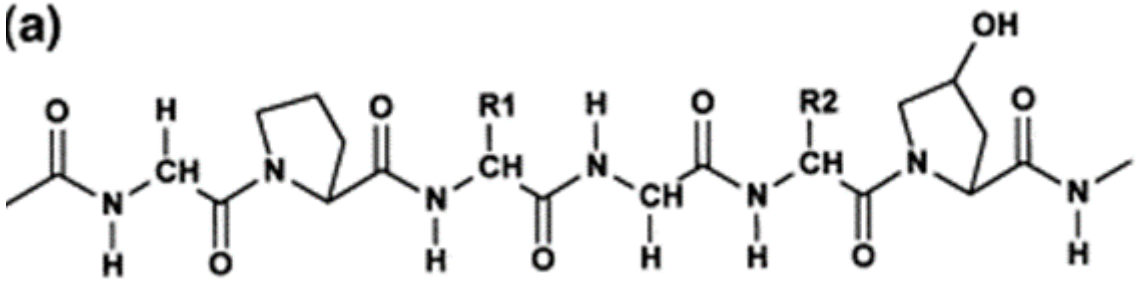

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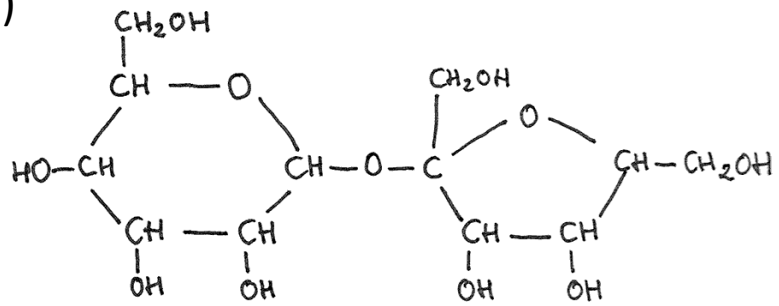

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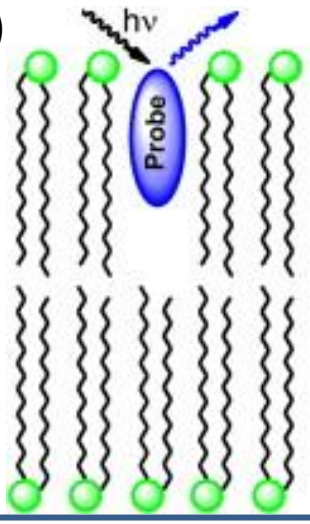

Socrative: 3. What is it?

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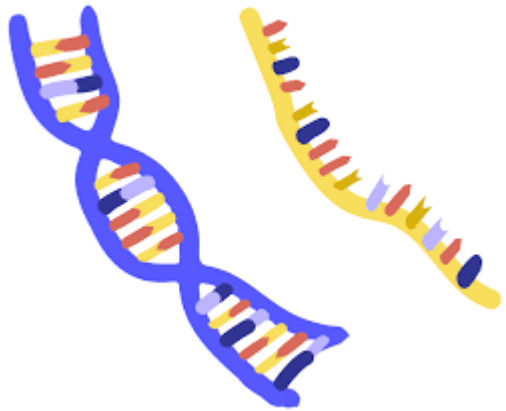

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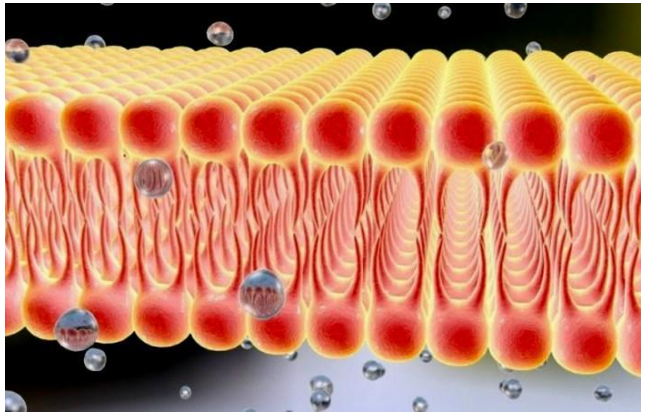

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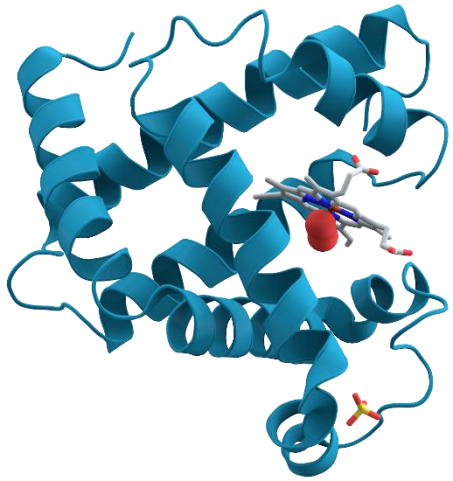

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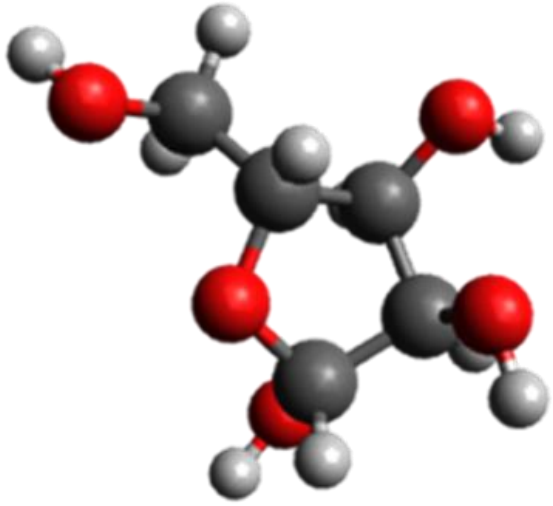


What is biochemistry?

Biochemistry studies not only biomolecules but also the relationships between their components, their transformation inside living organisms, and the regulation of these processes.

The aim of biochemistry is to study the chemical composition of living beings, the relationships between these materials, their transformation, the regulation and functioning of these processes, and, therefore, the repercussions they have on the physiology of organisms.

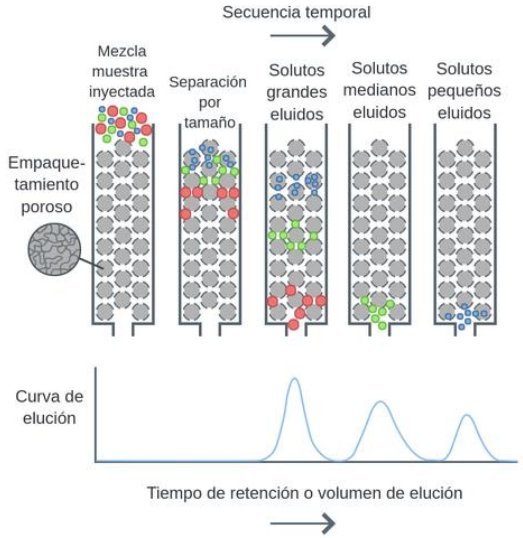
What laboratory techniques can you think of?

Content of this seminar

1. What is biochemistry?

2. Biochemical techniques
   2.1. Spectrophotometry
   Chromatography
   Electrophoresis
   - of DNA
   - of proteins

Techniques we are going to study:



Chromatography

Spectrophotometry

Electrophoresis

1. Spectrophotometry

What is the difference between spectroscopy and spectrophotometry?

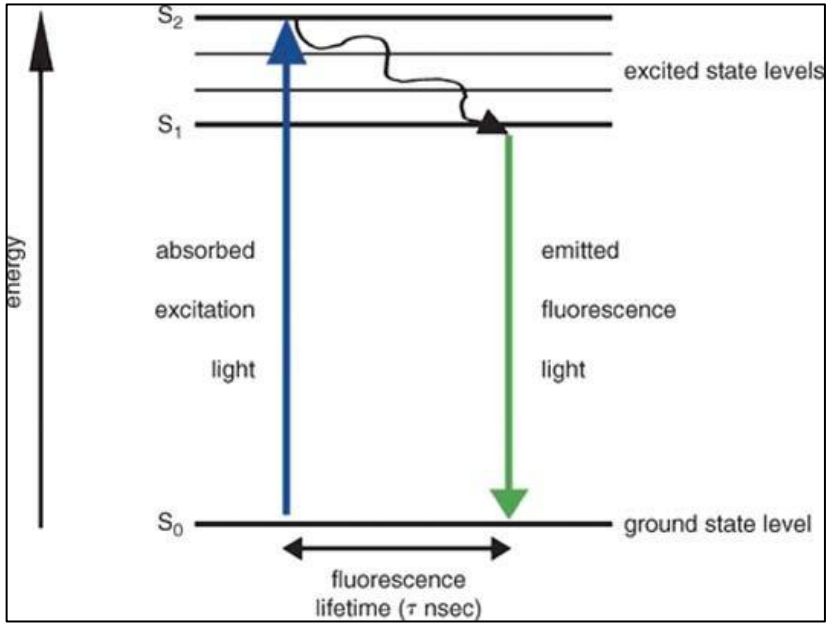
1. Spectrophotometry

Subject characteristics

Molecules can have different energy states. The lowest energy state is the fundamental state. They can reach an excited state by absorption of energy.

We can take advantage of this feature to obtain information from molecules using **techniques that tell us about changes in the excited state of the molecules**.

These changes can be effected by incident light.



1. Spectrophotometry

Spectroscopy vs. spectrophotometry

Spectroscopy and spectrophotometry exploit these qualities of interaction between energy sources in the form of light and matter.

<u>Absorption spectroscopy</u>: a variety of techniques using the interaction of electromagnetic radiation with matter.

1. Spectrophotometry

Spectroscopy vs. spectrophotometry

Spectroscopy is the study of the absorption and emission of photons by matter when electromagnetic radiation passes through it.

Light is visible electromagnetic radiation (between 380-770 nm) and is made up of packets of energy.

The energy (E) associated with electromagnetic radiation is directly related to the frequency (ν), and inversely related to the wavelength (λ) by Planck's constant (h) (c=speed of light):

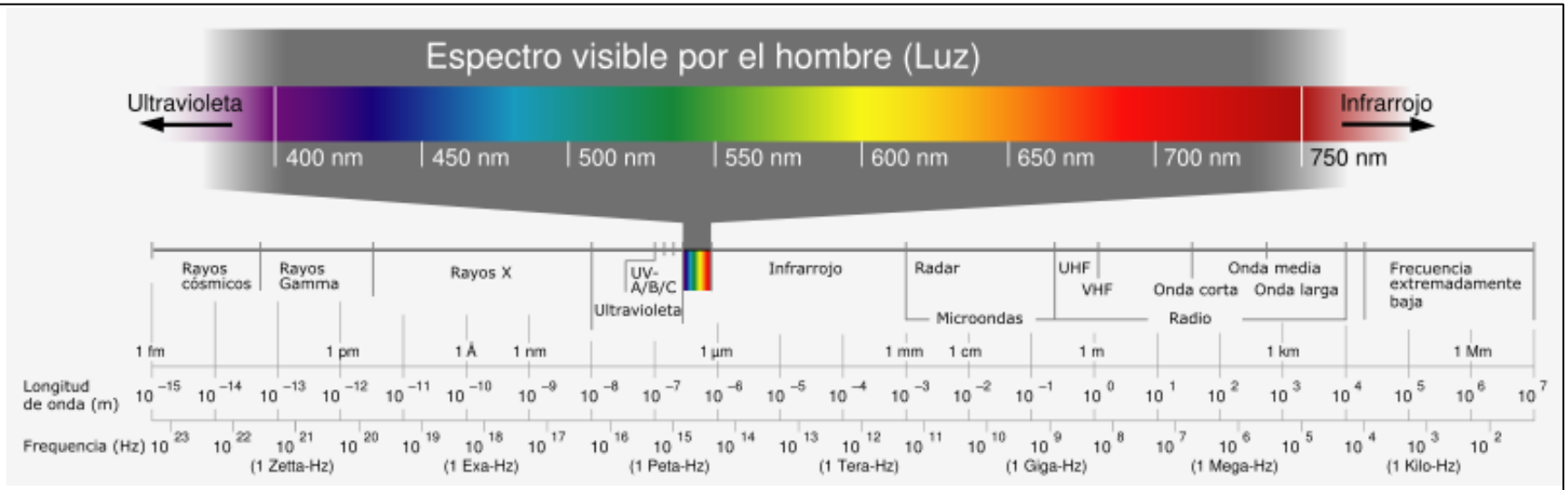
$$\text{(Energy) } E = h\nu = h(c/\lambda)$$

h= Planck's constant
ν= wave frequency
c= speed of light
λ= wavelength

$\lambda = c/\nu$ with $c = 3.10^8$ m/s, which is the speed of light in a vacuum.

