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Faculté des Sciences de la Nature & de la Vie
Département des Sciences Biologiques

Attestation de production pédagogique

Nous, Présidente du Comité Scientifique du Département des Sciences Biologiques, attestons que l'enseignante :

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Grade : Maître de Conférences Classe B

A élaboré un polycopié pédagogique en anglais intitulé « *Methodes and Techniques of Biochemical Analys I* ». Destiné aux étudiants du niveau : 3ème année Licence Biochimie du département des Sciences Biologiques.

Le présent document a été soumis à une expertise sous l'égide du CSD du département, lequel a émis un avis favorable à son édition et à sa diffusion.

Fait à Adrar le 28/04/2026

La Présidente du CSD

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En foi de quoi, la présente attestation est délivrée pour servir et valoir ce que de droit

Ministry of Higher Education and Scientific Research

Ahmed DRAIA University, Adrar

Faculty of Natural and Life Sciences

Department of Biological Sciences

Educational Handout

Title

Methods and Techniques of Biochemical Analysis I

Prepared by : ATTOU Amina

Course intended for Bachelor's students

Specialty and level: 3rd year Bachelor’s degree in Biochemistry

Academic year : 2025-2026

Foreword

This module, titled **"Biochemical Analysis Methods and Techniques I,"** constitutes the methodological cornerstone of your Biochemistry Bachelor's degree. Offered within the Methodological Teaching Unit (UEM1), it carries 5 credits and a coefficient of 3, underscoring its fundamental importance in the curriculum of a Bachelor's degree in Biochemistry.

The central aim of this course is to equip the student with a complete and essential toolkit for tackling any analytical challenge in the life sciences. This module is dedicated to acquiring practical and theoretical skills.

The module explore two major pillars:

- Sample preparation and fractionation methods (centrifugation, dialysis, lyophilization...), which are the indispensable steps for obtaining clean, concentrated biological material prior to any analysis.
- The fundamental principles of spectroscopy (UV-Visible, Infrared, Atomic), which form the physical basis for most modern detection and quantification instruments.

By mastering these techniques, the student will be able to develop a critical analytical mindset and will learn to choose the most suitable method for a given problem, understand its limitations, and correctly interpret the results it provides.

This course builds upon the knowledge acquired in Biology, General Biochemistry, and Chemistry. It, in turn, serves as an indispensable foundation for practical laboratory work, final-year projects, and future professional life.

We wish to each student a stimulating exploration into the heart of the instruments and principles that make the invisible visible and discover the molecular language of life.

Teaching unit	**Semester volume (14-16 weeks)**	**Weekly hour volume**				**Coefficient**	**Credit**	**Evaluation**	
Methodology unit 1 : Analysis techniques		**Course**	**Tutorial**	**PW**	**others**			**CC (40%)**	**Exam (60%)**
Subject 1: Methods and Techniques of Biochemical Analysis I	**60H**	**1H30**	**/**	**2H30**	**65H**	**3**	**5**	**x**	**x**

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Abbreviations list :

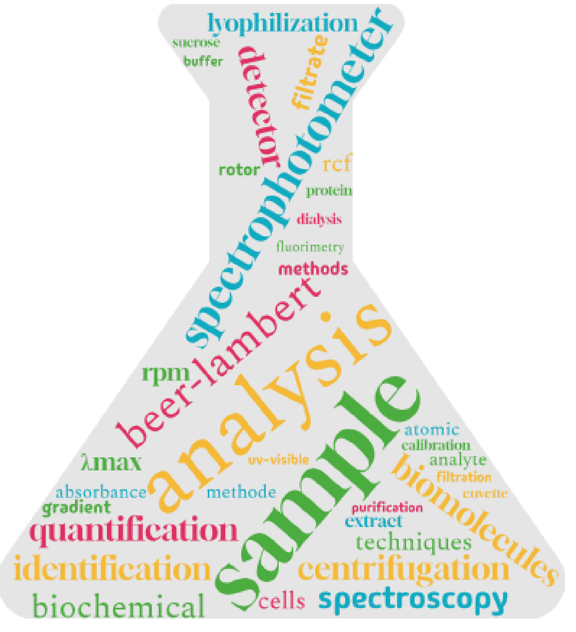
- **A** – Absorbance
- **AAS** – Atomic Absorption Spectrometry
- **AES** – Atomic Emission Spectroscopy
- **AO** – Atomic Orbital
- **DNA** – Deoxyribonucleic Acid
- **ELISA** – Enzyme-Linked Immunosorbent Assay
- **ETAAS** – Electrothermal Atomic Absorption Spectrometry
- **FAAS** – Flame Atomic Absorption Spectroscopy
- **FAES** – Flame Atomic Emission Spectroscopy
- **FIR** – Far Infrared
- **FT-IR** (or **FTIR**) – Fourier Transform Infrared
- **GFAAS** – Graphite Furnace Atomic Absorption Spectrometry
- **HCL** – Hollow Cathode Lamp
- **ICP** – Inductively Coupled Plasma
- **ICP-OES** – Inductively Coupled Plasma Optical Emission Spectrometry
- **IR** – Infrared
- **KBr** – Potassium Bromide
- **LED** – Light Emitting Diode
- **MIR** – Mid Infrared
- **MO** – Molecular Orbital
- **MRI** – Magnetic Resonance Imaging
- **MWCO** – Molecular Weight Cut-Off
- **NADH** – Nicotinamide Adenine Dinucleotide (reduced form)
- **NIR** – Near Infrared
- **PCR** – Polymerase Chain Reaction
- **PMT** – Photomultiplier Tube
- **PTFE** – Polytetrafluoroethylene
- **PVDF** – Polyvinylidene Fluoride
- **RCF** – Relative Centrifugal Force
- **RPM** – Revolutions Per Minute
- **S** – Svedberg (sedimentation coefficient unit)
- **T** – Transmittance
- **UV** – Ultraviolet
- **UV-Vis** (or **UV/Vis**) – Ultraviolet-Visible
- **Vis** – Visible

Introduction

This module, spanning in semester 5 of the Biochemistry Bachelor's degree, is designed to provide students with a solid theoretical and practical foundation in the core analytical methodologies that underpin modern biochemical research and diagnostics.

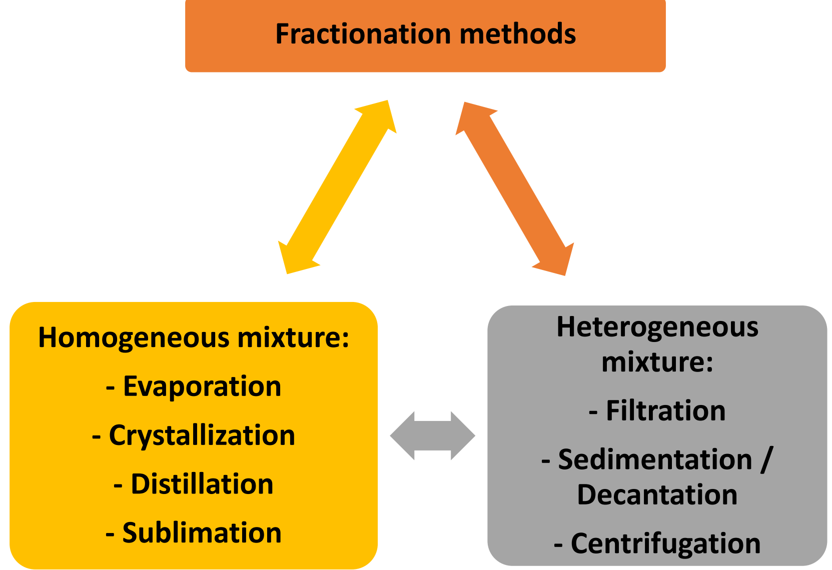
The module is structured to guide students from fundamental sample preparation techniques to sophisticated instrumental analysis. In this semester 5, the focus is on essential preparatory and spectroscopic methods. Students will explore standard laboratory techniques for fractionating, concentrating, and purifying biological samples, including filtration, centrifugation, and lyophilization. This foundation is crucial for ensuring sample integrity prior to analysis. The module then delves deeply into the principles and applications of spectroscopy. Students will learn the fundamental physics of light-matter interactions and apply this knowledge to various spectroscopic techniques, such as UV-Vis, Infrared (IR), Atomic Absorption (AAS), and fluorimetric analyses. These methods are indispensable for the identification and quantification of biomolecules.

By integrating these two core areas—spectroscopic analysis and separation science—this module equips future biochemists with the essential toolkit to isolate, identify, characterize, and quantify biomolecules. Mastery of these techniques is vital for work in diverse fields, including medical diagnostics, pharmaceutical development, quality control in the food industry, and fundamental biomedical research.



I/ Common Fractionation Methods

- **Fractionation** is a process for separating a mixture into several successive fractions with different properties.
- Fractionation techniques are based on differences in the properties of the component species of the original mixture.
- These properties may be physical or chemical.



1.1 Filtration methods

a.Filtrationdefinition:

Filtration is a physical or mechanical separation process that removes solid particles from a fluid (liquid or gas) by passing it through a porous medium, called a filter medium. The particles, collectively called the **retentate** or residue, are retained on the medium, while the clarified fluid, called the **filtrate**, passes through.

b.Purpose:

The primary goal of filtration is to purify, clarify, or isolate. Its applications are vast and include:

- **Purification:** Producing potable water by removing particulates and microorganisms.
- **Clarification:** Clearing turbid solutions in laboratories (e.g., using filter paper) or in industries like winemaking and pharmaceuticals.
- **Sterilization:** Removing bacteria and cells using membrane filters with pore sizes smaller than the microorganisms (0.2 µm).
- **Solid Recovery:** Isolating a valuable solid product from a suspension, such as a crystallized pharmaceutical compound.

c.Principle:

The fundamental principle is **size-exclusion (or sieving)**. The filter medium contains pores of

a specific size. Particles in the fluid larger than these pores are physically retained on or within the medium, while the fluid and dissolved/smaller solutes pass through. This is primarily a physical, not chemical, separation. Common filter media include filter paper, sintered glass, sand beds, and polymeric membranes.

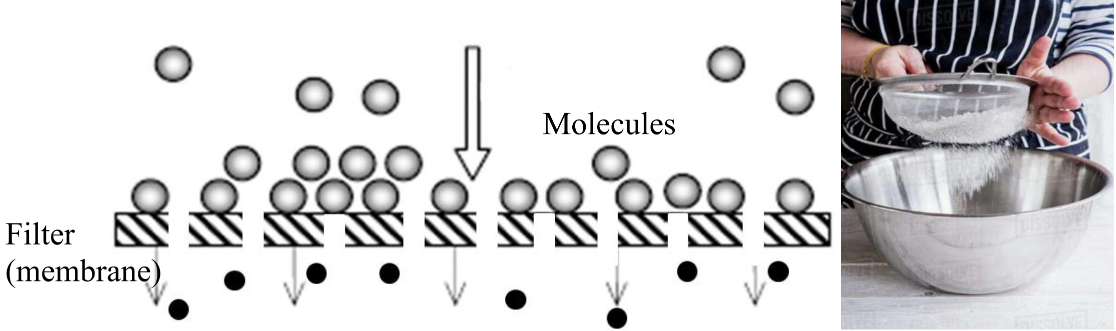


***Figure 1:** filtration principle*

d. Accompanying Phenomena Affecting Filtration

While the ideal principle is simple sieving, two key phenomena often accompany the process and significantly impact its efficiency and performance: **clogging (or fouling)** and **adsorption**.

- **Clogging (Fouling)**

• **Description:** Clogging is the gradual reduction in filtration flow rate and efficiency due to the accumulation of retained particles within the pores or on the surface of the filter medium.

• **Mechanism:**

Pore Blocking: Particles lodge directly in the pores, physically narrowing or sealing them.

Cake Formation: Retained particles build up on the filter surface, forming a "filter cake." While this cake can act as a secondary, finer filter, it also creates an additional layer of resistance to fluid flow.

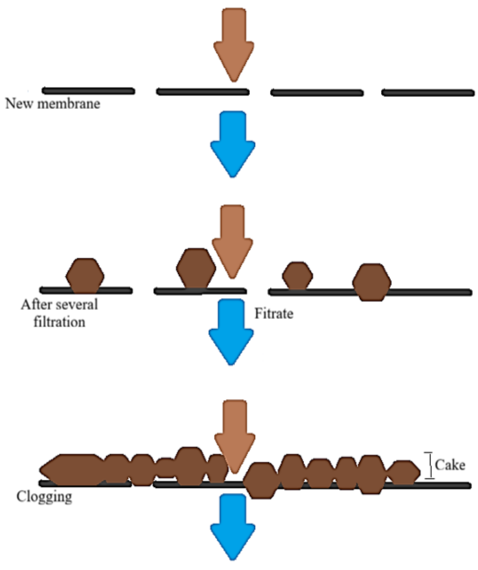


***Figure 2:** clogging phenomenon*

• **Consequences:** Increased pressure drop across the filter, decreased filtrate flow rate, reduced filter lifetime, and potential deformation or rupture of the filter medium if pressure builds excessively.

• **Mitigation:** Strategies include using pre-filters to remove larger particles, employing backwashing (reversing flow) to dislodge cake, selecting filter media with appropriate pore size and surface characteristics, and applying chemical or thermal cleaning regimens.

- **Adsorption**

- **Description:** Adsorption is a surface phenomenon where dissolved molecules (solutes, ions, or even small colloids) in the fluid adhere to the surface of the filter medium or the retained particles (filter cake) via physical or chemical interactions, *even though they are small enough to pass through the pores*.
- **Mechanism:** The adhesion is driven by intermolecular forces such as van der Waals forces, electrostatic attraction, or hydrophobic interactions. Activated carbon filters operate primarily on this principle, not sieving.
- **Consequences:**
  - **Unintended Removal:** Desired components in the filtrate may be lost.
  - **Filter Poisoning:** The active sites on the filter medium become saturated, reducing its effectiveness.
  - **Sample Contamination:** Molecules adsorbed from previous filtrations can later desorb and contaminate a new sample.
- **Solution:** Selecting a chemically inert filter medium (e.g., certain polymers like PTFE for non-polar adsorption issues), pre-saturating the filter with an inert solution, or adjusting the pH/ionic strength of the sample to minimize solute-medium interactions.

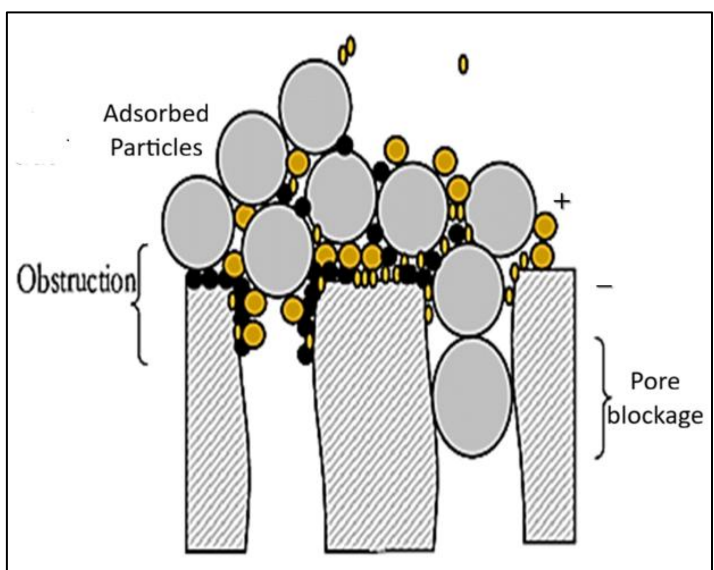


Figure 3: *adsorption phenomenon*

e. Filtration equipment

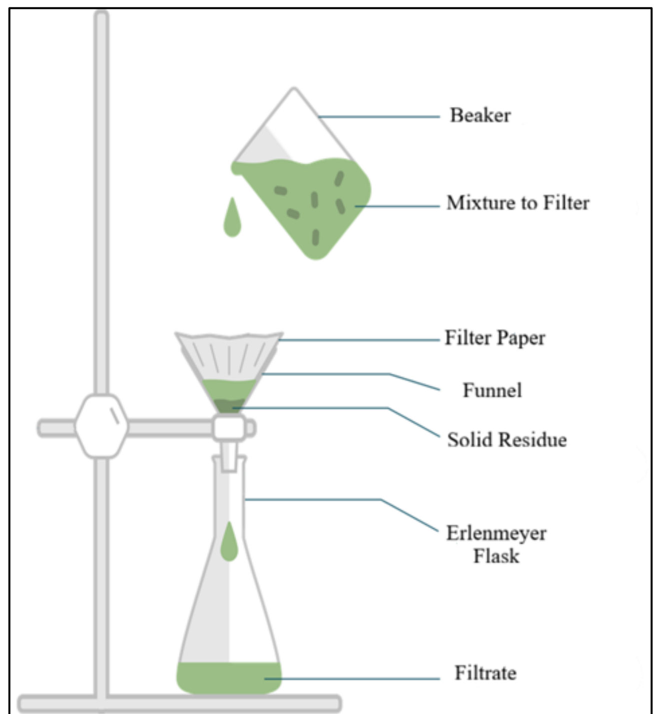


Figure 4: *laboratory equipement for gravity filtration*

➢ **Filters :** There are two types of filters. They can be used separately or in combination, depending on the requirements.

- **Depth Filters:** These are composed of fibrous substances (paper, asbestos, cellulose, cotton, glass fiber) or agglomerated substances (sintered glass, sand, charcoal). The efficiency of a depth filter increases with its thickness and decreases with an increase in the pressure applied to the filter.

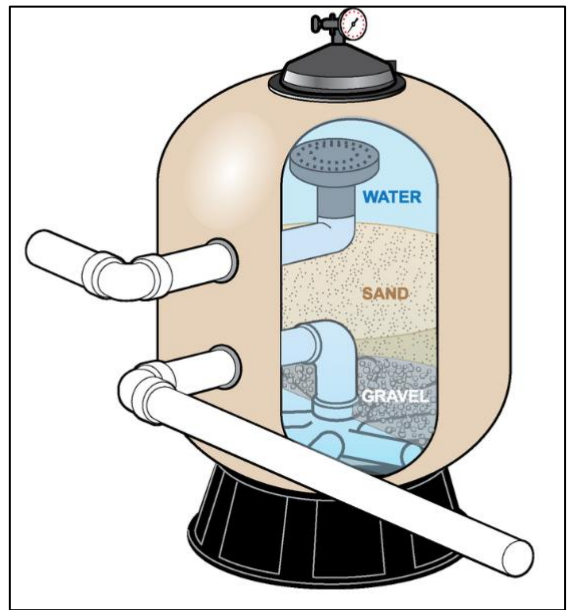


Figure 5: *sand filter*

- **Surface Filters** (like a sieve or a polymeric membrane) work primarily by retaining particles on their *surface* based on a strict size-exclusion (sieving) principle.

