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Abdelhamid Ibn Badis University of Mostaganem
Faculty of Natural and Life sciences
Department of food sciences

Cours Handbook
Biochemical Analysis Techniques

Intended for Third-Year Undergraduate Students (L3)

Specialty: Food, Nutrition, and Pathologies

Presented by :
Dr. ALACHAHER Fatima Zohra
Lecturer B

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CHAPI. CHROMATOGRAPHIC METHODS

1. Introduction

General Principle

Biochemical analysis techniques constitute a set of essential tools for the study, identification, and quantification of compounds of biological interest. They are widely used in numerous fields such as analytical biochemistry, molecular biology, microbiology, the pharmaceutical industry, the food industry, and quality control (Pool, 2019).

The analysis of biological systems is hampered by the complexity of the matrices studied, characterized by the coexistence of numerous molecules of varied physicochemical natures, sometimes present at very low concentrations. In this context, separation methods play a crucial role, as they allow for the isolation of analytes prior to their identification and quantification (Snyder *et al.,* 2019).

Among these methods, chromatography has established itself as a reference technique due to its versatility, precision, and wide range of applications. It is based on well-defined physicochemical principles, enabling the efficient separation of the components of a complex mixture, for both analytical and preparative purposes.

Chromatography, a physicochemical analysis method, is a technique used for the separation, identification, and purification of the components of a mixture. The separation of the mixture's components (the solutes) is achieved by dragging a mobile phase (liquid or gas) along a stationary phase (solid or fixed liquid), thanks to the selective (re)partition of the solutes between these two phases. Each solute is therefore subjected to a retention force (exerted by the stationary phase) and a mobility force (due to the mobile phase) (Dong, 2021).

CHAP I. CHROMATOGRAPHIC METHODS

The term "chromatography" was coined by Mikhail Tswett in 1906 to describe a technique for separating plant pigments (chlorophylls and carotenoids) using columns filled with an adsorbent substance (chalk). He named this separation phenomenon chromatography (from the Greek words khrôma, color, and graphein, writing), which he defined as the graphic recording of colors (Kazakevich & Lobrutto).

Chromatography encompasses a set of analytical techniques based on the differential distribution of the components of a mixture between two phases in contact: a stationary phase and a mobile phase. The mobile phase, liquid or gaseous, moves through the stationary phase, carrying the analytes at different speeds depending on their interactions with the two phases. The separation of the compounds results from the establishment of a distribution equilibrium between the stationary and mobile phases. Each component of the mixture migrates in the chromatographic system according to a characteristic time, a function of its induced physico-chemical properties (structure, molecular mass, polarity) and its relative affinity for the stationary phase (Gidding, 2018).

I.2. Distribution coefficient

The distribution of an analyte A between the two phases is expressed by the distribution coefficient (K):

$$K = \frac{C_S}{C_M}$$

where:

Cs represents the concentration of analyte A in the stationary phase,

C_M represents the concentration of analyte A in the mobile phase.

K value reflects the intensity of the interactions between the analyte and the stationary phase.

A high coefficient corresponds to significant retention and slow migration, while a low coefficient indicates a preferential affinity for the mobile phase and rapid elution. Partition Principle and Related Techniques

Chromatography falls within a general framework of separation techniques based on a partitioning phenomenon between two phases. This principle is also used in several physicochemical processes employed in analysis:

• **Chromatography**: partitioning between a stationary phase and a mobile phase

• **Extraction**: separation between two immiscible liquid phases

• **Crystallization:** partitioning between a liquid phase and a crystalline solid phase

• **Distillation**: partitioning between a liquid phase and a gaseous phase

• **Sublimation**: partitioning between a solid phase and a gaseous phase

These methods are based on comparable thermodynamic equilibria, but differ in the nature of the phases involved and their fields of application (Grob & Barry, 2019).

The Role of Chromatography in Biochemical Analysis

In biochemical analysis, chromatographic methods play a central role due to their selectivity, sensitivity, and reproducibility. They are also well-suited for both qualitative and quantitative analysis of simple or complex biological compounds, as well as for purification processes (Tswett, 1906).

Chromatographic principles form the basis of widely used techniques such as thin-layer chromatography, liquid chromatography, and gas chromatography, as well as specialized methods adapted for the analysis of biomolecules (Tswett, 1906).

2. Different Chromatographic Methods

2.1. Classification According to the Nature of the Phases

2.1.1. Stationary Phase (Fixed Phase)

The stationary phase is the fundamental element of the chromatographic system. It can be solid or liquid, and its choice determines the mechanism for separating the components of the mixture.

When the stationary phase is solid, it is generally composed of materials such as silica or alumina, often chemically modified. These supports exhibit adsorptive properties that allow the separation of analytes based on their surface interactions. Solid phases are used either as column packing (gravity chromatography, high-performance liquid chromatography – HPLC) or as a thin layer deposited on glass, aluminum, or plastic plates (thin-layer chromatography).

The stationary phase can also be liquid, immobilized on an inert solid support, or it can be a grafted phase, resulting from the covalent attachment of carbon chains to a mineral support.

For example, in paper chromatography, the stationary phase consists of water adsorbed by the cellulose fibers of the paper. In gas chromatography, the phase

The stationary phase is a low-volatility, thermally stable liquid that impregnates a porous solid support (Antonot *et al.*, 1998).

2.1.2. Mobile Phase

The mobile phase is the fluid that carries the analytes through the stationary phase. It can be gaseous or liquid, depending on the chromatographic technique used. When the mobile phase is a gas, the technique is called gas chromatography (GC). In this case, the mobile phase is called the carrier gas. When the mobile phase is a liquid, the separation is performed

using liquid chromatography, including paper chromatography, thin-layer chromatography, and column chromatography. The mobile phase is then called the eluent (Antonot *et al.*, 1998).

Depending on the nature of the mobile phase, the main chromatographic methods are classified as follows:

• Liquid Chromatography (LCP)

• Gas Chromatography (GC)

• Supercritical Fluid Chromatography (SFC)

Supercritical fluid chromatography is a technique in which the mobile phase consists of a fluid heated to critical temperature and pressure conditions. Above the critical temperature, the fluid can no longer be liquefied by increasing the pressure and exhibits properties intermediate between those of a gas and a liquid.

The most commonly used supercritical fluid is carbon dioxide (CO_2). Its low viscosity and high diffusivity allow for significantly faster analysis speeds than those achieved with liquid chromatography, with analysis times of generally 5 to 10 times shorter. Furthermore, these properties facilitate coupling with sensitive detectors such as mass spectrometers or light scattering detectors (Cuq, 2007).

2.2. Classification according to the nature of the phenomena involved

This classification is based on the separation mechanism resulting from the physicochemical interactions between the analytes and the stationary phase. Depending on the predominant phenomenon, chromatographic methods are distinguished by mechanisms of adsorption, partitioning, ion exchange, size exclusion, or specific interactions.

The following are the main categories of chromatography:

2.2.1. Partition Chromatography

Partition chromatography relies on the difference in solubility of the compounds to be separated between two perfectly miscible fluid phases. It is classically illustrated by paper chromatography, where the stationary phase consists of a liquid immobilized on an inert solid support.

The mobile phase, liquid or gas, flows through the system and transports the analytes. The separation depends primarily on the partition coefficient of each solute between the stationary and mobile phases. Compounds with greater solubility in the mobile phase migrate more rapidly than those preferentially retained by the stationary phase.

The polarity of the phases is also a determining parameter. In high-performance liquid chromatography (HPLC), the use of nonpolar or slightly polar stationary phases combined with a polar mobile phase (water or a water-methanol mixture) corresponds to reversed-phase partition chromatography (Antonot et al., 1998).

2.2.2. Adsorption Chromatography

Adsorption chromatography is a classic separation technique, performed on columns or thin layers, based on the interactions between analytes and a solid stationary phase. The separations are based on the polarity of the compounds, linked to the presence of dipoles, which determines the intensity of the interactions with the stationary phase. The eluting power of the mobile phase depends on its polarity. Solvents can be classified by increasing polarity, forming an eliotropic series, ranging from nonpolar solvents (petroleum ether, cyclohexane) to highly polar solvents (methanol, water, ethanoic acid). Adsorbents are classified according to the increasing intensity of their interactions with polar compounds. Among them, silica gel and alumina are the most commonly used. In general, the more active an adsorbent is, the greater its retention of polar compounds. The activity of the adsorbent depends in particular on its water content, a low hydration thanks to the number of available adsorption sites (Antonot *et al.*, 1998).

Figure 1. Principle of adsorption in silica chromatography

2.2.3. Size Exclusion Chromatography

Size exclusion chromatography relies on the use of a stationary phase made of a porous polymer, the pore size of which is adapted to the size of the molecules to be separated. It functions as a molecular sieve, also known as selective permeation. In this technique, the characteristic parameter is the diffusion coefficient, which replaces the partition coefficient. Depending on the nature of the mobile phase, it is referred to as gel filtration (aqueous mobile phase) or gel permeation (organic mobile phase). The pore diameter is a specific property of each gel. When a mixture of solutes passes through the gel, the large molecules, excluded from the pores, are eluted first. Medium or small molecules penetrate the pores and migrate more slowly. The separation thus takes place according to an inverse order of molar masses, with the highest being elected first (Antonot *et al.*, 1998).

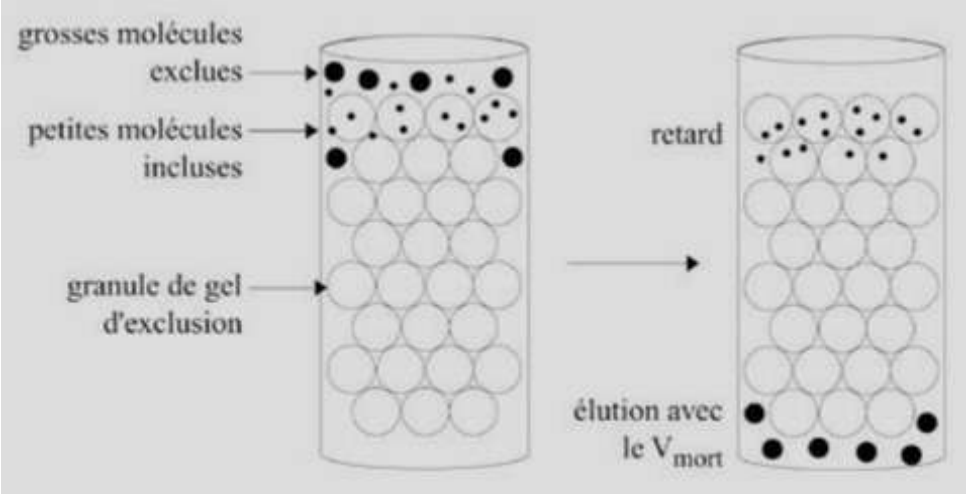

Figure 2. Molecular sieving

2.2.4. Affinity Chromatography

Affinity chromatography relies on the immobilization, on an inert solid support, of a specific ligand capable of selectively interacting with the molecule to be isolated. This ligand-molecule interaction results in the specific binding or retention of the analyte on the stationary phase. Elution is achieved by the introduction of a solvent or by modifying the physicochemical conditions, favoring the dissociation of the ligand-macromolecule complex. This technique is primarily used in biology for protein purification, as well as for the separation of cells, viruses, and nucleic acids (Safi, 2009).

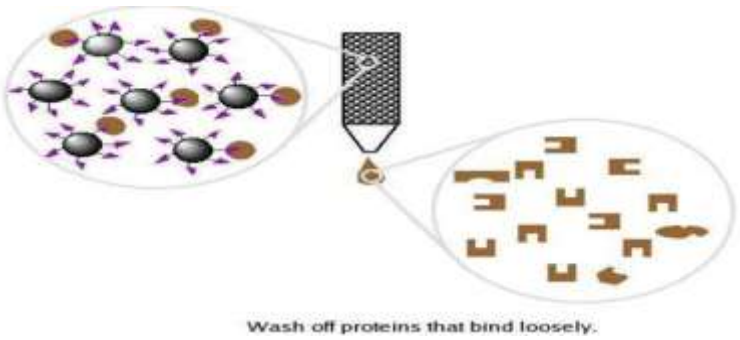

Figure 3. Affinity chromatography

2.2.5. Ion-Exchange Chromatography

Ion-exchange chromatography is a widely used analytical and preparative technique for the separation, purification, and analysis of charged molecules, particularly biomolecules such as proteins, peptides, amino acids, and certain metabolites. The fundamental principle of this method is based on the net electrical charge difference carried by the molecules to be separated (Carta & Jungbauer, 2010).

The stationary phase consists of an insoluble solid support, generally a polymeric resin, to which ionizable functional groups are attached. These groups can be negatively or positively charged, giving the resin the role of an ion exchanger. When the groups on the resin are negatively charged for example $–SO_3^-$ or $–COO^-$), the resin acts as a cation exchanger, while when they are positively charged (for example $–NH_3^+$), it acts as an anion exchanger (Regnier, 1987).

Ionized molecules are present in the mobile phase, interacting with the stationary phase through electrostatic forces (ionic interactions). These interactions are reversible and depend primarily on the net charge of the solute, the pH of the medium, the ionic strength of the solution, and the nature of the functional groups in the ion exchanger. Thus, compounds with a charge opposite to that of the resin will be retained, while weakly charged molecules or those with the same charge will be eluted more rapidly (Carta & Jungbauer, 2010).

Ion exchangers are capable of reversibly exchanging their mobile counterions (e.g., Na^+ , Cl^-) with the ions carried by the solutes dissolved in the mobile phase. This property allows for precise control of the separation, which can be modulated by varying the pH or salt concentration (ionic gradient), resulting in the progressive elution of molecules according to their affinity for the stationary phase (Fig. 4).

Thus, ion exchange chromatography relies on reversible ionic interactions between the stationary phase (ion exchanger), exchangeable counter-ions and charged solutes, allowing efficient and selective separation of compounds according to their electrical charge (Regnier, 1987).

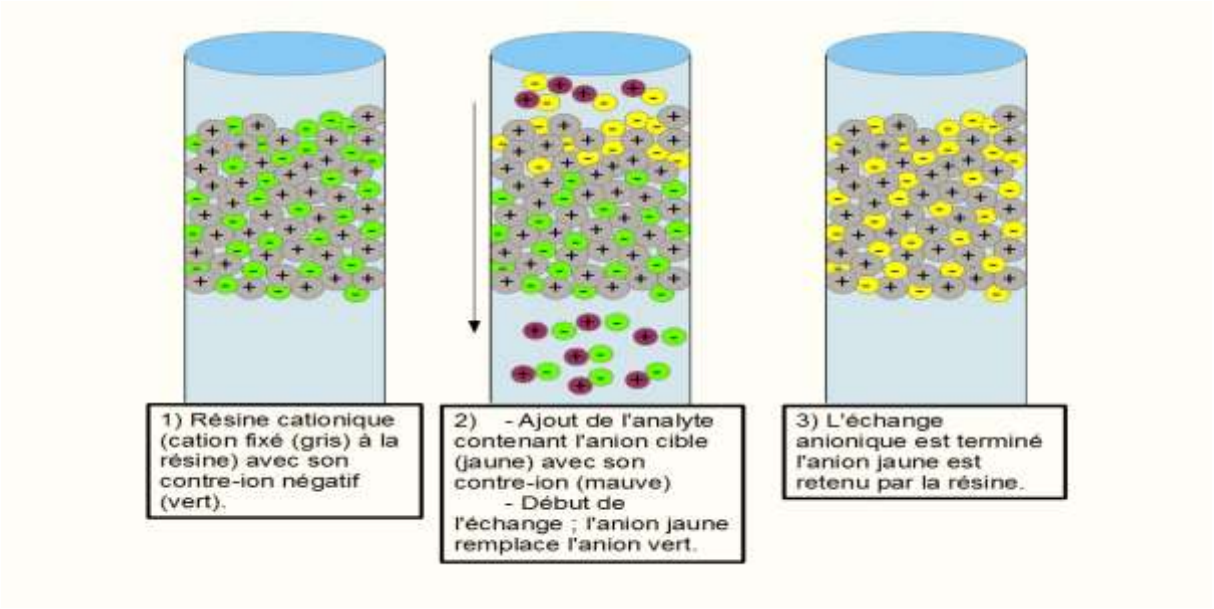

Figure 4. Diagram of steps of anion exchange

In anion-exchange chromatography, separation relies on the use of an anion-exchange resin, characterized by the presence of positively charged functional groups permanently attached to the stationary phase (step 1). These groups initially carry a mobile counterion (E^-), electrostatically associated with the fixed charge of the resin (Weiss, 2004).

After the sample is introduced into the column, the anions present in the mobile phase (analytes) compete with the counterions on the resin. This interaction leads to the progressive replacement of the initial counterion by the negatively charged solute ion (step 2). When the counterion at the exchange site is completely replaced by the analyte anion (step 3), the analyte is temporarily retained on the resin by electrostatic interactions with the positively charged fixed charges (Rocklin & Paul). The different anions present in the sample do not

have the same affinity for the stationary phase. Consequently, they are retained for varying durations within the chromatographic column, leading to their separation.

Thus, upon injection of a mixture containing two distinct anions, denoted A^- and B^-, each establishes a reversible exchange equilibrium with the resin according to the following reactions (Martens & Frankenberger, 1992).

$$K = \frac{[X^-]_s * [E^-]_m}{[E^-]_s * [X^-]_m}$$

$[X-]_{ms}$: Concentration of sample ions in the mobile phase (*m*) or in the stationary phase (*s*).

$[E-]_{ms}$: Concentration of bicarbonate in the mobile phase (*m*) or in the stationary phase (*s)*.

2.3. Classification According to the Technique Used

This classification is based on the experimental configuration of the chromatographic apparatus. Depending on the technique used, we distinguish:

• Column chromatography, in which the stationary phase is contained within a column and separation is achieved by the flow of the mobile phase through it.

• Surface chromatography, where the stationary phase is arranged on a support plane, as in paper chromatography or thin-layer chromatography.

2.3.1. Column Chromatography

Column chromatography is primarily a preparative technique, allowing the separation and isolation of components from relatively large samples (up to several grams). Unlike other chromatographic methods, it can process large quantities, but it has some drawbacks:

• High solvent consumption,

• Generally long elution time,

• Detection of compounds requiring constant attention.

The method is suitable for purifying small quantities of product when operating conditions are optimized, but its development remains empirical and often requires several trials (Antonot *et al.*, 1998).