1. Spectrophotometry

Spectroscopy vs. spectrophotometry



Spectrum type — Electronic (UV-VIS)

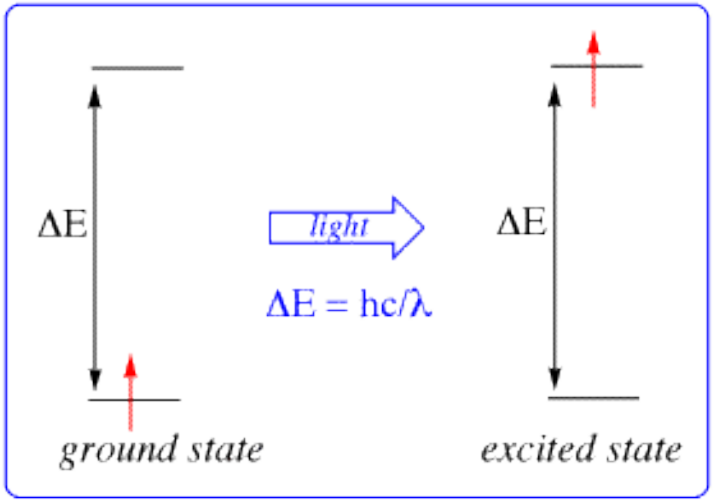
Molecular element involved — Valence electron orbitals

Light is produced when an atom receives energy and excites its electrons which move into higher energy orbitals. Electrons are unstable in higher energy shells if there are unoccupied lower energy levels and when they decay they emit photons whose frequencies fall in the range of frequencies associated with (visible) light.

1. Spectrophotometry

Spectroscopy vs. spectrophotometry

The energy of a photon or quantum of light corresponds to the energy difference between two quantum states.



The ground state (E_0) of a substance is defined by the electronic structure it possesses in the **naturally preferred lowest energy level**. Its electrons will remain in the **ground state as** long as the atom does not receive energy, in which case they will go to an excited state (E_1 or E_2)

$$\Delta E = h\nu = h\,(c / \lambda)$$

Depende de la naturaleza de la sustancia

Energía de la radiación absorbida o emitida

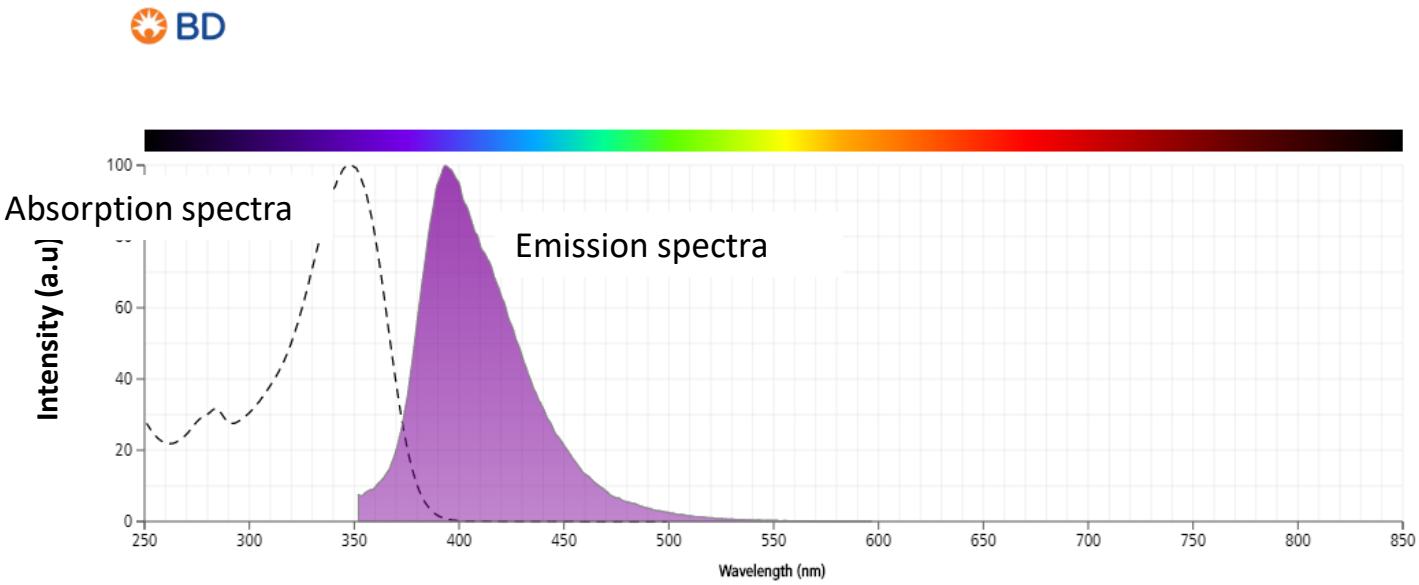
When radiation of energy ***E*** is incident on a sample, some of its photons can be absorbed, provided that the energy difference between the ground state of the sample molecules and one of the excited states is equal to the energy of the radiation. The radiation exiting the sample will have less intensity (fewer photons).

1. Spectrophotometry

Spectroscopy vs. spectrophotometry

Absorption and Emission Spectra

The absorption spectrum is the fraction of electromagnetic radiation that a substance absorbs in a range of frequencies and is, in a sense, the opposite **of the emission spectrum** (image below), which is the fraction of electromagnetic radiation that it emits when it is excited and returns to its ground state.



Absorption and emission spectra of a sample

1. Spectrophotometry

Spectroscopy vs. spectrophotometry

Spectroscopic analytical technique that measures the **amount of light absorbed by a solution. The amount of light is used** to determine the **concentration of the substance dissolved in it.**

The molecules that absorb this light are known as **chromophores**. A chromophore is a **molecule that has electrons capable of absorbing photons** from the visible spectrum of electromagnetic radiation.

What *chromophores* do proteins have?

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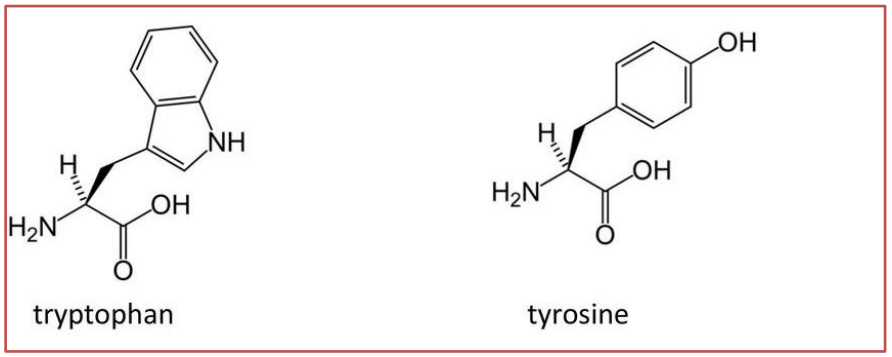
Peptide bonds: λ_{max} 200 nm
Aromatic amino acids: tryptophan and tyrosine: λ_{max} 250-300 nm
Prosthetic groups (Haem / heme group).

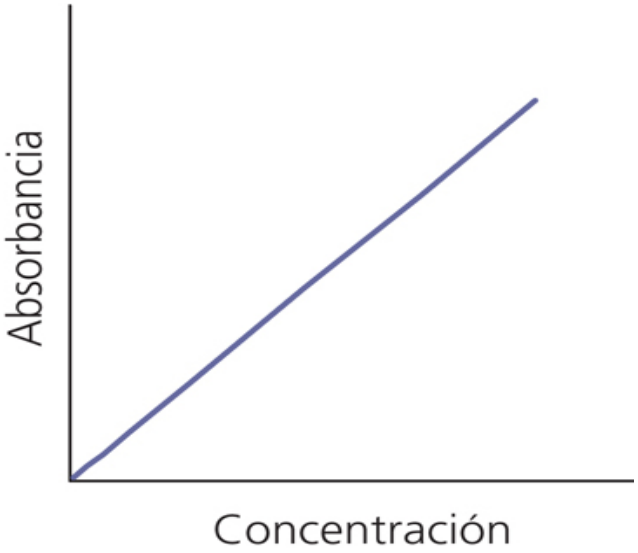


1. Spectrophotometry

Spectroscopy vs. spectrophotometry

Lambert-Beer law: the absorbance of light from a chromophore is proportional to the concentration

$$A = \varepsilon \cdot C \cdot l$$



What is a standard curve?

1. Spectrophotometry

Spectroscopy vs. spectrophotometry

Lambert-Beer law: the absorbance of light from a chromophore is proportional to the concentration

$$A = \varepsilon \cdot C \cdot b$$

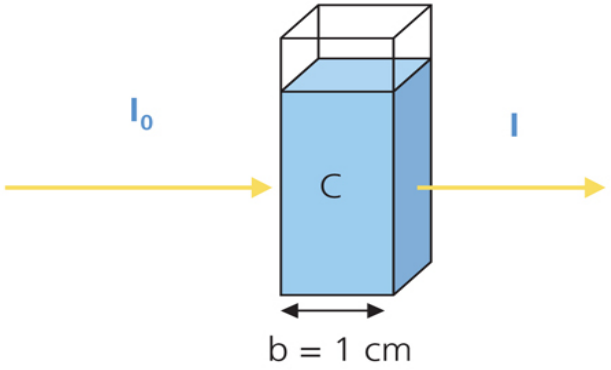
The absorbance is linearly dependent on the concentration of the chromophore:

The incident light intensity (I_o) in a solution is related to the transmitted light intensity (I) according to the equation:

ε *Molar extinction coefficient (M^{-1} cm^{-1}): is a constant for each substance, it defines how strongly a substance absorbs light at a given wavelength.*

C Concentration (M)

b Distance traversed by light (cm)



1. Spectrophotometry

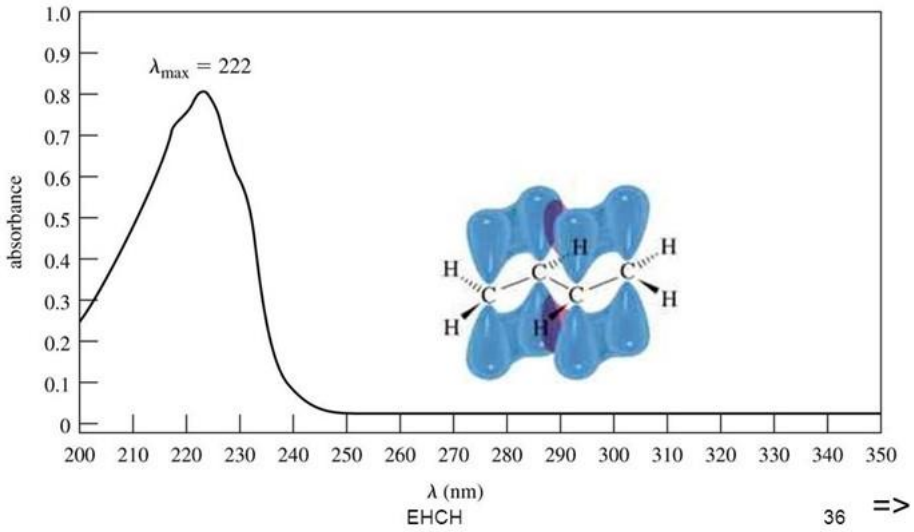
Absorption spectra

What is an absorption spectrum?