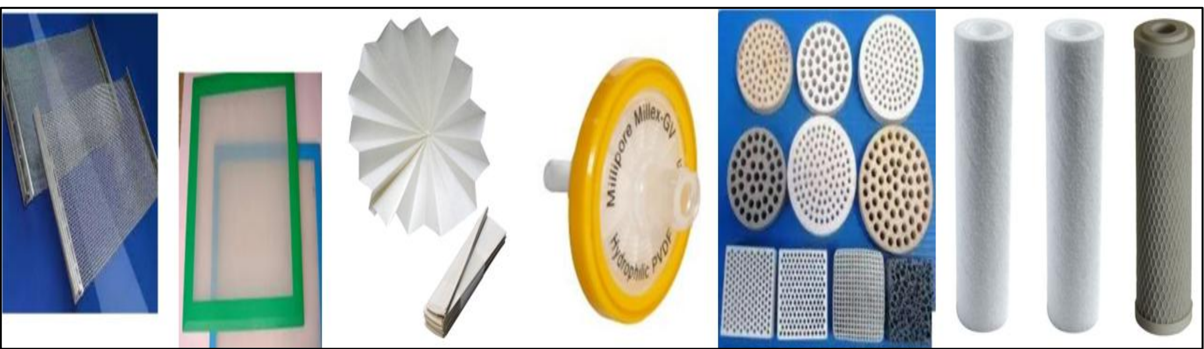


Figure 6: *several types of surface filters*

Surface filters types :

- **Filter Papers:** The type of paper differs depending on whether the desired filtration is **fast** (Whatman® No. 4 or 90), **medium speed** (Whatman® No. 1 or 7), or **slow** (Whatman® No. 5 or 6).
- **Glass Fiber or Borosilicate Glass Filters:** These are circular and are always used flat on a Büchner funnel or on a funnel with a fritted disc. Their pores are narrower than those of paper filters.
- **Fritted Plates (or Fritted Discs):** These are porous plates made of sintered glass powder, fitted into a funnel, and allow for vacuum filtration. Their cleaning requires washing with **sulfochromic acid** (a chromic acid-sulfuric acid mixture, now largely replaced by safer alternatives) and rinsing with distilled water.
- **Millipore Membranes:** Made from polymerized cellulose esters containing a large number of calibrated pores (0.025 to 8 µm in diameter, filter thickness: 0.15 mm).
- Membranes made of **nitrocellulose** or **PVDF** (polyvinylidene fluoride) adsorb proteins and nucleic acids.
- **Cellulose acetate** membranes are suitable for aqueous and alcoholic media.
- Membranes made of **polycarbonate** or **nylon** are suitable for aqueous and organic solutions.
- **Polyamide** membranes are suitable for solvents and alkaline solutions.
- **Fluoropore** (PTFE) membranes are adapted for gas filtration.
- **Screen Filters:** They retain all particles larger than the pore size of the filter, which must be calibrated (the most common pore diameters are 0.8, 0.45, and 0.22 µm). They are made of cellulose, cellulose acetate, nitrocellulose, Teflon (PTFE), etc. In these

membranes, approximately 35% of the material is occupied by the polymer matrix and the remainder by the pores.

- **Funnels :**

Laboratory funnels are essential tools for guiding liquids and granular solids during transfers, filtration, or separations. The most common is **the ordinary (simple) glass funnel,** used for pouring, filtering with paper, or supporting powdered reagents. **Specialized funnels** are designed for more precise tasks. These include the **Büchner funnel** (used with a vacuum for fast solid-liquid separation), the separatory funnel (for immiscible liquid-liquid extractions), the dropping funnel (allowing controlled addition of reagents), and the powder funnel (with a wider stem to prevent clogging when transferring solids). Each variant enhances a specific laboratory procedure for greater efficiency, safety, or control.

Figure 7: *Funnels (from left to right : Buchner, Hirsch, ordinary)*

f. Filtration process :

we can classify filtration processes according to several creteria : device used or pore size.

- **Classification according to the device used:**

- **Gravity filtration**

This method uses a filter and a container to receive the filter. The simplest device for this method is a simple funnel fitted with filter paper placed on an Erlenmeyer flask to collect the filtrate (Figure 4). This type of filtration has some drawbacks: filtration is relatively slow, difficulty in recovering the isolated solid phase.

- **Vacuum Filtration**

The speed of filtration is increased by creating a vacuum (reduced pressure) downstream from the filter material. The vacuum is applied inside the vacuum flask; therefore, the pressure above the funnel (atmospheric pressure) is greater than the pressure inside the flask, which "pushes" the liquid through into the flask. The funnel is typically fitted to the flask using a rubber adapter, which will form a tight seal against both the flask and the funnel once the vacuum is established.

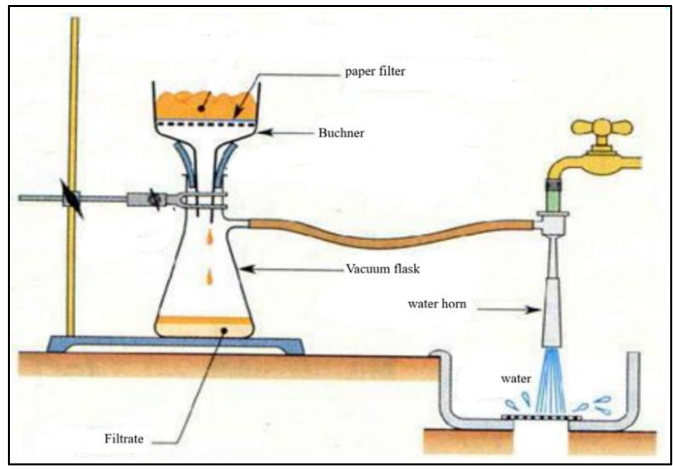


Figure 8: *vacuum filtration device*

- **Pressure Filtration**

Filtration speed is increased by applying pressure to the liquid to be filtered upstream of the filter material, which is represented by a filter membrane. This pressure filtration system with filter membranes also exists in the form of filter cartridges (e.g., Millipore), which can be attached to a syringe—a practical method for filtering small volumes of solution.

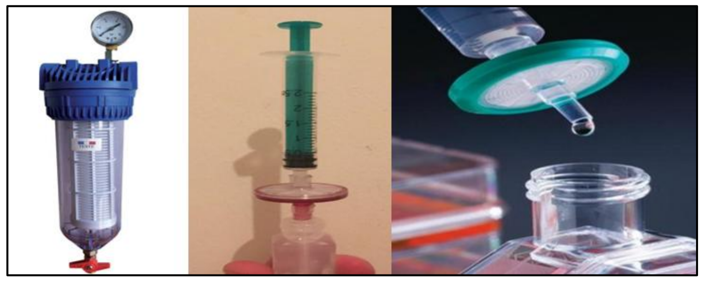


Figure 9: *pressure filtration devices*

- **Classification according to the pore size :**

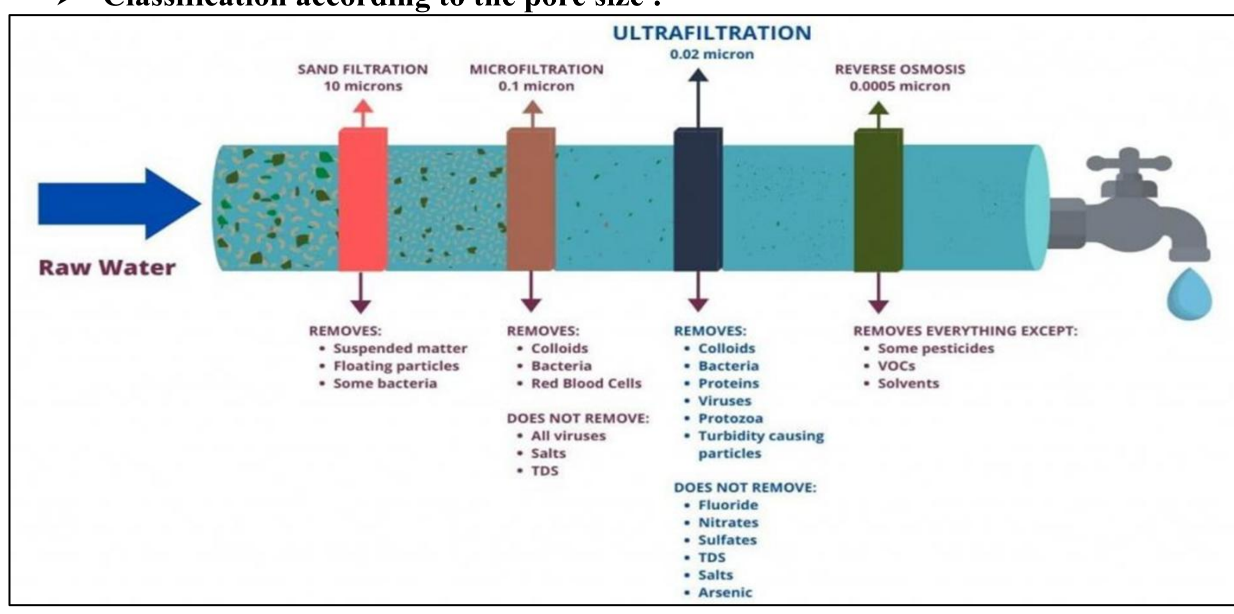


Figure 10: *filtration types according to pore size and their applications*

Table 1: applications of filtration

Ultrafiltration	Microfiltration	Nanofiltration
• Used for separating fluids rather than concentrating them (reverse osmosis or nanofiltration). • Purification of wastewater containing fats (oil, grease, etc.) before discharge into the sewer system, • Purification of food liquids containing microorganisms (pasteurization of fruit juices), • Separation of water from sugars, alcohols, and dissolved organic matter, • Production of protein concentrates from milk and whey, • Recovery of metallic particles, • Treatment of oil-in-water emulsions, • Recovery of paint, dye, and primer.	• Cold sterilization of beverages and pharmaceutical products • Clarification of fruit juices, wines, and beers • Wastewater and effluent treatment • Separation of oil/water emulsions • Water pretreatment for nanofiltration or reverse osmosis.	• Removal of pesticides from groundwater • Removal of heavy metals from wastewater.

g. Dialysis :

Dialysis is a technique used to separate substances based on their ability to pass through the pores of a semi-permeable membrane, known as a dialysis membrane.

It applies to macromolecules—such as proteins, DNA, polymers, and antibodies—as well as smaller biological molecules like peptides.

Dialysis membranes commonly used in biochemistry are typically shaped as a cylinder sealed at both ends, containing the solution to be dialyzed. This cylinder is called a dialysis tube or dialysis sac and is placed in a container holding the dialysis buffer or external solution.

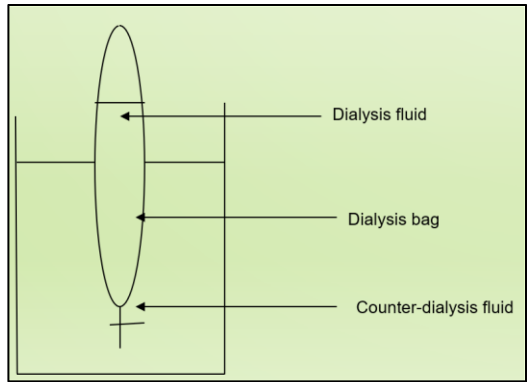


***Figure 11:** dialysis device*

Typically, modified cellulose membranes are used, which may be transparent or opaque, and either rigid or flexible. They are available in a wide range of sizes and molecular weight cut-offs (MWCO).

Cellulose ester membranes are particularly well-suited for proteins because they exhibit low affinity for them and are more tolerant toward alcohols and solvents.

PVDF (polyvinylidene fluoride) membranes are highly hydrophobic and resistant to most organic solvents, aqueous acids, and bases.

Dialysis membranes may contain glycerol, sulfur compounds, and heavy metals, which must be removed beforehand by soaking.

Principle of Dialysis

Dialysis is based on the principles of diffusion across a permeable or semi-permeable membrane. Two mechanisms are involved in this process:

- First, diffusible molecules will cross the membrane along the concentration gradient. There will therefore be a net movement of molecules from the more concentrated side toward the less concentrated side. At equilibrium, the concentrations of each diffusible species will be equal on both sides.
- Another phenomenon occurring simultaneously with the movement of small molecules is **osmosis**. Indeed, since the content of the dialysis sac is often more concentrated at the beginning of the process than the dialysate, water will enter the sac by osmosis. However, as particles diffuse out of the sac, the concentration on both sides of the membrane will equalize, and osmosis will cease.

Several factors can significantly influence the rate of dialysis:

- Membrane thickness;
- Membrane surface area;
- pH of the solution to be dialyzed and of the dialysate;
- Temperature.
- The addition of certain substances can also alter the dialysis rate. For example, urea acts on proteins by dissociating specific structures, which reduces the apparent molecular radius.

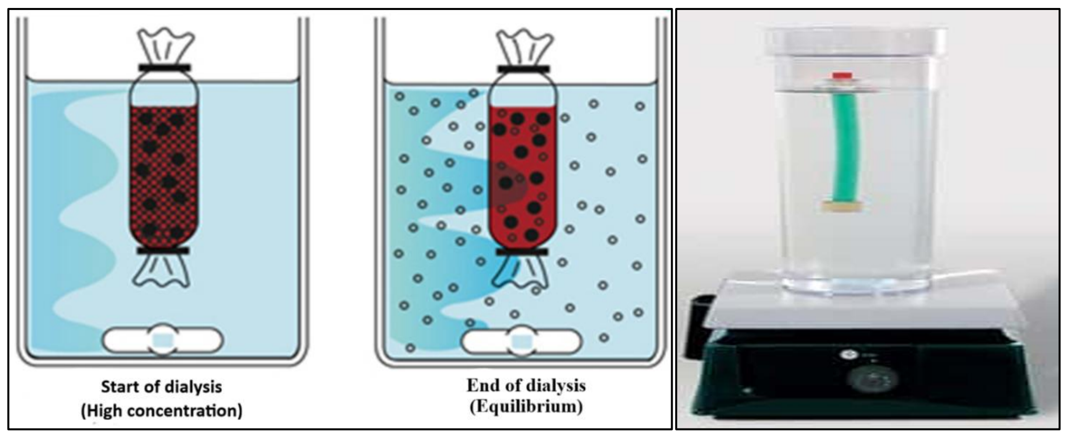


Figure 12: *Dialysis device*

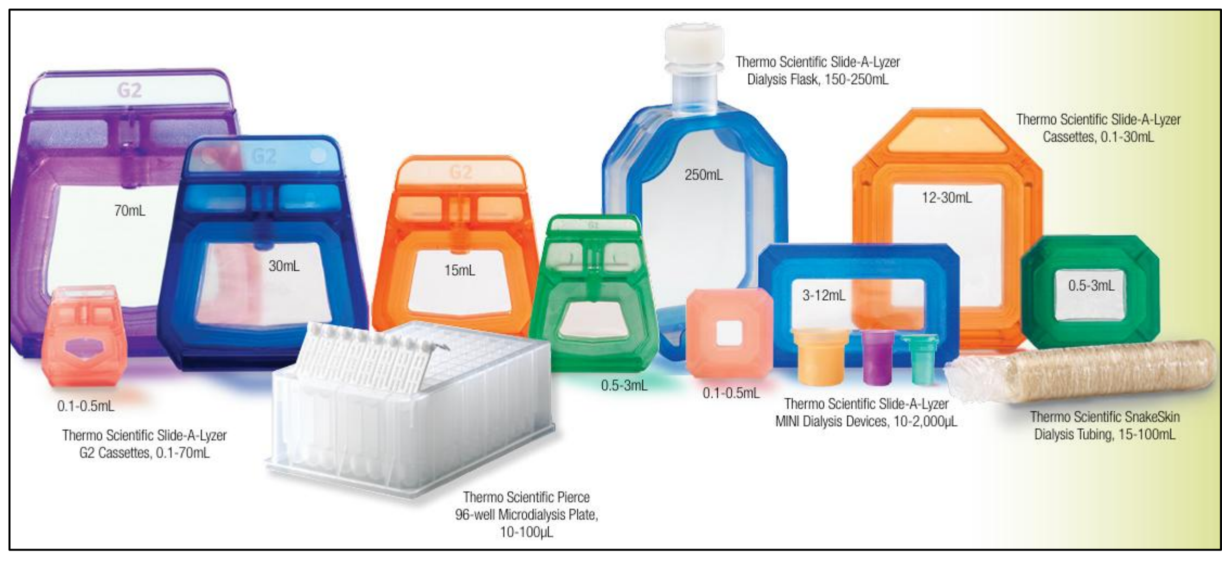


Figure 13: *Innovative dialysis devices*

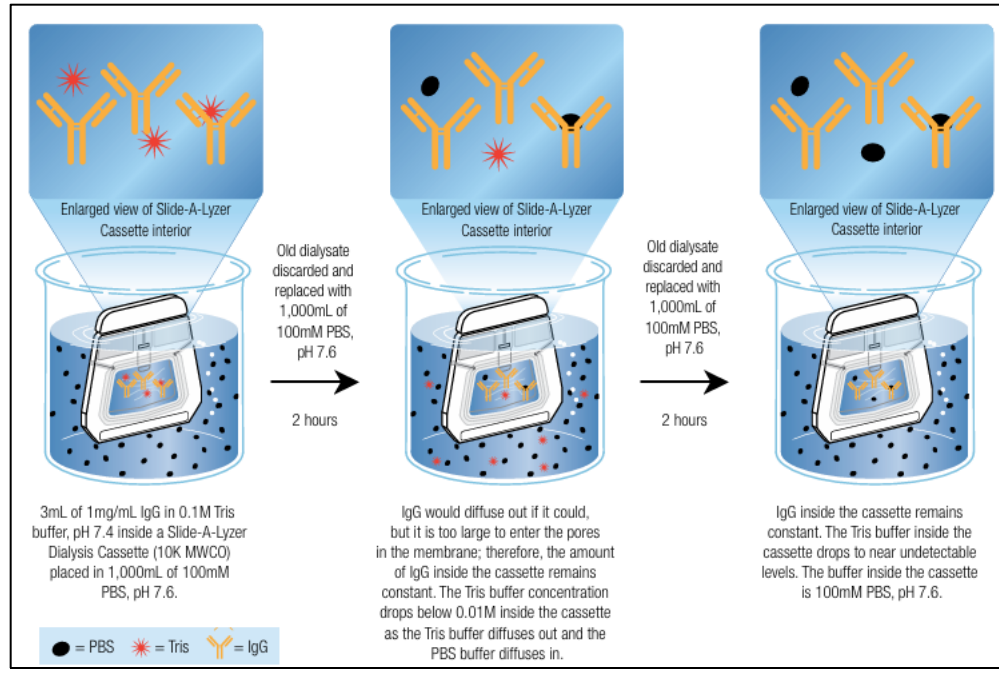


Figure 14: *how new dialysis cassette works*

- Electrodialysis : The removal of mineral salts can be achieved using this method. Two peripheral compartments are separated from the central compartment by semi-permeable membranes. Applying a direct current of a few milliamperes allows anions to migrate toward the cathode and cations toward the anode. Mineral ions are thereby eliminated without loss of organic products. However, the heat generated by the current prevents the use of this method with heat-labile (thermosensitive) substances.

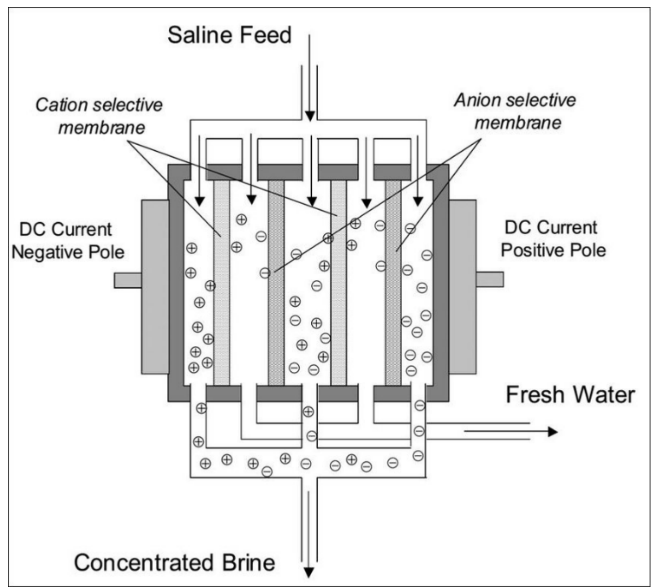


Figure 15: *diagram of electrodialysis*

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1.2 Centrifugation and ultracentrifugation methods

Many experiments in biochemistry require other methods of separation and fractionation. By subjecting samples to strong acceleration forces, separation occurs based on the density and mass of the mixture's components. Upon completion of these techniques, the result is:

- A **sediment** composed of denser, compacted material at the bottom of the tube,
- And a **residual liquid** above the sediment.

The physical phenomena employed and the resulting forces characterize two fractionation processes used in the laboratory:

• **Sedimentation** uses the Earth's gravitational acceleration field.

• **Centrifugation** uses a centrifugal acceleration field.

a. Sedimentation : is an analytical technique used to separate a solid within a liquid, or one liquid within another immiscible liquid of different density. This separation can occur naturally under the influence of gravity. When the mixture consists of a substance much denser than the liquid, the process is called **decantation**.