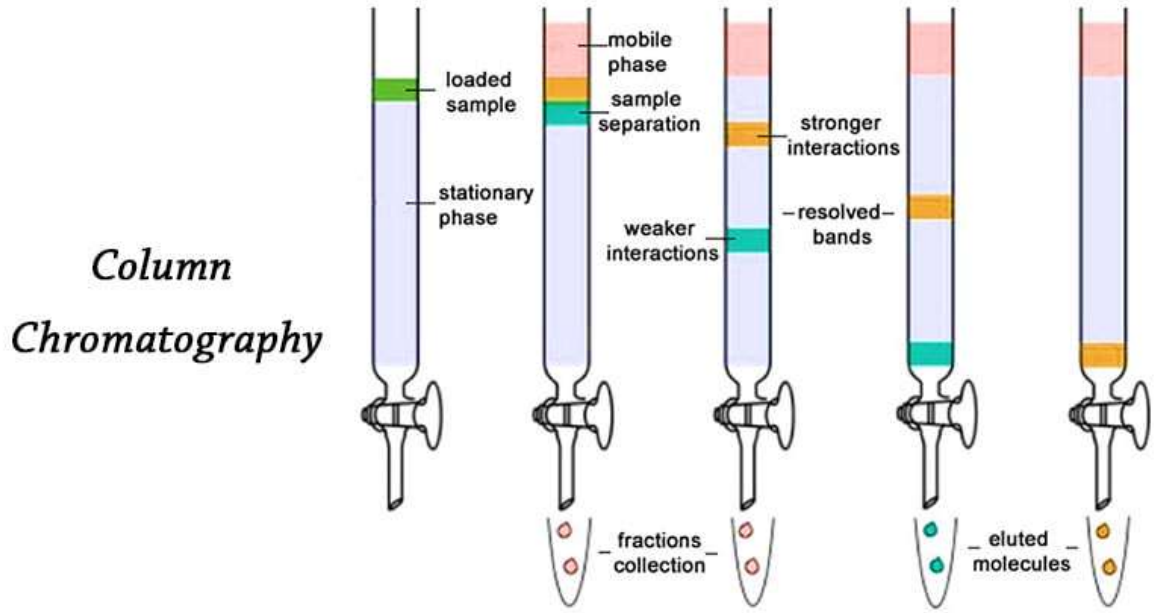

Figure 5. Column chromatography

Equipment and Parameters

• Support: selection based on chemical composition, particle size, and physical state (dry or wet).

• Column: shape (height/diameter ratio), size, and preparation (support homogeneity, filling, storage at low pressure or in a cold chamber).

• Mobile phase and pump: preparation, flow rate control, and pressure stability.

• Sample introduction: injection into the column.

• Elution: gradient or isocratic system (discontinuous or continuous gradient).

• Detector: identification and quantification of solutes.

• Computer system: command, control, data storage, and processing.

Among the main column chromatography methodologies are:

• Low-pressure chromatography: a generally manual, preparative technique suitable for separating and isolating components from small sample quantities (Dog, 2021).

•Medium- and high-pressure chromatography: an automated and computerized technique based on sophisticated equipment. It is used for both analytical and industrial applications, with precise control of flow rate, pressure, and elution thanks to computer systems (Snyder, 2009).

2.3.2. Gas Chromatography (GC)

Gas chromatography is a partitioning and/or adsorption technique that uses a mobile gas phase as the carrier for the mixture to be analyzed and a stationary phase consisting of a solid support, impregnated or not with a fixed liquid.

Two main modes are distinguished:

• Gas-liquid chromatography: inert gas as the mobile phase and viscous liquid as the stationary phase.

• Gas-solid chromatography: solid material as the stationary phase.

Separation is based on the retention time of the analytes in the column, a characteristic parameter of each compound (Antonot *et al.*, 1998).

A typical GC system generally includes the following components:

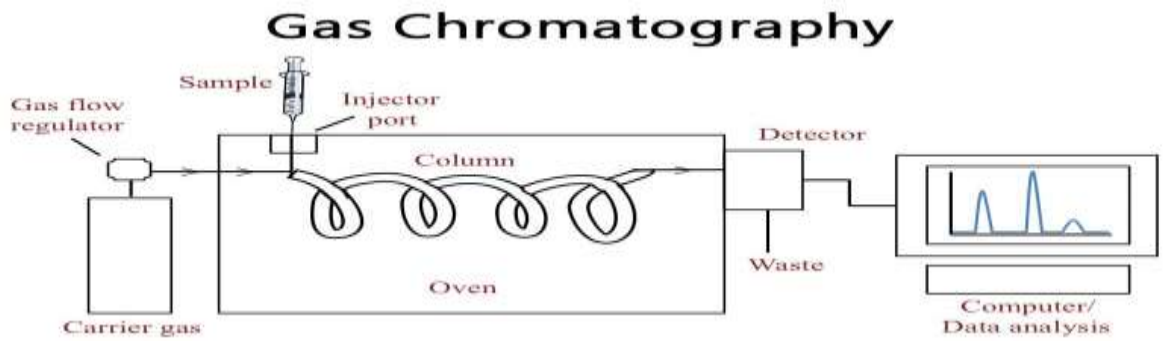

Figure 6. Diagram Gas Chromatography (GC)

Carrier Gas Reservoir

The carrier gas must be inert, high-purity, low-viscosity, and compatible with the detection system (thermal conductivity). The most commonly used gases are nitrogen, argon, helium, and hydrogen.

It is stored in high-pressure cylinders and regulated by pressure regulators to achieve pressures of 1 to 4 bar in the chromatographic system. The carrier gas flow rate, controlled by regulators, directly influences the separation quality and varies according to the column diameter (Antonot *et al.*, 1998).

Injector

Allows the introduction of the liquid sample, which must be vaporized instantly before entering the column. Its temperature is generally 20°C above that of the least volatile compound. The preheated gas carrier passes through a heated chamber sealed by a septum. The sample is injected using a syringe, with the needle tip positioned below the level of the gas carrier.

The injection must be rapid and of low concentration to avoid image enlargement. The injection chamber ensures both the vaporization of the sample and homogeneous mixing with the carrier gas. For low-volatility compounds, chemical derivatives are used to make them volatile and stable: silylation (trimethylsilane), alkylation, or acylation (Antonot *et al.*, 1998).

Column

The column is the central element of gas chromatography. It is generally a metal or glass tube, with an internal diameter on the order of a millimeter, containing the stationary phase:

• Liquid phase adsorbed onto a carefully calibrated, inert solid support (e.g., crushed brick, alumina).

• Solid phase, possibly grafted onto the support.

There are two main types:

- Packed columns: glass or stainless-steel tubing, internal diameter 2–6 mm, length 1–10 m. The support consists of 60–70 μm grains (refractory material or silica), impregnated with a low-volatility liquid (~10% of the support mass).

- Capillary columns: metal, glass, fused silica, or quartz tubes, internal diameter 0.2–0.5 mm, length 50–100 m. The stationary phase is fixed to the wall as a thin film, with a central channel widely open to minimize pressure losses and facilitate the passage of the gas carrier.

Conventional adsorbents are primarily mineral (activated carbon, alumina, molecular sieves). They are essential for gas analysis, as they are often poorly soluble in the stationary phase. However, their slow desorption can cause photographic streaking. High molecular weight organic adsorbents, such as styrene-divinylbenzene copolymers, are also used, enabling a wide range of analyses (Antonot *et al.,* 1998).

Performance of GC Columns and Detectors

• Stabilization and Maintenance: Before any analysis, the instrument must be stabilized in terms of carrier gas flow rate and temperature for at least two hours. When not in use, the columns must be capped to prevent moisture and oxidation of the stationary phase.

• Temperature and Stationary Phase: A lower temperature improves separation but increases analysis time, and the choice of stationary phase (polar or nonpolar) must correspond to the nature of the substances to be separated. The carrier gas, usually helium or nitrogen, carries the analytes through the column. At each point, an equilibrium is established between the stationary phase and the mobile phase.

• Thermostatic control: All components (column, injector, detector) are housed in thermostatically controlled chambers, programmed according to the nature of the sample and the instrument's components (Antonot *et al.,* 1998).

Detectors convert changes in the physical or chemical properties of the carrier gas into electrical signals, which are amplified and recorded as baseline images.

The most common detectors are:

• Heat-sensitive material detector (HSP),

• Flame ionization detector (FID),

• Electron capture detector (ECD),

• Photoionization detector,

• Flame and atomic emission photometry,

• Mass spectrometer.

The detector temperature is generally the same as that of the injector (Antonot *et al.*, 1998).

2.3.3. High-performance liquid chromatography (HPLC)

High-performance liquid chromatography allows for optimal selectivity, reduced analysis time, and high detection sensitivity. Every HPLC instrument always includes the following basic components:

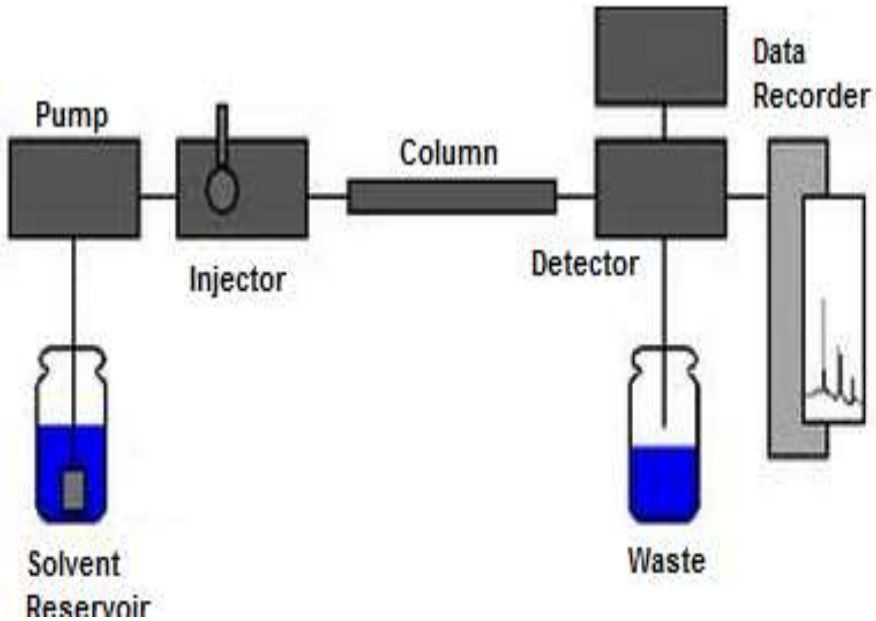

Figure 7. Principle of HPLC

Mobile Phase Reservoir

The reservoir contains a sufficient quantity of the mobile phase (eluent). Several bottles can be used for solvents of different polarities, allowing for the creation of elution gradients using the metering pump. The use of a closed vessel prevents evaporation, particularly for highly volatile solvents, and allows operation in the presence of an inert gas (Safi, 2009).

Pumping System

The pump, equipped with a gradient system, allows for programming the mobile phase composition. It can operate:

• in isocratic mode, with a pure solvent or a mixture of constant composition;

• in degraded mode, with a mixture of solvents whose composition varies over time.

Modern pumps offer a variable flow rate, from a few microliters per minute to several milliliters per minute.

Injection Valve

The injector typically uses a sampling loop (e.g., Rheodyne valve). Loops are available in various volumes (e.g., 20 µL), selected according to the column size and analyte concentration. This system ensures a constant injection volume, essential for quantitative analysis (Safi, 2009).

Columns

HPLC columns are generally made of stainless steel, with a length of 10 to 30 cm and an internal diameter of 4 to 10 mm. Newer microcolumns have a diameter of 1 to 4.6 mm and a length of 3 to 7.5 cm, packed with 3 to 5 µm particles, offering speed and low solvent consumption—a significant economic advantage.

To extend their lifespan, columns are often preceded by a pre-column or guard column (approximately 1 cm), which retains impurities. This is replaced periodically, and it is recommended to filter samples through a <0.5 μm filter before injection (Safi, 2009).

Detectors

The detector provides a continuous electrical signal reflecting change in the composition of the eluate at the column outlet, allowing the passage of compounds to be monitored. Common detectors include: UV-visible spectrometers, electrochemical detectors, conductimetric detectors, radioactive detectors, fluorimetric detectors, refractometric detectors, and mass spectrometers.

Mobile Phase

In HPLC, it is recommended to use specifically prepared solvents, always degassed and filtered before use. Degassing can be performed by ultrasound or bubbling with an inert gas (nitrogen, argon, helium) to ensure reproducibility and prevent baseline oscillations. Despite the high quality of the solvents, filtration through a 0.4 μm filter is advised to prevent orifice clogging.

The most common mixtures are: methanol–water, acetonitrile–water, and tetrahydrofuran–water. A high proportion of water is often used initially, and the organic solvent is then gradually increased until optimal separation is achieved. When a constant composition does not allow for good resolution, graded elution is used, with a gradual increase in the strongest solvent. Programmable instruments allow for the creation of these elution gradients (Safi, 2009).

Stationary Phase

HPLC stationary phases are varied and can be used for ion exchange or gel permeation, offering numerous applications. Silica is commonly used in solid-liquid chromatography for organic compounds with a mass < 2000 and for separating components

with different functional groups. However, it is less efficient for weakly polar compounds and requires more organic solvent. Partition chromatography is more common, particularly with weakly polar grafted stationary phases, allowing the use of aqueous mobile phases containing little organic solvent.

These phases, obtained by silanization of the hydroxyl groups of silica, are used in reversed-polarity chromatography, where weakly polar organic compounds are retained more strongly by the stationary phase than by the polar solvent (Safi, 2009).

3. Electrophoresis

Electrophoresis is a method for separating, characterizing, and purifying electrically charged particles, widely used in biochemistry and molecular biology for proteins and nucleic acids.

3.1. Principle

Charged molecules, in solution or suspension, migrate under the influence of an electric field applied by a DC power supply. The migration support is impregnated with a conductive buffer, the polarity of which depends on the two electrodes.

The migration rate depends on the specific characteristics of the molecules and the experimental conditions, allowing their separation:

• Cations (+): attracted towards the cathode (negative electrode).

• Anions (-): attracted towards the anode (positive electrode).

The samples are deposited on a support, each end of which is immersed in a buffer solution containing an electrode. When the power supply is activated, the charged molecules migrate towards the electrode with the opposite polarity to their charge, thus ensuring their separation (Barani, 2007).

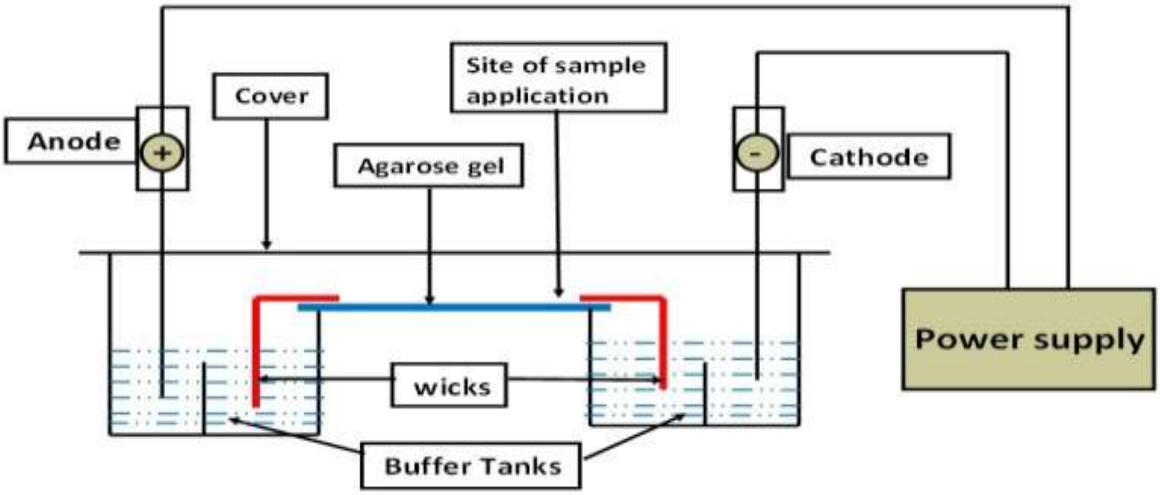

Figure 8. Electrophoresis device

Electrophoresis Supports

• Liquid-Vendor Electrophoresis

Used for very large particles (cells, organelles), this technique is now almost obsolete. The electric field is applied by a direct current generator, and the support is a suitable buffer whose ions conduct the current from one electrode to the other.

• Zone Electrophoresis (Porous Supports)

Allows for a clear separation of charged molecules into bands. The supports can be:

• Paper or cellulose acetate

• Semi-solid gels (agarose, polyacrylamide)

Support Setup

• Horizontal: For paper or cellulose acetate, the support is a long, narrow strip, the ends of which are immersed in an electrode pad.

• Vertical: For gels (polyacrylamide or agarose), the gel is poured between two glass plates with wells for sample application. Each end is in contact with an electrolytic pad, allowing current to flow.

Types of gel electrophoresis: agarose gel, polyacrylamide (PAGE), pulsed field, two-dimensional, and immunoelectrophoresis. Conditions can be denaturing (SDS, urea) or non-denaturing, depending on the application (Barani, 2007).

3.2. Paper and Cellulose Acetate Electrophoresis

• Migration is primarily based on the overall charge of the molecules.

• Basic medium: proteins carry a negative charge.

Procedure

- The ends of the strip are immersed in a conductive buffer (pH ≈ 9.2).
- The sample is dissolved in a conductive solution.
- An electric field is applied, and the sample migrates along the strip.
- The migration rate depends on the charge and size of the molecules.
- Proteins are stained (e.g., Ponceau red), and the optical density of the bands is analyzed; the intensity of the staining is proportional to the protein concentration.

3.3. Gel Electrophoresis (Semi-Solid Supports)

Gel electrophoresis separates molecules according to their charge-to-mass ratio by combining electrophoretic mobility and the sieving effect of the gel, as the pores limit the migration rate. The velocity of a charged particle in the gel is given by:

$$V = \frac{qE}{f}$$

where is the particle charge, is the electric field, and is the particle/solvent friction coefficient. The mobility u in a gel can be described by:

$$\log u = \log u_0 - K_r C$$

where u_0 is the mobility in liquid medium, KrC is the retardation coefficient related to molecular mass, and Cla is the gel concentration. Thus, the migration rate also depends on the molecular mass of the molecules.

Supports used:

Agarose: a natural gel extracted from red algae (Gracilaria), with large pores, suitable for separating very large molecules (proteins >500 kDa, nucleic acids >1500 bp).

Polyacrylamide: obtained by polymerizing acrylamide with bis-acrylamide, forming linear side chains. Ideal for proteins or small nucleic acid fragments (<1000 bp), such as oligonucleotide purification or DNA sequence determination.

Starch: sometimes used for certain specific applications (Barani, 2007).

3.3.1. Polyacrylamide gel electrophoresis (PAGE)

PAGE allows the separation and purification of protein mixtures, as well as the determination of the size and subunit composition of a protein.

Protein migration depends on their net charge (positive or negative, depending on their amino acid composition), their size and shape, the applied voltage, and the buffer viscosity.

This technique is tending to replace paper or cellulose acetate electrophoresis for protein analysis.

3.3.2. SDS-PAGE Electrophoresis

SDS-PAGE is a denaturing version of PAGE, performed in the presence of the detergent Sodium Dodecyl Sulfate (SDS). This anionic detergent homogenizes the negative charge of proteins and allows their separation primarily according to molecular size, depending on their charge involved or their conformation (Barani, 2007).