1. Recording of *A* at varying wavelengths λ.

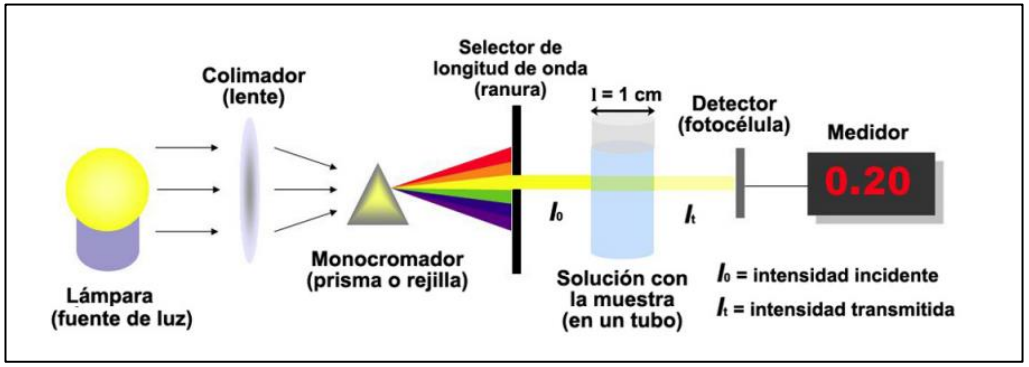
2. It gives rise to broad bands characterised by an absorption maximum (λ_{max}).

3. Chromophore-specific: each chromophore has its own characteristic spectrum.



1. Spectrophotometry

Application: Determination of concentrations



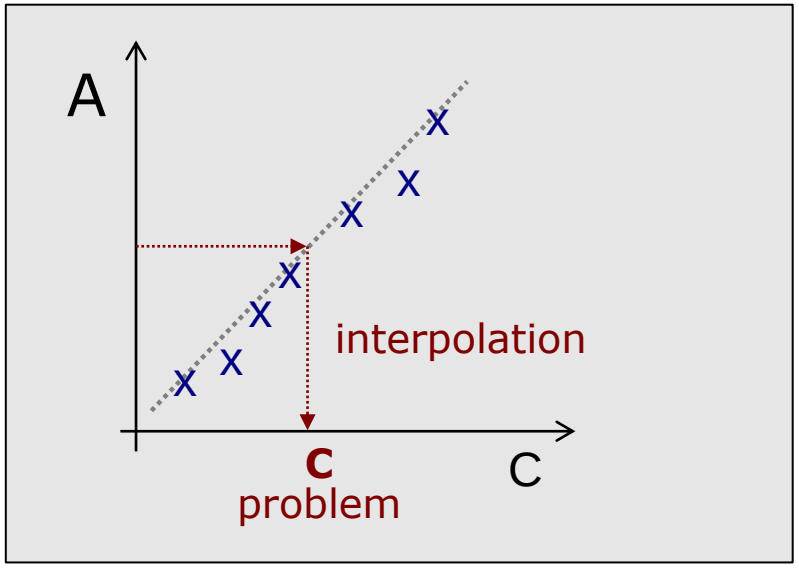
If e of the substance is known at a **characteristic λ: <u>direct application of the Lambert-Beer law</u>**

Measurement of A at the characteristic λ

$$A = \varepsilon \cdot C \cdot b$$

1. Spectrophotometry

Application: Determination of concentrations

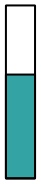


If **ε** is not known but the substance absorbs at a **characteristic λ** and a **purified substance (standard) is available.**

Preparation of a standard line at the characteristic λ

The absorbance value (A) of the test sample is projected from the ordinate axis to the standard line and from the standard line to the abscissa axis to determine the concentration (C) as shown in the diagram.

The projection point on the line must be within the parameters of the line and between experimental points (interpolation).



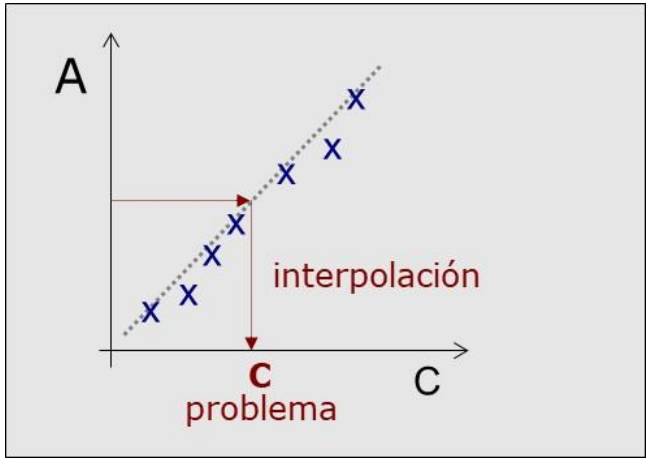
Problem

C = ?

A = 0.47

1. Spectrophotometry

Application: Determination of concentrations



Problem

C = ?

A = 0.47

If the protein is part of a complex mixture, but has a characteristic chromophore (e.g. haemoglobin, with a known λ) and a purified protein standard is available.

Preparation of a standard line for the characteristic λ of the chromophore

If the protein does not have a characteristic chromophore (e.g. albumin) but the protein is pure in the sample and a purified protein standard is available.

Preparation of a standard line at a characteristic λ for proteins (e.g. 280 nm)

If the protein does not have a characteristic chromophore and is part of a complex mixture, we cannot measure its concentration.

What do we do?

1. Spectrophotometry

Application: Determination of concentrations

How do we measure a protein if it has no chromophore and is part of a complex mixture of proteins?

The normal blood protein value is between 60-83 g/L. Construct the calibration curve (having a BSA solution of 300 mg/mL) for the comparison of normal samples and those in pathological conditions of hyperproteinemia or hypoproteinemia, so that with 200 mL of sample we can determine the amount of blood protein present. Titration would be carried out in a final volume of 1 mL via the Bradford method by adding 0.2 mL of Bradford reagent.

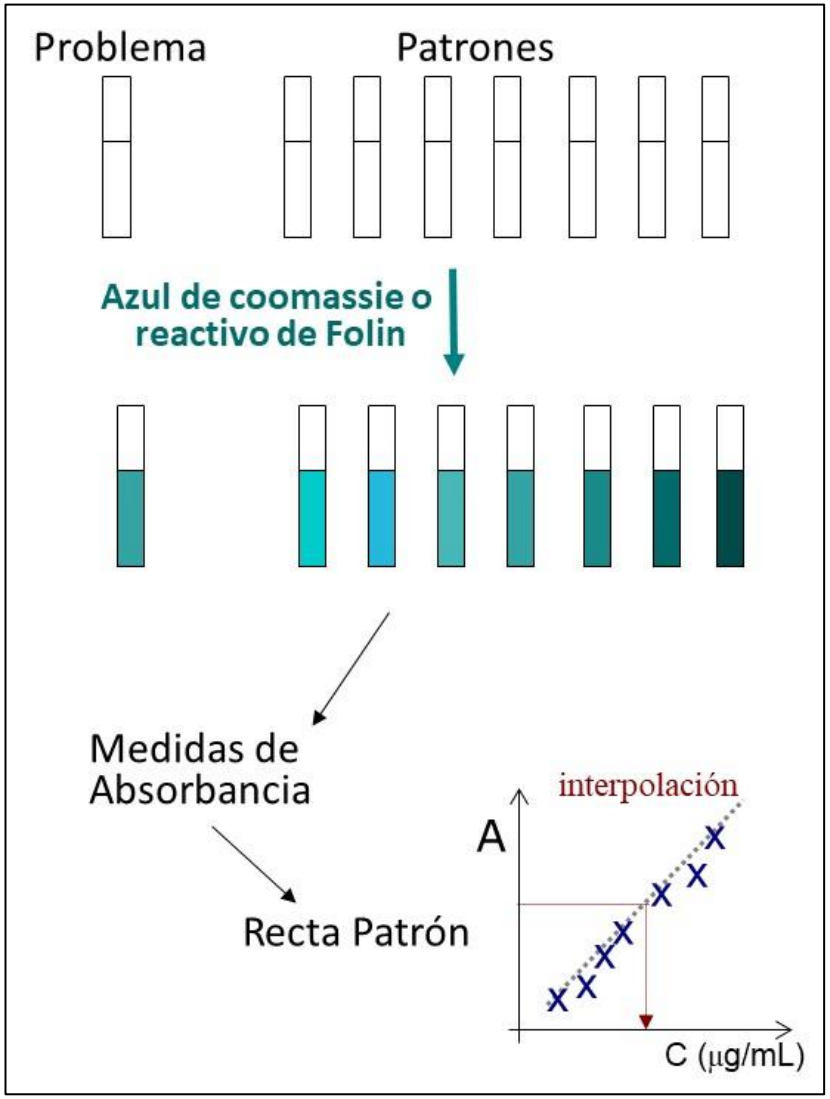
Tube	1				5			8		
	STANDARD CURVE									
BSA (mL)									--	--
[BSA] (mg/mL)									--	--
Sample (mL)									0.2	0.2
Water (mL)									0.6	0.6
Bradford (mL)									0.2	0.2

1. Spectrophotometry

Application: Determination of concentrations

The concentration of any protein mixture can be measured with external chromophores that regularly bind to the peptide backbone (e.g. Coomassie brilliant blue in the Bradford method) or by the addition of a reagent that generates chromophores proportionally to the protein concentration (e.g. Folin-Ciocalteu reagent, or simply Folin, in the Lowry method) and a standard of any protein which is available.

Preparation of a line with the standard at the characteristic λ of the chromophore added (e.g. 595 nm in the Bradford method) or generated (e.g. 660 nm in the Lowry method) following the procedure shown in the diagram.



2. Chromatography

What is chromatography?

Definition:

Technique for the separation of the components of a mixture. The mixture passes through a **stationary phase** transported by a **mobile phase.**

The stationary phase: components with certain characteristics.