When the density of the substance is lower, sedimentation is hindered, requiring the application of more intense force fields.

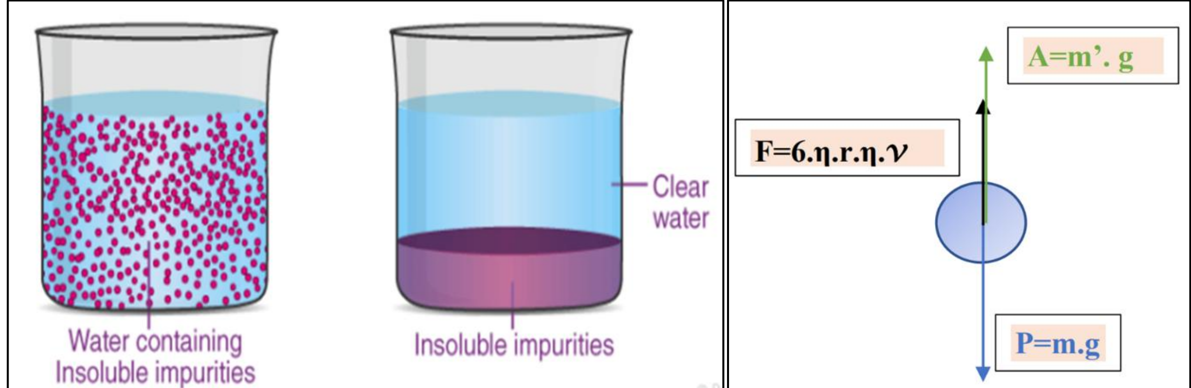


Figure 16: *sedimentation process and forces subjected on particles*

Each particle in the mixture will be subjected to several forces:

- **The force of gravity (weight)**: dependent on its mass and gravity : $P = m \cdot g$
- **The frictional force** F: determined by Stokes' law : $F = 6\pi r\eta v$

  where:

  η = viscosity of the liquid,

  v = sedimentation velocity,

  r = radius of the molecule,

  $6\pi r\eta$ = frictional coefficient.

- **The Archimedes' buoyancy force** A: $A = m' \cdot g$

  where m' = mass of the fluid displaced by the volume v.

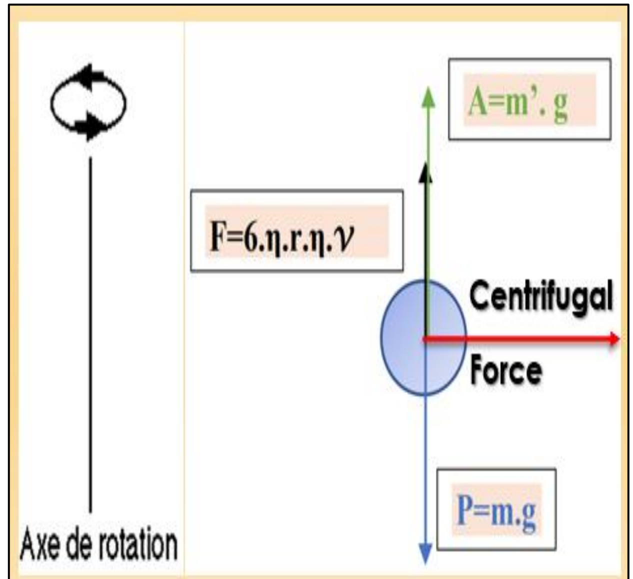
If $P > A + F$: the molecule settles to the bottom.

If $P \leq A + F$: little or no movement occurs; the particle remains in solution.

b. Centrifugation : is a technique that uses centrifugal force to separate fluids of different densities or to isolate solid particles suspended in a fluid. It allows the components of a mixture to be separated by spinning it at high speed. The device used for this purpose is called a **centrifuge**.

Centrifugal force is generated by a rotor spinning at high speeds.

- from 6 to 10,000 revolutions per minute (RPM) for regular centrifugation,
- and up to 75,000 RPM for **ultracentrifugation** enabling the sedimentation of ultra-microscopic particles.



The velocity at which these particles move is proportional to:

- The gravitational (centrifugal) force to which the particle is subjected,
- The mass of the particle,
- The difference between the density of the particle and that of the solvent,

and inversely proportional to:

- The friction with the medium, which depends on the size and geometry of the particles.

Figure 17: *forces subjected on centrifuged particles*

- **Relative Centrifugal Force (RCF),** often expressed in "× g," represents the actual separation force exerted on a sample in a centrifuge rotor. Unlike rotations per minute (RPM), which only indicates rotational speed, RCF accounts for both the rotor's radius and its rotational speed, providing a standardized measure of the effective centrifugal acceleration. The relationship between RCF and RPM is defined by the formula:

  RCF= 1,119. 10 .(rpm)^2 .r

  rpm: expresses the rotational speed, in revolutions per minute, x or r: distance to the axis of rotation, radius of rotation of the rotor (in cm)

- During centrifugation, the **sedimentation velocity** of a particle depends on: its mass, its volume, the density of the solvent (which determines the Archimedes' buoyant force), the acceleration to which it is subjected, as well as the frictional forces associated with its movement through the solution forces that depend on the size and shape of the particle. **Sedimentation Coefficient** : $s = v_t / r\omega^2 = m / 6\pi\eta r$ expressed in svedbergs (S), where 1 S = 10^{-13} seconds.
- Another method: use a nomogram. One can calculate the rotation speed (in RPM) to achieve a given acceleration. $rpm = \sqrt{\frac{RCF}{1{,}119 \cdot 10^{-5} \cdot r}}$

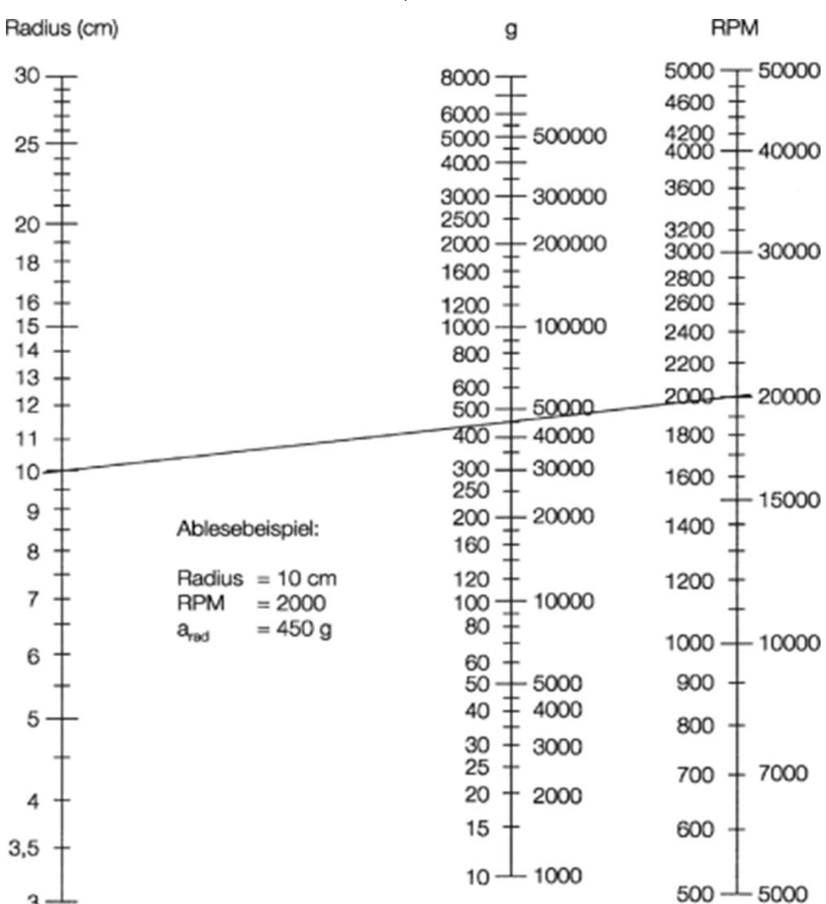


Figure 18: *nomogram*

c. Centrifuge : is a machine equipped with a rotating shaft enclosed within a centrifugation chamber, fitted with tubes designed to hold mixtures.

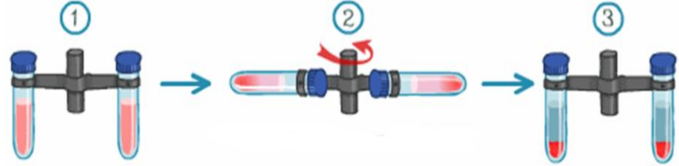
Except for benchtop centrifuges, which have relatively limited rotation speeds and typical usage times, it is necessary to prevent sample heating. For this purpose, the chamber is refrigerated. The primary limitation that determines the rotor's rotation speed is, of course, the strength of the motor that drives it. The heavier and larger the rotor, the greater the effort the motor must deliver.

Tableau 2: centrifuge types

Benchtop (Tabletop) Centrifuges: The simplest models, often called clinical centrifuges, can achieve low relative centrifugal forces (1000 to 3000 × g) at relatively low rotation speeds. Some models are refrigerated, while others are not.	
Floor-Standing Centrifuges: These devices are somewhat more complex. They can achieve rotation speeds on the order of 30,000 RPM. All models are refrigerated. These centrifuges are capable of processing large volumes. Some rotors can even hold four or six 250 mL bottles.	
Ultracentrifuges: These are complex and expensive devices capable of achieving very high accelerations (up to 300,000 × g) by spinning rotors at extremely high speeds (up to 85,000 RPM). All models are refrigerated. The rotors typically hold only about ten tubes of 40 mL each.	
Microcentrifuges: These are specifically designed for micro-volumes. The microcentrifuge tubes are small conical tubes, typically 1.5 mL in volume and made of polypropylene. Centrifuges of this type can be refrigerated and achieve accelerations in the range of 12,000–15,000 × g.	

Tableau 3: rotors types

Fixed-angle rotors: They are made from blocks of metal (aluminum, titanium) with drilled holes inside, inclined at a fixed angle relative to the horizontal plane—typically between 15° and 35°, depending on the model. Centrifuge tubes are placed into these wells. These rotors are relatively compact and can be spun at high speeds more easily due to their relatively short radius. Particles will sediment mainly along the wall of the tube.	

Swinging bucket rotors: The buckets are mounted on hooks or a pivoting mechanism. Under the effect of centrifugal force, the buckets and tubes reorient themselves and move into a horizontal position. Particles can therefore sediment directly to the bottom of the tube without ever striking the tube walls. The main disadvantage of this type of rotor is that it cannot reach very high speeds, as the buckets in the horizontal position greatly increase the rotor's effective radius. This kind of rotor is mainly used for centrifugation in **discontinuous or continuous gradients**. 	
Vertical rotors are much less common and are primarily used for **isopycnic or zonal gradient** separations.	

d. Centrifugation process types

d.1. Differential Centrifugation

Differential centrifugation is a separation technique used to isolate different types of particles (such as organelles or macromolecules) from a mixture based primarily on their size and mass.

The principle : the process works by subjecting the initial mixture to a series of centrifugation steps at progressively higher speeds (and thus higher centrifugal forces) and longer durations, so particles are separated in order of decreasing size/mass

- **First, low-speed spin:** The mixture is centrifuged at a relatively low acceleration. The largest and heaviest particles (e.g., whole cells, nuclei) sediment fastest and form a pellet at the bottom of the tube.
- **Separate supernatant:** The liquid above the pellet, called the supernatant, is carefully removed. This supernatant now contains all the smaller, lighter particles that did not sediment at that force.

- **Repeat at higher speeds:** This supernatant is then transferred to a new tube and centrifuged again, but at a higher speed. The next-largest particles (e.g., mitochondria, chloroplasts) will now pellet.
- **Progressive isolation:** This cycle is repeated several times, each time with the *new* supernatant and at increasingly higher speeds, sequentially pelleting smaller and smaller particles (e.g., microsomes, vesicles, ribosomes).

This is a fundamental technique in cell biology for the crude fractionation of cellular components (subcellular fractionation), such as separating nuclei, mitochondria, and ribosomes from a cell homogenate.

d.2. Equilibrium Centrifugation (Isopycnic Centrifugation)

In equilibrium centrifugation, the different components reach a stable position where they no longer move, because they are in equilibrium.
Equilibrium is reached when the density of a particle equals the density of the solvent, which means that the gravitational (centrifugal) force is equal to the buoyant force (Archimedes' principle).

Therefore, a solvent is used whose density varies depending on the position in the tube, this is called a gradient, and the centrifugation is known as density gradient centrifugation.

To obtain solutions of varying densities, the most common method is to use solutions with increasing concentrations of sucrose (sucrose gradient) or cesium chloride (cesium chloride gradient).

e. Ultracentrifugation

is a centrifugation method aimed at separating very fine particles dispersed in a liquid of nearly equal density. For this separation to occur, the centrifuge's rotational speed must exceed 15,000 revolutions per minute and can reach up to 75,000 RPM.

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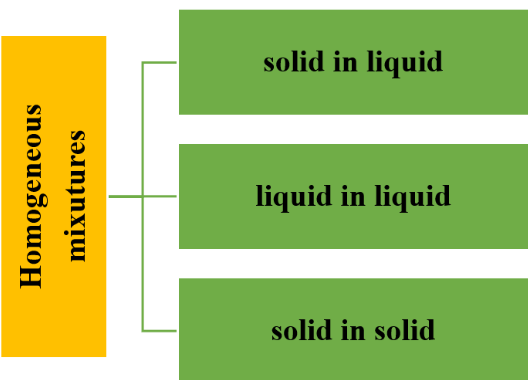
1.3 Concentration, evaporation, and lyophilization methods

a. Concentration used for fractionation of homogeneous solutions.



- **Case of a liquid-liquid mixture (total miscibility)**

Simple Distillation (Separation by Phase Change):

A technique used to separate two volatile components of a mixture that have sufficiently different boiling points. It involves heating the mixture until it boils (vaporization). The vapors produced rise through the distillation column and enter the condenser, where they liquefy. The liquid collected after condensation is called the **distillate**.

Fractional Distillation

Allows for the separation of volatile components in a mixture whose boiling points are close. The separation occurs in several stages, hence the name "fractional." This separation is achieved through a series of vaporizations and condensations characterized by an exchange of material between an ascending vapor phase and a descending liquid phase.

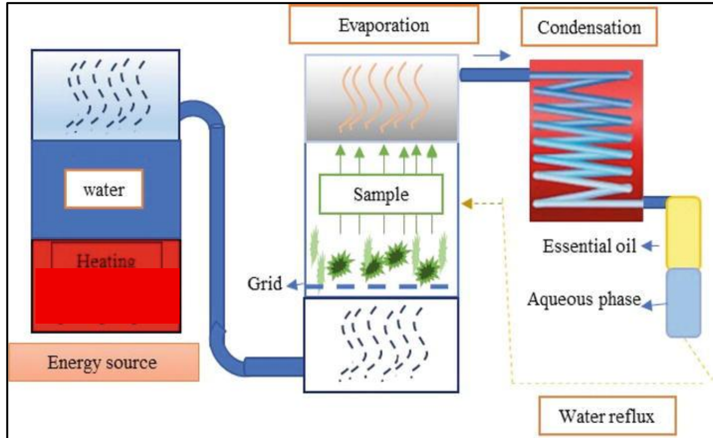


Figure 19: *distillation of essential oils*

- **Case of solid in solid mixture :**

Sublimation (Separation by Phase Change)

Its principle consists of heating the mixture to induce sublimation and cooling the vapors of the sublimed compound through contact with a cold surface. This method is possible if one of the

solids is significantly more volatile than the other. For mixtures that do not contain a solid that sublimes at room temperature, the principle involves lowering the temperature and pressure so that only one of the components passes directly from the solid to the gaseous state.

- **Case of a solid-liquid mixture (solid completely dissolved in a liquid)**

Crystallization

Crystallization involves isolating a product in the form of solid crystals from a uniform (homogeneous) solution. This is achieved through several techniques:

- **Reducing the solubility** of the product in solution by lowering the temperature of the solution. As the solubility decreases, the product crystallizes.
- **Concentrating the product** in solution (as with seawater) by evaporating the solvent. The solution becomes saturated, causing the dissolved product to crystallize.
- **Concentrating the product** by adding an **anti-solvent** (which does not dissolve, or only poorly dissolves, the product to be crystallized) that is immiscible with the original solvent. At the interface between the two liquids, the product crystallizes.

Evaporation is used when the solution consists of a solid dissolved in a liquid. If the liquid in the solution is volatile, the liquid is eliminated by heating the solution, allowing the solid substance to be recovered.

b. Lyophilization : also known as **freeze-drying**, is a process that involves removing water from food or solvents from a sample to render it stable at room temperature and to facilitate its preservation.

Lyophilization comprises three steps: designed to remove water from a sensitive sample while preserving its structure and biological activity. First, the sample is **frozen** at a very low temperature, turning its water into ice. Next, during **primary drying**, a vacuum is applied, and the temperature is carefully raised, allowing the ice to sublimate directly from solid to vapor without passing through a liquid phase. Finally, **secondary drying** removes any remaining bound water molecules by slightly increasing the temperature under continued vacuum, resulting in a stable, dry product that can be easily stored and reconstituted.

Figure 20: *freeze dryer*

c. Evaporation is the gradual change of state from liquid to gas. This phenomenon is therefore a progressive vaporization which has the effect of absorbing heat and thus reducing the temperature of the environment.

Vacuum evaporation is a concentration technique that involves introducing a liquid, first brought to its boiling point, into a vacuum chamber. Any further heat supplied to the liquid will then cause part of the water to vaporize.

A device that allows evaporation under vacuum in the laboratory is the rotary evaporator.

Figure 21: *rotary evaporator*

1.4 Protein precipitation by salting out

A commonly used approach to purify one or more proteins is to precipitate them (selectively reduce their solubility in water).

Protein precipitation is widely used in the downstream processing of biological products to concentrate proteins and purify them from various contaminants. For example, in the biotechnology industry, protein precipitation is used to remove contaminants commonly found in blood.

The solubility of proteins in aqueous buffers depends on the distribution of hydrophilic and hydrophobic amino acid residues on the protein's surface. Hydrophobic residues are mainly located in the core of globular proteins, but some exist in patches on the surface. Proteins that have a high content of hydrophobic amino acids on their surface have low solubility in an aqueous solvent.

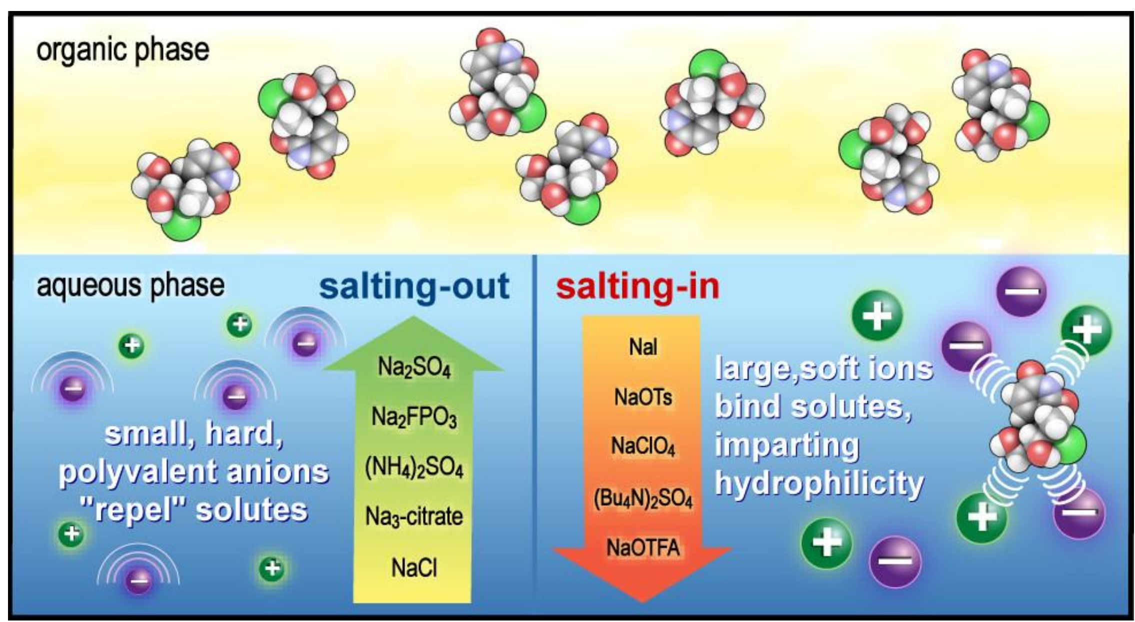


***Figure 22:** salting out/salting in*

Salting out is a classic protein purification technique based on reducing protein solubility in water by adding high concentrations of neutral salts, most commonly **ammonium sulfate**. At high ionic strength, the salt ions compete with proteins for water molecules, effectively "stealing" the hydration shell. This exposes the hydrophobic patches on protein surfaces, leading to protein-protein aggregation and precipitation. Since different proteins precipitate at different salt concentrations, this method can provide a useful initial fractionation and concentration step before more refined techniques like chromatography.