Protein Separation by Size (SDS-PAGE)

To separate proteins solely by size, use a separation gel (6–15% Polyacrylamide) containing SDS. On top, pour a concentrated gel (stacking gel, 3–5%):

• Ensure homogeneous sample entry into the separation gel.

• Refine the bands before their migration to the resolving gel.

Molecular weight determination is performed by comparing the proteins to standards of known mass.

Proteins can be visualized in the gel using:

• Coomassie blue

• Silver nitrate (more sensitive)

• Fluorescent dyes

For specific detection, labeled antibodies are used, as in Western blotting (Barani, 2007).

3.3.3. Two-Dimensional (2D) Electrophoresis

One-dimensional SDS-PAGE electrophoresis can separate fewer than 50 proteins and has limitations when protein bands are very close together or overlap. To overcome this problem, two separation modes are combined in a two-dimensional (2D) process, enabling the separation of more than 1,000 different proteins.

Dimension 1: Charge Separation (Isoelectric Focusing, IEF)

• The net charge of a protein varies with pH and becomes zero at its isoelectric point (pI).

• Each protein migrates until it reaches its pI, where it stops.

• Separation is carried out in a narrow polyacrylamide gel with a pH gradient, obtained from ampholytes (small polycarboxylic and polyamine peptides), under the action of a strong electric current.

Dimension 2: Size Separation (SDS-PAGE)

• The gel containing the proteins separated by FIE is placed perpendicularly in an SDS-PAGE gel.

• The proteins are then separated according to their size, thus completing the resolution obtained by the first dimension (Barani, 2007).

3.4. Agarose Gel Separation

3.4.1. Agarose Gel Electrophoresis

Agarose gel electrophoresis is primarily used for the separation of nucleic acids. These macromolecules are polyanionic, with a uniform relative charge, making size and structure the main separation criteria. The gel acts as a molecular sieve: the smaller the molecules, the faster they migrate through the pores of the gel.

For example, supercoiled plasmid DNA will migrate faster than circular or linearized plasmid DNA. A higher agarose concentration reduces pore size, allowing the separation of smaller fragments.

Migration Monitoring:

• To prevent sample loss, migration must be controlled.

• Samples are mixed with two indicator dyes:

- Bromophenol blue: migration similar to a 300 bp DNA fragment (small fragments).

- Xylene cyanol: migration similar to a 4,000 bp DNA fragment (large fragments).

DNA Visualization and Quantification:

• DNA bands are visualized with ethidium bromide (ETB), which intercalates between bases and fluoresces orange under UV light (200–300 nm).

• Quantification is performed by comparing the fluorescence intensity of the samples with that of DNA standards of known mass. The detection threshold is a few nanograms (Barani, 2007).

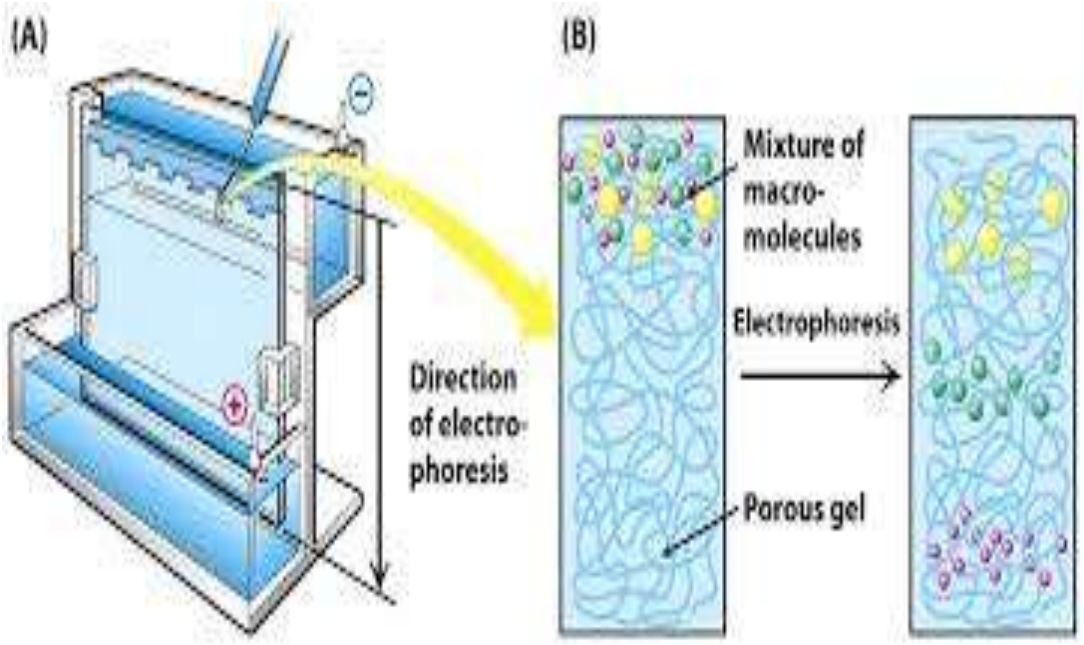

Figure 9. Electrophoretic Techniques

3.4.2. Pulsed-Field Gel Electrophoresis (PFGE)

Pulsed-Field Gel Electrophoresis (PFGE) is a variant of agarose gel electrophoresis that allows the separation of very large DNA molecules (from several tens of kilobases to several megabases), which are impossible to separate by conventional electrophoresis.

The principle is based on the application of a non-constant electric field, the direction and intensity of which vary periodically. These changes in direction force the long DNA molecules to reorient themselves within the gel, a process that is slower the larger the molecule. This difference in reorientation time allows separation according to molecular size.

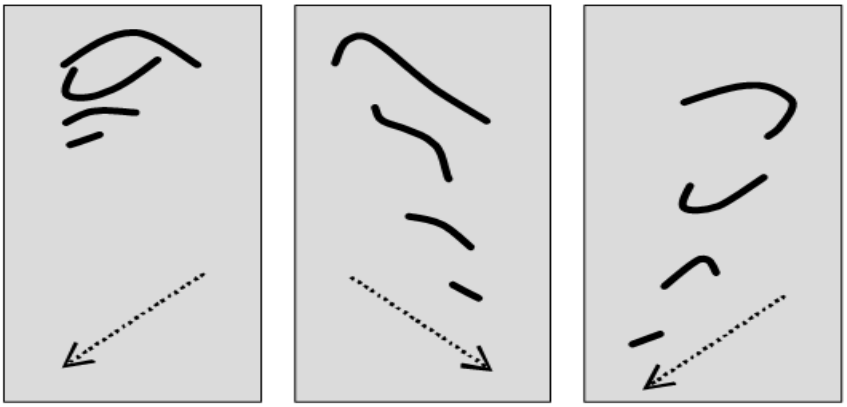

Figure 10. Pulsed field electrophoresis.

Experimental Support and Conditions

Separation is performed on a low-concentration agarose gel, which provides large pores suitable for macromolecules. DNA is generally prepared as agarose plugs to prevent mechanical fragmentation.

The electric field is generated by a specific device capable of producing pulses at different angles (generally 120°) and according to programmable cycles.

The duration of the pulses, the applied voltage, the temperature, and the total migration time are key parameters for resolution.

Performance and Applications

PFGE allows the separation of DNA from 50 kB to over 10 MB, with excellent resolution. It is considered a reference method for the analysis of large DNA fragments.

Its main applications include:

• molecular genetics,

• physical mapping of genomes,

• analysis of bacterial and fungal chromosomes,

• molecular epidemiology and typing of bacterial strains,

• the study of chromosomal rearrangements.

After migration, the DNA is visualized by ethidium bromide staining or alternative fluorescent dyes, followed by observation under UV light. The size of the fragments is estimated by comparison with molecular weight markers adapted for PFGE.

3.4.3. Immunoelectrophoresis

Immunoelectrophoresis is an analytical technique combining the principles of electrophoresis and immunodiffusion. It allows the separation, identification, and characterization of biological macromolecules, primarily antigenic proteins, using specific antigen-antibody reactions.

First, the proteins in a mixture are separated by gel electrophoresis (most often agarose gel) based on their electrical charge and mobility. After migration, a channel is cut parallel to the migration bands and filled with a specific antiserum. The antigens and antibodies then diffuse toward each other in the gel.

When they meet at equivalent concentrations, they form insoluble immune complexes, visible as precipitation lines. Each precipitation arc corresponds to a specific antigenic protein.

Experimental Support and Conditions

Immunoelectrophoresis is generally performed on an agarose gel under non-denaturing conditions to preserve the antigenic activity of the proteins. The choice of buffer, pH, and gel concentration is essential to ensure good migration and efficient diffusion of the reagents (Barani, 2007).

Applications

This technique is primarily used for:

• qualitative analysis of serum proteins,

• identification of immunoglobulins,

• the study of protein abnormalities,

• protein purity testing,

• certain applications in clinical diagnostics and immunology.

CHAPTER II. SPECTROSCOPIC ABSORPTION METHODS

CHAPTER II. SPECTROSCOPIC ABSORPTION METHODS

1. General Principle

Spectroscopy encompasses all analytical techniques based on measuring the absorption, emission, or scattering of electromagnetic radiation by matter. The electromagnetic spectrum is very broad and covers a wide range of wavelengths, from gamma rays to radio waves. In most spectroscopic methods, the sample interacts with electromagnetic radiation whose energy is modified by the atoms or molecules constituting the substance under study. This interaction, which can result in absorption, re-emission, or scattering of the radiation, is the basis for different spectroscopic techniques, each associated with a specific region of the electromagnetic spectrum (Maarouf, 2005).

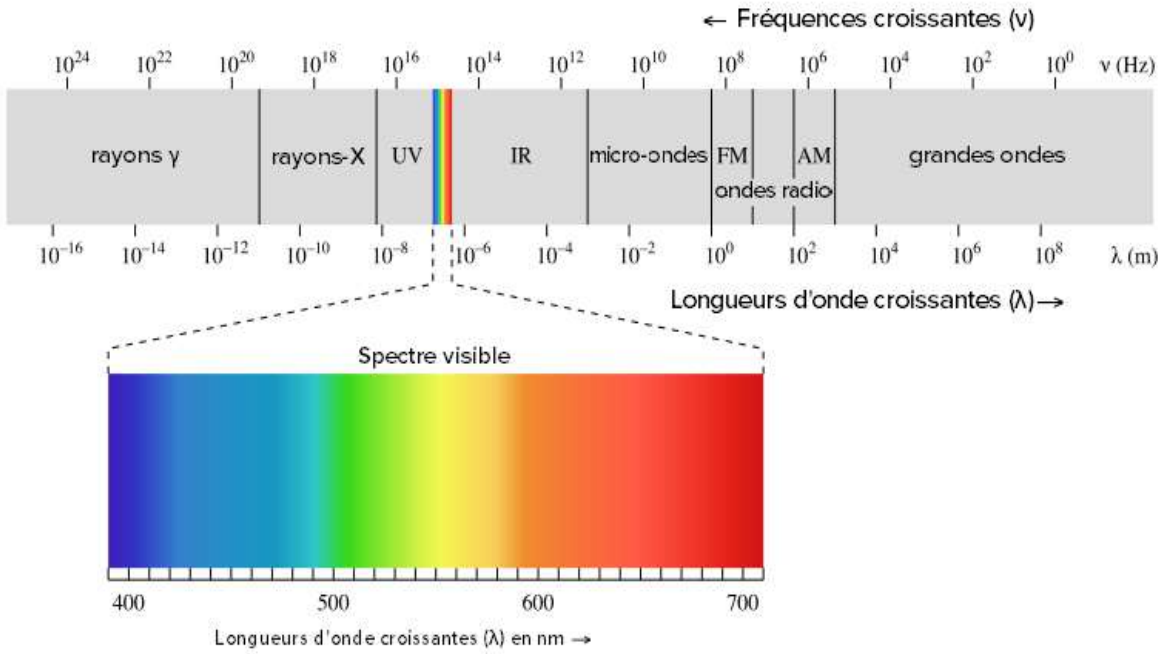

Figure 11. Electromagnetic spectrum.

2. Beer-Lambert Law

Beer-Lambert Law establishes a quantitative relationship between the absorbance of a solution and the concentration of the absorbing species, and forms the basis of UV-Visible absorption spectrophotometry (Skoog *et al.*, 2009). When monochromatic radiation passes through a homogeneous solution, the absorbance A is proportional to the concentration C of the absorbing species and the optical path length l, according to the following relationship:

$$\boldsymbol{A = \varepsilon\, l\, C}$$

where ε represents the molar extinction coefficient, characteristic of the substance and the wavelength used. This law is applicable to dilute, chemically stable solutions illuminated by monochromatic radiation, in the absence of scattering or fluorescence phenomena. It is widely used for the quantitative determination of chemical and biological compounds by establishing calibration curves that depend on absorbance at concentration (Harris, 2010).

3. UV-Visible Spectroscope

The UV-Visible spectroscope is an analytical instrument used to measure the absorbance or transmission of a substance in the ultraviolet (200–400 nm) and visible (400–800 nm) ranges of the electromagnetic spectrum. It is based on the interaction between UV-Visible radiation and the electrons of molecules, whose electronic transitions are responsible for absorption. This technique is widely used in chemical, biochemical and biological analysis for the identification and quantification of many compounds (Skoog *et al.*, 2009).

A UV-Visible spectroscope essentially consists of a light source (deuterium lamp for UV and tungsten-halogen lamp for visible light), a monochromator (prism or diffraction grating) allowing the selection of a precise wavelength, a sample compartment generally containing a quartz or glass cuvette depending on the spectral range, a detector converting the

light signal into an electrical signal, and a processing system ensuring the display and recording of results.

Monochromatic light beam passes through the solution to be analyzed, and the decrease in intensity of the incident radiation due to absorption is measured. The absorbance obtained is directly related to the concentration of the absorbing species according to Beer-Lambert's law, which allows for reliable quantitative analyses.

Modern UV-Visible spectroscopes can operate in single beam or double beam mode, the latter offering better stability and automatic correction of variations in the light source (Harris, 2010).

4. Infrared Absorption Spectrophotometry

Infrared (IR) absorption spectrophotometry is a technique particularly well-suited to the analysis of organic compounds, as the bonds corresponding to the different functional groups possess specific vibrational energies and absorb infrared radiation at characteristic frequencies. Unlike UV-Visible spectra, which are generally simple and contain a limited number of bands, infrared spectra are often complex, consisting of numerous absorption photos. Although only a portion of these bands can be interpreted accurately, they nevertheless provide essential information about the molecular structure. Due to the wealth of information it provides, infrared spectroscopy is one of the major analytical methods for the identification of chemical compounds. Each substance exhibits a nearly specific infrared spectrum, which, by comparison with reference spectra, allows for the identification of compounds, the detection of impurities, and, in some cases, the performance of quantitative analyses.

The study of the infrared spectrum of an unknown substance thus makes it possible to highlight the functional groups present and contributes effectively to the elucidation of its structure (Marouf, 2005).

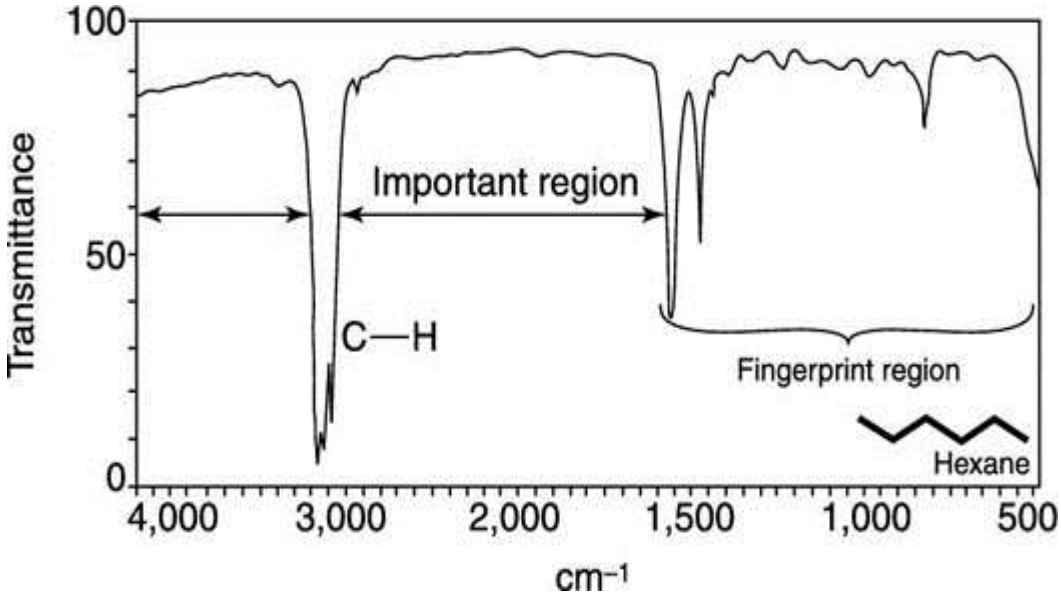

Figure 12. Identification of functional groups by infrared spectroscopy

4.1. Principle of Fourier Transform Infrared (FTIR) Spectrophotometry

Fourier Transform Infrared (FTIR) spectrophotometry is a modern evolution of classical infrared spectroscopy. It relies on the use of a Michelson interferometer, which allows for the simultaneous analysis of all wavelengths of infrared radiation, unlike dispersive spectrometers that operate wavelength by wavelength.

The infrared radiation emitted by the source is split into two beams by a semi-reflective beam splitter. One beam is reflected by a fixed mirror, the other by a moving mirror. The two beams then recombine to produce an interferogram, which contains all the spectral information of the signal. After interaction with the sample, this interferogram is recorded by the detector and then mathematically transformed into an infrared spectrum using the Fourier transform.

FTIR offers several major advantages: high analysis speed, improved sensitivity, excellent spectral resolution, and increased reproducibility. It is widely used for the identification

of organic compounds, functional group analysis, quality control, and the structural study of materials and biomolecules (Skoog *et al.*, 2009; Marouf, 2005).

Table 1. Main functional groups identified by infrared spectroscopy

functional Group	Bond Characteristic	Absorption range(cm^{-1})	Band Appearance
Alcohols / Phénols	O–H (elongation)	3200 – 3600	Broad, intense
Acides carboxyliques	O–H (elongation)	2500 – 3300	Very broad
Alcohols / Ethers	C–O (elongation)	1000 – 1300	Medium
Amides	N–H (elongation)	3300 – 3500	Medium
Amines	N–H (élongation)	3300 – 3500	Narrow
Alcènes	=C–H (elongation)	3020 – 3100	Weak to medium
Alcanes	C–H (elongation)	2850 – 2960	Medium
Carbonyls (general)	C=O (elongation)	1650 – 1750	Intense
Aldehydes	C=O	1720 – 1740	Intense
Ketones	C=O	1705 – 1725	Intense
Esters	C=O	1735 – 1750	Intense
Carboxylic Acids	C=O	1700 – 1725	Intense
Amides	C=O (amide I)	1630 – 1690	Intense
Alkynes	C≡C	2100 – 2260	Low to medium
Nitriles	C≡N	2210 – 2260	Intense
Aromatics	C=C	1450 – 1600	Medium
Halides	C–Cl	600 – 800	Low
Halides	C–Br	500 – 600	Low

The region between 1500 and 500 cm^{-1}, called the fingerprint region, is characteristic of each molecule and allows its identification by comparison with reference spectra, even when the functional groups are similar.