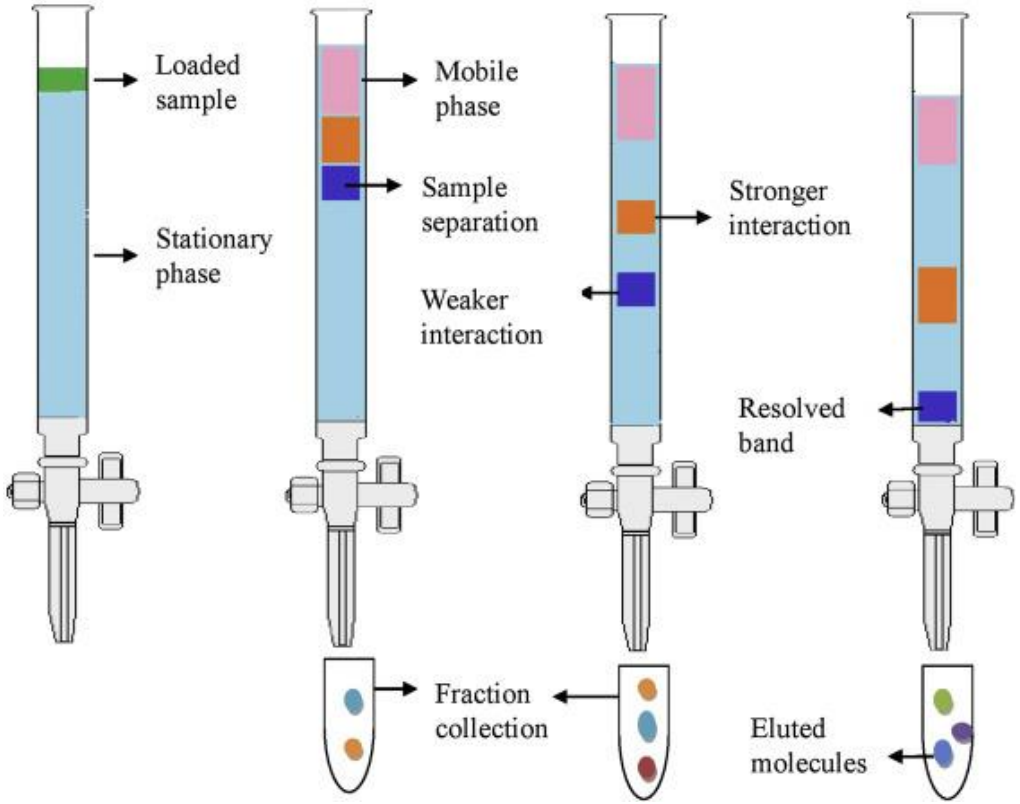
The individual components of the mixture **advance unevenly due to differing interaction** with the stationary phase.

- **Mobile phase** ≅ eluent
- **Stationary phase** ≅ chromatographic bed
- **Elution**: The elution of the eluent and the molecules dissolved in it.

2. Chromatography

2.1. *Column liquid chromatography*

The mobile phase is liquid and the stationary phase is contained in a column.



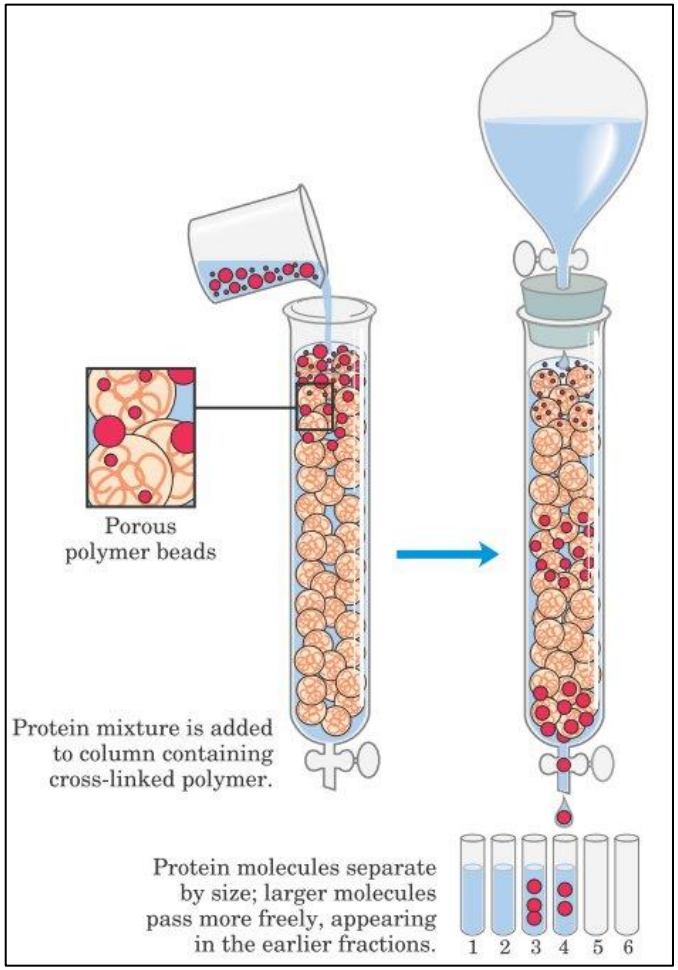
Joe M. Regenstein, Carrie E. Regenstein, in Food Protein Chemistry, 1984

2. Chromatography

2.1. Column liquid chromatography

a. Gel filtration or molecular exclusion

Separation of the components of a mixture by size, so that small molecules lag behind large molecules.



From Lehninger, A. L., Nelson, D. L., and Cox, M. M. Lehninger Principles of Biochemistry. W. H. Freeman, (2005).

2. Chromatography

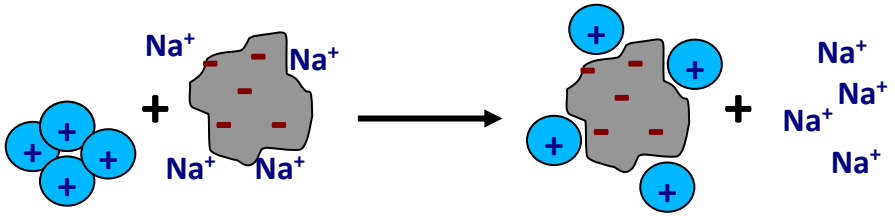
2.1. Column liquid chromatography

b. Ion exchange chromatography

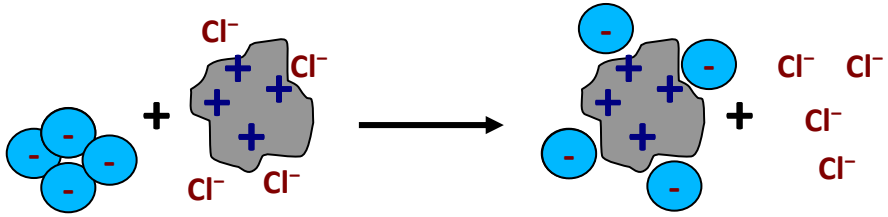
The components of a mixture are separated due to their differential interaction with a charged stationary phase. Depending on the charge of the stationary phase:

- **anion** exchange (positively charged)
- **cation** exchange (negative charge)

Cation exchange



Anion exchange



2. Chromatography

2.1. Column liquid chromatography

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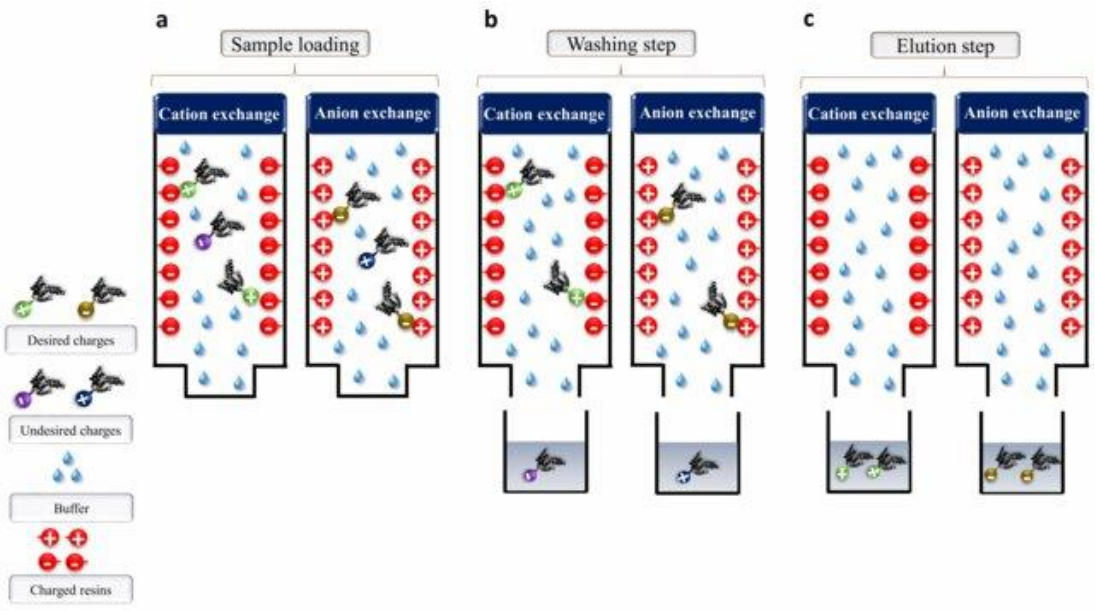
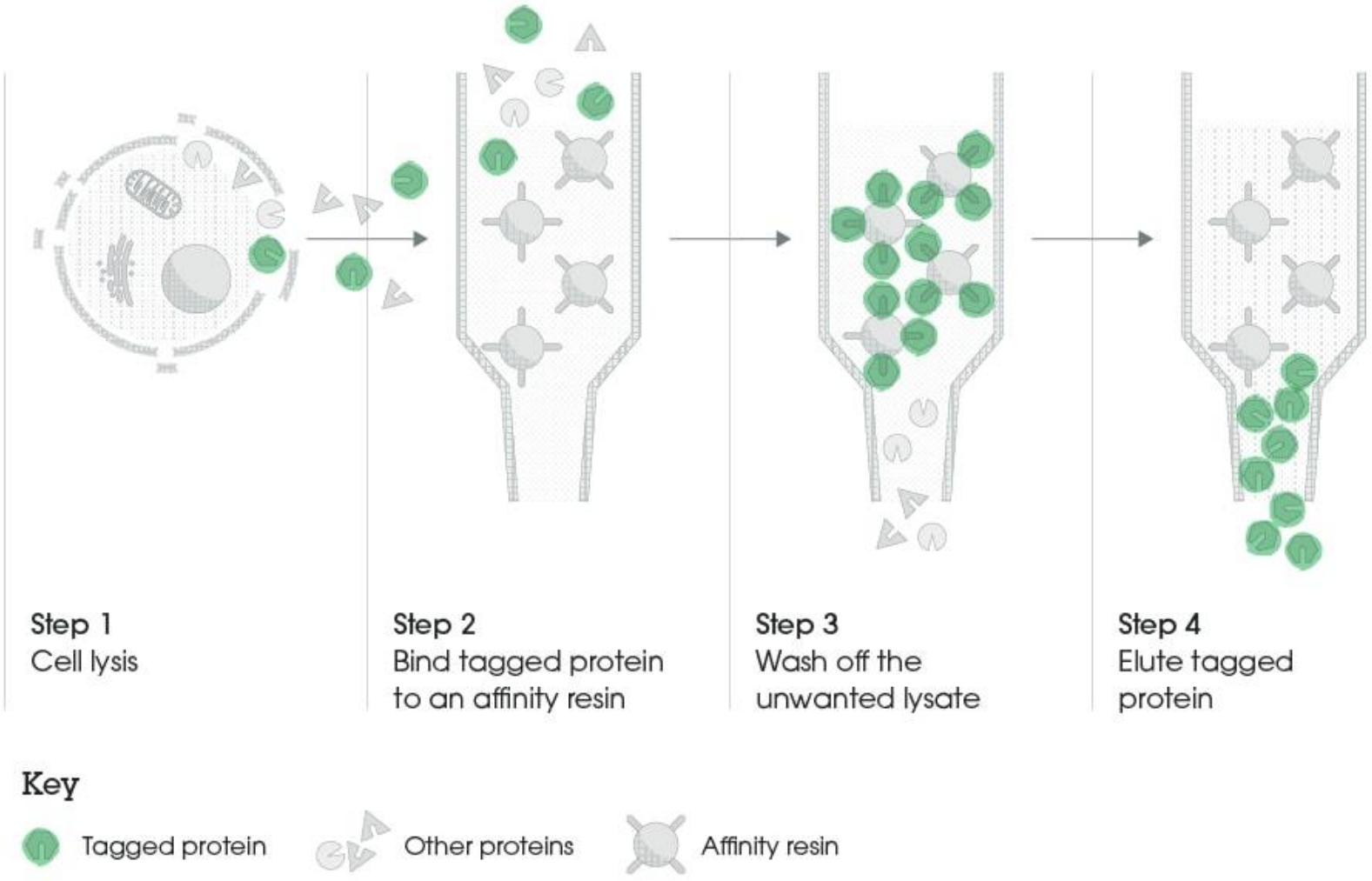