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II/ Spectroscopic Techniques

2.1 Fundamental principles

What is Spectroscopy?: Spectroscopy is the study of electromagnetic radiation emitted, absorbed, or scattered by atoms or molecules. It provides information about the identity, structure, and energy levels of atoms and molecules by analyzing how electromagnetic radiation interacts with matter.

Spectroscopy explains many phenomena that constantly surround us: the color of our clothes, the color of the sky.
In laboratories, it enables:

- identification of molecules
- determination of structures
- study of reaction kinetics
- determination of reaction mechanisms
- quantitative analysis (assays)
- medical analyses (MRI, scintigraphy, mammography, etc.).

2.2 Spectral lines and effects

What is Radiation?

Wave Nature: Electromagnetic radiation (or electromagnetic radiation) is a wave composed of two oscillating fields: an electric field E and a magnetic field H, which are both perpendicular to each other and perpendicular to the direction of propagation.
An electromagnetic radiation is characterized by its frequency (ν), its wavelength (λ), or its wavenumber.

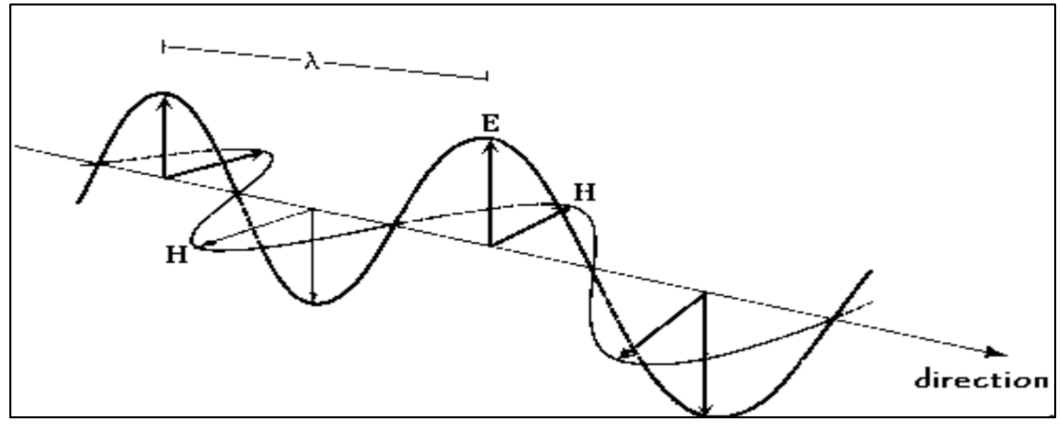


Figure 23: *electromagnetic waves*

The entire range of radiation constitutes the electromagnetic spectrum.

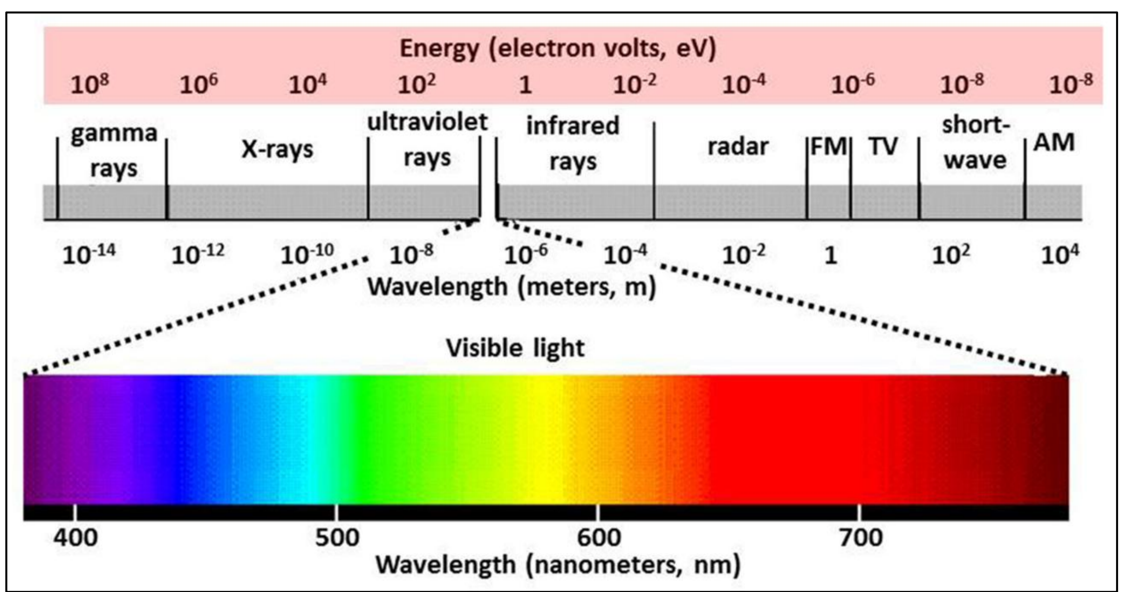


Figure 24: *electromagnetic spectrum*

What is a Spectrum?

A spectrum is the image obtained by decomposing light using a prism or a diffraction grating. Spectrum = a graph representing absorption, emission, or fluorescence as a function of wavelength.

- **Continuous spectrum** – a continuous band of wavelengths (e.g., sunlight).
- **Discontinuous spectrum:**
  - **Line spectrum** – produced by atoms.
  - **Band spectrum** – produced by molecules.

The energy of radiation is related to the aforementioned quantities by Planck's fundamental relation.

- **Corpuscular Nature:** The wave nature of light alone cannot explain the phenomena of interaction between light and matter.
- Planck and later Einstein proposed the quantum theory: **"Light is composed of energy grains: photons."**
- A photon is a particle that propagates at the speed of light and possesses a quantum of energy: $E = h\nu$, where **h** is Planck's constant.

2.3 Principles of emission and absorption

Energy exchanges between matter and radiation can only occur in discrete quanta: $\Delta E = h\nu$. Four fundamental processes underlie spectroscopic phenomena: absorption, spontaneous emission, stimulated emission (as in lasers), and scattering.

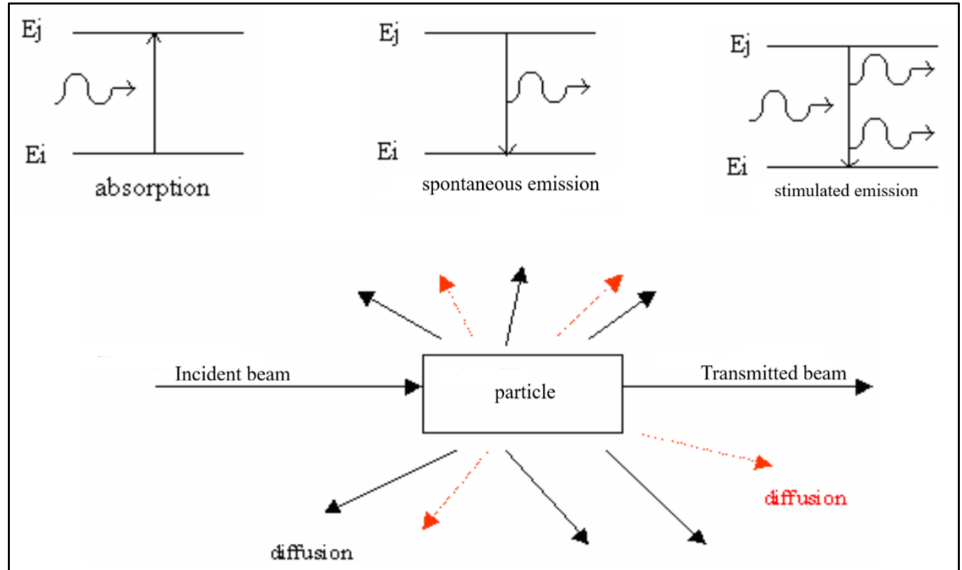


Figure 25: *wave-particle interaction*

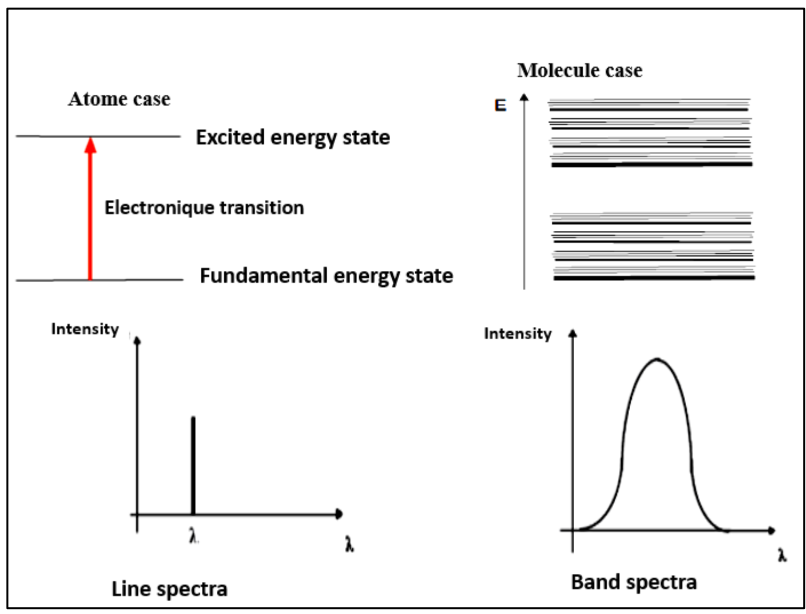


Figure 26: *excitation and spectrum*

a. Emission and Absorption : absorption of a certain amount of energy followed by the process of de-excitation, accompanied by the emission of a photon, so there is a relation between frequency domains and excitation process.

Energy can be exchanged between matter and radiation in two ways:

Emission: Under certain conditions, matter can emit radiation. This is the case, for example, with all light sources: the sun, incandescent bulbs, flames, fluorescent tubes, fireflies, etc.

Absorption: The energy of radiation can be absorbed by matter. Examples include the warming of an object in the sun, the absorption of X-rays by dense parts of our body, and the phenomenon of color. This absorption can have chemical effects by triggering chemical reactions.

Tableau 4: *excitation process relation with frenquency*

Frequency Domain	Excitation Process
X-Rays (RX)	Inner electrons
Ultraviolet (UV) domain	Valence electrons
Visible domain	Valence electrons
Infrared (IR) domain	Bond vibrations
Microwave (μW) domain	Rotation
Television (TV) waves/ Radio waves	Nuclear spins

b. Emission Line Spectrum

A hot gas emitting specific colors only emits at certain wavelengths, creating luminous bands. This pattern serves as the unique signature of a chemical element.

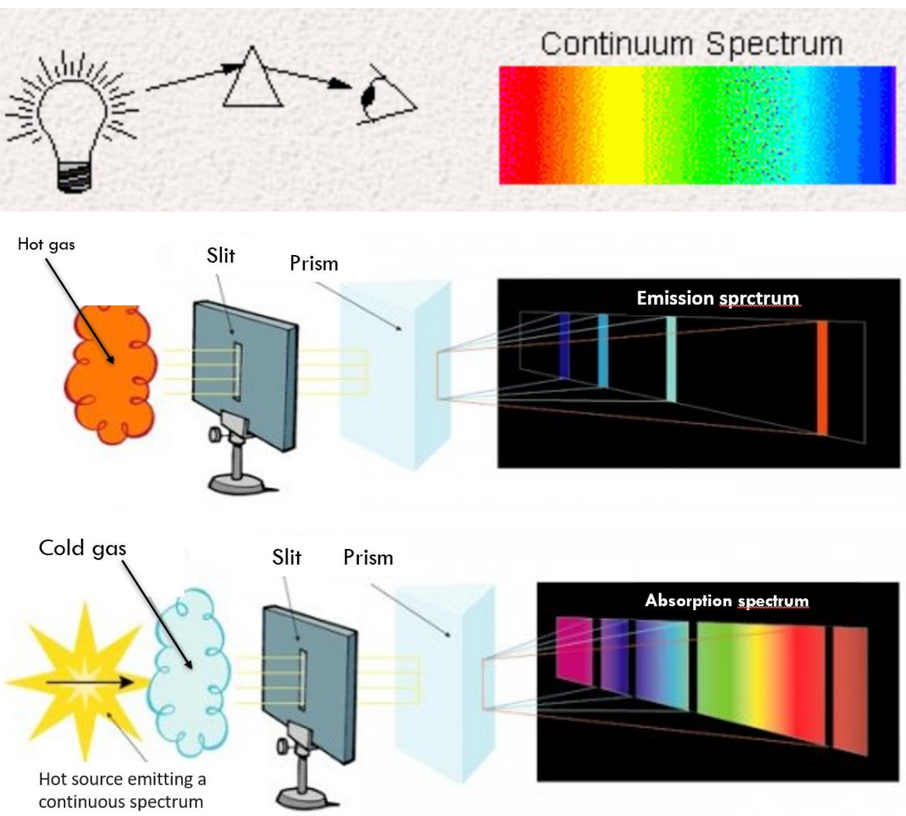


Figure 27: *continuum, emission and absorption spectra*

c. Absorption Line Spectrum

In this case, white light passes through a gas. Upon exiting, certain wavelengths have been absorbed, resulting in the appearance of dark lines in the spectrum. For a given chemical element, the missing lines in this absorption spectrum correspond exactly to the lines present in the emission line spectrum of that same element.

2.4 Excitation and atomization sources

Excitation and atomization sources are responsible for preparing a sample for analysis by converting it into free atoms or molecules and promoting them to higher energy states. Common sources include flames (e.g., air-acetylene), which thermally atomize samples for atomic absorption; electrical discharges (e.g., arcs, sparks), used to vaporize and excite solid samples; and plasmas (e.g., Inductively Coupled Plasma or ICP), which provide extremely high temperatures for efficient atomization and excitation in emission spectroscopy. For molecular techniques, sources like hollow cathode lamps (for AA) or lasers provide specific, intense wavelengths to excite electronic or vibrational states, with the choice of source depending on the required energy, sample type, and analytical technique.

2.5 Instrumentation and measurement

Instrumentation

A basic spectroscopic instrument consists of several key components: a stable radiation source (e.g., deuterium lamp for UV, Nernst glower for IR), an atomizer/excitation source (as above), a wavelength selector (such as a prism, diffraction grating, or interferometer) to disperse light, a sample compartment, and a detector (e.g., photomultiplier tube, charge-coupled device - CCD) to convert light intensity into an electrical signal. In modern instruments like FT-IR spectrometers or ICP-OES systems, these components are integrated with sophisticated optics and computers to control the experiment, scan wavelengths, and process the raw spectral data.

Measurement

The core measurement in spectroscopy is the quantification of how radiation interacts with the sample—typically how much light is **absorbed, emitted, or scattered** at specific wavelengths. The detector's signal is processed and related to the analyte's concentration via fundamental laws like the **Beer-Lambert Law** for absorption. The process involves calibration with standards, background correction, and signal averaging to improve accuracy. The final output is a **spectrum** (a plot of intensity vs. wavelength, wavenumber, or frequency), which serves as a unique fingerprint for qualitative identification, while the intensity at a selected wavelength provides the basis for quantitative analysis.

2.6 Absorption spectrometric analyses

2.6.1 UV/Visible absorption spectrometry

Absorption spectroscopy in the UV and visible range is based on the property of molecules to absorb light radiation at specific wavelengths. "UV/Vis" refers to a portion of the spectral range extending from the near ultraviolet to the very near infrared, i.e., between 180 and 1100

nm. It also includes radiation detectable by the human eye.

This spectral range provides little structural information, but it has many applications in quantitative analysis.

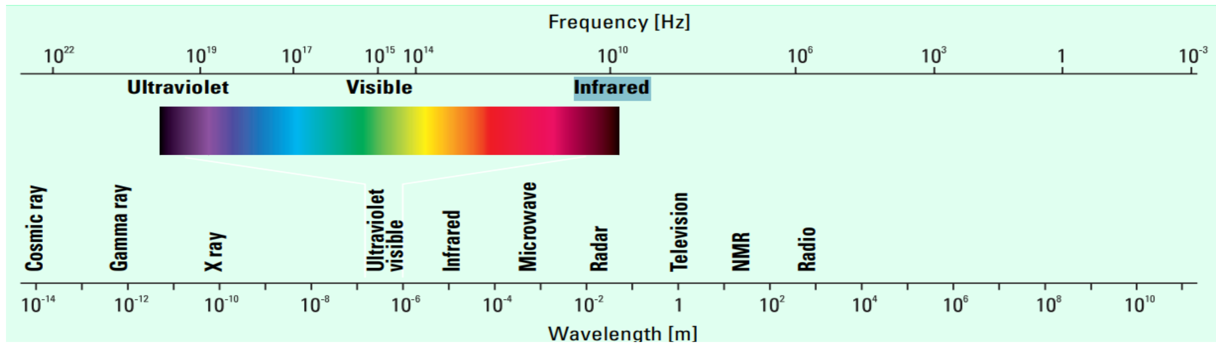


Figure 28: *UV/visible wave length*

The UV-Visible range extends approximately from 180 to 1100 nm.

- Very near infrared: 800 - 1100 nm.
- Visible: 400 - 800 nm.
- Near-UV: 200 - 400 nm.
- Far-UV: 10 - 200 nm.

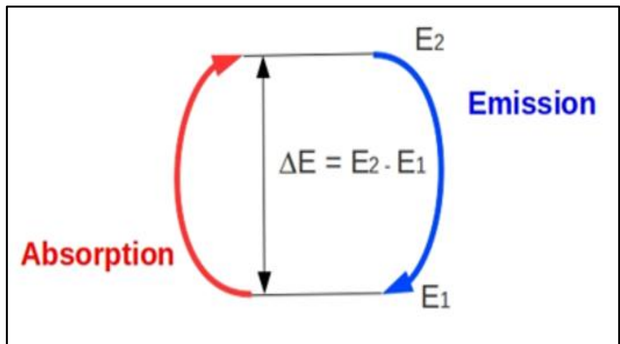


Figure 29: *absorption and emission process*

In this context of spectroscopy, the principle of **color complementarity**, often illustrated by models like the **Ostwald star**, provides a direct visual and analytical link between the color a substance exhibits and the specific wavelengths of light it absorbs. When a molecule absorbs light in a particular region of the visible spectrum (e.g., blue light around 450 nm), the human eye perceives the complementary color (in this case, orange-yellow). This observed color is the result of the transmitted or reflected light—the wavelengths that were *not* absorbed. Therefore, a substance's color serves as a qualitative indicator of its absorption spectrum. In quantitative UV-Vis spectroscopy, this relationship is formalized by the **Beer-Lambert law**, which states that the absorbance at a given wavelength is directly proportional to the concentration of the absorbing species. Thus, understanding complementarity helps bridge the gap between a simple

visual observation and precise photometric measurement, allowing chemists to select the optimal wavelength for analysis based on the sample's perceived color.