5. Emission Spectroscopy: Fluorometry

Fluorometry is based on the inverse phenomenon of spectrophotometric absorption. When a compound is exposed to electromagnetic radiation of a specific wavelength, it reacts to a higher energy state. After a very short time (on the order of 1 to 10 nanoseconds), it returns to its ground state by emitting light.

The emitted energy is less than the absorbed energy, which results in an emission wavelength λ' that is longer than the excitation wavelength λ. The intensity of fluorescence is proportional to the concentration of the compound in the sample, which allows for very sensitive quantitative measurements, often superior to those obtained by absorption spectrophotometry (Marouf, 2005).

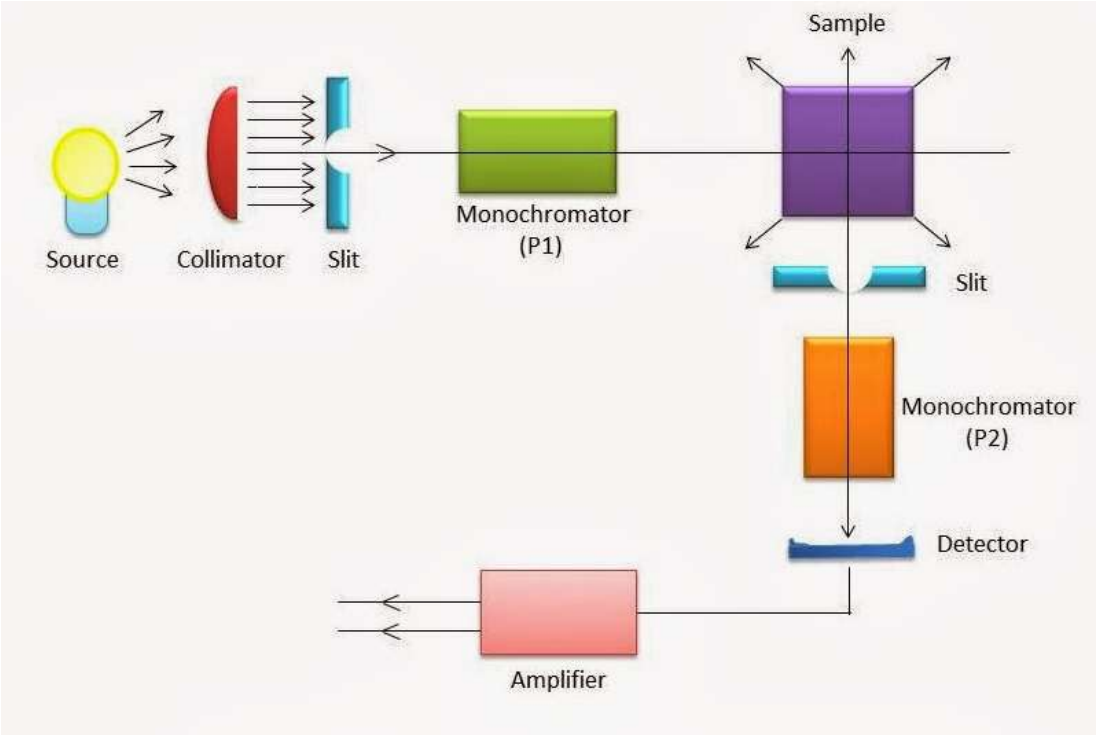

Figure 13. General diagram of a spectrofluorimeter

Fluorimetric Apparatus

A conventional spectrofluorimeter comprises the following components:

• Light source: often a xenon or mercury discharge lamp, producing intense and stable radiation covering the required excitation wavelengths.

• Monochromators: placed at the cell's inlet and outlet, they allow selection of the excitation wavelength and filter the emission wavelength to avoid noise and interference.

• Sample cell: the solution containing the fluorophore is placed in a cuvette transparent to the wavelengths used (glass or quartz).

• Detector: converts the emitted light into an electrical signal proportional to the fluorescence intensity. Detectors used are generally photomultiplier tubes or photodiodes.

• Data acquisition and analysis system: records the intensity as a function of wavelength and allows for qualitative and quantitative analysis.

Main applications

• Quantification of biomolecules: proteins (e.g., tryptophan), nucleic acids (DNA/RNA), fluorescent vitamins, and coenzymes.

• Enzyme monitoring: use of fluorescent substrates or products to study enzyme activity.

• Detection of contaminants: heavy metals and pollutants labeled with specific fluorophores.

• Structural analysis: study of molecular interactions, protein conformation, and ligand-receptor binding (Marouf, 2005).

6. Polarimetry

Polarimetry is an optical technique that measures the rotation of the plane of polarization of light as it passes through an optically active substance, usually chiral molecules. This property is typical of many organic compounds, including sugars, amino acids, and some alkaloids (Polavarapu, 2017).

Principle

When a beam of plane-polarized light passes through a solution containing an optically active compound, the light undergoes a rotation of the plane of polarization, either clockwise (dextrorotatory, +) or counterclockwise (levorotatory, -). The angle of rotation α depends on:

The length of the tube containing the solution (l, in dm)

The concentration of the sample (c, in g/mL)

The chemical nature and specific rotation of the substance ([α])

The relationship is given by Biot's formula:

$$[\alpha] = \frac{\alpha}{l \times c}$$

This method allows for the direct quantification of optically active substances and can also be used to determine the purity and concentration of chiral solutions.

Instrumentation

A conventional polarimeter includes:

• Light source: generally a sodium lamp emitting monochromatic light (589 nm).

• Polarizer: transforms the incident light into plane-polarized light.

Sample tube: filled with the solution to be analyzed.

• Analyzer: detects the angle of rotation of the plane of polarization after passing through the sample.

Reading system: optical or electronic, allowing the measured angle to be recorded.

Applications

• Determination of sugars and carbohydrates in the food and pharmaceutical industries.

• Determination of the purity and concentration of amino acids and other chiral compounds.

• Analysis of asymmetric chemical reactions in organic chemistry and biochemistry.

7. Atomic Spectroscopy

Atomic spectroscopy focuses on the study of isolated atoms and their interaction with electromagnetic radiation. It primarily comprises two techniques used in analysis for the detection and quantification of elements at low concentrations: atomic absorption spectroscopy (AAS) and atomic emission spectroscopy (AES) (Becker, 2007).

7.1. Principle

Atomic Absorption Spectroscopy (AAS): The sample is atomized, and the free atoms absorb energy in the form of photons at characteristic wavelengths. The absorbed energy is then dissipated as heat or kinetic energy. Measuring absorbance allows for the quantitative determination of the concentration of the elements present.

Atomic Emission Spectroscopy (AES): Atoms are excited by a thermal source (flame, plasma). They then return to their ground state by emitting light at characteristic wavelengths. The intensity of this emission is proportional to the concentration of the element in the sample. These techniques are particularly well-suited to the quantitative analysis of metallic elements and trace minerals in various matrices.

7.2. Difference between atomic absorption and emission

Light emitted by a polychromatic source such as a lamp produces a continuous spectrum after being dispersed by a prism.

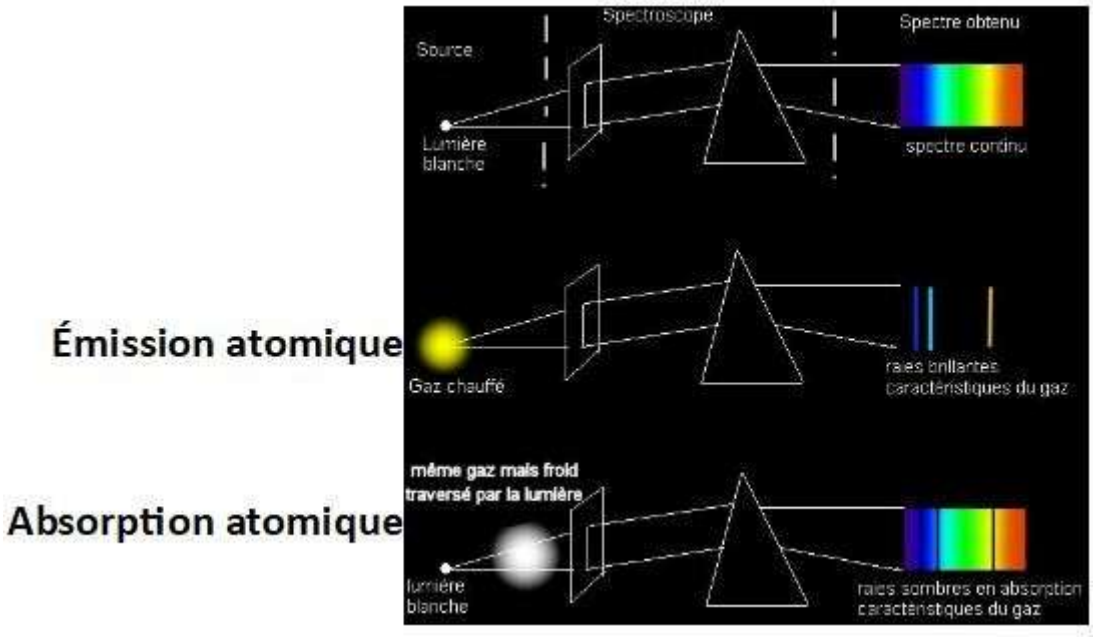

Figure 14. Kirchhoff line reversal experiment

A. Atomic Emission

When the lamp is replaced by a Bunsen burner into which NaCl is projected, a line spectrum is obtained, corresponding to the few sodium atoms that have passed into an excited state and emit light upon returning to their ground state.

B. Atomic Absorption

This time, the Bunsen burner is placed in the path of the polychromatic light. A spectrum is then obtained in which dark lines can be distinguished: the atoms that remained in their ground state in the flame absorb light at specific wavelengths (Becker, 2007).

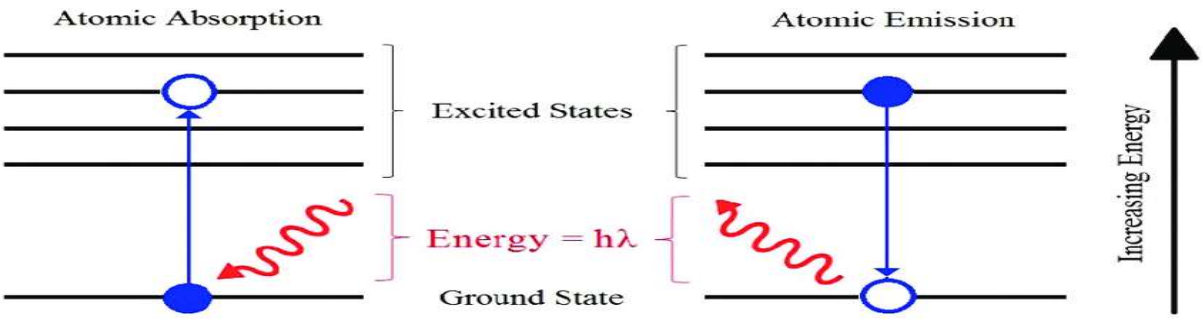

Figure 15. The difference between atomic absorption and emission

7.3. Operating Principle

In flame emission spectrometry techniques, the flame plays a crucial role by ensuring the dissociation of molecules in the analyzed medium, leading to the release and vaporization of the constituent atoms. Under the influence of the flame's high temperature, a fraction of these atoms are within reach of an excited electronic state.

The relative population of atoms between the different electronic energy levels is governed by the Maxwell-Boltzmann distribution law, which describes the influence of temperature on the probability of electronic transitions. This law allows us to relate the number of excited atoms to the flame temperature and thus constitutes the theoretical basis for the intensity of the radiation emitted when the atoms return to their ground state (Boumans, 1983).

$$NE / N0 = ge^{-\Delta E}$$

Where:
T ► Absolute temperature (in Kelvin)
g ► Integer depending on the quantum numbers of each element (degeneration factor)
ΔE ► Energy difference between the ground state and the excited state (in joules)
k ► Boltzmann constant (1.38×10^{-23} J·K^{-1})

It gives the ratio of the number of atoms in the ground state to the number of atoms in the ground state. Note that the majority of atoms remain in the ground state; this ratio is therefore small.

CHAPTER II. SPECTROSCOPIC ABSORPTION METHODS

A. Fundamentals of Measurement Methods by Atomic Absorption

The atoms in the solution are thermally atomized by a flame or in a furnace. The majority of these atoms are in their ground state. Their number can be calculated using the Maxwell-Boltzmann distribution.

$$(N1/N0 = g.e\ [-\Delta E/kT]).$$

In their ground state, they can absorb the energy of a beam of light. These absorptions occur at wavelengths characteristic of the element, according to the relation

$$\Delta E=h\nu$$

With ΔE being the energy difference between the ground state and the excited state.

B. Atomization

In atomic absorption spectrometry, the sample is generally introduced into a graphite furnace, consisting of a graphite tube through which an electric current flows. This current causes a rapid and controlled temperature rise, allowing the successive vaporization and then atomization of the element to be analyzed. The atoms thus formed are predominantly in the ground state, an essential condition for the absorption of the incident monochromatic radiation.

Before any quantitative analysis, calibration of the atomic absorption spectrometer is necessary, using standard solutions of known concentrations, to establish the relationship between the measured signal and the concentration of the element being studied (Boumans, 1983).

C. Beer-Lambert Law Measurements

Atomic absorption spectrometry measurements are based on the relationship between the concentration of the element and the amount of light absorbed by atoms remaining in their ground state. This relationship is described by Beer-Lambert's law, which expresses the decrease in the intensity of the light beam as it passes through the absorbing medium.

$$A = \varepsilon cl = \log(I_0/I_t) = \log(1/T) = -\log T$$

$A = \log (I_0 / I_t) = kC$

Where:

A: absorbance

T: transmittance

I0: intensity of the incident beam

It: transmitted intensity

C: concentration

k: proportionality fraction

L: flame length

ε: molar absorption coefficient, which depends on the wavelength and the absorbing atom

Atomic absorption allows the quantification of a greater number of elements than flame photometry.

7.4. Theoretical Aspects of Atomic Absorption and Emission

• Beer-Lambert Law

In atomic absorption spectrometry, the measured quantity is absorbance, which is proportional to the concentration of the element:

A= K.c

where:

A: absorbance

c: concentration of the element

k: characteristic coefficient of the element for the considered wavelength

In atomic emission spectrometry, the measured quantity is the intensity of the radiation emitted by the excited atoms:

$$Ie = K.c$$

Ie: Intensity of the emitted radiation

c: Concentration of the element

k: Coefficient specific to each element for the chosen wavelength.

Measurements are taken at a wavelength specific to the element being analyzed. Linearity is only verified for low concentrations.

• Sample Preparation

To determine the composition of the samples to be analyzed, it is necessary to perform mineralization to remove organic compounds.

Table 2. Mineralization Processes – Advantages and Disadvantages

Procedure	Adventages	Disavantages
Dry ashing	Low cost Sample weight may be increased	Loss of volatile elements Risk of contamination Formation of insoluble silicates
Wet digestion	Fewer losses Reduced contamination	Hazardous acids Very time consuming
Microwave-assisted digestion	Rapid No loss of volatile elements Minimal contamination	Expensive equipment

7.5. Atomic Absorption Spectrometry

Principle of Atomic Absorption

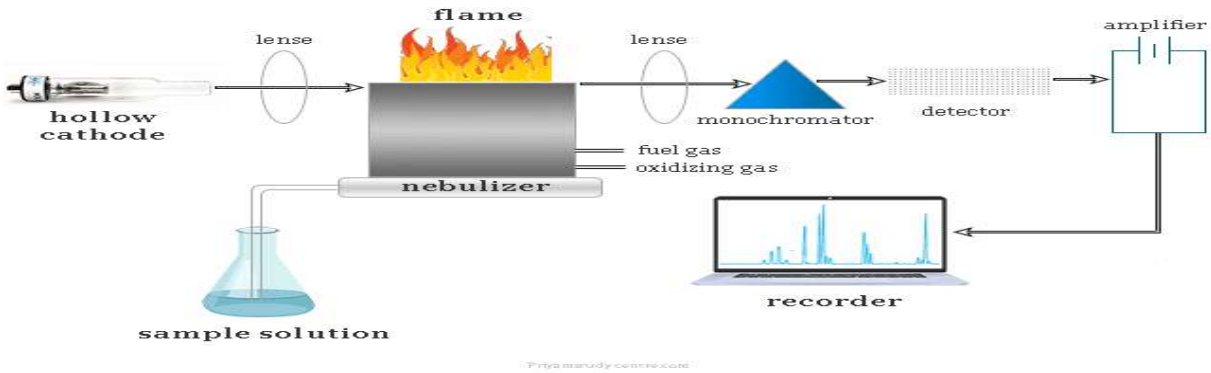

Figure 16. Principle of atomic absorption spectrometry

- The sample is reduced to atomic vapor.

- Atoms in their ground state absorb specific radiation.

- Absorbance is proportional to the quantity of atoms of the element being analyzed. In atomic absorption spectroscopy (AAS), atomic vapors are obtained by:

- Atomization by nebulization in a flame

- Electrothermal atomization

Instrumentation

An atomic absorption spectrophotometer generally includes a light source, an atomizer (atom generator), a monochromator, a detector, and a display or data logger.

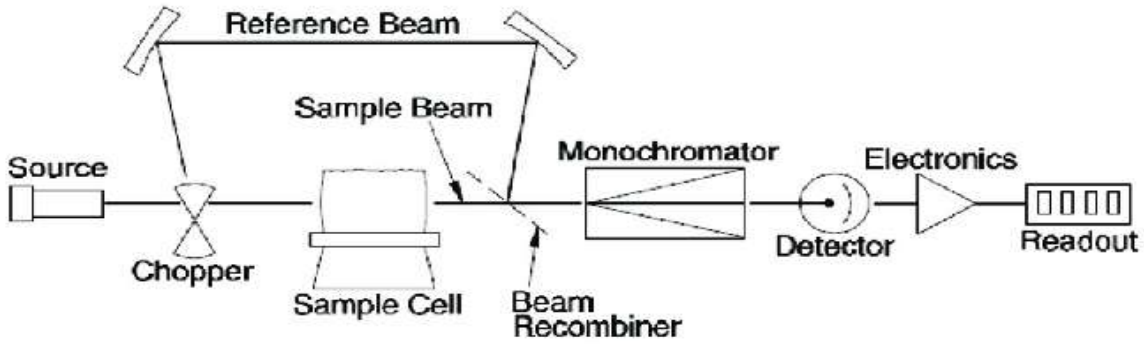

Figure 17. Diagram of an atomic absorption spectrometer

Light Sources

It must emit the resonance line of the element to be analyzed, which will be absorbed if the element is in its atomic state along the optical path.

- The resonance line corresponds to the most probable allowed transition (E1-E0); it is characteristic of the absorbing element.