Opportunities and challenges of the tag-assisted protein purification techniques: Applications in the pharmaceutical industry, November 2020 Biotechnology Advances 45:107653

2. Chromatography

2.1. Column liquid chromatography

c. Affinity chromatography



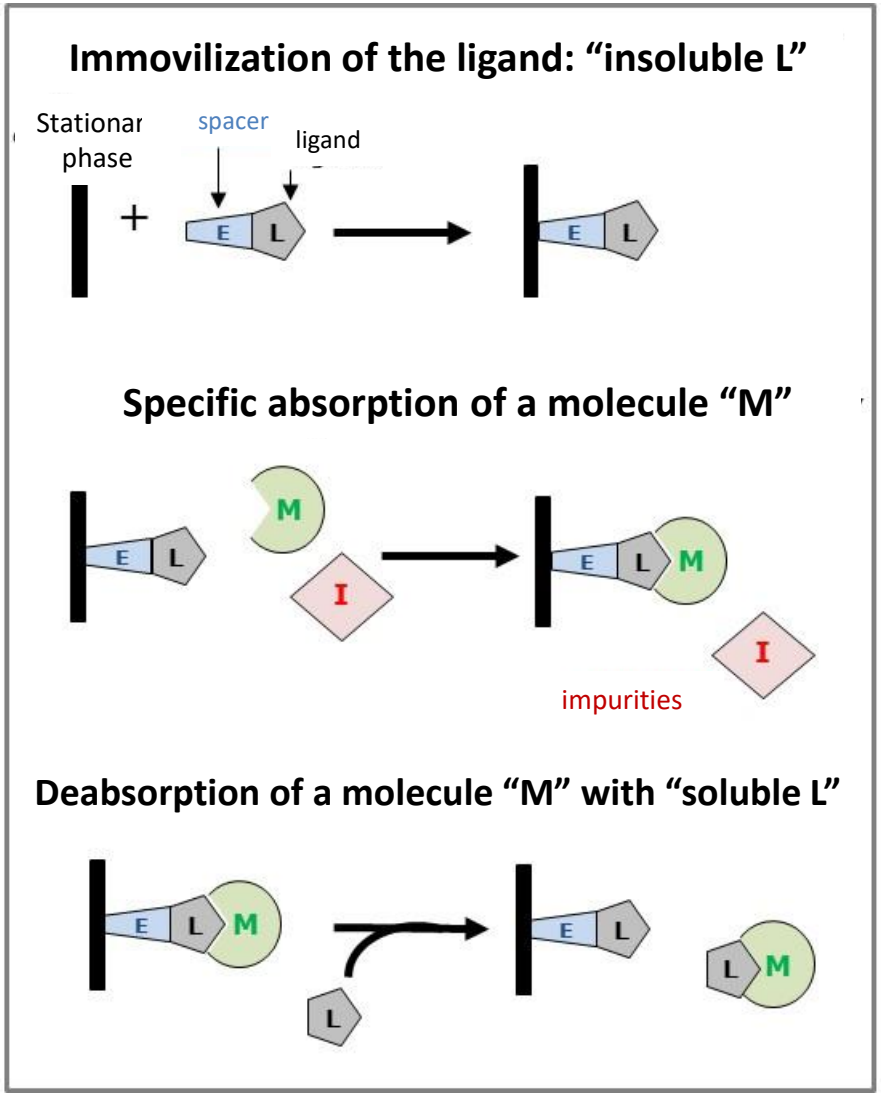
www.abcam.com/proteins/affinity-tags

2. Chromatography

2.1. Column liquid chromatography

c. Affinity chromatography

- Separation of the components of a mixture by a **specific and reversible interaction** with ligands immobilised in the stationary phase.
  - Enzyme purification:
    - Insoluble or soluble L: substrate analogue
  - Protein purification: ***Immunoaffinity***
    - Insoluble L: antibody
    - Soluble L: synthetic peptide



2. Chromatography

2.1. HPLC chromatography: Analytical technique

HPLC: High Performance Liquid Chromatography

Also known as high-pressure liquid chromatography, it is VERY accurate. Characteristics:

1. Small spherical particles that are very resistant to pressure and have very large pores that allow for a large number of chromatographic interactions.
2. It is fast, reproducible and automatic.
3. It is usually **reverse phase**, so that **hydrophobic interactions occur.** The eluent is more polar than the stationary phase.

It is widely used in clinical biochemistry laboratories. Applications:

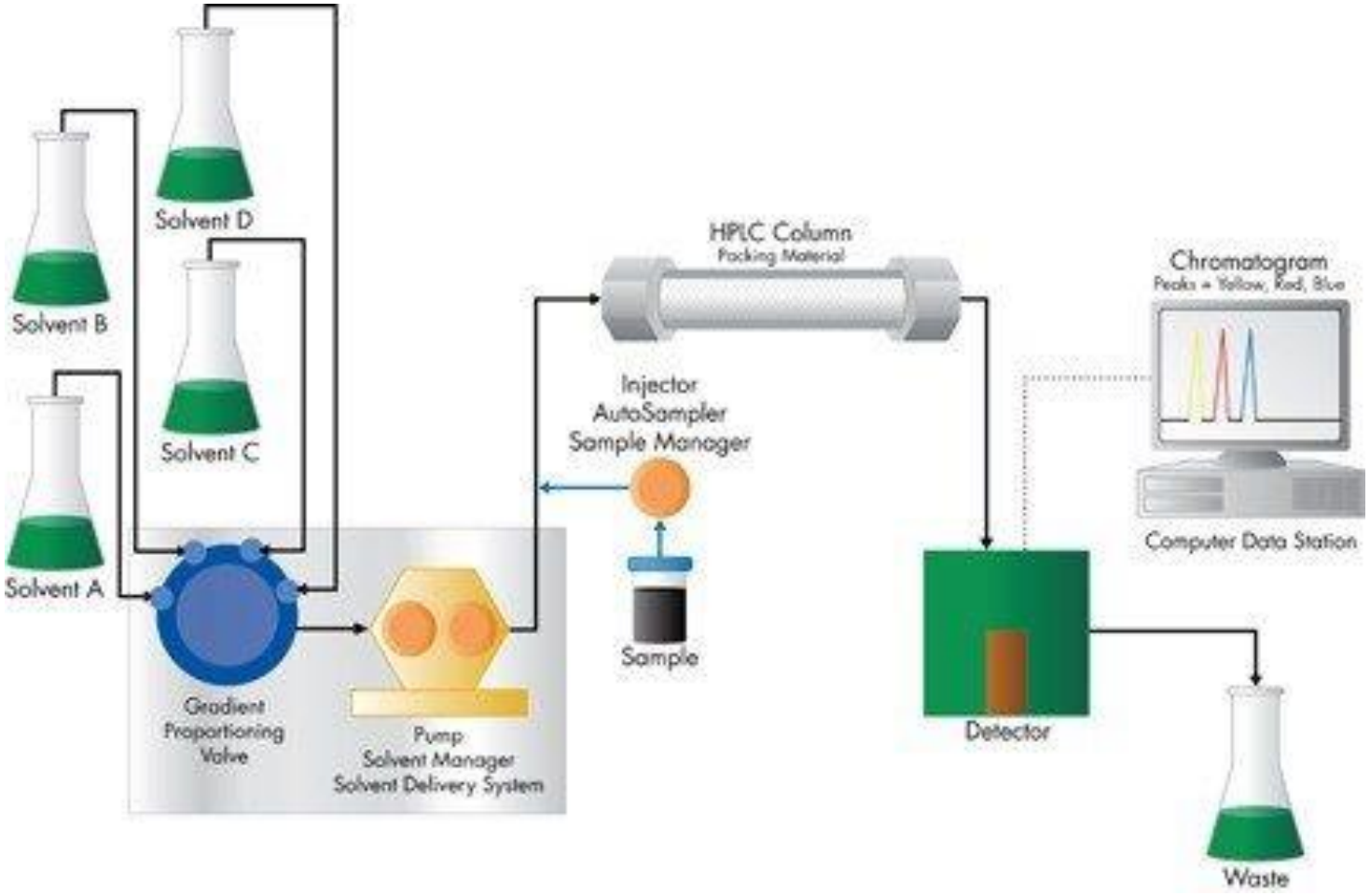
- **Forensic chemistry**: for the separation of drugs, poisons, detection of alcohol in blood, detection of narcotics, etc.
- **Clinical medicine**: separation of bile acids, metabolites, drugs, etc.

2. Chromatography

2.1. HPLC chromatography: analytical technique

2. Chromatography

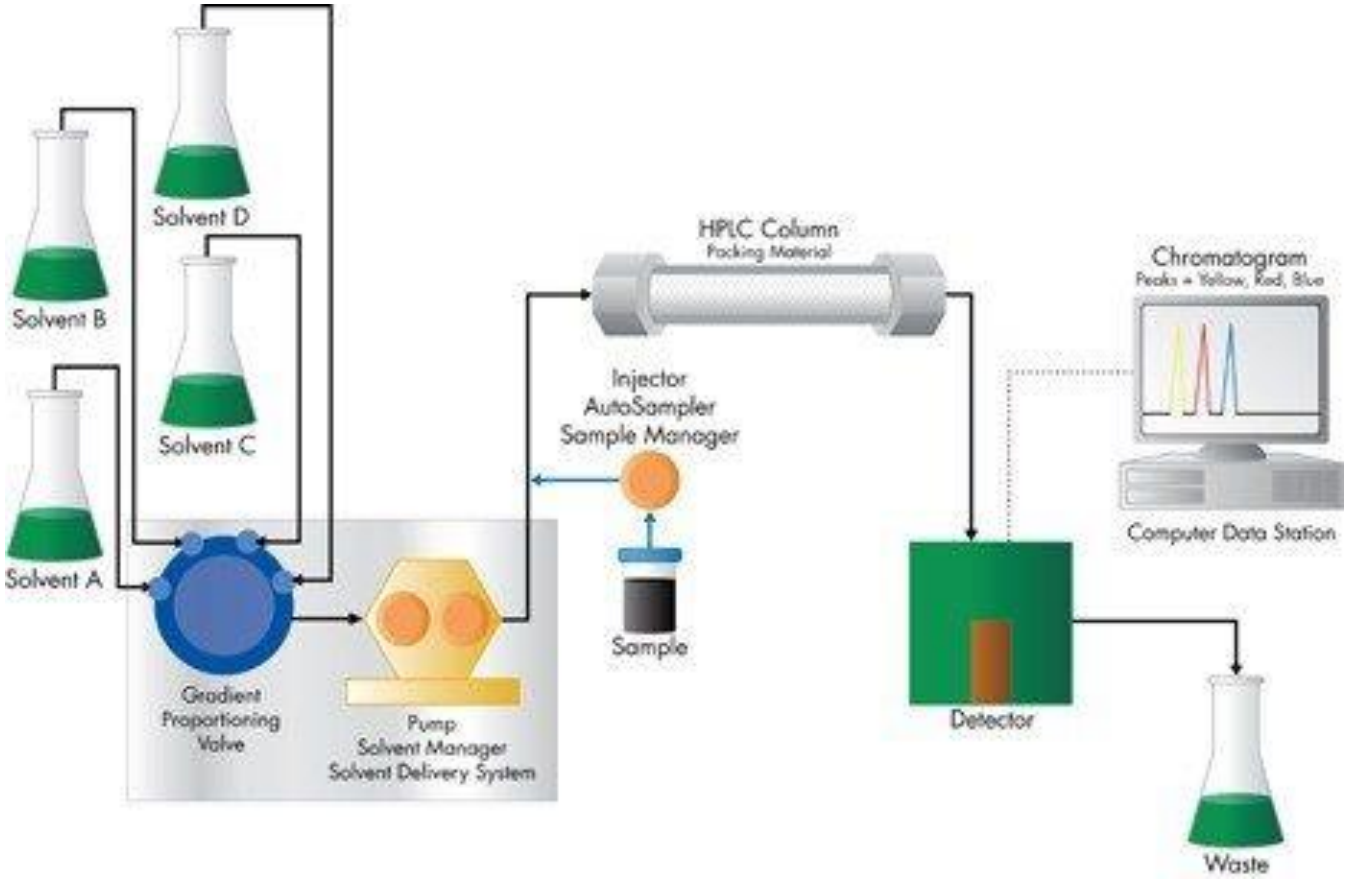
2.1. HPLC chromatography: analytical technique



https://www.waters.com/nextgen/es/es/education/primers/beginner-s-guide-to-liquid-chromatography/identifying-and-quantitating-compounds.html

2. Chromatography

2.1. HPLC chromatography: analytical technique



The **stationary phase** is usually a non-polar phase, using silica gels.