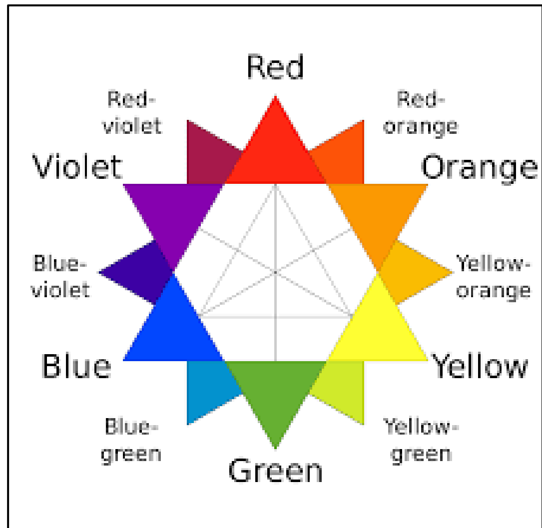


Figure 30: *Ostwald star and color complementarity*

2.6.2. UV/Visible spectroscopy

The operating principle of a spectrometer is relatively simple. A beam of light from a visible and/or UV light source is separated into its individual wavelength components by a prism or diffraction grating. Each monochromatic beam (single wavelength) is then split into two beams of equal intensity by a half-mirror device. The **sample beam** passes through a small transparent container (cuvette) holding a solution of the compound being studied in a transparent solvent. The other beam, the **reference beam**, passes through an identical cuvette containing only the solvent. The intensities of these light beams are then measured by electronic detectors and compared. The intensity of the reference beam is defined as (**I_0**). The intensity of the sample beam is defined as (**I**).

- If the sample does **not** absorb light at a given wavelength, then **$I = I_0$**.
- However, if the compound in the sample absorbs light, then **I is less than I_0**, and this difference can be plotted on a graph against the wavelength.

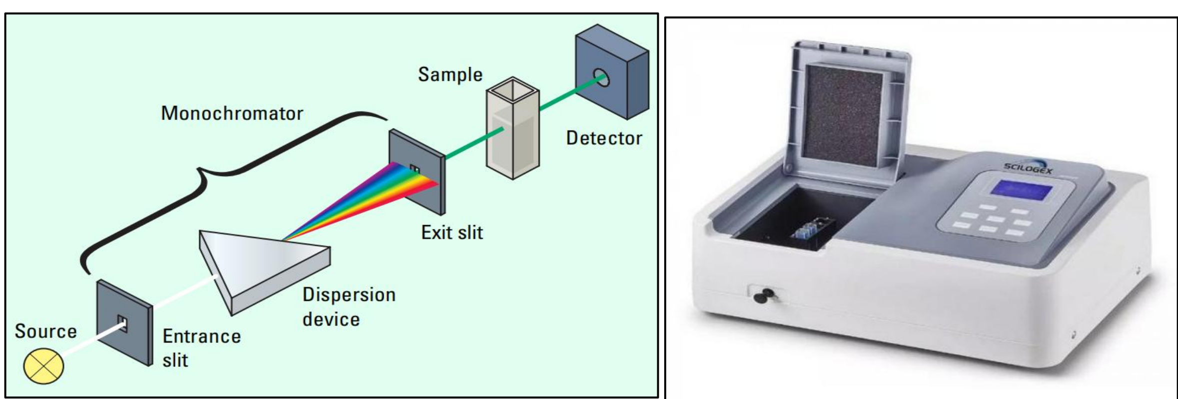


Figure 31:*UV/Visible spectrophotometer*

a. The components of a UV/Visisble spectrophotometer

A standard UV-visible spectrometer is composed of several key components that work in sequence to measure the absorption of light by a sample. The process begins with a **radiation source**, typically a combination of a deuterium lamp (for the UV range) and a tungsten or halogen lamp (for the visible range), which emits a broad spectrum of light. This polychromatic light then passes through a **monochromator** (containing a prism or, more commonly, a diffraction grating), which disperses the light and allows the selection of a specific, narrow wavelength band. In a classic **double-beam** design, this monochromatic beam is split by a beam splitter into two paths: one passes through the **sample compartment** containing the analyte in a transparent cuvette, while the other passes through a reference cuvette containing only the solvent. Both beams are then directed onto a **detector**, such as a photomultiplier tube (PMT) or a photodiode, which converts the light intensity into an electrical signal. Finally, a **signal processor and data system** (computer) compares the two signals, calculates the absorbance using the Beer-Lambert law ($A = \log (I_0/I)$), and outputs the result as a spectrum (absorbance vs. wavelength) for analysis. This integrated setup ensures precise and stable quantitative measurements.

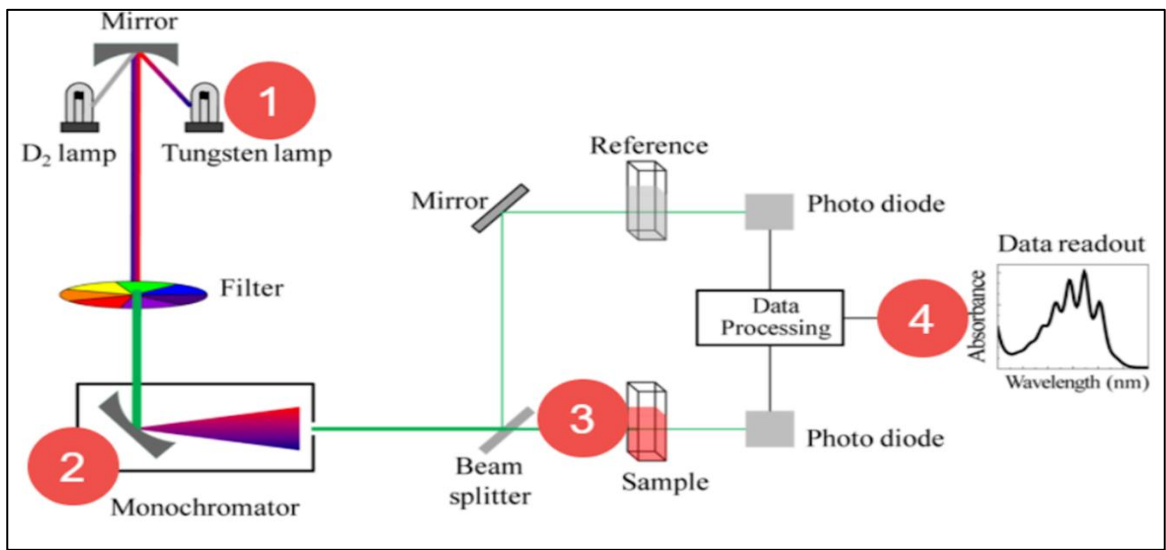


Figure 32: *schematic spectrophotometer*

- **Sample cuvette considerations across the spectrum:**

- **Far Ultraviolet (Vacuum UV):** For wavelengths between 10 - 200 nm (energy: 1200 to 600 kJ·mol^{-1}). In this region, atmospheric oxygen (O_2) and carbon dioxide (CO_2) absorb radiation, as do glass and quartz. Measurements require special apparatus and must be performed in a nitrogen atmosphere. Consequently, this type of spectroscopy is not commonly used in routine analysis.

- **Near Ultraviolet:** For wavelengths between 200 and 400 nm (energy: 600 to 300 $kJ \cdot mol^{-1}$). At these wavelengths, glass absorbs light, but air and quartz are transparent.
- **Visible:** For wavelengths between 400 and 800 nm. At these wavelengths, glass no longer absorbs, and this region corresponds roughly to the radiation detectable by the human eye.

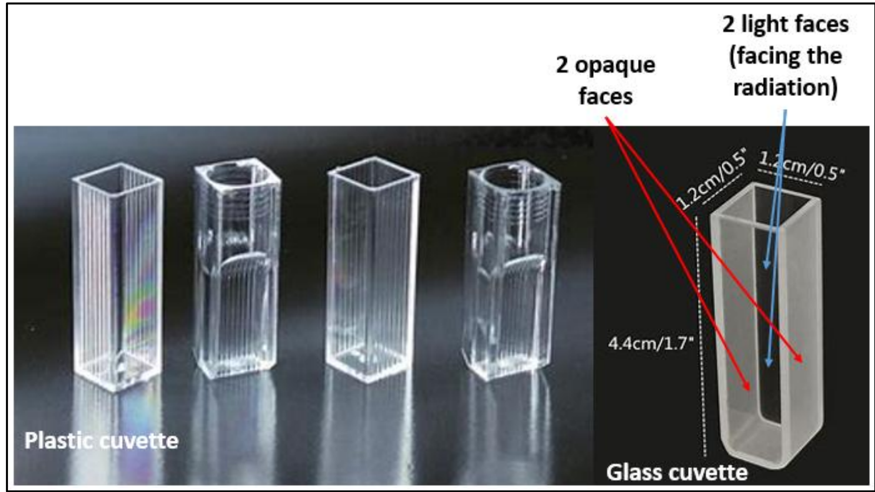


Figure 33: *cuvette types*

d. Spectrophotometer categories :

The single-beam spectrophotometer: The light beam passes through only one cuvette at a time. This type of setup requires frequent zero absorbance adjustments. This adjustment must be performed for each wavelength (because the energy emitted by the source, as well as the amount of light absorbed by the solvent, vary with wavelength). (figure 30)

The double-beam spectrophotometer: The light beam alternately illuminates the measurement and reference cuvettes by means of a rotating mirror.

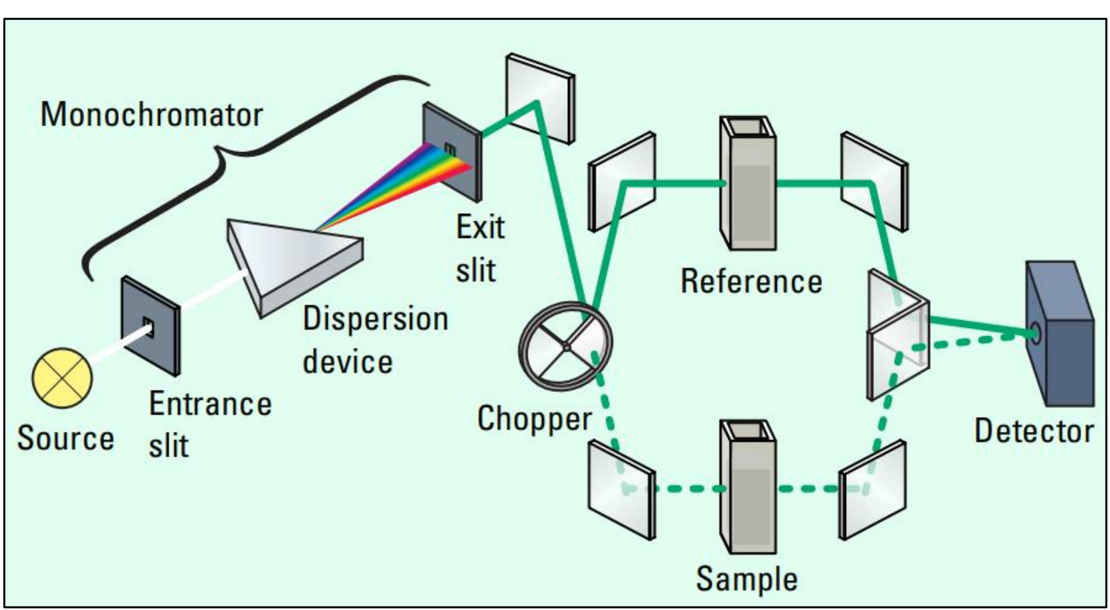


Figure 34: *double beam spectrometer*

e. **Applications**

- **The maximum absorption wavelength** (λ_max) is the specific wavelength within the UV or visible spectrum at which a given molecule or substance absorbs light most strongly. It serves as a unique identifier or "spectral fingerprint" for a compound, as the electronic transitions within its structure are highly specific. In quantitative analysis using UV-Vis spectroscopy, measurements are taken at λ_max to achieve the highest sensitivity and accuracy, as dictated by the Beer-Lambert law, which relates absorbance to concentration. This wavelength is intrinsically linked to the color we perceive; a substance absorbing strongly in the blue region (around 450 nm) will appear orange-yellow, demonstrating the principle of color complementarity. Determining λ_max is therefore the essential first step in both identifying an unknown compound and precisely measuring its amount in solution.

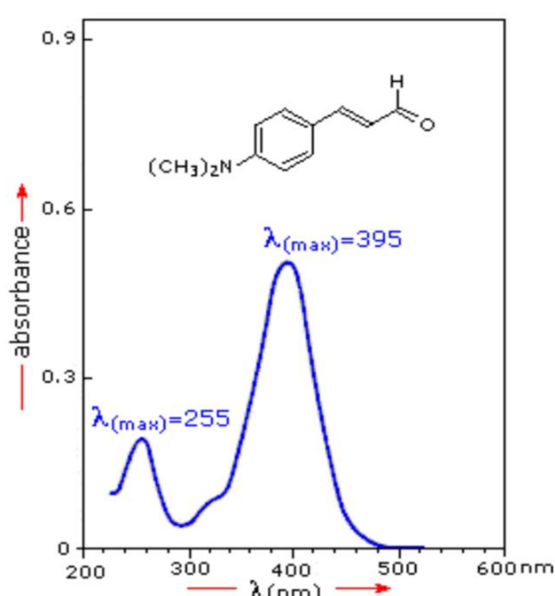


Figure 35: *maximum absorption wavelength*

If the maximum absorbance corresponds to a wavelength within the ultraviolet region (200–400 nm), then the substance appears colorless to the eye.
If λ_max falls within the visible region (400–800 nm), then the chemical species exhibits the complementary color of the wavelength corresponding to λmax.

Absorption can be expressed as:

Transmittance : $T = I/I_0$ or $\%T = (I/I_0) \times 100$

Absorbance : $A = -\log(T) = \log(I_0/I)$

- If no absorption occurs, $T = 1.0$ and $A = 0$.
- Most spectrometers display absorbance on the vertical axis, and the typical observed range for A is from 0 (100% transmission) to 2 (1% transmittance).

A **transparent medium** corresponds to $T = 100\%$ (or $A = 0$), and an **opaque medium** corresponds to $T = 0$.

- **Beer-Lambert Law**

- In 1729, **P. Bouguer** showed that the fraction of light absorbed in a medium depends on the path length traveled (**L** or **l**).
- **Lambert** later rediscovered this fact, which is mathematically expressed as:

$$I = I_0 \cdot \exp(-k' \cdot l)$$

- In 1852, **August Beer** demonstrated that the variation in intensity also depends on concentration:

$$I = I_0 \cdot \exp(-k \cdot l \cdot c)$$

This allowed UV/VIS spectroscopy to be used as a quantitative analytical method. Their combined principle is known as the **Beer-Lambert law**.

$$A = -\log(T) = \log I_0 / I = \varepsilon \, L \, C$$

A: Absorbance (formerly called optical density, O.D.), a dimensionless quantity.

ε: The molar extinction coefficient (or molar absorption coefficient) if concentration is expressed in mol/L (unit: $L \cdot mol^{-1} \cdot cm^{-1}$); or the mass absorption coefficient if concentration is expressed in g/L (unit: $L \cdot g^{-1} \cdot cm^{-1}$). It depends on the nature of the absorbing species, the wavelength, and the temperature.

l: The path length (cuvette width) in cm.

C: Concentration of the molecule in solution.

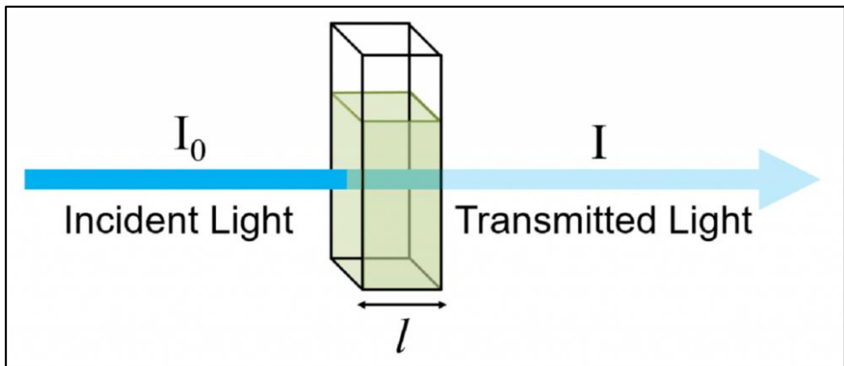


***Figure 36:** transmission of light through a sample solution in cuvette*

Note : The Beer-Lambert Law is additive: For any wavelength, the absorbance of a mixture is equal to the sum of the absorbances of each component of the mixture.

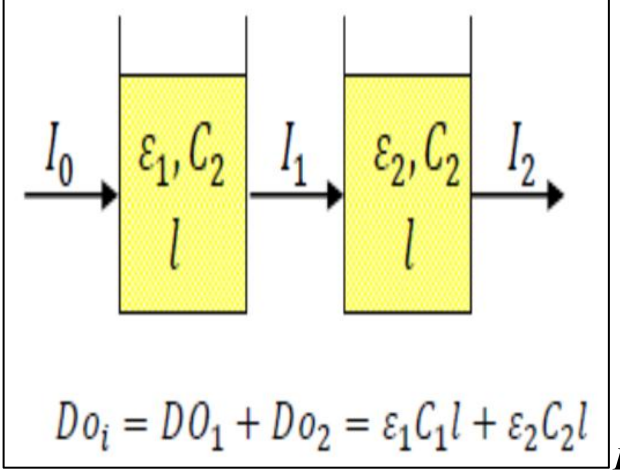


***Figure 37:** additivity of absorbance*

- **Validity Conditions of the Beer-Lambert Law**

This law is only valid if the following conditions are met:

➤ The light used must be **monochromatic**;

➤ Concentrations must be **low**;

➤ The solution must be **neither fluorescent nor heterogeneous**;

➤ The solute must not undergo **photochemical transformations**;

➤ The solute must not form **variable associations with the solvent**.

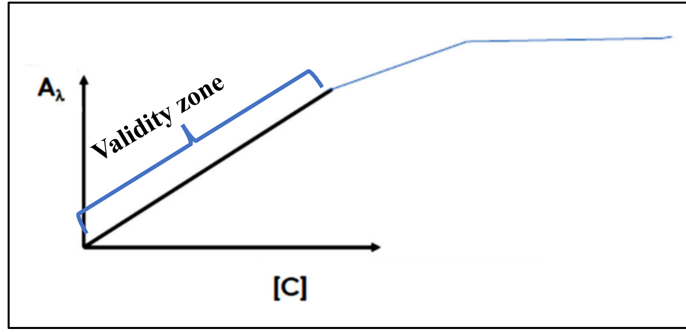


Figure 38: *validity of Beer Lambert law*

- **Parameters influencing absorbance :**

Influence of time: For many colored compounds, absorbance changes over time. When developing an assay, it is necessary to study absorbance variations from the moment the coloration develops: $A = f(T)$.

Influence of temperature: Calibration curves are only valid at a given temperature, and absorbance variations can sometimes be significant depending on temperature.

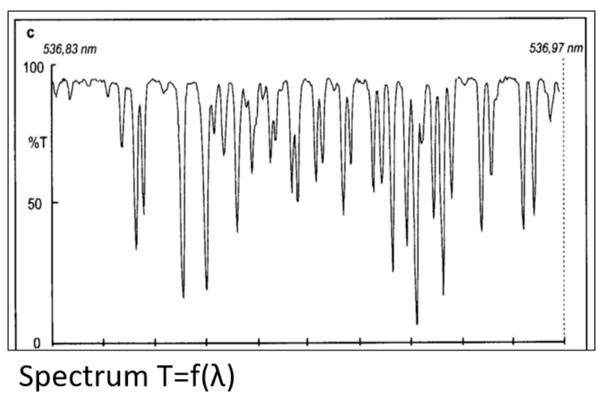
Influence of pH: The pH of the medium in which the solute is dissolved can have a strong effect on the spectrum.

Solvents: The choice is wide (water, ethanol, methanol, hexane, etc.). Only cuvette compatibility and the solvent's own absorption spectrum can be limiting factors in this choice.