- Sources made of the element itself.

- Hollow cathode lamp (one lamp per element to be analyzed).

A. Hollow Cathode Lamp

A hollow cathode lamp consists of a tube containing a filling gas (a noble gas such as argon or neon), an anode, generally made of tungsten wire, and a hollow cathode containing the element whose emission spectrum is to be used.

Since the potential difference between the anode and cathode is a few hundred volts, an ionization phenomenon occurs inside. The ions, accelerated by the electric fields, strike the cathode, acquiring sufficient kinetic energy to dislodge metallic atoms from the cathode and excite them. These excited atoms then emit a spectrum characteristic of the cathode and the filling gas.

B. Electrodeless Discharge Lamp

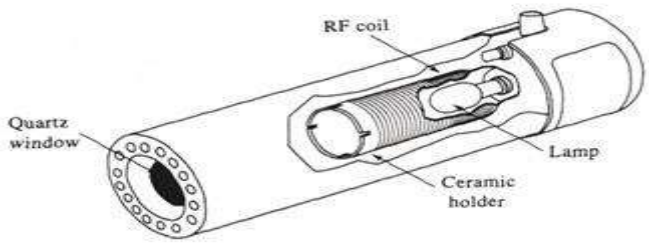

Figure 18. Electrodeless discharge lamp

When current flows through the coil, a magnetic field is created, ionizing the inert gas and exciting the atoms, which then emit their characteristic spectrum.

• Atom Generator

The atom generator, usually consisting of a flame, ensures the nebulization and atomization of the sample. This device transforms the element to be analyzed into free atoms in their ground state, an essential condition for the absorption of monochromatic radiation.

The light emitted by the hollow cathode lamp is the most commonly used light source in atomic absorption spectrometry.

The flame provides both the thermal energy necessary for the dissociation of molecules and the medium that allows the formation of atoms capable of absorbing incident photons at a wavelength specific to the element being studied.

It is essential that the gases or vapors that will absorb the light beam be in an atomic state; the most commonly used atomization devices are the flame and the graphite furnace. The sample solution is drawn in by a nebulizer and vaporized as an aerosol in a premixing chamber where it is mixed with the fuel and oxidizing gases. This mixture is then transported to the burner head where combustion and atomization of the sample occur.

A- The Flame

B. Graphite Furnace

The graphite tube is placed inside an electric furnace. An automatic injection system precisely injects a few microliters (µL) of the analyzer solution directly into the tube (diagram below).

The tube is then subjected to a specific heating program, adapted to the nature of the element being studied. This thermal program generally comprises four successive stages: drying, pyrolysis (or mineralization), atomization, and cleaning. Each stage is carried out at a

specific temperature and for a specific duration to ensure efficient atomization of the element while minimizing interference and analytical losses.

• Monochromator

A radiation-splitting system that isolates the radiation of interest for analysis.

• Radiation detection and signal processing

Its role is to convert the signal into absorbance. There are three types of detectors:

- Thermal detectors

- Pyroelectric detectors

- Photomultiplier tubes

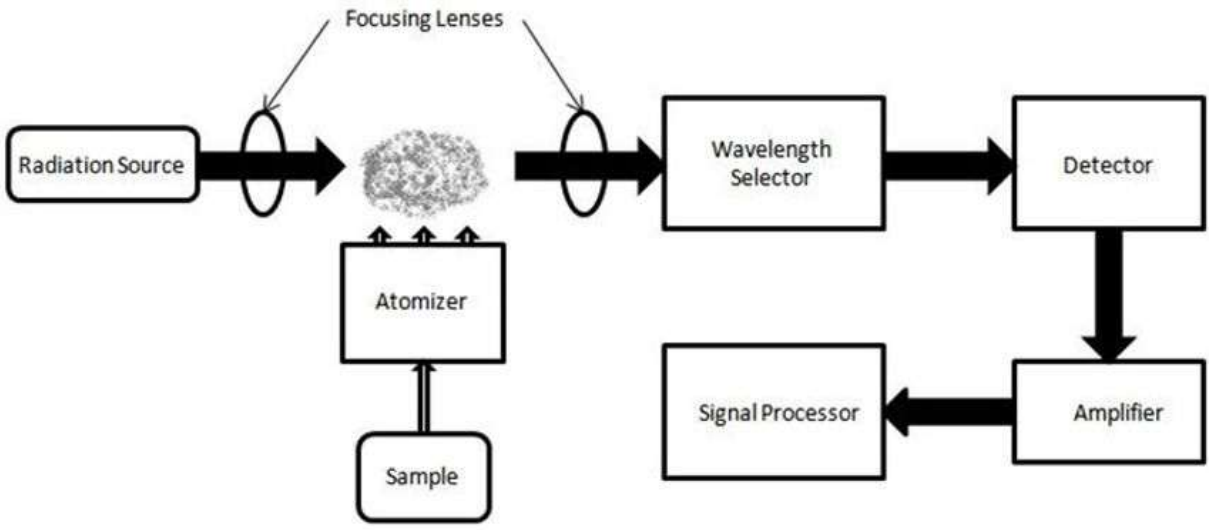

Figure 19. Atomic absorption spectrometry equipment

7.6. Flame Atomic Absorption Spectrometry

The atomizer is a flame supplied by a laminar slit burner.

• The Atomizer

The atomizer consists of a laminar slit burner, fueled by a carefully controlled fuel/oxidizer mixture to ensure a stable and homogeneous flame.

The liquid sample is drawn in and then atomized by a pneumatic atomizer, which breaks it into fine droplets forming an aerosol.

The aerosol thus generated is then directed to a nebulization chamber, where the larger droplets are removed. This step improves the reproducibility and efficiency of the atomization by allowing only the finest particles to reach the flame.

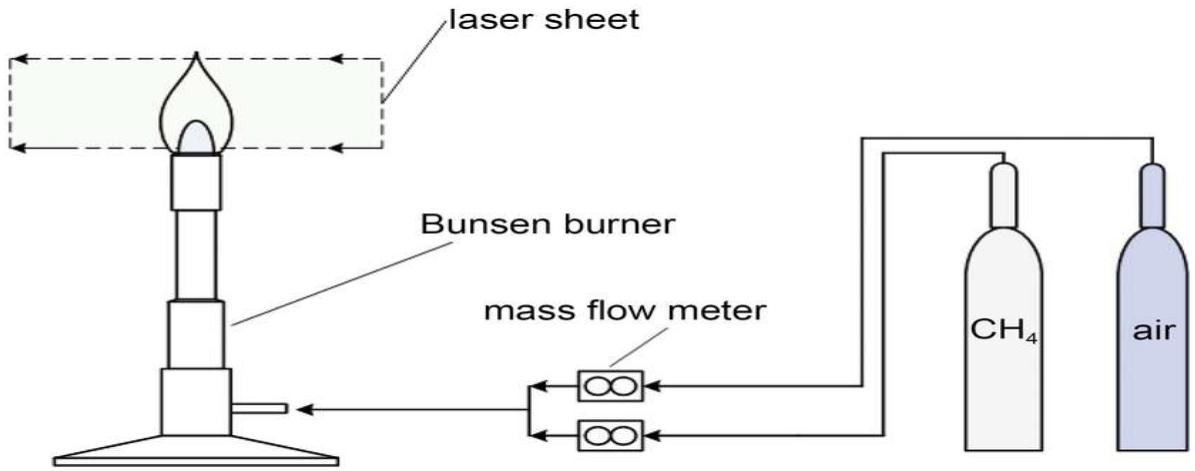

Figure 20. Laminar flow burner

• The flame

The flame produced is laminar; its temperature depends on the nature of the fuel/oxidizer mixture.

Table 3. Limiting temperatures of some fuel/oxidizer mixtures

Fuel/Oxidizer Mixture	Maximum Temperature (K)
Butane/Air	2200
Acetylene/Air	2600
Acetylen/nitrous oxide (N20)	3000
Acetylene/Oxygen	3400

Phenomena occurring in the flame

The sample in solution is nebulized at the base of the flame in the form of an aerosol.

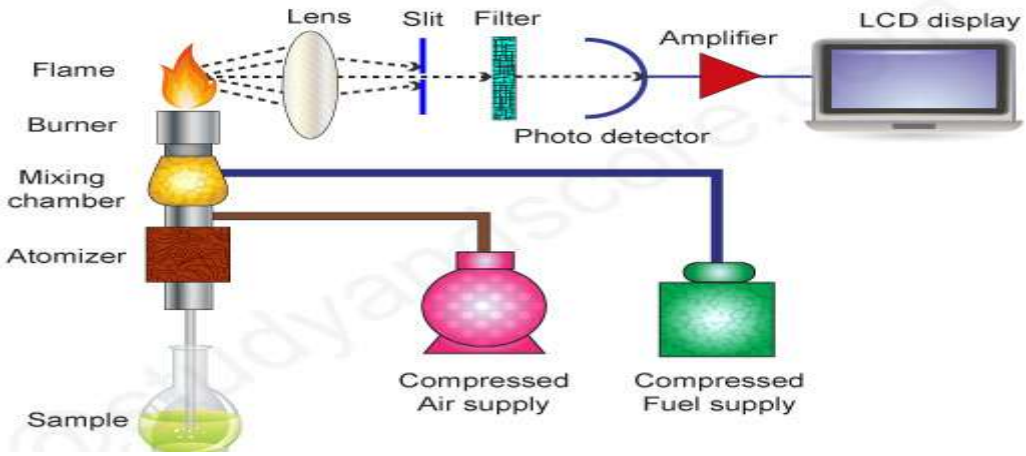

Figure 21. Phenomena occurring in the flame

Excitation and ionization of atoms are undesirable phenomena in atomic absorption spectrometry because they lead to a decrease in the number of atoms present in their ground state, which are the only ones capable of absorbing the incident radiation. This reduction results in a decrease in analytical sensitivity.

Ionization can, however, be limited by the addition of ionization buffers, which maintain a high concentration of electrons in the atomization medium and thus preserve the presence of atoms in their neutral form.

7.7. Electrothermal Atomic Absorption Spectrometry

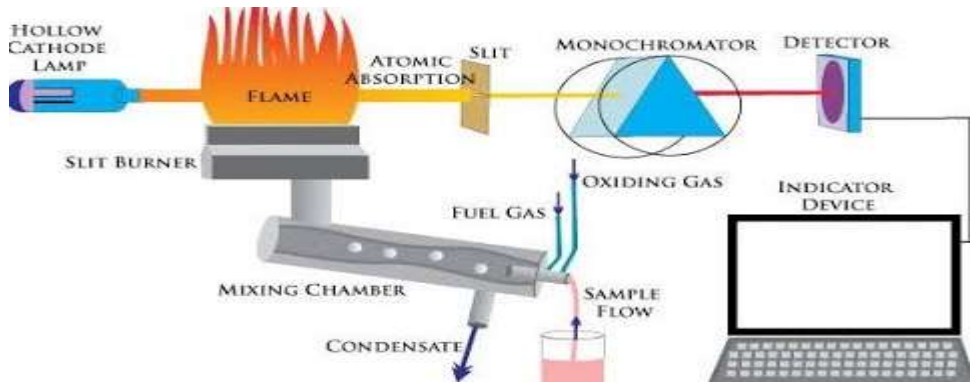

Figure 22. Electrothermal atomic absorption spectrometry equipment

• Temperature Program

The graphite furnace is heated according to a temperature program specific to each analyte, generally consisting of four successive stages. This program ensures efficient atomization while minimizing analytical losses and interferences.

The main stages are:

Drying: removal of the solvent at low temperature.

Pyrolysis (or mineralization): decomposition of the matrix without loss of the analyte.

Atomization: rapid temperature increase to form free atoms in their ground state.

Cleaning: removal of residues to prepare the furnace for the next analysis.

• Interferences

Interferences that can disrupt atomic absorption spectroscopy can be classified into four categories: chemical, physical, ionization, and spectral.

Chemical Interferences

Chemical interferences arise from the difficulty in atomizing certain species or from the formation of refractory compounds, such as oxides or low-volatility salts. For example, calcium in the form of $CaCl_2$ is more readily atomized than calcium in the form of $Ca_3\ (PO_4\ )_2$.

To correct for these types of interferences, calibration and assay must be performed under identical chemical conditions, specifically by using the same salt form of the element being studied to prepare the calibration curve.

Physical Interference

Physical interference is related to the physical properties of solutions, such as density, surface tension, or the presence of high ion concentrations. These factors can cause light scattering in the flame, disrupting the absorbance measurement.

Correction can be performed by a differential measurement:

At the resonance line wavelength: atomic absorption and scattering are present.

At a wavelength far from the resonance line: the element no longer absorbs, but scattering remains.

The difference between the two measurements provides the true absorbance of the element being analyzed.

Ionization Interference

Ionization interference occurs when the analyte is a readily ionizable element. The ionized atoms no longer participate in the absorption process, which reduces the analytical signal. The presence of another, more readily ionizable element can alter the ionization equilibrium of the analyte.

This type of interference can be limited by adding an ionization buffer, designed to increase the electron concentration and maintain the analyte in an atomicly neutral state, thereby improving the measured absorbance.

Spectral Interference

Spectral interference occurs at the atomization source and involves measuring absorbance by the superposition of spectral lines. It results from the coincidence of the spectral line of the element being analyzed with that of another element present in the sample.

These interferences generally result in a shift in the calibration curve obtained in a complex medium compared to that established in a simple medium (additive interferences). Their correction can be performed during the preliminary instrument settings by adjusting the condition $\log(I_0 /I) = 0$ in the absence of a sample.

Characteristic Quantities

Sensitivity

Sensitivity, also called characteristic concentration, corresponds to the concentration (expressed in $mg \cdot L^{-1}$) that produces an absorption of 1%, or an absorbance of 0.0044.

Limit of Detection

The limit of detection is defined by the IUPAC (International Union of Pure and Applied Chemistry) as the minimum analyte concentration that produces an analytical signal equal to three times the baseline noise, measured in the absence of analyte.

Atomic Absorption Assay

The calibration curve can be established using two main methods:

Direct calibration: used for simple matrices containing a single analyte.

Method of standard additions: applied to complex or unknown matrices to compensate for matrix effects.

7.8. Applications

Atomic absorption spectrometry is primarily a quantitative analytical method, particularly well-suited for determining trace elements. It is distinguished by its high sensitivity, high precision, rapid analysis, small sample size, and the ease of preparing standard solutions (Welz & Sperling, 1999).

Its areas of application include:

Environmental analysis: soils, plants, water.

Food and beverage analysis.

Metallurgy and petrochemicals: ceramics, glass, alloys, petroleum.

Medicine and biology: heavy metal analysis in hair, nails, and biological tissues; determination of calcium, sr, and zinc in bones.

Pharmaceutical industry: testing of raw materials and finished products; identification and quantification of mineral active substances.

Testing of elemental impurities and heavy metals originating from: catalysts and metallic reagents used in synthesis, production and transfer lines, packaging, the environment, and solvents used for cleaning.

8. Nuclear Magnetic Resonance (NMR) Spectroscopies

Nuclear magnetic resonance (NMR) is a spectroscopic method of great importance, providing access to precise information on the structure of compounds, whether in the liquid or solid state. It also provides essential data on the physicochemical properties of a system, including the arrangement of atoms and the local environment of the nuclei (nature of neighboring atoms).

This analytical technique is distinguished by its high sensitivity and reliability, which explains its widespread use in both structural and quantitative analysis. The NMR phenomenon is based on the selective absorption of energy by nuclei possessing a magnetic moment when they are placed in a strong magnetic field and subjected to appropriate electromagnetic irradiation (Claridge, 2016).

8.1. Properties of Nuclei

Atomic nuclei are characterized by several fundamental properties, namely: mass, electric charge, spin, and magnetic moment.

- **Nucleus Mass**

The mass of a nucleus corresponds to the sum of the masses of all its nucleons. It therefore depends directly on the number of protons (Z) and the number of neutrons (N) constituting the nucleus.

- **Electric Charge**

Electric charge is an essential property of matter, responsible for interactions via electromagnetic fields. We distinguish between two types of charge: positive and negative. Charges of the same sign repel each other, while those of opposite signs attract each other. In ordinary matter, positive and negative charges are generally balanced, resulting in electrical neutrality.

- **Spin**

Spin is an indirect property of particles such as electrons and atomic nuclei. It can be likened to an internal angular momentum, comparable to the particle's rotation about its own axis. Like all quantum quantities, spin is quantized and can only take integer or half-integer values of Planck's constant.

Some nuclei have zero spin (generally those with an even mass number), such as carbon-12 or oxygen-18.

Other nuclei possess non-zero spin, which gives them a nuclear magnetic moment, such as the hydrogen nucleus, which consists of a single proton.

- **Nuclear Magnetic Moment**

Due to its charge and spin, the nucleus behaves like a tiny magnet. Indeed, any rotating charged particle generates a magnetic field. Thus, the combination of nuclear charge and spin leads to the appearance of a magnetic field specific to the nucleus. This property is described by the magnetic moment, represented by a vector oriented along the north-south axis of the nuclear magnet (Claridge, 2016).

8.2. Principle of NMR

NMR spectroscopy is based on the study of transitions between two very close energy levels of a nucleus subjected to an external magnetic field. When a hydrogen nucleus is placed in a magnetic field H_0 , its spin magnetic moments tend to align with this field.

In the case of spin nuclei I = 1/2, the spin magnetic quantum number can take two values:

ms = +½, corresponding to the most stable state, aligned in the same direction as H_0 ;

ms = −½, corresponding to a less stable state, oriented in the opposite direction to H_0 and sparsely populated.

The ratio between these two populations remains very close to unity, resulting in a weak overall magnetization denoted M_0 (Fig. 22). A transition between these two energy levels can be induced by the absorption of electromagnetic energy, giving rise to a resonance phenomenon. On a macroscopic scale, the population difference between the spin states translates into a residual magnetization oriented along the H_0 field.

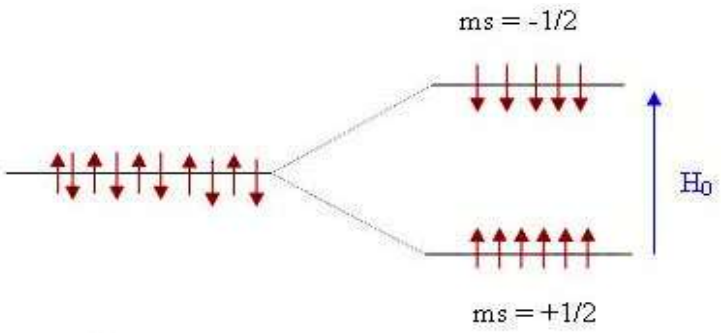

Figure 23. Representation of the polar coordinates of a nucleus H.

When a nucleus is placed in an external magnetic field H_0 , its magnetic moment is preferentially oriented along this field. However, due to its induced rotation, the nucleus does not remain stationary: it undergoes precession around the axis of the magnetic field (Fig. 23). The magnetic moment then acquires an angular velocity directly proportional to the intensity of the applied field, given by the relation:

$$\omega_0 = \gamma B_0 .$$

To induce a reversal of the magnetic moment, it is necessary to apply a second magnetic field H_1 , oriented perpendicular to μ, so as to generate a force capable of reversing it, according to Lorentz's rule, also known as the three-finger rule.