The typical **mobile phase** is a mixture of an aqueous solution and a hydrophobic solvent, such as acetonitrile or methanol.

3. Electrophoresis

Definition: Separation of the components of a mixture by using an electric current to move it through a matrix.

The electrophoretic mobility of a molecule through an **unrestricted** matrix depends only on its **charge density.**

The electrophoretic mobility of a molecule when passing through a matrix **that restricts** its progress depends on its **charge density, its size and its shape.**

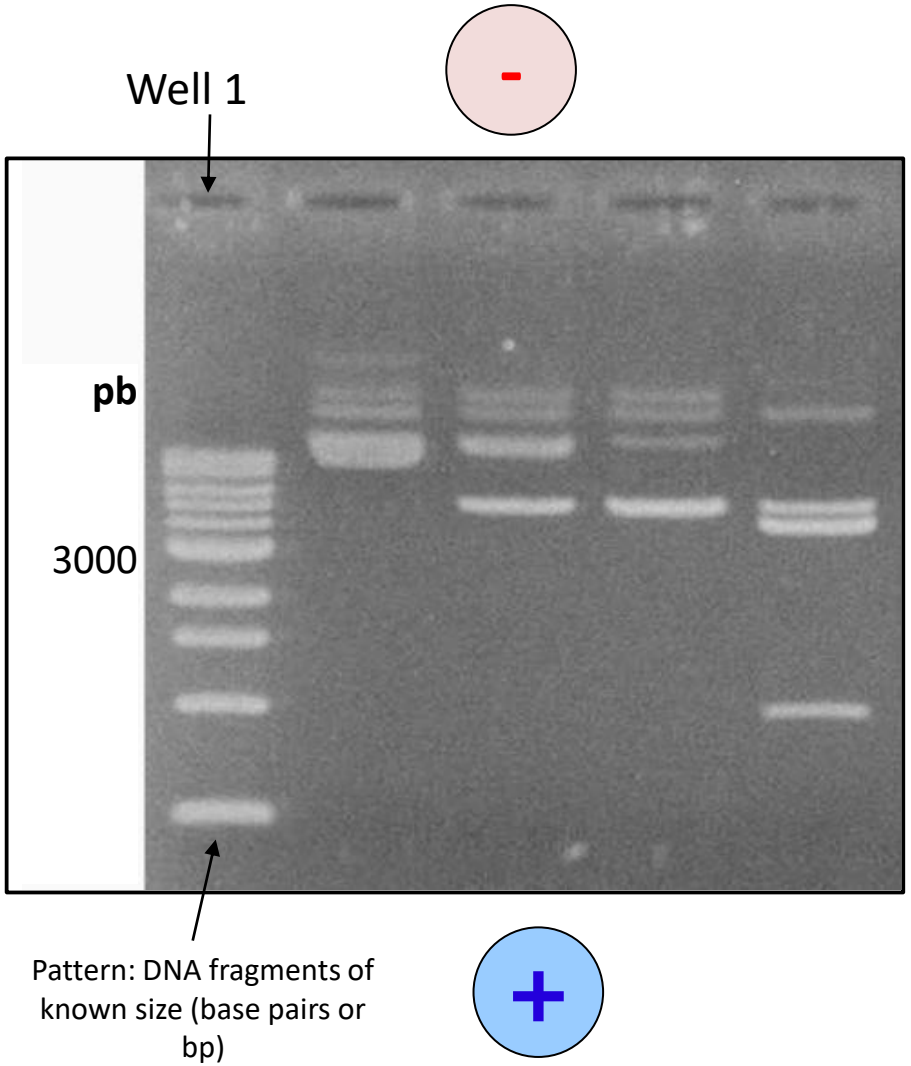
Medios no restrictivos	Medios restrictivos	
Papel Acetato de celulosa	Gel de almidón Gel de agarosa	Gel de poliacrilamida Gel de agarosa + poliacrilamida
separación principalmente por carga	Separación por carga, tamaño y forma	

3. Electrophoresis

3.1. Agarose gel electrophoresis of DNA

- **Agarose gels** restrict the migration of **DNA fragments**

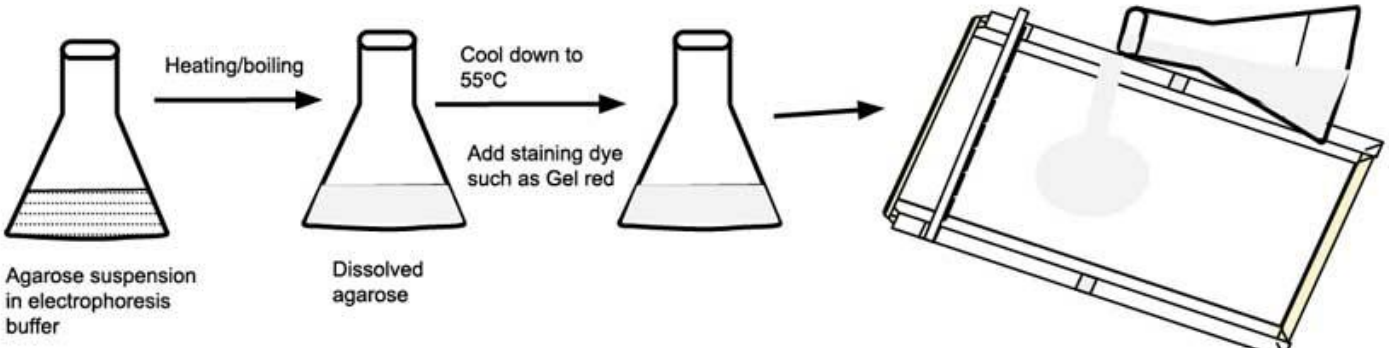
The DNA fragments have the same negative charge density and share the same shape, so they are **separated by their size** (number of base pairs) as they migrate towards the anode.