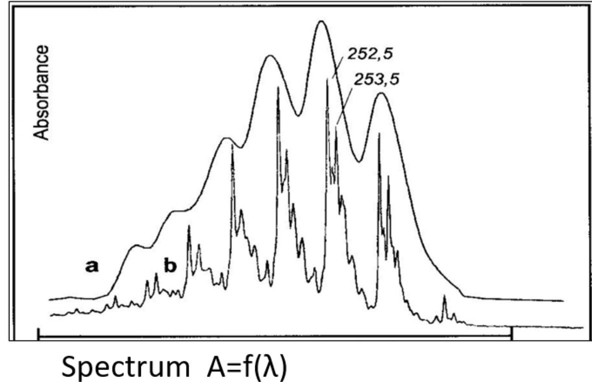
- **Qualitative application :**

Absorption in the UV/visible domain is due to the transition from one electronic energy level to another, higher one, along with changes in vibrational and rotational levels. During this process, an electron moves from one molecular orbital to another of higher energy.

UV/Visible spectrometers allow for obtaining the spectrum of analyzed compounds in the form of a plot of **transmittance** or **absorbance** as a function of **wavelength**, which is marked on the x-axis.



Spectrum T=f(λ)



Spectrum A=f(λ)

Figure 39: *UV/Visible spectra*

- **Electronic Transitions of Organic Compounds**

Organic compounds constitute the majority of studies conducted in UV/visible spectroscopy. The observed transitions originate from electrons in **σ or π bonds** and from **non-bonding electron pairs (n electrons)** on atoms such as H, C, N, and O.

Electronic transitions involve the promotion of electrons from filled, low-energy bonding molecular orbitals (MOs) to higher-energy, unfilled (vacant) antibonding molecular orbitals (MOs*).

- **The nature of an absorption band is related to the molecular orbitals (MOs) involved and the molar absorption coefficient ε ($L \cdot mol^{-1} \cdot cm^{-1}$) calculated at λ_max.** Molecular orbitals (MOs), constructed from atomic orbitals (AOs) *s* and *p*, are of different types:

- **σ (sigma)** – constructed from AOs with axial overlap;
- **π (pi)** – constructed from AOs with lateral overlap;
- **n** – non-bonding MOs;
- **σ*** – antibonding MOs;
- **π*** – antibonding MOs.
- Non-bonding orbitals correspond to the lone electron pairs (lone pairs) of atoms such as O, N, S, and Cl.

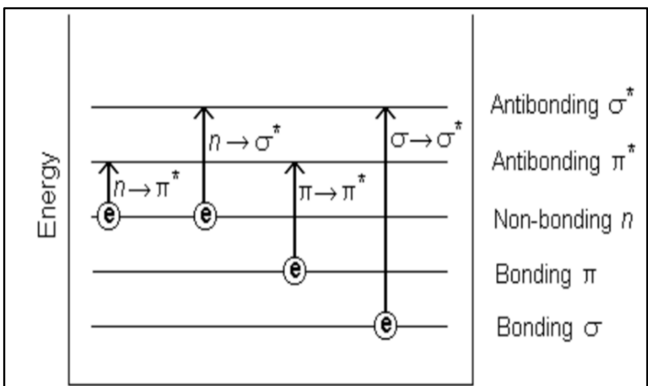


Figure 40: *origin of absorption*

- **Electronic transition :**

In UV-Visible spectroscopy, organic compounds absorb light when electrons are promoted from ground-state molecular orbitals to higher-energy excited-state orbitals. The most relevant transitions involve **σ (sigma)**, **π (pi)**, and **n (non-bonding)** electrons. Here is a breakdown of the primary electronic transitions observed:

. σ → σ Transition*

- **Energy Required:** Very High
- **Typical Wavelength:** < 150 nm (Vacuum/Far UV)
- **Molar Absorptivity (ε):** Often high, but rarely measured
- **Description:** Involves electrons in **single bonds** (C–C, C–H). The energy required is so high that it falls outside the standard UV-Vis range. These transitions are typically only studied with specialized equipment under vacuum.
- **Example:** Alkanes like methane or ethane.

. n → σ Transition*

- **Energy Required:** High
- **Typical Wavelength:** 150–250 nm
- **Molar Absorptivity (ε):** Low to moderate (10–100 $L \cdot mol^{-1} \cdot cm^{-1}$)
- **Description:** Involves the promotion of a **non-bonding electron** (lone pair) from an atom like O, N, or S to an antibonding sigma orbital. Often observed as a weak, broad band. Can be obscured by more intense transitions.
- **Example:** Saturated compounds with heteroatoms (e.g., water, methanol, amines like CH_3NH_2).

. π → π Transition*

- **Energy Required:** Moderate to Low (depends on conjugation)
- **Typical Wavelength:** Varies widely:
    - Isolated π bond: ~160–180 nm
    - Conjugated dienes: ~220–250 nm
    - Aromatic systems: ~250–280 nm
    - Extended polyenes/conjugation: >300 nm (visible region)
- **Molar Absorptivity (ε): Very High** (10,000–100,000+ $L \cdot mol^{-1} \cdot cm^{-1}$)
- **Description:** The most important and intense transition for analysis. Involves electrons in **π bonds** (C=C, C=O). The energy gap decreases dramatically with **conjugation**, causing a **bathochromic shift** (red shift) to longer wavelengths. This is why carotenes (highly conjugated) are colored.

- **Example:** Ethene (isolated π), butadiene (conjugated), benzene, beta-carotene.

. n → π Transition*

- **Energy Required:** Low
- **Typical Wavelength:** 270–350 nm
- **Molar Absorptivity (ε): Very Low** (10–100 $L \cdot mol^{-1} \cdot cm^{-1}$)
- **Description:** Involves promoting a **non-bonding electron** (lone pair) to an antibonding π orbital. This is a **symmetry-forbidden** transition, resulting in weak absorption bands. Sensitive to solvent polarity: a **hypsochromic shift** (blue shift) occurs in polar protic solvents due to hydrogen bonding stabilizing the n orbital.
- **Example:** Carbonyl compounds like acetone (λ_max ~280 nm, ε ~15), aldehydes, ketones.

- **Exemple of Chromophore group:** An unsaturated covalent group responsible for light absorption. Examples: C=C, C=O, C=N, C≡C, C≡N, etc. A **chromophore** is the light-absorbing part of a photopigment.
- The photopigment is **retinal** (a form of vitamin A). Upon **light absorption**, the retinal changes its structure, causing it to detach from the opsin protein (Opsin is the protein component of a photoreceptor pigment). This chemical change triggers the visual response.
- **Auxochrome group:** A saturated group (a group of ionizable atoms) that, by its effect, modifies the absorption of a chromophore group. Consequently, it can change the frequency, and therefore the absorption wavelength, of this chromophore. These auxochrome groups increase electron delocalization, thereby altering absorption energies and molar extinction coefficients. Example: OH, NH_2, Cl.
- **Bathochromic effect:** Shift of absorption bands toward longer wavelengths.
- **Hypsochromic effect:** Shift of absorption bands toward shorter wavelengths.
- **Hyperchromic effect:** Increase in absorption intensity.
- **Hypochromic effect:** Decrease in absorption intensity.

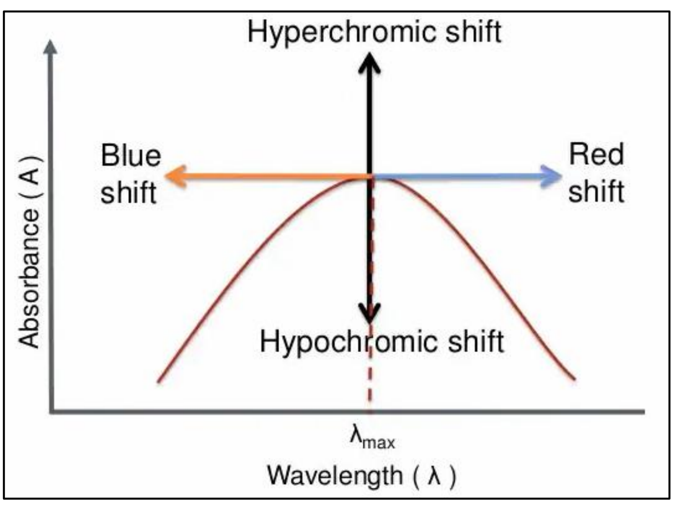


Figure 41: *shifts of absorption maximum*

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2.6.3 IR absorption spectrophotometry

Infrared radiation (IR) is defined as electromagnetic radiation of the same nature as visible light. However, its wavelengths are too long to be detected by the human eye. Ranging from 800 nm to 1 mm, they are longer than those of visible light and shorter than those in the microwave region. (figure 27)

Infrared spectroscopy is one of the most widely used spectroscopic tools for characterizing molecules.

Infrared (IR) spectroscopy studies molecular vibrations when molecules are irradiated by electromagnetic waves within the infrared domain: approximately 0.8 to 1000 µm. This spectral region is divided into:

- **Near-IR:** 0.8 – 2.5 µm / 13,300 – 4,000 cm^{-1}
- **Mid-IR:** 2.5 – 25 µm / 4,000 – 400 cm^{-1}
- **Far-IR:** 25 – 1000 µm / 400 – 10 cm^{-1}

a. Principle : The success of this technique lies in its rapid characterization capability and its sensitivity to existing molecules.

The basic principles of IR spectroscopy are very similar to those governing UV-visible spectroscopy. The difference lies in the energy levels involved in this technique: IR spectroscopy deals with molecular vibrational energies.

b. Molecular vibrations

Diatomic molecules (H–H, H–Cl, C=O, etc.) vibrate in only one way: they move closer together and farther apart, as if connected by a spring. This is called the **valence (stretching) vibration**. A diatomic molecule can therefore be represented as two masses (m_A and m_B) connected by a spring with a force constant **k** and a length **r**, which stretches and compresses at a certain frequency **ν**. The mathematical model used is that of a **harmonic oscillator**.

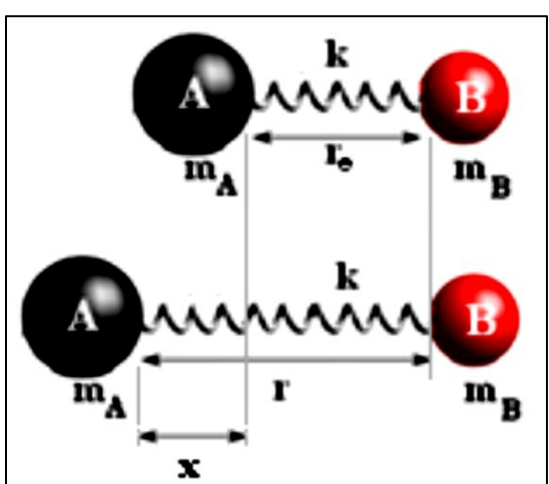

Figure 42: *schematic diatomic molecule*

Within the **harmonic oscillator approximation**, the fundamental vibrational frequency **ν** is given by the relation (Hooke's law):

$$\nu = \frac{1}{2\pi}\left(\frac{k}{\mu}\right)^{1/2} \qquad \mu = \frac{m_A \quad m_B}{m_A + m_B}$$

This frequency depends on k and μ :

Effect of k: The vibration frequency is proportional to the force constant k.

Effect of μ: The vibration frequency is inversely proportional to the reduced mass μ.

c. Vibration types :

The absorption of infrared (IR) radiation causes a molecule to vibrate by altering the angles and lengths of its bonds. There are two main modes of vibration:

- **Stretching vibration** : which corresponds to the extension of a bond A–B, denoted as ν(A–B), they occur when two atoms periodically move closer together or farther apart along their shared axis. These vibrations take place at high wavenumbers. There are two types of stretching vibration:
  - **Symmetrical stretching**, denoted as ν_s
  - **Asymmetrical stretching**, denoted as ν_{as}
- **Bending (or deformation) vibration**, which corresponds to a change in a bond angle, denoted as δ(A–B). These vibrations are observed at low wavenumbers. Different types of bending vibrations are possible: **in-plane** and **out-of-plane** :

- **Scissoring** (shearing): a vibration in which the two bonds move in opposite directions around an axis.
- **Rocking**: a vibration in which the two bonds move simultaneously in the same direction around an axis. These two modes are referred to as **in-plane**, because the plane of symmetry is maintained.
- **Wagging**: a vibration in which the bonds move simultaneously in the same direction, varying angles BAC and BAD, **out-of-plane**.
- **Twisting**: a vibration in which the bonds move in opposite directions, varying angles BAC and BAD.

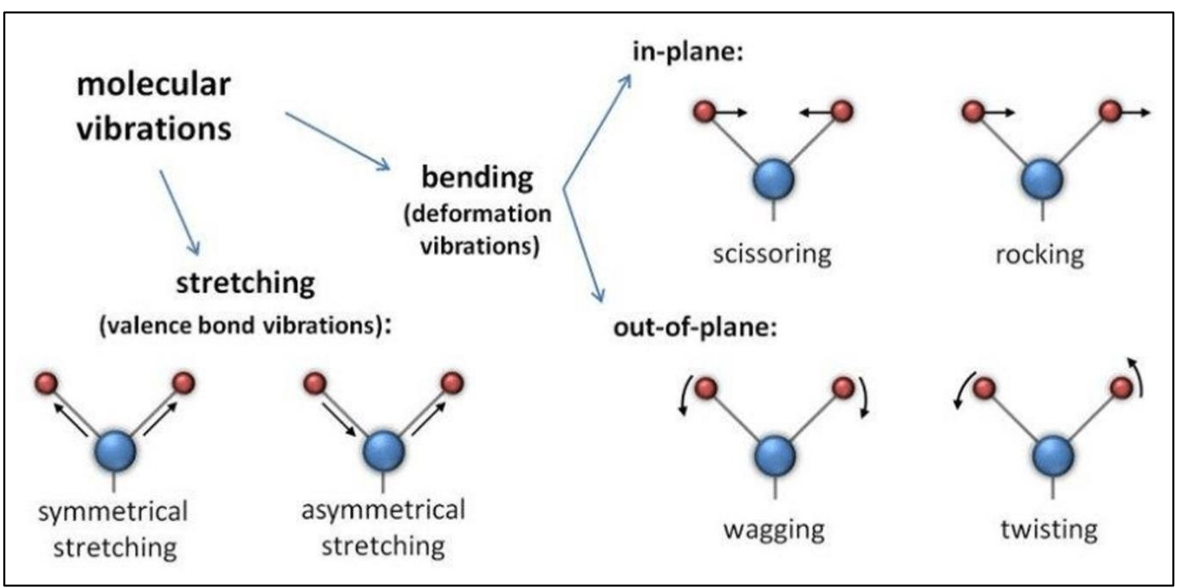

Figure 43: *molecular vibrations*

d. Instrumentation

There are two main types of infrared spectrometers: **scanning** and **Fourier transform spectrometers**.

A scanning infrared spectrometer is the classic design, similar to the spectrophotometers used in UV-visible spectroscopy. A Fourier transform infrared (FTIR) spectrometer is fundamentally identical in purpose to a scanning spectrometer, but the dispersive system is replaced by an interferometer (typically a Michelson interferometer) whose position is precisely adjusted using a laser.

Both types of spectrometers are composed of the following essential elements:

- A **source** of infrared radiation.
- A compartment for the **sample**.
- A **dispersive system** (a monochromator in a scanning instrument) or an **interferometer** (in an FTIR).
- A **detector**.

e. Description of the operation of an IR spectrometer

The choice of source depends on the infrared region one wishes to work in. In most cases, work is carried out in the region called the mid-infrared, between 4000 and 400 cm^{-1}.

The polychromatic IR radiation from the source is first split into two equivalent beams. One beam is focused on a reference cell and the other on a cell containing the sample. The latter then passes through the sample compartment and, via a rotating sector mirror, is recombined with the reference beam.

The monochromator most commonly consists of a grating capable of dispersing the incident radiation into its various wavelengths. This grating rotates continuously to focus each wavelength one after another onto a detector.

This detector senses temperature variations and converts them into intensity variations. This difference in intensity then easily yields the transmittance (T), which is usually expressed as a percentage.

The intensity of the beam reaching the detector is recorded as an interferogram. This interferogram is then processed by Fourier Transform—a mathematical process that decomposes a complex, time-dependent signal into a sum of simple signals of known frequencies.

Overall, for both types of spectrometers, the sources and detectors can be the same.

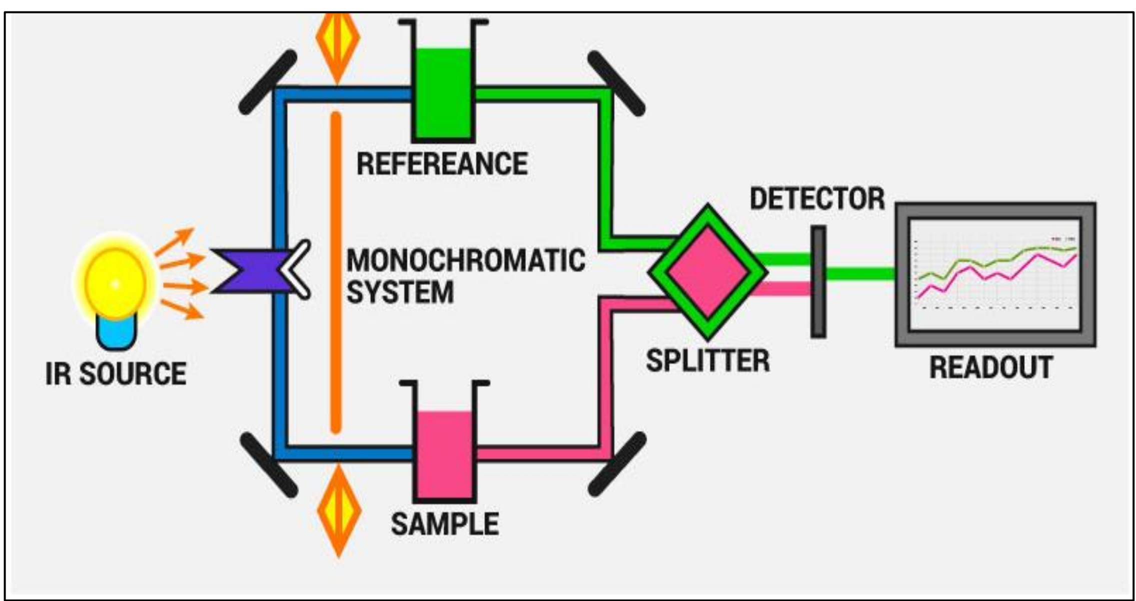

Figure 44: *Infrared spectroscopy*

f. Sample Preparation:

The sample can be solid, liquid, or gas. Depending on its state, different techniques are used: KBr pellets, liquid cells, or depositing a drop of liquid between two KBr (potassium bromide) plates.

For Solids:

A homogeneous mixture of KBr containing about 1% of the sample is finely ground, placed in a mold, and subjected to very high pressure. It is then removed from the mold in the form of a very thin pellet.

For Liquids:

Non-viscous, non-volatile liquids are introduced into a sealed cell of a specific pathlength. Liquids can also be deposited between two KBr plates.