If the field H_1 is constant, this condition is satisfied only once per rotation cycle of the nucleus. It therefore becomes essential to produce a rotating field H_1 . In practice, this rotating field is obtained using a solenoid carrying an alternating current. The angular velocity of the simulated field ω_1 must then be equal to ω_0 .

When this condition is met, the resonance phenomenon occurs, causing the macroscopic magnetization M_0 to flip. The resonance frequency is thus directly proportional to the intensity of the applied magnetic field. Consequently, any perturbation of the local field due to the electronic environment of the nucleus slightly alters the resonance frequency in question. This phenomenon is known as the screening effect. The NMR signals are then expressed on a specific scale called the chemical shift (Keeler, 2010).

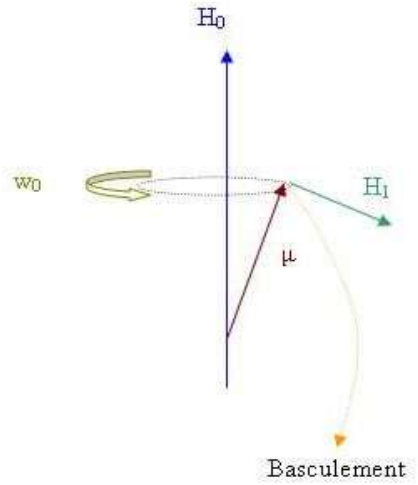

Figure 24. Magnetic field switching

8.3. NMR Signal

The recording of the NMR signal depends directly on the device used to emit the radiofrequency pulse. The magnetic field B_1 is generated by the passage of an electric current through a solenoid-type coil.

When the B_1 pulse is interrupted, the spin system tends to return to its equilibrium state. This return to equilibrium is accompanied by the emission of an electromagnetic field, which in turn induces an electric current in the same coil.

Thus, the coil plays a dual role, ensuring both the emission of the radiofrequency pulse and the detection of the signal emitted by the sample. The current induced in the coil corresponds to the NMR signal actually recorded and analyzed (Keeler, 2010).

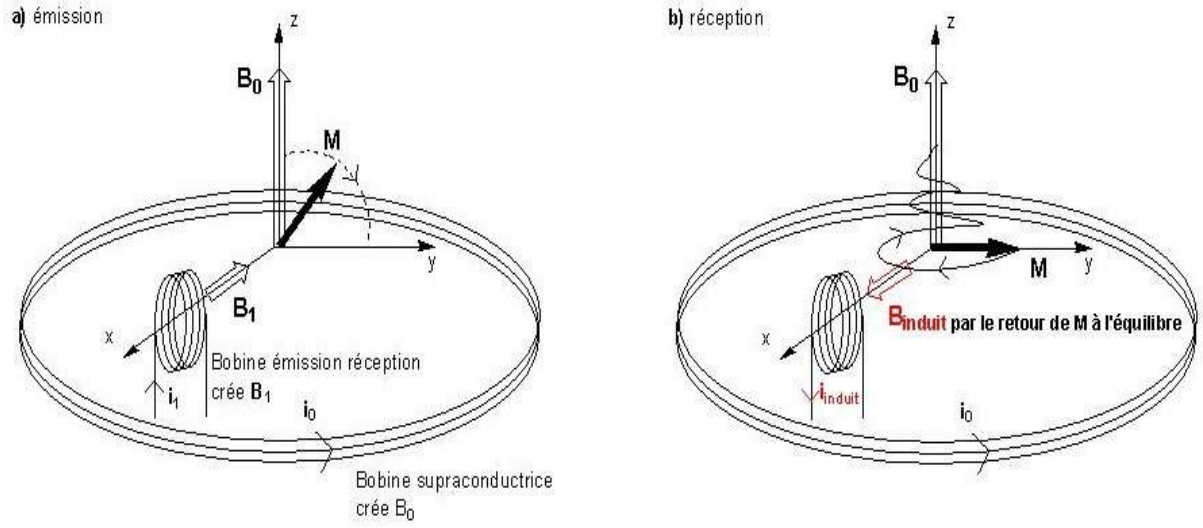

Figure 25. Signal transmission - reception.

The representation of the induced current as a function of time corresponds to the signal called FID (Free Induction Decay), or "free precession signal." For a simple system, such as an isolated proton, this signal has a relatively elementary shape: it is a sinusoid whose amplitude decreases exponentially (Fig. 24a).

However, when several nuclei are involved, the shape of the signal quickly becomes more complex. The FID then results from the superposition of several damped sinusoids, each with its own characteristics. This complexity makes the raw signal difficult, if not impossible, to interpret directly (Fig. 24b).

In order to extract usable information from this time-domain measurement, it is necessary to perform mathematical processing of the signal. This relies on the application of the Fourier transform, which allows us to move from the time domain to the frequency domain (Keeler, 2010).

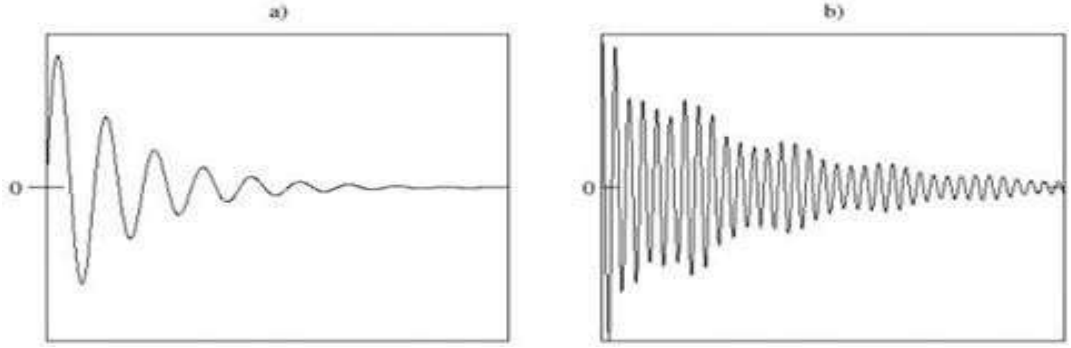

Figure 26. a) FID of an elementary system (an isolated proton); b) FID of a complex system.

Signal Processing by Fourier Transform

The Fourier transform (FT) is a fundamental mathematical tool for analyzing a signal defined in the time domain f(t) by determining its constituent frequency components. Figure 19 illustrates the application of the FT to simple periodic signals, such as the functions cos(t), cos(2t), and their superposition.

The analysis shows that the Fourier transform of cos(t) results in a single peak centered at frequency 1, while that of cos(2t) yields a peak located around frequency 2. When these two functions are added together, the resulting FT of the signal shows two distinct peaks, positioned at frequencies 1 and 2, respectively. This representation allows for the immediate identification of the frequencies present in the signal.

In such a simple case, it is still possible to access this information by directly examining the time-domain signal. However, for complex signals such as the FID illustrated in Figure 20, direct interpretation becomes impossible. The use of the Fourier transform then proves essential to correctly analyze the experimental data.

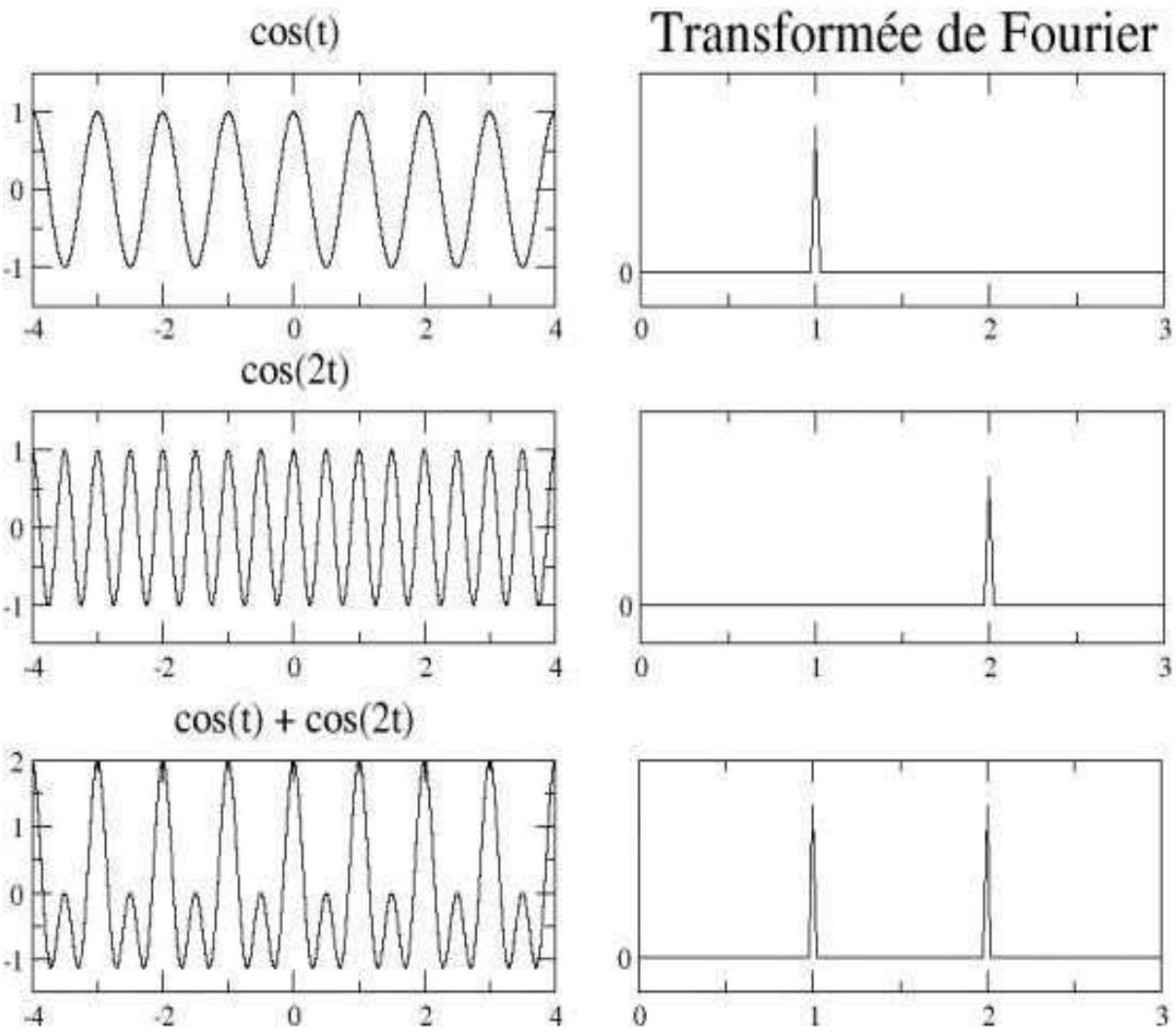

Figure 27. Fourier Transform

Applying the Fourier transform to the FID of a complex system allows us to highlight the different components of the signal. Each contribution is manifested by a peak centered on its characteristic frequency, while the area associated with this peak is directly related to the intensity of the corresponding contribution.

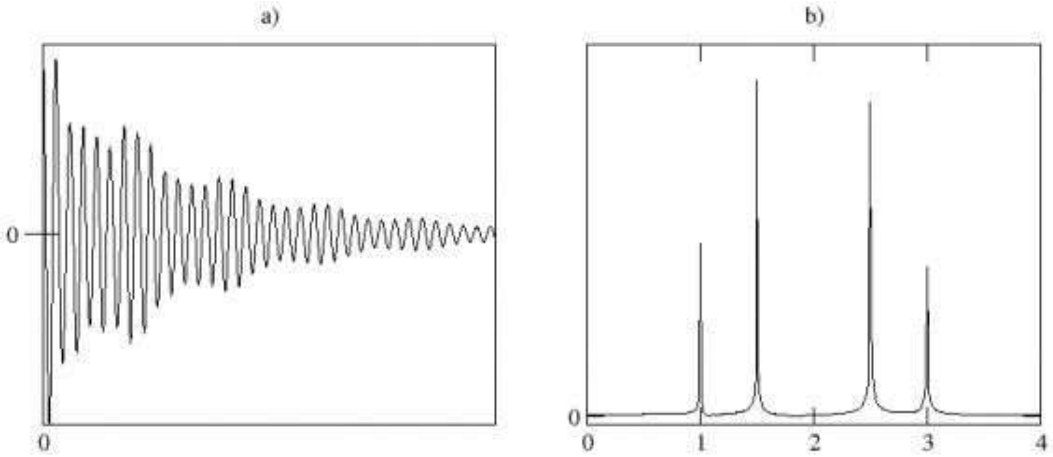

Figure 28. FID of a complex system and its Fourier transform.

Chemical analysis by NMR

The application of the Fourier transform allows the raw NMR signal to be converted into a usable spectrum. To illustrate this principle, we can consider the example of a simple molecule, ethanol (CH_3 CH_2 OH). The structural diagram of this molecule highlights the connectivity between the different atoms that compose it (Fig. 28).

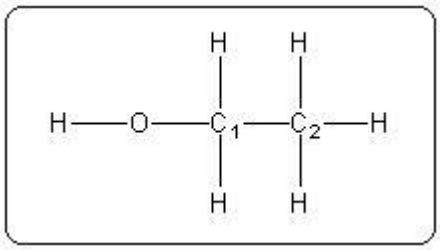

Figure 29. Structural formula of ethanol.

Ethanol has six hydrogen atoms that are active in NMR (for simplicity, we refer to them as "protons"). These six protons are subjected to identical B0 and B1 fields; therefore, they should exhibit the same response to the B1 impulse, and the resulting signal should be the sum of the six identical individual signals.

In ethanol, there are three different groups of protons, located at C1, C2, and O, respectively. Each proton within a given group is subjected to an identical local field, but the three local fields are different (Fig. 22). This results in a different response to the B1 impulse for each group, which explains the existence of three distinct clusters. The substructure of these clusters is due to interconnected nuclear spins within the different groups.

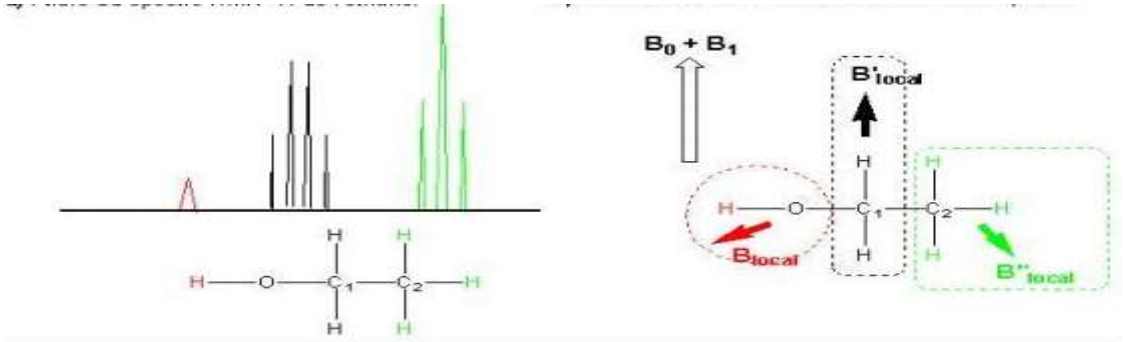

Figure 30. NMR spectrum of ethanol (simulation).

8.4. Apparatus

From an instrumental point of view, recording a nuclear magnetic resonance (NMR) spectrum requires sophisticated equipment due to the very low intensity of the detected signal (Fig. 23). The Fourier transform NMR (FTNMR) spectrometer essentially relies on three major components:

A magnet producing an intense and homogeneous magnetic field, a radio frequency emission system, and a signal reception system (Fig. 24).

The sample is introduced into a borosilicate glass tube, generally 15 cm long and 5 or 10 mm in diameter, and then dissolved in a suitable solvent, ideally free of atoms corresponding

to the isotope being studied. For the analysis of proton (^1H) spectra, the most commonly used solvent is deuterated chloroform ($CDCl_3$). This choice is justified by several advantages: the chloroform proton is replaced by deuterium, which limits spurious signals, makes the solvent economically accessible and allows the use of deuterium as an internal reference for stabilizing the magnetic field.

Indeed, deuterium resonates at a frequency distinct from that of the proton and serves to lock the magnetic field, ensuring both its temporal stability and spatial homogeneity. These two parameters are crucial, as any temporal drift or inhomogeneity of the magnetic field can lead to a broadening of spectral lines, potentially altering or even obscuring the fine coupling structures. Radiofrequency radiation is emitted by a coil located within the NMR probe, which also allows, in some cases, the detection of the induced signal. Depending on the instrument configuration, emission and reception can be performed by a single coil or by separate coils. The collected signal is then amplified, electronically processed, and converted into a usable spectrum, enabling the precise determination of resonance frequencies and the structural interpretation of the analyzed compounds.

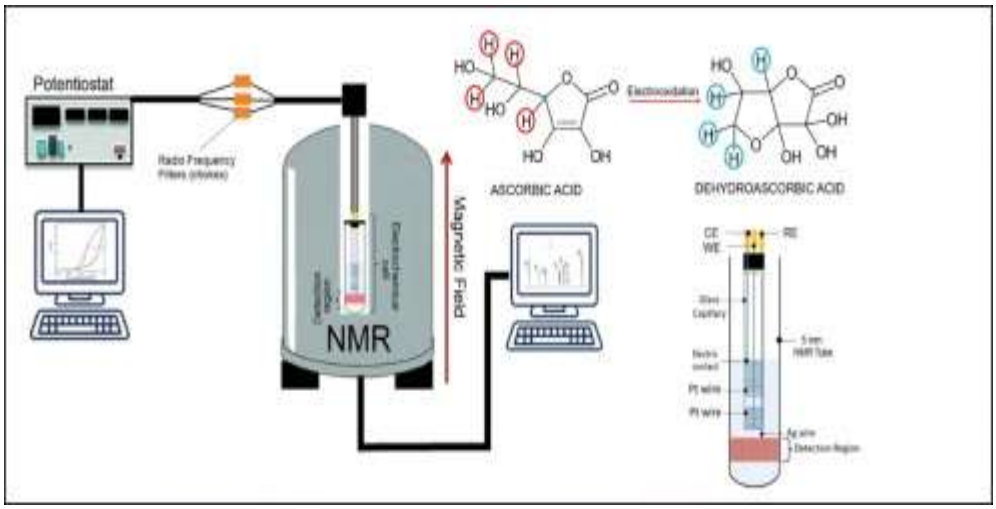

Figure 31. An NMR spectrometer

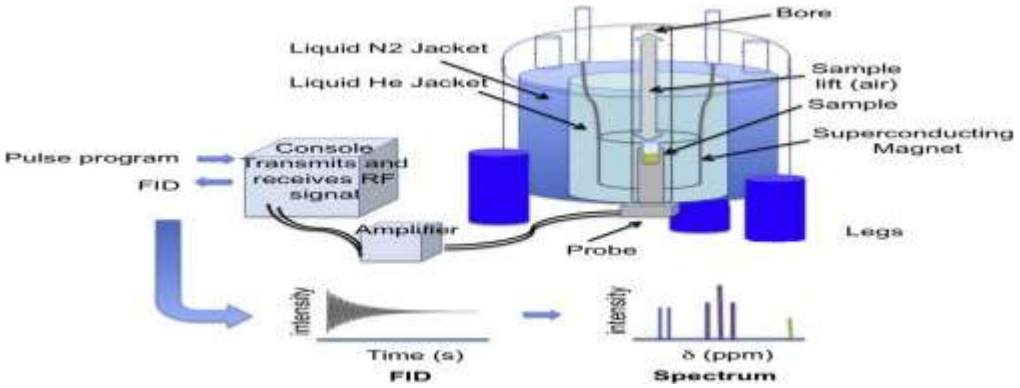

Figure 32. Schematic of an NMR spectrometer with separate transmitting and receiving coils

8.5. Applications

Nuclear magnetic resonance (NMR) has a wide range of applications in numerous scientific and technological fields. It is a reference tool for the structural analysis of organic substances in solution, the characterization of organic and inorganic materials in the solid state, and for magnetic resonance imaging (MRI) techniques.