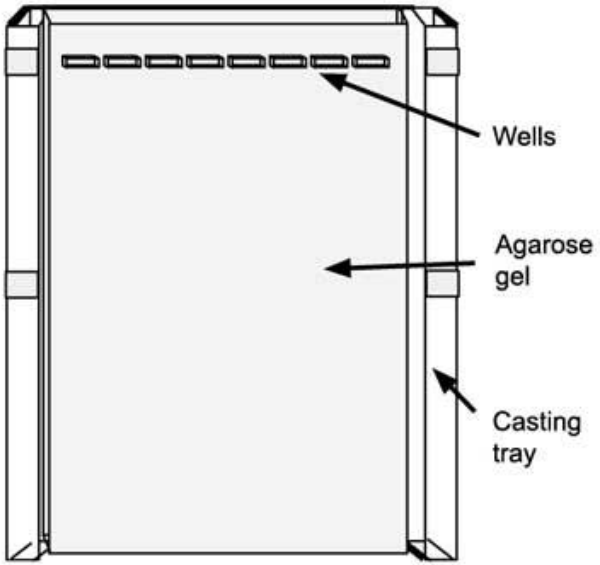


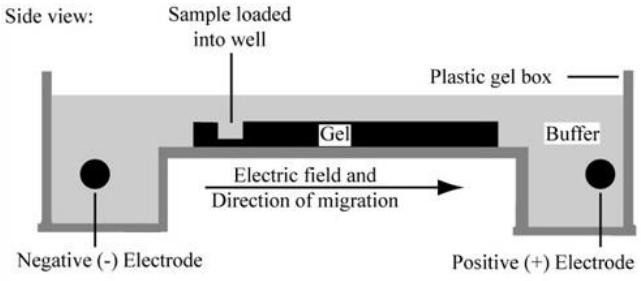
3. Electrophoresis

3.1. Agarose gel electrophoresis of DNA

Agarose gel preparation



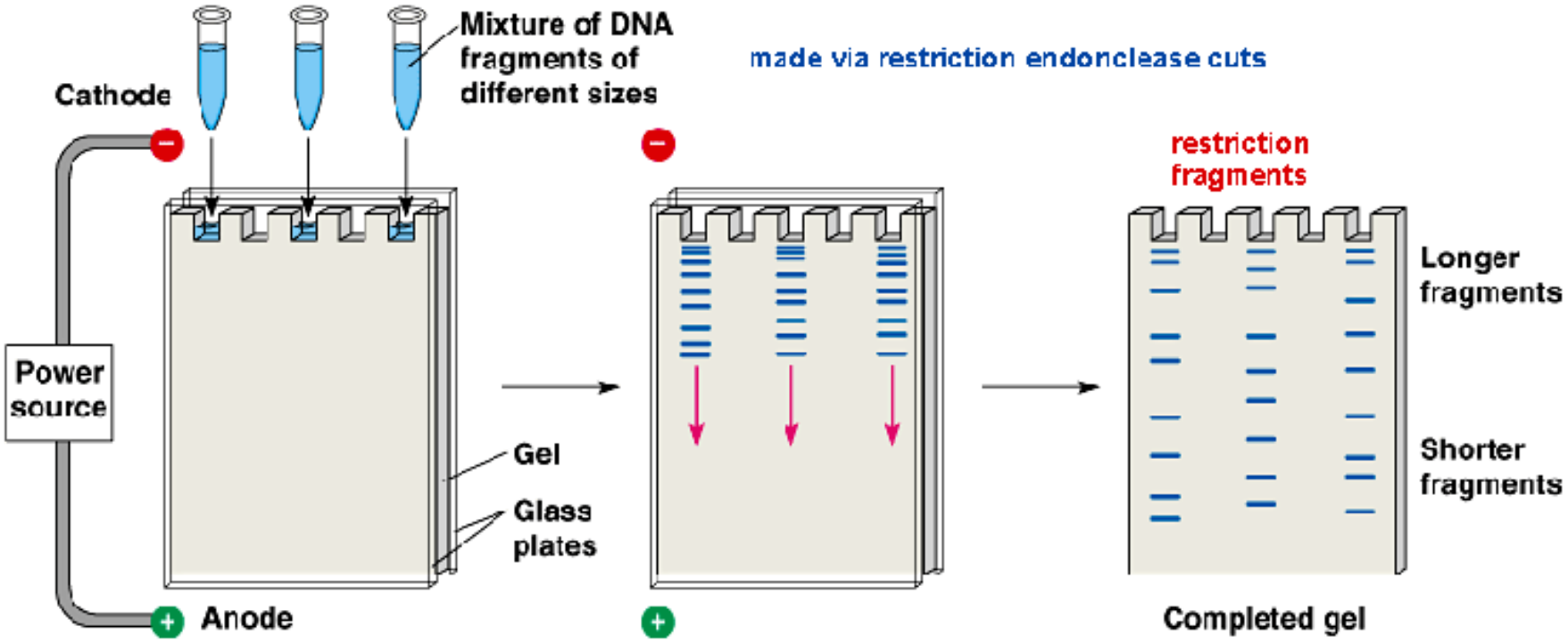




3. Electrophoresis

3.1. Agarose gel electrophoresis of DNA

Electrophoresis (mobility) of the sample

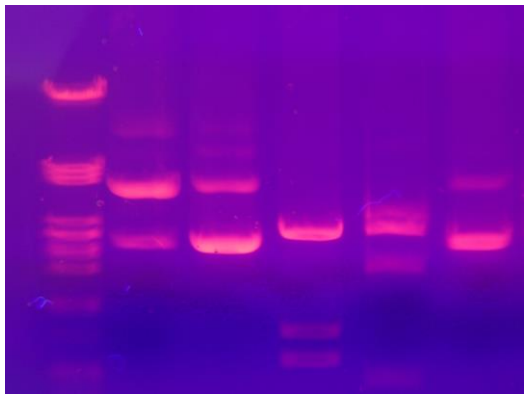


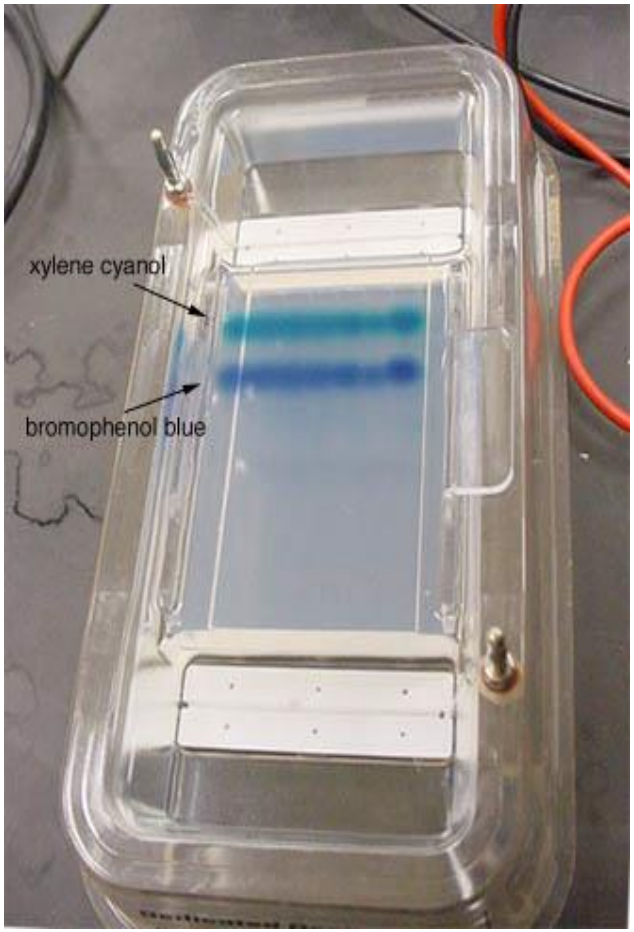
3. Electrophoresis

3.1. Agarose gel electrophoresis of DNA

Staining:

1. Electrophoresis control (bromophenol blue)
2. Post-electrophoresis DNA visualisation (apolar dye emitting fluorescence under UV light)





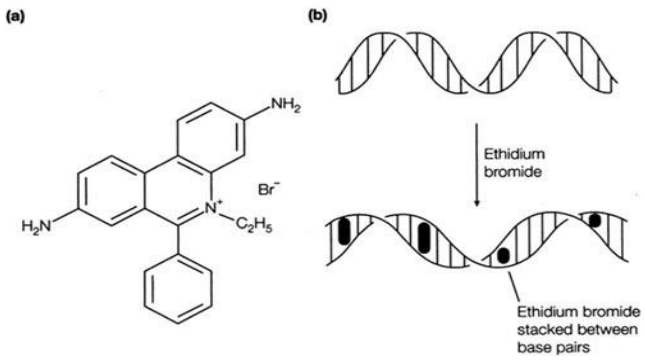
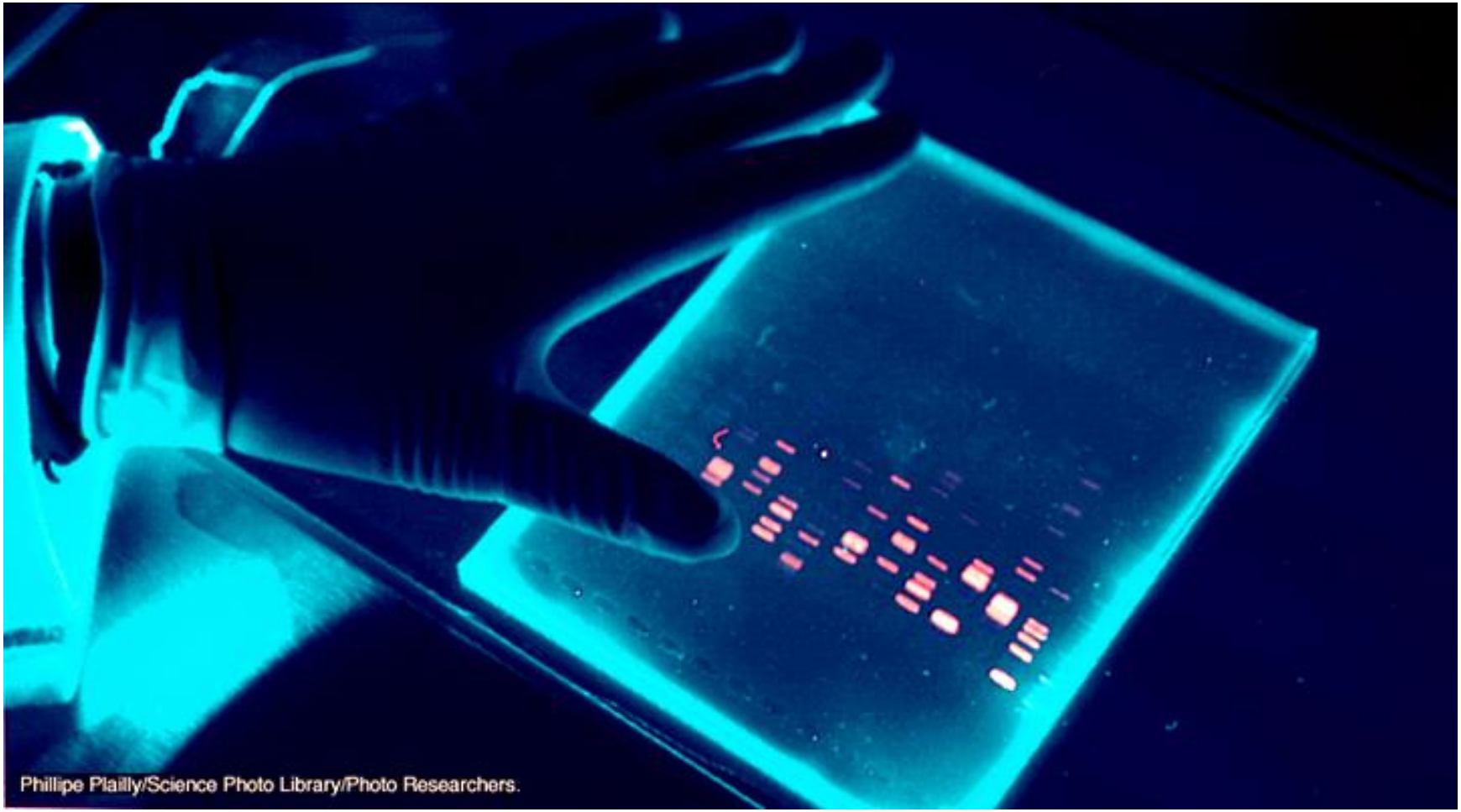


Fig. 3. (a) Ethidium bromide; (b) the process of intercalation, illustrating the lengthening and untwisting of the DNA helix.

3. Electrophoresis

3.1. Agarose gel electrophoresis of DNA

Visualisation with ultraviolet light



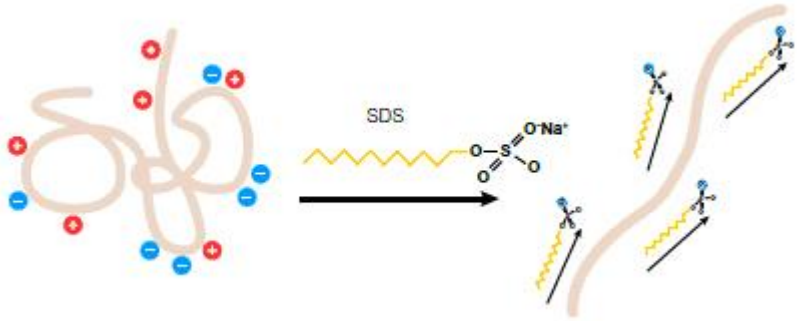
3. Electrophoresis

3.2. Protein electrophoresis using acrylamide gels (PAGE)

Polyacrylamide gels restrict the advancement of polypeptide chains that have a different charge density, shape and size.

To achieve the electrophoretic separation of the components of a mixture for analytical purposes, an **anionic detergent** (sodium dodecyl sulphate or SDS) is commonly added to the mixture, which readily binds to the polypeptide chains.

Denatured protein bound to SDS

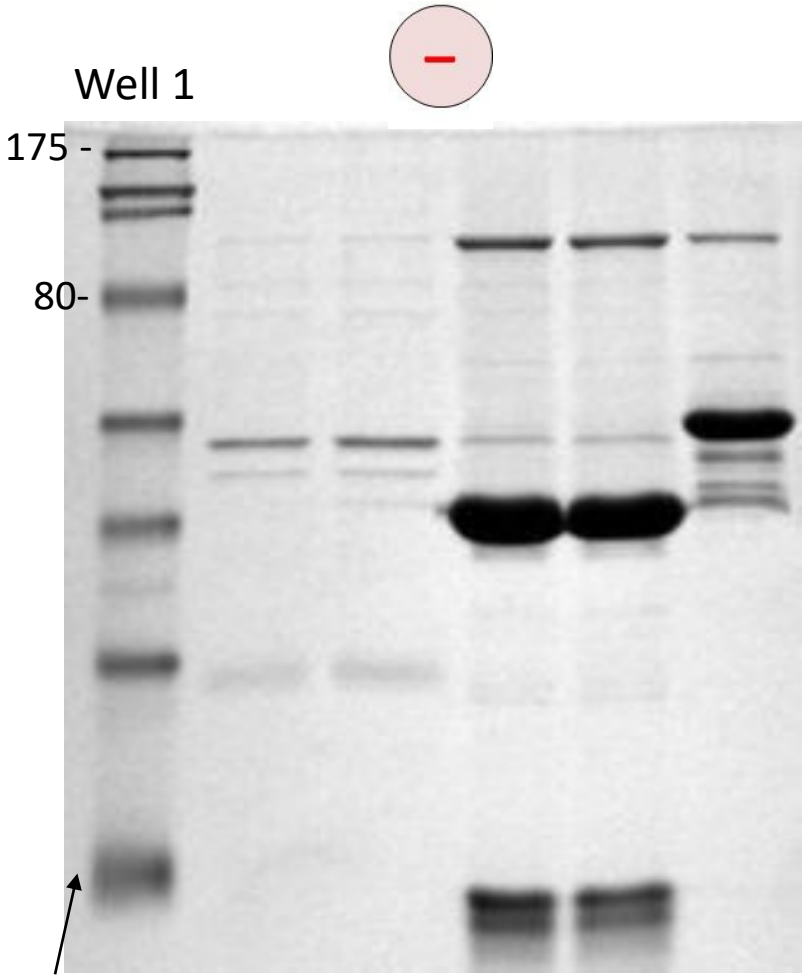


SDS molecules bind to proteins, while changing their quaternary, tertiary and secondary structures.