For Gases:

Gases are introduced into a cell with a larger volume than those used for liquids.

g. Infrared spectroscopy results :

For infrared spectra, the following parameters are represented:

- **Transmittance T**, or the intensity of light transmitted by the analyzed sample, on the y-axis (expressed as a percentage).
- **Wavenumber σ** on the x-axis, is the reciprocal of wavelength λ ($\sigma = 1/\lambda$), it is generally expressed in cm^{-1}**.**

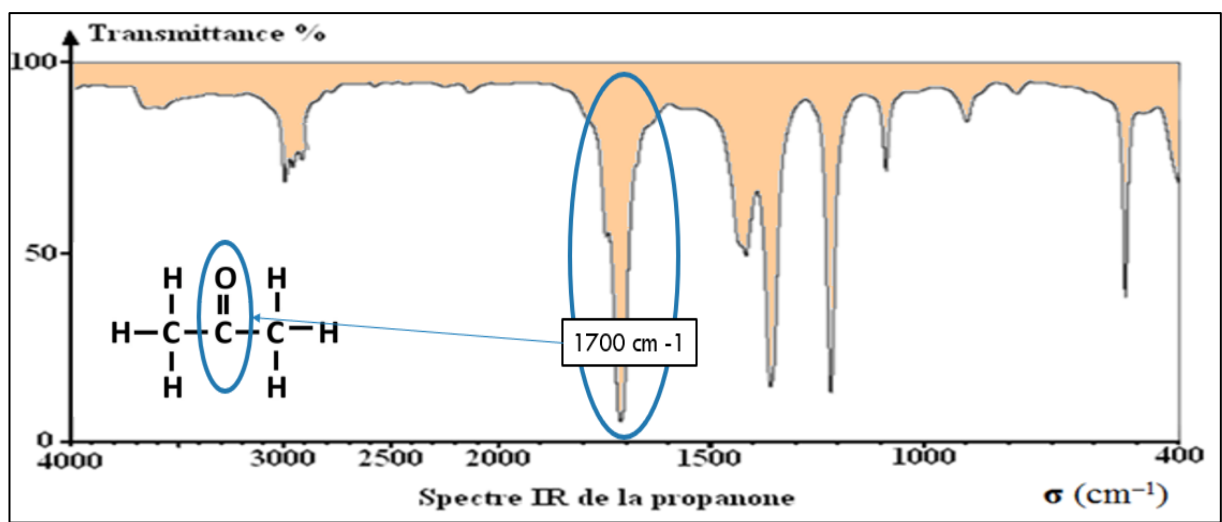

Figure 45: *propanone infrared spectrum*

Infrared Spectra and characteristic bond absorptions

- **The region from 4000 to 1300 cm⁻¹**

  This is called the **functional group region**. Characteristic stretching bands of functional groups appear in this domain, such as O-H (~3500 cm^{-1}), C=O (~1700 cm^{-1}), etc.

- **The region from 900 to 650 cm⁻¹**

  This region characterizes the **molecular framework**. Aromatic compounds show intense bands in this area.

- **The intermediate region from 1300 to 900 cm⁻¹**

  This is called the **fingerprint region**. Absorption in this zone is very complex, arising from combined vibrational interactions. It is unique for each molecular species and serves as a molecular "fingerprint."

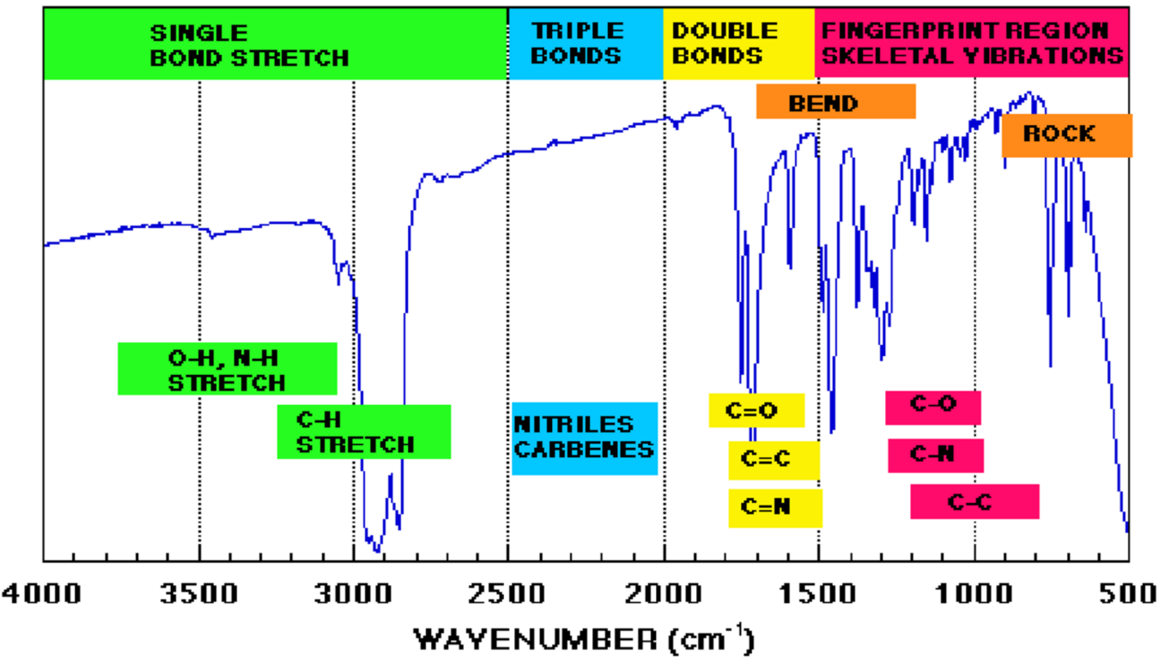

Figure 46: *characteristic bond regions in IR spectrum*

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2.7 Emission spectrometric analyses

Atomic emission is the release of electromagnetic radiation resulting from the de-excitation of atoms or molecules that have been excited by a sufficient supply of energy.

Example:

- If common table salt (sodium chloride) is introduced into a natural gas flame, a characteristic yellow sodium emission appears.
- A potassium salt will produce a characteristic orange emission.

The atomization and subsequent excitation of atoms in a sample to be analyzed are achieved by transfer to very high temperatures.

Using a flame or plasma, where the temperature is extremely high, the objective is to break down molecular structures and convert the element to be analyzed (at least partially) into an atomic vapor. Under the effect of these elevated temperatures, some of the atoms will become excited, causing their electrons to move to higher energy levels.

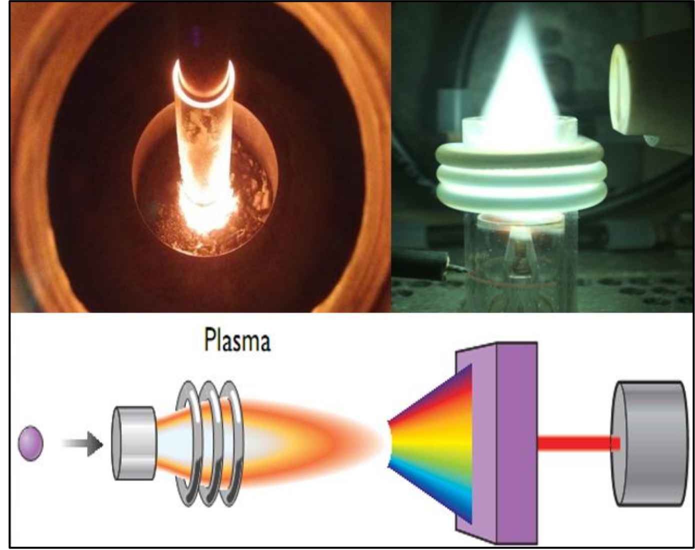

Figure 47: plasmas

Figure 48: flame

The role of the flame or plasma in optical emission analysis is:

- to break molecular bonds,
- to produce free ions and atoms,
- and to excite these particles.

2.7.1 Spectrophotometry by electrical excitation

refers to analytical techniques, primarily in Atomic Emission Spectroscopy (AES), where atoms are excited using electrical energy rather than thermal energy from a flame. In this method, a sample is introduced into a high-energy electrical source—such as an electric arc, spark, or inductively coupled plasma (ICP). This intense electrical field vaporizes the sample into its constituent atoms and provides sufficient energy to promote their electrons to higher, unstable energy states. As these excited electrons relax back to their ground state, they emit photons at wavelengths characteristic of each element. The emitted light is then dispersed by a monochromator or polychromator and detected, allowing for the simultaneous qualitative identification and quantitative measurement of multiple elements in the sample. This technique is highly sensitive and is widely used for trace metal analysis in materials science, environmental monitoring, and metallurgy.

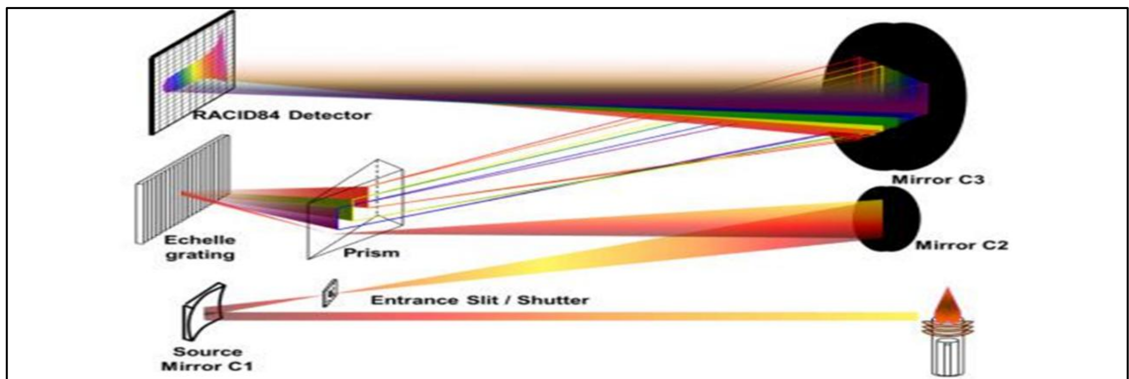

Figure 49: *plasma source emission spectrometry*

2.7.2 Spectrophotometry by flame excitation

is a foundational technique in atomic spectroscopy, most notably employed in Flame Atomic Emission Spectroscopy (FAES) and Flame Atomic Absorption Spectroscopy (FAAS). In this method, a liquid sample is first converted into a fine aerosol using a nebulizer. This mist is then introduced into a controlled, high-temperature flame (typically fueled by air-acetylene or nitrous oxide-acetylene mixtures). Within the flame, the solvent evaporates, and the analyte compounds are broken down into free, gaseous atoms in their ground state. For emission analysis, the thermal energy of the flame itself is sufficient to excite a fraction of these atoms. As they return to lower energy levels, they emit photons at their characteristic wavelengths. This emitted light is passed through a monochromator to isolate the specific wavelength and

measured by a detector. The intensity of the emission is directly proportional to the concentration of the element in the sample. While less energetic than plasma-based electrical excitation, flame excitation remains a robust, relatively simple, and cost-effective method for the routine determination of alkali and alkaline earth metals (like sodium, potassium, and calcium) in clinical, environmental, and agricultural samples.

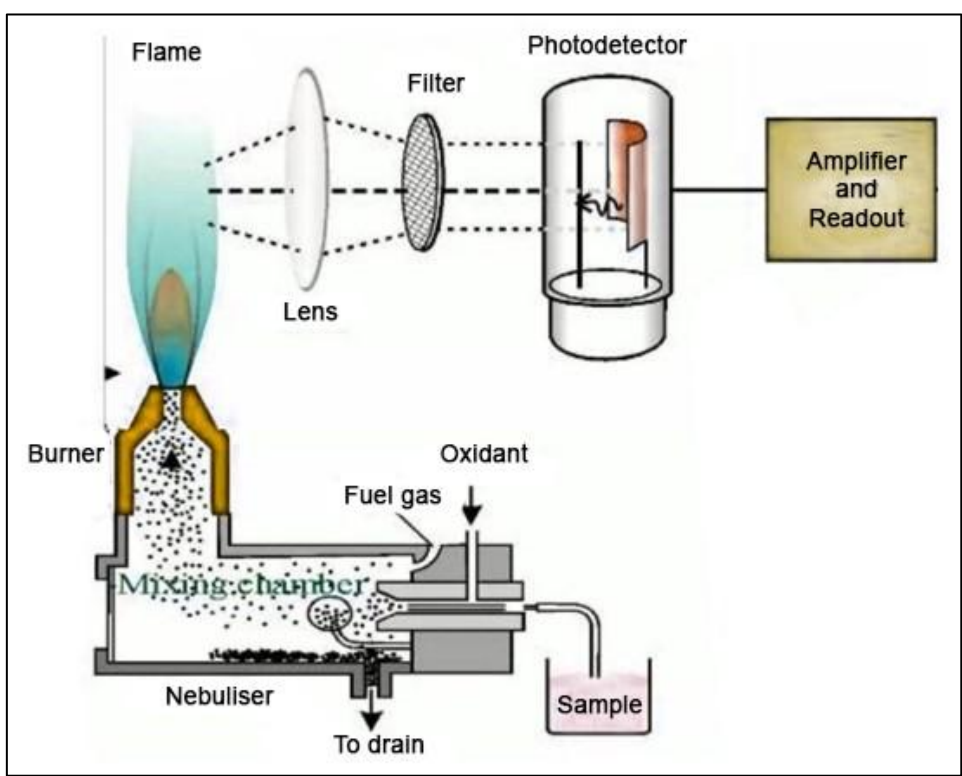

Figure 50: *spectrometry flame excitation*

2.7.3 Fluorimetric assays

are highly sensitive analytical techniques that measure the intensity of light emitted by a molecule (fluorescence) after it has absorbed light at a specific wavelength. The principle relies on a molecule's fluorophore—a structure that can be excited by photons and subsequently re-emits light at a longer, lower-energy wavelength upon returning to its ground state, a phenomenon known as the Stokes shift. Instrumentally, a fluorimeter directs an excitation beam from a powerful source (such as a xenon lamp or LED) through a primary monochromator to select the optimal excitation wavelength. The emitted fluorescence, typically at a 90° angle to the excitation path to avoid interference from the source light, passes through a second monochromator before being detected by a photomultiplier tube (PMT). This perpendicular geometry, combined with measurement of emission rather than absorption, grants fluorimetry exceptional sensitivity and specificity, often detecting compounds at concentrations 100–1000 times lower than UV-Vis absorption methods. In biochemistry, fluorimetry is indispensable for

quantitative assays of compounds with native fluorescence (e.g., NADH, aromatic amino acids) and for applications using fluorescent dyes or tags in immunoassays, DNA sequencing, enzyme activity studies, and live-cell imaging.

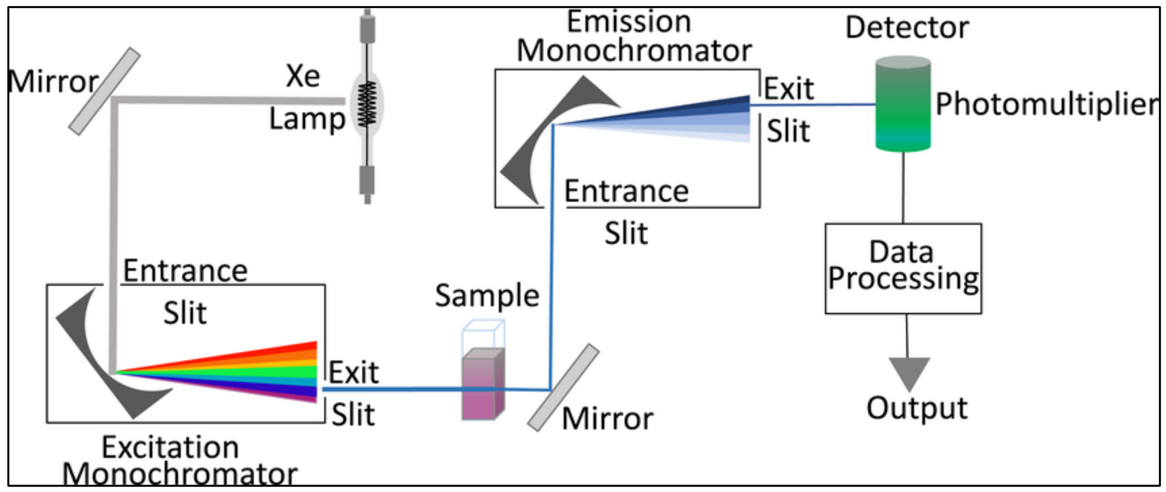

Figure 51: *fluorescence spectrophotometry*

2.8 Atomic absorption spectrometry (AAS)

is a highly sensitive and selective analytical technique used to determine the concentration of specific metallic and metalloid elements in a sample. It is based on the absorption of light by free, ground-state atoms in the gaseous phase.

AAS is a quantitative spectro-analytical method that measures the amount of light absorbed at a characteristic wavelength by free atoms generated from the sample. The degree of absorption is directly proportional to the concentration of the target element in the sample.

a. Principle

The core principle relies on the **Beer-Lambert Law** and the unique electronic transitions of atoms:

- **Atomization:** The sample is converted into a cloud of free, ground-state atoms, typically using a flame or an electrically heated graphite furnace.
- **Resonance Radiation:** A hollow cathode lamp (HCL) emits light at a very specific wavelength that corresponds to the energy needed to excite an electron from the ground state to a higher energy level in the target element.
- **Absorption:** As this precisely tuned light beam passes through the cloud of atoms, the ground-state atoms absorb photons, causing their electrons to become excited. This reduces the intensity of the transmitted light.
- **Quantification:** The amount of light absorbed is measured. Since each element has unique resonance wavelengths, the technique is highly selective. The absorbance is

proportional to the concentration of the element in the sample, allowing for precise quantitative analysis.

Formula: $A = \varepsilon \cdot l \cdot c$

(where A is absorbance, ε is the molar absorptivity, l is the path length through the atom cloud, and c is the concentration of the absorbing atoms).

b. Instrumentation

A typical AAS instrument consists of the following key components, operating in sequence:

- Radiation Source (Hollow Cathode Lamp - HCL): Emits a **narrow, intense beam of light** specific to the element being analyzed. The cathode is made of or coated with the pure element of interest.

- Sample Introduction & Atomization System:

* **Nebulizer:** Converts the liquid sample into a fine aerosol.

* **Atomizer:** The central component that produces free atoms.

* **Flame AAS (FAAS):** Uses a premix burner and a flame (air-acetylene or nitrous oxide-acetylene) to thermally atomize the sample. Robust but requires larger sample volumes.

* **Electrothermal AAS (ETAAS or Graphite Furnace AAS, GFAAS):** Uses a small graphite tube heated electrically to very high temperatures in a programmed sequence (drying, ashing, atomization). It is far more sensitive and requires only microliter sample volumes.

- Monochromator: A diffraction grating or prism that isolates the **specific analytical wavelength** emitted by the HCL from other background emissions (e.g., from the flame or furnace).

- Detector: Usually a **photomultiplier tube (PMT)** that converts the transmitted light intensity into an electrical signal.

- Signal Processor & Readout System: An amplifier and computer system that processes the detector's signal, calculates absorbance ($A = \log(I_0/I)$), and displays the result as a concentration reading, often using a calibration curve.

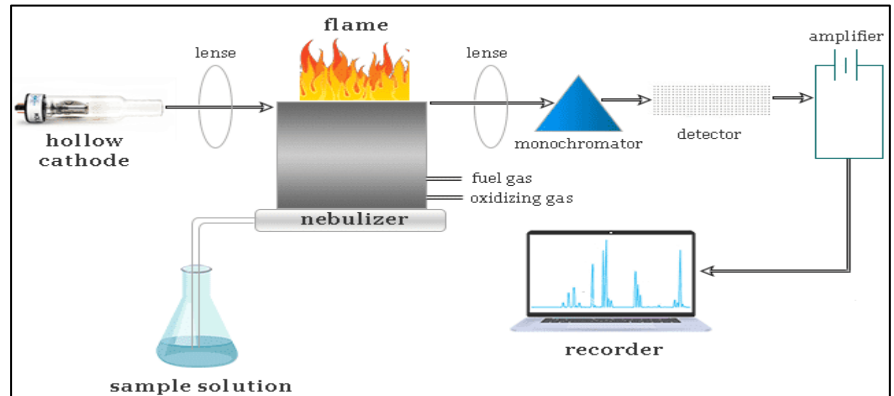

***Figure 52:** AAS*

- **Application domains :**

UV-Vis spectroscopy is indispensable in biochemistry and pharmaceuticals for quantifying nucleic acids (A260) and proteins (A280), monitoring enzyme kinetics, and ensuring drug purity.

IR spectroscopy serves as a structural elucidation tool in organic chemistry and material science, identifying functional groups, characterizing polymers, and detecting pollutants.

Atomic spectroscopy (AAS, AES, ICP) is the cornerstone of trace metal analysis in environmental monitoring (soil/water testing), clinical toxicology (blood lead levels), food safety, and metallurgy.