Liquid NMR is a fundamental method for the structural study of small organic molecules in solution, whether of natural or synthetic origin. It is also widely used for the analysis of soluble macromolecules such as proteins, nucleic acids, polysaccharides, and synthetic polymers. This technique provides access to detailed information on molecular structure, conformational dynamics, and intermolecular interactions.

Solid-state NMR is particularly well-suited to the study of amorphous or weakly crystalline materials, such as glasses and insoluble natural or synthetic polymers. It is an essential complementary approach to X-ray crystallography, especially when the latter is limited by the absence of crystalline order. Related methods are also used for the analysis of complex heterogeneous samples, such as solid supports in organic synthesis or soils, in the context of environmental studies and materials characterization. Magnetic resonance imaging

(MRI), as implemented in a conventional NMR laboratory, generally corresponds to micro-MRI. This technique is suitable for studying samples on the order of centimeters and offers a spatial resolution of up to a few tens of micrometers. It has applications in both materials analysis and the study of biological tissuespa rticularly for monitoring and evaluation, at the tissue level, of therapeutic strategies and pathophysiological processes (Levitt, 2008).

Table 4. Summary of the main spectroscopic methods (Marouf, 2005)

Method	Principle	Detector	Applications
UV-Vis Spectroscopy	Measurement of light absorption in the UV and visible regions by molecules, associated with electronic transitions ($\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$)	Photometer or spectrophotometer UV-Vis	Quantitative analysis of organic compounds, monitoring of chemical reactions
Fluorometry	Measurement of the fluorescence of a compound excited by radiation, emission at $\lambda' > \lambda$ incident	Fluorometer	Highly sensitive detection of trace amounts of fluorescent compounds
Infrared spectroscopy (IR)	Absorption of IR radiation by specific chemical bonds, revealing functional groups	Infrared Spectrometer	Identification of functional groups and structural analysis of organic molecules
Polarimetry	Measurement of the rotation of the plane of polarization of light by optically active substances	Polarimeter	Analysis of sugars, amino acids, and chiral compounds
Atomic absorption spectroscopy(AAS)	Absorption of radiation by thermally excited free atoms	Atomic Absorption Spectrophotometer	Determination of metallic elements at low concentrations
Atomic Emission Spectroscopy (AES)	Excitation of atoms by heat, emission of characteristic photons	Emission Spectrometer	Quantitative determination of metallic elements
Nuclear Magnetic Resonance (NMR)	Measuring the energy required to change the alignment of magnetic cores in a field	Spectromètre RMN	Molecular structure elucidation and identification of chemical groups

CHAPTER III. SEPARATION METHODS

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1. Dialysis

Dialysis is a purification technique that separates molecules according to their size by diffusion through a semi-permeable membrane. It is particularly used to concentrate biological solutions or to remove salts and small molecules from a protein solution (Nelson & Cox, 2021).

Dialysis membranes are often in the form of tubes closed at both ends ("sausages") and contain the liquid to be treated. They are immersed in a counterdialysis fluid (water or a suitable solvent). Small molecules gradually pass through the membrane, while large molecules remain in the internal compartment until equilibrium is reached.

The diffusion rate depends on several factors: the nature of the molecule, temperature, agitation, pH, and exchange surface area. Although simple and inexpensive, dialysis is a relatively lengthy process, which can be accelerated by regularly renewing the solvent (Scopes, 1994; Marouf, 2005).

2. Electrodialysis

Electrodialysis is a variant of dialysis that uses an electric field to enhance ion transport across the membrane. A weak current (a few mA) causes anions to move toward the cathode and cations toward the anode, allowing the selective removal of mineral ions without loss of organic compounds (Baker, 2012).

The device comprises three compartments: the central compartment contains the fluid to be treated and is separated from the lateral compartments by semi-permeable membranes. These compartments contain the anode and cathode, respectively. Ions are attracted to the electrodes corresponding and concentrated outside the central compartment. A low voltage, generally around 2 volts, is sufficient to ensure separation (Nelson & Cox, 2021).

Electrodialysis is used for the desalination of brackish water with a salinity below 500 $mg \cdot L^{-1}$, above which reverse osmosis becomes more efficient (Baker, 2012). It is also used for

the treatment of dairy industry by-products, particularly whey from cheese and casein production, offering an alternative to biological processes and ion-exchange resins for the denitrification and defloration of drinking water (Marouf, 2005; Nelson & Cox, 2021).

In electroplating, electrodialysis allows the treatment of effluents, such as rinse water, by concentrating and recovering the deposition baths for reuse (Baker, 2012). However, the temperature rise induced by the passage of current limits its application to heat-sensitive products, requiring specific conditions to prevent their degradation (Scopes, 1994).

3. Osmosis

Osmosis is a fundamental physicochemical phenomenon that corresponds to the spontaneous movement of a solvent, generally water, across a semi-permeable membrane separating two solutions of different molecular concentrations. This membrane allows only the solvent to pass through but retains the solutes. The osmotic flow occurs naturally from the solution with the lower solute concentration to the solution with the higher concentration, until an equilibrium is established, characterized by a pressure exerted on the more concentrated solution, called osmotic pressure (P_o). During this process, the solute(s) do not cross the membrane and remain confined in their respective compartment.

Osmosis can also be used as a method for concentrating solutions when the membrane used is completely impermeable to solutes. This is particularly the case for osmotic dialysis against a macromolecular solution, such as those containing polyvinylpyrrolidone (PVP) or polyethylene glycol (PEG). PEG is the most commonly used because it is

commercially available with high molecular weights (around 30,000 Da), which significantly limits its diffusion through the membrane and prevents contamination of the concentrate.

In this technique, the solution to be concentrated is placed in a cellulose coil (dialysis membrane) and then immersed in a concentrated PEG solution (generally 60%) for a period ranging from 4 to 24 hours, depending on the desired concentration factor. The operation is usually carried out at a low temperature (4°C) to preserve the integrity of sensitive compounds and limit unwanted exchanges. It is essential to accurately measure the volume of the solution before and after concentration in order to determine the concentration factor obtained (Marouf, 2005).

Reverse Osmosis

Reverse osmosis is a very fine membrane separation technique based on the transfer of water across a semi-permeable membrane, but in the opposite direction to that of natural osmosis. This phenomenon is made possible by the application of a high artificial osmotic pressure, generally between 30 and 80 bar, which exceeds the natural osmotic pressure and forces the solvent to migrate from the more concentrated solution to the less concentrated solution.

This process retains the majority of dissolved particles and low molecular weight contaminants, such as dissolved solids, mineral salts, bacteria, and certain organic molecules.

The required osmotic pressure increases with the concentration of the solution and decreases when the molar mass of the solutes is high. One of the major advantages of reverse osmosis is that it allows the concentration and purification of solutions without raising the temperature, which is particularly important for heat-sensitive compounds.

The main drawback of this method is its low flow rate, which can, however, be compensated for by using large-surface-area membranes. Reverse osmosis membranes

generally have a cutoff pressure between 150 and 1000 Da. There are two main types of membranes:

Cellulosic membranes, characterized by high permeability and selectivity, but susceptible to chemical hydrolysis. Their use is limited to temperatures below 40 °C, a pH range between 3 and 8, and pressures not exceeding 60 bar.

Organic polymer membranes, particularly those based on polysulfones, offer superior resistance to extreme pH levels and temperatures up to 70°C. Their flow rate is generally limited to approximately 150 L/h/m². Polyamide membranes are also widely used; they allow the removal of 90 to 98% of inorganic elements, non-ionic compounds, and organic molecules with a molecular weight greater than 100 Da.

Water produced by reverse osmosis is of very high quality and has numerous applications in various sectors. This technique is primarily used for water treatment and solution concentration, particularly for:

• Desalination of brackish or salt water for the production of drinking water.

• Preparation of ultrapure water for the electronics and pharmaceutical industries.

• The dairy industry (concentration of whey, skimmed milk, and whole milk).

• Concentration of active substances such as antibiotics, amino acids, and vitamins.

• Purification and concentration of flavorings (Marouf, 2005).

4. Filtration

Filtration consists of separating substances according to their size or nature by passing them through a suitable filter.

Types of filters

• Depth filters

These filters are made of fibrous materials (cellulose, cotton, fiberglass, asbestos) or agglomerated substances (fried glass, sand, charcoal), forming a network of internal channels. Their filtration capacity depends on the filter thickness and the applied pressure. The most common forms are flat or pleated paper filters, fiberglass filters, filter tubes, phase separator papers, and sintered plates for vacuum filtration.

• Screen filters

These are finely chopped sheets of cellulose nitrate or polyvinyl fluoride, used to trap particles on their surface.

Applications

• Filtration by trapping particles on the surface (prefiltration).

• Sterilizing filtration, for example, for rinsing cell cultures (newt).

• Use in cytology with immersion oil to clear the filters.

• Indirect enumeration of microorganisms by filtering large volumes of lightly contaminated products.

• Air filtration (HEPA filters) (Guevara, 2012; Mulder, 2001).

5. Ultrafiltration

Ultrafiltration is a semi-permeable membrane filtration technique that uses a pressure difference (transmembrane pressure) to separate substances according to their molecular size. The membranes act as molecular filters.

Principles

Separation is based on molecular size: only molecules smaller than the membrane pores pass through.

Methods

• Membrane Ultrafiltration

This is performed in specialized cells (e.g., Diaflo), with nitrogen pressurization or centrifugation. Applications: concentration of molecules, study of macro-micromolecular bonding, virus isolation, and column chromatography monitoring.

b) Ultrafiltration using Hollow Fiber Cartridges:

• MW > 30,000 Da: HFU

• MW > 5,000 Da: HFD

• MW > 200 Da: HFO

• Gas: HFG

Setup can be in macrotubes, beakers, or T-tubes. Precautions are necessary: pre-rinsing, pre-filtration, pH and temperature control, limiting the use of organic solvents, and cleaning before reuse.

Applications

- Desalting and concentration of biological liquids.

• Purification and concentration of macromolecule solutions (10^3 – 10^6 Da), particularly proteins.

- Ultrafiltration differs from microfiltration and nanofiltration primarily in the size of the molecules retained (pores from 0.001 to 0.1 μm) (Guevara, 2012; Mulder, 2001).
- Protein fractionation according to molecular weight cut-off (MWCO), enabling selective separation of protein mixtures.
- Pre-treatment and post-treatment step in chromatographic processes, reducing sample volume and improving resolution.
- Wastewater treatment and water recycling, particularly for the removal of organic matter and macromolecular pollutants.

- Food and industry dairy applications, including milk protein concentration, whey protein recovery, and lactose removal.
- Biotechnology and pharmaceutical industries, for downstream processing of enzymes, vaccines, antibodies, and therapeutic proteins.

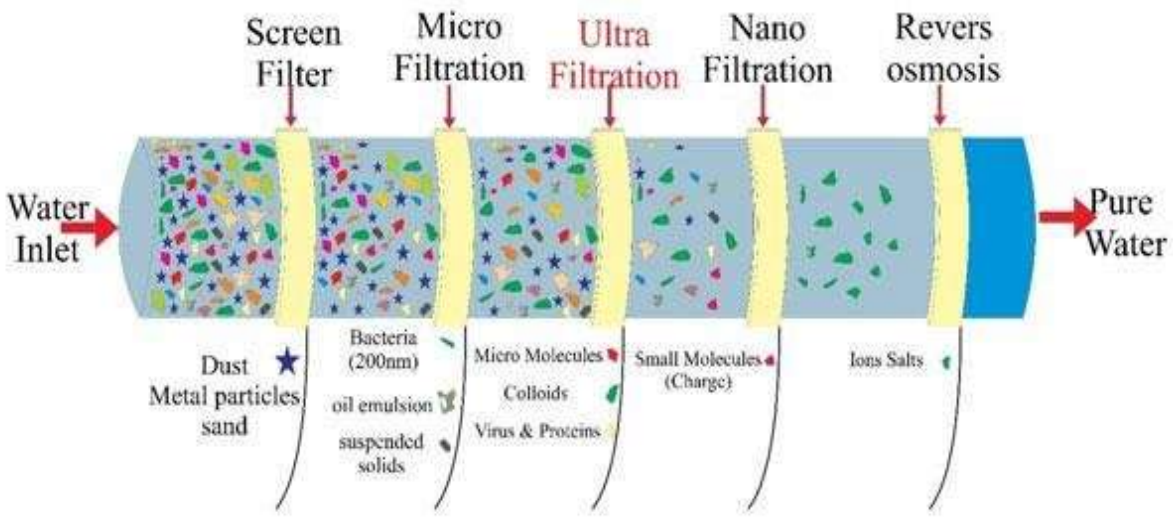

Figure 33. The different levels of filtration

6. Centrifugation

Centrifugation is a technique for separating particles from a solution, used in biology to isolate cells, organelles (mitochondria, chloroplasts), and large molecules (proteins, nucleic acids, viruses, etc.). The separation is based on the size and density of the particles: the heavier and denser components experience a greater centrifugal force and settle to form a pellet, while the lighter particles remain in suspension, forming the supernatant.

Centrifugation accelerates natural sedimentation by replacing the acceleration due to gravity ($g = 9.81\ m/s^2$) with a centrifugal force generated by the rapid rotation of a rotor. Conventional centrifuges generally reach less than 10,000 rpm, while ultracentrifuges can

exceed 100,000 rpm, allowing the sedimentation of particles or molecules with a mass >10,000 Da, often at refrigerated temperatures (Marouf, 2005).

The rotors used come in three main types:

• Fixed-angle: for simple separations between pellet and supernatant. The tubes, inclined at 15 to 40°, allow the particles to migrate to the bottom under the effect of centrifugal acceleration. This type is used for sequential separations at increasing speeds.

• Moving-cup: allows for finer separations because the tubes orient themselves parallel to the centrifugal field. This system is ideal for separating particles according to their density, often with density gradients.

• Vertical: less commonly used, but suitable for certain specialized applications (Marouf, 2005).

7. Preparative Ultracentrifugation

Preparative ultracentrifugation is a separation technique used for the isolation and purification of specific biological particles, including cell organelles and macromolecules. It relies on the application of high centrifugal forces, allowing the sedimentation of constituents according to their physical characteristics, primarily their size, shape, and density.

7.1. Differential Centrifugation

Differential centrifugation is the most practical method used in preparative ultracentrifugation. It allows the progressive separation of different intracellular structures from an initial cell homogenate, obtained after cell lysis, through a series of centrifugations at increasing speeds and durations.

The principle is based on the fact that larger, denser particles settle at low speeds, while smaller particles require higher centrifugal forces and longer precipitation times. Thus, during an initial low-speed centrifugation (approximately 1,000 to 2,000 g for 5 to 10

minutes), cell nuclei settle and form a pellet. The supernatant is then subjected to successive centrifugations at higher speeds.At intermediate centrifugal forces (approximately 10,000 to 15,000 g for 10 to 15 minutes), mitochondria and, in the case of plant cells, chloroplasts, settle. At this same speed range, after a longer time (approximately 30 minutes), microsomes can also sediment, which correspond to small vesicles resulting from the fragmentation of the membranes of the endoplasmic reticulum and the Golgi apparatus during homogenization.

Very small particles, such as ribosomes, require much higher centrifugal forces, on the order of 100,000 g, applied for one to two hours, to be collected as a pellet. The final supernatant then contains essentially soluble cytosolic material.

It should be emphasized that the speed and time values mentioned are indicative and may vary depending on the type of rotor, the apparatus used, and the characteristics of the membranes and cellular components, whose composition directly influences density. Furthermore, certain organelles or particles of different natures may exhibit similar sedimentation conditions, making their separation incomplete by differential centrifugation alone. In this case, complementary methods, such as equilibrium centrifugation on a density gradient (e.g., a sucrose gradient), are necessary to achieve finer separation, particularly of the Golgi apparatus compartments. This type of centrifugation is generally carried out using fixed-angle rotors (Marouf, 2005).

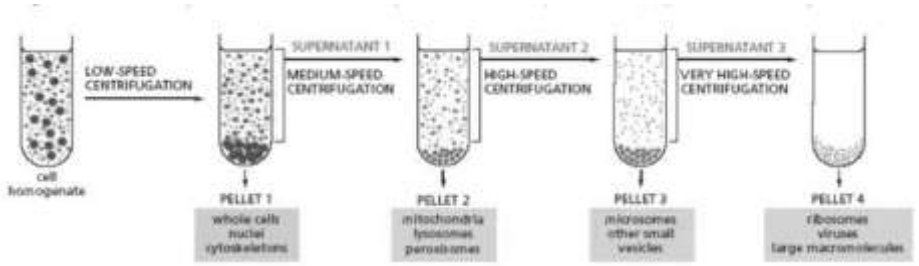

Figure 34. Differential centrifugation of a cell homogenate

7.2. Density Gradient Centrifugation

Density gradient centrifugation is a fine separation technique used to fractionate biological particles or macromolecules exhibiting subtle differences in density, size, or sedimentation coefficient. It relies on the prior establishment of a continuous or discontinuous density gradient within the centrifuge tube.

The medium constituting the gradient consists of a low molecular weight solute (such as sucrose, cesium chloride, or Percoll) dissolved in a solvent compatible with the sample par ticles, which are in suspension. This density gradient primarily plays an auxiliary role: it stabilizes the separated fractions by limiting diffusion phenomena and, in particular, the formation of convection currents that could disrupt the separation achieved during centrifugation.

Unlike differential centrifugation, where particles simply sediment according to their size and mass, density gradient centrifugation improves separation resolution by keeping the components within well-defined zones of the gradient. This technique is particularly well-suited for the purification of cellular organelles, macromolecular complexes, or viral particles.

There are two main density gradient centrifugation methods, distinguished by the separation mechanism involved:

Zonal centrifugation

Zonal centrifugation, also called zonal centrifugation, is a density gradient centrifugation technique widely used for separating macromolecules and biological particles based on their sedimentation coefficient.