The SDS-bound polypeptides have identical charge density (negative) and shapes, so that their size is what differentiates their progress towards the anode.

3. Electrophoresis

3.2. Protein electrophoresis using acrylamide gels (PAGE)

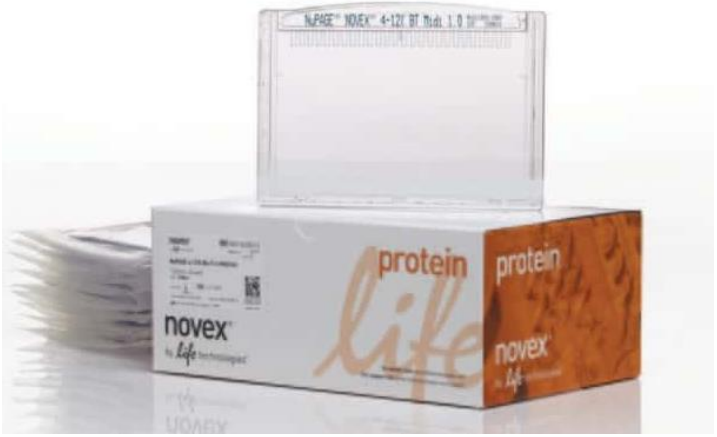


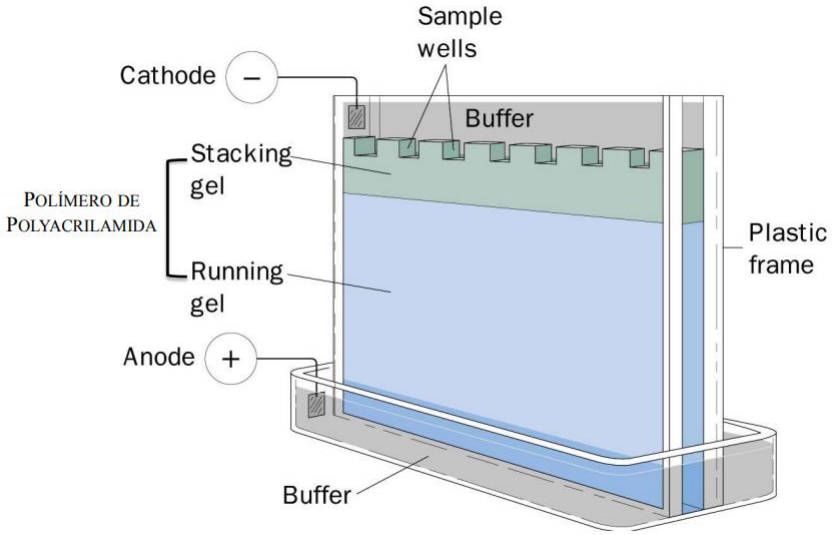
Pattern or standard: polypeptides of known molecular size (Kda)

3. Electrophoresis

3.2. Protein electrophoresis using acrylamide gels (PAGE)

Acrylamide gel preparation

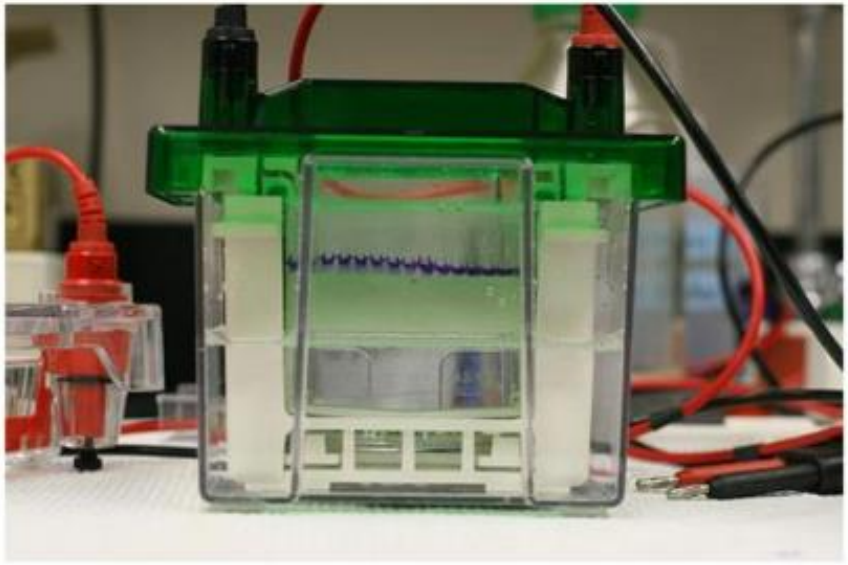


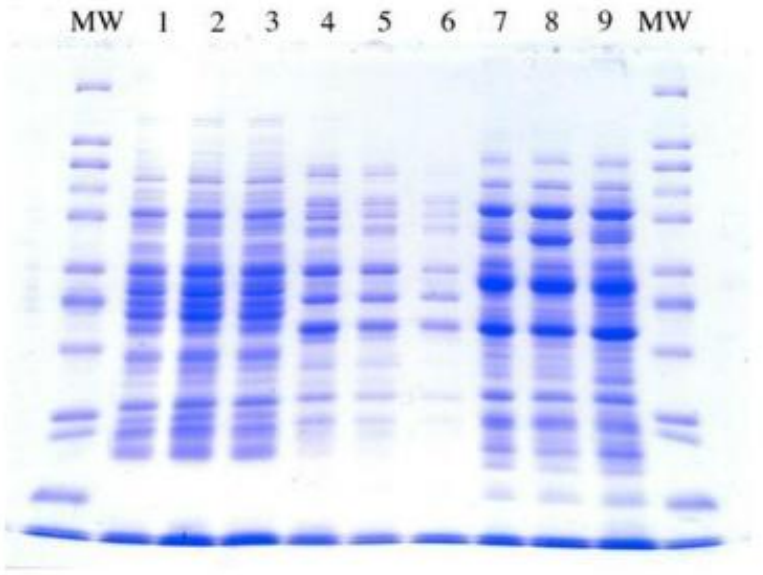


3. Electrophoresis

3.2. Protein electrophoresis using acrylamide gels (PAGE)

Electrophoresis (mobility) of the sample and visualisation





Coomassie brilliant blue stain, silver stain (more sensitive), etc.

3. Electrophoresis

3.3. Protein electrophoresis using cellulose acetate

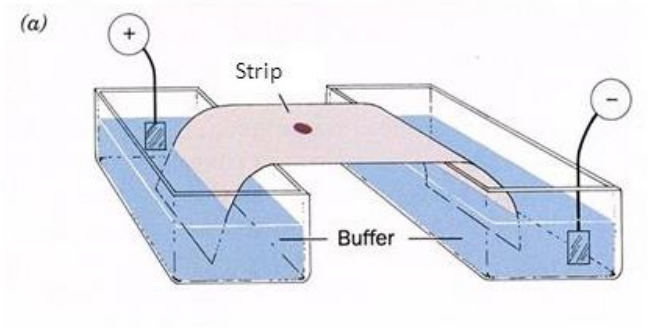
- Proteins are separated by their **charge density** (charge/mass).
  - **Their net charge depends on:**
    - The **ionisable residues** in their sequence
      - Can have + charge : Lys, Arg, His
      - Can have - charge : Asp, Glu, Tyr, Cys
    - **Of the pH of the medium**
      - Isoelectric point (*pI*)*:* pH at which the net charge is 0
        - » pH > pI - charge
        - » pH < pI + charge

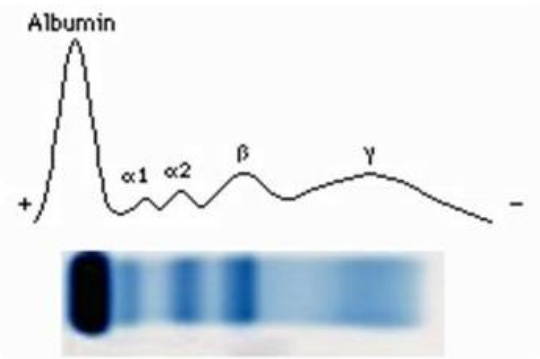
3. Electrophoresis

3.2. Protein electrophoresis using cellulose acetate

Example: serum protein proteinogram

- Performed at pH > pI normally pH= 8.6
  - Negatively charged proteins (migration to the anode)
- Five bands in characteristic proportions
  - Diagnostic value

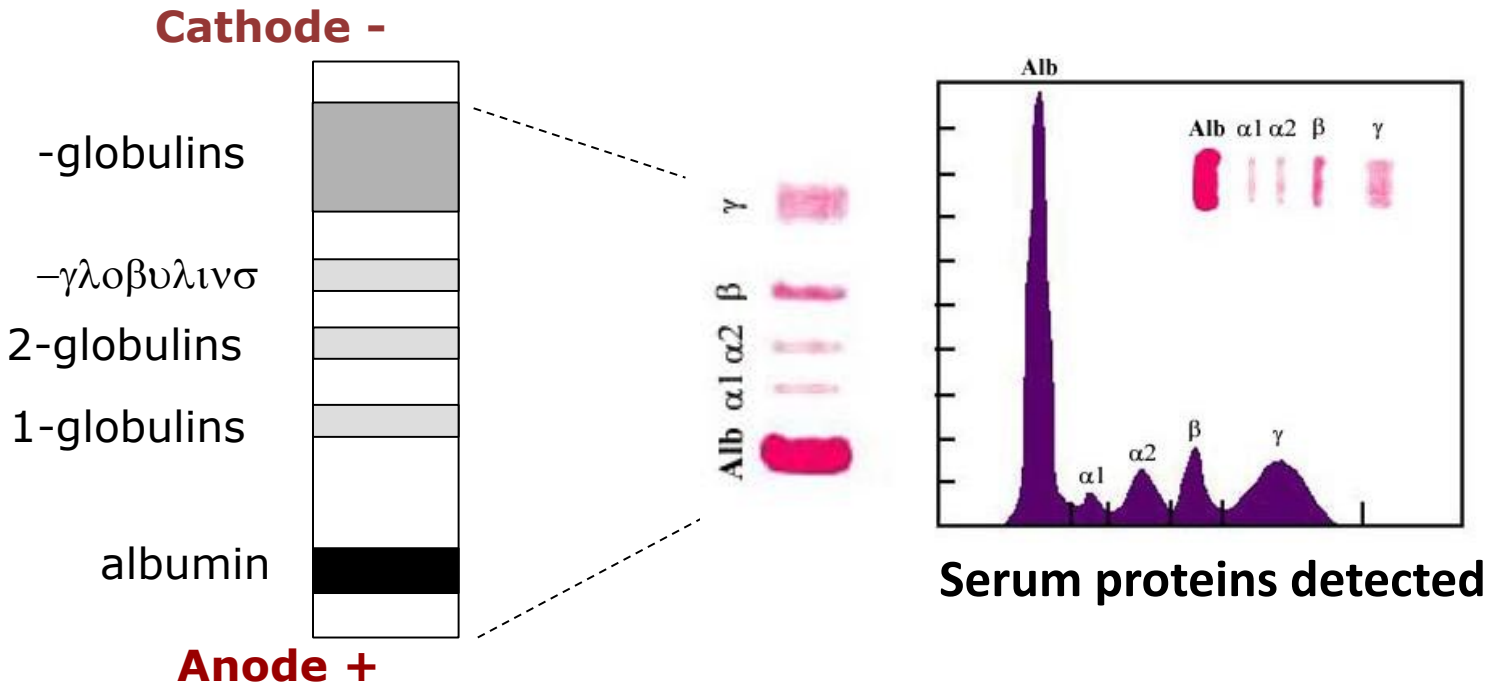




3. Electrophoresis

3.2. Protein electrophoresis using cellulose acetate

Example: serum protein electrophoresis



Much more detail in practical session 5