Fluorimetry, with its exceptional sensitivity, enables advanced applications such as DNA sequencing, immunoassays (ELISA), cellular imaging, and real-time PCR. Together, these methods provide the foundation for qualitative identification, quantitative measurement, and dynamic process monitoring in research, quality control, and diagnostic laboratories worldwide.

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Conclusion

The module **"Biochemical Analysis Methods and Techniques I"** provides the indispensable foundation for any aspiring biochemist or life science researcher. By journeying from basic sample preparation to advanced instrumental analysis, it equips the student with the most relevent infromations about analytical methods.

The path to discovery begins with separation and purification: isolating the molecule of interest from the complex matrix of life using centrifugation, dialysis, filtration…. Then the course focuses on how spectroscopy (UV-Vis, IR, Atomic) acts as the primary sense, allowing to identify molecules by their interaction with light and quantify them with precision.

Ultimately, this module teaches a fundamental principle: the choice of technique is dictated by the biological question. Whether monitoring a reaction kinetics, purifying an enzyme, or quantifying a trace metal, the student now possess the conceptual map to navigate the vast landscape of bioanalytical tools. This knowledge is the critical link between theoretical biochemistry and impactful laboratory work, forming the core skill set for innovation in research, diagnostics, and biotechnology

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Annexes

1/ Glossary of Key Terms

A

- **Absorbance (A):** A dimensionless measure of the amount of light absorbed by a sample. Calculated as $A = \log_{10}(I_0/I)$, where I_0 is incident light intensity and I is transmitted light intensity.
- **Absorption Spectrum:** A plot of absorbance (or transmittance) of light by a substance as a function of wavelength or frequency.
- **Adsorption:** A surface phenomenon where molecules (solutes, ions) adhere to the surface of a solid material (like a filter) via physical or chemical interactions.
- **Atomic Absorption Spectrometry (AAS):** An analytical technique for determining the concentration of specific metallic elements by measuring the absorption of light by free, ground-state atoms in a gaseous phase.
- **Auxochrome:** A functional group attached to a chromophore that modifies its light absorption properties (e.g., shifts the wavelength, increases intensity).

B

- **Bathochromic Shift (Red Shift):** A shift of an absorption band to a longer wavelength.
- **Beer-Lambert Law:** The fundamental law of absorption spectroscopy stating that absorbance is directly proportional to the concentration of the absorbing species and the path length: $A = \varepsilon \cdot l \cdot c$.
- **Bending Vibration:** In IR spectroscopy, a molecular vibration that involves a change in bond angle rather than bond length (e.g., scissoring, rocking).

C

- **Centrifugation:** A separation technique that uses centrifugal force to separate components of a mixture based on their density, size, or shape.
- **Chromatography:** (Mentioned as future topic) A set of separation techniques used to separate mixtures based on differential partitioning between a mobile phase and a stationary phase.
- **Chromophore:** A part of a molecule responsible for its color, absorbing light in the UV-Visible region due to electronic transitions (e.g., C=C, C=O).
- **Clogging (Fouling):** The reduction in filtration efficiency and flow rate due to the accumulation of particles within or on the surface of a filter medium.
- **Cuvette:** A small, transparent container (often made of quartz, glass, or plastic) used to hold liquid samples in spectroscopic analysis.

D

- **Dialysis:** A separation technique that uses a semi-permeable membrane to remove small molecules (salts, impurities) from a solution containing larger molecules (proteins, DNA) based on differential diffusion rates.
- **Differential Centrifugation:** A centrifugation method where a homogenate is centrifuged at progressively higher speeds to separate cellular components (nuclei, mitochondria, ribosomes) based on their sedimentation rates.

E

- **Electrodialysis:** A dialysis technique that uses an electric field to accelerate the removal of charged ions through ion-selective membranes.
- **Emission Spectrum:** A plot of the intensity of light emitted by an excited substance as a function of wavelength. It serves as a unique fingerprint for elements.
- **Extinction Coefficient (ε):** A measure of how strongly a chemical species absorbs light at a given wavelength. It is used in the Beer-Lambert law (units: $L \cdot mol^{-1} \cdot cm^{-1}$).

F

- **Filtration:** A physical separation process that removes solid particles from a fluid by passing it through a porous medium (filter).
- **Fluorimetry:** A highly sensitive spectroscopic technique that measures the intensity of fluorescence emitted by a molecule after it absorbs light.
- **Fourier Transform Infrared (FTIR) Spectrometer:** A type of IR spectrometer that uses an interferometer and Fourier Transform mathematics to obtain spectra, offering higher speed and sensitivity compared to dispersive instruments.
- **Fractionation:** A general process for separating a mixture into its individual components or fractions based on differences in their properties.

H

- **Hollow Cathode Lamp (HCL):** The radiation source in Atomic Absorption Spectrometry (AAS), emitting a specific, narrow wavelength characteristic of the element being analyzed.
- **Hydrophobic Interaction:** A non-covalent interaction between non-polar molecules or groups that aggregate in an aqueous environment to minimize contact with water. Influences protein precipitation.
- **Hyperchromic Effect:** An increase in the intensity of an absorption band.
- **Hypochromic Effect:** A decrease in the intensity of an absorption band.
- **Hypsochromic Shift (Blue Shift):** A shift of an absorption band to a shorter wavelength.

I

- **Infrared (IR) Spectroscopy:** A spectroscopic technique that studies molecular vibrations induced by the absorption of infrared radiation. Used for identifying functional groups and characterizing molecular structure.
- **Isopycnic Centrifugation (Equilibrium Centrifugation):** A centrifugation method where particles separate based on their buoyant density in a density gradient, migrating until they reach a zone where their density equals that of the medium.

L

- **λmax (Lambda max):** The specific wavelength at which a substance exhibits its maximum absorbance.
- **Lyophilization (Freeze-Drying):** A dehydration process where a sample is first frozen and then placed under a vacuum to allow the frozen solvent (usually water) to sublime directly from the solid to the gas phase.

M

- **Molar Absorptivity:** See *Extinction Coefficient*.
- **Monochromator:** An optical device (using a prism or diffraction grating) that selects a narrow band of wavelengths from a broader light source.

N

- **Nebulizer:** A device used in flame AAS to convert a liquid sample into a fine aerosol mist for introduction into the flame.

P

- **Protein Precipitation (Salting Out):** A purification technique where high concentrations of salt (e.g., ammonium sulfate) are added to a protein solution to reduce protein solubility, causing them to aggregate and precipitate.

R

- **Relative Centrifugal Force (RCF):** The actual separation force exerted on a sample in a centrifuge rotor, expressed in multiples of gravitational force (× g). It is more meaningful than rotor speed (RPM) as it accounts for rotor radius.
- **Rotor:** The rotating unit of a centrifuge that holds the sample tubes. Types include fixed-angle, swinging-bucket, and vertical rotors.

S

- **Salting Out:** See *Protein Precipitation*.
- **Sedimentation:** The process by which particles settle out of a suspension under the influence of gravity or centrifugal force.
- **Sedimentation Coefficient (s):** A measure of a particle's sedimentation rate in a centrifugal field, expressed in Svedberg units (S).
- **Spectrophotometer:** An instrument that measures the intensity of light as a function of its wavelength. Used in UV-Vis, IR, and fluorescence spectroscopy.
- **Spectroscopy:** The study of the interaction between electromagnetic radiation and matter, used to identify, quantify, and characterize substances.
- **Stokes' Law:** Describes the frictional force (drag) on a spherical particle moving through a viscous fluid. Used in calculations of sedimentation velocity.
- **Stretching Vibration:** In IR spectroscopy, a molecular vibration where the distance between two atoms increases and decreases, changing the bond length.

T

- **Transmittance (T):** The fraction of incident light that passes through a sample. $T = I / I_0$, often expressed as a percentage (%T).

U

- **Ultracentrifugation:** Centrifugation at very high speeds (typically > 50,000 RPM) to separate very small particles like ribosomes, viruses, or macromolecules.
- **Ultrafiltration:** A pressure-driven filtration technique using membranes with very small pores to separate macromolecules from solvents and smaller solutes based on size.
- **UV-Visible (UV-Vis) Spectroscopy:** A spectroscopic technique that measures the absorption of ultraviolet and visible light by molecules, primarily used for quantitative analysis and studying electronic transitions.

V

- **Vibrational Transition:** A change in the vibrational energy state of a molecule, induced by the absorption of infrared radiation.

2/ Pratical works :

- **PWn°1 : Filtration**

This is a widely used mechanical method in the fields of agri-food, pharmaceuticals, chemistry, etc.

A technique for separating the phases of a mixture containing:

- A liquid or gaseous phase
- And a solid phase,

by passage through a porous element (porous membrane) which constitutes the filter. It retains the solid phase called the **retentate**, while allowing the purified liquid called the **filtrate** to flow freely.

Principle:

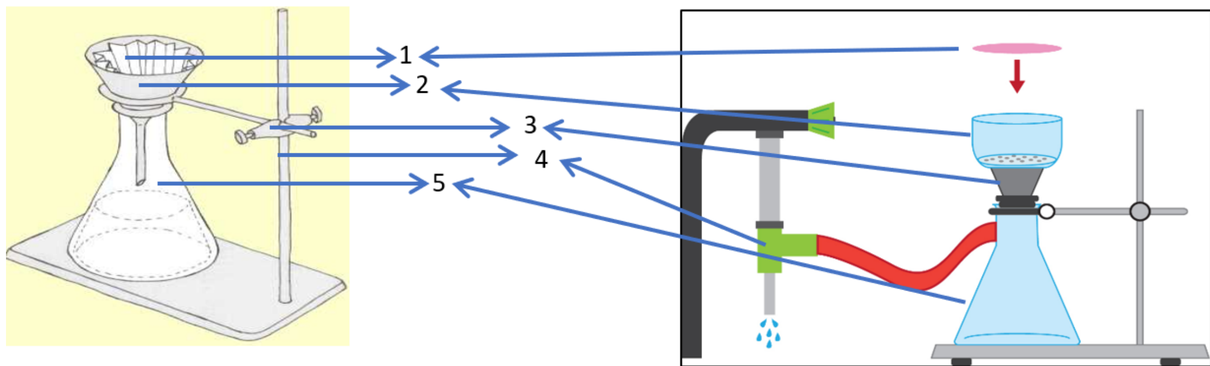
It is based on the size difference between the particles to be separated and the porosity of the filter medium. The pressure difference forces the liquid to pass through the filter while the solid particles remain on the surface.

Filtration Equipment:

Filters, funnels, Erlenmeyer flasks, glass rod, vacuum pump (Buchner flask), water aspirator, Buchner funnel, rubber adapter, vacuum tubing, syringe, syringe filter (tip filter).

Procedure:

- In two beakers, mix 10 ml of NaOH solution and 10 ml of $CuSO_4$ solution. What is the nature of each separate solution and of the mixture?
- Set up the two filtration devices: gravity filtration and vacuum filtration. Weigh the filters before starting the manipulation.
- Perform the filtration test. What do you observe?
- Dry the obtained residues and weigh them. Conclude.
- Label the devices used.



Gravity Filtration **Pressure Filtration**

- Filter the colored solution using a tip filter. Compare the starting solution and the obtained filtrate. What do you observe?
- Read the pore diameter of the filter. What conclusion can you draw about the molecules retained by the filter?

- **PWn°2 : Solution Preparation / Differential Centrifugation and Sucrose Density Gradient Centrifugation**

Part I: Solution Preparation

In chemistry, a solution is a homogeneous mixture resulting from the dissolution of one or more solute(s) (dissolved chemical species) in a solvent.

Molar concentration (M) indicates the amount of solute substance in one liter of solvent. It is the ratio of the amount of solute substance (n), expressed in moles, to the volume (V) of the solution. It is expressed in $mol.L^{-1}$.
M = n / V

Mass concentration (Cm) corresponds to the ratio of the mass of the dissolved solute to the total volume of the solution. Cm = m / V

Concentration expressed as a percentage is a measure of the amount of solute dissolved in 100 units of solution, calculated as volume percentage (% v/v) or mass percentage (% m/m) of a solution.

In a **dilution** operation, the amount of substance does not change, only the volume varies.

Materials Used:

Sucrose powder, Balance; weighing boat; spatula; volumetric flasks; stoppers for flasks; graduated pipettes and pipettor; micropipettes, beakers.

Preparation of Sucrose Solutions:

- Prepare 100 ml of a 60% and a 40% (m/v) sucrose solution.
- **Calculate the molar concentrations of the prepared solutions??**
- Prepare 50 ml of daughter solutions at 3%, 10%, and 25% sucrose by dilution from the 60% solution.
- **How do you proceed?**
- Label and store the solutions.

Preparation of Plant Extracts:

- Cut the samples (basil leaf, carrot, and tomato) into pieces and grind each in a mortar, first dry, then add a few milliliters of 3% sucrose.
- Filter using a compress and collect the extracts.
- Label and store the extracts.
- **What is the purpose of grinding and solubilization in the 3% sucrose solution??**

Part II: Centrifugation

Centrifugation is a separation technique that allows recovery of a precipitate (pellet) + a supernatant. The apparatus that performs centrifugation is called a centrifuge. Centrifugation allows the separation of constituents of varying size, mass, and density contained in a liquid.

Differential Centrifugation: Technique applied to the separation of organelles having significantly different and high sedimentation constants. It is the application of centrifugation forces of increasing intensity which allows the separation of organelles of smaller and smaller size and/or density that sediment at the bottom of the tube as a pellet, during successive centrifugations.

Materials: Centrifuge + Centrifuge tubes, 2 ml Pipettes + pipettor, micropipette.

Experiment:

1. Grind a few basil leaves with a few ml of 3% sucrose.
2. Start the first centrifugation: 800 rpm for 5 min.
3. Recover the supernatant (1) in another tube.
4. Start the second centrifugation of supernatant (1): 3000 rpm for 5 min.
5. Recover the supernatant (2).
6. Start the third centrifugation of supernatant (2): 10000 rpm for 5 min.

- Compare the pellets and supernatants recovered at each centrifugation?

Density Gradient Centrifugation:

1. Prepare a density gradient in three centrifuge tubes using the sucrose solutions: 10%, 25%, 40%, and 60%, proceeding as follows:
2. Place 1.5 ml of 60% sucrose in each tube.
3. Carefully add 1.5 ml of 40% sucrose along the tube walls (avoid agitation to ensure two distinct phases visible to the naked eye).
4. Add 1.5 ml of 25% sucrose (avoid agitation to ensure three distinct phases visible to the naked eye).
5. Add 1.5 ml of 10% sucrose (avoid agitation to ensure four distinct phases visible to the naked eye).
6. Add 0.5 ml of each extract (basil leaf, carrot, and tomato extract) to each tube.
7. Start centrifugation at 5000 rpm for 10 min, ensuring balance between the tubes.
8. After centrifugation, recover the tubes. **Describe the results obtained?**

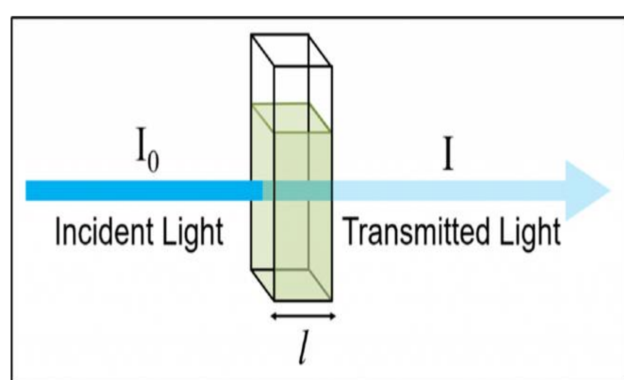
- **PW n°3 : Molecular Absorption Spectrophotometry (UV-Visible)**
- **Determination of the Maximum Wavelength of a Colored Compound and Isosbestic Point**

Spectrophotometry is a qualitative and quantitative analysis technique for substances that absorb electromagnetic radiation with wavelengths between 200 and 900 nm using the type of apparatus employed. When a substance absorbs in the visible region (400 nm < λ < 700 nm), the eye perceives only the non-absorbed radiations when looking at this substance, which is why it appears colored, of the complementary color to that of the absorbed radiation.

Beer-Lambert Law

An electromagnetic radiation passing through a medium undergoes several modifications. One part of the beam is reflected, another is absorbed and transformed into heat through interaction with matter, and the remainder passes through the medium subjected to the radiation. These wave-matter interactions are specific to the radiation involved and the chemical element subjected to the radiation.

Let a parallel beam of monochromatic light (of wavelength λ) with intensity I_0 pass through an absorbing solution of concentration C over a cuvette path length of 1 cm. The intensity of the emerging beam is I.



T=I/I0 (0 < T < 100%)

A= -log T= -log I/I0

Beer Lambert law : $A= \varepsilon_{(\lambda)}.l.C$

C: concentration of the solute in the measured solution; l: optical path length (cm); A: absorbance;

$\varepsilon(\lambda)$: molar absorption coefficient (function of the nature of the substance, the wavelength of light λ, the nature of the solvent, and temperature T.

Procedure

An Isosbestic Point corresponds to a wavelength where the molar extinction coefficients are equal for all species in solution.

By working at this wavelength, one can thus more easily estimate solute concentrations.

The $A(\lambda) = f(\lambda)$ curve, which represents absorbance as a function of wavelength, is called the sample's spectrum.

a- Prepare a 100 mg/L solution of a food dye "Sin 110";

b- Prepare 100 mL of a 1 mg/L daughter solution by diluting the stock solution (100 mg/L);

c- Divide the daughter solution into four equal volumes (25 mL) and place them in 50 mL beakers;

d- Adjust the pH of each solution in the beakers to: pH = 2.5, natural (unadjusted), and 10;

e- Each time, place the food dye sample in the spectrophotometer cuvette after rinsing it with the same solution;

f- Perform a wavelength scan from 200 to 600 nm using water as a blank;

Questions

1. On what principle are spectral methods based?
2. What is meant by a monochromatic beam?
3. Draw up the concentration/absorbance table and plot the sample's spectrum; determine the maximum wavelength (λ_{max}) of the food dye.
4. Plot the curves of absorbance variation as a function of wavelength at different pH values and identify the isosbestic point.
5. What is the absorption range of the food dye used? Justify.

- **PWn°4 : Molecular Absorption Spectrophotometry (UV-Visible)**
- **Establishment of a Calibration Curve**

Determination of the Concentration of a Substance in a Sample

Assay is a technique that allows the determination of the molar concentration of a chemical species dissolved in a solution. There are several methods to assay a species in solution; one can perform:

• either a **calibration-based assay**;
• or a **titration assay**.

These techniques are very important in many industries: for example, assay techniques are used in medicine to quantify drugs or in the agri-food industry to measure the amounts of solutes to incorporate into beverages, or to assay different types of biomolecules in plant extracts...

A colorimetric assay corresponds to one that can be performed when a chemical reaction produces a colored product and if the intensity of the coloration is proportional to the concentration of the chemical element to be assayed. Consequently, the reaction is logically based on the Beer-Lambert law, and the concentration of the chemical species affects a physical quantity (such as **absorbance**, conductivity, etc.) that can be measured.

Calibration relies on the use of solutions (called standard solutions) that contain the chemical species to be assayed at different known concentrations.

Part I: Calibration Curve

Procedure: Prepare the Sin 110 dye solutions according to the following table and measure the absorbance:

N° diluted solution	0	1	2	3	4	5	6
V of the diluted solution (ml)	25						
V_0 from the stock solution (500mg/L) (ml)	0	0.5	1	2	5	10	25
V water (ml)							
C dye (mg/L)							
A (λ)							

1. Complete the table.
2. Plot the calibration curve (A = f(C)). Using graph paper. Interpret the curve.
3. Is the Beer-Lambert law valid?
4. In this case, specify the validity interval of the Beer-Lambert law.

Part II: Determination of the Concentration of Sin 110 Dye in Food Samples:

Procedure: Consider the following solutions:

N° of solution	**1**	**2**	**3**	**4**
C (mg/L)				
A (λ)				

1. Complete the table.
2. Describe the method followed to determine the unknown concentration of the samples. (Propose two methods)