It relies on the use of a continuous density gradient, generally composed of sucrose, the concentration of which gradually increases from the top to the bottom of the centrifuge tube.

In practice, a continuous sucrose gradient (for example, from 5 to 45%) is prepared in advance in a plastic centrifuge tube using a device that progressively mixes a concentrated sucrose solution with water, in a decreasing ratio as the tube is filled. This process ensures a gradient in which the density is highest at the bottom of the tube and lowest at its surface.

A small volume of the sample to be fractionated is then carefully deposited on the surface of the pre-formed gradient to avoid disturbing the density profile. The tube is then subjected to high-speed centrifugation for a duration and at a centrifugal force determined according to the nature of the particles to be separated and the desired experimental objective.

The separation achieved relies on the differences in sedimentation rates of particles across the gradient. This rate depends primarily on the sedimentation coefficient, which is itself influenced by the mass, size, and shape of the macromolecules. Since shape affects sedimentation rate more than density, zonal centrifugation allows for the efficient separation of similarly shaped macromolecules mainly based on their mass. During centrifugation in a moving-bucket rotor, the tubes adopt a horizontal position, which promotes the regular migration of different species as distinct bands or zones along the gradient.

After centrifugation, the fractions are recovered by piercing the bottom of the tube with a needle and then collected sequentially using a fraction collector. The distribution of proteins in the gradient can be determined by measuring the absorbance at 280 nm for each fraction, thus allowing the macromolecules to be classified according to their apparent mass. Alternatively, the tube can be frozen and then sliced into thin sections for further analysis.

Zone centrifugation is primarily used for the separation and analysis of DNA and RNA molecules, as well as ribonucleoprotein complexes, based on their size and sedimentation coefficient (Marouf, 2005).

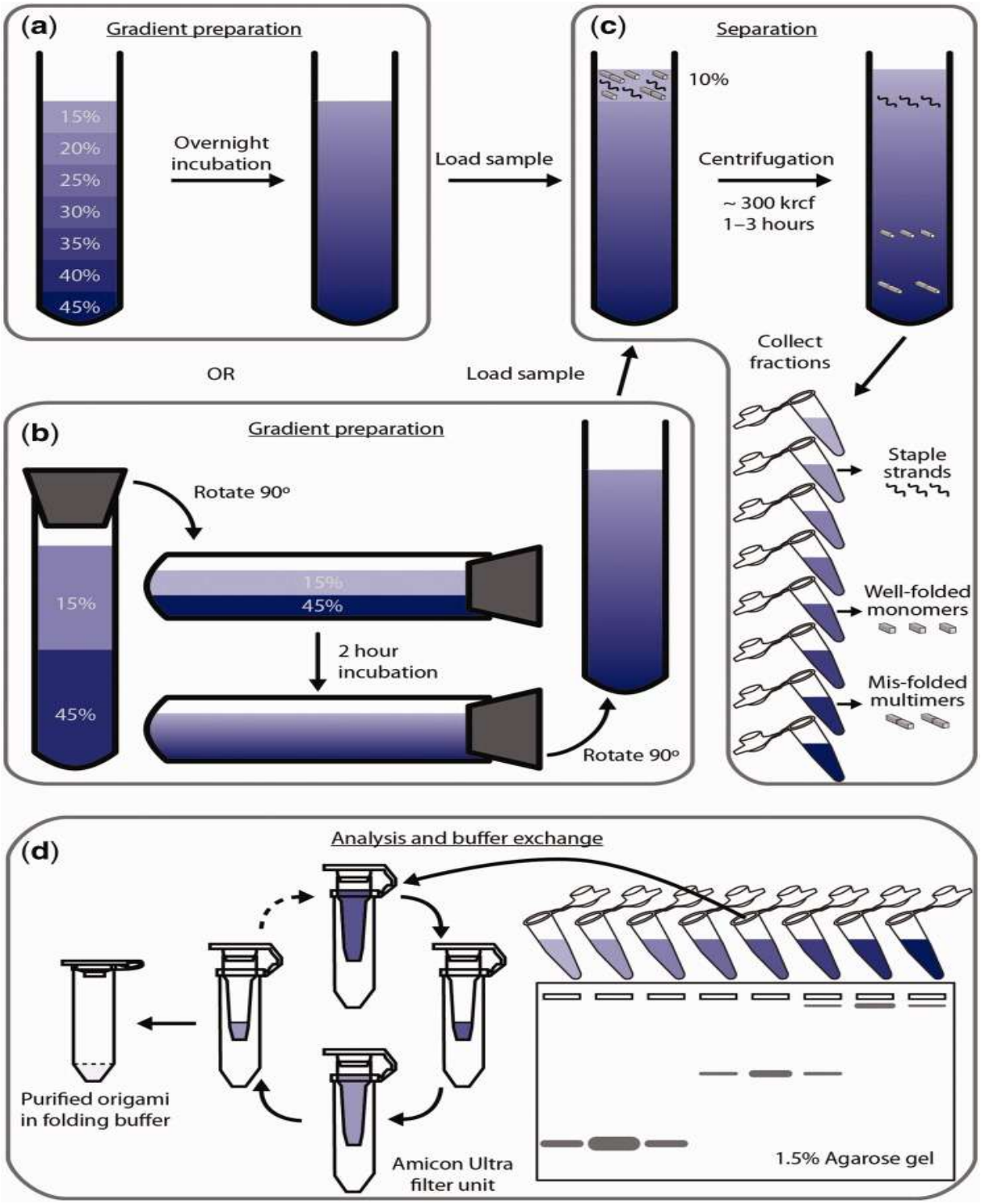

Figure 35. Zonal centrifugation.

8. Sedimentation

Sedimentation is a physical process that separates particles suspended in a fluid under the influence of gravity or centrifugal force. Heavier or denser particles settle more quickly, while lighter particles remain suspended. The sedimentation rate of a spherical particle in a fluid can be estimated using Stokes' Law:

$$v = \frac{2r^2(\rho_p - \rho_f)g}{9\eta}$$

Let be the particle radius, ρ_p and ρ_f the particle and fluid densities, the acceleration due to gravity, and η the fluid viscosity (Cussler, 2009).

In biology, sedimentation is used to separate cells, organelles, or macromolecules according to their size and density. It can be accelerated by centrifugation, allowing the separation of small or low-density components that would be too slow to sediment naturally (Alberts et al., 2014). Density gradients are often used to improve resolution, particularly for isolating specific organelles or subcellular particles (Sheehan & Garbett, 2018).

Table 5. Comparison of the main separation and fractionation methods

Method	Principle	Applications	Advantages	Limitations
Dialysis	Selective diffusion of molecules through a semi-permeable membrane according to size	Concentration of biological solutions, desalting, protein purification	Simple, low-cost, effective for macromolecules	Time-consuming, low separation rate
Electrodialysis	Transport of ions through a membrane under an electric field	Desalination of brackish water, whey treatment, denitration of drinking water	Faster than dialysis, ion-selective	Thermolabile products may be affected; electrical energy cost
Filtration (Microfiltration)	Selective passage of particles through a filter based on size	Sterile filtration, liquid clarification, HEPA filtration for air	Fast, simple, low-cost	Does not separate dissolved molecules
Ultrafiltration	Separation through a semi-permeable membrane under transmembrane pressure according to molecular size	Protein concentration, virus isolation, chromatographic monitoring	Efficient separation of macromolecules (10^3–10^6 Da)	Requires specific membranes; sensitive to pressure and pH
Centrifugation / Ultracentrifugation	Separation based on size and density using centrifugal force	Separation of cells, organelles, proteins, viruses	Fast, adaptable to density gradients, refrigerated operation possible	Expensive equipment, temperature sensitivity, risk of degradation of thermolabile products
ColumnChromatography	Separation by diffusion, adsorption, or partition based on affinity for the stationary phase	Purification of compounds, separation of complex mixtures	Allows isolation of gram-scale quantities; flexible	High solvent consumption, long processing time, empirical optimization
High-Performance Liquid Chromatography (HPLC)	Mobile phase forced under pressure through a column packed with fine particles	Rapid separation and quantitative analysis; purification	High selectivity, high sensitivity, automation possible	High cost of solvents and columns; requires high-purity solvents
Gas Chromatography (GC)	Separation based on retention time in a column with a stationary phase and a carrier gas	Analysis of volatile compounds	Very high sensitivity, excellent resolution	Samples must be volatile; expensive instrumentation
Gel Electrophoresis (PAGE, SDS-PAGE, Agarose)	Separation according to charge and/or molecular size under an electric field	Analysis and purification of proteins and nucleic acids	High resolution, specific visualization possible	Limited to charged molecules; variable migration time

Chapter IV. Isotopic Methods

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1. Isotopes

Isotopes are atoms of the same chemical element that possess an identical atomic number (Z), corresponding to the same number of protons in the nucleus, but differ in their number of neutrons (N). Consequently, isotopes have different mass numbers (A = Z + N). While the nuclear composition varies, the electronic configuration remains unchanged, which explains why isotopes exhibit nearly identical chemical behavior. However, the difference in mass leads to significant variations in physical properties, such as density, diffusion rate, vibrational frequencies, and melting or boiling points.

The existence of isotopes reflects the balance between nuclear forces within the atom. Variations in neutron number affect the nuclear binding energy and, in some cases, result in nuclear instability. Isotopes are therefore classified into stable isotopes and radioactive isotopes (radioisotopes) based on their nuclear stability.

1.1 Stable Isotopes

Stable isotopes, such as ^{12}C, ^{13}C, ^{15}N, ^{18}O, and ^{2}H (deuterium), do not undergo radioactive decay and remain unchanged over geological timescales. Their stability makes them particularly valuable as non-radioactive tracers, allowing safe and precise investigations in laboratory, environmental, and industrial contexts.

Stable isotope techniques are widely applied in:

Chemical reaction mechanism studies, where isotopic substitution reveals bond-breaking and bond-forming steps (kinetic isotope effects),

Metabolic and biochemical pathway analysis, including carbon and nitrogen fluxes in living organisms,

Environmental and ecological studies, such as tracing nutrient cycling, water sources, and pollution pathways,

Food authenticity and quality control, enabling the determination of geographical origin, adulteration, and production methods,

Isotopic fractionation studies, where mass-dependent differences during physical or biochemical processes lead to characteristic isotopic signatures.

Because stable isotopes do not emit radiation, they pose no radiological risk, making them suitable for studies involving humans, animals, and ecosystems.

1.2 Radioactive Isotopes (Radioisotopes)

Radioactive isotopes are characterized by nuclear instability, resulting from an unfavorable ratio of neutrons to protons. To reach a more stable configuration, these nuclei undergo spontaneous radioactive decay, accompanied by the emission of ionizing radiation. The main types of radiation include:

Alpha particles (α), consisting of helium nuclei, with high ionizing power but low penetration depth,

Beta particles (β^- or β^+), corresponding to electrons or positrons emitted during neutron–proton transformations,

Gamma rays (γ), high-energy electromagnetic radiation often emitted following alpha or beta decay.

This emitted radiation can be detected with exceptional sensitivity, allowing radioisotopes to be traced even at extremely low concentrations. As a result, radioisotopes are powerful tools in:

- **Biological labeling**, including DNA, RNA, proteins, and hormones,
- **Chemical kinetics**, enabling the monitoring of reaction rates and intermediate formation,

- **Pharmacokinetics and drug distribution studies**, tracking absorption, distribution, metabolism, and excretion,
- **Environmental tracing**, such as groundwater movement, sediment transport, and contaminant dispersion,
- **Medical diagnostics and therapy**, particularly in nuclear imaging (PET, SPECT) and radiotherapy.

Despite their usefulness, radioisotopes require strict safety measures, including shielding, controlled handling, and waste management, due to their ionizing radiation.

1.3 Analytical and Scientific Importance of Isotopes

The unique combination of chemical equivalence and physical distinguishability makes isotopes indispensable in modern science. Their applications extend across multiple disciplines, including:

Radiometric dating, such as ^{14}C dating for archaeological and geological samples,

Quantitative labeling of biomolecules, providing precise measurements of synthesis and degradation rates,

Monitoring of chemical and biochemical reactions under real conditions,

Mass transfer, diffusion, and transport studies in porous or complex systems,

Medical diagnostics and therapeutic applications, particularly in oncology and metabolic disorders.

Through both stable and radioactive forms, isotopes provide unparalleled insight into mechanisms, dynamics, and transformations that are otherwise inaccessible by conventional analytical methods (Krane, 1987; Choppin et al., 2013).

2. Fundamental Laws of Radioactivity

Radioactivity is a spontaneous and intrinsic nuclear phenomenon by which an unstable atomic nucleus transforms into a more stable configuration through the emission of particles and/or electromagnetic radiation. This process results from an imbalance between the nuclear forces that bind protons and neutrons within the nucleus. Importantly, radioactive decay is random at the level of individual nuclei, yet highly predictable at the macroscopic scale when considering a large population of radioactive atoms.

2.1 Law of Radioactive Decay

The fundamental law governing radioactive decay describes the decrease in the number of radioactive nuclei as a function of time. This decay follows a first-order kinetic law, expressed by the exponential equation:

$$N(t)=N0e-\lambda t$$

where:

N(t) is the number of radioactive nuclei remaining at time t,

$\mathbf{N_0}$ is the initial number of radioactive nuclei at time t = 0,

λ is the decay constant, a characteristic parameter specific to each radioisotope,

t is the elapsed time.

The decay constant λ represents the probability per unit time that a given nucleus will decay. It is independent of external factors such as temperature, pressure, chemical environment, or physical state of the element, emphasizing the purely nuclear nature of radioactive processes.

2.2 Half-Life Concept

A key parameter derived from the decay constant is the half-life ($T_{1/2}$), defined as the time required for half of the radioactive nuclei in a sample to decay. The relationship between the half-life and the constant decay is given by:

$$T1/2=\lambda ln2$$

Activity is expressed in becquerels (Bq), where 1 Bq corresponds to one nuclear disintegration per second. Historically, the curie (Ci) was also used, where 1 Ci = 3.7×10^{10} Bq. Because activity is directly proportional to the number of radioactive nuclei present, it decreases exponentially with time in the same manner as N(t). Activity is a crucial parameter for:

- Quantifying radioactive samples,
- Designing tracer experiments,
- Ensuring compliance with radiation safety regulations,
- Calculating administered doses in nuclear medicine.

2.4 Statistical Nature of Radioactive Decay

Although the decay of an individual nucleus is unpredictable, radioactive decay obeys statistical laws when large numbers of nuclei are involved. The exponential decay law arises from the constant probability of decay per unit time. As a consequence:

- Radioactive decay cannot be accelerated or slowed by external means,
- Measurements are subject to statistical fluctuations, especially at low activity levels,
- Counting errors follow Poisson statistics, which must be considered in quantitative analysis.

2.5 Practical Implications and Applications

The fundamental laws of radioactivity provide the theoretical basis for numerous scientific and technological applications, including:

- Prediction of isotope behavior over time, essential for storage, handling, and disposal of radioactive materials,
- Calculation of radioisotope concentrations required for analytical, biological, or medical experiments,
- Radiometric dating, enabling age determination of archaeological, geological, and environmental samples,
- Tracer studies in chemistry, biology, and environmental sciences,
- Dosimetry and radiation protection, ensuring safe use of radioactive sources.

By combining decay equations, half-life data, and activity measurements, researchers can design experiments with optimal sensitivity while minimizing radiation exposure (Krane, 1987; Choppin *et al.*, 2013).

3. Measurement Technology

The detection and quantification of radioactive isotopes require the use of specialized measurement technologies specifically adapted to the type, energy, and intensity of the emitted radiation. Since alpha (α), beta (β), and gamma (γ) radiations differ significantly in mass, charge, penetration power, and interaction mechanisms with matter, no single detector is suitable for all applications.

3.1 Gas-Filled Detectors

Geiger–Müller (GM) counters are among the most widely used instruments for detecting ionizing radiation. They consist of a gas-filled tube with a central anode wire and

operate at high voltage. When ionizing radiation enters the detector, it ionizes the gas molecules, triggering an avalanche of charged particles that produces a measurable electrical pulse. GM counters are highly sensitive and robust, making them suitable for radiation monitoring and contamination surveys. However, they do not provide information on the energy of the radiation or allow discrimination between different types of particles.

Proportional counters operate at lower voltages than GM counters and generate electrical pulses proportional to the energy deposited by the incident radiation. This property allows partial discrimination between alpha, beta, and gamma radiation and enables quantitative measurements of particle energy. Proportional counters are particularly useful for detecting low-energy beta emitters and for applications requiring energy resolution and improved analytical precision.

3.2 Scintillation and Gamma Spectrometry

For the detection of gamma radiation, gamma spectrometry is the method of choice. It is based on the interaction of high-energy photons with scintillator materials, which emit visible light when excited by ionizing radiation. Common scintillation crystals include sodium iodide doped with thallium [NaI(Tl)], which offers high detection efficiency and good sensitivity.

The light emitted by the scintillator is collected by a photomultiplier tube (PMT), where it is converted into an electrical signal. The amplitude of this signal is proportional to the energy of the incident gamma photon, allowing the construction of an energy spectrum. Gamma spectrometry enables:

- Precise identification of radionuclides based on their characteristic gamma energies,
- Quantitative determination of isotope concentrations,

- Analysis of complex radioactive mixtures in environmental, medical, and industrial samples.

3.3 Factors Affecting Measurement Accuracy

The efficiency and accuracy of radioactive measurements depends on several critical factors, including:

- Type and energy of the emitted radiation, which influences detector choice and response,
- Detector efficiency and geometry, affecting counting statistics,
- Shielding against background radiation, typically using lead or other dense materials,
- Sample preparation and handling, to avoid losses, contamination, or self-absorption effects,
- Calibration and quality control, ensuring reliable and reproducible measurements.

Careful optimization of these parameters is essential for achieving accurate, sensitive, and reproducible results in radioisotope detection and analysis
(Knoll, 2010).

4. Liquid and Solid Scintillation

Scintillation is a physical phenomenon in which a material undergoes ionizing radiation to emit light. This light emission is then converted into an electrical signal by a photomultiplier tube. In liquid scintillation, used primarily to detect low-energy beta particles, the radioactive sample is mixed with a scintillation liquid containing an organic solvent and a fluorine. The light emitted by the interactions of the beta particles with the fluid is collected and transformed into a signal proportional to the radioactive activity. This technique offers high detection efficiency and low background noise, making it ideal for biological and

metabolic applications, such as tracking labeled nucleic acids or enzyme kinetic studies (Knoll, 2010).

Solid-state scintillation relies on scintillator crystals, such as NaI(Tl) or CsI(Tl), to detect high-energy gamma and beta radiation. In this case, the crystal emits photons proportional to the energy of the incident radiation, which are then detected by a photomultiplier tube. This technique is widely used for gamma spectrometry, dosimetry, and nuclear medicine. Solid-state scintillation allows for precise and quantitative detection of high-energy radiation and offers the advantage of good energy resolution, essential for the identification and quantification of specific isotopes in complex mixtures (Knoll, 2010).

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